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Engineered Ribosomes for Basic Science and Synthetic Biology

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Abstract

The ribosome is the cell's factory for protein synthesis. With protein synthesis rates of up to 20 amino acids per second and at an accuracy of 99.99%, the extraordinary catalytic capacity of the bacterial translation machinery has attracted extensive efforts to engineer, reconstruct, and repurpose it for biochemical studies and novel functions. Despite these efforts, the potential for harnessing the translation apparatus to manufacture bio-based products beyond natural limits remains underexploited, and fundamental constraints on the chemistry that the ribosome's RNA-based active site can carry out are unknown. This review aims to cover the past and present advances in ribosome design and engineering to understand the fundamental biology of the ribosome to facilitate the construction of synthetic manufacturing machines. The prospects for the development of engineered, or designer, ribosomes for novel polymer synthesis are reviewed, future challenges are considered, and promising advances in a variety of applications are discussed.

1. INTRODUCTION

r-proteins: ribosomal proteins

rRNA: ribosomal RNA

aa-tRNA: aminoacyl-transfer RNA

aaRS: aminoacyl-transfer RNA synthetase

tRNA: transfer RNA

EF-Tu: elongation factor Tu

The ribosome is a ribonucleoprotein machine composed of RNA and proteins. In all kingdoms of life, the ribosome is made up of one large and one small subunit. In bacteria, these correspond to the 50S large subunit and the 30S small subunit. The 50S subunit of the *Escherichia coli* ribosome is composed of 33 ribosomal proteins (r-proteins), the 23S ribosomal RNA (rRNA), and the 5S rRNA. The corresponding 30S subunit is composed of 21 r-proteins and the 16S rRNA (1).

One can think of the ribosome as the catalytic workhorse of the cell, because it carries out the polymerization of α-amino acids into polypeptides called proteins. To achieve this function, the ribosome has several tasks. These tasks include accommodating aminoacyl-transfer RNA (aa-tRNA) monomers, decoding messenger RNA (mRNA), catalyzing polypeptide synthesis, and excreting polypeptides (2). The shape along with the physiochemical and dynamic properties of the ribosome have been evolutionarily optimized to polymerize ∼20 different canonical amino acids. With protein elongation rates of up to 20 amino acids per second in bacteria (3) at an error rate of approximately 1 in 10,000 (4), the extraordinary synthetic capability of the protein biosynthesis system has driven extensive efforts to harness it for societal needs in areas as diverse as energy, materials, and medicine. For example, recombinant protein production has transformed the lives of millions of people through the synthesis of biopharmaceuticals like insulin and industrial enzymes like subtilisin. In nature, however, only limited sets of protein monomers are used, 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. For instance, sequence-defined polypeptides made of protease-resistant monomers (e.g., D- and β-amino acids) could lead to antimicrobial drugs that combat rising antibacterial resistance. Such sequence-defined molecules have never been synthesized before by the ribosome. Yet their synthesis would redefine biomanufacturing and could become a major driver of global innovation and economic growth.

Unfortunately, expanding the range of genetically encoded chemistry into proteins presents a complex and formidable challenge for many reasons. One of the main challenges is that modifying the ribosome is a systems-level task. This is because many factors are involved in protein synthesis that must be tuned (**Figure 1**). These factors include (*a*) aa-tRNA synthetases (aaRSs), which covalently link amino acid monomers with their cognate transfer RNA (tRNA) delivery vehicles; (*b*) aa-tRNA monomers that decode codons; and (*c*) elongation factor Tu (EF-Tu), which delivers a correctly activated monomer (aa-tRNA) to the ribosome and also serves as a gateway to ensure sequence fidelity. Another challenging constraint is that the ribosome is required for life, which restricts the mutations that can be made to ribosomes. In practice, these constraints have made the natural ribosome essentially unevolvable, and so far, there has not been a generalizable method for designing and modifying the ribosome to work with substrates beyond those found in nature. Despite these challenges, new tools are emerging to derive general paradigms for engineering translation systems for basic science and advanced applications.

In this review, we summarize the growing body of work on the design and engineering of bacterial ribosomes, with a focus on *E. coli*, for applications in both basic and synthetic biology research. We review four key categories in ribosome design and engineering: (*a*) the parts and pieces of the ribosome and the translation system; (*b*) engineered ribosomes for basic biology; (*c*) engineered ribosomes for synthetic biology; and (*d*) the progress, prospects, and challenges of designing and engineering ribosomes. Along the way, we summarize the numerous methodologies that have been developed to advance ribosome engineering. Finally, we highlight how ribosome engineering can impact fields as diverse as materials, energy, and medicine and end with future outlooks on the field.

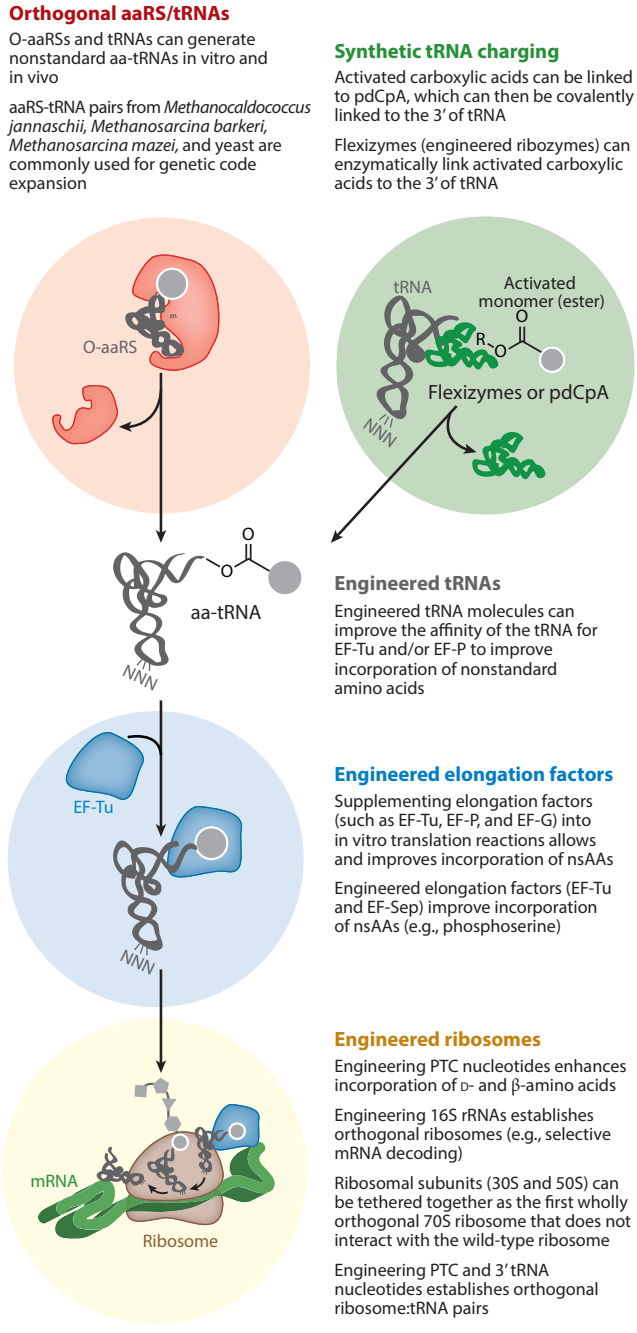

Figure 1

Ribosome engineering in context: protein translation in a cell. Free transfer RNAs (tRNAs) are charged (aminoacylated) with amino acids by the aminoacyl-tRNA synthetase (aaRS). Aminoacyl-tRNA molecules can also be generated in vitro using Flexizymes or pdCpA. Next, the aminoacyl-tRNA molecule is delivered to the ribosome by elongation factor Tu (EF-Tu). The tRNA, whose anticodon loop corresponds to a messenger RNA (mRNA) codon, delivers the amino acid to the ribosome, where it is polymerized into a protein. Abbreviations: nsAA, nonstandard amino acid; PTC, peptidyl transferase center.

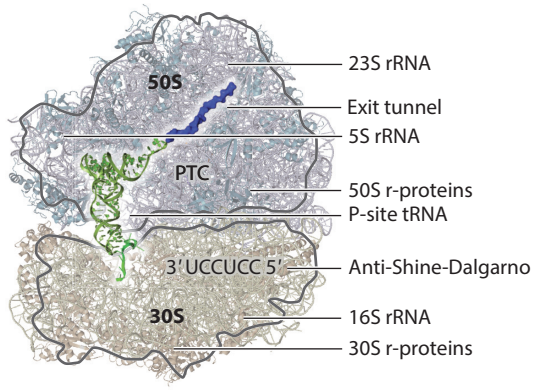

Figure 2

Map of the ribosome's parts. The ribosome is a complex machine composed of numerous parts. Some of the integral parts are mapped here. These include 3 different ribosomal RNA (rRNA) molecules (5S rRNA and 23S rRNA in the large subunit are depicted in grey, and 16S rRNA in the small subunit is depicted in wheat); ribosomal proteins (r-proteins) (the 33 large subunit r-proteins are depicted in teal, and the 21 small subunit r-proteins are depicted in orange); the catalytic peptidyl transferase center (PTC); the A-, P-, and E-sites; and the anti-Shine-Dalgarno sequence for decoding. Figure rendered using PyMol and PDB ID 4V5H.

