Fidelity of translation initiation in bacteria: an initiator tRNA-centric view

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Abstract

Translation of messenger RNA (mRNA) in bacteria occurs in the steps of initiation, elongation, termination and ribosome recycling. The initiation step comprises multiple stages and uses a special transfer RNA (tRNA) called initiator tRNA (i-tRNA), which is first aminoacylated and then formylated using methionine and N ^{10}-formyl-tetrahydrofolate (N ^{10}-fTHF), respectively. Both methionine and N ^{10}-fTHF are produced via one-carbon metabolism, linking translation initiation with active cellular metabolism. The fidelity of i-tRNA binding to the ribosomal peptidyl-site (P-site) is attributed to the structural features in its acceptor stem, and the highly conserved three consecutive G-C base pairs (3GC pairs) in the anticodon stem. While the acceptor stem region is important in formylation of the amino acid attached to i-tRNA and its initial binding to the P-site, the 3GC pairs are crucial in transiting i-tRNA through various stages of initiation. We utilized the feature of 3GC pairs to investigate the nuanced layers of scrutiny that ensure fidelity of translation initiation through i-tRNA abundance and its interactions with the components of the translation apparatus. We discuss the importance of i-tRNA in the final stages of ribosome maturation, as also the roles of the Shine-Dalgarno sequence, ribosome heterogeneity, initiation factors, ribosome recycling factor and coevolution of the translation apparatus in orchestrating a delicate balance between the fidelity of initiation and/or its leakiness to generate proteome plasticity in cells to confer growth fitness advantages in response to the dynamic nutritional states.

Fidelity of translation initiation in bacteria: an initiator tRNA-centric view

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(tRNA) called initiator tRNA (i-tRNA), which is first aminoacylated and then formylated using methionine and N^{10}-formyl-tetrahydrofolate (N^{10}-fTHF), respectively. Both methionine and N^{10}-fTHF are produced via one-carbon metabolism, linking translation initiation with active cellular metabolism. The fidelity of i-tRNA binding to the ribosomal peptidyl-site (P-site) is attributed to the structural features in its acceptor stem, and the highly conserved three consecutive G-C base pairs (3GC pairs) in the anticodon stem. While the acceptor stem region is important in formylation of the amino acid attached to i-tRNA and its initial binding to the P-site, the 3GC pairs are crucial in transiting i-tRNA through various stages of initiation. We utilized the feature of 3GC pairs to investigate the nuanced layers of scrutiny that ensure fidelity of translation initiation through i-tRNA abundance and its interactions with the components of the translation apparatus. We discuss the importance of i-tRNA in the final stages of ribosome maturation, as also the roles of the Shine-Dalgarno sequence, ribosome heterogeneity, initiation factors, ribosome recycling factor and coevolution of the translation apparatus in orchestrating a delicate balance between the fidelity of initiation and/or its leakiness to generate proteome plasticity in cells to confer growth fitness advantages in response to the dynamic nutritional states.

1. Introduction

The cellular protein synthesis, a key process in gene expression, is orchestrated by multiple players including ribosomes, mRNAs, tRNAs and the translation factors. Bacterial ribosomes synthesize proteins in four distinct steps of initiation, elongation, termination, and ribosome recycling (**Fig. 1**) using a distinct set of translation factors for each step (Gold 1988; Laursen et al. 2005). The initiation step uses three initiation factors, IF1, IF2 and IF3 and employs, almost exclusively, a special tRNA called initiator tRNA ($tRNA^{fMet}$ or i-tRNA) (Gualerzi et al. 2014; Gualerzi and Pon 1990). In all organisms, i-tRNA is charged with methionine (Met-i-tRNA) by methionyl-tRNA synthetase (Meinnel et al. 1990). In bacteria and eukaryotic organelles (mitochondria and chloroplasts), the Met-i-tRNA is then formylated by formylmethionine transferase (Fmt) to fMet-i-tRNA (also referred to simply as i-tRNA) using N^{10}-formyltetrahydrofolate (N^{10}-fTHF) as the formyl group donor (Kozak 1983; Shetty and Varshney 2021). Thus, i-tRNA mediated initiation with Met (or fMet), irrespective of the nature of the initiation codon, is also conserved in all domains of life (Kozak 1983). The initiation step is meticulously regulated (Gold 1988; Gualerzi and Pon 1990). It not only ensures conservation of cellular energy but also prevents toxicity that may result from accumulation of faulty proteins in the cell. The canonical pathway of initiation, a major mechanism in bacteria (Höfig et al. 2019), begins with the binding of i-tRNA and mRNA on 30S subunit (30S) in the presence of the three initiation factors by forming a 30S pre-initiation complex (30S PIC). The 30S PIC then rearranges to 30S IC to allow accurate recognition of initiation codon in the mRNA by i-tRNA anticodon in the P-site. A 70S complex, competent to transit into the elongation step, is then formed by joining of 50S ribosomal subunit (50S) and release of the initiation factors. The initiation factors play essential roles in initiation. IF1 facilitates the functions of IF2 and IF3, and its 30S binding blocks the A-site. IF2, because of its affinity to the formyl group in fMet-i-tRNA facilitates its localization to the P-site. Also, as IF2 has affinity to 50S (Boileau et al. 1983; Heimark et al. 1976; La Teana, Gualerzi, and Dahlberg 2001), it helps its docking to 30S IC. IF3, on the other hand, increases off-rates of tRNAs that bind to 30S (Antoun et al. 2006a; Haggerty and Lovett 1997; Hartz et al. 1990; Hartz, McPheeters, and Gold 1989; Meinnel et al. 1999; Sussman, Simons, and Simons 1996; Tedin et al. 1999) and also serves as an anti-association factor, which allows proper assembly of 30S IC prior to joining of 50S (Dallas and Noller 2001; Godefroy-Colburn et al. 1975; Karimi et al. 1999; Sacerdot et al. 1996). The rate at which 50S joins 30S IC is crucial for accuracy of initiation. Faster rates of subunit docking may lead to inaccurate initiation because of trapping incorrect tRNA at the P-site (Antoun et al. 2006b, 2006a). The selection of i-tRNA at the P-site is a regulated process, and various ribosomal elements add to the fidelity of i-tRNA binding. For example, 16S rRNA bases A1339 and G1338 monitor the G_{29}-C_{41} and G_{30}-C_{40} base pairs (the first two GC pairs) by type-I and type-II A-minor interactions, respectively (Selmer et al. 2006). Methylations of G966 and C967 to m^2G966 and m^5C967 by RsmD and RsmB, respectively, contribute to the interactions between i-tRNA anticodon and the initiation codon in the mRNA (Arora, Bhamidimarri, Weber, et al. 2013; Selmer et al. 2006; Seshadri et al. 2009). Likewise, methylations of A1518 and A1519 to m^6A1518 and m^6A1519, respectively in 16S rRNA by RsmA enhance the fidelity of i-tRNA binding (Demirci

et al. 2010). The modifications at A1518 and A1519 are also important for 30S biogenesis (Connolly, Rife, and Culver 2008; Demirci et al. 2010). In addition, the C-terminal tails of ribosomal proteins (r-proteins), uS9 and uS13 that extend into the P-site, contribute to i-tRNA selection (Arora, Bhamidimarri, Weber, et al. 2013). The r-protein, uS12, despite being a resident of the A-site contributes to i-tRNA selection and the fidelity of translation initiation (Datta, Pillai, et al. 2021; Datta, Singh, et al. 2021; Hoang, Fredrick, and Noller 2004; Selmer et al. 2006).

