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Playing with the molecules of life

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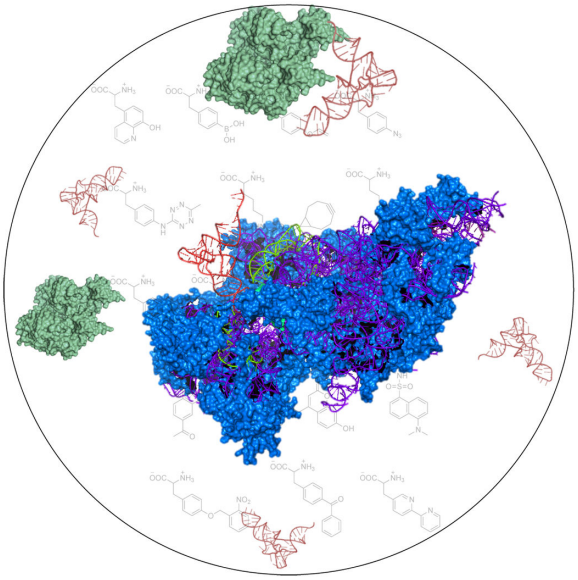
Abstract

Our understanding of the complex processes of living organisms at the molecular level is growing exponentially. This knowledge, together with a powerful arsenal of tools for manipulating the structures of macromolecules, is allowing chemists to harness and reprogram the cellular machinery. Here we review one example in which the genetic code itself has been expanded with new building blocks that allow us to probe and manipulate the structures and functions of proteins in ways previously unimaginable

Graphical abstract



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Author Contributions

The manuscript was written through contributions of all authors. /All authors have given approval to the final version of the manuscript.

Keywords

Noncanonical amino acid: any amino acid that is structurally different from the 20 endogenously encoded amino acids; Translation: process by which the genetic material in the form of RNA is converted into a protein context; Ribosome: cellular organelle that is the site of translation; Aminoacyl-tRNA Synthetase: protein responsible for recognizing an amino acid and cognate tRNA, resulting in the aminoacylation of the tRNA; Bioothogonal: a process that occurs under physiological conditions by does not react with endogenous systems; Photocaging: the installation of a photo-protecting group often leading to the inactivation of a molecule until the group is remove via light irradiation; Bioconjugation: a process involving the linking of a biological molecule to another species (biomolecule, small molecule, surface, etc); Post-translational modification: alteration of natural amino acid structure after it has been incorporated into a protein

1. Introduction

The genetic code is conserved across virtually every species – a total of 61 triplet codons provide the genetic information to encode the 20 canonical amino acids. It is remarkable that the limited set of functional groups contained in these building blocks enable proteins to carry out most of the complex processes of living organisms. The ability to add noncanonical amino acids (ncAAs) with novel structures and properties to the code might therefore allow the biosynthesis of proteins with new or enhanced functions, and provides the opportunity to probe the structure and functions of these macromolecules with unparalleled chemical precision.

This review overviews the recombinant technology developed for the site-specific incorporation of ncAAs into proteins, as well as recent advances that expand its scope. We then highlight illustrative examples of the use of ncAAs to control, evolve, and understand protein function.

2. Expanding the Genetic Code

2.1. Generating bio-orthogonal translational machinery

Several key components are required to genetically encode noncanonical amino acids with high efficiency and fidelity in a host organism (Figure 1A).[1–4] The first component is a reassigned codon that encodes the ncAA. Frequently, the amber nonsense codon (TAG) is selected due to its less frequent utilization as one of the three stop codons; however, other nonsense or four base frameshift codons have also been used for this purpose.[5–10] Another requirement is an orthogonal aminoacyl-tRNA synthase (aaRS)/tRNA pair capable of recognizing the desired ncAA and incorporating it in response to the appropriate codon.[11–16] The orthogonality of this pair must be such that the aaRS does not recognize endogenous tRNAs (84 in *Escherichia coli*,) or amino acids, and only aminoacylates its cognate tRNA; additionally, the tRNA must not act as a substrate for any endogenous aaRSs.[3, 17] The final key component is the ncAA itself, which can often be directly supplemented to the growth media, taken up as a dipeptide precursor through a transporter,[18] or biosynthesized by the host.[19] An elegant example of the synthesis of all these components was the genetic encoding of *p*-aminophenylalanine in engineered bacteria that contained the orthogonal

aaRS/tRNA pair, an amber codon inserted into the myoglobin sequence, and a gene cluster from *S. venezuelae* that enables the bacterial biosynthesis of *p*-aminophenylalanine to afford a 21 amino acid synthetic organism.[19]

2.1.1. Aminoacyl-tRNA synthase evolution—Recognition of a desired ncAA by the aaRS is often engineered using structure-based approaches in combination with powerful double-sieve selection schemes (Figure 2).[3, 21–27] This process typically begins with analysis of the crystal structure of the aaRS active site to identify those residues that contribute to specificity, followed by random mutagenesis of these sites to generate large aaRS libraries. The library is then subjected to positive selection in the presence of the desired ncAA (typically linked to suppression of an amber codon at a permissive site in a protein conferring antibiotic resistance) to ensure that only aaRSs that recognize and aminoacylate the desired ncAA proffer survival.[23] The survivors are then subjected to a negative selection in the absence of the ncAA (e.g., suppression of amber codons at permissive sites in a lethal gene product) to ensure survival in the positive selection step is not a result of aminoacylation with any natural host amino acid.[23] This process is repeated for several rounds to afford an aaRS that is highly selective for the ncAA over any endogenous amino acids.[28] A number of other selection and screening systems have also been developed, but all typically require both positive and negative screening or selection steps.[29, 30] One recent exciting example involves the use of rapid phage-assisted continuous evolution (PACE) methodology to evolve highly efficient and selective aaRSs.[31] The x-ray crystal structures of a number of these evolved aaRSs reveal remarkable plasticity in the amino acid binding site such that a limited number of mutations can substantially alter the active site structure and specificity.[32, 33] More recently, it has been discovered that as a consequence of other ncAAs being absent from the negative selection, some aaRSs exhibit a degree of polyspecificity towards structurally related ncAAs.[34–41] One such aaRS, evolved to encode *p*-cyanophenylalanine in *E. coli*, is capable of incorporating over 20 aromatic amino acid derivatives.[34, 37] Another such pair is the PylRS/$tRNA_{CUA}$ pair from certain methanogens (notably *Methanosarcina barkeri* (*Mb*), and *Methanosarcina mazei* (*Mm*)).[35, 42, 43]

Using the above methods a large number (>150) of ncAAs with distinct structures and functions have been genetically encoded in prokaryotes and eukaryotes.[44–46] These include metal chelating[47–49] and fluorescent amino acids,[50–53] a variety of photo-crosslinkers,[54–58] amino acids with altered pKas and redox properties,[36, 59–62] amino acids with bioorthogonal chemical reactivity,[12, 59, 63–71] and post-translationally modified amino acids and their stable analogues.[18, 26, 72–77] Whereas many of the reported ncAAs are structural variants of canonical amino acid side chains, it has also been demonstrated that the amino acid backbone can be modified as well. These modifications include the incorporation of α-hydroxy acids,[78–80] *N*-methyl amino acids,[78] and α-α -disubstituted amino acids, albeit the latter two amino acids involved an *in vitro* translation method.[78] Recently, β-amino acids were genetically encoded, specifically β-phenylalanine analogs were introduced into a protein using mutant ribosomes and an orthogonal aaRS/tRNA pair.[81]

2.1.2. Ensuring orthogonality—The selection of an appropriate aaRS/tRNA pair to encode an ncAA is dictated by the host organism requirements to achieve orthogonality. Early experiments relied on an engineered *Methanococcus jannaschii* (*Mj)* TyrRS/$tRNA_{CUA}$ pair that is orthogonal in *E. coli* and other bacteria largely due to distinct acceptor stem recognition sequences.[16, 23, 82] Since these initial studies, TyrRS/tRNA, LeuRS/tRNA, $TrpRS/tRNA_{,,}$ SerRS/tRNA, AspRS/tRNA, GluRS/tRNA, LysRS/tRNA and ProRS/tRNA pairs that are orthogonal in either prokaryotic and/or eukaryotic cells have been generated.[8, 9, 15, 22, 46, 83–88] $PylRS/tRNA_{CUA}$ pairs from certain methanogens (notably *Methanosarcina barkeri* (*Mb*), and *Methanosarcina mazei* (*Mm*)) that are orthogonal in both bacteria and eukaryotic cells have been developed.[24, 42, 43] These latter pairs are especially advantageous as they allow aaRSs to be evolved in *E. coli* prior to transfer of the machinery to more diverse eukaryotic hosts. Utilizing these orthogonal pairs, ncAAs have been encoded in *B. cereus,*[89] *P. pastoris,*[90] *C. elegans,*[91, 92] *D. melanogaster,*[93] *A. thaliana,*[94] Zebrafish embryos,[95] and the mouse.[96–99] More recently, it has been demonstrated that a native Trp aaRS/tRNA pair in *E. coli* can be functionally replaced with a counterpart from yeast, and the liberated Trp pair can be used to encode ncAAs in bacteria.[86] Additionally, orthogonal aaRS/tRNA technologies have been used to incorporate ncAAs into proteins in mammalian cell lines at gm/L scale employing transient expression methods.[100-102] Viral vectors have allowed the ncAA machinery to be delivered efficiently into primary cells, as well as tissues,[96, 103, 104] where it was used among other applications to monitor voltage-sensitive changes in response to membrane depolarization events in neural cells.[100]

2.1.3. Recent Advances—A variety of strategies have been reported to further improve the efficiency and specificity of ncAA incorporation into proteins, including mutations to the aaRS, tRNA, ribosomal peptidyl transferase and elongation factor.[13, 17, 104–110] Moreover, aaRS and tRNA expression levels have been modulated in order to facilitate high-level expressions of proteins containing ncAAs.[13, 104, 105, 111–113] These alterations have led to ncAA-incorporation on multigram/L levels in large scale bacterial fermentation, and gram/L scale in stable CHO cell lines as demonstrated in the production of ncAA containing pegylated proteins and antibody-drug conjugates (ADCs).[111]

An exciting recent advance is the ability to incorporate more than one ncAA into a protein sequence with the ultimate challenging goal of the mRNA template-directed biosynthesis of monodisperse biopolymers made up of synthetic building blocks. Toward this end several *E. coli* strains have been generated that either conditionally or constitutively remove release-factors (RF1 in *E. coli* and eRF1 in eukaryotes) that terminate polypeptide synthesis in response to specific nonsense codons, in order to improve suppression efficiencies.[75, 114–116] Orthogonal bacterial ribosomes that are directed to an orthogonal message, by the incorporation of a mutant 16S rRNA into their small subunit (and therefore not essential to the cell) have also been created (Figure 3).[117, 118] One such orthogonal ribosome that no longer recognizes RF1 was discovered by directed evolution, and enables the efficient incorporation of an ncAA in response to amber codons at multiple sites in a single polypeptide.[119] Another approach involves recoding the genome such that some or all of the amber codons have been replaced by the ochre nonsense codon TAA in an effort to remove potential read-through of endogenous termination signals.[120–122] These strains, which have

