Transfer RNAs: diversity in form and function

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ABSTRACT
As the adaptor that decodes mRNA sequence into protein, the basic aspects of tRNA structure and function are central to all studies of biology. Yet the complexities of their properties and cellular roles go beyond the view of tRNAs as static participants in protein synthesis. Detailed analyses through more than 60 years of study have revealed tRNAs to be a fascinatingly diverse group of molecules in form and function, impacting cell biology, physiology, disease and synthetic biology. This review analyzes tRNA structure, biosynthesis and function, and includes topics that demonstrate their diversity and growing importance.

Genetic information is passed from DNA to RNA to protein through the processes of transcription and translation. Central to the information flow from RNA to protein are transfer RNA (tRNA) molecules [1]. tRNAs are the vehicle that bring amino acids to the growing polypeptide chain at the ribosome and read the three base codons that define protein sequence [2–5]. At the textbook level, tRNA structure and function can be described quite simply. In reality, they are significantly more complex, with nuances highly relevant to disease, evolution and biotechnology. This review will focus on the molecular biology of tRNAs, assembling some of the information that is not discussed in depth when considering general genetic information flow. Topics central to all organisms will be discussed but there will be specific focus on eukaryotic cytosolic tRNAs. The breadth of the review has meant that we have been unable to reference all of the relevant literature; however, in each section, we refer to more detailed review articles.

Structure
Holley et al. [6] determined the first tRNA sequence, a 77 base tRNA Ala from yeast. Overall the length of canonical tRNAs varies from 76 to 90 bases [reviewed in 7]. This gives the impression that they are small molecules; however, their average mass is greater than 26,000 g/mol, the approximate mass of a 230 amino acid residue protein. Their structure is defined by internal base pairing that results in a stem-loop pattern when depicted in two dimensions (Fig. 1A). From 5’ to 3’, the stem-loop structures are the acceptor stem, the D-arm, anticodon stem, variable loop and T-arm. The D-loop is named after the modified dihydrouridine base present in the loop and is responsible for stabilizing the tRNA tertiary structure [8]. The T-arm, also known as the TΨC arm, is named after the presence of the universally conserved modified bases thymidine, pseudouridine and cytidine [6] and is responsible for facilitating interactions with the ribosome [9]. As suggested by the name, the length of the variable arm is variable. In eukaryotes, it is most prominent for the serine (tRNA Ser), selenocysteine (tRNA Sec) and leucine (tRNA Leu) tRNAs. Longer variable arms are also found in eubacterial and organellar tRNA373 and in a systematic search for other variable arm containing tRNAs, Hamashima et al. [10] identified 253 additional non-serine or non-leucine tRNAs with an extended variable arm from a range of eukaryotic organisms.

tRNAs have two key single-stranded regions required for translation: the three base anticodon determines translation specificity by base pairing with the mRNA codon and the 3’ acceptor CCA is the site of covalent amino acid attachment in an ester linkage. The enzymes responsible for aminoacylation, or charging, of an amino acid onto its cognate tRNAs are called aminoacyl-tRNA synthetases [aARS]. The specificity of aminoacylation is critical because the ribosome does not read the amino acid on the tRNA. As demonstrated by Chapeville et al. [11], the ribosome will incorporate a non-coded amino acid if the tRNA is incorrectly aminoacylated. The base 5’ to the terminal CCA is also not base paired. It is referred to as the discriminator base because of its importance in determining aminoacylation specificity [12].

In three dimensions, tRNAs fold into an L-shaped structure, primarily through intramolecular tertiary interactions of the T- and D-arms (Fig. 1B; [13,14]). At one end of the L is the amino acid acceptor branch and at the other is the anticodon branch. The amino acid acceptor branch is formed from interactions between the acceptor stem, generally seven base pairs, and T-stem, generally five base pairs. It is this 12 base pairs in a 7/5 configuration that is recognized by the elongation factor EF1A in eukaryotes and archaea and EF-Tu in bacteria [15]. The anticodon branch consists of the anticodon and D-arms. The region where the branches meet is referred to as the elbow [16]. It should be noted that whereas the L-shaped structure of the
tRNA is generally conserved across organisms, the exact structural details are not [reviewed in 17, 18]. For example, both bacterial and eukaryotic tRNA Sec have a 13 base-pair acceptor branch [19,20]. There are even functional mitochondrial tRNAs that lack one or both of the D- or T-arms [21-24]. Other proteins and RNA molecules adopt the canonical L shape to interact with translational machinery in a process known as tRNA mimicry. For example, some RNA viruses have internal ribosome entry sites (IRES) to direct translation of overlapping open reading frames, where a pseudoknot RNA structure within the viral RNA transcript adopts a tRNA-like structure to recruit the host ribosome without the need for an initiator tRNA Met [reviewed in 25]. In other cases, proteins such as elongation factor G, responsible for translocation of the tRNA and mRNA through the ribosome, and the release factor proteins, responsible for translation termination, adopt tRNA-like structures to facilitate interactions with the ribosome (Fig. 1C; reviewed in 26, 27).

tRNAs that accept the same amino acid are called isoacceptors. They differ in their anticodon sequence reflecting that all amino acids other than methionine and tryptophan are coded for by more than one codon. Generally, tRNA sequences in the same isoacceptor family are similar but not identical. There are 21 types of isoacceptors, one for each of the 20 standard amino acids and one for selenocysteine. The three stop codons are represented by release factor proteins that terminate translation [28], rather than tRNAs; though it should be noted that stop codons have been reassigned to encode amino acids in some organisms [reviewed in 29]. Not all of the 61 amino acid encoding codons have a corresponding tRNA with a completely complementary anticodon sequence. Wobble at the third position of the codon allows for decoding of all codons (discussed below). tRNAs with the same anticodon and thus decode the same set of codons are called isodecoders. tRNAs in the same isodecoder family differ in their primary sequence at sites other than the anticodon.

The number of tRNAs found in different organisms varies widely; for example, Saccharomyces cerevisiae and humans contain 275 and 416 high confidence tRNA encoding genes, respectively [30, http://gtrnadb.ucsc.edu]. Because of wobble, 32 tRNA species are sufficient to decode all 61 codons. There are, however, many different isodecoders per organism, each often having multiple genomic copies. The additional copies provide redundancy in case of a loss of function mutation and a buffer should a mutation result in noncognate decoding [31]. These multiple copies provide the raw material for genetic code evolution.
tRNAs have a standard numbering ending with the terminal 3′ CCA being numbers 74, 75 and 76 (Fig. 1A; [32]). Base 1 in the acceptor arm is the 5′ base that pairs with base 72. tRNAHis has an additional 5′ base, which is added post-transcriptionally, numbered 0 but sometimes referred to as −1 (discussed below). Bases with conserved function or invariant nucleotides always have the same number. Most notable of these are the three anticodon positions, which are always numbered 34, 35 and 36. Other constant bases include two invariant guanine residues at positions 18 and 19 in the D-loop and 1ΨC residues at positions 54, 55 and 56. Additional bases found after a conserved position are designated with letters following the conserved position number (e.g. 20A and 20B). The discriminator base is numbered 73. The variable arm begins with bases 44 and 45 and ends with bases 46, 47 and 48. In tRNAs with extended variable arms, mainly tRNA^{Sec}, tRNA^{Sec} and tRNA^{Leu}, additional bases are numbered after position 45 and designated with an e. They are not numbered sequentially but rather numbered in a way that corresponds to their base-pairing partner. For example, position e11 would be the first extended variable arm base and base pair with the last extended variable arm base, position e21. The single-stranded loop region in an extended variable arm is numbered as single digits beginning with e1. Full tRNA numbering can be found in [32].

