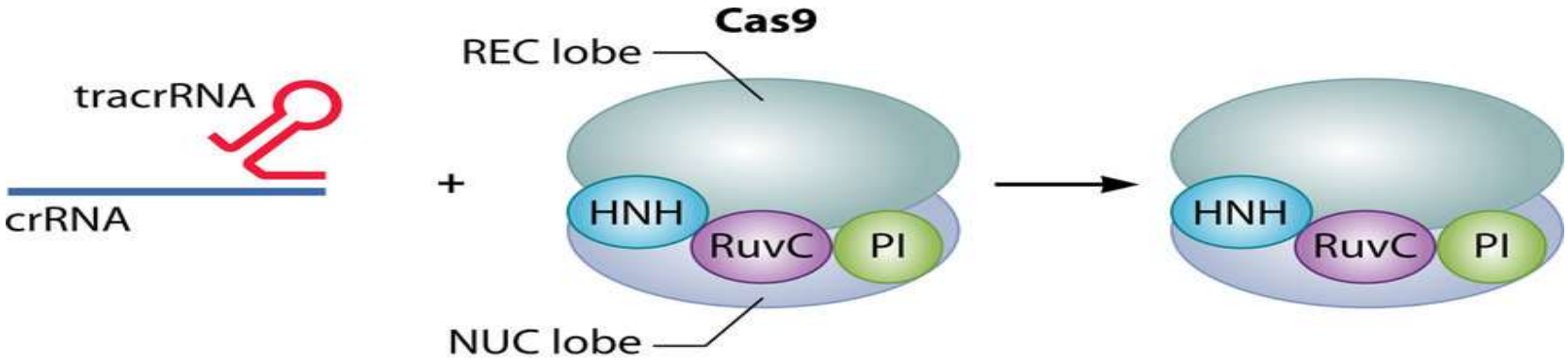
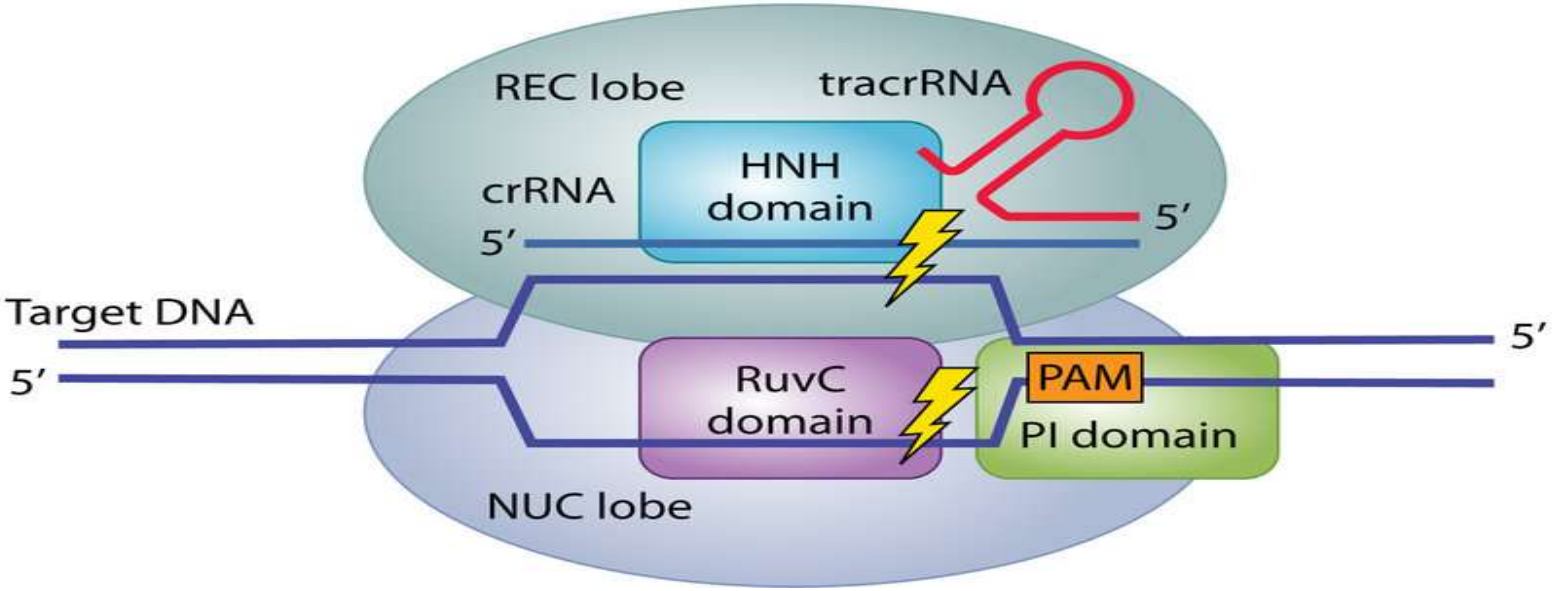
IN THEE NAME OF GOD

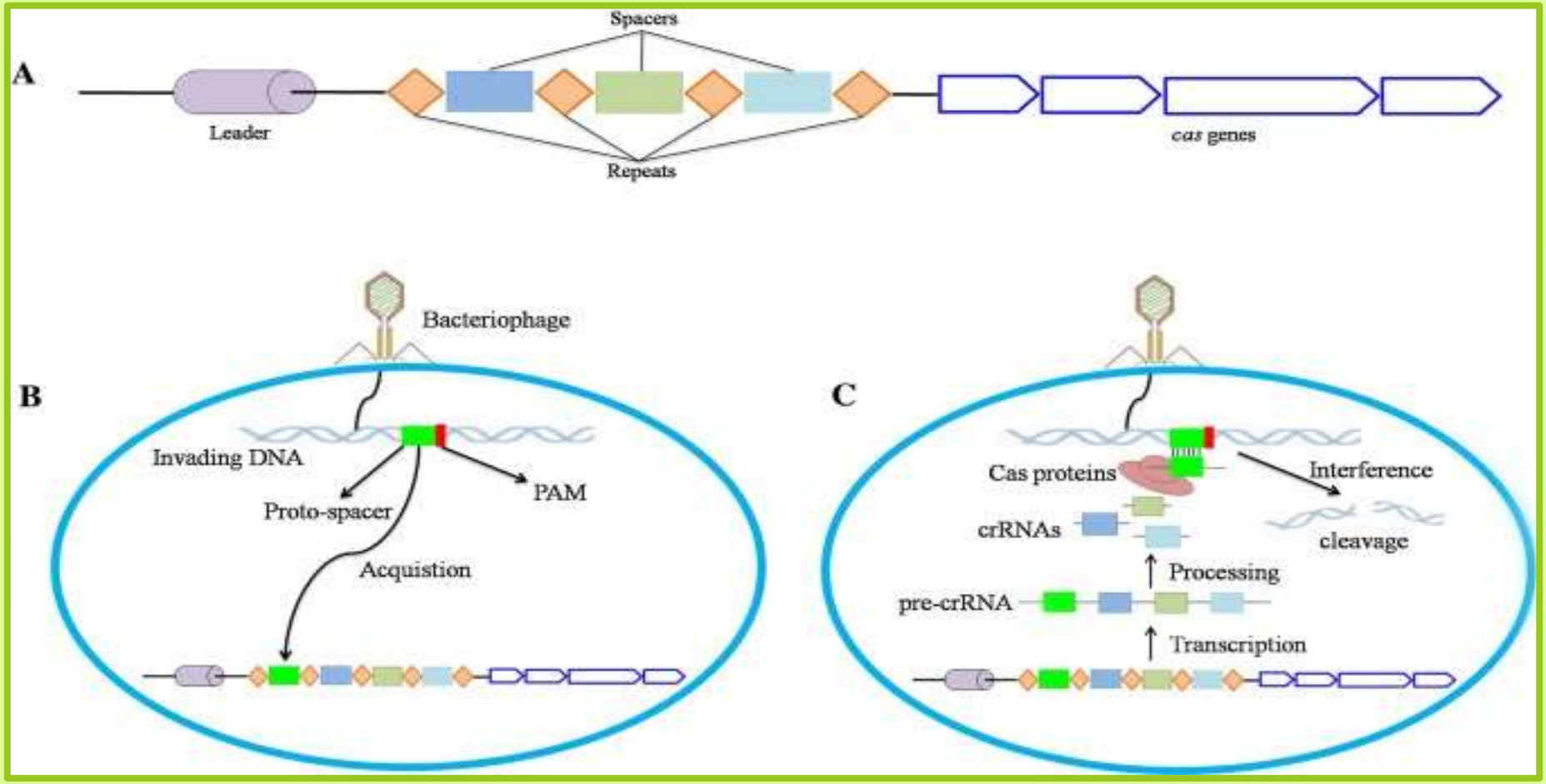
WHAT IS CRISPR?

