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tRNA Biology in Trypanosomes

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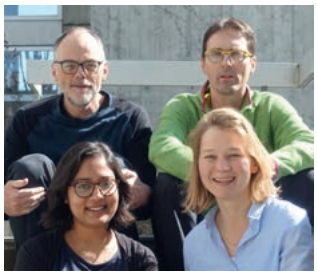
Abstract: Besides their medical importance, the parasitic protozoan *Trypanosoma brucei* and its relatives are experimentally highly accessible model systems for many cell biological processes. Trypanosomes are phylogenetically essentially unrelated to the popular model eukaryotes, such as yeast and animals, and thus show several unique features, many of which are connected to RNA. Here we review the tRNA biology of trypanosomes. Even though tRNAs were already discovered 60 years ago, owing to current technological advances in the field, research on tRNA biology has seen a Renaissance in recent years. First we discuss the extensive mitochondrial tRNA import process and the consequences it has for the parasite. Next we focus on trypanosomal aminoacyl-tRNA synthetases, some of which may be exploited as drug targets. Furthermore, we summarize what is known about trypanosomal tRNA modifications in both the cytosol and the mitochondrion. Finally, we provide an overview on the emerging field of tRNA-derived fragments and their possible function as translation regulators.

Keywords: Aminoacyl-tRNA synthetases · tRNA · tRNA-derived fragments · tRNA modification · *Trypanosoma brucei*



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1. Introduction

The parasitic protozoan *Trypanosoma brucei* and many of its relatives are the causative agents of devastating diseases in both humans and animals.[1] However, in addition to their clinical importance trypanosomes have also proven to be excellent model systems for eukaryotic cell biology. There are two main reasons for this: (i) they are phylogenetically only remotely related to essentially all popular eukaryotic model systems such as yeast, mammals and other animals[2] and (ii) they are an experimentally highly amenable system. Thus, molecular genetic methods such as gene replacement by homologous recombination, tight control of gene expression by the tetracycline repressor, inducible highly efficient RNAi, *etc.* are being used on a routine basis since many years.[3]

Decades of research with trypanosomes has led to a number of landmark discoveries that had impact on cell and molecular biology in general.[4] In the field of RNA biology, they include that RNA polymerase II transcription of protein coding genes is polycistronic, constitutive and strictly coupled to trans-splicing to an identical leader sequence.[5] Another example is RNA editing, the post-transcriptional change of an mRNA sequence, which was originally discovered in the mitochondrion of trypanosomatids.[6] Today we know that RNA editing is a ubiquitous process, although its mechanisms vary in the different systems.[7] The aim of this review is to summarize another well studied but less known aspect of trypanosomal RNAs, namely the biology of its tRNAs.

The *T. brucei* genome encodes 50 tRNA genes specifying 40 different isoacceptors. Most trypanosomal tRNA genes are found in clusters of two to five tandemly repeated genes showing both sense and anti-sense orientations.[8] As in other eukaryotes, tRNA genes are transcribed by RNA polymerase III. The $tRNA^{Tyr}$ is the only one that requires splicing for its maturation.[9] Its 11 nucleotide intron is removed by orthologues of the eukaryotic-type tRNA splicing system in the cytosol.[10] Unexpectedly, splicing of the $tRNA^{Tyr}$ depends on a single G to U and several G to A editing events within the intron.[11]

In summary, trypanosomal tRNAs look like bona fide eukaryotic tRNAs, there is nothing unusual about them. However, if we look at their genetic origin, the situation looks different. Whereas tRNAs involved in mitochondrial translation are normally encoded on the organellar genome, this is not the case in trypanosomes whose mitochondria completely lack tRNA genes.

2. Mitochondrial tRNA Import (Fig. 1)

18 proteins are encoded on the mitochondrial genome of trypanosomes and produced within the organelle. In order to compensate for the lack of tRNA genes, trypanosomal mitochondria therefore must import all tRNAs from the cytosol in quantities sufficient to support mitochondrial translation.[12] Mitochondrial tRNA import is widespread but has experimentally been analyzed in few systems only.[13] The number of tRNAs postulated to be imported is highly variable. Whereas many systems lack some mitochondrial tRNA genes, they have retained at least a few tRNA genes in the mitochondrial genome. Thus, the complete lack of mitochondrial tRNA genes as seen in trypanosomes is unusual and makes trypanosomes an excellent system to study tRNA import into mitochondria.

2.1 *Specificity of tRNA Import*

In trypanosomes, the set of mitochondrial tRNAs completely overlaps with the set that is found in the cytosol. Thus, there are single nuclear gene products which code for both cytosolic and mitochondrial tRNAs. In all cases, the major fraction of a given tRNA is found in the cytosol, where it functions in cytosolic translation, and only a small fraction – between 1 and 14% depending on the tRNA species – is imported into mitochondria, where it is used for mitochondrial protein synthesis.[8c,12] There are no nuclear genes coding for mitochondria-specific tRNAs, not in trypanosomes and also not in all other systems shown to import mitochondrial tRNAs. However, while all mitochondrial tRNAs from trypanosomes are imported from the cytosol, there are two cytosol-specific tRNAs: the initiator $tRNA^{Met\text{-}i}$[14] and the $tRNA^{Sec}$.[15] The eukaryotic-type initiator $tRNA^{Met\text{-}i}$ could not possibly function in the context of the bacterial-type mitochondrial translation system and selenocysteine insertion systems are not found in organelles, it therefore makes sense that these two tRNAs stay in the cytosol.

What determines the specificity of tRNA import? It has been shown that the genomic context from which the tRNA is expressed is not important for mitochondrial import.[16] Moreover, there is no feature specific for trypanosomal tRNAs that promotes their import, because a yeast and a human tRNA were also imported when expressed in trypanosomes.[16,17] The targeting signal for tRNAs was identified by expressing chimeras between the cytosol-specific $tRNA^{Met\text{-}i}$ and the in part imported elongator $tRNA^{Met\text{-}e}$, which differ by 26 nucleotides only.[18] The results showed that a switch of two adjacent base pairs in the T-stem of the elongator $tRNA^{Met\text{-}e}$ or initiator $tRNA^{Met\text{-}i}$ was sufficient to prevent or induce mitochondrial import of the corresponding tRNA variants. Interestingly, the two nucleotide pairs in the initiator $tRNA^{Met\text{-}i}$ overlapped with the antideterminants for eukaryotic elongator factor 1a (EF1a) binding as defined in other systems.[15] This suggests that for a tRNA to be imported into mitochondria, it needs to interact with EF1a. Indeed, as the $tRNA^{Sec}$ does not interact with EF1a but has its own elongation factor this also explains the exclusive cytosolic localization of the trypanosomal $tRNA^{Sec}$. In line with this, a variant of the $tRNA^{Sec}$ in which the anti-determinant for EF1a binding (the eighth base pair in the acceptor stem) was mutated and binding of EF1a was induced resulted in mitochondrial import of the variant $tRNA^{Sec}$.[15] The direct requirement for EF1a for mitochondrial tRNA import was finally demonstrated by the fact that its ablation, in contrast to ablation of initiation factor 2 (IF2), abolished mitochondrial tRNA, whereas the effects on translation were identical after ablation of either of the two translation factors.[15] Thus for a tRNA to be imported into mitochondria of trypanosomes it first needs to interact with EF1a in the cytosol. Since only aminoacylated tRNAs interact with EF1a, this strongly suggests that, unlike initially proposed,[19] tRNAs are imported as fully processed and aminoacylated molecules.[20] The precise role EF1a plays in tRNA import remains unclear. The fact that import of tRNA is not affected by ablation of IF2 and therefore does not depend on cytosolic translation suggests a direct role of EF1a that is not related to translation. Since EF1a is a cytosolic protein that is not imported into mitochondria it could be that it brings the tRNA to the vicinity of mitochondria. Having said that, the requirement of EF1a is not absolute, since a $tRNA^{Tyr}$ variant, that is deficient in splicing and cannot be aminoacylated and therefore presumably does not interact with EF1a, nevertheless is imported, albeit less efficiently.[21]

Besides the import specificity an important question is, how the extent of import, which varies by a factor of ten or more between different tRNAs,[8c] is determined. Presently we do not have an answer to this question.

