



Repurposing ribosomes for synthetic biology

Yi Liu^{1,2,3}, Do Soon Kim^{2,3} and Michael C Jewett^{1,2,3}

The translation system is the cell's factory for protein biosynthesis, stitching together hundreds to thousands of amino acids into proteins, which are required for the structure, function, and regulation of living systems. The extraordinary synthetic capability of this system, which includes the ribosome and its associated factors required for polymerization, has driven extensive efforts to harness it for societal use in areas as diverse as energy, materials, and medicine. A powerful example is recombinant protein production, which has impacted the lives of patients through the synthesis of biopharmaceuticals such as insulin. In nature, however, only limited sets of monomers are utilized, thereby resulting in limited sets of biopolymers (i.e., proteins). Expanding nature's repertoire of ribosomal monomers could yield new classes of enzymes, therapeutics, materials, and chemicals with diverse, genetically encoded chemistry. Here, we discuss recent progress towards engineering ribosomes both *in vivo* and *in vitro*. These fundamental and technical breakthroughs open doors for advanced applications in biotechnology and synthetic biology.

Addresses

¹ Interdisciplinary Biological Sciences Graduate Program, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA

² Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA

³ Center for Synthetic Biology, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA

Corresponding author: Jewett, Michael C (m-jewett@northwestern.edu)

Current Opinion in Chemical Biology 2017, 40:87–94

This review comes from a themed issue on **Synthetic biology**

Edited by **Tom Ellis** and **Michael Jewett**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 1st September 2017

<http://dx.doi.org/10.1016/j.cbpa.2017.07.012>

1367-5931/© 2017 Elsevier Ltd. All rights reserved.

Introduction

The translation apparatus is the cell's factory for protein biosynthesis, stitching together L- α -amino acid substrates into sequence-defined polymers (i.e., proteins) according to a defined genetic template. With protein synthesis rates of up to 20 amino acids per second at an accuracy of 99.99% [1,2], the extraordinary catalytic capacity of the translation apparatus has attracted extensive efforts to repurpose it for novel functions [3–6]. Previous

pioneering works have shown site-specific incorporation of more than 150 non-canonical amino acids (ncAAs) into proteins using an engineered translation apparatus to generate biological insights and new applications [3–5]. As a result of these impressive efforts, expanding the genetic code has emerged as a major opportunity in synthetic and chemical biology [7–10].

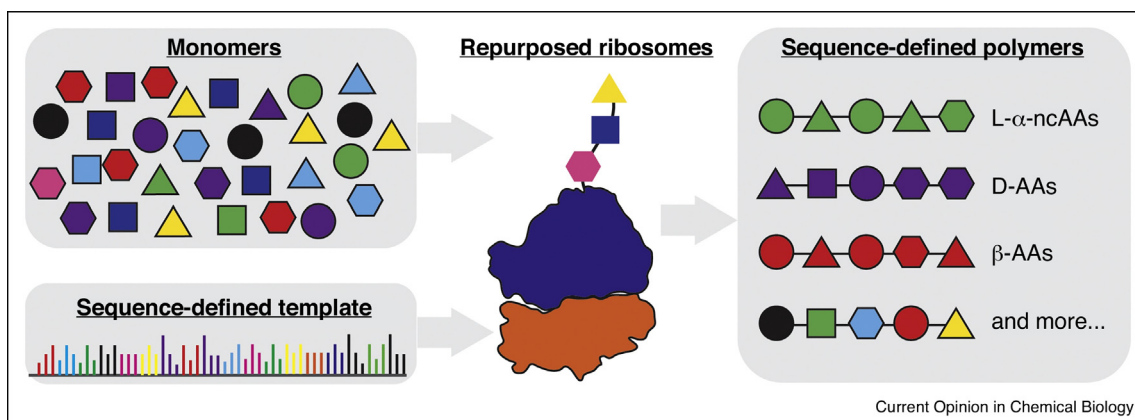
Unfortunately, expanding the range of genetically encoded chemistry into proteins presents a complex and formidable challenge for several reasons [3]. First, the components of engineered orthogonal translation systems that specifically utilize ncAAs suffer poor enzymatic efficiencies relative to native translational machinery [3]. Second, the focus of most engineering efforts remains on evolving orthogonal translation system components by targeting only a small number of individual parts, rather than coordinately tuning and optimizing all biological components involved in the complex system of protein biosynthesis [11**]. Third, certain biological constraints — especially that of the ribosome, the protein polymerase core of the translation apparatus — limit the scope of ncAA diversity. Because ribosome function is necessary for life, cell viability restricts the changes that can be made to ribosomes for expanding the range of substrates beyond those found in nature.

This review focuses on recent developments in repurposing the translation system for novel functions, with a focus on engineering the bacterial *Escherichia coli* ribosome (Figure 1). We first describe the state-of-the-art in the ribosome's ability to process ncAAs. Next, we highlight recent progress towards engineering ribosomes both *in vivo* and *in vitro*. We end with a discussion of current challenges in the field and provide commentary on future opportunities.

