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Review

Engineering molecular translation systems

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SUMMARY

Molecular translation systems provide a genetically encoded framework for protein synthesis, which is essential for all life. Engineering these systems to incorporate non-canonical amino acids (ncAAs) into peptides and proteins has opened many exciting opportunities in chemical and synthetic biology. Here, we review recent advances that are transforming our ability to engineer molecular translation systems. In cell-based systems, new processes to synthesize recoded genomes, tether ribosomal subunits, and engineer orthogonality with high-throughput workflows have emerged. In cell-free systems, adoption of flexizyme technology and cell-free ribosome synthesis and evolution platforms are expanding the limits of chemistry at the ribosome's RNA-based active site. Looking forward, innovations will deepen understanding of molecular translation and provide a path to polymers with previously unimaginable structures and functions.

INTRODUCTION

The ribosome is the catalytic core of molecular translation, stitching together natural amino acid substrates into sequence-defined polymers (i.e., proteins) from a genetic template. For decades, the ribosome has been exploited to site-specifically incorporate >200 non-canonical amino acids (ncAAs) into proteins. These ncAAs include non-canonical α-amino acids (e.g., p-azidophenylalanine), as well as cyclic (Katoh and Suga, 2020; Lee et al., 2020c), backbone-extended (e.g., β-, γ-, δ-) (Fujino et al., 2016; Katoh et al., 2020; Lee et al., 2020b; Takatsuji et al., 2019), N-alkylated (Iwane et al., 2016; Kawakami et al., 2013), and oligomeric analogs (Tsiamantas et al., 2020), among others. Site-specific incorporation of such diverse chemical entities into peptides and proteins has yielded new biopolymer structures and functions (e.g., antibody-drug conjugates and macrocyclic foldamer–peptide drugs).

While these pioneering efforts have opened new application spaces, the potential of the translational apparatus remains underexploited. The molecular translation apparatus has evolved over billions of years to prefer α-L-amino acid substrates and to polymerize amide bonds. As such, numerous classes of ncAAs remain poorly compatible with the natural translation apparatus, and several roadblocks have made alteration of the natural translation system difficult. First, engineering molecular translation is the ultimate systems biology challenge. It is difficult to coordinately tune all of the cellular machinery necessary to site-specifically introduce ncAAs into proteins with high efficiency (e.g., ribosomes, transfer RNAs [tRNAs], aminoacyl-tRNA synthetases [aaRSs], and elongation factors). Second, creating new genetic codes with free codons that can be reassigned to ncAAs is challenging, especially in cells. This is because the translation apparatus has been delicately tuned by evolution to use each of its 64 codons for a defined purpose. Third, engineering the ribosome is particularly problematic: maintaining cell viability restricts exploring ribosome sequence mutations and non-canonical substrates; the ribosome sequence design space is endless; and we have only a primitive understanding of how to rationally redesign ribosomal RNA (rRNA) at the ribosome's active site to alter structure and function.

To address these complex issues, new tools and insights are needed. Working both *in vitro* and *in vivo*, novel strategies are emerging that interweave and advance state-of-the-art laboratory evolution, genome synthesis, genome engineering, and synthetic biology technologies to repurpose the translation apparatus for systems and synthetic biology. In this review, we discuss these recent advances. First, we provide background on the biological parts necessary for molecular translation. We then describe efforts to engineer these components for upgrading protein synthesis with new properties, with a focus on cellular systems. Finally, we transition to *in vitro* systems that have greater engineering flexibility and highlight recent efforts to expand the scope of chemical substrates for ribosome-directed polymerization. We focus on bacterial systems derived from *Escherichia coli* (*E. coli*), while noting that several recent reviews have focused on the role of orthogonality for genetic code expansion and the incorporation of ncAAs in other systems as well (Arranz-Gibert et al., 2018; Chin, 2017; Hammerling et al., 2020b; Tharp et al., 2020)

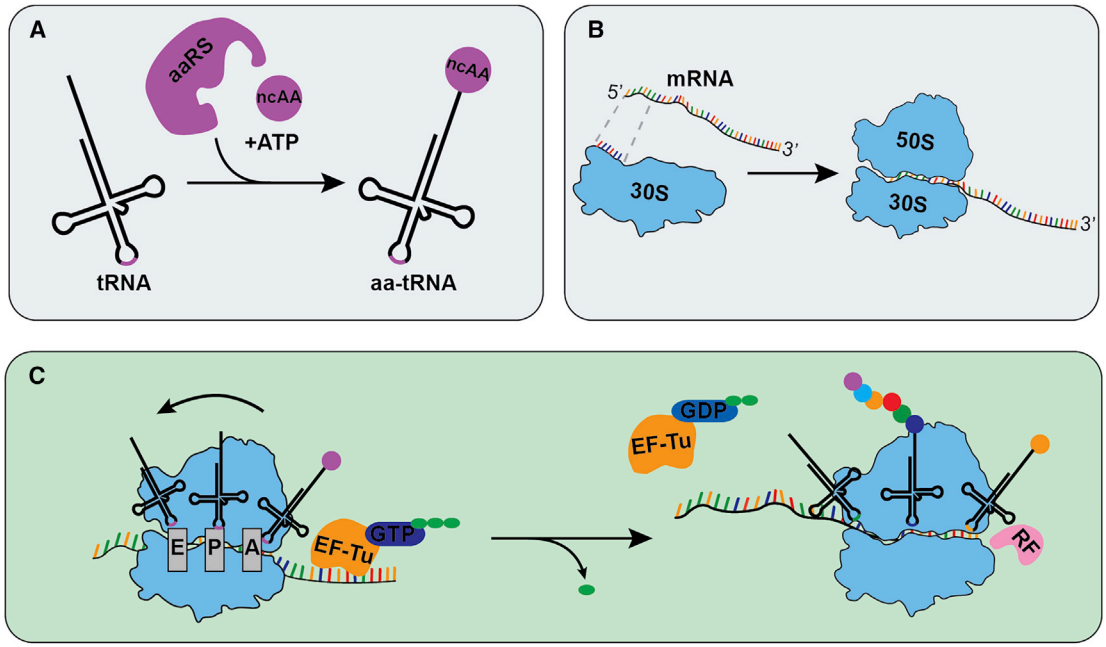

Figure 1. Overview of essential orthogonal translational system parts

(A) A specific aaRS (magenta) charges its cognate tRNA with the matching ncAA (magenta circle).

(B) The ribosome must associate with the mRNA transcript, using interactions based primarily on the SD sequence at the 5′ end of the mRNA and the anti-SD sequence of the 16S rRNA (shown).

(C) EF-Tu (with guanosine 5′-triphosphate [GTP], shown as three green circles) forms a ternary complex with the aminoacylated tRNA, accessing the A-site of the ribosome, upon which it is translocated into the P-site and finally the E- site, resulting in the production of a peptide sequence that emerges from the exit tunnel. Once the codon-anticodon interaction has been confirmed to be correct, GTP bound to EF-Tu is hydrolyzed to yield GDP and Pi. Release factors (RFs) associate with the ribosome to terminate elongation.

Biological parts for orthogonal translation systems

DNA replication, transcription, and translation are the basis of the central dogma processes that are responsible for living systems. In order to engineer novel biological systems for applications in biotechnology such as medicine (Costa et al., 2019; Freidinger, 2009; Vinogradov et al., 2019), genetically encoded materials (Davis and Chin, 2012; Rogers et al., 2018), and biocontainment (Ma and Isaacs, 2016; Mandell et al., 2015; Rovner et al., 2015), the ability to control these processes is essential. However, the machinery that enables the central dogma processes is highly interconnected, and engineering of single components can have unintended downstream effects that decrease the ability to support life. Orthogonal systems, both *in vivo* and *in vitro*, are a solution to this challenge, as they provide a way to build systems that work in the context of a complex biological environment without negatively affecting cellular or engineering goals (Liu et al., 2018). This is especially important in molecular translation systems, as there are a large number of biological parts involved, and cross-reactivity with native components poses a challenge to creating systems that open the door to new chemistries and materials.