TAG or TAGN (N=A, G, C, T) uniquely assigned to the ncAA, have been shown to enhance ncAA incorporation in response to the quadruplet codon TAGA, which is derived from and competes with RF1 recognition of the amber codon (TAG).[5]

There is also interest in the incorporation of multiple distinct amino acids into a single protein, which requires aaRS/tRNA pairs that are mutually orthogonal and orthogonal to the host aaRS/tRNA pairs.[9] Recently, a new expression cassette was engineered for bacterial expression that affords two aaRS/tRNA pairs (*M. jannaschii* and *M. barkeri*) that meet this requirement (recognizing TAG and TAA codons, respectively).[6] This methodology allowed for the incorporation of two reactive ncAAs harboring either a ketone or azide, respectively, and also the generation of a fluorescence resonance energy transfer (FRET) pair within the same reporter protein.[17, 123] The synthesis of unnatural biopolymers with 3 or more ncAAs requires one new orthogonal aaRS/tRNA pair per ncAA, and has catalyzed efforts to discover new orthogonal aaRS/tRNA pairs and repurpose additional codons.[6, 124–131] To this end it has been shown that existing orthogonal pairs can be used as starting points to generate additional mutually orthogonal pairs by directed evolution.[87, 132] These new tRNA/aaRS pairs have allowed multiple, different amino acids to be incorporated in both *E. coli*[123, 126, 133] and eukaryotic systems.[134, 135] These experiments suggest that the number of orthogonal pairs that can be discovered from natural sequence diversity does not place a limit on the number of distinct building blocks that may be simultaneously genetically encoded in cells. An active area of research now focuses on reassigning degenerate codons in the genetic code to encode additional ncAAs.[124, 136]

3. Applications of Non-Canonical Amino Acids

While the technology development associated with ncAA incorporation has stimulated efforts to create other bioorthogonal cellular systems, the application of these methodologies illustrate the true utility of this technology.[3, 127, 129, 137–139] To date, ncAAs have been employed in a large number of studies of protein structure and function, and also to alter or enhance protein function. In addition, the recombinant introduction of ncAAs into proteins is allowing the development of new therapeutics and diagnostics.

3.1. Probing protein structure and function

The ability to expand the genetic code beyond the 20 common amino acids facilitates the site-specific introduction of noncanonical amino acids with altered structures and functions to better understand protein function both *in vitro* and in living cells with minimal perturbation to protein structure. These ncAAs include residues with altered pKas for mechanistic studies, isotopic labels for infrared and NMR studies, photocrosslinkers for mapping biomolecular interactions in living cells, heavy atoms for X-ray crystallography, and spin labels and fluorescent side chains for EPR and optical applications, respectively. While ncAAs probes have been used in numerous studies, below we highlight instructive examples of their use.

3.1.1 Altering pKa and redox potential—Electron-withdrawing or donating substituents allow one to alter the acidity, basicity and redox potential of canonical amino acids (Figure 4).[36, 61, 62, 140–146] For example fluorinated tyrosine analogues served as

effective EPR probes to monitor long-lived tyrosyl radicals in the complex mechanism of ribonucleotide reductase, and better understand the role of conserved tyrosine residues in the prevention of undesirable radical chemistry.[36, 147] These studies complemented previous semisynthetic studies employing nitrotyrosine[140] and aminotyrosine,[143] which were used to investigate the kinetics of radical intermediate formation within these ribonucleotide reductases.

3.1.2 Protein crosslinking to map protein interactions—An arena where ncAAs have found widespread use is in the field of protein crosslinking, both in vitro and inside living cells. Multiple crosslinking ncAAs have been genetically encoded including aryl azides,[59] aryl haloketones,[148–150] benzophenones,[54] aryl carbamates,[151] isothiocyanates,[152] and diazirines[56, 58, 153] to covalently crosslink interacting proteins and protein-nucleic acid complexes. This approach has been extensively used to map transient protein interactions that elude detection by other methods such as immunoprecipitation or are unstable outside the context of a living cell. Depending on the chemistry employed, crosslinking can occur via either direct chemical reaction with residues in close proximity or by photoactivation. One example using a benzophenone containing ncAA (which inserts relatively nonselectively into C-H bonds) was the identification of key protein-protein interactions necessary for lipopolysaccharide (LPS) transport to bacterial outer membranes (Figure 5).[154] This same ncAA was also employed to afford spatiotemporal control over the mapping of histone 2A interactions in yeast, elucidating key proteins involved in the histone modification cascade that induces mitosis.[155] The utility of this cross-linking ncAA has been further expanded via modification with an alkynyl handle for subsequent reaction, facilitating rapid isolation of cross-linked products.[156] More recently, new ncAA photocrosslinkers have been developed that can be chemically cleaved following crosslinking, further simplifying the identification of a target protein by mass spectrometry.[153] In addition, chemical crosslinkers have also been generated that rely on the nascent chemical reactivity of lysine and cysteine residues with nearby electrophilic ncAAs to afford covalent crosslinks between proteins.[148, 151, 157] Other examples of the use of photocrosslinking ncAAs include the identification of G-protein coupled receptor activating ligands,[158, 159] acid chaperone associated proteins in pathogens,[160] the binding site of antidepressant drugs in the serotonin transporter,[161] and the interacting partners of short open reading-frame encoded peptides.[162] An important new direction is the encoding of crosslinking agents in whole organisms to explore cell-cell interactions.

3.1.3. Genetically encoded fluorescent amino acids—Genetically encoded fluorescent ncAAs have been used both in vitro and in living cells to label proteins with fluorescent probes. This approach has advantages over strategies that rely upon the reactivity of canonical amino acids such as Cys and Lys, as well as genetic fusions, due to the high level of control over the labeling site within a protein and minimal perturbation to protein structure and function. Several fluorescent ncAAs, including coumarin,[50, 163] dansyl,[53] naphthyl,[51] terphenyl,[164] and prodan derivatives[52, 165] have been genetically encoded in both prokaryotes and eukaryotes (Figure 6). The fluorescence of some of these ncAAs is environmentally sensitive and can be used to probe cellular localization, biomolecular interactions, posttranslational modifications, and residue exposure to solvent.[52] For example,

fluorescent ncAAs were used to detect the binding of glutamine to glutamine binding protein,[166] and the phosphorylation of STAT3 .[167] The prodan derivative, ANAP, has also been used as a partner to generate a FRET pair within mammalian cells to investigate protein proteolysis and protein conformational changes.[168, 169] While the majority of genetically encoded fluorophores are excited at shorter wavelengths and provide a convenient way to site-specifically label proteins for in in vitro studies, some directly encoded fluorophores have also been used for imaging proteins in live cells[52, 163]. An important direction for future research is the genetic encoding of longer wavelength, photostable fluorescent ncAAs.

3.1.4. Infrared probes—The incorporation of deuterium,[170] cyano,[171] nitro,[172] and azido[59] IR active probes into proteins introduces unique vibrational modes that do not overlap those present in the canonical amino acids. These localized probes have facilitated investigations of enzymatic catalysis,[170] solvent exposure[173, 174] and receptor activation using IR spectroscopy.[175, 176] In one example, azidophenylalanine was introduce ed into rhodopsin at distinct sites within the transmembrane helices allowing for helix movements to be monitored when the receptor is activated by light.[175] In a second example, deuterium probes were introduced via a photocaged tyrosine derivative (which upon photodeprotection installed 2,3,4,5-D_4 tyrosine) at specific sites in the active site of dihydrofolate reductase. IR spectroscopy allowed one to monitor conformational changes in the presence of different ligands that correspond to different states along the catalytic pathway.[170] These studies have provided useful information regarding protein structure and function and all relied upon the novel functionality of ncAAs.

3.1.5. Spin-label and NMR probes—The introduction of spin labels into protein affords site-specific EPR probes, and is traditionally accomplished via reaction of a chemically reactive spin label with sulfhydryl groups. This approach has limitations, as there can be multiple cysteine residues in a protein and they often play a role in protein folding. Early examples involved the incorporation of *p*-acetylphenylalanine, a ketone containing ncAA with bioorthogonal chemical reactivity, which can be conjugated to a nitroxide group via an oxime ligation.[177] Recently, a spin label has been directly incorporated into proteins, facilitating in-cell EPR studies of endogenous proteins.[178, 179]

Two approaches have been developed to use ncAAs as site-specific NMR probes. The first involves genetically encoding a ^{15}N-labeled tyrosine that is caged with a nitrobenzyl moiety, which upon uncaging by exposure to UV light results in the site-specific incorporation of ^{15}N-tyrosine.[180] A second approach is based on the site specific incorporation of close analogues of canonical amino acids possessing isotopic labels, for example, O-methyl-phenylalanine containing ^{13}C and/or ^{15}N, or a trifluoro derivative of the same amino acid possessing ^{19}F.[180–185] Both strategies were used to map the binding site of a fatty acid synthase with a model ligand, as well as active site conformational changes that occur upon ligand binding.[180] Fluorinated amino acid analogues, due to their increased signal sensitivity, have also been used to monitor protein conformational changes in living cells, for example to monitor phosphorylation induced conformational shifts in arrestin-1 during cell signaling.[183]

3.1.6 Environmental sensors—Several ncAAs are capable of either specifically coordinating, or directly reacting with analytes to act as sensors. One successful approach involved introducing ncAAs into green fluorescent protein (GFP) at sites that altered either the wavelength or intensity of protein fluorescence in the presence of an external stimulus (Figure 7).[186] Specifically, GFP containing either L-3,4-dihydroxyphenylalainine (DOPA)[187] or 8-hydroxyquinoline alanine (HqA)_[188] at residue 66 within the fluorophore was used to detect biologically relevant transition metals including Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+}. More recently, a *p*-vinylphenylalanine was incorporated at this same site to act as a turn-on probe for the detection of mercuric ions in bacteria.[189] In addition to ions, this approach has been employed for the detection of H_2O_2 with a boronate ncAA,[190] H_2S with a azidophenylalanine ncAA,[191] and sirtuins using an actylated lysine which can be cleaved by these enzymes.[192] Finally, the *p*-boronophenylalanine has also been exploited to detect peroxynitrite in mammalian cells in order to monitor the production of this cell-signaling molecule at physiologically relevant concentrations.[193, 194] In addition ncAAs have been incorporated at residue 66 in GFP to alter the fluorescence properties of the protein.[37, 190, 195–198], and into the coelenterazine binding site of aequorin to dramatically red-shift the bioluminescence of the interaction to afford *in vivo* bioluminescence reporters in mice.[197]

3.2. Site-specific protein conjugation

A general strategy for site-specifically modifying proteins with synthetic moieties (e.g., biophysical probes, drugs, PEGs, oligonucleotides, etc.) involves installing a chemically (or enzymatically) reactive amino acid at the desired site and selectively conjugating the side chain to a reaction partner linked to the molecule of interest. [199] Cysteine and lysine residues are the most commonly targeted canonical amino acids, and typically modified with electrophiles. However, there are often multiple such residues in a protein resulting in heterogeneous labeling, and Cys residues may also be required for protein folding and function. The site-specific incorporation of ncAAs with unique chemical reactivity allows the selective modification of proteins with medicinal chemistry-like precision. This approach requires ncAAs with bioorthogonal chemical reactivity, i.e., unreactive with the canonical amino acids or metabolites in the cell. The most common bioorthogonal ncAAs that have been encoded have keto,[200] azido[59] and acetylenic side chains (Figure 8).[42, 63, 201, 202] Azide and alkynyl ncAAs undergo highly selective copper-catalyzed 1,3-dipolar cycloaddition reactions and have been generally used for in vitro applications.[203, 204] However, strained alkynes have recently been genetically encoded to afford copper-free “click” reactions that can be used in living cells.[205–207] Target proteins are efficiently and selectively labeled and background nonspecific proteome labeling is minimal or undetectable, despite a significant number of endogenous genes that terminate in amber codons[208]. The use of Cys and an ncAA, or two ncAAs, with bio-orthogonal reactivity allows the site-specific labeling of proteins with two different moieties via sequential or one-pot bioorthogonal reactions for applications such as Forster resonance energy transfer (FRET) measurements[17, 133, 136] with excellent dynamic range[209].

