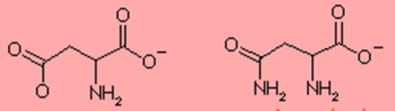
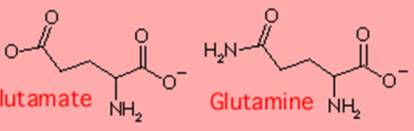


Incorporazione in proteine di
aminoacidi non naturali

Acidic and amide side chains

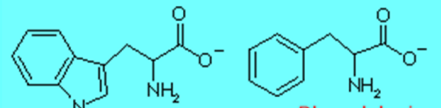


Aspartate Asparagine



Glutamate Glutamine

Aromatic side chains

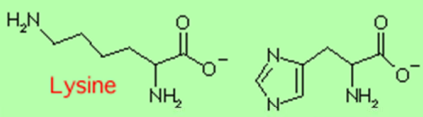


Tryptophan Phenylalanine

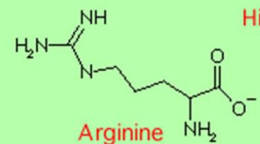


Tyrosine

Basic side chains

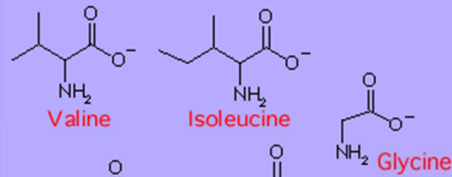


Lysine Histidine

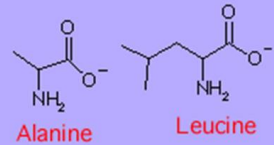


Arginine

Aliphatic side chains

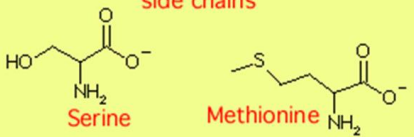


Valine Isoleucine Glycine

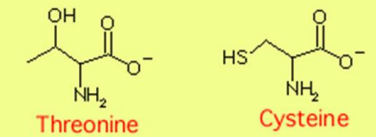


Alanine Leucine

Hydroxyl or sulfur-containing side chains

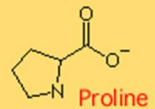


Serine Methionine



Threonine Cysteine

Cyclic side chain



Proline

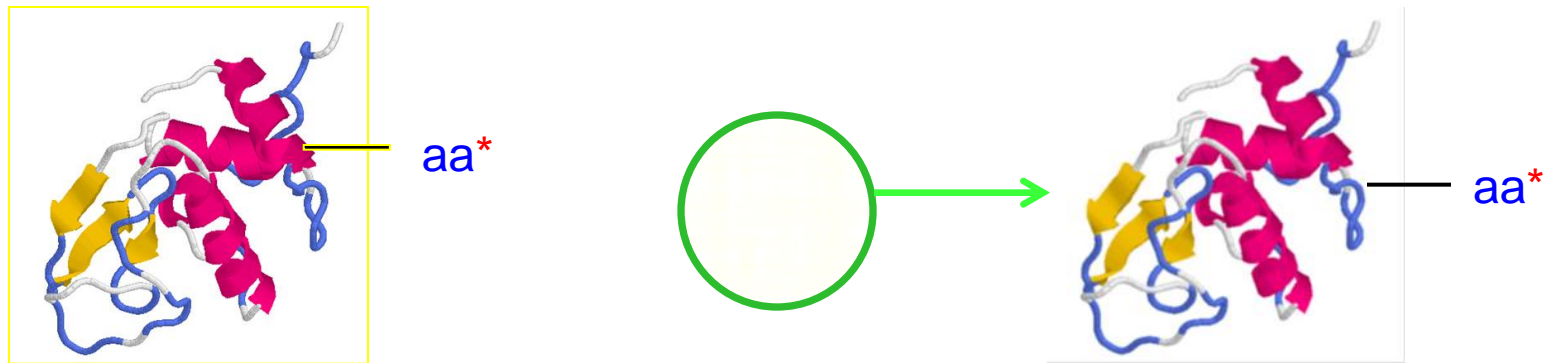
La catena laterale dei 20 amminoacidi naturali manca di gruppi elettrofili non permette reazioni chimiche precise

Incorporazione di aminoacidi non naturali

- Sonde fluorescenti
- modifiche post traduzionali
- marcatura per fotoaffinità
- biotina
- chelanti per metalli

Metodi per incorporare aminoacidi non naturali

Selettiva modifica
chimica di un aminoacido



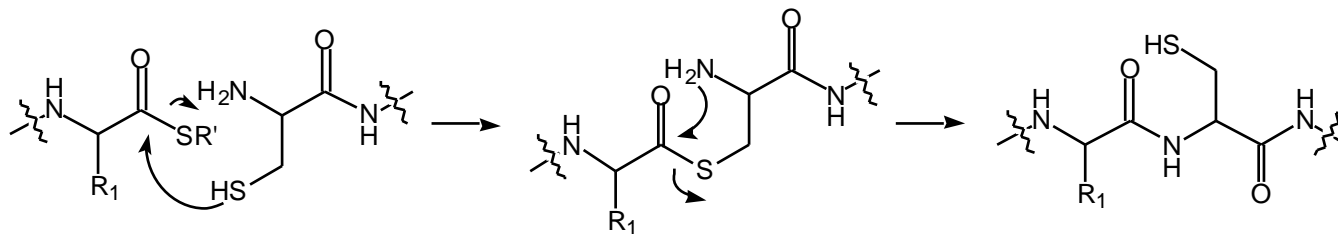
Come si può modificare
selettivamente un
amminoacido?

Methods for Incorporation of Unnatural Amino Acids into Proteins

- Sintesi chimica

limitata a 30-50 residui

ligazione del frammento



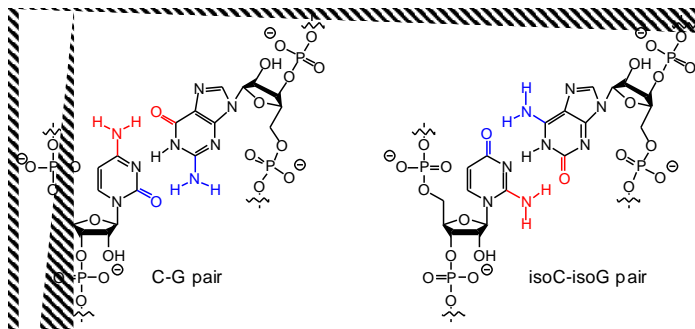
Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1997**, 266, 776-779.

- Modifica Post-traduzionale tramite reazioni chimiche o enzimatiche

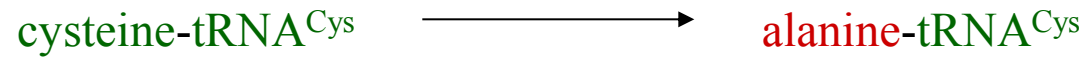
Metodi per Incorporare Aminoacidi modificati in Proteine

- *In vivo* – crescita in un mezzo che contenga l'aminoacido modificato
- *In vitro* – modifica di tRNA già acilati lisina/cisteina
- alterazione dell'anticodone
4 basi o 5 basi

- inserimento di nucleotidi modificati



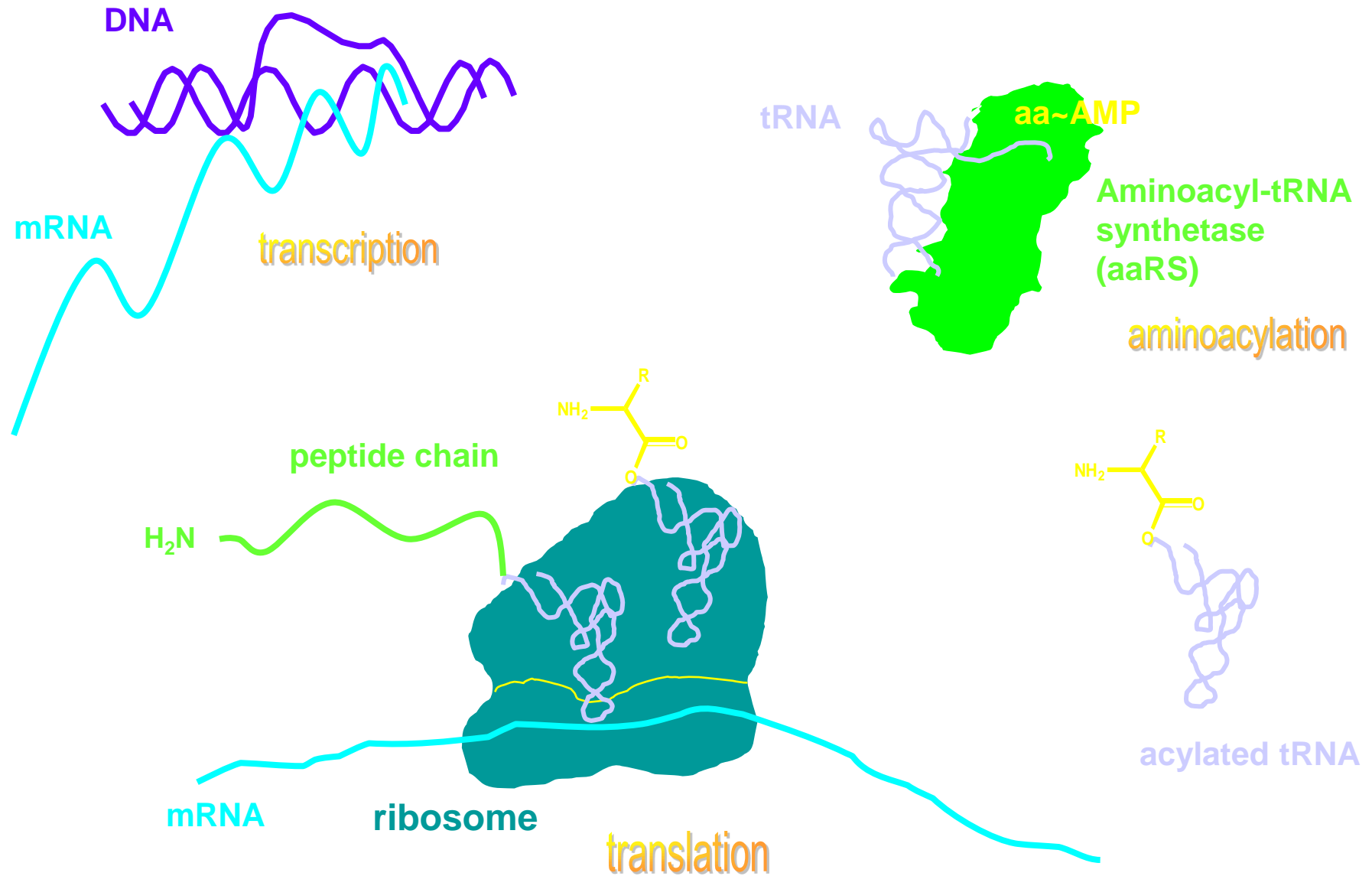
“Misacylation” del tRNAs



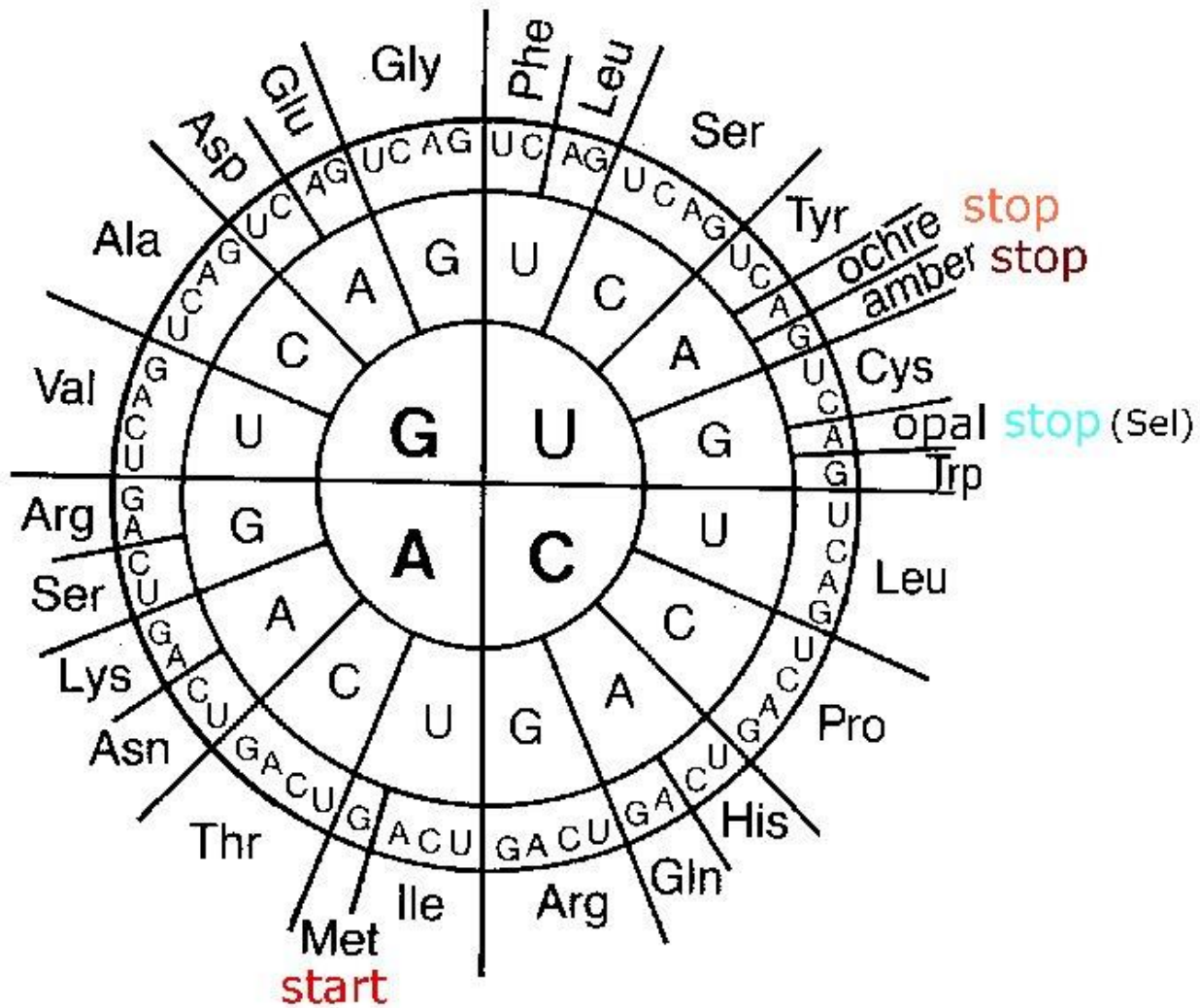
Chapeville, F.; Lipmann, F.; von Ehrenstein, G.; Weisblum, B.; Ray, W. J.; Benzer, S. *Proc. Natl. Acad. Sci.* **1962**, *48*, 1086-1092.
von Ehrenstein, G.; Weisblum, B.; Benzer, S. *Proc. Natl. Acad. Sci.* **1963**, *49*, 669-675.

