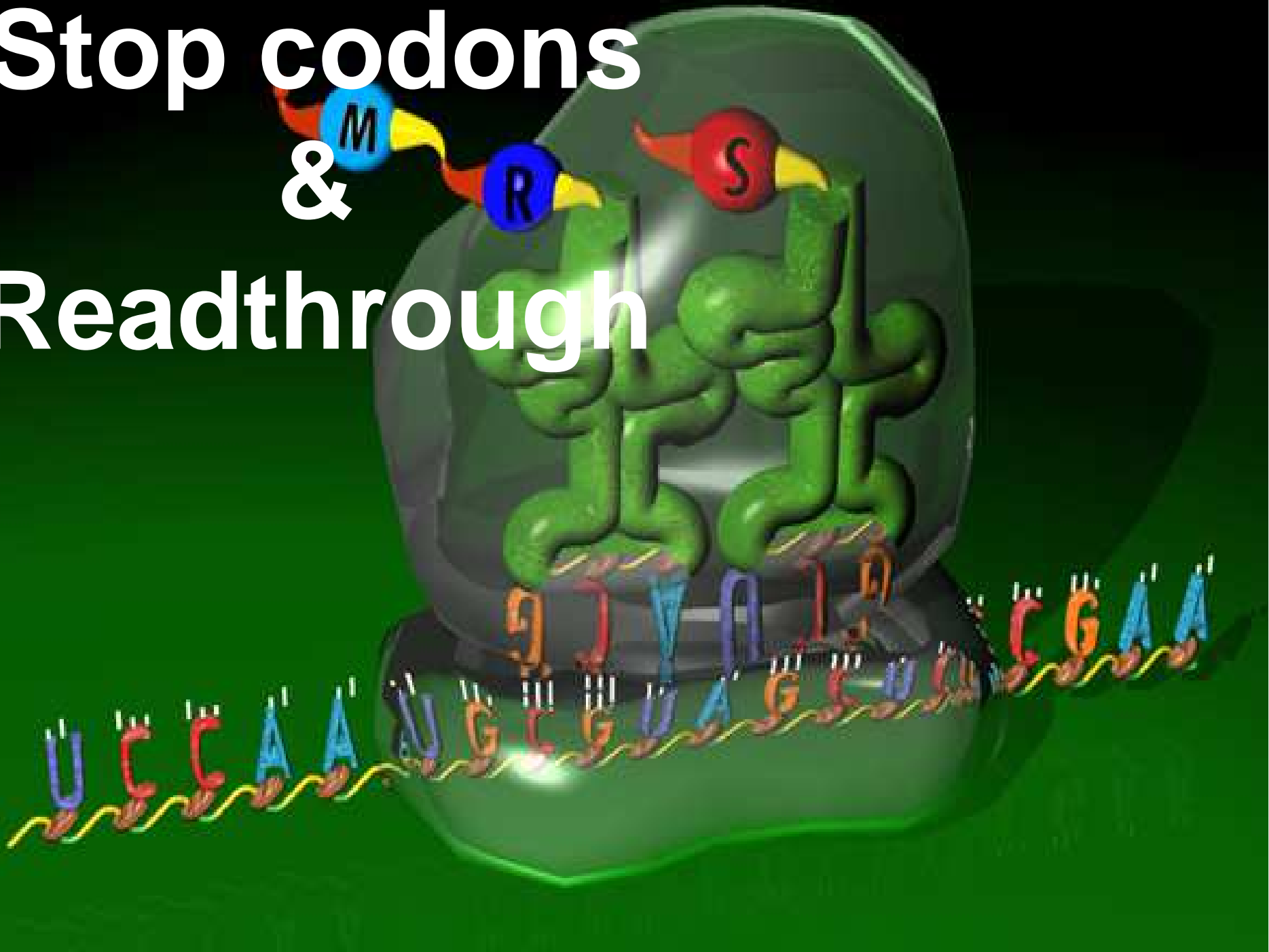


Stop codons & Readthrough



Fidelity in protein synthesis

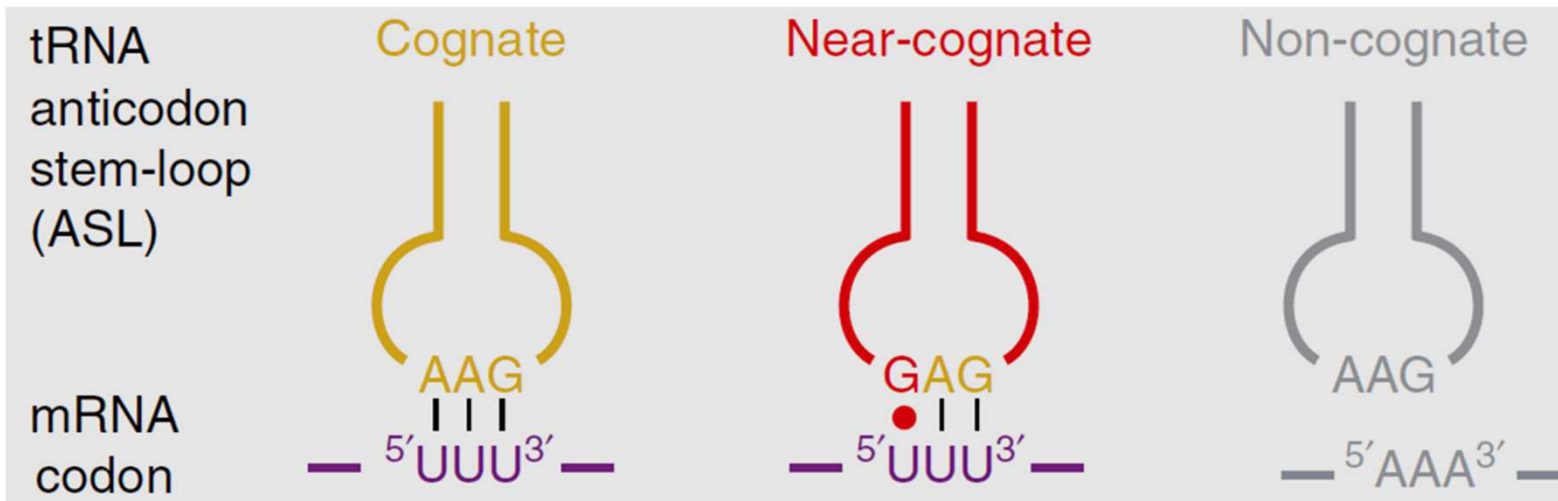
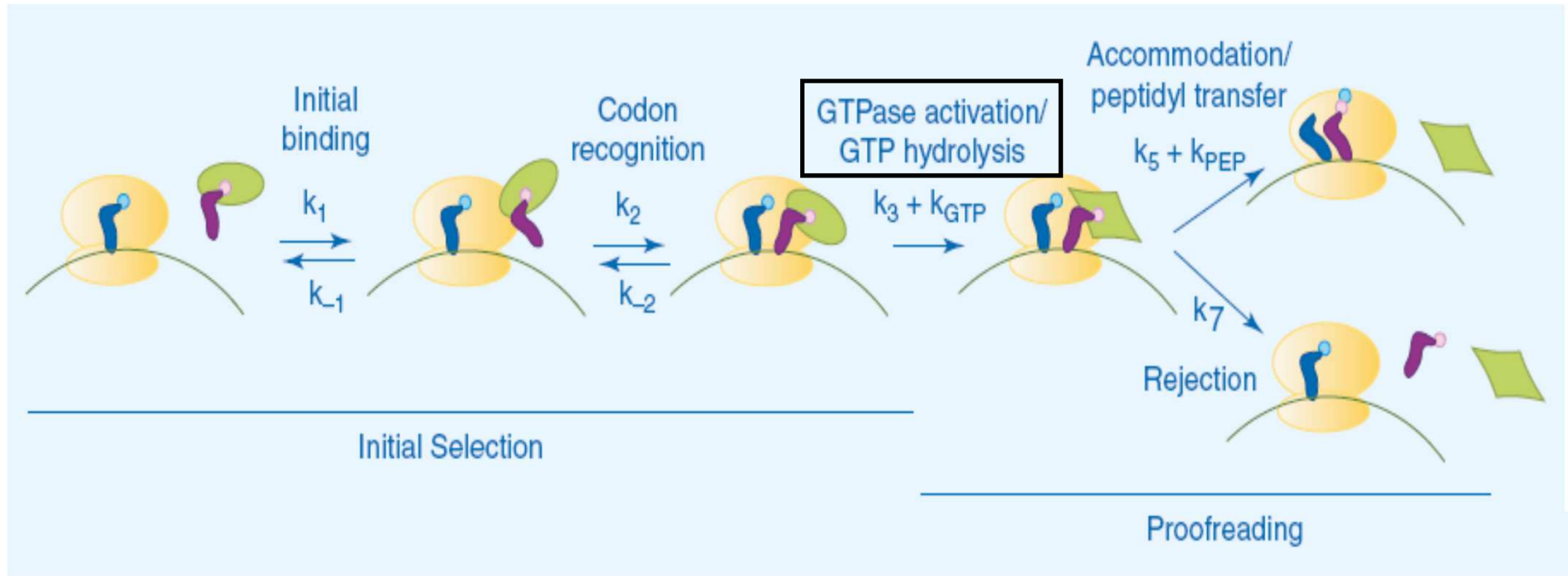
DNA replication and transcription are based on complementarity and correctly matched base-pairing.

During translation, each tRNA is covalently bound to an amino acid in order to be accommodated in the ribosomal A-site due to **correspondence of codon (on mRNA), anticodon (on tRNA), and amino acid.**

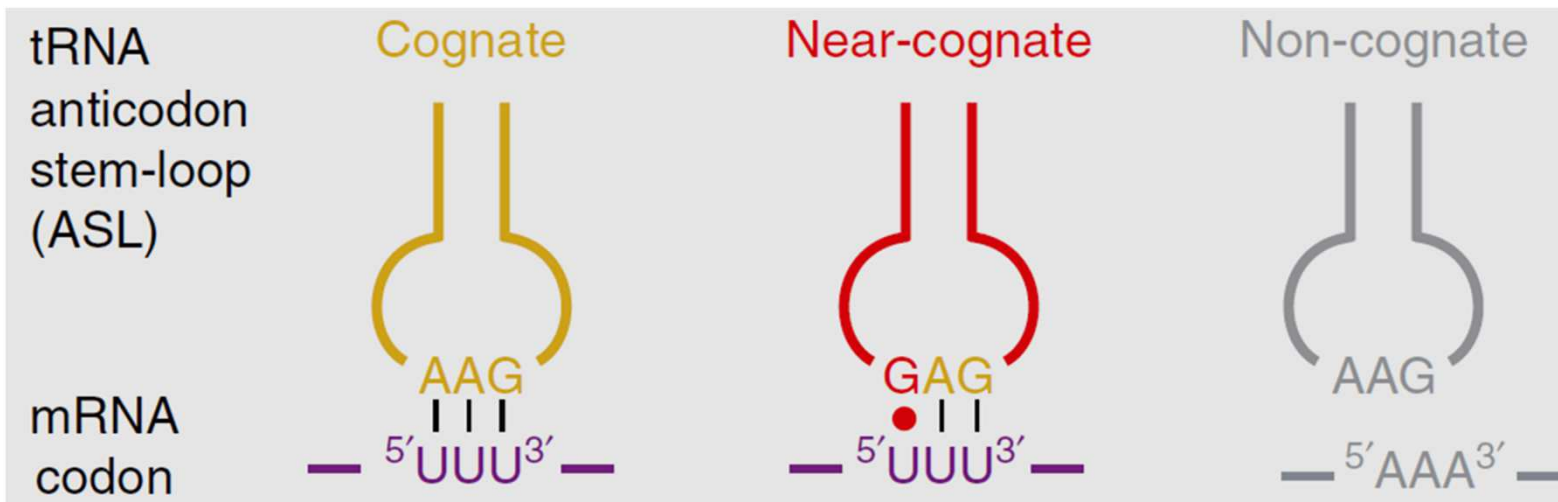
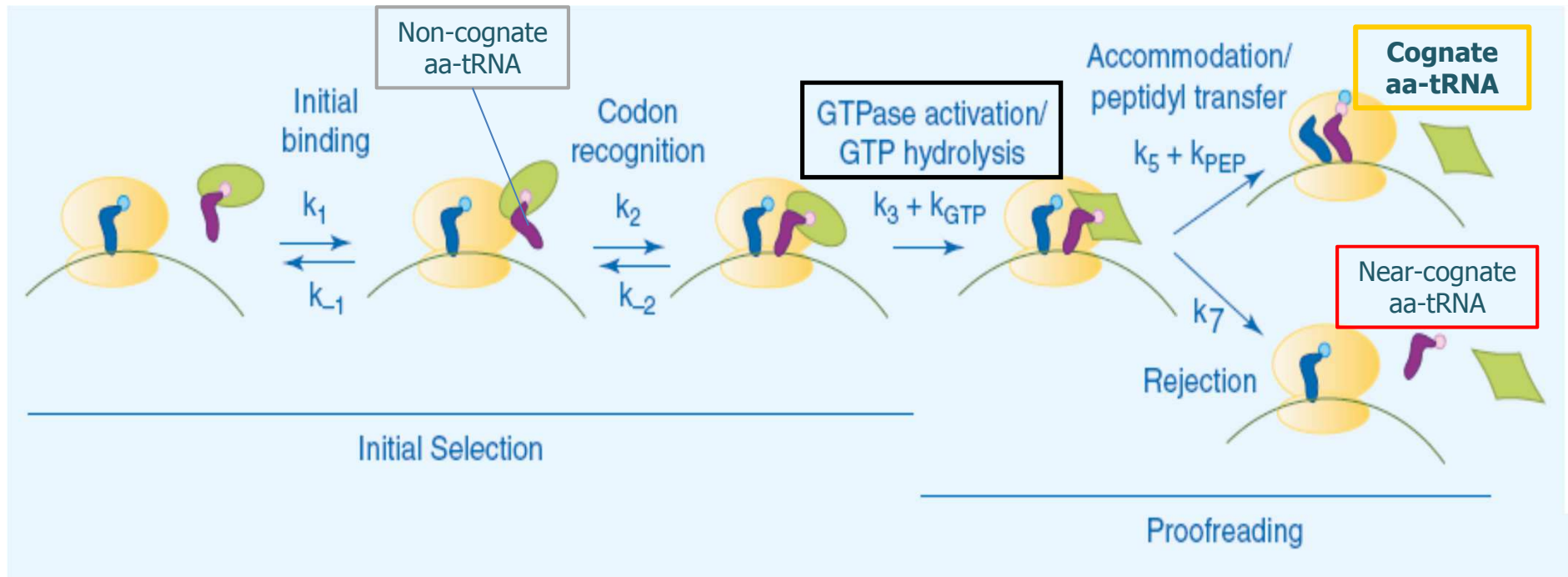
Three strategies ensure a balance between velocity (3-5 aa/sec) and accuracy (**error rate $\sim 10^{-4}$**):

1. Editing (tRNA/amino acid) ----- Aa-tRNA synthetase
2. Kinetic proofreading (codon/anticodon) ----- Ribosome
3. Induced fit (codon/anticodon) ----- Ribosome

Kinetic proofreading



Kinetic proofreading



Translational reprogrammed genetic decoding (RECODING) during protein synthesis

Recoding: regulatory mechanisms of protein expression that include several non-canonical events, opposite to the DNA → RNA → Protein central dogma of biology

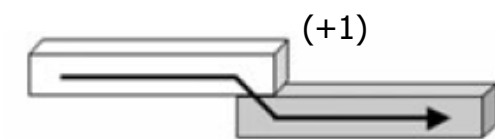
Recoding was found to be associated to elongation and termination phases:

- Elongation phase
- +1 Frameshifting
 - -1 Frameshifting
 - Ribosome hopping

- Termination phase
- Stop codon **Readthrough**
(frequency: **10^{-4}**)

Translational reprogrammed genetic decoding (RECODING) during protein synthesis

Recoding: regulatory mechanisms of protein expression that include several non-canonical events, opposite to the DNA → RNA → Protein central dogma of biology



(+1)

Elongation phase

Programmed frameshifting

Ribosomes switch to an alternative frame (± 1) at a specific shift site



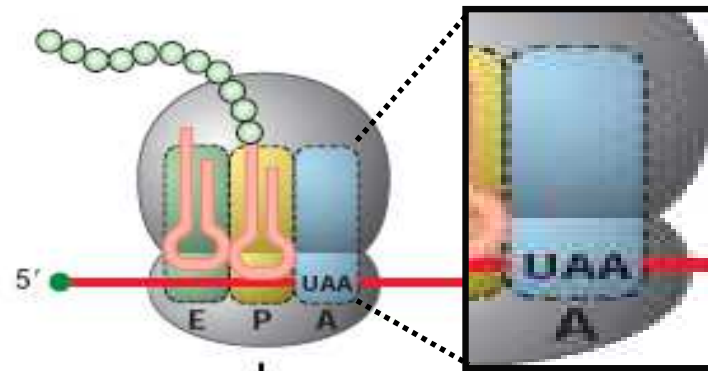
(-1)



