Potential vs Challenges of Expanding the Protein Universe With Genetic Code Expansion in Eukaryotic Cells

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Abstract

Following decades of innovation and perfecting, genetic code expansion has become a powerful tool for *in vivo* protein modification. Some of the major hurdles that had to be overcome include suboptimal performance of GCE-specific translational components in host systems, competing cellular processes, unspecific modification of the host proteome and limited availability of codons for reassignment. Although strategies have been developed to overcome these challenges, there is critical need for further advances. Here we discuss the current state-of-the-art in genetic code expansion technology and the issues that still need to be addressed to unleash the full potential of this method in eukaryotic cells.

Proteins, fundamental functional units of the living cell, are built from 22 canonical amino acids, of which 20 are most frequently used.[1,2] 61 codons, each composed of different combinations of 3 nucleotides, encode these amino acids and 3, referred to as stop codons, terminate translation.[3] It is quite remarkable to note the diversity that nature has achieved with only 22 building blocks, highlighting the untapped potential of expanding the repertoire of amino acids. Decades of research on genetic code expansion (GCE) technology have made it possible to incorporate over 500 noncanonical amino acids (ncAAs) of desired functionality into proteins of interest (POI),[4] opening a plethora of scientific avenues. GCE research, which began with the synthesis of modified proteins by *in vitro* ligation of ncAAs to tRNA, has evolved today to include sophisticated techniques that allow hijacking of the host translational machinery.[5,6] In the following sections we introduce the general mechanism of GCE and its applications. Further we discuss some of the most pressing challenges as well as the strategies to address them. Figure 1 serves as the pictorial representation of these challenges and throughout this article bold texts have been used to refer to subpanels in Figure 1.

GCE is an extremely powerful *in vivo* protein modification tool which enables the incorporation of tailor-made ncAAs into POIs. One of the common methods to do so is by utilizing orthogonal aminoacyl-tRNA synthetase (aaRS)/tRNA pairs to reassign codons to ncAAs.[7] Orthogonal refers to the requirement that neither the synthetase nor the tRNA cross-react with any endogenous aaRS/tRNA pairs of the host organism. Some aaRS/tRNA pairs from archaea are orthogonal to both prokaryotic and eukaryotic systems and hence find widespread application in GCE. One of the most commonly used systems involves derivatives of the pyrrolysine RS (PylRS)/tRNA pair from *Methanosarcina mazei*[8] which can reassign an amber (TAG) stop codon to incorporate

