Gene Technology Hot Paper

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Genetic Incorporation of Multiple Unnatural Amino Acids into Proteins in Mammalian Cells**

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The ability to genetically incorporate unnatural amino acids (UAAs) at specific sites in the proteome of living cells provides a powerful tool to both investigate and engineer protein structure and function.[1] A reassigned nonsense or frameshift codon is used to encode the UAA of interest, and an orthogonal aminoacyl-tRNA synthetase/tRNA (aaRS/tRNA) pair specific for the UAA delivers the latter cotranslationally into the target protein.[1] This technology has been used to genetically encode a large number of diverse amino acids, including chemically and photochemically reactive amino acids, biophysical probes, metal-ion chelators and redox-active amino acids in *E. coli*, *S. cerevisiae*, *C. elegans*, plant, and mammalian cells.[1,2] Optimization of the various components of this system in *E. coli* has resulted in a significant enhancement in suppression efficiency, allowing the incorporation of multiple UAAs in the same polypeptide chain, as well as two different UAAs into a single protein.[3]

Unfortunately, suppression systems in mammalian cells are generally less efficient than in *E. coli*.[2d] Furthermore, the incorporation of multiple distinct UAAs into one protein in mammalian cells requires the development of mutually orthogonal aaRS/tRNA pairs capable of suppressing different nonsense/frameshift codons. Here we show that a previously developed enhanced suppression system can be used to incorporate *O*-methyltyrosine (OMeY, Figure 1b) in good yields at up to three different sites in an enhanced green fluorescent protein (EGFP) in HEK293T cells. We also show that the orthogonal PylRS/$tRNA_{UUA}^{Pyl}$ pair can efficiently suppress the ochre (TAA) nonsense codon in mammalian cells, and in conjunction with the amber (TAG) suppressing

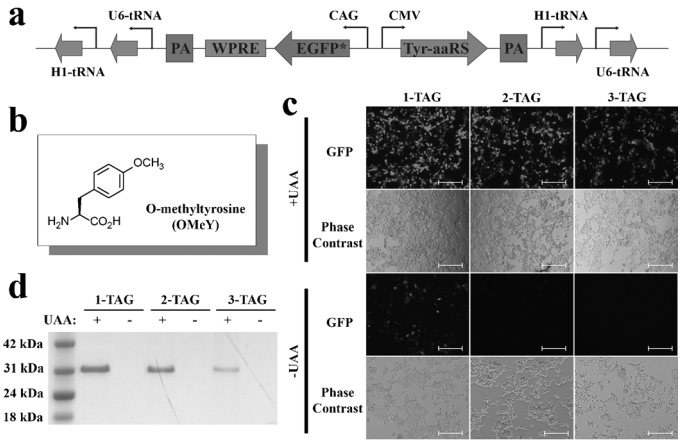

Figure 1. Incorporation of one UAA into multiple sites of EGFP expressed in mammalian cells. a) pAcBac2.tR4-OMeYRS/GFP* encodes a CAG-promoter-driven mutant EGFP expression cassette, *Ec*TyrRS gene driven by CMV promoter, and two 2X-$tRNA_{CUA}^{Tyr}$ cassettes in two different orientations. b) Structure of *O*-methyltyrosine (OMeY). c) Expression of the following EGFP mutants in HEK293T cells analyzed by fluorescence microscopy in the presence (+UAA) or absence (-UAA) of 1 mM OMeY. Scale bar = 500 μm. d) Expression of the aforementioned EGFP mutants analyzed by SDS-PAGE in the presence (+) or absence (−) of 1 mM OMeY.

*Ec*TyrRS/$tRNA_{CUA}^{Tyr}$ pair can be used to incorporate two different UAAs into distinct sites of the same protein. The utility of this technology was demonstrated by generating full-length anti-HER2 antibody conjugated to auristatin and the fluorophore Alexa Fluor 488 to obtain a defined antibody–drug–fluorophore conjugate.

