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Review Article

Using genetically incorporated unnatural amino acids to control protein functions in mammalian cells

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Genetic code expansion allows unnatural (non-canonical) amino acid incorporation into proteins of interest by repurposing the cellular translation machinery. The development of this technique has enabled site-specific incorporation of many structurally and chemically diverse amino acids, facilitating a plethora of applications, including protein imaging, engineering, mechanistic and structural investigations, and functional regulation. Particularly, genetic code expansion provides great tools to study mammalian proteins, of which dysregulations often have important implications in health. In recent years, a series of methods has been developed to modulate protein function through genetically incorporated unnatural amino acids. In this review, we will first discuss the basic concept of genetic code expansion and give an up-to-date list of amino acids that can be incorporated into proteins in mammalian cells. We then focus on the use of unnatural amino acids to activate, inhibit, or reversibly modulate protein function by translational, optical or chemical control. The features of each approach will also be highlighted.

Introduction

Knowledge of protein function is of pivotal importance to life science research. It can guide conventional drug development programmes and lead to novel strategies to address currently non-targetable systems [1–3]. In order to understand the precise role and interacting network of a protein, it is essential to analyse it within its native environment. For a mammalian protein, its function often also depends on its host cell (e.g. cell type and cell cycle stage), specific subcellular location and post-translational modifications. In addition, a protein of interest often exists in the presence of other closely related homologues (e.g. proteins within the same family), making it difficult to decipher the precise function of a specific protein in cells. Targeting the protein by small-molecule inhibition is often not possible in these cases, as protein homologues will also be affected. To tackle this problem, over the last two decades there has been a drive to develop and refine the technique of genetic code expansion which allows researchers to exploit the cellular translation machinery for site-specific incorporation of unnatural (non-canonical) amino acids into target proteins [4–14]. Consequently, this enables the use of building blocks beyond the 20 canonical amino acids and incorporation of unnatural amino acids with unprecedented functionality into target proteins in live cells. The repurposing of the translational machinery by this approach has paved the way for revealing the functions of proteins under physiological conditions [15–19]. For example, the technique can be used to site-specifically introduce an unnatural amino acid into the homologue of interest, whereby unique functionality (on the unnatural amino acid) can be used for selective activation, inhibition, or reversible regulation of the target homologue [7].

At the molecular level, the mechanism of protein translation is highly conserved in all organisms, where the cellular machinery 'translates' every nucleotide triplet as a codon consecutively on the mRNA
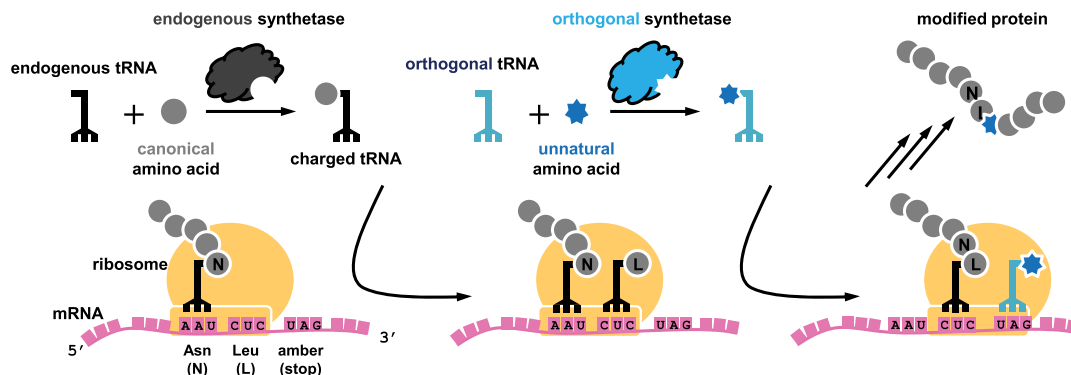
Figure 1. Mechanism of genetic code expansion for site-specific incorporation of an unnatural amino acid by amber suppression
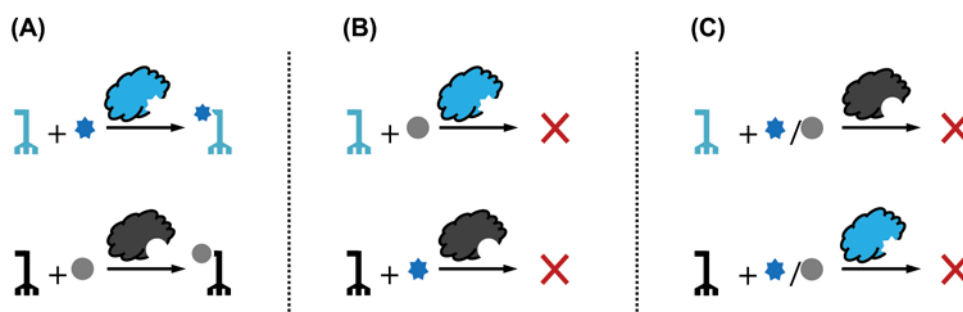

Figure 2. Allowed and not allowed reactivities between the orthogonal and endogenous aaRS/tRNA pairs
(**A**) Matching amino acid and aaRS/tRNA pairs; (**B**) mismatched amino acids; (**C**) mismatched aaRS/tRNA pairs.

into the corresponding amino acid. In nature, the endogenous aminoacyl-tRNA synthetase (aaRS)/tRNA pairs within the cell decode 61 of the total 64 codons to 20 canonical amino acids. The remaining three codons (UAG, UGA and UAA) are used for translation termination, and hence they are also known as 'stop' codons. In order to achieve site-specific incorporation of an unnatural amino acid, an orthogonal aaRS/tRNA pair is needed, which must decode a codon that does not correspond to any canonical amino acid, a so-called blank codon (Figure 1). Stop codons are most commonly used as a blank codon in genetic code expansion, and decoding of a stop codon is known as 'suppression' because it suppresses the translation termination. The amber stop codon (UAG) is often used as the blank codon due to its minimal occurrence in most organisms.

Within the concept of genetic code expansion, 'orthogonality' refers to the non-reactivity of the orthogonal aaRS/tRNA pair with the endogenous pair and canonical amino acids in the host cell. The orthogonal synthetase must only acylate the orthogonal tRNA with the designated unnatural amino acid; neither canonical amino acids nor endogenous tRNAs are substrates of the orthogonal synthetase; similarly, neither the unnatural amino acid nor orthogonal tRNA is a substrate of the endogenous synthetases (Figure 2).

Besides the amber codon, other stop codons [20–26] and different four-nucleotide codons [27,28] have been used as a blank codon. The use of four-nucleotide codons expands the theoretical codon numbers from 4^3 (64) to 4^4 (256) so that multiple different unnatural amino acids can be incorporated at the same time. However, decoding a four-nucleotide codon by the ribosome is less efficient than decoding the normal three-nucleotide codons. Although this issue has been addressed in *Escherichia coli* through ribosome engineering [29–31], the lower efficiency in decoding four-nucleotide codons remains an issue in mammalian systems [27,28].

To date, many unnatural amino acids (**1–110**, Table 1) can be site-specifically incorporated into proteins produced by mammalian cells using genetic code expansion [5,32]. While the amino acids are structurally diverse, the majority of them can be incorporated through only a few orthogonal synthetases and their mutants. The Pyrrolysyl-tRNA synthetase (PylRS)/tRNA^{Pyl} pairs from archaea species *Methanosarcina barkeri* (*Mb*) and
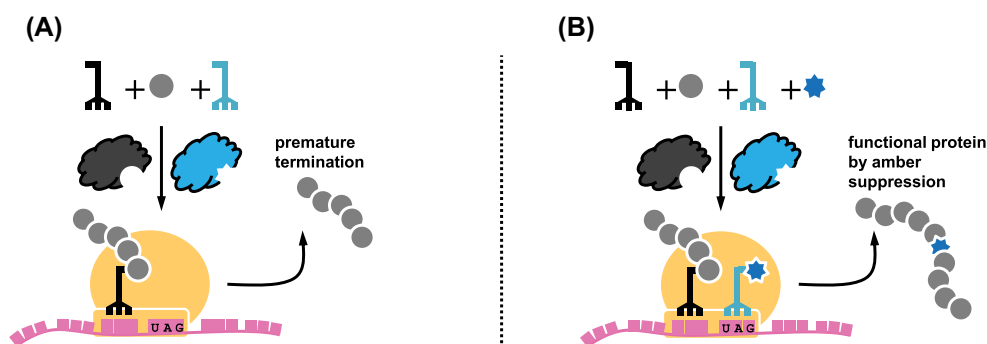
Figure 3. Use of amber suppression to switch on protein production
(**A**) Absence of the unnatural amino acid leads to recognition of the UAG codon for translation termination. (**B**) Addition of the unnatural amino acid leads to amber suppression and successful production of the full-length and functional protein.

Methanosarcina mazei (*Mm*) have proven to be extraordinarily useful pairs [4]. The tRNA^{Pyl} naturally recognises the UAG codon and thus engineering of this tRNA is not needed. In addition, this pair is orthogonal in both *E. coli* and mammalian cells; hence, it facilitates the engineering of PylRS in *E. coli* and subsequently using the engineered PylRS mutant for incorporation of the designated unnatural amino acid in mammalian systems. As shown in Table 1, a wide range of amino acids has been incorporated into proteins in mammalian cells through only a few point mutations on the *PylRS* gene.

Many engineered *E. coli* aaRS/tRNA pairs have also been used as orthogonal pairs in mammalian cells. The most successful ones are the *E. coli* tyrosine, leucine and tryptophan pairs [33]. However, as all these synthetases naturally recognise a canonical amino acid, it is necessary to abolish their natural activity towards the canonical amino acid and to recognise only the designated unnatural amino acid. As it is technically difficult to perform directed evolution in mammalian cells due to low efficiency in transfection and screening, synthetase engineering is normally carried out in *E. coli* [34,35] or yeast [15,36,37] so that large mutant libraries can be easily screened. It is also necessary to modify the *E. coli* tRNA so that it decodes a blank codon instead of a codon corresponding to a canonical amino acid.
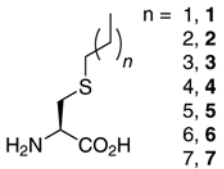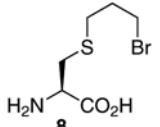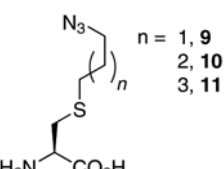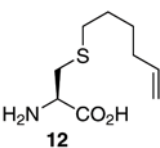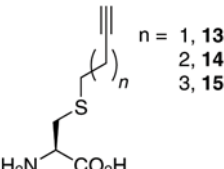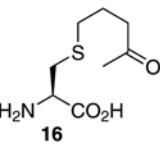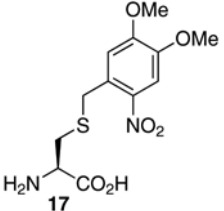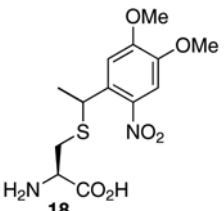
Based on the simplicity of the established methodology [38–40] and the promiscuity of many orthogonal synthetases towards different unnatural amino acids (*vide infra*) [41], the number of genetically incorporable unnatural amino acids has steadily increased. In addition, some orthogonal aaRS/tRNA pairs are mutually orthogonal [21,24–26,42] and can be used at the same time to incorporate multiple different unnatural amino acids into a protein of interest.

As recent reviews cover fundamental aspects of genetic code expansion [4,6,9,13], the engineering of new orthogonal synthetases [8], and general [5,10,11,14] or specific [7,12] applications of genetic code expansion in eukaryotic systems, we will focus on recent advances and applications of genetic code expansion for controlling protein function in mammalian cells through translational, optical or chemical means.
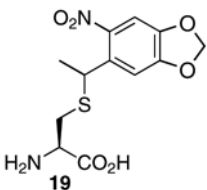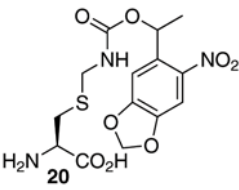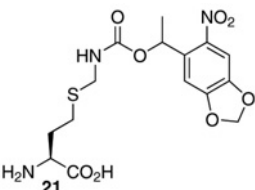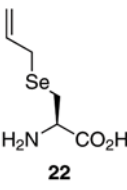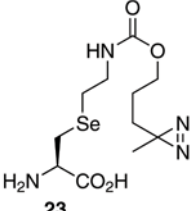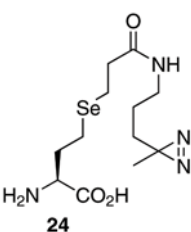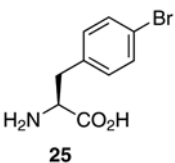
Translational control by amber suppression

Genetic code expansion by unnatural amino acid incorporation in response to an amber stop codon provides the simplest way to 'switch on' protein production. In this case, an amber stop codon is placed into the gene of interest (Figure 3) for incorporation of the unnatural amino acid into a permissive site of the target protein [43]. In the absence of the designated unnatural amino acid, protein translation stops prematurely at the amber stop codon, generating truncated and non-functional protein product and thus giving an effect as a nonsense mutation (Figure 3A). In the presence of the unnatural amino acid, the orthogonal tRNA is acylated and decodes the amber codon, leading to amber suppression and generation of full-length, functional protein product (Figure 3B). Thus, simple addition of the unnatural amino acid into the growth medium 'switches on' the protein production and function [44]. In contrast with commonly used systems for inducible mammalian protein expression (e.g. the tetracycline transcriptional transactivation) [45], the lag time is shorter in the translational control by amber suppression (i.e. time for translation and folding) than the gene activation approaches (i.e. time for transcription, mRNA processing, translation and folding). Amber suppression also allows a more stringent control, as the background activity (if any) can be further

Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date

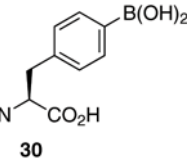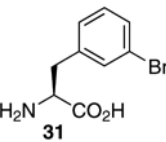| Amino acid	aaRS	Mutations	tRNA	Application
Cysteine and selenocysteine derivatives				
n = 1, **1**; 2, **2**; 3, **3**; 4, **4**; 5, **5**; 6, **6**; 7, **7**	*Ec*LeuRS [24]	M40I T252A Y499I Y527A H529G	*Ec*tRNA$_{CUA}^{Leu}$	Method development
8	*Ec*LeuRS [24]	M40I T252A Y499I Y527A H529G	*Ec*tRNA$_{CUA}^{Leu}$	Method development
n = 1, **9**; 2, **10**; 3, **11**	*Ec*LeuRS [24]	M40I T252A Y499I Y527A H529G	*Ec*tRNA$_{CUA}^{Leu}$	Method development
12	*Ec*LeuRS [24]	M40I T252A Y499I Y527A H529G	*Ec*tRNA$_{CUA}^{Leu}$	Method development
n = 1, **13**; 2, **14**; 3, **15**	*Ec*LeuRS [24]	M40I T252A Y499I Y527A H529G	*Ec*tRNA$_{CUA}^{Leu}$	Method development
16	*Ec*LeuRS [24]	M40I T252A Y499I Y527A H529G	*Ec*tRNA$_{CUA}^{Leu}$	Method development
17	*Ec*LeuRS [71]	M40G L41Q T252A Y499L Y527G H537F	*Ec*tRNA$_{CUA}^{Leu}$	Photoactivation
18	*Ec*LeuRS [72]	M40G L41Q Y499L Y527G H537F	*Ec*tRNA$_{CUA}^{Leu}$	Photoactivation
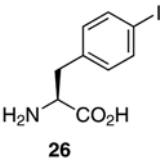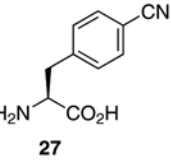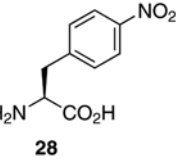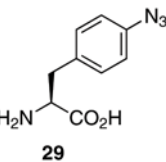
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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

