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Tethered Ribosomes: Toward the Synthesis of Nonproteinogenic Polymers in Bacteria

Andreea Stan[+[a]] and Clemens Mayer*[a]

The ribosome is the core element of the translational apparatus and displays unrivaled fidelity and efficiency in the synthesis of long polymers with defined sequences and diverse compositions. Repurposing ribosomes for the assembly of nonproteinogenic (bio)polymers is an enticing prospect with implications for fundamental science, bioengineering and synthetic biology alike. Here, we review tethered ribosomes, which feature inseparable large and small subunits that can be evolved for novel function without interfering with native translation. Following a tutorial summary of ribosome structure, function, and biogenesis, we introduce design and optimization strategies for the creation of orthogonal and tethered ribosomes. We also highlight studies, in which (rational) engineering efforts of these designer ribosomes enabled the evolution of new functions. Lastly, we discuss future prospects and challenges that remain for the ribosomal synthesis of tailor-made (bio)polymers.

1. Introduction

The genetic code is the set of rules used by living cells to translate information encoded within genetic material (DNA or RNA sequences) into proteins.[1] The standard genetic code comprises 64 triplet codons, of which 61 translate for the 20 canonical amino acids and three nonsense codons terminate translation. The promise of bestowing proteins with new functionalities has fueled efforts to enable the ribosomal incorporation of noncanonical amino acids (ncAAs) with distinct properties.[2] Common genetic-code expansion strategies include the global reassignment of rare sense codons (i.e., the AUG start codon),[3] the suppression of stop codons by orthogonal amino-acid tRNA synthetase/tRNA pairs (=orthogonal translation systems, OTSs),[4] or translation through four-base and five-base codons.[5] Combined, these strategies have enabled the cellular incorporation of >150 ncAAs into proteins,[6] demonstrating that the genetic code Crick once referred to as *frozen accident* remains highly malleable today.[7]

In general, genetic-code expansion strategies take advantage of the ribosome displaying high substrate promiscuity, readily accepting (heavily) modified α-amino acids as well as translating a handful of α-hydroxy, D- or β-amino acids has.[8] While in reality often only a single noncanonical building block is (repeatedly) incorporated into a given polypeptide, increasing both the number and type of monomeric units that can be used in ribosomal translation is an enticing prospect for the preparation of tailor-made (bio)polymers (Figure 1).[9] For once, relying on the ribosome for the construction of sequence-specific (bio)polymers contrasts their chemical syntheses, which make use of iterative cycles of coupling and deprotection steps.[10] Moreover, making unnatural polymers genetically encodable could allow for unprecedented opportunities to finetune polymeric structures and properties by directed evolution.[11] Specifically, using cycles of mutagenesis and selection could tailor a polymer's backbone and its side chains and, as a result, pave the way toward diverse applications in bioengineering, synthetic biology, biomedicine and biotechnology.

Figure 1. A modified central dogma, in which an engineered ribosome enables the synthesis of tailor-made (bio)polymers featuring unnatural building blocks.

Unfortunately, many unnatural monomeric units that could be employed for the ribosomal synthesis of sequence-specific, nonproteinogenic (bio)polymers are not recognized as cognate substrates by the native translational machinery.[12] Critically though, recent studies have demonstrated that randomizing key regions required for peptide-bond formation can elicit ribosome variants with an expanded substrate scope. Specifically, subjecting cells harboring ribosome libraries to puromycin derivatives featuring altered amino acids enabled the isolation of clones uniquely sensitive to these analogs.[13] Moreover, isolated ribosome variants often enabled the incorporation of amino acid analogs similar to those present in the modified puromycin. Combined, these studies facilitated the synthesis of proteins containing β-amino acids, cyclic amino acids, as well as dipeptides and dipeptomimetics.[8d,14]

Nevertheless, many mutations that could significantly expand the scope of monomers accepted by the ribosome remain inaccessible applying modified puromycin derivatives,

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as alterations of the ribosome structure often prove lethal (vide infra). To address this challenge, a series of recent manuscripts has described orthogonal and tethered ribosomes that can process mRNA in parallel with, yet independent of endogenous ribosomes. In this review, we highlight these ribosome-engineering efforts, which have the potential to enable the sequence-selective synthesis of diverse (bio)polymers. With a broad chemical biology audience in mind, we will first discuss mechanistical and structural aspects of the ribosome and the assembly processes that are critical for engineering the translational apparatus for new building blocks. From there, we will highlight recent efforts of designing, characterizing and evolving orthogonal and tethered ribosomes for the synthesis of polymers in *Escherichia coli*.

2. Ribosome Structure, Function, and Biogenesis

The bacterial ribosome is a 2.5 megadalton molecular machine and the core element of the translational apparatus of the cell.[15] Consistent with its central role in biology, the ribosome displays high efficiency (~16 amino acids per second) and fidelity (error rate of ~$1:10^3$–10^4) for the synthesis of proteins of varying length and amino acid compositions.[16] Ribosomes build up proteins by sequentially catalyzing peptide bond formations between amino acylated tRNA adapter molecules that are recruited in response to triplet codons located on mRNA molecules (Figure 2A).[17] Although the ribosome is made up from more than 50 proteins and only three ribosomal rRNAs (the 5S, 16S and 23S rRNA),[15] it is a ribozyme at heart.[18] In fact, only two ribosomal proteins (L16 and L27) have thus far been confirmed to interact with the tRNA substrates and thus could have a potential role in the peptidyl transfer reaction.[19] While the catalytic properties of the ribosome arise in part from providing well-positioned nucleobases for transition-state stabilization,[20] the observed rate accelerations predominantly result from correctly positioning the reactive ester and amine moieties of the participating substrates (= entropy trap).[21]

Structurally, prokaryotic ribosomes (70S) are nucleoprotein particles formed from two main components: the small (30S) and the large (50S) subunit (Figure 2A). While the former contains the 16S rRNA and 21 ribosomal proteins,[22] the latter is made up from an assembly of the 23S and 5S rRNA as well as 33 proteins.[23] Functionally, the two subunits also perform distinct, yet coordinated roles during translation, where the small subunit is responsible for mRNA recognition/decoding.[24] Conversely, the large subunit harbors the peptidyl-transferase center (PTC) and orchestrates the conformational changes required for the sequential processing of amino-acylated tRNA molecules.[25] During translation, a given tRNA transits three sites that are (largely) located within the large subunit (Figure 2A): 1) the A-site accepts loaded tRNAs, 2) the P-site holds the tRNA-nascent peptide chain, and 3) the E-site houses processed tRNAs prior to their departure from the ribosomal complex.[26] Peptide-bond formation takes place in the PTC, which is located at the interface of the A- and P-sites. Specifically, recognition of the acceptor stem of a loaded tRNA induces conformational changes that bring the substrates into close proximity of each other.[27] While the exact nature of the required proton shuffling reactions remain doubtful, the nucleophilic attack and deprotonation of the α-amine are likely taking place in a single rate-determining step, which is coordinated by the O2′ hydroxy group of the A76 tRNA in the P-site, nucleobases from the 23S rRNA, a structured water network, and potentially the N-terminal amine of L27 (Figure 2B).[20,28] Lastly, the nascent peptide chain leaves the ribosomal complex through the exit tunnel (Figure 2A), which stretches from the PTC to the surface of the ribosome. This hollow structure is largely composed of the 23S rRNA but has significant contributions from proteins L4, L22, and L39e, and its exit is encircled by several ribosomal proteins.[29]

