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Review Article

Knowing Right From Wrong: Quality Control of Ribosome Biogenesis in Eubacteria

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Ribosomes are amongst the largest molecular machines in any living cell, which consist of three ribosomal RNAs (rRNAs) over 50 ribosomal proteins (r-proteins). In bacteria they have a small subunit (30S) and a large subunit (50S) which together form a functional 70S ribosome, which translates the information in a messenger RNA (mRNA) into a polypeptide sequence. Translation occurs in multiple steps such as initiation, elongation, termination and recycling. The fidelity of these steps depends on the faithful assembly of the ribosome itself. The synthesis of ribosomes involves various processes such as folding and processing of rRNA, ordered loading of ribosomal proteins and modification of various critical residues in them. For accuracy of protein synthesis, a cell must ensure that the immature particles are kept away from participating in active translation. And, once the ribosomes have been assembled, the cells must have a quality check to ensure the accuracy of assembled particles. Defect in any of these processes will result in the occurrence of defective ribosomes in translating pool, which in turn may lead to production of mis-translated proteins. In humans, genetic disorders of ribosomopathies occur because of deficiencies in ribosome biogenesis and quality control. Thus, it is crucial to understand the hitherto obscure mechanisms of quality control of ribosome biogenesis. This review is an attempt to provide an overview of our understanding of quality control mechanisms in ribosome biogenesis of prokaryotes.

Keywords: Quality Control; Biogenesis; Bacteria

Introduction

Ribosomes are one of the most complicated biological machines whose assembly process still remains elusive. In eubacteria, ribosome biogenesis requires transcription of rRNAs, stoichiometric translation of r-proteins, folding of rRNA, binding of r-proteins on to the rRNA, and modifications of several residues of rRNA as well as r-proteins before the assembled ribosomes are ready for their participation in active translation {reviewed in (Kaczanowska and Ryden-Aulin 2007; Shajani *et al.,* 2011; Sergeeva *et al.,* 2014)}. In any living cells, ribosomes form a major bulk of the cellular content and utilize major portion of cellular energy for its synthesis (Tissieres and Watson 1958). More than 80% of cellular RNA is composed of rRNA and synthesis of r-proteins also occupies the large proportion of translation machinery. Further, ribosome biogenesis requires various GTP dependent assembly factors which help in correct folding of the r-proteins as well as rRNA (Shajani *et al.,* 2011). Minor modulation of ribosomal levels can impact cellular energy status. Therefore, all cells tightly regulate their rate of synthesis of ribosomes depending on the external conditions.

In eubacteria, ribosomes are composed of two subunits: a small subunit (30S) and a large subunit (50S). The functional 30S and 50S subunits catalyse the process of protein synthesis by forming the 70S complex. In general, translation of an mRNA begins with the binding of 30S subunit along with initiation factors (IFs) such as IF1 and IF3, to the mRNA in a region consisting of the start codon AUG, and the sequences upstream to it that include Shine Dalgarno (SD) sequence (AGGAGGU). The process is facilitated by the interaction of the SD sequence in mRNA with the anti-Shine Dalgarno (anti-SD)

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sequence found in the 3' terminal of the 16S rRNA (Steitz 1973; Shine and Dalgarno 1975; Steitz and Jakes 1975). A special transfer RNA (tRNA), initiator tRNA (i-tRNA) which carries formylated methionine (fMet-tRNA^{fMet}), binds to the P-site of the small subunit with the help of IF2. This is followed by joining of large subunit and followed by release of all the three IFs to form an elongation competent 70S complex. The 70S complex, in addition to the P-site that harbors the i-tRNAs, contains an entry site (A-site) for binding aminoacylated elongator tRNA as well as an exit site (E-site) where the deacylated tRNA binds prior to its departure from the ribosome. The elongation factor Tu (EF-Tu) brings the aminoacylated tRNAs into the A-site. Following the accommodation of correct aminoacyl-tRNA into the A-site, peptide bond formation between the aminoacid/peptide in the P-site tRNA and the aminoacid on the A-site tRNA, is catalysed by Peptidyl Transferase Centre (PTC) in the 50S subunit. The peptide bond formation results in a hybrid state of the tRNAs (the A/A and P/P state tRNAs move into the A/P and P/E sites on the 30S/50S subunits), as a result of an anti-clockwise rotation of the 30S subunit. Subsequently, the elongation factor G (EF-G) binding returns the ribosomes into a classical state and the tRNAs into P/P and E/E sites) making the A–site available to accept the new aminoacylated elongator tRNA for elongation. Several rounds of such a cycle continue to synthesize a chain of amino acids called polypeptide which passes through the exit tunnel present in the 50S subunit, till the ribosome reaches the stop codon, which lacks a cognate tRNA to recognize them. The release factors (RF1 and RF2) recognize these stop codons and catalyse the chain termination reaction to release the polypeptide from the P-site tRNA. Subsequently, RRF and EF-G bind to these post-termination complexes and split the subunits so that they can start a new cycle of translation (Ramakrishnan 2002; Schmeing and Ramakrishnan 2009).

