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Repurposing the translation apparatus for synthetic biology

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Abstract

The translation system (the ribosome and associated factors) is the cell's factory for protein synthesis. The extraordinary catalytic capacity of the protein synthesis machinery has driven extensive efforts to harness it for novel functions. For example, pioneering efforts have demonstrated that it is possible to genetically encode more than the 20 natural amino acids and that this encoding can be a powerful tool to expand the chemical diversity of proteins. Here, we discuss recent advances in efforts to expand the chemistry of living systems, highlighting improvements to the molecular machinery and genomically recoded organisms, applications of cell-free systems, and extensions of these efforts to include eukaryotic systems. The transformative potential of repurposing the translation apparatus has emerged as one of the defining opportunities at the interface of chemical and synthetic biology.

Background

Proteins represent a crucial class of biomolecules, universally employed by *all* living organisms to fulfill essential structural, functional, and enzymatic roles necessary to support life. In nature, these polymers are composed generally of twenty natural amino acid (AA)

building blocks, which can be combined in a near-infinite number of combinations to generate an impressive level of structural and functional diversity (Figure 1). However, many interesting chemistries cannot be accessed using only these natural building blocks; accordingly, for some time there has been an interest in the incorporation of nonstandard amino acids (nsAAs) featuring novel functional sidegroups to expand the repertoire of protein functions.

Broadly speaking, nsAAs can be divided into two classes. *Synthetic nsAAs* are chemically synthesized, and can bear little resemblance to their naturally occurring counterparts. *Posttranslational modifications* (PTMs) are modified derivatives of canonical amino acids. In recent years, two distinct approaches for the incorporation of nsAAs into proteins have emerged (Figure 1(a)). One such approach is *global suppression*. This method uses auxotrophic strains that are incapable of synthesizing a particular AA. When grown in the presence of a nsAA that bears close structural resemblance to the ‘missing’ AA, the organism's native translational machinery incorporates the nsAA instead [1,2]. An alternative approach uses orthogonal translation systems (OTSs) to genetically encode an nsAA of interest site-specifically by reassignment of codons, typically the amber stop codon (TAG) in a strategy known as *amber suppression* [3].

To date, >150 nsAAs have been incorporated by OTSs into polypeptides [4] for a wide range of applications including the introduction of bioorthogonal handles for protein tagging [5,6], alteration of protein stability [7,8], monitoring of protein localization, and genetic encoding of PTMs [9,10–12]. As a result of these impressive efforts and the transformative potential to construct bio-based products beyond natural limits, expanding the genetic code has emerged as one of several major defining opportunities and points of synergy in chemical and synthetic biology.

This review focuses on recent developments in repurposing the translation system for novel functions, with a focus on codon reassignment. We first examine development of the molecular machinery at the heart of genetic code expansion. Next, we discuss nsAA incorporation in several contexts, including whole-genome recoding, prokaryotic and eukaryotic systems *in vivo* as well as *in vitro*. We end with a discussion of current challenges in the field and provide commentary on future opportunities.

Genetic code expansion using OTSs

Amber suppression seeks to ‘hijack’ the amber translational stop codon (TAG), recoding it into a sense codon corresponding to a nsAA of interest. Generally, this is accomplished using a *suppressor tRNA* that has been mutated to decode the amber codon and an *aminoacyl-tRNA synthetase* (aaRS) that has been mutated to accept the nsAA of interest and covalently load it onto the suppressor tRNA. These components are typically sourced from distant archeal species to ensure orthogonality to host translation machinery, undergoing directed evolution to improve their compatibility with a new nsAA and enable its site-specific incorporation into proteins.