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

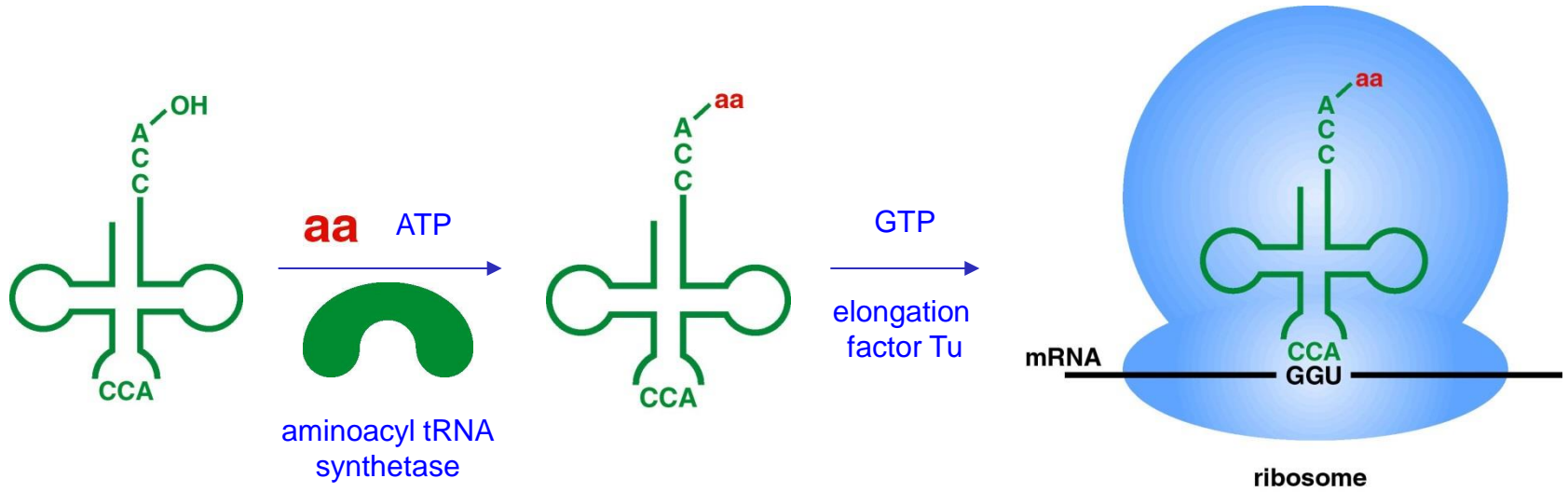
Sintesi proteica



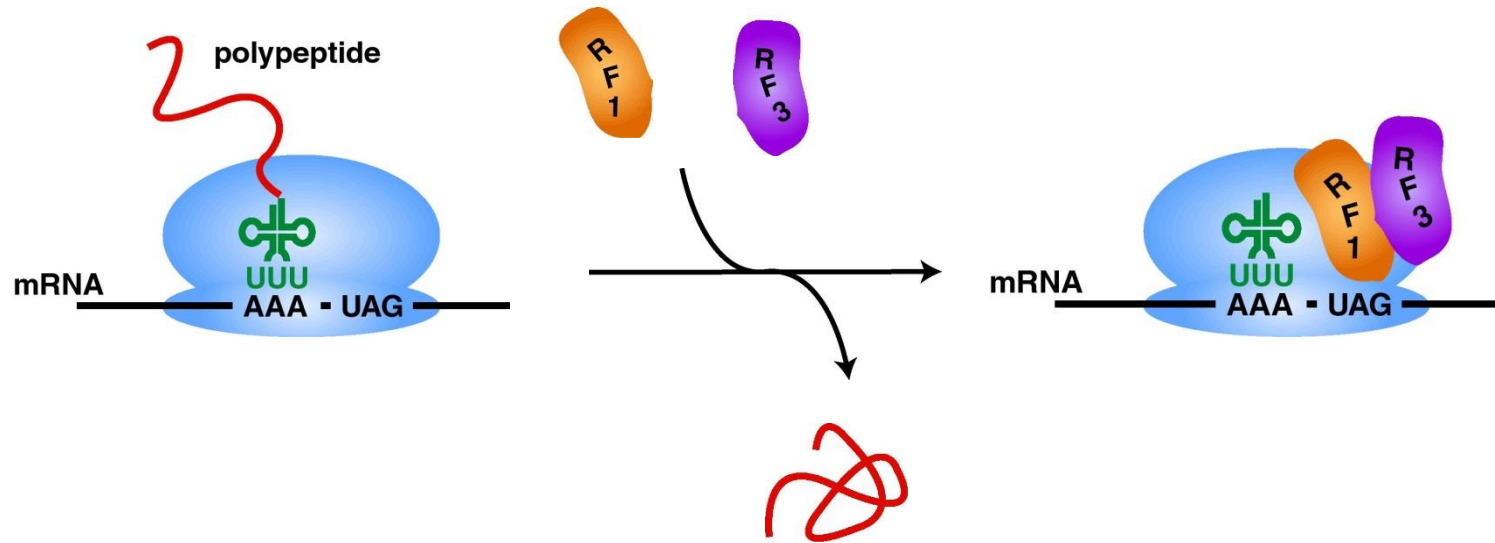
“Codice genetico”



Traduzione

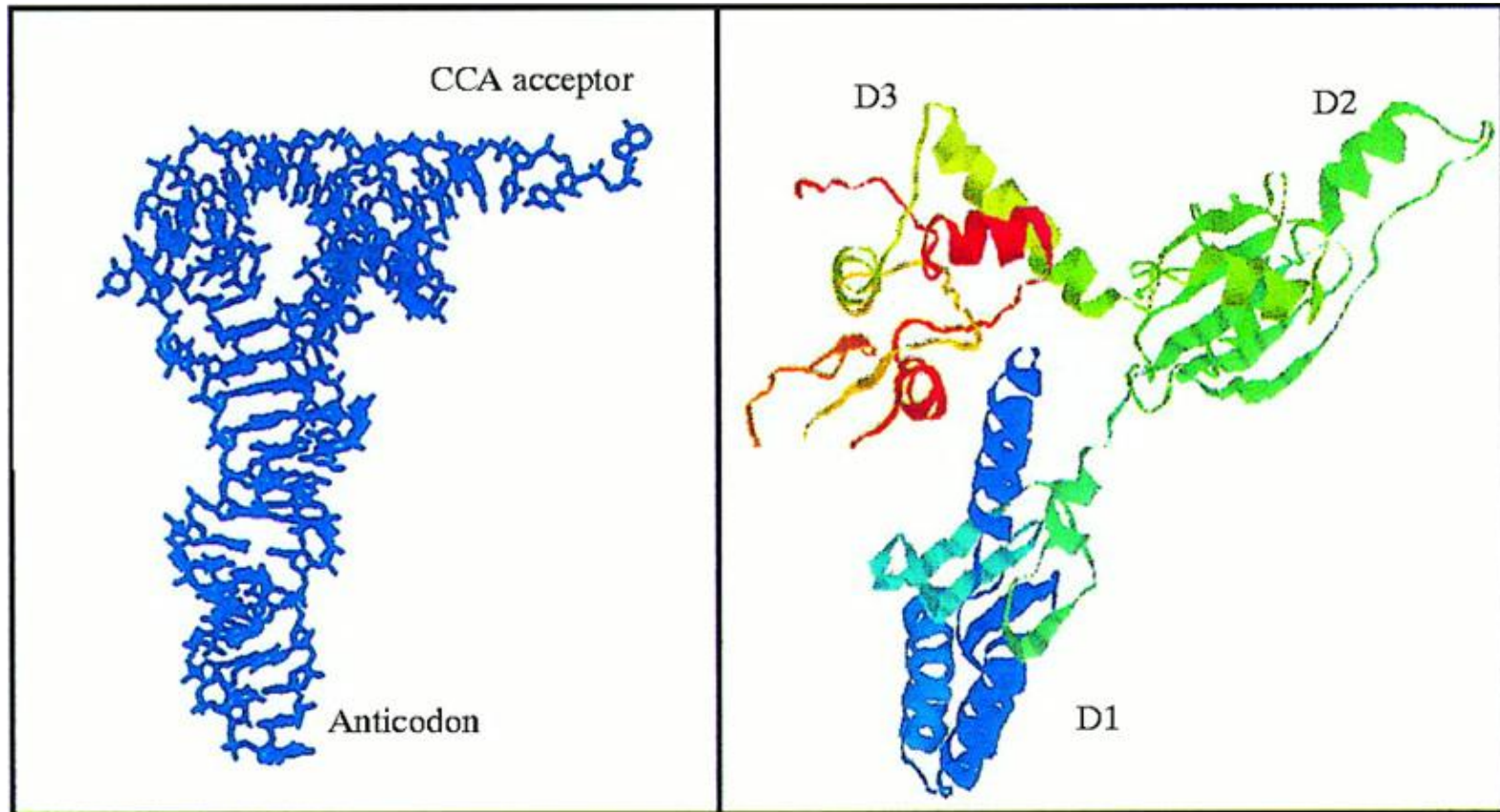


Terminazione

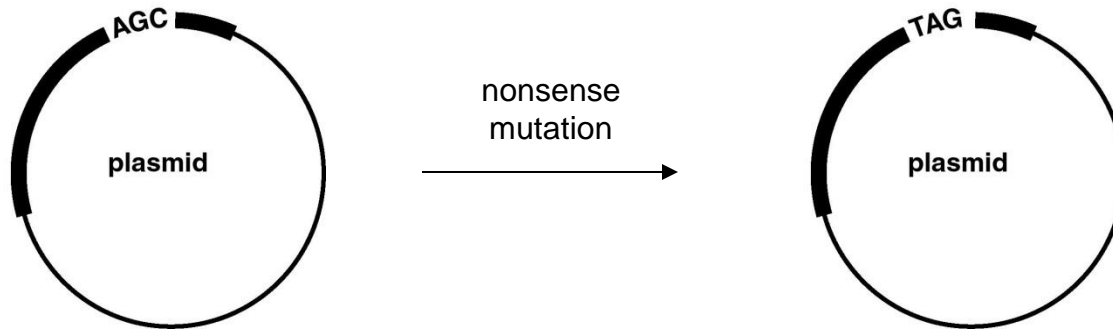


Amber (UAG), Opal(UGA), Ochre(UAA)

Yeast tRNA^{Phe} e RF1 (Release Factor) umano



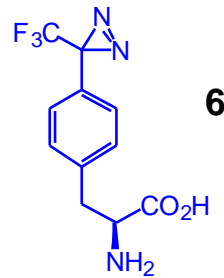
Nonsense Mutation



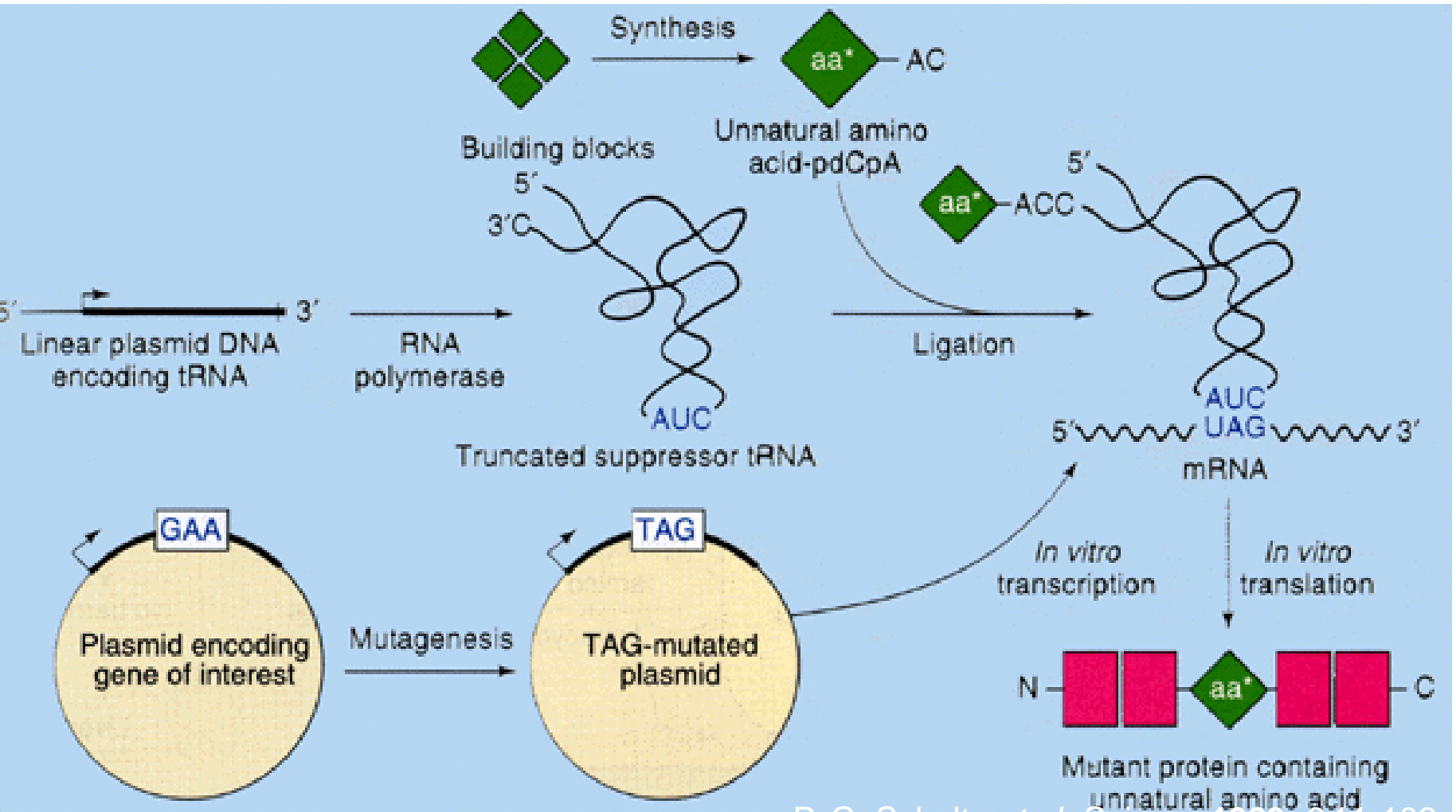
- proteina troncata e non funzionale

Prima evidenza della possibilità di usare Soppressori Nonsense per inserire in una catena polipeptidica aminoacidi modificati