Ribosome hopping

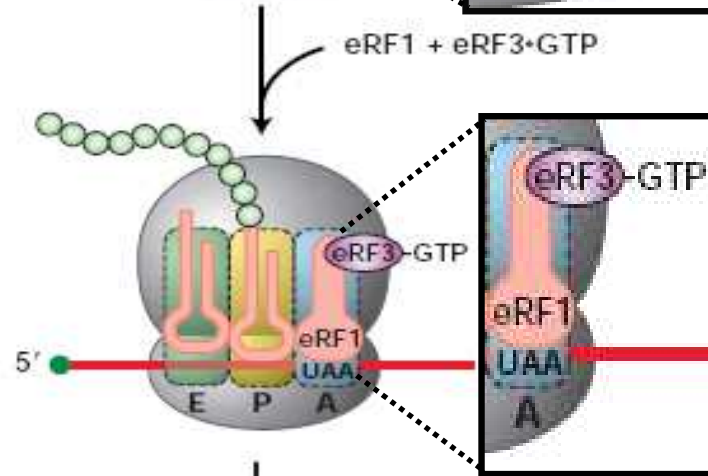
Ribosomes suspend translation at a certain site and then resume translation downstream

Translation termination

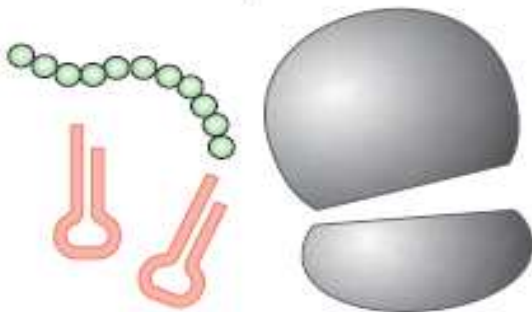
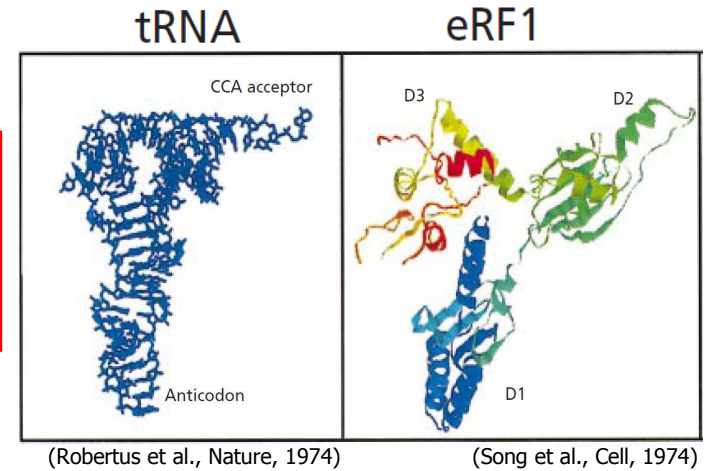


In-frame stop codon (UAG, UGA, UAA) enters the ribosome A-site

Recognition of the stop codon by release factor eRF1

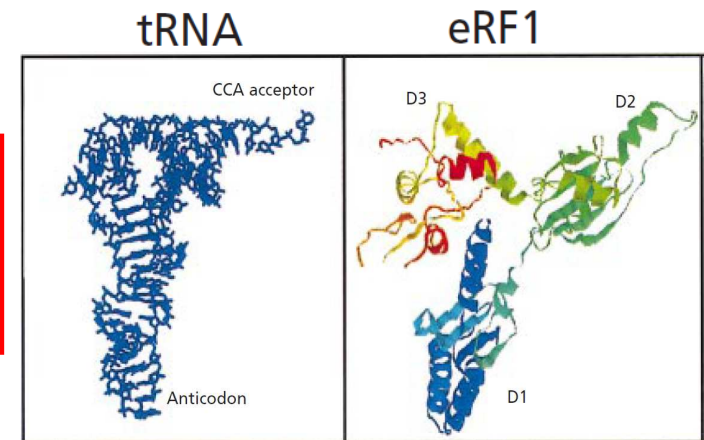
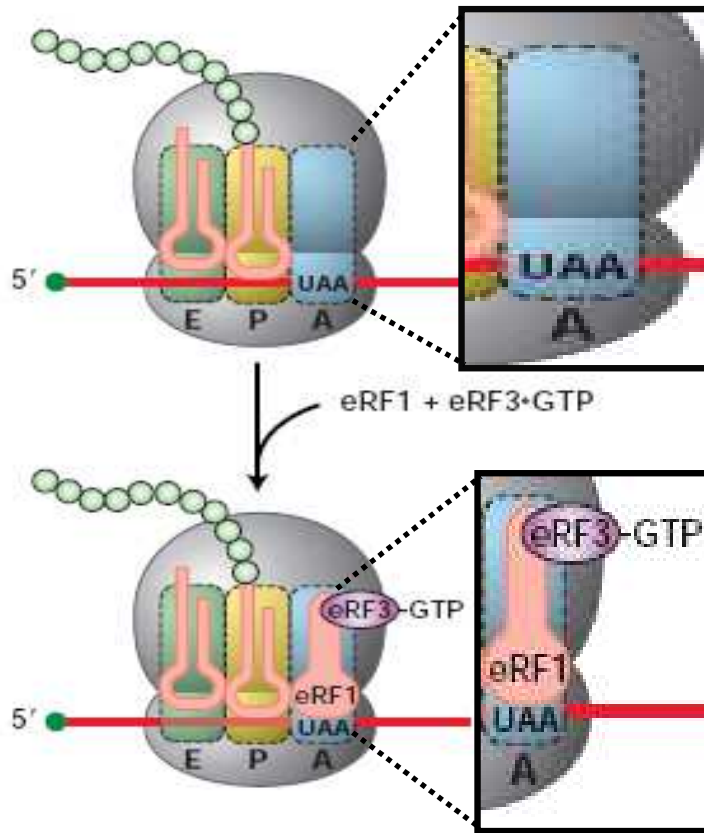


Molecular mimicry and competition



Hydrolysis of the P-site-bound peptidyl-tRNA and release of the nascent polypeptide

Translation termination



Termination phase



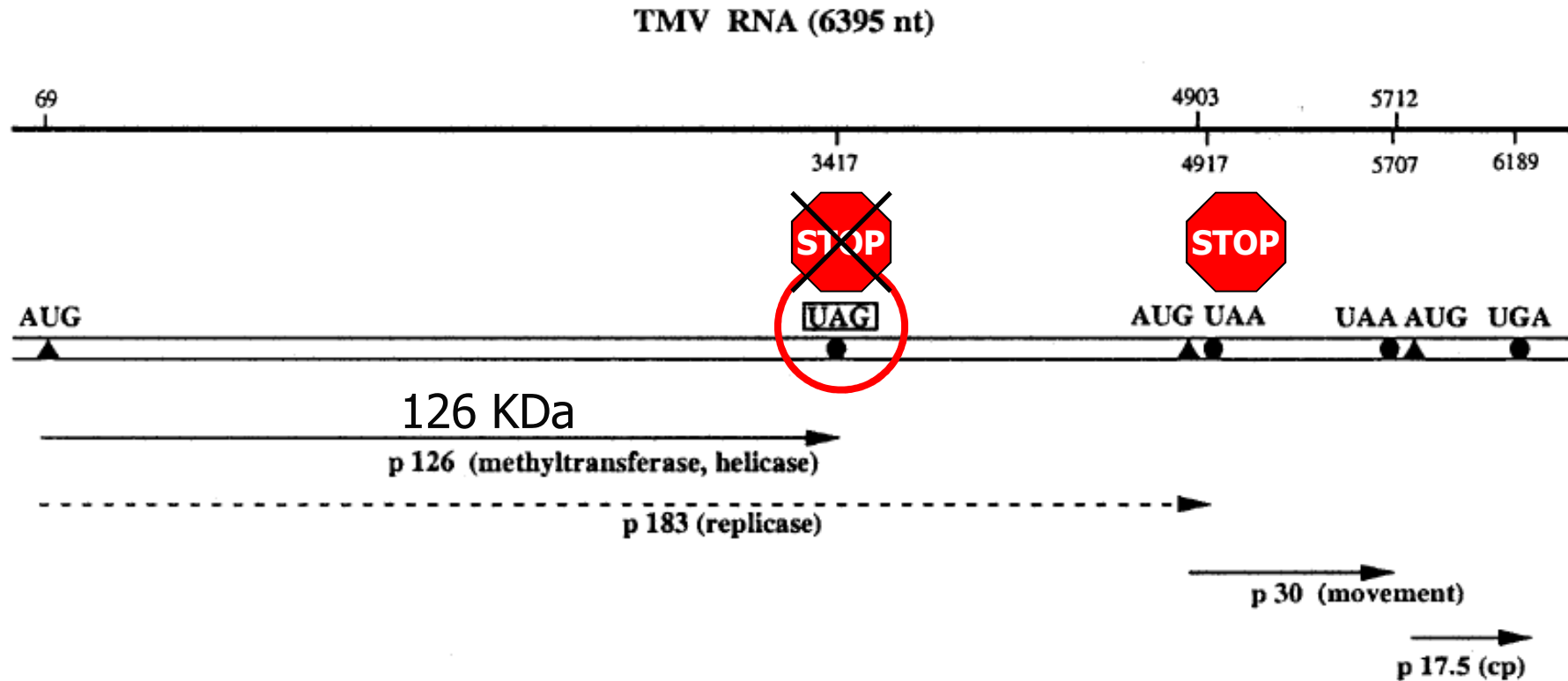
Stop codon readthrough

Translation is continued beyond the stop codon

Translational READTHROUGH

A regulatory mechanism of gene expression, extensively used by ssRNA viruses, which provides the differential production of more than one polypeptide from a single mRNA

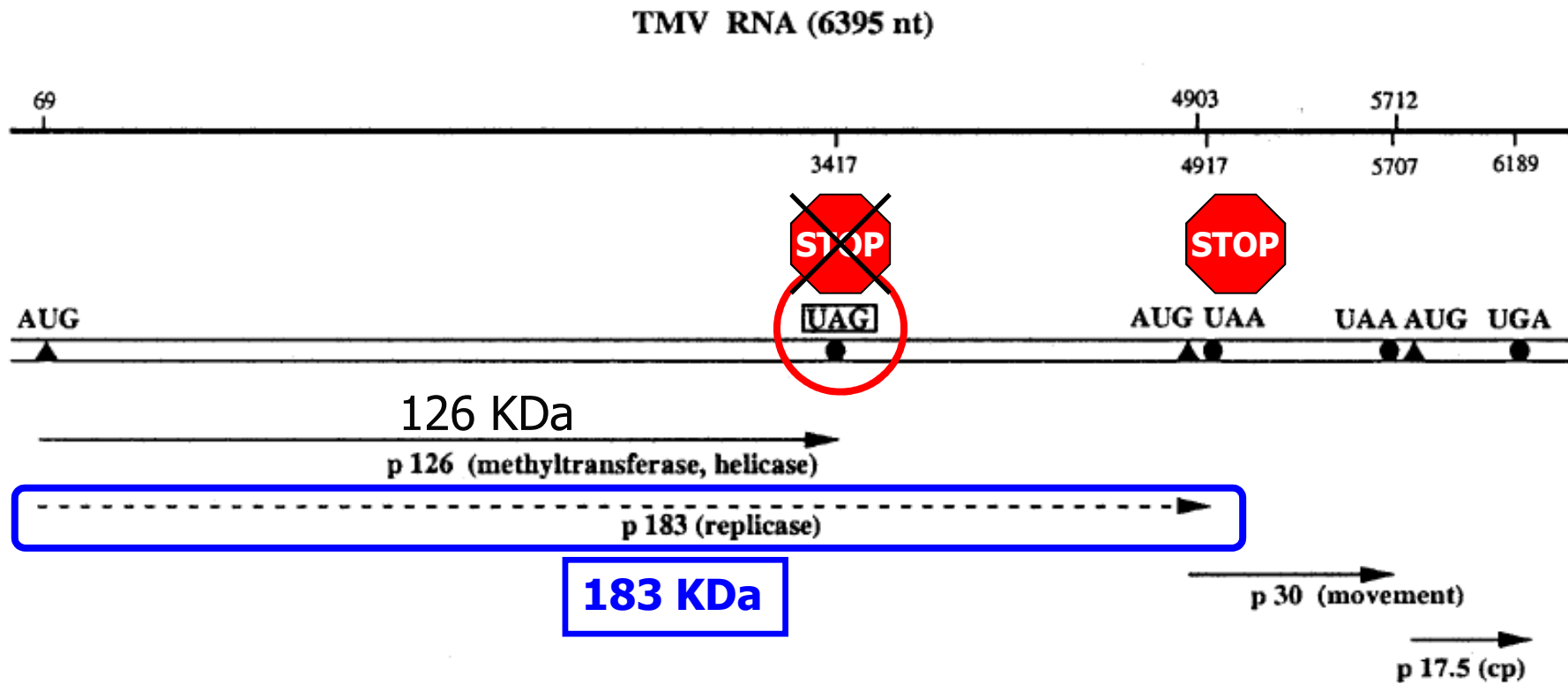
Virus (small genome) → **Readthrough** → Expansion of genetic information




Translational READTHROUGH

A regulatory mechanism of gene expression, extensively used by ssRNA viruses, which provides the differential production of more than one polypeptide from a single mRNA

Virus (small genome) → **Readthrough** → Expansion of genetic information



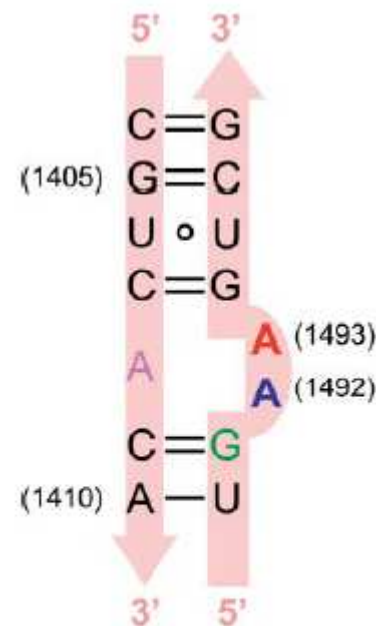
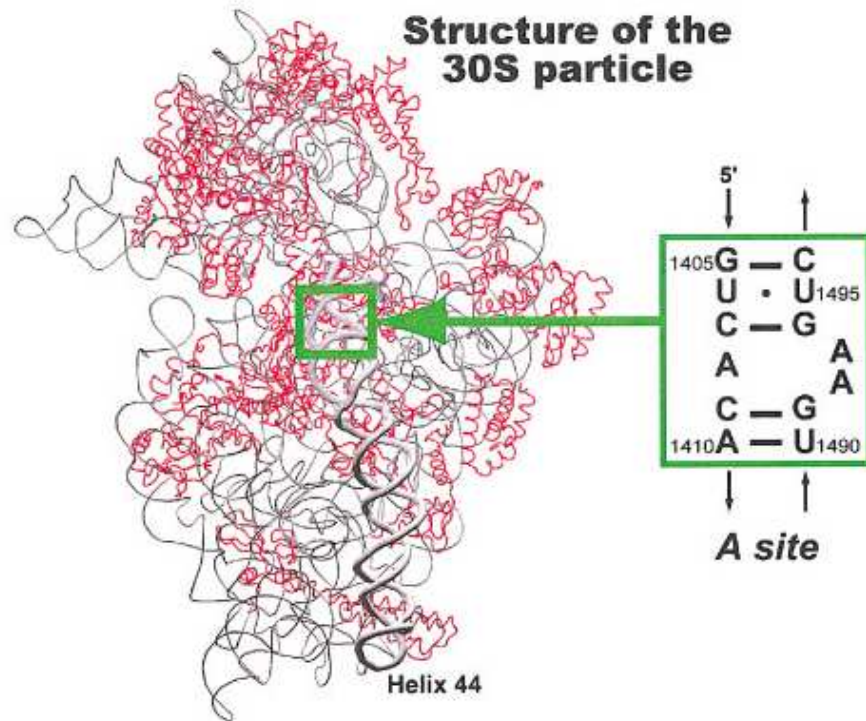
Termination codons can be "Leaky" stop signals

Virus	Genus	"Leaky" termination codon	Readthrough product/function
Enterobacteria phage Q β	Allolevivirus	UGA	Coat protein extension; assembly
Murine leukemia virus (MuLV)	Gammaretrovirus	UAG	Reverse Transcriptase
Sindbis virus (SIN)	Alphavirus	UGA	Replicase
Tomato bushy stunt virus (TBSV)	Tombusvirus	UAG	Replicase
Carnation mottle virus (CarMV)	Carmovirus	UAG	Replicase
Tobacco necrosis virus (TNV)	Necrovirus	UAG	Replicase
Maize chlorotic mottle virus (MCMV)	Machlomovirus	UAG	Replicase
Barley yellow dwarf virus (BYDV)	Luteovirus	UAG	Coat protein extension; aphid transmission
Potato leafroll virus (PLRV)	Poterovirus	UAG	Coat protein extension; aphid transmission
Pea enation mosaic virus (PEMV) RNA-1	Enamovirus	UGA	Coat protein extension; aphid transmission
 Tobacco mosaic virus (TMV)	Tobamovirus	UAG	Replicase
Tobacco rattle virus (TRV) RNA-1	Tobravirus	UGA	Replicase
Peanut clump virus (PCV) RNA-1	Pecluvirus	UGA	Replicase
Soil-borne wheat mosaic virus (SBWMV) RNA-1	Furovirus	UGA	Replicase
Soil-borne wheat mosaic virus (SBWMV) RNA-2		UGA	Coat protein extension; fungus transmission
Potato mop-top virus (PMTV) RNA-1	Pomovirus	UGA	Replicase
Potato mop-top virus (PMTV) RNA-3		UAG	Coat protein extension
Beet soil-borne virus (BSBV) RNA-1	Pomovirus	UAA	Replicase
Beet soil-borne virus (BSBV) RNA-2		UAG	Coat protein extension
Broad bean necrosis virus (BBNV) RNA-2	Pomovirus	UAA	Coat protein extension
Beet necrotic yellow vein virus (BNYVV) RNA-2	Benyvirus	UAG	Coat protein extension; fungus transmission
Turnip yellow mosaic virus (TYMV)	Tymovirus	UAG	Replicase extension ?

