We describe a new expression system for efficient non-canonical amino acid mutagenesis in cultured mammalian cells by using the pyrrolysine tRNA synthetase/tRNA\textsubscript{CUA\textsuperscript{Pyl}} pair. A significant improvement in the incorporation of non-canonical amino acids into proteins was obtained by combining all the required genetic components into a single and compact vector that can be efficiently delivered to different mammalian cell lines by conventional transfection reagents.

The chemical diversity of genetically encoded and ribosomally synthesized proteins is naturally limited to the chemical groups found within the 20 canonical amino acids (22 if including selenocysteine and pyrrolysine). This chemical repertoire can be expanded by genetically encoding the incorporation of non-canonical amino acids (NCAAs) by using genetic code expansion methods.\cite{1–3} Current technologies usually use the amber stop codon (TAG) together with an orthogonal pair of amber suppressor tRNA\textsubscript{CUA} and aminoacyl-tRNA synthetase, but the use of other stop codons or frame-shift codons has also been demonstrated.\cite{4–6} A frequently used aminoacyl-tRNA synthetase/tRNA\textsubscript{CUA} pair is the pyrrolysyl-tRNA synthetase (PylRS)/tRNA\textsubscript{CUA\textsuperscript{Pyl}} from Methanosarcina species; this pair was found to be inert (orthogonal) to synthetases and tRNAs in E. coli, yeast, mammalian cells, and multicellular organisms.\cite{1, 3, 7–10} By using this system, dozens of NCAAs have been added to the common genetic code, including amino acids with reactive groups for bio-orthogonal labeling, photo-cleavable protecting groups, and amino acids carrying natural post-translational modifications.\cite{3, 10–13}

One exciting application of genetic code expansion technology is the expression of proteins with site-specifically incorporated NCAAs in mammalian cells.\cite{14, 15} However, this can be limited by low suppression efficiency in mammalian cells, and therefore low expression levels of the recombinant protein. The efficiency of NCAAC incorporation in response to an in-frame stop codon (i.e., suppression efficiency) depends on several factors, such as aminoacylation efficiency of the orthogon-
pression of foreign tRNA in mammalian cells.\textsuperscript{14} Although the intracellular level of suppressor tRNA is a limiting factor in NCAA mutagenesis, increased levels of tRNA can have toxic effects and interfere with downstream processes. In addition, the use of a two-plasmid-based system can limit the integration of NCAA mutagenesis methods with other methodologies and assays; for example, dual labeling of proteins, expression of more than one labeled protein, or assays (such as the luciferase assay) that require co-transfection with other plasmids.

Schultz and co-workers\textsuperscript{21} demonstrated enhanced NCAA mutagenesis in mammalian cells by using a single-plasmid-based baculovirus expression system; this plasmid was subsequently used with chemical transfection methods.\textsuperscript{22} Although significantly improving NCAA mutagenesis, baculovirus expression systems are less common in research laboratories. Importantly, combining all the required components on one plasmid resulted in a relatively large construct (> 12 kb, not including the gene of interest).\textsuperscript{21} The use of a large plasmid in transient transfection methods can reduce transfection efficiency and expression level, as well as limit the size of the protein to be studied.

Our goal, therefore, was to design a minimal expression vector for efficient NCAA mutagenesis by using the PyrS/tRNA\textsubscript{Cua}\textsuperscript{Pyl} pair. To facilitate the use of this vector, it would have to be small enough to allow the expression of large proteins by common cloning techniques and chemical transfection methods. The small size of the plasmid might also enable efficient co-transfection with additional plasmids, including co-transfection with a similar vector for the expression of two different proteins. In addition, the designed plasmid must have a selection marker for stable transfection in mammalian cells.

