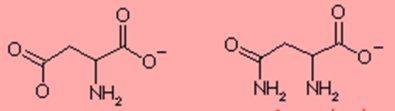
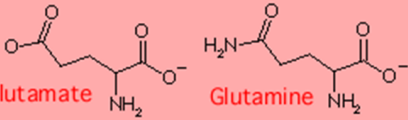


Incorporazione in proteine di
aminoacidi non naturali

Acidic and amide side chains

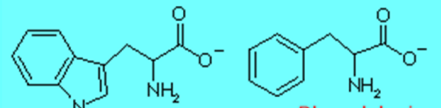


Aspartate Asparagine



Glutamate Glutamine

Aromatic side chains

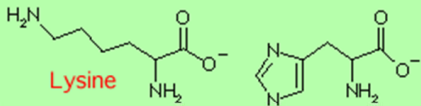


Tryptophan Phenylalanine

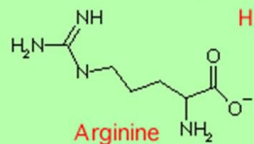


Tyrosine

Basic side chains

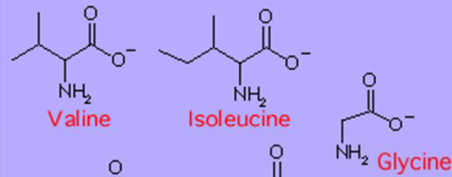


Lysine Histidine

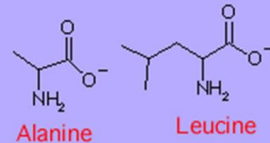


Arginine

Aliphatic side chains

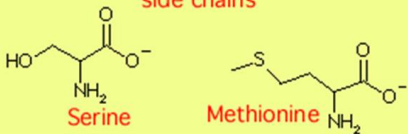


Valine Isoleucine Glycine

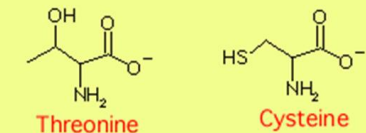


Alanine Leucine

Hydroxyl or sulfur-containing side chains

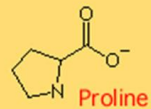


Serine Methionine



Threonine Cysteine

Cyclic side chain



Proline

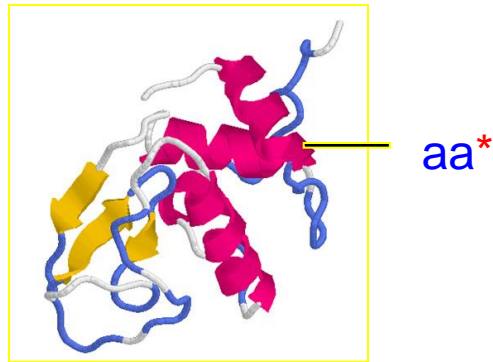
La catena laterale dei 20 amminoacidi naturali manca di gruppi elettrofili non permette reazioni chimiche precise

Incorporazione di aminoacidi non naturali

- Sonde fluorescenti
- modifiche post traduzionali
- crosslinker
- biotina
- chelanti per metalli

Metodi per incorporare aminoacidi non naturali

Selettiva modifica
chimica di un aminoacido



- Come si può modificare selettivamente un aminoacido

Modifica Post-traduzionale tramite reazioni chimiche o enzimatiche

- Sintesi chimica dei peptidi di 30-50 residui + ligazione

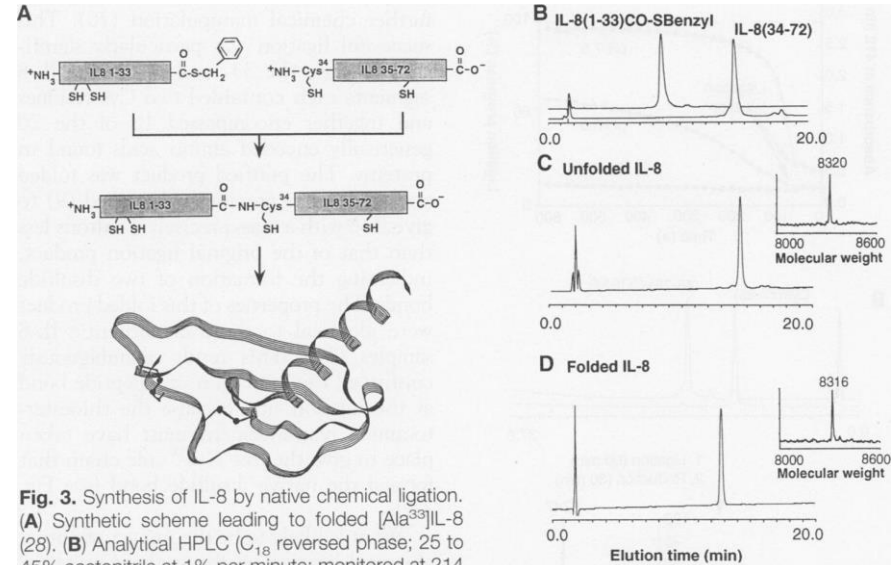
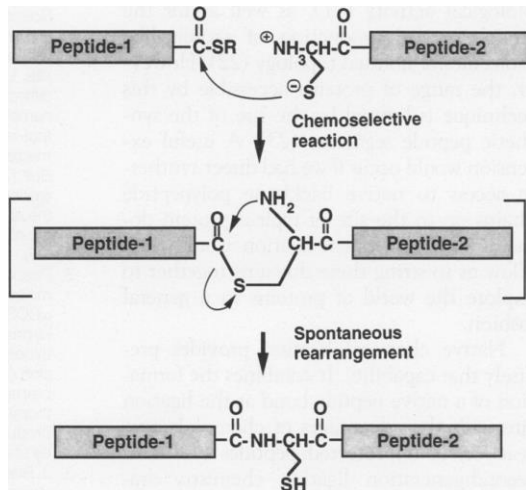
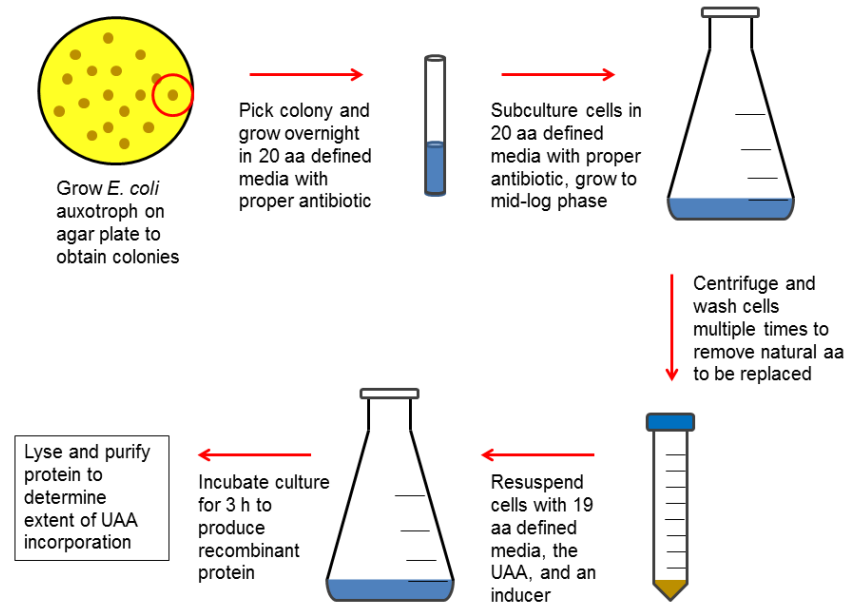


Fig. 3. Synthesis of IL-8 by native chemical ligation. **(A)** Synthetic scheme leading to folded [Ala³³]IL-8 (28). **(B)** Analytical HPLC (C₁₈ reversed phase; 25 to 45% acetonitrile at 1% per minute; monitored at 214 nm) of the synthetic peptide segments (29), IL-8(1-33)αCOSBzyl and IL-8(34-72), each shown before reaction was initiated (30). **(C)** Analytical HPLC under the same conditions of the purified ligation product, IL-8(1-72)(SH)₄, in fully reduced form. (Inset) Electrospray mass spectrum (raw data displayed as a single charge state): observed molecular mass 8319.8 daltons; calculated molecular mass (average isotope composition), 8319.8 daltons. **(D)** Air oxidation of the purified 1-72 ligation product to form the folded [Ala³³]IL-8 molecule, shown after HPLC purification. The earlier elution of the folded, disulfide cross-linked native protein compared with the reduced polypeptide is typical (9). Folding and oxidation conditions: polypeptide at 0.2 mg/ml, 1 M guanidine-HCl, pH 8.5 tris buffer, and vigorous stirring in air at ambient temperature. (Inset) Electrospray mass spectrometry of the oxidized and folded synthetic IL-8 (raw data displayed as a single charge state). Observed molecular mass, 8315.6 daltons; calculated molecular mass (average isotope composition), 8315.8 daltons.

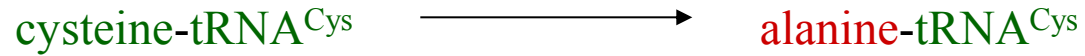
Metodi per incorporare aminoacidi modificati in proteine

In vivo – sostituzione residuo specifica:
crescita in un mezzo che contenga
l'aminoacido modificato che deve essere
strutturalmente simile a quello naturale
(selenometionina/metionina o aa marcati
con ^{15}N)



- *In vitro e in vivo* – sostituzione sito specifica
- mediante l'uso di tRNA soppressori non senso

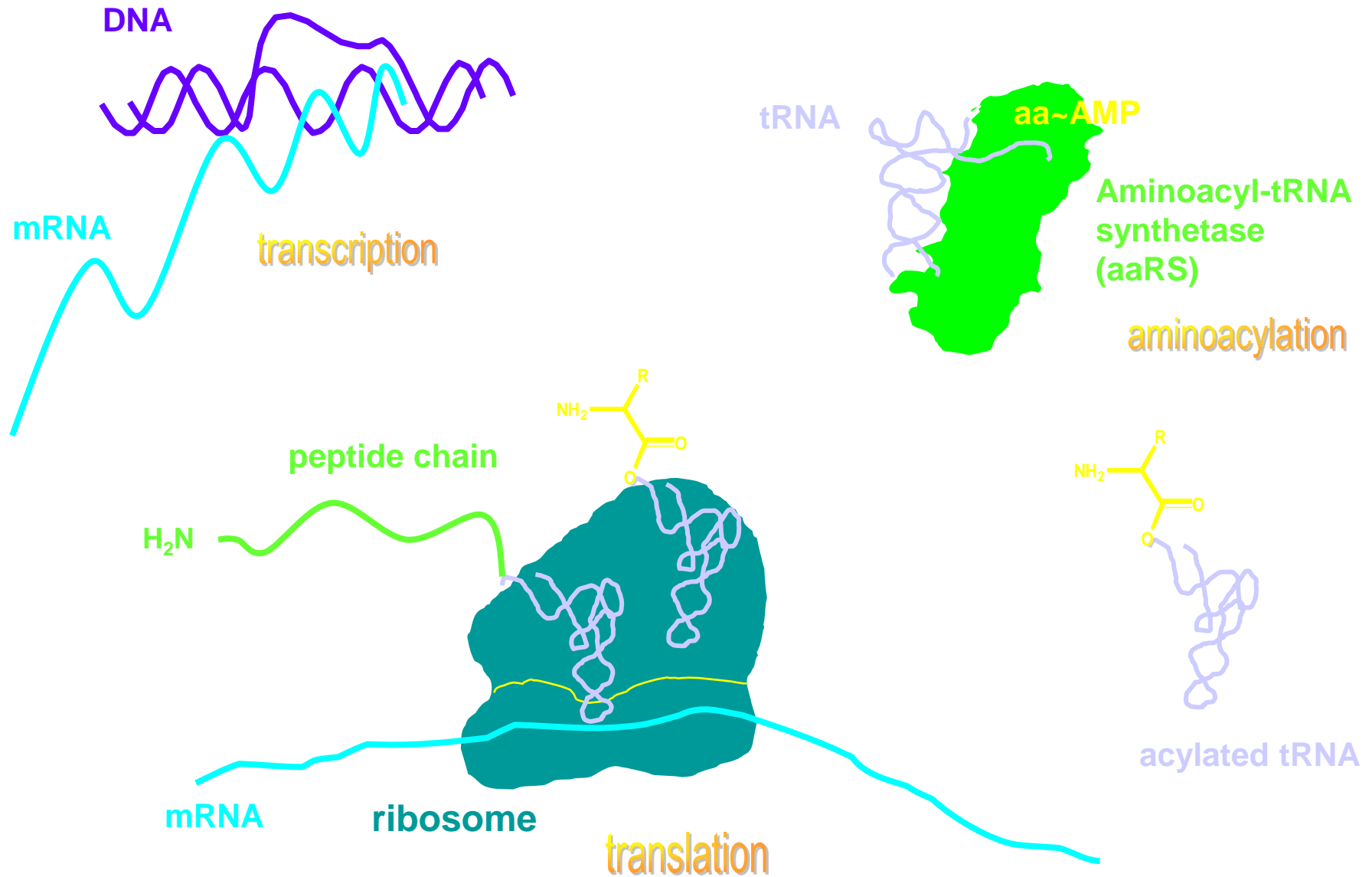
“Misacylation” del tRNAs



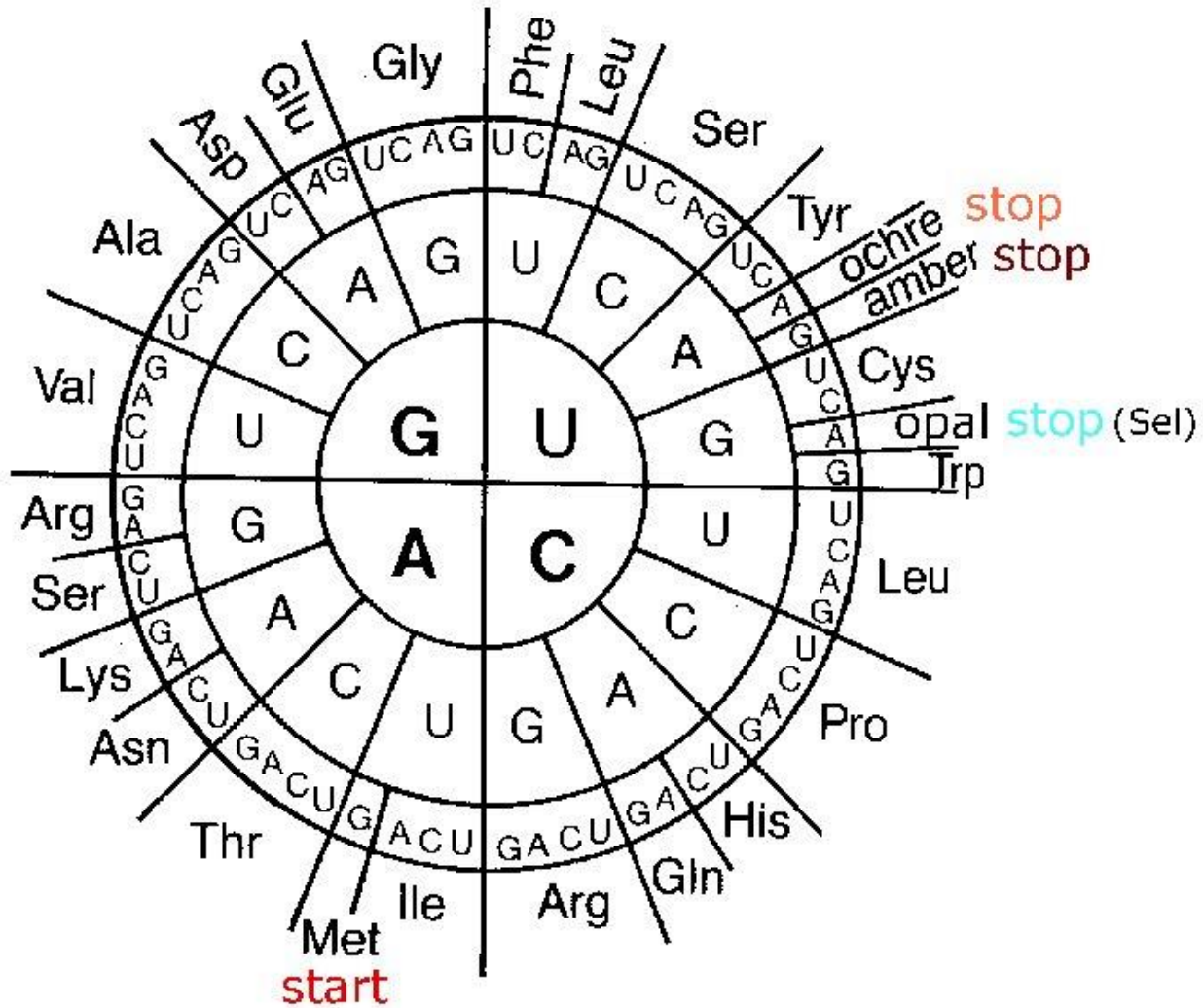
Chapeville, F.; Lipmann, F.; von Ehrenstein, G.; Weisblum, B.; Ray, W. J.; Benzer, S. *Proc. Natl. Acad. Sci.* **1962**, *48*, 1086-1092.
von Ehrenstein, G.; Weisblum, B.; Benzer, S. *Proc. Natl. Acad. Sci.* **1963**, *49*, 669-675.