Cas9 is an RNA-guided DNA endonuclease enzyme

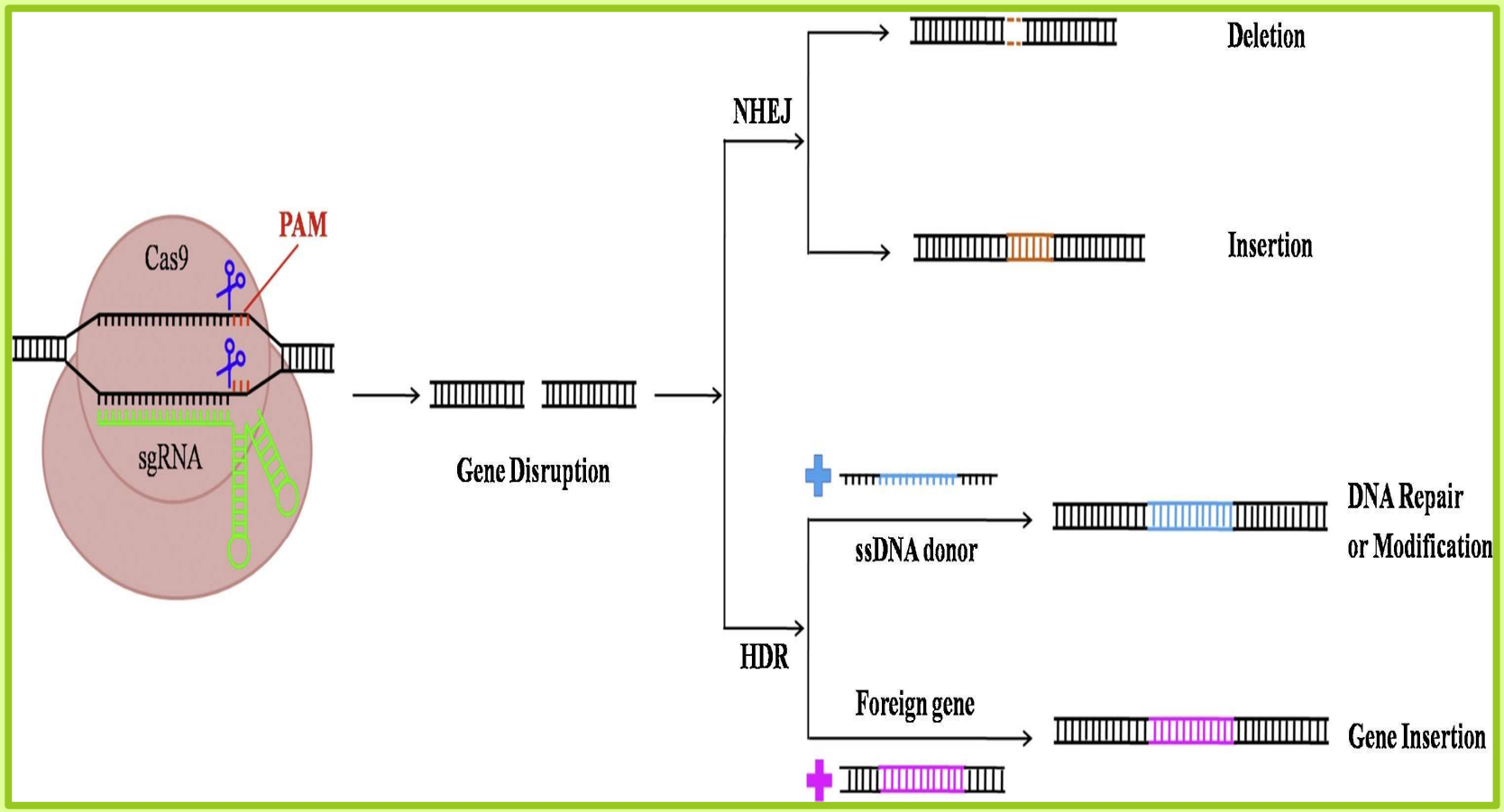


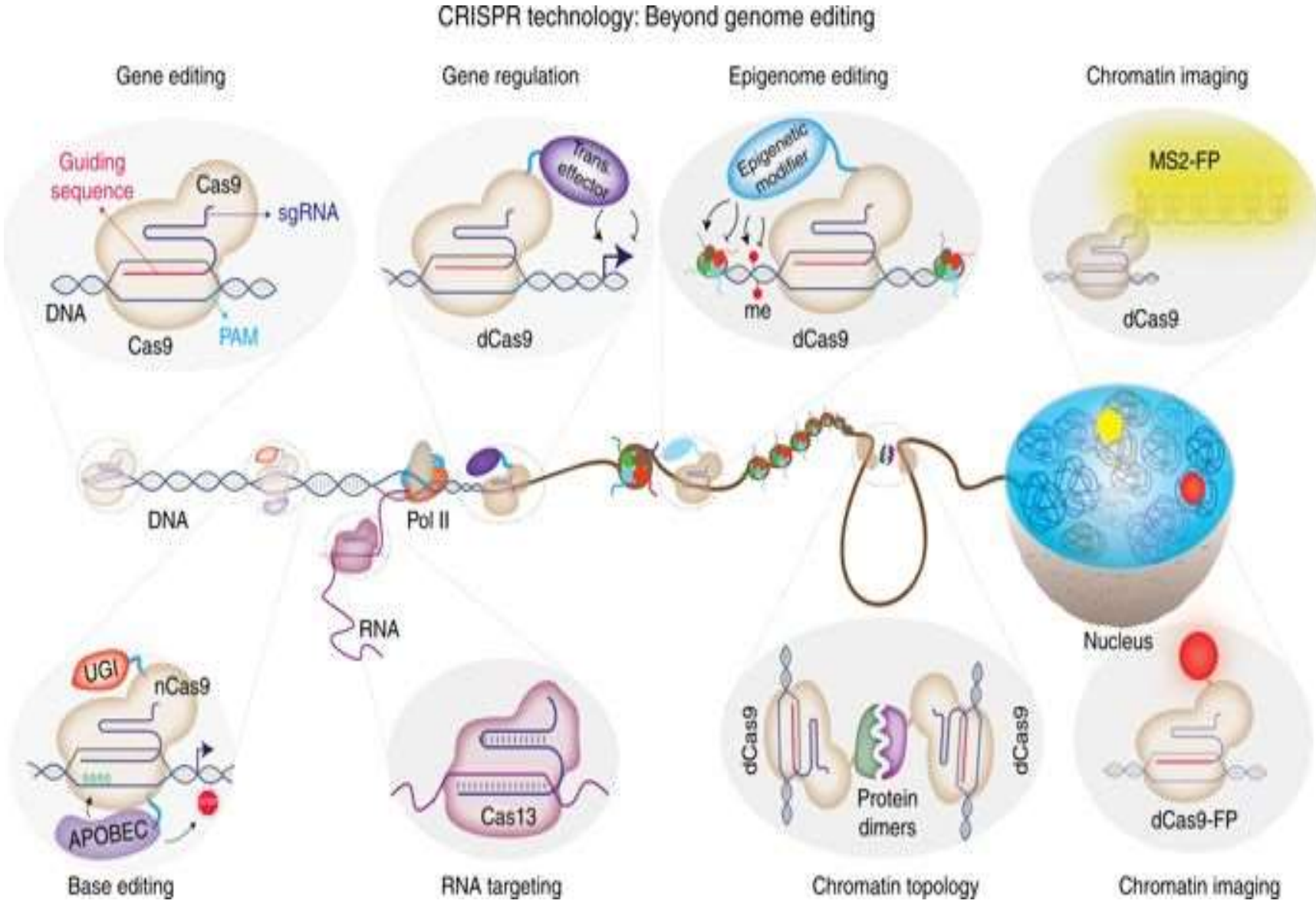


ORIGIN OF CAS9

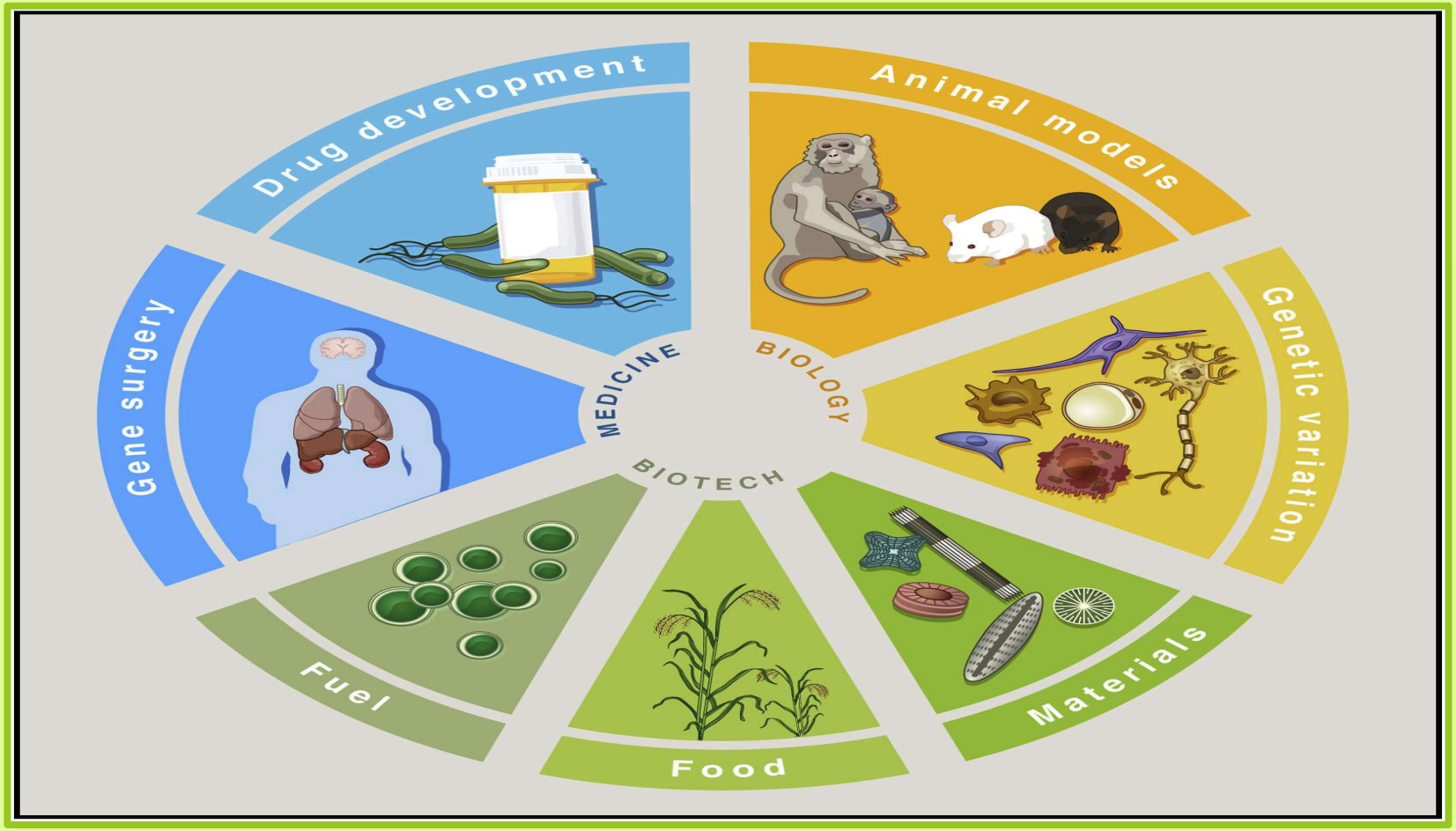


Application of cas9 in the genome editing

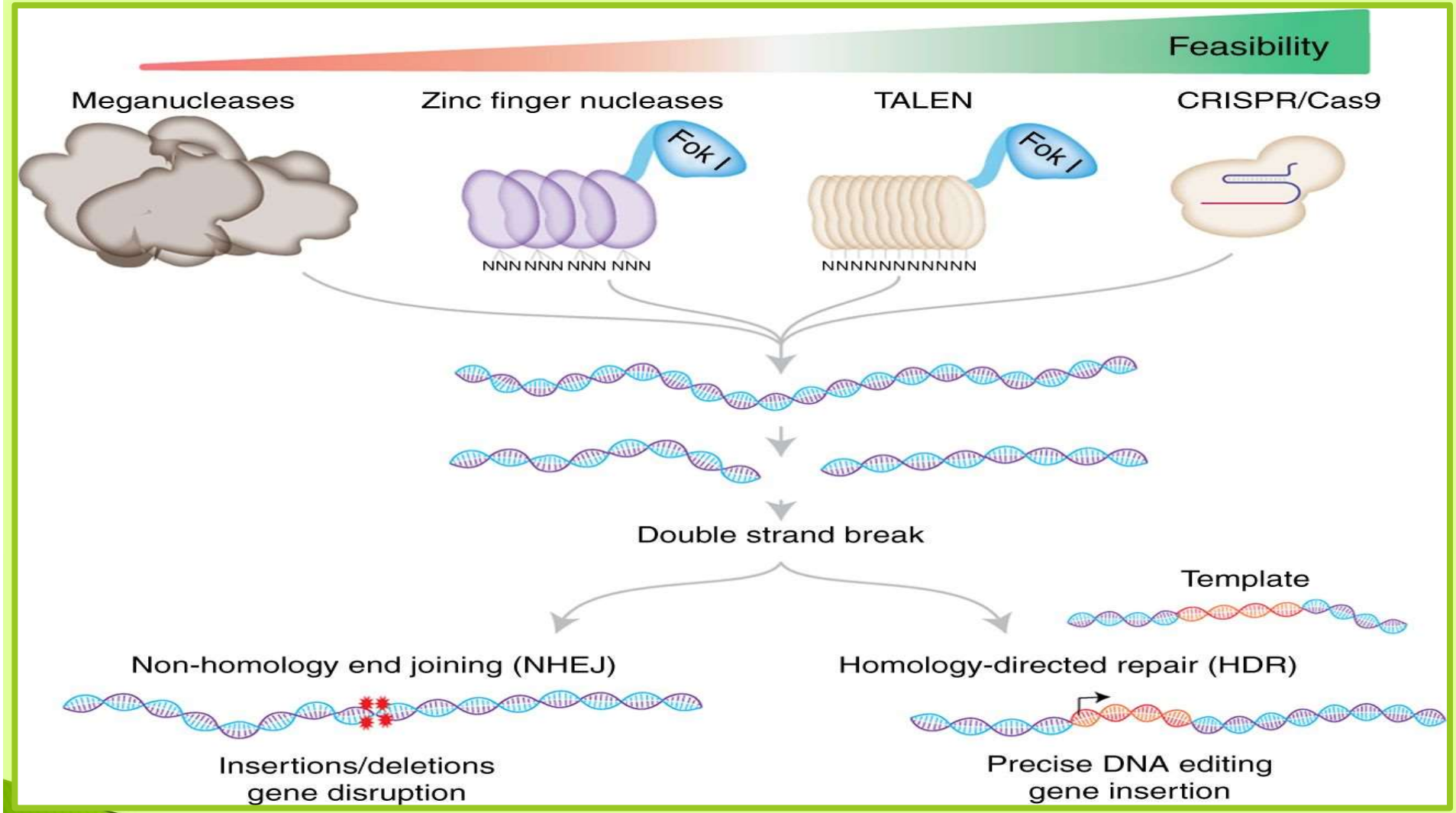



CRISPR technology: Beyond genome editing
Gene editing
Gene regulation
Epigenome editing
Chromatin imaging
Guiding sequence
Cas9
sgRNA
DNA
PAM
Cas9
Trans effector
dCas9
Epigenetic modifier
me
dCas9
MS2-FP
dCas9
DNA
Pol II
RNA
Nucleus
UGI
nCas9
APOBEC
Cas13
dCas9
Protein dimers
dCas9
dCas9-FP
Base editing
RNA targeting
Chromatin topology
Chromatin imaging