Recently, we reported a mammalian suppression system that offers a significant improvement in the efficiency of UAA incorporation, raising the possibility of its use for the expression of proteins containing multiple UAAs.[4] This system encodes optimized expression cassettes for the aaRS/tRNA pair and the nonsense mutant of the target gene within one plasmid, which can be used to deliver these genetic elements into mammalian cells either using a baculovirus expression vector, or directly by transient transfection.[4] To evaluate the ability of this system to suppress multiple amber nonsense codons in the same polypeptide, we used pAcBac2 plasmids (Figure 1a) harboring an enhanced green fluorescent protein (EGFP) expression cassette encoding from one to three TAG codons at permissive sites: pAcBac2.tR4-OMeYRS/GFP*-1 (Tyr39TAG), pAcBac2.tR4-OMeYRS/GFP*-2 (Tyr39TAG, Tyr151TAG), and pAcBac2.tR4-OMeYRS/GFP*-3 (Ser28TAG, Tyr39TAG, Tyr151TAG). This plasmid also encodes two copies each of the *E. coli* and

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Bacillus stearothermophilus $tRNA_{CUA}^{Tyr}$, and a polyspecific *Ec*TyrRS mutant, originally evolved to charge OMeY, that has been used to encode several UAAs in mammalian cells with high fidelity and efficiency.[4]

These plasmids, encoding the EGFP variants and the amber suppressor aaRS/tRNA pair, were transfected into HEK293T cells, and EGFP expression in the presence or absence of OMeY (1 mM) was monitored by fluorescence microscopy (Figure 1c). Robust expression was observed for all three mutants only in the presence of the UAA (Figure S1a and b in the Supporting Information). All mutant EGFP proteins were isolated from 10^6 HEK293T cells by Ni^{2+}-NTA affinity chromatography and analyzed by SDS-PAGE and ESI-MS (Figures 1d and S2), which confirmed the incorporation of the expected number of OMeY in each case. The yields of isolated single, double, and triple OMeY mutants of EGFP from 10^6 HEK293T cells were 7.1 μg, 4.0 μg, and 1.3 μg, respectively.

The system described above enables the expression of proteins incorporating a single UAA at multiple sites. To incorporate multiple, distinct UAAs into a protein, two different nonsense and/or frameshift codons must be suppressed with mutually orthogonal aaRS/tRNA pairs with unique UAA specificities (Figure 2a). Recently, it was shown that the lack of anticodon recognition by PylRS allows the PylRS/$tRNA^{Pyl}$ pair to efficiently suppress both the ochre (TAA) and the opal (TGA) nonsense codons in *E. coli*.[3a,5] Furthermore, the ochre-suppressing PylRS/$tRNA_{UUA}^{Pyl}$ pair was used together with an orthogonal TyrRS/$tRNA_{CUA}^{Tyr}$ (derived from *M. jannaschii*) to incorporate two distinct UAAs into a single protein in *E. coli*.[3a] To investigate if the PylRS/$tRNA^{Pyl}$ pair is also able to suppress other nonsense codons in mammalian cells, anticodon variants of the *Methanosarcina mazei* pyrrolysyl tRNA ($MmtRNA^{Pyl}$) were generated. A plasmid (pShax-Pyl) encoding four tandem copies of the tRNA expression cassette behind the U6 promoter, as well as the *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase (MbPylRS) gene expressed from a CMV promoter, was constructed (Figure 2b). To evaluate the amber, opal, or ochre suppression activity of these PylRS/$tRNA^{Pyl}$ pairs, pEGFP reporter plasmids were generated encoding an EGFP gene harboring either an amber, opal, or ochre codon at a permissive site (Tyr39). The pShax-Pyl suppressor plasmids were co-transfected into HEK293T cells with the appropriate pEGFP reporter plasmid and the expression levels of full-length EGFP were monitored by fluorescence microscopy in the presence and absence of 1 mM ε-*tert*-Boc-lysine (eBK; Figure 2c). Robust expression of EGFP was observed in each case only in the presence of the UAA, demonstrating the ability of the MbPylRS/$MmtRNA^{Pyl}$ pairs to suppress all three nonsense codons in mammalian cells with good efficiency and fidelity (Figure S4a and b).