Once 70S complex is formed, it is competent to transit into the elongation step to translate the remainder of the open reading frame (ORF) in the mRNA by recruiting aminoacyl-tRNAs (in complex with elongation factor Tu, EFTu) in the ribosomal A-site (A-site) as directed by the ORF. Accommodation of aminoacyl-tRNA in the A-site triggers peptide bond formation between the amino acid it (A-site tRNA) carries and the peptide (or formyl-amino acid) the P-site tRNA harbours, positioning the two tRNAs in the P/E and A/P hybrid states. The hybrid state of the ribosome so formed is brought back to the classical state by the translocation step facilitated by elongation factor G (EFG) to accommodate the next codon in the A-site. The elongation cycles repeat until a termination codon is positioned in the A-site for its recognition by a release factor (RF1 or RF2) to release the peptide chain from the P-site peptidyl-tRNA. The RF1/RF2 are recycled by RF3 (Freistroffer et al. 1997) followed by recycling of the post-termination complex (ribosome, mRNA, tRNA) by the concerted action of ribosome recycling factor (RRF), EFG and IF3 to enable a new cycle of initiation (Borg, Pavlov, and Ehrenberg 2016; Prabhakar et al. 2017). Despite cells having evolved with diverse mechanisms to uphold the accuracy and efficiency of different translation steps, the translating ribosomes often encounter stalls during elongation. The stalled ribosomes are recycled by tmRNA (SsrA) mediated trans-translation (Keiler, Waller, and Sauer 1996), alternate ribosome rescue mechanisms that use ArfA and ArfB (Abo and Chadani 2014) or other less understood processes that release (drop-off) peptidyl-tRNAs from ribosomes. Accumulation of peptidyl-tRNAs in cell is toxic as it sequesters tRNAs and makes them unavailable for the elongation step. The cells avoid accumulation of peptidyl-tRNAs by possessing peptidyl-tRNA hydrolase (Pth), which cleaves the ester bond between the peptide and tRNA (Menninger 1978, 1979; Menninger et al. 1983; Singh et al. 2005, 2008; Singh and Varshney 2004).

The details of the various steps in protein synthesis and the translation apparatus outlined above have been extensively investigated by genetic, biochemical, and structural approaches, to unravel the fundamental mechanisms since the history of molecular biology. In addition, the three-dimensional structural determinations of ribosomes providing snapshots of the translational events in atomic details, in the relatively more recent times, have remarkably advanced our understanding of the ribosome function (Green and Noller 1997; Jobe et al. 2019; Korostelev 2022; Noller 2005; Noller et al. 2017; Noller, Donohue, and Gutell 2022; Ramakrishnan 2002, 2011; Samhita and Varshney 2010; Schmeing and Ramakrishnan 2009; Voorhees and Ramakrishnan 2013). The role of changes in the ribosomal composition (ribosomal heterogeneity) has also been implicated in differential translation of mRNAs (Byrgazov, Vesper, and Moll 2013; Genuth and Barna 2018; Shi et al. 2017; Zhang et al. 2022). However, much remains to be probed to understand the intricacies of the ribosome function, regulation, and the evolution of the translation apparatus to respond to the changes in the cellular physiology/metabolic state.

We have investigated the regulation of translation initiation in bacteria, using molecular genetics approaches employing analysis of*E. coli* suppressors which allow initiation with a mutant i-tRNA defective for its participation in initiation. The findings have allowed us to address the evolutionary significance of the special features of i-tRNA and its interactions with the translation apparatus including the ribosome biogenesis factors. Further, the studies have revealed that execution of faithful translation initiation depends critically on one-carbon metabolism. However, to elaborate on these findings, it is important to introduce the special features of i-tRNA and the design of the *in vivo* assay system used in the suppressor analysis.

2. Special features of i-tRNA

Organisms possess two distinct methionine tRNAs, the i-tRNA and elongator $tRNA^{Met}$. Both the tRNAs possess CAU anticodons. The i-tRNA decodes the initiation/start codons (AUG or a related codon), and elongator $tRNA^{Met}$ decodes the AUG codons found at internal positions in ORF. Like other tRNAs, these

tRNAs possess typical clover-leaf secondary structures. However, i-tRNAs harbour at least three distinct structural features that distinguish them from the elongator tRNAs (**Fig. 2A**). Firstly, there exists a Watson-Crick mismatch between nucleotides 1 and 72 (C_1x A_{72} in *E. coli*) at the top of the acceptor stem (in contrast to the Watson-Crick base pairs found in elongator tRNAs at this position). The 1x72 pair mismatch is crucial for, (i) recognition of i-tRNA by Fmt (along with G_2-C_{71}, C_3-G_{70}, and A_{11}-U_{24} base pairs) for formylation of the amino acid attached to it; (ii) prevention of fMet-i-tRNA (a form of peptidyl-tRNA) from hydrolysis by peptidyl-tRNA hydrolase (Pth), whose role is to recycle tRNAs from peptidyl-tRNAs (Das and Varshney 2006); and (iii) avoidance of its binding to EFTu and the ambiguity of its participation at the step of elongation (Dutka et al. 1993; Guillon et al. 1992; Hansen et al. 1986; Lee et al. 1992; Lee, Seong, and RajBhandary 1991; Ramesh et al. 1997; Schulman and Pelka 1975; Seong, Lee, and RajBhandary 1989; Seong and RajBhandary 1987; Varshney et al. 1991). Secondly, the bacterial i-tRNAs possess a Pu_{11}-Py_{24} base pair (unlike Py_{11}-Pu_{24} base pair in elongator tRNAs). This position has been shown to impact formylation of the amino acid attached to i-tRNA (Lee et al. 1991). Thirdly, all i-tRNAs possess a highly conserved feature of three consecutive GC base pairs (G_{29}-C_{41}, G_{30}-C_{40}, G_{31}-C_{39}, also stated as GC/GC/GC or 3GC pairs) in the anticodon stem (**Fig. 2A, i**), which enable its direct binding to the P-site (Mandal et al. 1996; Marck and Grosjean 2002; Mayer et al. 2001; Varshney, Lee, and RajBhandary 1993). These special features of i-tRNA not only distinguish it from elongator $tRNA^{Met}$ **(Fig. 2A)** but also, along with IFs, allow it to outcompete elongator tRNAs from binding into the P-site. While the 3GC pairs in i-tRNA anticodon stem are highly conserved in all domains of life, the first and third GC pairs (G_{29}-C_{41}, and G_{31}-C_{39}) have been found to be replaced by A-U or G-U pairs in organisms such as mycoplasma, and α-proteobacterial species (Ayyub et al. 2018; Dong et al. 2014; Samhita, Shetty, and Varshney 2012). The middle one (G_{30}-C_{40}) is the most crucial GC pair in the bacterial i-tRNAs. In addition, it was shown that the first and the third GC pairs are dispensable even in *E. coli* (Samhita et al. 2012). In fact, even the second G-C pair could be changed to a G-U pair in some sequence contexts (Samhita et al. 2012; Shetty et al. 2017). Although it should also be said that the mutations in the 3GC pairs do not support a healthy growth of *E. coli* (Shetty et al. 2017).