**tRNA biosynthesis**

**Transcription**

In eukaryotes, tRNAs are transcribed by the relatively α-amanitin resistant RNA polymerase III [RNAPIII; 33–35, reviewed in 36]. RNAPIII is a 17-subunit enzyme with its two largest subunits resembling β and β′ from bacterial RNA polymerase [37]. RNAPIII transcribes other small noncoding RNAs including 5S rRNA, U6 snRNA of the spliceosome and the RNA component of both RNase P and the signal recognition particle [reviewed in 38]. The tRNA genes are highly expressed at approximately 300,000 copies per cell and on par with ribosomal RNA genes [39]. The elements required to transcribe tRNA genes, known as the A box (consensus sequence TRGYNNARNNG) and B box (consensus sequence RGTTCAACTCC), are internal to the tRNA gene within the sequences encoding the D- and T-arms [40–42]. The spacing between the A and B box varies greatly depending on whether a tRNA encoding gene contains an intron and the length of the variable arm. Segall et al. [43] were the first to characterize the factors required for RNAPIII transcription at tRNAs. They identified two column fractions they termed B and C that allowed in vitro transcription of tRNA genes. Further purification of the activities in these fractions led to the designations TFIIIB and TFIIIC. Transcription is initiated with the binding of TFIIIC to the A and B box (Fig. 2: [44,45]). Bound TFIIIC recruits TFIIIB to the 5′ side of the gene [46,47]. One of the three protein subunits of TFIIIB is the TATA-binding protein [48,49]. As part of the TFIIIC-TFIIIB-DNA ternary complex, TFIIIB recruits RNAPIII [50] and participates in promoter melting with the C37 subunit of RNAPIII [51,52], whether TFIIIC is displaced during elongation is unclear. Turowski et al. [53] suggest that it is displaced first from the A box then the B box as transcription proceeds. Transcriptional termination requires a stretch of five or more T residues on the non-template strand [54] and three subunits of RNAPIII [55]. Transcriptional re-initiation, which allows enhanced transcription of tRNA genes, is thought to occur by TFIIIB maintaining contact with RNAPIII through the termination process [56, reviewed in 57]. The exception to the basic mechanism described above is tRNA^{Sec}. In most eukaryotes, tRNA^{Sec} recruits RNAPIII through an internal B box and upstream elements also found in U6 snRNA genes [see, for example, 58]. The distinction is even clearer in trypanosomes where tRNA^{Sec} is transcribed by RNA polymerase II [59].

![Figure 2](image-url) tRNAs are transcribed by RNA polymerase III. The internal A- and B-box sequences are recognized by TFIIIC (i). Bound TFIIIC leads to the recruitment of TFIIIB which binds upstream of the tRNA gene and recruits RNAPII (ii). TFIIIB and RNAPII melt the promoter, leading to transcription of the tRNA (iii and iv). Termination is signalled by a string of thymine residues on the non-templated strand (v). Transcriptional re-initiation, where RNAPIII is recycled to the transcriptional start site, can enhance transcription of tRNA genes (iv).
RNAPIII transcription is regulated by Maf1, which inhibits transcriptional re-initiation in response to nutrient stress [60]. Maf1 is regulated by phosphorylation from a range of kinases including TORC1 [61,62]. Interestingly, Gerber et al. [63] recently demonstrated that in HEK293 cells, RNA polymerase II transcription through a subset of tRNA encoding genes inhibits RNAPIII transcription in a Maf1 dependent fashion. Also in mammalian cells, RNAPIII is regulated by Myc [reviewed in 64], p53 [65] and Rb [66], emphasizing the importance of RNAPIII transcription in cell cycle control and disease processes.

**tRNA processing**

After transcription, pre-tRNA transcripts are processed (Fig. 3A). Transcription results in a 5′ leader of approximately 10 bases that is removed by RNase P, the first transacting ribozyme identified [67]. Though there are exceptions [68], the canonical eubacterial, archaeal and eukaryotic RNase P is a ribonucleoprotein where the RNA plays a catalytic role [69–71]. Cleavage of the scissile phosphodiester bond leaves the 5′ end of the RNA with a 3′-OH and the 3′ end of the RNA with a 5′-phosphate [69, reviewed in 72]. In eukaryotes, cleavage of pre-tRNA leader sequences by RNase P requires base pairing at the first and second base pairs in the acceptor stem and generates an acceptor stem with the canonical seven base pairs [73–75]. The 3′ ends of pre-tRNAs are a short U tract [reviewed in 76]. This U tract is the binding site for the La protein, which protects the 3′ end of the transcript from exonuclease digestion and assists pre-tRNA folding [77, reviewed in 78]. The 3′ endonuclease RNase Z cleaves after the discriminator nucleotide leaving a 3′-OH [79]. Both 5′ and 3′ cleavage occur in the nucleus. It should be noted that in eukaryotes, the 3′ exonucleases Rex1p and Rpr6p process pre-

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**Figure 3.** Lifecycle of a tRNA. (A) tRNAs are transcribed (i) with 5′ leader and 3′ trailer sequences that are cleaved by RNase P and RNase Z respectively (ii). After trimming, partial modification (iii), indicated by the red circles, and addition of the terminal CCA residues (iv) occur in the nucleus. In yeast, the tRNA is exported out of the cytoplasm by Los1 (v) and if the tRNA contains an intron, it is spliced on the mitochondrial surface (vi). In mammals, splicing occurs before nuclear export. In the cytoplasm, additional modifications may be added (vii). tRNAs can then be aminoacylated by their cognate aminoacyl-tRNA synthetase (viii) or be re-imported (ix) by Mtr10 into the nucleus for further modification (x) and quality control through nuclear aminoaacylation (xi). Re-export of tRNAs is facilitated by both Los1 and an MnsS dependent pathway that specifically recognizes mature tRNAs (xii). Once aminoacylated, tRNAs are used in translation (xiii). (B) Quality control mechanisms can lead to the degradation of tRNAs. After transcription, nuclear surveillance (i) monitors tRNAs for end maturation and specific modifications. Improper tRNAs are polyadenylated by the TRAMP complex and degraded by the nuclear exosome. After processing and export, tRNAs are monitored and degraded through the rapid tRNA pathway by the 5′-3′ exonuclease Xrn1 in the cytoplasm (ii) and Rat1 in the nucleus (iii).
tRNA trailer sequences under some conditions [80,81], while processing for bacterial tRNAs with an encoded 3’ CCA uses a different mechanism. Following 3’ cleavage, the CCA adding enzyme [ATP (CTP): tRNA nucleotidyltransferase] catalyzes the addition of the CCA terminus or any nucleotides required to complete the CCA terminus, without the need of a template [82]. In organisms where the CCA is encoded in the genome, the enzyme repairs 3’ termini [83].

tRNA$^{\text{His}}$ has an additional G nucleotide at its 5’ end that is not genomically encoded [reviewed in 84]. In eukaryotes, this is accomplished after processing by Thg1 enzymes, which use a 3’-5’ addition reaction to add the G residue [85, 86, reviewed in 87]. The 3’-5’ reaction is the reverse of other known DNA and RNA polymerases. The reaction occurs in a multistep process which begins with adenylation of the processed 5’ end in a high energy 5’-5’ linkage [86,88]. The 3’-OH of GTP then attacks the pyrophosphate of the adenylated tRNA, leaving a 5’ triphosphate, which Thg1 removes leaving a single 5’ phosphate. Thg1 specificity for tRNA$^{\text{His}}$ occurs through recognition of the GUG anticodon [89].

tRNAs are highly modified, with approximately 12% of nucleotides bearing a modification (Fig. 4A; reviewed in 90). Currently, 93 unique tRNA modifications have been identified from more than 700 diverse tRNA sequences [https://mods.rna.albany.edu/; 91]. Many of these modifications are broadly conserved in bacteria, eukaryotes and archaea; for example, the ribothymidine in the T-stem and dihydrouridine in the D-loop. *Saccharomyces cerevisiae* tRNAs contain 25