Repurposing ribosomes

The *E. coli* 70S ribosome is a ~2.4 MDa macromolecular machine made up of two subunits, the 50S large subunit, comprised of 33 ribosomal proteins (r-proteins), 23S ribosomal RNA (rRNA), and 5S rRNA, and the 30S small subunit, comprised of 21 r-proteins and 16S rRNA [12]. The 16S rRNA of the 30S subunit accommodates ribosomal monomers, aminoacyl-transfer RNAs (aminoacyl-tRNAs), and decodes mRNA. The 23S rRNA of the 50S subunit primarily makes up the peptidyl transferase center, which catalyzes the polymerization of amino acids into proteins via peptide bonds, and the nascent peptide exit tunnel, through which newly synthesized proteins leave the ribosome. Previous works have shown the possibility of using natural ribosomes to site-specifically

Figure 1



Engineering the translation apparatus to manufacture sequence-defined polymers. Expanding the repertoire of ribosome substrates and functions has the potential to not only create novel biopolymers, but also open new areas of research in materials science and synthetic biology.

incorporate ncAAs into polypeptides, including α,α -disubstituted amino acids, D-amino acids, β -amino acids, N-alkyl amino acids, and N-methyl amino acids [13–16,17*,18–20,21*,22*]. Since sequence defines structure and structure defines function, expanding the repertoire of ribosome substrates and functions has the potential to create polymers with even greater functional breadth. For example, the ribosome has been shown to be capable of producing polymers with non-peptide backbones such as polyesters. Over forty years ago, Fahrenstock and colleagues first demonstrated that the ribosome polymerizes phenyllactic acid in a template-directed manner [23]. More recently, Suga and colleagues synthesized polyesters up to ~ 10 units long containing up to three different side chains by genetic code reprogramming [24*,25].

Despite these advances, engineering the translation machinery can be hampered by low efficiencies of the ribosome to accept non-proteinogenic building blocks. The structural, physiochemical, and dynamic properties of the ribosome have been evolutionarily optimized to translate native proteins from the 20 canonical amino acids. To address this concern, the ribosome can be manipulated through directed evolution to accommodate non-canonical monomers, although there are relatively few examples. Hecht and colleagues evolved the peptidyl transferase center to enable exotic monomer incorporation, such as D-amino acids [26,27*] and β -amino acids [18,19]. More recently, Czekster and colleagues generated additional ribosome mutations to show, for the first time, the ability to incorporate these exotic monomers in a living cell [28*]. Even with site-directed evolution of the ribosome, efficiencies can be low. How might the efficiencies and utilities of engineered ribosomes be improved? There are some recent indications that modifying non-ribosomal factors involved in translation can

also lead to improved non-canonical monomer incorporation. For example, Suga and colleagues recently showed that modifying the concentrations of translation factors (e.g., Initiation Factor 2, Elongation Factor Tu, and Elongation Factor G) and using tightly binding elongator tRNAs can enhance multi-site incorporation of D-amino acids for the synthesis of novel macrocyclic peptides [21*]. Similarly, Huang *et al.* demonstrated that multiple D-amino acid incorporation could be enabled by using Elongation Factor P to resolve peptidyl transferase stalling (biorxiv.org/content/early/2017/04/10/125930).

Although the ribosome can feasibly accept non-canonical building blocks and be tolerant of modifications that enable increased promiscuity, constraints imposed by living cells have hindered efforts to engineer ribosomes effectively. In particular, dominant growth defects caused by mutations in the ribosome can preclude identification of mutants that confer desired functions [29,30]. This has motivated the need to develop new technology platforms for engineering ribosomes both *in vivo* and *in vitro*, which we describe next.

Engineering ribosomes *in vivo*

Expanding the decoding and catalytic capabilities of the ribosome is often at the expense of diminishing its endogenous function in protein synthesis. To bypass this limitation, recent developments in cells have focused on the creation of specialized ribosome systems. The concept is to create an independent, or *orthogonal*, translation system within the cell while wild-type ribosomes continue to synthesize genome-encoded proteins to ensure cell viability and productivity. The orthogonal ribosome is thus excluded from the production of endogenous polypeptides and, ideally, exclusively translates specific, targeted mRNA(s). Therefore, the orthogonal translation apparatus can be engineered to carry out new functions,

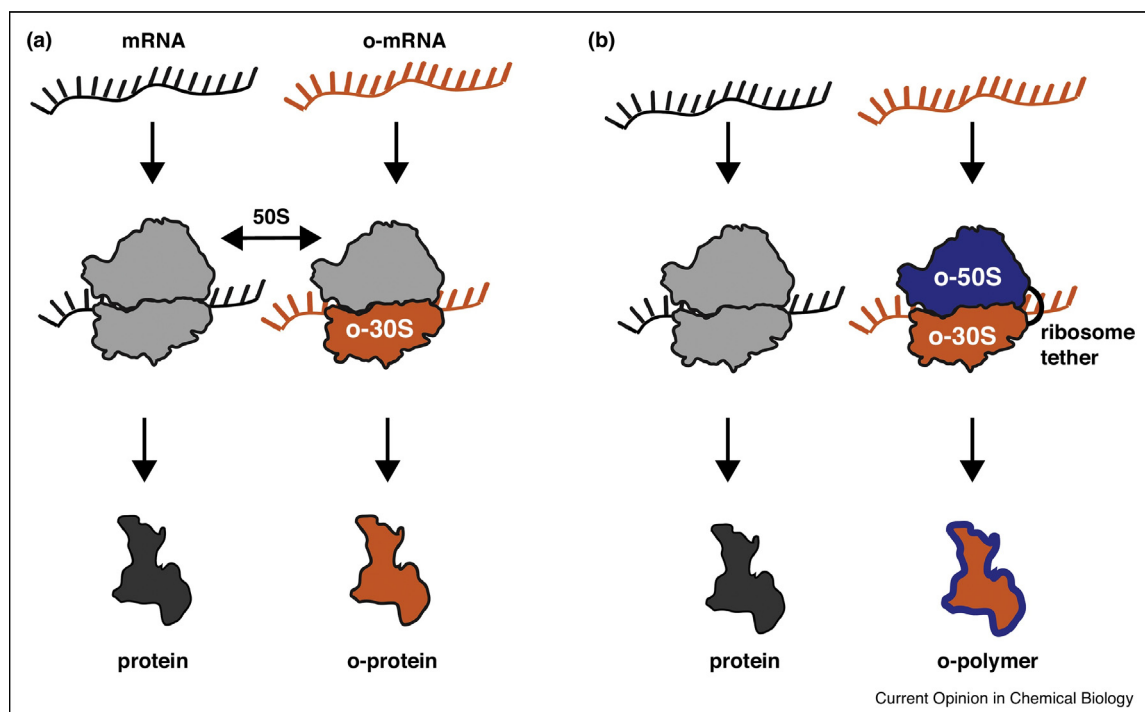
even if such modifications may negatively affect the operation of the orthogonal ribosome in normal translation. The principle of orthogonality is achieved through the bacterial small ribosomal subunit. Recognition of the start codon in bacteria relies on complementary interactions between the purine-rich Shine-Dalgarno (SD) region, which precedes the initiator codons of bacterial open reading frames, and the pyrimidine-rich ‘anti-SD’ (aSD) sequence at the 3′-end of the 16S rRNA in the 30S subunit [31]. Thus, by modifying the SD sequence of an mRNA and the corresponding aSD sequence in 16S rRNA, it is possible to create specialized ribosomes capable of translating only a specific kind of engineered mRNAs, while simultaneously excluding translation of endogenous cellular mRNAs (Figure 2a). Initially, the concept of orthogonal ribosomes was pioneered by Hui and deBoer [32–34], and then was extended and improved in several ways by Chin and colleagues. Rackham and Chin, for example, generated random mutagenesis libraries of possible 16S rRNA and mRNA pairs with complementary SD/aSD sequences, then selected for pairs that can robustly and exclusively translate orthogonal message without crosstalk with native translation processes [35**]. Chubiz and Rao developed a computational model to

