

# Improving orthogonal tRNA-synthetase recognition for efficient unnatural amino acid incorporation and application in mammalian cells†‡

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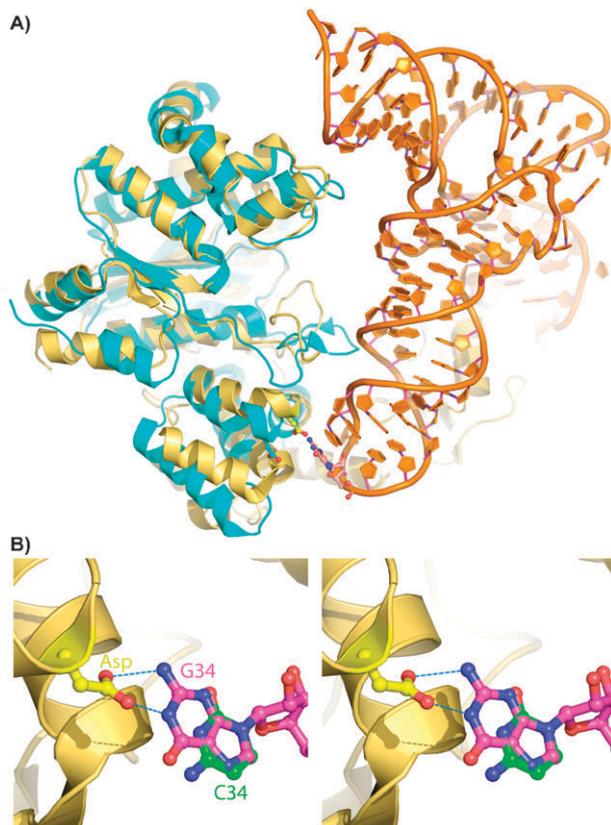
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**Optimizing the anticodon recognition between orthogonal tRNA and synthetase significantly increased the incorporation efficiencies of various unnatural amino acids in mammalian cells, and the enhanced incorporation enabled efficient photocrosslinking of interacting proteins in mammalian cells.**

Unnatural amino acids have been genetically encoded in *Escherichia coli*, yeast, and mammalian cells using orthogonal tRNA-synthetase pairs and unique codons.<sup>1</sup> This technology enables novel chemical and physical properties to be selectively introduced into proteins directly in live cells, and thus have great potential for addressing molecular and cell biological questions in the native cell settings. While the incorporation efficiency of unnatural amino acids is high in *E. coli* and yeast,<sup>2</sup> it is currently low in mammalian cells. Inefficient incorporation results in reduced yield of proteins containing the unnatural amino acid, which may not be sufficient to perform the desired function and to be detected. In addition, as stop codons are the most frequently used to encode unnatural amino acids, low incorporation efficiency often leads to an increase in truncated protein products, which may negatively interfere with the function of the full-length target protein. Therefore, efficient incorporation of unnatural amino acids is critical for their effective application in mammalian cells. Efforts to improve the efficiency have been focused on optimizing the expression of the orthogonal tRNA and synthetase;<sup>2-4</sup> another important aspect is to increase the affinity of the orthogonal tRNA toward the orthogonal synthetase, which has not been addressed in mammalian cells. Here we show that the recognition of the orthogonal tRNA by the synthetase could be enhanced by engineering the anticodon-binding domain of the synthetase. The mutation identified could be generally transplanted to multiple synthetases specific for different unnatural amino acids to improve their incorporation efficiencies in mammalian cells. Using the enhanced synthetase specific for *p*-azidophenylalanine (Azi), we performed site-specific photocrosslinking of interacting proteins in mammalian cells with high efficiency.

The efficiency of an orthogonal synthetase in charging the orthogonal tRNA with an unnatural amino acid depends on

the affinity of the synthetase toward both the unnatural amino acid and the tRNA. To decode the amber stop codon UAG used for specifying the unnatural amino acid, the anticodon of the orthogonal tRNA has to be changed to CUA. This change would decrease the affinity of the tRNA toward its cognate orthogonal synthetase, because the anticodon is a major recognition element of most synthetases in distinguishing tRNAs.<sup>5</sup> The *E. coli* tRNA<sup>Tyr</sup><sub>CUA</sub>-tyrosyl tRNA synthetase (TyrRS) pair has been the candidate for evolving orthogonal tRNA-synthetase pairs for incorporating various unnatural amino acids in yeast and mammalian cells.<sup>2-4</sup> We thus decided to improve the recognition of the *E. coli* TyrRS toward its tRNA<sup>Tyr</sup><sub>CUA</sub>.