![Figure 4](https://mods.rna.albany.edu/) (A) Heat map showing modification frequency for each position in the tRNA on the 2D (left) and 3D (right) tRNA structures. Modification data for 715 unique tRNA sequences from archaea, bacteria and eukaryotes accessed from the MODOMICS database [http://genesi. pl/modomics/; 282]]. Circular bases are not modified in any of the 715 unique tRNA sequences in the database. (B) tRNA modifications found across all *S. cerevisiae* tRNAs [pseudouridine (Ψ); 2’-O-methylcytidine (Cm); 2’-O-methyladenosine (Am); 1-methylguanosine (m$^\text{G}$); N2-methylguanosine (m$^\text{G}$); N4-acetylcytidine (ac$^\text{C}$); dihydrouridine (D); 2’-O-methylguanosine (Gm); N2,N2-dimethylguanosine (m$^\text{2,2}$G); 3-methylcytidine (m$^\text{C}$); inosine (I); 5-methylcytidine (m$^\text{C}$); 5-methoxy carbonylmethyl-methyluridine (mcm$^\text{3}$U); 5-methoxy carbonylmethyl-2-thiouridine (mcm$^\text{3}$sU); 5-carbamoylmethyluridine (ncm$^\text{1}$U); 5-carbamoylmethyl-2’-O-methyluridine (ncm$^\text{1}$Um); 1-methylinosine (m$^\text{I}$); N6-isopen teryl adenosine (l$^\text{A}$); wybutosine (YW); N6-threo nitly carbamoyladenosine (l$^\text{A}$); 2’-O-methyluridine (Um); 7-methylguanosine (m$^\text{G}$); ribothymidine (RT); 1-methyladenosine (m$^\text{A}$); 2’-O-ribosyladenosine phosphate (Ar(p))]. The nucleobases of select-modified nucleotides are shown on the right.
Transport in and out of the nucleus

Eukaryotic tRNAs are shuttled in and out of the nucleus through the nuclear pore complex [97, reviewed in 98]. Primary export after synthesis requires a dedicated member of the β-importin family, Los1 in yeast and Xpo1 in vertebrates [99–101]. Los1/Exportin-t directly binds tRNAs in a Ran-GTP-dependent fashion [102–106]. Xpo1 is regulated by the small GTPase Ran and binds tRNAs in the nucleus in its GTP-bound form [reviewed in 107]. Binding requires a mature tRNA backbone and aminoacylation stem with processed 5‘ and 3‘ ends [103] but does not require intron removal [101,102,108]. Upon translocation through the nuclear pore, GTP hydrolysis results in tRNA release into the cytoplasm. Crm1 and Mex67-Mtr2 were identified in a genome-wide screen in yeast for other genes involved in primary tRNA nuclear export and participate in parallel pathways [109,110].

Retrograde transport of mature tRNAs back into the nucleus occurs constitutively in some organisms and in response to stress and nutrient deprivation [111–116, reviewed in 117]. The β-importin family member Mtr10 and interestingly the heat shock protein Hsp70 have been implicated in the process [118,119]. Constitutive retrograde transport is necessary for S. cerevisiae because the intron-containing tRNA(phe) is spliced on the surface of mitochondria (see below) and subsequently subject to G37 methylation in the nucleus [120]. The intron-containing tRNA(phe) is not a substrate for modification. Similarly, in Trypanosoma brucei, tRNA(3′-G) modification by tRNA-guanine transglycosylase occurs after retrograde transport of the spliced tRNA [121]. Schwenger et al. [122] recently demonstrated that in HeLa cells, tRNA(Sec) which is integral to redox control because of its requirement in the synthesis of selenoproteins, is subject to retrograde transport regulated by the integrated stress response pathway.

Re-export of tRNAs to the cytoplasm involves three pathways [reviewed in 123]. Two of these are shared with the export of primary tRNAs, the Los1/Xpo1 pathway and the Mex67-Mtr2 pathway. The third, an Msn5 dependent pathway, appears specialized for re-export of mature tRNAs since it is unable to transport unspliced tRNA transcripts [118]. Re-export is also partially dependent on nuclear aminoacylation of tRNAs [108,124,125]. Conditional mutants of aaRSs or the CCA adding enzyme or drugs that inhibit aminoacylation [see, for example, 126] lead to mature tRNAs accumulating in the nucleus. This likely functions as a quality control mechanism to ensure the export of functional tRNAs.

Splicing

A subset of tRNA genes in all three kingdoms of life contain introns [127]. In eukaryotes, the intron is positioned primarily following base 37 and does not affect the overall structure of the tRNA [reviewed in 128]. Splicing requires a tRNA-splicing endonuclease known as the Sen complex [129,130] and a tRNA ligase [131,132]. To allow flexible sequence selection, the Sen complex measures the distance between the body of tRNA and the splice sites [133,134]. In vertebrates, splicing occurs in the nucleus [129,135]; whereas in yeast the splicing endonuclease is located on the surface of the mitochondrion [136]. Cleavage by the Sen complex results in a 2′,3′ cyclic phosphate on the 5′ exon and a 5′-OH on the 3′ exon [137,138]. How these are treated depends on the organism. In yeast, Trl1 ligates the two exons together by opening the 2′,3′ cyclic phosphate, phosphorylating the 5′ terminus of the 3′ exon with GTP, activating the 5′ terminus with AMP and finally joining the two exons together [131,132]. Trl1 is also required to splice the HAC1 transcript in the unfolded protein response [139]. In vertebrates, tRNA exons are ligated directly by 5′-3′ ligation catalysed by a complex containing HSPC117 [140].

tRNA turnover

Mature tRNAs are stable, with half-lives around 2 to 3 days in eukaryotes [141–143]. Nonetheless, two main tRNA degradation pathways remove partially processed, hypomodified or misfolded tRNAs (Fig. 3B; reviewed in 144). In the first pathway, the TRAMP (Tf4 Ai2 Mtr4 polyadenylation) complex monitors pre-tRNAs and hypomodified tRNAs in the nucleus and targets them for degradation by the nuclear exosome. The pathway was recognized by Anderson et al. [145], who demonstrated that initiator tRNA(Met) lacking methylation at A58 is unstable. The components required were identified genetically [146, 147] when the temperature-sensitive lethality of a trn6 mutant, encoding the tRNA 1-methyladenosine methyltransferase responsible for initiator tRNA(Met) modification, was suppressed by deleting the nuclease subunit of the nuclear exosome or the poly(A) polymerase of the TRAMP complex. Hypomodified tRNAs are 3′ polyadenylated, which signals degradation by the nuclear exosome in a 3′ to 5′ manner. This pathway also monitors the 3′ ends of pre-tRNAs, where the loss of accurate 3′ end maturation leads to polyadenylation of select tRNA substrates and degradation by the nuclear exosome [80,81]. A large-scale study of nuclear exosome catalytic mutants performed by Gudipati et al. [148] suggests that up to 50% of pre-tRNAs are degraded by this pathway.

The second tRNA decay pathway, the rapid tRNA decay (RTD) pathway, monitors specific mature tRNAs and degrades hypomodified or unstable tRNAs in a 5′ to 3′ manner. tRNAs targeted by the RTD pathway include hypomodified tRNA(Val) lacking 7-methylguanosine and 5-methylcytidine [149] and tRNA(ser)(UGA) and tRNA(ser)(CGA) lacking 2′-O-methyluridine and 4-acetylcytidine or 5-methylcytidine and 2′-O-methylguanosine [150–152]. In addition, tRNAs that are structurally unstable are targeted by this pathway [153–156]. Degradation requires the 5′-3′ exonucleases Rat1 or Xrn1 [150].

unique modifications that occur at 36 positions (Fig. 4B). These modifications include the addition of methyl groups to the ribose 2′-OH or nucleobases, acetylation of nucleobases, modification of uridine to pseudouridine and dihydrouridine, deamination of adenine to inosine and addition of threonyl-carbamonyl and isopentenyl groups to adenine, among others. Modification occurs at many stages of tRNA biosynthesis [reviewed in 92], including prior to processing in the nucleus [93–95]. As such, some of the modifying enzymes are located in the nucleus, others in the cytoplasm and some have isozymic forms located in both [96]. A detailed discussion of the functions of tRNA modifications is presented below.
tRNAs can also be degraded through endonucleolytic cleavage. This often occurs in response to stress and involves cleavage near or within the anticodon loop to generate tRNA half molecules that can be signalling molecules (discussed further below: Other functions of tRNAs).