The above mentioned simplified scheme suggests that the fidelity of translation of an mRNA into a polypeptide chain absolutely depends on the proper functioning of ribosomes. Thus, ribosomes have multiple functions such as start codon selection at the P-site, monitoring codon:anticodon interaction at the decoding centre, peptide bond formation in the PTC and facilitating proper binding of several translation factors. Further, these distinct functions involve different regions of the ribosomes. Thus, assembly and the quality control of this multi-functional complex is highly crucial and the cell needs to ensure proper maturation of the ribosomes. The organisms have evolved with quality control mechanisms to monitor quality of the assembled ribosomes. Various assembly factors, modification enzymes, as well as rRNA processing enzymes assist in the proper assembly of the ribosomes. Any deficiencies in these processes may produce ribosomes that fail to maintain the fidelity of protein synthesis and cause the cellular toxicity/death (Roy-Chaudhuri *et al.,* 2010). Defects in ribosome biogenesis impart cold sensitivity to *Escherichia coli*, and may affect bacterial virulence and sensitivity to various drugs (Bjorkman *et al.,* 1999; Borg *et al.,* 1999; Champney 2006; Ilina *et al.,* 2013; Phunpruch *et al.,* 2013). In humans, genetic defects resulting in imperfect ribosome biogenesis are the cause of various ribosomopathies (Xue and Barna 2012, Ellis 2014, Trainor and Merrill 2014, Shi and Barna 2015).

Ribosome Biogenesis in Eubacteria

The 30S subunit is composed of 16S rRNA (1542 nucleotides) and 21 r-proteins (S1-S21). At least 11 nucleotides of the 16S rRNA are known to be modified. The 50S subunit is composed of a 23S rRNA of 2904 nucleotides and a 5S rRNA of 120 residues along with around 33 r-proteins (L1-L36). So far around 24 prominent modifications of 23S rRNA residues are known {reviewed in (Decatur and Fournier 2002; Shajani *et al.,* 2011)}. Further, r-proteins also undergo post-translational modifications; such as methylation in case of S11, L3, L11, L7/L12, L16 and L33, acetylation in case of S5, S18 and L7 and methylthiolation in S12 {reviewed in (Nesterchuk *et al.,* 2011)}. In bacteria, all the 3 types of rRNAs (16S, 23S and 5S) are transcribed together in *rrn* operon in the evolutionarily conserved order of 16S-23S-5S rRNA, possibly to maintain their stoichiometric balance (Fig. 1). The transcripts possess upstream and downstream flanking sequences, as well as spacer regions between 16S and 23S rRNA, as well as, 23S and 5S rRNA, which in turn may contain tRNA genes (Ikemura and Nomura 1977; Morgan *et al.,* 1978; Jinks-Robertson *et al.,* 1983; Gourse *et al.,* 1985). *E. coli* has 7 such operons while slow growing bacteria such as *Mycobacterium smegmatis* have 2 *rrn*

operons and *M. tuberculosis* has only 1 *rrn* operon. The *rrn* operon copy numbers have been associated with differential survival strategies in bacterial populations in different ecological niches (Klappenbach *et al.,* 2000). In *E. coli*, high copy number of *rrn* operons is favoured in nutrient rich conditions, while a low copy number in nutrient poor conditions (Gyorfy *et al.,* 2015). Furthermore, the 7 operons of rRNA are not completely identical, they possess micro heterogeneities in 16S and 23S rRNA sequences whose functional significance is still unclear. The expression of individual operons is shown to vary in different conditions and it is speculated that these variants might have a role in specialized translation to favour the expression of stress response genes (Condon *et al.,* 1992, Asai *et al.,* 1999, Yap *et al.,* 1999, Hillebrand *et al.,* 2005, Maeda *et al.,* 2015).