**Fig. 1** (A) Superposition of *E. coli* TyrRS (PDB ID 1X8X, cyan) on *T. thermophilus* tRNA<sup>Tyr</sup>-TyrRS complex (PDB ID 1H3E, yellow and orange). Only one subunit of the dimeric TyrRS and one tRNA are shown. Base G34 on the tRNA and the interacting Asp on TyrRS are represented as ball-and-stick. (B) Recognition of G34 by Asp259 in the *T. thermophilus* tRNA<sup>Tyr</sup>-TyrRS complex. C34 was modeled to show the gap after G34C change.

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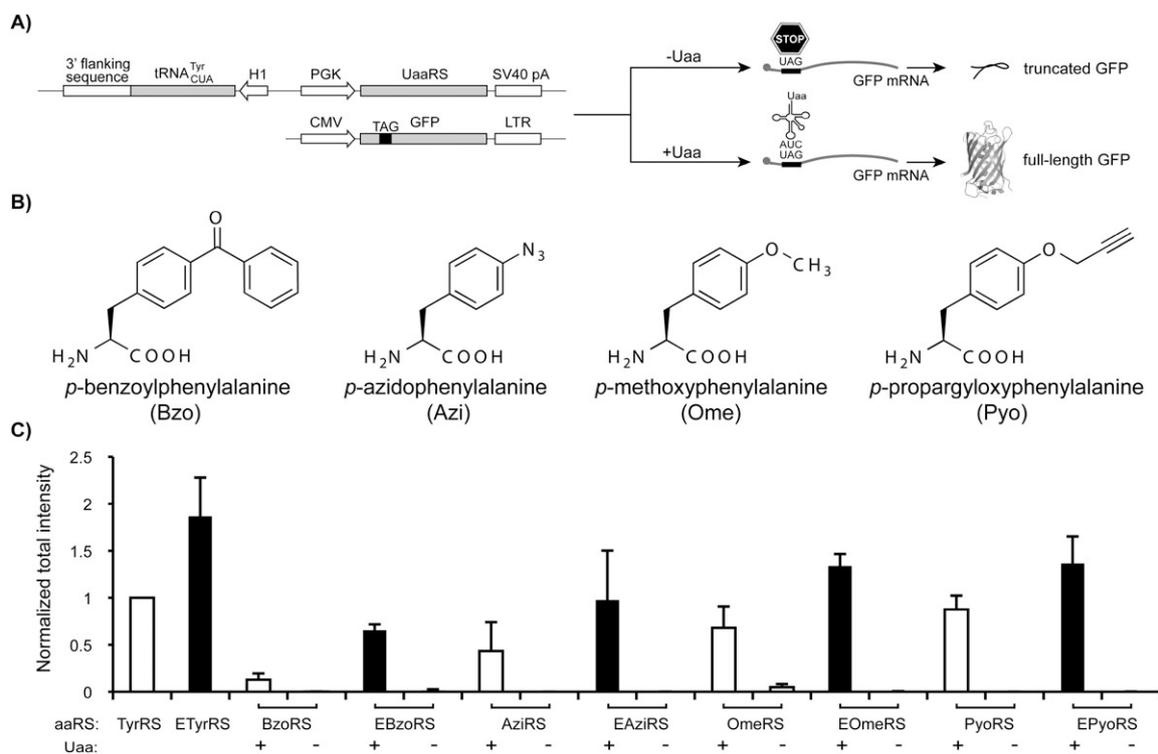
‡ Electronic supplementary information (ESI) available: Materials, experimental methods and links to view structures in FirstGlance. See DOI: 10.1039/b904228h