rationally design orthogonal SD/aSD sequences in order to explore a larger mutational space, with similar results as they discovered several orthogonal 16S and mRNA pairs without toxicity effects on the host strain [36].

Leveraging these advances, orthogonal 30S subunits have been engineered to preferentially bind to the amber suppressor tRNA over RF-1 to improve ncAA incorporation efficiency [37], select for opal suppressor tRNA over RF-2 [38], decode quadruplet codons towards genetic code expansion [39**], and enable orthogonal transcription-translation networks [40]. These innovations have enabled the creation of engineered ribosomes with altered substrate preferences *in vivo*. However, until two years ago, such techniques have been limited to the 30S small subunit because 50S large subunits freely exchange between pools of native and orthogonal 30S subunits. This has previously constrained the engineering potential of the large subunit, which includes functionally important domains such as the peptidyl transferase center and the exit tunnel.

Recently, the covalent linking of the core ribosomal RNA of the large and small subunits of the orthogonal ribosome

Figure 2



Approaches to orthogonal ribosomes. **(a) 30S orthogonality.** 30S subunits (o-30S), with the anti-Shine-Dalgarno (aSD) sequence of its 16S rRNA modified to bind only to RNA messages (o-mRNA) with the complementary Shine-Dalgarno (SD) sequence, translate proteins (o-protein) orthogonal and parallel to the native translation process (gray). However, the 50S subunit is allowed to freely associate between native 30S and o-30S. **(b) A fully orthogonal 70S.** 50S and 30S subunits are connected by an RNA tether, preventing free association of native and orthogonal species.

has enabled the first completely orthogonal ribosome-mRNA system where mRNA decoding, catalysis of polypeptide synthesis, and protein excretion can all be optimized for new substrates and functions [41^{**},42^{*},43]. Orelle and colleagues described the first successful construction of a ribosome with covalently tethered subunits, termed Ribo-T, capable of carrying out protein synthesis (Figure 2b) [41^{**}]. Specifically, they engineered a ribosome whose core 16S and 23S rRNAs form a single chimeric molecule with a covalent connection between the 23S rRNA termini within the loop of helix 101 (H101) and the apex loop of the 16S rRNA helix 44 (h44). Not only could this hybrid rRNA support the assembly of a functional ribosome in a cell, but surprisingly Ribo-T could also maintain bacterial growth even in the absence of wild-type ribosomes. Orelle and colleagues also used Ribo-T to create a fully orthogonal ribosome-mRNA system [41^{**}]. They demonstrated its evolvability by selecting otherwise dominantly lethal rRNA mutations in the peptidyl transferase center that facilitated the translation of problematic protein sequences. Fried and colleagues also demonstrated a linked ribosome design capable of sequestering dominant lethal mutations in a fully orthogonal ribosome using a different set of linkers, or ‘staples’ [42^{*}]. Collectively, these findings uncover new directions in biomolecular engineering and synthetic biology. Looking forward, tethered ribosomes can be used for exploring poorly understood functions of the ribosome (e.g., antibiotic resistance mechanisms), enabling orthogonal genetic systems, and engineering ribosomes with altered chemical properties. Future challenges include resource re-allocation of the cell’s translational capacity and shared ribosomal protein pool, as well as optimizing the expression levels of tethered ribosomes in the background of high concentrations of endogenous ribosomes.