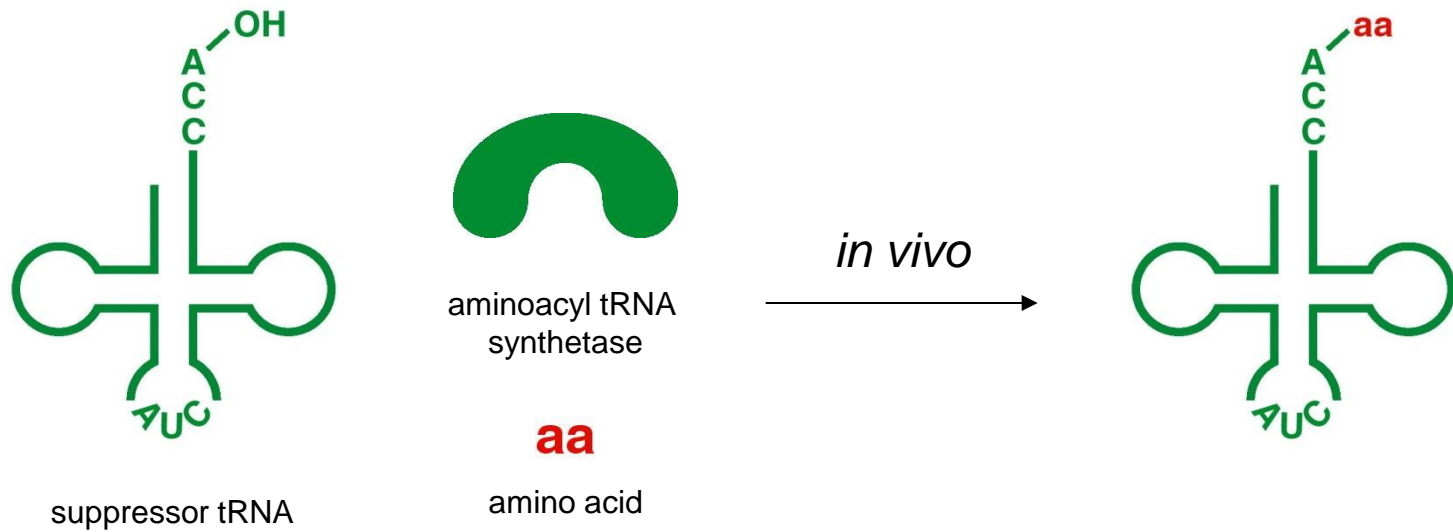
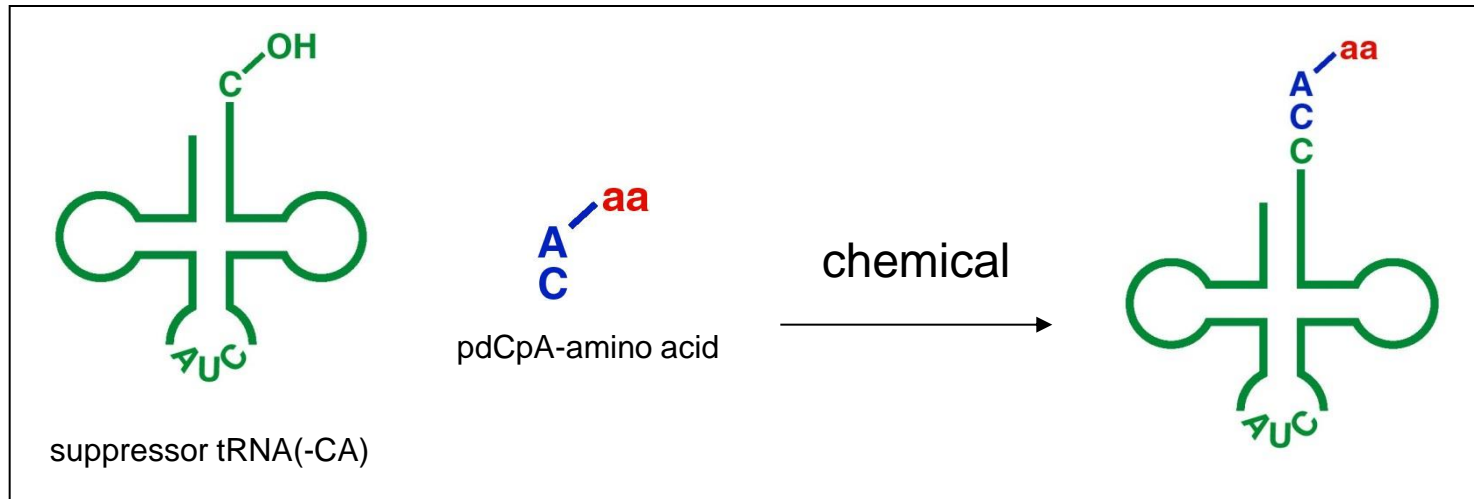
Introduzione della diazirina in un sito che portava la mutazione amber
Mediante modifica chimica del suppressor tRNA acilato



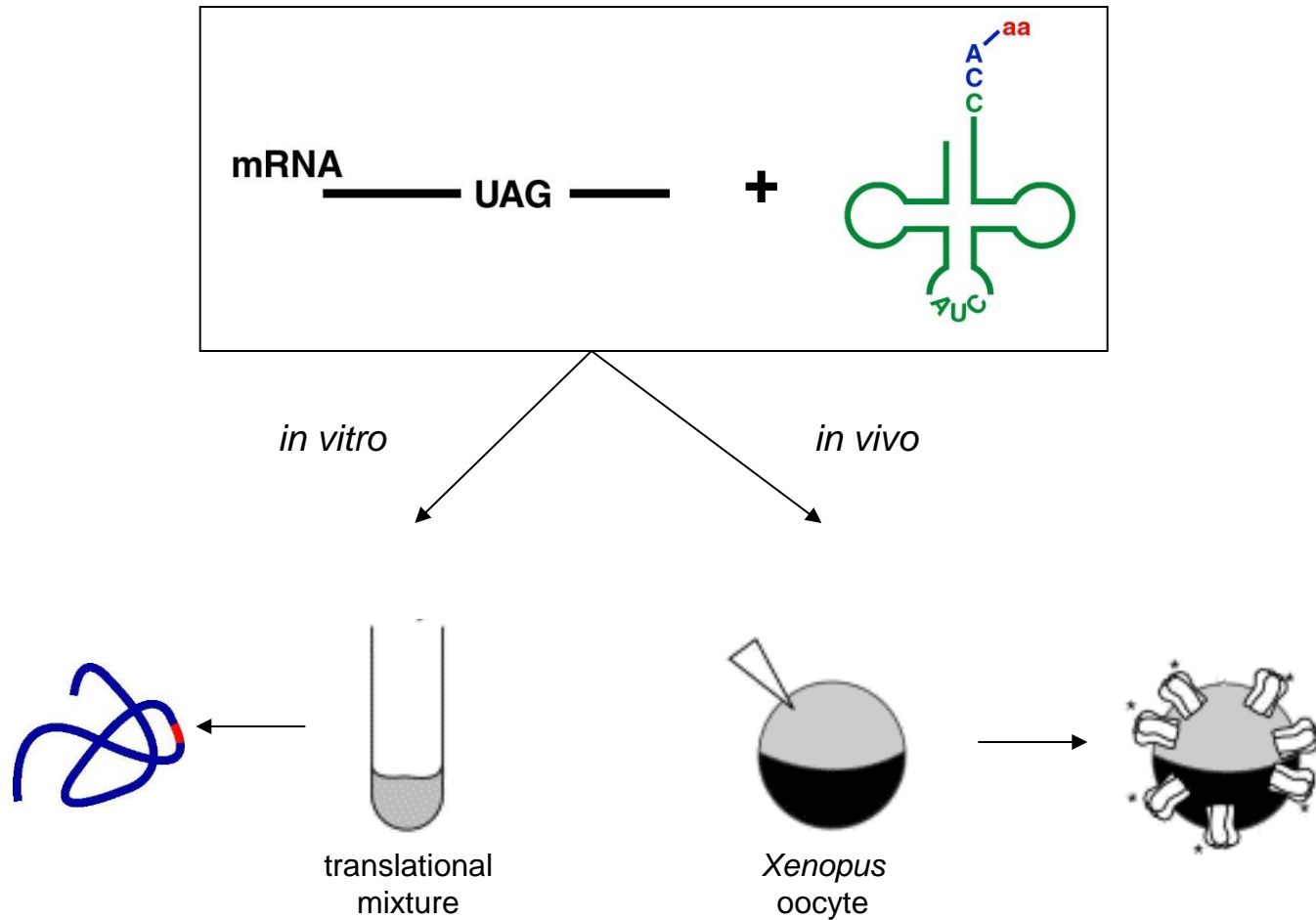
In vitro



Misacylation of Suppressor tRNAs



In vitro and *in vivo* Systems to Produce Protein

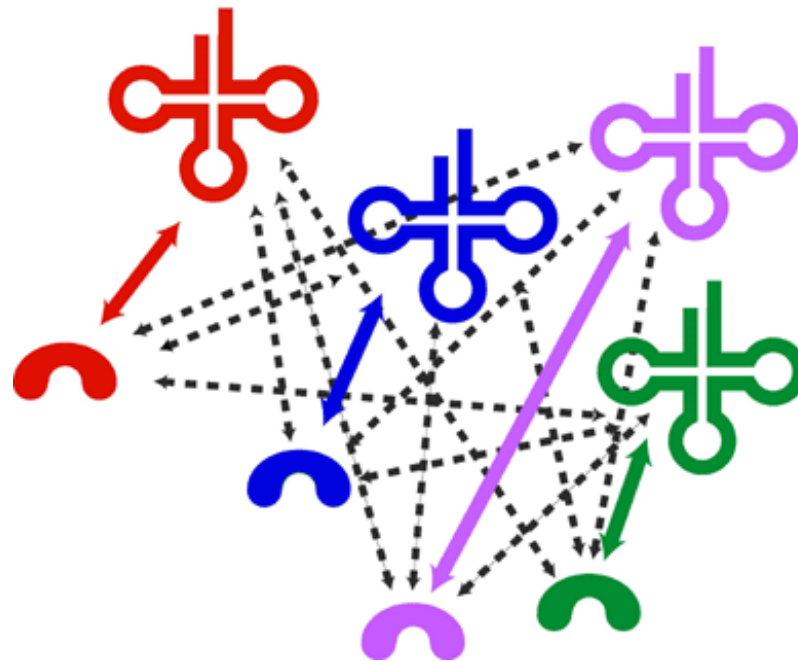


Thorson, J. S.; Cornish, V. W.; Barrett, J. E.; Cload, S. T.; Yano, T.; Schultz, P. G. *Methods Mol. Biol.* **1998**, 77, 43-73.
Dougherty, D. A. *Curr. Opin. Chem. Biol.* **2000**, 4, 645-652.

Incorporazione di aminoacidi nonnaturali in vivo

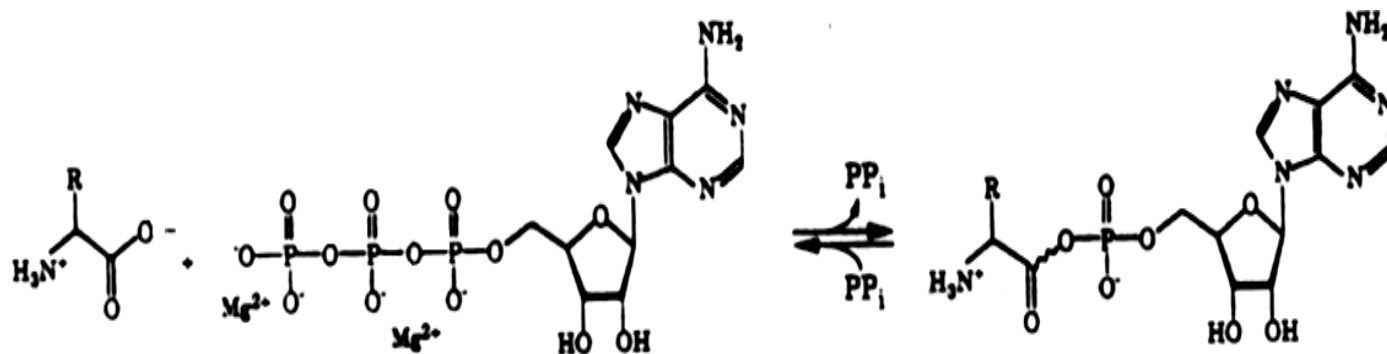
In Vivo Misacylation

- 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**



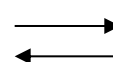
amino acid

ATP

aminoacyl adenylate

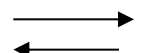
Amminoacil AMP
(amminoaciladenilato)

+ tRNA



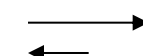
AMP + amminoacil-tRNA

ATP+amminoacido+tRNA



Amminoacil-tRNA+ AMP+ PPi

PPi



2Pi

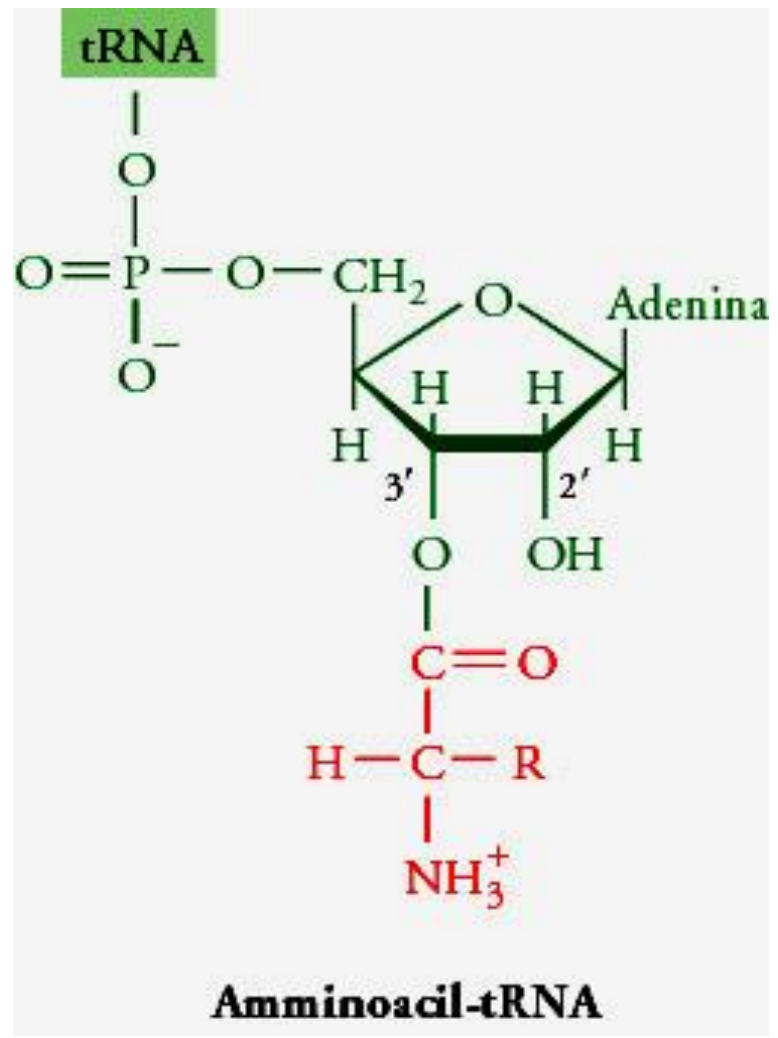
Esistono due classi tRNA sintetasi che si differenziano per:

