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Semisynthetic tRNA Complement Mediates *in Vitro* Protein Synthesis

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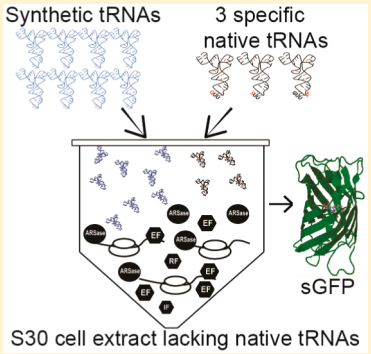
Supporting Information

ABSTRACT: Genetic code expansion is a key objective of synthetic biology and protein engineering. Most efforts in this direction are focused on reassigning termination or decoding quadruplet codons. While the redundancy of genetic code provides a large number of potentially reassignable codons, their utility is diminished by the inevitable interaction with cognate aminoacyl-tRNAs. To address this problem, we sought to establish an *in vitro* protein synthesis system with a simplified synthetic tRNA complement, thereby orthogonalizing some of the sense codons. This quantitative *in vitro* peptide synthesis assay allowed us to analyze the ability of synthetic tRNAs to decode all of 61 sense codons. We observed that, with the exception of isoacceptors for Asn, Glu, and Ile, the majority of 48 synthetic *Escherichia coli* tRNAs could support protein translation in the cell-free system. We purified to homogeneity functional Asn, Glu, and Ile tRNAs from the native *E. coli* tRNA mixture, and by combining them with synthetic tRNAs, we formulated a semisynthetic tRNA complement for all 20 amino acids. We further demonstrated that this tRNA complement could restore the protein translation activity of tRNA-depleted *E. coli* lysate to a level comparable to that of total native tRNA. To confirm that the developed system could efficiently synthesize long polypeptides, we expressed three different sequences coding for superfolder GFP. This novel semisynthetic translation system is a powerful tool for tRNA engineering and potentially enables the reassignment of at least 9 sense codons coding for Ser, Arg, Leu, Pro, Thr, and Gly.



INTRODUCTION

Transfer (t) RNAs play a central role in genetic decoding, and have been extensively engineered to create new connections between codons and amino acids, thereby expanding the genetic code.[1] This process, also known as codon reassignment, is frequently used for site-specific incorporation of non-natural amino acids (nnAAs) into proteins. Such genetic engineering can endow proteins with novel activities and enable protein immobilization, conjugation, and labeling that cannot be achieved with conventional biochemical methods.[2] The site-selective incorporation of a single nnAA at one or multiple selected positions is well established and relies predominantly on nonsense codon reassignment.[3−5] However, the site-selective insertion of two or more nonidentical nnAAs is still a challenging and active area of research.[6−9] The leading strategies for orthogonal encoding of at least two nnAAs rely on a nonsense codon reassignment combined with the use of quadruplet codons.[8] However, the limited number of available nonsense codons, the unfavorable competition with release factors, and the low efficiency of correct quadruplet suppression underpin the need for new strategies of codon orthogonalization.

Degeneration of the genetic code presents a large source of potentially reassignable orthogonal sense codons.[10] Previously, the degeneracy of Phe codons was exploited by retaining UUC codon for Phe and reassigning the UUU codon to naphthylalanine using engineered yeast phenylalanyl-tRNA and the respective aminoacyl-tRNA-synthetase (aaRS) mutant.[11,12] While the authors reported 80% nnAA incorporation efficiency in a Phe-auxotrophic *Escherichia coli* strain grown in Phe-depleted media, this approach is likely to have limited applicability. This is due to difficulties in engineering cells in which wobble decoding by native tRNAs could be effectively prevented and replaced by an heterogeneous tRNA/aaRS/nnAA system.

Compared to *in vivo* systems, *in vitro* protein expression systems are more attractive platforms for sense codon reassignment due to their superior control over the levels and identities of the translation reaction components.[13] Therefore, we hypothesized that a selective depletion of tRNA isoacceptors for amino acids encoded either by mixed codon families or by the codon families with high wobble restrictions could free the respective codons for decoding with their corresponding orthogonal tRNAs.

The major obstacle in reassigninging sense codons is the competition between synthetic tRNAs with and the endogenous tRNAs[14,15] However, an earlier study found similar suppression efficiency of the GUA sense codon compared to *amber* codons in rabbit reticulocyte lysate, indicating that sense codon suppression could potentially expand the number of reassignable codons.[10] The authors also reported that sense codons corresponding to low-abundance cognate tRNAs display higher suppression efficiencies than other codons.

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Thus, elimination of competing tRNAs from the translation lysate could enable reassignment of multiple sense codons. Progress in this direction has been made using a reconstituted PURE *E. coli in vitro* translation system,[16,17] wherein omitting the amino acids and aminoacyl-tRNA transferases responsible for a specific codon box allows the reassignment of this codon box by supplementing the translation system with precharged tRNAs bearing the respective anticodons. This system has been successfully exploited to express and select peptides with a range of nnAAs. However, incomplete amino acid vocabulary makes this approach incompatible with sequences that include all natural amino acids. We conjectured that this problem could be solved by depleting all tRNAs from the lysate followed by replenishment with a synthetic tRNA subset. Such a subset could be formed *in vitro* from the transcribed tRNAs lacking the isoacceptor species for the codons from mixed codon boxes or codons with high wobble restrictions. This assembly of mixed codons could then be reassigned and used in the synthesis of proteins with unrestricted amino acid compositions. Theoretically, this approach realizes the opportunity to reassign all 61 sense codons. The proposed sense codon reassignment approach hinges on the ability of synthetic tRNAs to support *in vitro* translation reactions. This is an important issue as tRNAs are known to undergo more than 85 secondary modifications,[18] with some of the identified tRNA modifications shown to affect tRNA folding, structure, and function.[19,20] However, only tRNAs for Ile, Glu, and Lys require modifications for efficient aminoacylation by their respective aaRSes.[21] T7 RNA polymerase-synthesized tRNAs (t7tRNAs) are widely used in cell-free translation systems for genetic reprogramming[2] or genetic code simplification.[22] In the latter case, several codons were reassigned to Ser or Ala by grafting the heterologous anticodons into Ser or Ala tRNAs respectively,[22,23] and the results strongly suggested that, at least in some cases, t7tRNAs could functionally replace endogenous tRNAs. Although the ability of bacterial and eukaryotic t7tRNAs to undergo aminoacylation *in vitro* has been extensively studied, their ability to support protein synthesis was tested only in a few cases.[15,24−26] Therefore, the systematic analysis of t7tRNA functionality in protein translation is still outstanding.

Here, we utilized an *E. coli* cell-free translation system[20] depleted of endogenous tRNAs for the systematic analysis of t7tRNA functionality.[27,28] By combining this system with a novel peptide expression assay, we could measure the codon-decoding efficiency of the individual tRNA isoacceptors and thereby create a fully functional cell-free system with a defined and predominantly synthetic tRNA complement.

RESULTS

***In Vitro* Synthesis of 48 *E. coli* tRNA Species.** To construct a synthetic *E. coli* tRNA complement capable of supporting protein translation, we conducted *in vitro* runoff transcription[29,30] on 48 DNA templates harboring t7 promoter followed by the corresponding tRNA-coding sequences (Supporting Information Figure S1).