Engineering ribosomes *in vitro*

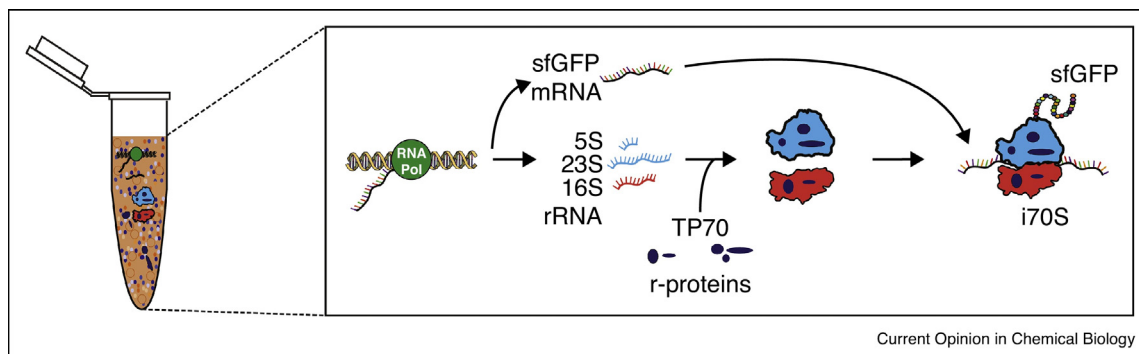
As a complement to engineering ribosomes *in vivo*, *in vitro* approaches offer potential advantages to precisely control the reaction environment in a manner that may allow for the isolation of certain mutant ribosomes not possible in cells, such as those in non-physiological pH, temperature, and redox levels. *In vitro* translation systems, both reconstituted from purified parts or from crude cell lysates, have shown promise for applications stemming from recent advances that alleviate possible ribosomal limitations and increase yields [44–47]. *In vitro* reconstitution of *E. coli* ribosomes from their individual components was first achieved over 40 years ago [48,49]. Despite relatively efficient reconstitution with natural components, the use of *in vitro* transcribed rRNA, which lack naturally occurring post-transcriptional modifications, is less efficient than *in vivo* transcribed versions [50,51]. This is a particular concern for the *E. coli* 50S subunit, where peptidyl transferase activity from reconstituted 50S subunits using *in vitro* transcribed 23S rRNA

is diminished ~10,000-fold relative to those with naturally derived 23S rRNA. Low reconstitution efficiencies from *in vitro* transcribed rRNA have represented one of the most significant bottlenecks to *in vitro* ribosome engineering.

Several efforts are underway to move beyond previous limitations. One method is to leverage purified translation systems, such as the PURE system with no endogenous ribosomes, to build new ribosomes in ways similar to strategies used in cells [52–54]. In a key milestone this year, Li and colleagues showed that ribosomal proteins generated *in vitro* could be used alongside *in vitro* transcribed rRNA to build a functional small ribosomal subunit [54]. In another approach, Jewett *et al.* developed an integrated synthesis, assembly, and translation method, termed iSAT, that enabled efficient one-step co-activation of rRNA transcription, assembly of transcribed rRNA with native ribosomal proteins into *E. coli* ribosomes, and ribosomal synthesis of functional proteins in a ribosome-free S150 extract [55] (Figure 3). Notably, iSAT mimics co-transcription of rRNA and ribosome assembly as it occurs in cells.

The iSAT system’s utility was improved over the past four years, where the activity of iSAT was increased by more than three orders of magnitude through optimization of extract preparation methods, rRNA transcription turning, substrate limitation alleviations, and the use of macromolecular crowding and reducing agents [55–58]. In one instance, Fritz and Jewett increased transcriptional efficiency through 3’-modifications in rRNA gene sequences, optimized plasmid and polymerase concentrations, and demonstrated the use of a T7-transcribed rRNA operon for stoichiometrically balanced rRNA synthesis and native rRNA processing [56]. These modifications produced a 45-fold improvement in iSAT protein synthesis activity. In another advancement, Liu and colleagues determined substrate depletion and toxic byproduct accumulation to be causes of reaction termination in iSAT, and alleviated these constraints using a semi-continuous reaction format [57]. Another study demonstrated that macromolecular crowding and reducing agents (6%, w/v, Ficoll 400, and 2 mM DTBA) yielded approximately a five-fold increase in overall iSAT protein synthesis activity [58]. By utilizing a fluorescent RNA aptamer, fluorescent reporter proteins, and ribosome sedimentation analysis, Fritz and colleagues showed that crowding agents increased iSAT yields by enhancing translation while reducing agents increase rRNA transcription and ribosome assembly [58]. These efforts demonstrated that iSAT ribosomes possess ~70% of the protein synthesis activity compared to *in vivo*-assembled *E. coli* ribosomes, which surpasses an important benchmark: iSAT ribosomes are now capable of translating >8,000 peptide bonds per ribosome — enough peptide bonds for the translation of a complete

Figure 3



Integrated synthesis, assembly, and translation (iSAT) method for ribosome construction *in vitro*. In cell-free, ribosome-free S150 extract, reporter mRNA and rRNA are transcribed by RNA polymerase (RNA Pol) and the rRNA assembled into ribosomal subunits with added total r-proteins of the 70S (TP70). iSAT-assembled ribosomes then translate the reporter, here superfolder green fluorescent protein (sfGFP), as a measure of ribosome activity.