Another commonly employed ncAA for bioorthogonal conjugations is *p*-acetylphenylalanine, which can be selectively modified at lower pH with highly efficient

oxime formation reactions.[200] This reaction has been used to generate site-specific drug, polyethylene glycol (on kilogram commercial scale), peptide and oligonucleotide-protein conjugates, and also to conjugate a large number of probes to proteins.[20, 111, 210–218] The high selectivity of this reaction results in well-defined conjugates that do not significantly affect the intrinsic properties of a protein, in contrast to more nonspecific approaches. This advantage was demonstrated with the generation of a DNA-antibody conjugate for immuno-PCR: an anti-Her2 antibody/DNA conjugate was more sensitive with lower background when used to detect Her2 positive cells in complex blood mixtures relative to nonspecific lysine conjugates (Figure 8).[215]

Considerable effort has recently been devoted to expand the toolbox of genetically encoded ncAAs with bioorthogonal chemical reactivity. Alkenyl ncAAs have been used in bioorthogonal methathesis reactions to conjugate carbohydrates to proteins:[219, 220] hydroxytryptophan residues have been employed for azo-couplings,[221] cyclopropenone and azide residues for reactions with phosphines,[222, 223] alkynyl amino acids have been exploited in Sonogoshira,[224, 225] Glaser-Hay,[226] and Cadiot-Chodkewitz bioconjugation reactions;[227] and *p*-boronophenylalanine and *p*-iodophenylalanine have been used in Suzuki and other transition metal-based couplings.[228–231]

An important recent advance has been the encoding of new bioorthogonal reactive side chains that undergo rapid and selective labeling reactions in the cell. The importance of reaction rate has been increasingly appreciated, and reactions with rate constants that approach those of enzymatic mediated labeling reactions have been developed.[204] One such reaction is the inverse electron demand Diels-Alder reaction between strained alkenes or alkynes and tetrazines (Figure 9). The rate constants for some tetrazine reactions with alkenes and alkynes can exceed 10^4 $M^{-1}s^{-1}$, and several alkenes, alkynes and tetrazines have now been recombinantly introduced into proteins and used for rapid, site-specific, live cell protein labeling.[65, 68, 233–236] Live cell labeling and super resolution imaging have been further facilitated by the development of bright small molecule fluorophores that enter mammalian cells and have minimal background fluorescence.[208, 237] While the refinement and optimization of labeling and imaging approaches is still on going, early examples of applications that are not possible by other methods have already emerged.[238–240]

Another application of ncAAs with bioorthogonal handles is their use in protein immobilization to a surface for a wide range of industrial and diagnostic uses. Immobilization typically leads to increased protein stability, tolerance to organic solvents, and recyclability of enzymes (Figure 8).[241] ncAAs provide a means to both covalently link proteins to a surface, as well as control the site of immobilization to prevent protein inactivation or heterogeneous immobilization. To date, ncAAs have been used for the immobilization of proteins to nanotubes, magnetic beads, and solid-supported resins and have been shown to confer increased stability to the immobilized protein.[232, 242–245] Further exploration and optimization of these immobilization technologies has the potential to make significant advances to both materials chemistry and therapeutic diagnostics.

3.3 Control of protein function

The ability to control protein activity chemically or with light is useful for spatially and temporally activating biological processes in living cells. One such strategy involves the blocking of essential amino acid side chains with a removable protecting group (Figure 10). This moiety blocks normal side chain function until removed by an external stimulus. Many analogs of natural amino acids (especially lysine, serine, cysteine, and tyrosine) containing a photo-removable protecting group have been genetically encoded in both prokaryotic and eukaryotic cells and organisms.[246–249] These photocaged ncAAs have been used to control kinase, protease, intein, and other enzyme activities, nuclear localization, virus-host interactions, and cell signaling cascades.[102, 163, 240, 250–255]

One example of the use of this technology is the photoactivation of Cas9 using a photocaged lysine residue.[256] The photocaged CRISPR/Cas9 system was used to both silence and activate exogenous reporter genes in mammalian cell culture, as well silence endogenous CD71, a receptor linked to leukemia and lymphoma. The regulation of this system in living cells has many potential applications in the rapidly developing field of gene editing. In another example, recombinant incorporation of a photocaged serine into the transcription factor Pho4 in yeast blocked phosphorylation and subsequent nuclear export until removal of the caging group with 400nm light, allowing the kinetics of these processes to be followed.[257] A final example of light-regulated in vivo protein function involved photocaging a lysine residue within an isocitrate dehydrogenase mutant known to produce the onco-metabolite 2-hydroxygluterate (2-HG).[258] Light activation of this oncogenic protein provided insights into the role of 2-HG in oncogenic activation.

The reversible photoregulation of protein activity has been accomplished through the genetic encoding of a photo-switchable azobenzene ncAA. This ncAA has been used to photoregulate enzyme activity by reversibly blocking the active site[247] or by moving a tethered inhibitor in and out of the active site by *cis-trans* photoisomerization of the azobenzene moiety.[259]

While light is a useful external stimulus for protein activation, it has also been demonstrated that small molecule activators can be used in conjunction with ncAAs to modulate protein function in cells. A recent study incorporated a cyclooctene lysine derivative into a reporter luciferase protein in mammalian cells.[260] Incubation with a tetrazine induced a Diels-Alder reaction that led to the removal of the cyclooctene cage, restoring wild-type lysine and consequently fluorescence. Palladium-catalyzed allene decaging[261] and phosphine-mediated decaging of azides via a Staudinger reduction have also been employed to chemically modulate protein function in vivo.[262]

3.4. Post-translational modification

Protein post-translational modifications are ubiquitous in biology and control cellular processes ranging from signal transduction and transcription to cell division and protein degradation. It is relatively difficult to generate site-specific PTMs in proteins- the enzyme responsible for the modifications may be unknown, may not be site-specific, or the modifications may be removed in cells or lysates. Consequently, the ability to genetically

encode post-translationally modified amino acids, or stable analogues thereof provides a useful tool to better understand and characterize individual PTMs in proteins. A number of post-translational modifications have been genetically encoded, including phosphoserine, phosphothreonine, phosphotyrosine, sulfotyrosine, nitrotyrosine, and numerous epigenetic lysine modifications.[18, 26, 60, 71, 74–77, 240, 263–266] Stable mimicks of posttranslational modifications, such as phosphonotyrosine and carboxymethyl-phenylalanine have also been genetically encoded.[72, 75, 267] Incorporation of this latter phosphotyrosine mimetic into the arginine methyltransferase PRMT1 facilitated analysis of how phosphorylation alters the binding of this protein to its substrates.[268] Phosphonotyrosine was recently genetically incorporated into proteins by exploiting the polyspecificity of the previously evolved carboxymethyl-phenylalanine aaRS, and a dipeptidyl transporter to transport this negatively-charged amino acid into cells.[269] Subsequent incorporation of phosphonotyrosine into human Abl1 at specific residues allowed determination of the binding affinities of the various phosphorylated forms of the protein to its substrates. Additionally, 3-nitrotyrosine has been genetically encoded, and used to gain new insights into post-translational modifications that modulate inflammatory responses and mimic disease-state processes.[60]

Given the polyspecificity of the pyrrolysyl-aaRS towards a variety of lysine modifications, a number of labs have genetically encoded post-translationally modified lysines, including ε-*N*-2-hydroxyisobutylyl-lysine, ε-*N*-pivaloyl-lysine, ε-*N*-methyl-lysine, and ε-*N*-crotonlyl-lysine amongst others, as a means to study these post-translational modifications in a variety of proteins. For example these ncAAs have also been exploited to characterize the effects of ubiquitination and histone methylation on gene regulation.[71, 270] Finally, *E. coli* has been engineered for the recombinant production of ribosomally synthesized posttranslationally modified peptides (RIPPs) that contain ncAAs, and analogues of the lanthipeptide nisin with altered ring structures and sizes have been produced.[271–275]

3.5 Therapeutic applications

The ability to site-specifically modify proteins using bioorthogonal ncAAs greatly facilitates the generation of homogeneous therapeutic proteins whose structures are precisely controlled, much like the medicinal chemists ability to precisely control the structures of small molecule therapeutics. Recent efforts have focused on introducing amino acids with bio-orthogonal reactivity into cytokines, growth factors, antibodies and antibody domains, providing a route to their site-specific conjugation with diverse moieties.[276, 277] Pegylated proteins, antibody-drug conjugates (ADCs), antibody-antisense oligonucleotide conjugates and bispecific antibodies have been created for a variety of clinical indications[111, 278112, 218, 279–283], and a number of these new therapeutics have been approved or are showing positive results in clinical trials. Importantly, it has been shown that by controlling both the stoichiometry and site of conjugation of a PEG or drug on a target protein one can optimize both half-life and potency in a manner that is difficult to achieve with less specific chemistries. Moreover, it has been possible to conjugate small molecules that bind tumor antigens (e.g., DUPA and folate which selectively target PSMA and folate receptor, respectively[281, 284]) site-specifically to anti-CD3 antibodies to generate extremely potent semisynthetic bispecific antibodies, which in the case of the DUPA-anti-CD3 conjugate show impressive efficacy in preclinical models of metastatic prostate cancer

(Figure 11).[284] Recently a modular strategy for chimeric antigen receptor (CAR)-T cell therapy was created in which a chimeric T cell receptor was created that binds FITC, and FITC was selectively conjugated to an aryl ketone containing ncAA selectively incorporated into an antibody specific for a tumor antigen. The resulting 'switch' molecules bridge the CAR-T cell and the tumor cell and their structures can again be precisely controlled to optimize formation of the immunological synapse. This approach enabled dose dependent tumor clearance[285] with minimal cytokine release using a universal CAR-T cell that can be adapted to distinct tumor antigens by appropriate switch molecules.

Introducing ncAAs with immunogenic side chains, such as nitro- or sulfo-tyrosine, into proteins has been used to break immunological tolerance to the corresponding native protein. The immunogenic side chain forms a neoepitope that elicits a T cell response and leads to a cross-reactive antibody response to the native proteins (Figure 10).[286] Indeed, immunization of mice with a murine TNF that contains a single nitrotyrosine led to neutralizing antibodies to native TNF that were protective in a LPS mouse challenge model. [287] These studies provide a route to breaking tolerance against specific native proteins and, support the view that tyrosine nitration, which naturally results from viral infection and inflammation, could contribute to autoimmunity.

