

Chapter 10

Biogenesis and Evolution of Functional tRNAs

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Abstract Faithful translation of genomes into proteomes depends, mainly, on the activity of transfer RNA (tRNA) as universal adaptor, as insightfully predicted by Crick. The central role of this relatively simple oligonucleotide depends upon a very large number of intermolecular interactions, some of which require that tRNAs maintain a constant general structure, while others depend on specific features that discriminate any given tRNA from the rest. Posttranscriptional modifications that increase the chemical diversity contained in the nucleotides of tRNAs can serve both purposes. Chemical modifications of tRNAs, thus, come in two general flavors: those that help to maintain the general shape of the molecule, and those that improve its interactions with one or more of its many molecular partners. Although the function of most of the chemical modifications known to occur in tRNAs remain unknown, up-to-date knowledge allows us to analyze the majority of them in some model organisms, including *Saccharomyces cerevisiae*. Here we will review our current understanding on the function of tRNA modifications, generally dividing them into two families: those that are likely to influence the structure of tRNA, and those that may play a role in the codon-anticodon interaction at the decoding center of the ribosome.

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Introduction

Faithful translation of genomes into proteomes depends, mainly, on the activity of transfer RNA (tRNA) as universal adaptor, as insightfully predicted by Crick (Crick 1963). The central role of this relatively simple oligonucleotide depends upon a very large number of intermolecular interactions, some of which require that tRNAs maintain a constant general structure, while others depend on specific features that discriminate any given tRNA from the rest. Posttranscriptional modifications that increase the chemical diversity contained in the nucleotides of tRNAs can serve both purposes.

Chemical modifications of tRNAs, thus, come in two general flavors: those that help to maintain the general shape of the molecule, and those that improve its interactions with one or more of its many molecular partners. Although the function of most of the chemical modifications known to occur in tRNAs remain unknown, up-to-date knowledge allows us to analyze the majority of them in some model organisms, including *Saccharomyces cerevisiae*. Here we will review our current understanding on the function of tRNA modifications, generally dividing them into two families: those that are likely to influence the structure of tRNA, and those that may play a role in the codon-anticodon interaction at the decoding center of the ribosome.

The structural role played by chemical modifications in tRNA was clearly demonstrated by Kowalak and colleagues (Kowalak et al. 1994), who showed that, in the hyperthermophile *Pyrococcus furiosus*, the levels of tRNA modifications progressively increased with culture temperature. Most of the modifications that exhibited an apparent role in the adaptation to extreme temperatures are, in fact, unique to the archaeal hyperthermophiles. Similarly, in yeast, modifications are used to improve tRNA stability. For example, mutations that affect the activity of Trm6/Trm61, responsible for the formation of 1-methyl adenosine, lead to a reduction in steady-state levels of mature tRNA^{iMet} due to a decrease in the stability of the molecule (Anderson et al. 1998).

More challenging remains the analysis of the function of modifications in the anticodon, given the complexity of the interactions that depend on the structure of this region of tRNA. In this regard, the analysis by mass spectrometry in yeast of general variations in tRNA modifications upon stress induction has revealed a complex and fluid pattern of adaptation that involves several modifications and positions in the tRNA (Chan et al. 2010, 2012). These ground-breaking experiments have demonstrated that the levels of certain modification enzymes are regulated as a response to specific insults, possibly because their activity is important in the control of the cellular responses to that particular stress. In the coming years we expect that these regulatory mechanisms will become clear, not only from the point of view of the regulation of the expression of these enzymes, but also in terms of the specific roles that each of the involved modifications play in the shaping of the genetic program and the proteome.

The connections described between tRNA modifications and human disease continue to grow (Torres et al. 2014) and, with them, the interest in the functional role played by these chemical groups in cellular homeostasis. Particularly, exciting is the possibility that anticodon modifications may play a role in the regulation of translation of certain genes. We have recently shown that highly expressed genes in yeast are enriched in codons whose translation requires tRNAs with inosine at position 34 (I34) in the anticodon (Novoa et al. 2012). I34 is generated after deamination of A34 by the enzyme adenosine deaminase acting on tRNA (ADAT), which is one of the few modifications known to be essential in yeast (Gerber and Keller 1999). The open question now is deciphering which genetic programs might be controlled by variations in levels of tRNA modifications, and what are the dynamics of the same modifications in tRNA populations.

tRNA Biogenesis

tRNA biogenesis is a complex process that leads to the production of mature and functional tRNA molecules. There are many proteins involved in this process which spans along several steps and cellular compartments. The entire biosynthetic pathway comprises the following key steps: transcription of the pre-tRNA molecule from its gene, removal of the 5' leader and 3' trailer sequences, addition of a 3'-terminal CCA motif, splicing of introns that may be present and chemical modification of particular nucleoside residues (Fig. 10.1).

In eukaryotes, tRNAs can be nuclear- or organelle-encoded (i.e., encoded in genome containing organelles such as mitochondria and chloroplasts). This section is focused on cytosolic tRNAs produced from nuclear genes. We will mainly focus on the process of tRNA biogenesis in *S. cerevisiae*, but the most relevant differences with other organisms will be mentioned where appropriate.