Amino acid	aaRS	Mutations	tRNA	Application
19	*Mb*PylRS [73,74]	N311Q C313A V366M	MbtRNA$^{Pyl}_{CUA}$	Photoactivation
20	*Mb*PylRS [75]	M241F A267S Y271C L274M	MbtRNA$^{Pyl}_{CUA}$	Photoactivation
21	*Mb*PylRS [75]	M241F A267S Y271C L274M	MbtRNA$^{Pyl}_{CUA}$	Photoactivation
22	*Mb*PylRS [76]	C313W W382T	MbPyltRNA	Method development
23	*Mb*PylRS [40]	L274A C313S Y349F	MbtRNA$^{Pyl}_{CUA}$	Photocrosslinking
24	*Mb*PylRS [40]	L274A C313S Y349F	MbtRNA$^{Pyl}_{CUA}$	Photocrosslinking
Phenylalanine derivatives				
25	*Ec*TyrRS [77]	Y37V D182S F183M D265R	EctRNA$^{Tyr}_{CUA}$ BstRNA$^{Tyr}_{CUA}$	Method development
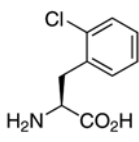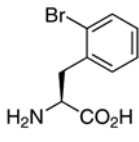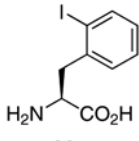
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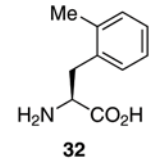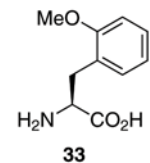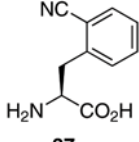**Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)**

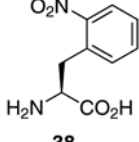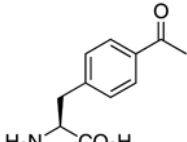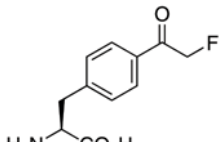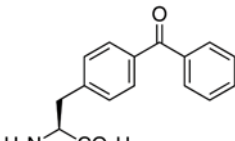| Amino acid	aaRS	Mutations	tRNA	Application
26	EcTyrRS [35,37,43,77]	Y37V D182S F183M D265R [77] Y37I D182S F183M [37,43] Y37V D165G D182S F183M L186A D265R [35]	EctRNA$_{CUA}^{Tyr}$ [35,77] BstRNA$_{CUA}^{Tyr}$ [37,43,77]	Method development [35,37,77] Protein engineering [43]
27	EcTyrRS [77]	Y37V D182S F183M D265R	EctRNA$_{CUA}^{Tyr}$ BstRNA$_{CUA}^{Tyr}$	Method development
28	EcTyrRS [77]	Y37V D182S F183M D265R	EctRNA$_{CUA}^{Tyr}$ BstRNA$_{CUA}^{Tyr}$	Method development
29	EcTyrRS$_{CUA}$ [16,17,35,37,38,43,49, 55,68,77–96] EcTyrRS$_{UCA}$ [25]	Y37L D182S F183A L186A D265R [78,81,84,85] Y37V D182S F183M D265R [77,90] Y37L D182S F183M L186A [16,17,37,38,43,49,55,68,79, 80,82,83,86–89,91–96] Y37V D165G D182S F183M L186A D265R [35]	EctRNA$_{CUA}^{Tyr}$ [35,77,78,81,86,90] BstRNA$_{CUA}^{Tyr}$ [16,17,37,38,43,49,55,68,77,79, 80,82–85,87–96] EctRNA$_{UCA}^{Tyr}$ BstRNA$_{UCA}^{Tyr}$	Bioorthogonal labelling [38,79,83,87–89,96] Method development [17,25,35,37,77,78,90] Photocrosslinking [38,68,81,84,85,91–95] Protein engineering [43,49,55,83] Spectroscopic probe [16,80,82,86]
30	EcTyrRS [35,97]	Y37V D182S F183M Y37I D182S F183M D265R [97] Y37S D182S F183A L186E D265R [35] Y37G D182S F183I L186E D265R [35] Y37S D182S F183I L186E D265R [35]	EctRNA$_{CUA}^{Tyr}$ [35,97] BstRNA$_{CUA}^{Tyr}$ [97]	Method development [35] Spectroscopic probe [97]
31	MmPylRS [50]	L301M Y306L L309A C348F	MmtRNA$_{CUA}^{Pyl}$	Method development
32	MmPylRS [98]	N346A C348A	MmtRNA$_{CUA}^{Pyl}$	Method development
33	MmPylRS [98]	N346A C348A	MmtRNA$_{CUA}^{Pyl}$	Method development
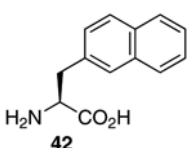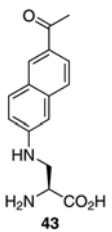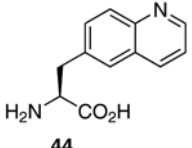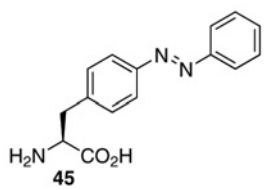
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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

Amino acid	aaRS	Mutations	tRNA	Application
34	*Mm*PylRS [98]	N346A C348A	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
35	*Mm*PylRS [98]	N346A C348A	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
36	*Mm*PylRS [98]	N346A C348A	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
37	*Mm*PylRS [98]	N346A C348A	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
38	*Mm*PylRS [98]	N346A C348A	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
39	*Ec*TyrRS$_{CUA}$ [21,35,37,43,77,83,87,90,99,100] *Ec*TyrRS$_{UCA}$ [25]	Y37I N165G D182G F183M L186A [83,99] Y37I D182G F183M L186A [37,43,87,100] Y37V D182S F183M D265R [21,77,90] Y37V D165G D182S F183M L186A D265R [35] Y37V D182S F183M	*Ec*tRNA$^{Tyr}_{CUA}$ [21,35,77,90] *Bs*tRNA$^{Tyr}_{CUA}$ [37,43,77,83,87,99,100] *Ec*tRNA$^{Tyr}_{UCA}$ *Bs*tRNA$^{Tyr}_{UCA}$	Bioorthogonal labelling [83,87,100] Method development [25,35,37,77,83,90,99] Protein engineering [21,43]
40	*Ec*TyrRS [84,101]	Y37I D182G F183M L186A D265R	*Bs*tRNA$^{Tyr}_{CUA}$	Chemical crosslinking [84,101] Method development [101]
41	*Ec*TyrRS [15,37,55,78,85,92,94,95,99,102–105] *Mm*PylRS [106]	Y37G D182G L186A D265R [78,85,103] Y37G D182G L186A [15,55,92,94,95,99,102,104,105] Y37G D182G F183Y L186M [37] A302T N346T C348T W417C [106]	*Ec*tRNA$^{Tyr}_{CUA}$ [15,78,103] *Bs*tRNA$^{Tyr}_{CUA}$ [37,55,85,92,94,95,99,102,104,105] *Mm*tRNA$^{Pyl}_{CUA}$ [106]	Mechanistic studies [15] Method development [37,78,95,99,103,106] Photocrosslinking [85,92,94,102,104,105] Photoinhibition [55]
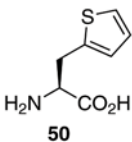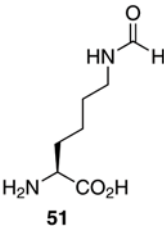
Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

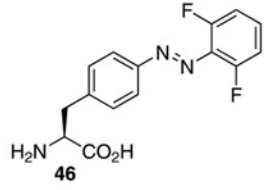| Amino acid	aaRS	Mutations	tRNA	Application
 42	*Mm*PylRS [106]	A302T N346G C348T V401I W417Y	*Mm*tRNA$_{CUA}^{Pyl}$	Method development
 43	*Ec*LeuRS [107–109]	L38F M40G L41P Y499V Y500L Y527A H537E L538S F541C A560V	*Ec*tRNA$_{CUA}^{Leu}$	Method development [107,108] Spectroscopic probe [109]
 44	*Mm*PylRS [110]	N346Q C348S V401G W417T	*Mm*tRNA$_{CUA}^{Pyl}$	Spectroscopic Probe
 45	*Mb*PylRS [111]	L270F L274M N311G C313G Y349F	*Mb*tRNA$_{CUA}^{Pyl}$	Photoswitching
 46	*Mb*PylRS [111]	L270F L274M N311G C313G Y349F	*Mb*tRNA$_{CUA}^{Pyl}$	Photoswitching
 47	*Mb*PylRS [111]	L270F L274M N311G C313G Y349F	*Mb*tRNA$_{CUA}^{Pyl}$	Photoswitching
 48	*Mm*PylRS [56,112]	A302T L309S N346V C348G	*Mm*tRNA$_{CUA}^{Pyl}$	Method development [112] Photoswitching [56]
Histidine derivatives				
 49	*Ma*PylRS [26] *Mb*PylRS [113]	L121M L125I Y126F M129A V168F L270I Y271F L274G C313F Y349F	*Ma*tRNA$_{CUA}^{Pyl}$ *Mb*tRNA$_{CUA}^{Pyl}$	Method development

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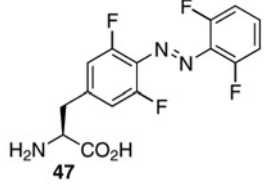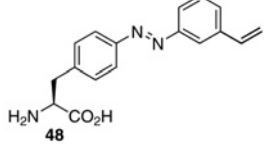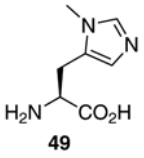## Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

Amino acid	aaRS	Mutations	tRNA	Application
50	*Mb*PylRS [113]	L270I Y271F L274G C313F Y349F	*Mb*tRNA$_{CUA}^{Pyl}$	Method development
Lysine derivatives				
51	*Mb*PylRS [114]	L266M L270I Y271F L274A C313F	*Mb*tRNA$_{CUA}^{Pyl}$	Method development
52	*Mb*PylRS [115–117] *Mm*PylRS [44,50,118]	D76G L266V L270I Y271F L274A C313F [115] D76G L266M L270I Y271F L274A C313F [116,117] L305I Y306F L309A C348F [118] L301M Y306L L309A C348F [44,50]	*Mb*tRNA$_{CUA}^{Pyl}$ *Mm*tRNA$_{CUA}^{Pyl}$ [50,118] *Mm*tRNA$_{UUA}^{Pyl}$ [44]	Method development [44,50,115,117,118] Spectroscopic probe [116]
53	*Mm*PylRS [50]	L301M Y306L L309A C348F	*Mm*tRNA$_{CUA}^{Pyl}$	Method development
54	*Mb*PylRS [119]	D76G L266M L270I Y271F L274A C313F	*Mb*tRNA$_{CUA}^{Pyl}$	Method development
55	*Mb*PylRS [77,120]	L274A C313F Y349F [120] wt [77]	*Mb*tRNA$_{CUA}^{Pyl}$	Method development

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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)
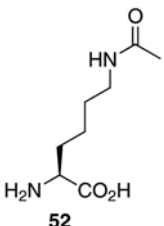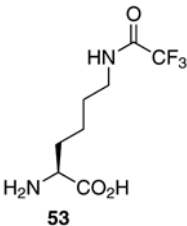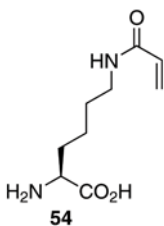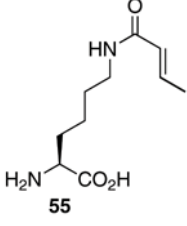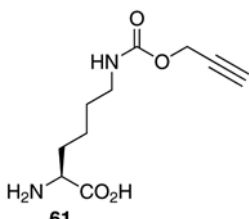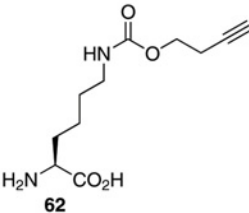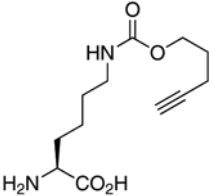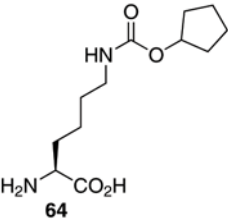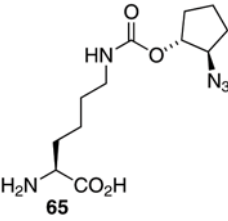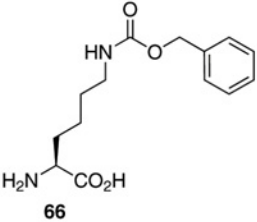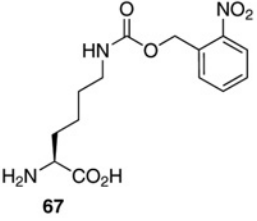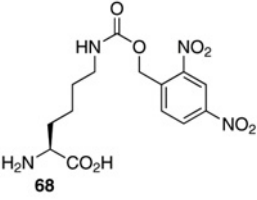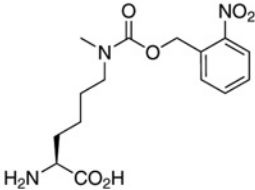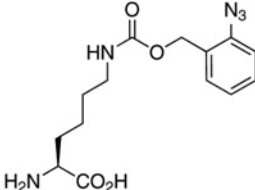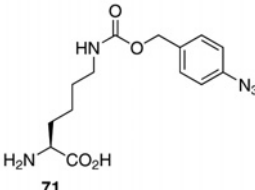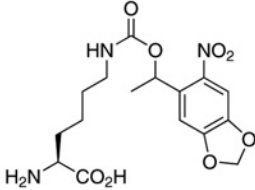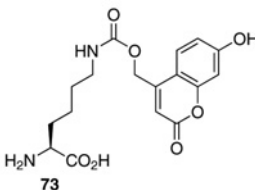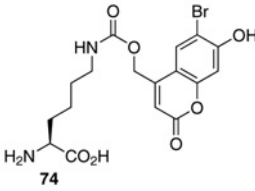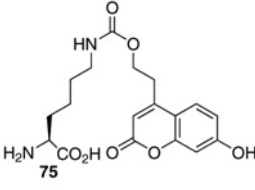
Amino acid	aaRS	Mutations	tRNA	Application
56, 57	*Mb*PylRS [121]	Y271M L274A C313A	*Mb*tRNA$^{Pyl}_{CUA}$	Photocrosslinking
58	*Mm*PylRS [122]	Y306V L309A C348F Y384F	*Mm*tRNA$^{Pyl}_{CUA}$	Photocrosslinking
59	*Ma*PylRS [26] *Mb*PylRS [21,25,44,125,129,130] *Mm*PylRS [22,26,44,47,69,73,77,106, 115,117,118,123,124,126–128]	wt wt wt	*Ma*tRNA$^{Pyl}_{CUA}$ *Mb*tRNA$^{Pyl}_{CUA}$ [25,44,125,129,130] *Mb*tRNA$^{Pyl}_{UUA}$ [44] *Mb*tRNA$^{Pyl}_{UCA}$ [25,44] *Mm*tRNA$^{Pyl}_{CUA}$ [21,22,26,47,69,73,77, 106,115, 118,123,124,126,127] *Mm*tRNA$^{Pyl}_{CUA}$ *B*U25C*B* [22,117,128] *Mm*tRNA$^{Pyl}_{UUA}$ [21,44] *Mm*tRNA$^{Pyl}_{UCA}$ [21]	Method development [21,22,25,26,44,47,69,73,77,106, 115,117,118,123–130]
60	*Mb*PylRS [21,24,25,48,77,131–137] *Mm*PylRS [127]	Wt [21,24,25,48,77,132–136] L274A C313S Y349F [131] Y349F [137] wt	*Mb*tRNA$^{Pyl}_{CUA}$ [25,77,131] *Mb*tRNA$^{Pyl}_{UCA}$ [24,25,48,132–136] *Mm*tRNA$^{Pyl}_{CUA}$ [127,137] *Mm*tRNA$^{Pyl}_{UUA}$ [21]	Bioorthogonal labelling [127,131,137] Imaging [136] Method development [24,25,77,134,137] Protein engineering [21,48,132,133,135–137]
61	*Mb*PylRS [57,69,77,138,139] *Mm*PylRS [69,129,131]	wt wt	*Mb*tRNA$^{Pyl}_{CUA}$ *Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling [131,139] Chemical decaging [57] Imaging [69,129,138] Method development [69,77]
62	*Mb*PylRS [25,130] *Mm*PylRS [130]	wt wt	*Mb*tRNA$^{Pyl}_{CUA}$ [25] *Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling [130] Method development [25]

Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

Amino acid	aaRS	Mutations	tRNA	Application
63	*Mb*PylRS [131,140]	L274A C313S Y349F	*Mb*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling
64	*Mm*PylRS [141]	wt	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
65	*Mb*PylRS [140]	wt	*Mb*tRNA$^{Pyl}_{CUA}$	Method development
66	*Mm*PylRS [118,142,143]	R61K G131E L309A C348V Y384F [118] Y306A Y384F [142] R61K G131E Y306A Y384F [143]	*Mm*tRNA$^{Pyl}_{CUA}$ [118,142] *Mm*tRNA$^{Pyl}_{CUA}$*B*U25C [143]	Method development [118,142,143]
67	*Mb*PylRS [140] *Mm*PylRS [61,141,144]	Y271I L274A C313A Y349F Y306M L309A C348A Y384F	*Mb*tRNA$^{Pyl}_{CUA}$ *Mm*tRNA$^{Pyl}_{CUA}$	Method development [140,141] Photoactivation [61,144]
68	*Mb*PylRS [145]	Y271M L274T C313A Y349F	*Mb*tRNA$^{Pyl}_{CUA}$	Method development
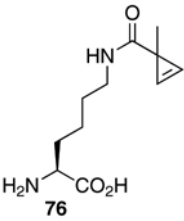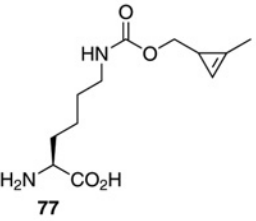
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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)
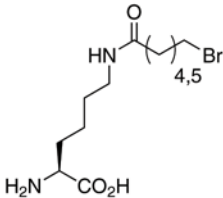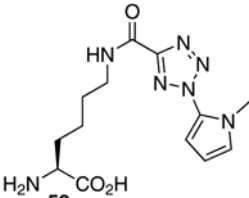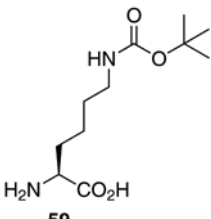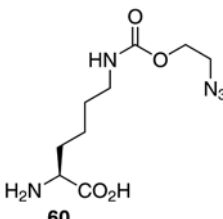
Amino acid	aaRS	Mutations	tRNA	Application
69	*Mb*PylRS [146]	Y271I 274M C313A	*Mb*tRNA$_{CUA}^{Pyl}$	Method development
70	*Mb*PylRS [63]	Y271A Y349F	*Mb*tRNA$_{CUA}^{Pyl}$	Chemical decaging
71	*Mb*PylRS [62]	L274A C313S Y349F	*Mb*tRNA$_{CUA}^{Pyl}$	Bioorthogonal labelling Chemical decaging
72	*Mb*PylRS [66,67,69,75,125,147–152]	M241F A267S Y271C L274M [66,67,69,75,125,147–152]	*Mb*tRNA$_{CUA}^{Pyl}$ [66,67,69,75,125,147–152] *Mb*tRNA$_{CUA}^{Pyl}$ U25C [66]	Method development [69] Photoactivation [66,67,75,125,147–152]
73	*Mb*PylRS [153]	Y271A L274M	*Mb*tRNA$_{CUA}^{Pyl}$	Photoactivation
74	*Mb*PylRS [153]	Y271A L274M	*Mb*tRNA$_{CUA}^{Pyl}$	Photoactivation
75	*Mb*PylRS [153]	Y271A L274M	*Mb*tRNA$_{CUA}^{Pyl}$	Method development
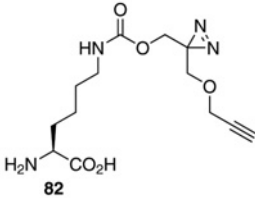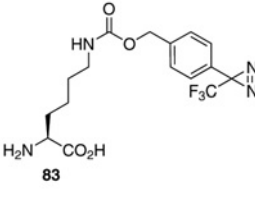
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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

Amino acid	aaRS	Mutations	tRNA	Application
76	*Mb*PylRS [154]	L266M L270I Y271L L274A C313	*Mb*tRNA$^{Pyl}_{CUA}$	Method development
77	*Mb*PylRS [24,25] *Mm*PylRS [22,69,115,123,155,156] *Mx1201*PylRS [155]	wt Wt [22,69,115,123,155,156] Y306A Y384F [155] wt	*Mb*tRNA$^{Pyl}_{CUA}$ [25] *Mb*tRNA$^{Pyl}_{UCA}$ [24] *Mm*tRNA$^{Pyl}_{CUA}$ *B*U25C*B* [22,155] *Mm*tRNA$^{Pyl}_{CUA}$ [69,115,123,156] *M*x1201tRNA$^{Pyl}_{CUA}$ *M*x1201tRNA$^{Pyl}_{CUA}$ C41CA	Imaging [123] Method development [22,24,25,69,115,155,156]
78	*Mm*PylRS [124]	wt	*Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling
79	*Mb*PylRS [77,157,158] *Mm*PylRS [155,159]	Wt [77,158] L274M 313A Y349F [157] Y306A Y384F	*Mb*tRNA$^{Pyl}_{CUA}$ *Mm*tRNA$^{Pyl}_{CUA}$ [159] *Mm*tRNA$^{Pyl}_{CUA}$ U25C [155]	Method development [77,155,159] Photocrosslinking [157,158]
80	*Mm*PylRS [159]	Y306A Y384F	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
81	*Mb*PylRS [132,140,160]	L274A C313S Y349F	*Mb*tRNA$^{Pyl}_{CUA}$	Method development [140] Photocrosslinking [132,160] Protein engineering [132]
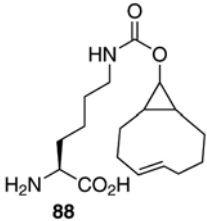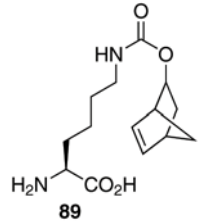
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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

Amino acid	aaRS	Mutations	tRNA	Application
82	*Mm*PylRS [159]	Y306A Y384F	*Mm*tRNA$_{CUA}^{Pyl}$	Method development
83	*Mm*PylRS [142]	Y306A Y384F	*Mm*tRNA$_{CUA}^{Pyl}$	Photocrosslinking
84	*Mm*PylRS [143]	R61K G131E Y306A Y384F	*Mm*tRNA$_{CUA}^{Pyl}$ *B*U25C	Photocrosslinking
85	*Mm*PylRS [18,39,59–61,123,155,161–166] *Mx1201*PylRS [155]	Y306A Y384F [18,39,59–61,123,155,161–166] Y126A	*Mm*tRNA$_{CUA}^{Pyl}$ [18,39,59–61,123,161–166] *Mm*tRNA$_{CUA}^{Pyl}$ *B*U25C*B*[155] *M*x1201tRNA$_{CUA}^{Pyl}$	Imaging [123,161,162,164,166] Chemical decaging [18,59–61] Chemical crosslinking [163] Method development [155,165] Protein labelling [39]
86	*Mb*PylRS [123,167,168] *Mm*PylRS [124,169]	Y271A L274M C313A Y306A Y384F [169] Y306A L309M C348A [124]	*Mb*tRNA$_{CUA}^{Pyl}$ *Mm*tRNA$_{CUA}^{Pyl}$	Bioorthogonal labelling [124,167] Imaging [123,168] Method development [169]
87	*Mm*PylRS [165]	Y306A Y384F	*Mm*tRNA$_{CUA}^{Pyl}$	Method development

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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

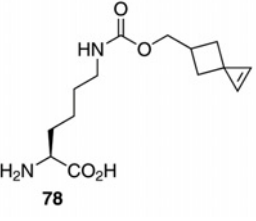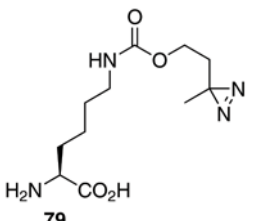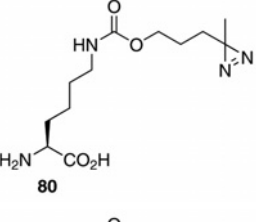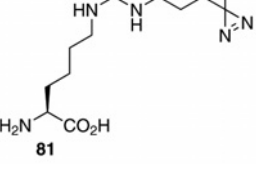| Amino acid	aaRS	Mutations	tRNA	Application
88	*Mb*PylRS [167,168] *Mm*PylRS [169]	Y271A L274M C313A Y306A Y384F	*Mb*tRNA$^{Pyl}_{CUA}$ *Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling [167] Method development [167,169]
89	*Mb*PylRS [24] *Mm*PylRS [39,127,131,169]	wt Wt [127,131] Y306A Y384F [39,169]	*Mb*tRNA$^{Pyl}_{UCA}$ *Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling [39,127,131] Method development [24,169]
90	*Mm*PylRS [169]	Y306A Y384F	*Mm*tRNA$^{Pyl}_{CUA}$	Method development
91	*Mm*PylRS [39,161,166,169,170]	Y306A Y384F	*Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling [39] Imaging [161,166,170] Method development [169]
92	*Mb*PylRS [19,64,140,168,171,172] *Mm*PylRS [39,123,128,131,155,159,161, 165–167]	Y271M L274G C313A [19,64,168,171,172] M241F A267S Y271C L274M [140] Y306A 384F [39,123,128,131,155,159, 161, 165–167]	*Mb*tRNA$^{Pyl}_{CUA}$ [64,140,168,171,172] *Mb*tRNA$^{Pyl}_{CUA}$U25C [19] *Mm*tRNA$^{Pyl}_{CUA}$ [39,123,128,131,159,161, 165–167] *Mm*tRNA$^{Pyl}_{CUA}$*B*U25C [155]	Chemical inhibition [64] Bioorthogonal labelling [39,131,167] Imaging [123,128,161,166,168,171,172] Method development [155,159,165] Protein engineering [140] Spectroscopic probe [19]
Tryptophan derivatives				
93	*Ec*TrpRS [34]	S8A V144S V146A S8A V144G V146C	*Ec*tRNA$^{Trp}_{CUA}$	Method development

Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)
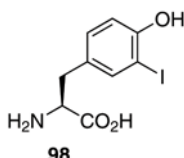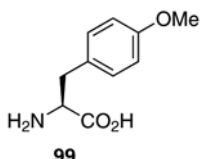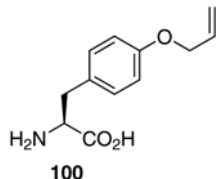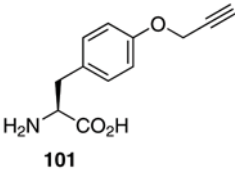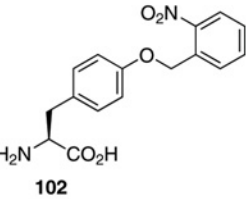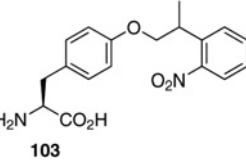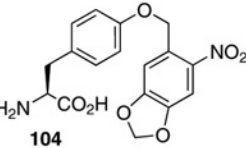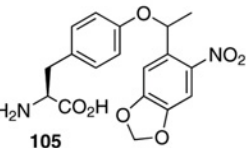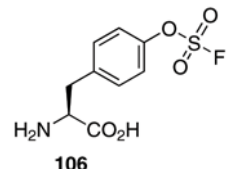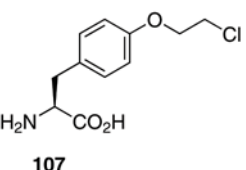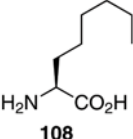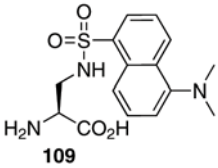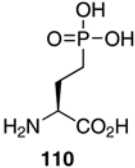
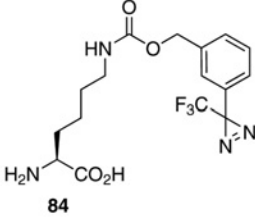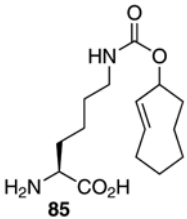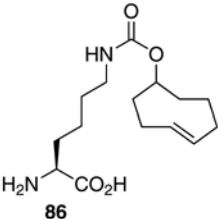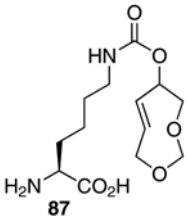| Amino acid	aaRS	Mutations	tRNA	Application
94	*Ec*TrpRS [34]	S8A V144S V146A	EctRNA$_{CUA}^{Trp}$	Method development
95	*Ec*TrpRS [34]	S8A V144G V146C	EctRNA$_{CUA}^{Trp}$	Method development
96	*Ec*TrpRS [34]	S8A V144G V146C	EctRNA$_{CUA}^{Trp}$	Method development
97	*Ec*TrpRS [34]	S8A V144G V146C	EctRNA$_{CUA}^{Trp}$	Method development
Tyrosine derivatives				
98	EcTyrRS [46]	Y37V Q195C	BstRNA$_{CUA}^{Tyr}$	Method development
99	*Ec*TyrRS$_{CUA}$ [15,21,35,37,77,78,90] *Ec*TyrRS$_{UCA}$ [25]	Y37T D182T F183M D265R [78] Y37V D182S F183M [37] Y37V D182S F183M D265R [21,77,90] Y37T D182T F183M [15] Y37V D165G D182S F183M L186A D265R [35] Y37V D182S F183M	EctRNA$_{CUA}^{Tyr}$ [15,21,35,77,78,90] BstRNA$_{CUA}^{Tyr}$ [21,37,77,90] EctRNA$_{UCA}^{Tyr}$ BstRNA$_{UCA}^{Tyr}$	Mechanistic studies [15] Method development [21,25,35,37,77,78,90]
100	*Ec*TyrRS [77]	Y37V D182S F183M D265R	EctRNA$_{CUA}^{Tyr}$ BstRNA$_{CUA}^{Tyr}$	Method development