Incorporazione di aminoacidi modificati mediante l'uso di
tRNA soppressori nonsense

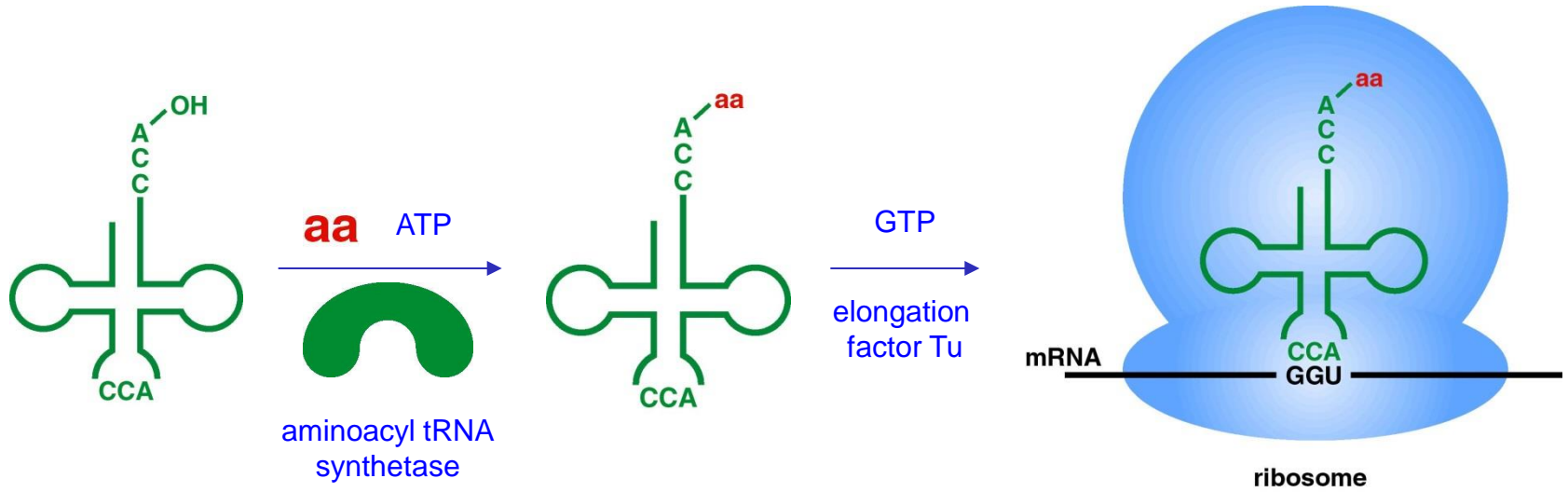
Sintesi proteica



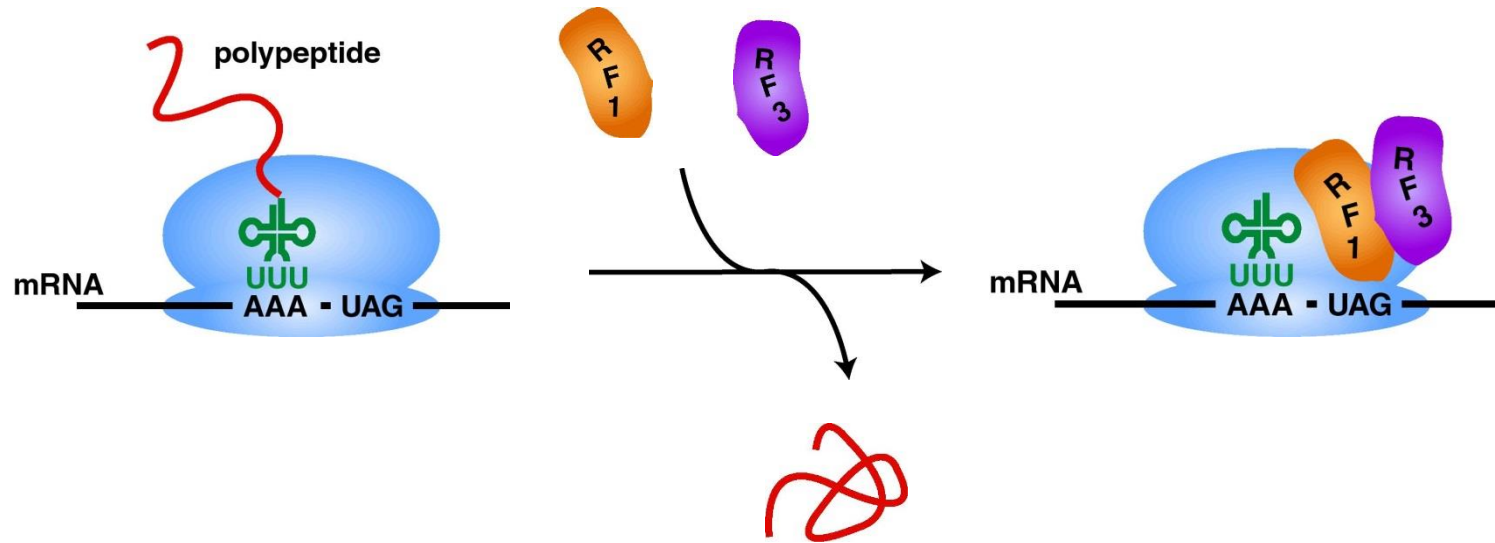
“Codice genetico”



Traduzione



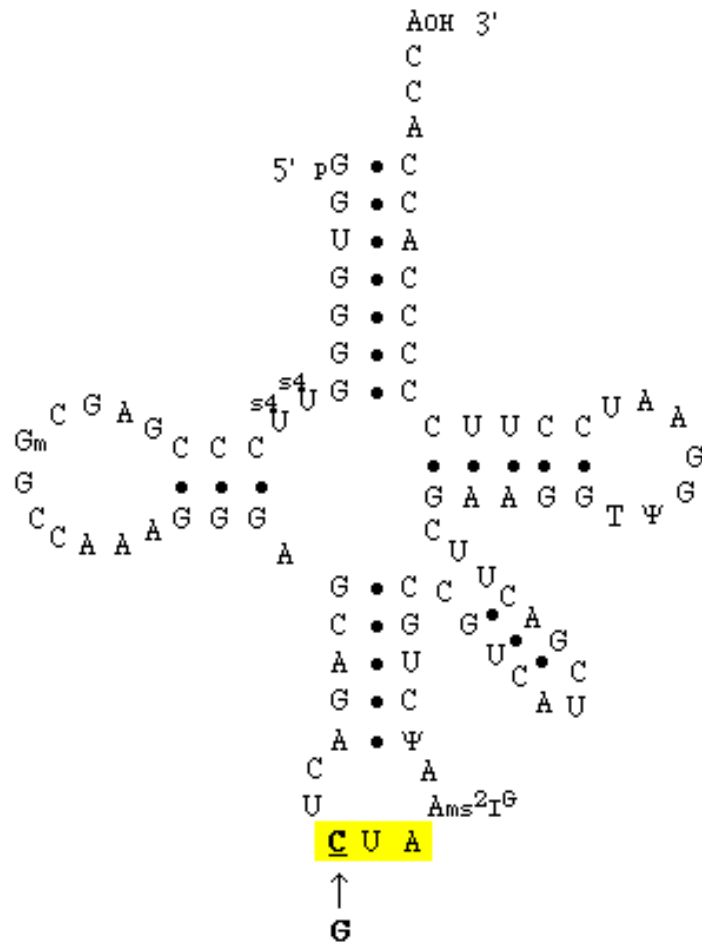
Terminazione



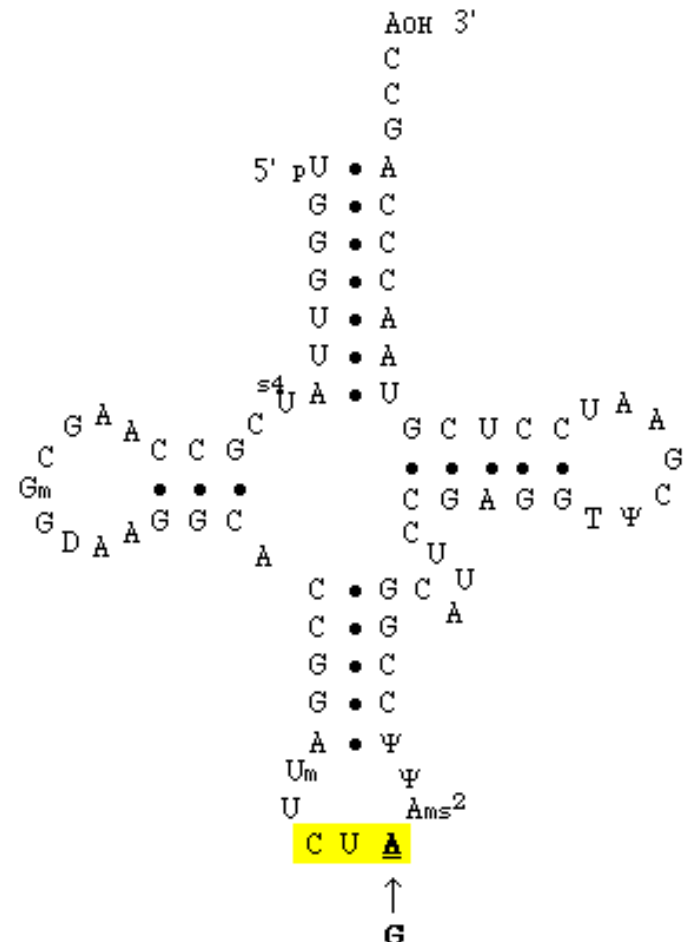
Amber (UAG), Opal(UGA), Ochre(UAA)

tRNAs Soppressori

supF tRNA Amber (UAG) suppressor
= *tyrT* mutation

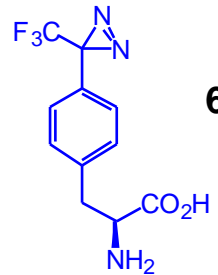


supE tRNA Amber (UAG) suppressor
= *glnV* mutation



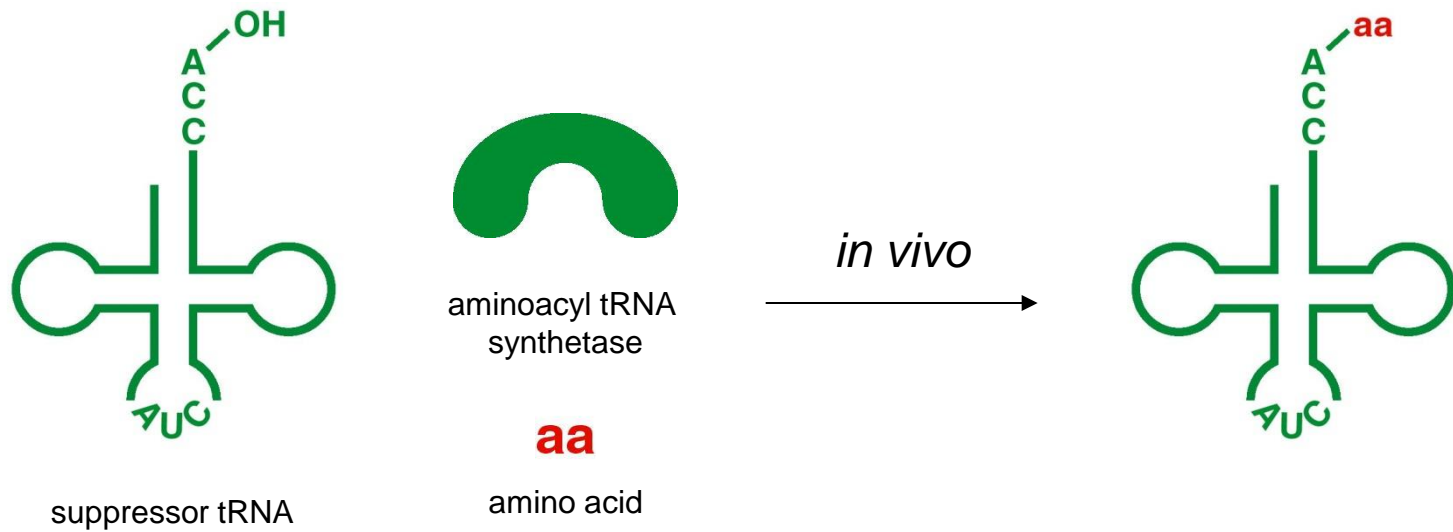
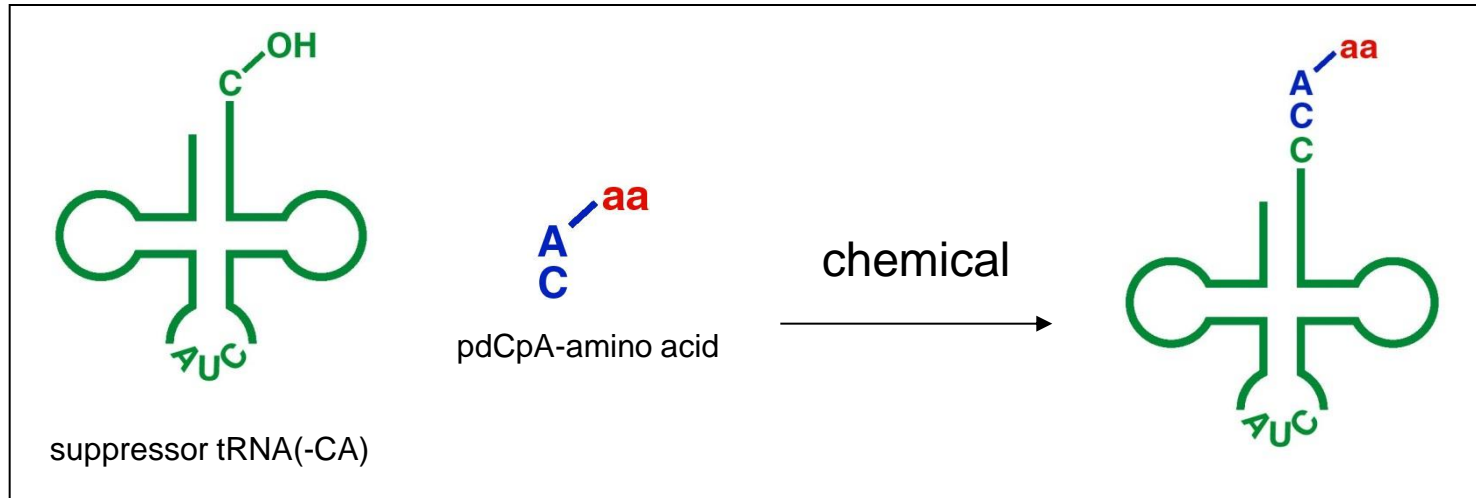
Prima evidenza della possibilità di usare soppressori nonsense per inserire in una catena polipeptidica aminoacidi modificati