Directed evolution is the most widely used approach for the generation of novel OTS components [13–15] (Figure 2). These efforts start with the selection of a scaffold aaRS/tRNA pair. To date, several aaRS/tRNA pairs have been used in the creation of new OTSs. The *Methanocaldococcus jannaschii* TyrRS/tRNA^{Tyr} pair is arguably the most common pair used, but is generally limited to aromatic amino acids and is not orthogonal in eukaryotes [4,15,16*]. The PylRS/tRNA^{Pyl} pair from *Methanosarcina* species (*M. mazei*, *M. barkeri*) has shown compatibility with eukaryotic systems [16*,17], and is an especially attractive starting point for evolution as the native PylRS natively demonstrates polysubstrate specificity [18] and tRNA^{Pyl} natively decodes the amber codon [19]. Other starting components have included the *o*-phosphoserine (Sep)RS from *Methanococcus maripaludis* [9*] and the TrpRS/tRNA^{Trp} pair from *Saccharomyces cerevisiae* [20]. The TyrRS/tRNA^{Tyr} and LeuRS/tRNA^{Leu} pairs from *Escherichia coli* have also been used in OTS development for use in higher organisms [21,22]. After selecting a scaffold pair, crystal structural data is commonly used to identify specific residues in the aaRS that interact with the amino acid and the tRNA [13,23]. These residues are randomized to create a library of mutant aaRS variants *in vitro* and subsequently transformed *in vivo*. Finally, alternating rounds of selection identify mutant variants that are both functional and orthogonal to native machinery [13,15]. Negative selections eliminate variants that can charge the *o*-tRNA with native amino acids based on the synthesis of a toxic gene (e.g., barnase) in the absence of the cognate nsAA. Positive selections in the presence of the nsAA isolate variants that can charge the nsAA of interest onto the *o*-tRNA based on the suppression-dependent synthesis of a selectable marker (e.g., antibiotic resistance gene) or reporter (e.g., GFP). By subjecting ‘winners’ to alternating rounds of these screens, a nsAA-specific aaRS/tRNA pair can be identified.

While most new OTSs have focused on the generation of new nsAA–aaRS–tRNA pairs, some efforts have expanded OTSs to include additional components. In an exemplary study, incorporation of Sep was *only possible* after the development of a Sep-specific elongation factor [9*]. More recently, researchers were able to increase incorporation efficiency of selenocysteine (Sec) to >90% by engineering an elongation factor optimized for Sec [24]. nsAA incompatibility with the ribosome may also be an impediment to incorporation [25]. Platforms for ribosomal engineering and evolution will be integral to the elucidation and optimization of ribosome/nsAA interactions. Two recent approaches permit construction of modified ribosomes by decoupling organism fitness from ribosome function. In the first, researchers engineered a 30S subunit that is made orthogonal to natural components by a mutant 16S rRNA; this orthogonal subunit can be mutated to evolve novel function without impairing host viability [26,27**]. In a parallel approach, a system for the *in vitro* assembly of functional modified ribosomes has been developed [28–30]. We still await the development of a fully orthogonal ribosome *in vivo*.

Prokaryotic strain engineering tailored for genetic code expansion

Historically, nsAA incorporation via natural codon suppression has been limited by native translational components that have evolved essential function to faithfully decode all codons within an open reading frame. In the case of amber suppression, *release factors* are a class of proteins responsible for facilitating the termination of translation in response to ribosomal

stalling at a stop codon. Competition between loaded suppressor tRNAs and release factor proteins at amber codons meant to encode nsAAs severely hinders successful suppression, with release factor activity resulting in the premature truncation of most of the protein product [31]. Resultant yields of the target nsAA-containing protein under these conditions are very low, especially for proteins containing multiple instances of the same nsAA [32*]. Conversely, the presence of OTSs on high copy plasmids in the presence of high concentrations of nsAA *in vivo* can also drive the incorporation of nsAAs at >300 amber codons that terminate native genes, resulting in cellular toxicity [33**].

Consequently, much effort has gone into the elimination of release factor activity to improve nsAA incorporation. Initial attempts to outright delete the essential gene *prfA* that encodes release factor 1 (RF1) were stymied by cell inviability. Several early approaches, including release factor engineering [34] and supplementation *in trans* with partially recoded versions of the amber-dependent essential genes [35] permitted subsequent removal of *prfA* from the organism. More recent efforts have recoded the genome of *E. coli*. In such efforts, researchers edited the amber-dependent essential genes to terminate instead with the synonymous ochre codon (TAA) and deleted *prfA* from the organism. Incorporation of Sep was greatly enhanced in one such RF1-deficient recoded strain [36]. More recently, a recoded RF1-deficient strain was used in the preparation of cell lysates to improve the incorporation of the nsAA *p*-propargyloxy-L-phenylalanyl (pPaF) *in vitro* [32*,37].