Aminoacylation

Aminoacyl-tRNA synthetases (aaRS) are the enzymes that couple amino acids to tRNAs and thus allow tRNA molecules to serve as adaptors in the central dogma [157]. Each tRNA isoacceptor family is aminoacylated by a specific aaRS [reviewed in 158]. The reaction requires specific recognition of an amino acid and a tRNA. In the first step of the two-step reaction, the amino acid is condensed with ATP to form an aminoacyl adenylate. Interestingly, the aaRSs were first identified for this step in the reaction [e.g. 159, see 2]. In the second step, the amino acid is transferred in a high energy ester linkage to the 2'- or 3'-OH of the terminal adenosine ribose [reviewed in 160]. In solution, the 2'- and 3'-linked amino acid transacylates to the neighbouring -OH [161] with translation elongation factor EF-Tu/EF1A binding stabilizing the preferred 3'-linked form [162].

In general, each aaRS is specific for one amino acid and one tRNA isoacceptor group, but there are exceptions. SerRS serylates the different tRNA\(^{\text{Ser}}\) isoacceptors and tRNA\(^{\text{Sec}}\) with serine [163,164]. Serylated tRNA\(^{\text{Sec}}\) is then converted to Sec-tRNA\(^{\text{Sec}}\) enzymatically [165-168]. tRNA\(^{\text{Sec}}\) has a UCA anticodon and is selectively inserted at UGA stop codons. Bacteria have a relatively common mechanism that differs from that found in archaea and eukaryotes [reviewed in 169]. Another example of an aaRS charging multiple isoacceptor groups occurs in several eubacteria and archaea that lack GlnRS and AsnRS [170]. In these cases, tRNA\(^{\text{Gln}}\) and tRNA\(^{\text{Asn}}\) are charged by GluRS and AspRS, respectively, with glutamate and aspartate [171]. A glutamine-dependent amidotransferase (AdT) then converts Glu-tRNA\(^{\text{Gln}}\) and Asp-tRNA\(^{\text{Asn}}\) to Gln-tRNA\(^{\text{Gln}}\) and Asn-tRNA\(^{\text{Asn}}\), respectively [172]. Sauerwald et al. [173] described an unusual circumstance in methanogenic archaea where Cys-tRNA\(^{\text{Cys}}\) is synthesized in a two-step pathway that does not involve an aaRS specific for cysteine. Rather, O-phosphoserine (Sep) is first transferred to tRNA\(^{\text{Cys}}\) by SepRS. Sep-tRNA\(^{\text{Cys}}\) is then converted to Cys-tRNA\(^{\text{Cys}}\) by Sep-tRNA\(^{\text{Cys}}\)-tRNA synthase (SepCysS). This pathway is likely more broadly conserved as bioinformatic analysis has revealed SepRS and SepCysS in other archaea and bacteria [174].

The sequences that allow tRNA recognition by a specific aaRS are called identity elements. Identity elements consist of single nucleotides, nucleotide pairs and structural motifs [175,176]. For many tRNAs, the anticodon is a principal identity element making direct contact with the aaRS [e.g. 177]. This recognition provides a direct link between the anticodon and its amino acid assignment [178]. However, other specific nucleotides in the acceptor stem provide essential structural characteristics for aaRS recognition and aminoacylation [179–181]. In particular, base 73, the discriminator base positioned directly 5' of the terminal CCA, is a common point of recognition. The additional G at −1 of tRNA\(^{\text{His}}\) is essential for charging by HisRS [182,183].

For tRNA\(^{\text{Ala}}\), tRNA\(^{\text{Ser}}\) and tRNA\(^{\text{Sec}}\) the anticodon plays no role in recognition by their cognate aaRSs. In some organisms (e.g. S. cerevisiae and humans) this applies to a lesser extent for tRNA\(^{\text{Met}}\) as well. For tRNA\(^{\text{Ala}}\), the principal identity element is a G3:U70 base pair in the acceptor stem [184–187]. In fact, including a G3:U70 base pair in non-alanine isoacceptors is sufficient for their charging by AlaRS [e.g. 188, 189]. This may explain the relative absence of G3:C70 or A3:T70 base pairs in non-alanine tRNAs, as a single mutation would change coding specificity [190]. For SerRS, the major feature recognized in both tRNA\(^{\text{Ser}}\) and tRNA\(^{\text{Sec}}\) is the variable arm, with both the length and composition being important [191–194]. For these tRNAs, a change in the anticodon to a sequence designating another amino acid will lead to a coding error (discussed below: tRNA-dependent mistranslation).

Ten aaRSs have pre- and post-transfer editing mechanisms to distinguish between similar amino acids and correct aminoacylation errors [IleRS, PheRS, ValRS, LeuRS, MetRS, ThrRS, AlaRS, ProRS, LysRS and SerRS; reviewed in 195]. Baldwin et al. [196] discovered the first example of editing when they observed that IleRS can form Val-AMP, but addition of tRNA\(^{\text{ile}}\) results in its hydrolysis. Further studies from Eldred and Schimmel [197] and Yarus [198] demonstrated post-transfer editing where Val-tRNA\(^{\text{Val}}\) and Ile-tRNA\(^{\text{Ile}}\) were hydrolysed by IleRS and PheRS, respectively. Pre-transfer editing was later observed by Fersht [199] for activated Val-AMP by IleRS using fast-kinetic studies.

Structural studies by Nureki et al. [200] revealed that editing occurs in a separate domain of the aaRS, supporting the double-sieving model proposed by Fersht [199]. The first sieve is the aaRS active site, which activates only cognate, isosteric or smaller amino acids. The second sieve is the editing site, which rejects cognate amino acids based on hydrophobicity, but hydrolyzes mis-activated or mischarged amino acids. Similar to aaRSs, mischarged tRNAs are recognized through identity elements within the tRNA. For example, LeuRS and AlaRS recognize the elbow region of tRNA\(^{\text{Leu}}\) and the G3:U70 identity element of tRNA\(^{\text{Ala}}\), respectively, for both aminoacylation and post-transfer editing [201–203]. In other cases, the elements for editing are distinct from those for aminoacylation. For example, the D-loop of tRNA\(^{\text{ile}}\) is essential for editing of mischarged Val-tRNA\(^{\text{ile}}\), but mutation of the D-loop does not affect aminoacylation [204,205].

In addition to aaRS editing domains, separate trans-acting editing proteins exist. These include D-Tyr-tRNA\(^{\text{A}3}\) that hydrolyzes D-Tyr and other D-amino acids in many species [206–208]. Other trans-editing proteins target mischarged L-amino acids; for example, bacterial YbaK hydrolyzes mischarged tRNA\(^{\text{Pro}}\) [209,210]. Other similar free-standing enzymes edit tRNA\(^{\text{Ala}}\) and tRNA\(^{\text{Thr}}\) [211,212].

It should be noted that aaRSs act in other key cell signaling pathways beyond protein synthesis [reviewed in 213]. The multiple functions of aaRSs are perhaps not surprising because they were evolutionarily among the first group of enzymes to acquire specificity for amino acid binding, the synthetases also interact with the principal cell energy source, ATP, and specifically recognize a charged or uncharged isoacceptor group of tRNAs. Aminoacyl-tRNA synthetases are thus in a unique position to monitor cellular nutritional and energy status.
Decoding at the ribosome

After aminoacylation, elongator aminoacyl-tRNAs (aa-tRNA) are recognized by GTP-bound EF-Tu in bacteria or EF1A in archaea and eukaryotes and brought to the ribosome for amino acid incorporation during the elongation stage of translation (Fig. 5; [214–216, 9, 15, 217]). At the ribosome, speed and accuracy in protein synthesis are achieved by rapidly sampling many aa-tRNAs and assessing the stability of each codon–anticodon interaction.