Introduzione della diazirina in un sito che portava la mutazione amber
Mediante modifica chimica del tRNA acilato

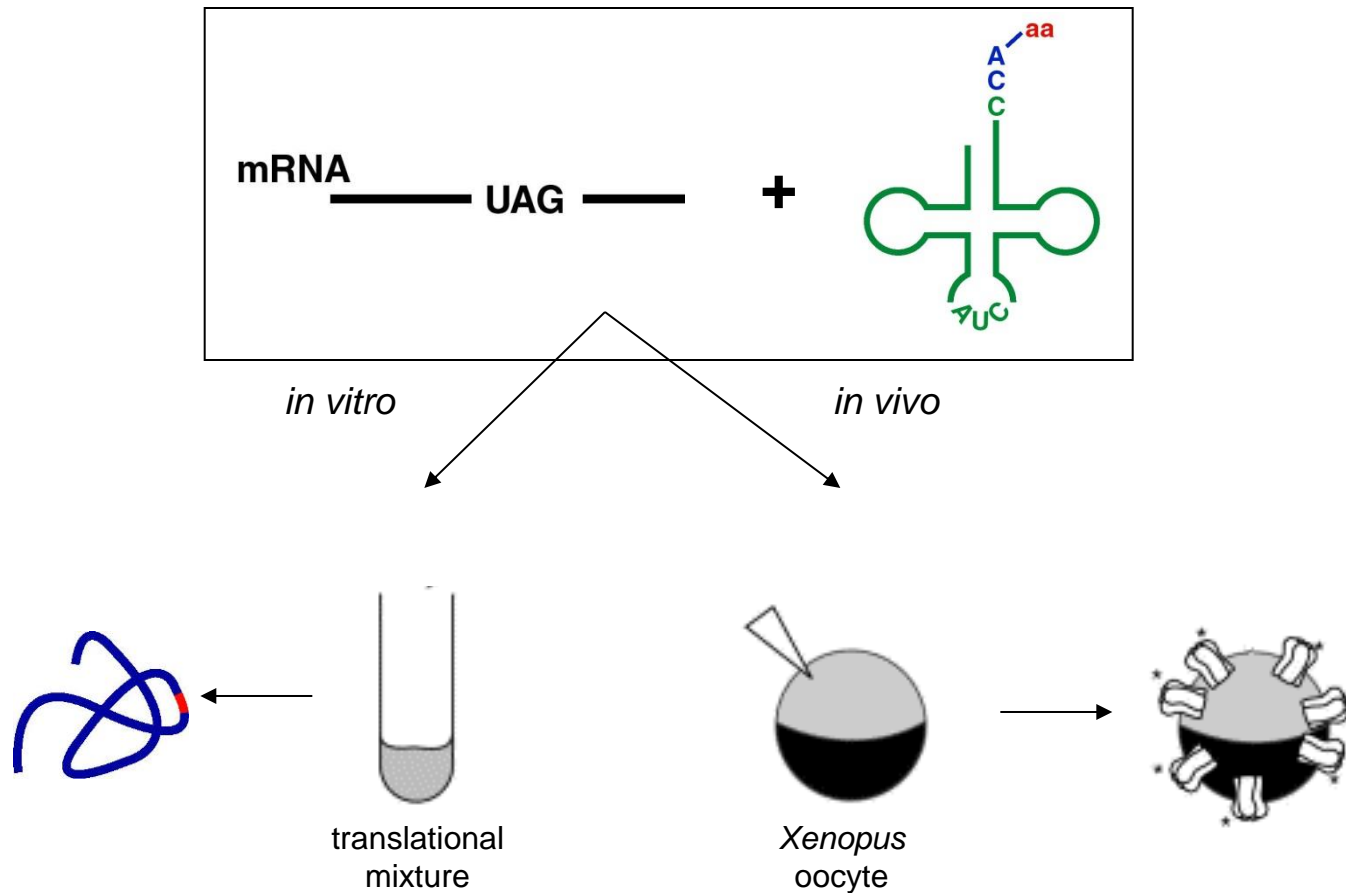


Shih, L. B.; Bayley, H. *Anal. Biochem.* **1985**, *144*, 132-141.

Misacylation of Suppressor tRNAs

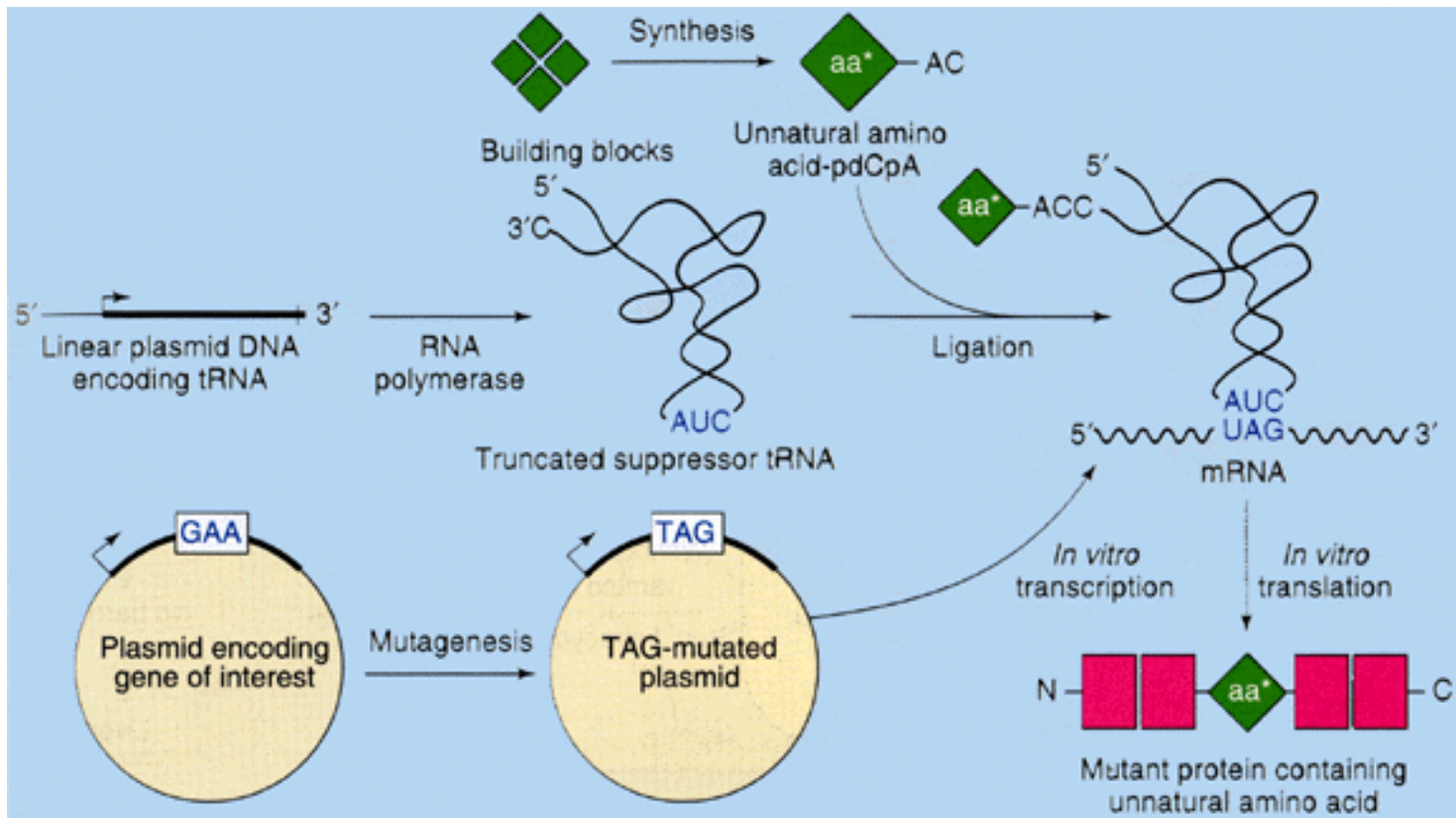


Produzione di proteine con aa modificati *in vitro* e *in vivo* con tRNA acilato chimicamente

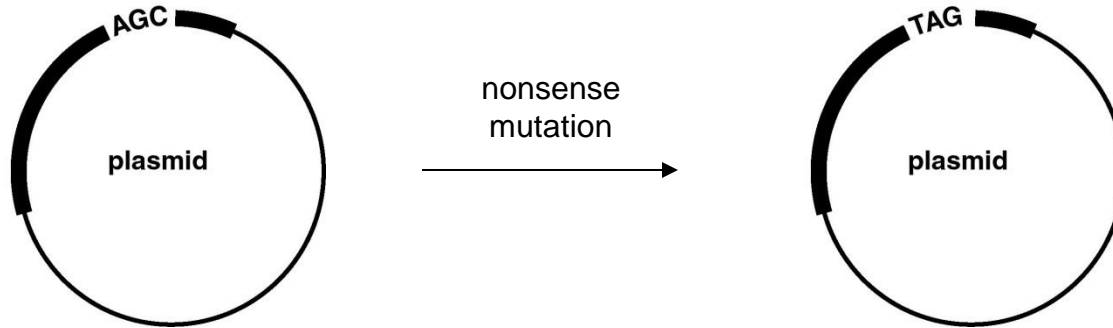


Bassa resa perchè il tRNA non può essere riacilato in vivo.

In vitro



Nonsense Mutation

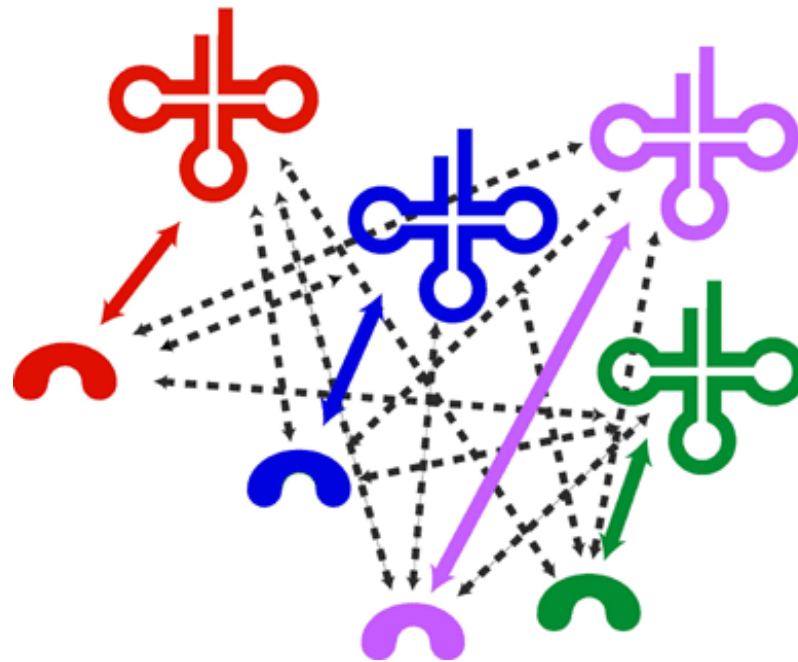


- proteina troncata e non funzionale

Incorporazione di aminoacidi nonnaturali in vivo

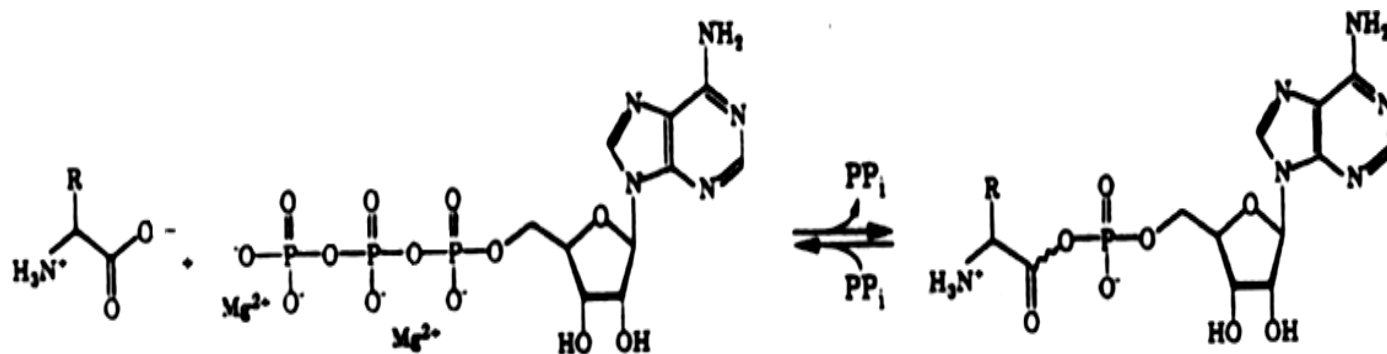
In Vivo Misacylation

- L' aminoacido modificato non deve essere tossico e deve poter entrare nella cellula
- Il “Suppressor tRNA” deve essere acilato solo dalla sintetasi eterologa (orthogonal tRNA/synthetase pair)



Gli aminoacidi vengono attivati mediante adenilazione

La formazione dell' **amminoaciladenilato** è catalizzata dall'**amminoacil-tRNA sintetasi**



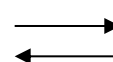
amino acid

ATP

aminoacyl adenylate

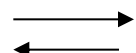
Amminoacil AMP
(amminoaciladenilato)

+ tRNA



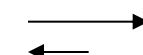
AMP + amminoacil-tRNA

ATP+amminoacido+tRNA



Amminoacil-tRNA+ AMP+ PP_i

PP_i



2Pi

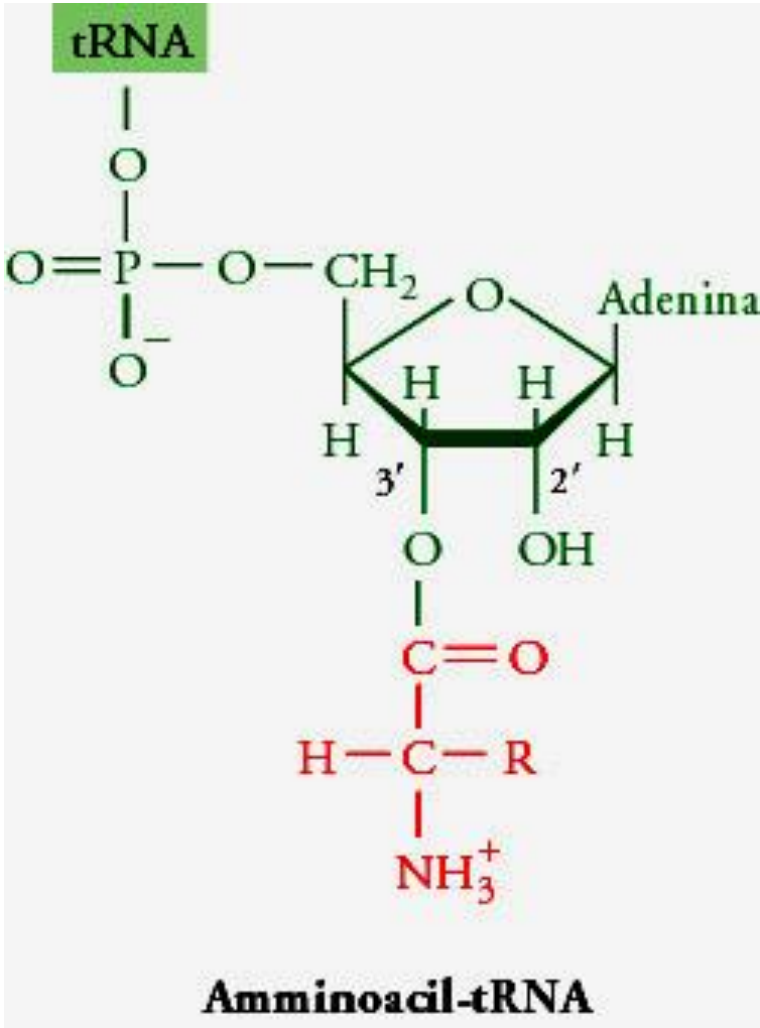
Esistono due classi tRNA sintetasi che si differenziano per:

- **Riconoscimento dell'anticodone**

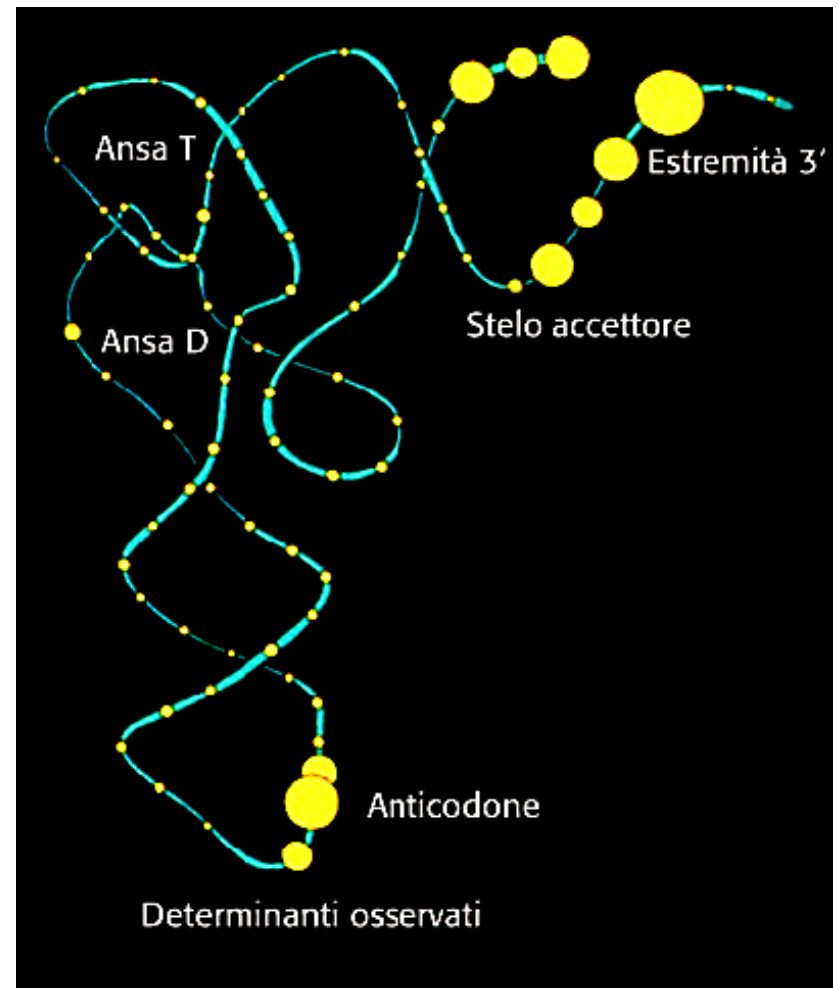
Enzimi di classe II non interagiscono con l'anticodone

- **Sito di amminoacilazione** Enzimi di Classe I caricano l'amminoacido sull'OH 2'

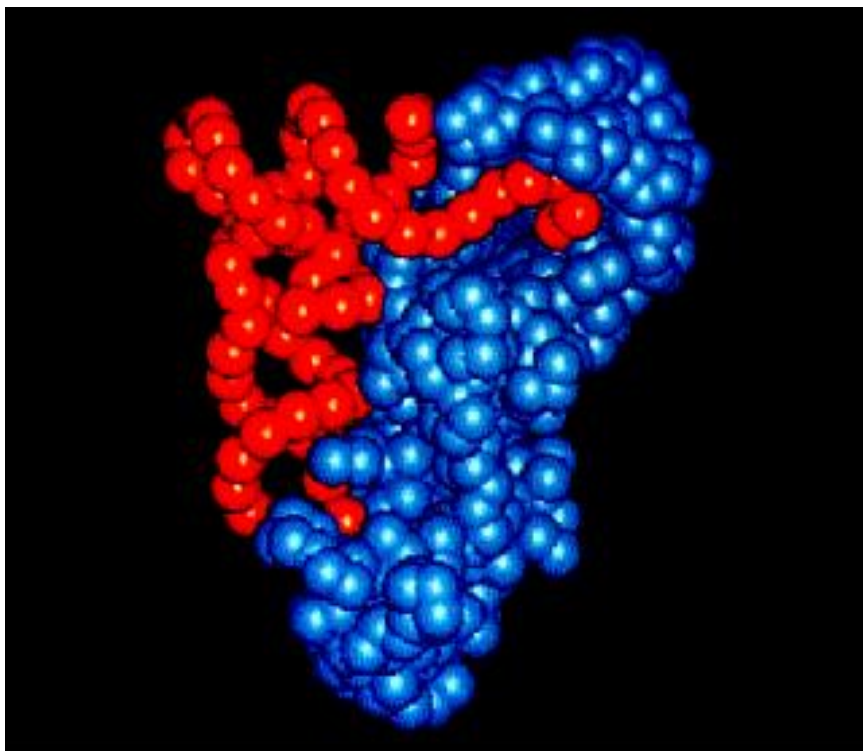
- **Struttura**



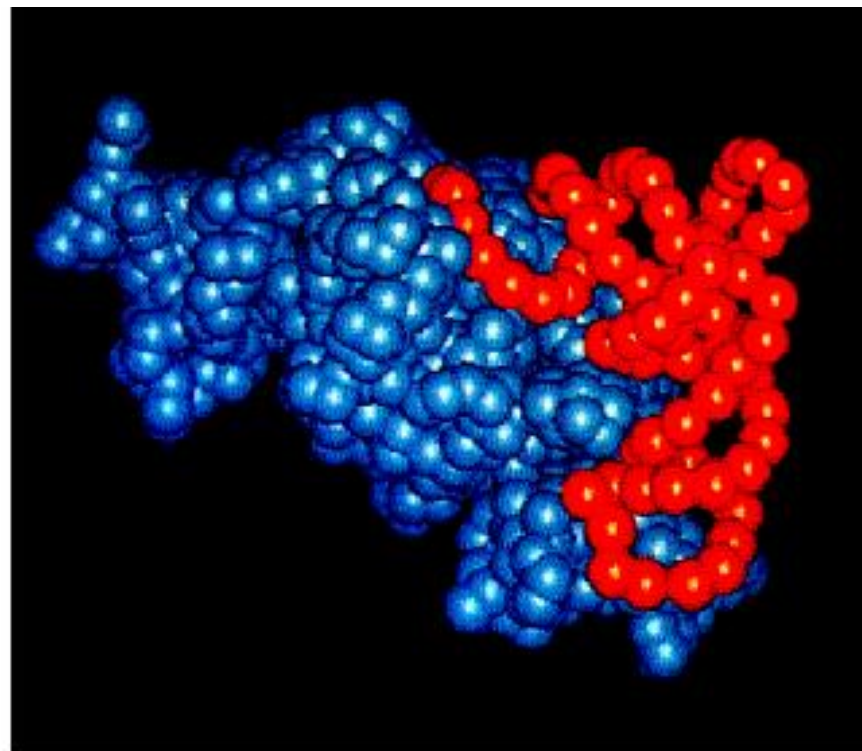
Le sintetasi riconoscono le anse dell'anticodon e gli steli accettori delle molecole di tRNA



Le due classi di tRNA sintetasi legano lati diversi del tRNA

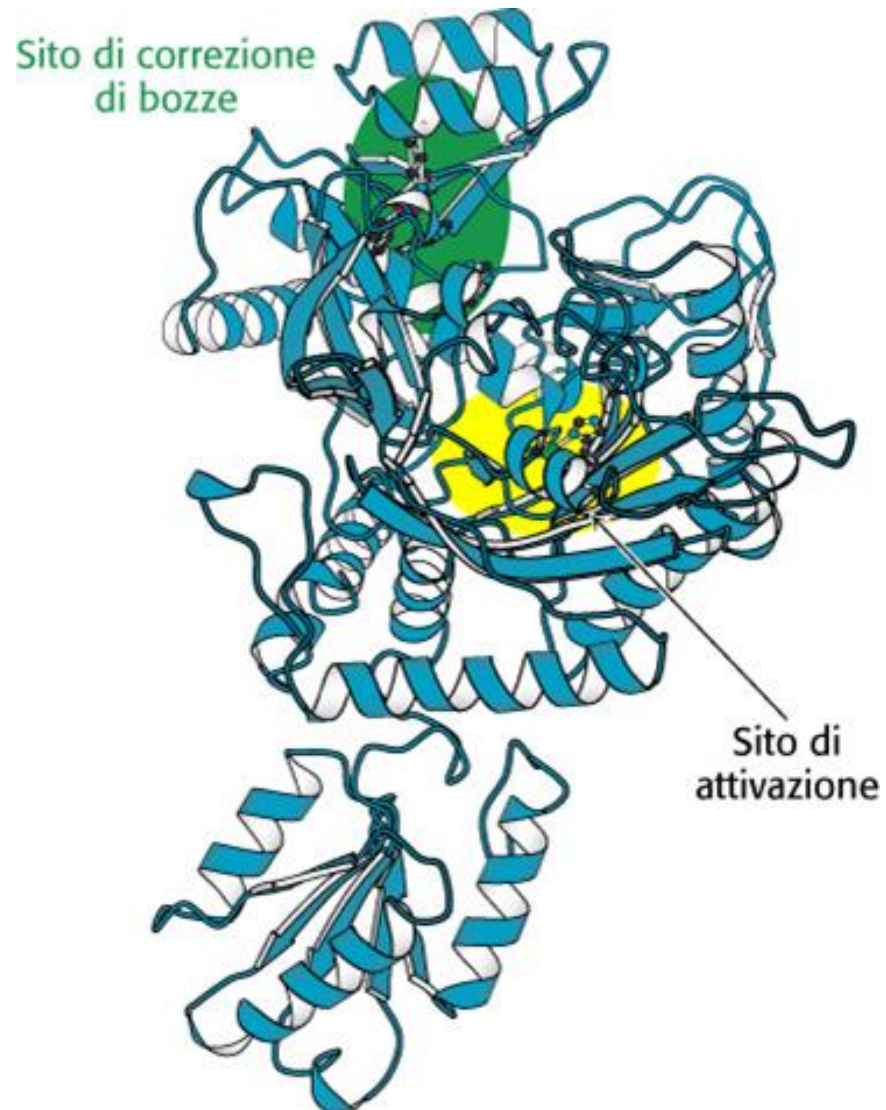


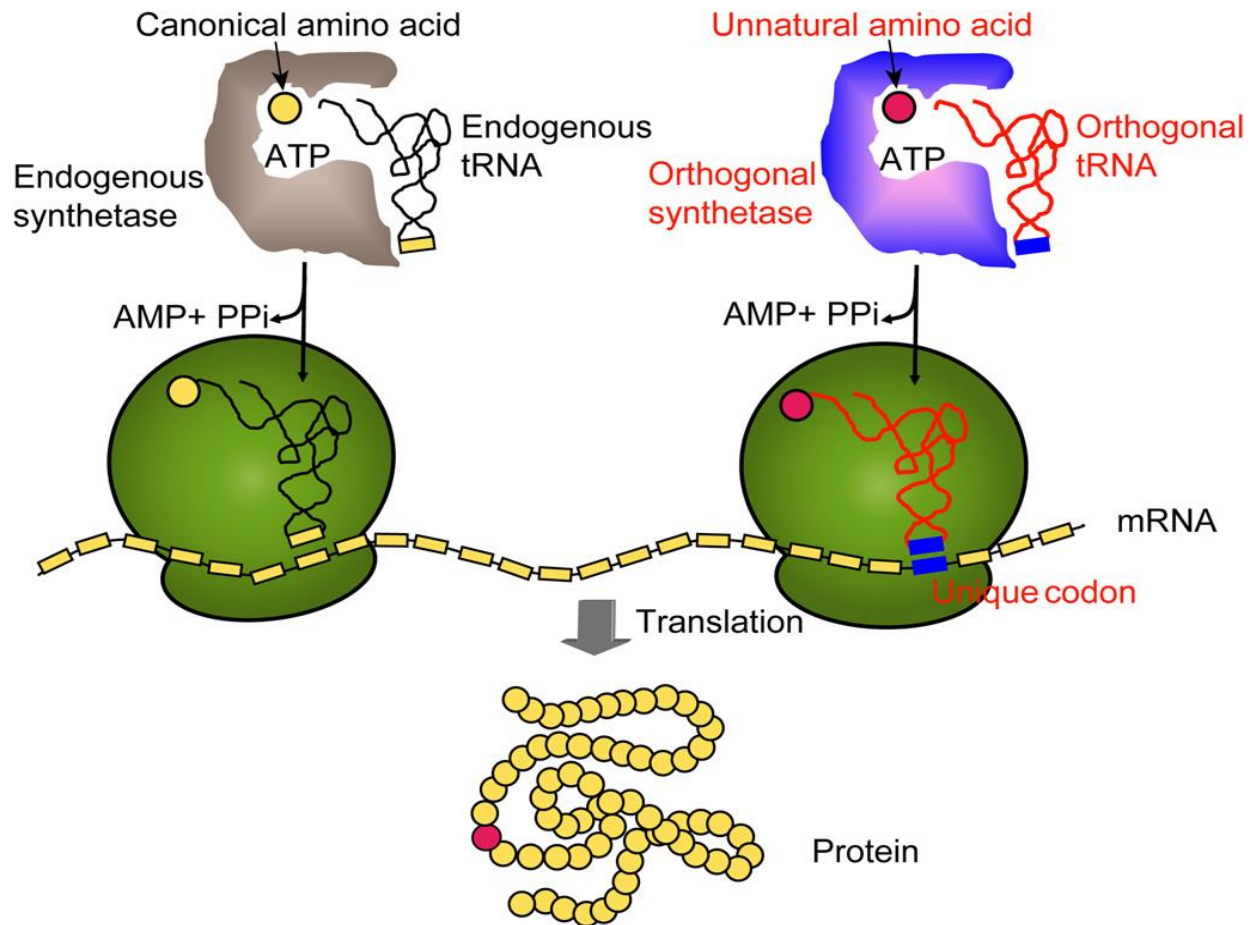
(a)



(b)