2. THE RIBOSOME'S PARTS

To design and engineer a machine as complex as the ribosome for novel purposes, it is critical to first understand the various functional moieties. From the structural and interwoven proteins, to the subsequent RNA helices and loops, to the active site of the machine, identifying and characterizing the pieces at play before manipulating them is a critical requirement. Below, we have summarized the key ribosomal parts whose understanding will facilitate engineering and design.

2.1. Nucleic Acids of the Ribosome: Ribosomal RNA

The bacterial ribosome has three rRNAs. The three rRNAs that constitute the *E. coli* ribosome (5S, 16S, and 23S) are necessary for ribosomal structure and function (**Figure 2**). Noller and colleagues (5–7) solved the sequences of the DNA genes encoding the 16S, 23S, and 5S rRNAs, establishing a foundation in our molecular understanding of rRNA molecules. After the discovery of catalytic RNA (8, 9), biochemical evidence proposing the role of 23S RNA in peptidyl transferase (10), and finally, a high-resolution crystal structure of the 50S subunit (11), the ribosome was declared a ribozyme that catalyzes peptide bond formation via an RNA-based mechanism. Additionally, Steitz and colleagues (11) established that no protein moiety resides within several angstroms of the peptidyl transferase center (PTC), confirming RNA catalysis.

Of the three rRNAs, the 5S rRNA, which is approximately 120 nucleotides in length, is arguably the least understood. The 5S rRNA resides on almost all ribosomes, with the exception of some fungal mitochondrial ribosomes, higher animals, and certain protists (12) (**Figure 2**). Structurally, this rRNA molecule can bind proteins and other molecules, suggesting that it is involved with and connected to several translation elements. Functionally, it has been proposed that the 5S rRNA interacts with key domains of the ribosome, including domain V containing the active site and GTPase-associated domains (13). Although many studies have been carried out to characterize this molecule, details surrounding its activity and specific mechanism are still unclear. As we describe in Section 5, future work still remains to understand this ribosomal component.

The 16S rRNA makes up the small (30S) subunit (**Figure 2**) and is involved in early and intermediate steps of translation, including translation initiation and decoding. During translation initiation, the 3′ end of the 16S rRNA (the anti-Shine-Dalgarno sequence, as discussed in the following subsections) base pairs with a purine-rich element of mRNA (the Shine-Dalgarno sequence), which is positioned upstream of the start codon (14). Importantly, the 16S rRNA also interacts with initiation factors, impacting the kinetics of translation initiation (15). Regarding translation decoding, the 16S rRNA possesses the site of codon-anticodon interactions, wherein 16S nucleotides G530, A1492, and A1493 effectively stabilize base pairing formed between tRNA anticodons and the mRNA (16). Methyl-modifications and mutations to 16S rRNA modulate and impact translation fidelity and kinetics (17).

The 23S rRNA makes up the 50S subunit, which is responsible for accommodation, peptidyl transferase, and protein excretion. Biochemical studies and crystal structures of the ribosome have shown that many of these key activities are mediated through specific nucleotides within the 23S rRNA (**Figure 2**). Over the past approximately two decades, crystal structures have played an increasing role in our understanding of how the ribosome works, especially the PTC. The groundbreaking high-resolution crystal structures were solved using a small molecule that mimics the tetrahedral intermediate in peptide bond formation and inhibits peptidyl transferase activity (11, 18). In these studies, the structures elegantly illustrated that the ribosome specifically positions the 3′ terminus CCA ends (which are acylated to amino acids) of the A- and P-site tRNAs via interactions with the 23S rRNA at highly conserved A- and P-loops, respectively (11). The structure also suggests that rRNA residues stabilize the intermediate state of the tRNA-peptide complex. Additionally, the authors' structure implies the involvement of two key nucleotides in peptide bond formation: A2451 and G2447. Steitz and colleagues (11) found that the N3 of nucleotide A2451 resides in closest proximity (3 Å) to the peptide bond being synthesized, thus playing a key role in monomer positioning for translation elongation.

The insights gained regarding rRNAs have had significant impacts on both basic biology and engineering. Understanding the significance of the three rRNAs in protein synthesis has resulted in growing research interest to manipulate and design parts of this rRNA for purification and novel functions. With the plethora of rRNA knowledge in hand, we can now begin to imagine engineering these rRNA components. Because sequence defines structure, and structure defines function, we can begin from our knowledge of rRNA sequences to understand how we might design rRNA with new functions. In the following discussion of Section 2, we aim to describe in more detail the remaining parts of the ribosome and their mechanisms of action.

2.2. The Polypeptides of the Ribosome: Ribosomal Proteins

The *E. coli* ribosome contains 54 r-proteins that are synthesized in cells under tight regulation to ensure stoichiometric expression (19). The small 30S subunit contains 21 r-proteins, whereas the 50S subunit contains 33 r-proteins (1). R-proteins play essential, and sometimes mysterious, roles in ribosome assembly and function. These proteins have attracted the attention of both basic and synthetic biologists who are interested in not only understanding their function in translation but also leveraging our understanding for novel purposes.

The functions of r-proteins range from structural support, to mRNA decoding, to chaperone and cofactor binding, and to catalytic activity near the PTC (20). Mutations in r-proteins convey resistance to antibiotics through direct and indirect disruption of antibiotic binding sites (21, 22), but the precise roles of all proteins in translation have yet to be fully elucidated. Although we know that protein synthesis is catalyzed by rRNA, this catalytic activity still requires certain r-proteins (23). Conversely, whereas the *E. coli* ribosome is said to include 54 r-proteins, ribosomal mutants

have been found that lack certain r-proteins, and gene knockout studies have illustrated that 22 r-proteins can be individually deleted from the *E. coli* genome without lethality (24, 25). Work from Noller and colleagues (26) has suggested a minimum number of macromolecules required for peptidyl transferase, specifically the 23S rRNA and r-proteins L2 and L3. L2 and L3 are near the PTC in the assembled ribosome (27–29). Additionally, Schulze, Nierhaus, and Mankin demonstrated via single-omission tests that L2, L3, and L4 are essential for peptidyl transferase activity (30, 31).

Knowledge gained from r-protein studies has helped demystify their contributions to protein translation, ribosome assembly, antibiotic resistance, and more. With this knowledge in hand, many scientists have begun using these proteins for purification, tagging, dynamics studies, and reconstitution. Section 3 of our review, titled Engineered Ribosomes for Advancing Basic Biology, further elaborates on this progress. Next we discuss the components of the ribosome involved in translation initiation, monomer accommodation, peptide elongation, and peptide release.

2.3. The Site of Translation Initiation: Shine-Dalgarno and Anti-Shine-Dalgarno Sequences

In bacteria, one mechanism through which open reading frames in mRNAs are recognized and translated by ribosomes includes the interaction of two complementary sequences: the Shine-Dalgarno (SD) and anti-Shine-Dalgarno (aSD) sequences. Both translation initiation and translation efficiency are controlled by these sequences (32). The SD sequence is found approximately 9 bases upstream from the start codon in *E. coli* mRNA sequences. Its complementary aSD sequence is part of the highly conserved 3′ end of the small subunit 16S rRNA (14) (**Figure 2**). The SD sequence in the mRNA interacts with the aSD region of the 16S rRNA such that the mRNA-rRNA base pairing facilitates the initiation of protein translation and synthesis.

Extensive work has been carried out to support, define, and characterize the SD:aSD interactions. For example, by analyzing data from experiments measuring translation activity across different SD:aSD pairs, Hockenberry and coworkers (32) found that translation efficiency is maximized for sequences with intermediate aSD binding strengths. Additionally, numerous publications have demonstrated that mutations altering mRNA sequence complementarity and binding have significant impacts on translation efficiency and ability (33–35). Finally, complete substitution of the SD sequence with a non-SD sequence was demonstrated to completely abolish mRNA translation (36).

2.4. The Ribosome's Catalytic Peptidyl Transferase Center: A-, P-, and E-Sites

Following translation initiation via the SD:aSD sequences, protein translation enters the elongation phase. During elongation, both the incoming amino acid and the nascent peptide are simultaneously bound to tRNA molecules within the ribosome's active site (**Figure 2**). There exist three binding sites for tRNA molecules in the ribosome: the A-site, which accepts the incoming aa-tRNA; the P-site, which holds the elongating peptidyl chain attached to tRNA; and the E-site, through which the deacylated tRNA exits the ribosome. These three binding sites account for the codon-anticodon (mRNA-tRNA) interaction; the precise arrangement of tRNA acceptor and donor arms during peptide bond formation; and the successive movement, or translocation, of mRNA relative to the ribosome (10).

In the first step of translation, aa-tRNA is delivered to the A-site of the ribosome as a complex with EF-Tu and GTP. The tRNA anticodon associates with the complementary codon in the 30S A-site, whereas the aminoacyl acceptor end (CCA) remains bound to EF-Tu.

The codon-anticodon pairing and binding instigates a conformational change in EF-Tu, subsequently leading to GTP hydrolysis. This hydrolysis triggers EF-Tu to dissociate from the ribosome, releasing the aminoacyl end of the tRNA molecule to fully enter the A-site of the large 50S subunit. This process is known as accommodation (37).