Elongation begins with the mRNA-independent binding of the aa-tRNA to the ribosome with EF-Tu and GTP [218]. Ternary complex interactions are mediated through EF-Tu and ribosomal protein L7/L12 [219]. The accuracy of the codon–anticodon interaction is assessed during two steps in the decoding centre. During the initial selection step, three universally conserved bases within the 16S rRNA, or 18S rRNA in eukaryotes, monitor the codon–anticodon interaction through the minor groove of the codon–anticodon helix [220–223]. EF-Tu also interacts with the tRNA acceptor stem and amino acid during these steps to protect the labile aminoacyl ester bond and prevent hydrolysis [224]. The correct codon–anticodon interaction, particularly at the first two positions of the codon, provides the free energy for conformational changes in the small ribosomal subunit, known as domain closure [225,226]. Domain closure activates EF-Tu GTPase activity, leading to GTP hydrolysis [227]. Near-cognate codon–anticodon pairings, with G-U mismatches in the first or second codon position, adopt base pairs similar to Watson-Crick G-C pairs and induce domain closure similarly to cognate interactions [228,229].

After domain closure and GTP hydrolysis, GDP bound EF-Tu is released from the aa-tRNA, freeing the aminoacyl group for interactions with the peptidyl transferase centre of the ribosome [230]. During the second specificity step, known as proofreading, near-cognate tRNAs dissociate from the ribosome [231]. After EF-Tu release, the tRNA is less tightly bound to the ribosome and codon–anticodon interactions influence tRNA accommodation into the peptidyl transferase centre. Both the anticodon loop and the elbow region of the tRNA are monitored by either rRNA or a combination of rRNA and protein, respectively, to determine accommodation [232]. Once the 3′ end of the tRNA moves into the peptidyl transferase centre, peptide bond formation rapidly occurs [227].

Traditional Watson-Crick base pairing is not required at all three codon positions for an amino acid to be incorporated into a growing polypeptide chain [reviewed in 233]. Crick first identified that G-U wobble base pairing occurs in the third codon position and that only 32 tRNA species were required to decode 61 sense codons [234]. Early experimental evidence demonstrated codon misreading and subsequent missense errors due to G-U wobble interactions [235–237]. In bacteria and eukaryotes, G-U codon–anticodon mismatches at the middle position are the most frequent errors causing amino acid misincorporation [238–240]. In vitro studies of G-U mismatches demonstrate that they are less destabilizing to the codon–anticodon helix than other non-Watson-Crick mismatches [241,242]. Pyrimidine–pyrimidine mismatches, such as U-U codon–anticodon pairing at the

Figure 5. Aminoacyl-tRNA at the ribosome. Aminoacyl-tRNAs are recognized by GTP-bound EF-Tu in bacteria or EF1A in eukaryotes and recruited to the elongating ribosome in an mRNA-independent manner. During initial selection (i), codon–anticodon interactions are monitored by the ribosome and non-cognate tRNAs are rejected. A cognate codon–anticodon interaction triggers GTP hydrolysis by EF-Tu/EF1A and a conformational change that puts the aminoacyl branch of the tRNA into the peptidyl transferase centre. Proofreading (ii) occurs as a second check to ensure proper codon–anticodon pairing. Here, near-cognate matches that may have made it past initial selection are rejected. A cognate codon–anticodon interaction triggers peptide bond formation (iii) and the amino acid is added to the growing polypeptide chain. Translocation (iv) moves the tRNA containing the polypeptide chain into the P site and the cycle repeats.
first codon position and third position, or C-U codon–anticodon pairing at the third position, also cause errors in translation [239,240,243]. Recently, Pernod et al. [283] investigated codon–anticodon interactions that are efficiently accepted by the eukaryotic ribosome in a high-throughput screening experiment using an IRES from the Cricket Paralysis Virus. This virus contains a pseudoknot that structurally and functionally mimics the codon–anticodon helix in the ribosome. They identified 59 near-cognate codon–anticodon interactions with one or two mismatches that permitted translational elongation. In their setup using unmodified RNA, the ribosome accepted G/U, G/A, C/A, C/U, A/A, C/C and G/G mismatches, with the majority occurring at the third position of the codon.

Superwobble, where an unmodified U in the wobble anticodon position reads all four nucleotides in the third codon position, also occurs in bacterial and organellar genomes that do not encode the full set of tRNAs required to read all codons. Rogalski et al. [244], demonstrated that of the two tRNA species that decode all four GGN glycine codons in chloroplasts, only tRNA$^{\text{Gly}}_{\text{UCC}}$ was required. In other cases, modifications to nucleotides in the anticodon, as discussed above, can alter decoding. For example, in S. cerevisiae a currently unknown modification of tRNA$^{\text{Pro}}_{\text{UGG}}$ allows it to decode all four CCN proline codons [31].

**Impact of tRNA modifications**

Modifications play roles in all aspects of tRNA function, from structure and stability to aminoacylation and decoding at the ribosome. Modifications that affect tRNA stability or folding tend to occur in the body of the tRNA, outside of the anticodon stem-loop. As compared to unmodified tRNAs transcribed in vitro, native modified tRNAs show increased melting temperature, stabilized tertiary interactions and increased overall stability [245-252]. A role for modifications in stabilizing tertiary interactions is suggested by findings that the number of modifications positively correlates with growth temperature [111,253-255]. Methylation of nucleobases at the junctions between the acceptor stem and D-arm or between the D-arm and anticodon stem-loop interrupt RNA duplex formation and facilitate the three-dimensional L-shape tRNA fold, as the methyl group on the Watson-Crick face of the nucleobase prevents base pairing [reviewed in 256]. Other studies show that pseudouridine stabilizes base-stacking interactions [257-259], while dihydrouridine in the D-arm enhances tRNA flexibility for organisms that grow optimally at low temperature [260-262]. Modifications also stabilize the secondary structure. For example, Nobles et al. [263] found that adding any one of the highly conserved T-arm modifications (U54 modified to ribothymidine, C49 modified to 5-methylcytidine or U55 modified to pseudouridine) to an otherwise unmodified transcript significantly increased the interaction between the T-arm and D-arm.

Interestingly, deleting genes encoding the enzymes responsible for many of the modifications outside of the anticodon loop of the tRNA does not lead to abnormal growth phenotypes, likely because of redundancy among the modifications that stabilize tRNA structure [reviewed in 90]. Only the removal of multiple genes leads to growth defects. For example, deleting either yeast TRM8 or TRM82, which methylate G46 [264], results in no obvious growth defect. When removed in combination with genes that encode the enzymes that methylate cytosine or guanine [TRM4 or TRM10; 265, 266] or that modify uracil to pseudouridine or dihydrouridine [PUS7, DUS1, DUS2, DUS3 or DUS4; 267-269], synthetic slow or synthetic lethal interactions are observed [149]. Whipple et al. [156], discovered that the absence of 7-methylguanine and 5-methylcytidine or 2’-O-methyluridine and N4-acetylicytidine modifications in the tRNA body triggers tRNA degradation by the RTD pathway due to decreased stability in the acceptor and T stems.

The highest density of tRNA modifications occurs in the anticodon stem-loop (ASL). Positions 32, 34, 37 and 38 are all frequently modified and have a major impact on growth, due to their role in translation and decoding at the ribosome. Modifications at position 34, the wobble position, can expand or restrict decoding potential [reviewed in 270]. Structural studies have shown that U34 modification, with moieties such as 5-oxyacetic acid, 5-methylaminomethyl or 2-thio, stabilize and pre-structure the ASL by facilitating intramolecular hydrogen bonds that decrease the entropic cost of codon–anticodon binding and allow U34 pairing with codons ending in C or U [271,272]. Adenine at position 34 is nearly always modified to inosine, which expands decoding to A, U or C [reviewed in 273]. Other modifications at position 34 restrict decoding to prevent mistranslation. For example, some bacteria decode AUA codons with tRNA$^{\text{Leu}}_{\text{CAU}}$. Lysidine modification of cytidine at position 34 changes the base-pairing preference from G to A [274]. This prevents misrecognition of the near-cognate AUG codon which codes for methionine. Modifications of uridine to 2-thiouridine at position 34 similarly restrict codon reading by altering the pucker of the ribose sugar and glycosidic angle leading to preferential binding of A rather than G [275-277]. Interestingly, Gupta et al. [278] have linked the thiolation of U34, which is seen in tRNA$^{\text{Glu}}_{\text{Gln}}$, tRNA$^{\text{Gln}}_{\text{Gln}}$ and tRNA$^{\text{Lys}}_{\text{Lys}}$ in yeast, to broad aspects of cell metabolism that control growth and cell cycle progression. They demonstrated that U34 thiolation diverts carbon metabolism from the pentose-phosphate pathway and nucleotide synthesis towards storing carbohydrates trehalose and glycogen in response to low methionine levels. The effect occurs with only small changes in translation but is achieved through limiting intracellular phosphate metabolism. Modification of U34 also maintains proteome integrity by optimizing codon translation speed and regulating co-translation protein folding [279].

