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Efficient Multisite Unnatural Amino Acid Incorporation in Mammalian Cells via Optimized Pyrrolysyl tRNA Synthetase/tRNA Expression and Engineered eRF1

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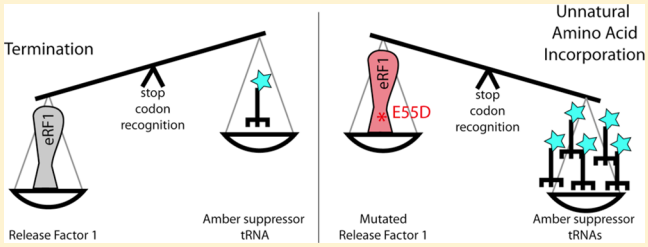
Supporting Information

ABSTRACT: The efficient, site-specific introduction of unnatural amino acids into proteins in mammalian cells is an outstanding challenge in realizing the potential of genetic code expansion approaches. Addressing this challenge will allow the synthesis of modified recombinant proteins and augment emerging strategies that introduce new chemical functionalities into proteins to control and image their function with high spatial and temporal precision in cells. The efficiency of unnatural amino acid incorporation in response to the amber stop codon (UAG) in mammalian cells is commonly considered to be low. Here we demonstrate that tRNA levels can be limiting for unnatural amino acid incorporation efficiency, and we develop an optimized pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ expression system, with optimized tRNA expression for mammalian cells. In addition, we engineer eRF1, that normally terminates translation on all three stop codons, to provide a substantial increase in unnatural amino acid incorporation in response to the UAG codon without increasing readthrough of other stop codons. By combining the optimized pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ expression system and an engineered eRF1, we increase the yield of protein bearing unnatural amino acids at a single site 17- to 20-fold. Using the optimized system, we produce proteins containing unnatural amino acids with comparable yields to a protein produced from a gene that does not contain a UAG stop codon. Moreover, the optimized system increases the yield of protein, incorporating an unnatural amino acid at three sites, from unmeasurably low levels up to 43% of a no amber stop control. Our approach may enable the efficient production of site-specifically modified therapeutic proteins, and the quantitative replacement of targeted cellular proteins with versions bearing unnatural amino acids that allow imaging or synthetic regulation of protein function.



INTRODUCTION

Genetic code expansion has allowed the site-specific incorporation of more than 100 unnatural amino acids into proteins. However, the utility of these approaches may be limited by the efficiency with which unnatural amino acids are incorporated into proteins. The efficient, co-translational, site-specific incorporation of unnatural amino acids into proteins will enable emerging approaches for creating site-specifically modified recombinant proteins,[1,2] as well as strategies to precisely control and image protein function *in vivo*,[3,4] and many other approaches in which designer unnatural amino acids are used to control or report on protein function.

Orthogonal aminoacyl-tRNA synthetase/tRNA pairs direct the incorporation of unnatural amino acids, most commonly in response to the amber stop codon (UAG). The efficiency of unnatural amino acid incorporation is defined both by (i) the intrinsic efficiency with which the orthogonal synthetase/tRNA pair enables aminoacylation and translational elongation in response to a UAG codon in the A site of the ribosome, and (ii) the efficiency with which release factors compete with the aminoacylated orthogonal $tRNA_{CUA}$ to terminate protein synthesis.

The pyrrolysyl-tRNA synthetase(PylRS)/$tRNA_{CUA}$ pair is arguably the most useful pair to be developed for genetic code expansion because (i) it is orthogonal in a range of hosts including *Escherichia coli*, yeast, mammalian cells, *Caenorhabditis elegans*, and *Drosophila melanogaster*; (ii) PylRS does not recognize the common 20 amino acids; (iii) PylRS does not recognize the anticodon of its cognate $tRNA_{CUA}$; (iv) the active site of PylRS accommodates a range of unnatural amino acids bearing useful functional groups without the need for directed evolution; (v) the active site of PylRS can be evolved to recognize structurally diverse unnatural amino acids bearing a range of useful functional groups in *E. coli*; and (vi) the synthetase variants discovered in *E. coli* may be used in diverse eukaryotic hosts, where directed evolution of synthetases is challenging to implement.[5]

While unnatural amino acid incorporation is currently less efficient in mammalian cells than in *E. coli*, a systematic study of the effects of synthetase and tRNA promoter and copy number on unnatural amino acid incorporation efficiency has not been performed. Moreover, a direct comparison of the efficiency of

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natural translation to the efficiency of unnatural amino acid incorporation into proteins in mammalian cells has not been performed.

