Studies on RNA Modification and Editing in *Trypanosoma brucei*

**DISSERTATION**

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By

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Abstract

RNA editing and RNA modification are chemical processes often essential for the survival of self-replicating organisms. RNA editing enables any RNA species to expand its decoding capacity or alter its coding potential using post-transcriptional enzyme catalyzed reactions. RNA modifications are equally important and can optimize the function as well stabilize many types of RNA. *Trypanosoma brucei* has provided ground-breaking discoveries in the field of RNA editing and modification which have gone against biological norms. A subset of tRNAs undergo adenosine to inosine (A to I) editing of their anticodon which permits decoding of up to three codons with a single tRNA. A few of these tRNAs are additionally edited from cytidine to uridine (C to U) in their anticodon stem at position 32 and further methylated at this same position. *T. brucei* possess two homologs of the 3-methylcytidine methyltransferase which catalyzes methylation of C or U at position 32. We have shown only one homolog is responsible for position 32 modification in this organism and my work has focused on the importance, and begun to elucidate, the biological function of the homologous 3-methylcytidine methyltransferase which we have named MTase37.

Chapter 2 focuses on the importance of MTase37 for cytokinesis and ribosome stability and/or assembly. Using RNA interference (RNAi) I have determined MTase37 is specifically important for the stability and/or biogenesis of the large ribosomal subunit.
MTase37 depletion causes loss of small ribosomal RNAs srRNA 4 and 5S from ribosomes. At the onset of an RNAi phenotype, cells show an increased number of flagella as well as increased nuclear and kinetoplastid DNA content. RNA processing is not affected in these cells, but we believe MTase37 plays an essential role in ribosome maturation acting as an RNA methyltransferase.

In continuing to build upon our understanding of MTase37 Chapter 3 focuses on its modification activity and impact on cellular processes at the molecular level. Using mass spectrometry, HPLC, TLC and RNAseq we analyzed tRNA and rRNA for changes in modifications upon depletion of MTase37. Although we were unable to observe changes in the modifications on rRNA we did discover that $\text{m}^1\text{A}_{58}$ is surprisingly increased on a subset of tRNAs following MTase37 RNAi.

The final chapter describes a genetic screen for tRNA editing activity designed in *Saccharomyces cerevisiae*. The screen was designed to work in multiple contexts using an exogenously expressed protein and tRNA from *T. brucei*. After constructing the genetic system I used a cDNA library of *T. brucei* genes to search for the C to U deaminase responsible for editing of mitochondrial tRNA$^{\text{Trp}}$. In a separate application of this system I investigated the amino acid changes to ADAT2 which might alter its activity from an A to I into a C to U deaminase. Neither applied system resulted in identification of our anticipated deaminases, but I was able to demonstrate the system will function if an active enzyme is expressed and generated building blocks for further application and optimization of the genetic screen in discovering deaminases.
Dedication

I wish to dedicate this document to my extraordinarily dedicated and loving mother, Patricia Taylor. She has been a source of great inspiration and support every day of my life. Her passion for family, dedication to her profession and desire to put others first are a constant reminder that success could not be possible without hard work and happiness.
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Publications


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**Field of Study**

Major Field: Microbiology
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Chapter 1: Introduction

1.1 Introduction to trypanosome biology

Trypanosomes are parasitic unicellular protozoa with a single flagellum and mitochondrion. When in the human host *T. brucei* resides in the blood stream but when within its insect vector *T. brucei* resides in the midgut. As members of the order Kinetoplastida, they are among the earliest diverging eukaryotes. These organisms are so named for their defining feature, the kinetoplast. This dense granular structure is found within the cell’s singular mitochondrion and possesses a unique network of mitochondrial DNA. The kinetoplastid mitochondrial genome is composed of two circular DNA species: maxicircles which are between 20 and 40kb at 10-30 copies per cell and minicircles which are between 0.5 and 1kb at several thousand copies per cell. Maxicircles contain “cryptic” genes which are transcribed into premature RNAs that must be edited, or post-transcriptionally recoded to generate mature translatable mRNAs. Editing occurs through the action of minicircle RNA products known as guide RNA (gRNA) and proteinaceous editing complexes (Benne et al. 1986). The editing process involves extensive removal or insertion of uridine residues within the mRNAs and only after exact insertion or deletion will the maxicircle mRNA products encode for functional protein.

Trypanosomes multiply by binary fission where each cell gives way to a new daughter cell in each successive round of cell division (Figure 1.1). Throughout cell
division, structures associated with the flagellum and kinetoplast are key checkpoints for cell cycle progression (Vaughan and Gull 2008).

Figure 1.1. Cell division in trypanosomes.

Trypanosomes possess one flagellum (F), one kinetoplast (K) and one nucleus (N). Upon entry into mitosis the flagellum (green) is first duplicated (blue) stemming from the basal body of the kinetoplast. Subsequently kinetoplastid DNA (small grey circles) is duplicated followed by nuclear DNA replication (large grey circles). The progression from 1F:1K:1N to 2F:1K:1N to 2F:2K:1N to 2F:2K:2N is typical of normal cell division. Upon reaching the 2F:2K:2N state two cells a formed by cleavage at the pole of the cell dividing the two flagella and segregating DNA content equally. (Image adapted from Ralston and Hill 2008).
As human parasites, trypanosomes must be able to change life cycles upon encountering a new environment in order to evade the human immune response. This life form change is accompanied by a number of intracellular metabolic and mRNA stability and/or expression changes. Upon infection of a human host through blood meal by the tsetse fly reservoir, African trypanosomes rapidly undergo a switch in cell type from procyclic form to blood stream form parasites. Specific alterations accompanying the life form switch include a cell-surface antigen switch from procyclin or procyclic acid repeat protein (PARP), into variant surface glycoprotein (VSG) upon human infection as well as repression of mitochondrial activity (Fenn and Matthews 2007).

1.2 Trypanosome importance to world health

Disease causing Trypanosomatid organisms of the genera Trypanosoma are endemic to sub-Saharan Africa (African trypanosomiasis or sleeping sickness) and the Americas (American trypanosomiasis or Chagas disease) where they cause debilitating diseases in humans, livestock and wild game. *Trypanosoma brucei* is carried by the tsetse fly where it resides and duplicates with in the midgut and salivary glands. Tsetse flies themselves become infected upon blood meal from an infected human or animal which itself was initially infected upon blood meal by and infected tsetse fly. Another species of trypanosome, *Trypanosoma cruzi*, is carried by the triatomine (also known as kissing or reduvid) bug which commonly infects humans by feces deposited on skin upon blood meal. Both *T. brucei* and *T. cruzi* are considered to be neglected parasites with associated neglected diseases by the Center for Disease Control (CDC). It is estimated that 300,000
people are currently infected with *T. cruzi* in the United States but show no symptoms to be aware of it (CDC). *T. brucei* is documented to infect between 7,000 and 10,000 new individuals each year in African countries where treatment and health care infrastructures each need to be developed. There is currently no curing treatment for either species of trypanosome and only one drug, pentamidine, is commercially available. However, this drug is minimally effective against later stages of the disease. A number of experimental drugs are available directly from the CDC to physicians for treatment, but these drugs are not yet cleared by the Food and Drug Administration (FDA).

Research done in the Alfonzo laboratory seeks to identify and understand essential pathways unique to trypanosomes, which may be exploited and targeted in further studies as a potential treatments. Much of the laboratory’s recent findings have demonstrated *T. brucei* utilizes unique approaches to achieve the same end result as other eukaryotes. Such examples include deamination of both adenosine to inosine and cytidine to uridine on a single tRNA and editing of the tRNA tyrosine intron which is essential for splicing (Rubio et al. 2013; Gaston et al. 2007; Rubio et al. 2007). My work, primarily described in this document, has investigated a protein predicted to be a 3-methylcytidine methyltransferase which we have named MTase37. It appears to be the result of a gene duplication throughout the kinetoplastid lineage as well as higher eukaryotes, some of which encoded multiple methyltransferase-like proteins. Importantly, an ortholog in human (methyltransferase-like protein 6) was shown to be an important driver in breast cancer (Gatza et al. 2014).
1.3 The central dogma of molecular biology

In 1958 Francis Crick made two propositions in his publication, “On Protein Synthesis”, both of which are still regarded as central notions in molecular biology today. These were that: first, the sequence of a nucleic acid chain specifies exactly the product coded for by its sequence of bases and second, this sequence information can be transferred from deoxyribose nucleic acid (DNA) bases into ribonucleic acid (RNA) and RNA to protein, but never from protein to nucleic acid (Figure 1.2). Together these form what we know today as the central dogma of molecular biology. In all cells both RNA and DNA are composed of combinations of four standard nucleotides (Figure 1.3). As information is translated from RNA into protein, 20 standard L-form amino acids are used in generating proteins (Figure 1.4). Following the central dogma of molecular biology, the incorporation of each nucleotide and each amino acid is defined by the exact sequence defined within the templating DNA or RNA.

Today we have a much deeper understanding of the enzymes and biochemical processes involved in the central dogma. Since 1958 there have been numerous discoveries which have led us to “modernize” Crick’s central dogma including the existence of RNA as genetic material and reverse transcription of RNA into DNA, but the overarching idea that protein cannot be translated in reverse into nucleic acid holds true. We also making significant discoveries about the regulation of the flow of information from DNA into RNA and RNA into protein through the use of high-throughput techniques. Proteins can regulate the transcriptional and translational activities through binding events or enzymatic modifications. This is a rapidly growing field known as epigenetic regulation.
Figure 1.2. The Central Dogma of Molecular Biology.

Full black arrows outline the original idea of the Central Dogma which stated DNA was transcribed into RNA which is translated into protein. Dashed arrows depict the flow of information from RNA to DNA by reverse transcriptase enzymes or RNA to RNA replication found in numerous viruses. Black and red ribbons represent sense or anti-sense strands of genetic information.
Figure 1.3. **Chemical structure of the standard DNA and RNA nucleotides.**

A) Nucleobase structures of purines [guanine (G) and adenine (A)] or pyrimidines [uracil (U), cytosine (C) and thymine (T)]. “R” denotes the point of covalent attachment to a ribose or deoxyribose base. B) Structures of the 5 carbon sugar bases of ribose and deoxyribose. “B” denotes the point of covalent attachment for nucleobases.
Figure 1.4. The 20 Standard Amino Acids.

Chemical structure and nomenclatures of each standard amino acid. Full names, three letter abbreviation and single letter code is listed under each structure.
1.4 Processing of RNAs in trypanosomes

Many cellular RNAs resulting from transcription are immature and require further processing before becoming functional. Essential processing steps include intron removal from both messenger RNA (mRNA) and transfer RNA (tRNA). In this process two exon sequences are joined simultaneously or immediately following removal of the intronic sequence. Ribosomal RNA (rRNA) does not contain true introns, but instead possess internal transcribed spacers (ITS) and external transcribed spacers (ETS) which must be processed out of the pre-rRNA transcript by endo- and exonucleases, generating mature rRNAs ready for final incorporation into the ribosome. *T. brucei* mRNAs do not adhere to typical eukaryotic trends for processing. First, there are very few intron containing mRNAs. Second, each mRNA receives an identical 5’ leader sequence known as the spliced leader (SL) RNA which is transferred to every mRNA by trans-splicing (Parsons et al. 1984). The spliced leader is encoded in a genomic region of about 200 repeats, each gene generating a 140 nucleotide RNA transcript which is further processed to possess a unique 5’ cap known as the “cap 4 structure” (Bangs et al. 1992). Each mRNA receives the 5’ most 37 nucleotides of the spliced leader RNA upon trans-splicing at a defined 5’ splice site within the 5’ untranslated region (UTR) of the mRNA (Parsons et al. 1984; Mair et al. 2000). In common with mRNAs of other eukaryotes, processing in *T. brucei* also includes addition of a canonical poly-A tail to the end of the 3’ UTR. The spliced leader, poly-A tail and UTRs are believed to be an important factor in regulation of gene expression within trypanosomes where no regulatory control of transcription has been identified.
1.4.1 Transfer RNA

Transfer RNA (tRNA) is required within cells to decode the information found in codons in mRNA, and deliver the appropriate amino acid for peptidyl transfer to a growing amino acid chain at the peptidyl transfer site of the ribosome. Each codon being a 3 nucleotide division of an mRNA contains one of the four standard nucleotides and provides 64 possible combinations to define amino acid incorporation (Figure 1.5). Three of the 64 possible combinations (UGA, UAA, UAG) are reserved for translation termination by ribosome release factors and typically have no complementary tRNA. There are, however, a few exceptions to this rule. The UGA codon may also define incorporation of the 21st amino acid, selenocysteine, in cytoplasmic translation but requires the selenocysteine insertion sequence for incorporation. In mitochondrial translation the UGA codon is decoded by a special tRNA tryptophan and consequently no release factor is present to recognize this codon. The UAG codon in some organisms encodes for the 22nd amino acid, pyrolysine. The remaining 61 codons are thus reserved to define the incorporation of the 20 standard amino acids. Although 61 tRNAs could perfectly decode the 61 codons, organisms never encode 61 distinct tRNAs and instead rely on “wobble” pairing to permit decoding of multiple codons for the same amino acid with a single tRNA. A few amino acids are encoded by only one or two codons for which a single tRNA anticodon is sufficient to decode up to two codons. For example, tRNA\textsuperscript{Phe}\textsubscript{GAA} can decode both UUU and UUC codons. In some cases there can be up to six codons defining the same amino acid. These codons rely on tRNA editing to change nucleotide identity of the anticodon. Editing increases the decoding capacity of a single tRNA and reduces the complement of
tRNAs which need to be genomically encoded. This type of tRNA editing will be discussed in depth in section 1.6.3.

The overall L shaped three-dimensional structure must be maintained between all tRNAs despite differences in sequence length and composition. All tRNAs share a common secondary structure, commonly depicted as a cloverleaf, which is comprised of approximately 76 nucleotides forming 4 paired stems and 3 unpaired loops with a 5’ terminal phosphate and 3’ terminal CCA nucleotides necessary for aminoacylation and peptidyl transfer (Kim et al. 1973; Xiong and Steitz 2004) (Figure 1.6). The three stem-loop structures have been named the D, anticodon and TψC stem-loops based on the presence of characteristic dihydrouridine, anticodon sequence and TψC residues present in all tRNAs. The overall structure is not entirely reliant upon its primary nucleotide sequence, but also upon post-transcriptional modifications which serve to contribute stability, fine tune structure and improve decoding (Motorin and Helm 2010; Gustilo et al. 2008; Khade et al. 2013; Jackman and Alfonzo 2012). Modification of tRNA will be the focus of section 1.6.1.

Each tRNA is transcribed from either nuclear, mitochondrial or chloroplast DNA using RNA polymerase III (RNA Pol III) in the nucleus or specialized polymerases within the organelles. In some eukaryotes only a subset of tRNAs are encoded within organelles and they therefore rely on a process known as tRNA import to supply the full complement of tRNAs for translation. Trypanosomes are an extreme case where no tRNA is encoded within the mitochondrial genome and therefore all tRNAs are transcribed within the nucleus, exported to the cytoplasm and finally a complete set is imported into the
Figure 1.5. The standard genetic code.

All 64 possible codons are listed with their respective amino acid denoted with three letter code. Stop codons (UAA, UAG, UGA) are highlighted in purple and the canonical start codon AUG (Met, methionine) is highlighted in orange.
Figure 1.6. **Secondary and tertiary structure of transfer RNA.**

A) The cloverleaf secondary structure of a generic tRNA with names of each stem and loop. The acceptor stem (purple) and CCA (yellow) is formed by pairing of 5’ and 3’ ends of the linear molecule. D stem and loop (red), TΨC step and loop (green) and anticodon stem (blue) are formed by hairpin pairing of consecutive sequence. The variable loop (orange) and anticodon (grey) are also depicted. B) Crystal structure of tRNA^Phe from *Saccharomyces cerevisiae*. Colors uses are the same colors used in A. In three-dimensional space the D and TΨC stem loops for tertiary interactions forming an L-shaped tRNA (Shi and Moore 2000).
mitochondrion. Nuclear tRNA synthesis utilizes promoter elements within the tRNA known as A and B boxes (Reviewed in Schramm & Hernandez, 2002). Processing of each pre-tRNA varies by species and tRNA identity, but processing may include trimming of 5’ leader and 3’ trailer ends, intron removal accompanied by splicing and finally enzymatic modification or editing of nucleotides (Figure 1.7).

Before a tRNA is equipped to depart the nucleus and enter into the cytoplasm it must have any 5’ leader and 3’ trailer ends removed. Removal of 5’ leader is accomplished by ribonuclease P (RNase P), a ribonucleoprotein endonuclease present in all domains of life (Chen and Pace 1997; Guerrier-Takada et al. 1983; Stark et al. 1978). Removal of the 3’-trailer is accomplished by a tRNA 3’ processing exonuclease, most commonly tRNase Z (reviewed in Maraia & Lamichhane, 2011). The order of processing is predominantly a 5’ before 3’ process due to inhibition of 3’ exonuclease activity in the presence of a 5’- leader with a few exceptions including Saccharomyces cerevisiae tRNA^{Trp} and Trypanosoma brucei tRNA^{Thr} (Kufel and Tollervey 2003; Gaston et al. 2007). For eukaryotic tRNAs with introns, splicing is carried out by a heterotetrameric tRNA splicing endonuclease complex, also known as the SEN complex, originally characterized in yeast (Trotta et al. 1997). In trypanosomes the only tRNA transcribed with an intron is tyrosyl-tRNA, for which the Alfonzo lab recently showed requires editing of at least two intronic nucleotides before splicing (Rubio et al. 2014). The importance of introns within tRNAs is not well understood, however in budding yeast the intron is required to convert U_{35} of the anticodon to pseudouridine (Johnson and Abelson 1983). Following splicing, two tRNA halves are generated which must be ligated together using a tRNA specific ligase.
Figure 1.7. Pathway of transfer RNA biogenesis.

In this schematic tRNA genes are transcribed in the nucleus by RNA Pol III (yellow) generating a tRNA which might contain a 5’ leader (red), 3’ trailer (blue) and/or intron (green). Cleavage and splicing are performed to generate a mature tRNA to which a CCA (orange) must be added at the 3’ end for aminoacylation.
Recently we have begun to characterize the endonuclease complex of *T. brucei* and have only identified 3 subunits which appear essential for activity rather than the expected 4 subunit complex as in other eukaryotes.

Additionally, tRNAs containing an encoded adenosine at position 34 (A_{34}) undergo editing by the dimeric enzyme complex adenosine deaminase acting on tRNAs, or ADAT2/3. This editing enzyme catalyzes the hydrolytic deamination of A_{34} forming inosine 34 (I_{34}). Specific tRNA editing and modification reactions will be the focus of section 1.6.

1.4.2 *Ribosomal RNA*

Ribosomal RNA (rRNA) is the most abundant RNA in the cell (approximately 70% of all RNA) and is transcribed by RNA polymerase I (RNA Pol I) centrally within the nucleus in a region known as the nucleolus. The primary function of this non-membrane bound nuclear subdivision is rRNA transcription and ribosome assembly. Within the nucleolus, regions of the chromosome containing the polycistronic ribosomal DNA (rDNA) repeats are extended and sites of rapid pre-rRNA transcription are formed. The pre-rRNA transcripts are processed in a sequential manner beginning with co-transcriptional cleavages and are further processed after transcription termination. After appropriately sized rRNAs are generated, they are heavily modified by enzymatic reactions. Ribosomal RNAs are assembled with ribosomal proteins into ribosomal subunits in a highly orchestrated pattern which involves hundreds of factors. Ribosomal RNAs may not be completely processed or modified until after subunits begin to form.
Eukaryotic rRNA processing is most well understood through studies using *Saccharomyces cerevisiae* and mammalian cells (Figure 1.8). Subtle differences have been observed in pre-rRNA processing between these two organisms and we could expect rRNAs of other organisms to also possess unique processing pathways. Trypanosome rRNA transcription occurs as in other eukaryotes. However, marked differences are seen beginning with processing of the pre-rRNA transcripts, which generates six large subunit rRNA pieces corresponding to the canonical 28S or 26S rRNA (Figure 1.9).

1.4.3 mRNA transcription and the spliced leader RNA

Gene expression in trypanosomes appears to occur without any regulation at the level of transcription as is commonly accomplished in other eukaryotes. Transcription of mRNAs is generally performed by RNA polymerase II (RNA Pol II) generating very long polycistronic directional mRNAs. The resulting polycistronic transcription unit is unusual for eukaryotes which typically initiate transcription at a promoter and terminate at a terminator sequence for each mRNA. There are, however, exceptions to the eukaryotic rules such as *Caenorhabditis elegans* where about 15% of protein coding genes are transcribed in polycistrons (Blumenthal et al. 2002; Zorio et al. 1994). Unlike prokaryotic operons of *trp* and *lac*, tandem genes in trypanosomes don’t encode for proteins acting in related metabolic pathways. No control of transcription initiation has been identified in these organisms and only a few promoters have been fully characterized including those of the spliced leader RNA (SL RNA), procyclic acidic repetitive protein (PARP), variant
Figure 1.8. Processing of *Saccharomyces cerevisiae* ribosomal RNA.

The rRNA operon of yeast generates a 35S precursor of 9.1 kilobases. Processing by cleavage is shown from 35S to mature rRNAs as indicated by grey arrows. Enzymes and nucleases involved in processing are not shown for simplicity. The 35S precursor is first cleaved at the 5’ ETS generating a 33S and then 32S precursor. The 32S precursor is subsequently separated into small subunit (20S) and large subunit (27S) precursors which are further matured into final rRNAs as precursors are moved from nucleolus to nucleoplasm to cytoplasm. The pathway for 27S processing as shown is the dominant pathway in cells and it should be noted that alternative processing pathways are shown to exist. (Diagram adapted from Woolford and Baserga 2013)
Figure 1.9. **Schematic for processing of *Trypanosoma brucei* ribosomal RNA.**

Transcription of ribosomal DNA (rDNA) produces a 9.7kb precursor. The diagram, drawn to scale, shows the 8 rRNAs of a mature ribosome as black boxes. Sizes of known intermediates are shown to the right or left of each intermediate. Between each rRNA is an internal transcribed spacer, ITS, labeled starting at ITS1 and ending with ITS7 from 5’ to 3’ (left to right) in the precursor. (Diagram adapted from Jensen et al. 2005).
surface glycoprotein (VSG) and ribosomal RNA (Das et al. 2005; Sherman et al. 1991; Janz and Clayton 1994). Interestingly transcription of PARP, VSG and rRNA is catalyzed by RNA Pol I which is typically reserved for rRNA rather than protein coding genes. Transcription initiation is believed to be regulated in part by chromatin alterations mediated by histone modification states (Siegel et al. 2005). To date, no obvious significance has been attributed to the presence of polycistronic mRNAs or the apparent lack of transcriptional control.

A lack of transcriptional control means gene expression could be controlled at the levels of mRNA processing from the primary transcript, mRNA stability or through differential translation initiation. Processing of a primary polycistronic transcript into individual mRNAs involves a unique trans-splicing reaction coupled with a common eukaryotic polyadenylation reaction. The trans-splicing reaction is essential to first cut out the mRNA from the primary transcript and also to provide the 39 nucleotide spliced leader RNA which is an essential element of translation initiation. This trans-splicing reaction is similar in mechanism to a cis-splicing reaction where a GU dinucleotide at the 5’ splice site, a AG dinucleotide at the 3’ splice site plus a poly pyrimidine tract are required. In both trans- and cis-splicing, catalysis involves two transesterification reactions but differ in spliceosome composition and intermediate structures of Y-branched and lariat structures, respectively. In trans-splicing the first transesterification occurs at the 3’ end of the 5’ exon, or spliced leader, and releases the 5’ exon while forming the Y-structured intermediate at branch point adenosine of the mRNA. The second transesterification reaction attaches the spliced leader exon to the 3’ splice site within the trypanosome mRNA.
(Reviewed in Gunzl 2010). In trypanosomes, cis-splicing is known to occur in only one mRNA, that of poly(A) polymerase (Mair et al. 1991). Since an intron is present in only one gene, and its presence is conserved among the kinetoplastids, it is possible that, like the intron of tRNA\textsuperscript{Tyr}, it may play a yet to be discovered function in the cell, perhaps functioning as a regulatory cis acting RNA.