These strain engineering efforts culminated with the recently reported completion of the first completely genomically recoded organism (GRO), an *E. coli*-derived strain that lacks all amber codons and RF1 [33**,38]. In this study, the authors systematically reassigned *all 321* native instances of the amber (TAG) codon to the ochre (TAA) codon and deleted the *prfA* gene (Figure 3). In the resulting strain (C321 A) the amber codon is orthogonal and unrecognized by the remaining translational machinery, freeing it for use as a dedicated codon for nsAA incorporation. This strain has demonstrated improved properties for incorporation of nsAAs [33**] and has more recently been engineered to depend on nsAAs as a 21st synthetic biochemical building block [39*,40*].

Beyond amber suppression, some effort has been made to access additional codons for nsAA assignment, such as the ‘ochre’ (TAA) [41] and ‘opal’ (TGA) stop codons [42]. In a parallel approach, researchers instead sought to access non-natural *quadruplet* codons to encode nsAAs [43,44]. Combined with amber suppression, quadruplet suppression has enabled the incorporation of multiple distinct nsAAs into a single polypeptide [45**,46,47].

Cell-free systems for genetic code expansion

Cell-free protein synthesis (CFPS) systems offer another approach for the incorporation of nsAAs (Figure 3). CFPS is the *in vitro* synthesis of proteins without using intact cells [48–50]. A lack of physical boundaries permits precise manipulation of reaction contents, simplifies product purification, and enables efficient incorporation of bulky/charged nsAAs that typically exhibit poor membrane permeability *in vivo* (e.g., pPaF) [51*]. Additionally, cell viability is no longer a constraint — reactions may be supplemented directly with purified OTS components, which have known toxicity effects *in vivo* [52]. These

advantages, when combined with recent improvements to chassis strains and energy regeneration systems that have enabled high-yielding (>1 g/L) [49] and long-lasting (>10 h batch mode) [37] synthesis, have led to increased interest in the use of CFPS for nsAA incorporation into proteins [32*,37,51*,53–55].

One particularly exciting area for CFPS is in genetic code reassignment. For instance, translation systems reconstituted from purified components [56] or crude cell extracts depleted of native tRNAs [57] can be selectively supplied with purified tRNAs to essentially create a custom genetic code with select sense codons left 'blank' for nsAA reassignment [58,59]. Alternatively, in a recent effort a 50S ribosomal subunit was developed with mutations at the peptidyl transferase center that abolish its ability to use several native tRNAs for translation [60]. The use of this ribosome together with tRNAs containing compensatory point mutations enabled reprogramming of the genetic code, providing proof of concept for a potential new strategy for genetic code rewriting and expansion *in vitro*. Finally, *in vitro* nsAA-incorporation efforts uniquely benefit from the use of self-aminoacylating tRNAs featuring *flexizyme* ribozymes to extend the genetic code without the need for laborious nsAA–aaRS–tRNA cognate pair development [61].

Genetic code expansion in eukaryotic systems

Amber suppression has been adapted to eukaryotic systems as a tool for the interrogation of cellular biology. Engineered orthogonal aaRS/tRNA pairs have been used to genetically encode nsAAs that modulate PTMs by photocaging lysine [62] and serine [63] residues, promote chromatin condensation with crosslinking residues [64], introduce chemical handles for fluorescent protein labeling and live cell imaging [65], among many other applications. Most often, OTS components are scaffolded on *M. mazei* PylRS/tRNA^{Pyl}, *E. coli* TyrRS/tRNA^{Tyr} or *E. coli* LeuRS/tRNA^{Leu} pairs, engineered in *E. coli* or *S. cerevisiae* [21], and then ported to mammalian vectors.