Application in biology



comparison

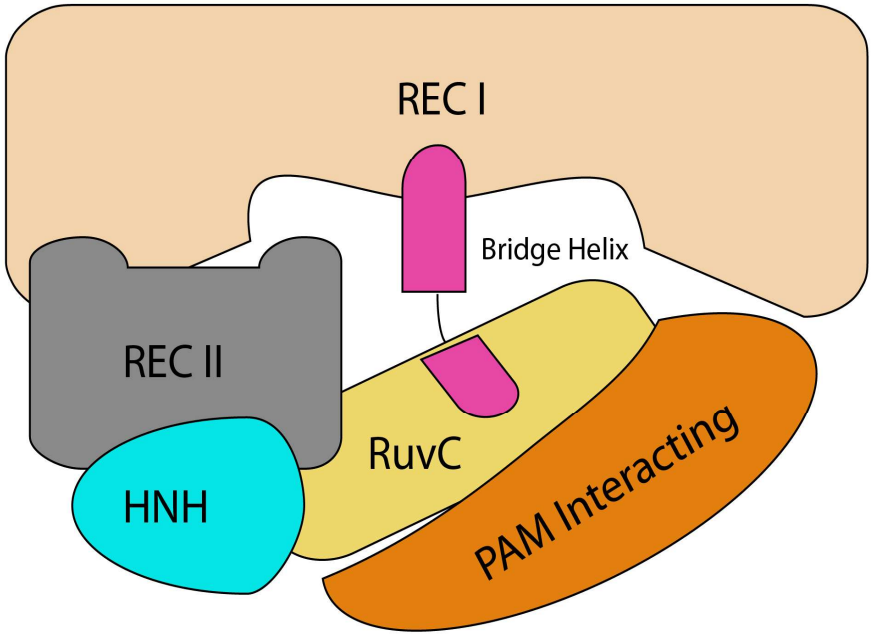

Feasibility
Meganucleases
Zinc finger nucleases
TALEN
CRISPR/Cas9
Fok I
Fok I
NNN NNN NNN NNN
NNNNNNNNNNN
Double strand break
Template
Non-homology end joining (NHEJ)
Homology-directed repair (HDR)
Insertions/deletions
gene disruption
Precise DNA editing
gene insertion

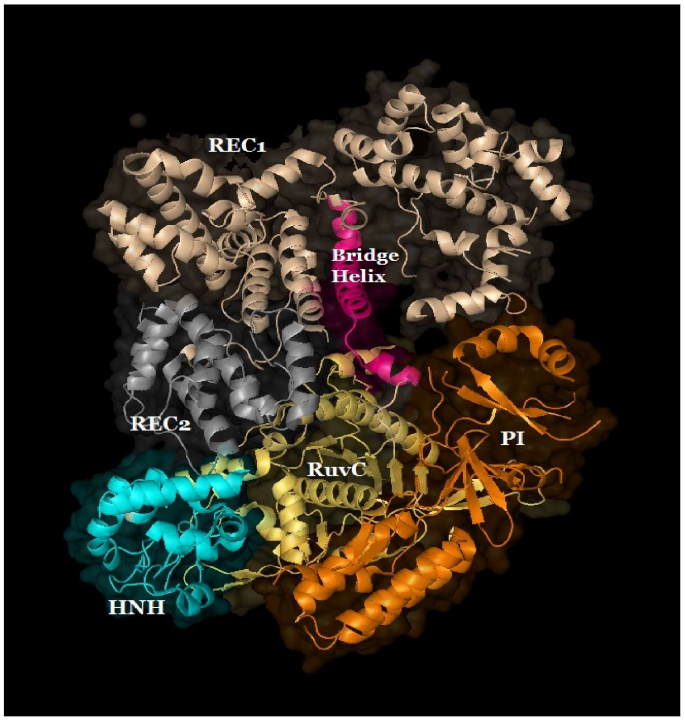
WHY PROTEIN?

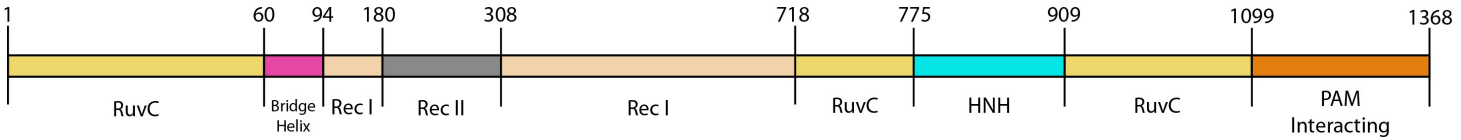
Cas9 Delivery Methods	pDNA	mRNA	Protein
			
High Efficiency	+++++	++++	++++
Low Cost	++++	++++	+++++
Specificity	+++++	++++	++++

Cas9 Delivery Formats	Protein	Viral	Plasmid	RNA
Insertional Mutagenesis	None	High	Moderate	None
Editing Fidelity	High	High	Moderate	Moderate
Off-Target Effects	Low	High	Moderate	Moderate
Immunogenicity	Low	High	Moderate	Moderate

cas9 is a Big protein: bad property







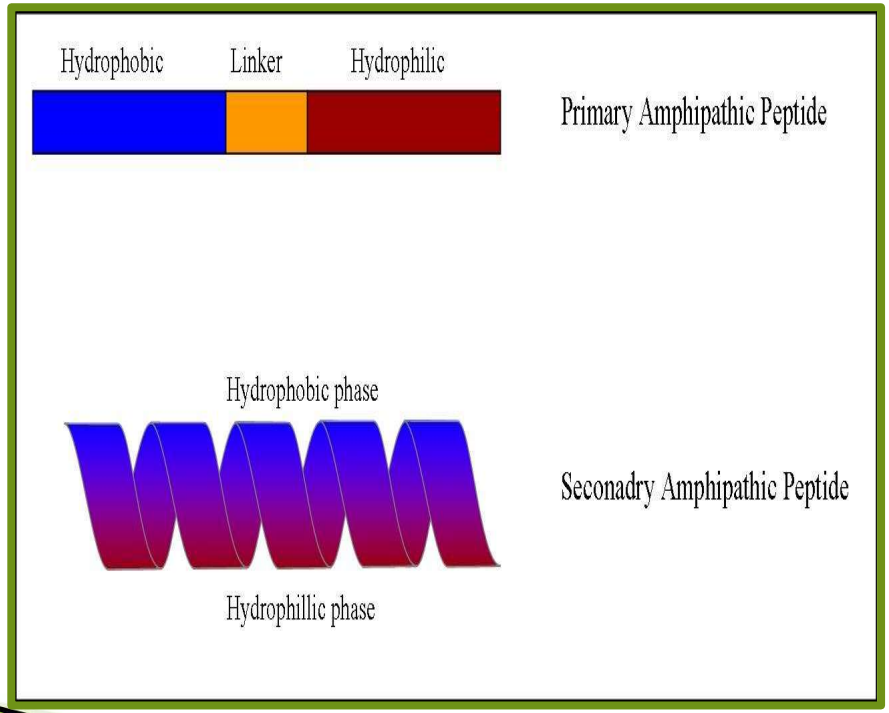
Protein-Based CRISPR Delivery Technologies

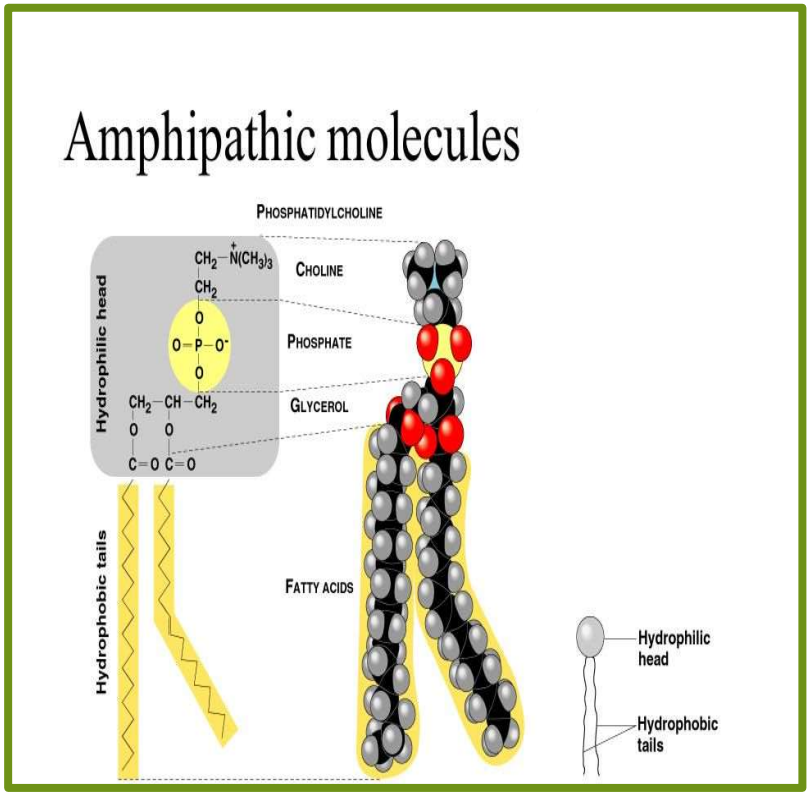
1) Lipid-Based Systems(cationic liposomes)
2) Polymer-Based Systems(Polyethyleneimine (PEI))
3) Nanoparticle-Based Systems
4) Cell-Penetrating Peptide-Based Systems

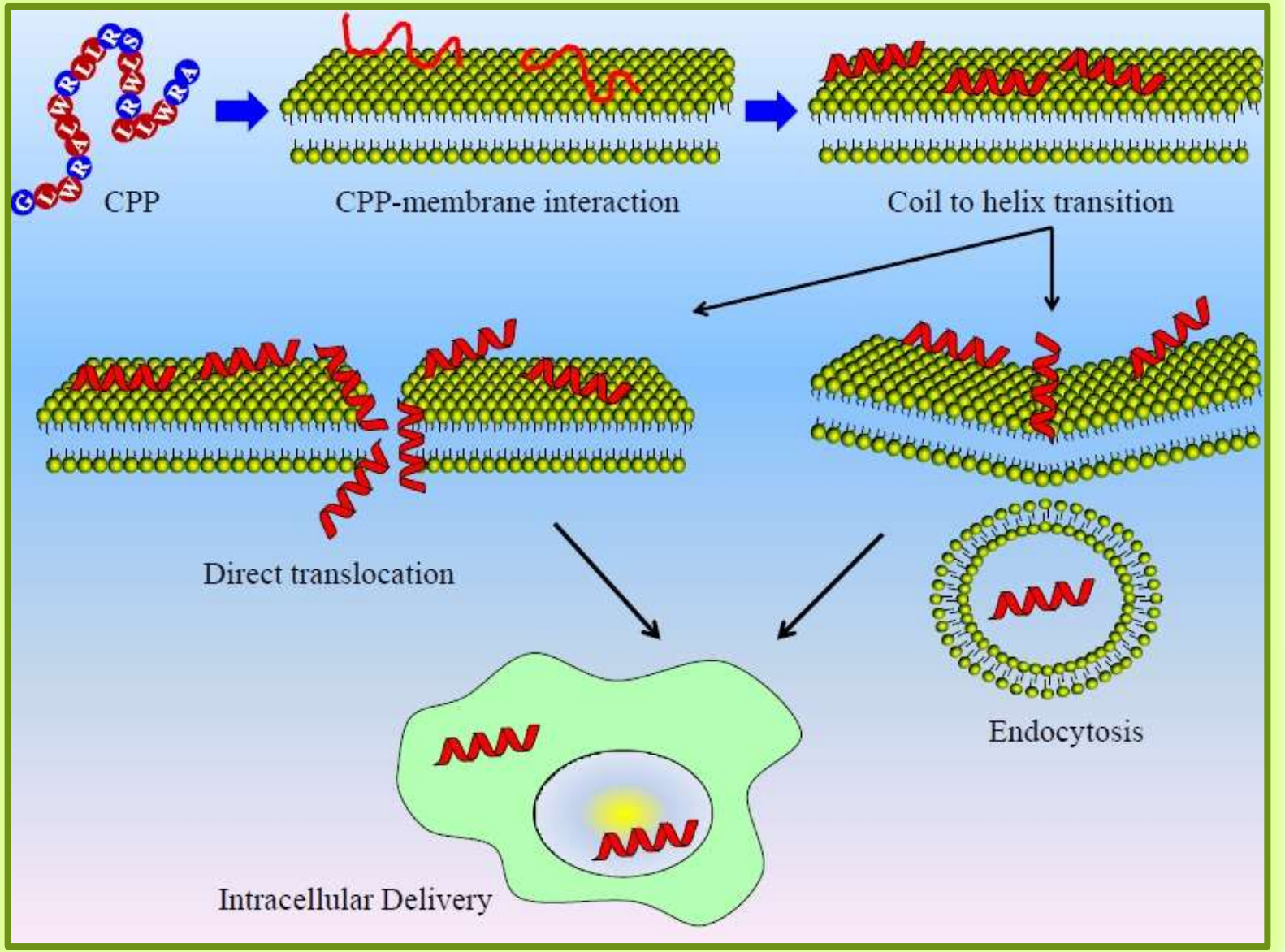
What is CPP?