As there is no crystal structure available for the *E. coli* tRNA<sup>Tyr</sup>-TyrRS complex, we superposed the *E. coli* TyrRS<sup>6</sup> onto the homologous *Thermus thermophilus* TyrRS in the tRNA<sup>Tyr</sup>-TyrRS complex of the latter<sup>7</sup> using the secondary-structure matching method.<sup>8</sup> The two TyrRS superposed well with an r.m.s.d. of 2.2 Å for the main chain atoms (Fig. 1A). Only G34 in the anticodon of tRNA<sup>Tyr</sup> needs to be changed to C34 to make tRNA<sup>Tyr</sup><sub>CUA</sub>. The G34 base of *T. thermophilus* tRNA<sup>Tyr</sup> is recognized by the carboxyl group of Asp259 in *T. thermophilus* TyrRS, which corresponds to Asp265 in *E. coli* TyrRS. Mutation of G34 to C34 would create a gap between the base and the Asp residue, disrupting the recognition (Fig. 1B). We thus mutated Asp265 in *E. coli* TyrRS to five amino acids with side chains longer than Asp (Tyr, Arg, Gln, Phe, and Leu) in the hope to restore the interaction with C34. These five mutants were first screened in yeast to test their ability to suppress the amber codon together with the *E. coli* tRNA<sup>Tyr</sup><sub>CUA</sub>. An amber stop codon was introduced into a permissive site (Lys5) in the HIS3 gene. If the *E. coli* tRNA<sup>Tyr</sup><sub>CUA</sub>-mutant TyrRS suppresses the amber codon, yeast cells will be able to make full length HIS3 protein and survive in media lacking histidine. Yeast harboring mutant Asp265Phe and Asp265Leu could not grow, suggesting these two mutations could abolish the recognition for the tRNA<sup>Tyr</sup><sub>CUA</sub>. The other three mutants grew as good as the wt TyrRS, and were

further characterized in mammalian cells using an *in vivo* fluorescence assay.

In this assay, genes for the *E. coli* tRNA<sup>Tyr</sup><sub>CUA</sub> and synthetase mutant to be tested were transfected into a stable clonal HeLa cell line, in which the green fluorescent protein (GFP) gene containing a premature UAG stop codon at a permissive site (Tyr39) was integrated into the genome (Fig. 2A).<sup>4</sup> Suppression of UAG39 by the tRNA<sup>Tyr</sup><sub>CUA</sub>-synthetase pair results in full-length fluorescent GFP. The total fluorescence intensity of cells measured by fluorescence-activated cell sorting (FACS) reports the incorporation efficiency and thus the affinity of the mutant synthetase for the tRNA<sup>Tyr</sup><sub>CUA</sub>. Compared with the wt TyrRS, the Asp265Gln and Asp265Tyr mutants decreased the fluorescence intensity to below 10%, while the Asp265Arg mutant increased the fluorescence intensity to 186%, indicating that the Asp265Arg mutant TyrRS (EwtYRS) has higher activity toward the tRNA<sup>Tyr</sup><sub>CUA</sub> than wt TyrRS (Fig. 2C).

To test if the Asp265Arg mutation could be transplanted to improve the incorporation efficiency of different unnatural amino acids in mammalian cells, we introduced this mutation to 4 synthetases that are derived from the *E. coli* TyrRS for the incorporation of unnatural amino acids Bzo, Azi, Ome, and Pyo, respectively (Fig. 2B).<sup>9</sup> The resultant mutant synthetases (referred to as enhanced synthetases EBzoRS, EAziRS, EOmeRS, and EPyoRS) were individually coexpressed with