The incorporation of unnatural amino acids by the PylRS/$tRNA_{CUA}$ pair in response to the amber stop codon may be limited by competition with the eukaryotic release factor 1 (eRF1)/eukaryotic release factor 3 (eRF3) complex that mediates translational termination in mammalian cells.[6−8] While in prokaryotes, the two eRF1 orthologs RF1 and RF2 selectively terminate protein synthesis at UAG/UAA and UGA/UAA codons, respectively,[9,10] eRF1 recognizes all three stop codons.[11] Thus, strategies developed in *E. coli* to enhance unnatural amino acid incorporation in response to the amber codon through selective disruption of RF1 function[12−16] cannot be extended to the mammalian system. Indeed, there are no reports of engineering the eukaryotic translational machinery to enhance the efficiency with which unnatural amino acids are site-specifically incorporated into proteins in mammalian cells using orthogonal synthetase/$tRNA_{CUA}$ pairs.

Here we define the efficiency of unnatural amino acid incorporation in mammalian cells relative to a natural translation control, allowing us to quantitatively benchmark improvements in unnatural amino acid incorporation efficiency. We demonstrate that expression of PylT (encoding Pyl $tRNA_{CUA}$) is limiting for unnatural amino acid incorporation in mammalian cells, and we optimize the PylRS/$tRNA_{CUA}$ expression system by increasing the levels of Pyl $tRNA_{CUA}$ expressed in mammalian cells. We engineer eRF1 to enhance unnatural amino acid incorporation in response to the amber stop codon without increasing readthrough of opal and ochre stops. We demonstrate that the optimized system, which combines the best PylRS/$tRNA_{CUA}$ expression system and an engineered eRF1, provides a 17- to 20-fold improvement in the yield of protein containing an unnatural amino acid incorporated in response to the amber stop codon, with optimized yields of protein approaching those for a no stop codon control. Moreover, the optimized system increases the yield of proteins incorporating unnatural amino acids at three sites from unmeasurably low levels to up to 43% of a no stop codon control.

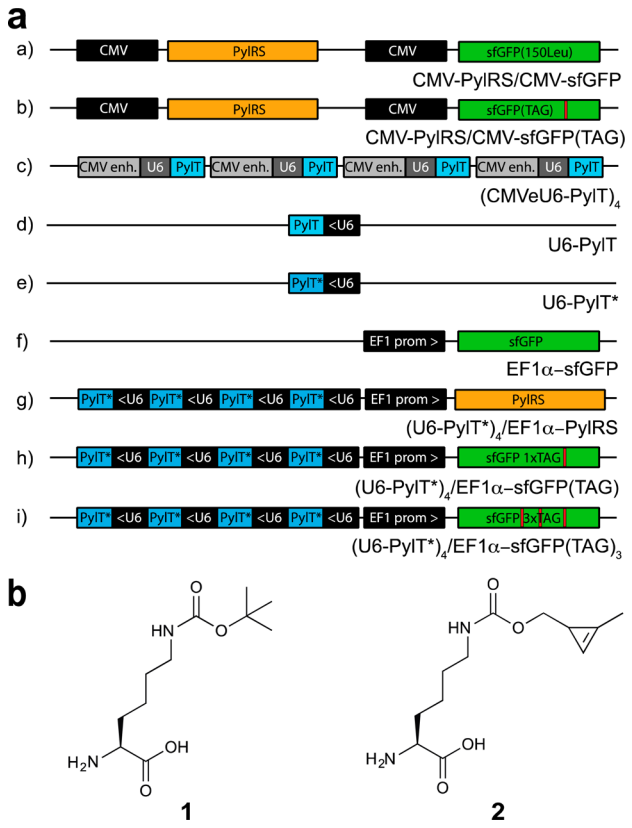
RESULTS

Increasing tRNA Levels Increases Unnatural Amino Acid Incorporation Efficiency. We optimized the expression levels of $tRNA_{CUA}$ to increase the efficiency of unnatural amino acid incorporation in mammalian cells. Investigators have used different PylRS and tRNA plasmids that vary the copy number of PylRS, $tRNA_{CUA}$ and the choice of promoters.[17−20] However, there are no reports that quantify the yields of proteins bearing unnatural amino acids incorporated with the PylRS/$tRNA_{CUA}$ pair in mammalian cells, nor are there reports that quantify the efficiencies of unnatural amino acid incorporation relative to the expression of a control protein expressed from a gene that does not contain an amber stop codon. These experiments are crucial for understanding how well unnatural amino acid incorporation in mammalian cells compares to natural protein synthesis.