This accommodated aa-tRNA quickly undergoes the peptidyl transferase reaction. In the first step of the reaction, the A- and P-site tRNA molecule acceptor arms are arranged in the 50S subunit through interactions with 23S rRNA nucleotides (38). Specifically, the CCA end (which carries the amino acid) of the P-site tRNA is base paired to G2251, G2252, and G2583, whereas the A-site tRNA CCA end is base paired to G2553 and A2450 (*E. coli* numbering). During this reaction, peptide bond formation takes place via the transfer of the growing peptidyl group from the CCA terminus of the P-site tRNA to the aminoacyl group attached to the CCA terminus of the A-site tRNA (37). This reaction is facilitated through interactions with conserved 23S rRNA bases located at the center of the PTC: A2451, U2506, U2585, C2452, and A2602 (11).

Finally, the now-empty tRNA that previously resided in the P-site translocates to the E-site, where it will subsequently exit the ribosome; the A-site tRNA, esterified to the growing peptide chain, translocates to the P-site. The E-site, although still not fully understood, contributes significantly to the fidelity and process of translation. Importantly, the E-site maintains the reading frame of the ribosome, permitting faithful translation of genetic information. Having a tRNA molecule in the E-site prevents loss of the ribosome's reading frame (39). The results of numerous structural biology breakthroughs have provided biochemists, engineers, and synthetic biologists with the foundation to probe the role of PTC nucleotides.

Each of the parts of the ribosome can be thought of as components of a machine. These components work harmoniously in a rhythmic dance, performing the task of decoding, synthesizing, and excreting the proteins of the cell. In the following sections, we discuss how the ribosome's parts have been designed and engineered for a better understanding of their roles in the overall process of translation and, furthermore, how these parts can be repurposed for novel functions.

3. ENGINEERED RIBOSOMES FOR ADVANCING BASIC BIOLOGY

Learning by building is a powerful approach for probing fundamental biological processes. These insights into basic biology permit the efficient engineering of these machines for novel purposes. From understanding how the ribosome assembles; to how it decodes, accepts, and processes tRNAs charged with amino acids; to the intricacies in how it dynamically rotates and moves—understanding how the ribosome functions represents basic biology discoveries that also embody subtle but significant engineering feats.

3.1. Reconstructing the Ribosome from Its Parts: Ribosome Reconstitution

Structure, function, and assembly studies of the ribosome have been greatly facilitated by reconstitution of both the large and small subunits. These advances have defined necessary components, elucidated key ribosomal mechanisms, and facilitated the construction of minimal cell systems.

The *E. coli* 30S ribosomal subunit was the first to be reconstituted. In 1968, Traub & Nomura (40) demonstrated that a mixture of the 30S r-proteins (TP30) could be reconstituted with 16S rRNA to form fully functional 30S subunits. This conventional 30S subunit reconstitution protocol involves a one-step incubation at 20-mM Mg^{2+} and 40°C (40). In 1999, Culver & Noller (41) improved the reconstitution of the small subunit, alleviating challenges in cost, time, resources, and cross-contamination associated with traditional reconstitution. They developed an ordered assembly protocol that allowed efficient reconstitution of 30S subunits using the purified

iSAT: integrated synthesis, assembly, and translation

S150: ribosome-free *Escherichia coli* crude cell extract

recombinant proteins. Their reconstitution method represented the first means for exhaustive mutational analysis of each r-protein of the small subunit (41). More recently, Maki & Culver (42) showed that 30S assembly can be facilitated at lower temperatures by chaperones.

The reconstitution and assembly of the large 50S subunit was not elucidated until after the 30S subunit. Owing to the increased number of r-proteins (32) and larger size of rRNA (the 23S rRNA is 2,904 nucleotides and the 5S rRNA is 120 nucleotides), bottom-up 50S assembly was significantly more complex compared with the small subunit; Nierhaus & Dohme (43) first achieved it in 1974, and it was extended in the following years (44). The conventional *E. coli* 50S subunit reconstitution protocol involves a nonphysiological two-step high-temperature incubation, first at 4-mM Mg^{2+} and 44°C and then at 20-mM Mg^{2+} and 50°C. Green & Noller (45) later carried out total reconstitution of the 50S subunit with an in vitro transcribed 23S rRNA, though it was inefficient.

Although studies using the conventional reconstitution approach with *E. coli* ribosomes have revealed key parts of today's knowledge regarding the assembly of the ribosomal subunits from the deconstruction and reconstitution procedures described above (46–48), inefficiencies in reconstitution make the construction and analysis of engineered variants difficult (49). For example, conventionally reconstituted 50S subunits made with in vitro transcribed 23S rRNA (lacking the naturally occurring posttranscriptional modifications) are up to 10,000 times less efficient in reconstitution than those using mature 23S rRNA as measured by the fragment reaction, where single peptide bonds are formed on isolated 50S subunits (45, 49). Furthermore, the nonphysiological two-step conditions for 50S assembly preclude coupling of ribosome synthesis and assembly in a single integrated system.

To overcome limitations in conventional reconstitutions, Jewett and coworkers developed the integrated synthesis, assembly, and translation (iSAT) method, which enables efficient one-pot coactivation of rRNA transcription and processing, assembly of rRNA with native r-proteins (TP70) into *E. coli* ribosomes, and synthesis of functional proteins from assembled ribosomes in a S150 crude cell extract lacking native ribosomes (**Figure 3**) (50). This work is significant

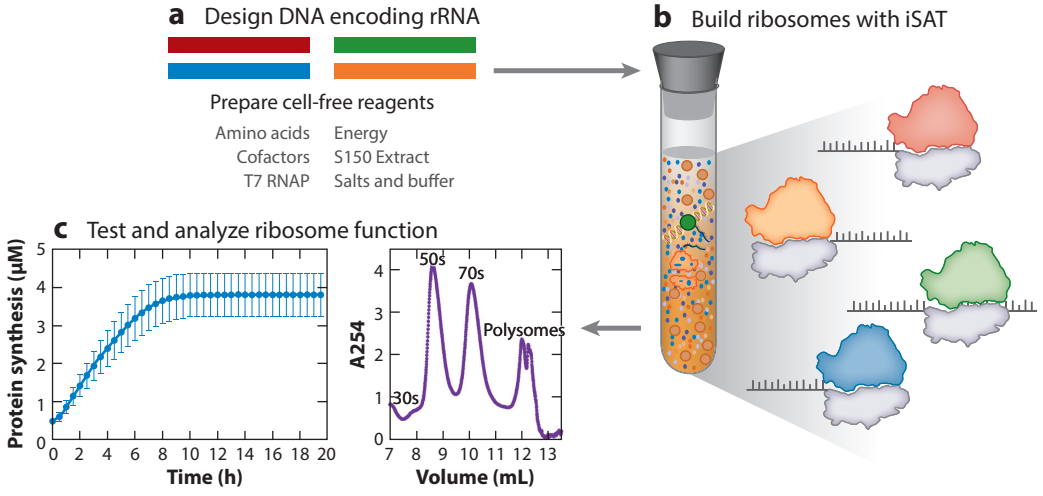

Figure 3

In vitro synthesis, assembly, and translation (iSAT) for ribosome design and engineering. (*a*) The iSAT method enables efficient one-pot co-activation of ribosomal RNA (rRNA) transcription, (*b*) assembly of rRNA with native ribosomal proteins into *Escherichia coli* ribosomes, and (*c*) synthesis and analysis of functional proteins from these ribosomes in a S150 extract lacking native ribosomes. This purely in vitro strategy for *E. coli* ribosome construction offers a powerful technique to study and exploit engineered ribosomes (50–52).

because it is the first demonstration in which rRNA from any organism has been co-synthesized and assembled into functional ribosomes in a single compartment in vitro. A novel feature of iSAT is that it mimics cotranscription of rRNA and ribosome assembly as it occurs in cells. iSAT activity has been improved >1,000-fold by optimizing extract preparation methods, tuning rRNA transcription levels, alleviating substrate limitations, and using macromolecular crowding and reducing agents (50–52). A key feature of this system is the ability to generate rRNA variants by simply changing the rDNA input, making it an attractive platform for probing the ribosome in vitro and engineering modified ribosomes. Indeed, the concept, carried out in a purified translation system, was recently extended to evolving the 16S rRNA (53).

Beyond engineering the ribosome, the in vitro construction of ribosomes is a topic of rapidly growing interest in systems and synthetic biology for designing and building minimal cells to understand the origins of life (54, 55). The idea is that one could enable self-replication by pooling together essential purified biological macromolecules, their genes, and their small-molecule substrates. Toward this goal, researchers in the Church lab reported on the construction of functional *E. coli* 30S ribosomal subunits using a defined, purified cell-free system lacking ribosomes (PURE Δribosome system) under physiological conditions (56). Using this system, the authors achieved high engineering flexibility, effectively permitting the screening and testing of ribosome biogenesis factors and assembly cofactors, and their impacts on and functions in ribosome assembly (57). Importantly, their method was used to demonstrate that 30S ribosomes can be constructed from only in vitro synthesized 30S r-proteins and in vitro transcribed 16S rRNA. Their small subunits assembled from in vitro synthesized parts resulted in ribosomes with 17% activity as compared with wild-type ribosomes. Additionally, the authors further demonstrated the synthesis of 50S r-proteins and the co-synthesis of 30S and 50S r-proteins in the PURE system. Thus, their integrated method can potentially be applied to construct ribosomes completely from the ground up, endowing even greater control over finely probing various parts of the ribosome.