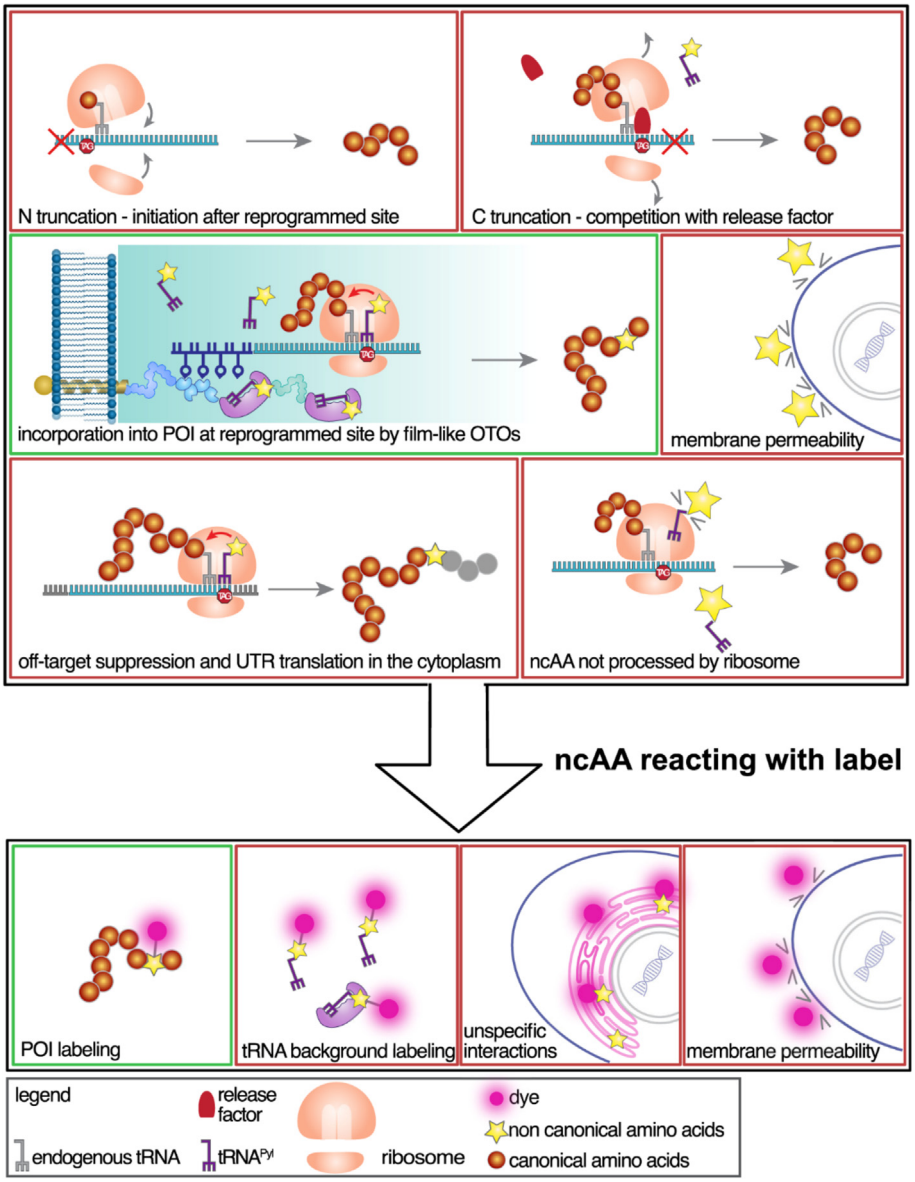

Figure 1. GCE mechanisms and challenges. The upper section of the figure depicts OTO enabled reassignment of the amber (TAG) codon (highlighted by the green frame) and the challenges that need to be overcome (red frames). The lower section of the figure describes possible outcomes when an ncAA containing POI is to be labelled *in vivo* by clickable fluorescent dyes. The desired outcome, in this case, is highlighted by the green frame.

ncAAs into a POI. Once encoded into the POI, the ncAAs equip the modified POI with new functions, enabling for example investigation of post-translational modifications (PTMs),[9–12] photo-control of protein functionality by photocaged chemical groups,[13] study of protein-protein interactions by photo-crosslinking moieties,[14] incorporation of heavy atoms for protein structure elucidation by X-ray crystallography and NMR spectroscopy[15,16] and click-reactions to synthetic dyes having compatible chemical handles[17], to name a few. It is noteworthy how GCE has profoundly minimized the size of fluorescent probes to a single site-specifically click-labelled synthetic dye molecule,[18] thereby significantly improving the resolution for visualizing subcellular protein structures by advanced microscopy. Currently with the emergence of the MINFLUX super-resolution microscopy technique it is possible to visualize structures at a resolution of 1–3 nm.[19] In combination with GCE, this technique could become pivotal in elucidating yet invisible subcellular structures.

Despite its wide-ranging applications across various fields of life sciences, the vast potential of GCE to synthesize completely artificial amino-acid-based biopolymers *in vivo*, remains largely unexplored. Competing against billions of years of evolution to entirely manipulate the translational

machinery of living systems is a herculean task, especially in the context of eukaryotic organisms. Thus, it is not surprising that GCE faces several limitations. While many of these have been addressed with great success in prokaryotes, directly applying the same strategies to eukaryotes is challenging due to the greater complexity of their cellular organization.