The characteristic architecture of the ribosome emerges from a complex and tightly regulated enzymatic process that facilitates the correct folding of rRNAs and their association with ribosomal proteins (Figure 2C).[30] This process, termed

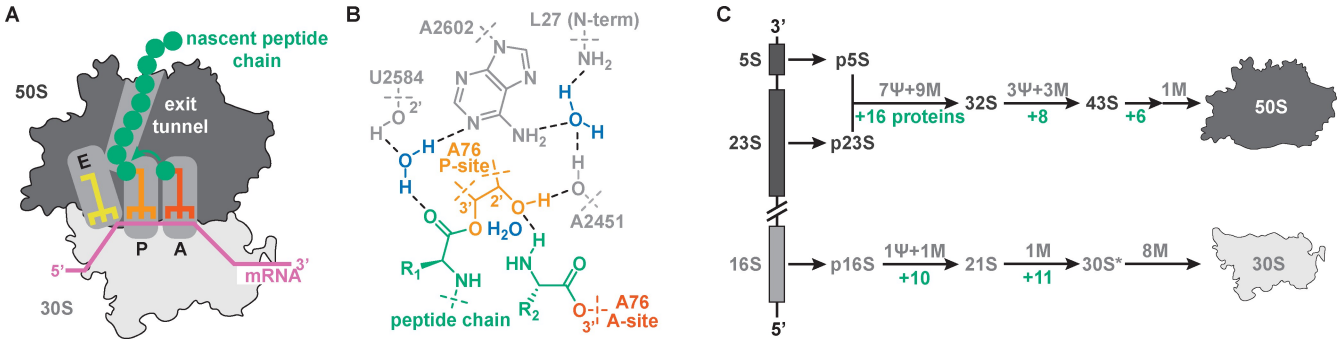

Figure 2. A) Schematic representation of the 70S bacterial ribosome. B) Schematic view of the PTC that is located at the interface of the A- and P-sites. The nucleophilic attack and proton-shuffling reactions are coordinated by the 2′-OH group of the A76 tRNA in the P-site, nucleobases from the 23S rRNA, a structured water network, and potentially the N-terminal amine of L27. C) Schematic representation of ribosome biogenesis. p5S, p23S, and p16S are precursors to their respective matured rRNAs. M and ψ indicate methylations and isomerization of uridine to pseudouridines, respectively. Mature 30S and 50S subunits assemble to 70S ribosomes upon binding to an mRNA.

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ribosome biogenesis, starts with the transcription of the 5S, 16S, and 23S rRNAs, which are located on a single transcript.[31] Concomitantly, ribosomal proteins are synthesized and undergo posttranslational modifications, of which the majority facilitate the correct association of ribosomal proteins with rRNA species and nucleoparticles during biogenesis.[32] In the next step, the primary transcript undergoes maturation processes that consist of nucleolytic processing (i.e., end trimming) and chemical modifications of nucleotides.[30] Methylations, isomerization of uridine to pseudouridine and the formation of dihydrouridine predominantly occur close to functional regions of the ribosome,[33] such as the decoding center on the small subunit and the PTC on the 50S subunit. Consequently, the deletion of enzymes responsible for installing these modifications can compromise ribosome activity and result in growth defects or cell death.[30a] Moreover, the installation of these modifications is dependent on the rRNA being folded correctly and therefore act as checkpoints in ribosome maturation (Figure 2C). With the PTC being one of the last structures to be assembled during biogenesis, any maturation defect that occurs upstream can render ribosomes dysfunctional. Indeed, while mutations in the 16S rRNA can compensate for the absence of chemical modifications, engineering functional ribosomes harboring unmodified 23s rRNA has not been achieved.[30] Lastly, in addition to ribosomal proteins, a number of RNA chaperones, RNA helicases and ribosome-dependent GTPases assist in the folding of the rRNA and, when overexpressed, can rescue ribosomes with folding defects.[34]

Considering that cell growth and survival depends on the rate of protein synthesis, ribosome biogenesis evolved to be a complex, yet efficient and well-orchestrated process. As such, any defects during this maturation process will impact the formation of the characteristic PTC architecture, which is required for accurate and efficient translation. When attempting to engineer ribosomes for the synthesis of nonproteinogenic (bio)polymers, it is therefore necessary to take into consideration all factors that contribute to the proper folding, assembly and function of this molecular machine.

3. Creating Orthogonal Ribosome Populations

In a cell, all mRNA molecules are translated by a single population of (functionally) identical ribosomes. The fact that mutations in conserved sites of rRNAs are often dominant-negative or lethal complicates efforts to alter and evolve endogenous ribosomes for the synthesis of nonproteinogenic polymers. Creating ribosome subpopulations that solely recognize and translate a dedicated subset of mRNAs has proven a means to overcome this hurdle.[35] Ideally, the separation into two distinct ribosome pools allows for introducing mutations throughout the ribosome subpopulation *without* negatively impacting the proteome synthesis performed by wild-type ribosomes.