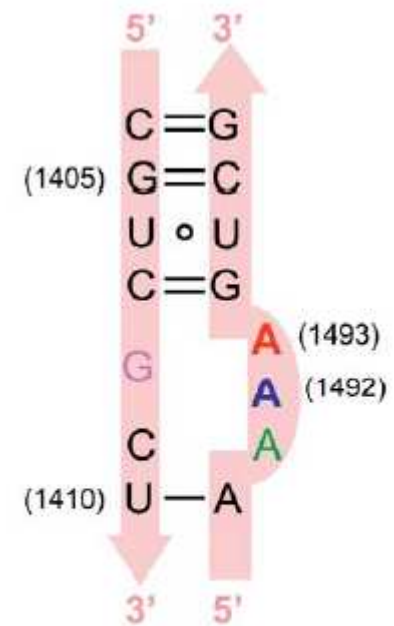
Ribosome Decoding Site

Region located within the **A-site** in the ribosomal small subunit

The Decoding Site contains two **adenine nucleotides (A1492 and A1493)** that **monitor codon/anticodon base pairing**



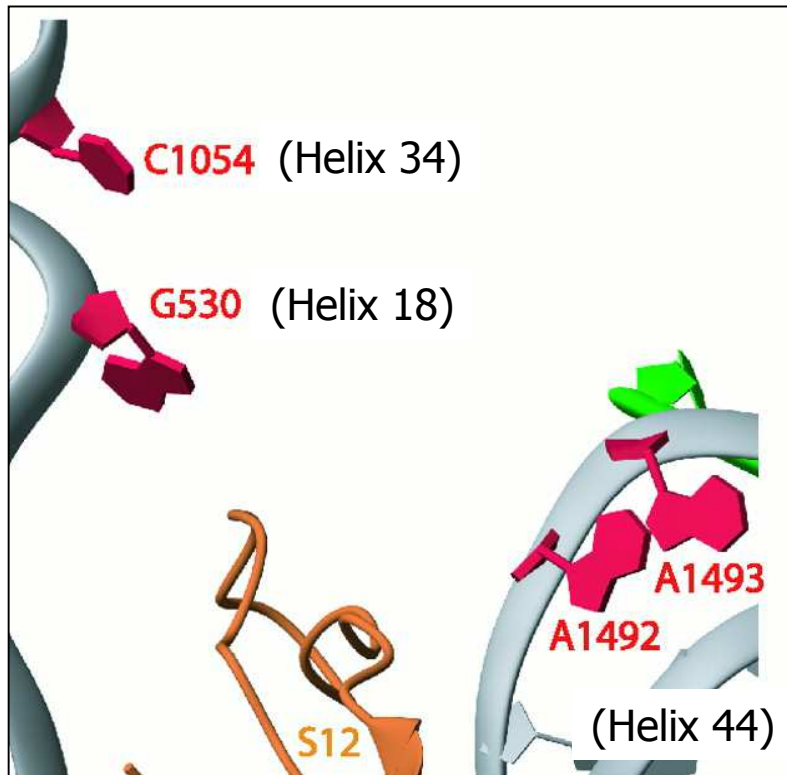
Prokaryotic 16S



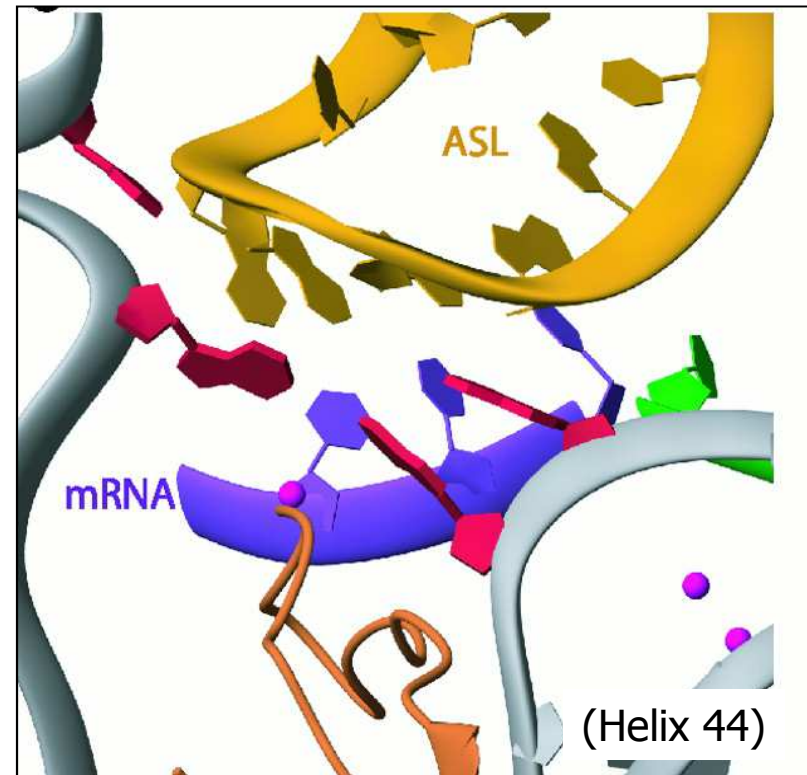
Eukaryotic 18S
cytoplasmic

The decoding site switches to a “closed” conformation when the correct (cognate) aa-tRNA enters the A-site

“open” conformation



“closed” conformation

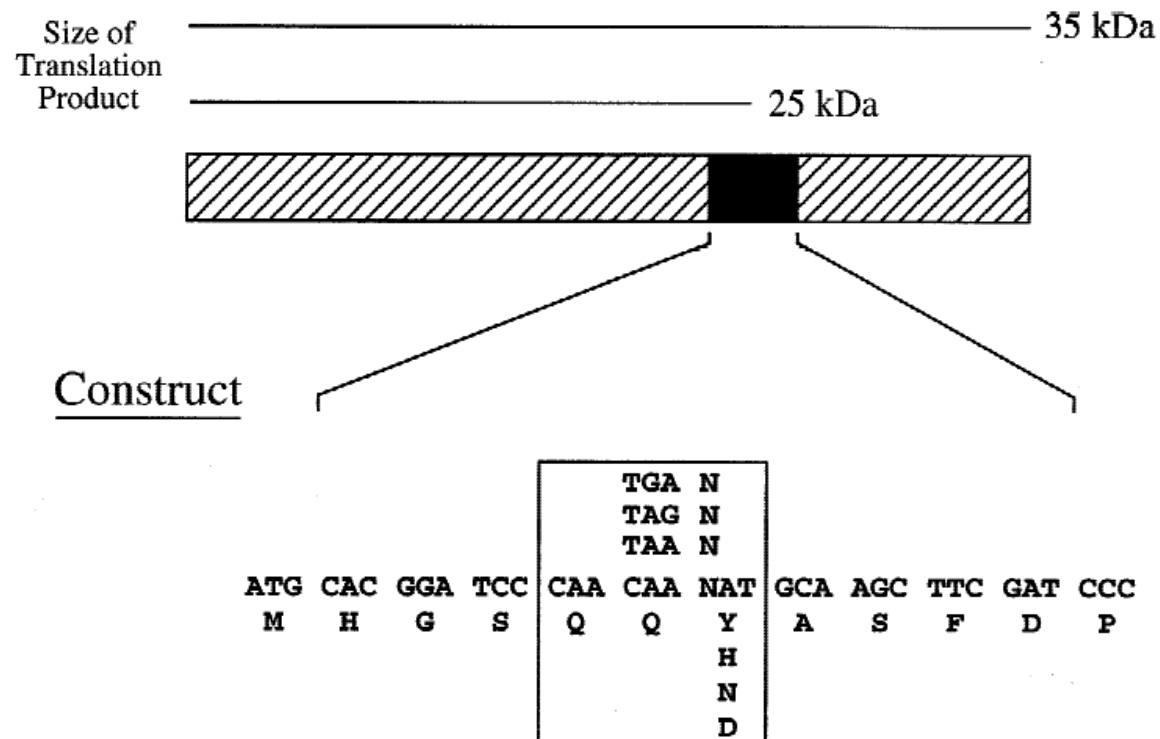


When the codon and a tRNA-ASL bind in the A-site, A1492 and A1493 flip out to monitor the codon-anticodon interaction

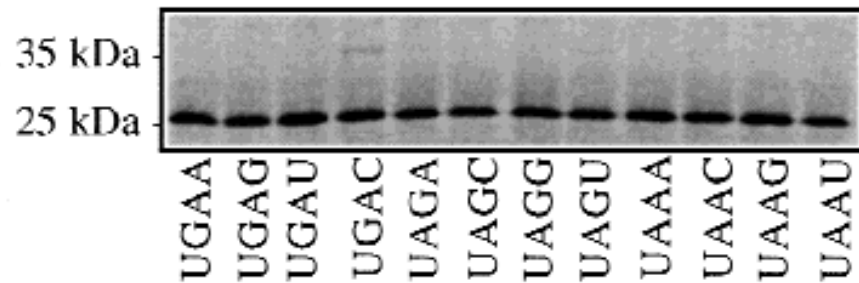
Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system

MARINA MANUVAKHOVA,^{1,3} KIM KEELING,² and DAVID M. BEDWELL^{1,2}

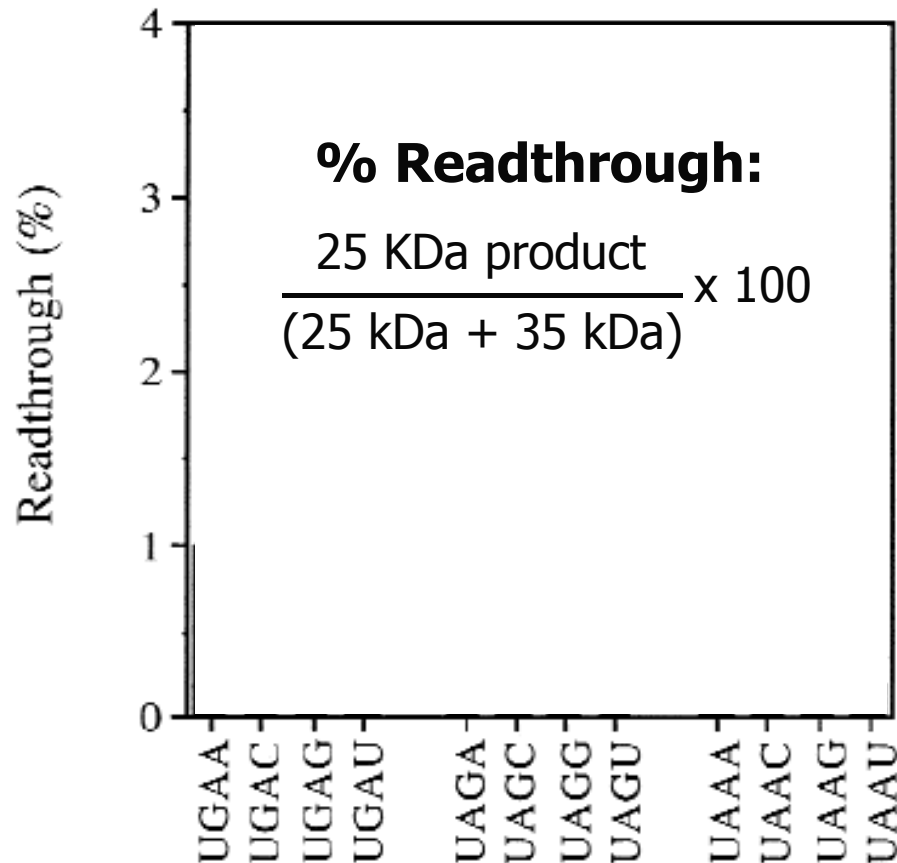
Experimental model: synthetic constructs bearing different termination signals are translated *in-vitro* in the presence of [³⁵S]-Met/Cys and the resulting [³⁵S]-labeled polypeptides are analyzed by SDS-PAGE



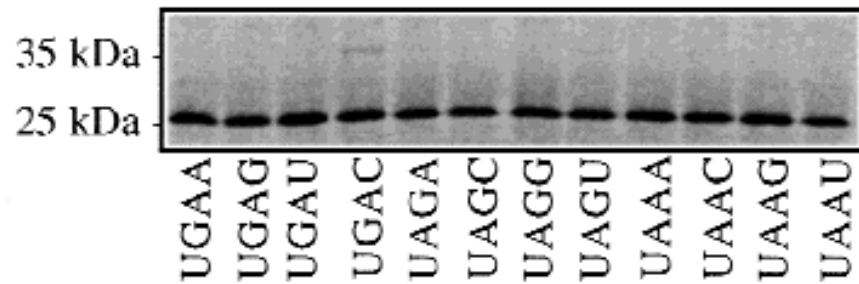
Readthrough is influenced by the sequence context



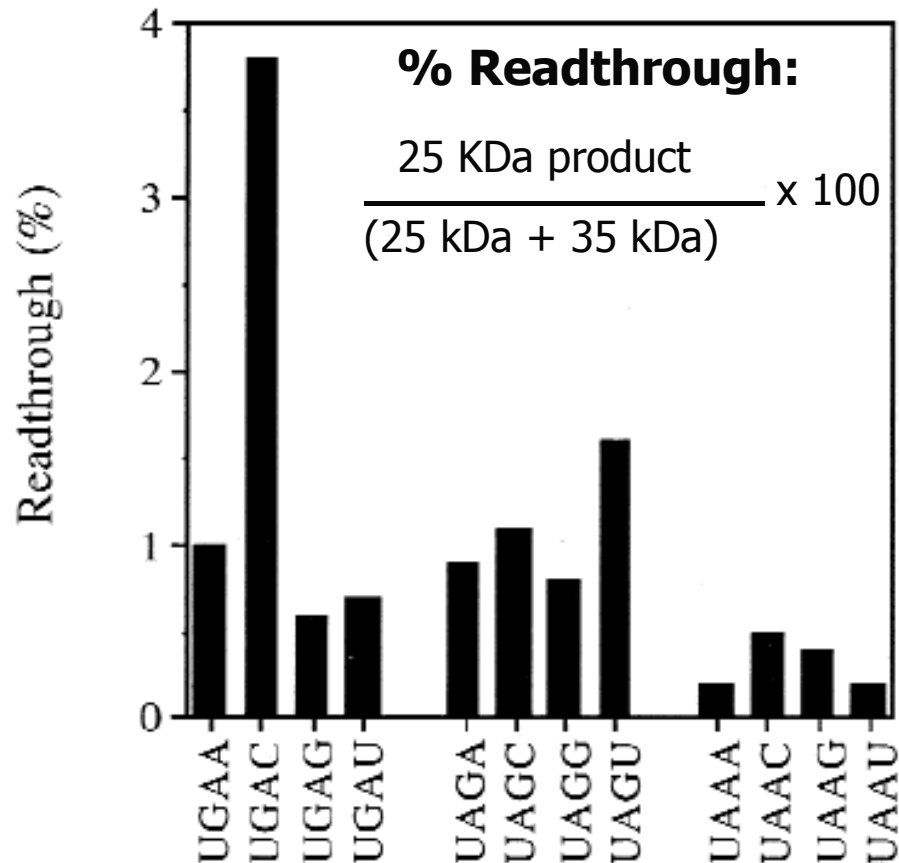
The termination signal is recognized as an extended **tetranucleotide** comprised of the stop codon and the **first nucleotide** that follows