2.2 *OM Proteins Required for tRNA Import*

The tRNA import process starts in the cytosol by binding of tRNAs to EF1a which explains the specificity of the process. However, the most difficult to understand step is how tRNAs, highly charged macromolecules of *ca.* 25 kDa mass, are translocated across the two mitochondrial membranes. Recently, some progress has been made regarding which proteins in the mitochondrial outer membrane (OM) are required for the process. This was possible because the OM proteome of *T. brucei* had been characterized.[22] It was therefore clear that whatever proteins are required for tRNA translocation across the OM, they must be among the 82 proteins making up the OM proteome. It had previously been shown that the most abundant OM protein, the voltage dependent anion channel (VDAC), unlike in the plant system,[23] is not required for tRNA import.[24] The same was the case for Sam50, the core subunit of the OM localized β-barrel protein insertase.[25] Next the subunits of the main trypanosomal protein translocase of the OM (TOM) were analyzed for their involvement in tRNA import. The trypanosomal TOM complex is unique in that only two of its seven subunits have orthologues in other eukaryotes.[26] It was therefore termed atypical TOM complex (ATOM).[27] Using inducible RNAi cell lines for individual ATOM subunits allowed to determine the effects the lack of each subunit has on protein and tRNA import, respectively. The results showed that while the ATOM core subunits (ATOM40, ATOM14, ATOM12 and ATOM11) were required for both protein and tRNA import, the two protein import receptors (ATOM46, ATOM69) were necessary for protein import but dispensable for tRNA import.[28] Thus, in cell lines ablated for ATOM46, ATOM69 or for both receptors at the same time, tRNA import could be uncoupled from protein import. Further experiments showed that plugging the ATOM40 import pore by a stuck precursor protein simultaneously inhibited protein and tRNA import.[28] These experiments show that both proteins and tRNAs use the β-barrel protein ATOM40 to translocate across the OM. However, unlike suggested in yeast,[29] tRNAs are not co-imported with proteins, since tRNA import still works in cell lines that cannot import proteins due to depleted protein import receptors. All results described above were obtained by *in vivo* studies and thus are physiologically relevant. Moreover, great care was taken to detect potential unwanted indirect effects. Thus, the mitochondrial proteomes of uninduced and induced ATOM subunit RNAi cell lines were analyzed to determine whether other OM proteins than the RNAi targets were also downregulated.[28] In summary, these analyses corroborated that the inhibition of tRNA import seen in the RNAi cell lines is a direct consequence of the lack of the corresponding ATOM subunits.

2.3 *IM Proteins Required for tRNA Import*

How tRNAs are transported across the mitochondrial inner membrane is less clear. An *in vivo* study has shown that ablation of Tim17, a core subunit of the non-canonical trypanosomal inner membrane protein translocase, affects import of newly synthesized tRNA and the same was the case for mitochondrial heat

shock protein 70 (mHsp70).[25] In both cases, inhibition of tRNA import was observed at a time point where the mitochondrial membrane potential was still intact. Moreover, in a different study mitochondrial membrane proteins were subjected to tRNA affinity purification, which recovered 44 proteins, including mHsp70.[30] Ablation of two trypanosomatid-specific proteins present in this list affected growth and inhibited import of newly synthesized tRNAs *in vivo*. When one of them was tagged and precipitated, it recovered many subunits of the trypanosomal TIM complex.[31] In summary, these results suggest that also in the case of the IM, tRNA import might require similar components to mitochondrial protein import. However, unlike for the ATOM subunits, the mitochondrial proteome of none of the RNAi cell lines was globally analyzed, thus indirect effects, while unlikely, cannot be excluded.

tRNA translocation across the IM has also been studied in *Leishmania tropica*.[32] It was claimed in these studies that a complex of six essential proteins, including the alpha subunit of the F1 ATPase (F1a) and the Rieske protein, mediates the process.[33] However, for the key publication implicating the F1a in tRNA import[32b] an editorial expression of concern has been published.[34] Moreover, it has been shown in two *in vivo* studies that F1a[25] and the Rieske protein[35] are not required for tRNA import in *T. brucei*. Thus, the *Leishmania* studies in question will not be further discussed here.

3. Aminoacyl-tRNA Formation

3.1 Dually Localized Aminoacyl-tRNA Synthetases

Non-plant eukaryotes in general contain a full cytosolic and a full mitochondrial set of aminoacyl-tRNA synthetases (aaRSs), although a few aaRSs might be dually targeted. Due to the lack of mitochondrial tRNA genes, the set of mitochondrial and cytosolic tRNAs are identical in trypanosomes with the exception of the cytosol-specific $tRNA^{Met\text{-}i}$ and the $tRNA^{Sec}$ (see above). It would therefore be expected that the same applies to the trypanosomal aaRSs. A survey of the *T. brucei* genome shows that this indeed