Notable hurdles hindering high-yielding GCE in eukaryotes which include but are not limited to, are, lack of promoter elements for optimal expression of GCE-specific tRNAs,[20] nonsense-mediated decay (NMD) pathway which degrades transcripts containing premature stop codons,[21] as well as competition against the endogenous translation termination machinery, the latter one leading to C-terminal truncations (Figure 1, **C truncation – competition with release factor**).[22] Another problem in eukaryotes is that the concept of an open reading frame is not as rigid as stated in some textbooks. Various start codons exist, as well as internal ribosome recognition elements.[46,47] If one places a stop codon close to the N-terminus, it is possible that protein translation is started after the stop codon, leading to a N-terminal truncation instead of encoding an ncAA at the anticipated site (Figure 1, **N truncation – initiation after reprogrammed site**).[48] Additionally it is also important to have high intracellular availability of the ncAA, for which the ncAA needs to be membrane permeable and metabolically stable enough to get incorporated during comparatively slow protein expression (Figure 1, **membrane permeability of ncAA**).[23]

Engineering of orthogonal aaRS/tRNA pairs comprises a significant part of the GCE optimization efforts. The cumulated effect of several strategies has resulted in some of today's most efficient GCE-specific tRNAs. Most bacterial tRNAs lack promoter elements necessary for transcription by RNA polymerase III in eukaryotes. Hence, appropriate promoters or optimized promoter elements have to be engineered to ensure adequate expression of bacterial tRNAs in eukaryotic hosts.[24,25] Furthermore, both rational design and evolutionary approaches have been employed to address the structural divergence between archaeal and mammalian tRNAs.[26–28] Synthetases derived from archaea and bacteria have undergone extensive evolution to produce new variants with specific affinities for different ncAAs,[29–32] as well as to generate mutually orthogonal aaRS/tRNA pairs for the simultaneous incorporation of distinct ncAAs.[33,34] Of course, the synthetase is not the only limiting factor for ncAA incorporation, as ultimately also the ribosomes need to accept the aminoacylated tRNA, which does impose a strict size criterion. This has led to the evolution of ncAAs that are only moderately bigger than the biggest natural amino acid, tryptophan (Figure 1, **ncAA not processed by ribosome**). Besides engineering of aaRS/tRNA-pairs, other strategies to improve GCE include development of host organism strains with impaired NMD pathway,[35,36] mutating eukaryotic release factor eRF1 to specifically hinder translation termination at the amber (TAG) stop codon[22] and modulation of the integrated stress response (ISR) pathway.[37] Cellular uptake of ncAAs can be improved by esterification of the carboxyl group since neutral ncAA molecules have higher membrane permeability.[23] This chemical modification has no effect on the final incorporation of the original ncAA in the POI due to hydrolysis of the ester bond by cellular esterases prior to translation. However ester hydrolysis can generate toxic byproducts that increase cell mortality, for example generation of formaldehyde from acetoxymethyl dansyl alanine. It has been shown that for multicellular organisms like *Caenorhabditis elegans*, manipulation of dipeptide transporters can be an alternative solution for increasing intracellular availability of ncAAs.[35]

GCE also suffers from a lack of mRNA selectivity, which means that along with the engineered stop codon in the mRNA of interest, there is also suppression of naturally occurring stop codons (even the rarest stop codon (amber TAG) in eukaryotes has ~20% abundance).[6,38] This lack of specificity can result in unintended modification of the host proteome, as natural untranslated regions (UTR) may erroneously get added to proteins when the translation is not adequately terminated (Figure 1, **off-target suppression and UTR translation in the cytoplasm**). The resulting proteome-wide unspecific ncAA incorporation can give rise to background labelling in case of imaging applications and adversely affect the signal-to-noise ratio.

In prokaryotes, this problem can be solved by multiple approaches. One of them is the development of orthogonal ribosomes that recognize and translate only the mRNA of interest using complementary Shine Dalgarno sequences.[39] Eukaryotes do not have Shine Dalgarno sequences, hence, orthogonal ribosomes are not applicable to these systems. Alternatively, the entire genome of the host organism can be engineered to eliminate selected codons, which subsequently become available for ncAA incorporation.[40,41] However such an approach represents a tremendous effort in case of eukaryotes due to their larger and more complex genomes. It is also possible to use codons derived from artificial base pairs for ncAA incorporation.[42,43] These base pairs do not naturally occur in the host genome, thereby mitigating the issue of unspecific GCE. Since none of the concepts mentioned above are easily transferable to eukaryotes, Reinkemeier *et al* aimed to confine the GCE-machinery in membraneless designer orthogonally translating organelles (OTO) built *in vivo*, borrowing concepts from phase separation.[44,45,6] The OTO was most