As previously mentioned gene expression may be controlled through altering mRNA stability. Furthermore, without transcriptional control there must exist a means of silencing mRNAs when a protein is not required, especially since the metabolic stage differs greatly between the two trypanosome developmental stages (insect vs. mammal stage). In support of this idea it is known that significant changes in gene expression accompany the switch from procyclic to bloodstream life form, but this study looked only at complete mRNA transcripts, not the activity of mRNA transcription or translation (Siegel et al. 2011). mRNA half-life is impacted by motifs within 3’ UTRs which serve to either enhance or decrease stability through protein binding and provide either protection to the mRNA or targets it for degradation. Multiple 3’ UTR sequence motifs were identified which enhance mRNA stability in procyclic form parasites (Siegel et al. 2011). Additionally, elements of the 5’ UTR of alpha tubulin mRNA are shown to modulate trans-splicing (López-Estraño et al. 1998). Without the SL RNA and 5’ cap from trans-splicing, translation is not possible and trans-splicing could therefore serve as an additional means of regulation. One final avenue not yet mentioned is alternative trans-splicing. Instead of exon inclusion or exclusion as is typical of eukaryotic mRNA which undergo cis-splicing, the trans-splicing reactions necessary in processing trypanosome mRNAs have been shown to exhibit
alternative splice acceptor sites (Nilsson et al. 2010). Up to 40% of transcripts, or 2,500 genes, exhibit alternative splicing and many appear to be life form regulated (Nilsson et al. 2010). This is important in at least one instance where alternative splicing of isoleucyl-tRNA synthetase generates two isoforms; one maintained in the cytoplasm and one targeted to the mitochondrion (Rettig et al. 2012). Notably, the mechanisms regulating alternative splicing and the downstream implications of alternatively spliced mRNAs remain to be elucidated and will inevitably be of significant importance in trypanosome biology.

1.5 The eukaryotic ribosome

The ribosome is a highly specialized and complex ribonucleoprotein (RNP) responsible for protein synthesis in living cells. The most unique component of this RNP is RNA-based catalysis, a major discovery which led to a Nobel Prize in Chemistry in 1989. From genus to genus, each ribosome maintains a very similar overall structure with conserved function despite possessing slight differences in protein length and composition. All domains of life share a common ribosome core structure of 3 rRNA molecules and 34 proteins. The eukaryotic ribosome contains around 80 ribosomal proteins and 4 rRNAs which are assembled into a structure of over 3 megadaltons. Recent structural studies have provided great insights into the molecular mechanisms of ribosome function which would not have been possible otherwise. Due to the large size of the ribosome, high-resolution structures are not easily achieved but despite these limitations, two structures of the S. cerevisiae ribosome at 4.2Å and 3.0 Å have provided us a clear understanding of how proteins and RNA interact within these complexes as well as important information on
protein synthesis and its regulation (Ben-Shem et al. 2010, 2011). Slightly lower resolution cryo-electron microscopy (cryo EM) studies are capable of ~5.5Å resolution permitting individual proteins and rRNAs to be resolved and identified using density differences. Although lower resolution, cryo EM has the advantage of not requiring a pure crystal for diffraction and instead freezes a heterogeneous population of ribosomes. Using this technique ribosomes from a diverse set of organisms have been investigated and show subtle differences in structure and composition.

Each component of the ribosome is assembled in single copy with the exception of stalk proteins L7/L12 (P1/P2 in prokaryotes) which can range from one to six copies per molecule (Diaconu et al. 2005). Shared among all ribosomes is a common core of 15 small subunit proteins, 19 large subunit proteins nucleotides (Reviewed in Wilson & Cate, 2012). Within this core are the functional centers which include the peptidyl transferase center, mRNA decoding site and tRNA binding sites. The two subunits which compose all ribosomes (regardless of the domain of life) are the small subunit (SSU) and large subunit (LSU) originally defined by their relative sizes. Each subunit has a distinct sedimentation rate which is used to define the subunit size. In eukaryotes the small subunit has a 40S sedimentation coefficient and the large subunit 60S sedimentation coefficient. Together, they compose what we know as the 80S ribosome (Figure 1.10). After transcription of the pre-rRNA transcript by RNA Pol I, processing must divide it into functional RNAs. The 5’ most RNA is the 18S rRNA, the only RNA component of the small subunit. The large subunit rRNA is generally a very long 26S or 28S rRNA that is complemented by a 5S and 5.8S rRNA. A few organisms do not follow suit when considering LSU rRNA composition
including *Euglena gracilis*, *Drosophila melanogaster* and the kinetoplastid organisms including *Trypanosoma brucei*. These organisms possess a LSU architecture composed of anywhere from 2 to 14 fragmented 26S/28S rRNAs. The 5S rRNA is unique in that it is the only rRNA transcribed in the nucleus by RNA Pol III and transported to the nucleolus for assembly.

At the subunit interface of the mature 80S ribosome are a number of intersubunit bridges mediated by protein-protein or protein-RNA interactions between the small and large subunits. These bridges provide a means of communication as well as physical connection between two independent molecules. Even though the number of bridges and complexity of interactions between the two subunits can vary between organisms their function is thought to be conserved. In *Saccharomyces cerevisiae*, bridges possess a substantial number of RNA modifications and interact with translation elongation factors and tRNA molecules, suggesting the high presence of modifications could be important in bridge formation and translation efficiency. Studies on bridge function using prokaryotic ribosomes have shown small subunit RNA (16S) modifications are present in 4 bridges and are essential for subunit association (Bock et al. 2015). This may suggest ribosome stability is determined, in part, by the large network of intersubunit interactions.
Figure 1.10. X-ray structure of *Saccharomyces cerevisiae* 80S ribosome.

A view looking through the A, P and E sites of ribosome. Small subunit proteins are grey, small subunit rRNA is green, large subunit protein is red and large subunit rRNA is blue or yellow (5S rRNA). The central protuberance of 60S subunit and 40S subunit are labeled for orientation. The structure was viewed and image was generated using Chimera, UCSF (Ben-Shem et al. 2011).
1.5.1 *Trypanosoma brucei* ribosome

The ribosome of *Trypanosoma brucei*, and presumably all kinetoplastids, has a unique rRNA and protein composition. As previously described, the large ribosomal subunit typically contains a long 26/28S rRNA, however, in some insects, protozoans, and vertebrates this molecules is nicked into two or more pieces (Jordan 1975; Stevens and Pachler 1972). The composition of *T. brucei* large subunit rRNA is remarkable in that it is fragmented into 6 pieces (Cordingley and Turner 1980). The same fragmentation is seen in *Trypanosoma cruzi* based on a cryo-EM structure and genomic sequence analysis (Gao et al. 2005). Perhaps the most extreme case of rRNA fragmentation is seen in *Crithidia fasciculata* where the large subunit broken into 13 fragments (Gray 1981).

The *T. brucei* ribosome, although superimposable with other eukaryotic ribosomes, possess a kinetoplastid specific domain (KSD), large rRNA expansion segments and r-protein expansion segments which together generate 4 entirely new intersubunit bridges (B_{Tb}-1 through B_{Tb}-4) as inferred by electron density (Figure 1.11) (Hashem et al. 2013). One bridge, B_{Tb}-3 is formed between SSU protein ES12S and LSU rRNA srRNA4 and is thought to be the strongest among the 4 new bridges based on its number of contact points and distance apart. The other 3 bridges are between proteins conserved among other eukaryotes. All 9 of the expansion segments present on both 40S and 60S subunits are larger than any previously described eukaryotic ribosome with the largest expansion segments being situated near the LSU L1 protein stalk and at the platform of the SSU.
Figure 1.11. **Comparison of Saccharomyces cerevisiae and Trypanosoma brucei ribosomes.**

**A**) Superimposition of *T. brucei* cryo-EM ribosome structure (blue) with yeast X-ray structure (grey). View is looking from A site through P and E sites. Central protuberance and head of 60S and 40S subunits are labelled for reference. **B**) Side of 60S subunit superimposition. Small ribosomal RNAs of *T. brucei* are colored: srRNA 1, black; srRNA 2, fuscia; srRNA 3, green; srRNA 4, yellow, 5S rRNA, pink. The kinetoplastid domain is colored red. **C**) A view of 60S subunit superimposition from the top. All labeling is as in panel **B**. (Hashem et al. 2013; Ben-Shem et al. 2011)
Of the 6 rRNA fragments, srRNAs 2-4 are involved in formation of what is domain VI in other eukaryotic ribosomes and fills the void left because of shortness in LSU-β rRNA. They each interact directly with the KSD and are held in place through extensions of proteins L14e and L31e.

1.5.2 Ribosome biogenesis

The assembly of individual ribosomal RNAs and protein into a functional ribosome is a complex and sequential process that depends on many factors. In yeast, to assemble 4 RNAs and 79 proteins there are over 200 assembly factors and 76 small nucleolar RNAs (snoRNAs) known (WooIford and Baserga 2013). Given the additional complexity present in the *T. brucei* ribosome this number is likely to be expanded to facilitate the processing and assembly of 5 additional rRNAs. We cannot discuss ribosome biogenesis without discussing the nucleolus, a non-membrane-bound sub-compartment of the nucleus where rRNA is transcribed and ribosomes begin to assemble. Ribosomal DNA is typically found in long tandem arrays of identical sequences upwards of 200 copies per genome and gives rise to pre-rRNA upon transcription. Extensive processing of transcribed pre-rRNA is required to generate mature rRNAs to assemble into the ribosome. The cleavage process of pre-rRNA was described in section 1.4.2 and will therefore be excluded from this section. Concurrent with processing occur chemical modifications of rRNA. Most of these modifications are 2’-O-ribose methylation or pseudouridylation and are catalyzed by small nucleolar ribonucleoproteins (snoRNPs), a complex of snoRNA and protein. In each snoRNP the RNA component is necessary to guide modification location by base pairing.
with the rRNA while proteins are required for enzymatic modification with each snoRNP guiding two modifications at distinct sequence sites. The complete modification map of *S. cerevisiae* rRNA has been elucidated and possesses 44 pseudouridines (catalyzed by H/ACA snoRNPs) and 67 2’-O-ribose methylations (catalyzed by box C/D snoRNPs). A small subset of these pseudouridine and 2’-O-ribose modifications are not guided by snoRNAs and are catalyzed by enzymes (Decatur and Schnare 2008; Lowe and Eddy 1999). Most of these modifications are present within important functional locations of the ribosome such as intersubunit bridges, ribosome decoding center or tRNA binding pockets and are therefore necessary for optimal ribosome structure and function (Decatur and Fournier 2002; Liang et al. 2007, 2009; Jack et al. 2011; Baxter-Roshek et al. 2007). In addition the snoRNP catalyzed modifications, snoRNP independent base modifications of rRNA are present in rRNA, although with lower abundance. There are 23 RNA base modifications known in rRNA from all domains of life (Table 1-1). Ten of these known base modifications are found in both humans and yeast and are believed to fine tune RNA-Protein or RNA-RNA interactions as well as structure during biogenesis and translation (Baxter-Roshek et al. 2007). One base modification, m^1^acp^3^ψ, is located within helix 31 in the decoding center of the ribosome at position 1191 of the 18S rRNA where it interacts with the P-site tRNA (Baudin-baillieu et al. 2009). The enzyme responsible for modification of m^1^acp^3^ψ is known along with that of three other 18S modifications m^7^G at 1575 (Bud23) and m^6^A at 1781 and m^6^A at 1782 (Dim1). The remaining 7 modifications (m^1^A at 645, m^2^U at 56, m^1^A at 2142, m^5^C at 2278, m^3^U at 2634, m^3^U at 2843, m^5^U at 2924) are on the 25S rRNA and have no enzyme attributed to their formation nor has the
## Table 1.1. RNA modifications found in eukaryotic ribosomal RNA

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Common name</th>
<th>Subunit presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>m^1A</td>
<td>1-methyladenosine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^6A</td>
<td>N^6-methyladenosine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>Am</td>
<td>2'-O-methyladenosine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^6_2A</td>
<td>N^6,N^6'-dimethyladenosine</td>
<td>SSU</td>
</tr>
<tr>
<td>m^3C</td>
<td>3-methylcytidine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^2C</td>
<td>5-methylcytidine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>Cm</td>
<td>2'-O-methylcytidine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>ac^4C</td>
<td>N^4-acetylcytidine</td>
<td>SSU</td>
</tr>
<tr>
<td>m^3C</td>
<td>N^4-methylcytidine</td>
<td>SSU</td>
</tr>
<tr>
<td>hm^2C</td>
<td>5-hydroxymethylcytidine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^1G</td>
<td>1-methylguanosine</td>
<td>SSU</td>
</tr>
<tr>
<td>m^2G</td>
<td>N^2-methylguanosine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^2G</td>
<td>7-methylguanosine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>Gm</td>
<td>2'-O-methylguanosine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^2_2G</td>
<td>N^2,N^2'-dimethylguanosine</td>
<td>SSU</td>
</tr>
<tr>
<td>Im</td>
<td>2'-O-methylinosine</td>
<td>Not assigned*</td>
</tr>
<tr>
<td>Ψ</td>
<td>Pseudouridine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>Um</td>
<td>2'-O-methyluridine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^3Ψ</td>
<td>1-methylpseudouridine</td>
<td>SSU</td>
</tr>
<tr>
<td>Ψm</td>
<td>2'-O-methylpseudouridine</td>
<td>SSU</td>
</tr>
<tr>
<td>m^3U</td>
<td>3-methyluridine</td>
<td>SSU, LSU</td>
</tr>
<tr>
<td>m^1acp^3Ψ</td>
<td>1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine</td>
<td>SSU</td>
</tr>
<tr>
<td>cm^5U</td>
<td>5-carboxymethyluridine</td>
<td>Not assigned*</td>
</tr>
</tbody>
</table>

* Identification did not separate the LSU from SSU. Therefore the presence of each modification cannot be definitively assigned to either the SSU or LSU.
importance of the modification been investigated. It is interesting to note that the majority of base modifications are within single-stranded loops and could therefore be important in loop stabilization or for facilitating stable tertiary interactions.

As rRNA processing progresses through transcription into the 35S precursor and finally into mature products, ribonucleoprotein complexes are formed around the RNA to presumably stabilize RNA structures in productive forms. In this way, binding of ribosomal proteins (r-proteins) and assembly factors may stabilize local folding in turn creating binding sites for distal RNAs or proteins allowing parallel folding events to join each other in building a useful final structure (Ramaswamy and Woodson 2009). Association of r-proteins with immature rRNAs follows a hierarchical order generating a 43S pre-RNP with small subunit 20S pre-rRNA and a 66S pre-RNP with the 27S pre-rRNA following initial cleavage events. Ever advancing technology allowing us to capture intermediary complexes and study single molecules has allowed us to understand the assembly and shows there are primary r-proteins which join the RNA very early in assembly. Following the primary r-protein association, secondary and tertiary r-proteins associate with pre-ribosomes, strengthening the complex as each group of proteins is added (Ferreira-Cerca et al. 2007; Woodson 2008; Sykes and Williamson 2009). Not all assembly occurs within the nucleolus, or even the nucleus. Export of pre-ribosomes occurs from the nucleus into the cytoplasm where the final proteins assemble and generate a functional ribosome (Strunk et al. 2011; Ferreira-Cerca et al. 2007; Saveanu et al. 2003; Ferreira-Cerca et al. 2005; Lo et al. 2010). In the case of yeast, as assembly progresses, there are over 200 assembly factors found among 43S or 66S pre-RNPs which assist in ribosome formation
(Reviewed in Woolford & Baserga, 2013). Many of the factors are conserved amongst eukaryotes and can be purified along with assembly intermediates. These factors are not all simply chaperones which bind to stabilize structures and then depart to act again, but rather are enzymes with specific duties during assembly. Such enzymes can include endonucleases and exonucleases for pre-rRNA maturation, rRNA and protein modifying enzymes, helicases, ATP- and GTPases, kinases and phosphatases. The mechanism for how these assembly factors are finally released from the ribosome is not well understood.

In rapidly dividing cells, a pre-ribosome is believed to be actively exported from the nucleus every 2 to 3 seconds (Warner 1999). The demand for ribosomes is therefore a large factor in what limits growth rate. In order to control the release of ribosome into the cytoplasm, the nucleus must check each particle’s assembly to avoid releasing incompletely assembled or improperly assembled subunits. The most important players in the export and surveillance pathways are: Nmd3, Mex67-Mtr2 complex, and Arx1. Nmd3 binds at the subunit interface, Arx1 binds at the peptide exit tunnel, and Mex67-Mtr2 the 5S rRNA at the central protuberance; together these proteins ensure the 60S subunit is structurally ready for export (Sengupta et al. 2010; Greber et al. 2012; Yao et al. 2007, 2008; Bassler et al. 2012; Bradatsch et al. 2012).

Once in the cytoplasm, subunits must complete final steps of maturation. These include final pre-rRNA processing, r-protein assembly, and assembly factor release. They must also not engage in translation until assembly is complete and this is accomplished by shielding the pre-40S subunit interface from association with initiation factors, tRNA and 60S subunits with 7 assembly factors (Strunk et al. 2011). Likewise, pre-60S subunits are
also blocked by Nmd3 and Tif6 proteins exported in complex from the nucleus (Valenzuela et al. 1982; Gartmann et al. 2010; Sengupta et al. 2010). It is thought that final steps in maturation include a “functional proofreading” step involving r-protein L10 sending the environment of peptidyl transferase center whereby Tif6 is release and the ribosome is ready for translation (Bussiere et al. 2012).

Finally, as in all intricate pathways, mistakes will occur and ultimately need to be observed, sequestered and resolved to avoid undesired side effects. A single missense amino acid incorporation in r-proteins or missense nucleotide incorporation in rRNA can lead to ribosomes with altered structure and decreased translational fidelity (Dinman 2009). Two independent complexes work together and are central in ribosome quality control: the TRAMP complex and the exosome. The exosome was originally thought to function in productive pre-rRNA processing but was later found to also take part in turnover of aberrant processing intermediates (Dez and Tollervey 2004; Allmang et al. 2000; Wery et al. 2009). Aberrant intermediates are detected by the TRAMP complex which includes an RNA binding protein, RNA helicase and a poly-(A) polymerase. The complex adds a short poly-A tail the 3’end of aberrant processing intermediates which targets it for destruction by the exosome (LaCava et al. 2005; Dez et al. 2006; Houseley et al. 2006). In T. brucei a stable TRAMP complex is believed to exist in addition to several additional poly(A) polymerases and terminal uridyl transferases (TUTases) (Etheridge et al. 2009).
1.5.3 Ribosome in translation

Translation occurs in three independent phases: initiation, elongation and termination. To initiate translation a pre-initiation complex (PIC) is formed by the association of many eukaryotic initiation factors (eIFs), RNA binding proteins, an initiator tRNA\textsuperscript{Met} and an mRNA to be translated. The complex undergoes ATP-dependent scanning to locate the AUG codon for initiation and transition into the elongation phase. Once an AUG codon is located, dissociation of eIFs is coupled with the association of eIF5B-GTP, an essential component of 60S recruitment. Upon hydrolysis of GTP, eIF5B can be released allowing the 60S subunit to join and elongation can begin (Hinnebusch 2014). The incorporation of one amino acid during each successive round of elongation relies upon the association of an aminoacyl tRNA in the A site delivered by eEF1A-GTP. GTP is then hydrolyzed and eEF1A is released, permitting peptidyl bond formation by the ribosome between the amino acids of the P and A site tRNAs. In order to translocate tRNAs between sites of the ribosome, a number of steps must occur. First eEF2-GTP must associate and undergo hydrolysis into eEF2-GDP. Subunits are then able to ratchet relative to one another moving tRNAs from the P to E and A to P sites, thus freeing the A site for the next round of elongation (Schneider-Poetsch et al. 2010). After successive rounds of elongation, the ribosome will encounter a stop codon (UAA, UAG, UGA) signaling the end of translation. The nascent peptide at this point remains attached to the tRNA of the P site is not able to let itself free without assistance from GTP-dependent ribosome release factors (eRFs). Release factors are tRNA shaped proteins which can recognize distinct codons and promote peptide hydrolysis from the P site tRNA. One final step required before initiation can be
re-initiated is ribosome recycling. After nascent chain release the 80S ribosome is still bound to mRNA with deacylated tRNA in the P site. Eukaryotes lack homologs of the extensively characterized prokaryotic ribosome recycling factor (RRF) the mechanism for release and recycling in the eukaryotic system has not been elucidated further (Dever and Green 2012).

1.6 RNA modification and editing

All RNAs are potential substrates to undergo post-transcriptional modifications, extending their chemical activity and imparting regulatory or auxiliary functions. With respect to size, tRNAs are the most extensively modified RNA of a cell, receiving an average of 12.6 modifications per 76 nucleotide molecule (Phizicky and Hopper 2010). The next most heavily modified are ribosomal RNAs which, in S. cerevisiae, receives 3 or 7 modifications to small or large subunit rRNA, respectively (Reviewed in Woolford & Baserga, 2013). There are at least 109 characterized chemical modifications to RNAs and additional modifications have been observed, but not completely characterized. Of these, 93 can be found in tRNAs across all species while 31 can be found in rRNA (Cantara et al. 2011). Both tRNA and rRNA require many of their modifications for proper structure and function. Other RNAs which are known to be modified include mRNA, snRNA, snoRNA, and tmRNA. Advances in high-throughput sequencing have recently revealed numerous RNA modifications which have implications in human diseases, regulation of gene expression and cell to cell signaling (Gu et al. 2014; Guy et al. 2015; Blanco et al. 2014; Yasukawa et al. 2001).
1.6.1 *Transfer RNA modifications*

Transfer RNA harbors the most concentrated and diverse set of modification compared to any other RNA molecule within a cell. Modifications on a tRNA within the anticodon stem are generally thought to be important in processes associated with translation including cognate tRNA selection and reading frame maintenance (Gustilo et al. 2008). On the other hand, modifications to the acceptor stem, TψC loop or D loop of a tRNA are regarded as structural modifications which can enable high variations in sequence composition among the whole complement of tRNAs to fold into a conserved L shape tRNA (Phizicky and Alfonzo 2010). The exact significance of each modified position on every tRNA is not yet clear and only select modifications are essential. Historically, tRNA modifications were difficult to study because they required isolation and purification of single tRNAs from total cellular RNA. Furthermore, a molecule of 76 nucleotides is likely to possess only one of the modifications of interest making detection of a low abundance nucleotide more difficult. The development of gene manipulation techniques coupled with more sensitive and robust detection methods has aided in the characterization of modifications, but there is still quite a ways to go. The most common means of analyzing modifications on any RNA today is through HPLC coupled with mass spectrometry (Su et al. 2014). The benefits of this method outweigh previous radioactivity or strictly mass spectrometry based investigations by saving time and labor while allowing for analysis of multiple modifications and providing information about nucleotide sequence with less sample input (Zhao 2004).
The modification set of a given tRNA in some cases determines whether the tRNA is destined for import into the mitochondrion or whether it stays in the cytoplasm. *T. brucei* is an extreme case where every tRNA used in mitochondrial translation must be imported from the cytoplasm providing an excellent system to study impact of modifications on tRNA partitioning within a cell. Our lab has studied import of tRNA in multiple contexts including thiolation of tRNA\textsubscript{Gln} and tRNA\textsubscript{Tyr} and G\textsubscript{37} methylation of several tRNAs at the N1 position (Paris et al. 2013; Wohlgamuth-Benedum et al. 2009; Rubio et al. 2008). Along these lines, modification events are known to be catalyzed in specific cellular compartments, sometimes requiring other modifications as pre-requisites for optimal enzyme activity. One tRNA modification, pseudouridine at position 35 of tRNA\textsuperscript{Tyr} anticodon requires the intron to be present for formation (Johnson and Abelson 1983). Surprisingly, in our studies of tRNA\textsuperscript{Tyr} of *T. brucei*, we observed no pseudouridine present in intron containing tRNA, suggesting the intron is not likely required for formation and adding complexity to the regulation of modification enzyme activity (Rubio et al. 2014).