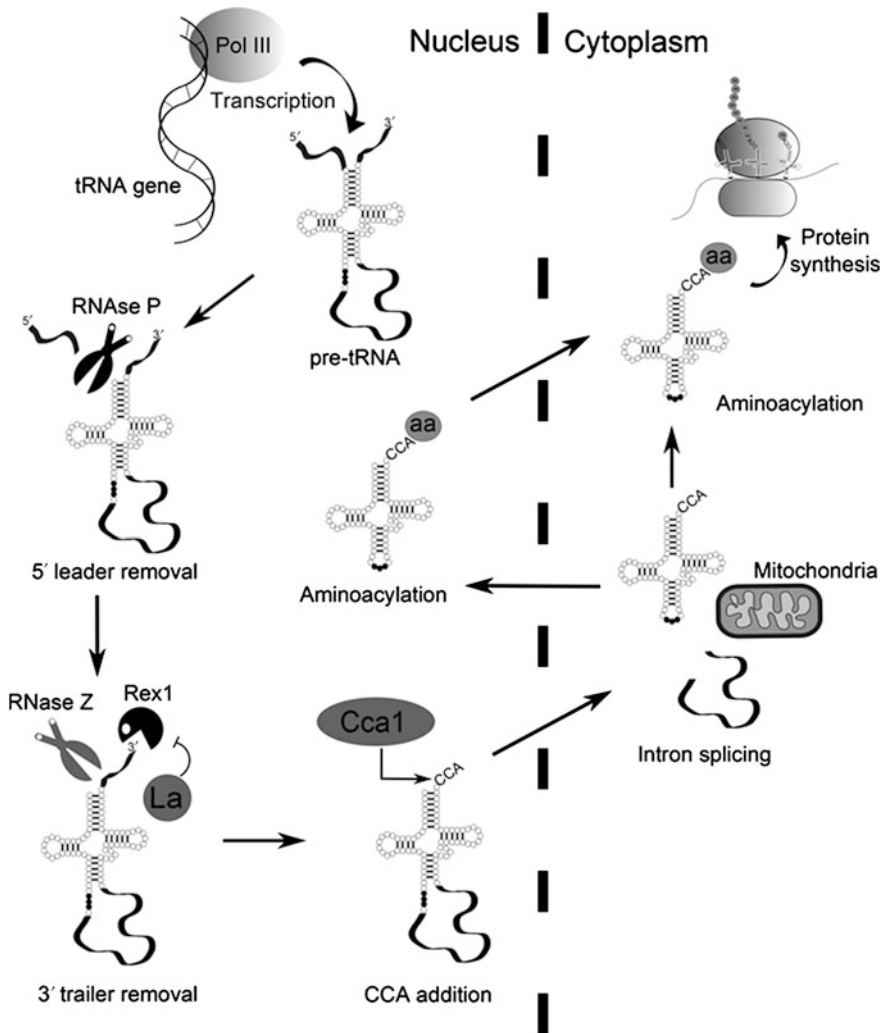


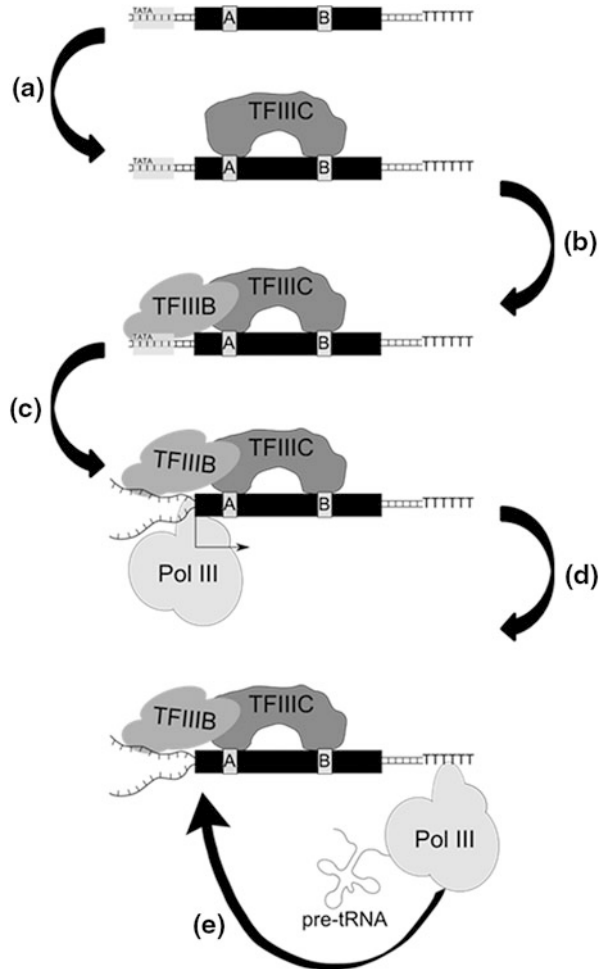
Fig. 10.1 tRNA biogenesis. tRNA biogenesis begins in the nucleolus, with the transcription of the pre-tRNA molecule. Afterward, the 5' leader sequence (*ribbon*) is removed by the RNase P ribonucleoprotein (*black scissors*). Then, RNase Z (*gray scissors*), Rex1 (*Pacman symbol*), and La protein (*gray circle*) perform the 3' trailer (*ribbon*) removal. The binding of La protein prevents the exonucleolytic activity of Rex1 and leads to endonucleolytic scission by RNase Z. Once the 5' and 3' tails have been removed, the CCA nucleotidyl transferase (*Cca1, gray ellipse*) adds the CCA tail. After that, the pre-tRNA molecule is transported to the cytoplasm. Intron splicing takes place at the mitochondrial outer membrane surface. The now mature tRNA molecule is aminoacylated by its cognate aminoacyl-tRNA synthetase (ARS) in the cytoplasm and incorporated to the protein synthesis. Alternatively, under certain conditions, the mature tRNA can be imported back into the nucleus, where it can be aminoacylated by a nuclear pool of ARSs and then re-exported to the cytoplasm. The pre-tRNA residues undergo several chemical modifications throughout the maturation process

tRNA Transcription

Nuclear tRNA genes are transcribed by RNA polymerase III (RNA pol III) and its associated machinery. In terms of promoter complexity and diversity, RNA pol III stands at an intermediate position compared to the rest of the RNA polymerases, since it is responsible for the transcription of a limited number of different RNA molecules: all the nuclear encoded tRNAs, the 5S ribosomal RNA (rRNA), U6 small nuclear RNA (snRNA), and many other nonprotein-coding RNAs (ncRNAs) (Willis 1993; Dieci et al. 2007; White 2011). Transcription by RNA pol III depends on several cis-acting elements (i.e., sequence elements) and some transcription factors (TFs). In the case of tRNA genes, the required cis-acting elements are two internal regions of the genes known as the A and B boxes. The A box is usually located about 10–20 bp downstream of the Transcription Start Site (TSS), and the B box normally sits at 30–60 bp downstream of the A box (Willis 1993; Dieci et al. 2007; Dumay-Odelot et al. 2010; Orioli et al. 2012). These sequences are involved in the correct positioning of the necessary transcription factor III C (TFIIIC), and the formation of the conserved D-loop and T-loop of the tRNA structure (Orioli et al. 2012). Additionally, flanking sequences upstream of the TSS are also important in some organisms, a TATA box being the most prevalent one (Geiduschek and Kassavetis 2001; Hamada et al. 2001). Although absent in *S. cerevisiae*, many tRNA promoters of *Schizosaccharomyces pombe* contain a TATA box at ~30 bp upstream of the TSS, which is essential for the expression of the tRNA gene (Hamada et al. 2001).

The transcription process begins with the binding of the multi-subunit TFIIIC to the A and B boxes of the tRNA gene. The B box is known to be the most important promoter element for such interaction as TFIIIC has been found bound to isolated B box elements, but not to isolated A box elements (Noma et al. 2006; Wallrath and Geyer 2006; Moqtaderi et al. 2010). However, isolated B boxes are usually nontranscriptionally active, with only few exceptions producing ncRNAs of yet unknown function (Olivas et al. 1997; Parrott and Mathews 2007; Parrott et al. 2011). Once TFIIIC is on the tRNA gene, it recruits a second essential transcription factor, TFIIIB, which is a multi-protein complex composed of three different subunits: TATA box Binding Protein (TBP), Brf1, and Bdp1 (Kassavetis et al. 1991; Willis 2002). The finding that TBP is a universal component for all of the nuclear transcription machineries highlighted their common origin and conservation (Hernandez 1993; Rigby 1993; White et al. 1992). Moreover, the Brf1 amino terminal part is also related with TFIIIB, a component of the RNA pol II machinery (Kassavetis et al. 1998). This conservation also extends to the polymerase itself, as five subunits are shared by the three main eukaryotic RNA polymerases (Carles et al. 1991; Vannini and Cramer 2012). Once TFIIIB is bound upstream of the TSS, it participates in the promoter opening and the recruitment of RNA pol III to start transcription (Acker et al. 2013). The transcription termination signal is relatively simple, consisting of a stretch of thymidine (T) residues in the coding sequence. The length of the stretch varies between species, being $\geq 4T$ in

Fig. 10.2 tRNA transcription. **a** tRNA transcription begins with the binding of the TFIIC complex to the A and B boxes in the tRNA gene sequence. **b** TFIIC recruits the TFIIB complex. TFIIB binds upstream the transcription start site (TSS), which may contain a TATA box. **c** TFIIB participates in the promoter opening and the recruitment of the RNA pol III (Pol III) complex, to initiate the transcription. **d** The transcription ends when Pol III finds the termination signal, consisting of a stretch of thymidines (T). **e** Finally, Pol III can undergo several rounds of reinitiation



vertebrates, $\geq 5T$ in *S. pombe*, and $\geq 6T$ in *S. cerevisiae*. It is worth noting that the efficiency of the termination signal can be strongly influenced by the surrounding sequences (Arimbasseri et al. 2013). That kind of weak terminators, when found between two transcription units, are thought to bring new opportunities for transcription regulation (Orioli et al. 2012). Finally, RNA pol III can undergo several rounds of reinitiation which, at least in fast growing cells, ensures the necessary yield of tRNAs (Dieci et al. 2013) (Fig. 10.2).

The study of the regulation of tRNA transcription is a relatively new field. The Maf1 protein was the first described and, to date, the only global negative regulator of RNA pol III transcription (Boguta et al. 1997). The activity of Maf1 is dependent on its phosphorylation state, thus connecting diverse unfavorable growth conditions with RNA pol III activity (Oficjalska-Pham et al. 2006; Roberts

et al. 2006). Regarding the RNA pol III activators, the Sub1 protein has a strong positive effect on RNA pol III transcription in vitro (Wang and Roeder 1998). Sub1 is the yeast homologue of the mammalian co-activator factor PC4, involved in the activation of many RNA pol II genes (Werten and Moras 2006; Malik et al. 1998; Ge and Roeder 1994). It has been shown that Sub1 participates both in the initiation and reinitiation of RNA pol III transcription (Tavenet et al. 2009). In addition to these two factors, some of the components of the RNA pol III transcription apparatus are phosphoproteins, and some kinases involved in their phosphorylation have been identified (Acker et al. 2013). Moreover, recent genome-wide studies established a link between chromatin state and RNA pol III activity (Brogaard et al. 2012; Mavrich et al. 2008). In this regard, it has been shown that several chromatin remodeling complexes such as FACT, Nhp6, and Isw2 are involved in RNA pol III transcription regulation, affecting in many cases the transcription of tRNA genes (see also Chap. 13). Likewise, the histone acetylation state also affects the expression of RNA pol III genes, thus linking histone acetylases and deacetylases to this process (Acker et al. 2013). Finally, many other proteins, most of them being known RNA pol II TFs, have been identified associated with RNA pol III genes by chromatin immunoprecipitation (ChIP) and subsequent genome-wide studies. However, their actual implication in the regulation of those RNA pol III genes remains unclear (Acker et al. 2013).

Trimming of the 5' Leader and 3' Trailer Ends

For most eukaryotic tRNA genes transcription starts some bases before and ends some bases after the limits of the mature tRNA, resulting in a pre-tRNA molecule with 5' leader and 3' trailer sequences. In *S. cerevisiae* the length of each of these extra sequences is about 12 bases, and its trimming is a stepwise process where the 5' leader excision usually precedes the 3' trailer removal (O'Connor and Peebles 1991; Hopper 2013) (Fig. 10.1).

The 5' end processing is carried out by the ribonucleoprotein RNase P. This ancient ribozyme is present in all the organisms and organelles and is thought to be a relic from the "RNA world", the proposed evolutionary age when the enzymatic reactions were conducted by nucleic acids instead of proteins (Ellis and Brown 2009). The RNase P enzymatic activity is carried out by its RNA component, conserved in all life domains. There are a few rare cases where RNase P RNA has been substituted by proteins, as in human mitochondrial RNase P or the nuclear and mitochondrial RNase P of higher plants (Holzmann et al. 2008; Walker and Engelke 2008; Thomas et al. 2000; Gutmann et al. 2012). Besides its contribution to tRNA processing, RNase P is also implicated in the maturation of other RNA precursors such as small nucleolar RNAs (snoRNAs) and the *HRA1* ncRNA in yeast (Coughlin et al. 2008; Samanta et al. 2006; Yang and Altman 2007).