Numerous biological parts are required to create orthogonal translation systems necessary for expanding the genetic code beyond the 20 canonical amino acids. In order for an ncAA to be successfully incorporated into a peptide by the ribosome, it must first be specifically charged onto a tRNA that decodes a codon in the mRNA that is reassigned to the ncAA (Figure 1A). This is typically done by an aaRS, though other strategies exist, as we discuss later (e.g., chemical acylation, ribozymes). The ribosome, which translates native mRNA, must be directed to translate orthogonal mRNA transcripts (Figure 1B). The aminoacylated tRNA must then be delivered to the A-site of the ribosome with the help of translation factors (e.g., elongation factor Tu [EF-Tu]) and accepted by the ribosome as a substrate for polymerization (Figure 1C). This engineered translation machinery is evolved to recognize an ncAA monomer and operate alongside the cell's natural translation apparatus in a parallel and independent fashion. It is "orthogonal" in the sense that it does not recognize natural amino acids or cross-react with native translational machinery. The development of a truly orthogonal translation system, therefore, requires the engineering of each of the parts that play a role in translating DNA to protein: codons for programming ncAAs, polymerases to translate DNA codons into mRNA transcripts, tRNAs, aaRSs, translation factors, and ribosomes. We next describe efforts to engineer these parts, starting with new genetic codes for codon reassignment.

Expansion of codon availability through genome recoding

As each of the 64 codons in the genetic code is assigned to one of the 20 canonical amino acids, the relationship that these codons have to their cognate amino acids and aaRSs must be severed in order to free the coding "channel" for ncAAs. A long-standing strategy to prepare available codons for ncAAs is to omit specific canonical amino acids and/or their cognate aaRSs,

leaving those omitted codons free to be paired with ncAAs. In cells, global replacement strategies of canonical amino acids by their non-canonical counterparts are used (Fang et al., 2018). The key idea is to use auxotrophic strains that are incapable of synthesizing a particular canonical amino acid. When grown in the presence of an ncAA that has a close structural resemblance to the ''missing'' canonical amino acid, the organism's native translational machinery incorporates the ncAA instead. In cell-free systems, such strategies for genetic code reprogramming are more straightforward (Forster et al., 2003; Josephson et al., 2005; Goto et al., 2011), as we discuss later.

Amber codon suppression is an alternative approach to global suppression strategies that has proven to be a powerful tool (Wals and Ovaa, 2014; Wang and Schultz, 2002; Xie and Schultz, 2006). The goal is to re-assign the meaning of the amber stop codon (TAG) such that it codes for the incorporation of an ncAA rather than termination of translation. Of the 64 triplet codons, there are three stop codons that lead to termination of peptide synthesis by recruiting release factors (RF1 and RF2). The amber codon is the least commonly used of the three stop codons in the *E. coli* genome, accounting for less than 7% (Korkmaz et al., 2014). Based on this analysis, Lajoie et al. constructed an *E. coli* strain that has all instances of TAG replaced with the synonymous TAA stop codon (called *C321 ΔA*), leaving the TAG codon free for non-canonical monomers (Lajoie et al., 2013). Importantly, this genetically recoded organism permitted the deletion of RF1 and complete reassignment of TAG to an ncAA (see also Isaacs et al., 2011; Johnson et al., 2011). Together, these engineering advances allowed the TAG codon to be converted from a nonsense codon (terminates translation) to a sense codon (incorporates amino acid of choice), provided that the appropriate translation machinery was present. Recently, engineering efforts have taken advantage of the redundancy of the genetic code to remove six sense codons (two arginine codons, two leucine, and two serine codons) from all protein-coding genes in the genome (Ostrov et al., 2016), in theory allowing for the incorporation of four ncAAs *in vivo*. This recoded strain, however, exhibited growth impairments of at least 25%; passaging this strain for over 1,000 generations to allow for evolutionary changes yielded a strain with a robust growth rate (Wannier et al., 2018). In a separate effort, the entire genome of *E. coli* was synthesized and recoded to replace all instances of TCG, TCA, and TAG with AGC, AGT, and TAA, respectively (Fredens et al., 2019). Notably, the strain arising from this *tour de force* effort retained viability even with the deletion of transfer RNA *serV*, which was previously considered essential. The ability to synthesize recoded genomes *de novo* opens a new era in the creation of orthogonal translation systems.

E. coli strains with synthetic genomes will require substantial re-engineering of tRNA charging systems. While strains could be engineered to use a quadruplet codon (Dunkelmann et al., 2020; Neumann et al., 2010; Sachdeva et al., 2014; Wang et al., 2014, 2012; Willis and Chin, 2018), the translation apparatus has been delicately tuned by evolution to perform optimally with a triplet codon. Thus, another powerful way to enable a significant increase in the number of possible unique codons that could be used to decode for non-canonical monomers involves expanding the genetic alphabet. In one example, an unnatural base pair (dNaM-dTPT3), which interacts favorably in the DNA double helix through complementary packing hydrophobic interactions, was developed (Li et al., 2014). This unnatural base pair can be transcribed *in vivo* to direct ncAA incorporation into proteins (Zhang et al., 2017b). Building off early innovations, a new semi-synthetic organism with 67 codons (Fischer et al., 2020) was recently constructed that uses the dNaM-dTPT3 base pair to create three additional codons for protein biosynthesis. In yet another direction, the number of letters in the DNA alphabet has been expanded to create the ''artificially expanded genetic information system'' (AEGIS) based on novel base pairs that retain DNA-like hydrogen bonding patterns. The AEGIS technology and its many applications have been developed over the past 20 years (Yang et al., 2010). During this time, the structures of the non-canonical synthetic nucleobases have been optimized with respect to their chemical stability and ability to be accepted by various enzymes routinely used in molecular biology. Recently, the design of four new nucleotide letters that can be incorporated into native DNA polymers without affecting the DNA double helix was shown, called ''hachimoji'' (Hoshika et al., 2019; Yang et al., 2007). Novel DNA monomers offer significant expansion of the tools available for increasing the number of free codon channels available for orthogonal translation systems (e.g., the four additional bases made available by this advance theoretically creates 512 potential codons [8^3]). Recently, there has been an increased effort to create extra base pairs to augment nucleic acid and protein function (Iwane et al., 2016; Katoh et al., 2020; Okamoto et al., 2016; Rogers et al., 2018; Takatsuji et al., 2019). Moreover, the possibility of creating an orthogonal DNA replication system dedicated exclusively to the replication of a DNA plasmid, that cannot be replicated by native host systems, for novel genetic codes is even within reach (Ravikumar et al., 2014, 2018).

The progress made in novel genetic code design necessitates the engineering of polymerases capable of transcribing DNA composed of non-canonical nucleotides. Directed evolution has been used to engineer DNA polymerases that can accept nucleotides that exhibit either non-canonical hydrogen bonding or no hydrogen bonding at all (Laos et al., 2013; Leconte et al., 2005). Libraries to generate these specialized polymerases have been built through error-prone PCR, the introduction of degenerate codons, gene shuffling between homologs, and phylogenetic analysis (Laos et al., 2014). Reconstructing evolutionary adaptive paths (REAP) is a method that was first applied to polymerase engineering. By mapping divergent motifs in homologous protein sequences, Cole et al. were able to use evolutionary data to inform library design and ultimately designed thirty DNA polymerase variants capable of incorporating non-canonical nucleosides (Cole and Gaucher, 2011). Taken together, these works set the stage to engineer organisms that decipher entirely alternative genetic codes free from the decoding needs of the host organism for the synthesis of sequence-defined polymers made entirely of non-native monomers.