1.6.2 *Ribosomal RNA modifications*

Modifications to ribosomal RNA are catalyzed co-transcriptionally and are heavily concentrated within functional regions of the ribosome, being fundamental in final ribosome function (Baudin-baillieu et al. 2009; Kos and Tollervey 2010). The majority of eukaryotic cytoplasmic rRNA modifications are either 2’-O-methylation of the ribose or pseudouridine (ψ), both of which are formed by small nucleolar RNA (snoRNA) complexed with protein forming ribonucleoprotein complexes (snoRNPs) (Watkins and
Bohnsack 2012). A select number of snoRNAs are targeted to ITS regions and are important in rRNA folding and processing reactions (Baudin-baillieu et al. 2009). There are two families of snoRNPs, differentiated by their guiding mechanisms important in directing modification. In both families, RNA plays a guiding role with protein being the catalytic factor. In this way, many modifications can be catalyzed by the same protein core and altered snoRNA molecules whereby genome complexity can be reduced. In archael systems, snoRNP homologs are known to exhibit activity on both rRNA and tRNA, showing a conserved mechanism for catalysis. Modifications to rRNA have been shown to play roles in maintaining translation fidelity and in at least one surprising case, the lack of a modification leads to increased fidelity (Baudin-baillieu et al. 2009).

Of the 10 base modifications identified in rRNA from *S. cerevisiae*, 7 are specific to the large subunit and 3 specific to the small subunit (Woolford and Baserga 2013). In contrast to the base modifications, ribose methylations and pseudouridylations are far more abundant and many of these modifications are localized to functional centers of the ribosome including peptidyl transferase center, decoding center and two intersubunit bridges involving Helix 69 and A-site finger (Baudin-baillieu et al. 2009; Liang et al. 2009; Baxter-Roshek et al. 2007). Using yeast genetics, each snoRNA directing a modification was individually deleted and shows minimal to no effects on growth, but combinations of deletion in functional regions show substantial growth defects which range from changes in growth rate to decreased ribosome production to reduced translation efficiency (King et al. 2003; Badis et al. 2003; Piekna-Przybylska et al. 2008; Liang et al. 2009). Together this suggests modifications are advantageous, and in some cases essential, for appropriate
processing of rRNA as well as faster and/or more accurate translation leading to faster growth rates without sacrificing fidelity.

1.6.3 RNA editing

In contrast to RNA modifications, RNA editing events are chemical reactions which alter the coding information of RNA at the sequence level when compared to the originating genomic DNA. There are two well characterized types of RNA editing: nucleotide substitution and insertion/deletion editing (Gray 2003). The most common and widely observed type of editing is nucleotide substitution editing, a reaction which proceeds through hydrolytic deamination of an amine containing nucleobase converting it into a new nucleotide (Carter 1995). Deamination editing is targeted to either adenosine forming inosine or cytosine forming uridine and has been characterized in the context of both mRNA and tRNA. The second type of editing is insertion/deletion editing first described in Trypanosoma brucei and Crithidia fasciculata when nucleotides were found in RNA that were not present in the mitochondrial DNA sequence (Benne et al. 1986). Insertion/deletion editing is essential for mitochondrial mRNAs of both organisms to generate the correct reading frame of translation and the exact number of U residues must be inserted or deleted at the correct position for a functional protein.

Other examples of RNA editing have been identified but are confined to small families of organisms. Similar to the trypanosome U insertion/deletion discovery, the slime mold Physarum polycephalum was found to insert cytidines at 54 locations in its mRNA to enable translation of functional proteins (Mahendran, Spottswood, Miller 1991).
Physarum was later found to possess more editing of its mitochondrial rRNA and tRNA using unconventional editing which inserts single C and U nucleotides and AG dinucleotides (Miller et al. 1993). In addition to nucleotide insertion to tRNAs in the Physarum mitochondria, tRNA\textsuperscript{Met} requires a G addition at its 5’ end which is not encoded by DNA (Gott et al. 2010). This discovery is similar to two other tRNA editing reactions present in Acanthamoeba castellanii and Saccharomyces cerevisiae. In A. castellanii 16 of 19 mitochondrial tRNAs undergo editing to restore mismatches in the acceptor stem of the tRNA for proper function (Lonergan and Gray 1993). In S. cerevisiae a G residue is added to the 5’ end of tRNA\textsuperscript{His} generating a G-1 nucleotide by a 3’ to 5’ polymerase, tRNA\textsuperscript{His} guanyltransferase (Thg1) (Gu et al. 2003). Recently, our laboratory has discovered a new editing event where non-canonical changes of G to A or A to T were consistently identified in the tRNA\textsuperscript{Tyr} intron of Trypanosoma brucei (Rubio et al. 2013). The exact mechanism of editing has yet to be elucidated, but the reaction is essential for splicing and edited positions are conserved throughout other kinetoplastid organisms.

1.6.3.1 Nucleotide substitution editing

As mentioned to in the beginning of this section, nucleotide substitution editing occurs through an enzymatic deamination reaction. On mRNAs, editing is carried out by the APOBEC, ADAR1 or ADAR2 family of enzymes. On tRNAs, editing of adenosine in the anticodon is catalyzed by the enzymes known Adenosine Deaminase Acting on tRNAs (ADATs). Regardless of the enzyme, each deamination reaction shares a common mechanism of catalysis utilizing the same catalytic active site amino acids, but differ in
overall protein architecture (Carter 1995). The catalytic residues necessary for catalysis are two cysteine and one histidine which coordinate Zn\(^{2+}\) as well as a glutamate to shuttle protons during nucleophilic attack by water (Carter 1995). The metal cofactor Zn\(^{2+}\) is required for catalysis to activate a water molecule for nucleophilic attack of the C6 amine of the purine ring and releases ammonia as inosine or cytosine is formed (Figure 1.12) (Betts et al. 1994; Carter 1995).

Editing of mRNA has been extensively studied in the context of apolipoprotein B (ApoB) mRNA which undergoes editing by apolipoprotein B editing complex 1 (APOBEC-1) (Tennyson et al. 1989b). APOBEC-1 deaminates cytosine 6666 to uridine and converts the CAA glutamine codon into a UAA stop codon generating a truncated protein product (Tennyson et al. 1989a). The ratio of full-length (Apo-B100) to shortened (Apo-B48) protein is important in lipid homeostasis in the small intestines of humans (Scott et al. 1989). It is believed that the main function of APOBEC-1 editing is for apoB, but transcriptome wide studies have shown at least 32 additional mRNA sequences undergo C to U editing by APOBEC-1 and are concentrated in 3’ UTRs (Rosenberg et al. 2011). A similar C to U editing process is that of AID, an activation induced deaminase important in immunoglobulin diversification (Muramatsu et al. 2000).

Another type of deamination editing which is also targeted to mRNA is intended to diversity proteins using a single transcript. This editing is carried out by adenosine deaminases acting on RNA, or ADARs (Paul and Bass 1998). Two of the ADARs, ADAR1 and ADAR2 are homodimeric enzymes specific for double stranded mRNA (Wong et al. 2001). Both ADARs deaminate adenosine to inosine and change the coding properties of
Figure 1.12. Mechanism for hydrolytic deamination of nucleosides.

A) A schematic showing the hydrolytic deamination of adenosine (left) to inosine (right). In the presence of water a deaminase with conserved histidine, cysteine and cysteine coordinates zinc. Water is activated for nucleophilic attack of N-C bond at C6. Glutamate acts as proton shuttle from water to the leaving group of ammonia (Carter 1995). B) A schematic for the hydrolytic deamination of cytidine (left) to uridine (right). The reaction mechanism is the same as in A except that N-C bond is broken at C4.
their target mRNAs, the glutamate receptor (GluR) in the brain and hepatitis delta virus (HDV) in the liver. ADAR activity is most concentrated to the brain where it is believed that 1 in 17,000 mRNA bases are converted to inosine (Paul and Bass 1998). Furthermore, almost 100% of a single CAG glutamine codon in three subunits of the glutamate receptor are changed to a CIG codon which changes its identity to arginine. Hypo-modified GluR is associated with seizures in mice, connecting the editing of glutamine to arginine to proper brain function (Brusa et al. 1995).

Our laboratory is most interested in editing in the context of tRNAs. The anticodon of A\textsubscript{34} containing tRNAs is deaminated to inosine in all domains of life. Anticodon deamination is important for expanding the decoding capacity of a tRNA, enabling one tRNA to decode up to three distinct codons and reducing the set of tRNAs that need to be genetically encoded. This decoding is referred to as “wobble pairing” where inosine pairs like a guanosine and can form non-standard base pairs with A, T and C. In eukaryotes, A\textsubscript{34} to I\textsubscript{34} is catalyzed by the heterodimeric enzyme ADAT2/3 (Gerber 1999). Initially described in the yeast Saccharomyces cerevisiae, ADAT2/3 is essential and conserved through Eukarya. Unexpectedly, its domain structure resembles that of a cytidine deaminase rather than an adenosine deaminase suggesting it may have evolved from an ancestral cytidine deaminase. In T. brucei ADAT2/3 deaminates 8 tRNAs containing A\textsubscript{34} and has additional deamination activity not described in other ADAT2/3 enzymes. In addition to A to I editing, T. brucei ADAT2/3 is capable of C to U deamination of single stranded DNA (ssDNA) \textit{in vitro} and C\textsubscript{32} to U\textsubscript{32} of select tRNAs \textit{in vivo} (Gaston et al. 2007; Rubio et al. 2007). Interestingly, some tRNAs undergo both C to U and A to I editing but
activity is separated between cytoplasm and nucleus within the cell. This situation is further complicated since our lab has found C\textsubscript{32} and U\textsubscript{32} can both be found in methylated states at differing levels depending on the tRNA species. The complexity of the editing and modification to these tRNAs has provided an exciting problem to study.

*T. brucei* and other kinetoplastids have a unique editing event restricted to tRNA\textsuperscript{Trp} of the mitochondrion. C to U editing of the anticodon is essential for decoding of UGA codons as tryptophan during mitochondrial translation (Alfonzo et al. 1999). The editing reaction is not comprehensive as subset of tRNA\textsuperscript{Trp} remains with a CCA anticodon while the remainder is UCA with each version requiring specific tryptophanyl-tRNA synthetases (Horn and So 2006). This mitochondrial editing is accompanied by mitochondrial specific modifications. One modification occurs at U\textsubscript{33} as s\textsuperscript{2}U\textsubscript{33} (Crain et al. 2002). The absence of this modification stimulates C to U editing, perhaps due to structural differences which favor C to U activity (Wohlgamuth-Benedum et al. 2009; Crain et al. 2002). A genetic screen designed to identify the enzyme responsible for C to U editing will be discussed in Chapter 4.

1.6.4 Importance of modification and editing in cellular processes and disease

RNA modification and editing events have direct implications in human health and disease. An APOBEC complex not discussed previously in this section, APOBEC3G, is important in protection against HIV in vertebrates (Harris 2008). APOBEC3G activity relies upon a small molecule, RN-18, along with an HIV factor, Vif, for APOBEC3G to have optimal mutagenesis activity towards HIV genome and promote host survival.
The importance of other deaminases has been discussed earlier in this section, where editing can provide a means of lipid homeostasis in the small intestine or control ion flow through the glutamate receptor in the brain.

A few known diseases are the result of genomic mutations which remove the target residue for modification and therefore affect tRNA stability or decrease decoding capacity. It is not clear whether the nucleotide change or lack of modification is the cause for disease however. A very fascinating and relatively recently investigated aspect of RNA modifications is the relationship between tRNA modifications and cellular stress. Lack of a the m$^5$C methylation catalyzed by Nsun2 protein has been linked with stress induced tRNA cleavage into 5’ and 3’ halves (Blanco et al. 2014). There are a number of neurodevelopmental disorders which result from genetic mutation of the Nsun2 protein and are believed to be the result of tRNA stress induced cleavage. In yeast, tRNA$^{\text{Lys}}_{\text{UUU}}$ requires the anticodon modification 5-methoxycarbonylmethyl-2-thiouridine (mcm$^5$s$^2$U) in the cytoplasm for proper mitochondrial function (Tigano et al. 2015). Stress has recently been shown to reprogram tRNA modification sets and tune translation, mainly through arginine and leucine codons (Gu et al. 2014). As we continue to understand the relationships between tRNA modifications and editing events with respect to changes in cellular stimuli we will be better suited in treating diseases resulting from disrupted regulation during stress or from genetic mutation.
Chapter 2: A 3-methylcytidine methyltransferase important for ribosome stability in *Trypanosoma brucei*¹

2.1 Introduction

The work in this chapter begins to define the cellular function of a 3-methylcytidine methyltransferase paralog in *T. brucei*. Unpublished work from our lab has identified the methyltransferase (Trm140) responsible for m^3^C formation at position 32 of three tRNAs (serine, arginine and threonine). This enzyme is homologous to the yeast m^3^C methyltransferase but is unique in that it requires the tRNA editing deaminase ADAT2/3 for activity. Furthermore, we have observed both editing (C to U) and methylation at position 32 of mature tRNAs resulting in C, U, m^3^C and m^3^U on these tRNAs. The function of the second 3-methylcytidine methyltransferase, an enzyme we have named MTase37, was the main focus of my research. In this chapter I show it is a nucleolar enzyme and appears essential for cell proliferation. Insights into its function were achieved mainly using an RNA interference (RNAi) cell line. I show that in addition to cell proliferation, MTase37 is important in maintaining ribosome stability. More specifically MTase37 affects the large ribosomal subunit which possessed a unique composition of fragmented rRNA. One of these rRNA fragments, srRNA 4, exhibits decreased levels following RNAi.

¹ The majority of data presented in this chapter was submitted for publication and is in review as of submission of this document. The citation is as follows: Fleming, I.M., Paris, Z., Gaston K.W., Balakrishnan, R., Fredrick, K., Rubio, M.T., Alfonzo, J.D. A tRNA methyltransferase paralog is important for ribosome stability and cell division in *Trypanosoma brucei*. In review.
Although we have been unable to identify an exact methylation target present within ribosomal RNA, we believe MTase37’s conserved AdoMet/S-adenosylmethionine binding domain is an essential component of this protein.

2.2 Results

2.2.1 *Trypanosoma brucei* possesses two discrete 3-methylcytidine methyltransferases

In an effort to identify the enzyme responsible for formation of 3-methylcytidine at C\(_{32}\) of tRNAs threonine, serine and arginine we utilized BLASTP with the C\(_{32}\) methyltransferase of *Saccharomyces cerevisiae* (GenBank: NP_014882.4) as the query sequence. Present in the annotated proteins of *T. brucei* are two putative proteins identified here using their TriTrypDB identifiers: Tb927.9.11750 and Tb927.10.1800. Through biochemical analyses, some of which will be presented in this chapter, we identified Tb927.10.1800 as the tRNA C\(_{32}\) methylase. Following the published nomenclature of the homologous enzyme in yeast we have named it TbTrm140 (Silva et al. 2011; Noma et al. 2011). A multiple sequence alignment of both *T. brucei* homologs with Trm140 of yeast shows that all key catalytic residues important for methylation in yeast are conserved in both *T. brucei* homologs (Figure 2.1). Each sequence also possesses a conserved AdoMet methyltransferase domain responsible for binding the methyl donor S-adenosylmethionine. Consequently we have named Tb927.9.11750 MTase37 as it is predicted to be 37kDa and possess all predicted residues necessary for methylation. The only major difference between both *T. brucei* homologs and Trm140 of yeast is the absence of the N-terminal actin-binding domain. However, this domain is dispensable for methylation activity in yeast.
The same BLASTP query using Trm140 of *S. cerevisiae* yields only a single high scoring hit (excluding enzyme isoforms of higher eukaryotes) for all eukaryotes not belonging to the Kinetoplastida group. An alignment of these sequences was generated using the MUSCLE algorithm and an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree was generated (Figure 2.2). Also included in the alignment were methyltransferase like protein 6 (METTL6) homologs from humans, mice and wild hogs as well as two well characterized proteins that belong to the RNA methyltransferase family, but are unrelated to m^3^C formation (Trm5 of *T. brucei* and Nop2 of *S. cerevisiae*) which served as outgroups. *T. brucei* TRM5, is a *bona fide* 1-methylguanosine tRNA methyltransferase for G^37^ of seven tRNAs and *S. cerevisiae* Nop2 is a nucleolar methyltransferase that catalyzes m^5^C at C^2870^ of 25S rRNA and is important in ribosomal RNA processing (Paris et al. 2013; Hong et al. 1997; Sharma et al. 2013). METTL6 proteins have no identified function but also possess a conserved methyltransferase domain and have been reported as drivers in cell proliferation in a subclass of breast cancers, linking methylation activity to carcinoogenesis (Gatza et al. 2014). 

Our phylogenetic tree reveals a polyphyletic relationship between m^3^C methyltransferases from numerous organisms and also suggests that a gene-duplication event gave rise to TbTrm140 and TbMTase37. 

In order to further explore the importance of both TbMTase37 and TbTrm140, we placed a portion of each coding sequence in the tetracycline inducible RNAi vector p2T7-177 and monitored growth of cells (Wickstead et al. 2002). Upon tetracycline addition to turn on RNAi, a pronounced slow-growth phenotype was evident for both proteins 6 days.
Figure 2.1. **Sequence alignment of 3-methylcytidine methyltransferases.**

A multiple sequence alignment of two *T. brucei* paralogs (TbTrm140 and TbMTase37) of Trm140 from *Saccharomyces cerevisiae* (ScTrm140). Black highlighting (*) indicates identical amino acids shared among the sequences, while amino acids highlighted in gray (:) or (.) denote conservative changes. The putative SAM-binding domain is indicated by the black filled rectangle labeled “AdoMet Binding Domain” and it is conserved in all three proteins.
Figure 2.2. Two paralogous proteins in *T. brucei* belong to a family of methyltransferases that includes mammalian METTL6.

A UPGMA tree was generated following multiple sequence alignment of *S. cerevisiae* Trm140 (NP_014882.4) with orthologous sequences from other organisms using MEGA6. Multiple sequence alignment was accomplished with the MUSCLE algorithm. The UPGMA tree was generated from the alignment with default parameters and 10,000 bootstrap replicates. Bootstrap values are displayed as percentages at each tree node.
post-induction compared to wild type and uninduced cells (Figure 2.3). To corroborate the down-regulation of each protein, protein lysates were prepared from cultures 6 days post-induction and analyzed by western blotting using either TbMTase37- or TbTrm140-specific antibody raised against a recombinant version of the proteins purified from *E. coli*. This experiment confirmed the reduction of protein levels compared to the uninduced control (Figure 2.3, bottom).

To confirm the effects of RNAi on C\textsubscript{32} methylation specifically on tRNA we prepared RNA from each RNAi knock-down and performed primer extension using a radioactive primer specific for tRNA\textsuperscript{Thr\textsubscript{AGU}} (Figure 2.4). The amount of read-through relative to reverse transcriptase stop at position 32 (8 nucleotides past the oligo) was calculated for each using a boxed density approach where percent read-through equals read-through density divided by read-through plus stop densities. This experiment provided the first evidence that MTase37 was not responsible for m\textsuperscript{3}C\textsubscript{32} and that TbTrm140 was the C\textsubscript{32} modifying enzyme *in vivo*.

2.2.2 Nucleolar localization of MTase37

To begin defining the role of MTase37 in the cell we used cell fractionation followed by western blot analysis, in addition to immunofluorescence microscopy, to determine the intracellular localization of this protein. The same experiments were also performed for Trm140 and used as a comparison. Western blot analysis of *T. brucei* sub-cellular fractions (total, nuclear and cytoplasmic) showed that both MTase37 and Trm140 are present in the nuclear fraction. In these experiments, antibodies against enolase and Nog1 were used as
Figure 2.3. RNAi generates a growth phenotype for both methyltransferase homologs.

**A)** Growth curve of wild type, uninduced (Tet -) and induced (Tet +) cell lines. Both Tet – and Tet + cell lines are *T. brucei* 29-13 cells transformed with the tetracycline-inducible dual T7 plasmid p2T7-177/TbMTase37 or p2T7-177/TbTrm140 integrated into genomic 177 repeat regions. RNAi was induced by addition of tetracycline to growth media and monitored every 24h. Six days post-induction (indicated by *) total protein extracts of each cell line were prepared. **B)** Western blots were performed using purified rabbit polyclonal antibodies against TbMTase37 and TbTrm140. Protein extracts from equal number of cells of wild type, Tet -, and Tet + were loaded per lane. The inset shows a representative western blot where “α-TbMTase37” and “α-Isd11” or “α-TbTrm140” and “α-enolase” refer to separate blots of the same membrane performed using antibodies against each protein. Isd11 and enolase proteins were used as a loading controls for each set of experiments.
Figure 2.4. *TbTrm140* affects position 32 methylation of tRNA<sup>Thr</sup><sub>AGU</sub>.

RNA from RNAi growth curve experiments (Figure 2.3) was prepared from wild type (WT), uninduced (-) and induced (+) cells. A radiolabeled primer specific for tRNA<sup>Thr</sup><sub>AGU</sub> was used for primer extension analysis. The specific stop signal produced during reverse transcription was quantitated in relation to the read-through signal for each lane. Percent read-through was calculated as read-through signal divided by read-through plus stop signals.
cytoplasmic and nuclear markers, respectively, and confirmed the purity of the nuclear fraction (Figure 2.5) (Jensen et al. 2003). The gene encoding MTase37 was cloned into the T. brucei tetracycline inducible protein expression vector pLEW79, which places three epitope-tags (Myc, His and Protein A) at the C-terminus of the protein. The resulting construct, pLew79-MTase37-MHA, was transfected into T. brucei 29-13 cells, and protein production was induced by addition of tetracycline. Immunofluorescence experiments with anti-His antibodies showed that MTase37 was restricted only to the central portion of the nucleus, suggesting nucleolar localization (Figure 2.5A, bottom). Including an antibody against a marker nucleolar protein NOG1 shows co-localization of the two proteins, again confirming the nucleolar localization of MTase37 (Jensen et al. 2003). Ideally, we would have performed the co-localization experiments with the anti-MTase37 antibody used in the western blot experiment; however, the fact that both anti-TbMTase37 and anti-Nog1 are rabbit antibodies precluded such analysis. To rule out the possibility that the observed fluorescence with anti-His antibody is non-specific, we also performed a control experiment with wild-type cells not expressing the His-tagged TbMTase37 and no signal was observed ruling out the possibility of non-specific antibody binding. Localization of Trm140 by immunofluorescence using an antibody specific to Trm140 shows complete co-localization with nuclear DNA stained by DAPI (Figure 2.5B). Together, these experiments provide strong evidence for the nuclear localization of both MTase37 and Trm140 with more specific sub-nuclear localization of MTase37 to what is believed to be the nucleolus.
Figure 2.5. Both methyltransferases are targeted to the nucleus, but MTase37 is a nucleolar protein.

