Hyperaccurate ribosomes for improved genetic code reprogramming

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Abstract

The reprogramming of the genetic code through the introduction of non-canonical amino acids (ncAAs) has enabled exciting advances in synthetic biology and peptide drug discovery. Ribosomes that function with high efficiency and fidelity are necessary for all of these efforts, but for challenging ncAAs, the competing processes of near-cognate readthrough and peptidyl-tRNA dropoff can be issues. Here we uncover the surprising extent of these competing pathways in the PURE translation system using mRNAs encoding peptides with affinity tags at the N- and C-termini. We also show that hyperaccurate or error restrictive ribosomes with mutations in ribosomal protein S12 lead to significant improvements in yield and fidelity in the context of both canonical AAs and a challenging α,α-disubstituted ncAA. Hyperaccurate ribosomes also improve yields for quadruplet codon readthrough for a tRNA containing an expanded anticodon stem-loop, although they are not able to eliminate triplet codon reading by this tRNA. The impressive improvements in fidelity and the simplicity of

introducing this mutation alongside other efforts to engineer the translation apparatus make hyperaccurate ribosomes an important advance for synthetic biology.

Keywords

Ribosome mutants, *in vitro* translation, quadruplet codons, non-canonical amino acids

Introduction

Reprogramming of the genetic code offers exciting opportunities for the introduction of novel non-canonical amino acids (ncAAs) for the controlled syntheses of non-natural biopolymers.[1–4] The introduction of ncAAs with backbone modifications is particularly exciting because it enables the creation of extremely diverse peptide libraries composed of peptides with remarkable drug-like properties.[5–7] Recent work has shown that the ribosome can tolerate novel backbone modifications including N-methyl, alpha-hydroxy, alpha-alpha disubstituted, D-, beta, and even certain aniline acids.[5,8] However, being able to tolerate a given ncAA does not mean that the translation apparatus can efficiently utilize these ncAAs, even if they can be charged effectively onto tRNA. For highly modified ncAAs, engineering of the tRNA, EF-Tu, and the ribosome are often required to improve efficiency.[9–12] The problem of inefficient translation is magnified in the context of peptide libraries where it leads to selective pressure against synthesis library members containing the ncAA.

For challenging ncAAs, competing processes can be significant hurdles for efficient incorporation.[13,14] Two of these–near-cognate misincorporation and peptidyl-tRNA dropoff–have been studied in vivo under conditions of amino acid starvation,[15–17] but these processes are likely to also manifest themselves with difficult to translate ncAAs *in vitro*. A careful inspection of the literature shows that the majority of the studies with difficult ncAAs utilize reconstituted *in vitro* translation reactions that are devoid of AA-tRNAs that are not required for the encoding mRNA[18] (presumably to minimize near-cognate misincorporation)

and have a C-terminal tag to remove sequences that result from misincorporations within the purification-tag region or peptidyl-tRNA dropoff.[19,20] While these strategies do lead to homogeneous peptides, they hide the underlying inefficiencies that compromise yield and fidelity. An ideal translation system would avoid these undesired side reactions altogether, leading to gains in the yield and efficiency of incorporation of ncAAs, enabling the reliable and predictable creation of diverse, highly-modified peptides and proteins.

In this work, we investigate the extent of undesired translation outcomes that diminish yield and fidelity with canonical and ncAAs, and show how error-restrictive, hyperaccurate ribosomes can lead to significant improvements in overall peptide yield and in the incorporation efficiency of ncAAs in the PURE translation system. We further examine the exploitation of this enhanced translation machinery to genetic code reprogramming using quadruplet codons.

Results and discussion:

In the presence of non-cognate aminoacyl tRNAs, the fidelity and relative yield of PURE *in vitro* translation reactions is reduced. We first set out to explore situations where near-cognate competition can be a problem in the PURE reconstituted *in vitro* translation system[21] , exemplified by templates that have a high requirement for a low abundance AA-tRNA, leading to depletion and potential competition from near-cognate AA-tRNAs.[19,22,23] We used a short, His6-tagged template (Figure 1a, mRNA 1), in which 6 of the 11 codons encode for Histidine. During *in vitro* translation reactions where all 20 AARS and AAs are included, we observe frequent substitution of glutamine for the histidine codons in the purified peptides (Figure 1b). (Gln is known to be a competent substitute for His in binding to nickel[24]). The misincorporations occur regardless of the total translation time (Supplementary figure S1) or translation buffer (Supplementary figure S2). Notably, this situation mirrors what is observed *in vivo* under conditions of histidine starvation.[16] A similar

situation can arise with ncAAs that are difficult substrates for the translation apparatus.[25] For example, we added α-MeCys-tRNA^{Val} at 12.5 mM to a translation reaction lacking Val/ValRS but containing only the other required AARS efficient translation of mRNA 1 (MetRS, HisRS, GluRS, ProRS) (Figure 1c).[26] Under these conditions, the MALDI-MS spectrum showed significant near-cognate readthrough by Glu-tRNA^{Glu}.

The presence of unused near-cognate AA-tRNAs also negatively affects the yields of *in vitro* translation reactions. We performed *in vitro* translation reactions in the presence of the minimal complement of AARS/AAs or all 20 AARS/AAs for mRNAs 2-5 (Figure 1d). In every case, inclusion of all 20 AARS/AAs led to a significant drop in yield. This drop in yield could not be rescued by additional EF-Tu[27] , showing that the AA-tRNA/EF-Tu/GTP ternary complex interaction was not the limiting factor (Supporting figure S3). These losses in yield have perhaps motivated the practice of excluding unused AA/AARS in PURE *in vitro* translation reactions.

The interference of near-cognate AA-tRNAs in these contexts led us to wonder if hyperaccurate, or error-restrictive, ribosomes could be a potential solution. These ribosomes were first identified through their ability to confer resistance to antibiotics, like streptomycin, that interact with the decoding center of the ribosome.[28,29] Many of these mutations occur within the ribosomal S12 protein, which is known to stabilize the codon:anticodon recognition elements within the mRNA and tRNA through a salt bridge,[30,31] thereby promoting transition to the closed form necessary for the elongation process to continue.