Assembly of the ribosome particles starts with the transcription of rRNA operon where folding of the rRNA and the binding of r-proteins occurs co-transcriptionally (Fig. 1). As the transcription progresses, the 3 species of rRNAs (16S, 23S and 5S) are cleaved and processed by various RNases. The r-proteins are categorized into 3 groups as primary, secondary and tertiary r-proteins based on their order of incorporation. Primary r-proteins directly interact with the rRNA and they are mostly incorporated during transcription. The binding of the primary r-proteins facilitates secondary r-proteins' binding which further bring about structural organization of ribosomes. Finally, tertiary r-proteins bind to form functional ribosomes. During these assembly steps, rRNA also undergoes various processing steps. Importantly, after the transcription, a double strand specific RNase, RNase III cleaves the primary transcript into 16S, 23S and 5S precursor rRNAs called 17S, pre-23S and 9S rRNA respectively, by recognizing the double helical structures formed in the spacer regions. The 17S rRNA retains unprocessed extensions of 110-115 nucleotides at the 5' end and 33 nucleotides at the 3' end, which are also called immature 16S regions and are further processed by somewhat functionally redundant RNases such as RNase II, RNase E, RNase G, RNase R, RNase PH and PNPase to form mature 16S rRNA (Sulthana and Deutscher 2013). The pre-23S rRNA has 3 or 7 nucleotides at the 5'end and 7 or 9 nucleotides at the 3' end and is processed by an unknown RNase and RNase T, respectively. The 9S rRNA contains 84 extra nucleotides at the 5' end and 42 nucleotides at the 3' end, which are further processed by RNase T and a hitherto unidentified RNase to form 5S rRNA.

Classical experiments of Nomura and others

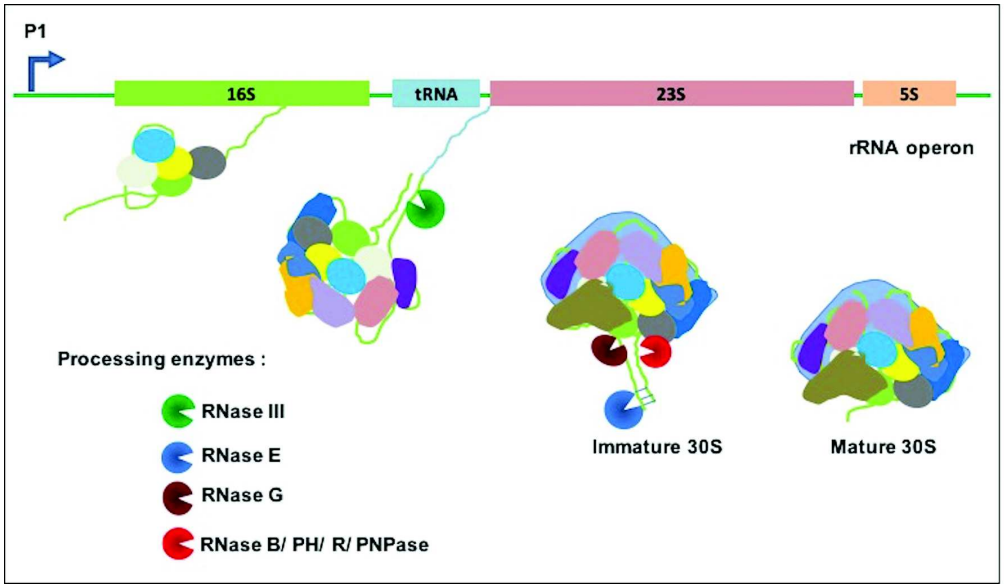

Fig. 1: Ribosome biogenesis in eubacteria. All the three rRNA species are transcribed from a single operon and the assembly of ribosomes starts co-transcriptionally. Secondary structures formed between the 5' end and the 3' end of 16S rRNA promote RNase III mediated cleavage of precursor 16S rRNA, which further undergoes maturation upon completion of the assembly process

have shown that ribosomes can self-assemble *in vitro* into functional particles if mature rRNAs and purified r-proteins are provided. The *in vitro* assembly is constrained by formation of intermediates which need further non-physiological conditions of temperature and salt concentrations to complete the assembly process (Traub and Nomura 1969; Traub and Nomura 1969; Nomura and Erdmann 1970; Held and Nomura 1973; Culver and Noller 1999; Mulder *et al.,* 2010). *In vivo*, various ribosome biogenesis factors might be facilitating transition of these intermediates into the mature ribosomes. *In vivo* analysis of 30S precursor particles using cryo electron microscopy (cryo-EM) suggested existence of early, intermediate and late assembly particles and show certain changes in the order of r-protein assembly. This is mostly because of the presence of various assembly factors in the cell as well as co-transcriptional assembly process, which permit assembly of 5' domain prior to 3' domain. Thus, it is difficult to precisely recapitulate the *in vivo* events of coordination between the transcription, assembly and processing of pre-rRNAs *in vitro* (Kaczanowska and Ryden-Aulin 2007).