mRNA selective when tethered to a membrane, so a steep gradient of the suppressor tRNA could be generated at the membrane. The mRNA of interest was selectively recruited in what were termed film-like OTOs by the interaction of RNA loops and cognate RNA-binding proteins. The OTOs have been shown to achieve more than an order of magnitude in mRNA selectivity than the cytoplasmic GCE system (Figure 1, **incorporation into POI at reprogrammed site by film-like OTOs** indicated by the membrane-anchored aaRS vs **off-target suppression and UTR translation in the cytoplasm**). So far, a strong impact of OTO technology on cell viability was not detected, but further research might be needed to characterize potential minor physiological effects.

To advance towards the *in vivo* synthesis of designer biopolymers, it is essential to move beyond the traditional three stop codons. This expansion is crucial for enabling the simultaneous incorporation of multiple distinct ncAAs, marking a significant step forward in the development of sophisticated biopolymer systems. Some of the possibilities include using artificial base pairs,[43,49] quadruplet codons[50] and repurposing the 61 sense codons. Besides eliminating unspecific GCE, artificial nucleic acid base pairs also have great potential in expanding the available set of codons. However these could so far only be used in *E.coli* for ncAA incorporation.[51] On the other hand, quadruplet codons have been found successful in mammalian cells and animal models like *C. elegans*.[52,53] Much effort has also been directed towards efficiently recoding sense codons. 61 sense codons encoding 20 amino acids allow for codon degeneracy. Taking advantage of this, selected sense codons have been freed up in synthetic *E.coli* strains by eliminating these codons from the entire genome.[41] Unlike stop codons, repurposing sense codons face the major challenge of competing with the abundant endogenous tRNAs, but it is possible and has recently been reported using, for example, rare codons like TCG codon.[54] However, it remains to be determined whether the efficiency of incorporation and the extent of off-target modifications are adequate for practical applications, such as fluorescence microscopy.

In vivo fluorescent labelling of ncAA-incorporated POIs can pose certain challenges which are detailed in the lower section of Figure 1 (**ncAA reacting with label**). Although synthetic dyes equipped with clickable chemical handles designed to react specifically with corresponding ncAAs are effective in minimizing background labeling, this strategy has inherent limitations. Cell permeable synthetic dyes often unspecifically bind to subcellular structures, especially membranes. A possible solution to this issue could be the development of fluorogenic dyes that become fluorescent only after reacting with its target.[55] However, it still leaves us with two other complications. During GCE, there is an excess availability of ncAAs in the cells, which are not incorporated into the POI and are free to react with the fluorescent dyes. Similarly, aminoacylated tRNAs that are not utilized by the ribosome can serve as reactive sites for clickable dyes, leading to background labeling. Nikic *et al.* showed that aminoacylated tRNAs bound to synthetases caused unspecific nuclear labelling, significantly hindering *in vivo* fluorescence imaging. It was discovered that a nuclear localization signal was encoded in the PylRS sequence, which led to its accumulation in the nucleus. The addition of a stronger nuclear export signal could prevent this undesirable nuclear accumulation of the tRNA-bound PylRS, making it accessible to the translation machinery and subsequently enabling reliable imaging applications.[56]

In conclusion, it can be said that while GCE research has come a long way, we are still quite far from unleashing the full potential of this technology. To advance, we must address challenges such as optimizing GCE machinery performance, mitigating the impact of competing host translational components like release factors and overcoming GCE-inhibiting pathways such as NMD and ISR. Additionally, enhancing mRNA specificity and expanding the availability of blank codons for ncAA incorporation are crucial. Ultimately, the convenience of the technology will be key to its widespread adoption. Clearly, the potential of GCE outweighs the effort that still needs to be made, but the effort has to be made to transform GCE into a technology that can genuinely revolutionize biology, biotechnology and medicine to achieve more possibilities and opportunities than evolution generated in billions of years.

DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 'R.B. declares no competing financial interest. E.A.L. holds several patents related to genetic code expansion and is a cofounder and consultant of Veraxa GmbH, a company specialized on generation of antibody drug conjugates via GCE.'.

