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In vitro genetic code reprogramming and expansion to study protein function and discover macrocyclic peptide ligands

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Abstract

The ability to introduce non-canonical amino acids into peptides and proteins is facilitated by working within in vitro translation systems. Non-canonical amino acids can be introduced into these systems using sense codon reprogramming, stop codon suppression, and by breaking codon degeneracy. Here, we review how these techniques have been used to create proteins with novel properties and how they facilitate sophisticated studies of protein function. We also discuss how researchers are using in vitro translation experiments with non-canonical amino acids to explore the tolerance of the translation apparatus to artificial building blocks. Finally, we give several examples of how non-canonical amino acids can be combined with mRNA-displayed peptide libraries for the creation of protease-stable, macrocyclic peptide libraries for ligand discovery.

Graphical Abstract

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Introduction of ncAAs in vitro.

The addition of non-canonical amino acids (ncAAs) to the genetic code has had a significant impact on the field of synthetic biology, allowing for the expansion of our understanding of protein structure and function, as well as the discovery of novel peptide ligands with properties not readily attainable via standard amino acids. To facilitate their use in vitro, researchers have worked to develop methods to introduce ncAAs into peptides and proteins via in vitro translation systems.

There have been three main approaches to alter or expand the genetic code in vitro (Fig. 1). Sense codon reprogramming allows for reassignment of a codon box to an ncAA (Fig. 1A). This approach typically utilizes reconstituted in vitro translation systems (like the PURE system [1]). The inherent flexibility of these systems allows researchers to omit a given amino acid or aminoacyl-tRNA synthetase (AARS), leading to tRNA(s) that are not charged with their cognate AA. The ncAA can then be integrated into the translation system, either by recognition of the ncAA by the cognate synthetase or, more commonly, addition of an ncAA-charged tRNA to cover the vacated codons. The ncAA-tRNA can be prepared by a variety of methods. Three common strategies include: the Flexizyme ribozyme [2], enzymatic charging using natural [3,4] or engineered synthetase promiscuity [5, 6, 7], or aminoacyl-dinucleotide ligation [8,9]. The second approach to genetic code expansion is utilization of stop codon suppression to introduce the ncAA (Fig. 1B). In this case, the system is supplemented with an ncAA-tRNA that recognizes a stop codon, typically the amber (UAG) codon. The suppressor tRNA can be generated using an orthogonal AARS-tRNA pair or can be created via chemical charging methods. Finally, working in vitro also affords opportunities to break sense codon degeneracy, which can enable significant genetic code expansion (Fig. 1C). Recently, Alexandrov has shown that many of the tRNAs in *E. coli* can be replaced with their in vitro transcribed counterparts, which function even though they lack post-transcriptional modifications [10]. By depleting native tRNAs and replacing them with in vitro transcribed ncAA-tRNAs, his group has been able to reassign amber and arginine codons to ncAAs [11]. Suga has used a similar strategy to achieve genetic code expansion. By creating synthetic tRNA^{Asn} with modified anticodons and charging these tRNAs with ncAAs via Flexizyme technology, they were able to split the valine, arginine and glycine codon boxes to generate a genetic code with 23 amino acids [12]. Although the limits of these methods have not yet been probed, a reasonable estimate is that a genetic code with over 30 building blocks could be constructed.

Scope of review.

In the rest of this review, we discuss applications of these three technologies to alter the genetic code in vitro. We have limited the scope of this review to cover research first published online from November 2015 to January 2018.

Improving stop codon suppression systems.

In vivo stop codon suppression systems with orthogonal AARSs and tRNAs are increasingly being used in synthetic biology and to probe protein function. In vitro translation

technologies are being used for rapid troubleshooting of these components to improve their activities and optimize the efficiency of ncAA introduction both in vitro and in vivo. For example, Forster has investigated the inefficiencies of the pyrolysyl tRNA (tRNA^{Pyl}) [13] and found that there is room for optimization on multiple levels. Bundy has used in vitro screening to rapidly investigate the efficiency of stop codon suppression of ncAA-tRNA^{Pyl}s at various positions within a protein [14]. Work by Alfonta has optimized suppression of both the amber and ochre (TAA) stop codons with aminoacyl-tRNAs bearing azides and alkynes [15]. Fan has employed biotinylated oligonucleotides to deplete near-cognate tRNAs for incorporation of ncAAs in response to amber stop codons. The fidelity of the system was improved, which suggests that competing tRNAs can be an issue that limits suppression efficiency [16]. Finally, Kubick has worked on incorporating ncAAs into cell free protein synthesis systems from insect [17] and mammalian cells [18]. Overall, these in vitro studies point to important features of the in vivo systems that can be optimized to improve efficiency.