Quality Control of Ribosome Biogenesis

In vivo, ribosomes pass through various assembly intermediates and their participation in translation may result in mis-translation. The cell must ensure that the immature ribosomal particles are kept unavailable for the translation process. The presence of assembly factors until the end of complete assembly might be just one way to achieve this. Further, after completion of assembly process, the cell must ensure that the naïve particles are not defective for any step of the translation process. The immature sequences of rRNAs play crucial roles in this quality control process. The processing of immature 16S rRNA is a stepwise process mediated by several RNases, although their physiological implication is still not clear (Fig. 1). *In vivo* 30S assembly occurs on immature 16S rRNA, although the presence of these regions interferes with the *in vitro* assembly process. The complete trimming of these extra nucleotides occurs at the late stage of ribosome assembly and hence, deficiencies in biogenesis factors, or the assembly process due to stress or otherwise, result in accumulation of ribosomes with immature rRNA ends (Culver 2003; Williamson 2003; Clatterbuck Soper *et al.,* 2013). It was shown that translating 70S particles with 30S subunit containing immature 16S rRNA sequences are degraded by the concerted action of YbeY and RNase R. YbeY binds to the 70S complex and recognizes the 3' end of immature 16S rRNA and degrades the defective ribosomes as part of the quality control process (Jacob *et al*., 2013). Thus, trimming of immature 16S sequences becomes highly crucial to avoid unwarranted degradation. The processes that trigger final trimming of these immature sequences for a proper ribosome assembly have remained unclear. Interestingly, our *in vivo* analyses in *E. coli* suggest that the first round of translation initiation and specifically, the i-tRNA might be providing this check point for the final stages of ribosome assembly, as well as quality control.

i-tRNA and its Unique Features

Translation initiation is the rate limiting step of protein synthesis and fidelity of start codon selection is vital for cellular survival. Initiation itself is a complicated process involving multiple steps such as binding of mRNA to the 30S subunit, recruitment of i-tRNA to the P-site to form 30S initiation complex, binding of 50S complex to form 70S initiation complex which undergoes GTP hydrolysis and conformational changes to release IFs and form elongation competent 70S complex (Fig. 2). Direct recruitment of i-tRNAs into the ribosomal P-site mainly depends on the two unique features of i-tRNAs: a C:A mismatch in the acceptor arm which guides formylation of the amino acid attached to the i-tRNA, and the three consecutive GC base pairs (3GC base pairs) in the anticodon stem (RajBhandary 1994). These two features are necessary to discriminate between the i-tRNA and elongator tRNAs, hence preventing mis-initiation from elongator tRNAs (Varshney *et al*., 1993). Formylation of the amino acid attached to i-tRNA enhances its affinity for IF2, favouring its targeting to the P-site (Varshney and Raj Bhandary 1992, Mangroo and Raj Bhandary 1995). The 3GC base pairs are universally conserved in all three domains of life with a few known exceptions in the mycoplasmal and rhizobial species. However, the variants in the mycoplasmas and rhizobial species were shown to be functional in *E. coli* as they could sustain *E. coli* lacking its own genomic i-tRNAs (Samhita *et al.,* 2012). Further, systematic mutagenesis of the 3GC base pairs suggested that among the three GC pairs, the middle GC pair is essential for the sustenance of *E. coli* while

the other two flanking GC pairs contribute for the efficient growth in stress conditions (Shetty *et al.,* 2017). Using *in vivo* polysome profile analyses, we showed that the 3GC base pairs are important for conversion of 30S initiation complex into the elongation competent 70S complex (Fig. 2). Presence of the extended SD sequence in mRNA could partially compensate for the defect in 3GC base pairs and allow i-tRNA transition into the 70S complex (Shetty *et al.,* 2014). Further, the 70S mode of translation initiation could bypass the requirement of 3GC base pairs suggesting a role of the 3GC base pairs in the formation of 70S complex from 30S initiation complex (Shetty *et al.,* 2014). Furthermore, while the increased amount of i-tRNAs can compensate for the lack of formylation (Nilsson *et al.,* 2006), they do not compensate for the lack of 3GC base pairs, indirectly implying that the formylation of i-tRNA is needed for the initial recruitment of i-tRNA to the P-site while the 3GC base pairs for the later stages of initiation (Shetty *et al.,* 2017). Therefore, the two unique features of i-tRNA function in sequential manner and bring about proof-reading steps to ensure initiation with the authentic i-tRNA.