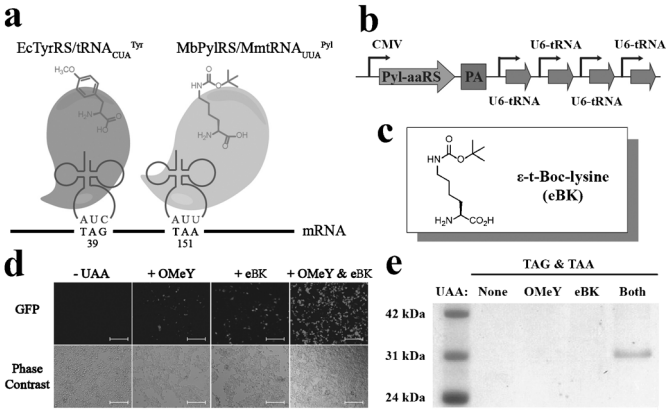

Figure 2. Incorporation of two distinct UAAs into EGFP expressed in HEK293T cells. a) *Ec*TyrRS/$tRNA_{CUA}^{Tyr}$ and MbPylRS/$MmtRNA_{UUA}^{Pyl}$ pairs were used to incorporate OMeY and eBK into EGFP in response to an amber (Tyr39TAG) and an orchre codon (Tyr151TAA), respectively. b) pShax-Pyl^{UUA} encodes a CMV-promoter-driven MbPylRS gene and four copies of $MmtRNA_{UUA}^{Pyl}$ behind the U6 promoter. c) Structure of ε-*tert*-Boc-lysine (eBK). d) Fluorescence and phase-contrast images of HEK293T cells co-transfected with pShax-Pyl^{UUA} and pAcBac2.tR4-OMeYRS/GFP^{dual}, in the presence of both UAAs (+OMeY and eBK), OMeY alone, eBK alone, or in the absence of both UAAs (-UAA). Scale bar = 500 μM. e) SDS-PAGE analysis of EGFP mutants isolated from 10^7 HEK293T cells under the aforementioned conditions.

Because of its higher suppression efficiency, we selected the ochre-suppressing MbPylRS/$MmtRNA_{UUA}^{Pyl}$ pair to be used along with the amber-suppressing EcTyrRS/$tRNA_{CUA}^{Tyr}$ pair for simultaneous incorporation of two distinct UAAs into a single protein in mammalian cells. To test the efficiency of this dual suppression system, we first constructed an EGFP reporter encoding an amber (Tyr39TAG) as well as an ochre (Tyr151TAA) mutation. This reporter was incorporated into the previously described pAcBac2 plasmid to generate pAcBac2.tR4-OMeYRS/GFP^{dual}, which also encodes the polyspecific OMeYRS/$tRNA_{CUA}^{Tyr}$ pair. The MbPylRS/$MmtRNA_{UUA}^{Pyl}$ pair was supplied separately on the pShax-Pyl^{UUA} vector. These plasmids were co-transfected into HEK293T cells and the expression of EGFP was determined in the presence of 1 mM each of OMeY and eBK. Identical control experiments were set up, where either one or both of the UAAs were omitted from the media. A high level of EGFP fluorescence was observed 48 hours after transfection only in the culture containing both UAAs (Figure 2d). While no EGFP fluorescence was observed in the absence of both UAAs, low levels of fluorescence could be detected in cultures containing only one of the UAAs (Figure 2d and e), likely because of a low level of readthrough of the single nonsense codon. Full-length EGFP was isolated from cultures containing both UAAs by Ni^{2+}-NTA affinity chromatography, and was characterized by SDS-PAGE and ESI-MS (Figures 2e and S2). The observed mass of 29758 Da confirms the incorporation of both OMeY and eBK into EGFP (Figure S2). The yield of the isolated EGFP double mutant was approximately 5 μg/10^7 cells, approximately 10% relative to the yield of the single EGFP mutant.