Traditional and engineered ribosome reconstitution efforts described above have been highly impactful on the ribosome field. Indeed, the ability to deconstruct and reconstruct the ribosome precisely defines its parts and functional moieties, which is crucial in future efforts to engineer it for novel purposes. Importantly, it changed the perspective with which scientists and engineers viewed the ribosome, from a black-box macromolecular machine to a precise machine with well-defined parts. In the text that follows, we describe further efforts to probe and understand those parts.

3.2. Probing the Ribosome's Active Site

The PTC is the active site of the ribosome. It resides in domain V of the 23S rRNA in the large 50S subunit and is composed solely of rRNA. The PTC is the site of peptide bond formation, tRNA positioning, peptide stalling, and antibiotic binding. This catalytic core holds many key functions and has been studied using biochemistry, genetics, structural biology, and engineering.

Many labs have studied the PTC and its role in translation using crystallography and structural studies, chemical crosslinking and foot printing, and genetics. These techniques represent the most established methods for probing the PTC and its role in peptide bond formation. Groundbreaking bacterial ribosome structures provided the first suggestions of the role of rRNA in translation (11). Pioneering work from the Noller lab implicated important contacts between the tRNA and nucleotides in both the large- and small-subunit rRNAs (58, 59). Other classical techniques for probing the PTC include puromycin assays, translation fidelity assays, nuclear magnetic resonance, ribosome foot printing, and sucrose gradient fractionation. Notably, engineering efforts in basic biology have probed the PTC through mutational studies. In 2006, Sato and coworkers

(60) carried out a comprehensive genetic selection revealing essential nucleotides in the PTC. Specifically, they leveraged systematic selection of functional sequences by enforced replacement, wherein they generated randomized rRNA libraries of critical regions in and around the PTC and then introduced these into *E. coli* cells. This study resulted in elucidation of essential bases within the PTC. However, like many mutational studies of the active site, dominant lethal phenotypes prevented complete mutational characterization. Toxic mutation sequences that did not produce transformants in vivo were not further investigated. Studies of these lethal mutations are critical, though, as they improve our understanding of the ribosome and antibiotic resistance and open doors to engineering efforts. For example, in their 2007 paper, Yassin & Mankin (61) leveraged deleterious mutations in the large ribosomal subunit to identify potential antibiotic binding sites.

All of the works described above have helped us gain insight into how the ribosome catalyzes peptide bond formation, translocation, and peptide release. In **Table 1**, we organized a noncomprehensive list of 23S rRNA active site nucleotides studied, how they were studied, and their proposed roles in translation. Basic biology studies of the PTC have been invaluable; however, investigations into the ribosome's PTC have so far been limited, primarily owing to the dominant lethal phenotype of many mutants, as described above. Dominant growth defects caused by mutations in the ribosome can preclude identification of mutants that confer desired functions. In Sections 4 and 5, we discuss more avenues for filling this gap in knowledge and facilitating engineering of the ribosome's active site. Below, we describe three important techniques for studying ribosome function, which have been crucial for enabling studies of rRNA mutants.

3.3. Specializing the Ribosome

To overcome the dominant lethality problem, Hui & de Boer (62) pioneered a specialized ribosome system using orthogonal SD (o-SD) sequences in specific reporter protein mRNAs that target orthogonal anti-SD sequences in 16S rRNA (**Figure 4**). The authors accomplished this by altering the SD sequence preceding the human growth factor gene from 5′ GGAGG (wild type) to 5′ CCTCC or 5′ GTGTG. Doing this prevented wild-type bacterial ribosomes from recognizing and translating these modified mRNAs. However, upon changing the aSD region in the 3′ end of the 16S rRNA gene from 5′ CCTCC (wild type) to 5′ GGAGG or 5′ CACAC, they could restore Watson-Crick base pairing between the complementary sequences and achieve significant expression of the human growth factor. This groundbreaking work not only opened the door to numerous basic biology studies but, importantly, also initiated in vivo ribosome engineering efforts, discussed below.

3.4. Isolating Ribosomes by Affinity Purification

As noted above, a purely in vitro strategy for ribosome construction offers a powerful solution for studying and exploiting engineered ribosomes. With the development of ribosome assembly and reconstitution, as well as high interest in ribosome mutational studies, researchers have become interested in an efficient, easy, and high-throughput method for purifying the bacterial ribosome. Particularly challenging aspects have been mutations in the rRNA of the PTC, which often confer dominant lethal phenotypes in *E. coli*. This makes it prohibitively difficult to synthesize and express pure populations of mutant ribosomes. Often, researchers have resorted to expressing mixed populations of mutant and wild-type ribosomes and subsequently parsed out the noise contributed by wild-type ribosomes. Recently, however, three approaches to selectively tag and purify mutant ribosomes from a mixed population have been developed.

Table 1 Proposed roles of select 23S rRNA nucleotides in translation

rRNA nucleotide	Proposed role in translation	Reference
A2451	■ First proposed to be involved in peptide bond formation. Crystal structures show that it is closest (3 Å) to the peptide bond being catalyzed. ■ Studies demonstrate that all base changes confer dominant lethal phenotype. ■ Proposed to be involved in peptide release based on kinetic studies. ■ Proposed to stabilize the ordered structure of the PTC.	Nissen et al. (11) Youngman et al. (64) Thompson et al. (154)
G2447	■ Proposed involvement in peptide bond formation. Believed to render catalytic properties to A2451. ■ G2447A and G2447C maintain cell viability. G2447A synthesizes protein at rates similar to wild type.	Nissen et al. (11) Thompson et al. (154)
G2251, G2252, and G2583	■ Base pairs with P-site tRNA to position tRNA acceptor arms.	Nissen et al. (11)
G2253 and A2450	■ Base pairs with A-site tRNA to position tRNA acceptor arms.	Nissen et al. (11)
A2451, U2506, U2585, C2452, and A2602	■ Proposed to be involved in peptide bond formation, based on crystal structure. ■ A2451, U2506, U2585, and A2602 proposed to be involved in peptide release based on kinetic studies. ■ Release factors may promote conformational rearrangements of U2585 to promote peptide release.	Nissen et al. (11) Youngman et al. (64) Schmeing et al. (155)
C2394	■ Contacts E-site tRNA. C2394G impacts ribosome's ability to bind deacylated tRNA to the E- and P/E-sites and reduces translation fidelity.	Schmeing et al. (156) Moazed & Noller (157)
G2112 and G2116	■ Contacts E-site tRNA.	Schmeing et al. (156) Moazed & Noller (157)
G2655	■ Part of the SRL, which interacts with and binds EF-G. ■ G2655C is lethal in vivo. Ribosomes carrying the G2655C mutation are defective in EF-G-driven translocation.	Leonov et al. (63)
A2450 and C2063	■ Proposed to form an A-C wobble base pair essential for tRNA translocation.	Chirkova et al. (158)
C2063, A2451, U2506, and A2602	■ Proposed (based on crystal structure) to surround the GGQ motif of RF2 during translation termination and peptide release. ■ Mutational studies of A2602 suggest that this nucleotide is most essential for hydrolysis.	Youngman et al. (64) Petry et al. (159) Polacek et al. (160)
C2573 and A2572	■ Mutations do not impact aa-tRNA accommodation, peptide bond formation, or fidelity of aa-tRNA selection. The mutations do impair RF2-catalyzed peptide release.	Burakovsky et al. (70)
A2058 and A2059	■ Mutations A2058G and A2059G confer resistance to macrolides such as clindamycin and erythromycin.	Sigmund & Morgan (161)
G2057	■ G2057A confers low-level resistance to erythromycin in *Escherichia coli*.	Ettayebi et al. (162)
A2062	■ A2062G confers clindamycin resistance in *E. coli* and macrolide resistance in other bacteria.	Cochella & Green (163)
A2503	■ Located at the start of the exit tunnel. ■ Involved in nascent-peptide recognition and ribosome stalling. ■ Only A2503G is viable; all other mutations are dominant lethal.	Vázquez-Laslop et al. (68)
U2609	■ U2609C mutation renders *E. coli* cells resistant to two ketolides: telithromycin and ABT-773.	Garza-Ramos et al. (164)

Abbreviations: aa-tRNA, aminoacyl-transfer RNA; PTC, peptidyl transferase center; rRNA, ribosomal RNA; SRL, sarcin-ricin loop; tRNA, transfer RNA.

