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Tuning tRNAs for improved translation

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Transfer RNAs have been extensively explored as the molecules that translate the genetic code into proteins. At this interface of genetics and biochemistry, tRNAs direct the efficiency of every major step of translation by interacting with a multitude of binding partners. However, due to the variability of tRNA sequences and the abundance of diverse post-transcriptional modifications, a guidebook linking tRNA sequences to specific translational outcomes has yet to be elucidated. Here, we review substantial efforts that have collectively uncovered tRNA engineering principles that can be used as a guide for the tuning of translation fidelity. These principles have allowed for the development of basic research, expansion of the genetic code with non-canonical amino acids, and tRNA therapeutics.

KEYWORDS

tRNA engineering, synthetic biology, translation, genetic code expansion, noncanonical amino acid, directed evolution, rational design, tRNA therapeutics

1 Introduction

tRNAs play an essential role in translation, mediating the conversion of nucleic acid templates into protein products. They facilitate this by binding a programmed amino acid substrate before transferring it to the ribosome for incorporation into a peptide. During this process, tRNAs make specific interactions with several binding partners: elongation factors, mRNA, and multiple sites within the ribosome. As such, tRNAs directly influence translation and are a prime scaffold for its engineering. This engineering primarily addresses three fundamental applications: i) basic research, ii) genetic code expansion (GCE) in synthetic biology, and iii) tRNA therapeutics.

Basic research on understanding protein synthesis has identified a plethora of information regarding tRNA sequence and structure. The specific elements in the tRNA sequence that promote or deter interaction with a binding partner are termed identity or anti-determinant elements, respectively. The interactions between tRNAs and their cognate aminoacyl-tRNA synthetase (aaRS) in particular have been extensively studied (Giegé and Eriani, 2023) and are an important first step in translation. Still, aminoacyl-tRNAs (aa-tRNAs) must be efficiently incorporated into the ribosome to complete translation. Natural translation machinery is flexible enough to efficiently incorporate the natural amino acids which contain a broad range of chemical properties. However, ribosomal incorporation of non-canonical amino acids (ncAAs), important for widespread applications in synthetic biology, drug discovery, and basic research, are inherently poor ribosomal substrates that can dramatically lower ribosomal translation efficiency. Chemical synthesis via non-ribosomal peptide synthetases (Walsh et al., 2013; Süssmuth and Mainz, 2017) and post-translational modification enzymes (McIntosh et al., 2009) are some alternate strategies to bypass ribosomal synthesis of these amino acid derivatives. However, these strategies are not compatible with screening vast libraries for structurally unique or medically relevant peptides, which is a possibility in a ribosome-based (Yamagishi et al., 2011; Goto and Suga, 2021; Katoh and Suga, 2022a). Through tRNA engineering, hundreds of traditionally ribosome-incompatible substrates have been permitted for ribosomal installation

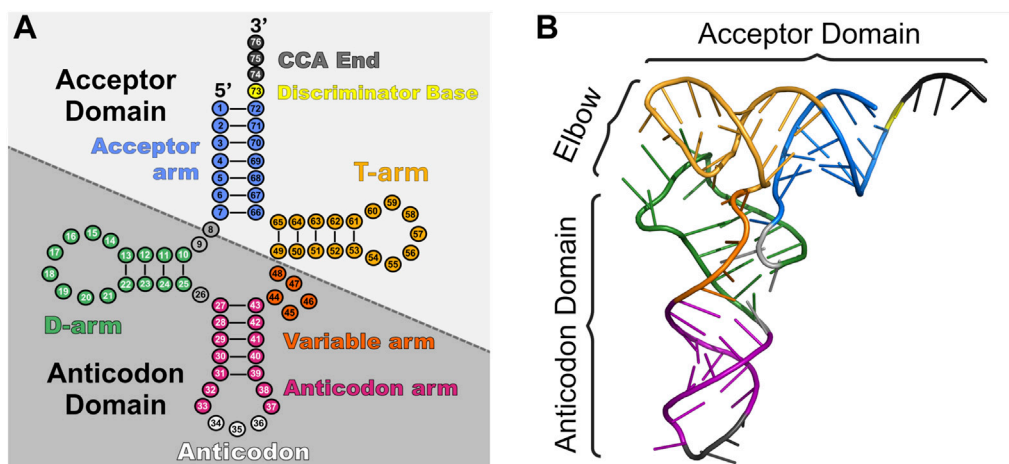


FIGURE 1
Schematic of tRNA structure. tRNAs consist of two major domains (acceptor domain and anticodon domain) and five stem-loop arms (acceptor arm (blue), D-arm (green), anticodon arm (pink and white), variable arm (orange), and T-arm (gold)) which play different roles in translation. **(A)** Cloverleaf 2D structure of tRNA^{Asp} (PDB: 6UGG) highlighting the major domains and regions. **(B)** 3D stick representation of tRNA^{Asp} with domains highlighted.

with varying levels of success (Kato and Suga, 2022a; Sigal et al., 2024). Additionally, nearly 10% and 50% of pathogenic genetic conditions result from aberrant stop codons (Mort et al., 2008) and missense mutations (Katsonis et al., 2014; Stenson et al., 2017), respectively. This has led to substantial research aimed towards developing modified tRNAs for efficient, orthogonal, and safe readthrough of improper gene sequences (Dolgin, 2022; Anastassiadis and Köhrer, 2023; Coller and Ignatova, 2024).

The process of tRNA engineering itself can be broadly divided into engineering for i) improving the orthogonality of an aaRS:tRNA pair, and ii) moderating the aa-tRNA's journey to and through the ribosome. Briefly, the former objective optimizes aaRS:tRNA interactions to ensure orthogonality and production of the desired aa-tRNAs (Soye et al., 2015; Tamaki et al., 2018; Melnikov and Söll, 2019; Krahn et al., 2020b; Ganesh and Maerkel, 2022; Kim et al., 2024). The latter objective optimizes aa-tRNA interactions with elongation factors, mRNA, and the ribosome to mediate initiation (Tharp et al., 2021b; Kato and Suga, 2023a; Kato and Suga, 2023c) or elongation fidelity (Uhlenbeck and Schrader, 2018; Sigal et al., 2024). Our review addresses tRNA engineering principles for the latter processes as a roadmap for modulating tRNA incorporation into the ribosome. We will discuss four major roadblocks involved in this endeavor: i) initial acceptance of an aa-tRNA into the ribosome (P-site for initiation, A-site for elongation), ii) proper codon-anticodon interaction, iii) peptide bond formation and peptidyl transfer efficiency, and iv) translocation of the tRNA. Through this discussion we will highlight the tRNA elements found to overcome these challenges.

1.1 tRNA structure

A tRNA's overall three-dimensional "L-shape" and specific nucleotide sequence dictates its interactions with binding partners and overall function. Canonical tRNAs vary between 76 and 100 nucleotides (Krahn et al., 2020a) and have a highly conserved "cloverleaf" secondary structure (Holley et al., 1965; Bolton and Kearns,

1975; Berg and Brandl, 2021) divided into five stem-loop arms based on internal base pairing: the acceptor stem, D-arm, anticodon arm, variable arm, and T-arm (Figure 1) (Giegé et al., 2012). The acceptor stem contains the 3' conserved CCA sequence for attachment of the amino acid and discriminator base that participates in conferring aaRS specificity. The D-arm is named after a conserved, modified dihydrouridine base that aids in stabilizing the tertiary tRNA structure (Maglott et al., 1999). The anticodon arm contains a three-base anticodon sequence for decoding the mRNA surrounded by other nucleotides that impact its efficiency and binding to aaRSs. The T-arm is named after its universally conserved thymine-pseudouridine-cytidine sequence (T_ΨC) that aids in ribosome and elongation factor interactions (Holley et al., 1965; Nissen et al., 1996). Finally, the variable arm varies in length and function, participating in binding of certain translation factors and/or impacting overall tRNA flexibility during mRNA translation (Sun and Caetano-Anollés, 2009; Krahn et al., 2020a; Prabhakar et al., 2022).

Structure conservation in tRNAs is also found in 3D (Figure 1), as they twist and fold into an L-shape (Kim et al., 1974; Giegé et al., 2012). This occurs by coaxial stacking of the acceptor stem and T-stem with the D-stem and anticodon stem. Furthermore, the variable loop and D-stem undergo intramolecular tertiary interactions with the D-loop and T-loop (Biela et al., 2023). This newfound L-shape consists of the acceptor domain (acceptor arm and T-arm), elbow region (D-loop and T-loop) (Zhang and Ferré-D'Amaré, 2016), and anticodon domain (D-stem, variable loop, and anticodon arm). The acceptor domain is primarily responsible for binding to its specific aaRS followed by the elongation factor (EF-Tu in prokaryotes, eEF1A in eukaryotes) for transport to the ribosome. The elbow region notably interacts with all three of the ribosome's tRNA binding domains and an extensive list of RNA and proteins that help mature and modify tRNAs (Zhang and Ferré-D'Amaré, 2016). Decoding of the mRNA occurs through the anticodon domain (Yarus et al., 1986).

In addition to conserved structural features, tRNAs are also heavily modified to further define their function, including all three stages of translation (Agris, 2008; Grosjean et al., 2010; Phizicky and Alfonzo, 2010; Phizicky and Hopper, 2010; Wang and Lin, 2023). In

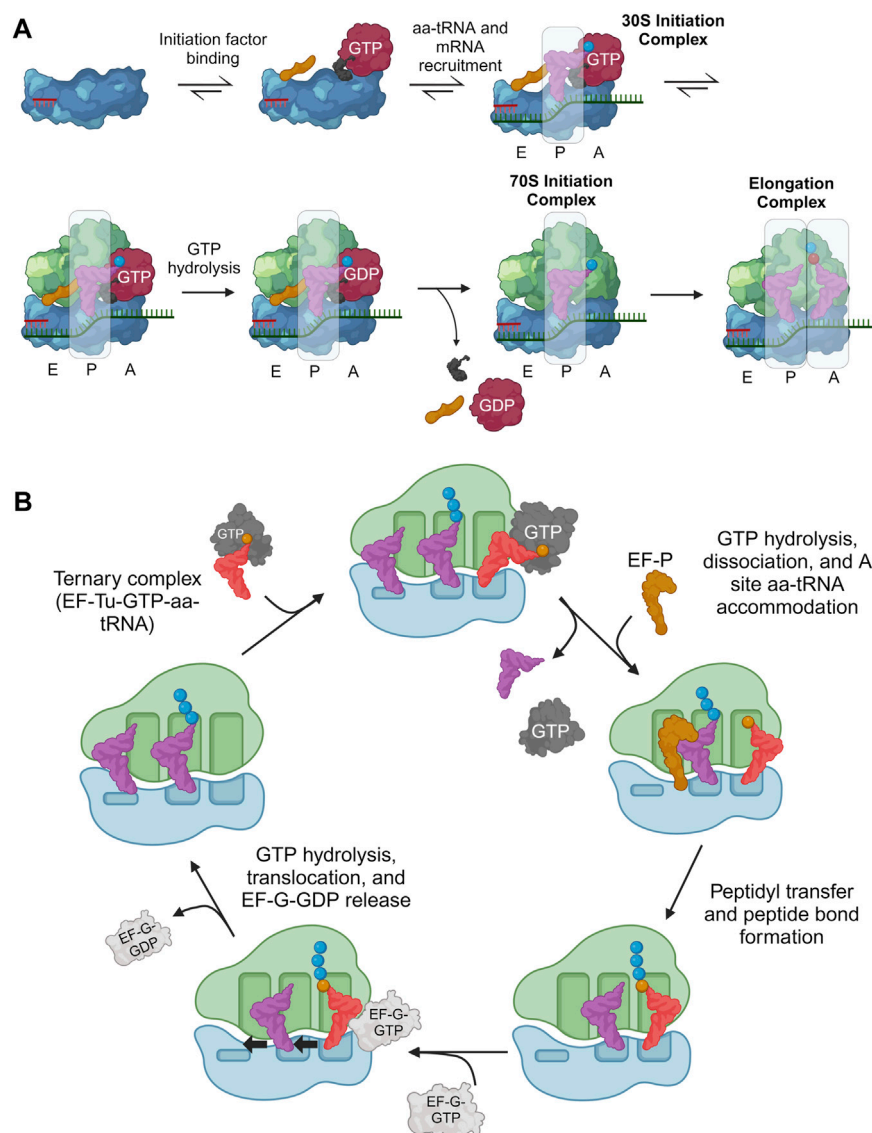


FIGURE 2

Overview of translation initiation and elongation highlighting important tRNA interactions. During translation, tRNA frequently interacts with both the ribosome and a host of translation factors, playing a central role in all major translational processes. **(A)** Initiation factors (gold, black, and red) help guide the initiator aa-tRNA into the ribosomal P-site to form the 30S initiation complex. Then, the initiation factors release and allow another aa-tRNA to accommodate into the A-site which catalyzes the first peptide bond to form, marking the beginning of elongation. **(B)** In translation elongation, the aa-tRNA must bind EF-Tu/eEF1A to be transported to the ribosomal A/T site. Codon-anticodon interactions between the aa-tRNA, mRNA, and ribosome ensure the correct aa-tRNA is selected before peptidyl transfer between the P-site and A-site tRNAs occur. Additionally, the P-site tRNA binds EF-P/eIF5A when tRNAs are charged with difficult ribosomal substrates to improve peptidyl transfer efficiency. Finally, EF-G/eEF2 interacts with the ribosome and A-site aa-tRNA to catalyze translocation. Created with BioRender.com.

fact, tRNAs are the most extensively modified cellular RNA with about 12% of nucleotides modified (an average of eight modifications per bacterial tRNA and 13 per eukaryotic (Zhang et al., 2022) and around 100 distinct modifications identified in total (Machnicka et al., 2014; Cappannini et al., 2024). Some bases like the 3' CCA end and discriminator base are never modified, while others are modification hotspots, such as nucleotides in the elbow region (Yared et al., 2024) and anticodon loop (Lorenz et al., 2017).