The above system should allow the site-specific incorporation of two uniquely reactive UAAs that can be separately functionalized without cross-reactivity into a single protein, which can then be selectively conjugated to two different molecules.[3] Both OMeYRS and MbPylRS exhibit substantial polyspecificity, and likely can be used to incorporate a keto- and azide-containing UAAs into proteins in mammalian cells, respectively (Figure 3b). Antibody–drug conjugates (ADCs) have emerged as a promising family of anticancer

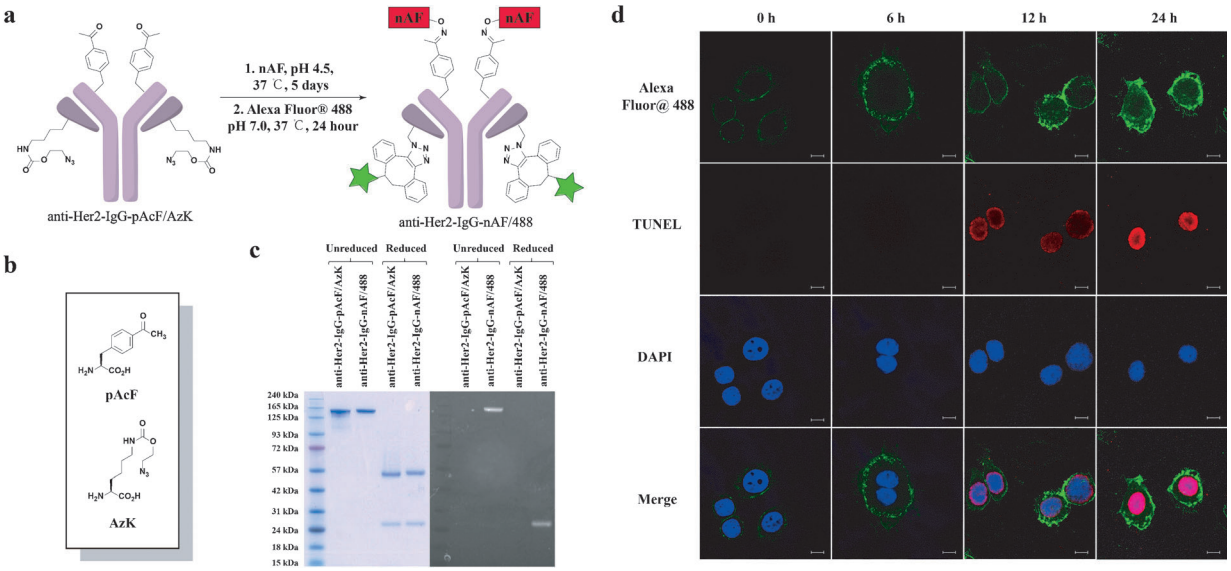

Figure 3. Preparation of anti-Her2-IgG site-specifically labeled with nAF and Alexa Fluor 488 and its internalization in SK-BR-3 cells. a) The pShamu-Her2$^{TAG/TAA}$ and pShax-Pyl^{UUA} vectors were co-transfected into Freestyle™ 293-F cells to express anti-Her2-IgG-pAcF/AzK. This mutant IgG was first coupled to an alkoxy-amine-derivatized nAF by oxime ligation, followed by coupling to an Alexa Fluor 488 DIBO alkyne by copper-free click reaction. b) Structures of pAcF and AzK. c) SDS-PAGE analysis of anti-Her2-IgG-pAcF/AzK and anti-Her2-IgG-nAF/488 under reducing and non-reducing conditions, visualized by UV transillumination (right) and coomassie staining (left). As expected, fluorescence was associated only with the light chain. d) Binding/internalization of anti-Her2-IgG-nAF/488 in SK-BR-3 cells visualized by confocal microscopy using the green fluorescence of AF488. Apoptosis induction was monitored by the TUNEL assay (red fluorescence). Cells were incubated with 10 nM anti-Her2-IgG-nAF/488 in DMEM media for 0, 3, 6, 12, 24 hours at 37 °C and processed for the TUNEL assay and stained with DAPI nuclear stain (blue fluorescence). Scale bar = 10 μM. DMEM = Dulbecco's Modified Eagle's Medium.