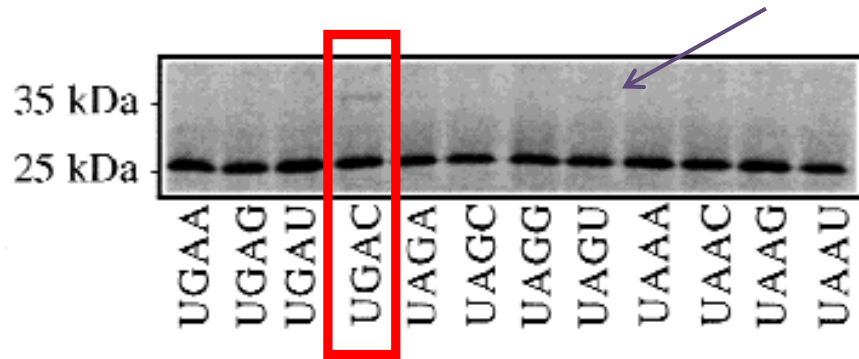
Readthrough is influenced by the sequence context



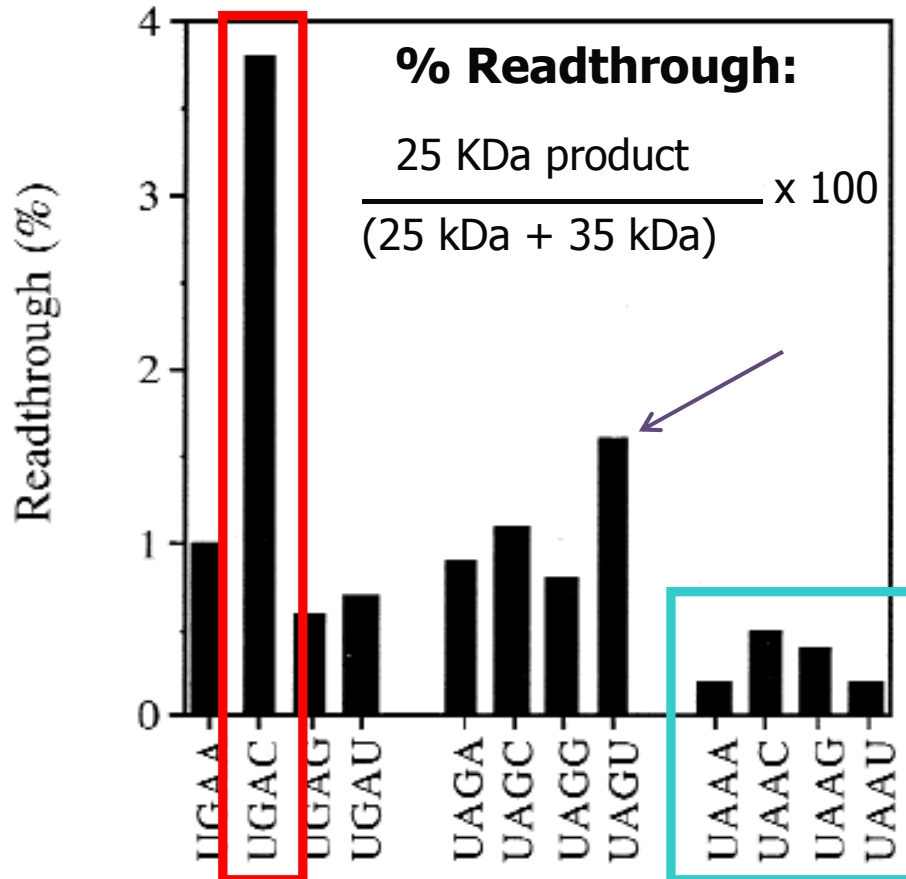
The termination signal is recognized as an extended **tetranucleotide** comprised of the stop codon and the **first nucleotide** that follows



Readthrough is influenced by the sequence context



The termination signal is recognized as an extended **tetranucleotide** comprised of the stop codon and the **first nucleotide** that follows



Basal Readthrough:

UGA-C 3-4%

Min termination efficiency

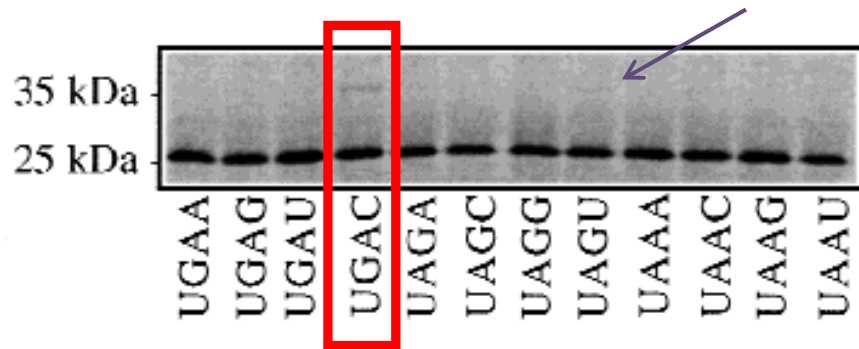
UAG-U 1.6%

Intermediate efficiency

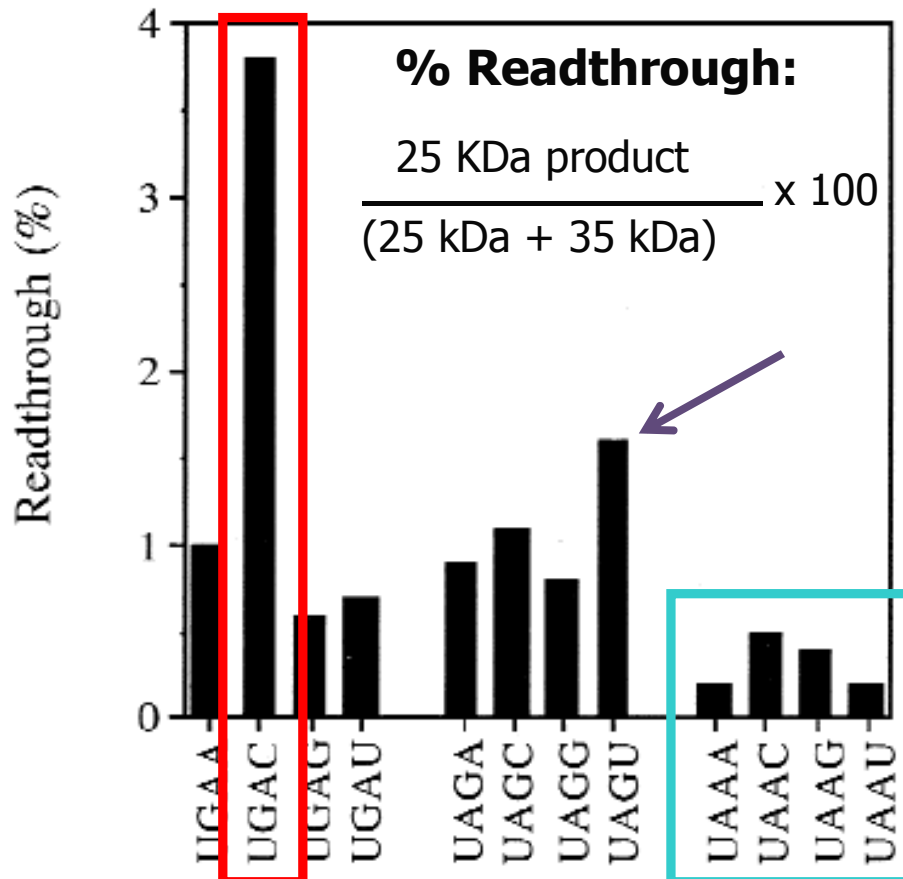
UAA(N) ~0.5%

Max termination efficiency

Readthrough is influenced by the sequence context



The termination signal is recognized as an extended **tetranucleotide** comprised of the stop codon and the **first nucleotide** that follows



Basal Readthrough:

UGA-C 3-4%

Min termination efficiency

UAG-U 1.6% Intermediate efficiency

UAA(N) ~0.5%

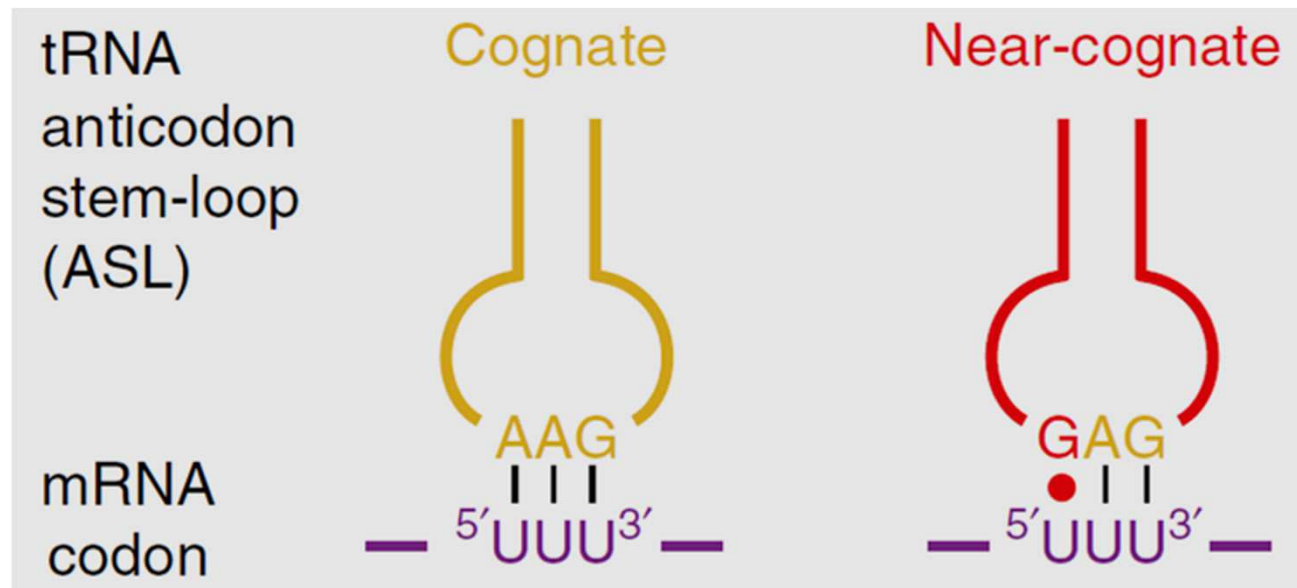
Max termination efficiency

Termination efficiency

UAA > UAG ≥ UGA

READTHROUGH

The occurrence of a basal readthrough prepares the ground for the use of molecules that are able to decrease the efficiency of translation termination, thus increasing the efficiency of readthrough itself



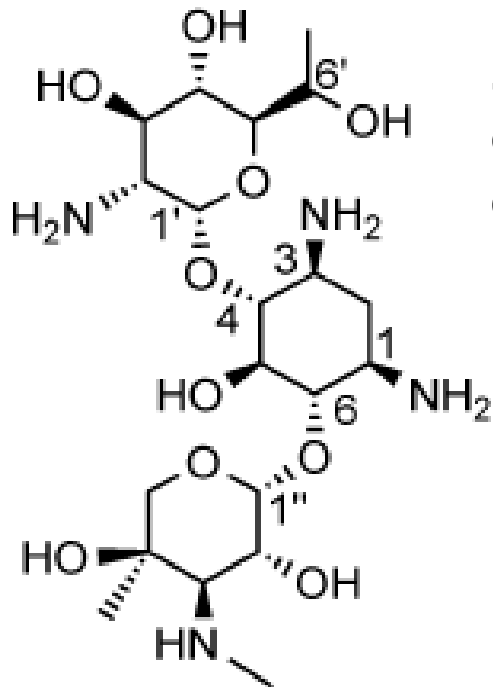
Aminoglycosides

A group of molecules belonging to the class of antibiotics

Aminoglycosides bind the decoding site within the A-site in the ribosomal small subunit

Mechanism of action

Stabilization of the decoding site (A1492 and A1493) in a conformation similar to that induced by the incorporation of a cognate tRNA (loss of proofreading capacity)

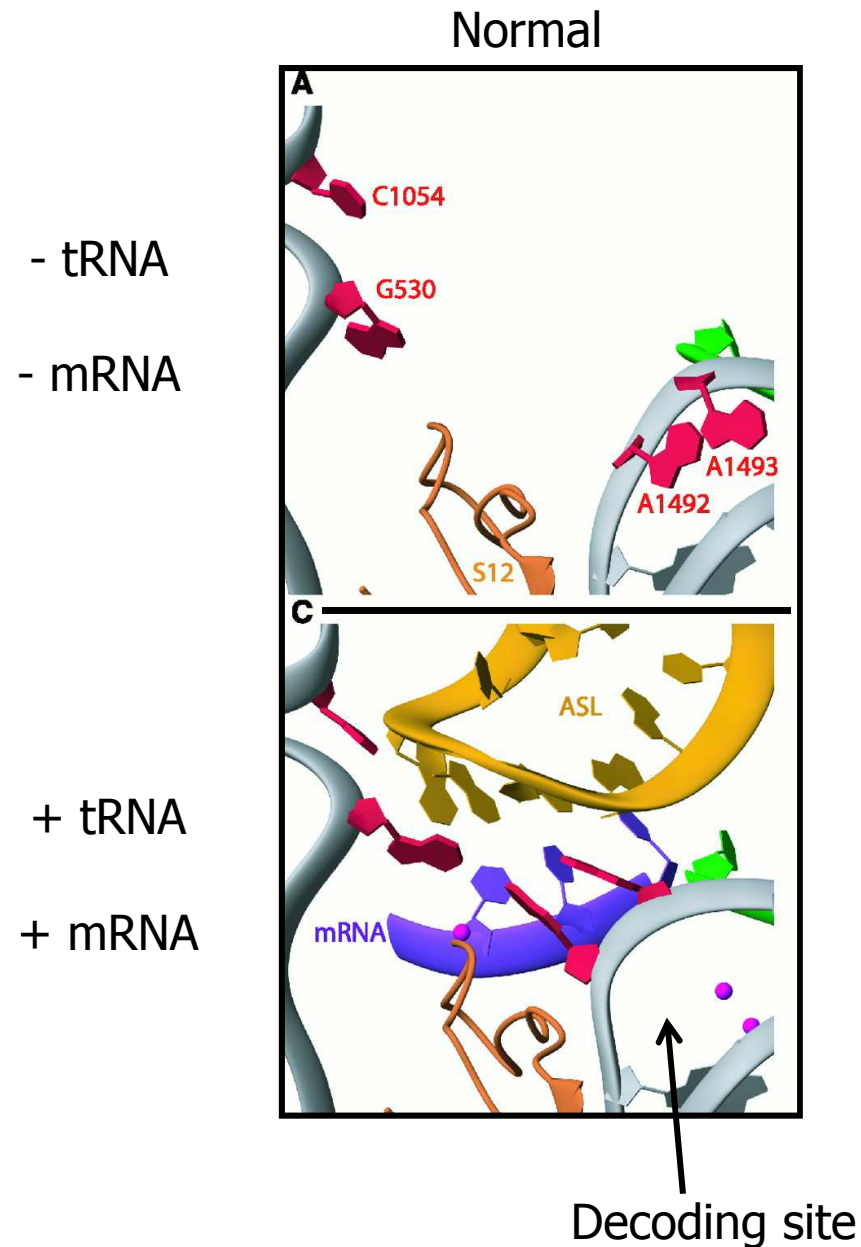


Geneticin (G418)