Our understanding of natural tRNA structure and its impact on translation is imperative to the design of an effective synthetic tRNA. This information lays the foundation for engineering strategies that have been implemented for this purpose.

1.2 Translation process

Our focus in translation is tRNA-based, guided in part by structural biology (Chua et al., 2022) which has elucidated many prokaryotic (Rodnina, 2018; Korostelev, 2022; Xu et al., 2022) and eukaryotic translational processes (Neelagandan et al., 2020; Blanchet and Ranjan, 2022; Zhang et al., 2023; Brito Querido et al., 2024). Studies have preferentially investigated the former, resulting in several tRNA engineering strategies developed for prokaryotes. We will focus on the processes of initiation (Figure 2A) and elongation (Figure 2B) as they are most relevant to tRNA engineering efforts.

Initiation of protein synthesis involves anchoring the ribosome onto an mRNA start codon (often AUG) and inserting the first aa-tRNA into the P-site. In bacteria, initiation is composed of two major processes (Milón and Rodnina, 2012; Roy et al., 2017): i) assembly of the 30S initiation complex (30SIC) which involves the binding of initiation factors (IF1, IF2, and IF3), initiator aa-tRNA, and the 30S subunit onto the start codon through interactions with the 30S Shine-Dalgarno (SD) RNA sequence, and ii) release of the initiation factors through the 50S subunit binding to the 30SIC. i) Initiation factors regulate translation initiation, requiring the initiator aa-tRNA, *N*-formylmethionine (fMet)-tRNA^{fMet}, to be accommodated into the ribosomal P-site and decode the corresponding AUG start codon (Rodnina, 2018). The GTPase IF2 plays a large role in this selection, through direct contacts with fMet-tRNA^{fMet} localized to the 3'-ACCAAC and the amino acid (Wu and RajBhandary, 1997; Guenneugues et al., 2000; Milon et al., 2010; Roy et al., 2017). ii) After accommodation, the 50S ribosomal subunit binds the 30SIC, catalyzing a series of conformational changes and ultimate release of initiation factors, to form the mature 70S initiator complex (70SIC) (Rodnina, 2018). As with many natural processes, there are exceptions to the general scheme of translation initiation including prokaryotic mRNA that lack a SD sequence, alternate starts codons (Tharp et al., 2020b) or the lack of a 5'UTR (Rodnina, 2018). However, the standard SD-led initiation at an AUG start codon, as described above, is almost exclusively explored with respect to initiator tRNA engineering.

Eukaryotic initiation resembles the path in prokaryotes with eIF1A, eIF2, and eIF1 homologous to bacterial IF1, IF2, and IF3, respectively (Brito Querido et al., 2024). In this process, the 43S pre-initiation complex (PIC) is formed through binding of initiation factors eIF1, eIF3, eIF1A, and eIF5, and a ternary complex [GTP-bound eIF2 complexed to the initiator aa-tRNA (Met-tRNA_i^{Met})] to the 40S subunit (Lapointe et al., 2022). In eukaryotes, there is no SD sequence, therefore the 43S PIC is recruited to the 5' end of an mRNA by the mRNA-bound eIF4F complex with the help of eIF4A and eIF4B to form the 48S PIC. The 48S PIC then scans the 5'UTR for a start codon (Wang J. et al., 2022). Upon recognition of this codon, most initiation factors are released, and the 60S subunit binds (catalyzed by eIF5B) to produce the 80SIC with a decoded AUG start codon in the ribosomal P-site (Brito Querido et al., 2024).

After translation initiation, the ribosome complex is ready to grow the nascent polypeptide chain. This is done through a well-conserved cyclic process to incorporate elongator tRNAs and form peptide bonds between amino acids (Dever et al., 2018). In prokaryotic systems, GTP-bound EF-Tu supports aa-tRNAs to the 70S ribosomal A/T-site where it decodes the exposed mRNA codon to promote tRNA accommodation into the ribosomal A-site and EF-Tu release. A peptide bond is then formed between the accommodated A-site and P-site aa-tRNAs (including de-acylation of the P-site tRNA) within the ribosomal peptidyl transferase center (PTC). Elongation factor P (EF-P) binds near the E-site to accommodate this reaction. Afterwards, the ribosome translocates along the mRNA by one codon (moving the tRNA species to the E- and P-sites, respectively), thereby opening the A-site to accept another aa-tRNA and repeat the elongation cycle (Xu et al., 2022). Elongation factor G (EF-G) binds near the A-site to disrupt codon-anticodon interactions and catalyze translocation (Liu et al., 2014; Rodnina et al., 2020). Finally, elongation factor

thermostable (EF-Ts) catalyzes GDP-to-GTP nucleotide exchange to recycle the EF-Tu used (Gromadski et al., 2002; Kavaliauskas et al., 2012).

Eukaryotic elongation is similar to that of prokaryotes. GTP-bound eEF1A binds an aa-tRNA and is transported to the eukaryotic 80S ribosome (Dever et al., 2018). Base pairing interactions between the aa-tRNA anticodon and A-site codon catalyzes GTP hydrolysis of eEF1A (Shao et al., 2016), leading to its dissociation from the ribosome and aa-tRNA accommodation (Jobe et al., 2019). Peptide bond formation and translocation is similar to that of prokaryotic systems (Ben-Shem et al., 2011). eIF5a (bacterial EF-P ortholog) also binds the E-site and interacts with the acceptor arm of the peptidyl-tRNA to promote favorable substrate positioning and efficient peptide bond formation (Gutierrez et al., 2013; Melnikov et al., 2016b; Shin et al., 2017). Translocation is enhanced by eEF2, and eEF1B (orthologs of EF-G and EF-Ts, respectively) to catalyze a similar nucleotide exchange as EF-Ts to renew eEF1A for another cycle of elongation (Dever et al., 2018).

2 Acceptor domain engineering for improved EF-Tu interactions

2.1 Basis of tRNA engineering for improved interactions with EF-Tu

Natural aa-tRNAs bind EF-Tu within a remarkably narrow affinity range which is necessary for unbiased transport of all amino acids to the ribosome and smooth mRNA translation (Louie et al., 1984; Ledoux and Uhlenbeck, 2008). This affinity is fine-tuned so that aa-tRNAs bind sufficiently to catalyze translation, but also dissociate at a rate fast enough to permit downstream peptide bond formation (Schrader et al., 2011; Jeong et al., 2014). Extensive research has determined the interface between EF-Tu and aa-tRNA (Figure 3A); importantly, the acceptor domain and amino acid moiety of aa-tRNAs are both responsible for binding EF-Tu (Kjeldgaard et al., 1993; Nissen et al., 1995; Nissen et al., 1996; Nissen et al., 1999; Sanderson and Uhlenbeck, 2007a; Schmeing et al., 2009; Kavaliauskas et al., 2012; Yikilmaz et al., 2014; Fischer et al., 2015; Johansen et al., 2018). Furthermore, both the tRNA sequence and amino acid structure work inversely together to achieve an affinity within this narrow range for optimal translation (Louie and Jurnak, 1985; LaRiviere et al., 2001; Asahara and Uhlenbeck, 2002; Asahara and Uhlenbeck, 2005; Dale et al., 2004; Dale and Uhlenbeck, 2005). Therefore, amino acids with weaker binding affinities are generally attached to tRNAs that have an acceptor domain with high affinity for EF-Tu and vice versa (Figure 3B). A series of mutagenesis-based experiments and computational modeling have further determined that base pairs 49:65, 50:64, and 51:63 within the T-stem determine the tRNA body's affinity to EF-Tu (Figure 3C), irrespective of the other acceptor domain nucleotides within the interface of EF-Tu (Sanderson and Uhlenbeck, 2007b; Eargle et al., 2008; Schrader et al., 2009; Schrader and Uhlenbeck, 2011). According to this research, these three T-stem base pairs contribute to EF-Tu affinity independently of each other, suggesting that one can accurately predict a bacterial tRNA's affinity for EF-Tu by summing the known contributions of the individual base pairs. Thus, engineering tRNA T-stems for increased EF-Tu affinity is a common, modular strategy