When streptomycin binds to the decoding center it stabilizes the closed-form of the ribosome even in the presence of near-cognate AA-tRNA causing translational errors[32] . However, a streptomycin resistant mutant S12 K42T, here called mS12, disrupts the salt bridge,[31] destabilizing this closed form. As a consequence, mS12 ribosomes are known to reject near-cognate tRNAs at a rate 7.5 fold higher than WT ribosomes[33] , yet they retain high activity.[34] These qualities seemed ideal for improving the issues with near-cognate readthrough highlighted in Figure 1. Using multiplex automated genome engineering

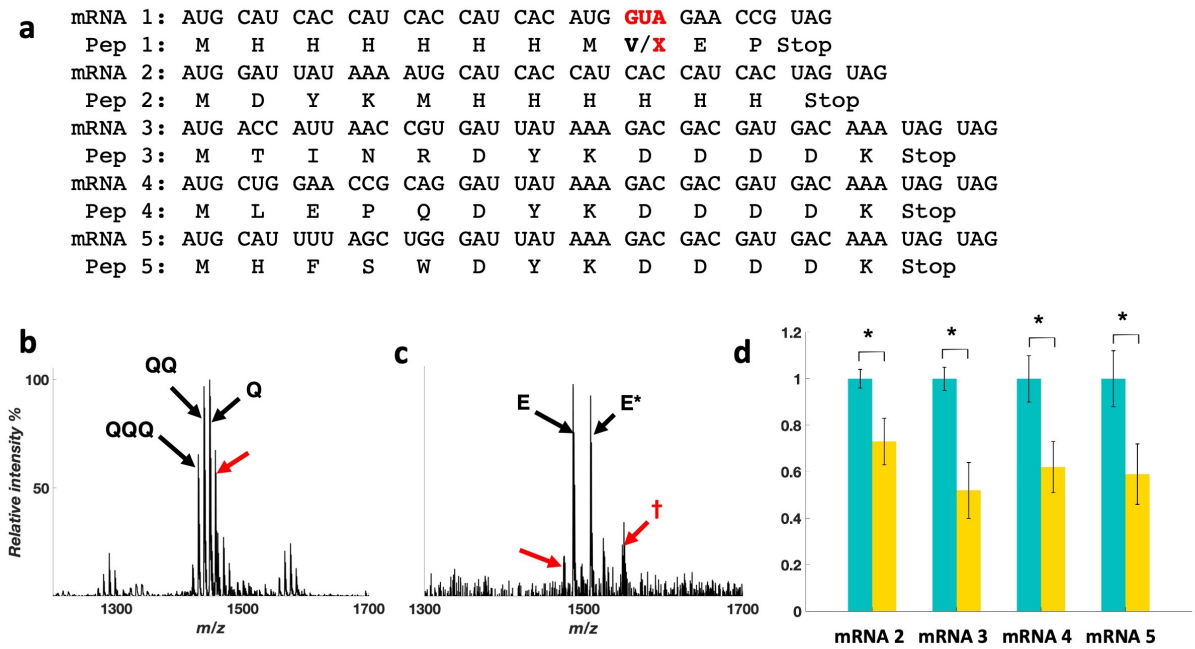

Figure 1: **Problems in *in vitro* translation with WT ribosomes.** a) mRNA templates used. b) MALDI-TOF spectra of mRNA 1 translated by WT ribosome in presence of GlnRS and Gln, Red arrow shows target peak, black arrows with 'n' number of Q show the 'n' number of Glutamine for Histidine misincorporations. c) *In vitro* translation of mRNA 1 in the presence of 12.5 μM αMeCys-tRNAVal. The major peak corresponds to near-cognate readthrough by Glu-tRNA^{Glu} (labeled as E = $[M = H]^+$, and E* = $[M + Na]^+$. The red arrows correspond to the desired peak with αMeCys incorporation and the red cross shows the desired peptide containing a disulfide with 2-mercaptoethanol. d) Relative yields of mRNAs 2-5 in a translation system with minimum AARS (in cyan) and all 20 natural AARS (in yellow), using WT ribosomes. Values represent averages and error bars are $\pm$ standard deviations, with n=6 (* : $p < 0.01$). Tables of the expected vs. calculated masses are given in SI table S3 and S4 for 1b and 1c respectively.

(MAGE)[35] we created K42T mutant *E. coli* A19 cells. The mutation was verified by sequencing (Supporting figure S4). We then compared purified mS12 ribosomes and WT ribosomes in a battery of tests, described in the following sections.

mS12 ribosomes resist losses in translational yields and fidelity in the presence of unused AA -tRNAs. We first posited that mS12 ribosomes would be resistant to the presence of increasing amounts of unused near-cognate AA-tRNAs. We repeated the experiments described above in Figure 1 with mS12 ribosomes. Unlike in the case of WT ribosomes, no misincorporation of Q was observed (Figure 2a), a dramatic improvement

compared to Figure 1b. We also repeated the analysis of templates 2-5 as in Figure 1d with mS12 ribosomes. In none of these templates did we observe erosion of yield in the presence of all 20 AARS/AAs (Figure 2b). To further explore the reason for this improvement, we pre-charged total *E. coli* total tRNA with the AARS/AA pairs not required for translation of mRNA 3 (Figure 2c, d). Increasing amounts of this pre-charged tRNA mix were then added to a translation reaction in the presence of the AARS/AAs required for the template. WT ribosomes showed decreases in relative peptide yield to almost no yield at the highest concentration added (500 μM). mS12 ribosomes were very resistant to losses of yield due to the presence of near-cognate AA-tRNAs, and only showed significant decreases in yield at 500 μM (Figure 2c). These trends were also reflected in the MALDI-MS analysis of the peptide products (Figure 2d). For mS12 ribosomes product peptide was still made cleanly at even at the highest concentration used, while the wild-type ribosomes show increasing losses of fidelity with added unused AA-tRNAs (Figure 2d). mS12 ribosomes also resisted loss of fidelity due to high magnesium concentrations (Supporting figure S5).