The introduction of amber codons into the genomes of viruses, including hepatitis D virus, HIV-1 and influenza A, has created viral genomes[289–291] that can only be replicated in cells that contain orthogonal aaRS/$tRNA_{CUA}$ pairs and their cognate ncAA. This strategy has enabled the creation of attenuated viruses that may be used for immunization. More recently, ncAAs have been used to create bacteria whose replication is strictly dependent on the presence of a specific ncAA at a functional site (e.g., catalytic, protein interface or metal ion binding site) in an essential protein. Such systems can be used for biological containment of engineered organisms. Immunization with these live conditional pathogens is expected to give robust immune responses, but the pathogen will die after the ncAA is depleted through rounds of replication in the host.[290] Importantly, revertants for some sites of suppression have yet to be isolated (reversion rates less than 10e-11). Clearly the use of ncAA technologies allows far more chemical control over the structures, and as a result, activities of therapeutic macromolecules.

3.6. Protein design and evolution with ncAAs

The addition of amino acids with novel properties to the genetic code presents new opportunities for the rational design of proteins with new or enhanced functions. Metal binding proteins are involved in a wide variety of cellular processes, but it has been challenging to design metal binding sites into proteins due, in part, for the need to precisely control the geometries of multiple amino acid side chains that chelate the metal.[292] The introduction of bipyridyl containing amino acids into proteins allows the design of metal ion binding sites that take advantage of the preorganization of the bidentate ligand for metal chelation. Computational design, using Rosetta, has enabled the generation and structural characterization of proteins and metallo-protein assemblies that bind metal ions with picomolar affinity.[292, 293] More recently, a protein was designed with Rosetta that binds the biphenyl side chain of an ncAA in a planar geometry which is stabilized by packing

interactions in the hydrophobic core of a thermophilic protein (Figure 12). Thus, the protein acts as a thermodynamic sink to make the transition state for biphenyl bond rotation kinetically persistent such that X-ray crystallography enabled direct observation of this transition state conformation.[294]

The ability to genetically encode noncanonical amino acids with chemically defined structures and properties has also allowed the *in vitro* evolution of proteins with novel or enhanced functions. For example, phage selections using genetically encoded sulfotyrosine and a germline antibody library containing a randomized CDR3 loop, led to the selection of high affinity sulfated antibodies that bind gp120, an HIV protein that naturally binds a sulfated receptor.[295] Phage display was also used to evolve a zinc finger transcription factor that had an unusual high spin-Fe(II) core containing a bipyridyl amino acid side chain,[296] and which bound its operator sequence with high affinity and selectivity. A bacterial-based selection scheme has also been used to identify ncAA containing cyclic peptides that inhibit HIV protease in an antibiotic based selection (using a protease sensitive antibiotic antiporter).[297] The highest affinity peptide to emerge incorporated a benzophenone containing ncAA that bound HIV protease through a Schiff base linkage to a surface Lys (Figure 13). More recently, beta-lactamase variants have been isolated using a growth-based selection from a library of mutants containing single amber codons at over half of the residues. One noncanonical amino acid substitution led to an enzyme with increased catalytic efficiency; x-ray crystallographic analysis suggested that the ncAA functioned by restricting the conformation of the active site to more efficiently stabilize the rate-limiting transition state.[298, 299] Similarly, a metA variant was isolated from a random ncAA library using a temperature dependent selection scheme that was stabilized by a keto-containing amino acid by a remarkable 23 °C, and likely involves formation of a ketone adduct to a nucleophilic side chain at the homodimer interface. Similar *in vitro* evolution experiments have demonstrated that ncAAs with long chain thiols can form extended disulfide crosslinks that lead to significant protein stabilization.[300] Another example was the evolution of T7 phage with an ncAA dependent growth advantage. The computational design and/or selection of variants of essential *E. coli* proteins that require a genetically encoded biphenyl or benzophenone amino acid for activity has provided an elegant strategy to make organism viability conditionally dependent on the presence of a ncAA.[301–303] These experiments suggest that an expanded genetic code can indeed provide unique solutions to evolutionary challenges faced by living organisms, and that this is a rich area for further study.

4. Conclusions

Precise and highly tailored structural perturbations to proteins are now possible through the genetic encoding of noncanonical amino acids. The ability to incorporate ncAAs with diverse structures and properties into proteins in cells and living organisms has provided unique opportunities to probe, image, control, rationally engineer and evolve protein structure and function, as well as to develop new therapeutics and approaches to biomaterials. The refinement of approaches for encoding and labeling amino acids containing bioorthogonal groups will be particularly important for coupling diverse functionalities at precise sites in proteins *in vitro* and *in vivo* as cell biological probes and precisely tailored therapeutic agents. In vitro evolution experiments will continue to reveal

how life with additional genetically encoded amino acids may evolve new or enhanced protein functions. The development of additional strategies to create or repurpose codons, expand the substrate scope of translation, and create a suite of mutually orthogonal aminoacyl-tRNA synthetase/tRNA pairs will further improve our ability to control protein structure at the building block level, and potentially generate biopolymers in which all the building blocks are unnatural. Finally, these studies underscore the exciting opportunities that now exist to synthesize complex molecular structures and functions through the rational manipulation of the cell's machinery using chemical and biological approached synergistically.

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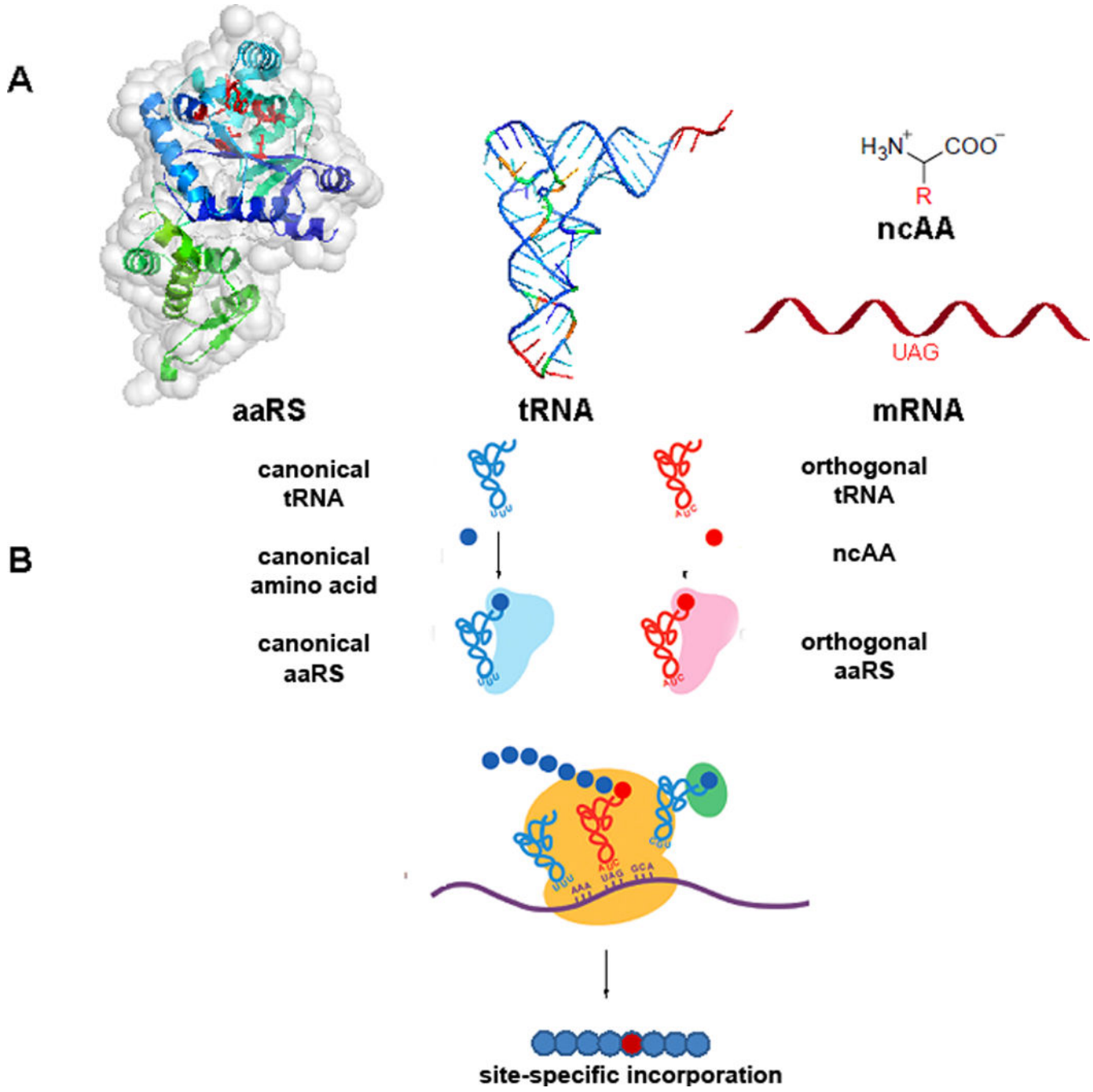

Figure 1.

Expansion of the genetic code. A) Requisite components for the site-specific incorporation of ncAAs into proteins. Red coloring indicates primary sites of mutation required to enable the efficient incorporation of the desired ncAA. B) Site-specific incorporation of ncAAs through engineering of the translational machinery. Standard translation occurs with endogenous tRNA/aaRS pairs and canonical amino acids (blue) on the ribosome (yellow). An orthogonal tRNA/aaRS pair and ncAA are added (red) and encoded by a nonsense or frameshift codon on the mRNA (purple). Adapted from Kim, C.H. *et. al* 2013.[20]