We based our expression vector on the pBudCE4.1 backbone (Invitrogen) that enables the co-expression of two proteins from two independent promoters: human cytomegalovirus (CMV) immediate-early promoter, and the human elongation factor 1α-subunit (EF-1α) promoter. The selection marker, Zeocin, can be used in both bacteria and mammalian cells, thereby eliminating the need for two selection markers. As a reporter gene, we expressed the mCherry-TAG-eGFP-HA construct suggested by Gautier et al.\textsuperscript{20} mCherry expression serves as a transfection control, whereas eGFP expression is detected only if an NCAA construct is incorporated to an in-frame TAG codon. First, we cloned wild-type Methanosarcina mazei PyrRS downstream of the CMV promoter and mCherry-TAG-eGFP-HA under the control of EF-1α promoter, thereby creating plasmid pBud-PyRS-mCherry-TAG-eGFP. Figure 1, plasmid e. Co-transfection of plasmids c and d led to a slight improvement in amber suppression efficiency compared to co-transfection of plasmids a and b (Figure 2, lane 1 vs. lane 5). Although plasmids a and c are based on different backbones, the improvement in amber suppression efficiency measured with plasmid c can be attributed to the use of the stronger EF-1α promoter (compared to CMV) for the expression of mCherry-TAG-eGFP-HA (Figure S1 in the Supporting Information).

In order to obtain high expression levels of tRNA\textsubscript{Cua}\textsuperscript{Pyl}, several copies of tRNA\textsubscript{Cua}\textsuperscript{Pyl} are commonly expressed (usually four), with each copy under the control of an independent promot-

![Figure 2](image-url) Improved NCAA mutagenesis in cultured mammalian cells. HEK293T cells were transfected with the indicated expression vectors and incubated in the presence (+) or absence (−) of 1 μM eGFP. In all experiments, cells were transfected with equal amounts of plasmid DNA. Protein yield was quantified by western blotting with anti-HA and anti-actin antibodies.
where NCAA mutagenesis efficiency is known to be very high. To demonstrate the advantage of our new expression system, we compared the incorporation level of 1 in four cell lines with known differences in NCAA mutagenesis efficiency (Figures 3 and S2). The improvement in NCAA incorporation in HEK293T, HCT116, and NIH3T3 cells was 12- to 20-fold. In HeLa cells, the improvement was even more dramatic, as incorporation of 1 by a two-plasmid-based mammalian expression system was below our detection limit.

In order to demonstrate the applicability of this expression vector to the study of biologically relevant systems, we incorporated Nε-acetyl-γ-lysine (2) into one position (and even two positions) in the tumor suppressor protein p53, exogenously expressed in a p53-null cell line (Figure 4).[26] Acetylation of p53 at Lys120 (and to some extent at Lys164) has been shown to modulate the transcriptional activity of p53.[27–29] The mechanisms by which p53 differentiates between different promoters as a function of acetylation are unknown. In order to demonstrate the potential of our expression system in the study of p53 acetylation-dependent DNA-binding specificity, we expressed wild-type p53 as well as K120TAG, K164TAG, and K120,164TAG p53 mutants in the HCT116-p53licing cell line

![Figure 3. Enhanced NCAA mutagenesis in cultured mammalian cells. The level of NCAA mutagenesis was estimated by using the mCherry-TAG-eGFP reporter. Protein was expressed in the presence or absence of 1 from plasmids a and b or plasmid e in cell lines A) HEK293T, B) HCT116, C) HeLa, and D) NIH3T3. Expressed mCherry-eGFP-HA was visualized by western blotting with antibodies against the C-terminal HA tag. Protein expression levels were quantified by densitometry (ImageJ) and normalized to actin (n = 3, mean ± SD).](image)

![Figure 4. Expression of site-specifically acetylated p53 in HCT116-p53licing cells. Wild-type or lysine-acetylated p53 was transiently expressed in HCT116-p53licing cells by using our expression system. Cells were incubated in the presence (+) or absence (−) of 2 (5 mM). Expression levels of acetylated p53 are compared to the expression level of exogenously expressed wild-type p53 (lanes 1 and 2, 50% of total protein) which is independent of 2. The improved expression system also enabled the expression of K120,K164 double-acetylated p53 (lanes 7, 8). ΔN'-p53 are known N'-truncated variants of p53.](image)

In summary, we have designed a compact single-plasmid-based expression system for efficient NCAA mutagenesis in cultured mammalian cells. The use of a single plasmid for amber suppression in cultured mammalian cells enhances the efficiency of NCAA mutagenesis by improving amber suppression as well as transfection efficiency (Figures S4 and S5). Moreover, our single-plasmid-based system will allow efficient co-transfection with additional plasmids, and the marker selection for mammalian cells can be used in stable transfections.

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