The 3' end processing is more complex and involves both endo- and exonuclease activities (Phizicky and Hopper 2010). In yeast, three proteins seem to drive

this process: Rex1, RNase Z, and La protein (Hopper 2013). The 3'-5' exonuclease activity is performed by Rex1, also involved in the processing of other ncRNAs (e.g., 5S rRNA, 5.8S rRNA, and snRNAs (van Hoof et al. 2000; Copela et al. 2008; Ozanick et al. 2009). The RNase Z (Trz1 in yeast) protein is in charge of the endonuclease activity, and processes both nuclear and mitochondrial pre-tRNAs (Chen et al. 2005; Daoud et al. 2012; Maraia and Lamichhane 2011). It has been proposed that the two aforementioned nucleases have differential access to the tRNA depending on the previous binding of the La protein (Lhp1), which prevents Rex1 activity and leads to endonucleolytic maturation by RNase Z (Yoo and Wolin 1997).

Additions to Pre-tRNA Ends: CCA and tRNA^{His} G₋₁

In eukaryotes, after the 3' trailer removal, the required 3' CCA sequence is added (Fig. 10.1). This process is accomplished by the CCA nucleotidyl transferase, Cca1 in yeast (Aebi et al. 1990), which is well conserved in different organisms. It is noteworthy that, in *Escherichia coli*, tRNA genes have the 3' CCA encoded in the genomic sequence and therefore do not need the nucleotidyl transferase activity. However, they do conserve a homologous gene that, when deleted, leads to a slow growth rate phenotype, suggesting a function in tRNA 3' end repair (Zhu and Deutscher 1987). The *CCA1* gene in yeast encodes for several splicing isoforms, each of them with distinct subcellular localization. It has been shown that the CCA adding activity is performed by the nuclear isoform (Wolfe et al. 1996), whereas the cytosolic isoform seems to be involved in tRNA end repair (potentially being the functional yeast equivalent to the *E. coli* enzyme) (Wolfe et al. 1994).

Another terminal nucleotide addition is necessary for the maturation of eukaryotic tRNA^{His}. After 5' end trimming, this tRNA undergoes the addition of a G to its 5' end (position -1 or G₋₁) in order to be efficiently aminoacylated by histidine-tRNA synthetase (HisRS) (Rudinger et al. 1994). The enzyme responsible for this activity is Thg1, which is an essential gene with 3'-5' nucleotide addition capacity, contrary to the regular nucleotide polymerization direction (Gu et al. 2003, 2005). While Thg1 null mutants are lethal, overexpression of both tRNA^{His} and HisRS under this genetic background can give a viable but unfit yeast strain (Preston and Phizicky 2010).

Intron Splicing

All eukaryotes and archaea sequenced to date carry introns in some of their tRNA genes, and their removal seems to be essential to accomplish the complete set of tRNAs. The length of introns is variable and, in fungi, ranges from just over 10 to about 60 nucleotides (Chan and Lowe 2009). The intron presence is not well

conserved throughout eukaryotes, with the exception of the tRNA^{Tyr}, which almost always contains introns. Perhaps the reason for this particular conservation lies in the fact that the intron processing is essential for subsequent modifications, as it was found in yeast (Johnson and Abelson 1983) (discussed in section “[Chemical modifications on tRNAs](#)”). The introns of all eukaryotic tRNA genes are always located between positions 37 and 38, whereas in archaea they can sometimes be located at other positions (Chan and Lowe 2009).

The splicing process of tRNA is simpler than mRNA splicing as carried out by the spliceosome (revised in [Chap. 2](#)), as there are less proteins involved. In yeast, the overall process consists of three steps, starting with the cleavage of the intron by the tRNA splicing endonuclease, resulting in two tRNA half molecules (Trotta et al. 1997, 2006). The second step is accomplished by the tRNA ligase (Trl1) and consists on the ligation of the two tRNA exons (Phizicky et al. 1986). This is a complex reaction that leaves a residual 2' phosphate at the splice junction that has to be removed by the last enzyme, the phosphotransferase Tpt1 (Spinelli et al. 1997).

One of the most striking and unexpected features of the yeast tRNA splicing machinery is its subcellular localization. Whereas in vertebrates this process is accomplished in the nucleus (Melton et al. 1980), in yeast it takes place in the cytoplasm (Yoshihisa et al. 2007) (Fig. 10.1). The localization of the three enzymes required for the splicing process described above gave a clue in this regard. In particular, the tRNA endonuclease is located on the outer part of the mitochondria membrane (Yoshihisa et al. 2003; Huh et al. 2003), whereas the tRNA ligase is located in the cytoplasm (Huh et al. 2003; Mori et al. 2010), and the phosphotransferase is distributed between nucleus and cytoplasm (Dhungel and Hopper 2012). Strong evidence indicates that the tRNA splicing occurs on the mitochondrial surface, as mutants of the tRNA endonuclease enzyme accumulated unspliced pre-tRNAs in the cytoplasm (Yoshihisa et al. 2007), as well as mutants of the tRNA nuclear export machinery were deficient in tRNA splicing (Hopper and Banks 1978; Hopper et al. 1980). Interestingly, experiments using yeast engineered to have the tRNA splicing machinery only in the nucleus reveal that, although they processed the tRNA correctly, they cannot grow without the mitochondrial component of the tRNA endonuclease. This result suggests that the peculiar localization of the tRNA splicing machinery in yeast could be explained by other functions in which this machinery may be involved (Dhungel and Hopper 2012).

Modifications During tRNA Maturation

Posttranscriptional modifications on the tRNA (that will be discussed in depth in section “[Chemical modifications on tRNAs](#)”) occur during its maturation process. Experiments carried out in *Xenopus* oocytes showed that the intricate series of modifications that pre-tRNA undergoes are introduced in an ordered manner (Nishikura and De Robertis 1981). This seems also to be true in yeast. For

example, in tRNA^{Phe}_{GAA}, some modifications can only occur in the presence of the tRNA intron (e.g., 5-methylcytidine at position 40); while others require a spliced tRNA (e.g., 2'O-methylcytidine, 2'O-methylguanosine, and 1-methylguanosine at positions 32, 34, and 37, respectively) (Jiang et al. 1997). However, some biochemical approaches suggest that the order of the tRNA modifications observed in vivo cannot be entirely explained by substrate specificity since it is usually possible to modify an in vitro produced tRNA molecule with the appropriate in vitro purified modification enzyme (Hopper and Phizicky 2003). Therefore, the subcellular localization of the tRNA modification enzymes seems to be an important factor in regulating the stepwise process of tRNA modification. In yeast, the subcellular localization of some tRNA modification enzymes is known. For example, N2, N2-dimethylguanosine at position 26 is produced in the nucleus by Trm1, and 2-methylguanosine at position 10 is made by Trm11 in the cytoplasm (Purushothaman et al. 2005; Rose et al. 1995).

Quality Control and tRNA Decay

tRNAs undergo several quality control mechanisms which ensure their correct function, as well as their regulated turnover during certain stress conditions. Two independent tRNA degradation pathways have been described: the nuclear exosome pathway and the rapid tRNA decay (RTD) pathway (Hopper 2013; Phizicky and Hopper 2010; Parker 2012).

The exosome pathway has been recently associated with the rapid degradation of approximately 50 % of pre-tRNA molecules (Gudipati et al. 2012). This pathway requires the action of the TRAMP (Trf4/Air2/Mtr4 polyadenylation) complex which, making use of its poly (A) polymerase activity, adds a poly (A) tail at the end of the tRNA to be degraded. Subsequently, this tRNA-TRAMP complex interacts with the nuclear exosome machinery leading to the tRNA degradation by 3'-5' exonucleolytic activity (Parker 2012). Defects in maturation, modification, and in the 3' end processing can be targeted by TRAMP and processed by the exosome (further discussed in section “[Chemical modifications on tRNAs](#)”) (Parker 2012).

The RTD pathway involves the activity of 5'-3' exonucleases located both in nucleus (the Rat1 exonuclease) and in cytoplasm (the Xrn1 exonuclease) (Chernyakov et al. 2008) (more details in [Chap. 7](#)). The Met22 protein, involved in the methionine biosynthetic pathway, also plays a role in the RTD pathway, thus connecting amino acid synthesis with tRNA turnover and quality control (Dichtl et al. 1997). This decay pathway seems to control the correct tertiary structure of certain tRNAs bearing modifications that stabilize their structure, since not all the tRNAs with the same modification are degraded by RTD (Whipple et al. 2011). The subcellular localization of the RTD pathway is still an open question, but it is likely that it takes place both in nucleus and in cytoplasm.