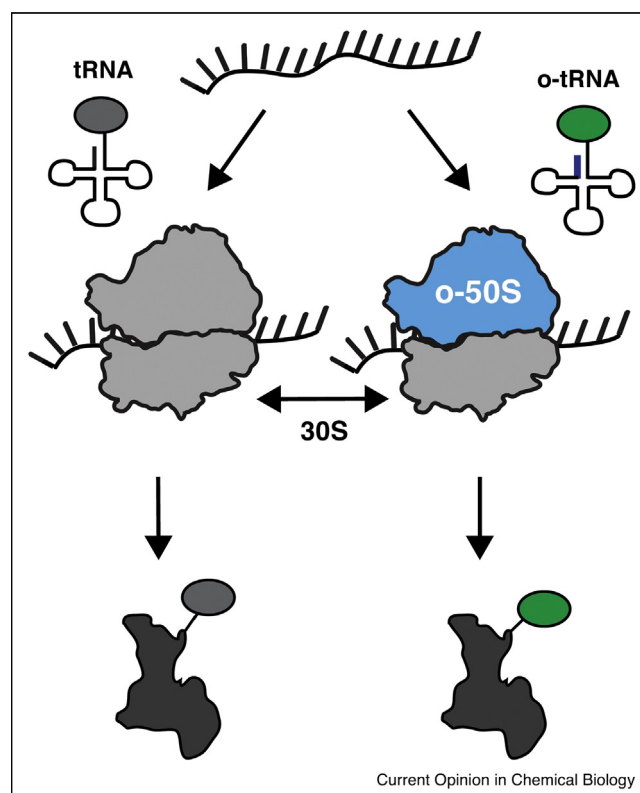
set of ribosomal proteins [55]. The iSAT system can also be encapsulated inside giant liposomes in a cell-like compartment which could facilitate *in vitro* evolution [59].

A key feature of the iSAT system is the ability to generate ribosomal variants by simply changing the DNA input, which allows for the facile construction of modified ribosomes with mutations in any desired domain. For example, ribosomes highly resistant to the lincosamide antibiotic clindamycin were readily constructed via targeted mutations in rRNA sequences [55], showcasing the ability of the iSAT system to generate functional modified ribosomes. Other approaches have shown the ability to evolve ribosomes *in vitro*. To select mutants of 23S rRNA, which contain the peptidyl transferase center, Cochella and Green developed a hybrid *in vivo/in vitro* approach [29]. Their strategy involved, first, *in vivo* assembly of tagged ribosomal mutants with variant 23S rRNAs (mutant rRNAs that are co-expressed with native rRNAs), second, isolation of tagged ribosomes by affinity purification, and third, *in vitro* selection of ribosome mutants using ribosome display. Employing this approach, the authors isolated functionally competent 23S rRNAs that were resistant to clindamycin and were not viable *in vivo* [29]. While this hybrid strategy enables *in vitro* selection of mutant ribosomes based on functional properties that cannot be accessed *in vivo*, it suffers from limited diversity resulting from the need to transform a mutant library of 23S rRNA genes into cells, as well as the challenge of separating mutant ribosome pools from native ones. This provides a robust opportunity to use iSAT for evolving modified ribosomes with altered substrate preferences.

Beyond modifying the active site of the ribosome for altered catalytic function, mutations elsewhere could allow the ribosome to use orthogonal tRNAs (Figure 4).

In pioneering work, Terasaka and colleagues demonstrated that the universally conserved 3'-terminal CCA sequence in tRNA molecules could be changed to CGA or GGA without loss of function, provided that three

Figure 4



Adapting the ribosome to unique tRNA pools. 50S subunits (o-50S) are modified in the 23S rRNA to bind only to orthogonal tRNA (o-tRNA) with a complementary sequence at the 3' tail. This can result in two species of protein translated from one RNA message. The 30S subunit freely associates between the two 50S species.

residues of the ribosomal peptidyl transferase center (P site G2251 and G2252, and A site G2553) were altered to retain base pairing with the tRNA terminus [60**]. To do this, they first generated synthetic tRNAs [61] bearing mutations at the 3' end at either one or two sites (C74G and/or C75G), and found that 3'-CGA-tRNA and 3'-GGA-tRNAs were not compatible with wild-type ribosomes and thereby orthogonal. Ribosomes with 50S subunits possessing complementary single (G2252C), double (G2251C and G2253C), and triple (G2251C, G2252C, and G2253C) mutations in the 23S rRNA were purified and their activity coupled with and without orthogonal tRNAs were assessed for translation using a flexible *in vitro* translation (FIT) system of purified parts [60**,62]. The authors found that the double mutant ribosome paired with 3'-CGA-tRNA was able to synthesize a separate species of peptide bearing an ncAA from a single mRNA, while wild-type ribosomes translated a peptide without an ncAA. This advancement suggests the possibility of establishing orthogonal coding channels for the biosynthesis of novel synthetic polymers capable of incorporating multiple ncAAs without crosstalk with canonical amino acids and the native translation system.

Conclusions

The construction of engineered ribosomes is poised to enable new opportunities to manufacture synthetic sequence-defined polymers that span vast structural and functional diversity, yet remain unattainable through existing methods in synthetic or biological chemistry. Despite these opportunities, many challenges remain. First, the fundamental constraints on the chemistry that the ribosome's RNA-based active site can carry out are unknown. By creating machines of translation that move beyond nature's processes and standard monomers, future work could elucidate a new understanding of the science of protein synthesis through construction. Second, the resolution of the crystal structure of the bacterial ribosome has provided newfound insights into the functional operation and mechanism of translation. Yet, repurposing ribosomes with new substrate preferences will require computational tools, as well as predictive models, to guide any fundamental redesign of the translation apparatus for new chemical activities. This is especially challenging given the size and structural complexity of the ribosome. Third, the ribosome is only one component of the translation apparatus and building wholly orthogonal translation systems with high specificity and activity for unique non-canonical substrates is complex, especially given its milieu of moving parts. New approaches for systematically engineering multiple components of the translation machinery (e.g., ribosomes, tRNAs, aminoacyl-tRNA synthetases, and elongation factors) concurrently as a cohesive unit, rather than in isolation, will provide synergistic opportunities to enhance multi-site incorporation of ncAAs into proteins.

Overcoming these challenges will have both short and long term benefits. In the short term, ribosome repurposing will deepen our understanding of translation, expand the genetic code in a unique and transformative way, and reveal how evolution has guided the structure and function of the ribosome. In the long term, engineering the translation apparatus will expand the range of genetically encoded chemistry in proteins and biopolymers, forging a broad range of innovative technologies that have the potential to transform synthetic biology.