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References

1. Nirenberg, M.W., Jones, O.W., Leder, P., Clark, B.F.C., Sly, W.S., Pestka, S., (1963). On the coding of genetic information. *Cold Spring Harb. Symp. Quant. Biol.* **28**, 549–557. https://doi.org/10.1101/SQB.1963.028.01.074.
2. Ambrogelly, A., Palioura, S., Söll, D., (2007). Natural expansion of the genetic code. *Nature Chem. Biol.* **3**, 29–35. https://doi.org/10.1038/nchembio847.
3. Crick, F.H.C., (1968). The origin of the genetic code. *J. Mol. Biol.* **38**, 367–379. https://doi.org/10.1016/0022-2836(68)90392-6.
4. Icking, L.-S., Riedlberger, A.M., Krause, F., Widder, J., Frederiksen, A.S., Stockert, F., Spädt, M., Edel, N., Armbruster, D., Forlani, G., Franchini, S., Kaas, P., Kırpat Konak, B.M., Krier, F., Lefebvre, M., Mazraeh, D., Ranniger, J., Gerstenecker, J., Gescher, P., Voigt, K., Salavei, P., Gensch, N., Di Ventura, B., Öztürk, M.A., (2024). iNClusive: a database collecting useful information on non-canonical amino acids and their incorporation into proteins for easier genetic code expansion implementation. *Nucleic Acids Res.* **52**, D476–D482. https://doi.org/10.1093/nar/gkad1090.
5. Heckler, T.G., Zama, Y., Naka, T., Hecht, S.M., (1983). Dipeptide formation with misacylated tRNAPhes. *J. Biol. Chem.* **258**, 4492–4495. https://doi.org/10.1016/S0021-9258(18)32650-4.
6. Reinkemeier, C.D., Girona, G.E., Lemke, E.A., (2019). Designer membraneless organelles enable codon reassignment of selected mRNAs in eukaryotes. *Science* **363**, https://doi.org/10.1126/science.aaw2644 eaaw2644.
7. Wang, L., Brock, A., Herberich, B., Schultz, P.G., (2001). Expanding the genetic code of *Escherichia coli*. *Science* **292**, 498–500. https://doi.org/10.1126/science.1060077.
8. Yanagisawa, T., Kuratani, M., Seki, E., Hino, N., Sakamoto, K., Yokoyama, S., (2019). Structural bgenetic-code expansion with bulky lysine derivatives by an engineered pyrrolasis for ysyl-tRNA synthetase. *Cell Chem. Biol.* **26**, 936–949.e13. https://doi.org/10.1016/j.chembiol.2019.03.008.
9. Niu, W., Guo, J., (2023). Co-translational installation of posttranslational modifications by non-canonical amino acid mutagenesis. *Chembiochem* **24**, https://doi.org/10.1002/cbic.202300039 e202300039.
10. Chen, J., Tsai, Y.-H., (2022). Applications of genetic code expansion in studying protein post-translational modification. *J. Mol. Biol.* **434**, https://doi.org/10.1016/j.jmb.2021.167424 167424.
11. Porter, J.J., Jang, H.S., Van Fossen, E.M., Nguyen, D.P., Willi, T.S., Cooley, R.B., Mehl, R.A., (2019). Genetically encoded protein tyrosine nitration in mammalian cells. *ACS Chem. Biol.* **14**, 1328–1336. https://doi.org/10.1021/acschembio.9b00371.
12. Beránek, V., Reinkemeier, C.D., Zhang, M.S., Liang, A.D., Kym, G., Chin, J.W., (2018). Genetically encoded protein phosphorylation in mammalian cells. *Cell Chem. Biol.* **25**, 1067–1074.e5. https://doi.org/10.1016/j.chembiol.2018.05.013.
13. Gautier, A., Nguyen, D.P., Lusic, H., An, W., Deiters, A., 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.
14. Yang, Y., Song, H., Chen, P.R., (2016). Genetically encoded photocrosslinkers for identifying and mapping protein-protein interactions in living cells. *IUBMB Life* **68**, 879–886. https://doi.org/10.1002/iub.1560.
15. Pearson, A.D., Mills, J.H., Song, Y., Nasertorabi, F., Han, G.W., Baker, D., Stevens, R.C., Schultz, P.G., (2015). Transition states. Trapping a transition state in a computationally designed protein bottle. *Science* **347**, 863–867. https://doi.org/10.1126/science.aaa2424.
16. Loh, C.T., Adams, L.A., Graham, B., Otting, G., (2018). Genetically encoded amino acids with tert-butyl and trimethylsilyl groups for site-selective studies of proteins by NMR spectroscopy. *J. Biomol. NMR* **71**, 287–293. https://doi.org/10.1007/s10858-017-0157-y.
