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GUEST EDITORIAL

Transfer RNA function and evolution

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Transfer RNA (tRNA) has a long-established role in protein synthesis. The tRNA molecule serves as an adaptor [1] between the genetic instructions written in nucleic acid sequences and the protein products encoded in genes. The review and original research articles featured in this special issue of *RNA Biology* focus on recent work that has revealed the central role of tRNA in the evolution and engineering of the genetic code and uncovered novel moonlighting functions of tRNAs in cells representing the diversity of life. These articles also highlight the expanding role for tRNAs in protein synthesis quality control and mistranslation. The issue concludes by examining aminoacyl-tRNA synthesis in disease and drug design.

1. tRNAs for synthetic biology

Natural proteins in organisms representing the diversity of life are normally made with the 20 canonical amino acids during translation, yet posttranslational modifications further diversify the amino acid pool in the proteome. An expanding host of chemical modifications, including phosphorylation, acetylation, and methylation have a profound impact on protein function, cellular signaling, and disease. At the same time, protein engineers dream of producing proteins with ever greater chemical and functional diversity than is accessible in naturally evolved cells.

For these reasons, genetic code expansion has emerged as a foundational method in synthetic biology. Engineering protein synthesis to accommodate additional amino acids beyond the 20 canonical building blocks has led to novel approaches to program or hardwire protein posttranslational modifications [2] and site-direct protein labelling [3] with a library of unnatural amino acids. These methods have been established in both cell-free systems [4] and in living cells from *Escherichia coli* to animal models and human cells [2,3].

A major goal in the field is to push protein synthesis to accommodate 22, 23 or eventually more genetically encoded amino acids in the same cell. Recent advances, reviewed in Tharp et al [3]., show that it is now possible to efficiently reassign 2 stop codons in *E. coli* to produce proteins labeled with two unnatural amino acids. Although the applications for production of proteins with 22 different amino acids are limitless [5], the authors highlight the ability to incorporate a Förster Resonance Energy transfer pair of fluorophores in a single protein. This technology will enable precise measurements of protein dynamics in the context of living cells.

In addition to reviewing novel approaches to expand the genetic code in cells, including orthogonal tRNA and ribosome engineering, Katoh et al. highlight the unparalleled freedom to manipulate the genetic code and protein synthesis in cell-free or in vitro translation systems [4]. In the context of a cell-free translation system, the population, abundance and nature of the aminoacyl-tRNA pool can be precisely modified. The approach relies on a minimal system of 32 pre-aminoacylated tRNAs that are programed to encode different combinations of natural and unnatural amino acids to achieve protein synthesis with 23 distinct amino acids in vitro. The methods were used to produce model proteins containing 3 unnatural amino acids or novel enzyme inhibitors that are macrocyclic peptides with 9 natural and 5 unnatural amino acids.

Although synthetic biologists have become adept at engineering the genetic code in living organisms, the code expanded as a result of natural evolution long ago. The first evidence of code expansion beyond the canonical 20 amino acids was found in the discovery of the 21st genetically encoded amino acid, selenocysteine [6]. The selenocysteine translation machinery is distinct in several fundamental aspects compared to normal protein synthesis. As Fu et al. point out, selenocysteine has no cognate aminoacyl-tRNA synthetase (AARS) and the formation of selenocysteinyl-tRNA^{Sec} requires tRNA-dependent formation of selenocysteine on the tRNA from a seryl-tRNA^{Sec} precursor. Because selenocysteine is a highly reactive amino acid that is chemically similar to cysteine, selenocysteine containing enzymes can be up to 100-fold more catalytically active than cysteine-containing counterparts. For this reason, selenocysteine is desirable for engineering redox enzymes or novel protein functions. Fu et al. reviewed the underlying molecular biology of translation with selenocysteine and they address challenges associated with engineering designer selenoproteins [7]. In a related study, Vargas-Rodriguez et al. uncovered an unusual or non-canonical

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$tRNA^{Cys}$ that co-opts the selenocysteine machinery to incorporate cysteine instead [8]. The authors characterized a novel $tRNA^{Sec}$-like $tRNA^{Cys}$ from *Desulfotomaculum nigrificans* that recodes UGA codons with cysteine instead of selenocysteine and dubbed this new molecule $tRNA^{ReC}$ to indicate its ability to recode with cysteine. This is just one example of how critical the tRNA is to defining codon meaning. Relatively few nucleotide changes in a tRNA can lead to unanticipated codon recoding or codon reassignment events in naturally evolved and engineered organisms.