•Riconoscimento dell'anticodone

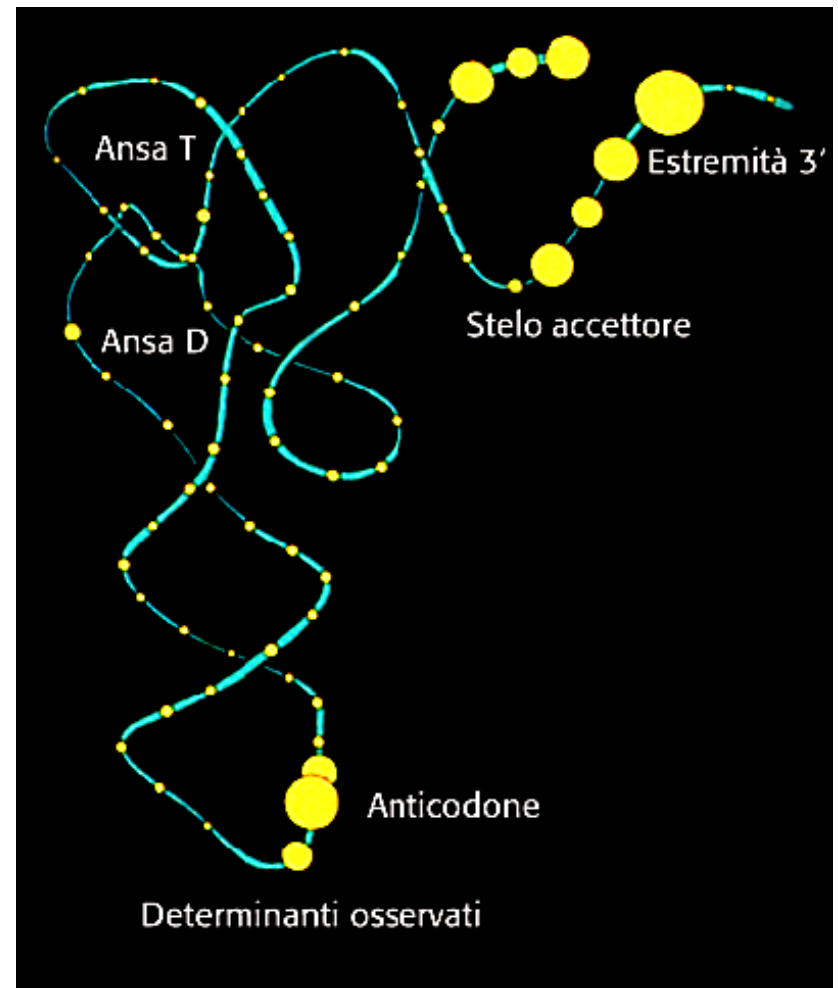
Enzimi di classe II non interagiscono con l'anticodone

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

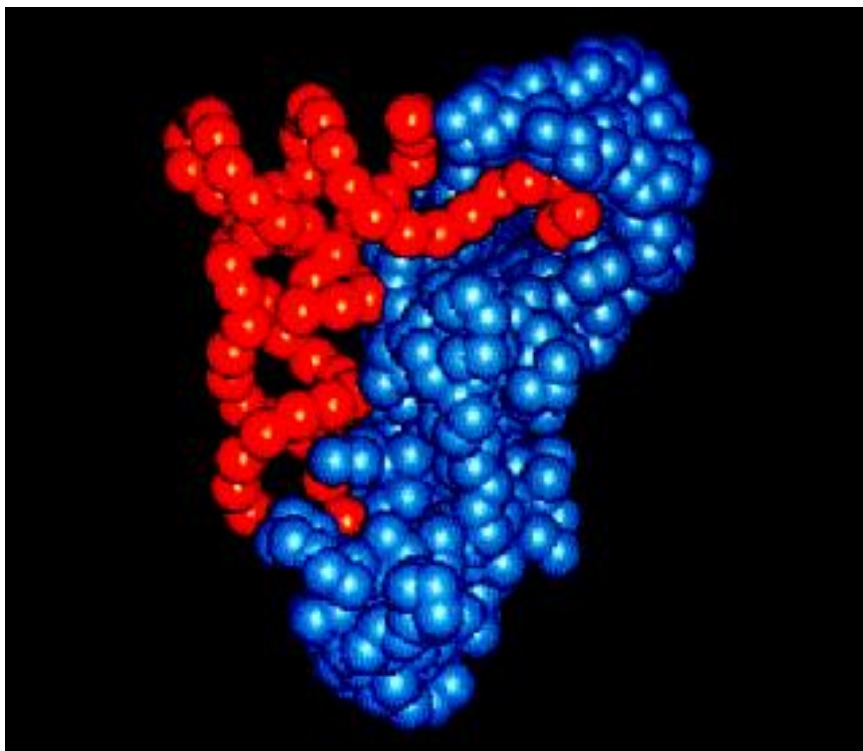
•Struttura



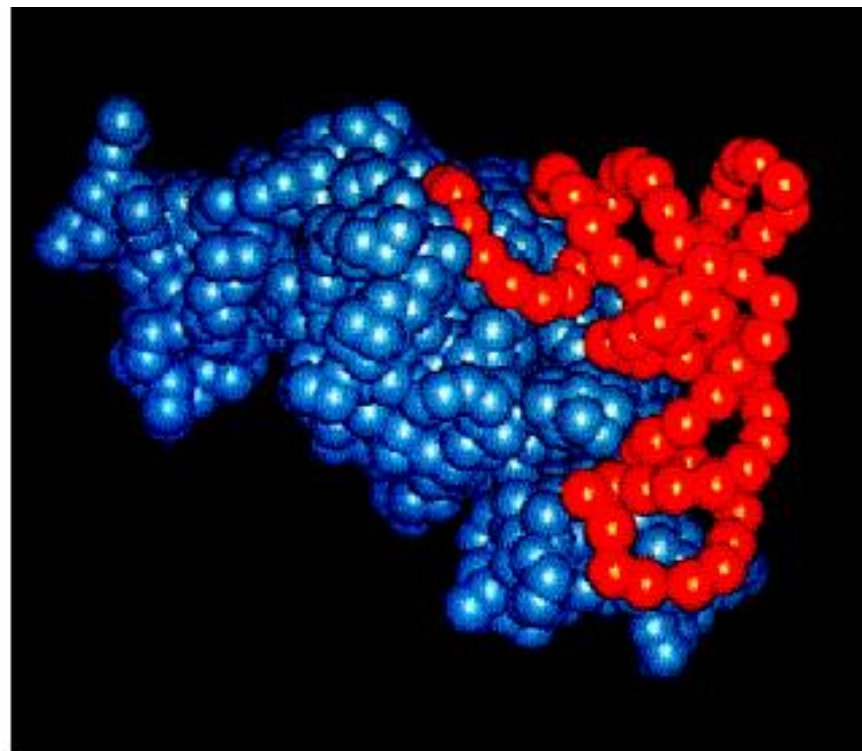
Le sintetasi riconoscono le anse dell'anticodon e gli steli accettori delle molecole di tRNA



Le due classi di tRNA sintetasi legano lati diversi del tRNA

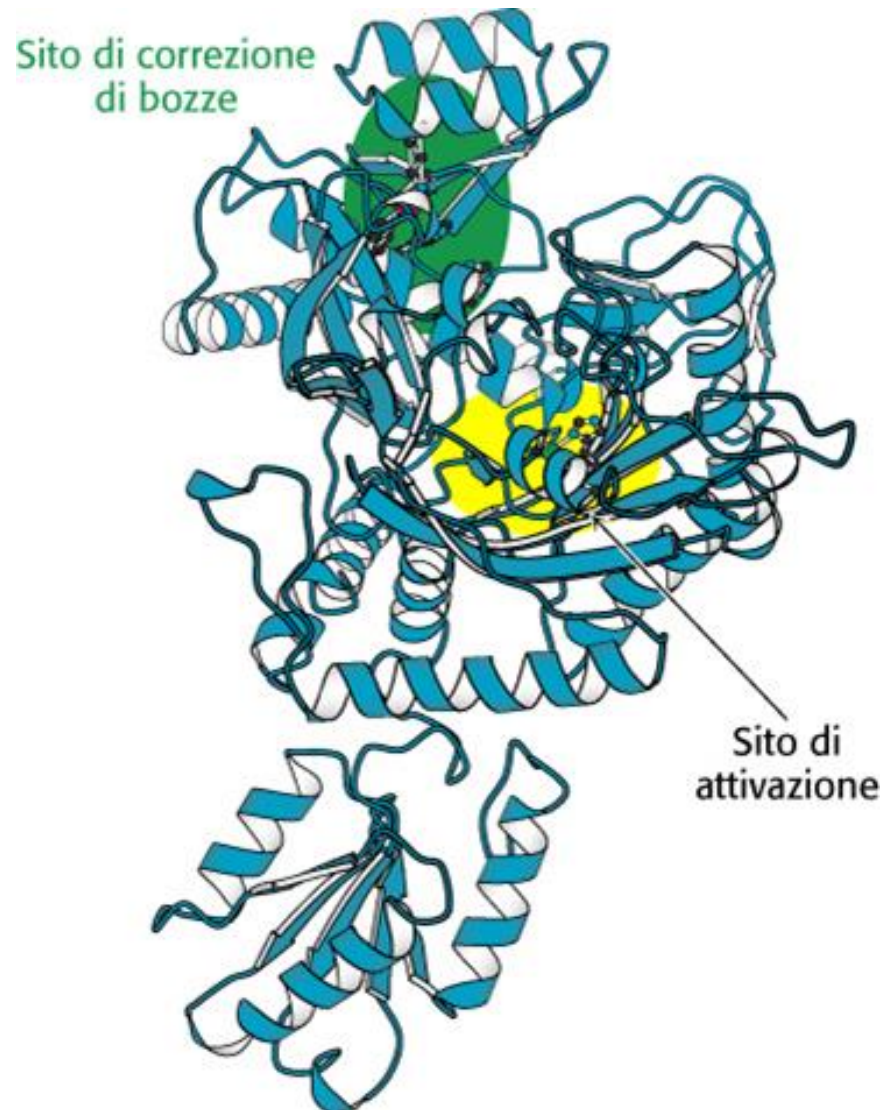


(a)



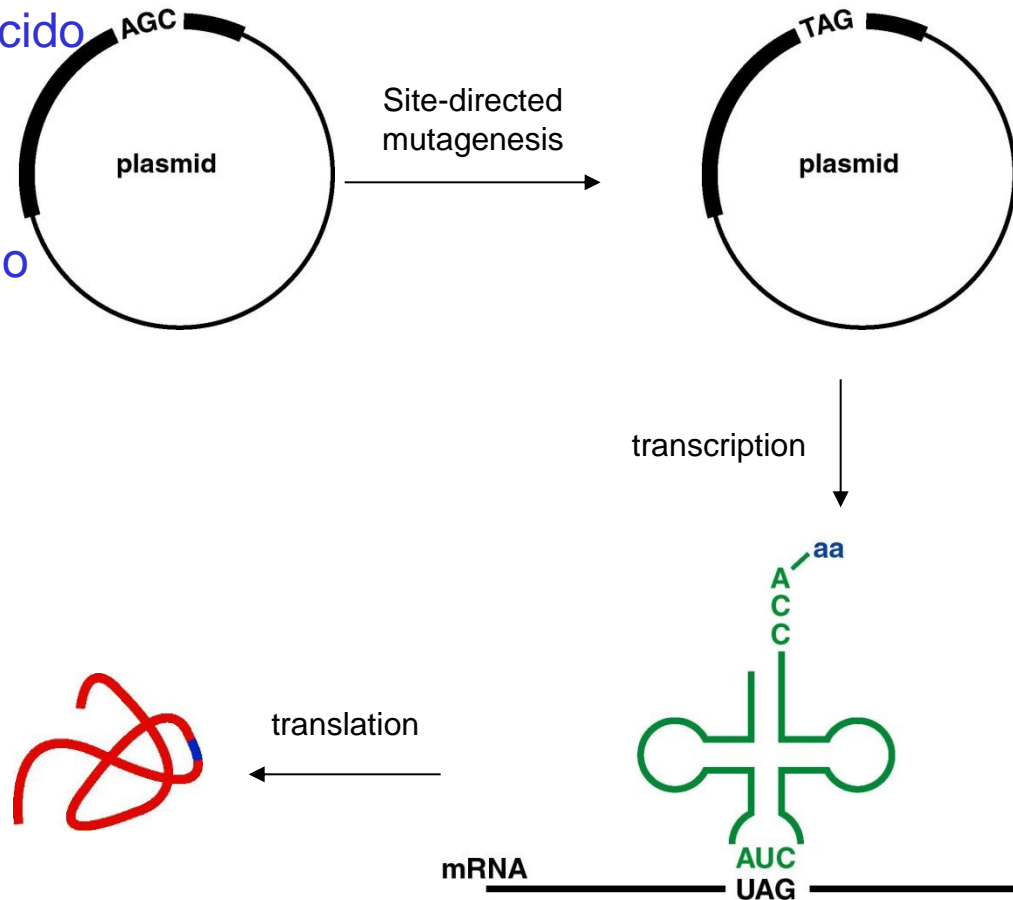
(b)

Alcune tRNA sintetasi hanno il sito per la correzione di bozze



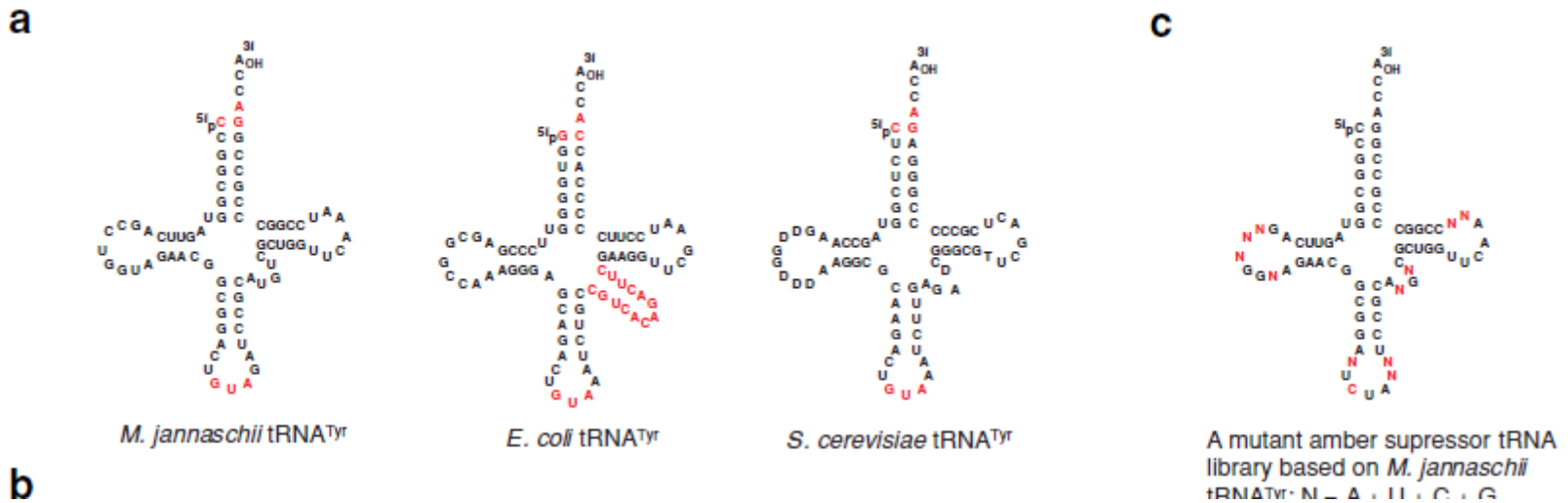
Nonsense Suppression Methodology

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



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

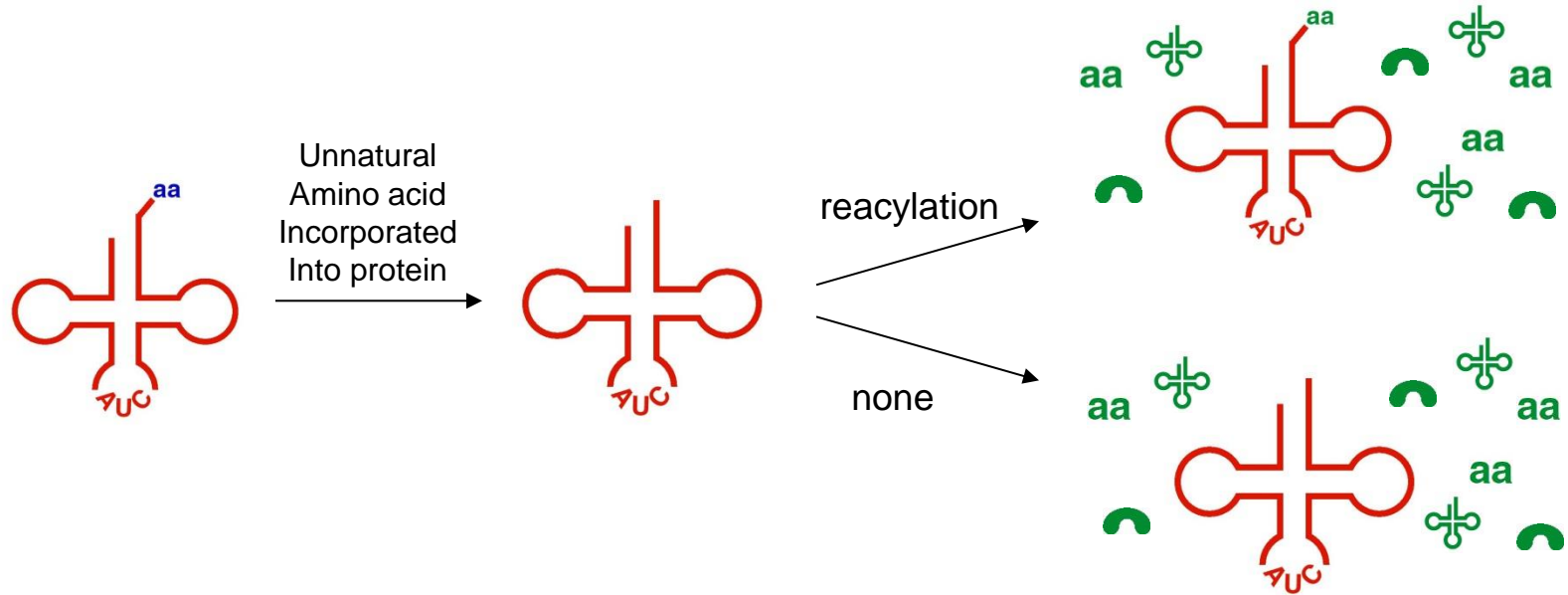
Le t-RNA sintetasi e tRNA degli eucarioti e degli archea sono simili tra loro e diverse da quelle di coli