mS12 ribosomes show increased full-length peptide yields. We wondered whether the impressive ability of mS12 ribosomes to tolerate unused AA-tRNAs would be extended to templates containing both a N- and C-terminal tag (Figure 3a). Purification via the N-terminal tag does not require that the full peptide be synthesized and allows identification of truncations, whereas the C-terminal purification quantifies only fully translated peptides. Translation reactions were carried out with both ribosomes and in presence of minimal AARS/AAs or all 20 AARS/AAs. Each translation reaction was split into two equal fractions: one was purified by the N-terminal His6 tag, and the other by the C-terminal FLAG tag. Surprisingly, even in the presence of minimal AARS/AAs, wild-type ribosomes showed large losses in yields when comparing C- vs. N-terminal capture (Figure 3b). This loss in yield was magnified in the presence of all 20 AARS/AAs. mS12 ribosomes, on the other hand, showed no significant losses in yields when the N- and C-terminal purification strategies were compared even in the presence of all 20 AARS/AAs (although the overall

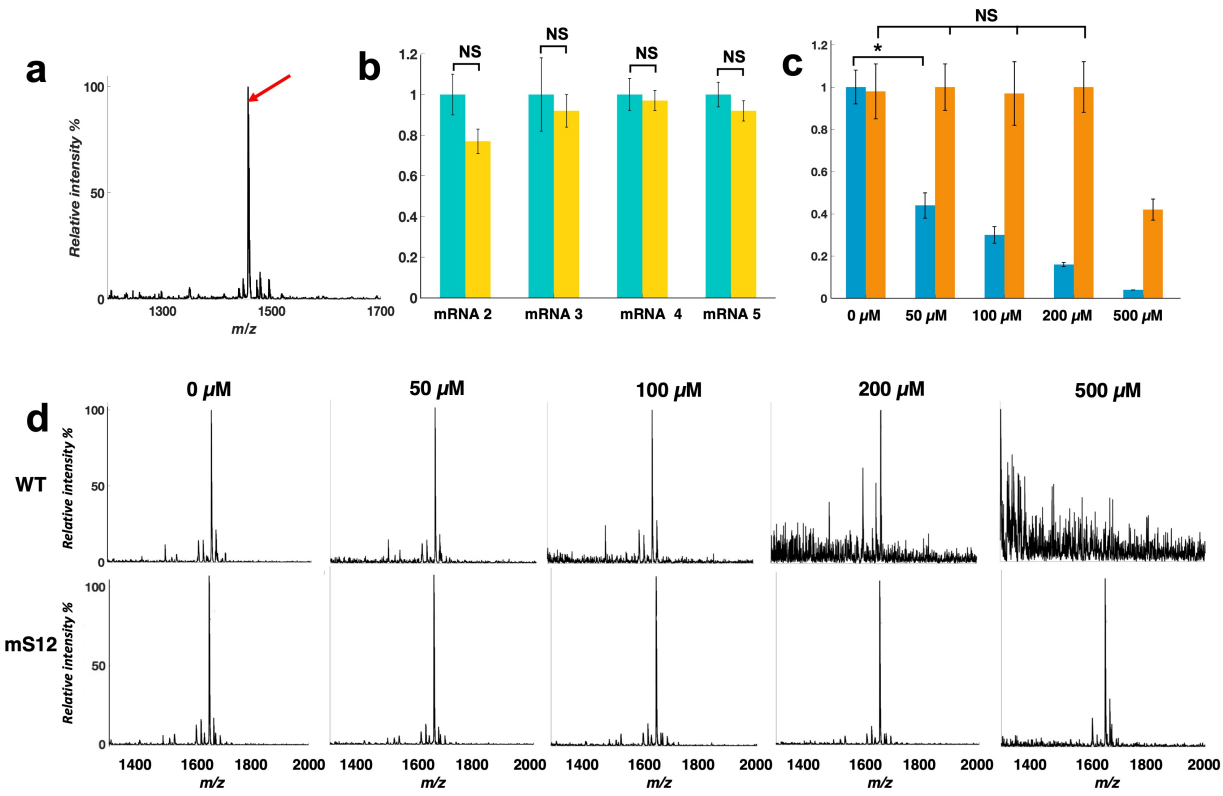

Figure 2: **mS12 ribosomes resist yield losses due to extraneous AARS/AA and extraneous ternary complexes.** a) MALDI-TOF spectra of mRNA-1 translated by mS12 ribosomes in presence of GlnRS and Gln, Red arrow shows target peak with no misincorporations. b) Relative yields of mRNAs 2-5 in a translation system with minimum AARS (in cyan) and all 20 AARS (in yellow), using mS12 ribosomes. Values represent averages and error bars are ± standard deviations, with n=6 (NS : not significant at $p < 0.05$). Panel c shows the relative translation yields of mRNA 3 corresponding to increasing amounts of extraneous AA-tRNA addition with wild-type (aqua) and mS12 ribosomes (orange): 0 μM, 50 μM, 100 μM, 200 μM, and 500 μM. Values represent averages and error bars are ± standard deviations, with n=5 (* : $p < 0.01$, NS: non significant), and were normalized to the yields of the 0 mM AA-tRNA added reactions. Panel d shows MALDI spectra for the same experiment. The expected peak is observed in all cases for the S12 ribosomes. Tables of the expected vs. calculated masses are given in SI table S5.

yield was lowered in the presence of all 20 AARS/AAs for this template, Figure 3b). The MALDI-MS under each condition was also consistent with the yield data and highlights the increased ability of mS12 ribosomes to produce full length peptides even in the presence of near-cognate AA-tRNAs (Figure 3c). mS12 ribosomes also gave higher absolute yields than WT ribsomes under all conditions, (Supporting figure S6). Taken together, the resistance of

mS12 ribosomes to misincorporations induced by near-cognate tRNA leads to higher fidelity and higher yielding PURE *in vitro* translation reactions.