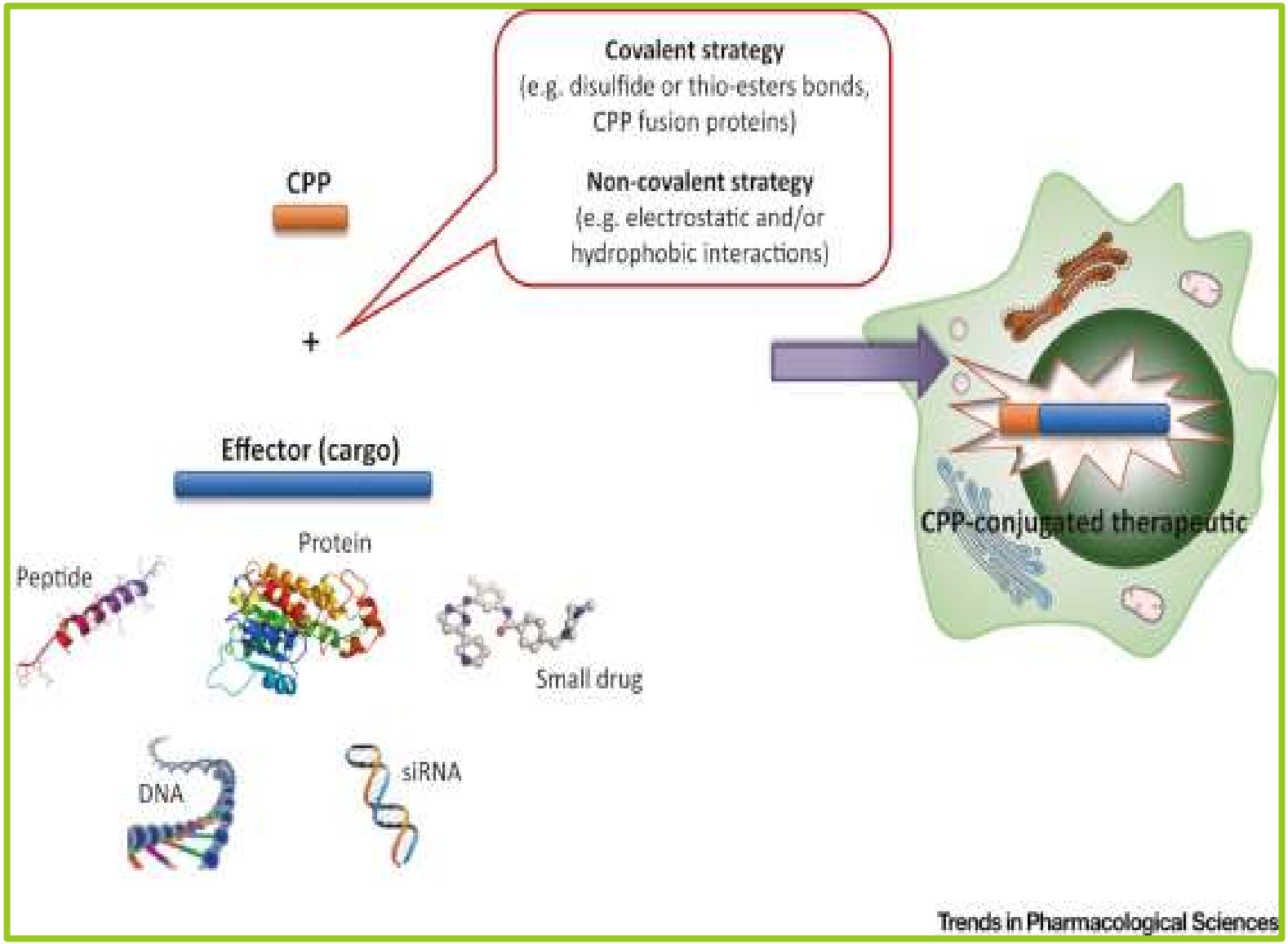
Cell-penetrating peptide(**CPP**)

- Short peptides <30
- Have alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids






CPP
CPP-membrane interaction
Coil to helix transition
Direct translocation
Endocytosis
Intracellular Delivery


Covalent strategy
(e.g. disulfide or thio-esters bonds, CPP fusion proteins)
Non-covalent strategy
(e.g. electrostatic and/or hydrophobic interactions)
CPP
+
Effector (cargo)
Peptide
Protein
Small drug
DNA
siRNA
CPP-conjugated therapeutic
Trends in Pharmacological Sciences

Object of study

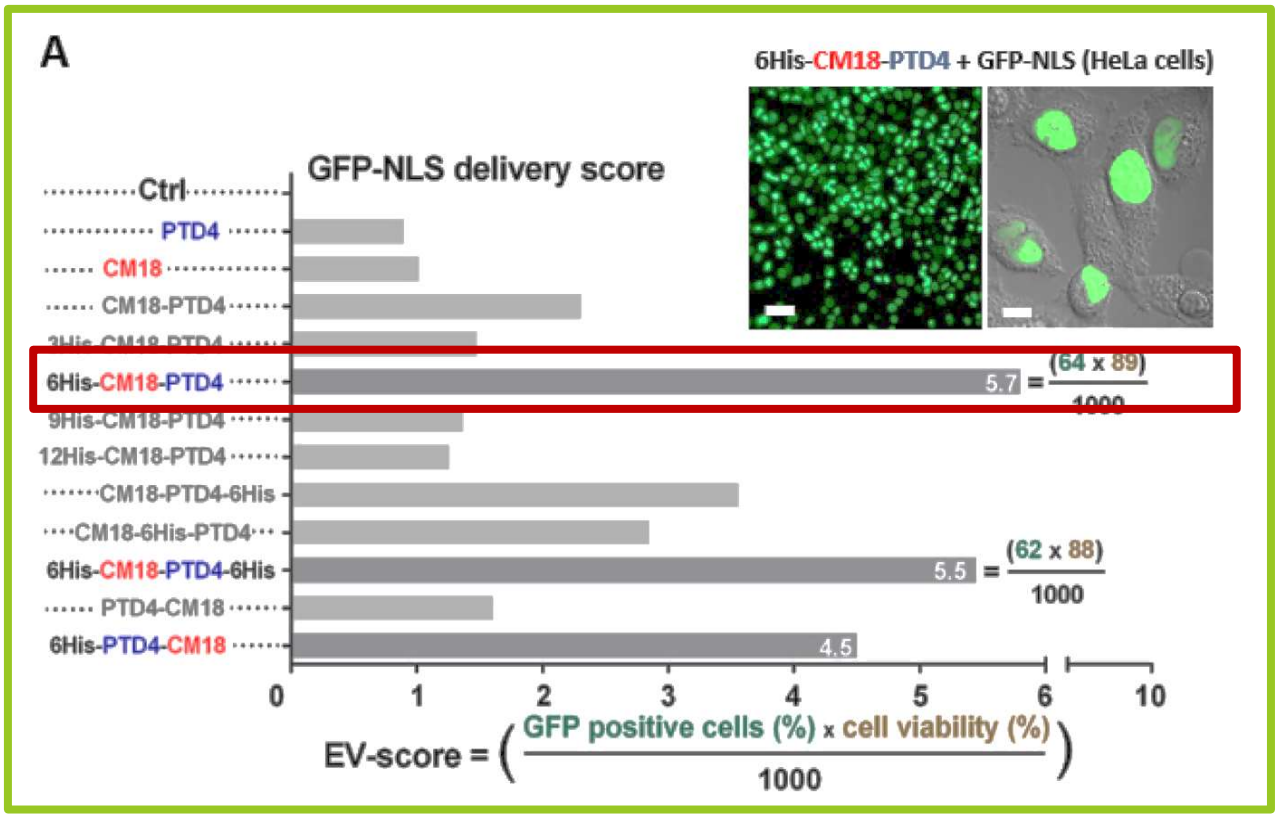
Design a delivery system for recombinant CRISPR-Cas9/Cpf1 protein by membrane permeabilizing amphiphilic peptide

1. 6x histidin reach domain
2. Endosoolytic peptide: CM18
3. Cell penetrating peptide: PTD4

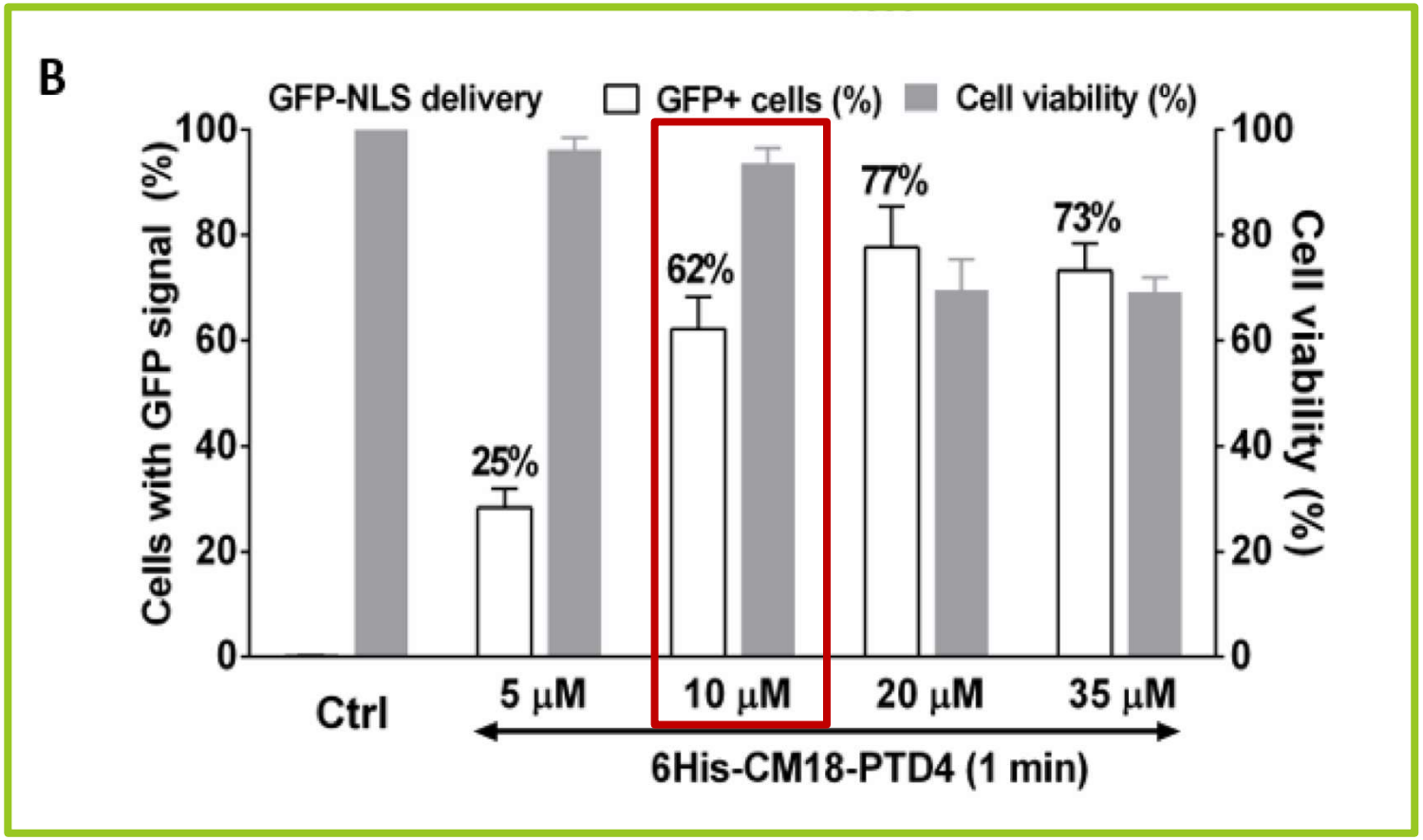
ASSAY
SAFELY AND EFFICIENCY OF DELIVERY BY GFP-NLS