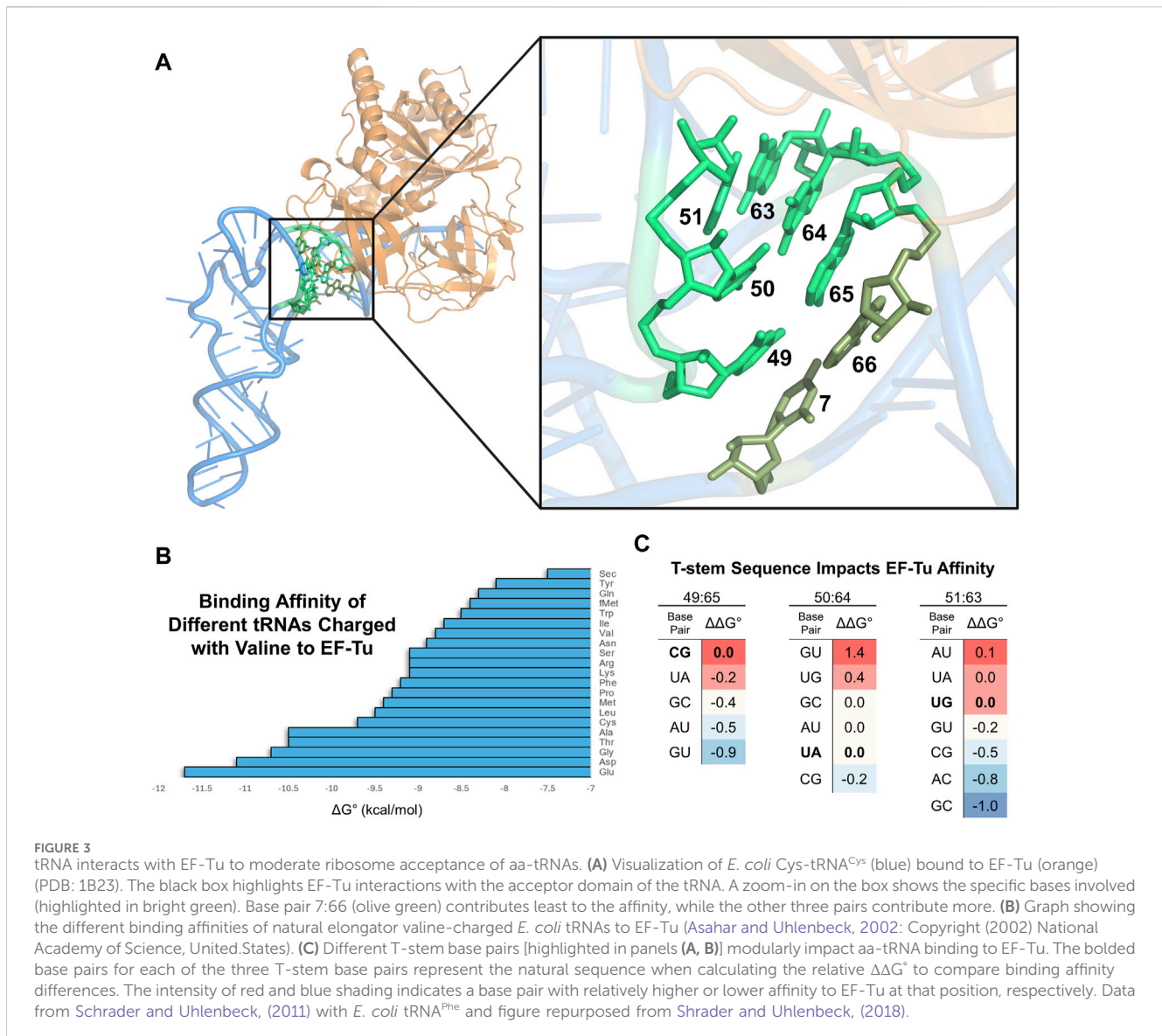


FIGURE 3

tRNA interacts with EF-Tu to moderate ribosome acceptance of aa-tRNAs. (A) Visualization of *E. coli* Cys-tRNA^{Cys} (blue) bound to EF-Tu (orange) (PDB: 1B23). The black box highlights EF-Tu interactions with the acceptor domain of the tRNA. A zoom-in on the box shows the specific bases involved (highlighted in bright green). Base pair 7:66 (olive green) contributes least to the affinity, while the other three pairs contribute more. (B) Graph showing the different binding affinities of natural elongator valine-charged *E. coli* tRNAs to EF-Tu (Asahar and Uhlenbeck, 2002; Copyright (2002) National Academy of Science, United States). (C) Different T-stem base pairs [highlighted in panels (A, B)] modularly impact aa-tRNA binding to EF-Tu. The bolded base pairs for each of the three T-stem base pairs represent the natural sequence when calculating the relative $\Delta\Delta G^\circ$ to compare binding affinity differences. The intensity of red and blue shading indicates a base pair with relatively higher or lower affinity to EF-Tu at that position, respectively. Data from Schrader and Uhlenbeck, (2011) with *E. coli* tRNA^{Phe} and figure repurposed from Schrader and Uhlenbeck, (2018).

to compensate for the incorporation of ncAAs that can be difficult ribosomal substrates (Figure 4).

2.2 Examples of improving EF-Tu binding

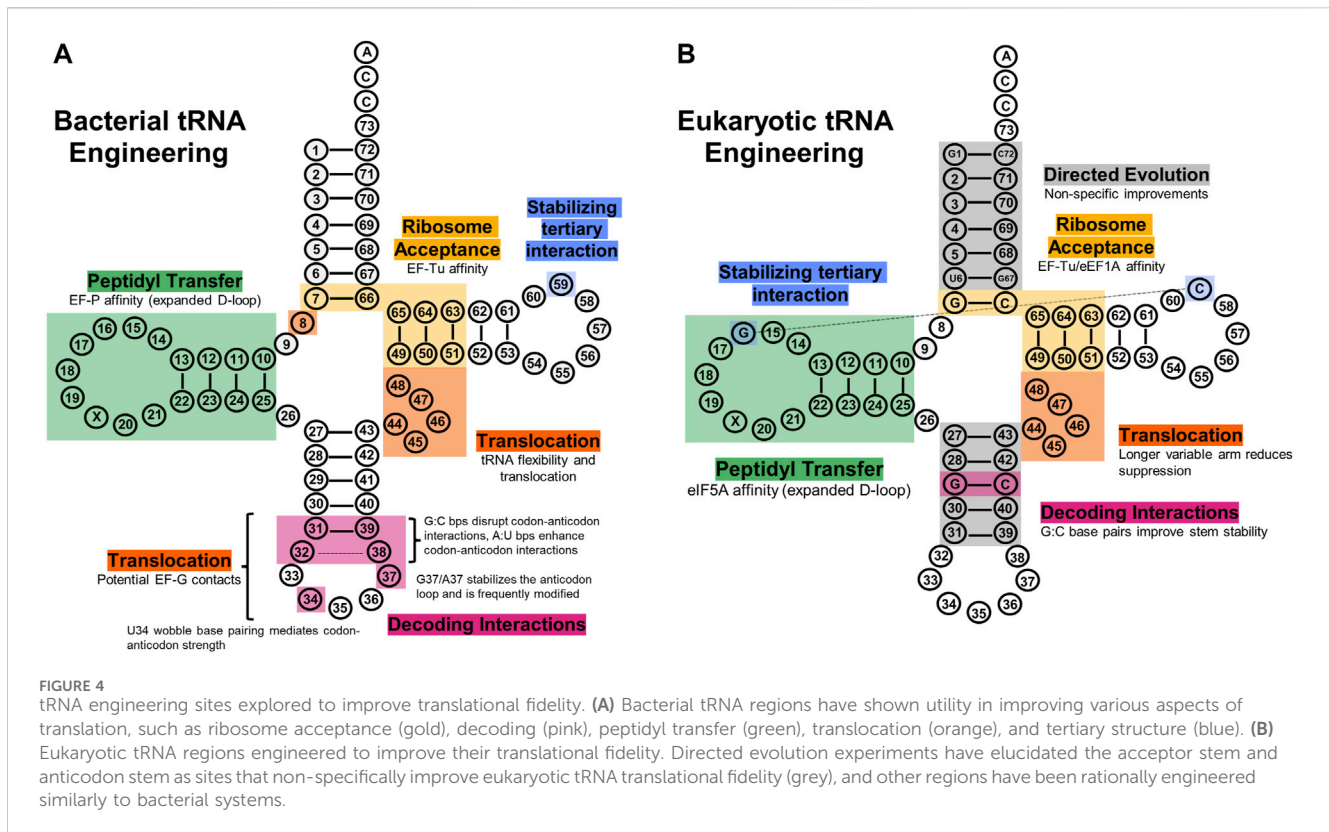
2.2.1 Canonical elongator tRNAs

As mentioned, canonical tRNAs have evolved to exhibit a wide range of affinities toward EF-Tu. Therefore, when engineering a tRNA for improved translational fidelity with a ncAA, it can be sufficient to use a natural tRNA with a high affinity for EF-Tu (e.g., tRNA^{Glu}). This strategy has notably allowed for the implementation of D-amino acids (AAs), which are pharmacologically relevant, (Feng and Xu, 2016; Melchionna et al., 2016; Wang L. et al., 2022), but notoriously difficult ribosomal substrates (Blackmond, 2010; Fujino et al., 2013; Englander et al., 2015; Fleisher et al., 2018; Liljeruhm et al., 2019; Melnikov et al., 2019). In one case, charging tRNA^{Gly} (fourth strongest affinity to EF-Tu) with various D-AAAs led to increased—but variable—single, double, and even triple

consecutive insertions (Achenbach et al., 2015). A similar approach utilized a tRNA^{Glu} (strongest natural EF-Tu affinity) variant (Terasaka et al., 2014) to efficiently incorporate up to 10 consecutive D-Ala or D-Ser amino acids into a peptide, and four or five consecutive D-AAAs into a macrocyclic peptide *in vitro*, particularly useful for drug discovery (Kato et al., 2017b; Goto and Suga, 2021). Although tRNA engineering alone demonstrated improved installation efficiency, optimization of IF2, EF-Tu, and EF-G concentrations further improved results—demonstrating the impact and complexity of other factors in cell-free protein synthesis.

2.2.2 *Methanocaldococcus jannaschii* tRNA^{Tyr} engineering

The *M. jannaschii* (*Mj*) tyrosyl-tRNA synthetase (TyrRS): tRNA^{Tyr} pair is commonly used for GCE in *Escherichia coli* (Xie and Schultz, 2006). After modifying loop regions of *Mj* tRNA^{Tyr} to be orthogonal in *E. coli* (Wang and Schultz, 2001), further research sought to improve its elongation efficiency. Specific nucleotides in the acceptor domain (2:71, 3:70, 6:67, and 7:66 in the acceptor stem,



and the entire T-stem) were selected based on a co-crystal structure of EF-Tu with Cys-tRNA^{Cys} (Nissen et al., 1999) and subsequently randomized (Guo et al., 2009). Several effective suppressor tRNA variants emerged which improved the incorporation of ncAAs. In particular, tRNA^{opt}_{CUA} was only modified at base pairs 49:65, 50:64, and 51:63, and found to be capable of installing six ncAAs (Young et al., 2010). Notably, the incorporation efficiency differed depending on the ncAA, which holds true with previous comments about the amino acid contacts affecting affinity with EF-Tu.

A separate investigation used a directed evolution approach to improve the ribosomal efficiency for installation of three 3-halo-tyrosines (Maranhao and Ellington, 2017). These efforts focused on modifying the acceptor and T-stem nucleotides that interact with EF-Tu: nucleotides 3, 6, 68, and 71 in the acceptor stem, and base pairs 49:65, 50:64, and 51:63 in the T-stem. Five evolved tRNAs demonstrated 12 to 20-fold higher suppression efficiency for single incorporation of all 3-halo-Tyr variants. However, in the process of evolution, these tRNAs lost varying degrees of orthogonality in the cell, demonstrating that engineering one aspect of a tRNA can impact other tRNA functions.

2.2.3 tRNA^{Pyl} engineering

Pyrrolysine (Pyl) is the 22nd proteinogenic amino acid found in archaea (Tharp et al., 2017) and some bacteria (Matassi, 2017) with its own incorporation machinery: pyrrolysyl-tRNA synthetase (PylRS) and its cognate tRNA (tRNA^{Pyl}). This system has been extensively researched for its use in GCE because of the promiscuity of the PylRS binding pocket and the orthogonality of the archaeal PylRS:tRNA^{Pyl} protein synthesis system in *E. coli*

(Tharp et al., 2017) and eukaryotic systems (Chin, 2014; de la Torre and Chin, 2021). Significant research has focused on expanding the substrate capacity of PylRS and engineering PylRS:tRNA^{Pyl} pairs to be mutually orthogonal (Wan et al., 2014; Beattie et al., 2023; Gong et al., 2023). However, others have focused on optimizing tRNA^{Pyl} for improved translational fidelity (Wang J. et al., 2016). In one case, rational engineering of the T-stem and acceptor stem in *Methanosarcinaeae barkeri* tRNA^{Pyl} resulted in improved binding to PylRS, and *E. coli* EF-Tu for incorporation of N(ε)-acetyl-L-lysine (AcK) (Fan et al., 2015). Small libraries of tRNA mutants were created at base pairs 2:71, 3:70, 6:67, and 7:66 in the acceptor stem, and 49:65 and 50:64 in the T-stem. It was found that changes to base pairs 7:66, 49:65, and 50:64, which are well-known EF-Tu affinity determinants (Asahara and Uhlenbeck, 2002), benefited from mutations. The best tRNA variant, tRNA^{Pyl-opt}, with G7:C66, U49:A65, and G50:C64 had a ~3-fold improvement in AcK incorporation into sfGFP and human histone H3. tRNA^{Pyl-opt} was also used to incorporate two different ncAAs simultaneously at different sites within a protein (Zheng et al., 2018). These results collectively demonstrate the effectiveness of T-stem engineering to incorporate ncAAs. Combined with its orthogonality in heterologous systems, tRNA^{Pyl} is a responsive platform that can be fine-tuned to efficiently accommodate various ncAAs.