Continued over

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Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)

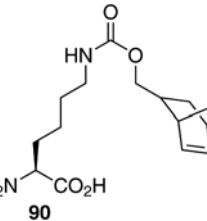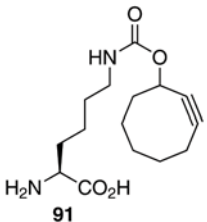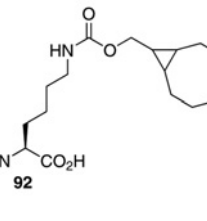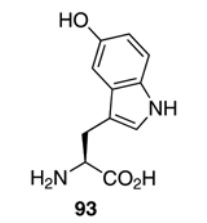| Amino acid	aaRS	Mutations	tRNA	Application
101	*Ec*TyrRS [25,35,37,77]	Y37V D182S F183M D265R [77] Y37S D182T F183M L186V [37] Y37V D182S F183M [25] Y37V D165G D182S F183M L186A D265R [35]	*Ec*tRNA$^{Tyr}_{CUA}$ [25,35,77] *Bst*RNA$^{Tyr}_{CUA}$ [37,77]	Method development
102	*Mb*PylRS [126,148,173]	L270F L274M N311G C313G Y349F [173] L270F L274M N311G C313G [126,148]	*Mb*tRNA$^{Pyl}_{CUA}$	Photoactivation
103	*Mb*PylRS [173]	L270F L274M N311G C313G Y349F	*Mb*tRNA$^{Pyl}_{CUA}$	Photoactivation
104	*Mb*PylRS [173]	L270F L274M N311G C313G Y349F	*Mb*tRNA$^{Pyl}_{CUA}$	Photoactivation
105	*Mb*PylRS [173]	L270F L274M N311G C313G Y349F	*Mb*tRNA$^{Pyl}_{CUA}$	Photoactivation
106	*Mm*PylRS [174]	N346T C348I Y384L W417K	*Mm*tRNA$^{Pyl}_{CUA}$	Bioorthogonal labelling
107	*Ec*TyrRS [35]	Y37V D182S F183M D265R Y37V D165G D182S F183M L186A D265R	*Ec*tRNA$^{Tyr}_{CUA}$	Method development
Miscellaneous unnatural amino acids				
108	*Ec*LeuRS [24,25]	M40I T252A Y499I Y527A H529G [24] E20K M40V L41S T252R Y499S Y527L H529G H537G [25]	*Ec*tRNA$^{Leu}_{CUA}$	Method development

Continued over

Table 1 Overview of unnatural amino acids that have been successfully incorporated into proteins in mammalian cells and used for a variety of applications to date (Continued)
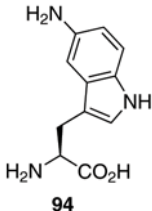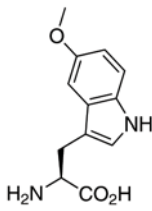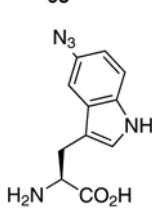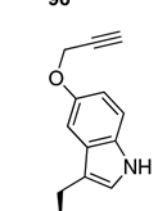
Amino acid	aaRS	Mutations	tRNA	Application
109	*Ec*LeuRS [15,103,175]	M40A L41N T252A Y499I Y527G H537T	*Ec*tRNA$_{CUA}^{Leu}$	Mechanistic studies [15,103] Method development [175]
110	*Mm*SepRS [176]	wt	*Mj*tRNA$_{CUA}^{Cys}$	Method development

Method development includes demonstration of incorporation, optimisation of incorporation efficiency, application as a control substrate, proof-of-principle of a technique for subsequent studies etc. Abbreviations: Ma, *Methanomethylophilus alvus*; wt, wild type.

minimised by including multiple amber codons into the gene of interest. The translational control by amber suppression approach is fully complementary to conventional genetic approaches (e.g. knockout, knockdown) that deplete a protein in cells to 'switch off' its function. In addition, the unnatural amino acid approach is reversible, as removing the unnatural amino acid in the growth medium will 'switch off' the translation of the protein of interest.

The translational switch-on process has been widely employed as a reporter system to test incorporation of new unnatural amino acids by using luminescent proteins like green fluorescent protein [46] or luciferase [20]. Upon successful incorporation, cells can emit light, whose intensity directly correlates to the unnatural amino acid incorporation efficiency. Apart from the reporter strategy, this approach has also been used to regulate function of other proteins, such as Cas9 for controllable gene editing in mouse embryos [47].
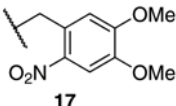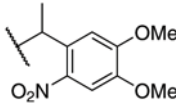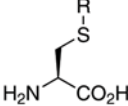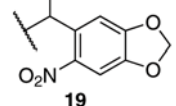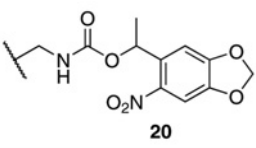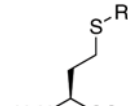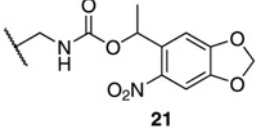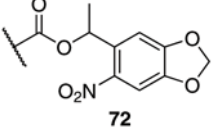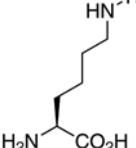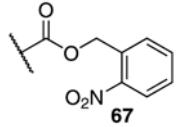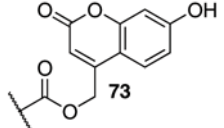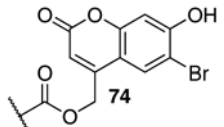
Besides the general use of the 'translational activation' approach to study protein function, this principle has been proven to be powerful in controlling virus replication [43,48,49]. By introducing TAG codons within the virus genes, viruses can only be generated using cell lines containing an orthogonal synthetase/tRNA$_{CUA}$ pair, and the resulting viruses are replication-incompetent in normal cells due to the lack of amber suppressor tRNA (Figure 4A) [43,48]. Such replication-incompetent viruses offer an additional tier of control for live-attenuated vaccines and significantly increase their safety. This concept has been further developed by including the genes encoding the orthogonal aaRS/tRNA pair into the viral genome (Figure 4B) [49]. In this case, viruses can be replicated in wild-type cells and the native hosts, as long as the unnatural amino acid is supplemented. Here, spatial control can also be achieved by local administration of the unnatural amino acid as demonstrated in examples of mice with an expanded genetic code [50,51]. Thus, the approach can be used for controlling viral vectors in gene therapy, where spatiotemporal virus replication and gene editing are highly desirable.

While the translational control approach is quite simple, the response is not instantaneous. There is always a lag time from when the unnatural amino acid is administered into the culture medium until the full-length protein is produced and folded. Similarly, depleting the unnatural amino acid in the growth medium will stop production of new proteins, but the protein function will only be completely switched off when all previously produced proteins are degraded in the cells. Thus, the kinetics of the switching off process largely depend on the half-life of the protein, so the response rate is the same as with genetic knockdown.

Light-induced activation or inhibition
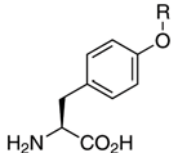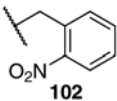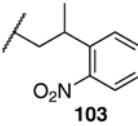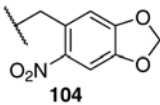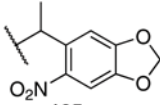
The slower kinetics of the translational control approach limit its applicability to study biological processes where fast response is needed. This can be addressed by using light to unmask or modify unnatural amino acids and subsequently regulate protein function. Depending on the nature of the light-responsive group, it is possible to either activate, inhibit, or reversibly switch on/off protein function (Table 2). Unnatural amino acids containing a photocage (i.e. a photolabile protecting group) [52] have been widely used for protein activation. When replacing a functionally critical amino acid residue with the corresponding photocaged amino acid, the target protein becomes inactive; upon

Table 2 Overview of photocaged unnatural amino acids that have been incorporated in mammalian cell systems for activating protein function upon irradiation with light of specific wavelength λ

Amino acid	Photocaging group (R)	System	Proteins	λ_{decag} (nm)
	17		eGFP, potassium channel Kir2.1 [71]	385
			eGFP [72]	
	18		eGFP, mCherry, *Npu* DnaE intein, Src kinase [72]	Long wavelength UV
	19	HEK293T	TEV protease [73,74], *Npu* DnaE intein [74]	
	20		sfGFP, luciferase [75]	365
	21	HEK 293T	sfGFP, luciferase [75]	365
	72	HEK293 [67,125] HEK293T [66,75,147–150,152] HEK293ET [151] HeLa [150,152]	Nuclear localisation peptide for subcellular localisation of SATB1 and FOXO3 transcription factors, and TEV protease [149]	350
			sfGFP, luciferase [75]	
			p53 transcription factor [125]	
			Isocitrate dehydrogenase [67]	
			Cas9 endonuclease [150]	
			Cre recombinase [148]	
			Capsid of adeno-associated virus 2 [147]	365
			T7RNA polymerase [152]	
			MEK1 kinase [151]	
			LCK kinase [66]	405
	67	HEK293T	Luciferase [144]	365
	73	HEK293T or CHO K1	eGFP and luciferase [153]	365 405
	74			365 405 760[1]

Continued over

Table 2 Overview of photocaged unnatural amino acids that have been incorporated in mammalian cell systems for activating protein function upon irradiation with light of specific wavelength λ (Continued)

Amino acid	Photocaging group (R)	System	Proteins	λ_{decag} (nm)
	102	HEK293 [126] HEK293T [148,173]	Cre recombinase [148]STAT1transcription factor [126]Luciferase [173]	
	103			365
	104	HEK293T	luciferase, TEV protease [173]	
	105			
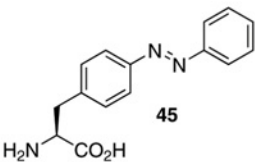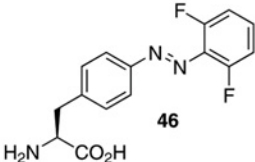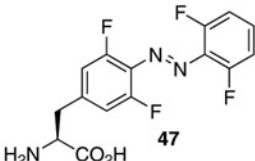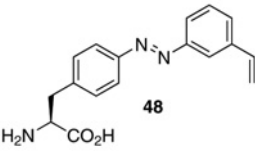
[1]Decaging is only achieved at this wavelength via a two-photon activation using a specialised multiphoton laser setup. Abbreviation: Npu, *Nostoc punctiforme*.

light irradiation, the photocage is removed, thereby restoring the protein's function. To date, photocaged cysteines (**17–21**), lysines (**67, 72–74**) and tyrosines (**102–105**) have been used to control enzyme function, intein splicing, protein subcellular localisation, virus–host interactions, and cell signalling cascades [13]. The light-activation approach is particularly useful for kinetic studies as it provides extreme spatiotemporal resolution. Spatial control can be achieved to even subcellular locations using focused light beams, which is virtually impossible when using the translational control approach. Theoretically, it is also possible to incorporate photocaged serine in mammalian cells as it has been demonstrated in yeast [53]. Therefore, the light-activation approach is applicable to regulate any protein that has a functionally critical cysteine, lysine, tyrosine, or serine residue in mammalian cells, including but not limited to kinases, DNA- and RNA-binding proteins, proteases, phosphatases, oxidoreductases, isomerases, and ubiquitin-modifying enzymes [54].

In contrast, the incorporation of a photocrosslinking amino acid can be used to inhibit protein function upon light irradiation [55]. In this case, a photocrosslinking amino acid is placed in the interior of the target protein. Upon light irradiation, a highly reactive functionality (e.g. radical, nitrene, carbene) is generated and reacted non-specifically with a nearby amino acid residue, causing cross-linking of the protein and subsequent abolishment of the protein's activity. The feasibility of this approach has been demonstrated with the use of *p*-benzoylphenylalanine (**41**) in the study of glutamate receptors, GluA1 and GluA2 [55]. When compared with the use of a photocaged amino acid, inhibiting a protein by photocrosslinking does not rely on the existence of a functionally critical residue, and thus theoretically, it can be used to investigate any protein in mammalian cells. Nevertheless, for each protein target it is necessary to screen a suitable site for placing the photocrosslinking amino acid. Protein variants containing the photocrosslinking amino acid must (i) behave in the same way as the wild-type protein (i.e. phenotypically silent) before light irradiation; and (ii) be fully inhibited after light irradiation causing the photocrosslinking. Due to these criteria, the screening process can be laborious and time-consuming.

In addition to light-induced activation and inhibition, reversible regulation of a protein function can be achieved through incorporation of a photoswitchable amino acid (Table 3). For example, **48**, containing an azobenzene functionality which undergoes reversible *cis-trans* isomerisation upon irradiation with blue and UV light, has been used to control the activity of a glutamate receptor [56]. However, the general applicability of this approach suffers from similar constraints as inhibition by photocrosslinking. Extensive screening is often needed to identify a suitable site for incorporation, such that the resulting protein variant is fully active or inactive upon irradiation with light of a specific wavelength. At the current state of the art, there is no guarantee that such a site can be found in the target protein.

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Table 3 Overview of photoswitchable unnatural amino acids that have been incorporated in mammalian cell systems used to modulate protein function upon irradiation with the given wavelengths λ

Amino acid	System	Proteins	$\lambda_{trans\text{-}cis}$ (nm)	$\lambda_{cis\text{-}trans}$ (nm)
45			355	450
46	HEK 293T	Luciferase [111]	530	405
47				
48		NMDAR glutamate receptor [56]	365	460

Overall, the use of light-responsive amino acids offers superior temporal control of protein function as the response is significantly faster (seconds) than the translational control by amber suppression (minutes to hours). Additionally, spatial control can be achieved at subcellular level, which is not possible with the translational approach. Generally, UV light at approximately 360 nm (i.e. UVA) is required (Tables 2 and 3) to induce the change (i.e. decaging, cross-linking, or isomerisation). However, UVA light has been shown to alter cellular signalling processes [57] or influence proper cellular function, if high intensity irradiation is applied (i.e. 50 J.cm^{-2}) [58]. Though not necessarily problematic, this has to be considered when planning to apply light-responsive unnatural amino acids. Thus, there is a trend to develop new functionalities that can be modulated by light of higher wavelengths [52]. In particular, red and near-infrared light (650–750 nm) are appealing because they cause no harm to cells even under excessive exposure, and they can penetrate tissues for *in vivo* applications. To date, coumarin-caged lysines (**73** and **74**) are the only genetically incorporable unnatural amino acids that can be decaged within these wavelengths, although by two-photon approach that requires a specialised multiphoton laser setup [52]. Nevertheless, with the continuous advances in light-responsive chemical functionalities and orthogonal aaRS engineering, it is expected that more unnatural amino acids with the desired photophysical properties can be incorporated through genetic code expansion.