17. Plass, T., Milles, S., Koehler, C., Szymański, J., Mueller, R., Wießler, M., Schultz, C., Lemke, E.A., (2012). Amino acids for Diels-Alder reactions in living cells. *Angew. Chem. Int. Ed.* **51**, 4166–4170. https://doi.org/10.1002/anie.201108231.
18. Schneider, A.F.L., Hackenberger, C.P.R., (2017). Fluorescent labelling in living cells. *Curr. Opin. Biotechnol.* **48**, 61–68. https://doi.org/10.1016/j.copbio.2017.03.012.
19. Schmidt, R., Weihs, T., Wurm, C.A., Jansen, I., Rehman, J., Sahl, S.J., Hell, S.W., (2021). MINFLUX nanometer-scale 3D imaging and microsecond-range tracking on a common fluorescence microscope. *Nature Commun.* **12**, 1478. https://doi.org/10.1038/s41467-021-21652-z.
20. Sharp, S., DeFranco, D., Dingermann, T., Farrell, P., Söll, D., (1981). Internal control regions for transcription of eukaryotic tRNA genes. *PNAS* **78**, 6657–6661. https://doi.org/10.1073/pnas.78.11.6657.
21. Amrani, N., Sachs, M.S., Jacobson, A., (2006). Early nonsense: mRNA decay solves a translational problem. *Nature Rev. Mol. Cell Biol.* **7**, 415–425. https://doi.org/10.1038/nrm1942.
22. Schmied, W.H., Elsässer, S.J., Uttamapinant, C., 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. Takimoto, J.K., Xiang, Z., Kang, J.-Y., 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.
24. Yin, H.-C., Chen, X.-Y., Wang, W., Meng, Q.-W., (2020). Identification and comparison of the porcine H1, U6, and 7SK RNA polymerase III promoters for short hairpin RNA expression. *Mamm. Genome* **31**, 110–116. https://doi.org/10.1007/s00335-020-09838-0.
25. Wang, Q., Wang, L., (2008). New Methods enabling efficient incorporation of unnatural amino acids in yeast. *J. Am. Chem. Soc.* **130**, 6066–6067. https://doi.org/10.1021/ja800894n.
26. Sakamoto, K., Hayashi, A., Sakamoto, A., Kiga, D., Nakayama, H., Soma, A., Kobayashi, T., Kitabatake, M., Takio, K., Saito, K., Shirouzu, M., Hirao, I., Yokoyama, S., (2002). Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. *Nucleic Acids Res.* **30**, 4692–4699.
27. Serfling, R., Lorenz, C., Etzel, M., Schicht, G., Böttke, T., Mörl, M., Coin, I., (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.
28. Jewel, D., Kelemen, R.E., Huang, R.L., Zhu, Z., Sundaresh, B., Cao, X., Malley, K., Huang, Z., Pasha, M., Anthony, J., van Opijnen, T., Chatterjee, A., (2023). Virus-assisted directed evolution of enhanced suppressor tRNAs in mammalian cells. *Nature Methods* **20**, 95–103. https://doi.org/10.1038/s41592-022-01706-w.
29. Chin, J.W., Cropp, T.A., Anderson, J.C., Mukherji, M., Zhang, Z., Schultz, P.G., (2003). An expanded eukaryotic genetic code. *Science* **301**, 964–967. https://doi.org/10.1126/science.1084772.
30. Pastrnak, M., Schultz, P.G., (2001). Phage selection for site-specific incorporation of unnatural amino acids into proteins *in vivo*. *Bioorg. Med. Chem.* **9**, 2373–2379. https://doi.org/10.1016/S0968-0896(01)00157-2.
31. Stieglitz, J.T., Van Deventer, J.A., (2022). High-throughput aminoacyl-tRNA SYNTHETASE engineering for genetic code expansion in yeast. *ACS Synth. Biol.* **11**, 2284–2299. https://doi.org/10.1021/acssynbio.1c00626.
32. Bryson, D.I., Fan, C., Guo, L.-T., Miller, C., Söll, D., Liu, D. R., (2017). Continuous directed evolution of aminoacyl-tRNA synthetases. *Nature Chem. Biol.* **13**, 1253–1260. https://doi.org/10.1038/nchembio.2474.
33. Willis, J.C.W., Chin, J.W., (2018). Mutually orthogonal pyrrolysyl-tRNA synthetase/tRNA pairs. *Nature Chem* **10**, 831–837. https://doi.org/10.1038/s41557-018-0052-5.