Pushing the boundaries of the translation apparatus.

Several groups have capitalized upon the ability to introduce ncAA-tRNAs in vitro to test the limits of the translation apparatus with respect to highly modified amino acid substrates (Fig. 2). While the translation system was shown to tolerate single D-amino acid substitutions in preceding years [19,20], achieving consecutive incorporation was only recently demonstrated by Katoh and Suga. This advance required using tRNA bodies with high affinity for elongation factor-Tu (EF-Tu) to improve incorporation efficiency [21]. To improve the peptidyl transfer rate, elements within the tRNA that are recognized by elongation factor-P (EF-P) were integrated [•22]. This could prove to be a general strategy to improve the incorporation of ncAA-tRNAs that are poor substrates for the wild-type ribosome. Murakami has demonstrated that standard β -homologs of many amino acids can also be incorporated into in vitro translation systems using wild-type ribosomes [23]. We recently demonstrated compatibility with cyclic β -amino acids, which are important for their ability to introduce conformational constraints in peptides, although their incorporation was template-dependent [•6]. We also found that α -methyl amino acids, useful for templating helical structures, are tolerated by the ribosome, but that the translation apparatus needs to be supplemented with additional aminoacyl-tRNA for efficient incorporation [•6]. We also showed that six consecutive 4-fluorohistidine residues could be incorporated, leading to a fluoro-His tag which binds to nickel resin even at low pH [24]. While the above studies focused on wild-type ribosomes, Hecht has taken a different tack and has selected for mutant ribosomes that can tolerate dipeptidyl-tRNAs, incorporating two amino acids in a single elongation step [25]. Although there is still room for further optimization, each of these recent advances has shown that the translation machinery is more pliable towards ncAAs than previously imagined.

In vitro synthesis of modified proteins.

In vitro translation has long been a dependable technique for preparation of small quantities of proteins. Because introduction of an ncAA into a protein in vitro does not require engineering a new AARS/tRNA pair, small scale studies of in vitro translated proteins with

ncAAs can be performed quickly using stop codon suppression. This approach allows proteins to be endowed with novel properties. For example, Hecht introduced 6-cyano tryptophan as a FRET pair to study the binding of several proteins to DNA labeled with fluorescent nucleobases [26]. Using the dipeptidyl-tRNA tolerant ribosomes, he incorporated aryl oxazole and thiazole dipeptide mimics into the GFP structure, leading to the formation of modified fluorophores and fluorescent proteins [27]. Introduction of ncAAs has also made its mark in the synthesis of radiolabeled proteins for PET imaging. In vitro translation in the presence of ^{18}F -4-fluoro-proline led to radiolabeled interleukin-8, which was employed by Yanai to detect tumors bearing IL8-receptors in mouse xenografts [28]. The inclusion of ncAAs can also contribute to the understanding of protein mechanisms and interactions. For example, Hecht created ncAAt-RNAs containing various lysine analogs to probe the importance of Schiff base formation of lysine 72 on the lyase activity of DNA polymerase β [29]. He also prepared phosphorylated I κ B- α (phosphotyrosine) and analyzed its reaction with NF- κ B [30]. Finally, Hecht has created alanine derivatives containing nucleobase side chains [31] and demonstrated that incorporation of these derivatives into a nucleic acid binding motif increased its affinity for *BCL2* i-motif DNA [•32]. As these examples demonstrate, in vitro stop codon suppression technologies have carved out an important niche for small scale studies of protein function.

Incorporating ncAAs into macrocyclic peptide libraries.

The small scale of in vitro translation experiments is sufficient, and in fact, ideal for ligand discovery projects. Coupling the PURE system or stop codon suppression with mRNA display is an extremely powerful approach that affords access to libraries containing over 10^{13} unique peptides containing ncAAs. With mRNA display, a peptide becomes co-translationally attached to the mRNA that encodes it, allowing for iterative cycles of selection based on binding affinity and amplification by PCR (Fig. 3). When combined with next-generation sequencing, deep pools of peptides can be rapidly analyzed for affinity [•33].