Small-molecule induced activation or inhibition

In addition to light, small molecules can also be used to unmask or modify unnatural amino acids and subsequently regulate protein function with prompt response. For example, several protecting groups can be removed bioorthogonally inside live mammalian cells, and these chemistries have been used to switch on protein function by genetic code expansion. Intracellular bioorthogonal reactions that have been used in this purpose include inverse electron demand Diels–Alder reactions [18,59–61], 1,3-dipolar cycloadditions [62], Staudinger reactions [63], and palladium-catalysed propargyl removal (Table 4) [57]. Currently, all of these have only been demonstrated in caged lysine molecules (**61**, **70, 71**, **85**) through a number of examples, including activation of luciferases, kinases, nucleases etc. Theoretically, all these protecting groups can be applied to other nucleophilic amino acids (e.g. cysteine, serine, threonine, tyrosine) subjected to successful engineering of the corresponding orthogonal synthetases.
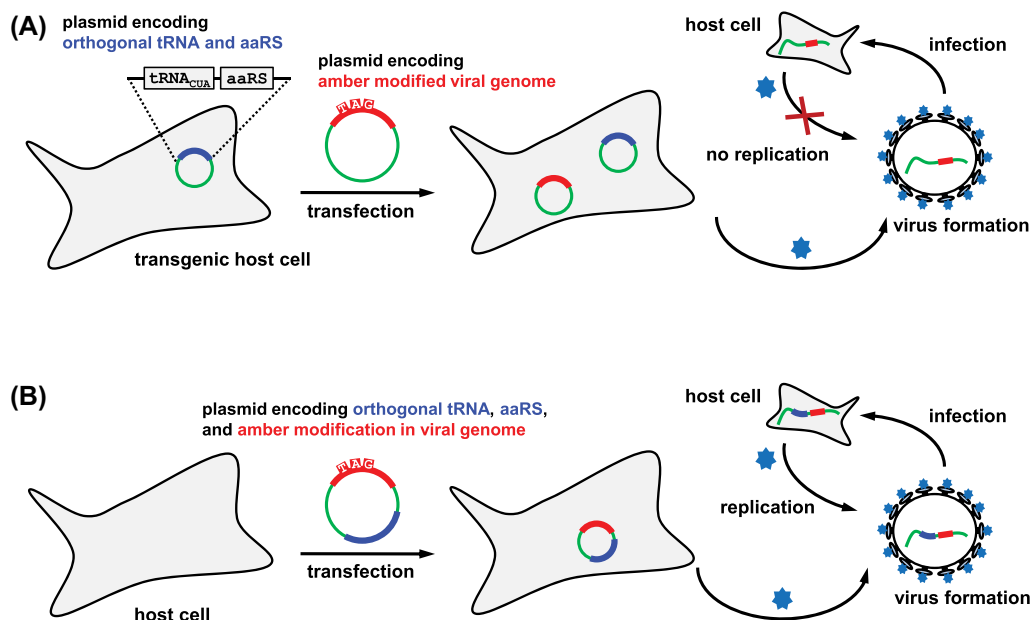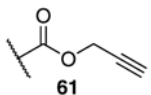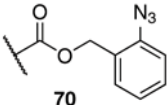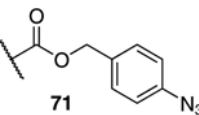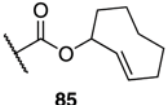
Figure 4. Translational activation approaches to control virus replication

(**A**) Use of genetic code expansion to control replication of an amber codon tagged virus within transgenic host cells containing the orthogonal tRNA/synthetase pairs [43,48]. (**B**) Use of genetic code expansion to control replication of an amber codon tagged virus within normal host cells with the orthogonal tRNA/synthetase pair gene encoded by the viral genome [49].

Table 4 Overview of bioorthogonally protected unnatural amino acids tested in mammalian cell systems and their deprotection conditions

Amino acid	System	Proteins	Reaction	Reagent
61	HeLa, CHO, HEK293T, IH3T3, Caco-2, A549, HeLa	GFP, OspF phosphothreonine lyase	Pd-catalysed Tsuji–Trost-like reaction	Pd(II) complexes [57]
70	HEK293T	eGFP, SATB1 transcription factor, Cre recombinase, Cas9 endonuclease	Staudinger	Various phosphines [63]
71	HEK293T	eGFR, luciferase, OspF phosphothreonine lyase, Src kinase	1,3-dipolar cycloaddition	*trans*-cyclooctenes [62]
85	HEK293T	GFP [61], Luciferase [59,61], MEK1 [18,60] and MEK2 [18] and FAK [60] and Src [60] kinases	Inverse electron demand Diels–Alder reactions	Tetrazines [18,59–61]

On the other hand, bioorthogonal amino acids (e.g. **77** and **92**, Table 5) have been used for rapid and selective inhibition of a specific enzyme in live mammalian cells [64]. In this case, a bioorthogonal amino acid is placed into the target enzyme without affecting the enzyme function. Upon contact with an inhibitor conjugate bearing the complementary bioorthogonal group, the enzyme variant is tethered to the conjugate and thus the enzyme activity is inhibited (Figure 5). The inhibition is exquisitely selective and can even discriminate between isoforms that differ by a single amino acid residue. Using this approach, selective inhibition of an intracellular kinase for which no selective small-molecule inhibitor exists was achieved. In addition, placing a photoswitchable moiety (i.e. azobenzene) into the inhibitor conjugate enables reversible modulation of enzyme activity by light.
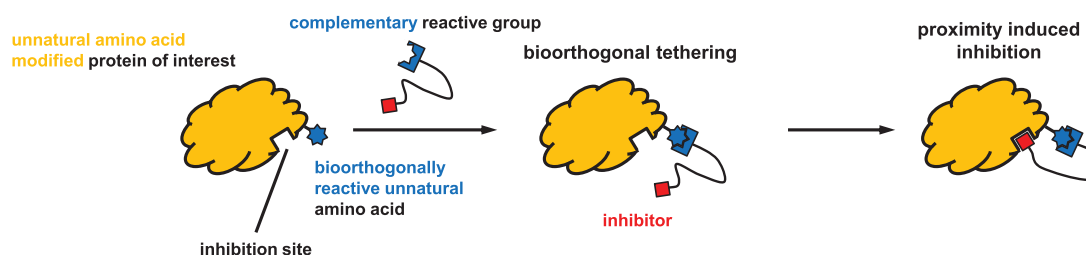
Figure 5. Example of the small-molecule approach to protein inhibition

Use of unnatural amino acid incorporation for selective inhibition of protein function by bioorthogonal tethering [64].

Table 5 Overview of bioorthogonally reactive unnatural amino acids that have been incorporated in mammalian cell systems used to deactivate protein function upon reaction with the specified reagents

Amino acid	System	Proteins	Reaction	Reagent
	HEK293T	MEK1 and LCK kinases		
		MEK1 and MEK2 kinases	inverse electron demand Diels–Alder reactions	Inhibitor–tetrazine conjugates [64]

Table 6 Comparison of protein function control approaches currently enabled by genetic code expansion

Approach	Temporal Control	Spatial control	Reversibility
Translational control	Yes, slow	Only by local administration	Yes, but high lag time
Optical control	Yes, fast	Yes, very high (to subcellular levels)	Yes, for photoswitchable amino acids
Chemical control	Yes, medium	Only by local administration	Yet to be established

In comparison with light-induced activation or inhibition, small molecules can be used to activate or inhibit the target protein in deep animal tissue or intact animals which are not easily accessible by light. However, as mentioned above, only a few reactions have so far been shown to be bioorthogonal with high reaction rates to allow fast response [65]. The extension of this methodology is therefore tied to the development of novel bioorthogonal reactions. In contrast to the use of light for activation or inhibition, the small-molecule approach, similar to the translational approach, only allows spatial control by using cell-compartment selective compounds or reactions, or local injections.

Conclusion

Genetic code expansion has matured into a technique that can be routinely used in mammalian systems. Controlling protein function is currently mostly achieved by translation, light, and small molecules. These methods have been summarised and discussed, and their features have been compared (Table 6). Translational control is arguably the easiest to perform but suffers from the longer response time (up to several hours). On the other hand, both light and small-molecule induced methods have a faster response (seconds to minutes). The light approach is particularly appealing where subcellular spatial resolution is needed. While all three approaches have shown promise, most of the reviewed applications are so far proof-of-principle studies. The dissemination of this technique could be enhanced by the community simplifying access to plasmids (e.g. through plasmid repository), standardising the reporting format of aaRS mutants with full sequencing information, and providing protocols with extensive details. Only general implementation of protein control by genetic code expansion in the wider scientific community to unravel new biological insights will truly demonstrate the power of these techniques [55,56,66–68]. Since genetic code expansion has also been recently demonstrated in mice [47,50,51,60,69,70], we foresee that optimisation will be tailored to target cells, tissues, and mammalian models and many of the aforementioned approaches will be applied *in vivo*, providing the complete native environment to study function of mammalian proteins. With further promotion and adaptation of genetic code expansion, this is to be expected.

Summary

- Genetic code expansion can now be routinely used for incorporation of more than 100 different unnatural amino acids in mammalian cells using only four orthogonal aaRS/tRNA pairs and their mutants as shown in Table 1.

- Protein function can be temporally regulated (activation or inhibition) by simple translational control, i.e. supplementation of the desired unnatural amino acid to allow full-length, functional protein production.

- More rapid control can be achieved by incorporating stimuli responsive amino acids which allow activation, inhibition, or reversible regulation of protein function by light or small molecules.

- Most of the approaches have been demonstrated in proof-of-principle studies, but are ready for adaptation by the broader scientific community.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

aaRS, aminoacyl-tRNA synthetase; Ec, *Escherichia coli*; Mx, *Methanomethylophilus alvus* Mx1201; PylRS, Pyrrolysyl-tRNA synthetase.

References

1 McDonnell, D.P., Wardell, S.E. and Norris, J.D. (2015) Oral selective estrogen receptor downregulators (SERDs), a breakthrough endocrine therapy for breast cancer. *J. Med. Chem.* **58**, 4883–4887, https://doi.org/10.1021/acs.jmedchem.5b00760

2 Petta, I., Lievens, S., Libert, C., Tavernier, J. and De Bosscher, K. (2016) Modulation of protein-protein interactions for the development of novel therapeutics. *Mol. Ther.* **24**, 707–718, https://doi.org/10.1038/mt.2015.214

3 van Montfort, R.L.M. and Workman, P. (2017) Structure-based drug design: aiming for a perfect fit. *Essays Biochem* **61**, 431–437, https://doi.org/10.1042/EBC20170052

4 Wan, W., Tharp, J.M. and Liu, W.R. (2014) Pyrrolysyl-tRNA synthetase: an ordinary enzyme but an outstanding genetic code expansion tool. *Biochim. Biophys. Acta* **1844**, 1059–1070, https://doi.org/10.1016/j.bbapap.2014.03.002

5 Dumas, A., Lercher, L., Spicer, C.D. and Davis, B.G. (2015) Designing logical codon reassignment - Expanding the chemistry in biology. *Chem. Sci.* **6**, 50–69, https://doi.org/10.1039/C4SC01534G

6 Leisle, L., Valiyaveetil, F., Mehl, R.A. and Ahern, C.A. (2015) Incorporation of non-canonical amino acids. *Adv. Exp. Med. Biol.* **869**, 119–151, https://doi.org/10.1007/978-1-4939-2845-3′7

7 Neumann-Staubitz, P. and Neumann, H. (2016) The use of unnatural amino acids to study and engineer protein function. *Curr. Opin. Chem. Biol.* **38**, 119–128

8 Wang, L. (2017) Engineering the genetic code in cells and animals: biological considerations and impacts. *Acc. Chem. Res.* **50**, 2767–2775, https://doi.org/10.1021/acs.accounts.7b00376

9 Italia, J.S., Zheng, Y., Kelemen, R.E., Erickson, S.B., Addy, P.S. and Chatterjee, A. (2017) Expanding the genetic code of mammalian cells. *Biochem. Soc. Trans.* **45**, 555–562, https://doi.org/10.1042/BST20160336

10 Debelouchina, G.T. and Muir, T.W. (2017) A molecular engineering toolbox for the structural biologist. *Q. Rev. Biophys.* **50**, e7, https://doi.org/10.1017/S0033583517000051

11 Chin, J.W. (2017) Expanding and reprogramming the genetic code. *Nature* **550**, 53–60, https://doi.org/10.1038/nature24031

12 Zhang, S., He, D., Lin, Z., Yang, Y., Song, H. and Chen, P.R. (2017) Conditional chaperone-client interactions revealed by genetically encoded photo-cross-linkers. *Acc. Chem. Res.* **50**, 1184–1192, https://doi.org/10.1021/acs.accounts.6b00647

13 Young, D.D. and Schultz, P.G. (2018) Playing with the molecules of life. *ACS Chem. Biol.* **13**, 854–870, https://doi.org/10.1021/acschembio.7b00974

14 Brown, W., Liu, J. and Deiters, A. (2018) Genetic code expansion in animals. *ACS Chem. Biol.* **13**, 2375–2386,
    https://doi.org/10.1021/acschembio.8b00520

15 Wang, W., Takimoto, J.K., Louie, G.V., Baiga, T.J., Noel, J.P., Lee, K.F. et al. (2007) Genetically encoding unnatural amino acids for cellular and neuronal
    studies. *Nat. Neurosci.* **10**, 1063–1072, https://doi.org/10.1038/nn1932

16 Ye, S., Zaitseva, E., Caltabiano, G., Schertler, G.F.X., Sakmar, T.P., Deupi, X. et al. (2010) Tracking G-protein-coupled receptor activation using
    genetically encoded infrared probes. *Nature* **464**, 1386–1390, https://doi.org/10.1038/nature08948

17 Zhu, S., Riou, M., Yao, C.A., Carvalho, S., Rodriguez, P.C., Bensaude, O. et al. (2014) Genetically encoding a light switch in an ionotropic glutamate
    receptor reveals subunit-specific interfaces. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 6081–6086, https://doi.org/10.1073/pnas.1318808111

18 Zheng, S., Fan, X., Wang, J., Zhao, J. and Chen, P.R. (2017) Dissection of kinase isoforms through orthogonal and chemical inducible signaling
    cascades. *ChemBioChem* **18**, 1593–1598, https://doi.org/10.1002/cbic.201700255

19 Baumdick, M., Gelleri, M., Uttamapinant, C., Beránek, V., Chin, J.W. and Bastiaens, P.I.H. (2018) A conformational sensor based on genetic code
    expansion reveals an autocatalytic component in EGFR activation. *Nat. Commun.* **9**, 3847, https://doi.org/10.1038/s41467-018-06299-7

20 Köhrer, C., Sullivan, E.L. and RajBhandary, U.L. (2004) Complete set of orthogonal 21st aminoacyl-tRNA synthetase-amber, ochre and opal suppressor
    tRNA pairs: concomitant suppression of three different termination codons in an mRNA in mammalian cells. *Nucleic Acids Res.* **32**, 6200–6211,
    https://doi.org/10.1093/nar/gkh959

21 Xiao, H., Chatterjee, A., Choi, S.H., Bajjuri, K.M., Sinha, S.C. and Schultz, P.G. (2013) Genetic incorporation of multiple unnatural amino acids into
    proteins in mammalian cells. *Angew. Chem. Int. Ed.* **52**, 14080–14083, https://doi.org/10.1002/anie.201308137

22 Schmied, W.H., Elsässer, S.J., Uttamapinant, C. and Chin, J.W. (2014) Efficient multisite unnatural amino acid incorporation in mammalian cells via
    optimized pyrrolysyl tRNA synthetase/tRNA expression and engineered eRF1. *J. Am. Chem. Soc.* **136**, 15577–15583,
    https://doi.org/10.1021/ja5069728