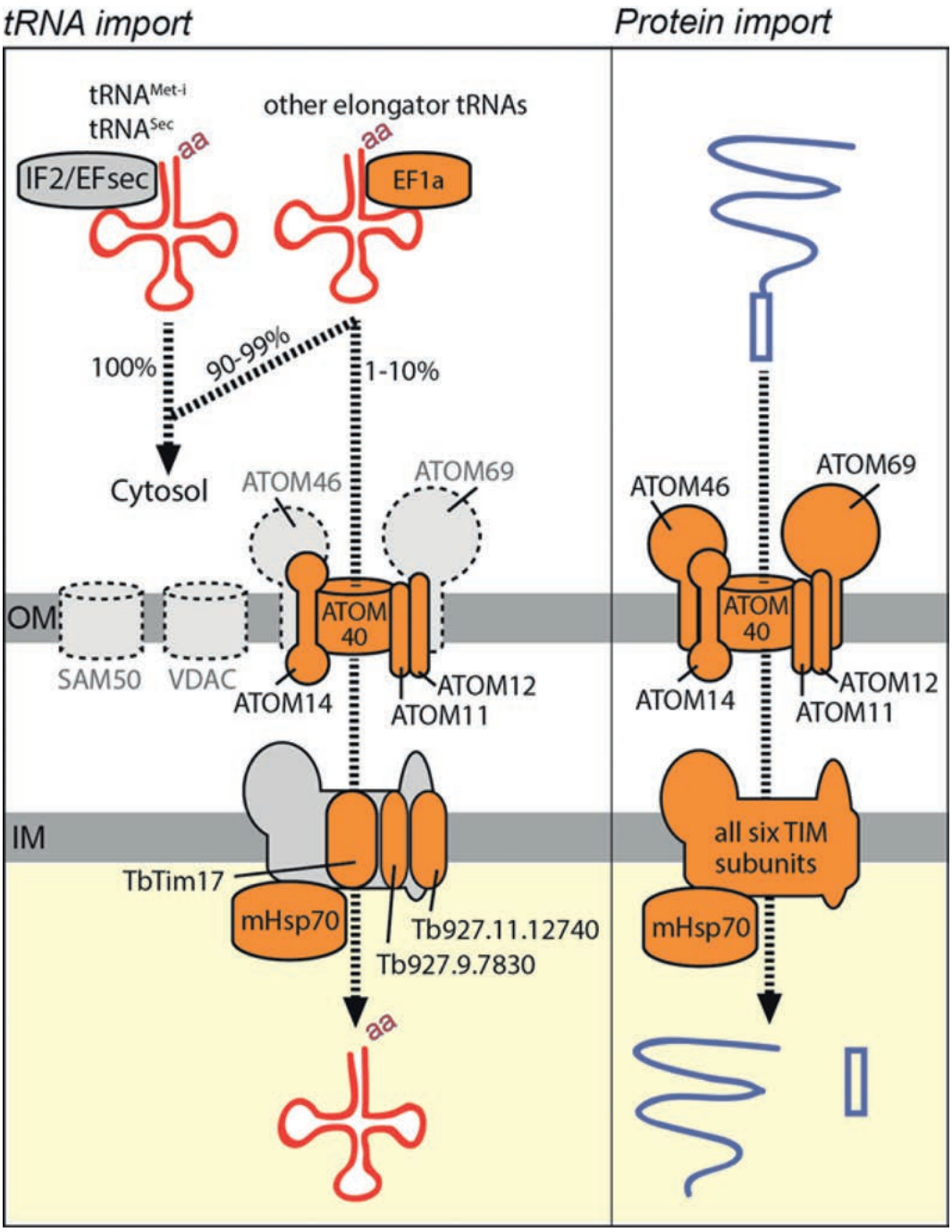

Fig. 1. Mitochondrial tRNA import and its connection to mitochondrial protein import in *T. brucei*. Left panel, mitochondrial tRNA import. Proteins experimentally shown to be essential for mitochondrial tRNA import are indicated in orange. Proteins experimentally shown *not* to be involved in tRNA import are indicated with broken lines and light grey. Binding to EF1a is a prerequisite for tRNA import and explains the specificity of the process. The cytosol-specific $tRNA^{Met\text{-}i}$ and $tRNA^{Sec}$ do not bind to EF1a but to initiation factor 2 (IF2) or selenoscysteine specific elongation factor (EFsec). tRNA import uses the pore of ATOM40 to translocate the OM but is independent of the two protein import receptors ATOM46 and ATOM69, indicating that tRNAs are not co-imported with proteins. Ablation of TbTim17 and mHsp70 inhibits tRNA import, other TIM complex components have not been tested. Tb927.11.12740 and Tb927.9.7830 have also been implicated in tRNA import and appear to be associated with the TIM complex. Right panel, mitochondrial protein import. All orange proteins were shown to be essential for mitochondrial protein import. This includes the two protein import receptors ATOM46 and ATOM69 which are dispensable for tRNA import.

is largely the case. It encodes for 23 different aaRSs, indicating that 17 aaRSs are encoded by single copy genes.[13d,36] Thus, we would expect that these 17 aaRSs function in both cytosolic and mitochondrial translation. Indeed, it has been experimentally verified for the cytosolic and mitochondrial GlnRS, GluRS[37] and TyrRS activities,[38] that each are encoded by a single nuclear gene. Typically, most of a given trypanosomal aaRS resides in the cytosol and only a minor fraction is imported. It can therefore be difficult to distinguish the small amount of aaRSs that is associated with the mitochondrion from cytosolic contamination. Recently, a new method termed importomics was introduced. It determined by SILAC proteomics the level of which mitochondrial proteins are reduced when import is abolished by ablation of the main OM protein translocase.[39] In this study, the levels of 16 mitochondria-associated, predicted to be dually targeted aaRSs (HisRS was not detected) were shown to be reduced in the absence of protein import, directly demonstrating that all trypanosomal aaRSs are dually localized.

The mechanism that allows dual targeting of trypanosomal aaRSs has only been solved for the IleRS.[40] Differential trans-splicing creates a long and a short variant of the enzyme. The protein product of the longer spliced variant has an amino-terminal presequence and therefore is found exclusively in mitochondria. Whereas the shorter spliced variant is translated to a cytosol-specific isoform that lacks the presequence.

3.2 *Aminoacyl-tRNA Synthetases with Distinct Mitochondrial and Cytosolic Isoforms*

While most trypanosomal aaRSs are dually localized, there are two distinct genes each for TrpRS,[41] LysRS[42] and AspRS,[43] which encode cytosol- and mitochondrion-specific enzymes, respectively. This is surprising, since the cytosolic and mitochondrial substrate tRNAs for these aaRSs, as is the case for all other trypanosomal aaRSs, derive from the same nuclear gene.[36,44]

The best understood cases are the two TrpRSs. They share 41% sequence identity, are both essential and of eukaryotic evolutionary origin. TrpRS1, the cytosolic version of the enzyme, aminoacylates cytosolic $tRNA^{Trp}$, but it cannot recognize the $tRNA^{Trp}$ after it has been imported into mitochondria.[41] The reason for this is, that due to the reassignment of the stop codon UGA to Trp inside mitochondria, the C at wobble position of the $tRNA^{Trp}$ gets edited to a U.[45] The edited mitochondrial $tRNA^{Trp}$ can now decode both the UGG Trp-codon as well as the UGA codon, which in the cytosol function as a stop. In addition, the imported $tRNA^{Trp}$ gets thiolated at the U at position 33. Both the edited U at the wobble position and the thiolated U33 act as antideterminants for charging by TrpRS1 as has been shown *in vitro*.[41] Thus, the re-assignment of the stop codon UGA to Trp in the mitochondrion requires two enzymes: i) an editing enzyme converting the wobble C to an U, whose nature is still unknown,[45] and ii) a mitochondria-specific TrpRS, termed TrpRS2, that has an extended substrate specificity and therefore can aminoacylate both cytosolic and imported $tRNAs^{Trp}$.[41]

While the existence of two trypanosomal TrpRSs can be linked to a mitochondrial genetic code variant, it is not known why trypanosomes have distinct cytosolic and mitochondrial AspRSs and LysRSs. The two AspRSs are of the eukaryotic-type and 43% identical. Biochemical assays show the cytosolic enzyme, AspRS1, is specific for the cytosolic $tRNA^{Asp}$ and that it cannot aminoacylate the imported versions of the $tRNA^{Asp}$. The mitochondrial enzyme, termed AspRS2, on the other hand can aminoacylate both cytosolic and imported $tRNA^{Asp}$.[43] This strongly suggests that even though cytosolic and mitochondrial $tRNA^{Asp}$ derive from the same genes, they are physically different, most likely due to a mitochondria-specific nucleotide modification of as yet unknown nature that is an antideterminant for the cytosolic AspRS1. The substrate specificity of the cytosolic and mitochondrial LysRS of trypanosomes has not been elucidated. However, it was shown that in order to be active, the C-terminal extension of the imported version of the enzyme (LysRS2) has to be removed.[42]

3.3 *Formation of Mitochondrial Formyl-Met-$tRNA^{Met\text{-}e}$*

All trypanosomal mitochondrial tRNAs are imported from the cytosol and therefore of the eukaryotic-type. However, they have to function in the context of the bacterial-type translation system of the mitochondrion. While for most tRNAs this is not problematic, the situation is different for the $tRNA^{Met}$ that functions in translation initiation, because this process works very differently in eukaryotic- and bacterial-type systems. In eukaryotes, it requires a dedicated initiator $tRNA^{Met}$ that has an A-T mismatch at the top of the acceptor stem, whereas in bacterial-type systems (including organelles) the initiator $tRNA^{Met}$ has a mismatch at the top of the acceptor stem.[46] Moreover, the bacterial-type $tRNA^{Met\text{-}i}$ not only needs to be aminoacylated by MetRS, but the methionine is further modified to formyl-methionine by a tRNA-dependent formyltransferase. Thus, translation in bacteria and organelles starts with formyl-methionine and only formylated $tRNA^{Met\text{-}i}$ is recognized by initiation factor 2 (IF2), the factor bringing the tRNA to the P-site of the ribosome.