Acknowledgements

This work was supported by the Army Research Office W911NF-16-1-0372 (to M.C.J.), National Science Foundation grant MCB-1716766 (to M.C.J.), the Air Force Research Laboratory Center of Excellence Grant FA8650-15-2-5518 (to M.C.J.), the David and Lucile Packard Foundation (to M.C.J.), and the Human Frontiers Science Program (RGP0015/2017). Y.L. is a Medical Scientist Training Program scholar under the MSTP NIH T32 training grant T32GM008152. D.S.K. is supported by the NSF Graduate Research Fellowship. We thank Jamie Cate, Rhiju Das, Andrew Ellington, Farren Isaacs, Shura Mankin, and Alanna Schepartz for ongoing scientific discussions. The U.S. Government is authorized to reproduce and distribute reprints for Governmental purposes notwithstanding any copyright notation thereon. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of Air Force Research Laboratory, Army Research Office, the Department of Defense, or the U.S. Government.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Bremer H, Dennis PP: **Modulation of chemical composition and other parameters of the cell by growth rate.** In *Escherichia coli and Salmonella typhirium: cellular and molecular biology*, 2nd ed.. Edited by Neidhart EA. 1996:1559.
 2. Zaher HS, Green R: **Fidelity at the molecular level: lessons from protein synthesis.** *Cell* 2009, **136**:746-762.
 3. O'Donoghue P, Ling J, Wang Y-S, Söll D: **Upgrading protein synthesis for synthetic biology.** *Nat Chem Biol* 2013, **9**:594-598.
 4. Forster AC, Tan Z, Nalam MN, Lin H, Qu H, Cornish VW, Blacklow SC: **Programming peptidomimetic syntheses by translating genetic codes designed de novo.** *Proc Natl Acad Sci U S A* 2003, **100**:6353-6357.
 5. Davis L, Chin JW: **Designer proteins: applications of genetic code expansion in cell biology.** *Nat Rev Mol Cell Biol* 2012, **13**:168-182.
 6. Wang L, Xie J, Schultz PG: **Expanding the genetic code.** *Annu Rev Biophys Biomol Struct* 2006, **35**:225-249.
 7. Hoesl MG, Budisa N: **Recent advances in genetic code engineering in Escherichia coli.** *Curr Opin Biotechnol* 2012, **23**:751-757.
 8. Liu CC, Schultz PG: **Adding new chemistries to the genetic code.** *Annu Rev Biochem* 2010, **79**:413-444.
 9. Des Soye BJ, Patel JR, Isaacs FJ, Jewett MC: **Repurposing the translation apparatus for synthetic biology.** *Curr Opin Chem Biol* 2015, **28**:83-90.
 10. Filipovska A, Rackham O: **Specialization from synthesis: how ribosome diversity can customize protein function.** *FEBS Lett* 2013, **587**:1189-1197.
 11. Amiram M, Haimovich AD, Fan C, Wang YS, Aerni HR, Ntai I, •• Moonan DW, Ma NJ, Rovner AJ, Hong SH et al.: **Evolution of translation machinery in recoded bacteria enables multi-site**

incorporation of nonstandard amino acids. *Nat Biotechnol* 2015, **33**:1272-1279.

This work highlights a significant advancement in expanding the genetic code for the incorporation of tens of multiple, identical ncAAs into a protein *in vivo*. The authors achieved this through engineering the aminoacyl-tRNA synthetase, a key enzyme in protein translation, and orthogonal tRNAs.