We first tested the efficiency of unnatural amino acid incorporation using plasmids *b* and *c* bearing a single copy of PylRS on a CMV promoter and four copies of $tRNA_{CUA}$ each driven by a U6 promoter with a CMV enhancer[17,19] (construct schematics are shown in Chart 1a). This system directed the incorporation of **1** (N^{ϵ}-[(*tert*-butoxy)carbonyl]-L-lysine), or **2** (N^{ϵ}-[((2-methylcycloprop-2-en-1-yl)methoxy)carbonyl]-L-lysine) (Chart 1b), both known and efficient substrates for the PylRS/$tRNA_{CUA}$ pair[21,22]) in response to an amber codon at position 150 in sfGFP[23] (CMV-sfGFP(TAG)) with efficiencies of 5% and 7% (Figure 1a,b); all incorporation efficiencies are reported as a percentage of sfGFP levels produced from an otherwise identical control construct bearing a leucine codon in place of the amber codon at position 150 (plasmid *a*, Chart 1). Next we replaced the four copies of $tRNA_{CUA}$ with a single copy of $tRNA_{CUA}$ on an optimized U6 promoter, leading to a small decrease in unnatural amino acid incorporation efficiency (plasmids *b* and *d*).

Unlike the original four-copy cassette (*c*), the new U6 $tRNA_{CUA}$ cassette (*d*) does not contain the CMV enhancer, and produces a precise 5′ end for the tRNA that does not require nuclease processing. Northern blots (Figure 1c) demonstrate that the levels of Pyl $tRNA_{CUA}$ produced from *d* are comparable to the levels produced from *c*. This indicates that the altered tRNA expression construct provides more copies of the tRNA per copy of the tRNA gene. Replacing $tRNA_{CUA}$ with a U25C variant[24] increased the incorporation efficiency slightly from 2.7−3.5% to 4.7−5.1% (plasmids *b* and *e*, Chart 1) and increased the $tRNA_{CUA}$ level.

Chart 1. Plasmid Constructs and Unnatural Amino Acids Used



(a) Schematics of vectors used. PylT is the gene encoding Pyl $tRNA_{CUA}$ and PylT* encodes the U25C variant. U6 indicates the U6 promoter, CMV is the CMV promoter, CMV enh is the 5′ enhancer fragment of CMV promoter, EF1 prom is the EF1*α* promoter. Red bars indicate location of amber stop codons. (b) Chemical structure of **1** (N^{ϵ}-[(*tert*-butoxy)carbonyl]-L-lysine) and **2** (N^{ϵ}-[((2-methylcycloprop-2-en-1-yl)methoxy)carbonyl]-L-lysine).

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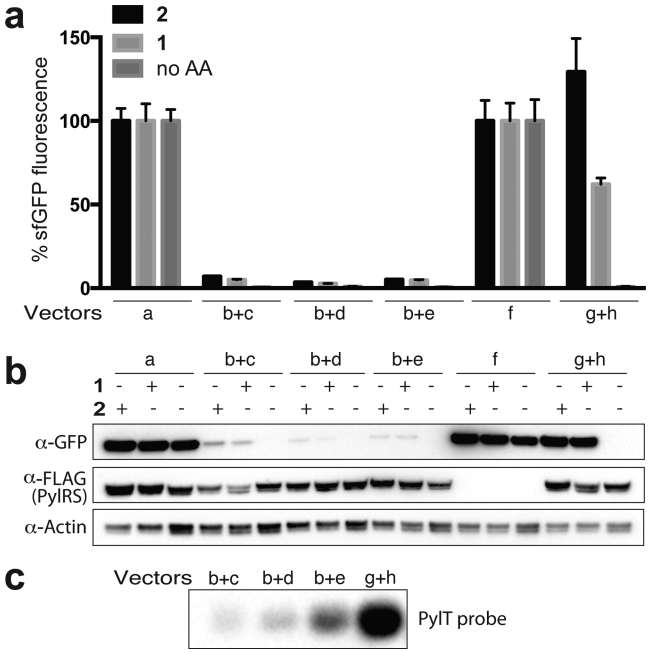

Figure 1. Optimizing PylRS/tRNA$_{CUA}$ expression vectors for the incorporation of unnatural amino acids in response to the TAG codon in mammalian cells. (a) Quantification of incorporation of **1** (2 mM) and **2** (0.5 mM) into superfolder green fluorescent protein (sfGFP) measured in a fluorescence assay. The indicated constructs were expressed transiently in HEK293T cells and sfGFP quantified in lysates by fluorescence at 520 nm, following excitation at 485 nm. A no amino acid control is included for each vector combination. The data are plotted as a percentage of the fluorescence exhibited by an equivalent sfGFP control plasmid with a leucine codon in place of a stop codon (construct *a* in Chart 1a). Data represent the mean ± SE of triplicates). (b) sfGFP yields in lysate visualized by Western blot. Equal amounts of cell lysate from cells transfected with the indicated vectors and grown in the presence of the indicated amino acid, or no amino acid, were immunoblotted with α-GFP, α-actin and α-FLAG antibodies. (c) Northern blot analysis of relative PylT/PylT* expression from constructs *b* + *c*, *b* + *d*, *b* + *e*, *g* + *h*, in the absence of amino acid. See Supporting Information Figure 1 for loading control.