Role of i-tRNA in Ribosome Biogenesis

E. coli encodes 4 copies of i-tRNAs, three in an operon called *metZWV* at 63.5' and one *metY* gene at 72' locus. In one of our previous genetic analysis it had been shown that a strain lacking expression of *metZWV* locus (Δ*metZWV*), which in turn causes 75% reduction in the total i-tRNA levels displays cold sensitive (poor growth at low temperature such as 22 ^{o}C) phenotype. This strain allowed enhanced initiation with the 3GC mutant i-tRNAs as also by the elongator tRNAs (Kapoor *et al.,* 2011, Samhita *et al.,* 2012, Samhita *et al.,* 2013). This led us to hypothesise that the high i-tRNA levels are also important to avoid binding of the elongator tRNAs to the P-site. Thus, upon decrease in i-tRNA levels, elongator tRNAs can enter the P-site and initiate. However, how elongator tRNAs bypass the requirement of the 3GC base pairs

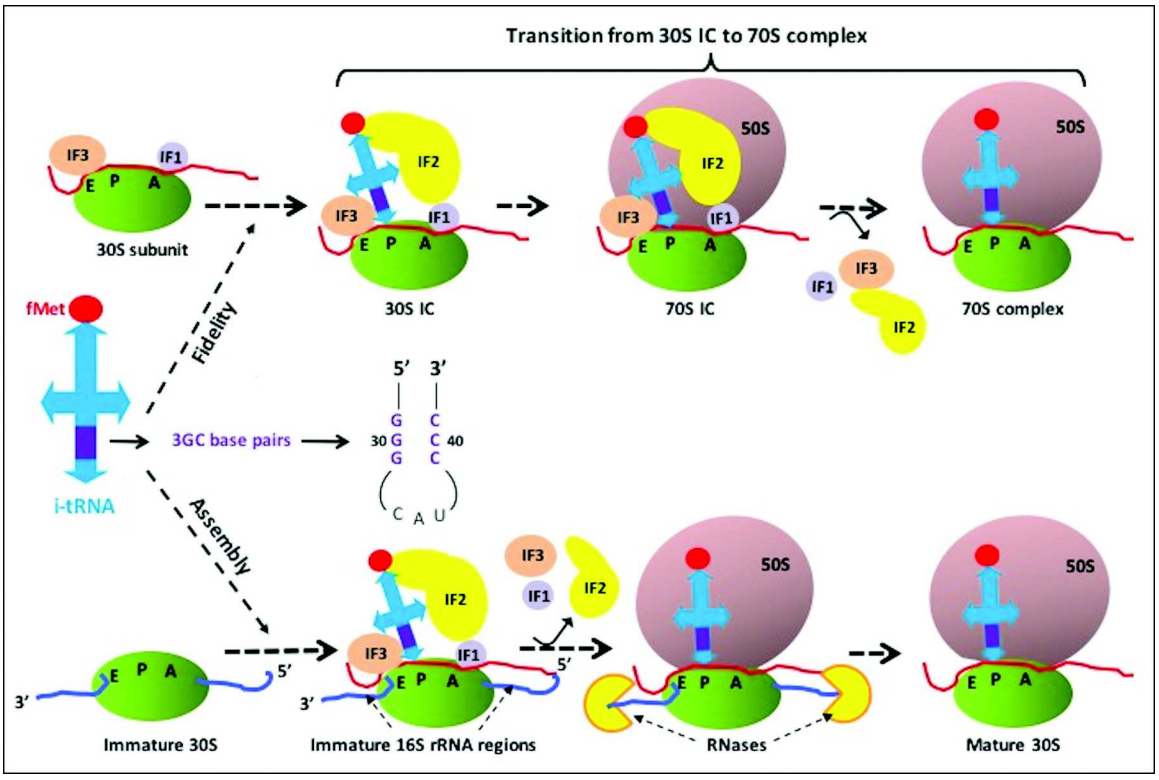

Fig. 2: Summary of role of the 3GC base pairs in the anticodon stem of i-tRNA in translation initiation and ribosome maturation. The 3GC base pairs are mainly involved in the transition of 30S initiation complex to 70S elongation competent complex. Among the 3 GC base pairs in the anticodon stem of i-tRNA, mid GC pair is essential for the functionality. The formation of first round of initiation complex serves as quality trigger for trimming of immature regions of the 16S rRNA by RNases