Tentativi di Produrre di tRNA soppressori / sintetasi a partire da Glutamil tRNA /sintetasi di Ecoli non hanno portato a risultati positivi

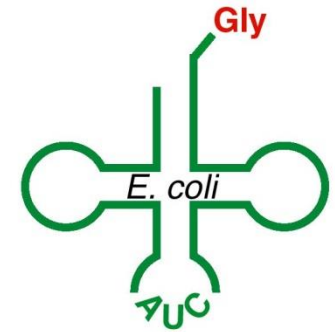
Selezione del tRNA Soppresore

- Non deve essere acilato da sintetasi endogene



Selezione dei tRNA Suppressor

- glycyl-tRNA sintetasi non ha attività di editing

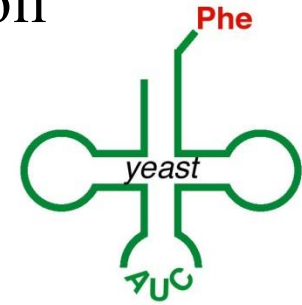


Fersht, A. R.; Dingwall, C. *Biochemistry* **1979**, *18*, 2627-2631.

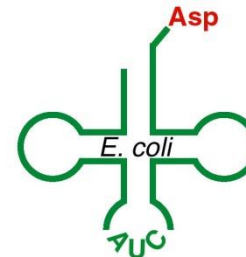
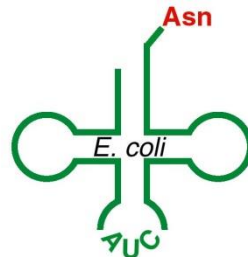
- Mutagenesi nello stelo accettore per eliminare il riconoscimento con la *E. coli* Gly sintetasi

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

tRNA soppressore di lievito da usare in Ecoli



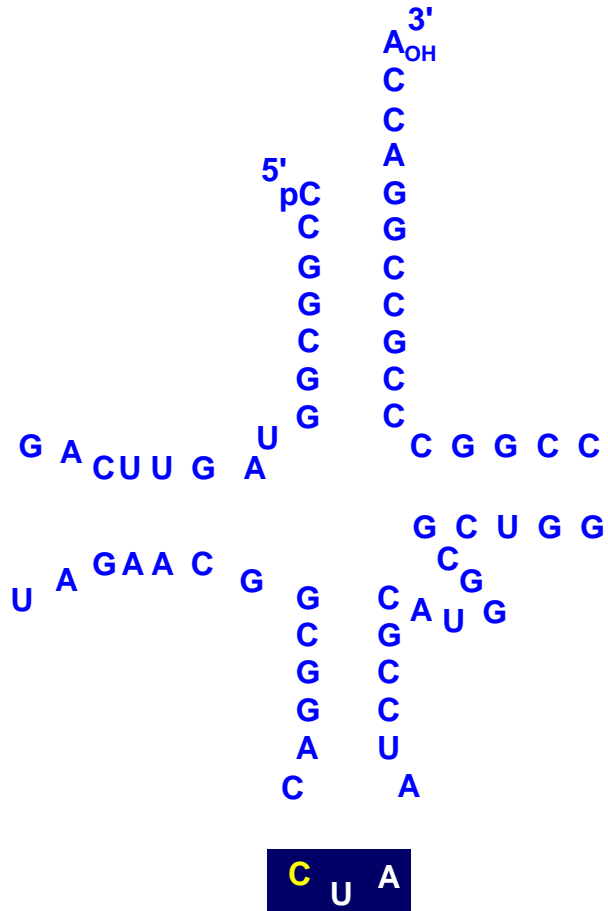
- *Non è riconosciuto dalla sintetasi Phe di E. coli*
- Bassa efficienza come soppressore
- *Bassa affinità per il ribosoma E. coli*
- aminoacidi polari non sono incorporati



Clod, S. T.; Liu, D. R.; Froland, W. A.; Schultz, P. G. *Chem. & Biol.* **1996**, 3, 1033-1038.

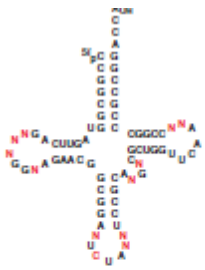
Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods in Enzymology* **1991**, 202, 301-336.

Sintetasi e tRNA *M. jannaschii*

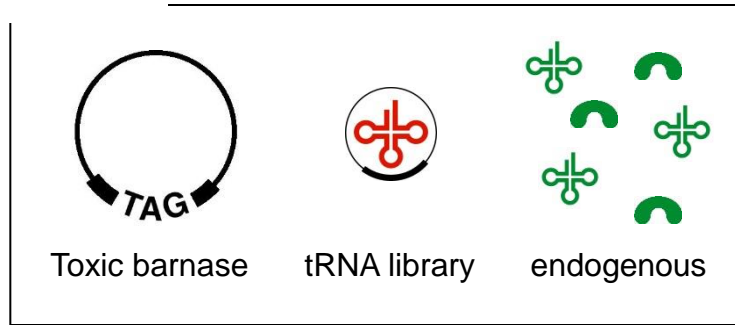
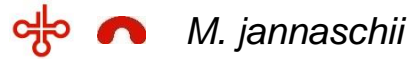


- *M. jannaschii* tRNA^{Tyr} è ortogonale alla sintetasi di *E. coli*
- *M. jannaschii* TyrRS è ortogonale a *E. coli* tRNAs
- *M. jannaschii* TyrRS non ha interazione con l'anticodone
- *M. jannaschii* TyrRS non ha attività di editing

Selezione per tRNA/Sintetasi

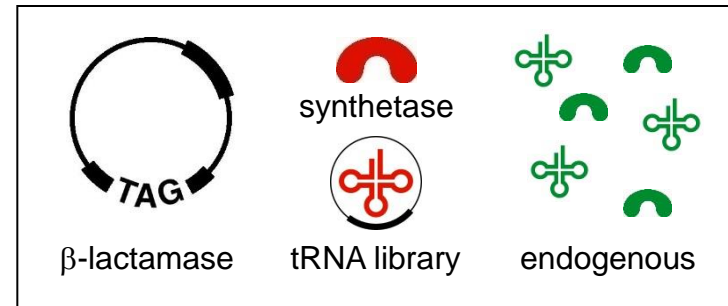


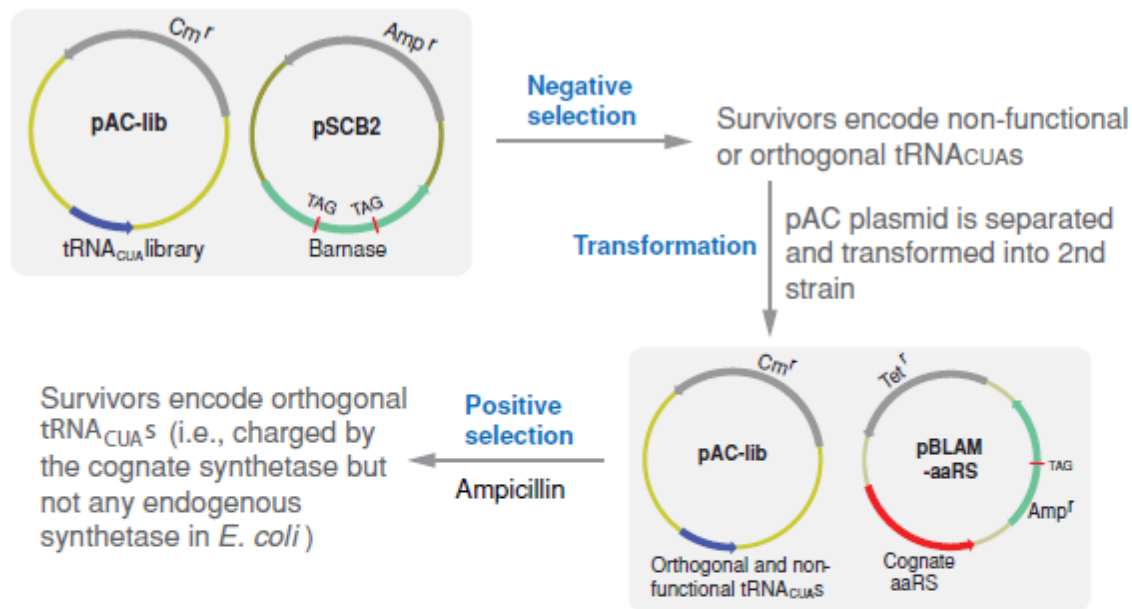
A mutant amber supressor tRNA library based on *M. jannaschii*
tRNA^{Tyr}: N = A + U + C + G



survivors

- orthogonal tRNAs
- non-functional tRNAs





d

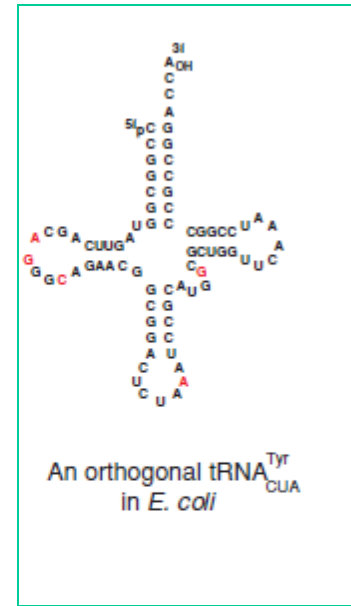
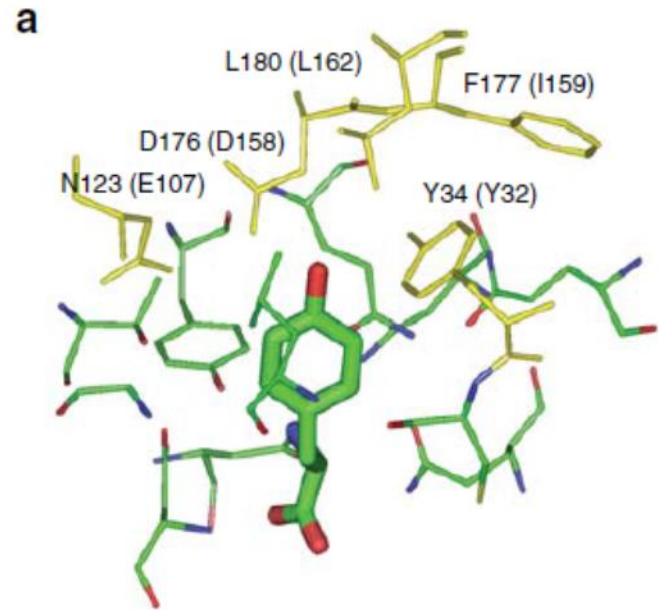


Figure 1

(a) Comparison of sequences of tRNA^{Tyr} from three different species. The major identity elements are in red. (b) Directed evolution of orthogonal amber suppressor tRNA_{CUA}^{Tyr} in *E. coli* by alternating negative and positive selections. (c) A library of amber suppressor tRNA^{Tyr} was generated by randomizing 11 nucleotides (red) of *M. jannaschii* tRNA_{CUA}^{Tyr} that do not interact directly with the cognate TyrRS. (d) The orthogonal amber suppressor *Mj* tRNA_{CUA}^{Tyr} (changed nucleotides are in red).