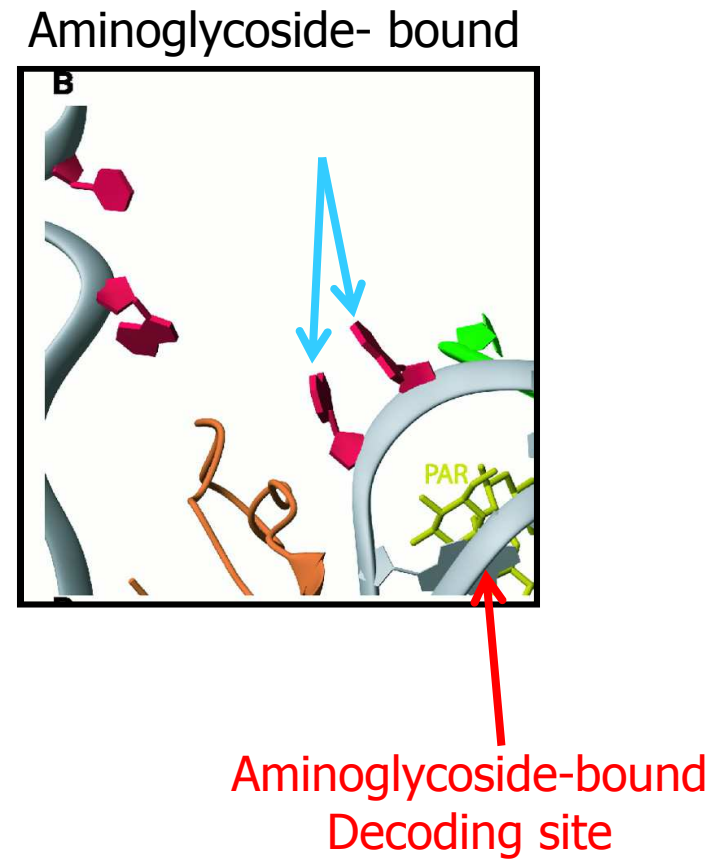
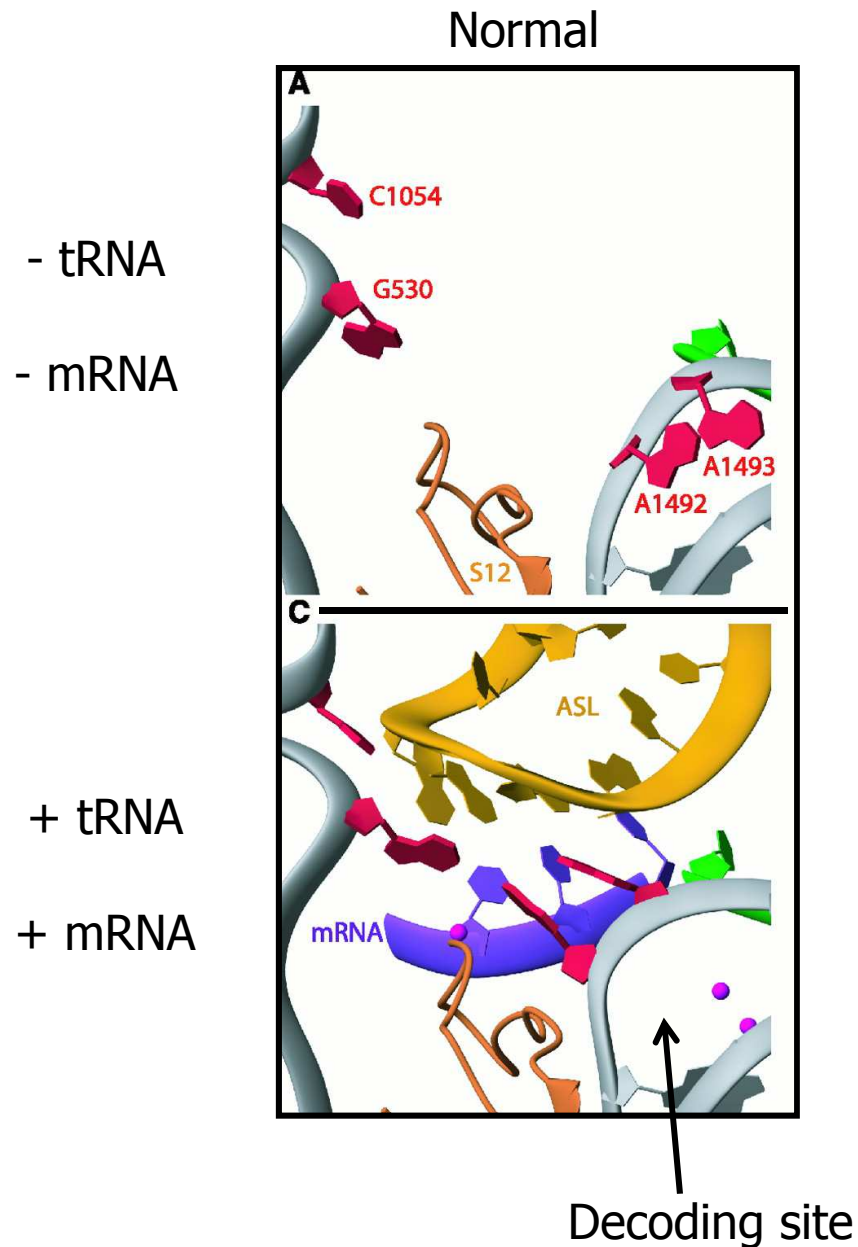
They alter the capacity of ribosome to discriminate between cognate and near-cognate aa-tRNA, thus leading to mis-incorporation of amino acids instead of binding release factors

near-cognate tRNA \approx cognate tRNA

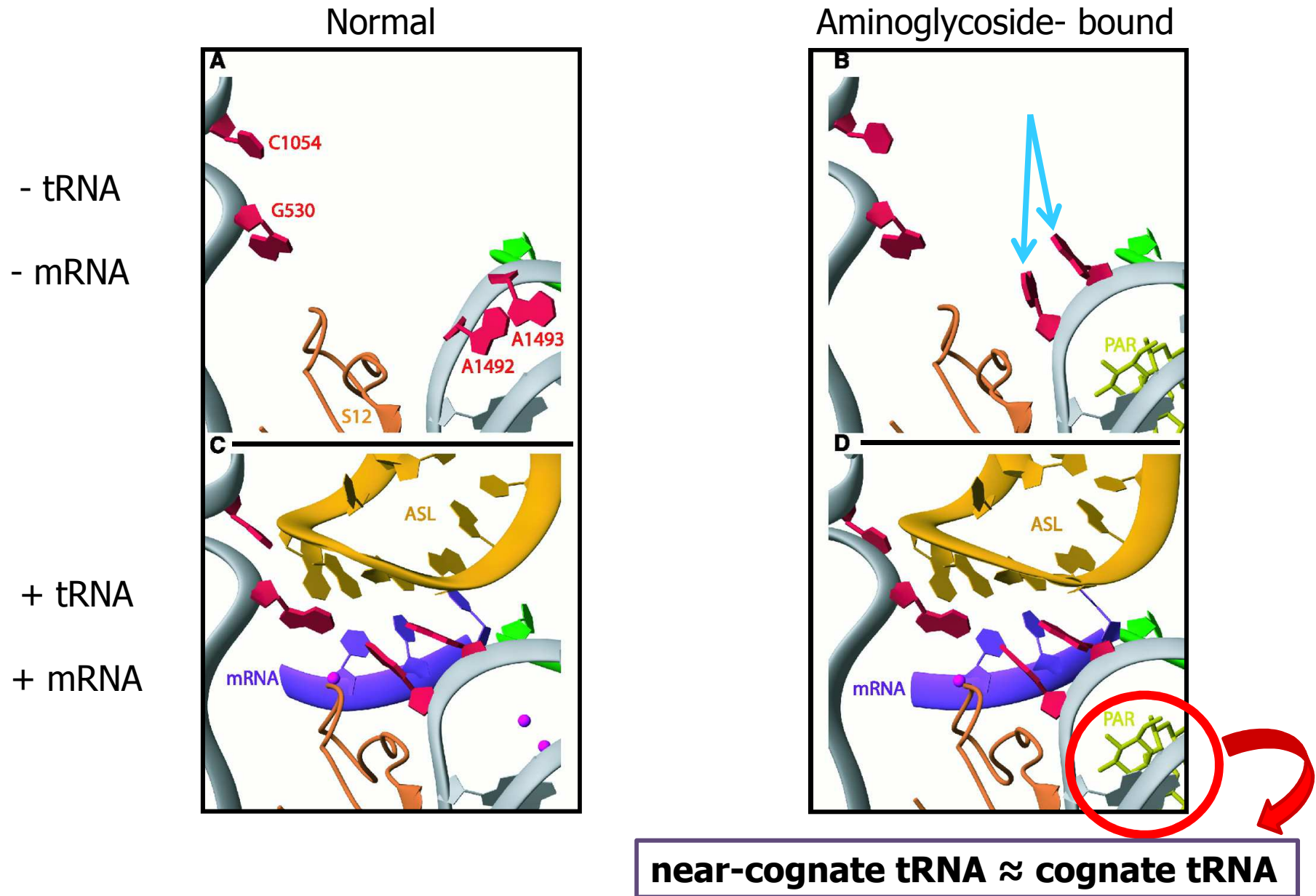
Aminoglycosides bind the decoding site and reduce ribosome fidelity



Aminoglycosides bind the decoding site and reduce ribosome fidelity

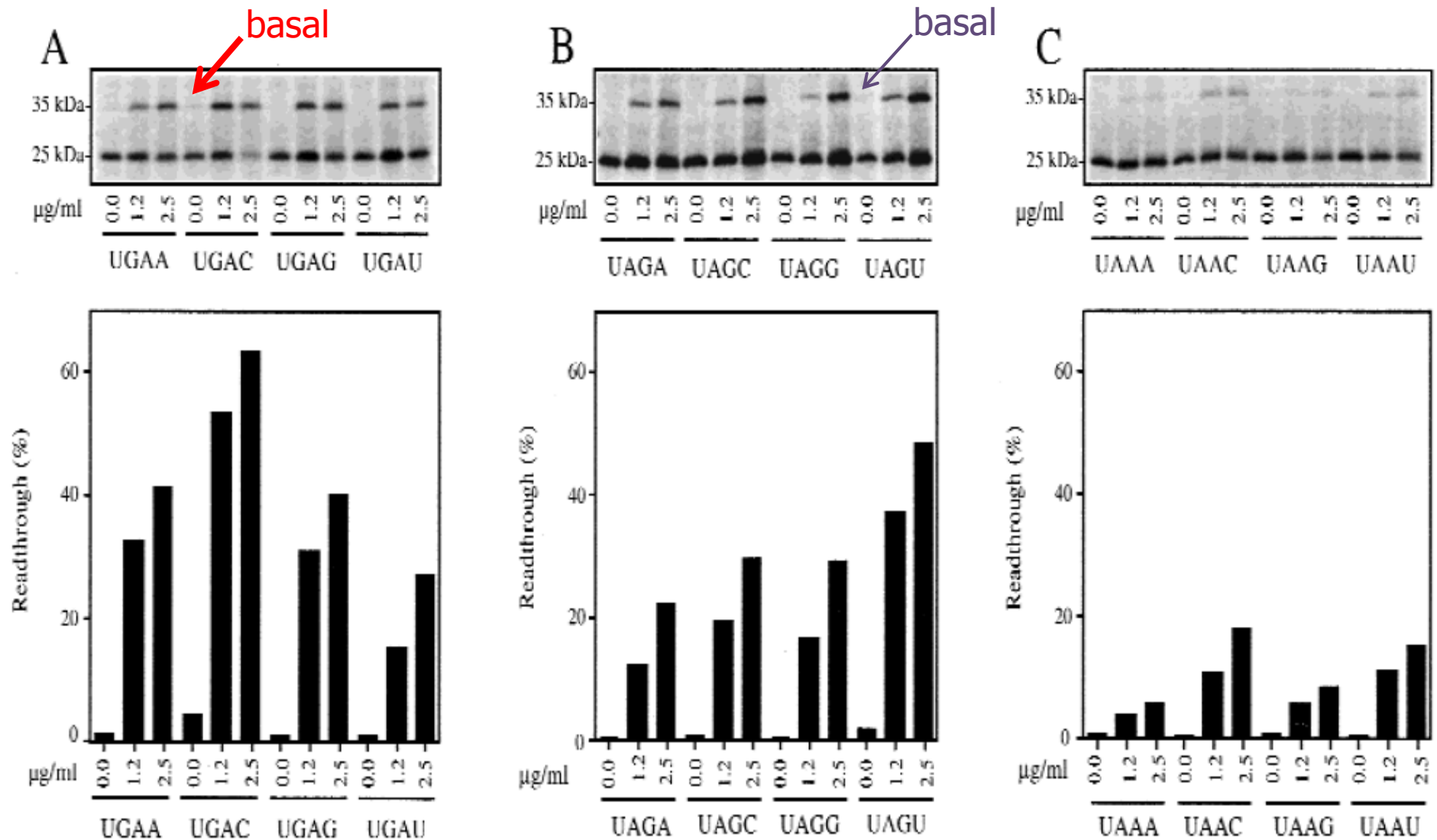


Aminoglycosides bind the decoding site and reduce ribosome fidelity



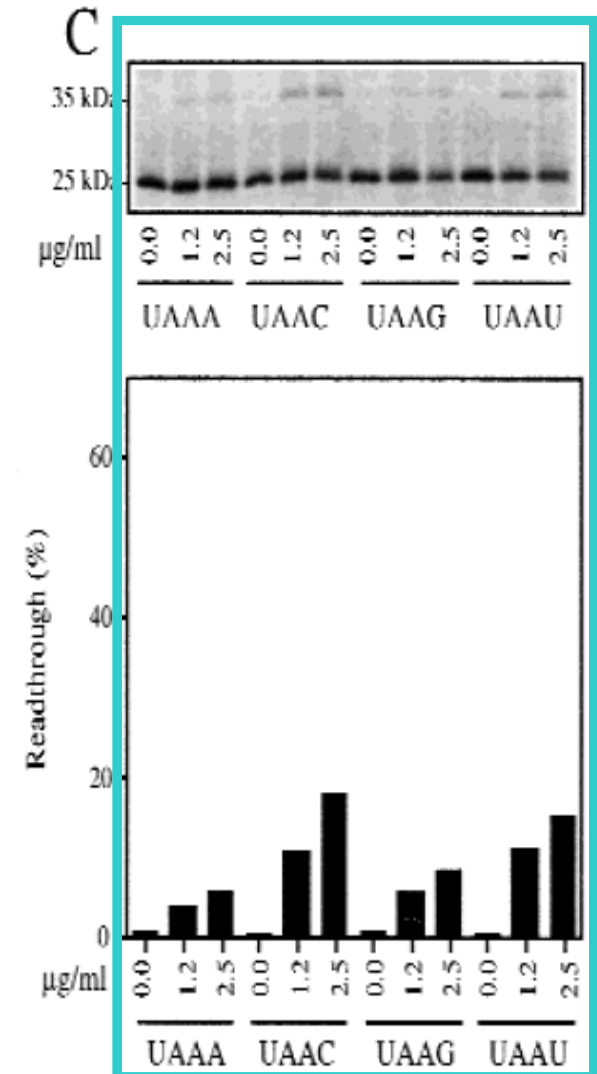
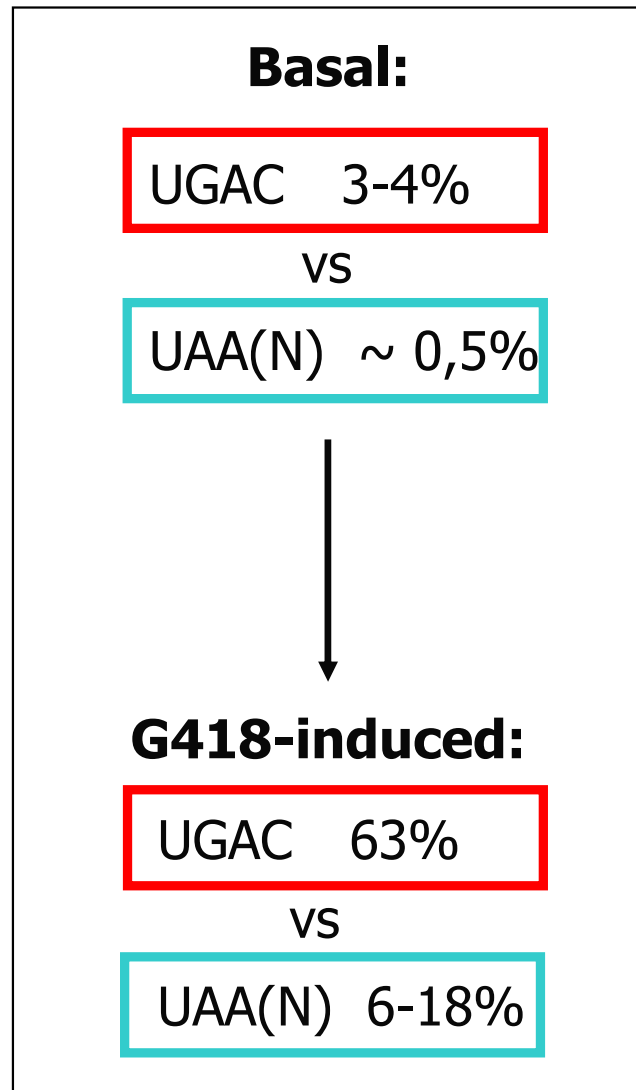
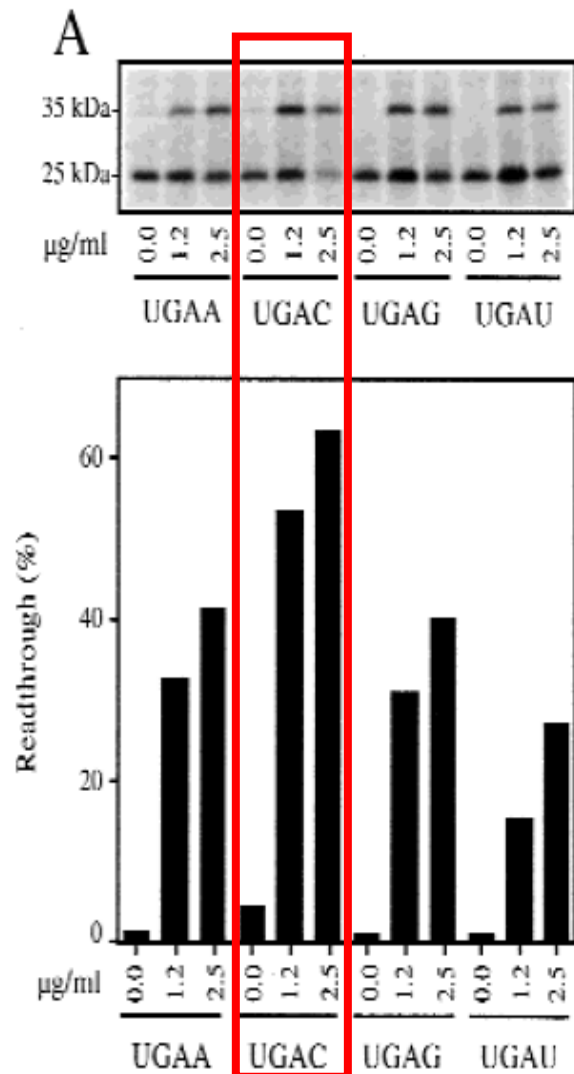
Treatment with aminoglycosides (G418) enhances readthrough

The context and/or stop codon with the **highest basal readthrough** (**UGAC** or **UGA** in general) display **the most efficient G418-induced readthrough**.