In addition to unicellular organisms and tissue culture, amber suppression with engineered PylRS/tRNA^{Pyl} derivatives has been demonstrated in the nematode *Caenorhabditis elegans* [66**]. Similar work has been successful in the fly *Drosophila melanogaster*, where nsAAs could be incorporated both site-specifically and tissue-specifically into proteins [67].

As with prokaryotic systems, competition with release factors remains a major barrier for efficient amber suppression in eukaryotes. Though orthogonal ribosomes or genome-wide codon reassignment have not yet been demonstrated in these systems, efforts are being made in this direction. Recently, a *de novo* synthesis of *S. cerevisiae* chromosome III included TAG → TAA stop codon reassignments [68**]. As this effort is extended to the remaining chromosomes, the resulting synthetic strain will lack native TAG codons, motivating the need to engineer the specificity of the single eukaryotic release factor to exclude amber recognition.

An additional challenge is native quality control machinery. Nonsense mediated decay (NMD) surveys transcripts for nonsense codons excessively distal from the 3' end,

triggering degradation in response to their presence [69]. Addressing this limitation, the pathway has been knocked out in *C. elegans* to boost amber suppression efficiency [66**].

A final consideration in these systems is the expression of the orthogonal tRNA, as eukaryotic RNA polymerase III is typically recruited to intragenic A and B-box promoter sequences. One solution has been to identify orthogonal aaRS/tRNA pairs containing A and B-like elements. For instance, *E. coli*'s TyrRS/tRNA^{Tyr} is orthogonal in *S. cerevisiae* and contains its consensus A and B-box [21]. However, OTSs scaffolded on PylRS/tRNA^{Pyl}, lack such intragenic sequences. Instead these tRNAs have been successfully expressed by co-opting dicistronic tRNA scaffolds such as tRNA^{Arg}_{UCU} [70], or by using the extragenic U6 RNA polymerase III promoter [71].

Current challenges and future outlook

Looking forward, three major challenges define the trajectory of this field: the development of more efficient OTSs, accession of more open coding channels to enable multi-site incorporation of multiple nsAAs, and the generation of OTSs engineered for more exotic nsAAs.

Engineered OTSs suffer poor enzymatic efficiencies relative to native translational machinery [25]. Overall, this represents a problem of insufficient aaRS evolution, as typically only 6–8 residues proximal to the amino acid binding pocket are mutagenized. Further diversification is limited by library size constraints — the randomization of six residues results in a library of nearly 10^8 members, quickly saturating standard screening techniques. The use of computational protein modeling may enable more rational engineering, guided by *in silico* binding predictions [72]. Further, during evolution OTSs are screened for the ability to suppress just a few codons in selectable markers, whereas native translational machinery faces a load of thousands of codons. Increasing the load on OTSs may promote the selection of more catalytically active enzymes. Amber suppression of native essential genes has proven to be an effective strategy to tie strain viability to nsAA incorporation [39*,40*], offering a potential route to drive protein evolution under more stringent selective conditions.

Multi-site incorporation of a single nsAA at high levels (>10) has remained elusive due to the use of codons that have their cognate translation components (e.g., RF1) out-competing the incorporation of the nsAA, as well as the impaired activity of OTSs. These limitations are compounded in efforts to achieve multi-site incorporation of *multiple distinct* nsAAs. This pursuit will require advances in our ability to suppress multiple codons simultaneously, and in the development of mutually orthogonal OTSs. *In vivo*, the field is currently limited to suppressing two distinct codons simultaneously. The use of nonstandard nucleotide bases [73] may enable crossing this barrier in the near future. Alternatively, radical recoding strategies that extend off the RF1-deficient GRO may provide a route to more codons. Even with more codons, new advances in orthogonal OTSs are needed. OTSs have been selected against interactions with *native* translational machinery, but not necessarily against other OTSs. This raises the potential for OTS cross-reactions when expressed simultaneously.

Work is being done to develop orthogonal tRNA acceptor stems [74] and additional aaRS/tRNA scaffolds to mitigate this cross-orthogonality.