23 Serfling, R., Lorenz, C., Etzel, M., Schicht, G., Böttke, T., Mörl, M. et al. (2018) Designer tRNAs for efficient incorporation of non-canonical amino acids
    by the pyrrolysine system in mammalian cells. *Nucleic Acids Res.* **46**, 1–10, https://doi.org/10.1093/nar/gkx1156

24 Zheng, Y., Mukherjee, R., Chin, M.A., Igo, P., Gilgenast, M.J. and Chatterjee, A. (2018) Expanding the scope of single- and double-noncanonical amino
    acid mutagenesis in mammalian cells using orthogonal polyspecific leucyl-tRNA synthetases. *Biochemistry* **57**, 441–445,
    https://doi.org/10.1021/acs.biochem.7b00952

25 Zheng, Y., Addy, P.S., Mukherjee, R. and Chatterjee, A. (2017) Defining the current scope and limitations of dual noncanonical amino acid mutagenesis
    in mammalian cells. *Chem. Sci.* **8**, 7211–7217, https://doi.org/10.1039/C7SC02560B

26 Beranek, V., Willis, J. C.W. and Chin, J.W. (2019) An evolved Methanomethylophilus alvus pyrrolysyl-tRNA Synthetase/tRNA pair is highly active and
    orthogonal in mammalian cells. *Biochemistry* **58**, 387–390, https://doi.org/10.1021/acs.biochem.8b00808

27 Niu, W., Schultz, P.G. and Guo, J. (2013) An expanded genetic code in mammalian cells with a functional quadruplet codon. *ACS Chem. Biol.* **8**,
    1640–1645, https://doi.org/10.1021/cb4001662

28 Taki, M., Matsushita, J. and Sisido, M. (2006) Expanding the genetic code in a mammalian cell line by the introduction of four-base codon/anticodon
    pairs. *ChemBioChem* **7**, 425–428, https://doi.org/10.1002/cbic.200500360

29 Neumann, H., Wang, K., Davis, L., Garcia-Alai, M. and Chin, J.W. (2010) Encoding multiple unnatural amino acids via evolution of a
    quadruplet-decoding ribosome. *Nature* **464**, 441–444, https://doi.org/10.1038/nature08817

30 Wang, K., Sachdeva, A., Cox, D.J., Wilf, N.M., Lang, K., Wallace, S. et al. (2014) Optimized orthogonal translation of unnatural amino acids enables
    spontaneous protein double-labelling and FRET. *Nat. Chem.* **6**, 393–403, https://doi.org/10.1038/nchem.1919

31 Schmied, W.H., Tnimov, Z., Uttamapinant, C., Rae, C.D., Fried, S.D. and Chin, J.W. (2018) Controlling orthogonal ribosome subunit interactions enables
    evolution of new function. *Nature* **564**, 444–448, https://doi.org/10.1038/s41586-018-0773-z

32 Xiao, H. and Schultz, P.G. (2016) At the interface of chemical and biological synthesis: an expanded genetic code. *Cold Spring Harbor Perspect. Biol.* **8**,
    a023945, https://doi.org/10.1101/cshperspect.a023945

33 Vargas-Rodriguez, O., Sevostyanova, A., Söll, D. and Crnković, A. (2018) Upgrading aminoacyl-tRNA synthetases for genetic code expansion. *Curr.
    Opin. Chem. Biol.* **46**, 115–122, https://doi.org/10.1016/j.cbpa.2018.07.014

34 Italia, J.S., Addy, P.S., Wrobel, C.J., Crawford, L.A., Lajoie, M.J., Zheng, Y. et al. (2017) An orthogonalized platform for genetic code expansion in both
    bacteria and eukaryotes. *Nat. Chem. Biol.* **13**, 446–450, https://doi.org/10.1038/nchembio.2312

35 Italia, J.S., Latour, C., Wrobel, C.J.J. and Chatterjee, A. (2018) Resurrecting the bacterial tyrosyl-tRNA Synthetase/tRNA pair for expanding the genetic
    code of both E. coli and eukaryotes. *Cell Chem. Biol.* **25**, 1304–1312, https://doi.org/10.1016/j.chembiol.2018.07.002

36 Chin, J.W., Cropp, T.A., Anderson, J.C., Mukherji, M., Zhang, Z. and Schultz, P.G. (2003) An expanded eukaryotic genetic code. *Science* **301**,
    964–967, https://doi.org/10.1126/science.1084772

37 Liu, W., Brock, A., Chen, S., Chen, S. and Schultz, P.G. (2007) Genetic incorporation of unnatural amino acids into proteins in mammalian cells. *Nat.
    Methods* **4**, 239–244, https://doi.org/10.1038/nmeth1016

38 Naganathan, S., Grunbeck, A., Tian, H., Huber, T. and Sakmar, T.P. (2013) Genetically-encoded molecular probes to study G protein-coupled receptors.
    J. Vis. Exp., https://doi.org/10.3791/50588

39 Nikić, I., Kang, J.H., Girona, G.E., Aramburu, I.V. and Lemke, E.A. (2015) Labeling proteins on live mammalian cells using click chemistry. *Nat. Protoc.*
    10, 780–791, https://doi.org/10.1038/nprot.2015.045

40 Yang, Y., Song, H., He, D., Zhang, S., Dai, S., Xie, X. et al. (2017) Genetically encoded releasable photo-cross-linking strategies for studying
    protein-protein interactions in living cells. *Nat. Protoc.* **12**, 2147–2168, https://doi.org/10.1038/nprot.2017.090

41 Young, D.D., Young, T.S., Jahnz, M., Ahmad, I., Spraggon, G. and Schultz, P.G. (2011) An evolved aminoacyl-tRNA synthetase with atypical
    polysubstrate specificity. *Biochemistry* **50**, 1894–1900, https://doi.org/10.1021/bi101929e

42 Willis, J.C.W. and Chin, J.W. (2018) Mutually orthogonal pyrrolysyl-tRNA synthetase/tRNA pairs. *Nat. Chem.* **10**, 831–837, https://doi.org/10.1038/s41557-018-0052-5

43 Wang, N., Li, Y., Niu, W., Sun, M., Cerny, R., Li, Q. et al. (2014) Construction of a live-attenuated HIV-1 vaccine through genetic code expansion. *Angew. Chem. Int. Ed.* **53**, 4867–4871, https://doi.org/10.1002/anie.201402092

44 Mali, P. and Katrekar, D. (2017) In vivo RNA targeting of point mutations via suppressor tRNAs and adenosine deaminases. *bioRxiv*

45 Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**, 1766–1769, https://doi.org/10.1126/science.7792603

46 Sakamoto, K., Hayashi, A., Sakamoto, A., Kiga, D., Nakayama, H., Soma, A. et al. (2002) Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. *Nucleic Acids Res.* **30**, 4692–4699, https://doi.org/10.1093/nar/gkf589

47 Suzuki, T., Asami, M., Patel, S.G., Luk, L.Y.P., Tsai, Y.-H. and Perry, A.C.F. (2018) Switchable genome editing via genetic code expansion. *Sci. Rep.* **8**, 10051, https://doi.org/10.1038/s41598-018-28178-3

48 Si, L., Xu, H., Zhou, X., Zhang, Z., Tian, Z., Wang, Y. et al. (2016) Generation of influenza A viruses as live but replication-incompetent virus vaccines. *Science* **354**, 1170–1173, https://doi.org/10.1126/science.aah5869

49 Yuan, Z., Wang, N., Kang, G., Niu, W., Li, Q. and Guo, J. (2017) Controlling multicycle replication of live-attenuated HIV-1 using an unnatural genetic switch. *ACS Synth. Biol.* **6**, 721–731, https://doi.org/10.1021/acssynbio.6b00373

50 Han, S., Yang, A., Lee, S., Lee, H.W., Park, C.B. and Park, H.S. (2017) Expanding the genetic code of Mus musculus. *Nat. Commun.* **8**, 14568, https://doi.org/10.1038/ncomms14568

51 Krogager, T.P., Ernst, R.J., Elliott, T.S., Calo, L., Beránek, V., Ciabatti, E. et al. (2018) Labeling and identifying cell-specific proteomes in the mouse brain. *Nat. Biotechnol.* **36**, 156–159, https://doi.org/10.1038/nbt.4056

52 Klán, P., Šolomek, T., Bochet, C.G., Blanc, A., Givens, R., Rubina, M. et al. (2013) Photoremovable protecting groups in chemistry and biology: reaction mechanisms and efficacy. *Chem. Rev.* **113**, 119–191, https://doi.org/10.1021/cr300177k

53 Lemke, E.A., Summerer, D., Geierstanger, B.H., Brittain, S.M. and Schultz, P.G. (2007) Control of protein phosphorylation with a genetically encoded photocaged amino acid. *Nat. Chem. Biol.* **3**, 769–772, https://doi.org/10.1038/nchembio.2007.44

54 The UniProt Consortium (2019) UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515, https://doi.org/10.1093/nar/gky1049

55 Klippenstein, V., Ghisi, V., Wietstruk, M. and Plested, A.J. (2014) Photoinactivation of glutamate receptors by genetically encoded unnatural amino acids. *J. Neurosci.* **34**, 980–991, https://doi.org/10.1523/JNEUROSCI.3725-13.2014

56 Klippenstein, V., Hoppmann, C., Ye, S., Wang, L. and Paoletti, P. (2017) Optocontrol of glutamate receptor activity by single side-chain photoisomerization. *Elife* **6**, e25808, https://doi.org/10.7554/eLife.25808

57 Li, J., Yu, J., Zhao, J., Wang, J., Zheng, S., Lin, S. et al. (2014) Palladium-triggered deprotection chemistry for protein activation in living cells. *Nat. Chem.* **6**, 352–361, https://doi.org/10.1038/nchem.1887

58 Wäldchen, S., Lehmann, J., Klein, T., van de Linde, S. and Sauer, M. (2015) Light-induced cell damage in live-cell super-resolution microscopy. *Sci. Rep.* **5**, 15348, https://doi.org/10.1038/srep15348

59 Li, J., Jia, S. and Chen, P.R. (2014) Diels-Alder reaction-triggered bioorthogonal protein decaging in living cells. *Nat. Chem. Biol.* **10**, 1003–1005, https://doi.org/10.1038/nchembio.1656

60 Zhang, G., Li, J., Xie, R., Fan, X., Liu, Y., Zheng, S. et al. (2016) Bioorthogonal chemical activation of kinases in living systems. *ACS Cent. Sci.* **2**, 325–331, https://doi.org/10.1021/acscentsci.6b00024

61 Fan, X., Ge, Y., Lin, F., Yang, Y., Zhang, G., Ngai, W.S. et al. (2016) Optimized tetrazine derivatives for rapid bioorthogonal decaging in living cells. *Angew. Chem. Int. Ed.* **55**, 14046–14050, https://doi.org/10.1002/anie.201608009

62 Ge, Y., Fan, X. and Chen, P.R. (2016) A genetically encoded multifunctional unnatural amino acid for versatile protein manipulations in living cells. *Chem. Sci.* **7**, 7055–7060, https://doi.org/10.1039/C6SC02615J

63 Luo, J., Liu, Q., Morihiro, K. and Deiters, A. (2016) Small-molecule control of protein function through Staudinger reduction. *Nat. Chem.* **8**, 1027–1034, https://doi.org/10.1038/nchem.2573

64 Tsai, Y.-H., Essig, S., James, J.R., Lang, K. and Chin, J.W. (2015) Selective, rapid and optically switchable regulation of protein function in live mammalian cells. *Nat. Chem.* **7**, 554–561, https://doi.org/10.1038/nchem.2253

65 Lang, K. and Chin, J.W. (2014) Bioorthogonal reactions for labeling proteins. *ACS Chem. Biol.* **9**, 16–20, https://doi.org/10.1021/cb4009292

66 Liaunardy-Jopeace, A., Murton, B.L., Mahesh, M., Chin, J.W. and James, J.R. (2017) Encoding optical control in LCK kinase to quantitatively investigate its activity in live cells. *Nat. Struct. Mol. Biol.* **24**, 1155–1163, https://doi.org/10.1038/nsmb.3492

67 Walker, O.S., Elsässer, S.J., Mahesh, M., Bachman, M., Balasubramanian, S. and Chin, J.W. (2016) Photoactivation of mutant isocitrate dehydrogenase 2 reveals rapid cancer-associated metabolic and epigenetic changes. *J. Am. Chem. Soc.* **138**, 718–721, https://doi.org/10.1021/jacs.5b07627

68 Simms, J., Uddin, R., Sakmar, T.P., Gingell, J.J., Garelja, M.L., Hay, D.L. et al. (2018) Photoaffinity cross-linking and unnatural amino acid mutagenesis reveal insights into calcitonin gene-related peptide binding to the calcitonin receptor-like receptor/receptor activity-modifying protein 1 (CLR/RAMP1) complex. *Biochemistry* **57**, 4915–4922, https://doi.org/10.1021/acs.biochem.8b00502

69 Ernst, R.J., Krogager, T.P., Maywood, E.S., Zanchi, R., Beránek, V., Elliott, T.S. et al. (2016) Genetic code expansion in the mouse brain. *Nat. Chem. Biol.* **12**, 776–778, https://doi.org/10.1038/nchembio.2160

70 Chen, Y., Ma, J., Lu, W., Tian, M., Thauvin, M., Yuan, C. et al. (2017) Heritable expansion of the genetic code in mouse and zebrafish. *Cell Res* **27**, 294–297, https://doi.org/10.1038/cr.2016.145

71 Kang, J.Y., Kawaguchi, D., Coin, I., Xiang, Z., O'Leary, D.D., Slesinger, P.A. et al. (2013) In vivo expression of a light-activatable potassium channel using unnatural amino acids. *Neuron* **80**, 358–370, https://doi.org/10.1016/j.neuron.2013.08.016

72 Ren, W., Ji, A. and Ai, H.-W. (2015) Light activation of protein splicing with a photocaged fast intein. *J. Am. Chem. Soc.* **137**, 2155–2158, https://doi.org/10.1021/ja508597d

73 Nguyen, D.P., Mahesh, M., Elsässer, S.J., Hancock, S.M., Uttamapinant, C. and Chin, J.W. (2014) Genetic encoding of photocaged cysteine allows photoactivation of TEV protease in live mammalian cells. *J. Am. Chem. Soc.* **136**, 2240–2243, https://doi.org/10.1021/ja412191m

74 Gramespacher, J.A., Stevens, A.J., Nguyen, D.P., Chin, J.W. and Muir, T.W. (2017) Intein zymogens: conditional assembly and splicing of split inteins via targeted proteolysis. *J. Am. Chem. Soc.* **139**, 8074–8077, https://doi.org/10.1021/jacs.7b02618

75 Uprety, R., Luo, J., Liu, J., Naro, Y., Samanta, S. and Deiters, A. (2014) Genetic encoding of caged cysteine and caged homocysteine in bacterial and mammalian cells. *ChemBioChem* **15**, 1793–1799, https://doi.org/10.1002/cbic.201400073

76 Liu, J., Zheng, F., Cheng, R., Li, S., Rozovsky, S., Wang, Q. et al. (2018) Site-specific incorporation of selenocysteine using an expanded genetic code and palladium-mediated chemical deprotection. *J. Am. Chem. Soc.* **140**, 8807–8816, https://doi.org/10.1021/jacs.8b04603