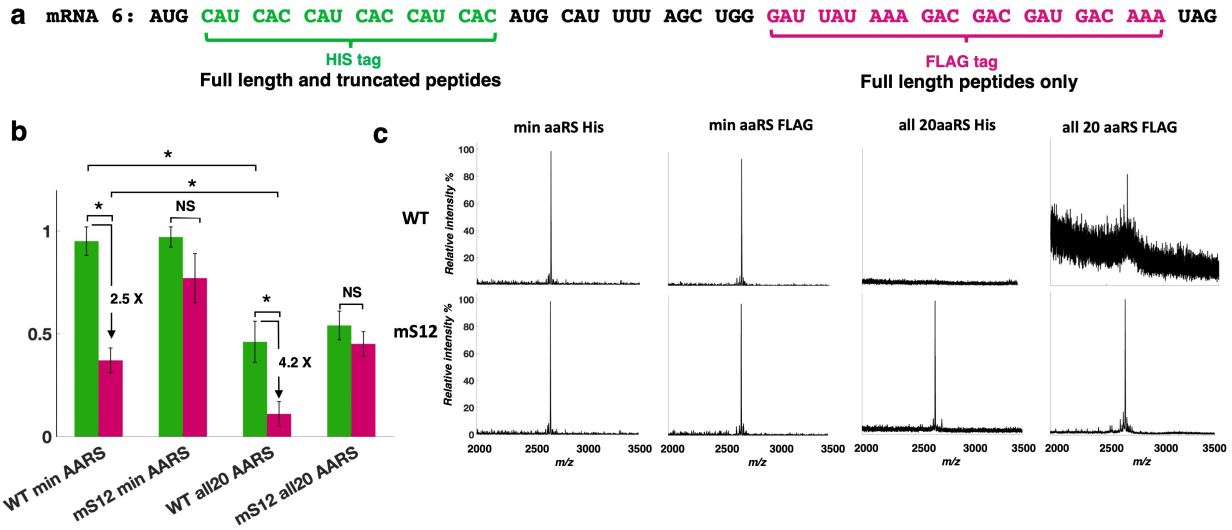

Figure 3: **mS12 ribosomes increase the yield of correct full-length peptides**. a) mRNA 6 encodes a peptide with both N- and C-terminal purification tags. b) Relative yields obtained for *in vitro* translation reactions containing WT and mS12 with both minimal and all 20 AARS conditions as purified by either the N-terminal His6 tag (green) or C-terminal FLAG tag (magenta). Values represent averages and error bars are ± standard deviations, with n=4 (* : $p < 0.005$, NS= not significant). c) MALDI-MS for experiments with WT ribosomes (top) or mS12 ribosomes (bottom) in the presence of only minimal synthetases (as purified by His6 and FLAG tag respectively) and all 20 synthetases as purified by His6 and FLAG tag respectively. Each MALDI-MS experiment was repeated at least 3 times and a representative spectrum is shown. Tables of the expected vs. calculated masses are given in SI table S6.

mS12 ribosomes improve the efficiency of incorporation of a challenging ncAA. Having seen improved fidelity and efficiency in canonical amino acid incorporation, we were interested to see if this efficiency could be translated to ncAAs. Among different ncAAs, αMeCys has been shown to be incorporated using an editing-deficient ValRS T222P [36] and is interesting because of its mimicry of a hydrocarbon staple.[26] As an α,α disubstitued analog; however, αMeCys has proven to be a difficult translation substrate (Figure 1c). We wondered if αMeCys could be cotranslationally incorporated with higher efficiency using mS12 ribosomes. To test this, $tRNA^{Val}_{GUA}$ was precharged with αMeCys using ValRS T222P and added at different concentrations (3.125 μM, 6.25 μM, 12.5 μM and

25 μM) into translation reactions conducted by WT and mS12 ribosomes with mRNA 1 (Figure 4). For WT ribosomes only 25 μM of αMeCys-tRNA^{Val} gave clean MALDI-TOF signals for the target peptide, whereas for mS12 the target peak was observed at as low as 6.25 μM of αMeCys-tRNA^{Val}. MALDI-TOF signals of WT ribosomes at concentrations below 25 μM (Figure 4) showed evidence of competition at the GUA codon by near-cognate Glu-tRNA^{Glu}. Notably, no misincorporations were observed in mS12 ribosomes even at the lower concentrations of αMeCys-tRNA^{Val}, showing that the mS12 ribosomes completely suppressed near-cognate competition in this context. The translation yields with mS12 ribosomes improved with increased αMeCys-tRNA^{Val} (Supporting figure S7) showing the expected dose-response. Taken together, the ability of mS12 ribosomes to eliminate misincorporations leads to improved fidelity and yields with this difficult, backbone-modified ncAA.

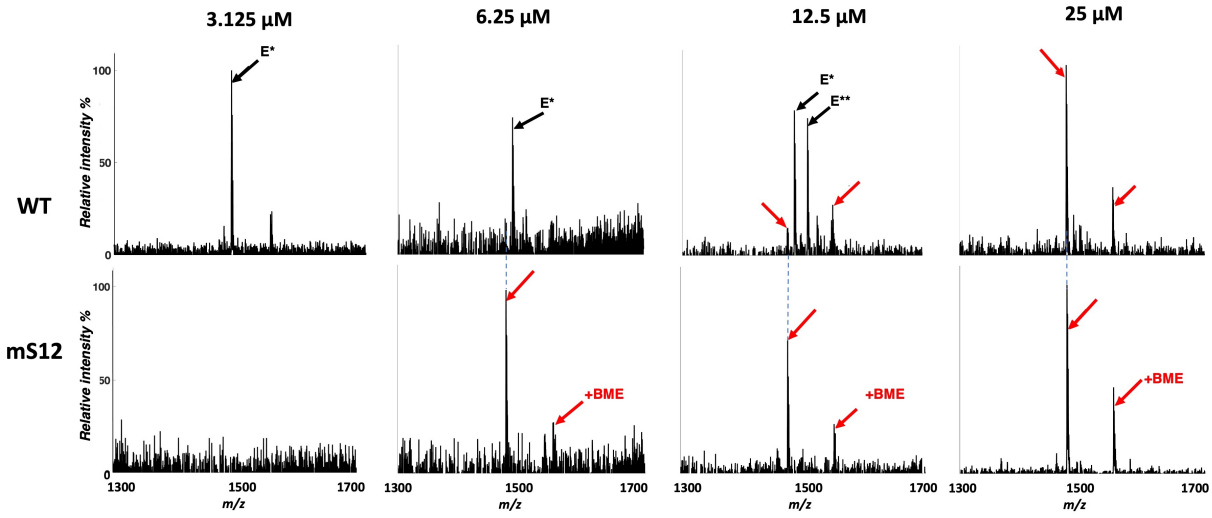

Figure 4: **mS12 ribosomes can improve the efficiency and accuracy of ncAA incorporation**. Val-GUA-tRNAs were precharged with α-MeCys by ValRS T222P and increasing concentrations were added in translation 3.125 μM, 6.25 μM, 12.5 μM, 25 μM, for WT and mS12 ribosomes. Note: the red arrows in MALDI-TOF spectra show the target peak for mRNA 1 with α-MeCys, $[M+H]^+$ =1474.661 and the BME disulfide adduct $[M+H]^+$ =1550.632. The black arrows indicate Glutamic acid incorporation instead of α-MeCys ($[M+H]^+$, E*) and its $[M+Na]^+$ peak (E**). Tables of the expected vs. calculated masses are given in SI table 7.