In contrast to the cytosol-specific initiator $tRNA^{Met\text{-}i}$, the cytosolic elongator $tRNA^{Met\text{-}e}$ is imported into mitochondria and it was shown that approximately 50% of it becomes formylated after import. The reaction is catalyzed by an unusual $tRNA^{Met}$ formyl transferase that has twice the mass of its orthologs in bacteria and eukaryotes and that selectively recognizes the imported $tRNA^{Met\text{-}e}$.[14] As shown with *in vitro* experiments using mitochondrial extracts, it does not formylate the eukaryotic initiator $tRNA^{Met\text{-}i}$ present in the cytosol. Thus, in *T. brucei* the single nucleus-encoded elongator $tRNA^{Met\text{-}e}$ has three different functions in two compartments.[47] It mediates translation elongation in the cytosol and is required for both initiation and elongation in mitochondrial translation. Thus, it is the formyl-group that is recognized by mitochondrial IF2 and that allows to discriminate between elongator and initiator function of the imported $tRNA^{Met\text{-}e}$.[48] Finally, as other systems, mitochondria of trypanosomes contain peptide deformylases that remove the formyl group from the N-termini of at least newly synthesized proteins.[49]

3.4 *Formation of Sec-$tRNA^{Sec}$*

Like all other tRNAs, eukaryotic $tRNA^{Sec}$ is transcribed by RNA polymerase III, although it requires extragenic promoter elements comparable to the U6 RNA. In trypanosomes, $tRNA^{Sec}$ genes are embedded within a large polycistron of protein-coding genes.[50] This is in contrast to all other tRNAs, which are clustered in between polycistrons. It was shown that the *T. brucei* $tRNA^{Sec}$ in line with its genomic location, unlike any other tRNA, is transcribed by RNA polymerase II.[50] Consequently, it requires an external promoter for ectopic expression outside the polycistron. A similar situation has been described for *Leishmania*.[51]

Unlike in bacteria, in which $tRNA^{Sec}$ is aminoacylated in two steps by the SerRS, creating Ser-$tRNA^{Sec}$, which in turn is converted into Sec-$tRNA^{Sec}$ by the selenocysteine synthase, eukaryotes and Archeae use a three-step pathway. Eukaryotic $tRNA^{Sec}$ first gets serylated by SerRS. However, unlike in bacteria, a phosphoseryl-$tRNA^{Sec}$ kinase (PSTK) phosphorylates the Ser on the $tRNA^{Sec}$ converting it into phospho-Ser-$tRNA^{Sec}$. Finally, phospho-Ser-tRNA:Sec-tRNA synthase (SepSecS) produces Sec-$tRNA^{Sec}$ as the final product.[52] Studies in *T. brucei* and its relatives have contributed to the characterization of the eukaryotic Sec-$tRNA^{Sec}$ formation pathway.[53] Using a combination of inducible RNAi and knockout cell lines allowed the first *in vivo* analysis of the complete Sec-$tRNA^{Sec}$ formation pathway in a eukaryote. Moreover, the trypanosomal PSTK and SepSecS were able to complement

for the absence of the Sec-tRNA^{Sec} formation in an *E. coli*.[54] The analysis in trypanosomes was greatly facilitated by the fact that neither of the three selenoproteins encoded in its genome[53b] is essential for growth of the parasite.[54,55] Thus, in contrast to the transcription of the tRNA^{Sec} gene, its aminoacylation pathway is highly conserved in all eukaryotes and Archaea.

3.5 *A Trypanosomal Multiple Aminoacyl-tRNA Synthetase Complex*

In eukaryotes, cytosolic aaRSs are generally organized in a multiprotein assembly called the multiple aminoacyl-tRNA synthetase (MARS) complex. In mammals, the MARS complex is composed of nine cytoplasmic aaRSs and three accessory proteins. The organization of aaRSs in MARS complexes in eukaryotes appears to improve the efficiency of tRNA-aminoacylation by tRNA channeling, which avoids tRNA diffusion in the cytoplasm and thus improves translation efficiency. Moreover, the MARS complex is also involved in translation regulation.[56] Recent work has shown that in *T. brucei* six cytoplasmic aaRSs together with three non-aaRSs proteins are also organized in a MARS complex in both bloodstream and insect stage forms.[44] Kinetic studies indicate that the trypanosomal MARS complex enhances the efficiency of tRNA-aminoacylation. Ablation of MCP2, one of the three non-aaRS proteins associated with the trypanosomal MARS complex, resulted in reduced growth of bloodstream form cells when grown in culture and in mice, respectively, indicating that the MARS complex might be a suitable target for drug development.[44]

3.6 *Trypanosomal Aminoacyl-tRNA Synthetases as Drug Targets*

As might be expected from their biological function, all tested trypanosomal aaRSs were essential in both the insect stage and the disease-causing bloodstream form.[44,57] Thus, aaRSs might also be suitable drug targets to combat the diseases caused by *T. brucei* and its relatives. This has motivated a number of studies with the aim to identify inhibitors of trypanosomal aaRSs as lead substances for drug development:

- A library of substances mimicking the lysyl adenylate complex has been produced using solid phase combinatorial synthesis and the resulting compounds were screened for inhibition of *in vitro* aminoacylation activity of *T. brucei* cytosolic LysRS (LysRS1). The screen identified three substances that inhibited LysRS1 in the low μM range.[58]
- The single trypanosomal MetRS shows more similarities to bacterial than to eukaryotic enzymes, suggesting it might be a good drug target.[44,59] A collection of aminoquinolone-based compounds, which are potent inhibitors of bacterial MetRSs, were synthesized and tested for inhibition of the MetRS and growth of bloodstream form of *T. brucei*. Some of the tested substances inhibited growth of *T. brucei* in the nM range, but did not interfere with growth of mammalian cells at high μM concentrations. The most potent compound delayed mortality in a murine model of trypanosomiasis.[59] In a different study, urea-based MetRS inhibitors were designed, synthesized and tested. This led to the discovery of low nM inhibitors with high selectivity towards the *T. brucei* enzyme when compared to the human one. One of them also inhibited growth of the parasite at μM concentration and another compound was shown to cross the blood brain barrier in mice. However, only limited suppressive action was observed in a murine model of trypanosomiasis.[60] A high-throughput screen of the 'NIH Molecular Libraries Small Molecule Repository' was also performed. It identified substances that inhibit trypanosomal MetRS and growth of *T. brucei*. The most potent one was subjected to structure-guided optimization, resulting in novel MetRS inhibitors with good potency.[61] Interestingly, it appears that resistance against the MetRS inhibitors described above develops only relatively slowly when compared to common anti-trypanosomal drugs. Moreover, unlike what was observed for other drugs, the resistance that was eventually obtained was due to overexpression of the target enzyme.[62] In yet another study, a large chemical library of 364'131 small molecules was screened to find inhibitors of MetRS activity. A counterscreen for toxicity and inhibition of growth of bloodstream form *T. brucei* were also performed, resulting in the identification of a number of compounds with sub μM potency.[63] MetRS was also investigated as a drug target in the trypanosomatid *Leishmania donovani*. High-throughput screening identified a highly potent inhibitor of the leishmanial enzyme which bound to the methionine pocket of MetRS. However, the compound was not active in an animal model of leishmaniasis.[64]
- One study targeted the single trypanosomal IleRS. It showed that analogues of the Ile-AMP efficiently inhibited the enzyme. Moreover, the molecules were shown to selectively kill *T. brucei* in culture and one compound was able to cure trypanosome-infected mice. This makes Ile-AMP analogues promising leads for drug development, especially since some of them are known to cross the blood brain barrier.[65]
- Using benzoxaborole as the core, a collection of compounds was tested for inhibition of the single trypanosomal LeuRS. The substances were optimized to target the editing site of LeuRS using a structural homology model of the enzyme. This led to the discovery of inhibitors with an IC_{50} of less than 1.6 μM that also inhibited growth of the parasites but not of mammalian cells.[66] In an extension of the study, docking-based virtual screening identified a new 2-pyrrolinone scaffold that inhibited the synthetic domain of trypanosomal LeuRS.[67]
- The activity of the single trypanosomal ThrRS, as well as growth of bloodstream trypanosomes, were inhibited by borrelidin, a natural product polyketide, in the nM and μM range, respectively.[57]
- Incubation of isolated, recombinant HisRS containing a bound histidine of *Trypanosoma cruzi* with 68 different cocktails from the medical 'Structural Genomics of Pathogenic Protozoa' fragment library identified 15 fragments that inhibited HisRS activity. Subsequently, their anticipated binding mode was confirmed crystallographically.[68] These substances may form the basis for the development of highly selective HisRS inhibitors in the future.

4. tRNA Modifications

4.1 *tRNA Molecules are Densely Decorated by Nucleoside Modifications*

All nucleic acids in a cell are subjected to chemical modifications as a post-replicative or post-transcriptional process. The biogenesis of these chemical modifications is driven by a vast number of highly specific enzymes capable of introducing diverse chemical functional groups on their nucleic acid substrates. RNA modification enzymes harbor a broad reaction repertoire including deaminations, isomerizations, glycosylations, thiolation, transglycosylation or methylations (reviewed in ref. [69]). Even more complex modifications are possible, in which sugars or amino acids are attached to the nucleobase base or ribose, respectively. While DNA modification is dominated by methylations, the chemical repertoire of post-transcriptional RNA alterations is significantly more diverse. Until today, about 150 unique RNA modifications have been characterized across all three domains of life.[70]

tRNA molecules are the most frequently targeted class for nucleic acids modifications and carry, on average, 13 modifications per molecule.[71] On average 17% of residues are post-transcriptionally altered, which is almost ten times more compared

to rRNA modifications (average 1–2% of residues are modified) (reviewed in ref. [69]). Nonetheless, there is a considerable quantitative variety of tRNA modifications between different organisms and even within similar tRNA isoacceptors. In general, modifications of tRNAs increase in abundance from bacterial and organellar to eukaryotic tRNAs. Not only does the density of post-transcriptionally altered residues vary but also the composition of modifications. Some of the chemical tRNA modifications are common at specific positions and show deep evolutionary conservation in all three domains. Other modifications are highly specific to a particular organism or even to a specific tRNA isoacceptor. A common set of 18 modifications in tRNAs are universally conserved and typically involve rather simple chemical alterations, such as the addition of a methyl group (reviewed in ref. [69]). Less conserved chemical modifications tend to be more complex in chemical composition and involve intricate biogenesis pathways. These more complex alterations, also called hypermodifications, are generally located in the anticodon loop of tRNAs (reviewed in ref. [72]). Experimental identification, mapping and functional characterization of RNA modifications have been challenging. However, new technical developments in mass spectrometry together with biophysical, biochemical and new genetic approaches led to a significant progress studying post-transcriptional modifications recently. Nevertheless, it still remains a challenging endeavor to uncover the functional relevance of a particular tRNA modification since they are often functionally interdependent on a network of neighboring nucleoside modifications.

4.2 *Functions of tRNA Modifications*

While it is often difficult to pinpoint the cellular role of an individual tRNA modification, global hypomodification was shown to correlate with decreased efficiency of protein biosynthesis, resulting in imbalances in proteostasis and thus contributing to several human diseases (reviewed in ref. [73]). Biological functions of tRNA modifications can be broadly grouped into three classes: those important for promoting and stabilizing the overall architecture of the tRNA structure (class I), those affecting mRNA decoding and translation efficiency (class II) and those influencing non-canonical tRNA functions (class III).[74] These non-canonical roles include tRNA fragment biogenesis, tRNA fragment function or serving as signaling molecules in bacterial stringent response or mammalian apoptosis regulation.

Effects of tRNA modifications on the three-dimensional L-shaped structure of tRNAs (class I) were shown to be very subtle. The overall tRNA structure was shown to fold correctly irrespective of the modification status of $tRNA^{Phe}$, including a fully formed anticodon stem-loop in *E.coli*.[75] However, minor variations became apparent in the angle between the anticodon stem-loop and the acceptor stem, which seemed to be correlated with the modification status.[75] In other examples, the influence of a particular tRNA modification on the three-dimensional architecture is more pronounced, as in the case of the highly conserved m^1A58, m^1A9 or m^5C38.[74,76] In all these cases, it seems that lack of these crucial methylations results in less compact tRNA folding, which renders these molecules prone to degradation by endonucleases. In general, modifications involved in adjusting subtle structural features are capable of either rigidifying the tRNA architecture or making it more flexible.[77] Both types of modification effects are essential for fine tuning tRNA stability, especially in response to environmental challenges. As an example, psychrophilic archaea harbor more flexibility-promoting modifications compared to thermophilic archaea.[78] Furthermore, the modification level of tRNAs were observed to change with increasing temperature and therefore maintaining a balance between stability and flexibility of the tRNA molecule depending on the temperature.[79]

The modifications belonging to class II are essential for translation fidelity. The importance of these modifications is highlighted by the fact that almost every tRNA is modified at position 34 (the wobble nucleotide) and position 37 (the nucleotide flanking the anticodon). Modifications at position 37 prevent frameshifting and help to stabilize the anticodon-codon interaction during translation in the A site of the ribosome (reviewed in ref. [69]). Recent ribosome profiling evidence suggests that the 5-methoxycarbonylmethyl-2-thiouridine modification at the wobble position also affects the speed of translation and thus modulates protein folding and proteostasis in Mammalia.[80] In some cases, the tRNA modification status can be highly dynamic and therefore serves a regulatory function. For example, chemical and temperature stress have been shown to influence modification levels at the wobble position,[81] thus potentially adjusting the cellular translatome during environmental challenges. In yeast it was demonstrated that during oxidative stress, an increase of the m^5C modification at the wobble position of $tRNA^{Leu}$(CAA) promotes the selective translation of mRNAs enriched for TTG codons. By this adaptive mechanism, proteins involved in the resilience towards oxidative stress get preferentially expressed.[81a] Additionally, tRNA modifications in pathogenic fungal and bacterial pathogens have been linked to modulate stress responses during infection.[82] For trypanosomatids, however, the role of dynamic tRNA modification during stress and infection remains to be elucidated.