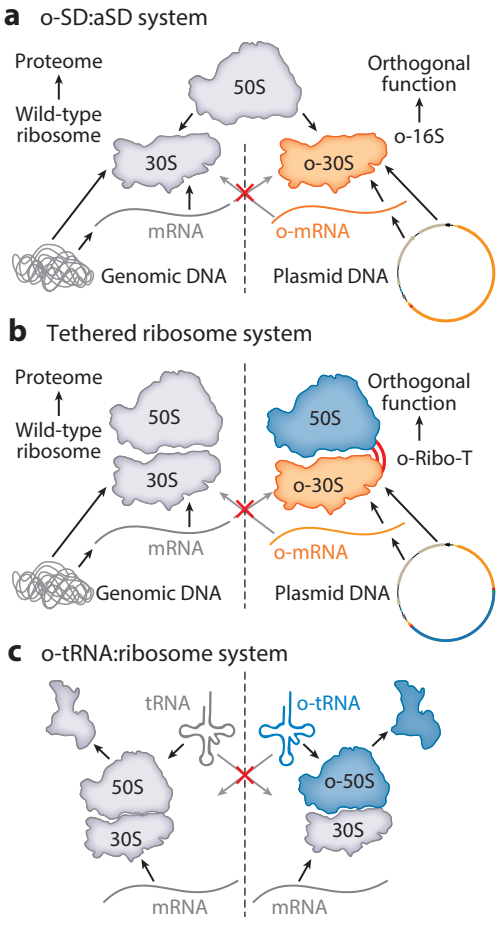

Figure 4

Orthogonal ribosomes. (*a*) For an orthogonal Shine-Dalgarno:anti-Shine-Dalgarno (o-SD:aSD) system, tuning of the SD sequence of the mRNA in conjunction with the aSD sequence of the 16S rRNA allows for orthogonal message decoding. Of note, the 50S subunit is not orthogonal and freely associates with both wild-type and orthogonal 30S subunits (36, 78). (*b*) In the tethered ribosome system, the entire ribosome, including the catalytic 50S subunit, is orthogonal and does not interfere with the wild-type ribosomes (151). (*c*) In the orthogonal transfer RNA (tRNA):ribosome system, tuning the interaction between the 3′ tRNA sequence and the ribosome's 23S rRNA establishes orthogonality to wild-type tRNAs and wild-type 23S rRNAs (153). Abbreviation: o-mRNA, orthogonal messenger RNA.

In 2003, Leonov and coworkers (63) developed a method based on site-directed incorporation of a streptavidin binding tag into functionally neutral sites of the large-subunit 23S rRNA. Using this tag, they successfully leveraged affinity chromatography to isolate ribosomes carrying a lethal G2655C mutation. The authors then used this technique to further demonstrate that G2655C plays a key role in interacting with EF-G in tRNA translocation in the ribosome. Youngman et al. (64) described the use of another affinity-tagging system for the purification of mutant ribosomes. They inserted a 17-nucleotide RNA hairpin that binds the MS2 phage coat protein into the terminal loop of *E. coli* 23S rRNA helix 98 (**Figure 5**). Using their purification system, they isolated and analyzed four universally conserved nucleotides in the PTC: A2451, U2506, U2585, and A2602. Using kinetic analyses, the authors found that each of these mutant ribosomes possesses

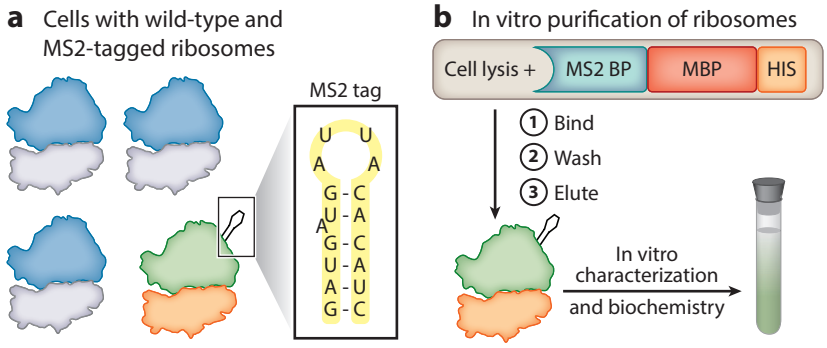

Figure 5

Cartoon depiction of the MS2 ribosome purification technique. (*a*) A 17-nucleotide RNA hairpin that binds the MS2 phage coat protein is engineered into the terminal loop of *Escherichia coli* 23S rRNA helix 98. (*b*) Upon lysing cells possessing the tagged ribosomes, binding the ribosomes to an affinity matrix, washing, and eluting, purified MS2-tagged ribosomes can be characterized and studied (64).

defects in peptide release, suggesting that these nucleotides are intimately involved in peptide release but not in peptide bond formation. Similarly, Hesslein and coworkers (65) leveraged a U1A protein-binding tag inserted onto the 23S rRNA to probe the conserved A2450-C2063 wobble pair within the PTC. Another example of ribosome purification came in 2009, when Ederth and coworkers (66) developed a novel strain of *E. coli* (JE28) by fusing the nucleotide sequence encoding a hexa-histidine affinity tag at the 3′ end of the single-copy *rplL* gene (encoding the large-subunit r-protein L12) in the *E. coli* MG1655 strain. The JE28 strain produces a homogeneous population of ribosomes with a (His)6-tag at the C termini of all four L12 proteins. Additionally, the authors developed and optimized a high-throughput method for a single-step purification of tetra-(hexa-His)-tagged 70S ribosomes from this strain using affinity chromatography. The authors further describe how this method can be adapted for purification of ribosomal subunits and mutant ribosomes (66).

Developments in ribosome purification and isolation have allowed studies into mutant ribosomes, defining the role of key rRNA nucleotides in translation. Future efforts to understand the impact of ribosomal mutations and develop multiply mutated ribosomes for novel purposes will undoubtedly leverage these techniques to isolate and probe new modified machines.

3.5. Developing and Using the Squires Strain

As described above, many studies on ribosomal structure and function have been carried out on the *E. coli* ribosome in vitro, which has enabled precise, well-controlled findings on ribosome structure and function. Verification of these findings in vivo enables further insight into ribosome function in a cell but can be hindered by the presence of wild-type ribosomes. In 1999, Asai et al. (67) successfully developed an *E. coli* strain (Δ7 prrn) commonly referred to as the Squires strain, in which all seven chromosomal rRNA operons were deleted and all rRNA was transcribed from a single plasmid–based rRNA operon. The plasmid containing the rRNA operon can be swapped with another plasmid coding for a variant rRNA operon, thereby allowing isolated studies of an rRNA variant (**Figure 6**). This strain has enabled study of the basic biology of engineered ribosomes in vivo.

In one example, Vázquez-Laslop et al. (68) leveraged this strain to study mutations of A2503 in the 23S rRNA. Specifically, the authors demonstrated that of the three possible mutations, only

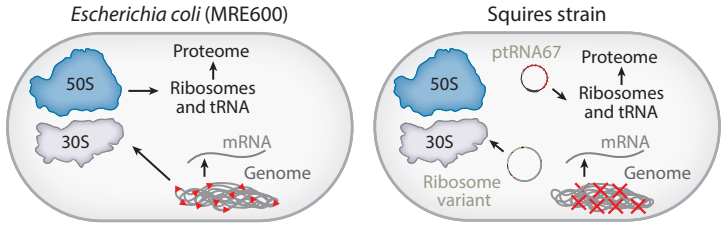

Figure 6

The Squires strain. The Squires strain (67) is an experimental system that can be used for studying and engineering ribosomes. In traditional *Escherichia coli* strains (such as MRE600), ribosomal RNA (rRNA) genes are highly repeated on the genome (*red triangles*). Ribosomes are synthesized based on seven genomic copies of the ribosomal operon. In the Squires strain, all seven chromosomal rRNA operons are deleted. A single *E. coli* rRNA operon carried by a multicopy plasmid (ptRNA67) supplies 16S rRNA and 23S rRNA to the cell. Using this strain, ribosome variants can be introduced into the Squires strain cells, and ptRNA67 removed. This permits the in vivo study of ribosome variants without the contamination of endogenous wild-type ribosomes. Abbreviation: mRNA, messenger RNA.

A2503G was capable of supporting cell growth in the absence of wild-type ribosomes. Additionally, Sahu et al. (69) used this strain to study the functional replacement of two highly conserved tetraloops in the bacterial ribosome. Using this strain, the authors estimated the frequency of missense error and nonsense error read-through, in addition to assessing whether mutated ribosomes could sustain the life of the cell. Furthermore, Burakovsky and coworkers (70) studied ribosome accommodation gate mutations using the Δ7 prrn strain. The authors designed a variety of mutations at C2573 and A2572 and found that mutations at these positions did not impact aa-tRNA accommodation, peptide bond formation, or the fidelity of aa-tRNA selection. However, the mutations did impair RF2-catalyzed peptide release.

The results of these studies, and many more, with the Squires strain demonstrate that it is indeed possible and important to assess the impacts of ribosome design and engineering in the context of the cell. The Squires strain has been invaluable in synthetic biology research, as described in the sections below.

3.6. Summary

Advances in basic biology have unlocked fundamental knowledge and developed tools for manipulating the ribosome. Synthetic biology, a burgeoning field of research, has been able to leverage these advances to develop new translation systems that address societal and scientific needs. In the sections below, we summarize advances and efforts in synthetic biology to design and engineer the ribosome.