mS12 ribosomes improve quadruplet codon suppression efficiency. There

has been a recent resurgence of interest in quadruplet codon suppression technologies[37,38] , which promise dramatic expansion of the genetic code. A major obstacle for the use of mixed quadruplet/triplet codons is that every quadruplet tRNA has to compete with a triplet tRNA for the same codon.[39] For this reason, most of the successful quadruplet tRNAs suppress triplet codons read by rare tRNAs or stop codons a part of their anticodon sequence.[40] Even in this context, suppression yields are quite poor, and the amount of full length proteins in the quadruplet system are a fraction of a percent relative to mRNAs with the corresponding triplet codon.[37] A second issue with quadruplet tRNAs relates to their dual ability to read both triplet and quadruplet codons.[37,39] Importantly, nearly all the literature dealing with quadruplet codons also focuses on C-terminal purification tags, which eliminate the ability to detect any unwanted products resulting from triplet codon reading.

Since the mS12 ribosomes demonstrate an enhanced ability to resist near-cognate readthrough, we were curious to see to what extent they could improve quadruplet codon reading by quadruplet tRNAs. As a proof of concept, we used the quadruplet tRNA qtRNA$^{Gly}_{CCCC}$, as it can be easily aminoacylated by GlyRS[41,42] and is known to be one of the most efficient qtRNAs[43] . mRNA 7 was designed such that the full-length peptide containing the FLAG tag is translated only when a qtRNA reads the quad codon as GGGG (Figure 5a). However, the template also contains a N-terminal His6 tag to track the yields and products of triplet codon readthrough either by near-cognate tRNAs or by qtRNA$^{Gly}_{CCCC}$ itself. We designed our template with a stop codon in the triplet reading frame three codons downstream from the triplet codon (Figure 5a) so we could easily analyze these unwanted products. Each reaction was supplemented with pre-charged 10 mM Gly-qtRNA$^{Gly}_{CCCC}$, and GlyRS was omitted from the reaction to prevent competition with cognate triplet tRNA^{Gly}. Translation reactions were carried out and split into two fractions. One was purified by the N-terminal His6 tag (green), and other with the C-terminal FLAG tag (magenta), (Figure 5). mS12 ribosomes gave significantly higher yields than wt ribosomes in both the N- and C-terminal capture conditions; little to no full-length product was observed

for the WT ribosomes. MALDI-MS analysis of the N-terminally captured products painted an interesting story. For the WT ribosomes, the predominant product produced was result of qtRNA$^{Gly}_{CCCC}$ reading the codon as a triplet (yellow arrow 1b, Figure 5c), with little to no quadruplet reading. For mS12 ribosomes, the major product was a peptide truncated prior to the quadruplet codon (black arrow, Figure 5c), consistent with peptidyl-tRNA dropoff at the quadruplet codon. mS12 ribosomes, however, also gave a significant amount of full-length product from reading the codon as a quadruplet, in addition to some triplet reading. As measured by the relative N- vs. C-terminal capture yields, for mS12 ribosomes 21% of the total yield was due to full length peptide, whereas the full length yield for WT ribosomes was only 5.3%. A similar set of experiments was performed with a mRNA template containing the triplet GGG codon (Supporting Figure S8). Both sets of ribosomes were able to use qtRNA$^{Gly}_{CCCC}$ to efficiently read the triplet codon. Taken together, these results highlight the enhanced ability of the mS12 ribosomes to use the quadruplet reading frame when presented with a quadruplet codon as compared to wt ribosomes.

In the experiments above we have shown that the inherent translational errors common in reconstituted ribosomal translation can be overcome using hyperaccurate mS12 ribosomes (Figure 2). The misincorporation events with WT ribosomes are caused by the pool of non- and near- cognate aminoacyl tRNAs competing with the preferred cognate complexes (Figure 1d and Figure 2c). This results in a mixture of peptides formed during translation and lowered yields (Figure 3). However, our data shows that mS12 ribosomes are able to resist these misincorporations and losses in yield. Using mS12 ribosomes we were able to achieve more efficient and high-fidelity incorporation of a difficult ncAA, αMeCys, at lower concentrations of ncAA-tRNAFigure 4. Quadruplet suppression is also improved with mS12 ribosomes Figure 5.

Extensive characterization has been done of the hyperaccurate mS12 ribosome (rpsL K42T). Compared to WT ribosomes, mS12 are better able to discriminate correct vs. near-cognate tRNA in vivo with their aggressive proofreading ability,[33,44] and our results in the