Recently tRNA modifications at positions 34 and 37 have been put into context of a structure- and energy-based view of the genetic code.[83] In this scenario, modifications at these two positions are especially crucial for stabilizing the thermodynamically weak AU-rich codon-anticodon pairs in the ribosomal A-site. This allows a uniform, balanced and accurate decoding at the ribosome despite the quite large differences in binding energies between strong (GC-rich) and weak (AU-rich) codons-anticodon duplexes.

The third class (III) of tRNA modifications involves those that affect 'non-canonical' functions of tRNA molecules. 'Non-canonical' is meant here in the sense of fulfilling biological roles that go beyond directly serving as substrates for protein biosynthesis.[74] It has been shown that certain modifications influence tRNA cleavage efficiency into smaller fragments and some even play a direct role in the biological function of tRNA fragments (see section 5.4).

4.3 *tRNA Modifications in Trypanosomatids*

Trypanosomatids are appreciated as divergent eukaryotes with a remarkable and unique RNA biology such as extensive mRNA editing in mitochondria (see Introduction). RNA editing is however not limited to mRNAs but was also described to occur on tRNA molecules (reviewed in ref. [84]). In trypanosomatids, two tRNA editing events were previously described, namely formation of inosine by deamination of adenosine (A to I editing) and cytidine deamination to yield uridine (C to U editing) in the anticodon loop.[45,85] Inosine at the first position of the anticodon allows the recognition of three different nucleotides at the third mRNA codon position (A, C, or U) and thus expands the decoding capacity of a tRNA.[86] Like in mammals, also trypanosomatids possess eight tRNAs with a genetically encoded adenosine at the wobble base (position 34) in the anticodon loop.[87] To enable the decoding of not only U-ending codons but also C-ending codons, this adenosine needs to be converted to an inosine for the expansion of the decoding capacity. A to I editing is crucial for viability in eukaryotes, since they lack tRNAs carrying a guanosine at the wobble position and therefore would not be able to decode C-ending codons in the absence of such an editing event. Additionally, the A to I tRNA editing events at position 34 are important determinants for their respective aminoacyl tRNA synthetases.[88] A to I editing is catalyzed by enzymes called Adenosine Deaminases Acting on tRNAs (ADATs). In trypanosomatids, they function as a heterodimer of two subunits, ADAT2 and ADAT3, and are very much alike cytidine deaminases.[89] A to I editing is not restricted to position 34 on

tRNAs but can also occur at position 37 neighboring the anticodon. Deamination at this position affects anticodon loop structure and is catalyzed by ADAT1.[89,90] C to U tRNA editing is less widespread in biology and has so far been described in trypanosomatids, marsupials, plants and archaea.[45,85b,91] tRNAs of trypanosomatids contain both C to U and A to I editing in one tRNA molecule simultaneously. Rubio and colleagues demonstrated the intertwinement of both editing reactions and the catalysis of both reactions by the same enzyme, namely the ADAT2/3 on the $tRNA^{Thr}$ (AGU). C to U formation at position 32 was shown to promote further conversion of A to I at the wobble position 34 in cytosolic tRNAs.[85b] More recently, an even deeper layer of complexity was uncovered by the involvement of a post-transcriptional cytosine methylation event at position 32 as a prerequisite for subsequent deamination to uracil. Prior to the C to U deamination reaction at this site in *T. brucei* $tRNA^{Thr}$, the cytosine nucleobase is methylated to 3-methylcytosine (m^3C) by the methyltransferase TRM140. Once this modification is present, m^3C is finally deaminated to 3-methyluridine (m^3U).[85a]

Another example of C to U editing in the anticodon loop of a tRNA is well described in *Leishmania tarentolae* and in *T. brucei*.[41,45] These C to U editing events solve the inability of nuclear encoded tRNAs to decode the codons from the degenerate mitochondrial genetic code inside the mitochondria of trypanosomatids. A very prominent example of codon reassignment is that of tryptophan codons. 88% of the tryptophan codons from the mitochondrial derived mRNA transcripts are encoded as UGA, which in cytoplasmic mRNAs typically represents one of the three canonical stop codons. However, the nucleus-encoded $tRNA^{Trp}$ contains a CCA anticodon that can successfully decode UGG sense codons but not UGA stop codons. The uniquely mitochondrial localized C to U editing of the first anticodon position to generate $tRNA^{Trp}$ (UCA) solves the mitochondrial decoding conundrum.[45] Additionally, $tRNA^{Trp}$ encounters extensive mitochondria-specific post-transcriptional modifications. One such example is the mitochondrial thiolation of U_{33} (s^2U_{33}) of $tRNA^{Trp}$ prior to C to U editing.[92] In *Leishmania* thiolation and editing levels were comparable (~50%) and due to the special proximity of these two events, it was hypothesized that U_{33} thiolation is required for C34 editing. However, it turned out in *T. brucei* that even though equivalent editing levels were detected as in *Leishmania*, 85% of $tRNA^{Trp}$ was thiolated. This demonstrates that in either case edited and unedited tRNAs were subject to U_{33} thiolation.[41] RNAi against the thiolation machinery in *T. brucei* increased the editing levels to almost 100%.[93] This data suggests that thiolation at position 33 acts as negative determinant for the C to U editing at the wobble position 34, which helps to maintain the ratios of edited and unedited tRNAs in *T. brucei*. However, this phenomenon is not commonly found in all trypanosomatids, since in *Leishmania* only the edited $tRNA^{Trp}$ is thiolated.[93] These findings of $tRNA^{Trp}$ highlight three important features of tRNA editing and tRNA modification in the context of the mitochondria. First, editing is essential to increase the decoding capacity in the mitochondria, second, editing is driven by the unique organellar localization of the editing enzyme and the specificity to $tRNA^{Trp}$ and third, post-transcriptional modifications can have an important function in the modulation of tRNA editing.

Beside thiolation of U_{33}, additional tRNA modifications have been described in trypanosomatids, which are specific for distinct cellular compartments. $tRNA^{Lys}$ (CUU), $tRNA^{Leu}$ (CAA) and $tRNA^{Tyr}$ (GUA), which are all encoded in the nuclear genome of *T. brucei*, were shown to possess a distinct modification pattern depending on their cellular localization in the cytosol or in the mitochondria.[94] Similarly, mitochondria-specific and cytosol-specific modifications were detected in *Leishmania tarentolae* in the anticodon loop of $tRNA^{Glu}$ (UUC). $tRNA^{Glu}$ in both compartments are decorated with a common mcmU34 modification at the wobble base, however, Suzuki and co-workers could identify a cytoplasm-specific 2-thiouridine modification and a unique mitochondria-associated 1'-O-methyl uridine modification.[95] Posttranscriptional alterations at position 37 with the very complex chemical modification wyosine (imG), wybutosine (yW) and hydroxywybutosine (OHyW) were thought to serve as discriminators of eukaryal/archaeal from bacterial $tRNA^{Phe}$ (GAA). However, recently wyosine and its derivatives were found in mitochondria of trypanosomatids.[96] This was unexpected since it was generally assumed that mitochondrial tRNA modifications would resemble more the bacterial pattern due to their close evolutionary relationship. Probably these complex wyosine derivatives at position 37 are beneficial for maintaining the reading frame in mitochondrial translation in the light of the U-rich nature of mRNA codons in trypanosomal mitochondria that result from the immense U insertion mRNA editing mechanism.