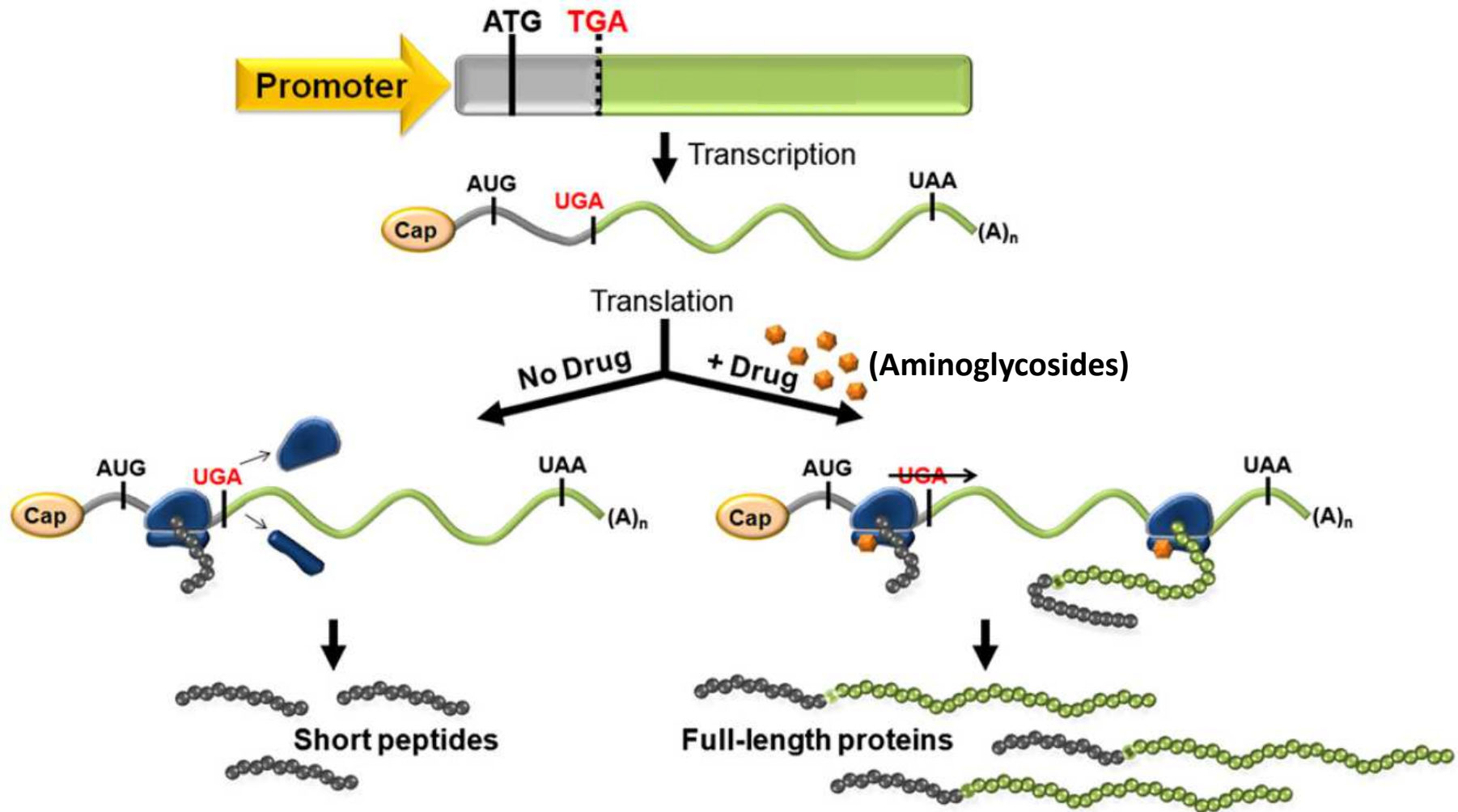
Treatment with aminoglycosides (G418) enhances readthrough

The context and/or stop codon with the **highest basal readthrough** (**UGAC** or **UGA** in general) are those with **the most efficient G418-induced readthrough**.

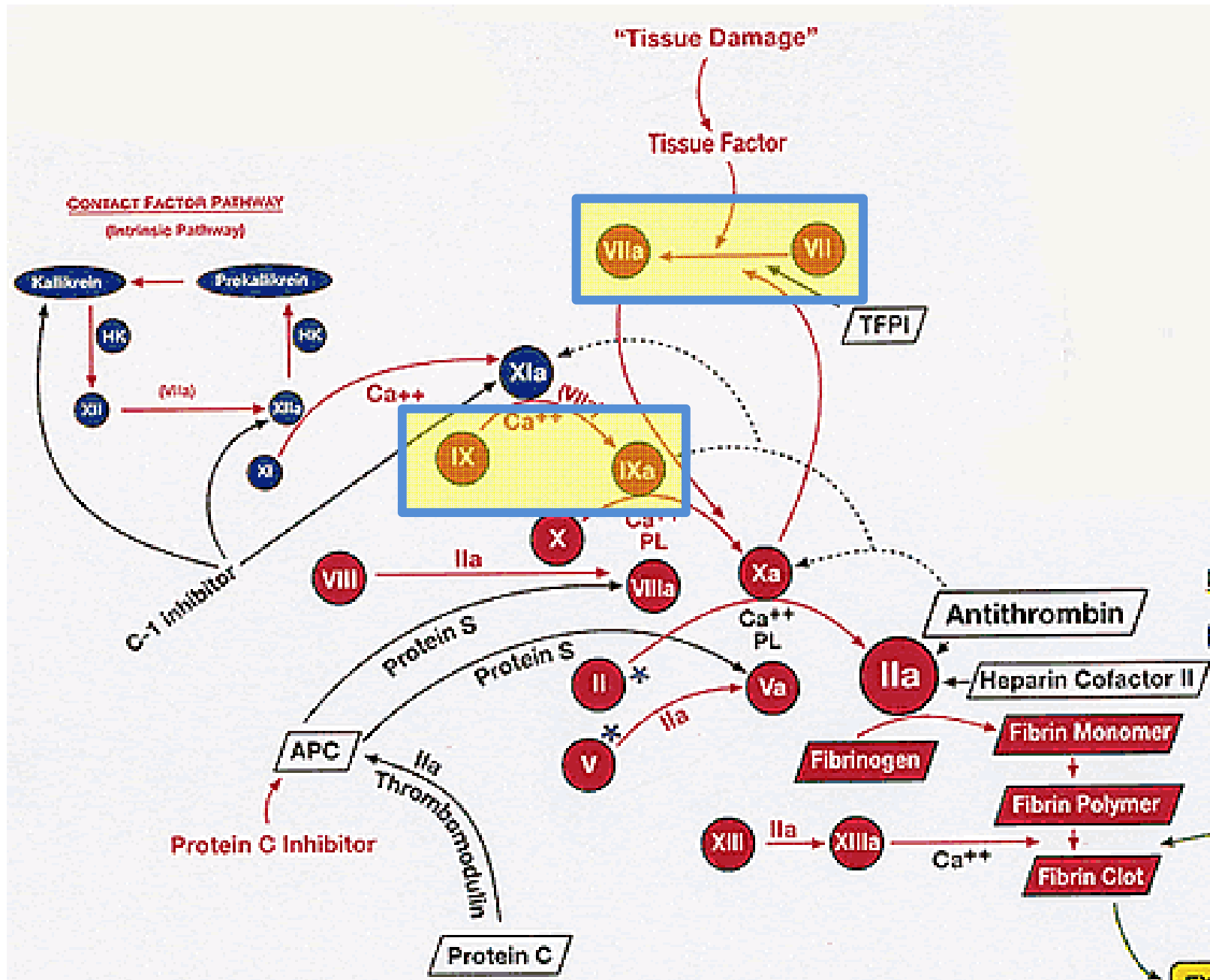


Parte II Metodologie Sperimentali

The readthrough process



The coagulation cascade



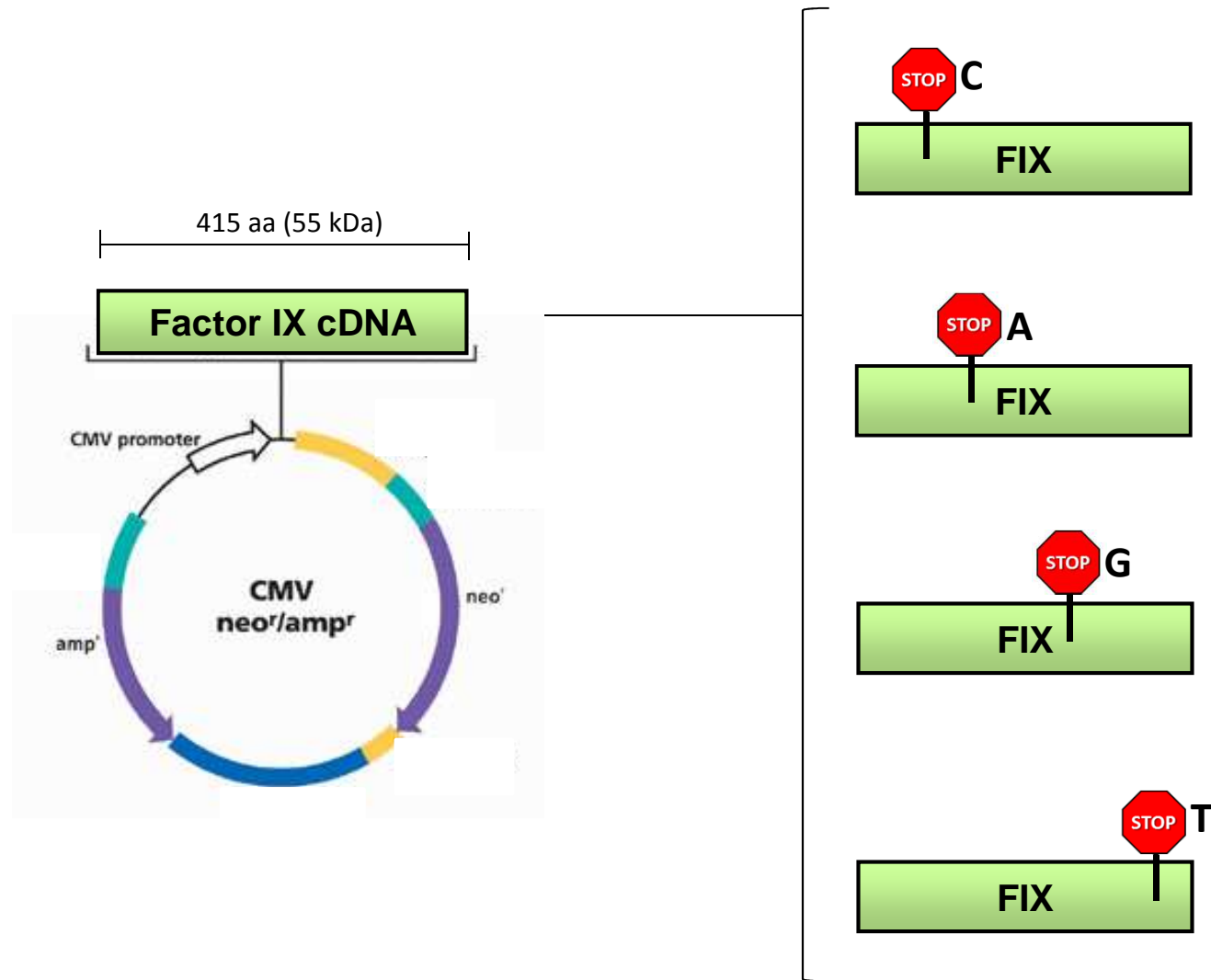
Workflow

1. Creation of expression vector(s)
2. Expression studies in eukaryotic cells
 - treatment of cells with readthrough-inducing drug
3. Analysis of protein isoforms
4. Evaluation of (pro-coagulant) activity

Workflow

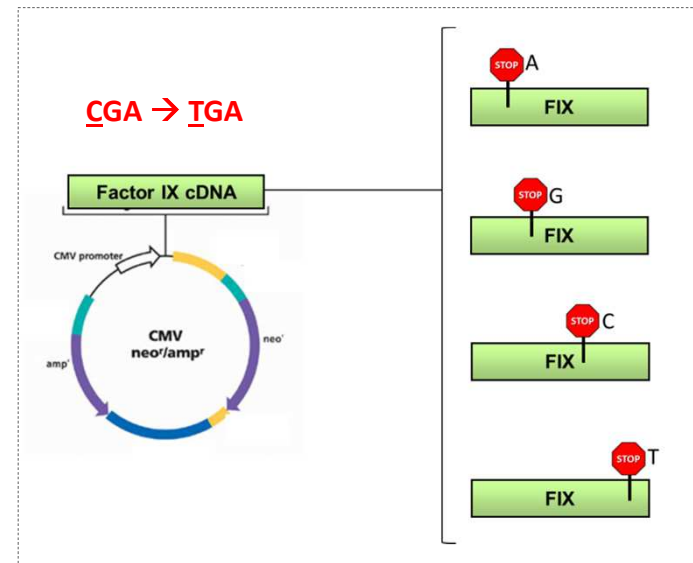
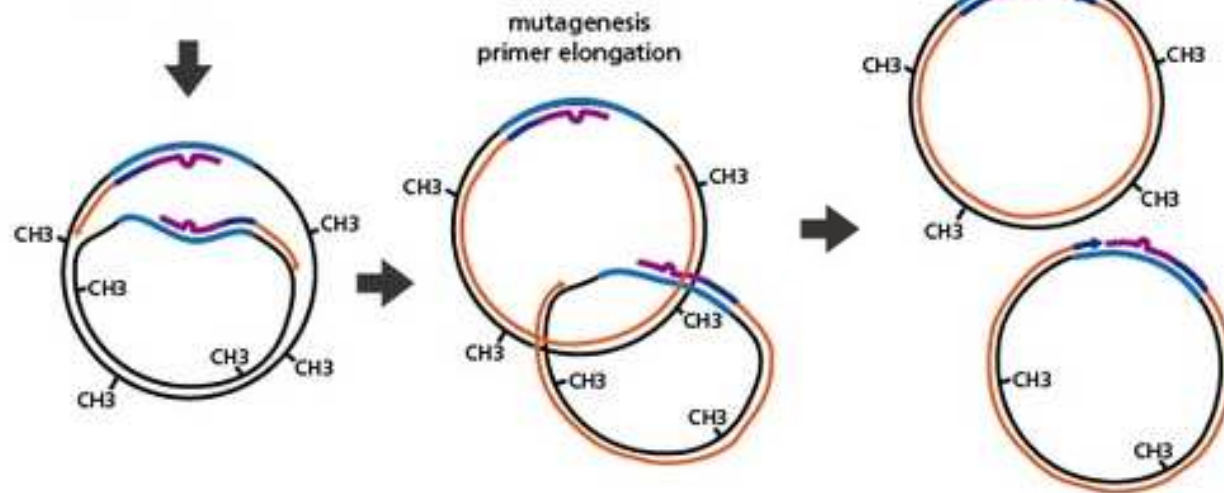
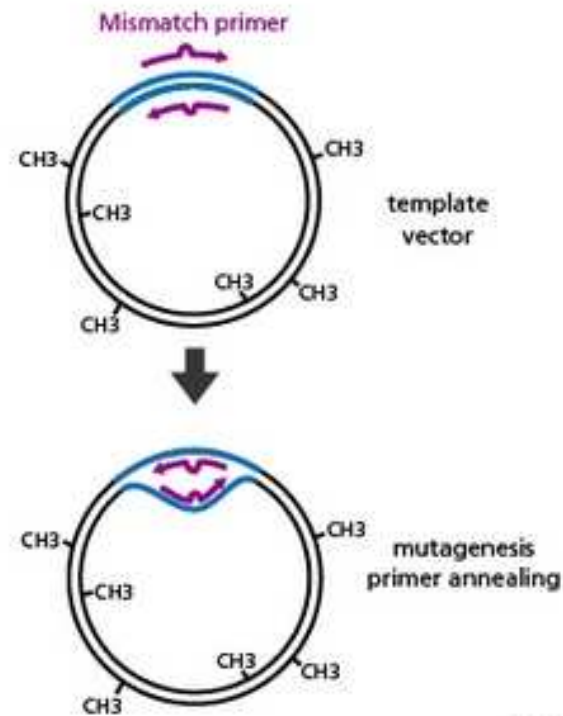
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1. Creation of expression vectors - Site-directed mutagenesis