T7tRNA transcripts were obtained in good amounts for all tRNAs except tRNAIle(GAU) and tRNATrp(CCA) (Figure 1). The sequences coding for these tRNAs start with adenosine, which is likely to cause high abortion rates in the early transcription phase. For these two tRNAs, a Hammerhead ribozyme (HHRz) coding sequence prefaced by a strong transcription start site was introduced upstream to the tRNA coding sequences to ensure efficient transcription followed by HHRz-mediated autoexcision (Supporting Information Figure S1).[31] Denaturing PAGE analysis revealed that more than 90% of the RNA precursor was cleaved to yield the desired tRNAs (Figure 1, Igau and Wcca). Although some tRNAs, such as tRNALeu(CAA) and tRNATyr, contain additional minor bands, we obtained a major species of the expected size in all cases.

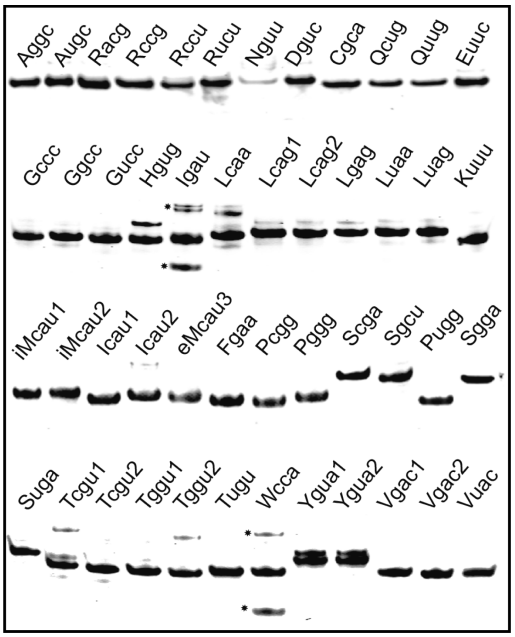

Figure 1. Denaturing PAGE analysis of 48 *in vitro* synthesized t7tRNA species. The t7tRNAs are denoted by the respective single letter amino acid code (uppercase) and the 5′-3′ anticodon triplet (lowercase). Polymorphic tRNA variants are indicated by the respective numerical indeces. tRNAs for Ile and Trp (Igau and Wcca) were generated through autocleavage of the HHRz-containing RNA precursor. The asterisks above and below the corresponding tRNA-bands denote the precursor and the excised HHRz, respectively. Initiator and elongator tRNAs (Met) are prefixed with "i" and "e", respectively.

***In Vitro* Peptide Expression Assay.** Next, we sought to devise an *in vitro* synthesis assay to evaluate the decoding efficiency of t7tRNAs. We conjectured that a reporter peptide corresponding to a short open reading frame (ORF) that uses a limited set of codons is advantageous over classical reporter proteins such as GFP or luciferase that require a full set of tRNAs for their synthesis.

To establish a multiplexed quantitative homogeneous peptide expression assay, we took advantage of a peptide biosensor recently developed by our group.[32] It is composed of an artificially engineered peptide binding domain known as the "affinity clamp"[33] and an autoinhibited tobacco vein mottling virus (TVMV) protease. Binding of an 8-amino acid ligand peptide RGSIDTWV (RGS-peptide) to the biosensor triggers a conformational change resulting in protease activation, which is detected through cleavage of a quenched fluorescent TVMV substrate peptide (Figure 2A). We demonstrated that as little as 50 nM of the peptide could be detected using this assay. The relationship between initial velocities of TVMV substrate cleavage (V_{max}) and the absolute concentration of the RGS-peptide was found to be linear in the 50−400 nM range (Figure 2B). Furthermore, the assay could be performed in an *E. coli*

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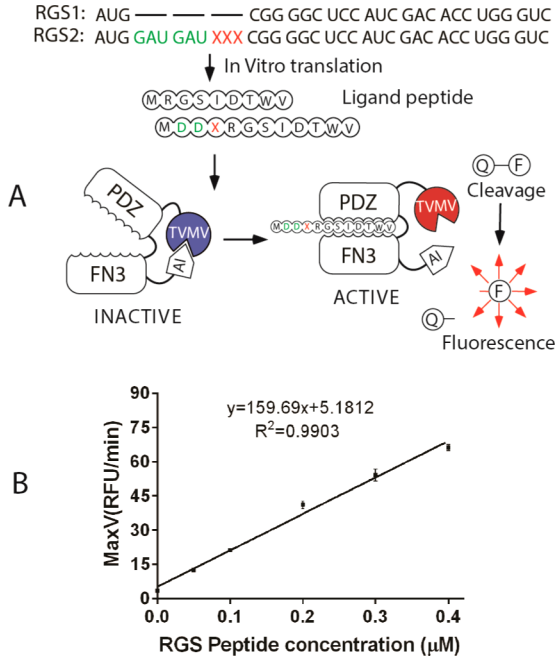

Figure 2. Principle and calibration of the affinity clamp peptide biosensor. (A) Schematic representation of experimental procedure. The RNA sequences at the top represent coding frames for RGS-peptide and its derivatives, RGS1 and RGS2, the latter comprising the "insulator" codons (green) followed by the test-codon triplet (XXX, red). The resulting peptide containing the constant eight-amino-acid C-terminus and variable N-terminus binds to the biosensor composed of an autoinhibited TVMV-protease, with PDZ and FN3 domains forming the affinity clamp. Peptide binding results in a conformational change of the affinity clamp that in turn dislodges the inhibitory peptide from the active site of TVMV leading to protease activation and subsequent cleavage of the quenched reporter substrate. Q and F denote the fluorescence quencher and fluorophore groups, respectively. (B) The calibration curve for RGS-peptide obtained by assaying different concentrations of synthetic peptide in the heat-denatured *E. coli* extract. The initial rates were plotted against peptide concentration and the averaged data of triplicate experiments were fitted using regression coefficients (R^2).

cell-free system, although this requires a cocktail of protease inhibitors to mitigate the endogenous proteolytic activity.

While binding of the RGS-peptide to the affinity clamp critically depends on a free C-terminus,[34,35] introducing additional amino acids at the N-terminus of the RGS-peptide is not expected to affect the clamp-to-peptide binding.[33] We confirmed this by testing two N-terminally extended synthetic peptides, GG RGSIDTWV and DD RGSIDTWV, in our assay (Supporting Information Figure S2), with the calibration curves for all three peptides displaying good linearity with $R^2 > 0.99$ (Figure 2B and Supporting Information Figure S2). We concluded that our expression assay was well suited to quantify the expression of RGS-derived peptides in the cell-free system and thus could be used to test the decoding efficiency for various isoacceptor/codon pairs. To reduce the influence of codons immediately downstream of the initiator AUG codon on translation initiation, two GAU codons were inserted between the initiator and the test codons to yield an RGS2 template (Figure 2A). Control experiments demonstrated that such a template mediated consistent peptide expression levels regardless of the test-codon upstream of the RGS-peptide coding sequence (Supporting Information Figure S3).