to form 70S complex is still not clear. Furthermore, what maintains the fidelity of initiation during starvation or other stress conditions where the rate of initiation is expected to go down is also an open question. Interestingly, *E. coli* with reduced i-tRNA levels (encoded by 3 genes as opposed 4) showed competitive advantage when co-cultured in nutrient poor conditions but not in nutrient rich conditions (Samhita *et al.,* 2014) indicating that there may be fitness advantage by reducing the global translation and the cells might cope better with stress, similar to observations of calorie restriction in yeast and other higher systems (Heilbronn and Ravussin 2003, Lin *et al.,* 2004, Mattison *et al.,* 2017). This also suggests the possibility of alternative initiation with elongator tRNAs contributing to the synthesis of novel peptides which might help in stress response and also provide fitness advantage (Shetty *et al.,* 2015).

The cold sensitive phenotype in *E. coli* was observed for many of the mutant strains defective for ribosome biogenesis (Kaczanowska and Ryden-Aulin 2007). However, the mutants grew efficiently at 37ºC. The exact reason behind this behaviour is not clear although it might be related to slower kinetics and trapping of assembly intermediates in wrong conformations due to mis-folding of RNA structures at the lower temperatures. In agreement, most of the ribosome assembly mutants accumulate precursor ribosomes and retain the immature rRNA sequences only at lower temperatures. *E. coli* with lower i-tRNA levels (Δ*metZWV* strain) also accumulates immature 16S rRNA at lower temperature. Further, over expression of i-tRNA mutant lacking 3GC base pairs (3GC mutant i-tRNA) in wild type *E. coli* phenocopies the Δ*metZWV* strain for both cold sensitivity as well as accumulation of immature 16S rRNA (Shetty and Varshney 2016). Over expression of 3GC mutant i-tRNA in Δ*metZWV* strain was highly toxic and accumulated high levels of immature 16S rRNA even at 37 ºC. These observations suggest that the i-tRNA, particularly the 3GC base pairs in the anticodon stem is crucial for the 30S ribosome biogenesis. However, the i-tRNA did not impact the processing of the 23S rRNA in the 50S subunit. Further, different combination of 3GC base pair mutations showed different degrees of accumulation of immature 16S rRNA, suggesting that a full complement of 3GC base pairs is needed for the efficient biogenesis of 30S. The i-tRNA binding is most likely mRNA dependent in the form of initiation complex as mutations in the anticodon of i-tRNAs with or without 3GC base pair mutations were unable to cause biogenesis defect.

Analysis of the 70S ribosome composition suggested no change in the major r-proteins except for small subunit protein S1 (Samhita *et al.,* 2013). The S1 r-protein was slightly low in 70S ribosomes of Δ*metZWV* strain. The S1 r-protein interacts loosely with the ribosomes and is the last protein to be incorporated into the 30S ribosome with the help of the S2 r-protein. This suggested that i-tRNAs might be needed for the late stage of the ribosome biogenesis. In agreement with this, over expression of 3GC mutant i-tRNA was highly toxic in strains defective for S9 r-protein or in the strains defective for the methylations (carried out by RsmB/D methyltransferases) in the 16S rRNA. Interestingly, expression of the 3GC mutant i-tRNA did not enhance the phenotype of the strain lacking RbfA, a well-known early 30S ribosome biogenesis factor in *E. coli* (Culver 2003, Clatterbuck Soper *et al.,* 2013). The C–terminal tail of S9 r-protein protrudes into the P-site of the 30S ribosome and the lack of three amino acids in the S9 tail (S9Δ3) confers cold sensitivity and ribosome biogenesis defect (Selmer *et al.,* 2006, Arora *et al.,* 2013). RsmD and RsmB methylates 966 and 967 positions of 16S rRNA respectively and their deficiencies affect ribosome biogenesis (Burakovsky *et al.,* 2012). Absence of S9 tail as well as lack of methylations at 966 and 967 positions affect i-tRNA selection in ribosomal P-site (Hoang *et al.,* 2004, Burakovsky *et al.,* 2012) and overexpression of wild type i-tRNA could rescue the biogenesis defect in these strains. It is noteworthy that mere overexpression of i-tRNA could rescue biogenesis defect caused by alteration in the r-proteins or rRNA modifications. In eukaryotes, mutations in the r-proteins are known to affect ribosome biogenesis and cause ribosomopathies such as Diamond Blackfan Anemia. It is interesting to probe whether enhanced i-tRNA levels can alleviate some of the biogenesis defects in eukaryotes.