Finally, certain biological constraints limit the scope of nsAA diversity. These include limitations on cell membrane permeability and steric incompatibility with the ribosome and other translational components such as elongation factors. Further engineering these components with cell-free systems or synthetic ribosomes is necessary for increasing the available nsAA chemical space.

Overcoming some of these technological barriers will better enable the creative potential of nonstandard protein development in producing the next wave of highly functionalized biomaterials and protein therapeutics [75] with broad applications in medicine, materials science and biotechnology.

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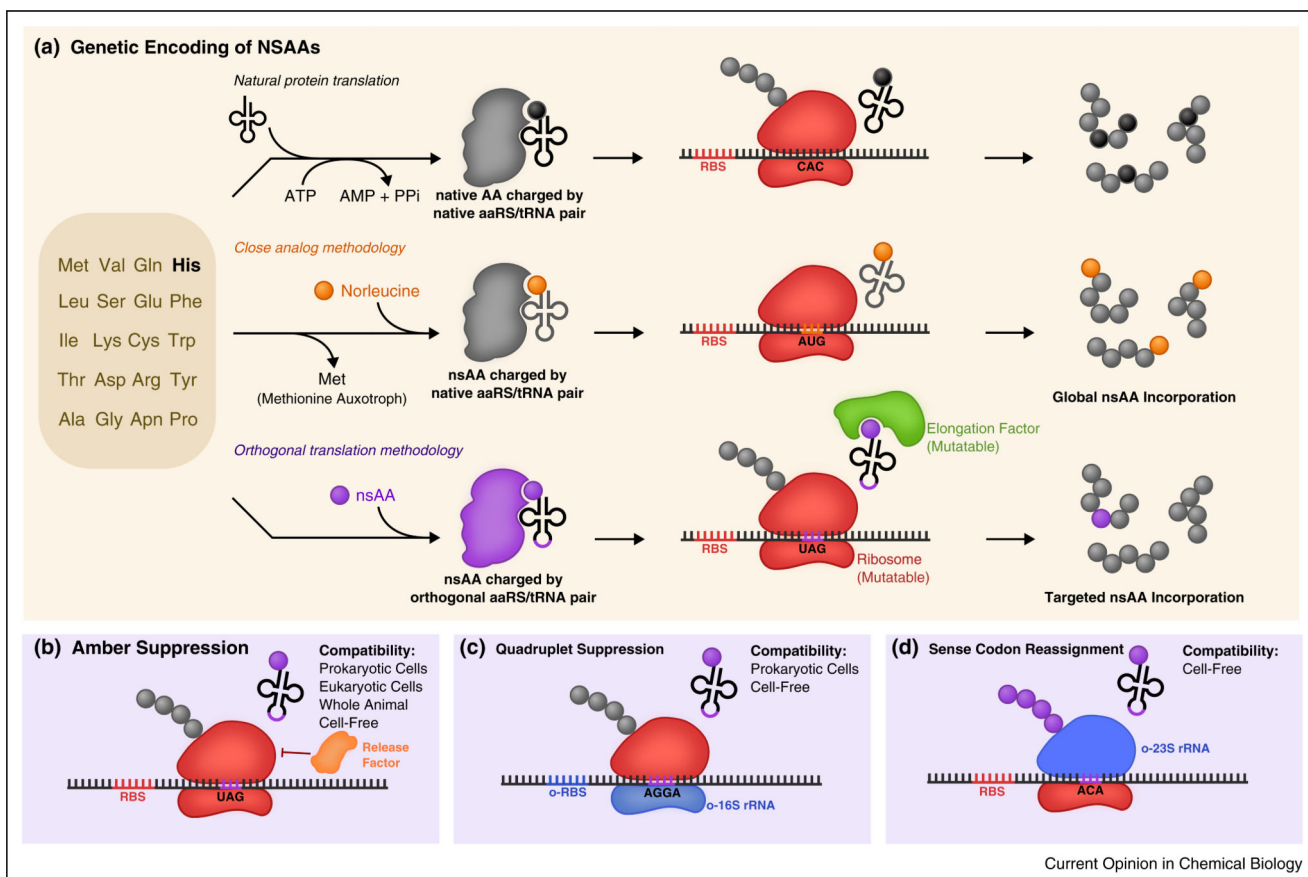
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**Figure 1.**