2. tRNA evolution and modification in microorganisms

In addition to their vital role in protein synthesis and synthetic biology applications, tRNAs are also involved in cellular processes beyond translation. These processes include lipid aminoacylation [9] and bacterial conjugation [10]. The contributions to the special issue in this area also focused on the role of tRNA modifications in wobble decoding [11] and bacterial cell death [12].

Bacteria have many mechanisms to resist antibiotic action and antibiotic resistance is an increasing challenge in the clinic. The tRNA-dependent aminoacylation of bacterial outer-membrane lipids confers increased virulence and resistance to cationic antimicrobial peptides. Fields et al. review the known pathways to lipid aminoacylation, highlighting how enzymes from a large family of aminoacyl-phosphatidylglycerol synthases utilize aminoacyl-tRNA substrates as the amino acid donor for lipid modifications to enhance antibiotic resistance [9].

Emerging evidence indicates a new role for tRNA genes in bacterial conjugation. Alamos et al. found that the acidophilic bacterium *Acidithiobacillus ferrooxidans* encodes 36 of its 95 tRNAs in an integrative-conjugative genetic element [13]. Indeed, Castillo et al. show that the integrases encoded in the conjugative element recognize the anticodon stem loop region in the tRNA gene for active and site-specific recombination [10].

Mature tRNAs are rich in post-transcriptional nucleotide base modifications. The modifications have important roles in translation fidelity and reading frame maintenance [12], tRNA stability and transport [14]. Modifications also occur in the anticodon. Rafels-Ybern et al. conducted a phylogenetic study to reveal the evolution of base modification of adenine to inosine (I) at position 34 (ref. 11). The A to I modification alters the ability of the tRNA to decode the 3rd nucleotide position of the codon. While A34 forms an ideal interaction with U, I34 recognizes U, C or A equally well. The function of I34 is to extend wobble decoding, which was reviewed in Agris et al. [15]. The I34 modification enables one tRNA to read three codons for the same amino acid. Although the modification is used commonly in eukaryotes, previous examples in bacteria were limited. The authors identified several potential I34 modification sites in tRNAs from the genomes of Firmicutes and Cyanobacteria [11].

Modifications can also require transport of the tRNA between cellular compartments. In a fascinating review, Kessler et al. describe how the transport of tRNAs between different cellular compartments impacts tRNA maturation and modification [16]. In seminal studies on the causative agent of sleeping sickness, *Trypanosoma brucei*, the same authors show how $tRNA^{Tyr}$ is transported to the cytoplasm where the intron in the immature tRNA is spliced. In a process called retrograde transport, the tRNA is then re-imported into the nuclease where the spliced tRNA is required for modification with queuosine [14].

Some tRNA modifications are conserved in all forms of life. The threonylcarbamoyladenosine (t6A) modification at position 37 is found in most tRNAs that decode ANN codons and it is important for stabilizing the codon anticodon duplex. The t6A modification at position 37, which is adjacent to the anticodon loop, was found to be an essential determinant for the activity of the anticodon nuclease PrrC from *Streptococcus mutans* [12]. The nuclease promotes bacterial cell death in conditions of stress or upon phage infection when it specifically cleaves the $tRNA^{Lys}_{UUU}$ anticodon loop.

3. Mistranslation and protein synthesis quality control

Correct aminoacylation of the tRNA pool is a pre-requisite for accurate production of the proteome in all life. Surprisingly, a perfect proteome is not a pre-requisite for cellular viability even in the context of human cells. Lant et al. demonstrated that a single tRNA mutant can lead to significant mistranslation in human cells [17]. This was accomplished by expressing an Ala accepting $tRNA^{Pro}$ G3:U70 variant in HEK 293 cells. The authors visualized a rate of ~ 3% mistranslation using a novel green fluorescent protein (D129P) reporter that fluoresces in response to mistranslation at proline codons. In contrast to previous studies in yeast [18], human cells in culture did not mount a detectable heat-shock response and tolerated the mistranslation without apparent impact on cell viability.