Modifications of the universal purine at position 37, 3’ of the anticodon, modulate the ASL structure by interfering with intra-ASL hydrogen bonding and also stabilize anticodon interactions through base stacking [280,281]. N6-isopentenyladenosine and N6-threonylcarbamoyladenosine often occur at position 37 for tRNAs that decode codons beginning in U or A, respectively [282]. These modifications stabilize A-U Watson-Crick pairs in the first codon position and prevent hydrogen bonding between U33 and A37, which would disrupt the anticodon stem [284, 285, reviewed in 286]. In tRNA$^{\text{Phe}}_{\text{GAA}}$ position 37 is similarly modified to
wybutosine [287]. Wyosine derivatives at position 37 enhance base stacking with adjacent bases to restrict flexibility of the anticodon loop, prevent frameshifting and enhance translational fidelity through stabilization of codon–anticodon interactions [288–290].

Other modified positions in the ASL synergistically act to alter decoding. For example, inosine at position 34 should allow decoding of A, U and C, but for bacterial tRNAArg modification of 2-thiocytidine at position 32 prevents I-A base pairing at the third codon position [291]. Pseudouridine at positions 32 and 39 stabilizes the open-loop structure required for decoding at the ribosomal A-site [259,292]. Modifications in the ASL also direct other modifications [reviewed in 293]. For example, in human and yeast tRNA^Phe, wybutosine formation at position 37 is stimulated by 2'-O-methylation of C32 and G34 [294–296].

Some modifications act as identity elements for recognition and aminoacylation by an aARS [176]. For example, inosine at position 34 of yeast tRNA^Leu enhances aminoacylation by IleRS compared to an unmodified adenosine [297]. In other cases, such as for yeast tRNA^Asp, methylation of G37 blocks aminoacylation by ArgRS to prevent mis-charging [298]. Other modifications influence tRNA interactions with the translational machinery; for example, ribosylation of A64 in initiator tRNA^Met prevents its interaction with EF1A, restricting function to only the initiation AUG codon [299].

tRNA modifications can be dynamic and regulate cellular processes in response to stimuli or changing cellular conditions. The role of U34 thiolation in the regulation of cell metabolism in response to starvation as discussed above is one example [278]. Another is the increase of 2'-O-methylcytidine, 5-methylcytidine and N2,N2-dimethylguanosine in response to hydrogen peroxide treatment [300]. Yeast lacking the enzymes required for these modifications are more sensitive to a variety of stresses, including hydrogen peroxide, sodium arsenite and the DNA damaging agent methyl methanesulfonate [300,301]. Begley et al. [301] found that Trm9 methyltransferase of U34 in tRNA^Arg^UCU and tRNA^Glu^UUC allows for efficient translation of genes involved in the DNA damage response. Similarly, upon exposure to oxidative stress, Trm4 methylates tRNA^Leu^CAA at the wobble position to enhance translation of Rpl22a, which is enriched in UUG codons [302]. In addition, treating yeast with oxidants and alkylating agents results in stress-specific changes in tRNA modifications that correlate with increased translation of specific transcripts required to respond to the stress [303].

These examples suggest that dynamic tRNA modification controls gene expression post-transcriptionally by altering tRNA decoding efficiency, resulting in enhanced translation of specific transcripts required to deal with changing environmental conditions [304,305]. Also supporting this model, Torrent et al. [306] demonstrated that in response to stress, yeast change their tRNA pool to favour the expression of stress response transcripts with select codon bias.

The importance of tRNA modification is underscored by the association of mutations in tRNA modifying enzymes with disease [reviewed in 307]. MELAS (Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) and MERRF (Myoclonic epilepsy with ragged-red fibres) were first associated with mutations in mitochondrial tRNAs that lead to hypomodification of U34 [308,309]. Mutations in genes encoding the enzymes responsible for these modifications, GTPBP3 and MTO1, result in similar outcomes [310,311]. FTSJ1 encodes a methyltransferase that modifies positions 32 and 34 on tRNA^Leu^, tRNA^Phe^ and tRNA^TTP [312,313]. Mutation of FTSJ1 is linked with intellectual disabilities [314,315]. Both mouse and fly models reveal that FTSJ1 mutations impact transcription, small RNA silencing and disease associations [312,316]. Other tRNA modification enzymes, NSUN2 [317], ADAT3 [318] and TRMT1 [319], are also implicated in intellectual disability. Related to this, mutations in the gene encoding the IKAP subunit of Elongator, a multi-subunit, multifunctional complex first described for its association with elongating RNA polymerase II [320], result in the neuropathy familial dystonia [321]. The mechanism involves Elongator’s role in the U34 modification of a number of tRNAs [reviewed in 322]. Interestingly, over-expression of NSUN2 [323], Elongator components [324, reviewed in 325] and other tRNA modifying enzymes are associated with cancer [reviewed in 326]. Some caution must be taken in interpreting how mutations in these modifying enzymes are acting, as many of these enzymes modify additional RNA substrates [reviewed in 327]. For example, NSUN2 modifies cytoplasmic tRNA, as well as mitochondrial tRNAs [328], mRNAs [329] and miRNAs [330].

tRNA-dependent mistranslation

Mistranslation, when an amino acid that differs from what is specified by the codon is inserted into a protein during synthesis, occurs naturally in all cells at frequencies ranging from 1 in 3000 to 1 in 10⁶ depending on the amino acid [331–334, reviewed in 233]. A number of factors contribute to mistranslation frequency, with many centring on tRNAs. Since the ribosome reads the codon and not the acylated amino acid, misacylation of tRNAs leads to mistranslation [11]. Mistranslation also results from codon–anticodon misreading by the ribosome through nonconventional base pairing at all three positions, as discussed in the previous section, and can be influenced by tRNA modification, which affects codon-anticodon pairing [271,335–339]. Furthermore, mistranslation increases upon protein overexpression, likely by altering the pools of aa-tRNAs when genes with rare codons are introduced [340,341].

Mistranslation should not be viewed simply as an error. Like protein modification, it provides diversity and is often an adaptive response. A well-studied example occurs in response to oxidative stress. Cysteine residues are often found within enzyme active sites or have regulatory roles [342]. Their oxidation often results in enzyme inhibition [for example, 343]. The Pan lab has shown that, in organisms including E. coli, yeast and mammals, mistranslation of methionine at non-methionine codons occurs at a frequency as high as 10% in response to oxidative stress [344–348]. These surface exposed methionines create a high concentration of oxidant scavengers that protect key cysteine residues in the protein [349].

Mistranslation is also a response to amino acid starvation. This was first documented in E. coli strains lacking a stringent response [350,351]. The stringent response requires the
alarmone guanosine tetraphosphate (ppGpp, Magic spot). ppGpp dramatically increases upon nutrient stress, with one consequence being the inhibition of translation [352, reviewed in 353]. In the absence of translational inhibition, specific amino acid substitutions appear when amino acids are depleted. Near-cognate substitutions such as leucine for phenylalanine [236,354] or lysine for asparagine [237] are observed. Starvation-induced increases in mistranslation are also observed in mammalian cells [189,351,355–357]. Interestingly, mistranslation makes cells more resistant to other stresses, perhaps by priming stress response pathways [358–360].