A) Western blot analysis was performed on total, nuclear (N) and cytoplasmic (C) protein fractions using antibodies against TbMTase37, TbTrm140, Nog1 (a nuclear/nucleolar marker) and Enolase (cytoplasmic marker). Anti-Enolase antibody (α-Enolase) was used as a control for cytoplasmic contamination in the nuclear fractions and anti-Nog1 antibody (α-Nog1) was used as a control for nuclear contamination of cytoplasmic fraction. B) Immunofluorescent localization using antibodies against NOG1 (α-NOG1) and anti-His antibody (α-His) to detect the His-tagged TbMTase37. Anti-TbTrm140 antibody was used to detect TbTrm140. DAPI (blue) was used to detect the nuclear (N) and mitochondrial kDNA (K). Fluorescent-labeled secondary antibodies were used to detect TbMTase37 (red) and NOG1 (green) or TbTrm140 (green). DIC represents light microscope view of a single cell. Merged images are superimpositions of each single combination or all three channels together performed with ImageJ (NIH). Yellow fluorescence is indicative of colocalization of NOG1 and TbMTase37.

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2.2.3  **MTase37 RNAi differentially impacts cellular RNA species**

To begin elucidating the role of TbMTase37 in the cell, total cellular RNA was purified from RNAi-induced, uninduced and wild-type cells and then analyzed by gel electrophoresis and Northern blot analysis. Ten micrograms of RNA from each treatment was electrophoresed and stained using ethidium bromide (Figure 2.6A). Comparison of each RNA species visible in each lane revealed a reduced level of one of the small sized (<230nt) LSU rRNA bands, fragment srRNA 4, in the RNA-induced sample. We also noticed an increase in RNAs corresponding to the size of either tRNA or srRNA which are both approximately 75 nucleotides and length and inseparable using an acrylamide gel. In light of these results we specifically examined individual cellular RNAs, including tRNA and rRNA by Northern hybridization with probes specific to each individual RNA species (Figure 2.6B). An RNA unrelated to cytoplasmic ribosomes, spliced leader RNA (SL RNA), was used as a loading control for signal normalization. In this experiment RNA samples were purified in triplicate from cells after 6 days of RNAi induction, when the growth and cytokinesis phenotypes first became evident. We found that, although different increases in the steady-state levels of some tRNAs were observed, there was a statistically significant (p<0.05) reduction in srRNA 4 (Figure 2.6C). Statistical analysis was performed using one sample t-test calculating the average normalized ratio of wild type to RNAi-induced signal for each RNA species compared to the expected value of 1.0 in the case of a completely functional ribosome which should possess one copy of each rRNA.
Figure 2.6. \textit{TbMTase37} RNAi affects steady-state RNA levels.

A) Total cellular RNA from wild type (W), uninduced (-) and induced (+) cells was analyzed by gel electrophoresis and Ethidium Bromide staining. B) Northern blot of RNA from A using radioactive oligonucleotide probes specific for each small rRNA species and a subset of cellular tRNAs were used to detect changes in steady-state RNA levels after RNAi induction. Cytoplasmic spliced leader RNA (SL RNA) was used as a normalization control, where changes in RNA levels were calculated by normalization of wild type and induced signals to SL. C) The normalized ratio of induced signal was divided by the normalized uninduced signal and graphed as “Fold Change”. Each bar represents an average of 3 independent replicates with error bars representing standard error of mean. Statistical analysis was performed using a one-sample t-test. An asterisk (*) represents a significant (p<0.05) difference from 1.0 (no change, dashed line).
2.2.4 MTase37 depletion leads to a reduction in polysome associated ribosomes

To study the effect on decreased srRNA 4 levels on the ribosome, the levels of 40S, 60S, 80S and polysome-associated ribosomes in wild-type and induced TbMTase37 RNAi cell lines were compared (Figure 2.7). Upon TbMTase37 depletion, the abundance of polysomes is reduced, 80S particles accumulate, and free 60S particles decrease. Gel electrophoretic analysis of rRNAs from the gradient fractions showed under-representation of srRNA 4, particularly in the 60S and 80S fractions. Notably, we never detected “half-mer” peaks characteristic of cells with initiation defects where only the 40S subunit is bound to an mRNA and is pushed along by trailing 80S ribosomes. It is important to note that all wild-type experiments looking at polysome profiles excluded the addition of tetracycline. Previous data from our lab suggests tetracycline, even at higher than inducing levels, does not affect T. brucei ribosomes.

We next evaluated the individual rRNA species present in each sucrose gradient fraction after a second round of ultracentrifugation to pellet ribosomal subunits or polysome complexes from sucrose. RNA was phenol extracted away from proteins and analyzed by Northern hybridization (Figure 2.8A). Oligonucleotide probes specific for each of the LSU rRNA species reveal a consistent ~1:1 ratio of all small rRNA species for wild-type cells. This trend, however, changes for MTase37 RNAi where levels of srRNA 4 are significantly underrepresented (p<0.05) and 5S rRNA levels are considerably underrepresented in 60S and 80S fractions (Fractions 5 and 6 in Figure 2.8C), but both are present at nearly stoichiometric levels in polysome-associated fractions (Fractions 7-10 in Figure 2.8C). Notably, since srRNA 4 is part of a polycistronic transcript, and only srRNA
4 levels decrease, we can rule out the possibility of a transcriptional component to such down-regulation and favor the hypothesis that it is destabilized or excluded in assembly following MTase37 RNAi. In addition, there is higher variability observed in relative abundances of the rRNA species after RNAi induction, consistent with incompletely assembled particles and/or decreased subunit stability. Quantification of the area under the curve for polysome traces from induced and wildtype gradients shows that the reduction of polysome associated ribosomes and increase of 80S sized particles are statistically significant (Figure 2.9). Area under the curve (AUC) was calculated by drawing a baseline across the three gradient traces for each treatment and then extrapolating peaks of 60S, 80S and polysome start and end to the baseline to establish quantifiable areas. The AUC for three individual A254 traces of wildtype and RNAi induced samples were calculated using the measurement feature of ImageJ (NIH) and statistics were generated using a two-tailed t-test. Statistical significance was established if p<0.05 in t-tests. Our results show that TbMTase37 plays an important role in generating functional, stable and translationally competent ribosomes.

2.2.5 MTase37 is important for large subunit stability, not rRNA processing

Next, we analyzed the subunit profiles for both wild type and TbMTase37 RNAi cells by lysis and sucrose gradient sedimentation in the absence Mg^{2+} to induce 80S dissociation into 40S and 60S subunits without dissociation of individual subunits into free RNA and protein (Figure 2.10) (Ball et al. 1973). Analysis of the resulting A254 traces revealed a 40% reduction in 60S subunits as compared with wild type ribosomes. In
Figure 2.7. Down-regulation of TbMTase37 leads to reduction in polysome-associated ribosomes and 60S subunit levels.

*T. brucei* lysates from wild type and RNAi cells were separated by sucrose gradient centrifugation (10-50% sucrose is shown) and fractions monitored by A$_{254}$. Peaks corresponding to 40S, 60S, 80S and polysome-associated ribosomes or subunits are as indicated. Polyacrylamide gels stained by ethidium bromide contain RNA from the fraction number indicated and the identity of the 6 small rRNA bands in the gel are indicated.
Figure 2.8. **Down-regulation of TbMTase37 leads to a reduction in the steady-state levels of srRNA 4 and 5S rRNAs.**

A) RNA isolated from the sucrose gradient fractions in Figure 2.7 were analyzed by Northern hybridization with oligonucleotide probes specific for each small ribosomal RNA fragment. Fraction numbers are as in Figure 2.7. **B)** Quantification of the signals in A, the signal of each rRNA in each fraction was first normalized as a percentage of total probe intensity across all fractions to exclude oligo hybridization differences. Relative signals were used to calculate the signal ratio of wild type to RNAi. Calculations were made as the percentage of signal for each rRNA in each fraction divided by the sum of all probe percentages in that fraction and multiplied by 6 (the total number of rRNA bands). The dashed line across each graph represents the expected 1:1 stoichiometry for all rRNAs within a given fraction.
Figure 2.9. **Down-regulation of TbMTase37 significantly reduces polysome associated ribosomes and increases free 80S subunits.**

The area under the curve for $A_{254}$ traces from polysome fractionation as in Figure 2.7 was calculated for wild-type and Tet + (RNAi induced) samples. AUC for 60S, 80S and polysome associated ribosomes was transformed to a percentage of total AUC for these three classifications. The mean percentage of three replicates is graphed where error bars represent standard deviation. Statistical analysis was performed using a two-sample t-test. An asterisk (*) represents a significant (p<0.05) difference from wild-type.
quantification the LSU:SSU ratio in wild type ribosomes was set equivalent to 1 and the reduction in RNAi samples was determined by comparison to this ratio. The possibility that dissociation of ribosomes by low Mg\textsuperscript{2+} leads to the loss of rRNA from the large subunit was excluded by pelleting polysome associated ribosomes and then dissociating the resulting 80S ribosomes into 40S and 60S subunits. The free subunits were then separated by a second round of gradient centrifugation and the rRNA composition between our input polysome associated ribosomes and 60S subunits following dissociation was compared by Northern blot (Figure 2.11). No difference in rRNA composition as observed before and after treatment. Thus, differences after RNAi cannot simply be caused by the lack of Mg\textsuperscript{2+} because wild-type ribosomes which were used for standardization were no affected by the removal of Mg\textsuperscript{2+}.

We reasoned that the decrease in 60S subunits could fundamentally be due to a defect in 60S assembly, stability and/or LSU rRNA processing. To address the impact of RNAi on rRNA processing, RNA from wild type, un-induced and induced TbMTase37 cells were analyzed by Northern hybridization using oligonucleotide probes specific for each of the 7 intergenic transcribed spacers (ITS) of the 35S pre-rRNA transcript (Figure 2-12A). We observed no aberrant processing intermediates for any of our ITS probes. In case low abundance intermediates escaped detection using Northern hybridization, we also performed qRT-PCR for 6 of the spacers and again observed no significant change in ITS levels between induced and wild-type cells (Figure 2.12B). Together these experiments suggest that TbMTase37 functions independently of the processing of the precursor transcript.
Figure 2.10. **MTase37 RNAi specifically impacts the large subunit.**

Ribosomes from wild type and induced TbMTase37 RNAi cells were dissociated into free subunits in the absence of Mg$^{2+}$ followed by separation through 7-27% sucrose gradients. To quantify percent reduction of 60S subunits after RNAi, the ratio of area under the curve for 60S to 40S was set to 1. The 60S:40S ratio for RNAi is the percent reduction compared to wild type. Error represents standard deviation of three independent replicates.
Figure 2.11. **Dissociation of 80S subunits does not cause loss of srRNA 4 upon MTase37 depletion.**

Polysome associated ribosomes were pelleted and suspended in Mg$^{2+}$-free buffer to dissociate subunits as in Figure 2.11. Dissociated subunits were separated through sucrose gradients and the rRNA composition of resulting 60S ribosomes was compared to input polysome associated ribosomes by Northern blot analysis. srRNA 1 was used as a control for comparing srRNA 4 levels.
After observing no effect of RNAi on rRNA processing we wanted to address the possibility that MTase37 acts as a large subunit assembly factor. To do this we performed western blot analysis on polysome gradient fractions from wild-type cells to observe co-migration of assembly-factors with assembling ribosomes (Figure 2.13). Samples of polysome fraction were loaded directly onto SDS-PAGE gels and transferred to nitrocellulose membranes without additional processing as was performed in RNA analyses described previously in this chapter. Using a control antibody to a known 60S subunit assembly factor Nog1, a nucleolar GTPase, we were able to detect migration of this protein beginning with 60S sizes particles through the polysome associated fractions as described previously (Jensen et al. 2003). When the same membrane was blotted for MTase37 using antibodies raised in rabbits we were only able to detect signal in the initial 3 fractions corresponding to small complexes. While this experiment does not exclude the possibility that MTase37 acts as an assembly factor, it suggests that MTase37 does not associate with large assembly intermediates and may act at earlier stages of assembly.

2.2.6 *MTase37 depletion dysregulates cell cycle control*

Upon RNAi induction and concomitant with the onset and progression of the observed growth phenotype which began after 6 days of induction, cells begin to exhibit abnormal shape and size. These abnormalities have not been observed in our studies using RNAi constructs against other methyltransferases nor for unrelated genes and was indicative of a cell division defect. Following RNAi induction cells were analyzed by fluorescent microscopy to visualize and enumerate flagellar structures, DNA content
Figure 2.12. **Reduction in TbMTase37 levels has no effect on pre-rRNA processing.**

A) Schematic of *T. brucei* rRNA processing from a polycistronic transcription unit into final mature rRNAs. The names of each mature rRNA in the operon are designated with “srRNA” representing small rRNA. The lengths of predominant intermediates are specified in kilobases. B) Northern blot analysis for internal transcribed spacers using RNA from wildtype (W), uninduced (-) and induced (+) cells. The ethidium bromide stained gel with the three large rRNA species visible (rightmost panel) shows equal RNA loading. Oligonucleotide probes specific for each internal transcribed spacer (ITS) were used to detect possible aberrant processing. C) The levels of 6 of the 7 ITS regions were analyzed by quantitative RT-PCR after RNAi treatment and plotted as a fold change from wild-type. All analyses were performed in triplicate. Bars represent the average change in replicates and error bars are standard error of mean. Fold change was calculated using the comparative $C_T$ method as described previously (Schmittgen and Livak 2008).
Figure 2.13. **MTase37 does not migrate with assembly intermediates through gradients.**

Western blot analysis of sucrose gradient fractions from polysome fractionation experiments. Gradient fractions were concentrated by centrifugation at high speed and separated by SDS-PAGE. Nog1 and TbMTase37 were detected in gradient fractions using antibodies specific for each protein. Fractions are labeled 1-17 and “in” denotes total cell input of gradient.
(nuclear and mitochondrial), cell shape, and mitochondrial integrity (Figure 2.14A). Our results revealed a defect in cytokinesis typified by multiple flagella, nuclei and mitochondrial DNA (kDNA, kinetoplast DNA) per cell, as well as loss of the mitochondrial reticulation characteristic of *T. brucei*. That these “abnormal” cells were alive was established by staining with the membrane potential-dependent dye MitoTracker. By 10 days post-induction at least 25% of cells showed the “abnormal” phenotype with respect to cell shape and intracellular structure (Figure 2.14B). To rule out the possibility that this phenotype is due to a general effect on translation following RNAi, protein lysates were prepared at 2 days intervals starting at the beginning of induction and analyzed by western blotting using antibodies specific to several proteins with diverse functions and unrelated to MTase37. This experiment confirmed that a defect in translation is not the cause for the observed phenotype (Figure 2.15). These results also indicate that MTase37 contributes in some way to cytokinesis in *T. brucei*. 
Figure 2.14. MTase37 RNAi cells exhibit dysregulated cell division.

A) Immunofluorescence was used to monitor changes in cellular morphology, mitochondrial shape and cellular DNA content. Wildtype, RNAi – and RNAi + cells collected at 6, 8, and 10 days post-induction are shown. DIC imaging was used to visualize flagellum and cell size. All cells were stained with MitoTracker (red) and DAPI (blue) to visualize mitochondrial shape and DNA content of either nuclei (N) or mitochondrial kDNA (K), respectively. Scale bars (white lines) represent 10µm. B) Characterization of cell-division structural signals. For each cell line and time point in A, at least 100 cells were scored for number of flagella (F), kDNA (K), and nuclear DNA (N) to assess percentage of “abnormal” cells which did not follow typical cell division progression of 1F:1K:1N, 2F:1K:1N, 2F:2K:1N, 2F:2K:2N and into 2 cells. Histogram bars are classified first as normal or abnormal. Abnormal cells are further classified by the structure(s) found to be abnormal in typical cell division progression.
Western blots were performed using purified rabbit polyclonal antibodies against several *T. brucei* proteins (Trm140, Isd11, Enolase and Prohibitin) as gauge for translational defects after MTase37 depletion. Protein extracts were generated from an equal number of wild type (W), RNAi –, and RNAi + cells at 2, 4, 6, 8 or 10 days of induction. An equal numbers of cells were lysed and loaded in the gel.
2.3 Discussion

In these studies we begun characterization of a putative methyltransferase from *T. brucei* and demonstrated its importance in the stability of the large ribosomal subunit and in maintaining the normal progression of cell division. Following the organism’s unusual nature, the *T. brucei* ribosome possesses rRNA expansion segments and protein extensions that form a kinetoplastid-specific domain (KSD). Predictably, this ribosome may require rRNA processing enzymes and assembly factors, such as MTase37, in addition to those already discovered. Novel chemical modifications to RNA or protein extensions can also not be excluded and may be important for molecular interactions. Supporting these notions, a number of essential 60S ribosome maturation factors have already been identified in *T. brucei* and all appear to be unique to the kinetoplastid family of organisms (Hellman et al. 2007; Jensen et al. 2005, 2003; Umaer et al. 2014; Sakyiama et al. 2013). We believe MTase37 to be another factor essential for ribosome function.

It is unlikely that TbMTase37, while resembling a methyltransferase, simply plays a chaperoning role in ribosome assembly beyond any enzymatic activity. We have tried to address the importance of TbMTase37’s conserved catalytic domain by creating a catalytically dead variant of this gene with a mutated SAM-binding domain; however, several attempts at *T. brucei* transformations failed. These mutations changed two essential glycine residues of the SAM-binding domain to alanine which was previously shown to be sufficient in abolishing enzyme activity of a tRNA methyltransferase (Alexandrov et al. 2005). Although this does not prove, it suggests that this version of the protein may exert a dominant negative effect and the methylation domain is an essential feature of MTase37.
Recombinant expression systems for both proteins and RNAi are notoriously leaky in *T. brucei*. Even in the absence of induction the catalytic mutant may be expressed at sufficient levels which lead to toxicity. Although we show MTase37 does not migrate with ribosome particles as other ribosome biogenesis factors do, it is conceivable that as an rRNA modification enzyme MTase37 may catalyze methyl transfer and quickly release the modified product. In this case we may never detect migration in a sucrose gradient but may detect interacting RNAs after irreversible crosslinking, an experiment that could be pursued next. Without MTase37 it seems as though srRNA 4 is destabilized. While there has been a report of functional ribosomes lacking a single rRNA, there are no reports of translationally competent ribosomes with sub-stoichiometric levels of multiple rRNA species (Dresios et al. 2003; Chaudhuri et al. 2007). In these studies we have strictly investigated the composition of ribosomes with respect to rRNA and have not looked at the protein composition. Learning more about the effects of RNAi on ribosomal protein composition may contribute additional insights into MTase37’s function and importance.

The loss of TbMTase37 and the resulting decrease in functional ribosomes likely induces nucleolar stress. In other eukaryotic systems depletion of ribosomal proteins or impairments to rRNA transcription and processing generates nucleolar stress and elicits a combination of p53 dependent and independent responses (James et al. 2014). *T. brucei* lacks the p53 protein as well as many other well-characterized cell cycle control proteins and has likely evolved new control mechanisms for stress responses and cell cycle signaling. A putative human methyltransferase METTL6 was recently identified as a key genetic driver or regulator of oncogenesis and was shown essential for cell proliferation
(Gatza et al. 2014). Sequence alignment of TbMTase37 and human METTL6 reveals a high degree of similarity even outside of the conserved methyltransferase domain. In fact, the similarity of MTase37 and METTL6 is higher than S. cerevisiae Trm140, the protein which initially led us to discovering MTase37. Our data for MTase37 correlates well with the predicted function and published importance of METTL6 in controlling cell proliferation. Consequently MTase37 could be a useful model for studying the importance of methyltransferases and RNA methylation in cell proliferation.

The accumulation of 80S ribosomes following the onset of RNAi could imply that what appear to us as 80S particles are actually defective or incomplete particles. One model for this accumulation is that without srRNA 4 and/or 5S rRNA these ribosomes are unable to engage in translation elongation as is evidenced from a decrease in polysome associated ribosomes. As defective ribosomes accumulate ribosome quality control pathways would be required to monitor and degrade the particles. The cryo-EM structure of the T. brucei ribosome shows srRNA 4 forms an intersubunit bridge novel to kinetoplastid ribosomes. Lack of this bridge may be what renders ribosomes inactive, although otherwise fully formed and resembling an 80S ribosome. It is possible that T. brucei srRNA 4 is required to be present for proper assembly of 5S rRNA into the ribosome or to induce a conformational change. Typically the 5S rRNA is assembled into the 66S pre-ribosome in the nucleolus as a ribonucleoprotein particle together with ribosomal proteins L18 and L5. Cryo-EM experiments from yeast have demonstrated that the 5S particle associates with the assembling ribosome in an orientation 180° rotated from its final confirmation and must be turned to stabilize the final structure prior to assembly (Leidig et al. 2014). In fact, two
trypanosome specific proteins, p34 and p37, were shown to be essential factors in 5S rRNA abundance and early assembly into the pre-ribosome cooperatively with ribosomal protein L5 (Wang et al. 2013; Hellman et al. 2007). In RNAi experiments against p34 and p37, cells exhibited similar morphological changes to those described in my work, supporting the connection with ribosome stability and rRNA abundance (Hellman et al. 2007). The putative methylation activity of MTase37 may modify srRNA 4 or another interacting rRNA(s), which in turn provides an essential docking point for interactions and facilitates assembly. This has been shown for nucleolar protein Nep1 of S. cerevisiae which is required for small subunit biogenesis as both a biogenesis factor and rRNA methyltransferase forming \( \text{m}^1\text{acp}^3\psi \) at U1191 of 18S rRNA (Meyer et al. 2011; Wurm et al. 2010).
Chapter 3: Exploring the cellular importance of MTase37 in *T. brucei*

3.1 Introduction

The apparent lack of transcriptional control of protein-coding genes in *T. brucei* suggests gene expression is regulated by pathways which exclude RNA polymerase activity. Protein-coding genes in eukaryotic systems are transcribed by RNA Polymerase II, a well-studied polymerase sensitive to the antibiotic α-Amanitin. Control of RNA Pol II activity is achieved through a variety of means which differ between organisms and include recruitment of transcription factors, histone modification, and DNA sequences present within the core promoter regions, including sequences upstream and downstream from the transcriptional start site (Fuda et al. 2009). Depending on the organism and the gene being regulated, the impact of these control mechanisms can vary drastically.

All mRNAs in trypanosomes receive an identical 5’ leader through a *trans*-splicing reaction, for which the donor molecule gains the name of spliced leader RNA (SL RNA) (Parsons et al. 1984). In common with other eukaryotic mRNAs, the spliced leader molecule provides an mRNA with the typical 5’-5’ linked m\(^7\)G cap. However, in contrast to most other organisms, modification of the mRNA cap does not stop at this first position and instead a “cap 4” structure is formed (Perry et al. 1987). The cap 4 structure gains its name because the first four nucleotides are extensively modified with a final sequence of its first 5 nucleotides as m\(^7\)Gpppm\(^{2,6}\)ApApCmpm\(^3\)Um (Bangs et al. 1992). Generation of the cap 4 structure is co-transcriptional, but surprisingly formation of the entire cap
structure is not essential for trans-splicing and, perhaps more importantly, for cell viability (Zamudio et al. 2006; Mair et al. 2000). In addition, poly-adenylated mRNAs with incomplete cap 4 structures have been identified (Zamudio et al. 2006). Together these data lend the possibility that control of gene expression at the translational level could be regulated through differential modification of the 5’ cap of mRNAs.