It is still not very clear whether both the 5' and the 3' immature ends of the 16S rRNA undergo maturation together or sequentially. The overexpression of 3GC mutant i-tRNA led to the accumulation of precursor 16S rRNAs which contain

both the 5' and 3' immature sequences. Interestingly, in polysome analysis upon 3GC mutant i-tRNA overexpression at lower temperature, increased 5' end immature signal was retained in the 30S population only while 3' immature signal was increased in both the 30S and 70S ribosome populations. This indicates that 5' end can be processed independent of the 3' end and the 16S rRNA with unprocessed 3' end can form 70S complexes. Further, 3GC mutant i-tRNA was also found in the 70S complexes at lower temperature. Thus, the lack of the 3GC base pairs in i-tRNA might be slowing down the processing of the 3' end of the 16S rRNA in 70S complex. The screen for genetic interactions of 3GC mutant i-tRNA with the various biogenesis factors and RNases involved in the rRNA processing suggested that 3' end maturation of 16S rRNA is the key step affected by the mutant i-tRNA. Interestingly, overexpression of the 16S rRNA 3' end processing enzymes such as RNase R and RNase PH could rescue the cold sensitivity as well as the accumulation of immature 16S rRNA indicating that the maturation of the 16S rRNA is the crucial step regulated by i-tRNA. Overexpression of YbeY which is mainly involved in the quality control step of degrading defective 70S ribosomes could also partially rescue the cold sensitivity as well as the ribosome maturation defect (Jacob *et al.,* 2013).

First round of Initiation as Quality Control Check for Ribosome Maturation

It is not very clear whether the final processing of the 30S ribosomes occurs at the 30S stage or 70S stage. The accumulation of the 3' end unprocessed 16S rRNA in 70S might imply that the processing might be happening at the 70S stage. Mutations in the initiation factors are known to impart cold sensitivity, indirectly suggesting the role of initiation complex formation in ribosome biogenesis. Overexpression of IF2 is toxic and causes severe cold sensitivity which can be rescued by overexpression of the wild type i-tRNA. Increased level of IF2 could bring elongator tRNAs to the P-site and interfere with processing. Lamotrigine, an anticonvulsant drug could bind to IF2 and cause defective assembly of the ribosomal subunits (Stokes *et al.,* 2014). Rescue of lamotrigine mediated cold sensitivity by i-tRNA, and the fact that both lamotrigine and i-tRNA bind to IF2, further supports the role of i-tRNA in ribosome biogenesis at the step of initiation. Overexpression of IF3 and RRF also negatively affected 16S maturation suggesting that 70S complex formation might be needed for the efficient maturation (Singh *et al.,* 2008; Seshadri *et al*., 2009). Similarly, mutant IF3 (*infC135*) as well as mutant RRF (LJ14) which allow initiation to occur with the 3GC mutant i-tRNA also rescue the biogenesis defect. As the factors that promote initiation with the 3GC mutant i-tRNa rescue ribosome maturation, it suggests that functional initiation or the proper conformation of the initiation complex is needed for the 16S processing to occur.

It is possible that the first round of initiation is key to decide the fate of ribosomes. The binding of i-tRNA might prompt release of certain ribosome biogenesis factors from the 30S subunit and/or the formation of 70S complex by joining of the 50S subunit might trigger such event. In accordance with this, the defect in biogenesis of 50S subunit also affects 30S subunit assembly (Hwang and Inouye 2006, Jiang *et al.,* 2006). Treatment with antibiotics affecting different stages of the translation process do accumulate defective ribosomes mainly because of imbalance between rRNA transcription and insufficiency of r-proteins due to translational inhibition (Siibak *et al.,* 2009, Siibak *et al.,* 2011). The analysis of maturation of 16S rRNA upon inhibition of both transcription (with rifampicin) and translation (antibiotics for different stages of translation) suggested that translation elongation per se may not be crucial for the ribosome maturation as chloramphenicol and puromycin did not affect the maturation of the 16S rRNA. Interestingly, tetracycline, which prevents binding of the A-site tRNA, inhibited the maturation of 16S rRNA after rifampicin treatment. It is possible that the maturation of 16S rRNA occurs upon formation of the elongation competent 70S initiation complex and the A-site is needed for the processing enzyme(s) to work. Analysis of binding of various RNases and other biogenesis factors in ribosomal sub-populations might provide greater insight into this process. It was also reported that tetracycline could inhibit i-tRNA binding to the ribosomal P-site (Geigenmuller and Nierhaus 1986). Recently, LepA, a conserved 70S bound GTPase, has been shown to be important for the late stage of 30S ribosome biogenesis, most likely involved in the quality control of the 70S initiation complex (Gibbs *et al.,* 2017). Based on these observations, it can be argued