Methods for genetic code expansion. **(a)** Two general paradigms exist for the genetic incorporation of nonstandard amino acids into proteins contrasted with the natural process of encoding the canonical amino acids. The close analog methodology complements a natural amino acid auxotrophy with a close nonstandard analog, enabling global protein labeling by native translational machinery. The orthogonal translation methodology introduces orthogonal translational machinery engineered to charge an orthogonal tRNA with a nonstandard amino acid, enabling site-specific targeted genetic incorporation. Certain nsAAs may require additional mutations in the elongation factor or the ribosome. **(b)** For targeted genetic incorporation, amber suppression is the most widely used technique. Competition with release factors limits efficiency, and methods are discussed to overcome this. **(c)** Quadruplet suppression can be performed with appreciable efficiency with the use of an engineered orthogonal 16S ribosomal subunit [27^{**}]. **(d)** Sense codons can be reassigned by using an orthogonal 23S ribosomal subunit, engineered to accept a synthetic set of tRNAs [60].

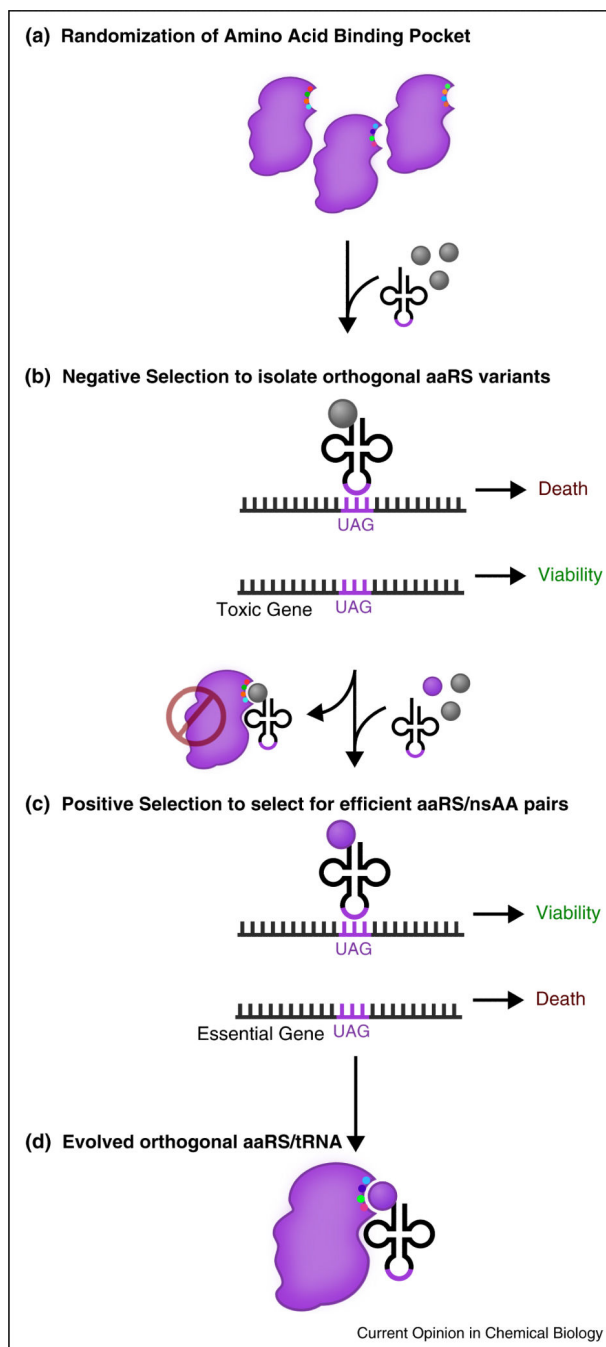


Figure 2. General methodology for engineering orthogonal translation systems for novel nsAAs. **(a)** Scaffold orthogonal aaRS/tRNA pairs are selected from distant organisms. Residues in the amino acid binding pocket of the aaRS are randomized. **(b)** A negative selection is performed to exclude aaRS variants capable of charging natural amino acids. In the absence of the nsAA, clones are selected by the inability to suppress a toxic gene. **(c)** A positive selection is performed to retrieve aaRS variants capable of charging the nsAA, selecting for the ability to suppress a selectable marker gene, such as an antibiotic resistance marker. **(d)**