Recent work in the mRNA display field has focused on the creation of macrocyclic peptide libraries containing ncAAs. Peptide cyclizations in the context of in vitro translated libraries are challenging, as the reactions must be high-yielding and compatible with all other library components (and, if performed co-translationally, with the translation buffer and proteins). There have been relatively few chemistries discovered so far that meet these criteria (Fig. 4). Roberts and co-workers demonstrated the first cyclization in mRNA-displayed libraries by alkylating two lysines via a bis-succinimidyl ester [34] (Fig. 4A). More recently, they have used this cyclization chemistry in combination with the addition of an N-methyl alanine or norvaline to a suppressor tRNA to select for protease-stable peptides [•35]. During the in vitro selection, a protease challenge step was applied to further eliminate those peptides that were unstable. The resulting cyclic peptides bound with high affinity and were orders of magnitude more stable in serum than their linear counterparts [•35].

Suga has pioneered co-translational cyclization of mRNA-displayed peptide libraries. By charging the initiator tRNA with an N-chloroacetyl amino acid rather than the standard formyl group, post-translational cyclization occurs with a downstream cysteine within the

library, forming a thioether linkage [36] (Fig. 4B). The advantage of this strategy is that the cyclization occurs in situ, with no further treatment required. The translation can also be initiated with D- or L-amino acids, N-acyl or N-alkyl amino acids, and even carboxylic acids without amino groups [39]. This technology has led to seminal discoveries of cyclic peptides with high affinity. Natsume exploited the promiscuity at the initiator position to discover cyclic peptides that bind to β -catenin that are hybrids of peptides and a single peptoid building block [39]. Using a D-tryptophan initiated library and a C-terminal cysteine flanked by variable numbers of amino acids, Suga uncovered fluorescent peptides with low nM affinity to the extracellular domain EpCAM and subsequently utilized them as imaging agents to detect EpCAM expressing cells [40]. They also identified a high-affinity ligand to Plexin B1 that inhibits its signaling cascade through its interaction with Sema4D [41]. Phosphoglycerate mutase inhibitors were also developed and were used to unravel the enzyme mechanism [42]. Suga has applied this system to Ebola virus inhibition by targeting the VP24–KPNA5 interaction, leading to tight binding protein-protein interaction (PPI) inhibitors with micromolar activity [43]. While none of the above inhibitors were reported to be cell permeable (and in most cases this was not essential for activity), recently Suga has used mRNA display to uncover a peptide that binds intracellularly to histone demethylases KDM4A-C. Although the initial hit was not protease-stable or cell-permeable, these characteristics could be augmented via iterative rational design [44,45]. Finally, Suga and Withers used in vitro selection to uncover lariat peptides that were potent glycosidase inhibitors, binding to pancreatic α -amylase with low nM affinity. The interesting co-crystal structure of the interaction showed that the lariat tail adopted a 3_{10} helical structure [46].

The third type of chemistry for the synthesis of cyclic peptide libraries, originally developed by Timmerman, is the dibromoxylene (DBx) cyclization that reacts with two cysteines [47] (Fig. 4C). This chemistry has been applied across multiple library platforms [48–51] and was first applied to mRNA-displayed peptide libraries in combination with ncAAs by Guillen-Schlippe and Szostak [37] in their seminal work to develop thrombin inhibitors. We recently built upon this work by combining DBx cyclization with click chemistry to create highly constrained bicyclic binders of streptavidin [38] (Fig. 4D). To introduce the second cycle, *p*-ethynyl-phenylalanine and γ -azido-homoalanine were introduced in place of phenylalanine and methionine, respectively. The resulting peptides were high nM binders and protease stable. The ability to create two orthogonal rings allows for the construction of overlapping (θ) cyclic structures of high rigidity, which should promote binding affinity alongside stability. With access to diverse ncAAs through genetic code reprogramming and more complex structures via macrocyclization, researchers are now able to routinely uncover high-affinity peptide and peptidomimetic ligands against their desired targets.

Future perspectives.