that the first round of functional initiation is needed for quality check of ribosomes, and final trimming of the 16S rRNA might be happening at the 70S stage. If the i-tRNA binding is defective due to assembly defect, trimming will not occur and the complex might be subjected to degradation by YbeY-RNase R complex.

If initiation is the key step for the quality control of the ribosome biogenesis, how quality control for other functions of the ribosome such as elongation and recycling is monitored? As said in the beginning the quality control process must ensure that the newly assembled ribosome is efficient for its all the distinct functions. It is well accepted that initiation is the most crucial step for proper synthesis of proteins. Further, it is possible that there may be other quality control machineries to take care of ribosomes defective in elongation or recycling, similar to degradation machineries found in eukaryotes. Also, we still do not know whether it is after the formation of functional initiation complex, that 70S undergoes the recycling process. Interestingly, in yeast it was proposed that the ribosomes undergo one round of subunit association and dissociation as quality check (Strunk *et al.,* 2012). Eukaryotes use eIF5B, an ortholog of bacterial IF2, to promote the association of pre-40S and 60S subunits to form an 80S subunit, prior to maturation of 20S rRNA to 18S rRNA, but this can occur in an i-tRNA and mRNA independent manner (Hinnebusch and Lorsch 2012; Strunk *et al.,* 2012).

It is still not clear how exactly, i-tRNA, especially its 3GC base pairs function as ribosome biogenesis factor. The rescue of S9 mutation and methylation defects in rRNA by i-tRNA overexpression might support that i-tRNA serves as a 'template' for correct structuring of the P-site elements by establishing contacts via its 3GC base pairs. Alternatively, the conformational changes that occur during the initiation process might themselves assist in the recruitment of processing enzymes. In the primary transcript, RNase III cleaves the double stranded regions formed by interaction between the 5' and 3' immature regions of the 16S rRNA (Dunn and Studier 1973; Nikolaev *et al.,* 1973). The SD-aSD interaction during the first round of initiation might help in melting the secondary structures between 5' and 3' end regions. Further, the exonuclease activity of RNase R might be prevented beyond the immature regions of the 16S rRNA by SD-aSD duplex formed by mRNA and the 3'-terminal of 16S rRNA in 70S complex. Interestingly, the SD-aSD interaction as well as i-tRNAs are known to affect domain movements around the region of 3' end maturation (Korostelev *et al.,* 2007). As 3GC base pairs interact with the universally conserved residues of the 16S rRNA (G1338 and A1339), which in turn can affect the domain rearrangement at h28 region, it is possible that the cross-talk between these regions govern the fidelity of the final processing (Lancaster and Noller 2005; Selmer *et al.,* 2006). Further work using cryo-EM and single molecule analyses may be needed to investigate the exact mechanism.

Conclusion

Ribosome biogenesis and its subsequent quality assurance still remains a major mystery. It is more evident that the immature sequences at the 5' and 3' end of 16S rRNA have a role in quality control of ribosome biogenesis. The trimming of these immature sequences is triggered by formation of 70S initiation complex. The universally conserved 3GC base pairs in the anticodon stem of i-tRNAs are necessary for efficient trimming of the 3' end immature sequence by RNases. The inability of defective ribosomes to form efficient initiation complex might lead to retention of immature signals and its subsequent degradation by RNases. This quality control step might be very crucial to avoid mis-translation from faulty ribosomes. How exactly these ribosomes are recognized and degraded needs further work. Interestingly, *E. coli* accumulates 30S subunits with immature sequences in stress conditions especially under cold stress. It will be exciting to analyse whether such ribosomal subunits with immature sequences have any physiological role in specialized translation of stress response mRNAs by providing differential initiation context.

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