Engineering of orthogonal tRNA charging systems

In vivo, orthogonal aaRSs that are capable of specifically charging their cognate tRNAs with an ncAA of choice are essential in order for the ncAA to be site-specifically incorporated into a peptide. The orthogonal aaRSs must not cross-react with any canonical amino acids or native tRNAs, requiring that the aaRSs

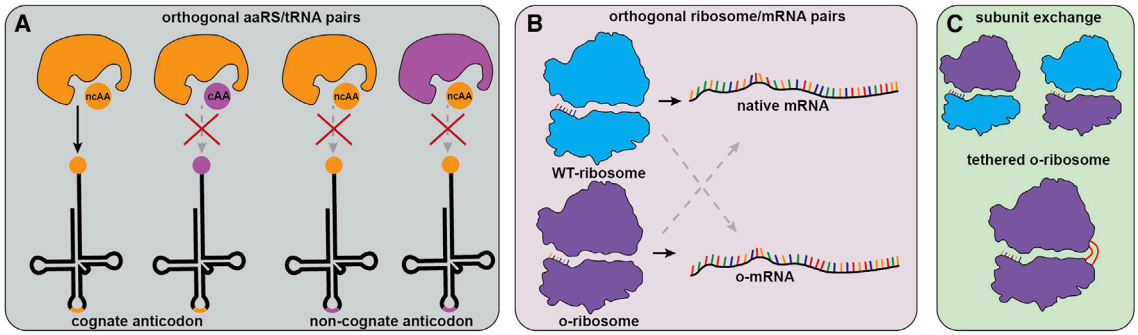

Figure 2. Design rules for engineering of orthogonal parts in translation

(A) aaRSs must be orthogonal to a specific amino acid and tRNA. Orthogonal aaRS (orange) must only charge their cognate tRNA (orange) with the ncAA (orange). Additionally, native synthetases (magenta) must not be able to charge the ncAA onto a native tRNA (magenta).

(B) Orthogonal ribosomes must interact only with o-mRNA. This can be controlled by engineering the anti-SD sequence of the 16S rRNA.

(C) Ribosome subunits can freely associate with either o-ribosome or wild-type (WT) ribosome subunits. Tethering of the two subunits can help to overcome this limitation.

are products of extensive protein engineering and optimization and/or derived from a sufficiently phylogenetically distant organism (often archaea) such that cross-reactivity is innately low (Figure 2A). To achieve the necessary properties for orthogonal translation systems, engineering strategies for tRNA charging systems require the use of both positive and negative selections for orthogonal pairs; a positive selection to ensure that the pair successfully can incorporate an ncAA at the amber codon and a negative selection to confirm that the pair is specific to the given ncAA and does not incorporate canonical amino acids. In *E. coli*, the most commonly used orthogonal aaRS/tRNA pairs are derived from archaea, such as *Methanocaldococcus jannaschii*, *Methanosarcina sp.*, and archaeal methanogens (O'Donoghue et al., 2013); these systems are based on the tyrosyl-tRNA synthetase, pyrrolysyl-tRNA synthetase (PylRS), and phosphoseryl-tRNA synthetase (SepRS) systems, respectively, and function well with structural analogs of their native substrates (Polycarpo et al., 2006). While the orthogonal aaRS/tRNA systems have been used to incorporate many ncAAs at the amber codon, including amino acids with photocrosslinking and redox active groups, keto and alkyne side chains, heavy atoms, and click-chemistry reactive groups (O'Donoghue et al., 2013; Zhang et al., 2005), these efforts are usually accompanied by a low acylation efficiency (Amiram et al., 2015; Bonieckl et al., 2008; Ling and Söll, 2010; Reynolds et al., 2010; Tanrikulu et al., 2009; Umehara et al., 2012) as compared to canonical amino acids.

To improve the incorporation efficiency, further optimization of these orthogonal parts is needed. This can be facilitated by using RF1-deficient strains to avoid competition in amber suppression (Lajoie et al., 2013), optimization of expression of the orthogonal tRNA-aaRS pair (O'Donoghue et al., 2013), elevated ncAA concentrations for high-yielding expression of full-length desired proteins (O'Donoghue et al., 2013), tuning interactions of aminoacyl-tRNAs with elongation factor Tu (EF-Tu) (Gan et al., 2017; Haruna et al., 2014), and new tools for evolution of such systems, including the use of genome engineering technologies (Amiram et al., 2015; Zhang et al., 2017a) and deep sequencing (Zhang et al., 2017a). In one recent example, a multiplexed automated genome engineering (MAGE) strategy was used to generate highly selective and orthogonal aaRS-tRNA pairs that enhance the insertion of ncAAs into proteins *in vivo*. Guided by aaRS three-dimensional crystal structures, mutations surrounding the amino acid side chain binding pocket and five residues at the aaRS-$tRNA_{CUA}$ anticodon interface were varied to generate $>10^9$ variants and evolve aaRS-tRNA pairs capable of multi-site ncAA incorporation. Notably, using the recoded strain lacking RF1, an aaRS demonstrated incorporation of 30 *p*-acetyl-L-phenylalanine and *p*-azido-L-phenylalanine into an elastin-like polypeptide (ELP) with yields near 50 mg/L (Amiram et al., 2015). This enabled synthesis of new classes of functional materials and applications in which the basic biopolymer structure is elaborated with pendant moieties to program physical properties with atomic-scale resolution (Costa et al., 2018, 2019).

While the aforementioned aaRS/tRNA pairs serve as powerful tools, the development of additional orthogonal pairs is desirable to enable the incorporation of multiple, distinct ncAAs in the same cell, especially with the emergence of novel genetic codes (Fredens et al., 2019; Fischer et al., 2020). One way to do this is to seek out additional archaeal tRNA charging systems that are similar to those in use. Along these lines, engineering novel mutually orthogonal aaRS/tRNA pairs has been a focus in this field. Recently, sequence-alignment methods identified pyrrolysyl-tRNA synthetases that lack the N-terminal domain (ΔNPylRS) and interact orthogonally with cognate ^{Pyl}tRNAs (Willis and Chin, 2018). Several ΔNPylRS/^{Pyl}tRNA pairs were evolved to recognize specific ncAAs in *E. coli*, resulting in the identification of two mutually orthogonal pairs. This strategy was further pursued to identify 18 additional orthogonal aaRS/tRNA pairs (Dunkelmann et al., 2020). In this same work, the authors engineered a triply orthogonal set of ΔNPylRS/^{Pyl}tRNA by co-evolving the tRNA and synthetase, resulting in incorporation of three distinct ncAAs in a single polypeptide.

Additional innovations are having an impact on the development of orthogonal aaRS/tRNA pairs. Phage-assisted continuous evolution (or PACE) has also been applied to engineer improved orthogonal aaRSs. Specifically, a chimeric *Methanosarcina spp.* PylRS was evolved to have 45-fold improved enzymatic efficiency (k_{cat}/K_M^{tRNA}) compared with the parent enzyme. This led to a 9.7-fold increase in protein yield (Bryson et al.,

2017). Of note, PACE can be applied to engineer virtually any protein that acts upon a DNA or RNA target. A computational method to predict whether a foreign tRNA sequence would be recognized by each *E. coli* synthetase was also recently developed. This approach allowed for testing of almost three million unique tRNAs, ultimately leading to the identification of five pairs that retain orthogonality in *E. coli* (Cervettini et al., 2020). Importantly, this study introduced high-throughput methods to detect tRNA acylation as well as orthogonality. In summary, multiple new synthetic biological approaches are paving the way for large-scale engineering of mutually orthogonal translation systems.