Another interesting facet of trypanosome biology is the presence of four distinct forms of the mRNA cap binding protein eIF4E, an essential component of eukaryotic translation initiation (Freire et al. 2011). None of these isoforms are capable of complementing a yeast knockout strain implying unique and species-specific mechanisms in their function (Yoffe et al. 2006). The apparent absence of transcriptional regulation may have brought about more complex translation initiation regulation which could be accomplished through differential binding affinities of translation initiation factors to distinct elements of 5’ and 3’ UTRs of mRNAs. One model for translational control suggests that trypanosomes may utilize each eIF4E isoform to recognize differentially modified capped mRNAs. Alternatively, each eIF4E isoform may recruit separate subsets of initiation factors which recognize different elements in the 3’ and/or 5’ UTRs.

In Chapter 2 we took a thorough look into how the absence of MTase37 in T. brucei affects ribosomes and cell division, but there remains the question of what aspect of MTase37 is directly responsible for the lower levels of srRNA 4 in ribosomes when MTase37 is depleted. In this chapter I will present work seeking to identify the modification target(s) of MTase37. We are also intrigued by the effect of MTase37 RNAi on cytokinesis and seek to better understand how and at what level this putative RNA
methyltransferase might be controlling cell proliferation. Using a set of single nucleoside analysis methods including thin layer chromatography, HPLC and mass spectrometry we investigated the modified nucleotides present in individual cellular RNAs and the effect of MTase37 depletion on each RNA species. After determining MTase37 protein depletion decreased ribosome stability and perhaps assembly, we decided to evaluate the impact of translation using a chloramphenicol acetyltransferase (CAT) reporter. Using this approach we determined there is differential expression of CAT depending on the identity of the promoter and 5’ UTR controlling CAT expression. Importantly no measurable difference in Pol II transcripts was observed. To enable the use of in vitro experiments to identify the substrate(s) of MTase37 and characterize catalytic activity I have established a purification scheme for recombinant expression from E. coli and native purification from T. brucei using 6X-His tagged expression cassettes. With the purified proteins, we have looked for RNA binding and modification activity on numerous tRNAs, Spliced leader RNA and rRNAs but were unable to detect obvious modification differences.

3.2 Results

3.2.1 Translation from the Pol I Procyclin promoter is elevated upon MTase37 RNAi

We were interested to understand how depletion of MTase37 affects translation after determining that its down-regulation affects the stability and/or biogenesis of the 60S subunit. To investigate the effects of RNAi on translation we utilized a chloramphenicol acetyltransferase (CAT) reporter stably transfected into the MTase37 RNAi cell line. The CAT reporter was inserted into the pABuro cloning vector which provided the promoter,
5’ UTR and 3’UTR of the procyclic acidic repeat protein, commonly known as PARP or procyclin. Procyclin is the main surface protein expressed in procyclic form trypanosomes and its promoter and regulation is one of the best studied in trypanosomes. It is also one of the few genes for which a distinguishable promoter has been identified. To validate the observations from pABuro-CAT activity assays, the promoter and 5’ UTR of procyclin was excised from pABuro-CAT at the KpnI and HindIII and replaced with the 500 nucleotides upstream of the transcriptional start site for Isd11, generating pABuro-Isd11 CAT. We chose Isd11, an iron-sulfur assembly protein, as a comparison because western blots suggested Isd11 levels do not change under MTase37 RNAi conditions and it would therefore provide us with a good control for comparison.

To evaluate the impact of RNAi on translation of CAT under the two UTRs, cells were grown in the presence of tetracycline for an increasing number of days as CAT enzyme activity was assessed (Figure 3.1).

The increase of CAT activity in pABuro-CAT cells correlates with the phenotype of RNAi induction where 6 days post-induction cells began to grow slower and exhibit abnormal cell division when quantifying the quantity of flagella and DNA per cell. This suggested the increase in activity is indeed the result of decreased levels of MTase37. However, based upon the data presented in Chapter 2, we expected to see a decrease in CAT activity due to decreased levels of polysome-associated ribosomes. No changes in activity were observed for the pABuro-Isd11 CAT construct and we therefore conclude that the increased CAT reporter levels are a result of either increased translation using the PARP 5’UTR, significantly increased mRNA levels or changes in mRNA stability. To
Figure 3.1. MTase37 differentially affects translation based upon 5' UTR identity.

A) A chloramphenicol acetyltransferase (CAT) reporter gene with the PARP promoter, 5’ UTR directly upstream was electroporated into cells containing the RNAi plasmid for MTase37. After 2, 4, 6, 8 or 10 days of RNAi induction cell lysates were prepared and CAT reporter activity was measured with FastCAT reagents (MolecularProbes). B) The CAT reporter from A was modified to contain the Isd11 5’ UTR and any promoter present (500 nucleotides upstream) instead of PARP promoter and UTR. As in A, lysates from cells were prepared and CAT activity was assessed.
begin to understand the level at which PARP promoter and UTR are affected by protein depletion we performed quantitative reverse transcription PCR (qRT-PCR) to evaluate relative changes in mRNA levels after induction (Figure 3-2).

Quantitative RT-PCR analysis revealed that, as expected, MTase37 mRNA is decreased two-fold upon RNAi induction. Neither PARP nor Isd11, the two genes from which UTRs were used for CAT transcription and translation, differed significantly (1 fold) upon MTase37 RNAi induction. The tRNA C32 methyltransferase homolog of MTase37, Trm140, which bears sequence similarity to MTase37 is also not affected by RNAi and serves as a control for off target effects in this analysis. Since steady-state mRNA levels are not changing, we can conclude that neither promoter activity nor differences in mRNA stability are the reasons for increased CAT activity. This offers us with the possibility that PARP translation is stimulated in the absence of MTase37 or in response to RNAi.

3.2.2 TAP purification of MTase37

In attempt to identify the modification target MTase37 we utilized a tandem affinity tagged (TAP) expression cassette for inducible expression of MTase37 with Myc, His and ProteinA epitope tags at the C-terminus. This construct, MTase37-TAP, is stably integrated into the T. brucei genome allowing for consistent and inducible expression of protein. Following 48 hours of induction by tetracycline, MTase37-TAP cells were lysed and proteins purified over Ni$^{2+}$ resin. Eluting proteins were silver stained to visualize the respective size and abundance of each species present in each fraction (Figure 3.3A). The same fractions were subjected to SDS-PAGE and transferred to nitrocellulose membrane
Figure 3.2. **Differential translation upon MTase37 RNAi is not due to changes in mRNA levels.**

RNA from induced and un-induced MTase37 RNAi was subjected to qRT-PCR analysis using primer pairs for PARP, Isd11, MTase37 and Trm140. Fold change in RNA levels after RNAi induction is plotted in relation to un-induced samples. Statistical significance was calculated using a one-sample t-test. An asterisk (*) denotes p<0.05.
for western blot analysis using His antibody (Figure 3.3B).

Silver staining is a highly sensitive technique, capable of detecting protein down to the 1 ng level. Western blotting, on the other hand, utilizes specific antibodies enabling detection of only the protein(s) of interest and can achieve as much as 10X more sensitivity than silver staining. In Figure 3.3, the silver stained gel shows one dominant band, present in all elution fractions, at the expected 55kDa size of MTase37-TAP. In addition, there is a smaller band of approximately 28kDa which is faintly visible in elution 1 but is nearly stoichiometric to MTase37 in elution fractions 2-5. The stoichiometry of this band could imply a 1:1 interaction of MTase37 with another protein, or possibly with an RNA. At 28kDa in size, if this second stained band is RNA, would correspond to a molecule of approximately 85 nucleotides in length. The western blot of Figure 3.3B shows the same 55kDa band observed in the silver stained gel and a faint, sub-stoichiometric band of approximately 23kDa. This experiment utilized α-His antibody to target the His portion of the TAP tag on MTase37-TAP. It is possible that the faint band of 23kDa is a degradation product of full length MTase37-TAP and we are observing the C-terminal half of the protein following cleavage.

We also wanted to look at any RNAs that co-purify with MTase37-TAP. Using the same cell line we isolated protein and subjected elutions to phenol extraction to remove protein followed by ethanol precipitation to collect purified RNA. RNA resulting from the final purification, and each step along the way, was then labeled at its 5’ end using γ-32P-ATP to enable visualization of relative sizes following separation on an acrylamide gel and exposure to a phosphor imaging screen. The results showed no significant enrichment for
Figure 3.3. TAP purified MTase37 has a protein partner.

A) Protein from MTase37-TAP preparations was separated on an SDS-PAGE gel and silver stained. Samples from left to right are protein marker, crude lysate (C), flow-through (F), wash (W), elutions 1-5. Sizes of marker bands are shown. B) The samples as in A were again separated by SDS-PAGE and then subjected to western blot analysis using α-His antibody. Size markers are indicated to the left.
any of the RNA species we could observe (Figure 3.4). To definitively say that MTase37 binds any of the ribosomal RNAs the experiment could be repeated using more stringent washing to remove all signals from the washing lane or by using crosslinking to purify only RNAs covalently bound to MTase37 after UV treatment.

3.2.3 Ribosomal RNA as a candidate target for MTase37

The focus of chapter 2 was the impact of MTase37 depletion on cellular health and RNAs which led us to conclude the lack of MTase37 impacts ribosome stability and/or biogenesis and seems to directly affect srRNA 4. In section 3.2.2, MTase37-TAP purifications showed us other rRNAs may also be bound by MTase37. To look deeper at the RNA modifications of each ribosomal RNA, wild type and induced RNAi cells were cultured and each ribosomal RNA was prepared from these cells. A number of methods were used to investigate the nucleotide composition of each species including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), mass spectrometry and RNAseq analysis.

Gel purified srRNA 4 from total RNA prepared from wild type and RNAi induced cells was digested into single nucleosides to be separated by TLC. In this approach RNA is first digested with non-specific nuclease RNase T2 producing individual nucleotides with 3’ phosphates. The resulting nucleotides are then phosphorylated at the 5’ end using γ-32P-ATP which
Figure 3.4. **Ribosomal RNA elutes with MTase37-TAP.**

Radiolabeled RNA from MTase37-TAP elutions, column wash, flow through and total cell lysates was visualized by exposure to phosphorimaging screen. Dominant ribosomal RNA bands are identified using srRNA nomenclature.
generates a radioactive nucleotide with 5’ and 3’ phosphates. For separation on TLC plates the 3’ phosphate must finally be removed using nuclease P1. Radioactive monophosphate nucleotides are spotted on silica coated TLC plates and separated in a two buffer system based upon chemical properties. Three buffers (A, B, C) with unique compositions are used in this analysis providing us the possibility to employ buffers in two-dimensional combinations of A-B and A-C (Grosjean et al. 2004). RNA from wildtype and induced samples were spotted on TLC plates, separated and exposed for comparison with known migration maps (Figure 3.5).

The separation of srRNA nucleotides shows the presence of all 4 standard RNA nucleotides in addition to 3 spots which we suspect correspond to modified nucleotides. Upon matching the TLC plates of Figure 3.5 to the central plate of known modification migrations it is apparent that more than the 7 already mentioned spots are present. These spots correspond to free phosphate (right edge) and dinucleotides (below and left of guanosine, G, in system A-C). There was no obvious difference in the modification set of wildtype compared to induced RNAi. This may reflect the requirement for modifications to maintain srRNA 4 stability and any RNA not receiving a modification from MTase37 is more quickly turned over by quality control pathways or nucleases due to lower stability and a short half-life.
Figure 3.5. Modified nucleotides present in purified srRNA 4 visualized by TLC.

Two-dimensional TLC analysis of purified srRNA 4 from wild type and induced cells. Post-labeling of digested nucleotides shows no difference between the two conditions. The top panel of TLC plates was separated in a system of Buffer A (isobutyric acid, 25% ammonium hydroxide and water [50:1.1:28.9]) and Buffer C (0.1M sodium phosphate pH 6.8, ammonium sulfate and n-propanol [100:60:2, v/w/v]). The bottom panel was separated in a system of Buffer A and Buffer B (hydrochloric acid, ddH2O, isopropanol [1.5:1.5:7, v/v/v]). Modification assignments in the middle square were made using published maps. Unknown nucleotides of 1, 2 and 3 correspond to no mapped spots. The intense black spot along the right side or bottom right corner of each TLC plate is free inorganic phosphate (P_i) released from Nuclease P1 treatment.
The modifications of srRNA 4 were also investigated using primer extension. In this experiment the purified rRNA was annealed with radiolabeled primers and extended from the 3’ end of the primer to the 5’ terminus of the rRNA. Strong secondary structures and modifications to the Watson-Crick face of any nucleotide commonly cause reverse transcriptase to stop extending, leaving a reverse transcribed product less than full length. When srRNA 4 was subjected to reverse transcription using a primer which anneals at the middle of the RNA, no prematurely terminated cDNA products exhibited a profile which differed from induced RNA implying there were no modifications to the Watson-Crick face at this region (Figure 3.6). However, when srRNA 4 was extended using a primer annealing to the 3’ end and allowing complete synthesis of a cDNA product differences were observed in at least 5 positions. These differences were likely due to structural changes arising from differences in modification state following RNAi. When considering the results in light of the primer extension with the oligo annealing at the middle of the RNA, any modification differences altering the structure most likely reside outside of the Watson-Crick face.

In a similar experiment to the TLC analysis for srRNA 4 as described previously in this section, each small rRNA species was digested with nuclease P1 and analyzed using HPLC with absorbance monitoring for nucleoside detection. The key differences of HPLC from TLC are the lack of radioactivity, removal of both 5’ and 3’ phosphate and separation in a liquid phase using a gradient buffer system under high pressure rather than
Figure 3.6. Primer extension on srRNA 4 shows no differences in modification, but differences in structure.

Primer extension reactions were performed on wild type and induced RNAi samples. Reactions used either a primer which anneals 4 nucleotides from the 3’ end, allowing full length extension, or a primer which anneals in the middle of rRNA. Closed black dots (●) denote nucleotide positions which show differences in modification or structure between the two samples.
solvent mobility against gravity. Each rRNA was purified and tested separately with the exception of srRNA 3. Since srRNA 3 has the same relative size as tRNAs it was excluded from this analysis as tRNA modifications would greatly skew our understanding of modifications specific to srRNA 3. The absorbance of nucleotides was monitored in real-time with a diode array detector which captured the absorbance in 1nm increments from 220nm to 350nm every 0.3 seconds (Figure 3.7). The resulting elution profiles reveal the expected 4 standard nucleotides (G, C, A and U) in highest abundance with other smaller peaks corresponding to less abundant nucleotides present in each rRNA species. The identity of each minor peak cannot be confirmed by HPLC analysis alone and will require a secondary method such as HPLC-MS, a technique which analyzes a portion of the material eluting from HPLC column by mass spectrometry in tandem with UV analysis. Using HPLC alone and combining the previous HPLC elution times of modified nucleosides with the known rRNA modifications presented in Table 1-1 we are able to exclude and predict the identity of some minor peaks. The relatively consistent jagged profile of peaks between G and A in the UV profiles are unlikely to all be nucleotide in nature and are probably a combination of nucleoside and contamination of samples obtained during the processing of RNA. The two most likely contaminants are urea or acrylamide carried over from RNA isolation procedures as samples were not subjected to filtration prior to analysis which would have removed these impurities.
Figure 3.7. **HPLC analysis of T. brucei rRNAs.**

Small ribosomal RNAs were digested by nuclease P1 and alkaline phosphatase for HPLC analysis. Absorbance was monitored over time at 254nm. The four main nucleosides (C, U, G, A) are labeled. Smaller peaks represent dinucleotides, modified nucleosides or debris from purification and possible identity of peaks is listed in dashed boxes based upon known HPLC elution times.
Figure 3.7 continued

**srRNA 2**

**5.8S**

continued
Figure 3.7 continued
3.2.4 Effects of MTase37 on global tRNA and rRNA modification

The same purified RNA from section 3.2.3 which was subjected to HPLC analysis was also analyzed at the nucleoside level by mass spectrometry. Included in this analysis was RNA from uninduced and induced cells as well as srRNA 3 mixed with total tRNA. Using nucleoside mass spectrometry, performed by the laboratory of Dr. Mark Helm, we hoped to better determine what RNAs were substrates for MTase37 and what modifications it might be catalyzing. After analyzing 8 specific nucleosides from all three treatment groups we again did not observe any significant changes in modifications from srRNA 4 upon RNAi induction (Figure 3.8). Despite this, we did observe a greater than 3 fold change m\(^6\)A levels in the srRNA3/tRNA analysis, specific to the induced RNAi sample.

In addition to mass spectrometry, the tRNA and srRNA 3 samples were subjected to RNA deep sequencing in attempt to detect changes in modification status based upon the propensity of reverse transcriptase to halt at locations of modification to the Watson-Crick face of nucleotides (Hauenschild et al. 2015). This recently described technique has the benefit of analyzing thousands of a single RNA species without individual purification. In addition to detecting halted events of reverse transcriptase, because sequencing of resulting cDNA is incorporated in addition to stopped events, this technique can also monitor positions where mismatched incorporation of nucleotides have occurred. Mismatch incorporation is an indication that modifications are present, but unable to halt the RT reaction instead causing a preferential, structural and 3’-sequence dependent incorporation of the incorrect nucleotides (Hauenschild et al. 2015).
When this RNAseq technique was applied to our purified tRNA there was a noticeable increase in m$^1$A$_{58}$ on a subset of tRNAs following MTase37 RNAi induction (Table 3-1). Surprisingly this increase was not observed the mass spectrometry analysis of Figure 3.8 and is likely because tRNAs were combined with srRNA 3 for MS analysis. RNAseq reads were successfully mapped to 16 of the 21 different tRNA species found in *T. brucei* covering a total of 30 isoacceptors. The profile of mismatch in matched reads above a coverage threshold at A$_{58}$ was analyzed for each isoacceptor. RNAseq profiles of RNA samples after induction were compared to wild-type or non-induced RNAi samples (Figure 3.9). Each tRNA was then classified into classes of “candidate”, “possible-candidate” or “non-candidate” based upon differences in mismatch ratio at A$_{58}$. Candidate targets had a mismatch ratio difference of ~0.15 while non-candidates exhibited no change in mismatch ratio. Possible-candidates were determined to be between 0 (no change) and 0.1 difference but not assigned as candidate or non-candidate because of uncertainty in significance of mismatch differences. For tRNA$^{\text{Gly}}$, a candidate target, the mismatch ratio in the wild type sample increased from 0.62 to 0.79, a difference of 0.17. The complete set of mapped reads and mismatch ratios from RNAseq for candidates, possible-candidate and non-candidates are included in Appendix A.
Figure 3.8. **Modified nucleotide profiles of tRNA and rRNA from wildtype and MTase37 depleted cells.**

Individual RNA species from wildtype (white bars), uninduced RNAi (grey bars) and induced RNAi (black bars) were digested to single nucleosides and analyzed by LC-MS/MS analysis. The relative abundance of each modified nucleoside is represented by intensity on the Y axis. RNA species analysed included tRNA, srRNA 3, 5S rRNA, srRNA 4, 5.8S rRNA, srRNA 2 and srRNA 1.
Figure 3.8 continued
Table 3.1. Candidate tRNAs with m^1A58 affected by MTase37 RNAi.

<table>
<thead>
<tr>
<th></th>
<th>Candidates</th>
<th>Non-candidates</th>
<th>Possible-candidates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>CGA, UGA, GCU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>CAC, AAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>CUC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>UUG, CUG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenocysteine</td>
<td>UCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>CUU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>UCC, GCC, CCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>AAU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>GUU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>AGC</td>
<td>UGC</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>CGG, AGG</td>
<td>UGG</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>UCG, CCU, UCU</td>
<td>CCG</td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
<td>GUC</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>UAG, AAG</td>
<td>CAG</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td>GCA</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td>CAU</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.9. **RNAseq profiles of candidate and non-candidate m\textsuperscript{1}A\textsubscript{58} tRNAs.**

Purified tRNA from wild-type (WT), ininduced (RNAi-) and induced (RNAi+) samples was subjected to RNASEq and mapped to the *T. brucei* strain 427 genome for sequence comparison. Grey bars in the background represent sequence coverage for each tRNA gene (left Y-axis). Colored bars represent positions where non-genomic nucleotides were returned in sequencing reads. Each colored bar represents a proportion of the total coverage in gray. Using the proportions at mismatch positions a mismatch ratio is calculated and displayed using a black circle (●, right Y-axis). Color definitions are yellow (G), green (A), red (T/U) and blue (C). Events considered to be over threshold are denoted by yellow triangles.
3.2.5 Recombinant expression of MTase37 using E. coli

To enable us to perform \textit{in vitro} assays with MTase37 and further investigate any activity we wanted to express the protein recombinantly from \textit{E. coli} to be purified using a 6xHis tag. MTase37 was cloned into the expression vector pET100D with N-terminal His tag. The conditions for expression and solubilization were optimized and expression levels for MTase37 are higher than any other \textit{T. brucei} protein we have purified previously. Inclusion of glycerol during elution and dialysis was essential in preventing undesired protein precipitation during dialysis which would otherwise lead to loss of >90\% of the soluble protein. Purified protein on an SDS-PAGE gel shows relatively few contaminants following a single Ni\textsuperscript{2+} column purification with MTase37 migrating at the expected size (Figure 3.10). Since the activity of this protein cannot be tested without knowing the substrate we are not able to determine if this purification method retains activity following purification. However, similar purification schemes for other \textit{T. brucei} methyltransferases (Trm5 and Trm140) with known targets do exhibit modification activity and provide promise that purification of MTase37 with this method will have activity.

Recombinantly expressed MTase37 was subjected to liquid chromatography on a Superdex 10/100 sizing column to determine its apparent size in solution. This experiment could also give an estimate on the amount of undesired proteins contaminating the preparation. Using a mixture of commercially available proteins of known size we are able to calibrate the retention time of our column and correlate time to a molecular weight. UV absorbance at 280nm is recorded over time and higher absorbance is a reflection of higher protein absorbance. Our purified MTase37 elutes from the column at an approximate
molecular size corresponding to 75kDa, the size of dimeric MTase37 each possessing a single His tag (Figure 3.11).