Evoluzione diretta di *Mj* TyrRS per selezionare tRNAsintetasi in grado di identificare diversi aa modificati



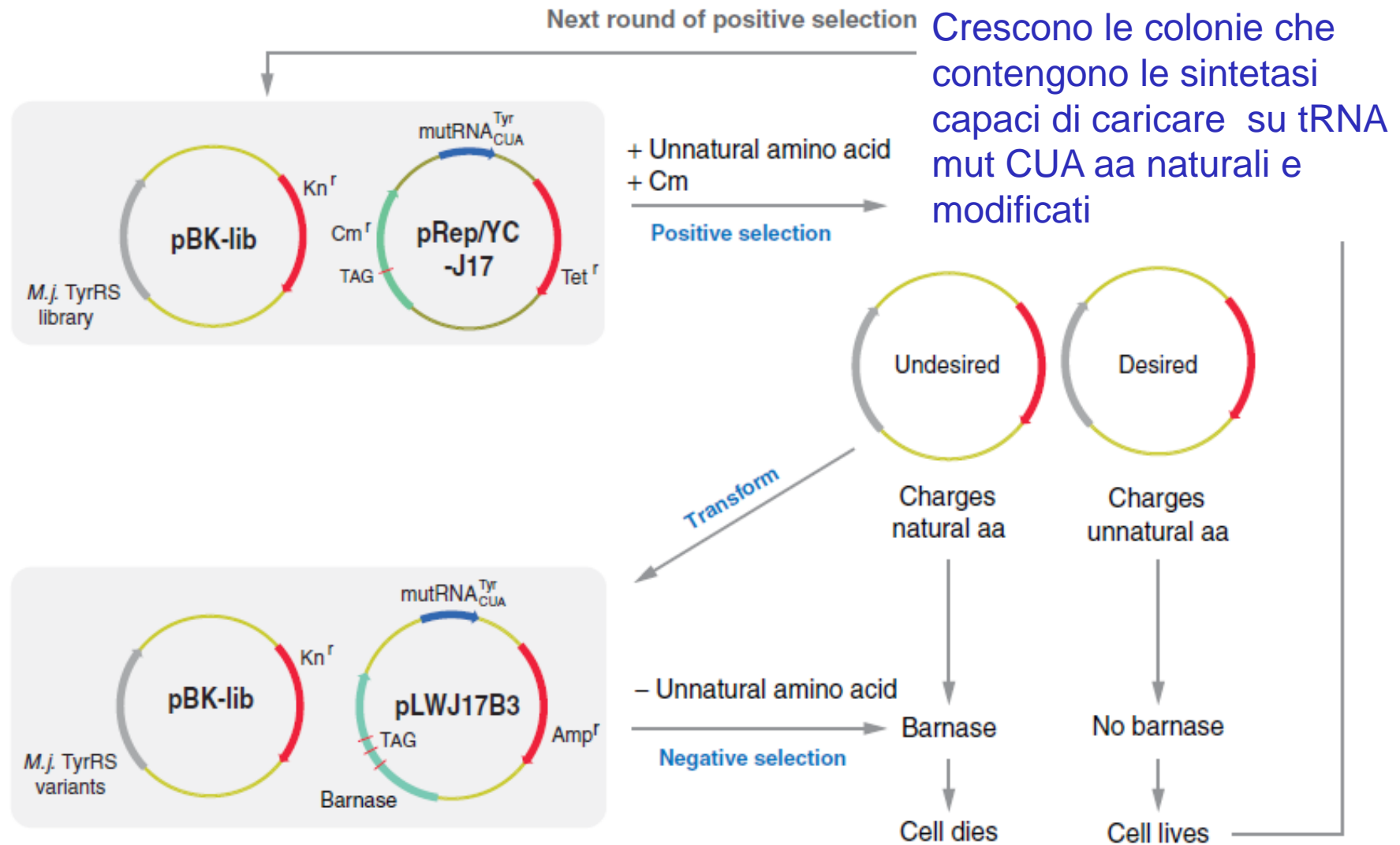
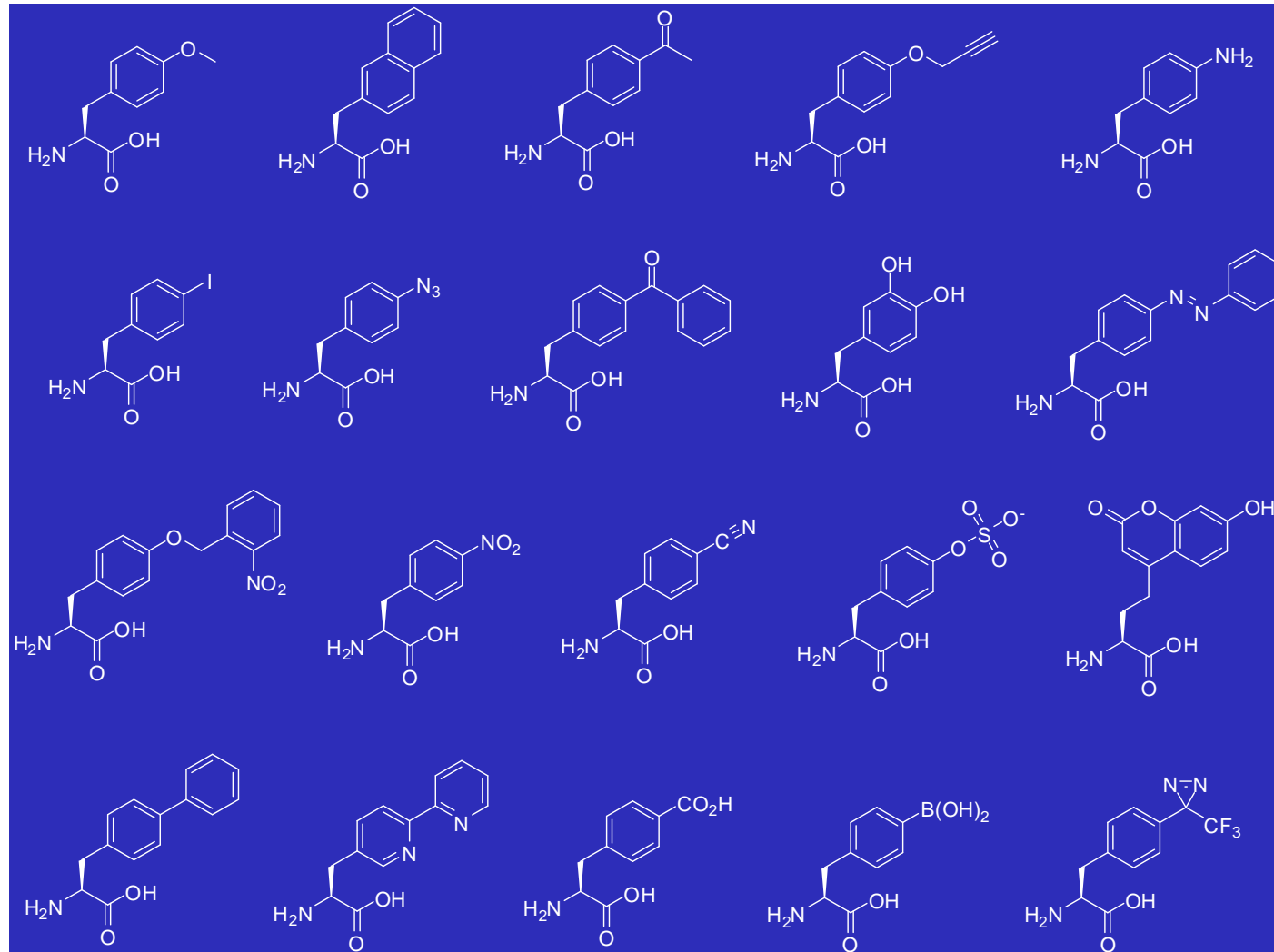


Figure 2

Modification of the amino acid specificity of an orthogonal *M. jannaschii* TyrRS (*Mj* TyrRS) in *E. coli*. (a) A library of *Mj* TyrRS mutants was generated by randomizing five residues (in parentheses) in the tyrosine binding site. (b) A general positive and negative selection scheme for evolving synthetase variants specific for an unnatural amino acid in *E. coli*. Cm, chloramphenicol.

Expanded Genetic Code in *E. coli*



Incorporazione del fotocrosslinker pbenzilfenilalanina (pBpa)

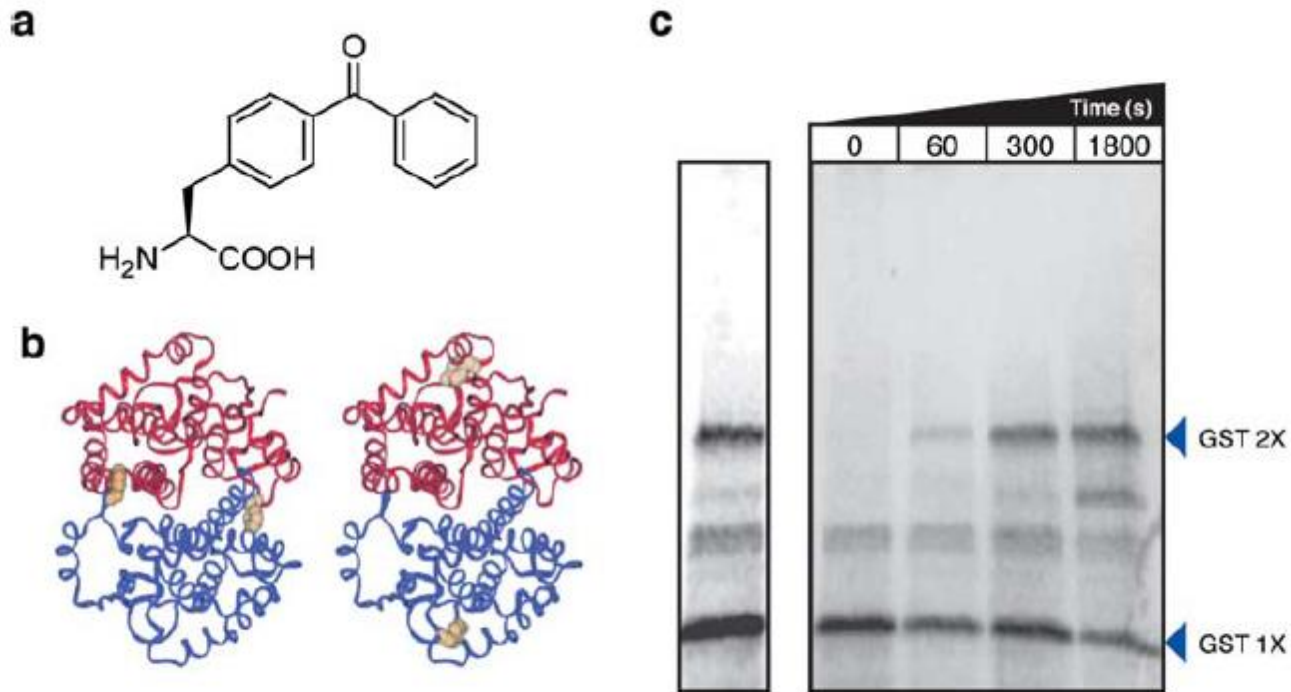
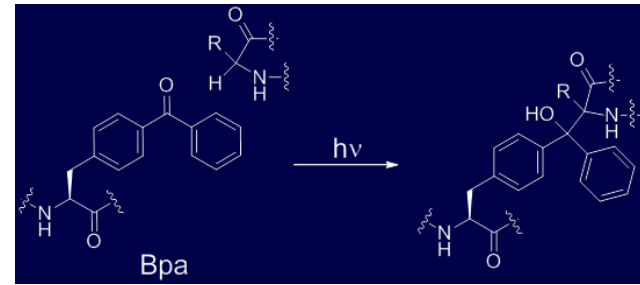
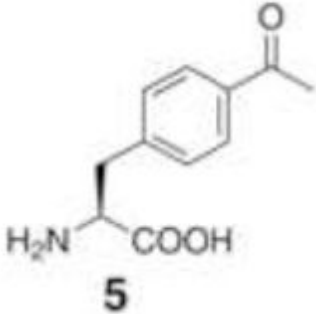


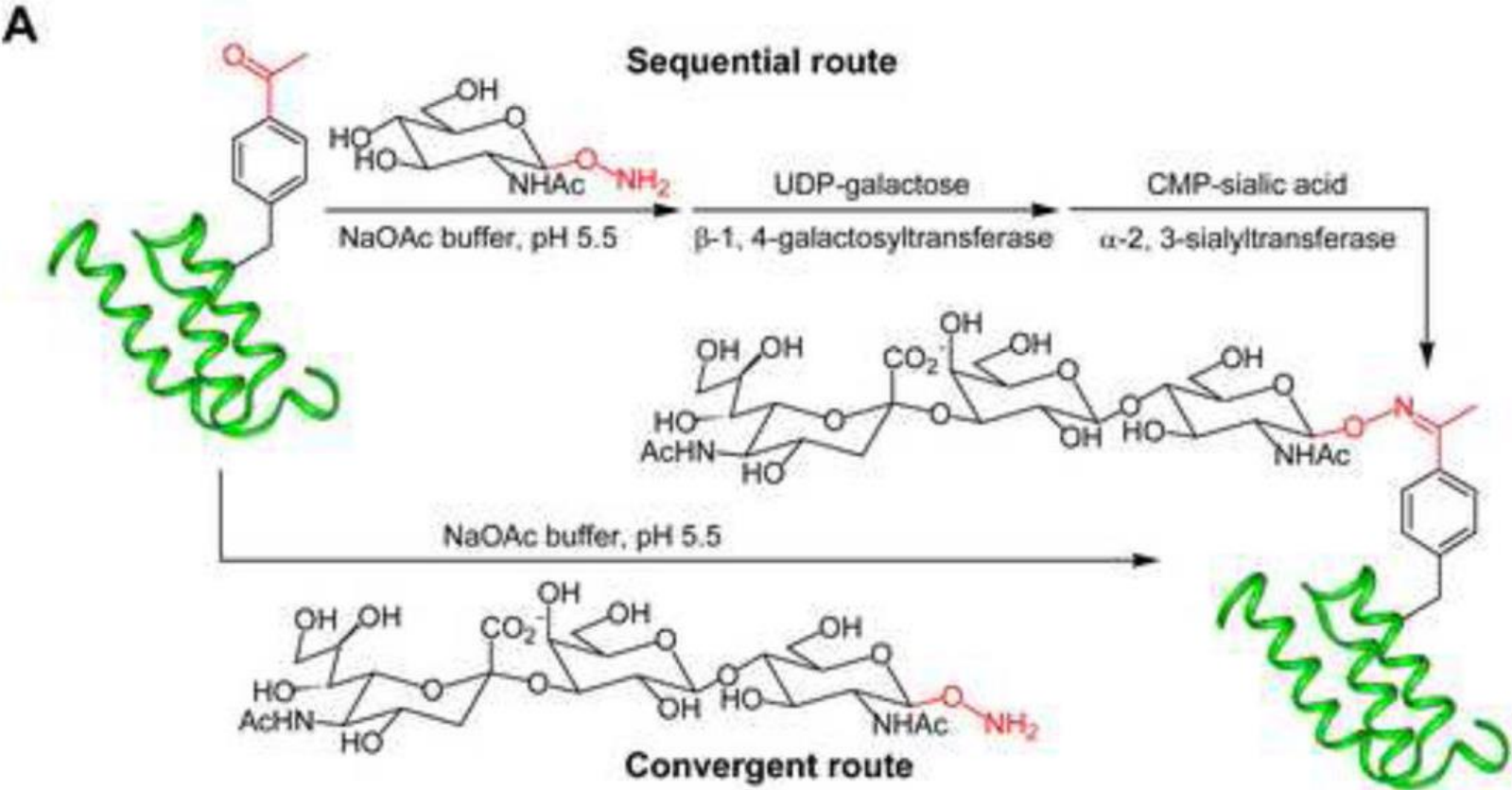
Figure 7

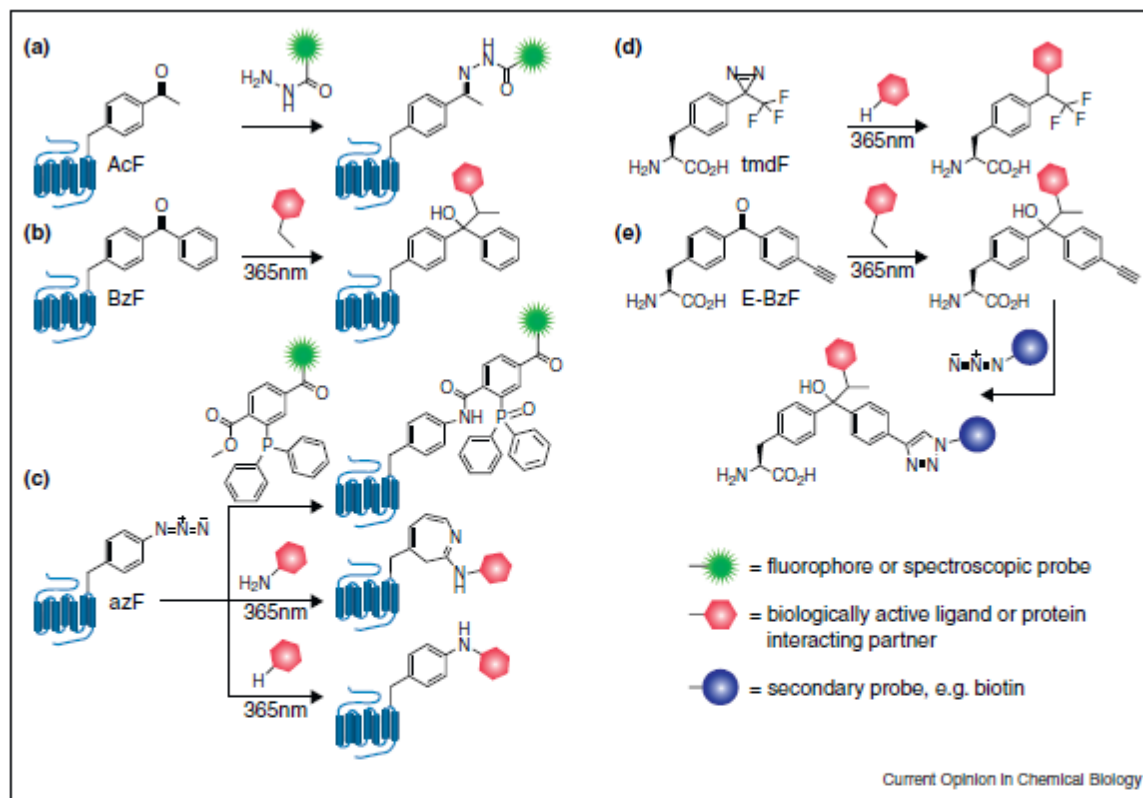
Site-specific incorporation of a photocrosslinker into proteins for mapping protein-protein interactions *in vivo*. (a) The chemical structure of *p*-benzoyl-L-phenylalanine (*p*Bpa). (b) Residue Phe-52 or Tyr-198 in *Schistosoma japonica* glutathione S-transferase (SjGST) was substituted by *p*Bpa. 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