In addition to those exonucleolytic tRNA decay pathways, tRNAs can also be degraded by Rny1, an RNase T2 endonuclease. Rny1 is located in membrane-bound compartments, mainly vacuoles, and its activity produces cleavage at the anticodon loop of tRNAs in certain stress conditions (MacIntosh et al. 2001; Thompson et al. 2008; Thompson and Parker 2009). The activity of Rny1 may take place in the cytoplasm, by release of the enzyme during stress or directly in the vacuole, where the tRNA could be imported by some kind of autophagy process (Parker 2012). The function of these tRNA halves generated by endonucleolytic tRNA cleavage is not well understood but it has been proposed to play a role in the autophagy process (Thompson et al. 2008; Andersen and Collins 2012).

tRNA Transport

tRNAs are transcribed in the nucleus and function in the cytoplasm or the mitochondria, thus a nuclear export mechanism is mandatory and expected. However, studies on the process of tRNA trafficking have revealed many surprises. In addition to being exported out of the nucleus, tRNAs can be re-imported back to the nucleus and then exported again using specific transport mechanisms (Fig. 10.1).

The tRNA nuclear export through the nuclear pores is mediated by the small GTPase Ran. Ran regulates the nuclear export by its association with several proteins of the importin- β family. In yeast, those importin- β members are Los1 (Hopper et al. 1980; Hellmuth et al. 1998; Sarkar and Hopper 1998) and Msn5 (Takano et al. 2005; Shibata et al. 2006; Murthi et al. 2010). Los1 is thought to be the major tRNA exporter, albeit not the only one as demonstrated by the viability of Los1 deletion mutants (Hopper et al. 1980; Hurt et al. 1987). The interaction of Los1 with tRNA is strongly affected by the tRNA tertiary structure as well as the maturation of the 5' and 3' termini (Arts et al. 1998; Lipowsky et al. 1999; Cook et al. 2009; Lee et al. 2011). Therefore, Los1 works also as a quality control protein, preventing the export of misfolded or improperly processed tRNAs. Furthermore, under several stress conditions or in the absence of fermentable carbon sources, the subcellular localization of Los1 changes from nuclear to cytoplasmic. In the cytosol Los1 triggers several response mechanisms, thus linking cell homeostasis to nuclear tRNA export (Qiu et al. 2000; Ghavidel et al. 2007). The other importin- β , Msn5, plays a minor role in tRNA export in vertebrates (Bohnsack et al. 2002; Calado et al. 2002) but it is clear that, at least in yeast and *Drosophila*, Msn5 serves as a bona fide tRNA nuclear exporter (Takano et al. 2005; Shibata et al. 2006; Murthi et al. 2010). In these two organisms, Msn5 has been related to the export of intronless tRNAs and also with the reexport from nucleus to cytoplasm of tRNAs that previously reentered the nucleus by the tRNA retrograde mechanism (see below) (Murthi et al. 2010). Importantly, Los1 and Msn5 are likely not the only tRNA nuclear exporters since the double mutant for both proteins is not lethal (Takano et al. 2005).

Since tRNA splicing in yeast occurs in the cytoplasm, new questions arose when mature tRNAs were found in the nucleus under certain conditions such as nutrient deprivation or inhibition of tRNA aminoacylation (Hopper 2013). To explain this phenomenon, a tRNA retrograde import has been proposed (Yoshihisa et al. 2003; Stanford et al. 2004). The factors involved in such mechanism are not yet well understood, but some data suggest the participation of the Ssa2 heat shock protein in a Ran independent manner (Takano et al. 2005), and the Mtr10 importin- β in a Ran dependent manner (Shaheen and Hopper 2005). The tRNA imported by this retrograde mechanism under stress conditions can later return to the cytoplasm again by a mechanism named tRNA reexport (Whitney et al. 2007). Moreover, in normal conditions, tRNAs can be aminoacylated in the nucleus by a nuclear pool of aminoacyl-tRNA synthetases and these aminoacylated tRNAs may also use the tRNA reexport machinery to reach the cytoplasm (Hopper 2013). Notably, some evidence suggests that both Los1 and Msn5 may be participating in this tRNA reexport process (Murthi et al. 2010).

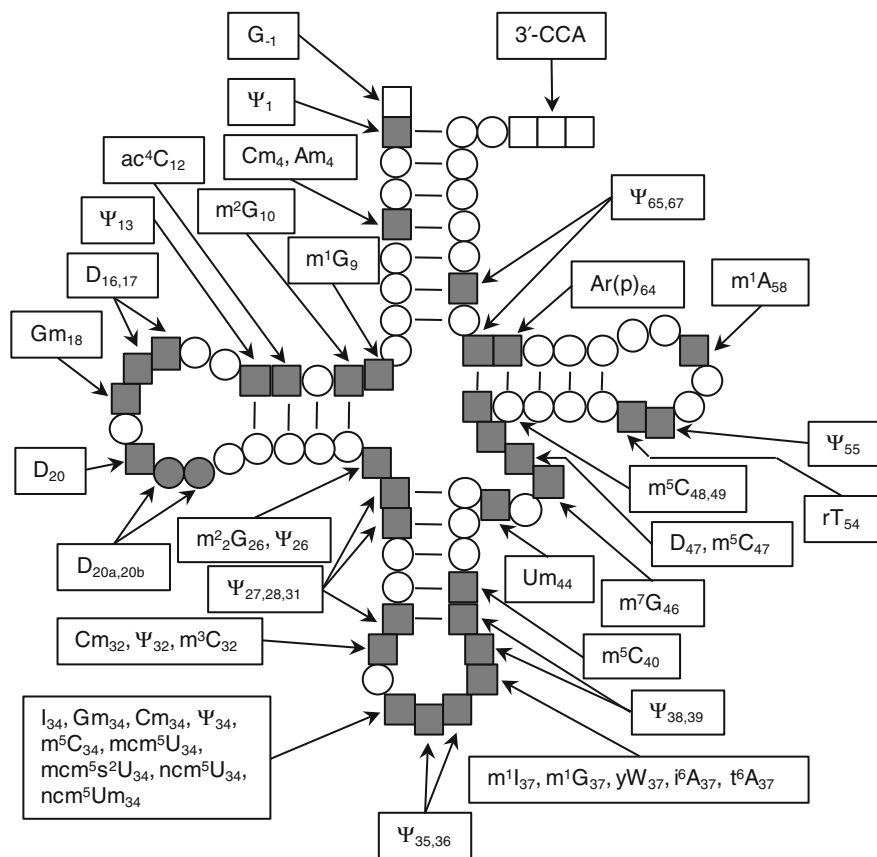
As mentioned above, some tRNAs can be exported into mitochondria. This has been observed for few nuclear encoded tRNAs in yeast (Rubio and Hopper 2011). Interestingly, yeast mitochondria encodes for the complete set of tRNAs necessary for the mitochondrial translation, so it has been proposed that those nuclear encoded tRNAs could be very important under stress situations. For example, yeast mitochondria use the tRNA^{Lys}_{UUU} to read AAA and AAG codons and that capacity is achieved by a modification at the wobble position 34 of the tRNA. Under stress conditions, this tRNA is hypomodified and the nuclear encoded tRNA^{Lys}_{UUU} seems to accomplish the task in the organelle (Kamenski et al. 2007).

Chemical Modifications on tRNAs

The primary sequence of tRNAs undergoes extensive posttranscriptional modifications. There are more than 60 different tRNA chemical modifications listed for eukarya (Cantara et al. 2011) and, for the majority of them, the enzymes responsible for such modifications have been characterized. The most detailed landscape on the chemistry and biology of tRNA modifications has been achieved in the budding yeast *S. cerevisiae*. In this section, we will use this organism as a model to describe the main tRNA modification enzymes (Fig. 10.3; Table 10.1) and their roles in biological processes.

Modifications Involving tRNA Methyltransferases

The most commonly found tRNA modifications are those catalyzed by tRNA methyltransferases (Trms). In *S. cerevisiae*, 18 different Trms have been described and these are sufficient for methylation of almost all known methylated tRNA residues (Townsend and Begley 2012).



Universally conserved tRNA modifications

Cm, Um, Gm, D, Ψ , m^5C , m^5U , m^3U , m^1G , m^7G , m^2G , m^2_2G , m^1A , m^6A , s^2U , ac^4C , t^6A

Fig. 10.3 Schematic representation of the tRNA clover-leaf secondary structure. Modifications present in *Saccharomyces cerevisiae* are shown. *Circles and Squares* nucleosides. *Lines* base pairing. *Empty circles* unmodified residues. *Empty squares* posttranscriptional addition of nucleosides. *Filled squares* modified residues. *Filled circles* nucleosides at positions 20a and 20b present in some tRNAs (these residues can be found modified). The additional *box* with modifications shown in bold corresponds to those modifications universally conserved in all domains of life (but not necessarily present in *S. cerevisiae*). Abbreviations: G_{-1} (addition of guanine at position -1), Ψ (pseudouridine), $Cm/Am/Gm/Um$ (2'-O-methylcytidine/adenosine/guanosine/uridine, respectively), $m^1G/m^1A/m^1I$ (1-methylguanosine/adenosine/inosine, respectively), m^2G (2-methylguanosine), m^2_2G (N2, N2-dimethylguanosine), m^7G (7-methylguanosine), m^3C (3-methylcytidine), m^5C (5-methylcytidine), mcm^5U (5-methoxycarbonylmethyluridine), mcm^5s^2U (5-methoxycarbonylmethyl-2-thiouridine), ncm^5U (5-carbamoylmethyluridine), ncm^5Um (5-carbamoylmethyl-2'-O-methyluridine), ac^4C (4-acetylcytidine), D (dihydrouridine), I (inosine), yW (wybutosine), i^6A (N6-isopentyladenosine), t^6A (N6-threonylcarbamoyladenine), rT (ribothymidine), $Ar(p)$ (2'-O-riboyladenosine phosphate). Underscript at the end of the modification indicates the affected residue(s)