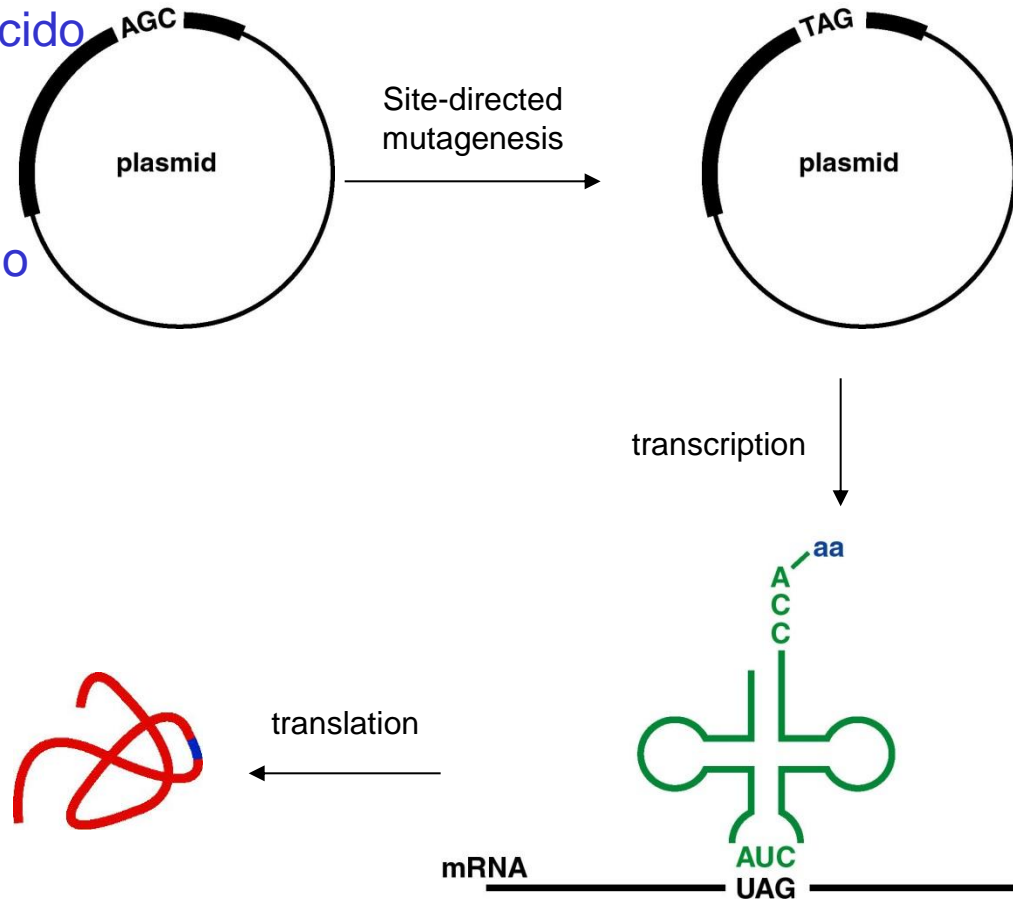
Alcune tRNA sintetasi hanno il sito per la correzione di bozze





Nonsense Suppression Methodology

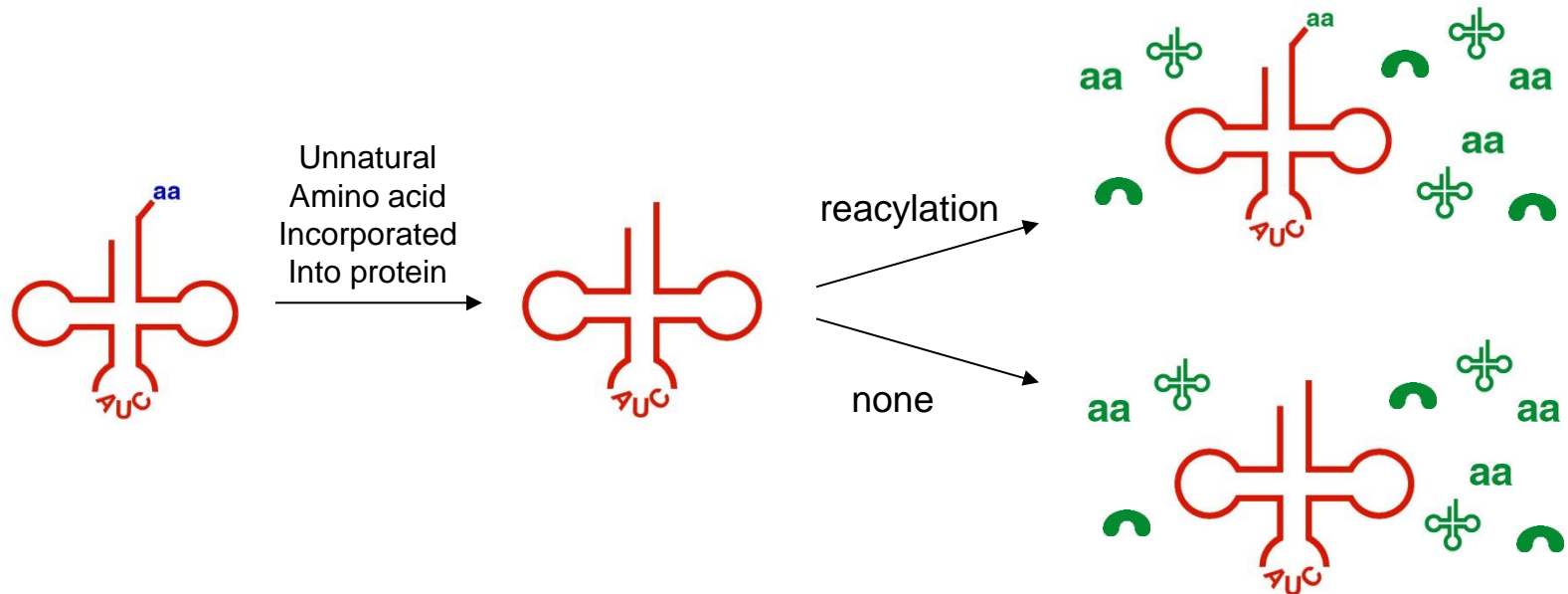
- mutazione non senso in un tRNA specifico per un determinato aminoacido modificato
- aminoacil sintetasi in grado di riconoscere e attivare l' aminoacido modificato
- inserimento del codone di stop nella posizione in cui si vuole inserire l' aminoacido modifica



Ricerca di una coppia tRNA /sintetasi in grado incorporare aminoacidi modificati

Selezione del tRNA Soppressore

- Non deve essere acilato da sintetasi endogene

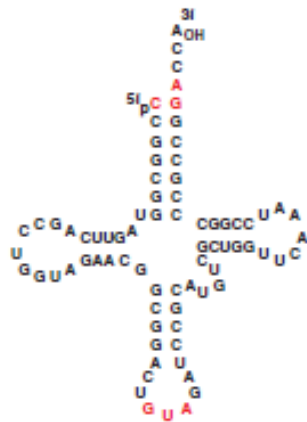


Tentativi di produrre coppie di tRNA soppressori / sintetasi a partire da Glutamil tRNA /sintetasi di E coli non hanno portato a risultati positivi

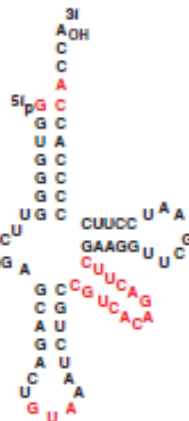
Ricerca di una coppia tRNA /sintetasi in grado incorporare aminoacidi modificati

tRNA degli eucarioti e degli archea sono simili tra loro e diverse da quelle di E.coli

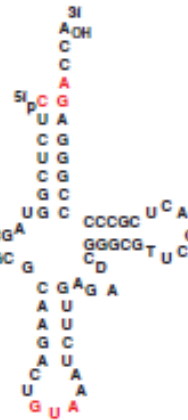
a



M. jannaschii tRNA^{Tyr}

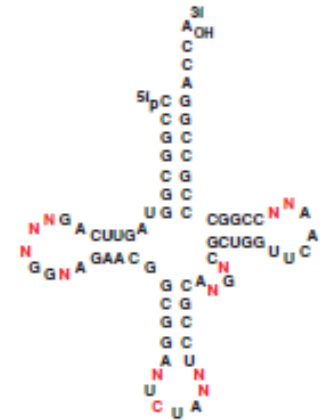


E. coli tRNA^{Tyr}



S. cerevisiae tRNA^{Tyr}

c

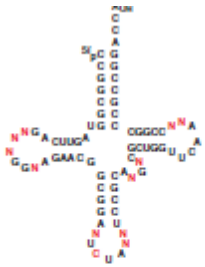


A mutant amber suppressor tRNA library based on *M. jannaschii*
tRNA^{Tyr}: N - A : U : C : G

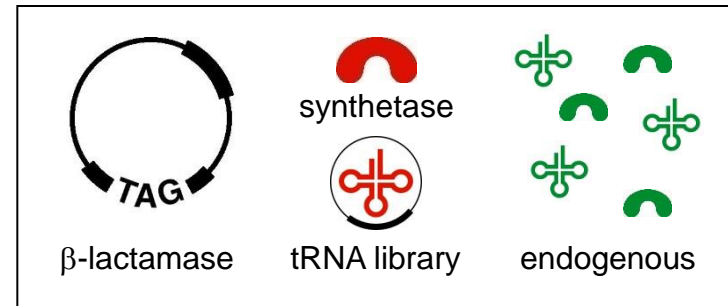
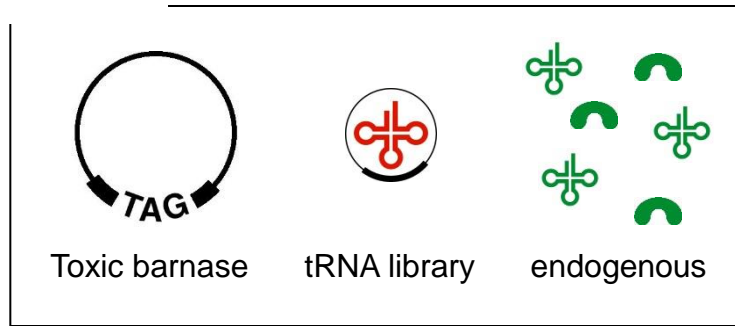
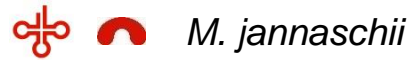
b

A 73 C1 G72 residui dello stelo accettore discriminanti
Library di tRNA con mutazioni random su 11 residui

Selezione per tRNA/tRNA sintetasi

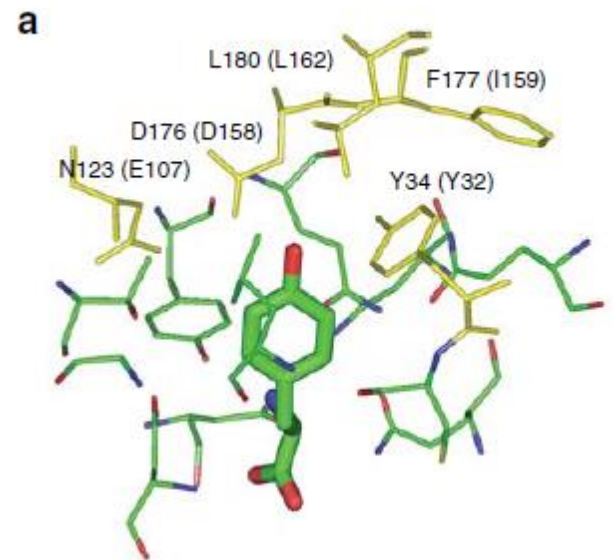
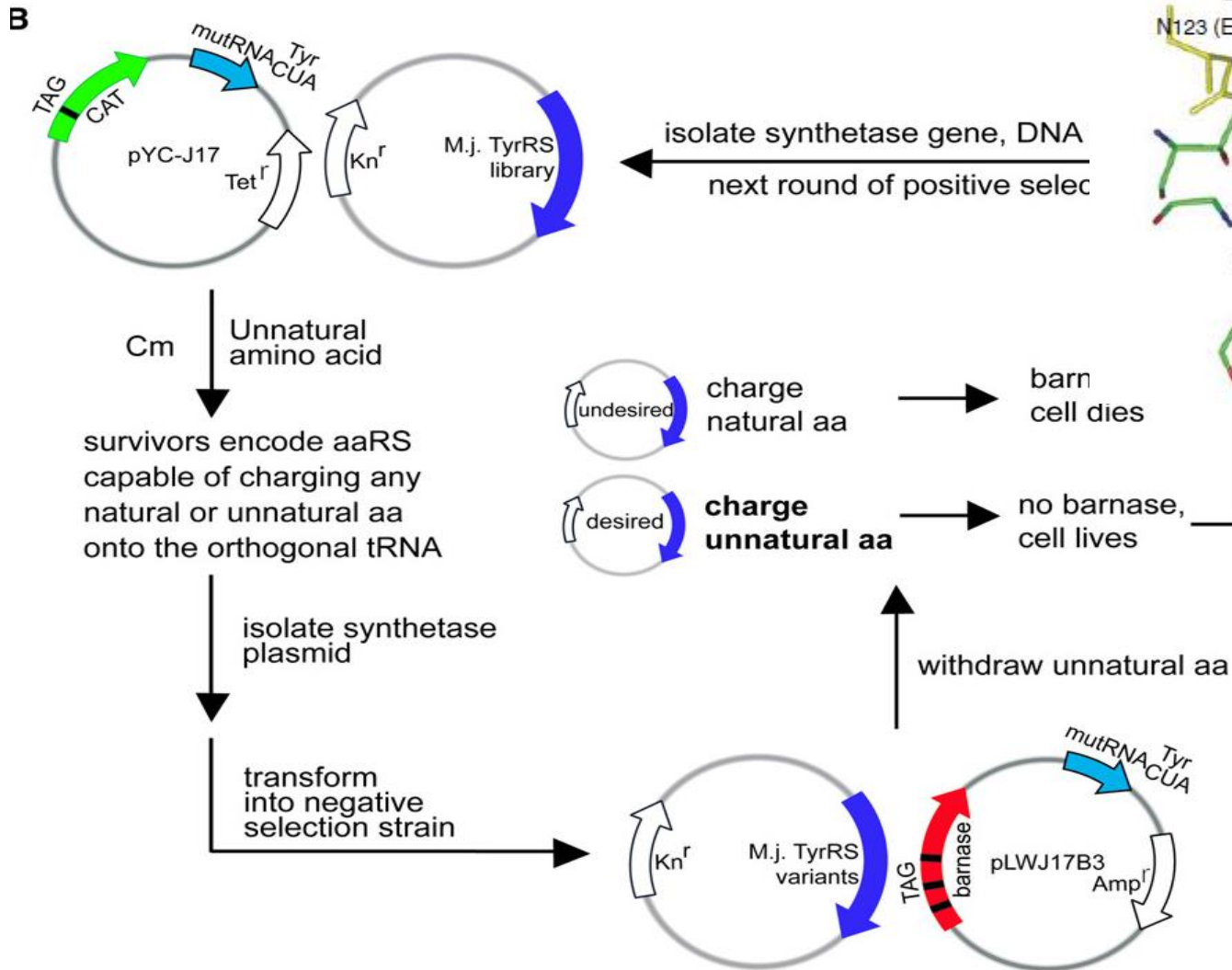


A mutant amber supressor tRNA library based on *M. jannaschii*
tRNA^{Tyr}: N = A + U + C + G



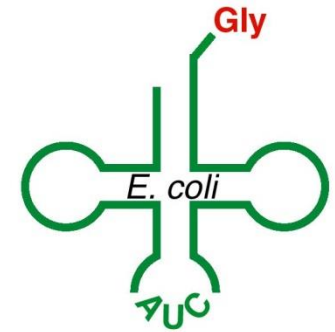
Selezione di sintetasi specifiche per amminoacidi modificati:

Costruzione di library di cDNA di tRNA sintetasi con mutazioni random su 5 residui della tasca di legame