Orthogonal translation factor engineering for improved delivery to active site

Once generated, aminoacyl-tRNAs are delivered to the ribosome by EF-Tu, where the anticodon of the tRNA is paired with the triplet codon of the mRNA. Among the many complexities of translation, the thermodynamic compensation interactions between EF-Tu and aminoacyl-tRNAs are carefully tuned. In recent years, engineering this interaction by modifying EF-Tu or orthogonal tRNAs has been shown to improve ncAA incorporation (Gan et al., 2017; Hansen et al., 1986; Haruna et al., 2014). This is typically a second-tier optimization for engineering molecular translation, once aaRS/tRNA pairs have been created. EF-Tu engineering has been used in three main ways: first, EF-Tu can be engineered to enable ncAA incorporation where it is otherwise not possible. In an exemplary study, incorporation of phosphoserine was possible only after the development of a Sep-specific elongation factor (Park et al., 2011). Second, EF-Tu can be engineered to increase efficiency. In one example, the incorporation efficiency of selenocysteine (Sec) was increased to >90% by engineering EF-Tu optimized for Sec (Deley Cox et al., 2019; Haruna et al., 2014). Elegant, continuous-evolution-selection schemes for Sec have also enhanced incorporation of this ncAA (Thyer et al., 2018). In another work, it was shown that the positive effects of an engineered EF-Tu in combination with an optimized orthogonal aaRS are not additive, suggesting that these parts must be engineered in concert in order for epistatic mutations to be identified (Gan et al., 2017). Third, computational methods based on evolutionary paths of EF-Tu variants have been applied to discover variants that expand substrate specificity and improve host fitness (Deley Cox et al., 2019). It has become clear that engineering the entire system of molecular translation, including EF-Tu, is important to enable highly efficient ncAA incorporation into proteins or biopolymers.

RIBOSOME ENGINEERING

The ribosome has evolved over billions of years to accelerate the rate of amide bond formation between α-amino acids by more than 10^7-fold (Sievers et al., 2004). In order to enable the incorporation of non-α-ncAAs and facilitate the synthesis of polymers comprised solely of such monomers, the ribosomal active site, or the peptidyl transferase center (PTC) will likely have to be redesigned. The PTC is a dynamic pocket that adjusts conformations and interaction of the 3′ terminal peptidyl group of the bound peptidyl-tRNA with the 5′ terminal group of the α-amino group and serves as proofreading for aminoacylated-tRNAs (Kosterlev et al., 2006).

Engineering the ribosome *in vivo* has key advantages, such as scalability and accessibility to many evolution strategies that take advantage of growth phenotype-based selections (i.e., live/dead selections). Several studies have helped to elucidate the function of specific PTC nucleotides in the context of amino acid discrimination (Englander et al., 2015) and in engineered ribosomes. For example, the Hecht group has shown that tRNAs charged with D-amino acids are discriminated against by the wild-type ribosome as the substrates are positioned in specific orientations in the PTC (Dedkova and Hecht, 2019). A mutational study based on this result yielded ribosomal mutants that enabled a significant improvement (4-fold) in D-amino acid incorporation (Dedkova et al., 2006). Similarly, a ribosome library mutagenizing positions 2,057–2,063 in combination with 2,496–2,501, or 2,502–2,507, or 2,582–2,588 was screened to identify a ribosome capable of β-amino acid incorporation *in vitro* (Dedkova et al., 2012). These mutant ribosomes have allowed incorporation of β-amino acids, D-amino acids, dipeptides, and oxazoles (Chen et al., 2019; Maini et al., 2015b). Further evolutionary studies showed that a ribosomal mutant with AGCGUGA at 2,057–2,063 and UGACUU at 2,502–2,507 could successfully incorporate β^3-Phe analogs *in vivo* (Melo Czekster et al., 2016). Analysis of this mutant via cryo-electron microscopy indicated that it exhibits substantial disordering of the PTC (Ward et al., 2019). Additionally, this mutant is not functional *in vitro*, likely a result of inefficient assembly, demonstrating that mutations in this highly dynamic region of the ribosome can have unintended effects on ribosome biogenesis.

Unfortunately, biological constraints, especially cell viability, have previously made it difficult to engineer the ribosome extensively to work with exotic substrates or novel polycondensation chemistries beyond those in nature. However, several ribosome-engineering platforms have recently been developed that are decoupled from cellular growth, providing new opportunities to expand the chemistry of life. The first key advance was the creation of orthogonal ribosomes. In order to separate ribosome pools, o-ribosome:o-mRNA systems have been developed and optimized by altering the Shine-Dalgarno (SD) sequence in tandem with the anti-SD motif at the 3′ end of the 16S rRNA (Figure 2B; Hui and de Boer, 1987; Rackham and Chin, 2005). A computational approach to designing orthogonal ribosome:mRNA pairs was later developed (Chubiz and Rao, 2008). While a useful tool, this method did not enable complete separation of ribosome pools: large subunits associate freely with small subunits, which negated any engineered specificity of the large subunit toward o-mRNAs, as engineered 23S rRNA (containing the PTC) were still able to associate with wild-type 30S subunits. This challenge was overcome by linking the two subunits of the engineered ribosome, creating the first fully orthogonal translation system (Figure 2C) (Orelle et al., 2015).

Tethered or stapled ribosomes (Aleksashin et al., 2020; Carlson et al., 2019; d'Aquino et al., 2018; Fried et al., 2015; Liu et al., 2017; Neumann et al., 2010; Orelle et al., 2015; Schmied et al., 2018; Wang et al., 2007; Yesselman et al., 2019) covalently tether the subunits of the ribosome by linking the core 16S and 23S rRNAs to form a single chimeric molecule. This can be