Some tRNA variants significantly increase the frequency of mistranslation. Many of these were first identified as suppressor mutations that compensate for nonsense or missense mutations in E. coli, yeast [reviewed in 361–363], mammalian cells [364] and archaea [365]. Intergenic suppressor mutations that had broad cellular consequences as a result of altering information flow from DNA to protein were termed informational suppressors [366]. A number of the original tRNA suppressor mutations were found based upon second-site mutations that restored tryptophan auxotrophy in alleles of trpA with nonsense [367] or missense mutations [368–370]. These studies identified, for example, a tRNA<sub>G2</sub> with a C26 to U base change that resulted in decoding of arginine codons with glycine. Similarly, missense and nonsense suppressor tRNAs have been identified in several eukaryotic organisms e.g. 188, 371–374).

Mistranslation results when the anticodon and aminoacylated tRNA are not a match defined by the genetic code. For example, mutations that result in a G3:U70 base pair in a tRNA cause mistranslation to alanine (Fig. 6A). These occur spontaneously in yeast within tRNA<sub>Pro</sub> and cause alanine insertion at proline codons [188]. The effect of the mistranslation caused by the tRNA variant is buffered by multiple copies of wild-type tRNA<sub>Pro</sub> with the frequency of mistranslation being approximately 3% [188]. Protein quality control mechanisms such as the heat shock response and proteasomal degradation of misfolded proteins allow cells to cope with the mistranslation with minimal phenotypic consequences [375,376]. As the anticodon plays no role in aminoacylation, tRNA<sub>Ser</sub> variants with anticodon changes similarly cause high levels of mistranslation. In fact, if they are not buffered by secondary mutations that reduce their functionality or by additional copies of the wild-type tRNA, they are lethal (Fig. 6B; [377]). We and others have demonstrated the insertion of serine at a number of different codons in yeast and mammalian cells [192,378,379]. The same type of anticodon variation in tRNA<sub>Ala</sub> or tRNA<sub>Leu</sub> will result in misincorporation of alanine or leucine, respectively. The impact of each of these tRNA variants on cells depends on both the extent of mistranslation and the type of substitution (Berg et al., in preparation). The threshold frequency at which cells no longer tolerate sustained mistranslation in yeast is approximately 8%, as compared to approximately 10% in E. coli [192,376]. The ability of tRNA variants to effectively fix mutations in protein-encoding genes [for example, 188, 380] raises their therapeutic potential in diseases resulting from missense or nonsense mutations [reviewed in 381].

The occurrence of other complex mechanisms that lead to mistranslation is perhaps best demonstrated in an example from E. coli. Hirsh [382] identified a tRNA<sup>TTP</sup> that inserts tryptophan at UGA stop codons. The tRNA contains a G to A change in the D-arm resulting in U-A rather than U-G base pair, demonstrating that tRNA properties independent of the anticodon can affect decoding. Kinetic and structural studies show that binding of the aa-tRNA with EF1A to the A site of the ribosome and interactions with the mRNA occurs in discrete steps that require flexibility in the tRNA structure [383–385]. The Hirsh mutation alters tRNA flexibility enabling codon–anticodon mismatching. Further studies have highlighted the dynamics of the tRNA throughout the translation process [386].

Interestingly, similar mutations within tRNA encoding genes that cause mistranslation in yeast are found in human tRNA genes [381,387]. Using a DNA capture array, we sequenced the tRNA genes from 84 individuals. On average each individual contained 65 variations from the reference genome with nine of these being found in less than 5% of the population. Included amongst these were 17 variants creating a G3:U70 base pair or anticodon changes that have a high potential to mistranslate. As mistranslation would increase proteotoxic stress, we have hypothesized that the tRNA variants may act as modifiers of disease, particularly diseases characterized by proteotoxic stress. Our findings in yeast reveal that mistranslating tRNA variants, even those with similar mistranslation frequency, impact cell growth, heat shock response and the cellular proteome differently and have different genetic interactions. From this we propose that individual tRNA variants may contribute to distinct diseases, depending on the type of mistranslation.

**Genetic code evolution**

Mutations in tRNA genes that cause mistranslation provide the material for genetic code evolution. The ambiguous intermediate theory for genetic code evolution predicts that tRNA mutations can lead to decoding of a single codon as two different amino acids [388]. In time, codons that cannot tolerate ambiguous decoding will be recoded to other synonymous codons. An example of this is seen in Candida species, where the CUG codon has been recoded from leucine to serine due to a serine tRNA variant that decodes leucine codons [389]. Early in this evolution, the CUG codon would have been decoded by both the novel tRNA<sub>Ser</sub> variant and the canonical tRNA<sub>Leu</sub>. This evolutionary intermediate has been retained in the related species, Ascoidea asiatica [390]. Over time, CUG codons that could not tolerate being decoded as serine were replaced by synonymous leucine codons and the tRNA<sub>Leu</sub> that decoded CUG codons was lost. In other yeast species, CUG is decoded as alanine [391,392].

More commonly, deviations from the ‘standard’ genetic code are seen in mitochondria [reviewed in 29]. In yeast mitochondria, CUN codons are decoded as threonine instead of leucine and the UGA stop codon is decoded as tryptophan [393,394]. In the case where the UGA stop codon is decoded as tryptophan, post-transcriptional editing or a mutation converts the tRNA<sup>TTP</sup> anticodon from CCA to UCA [395, 396].
Figure 6. tRNA variants can increase levels of mistranslation. (A) A _S. cerevisiae_ proline tRNA variant with a C70U mutation that creates a G3:U70 base pair in the acceptor stem is aminoacylated with alanine instead of proline, leading to alanine misincorporation [188]. The toxicity of mistranslation is buffered by other wild-type copies of tRNA[^3] that compete for decoding of CCA codons with the mistranslating tRNA variant. (B) A serine tRNA variant with a UGG anticodon is aminoacylated with serine, but decodes proline codons, leading to serine misincorporation. A secondary mutation of G26A dampens tRNA function allowing non-lethal levels of mistranslation [377].

Recent approaches combining large-scale sequencing, analysis of amino acid distributions at conserved positions and characterization of organellar tRNA complements have identified many additional mitochondrial genetic code deviations in diverse species arising from novel tRNA variants [397, 398].

Woese [399] has discussed how the genetic code has co-evolved with the translational machinery in primitive cells. Central to this is that translational accuracy could not have existed early in evolution and that ambiguous codon assignments were common. Mistranslation was thus an inherent property of the primordial cell. Woese [400] classifies any protein that arose from ambiguous translation as a statistical protein, that is a heterogeneous mixture of molecules, made from the same genetic template but differing at a subset of amino acid positions. As such, statistical proteins provided cells with a wider range of functions than would be achievable from perfect translation. As stated by Woese [401]: 'Statistical proteins form the basis of a powerful strategy for searching protein phase space, finding novel proteins.' Some degree of mistranslation has been maintained to provide rapid functional diversity in response to a changing environment, not unlike protein modification. Wang and Pan [402] demonstrated that mistranslation of methionine in response to high calcium levels creates statistical Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). In HEK293T cells, CaMKII produced under high calcium conditions has greater kinase activity than CaMKII produced under non-stress conditions. CaMKII constructs containing mutations of specific amino acids to methionine had different activation profiles and subcellular localizations. One can imagine other circumstances in development and differentiation where mistranslation could provide the required diversity. Furthermore, statistical proteins created with tRNA variants provide an approach to produce proteins in synthetic biology and biotechnology applications with a wider range of binding interactions or activities than the homogenous form. For example, mistranslation makes it possible to create an antibody pool with broadened specificity.
Genetic code expansion

From primordial times to the present, the potential of the proteome has increased by incorporating additional amino acids into proteins [29,403–406]. The addition of selenocysteine and pyrrolysine as the 21st and 22nd amino acids are two natural examples of the increased repertoire of chemistries that an expanded genetic code provides [407]. The genetic code does restrict the degree of expansion and as a result, post-translational modification has evolved as a mechanism to enhance protein function. However, the ability to expand protein chemistry with targeted genetic code expansion has advantages and has become a significant tool in modern synthetic biology [reviewed in 408–410]. Genetic code expansion is able to produce proteins with novel chemistries and thus also novel structures and functions. Moreover, non-canonical amino acids allow state-of-the-art biophysical, physiological and cellular biological experiments either directly or combined with chemoselective reactions to specifically label a protein [reviewed in 408]. Synthetically expanding the code requires an open or largely open codon (to date generally a stop codon), a non-canonical amino acid that can enter the cell, a tRNA that decodes the open codon and an aaRS that specifically ligates the non-canonical amino acid to the tRNA [411,412]. Each orthogonal translation system must be optimized to be compatible with the translational machinery, to enhance specificity and minimize competition with native factors [409]. Combined with the ability to make totally synthetic genomes where codons can be vacated and then reassigned [e.g. 413], and the development of an expanded genome alphabet [414,415], these strategies will allow the design of proteins and even organisms with unique potential.