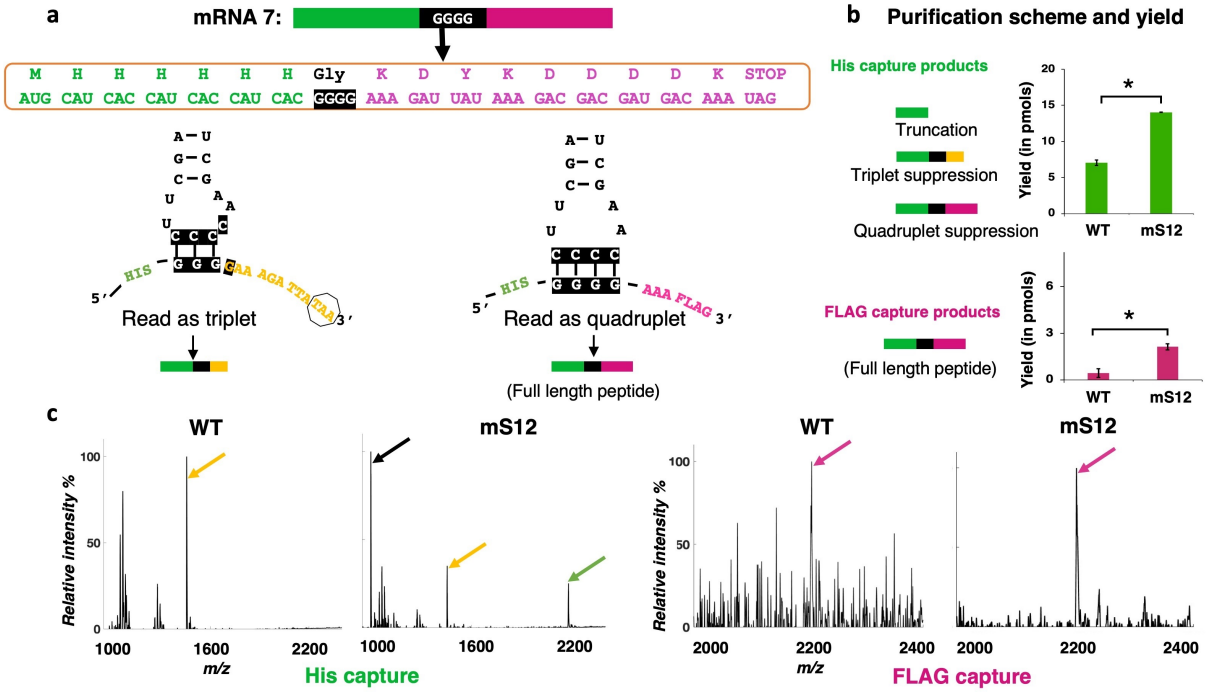

Figure 5: **Design of translation using templates mRNA 7 by ribosomes WT and mS12 for quadruplet codon suppression.** a) The design of mRNA 7 has GGGG codon with N-terminal His6 and C-terminal FLAG tags. When mRNA 7 is translated in presence of qtRNA$^{Gly}_{CCCC}$, it can either be read by the qtRNA as a triplet codon (left scheme) giving truncated peptide due to the stop codon in the triplet frame (circled) that is only captured in His6 purification or as quadruplet codon (right scheme) producing only full length peptides. b) Purification with His6 capture and FLAG capture and their expected products, alongside the yields obtained for each purification as produced by WT and mS12 ribosomes (green and magenta respectively). Translation experiments were performed with all of the components necessary to translate the full-length peptide MHHHHHHGKDYKDDDDK (2179.91, green in His6 capture, magenta in FLAG capture) and truncated peptide MHHHHHHGERL (1455.65, yellow arrow) and included 10mM Gly-qtRNA$^{Gly}_{CCCC}$. Values represent averages and error bars are ± standard deviations, with n= 4 or 6. (*: $p < 0.005$). In (c) MALDI spectra for WT and mS12 for respective captures. Note: Green and magenta arrows represent full length peptides from His6 and FLAG tag respectively. Yellow arrow: shorter peptide MHHHHHHGERL produced if quad codon is read as triplet. Black arrow: truncation seen before quadruplet codon elongation. Tables of the expected vs. calculated masses are given in SI table 8.

PURE translation system are consistent with this. Different studies have shown the effect of ternary complexes on speed and accuracy of decoding for WT ribosomes where non/near-cognate EF-Tu/GTP/AA-tRNA ternary complexes bearing near-cognate AA-tRNAs can compete with cognate complexes and slow the ternary complex GTP hydrolysis step.[45] Near-cognate complexes also can cause a local depletion of correct complex leading to misincorporation events and peptidyl-tRNA dropoff.[15] Our results comparing N- and C-terminal capture highlight that these issues are significant for WT ribosomes in PURE translation reactions, but are dramatically improved with the mS12 ribosomes (Figures 2 and 3). The lower yields for C-terminal vs N-terminal capture for WT ribosomes in the presence of unused AA-tRNAs are likely due to both enhanced peptidyl-tRNA dropoff and misincorporations that prevent correct translation of tags.

ncAAs have been incorporated using quadruplet tRNA and corresponding quadruplet codons, and this technology opens an array of codons expanded for breaking the genetic code.[38] Here we show that mS12 ribosomes can improve quadruplet codon suppression efficiency by qtRNA$_{CCCC}$, with the caveat that mS12 ribosomes do not enforce quadruplet codon reading in the presence of a GGG codon.

Although our current study has been limited to *in vitro* tests of fidelity with short peptides, one of the exciting advantages of mS12 ribosomes is that they can be easily adapted to many types of other experiments, including *in vivo* translation experiments, through MAGE mutagenesis of current strains. This will broaden opportunities to study the effect of error resistant systems in many aspects of synthetic biology, including genetic reprogramming through suppression[46] and sense codon reassignment strategies, or through combining the K42T mutation with designer ribosomes.[47,48] *In vitro*, it is also likely that the higher fidelity provided by mS12 ribosomes will lead to easier production of peptide libraries containing difficult to translate amino acids.

Materials and Methods

General : pORTMAGE-2 was a gift from Csaba Pál (Addgene plasmid # 72677).[35] PURE system proteins and ribosomes were prepared as described.[49] MALDI was performed to find the m/z value using a-cyano-4 hydroxy-cinnamic acid matrix solution for analysis of sample peptides using Voyager DE-Pro instrument with external standard calibration. RNA concentration was determined by using UV-Vis spectrophotometer to find the A260 value, which was input into a web tool (http://www.idtdna.com/calc/analyzer) along with the sample sequence to determine the total concentration of the sample. Amino acids were purchased from Fluka. Statistical significance was tested using unpaired t-test calculated from GraphPad (https://www.graphpad.com/quickcalcs/ttest2/).