Characterization of tRNA Depleted *E. coli* Cell-Free Translation System. To obtain tRNA-depleted *E. coli* S30 cell extract, we modified a previously published chromatographic tRNA depletion protocol.[28] In this procedure, the endogenous tRNAs bind to ethanolamine−Sepharose matrix, while other components required for protein synthesis remain in the flow-through. To obtain optimal tRNA depletion while retaining the translation efficiency, we optimized the potassium/magnesium concentration as well as the matrix to lysate ratio. The extent of tRNA depletion was evaluated by comparing eGFP expressions in the depleted lysate with or without adding the total native tRNA mixture. As can be seen in Figure 3A, no eGFP was

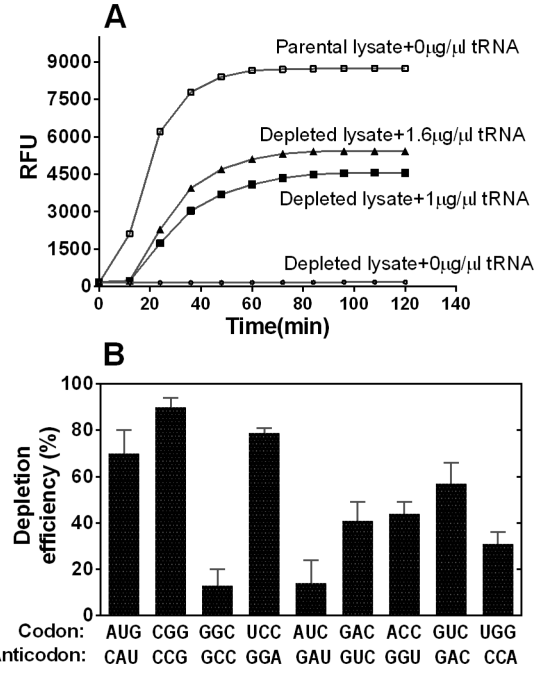

Figure 3. Cell-free translation of eGFP and RGS-peptide in tRNA-depleted *E. coli* lysate. (A) Native tRNA-dependent eGFP expression. (B) Analysis of the depletion efficiency of native tRNAs for each codon assessed by withholding individual t7tRNA from the t7tRNA mixture, mediating RGS peptide translation in the depleted lysate. The codon/anticodon pairs corresponding to t7tRNA that were individually excluded are indicated below the corresponding bars. Elongator t7tRNAs were supplemented to a final concentration of 0.8 μM, with the initiator t7tRNAiMet at 1.6 μM and the native tRNA mixture at 1 μg/μL final concentrations.

produced in the absence of native tRNAs, while the addition of total native tRNA mixture restored translation to 60% of the parental lysate level. A time lag of approximately 10 min was observed in tRNA-depleted lysates, possibly reflecting the time required for aminoacylation of the readded tRNAs.

Similar to eGFP, translation of a short RGS1 template (Figure 2A) was also tRNA-dependent. Both native tRNAs and a mixture of 9 codon-specific t7tRNA species (Supporting Information Table S1) restored translation to similar levels (data not shown).

The observation that synthetic tRNAs could support translation of RGS1 template was somewhat surprising considering that tRNAIle requires post-transcriptional modifications for efficient aminoacylation.[21] Hence, we performed a control experiment where we omitted individual t7tRNAs from the mixture and measured the translational activities in the

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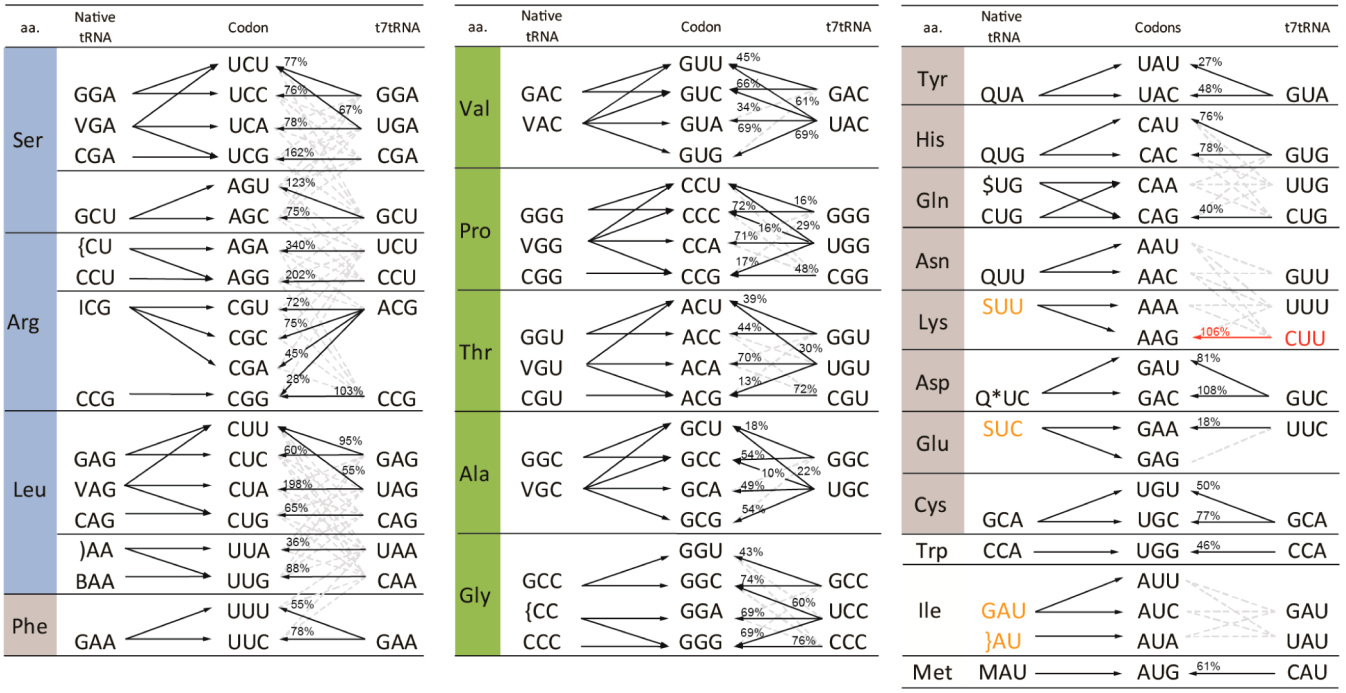