Creating tandem arrays, each containing four copies of U6 Pyl tRNA$_{CUA}$ (bearing U25C) and switching the promoter for the protein coding genes from CMV to EF1α (plasmids *g* and *h*, Chart 1) led to a substantial increase in sfGFP bearing **1** or **2**. In this system, amino acid **1** was incorporated in response to the amber codon in sfGFP with an efficiency of 62%, while **2** was incorporated with an efficiency of approximately 129%. Western blots demonstrate that changing the promoter of the protein coding genes to EF1α does not change the levels of PylRS (anti-FLAG *b* + *e* vs *g* + *h*, Figure 1b) or wt sfGFP (*a* vs *f* Figure 1b), demonstrating that neither PylRS levels nor maximal levels of sfGFP expression are substantially altered by changing to the EF1 α promoter. However, northern blots demonstrate that tRNA levels are much higher in this system than in all other systems tested, indicating that the large increases in unnatural amino acid incorporation efficiency we observe are correlated with an increase in tRNA level.

Ectopic Expression of Selected eRF1 Variants Does Not Increase Readthrough of Stop Codons. Next, we asked if we could further enhance unnatural amino acid incorporation efficiency, without increasing readthrough of other stop codons, by engineering eRF1. While the efficiency of unnatural amino acid incorporation was much improved with the optimized synthetase and tRNA system, we envisioned that eRF1 engineering might further improve this efficiency and allow us to efficiently incorporate unnatural amino acids at multiple sites in a protein.

We first identified amino acid positions in eRF1 that are reported to have an effect on termination at amber codons from genetic or biochemical studies.[25−30] These mutations are in the N-terminal domain of eRF1 (Figure 2a) that interacts with the stop codon on the mRNA within the ribosome. To assess the effect of the eRF1 mutants on translational termination in mammalian cells, we quantified suppressor tRNA independent readthrough at the amber, opal and ochre stop codon in HEK 293T cells and in HEK 293T cells bearing added, overexpressed human eRF1 and eRF1 mutants (Figure 2b). eRF1 forms a complex with eRF3, primarily mediated through the C-terminal domain on eRF1[37] that mediates translational termination. eRF3 is present in cells at levels comparable to endogenous eRF1, and therefore, eRF3 limits the number of termination complexes that may form.[35] Overexpression of eRF1 N-terminal domain mutants may bias (by mass action) these complexes toward containing the eRF1 mutants, thereby revealing the phenotype of the eRF1 mutations.

We introduced each eRF1 variant into cells (Figure 2b), and measured basal readthrough of stop codons, using three dual luciferase reporters (Figure 2c). Each reporter contained an N-terminal *Renilla* luciferase followed by a stop codon (amber, opal or ochre) and a C-terminal firefly luciferase.[31−34] The readthrough of the stop codons was between 0.08 and 0.12% (TAG 0.09%, TGA 0.12%, TAA 0.08%), providing a benchmark for further experiments. Ectopic overexpression of eRF1 led to a decrease in readthrough of all three stop codons (TAG 0.03%, TGA 0.07%, TAA 0.04%), consistent with the increased level of eRF1 in cells.[35] This decrease in readthrough is small, consistent with the levels of eRF3 being comparable to the levels of endogenous eRF1, and eRF3 levels limiting the number of functional termination complexes that can be formed.[35]

Introduction of eRF1 variants increased stop codon readthrough with respect to the introduction of wild-type eRF1. However, for all eRF1 mutants tested, except two, readthrough of all three stop codons was not increased above the levels found in the absence of ectopically expressed eRF1. We conclude that ectopic expression of most of the eRF1 variants tested does not increase readthrough of stop codons above basal levels.

The two eRF1 mutants, which increase readthrough of stop codons above levels normally found in cells, are eRF1 Δ100, a mutant that increases readthrough to 1.6% (TAA), 2% (TAG) and 15% (TGA), and the T122Q, S123F mutant[29] that selectively increases readthrough at TGA codons 2-fold. Reduction of endogenous eRF1 levels by shRNA increased basal readthrough for all three stop codons 2- to 3-fold.