Strains and MAGE Cycling Protocol. To do MAGE cycling A19 electrocompetent cells were transformed with pORTMAGE2. pORTMAGE-2 was a gift from Csaba Pál (Addgene plasmid # 72677).[35] Transformants were selected on LB^L agar + Ampicillin plates. MAGE cycling was initiated by inoculating transformed colony in 2mL LB^L media with individual colonies from a freshly streaked overnight plate. It was grown in glass tubes stationed in a rotator drum at 250 rpm at 30 °C. From stationary phase cells were diluted 100x and supplemented with more media and Amp (with a final volume of 20 mL), in a 100 mL glass flask. The incubator was set at 250 rpm and 30 °C. When the cultures reached an OD600 of 0.6–0.7, the tubes were moved to a 42 °C shaking water bath for 15 minutes to induce the expression of λ-Red proteins. Cells were then immediately chilled on ice for at least 5 min and subsequent made electrocompetent in 1 mL aliquots by repeated (at least twice) pelleting and re-suspension in 160 uL cold-sterile dH2O and kept cold until electroporation. Cells were electroporated with 1uL of 100 mM MAGE oligo in BioRad MicroPulser using a 1-mm gap cuvette (2.5 kV, 200 Ω, 25 μF). The electroporated cells were immediately added to 1 mL of warm TB-min media and recovered for 1 h, then 5 mL of RT TB media + Amp was added and grown until OD600 reached 0.7 at 250 rpm and 30 °C. Since the mutation leads to StrepR, transformed colonies were selected on a 100 mg/mL Streptomycin agar plate.

IDT sequencing was performed by using specific primers S12IDFWD and S12IDREV. 50 mL of culture was plated on a 100 mg/mL Streptomycin agar plate. Genomic sequencing was performed by PCR amplifying around rpsL gene mutation using specific primers S12IDFWD and S12IDREV (Table S1). The PCR product was purified using Qiaquick PCR purification Kit (#Cat:28104) and for sequencing it was sent to Eurofins for tube sequencing.

Preparation of A19 cells with the mS12 ribosomal mutation. Chemically competent A19 cells were transformed with pORTMAGE2 and were selected on LB agar + Ampicillin plates according to the described protocol.[35] MAGE cycling was initiated by inoculating transformed colony in 2mL LB^L media growing overnight at 30 °C to stationary phase. This culture was diluted 100X into 20 mL LB in a 100 mL flask shaking at 250 rpm and 30 °C. When the cultures reached an OD600 of 0.6–0.7, the tubes were moved to a 42 °C shaking water bath for 15 minutes to induce the expression of λ-Red proteins. Cells were then immediately chilled on ice for at least 5 min and subsequently made electrocompetent in 1 mL aliquots by repeated (at least twice) pelleting and re-suspension in 160 μL cold-sterile dH2O keeping on ice. Cells were electroporated with 1 mL of 100 μM MAGE oligo (Sequence in Supporting Table S1) in BioRad MicroPulser using a 1-mm gap cuvette (2.5 kV, 200 Ω, 25 μF). The electroporated cells were immediately added to 1 mL of warm TB-min media and recovered for 1 hours, then 5 mL of RT TB media + Amp was supplemented and grown until OD600 reached 0.7 at 250 rpm and 30 °C. Transformed cells were selected on a 100 μg/mL Streptomycin agar plate. Validation of the mutation was done by PCR amplification of the genomic DNA using primers: S12IDFWD and S12IDREV (sequences are in SI table S1).

Purification of Ribosomes. Purification of ribosomes was done according to the published protocols[49] for WT ribosomes using A19 cells, and for mS12 ribosomes using A19 cells transformed with mutated rpsL gene K42T as described above. The concentration of ribosomes was calculated using UV absorbance. Aliquots were stored in -80°C until used.

mRNA preparation. Universal T7 forward, and T7 reverse primers (1 μM each) were

mixed with pET12b plasmids containing the corresponding genes[19,20] (0.01μM), Q5 reaction buffer (1X), dNTPs (0.2mM), Q5 High-Fidelity DNA Polymerase (20 U/mL) and dH_2O to complete 500μL reaction and allowed to PCR amplify according to the recommended temperatures and time for 15 cycles. Then DNA was solvent extracted (unbuffered phenol) and ethanol precipitated. The DNA pellet was dissolved in 100 μL of water and mixed with a transcription mix composed by: Tris/Triton (40mM pH=7.8), Spermidine (2.5mM), $MgCl_2$ (25mM), Dithiothreitol (10mM), UTP/GTP/CTP/ATP (5mM each), extra GTP (4mM), RiboSafe RNase Inhibitor (0.2U/μL Bioline Cat#: C755H60), T7 Polymerase (0.2μM) and inorganic pyrophosphatase (1μg/mL). Transcription was incubated overnight and further purified through urea denaturing gel electrophoresis; the gel was crushed and soaked in 0.3 M KCl overnight and supernatant was filtered, and ethanol precipitated. The pellet was dissolved in water. Then mRNAs were quantified by UV absorbance and were diluted to 30 μM and stored at -20 ^{o}C until used. The RNA sequences and the expected $[M+H]^+$for each encoded peptide its sequence are shown in SI Table S2.

Charging of α-Methyl Cysteine onto $tRNA^{Val}_{GUA}$ using Val T222P. The procedure for each charging assay was conducted as previously described. Briefly, 20 mM of α-MeCys (pH adjusted to 7.4 with 3 M KOH) was incubated with 5 μM ValRS T222P for 120 min in charging assay buffer (30 mM HEPES-KOH pH 7.4, 15 mM MgCl2, 25 mM KCl, 2 mM 2-mercaptoethanol (BME), 6 mM ATP, 0.09 mg/mL bovine serum albumin (BSA, previously dialyzed into ddH_20), 0.02 units/μL inorganic pyrophosphatase (PPiase), 25 μM tRNAVal, ddH20 to 50 μL). Reactions were quenched by adding 0.1 volume 3M NaOAc, pH 5.2) followed by 25:24:1 phenol:chloroform:isoamyl alcohol pH 4.3 extraction followed by ethanol precipitation. The tRNA pellet was allowed to air dry and dissolved in 20 μL 1 mM KOAc (pH 5.5). Finally, the concentration of tRNA was measured using UV-Vis spectrophotometer and stored at -80^{o}C until used.