Figure 4. T7tRNA decoding table. Ser, Arg, and Leu, shaded in blue, are encoded by mixed codon family boxes from which two codons (N1N2N3) belong to a split and the other four to unsplit codon family boxes.[19] The 4- and 2-fold degenerate amino acids are shaded in green and gray, respectively. Native tRNAs and t7tRNAs are denoted by their respective anticodons (N34N35N36). The letters other than A/U/G/C in the native tRNA anticodons denote modified nucleosides.[55] The native and t7tRNA decoding patterns are indicated by arrow-lines from the left and right sides of the codon columns, respectively. Anticodons of the tRNAs specific for Lys, Glu, and Ile where modification either in anticodon or another part was essential for aminoacylation are highlighted in pink. Lys t7tRNA with U34 to C34 anticodon replacement based on tRNALys(UUU) is highlighted in red. The arrow-lines connecting t7tRNAs and the respective codons indicate the tRNA/codon combinations tested in the peptide biosensor assay. The dashed gray and black continuous arrow-lines correspond to <10% or ≥10% codon decoding efficiency, respectively. The associated number beside the black arrow-line indicates the calculated decoding efficiency of the t7tRNAs toward the analyzed codon as described in Supporting Information Figure S4. The N34 modifications include "V", uridine 5-oxyacetic acid; "{", 5-methylaminomethyluridine(mnm^{5}U); "$", 5-carboxymethylaminomethyl-2-thiouridine(cmnm^{5}s^{2}U); "S", 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U), ")"-5-carboxymethylaminomethyl-2′-*O*-methyluridine (cmnm^{5}Um);"B", 2′-*O*-methylcytidine (*Cm*); "M", N4-acetylcytidine (ac4C); "}", 2-lysidine (k2C); "I", Inosine; "Q", queuosine; and "Q*", glutamyl-queuosine.[55]

resultant reaction mixtures. As depicted in Figure 3B, when the individual t7tRNAs for AUG(iMet), CGG(Arg), or UCC(Ser) codons were independently withheld from the t7tRNA mixture, peptide expression decreased significantly. However, when the t7tRNAs for GGC(Gly), AUC(Ile), GAC(Asp), ACC(Thr), or UGG(Trp) were excluded, some residual expression was observed, probably due to native tRNA remnants in the lysate. The depletion efficiencies of tRNAs for CGG(Arg) and UCC(Ser) were 75−90%, indicating that these codons are more suitable for reassignment due to negligible amounts of the respective native tRNA isoacceptors remaining in the depleted lysate (with the tRNA depletion efficiency defined as 100% when no peptide was produced without the addition of selected tRNA).[36] Both of these codons belong to mixed codon boxes composed of two codon families, making them particularly promising candidates for the reassignment (see below).

Systematic Analysis of t7tRNA Functionality and Specificity. The developed assay provided a platform to systematically test the entire ensemble of t7tRNAs (Figure 2A). Yet, the initial experiments revealed residual amounts of some isoacceptors in the depleted lysate. These represented tRNAs that are abundant in *E. coli*, indicating a relationship between the depletion efficiency of individual tRNAs and their abundance (Supporting Information Table S2).[10,36] The incomplete depletion of endogenous tRNAs potentially complicates the functionality test by masking the signal from their t7tRNA counterparts. However, we observed that including two consecutive codons for a particular tRNA into the template significantly enhances the adverse effect of its depletion on the peptide translation efficiency. This is likely to reflect the changes in translation kinetics at reduced tRNA concentrations previously described for low-abundance tRNAs *in vivo*.[37] Therefore, we rescreened codons with incompletely depleted isoacceptors using a reporter peptide ORF harboring two consecutive target codons. The decoding efficiency was calculated as the mean value of both one- and two-codon templates (Supporting Information Figure S4). The ability of t7tRNAs to decode the 61 codons is summarized in Figure 4 and Supporting Information Figures S5−S7.

One important aspect to consider when interpretating the assay results is the extent to which t7tRNAs could undergo modifications in the lysate. For example, crude *E. coli* lysate was reported to mediate formation of pseudouridine in synthetic tRNAs.[38] Such modifications require only isomerase activity, but not the low molecular weight substrates or cofactors potentially present in our system.[39] However, the modifications on N34 and N37 involve up to 20 enzymes with relay chains, as well as multiple substrates and cofactors.[19] Therefore, such modifications are unlikely to emerge on the synthetic tRNAs in the crude lysate without significant optimization of the system. We experimentally address this issue later in the report (see below).

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Split Codon Family Boxes. Decoding of split codon families ending on U and C such as Ser, Phe, Tyr, His, Asn, Asp, and Cys is carried out by tRNAs with G or its modified form (Q) in the first anticodon position. The A- and G-ending codons are decoded either using modified uridine (Lys and Glu) or by adding isoacceptors with C in the first anticodon position (Arg, Leu, and Gln) (Figure 4 blue and gray shaded amino acids). Uridine in the former case is modified with various aminomethyl derivatives that restrict the recognition solely to A- and G-ending codons,[40] additionally supported by ribose 2′-*O*-methylation when U and C are in the first anticodon position of both Leu isoacceptors.

As compared to their native counterparts, t7tRNAs for Phe, His, Asp, and Cys were efficient (50−70%) in decoding both their cognate (C-ending) codon with Watson−Crick geometry (further referred to as "cognate-WC") (C-ending) and wobble (U-ending) codons. The t7tRNATyr(GUA) decoded UAC with ∼50% efficiency and UAU with less than 30%. T7tRNAHis(GUG), featuring an additional G-1C73 base pair, demonstrated an effective decoding of its cognate-WC CAC and wobble CAU codons. The t7tRNA lacking G-1 was not functional in restoring peptide translation (data not shown), presumably due to failure of the aminoacylation step.[41]

The t7tRNASer(GCU) recognized both AGC and AGU codons with 123 and 75% efficiency, respectively. For Leu and Arg, two tRNA isoacceptors are responsible for decoding each split codon box, and in our assay, both Leu t7tRNAs with UAA and CAA anticodons recognized only their cognate codons via clasical WC-base pairing. This is in agreement with a previously reported restricted mode of recognition by unmodified uridine,[42] albeit with efficiencies of 36 and 88%, respectively. The t7tRNAs for Arg with UCU and CCU anticodons demonstrated similar behavior in strictly recognizing their cognate-WC codons at 340 and 202% efficiency, respectively. The higher apparent activity of these t7tRNAs possibly reflected the low abundance of these isoacceptors in the native tRNA mixture.[36] Consistent with previous observations from the ribosome binding assay,[43] t7tRNAGln(UUG) could not decode its cognate-WC CAA codons. The t7tRNAGln(CUG) also could not decode CAA codons, although it could decode its cognate-WC CUG codon with 40% efficiency.

T7tRNAs for Glu(UUC), Ile(GAU), Asn(GUU), and Lys(UUU) failed to sustain peptide translation from the template comprising both their cognate-WC and wobble codons. Lack of modifications within the anticodon loops of Glu and Ile t7tRNAs was previously shown to prevent their aminoacylation, making them inactive in the peptide translation,[44,45] and t7tRNAAsn(GUU) prepared with or without the help of HHRz performed poorly in the reporter peptide synthesis. Although chimeric t7tRNALys, with the grafted anticodon and the discriminator base both derived from tRNAAsn, could be aminoacylated by AsnRS with Asn,[46] it still failed to support peptide expression in our assay (data not shown). In this regard, it was previously reported that tRNALys, with unmodified U34, failed to decode either of its codons due to the potential loss of structural order in the anticodon loop as well as poor stacking within the codon-anticodon duplex formed by three consecutive, least-overlapping A−U base-planes.[47,48] In our system, mutating U to C in the first anticodon position of t7tRNALys fully restored its decoding activity toward AAG-codon. This effect can potentially stem from the stronger stacking provided by cytidine within both the anticodon loop and the codon-anticodon helix, as well as from a higher affinity toward lysyl aaRS.[49]

T7tRNAs for Trp(CCA) and Met(CAU) decoded their cognate-WC codons with 46% and 61% efficiency, respectively.