In a first approximation, the selection of mRNAs by bacterial ribosomes is based on the presence of the Shine-Dalgarno (SD) sequence.[36] While not universally conserved, placing the SD sequence ~8 bases upstream to an AUG start codon, robustly enhances the initiation rate of translation.[37] mRNAs carrying the 5-nucleotide stretch, GGAGG, are recognized by a complementary sequence located close to the 3'-end of the 16s rRNA, termed the anti-SD (ASD) sequence, CCUCC (Figure 3A).[38] By installing complementary mutations in the ASD and SD sequences of a designed 16S rRNA and a matching mRNA encoding for the human growth hormone (hGH), Hui and coworkers were the first to exploit this recognition mode for the creation of orthogonal ribosomes (o-ribosomes).[39] Critically, hGH production in *E. coli* cells transformed with these constructs proved to be dependent on *specialized* 30S subunits with designed ASD sequences in their 16S rRNAs. Although these and other early studies served as a proof of principle for segregating two translation systems in vivo, the designer

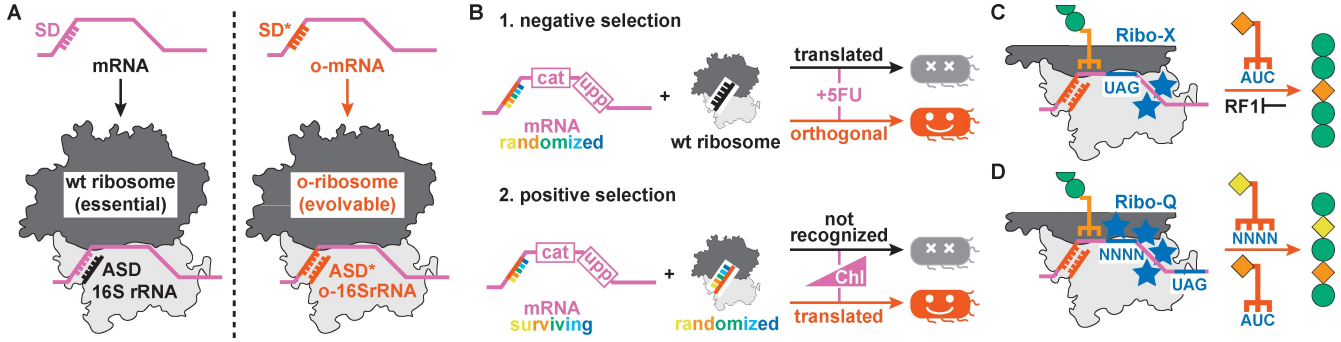

Figure 3. A) Ribosomal translation is initiated through the recognition of the SD sequence by the complementary ASD located on the 16S rRNA. A subpopulation of *orthogonal* ribosomes can be created by introducing matching mutations into ASD and SD sequences, resulting in SD*/ASD* pairs that are recognized independently to the transcripts processed by wild-type ribosomes. B) Identifying SD*/ASD* pairs through a dual selection strategy. 1) Negative selection: in the presence of 5-fluoruracil (5FU), the translation of mRNAs with SD* sequences that are recognized by wild-type ribosomes results in the production of lethal uracil phophoribosyltransferase (upp). 2) Positive selection: pairs of functional SD*/ASD* are selected based on their ability to produce chloramphenicol acetyl transferase (cat), which allows for survival in the presence of chloramphenicol (Chl). Increasing concentrations of Chl serve as a selection pressure to identify the best SD*/ASD* pairs from a randomized 16S rRNA library and the surviving mRNA constructs from the negative selection. C) Ribo-X features mutations (blue stars) in the 530-loop, which result in a decreased recognition of release factor 1 (RF1). As a result, Ribo-X reads through UAG stop codons, allowing the efficient incorporation of ncAAs. D) Ribo-Q features two additional mutations at the decoding center and enables the translation of quadruplet codons. Paired with the ability to suppress stop codons, Ribo-Q facilitates the site-selective incorporation of two distinct ncAAs.

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mRNAs and 16S rRNAs employed were often not fully orthogonal to the endogenous translational machinery.[40] As a result, the expression of orthogonal transcripts was partially sustained by wild type ribosomes and vice versa, which could impact cellular fitness and proliferation when attempting to engineer specialized 30S subunits.

To overcome this limitation, Rackham and Chin randomized SD and ASD sequences and leveraged biological selections to evaluate 4^7 (16,384) mRNAs against 4^8 (65,536) 16S rRNA variants (Figure 3B).[41] Three distinct classes of o-mRNA/o-ribosome pairs emerged from these efforts, which did not impact cellular fitness and could process information in parallel with, but independent of their wild-type progenitors. Capitalizing on the act that these o-ribosomes were *free to evolve*, a series of follow-up studies successively engineered the mRNA decoding centers of these o-16S rRNAs.[35] For example, introducing mutations to weaken the interaction of o-ribosomes with release factor 1 yielded Ribo-X, which was able to efficiently decode amber stop codons (UAG) with ncAAs loaded onto suppressor tRNAs (Figure 3C).[42] Another o-ribosome variant, Ribo-Q, was engineered to recognize diverse quadruplet codons and enabled the site-specific incorporation of multiple noncanonical building blocks into recombinant proteins (Figure 3D).[43]

Combined these studies demonstrate how creating and engineering independent subpopulations of orthogonal ribosomes can result in a surprising level of innovation in a catalyst as ancient and conserved as the ribosome. Despite the undeniable power of o-ribosome-engineering though, without the ability to selectively control the association between orthogonal 30S and designer 50S subunits, many key ribosomal functions cannot be tinkered with using this approach. As a case in point, the majority of the A-, P- and E-sites, as well as the PTC and the exit tunnel are made up from the 23S rRNA within the large subunit.[23,25]

4. Creating and Engineering Tethered Ribosomes

The requirements for controlling the stochastic association and exchange of large and small subunits makes the creation of *fully* orthogonal ribosomes particularly challenging. In addition of comprising a network of RNA-RNA interactions that spans approximately 6000 $Å^2$,[15a] the independent but coordinated assembly, rotation, and dissociation of the two subunits are all hallmarks of the translation process.[44] Challenging the *universal, bipartite* of the ribosome, two independent reports have recently demonstrated that ribosomal subunits can be linked through small RNA linkers (Figure 4A).[45] Astoundingly, these tethered (or stapled) ribosomes did not only retain good translational activities but also proved excellent starting points for expanding ribosomal function by engineering efforts that targeted the 23S rRNA.

4.1. Designing and validating tethered ribosomes

Designs aimed at creating functional ribosomes by fusing the 16S and 23S rRNAs can only be successful if they meet a strict set of criteria. For once, 16S-23S chimeras must be recognized by the vast majority of ribosomal proteins and enzymes involved in biogenesis to guarantee their proper processing, folding and assembly into functional entities.[30] This also includes the 5S rRNA, which is transcribed independently and must be able to associate in a stoichiometric fashion.[46] Additionally, the connecting RNA tether needs to be sufficiently flexible to not interfere with the coordinated movements of subunits that is required for initiation, elongation and chain termination during translation.[26] However, the flexibility and length of the linker also need to be limited in order to avoid its rapid degradation by endogenous RNAses and ensure the intramolecular (*cis*) rather than intermolecular (*trans*) assembly of large and small subunits (Figure 4B).