S1 Table
Peptide sequences and delivery efficiency

Domain(s)	Peptide or Shuttle agent	Amino acid (a.a.) sequence	a.a.	MW (kDa)	p.I.	Net Charge	Hydro-phobic moment (μ_H)	Delivery efficiency (Mean ± SD) (%)	Cell viability (Mean ± SD) (%)	Score (delivery / viability)
ELD	CM18	KWKLFKKIGAVLK VLTTG	18	2.03	10.60	+5	4.28	12.9 ± 1.3	85.1 ± 1.2	1.02
CPD	PTD4	YARAAARQARA	11	1.2	11.72	+3	2.44	1.1 ± 0.16	94 ± 4.5	0.9
ELD-CPD	CM18-PTD4	KWKLFKKIGAVLK VLTTGYARAAARQ ARA	29	3.217	11.76	+8	6.72	57.3 ± 5.3	40.3 ± 3.1	2.31
	3His-CM18-PTD4	HHHKWKLFKKIGA VLKVLTTG YARAAARQARA	32	3.63	11.76	+8	7.21	39.4 ± 0.5	39.2 ± 3.3	1.48
	6His-CM18-PTD4	**HHHHHHKWKLF KKIGAVLKVLTTG YARAAARQARA**	**35**	**4.039**	**11.76**	**+8**	**7.79**	**64.3 ± 3.2**	**88.6 ± 4.5**	**5.8**
	9His-CM18-PTD4	HHHHHHHHHKWK LFKKIGAVLKVLTT GYARAAARQARA	38	4.45	11.76	+8	7.92	36.7 ± 3.3	38.7 ± 3.1	1.37
	12His-CM18-PTD4	HHHHHHHHHHHH KWKLFKKIGAVLK VLTTGYARAAARQ ARA	41	4.86	11.76	+8	7.48	36.9 ± 4.3	33.4 ± 4.3	1.26
	CM18-PTD4-6His	**KWKLFKKIGAVL KVLTTGYARAAA RQARAHHHHHH**	**35**	**4.039**	**11.76**	**+8**	**6.13**	**61.7 ± 1.8**	**57.7 ± 4.2**	**3.56**
	CM18-6His-PTD4	KWKLFKKIGAVLK VLTTGHHHHHHYA RAAARQARA	35	4.04	11.76	+8	5.28	44.7 ± 1.5	63.9 ± 1.1	2.85
	6His-CM18-PTD4-6His	**HHHHHHKWKLF KKIGAVLKVLTTG YARAAARQARAH HHHHH**	**41**	**4.86**	**11.76**	**+8**	**7.5**	**62 ± 6**	**88.3 ± 4.1**	**5.45**
	PTD4-CM18	YARAAARQARAK WKLFKKIGAVLKV LTTG	29	3.217	11.76	+8	6.66	47.6 ± 2.6	33.9 ± 3.7	1.61
	6His-PTD4-CM18	**HHHHHHYARAAA RQARAKWKLFKK IGAVLKVLTTG**	**35**	**4.039**	**11.76**	**+8**	**7.66**	**53.7 ± 4.9**	**83.5 ± 5.7**	**4.5**



OPTIMAL ENTRY- VIABILITY



OPTIMAL CONCENTRATION

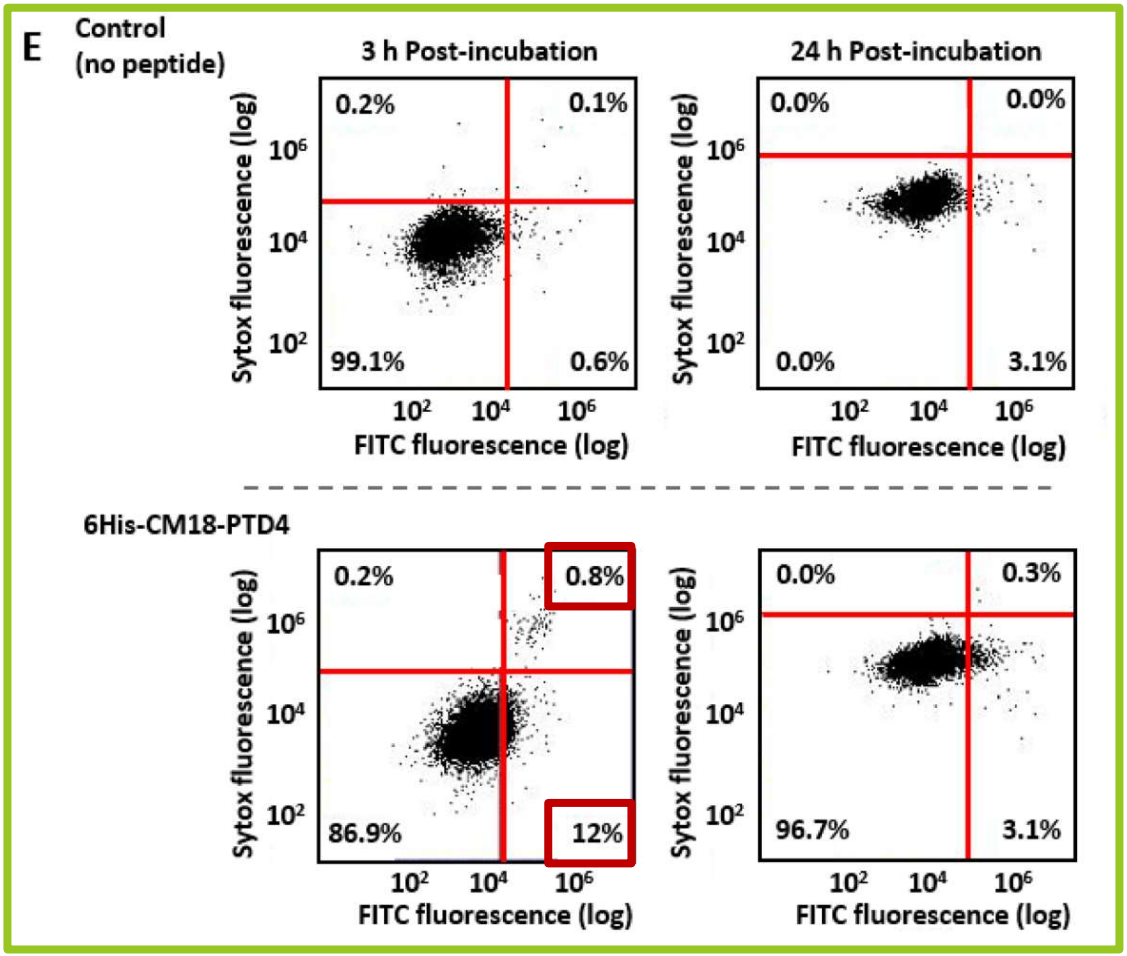
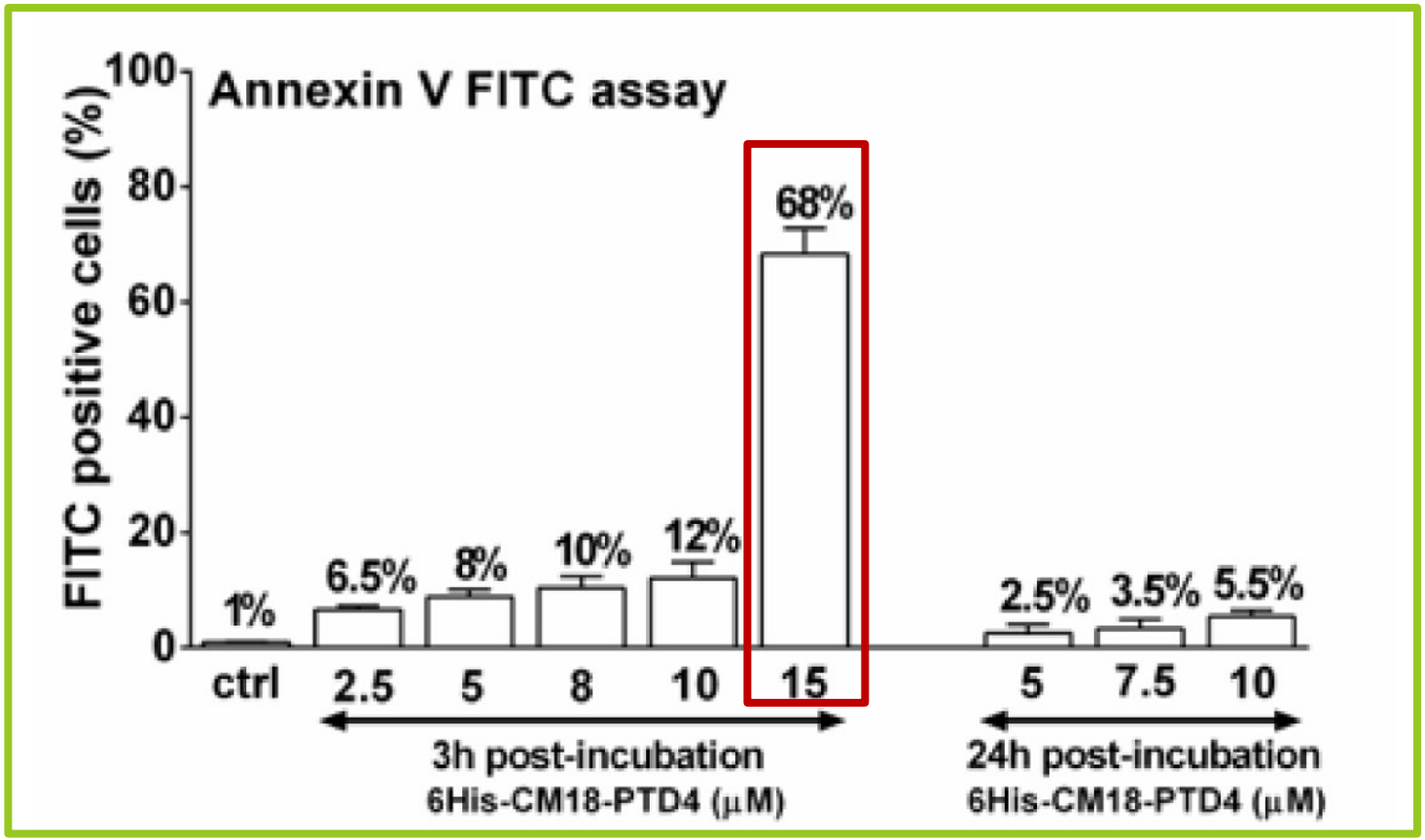


Fig 1.E. Cell viability assay with both the pre-apoptosis reporter FITC-Annexin V and the Syto red reagents