4. ENGINEERED RIBOSOMES FOR SYNTHETIC BIOLOGY

The precise and robust nature of the translation apparatus has attracted significant efforts to engineer it for compelling applications in energy, medicine, and materials (71–76). For example, establishing novel message-reading capabilities of the ribosome could result in the ability to run orthogonal genetic programs inside a living cell (77, 78), as well as expansion of the genetic code beyond the canonical triplet codon-anticodon model (79) or even the canonical four-base code (80). Given this promise, recent efforts to expand and enhance the function of the ribosome for synthetic biology applications have produced important milestones in ribosome engineering at

an accelerated pace. Below, we examine the ribosome and protein translation machinery that has been engineered for applications in synthetic biology (**Figure 1**).

nsAA: nonstandard amino acid

4.1. Engineering Ribosome-Associated Translation Machinery

Pioneering works have opened the possibility to overcome the sparse chemical space afforded by natural translation. These pioneering efforts have shown site-specific incorporation of more than 150 nonstandard amino acids (nsAAs) into proteins using an engineered translation apparatus to generate biological insights and new applications (81–83). The natural ribosome is also capable of producing polymers with nonpeptide backbones (e.g., polyesters) (84–86) and has even incorporated select exotic monomers (86–95). Photocaged (96), fluorescent (97), and bioorthogonally reactive (98, 99) monomers have provided new ways to study protein structure, dynamics, and posttranslational modifications (81, 100–110). Further, the generation of proteins with nonnatural chemistry has enabled the synthesis of novel therapeutics (103–105, 111, 112) and genetically encoded materials (102, 113–115). As a result of these impressive efforts, expanding the genetic code has emerged as a major opportunity in synthetic and chemical biology (100, 116–118).

Given the ribosome's flexibility in accommodating the ~20 different standard amino acid functional groups found in nature, the bulk of initial efforts in engineering the translation apparatus has focused on the three peripheral machines involved in translation: tRNAs, aaRSs, and elongation factors (119, 120). The key need is to prepare aa-tRNA substrates that can be polymerized by the ribosome bearing the nsAAs. This has been done using protein- and RNA-based enzymes. Often, the ability to generate nsAA-tRNA monomers is achieved by evolving orthogonal aaRS::orthogonal tRNA pairs. These orthogonal translation components do not recognize natural amino acids or cross-react with native translational machinery yet can deliver nsAA-tRNA monomers to the ribosome. To achieve this task, synthetic biologists frequently use the *Methanocaldococcus jannaschii* tyrosyl synthetase, *Methanosarcina barkeri/Methanosarcina mazei* pyrrolysyl synthetases, and the yeast tryptophanyl synthetase. In such efforts, the substrate preference of the amino acid binding pocket of an aaRS is modified, thereby generating a novel, orthogonal aa-tRNA molecule for incorporation into a protein. In one illustrative example, Amiram et al. used a multiplex automated genome engineering (MAGE) strategy (121, 122) to generate highly selective and orthogonal aaRS/tRNA pairs that enhance the insertion of nsAAs into proteins in vivo (123). Guided by the 3D crystal structures, they simultaneously varied 12 residues surrounding the amino acid side chain binding pocket and five residues at the aaRS-$tRNA_{CUA}$ anticodon interface to generate $>10^9$ variants (123). From this library, they isolated aaRS variants with up to 17-fold (*p*-acetyl phenylalanine: pAcF-m4) and 25-fold (*p*-azido phenylalanine: pAzF-m4) higher activity than either progenitor enzyme. Importantly, library diversity can be modified by increasing the number of MAGE cycles, changing the mutagenic ssDNA pool, targeting different aaRS regions, or changing the conditions applied during negative selection, without the need for plasmid reconstruction, retransformation, and subsequent loss of library diversity. Such efforts hold promise for future work in improving translation machinery outside of the ribosome for genetic code expansion.

As an alternative approach, Flexizymes can also be used to generate novel aa-tRNA molecules. Flexizymes are synthetic RNA molecules with catalytic capabilities (thereby classifying them as ribozymes), capable of acylating tRNA molecules with an activated amino acid monomer (124–127). This system requires activation of the monomer with a leaving group, such as cyano-methyl ester, dibenzyl ester, or chlorobenzyl thioester, and tolerates a large diversity of side chains and backbones of amino acid monomers being acylated onto the tRNA. Flexizymes recognize the 3′ end of a tRNA molecule (R-CCA-3′, where R is a G or an A depending on the tRNA) and acylate the terminal 3′ A with the activated monomer, forming the aa-tRNA molecule. Interestingly, they can

also act on activated monomers with alternate backbones, such as β-, D-, and N-alkyl amino acids, as well as hydroxy acids. This technology is less synthetically laborious than traditional chemical ligation with an activated nucleotide (pdCpA) and enables incorporation of many different nonstandard monomers into sequence-defined polymers. The Suga group has been instrumental in developing, implementing, and advancing Flexizyme technology and has so far developed three kinds of Flexizymes: aFx, dFx, and eFx. Furthermore, the Suga lab has demonstrated the synthesis of peptides containing α-L-nonstandard-, β-, D-, N-methyl-, N-acyl-, and N-alkyl-amino acids, as well as synthesis of sequence-defined polyesters (128–136), allowing researchers to stretch the bounds of possible substrates that can be used by the ribosome.

Beyond engineering the process of loading amino acids onto tRNAs, recent work has been carried out to study, understand, and engineer the tRNA molecule itself to facilitate nonstandard amino acid incorporation at single and multiple positions. In their 2017 paper, Katoh et al. (137) engineered the D-arm and T-stem of tRNA to enhance D-amino acid incorporation. Specifically, they leveraged their previous basic science breakthroughs to rationally design an optimal tRNA. In their first set of prior work, Katoh and coworkers (135) demonstrated that the T-stem sequence on aa-tRNAs modulates strength of interactions with EF-Tu, which can in turn be exploited to improve incorporation of nsAAs. In another breakthrough, a collaborative effort between the Suga and Rodnina labs demonstrated that translation factor EF-P alleviates ribosomal stalling through recognition of a D-arm motif found in $tRNA^{Pro}$ (138). EF-P effectively accelerates peptidyl transfer when a proline is being processed. Based on these two discoveries, Katoh and coworkers logically engineered tRNAs' D-arm and T-stem sequences to improve consecutive incorporation of D-amino acids as well as an α-disubstituted amino acid. The chimeric tRNA possessed the T-stem of $tRNA^{GluE2}$, which was previously shown to have a higher binding affinity to EF-Tu, and the D-arm of $tRNA^{Pro1}$. As a result of this engineering, the authors successfully synthesized two macrocyclic peptides possessing four and five consecutive D-amino acids.

4.2. Limitations of Wild-Type Ribosomes for Genetic Code Expansion

Although many types of monomers can be incorporated into a growing polymer chain by the ribosome, incorporation of backbone-modified monomers remains limited in the total peptide length and efficiency. Backbone-modified β- or D-amino acids face a challenge in the ribosome, specifically in the ribosome's catalytic active site and nascent peptide exit tunnel (135, 139). In certain cases, such as the naturally modified amino acid phosphoserine, engineered elongation factors (EF-Sep) aid the ribosome during the polymerization reaction for inefficiently accommodated substrates. For example, engineering of EF-Sep and the tRNA body has improved the binding and delivery of phosphoseryl-tRNA to the ribosome (140), hence enhancing incorporation of phosphoserine into proteins.

Modifying nonribosomal factors involved in translation aids in incorporation of challenging unique monomers as well. Proline, one of the naturally occurring amino acids, has been known to stall the ribosome (141). The presence of EF-P, a specialized elongation factor, effectively alleviates the stalling caused by stretches of amino acid sequences containing this monomer (142, 143). The pyrrolidine side chain present in proline presents a steric deviation from the other 19 amino acids, as the α-amino group is relatively displaced. Because the ribosome is hypothesized to catalyze peptide bond formation through careful positioning of the reactive moieties in the PTC (144) and the consequential lowering of the entropy of peptide bond formation (145), the spatial deviation as observed in proline processing stalls the ribosome. Proline-driven stalling of the ribosome may indicate that the ribosome is sensitive to the backbone geometry of the monomer substrate, which has been useful for alleviating ribosome stalling with backbone-modified nsAAs.

In 2016, Katoh and colleagues (135, 138) demonstrated that supplementing elongation factors (EF-G and EF-Tu) into in vitro translation reactions helps alleviate ribosome stalling, thus allowing multiple consecutive incorporations of D-amino acids by the ribosome. However, such approaches may face a fundamental limit in accommodation by the ribosome active site. For this reason, engineering the ribosome may represent a key target for expanding such monomers in the future. In the following section, we discuss examples of ribosome engineering efforts for expanding the genetic code and the chemistry of life.

4.3. Engineering the Ribosome's Active Site

The fundamental limits of the chemistry that the ribosome can carry out are currently unknown, but based on recent work from Englander and colleagues highlighting the discrimination of the PTC against D-amino acids (139), use of exotic monomers (e.g., β-, D-, or γ-amino acids) by the wild-type ribosome may be limited. Ribosome engineering may address this key limitation. Recent work by the Hecht and Schepartz groups mutagenized key residues in the ribosome's active site known to be involved, at least spatially, in peptide bond formation between the P-site and A-site aa-tRNA molecules (119, 146–148). Using puromycin-based screening, they isolated mutant sequences of the 23S rRNA that include residues 2057–2063 and 2492–2507 (**Figure 7**). Such mutant ribosomes have been isolated and demonstrated to increase incorporation of D- and β-amino acids, both in vitro and in vivo (119, 146–148). However, the isolated mutant ribosomes alone have not been shown to incorporate multiple or consecutive residues of backbone-modified monomers. Multiple, consecutive incorporation may trigger stalling of the ribosome that is not alleviated by the currently isolated mutant ribosomes, providing a future challenge. In addition, mutant ribosomes have been identified only for D- and β-amino acid incorporation, each with distinct 23S rRNA sequences. There may be no holy grail sequence of a mutant 23S rRNA that permits incorporation of all backbone-modified monomers, but rather, each exotic monomer may be best incorporated by a specific mutant ribosome. In this case, efforts in ribosome engineering should apply toward a variety of exotic monomers, given the evidence that each exotic monomer may require a distinct mutant ribosome for polymerization.