3. *In vivo* assay system for genetic analysis of initiation

To develop a plasmid based assay system for *in vivo* analyses of the structure function relationship of i-tRNA, chloramphenicol (Cm) acetyltransferase (CAT) gene was mutated to encode CAT_{am1} mRNA possessing UAG as initiation codon (in place of AUG) along with two other mutations at codons 2 and 5 to avoid background initiation (Fig. 2B, i) (Varshney and RajBhandary 1990). A corresponding change was also made in i-tRNA gene (*metY*) to encode an i-tRNA with CUA anticodon (i-$tRNA_{CUA}$) to allow it to pair with the UAG initiation codon in CAT_{am1} mRNA.*E. coli* harbouring CAT_{am1} gene (on a plasmid, $pCAT_{am1}$) gained resistance to Cm (Cm^R) only if it also simultaneously possessed i-tRNA gene (*metY* $_{CUA}$) to encode i-$tRNA_{CUA}$. The $pCAT_{am1}$*metY* $_{CUA}$ (harbouring both the CAT_{am1} and *metY* $_{CUA}$genes) provided an assay system for *in vivo* initiation (**Fig. 2B, i and ii**). Any mutants of i-$tRNA_{CUA}$that continue to confer Cm^R to *E. coli* , are functional as initiators; and those that are Cm sensitive (Cm^S) are non-functional as initiators. In this assay system, initiation occurred predominantly with fGln rather than with fMet (the CAU to CUA change in the anticodon, the mutant i-$tRNA_{CUA}$ becomes highly deficient for recognition by MetRS but it becomes a good substrate for GlnRS (Schulman and Pelka 1985; Seong et al. 1989)). Gln attached to i-tRNA is also an excellent substrate for Fmt, an enzyme that formylates the α-amino group of most amino acids attached to i-tRNA (Mayer et al. 2003). Importantly, the assay system showed that even though Met is evolutionarily conserved as an initiating amino acid, initiation can occur with other amino acids even *in vivo* . Further, the assay system showed that when the mismatch at 1-72 position is converted to a Watson-Crick pair, the mutant i-tRNA becomes a dual function tRNA that participates both at the initiation and elongation steps (Govindan, Ayyub, and Varshney 2018; Varshney et al. 1991). When the special features of i-tRNA found in its acceptor stem and anticodon stem were transplanted into elongator tRNAs, they gained initiator function (Varshney et al. 1993). Not surprisingly, when the i-tRNA 3GC pairs in the anticodon stem were changed with those found in the elongator $tRNA^{Met}$(U_{29}-A_{41}, C_{30}-G_{40}, A_{31}-?$_{39}$ or 3GC mutant i-tRNA or ua/cg/au; mutations shown in small letters), the i-tRNA mutant failed to function

in initiation **(Fig. 2B, iii)** despite its efficient aminoacylation and formylation (Das et al. 2008; Mandal et al. 1996; Shah et al. 2019).

4. Regulation of the fidelity of translation initiation and ribosome biogenesis

The failure of the 3GC mutant i-tRNA in initiation *in vivo* , formed the basis for our genetic screen to isolate suppressor mutations (in the genome) that allow initiation with the 3GC mutant i-tRNA (**Fig. 2B, iv**). A detailed characterisation and molecular analysis revealed the roles of, (i) i-tRNA gene copy number and its abundance; (ii) one-carbon metabolism, Fmt and FolD; and (iii) ribosomal components, ribosome biogenesis factors, translation factors and their coevolution; in the fidelity of translation initiation. In addition, the suppressors have provided newer insights into the process of ribosome biogenesis/maturation. We discuss these findings in the context of translation initiation, and ribosome biogenesis.

(i) Role of i-tRNA in translation initiation and ribosome biogenesis.

(a) Initiator tRNA gene copy numbers and its abundance in the fidelity of initiation: *E. coli* possesses four copies of i-tRNA genes. Of these, three are located at 63.5' as *metZWV* operon ($tRNA^{fMet1}$); and the fourth one, *metY*($tRNA^{fMet2}$) is located at 71.5' (Kenri, Imamoto, and Kano 1994). All four copies of i-tRNAs in *E. coli* B-strains are identical (Mandal and RajBhandary 1992). However, in the K-strains, $tRNA^{fMet2}$ harbours an A at position 46, whereas other i-tRNAs ($tRNA^{fMet1}$) harbour m^7G at this position (m^7G_{46}). The significance of retention of i-tRNAs with m^7G_{46} and A_{46} in*E. coli* K strains is unclear. Nonetheless, either of the loci is sufficient for survival of *E. coli* (Kenri, Imamoto, and Kano 1992; Tsuyoshi et al. 1991). The cellular abundance of i-tRNA is proportional to the copy number of i-tRNA genes (Kapoor, Das, and Varshney 2011). Deletion of *metZWV* reduces *E. coli* growth and confers cold sensitivity phenotype to it. However, deletion of*metY* has almost no effect on the growth of the strain. Samhita and colleagues found that *E. coli* strains with four i-tRNA genes outcompeted a strain with three i-tRNA genes when grown in rich medium. However, the opposite was true when they were grown in nutrient-poor conditions for a prolonged time (Samhita, Nanjundiah, and Varshney 2014). These observations suggested that i-tRNA genes, in bacteria, may be subjected to dynamic copy number changes. The presence of *E. coli* strains with five i-tRNA genes (HS) was reported in the gut (nutrient-rich) and with three i-tRNA genes (IAI39) was identified in urinary tract infections (nutrient-poor). In our genetic analyses, a severe depletion of i-tRNA in *E. coli* either by promoter down mutations (as present in a series of suppressor strains identified in our genetic screen) or by engineering a deletion of the entire*metZWV* locus (Kapoor et al. 2011), revealed a link between i-tRNA abundance and the fidelity of initiation. Depletion of cellular i-tRNA allowed initiation not only with the 3GC mutant i-tRNA and the noncanonical i-tRNAs lacking a full complement of 3GC pairs in the anticodon stem, but also with elongator tRNAs (**Fig. 3, ii**) (Kapoor et al. 2011; Samhita et al. 2013). Thus, a high abundance of canonical i-tRNAs is required not only to overcome the rate-limiting step of translation initiation (Gualerzi and Pon 1990; Laursen et al. 2005) but also to discriminate against binding of "i-tRNA like" or elongator tRNAs in the P-site (Kapoor et al. 2011; Samhita et al. 2013). These observations allowed us to then engineer the growth of *E. coli* exclusively on i-tRNAs lacking either the first GC pair, the third GC pair or both the first and the third GC pairs as found in some mycoplasma and rhizobium species (Samhita et al. 2012). In a recent study where all the components of the translation machinery and entire translatome of the human pathogen *Mycoplasma pneumoniae*(*Mpn*) were analysed, the abundance of i-tRNAs was found to be massive at 12.1% as opposed to ˜3% in *E. coli*(Dong, Nilsson, and Kurland 1996; Weber et al. 2023). Given that the anticodon stem of i-tRNA in *Mpn* harbours AU/GC/GU sequence instead of the canonical GC/GC/GC, the high abundance of i-tRNA could be the organism's way of outcompeting "i-tRNA like" or elongator tRNA binding in the P-site, mitigating loss of initiation fidelity.