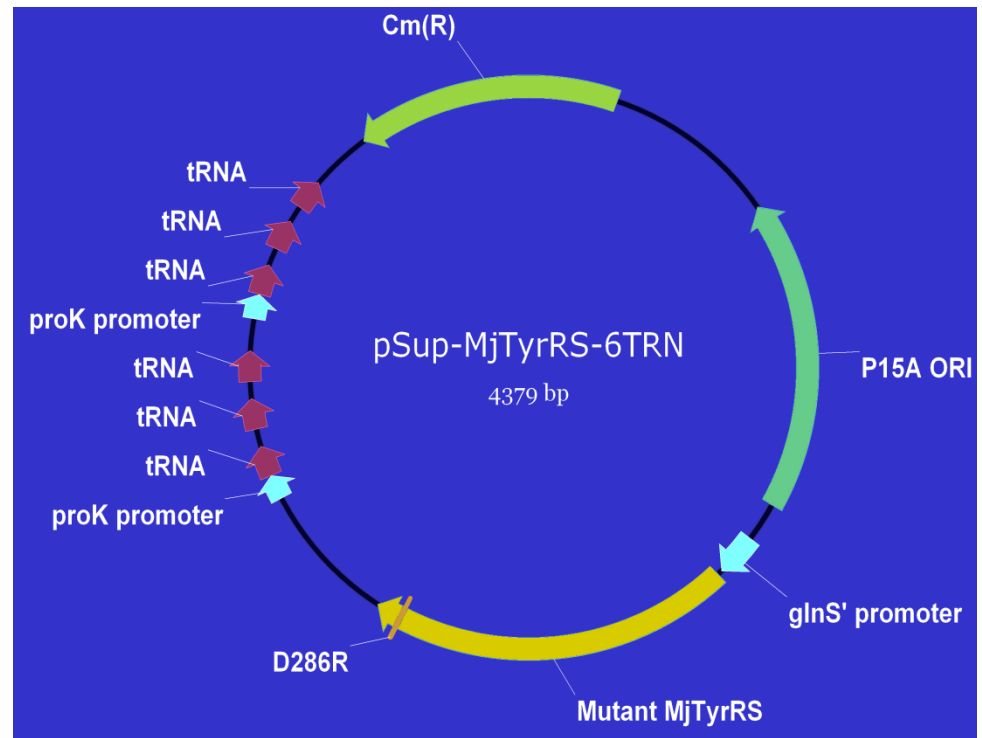


Representative UAAs used in GPCR structure–function studies. UAAs have been incorporated into expressed GPCRs (a–d) or synthetically incorporated into peptides to probe GPCR function (e). (a) *p*-Acetyl-L-phenylalanine (AcF) can react with a hydrazide or hydroxylamine derivative. (b) *p*-Benzoyl-L-phenylalanine (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-diazirinyl-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.

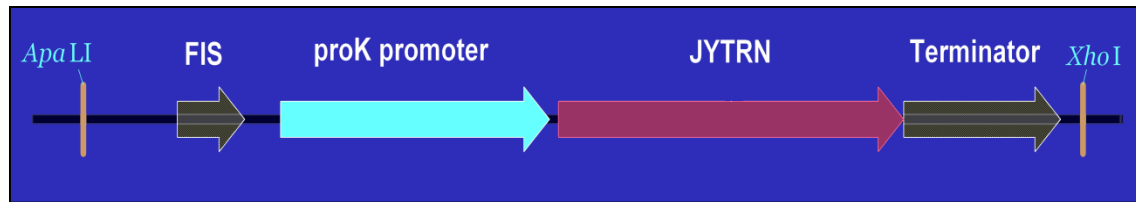
Metodi per ottimizzare la produzione di
di proteine con aminoacidi modificati in
E coli

Optimizing protein yields in *E. coli*

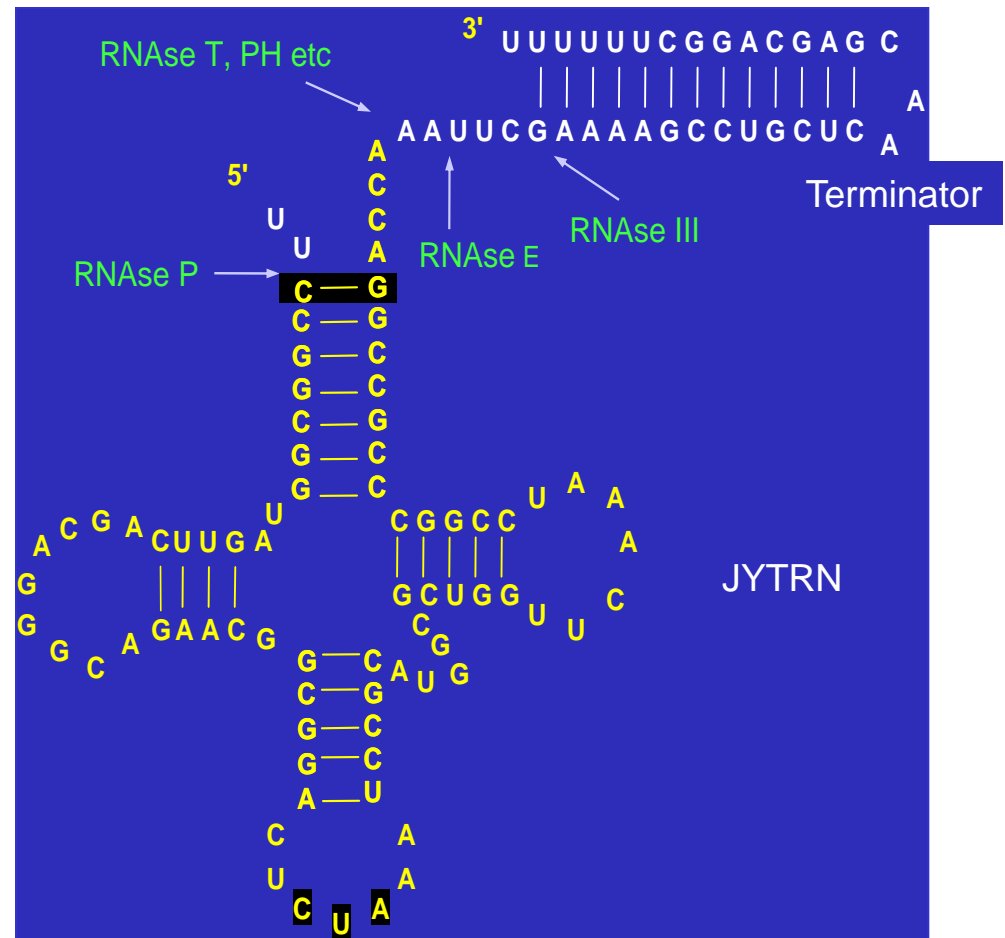
- *E. coli* prolyl-tRNA promoter and terminator for the amber suppressor tRNA
- Mutated form of the *glnS* promoter for the synthetase
- D286R substitution in the synthetase gene
- Multiple copies of the suppressor tRNA gene
- Yield of adiponectin (Glu123Bpa) mutant: 0.4g/L



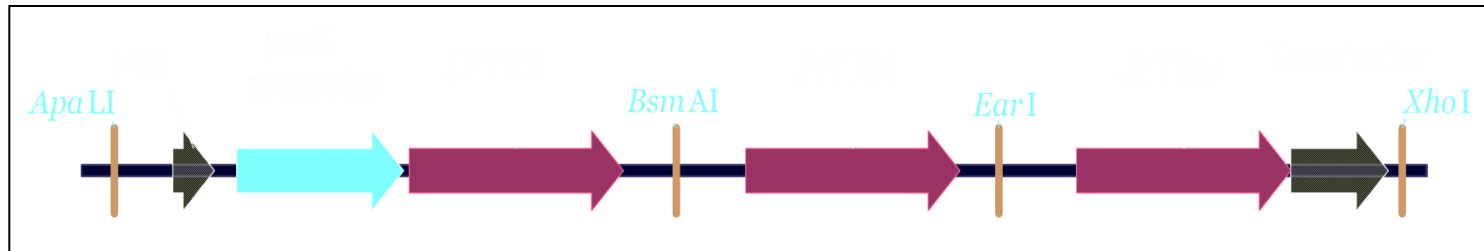
Mj tRNA expression cassette



- *E. coli* prolyl tRNAs have C1-G72 pair, which is major identity determinant of *Mj*tRNA – Important context for the precise tRNA processing
- *proK* tRNA is most frequently used in *E. coli*
- FIS enhances tRNA transcription

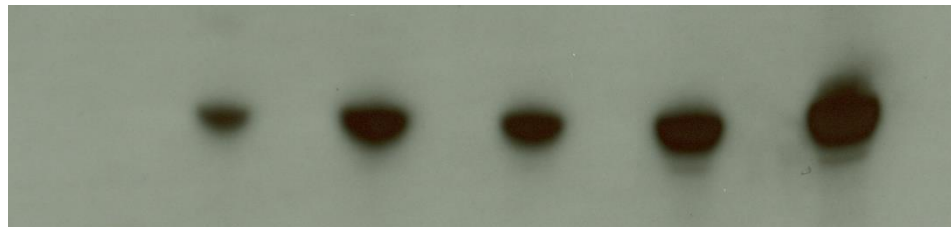


espressione di *MjtRNA*

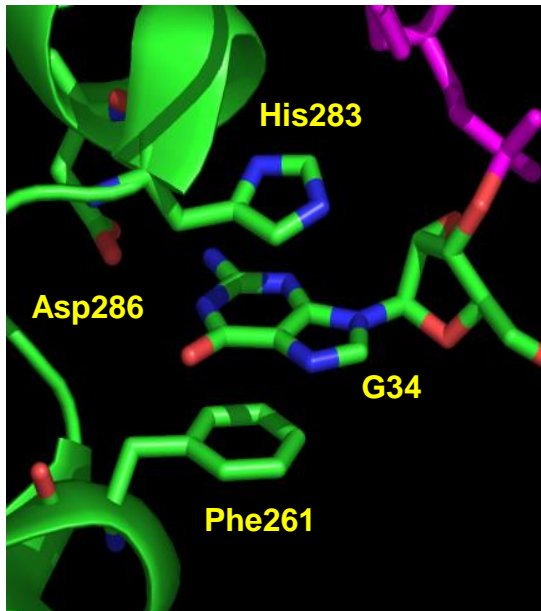


aaRS promoter	<i>glnS</i>	<i>glnS</i>	<i>glnS</i>	<i>glnS'</i>	<i>glnS'</i>	<i>glnS'</i>
tRNA promoter	-	<i>lpp</i>	<i>proK</i>	<i>proK</i>	<i>proK</i>	<i>proK</i>
tRNA copy #	0	1	1	1	3	6

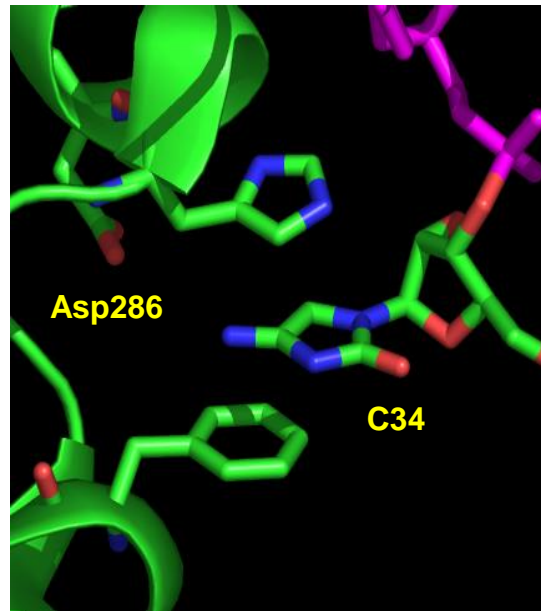
JYTRN →



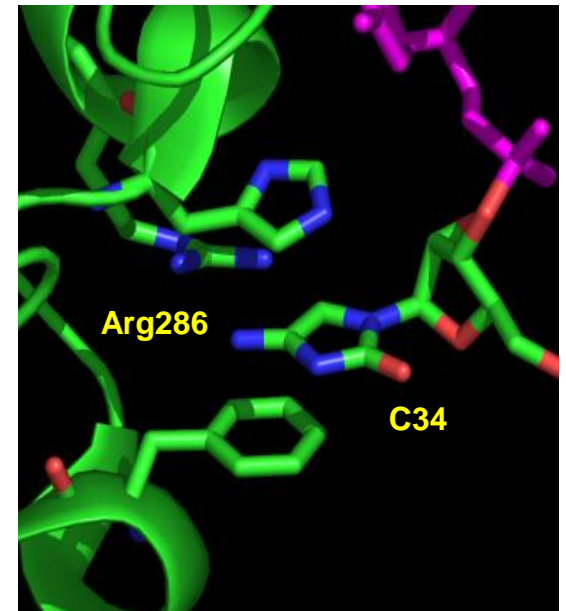
Asp286Arg (D286R) aumenta tRNA(CUA) binding affinity



$$K_m = 0.35 \mu\text{M}$$
$$k_{\text{cat}} = 0.19 \text{ s}^{-1}$$
$$k_{\text{cat}}/K_m \text{ (relative)} = 1$$