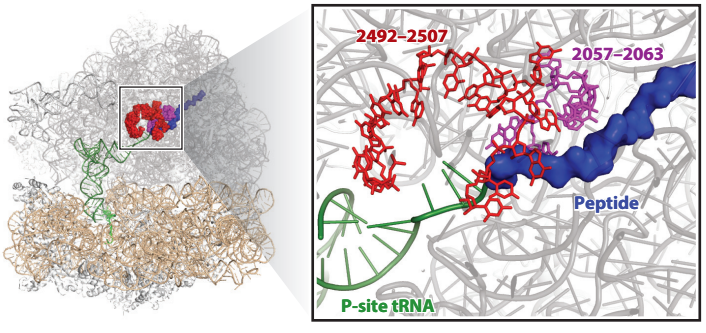

Figure 7

23S ribosomal RNA (rRNA) residues in the peptidyl transferase center engineered for new chemistries. Featured in work by both the Hecht and Schepartz groups, key residues in the 23S rRNA (2057–2063 and 2492–2507) are highlighted in magenta and red, respectively. The rest of the 23S rRNA and 16S rRNA are shown in gray and wheat, respectively. These key residues were selected for mutation based on their role in peptide bond formation and their vicinity to the 3′ end of both the A-site tRNA and P-site tRNA (*green*), which carries the activated amino acid monomer for polymerization. The messenger RNA (mRNA) is shown in lime for reference, near the bottom of the tRNAs (119, 146–148).

Efforts to engineer the ribosome's active site for novel functions remain among the most complex challenges synthetic biologists face, and the lessons learned from such efforts, including fundamental knowledge, novel function, and experimental techniques, can be leveraged to design and engineer parts of the ribosome beyond the PTC. Below, we describe a different approach to repurposing the ribosome, primarily in establishing orthogonal translation systems with orthogonal ribosomes. Orthogonal ribosomes may hold the key to accelerating ribosome design and engineering efforts, as well as unlocking new functions in ribosomes.

4.4. Orthogonal Ribosomes: Message Decoding, Transfer RNA Interactions, Subunit Tethering

Expanding the decoding and catalytic capabilities of the ribosome often occurs 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 the endogenous polypeptides and ideally exclusively translates only 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.

Initial efforts focused on the small 30S subunit of the bacterial ribosome. The small subunit is primarily responsible for decoding the mRNA message (14). As mentioned above, 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 population of engineered mRNAs, while simultaneously excluding them from translating endogenous cellular mRNAs. In recent years, the Chin lab has extended and improved the approach pioneered by Hui and de Boer in several important ways. For example, by testing various combinations of bases that comprise the SD (on the mRNA) and the aSD (on the 16S rRNA) sequences, Rackham & Chin (78) identified new pairs of SD:aSD sequences that enable robust orthogonal translation (**Figure 4**). Specifically, the authors identified o-SD sequences by randomizing the −13 to −7 positions in the mRNA and subjected this randomized library to negative selection, such that any o-SD sequences translated by wild-type ribosomes were eliminated. With the remaining sequences, a plasmid coding for the 16S rRNA was introduced, with randomization of eight nucleotides, including the aSD sequence, that are hypothesized to play a role in ribosome-mRNA interactions. Specifically, nucleotides 722, 723, and 1536–1541 of the 16S rRNA (*E. coli* ribosome numbering) were selected. In a positive selection, the gene for chloramphenicol acetyl transferase (CAT) coupled with each o-SD sequence, and variants of the 16S rRNA library best able to translate CAT, and therefore survive, were selected and identified. These identified pairs of o-SD:aSD sequences were instrumental in subsequent work evolving the ribosome for message decoding capabilities, such as more efficient incorporation of nsAAs at UAG stop codons (149) and decoding of quadruplet codons (79). Building upon orthogonal pairs of SD:aSD sequences, Chubiz & Rao (150) computationally identified additional o-SD:o-aSD pairs. The work is based on the hypothesis that SD:aSD interactions are driven primarily by thermodynamics (Watson-Crick pairing), and best-functioning o-SD:aSD sequence pairs would be less energetically favored to interact with the wild-type SD:aSD sequences while being favored to interact with each other.

Until two years ago, such techniques were limited to the 30S small subunit because the 50S large subunits freely exchange between pools of native and orthogonal 30S subunits. This previously

constrained the engineering potential of the large subunit. However, a significant advance in orthogonal ribosomes came with the advent of tethered ribosomes, in which the bipartite design of ribosomes conserved in all of nature was reconceptualized by linking the 16S and 23S rRNAs into a hybrid chimera (151). These ribosomes with tethered 50S and 30S subunits (termed Ribo-T) can successfully carry out protein synthesis. The construction of Ribo-T is based on identifying all viable circular permutants of the 23S rRNA in the Squires strain. Through this effort, new 5′ and 3′ ends of functional 23S rRNA constructs were identified. This identification was key because the native 5′ and 3′ ends of the 23S rRNA are not spatially close to the 16S rRNA 5′ and 3′ ends according to known crystal structures of the ribosome. After identification of functional circular permutants, helix H101 of the 23S rRNA and helix h44 of the 16S rRNA were identified as possible targets for tethering the rRNAs together. After optimization of the RNA linker, referred to as the tether, Ribo-T was shown to successfully conduct protein synthesis, and even to support cell growth in the Squires strain. This represented, for the first time, a completely in vivo orthogonal ribosome in which both subunits are isolated from the wild-type ribosome population (151). Importantly, Ribo-T can operate both as an orthogonal mRNA decoding ribosome that works alongside a wild-type population and as a wild-type mRNA decoding ribosome that can meet cellular demands for sustaining life (**Figure 4**). When operating as an orthogonal ribosome, the catalytically active 50S subunit is sequestered and isolated from the wild-type ribosome population, and therefore can harbor dominant lethal mutations in the 23S rRNA that were not possible in the previous state-of-the-art orthogonal ribosome system. Ribo-T offers an exciting opportunity for ribosome engineering, as it enables in vivo engineering of different parts of the ribosome, namely, the PTC, free from cell viability constraints. Subsequent to the development of Ribo-T, Fried and colleagues (152) described the development of a stapled ribosome, which also featured conjoined ribosomal subunits at H101 and h44. The stapled ribosome was capable of orthogonal message decoding.

Although the development of orthogonal Ribo-T–mRNA systems opens new opportunities for engineering ribosomes in vivo, in vitro systems still 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. For example, Terasaka and colleagues (153) engineered the interaction between tRNA molecules and the 23S rRNA in the ribosomal 50S subunit to establish orthogonal tRNA-ribosome pairs. In this work, the authors identified key residues in the tRNA 3′ end (C74 and C75) that interact with the PTC of the ribosome's 23S rRNA to deliver the aa-tRNA monomer. The corresponding residues in the 23S rRNA (G2251, G2252, and G2553) were mutated, and pairs of tRNA-ribosome variants were tested for peptide synthesis activity. Through screening, the double 23S rRNA mutant (G2251C/G2553C) and double-mutant tRNA (CGA 3′ end, deviated from the wild-type CCA 3′ end) were identified as orthogonal to wild-type ribosomes and tRNAs but active in translating when both the mutant ribosome and mutant tRNA are present (**Figure 4**). Of note, many aa-tRNA synthetases, responsible for generating the aa-tRNA monomer required for protein synthesis, recognize and require the CCA 3′ end of cognate tRNAs; therefore, this work is currently limited to Flexizyme or pdCpA-charged tRNAs for use in in vitro translation systems.

Taken together, recent works in orthogonal ribosomes have pushed the boundaries of ribosome engineering to new heights. They establish targeted populations of ribosomes for engineering that can operate in parallel with the cell's native ribosomes and thereby carry out functions that supplement or are completely separate from the synthesis of natural proteins. We can imagine that such populations of ribosomes can be engineered further and further away from natural functions, toward human-defined functions.

4.5. Summary

Ribosome design and engineering efforts for advancing synthetic biology have opened new doors for exploring poorly understood functions of the ribosome (e.g., antibiotic resistance mechanisms), enabling orthogonal genetic systems, and engineering ribosomes with altered chemical properties. These advancements are built upon knowledge and techniques from basic biology and in turn can help develop tools and insights for elucidating fundamental mechanisms of the ribosome. We expect basic and synthetic biology to become increasingly complementary and believe ribosome design and engineering is a research area keenly positioned for these advancements.

5. PERSPECTIVES, CONCLUSIONS, AND OUTLOOK

This review provides a broad examination of the innovations in our understanding, design, and engineering of the ribosome. The findings, systems, tools, and techniques described provide an overview of the current state of ribosome engineering but are not completely comprehensive. Below, we highlight future challenges and opportunities on the horizon.