The effect of the eRF1 Δ100 mutant on readthrough of all stop codons is expected, as the N-terminal domain, from which the residues are deleted, mediates recognition of all three stop codons in mRNA, but does not mediate interactions with eRF3.[36−39] The mutant is therefore predicted to form inactive complexes with eRF3, decreasing the number of functional eRF1/eRF3 complexes that can mediate termination. Similarly, the effects of shRNA against eRF1 on all stop codons are expected[40] since a decrease in eRF1 should lead to a decrease in termination on all stop codons.

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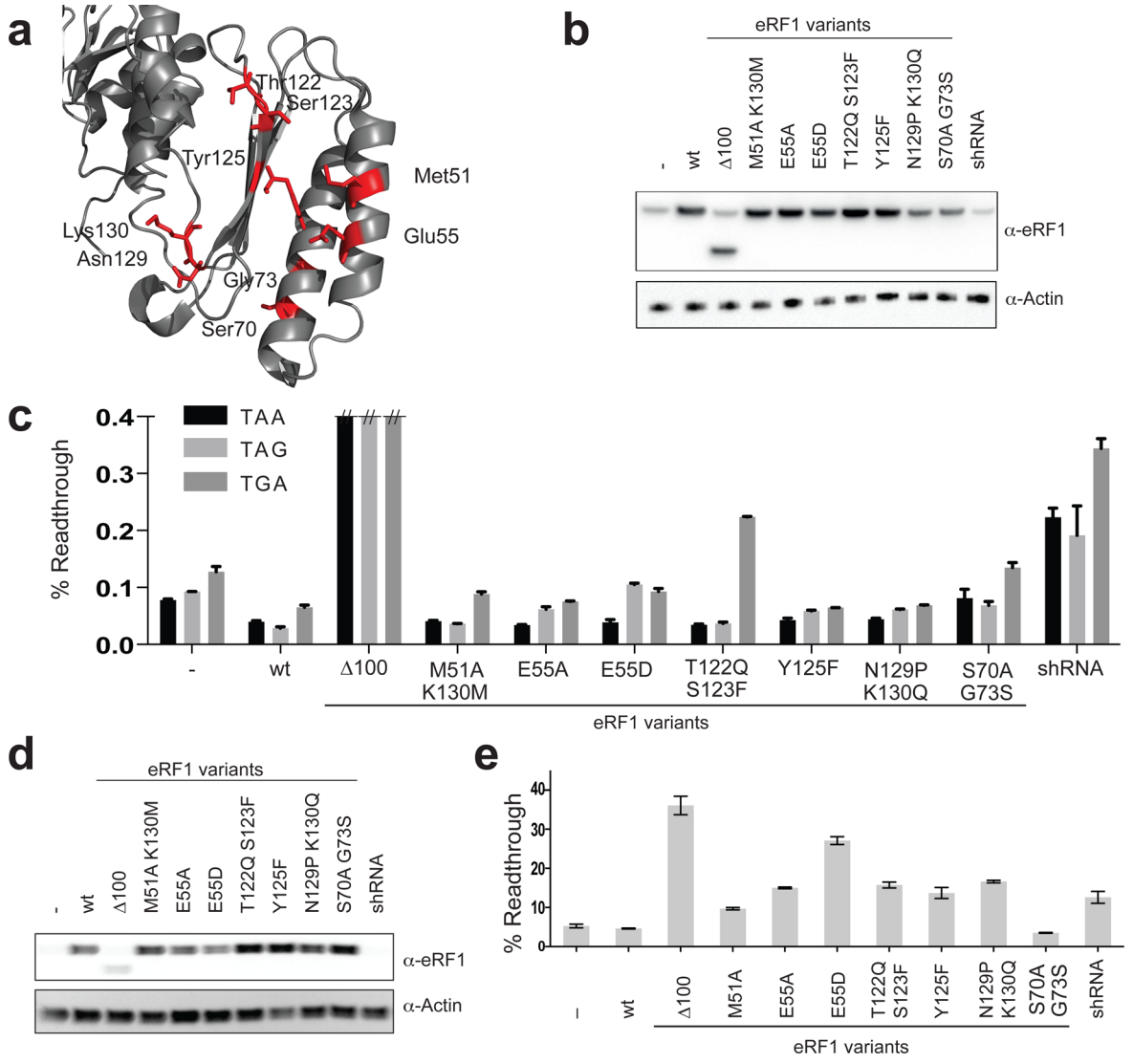