With a distance of >170 Å, the existing termini of the 23S and 16S rRNAs are too far apart to allow for their direct connection.[15a] However, it has been shown that the native 23S rRNA ends are proximal to each other and can first be joined and the resulting circular construct subsequently cleaved to generate new 5′ and 3′-ends without sacrificing ribosome function (=*circular permutation*, Figure 4C).[47] Moreover, in some organisms the 16S rRNA is split into multiple fragments or can feature long insertions.[48] Based on these observations, Orelle, Carlson and co-workers as well as later Fried et al. reasoned that circularly permutated 23S rRNA constructs might be inserted into the native 16S rRNA sequence of *E. coli* to yield a single-chain chimeric rRNA (Figure 4C).[45] Independently, both reports identified helix 101 (H101) on the 23S and helix 44 (h44) on the 16S rRNA as potential junction sites (Figure 4D). Not only are these helices in close proximity to each other (~3 nm), but their sequences are also less conserved than other regions of the rRNAs.[15b,49] Moreover, these helices are located distal from functional regions of the ribosome – albeit the upper portion of h44 is located closely to the ASD sequence – and are known to tolerate mutations and insertions in natural ribosomes.[48a,50] For the RNA linker joining H101 and h44, the groups pursued different strategies. While Orelle, Carlson and co-workers simply inserted a stretch of 8 and 9 adenine residues on the respective termini of the 23S rRNA,[45a] Fried et al. opted to join the rRNAs via the J5/J5a region from the *Tetrahymena* group 1 self-splicing intron (Figure 4D).[45b] While the adenine stretches likely remain unstructured, the RNA hinge employed by the latter can toggle between an extended and *U-turning* form[51] and, if necessary, compensate for the independent movement of subunits during translation.

Remarkably, both designs gave rise to tethered ribosomes that were functional when introduced into *E. coli*. Specifically, replacing endogenous rRNAs with 16S-23S chimeras that feature adenine linkers gave rise to viable cells (Figure 4E).[45a] While the initial transformants grew to low cell densities and exhibited poor recovery from the stationary phase, sequential passaging in liquid culture for ~100 generations gave rise to a clone that displayed significantly improved growth character-

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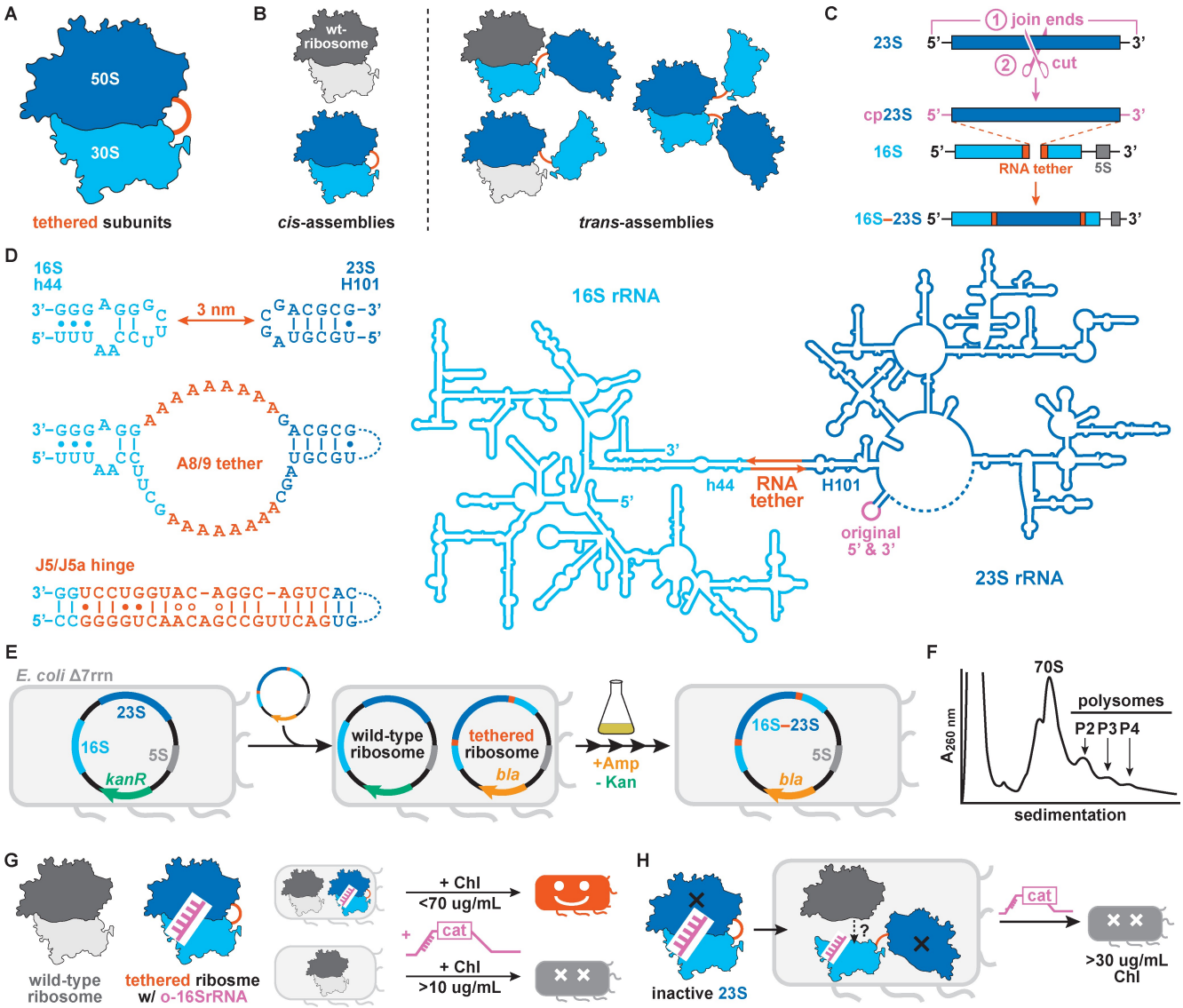