These observations raise a question if there are any natural means of regulating fidelity of translation initiation by regulating i-tRNA abundance in bacteria? Several lines of evidence suggest that *E. coli* could regulate the levels of its i-tRNA contents depending on the nutritional status. At least *in vitro* , ppGpp has been shown to downregulate expression of *metZWV* during the stringent response (Takahiro, Shunsuke, and Fumio 1988). Likewise, expression from*metY* may be regulated by cAMP-CAP a global regulator of transcription, as well as ArgR, a specific transcriptional regulator of arginine metabolism (Krin et al. 2003). Moreover, studies

have shown that if *E. coli* is deprived of leucine, the level of aminoacylated i-tRNAs decreases dramatically (Dittmar et al. 2005). Another mechanism of downregulating i-tRNA levels is based on the action of VapC toxin of the VapBC toxin-antitoxin module in *Shigella flexneri* 2a and the VapCLT2 of Salmonella enterica serovar Typhimurium LT2, both of which are site-specific tRNases that target 3GC pairs in the i-tRNA anticodon stem (Winther and Gerdes 2011). Based on our findings, such a depletion of i-tRNA will favour initiation with unconventional i-tRNAs or elongator tRNAs. In yeast, i-tRNA depletion prompts translation of GCN4, a nutritional stress transcription factor (Conesa et al. 2005). Further, the levels of initiator and elongator methionine tRNAs are negatively associated with cell proliferation versus quiescence (Kanduc 1997). Thus, it seems that downregulating i-tRNA levels could be a cellular response to overcome stress by promoting 'leakiness' in the translation apparatus.

(b) Initiator tRNA abundance and ribosome maturation: Another consequence of i-tRNA depletion we observed in *E. coli*was the gain of cold sensitive phenotype in the strains deleted for*metZWV* . We showed that the i-tRNA, more specifically its 3GC pairs, play a crucial role in the terminal stages of ribosome maturation by prompting trimming of the extra sequences at the 3' and 5' ends of the 17S precursor to produce mature 16S rRNA during the pioneering round of initiation **(Fig. 4)** . Based on the genetic interactions, the extra sequences may be trimmed by RNase R, RNase II, and RNase PH (Samhita et al. 2012; Shetty and Varshney 2016; Tsuyoshi et al. 1991). More recently, based on the analysis of lamotrigine toxicity (which targets IF2), we showed that this role of i-tRNA in maturation of 16S rRNA is mediated through IF2 and i-tRNA complex bound to the 30S. Also, lamotrigine mediated inhibition of ribosome biogenesis led to an increased accumulation of ribosome binding factor A (RbfA), a late stage ribosome biogenesis factor, on 30S (Singh et al. 2023).

In other investigations, we noted that overexpression of i-tRNA rescues biogenesis defects resulting from the deficiency of methylations at G966 and C967 in 16S rRNA in the P-site or deletion of the C-terminal residues of uS9 (S126, K127 and R128, or SKR) that impact i-tRNA binding (Arora, Bhamidimarri, Bhattacharyya, et al. 2013; Ayyub et al. 2018). The binding of i-tRNA may affect the 3' end region of 16S rRNA through conserved residues, for example, G1338 and A1339, which interact with the 3GC pairs for the accuracy of i-tRNA selection in initiation. The conformational changes induced in the ribosome during initiation might then signal the final processing of the 17S rRNA to 16S rRNA.

(ii) Role of one-carbon metabolism, Fmt and FolD in the fidelity of translation initiation.

One-carbon metabolism (OCM), a central pathway for synthesis of many amino acids, nucleotides, and folate species comprising one-carbon unit intermediates (formyl, methenyl, methyl, and methylene) impacts the rate and fidelity of translation in ways that include availability of amino acids and modifications of translation factors, tRNAs, rRNAs, mRNAs, and r-proteins. A detailed review of translational regulation by OCM was recently published (Shetty and Varshney 2021). Formylation of i-tRNA increases its affinity towards IF2, and contributes to its selection in the P-site in bacteria, mitochondria and chloroplasts (Majumdar et al. 1976; Sundari et al. 1976). Formylation of i-tRNA is carried out by Fmt, and a formyl group donor, N^{10}-fTHF (**Fig. 3, i**). Interestingly, Fmt can also utilize N^{10}-formyl dihydrofolate (N^{10}-fDHF) as a poor alternate for N^{10}-fTHF (Sah and Varshney 2023). The deletion of*fmt* (gene encoding Fmt) causes modest to severe growth defects in*Pseudomonas aeruginosa* and *E. coli* , respectively (Guillon et al. 1992; Newton, Creuzenet, and Mangroo 1999) and is not essential for survival of these bacteria. However, as we discuss in the following paragraphs, formylation of i-tRNA increases efficiency and accuracy of initiation (Lahry et al. 2020; Shah et al. 2019). Deficiency of Fmt (slow growth phenotype) can be rescued by overproducing i-tRNA (Nilsson et al. 2006; Shah et al. 2019; Shetty et al. 2016) suggesting that in initiation, the role of formylation is primarily at the early stages of i-tRNA binding to ribosome (Shetty and Varshney 2016).

In our genetic analyses, one of the suppressor strains (named B2) that allowed initiation with 3GC mutant i-tRNA revealed a mutation in*fmt* gene (*fmt* $_{\text{am274}}$) wherein a Gln codon (CAG) at position 274 was changed to an amber (UAG) codon, 42 codons prior to the naturally occurring termination codon. Earlier biochemical studies had shown that a deletion of even 20 amino acids from the C-terminal, rendered Fmt inactive (Gite et al. 2000; Gite and RajBhandary 1997; Ramesh, Gite, and RajBhandary 1998). Surprisingly, analysis

of the B2 suppressor showed that it produced full length Fmt, albeit to a level of less than 5% of the wild type strain. Given that the B2 suppressor was derived from *E. coli* KL16 lacking any elongator tRNA derived amber suppressors, it revealed that the 3GC mutant i-tRNA$_{CUA}$ (the only tRNA in cell with an amber reading anticodon) read the amber codon at position 274 to produce full length Fmt. We showed that diminished levels of cellular Fmt failed to quantitatively formylate i-tRNA in real time, and the unformylated i-tRNA (including the 3GC mutant) bound with EFTu to participate at the step of elongation (in spite of it having a CxA mismatch at the top of the acceptor stem). The B2 suppressor has evolved with an intriguing mechanism of autoregulation to ensure balanced availability of both the formylated and unformylated forms of i-tRNA (Shah et al. 2019). Any excess production of Fmt would formylate increased levels of i-tRNA to decrease the availability of unformylated i-tRNA to bind to EFTu (and to suppress amber codon in the *fmt* $_{am274}$ mRNA) lowering Fmt production, which in turn would result in restoring sufficient unformylated i-tRNA to bind to EFTu **(Fig. 5)** . The lack of quantitative formylation in real time in B2 explains how i-tRNA participates at the step of elongation. However, how does it allow initiation with the 3GC mutant i-tRNA, especially because the deficiency of formylation that applies to the cellular i-tRNA (wild type) will also apply to the 3GC mutant i-tRNA? We believe that a critical level of fMet-i-tRNA is required to saturate the available 30S in the cell. Deficiency of the formylated form of the cellular i-tRNA leaves behind a population of ribosomes which can now bind formylated fraction of the 3GC mutant i-tRNA (in the P-site) to allow its participation in initiation (**Fig. 3, iii**).