Other functions of tRNAs

There is vast complexity within an organism’s tRNA molecules related to the presence of isoacceptors and isodecoders as well as the numerous possible base modifications. tRNAs are also ancient molecules originating with the enzymatic synthesis of proteins, even likely predating the ribosome [see, for example, 416]. It is not surprising then that tRNAs have evolved functions outside their direct role in translation.

The first discovered function for tRNAs outside of translation was the enzymatically catalysed transfer of arginine from tRNA$^{ARG}$ to N-terminal Asp or Glu residues by Arg-tRNA-protein transferase [R-transferase; 417, 418]. Arginine is a primary destabilizing residue for the N-end rule signalling ubiquitylation then proteasomal turnover of the protein. Conjugation of arginine to these proteins or peptides increases their turnover in a regulated fashion [reviewed in 419]. Bacteria similarly transfer leucine or phenylalanine to N-terminal arginine or lysine residues, targeting their turnover by the ClpP protease [420]. In a somewhat related process, bacteria use a remarkable dual-purpose tRNA-mRNA, SsrA, to extend stalled peptides with a tag that then targets them for degradation [reviewed in 421]. Bacteria also use aa-tRNAs to transfer amino acids to peptides and lipids, in the synthesis of antibiotics and for the interbridge peptide synthesis of peptidoglycan [reviewed in 422, 423].

In eukaryotes, tRNAs regulate the cellular proteome in response to amino acid starvation. This has been studied in detail in yeast [reviewed in 424], whereupon starvation for one or more amino acids, uncharged tRNAs bind to the histidyl-tRNA synthetase (HisRS)-like domain in Gcn2 activating its kinase activity. Activated Gcn2 phosphorylates the eukaryotic translation initiation factor 2 (eIF2) derepressing the translation of GCN4 mRNA, which encodes the transcriptional activator responsible for inducing amino acid biosynthetic genes [425]. Phosphorylated eIF2 also decreases global translation allowing cells to reduce amino acid consumption while increasing amino acid biosynthesis [426]. The latter is a common mechanism shared with mammalian cells in response to stress.

The third function for intact tRNAs in mammalian cells is their priming of reverse transcriptase catalysed cDNA synthesis of retroviral RNA genomes for subsequent integration into the host [reviewed in 427]. The tRNA involved depends on the virus. HIV-1, HTLV-1 and Rous sarcoma virus use specific tRNA$^{3\prime\,Lys}$, tRNA$^{Pro}$ and tRNA$^{TTP}$ isoacceptors, respectively [428–430]. Similarly, reverse transcription of some retrotransposons is primed by specific tRNAs or tRNA fragments [see, for example, 431, reviewed in 432]. Interestingly, tRNA fragments also interfere with reverse transcription and retrotransposon mobility [433].

Recent studies have revealed the abundance of tRNA fragments and their roles in diverse cellular functions [434, reviewed in 435, 436]. Interestingly, Torres et al. [437] suggest that the differential expression of human tRNA isodecoders may relate to their roles as tRNA fragments rather than translation per se. Many tRNA fragments originate from cleavage of mature tRNAs rather than tRNA biogenesis intermediates [reviewed in 438]. Four types of tRNA-derived small RNAs can be produced from mature tRNAs (Fig. 7). tRNA halves are 31–40 nucleotides long and originate from cleavage within the anticodon loop by Rny1 in yeast [439] or angiogenin in mammals [440,441], generating 5’ and 3’ halves. Smaller tRNA fragments (tRFs) are generated by Dicer dependent and independent mechanisms [442–444] in either the D-loop, giving rise to a 5’ tRF, or in the T-loop, giving rise to a 3’ CCA tRF. Specific tRNA fragments are observed in response to numerous types of stress including starvation [445], oxidative stress [441,446,447] and hypoxia [440]. As a result, many of the functions ascribed to tRNA fragments relate to stress response [reviewed in 448]. Both intact tRNAs and tRNA halves bind cytochrome C, blocking caspase activation and reducing stress-responsive apoptosis [449, 450, reviewed in 451]. tRNA fragments also regulate translation. Stress-responsive translational inhibition occurs as a result of tRNA fragment interactions with the translational machinery [441,446,452–454], as well as through binding of tRNA fragments to complementary sites in the mRNA [444,455]. Other processes, including modulating the cellular response to DNA damage, are regulated by specific tRNA fragments [456].

The ability of tRNA fragments to regulate gene expression extends to epigenetic inheritance. tRNA fragments are abundant in mammalian sperm [457]. Sharma et al. [458] show...
that the composition of tRNA fragments in mammalian sperm is diet dependent. These tRNA fragments regulate the expression of approximately 70 genes associated with the long terminal repeat of the retroelement MERVL in the resulting embryo. Chen et al. [459] observed inheritance of metabolic disorders upon injection of sperm derived tRNA fragments from mice fed a high-fat diet into normal zygotes. These effects were unrelated to changes in DNA methylation at CpG-enriched sequences.

Sharma et al. [458,460] demonstrate tRNA fragments in sperm arise from the fusion of small extracellular vesicles from the epididymal epithelium during spermatogenesis. tRNAs thus have a role in extracellular signalling and regulation [reviewed in 461, 462]. A related example comes from Chiu et al. [463], who demonstrate that activated T cells secrete tRNA fragments that repress T cell activation. Extracellular tRNAs and tRNA fragments are also generating great interest for their diagnostic potential as they are found in biofluids [e.g. 464–466, reviewed in 467]. The exact RNA composition in extracellular vesicles is diverse and includes many classes of small noncoding RNAs [468,469]. Interestingly, Shurtleff et al. [470] find that mature tRNAs are abundant in the vesicles released from HEK293T cells. The importance of extracellular vesicles in cellular processes such as myofiber repair [reviewed in 471] and in disease [472] suggests that extracellular tRNAs have a broad role in mammalian physiology.

Given the importance of RNA fragments in a number of aspects of gene regulation, it is not surprising that tRNA fragments have significant roles in development and disease [473–475]. In a fascinating report suggesting the therapeutic potential of tRNA fragments, Goodarzi et al. [476] find that tRNA fragments displace known oncogenic transcripts from the RNA binding protein YBX1, decreasing their stability and expression, and as a result suppressing metastatic progression.

**Concluding remarks**

tRNAs were the solution to the problem of how to convert the four bases found in the genetic code to protein sequence composed of 20 building blocks. With differences in sequence and modifications, the potential diversity in the cellular tRNA complement is tremendous. The diversity of form affects function by altering aminoacylation, stability and base pairing. The diversity also provides distinct molecules for intracellular and intercellular signalling and regulation. The number of genetic copies for each isoacceptor family has many implications. Few individual tRNA genes are essential [for an exception see 477] and the abundance of tRNA genes provides the raw material for genetic code evolution. Many tRNA variants occur within genomes; these include variants that are neutral or result in loss or gain of function. tRNAs play a central role in biology, it is not surprising tRNA variants contribute to disease [381]. But, by restoring protein sequence, mistranslating tRNA variants have the potential to treat diseases resulting from missense mutations. The use of tRNA variants in engineering of novel proteins and biopolymers is further evidence of the central role of tRNA biology.

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