12. Schuwirth BS, Borovinskaya MA, Hau CW, Zhang W, Vila-Sanjurjo A, Holton JM, Cate JH: **Structures of the bacterial ribosome at 3.5 Å resolution.** *Science* 2005, **310**:827-834.
 13. Ellman JA, Mendel D, Schultz PG: **Site-specific incorporation of novel backbone structures into proteins.** *Science* 1992, **255**:197-200.
 14. Kawakami T, Murakami H, Suga H: **Ribosomal synthesis of polypeptoids and peptoid-peptide hybrids.** *J Am Chem Soc* 2008, **130**:16861-16863.
 15. Guo J, Wang J, Anderson JC, Schultz PG: **Addition of an alpha-hydroxy acid to the genetic code of bacteria.** *Angew Chem Int Ed* 2008, **47**:722-725.
 16. Subtelny AO, Hartman MCT, Szostak JW: **Ribosomal synthesis of n-methyl peptides.** *J Am Chem Soc* 2008, **130**:6131-6136.
 17. Englander MT, Avins JL, Fleisher RC, Liu B, Efraim PR, Wang J, Schulten K, Leyh TS, Gonzalez RL, Cornish VW: **The ribosome can discriminate the chirality of amino acids within its peptidyl-transferase center.** *Proc Natl Acad Sci USA* 2015, **112**:6038-6043.
- Through careful investigation using biochemical assays, the authors elucidated the limitation of the ribosome's peptidyl transferase center to process D-amino acids in translation. This work highlighted the need to either engineer the ribosome's peptidyl transferase center or otherwise alleviate ribosome stalling to achieve processing of non-L-amino acids.
18. Maini R, Nguyen DT, Chen S, Dedkova LM, Chowdhury SR, Alcalá-Torano R, Hecht SM: **Incorporation of beta-amino acids into dihydrofolate reductase by ribosomes having modifications in the peptidyltransferase center.** *Bioorg Med Chem* 2013, **21**:1088-1096.
 19. Maini R, Chowdhury SR, Dedkova LM, Roy B, Daskalova SM, Paul R, Chen S, Hecht SM: **Protein synthesis with ribosomes selected for the incorporation of β-amino acids.** *Biochemistry* 2015.
 20. Goto Y, Murakami H, Suga H: **Initiating translation with D-amino acids.** *RNA* 2008, **14**:1390-1398.
 21. Katoh T, Tajima K, Suga H: **Consecutive elongation of D-amino acids in translation.** *Cell Chem Biol* 2017, **24**:46-54.
- This work highlighted the flexibility of *in vitro* translation systems in alleviating ribosomal limitations through the supplementation of translation components such as elongation factor Tu, elongation factor G, and initiation factor 2. Additionally, engineering the tRNA body to more tightly bind EF-Tu allowed the authors to demonstrate consecutive incorporation of backbone-modified amino acids, a feat previously unachievable.
22. Fujino T, Goto Y, Suga H, Murakami H: **Ribosomal synthesis of peptides with multiple beta-amino acids.** *J Am Chem Soc* 2016, **138**:1962-1969.
- Building upon methods of reprogramming the genetic code for ncAA incorporation, the authors demonstrated the ability of the ribosome to synthesize a peptide containing multiple backbone modified beta-amino acids, although consecutive incorporation was shown to be difficult.
23. Fahnstock S, Rich A: **Ribosome-catalyzed polyester formation.** *Science* 1971, **173**:340-343.
 24. Ohta A, Murakami H, Higashimura E, Suga H: **Synthesis of polyester by means of genetic code reprogramming.** *Chem Biol* 2007, **14**:1315-1322.
- Using a purified *in vitro* translation system, the authors demonstrated the ribosome's ability to carry out sequence-defined polymerization of non-peptide backbone polymers. Specifically, the authors produced sequence-defined polyesters using tRNAs synthetically charged with hydroxy acids.
25. Ohta A, Murakami H, Suga H: **Polymerization of alpha-hydroxy acids by ribosomes.** *ChemBiochem* 2008, **9**:2773-2778.
 26. Dedkova LM, Fahmi NE, Golovine SY, Hecht SM: **Enhanced D-amino acid incorporation into protein by modified ribosomes.** *J Am Chem Soc* 2003, **125**:6616-6617.
 27. Dedkova LM, Fahmi NE, Golovine SY, Hecht SM: **Construction of modified ribosomes for incorporation of D-amino acids into proteins.** *Biochemistry* 2006, **45**:15541-15551.
- Through *in vivo* generation of modified ribosomes with *in vivo* and *in vitro* tests of their activity, this work highlights an innovative approach to identifying ribosomes with modified peptidyl transferase centers and improved capacities for incorporating D-amino acids into proteins. The approach showcased here was successfully extended to other backbone-modified amino acids.
28. Melo Czekster C, Robertson WE, Walker AS, Soll D, Schepartz A: **In vivo biosynthesis of a beta-amino acid-containing protein.** *J Am Chem Soc* 2016, **138**:5194-5197.
- The authors demonstrated the ability of the phenylalanine-tRNA synthetase to mischarge a tRNA with beta-amino acids and synthesize a model protein, DHFR, with a single instance of the beta-amino acid. This is the first demonstration of *in vivo* biosynthesis of a beta-amino acid-containing protein.
29. Cochella L, Green R: **Isolation of antibiotic resistance mutations in the rRNA by using an in vitro selection system.** *Proc Natl Acad Sci U S A* 2004, **101**:3786-3791.
 30. Chin JW: **Expanding and reprogramming the genetic code of cells and animals.** *Annu Rev Biochem* 2014, **83**:379-408.
 31. Shine J, Dalgarno L: **The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites.** *Proc Natl Acad Sci U S A* 1974, **71**:1342-1346.
 32. Hui A, Jhurani P, de Boer HA: **Directing ribosomes to a single mRNA species: a method to study ribosomal RNA mutations and their effects on translation of a single messenger in Escherichia coli.** *Methods Enzymol* 1987, **153**:432-452.
 33. Hui AS, Eaton DH, de Boer HA: **Mutagenesis at the mRNA decoding site in the 16S ribosomal RNA using the specialized ribosome system in Escherichia coli.** *EMBO J* 1988, **7**:4383-4388.
 34. Hui A, de Boer HA: **Specialized ribosome system: preferential translation of a single mRNA species by a subpopulation of mutated ribosomes in Escherichia coli.** *Proc Natl Acad Sci U S A* 1987, **84**:4762-4766.
 35. Rackham O, Chin JW: **A network of orthogonal ribosome × mRNA pairs.** *Nat Chem Biol* 2005, **1**:159-166.
- By tuning the interaction between the Shine-Dalgarno sequence in the mRNA and the anti-Shine-Dalgarno sequence in the 16S rRNA, the authors identified orthogonal SD/a-SD pairs that minimize cross-talk with the wild-type ribosomes and cellular mRNAs. This work laid the foundation for future ribosome engineering efforts in cells, and provided a key handle for evolution schemes involving the 16S rRNA.
36. Chubiz LM, Rao CV: **Computational design of orthogonal ribosomes.** *Nucleic Acids Res* 2008, **36**:4038-4046.
 37. Wang K, Neumann H, Peak-Chew SY, Chin JW: **Evolved orthogonal ribosomes enhance the efficiency of synthetic genetic code expansion.** *Nat Biotechnol* 2007, **25**:770-777.
 38. Thyer R, Filipovska A, Rackham O: **Engineered rRNA enhances the efficiency of selenocysteine incorporation during translation.** *J Am Chem Soc* 2013, **135**:2-5.
 39. Neumann H, Wang K, Davis L, Garcia-Alai M, Chin JW: **Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome.** *Nature* 2010, **464**:441-444.
- This represents a key milestone in orthogonal ribosomes *in vivo*. In this work, the authors showed the ability to mutate key residues in the 16S rRNA responsible for decoding the mRNA-tRNA interface, and identified mutant small subunit designs that allowed for improved decoding of quadruplet codons.
40. An W, Chin JW: **Synthesis of orthogonal transcription-translation networks.** *Proc Natl Acad Sci U S A* 2009, **106**:8477-8482.
 41. Orelle C, Carlson ED, Szal T, Florin T, Jewett MC, Mankin AS: **Protein synthesis by ribosomes with tethered subunits.** *Nature* 2015, **524**:119-124.