77 Chatterjee, A., Xiao, H., Bollong, M., Ai, H.-W. and Schultz, P.G. (2013) Efficient viral delivery system for unnatural amino acid mutagenesis in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 11803–11808, https://doi.org/10.1073/pnas.1309584110

78 Takimoto, J.K., Adams, K.L., Xiang, Z. and Wang, L. (2009) Improving orthogonal tRNA-synthetase recognition for efficient unnatural amino acid incorporation and application in mammalian cells. *Mol. BioSyst.* **5**, 931–934, https://doi.org/10.1039/b904228h

79 Smits, A.H., Borrmann, A., Roosjen, M., van Hest, J.C. and Vermeulen, M. (2016) Click-MS: tagless protein enrichment using bioorthogonal chemistry for quantitative proteomics. *ACS Chem. Biol.* **11**, 3245–3250, https://doi.org/10.1021/acschembio.6b00520

80 Ye, S., Huber, T., Vogel, R. and Sakmar, T.P. (2009) FTIR analysis of GPCR activation using azido probes. *Nat. Chem. Biol.* **5**, 397–399, https://doi.org/10.1038/nchembio.167

81 Coin, I., Perrin, M.H., Vale, W.W. and Wang, L. (2011) Photo-cross-linkers incorporated into G-protein-coupled receptors in mammalian cells: a ligand comparison. *Angew. Chem. Int. Ed.* **50**, 8077–8081, https://doi.org/10.1002/anie.201102646

82 Chen, S., Chen, Z.-j., Ren, W. and Ai, H.-W. (2012) Reaction-based genetically encoded fluorescent hydrogen sulfide sensors. *J. Am. Chem. Soc.* **134**, 9589–9592, https://doi.org/10.1021/ja303261d

83 Naganathan, S., Ye, S., Sakmar, T.P. and Huber, T. (2013) Site-specific epitope tagging of G protein-coupled receptors by bioorthogonal modification of a genetically encoded unnatural amino acid. *Biochemistry* **52**, 1028–1036, https://doi.org/10.1021/bi301292h

84 Coin, I., Katritch, V., Sun, T., Xiang, Z., Siu, F.Y., Beyermann, M. et al. (2013) Genetically encoded chemical probes in cells reveal the binding path of urocortin-I to CRF class B GPCR. *Cell* **155**, 1258–1269, https://doi.org/10.1016/j.cell.2013.11.008

85 Wang, W., Li, T., Felsovalyi, K., Chen, C., Cardozo, T. and Krogsgaard, M. (2014) Quantitative analysis of T cell receptor complex interaction sites using genetically encoded photo-cross-linkers. *ACS Chem. Biol.* **9**, 2165–2172, https://doi.org/10.1021/cb500351s

86 Chen, Z.J. and Ai, H.-W. (2014) A highly responsive and selective fluorescent probe for imaging physiological hydrogen sulfide. *Biochemistry* **53**, 5966–5974, https://doi.org/10.1021/bi500830d

87 Tian, H., Naganathan, S., Kazmi, M.A., Schwartz, T.W., Sakmar, T.P. and Huber, T. (2014) Bioorthogonal fluorescent labeling of functional G-protein-coupled receptors. *ChemBioChem* **15**, 1820–1829, https://doi.org/10.1002/cbic.201402193

88 Naganathan, S., Ray-Saha, S., Park, M., Tian, H., Sakmar, T.P. and Huber, T. (2015) Multiplex detection of functional G protein-coupled receptors harboring site-specifically modified unnatural amino acids. *Biochemistry* **54**, 776–786, https://doi.org/10.1021/bi501267x

89 Tian, H., Sakmar, T.P. and Huber, T. (2015) Micelle-enhanced bioorthogonal labeling of genetically encoded azido groups on the lipid-embedded surface of a GPCR. *ChemBioChem* **16**, 1314–1322, https://doi.org/10.1002/cbic.201500030

90 Zheng, Y., Lewis, Jr, T.L., Igo, P., Polleux, F. and Chatterjee, A. (2017) Virus-enabled optimization and delivery of the genetic machinery for efficient unnatural amino acid mutagenesis in mammalian cells and tissues. *ACS Synth. Biol.* **6**, 13–18, https://doi.org/10.1021/acssynbio.6b00092

91 Koole, C., Reynolds, C.A., Mobarec, J.C., Hick, C., Sexton, P.M. and Sakmar, T.P. (2017) Genetically encoded photocross-linkers determine the biological binding site of exendin-4 peptide in the N-terminal domain of the intact human glucagon-like peptide-1 receptor (GLP-1R). *J. Biol. Chem.* **292**, 7131–7144, https://doi.org/10.1074/jbc.M117.779496

92 Grunbeck, A., Huber, T., Sachdev, P. and Sakmar, T.P. (2011) Mapping the ligand-binding site on a G protein-coupled receptor (GPCR) using genetically encoded photocrosslinkers. *Biochemistry* **50**, 3411–3413, https://doi.org/10.1021/bi200214r

93 Ray-Saha, S., Huber, T. and Sakmar, T.P. (2014) Antibody epitopes on g protein-coupled receptors mapped with genetically encoded photoactivatable cross-linkers. *Biochemistry* **53**, 1302–1310, https://doi.org/10.1021/bi401289p

94 Grunbeck, A., Huber, T., Abrol, R., Trzaskowski, B., Goddard, III, W.A. and Sakmar, T.P. (2012) Genetically encoded photo-cross-linkers map the binding site of an allosteric drug on a G protein-coupled receptor. *ACS Chem. Biol.* **7**, 967–972, https://doi.org/10.1021/cb300059z

95 Rannversson, H., Andersen, J., Sorensen, L., Bang-Andersen, B., Park, M., Huber, T. et al. (2016) Genetically encoded photocrosslinkers locate the high-affinity binding site of antidepressant drugs in the human serotonin transporter. *Nat. Commun.* **7**, 11261, https://doi.org/10.1038/ncomms11261

96 Park, M., Sivertsen, B.B., Els-Heindl, S., Huber, T., Holst, B., Beck-Sickinger, A.G. et al. (2015) Bioorthogonal labeling of ghrelin receptor to facilitate studies of ligand-dependent conformational dynamics. *Chem. Biol.* **22**, 1431–1436, https://doi.org/10.1016/j.chembiol.2015.09.014

97 Chen, Z.-j., Ren, W., Wright, Q.E. and Ai, H.-W. (2013) Genetically encoded fluorescent probe for the selective detection of peroxynitrite. *J. Am. Chem. Soc* **135**, 14940–14943, https://doi.org/10.1021/ja408011q

98 Tharp, J.M., Wang, Y.S., Lee, Y.J., Yang, Y. and Liu, W.R. (2014) Genetic incorporation of seven ortho-substituted phenylalanine derivatives. *ACS Chem. Biol.* **9**, 884–890, https://doi.org/10.1021/cb400917a

99 Ye, S., Köhrer, C., Huber, T., Kazmi, M., Sachdev, P., Yan, E.C. et al. (2008) Site-specific incorporation of keto amino acids into functional G protein-coupled receptors using unnatural amino acid mutagenesis. *J. Biol. Chem.* **283**, 1525–1533, https://doi.org/10.1074/jbc.M707355200

100 Axup, J.Y., Bajjuri, K.M., Ritland, M., Hutchins, B.M., Kim, C.H., Kazane, S.A. et al. (2012) Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 16101–16106, https://doi.org/10.1073/pnas.1211023109

101 Xiang, Z., Ren, H., Hu, Y.S., Coin, I., Wei, J., Cang, H. et al. (2013) Adding an unnatural covalent bond to proteins through proximity-enhanced bioreactivity. *Nat. Methods* **10**, 885–888, https://doi.org/10.1038/nmeth.2595

102 Hino, N., Okazaki, Y., Kobayashi, T., Hayashi, A., Sakamoto, K. and Yokoyama, S. (2005) Protein photo-cross-linking in mammalian cells by site-specific incorporation of a photoreactive amino acid. *Nat. Methods* **2**, 201–206, https://doi.org/10.1038/nmeth739

103 Shen, B., Xiang, Z., Miller, B., Louie, G., Wang, W., Noel, J.P. et al. (2011) Genetically encoding unnatural amino acids in neural stem cells and optically reporting voltage-sensitive domain changes in differentiated neurons. *Stem Cells* **29**, 1231–1240, https://doi.org/10.1002/stem.679

104 Murray, C.I., Westhoff, M., Eldstrom, J., Thompson, E., Emes, R. and Fedida, D. (2016) Unnatural amino acid photo-crosslinking of the IKs channel complex demonstrates a KCNE1:KCNQ1 stoichiometry of up to 4:4. *Elife* **5**, e11815, https://doi.org/10.7554/eLife.11815

105 Valentin-Hansen, L., Park, M., Huber, T., Grunbeck, A., Naganathan, S., Schwartz, T.W. et al. (2014) Mapping substance P binding sites on the neurokinin-1 receptor using genetic incorporation of a photoreactive amino acid. *J. Biol. Chem.* **289**, 18045–18054, https://doi.org/10.1074/jbc.M113.527085

106 Lacey, V.K., Louie, G.V., Noel, J.P. and Wang, L. (2013) Expanding the library and substrate diversity of the pyrrolysyl-tRNA synthetase to incorporate unnatural amino acids containing conjugated rings. *ChemBioChem* **14**, 2100–2105, https://doi.org/10.1002/cbic.201300400

107 Chatterjee, A., Guo, J., Lee, H.S. and Schultz, P.G. (2013) A genetically encoded fluorescent probe in mammalian cells. *J. Am. Chem. Soc.* **135**, 12540–12543, https://doi.org/10.1021/ja4059553

108 Mitchell, A.L., Addy, P.S., Chin, M.A. and Chatterjee, A. (2017) A unique genetically encoded FRET pair in mammalian cells. *ChemBioChem* **18**, 511–514, https://doi.org/10.1002/cbic.201600668

109 Park, S.-H., Ko, W., Lee, H.S. and Shin, I. (2019) Analysis of protein–protein interaction in a single live cell by using a FRET system based on genetic code expansion technology. *J. Am. Chem. Soc.* **141**, 4273–4281, https://doi.org/10.1021/jacs.8b10098

110 Fu, C., Kobayashi, T., Wang, N., Hoppmann, C., Yang, B., Irannejad, R. et al. (2018) Genetically encoding quinoline reverses chromophore charge and enables fluorescent protein brightening in acidic vesicles. *J. Am. Chem. Soc.* **140**, 11058–11066, https://doi.org/10.1021/jacs.8b05814

111 Luo, J., Samanta, S., Convertino, M., Dokholyan, N.V. and Deiters, A. (2018) Reversible and tunable photoswitching of protein function through genetic encoding of azobenzene amino acids in mammalian cells. *ChemBioChem* **19**, 2178–2185, https://doi.org/10.1002/cbic.201800226

112 Hoppmann, C., Lacey, V.K., Louie, G.V., Wei, J., Noel, J.P. and Wang, L. (2014) Genetically encoding photoswitchable click amino acids in Escherichia coli and mammalian cells. *Angew. Chem. Int. Ed.* **53**, 3932–3936, https://doi.org/10.1002/anie.201400001

113 Xiao, H., Peters, F.B., Yang, P.Y., Reed, S., Chittuluru, J.R. and Schultz, P.G. (2014) Genetic incorporation of histidine derivatives using an engineered pyrrolysyl-tRNA synthetase. *ACS Chem. Biol.* **9**, 1092–1096, https://doi.org/10.1021/cb500032c

114 Wang, T., Zhou, Q., Li, F., Yu, Y., Yin, X. and Wang, J. (2015) Genetic incorporation of N(epsilon)-Formyllysine, a new histone post-translational modification. *ChemBioChem* **16**, 1440–1442, https://doi.org/10.1002/cbic.201500170

115 Elsässer, S.J., Ernst, R.J., Walker, O.S. and Chin, J.W. (2016) Genetic code expansion in stable cell lines enables encoded chromatin modification. *Nat. Methods* **13**, 158–164, https://doi.org/10.1038/nmeth.3701

116 Xuan, W., Yao, A. and Schultz, P.G. (2017) Genetically encoded fluorescent probe for detecting sirtuins in living cells. *J. Am. Chem. Soc.* **139**, 12350–12353, https://doi.org/10.1021/jacs.7b05725

117 Cohen, S. and Arbely, E. (2016) Single-plasmid-based system for efficient noncanonical amino acid mutagenesis in cultured mammalian cells. *ChemBioChem* **17**, 1008–1011, https://doi.org/10.1002/cbic.201500681

118 Mukai, T., Kobayashi, T., Hino, N., Yanagisawa, T., Sakamoto, K. and Yokoyama, S. (2008) Adding l-lysine derivatives to the genetic code of mammalian cells with engineered pyrrolysyl-tRNA synthetases. *Biochem. Biophys. Res. Commun.* **371**, 818–822, https://doi.org/10.1016/j.bbrc.2008.04.164

119 Li, F., Zhang, H., Sun, Y., Pan, Y., Zhou, J. and Wang, J. (2013) Expanding the genetic code for photoclick chemistry in E. coli, mammalian cells, and A. thaliana. *Angew. Chem. Int. Ed.* **52**, 9700–9704, https://doi.org/10.1002/anie.201303477

120 Kim, C.H., Kang, M., Kim, H.J., Chatterjee, A. and Schultz, P.G. (2012) Site-specific incorporation of epsilon-N-crotonyllysine into histones. *Angew. Chem. Int. Ed.* **51**, 7246–7249, https://doi.org/10.1002/anie.201203349

121 Cigler, M., Müller, T.G., Horn-Ghetko, D., von Wrisberg, M.K., Fottner, M., Goody, R.S. et al. (2017) Proximity-triggered covalent stabilization of low-affinity protein complexes in vitro and in vivo. *Angew. Chem. Int. Ed.* **56**, 15737–15741, https://doi.org/10.1002/anie.201706927

122 Tian, Y., Jacinto, M.P., Zeng, Y., Yu, Z., Qu, J., Liu, W.R. et al. (2017) Genetically encoded 2-aryl-5-carboxytetrazoles for site-selective protein photo-cross-linking. *J. Am. Chem. Soc.* **139**, 6078–6081, https://doi.org/10.1021/jacs.7b02615

123 Peng, T. and Hang, H.C. (2016) Site-specific bioorthogonal labeling for fluorescence imaging of intracellular proteins in living cells. *J. Am. Chem. Soc.* **138**, 14423–14433, https://doi.org/10.1021/jacs.6b08733

124 Ramil, C.P., Dong, M., An, P., Lewandowski, T.M., Yu, Z., Miller, L.J. et al. (2017) Spirohexene-tetrazine ligation enables bioorthogonal labeling of class B G protein-coupled receptors in live cells. *J. Am. Chem. Soc.* **139**, 13376–13386, https://doi.org/10.1021/jacs.7b05674

125 Gautier, A., Nguyen, D.P., Lusic, H., An, W., Deiters, A. and Chin, J.W. (2010) Genetically encoded photocontrol of protein localization in mammalian cells. *J. Am. Chem. Soc.* **132**, 4086–4088, https://doi.org/10.1021/ja910688s

126 Arbely, E., Torres-Kolbus, J., Deiters, A. and Chin, J.W. (2012) Photocontrol of tyrosine phosphorylation in mammalian cells via genetic encoding of photocaged tyrosine. *J. Am. Chem. Soc.* **134**, 11912–11915, https://doi.org/10.1021/ja3046958