Unsplit Codon Family Boxes. With the only exception of Arg, the standard subset of 2 or 3 isoacceptors bearing G, U, and/or C in the first anticodon is employed in bacteria to decode 8 unsplit codon family boxes. Here, G34 pairs with C- and U- and C34 to exclusively mediate the decoding of G-ending synonymous codons (Figure 4 blue and green shadings). In the peptide synthesis assay, all t7tRNA isoacceptors with G or C in the first anticodon position demonstrated specific recognition of their cognate-WC codons with efficiencies of 40−160% (Figure 4). In all native tRNA isoacceptors except tRNAGly(UCC), U34 carries a 5′-oxyacetic acid modification which extends recognition beyond its cognate-WC A-ending[50] to G-, U-, and C-ending codons for Val, Pro, and Ala by partially altering the nucleoside sugar pucker geometry.[51,52]

Furthermore, t7tRNALeu(UAG) and t7tRNASer(UGA), which are presumably devoid of modifications in the translation reaction, show strong preferences for A- and to a lower degree U-, but fail to recognize G- and C-ending codons.[24] The relative efficiency for tRNASer(UGA) decoding two consecutive UCU codons at 1.6 μM was ∼10%, while an increase in the isoacceptor concentration to 6.4 μM resulted in ∼70% decoding efficiency (Supporting Information Figure S5A). This finding can be easily rationalized considering the lack of some post-transcriptional modifications in the tRNA body (including the anticodon loop) leads to either a reduction in affinity toward aaRS or a higher rate of dissociation of the codon−anticodon interaction and/or tRNA accommodation.[53] Both of these effects could, at least partially, be compensated by the increase in tRNA concentration.

Surprisingly, t7tRNAs for Val, Pro, Thr, and Ala with most likely unmodified U34 displayed a similar codon-reading pattern to their native counterparts; i.e., these t7tRNAs not only efficiently decoded their cognate-WC A-ending codons, but also to a lower degree the U- and C-ending ones.[54] Decoding of G-ending codons features strong U34-G3-mediated recognition for Val and Ala, which lack C34-bearing back-up isoacceptors. This contrasts the inefficient U34-G3-mediated recognition for Pro and Thr (Figure 4) that is possibly mediated by the cognate-WC isoacceptors.

Native tRNAGly(UCC) differs from other tRNAs decoding unsplit boxes with U in the first anticodon position, in that it carries aminomethyl modifications at U34 (see above), which is characteristic of tRNAs decoding split codon boxes.[50] T7tRNAGly(UCC) is an exception to the above-described correlation as it effectively decodes C- and G-ending codons despite the existence of C34 isoacceptor for the cognate-WC decoding of the latter.

As mentioned above, decoding of four Arg codons from the unsplit family box in bacteria is unusual because it relies on two isoacceptors, one of which carries an inosine modification more common in eukaryotes. This modification enables decoding of A-, U-, and C-ending codons via base pairing with wobble and WC-geometries, respectively. The unmodified anticodon stem-loop of t7tRNA(ACG) showed almost the same affinity to its cognate-WC codon CGU, but was inefficient in binding to its wobble CGC and CGA codons.[56] In our study, t7tRNAArg-(ACG) could efficiently decode not only U-, but also C-, A-, and, to a lower extent, G-ending codons.

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The experiments described thus far demonstrate that the synthetic tRNAs could functionally replace their native counterparts *in vitro* for 17 amino acids. Importantly, for all tested t7tRNAs, no cross-recognition was observed either for codons from the same family coding different amino acids (Figure 4, Ser and Arg, Leu and Phe, His and Gln, Asn and Lys) or for synonymous codons belonging to different families with the same mixed codon boxes such as Ser, Arg, and Leu. Analyzing these three amino acids with split codon families joined with unsplit codon boxes is particulary interesting because the former and the latter possess nonoverlapping decoding patterns and represent potentially reassignable codons.

Isolation of Native tRNAs for Glu, Asn, and Ile from the *E. coli* Native tRNA Mixture. Our results demonstrated that the majority of amino acids could be incorporated into protein by using *in vitro*-transcribed tRNAs. To reconstitute a tRNA mixture capable of supporting translation of proteins containing all 20 canonical amino acids, we needed to efficiently decode codons for Glu, Asn, and Ile. To this end, we decided to purify native tRNAs specific for these amino acids from the native tRNA mixture by DNA/RNA hybridization chromatography.[57,58] We tested several immobilization strategies and obtained the best results by coupling 3′-aminated oligonucleotides to NHS-sepharose (Supporting Information Figure S8). All three specific tRNAs for Glu, Asn and Ile were successfully obtained at good purity from the native tRNA mixture by selective hybridization with oligonucleotides complementary to the D-loop and the anticodon loop of the target tRNA (Supporting Information Table S3). The tRNAs were eluted from the matrix by thermal denaturation and were shown to be of >90% purity by denaturing PAGE analysis (Figure 5A).

The functionality of purified native tRNAs for Glu(SUC), Asn(QUU), and Ile(GAU) was tested as described above using templates harboring their cognate-WC or wobble codons. Two templates with consecutive GAA or GAG codons were employed to test the functionality of purified tRNAGlu(SUC), which, as shown in Figure 5B, could efficiently decode both codons. Similarly, the purified native tRNAAsn(QUU) restored the translation of templates harboring either AAU or AAC codons. The purified native tRNAIle(GAU) could only decode AUU codons with 40% efficiency, possibly due to inefficient refolding after denaturation or coisolation of the under-modified isoacceptor variant.

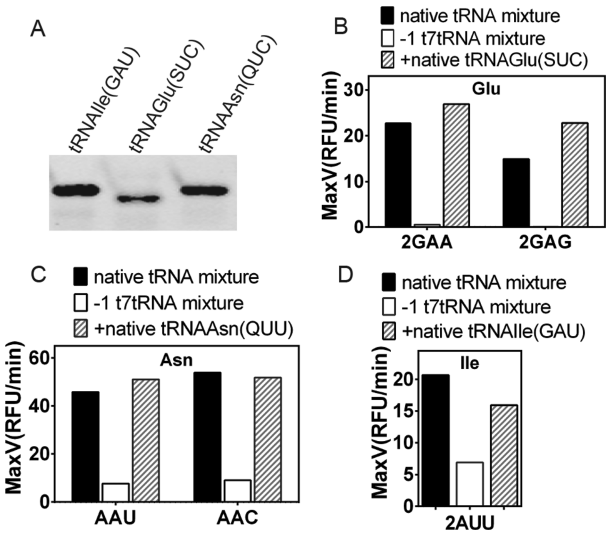

Figure 5. Analysis of the purified native tRNAs for Ile, Glu and Asn. (A) Analysis of purified tRNAs on the denaturing PAGE stained with SYBR green. (B–D) The activities of purified native tRNAGlu (B), tRNAAsn (C), and tRNAIle(GAU) (D) analyzed by the peptide expression assay. The final concentration of native tRNAs in the assay was 1.6 μM.