Adding ncAAs to the genetic code by reprogramming, stop codon suppression, or by breaking codon degeneracy has proven to be a powerful strategy to study the limits of the translation apparatus and to modify and study protein function. Efforts to extend these insights to creation of organisms with expanded genetic codes are exciting and ongoing [52]. However, the most promising use of the technology in vitro lies in the power of creation of diverse peptide libraries. The chief challenge facing peptide library technologies is that

larger peptides are often not cell permeable and therefore cannot be used for disruption of undruggable intracellular protein-protein interactions. While recent discoveries of highly penetrant carrier peptides [53] could perhaps lead to a general solution, genetic code expansion will also play an important role. The ability to increase the number of unique building blocks by breaking codon degeneracy will allow more diversity to be packed into shorter sequences that are more likely to be cell permeable. Moreover, through engineering, the ribosome is becoming increasingly tolerant to building blocks with highly modified structures, opening the door to future ribosomal biosynthesis of peptidomimetic sequences. The future promise of this technology is the synthesis of highly diverse, protease-stable, and cell-permeable libraries suitable for rapid drug discovery.

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References

1. Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, Ueda T: Cell-free translation reconstituted with purified components. *Nat Biotechnol* 2001, 19:751–755. [PubMed: 11479568]
 2. Murakami H, Ohta A, Ashigai H, Suga H: A highly flexible tRNA acylation method for non-natural polypeptide synthesis. *Nat Methods* 2006, 3:357–359. [PubMed: 16628205]
 3. Hartman MCT, Josephson K, Szostak JW: Enzymatic aminoacylation of tRNA with unnatural amino acids. *P Natl Acad Sci Usa* 2006, 103:4356–4361.
 4. Fan C, Ho JML, Chirathivat N, Söll D, Wang Y-S: Exploring the Substrate Range of Wild-Type Aminoacyl-tRNA Synthetases. *ChemBioChem* 2014, 15:1805–1809. [PubMed: 24890918]
 5. Richardson CJ, First EA: Hyperactive editing domain variants switch the stereospecificity of tyrosyl-tRNA synthetase. *Biochemistry* 2016, 55:2526–2537.
- The authors of this work demonstrate a remarkable promiscuity of an editing deficient AARS to ncAAs. They also show, for the first time, that cyclic β -amino acids are compatible with the translation apparatus.
- [PubMed: 27064538]
6. Iqbal ES, Dods KK, Hartman MCT: Ribosomal incorporation of backbone modified amino acids via an editing-deficient aminoacyl- tRNA synthetase. *Org Biomol Chem* 2018, doi:10.1039/c7ob02931d.
 7. Young DD, Young TS, Jahnz M, Ahmad I, Spraggon G, Schultz PG: An evolved aminoacyltRNA synthetase with atypical polysubstrate specificity. *Biochemistry* 2011, 50:1894–1900.
 8. Heckler TG, Chang LH, Zama Y, Naka T, Chorghade MS, Hecht SM: T4 RNA Ligase Mediated Preparation of Novel “Chemically Misacylated” tRNAs. *Biochemistry* 1984, 23:1468–1473. [PubMed: 6372858]
 9. Robertson SA, Ellman JA, Schultz PG: A general and efficient route for chemical aminoacylation of transfer RNAs. *J Am Chem Soc* 1991, 113:2722–2729.
 10. Cui Z, Stein V, Tnimov Z, Mureev S, Alexandrov K: Semisynthetic tRNA complement mediates in vitro protein synthesis. *J Am Chem Soc* 2015, 137:4404–4413. [PubMed: 25822136]
 11. Cui Z, Mureev S, Polinkovsky ME, Tnimov Z, Guo Z, Durek T, Jones A, Alexandrov K: Combining Sense and Nonsense Codon Reassignment for Site-Selective Protein Modification with Unnatural Amino Acids. *ACS Synth Biol* 2017, 6:535–544. [PubMed: 27966891]
 12. Iwane Y, Hitomi A, Murakami H, Katoh T, Goto Y, Suga H: Expanding the amino acid repertoire of ribosomal polypeptide synthesis via the artificial division of codon boxes. *Nat Chem* 2016, 8:317–325.

The authors replace the standard commercial tRNA mix with in vitro transcribed versions. This strategy allows them to rapidly divide codon boxes for the introduction of 23 amino acids and highlights the potential of dividing codon boxes for the expansion of the genetic code.