Figure 2. Effect of mutations in eRF1 on stop codon readthrough, and incorporation of **1** (2 mM) using the PylRS/tRNA$_{CUA}$ pair. (a) eRF1 positions mutated in this study. Structure of the N-terminal domain from eRF1 (PDB ID:3E1Y);[29] the residues mutated in this study are in red. (b) Human eRF1 variants are expressed following transient transfection of HEK 293T cells with peRF1 (*X*), where *X* designates the mutations introduced, and CMV-PylRS/CMV-DLR(TAG). The negative control (−) detects endogenous eRF1; shRNA is a knockdown of endogenous eRF1. (c) Readthrough of all three stop codons is determined by the expression of a *Renilla*-TAG-firefly luciferase reporter and eRF1 variants in HEK293T cells in the absence of a suppressor tRNA. CMV-PylRS/CMV-DLR(TAG) (or the corresponding TAA, TGA or serine codon variant) was transiently transfected into cells, expression levels determined after 20 h. TAG, TAA, or TGA readthrough was normalized against data from the serine codon (TCC) containing construct. Data represents the mean ± SE of quadruplet measurements. The negative control (−) detects endogenous eRF1, shRNA is a knockdown of endogenous eRF1. Wt is human eRF1 recoded with *D. melanogaster* codon useage. Data for the Δ100 mutant are off scale; the values are 1.6% (TAA), 2% (TAG) and 15% (TGA). (d) Transient transfection of HEK 293T cells with peRF1 (*X*), where *X* designates the mutations introduced, plasmid *c* expressing PylT from a U6 promoter (Chart 1a) and CMV-PylRS/CMV-*Renilla*-TAG-firefly, a version of plasmid *a* (Chart 1a) in which sfGFP is replaced by *Renilla*-TAG-firefly. The negative control (−) detects endogenous eRF1; shRNA is a knockdown of endogenous eRF1. Equal amounts of cell lysate were immunoblotted with *α*-eRF1 and *α*-actin antibodies. (e) eRF1 (*X*) variants increase unnatural amino acid incorporation in response to an amber stop codon using the pyrolysyl tRNA/synthetase pair. HEK293T cells were transfected as described for panel d, and grown in the presence of 1 mM amino acid **1**, and measurements made after 20 h. Percent readthrough was measured relative to a *Renilla*-TCC-firefly reporter bearing a serine codon in place of the amber stop codon.

eRF1 Mutants Increase Unnatural Amino Acid Incorporation Efficiency. To investigate the effects of eRF1, eRF1 mutants and shRNA on unnatural amino acid incorporation, we transfected cells with the relevant eRF1 mutant (Figure 2d). Each sample was also provided with the dual luciferase reporter of amber suppression, a single copy of the orthogonal pyrrolysyl tRNA- synthetase (PylRS)/tRNA$_{CUA}$ pair (the arrangement shown as *b* + *d* in Chart 1, but with a dual luciferase reporter replacing sfGFP). We used this system to maximize the dynamic range with which we could measure the enhancement provided by eRF1 variants. The amino acid **1** was added to all cells. In one case, an shRNA construct targeting endogenous eRF1[40] was added allowing us to compare the effects of ectopically expressed eRF1 mutants to knocking down endogenous eRF1.

The dual luciferase assay was used to determine the effects of eRF1 on unnatural amino acid incorporation efficiency (Figure 2e). In the absence of ectopically expressed release factor, the

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efficiency of unnatural amino acid incorporation was approximately 5.3% in this assay. The incorporation efficiency was decreased slightly upon ectopic expression of wild-type release factor, and increased to 13% upon shRNA knockdown of endogenous eRF1. The efficiency of incorporation for **1** increased in the presence of all mutant release factors except the S70A, G73S mutant. This mutant was described previously as a bipotent UAR specific eRF1.[28,41]

Two eRF1 mutants led to the most efficient unnatural amino acid incorporation: eRF1 (E55D), 27%; and eRF1 (Δ100), 36%. The incorporation efficiencies with the Δ100 mutant and the E55D mutant are 5- to 7-fold greater than the incorporation efficiency in cells that do not contain ectopically expressed release factor. Interestingly, while strongly enhancing amber readthrough in the presence and absence of the PylRS/$tRNA_{CUA}$ pair, the eRF1Δ100 mutant significantly reduced the total amount of luciferase produced in both situations, consistent with a drastic disruption of termination at all three stop codons having global effects on translation efficiency (Supporting Information Figure 2) In addition, the Δ100 mutant leads to readthrough of all three stop codons in the absence of suppressor tRNAs; therefore, we did not investigate this release factor mutant further. We focused further work on eRF1 (E55D). This release factor mutant was previously identified in an in vitro assay in rabbit reticulocyte extract (based on its ability to efficiently remove formyl-methionine from the initiator tRNA in the P site of the ribosome in response to an ochre or opal codon, but not an amber codon, in the A site).[25]