One key challenge is the ability to effectively and efficiently evolve ribosomes for new functions. These new functions could include, for example, the production of mirror-image polypeptides that have commercial relevance as peptidomimetic drugs, as novel chiral catalysts, in enzyme resistance, and for racemic crystallography. Other developments may be in the synthesis of sequence-defined polymers for functional materials that cannot be made using existing chemistry. However, enabling new functions in the ribosome is a significant challenge, a challenge that is exacerbated by cell viability constraints, especially in efforts to redesign and reshape parts of the ribosome critical to protein synthesis activity, such as the PTC. Improvements and innovations in in vivo and in vitro ribosome evolution platforms will provide scientists and engineers with unprecedented tools to understand how this machine has evolved, while providing a platform for rapidly evolving novel ribosomes.

Second, current tools in RNA modeling and computational design are not yet capable of designing a ribosome de novo, given the complexity of the ribosome's structure and assembly. Advancements in such tools, as well as freely available online resources (**Table 2**), would allow engineers to rationally design and repurpose the ribosome. The combined efforts of basic, synthetic, and computational biologists would permit the rapid and efficient identification of rRNA targets and the subsequent engineering of those targets.

Another opportunity for investigation lies in the understanding and engineering of the 5S rRNA. As mentioned above, many uncertainties still surround this molecule. What exactly is its role, and why might it be required for translation? Why has the ribosome evolved to have this small and separate rRNA? Engineering efforts carried out with this molecule may reveal the role of this rRNA in translation and polypeptide synthesis.

Broadly, we must continue to build our toolkit. We still lack complete characterization of translation machines. For instance, if one's goal is to incorporate a nsAA with specific characteristics (e.g., long flexible backbone, mirror-image monomer, or specific pKa), what parts should be used to incorporate the monomer into a growing peptide? Which synthetase should be used? Should chemical ligation or Flexizymes be leveraged? We have useful parts and pieces, but they still require characterization (**Table 3**). Thus, these questions remain unanswered, and a thorough characterization of the parts and how or when they are to be used will provide a translation machinery toolkit for basic and synthetic biologists.

Finally, a grand challenge remains in developing and understanding rules and patterns in designing the ribosome. What does it mean to be a ribosome designer? Currently, studies are

Table 2 Freely available online tools, software, and resources for aiding ribosome design and engineering efforts

Resource	Description and potential use
The Center for Molecular Biology of RNA: RNA Center Software	■ A freely available center for RNA software ■ Possesses databases, tools for editing and manipulating rRNA secondary structure, plug-ins for working with ribosome crystal structure files, and links to software for producing sequence logos of RNA sequences
RiboKit (Das Lab, Stanford University)	■ PyMol commands for various RNA manipulation tools ■ RiboVis: a set of useful and short Python functions for rendering RNA and proteins in PyMol ■ RNAMake: a toolkit for designing and optimizing RNA 3D structure; allows alignment between RNA motifs ■ BIERS: Best Inference Engine for RNA Structure
RNAstructure: webservers for RNA secondary structure prediction	■ DuplexFold: predicts the lowest–free energy structure for two interacting sequences, not allowing intramolecular base pairs ■ AllSub: generates all possible low–free energy structures for a nucleic acid sequence ■ Bifold: predicts the lowest–free energy structure for two interacting sequences, allowing intramolecular base pairs
PDB	■ A free archive containing structures of ribosomes (as well as other proteins, nucleic acids, and complex assemblies)
Ribosome Binding Site Calculator (Salis Lab, Pennsylvania State University)	■ Predicts the translation initiation rates of natural bacterial mRNA sequences (validated in many bacterial hosts) and designs synthetic ribosome binding sites for targeted translation initiation rates
RiboVision (Georgia Institute of Technology)	■ Ribosome information viewer, including domain/helix definitions, protein contacts, base pairing, and sequence conservation ■ Archive of rRNA secondary structures annotated with precomputed data, such as domain/helix labels, possible contacts, and conservation ■ Pending update to RiboZones, comprehensive suite of ribosome tools and data sets
Comparative RNA Website (CRW, University of Texas at Austin)	■ Collection of RNA data sets, focusing in comparative analysis (e.g., structure models, sequence alignment) ■ Contains significant metadata on rRNAs yet to be analyzed in detail
Silva: high-quality ribosomal RNA database (Denmark)	■ Large database of rRNA sequences (both prokaryotic and eukaryotic) ■ Both curated/annotated and raw data ■ Tools for phylogenetic analysis of ribosomal RNA sequences, including graphically oriented software (ARB)

Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA.

limited to a few nucleotides (**Table 1**) and frequently explore only single or double mutants. Although these studies have yielded invaluable insights on the role of key nucleotides in ribosome function, fully comprehensive maps of ribosomal variants do not currently exist. Furthermore, the ribosome as a machine works through a dynamic consortium of rRNAs. A platform to enable engineering the ribosome beyond single, double, or even triple rRNA changes across an expansive section of the ribosome would enable transformative engineering opportunities for both basic and synthetic biology applications. Expansive characterization of ribosomal variants will enable a thorough understanding of the form and function of the ribosome's domains, helices, and nucleotides. This complete characterization will additionally provide the groundwork and footing for ribosome evolution targets by providing rules for permissible alterations to this machine.

Table 3 Summary of translation apparatus parts, pieces, and tools for incorporating various nonstandard monomers

Part/piece/tool	Description and use
Engineered tRNAs	■ Designated orthogonal tRNA for acylation with nonstandard monomer. Should not interact with cellular tRNAs and aaRSs. ■ Often paired with orthogonal aaRSs from *Methanocaldococcus jannaschii*, *Methanosarcina barkeri*, *Methanosarcina mazei*, or the mutated yeast tryptophanyl-tRNA synthetase. ■ $tRNA^{GluE2}$ possesses a high affinity for EF-Tu and thus improves incorporation of D-amino acids. ■ A chimeric tRNA bearing the D-arm of $tRNA^{Pro1}$, and the T-stem of $tRNA^{GluE2}$ has been used to further improve incorporation of multiple D-amino acids.
Engineered aaRS	■ Specifically acylate orthogonal tRNAs with a nonstandard amino acid. ■ The tyrosyl-tRNA (*M. jannaschii*), pyrrolysyl-tRNA (*M. barkeri/M. mazei*), and yeast phenylalanyl-tRNA synthetases have been popular starting points for engineering. ■ Works both in vivo and in vitro.
Flexizymes	■ Ribozymes capable of charging tRNAs with monomers containing a carboxylic acid moiety. ■ Does not require primary amine moiety, capable of charging non-amino acids onto tRNA. ■ tRNAs are charged with Flexizymes in vitro then supplemented into translation reactions (genetic code reprogramming). ■ Enabled incorporation of hydroxy acids, β-amino acids, D-amino acids, *N*-acyl/*N*-methyl/*N*-alkyl amino acids, and nonstandard α-amino acids.
pdCpA	■ Activated nucleotide that can be used in chemoenzymatic synthesis of nonstandard monomer-tRNAs. ■ Attached to nonstandard monomer in synthetic in vitro reaction, then ligated to 3′ end of tRNA using ligase enzyme.
EF-Tu/EF-Sep/EF-G/EF-P	■ EF-Tu with an altered amino acid binding pocket allows for site-specific incorporation of Sec into proteins at the UAG codon. ■ EF-Sep is an engineered EF-Tu with an amino acid binding pocket specific to phosphoserine and allows direct incorporation of phosphoserine into proteins. ■ EF-G is proposed to play a crucial role in tRNA translocation in the ribosome and polypeptide elongation, and supplementing EF-G to in vitro translation reactions enables consecutive and improved incorporation of D-amino acids into proteins. ■ EF-P has been used to incorporate multiple D-amino acids into proteins. When used with a tRNA molecule bearing the D-arm of $tRNA^{Pro1}$, D-amino acid incorporation is improved.
Orthogonal SD:aSD pairs	■ Engineering the 16S rRNA's aSD and the corresponding mRNA SD sequence makes possible orthogonal translation, where the orthogonal ribosome selectively translates orthogonal mRNAs. ■ Orthogonal 30S subunits enable selective engineering of ribosome demonstrating improved amber suppression and quadruplet codon decoding for nonstandard amino acid incorporation.
Tethered ribosomes	■ Tethered ribosomes (Ribo-T) link the 23S rRNA and 16S rRNA through RNA tethers, forming a chimeric 16S/23S rRNA-based ribosome. ■ This establishes orthogonal ribosomes, where message decoding and peptide bond formation are sequestered from the wild-type ribosome population. ■ Enables in vivo engineering of both small and large subunits of ribosomes without being hindered by cell-viability constraints.
Schepartz and Hecht ribosomes	■ Engineered ribosomes featuring mutations in 23S rRNA residues that enable improved incorporation of β-amino acids (Schepartz in vivo and Hecht in vitro) and D-amino acids (Hecht). ■ Ribosomes screened/selected using puromycin as a tRNA mimic.

Abbreviations: aaRS, aminoacyl-tRNA synthetase; EF-Tu, elongation factor Tu; mRNA, messenger RNA; Sec, selenocysteine; tRNA, transfer RNA.

FUTURE ISSUES

1. What rapid in vivo and in vitro ribosome evolution platforms can we develop to bypass dominant lethal phenotypes while evolving ribosomes with new functions?
2. How can we leverage computational RNA tools to design a ribosome?
3. How can we begin cataloging engineered parts for incorporating user-defined, exotic monomers into a growing peptide?
4. What is the role of the 5S rRNA, and why might it be required for translation?
5. Are there rules and/or patterns for designing and engineering ribosomes?
6. How can we use evolution in the context of engineering design?

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