Figure 4. A) Tethered (or stapled) ribosomes result from connecting the 16S and 23S rRNAs via a short RNA linker. B) The RNA linker must be rigid enough to facilitate the assembly of subunits from the same molecule (*cis*), rather than allow the free exchange of subunits between wild-type and/or tethered ribosomes (*trans* assembly). C) Single-chain 16S-23S hybrids can be generated by 1) circular permutation of the 23S rRNA and 2) its insertion into the 16S rRNA sequence, which features an RNA tether (in red). D) Sequences, structures and location of helices 44 and 101, which are used as insertion sites to generate tethered ribosomes. Linker sequences to connect subunits are shown in red, for the J5/J5a hinge, the structure of the closed form is shown. E) An *E. coli* strain, for which all 7 copies of ribosomal genes (rrn) are knocked out can be used to introduce tethered ribosomes in place of the endogenous variant. Limited proliferation in the presence of ampicillin results in viable cells that only harbor tethered ribosomes. F) Isolation of tethered ribosomes from cells followed by sucrose gradient fractionation demonstrates that linked subunits are active and associate with the heavy polysomal (P2-4) fractions. G) Incorporation of an o-ASD enables tethered ribosomes to function in concert with wild-type ribosomes in cells. *E. coli* cells featuring linked subunits can proliferate in the presence of a higher concentration of chloramphenicol (Chl), when an orthogonal mRNA bearing a chloramphenicol acetyltransferase gene (cat) is provided. H) Tethered ribosomes with inactivated large subunits can only survive under less stringent conditions, indicative of a reduced cross assembly between the orthogonal 30S and wild-type 50S subunits in cells.

istics. Shorter doubling times for this variant were ascribed to compensating mutations in the *E. coli* genome, rather than alterations in the introduced single-chain rRNA. Notably, sucrose gradient fractionation experiments followed by PAGE analyses, confirmed that linked subunits remained intact during biogenesis and associated with the heavy polysomal fractions (Figure 4F). Combined, these results showed the tethered ribosome was responsible for proteome synthesis and displayed ~50% of the activity when compared to its progenitor.

To demonstrate that subunits stapled by an RNA hinge can function alongside endogenous ribosomes in cells, Fried and co-workers inserted their circularly permutated 23S rRNA into an orthogonal-16S rRNA.[45b] When *E. coli* cells were provided with a matching o-mRNA encoding for a chloramphenicol acetyltransferase gene, cells producing tethered ribosomes allowed for growth at significantly higher antibiotic concentrations (70 μg/mL) than those unable to decode the orthogonal message (< 10 μg/mL, Figure 4G). To pinpoint whether the

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observed activity was dependent on the *cis* assembly of the covalently linked subunits, the authors introduced mutations into the stapled 23S rRNA that severely hinder protein synthesis.[52] *E. coli* cells transformed with these constructs could only proliferate in the presence of <30 μg/mL chloramphenicol, thereby confirming an active role of the tethered 23S rRNA in translation of the o-mRNA (Figure 4H). Furthermore, cells producing these compromised tethered ribosomes displayed a negligible increase in doubling times, thus contrasting the dominant-negative effect of these mutations in endogenous ribosomes.[52] Notably, performing comparable experiments with ribosomes linked via adenine stretches also enabled the production of tethered ribosomes featuring otherwise lethal mutations.[45a] Combined, these results are consistent with the reduced association of stapled, large subunits and endogenous small subunit (Figure 4B), as such a *trans* assembly would negatively impact proteome synthesis and inhibit growth.

Overall, these studies highlight that, despite its highly conserved architecture, the ribosome can tolerate major structural modifications without the concomitant loss of activity. Somewhat surprisingly, introducing a short RNA tether or staple proved a straightforward, yet effective strategy to create functional 16S-23S rRNA chimeras and exert control over the otherwise stochastic assembly of large and small subunits.

4.2. Characterizing and optimizing tethered ribosomes

While these results were encouraging, a more thorough characterization revealed some notable shortcomings of these first-generation, tethered ribosomes. For example, a detailed study by Aleksashin and co-workers pinpointed defects in the biogenesis process as the main reason for lower performances of tethered ribosomes with adenine-linkers.[53] Specifically, sucrose gradient fractionation experiments indicated that their maturation stalls at later stages of the assembly (Figure 5A). Analysis of rRNAs and associated proteins in the stalled fractions confirmed the accumulation of non-functional intermediates characterized by 1) lower modification rates of key rRNA residues, 2) a diminished association with ribosomal proteins and 3) an incomplete trimming of rRNA ends (Figure 5B). Posttranscriptional modifications were found to be under-