An Optimized System for Incorporating Multiple Unnatural Amino Acids. Next, we combined the optimized synthetase and tRNA system and the E55D mutant of eRF1 (Figure 3). We find that the addition of eRF1 (E55D) to cells containing the PylRS/$tRNA_{CUA}$ pair, grown in the presence of **1**, increases the incorporation of **1** into sfGFP(TAG) from 62% to 85% (Figure 3a). Similarly, the addition of the eRF1 (E55D) increases the efficiency with which **1** is incorporated into $sfGFP(TAG)_3$, that contains amber stop codons at positions 101, 133 and 150 of GFP, from 5% to 12% (Figure 3a,b). The yield of sfGFP-**1** from sfGFP(TAG) was 0.65 μg from 10^5 cells, while the yield of sfGFP-$(\mathbf{1})_3$ from $sfGFP(TAG)_3$ was 0.1 μg per 10^5 cells (Supporting Information Figure 3; all yields are quoted per number of cells seeded, and were measured 48 h after transfection).

We find that the addition of the eRF1 (E55D) to cells containing the PylRS/$tRNA_{CUA}$ pair, grown in the presence of **2**, increases the incorporation of **2** into sfGFP(TAG) from 129% to 157%, and that the addition of the eRF1 (E55D) quadruples the efficiency of producing of sfGFP-$(\mathbf{2})_3$ from $sfGFP(TAG)_3$ from 11% to 43% (Figure 3c,d). The yield of sfGFP-**2** from sfGFP(TAG) was 1.76 μg per 10^5 cells, while the yield of sfGFP-$(\mathbf{2})_3$ from $sfGFP(TAG)_3$ was 0.49 μg per 10^5 cells (Supporting Information Figure 3).

Full-length sfGFP was purified from cell lysates containing the optimized system (Figure 4a). Electrospray ionization mass spectrometry demonstrated the site-specific incorporation of one and three molecules of **1** and **2** into sfGFP from sfGFP(TAG) and $sfGFP(TAG)_3$ respectively (Figure 4b, Supporting Information Figure 4). This data, in combination with the no amino acid controls in Figures 4a demonstrate the high fidelity incorporation of unnatural amino acids in the presence of eRF1 (E55D).

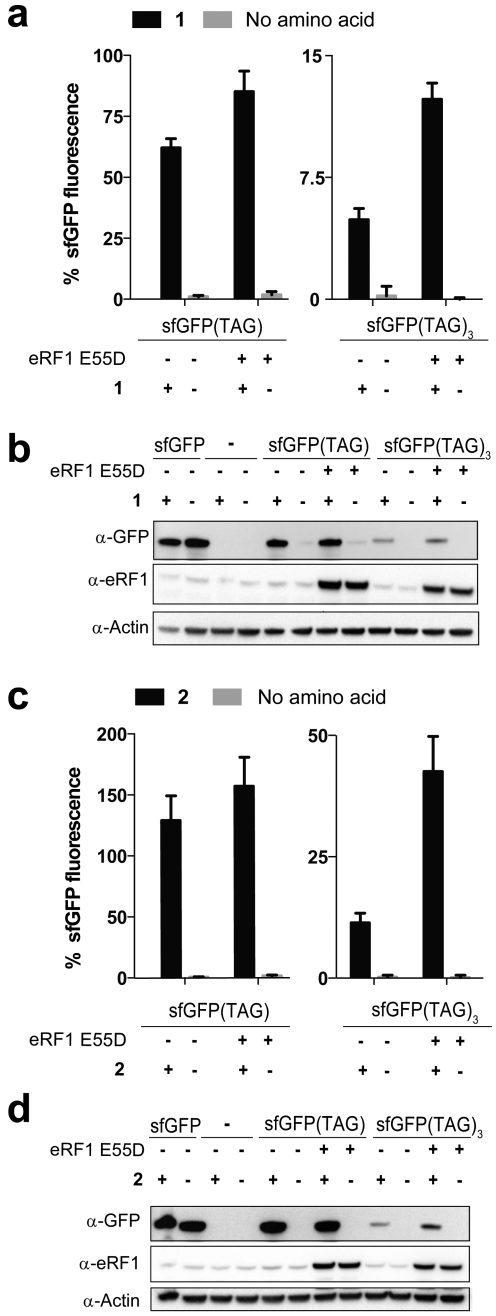

Figure 3. Combining eRF1 E55D with an optimized PylRS/$tRNA_{CUA}$ pair expression system enables efficient incorporation of multiple unnatural amino acids into recombinant proteins in mammalian cells. (a) Plasmids g, h (or i, Chart 1a) and eRF1 E55D were transiently transfected into HEK293T cells, and grown in the presence or absence of 2 mM amino acid **1** for 48 h. Full-length sfGFP was quantified in cell lysate at 520 nm, following excitation at 485 nm. Data represents the mean ± SE of four independent measurements. (b) Western blots from lysates. (c) As in panel a, but using 0.5 mM amino acid **2**. (d) Western blots from lysates.