2.2.4 tRNA^{Sec} engineering

Selenocysteine (Sec) is the 21st genetically encoded amino acid, found in all three domains of life, and can imbue proteins with unique properties such as overoxidation resistance, diselenide bond formation, and ability to be chemically modified (Arnér, 2010; Reich

and Hondal, 2016; Mousa et al., 2017). Unlike the other natural amino acids, the mechanism for incorporating Sec into the ribosome involves additional steps and translation factors, including a specialized elongation factor, which makes site-specific Sec incorporation into proteins a formidable challenge (Yoshizawa and Böck, 2009; Chung and Krahn, 2022; Wright and O'Donoghue, 2024). The extended, 8 bp acceptor stem of tRNA^{Sec} is a distinguishing feature that allows recognition by SelB and was originally hypothesized to prevent it from binding to EF-Tu (Baron and Böck, 1991). It was later shown that EF-Tu is capable of binding extended acceptor stems, albeit at a lower affinity, and rather it is the antideterminant sequence in tRNA^{Sec} that specifically blocks EF-Tu binding (Rudinger et al., 1996). The antideterminant sequence in tRNA^{Sec} consists of base pair C7:G66 in the acceptor stem and base pairs G49:U65 and C50:G64 in the T-stem—the same base pairs that are modified to improve EF-Tu affinity in other tRNAs. Thus, these base pairs act as a double-edged sword for EF-Tu binding; they can drastically improve and inhibit tRNA affinity for EF-Tu (Rudinger et al., 1996; Giegé and Eriani, 2023).

With this information, it suggests that tRNA^{Sec} could be engineered to be recognized by EF-Tu instead of SelB. This would open the possibility for overexpression of selenoproteins and site-specific installation of Sec to harness its enhanced chemical properties. To achieve this, hybrid tRNAs were created between tRNA^{Sec} and tRNA^{Ser} (tRNA^{UTu}, tRNA^{SecUx}, tRNA^{UTuX}, and tRNA^{UTuT} variants) by several groups. These hybrid tRNAs successfully demonstrated the possibility of EF-Tu-mediated Sec insertion (Aldag et al., 2013; Miller et al., 2015; Thyer et al., 2015; Fan et al., 2018).

The earliest example (tRNA^{UTu}, Aldag et al., 2013) utilized tRNA^{Ser} as the major scaffold; it retained the recognition elements for EF-Tu (G7:C66, C49:G65, and A50:U64), and incorporated the rest of the extended acceptor stem of tRNA^{Sec} necessary for Sela recognition (to convert Ser-tRNA^{Sec} to Sec-tRNA^{Sec}). This created a 13 bp acceptor domain conducive for Sela recognition, but with EF-Tu identity elements already present in tRNA^{Ser} to mimic the translation pathway of canonical AAs. Although a great leap forward in simplifying Sec's translation pathway, this tRNA resulted in low selenoprotein yield and significant serine misincorporation (as EF-Tu still recognizes Ser-tRNA^{UTu}) (Aldag et al., 2013). Optimizing Ser to Sec conversion became the focus of subsequent rational tRNA engineering efforts that were outside of the acceptor domain (tRNA^{UTuX}, Miller et al., 2015).

Another group utilized *E. coli* tRNA^{Sec} as a scaffold to design a tRNA with heightened EF-Tu binding affinity (Thyer et al., 2015). They accomplished this through tuning the antideterminant sequence (base pairs 7:66, 49:65, and 50:64) known to govern EF-Tu affinity (Rudinger et al., 1996; Schrader et al., 2009; Schrader and Uhlenbeck, 2011). Additionally, the authors hypothesized that tRNA^{UTu} demonstrated Ser misincorporation due to reduced Sela contacts with the D- and T-loops of tRNA^{Sec} as shown in the crystallized Sela structure (Itoh et al., 2013a; Thyer et al., 2015). Results indicated that G7:C66, U49:G65, and C50:U64 modifications on the tRNA^{Sec} scaffold (tRNA^{SecUx}) greatly improved EF-Tu affinity and subsequent Sec insertion efficiency compared to tRNA^{UTu}.

Later, another group re-engineered tRNA^{UTu} by creating a small library of variants combining different tRNA^{Ser} and tRNA^{Sec} sequence segments. The segments tested were the acceptor stem, T-stem, and nucleotide 59 in the T-loop from either tRNA^{Ser} or tRNA^{Sec}. Nucleotide 59 was tested because it is a conserved base (Romby et al., 1987; Lowe and Chan, 2016) relevant for maintaining 3D L-shape (Sanbonmatsu et al., 2005; Pan et al., 2008; Itoh et al., 2013b) and Sela binding (Fischer et al., 2016). This strategy resulted in many tRNA variants with modified T-stems, increasing their affinity to EF-Tu (Lajoie et al., 2013). However, the best performing tRNA (tRNA^{UTuT6}) was formed by only changing A59C from the original tRNA^{UTu} (Fan et al., 2018). This suggests that other T-arm nucleotides can contribute to translational fidelity, perhaps by improving interactions with SerRS, Sela, EF-Tu, and/or improved tertiary tRNA structure. The unique batch of tRNA variants from this study provided an unclear pattern on how to increase EF-Tu affinity.

Combined, these efforts demonstrate that Sec translation can be rewired for elongation by EF-Tu through focused engineering on the tRNA acceptor domain. However, the efficiency of these changes is limiting, most likely due to the fact that tRNA^{Ser} and tRNA^{Sec} have evolved for specific interactions with their translation machinery. Therefore, as discussed below, there are alternative strategies that can be taken to increase translation efficiency.

2.3 The curious case of EF-Ts

EF-Ts canonically function by binding EF-Tu-GDP and catalyzing GDP dissociation, allowing EF-Tu to rebound GTP and deliver another aa-tRNA to the ribosome (Miller and Weissbach, 1970; Weissbach et al., 1970; Miyajima and Kaziro, 1978; Pedersen et al., 1978; Romero et al., 1985; Wang et al., 1997). However, there is also research indicating that EF-Ts may have a more direct role in elongation by interacting directly with the aa-tRNA (Romero et al., 1985; Schwartzbach and Spremulli, 1991; Bubunenko et al., 1992) by i) regulating ternary complex formation and dissociation in response to cellular growth or stress signals, and ii) forming quaternary complexes that dramatically increase the rate of protein synthesis (Burnett et al., 2013; Burnett et al., 2014). Although there is no solved structure for aa-tRNA-EF-Tu-GTP-EF-Ts, it has been suggested that direct interactions between EF-Ts and the ternary complex are possible (Kawashima et al., 1996; Gromadski et al., 2002; Burnett et al., 2013) and may bind the aa-tRNA acceptor stem and elbow region in a conformation similar to the previously characterized *E. coli* EF-Tu-EF-Ts complex (Kawashima et al., 1996). It is unclear exactly how EF-Ts may interact with the complexed aa-tRNA, and whether tRNA engineering to improve favorable quaternary interactions with EF-Ts is a viable strategy for improving translational fidelity.

3 Improving codon-anticodon interactions

After the ternary complex has delivered aa-tRNA to the ribosome, the complex undergoes a series of interactions to ensure proper acceptance into the A-site for peptidyl transfer

(Rodnina et al., 2017). Decoding efficiency essentially relies on a careful balance of codon-anticodon interaction strength and the ability of tRNA to adopt a proper conformation, both of which have been evolutionarily tuned, in part by tRNA modifications, to accommodate different codons and aa-tRNA combinations (Lorenz et al., 2017; Uhlenbeck and Schrader, 2018). The principles guiding this idiosyncratic tuning are less complete than the principles that have been shown to guide EF-Tu-related tRNA engineering efforts (Figure 4). Regardless, there has still been an exciting amount of information hinting at tRNA engineering principles to help improve their decoding mechanics, focused overwhelmingly on bacterial systems.

3.1 Engineering the anticodon arm for improved translational fidelity

While the anticodon sequence itself determines a substantial proportion of the codon-anticodon interactions within the ribosome (Rozov et al., 2016; Loveland et al., 2017; Rodnina, 2023), it is also well-known that surrounding nucleotides and base pairs within the anticodon arm influence these dynamics to uniformly tune the different strengths of codon-anticodon interactions to ensure uniform decoding efficiency and rate of mRNA translation (Olejniczak et al., 2005; Olejniczak and Uhlenbeck, 2006; Ledoux and Uhlenbeck, 2008). This covariance between the anticodon sequence and other anticodon arm elements is known as the “extended anticodon” (Yarus, 1982; Yarus et al., 1986; Raftery and Yarus, 1987; Kleina et al., 1990; Auffinger and Westhof, 2001; Murakami et al., 2009; Schmeing et al., 2011; Shepotinovskaya and Uhlenbeck, 2013; Cervettini et al., 2020). Specifically, it has been shown that the identities of positions 32, 37, 38, and at least two base pairs in the anticodon stem are well-conserved (Saks and Conery, 2007).

The flexibility of the anticodon stem-loop, largely influenced by base pair 31:39 and non-canonical interactions between nucleotide 32:38 (Auffinger and Westhof, 1999), impacts this decoding efficiency and codon recognition specificity (Olejniczak et al., 2005; Ledoux and Uhlenbeck, 2008; Sigal et al., 2024). “Stiffer” anticodon arms result from G:C base pairs at these positions, generally distorting the canonical anticodon loop conformation and disrupting the binding of GC-rich codons (Olejniczak et al., 2005; Olejniczak and Uhlenbeck, 2006; Murakami et al., 2009), whereas weaker interactions, including the rare A32:U38 interaction, allows easier codon-anticodon base pairing (Ledoux et al., 2009; Schmeing et al., 2011; Grosjean and Westhof, 2016). Therefore, tRNA sequences are seemingly tuned for optimal bending in the ribosome to off-set the strengths of different codon-anticodon interactions (Shepotinovskaya and Uhlenbeck, 2013).

A more recent example (Katoh and Suga, 2024) leveraged these principles to further improve the translational efficiency of an engineered tRNA platform (discussed below) to incorporate especially difficult ribosomal substrates that had eluded previous tRNA engineering efforts (Lee et al., 2022). Crucially, screening the anticodon stem of the scaffold tRNA^{Pro1E2} (Katoh et al., 2017a) led to different tRNA^{Pro1E2} variants each uniquely

optimized to one of five different anticodon sequences. Then, anticodon loop mutations at positions 32, 33, 37, and 38 were introduced according to the native *E. coli* anticodon sequences leading to further improvement of ncAA incorporation efficiency. Combined, these improvements allowed for multi-site incorporation of β AAs at five different codons *in vitro* and the first reported ribosomal installation of 10 consecutive β -homophenylglycine (β Phg) residues. The G:C or C:G base pair at 31:39 was conserved across all codons, along with a conserved A37.

However, it is worth noting that mutational, structural, and bioinformatic has also identified positions dispersed throughout all arms of bacterial tRNAs that influence proper bending and decoding specificity (Saks and Conery, 2007; Schmeing et al., 2011; Shepotinovskaya and Uhlenbeck, 2013). In this way, the “extended anticodon” truly extends throughout the entire tRNA, potentially complicating tRNA engineering efforts when focusing on multiple tRNA regions.

3.2 Anticodon arm modifications influence decoding

tRNAs are frequently post-transcriptionally imbued with diverse modifications that drastically impact their structure and function (Lorenz et al., 2017; Suzuki, 2021; Cappannini et al., 2024; Yared et al., 2024). tRNAs exhibit diverse modifications in all arms, but they are most frequent at the tRNA core and anticodon arm (Cantara et al., 2011; Smith et al., 2024). Anticodon arm modifications in particular have thus been targets for uncovering tRNA translation fidelity principles as an “unrealized genetic code” to complement the tRNA sequence itself (Agris et al., 2017). In particular, modifications at positions 32, 34, and 37 are frequent and chemically varied, especially at the first two positions (Jühling et al., 2009).