Annexin V FITC assay
FITC positive cells (%)
100
80
60
40
20
0
1%
6.5%
8%
10%
12%
68%
2.5%
3.5%
5.5%
ctrl
2.5
5
8
10
15
5
7.5
10
3h post-incubation
6His-CM18-PTD4 (μM)
24h post-incubation
6His-CM18-PTD4 (μM)

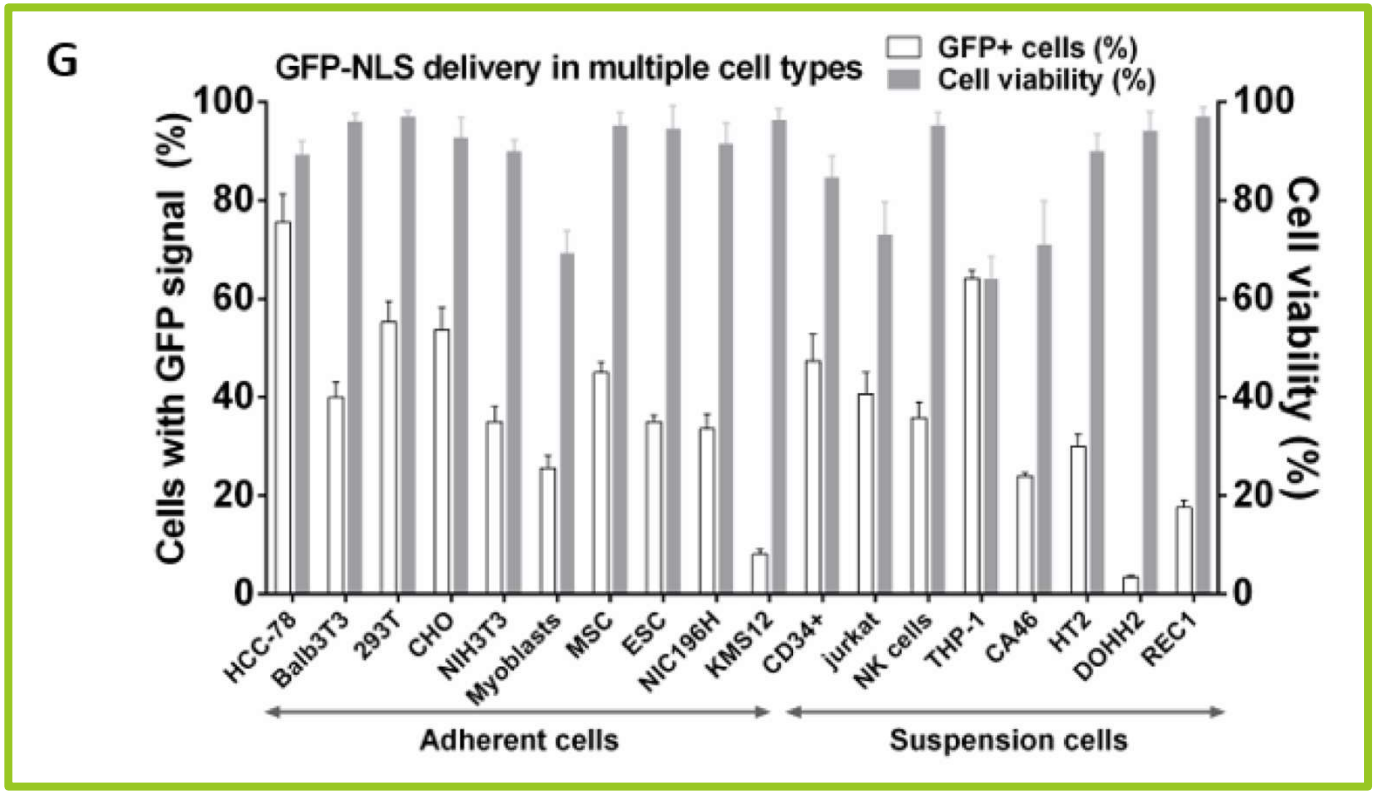
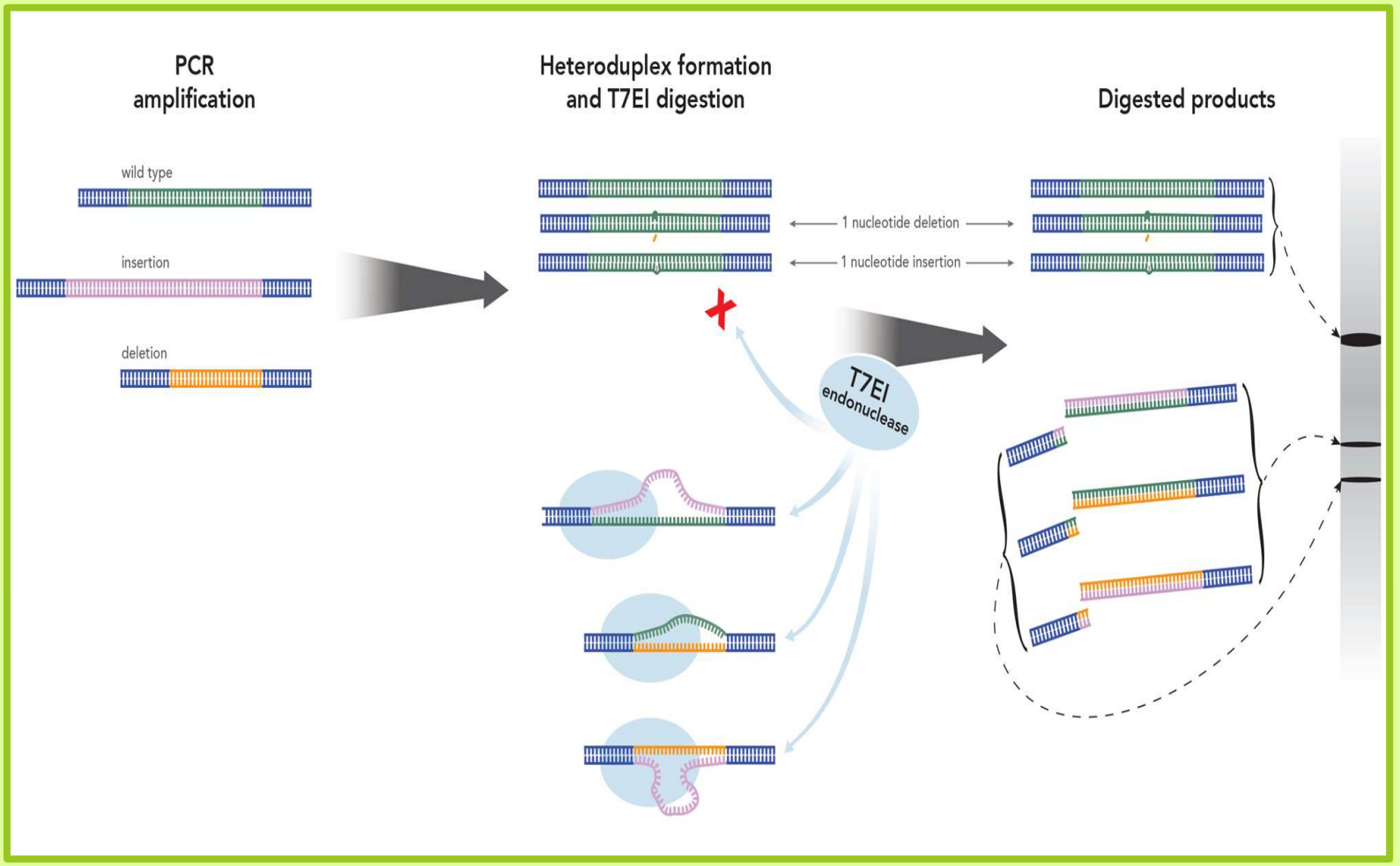
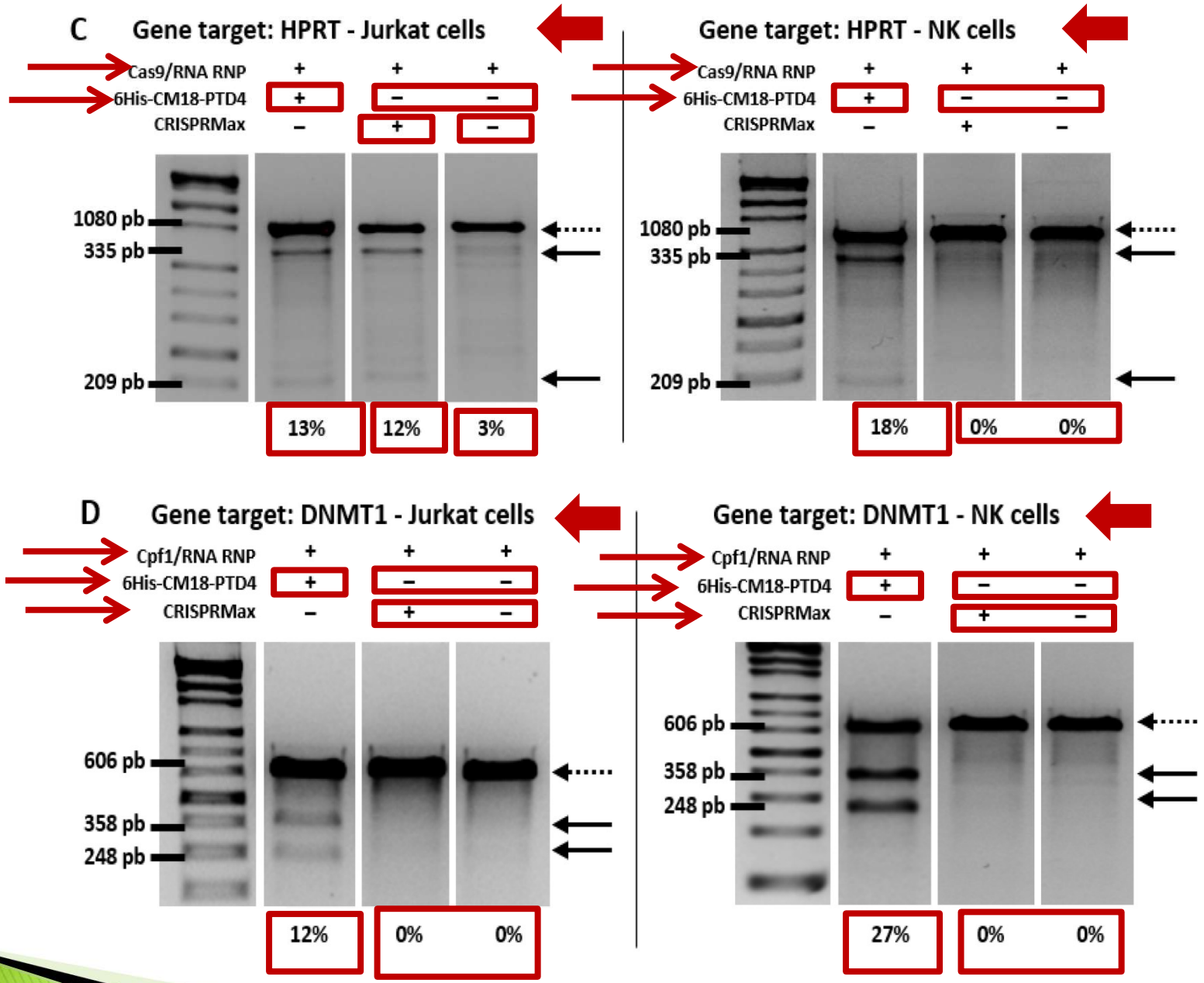


Fig 1.G. GFP-NLS (10 μM) delivery efficiency and related cell viability in multiple mammalian cells after 1 min incubation of 6His-CM18-PTD4 (10 μM) with adherent cells or of 6His-CM18-PTD4 (5 μM) with cells in suspension