[PubMed: 27001726]

13. Wang J, Kwiatkowski M, Forster AC: Kinetics of tRNA^{Pyl}-mediated amber suppression in *Escherichia coli* translation reveals unexpected limiting steps and competing reactions. *Biotechnol Bioeng* 2016, 113:1552–1559. [PubMed: 26705134]
 14. Schinn S-M, Bradley W, Groesbeck A, Wu JC, Broadbent A, Bundy BC: Rapid in vitro screening for the location-dependent effects of unnatural amino acids on protein expression and activity. *Biotechnol Bioeng* 2017, 114:2412–2417. [PubMed: 28398594]
 15. Ozer E, Chemla Y, Schlesinger O, Aviram HY, Riven I, Haran G, Alfonta L: In Vitro Suppression of Two Different Stop Codons. *Biotechnol Bioeng* 2017, 114:1065–1073. [PubMed: 27882539]
 16. Gan Q, Fan C: Increasing the fidelity of noncanonical amino acid incorporation in cell-free protein synthesis. *Biochim Biophys Acta - Gen Subj* 2017, 1861:3047–3052. [PubMed: 27919800]
 17. Quast RB, Ballion B, Stech M, Sonnabend A, Varga BR, Wüstenhagen DA, Kele P, Schiller SM, Kubick S: Cell-free synthesis of functional human epidermal growth factor receptor: Investigation of ligand-independent dimerization in Sf21 microsomal membranes using non-canonical amino acids. *Sci Rep* 2016, 6:34048. [PubMed: 27670253]
 18. Stech M, Nikolaeva O, Thoring L, Stöcklein WFM, Wüstenhagen DA, Hust M, Dübel S, Kubick S: Cell-free synthesis of functional antibodies using a coupled in vitro transcription-translation system based on CHO cell lysates. *Sci Rep* 2017, 7:12030. [PubMed: 28931913]
 19. Achenbach J, Jahnz M, Bethge L, Paal K, Jung M, Schuster M, Albrecht R, Jarosch F, Nierhaus KH, Klussmann S: Outwitting EF-Tu and the ribosome: translation with D-amino acids. *Nucleic Acids Res* 2015, 43:5687–98. [PubMed: 26026160]
 20. Fujino T, Goto Y, Suga H, Murakami H: Reevaluation of the D-amino acid compatibility with the elongation event in translation. *J Am Chem Soc* 2013, 135:1830–1837.
 21. Katoh T, Tajima K, Suga H: Consecutive Elongation of D-Amino Acids in Translation. *Cell Chem Biol* 2017, 24:46–54. [PubMed: 28042044]
 22. Katoh T, Iwane Y, Suga H: Logical engineering of D-arm and T-stem of tRNA that enhances D-amino acid incorporation. *Nucleic Acids Res* 2017, 45:12601–12610.
- The authors show that adding EF-P recognition elements to difficult to translation AA-tRNAs improve efficiency. This may be a general strategy for incorporating many ncAAs that have shown low efficiency.

[PubMed: 29155943]

23. Fujino T, Goto Y, Suga H, Murakami H: Ribosomal Synthesis of Peptides with Multiple β -Amino Acids. *J Am Chem Soc* 2016, 138:1962–1969. [PubMed: 26807980]
24. Ring CM, Iqbal ES, Hacker DE, Hartman MCT, Cropp TA, Forster AC, Blacklow SC: Genetic incorporation of 4-fluorohistidine into peptides enables selective affinity purification. *Org Biomol Chem* 2017, 15:4536–4539. [PubMed: 28517015]
25. Maini R, Dedkova LM, Paul R, Madathil MM, Chowdhury SR, Chen S, Hecht SM: Ribosome-Mediated Incorporation of Dipeptides and Dipeptide Analogues into Proteins in Vitro. *J Am Chem Soc* 2015, 137:11206–11209. [PubMed: 26301427]
26. Talukder P, Chen S, Roy B, Yakovchuk P, Spiering MM, Alam MP, Madathil MM, Bhattacharya C, Benkovic SJ, Hecht SM: Cyanotryptophans as Novel Fluorescent Probes for Studying Protein Conformational Changes and DNA-Protein Interaction. *Biochemistry* 2015, 54:7457–7469. [PubMed: 26618501]
27. Chowdhury SR, Chauhan PS, Dedkova LM, Bai X, Chen S, Talukder P, Hecht SM: Synthesis and evaluation of a library of fluorescent dipeptidomimetic analogues as substrates for modified bacterial ribosomes. *Biochemistry* 2016, 55:2427–2440. [PubMed: 27050631]