Table 10.1 Genes encoding for enzymes that modify tRNA residues posttranscriptionally in *Saccharomyces cerevisiae*

Gene	Posttranscriptional modification	Position on tRNA	Target tRNA ^a	Essential/not essential gene ^b
CCAI	CCA	3'-end	All tRNAs	Essential
DUS1	D	16, 17	<p><i>Pos 16:</i> Ala(AGC), Arg(ACU), Arg(ACG), Asn(GUU), Asp(GUC), Cys(GCA), Gly(GCC), Gly(UCC), His(GUG), Ile(AAU), Ile(UAU), Leu(UAG), Leu(UAA), Lys(CUU), Lys(UUU), Met(CAU), Phe(GAA), Pro(UGG), Ser(CGA), Ser(UGA), Ser(AGA), Thr(AGU), Trp(CCA), Tyr(GUA), Val(AAC)</p> <p><i>Pos 17:</i> Asn(GUU), Ile(AAU), Lys(CUU), Phe(GAA), Thr(AGU), Arg(UCU)</p>	Not essential
DUS2	D	20	<p>Ala(AGC), Arg(ACG), Asn(GUU), Asp(GUC), Cys(GCA), Gly(GCC), Gly(UCC), His(GUG), Ile(AAU), Ile(UAU), Leu(UAG), Leu(CAA), Leu(UAA), Pro(UGG), Ser(CGA), Ser(UGA), Ser(AGA), Thr(AGU), Trp(CCA), Tyr(GUA), Val(UAC), Val(AAC)</p>	Not essential
DUS3	D	47	<p>Ala(AGC), Arg(ACU), Arg(ACG), Asn(GUU), Cys(GCA), Ile(AAU), Ile(UAU), Lys(CUU), Met(CAU), Thr(AGU), Trp(CCA), Tyr(GUA), Val(UAC), Val(CAC), Val(AAC)</p>	Not essential
DUS4	D	20a, 20b	<p>Asn(GUU), Glu(CUC), Gly(UCC), His(GUG), Ile(AAU), Ile(UAU), Leu(UAG), Ser(CGA), Ser(UGA), Ser(AGA), Tyr(GUA), Val(UAC), Val(CAC), Val(AAC)</p>	Not essential

(continued)

Table 10.1 (continued)

Gene	Posttranscriptional modification	Position on tRNA	Target tRNA ^a	Essential/not essential gene ^b
⁵ IK11, IK13, ELP2-4, ELP6, KTH11-14, SIT4, SAP185, SAP190	"cm ^{5m} " modification on mcm ⁵ U, mcm ⁵ s ² U, ncm ⁵ U and ncm ⁵ Um	34	Cm ⁵ (mcm ⁵ U): Arg(UCU), Gly(UCC) Cm ⁵ (mcm ⁵ s ² U): Lys(UUU), Gln(UUG), Glu(UUC) Cm ⁵ (ncm ⁵ U): Ala(UGC), Pro(UGG), Ser(UGA), Thr(UGU), Val(UAC)	Only IK11 essential
MOD5	I ⁶ A	37	Cm ⁵ (ncm ⁵ Um): Leu(UAA) Cys(GCA); Ser(CGA); Ser(UGA); Ser(AGA); Tyr(GUA); Gly(UCC);	Not essential ^c
NFS1, ISU1, ISU2, CFD1, NBP35, CIA1, URM1, UBA4, NCS2, NCS6, TUM1	"s ^{2m} " modification on mcm ⁵ s ² U	34	NCS2 and NCS6: Glu(UUC), Lys(UUU), Gln(UUG)	Not essential
PUS1	Ψ	1, 26, 27, 28, 34, 35, 36, 65, 67	Pos 1: Arg(ACG) Pos 26: Trp(CCA) Pos 27: Arg(ACU), Arg(ACG), Glu(CUC), Ile(UAU), Lys(CUU), Lys(UUU), Met(CAU), Trp(CCA), Val(UAC), Val(CAC) Pos 28: Lys(UUU), Trp(CCA), Val(CAC) Pos 34: Ile(UAU) Pos 35: Tyr(GUA) Pos 36: Ile(UAU) Pos 65: Trp(CCA) Pos 67: Ile(UAU), Lys(UUU)	Not essential

(continued)

Table 10.1 (continued)

Gene	Posttranscriptional modification	Position on tRNA	Target tRNA ^a	Essential/not essential gene ^b
PUS3	Ψ	38, 39	<i>Pos 38:</i> Ala(AGC), Gly(GCC), His(GUG), Thr(UAG) <i>Pos 39:</i> Arg(ACU), Arg(ACG), Arg(UCU), Asn(GUU), Cys(GCA), His(GUG), Leu(CAA), Leu(UAA), Lys(CUU), Met(CAU), Phe(GAA), Ser(AGA), Ser(CGA), Ser(UGA), Thr(AGU), Trp(CCA), Trp(UCA), Tyr(GUA)	Not essential ^c
PUS4	Ψ	55	Ala(AGC), Arg(ACU), Arg(ACG), Arg(UCU), Asn(GUU), Asp(GUC), Cys(GCA), Glu(CUC), Gly(GCC), Gly(UCC), His(GUG), Ile(AAU), Ile(GAU), Ile(UAU), Leu(UAG), Leu(UAA), Lys(CUU), Lys(UUU), Met(CAU), Phe(GAA), Pro(UGG), Ser(CGA), Ser(UGA), Ser(AGA), Ser(GCU), Thr(AGU), Thr(UAG), Trp(CCA), Trp(UCA), Tyr(GUA), Val(AAC), Val(CAC), Val(UAC)	Not essential
PUS6	Ψ	31	Met(CAU), Gly(UCC), Ile(GAU), Leu(UAA), Lys(UUU), Ser(GCU), Thr(UAG)	Not essential
PUS7	Ψ	13, 35	<i>Pos 13:</i> Asp(GUC), Glu(CUC), Gly(GCC), Gly(UCC), His(GUG), Pro(UGG), Val(CAC), Val(AAC)	Not essential
PUS8	Ψ	32	<i>Pos 35:</i> Tyr(GUA) Asp(GUC), Cys(GCA), Gly(GCC), His(GUG), Leu(UAG), Leu(CCA), Pro(UGG), Ser(AGA), Val(UAC), Val(CAC), Val(AAC)	Not essential
RIT1	Ar(p)	64	Initiator tRNA Met	Not essential
^d SUA5	t ^o A	37	All tRNAs decoding ANN codons bearing A37	Not essential ^c

(continued)

Table 10.1 (continued)

Gene	Posttranscriptional modification	Position on tRNA	Target tRNA ^a	Essential/hot essential gene ^b
TAD1	I	37	Ala(AGC)	Not essential
TAD2, TAD3	I	34	Ala(AGC), Arg(ACG), Ile(AAU), Ser(AGA), Thr(AGU), Val(AAC), Pro(AGG)	Both genes essential
TAN1	ac ⁴ C	12	Leu(UAG), Leu(CAA), Leu(UAA), Ser(CGA), Ser(UGA), Ser(AGA)	Not essential ^c
THG1	G	-1 (5'end)	His	Essential
TRM1	m ² G	26	Ala(AGC), Arg(ACG), Arg(ACU), Asn(GUU), Leu(CAA), Leu(UAA), Leu(UAG), Lys(CUU), Lys(UUU), Met(CAU), Phe(GAA), Pro(UGG), Ser(AGA), Ser(CGA), Ser(UGA), Thr(AGU), Thr(UAG), Tyr(GUA), Val(CAC)	Not essential
TRM2	m ⁵ U	54	Arg(ACU), Arg(ACG), Asn(GUU), Asp(GUC), Cys(GCA), Glu(CUC), Gly(GCC), Gly(UCC), His(GUG), Ile(AAU), Ile(UAU), Leu(CAA), Leu(UAA), Leu(UAG), Lys(CUU), Lys(UUU), Met(CAU), Phe(GAA), Pro(UGG), Ser(CGA), Ser(UGA)	Not essential
TRM3	Gm	18	Leu(UAG), Leu(CAA), Leu(UAA), His(GUG), Tyr(GUA), Ser(AGA), Ser(CGA), Ser(UGA), Trp(CCA)	Not essential
TRM4	m ⁵ C	34, 40, 48, 49	Pos 34: Leu(CUA) Pos 40: Phe(GAA) Pos 48: Asn(GUU), Cys(GCA), Ile(AAU), Ile(UAU), Leu(CAA), Leu(UAA), Leu(UAG), Lys(UUU), Met(CAU), Ser(AGA), Ser(CGA), Ser(UGA), Thr(AGU), Tyr(GUA) Pos 49: Arg(ACG), Asp(GUC), Glu(CUC), Gly(GCC), His(GUG), Phe(GAA), Val(AAC), Val(CAC), Val(UAC)	Not essential