DELIVER FUNCTIONAL COMPLEXES IN HARD TO MODIFY NK CELL

Functional assay of cas9 in the gene editing by T7 endonuclease I




C
Gene target: HPRT - Jurkat cells
Cas9/RNA RNP
6His-CM18-PTD4
CRISPRMax
1080 pb
335 pb
209 pb
13%
12%
3%
Gene target: HPRT - NK cells
Cas9/RNA RNP
6His-CM18-PTD4
CRISPRMax
1080 pb
335 pb
209 pb
18%
0%
0%
D
Gene target: DNMT1 - Jurkat cells
Cpf1/RNA RNP
6His-CM18-PTD4
CRISPRMax
606 pb
358 pb
248 pb
12%
0%
0%
Gene target: DNMT1 - NK cells
Cpf1/RNA RNP
6His-CM18-PTD4
CRISPRMax
606 pb
358 pb
248 pb
27%
0%
0%

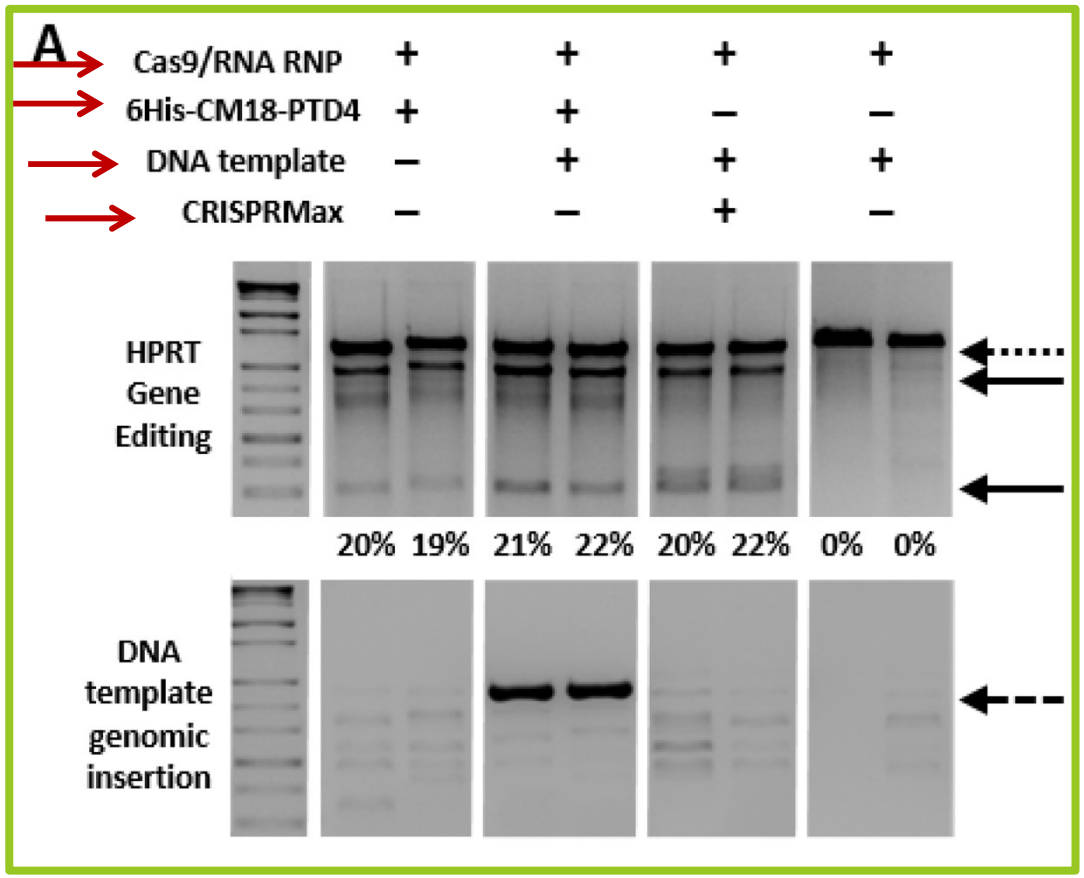


Fig 4.A. 48 hours after CRISPR RNPs deliveries, (A) the genomic insertion of a DNA template (72 bp) in the Cas9-edited HPRT gene was confirmed by PCR amplification with specifically designed primers.

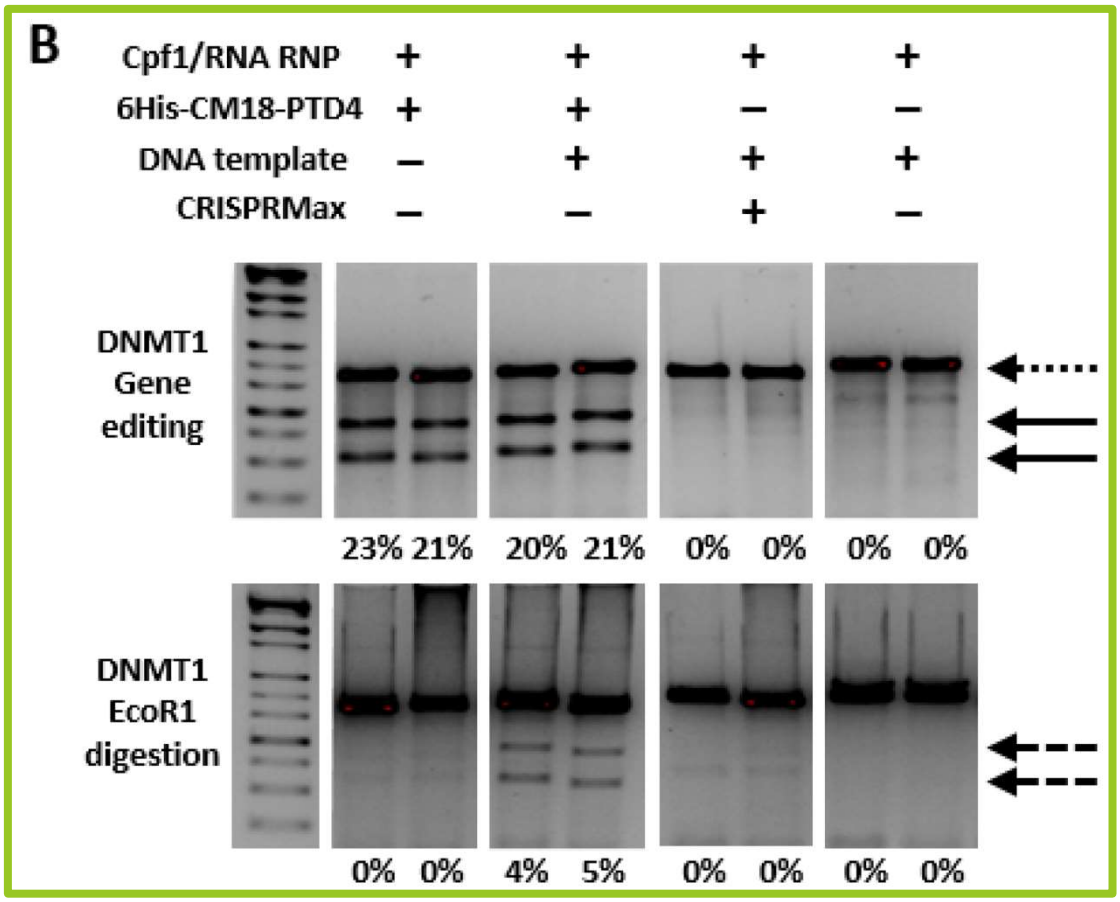
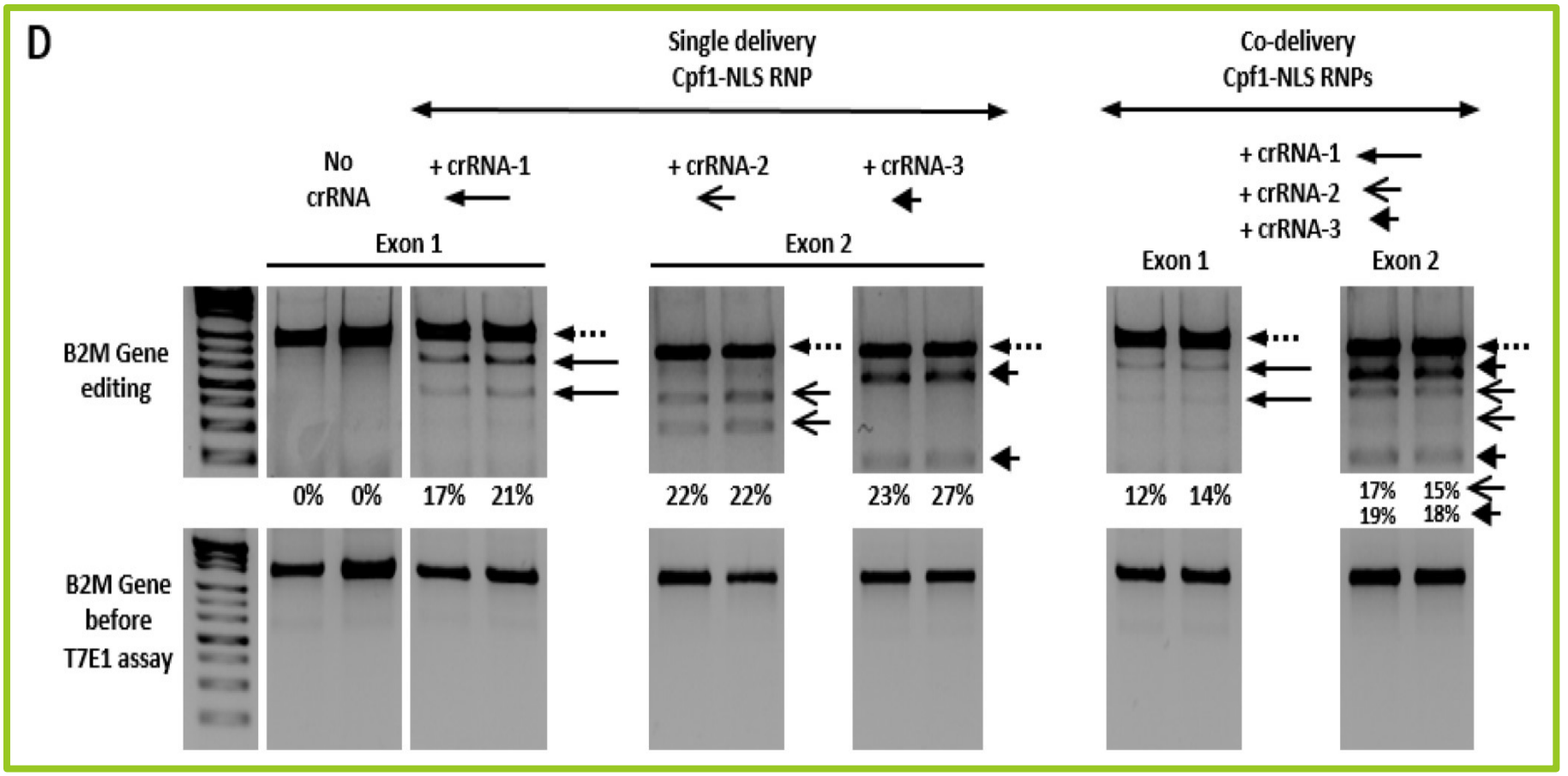


Fig 4.B. The genomic insertion of a DNA template (76 bp) containing an EcoR1 site in the Cpf1-edited DNMT1 gene was confirmed by exposing DNMT1 PCR product to a EcoR1 restriction enzyme (dashed arrow).