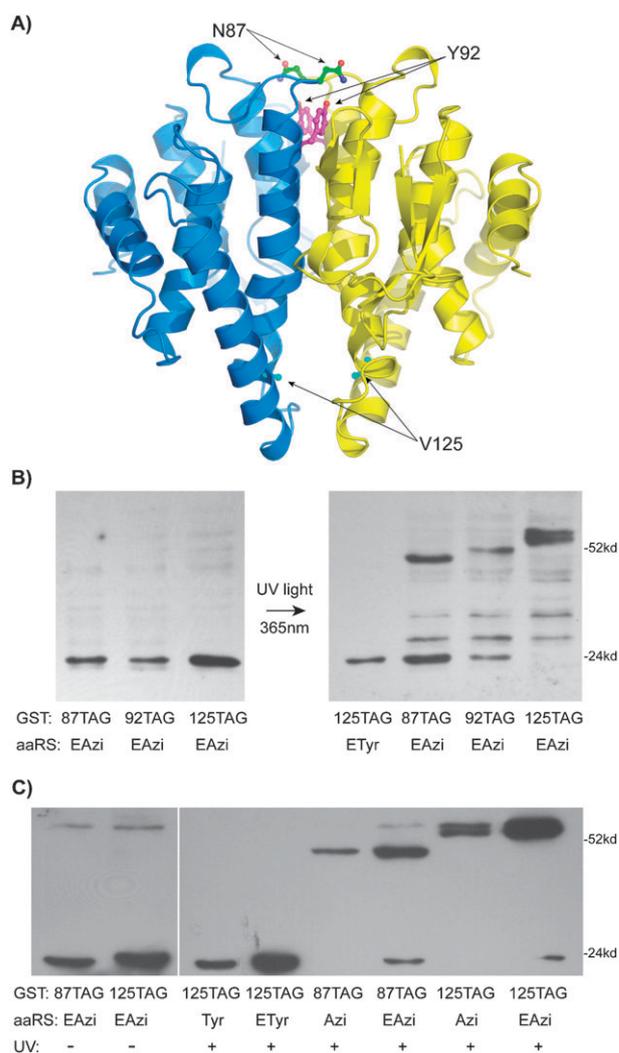
**Fig. 2** Enhanced mutant synthetases increased the incorporation efficiency of various unnatural amino acids in mammalian cells. (A) An *in vivo* fluorescence assay for measuring the amber suppression efficiency of the expressed orthogonal tRNA-synthetase. Uaa: unnatural amino acid. (B) Structures of the unnatural amino acids. (C) Fluorescence assay results for the incorporation efficiency of different synthetases. Error bars represent sem  $n = 3$ . Mutants used here were: EBzoRS (Y37G, D182G, L186A, and D265R), EAziRS (Y37L, D182S, F183A, L186A, and D265R), EOmeRS (Y37T, D182T, L183M, and D265R), and EPyoRS (Y37G, D182S, F183M, and D265R). All mutations are in reference to the wt *E. coli* TyrRS.

the *E. coli* tRNA<sup>Tyr</sup><sub>CUA</sub> in the clonal GFP reporter HeLa cell line in the absence and presence of the unnatural amino acid. The incorporation efficiency was quantified with the total fluorescence intensity of cells using FACS (Fig. 2C). In comparison to the original synthetases, all four enhanced mutant synthetases improved the incorporation efficiency of their respective unnatural amino acids, with the increase ranging from 1.6 fold to 5.2 fold. The EOMeRS and EPyoRS could incorporate the corresponding unnatural amino acid more efficiently than the wt *E. coli* TyrRS. Importantly, the background misincorporation in the absence of the unnatural amino acid did not increase significantly, suggesting that the Asp265Arg mutation did not affect the amino acid specificity of the wt and evolved synthetases. That all four tested mutants showed improvement suggests that the Asp265Arg mutation can generally be transplanted to more TyrRS-derived synthetases.

Prokaryotic TyrRS differ distinctly from archaeal and eukaryotic TyrRS in anticodon recognition.<sup>10</sup> In prokaryotic tRNA<sup>Tyr</sup>, base ψ35 is flipped oppositely to G34 and A36 and recognized by the extra C-terminal domain characteristic of prokaryotic TyrRS, and base G34 is stacked directly on base A36. In contrast, in archaeal and eukaryotic tRNA<sup>Tyr</sup>, all three anticodon bases are in the same side, and G34 is stacked with hydrophobic residues of the TyrRS. Although G34 is recognized by the carboxyl group of Asp in both prokaryotic and archaeal/eukaryotic TyrRS, the helices in which this Asp residue locates do not correspond. By mutating the residue interacting with G34, Kobayashi *et al.* showed that the initial aminoacylation rate of the cognate tRNA<sup>Tyr</sup><sub>CUA</sub> by the archaeal *Methanococcus jannaschii* TyrRS can be increased.<sup>11</sup> Using the *in vivo* translational assay, we demonstrated here that, despite the different anticodon recognition mode, the prokaryotic TyrRS could also be optimized for its cognate tRNA<sup>Tyr</sup><sub>CUA</sub> that lead to higher incorporation efficiency for Tyr and various unnatural amino acids into proteins.