Although a perfect proteome is not required for cellular viability, the cell contains several mechanisms to monitor and respond to errors in protein synthesis. Accuracy in translation requires that the AARS discriminate one amino acid from the 20 or 21 proteinogenic amino acids, but the AARSs must also discriminate against the hundreds of non-genetically encoded amino acids present as metabolites in the cell. While the active site of some AARSs is sufficient to discriminate one amino acid from many others, there are several AARSs that require either an in cis or in trans editing activity to hydrolyze misacylated-tRNA species and prevent mistranslation. Bacusmo et al. characterized a novel activity of the trans-editing protein ProXp-x from *Rhodopseudomonas palustris*. Although the domain was known to edit mischarged Ala-$tRNA^{Pro}$, the authors found that the trans-editing factor prevents mistranslation with α-aminobutyrate (Abu) [19]. Abu occurs naturally in the cell as a metabolic intermediate to isoleucine, threonine, and aspartic acid biosynthesis. The *R. palustris* ProRS was competent in aminoacylation with Abu, and ProXp-x readily deacylated Abu-$tRNA^{Pro}$. The data reveal the evolution of mechanisms to defend the cell against mistranslation with non-proteinogenic amino acids that are produced as metabolic intermediates.

Cellular stress impacts the cell's ability to tolerate mistranslation. In a timely review, Sørensen et al. reveal the impact of cellular stress on the synthesis, stability, and degradation of tRNAs [20]. Given that cellular mechanisms exist to respond

to misacylation and mistranslation, the question remains as to how the cell senses and responds to changes in the aminoacyl-tRNA pool. Mohler et al. applied systems level phenotypic microarray experiments in yeast to identify changes in cells with defective protein synthesis quality control [21]. These yeast cells contain an editing defective phenylalanyl-tRNA synthetase that mis-incorporates tyrosine at phenylalanine codons leading to a phenotypic defect under conditions of amino acid stress. In previous seminal studies (reviewed in [22]), this group found that tRNA aminoacylation status is monitored by both the general amino acid control (GAAC) and target of rapamycin (TOR) stress response pathways. The results support that mechanism that GAAC and TOR pathways interact via the deacylated tRNA monitoring protein, Gcn2p. Gcn2p is protein kinase that is activated upon binding de-acylated tRNA, which then leads to downstream activation of the GAAC and subsequent biosynthesis of amino acids. The TOR pathway monitors amino acid viability and via phosphorylation of Gcn2p the TOR pathway turns off the GAAC in conditions of amino acid abundance and the phosphatase Sit4p dephosphorylates Gcn2p and activates the GAAC when amino acids are limiting. Moher et al. found that in cells with defective protein synthesis quality control, the normal cellular mechanisms to sense amino acid limitation are unable to regulate the GAAC properly and thus cannot appropriately respond to nutrient deprivation.

As noted above the expression levels of tRNAs and their aminoacylation states are known to impact translation fidelity, but unique features of tRNAs are also important for maintaining the proper codon reading frame during protein synthesis. Using detailed biophysical measurements [23], Roy et al. compared the stability of the complex formation between 30S ribosome subunit, mRNA and the initiator or elongator $tRNA^{Met}$. Using mutagenesis, the authors uncovered three G-C basepairs in the acceptor stem of the initiator $tRNA^{Met}$ were required for proper positioning of the mRNA in the correct reading frame.

Like all RNAs, tRNAs are subject to damage and degradation as a result of cellular nuclease activities. What is the fate of damaged tRNAs and are there mechanisms to repair tRNA? This underexplored area of tRNA biology was highlighted in work from Desai et al. that identified a novel extended tRNA repair activity catalyzed by the reverse polymerase Thg1 [24]. Thg1 is a tRNA editing enzyme that functions to add a critical identity element for histidyl-tRNA synthetase in the form of a 3′-addition of a $G^{-}[1]$ residue. The authors demonstrated that Thg1 homolog from archaeon *Ignicoccus hospitalis* catalyzed a template dependent repair via reverse (3′ to 5′) nucleotide polymerization of a tRNA molecule lacking 15 residues from its 3′ terminus. This is the longest template-dependent reverse polymerization reported in the literature, revealing the potential for cells to repair damaged or truncated tRNAs.