Selezione dei tRNA Suppressor

- glycyyl-tRNA sintetasi non ha attività di editing



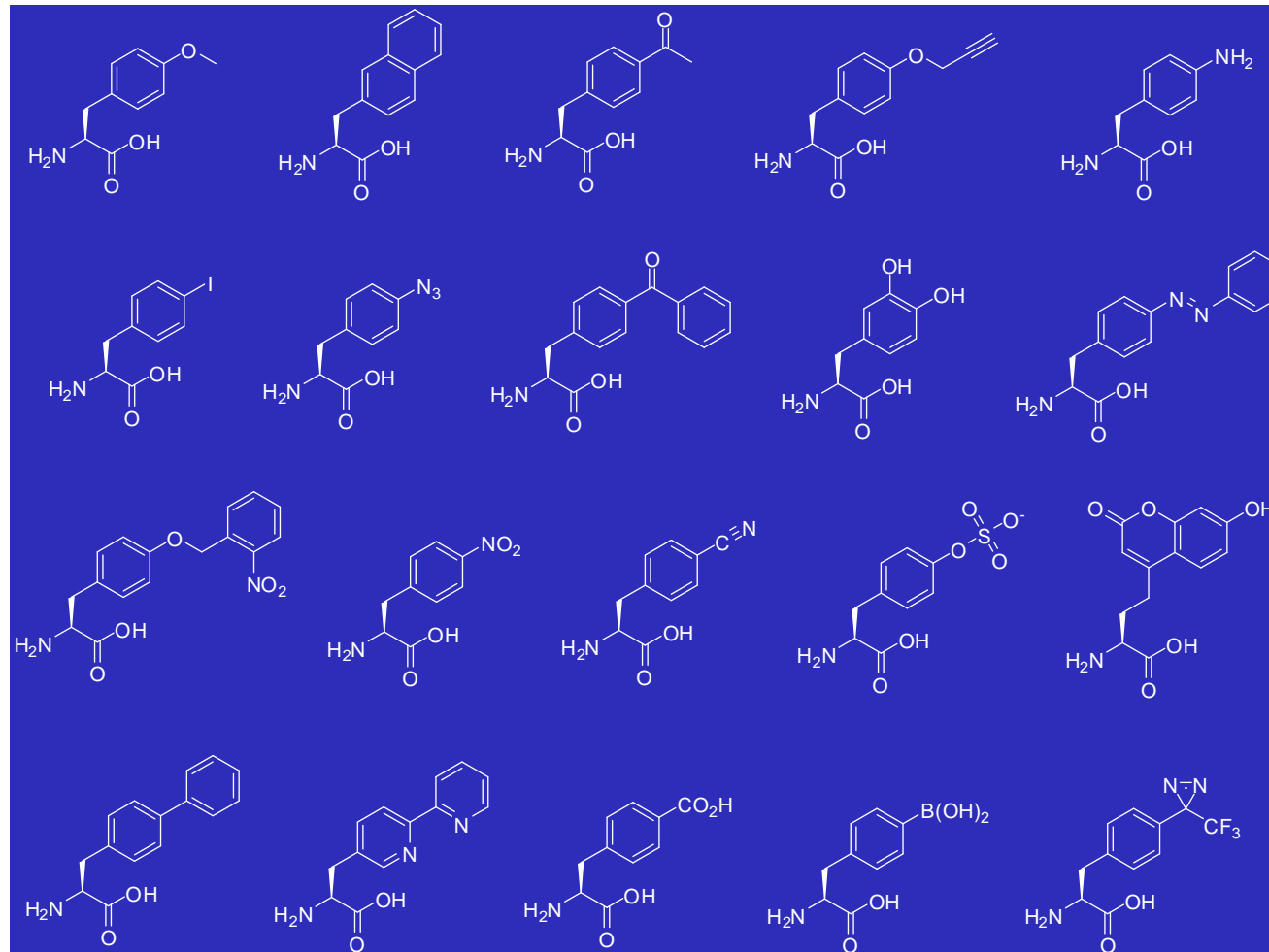
Fersht, A. R.; Dingwall, C. *Biochemistry* **1979**, *18*, 2627-2631.

- Mutagenesi nello stelo accettore per eliminare il riconoscimento con la *E. coli* Gly sintetasi

Bain, J. D.; Diala, E. S.; Glabe, C. G.; Wacker, D. A.; Lyttle, M. H.; Dix, T. A.; Chamberlin, A. R. *Biochemistry* **1991**, *30*, 5411-5421

Expanded Genetic Code in *E. coli*

Amminoacidi non naturali correlati strutturalmente alla tirosina



tRNA/ tRNA sintetasi in lievito

Il ${}_{\text{CUA}}\text{tRNA}^{\text{Tyr}}$ non si esprime con efficienza

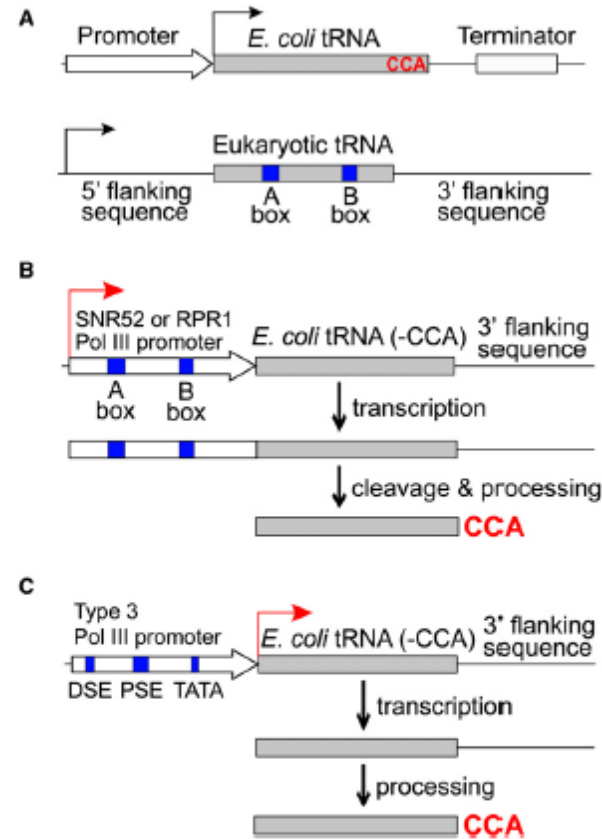


Figure 4. General Methods for Efficient Expression of Prokaryotic tRNAs in Eukaryotic Cells by Using Special Pol III Promoters
(A) Gene elements for tRNA transcription in *E. coli* and eukaryotic cells.
(B) Expression of prokaryotic tRNAs in yeast by using an external SNR52 or RPR1 Pol III promoter, which contains the consensus A- and B-box sequences and is posttranscriptionally cleaved from the primary transcript.
(C) A type-3 Pol III promoter, such as the H1 promoter, with a defined transcription initiation site is used to express prokaryotic tRNAs in mammalian cells.

Incorporazione del fotocrosslinker pbenzilfenilalanina (pBpa)

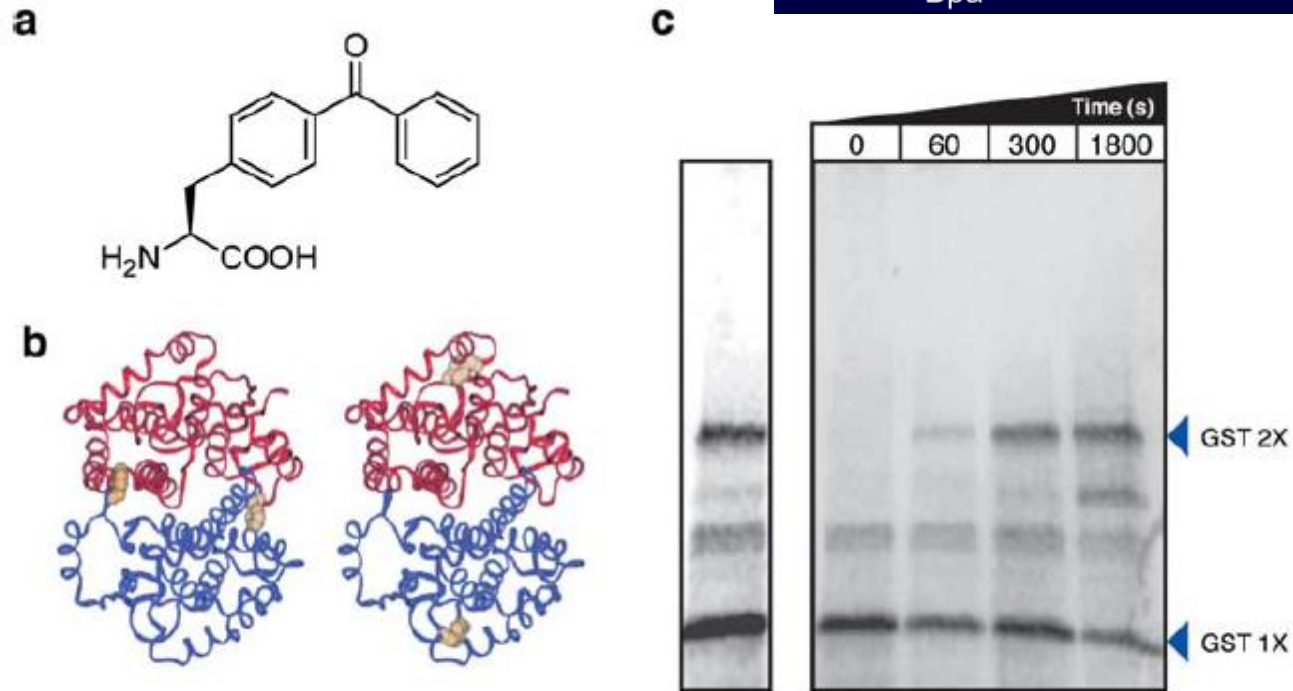
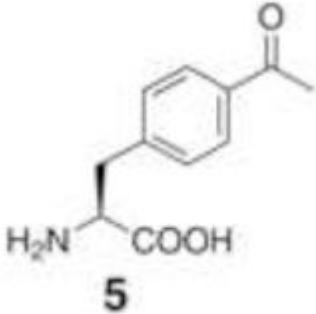


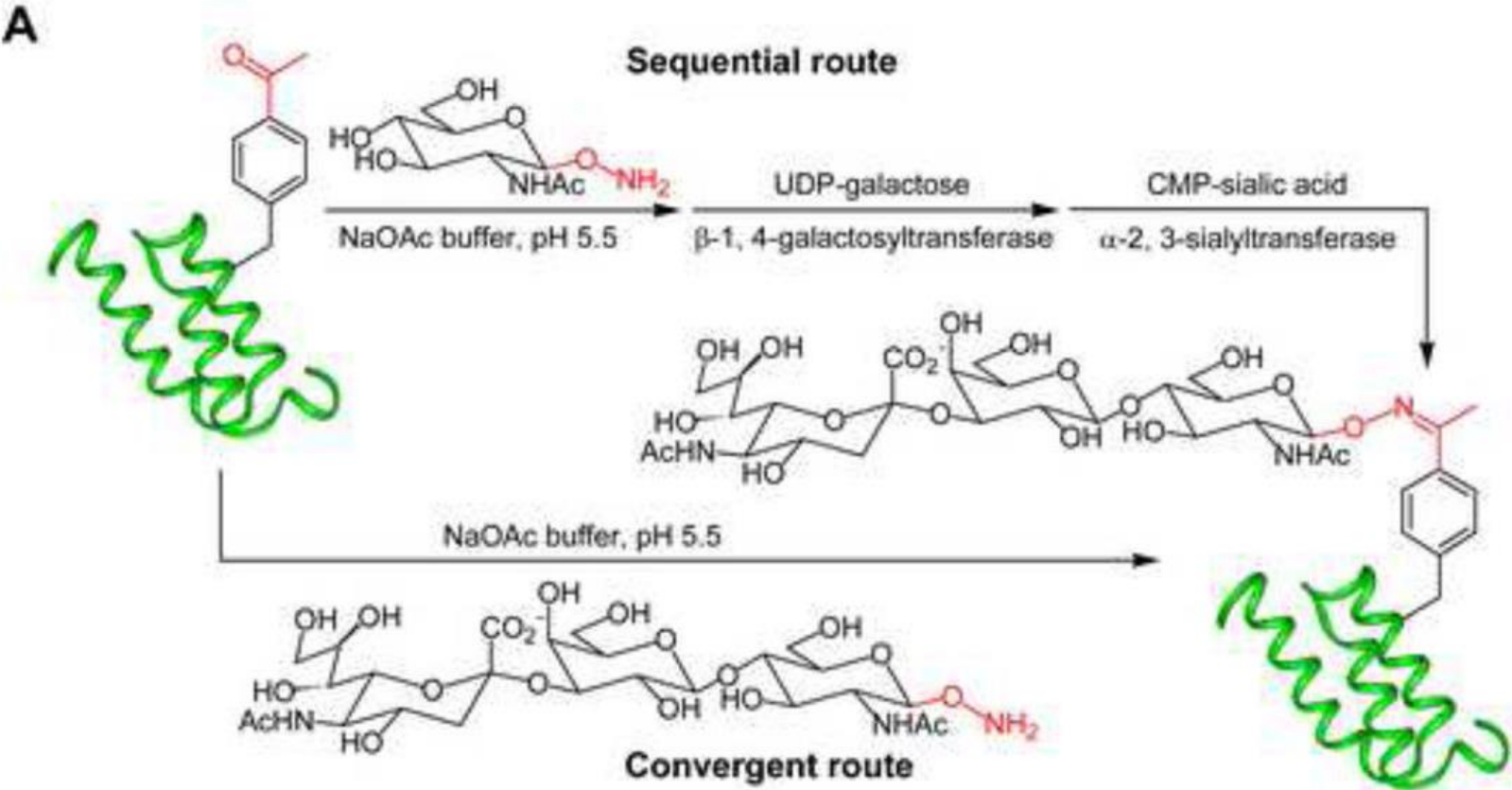
Figure 7

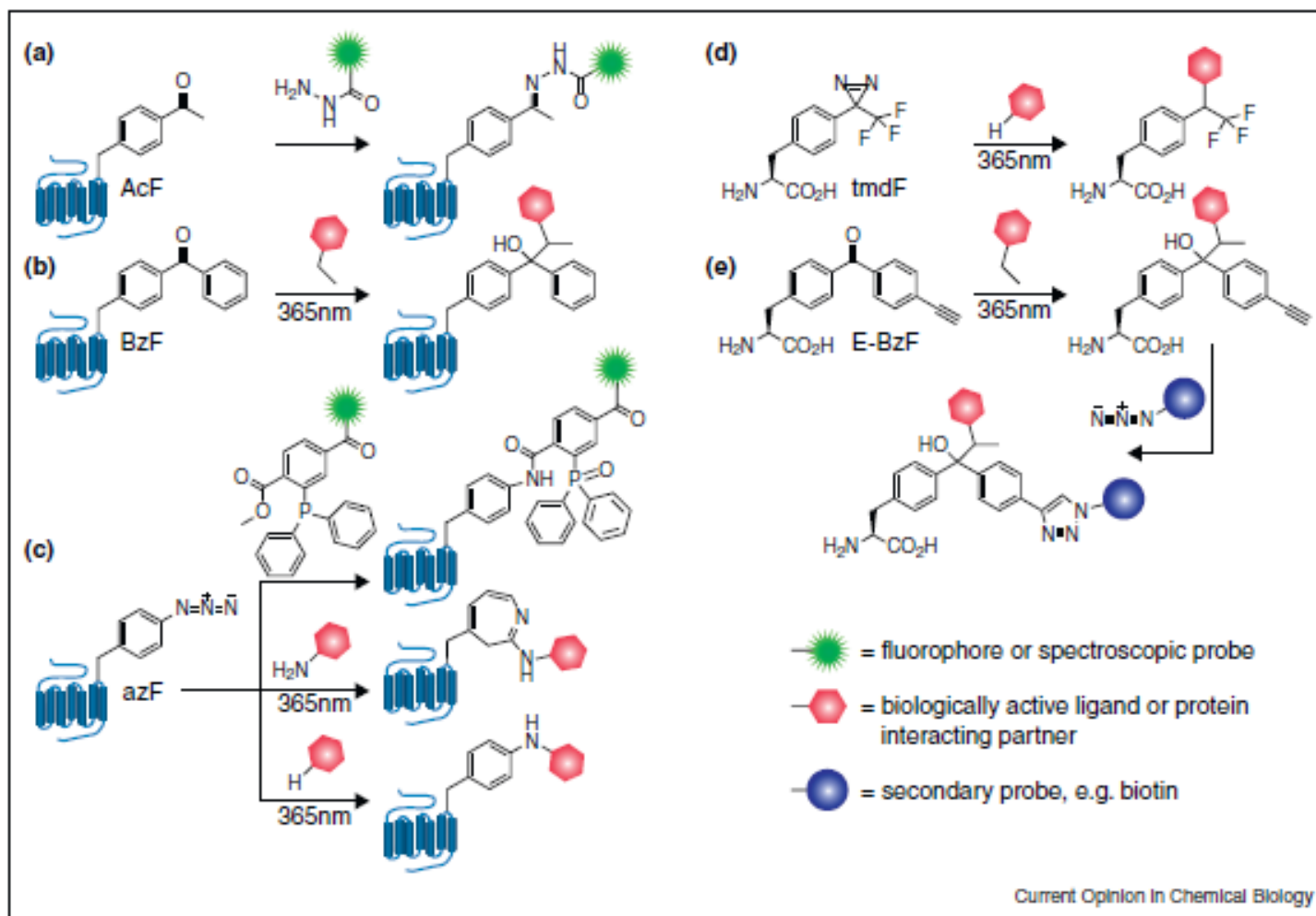
Site-specific incorporation of a photocrosslinker into proteins for mapping protein-protein interactions in vivo. (a) The chemical structure of *p*-benzoyl-L-phenylalanine (*p*Bpa). (b) Residue Phe-52 or Tyr-198 in *Schistosoma japonica* glutathione S-transferase (SjGST) was substituted by *p*Bpa. Monomers of the dimer are shown in blue and red. The side chain of Phe-52 is shown in orange for each monomer (*left*). The side chain of residue Tyr-198 is shown in orange (*right*). (c) The covalent dimerization of SjGST (Phe52*p*Bpa) in vivo upon irradiation at 365 nm.