D
Single delivery
Cpf1-NLS RNP
Co-delivery
Cpf1-NLS RNPs
No
crRNA
+ crRNA-1
+ crRNA-2
+ crRNA-3
+ crRNA-1
+ crRNA-2
+ crRNA-3
Exon 1
Exon 2
Exon 1
Exon 2
B2M Gene
editing
0% 0% 17% 21%
22% 22%
23% 27%
12% 14%
17% 15%
19% 18%
B2M Gene
before
T7E1 assay

investigate the delivery mechanism and endosomolytic activity of 6His-CM18-PTD4

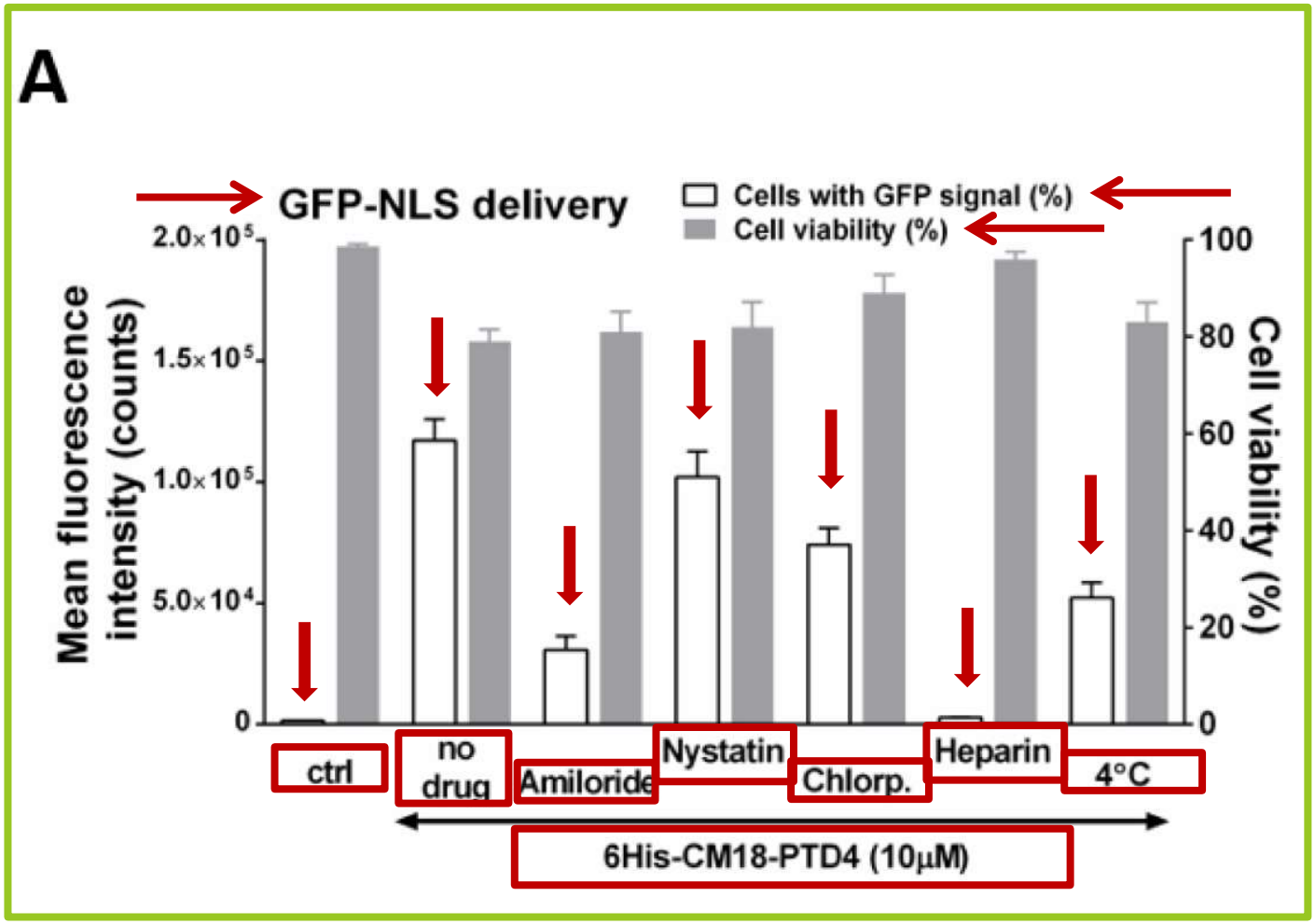
endocytosis inhibitors that target specific pathways:

- amiloride is a macropinocytosis blocker
- nystatin is a caveolae-dependent endocytosis blocker
- chlorpromazine is a clathrin-mediated endocytosis blocker.

all forms of endocytosis were inhibited by

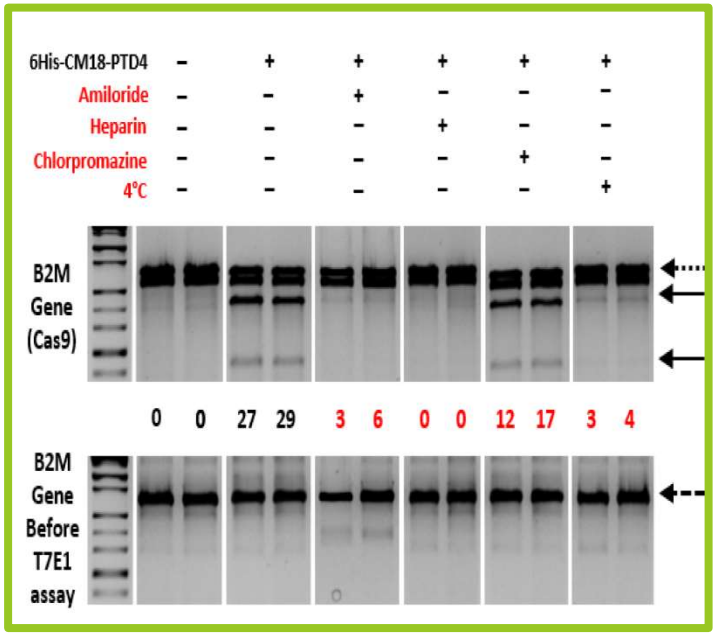
- exposing HeLa cells to low temperature.
- heparin, a close structural homologue

of heparan sulfate proteoglycans (HSPGs), to compete with 6His-CM18-PTD4 for cell penetration mediated by HSPG at the cell surface

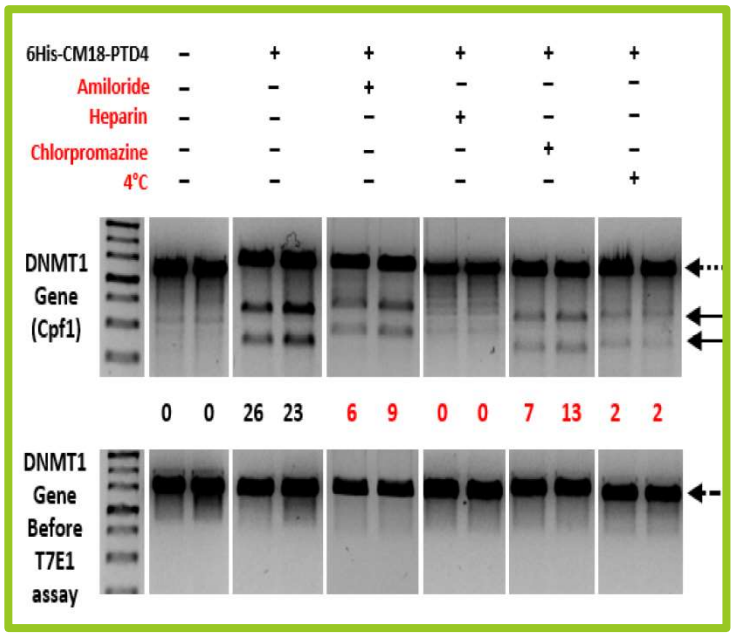


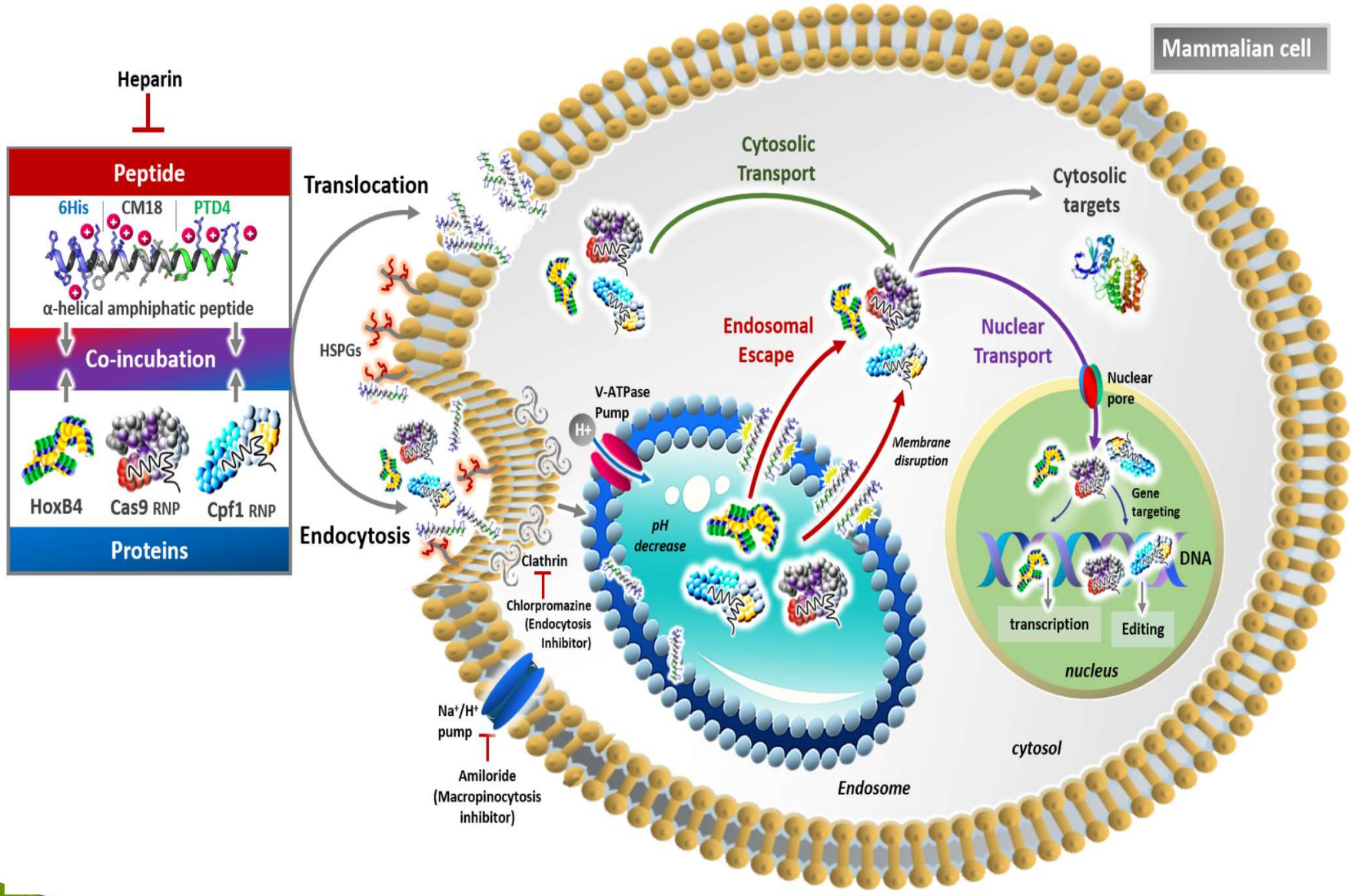
This partial loss of protein signal at low temperature suggests that 6His-CM18-PTD4 activates both translocation and endocytosis mechanisms.

E


6His-CM18-PTD4 – + + + + +
Amiloride – – + – – –
Heparin – – – + – –
Chlorpromazine – – – – + –
4°C – – – – – +
B2M Gene (Cas9)
0 0 27 29 3 6 0 0 12 17 3 4
B2M Gene Before T7E1 assay

F


6His-CM18-PTD4 – + + + + +
Amiloride – – + – – –
Heparin – – – + – –
Chlorpromazine – – – – + –
4°C – – – – – +
DNMT1 Gene (Cpf1)
0 0 26 23 6 9 0 0 7 13 2 2
DNMT1 Gene Before T7E1 assay


Heparin
Peptide
6His
CM18
PTD4
α-helical amphiphatic peptide
Co-incubation
HoxB4
Cas9 RNP
Cpf1 RNP
Proteins
Translocation
Endocytosis
Mammalian cell
Cytosolic Transport
Cytosolic targets
HSPGs
Endosomal Escape
Nuclear Transport
V-ATPase Pump
H+
pH decrease
Membrane disruption
Nuclear pore
Gene targeting
DNA
transcription
Editing
nucleus
Clathrin
Chlorpromazine (Endocytosis Inhibitor)
Na+/H+ pump
Amiloride (Macropinocytosis inhibitor)
cytosol
Endosome


BE HAPPY