The most well-known examples of post-transcriptional modifications impacting decoding are those that occur at position 34, the “wobble” base (Crick, 1966; Agris, 1991; Demeshkina et al., 2012). In some cases, the modification status at position 34 expands wobble pairing to all four mRNA nucleotides at the third codon position (Rogalski et al., 2008; Bloom-Ackermann et al., 2014). Other modifications have been shown to impact decoding specificity or even completely disable codon binding altogether (Kurata et al., 2008; Kumbhar et al., 2013; Nedialkova and Leidel, 2015; Nakano et al., 2016; Song et al., 2022). A34 is also almost universally modified to inosine to expand decoding potential to C, U, and A (Crick, 1966). Position 37 does not directly interact with the codon, but is often a purine frequently modified to stabilize Watson-Crick base pairing at the first two positions of a codon (Väre et al., 2017). Moreover, position 32 modifications generally help to create non-canonical interactions at base pair 32:38 to stabilize the U-turn structural motif of the anticodon arm relevant for ribosomal A-site acceptance and codon discrimination (Auffinger and Westhof, 1999; Cantara et al., 2012; Väre et al., 2017). In addition to the anticodon loop, tRNAs are also often simultaneously modified at anticodon arm positions. These modifications are connected with the anticodon sequence, synergistically functioning to alter tRNA structure and decoding

Quadruplet tRNA Engineering

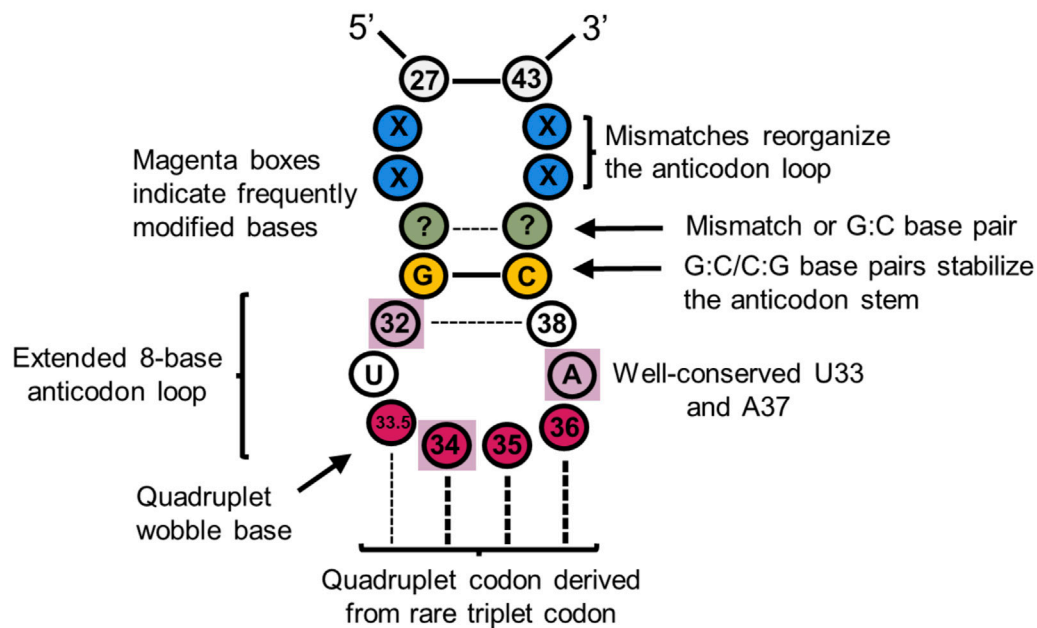


FIGURE 5

tRNA sites explored in quadruplet tRNAs to improve translational fidelity. Efficient quadruplet tRNAs have a well-conserved G:C/C:G base pair adjacent to the anticodon loop (yellow), A37 and U33 residues, and base pair mismatches near the beginning of the anticodon stem (blue) which adjusts the conformation of tRNAs. Frequently post-transcriptionally modified residues (purple boxes) also impact translational fidelity, although position 33.5 base pairing with a quadruplet codon is not necessary.

efficiency (Nishimuka, 1972; Jühling et al., 2009; Cantara et al., 2012; Grosjean and Westhof, 2016; Väre et al., 2017). However, the exact impact of adding or removing a modification across different tRNAs is not well understood, and some unmodified tRNAs can still function (Phizicky and Alfonzo, 2010; Lorenz et al., 2017). One heuristic approximation is that GC-rich decoding tRNAs are modified to reduce binding affinity whereas AU-rich decoding tRNAs are modified to improve codon binding (Uhlenbeck and Schrader, 2018; Sigal et al., 2024). One recent example improved the UAG decoding efficiency of a previously engineered ^{opt}tRNA_{CUA}^{Tyr} (Young et al., 2010) by replacing adenine with 2, 6-diaminopurine (D) at positions 31 and 36 (Mala and Saraogi, 2022). D is similar to adenine but has an added C₂ amino group, enabling the formation of an additional hydrogen bond in the D-U base pair compared to A-U pairing, which strengthens codon-anticodon interactions and stabilizes RNA duplexes (Strobel et al., 1994).

It has also been hypothesized that the overreliance on synthetic tRNAs that lack post-transcriptional modifications limits tRNA activity (McFeely et al., 2022); it was recently shown that wild-type (i.e., fully modified) tRNAs significantly outcompeted their synthetic counterparts (3 to 4-fold improvement) for the same cognate sense codons (McFeely et al., 2022). Interestingly, heterologous tRNAs such as tRNA^{Pyl}-^{opt} can also be engineered to reduce dependence on post-transcriptional modifications and improve suppression efficiency (Baldrige et al., 2018).

Combined, tRNA modification engineering will almost certainly aid in producing more effective synthetic tRNAs, although more research is needed to better define fundamental principles.

3.3 Engineered quadruplet codons efficiently expand the genetic code

Significant research efforts have focused on engineering triplet codons for improved translational fidelity. However, there are limitations for genetic code expansion because triplet codons result in only 64 possible codon combinations, which traditionally encode for 20 proteinogenic AAs (plus Sec and Pyl) and three stop codons. Therefore, there has been significant research efforts toward engineering quadruplet codon-decoding tRNAs—tRNAs that decode four base codons on the mRNA—to vastly expand the available number of empty codons (up to 256 free codons) (Figure 5). Efficient quadruplet codons are also inherently orthogonal to the 64 triplet codons, thereby not requiring codon reassignment.

Such quadruplet decoding, or “programmed frameshifts,” occur in nature, often with an extended 8-base anticodon loop as opposed to the canonical 7-base loop (Riyasaty and Atkins, 1968; Riddle and Roth, 1970; Yourno and Kohno, 1972; Riddle and Carbon, 1973; Bossi and Smith, 1984; Farabaugh, 1996; Moore et al., 2000a; Walker and Fredrick, 2006; Atkins and Björk, 2009; Mohanta et al., 2023). Many experiments have since demonstrated that inserting a single nucleotide into the anticodon loop of a tRNA to create an 8-base anticodon results in moderate quadruplet decoding *in vitro* and *in vivo*, especially in combination with engineered synthetases (Curran and Yarus, 1987; Ma et al., 1993; Hohsaka et al., 1996; Hohsaka et al., 1999; Hohsaka et al., 2001; Moore et al., 2000b; Magliery et al., 2001; O’Connor, 2002; Ohtsuki et al., 2005; Taki et al., 2006; Wan et al.,

2010; Chatterjee et al., 2012; Hankore et al., 2019; Dunkelmann et al., 2020; Dunkelmann et al., 2021; DeBenedictis et al., 2022). In a recent report, it was determined that nearly a third of the 20 bacterial tRNA isoacceptors can demonstrate modest quadruplet decoding activity after carrying the insertion of a single nucleotide insertion in the anticodon loop in *E. coli* (DeBenedictis et al., 2022). Additionally, while experiments with quadruplet *M. barkeri* tRNA^{Pyl} using a 7-base anticodon loop were unsuccessful, expanding them to 8-bases provided an avenue for quadruplet decoding (Wang N. et al., 2016). One potential issue with anticodon arm engineering is that the anticodon loop can be an essential identity element for binding respective aaRSs (Melnikov and Söll, 2019). Therefore, engineering the anticodon arm for improved decoding mechanics can also alter aminoacylation specificity and efficiency. Many engineered quadruplet tRNAs thus originate from tRNA^{Pyl} scaffolds as PylRS is known not to interact with the anticodon loop (Nozawa et al., 2009).

Designing quadruplet anticodon sequences derived from rarely-used triplet codons can help with endogenous machinery competing to perform triplet decoding as a general quadruplet tRNA engineering principle (Guo and Niu, 2022). In one case, a GGGU quadruplet tRNA was outcompeted by inserting Gly at the CCC codon in *Xenopus* oocytes (Shafer et al., 2004). In contrast, deriving quadruplet tRNAs from rarely used three base codon sequences in *E. coli* found more success in quadruplet decoding efficiency (Hohsaka et al., 2001). Interestingly, an *in vivo* library of *E. coli* tRNA^{Ser} with randomized 8- or 9-base anticodon loops containing various quadruplet codons (NNNN) found that the most efficient tRNAs decoded CCCU, AGGA, UAGA, and CUAG codons—all of which are derived from rare triplet codons (Magliery et al., 2001). This strategy has also served to successfully incorporate ncAAs in *E. coli* (Anderson et al., 2004; Chatterjee et al., 2014).

As the anticodon arm (and entire tRNA, to an extent) often coevolves with its anticodon sequence, further modification of the anticodon arm outside of the quadruplet anticodon has been shown to improve decoding efficiency. In *E. coli* and mammalian cells alike, *in vitro* evolution of the entire tRNA anticodon arm generated mutants with efficient incorporation of ncAAs at the AGGA codon (Niu et al., 2013). Importantly, sequence convergence of base pairs G28:C36 and G27:C37, along with some base pair mismatch at 26:38 was observed. Combined, this sequence was thought to restructure the anticodon arm to help with A-site binding and translocation. Another investigation (Wang et al., 2014) evolved a series of *M. barkeri* tRNA^{Pyl} mutants for efficient quadruplet decoding with ncAAs using an engineered ribosome (ribo-Q1) (Neumann et al., 2010). tRNAs were randomized at positions 29–33 and 37–41 for four quadruplet codons (AGGA, AGTA, TAGA, and CTAG). The most efficient mutants all shared a G:C or C:G base pair adjacent to the anticodon loop, even though the size of the anticodon loop varied. Position A37 was also conserved among all efficient mutants, which is associated with reading frame maintenance (Agris, 2004). Follow up research varied the same regions in UAGN-decoding *M. mazei* tRNA^{Pyl} to improve quadruplet decoding efficiency and uncover a better mechanistic understanding of quadruplet decoding in *E. coli* (Wang N. et al., 2016). This led to significant quadruplet decoding efficiency improvements and incorporation of ncAAs with two PylRS variants, though quadruplet codon orthogonality was not

obtained. Most strikingly, all observed tRNA mutants contained U33.5 regardless of the UAGN codon they decode, validating the previously hypothesized idea that strict Watson-Crick base pairing at all four positions is not required for quadruplet decoding (Guo and Niu, 2022). It is possible that U33.5 impairs interactions at position 32:38 to expand the anticodon loop and permit quadruplet decoding (Maehigashi et al., 2014; Wang N. et al., 2016) and/or preserves an essential U-turn structure in the anticodon loop necessary for translocation (Phelps et al., 2002; Phelps and Joseph, 2005). Importantly, all mutants contained A31G and U39C mutations, resulting in a new G:C base pair which strengthens the anticodon stem, but most mutants did not have Watson-Crick base pairing at pairs 29:41 or 30:40 which may restructure the anticodon arm for more efficient quadruplet decoding. It was also noted that position 33 and 37 were never mutated. While not specifically evolved for mammalian cells, these quadruplet tRNAs were shown to be effective in incorporating a ncAA in HEK 293T cells, suggesting that anticodon arm engineering strategies are applicable to a wide range of systems (Chen et al., 2018). Additional research (Mills et al., 2021) screened examples of these previously-engineered quadruplet-decoding tRNA^{Pyl} variants (Niu et al., 2013; Wang et al., 2014; Wang et al., 2016 N.; Chen et al., 2018) in HEK293 cells, and found that two of the tRNA variants (one evolved for mammalian cells, one with a simple quadruplet anticodon change) were suitable for use in ncAA implementation. Notably, engineered quadruplet tRNAs across all experiments were generally most effective only in the organism that were designed in.

A more recent investigation sought to improve different quadruplet *E. coli* tRNA suppression efficiencies through directed evolution (DeBenedictis et al., 2021) at the UAGA-codon, permitting multiplex decoding of four distinct quadruplet codons within a single reporter protein. It was found that mutations in the anticodon loop (positions 32 and 38), variable arm, central loop, D-stem, and acceptor stem improved quadruplet coding efficiency up to 80-fold. However, there was no clear consensus for these mutations. In a follow up experiment, focused libraries at positions 32, 37, and 38 further improved translation efficiency for different quadruplet codons in *E. coli* (DeBenedictis et al., 2022). Individual libraries for specific codons demonstrated enrichment for a specific nucleotide at one or more positions, suggesting significant covariance between the anticodon sequence and other regions of the anticodon arm. Additionally, there was a pronounced preference for base A37 across all codon libraries.