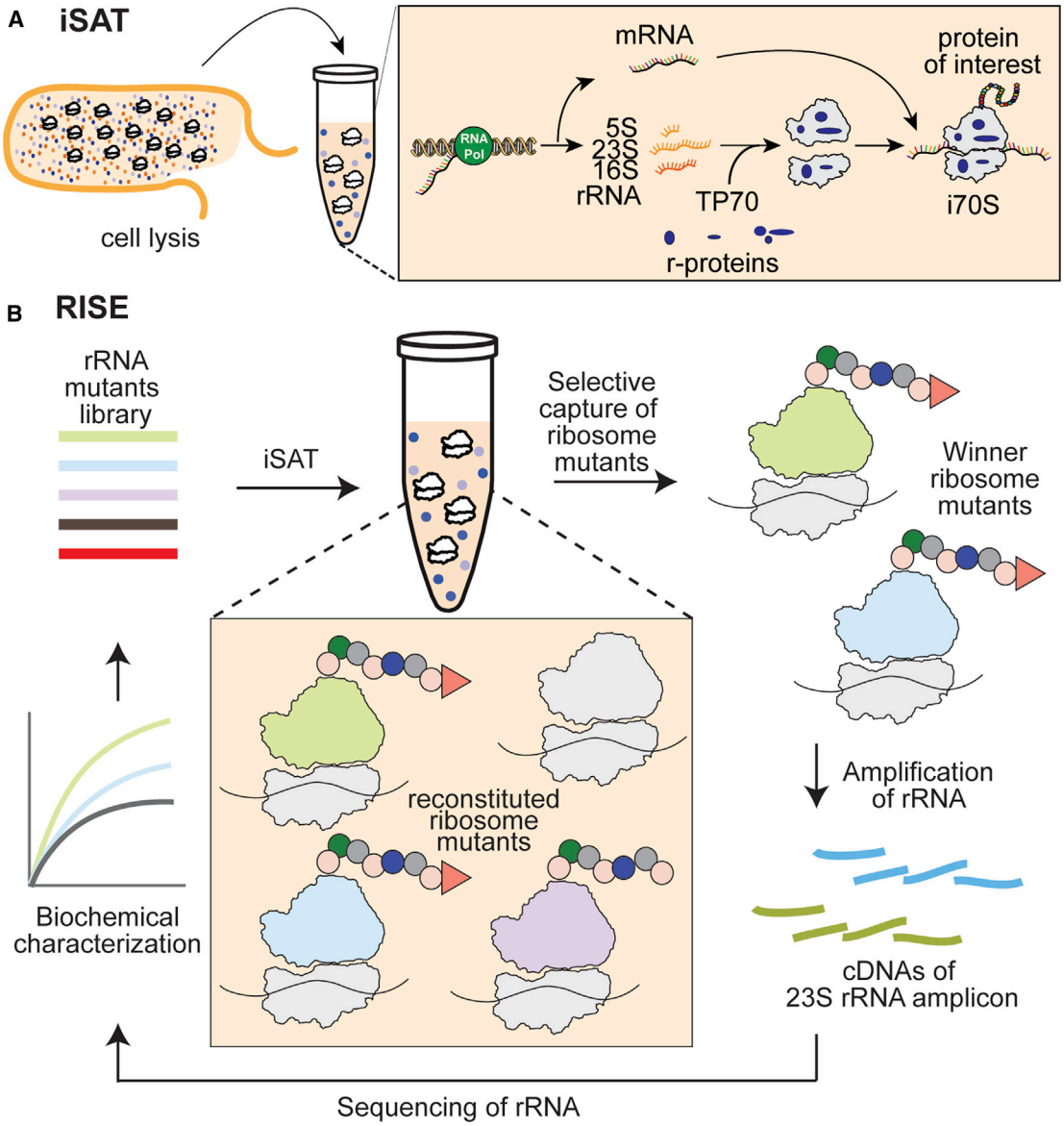

Figure 3. *In vitro* ribosome synthesis and evolution

(A) iSAT method of *in vitro* ribosome construction. DNA template encoding the rRNA operon and reporter gene are mixed with purified ribosomal (r-) proteins (TP70) and T7 RNA polymerase (RNAP) in a cell-free, ribosome-free S150 crude extract. T7 RNAP transcribes 23S, 16S, and 5S rRNA from DNA template, as well as protein encoded in the mRNA. As rRNA is being transcribed, TP70 r-proteins bind and assemble into 50S and 30S subunits, which then bind to mRNA, forming the 70S ribosome and the translating active protein of interest as a reporter of ribosome activity.

(B) The RISE system selecting mutant ribosomes *in vitro* from a DNA library. By selectively capturing rRNA mutants capable of synthesizing desired polymers, RISE can enrich a target 23S rRNA gene from a library.

used to create a sub-population of orthogonal ribosomes in cells that is available for engineering and is independent of wild-type ribosomes that support cell life. For example, Orelle et al. first demonstrated the utility of this system for ribosome evolution by introducing mutations into the tethered ribosome's PTC that improved translation of a problematic protein and would otherwise have been dominantly lethal (Orelle et al., 2015). More recently, the ribosomal subunit linker sequences were optimized to improve activity such that the tethered ribosome could support cellular growth at rates comparable to the wild-type ribosome (Carlson et al., 2019; Schmied et al., 2018). Importantly, both tethered and stapled ribosomes have now been shown to be functionally isolated and do not cross assemble to form hybrids. Orthogonal ribosomes have also since been used to design a "flipped" orthogonal system in which the tethered ribosomes translate the proteome and leave the untethered ribosome available for engineering and translation of o-mRNAs (Aleksashin et al., 2020). Notably, this system can be used to introduce mutations that would otherwise be dominantly lethal into the untethered ribosome's rRNA, allowing for evolution of the ribosome to incorporate previously inaccessible ncAAs and produce novel proteins.

In vivo-based ribosome-engineering strategies offer much potential but present their own challenges associated with cell viability and the requirement that ncAAs must permeate the cell membrane. *In vitro*, or cell-free, strategies have thus also emerged for ribosome synthesis and evolution. The *in vitro* synthesis, assembly, and translation (iSAT) system generates highly active *E. coli* ribosomes (Figure 3A), including the production of modified ribosomes containing 23S rRNA mutations (d'Aquino et al., 2020; Fritz et al., 2015; Fritz and Jewett, 2014; Hammerling et al., 2020a; Jewett et al., 2013; Liu et al., 2015). iSAT has also been shown to be compatible with ncAA incorporation (Liu et al., 2021) into proteins. By combining iSAT with ribosome display, *in vitro*-directed evolution of the ribosome is possible in a platform called "RISE" (Figure 3B), demonstrating the importance of epistatic interactions in selecting new functions (Hammerling et al., 2020a). The small subunit of the ribosome can also be engineered (Murase et al., 2018) and efforts are underway to enable mirror-image polymerization (Jiang et al., 2017; Xu et al., 2017).

A new frontier for ribosome engineering may include integrating computational design. In one recent example, statistical coupling analysis (SCA), a method previously only applied to proteins, was applied to the ribosome to better understand coevolving RNA bases in the 23S rRNA (Walker et al., 2020). In a different direction, computational methods for 3D RNA design and Eterna, a crowdsourcing/machine-learning platform involving >200,000 global participants, are emerging as potential tools to facilitate ribosome design. Surprisingly, players of Eterna consistently outperform most RNA-folding algorithms and can be used to improve computational RNA design (Koodli et al., 2019; Lee et al., 2014). Recently, Eterna based on player-proposed rules and stringent experimental tests was shown to be the best available algorithm for RNA secondary structure prediction (Wayment-Steele et al., 2020). Eterna has also started a ribosome pilot challenge (https://eternagame.org/labs/9162726), which could lead to new approaches for ribosome design.

Genetic code reprograming *in vitro*

While orthogonal translation platforms are frequently developed *in vivo*, recent improvements in cell-free systems, along with the open reaction environment, have matured *in vitro* systems as a powerful tool for genetic code reprogramming (Carlson et al., 2012; Hodgman and Jewett, 2012; Silverman et al., 2020; Swartz, 2018). The key idea is that precise and complex biomolecular transformations can be conducted in crude cell lysates or purified systems without intact cells. Cell-free platforms are being used for both probing and designing cellular function and manufacturing. These two main approaches include the reconstituted protein synthesis using recombinant elements, or "PURE" system (Suga, 2018; Tuckey et al., 2014; Whittaker, 2013), and cell-extract-based systems (Albayrak and Swartz, 2013, 2014; Hammerling et al., 2020b; Silverman et al., 2020). In short, the PURE system is a reconstituted *E. coli* cell-free translation system supplemented with the total endogenous tRNA and synthetic aminoacyl-tRNA charged with ncAA. The approach has been simplified in recent years (Contreras-Llano et al., 2020; Lavickova and Maerkl, 2019; Shepherd et al., 2017; Villarreal et al., 2018) and provides the greatest flexibility for genetic code reassignment. The *E. coli* cell-extract-based system is prepared from the clarified cell lysate, supplemented with additional substrates and salts required for transcription and translation, such as cofactors, energy sources, and salts (Bogart et al., 2021; Hershewe et al., 2020; Karim et al., 2020; Silverman et al., 2020).

A technical renaissance has driven several technological improvements in extract-based approaches for engineering translation. Here, we highlight two. First, a bacterial cell-free protein synthesis platform was developed based on the aforementioned genomically recoded *E. coli* strain lacking RF1 (C321 ΔA) and optimized for high-level *in vitro* protein production using amber suppression. Using this platform, protein synthesis performance could be enhanced to 1.7 g/L in batch reactions and 40 ncAA incorporations into an ELP with high accuracy (>98%; Martin et al., 2018). This was further increased by integrating T7 RNA polymerase into the genome to obtain yields >2.5 g/L with site-specifically introduced ncAAs (Des Soye et al., 2019). Second, while extract systems are highly efficient at making proteins with ncAAs, they are constrained by the genetic code of the host organism, which limits codon reassignment. Several approaches have now addressed this. In one approach, the entire tRNA pool can be depleted and then re-configured (Ahn et al., 2006; Salehi et al., 2017). In another, specific tRNAs can be sequestered with synthetic oligonucleotides that hybridize to the anticodon loop of tRNA (Cui et al., 2017; Cui et al., 2018).