Semisynthetic Protein Translation System. We obtained at least one purified functional tRNA for each of the 20 canonical amino acids. To test whether this simplified tRNA complement could support synthesis of a full-length protein, we synthesized three DNA templates encoding for superfolder GFP(sGFP)[59] with variable codon compositions. These templates were designed to exclude codons inefficiently decoded by synthetic tRNAs such as CCU(Pro), UAU(Tyr), CAA(Gln), and AAA(Lys) (Supporting Information Tables S4–S6). The templates were expressed in tRNA-depleted lysate supplemented with different semisynthetic tRNA mixtures (Supporting Information Tables S4–S6), which supported translation of all three templates with efficiencies comparable to the native tRNA mixture (Figure 6A).

To ensure the sGFP expresion was a direct result of supplementation with semisynthetic tRNAs and to reconfirm the functionality of the individual tRNAs, we formulated tRNA mixtures lacking individual tRNAs. We then analyzed the ability of these mixtures to support synthesis of sGFP templates in a tRNA-depleted cell-free system and observed a reduction in translation ranging from several-fold to orders of magnitude (Figure 6B). Similar to the results obtained by peptide expression assay, including the t7tRNAs coding for UCG(Ser), CGG and AGG(Arg), UUG(Leu), GGA(Gly), CCA(Pro), ACA(Thr), GUG(Val), AAG(Lys), and UUC(Phe), the semisynthetic tRNA mixtures restored sGFP expression, thus reconfirming the functionality of the corresponding t7tRNAs. However, in contrast to the RGS-peptide expression profile, residual amounts of native tRNAs for CUA and AGC codons proved to be sufficient for sGFP expression. When only one CUA or AGC codon was present in ORF sequence of the reporter peptide, the presence of t7tRNA(UAG) and t7tRNA-(GCU) in the mixture restored peptide expression to 92 and 72%, respectively (Supporting Information Table S2). This inconsistency possibly reflected a higher concentration of peptide transcripts as well as higher turnover rates of peptide translation compared to sGFP. In the former case, the number of elongating complexes could possibly surpass the number of native tRNAs remaining in the lysate, while in the latter case the same tRNA could be tunneled within the same polysomal unit.[37,60]

When just one codon was present in the ORF, sGFP expression appeared to be more sensitive to the residual amounts of corresponding native tRNAs in the depleted lysate. Unlike for the CUA and AGC codons, the expression level of sGFP with only one AGG codon per ORF decreased by ~90% when t7tRNA for AGG was excluded. This makes AGG the most promising codon for reassignment even without further optimizing the depletion of native tRNAs.

The observation that full-length protein could be expressed in our cell-free system prompted us to probe the role of tRNA modificaitons in protein translation. As discussed above, even though the majority of tRNAs in our system were synthetic, they could potentially undergo partial editing or modifications in the context of a translationally active lysate.

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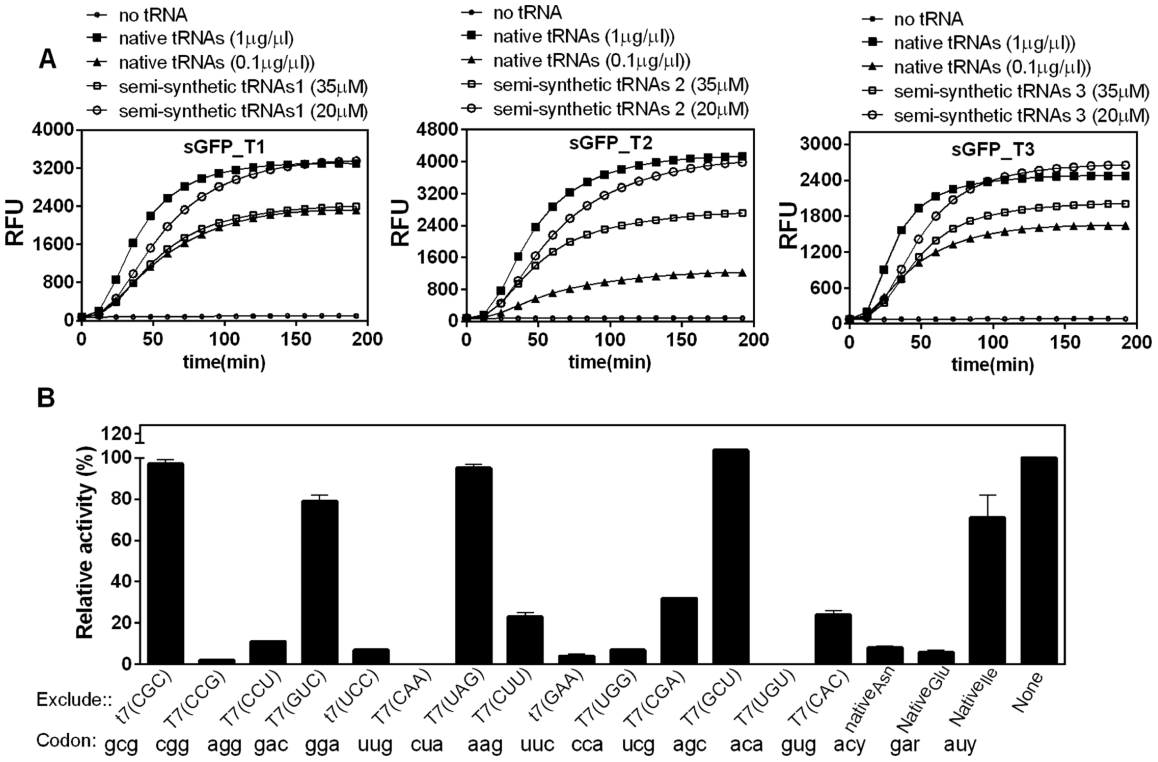

Figure 6. sGFP expression in semisynthetic *in vitro* translation system. (A) DNA templates for three sGFP ORFs of various codon compositions were expressed in tRNA-depleted lysate programmed with semisynthetic tRNA complements or native tRNA mixture at different concentrations. (B) Expression of sGFP_T2 template in tRNA-depleted lysate supplemented with semisynthetic tRNA mixtures lacking the indicated t7tRNAs. Corresponding codons are shown below the tRNAs where y stands for U or C and r for A or G.

To test the effect of such putative modifications on the functionality of t7tRNAs, we repeated the above experiment using the reconstituted PURE *in vitro* translation system, which presumably lacks tRNA processing and modification activities. We found that semisynthetic tRNAs sustain protein expression—yet, we observed that the relative translation efficiency of the PURE system supplemented with semisynthetic tRNAs was only 60% compared to the native tRNA complement. This is in contrast to our observation that both native and synthetic tRNA complements performed almost equally well in depleted lysates. On the one hand, this indicates that unmodified synthetic t7tRNAs could sustain protein synthesis. On the other hand, the observed reduction in efficiency may reflect additional post-transcriptional processing of at least some t7tRNAs in the S30 extract, but not in the PURE system. Addressing this issue conclusively would require testing the functionality of individual t7tRNAs in the PURE system. This is not straightforward due to the surprisingly high levels of contaminating native tRNAs (Figure 7).