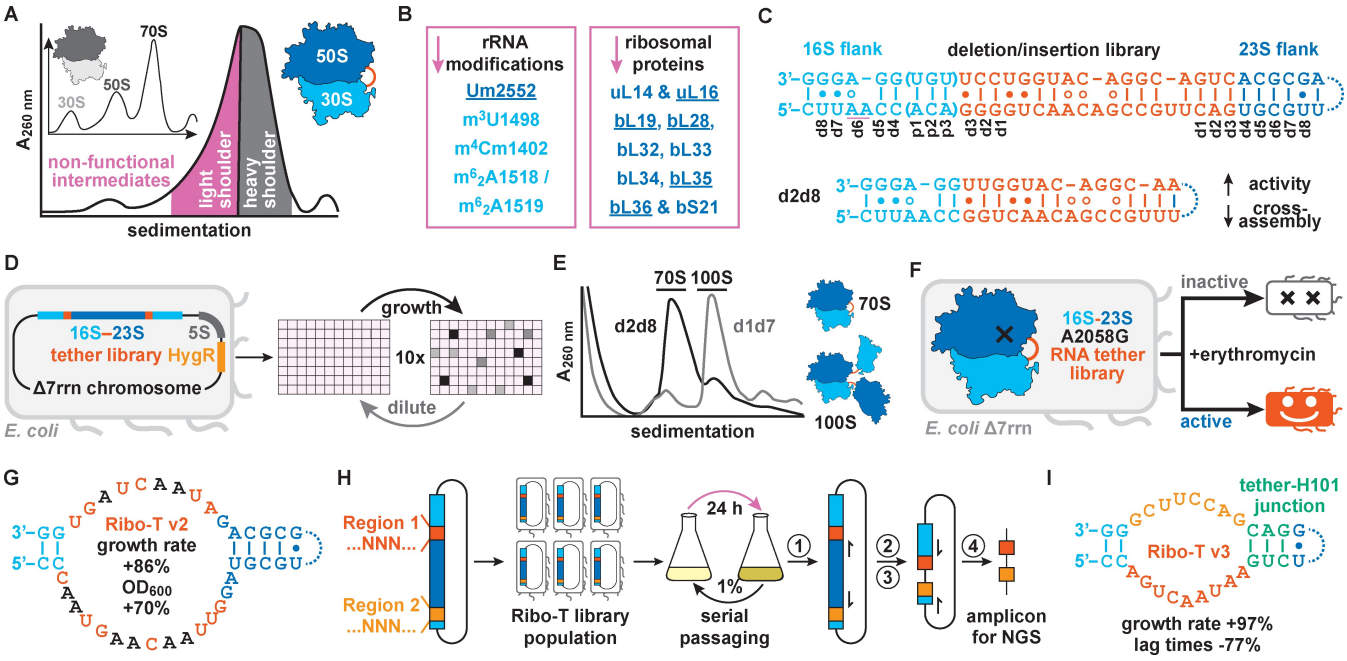

Figure 5. A) Sucrose fractionation experiments of adenine-tethered ribosomes result in a broad peak, with a lighter shoulder being indicative of an accumulation of nonfunctional, late-stage intermediates. The insert shows typical results for ribosomes with separate subunits. B) Analysis of rRNAs and associated proteins in stalled intermediates reveals an under-representation of several RNA modifications and ribosomal proteins. Methylation of U2552 in the heavy chain was pinpointed as a critical residue that affects the association with several ribosomal proteins. Those underlined have also been found to be under-represented in an independent study of wild-type ribosomes that lack Um2552. C) In a library of tethered ribosomes featuring the J5/J5a hinge, deletions (d) and insertions (p) are systematically explored at both the 16S and 23S flank. The d2d8 variant was found to possess higher activities and a lower cross-assembly with endogenous subunits. D) Genomic replacement of chromosomal rRNAs with d2d8 yields an *E. coli* strain with retarded growth rates. Automated parallel evolution of *E. coli* harboring d2d8 as the sole ribosome was performed by 10 growth/dilution cycles in 96-well plates. In a population, variants with genomic mutations that result in faster doubling times will outgrow those featuring nonbeneficial mutations. E) The d2d8 variant shows a strong preference for *cis* assembly, as judged by sucrose fractionation experiments, while other variants, such as d1d7 assemble predominantly in *trans*. F) Introducing the mutation A2058G into the 23S rRNA bestows erythromycin resistance and enables the selection of functional 16S-23S chimeras on solid support. G) Tether sequence of Ribo-T v2 that resulted after optimization of the original adenine linkers (Figure 4D). H) Further optimization of Ribo-T v2 through EVOLINK. First, tether sequences and/or the H101-tether junction are randomized and 16S-23S chimeras are allowed to compete on the population level. Tethered ribosomes that allow faster growth are then identified by 1) isolating plasmids from selection cultures, 2), 3) performing a PCR and ligation to join distant regions, and 3) a second PCR to generate amplicons of <300 bps for next-generation sequencing (NGS). **I:** The identified Ribo-T v3 features a shorter RNA tether and a designed tether-H101 junction to allow for the independent folding of the two domains.

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represented in both subunits, from which the incomplete/delayed 2'-methylation at residue U2552 in the 23S rRNA is thought to be a major bottleneck for the assembly of translationally active, tethered ribosomes. Given its critical role in the RNA element responsible for positioning the aminoacyl-tRNA in the PTC A-site,[54] 2-methylation of U2552 by the enzyme RlmE represents an important checkpoint in ribosome biogenesis.[55] Specifically, cells lacking or featuring a deactivated variant of this dedicated methylase show severe growth defects due to the accumulation of 50S subunit intermediates that lack specific ribosomal proteins. Notably, proteomics measurements identified that some of these ribosomal proteins were also underrepresented in stalled intermediates of tethered ribosome (Figure 5B). Although the altered molecular architecture and presence of the RNA linker clearly impedes ribosome biogenesis, once tethered ribosomes are fully assembled, they are highly active in translation. Indeed, ribosome profiling (Ribo-seq) data identified a somewhat increased relative occupancy of tethered ribosomes at the start and stop codons as the only notable differences to their wild-type progenitors. Thus, it appears that the tether affects primarily steps of translation that require subunit joining (initiation) or separation (termination/recycling), while having little effect on the relative mobility of subunits during the elongation stage of protein synthesis.

In a complementary study, Schmied, Tnimov, Uttamapinant, and co-workers investigated the propensity of ribosomes tethered via the J5/J5a region from the *Tetrahymena* group 1 self-splicing intron to cross-assemble with endogenous ribosomal subunits.[56] Anticipating that the initial RNA staple was not optimal, the authors prepared a panel of 107 tethered ribosomes that systematically probed deletions and/or insertions in h44 and H101 (Figure 5C). Consistent with facilitating translocation and rotation of one subunit with respect to the other, these alterations gave rise to tethered ribosomes with vastly different activities and propensities for cross-assembly with endogenous subunits. One variant featuring two and eight deletions (d2d8) in h44 and H101, respectively, displayed high activities and low cross-assembly coefficients (Figure 5C). Moreover, cells featuring the d2d8-variant as their sole ribosomes were viable and sequential, parallel passaging gave rise to *E. coli* strains that grew with growth rates comparable to those observed with the wild-type ribosome (Figure 5D). Lastly, sucrose gradient analysis also revealed that the d2d8-variant predominantly assembles in *cis*, while other deletion combinations did show a strong preference to form dimers of tethered ribosomes in vivo (Figure 5E).

Similarly, finetuning the linker sequence has also resulted in more efficient tethered ribosomes with adenine stretches. In two follow-up studies Carlson, d'Aquino and co-workers first aimed to optimize composition of the A8 A9 tether,[57] while Kim, Watkins et al. then set out to improve the sequence connecting the linker to H101.[58] In these experiments, native ribosomes of cells were replaced with tethered constructs that featured an A2058G mutation in their 23S rRNA sequence. As this mutation conferred erythromycin resistance, functional, 16S-23S chimeras on erythromycin-containing could readily be identified on agar plates. In the first study, a limited number of surviving colonies were selected and their growth rates determined. The 15 best performing tether sequences were allowed to compete in liquid culture, with one particular tether becoming dominant in the population after three serial passages (Figure 5F). Upon further characterization, this variant, termed Ribo-T v2,[57] exhibited an up to 86% faster growth rate and a 70% increase in maximum OD_{600}, when compared to parent tethered ribosome featuring adenine stretches.

Nevertheless, the evolution of Ribo-T v2 was limited in scope as it relied on clonal isolation and functional testing. In part, this bottleneck can be ascribed to the fact that the randomized tethers are far apart in the sequence, thus complicating next-generation sequencing (NGS) efforts (limited to read lengths of ~300 nt) to identify the best combination(s) in vast combinatorial libraries. To address this bottleneck, Kim, Watkins et al. developed EVOLINK,[58] a three-step process that uses a PCR, ligation and a second PCR to contract formerly distant regions on a plasmid into a continuous next-generation sequencing read. Critically, EVOLINK allowed *E. coli* populations featuring vast libraries of tethered ribosome variants to compete in liquid cultures, with those providing a growth advantage to their cells outcompeting less-active variants over consecutive growth-dilution cycles (=serial passaging, Figure 5H). Applying four design-build-test-analyze cycles, in which library designs are informed by NGS results and structural modelling, the group identified Ribo-T v3 (Figure 5I).[58] In comparison to Ribo-T v2, cells harboring this version as their sole ribosomes displayed a two-fold improvement in doubling times and 77% shorter lag-times when grown in M9 minimal media. Besides an optimized and shorter tether sequence, Ribo-T v3 also features a junction between H101 and the tether, which is predicted to enable the independent folding of these two elements.

