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New Functions *In Vivo* is Genetic Code Expansion

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Description

While these pioneering efforts have opened new application spaces, the potential of the translational apparatus remains underexploited. The molecular translation apparatus has evolved over billions of years to prefer α-L-amino acid substrates and to polymerize amide bonds. As such, numerous classes of ncAAs remain poorly compatible with the natural translation apparatus, and several roadblocks have made alteration of the natural translation system difficult. First, engineering molecular translation is the ultimate systems biology challenge. It is difficult to coordinately tune all of the cellular machinery necessary to site-specifically introduce ncAAs into proteins with high efficiency (e.g., ribosomes, transfer RNAs (tRNAs), aminoacyl-tRNA synthetases (aaRSs), and elongation factors). Second, creating new genetic codes with free codons that can be reassigned to ncAAs is challenging, especially in cells. This is because the translation apparatus has been delicately tuned by evolution to use each of its 64 codons for a defined purpose. Third, engineering the ribosome is particularly problematic: maintaining cell viability restricts exploring ribosome sequence mutations and non-canonical substrates; the ribosome sequence design space is endless; and we have only a primitive understanding of how to rationally redesign ribosomal RNA (rRNA) at the ribosome's active site to alter structure and function.

Ribosome Engineering

Numerous biological parts are required to create orthogonal translation systems necessary for expanding the genetic code beyond the 20 canonical amino acids. In order for an ncAA to be successfully incorporated into a peptide by the ribosome, it must first be specifically charged onto a tRNA that decodes a codon in the mRNA that is reassigned to the ncAA. This is typically done by an aaRS, though other strategies exist, as we discuss later (e.g., chemical acylation, ribozymes). The ribosome, which translates native mRNA, must be directed to translate orthogonal mRNA transcripts. The aminoacylated tRNA must then be delivered to the A-site of the ribosome with the help of translation factors (e.g., elongation factor Tu [EF-Tu]) and accepted by the ribosome as a substrate for polymerization. This engineered translation machinery is evolved to recognize an ncAA monomer and operate alongside the cell's natural translation apparatus in a parallel and independent fashion. It is "orthogonal" in the sense that it does not recognize natural amino acids or cross-react with native translational machinery. The development of a truly orthogonal translation system, therefore, requires the engineering of each of the parts that play a role in translating DNA to protein: codons for programming ncAAs, polymerases to translate DNA codons into mRNA transcripts, tRNAs, aaRSs, translation factors, and ribosomes. We next describe efforts to engineer these parts, starting with new genetic codes for codon reassignment.

Peptidyl Transferase Center

The ribosome has evolved over billions of years to accelerate the rate of amide bond formation between α-amino acids by more than 107-fold. In order to enable the incorporation of non-α-ncAAs and facilitate the synthesis of polymers comprised solely of such monomers, the ribosomal active site, or the peptidyl transferase center (PTC) will likely have to be redesigned. The PTC is a dynamic pocket that adjusts conformations and interaction of the 3′ terminal peptidyl group of the bound peptidyl-tRNA with the 5′ terminal group of the α-amino group and serves as proofreading for aminoacylated-tRNAs.

In vivo, orthogonal aaRSs that are capable of specifically charging their cognate tRNAs with an ncAA of choice are essential in order for the ncAA to be site-specifically incorporated into a peptide. The orthogonal aaRSs must not cross-react with any canonical amino acids or native tRNAs, requiring that the aaRSs are products of extensive protein engineering and optimization and/or derived from a sufficiently phylogenetically distant organism (often archaea) such that cross-reactivity is innately low. To achieve the necessary properties for orthogonal translation systems, engineering strategies for tRNA charging systems require the use of both positive and negative selections for orthogonal pairs; a positive selection to ensure that the pair successfully can incorporate an ncAA at the amber codon and a negative selection to confirm that the pair is specific to the given ncAA and does not incorporate canonical amino acids.

This can be used to create a sub-population of orthogonal ribosomes in cells that is available for engineering and is independent of wild-type ribosomes that support cell life. For example, Orelle et al. first demonstrated the utility of this system for ribosome evolution by introducing mutations into the tethered ribosome's PTC that improved translation of a problematic protein and would otherwise have been dominantly lethal.

In vivo-based ribosome-engineering strategies offer much potential but present their own challenges associated with cell viability and the requirement that ncAAs must permeate the cell membrane. In vitro, or cell-free, strategies have thus also emerged for ribosome synthesis and evolution.

More recently, the ribosomal subunit linker sequences were optimized to improve activity such that the tethered ribosome could support cellular growth at rates comparable to the wild-type ribosome. Importantly, both tethered and stapled ribosomes have now been shown to be functionally isolated and do not cross assemble to form hybrids. Orthogonal ribosomes have also since been used to design a “flipped” orthogonal system in which the tethered ribosomes translate the proteome and leave the untethered ribosome available for engineering and translation of o-mRNAs. Notably, this system can be used to introduce mutations that would otherwise be dominantly lethal into the untethered ribosome's rRNA, allowing for evolution of the ribosome to incorporate previously inaccessible ncAAs and produce novel proteins.