Formylation of i-tRNA depends on the availability of both the Fmt and N^{10}-fTHF. As already shown decreased Fmt confers dual functionality to i-tRNA and allows initiation with the 3GC mutant i-tRNA. Would decreased levels of cellular N^{10}-fTHF also confer a similar phenotype? N^{10}-fTHF is produced via OCM by the action of a bifunctional enzyme, folate dehydrogenase-cyclohydrolase (FolD), an essential enzyme in *E. coli* . FolD converts 5, 10 CH_2-THF to 5, 10 CH-THF (by its dehydrogenase activity), which is then converted to N^{10}-fTHF (by its cyclohydrolase activity). In our genetic screen, we isolated three different strains having point mutations in their *folD* genes viz., G122D (Das et al. 2008), C58Y (Sah and Varshney 2015), and P140L (Lahry et al. 2020) compromising the FolD activities, lowering the levels of N^{10}-fTHF and the rate of formylation of i-tRNAs (Lahry et al. 2020). The FolD mutants mimicked the phenotype of the strain with Fmt deficiency (B2 suppressor). Interestingly, the strength of the phenotype correlated directly with the deficiency of FolD activity of the alleles (Lahry et al. 2020). Also, inhibitors of FolA (dihydrofolate reductase, which converts DHF to THF) such as trimethoprim (TMP) led to decreased levels of formylation of i-tRNA and in loss of fidelity of initiation allowing initiation with the 3GC mutant i-tRNA. Interestingly, we noticed that mutant *folD* strains were hypersensitive to TMP. Likewise, our earlier investigations with the *folD122* allele (FolD, G122D) showed that the strain suffered from the deficiencies of Met and S-adenosylmethionine (SAM). A deficiency of SAM would result in deficiency of methylations in rRNA. We showed that deletion of methyltransferases that methylate 16S rRNA do lead to compromised fidelity of i-tRNA selection on the ribosome (Das et al. 2008).

The mechanism underlying the dual function of mammalian mitochondrial $tRNA^{Met}$ at both the initiation and elongation steps was thought to be by incomplete formylation of this tRNA population (Takeuchi et al. 1998, 2001). Thus, the fact that i-tRNA functions both at the initiation and elongation steps in the Fmt or FolD deficient strains (Lahry et al. 2020; Shah et al. 2019) inspired us to use i-tRNA mutants deficient in formylation to sustain *E. coli* for its total load of initiation (all 4 i-tRNA genes, *metZWV* and *metY* were deleted) and elongation (both elongator $tRNA^{Met}$genes, *metT* and *metU* were deleted). This study led to identification of i-tRNA mutants that resembled human mitochondrial i-tRNA in their acceptor stem sequences (Govindan et al. 2018).

(iii) Role of ribosomal components, ribosome biogenesis factors, translation factors and their coevolution in the fidelity of initiation and/or ribosome biogenesis.

(a) 16S rRNA (anti Shine-Dalgarno sequence) and the accuracy of i-tRNA selection in P-site: The interaction between the Shine-Dalgarno (SD) sequence often found upstream of the initiation codon in mRNAs, and anti-SD (aSD) sequence in the 3' end of 16S rRNA, facilitates binding and placement of mRNA on 30S, and this

interaction has a significant impact on the efficiency of initiation. An optimal strength of SD-aSD interaction is crucial for efficient and accurate initiation. Though previously believed that a high affinity between SD-aSD promotes translation initiation (Lim, Furuta, and Kobayashi 2012; Ma, Campbell, and Karlin 2002; Schurr, Nadir, and Margalit 1993; Starmer et al. 2006), it was later suggested that a sub-optimal level of SD-aSD affinity, which often occurs in *E. coli* , increases the efficiency of translation (Osterman et al. 2013; Wei, Silke, and Xia 2017). The correlation between the length of the SD sequence and the efficiency of translation follows a bell-shaped curve, with a maximum initiation efficiency in the range of 4-6 base pairs (Chen et al. 1994; Ma et al. 2002; Vimberg et al. 2007). Our suppressor analysis revealed that a C to T mutation at position 1536 towards the 3' end of the 16S rRNA (in*rrsC* operon) resulting in an extended SD-aSD interaction of 8 bp (as opposed to the original 6 bp) with the reporter CAT_{am1} mRNA, impaired scrutiny of i-tRNA, enabling initiation with the 3GC mutant i-tRNA (Shetty et al. 2014). This extended SD-aSD interaction of 8 bp mimics the conditions used in determining the X-ray crystal structure of the bacterial ribosome bound to the fMet-i-tRNA and mRNA (Selmer et al. 2006) wherein a weaker interaction of G1338 and A1339 was observed with the 3GC pairs. The extended SD-aSD interaction triggers a >2 movement between the h26 and h28 of 16S rRNA, resulting in a change in the position of G1338 and A1339 (Korostelev et al. 2007; Selmer et al. 2006; Shetty et al. 2014). Thus, the SD-aSD context and its extent of the base pairing decide not only the rate of initiation but also the fidelity of initiation.

(b) Importance of ribosome maturation in accuracy of initiation: In the section on 'Initiator tRNA abundance and ribosome maturation', we described a novel role of i-tRNA particularly the 3GC pairs during the pioneering round of initiation, in the terminal stages of ribosome maturation. The work showed that the ribosomes when not completely mature (for example because of deletion of *metZWV*) use the 3GC mutant i-tRNA for initiation. Ribosome biogenesis/maturation is an elaborate process requiring RNA/RNA, RNA/protein, and protein/protein interactions to process precursor rRNA molecules, nucleoside modifications, structural rearrangements, and interaction of r-proteins. A number of biogenesis factors are also required to assemble mature ribosomes *in vivo* (Kaczanowska and Rydén-Aulin 2007; Shajani, Sykes, and Williamson 2011). Interestingly, one of the suppressor strains (named A18) allowed us to uncover a new role for RluD in ribosome biogenesis and fidelity of initiation. RluD binds 50S and modifies U1911, U1915, and U1917 in H69 of 23S rRNA to pseudouridines. In A18, RluD suffered an E265K mutation in its C-terminal tail. This mutation causes no detectable change in the biochemical activity of RluD as pseudouridine synthase but allows initiation with the 3GC mutant i-tRNA (Lahry et al. 2022). An earlier study where a catalytically dead RluD was used, inferred an alternate function of RluD in rescue of*E. coli* growth (Gutgsell et al. 2001). The RluD (E265K) results in increased association of RbfA to 30S. The data suggested that the C-terminal tail of RluD facilitates release of RbfA from the 30S**(Fig. 4)** . The findings not only suggest the role of RluD in the fidelity of translation initiation via promoting 30S biogenesis but also resolve a long-held hypothesis of the alternate function of RluD. RbfA bound to 30S may affect the scrutiny of the 3GC pairs in the P-site possibly via G1338 and A1339 (Korostelev et al. 2007; Shetty et al. 2014) to allow initiation even with an i-tRNA mutated in its 3GC sequence. Interestingly, IF3 has also been shown to release RbfA from 30S (Sharma and Woodson 2020).