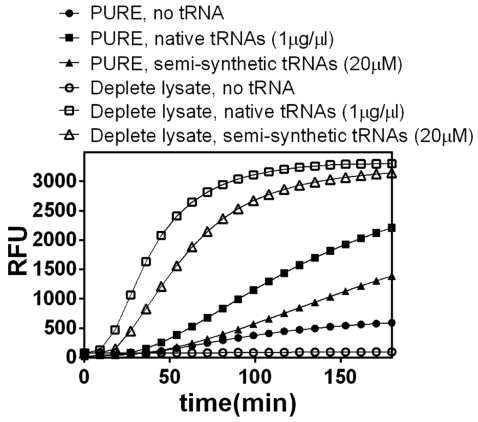

Figure 7. Expression of sGFP_T1 in the PURE *in vitro* translation system and in the tRNA-depleted S30 lysate. *In vitro* translation experiments were performed without tRNA(circles), with native tRNA (squares) or with semisynthetic tRNAs (triangles).

DISCUSSION

In this work, we established an approach for the systematic analysis of individual tRNA functions using an *in vitro* translation system depleted of endogenous tRNAs. This was achieved by developing a nonradioactive assay to quantify the *in vitro* expression of a reporter peptide in tRNA-depleted *E. coli* cell-free translation systems. We demonstrated that the depleted lysate retained more than 60% of the activity of the parental lysate. Although the residual tRNA pool in depleted lysate could not sustain eGFP and RGS-peptide expression, we found that a subset of native tRNAs was not fully depleted. The developed peptide biosensor assay allowed us to estimate the depletion level of tRNA isoacceptors relative to their codons and revealed a correlation between the depletion efficiency of individual tRNAs and their abundance. Importantly, RGS1-peptide expression was also observed in a fully recombinant *E. coli* PURE system[61] primed with t7tRNA mixtures lacking individual tRNAs (data not shown). Furthermore, the PURE system assembled without exogeneous tRNAs could support the expression of full-length GFP (Figure 7), indicating presence of the entire spectrum of contaminating tRNAs. This suggests that residual tRNAs most likely copurify with aaRSs or other components of the translational machinery.[16]

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Therefore, efficient tRNA depletion from *in vitro* translation systems remains a challange.

To distinguish the activity of t7tRNAs from the endogenous tRNA background activity, we utilized the observation that two identical, consecutive test-codons significantly sensitized the assay to the depletion of specific tRNAs. This enabled us to analyze the functionality of synthetic versions of all 48 *E. coli* tRNA species in our tRNA-depleted lysate.

Our results demonstrate that most of the synthetic tRNAs were efficient in supporting protein/peptide translation (Figure 4-7). Furthermore, most of the t7tRNAs corresponding to 2- or 4-fold-degenerate amino acids decoded their cognate-WC codons with high or medium efficiency and their wobble codons with medium or low efficiency (Figure4). In contrast, the t7tRNAs for Asn, Gln(CAA), Ile(AUC), Glu, and Lys were found to be nonfunctional in the affinity clamp assay.

Even though we could not exclude the partial editing and modification of occurring in the crude translation system, it appears unlikely that the N34 and N37 modifications, which require the activity of multiple enzymes, would occur efficiently. This notion is supported by the observation that incorporation of Ile, Glu, and Lys could not be supported by synthetic tRNAs, and accords with previous studies showing that modified nucleotides served as key molecular recognition features for their cognate aaRSs.[21] It was reported earlier that tRNALys-(UUU) lacking modifications outside the anticodon loop undergoes aminoacylation at 140-fold lower efficiency,[62] yet conversion of U to C in the first anticodon position restored its activity toward the AAG codon. In addition to Lys(UUU), a number of t7tRNA transcripts or their corresponding anticodon stem-loops such as Arg(UCU), Ala(UGC), Cys-(GCA), Glu(UUC), and Gln(UUG) have also failed in the ribosome-mediated codon binding assay.[50] With the exception of Glu(UUC) and Gln(UUG), three remaining t7tRNAs were translationally active in the affinity clamp assay. In particular, t7tRNAArg(UCU) demonstrated a 3-fold higher activity compared to its homologue, which is presumably under-represented in total native tRNA mixtures (Figure 4). Overall, the codon−anticodon interaction matrix depicted in Figure 4 shows highly similar codon recognition patterns between native and t7tRNAs, which are most likely devoid of modifications within anticodon loops. For instance, U34 in the first anticodon position of t7tRNAs decodes not only its cognate A and G, but also U and C in the third codon position with similar reading patterns to that of cmo5U in the native tRNAs for Ser, Leu, and Gly, and with an identical pattern in tRNAs for Val, Pro, Thr, and Ala. From the structural and kinetic data, it appears that the intrinsic stability of the codon-anticodon helix is less important than its proper geometry, which is sensed by the ribosome.[63,56,57] These studies thus imply that the net affinity between the codon−anticodon duplex and the ribosome induced 30S closing around the decoding center, thereby promoting tRNA accommodation and triggering the down-stream steps that lead to peptide-bond formation.[54] In the current study, the highly mosaic pattern of U34−N3 interactions and the lack of noncognate cross-recognition in the split codon boxes supports the idea of higher order contextuality in the tRNA body providing an additional check-point for accurate and productive decoding.[56,57]

The decoding preferences shown here provide a valuable guide for identifying "orthogonal" vs "native" codon pairs from the synonymous codons for a particular amino acid. Such pairs can either be created from the codons of different families of 6-fold-degenerate amino acids or from those derived from the unsplit codon family boxes with high wobble restrictions such as Arg, Ser, and Leu and Pro, Thr, and Gly (Supporting Information Table S7). This work suggests that the AGG-codon, for which native tRNA was depleted almost completely, is potentially easier to reassign than all the other codons.

We showed here that all amino acids except Ile, Glu, and Asn could be decoded by synthetic tRNAs, and that native tRNAs for these three amino acids were purified to homogeneity in a functional form. We demonstrated that the tRNA complement reconstituted with synthetic tRNAs and three specific native tRNAs could support *in vitro* synthesis of sGFP to comparable levels achieved with the native tRNA mixture. Although the full tRNA depletion remains a challenge, our results using the PURE system provide a clue to the origin of the contaminating tRNA pool.

Improved tRNA depletion protocols in combination with semisynthetic tRNA complements would enable reassigning sense codons in peptides and proteins, thereby significantly expanding the toolbox of synthetic biologists and protein engineers. Further, the developed peptide expression assay in combination with the PURE system enables the impact of individual tRNA modifications on their functionality to be dissected. This should in turn answer a long-standing question regarding the extent to which such modifications need to be maintained in the effort to construct the minimal cell.[64] Finally, the presented approach is not confined to *E. coli*, and can be transferred onto eukaryotic cell-free expression systems.

MATERIALS AND METHODS

Peptide Biosensor (Affinity Clamp) Assay. The reporter peptides of different sequences, RGSIDTWV, GGRGSIDTWV, DDRGSIDTWV, and the fluorescently quenched TVMV substrate peptide (5-amino-2-nitrobenzoic acid -ETVRFQSK-7-methoxycoumarin-4-yl), were synthesized by Mimotopes. A fusion of autoinhibited protease and the affinity clamp (peptide biosensor) was purified by Ni^{2+}-NTA affinity chromatography and stored in 50 mM Tris-HCl, 1 M NaCl, 5 mM EDTA, 2 mM TCEP, ans 10% glycerol buffer (pH 8.0).