While advances in crude-extract-based systems are opening opportunities in biomanufacturing, the PURE system offers the greatest freedom of design. This is because the user defines all of the elements in the PURE system and the concentrations of individual components (e.g., tRNA, aaRS, and engineered EF-Tu) can be increased or decreased at will. For example, optimizing the concentration of translation factors in a cell-free protein synthesis platform, such as elongation factor P (EF-P), a bacterial translation factor that accelerates peptide bond formation between consecutive proline residues, has been shown to improve the compatibility of translation for some ncAAs. This ease of customization, however, comes at a cost: the price of expressing protein in a PURE system is many times more expensive than in a crude-extract-based system (Jewett and Forster, 2010). Protein/peptide yields are also lower (Table 1). In several studies, supplementing EF-P and an engineered $tRNA^{Pro}$ (the sequence of the T-stem and D-arm motifs that interact with EF-Tu and EF-P, respectively, are modified) into the PURE reaction could be an efficient means to improve the incorporation of ncAA (e.g., β- and γ-amino acids) (Katoh and Suga, 2020; Katoh et al., 2020, 2016; Lee et al., 2020c).

Expanding the scope of ribosome-mediated polymerization with flexizyme

The PURE system allows for the addition of tRNAs that have been pre-charged with ncAAs (Figure 4A). There are several strategies to synthesize ncAA-tRNAs complexes, or "mis-acylated" tRNAs. As mentioned above, aaRSs can be evolved for ncAAs but directed evolution strategies require ncAAs to be permeable to cell membranes and have been, so far, generally confined to a range of amino acid analogs that resemble natural ones. Chemical acylation is another approach, but it is technically laborious (Yamanaka et al., 2004). The most promiscuous and simple approach to create these mis-acylated tRNAs beyond their cognate amino acids is the ribozyme-mediated reaction based

Table 1. Yield comparison of *in vitro* and *in vivo* protein production platforms

System		ncAA	# ncAAs	Protein yield	Protein	Reference
In vitro	cell-extract based	pAcF	40	96 mg/L	ELP	(Martin et al., 2018)
		pAcF	1	2,100 mg/L	sfGFP	(Des Soye et al., 2019)
		pAzF/pPaF	6	70 mg/L	sfGFP	(Albayrak and Swartz, 2014)
		pAzF/pPaF	1	900–1,700 mg/L	sfGFP	(Albayrak and Swartz, 2013)
		canavanine	6	0.25 mg/L	EGFP	(Worst et al., 2015)
	PURE	βhM/βhF	7	0.09 mg/L	Peptide	(Katoh and Suga, 2018)
		βhM/βhF	3	2 mg/L	Peptide	Katoh and Suga 2018)
		fluorescent amino acids	1–3	~100 mg/L	Peptide	(Lee et al., 2021)
In vivo		pAzF	30	50 mg/L	GFP	(Amiram et al., 2015)
		canavanine	7	0.1 mg/L	EGFP	(Worst et al., 2015)
		pAcF	1	30 mg/L	ELP	(Costa et al., 2019)
		pAzF	4	50 mg/L	ELP	(Costa et al., 2018)
		pAcF	3	5 mg/L	GFP	(Chatterjee et al., 2013)
		pAcF	1	27 mg/L	EGFP	(Johnson et al., 2011)

Yields from selected *in vitro* (cell-free lysate and PURExpress) and *in vivo* reported works, illustrating a high variance of protein yield as a function of the protein being produced as well as the ncAA being incorporated.

on flexizyme (Fx)(Goto et al., 2011; Goto and Suga, 2009; Lee et al., 2019; Morimoto et al., 2011). Below, we describe the development of the Fx system and substrate design rules for Fx-mediated acylation.

Fx is an aaRS-like ribozyme capable of catalyzing acylation of amino acids onto RNA. The RNA-world hypothesis suggests that in a primitive translation system, before the advent of sophisticated protein-based enzymes, tRNA acylation could conceivably have been catalyzed solely by RNA enzymes. Fx was developed by building upon efforts that used "SELEX" (systematic evolution of ligands by exponential enrichment) (Tuerk and Gold, 1990) to evolve a ribozyme that re-acylates a dipeptide (biotinylated Phe) molecule, covalently conjugated on the 3′ end of a short RNA donor (5′-CAACCA-3′), to its own 5′ end (Lohse and Szostak, 1996; Figure 4B; Lee et al., 2000). Fx can reversibly accelerate the catalytic reaction and accept the dipeptide substrate from the 5′ end of the ribozyme to the 3′ end of the RNA (Figure 4C). In this system, amino acids (or more exotic monomers) are esterified to a chemical leaving group, often a cyanomethyl moiety. Then, Fx recognizes both this leaving group and a tRNA molecule to catalyze the esterification of the 3′ hydroxyl of the tRNA.

The crystallization study of Fx covalently linked to a tRNA mimic (microhelix tRNA, mihx) elucidated the molecular interactions involved around mihx and the substrate, cyanomethylester (CME)-activated phenylalanine (Phe). Importantly, the crystal structure (Xiao et al., 2008) suggested that the aromatic side chain of Phe stacks against the terminal J1a/3 base pair of an Fx recognition pocket and the carbonyl (Figure 4C). Due to the use of Phe in the development of the Fx, this first generation of Fx preferentially charges aromatic amino acids (e.g., Phe or Tyr) activated with CME to particular tRNAs used for selection. Moreover, the crystal structure suggested that any activated esters, irrespective of the side chain, can be charged to tRNA by Fx, provided they have an aromatic moiety on the leaving group. This study drove efforts to create other types of activated esters or thioesters; dinitrobenzyl ester (DNB), chlorobenzyl thioester (CBT), and (2-aminoethyl)-amidocarboxybenzyl thioester (ABT), which led to an explosion in the scope of ncAA substrates compatible with Fx chemistry (Figure 4D). Due to this characteristic mechanism, Fx can charge virtually any α-amino acid to tRNA, provided that the side chain of α-amino acid is suitably protected or deprotected in the ester synthesis step. After a few more significant changes in length and sequence through *in vitro*-directed evolution over the past decade, Fx has improved to catalyze tRNA charging reactions more efficiently. A set of three Fxs (eFx, 45 nt; dFx, 46 nt; and aFx, 47 nt) (Passioura and Suga, 2014) is currently used, and the average kinetics of Fxs for tRNA charging reactions has advanced to $k_{cat}/K_M = 1.8 \times 10^6\ s^{-1} \cdot M^{-1}$. Of note, the first generation of Fx was 70 nt in length, with $k_{cat}/K_M = 5.6 \times 10^4\ s^{-1} \cdot M^{-1}$ (Morimoto et al., 2011).

The crystal structures (Xiao et al., 2008) and several follow-up studies (Goto et al., 2011; Lee et al., 2019; Maini et al., 2016) have proven that the aminoacylation reaction occurring in the catalytic site of Fx is a nucleophilic acyl substitution. More specifically, the leaving group of the activated ester is replaced by the attack of 3′ hydroxyl group of tRNA positioned to the carbonyl carbon of the substrate (Figure 4C).