28. Harada R, Furumoto S, Yoshikawa T, Ishikawa Y, Shibuya K, Okamura N, Ishiwata K, Iwata R, Yanai K: Synthesis and characterization of 18F-interleukin-8 using a cell-free translation system and 18F-fluoro-L-proline. *J Nucl Med* 2016, 57:634–640. [PubMed: 26742712]
 29. Daskalova SM, Bhattacharya C, Dedkova LM, Hecht SM: Probing the Flexibility of the Catalytic Nucleophile in the Lyase Catalytic Pocket of Human DNA Polymerase β with Unnatural Lysine Analogues. *Biochemistry* 2017, 56:500–513. [PubMed: 28005340]
 30. Chen S, Maini R, Bai X, Nangreave RC, Dedkova LM, Hecht SM: Incorporation of Phosphorylated Tyrosine into Proteins: In Vitro Translation and Study of Phosphorylated I κ B- α and Its Interaction with NF- κ B. *J Am Chem Soc* 2017, 139:14098–14108. [PubMed: 28898075]
 31. Talukder P, Dedkova LM, Ellington AD, Yakovchuk P, Lim J, Anslyn E V, Hecht SM: Synthesis of alanyl nucleobase amino acids and their incorporation into proteins. *Bioorganic Med Chem* 2016, 24:4177–4187.
 32. Bai X, Talukder P, Daskalova SM, Roy B, Chen S, Li Z, Dedkova LM, Hecht SM: Enhanced Binding Affinity for an i-Motif DNA Substrate Exhibited by a Protein Containing Nucleobase Amino Acids. *J Am Chem Soc* 2017, 139:4611–4614.
- In this paper, the authors introduce nucleobase amino acids into a protein and show enhanced, but modest DNA-binding affinity increases. These nucleobase amino acids promise to endow peptides and proteins with the ability to recognize DNA sequences with high selectivity and affinity.
- [PubMed: 28263595]
33. Jalali-Yazdi F, Huong lai L, Takahashi TT, Roberts RW: High-Throughput Measurement of Binding Kinetics by mRNA Display and Next-Generation Sequencing. *Angew Chemie - Int Ed* 2016, 55:4007–4010.
- The authors uses next-generation sequencing to solve on of the bottlenecks in peptide discovery by mRNA display--determining the affinity of the thousands of binders. Next-generation sequencing allows them to find high-affinty peptides that otherwise would have been neglected due to their low abundance in the selection.
34. Millward SW, Takahashi TT, Roberts RW: A General Route for Post-Translational Cyclization of mRNA-displayed Libraries. *J Am Chem Soc* 2005, 127:14142–14143. [PubMed: 16218582]
 35. Fiacco S V, Kelderhouse LE, Hardy A, Peleg Y, Hu B, Ornelas A, Yang P, Gammon ST, Howell SM, Wang P, et al.: Directed Evolution of Scanning Unnatural-Protease-Resistant (SUPR) Peptides for in Vivo Applications. *ChemBioChem* 2016, 17:1643–1651.
- In this paper, the authors challenge the mRNA-displayed peptide library containing a 21st N-methyl amino acid with a cocktail of proteases to select for protease resistance peptides. This creative approach allowed them to directly select peptides that were of high affinity and serum stable.
36. Goto Y, Ohta A, Sako Y, Yamagishi Y, Murakami H, Suga H: Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. *ACS Chem Biol* 2008, 3:120–129. [PubMed: 18215017]
 37. Guillen Schlippe Y V, Hartman MCT, Josephson K, Szostak JW: In vitro selection of highly modified cyclic peptides that act as tight binding inhibitors. *J Am Chem Soc* 2012, 134:10469–10477. [PubMed: 22428867]
 38. Hacker DE, Hoinka J, Iqbal ES, Przytycka TM, Hartman MCT: Highly Constrained Bicyclic Scaffolds for the Discovery of Protease-Stable Peptides via mRNA Display. *ACS Chem Biol* 2017, 12:795–804.
- This in vitro selection paper demonstrates that two, orthogonal cyclizations can be performed on mRNA-displayed peptide libraries. The authors use next-generations sequencing and computational clustering to identify the selection winners. The resulting, highly constrained binders to streptavidin were highly stable to digestion by chymotrypsin.
- [PubMed: 28146347]
39. Kawakami T, Ogawa K, Hatta T, Goshima N, Natsume T: Directed Evolution of a Cyclized Peptoid-Peptide Chimera against a Cell-Free Expressed Protein and Proteomic Profiling of the