The selected orthogonal translation system consists of the orthogonal aaRS, tRNA, and nsAA.

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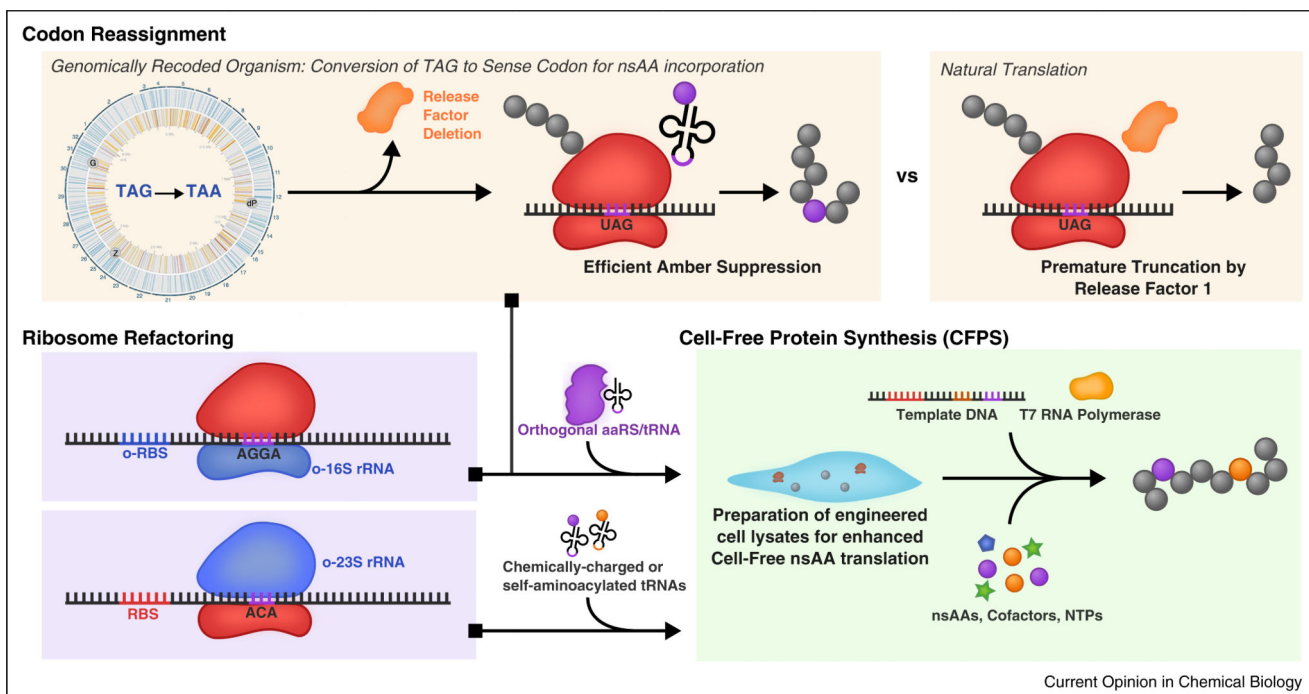


Figure 3. Genomically recoded organisms (GROs), strain engineering and *in vitro* nsAA translation. A genomically recoded organism reassigns the TAG stop codon to an open sense coding channel for enhanced nsAA amber suppression. Ribosomal engineering promotes other classes of codon suppression. These engineered strains can be used *in vivo* and via cell lysate, to develop *in vitro* translation systems with open coding channels for the production of nsAA-containing proteins.