drugs that selectively deliver toxic drugs to malignant cells.[6] Previously, we showed that site-specific conjugation of drug molecules to a uniquely reactive UAA residue in an antibody allows the generation of homogeneous ADCs with improved preclinical efficacy and pharmacokinetics relative to those generated by non-specific conjugation chemistry.[6a,e] Incorporation of two mutually orthogonal chemical conjugation sites into an antibody should allow the simultaneous attachment of a drug and a fluorescent and/or PET probe at two distinct sites, or two different drug molecules. To explore this possibility, we used the previously described herceptin–auristatin (anti-Her2-IgG-nAF) conjugate as a model system.[6a,7] We introduced an amber and an ochre mutation in the heavy (HC-Ala121TAG) and the light chain (LC-Val110TAA), respectively, of anti-Her2-IgG for a better suppression yield. pShamu-Her2$^{TAG/TAA}$ vector was generated encoding both the OMeYRS/tRNA$_{CUA}^{Tyr}$ pair and the mutant antibody expression cassettes. This plasmid was co-transfected into Freestyle™ 293-F cells by transient transfection with pShax-Pyl^{UUA} vector encoding the ochre-suppressing PylRS/tRNA$_{UUA}^{Pyl}$ pair. The TAG in the heavy chain was suppressed with a *p*-acetylphenylalanine (pAcF; Figure 3b) charged by OMeYRS, while the TAA in the light chain was suppressed with an azido-lysine (AzK; Figure 3b) charged by MbPylRS. Folded, full-length mutant protein (100 μg L^{-1}) was purified by protein L-affinity chromatography and analyzed by SDS-PAGE and ESI-MS analysis (Figures 3c and S3), which confirmed the incorporation of both pAcF and AzK. Anti-Her2-IgG-pAcF/AzK was then first coupled to the alkoxy-amine-derivatized nAF in 100 mM ammonium acetate buffer (pH 4.5) at 37 °C. Upon completion of the reaction, the resulting conjugate was buffer-exchanged into PBS, and Alexa Fluor 488-DIBO alkyne was conjugated to the azido group by a Cu-free click reaction (Figure 3a). The resulting dual-labeled antibody was further purified by a desalting column. The two-step conjugation reaction afforded the antibody-nAF–Alexa Fluor 488 conjugate (anti-Her2-IgG-nAF/488) in greater than 90% conjugation yield, as observed by ESI-MS analysis (Figures 3c and S3).

With the fluorophore-labeled ADC in hand, we investigated its uptake and ability to induce apoptosis in the Her2^{+} breast cancer cell line SK-BR-3. Cells were incubated with 10 nM anti-Her2-IgG- nAF/488 for different periods of time. As a control, we performed identical experiments with the Her2^{-} breast cancer cell line MDA-MB-468. While fluorescence imaging of SK-BR-3 showed time-dependent binding of anti-Her2-IgG-nAF/488, MDA-MB-468 cells did not exhibit any associated fluorescence, even after prolonged incubation (Figures S5 and S6). Next, we analyzed the cellular uptake of the modified antibody by laser-scanning confocal microscopy, which showed rapid binding of the anti-Her2-IgG-nAF/488 to SK-BR-3 cell surface followed by slow internalization (Figures 3d and S6). While cell-surface-associated fluorescence was detected upon a very short incubation period (10 min), significant internal fluorescence was observed only after 6 hours (Figure S6). To correlate these observations with the induction of apoptosis, a TUNEL assay was performed on SK-BR-3 cells incubated with anti-Her2-IgG-nAF/488 at different time points.[8] The earliest appearance of an apoptosis-specific signal was observed after 12 hours of

incubation, which grew much stronger after 24 hours (Figure 3d). Thus, the selective labeling of an antibody with both a drug and fluorophore provides a direct and general method to probe the binding and internalization of various ADCs.

In summary, we have demonstrated that mutant proteins can be efficiently expressed in mammalian cells that incorporate either a single or two different UAAs at multiple distinct sites. The use of mutually orthogonal *Ec*TyrRS/$tRNA_{CUA}^{Tyr}$ and MbPylRS/$tRNA_{UUA}^{Pyl}$ pairs to suppress amber (TAG) and ochre (TAA) nonsense codons, respectively, allowed the incorporation of two uniquely reactive UAAs (with keto and azido side chains), which were subsequently functionalized with a toxic drug and a fluorophore. The ability to produce antibodies with distinct, uniquely reactive sites should provide a general method for generating ADCs containing two distinct drugs or a drug and imaging agent. Because the polyspecific tyrosyl (*E. coli*) and pyrrolysyl aaRS/tRNA pairs have been used to genetically encode a number of UAAs with useful functionalities in eukaryotic cells, the ability to incorporate multiple UAAs into proteins in mammalian cells expands the scope and the potential applications of this technology.

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