Quadruplet decoding has also been utilized to incorporate ncAAs in animals; to adapt quadruplet decoding ncAA incorporation in *C. elegans*, hybrid tRNAs containing the UCUA anticodon were created and demonstrated significant improvement in ncAA incorporation efficiency. They used *M. mazei* tRNA^{Pyl} variants already known to interact well with *C. elegans* translational machinery in triplet codon ncAA incorporation (Serfling et al., 2018; Davis et al., 2021) as a starting scaffold while overlaying anticodon arm modifications previously identified in (Niu et al., 2013; Wang N. et al., 2016).

With many mutational and structural studies conducted elucidating several anticodon arm engineering principles thus far, engineering quadruplet codons with sufficient translational fidelity is now more feasible than ever before, bringing us closer to a drastically expanded genetic code.

4 Improving peptide bond formation and peptidyl transfer

Aminoacylated tRNAs undergo innumerable interactions with translation factors and the ribosome on their journey to translating a polypeptide. After transport, initial acceptance, and codon-anticodon interactions, the P-site peptidyl-tRNA amino acid chain is transferred to the A-site aa-tRNA through a peptidyl transfer reaction. It is hypothesized that the ability of a nCAA to go through the peptidyl transfer (PT) reaction pathway is just as important—if not more important—than its nCAA's affinity to EF-Tu (Katoh et al., 2017a).

Pro is a difficult ribosomal substrate during PT due to its rigid, bulky structure; and in this way, mimics nCAAs which are predominantly ill-suited for ribosomal translation. Pro directly slows down peptide bond formation compared to other AAs, with consecutive Pro residues leading to ribosomal stalling (Muto and Ito, 2008; Wohlgeuth et al., 2008; Pavlov et al., 2009; Doerfel et al., 2013; Peil et al., 2013; Ude et al., 2013; Starosta et al., 2014; Woolstenhulme et al., 2015; Melnikov et al., 2016a; Huter et al., 2017) and even protein truncation (Menninger, 1976; Cruz-Vera et al., 2004; Gonzalez de Valdivia and Isaksson, 2005; Muto and Ito, 2008). However, elongation factor P (EF-P) is an essential and well-conserved translation factor that alleviates ribosomal stalling caused by difficult ribosomal substrates like Pro and Gly (Katoh and Suga, 2018; Hummels and Kearns, 2020). EF-P binds on the ribosome between the E and P sites to interact with the P-site peptidyl-tRNA and stabilize its 3' CCA end for favorable PTC positioning (Hummels and Kearns, 2020), which circumvents the inherently low amino acid reactivity and restores canonical transfer efficiency (Doerfel et al., 2015; Melnikov et al., 2016a).

EF-P specifically recognizes a unique nine nucleotide D-loop and stable 4 bp D-stem sequence observed in canonical tRNA^{Pro} (Blaha et al., 2009; Katoh et al., 2016). Thus, researchers have begun implementing the D-arm sequence of tRNA^{Pro} in engineering efforts to recruit EF-P and promote efficient PT for nCAAs (Figure 4). Many Pro-like nCAAs have since been attached to tRNA^{Pro} derivatives (Doerfel et al., 2015; Katoh et al., 2016), and were more efficiently incorporated in an EF-P-dependent manner. In another investigation, non-Pro-like nCAAs also significantly benefitted from this strategy when charged onto a tRNA^{Pro} derivative (Katoh et al., 2017a). Thus, optimizing tRNA structure for EF-P binding can improve ribosomal incorporation of a variety of nCAAs. Results in tRNA engineering efforts for efficient EF-P recruitment will vary according to the nascent peptide length (Gonzalez de Valdivia and Isaksson, 2005) and surrounding sequence context (Peil et al., 2013; Elgamal et al., 2014; Starosta et al., 2014). Generally, it was also shown that the more difficult a substrate is to ribosomally incorporate, the more it benefits from EF-P-dependent tRNA engineering. Further optimization of tRNA affinity for EF-P is tricky because the full mechanism of EF-P-mediated nCAA incorporation is unknown (Hummels and Kearns, 2020; Katoh et al., 2020). More intricate biochemical details on this process will likely elucidate a further comprehensive guide for this tRNA engineering strategy.

5 Improving multiple tRNA binding partner interactions simultaneously

5.1 tRNA^{Pro1E2} revolutionizes ribosomal nCAA incorporation

An interesting feature of tRNAs is their ability to be modularly engineered for simultaneous tuning of different translational processes. Such an idea was recently exploited to produce tRNA^{Pro1E2}, a chimera between tRNA^{Pro} and tRNA^{Glu} (Katoh et al., 2017a) that allowed for simultaneous improvement of both EF-P and EF-Tu interactions. This tRNA transferred the T-stem and elements of the acceptor stem from tRNA^{Glu} (high natural EF-Tu affinity tRNA) onto the tRNA^{Pro} scaffold (optimized recruitment of EF-P) to synergistically improve the translational fidelity of difficult ribosomal substrates *in vitro* with optimized IF2, EF-G, EF-Tu, EF-Ts, and EF-G concentrations. In the presence of EF-P, tRNA^{Pro1E2} significantly outperformed other engineered tRNAs with similar high EF-Tu affinity (like tRNA^{GluE2}) to incorporate D-AAAs (Katoh et al., 2017a).

In light of this research, tRNA^{Pro1E2} has recently been used as an updated GCE scaffold tRNA to improve the installation of a staggering number of translation-incompatible substrates *in vitro* for a number of applications (Lee et al., 2020a; Lee et al., 2020b; Lee et al., 2021b; Lee et al., 2022; Hammerling et al., 2020; Tsiamantas et al., 2020; Katoh and Suga, 2022b; Katoh and Suga, 2022a; Katoh and Suga, 2023b). As discussed above, this platform has been recently updated by adjusting the anticodon arm according to the anticodon sequence for further improving *in vitro* nCAA incorporation (Katoh and Suga, 2024), demonstrating the modular tunability of different tRNA regions.

5.2 Non-specific tRNA engineering with directed evolution

Directed evolution offers a strategy to engineer tRNAs by linking tRNA incorporation efficiency to an organism's survival, helping to bypass the difficulties of rational design (Cobb et al., 2013; Miller et al., 2020; Wang et al., 2021). However, a common drawback of directed evolution is that it may be difficult to decode the results and extract general engineering principles.

In one notable example, compartmentalized partnered replication (CPR) (Abil et al., 2017), was used to co-evolve the *Saccharomyces cerevisiae* TrpRS:tRNA^{sup} pair for improved site-specific incorporation of the nCAA 5-hydroxy-L-tryptophan (5HTP) both *in vitro* and *in vivo* (Ellefson et al., 2014). Libraries targeting the anticodon stem, acceptor stem, or loop sequences (variable loop, D-loop, and one nucleotide in the T-loop) were generated independently. After 10 rounds of evolution, one tRNA variant ("40A") demonstrated ~12-fold better stop codon suppression than the original tRNA. The 40A variant contained one nucleotide change each in the D-loop, variable loop, and T-loop (U16G, G43U, U58G, respectively), highlighting the interesting solutions that directed evolution approaches often reveal.

Other examples of directed evolution are included in this review and detailed in other sections as they are often localized to specific tRNA regions. Nonetheless, broad directed evolution may serve to

help implement extremely difficult ncAAs or optimize overall tRNA structure in ways that may not be feasible to rationally derive.

6 Translocation through the ribosome

After a new peptide bond is formed and the nascent polypeptide chain is transferred to the A-site, efficient translocation of the A- and P-site tRNAs to the P- and E-sites, respectively, must occur to mark a completed cycle of elongation. Recently, tRNA engineering efforts have sought to explore the modulation of this process.

6.1 Selenocysteine tRNA engineering for improved translocation

Great strides regarding *in vivo* Sec insertion have been made by engineering tRNA^{UTu}, tRNA^{UTuX}, tRNA^{SecUx}, and tRNA^{UTuT} variants as previously discussed (Aldag et al., 2013; Miller et al., 2015; Thyer et al., 2015; Fan et al., 2018). However, Sec insertion efficiency and overall protein yields in these rewired Sec insertion pathways were still relatively low (Chung et al., 2022). EF-Tu only minimally binds tRNA with a 13 bp acceptor domain, even with a high-affinity antideterminant sequence (Rudinger et al., 1996). Therefore, exhaustive engineering efforts with tRNA^{Sec} and tRNA^{Ser} scaffolds could not reconcile the inherent discrepancy between the 13 bp acceptor domain necessary for Sela binding (but efficiently binds the elongation factor SelB (Fischer et al., 2016) and the 12 bp acceptor domain preferred for EF-Tu binding (Ohtsuki et al., 2005) in the rewired pathway.

Thus, a metagenomic search for EF-Tu-recognizing, tRNA^{Sec}-like tRNAs revealed a uniquely structured class of tRNAs that demonstrated efficient Sec incorporation: allo-tRNAs (Mukai et al., 2017). They contain a longer D-stem (6 bp), smaller D-loop (4 bases), and a unique acceptor domain consisting of a 9 bp acceptor stem and 3 bp T-stem. One Ser-isoacceptor of interest, dubbed allo-tRNA^{UTu1}, demonstrated efficient EF-Tu-mediated Sec installation when combined with *Aeromonas salmonicida* Sela (Mukai et al., 2018), which recognizes a 12-bp acceptor domain (Mukai et al., 2016). Further engineering of the tRNA^{UTu1} D-arm to improve Sela binding showed a significant improvement in Sec insertion. This tRNA and others (allo-tRNA^{UTu2D}) have since been reliably used for downstream applications (Evans et al., 2021; Evans et al., 2024; Morosky et al., 2023).

However, as a non-native *E. coli* ribosome substrate, the suitability and processivity of these allo-tRNAs during protein synthesis on the bacterial ribosome was studied via single-molecule FRET and cryo-EM (Prabhakar et al., 2022). It was determined that tRNA^{UTu1} was able to induce on-par suppression efficiency and decoding time compared to native suppressor tRNA^{supD}. However, single-molecule FRET analysis indicated that an overwhelming amount of ribosomal disassembly (64%) and low translational levels of processivity (21%) occurred after A-to P-site translocation. Structural determination of tRNA^{UTu1} in the bacterial ribosome determined that the long variable arm of tRNA^{UTu1} must undergo significant variable arm repositioning to avoid clashing with the ribosomal A-site finger (ASF) during translocation. The ASF is a region of the 23S rRNA that directly interacts with the

A-site tRNA elbow, T-loop (Nguyen and Whitford, 2016), and amino acid during decoding to attenuate translocation and maintain overall translational fidelity (Yusupov et al., 2001; Komoda et al., 2006; Nguyen and Whitford, 2016; Nguyen et al., 2017). Specifically, interactions between bases C8 and A45 within the central loop were hypothesized to overly stabilize the variable arm and cause steric hindrance with the ASF (Figure 4). A single C8A mutation on tRNA^{UTu1} disrupted the variable arm stability and restored post-suppression codon survival *in vivo* to canonical efficiency (Prabhakar et al., 2022).

tRNA flexibility, especially stemming from the tRNA elbow (interactions between the D-arm, T-arm, and variable arm) clearly plays an important role in dictating the aa-tRNA's journey through the ribosome (Frank et al., 2005; Zhou et al., 2014). More detailed research focused on the tRNA interactions through translocation and translation at large is emerging in prokaryotic systems (Ling and Ermolenko, 2016; Korostelev, 2022), which may aid in uncovering broad tRNA engineering principles to mediate this process.

6.2 The interconnected roles of EF-P and EF-G in translocation

Translocation is in large part mediated by the GTPase activity of elongation factor G (EF-G) in prokaryotes (Rodnina et al., 1997; Liu et al., 2011; Poulis et al., 2023) or eEF2 in eukaryotes (Kaul et al., 2011; Djumagulov et al., 2021; Milicevic et al., 2024). EF-G binds to the A-site to transfer deacylated P/E-site tRNA and peptidyl A/P-site tRNA to the E and P-sites, respectively. During states of slowed peptide bond formation and peptidyl transfer from inefficient substrates and/or excessive EF-G concentrations, EF-G is more likely to pre-maturely catalyze translocation before complete peptidyl transfer (mistranslocation). This can then induce drop-off of the aberrant P-site peptidyl tRNA from the ribosome, and in some cases, reinitiation of the aa-tRNA with active ribosomes to produce C- and N-terminally truncated proteins, respectively (Kang and Suga, 2011; Katoh et al., 2017a; Katoh et al., 2017b).