To test if the enhanced mutant synthetases would facilitate the application of unnatural amino acids in mammalian cells, we used the EAziRS to incorporate Azi into glutathione S-transferase (GST) and attempted photocrosslinking in HEK293T cells. *E. coli* GST is homodimeric.<sup>12</sup> Three sites at the dimer interface, Asn87 in a loop and Tyr92 and Val125 in α-helices were chosen for incorporating Azi (Fig. 3A). GST genes with TAG mutation at these sites and a C-terminal His6 tag were cotransfected with the orthogonal tRNA<sup>Tyr</sup><sub>CUA</sub>-EAziRS pair in HEK293 cells, and the cells were grown in the presence of 1 mM of Azi. Photocrosslinking was performed by exposing the cells under 365 nm UV light for 10 min. Cell lysates were separated on SDS-PAGE and probed with an antibody against the His6 tag. As shown in Fig. 3B, GST monomers (23 kD) could be detected for all three mutants before UV exposure. After photocrosslinking, higher molecular weight bands around 46 kD emerged, accompanied by the intensity decrease of the monomer band. Based on the intensity ratio of these bands, the crosslinking efficiency was calculated to be 33%, 40%, and 98% for site 87, 92, and 125, respectively.

Several faint bands between 23 kD and 46 kD were observed on the GST-His6 blot, which can be due to proteins recognized



**Fig. 3** Photocrosslinking in mammalian cells using Azi. (A) Crystal structure of *E. coli* GST (PDB ID 1A0F) with three sites for Azi incorporation labeled. (B) Western blot analysis of GST mutants using a penta-His antibody before and after photocrosslinking. (C) Western blot analysis of GST mutants using an anti-FLAG antibody before and after photocrosslinking.

nonspecifically by the penta-His antibody or non-GST proteins crosslinked to GST. To distinguish this, we replaced the His6 tag with the FLAG tag at the C-terminus of GST, and probed the blot with an anti-FLAG antibody after photocrosslinking (Fig. 3C). These bands disappeared, indicating that they are caused by the nonspecificity of the penta-His antibody. Compared with the original TyrRS and AziRS, the ETyrRS and EAziRS increased GST protein yields to 2.2 fold and ~2.3 fold, respectively. More crosslinked GST products were detected in the EAziRS than the AziRS samples (2.1 fold for 87TAG and 1.8 fold for 125TAG). Interestingly, crosslinked GST migrated at different positions in the gel depending on where Azi was incorporated. One possible explanation is that the crosslinking of GST dimer with 2 Azis generates an interlocked polypeptide with a core and 4 arms. Different Azi incorporation sites will change the core size and arm length in the crosslinked product, thus leading to different

mobility in gel. This effect may be analogous to how disulfide bonds influence protein mobility in nonreducing electrophoresis conditions. Another possibility to be excluded by mass spectrometry is that GST was crosslinked to other proteins.

These results demonstrate the feasibility of using genetically encoded Azi for detecting protein–protein interactions directly in live mammalian cells. Bzo, but no Azi, has been used to crosslink proteins in mammalian cells before.<sup>13</sup> Benzophenone can be photoactivated at wavelength longer than aryl azides (350–365 nm vs. <330 nm).<sup>14</sup> However, our results showed that Azi could also be activated at the long wavelength 365 nm for crosslinking. One advantage of Azi over Bzo is its smaller size. The bulky side chain of Bzo is more likely to disrupt the protein interaction under study. In the Azi crosslinking experiment, crosslinked products were detected from cell lysate directly. The reported Bzo experiments used immunoprecipitation to enrich the target proteins for detection.<sup>13</sup> Although crosslinking efficiency also depends on target protein and interaction strength, our results suggest that Azi incorporated using the EAziRS enables efficient photocrosslinking in mammalian cells and should prove useful for detecting protein–protein interactions *in vivo*.

In summary, we demonstrated that the recognition of *E. coli* TyrRS for its cognate tRNA<sub>CUA</sub><sup>Tyr</sup> could be optimized by engineering the anticodon-binding region. This optimization could be transplanted to multiple TyrRS-derived synthetases, and the resultant enhanced synthetases improved the incorporation efficiency of various unnatural amino acids in mammalian cells. This strategy should also be generally applicable to other orthogonal tRNA–synthetase pairs, such as the *E. coli* tRNA<sup>Lue</sup>–LeuRS pair and the *Methanosarcina mazei* tRNA<sup>Pyl</sup>–PylRS pair. We also showed that an enhanced synthetase allowed us to perform *in vivo* photocrosslinking in mammalian cells with high efficiency, which should help detect protein-involved interactions in native cellular surroundings. Efficient incorporation of unnatural amino acids in mammalian cells should enable the effective application of these novel amino acids in addressing a broad range of molecular and cellular biological questions.

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