(continued)

Table 10.1 (continued)

Gene	Posttranscriptional modification	Position on tRNA	Target tRNA ^a	Essential/hot essential gene ^b
TRM5	m ¹ G, m ¹ I, yW	37	<i>m¹G</i> : Asp(GUC), His(GUG), Leu(CAA), Leu(UAA), Leu(UAG), Phe(GAA), Pro(UGG), Ser(UGA), Thr(UAG) <i>m¹I</i> : Ala(AGC) <i>yW</i> : Phe(GAA)	Not essential ^c
TRM6, TRM61	m ¹ A	58	Arg(ACG), Arg(ACU), Asn(GUU), Cys(GCA), Ile(AAU), Ile(UAU), Leu(UAA), Leu(UAG), Lys(CUU), Lys(UUU), Met(CAU), Phe(GAA), Pro(UGG), Thr(AGU), Trp(CCA), Tyr(GUA), Val(AAC), Val(CAC), Val(UAC)	Both genes essential
TRM7	Cm, Gm, "Um" modification on ncm ⁵ Um	32(Cm), 34(Cm,Gm, Um)	<i>Cm Pos 32</i> : Leu(UAA), Phe(GAA), Trp(CCA) <i>Cm Pos 34</i> : Trp(CCA) <i>Gm</i> : Phe(GAA) <i>Um(ncm⁵Um)</i> : Leu(UAA)	Not essential ^c
TRM8, TRM82	m ⁷ G	46	Cys(GCA), Ile(UAU), Lys(CUU), Lys(UUU), Met(CAU), Phe(GAA), Pro(UGG), Trp(CCA), Val(AAC), Val(CAC)	Not essential
TRM9, TRM112	"m" modification on mem ⁵ U and mcm ⁵ s ² U	34	<i>m(mcm⁵U)</i> : Arg(UCU) <i>m(mem⁵s²U)</i> : Glu(UUC)	Not essential ^c
TRM10	m ¹ G	9	Ala(AGC), Arg(ACG), Arg(UCU), Cys(GCA), Gly(CCC), Gly(GCC), Ile(AAU), Lys(CUU), Met(CAU), Pro(UGG), Thr(AGU), Thr(CGU), Trp(CCA), Val(AAC), Val(UAC)	Not essential
TRM11, TRM112	m ² G	10	Arg(ACG), Arg(ACU), Asn(GUU), Ile(AAU), Ile(UAU), Leu(CAA), Leu(UAA), Leu(UAG), Lys(CUU), Met(CAU), Phe(GAA), Thr(AGU), Trp(CCA), Tyr(GUA), Val(CAC), Val(UAC)	Not essential

(continued)

Table 10.1 (continued)

Gene	Posttranscriptional modification	Position on tRNA	Target tRNA ^a	Essential/hot essential gene ^b
TRM13	Am, Cm	4	<i>Am</i> : His(GUG) <i>Cm</i> : Gly(GCC), Pro(UGG)	Not essential
TRM44	Um	44	Ser	Not essential
TRM140	m ³ C	32	Ser(AGA), Thr(AGU)	Not essential
TYW1-4	yW	37	Phe(GAA)	Not essential

^a All tRNA substrates (cytoplasmic or mitochondrial) that have been validated experimentally according to Modomics (Machnicka et al. 2013) are indicated

^b Data obtained from the Saccharomyces Genome Database (Cherry et al. 2012). See also (Phizicky and Hopper 2010; Novoa and Ribas de Pouplana 2012)

^c Components of the Elongator complex or associated to the Elongator complex

^d Component of the ECK/KEOPS complex

^e While these genes are not essential, yeast null mutants show several defects such as temperature sensitivity, slow growth, paramomycin sensitivity, or general sickness

Interestingly, while methylation of tRNA nucleotides is important for tRNA structure, function, and stability (see below), complete deletion of individual Trm genes in *S. cerevisiae* generally results in only mild phenotypes, or phenotypes under particular stress conditions. The exception to this is deletion of the Trm6/Trm61 complex, in charge of producing 1-methyladenosine (m^1A) at position 58 of many eukaryotic tRNAs, which was shown to be essential in budding yeast (Anderson et al. 2000). The deletion of *TRM5*, that catalyzes the formation of 1-methylguanosine (m^1G), 1-methylinosine (m^1I), and the first methylation step for synthesis of wybutosine (yW) (all at position 37 of the tRNA) results in dramatic growth defects but it is not an essential gene (Bjork et al. 2001).

An important observation regarding the methylation of tRNAs came from Chan and colleagues who showed that the methylation pattern for a given tRNA substrate can significantly change upon cell treatment with toxic chemical stimulants such as hydrogen peroxide (Chan et al. 2010). This is consistent with observed phenotypes in different yeast Trm null mutants when put under stress. Trm9 forms a complex with Trm112 to catalyze the last methylation step for 5-methoxycarbonylmethyluridine (mcm^5U) or 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) at position 34 of different tRNAs. Deletion of *TRM9* in *S. cerevisiae* results in increased sensitivity to DNA damage (Begley et al. 2007) and to paromomycin, a translational inhibitor, when cells are grown at 37 °C (Kalhor and Clarke 2003). Likewise, deletion of *TRM4*, responsible for 5-methylcytidine (m^5C) modification, confers hypersensitivity to oxidative stress (Chan et al. 2012).

Elongator is a highly conserved protein complex composed of 6 proteins (Elp1-6 in yeast) that has been associated with numerous cellular processes such as histone acetylation, regulation of exocytosis, transcriptional elongation, and others (Phizicky and Hopper 2010). The Elongator complex is also required for the early steps in the modification of U34, therefore affecting many derived modifications bearing a 5-carboxymethyl core structure (Huang et al. 2005). In *S. pombe* lack of a functional Elongator complex affects mcm^5s^2U modification at position 34 of tRNAs and results in enhanced sensitivity to hydrogen peroxide (Fernandez-Vazquez et al. 2013).

Some yeast *TRM* deletions have severe consequences when combined with the deletion of additional tRNA modification enzymes. This is the case for mutants missing Trm8/Trm82, the enzymes responsible for formation of 7-methylguanosine (m^7G) at position 46 in tRNAs. *S. cerevisiae* lacking the *TRM8* and *TRM82* genes can grow normally under standard laboratory conditions, but are temperature-sensitive when grown in synthetic media containing glycerol (Alexandrov et al. 2005). However, under this deletion background, cells also deleted for other non-essential tRNA modification enzymes such as Trm4, Pus7, and Dus3, among others, showed distinct temperature-sensitive growth defects. Moreover, a mechanism for rapid tRNA degradation has been reported for endogenous tRNA_{ACC}^{Val} in *S. cerevisiae* strains double-deleted for *TRM8* and *TRM4* (Alexandrov et al. 2006). Similarly, yeast strains lacking the nonessential enzymes Trm44 (in charge of 2'-O-methylating uridine at position 44 in tRNA^{Ser}) and Tan1 (tRNA cytidine N4

acetyltransferase) show growth defects, while mutant strains for each single deletion do not. The growth phenotype under this double-deletion background, was attributed to reduced levels of tRNA^{Ser}_{CGA} and tRNA^{Ser}_{UGA} (Kotelawala et al. 2008), suggesting a role for tRNA modifications in tRNA stability.

Other tRNA Modifications

Beyond tRNA methyltransferases, there are several other tRNA modification enzymes that play crucial roles in tRNA biology. These include deamination enzymes, enzymes involved in thiolation, and acetyltransferases and isomerases, among others. Here, we will give a brief description of the most relevant ones and those that have been most widely studied.

Adenine (A) to Inosine (I) conversion is catalyzed by Adenosine Deaminases acting on tRNAs (ADATs), and occurs mainly at two different positions in tRNAs: the wobble position 34 and position 37 adjacent to the anticodon. In all eukaryotes, deamination of A37 is catalyzed by ADAT1 while deamination of A34 is catalyzed by the heterodimeric enzyme ADAT2/ADAT3. The homologues for these enzymes in yeast are named Tad1, Tad2, and Tad3, respectively. The roles for A-to-I modification vary with the position in the tRNA, with I37 likely influencing tRNA anticodon loop structure (Gerber et al. 1998; Grosjean et al. 1996) and I34 being more directly involved in codon-anticodon recognition (Crick 1966; Novoa et al. 2012). I37 is present only on eukaryotic tRNA^{Ala} and is further modified to m¹I by an S-adenosyl-L-methionine-dependent methyltransferase. In *S. cerevisiae*, it was shown that Tad1 is capable to specifically modify A37 but not A34 and that *TAD1* null mutants were viable and showed no slow growth phenotype at 30 °C (Gerber et al. 1998).

While adenine can only pair efficiently with uridine; inosine can pair with uridine, adenine, and cytosine. This has raised the possibility that I34 plays a crucial role in expanding the number of codons a single tRNA can recognize. Indeed, very recently a role in shaping the genome structure and codon usage has been suggested for ADAT2(Tad2)/ADAT3(Tad3) in eukaryotes as an improved correlation between tRNA gene copy number and codon usage could be achieved when the effect of the I34 modification was considered in the analysis (Novoa et al. 2012). Consistent with this critical functional role for this modification, both *TAD2* and *TAD3* genes were shown to be essential in *S. cerevisiae* (Gerber and Keller 1999).