As molecular-level understanding about how Fx works has emerged, so too have design rules to guide non-canonical monomers for Fx-catalyzed acylation. In one recent study, four chemically diverse non-canonical scaffolds (phenylalanine, benzoic acid, heteroaromatic, and aliphatic acid analogs) were investigated for the compatibility of their substrates with the three flexizymes (Lee et al., 2019). Based on the molecular characteristics and yields in Fx-catalyzed reaction in several studies (Lee et al., 2021, 2020b, 2019, 2020c; Passioura and Suga, 2014; Rogers et al., 2018; Tsiamantas et al., 2020), several general substrate design rules were identified for efficient tRNA charging (Figure 4E). First, substrates with a similar molecular structure to

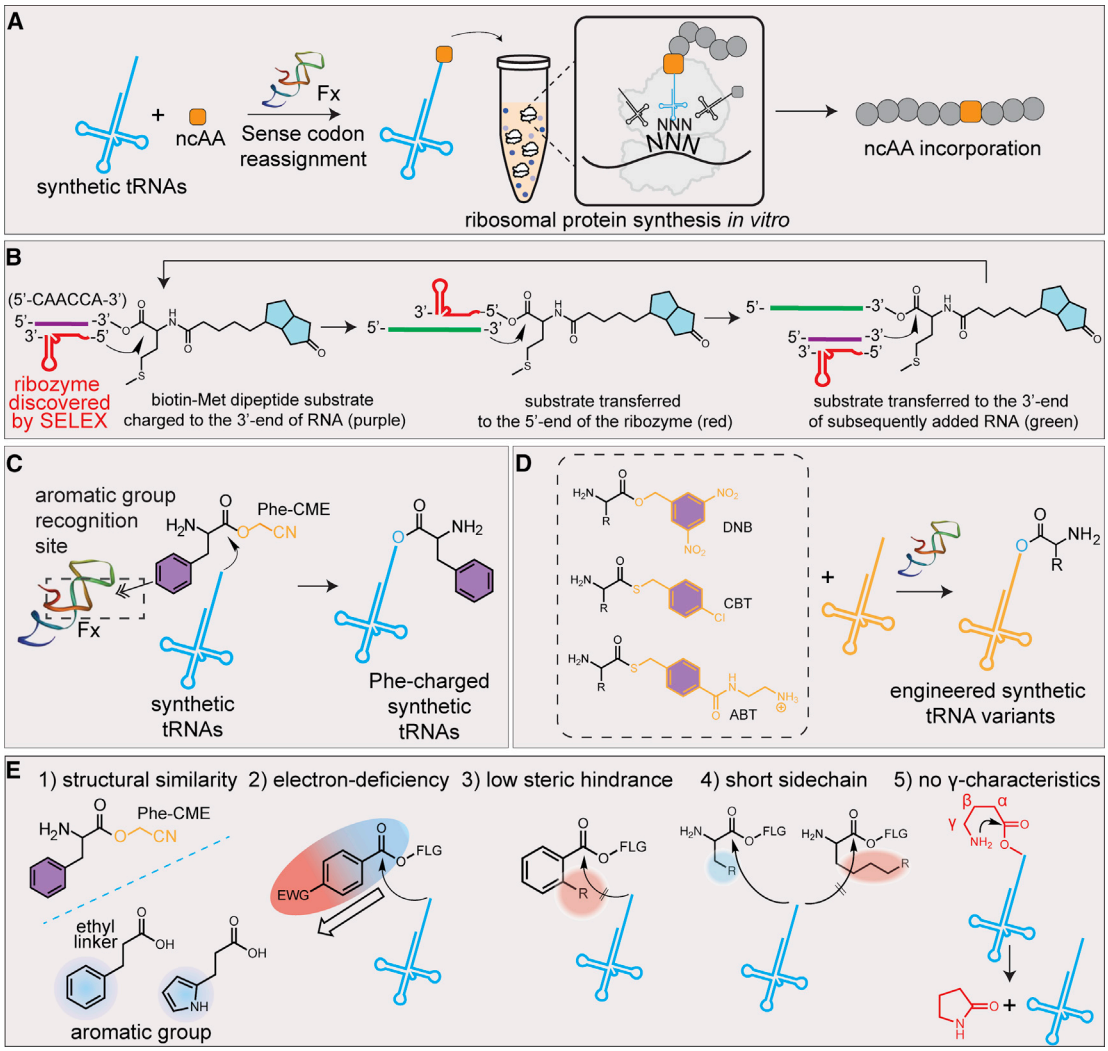

Figure 4. Genetic code reprogramming via Fx

(A) Fx-mediated tRNA charging of an ncAA and ribosome-mediated incorporation *in vitro*.

(B) Origin of flexizyme. The ribozyme (red) discovered to be capable of performing a reversible amino acid transfer reaction across two different RNAs has been advanced to three flexizymes (eFx, dFx, and aFx), to date, and is used for acylation of substrates bearing a carboxylic or thionyl acid.

(C) Mechanism of flexizyme-mediated tRNA charging. The tRNAs used in this system are orthogonal to endogenous tRNAs. Fx recognizes aromatic groups either on the side chain or on the leaving group of a substrate, which is a key discovery to expand the scope of chemical substrates.

(D) Structure of other alternative activated amino acid substrates that provide an aromatic group on the leaving groups.

(E) Five substrate design rules identified for efficient Fx-mediated acylation.

Phe return higher acylated tRNA. Specifically, aromatic moieties with a 2-carbon linker to carboxylate, for example, hydrocinnaminc acid, produce acylated tRNA in high yields due to the fact that Fx was initially selected with Phe. Second, substrates that increase the electrophilicity of the carbonyl carbon offer more acylated tRNA. Specifically, substrates with an electron-withdrawing group (e.g., nitrobenzyl) are more suitable for Fx-mediated acylation reaction than substrates with an electron-donating group (e.g., hydroxybenzyl or methoxybenzyl). Third, substrates with low steric hindrance around the carbonyl group facilitate the acylation reaction more efficiently than substrates with a bulky moiety. Fourth, in the case of side-chain-modified amino acid substrate charging, substrates with a bulky functional group on a short side chain (e.g., diaminopropionic acid, n = 1, where n is the number of carbons of the amino acid side chain) present less steric hindrance than the amino acid with a long side chain (e.g., Lys, n = 4). Finally, linear γ-amino acids do not remain charged to tRNA, as the monomer undergoes a deleterious lactam formation on the tRNA.

The Fx-driven genetic code reprogramming approach has dramatically expanded the palette of monomers for use in ribosome-mediated polymerization. Taken together, numerous non-canonical substrates have now been acylated to tRNA by Fx and subsequently incorporated site-specifically into a peptide in a reconstituted PURE system. The substrates include α- (Obexer et al., 2017), β- (Fujino et al., 2016), γ- (Ohshiro et al., 2011), D-amino acids (Katoh et al., 2017), cyclic (Katoh and Suga, 2020; Lee et al., 2020c), N-alkylated (Iwane et al., 2016; Kawakami et al., 2013), fluorescent amino acid analogs