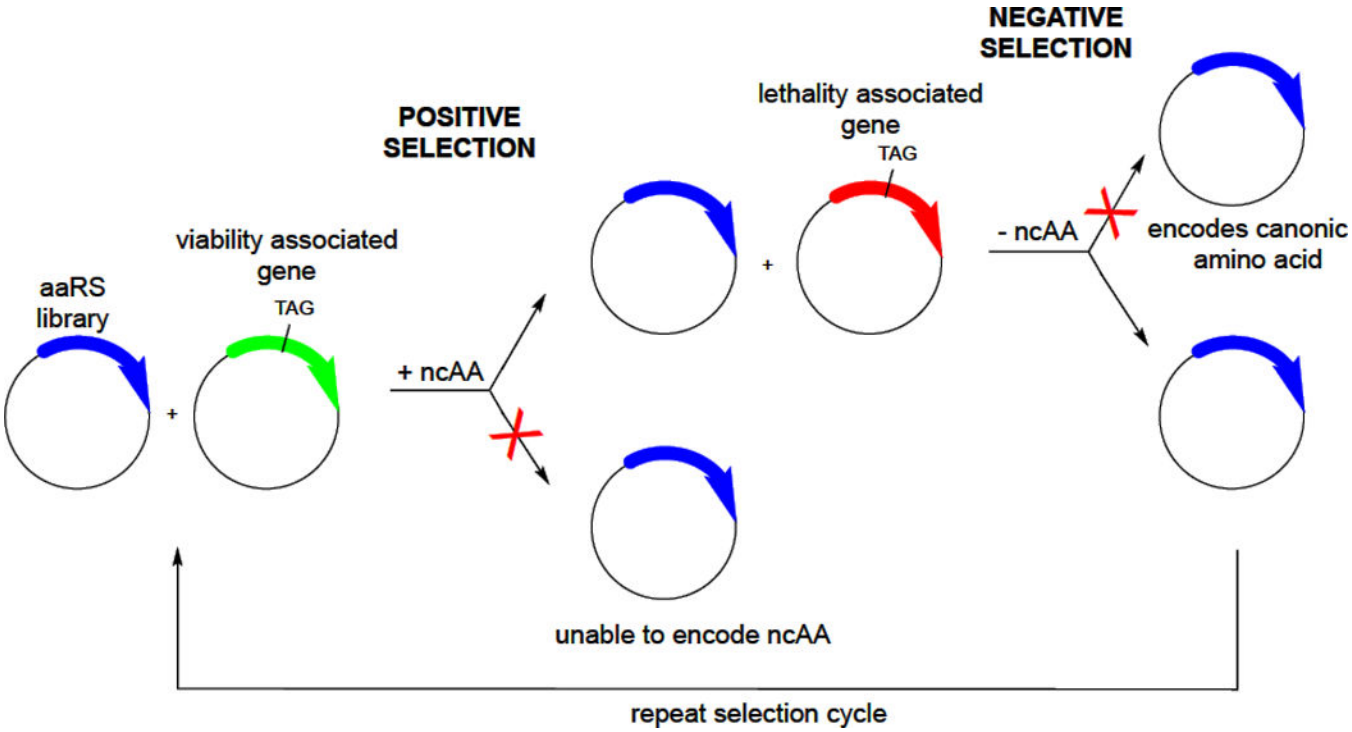

Figure 2.
Standard protocol for generation of an aaRS to encode ncAAs.

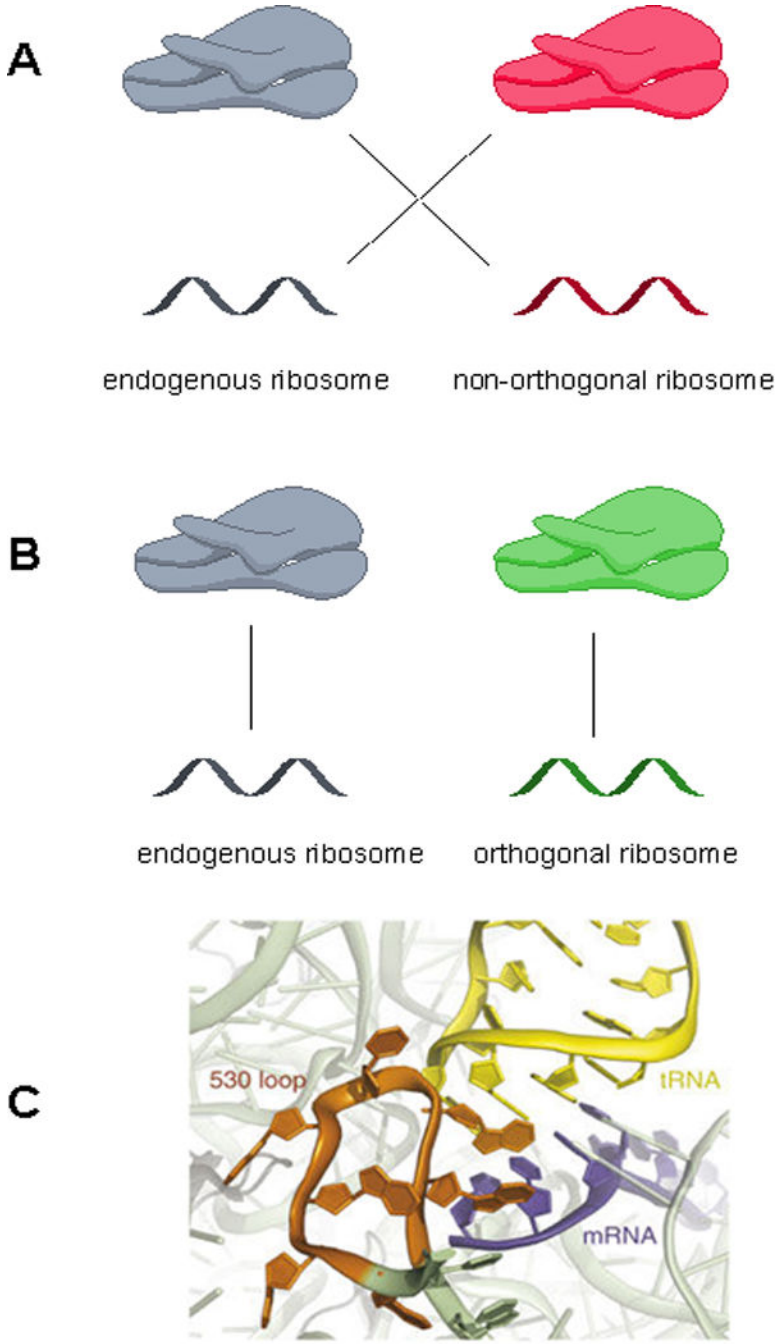

Figure 3.

Generation of an orthogonal ribosome. A) A non-orthogonal ribosome allows for cross talk between the two mRNAs, not providing efficient incorporation of ncAAs. B) An orthogonal ribosome where the endogenous system (grey) and the engineered ribosome and mRNA (green) exhibit no cross-reactivity. C) Crystal structure of the rRNA (orange), mRNA (purple) and tRNA (yellow), illustrating the key 530 loop within the ribosome that was subjected to mutagenesis to afford an orthogonal ribosome.[119]

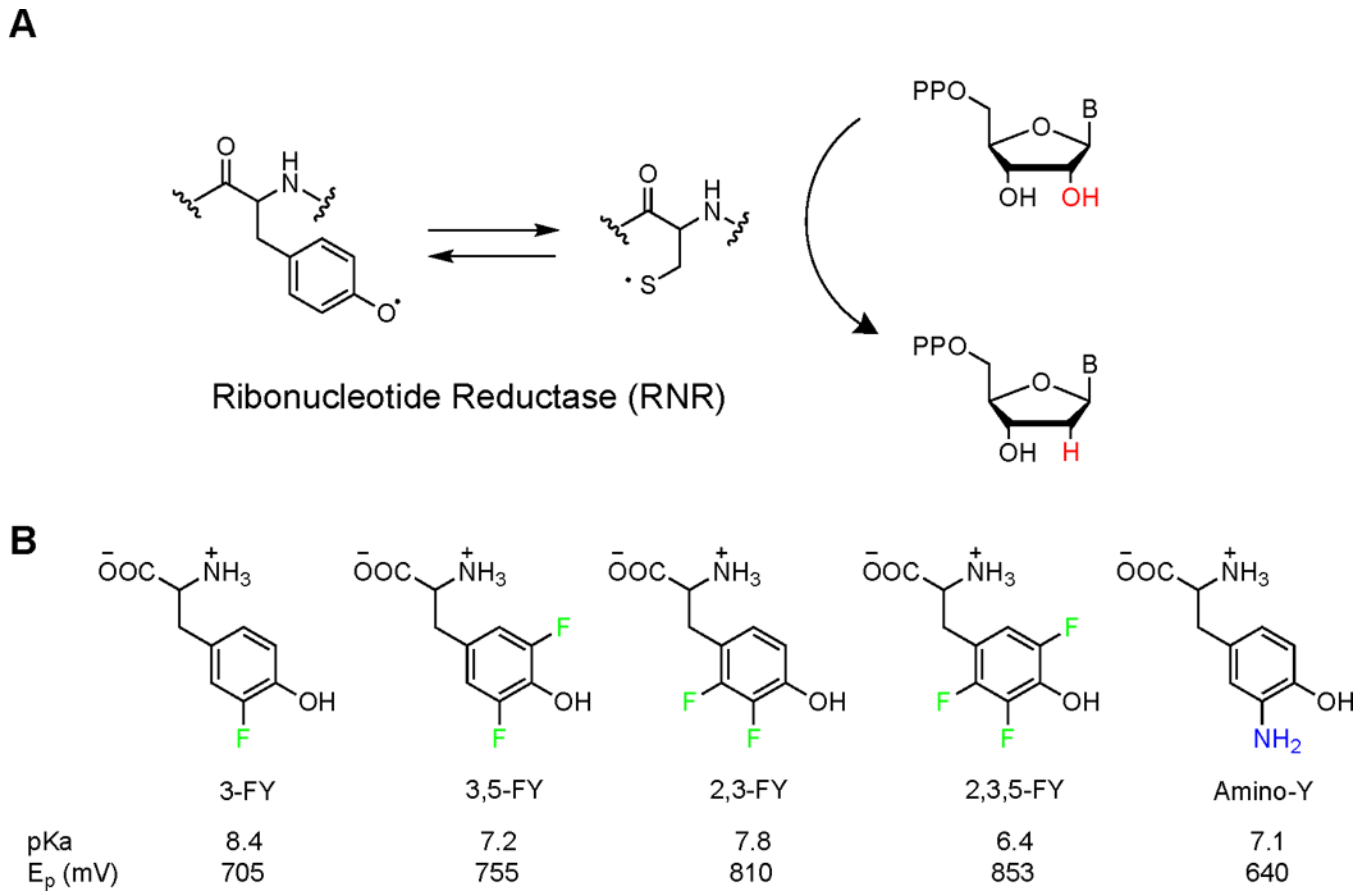

Figure 4.

Modulation of pKa and redox potential of tyrosine residues. A) The ribonucleotide reductase reaction converting ribose to deoxyribose relies upon a catalytic cysteine radical. The generation of this radical is dependent on radical formation on several key tyrosine residues. Altering the pKas and redox potentials of these residues affords key insights into the catalytic mechanism. B) Examples of ncAAs conferring altered tyrosine pKas and reduction potentials (E_p) that have been employed in the study of ribonucleotide reductase.