Since we didn’t yet have a substrate identified for MTase37, to begin testing for activity we used individual purified rRNAs and total tRNA. Through an electrophoretic mobility shift assay (EMSA) where each RNA was incubated with purified protein and then resolved in a non-denaturing gel we looked for complexes which result from protein binding to RNA and used this as an indication that bound RNA may be a target for modification. In this experiment each RNA is radiolabeled and free RNA will run faster through the gel than RNA complexed with protein. Although not a comprehensive analysis of RNA binding by MTase37, it appears as if MTase37 has affinity for each RNA we tested (Figure 3.12). Further investigation of binding specificity and competitive binding assays will be informative in determining if binding is biologically relevant or if the binding we observe is an artifact of including excess protein in the reactions.
Figure 3.10. **SDS-PAGE gel of recombinant MTase37 from *E. coli*.**

A Coomassie stained gel of fractions from each step of the purification. The migration of known molecular weight standards is marked in kilodaltons (kDa).
Figure 3.11. **MTase37 is a homodimer in solution.**

$A_{280}$ trace from Superdex 10/100 sizing column separation of recombinantly purified MTase37. The single buffer used was composed of 50mM Tris pH 7.5 and 100mM KCl. Maximum absorbance values of known proteins is denoted along the top of the graph and was used in determining molecular size based on column void and column volumes. Monomer and dimer forms of MTase37 are identified on top of each absorbance peak.
Figure 3.12. **MTase37 binds both rRNA and tRNA.**

Electrophoretic mobility shift assay (EMSA) using a native polyacrylamide gel was used to separate free radiolabeled RNA from radiolabeled RNA bound by protein. In each pair of lanes the lower band present in lanes marked “MTase37 –“ is unbound RNA and bands shifted to the top of the gel in “MTase37 +” lanes are indicate protein bound RNA.
3.3 Discussion

Because we lack a known target for MTase37, it is difficult to characterize its enzymatic activity in depth. Nevertheless, experiments on cells or with cell lysates comparing normal growth to protein depleted growth can provide clues into what its activity might be or might be related to. The observation that translation is up-regulated from the PARP UTR following RNAi was contrary to our expectation but sheds light on MTase37’s importance in cytokinesis as initially investigated in Chapter 2. Because PARP is the major surface protein of procyclic form parasites, the only life form we currently study in the lab, it would be interesting to investigate the impact of RNAi in blood-stream form parasites where the major surface proteins are variant surface glycoproteins (VSGs). We might expect to observe a similar result knowing its importance to the ribosome, but may also observe unexpected results where MTase37 has no impact on translation of VSGs. The connections between MTase37, cytokinesis and translation will be a fascinating problem to study further. No mechanism has been described for the switch from VSG to PARP expression during a switch of life stages for T. brucei. MTase37 could play a role in this regulation. Considering that RNAi of MTase37 leads to an increase of translation from the PARP UTR it could be that MTase37 represses PARP expression and induces VSG expression. A more global examination of the changes in protein and mRNA levels following RNAi in both procyclic and blood stream form parasites will enable us to build a better model to explain the importance of a putative methyltransferase in the regulation of surface protein expression.
Identifying the methylation target has been a trial and error approach for us thus far. Many of our newest ideas for what its target might be have been tested and proven false, but through the process we have gained a great deal of knowledge and ideas for further experimentation. MTase37 appears to have a protein partner which purifies with it, the identity of which is unknown. TLC experiments clearly show 3 nucleotides for which known modification maps and published works share no common identity for. Identifying the exact identity of the proteins which co-purify and nucleotides present in *T. brucei* rRNAs will be a valuable next step in elucidating its function. To identify the exact target of MTase37, crosslinking experiments and more stringent RNA co-purification experiments with a large volume of cells should be pursued so that RNAs can be sequenced and identified. Our tRNA RNAseq results begin to explain the increase in tRNA sized RNAs following RNAi observed by ethidium bromide staining gels as initially presented in Chapter 2. The increase of modification was unexpected and it is unlikely that MTase37 is catalyzing this reaction. However, there is been growing interest in the tRNA modification field around connections between stress responses and tRNA modification. Two possible explanations are that increased tRNA modifications stabilize tRNAs or that tRNA transcription is increased during stress induced by RNAi of MTase37. This could be the first description of a tRNA which undergoes modification during stress in *T. brucei*. Application of this RNAseq method to ribosomal RNAs would be a valuable addition to our knowledge of ribosomal RNA modifications in *T. brucei* and could help to identify the target position of MTase37 modification.
Chapter 4: A genetic screen to assess tRNA editing activity \textit{in vivo}

4.1 Introduction

The genetic code presented in Chapter 1 was stated as the “universal genetic code”, but there are exceptions to many biological “rules”, and the genetic code of mitochondria and chloroplasts are two such exceptions. In the trypanosome mitochondrion, every tRNA to decode mitochondrial mRNAs must travel from the nucleus, the site of transcription, to the cytoplasm and then into the mitochondrion through import pathways. One tRNA, tRNA\textsubscript{Trp}, is imported with a canonical CCA anticodon sequence which is perfectly able to decode the UGG tryptophan codon. However, the mitochondrial genetic code also uses the UGA codon to code for tryptophan but no tRNA is present in either the nuclear or mitochondrial genome to decode it. Editing of the CCA anticodon to a UCA anticodon to permit decoding of both UGG and UGA codons as tryptophan was described in 1999, but the enzyme responsible has not been identified (Alfonzo et al. 1999). The mitochondrial genome of \textit{T. brucei} is highly reduced and contains only 12 protein-coding sequences, all of which are essential for cellular respiration. Additional proteins must be imported after translation in the cytoplasm for full mitochondrial function. Therefore, it is reasonable to assume the deaminase required for C to U editing of tRNA\textsubscript{Trp} in the mitochondrion is also imported and presents an exciting, yet challenging, opportunity to potentially identify a new class of C to U editing enzymes.
An interesting aspect of deaminase enzymes is the differences observed in amino acid sequence motifs and how these motifs, which can be used as a predictor for enzymatic function, do not always define enzyme activity correctly. The A to I tRNA editing deaminase ADAT2/3 has sequence motifs which would predict its activity as a C to U deaminase. Biochemical evidence from our lab, and others, has extensively characterized the A to I editing activity of tRNAs by ADAT2/3. Published and unpublished work from our laboratory also describes C to U activity of the ADAT2/3 heterodimer which is capable of C to U deamination on single-stranded DNA, double-stranded E. coli genomic DNA and select T. brucei tRNAs in vivo (Rubio et al. 2007). These observations have led us to very interesting questions about how one enzyme can possess both A to I and C to U deamination activity. Since its protein sequence predicts it to be a C to U deaminase, ADAT2/3 is a perfect model enzyme to understand how an A to I deaminase may have evolved from an ancestral C to U deaminase. Knowledge about what residues alter substrate specificity could be applied in rational design of enzymes, in vivo systems and therapeutic treatments as well as providing better rules for protein prediction algorithms.

In addition to C to U editing of tRNA\textsuperscript{Trp}, unexpected C to U tRNA editing events have been described in the context of nearly all plant mRNAs as well as some tRNAs of marsupials, plants and archaea. In the mitochondria of plants (including beans, potatoes and some flowering plants) the mitochondrial tRNA\textsuperscript{Phe} gene encodes for a C at position 4 and is unable to form a Watson-Crick base pair with the complementary nucleotide, A\textsubscript{69}, in the acceptor stem of this tRNA (Maréchal-Drouard et al. 1993). Although C to U editing of tRNA\textsuperscript{Phe} is not essential for aminoacylation, it is likely required for processing of
precursor transcripts (Maréchal-Drouard et al. 1996). In addition to editing at position 4 of tRNA$^{\text{Phe}}$, tRNA$^{\text{Cys}}$ undergoes a C to U editing event. This editing is targeted to the anticodon stem where it changes opposing bases from C-U into U-U (Binder et al. 1994). Similar C to U editing was observed in coniferous trees where tRNA$^{\text{His}}$ undergoes three C to U editing events which again correct for C-A mismatches in the acceptor stem, D stem and anticodon stem (Maréchal-Drouard et al. 1996).

Marsupial editing of mitochondrial tRNA$^{\text{Asp}}$ again changes a C to U, but unlike those events described to this point, editing of tRNA$^{\text{Asp}}$ changes its identity from tRNA$^{\text{Asp}_{\text{GCC}}}$ into tRNA$^{\text{Gly}_{\text{GUC}}}$ (Borner et al. 1996). The switch in tRNA identity is essential because no tRNA$^{\text{Gly}}$ is encoded within the mitochondrial genome and editing is used to alter the substrate specificity of the two aminoacyl tRNA synthetases which charge tRNA$^{\text{Asp}}$ with aspartic acid and tRNA$^{\text{Gly}}$ with glycine. Following editing of C$^{35}$ to U$^{35}$, G$^{34}$ is able to be modified, by nucleotide exchange, to form queuosine (Q) and yield a final anticodon of Q$^{34}$U$^{35}$C$^{36}$ (Mörl et al. 1995). In describing the incorporation of queuine into tRNA$^{\text{Gly}}$, no tRNA with C$^{35}$ and Q$^{34}$ present in the same tRNA could be detected implying an order of events in the maturation of this tRNA and strengthening a connection between editing and modification.

Thirty of the 34 Methanopyrus kandleri tRNA genes are encoded with C$^{8}$ but it is well established that U$^{8}$ is important in forming a Hoogsteen base pair with A$^{14}$, an important contact for maintaining the conserved L-shaped structure (Romby et al. 1985). To restore U$^{8}$-A$^{14}$ pairing, archaea use a cytidine deaminase known as CDAT8 to deaminate C$^{8}$ to U$^{8}$ (Randau et al. 2009). The purpose of encoding 30 tRNAs which require
enzymatic conversion before properly functioning in translation is not clear. It is possible that as an extremophile M. kandleri requires higher genomic GC content to survive temperatures over 100°C and thus evolved the ability to edit tRNAs while maintaining a genome with higher GC composition. The identification of CDAT8, which shares several functional domains characterized in other ADAT proteins, could aid in identification of the cytidine deaminases responsible for the other C to U editing events described here. Despite being first described over 20 years ago, the C to U editing events described in Eukarya lack any identified enzyme attributed to their function and present an excellent opportunity to the field of RNA editing.

The work presented in this chapter seeks to identify the mitochondrial C to U editing enzyme for T. brucei tRNA^Trp^ and to gain an understanding of what amino acid changes have led ADAT2/3 to by prediction be a C to U editing enzyme, yet possess A to I editing activity. To this end I have designed a multifunctional genetic screening system to detect tRNA anticodon deamination events in vivo. The approach involves exogenous expression of T. brucei tRNA^Trp^ containing a CCA anticodon in S. cerevisiae. With a CCA anticodon this tRNA requires editing of C34 to U34 in order to suppress a UGA codon present within an essential respiratory gene. Previous work from our lab has studied modification of tRNA^T^ and it is apparent that C to U is a mitochondrial specific event which comes from this nuclear encoded tRNA^Trp^ gene. We believe that expressing deaminase mutants or a T. brucei cDNA library will allow us to detect enzymes active on the exogenously expressed tRNA. Further characterization of the DNA sequences generating expression and
permitting growth as well as the nature of the RNA sequence(s) after editing should allow us to develop a model for evolution of ADAT2/3 or identify the elusive tRNA\textsuperscript{Trp} deaminase.

4.2 Results

4.2.1 Bioinformatics query identifies putative RNA deaminases

In developing a genetic screening system, we reasoned that because all deaminases identified to date possess conserved deaminase catalytic residues conserved in motifs of HAE (or CAE) separated from PC and C where PC and C are divided by exactly 2 or approximately 70 amino acids, we could first use a bioinformatics query to identify \textit{T. brucei} proteins possessing these essential motifs. After identifying putative coding sequences we would be able to generate an RNA interference vector specific for each gene and assess the resulting phenotype. There are three distinct RNA deaminase motifs depending on the tRNA or mRNA position and nucleotide identity (Figure 4.1). A tRNA-specific adenosine deaminase for position 34 in eukaryotic organisms is a heterodimeric enzyme, while for position 37 it is likely a homodimeric enzyme with a different motif. Provided that ADAT2/3 possesses both types of activity we decided not to limit our search solely basing our criteria on one motif and instead encompass all characterized deaminase domains. In total, three queries were submitted to the European Molecular Biology Open Software Suite (EMBOSS) using the application patmatdb which uses the PROSITE pattern language to search for amino acid motifs in input amino acid sequences (Rice et al. 2000). Two of the submitted queries allowed for up to 600 amino acids between essential HAE or CAE and PCXXC and only differed in whether PCXXC was encountered before
or after HAE or CAE in sequence. Although previously identified deaminases have never shown the PCXXC motif to precede the HAE motif, we did not want to limit ourselves and decided include it as an option. Input for these queries, following PROSITE language, was either [CH]AEX(1-600)PCXXC or PCXXCX(1-600)[CH]AE. In the PROSITE language, X is defined as any amino acid and parenthetic numbers immediately following are used to define how many random amino acids might be in the sequence. Similarly, letters inside brackets permit only these defined amino acids to be the results, which in the case of this query are cysteine or histidine. A third query used was more specific and searched only for predicted proteins possessing CAE or HAE in addition to separated PC and C residues. PC was allowed to be separated from the subsequent C by between 55 and 85 nucleotides using the query input of [CH]AEX(1-600)PCX(55-85)C. All queries used the 9th version of translated predicted protein coding transcripts from the TriTrypDB database when identifying putative deaminases (Aslett et al. 2010). In total, 18 potential deaminases were discovered between the first two search methods using the defined PCXXC motif (Table 4-2). The search results for the divided PC and C motif were much more abundant and returned a total of 151 results. In all cases the length of the possible protein hits was restricted to greater than or equal to 50 amino acids.

We could be confident the query is successful in identifying deaminases because of identification of the tRNA deaminase subunit ADAT2 that our lab has extensively studied. For each putative deaminase hit, mitochondrial targeting predictions were performed using two web servers for prediction: TargetP and MitoProt (Emanuelsson et al. 2000; Claros and Vincens 1996). Both web servers predict localization using N-terminal sequences of the
A schematic highlighting the spatial arrangement of conserved amino acids involved in Zn$^{2+}$ coordination (N-terminal histidine and C-terminal cysteines) or proton shuttling (N-terminal glutamate) for three classes of deaminases. The “PC (spacer) C” motif is typical of adenosine deaminases while PCXXC motif is typical of cytidine deaminases.
Table 4.1. **Candidate cytidine deaminase proteins from EMBOSS query.**

EMBOSS query hits are tabulated by TriTrypDB identifier number and TriTrypDB annotated description. Mitochondrial targeting predictions are listed from entire predicted amino acid sequences.

<table>
<thead>
<tr>
<th>TriTrypDB Identifier</th>
<th>Description</th>
<th>TargetP</th>
<th>MitoProt</th>
</tr>
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<tbody>
<tr>
<td>Tb927.11.6690</td>
<td>Protein kinase, putative</td>
<td>0.062</td>
<td>16.09</td>
</tr>
<tr>
<td>Tb927.11.260</td>
<td>Intraflagelluar transport protein 144</td>
<td>0.084</td>
<td>19.73</td>
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<td>Tb927.11.13170</td>
<td>Hypothetical protein, conserved</td>
<td>0.221</td>
<td>27.15</td>
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<td>Ubiquitin ligase, putative</td>
<td>0.173</td>
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<tr>
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<td>Hypothetical protein, conserved</td>
<td>0.250</td>
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<td>Tb927.10.8390</td>
<td>Concanavalin A-like lectin, putative</td>
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<td>1.01</td>
</tr>
<tr>
<td>Tb927.9.3000</td>
<td>Cytidine deaminase, putative</td>
<td>0.446</td>
<td>60.69</td>
</tr>
<tr>
<td>Tb927.8.4180</td>
<td>tRNA-specific adenosine deaminase (ADAT2)</td>
<td>0.055</td>
<td>0.75</td>
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<tr>
<td>Tb927.8.1790</td>
<td>Hypothetical protein, conserved</td>
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<tr>
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<td>Beta-ketoacyl-ACP reductase 2</td>
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<td>Tb927.8.6490</td>
<td>Protein kinase, putative</td>
<td>0.426</td>
<td>19.84</td>
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</table>
protein, but output is received in different formats. For TargetP the higher the value, i.e. closer to 1.0, the stronger the prediction. With MitoProt, the higher the value, i.e. closer to 100, the stronger the prediction. It is important to note that neither algorithm is tailored to the mitochondrial targeting sequences used in *T. brucei* and the output is merely a prediction. These predictions could be improved by using the localization sequences of known mitochondrial proteins as predictors in the algorithms. Neither program returns a value that is a true percentage. Instead, each is simply a mathematical score. Looking at the mitochondrial targeting predictions for ADAT2, a protein we know to be nuclear and cytoplasmic, but not mitochondrial, in localization, TargetP predicts 0.055 and MitoProt predicts 0.75. Using these algorithms we would predict ADAT2 is not targeted to the mitochondrion as both values are at the lower end of prediction scale.

Looking at the individual results of our bioinformatics search, one protein stood out as our first testable candidate tRNA\(^{\text{Trp}}\) deaminase. This protein, Tb927.9.3000, is annotated as a cytidine deaminase of 182 amino acids. The predicted deaminase motif encoded for is CAE PCGFC, where essential residues used in the initial query are underlined. The mitochondrial prediction values were 0.446 (TargetP) and 60.69 (MitoProt) were among the highest in the search results. To determine if this protein affects editing of C\(_{34}\) in the mitochondrion we designed an RNAi vector using p2T7-177 with the entire CDA coding region inserted between opposing T7 promoters. Upon the addition of tetracycline T7 transcription is de-repressed, double stranded RNA is transcribed and RNAi is considered “ON”. The growth of cells with and without RNAi induction was monitored once daily to assess the effect of protein depletion on cellular health. After 13 days of RNAi induction,
no growth phenotype was evident despite confirming the mRNA for this putative CDA was depleted (Figure 4.2). To eliminate the possibility that tRNA editing is in fact affected despite the absence of a growth phenotype we performed reverse transcription followed by PCR amplification (RT-PCR) using primers specific for tRNA$_{Trp}$ and total cellular RNA isolated at the end of the growth curve (Figure 4.3). Since this approach uses total RNA which will include a large amount of cytoplasmic tRNA$_{Trp}$ expected to possess a CCA anticodon our results would either need to be extensively sequenced to detect low levels of C to U activity specific for mitochondrial tRNAs or PCR products would need to somehow be distinguished based on C to U activity. Fortunately, after conversion into double stranded DNA by PCR, tRNA$_{Trp}$ has a restriction endonuclease site (HinfI, 5’-GANTC-3’) which encompasses at the most 3’ residue, anticodon C$_{34}$. Upon deamination this restriction site is destroyed permitting us to differentiate full-length PCR product as the edited tRNA from two individual fragments representing the 5’ and 3’ halves after restriction. The RT-PCR and HinfI digestion reactions from wild type, induced and uninduced RNAi samples reveals no difference in resistance to endonuclease restriction and suggests this putative cytidine deaminase is not the enzyme responsible for C to U formation of tRNA$_{Trp}$.

4.2.2 Design of a multi-functional genetic screen for A to I and C to U deaminases

To enable relatively high-throughput screening of randomly mutagenized coding sequences or cDNA libraries we wanted to design a genetic screening system to detect tRNA editing activity in vivo. We tried to design the screening system in a manner that would enable detection of more than one type of editing. Initially we believed that choosing
Figure 4.2. **RNAi against Tb927.9.3000 does not produce a growth phenotype.**

**A)** Growth curve of wild type, uninduced (Tet -) and induced (Tet +) cell lines. Both Tet – and Tet + cell lines are *T. brucei* 29-13 cells transformed with the tetracycline-inducible dual T7 plasmid p2T7-177/CDA.9.3000 integrated into genomic 177 repeat regions. RNAi was induced by addition tetracycline to growth media and monitored every 24h. **B)** RNA isolated after 13 days of RNAi was DNase treated and subjected to reverse transcription (RT) plus PCR. The products from PCR reactions on RT- and RT+ samples were separated on a gel and visualized with ethidium bromide. A 100 basepair DNA ladder was used for size confirmation.
Figure 4.3. Digestion of tRNA\textsuperscript{Trp} reveals no changes to tRNA editing after Tb927.9.3000 RNAi.

Reverse transcription (RT) and PCR using oligos specific for tRNA\textsuperscript{Trp} were used to generate cDNA. Resulting cDNA from Tet – and Tet + cell lines was digested by HindIII and separated on an acrylamide gel. Full-length product remaining after digestion was gel purified, PCR amplified and digested again with HindIII to confirm the edited state of the remaining product.
a color based assay such as LacZ activity based upon blue/white screening in *E. coli* would be the best option for the screen. *E. coli* is fast growing and could provide overnight results upon protein induction. The blue/white screen would also be semi-quantitative in nature based on the relative intensity of blue in plate colonies. There is of course a downfall with a color based screen which is that low levels of activity may not be detected by eye. The screen would also have to be able to express exogenous tRNA, the substrate for enzyme activity. In addition to tRNA expression we would also require the tRNA be functional within the cell; that is to say the ability of a tRNA to be aminoacylated and then aminoacylated tRNA to pair with mRNA codons during translation and add a tRNA to the growing amino acid chain.

Although conceptually simple, designing the screen in *E. coli* was not going to function as desired based upon the fact that our positive control tRNA, already edited tRNA<sup>Trp</sup><sub>UCA</sub>, could not provide suppression of an in-frame stop codon of LacZ. This implied that tRNAs of *T. brucei* are either not substrates for translation or are not charged by aminoacyl-tRNA-synthetases in *E. coli*, and perhaps other bacteria. To get around this issue we decided to use *Saccharomyces cerevisiae* as our system for expression and library screening. There were fortunately at least two existing *S. cerevisiae* strains which harbored in-frame UGA stop codons in essential mitochondrial genes. One of the strains, SR128-3C has an A to G mutation of nucleotide 656 generating an in-frame UGA stop codon in the place of a UGG tryptophan codon in *coq6* (Gin et al. 2003). The other strain, BS5-5D, has a C to U mutation at position 406 of *cbs2* changing it from a CGA arginine codon to UGA stop codon (Hsieh et al. 2004). In both cases overexpression of *S. cerevisiae* tRNA<sup>Trp</sup><sub>CCA</sub>
was sufficient to suppress the stop codon and permit respiratory growth on glycerol after 10 to 12 days at 30°C.

To validate that expression of *T. brucei* tRNA$_{Trp}$ without editing could not suppress a UGA codon but an edited tRNA$_{Trp}$ could suppress the UGA codon, we transformed strain BS5-5D with plasmid pRS42K containing the *T. brucei* tRNA$_{Trp}$ gene with either a CCA or UCA anticodon and observed growth on glycerol media after 10 days (Figure 4.5). As controls, pRS42K without a tRNA insert was used alongside p4TTR, the plasmid which harbors *S. cerevisiae* tRNA$_{Trp}^{CCA}$ and was used in the original study describing this strain (Hsieh et al. 2004). We found that *T. brucei* tRNA$_{Trp}^{CCA}$ was not able to suppress the UGA codon while tRNA$_{Trp}^{UCA}$ was able to suppress the UGA codon (Figure 4.5).

### 4.2.3 Screening a cDNA library to identify the mitochondrial tryptophanyl tRNA C$_{34}$ deaminase

Equipped with a functional screening system the obvious next step was to introduce protein coding sequences to begin the search for editing activity. A previously generated cDNA library of *T. brucei* genes was provided to us by Dr. Patrice Hamel who originally obtained it from the laboratory of Dr. Ralph T. Schwarz. The cDNA library was generated from poly-A$^+$ total RNA of *T. brucei* strain 427 using commercially available cDNA synthesis reagents and placing cDNA inserts at the EcoRI and XhoI restriction sites of pRS416-Met (Mazhari-tabrizi et al. 1996; Mumberg et al. 1994). The use of pRS416-Met which contains the MET25 promoter and CYC1 terminator for the transcription of cDNA inserts requires that growth media lack methionine in order for gene expression to be
Figure 4.4. **Schematic for the operation and selection of deamination genetic screen.**

As designed and implemented in this research the genetic screen uses *S. cerevisiae* strain BS5-5D which harbors a *cbs2-223* mutation. Due to this nonsense mutation BS5-5D alone is unable to grow on respiratory media (e.g. synthetic glycerol media). The inability to grow by respiration in this cartoon is denoted by a red mitochondrion inside a tan colored budding yeast. Addition of *T. brucei* tRNA^{Trp} with CCA anticodon is unable to suppress the UGA nonsense codon of *cbs2-223*. Addition of tRNA^{Trp}_{UCA} enabled UGA decoding and the yeast is able to grow by respiration (green mitochondrion). If a tRNA^{Trp}_{CCA} is combined with a library plasmid (green circle) encoding a deaminase capable of deaminating CCA to UCA respiratory growth should also be permitted.
Figure 4.5. tRNA$^{Trp}_{UCA}$ suppresses the respiratory growth phenotype of BS5-5D strain.