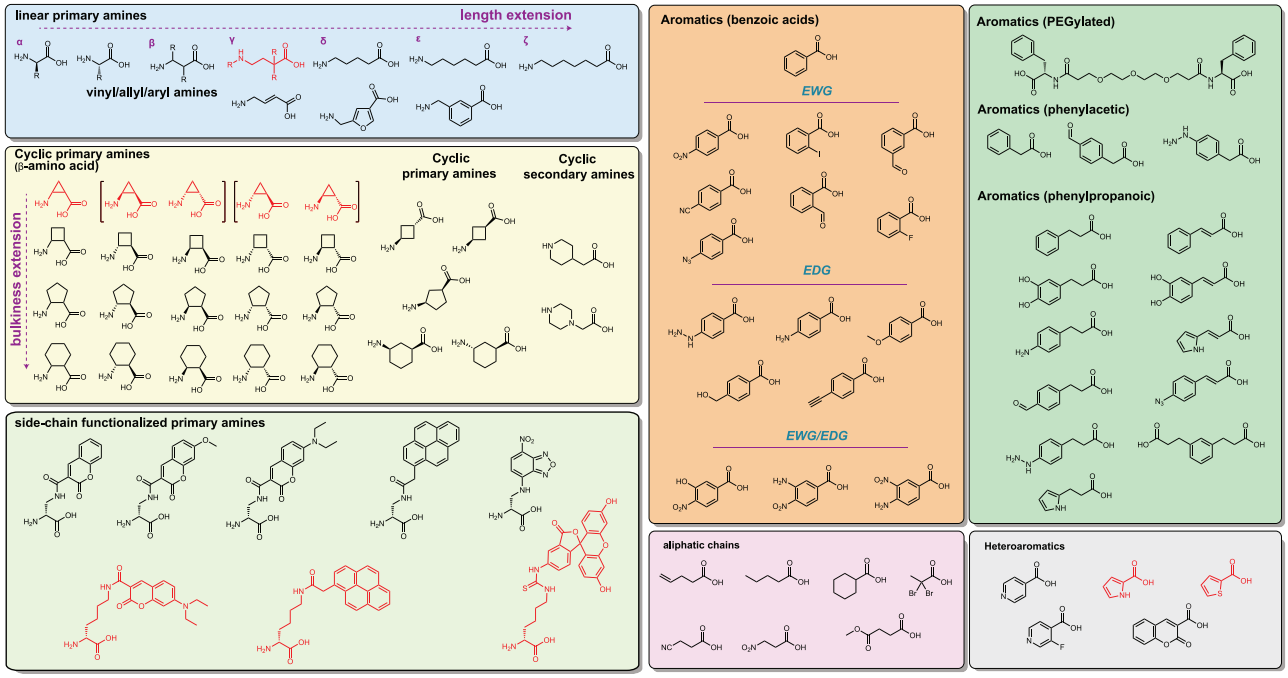

Figure 5. Novel architectures of non-canonical substrates designed for ribosome-mediated site-specific incorporation into peptides *in vitro*
Substrates in red were not charged to tRNA due to deleterious lactam formation (γ-amino acid), increased bulkiness (side-chain-functionalized primary amine), or electron-rich characteristics (heteroaromatics), providing insights to establishing the five substrate design rules that predictably guide the search for non-canonical substrates for flexizyme-mediated tRNA charging.

(Lee et al., 2021), non-amino (aromatic, aliphatic) acids (Lee et al., 2019), foldamers (Katoh et al., 2020; Rogers et al., 2018), oligomeric peptides (Tsiamantas et al., 2020), malonyl (polyketide-like) acids (Ad et al., 2019), hydroxyacids (Ohta et al., 2007, 2008), and thioacids (Takatsuji et al., 2019).

Pushing the bounds of non-canonical substrate length and bulkiness, backbone-extended monomers have recently been used to gain insights into the ribosome's RNA-based active site. For length, site-specific incorporation of long-chain-carbon (e.g., δ-, ε-, and ζ-) amino acids into peptides has been shown (Lee et al., 2020b), which could potentially form polyamide fibers (e.g., nylon or Kevlar) if efficiently polymerized by the ribosome. For bulkiness, a recent study demonstrated ribosome-mediated polymerization of cyclic β-amino acids into peptides with 3-, 4-, 5-, and 6-membered bulky rings on the α- and β-carbon (Lee et al., 2020c). These bulky rings had different stereochemistry, which could produce various helical peptides with different turn characteristics when incorporated into a polymer. In summary, Fx-enabled studies reveal that the ribosome is capable of polymerizing a wide array of substrates, especially at the N terminus (Figure 5), unveiling new substrate classes for chemical and synthetic biology studies.

Systems engineering needs for the synthesis of genetically encoded chemical polymers

N-terminal incorporation of non-canonical monomers at translation initiation provides the greatest flexibility for genetic code expansion strategies (Tharp et al., 2020). Indeed, the ribosome and other translation factors are highly tolerant of the physical and chemical properties of N-terminal ncAAs such as oligomeric length, steric bulkiness, hydrophilicity, and reactivity of functional groups. At the initiation step of translation when Met is loaded to fMet tRNA, it is delivered to the small submit (30S) with the initiation factors, and then the large subunit (50S) is assembled for the peptide elongation. Due to this sequential assembly mechanism, ncAAs or other monomers charged on the initiating tRNA can be directly positioned to the P-site through the exit tunnel where no growing polypeptide chain occupies for the polymerization reaction. This opens up space for non-amino acid groups, meaning that a greater variety of substrates can be imagined, because the monomer does not necessarily have to contain an amine or nucleophile to initiate the peptide chain.

The ribosome is less tolerant of C-terminal ncAA incorporation. This is because the C-terminal incorporation of a monomer involves chain extension, requiring more precise alignment of substrates in the ribosome's active site. Although inefficiently, β-amino acids (Dedkova et al., 2006, 2012; Dedkova and Hecht, 2019; Maini et al., 2015a), cyclic β-amino acids (Fujino et al., 2016; Katoh et al., 2020; Lee et al., 2020c), α-D-amino acids (Dedkova et al., 2003, 2006; Katoh et al., 2017), α-hydroxy acids (Ohta et al., 2007), α-thioacids (Takatsuji et al., 2019), and cyclic γ-amino acids (*cis*- and *trans*-3-aminocyclobutane carboxylic acid, Figure 5, Lee et al., 2020b) have been successfully incorporated into the C terminus of a peptide by the ribosome. In some cases, engineered ribosomes that address space, orientation, and reactivity challenges inherent to non-canonical substrates, were needed. This limited pool of substrates for the C-terminal incorporation is likely because of the entropy trap effect (Leung et al., 2011; Sievers et al., 2004) that only enables formation of

covalent bonds (e.g., amide, ester, or thioester) between the substrates bearing a short flexible carbon-chain backbone or optimal nucleophiles on the same geometry with α-amino acid. This major confounding factor hampering polymerization of non-canonical monomers into the C terminus may be addressed by systems-level engineering of all biological parts involved in the complex system of protein biosynthesis, including the tRNA, EF-Tu, EF-P, and ribosome, as discussed above.

Conclusions

Systems-level engineering of efficient orthogonal translation systems is driving innovation in an effort to expand the genetic code. Indeed, studying how to coordinately tune all orthogonal translation components—tRNAs, aaRSs, EF-Tu, EF-P, mRNA, ribosomes, and the resulting polymerized product—for new functions is teaching us basic biology. It also opens the door to the biological production of polymers with previously unimaginable structures and functions.

We anticipate three key areas of near-term growth. First, given the seemingly endless possibilities of modifying the machinery to make it efficient, we anticipate that computational approaches, driven by artificial intelligence, machine learning, and online gaming (e.g., Eterna) will facilitate systems-level design. Second, we expect that exploration of natural and engineered aaRS systems, including novel chemical moieties, network-level dynamics, and genomically recoded organisms will lead to new capabilities. With building genomes within reach, it is exciting to consider opportunities to recode organisms and the possibilities this unlocks. Third, we expect that new ribosome-engineering platforms will make the ribosome a compelling target for tuning designer chemistries and novel substrates. One tantalizing idea is to assess whether the ribosome's active site can be re-sculpted to promote alternative chemical reactions that would result in the formation of non-amide bonds.

Looking forward, we expect that engineering molecular translation will lead to peptidomimetic drugs comprising backbone-modified analogs with increased stability and decreased immunogenicity; polyketides that can act as anticancer drugs or combat rising antibiotic resistance, which threatens loss of 10 million lives per year by 2050; and to new functional materials (e.g., aramids underlying bulletproof materials, and biodegradable plastics). If successful, redesigning translation could enable researchers to begin evolving such non-protein medicines and materials in the ways that we (and nature) evolve proteins.

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DECLARATION OF INTERESTS

M.C.J. has a financial interest in SwiftScale Biologics, Design Pharmaceuticals, and Pearl Bio. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies. All other authors declare no competing interests.

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