Typically the affinity clamp assay was carried out in buffer A of 50 mM Tris-HCl, 1 M NaCl, 1 mM DTT, and 0.5 mM EDTA (pH8.0), supplemented with 1 μM of peptide biosensor and 15 μM of TVMV substrate peptide. RGS-peptides were used either as a solution in the buffer or in the context of *in vitro* translation, and the reaction progress was monitored by exciting the sample at 330 nm and recording the fluorescence changes at 405 nm for 1 h using the Synergy plate reader. A calibration plot was generated to establish the relationship between initial rates of substrate cleavage (V_{max}) and known concentrations of the control peptide. Samples were assayed in triplicate.

To quantify the RGS peptide and its derivatives in a cell-free translation reaction, the S30 *E. coli* cell extract formulated for coupled transcription−translation and supplemented with x2 protease inhibitor cocktail (Roche) was primed with the desired peptide-coding DNA template and incubated at 32 °C for 1 h. After translation, NaCl was added to the reaction mixture to the final concentration of 1 M followed by incubation at 65° for 10 min to inactivate the endogenous proteases otherwise competing with TVMV-protease for the substrate peptide. After centrifugation at 10 000 rpm for 5 min, 10 μL of supernatant was used for the affinity clamp assay as described above. In the context of *in vitro* translation reactions, the calibration plot was obtained by supplementing different amounts of synthetic peptides into the cell-free translation reaction lacking the DNA template.

Preparation of tRNA-Depleted Lysate. The s30 *E. coli* extract was prepared from BL21(DE3)GOLD as described previously[27] and stored frozen in 10 mM Tris-acetate (pH 8.2), 14 mM $Mg(OAc)_2$, 0.6

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mM KOAc, and 0.5 mM DTT buffer at −80° before the tRNA-depletion procedure.

For tRNA depletion, 2.5 mL of s30 extract was rebuffered on an NAP-25 column (GE healthcare) equilibrated with buffer B of 25 mM KCl, 10 mM NaCl, 2.1 mM $Mg(OAc)_2$, 0.1 mM EDTA, 10 mM Hepes-KOH (pH7.5), and 120 mM KOAc. After rebuffering, the lysate was incubated with 0.8 mL settled ethanolamine−Sepharose matrix, prepared according to previous procedures,[28] at 4 °C for 30 min on an orbital shaker. Following the incubation, the supernatant was collected and the matrix was washed with 1 mL of buffer B containing 180 mM KOAc. The flow-through was combined with the supernatant from the previous step to yield the tRNA-depleted lysate, snap frozen, and stored at −80 °C.

The cell-free translation reactions in the S30 lysate were performed at 32 °C following the standard protocol[27] using 30 nM DNA template and $Mg(OAc)_2$ at 10 mM final concentration. The PURExpress Δ (aa, tRNA) Kit (E6840S) was purchased from NEB and used according to the manufacturer's instructions.

Construction of tDNAs and Plasmids for Peptide and sGFP Expression. The coding sequences for tRNAs were obtained from the Genomic tRNA database (GtRNAdb).[29] The DNA templates (tDNAs) were synthesized by 3-step PCR (Supporting Information Figure S1).

All DNA templates coding for peptide and sGFP were constructed based on pOPINE-eGFP plasmid (GenBank: EF372397.1). To construct peptide-coding DNA templates, two complementary oligonucleotides harboring NcoI and NotI restriction site overhangs were used to assemble the ORFs of the desired peptides. The concentration of oligonucleotides was adjusted to 100 μM, mixed in water at a 1:1 molar ratio followed by heating at 95 °C for 5 min, and then slowly cooled down to room temperature for annealing. The pOPINE-eGFP plasmid vectors were digested by NcoI and NotI, combined with the annealed oligonucleotides, and ligated using T4 DNA ligase. The positive clones were verified by Sanger sequencing (AGRF Brisbane).

The fragments coding for sGFP ORFs with various codon biases denoted as sGFP_T1, sGFP_T2, and sGFP_T3 were synthesized as G-blocks by IDT and cloned into the pOPINE-based plasmid following the standard Gibson cloning procedure.

T7tRNA Synthesis and Purification. Standard runoff t7 transcription reactions were performed at 32° for 2 h in 40 mM Hepes-KOH (pH 7.9), 18 mM $Mg(OAc)_2$, 2 mM Spermidine, 40 mM DTT, 5 mM each rNTP containing 0.25 μM DNA template, 10 μg/mL T7 polymerase, and 0.25 U/mL yeast inorganic pyrophosphatase. For the synthesis of tRNAHis, the transcription reaction was first supplemented with 5 mM of each rATP/rCTP/rUTP and 6.8 mM rGMP for 5 min, followed by addition of 1.7 mM rGTP and incubation for 2 h. The DNA template for tRNAHis contained an additional G corresponding to −1 position in tRNA. After transcription, the reactions were diluted 5-fold into buffer C (125 mM NaOAc pH 5.2, 0.25 mM EDTA). The tRNA transcripts were purified by affinity chromatography using ethanolamine−Sepharose matrix. For 1 mL of transcription reaction, 0.2 mL of settled matrix was used. Following the 1-h incubation of the slurry at 4 °C, the matrix with bound tRNAs was extensively washed with buffer C containing 200 mM NaOAc. t7tRNAs were eluted from the matrix into buffer C containing 2 M NaOAc. tRNA was ethanol precipitatated and the pellets were dissolved in tRNA buffer containing 1 mM $MgCl_2$ and 0.5 mM NaOAc (pH 5.0).

sGFP Expression by Semisynthetic tRNA Mixture. Three ORFs with variable synonymous codon compositions coding for sGFP were synthesized commercially and cloned into pOPINE plasmid. Template 1 (T1) had the highest codon variation, including five different synonymous codons coding for Leu, four for Val, and three for Pro, Arg, Ser, and Thr (Supporting Information Table S4). Template 2 (T2) was designed to deliver the highest codon biases with only two synonymous codons used to encode Ser, Arg, and Leu, and one codon used to encode Val, Pro, Thr, Ala, and Gly (Supporting Information Table S5). Template 3 (T3) featured a medium codon variety with two codons for Ser and Arg, and several codons for Leu, Val, Pro, Thr, Ala, and Gly, as in T1 (Supporting Information Table S6). The proportions of individual tRNAs in the semisynthetic tRNA mixtures were roughly proportional to their codon abundance in the sGFP ORF sequences, except for codons occurring more than 10 times and those corresponding to the least-depleted native tRNAs. These t7tRNAs in the semisynthetic mixtures were taken at reduced proportions relative to their codon usage shown in Supporting Information Tables S4−S6. Production of sGFPs corresponding to T1−3 in the translation reactions with semisynthetic tRNA complement was monitored on a fluorescence plate reader for 3 h at 485 nm excitation and 528 nm emission wavelengths.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details on preparation and functionality test of synthetic tRNAs, purification of specific tRNAs from total native tRNA mixture, semisynthetic tRNA reconstitution for sGFP expression are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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