Both EF-G and the ribosome make important contacts with aa-tRNAs (Gao et al., 2009; Liu et al., 2011; Carbone et al., 2021), and thus engineering to improve these contacts presents itself as an avenue to mediate translational efficiency (Figure 4). However, simply maximizing an aa-tRNA's EF-G affinity is not appropriate as it can lead to mistranslocation. Recent research (Tajima et al., 2022) approximates the "default" state of cellular protein synthesis as a state with high peptidyl transfer efficiency and low translocation frequency. This offers an initial conceptual framework for balancing these processes, the specifics of which are still unknown.

7 tRNA engineering in eukaryotic systems

Prokaryotic tRNAs have been easier to engineer than eukaryotes due to the relative simplicity of prokaryotic translation and a plethora of structural and mutagenic investigations mapping specific tRNA contacts with translation factors and the ribosome. Eukaryotic translation is still well-researched (Korostelev, 2022),

though engineering specific sequences in tRNAs for enhanced translational outcomes in eukaryotes is less documented and has elucidated less guiding principles (Figure 4B). For example, specific bases in the T-stem and D-stem of tRNAs are known to impact binding with EF-Tu affinity and EF-P, respectively, with supporting structural determinations (discussed above). However, tRNA sequence-dependent affinity to equivalent eukaryotic factors eIF1A and eIF5A is considerably less understood. Regardless, research has still been aimed at optimizing tRNAs in various eukaryotic systems (Figure 4B). Current efforts have been focused on the Pyl and Sec insertion systems to improve their activities in eukaryotes.

7.1 Eukaryotic directed evolution

Effective aaRS:tRNA pairs from bacteria and archaea are often incorporated into higher eukaryotes because of inherent orthogonality, but this can lead to sub-optimal translational efficiency due to the presence of different translation machinery. Directed evolution methods have been established in *E. coli* to overcome this barrier, but an equivalent method for mammalian systems had not existed until recently (Jewel et al., 2023).

Such a directed evolution approach for mammalian systems, known as virus-assisted directed evolution (VADER), was recently developed (Jewel et al., 2023). Targeted evolution of the acceptor and T-stem regions for *Methanosarcinae mazei* tRNA^{Pyl}, and the acceptor stem of *E. coli* tRNA^{Tyr}, led to significant host-specific suppression efficiency improvement (Jewel et al., 2023). It was determined that the acceptor stem sequences made a significant impact on mammalian efficiency while the best T-stem sequences mirrored the wild-type sequence for tRNA^{Pyl} variants. Broadly, in tRNA^{Pyl} and tRNA^{Tyr} alike, the acceptor stems were significantly more G:C rich, with G residues preferentially on the 5' side of the acceptor stem. However, understanding the reason for sequence preferences requires a better understanding of tRNA biology in mammalian cells. To begin uncovering the basis of these improvements, the authors compared the activity in *E. coli* of their best-performing tRNA^{Pyl} (A2.1) with wild-type *M. mazei* tRNA^{Pyl}. It was shown that while A2.1 performed better in HEK293T, these improvements had no significant effect on its activity in *E. coli*. This emphasizes that the protein synthesis machinery differs between hosts, and tRNA sequences improved in one host cannot necessarily be transferred to others. Follow-up research identified a tRNA^{Pyl} variant, PyOtR, with markedly improved activity over A2.1 in mammalian cells (Jewel et al., 2024). PyOtR contained a similarly GC-rich acceptor stem, with a bias for 5' G residues, but also demonstrated an essential G5:U68 wobble pair—usually detrimental in tRNA acceptor stems (Santoro et al., 2003). Another study also recently used this technology to evolve the acceptor stem of *E. coli* tRNA^{Leu} for ncAA incorporation in eukaryotes (Huang et al., 2024). *E. coli* tRNA^{Leu} is a well-known platform for ncAA incorporation in eukaryotic systems, but typically suffered from low activity (Chin, 2017; Italia et al., 2017; Young and Schultz, 2018). The two best-performing mutants from this study contained G1:C72 and U6:G67 in the acceptor stem which led to substantial improvement in the incorporating nCAAs. The basis behind why these mutations

improve translational fidelity in mammalian systems is still unclear, but the ability to generate and screen large tRNA libraries will likely guide research efforts to uncover key eukaryotic interactions and refined tRNA engineering principles.

7.2 tRNA^{Pyl}

Archaeal tRNA^{Pyl} is significantly divergent from canonical tRNAs, containing a smaller D-loop, variable loop, and longer anticodon stem. This unique structure confers orthogonality in other domains of life which makes them a scaffold for GCE in bacteria and eukaryotes alike (Wan et al., 2014; Tharp et al., 2017; Krahn et al., 2024). However, implementing the Pyl system into mammalian cells had generally resulted in modest protein yields (Serfling et al., 2018). It was known from previous research with tRNA^{Leu} that replacing anticodon stem region G:U wobble pairs with Watson-Crick base pairs improved tRNA activity (Anderson and Schultz, 2003), so this same idea was applied to modestly improve the translational fidelity of *M. mazei* tRNA^{Pyl} in both *E. coli* (Chatterjee et al., 2013) and HEK293T cells (Schmied et al., 2014).

More recent work screened small collections of rationally designed *M. mazei* tRNA^{Pyl} variants to improve recognition by endogenous mammalian translation machinery. This was done by incorporating conserved human tRNA sequence patterns (Jühling et al., 2009; Giegé and Frugier, 2013; Suzuki et al., 2020) from mammalian tRNAs. These variants yielded more robust incorporation of one or several nCAAs into a G-protein coupled receptor in mammalian cells although the exact mechanism(s) of action are unknown. The top mutants demonstrated considerable sequence variability, but there were still some hints at potential eukaryotic tRNA engineering principles. The top mutants contained a specific, stabilizing G19:C56 tertiary interaction (“hinge”) between the D- and T-loops broadly conserved in tRNAs (Serfling et al., 2018; Berg and Brandl, 2021). Additionally, base pair G7:C66 (opposed to wild-type C7:G66) improved tRNA efficiency, a similarity found in bacteria (Guo et al., 2009; Fan et al., 2015). This base pair is known to impact aa-tRNA binding affinity to EF-Tu, hinting at the idea that tRNAs interact with EF-Tu in a similar way as eIF1A. This is further supported by evolution of a G7:C66 sequence in efficient tRNA^{Pyl} variants from a directed evolution experiment in mammalian cells (Zhao et al., 2021). These experiments importantly demonstrate the ability of tRNAs to be adapted for use in organisms with heterologous protein synthesis machinery, although the details of the specific eukaryotic protein synthesis mechanisms and tRNA engineering principles are only beginning to be determined.

7.3 tRNAs for inserting Sec

While many organisms contain Sec machinery, this machinery is widely absent in the Fungi kingdom (Mariotti et al., 2019). For the Pyl system, tRNA^{Pyl} has been modified to resemble mammalian tRNAs to improve interactions with mammalian translation machinery (discussed above). Recent Sec research used the opposite approach to produce Sec-containing proteins in yeast; *S. cerevisiae* tRNA^{Ser} isoacceptor (tRNA-Ser-AGA-1-1, (Chan and Lowe, 2016) was modified to resemble a Sec tRNA. Through this

strategy, a synthetic tRNA was generated that is compatible with yeast translation machinery and capable of Sec biosynthesis when artificially provided with the additional machinery (Hoffman et al., 2023). Many of these tRNA mutations were designed primarily to increase the efficiency of aminoacylation with Sec (i.e., tuning the tRNA sequence for binding to yeast SerRS and heterologous *A. salmonicida* SelA). However, it was shown that a G59U mutation (critical for maintaining 3D structure), was important for Sec insertion, and this engineered tRNA was capable of eEF1A-mediated delivery to the ribosome. Future structural and mutational research on this system will almost certainly uncover new rational tRNA engineering principles to improve translational fidelity in yeast.

8 Initiator tRNA engineering

Translation begins with some form of a methionine amino acid in all domains of life (Bhattacharyya and Varshney, 2016). A protein's N-terminus is also integral for regulating co-translational modifications and protein half-lives (Varshavsky, 2011), therefore important for site-specific amino acid implementation. Initiation with non-canonical initiation substrates can often induce truncated proteins lacking the first amino acid (Katoh and Suga, 2023a); however, mis-charging of *E. coli* tRNA^{Met} with other amino acids has regularly demonstrated diverse ncAA incorporation at the N-terminus of proteins without extensive tRNA engineering (Chattapadhyay et al., 1990; Goto et al., 2008a; Goto et al., 2008b; Rogers et al., 2018; Ad et al., 2019; Tharp et al., 2020b). This simply requires utilizing UAG-recognizing initiator tRNAs; otherwise, an engineered AUG-recognizing initiator tRNA will compete with natural, Met-inserting initiator tRNAs at AUG start codons, resulting in misincorporation. (Tharp et al., 2020b).

In bacteria, tRNA^{Met} is charged with formylated methionine (fMet) as a positive determinant for binding IF2 and a negative determinant for binding EF-Tu (Faulhammer and Joshi, 1987; Wang et al., 2015; Shah et al., 2019). A 1:72 bp mismatch in *E. coli* confers the conserved identity element for methionine formylation by methionyl-tRNA transformylase, exclusion of the tRNA from the ribosomal A-site, and resistance to peptidyl-tRNA hydrolase (Mandal et al., 1996). The anticodon stem also contains three G:C base pairs (G29:C41, G30:C40, and G31:C39) critical for P-site binding in both prokaryotic and eukaryotic initiator tRNAs (Seong and RajBhandary, 1987; Mandal et al., 1996; Rasmussen et al., 2009). Crucially, these two features can be transplanted to an elongator tRNA to convert it into an initiator tRNA (Varshney et al., 1993). Also important for formylation are base pairs 2:71 and 3:70 in the acceptor stem, and 11:24 in the D-stem (RajBhandary, 1994; Rasmussen et al., 2009; Lee B. S. et al., 2021). Lastly, G29:C41 and G30:C40 base pairs in the anticodon stem are required for efficient protein synthesis (Mandal et al., 1996). In eukaryotes, the initiator tRNA is charged with methionine (instead of fMet), although the tRNA structure differs from canonical tRNA^{Met}. Current information also shows that the initiator tRNA contains a specific A1:U72 base pair required for eIF2 binding (Rasmussen et al., 2009).

tRNA engineering efforts for enhancing eukaryotic initiation have not yet been shown, but recent research has explored prokaryotic initiator tRNA engineering (Figure 6). Non-canonical initiation in prokaryotes has been shown both *in vitro* and *in vivo*, likely paving

the way for future eukaryotic experimentation. One concern with incorporating ncAAs at the N-terminus is drop-off-reinitiation, where the aminoacyl initiator tRNA drops off from the ribosome and then translation is reinitiated with the second aa-tRNA in the A-site, resulting in an N-terminally truncated protein (Katoh and Suga, 2023a). Thus, tRNA engineering has been utilized to enhance initiator aa-tRNA activity in the ribosome. Recently, a bacterial initiator tRNA (tRNA^{Met2}) was engineered for *in vitro* incorporation of various ncAAs by increasing its affinity for EF-P while retaining core initiator tRNA function (Katoh and Suga, 2023c). EF-P functions in initiation to catalyze the peptide bond between the first and second amino acid (Blaha et al., 2009), although its potential role in improving translation initiation with ncAAs was unexplored. IF3 also functions in initiation, recognizing and positioning initiator tRNA into the P-site for start codon recognition (Hussain et al., 2016; Kaledhonkar et al., 2019). From this knowledge, a chimeric tRNA (tRNA^{iniP}) was engineered from canonical initiator tRNA^{Met2} and tRNA^{Pro1} (previously engineered for improved EF-P binding (Katoh et al., 2016) while retaining three G:C base pairs in the anticodon stem (G29:C41, G30:C40, and G31:C39) for IF3 recognition in initiation (Katoh and Suga, 2023c). tRNA^{iniP} contained other nucleotides from tRNA^{Pro1} (the D-arm motif for EF-P recognition, base pairs 27:43 and 28:42 in the anticodon stem, base pairs 49:65 and 50:64 in the T-stem, and nucleotide G46) as a result of screening different combinations of these chimeric tRNAs. The D-arm conferred EF-P affinity and the T-stem mutations likely fine-tuned EF-Tu affinity for improved translational fidelity, but the specific functions of the variable arm and anticodon stem mutations are not entirely obvious. Furthermore, the concentrations of IF3, EF-G, ribosome recycling factor, and sequences of the codon and SD were all optimized to help reduce drop-off-reinitiation. These mutations led to tRNA^{iniP} demonstrating nearly 1,000-fold improvement in peptide expression with a non-canonical initiation substrate compared to a conventional initiator tRNA (Katoh and Suga, 2023c).