4. Aminoacyl-tRNA synthesis in human diseases

Given the central role of protein synthesis in all cells, defects in aminoacyl-tRNA synthesis that can cause translation defects are strongly implicated in human diseases [25]. Ognjenovic et al. reviewed the role of AARSs in diseases of the nervous system [26]. Due to defects in protein synthesis and non-canonical activities of AARSs, mutations in half of the cytosolic and most of the mitochondrial AARSs are implicated in neurodegeneration or other disorders of the nervous system. The authors highlight a number of examples illustrating the causative link between AARSs mutants and diseases, including Leukoencephalopathies, early-onset brain disorders, and sensorineural disorders [26].

Although protein synthesis has well known roles in cancer, Kwon et al. investigated the impact of tRNA expression levels on cellular signaling pathways that drive oncogenesis [27]. In human cells under amino acid starvation, overexpression of $tRNA^{Leu}$ was found to lead to increase proliferation as a result of activation of the ribosomal S6 kinase and mitogen-and stress-activated protein kinase signaling pathways, which already have well-established roles in tumorigenesis [27]. In cell lines over-expressing $tRNA^{Leu}$, the authors used mass spectrometry to identify Her2-binding protein 1 (EBP1) as a binding partner of the un-aminoacylated $tRNA^{Leu}$. EBP1 normally binds the EGF receptor Her2 (also known as ErbB2) and disrupts its activating interaction with ErbB3, which results in suppression of cell proliferation. The authors suggest that $tRNA^{Leu}$ competes with Her2 for binding to EBP1, which leads to Her2 activation and stimulation of a proliferative transcription program. In agreement with this idea, the authors identified a positive correlation between $tRNA^{Leu}$ and Her2 expression in tissue samples from breast cancer patients.

Angiognesis, the ability to form and regulate the growth of blood vessels, is a vital process in development and has relevance to cancer and other human diseases. The human tryptophanyl-tRNA synthetase (TrpRS) is subject to proteolytic cleavage that removes the N-terminal 93 amino acids, resulting in the T2-TrpRS variant, which has anti-angiogenic activity due to its ability to interact with VE-cadherin. T2-TrpRS binds to VE-cadherin in endothelial cell-cell junctions, inhibiting the growth of new blood vessels [28]. TrpRS is known to bind zinc, which has anti-angiogenic properties as well. The authors provided biochemical and structural data indicating that zinc binding leads to a TrpRS confirmation that is more susceptible to proteolytic cleavage and production of the T2-TrpRS variant. The data suggest a novel link between T2-TrpRS and zinc in inhibiting angiogenesis.

Since tRNAs and aminoacyl-tRNA synthesis have both established and emerging roles in major human diseases, the final contributions to this special issue highlight approaches for drug discovery that target the proofreading and aminoacylation activities of tRNA synthetases. Grube et al. introduced a novel and high-throughput approach to monitor editing activity of AARSs [29]. Ho et al reviewed literature to provide an overview of how to use the unique co-evolution of AARSs and tRNAs to identify druggable AARSs for novel antibiotic development [30]. These contributions point the way forward as the tRNA field moves from identifying the role of protein synthesis defects in disease to translating these discoveries to breakthrough therapies in the clinic.

5. Advancing the frontiers in tRNA biology

At the conclusion of this special issue, we are awestruck by the expanding universe of tRNA biology and the ability of tRNA biologists, and our contributors in particular, to use tRNA as a window to reveal new biological complexity, and to engineer

and target tRNAs for applications in synthetic biology and medicine. The foundational discoveries on the role of tRNA in protein synthesis were only a beginning. From our perspective, studies on tRNA evolved a diverse and expanding research field and we only wonder where it will take us next.

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