Interacting Proteins to Create a Protein-Protein Interaction Inhibitor. *ACS Chem Biol* 2016, 11:1569–1577. [PubMed: 27010125]

40. Iwasaki K, Goto Y, Katoh T, Yamashita T, Kaneko S, Suga H: A Fluorescent Imaging Probe Based on a Macrocyclic Scaffold That Binds to Cellular EpCAM. *J Mol Evol* 2015, 81:210–217. [PubMed: 26553371]

41. Matsunaga Y, Bashiruddin NK, Kitago Y, Takagi J, Suga H: Allosteric Inhibition of a Semaphorin 4D Receptor Plexin B1 by a High-Affinity Macrocyclic Peptide. *Cell Chem Biol* 2016, 23:1341–1350. [PubMed: 27984026]

42. Yu H, Dranchak P, Li Z, Macarthur R, Munson MS, Mehzabeen N, Baird NJ, Battalie KP, Ross D, Lovell S, et al.: Macrocyclic peptides delineate locked-open inhibition mechanism for microorganism phosphoglycerate mutases. *Nat Commun* 2017, 8:14932

In this paper, the authors use mRNA display to find a high-affinity cyclic peptide that binds selectively to histone demethylases KDM4A-C. They used structure, guided rational design to improve the permeability and stability of the compound in cells. The breadth of the work demonstrates strategies needed and the potential of mRNA-displayed peptide libraries for development of inhibitors of intracellular targets.

[PubMed: 28368002]

43. Song X, Lu L, Passioura T, Suga H: Macrocyclic peptide inhibitors for the protein–protein interaction of Zaire Ebola virus protein 24 and karyopherin alpha 5. *Org Biomol Chem* 2017, 15:5155–5160. [PubMed: 28574091]

44. Kawamura A, Münzel M, Kojima T, Yapp C, Bhushan B, Goto Y, Tumber A, Katoh T, King ONF, Passioura T, et al.: Highly selective inhibition of histone demethylases by de novo macrocyclic peptides. *Nat Commun* 2017, 8:14773. [PubMed: 28382930]

45. Passioura T, Bhushan B, Tumber A, Kawamura A, Suga H: Structure-activity studies of a macrocyclic peptide inhibitor of histone lysine demethylase 4A. *Bioorganic Med Chem* 2018, 26:1225–1231.

46. Jongkees SAK, Caner S, Tysoe C, Brayer GD, Withers SG, Suga H: Rapid Discovery of Potent and Selective Glycosidase-Inhibiting De Novo Peptides. *Cell Chem Biol* 2017, 24:381–390. [PubMed: 28262556]

47. Timmerman P, Beld J, Puijk WC, Meloen RH: Rapid and quantitative cyclization of multiple peptide loops onto synthetic scaffolds for structural mimicry of protein surfaces. *ChemBioChem* 2005, 6:821–824. [PubMed: 15812852]

48. Heinis C, Rutherford T, Freund S, Winter G: Phage-encoded combinatorial chemical libraries based on bicyclic peptides. *Nat Chem Biol* 2009, 5:502–507. [PubMed: 19483697]

49. Peraro L, Siegert TR, Kritzer JA: Conformational Restriction of Peptides Using Dithiol Bis-Alkylation. *Methods Enzymol* 2016, 580:303–332. [PubMed: 27586339]

50. White ER, Sun L, Ma Z, Beckta JM, Danzig BA, Hacker DE, Huie M, Williams DC, Edwards RA, Valerie K, et al.: **Peptide Library Approach to Uncover Phosphomimetic Inhibitors of the BRCA1 C-Terminal Domain.** *ACS Chem Biol* 2015, 10:1198–1208. [PubMed: 25654734]