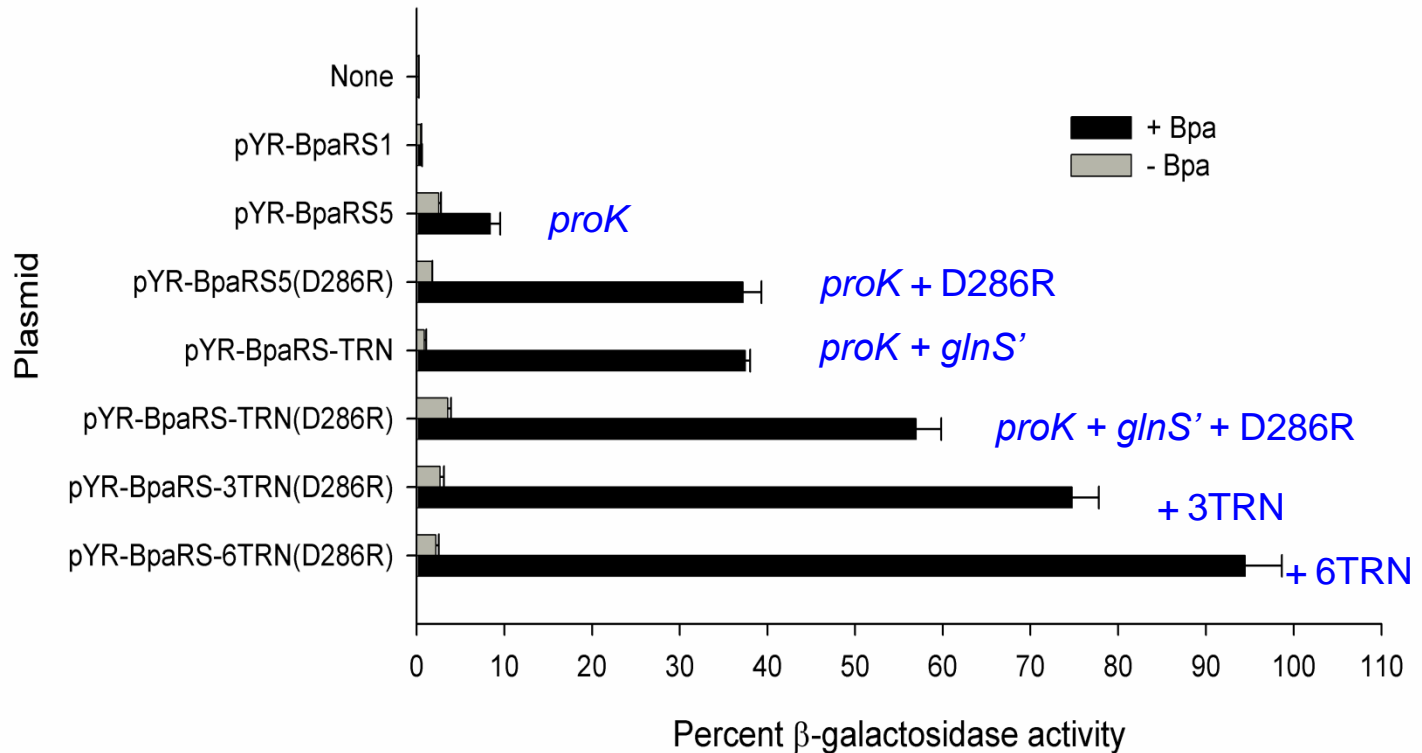


$$K_m = 39 \mu\text{M}$$
$$k_{\text{cat}} = 0.070 \text{ s}^{-1}$$
$$k_{\text{cat}}/K_m \text{ (relative)} = 0.0033$$

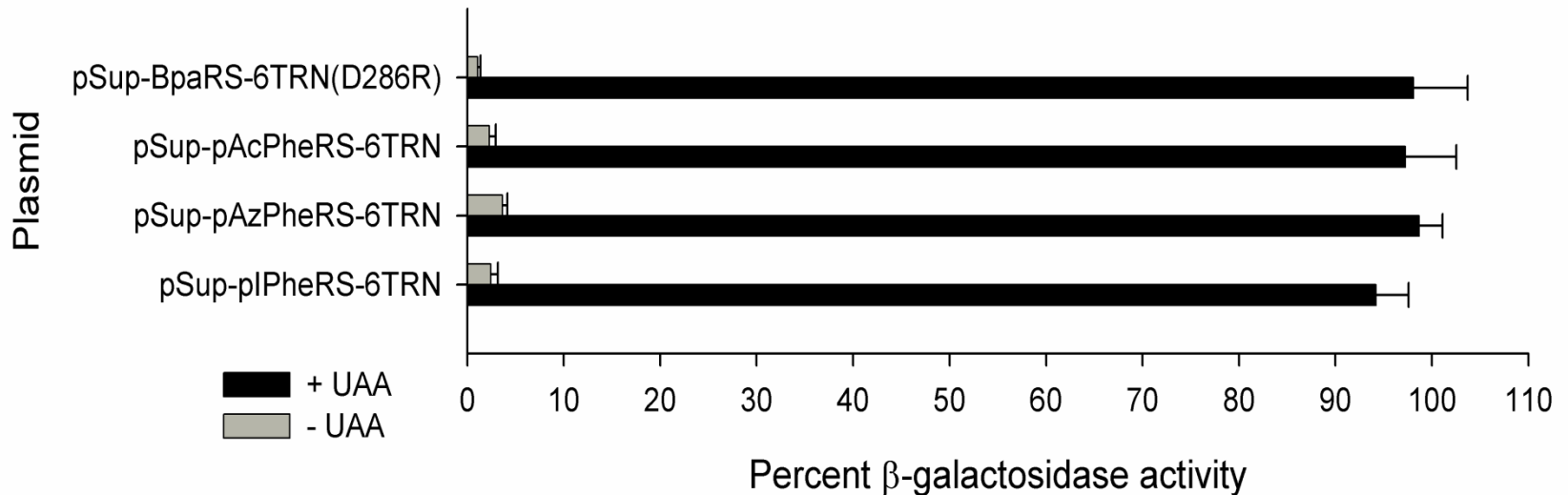
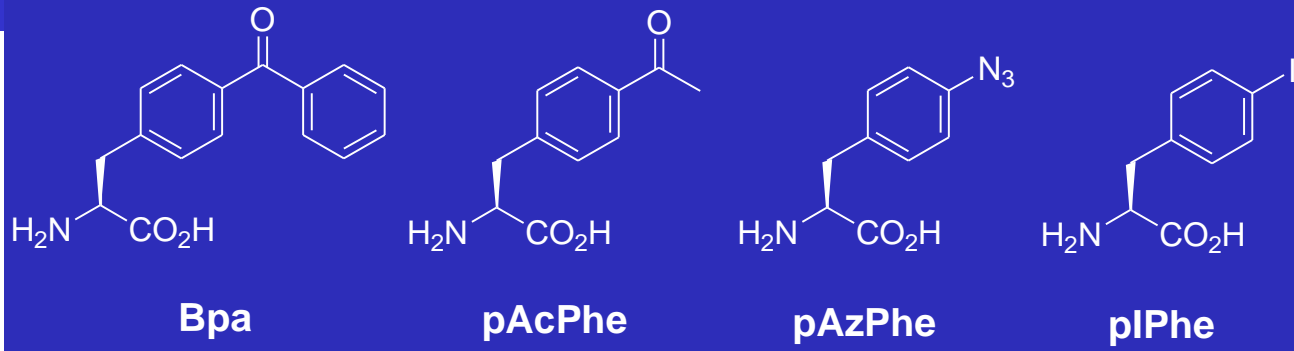


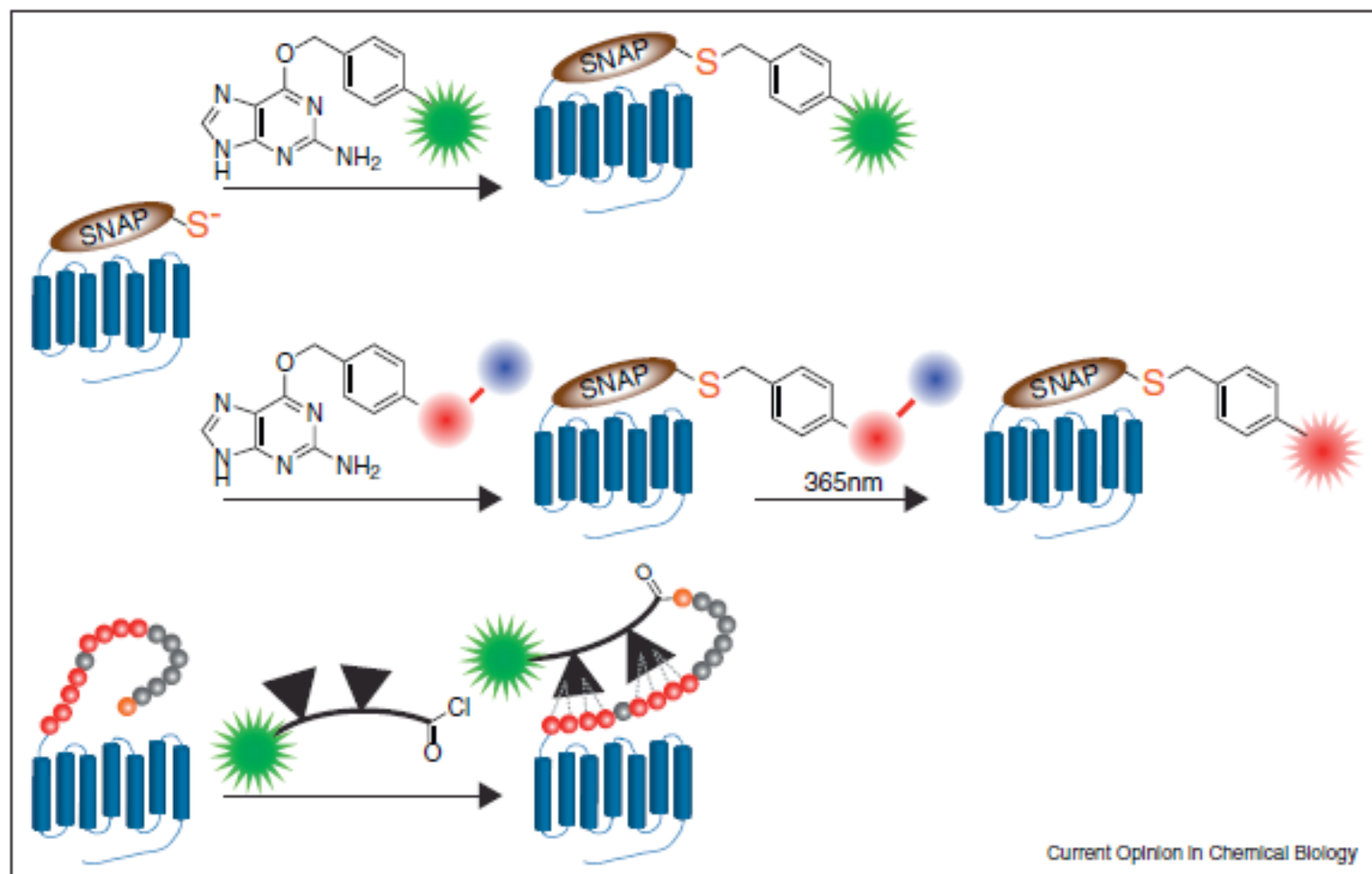
$$K_m = 0.68 \mu\text{M}$$
$$k_{\text{cat}} = 0.079 \text{ s}^{-1}$$
$$k_{\text{cat}}/K_m \text{ (relative)} = 0.22$$

β-Galactosidase assay for suppression efficiency



Incorporazione differenti aminoacidi modificati

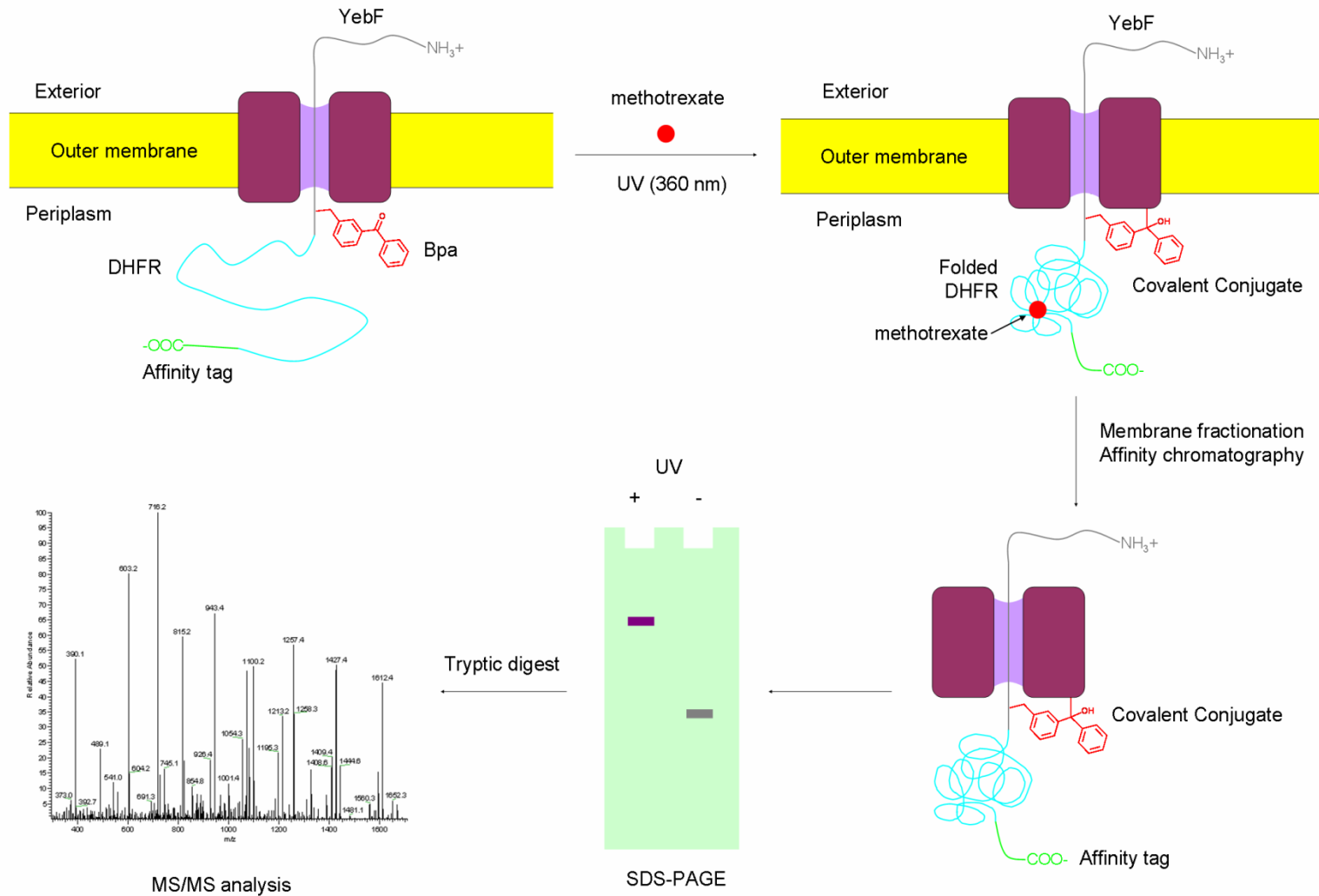




Current Opinion in Chemical Biology

to covalent labeling methods can be used in combination with UAA mutagenesis. **(a)** In SNAP-tag technology, GPCRs are fused to a 20-kDa tag that reacts with O^6 -benzylguanine derivatives carrying a fluorophore or a photo-convertible probe with a donor and acceptor separated by a photo-cleavable linker (red bond). **(b)** A mutant receptor with a CA6D4x2 tag binds to a tetra Zn(II) compound tethered to a fluorescence probe.

Strategy to identify the YebF transporter



Unnatural protein medicinal chemistry