5. The Emerging Biology of tRNA-derived Fragments

5.1 *tRNA Cleavage is Abundant and Conserved*

Over the last six decades tRNAs have been primarily described and viewed as universally conserved components of the translation machinery delivering activated amino acids to the ribosome and thus connecting the genetic triplet code with the amino acid sequence of proteins. A substantial change in our understanding of the complexity of tRNA biology emerged with the recognition that RNA molecules (coding and non-protein-coding) with well-identified cellular functions can act as precursors for post-transcriptional fragmentation, generating a further novel class of functional RNA species.[97] First evidence for abundant tRNA fragments was reported in human tumor tissues in 1977.[98] Subsequently, tRNA-derived cleavage products were described in *Escherichia coli* in response to T4 virus infection by the endonuclease PrrC.[99] This PrrC endonuclease cleaves tRNAs in their anticodon loop and therefore depletes the pool of mature tRNAs. As a consequence, the infected cell reduces translation of T4 proteins and therefore hampers virus proliferation. At that time, these cleavage entities were considered to be non-functional tRNA degradation products. This view changed during the last years due to the combination of high-throughput sequencing techniques and dedicated functional analyses. Recently, the field has witnessed the discovery of an unexpected functional diversity of tRNA-derived RNA fragments (typically abbreviated tRFs or tdRs) across all kingdoms of life. Today, tdRs are recognized as a rapidly expanding class of regulatory ncRNAs involved in various cellular functions with crucial roles in health and disease.[74,100]

5.2 *Structural and Functional Heterogeneity of tRNA-derived RNA*

Contrary to other classes of small regulatory ncRNAs, such as mi/siRNAs, tdR biogenesis and function is remarkably heterogeneous in various aspects. Firstly, tRNA cleavage occurs at different positions and involves distinct tRNA isoacceptors. Fragmentation generates tdRs of various sizes ranging from tRNA halves (size 30–35 nt) to smaller tRNA fragments (~14–26 nt) deriving from both the 5' or 3' termini of the full-length tRNA molecule. The intracellular stability of the two produced tdRs can be significantly different and can even change within the same organism. Additionally, tdRs can be generated from either the pre-tRNA or from the fully processed mature tRNA containing the post-transcriptionally added 3' CCA-tail in eukaryotes. Moreover, tdRs have been described to originate from trimmed mature tRNAs without containing the 3' CCA-tail.[101] Not only can the 3' termini differ but also the chemical nature of the 5' termini and thereby further increase the diversity of tdRs.[102] The vast amount of cleaving options within a tRNA molecule highlights only one aspect of the tdR heterogeneity. Secondly, another layer of tdR complexity is

achieved by the alternative expression levels even within the same organism. tdR abundance can vary enormously in different tissues of an organism, during development and can heavily depend on environmental stimuli. Initially the phenomenon of tRNA cleavage has been described in the context of stress response. This stress-induced tdR production is widespread and has been observed in archaea,[103] bacteria,[99] uni-[101,104] and multicellular eukaryotes.[105] However, more recent studies have demonstrated that tdRs can also be constitutively present,[101a,106] thus highlighting the potential also for housekeeping roles. Thirdly, not only are tdRs highly heterogeneous in their structure and expression profiles but they also vary significantly in their cellular roles. tdRs have been reported to be involved in a plethora of biological functions including the regulation of transcription, translation, stress granule formation, apoptosis, cell proliferation, RNAi, vesicle-mediated intercellular communication, intergenerational inheritance, retrotransposons mobility (reviewed in ref. [100a]) and ribosome biogenesis.[107]

5.3 *tRNA-derived RNAs in Trypanosomatids*

Lately, tdRs have also been characterized and their biology studied more extensively in trypanosomatids. During their complex life cycles these parasites have to adapt to different environmental challenges such as temperature shock, nutritional stress, oxidative stress and pH shift (reviewed in ref. [108]). These changes are concerted with the rearrangement of gene expression. Since transcription regulation is essentially absent in trypanosomes, they are crucially dependent on post-transcriptional regulation mechanisms to modulate gene expression.[109] Whereas well-characterized post-transcriptional mechanisms of gene regulation such as the RNA interference (RNAi) pathways are involved in most eukaryotes, the protozoan parasites *Trypanosoma cruzi*, *Leishmania major*, *Leishmania donovani* and *Plasmodium falciparum* are deficient in the RNAi pathway (reviewed in ref. [110]). In contrast, *Trypanosoma brucei* possesses an endogenous siRNAs-guided RNAi pathway, however, the miRNA-guided translation regulation seems to be absent.[111] Therefore, tdRs might represent a means for post-transcriptional regulation in the absence of transcriptional control along with the lack of a complete RNAi pathway in most of the trypanosomatids. Indeed, tdRs were identified in several trypanosomatids under different life stages and stress conditions.[101a,104a,b,106] The presence of tdRs in trypanosomatids was first reported in unstressed and nutritionally stressed *T. cruzi* cells by Garcia-Silva and colleagues.[104a] The authors constructed a 20-35 nt cDNA library and sequenced 348 clones. 26% of these clones were derived from tRNA and 60% from ribosomal RNA. Predominantly the tdRs derived from the 5' half of tRNAs and over 90% of tdRs originated from only three tRNA isoacceptors ($tRNA^{Asp(GUC)}$, $tRNA^{Glu(CUC)}$ and $tRNA^{Glu(UUC)}$). Additionally, the nutritional stress condition stimulated tRNA cleavage. This implies that tRNA cleavage is accentuated under nutritional stress in *T. cruzi* and 5' tRNA halves are generated from a constricted pool of tRNA isoacceptors. Later on, another independent cDNA library in *T. cruzi* was analyzed by Franzen *et al*.[106] In this study 73% of the tdRs reads derived from the 3' halves including the post-transcriptionally added CCA-tail. These two transcriptome studies in *T. cruzi* illustrate the asymmetric stability of cleaved tRNAs within the same organism, depending on the stress condition and its life stage. Franzen and colleagues detected the majority of tRNA halves deriving from the 3' end of mature tRNAs of epimastigote cells, with a preference for $tRNA^{His}$. However, upon nutritional stress, 98% of identified tRNA halves derived from the 5' termini, mainly from $tRNA^{Glu}$ and $tRNA^{Asp}$.[104a] This tdR state changed markedly in the metacyclic life stage, when 3' tRNA halves dominated with yet again a different preference for tRNA species.[104a] A similar picture became evident by deep-sequencing of a cDNA library prepared from ribosome-associated small ncRNAs under various stress and life stage conditions in *T. brucei*.[101a] The majority of tdRs belonged to tRNA halves and derived from the 5' part. The abundance of tRNA halves varied during different environmental stimuli and was specific to unique tRNA isoacceptors.[101a] Hence, tRNA cleavage into halves results upon various growth and stress conditions and is tRNA species-dependent. Not only were tdRs identified as a part of the non-coding cellular transcriptome but also in their respective secretomes in *Trypanosoma cruzi, Leishmania donovani* and *Leishmania braziliensis*.[104a,b] These protozoan parasites were shown to release RNA-containing extracellular vesicles that also include tdRs. The secretome study of both *Leishmania* species revealed a distinct population of tdRs compared to the intracellular tdRs population.[104b]

5.4 *Biogenesis of tRNA-derived RNAs in Trypanosoma*

As previously mentioned, the biological functions of tdRs are highly diverse. However, also the biogenesis pathways of the tdRs observed across different organisms are very heterogeneous. In yeast, Rny1, a member of the RNase T2 family, is responsible for the production of oxidative stress induced tRNA cleavage in the anticodon loop to generate tRNA halves.[112] In mammalian cells, stress-induced tRNA halves are generated by angiogenin, an RNase A-type enzyme.[105b] Trypanosomatids lack an angiogenin or an Rny1 homolog, implying an alternative biogenesis pathway. In plants and humans, it has been proposed that tdRs can also be produced by Dicer, a central endonuclease of the si/miRNA machinery.[113] Since a few members of the trypanosomatid family partially or totally lost the RNAi pathway, the Dicer homologs still present might be involved in the non-canonical tdRs biogenesis. However, RNAi experiments against the two Dicer-like proteins (TbDCL1 and TbDCL2) did not affect tRNA fragmentation in the anticodon loop of *T. brucei*.[101a] It seems that the tdR biogenesis in trypanosomes is unique and awaits identification of the involved nucleases.