Two different strategies have been applied to adapt the *Mj* tRNA^{Tyr}:TyrRS system for translation initiation: i) engineering initiator tRNAs for *Mj* TyrRS recognition while retaining initiator tRNA sequence motifs (Tharp et al., 2020a; 2021a), and ii) repurposing elongator *Mj* tRNA^{Tyr} as an initiator tRNA (Lee B. S. et al., 2021).

The first strategy modified *E. coli* initiator tRNA^{Met} for recognition by *Mj* TyrRS while retaining initiator tRNA sequence motifs to orthogonally incorporate diverse ncAAs *in vivo* (Tharp et al., 2020a). Nucleotide A73 and the C1:G72 base pair are sufficient for *Mj* TyrRS recognition and were transplanted into *E. coli* tRNA^{Met} (resulting in *itRNA*^{Ty2}). Using *Mj* TyrRS and TyrRS variants, 16 different ncAAs were encoded during initiation at the UAG nonsense codon *in vivo* for the first time. Interestingly, follow-up work demonstrated an *itRNA*^{Ty2} mutant (recoded to decode UAU codons) can selectively install ncAAs at the artificial UAU initiation codon while canonical machinery predominantly implements Tyr at repurposed elongator UAU codons (sense codon reassignment) (Tharp et al., 2021a). This importantly demonstrates the inherent orthogonality between initiator and elongator tRNAs, although it is not always exclusive (Tharp et al., 2021a). In the second strategy, *Mj* tRNA^{Tyr} was converted into an amber-suppressing initiator tRNA using *E. coli* initiator tRNA identity elements (Lee B. S. et al., 2021). Here, *Mj* tRNA^{Tyr}_{CUA} was engineered to contain a C1:A72 base pair mismatch (critical for methionyl-tRNA transformylase interactions), three G:C

Initiator tRNA Engineering

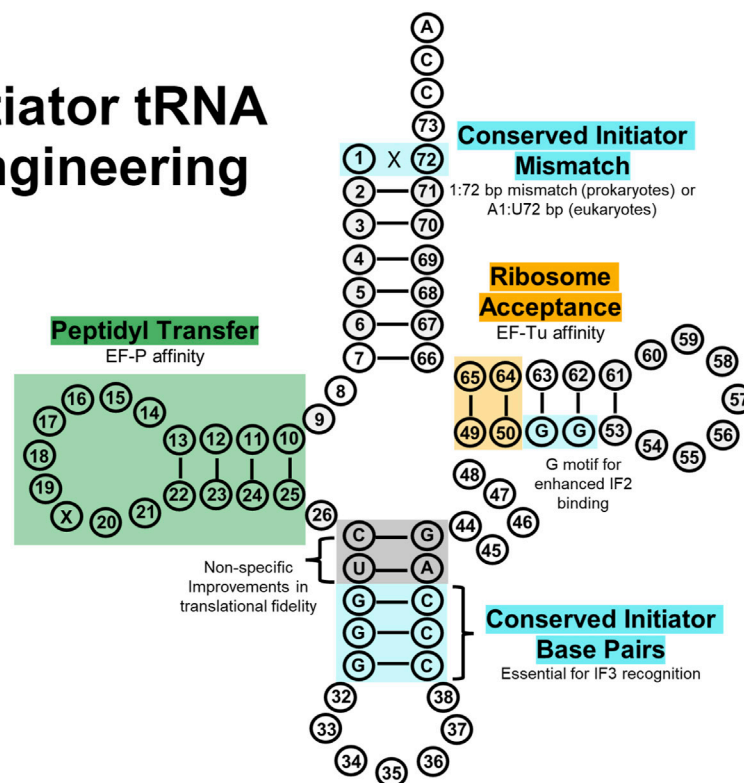


FIGURE 6

Initiator tRNA engineering sites. Initiator tRNAs contain unique sequence features (light blue) to encourage proper aminoacylation and binding to the ribosome P-site, as opposed to elongator tRNAs which bind the A-site. Sequences governing EF-Tu (green) and EF-P (orange) affinity have been carried over from elongator tRNA engineering research to improve initiator tRNA fidelity. Additionally, directed evolution has determined some non-specific improvements (grey) in the anticodon stem that further improve fidelity, including a G51-G52 motif in the T-stem that enhances IF2 binding to help position initiator tRNAs in the ribosomal P-site.

pairs in the anticodon stem (for binding the 30S P-site), and a G51-G52 motif for enhanced IF2 binding (Rasmussen et al., 2009). This resulted in *Mj*-itRNA-2, which, when combined with overexpression of methionyl-tRNA transformylase, efficiently encoded a nCAA into the amber start codon but not in identical elongation amber codons (Lee B. S. et al., 2021).

9 Therapeutic applications of tRNA engineering

The aberrant generation of a stop codon in a gene is the cause of nearly 11% of all human genetic disorders (Mort et al., 2008). As such, recent research has sought to develop safe and efficient suppressor tRNAs to readthrough pathogenic stop codons and rescue disrupted mRNAs and truncated proteins (Porter et al., 2021; Awawdeh et al., 2024; Coller and Ignatova, 2024). tRNA suppression therapy is at an early stage of development, with most research at the pre-clinical phase. Still, tRNA therapies compliment recent advances in the broader field of RNA therapeutics (like mRNA-based vaccines) and synthetic biology regarding GCE. These tRNA therapies give rise to unique medical benefits and inhibitions compared to the extensively researched genetic engineering toolbox of various CRISPR- and CRISPR-adjacent platforms (Terns, 2018; Petraitytė et al., 2021; Chehelgerdi et al., 2024). Certainly, engineering therapeutic suppressor tRNAs for proper

efficiency during elongation (or initiation) is a necessity and evidenced by several recent start-up companies developing tRNA therapeutics (Dolgin, 2022; Anastassiadis and Köhrer, 2023). Although outside the scope of this review, an excellent recent review is provided which details tRNA therapeutics and delivery (Ward et al., 2024).

The simplest and most common approach for alleviating PTCs is to supply individuals with a natural suppressor tRNA containing a repurposed anticodon to readthrough the PTC. This allows for the correct amino acid to be inserted and alleviate translation termination. Recently, a high-throughput, cell-based assay has been developed to screen such anticodon-engineered tRNAs for efficient suppression: ACE-tRNA (Lueck et al., 2019; Ko et al., 2022; Lin and Glatt, 2022). In this way, effective tRNAs must be sufficiently recognized by the protein synthesis machinery. However, the efficiency of these identified anticodon-engineered suppressor tRNAs was modest and may only be applicable to diseases with a low therapeutic threshold (Zhou et al., 2012; Bordeira-Carriço et al., 2014; Lueck et al., 2019; Porter et al., 2021; Coller and Ignatova, 2024). This is because i) a significant amount of tRNA isoacceptors lose activity when their anticodon arms are altered (Lueck et al., 2019), ii) correct positioning within the decoding center is compromised (Westhof et al., 2022) and cannot outcompete the nonsense-mediated mRNA decay pathway (Albers et al., 2023), and iii) anticodon-engineered tRNAs can be prone to incorporating the wrong amino acid (Kaiser et al., 2020).

These results still suggest that anticodon-modified tRNAs have some utility, and combining it with modifications to other regions of the tRNA can improve translational fidelity (Ogawa et al., 2011; Ogawa et al., 2012). Thus, other research has focused on holistically designing therapeutic suppressor tRNAs for improved translational efficiency. Bacterial tRNAs have been repurposed to efficiently and exclusively install Ala at a UGA PTC in *E. coli* (Albers et al., 2021). These tRNAs were designed *in silico* and programmed to have the suppressor anticodon and maintain key tertiary interactions for recognition by AlaRS, while varying the other nucleotides. However, these *de novo* tRNAs only displayed significant *in vivo* suppression when further engineered to contain an optimized T-stem for EF-Tu binding. In this case, the tRNA^{Glu} T-stem was transplanted to accommodate the destabilizing effect of Ala with EF-Tu. Testing of further variants using the D-arm from tRNA^{Pro} for enhanced EF-P affinity displayed modest impact in a variant-specific manner. Furthermore, modifications to lengthen the variable arm decreased suppression. In some studies, a long variable arm is associated with higher suppression, however the sequence of the tRNA core also plays a role in the efficiency of mRNA translation (Geslain and Pan, 2010; Chung and Krahn, 2022; Prabhakar et al., 2022). Structural determination of the most effective variant (t1A3T2) in the ribosome also suggested that engineered suppressor tRNAs still undergo a similar decoding mechanism as canonical sense codons, further confirming on a structural level that research surrounding canonical sense codon translation can be applied to further develop tRNA therapeutics.

Follow-up work used similar engineering principles to convert native human tRNAs into efficient suppressor tRNAs in mammalian cells for treating readthrough of the cystic fibrosis transmembrane conductance regulator gene *CFTR* (Albers et al., 2023). Three families of human tRNAs were engineered which resulted in considerably improved *CFTR* PTC suppression in mammalian cell lines and patient-derived human nasal epithelial cells at different stop codons. Similar suppression improvement was also shown when tRNAs were encapsulated in liquid nanoparticles (LNPs) and delivered to mice. The researchers importantly designed these tRNAs based on known bacterial tRNA engineering principles; the T-stem was modified for improved interactions with eEF1A (Andersen et al., 2000; Schrader et al., 2011) and the anticodon arm was modified to supplement decoding (Yarus et al., 1986; Nguyen et al., 2020). Importantly, suppression efficacy was dependent on both the stop codon sequence and sequence context surrounding the PTCs. Optimizing tRNAs for sequence context (in both therapeutic and synthetic biology applications) is an important variable that must be considered in tRNA design. As such, it has been determined that PTC-upstream translation velocity (using Ribo-seq data) can be modeled to accurately predict suppression fidelity at different PTCs when designing engineered suppressor tRNAs (Bharti et al., 2024).

Similar strategies may also be applied for missense mutations which account for a large portion of pathogenic human diseases, and are seemingly used naturally to compensate for environmental stress in bacteria (Schuntermann et al., 2023). Such missense-correcting tRNAs are charged with an amino acid but programmed to read another amino acid's cognate anticodon. Missense-correcting-tRNAs have been recently explored in mammalian cells and are likely to benefit from further engineering to improve translational fidelity (Bily et al., 2024; Hou et al., 2024).

10 Discussion

tRNAs play a role in every step of translation, often by directly interacting with the ribosome and other translation factors. As such, substantial research has mapped the interactions of tRNA sequence segments and even post-transcriptional modifications with these binding partners. Combined, this results in a robust “map” that can guide tRNA engineering efforts to mediate each of the key steps in translation, or several steps in parallel due to the modularity of different tRNA regions. With significant advances in structural and computational biology, a greater mechanistic understanding of the various roles of tRNA in translation will certainly uncover more rational engineering principles. Artificial intelligence and machine learning technologies in academia (Coller and Ignatova, 2024) and industry (e.g., <https://www.alltrna.com/>) alike are just beginning to trailblaze a new frontier in tRNA engineering research by predicting biomolecular structures and interactions to simulate and guide tRNA engineering efforts. Specifically, the recent release of AlphaFold3 newly allows for the prediction of protein-nucleic acid interactions which will certainly progress tRNA engineering research by offering another tool for illuminating tRNA interactions throughout protein synthesis (Abramson et al., 2024). Additionally, powerful directed evolution approaches adapted to prokaryotic and eukaryotic systems alike have dramatically improved the translational fidelity of tRNAs, bypassing the complexity of rational design. tRNA engineering has thus shined as a robust tool for applications in basic research, synthetic biology, and therapeutics.

Author contributions

JW: Investigation, Visualization, Writing—original draft, Writing—review and editing. JD: Visualization, Writing—original draft. AB: Writing—original draft. NK: Conceptualization, Funding acquisition, Supervision, Writing—review and editing.

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Conflict of interest

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