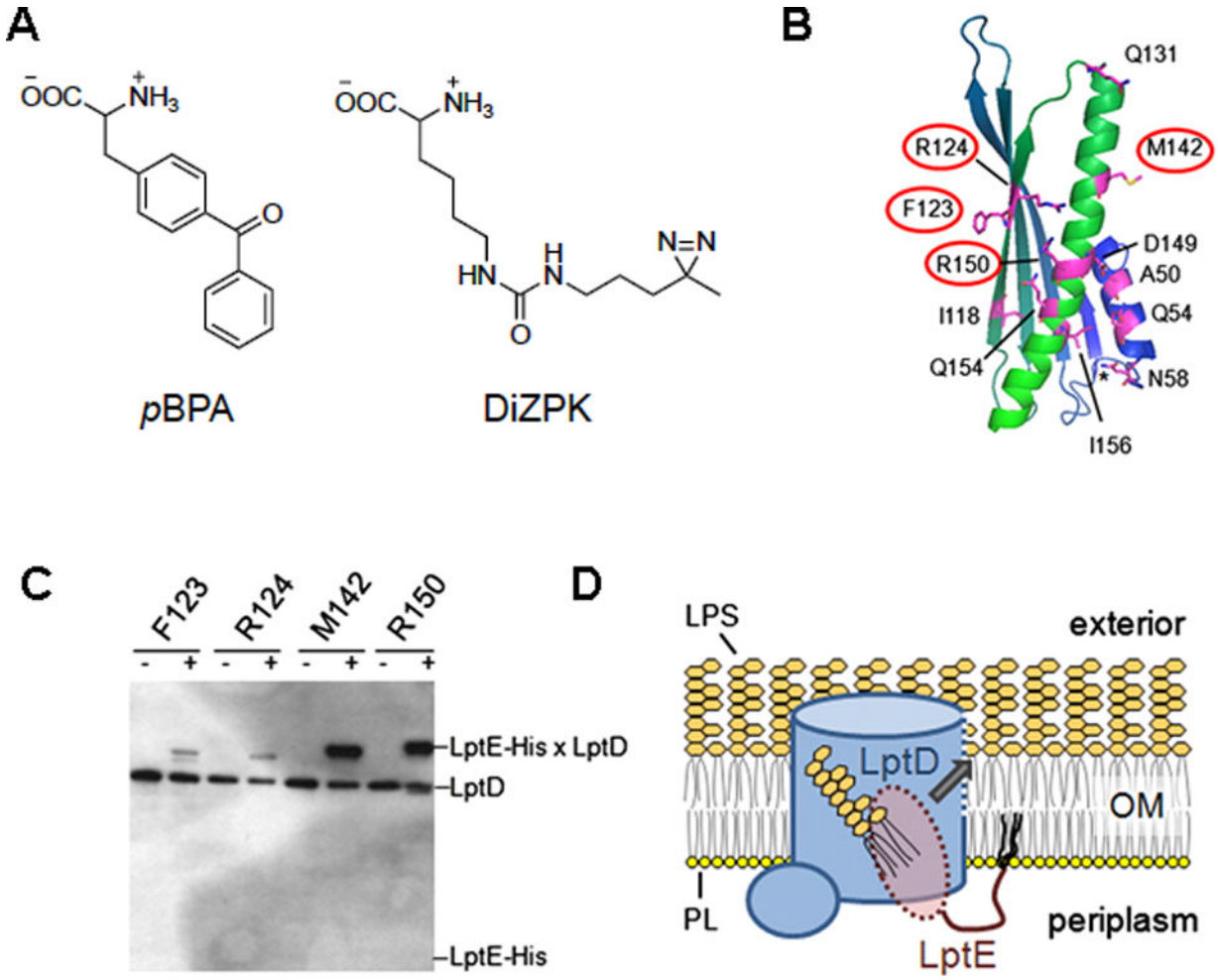

Figure 5.

Applications of photocrosslinking ncAAs. A) Structures of two common photocrosslinking ncAAs, *p*-benzoylphenylalanine and (3-(3-methyl-3H-diazirine-3-yl)-propaminocarbonyl-N^{ε}-lysine. B) Structure of the lipopolysaccharide transport protein E (LptE) with the sites of ncAA incorporation circled in red. C) Example of a photocrosslinking gel employing the benzophenone ncAA at multiple residues of the LptE protein associated with lipopolysaccharide transport. Gel shifts observed in the irradiated (+) samples indicate a crosslinking event with LptD. Proposed model of the LptD/LptE association for lipopolysaccharide transport established by the crosslinking experiments. Adapted from Freinkman, E. et al. 2011.[154]

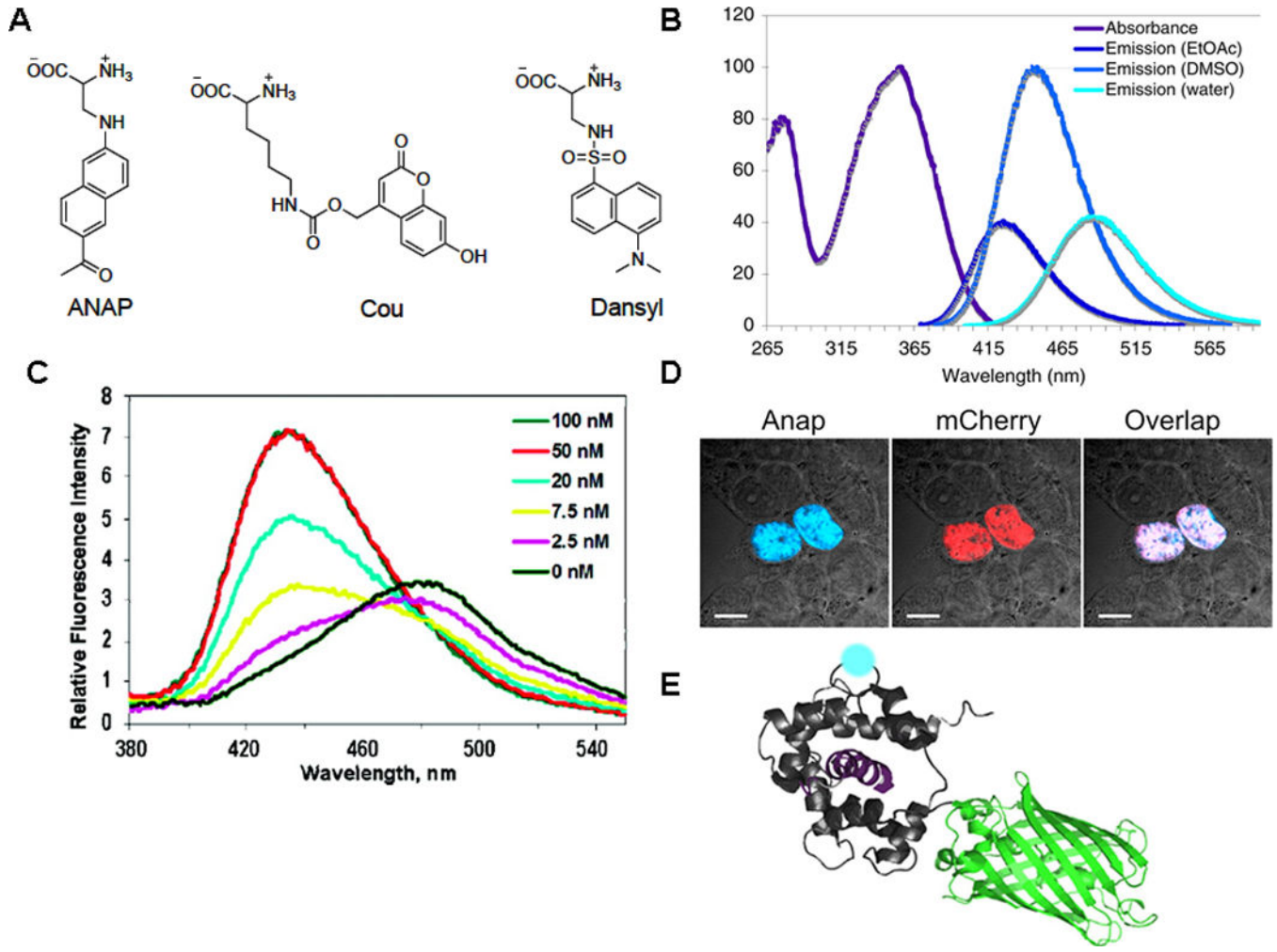

Figure 6.

Genetically encoding fluorescent amino acids. A) Structures of fluorescent ncAAs based on common fluorophores. B) Solvent sensitivity of ANAP fluorescence.[52] C) Use of ANAP in a glutamine binding protein as a site-specific probe to detect glutamine binding by fluorescence.[166] D) Demonstration of the use of an ANAP to track protein localization; the ncAA was incorporated into histones resulting in fluorescence only in nuclei. [52] E) A FRET pair prepared via site-specific incorporation of ANAP (blue circle) in a fusion construct with GFP. The presence of ANAP obviates the need for a second fluorescent fusion protein.[168]

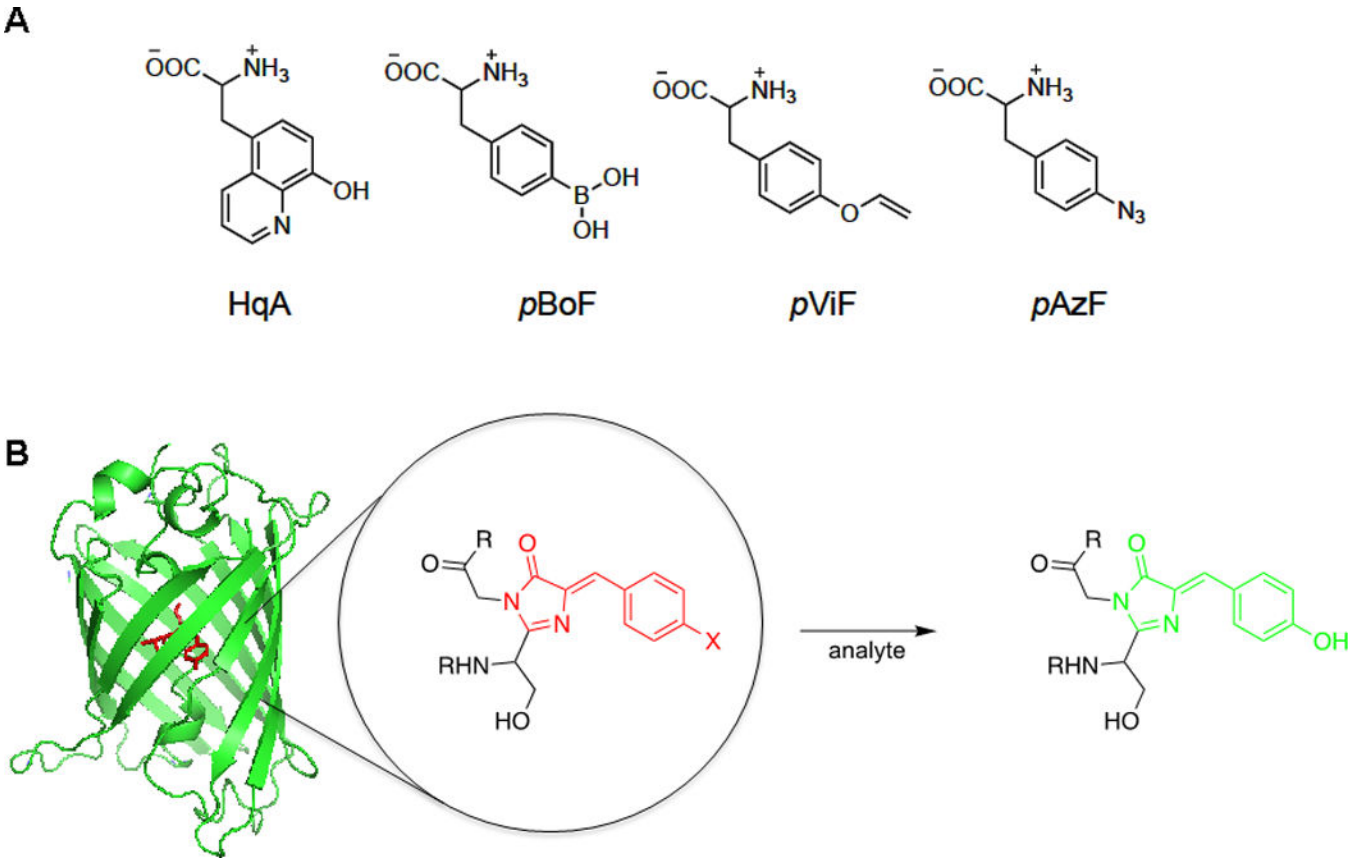

Figure 7.