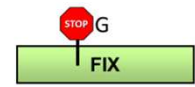
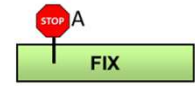
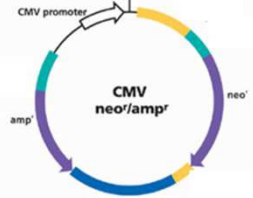
Nonsense mutation inserted: **CGA → TGA**
+ C-A-G-T (4th nucleotide)

1. Creation of expression vectors - Site-directed mutagenesis



CGA → IGA

Factor IX cDNA

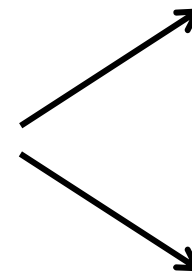
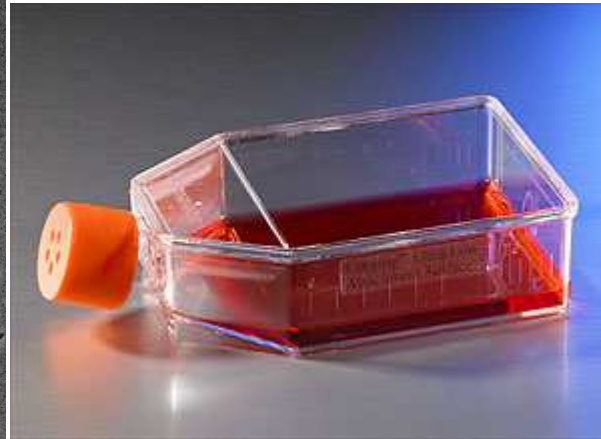
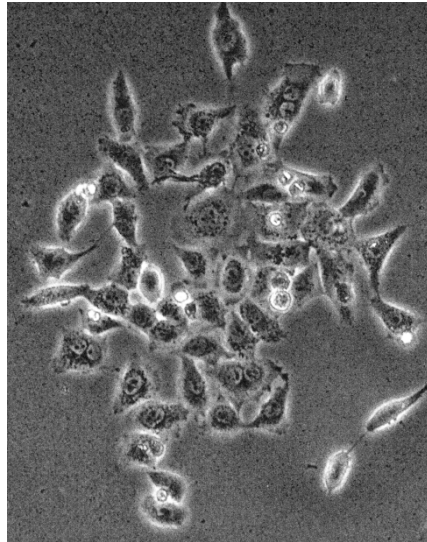


Workflow

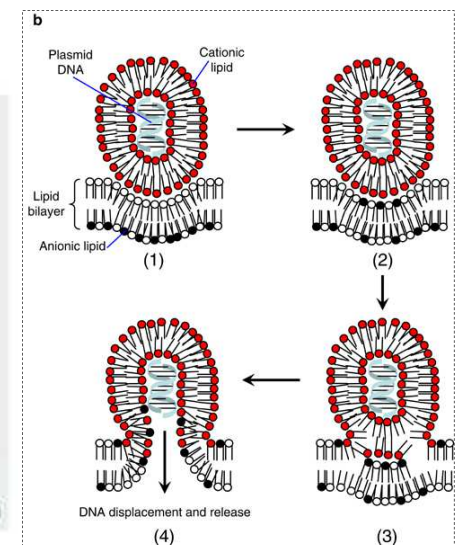
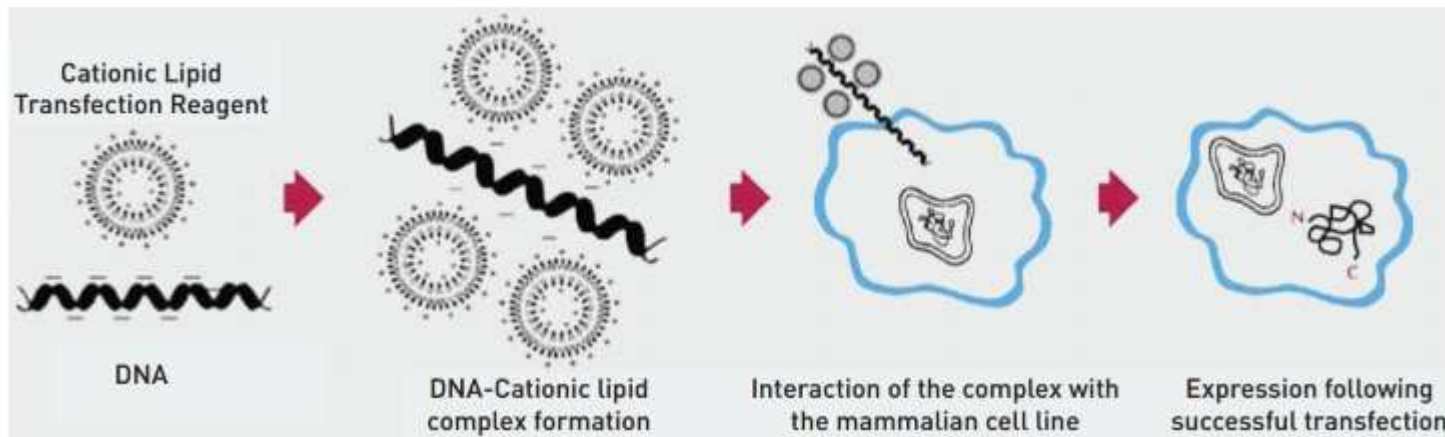
1. Creation of expression vector(s)
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2. Expression studies in eukaryotic cells - Transfection

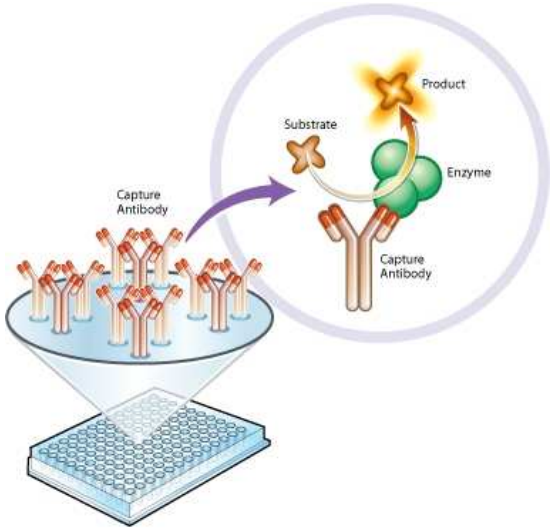
Human Embryonic Kidney (HEK) cells



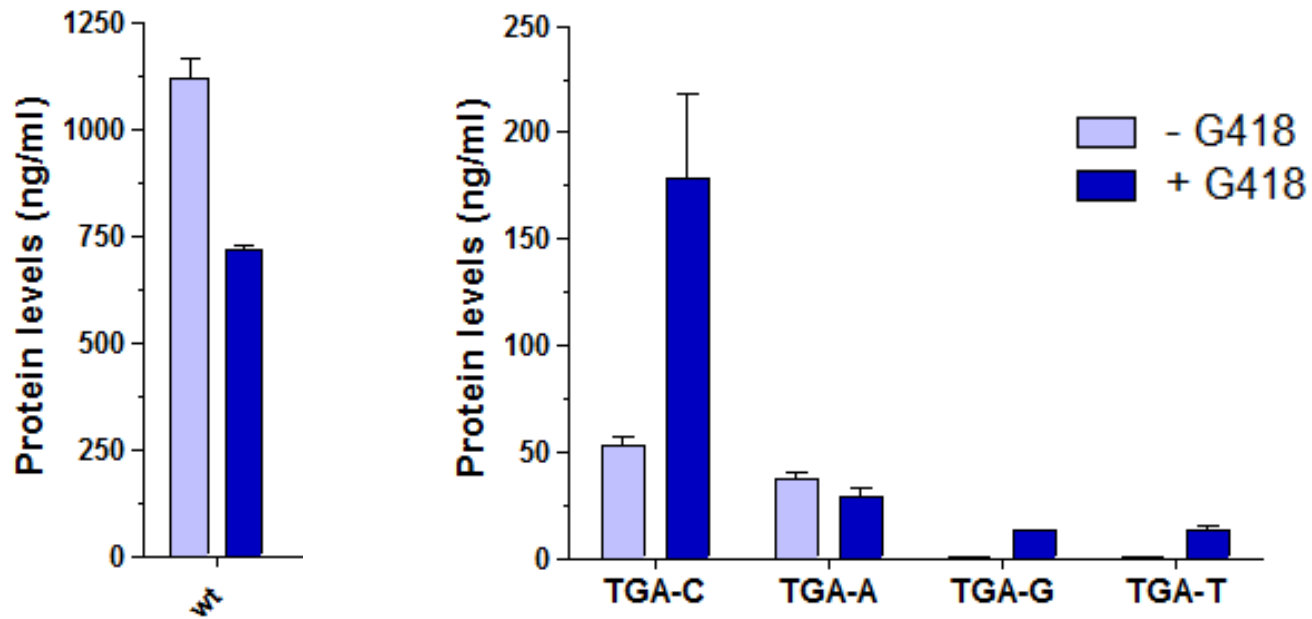
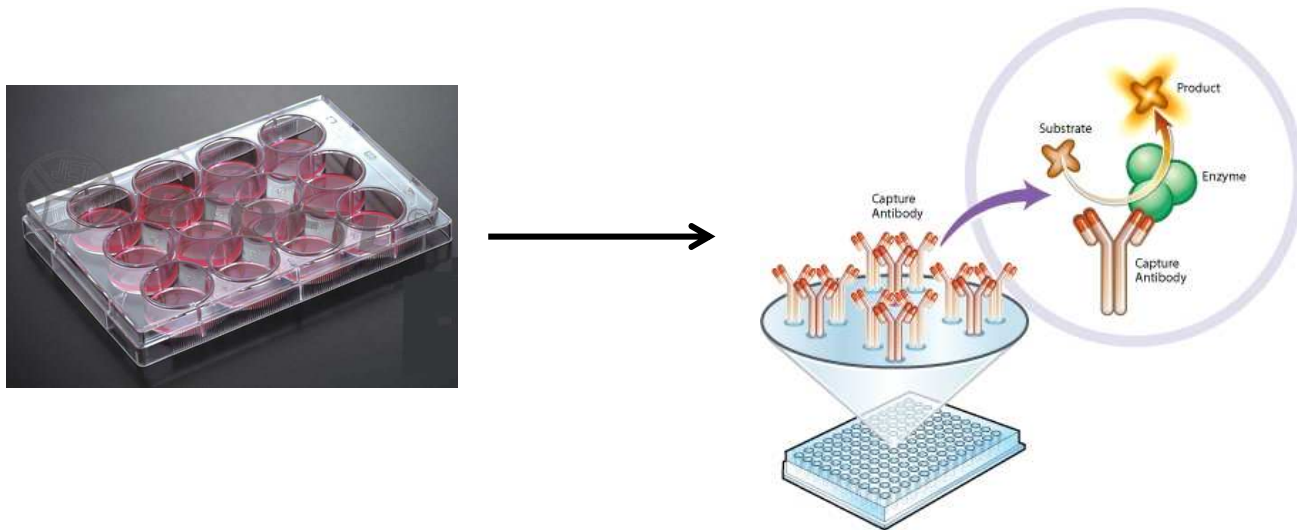
Lipofection



2. Expression studies in eukaryotic cells - ELISA



2. Expression studies in eukaryotic cells - ELISA



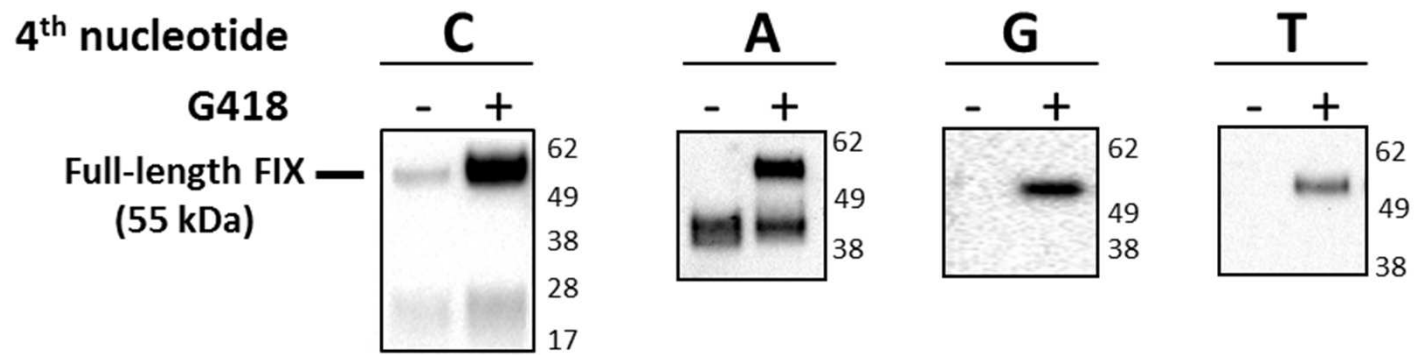
.....Readthrough?????

Workflow

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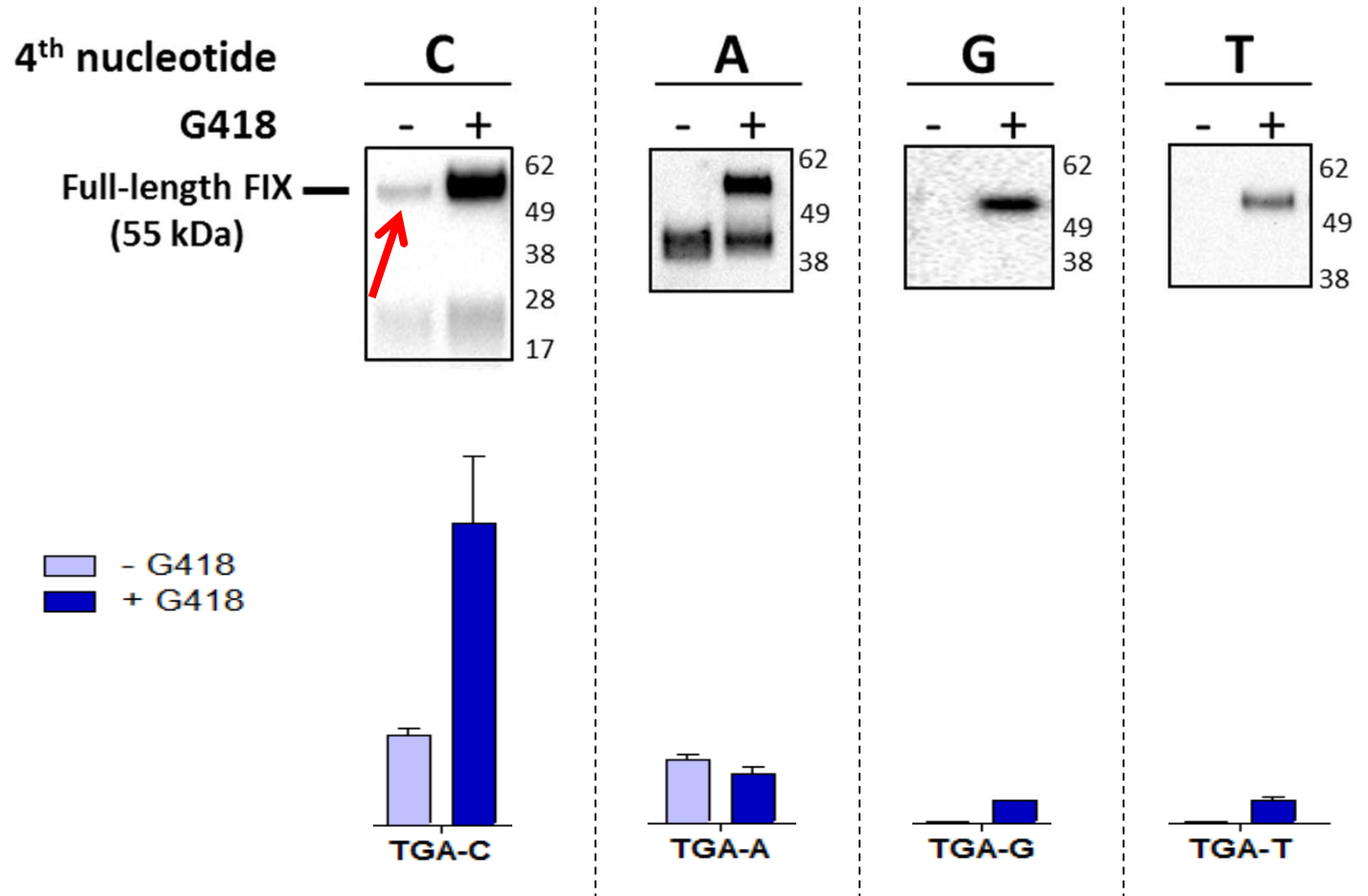
3. Analysis of protein isoforms – Western blotting