Thiolation is another very important modification that is found at U34 of several tRNA species resulting in 2-thiouridine (s²U) (Bjork et al. 2007). In *S. cerevisiae*, this modification is catalyzed by the complex Ncs2/Ncs6 for cytosolic tRNAs and by Mtu1 for mitochondrial tRNAs. Following thiolation, U34 is usually further modified at the C5-position of the pyrimidine ring with other chemical groups. The combination of all of these modifications at U34 may have consequences for aminoacylation, translation efficiency and fidelity (Jackman and Alfonzo 2013).

Wybutosine (γ W37) is present at position 37 of tRNA^{Phe}. It is a guanosine derivative resulting from several chemical modification steps. In *S. cerevisiae*, the reactions are catalyzed by 4 different enzymes Tyw1, Tyw2, Tyw3, and Tyw4 that produce a methyl imidazole ring, addition of an α -amino- α -carboxypropyl group, methylation of guanosine N3, and methylation of the α -carboxy group and methoxycarboxylation of the α -amino group; respectively. While this modification seems to be important for reading frame maintenance, none of the enzymes responsible for γ W37 were reported to be essential in yeast (Phizicky and Hopper 2010).

Isomerization occurs for uridine at several positions in tRNAs to give rise to pseudouridine. This reaction is catalyzed by tRNA pseudouridine synthases (Pus). In *S. cerevisiae*, there are 6 different Pus that act on different tRNA substrates and different tRNA residues (Phizicky and Hopper 2010). Disruption of *DEG-1*, the gene encoding for Pus3 that modifies uridine at position 38 and 39 in yeast, was shown to reduce growth rate, especially at 37 °C (Lecoite et al. 1998).

N6-threonylcarbamoyl adenosine (t^6 A) modification at position 37 of tRNAs is one of the few modifications at the anticodon loop that is present in all kingdoms of life. It is a complex modification involving several enzymes out of which some remain unknown for eukaryotes and archaea (El Yacoubi et al. 2012). In *S. cerevisiae*, the gene *SUA5* responsible for the t^6 A modification was shown to be dispensable; however, null strains for this gene showed severe growth phenotypes (El Yacoubi et al. 2009).

Role of tRNA Modifications in Structure, Function, and Stability of tRNAs

The role of tRNA modifications in tRNA structure and function has been recently reviewed (Jackman and Alfonzo 2013). Notably, while tRNAs are heavily modified by many different enzymes involving several types of chemical reactions, the overall result for such modifications in tRNA function seems to be rather subtle. Here, we will describe some of the most visible effects that tRNA modifications can have on tRNA structure, function, and stability.

Modifications in the region of the anticodon loop can affect protein translation. In general, modifications at position 37 sterically block Watson-Crick pairing and can influence frameshifting. For example, isopentenyl adenosine (i^6 A) modification at position 37 in *S. pombe* was shown to increase the affinity of tRNAs for cognate codons while decreasing affinity for noncognate codons (Lamichhane et al. 2013). Likewise, the presence of m^1 G at position 37 was shown to prevent frameshifting in *Salmonella typhimurium* (Bjork et al. 1989) and was likely the cause for the observed growth phenotypes upon deletion of *TRM5* in *S. cerevisiae* (Bjork et al. 2001). Similarly, Urbonavicius and colleagues showed that Trm5 methylation was necessary for proper in-frame translation affecting +1 frameshifting but not -1 frameshifting (Urbonavicius et al. 2001, 2003). Wybutosine 37, like other purine bases at position 37, helps stabilize codon-anticodon pairing by providing base stacking interactions within the A site of the ribosome (Konevega et al. 2004) and has been

shown to prevent -1 frameshifts in yeast (Waas et al. 2007). Finally, translation initiation defects have been reported for cytoplasmic tRNAs lacking the t⁶A37 modification (Lin et al. 2010; El Yacoubi et al. 2011).

Modifications at position 34 of the tRNA can also affect translational fidelity; one such example in *S. cerevisiae* is the role of Trm9 modifications in a particular set of transcripts where it prevents protein synthesis errors and the triggering of protein stress response pathways (Patil et al. 2012). However, modifications at position 34 are considered more important for decoding than for misreading, as exemplified by the I34 modification mentioned above and by the thiolation of uridine (and posterior modifications) recently reviewed by Phizicky and Hopper (Phizicky and Hopper 2010).

Some modifications play key roles in tRNA structure and stability. These modifications are usually located at the core of the tRNA and either rigidify or increase flexibility of its structure. For example, pseudouridines keep their ribose rings in the strong binding C3'endo conformation, while dihydrouridines keep the sugar pucker in a DNA-like C2'endo conformation (Giege et al. 2012). The C3'endo sugar conformation can also protect the RNA from endogenous nucleases. Thermostability of tRNAs due to particular modifications has been well documented in bacteria, where some of these modifications are exclusive of thermophilic species (Motorin and Helm 2010); it remains to be explored whether novel thermostable tRNA modifications are present in thermophilic fungi. In this respect an in vitro comparison between a tRNA from *Humicola lanuginosa* and *Candida utilis* did not show major differences in melting temperatures; however, the tRNA from *H. lanuginosa* was more resistant to RNase A treatment, suggesting a more compact tRNA tertiary structure (Joshi and Cherayil 1987). Finally, modifications can also promote ion binding, as is the case of m⁵C in yeast tRNA^{Phe}, which helps binding of magnesium at a distal site in the tRNA, thus promoting magnesium dependent conformational shifts (Chen et al. 1993).

Some modifications are critical to prevent the tRNA from entering certain degradation pathways. This has been exemplified previously when discussing *TRM8/TRM4* double mutants (Alexandrov et al. 2006; see above). Another example is the role for Trm2 in *S. cerevisiae* in stabilizing tRNA^{Ser}_{CGA}; although in this case the need for m⁵U at position 54 did not seem to be the main factor affecting tRNA stability (Johansson and Bystrom 2002). As mentioned before, yeast tRNA^{Ser}_{CGA} and tRNA^{Ser}_{UGA} can be destabilized in the absence of the enzymes responsible for methyluridine at position 44 (Trm44) and N4-acetylcytidine at position 12 (Tan1) (Kotelawala et al. 2008). Finally, in *S. cerevisiae*, aberrant pre-tRNA^{iMet} transcripts lacking (m¹A) at position 58 are recognized by the TRAMP complex (discussed above) and targeted for degradation (Phizicky and Hopper 2010).

There are also examples of modifications that serve as identity elements for the tRNA. We have already discussed the role of G₁ on amino acid charging by HisRS (Rudinger et al. 1994). Also, m¹G at position 37 of tRNA^{Asp} has been described as an antideterminant for ArgRS (Putz et al. 1994). Lastly, the 2'O-

ribosyl phosphate modification at position 64 of the initiator tRNA in *S. cerevisiae*, catalyzed by Rit1, discriminates the initiator tRNAs from the elongator tRNAs during protein synthesis (Astrom and Bystrom 1994).

Mounting evidence now suggests that some tRNA modifications may have several roles and be linked to complex phenotypes and even human diseases (Torres et al. 2014). While some modifications may affect several tRNAs and general protein translation; others may affect single tRNAs and the reading of specific codons, leading to translation defects on specific proteins enriched in such codons (Novoa and Ribas de Pouplana 2012). For example, in yeast exposed to hydrogen peroxide, an increase in m⁵C modification at the wobble position by Trm4 was observed in tRNA_{CAA}^{Leu} leading to selective translation of mRNAs enriched in the TTG codon (Chan et al. 2012). On the other hand, general translational defects might be the cause of the multiple phenotypes associated with the Elongator complex. This complex is involved in the formation of mcm⁵s²U, and has been associated with different roles such as acetylation of histones H3 and H4, transcriptional elongation, regulation of exocytosis, filamentous growth signaling, and telomeric gene silencing (Phizicky and Hopper 2010; El Yacoubi et al. 2012). Similarly, the KEOPS (Kinase, Endopeptidase, and Other Small Proteins)/ECK (Endopeptidase-like kinase associated to transcribed chromatin) complex has been associated with multiple cellular functions such as transcription, telomere maintenance, and chromosome segregation and have recently been shown to be required for formation of t⁶A34 modification (Srinivasan et al. 2011).

tRNA Modifications and Evolution

As discussed, the complete set of modifications of any given tRNA is involved in three different aspects of tRNA function: folding, molecular recognition by proteins or RNAs, and codon-anticodon pairing. Out of the more than a hundred possible modifications only 17 are universally present in all domains of life (Grosjean 2009) (Fig. 10.3), and a clear tendency in evolution to increase the number and complexity of tRNA modifications can be seen from Archaea to bacteria, and to eukarya (Jackman and Alfonzo 2013).