Combined, these studies elegantly demonstrate that some problems of first-generation tethered ribosomes, such as low activities, cross assembly with endogenous subunits or the tendency to form dimers, can be overcome through careful optimization of their linker sequences. While initial designs performed inferior to their endogenous progenitors, detailed biochemical analyses can provide critical insights into assembly defects. Attempts of improving the performance of adenine tethered ribosomes initially focused on rescuing non-functional intermediates by overexpressing proteins which alleviate assembly defects in wild-type ribosomes. Although the proteins tested as far (i.e., Der, ObgE, DbpA, CsdA, SrmB, KsgA, or RelE) did not improve the maturation of tethered ribosomes, there are several other ribosomal biogenesis and assembly factors to be examined (e.g., Hfq or IF3).[30] Conversely, optimizing the linker structure has already proven a means to engineer a new generation of tethered ribosomes with improved characteristics. Thus, it will be interesting to see whether the limited evolution that focused on the tether region has been sufficient to restore the biogenesis defects observed for their predecessors or further engineering will be necessary to guarantee an optimal activity.

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4.3. Engineering the large subunit of tethered ribosomes

With its highly conserved structure and its central role in translation, probing the characteristics of the PTC has long been restricted to a small set of nonlethal mutations.[59] Nevertheless, overcoming this limitation is critical to harness the full catalytic potential of the ribosome. Specifically, engineering the PTC could not only shed new light on the translation process per se, but is also necessary to introduce gain-of-function mutations that allow for the template-controlled polymerization of nonproteinogenic building blocks.[60] Tethered ribosomes, therefore, provide a unique opportunity toward these goals as they can function in parallel yet (largely) independent of the endogenous translation machinery. Consequently, a number of studies have begun to probe functional regions of tethered ribosomes – including the PTC at the heart of the 50S subunit – to elucidate *gain-of-function* mutations.

As a proof-of-principle, Orelle, Carlson and co-workers engineered the original A8/A9 tethered ribosome to translate the SecM polypeptide.[45a] Specifically, translation of this amino-acid sequence is problematic and typically stalls, due to the interaction of the SecM's nascent peptide chain with the ribosomal exit tunnel.[61] This interaction impairs PTC function by preventing the transfer of the 165-amino acid long peptide to the incoming prolyl-transfer-RNA. While mutations along the exit tunnels have been shown to alleviate stalling events,[61b,62] Orelle and co-workers surmised that otherwise, lethal mutations at the PTC A-site could result in efficient translation by tethered ribosomes. Toward this end, the authors first incorporated an orthogonal ASD into their linked 16S-23S chimeras. Next, they prepared a library of 16 variants that featured all dinucleotide combinations at positions 2451–2452, which are located at the amino acid binding pocket. The ability to prevent stalling upon *secM* translation was evaluated with an orthogonal reporter, in which the problematic sequence was fused in frame with a *lacZ* gene (Figure 6A). Thus, cells harboring tethered-ribosome variants that remained functional and displayed reduced stalling propensities could easily be identified by blue-white screening. This approach revealed one mutation (A2451C), which when introduced into tethered ribosomes displayed comparable readthrough frequencies as those provided by previously identified mutations in the exit tunnel. As such, this study provided proof-of-principle to employ orthogonal, tethered ribosomes for the identification of otherwise lethal, gain-of-function mutations.

Having engineered tethered ribosomes for controlled subunit association, Schmied, Tnimov, Uttamapinant, and co-workers aimed to further evolve their d2d8 variant to translate polyproline sequences – a task natural ribosomes are unable to perform.[56] Polyproline sequences present a number of challenges for translation, such as the poor accommodation of prolyl-tRNAs by the PTC, a retarded rate for peptide-bond formation, and clashes between the nascent proline chain and the exit tunnel.[63] In cells, ribosomes overcome these obstacles by recruitment of the elongation factor P (EF-P), which facilitates peptide-bond formation and elongation of consecutive proline residues.[64] Remarkably, engineering the PTC and exit tunnel of d2d8 enabled the translation of proline stretches in absence of EF-P. For this, the authors employed an EF-P knockout strain to construct a selection platform that featured a Pro3-sequence in frame with a chloramphenicol acetyltransferase gene (Figure 6B). As such, d2d8 variants that could read through the proline stretch could be identified by their ability to proliferate in the presence of higher chloramphenicol concentrations. After two rounds of directed evolution – a first one targeting 10 positions in the PTC and a second one probing 7 positions in the exit tunnel – a variant, d2d8-5, featuring 15 mutations emerged. Notably, this engineered tethered ribosome was able to translate proline-rich sequences of varying lengths and contexts at levels approaching those mediated by EF-P.

A related, but conceptually distinct approach to probe the function of ribosomes with *separate* subunits was recently introduced by Aleksashin and co-workers. In their Orthogonal