Growth on synthetic glycerol media of BS5-5D yeast harboring either an empty plasmid (42K), *S. cerevisiae* high-copy tRNA$^{Trp}_{CCA}$ (4TTR), *T. brucei* high-copy tRNA$^{Trp}_{CCA}$ (42K CCA Trp) or *T. brucei* high-copy tRNA$^{Trp}_{UCA}$ (42K UCA Trp).
de-repressed. The library was originally generated to study *T. brucei* glycosyltransferases using heterologous complementation in yeast and was successful at identifying the dilichol phosphate mannose synthase (Mazhari-tabrizi et al. 1996). The mannose synthase discovery used an approach similar to our approach. They began with a temperature sensitive yeast strain with no Dol-P-Man synthase activity and after screening 125,000 colonies came up with 12 colonies which were no longer temperature sensitive. Only one clone was able to rescue the temperature sensitive phenotype after retransformation of the starting strain, 0.0008% of all colonies.

To begin using the pRS416-Met cDNA library we needed to amplify the plasmid library to obtain a larger starting pool of plasmids. In this process it was important to evaluate the diversity of cDNA insert sizes before and after amplification to ensure that amplification did not enrich for only a select few cDNAs or sizes of cDNA inserts. To begin, 10ng of starting material was transformed into competent cells and 10 colonies were selected for restriction digestion analysis of cDNA inserts (Figure 4.6B). The released inserts were separated on an agarose gel and visualized under UV light following ethidium bromide staining. The results showed a range of size diversity and we considered it safe to proceed with the amplification procedure. Using electroporation MR32 electrocompetent cells were transformed with plasmid DNA. A total of 600,000 bacterial colonies were scrapped from plates, grown in liquid to an O.D. of 0.4 and lysed for plasmid isolation. Finally, to ensure diversity of the resulting amplified library colonies were again chosen at random following the same procedure as the pre-amplification check (Figure 4.6B). The
results of our post-amplification check again showed a range of insert sizes and initiating library screening was not going to be biased using our amplified material.

The amplified cDNA library was screened for tRNA^{Trp} deaminase activity using yeast strain BS5-5D previously transformed with pRS42K-TbTrpCCA. Yeast transformed following the method of yeast electroporation to obtain maximal transformation efficiency. After electroporation yeast were plated on minimal media -ura supplemented with 200µg/mL gentamicin (G418). In total 300,000 colonies were selected on –ura +G418 media from cDNA library electroporations and subsequently replica plated onto synthetic ethanol/glycerol media without methionine and supplemented with G418 to select for cDNA inserts which provide deaminase activity sufficient in allowing suppression of the UGA codon in mitochondrial cbs2 mRNA. After at least 10 days of growth colonies began growing and in total 12 respiratory positive (Resp⁺) colonies were selected for further analysis. Growth on respiratory media generated substantially smaller colonies than fermentative growth on YPDA media, likely due to reduced growth rate (Figure 4.7).

The 12 Resp⁺ colonies selected were grown in liquid culture for biochemical analysis. Cultures of minimal media lacking methionine and uracil which would induce expression of cDNA genes were harvested for RNA analysis and minimal media -ura cultures were grown for plasmid isolation and characterization. To isolate RNA from yeast, cells were suspended in Tris-EDTA supplemented with 0.5% SDS and combined with equal volume water saturated phenol. After 10 seconds of vortexing and 45 minutes at 65°C the aqueous phase was isolated, chloroform treated and ethanol precipitated providing total cellular RNA. Isolated RNA was finally treated with RQ1 DNase to remove DNA from
Figure 4.6. *T. brucei* cDNA library shows diversity both pre- and post-amplification.

Plasmids from cDNA library transformation pre-amplification (A) and post-amplification (B) were purified from MR32 *E. coli* by alkaline miniprep and digested. The inserts released from pRS416-Met were visualized on an agarose gel and stained with ethidium bromide.
the preparation. To investigate the anticodon editing state of tRNA$_{\text{Trp}}$ we used reverse transcription followed by PCR to generate a double stranded cDNA of a collection of T. brucei tRNA$_{\text{Trp}}$ molecules. An oligonucleotide specific for T. brucei tRNA$_{\text{Trp}}$ was used that is not able to anneal to tRNA$_{\text{Trp}}$ of S. cerevisiae. Reverse transcription from this primer was followed by PCR to amplify the pool of cDNA molecules. Instead of sequencing the resulting pool we utilized restriction digestion by the enzyme HinfI which recognizes the palindromic sequence 5’ GANTC 3’ which is present in the anticodon stem of T. brucei tRNA$_{\text{Trp}}$ molecules and destroyed by editing of C$_{34}$ to U$_{34}$. The digested products are resolved on a polyacrylamide gel and restriction resistant full-length bands are indicative of editing activity. Of the 12 Resp$^+$ clones that were tested, no clone showed full-length product after HinfI digestion that was more intense than a digestion of a control PCR from genomic DNA (Figure 4.8).

In addition to RT-PCR analysis of RNA, plasmids purified from the Resp$^+$ colonies were transformed into the initial BS5-5D strain with pRS42K-TbTrpCCA to replicate the switch from Resp$^-$ to Resp$^+$ phenotype based upon the presence of the cDNA library plasmid. In all 12 cases, the cDNA library plasmid was unable to transform BS5-5D into a Resp$^+$ strain. This experiment demonstrated that the 12 Resp$^+$ colonies isolated from respiratory growth were either the result of changes to the UGA codon which no longer conferred translation termination ability or they were result of suppressor mutations to another gene of the S. cerevisiae genome.
Figure 4.7. Growth of a cDNA library clone on respiratory media.

The growth of one Resp⁺ clone from cDNA library screening on rich media, rich media with antibiotic and respiratory media (EtOH/Gly). BS5-5D is the starting strain, “+tRNA_{Trp}^{CCA}” denotes BS5-5D with tRNA_{Trp}^{CCA} and “+ cDNA clone” denotes BS5-5D with both tRNA_{Trp}^{CCA} and a cDNA library plasmid. Spots of growth from left to right represent 10-fold dilutions of culture.
Figure 4.8. **Diagnostic digestion of RT-PCR products shows no editing of tRNA$^{\text{Trp}}$.**

**A)** RNA from Resp$^+$ clones 1 through 8 was purified and subjected to reverse transcription (RT) plus PCR using primers specific for *T. brucei* tRNA$^{\text{Trp}}$. Gels of RT- and RT+ PCRs show no genomic contamination of DNase treated RNA (RT-) and RT+ shows successful amplification of tRNA$^{\text{Trp}}$. On the right are HinfI digestions of RT+ PCR products to look for edited (restriction resistant) products. The sizes of expected products are full-length (91), 5' half (37), 3' half (54). **B)** The same analysis as in A except for Resp$^+$ clones 9 through 12. In the experiments “G” represents *T. brucei* genomic DNA used as a size control. “O” represents a PCR reaction with only oligos, no template. “NE” represents no enzyme treatment. Numbers “1” through “12” represent Resp$^+$ clones.
4.2.4 Switching ADAT2/3 specificity from an A to I into C to U deaminase

In a separate application of the genetic screening system we wanted to understand what amino acid changes to ADAT2 and/or ADAT3 would alter its activity from an A to I deaminase into a C to U deaminase given its apparent evolution from a C to U deaminase based upon amino acid motifs. The selection of positive clones in this application was still based on respiratory growth, but the media used in selection was changed based on the regulation of cloned protein coding sequences.

Protein coding sequences of ADAT2 and ADAT3 were mutagenized by mutagenic PCR using Taq polymerase in the presence of Mn$^{2+}$ and Mg$^{2+}$ at 0.4mM and 5mM, respectively. The concentration of Mg$^{2+}$ was titrated specifically for the ADAT2 template and 5mM was chosen because it generated an average of 2-3 amino acid changes per coding sequence. The PCR products generated were run on an agarose gel and the band of appropriate size was extracted by smashing the gel, adding phenol and freeze-thawing the mixture to obtain the aqueous phase by centrifugation which was concentrated by ethanol precipitation and ready for ligation to the plasmid. The plasmids used for expression of ADAT2 and ADAT3 were pRS426 and pRS425, respectively. Each plasmid was linearized in preparation for gap repair recombination of plasmid with PCR product after electroporation into *S. cerevisiae*. Gap repair recombination cloning inside the yeast strain was chosen because it is highly efficient and does not require sub-cloning steps in *E. coli* once a linearized vector and PCR product are produced. The expression of ADAT2 was verified in a derivative of pRS426, pIGinA, by fusing it at the C-terminus to a tandem GFP cassette (Butterfield-Gerson et al. 2006). When grown in presence of 0.1% galactose and
absence of glucose expression of ADAT2 is turned be “ON” and GFP signal can be observed by fluorescence microscopy (Figure 4.9). The application of this GFP expression cassette confirms that ADAT2 is indeed expressed from the GAL1 promoter upstream, but because GFP could interfere with ADAT2 function in the screen we chose to exclude the tandem GFP cassette from the vector during library generation and use only pRS426.

Recombination in *S. cerevisiae* of PCR product with plasmid following electroporation provided us with an estimated 526,800 colonies, determined by enumeration from a dilution of the plated electroporations. All colonies first selected on minimal media lacking leucine and supplemented with G418 at 200µg/ml and then replica plated onto synthetic glycerol minimal media supplemented with 0.1% galactose for ADAT2 induction and G418. The number of Resp⁺ colonies resulting from this screen totaled to 4 (Figure 4.10). Sequencing of one clone showed mutation of active site glutamate to arginine. RT-PCR experiments, as was performed for cDNA library clones, using RNA isolated from liquid cultures grown with 2% galactose revealed no editing activity of tRNAs.
Figure 4.9. **Expression of ADAT2-GFP in *Saccharomyces cerevisiae*.**

Yeast with plasmid pRS426-ADAT2-GFP were induced for expression by growth on 0.1% galactose and imaged by fluorescent microscopy. GFP signal was detected using 488nm laser. Phase and GFP signals were merged using ImageJ. GFP signal which does not perfectly align with phase contrast cells is due to diffusion of suspension cells on the microscope slide. Cells which appear to have no GFP signal have likely lost the expression plasmid after overnight growth in non-selective media.
The growth of one Resp\(^+\) clone from ADAT2 mutagenesis screening on rich media (YPD), rich media with antibiotic (YPD+G418), minimal media with antibiotic (-LEU+G418) and respiratory media (YPGly + 0.1% Gal). The identity of each strain plated is indicated in the box to right. BS5-5D is the starting strain, “+ tRNA\(^{Trp}\)\(_{CCA}\)” denotes BS5-5D with tRNA\(^{Trp}\)\(_{CCA}\). The bottom box represents BS5-5D with tRNA\(^{Trp}\)\(_{CCA}\) and the pRS426 vector with insert as indicated. Spots of growth from left to right represent 10-fold dilutions.
4.3 Discussion

We believe the genetic screen for RNA editing generated and described in this chapter will be a highly useful tool for studying editing reactions in the context of C to U and A to I reactions, but also in other tailored applications. General changes to the system described might include alteration of the tRNA being expressed or changes in stop codon identity which provide added flexibility in screen implementation. Using current research in our laboratory as an example one might envision expressing *T. brucei* tRNA$^{\text{Tyr}}$, the only tRNA in this organism which possesses an intron, and investigating how changes to intron sequence identity alters splicing efficiency. This will be important because we have described non-canonical editing activity to the intron which is essential for splicing but do not yet understand how or why editing occurs (Rubio et al. 2014). In such permutation of the genetic screening system, tRNA$^{\text{Tyr}}$ would have to be expressed with an altered anticodon sequence which recognizes either the UGA stop codon existing in *cbs2*. Changes to the genomic codon requiring suppression could also be changed to expend functionality for tRNA$^{\text{Tyr}}$ and other tRNAs desired to be studied. There is also the opportunity to try and detect the protein(s) responsible for tRNA$^{\text{Tyr}}$ editing by combining a tRNA$^{\text{Tyr}}$ variant with any *T. brucei* cDNA library. This is just one example of how the screen may be applied in other contexts using the “awesome power of yeast genetics”.

In our iterations of the genetic screen we were unable to identify a protein responsible for tRNA$^{\text{Trp}}$ editing or build a model for the evolution of ADAT2/3 from what is believed to be a cytidine deaminase into an adenosine deaminase. Application of the Clark-Carbon equation which estimates the total number of clones or colonies required to
obtain one of every gene in a genome of known size suggests that to be 99% confident all
genes are covered 75,207 clones would need to be screened (Clarke and Carbon 1976). In
our screen 300,000 clones were screened providing over 99.999% certainty that the gene
responsible would be uncovered. There are a number of explanations for why we didn’t
find the target gene we were hoping to find. It could be that the gene was not represented
in the original portion of the cDNA library which we received and amplified. To be certain
we were able to select a complementing gene from the library we used yeast strain BS5-
5D, a leucine auxotroph, and successfully complemented it with leu2 of T. brucei. This
gene is considered a housekeeping gene and would be expected to have many mRNAs
present to ensure sufficient Leu2 is available within the cell at any given time. Along these
lines, a deaminase with a function desired to be localized only within the mitochondria may
have its mRNA present at low levels to avoid undesired deamination of cytoplasmic
tRNA\textsuperscript{Trp}. We must also consider that although transcription of protein-coding genes is not
regulated there could be post-transcriptional regulation which may affect the relative
amount of deaminase mRNA present to be incorporated into the cDNA library. It is also
possible that, like ADAT2/3, the tRNA\textsuperscript{Trp} deaminase is a multi-subunit enzyme or requires
an accessory protein. In this case, the screen would never result in successful identification
of the deaminase unless S. cerevisiae had the complementing protein to the cDNA
expressed from the library plasmid. One might try to combine two cDNA library
constructions from two different selectable markers to find activity if the deaminase is a
heterodimeric enzyme. Of course this scenario requires that the appropriate cDNA for each
subunit ends up in the exact same yeast cell following electroporation, pushing the number
of clones which need to be screen for 99% certainty from 75,207 to 5.6x10^9. Even if we desired 95% coverage the number of clones would be 2.39x10^9. Hence, in the case that a multi-subunit enzyme is the active protein the genetic screen is unlikely to be the successful identification technique.

The 12 colonies which we isolated as Resp^+ from our genetic screen did not show any editing activity from RT-PCR experiments on cellular RNA. The cDNAs of these colonies may have coded for a completely irrelevant protein and the strains emerged due to suppressive mutations of the genome or reversion of the UGA codon to a sense codon. It is unlikely that the cDNA inserts may have contained a polycistronic mRNA which contained protein coding sequences in addition to tRNA \textsuperscript{Trp} and increased tRNA \textsuperscript{Trp,CCA} expression was sufficient for suppression. Even though \textit{S. cerevisiae} tRNA \textsuperscript{Trp} could suppress the UGA codon without editing, tRNA \textsuperscript{Trp} in the \textit{T. brucei} genome is not flanked by any protein coding genes on the negative strand of the genome and is unlikely to be in a polycistron. Another possibility is that a \textit{T. brucei} homolog of the mutated gene in BS5-5D, \textit{cbs2}, is expressed and complements the yeast mutation. However no proteins are currently annotated as a cytochrome B activator or CBS2 and a BLAST search against \textit{T. brucei} using the yeast sequence returns no hits suggesting \textit{T. brucei} does not encoded a CBS2 homolog.

To improve upon this screen a new cDNA library could be generated under conditions of high mitochondrial activity when deaminated tRNA \textsuperscript{Trp} would be required at high levels within the mitochondrion. If the mRNA is regulated post-transcriptionally this approach may increase its abundance and, at the same time, increase its representation in
the assemble library. Instead of blindly creating a cDNA library it would productive to generate expression vectors for each of the 18 genes listed in Table 4.1 as these genes are known to possess the cytidine deaminase motifs.

In trying to generate a model for ADAT2/3 evolution from an ancestral deaminase we made a number of assumptions which may have limited the chances of successfully identifying active mutant proteins. First, we hypothesized that 2-3 amino acid mutations will be sufficient to switch activity from A to I into C to U. Although this may be the case it could also require many mutations to alter activity. In this case, investigating multiple concentrations of Mg$^{2+}$ to evaluate the effect of a range of amino acid changes might be more fruitful. It may also be necessary to not only mutate ADAT2 but also mutate ADAT3, a subunit which we know to play a structural role in catalysis (Spears et al. 2011). By screening over 600,000 colonies of mutants with 2-3 amino acid mutations per coding sequence we can estimate sampling 6.029x10$^5$ unique full length proteins using the algorithm Programme for Estimating Diversity in Error-prone PCR Libraries (PEDEL-AA) (Firth and Patrick 2008). ADAT2 is 225 amino acids in length allowing for 1x10$^{47}$ possible combinations amino acids and meaning we have only sampled about 10% of possible combinations. Another reason we may not have found an active mutant enzyme is the lack of proper substrate. In our screen all yeast tRNAs and only one T. brucei tRNA (tRNA$^{Trp}$) were available for mutation. In future efforts it would be valuable to either add additional tRNAs to the system, or switch them out singly, with a focus mainly on the 8 current substrates for ADAT2/3. In light of all of these possible future directions, one final
avenue to increase the chances of uncovering active deaminases using this screening system is to simply screen more clones.
Chapter 5: Concluding remarks

Until the completion of the studies presented here, nothing was known about why an organism would require two paralogous 3-methylcytidine methyltransferases. Perhaps we still don’t know exactly why two such enzymes are necessary for normal growth, but we are now equipped with a wealth of knowledge on the importance of possessing a second 3-methylcytidine homolog for *T. brucei*. Our interest in MTase37 began with the observation that RNAi causes cells to morph into very large cells and possess multiple nuclei, a phenotype our lab has never seen when performing RNAi of other methyltransferases but has been observed with other ribosome assembly factors (Hellman et al. 2007). This observation raised many questions and prompted many experiments, the majority of which we have tried to address using the biochemical techniques I have described. We have established that one rRNA, srRNA 4, of the large ribosomal subunit is present at lower levels in 60S and 80S ribosomes when MTase37 is depleted. Since we were never able to see an effect on the small ribosomal subunit this could imply *T. brucei* requires species specific ribosome biogenesis factors for 60S biogenesis and stability.

The unique ribosome architecture of kinetoplastid organisms presents an opportunity to study ribosome biogenesis factors which may ultimately provide excellent drug targets for *T. brucei* and other trypanosomes. Our observation that srRNA 4 possess two apparently modified nucleotides which have not been mapped by TLC opens a new
opportunity to investigate the modifications of all *T. brucei* ribosomal RNAs further. Perhaps there is a collection of modifications present within the other fragmented large subunit rRNAs that have not been described in other organisms and are important for optimal ribosome stability and function in *T. brucei*. Given that srRNA 4 is a part of a fragmented large subunit RNA and participates in a very strong species-specific intersubunit bridge one may envision that RNA modifications are extremely important in maintaining the structural integrity of the large subunit rRNA fragments.

Another fascinating aspect of MTase37 is its connection with cell-cycle regulation and how it influences translation based on 5’UTR identity. I argue that MTase37’s connection to the cell cycle falls back to its impact on the ribosome, which is likely “sensed” before division. To ensure that successful cell division results in cells capable of sustained protein synthesis, a threshold level of ribosome may be monitored by measuring levels of ribosomal RNA, factors binding to assembled ribosomes, nucleotide levels or another high-abundance molecule. Following MTase37 RNAi there is likely a stress response generated from low ribosome levels which may play a role in the incomplete cell cycle. Why and how these cells continue to duplicate genomic DNA and produce additional flagella without splitting into two cells is a question which remains to be answered.

The differential impact of RNAi on translation from two separate UTRs is another intriguing problem to be studied further. If and when resources permit, a global study of transcription and translation following RNAi may reveal groups of proteins or mRNAs which are down- or upregulated. Importantly, this type of study has the potential to reveal
a methylation event which regulates mRNA stability or translation. Such a finding would be a landmark discovery in the trypanosome system.

The generation of a genetic screening systems for RNA editing should be a useful tool in the Alfonzo laboratory’s investigations of RNA editing. Even though my application of this genetic screen did not succeed in identifying an elusive tRNA deaminase or ADAT2/3 mutants capable of C to U editing, I hope that future variations might get closer to the answers. The bioinformatics analysis of deaminase domains presented in Chapter 4 may provide valuable direction for other proteins to be studied in isolation as it is clear that identifying the tRNA$^{\text{Trp}}$ deaminase will be a monumental finding in the RNA editing field.
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Appendix A: RNAseq profiles for $\text{m}^1\text{A}$ mismatch
Figure A.1. RNAseq profiles of candidate, possible-candidate and non-candidate m$^1$A58 tRNAs
Figure A.1 continued
Figure A.1 continued
Figure A.1 continued
Figure A.1 continued
Figure A.1 continued
Figure A.1 continued
**Possible Candidates**

Figure A.1 continued

- *tRNA^Met*<sub>CAU</sub>
  - Wild type
  - RNAi -
  - RNAi +

- *tRNA^Cys*<sub>GCA</sub>
  - Wild type
  - RNAi -
  - RNAi +

- *tRNA^Leu*<sub>CAG</sub>
  - Wild type
  - RNAi -
  - RNAi +

continued
Figure A.1 continued

Non-candidates

tRNA_{UAG}

Wild type

RNAi -

RNAi +

tRNA_{AAG}

Wild type

RNAi -

RNAi +

tRNA_{GUC}

Wild type

RNAi -

RNAi +

continued
Figure A.1 continued
Appendix B: Methods and materials
B.1 One Step Yeast Transformation

This protocol was provided by Dr. Patrice Hamel and was adapted from Chen et al. (1992) Current Genetics 21, pages 83-84.

1) Prepare microcentrifuge tubes with 90 µl of 1S buffer and 5 µl of SS carrier DNA 10 mg/ml (salmon or herring sperm) which has been denatured for 15 min at 100°C and kept on ice.

2) Add yeast cells in stationary phase to the 1S buffer (centrifuge 0.1 ml of a liquid culture in stationary phase $5.10^8$ cells/ml so $\approx 5.10^7$ cells or collect with a loop the cells at the periphery of two to three 20 µl drops of liquid culture that have grown overnight on a YPDA plate).

3) Add the DNA (50 ng to 1 µg for plasmid) and mix by pipetting gently.

4) Heat shock the tubes for 30 min to 1h at 45°C and spread the cells (centrifugation and washing are not necessary) on a selective medium.

1S buffer (1 ml):

- 0.8 ml of PEG 3350 50%
- 0.1 ml Li Acetate 2N
- 0.1 ml DTT 1M

Sterilize by filtration and store at -20°C
B.2 Electroporation of Yeast


1) Grow *Saccharomyces cerevisiae* in a 20mL overnight culture to stationary phase (OD$_{600}$ of ~3)
2) Inoculate 100mL of fresh YPD with enough overnight culture to reach OD$_{600}$ of ~ 0.3
3) Monitor growth until OD$_{600}$ is ~1.6 and collect by centrifugation
4) Wash pellet 2X with 50mL of cold water and followed by one wash with 50mL electroporation buffer
5) Condition cells by shaking 225rpm for 30 minutes at 30°C in 20mL of 0.1M LiOAc, 10mM DTT
6) Wash once with 50mL electroporation buffer and suspend in 100-200µL of the same buffer to reach a total volume of 1mL. This should be approximately 1.6 x 10$^9$ cells/mL.
7) Load sterile cuvettes with 1µg of vector. If using gap-repair, 1µg linearized vector + 3µg of insert (1:3 ratio)
8) Electroporate cells at 2.5kV, 25µF, 180Ω using a 0.2cm cuvette. The time constant should be somewhere between 3ms and 4.5ms
9) Suspend electroporation in 10mL of a 1:1 mix of 1M sorbitol and YPD media. Incubate 30°C for 1h
10) Pellet cells and culture on selective media. To quantify the number of transformants, plate serial dilutions and count after 3 days.