34. Osgood, A.O., Zheng, Y., Roy, S.J.S., Biris, N., Hussain, M., Loynd, C., Jewel, D., Italia, J.S., Chatterjee, A., (2023). An efficient opal-suppressor tryptophanyl pair creates new routes for simultaneously incorporating up to three distinct noncanonical amino acids into proteins in mammalian cells. *Angew. Chem. Int. Ed. Eng.* **62**, https://doi.org/10.1002/anie.202219269 e202219269.
35. Parrish, A.R., She, X., Xiang, Z., Coin, I., Shen, Z., Briggs, S.P., Dillin, A., Wang, L., (2012). Expanding the genetic code of caenorhabditis elegans using bacterial aminoacyl-tRNA synthetase/tRNA Pairs. *ACS Chem. Biol.* **7**, 1292–1302. https://doi.org/10.1021/cb200542j.
36. Greiss, S., Chin, J.W., (2011). Expanding the genetic code of an animal. *J. Am. Chem. Soc.* **133**, 14196–14199. https://doi.org/10.1021/ja2054034.
37. Sushkin, M.E., Koehler, C., Lemke, E.A., (2023). Remodeling the cellular stress response for enhanced genetic code expansion in mammalian cells. *Nature Commun.* **14**, 6931. https://doi.org/10.1038/s41467-023-42689-2.
38. Bartoschek, M.D., Ugur, E., Nguyen, T.-A., Rodschinka, G., Wierer, M., Lang, K., Bultmann, S., (2021). Identification of permissive amber suppression sites for efficient non-canonical amino acid incorporation in mammalian cells. *Nucleic Acids Res.* **49**, https://doi.org/10.1093/nar/gkab132 e62.
39. Rackham, O., Chin, J.W., (2005). A network of orthogonal ribosome·mRNA pairs. *Nature Chem. Biol.* **1**, 159–166. https://doi.org/10.1038/nchembio719.
40. Isaacs, F.J., Carr, P.A., Wang, H.H., Lajoie, M.J., Sterling, B., Kraal, L., Tolonen, A.C., Gianoulis, T.A., Goodman, D. B., Reppas, N.B., Emig, C.J., Bang, D., Hwang, S.J., Jewett, M.C., Jacobson, J.M., Church, G.M., (2011). Precise manipulation of chromosomes in vivo enables genome-wide codon replacement. *Science* **333**, 348–353. https://doi.org/10.1126/science.1205822.
41. Fredens, J., Wang, K., de la Torre, D., Funke, L.F.H., Robertson, W.E., Christova, Y., Chia, T., Schmied, W.H., Dunkelmann, D.L., Beránek, V., Uttamapinant, C., Llamazares, A.G., Elliott, T.S., Chin, J.W., (2019). Total synthesis of *Escherichia coli* with a recoded genome. *Nature* **569**, 514–518. https://doi.org/10.1038/s41586-019-1192-5.
42. Bain, J.D., Switzer, C., Chamberlin, R., Benner, S.A., (1992). Ribosome-mediated incorporation of a non-standard amino acid into a peptide through expansion of the genetic code. *Nature* **356**, 537–539. https://doi.org/10.1038/356537a0.
43. Hirao, I., Ohtsuki, T., Fujiwara, T., Mitsui, T., Yokogawa, T., Okuni, T., Nakayama, H., Takio, K., Yabuki, T., Kigawa, T., Kodama, K., Yokogawa, T., Nishikawa, K., Yokoyama, S., (2002). An unnatural base pair for incorporating amino acid analogs into proteins. *Nature Biotechnol.* **20**, 177–182. https://doi.org/10.1038/nbt0202-177.
44. Reinkemeier, C.D., Lemke, E.A., (2021). Dual film-like organelles enable spatial separation of orthogonal eukaryotic translation. *Cell* **184**, 4886–4903.e21. https://doi.org/10.1016/j.cell.2021.08.001.
45. Reinkemeier, C.D., Lemke, E.A., (2022). Condensed, microtubule-coating thin organelles for orthogonal translation in mammalian cells. *J. Mol. Biol.* **434**, https://doi.org/10.1016/j.jmb.2022.167454 167454.
46. Cao, X., Slavoff, S.A., (2020). Non-AUG start codons: expanding and regulating the small and alternative ORFeome. *Exp. Cell Res.* **391**, https://doi.org/10.1016/j.yexcr.2020.111973 111973.
47. Bhatter, N., Dmitriev, S.E., Ivanov, P., (2024). Cell death or survival: Insights into the role of mRNA translational control. *Semin. Cell Dev. Biol.* **154**, 138–154. https://doi.org/10.1016/j.semcdb.2023.06.006.
48. Joest, E.F., Winter, C., Wesalo, J.S., Deiters, A., Tampé, R., (2022). Efficient amber suppression via ribosomal skipping for in situ synthesis of photoconditional