DISCUSSION

We have defined the efficiency of unnatural amino acid incorporation relative to a natural translation control, allowing us to quantitatively benchmark improvements in unnatural amino acid incorporation efficiency. The optimized system we have created provides a 17- to 20-fold improvement in unnatural amino acid incorporation efficiency with amino

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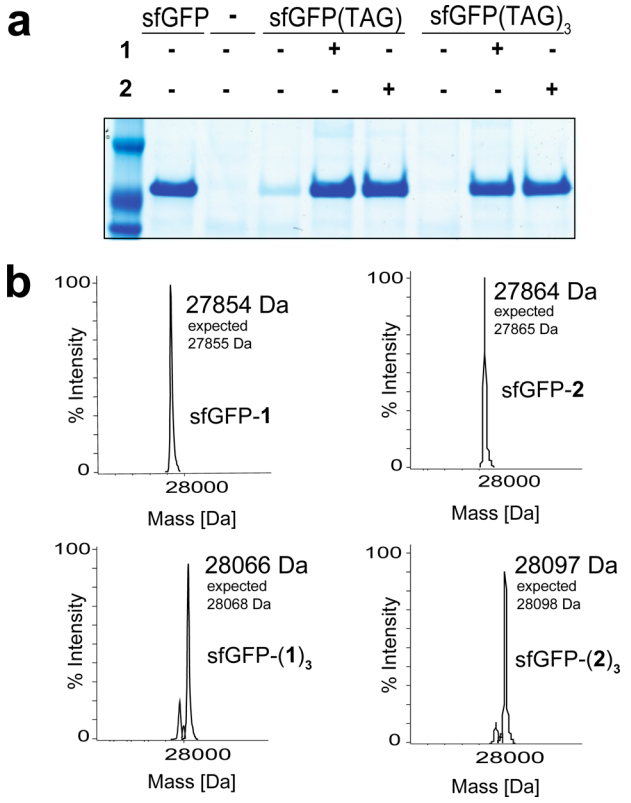

Figure 4. Expression, purification and characterization of recombinant sfGFP incorporating one or three unnatural amino acids (a). Plasmids g, h (or i, Chart 1a) and eRF1 E55D were transiently transfected into HEK293T cells, and grown in the presence or absence of 2 mM amino acid **1** or 0.5 mM amino acid **2** for 48 h. Full-length sfGFP was purified using anti-GFP resin. (b) Electrospray ionization mass spectrometry confirms the quantitative incorporation of unnatural amino acids **1** and **2**, at one or three sites in sfGFP (see also Supporting Information Figure 4).

acids **1** and **2** in sfGFP. For amino acid **1**, the incorporation efficiency is increased from 5% to 85%, while for amino acid **2**, the incorporation efficiency is increased from 7% to 157% of a no stop codon control. Moreover, the optimized system increases the yield of proteins incorporating **1** and **2** at three positions from unmeasurably low levels to 12% and 43% of a no stop control, respectively.

Two factors contribute to the dramatic improvement in unnatural amino acid incorporation: the optimization of $tRNA_{CUA}$ levels to optimize PylRS/$tRNA_{CUA}$ expression; and the development and use of engineered eRF1 variants. While the incorporation of unnatural amino acids is quite efficient in response to a single amber codon using the optimized PylRS/$tRNA_{CUA}$ system alone, the efficiency is further improved by the addition of eRF1 (E55D). The effect of the eRF1 mutant on unnatural amino acid incorporation is more dramatic when incorporating unnatural amino acids at multiple sites, increasing the yield of protein containing amino acid **1** at three sites, 2- to 3-fold and the yield of protein containing **2** at three sites, 4-fold.

Our data demonstrate that unnatural amino acid incorporation in mammalian cells can be very efficient. Moreover, our data provide the first demonstration that, despite eRF1 recognizing all three stop codons, it is possible to engineer eRF1 to selectively enhance the efficiency of unnatural amino acid incorporation in eukaryotic cells in response to the amber stop codon, without increasing readthrough of opal or ochre stop codons. Future work will further investigate the generality of the approaches we report for the efficient incorporation of diverse unnatural amino acids at diverse sites in diverse proteins in eukaryotic cells.

We anticipate that our approach may enable the efficient production of site-specifically modified therapeutic proteins, as well as the quantitative replacement of targeted cellular proteins with versions bearing unnatural amino acids that allow imaging or synthetic regulation of protein function.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental procedures, plasmid and primer sequences, and additional supplementary figures. 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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