Using ncAAs as environmental sensors for biologically relevant analytes. A) Structures of ncAAs commonly employed in environmental sensors, including metal binders, H_20_2, H_2S, and $ONOO^-$ sensitive functionalities. B) General method for the development of an “on” sensor. Incorporation of the ncAA within the GFP chromophore alters or quenches fluorescence, however upon coordination or reaction with a desired analyte, the group is removed or fluorescence is shifted.

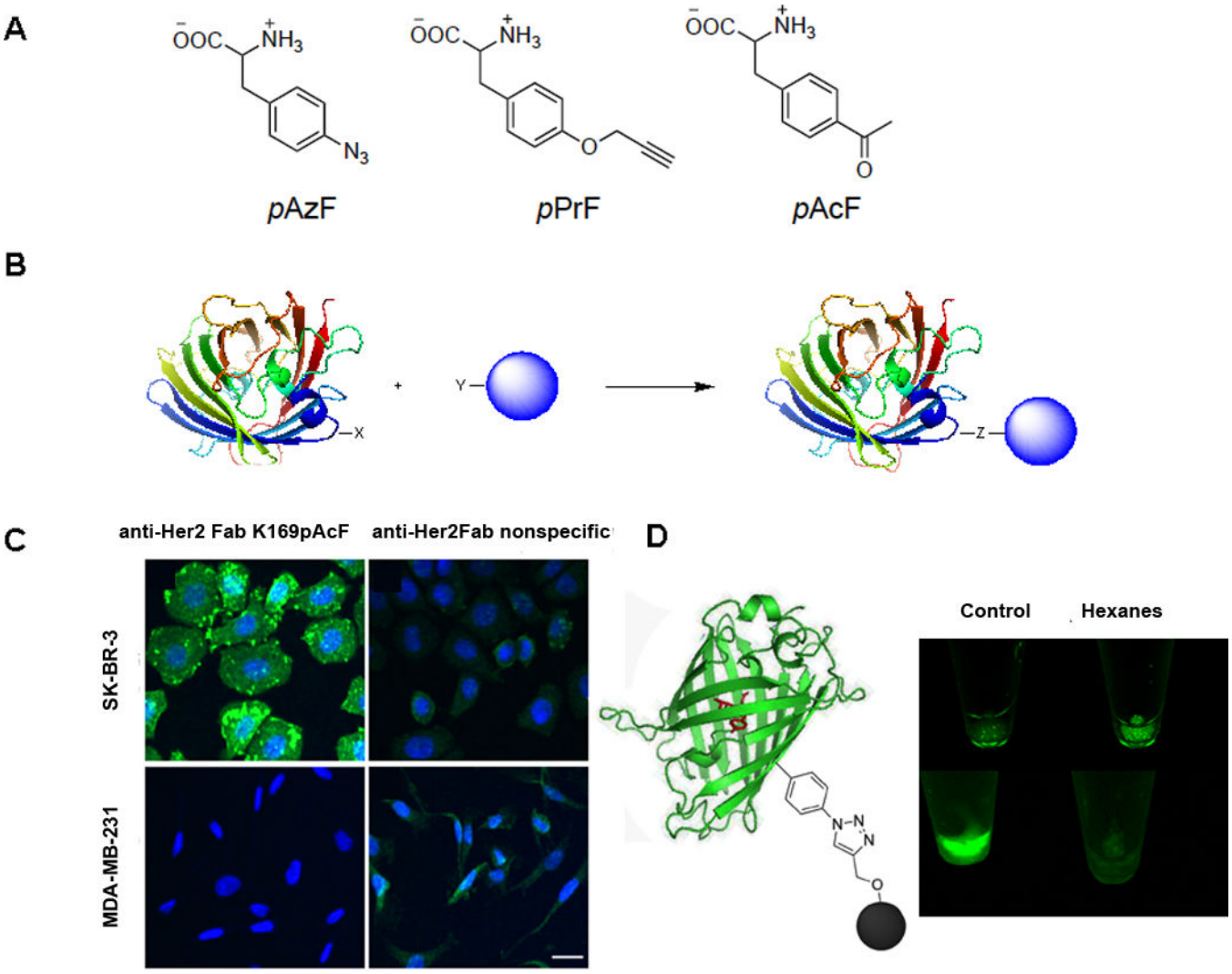

Figure 8.

Bioorthogonal conjugation with ncAAs. A) Common structures of ncAAs used in bioconjugations: *p*-azidophenylalanine (*p*AzF), *p*-propargyloxyphenylalanine (*p*PrF), and *p*-acetylphenylalanine (*p*AcF). B) General scheme of a bioorthogonal conjugation. The protein harboring an ncAA with unique reactivity is subjected to appropriate reaction conditions with another molecule (protein, DNA/RNA, surface, small molecule, etc; blue sphere) that possesses a chemical functionality that will only react with the ncAA and no other biological molecules. C) Incorporation of *p*-acetylphenylalanine into a Fab for Her2 followed by oxime ligation with DNA. This bioconjugate facilitates immuno-PCR with significantly higher levels of sensitivity than nonspecifically labeled antibodies.[215] D) By site-specifically incorporating *p*-azidophenylalalanine GFP can be site-specifically immobilized on a solid support, conferring a higher degree of protein stability in non-aqueous solvents.[232]

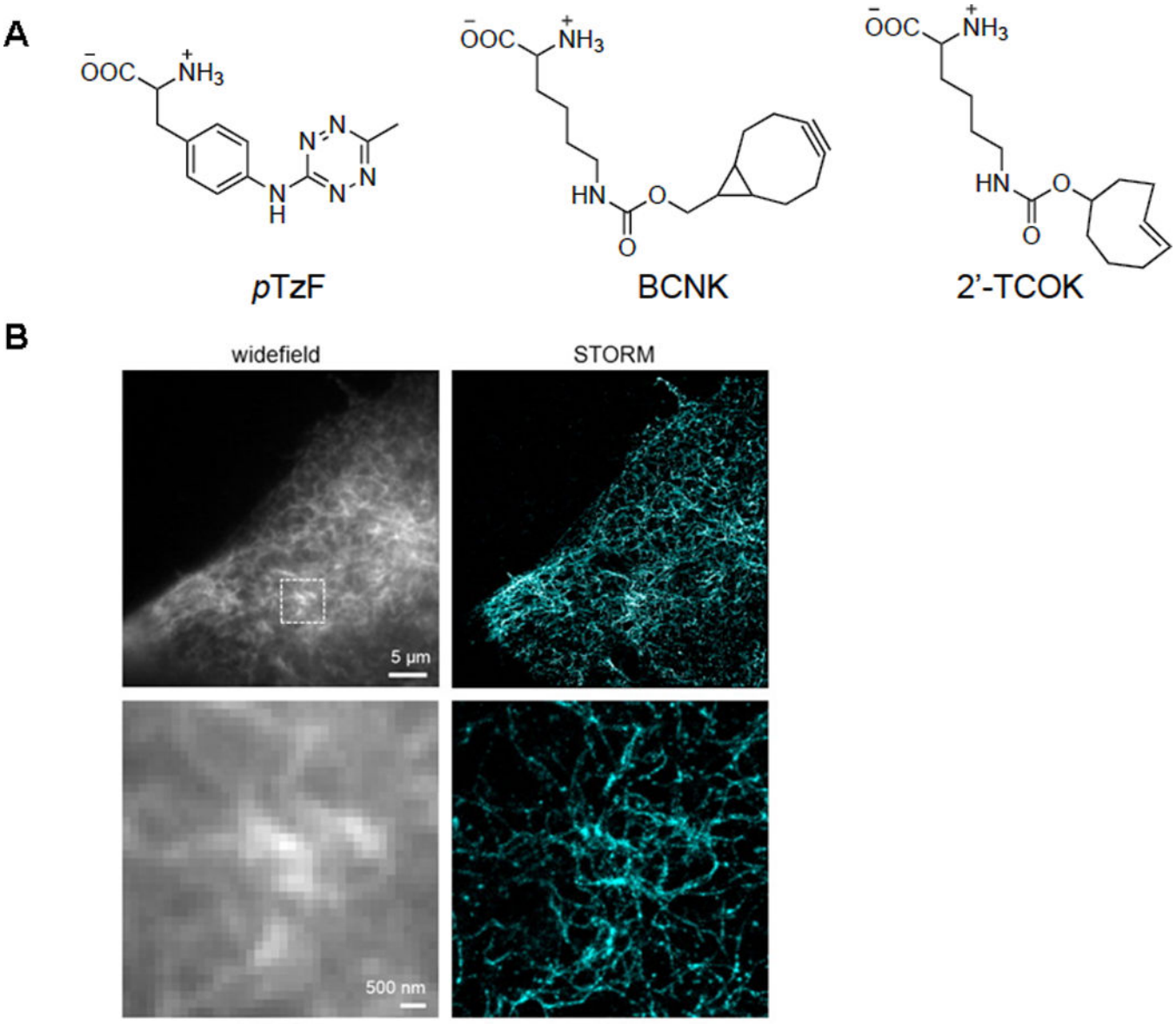

Figure 9.