51. Hacker DE, Almohaini M, Anbazhagan A, Ma Z, Hartman MCT: Peptide and peptide library cyclization via bromomethylbenzene derivatives. *Methods Mol Biol* 2015, 1248:105–117. [PubMed: 25616329]

52. Chin JW: Expanding and reprogramming the genetic code. *Nature* 2017, 550:53–60. [PubMed: 28980641]

53. Qian Z, Dougherty PG, Pei D: Targeting intracellular protein–protein interactions with cell-permeable cyclic peptides. *Curr Opin Chem Biol* 2017, 38:80–86. [PubMed: 28388463]

Highlights.

- The genetic code can be enhanced or expanded in vitro using sense codon reprogramming, stop codon suppression, and breaking of codon degeneracy.
- A variety of ncAAs can now be incorporated via the translation apparatus, including D-amino acids, dipeptides, β -amino acids, and α -methyl amino acids.
- Use of in vitro translation to introduce ncAAs into proteins enables rapid access to proteins endowed with enhanced and novel properties.
- Various cyclization chemistries can be utilized with in vitro translated ncAAs to generate macrocyclic peptide libraries, allowing for development of protease-stable, peptide ligands.

A Sense codon reprogramming

1st	2nd				3rd	
	U	C	A	G		
U	Phe	Ser	Tyr	Cys	U	
			STOP	STOP	C	
	Leu	Pro	STOP	Trp	A	
					G	
C	Leu	Pro	His	Arg	U	
						C
						A
			Gln			G
A	Ile	Thr	Asn	Ser	U	
					C	
	Met	Thr	Lys	Arg	A	
					G	
G	ncAA	Ala	Asp	Gly	U	
						C
			Glu			A
						G

Total: 20 amino acids **B Stop codon suppression**

1st	2nd				3rd
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
			STOP	STOP	A
	Leu	Pro	ncAA	Trp	G
C			His	Arg	U
	A	Ile			Thr
G			Met	Lys	
	G	Val			Ala
G			Glu	Gly	
	G	G			G
G			G	G	
	G	G			G

Total: 21 amino acids **C Breaking codon degeneracy + sense codon reprogramming**

1st	2nd				3rd
	U	C	A	G	
U	Phe	ncAA	Tyr	Cys	U
	ncAA	Ser	STOP	STOP	A
			STOP	Trp	G
C	ncAA	Pro	His	ncAA	U
	ncAA	ncAA	Gln	ncAA	C
					A
A	Ile	Thr	Asn	ncAA	U
		ncAA	Lys	Arg	C
	Met				G
G	ncAA	Ala	Asp	Gly	U
	Val	ncAA	Glu	ncAA	C
					A

Total*: 30 amino acids **Figure 1. Three strategies for introducing ncAAs in vitro.**

With sense codon reprogramming (A) an ncAA-charged tRNA is substituted for a standard AA-tRNA. For stop codon suppression (B), the genetic code is expanded with an ncAA-tRNA that suppresses the amber stop codon. When codon degeneracy is broken (C), a codon box is split, allowing expansion of the genetic code. *For sense codon reprogramming, the practical limit for genetic code expansion is around 30 amino acids due to overlapping tRNA recognition

Figure 2.
Types of backbone modified amino acids that have been incorporated using in vitro translation.

Figure 3. In vitro selection by mRNA display.

A library of mRNAs is first either ligated or photocrosslinked (shown here with a blue oval) to a puromycin-containing linker. The puromycin-labeled mRNA library is used as a template along with a customized pool of amino acids in an in vitro translation reaction. The resulting mRNA-peptide fusion library can be used directly or cyclized prior to capture onto beads coated with protein of interest (POI). The bound mRNA peptide fusions are amplified using RT-PCR and the selection cycle continues.

Figure 4. Four strategies for cyclization of mRNA displayed-peptide libraries.

(A) Roberts used a bis-succinimide linker to covalently link a lysine to the N-terminus [34].

(B) Suga's strategy used a chloroacetyl group that co-translationally reacts with a

downstream cysteine [36]. (C) Two cysteines can also be cyclized with DBx [37]. (D)

Hartman utilized DBx and click chemistry to prepare constrained bicyclic peptides[•38].