nanobodies. *ACS Synth. Biol.* **11**, 1466–1476. https://doi.org/10.1021/acssynbio.1c00471.
49. Seo, Y.J., Hwang, G.T., Ordoukhanian, P., Romesberg, F. E., (2009). Optimization of an unnatural base pair toward natural-like replication. *J. Am. Chem. Soc.* **131**, 3246–3252. https://doi.org/10.1021/ja807853m.
50. Chen, Y., Wan, Y., Wang, N., Yuan, Z., Niu, W., Li, Q., Guo, J., (2018). Controlling the replication of a genomically recoded HIV-1 with a functional quadruplet codon in mammalian cells. *ACS Synth. Biol.* **7**, 1612–1617. https://doi.org/10.1021/acssynbio.8b00096.
51. Zhang, Y., Ptacin, J.L., Fischer, E.C., Aerni, H.R., Caffaro, C.E., San Jose, K., Feldman, A.W., Turner, C.R., Romesberg, F.E., (2017). A semi-synthetic organism that stores and retrieves increased genetic information. *Nature* **551**, 644–647. https://doi.org/10.1038/nature24659.
52. Xi, Z., Davis, L., Baxter, K., Tynan, A., Goutou, A., Greiss, S., (2022). Using a quadruplet codon to expand the genetic code of an animal. *Nucleic Acids Res.* **50**, 4801–4812. https://doi.org/10.1093/nar/gkab1168.
53. Niu, W., Schultz, P.G., 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.
54. Ding, W., Yu, W., Chen, Y., Lao, L., Fang, Y., Fang, C., Zhao, H., Yang, B., Lin, S., (2024). Rare codon recoding for efficient noncanonical amino acid incorporation in mammalian cells. *Science* **384**, 1134–1142. https://doi.org/10.1126/science.adm8143.
55. Si, D., Li, Q., Bao, Y., Zhang, J., Wang, L., (2023). Fluorogenic and cell-permeable rhodamine dyes for high-contrast live-cell protein labeling in bioimaging and biosensing. *Angew. Chem. Int. Ed.* **62**, e202307641.
56. Nikić, I., Estrada Girona, G., Kang, J.H., Paci, G., Mikhaleva, S., Koehler, C., Shymanska, N.V., Ventura Santos, C., Spitz, D., Lemke, E.A., (2016). Debugging eukaryotic genetic code expansion for site-specific click-PAINT super-resolution microscopy. *Angew. Chem. Int. Ed. Eng.* **55**, 16172–16176. https://doi.org/10.1002/anie.201608284.