Produzione di proteine glicosilate in E coli



p-acetilfenilalanina





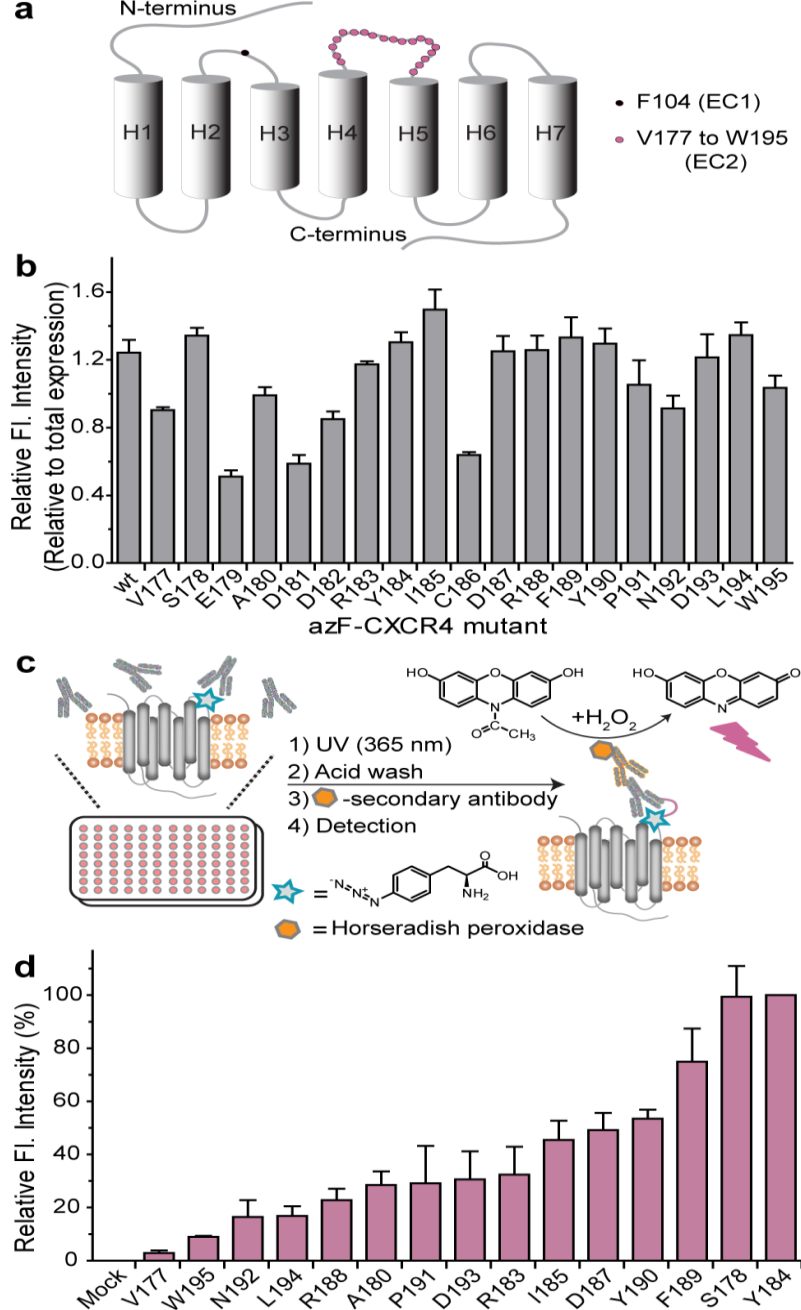
Representative UAAs used in GPCR structure–function studies. UAAs have been incorporated into expressed GPCRs (a–d) or synthetically incorporated into peptides to probe GPCR function (e). (a) *p*-Acetyl-L-phenylalanine (AcF) can react with a hydrazide or hydroxylamine derivative. (b) *p*-Benzoyl-L-phenylalanine (BzF) serves as a useful photoactivatable crosslinker when exposed to UV light. (c) *p*-Azido-L-phenylalanine (azF) can undergo a Staudinger–Bertozzi ligation reaction with triaryl phosphine derivatives or can react with primary amines or aliphatic hydrogens when photoactivated with UV light. Additionally azF has been used as an IR-probe. (d) *p*-Trifluoromethyl-diazirine-L-phenylalanine (tmdF), a smaller photo-labile amino acid. (e) *p*-Ethynylbenzoyl-L-phenylalanine (E-BzF), the clickable group allows for a crosslinked product to be labeled with an azido-containing probe.

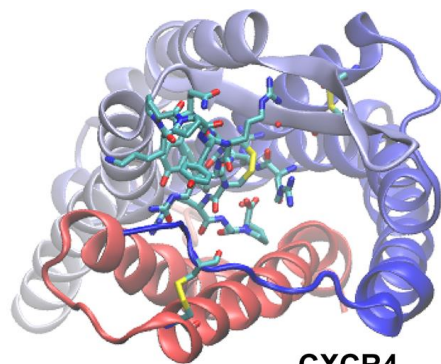
Antibody Epitopes on G Protein-Coupled Receptors Mapped with Genetically Encoded Photoactivatable Cross-Linkers

Sarmistha Ray-Saha, Thomas Huber, and Thomas P. Sakmar*

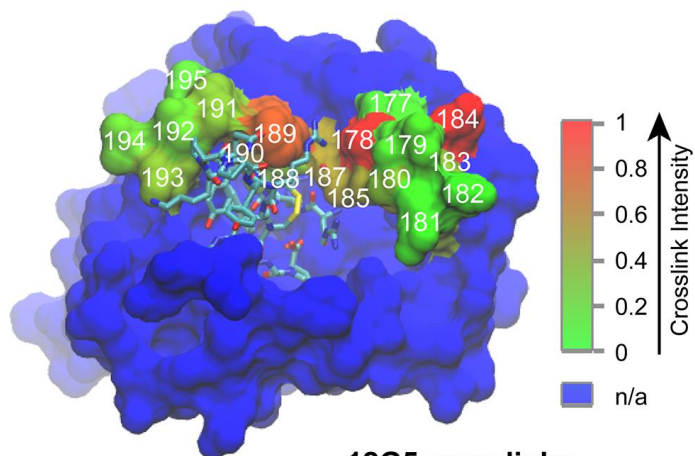
Laboratory of Chemical Biology and Signal Transduction, The Rockefeller University, 1230 York Avenue, New York, New York 10065, United States

Supporting Information



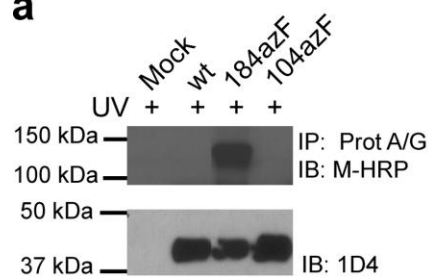


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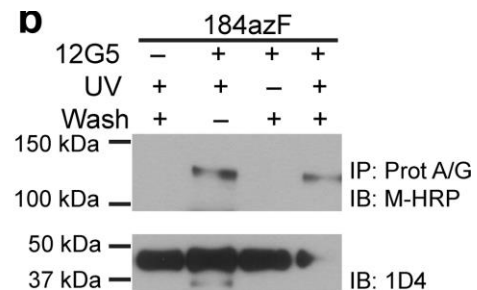


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In Vivo Expression of a Light-Activatable Potassium Channel Using Unnatural Amino Acids

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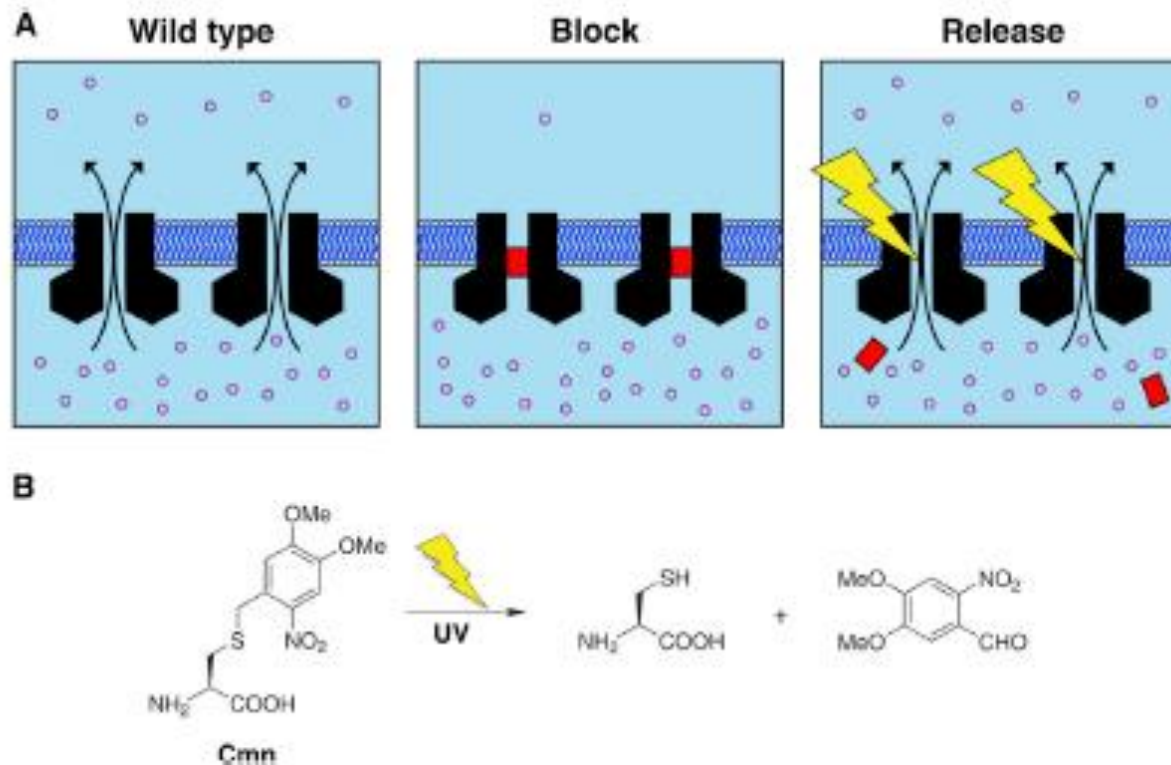
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Cmn 4,5-dimethoxy-2-nitrobenzyl-cysteine