The authors built a ribosome made of covalently tethered subunits, breaking the assumption that the separate but interacting nature of ribosomal subunits was required for successful protein synthesis. The tethered ribosome, Ribo-T, was used to create a fully orthogonal ribosome-mRNA system. Ribo-T provides a key tool for ribosome engineering in vivo as dominant lethal mutations in Ribo-T do not perturb cell viability.

42. Fried SD, Schmied WH, Uttamapinant C, Chin JW: **Ribosome subunit stapling for orthogonal translation in *E. coli***. *Angew Chem Int Ed Engl* 2015, **54**:12791-12794.
- This work demonstrates the ability to create a fully orthogonal ribosome in cells. It is used to sequester otherwise dominantly lethal mutations.
43. Carlson ED: **Creating Ribo-T: (Design, Build, Test)ⁿ**. *ACS Synth Biol* 2015, **4**:1173-1175.
44. Carlson ED, Gan R, Hodgman CE, Jewett MC: **Cell-free protein synthesis: applications come of age**. *Biotechnol Adv* 2012, **30**:1185-1194.
45. Hong SH, Kwon YC, Jewett MC: **Non-standard amino acid incorporation into proteins using *Escherichia coli* cell-free protein synthesis**. *Front Chem* 2014, **2**:34.
46. Jewett MC, Calhoun KA, Voloshin A, Wu JJ, Swartz JR: **An integrated cell-free metabolic platform for protein production and synthetic biology**. *Mol Syst Biol* 2008, **4**:220.
47. Jewett MC, Swartz JR: **Mimicking the *Escherichia coli* cytoplasmic environment activates long-lived and efficient cell-free protein synthesis**. *Biotechnol Bioeng* 2004, **86**:19-26.
48. Nierhaus KH, Dohme F: **Total reconstitution of functionally active 50S ribosomal subunits from *Escherichia coli***. *Proc Natl Acad Sci U S A* 1974, **71**:4713-4717.
49. Traub P, Nomura M: **Structure and function of *E. coli* ribosomes. V. Reconstitution of functionally active 30S ribosomal particles from RNA and proteins**. *Proc Natl Acad Sci U S A* 1968, **59**:777-784.
50. Green R, Noller HF: **In vitro complementation analysis localizes 23S rRNA posttranscriptional modifications that are required for *Escherichia coli* 50S ribosomal subunit assembly and function**. *RNA* 1996, **2**:1011-1021.
51. Semrad K, Green R: **Osmolytes stimulate the reconstitution of functional 50S ribosomes from in vitro transcripts of *Escherichia coli* 23S rRNA**. *RNA* 2002, **8**:401-411.
52. Jewett MC, Forster AC: **Update on designing and building minimal cells**. *Curr Opin Biotechnol* 2010, **21**:697-703.
53. Li J, Gu L, Aach J, Church GM: **Improved cell-free RNA and protein synthesis system**. *PLoS ONE* 2014, **9**:e106232.
54. Li J, Haas W, Jackson K, Kuru E, Jewett MC, Fan ZH, Gygi S, Church GM: **Cogenerating synthetic parts toward a self-replicating system**. *ACS Synth Biol* 2017, **6**:1327-1336.
55. Jewett MC, Fritz BR, Timmerman LE, Church GM: **In vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation**. *Mol Syst Biol* 2013, **9**:678.
56. Fritz BR, Jewett MC: **The impact of transcriptional tuning on in vitro integrated rRNA transcription and ribosome construction**. *Nucleic Acids Res* 2014, **42**:6774-6785.
57. Liu Y, Fritz BR, Anderson MJ, Schoborg JA, Jewett MC: **Characterizing and alleviating substrate limitations for improved in vitro ribosome construction**. *ACS Synth Biol* 2015, **4**:454-462.
58. Fritz BR, Jamil OK, Jewett MC: **Implications of macromolecular crowding and reducing conditions for in vitro ribosome construction**. *Nucleic Acids Res* 2015, **43**:4774-4784.
59. Caschera F, Lee JW, Ho KK, Liu AP, Jewett MC: **Cell-free compartmentalized protein synthesis inside double emulsion templated liposomes with in vitro synthesized and assembled ribosomes**. *Chem Commun (Camb)* 2016, **52**:5467-5469.
60. Terasaka N, Hayashi G, Katoh T, Suga H: **An orthogonal ribosome-tRNA pair via engineering of the peptidyl transferase center**. *Nat Chem Biol* 2014, **10**:555-557.
- This work established an example of an orthogonal 50S large subunit by engineering the interaction between a conserved sequence in the tRNA and key residues in the 23S rRNA. The authors achieved selective translation of a hexapeptide containing ncAAs through genetic code reprogramming in a purified *in vitro* translation system.
61. Saito H, Kourouklis D, Suga H: **An in vitro evolved precursor tRNA with aminoacylation activity**. *EMBO J* 2001, **20**:1797-1806.
62. Goto Y, Katoh T, Suga H: **Flexizymes for genetic code reprogramming**. *Nat Protoc* 2011, **6**:779-790.