1



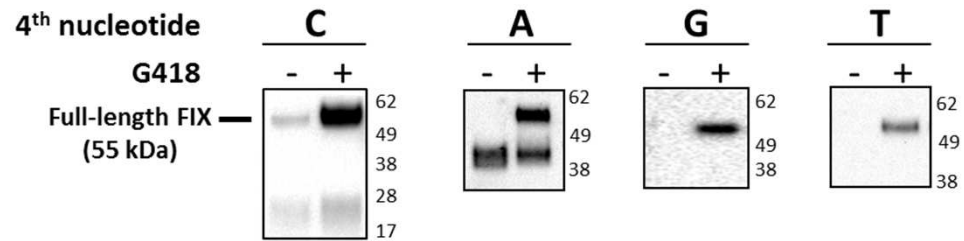
3. Analysis of protein isoforms – Western blotting

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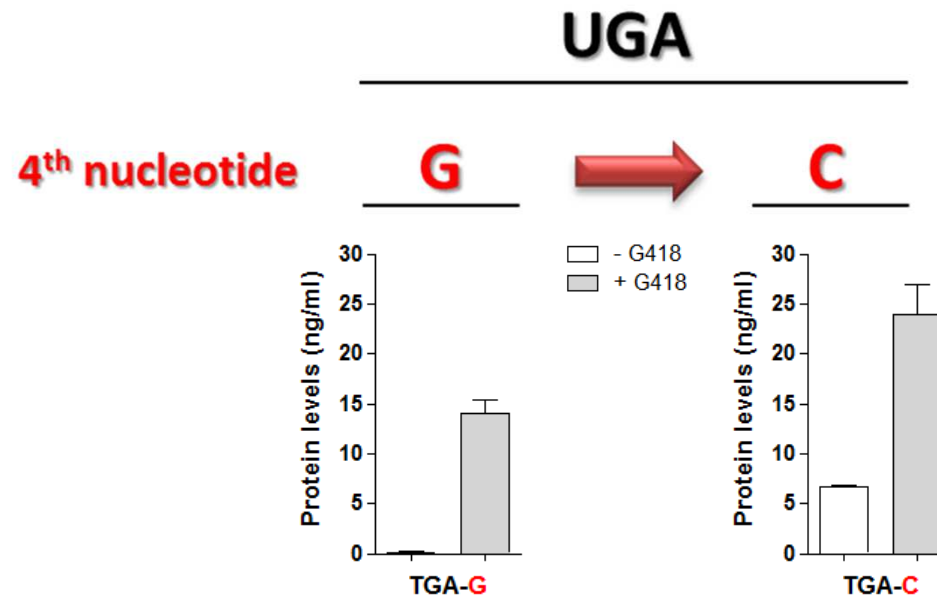


3. Analysis of protein isoforms – Sequence context effect

1

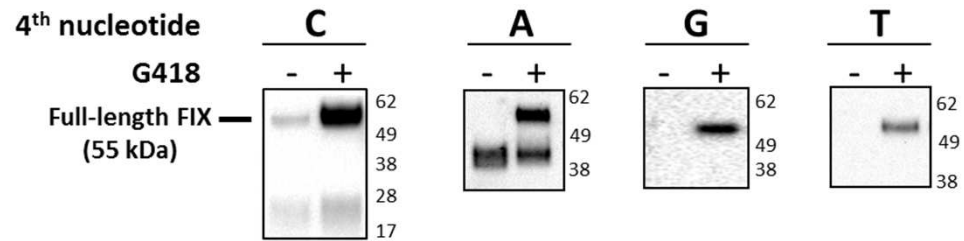


2

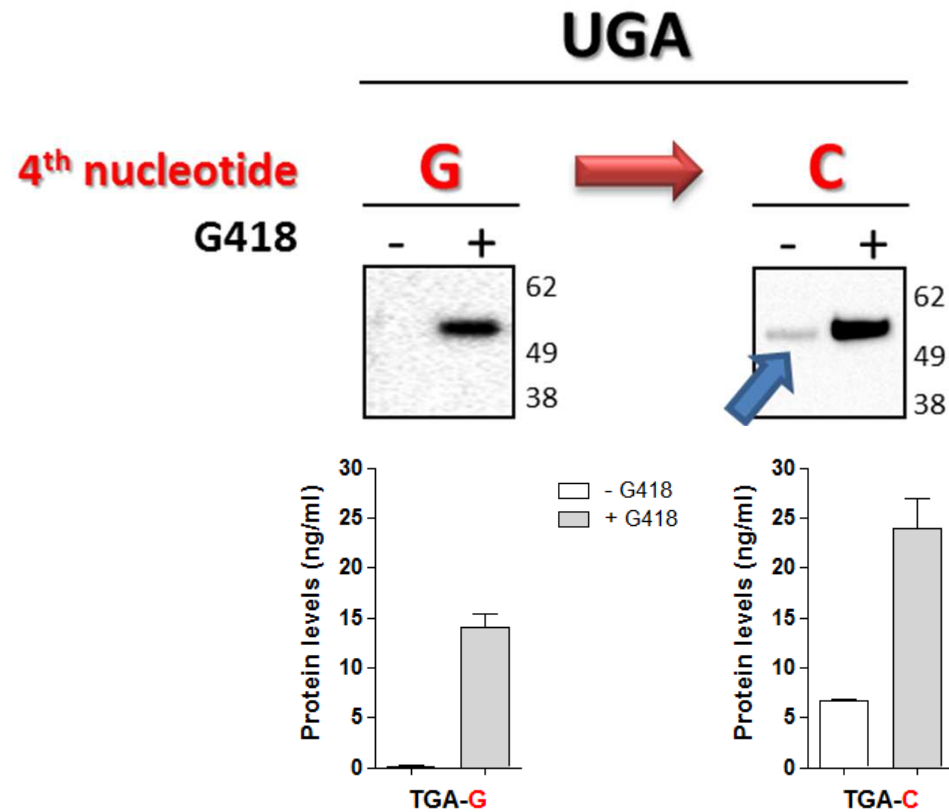


3. Analysis of protein isoforms – Sequence context effect

1



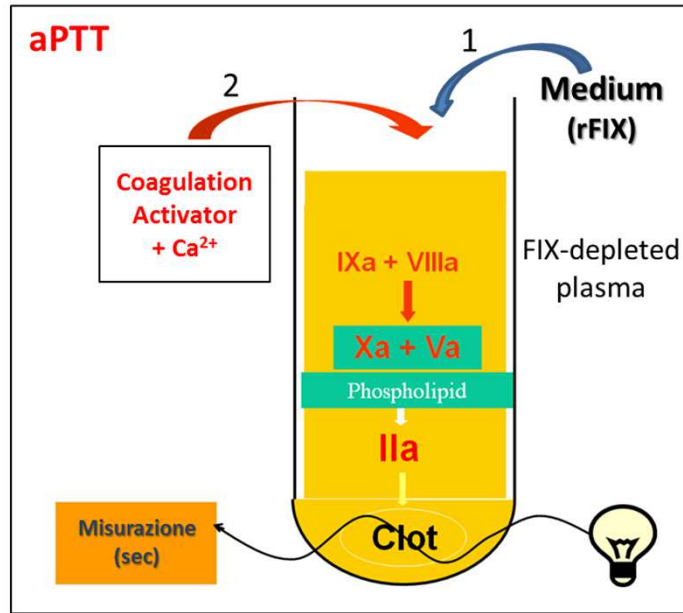
2



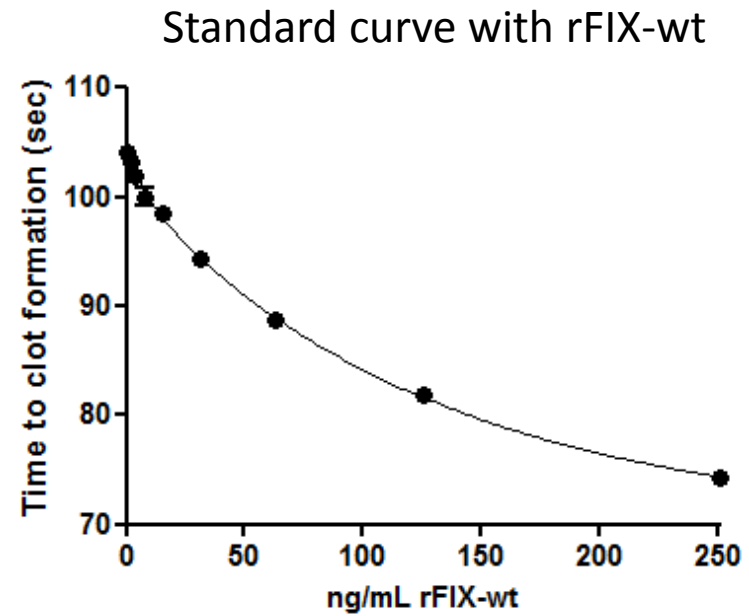
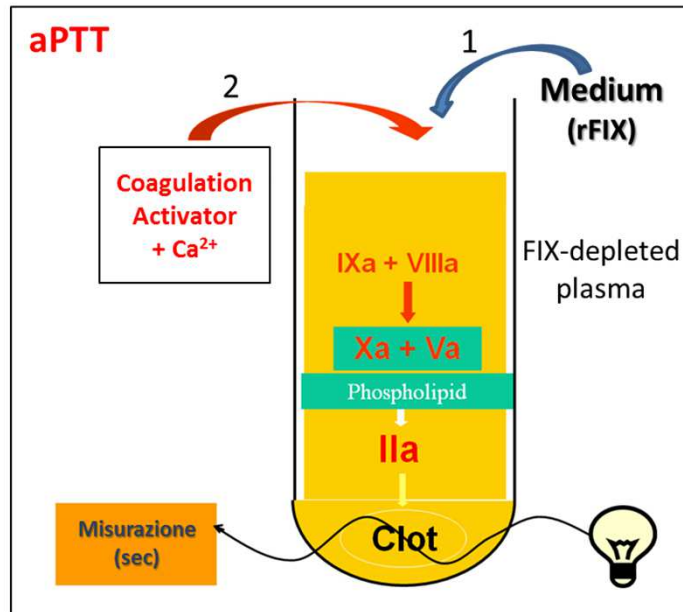
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4. Evaluation of (pro-coagulant) activity

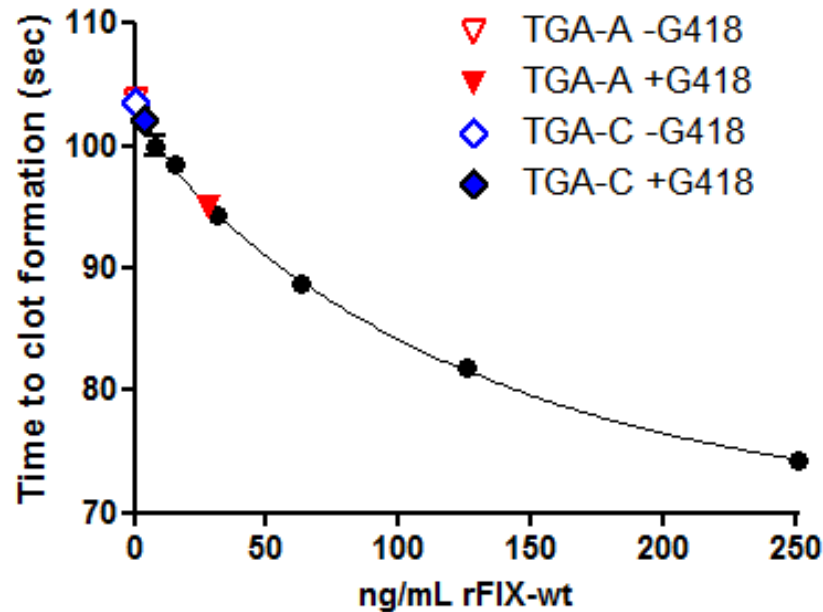
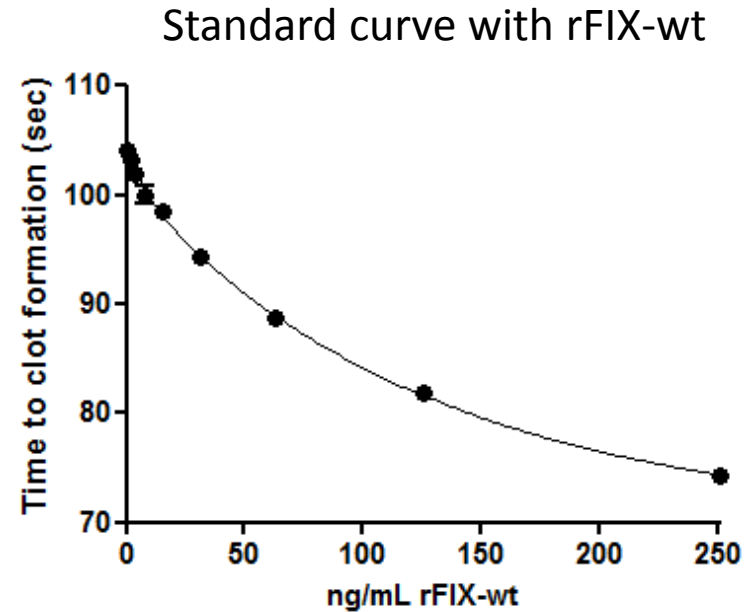
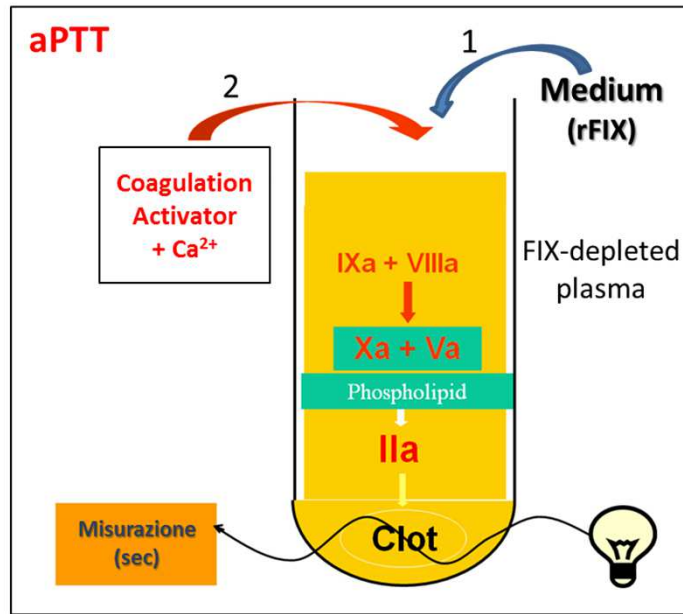
4. Evaluation of (pro-coagulant) activity



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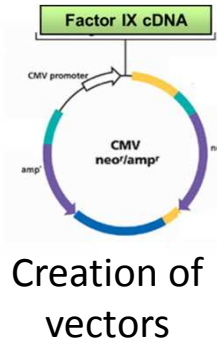
4. Evaluation of (pro-coagulant) activity



Despite TGA-C displayed the most efficient readthrough, the increase in activity after treatment is low....

Why??

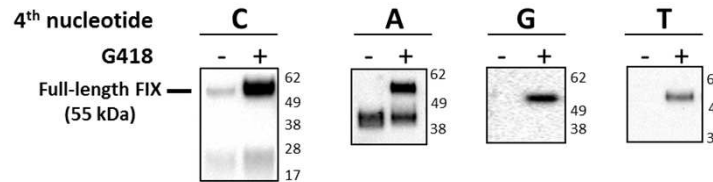
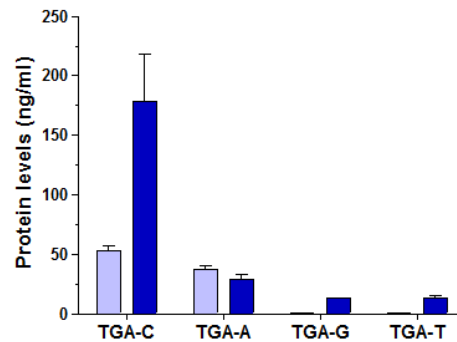
Summary



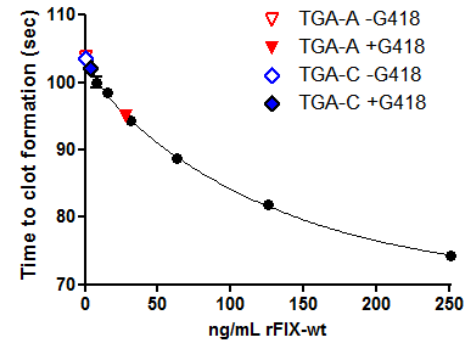
Creation of vectors



In-vitro expression



Evaluation of protein levels and isoforms



Evaluation of activity