In-vitro translation experiments. Experiments were carried in either min aaRS conditions or in presence of all 20 aaRS. Translation started with the mix of the

following components: dH_2O, TS-Solution (HEPES-KOH pH=7.6 (50 mM), potassium acetate (100 mM), Magnesium acetate (6mM), Spermidine (10mM), Dithiothreitol (1mM), Creatine Phosphate (20mM), 10-Formyltetrahydrofolate (100μM), ATP/GTP (1.5mM each, potassium exchanged), total *E. coli* tRNA (100mg/mL deacylated and dialyzed overnight against 50mM Tris/HCl pH=9)), Factor Mix (Ef-G (0.52 μM), IF-1 (2.7 μM), IF-2 (0.4 μM), IF-3 (1.5 μM), MTF (0.6 μM), Rf-1 (0.3 μM), Rf-3 (0.17 μM), RRF (0.5 μM)), Ef-Tu (10 μM), Ef-Ts (4.1 μM), Ribosomes (1.2 μM), amino acids in the template but methionine (0.1 mM each), aminoacyl-tRNA synthetases (0.1-1 μM), inorganic pyrophosphatase (1μg/mL), Creatine Kinase (4 μg/mL), Myokinase from rabbit (3 μg/mL) (Sigma-Aldrich Cat#: M3003-2.5KU), Nucleoside 5′-Diphosphate Kinase (1.1 μg/mL) (Sigma-Aldrich Cat#: N2635-100UN) unless otherwise mentioned. To the resulting mix, methionine (10 μM) and ^{35}S-methionine (optional) (0.1μM) were added simultaneously, and translation was initiated by addition of desired mRNA templates (1μM). For negative controls the template was left out and supplemented with equal volume of DI water. A final volume of 30 μL was used for a single reaction unless otherwise mentioned. The reaction mixtures were incubated for 1 hour at 37°C and quenched with a 3X volume wash buffer (50mM Tris-HCl, 300mM NaCl).

Affinity Purification of translation reactions using magnetic beads. The translation reactions were carried out on 96 well-PCR plate (Genessee scientific Cat #: 24-300). To the quenched translation reaction, 10 μL of Anti-FLAG® M2 Magnetic Beads (Millipore Sigma Cat#: M8823-5mL) or 40 μL Ni-NTA magnetic beads (NEB S1423L) were added and allowed to bind for 1 h at rt in a tumbler. A homemade 3-D printed 96 Well Magnetic Bead Separator Plate was used to collect the beads and the supernatant was removed using multichannel pipette. The beads were washed 3 x 200 μL to get rid of any residual nonbinders. Elution was done by incubating the beads 15 min with 50 μL of 1% aq trifluoroacetic acid at rt and subsequently separating the beads again as described. The solution was collected and analyzed by either MALDI or scintillation counting. For MALDI analysis, the peptide was desalted by using StageTips prepared in house[50] prior to spotting

on the MALDI plate.

Unused AA-tRNA supplemented translation assay. *E. coli* tRNA was precharged using the AA/AARS that were not needed to translate the given template. Here, Ala/AlaRS, Cys/CysRS, Glu/GluRS, Phe/PheRS, Gly/GlyRS, His/HisRS, Leu/LeuRS, Pro/ProRS, Gln/GlnRS, Ser/SerRS, Val/ValRS, Trp/TrpRS were used in the charging assay procedure as described above. 1 mM of each amino acid and 0.4-1 μM of individual AARS were used. Precharging was carried out for 1 hr, an the tRNA was extracted from the mixture with phenol/chloroform extraction followed by ethanol precipitation. The total tRNA concentration was determined using the UV-vis spectrophotometry using expected average molecular weight of *E. coli* total tRNA of 25330 Da. This tRNA mixture was added to the *in vitro* translation reactions at the specified concentrations.

α-Methyl cysteine incorporation and translation assay. Translation was conducted as described above except pre-charged αMeCys-tRNA$^{Val}{}_{GUA}$ was added to the translation mixture at the specified concentrations and Val/ValRS was omitted.

qtRNA supplemented translation assay. qtRNA$^{Gly}{}_{CCCC}$ or tRNA$^{Gly}{}_{CCC}$ were precharged with Gly/GlyRS using the general protocol as described above for the charging of αMeCys, except Gly was used at 1 mM final concentration. Gly-qtRNA$^{Gly}{}_{CCCC}$ and Gly-tRNA$^{Gly}{}_{CCC}$concentrations were determined using a UV-vis spectrophotometer and the calculated extinction coefficients. Glycine and GlyRS were excluded in the translation reaction. The translation itself was carried out a described above, except the volume was doubled to 60 μL, which was split into two equal portions after 1 hr of reaction incubation time. This was followed by parallel purification of each portion using either 40 μL Ni-NTA agarose or 10 μL Anti-FLAG® M2 agarose beads (Sigma-Aldrich Cat#: A2220-1ML). The beads were added to the quenched translation mixture and binding was allowed to proceed for 1 h at rt in a tumbler. The beads were washed with 50mM Tris-HCl, 300mM NaCl (2 x 400 μL) in a filter tube. 50 μL of 1% TFA was added to the beads and this solution was allowed to sit for 15 min at rt before centrifugation. The flow through was collected and

analyzed by either MALDI or scintillation counting. For MALDI analysis, the peptide was desalted by using StageTips prepared in house[50] .

Conflict of Interest

The authors have submitted a provisional patent application covering the use of mS12 ribosomes in *in vitro* translation.

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Author Contributions

B. Shakya: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **O. G. Joyner:** Resources, Investigation. **M. C. T. Hartman:** Conceptualization, Methodology, Writing - Review & Editing, Visualization, Supervision, Project Administration, Funding acquisition.

Supporting Information.

The supporting information is available free of charge at

- 8 figures highlighting supplemental *in vitro* translation yields and fidelity and the validation of the mS12 mutation

- 10 tables showing primers and expected and observed MALDI-MS values from *in vitro* translation experiments.

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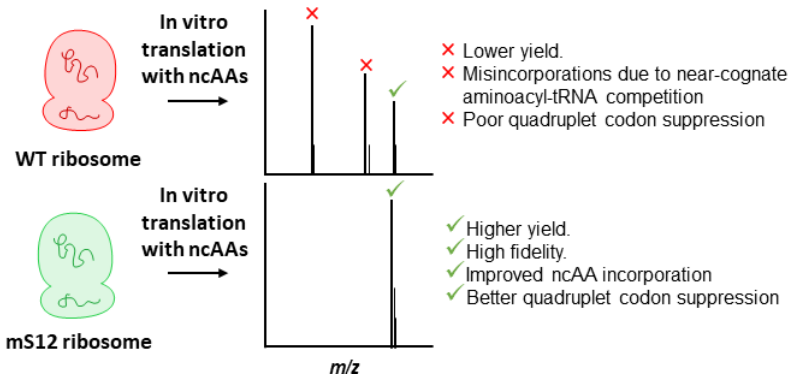

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