The modifications that play a role in tRNA folding are thought to influence subtle features of the structure of tRNA, rather than having a major impact on the folding of the molecule. Crystallographic studies comparing an unmodified tRNA^{Phe} from *E. coli* to the fully modified tRNA^{Phe} from yeast revealed only slight structural changes, with no effect, for example, on the anticodon conformation (Byrne et al. 2010). Structural modifications are generally thought to contribute to the rigidity or flexibility of the tRNA molecule (Giege et al. 2012), and tend to be conserved in organisms that can particularly benefit from such features as those living in extreme temperature conditions (Edmonds et al. 1991; McCloskey et al. 2001).

Recognition by enzymes and RNAs involved in the maturation, aminoacylation, or transit through the ribosome of the tRNA can also require specific modifications. A key molecular recognition of tRNAs takes place during their aminoacylation by aminoacyl-tRNA synthetases (ARS). This activity translates the genetic code from triplet information to amino acid side chain, and constitutes the main check point to maintain the fidelity of the whole process of gene translation. Indeed, some unmodified tRNA sequences would be too similar to be easily distinguished by ARSs directly, and depend on modifications to prevent misacylation. An example of this situation is the bacterial tRNA^{Ile}_{CAU}. This tRNA can be easily misacylated by methionyl-tRNA synthetase (MRS), but this reaction is prevented by the modification of its C34 into lysidine (L) by lysidine-tRNA synthase (TilS) (Muramatsu et al. 1988; Soma et al. 2003). Interestingly, many of these identity marks were established late in evolution and are not conserved among species. For example, in the bacteria *Mycoplasma penetrans* L34 is not required for MRS rejection of tRNA^{Ile}_{CAU} which, instead, depends upon interactions with the acceptor stem (Jones et al. 2008, 2013).

Interestingly, tRNA misacylation is a physiological feature of some yeast tRNAs, which can be charged with two amino acids by different ARS (Moura et al. 2010). Indeed, a *C. albicans* tRNA^{Ser}_{GAC} is charged normally with serine by seryl-tRNA synthetase (SRS), but is also charged with leucine by leucyl-tRNA synthetase (LRS) (Moura et al. 2010). It should be noted that this phenomenon results in a statistical proteome where, under normal circumstances, 3 % of all coded serines are replaced by leucines in a random manner. Remarkably the levels of mistranslation can increase dramatically as a response to environmental factors, resulting in important changes in cell adhesion and colony structure (Silva et al. 2007). *C. albicans* has evolved to tolerate this adaptive strategy, and the vast majority of CUG codons in its genome code for surface amino acids in order to minimize the effect of this decoding flexibility upon protein structure (Rocha et al. 2011).

Base modifications in the anticodon loop are of utmost importance due to their potential influence in codon-anticodon recognition. In fact, the most intensively modified part of the tRNA is the anticodon loop and, within it, the most often altered positions are positions 34 and 37 (Chan and Lowe 2009). Indeed, in the genome of the bacterial endosymbiont *Candidatus, Riesia pediculicola*, which is characterized by its extreme gene reduction, the only tRNA modification enzymes conserved are those responsible for modifications at positions 34, 37, 38, and 39 (de Crecy-Lagard et al. 2012). The tRNA wobble nucleotide is usually modified and such modifications are essential for tRNA decoding. Interestingly, when looking at the three domains of life, it is clear that not all modifications are universal. For example, Cm, s²U, Gm, and Um are found in eukarya, bacteria, and archaea, but s²Um is present only in archaea, mcm⁵s²U is present only in eukarya, and cmnm⁵s²Um is present only in bacteria (Grosjean et al. 2010). This implies that different species have developed different decoding strategies for some anticodon-codon pairings.

The relatively good correlation between tRNA gene content and tRNA abundance implies that, to optimize translation, a co-evolution probably exists between tRNA gene content in the genome and the activities of the enzymes that modify the anticodon loop. A balance is necessary between tRNA abundance and the introduced anticodon modifications to ensure proper translation of the degenerate genetic code in a given codon usage context. Studies from Grosjean and coworkers have proposed different decoding strategies to explain the genomic composition of tRNAs genes based on the sequences and tRNA modifications specifically at the wobble position (Grosjean et al. 2010). Interestingly, in all the organisms checked, an A34NN anticodon never coexists with a G34NN anticodon decoding for the same amino acid. In Archaea, only the G34NN are used to read the pyrimidine-ending codons (G at position 34 of the tRNA and C or U at the third position of the codon). In bacteria the same usually applies, except for the case of tRNA^{Arg}, using an A34NN anticodon instead of a G34NN. The A34 in this anticodon is modified to inosine (I) by the homodimeric TadA enzyme, specific for tRNA^{Arg}_{ACG}, and thus can perform I34:C3, I34:U3, and less efficiently I34:A3 wobble pairings (Murphy and Ramakrishnan 2004; Wolf et al. 2002).

Other unmodified A34NN tRNAs in bacteria such as tRNA^{Thr} from *M. capricolum*, or tRNA^{Leu} from *M. synovia*, are very rare and are used to decode the four synonymous codons of their boxes (Lim 1995; Grosjean et al. 2010). On the other hand, in eukarya an A34 is systematically used instead of a G34 for the four box tRNA sets, except in the case of tRNA^{Gly}. All of these A34 are also modified to I, but in this case by the heterodimeric complex Tad2/Tad3 in yeast and ADAT2/ADAT3 in other eukaryotes as mammals (Gerber and Keller 1999). We have recently proposed that the evolution of modification enzymes acting at this wobble position played a central role in the difference in tRNA gene content, and the codon usage, of bacteria and eukarya (Novoa et al. 2012). Moreover, we showed that in these species, a good correlation between tRNA gene copy number and codon usage can only be found when the effect of these modifications, catalyzed by hetADATs (I34 modification) in eukarya and by uridine methyltransferases in bacteria, are taken into account (Novoa et al. 2012).

While there are different decoding strategies in the different kingdoms, some decoding strategies are universally conserved. This is the case, for example, for the preferential usage of U₃₄NN tRNAs for decoding synonymous codons on amino acids in the 4 box set; or the use of U₃₄NN or G₃₄NN tRNAs to decode the purine-ending or pyrimidine-ending codons, respectively, on amino acids in the 2 box split set (Grosjean et al. 2010). In this latter case, wobble modifications are usually required and are catalyzed by phylogenetically unrelated enzymes in different organisms. An interesting example is that of s²U34 modification. This modification is universally conserved but the enzymes responsible for its synthesis evolved independently in bacteria and eukaryotes. Extant eukaryotes, however, contain both a bacterial- and an eukaryal modification system that act, respectively, in the mitochondria and in the nucleus (Jackman and Alfonzo 2013).

Several examples exist of convergent evolution involving tRNA modification enzymes. For instance, the chemical modification m¹G37 is highly conserved in bacteria, eukarya, and archea; however, it is catalyzed by TrmD (in bacteria) and by Trm5 (in eukarya and archea), two evolutionary unrelated enzymes (Christian and Hou 2007). A more extreme case is that of the t⁶A37 modification which is also universally conserved in all kingdoms but the synthesis pathways differ dramatically among different species. On one hand, a set of core enzymes are conserved in all organisms for the synthesis of t⁶A37; and on the other hand, specific kingdom-specific or even organelle-specific enzymes are required to complete the synthesis pathway for this modification (El Yacoubi et al. 2012).

Just as different enzymatic solutions exist for the same modification, members of the same enzyme family may catalyze different modification reactions in different organisms. Such is the case of the RlmD family, which in *E. coli* catalyze the formation of m⁵U in the 23S rRNA (Madsen et al. 2003), but in archea is responsible for the formation of m⁵U in tRNAs (Urbonavicius et al. 2008). This is just one of several cases for divergent functional evolution for tRNA modification enzymes. This phenomena limits our ability to predict the function of homologous enzymes from different organisms; and the experimental validation of each enzyme candidate for the catalysis of a given tRNA modification is crucial. Moreover, as we move on to more complex organisms, the number of co-existing genes coding for potential tRNA modification enzymes tends to increase. For example, humans have more Trm homologues than yeast (Townes and Begley 2012), possibly because such enzymes in humans have a different set of substrates.

The reason why some tRNA modifications are conserved while others are not remains unclear. While there are 61 codons for decoding all 20 proteinogenic amino acids, not all possible 61 tRNA species have been found in any organism. As explained above, different species resolve this issue by relying on different tRNA anticodon modifications, especially that of position 34, that allow tRNAs to recognize more than one codon. It has been proposed (Grosjean et al. 2010) that the lack of some tRNA species could be a trait from the Last Universal Common Ancestor (LUCA). According to this theory, LUCA would have only incorporated a subset of the 20 common proteinogenic amino acids to its genetic code. Therefore, the decoding capacity of its tRNAs did not rely on position 34 of the anticodon. After the split of the three domains, organisms gradually incorporated new amino acids to the genetic code, and that lead to the split of several four codon boxes into 2/2 or 3/1 alternatives. To maintain a faithful and efficient translation, these organisms evolved different decoding strategies using tRNA anticodon modifications to fine tune the decoding capacity of their tRNAs. It should be noted that this evolutionary scenario is also possible if one considers LUCA to have incorporated all the 20 amino acids, but with a certain degree of inefficiency or translation error. Posterior and independent solutions would be found in each domain of life to improve the efficiency and accuracy of the system.

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