Live cell imaging using ncAAs. A) Common structures of genetically encoded ncAAs for non-cytotoxic and rapid live cell imaging. Using either standard 1,3-cycloadditions with strained alkynes or Diels-Alder type reactions with a tetrazine and a strained alkyne or alkene, reactions can be performed within living cells. B) Genetic incorporation of the strained alkyne into vimentin for super-resolution, live cell imaging of proteins.[208]

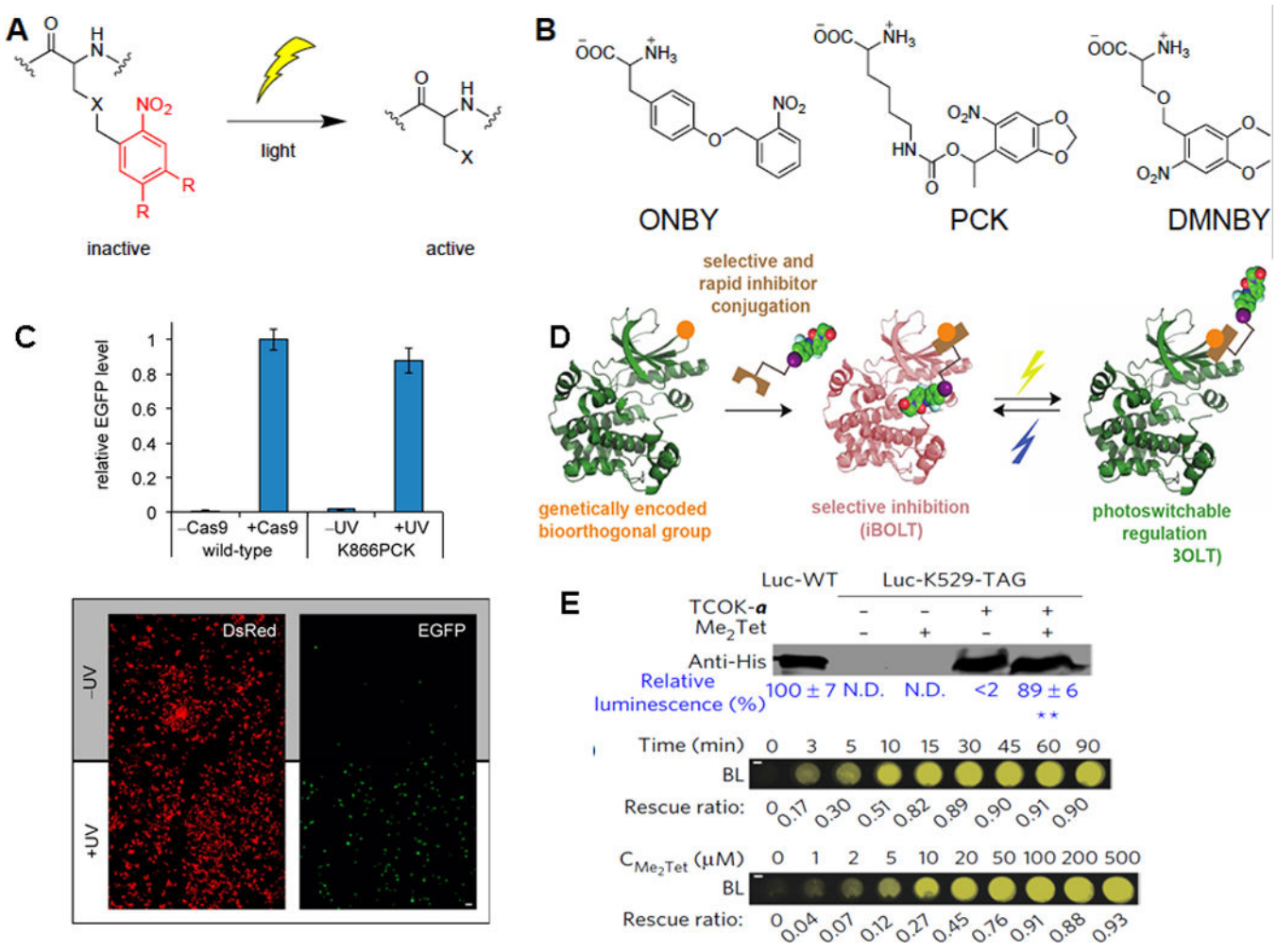

Figure 10.

Photocontrol over protein function with ncAAs. A) Photocaging strategy for activation of protein function with light. Typically a key residue is substituted with a caged ncAA, rendering the protein of interest non-functional. Upon brief irradiation with non-cytotoxic light, the caging group is removed to afford wild-type protein. X = O,N,S; R = H, O, OMe B) Photocaged tyrosine, lysine and serine amino acids with variations of the common *o*-nitrobenzyl caging group. C) Photocaging of the CRISPR/Cas9 system results in inactivity of the Cas9 protein, until brief irradiation with light restores its function close to WT levels, facilitating the expression of a reporter GFP plasmid.[256] D) Photoswitchable regulation of protein function via reaction of a specifically placed ncAA with a ligand harboring an azobenzene based linker. Light irradiation of different wavelengths results in *cis/trans* isomerization, blocking or exposing the active site.[259] E) Genetically-encoded decaging of a strained cycloalkene via reaction with a tetrazine, restoring a lysine residue and protein function. Proof-of-concept experiments were performed via caging luciferase, quenching luminescence until the tetrazine reagent is added.[260]

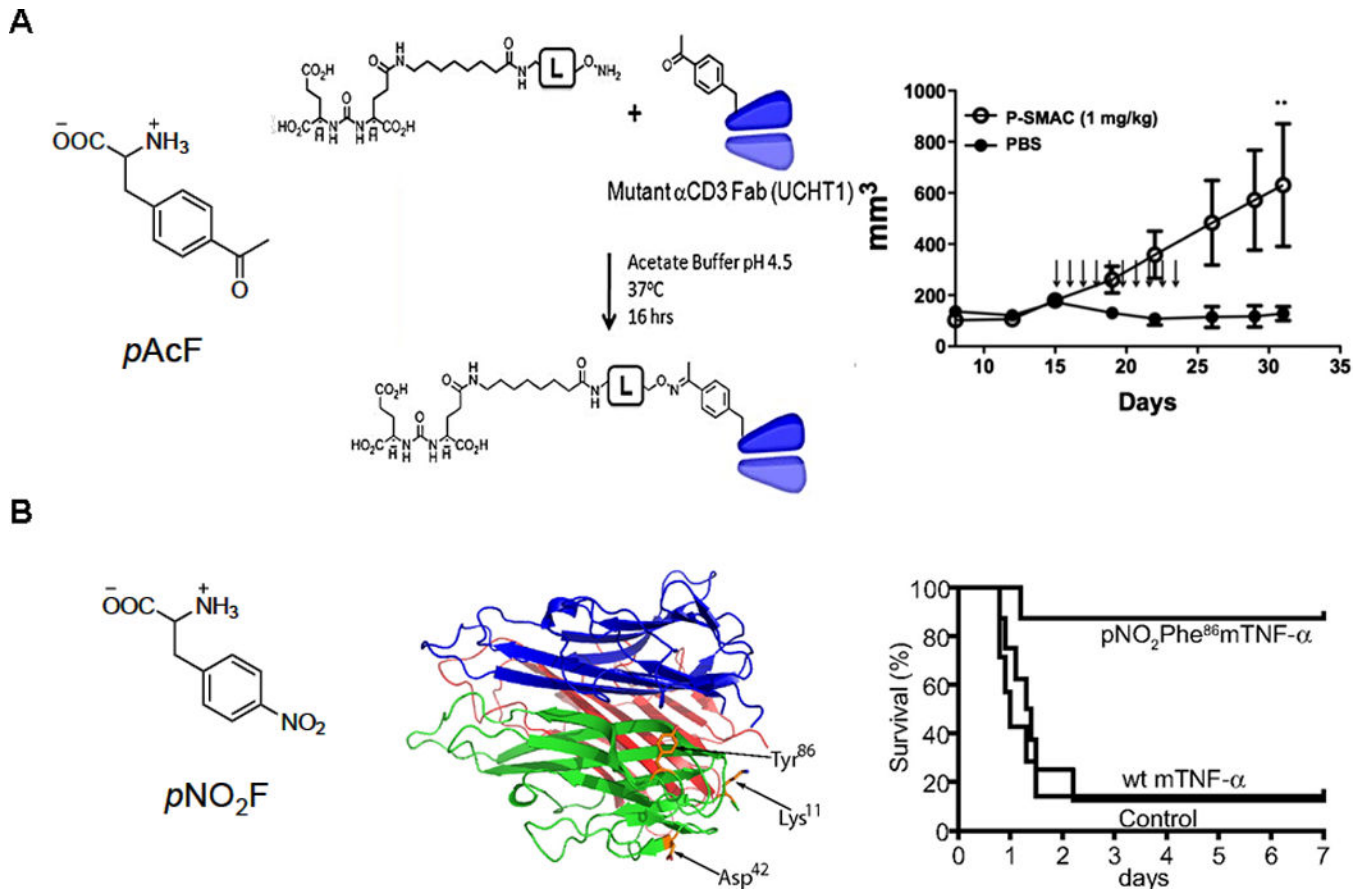

Figure 11.

Therapeutic applications of ncAAs. A) Incorporation of *p*-acetylphenylalanine into an anti-CD3 Fab followed by oxime ligation with an aminooxy-modified DUPA produces a bispecific agent for the treatment of prostate cancer. Treatment of mice in tumor xenografts resulted in complete tumor killing.[288] B) Example of using ncAAs to break immunological tolerance. The ncAA, *p*-nitrophenylalanine was incorporated into mTNF-α at distinct sites and used to immunize mice, resulting in increased survival relative to both the WT protein and a control in a endotoxemia mouse model.[287]

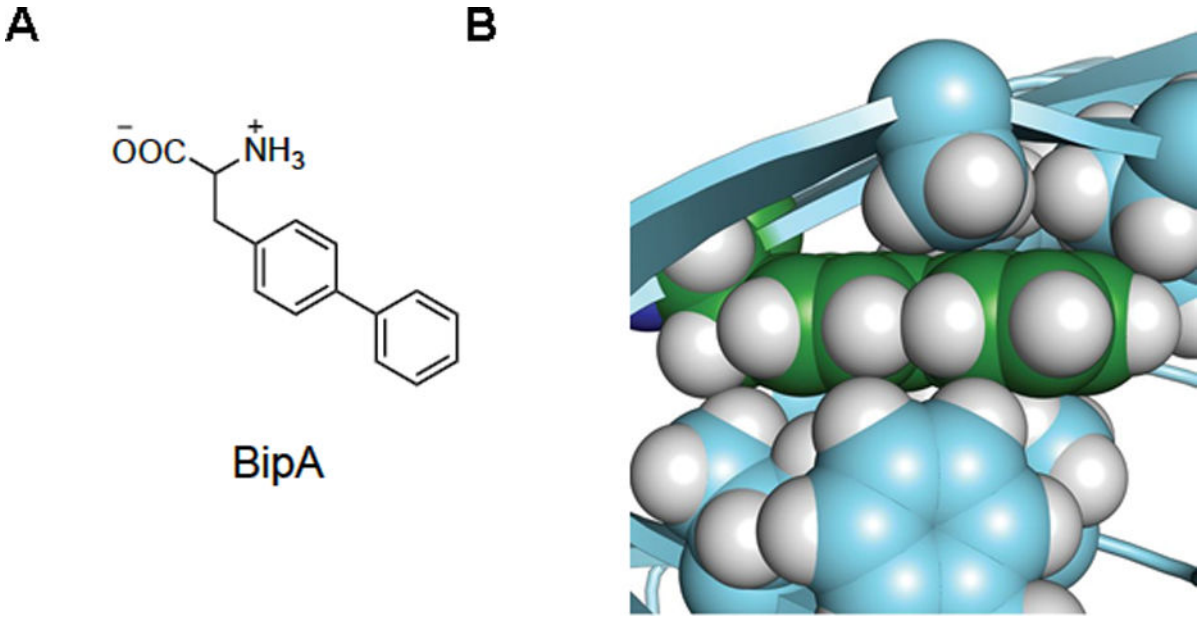

Figure 12.

Computational design of ncAAs containing proteins. A) Structure of *p*-phenylphenylalanine (BipA) used in the model study B) Space filling model of the interactions of the biphenyl (green) locked into its planar transition state with the designed pocket.[294]