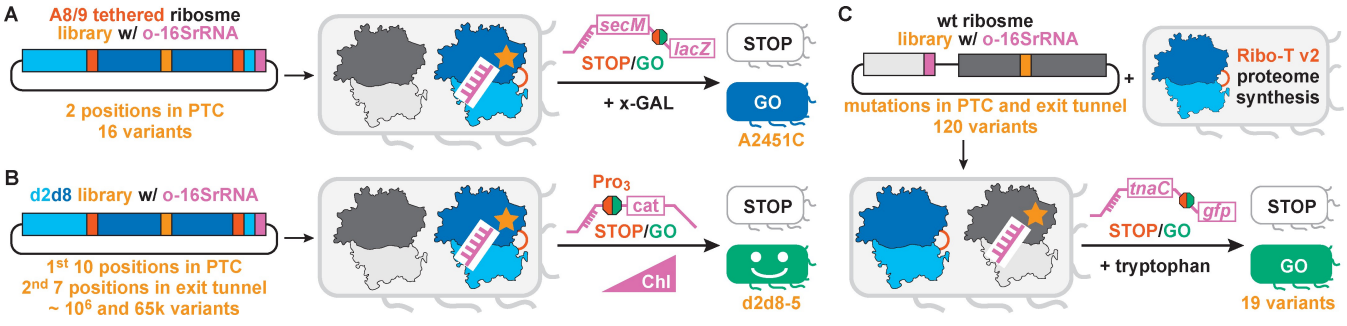

Figure 6. A) Engineering the A8/A9 tethered ribosome to alleviate SecM stalling. Introduction of an o-ASD and randomization of positions 2451 and 2452 results in 16 orthogonal, tethered ribosome variants. Their ability to alleviate SecM stalling was assessed by an o-mRNA encoding for a SecM-lacZ fusion, which enables readthrough efficiencies to be determined by blue/white screening on solid media. B) Selecting d2d8 variants that can translate polyproline sequences in absence of EF-P. Libraries targeting 10 positions in the PTC or 7 positions in the exit tunnel were successively probed for their ability to translate an o-mRNA bearing a Pro3-chloramphenicol acetyltransferase fusion in an EF-P knockout strain. d2d8 variants able to decode the polyproline stretch confer survival in the presence of higher chloramphenicol concentrations. C) Engineering ribosome with independent subunits to alleviate tryptophan-induced TnaC stalling events. In the OSYRIS system, tethered ribosomes are responsible for proteome synthesis, while orthogonal ribosomes with separate subunits are probed for gain-of-function mutations. A total of 120 23S variants that feature mutations in the PTC and the exit tunnel were evaluated to translate a TnaC-GFP fusion protein. Nucleotide substitutions that proved beneficial were identified by an increase in GFP fluorescence.

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translation SYstem based on Ribosomes with Isolated Subunits (OSYRIS), the roles of tethered and wild-type ribosomes are flipped.[65] Specifically, RIbo-T v2 is employed for proteome synthesis, while the introduction of o-ASD into the native 16S rRNA enables engineering of o-30S and 50S subunits without impacting cellular fitness (Figure 6C). Given that the RNA linker in their tethered ribosomes affects biogenesis and translation steps, the ability to engineer separate subunits could allow for innovations that are inaccessible for tethered constructs. As a proof of concept, the orthogonal, two-subunit ribosome in OSYRIS was engineered to overcome TnaC-mediated termination arrest.[66] Specifically, at high tryptophan concentrations termination of the *tnaC* stop codon is severely stalled by unfavorable interactions between the nascent TnaC polypeptide and rRNA nucleotides of the exit tunnel and the PTC.[67] A library of 120 23S rRNA variants featuring point mutations in these regions was constructed, and ribosomes that promoted efficient termination were identified by monitoring the translation levels of a TnaC-GFP reporter construct (Figure 6C). In total, 19 library members retained good translation activity and significantly decreased the efficiency of TnaC-dependent stalling (twofold compared to wild-type). Notably, these variants revealed a number of mutations that – due to their negative impact on cellular survival – had not been previously known to improve *tnaC* termination for natural ribosomes.[66a,67a] Furthermore, engineered 50S nucleoparticles could be isolated and reconstituted with wild-type small subunits to validate their ability to terminate TnaC in vitro. Indeed, for all tested variants, termination efficiencies in vitro correlated with those observed in the cell-based assay, thereby confirming the ability of otherwise lethal mutations in the 50S subunit to alleviate stalling.

Combined, these studies demonstrate that the controlled assembly of ribosomal subunits, allows for engineering the core elements of the translational apparatus. Having the ability to introduce, isolate and study the effect of otherwise detrimental mutations in functional parts of the large ribosomal subunit has already identified solutions to prevent stalling events when faced with problematic sequences. Notably, an extensive engineering campaign of d2d8 gave rise to a tethered variant with the general ability to translate polyproline sequences in absence of EF-P, a task that ribosomes with two subunits are unable to perform. Overall, we surmise that the efforts highlighted in this section take a first, critical step toward engineering ribosomes for the sequence-specific synthesis of non-proteinogenic biopolymers.

5. Conclusions and Future Prospects

In summary, recent advances in the design and engineering of single-chain 16S-23S chimeras has given rise to functional ribosomes with linked subunits. These tethered constructs can sustain cellular survival by themselves or operate in parallel with but (largely) independent of the endogenous ribosomes. Further characterization of tethered ribosomes has pinpointed defects in biogenesis processes as the main contributor to their overall decreased efficiencies. Conversely, optimizing the RNA tether and surrounding sequences proved fruitful for engineering tethered ribosome variants that display higher activities and less cross-assembly with endogenous subunits. Lastly, engineering ribosomes with linked subunits to expand ribosome functionality has identified mutations in the PTC and exit tunnels that alleviate stalling events for sequences that are otherwise difficult to translate.

However, several challenges remain before tethered ribosomes will be able to perform the on-demand synthesis of sequence-defined, nonproteinogenic polymers. First, while single residues of diverse monomeric building blocks are readily accepted by the ribosome, it remains unclear whether altering the PTC architecture will allow their ribosome-mediated oligo/polymerization. Tethered ribosomes offer a unique opportunity to identify variants for the sequence-specific synthesis of peptidomimetics (e.g., peptoids, β-peptides, γ-peptides) or more exotic polymers composed of aromatic foldamers or polyketide-like 1,3-dicarbonyl monomers. Second, in order to direct ribosome function to a particular set of monomers, it will be necessary to develop reliable computational tools to predict gain-of-function mutations. The rise of machine-learning algorithms and the considerable amount of existing data concerning ribosome structure and function should provide an ideal starting point for such efforts. Third, directed evolution campaigns will be dependent on efficient high-throughput screens/selections to effectively navigate the vast and combinatorial sequence space. Critically, as tethered ribosomes venture farther from their natural counterparts, the introduction of compensating mutations in the rRNA or the selection of ribosomal proteins specific for tethered subunits will become necessary to ensure the proper processing and functional assembly of tethered ribosomes.

Lastly, to create designer organisms for the on-demand synthesis of nonproteinogenic polymers, the creation of functionally diverse, tethered ribosomes does not suffice. Instead, they must be part of a wider translational apparatus that encompasses additional elements, such as codons, tRNAs, aminoacyl-tRNA synthetases and other translation factors. These additional elements could be made orthogonal and specific to tethered ribosomes through their known interfaces (such as the A- and P-site fingers, the Sarcin-Ricin loop, or E-site of the 23S rRNA)[68] with the rRNA. As all translational factors directly or indirectly interact with rRNA, tethered ribosomes present a common anchor for future engineering strategies that are aimed at creating a parallel translation system.

In conclusion, tethered ribosomes have taken a first, but critical step toward the synthesis of sequence-specific polymers. While efforts toward this goal are still in its infancy, we are confident that the recent advances highlighted in this review will open new perspectives for engineering ribosome function for diverse applications in synthetic biology.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: molecular evolution · ribosome engineering · RNA structure · sequence-specific polymers · synthetic biology

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