(c) Initiation factors in accuracy of i-tRNA selection: Initiation is assisted by three essential initiation factors, IF1, IF2, and IF3 which scrutinize the fidelity of translation initiation. The ribosome-bound IF2 recruits i-tRNA to 30S (Milon et al. 2010). IF1 prevents untimely access of tRNAs to the A-site and aids in positioning of IF2 bound i-tRNA in the P-site in P/I (the peptidyl/initiation) state, forming the 30S PIC (Antoun et al. 2006b, 2006a). Although IF1 and IF2 are independent proteins in bacteria, we showed that an insertion of 37 amino acids in IF2 (in mammalian mitochondria) substitutes for the independent requirement of IF1 in *E. coli*(Gaur et al. 2008). In case of IF3, it has been shown that it not only serves as an anti-association factor (preventing association of 30S and 50S in the absence of an mRNA) but also as a proof-reader of the 3GC pairs in i-tRNA on the 30S P-site (Ayyub, Dobriyal, and Varshney 2017; O'Connor et al. 2001). Biochemical analysis suggested that all of its (IF3) known activities (anti-association of the ribosomal subunits, prevention of pseudo-initiation complex formation, and prevention of initiation from leaderless mRNAs and non-canonical initiation codons) can be discharged by the C-terminal globular domain (CTD)

alone (Dallas and Noller 2001; Godefroy-Colburn et al. 1975; Gualerzi and Pon 1990; Hartz et al. 1990; O'Connor et al. 2001; Singh et al. 2005; Tedin et al. 1999), and the globular N-terminal domain (NTD) was deemed to provide stability of binding to IF3 (Petrelli et al. 2001). Our *in vivo*studies on the individual domains led to characterization of a crucial role of the NTD in the fidelity i-tRNA selection (Ayyub et al. 2017). Subsequently, we showed that IF3 NTD interactions with the elbow region of i-tRNA are crucial for the movements of the two domains of IF3 and in the fidelity of P-site binding of i-tRNA (Singh et al. 2022). Other studies revealed that the genetic interactions between uS12 and IF3 also play a role in i-tRNA selection (Datta, Singh, et al. 2021).

(d) Coevolution of the translation apparatus: Initiator tRNAs in mycoplasma and rhizobia species possess variations in the 3GC sequence in their i-tRNA anticodon stems. For example, variations like A_{29}-U_{41}, G_{30}-C_{40}, G_{31}-C_{39} (AU/GC/GC); G_{29}-C_{41}, G_{30}-C_{40}, G_{31}-U_{39} (GC/GC/GU) or A_{29}-U_{41}, G_{30}-C_{40}, G_{31}-U_{39} (AU/GC/GU), are of common occurrence in these organisms **(Fig. 6A)** . Computational analysis showed that unconventional nature of the 3GC pairs in these i-tRNAs is accompanied with changes in other components of the translation apparatus. The conserved C-terminal tail (SKR) of uS9 is represented by TKR sequence in mycoplasma, and uS13 possesses a longer C-terminal tail. A conserved R131 position in IF3, is represented by P, F or Y in mycoplasma harbouring i-tRNA with AU/GC/GU pairs. Initiation with non-AUG codons is also common in mycoplasma. When these features (P131 in IF3, TKR at C-terminal tail of uS9, and longer C-terminal tail of uS13) were tested in *E. coli* (Ayyub et al. 2018), they facilitated initiation with i-$tRNA_{AU/GC/GU}$ and 3GC mutant i-tRNA.

Additional understanding of coevolution of the translation apparatus, particularly the interplay between i-tRNA, IF3 and uS12 came from the homologous system of *E. coli* . We earlier showed that *E. coli* lacking native i-tRNA genes could be sustained on i-$tRNA_{cg/GC/cg}$ **(Fig. 6A)** . However, these strains grew poorly (Shetty et al. 2016). We later discovered that for sustenance on i-$tRNA_{cg/GC/cg}$ as the only source of i-tRNA, *E. coli* required V93A mutation in IF3. When this slow growing *E. coli* strain was repeatedly sub-cultured to acquire suppressor mutations to enable faster growth, it came up with additional mutations of either V32L or H76L in uS12. The V93A mutation in IF3 was the initial requirement to decrease the stringency of i-tRNA selection at the P-site, essential for survival with the only available i-$tRNA_{cg/GC/cg}$, at the cost of compromised overall growth rate. The succeeding mutations in the genome that enhance growth, occurred in a r-protein, uS12 (V32L or H76L). This led to the discovery of cooperation between uS12, IF3 and i-tRNA for faithful initiation and a crosstalk between uS12, RRF, EFG and Pth (recycles tRNAs from peptidyl-tRNAs by hydrolysing the ester bond between the peptide and tRNA) modulating ribosome recycling to fine-tune the overall fidelity of translation initiation (Datta, Pillai, et al. 2021). The aforementioned systematic evolution demonstrated a paradigm for coevolution of the translation apparatus (**Fig. 6B**).

(e) Ribosome recycling factor and the fidelity of initiation: Ribosome recycling, a vital step in protein synthesis, dissociates the post-termination complexes to make the ribosomal subunits available for another round of initiation (Kiel, Kaji, and Kaji 2007). We and others showed that the specific interactions between RRF and EFG, the two key factors involved at this step, are crucial in dissociation of the complexes consisting of mRNA-bound ribosomes harbouring deacylated tRNA (Fujiwara et al. 2004; Ito et al. 2002; Rao and Varshney 2001). Subsequently, it was shown that IF3 also plays an active role in ribosome recycling (Singh et al. 2005). The genetic interactions between RRF and Pth (Das and Varshney 2006) were seen in several independent investigations (Seshadri et al. 2009; Singh et al. 2005, 2008; Singh and Varshney 2004). These interactions strongly suggest that RRF, EFG and IF3 can act together on the pre-termination ribosomal complexes to release peptidyl-tRNAs. On the other hand, the structural investigations inferred that pre-termination ribosomal complexes are not optimal substrates for RRF binding (Schmeing and Ramakrishnan 2009; Weixlbaumer et al. 2008). However, given that RRF levels are high in cell (˜20 μM) (Prabhakar et al. 2017), and multiple GTP molecules are often hydrolysed in carrying out a single round of recycling (Borg et al. 2016), it is feasible that the pre-termination ribosomal complexes, at least the ones carrying short peptidyl-tRNAs (e. g., at the interface of initiation and elongation steps or in early elongation), may be utilized as substrates by RRF (**Fig. 7**).

In this context, would recycling of the incorrectly assembled 70S complexes (carrying formyl-aminoacyl-i-tRNA) or the ones wherein the initial codons in the ORF were incorrectly decoded, by RRF increase the accuracy of initiation? Evidence for this came from our earlier studies where we genetically combined the *folD122* allele (having G122D mutation in FolD) with the *frr* ^{ts} allele (temperature sensitive allele of RRF from *E. coli* LJ14) (Seshadri et al. 2009). As already described, *folD122* allows initiation with the 3GC mutant i-tRNA. Interestingly, when we transduced *E. coli* LJ14 strain (reduced level of RRF) with *folD122* allele, we noted increased initiation with the 3GC mutant i-tRNA, suggesting that deficiency of RRF allowed increased accommodation of the 3GC mutant i-tRNA in the ribosomal P-site (Singh et al. 2008) showing that RRF plays a role in accuracy of i-tRNA selection. Furthermore, we noted that uS12 (through its PNSA loop), shows genetic interaction with RRF and Pth, which are important in the fidelity of translation (Datta, Pillai, et al. 2021).