**Electroporation buffer:**
- 1M sorbitol
- 1mM CaCl$_2$

Sterilize by autoclaving and store at room temperature
B.3 Yeast Transformation of Libraries

This protocol was provided by Dr. Patrice Hamel was communicated by Dr Y Surdin-Kerjan (CGM, CNRS, France)

1) Prepare a fresh pre-culture of the strain you wish to transform (overnight).
2) Inoculate 100 ml YPDA culture in order to reach OD<sub>600nm</sub> ≈ 0.5 to 1 the next day.
3) Centrifuge the cells at room temperature (5000 g for 10 min).
4) Wash the cells in 10 ml sterile water and centrifuge at 5000 g for 10 min.
5) Wash the cells in 10 ml solution A (freshly prepared) and centrifuge at 5000 g for 10 min.
6) Resuspend the pellet in 450 µl of solution A and incubate at 28°C for 30 min.
7) For each transformation, add in a microfuge tube:
   - 200 µl cells
   - 200 µg carrier DNA (denatured 20 min by boiling and kept on ice)
   - 1 to 5 µg DNA
   - 1.2 ml solution B
8) Invert the tube to mix and incubate 15 min at 28°C.
9) Heat shock the cells at 42°C for 40 min.
10) Centrifuge at 3000 rpm for 30 sec and discard the supernatant.
11) Wash the cells in 1 ml sterile water and centrifuge at 3000 rpm for 30 sec.
12) Resuspend the cells in 5 ml YPDA (15 ml Falcon tube) and let the transformants recover for 1h30.
   
   *If an antibiotic resistance selection is used, incubate 4h.*
13) Centrifuge at 4500 rpm for 5 min and remove the supernatant.
14) Wash the cells with 5 ml SD (minimum medium) and centrifuge 4500 rpm for 5 min.
15) Resuspend the pellet with 500 µl SD and spread on selective medium:
   - 1 plate 1/1000 dilution (~100-120 µl/plate) for enumeration
   - 5 plates non diluted (~100-120 µl/plate)

*Solution A:*
- Li Acetate 10x 10 ml
- TE 10x 10 ml
- Sterile Water 80 ml

*Solution B:*
- Li Acetate 10x 1 ml
Solutions A and B need to be freshly prepared and sterilized by filtration.

Li Acetate 10x:
1M Lithium Acetate in water.
Bring to pH 7.5 by using acetic acid
Sterilize by filtration and store at room temperature

TE 10x:
100 mM Tris-Cl
10 mM EDTA, pH 8.
Bring to pH 8 by using HCl
Sterilize by filtration or autoclaving and store at room temperature
B.4 Isolation of Genomic DNA from Yeast


1) Transfer 1.5 ml of liquid culture of yeast grown for 20 - 24 h at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose) into a microcentrifuge tube. Pellet cells by centrifugation at 20,000 × g for 1-5 minutes.

2) Add 200 µl of Harju- buffer

3) Immerse tubes in a dry ice-ethanol bath for 2 minutes,

4) Transfer to in a 95°C water bath for 1 minute.

5) Repeat the last two steps

6) Vortex 30 seconds.

7) Add 200 µl of chloroform and vortex 2 minutes.

8) Centrifuge 3 minutes at room temperature, 20,000 × g.

9) Transfer the upper aqueous phase to a microcentrifuge tube containing 400 µl ice-cold 100% ethanol. Mix by inversion or gentle vortexing.

10) Incubate at room temperature, 5 minutes. Alternatively, precipitate DNA at -20°C to increase yield.

11) Centrifuge 5 minutes at room temperature, 20,000 × g.

12) Remove the supernatant with a pulled Pasteur pipette by vacuum aspiration.

13) Wash the pellet with 0.5 ml 70% ethanol

14) Centrifuge 5 minutes at room temperature, 20,000 × g.

15) Remove supernatant.

16) Air-dry the pellets at room temperature or for 5 minutes at 60°C in a vacuum dryer.

17) Resuspend in 25- 50 µl TE (pH 8.0)] or water. Samples obtained directly from plates should be resuspended in a 10 µl volume, because the yield will be smaller. 0.25 µl RNase cocktail should be added to the samples used for Southern blot hybridization (final concentration 0.125 U RNase A, 5 U RNase T1).

Harju- Buffer:
2% Triton X-100
1% SDS,
100 mM NaCl
10 mM Tris-HCl, pH 8.0,
1 mM EDTA
**B.5 Extraction of library DNA from *E. coli*.**

Protocol provided by Dr. Patrice Hamel.

1. Resuspend the cell pellet of 100,000-150,000 bacterial clones (or 500 ml of culture at OD\textsubscript{600nm} = 0.4) in 18 ml of solution I.
2. Vortex to resuspend well the cell pellet and transfer into 4 Nalgene tubes (4.5 ml/tube).
3. For each tube, add 500 µl of lysozyme 10 mg/ml (solution I) and 50 µl of RNase A 10 mg/ml. Let it stand for 5 min at room temperature.
4. Add 10 ml of solution II freshly prepared.
   Mix by inverting (do not vortex) and incubate 10 min on ice.
5. Add 5 ml of solution III.
   Mix by inverting and incubate 5 min on ice.
6. Centrifuge 25 min at 4°C at high speed (25,000 g).
7. Transfer the supernatant into a fresh tube. If the supernatant is cloudy, re-centrifuge for 20 min and save the supernatant.
   *Avoid as much as you can to pipet any white precipitate at this stage.*
8. Add 0.6 volume of isopropanol and mix by inverting.
9. Centrifuge at high speed for 15 min at 4°C and discard the supernatant.
10. Wash the pellet in 2.5-5 ml of 70% ethanol and re-centrifuge for 10 min.
11. Remove ethanol and dry the pellet (air or speed vacuum).
12. Resuspend all the pellets in 4 ml of sterile water and transfer into a fresh tube.
13. Add equal volume of phenol/chloroform/isoamyl alcohol (4 ml) and vortex.
14. Centrifuge 10 min at 5,000 rpm and transfer the aqueous phase into a Nalgene tube.
15. Add 0.25 volume (1 ml) of 4M NaCl and 1.25 volume (5 ml) of 13% PEG 8,000.
16. Mix and incubate at least 30 min at 4°C.
   *Overnight incubation will increase the yield of DNA recovery.*
17. Centrifuge at high speed for 15 min at 4°C and remove carefully the supernatant.
18. Wash with 10 ml of 70% ethanol and re-centrifuge for 10 min.
19. Remove ethanol and dry the pellet (air or speed vacuum).
20. Resuspend the pellet in 500 µl of water.
Solution I:
50 mM glucose
10 mM EDTA
25 mM Tris-HCl, pH 8

Solution II:
0.2 M NaOH
1% SDS

Solution III:
3 M potassium acetate, pH 4.8
5M Acetic Acid

Solution I and III are kept at 4°C. Solution II is stored at room temperature.
B.6 Electrotransformation of bacteria

This protocol was provided by Dr. Patrice Hamel and was communicated by Pr. L. Th1ny-Meyer, Zurich, Switzerland.

1) Thaw electrocompetent cells on ice and SOC medium at room temperature.
2) Place sterile electroporation cuvette on ice.
3) Mix 40 µl of electrocompetent cells with 1-2 µl of DNA.
4) Incubate for 1 min on ice and transfer into a cold electroporation cuvette.
5) Set the Gene Pulser apparatus at 25 pF, 200 O and 2.5 kV (with 0.2 cm cuvettes) or 1.5-1.8 kV (with 0.1 cm cuvettes).
6) Place the cuvette in the chamber and pulse until hearing a beep.
7) Remove the cuvette, transfer the cells in 1 ml of SOC medium (microfuge tube).
8) Incubate at 37°C for 1h.
9) Plate on selective medium.

SOC medium:
Yeast extract 0.5% (w/v)
Tryptone 2% (w/v)
NaCl 10mM
KCl 2.5mM
MgCl₂ 10mM
MgSO₄ 20mM
Glucose 20mM
B.7 RNA Isolation by Guanidine Extraction

1) Spin the cells down (3000 RPM) and wash with 1X PBS
2) Resuspend the pellet in 500µl Solution D and vortex.
   a. Can be stored at -20°C here
3) Add 50µl 2M NaOAc pH 4.0 and 500 µl water saturated phenol
4) Vortex 1 minute and place on ice for 10 min.
5) Centrifuge 12,000 RPM at 4°C for 15 min.
6) Transfer supernatant into a new tube and add equal volume of isopropanol and 1µl glycogen
7) Store at -20°C 20 minutes to overnight or longer
8) Centrifuge max speed for 30 minutes at 4°C
9) Wash with 70 % ethanol
10) Dissolve pellet in 100-200µl water
11) Extract with 0.5vol of phenol (Tris pH 8.0) and 0.5vol of chloroform/IAA
12) Precipitate with 2vol of ethanol, 0.1vol 3M NaOAc, 1 µl glycogen
13) Store at -20°C 20 minutes to overnight
14) Centrifuge max speed for 30 minutes at 4°C
15) Resuspend in 20-200 µl water

Solution D:
4M guanidine isothiocyanate
25mM sodium citrate pH 7.0
B.8 High-resolution polyacrylamide gel for RNA

1) Pipet 10 ml of 8M urea/8% acrylamide into 15 ml tube
2) Add 60 μl 10% APS and 10 μl TEMED
3) Mix well and pour quickly into small protein apparatus with 1.5 mm spacers
4) Incubate 10 μg of total and 2.5-5 μg of mitochondrial RNA in 50% urea loading dye at 70°C for 2 min and load on the gel
5) Run gel in 1X NNB 100V for approximately 2 hours
6) Incubate the gel in 1X NNB+EtBr to visualize RNA
7) Wet blot transfer to Zeta probe membrane (Biorad) in 0.5X NNB, 70V – 143 mA, 1h
8) UV crosslink (auto-crosslink)

8M Urea/8% acrylamide (500ml):
210g Urea
100ml 40% acrylamide
B.9 Formaldehyde agarose gel electrophoresis

Preparation of denaturing agarose gel

1) Heat 1g of agarose in 72ml of water until dissolved and cool until 60°C
2) Add 10ml 10X MOPS running buffer and 10ml of 37% (12.3M) formaldehyde
3) Pour gel into casting tray with comp and allow to cool until gel has formed

Preparing samples

1) Add 2 volumes of 1.5X formaldehyde loading dye to 1-3μg of RNA
   Note: The ratio of dye to RNA may need to be optimized
2) Heat samples at 70°C for 5-15 minutes

Loading and running gel

1) Assemble gel and gel tank according to specific manufacturer instructions
2) Add sufficient 1X MOPS running buffer to cover the top of the gel
3) Load sample into gel
4) Electrophorese at 5V/cm of gel length until bromophenol blue dye has migrated at least half the length of the gel.
5) Visualize with UV light

10X MOPS buffer:
200mM MOPS, pH 7.0
80mM Sodium Acetate
10mM EDTA pH 8.0
Store at room temperature protected from light

1.5X Formaldehyde load dye:
150μl formamide
52.5μl formaldehyde
39μl 10X MOPS
0.75μl 5mg/ml ethidium bromide

Make fresh!
B.10 Northern blot

1) Pre-hybridize membrane 30-60 minutes in HYB solution inside glass tube

2) Prepare gamma labeled oligonucleotide by 60 minutes incubation at 37°C
   
   1 µl 40 µM oligo
   1 µl 10X PNK buffer
   1 µl PNK
   1 µl γ-³²P-ATP
   6 µl water

3) G25 purify labeled oligo with G25 column

4) Denature purified oligo by heating 100°C 5min and rapidly cool on ice

5) Hybridize overnight in an rotating oven

6) The next day, rinse membrane and bottle with WASH1 without Denhards solution

7) Wash with WASH1 20 minutes

8) Pour out WASH1 and wash 20 minutes more with WASH2

9) Please membrane on wet filter paper and wrap in plastic wrap

10) Expose to Phosphoimager screen overnight

11) Develop with scanner

12) Strip membrane by boiling 2 x 20minutes in stripping solution to completely cover membrane

HYB solution (500ml):
125 ml 20X SSC
10ml 1M phosphate buffer pH 7.2
35g SDS
5ml 100X Denhards solution
5ml salmon sperm DNA (100mg/ml)
Water up to 500ml

WASH1:
75ml 20X SSC
125ml 20% SDS
12.5ml NaH₂PO₄ pH 7.5
237.5ml water

**WASH1 + Denhards:**
- 135ml WASH1
- 15ml 100X Denhards

**100X Denhards:**
- 2% Ficoll 400
- 2% Polyvinylpyrrolidone
- 2% Bovine Serum Albumin (BSA)

**WASH2:**
- 50ml 20X SSC
- 50ml 20% SDS
- 900ml water

**Stripping solution:**
- 5ml 20X SSC
- 5ml 20% SDS
B.11 Two-dimensional TLC

*RNase T2 digestion*

Incubate reaction at 37°C overnight and then dry to a pellet in SpeedVac with heat.

**Reaction:**
- tRNA
- 10X T2 buffer
- RNaseT2
- Water

*Kinase Reaction*

Radiolabel the 5’ end of free nucleotides with gamma ATP and kinase after suspending pellet in 7µl of water. Incubate 1 to 3 hours.

**Reaction:**
- 7µl nucleotides from T2 digestion
- 1.5µl 10X kinase buffer
- 1.5µl 10X BSA
- 4µl Gamma ATP
- 1µl Kinase

*Glycerokinase treatment*

Transfer extra gamma ATP to glycerol using glycerokinase for better resolution in TLC. After the reaction, extract aqueous phase using chloroform (aqueous is top) followed by water saturated ether (aqueous is bottom). Finally, SpeedVac the sample to a pellet.

**Reaction:**
- 3µl 0.2M glycerol
- 1µl glycerokinase
- 15µl kinase reaction
- 8µl water

*P1 digestion*

Remove 3’ phosphates from nucleotides to generate 5’phosphate nucleotides with 3’ hydroxyl termini. After the reaction dry to a pellet in SpeedVac.

**Reaction:**
- Pellet suspended in 8µl water
- 1µl 10X P1 buffer
- 1µl Nuclease P1
Running TLC

1) Spot a small volume (2µl up to 10µl) 1cm from the bottom and 1cm from the left side of a TLC plate of 9.95x9.95cm.
2) Develop in buffer system of choice from the bottom to top and dry completely before rotating 90° counterclockwise and developing in the second dimension (left to right with respect to original spot).
3) Dry plate and expose overnight.
B.12 Silver staining protein gel


1) Fix protein gel by incubating in fixative solution for 30 to overnight
2) Rinse gel 4 x 5 minutes in milli-Q water to remove all acid
3) Sensitize gel by incubating in Solution A for 5 minutes
4) Rinse gel 4 x 5 minutes in milli-Q water
5) Incubate gel in Solution B for 20-40 minutes
6) Pour off silver containing Solution B in a dedicated waste container
7) Rinse very quickly with 3 times milli-Q water to remove minimal silver from the gel
8) Incubate gel in Solution C until staining develops
   Monitor closely, it can develop very quickly!
9) Quench staining by removing Solution C and washing 2 times in 1% acetic acid

Fixative:
40% Ethanol
10% Acetic Acid

Solution A:
0.02% Na₂S₂O₅·5H₂O

Solution B:
0.1% AgNO₃

Solution C:
6% Na₂CO₃
0.02% Formaldehyde
0.0004% Na₂S₂O₅·4H₂O
**B.13 Immunofluorescent microscopy with *T. brucei***

*Count cells and Harvest*

1) Before beginning, bring fresh growth media to room temperature
2) Using a hemocytometer, count the number of cells/ml of culture.
3) Spin down 2 x 10⁷ to 1 x 10⁸ total cells at 1300 x g for 10 min at RT in microfuge tube
4) Resuspend in 1 mL of fresh media appropriate for the cell line and transfer to a fresh culture flask with 9mL of the same fresh appropriate media

*Mitotracker staining*

1) Add 4µL of 500µM Mitotracker stock solution
2) Incubate at 27°C shaking for 30 minutes
3) Spin cells 1300 x g for 10 min at room temperature
4) Wash with 2mL of 1X PBS and spin 1300 x g for 10 minutes at RT once more
5) Pipet away supernatant and gently suspend in 200µL of 1X PBS

*Fix cells to slide*

1) Add 200µL of 7.4% formaldehyde made fresh by dilution of 2mL 37% stock with 8mL 1X PBS
2) Mix tube well by inversion, do not vortex
3) Apply 50µL of fixed cells to a microscope slide and spread to make approximately 2cm x 2cm area.
4) Incubate 15 minutes at room temperature in a humid chamber (any sort of box with a moist towel not in contact with the slide will suffice)
5) Remove formaldehyde/cell mixture and wash 3X with 100µL of 1X PBS
   a. To wash – slowly pipette PBS onto the cloudy area. Tilt the slide to ~45° angle and pipette away liquid from the lowest point. Repeat for all washes.

*Permeabilize cells*

1) Pipette to the 2cm x 2cm area of the slide 100µL of 0.1% Triton X-100 made by dilution with PBS and incubate 10 minutes in humid chamber
2) Remove Triton/buffer and wash 3X with 1X PBS

*Block cells*

1) Block cells 45-60 minutes with 100µL of 5.5% FBS in 1X PBS+0.05% Tween
2) Remove buffer and wash 2X with 1X PBS
**Primary Ab**

1) Add 50µL of 1°Ab at desired dilution in 3% BSA in 1X PBS-0.05% Tween
   Usually 1:200 dilution is sufficient for commercial antibodies
2) Incubate 1 hour at RT in humid chamber
3) Remove buffer and antibody, wash 3X with 100µL of 1X PBS-Tween and 2X with 100µL 1X PBS

**Secondary Ab**

1) Add 50µL of 2°Ab at desired dilution with 3% BSA in 1X PBS-T
   a. If using Mitotracker Red and DAPI, secondary must be FITC
   b. Usually 1:400 dilution is sufficient for commercial antibodies
2) Incubate 1 hour at RT in humid chamber
3) Remove buffer and antibody, wash 3X with 100µL of 1X PBS-Tween and 2X with 100µL 1X PBS

**DAPI stain**

1) Dilute DAPI stock 1:1000 in 1X PBS
2) Add 100µL of diluted DAPI to the slide and incubate 1 minute
3) Wash 2X with 1X PBS
4) Remove final liquid and allow to completely air dry

**Mount cells**

1) Add a small amount of mounting media (VectaShield) on top of slide area with cells (~15µL, two drops from a Pasteur pipette)
2) Cover with coverslip
3) Using a paper towel, gently press coverslip to slide and remove all bubbles from underneath/edges. A little bit of mounting media will ooze out the sides
4) Seal edges with a very thin layer of nail polish
5) Allow slide to completely dry for about 30 minutes in complete darkness

**Visualize**

1) Use fluorescent microscope to visualize cells on slide for red, blue, green, etc. channels according to antibodies and dyes used above
B.14 Polysome analysis of *T. brucei* ribosomes

1) Supplement 5 x 10^8-10^9 cells in log phase growth (0.5-1.0 x 10^7 cells/ml) with 100µg/mL cyclohexamide (final concentration) for 10 minutes while shaking at 27°C

2) Centrifuge 5 minutes at 5,000rpm and wash with 15ml of ice-cold phosphate buffered saline (PBS) supplemented with 100µg/mL cyclohexamide (final concentration)

3) Centrifuge again and resuspend in 790µl of polysome buffer supplemented with 100µg/ml cyclohexamide, 2mM DTT, 1X protease inhibitor cocktail, 1µL RNasin (20 U/µL)

4) Lyse cells by adding 10μl of 10% NP-40 (0.25% final concentration) and incubate 5 min on ice

5) Centrifuge lysates at 15,000 x g at 4°C for 10 minutes

6) Remove supernatants into a new tube and take an A_260 measurement of a 1:200 dilution to determine gradient loading volume

7) Load 2-3mg of RNA supernatant on a linear 10-50% sucrose gradient (11ml in polysome buffer) and centrifuge at 4°C for 2h at 36,600 RPM in SW41 Ti rotor (Beckman)

8) After centrifugation harvest gradients from the top to bottom using a gradient fractionator measuring A_254nm throughout the harvest. Gradients can be pushed up with a 55% sucrose cushion and fractions are collected manually following A_254nm reading in cuvette

9) Ribosomes in each fraction can be pelleted by centrifugation for 2 hours at 45,000 rpm in Type 50.2 Ti rotor (Beckman) using polypropylene tubes. Collected samples (~1mL) must be diluted using polysome buffer to allow pelleting without dissociation of ribosomes/subunits, especially in high sucrose fractions.

**Polysome Buffer:**
- 10mM Tris-HCl pH 7.5
- 300mM KCl
- 10mM MgCl₂

**Notes:**
1) 50mL of mid-log culture is sufficient to produce a good profile with 2-3mg per gradient
2) I used ISCO UA-6 UV Vis detector and Type 11 optical unit with sensitivity set at 2.0 and paper speed of 60cm/hr for a 12 ml tube with at most 3mg loaded – this produced a nice profile of 40S, 60S, 80S and polysomes and no peaks over the maximum of the machine.
3) The pelleted ribosome containing fractions will often have visible pellets. Resuspend and phenol extract the resulting pellets to isolate RNA. Concentrations are usually over 1µg/µl for a 20µL resuspension.
References
Jensen BC et al. The NOG1 GTP-binding Protein Is Required for Biogenesis of the 60 S Ribosomal Subunit. JBC 2003 276(34) 32204-32211.
B.15 Subunit analysis of *T. brucei* ribosomes

1) Harvest cells by centrifugation 5 minutes at 5,000 rpm
2) Wash cells with 15ml of ice-cold phosphate buffered saline (PBS) and centrifuge again 5 minutes at 5,000 rpm
3) Resuspend pelleted cells in 790µl of dissociating polysome buffer supplemented with 2mM DTT, 1X protease inhibitor cocktail, 1µL RNasin (20 U/µL)
4) Lyse cells by adding 10µl of 10% NP-40 (0.25% final concentration) and incubate 5 minutes
5) Centrifuge lysates at 15,000 x g at 4°C for 10 minutes
6) Remove supernatants into a new tube and take an A_{260} measurement of a 1:200 dilution to determine gradient loading volume
7) Load 0.5mg of RNA supernatant on a linear 7-27% sucrose gradient in dissociating polysome buffer by centrifugation at 36,600 rpm and 4°C for 3.5hrs in SW41 Ti rotor (Beckman)
8) Harvest gradients from the top to bottom using a gradient fractionator measuring A_{254nm} throughout the harvest. Gradients can be pushed with a 30% or higher sucrose cushion. Fractions are collected manually following the A_{254nm} reading in cuvette

**Dissociating polysome buffer:**

10mM Tris-HCl pH 7.5
300mM KCl

**Notes:**

1) I used ISCO UA-6 UV Vis detector and Type 11 optical unit with sensitivity set at 2.0 and paper speed of 60cm/hr for a 12 ml tube with at most 0.5mg loaded
2) It is also possible to use 1M KCl instead of leaving out Mg^{2+} for dissociation but this makes samples very salty for further manipulation
3) Fractions can be diluted and pelleted by centrifugation at 45,000 rpm 2hrs in Type 50.2 Ti rotor (Beckman) using polypropylene tubes with metal caps and rubber inserts. Ribosome pellets are often visible. Suspend pelleted ribosomes in polysome buffer. Flash freeze in liquid N\textsubscript{2} to store at -80°C.