In light of the discovery that tRNA fragmentation increases upon stress exposure, another pending question to be addressed concerns the role of post-transcriptional nucleoside modifications on tRNA molecules for tdR biogenesis. In Drosophila and human cells, it has been shown that indeed the presence of particular tRNA modification affects the production of tdRs.[114] The DNA methyltransferase Dnmt2 methylates three tRNAs in the anticodon loop in flies. Drosophila Dnmt2 mutants were less viable under stress conditions and the three unmodified tRNAs were more susceptible to cleavage by angiogenin. Schaefer and colleagues demonstrated that stress-induced tRNA cleavage was indeed Dnmt2-dependent, whereas the Dnmt2-mediated methylation protected tRNAs against stress-induced RNase cleavage.[114] Another hint that modifications influence tdR biogenesis comes from an example in human disease.[115] The lack of tRNA methylation positively affected the production of tdRs and reduced protein translation rates. This effect contributed to human neurological disorders *via* stress-induced tRNA fragmentation.[115] It is therefore conceivable that some tRNA modifications are not necessarily required for canonical tRNA function (class III modifications; see 5.2.), but might be crucial for tdR biogenesis and/or tdR functionality (reviewed in ref. [74]). Indeed, a recent study demonstrated that a particular tdR in human stem cells can only fulfil its biological role in case it carries pseudouridines.[116] Such a requirement for post-transciptional modification for tdR function, however, cannot be generalized. The $tRNA^{Thr}$ 3' half shown to stimulate *T. brucei* translation neither depends on modifications for its biogenesis, nor for its regulatory function on the ribosome.[101a]

5.5 *Function of tRNA-derived RNA in Trypanosoma*

The biological function of most of the identified tdRs in trypanosomatids remained largely ambiguous up to date. Owing to the extreme functional heterogeneity of tdRs (see section 5.2), the mere identification of a tRNA-derived sequence in RNomes

impedes direct conclusions to be made about its biological significance. As a consequence, dedicated experiments for basically every individual tdR are required for unraveling its biological relevance. In trypanosomatids, this was achieved for only a handful of tdRs. The so far characterized tdRs are involved in diverse functions such as translation regulation,[101a] RNA interference,[117] intercellular communication[118] and intergenerational inheritance.[118] A very recent publication in *T. brucei* revealed a novel layer of post-transcriptional regulation during stress recovery, driven by a ribosome-associated tRNA half.[101a] A deep-sequencing approach revealed a 3' derived $tRNA^{Thr}$ half, which accumulates upon nutritional stress in the procyclic form of *T. brucei*. This respective tRNA half associates with ribosomes and polysomes and was shown to globally stimulate translation during stress recovery by facilitating mRNA loading during translation initiation. The $tRNA^{Thr}$ 3' half is the latest addition to an emerging class of ribosome-associated small ncRNAs (rancRNAs) present in all three domains of life.[119] RancRNAs were demonstrated to associate directly with the translation machinery and have the potential for acting as first wave stress regulators.[120] The $tRNA^{Thr}$ 3' half is not the first tdR known to stimulate protein synthesis. In 2017, Kim and colleagues reported on a 22 residue long tdR capable of fostering translation of at two ribosomal protein mRNAs and in the end fine-tune the number of ribosomes in mammalian cells.[107] While tdR-mediated translation stimulation has been described only very recently, global inhibition of protein synthesis involving tdRs was first reported already in 2009.[121] Two stress-induced tRNA 5' halves (5' $tRNA^{Ala}$ and a 5' $tRNA^{Cys}$) were shown to inhibit translation and promote stress granule formation. From a mechanistic point of view, these tdRs were shown to contain a specific 4-G-quadruplex structure[122] and were able to interfere with the eIF4F initiation complex and therefore reduce translation initiation.[105b] Additionally, stress granule assembly was induced by direct association with the effector protein YB-1.[123] The formation of stress granules upon a global translation arrest is a general feature for eukaryotic cells and does not exclude trypanosomatids. For example, stress granules were observed upon heat-shock, which leads to decreased polysome and mRNA levels in *T. cruzi*.[124] However, stress granule formation was not affected by the recently described *T. brucei* $tRNA^{Thr}$ 3' half.[101a]

Another function of tdRs, namely the involvement in the RNA interference pathway, was described in plants, fungi and animals. Various publications revealed that tdRs can directly participate as guide RNAs in the RNAi machinery. Some unicellular protozoan belonging to the family of trypanosomatids, such as *T. cruzi*, *L. major* and *L. donovani*, lack or possess an extensively simplified machinery involved in the RNA interference pathway (reviewed in ref. [110]). This raises the question whether tdRs might be involved in an alternative small RNA-mediated pathway to regulate gene expression post-transcriptionally. Garcia-Silva and colleagues co-purified a considerable number of small ncRNAs, with the second biggest fraction deriving from tRNAs, bound to the sole member of the Argonaute proteins, namely TcPIWI-tryp in *T. cruzi*.[117] However, despite the demonstration of an interaction between tdRs and an argonaute protein in *T. cruzi*, functional evidence for the involvement of tRNA fragments in RNAi is largely absent. The tdR-argonaute complexes were shown to be recruited to specific cytoplasmic granules, with a local distinction between 5'- and 3'-derived tRNA half complexes inside the cellular body.[117] Later on, these granules were identified as nutritional storage organelles, so-called reservosomes. These reservosomes store nutrients that derive from the extracellular media by a vesicle mediated engulfment process in *T. cruzi*.[118] Garcia-Silva and colleagues demonstrated the shedding event of vesicles containing tdRs and the argonaute protein TcPIWI-tryp as cargo during nutritional stress.[118] These tdRs-containing vesicles were subsequently engulfed by mammalian cells and other parasites and stored in reservosomes. This data uncovers yet another layer of biological relevance of tdRs in trypanosomatids. In particular, tdRs can be involved in cell-to-cell communication between parasites or even a cross-kingdom transfer to mammalian cells. These vesicles were secreted by nutritionally stressed *T. cruzi* parasites and induced epigenetic changes in the susceptible mammalian cells. During early stage of invasion, gene expression changes were observed in murine cardiomyocytes, favoring the infection process from *T. cruzi* in mammalian cells. Incubation with the extracellular vesicles fraction of nutritionally stressed *T. cruzi* induced remodeling of the cytoskeleton and the extracellular matrix, along with the expression of proinflammatory cytokines and other genes involved in the immune response.[118]

These new insights into tdRs in trypanosomatids cover a broad range of cellular function and add to the multifaceted regulatory potential of tdRs across all domains of life. Up to date, the biological relevance of many more identified tdRs awaits to be uncovered in the family of trypanosomatids. This might help to understand the peculiar RNA biology of trypanosomatids, especially for uncovering the hidden layer of post-transcriptional gene regulation.

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