More importantly, as mentioned, in our coevolution study involving initiation with the i-tRNA$_{cg/GC/cg}$, we noted that in the background of IF3 (V93A), while H76L mutation in uS12 improved the accuracy of i-tRNA selection, the V32L mutation compensated for the reduced fidelity of i-tRNA selection by ensuring a fidelity check by enhanced RRF function (Datta, Singh, et al. 2021). This investigation highlighted the importance of the genetic interactions between i-tRNA, IF3, uS12, EF-G, RRF, and Pth in maintaining the overall fidelity of initiation. It is satisfying that a more recent investigation further supports the role of RRF in peptidyl-tRNA drop-off in early stages of elongation as an additional quality control measure to maintain faithful translation (Nagao et al. 2023).

5. Conclusions and perspectives

The use of Met or fMet for initiation connects protein synthesis with OCM, whereby an energy rich state (higher flux of Met and N^{10}-fTHF) of the cell would favour translation initiation, and an energy depleted state (lower flux of Met and N^{10}-fTHF) would downregulate initiation. Such a regulation averts the cell from undertaking the highly energy expensive process of protein synthesis under energy deficient conditions and avoids accumulation of incomplete proteins and, toxicity to the cell. On an application front, the connection between translation initiation and OCM offers improved ways of inhibiting bacterial growth. The FolD mutant strains are hypersensitive to further perturbation of OCM by TMP (inhibitor of dihydrofolate reductase) (Lahry et al. 2020) suggesting that augmentation of the age-old sulfa drugs (which impact production of DHF by targeting dihydropteroate synthase) with FolD inhibitors could be an important antibacterial strategy.

In bacteria and the eukaryotic organelles, formylation of i-tRNA directs it to the initiation step and prevents its binding to EFTu. The lack of formylation of i-tRNA in real time leads to its binding to EFTu and its participation at the step of elongation (Shah et al. 2019). These observations allow better understanding of how a single $tRNA^{Met}$ (with features of i-tRNA) participates at the steps of initiation and elongation in mammalian mitochondria (Govindan et al. 2018). In eukaryotes (cytosol), the presence of A_1:U_{72}, and the structural features in TψC arm of mammalian i-tRNA, and the 2'-O-phosphoribosyl modification at position 64 in yeast i-tRNA avoid i-tRNA binding to eEF1A (Desgrès et al. 1989; Drabkin, Estrella, and Rajbhandary 1998). Together with the manner in which i-tRNA is delivered to the P-site in eukaryotes (or in archaea), allows them to do away with the requirement of formylation (Benelli and Londei 2011; Jackson, Hellen, and Pestova 2010). In fact, formylation of i-tRNA in eukaryotes is detrimental (Kim et al. 2018; Ramesh, Köhrer, and RajBhandary 2002). Separately, our studies (Shah et al. 2019; Shetty et al. 2016; Shetty and Varshney 2016) and those of others (Nilsson et al. 2006) showed that the lack of formylation in bacteria can be rescued by increased abundance of i-tRNA in cell. Thus, together with the observations in eukaryotes, it may well be that in bacteria, formylation may have no additional functions beyond the initial recruitment of i-tRNA to the ribosomes.

However, the feature of the 3GC pairs in the anticodon stem, highly conserved in all i-tRNAs, not only facilitates i-tRNA binding to ribosome but also helps in stabilizing its interactions in the P-site during the various stages of initiation that convert 30S PIC to 70S complex competent to transit to elongation step. Importantly, the 3GC pairs are also crucial in the release of IF3 from the 70S complex (Shetty et al. 2017) and

in the final maturation of 17S rRNA to 16S rRNA (Shetty and Varshney 2016). However, at least in a reporter system, the strict requirement of the 3GC pairs is functionally compensated for by an extended interaction between the SD and aSD sequences (Shetty et al. 2014). The G1338 and A1339, which establish A-minor interactions with the 3GC pairs (Lancaster and Noller 2005; Selmer et al. 2006) and the methylations of 16S rRNA nucleosides (Arora, Bhamidimarri, Bhattacharyya, et al. 2013; Das et al. 2008; Seshadri et al. 2009) are responsible for the functions of the 3GC pairs. Nonetheless, for a better understanding of the various roles of the 3GC pairs, knowledge of the dynamics of these interactions (or the network of interactions) is essential.

The bacteriophages producing ribonuclease toxins targeting i-tRNA, or the stress/starvation conditions, may deplete i-tRNA levels in bacteria. The deficiency of i-tRNA impacts ribosome maturation. Nutritional deficiency may also limit S-adenosyl-methionine (SAM) levels impacting methylations in rRNA and r-proteins, leading to heterogeneity in the ribosomes, which may influence proteome diversity (for example, by initiation with elongator tRNA) **(Fig. 8)** . However, a direct connection between the levels of i-tRNA or the heterogeneity of methylations of the rRNA nucleosides, and the changes in proteome has not been made possibly because the changes are subtle.

The role of RluD in 30S maturation was serendipitous, and the precise mechanism of how it releases RbfA from 30S remains unknown. RluD may do so by interacting directly with the 30S or that its binding to H69 (50S) may affect RbfA release during its (50S) docking onto the 30S in the pioneering round of initiation. The latter is explicable by the structure of the RluD docked 50S subunit (Vaidyanathan, Deutscher, and Malhotra 2007). In this model, the C-terminal tail of RluD protruding from the 50S would contact the 30S subunit to release RbfA before actual subunit joining. Further, biochemical and structural studies are required to explore the detailed molecular mechanisms of the role of RbfA in lowering the fidelity of initiation, and for the role of RluD (or IF3) in RbfA release from 30S.

Finally, an area of research where our understanding is inadequate, is the role of RRF in the fidelity of initiation. Genetic studies have clearly shown a connection between RRF, uS12, IF3 and Pth (Das and Varshney 2006; Datta, Pillai, et al. 2021; Singh and Varshney 2004). Interestingly, role of RRF in fidelity of initiation provides with a novel mechanism to not only ensure correct assembly of 70S complexes but also in the scrutiny of its transition into the elongation step by acting on the early-stage elongation complexes. Though the events of translation initiation are well characterized, this review documents novel findings that have furthered our understanding of the intricacies of faithful translation initiation and highlights the caveats that could be explored in future for a comprehensive understanding of the same.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figures legends:

Fig. 1: An overview of the different steps in bacterial translation. The i-tRNA and mRNA assemble on 30S ribosome bound with IF1, IF2 and IF3 to form 30S pre-initiation complex (PIC) which then converts into 30S IC. A circle on each tRNA indicates charging by the cognate amino acid and the star indicates its formylation. The i-tRNA transits from a P/I to a P/P state during its accommodation in the 70S resulting in an elongation competent 70S complex. The 70S complex then enters the repetitive cycles of peptide bond formation to extend the peptide chain by one amino acid each time with the help of elongation factors. When the A-site is presented with a stop codon in the mRNA, termination occurs with the help of release factors 1/ 2 and 3, releasing the nascent protein. The mRNA bound ribosome harbouring deacylated tRNA is then recycled by the concerted action of EF-G, RRF and IF3.