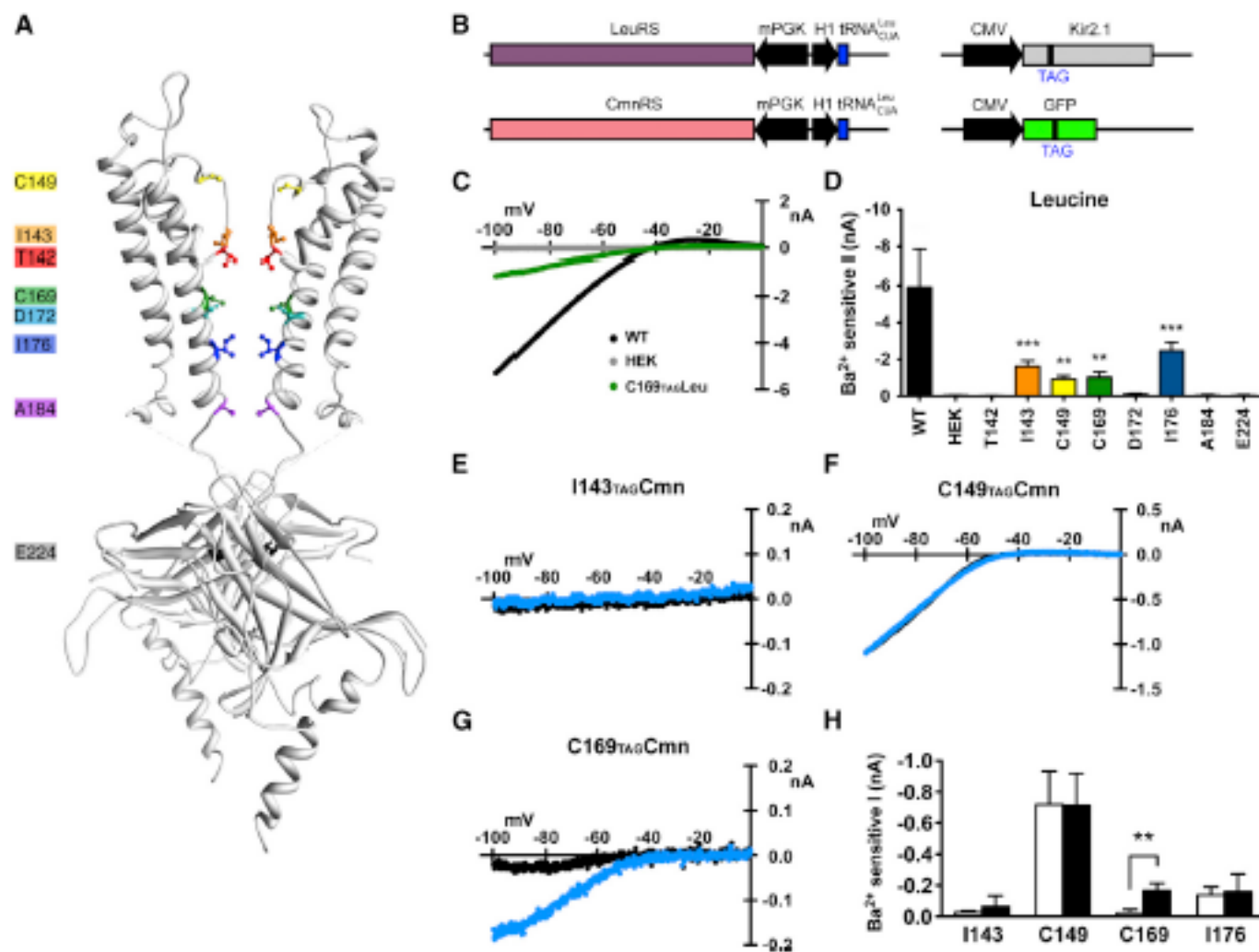
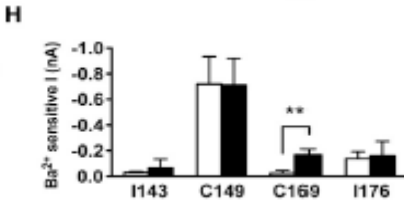
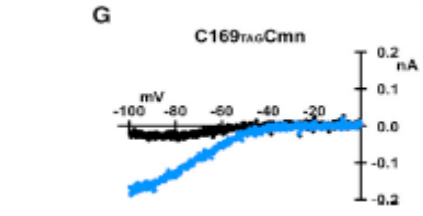
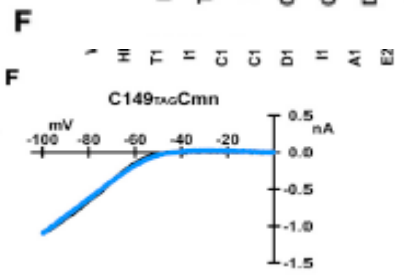
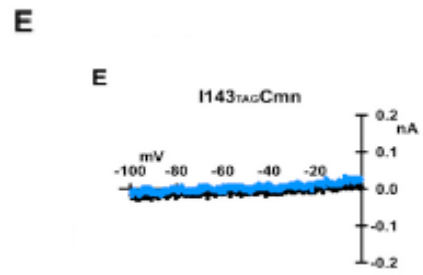
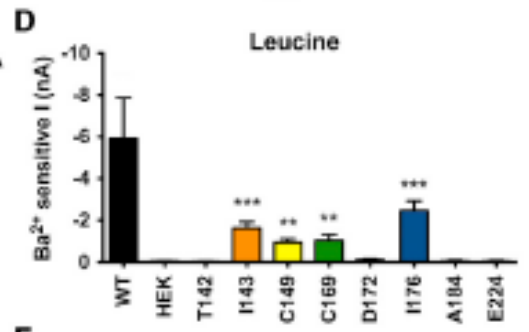
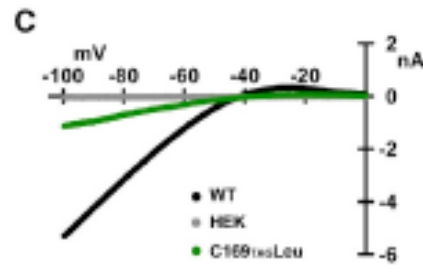
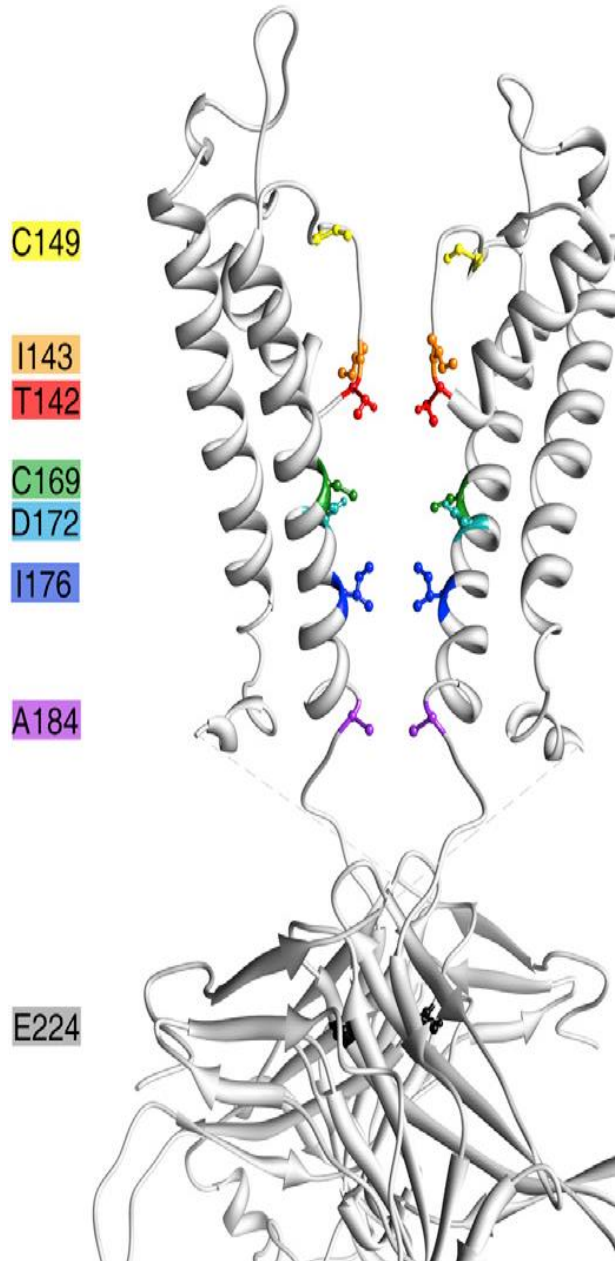


Figure 2. Identification of a Critical Site in Kir2.1 for Cmn Incorporation that Enables Photoactivation



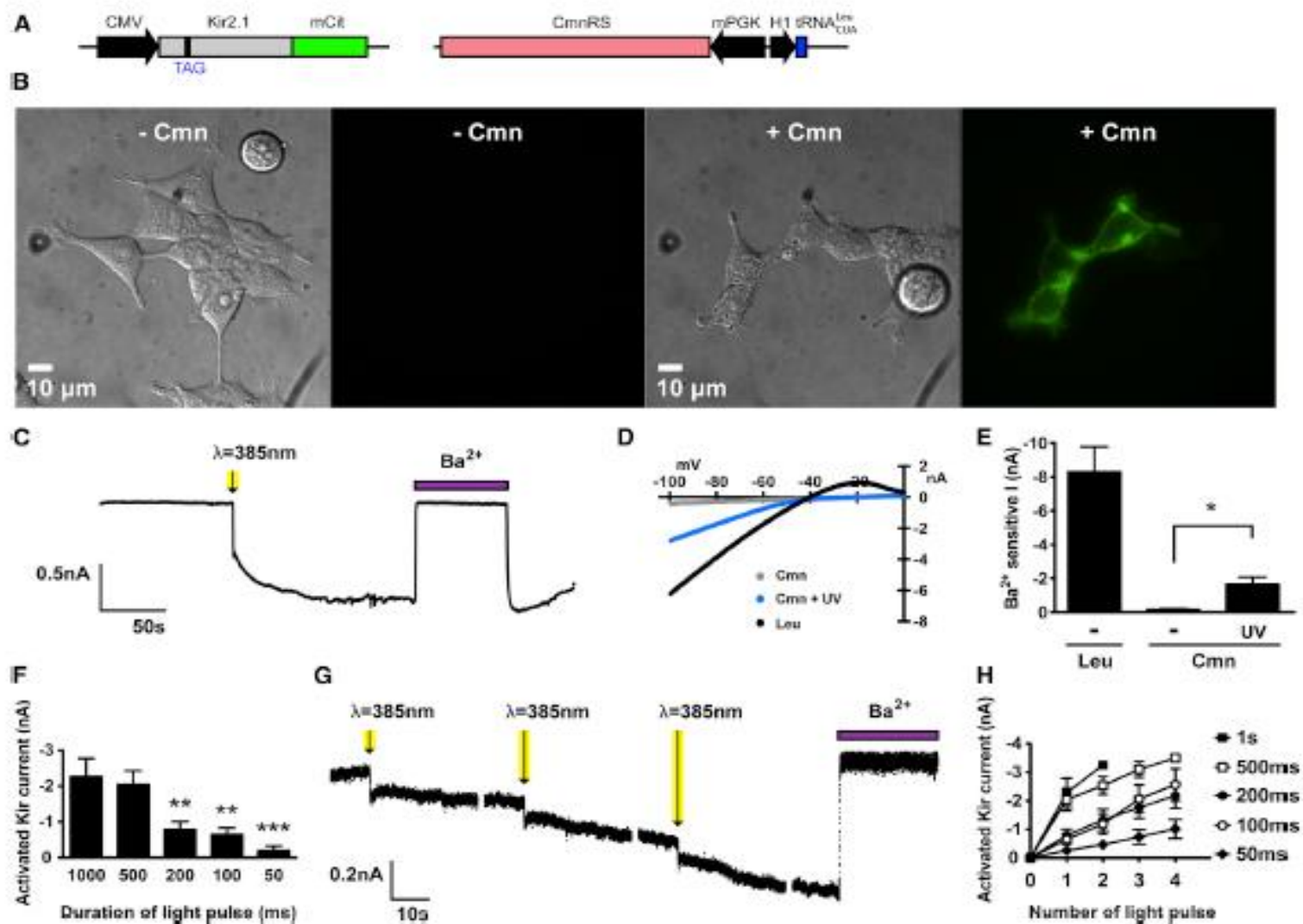
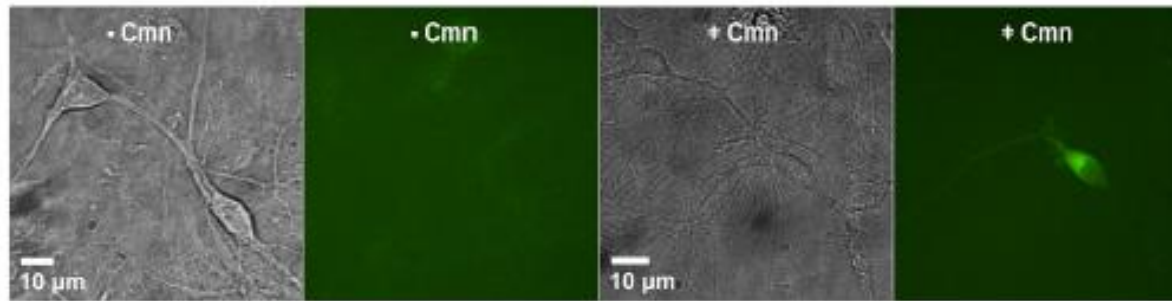


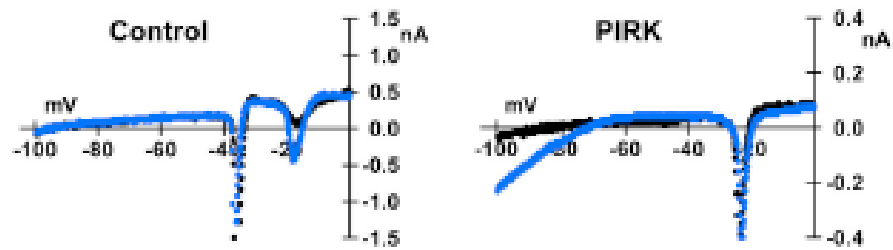
Figure 3. Light-Dependent Activation of PIRK (Kir2.1_C169_{TAG}Cmn) Expressed in HEK293T Cells

Rat hippocampal primary neurons

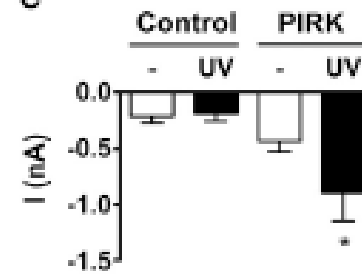
A



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C



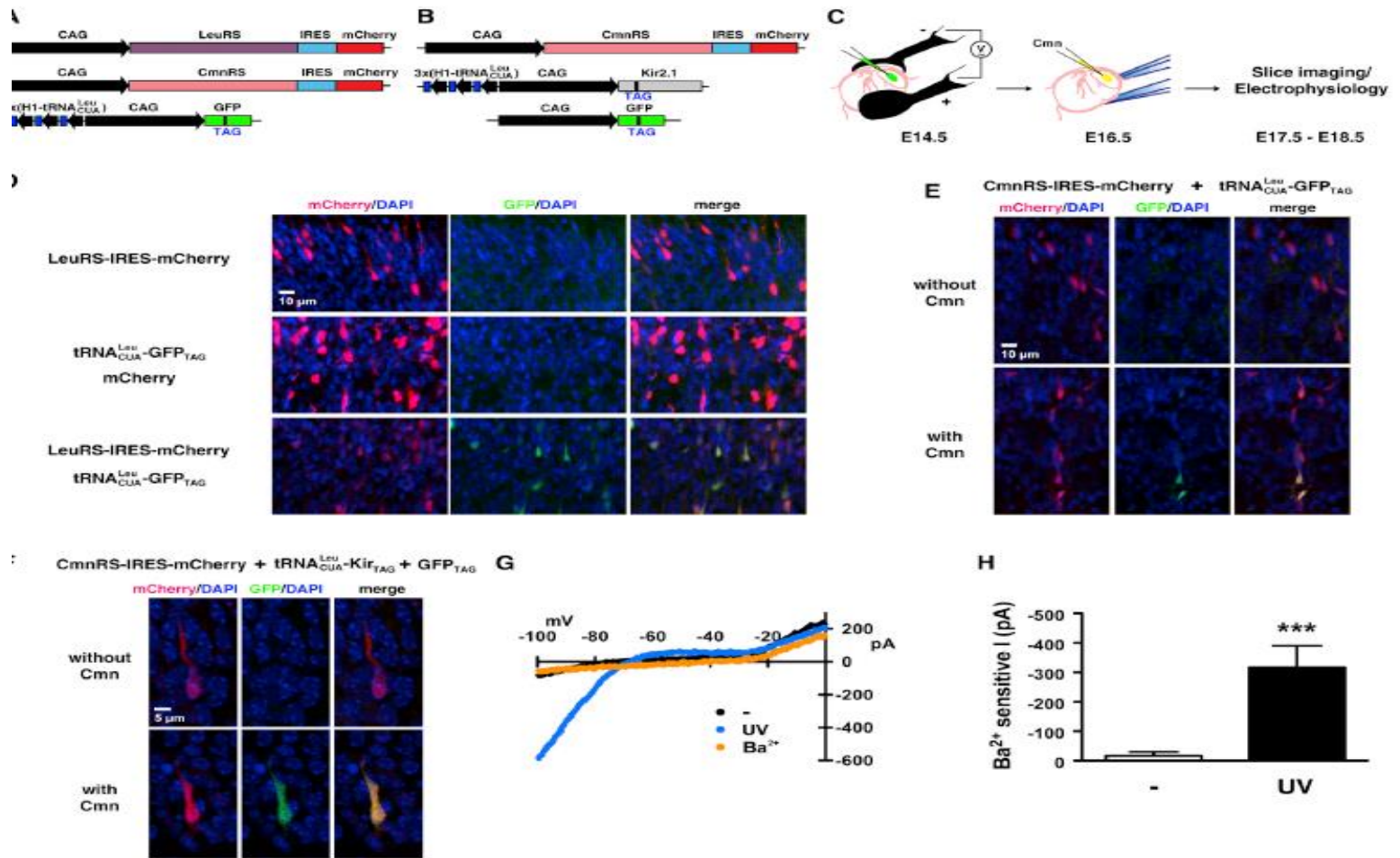


Figure 6. In Vivo Expression of PIRK Channels in the Mouse Neocortex

1) Validation plasmid set: one plasmid for *LeuRS* or for *CmnRS*, under the control of CAG promoter and coexpressed with *mCherry* via IRES sequence; one plasmid encoding *GFP_Y182TAG* under the control of the CAG promoter. Three copies of *tRNA^{Leu}_{CUA}* driven by the H1 promoter were combined with the *FP_Y182TAG* to increase incorporation efficiency. Green fluorescence indicates suppression of amber codon by Leu or Cmn. Red fluorescence indicates successful gene delivery of synthetase in vivo.

2) PIRK expression plasmid set: one plasmid for *CmnRS*, under the control of CAG promoter and coexpressed with *mCherry* via IRES sequence; one plasmid for *ir2.1_C169TAG* coupled with three copies of *tRNA^{Leu}_{CUA}*; one plasmid for *GFP_Y182TAG*. Green and red fluorescence indicates successful expression of all three plasmids and Cmn incorporation.

3) Cartoon shows experimental procedure for PIRK expression in vivo. Gene constructs in (B) were injected into the mouse neocortex (E14.5) and electroporated in utero. Two days later, Cmn was injected to the brain. Slice imaging and electrophysiological assay were performed on E17.5–E18.5.

4) Fluorescence images of mice embryonic cortical plates showing the successful incorporation of Leu into *GFP_{TAG}* in vivo. Top: CAG-*LeuRS*-IRES-*mCherry*