127 Lang, K., Davis, L., Torres-Kolbus, J., Chou, C., Deiters, A. and Chin, J.W. (2012) Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nat. Chem.* **4**, 298–304, https://doi.org/10.1038/nchem.1250

128 Aloush, N., Schvartz, T., König, A.I., Cohen, S., Brozgol, E., Tam, B. et al. (2018) Live cell imaging of bioorthogonally labelled proteins generated with a single pyrrolysine tRNA gene. *Sci. Rep.* **8**, 14527, https://doi.org/10.1038/s41598-018-32824-1

129 Schneider, N., Gabelein, C., Wiener, J., Georgiev, T., Gobet, N., Weber, W. et al. (2018) Genetic code expansion method for temporal labeling of endogenously expressed proteins. *ACS Chem. Biol.* **13**, 3049–3053, https://doi.org/10.1021/acschembio.8b00594

130 Li, N., Ramil, C.P., Lim, R.K. and Lin, Q. (2015) A genetically encoded alkyne directs palladium-mediated protein labeling on live mammalian cell surface. *ACS Chem. Biol.* **10**, 379–384, https://doi.org/10.1021/cb500649q

131 Yang, Y., Lin, S., Lin, W. and Chen, P.R. (2014) Ligand-assisted dual-site click labeling of EGFR on living cells. *ChemBioChem* **15**, 1738–1743, https://doi.org/10.1002/cbic.201400057

132 Zheng, Y., Yu, F., Wu, Y., Si, L., Xu, H., Zhang, C. et al. (2015) Broadening the versatility of lentiviral vectors as a tool in nucleic acid research via genetic code expansion. *Nucleic Acids Res.* **43**, e73, https://doi.org/10.1093/nar/gkv202

133 Zhang, C., Yao, T., Zheng, Y., Li, Z., Zhang, Q., Zhang, L. et al. (2016) Development of next generation adeno-associated viral vectors capable of selective tropism and efficient gene delivery. *Biomaterials* **80**, 134–145, https://doi.org/10.1016/j.biomaterials.2015.11.066

134 Zhang, Z., Xu, H., Si, L., Chen, Y., Zhang, B., Wang, Y. et al. (2017) Construction of an inducible stable cell line for efficient incorporation of unnatural amino acids in mammalian cells. *Biochem. Biophys. Res. Commun.* **489**, 490–496, https://doi.org/10.1016/j.bbrc.2017.05.178

135 Yao, T., Zhou, X., Zhang, C., Yu, X., Tian, Z., Zhang, L. et al. (2017) Site-specific PEGylated adeno-associated viruses with increased serum stability and reduced immunogenicity. *Molecules* **22**, 1155, https://doi.org/10.3390/molecules22071155

136 Katrekar, D., Moreno, A.M., Chen, G., Worlikar, A. and Mali, P. (2018) Oligonucleotide conjugated multi-functional adeno-associated viruses. *Sci. Rep.* **8**, 3589, https://doi.org/10.1038/s41598-018-21742-x

137 Kelemen, R.E., Mukherjee, R., Cao, X., Erickson, S.B., Zheng, Y. and Chatterjee, A. (2016) A precise chemical strategy to alter the receptor specificity of the adeno-associated virus. *Angew. Chem. Int. Ed.* **55**, 10645–10649, https://doi.org/10.1002/anie.201604067

138 Zhang, J., Yan, S., He, Z., Ding, C., Zhai, T., Chen, Y. et al. (2018) Small unnatural amino acid carried raman tag for molecular imaging of genetically targeted proteins. *J. Phys. Chem. Lett.* **9**, 4679–4685, https://doi.org/10.1021/acs.jpclett.8b01991

139 Luöhmann, T., Jones, G., Gutmann, M., Rybak, J.-C., Nickel, J., Rubini, M. et al. (2015) Bio-orthogonal immobilization of fibroblast growth factor 2 for spatial controlled cell proliferation. *ACS Biomater. Sci. Eng.* **1**, 740–746, https://doi.org/10.1021/acsbiomaterials.5b00236

140 Lin, S., Yan, H., Li, L., Yang, M., Peng, B., Chen, S. et al. (2013) Site-specific engineering of chemical functionalities on the surface of live hepatitis D virus. *Angew. Chem. Int. Ed.* **52**, 13970–13974, https://doi.org/10.1002/anie.201305787

141 Chen, P.R., Groff, D., Guo, J., Ou, W., Cellitti, S., Geierstanger, B.H. et al. (2009) A facile system for encoding unnatural amino acids in mammalian cells. *Angew. Chem. Int. Ed.* **48**, 4052–4055, https://doi.org/10.1002/anie.200900683

142 Yanagisawa, T., Hino, N., Iraha, F., Mukai, T., Sakamoto, K. and Yokoyama, S. (2012) Wide-range protein photo-crosslinking achieved by a genetically encoded N(epsilon)-(benzyloxycarbonyl)lysine derivative with a diazirinyl moiety. *Mol. Biosyst.* **8**, 1131–1135, https://doi.org/10.1039/c2mb05321g

143 Kita, A., Hino, N., Higashi, S., Hirota, K., Narumi, R., Adachi, J. et al. (2016) Adenovirus vector-based incorporation of a photo-cross-linkable amino acid into proteins in human primary cells and cancerous cell lines. *Sci. Rep.* **6**, 36946, https://doi.org/10.1038/srep36946

144 Zhao, J., Lin, S., Huang, Y., Zhao, J. and Chen, P.R. (2013) Mechanism-based design of a photoactivatable firefly luciferase. *J. Am. Chem. Soc.* **135**, 7410–7413, https://doi.org/10.1021/ja4013535

145 Ren, W., Ji, A., Wang, M.X. and Ai, H.-W. (2015) Expanding the Genetic Code for a Dinitrophenyl Hapten. *ChemBioChem* **16**, 2007–2010, https://doi.org/10.1002/cbic.201500204

146 Groff, D., Chen, P.R., Peters, F.B. and Schultz, P.G. (2010) A genetically encoded epsilon-N-methyl lysine in mammalian cells. *ChemBioChem* **11**, 1066–1068, https://doi.org/10.1002/cbic.200900690

147 Erickson, S.B., Mukherjee, R., Kelemen, R.E., Wrobel, C.J.J., Cao, X. and Chatterjee, A. (2017) Precise photoremovable perturbation of a virus–host interaction. *Angew. Chem. Int. Ed.* **56**, 4234–4237, https://doi.org/10.1002/anie.201700683

148 Luo, J., Arbely, E., Zhang, J., Chou, C., Uprety, R., Chin, J.W. et al. (2016) Genetically encoded optical activation of DNA recombination in human cells. *Chem. Commun.* **52**, 8529–8532, https://doi.org/10.1039/C6CC03934K

149 Engelke, H., Chou, C., Uprety, R., Jess, P. and Deiters, A. (2014) Control of protein function through optochemical translocation. *ACS Synth. Biol.* **3**, 731–736, https://doi.org/10.1021/sb400192a

150 Hemphill, J., Borchardt, E.K., Brown, K., Asokan, A. and Deiters, A. (2015) Optical control of CRISPR/Cas9 gene editing. *J. Am. Chem. Soc.* **137**, 5642–5645, https://doi.org/10.1021/ja512664v

151 Gautier, A., Deiters, A. and Chin, J.W. (2011) Light-activated kinases enable temporal dissection of signaling networks in living cells. *J. Am. Chem. Soc.* **133**, 2124–2127, https://doi.org/10.1021/ja1109979

152 Hemphill, J., Chou, C., Chin, J.W. and Deiters, A. (2013) Genetically encoded light-activated transcription for spatiotemporal control of gene expression and gene silencing in mammalian cells. *J. Am. Chem. Soc.* **135**, 13433–13439, https://doi.org/10.1021/ja4051026

153 Luo, J., Uprety, R., Naro, Y., Chou, C., Nguyen, D.P., Chin, J.W. et al. (2014) Genetically encoded optochemical probes for simultaneous fluorescence reporting and light activation of protein function with two-photon excitation. *J. Am. Chem. Soc.* **136**, 15551–15558, https://doi.org/10.1021/ja5055862

154 Yu, Z., Pan, Y., Wang, Z., Wang, J. and Lin, Q. (2012) Genetically encoded cyclopropene directs rapid, photoclick-chemistry-mediated protein labeling in mammalian cells. *Angew. Chem. Int. Ed.* **51**, 10600–10604, https://doi.org/10.1002/anie.201205352

155 Meineke, B., Heimgartner, J., Lafranchi, L. and Elsässer, S.J. (2018) Methanomethylophilus alvus Mx1201 provides basis for mutual orthogonal pyrrolysyl tRNA/Aminoacyl-tRNA synthetase pairs in mammalian cells. *ACS Chem. Biol.* **13**, 3087–3096, https://doi.org/10.1021/acschembio.8b00571

156 Elliott, T.S., Townsley, F.M., Bianco, A., Ernst, R.J., Sachdeva, A., Elsässer, S.J. et al. (2014) Proteome labeling and protein identification in specific tissues and at specific developmental stages in an animal. *Nat. Biotechnol.* **32**, 465–472, https://doi.org/10.1038/nbt.2860

157 Ai, H.-W., Shen, W., Sagi, A., Chen, P.R. and Schultz, P.G. (2011) Probing protein-protein interactions with a genetically encoded photo-crosslinking amino acid. *ChemBioChem* **12**, 1854–1857, https://doi.org/10.1002/cbic.201100194

158 Chou, C., Uprety, R., Davis, L., Chin, J.W. and Deiters, A. (2011) Genetically encoding an aliphatic diazirine for protein photocrosslinking. *Chem. Sci.* **2**, 480–483, https://doi.org/10.1039/C0SC00373E

159 Hoffmann, J.E., Dziuba, D., Stein, F. and Schultz, C. (2018) A bifunctional noncanonical amino acid: synthesis, expression, and residue-specific proteome-wide incorporation. *Biochemistry* **57**, 4747–4752, https://doi.org/10.1021/acs.biochem.8b00397

160 Zhang, C., Lu, J., Su, H., Yang, J. and Zhou, D. (2017) Fatty acid synthase cooperates with protrudin to facilitate membrane outgrowth of cellular protrusions. *Sci. Rep.* **7**, 46569, https://doi.org/10.1038/srep46569

161 Nikić, I., Plass, T., Schraidt, O., Szymański, J., Briggs, J.A., Schultz, C. et al. (2014) Minimal tags for rapid dual-color live-cell labeling and super-resolution microscopy. *Angew. Chem. Int. Ed.* **53**, 2245–2249, https://doi.org/10.1002/anie.201309847

162 Nikić, I., Estrada Girona, G., Kang, J.H., Paci, G., Mikhaleva, S., Koehler, C., Shymanska, N.V. et al. (2016) Debugging eukaryotic genetic code expansion for site-specific Click-PAINT super-resolution microscopy. *Angew. Chem. Int. Ed.* **55**, 16172–16176, https://doi.org/10.1002/anie.201608284

163 Rutkowska, A., Plass, T., Hoffmann, J.E., Yushchenko, D.A., Feng, S. and Schultz, C. (2014) T-CrAsH: a heterologous chemical crosslinker. *ChemBioChem* **15**, 1765–1768, https://doi.org/10.1002/cbic.201402189

164 Hoffmann, J.E., Plass, T., Nikić, I., Aramburu, I.V., Koehler, C., Gillandt, H. et al. (2015) Highly stable trans-cyclooctene amino acids for live-cell labeling. *Chem. Eur. J.* **21**, 12266–12270, https://doi.org/10.1002/chem.201501647

165 Kozma, E., Nikić, I., Varga, B.R., Aramburu, I.V., Kang, J.H., Fackler, O.T. et al. (2016) Hydrophilic trans-cyclooctenylated noncanonical amino acids for fast intracellular protein labeling. *ChemBioChem* **17**, 1518–1524, https://doi.org/10.1002/cbic.201600284

166 Sakin, V., Hanne, J., Dunder, J., Anders-Össwein, M., Laketa, V., Nikic, I. et al. (2017) A versatile tool for live-cell imaging and super-resolution nanoscopy studies of hiv-1 env distribution and mobility. *Cell Chem. Biol.* **24**, 635–645, https://doi.org/10.1016/j.chembiol.2017.04.007

167 Lang, K., Davis, L., Wallace, S., Mahesh, M., Cox, D.J., Blackman, M.L. et al. (2012) Genetic encoding of bicyclononynes and trans-cyclooctenes for site-specific protein labeling in vitro and in live mammalian cells via rapid fluorogenic Diels-Alder reactions. *J. Am. Chem. Soc.* **134**, 10317–10320, https://doi.org/10.1021/ja302832g

168 Uttamapinant, C., Howe, J.D., Lang, K., Beránek, V., Davis, L., Mahesh, M. et al. (2015) Genetic code expansion enables live-cell and super-resolution imaging of site-specifically labeled cellular proteins. *J. Am. Chem. Soc.* **137**, 4602–4605, https://doi.org/10.1021/ja512838z

169 Plass, T., Milles, S., Koehler, C., Szymański, J., Mueller, R., Wießler, M. et al. (2012) Amino acids for Diels-Alder reactions in living cells. *Angew. Chem. Int. Ed.* **51**, 4166–4170, https://doi.org/10.1002/anie.201108231

170 Xue, L., Prifti, E. and Johnsson, K. (2016) A general strategy for the semisynthesis of ratiometric fluorescent sensor proteins with increased dynamic range. *J. Am. Chem. Soc.* **138**, 5258–5261, https://doi.org/10.1021/jacs.6b03034

171 Borrmann, A., Milles, S., Plass, T., Dommerholt, J., Verkade, J.M., Wiessler, M. et al. (2012) Genetic encoding of a bicyclo[6.1.0]nonyne-charged amino acid enables fast cellular protein imaging by metal-free ligation. *ChemBioChem* **13**, 2094–2099, https://doi.org/10.1002/cbic.201200407

172 Schvartz, T., Aloush, N., Goliand, I., Segal, I., Nachmias, D., Arbely, E. et al. (2017) Direct fluorescent-dye labeling of alpha-tubulin in mammalian cells for live cell and superresolution imaging. *Mol. Biol. Cell* **28**, 2747–2756, https://doi.org/10.1091/mbc.e17-03-0161

173 Luo, J., Torres-Kolbus, J., Liu, J. and Deiters, A. (2017) Genetic encoding of photocaged tyrosines with improved light-activation properties for the optical control of protease function. *ChemBioChem* **18**, 1442–1447, https://doi.org/10.1002/cbic.201700147

174 Wang, N., Yang, B., Fu, C., Zhu, H., Zheng, F., Kobayashi, T. et al. (2018) Genetically encoding fluorosulfate-l-tyrosine to react with lysine, histidine, and tyrosine via SuFEx in proteins in vivo. *J. Am. Chem. Soc.* **140**, 4995–4999, https://doi.org/10.1021/jacs.8b01087

175 Takimoto, J.K., Xiang, Z., Kang, J.Y. and Wang, L. (2010) Esterification of an unnatural amino acid structurally deviating from canonical amino acids promotes its uptake and incorporation into proteins in mammalian cells. *ChemBioChem* **11**, 2268–2272, https://doi.org/10.1002/cbic.201000436

176 Beránek, V., Reinkemeier, C.D., Zhang, M.S., Liang, A.D., Kym, G. and Chin, J.W. (2018) Genetically encoded protein phosphorylation in mammalian cells. *Cell Chem. Biol.* **25**, 1067–1074, https://doi.org/10.1016/j.chembiol.2018.05.013