Figure 2: (A) Clover-leaf structures of i-tRNA, 3GC mutant i-tRNA, and elongator tRNA$^{\mathbf{Met}}$. (i) fMet-i-tRNA, (ii) fGln-i-tRNA$_{\mathrm{CUA/ua/cg/au}}$, (iii) Met-tRNA$^{\mathrm{Met}}$. The unique structural features of i-tRNA are highlighted. For details, see the text. **(B)***In vivo* **assay for initiation and isolation of suppressor strains that allow initiation with the 3GC mutant i-tRNA.** (i) *E. coli* harbouring pCAT$_{\mathrm{am1}}$ produces CAT$_{\mathrm{am1}}$reporter mRNA but it cannot be translated as the cells lack i-tRNA to pair with the UAG start codon and the cells are Cm$^{\mathrm{S}}$. (ii) *E. coli* harbouring pCAT$_{\mathrm{am1}}$*metY* $_{\mathrm{CUA}}$ make CAT$_{\mathrm{am1}}$ mRNA, and i-tRNA$_{\mathrm{CUA}}$ (from the CAT$_{\mathrm{am1}}$ and *metY* $_{\mathrm{CUA}}$ gene present on the plasmid). The CUA anticodon of i-tRNA$_{\mathrm{CUA}}$ pairs with UAG initiation codon in CAT$_{\mathrm{am1}}$ mRNA, and translation of CAT$_{\mathrm{am1}}$ mRNA results in production of CAT to confer Cm$^{\mathrm{R}}$ to the cells. (iii) *E. coli*harbouring pCAT$_{\mathrm{am1}}$*metY* $_{\mathrm{CUA/ua/cg/au}}$ makes CAT$_{\mathrm{am1}}$ reporter mRNA and the 3GC mutant i-tRNA (i-tRNA$_{\mathrm{CUA/ua/cg/au}}$) from the respective genes but because of the mutations in the 3GC pairs, the i-tRNA$_{\mathrm{CUA/ua/cg/au}}$ fails to initiate from the UAG initiation codon of CAT$_{\mathrm{am1}}$ mRNA and the cells are Cm$^{\mathrm{S}}$. (iv) Same as (iii) except that a suppressor mutation in the host genome (yellow asterisk) facilitates i-tRNA$_{\mathrm{CUA/ua/cg/au}}$ to initiate from the UAG start codon of CAT$_{\mathrm{am1}}$ mRNA and the cells become Cm$^{\mathrm{R}}$.

Figure 3: Fidelity of initiation depends on the availability of a 'critical level' of fMet-i-tRNA . **(i)** In wild type *E. coli* an adequate amount of Met-i-tRNA is available, which is rapidly formylated to fMet-i-tRNA by the sufficiency of N^{10}-fTHF and Fmt. The presence of fMet-i-tRNA above a critical threshold ensures occupancy of P-sites on all the available 30S avoiding binding of non-canonical i-tRNAs disallowing initiation with them. **(ii)** A strain in which expression of canonical i-tRNAs is reduced, or **(iii)** Met-i-tRNA is not formylated in real time due to the decreased levels of Fmt or N^{10}-fTHF, the amount of available fMet-i-tRNA is inadequate (below critical level) to occupy all available 30S P-sites, failing to avoid binding of non-canonical i-tRNAs (or elongator tRNAs) and initiation with them.

Figure 4: Ribosome biogenesis in the fidelity of translation initiation: In wild type *E. coli* , a canonical i-tRNA (with intact 3GC pairs) facilitates the ultimate steps of maturation of 16S rRNA (in 30S) in 70S ribosome by inducing trimming of the extra sequences at the 5' and the 3' ends by appropriate RNases. Conversely, the binding of non-canonical 3GC mutant i-tRNA with wild type anticodon, CAU blocks the final maturation of 16S rRNA leading to immature ribosomes. Similarly, RluD plays a crucial role in efficient release of RbfA from 30S subunit. However, the mutant RluD$_{\mathrm{E265K}}$fails in efficient release of ribosome binding factor A (RbfA) from 30S and allows translation initiation with non-canonical i-tRNAs (3GC mutant i-tRNA).

Figure 5: Role of Fmt in participation of i-tRNA at the steps of initiation and elongation. In wild type bacteria, expression of Fmt facilitates rapid formylation of i-tRNAs and their preferential participation in initiation. However, the reduced level of Fmt leads to slow formylation of i-tRNAs resulting in availability of unformylated i-tRNA population. The unformylated i-tRNA can bind with EF-Tu and participate at the step of elongation. Therefore, enough Fmt levels are important to avoid involvement of i-tRNA in elongation.

Fig. 6: Coevolution of the translation apparatus to optimize translation initiation with i-

tRNA variants. A. Clover leaf structure of the i-tRNA in *E. coli* highlighting the 3GC pair (light olive green). *Mycoplasma* sp. harbour three variants of the 3GC pairs in the anticodon stem namely A_{29}-U_{41}, G_{30}-C_{40}, G_{31}-C_{39} (AU/GC/GC); G_{29}-C_{41}, G_{30}-C_{40}, G_{31}-U_{39} (GC/GC/GU) or A_{29}-U_{41}, G_{30}-C_{40}, G_{31}-U_{39} (AU/GC/GU). Another i-tRNA mutant investigated contained c_{29}-g_{41}, G_{30}-C_{40}, c_{31}-g_{39} (cg/GC/cg) in the anticodon stem. **B.** Cryo-EM structure (PDB ID:5LMQ) of the mRNA (blue) bound 30S PIC (grey) along with IF3 (cyan) highlighting the relative molecular positions of the ribosomal proteins shown to directly (uS13 in red and uS9 in green) or indirectly (uS12 in magenta) play a role in moderating the fidelity of initiation by scrutinizing the 3GC pairs of the i-tRNA (brick-red) bound at the ribosomal P-site. The 16S rRNA is depicted in grey. Structure modified using PyMOL software.

Fig. 7: Fidelity of initiation by recycling of ribosomes at the elongation competent 70S complex stage or early in the elongation step. The canonical pathway of initiation and its transition into the elongation step (grey arrows) follows the stages of 30S IC formation and its transition into the 70S complex and the early stages of elongation cycles. However, in the cases where translation proceeded with the incorrectly assembled 70S complex or errors arising in early stages of elongation, the ribosomes may become a substrate for disassembly by the action of RRF, EFG and IF3 (orange dashed arrows).

Fig. 8: The initiator tRNA-centric view of faithful translation. Schematics showing that under the conditions of sufficiency of i-tRNA, Fmt and N^{10}-fTHF levels, the translation initiation occurs with high fidelity. However, under the deficiency of one or more of these (i-tRNA, Fmt or N^{10}-fTHF) the fidelity of initiation is relaxed to allow initiation with the non-canonical i-tRNAs or elongator tRNAs. The consequences of the high and compromised fidelity of initiation have been indicated.

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