MOLECULAR TOOLS TO EDIT THE GENOME

APPLICATION OF ZFN TECHNOLOGY

Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery

nature biotechnology

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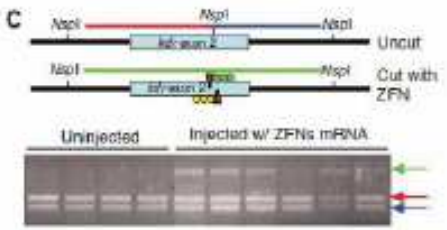
Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases

Xiangdong Meng[1,4], Marcus B Noyes[1,2], Lihua J Zhu[1], Nathan D Lawson[1,3] & Scot A Wolfe[1,2]



Knockout Rats via Embryo Microinjection of Zinc-Finger Nucleases

Science

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Table 1 Genomic sequences targeted with ZFNs

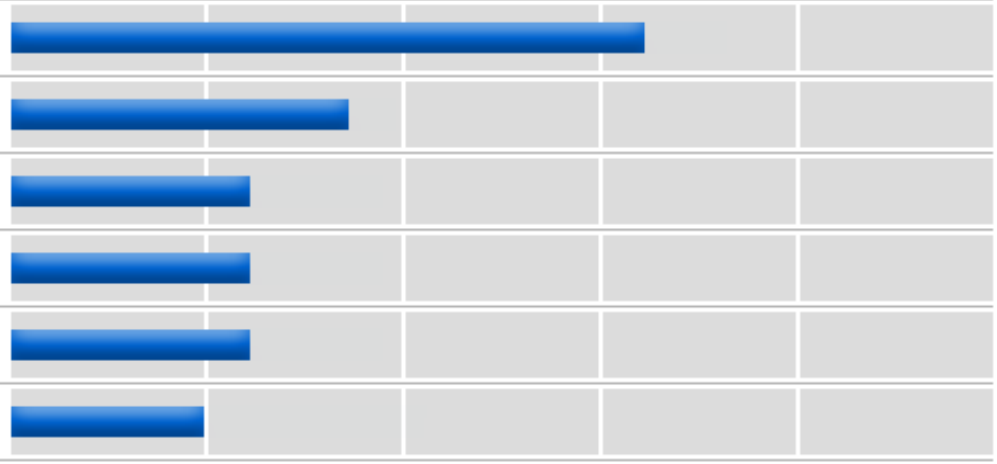
Organism/cells	Gene name	Reference
Human T cells	*CCR5*	Perez *et al.*[8]
CHO cells	*DHFR*	Santiago *et al.*[9]
Various human cells	*IL-2Rγ*	Lombardo *et al.*[10]
		Moehle *et al.*[11]
HEK293 cells	*VEGF-A*	Maeder *et al.*[12]
HEK293 cells	*HoxB13*	Maeder *et al.*[12]
HEK293 cells	*CFTR*	Maeder *et al.*[12]
Tobacco	*SuRA*	Maeder *et al.*[12]
Zebrafish	*ldlr*	Meng *et al.*[7]
Zebrafish	*golden*	Doyon *et al.*[6]
Zebrafish	*no tail*	Doyon *et al.*[6]
Drosophila	*rs*	Beumer *et al.*[13]
Drosophila	*ly*	Beumer *et al.*[13]
Drosophila	*ms*	Beumer *et al.*[13]

Table 1 Potential applications of zinc finger nucleases

Experimental uses	Drug development
Create knockout genes (cell lines, primary cells, transgenic animals)	Create humanized cell lines
Create point mutations or small deletions in permanent or primary cell lines	Create cell lines for drug target validation
Improve efficiency of gene targeting in ES cells	Create cell lines for high-throughput screening for novel compounds
Create targeted transgenics with insertions into precise genomic locations	
Genome manipulation in model organisms currently without gene targeting mechanism (worms, zebrafish)	

Therapeutics
Correction of genes in monogenic diseases (e.g., Huntington disease)
Inserting genes into precise (safe and permissive) locations for correcting complex mutations (hemophilia A) and introducing RNAi, for example
Altering alleles; for example, the CCR5 gene to create resistance to HIV.
Designer immunotherapeutics
Modification of stem cells

Program	Lead Indication	Research	Pre-clinical	Phase 1	Phase 2	Phase 3
SB-728	HIV / AIDS					
Shire	Hemophilia					
Shire	Huntington's Disease					
Multiple	Hemoglobinopathies					
Multiple	Lysosomal Storage Disorders					
Multiple	Other Monogenic Diseases					



http://www.sangamo.com/pipeline/index.html

28.03.2011

Terapia per la DMD

Nuove prospettive alla correzione di mutazioni genetiche all'origine della Distrofia Muscolare Duchenne (DMD)

di Matteo Bovolenta | *Ferrara 05/06/1980 / bvlmtt@unife.it PhD in Medical Genetic Università di Ferrara*

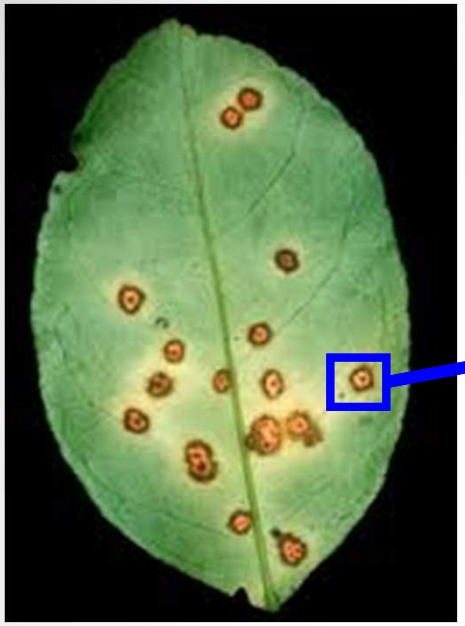
TR35 PROGETTO GIOVANI INNOVATORI
INNOVATORE DELL'ANNO 2011
Matteo Bovolenta

La Distrofia Muscolare Duchenne (DMD) è la patologia muscolare più comune nell'infanzia, caratterizzata da riduzione della massa muscolare, stanchezza, miocardiopatia, crescita ritardata. DMD è causata da mutazioni genetiche, che a tutt'oggi non è stato possibile correggere in modo sicuro ed efficace. Una tecnologia particolarmente promettente è basata sulla ricombinazione omologa HR (Homologous Recombination) che è nota come gene targeting, ma che fino a oggi non è stato possibile applicare in un contesto terapeutico.

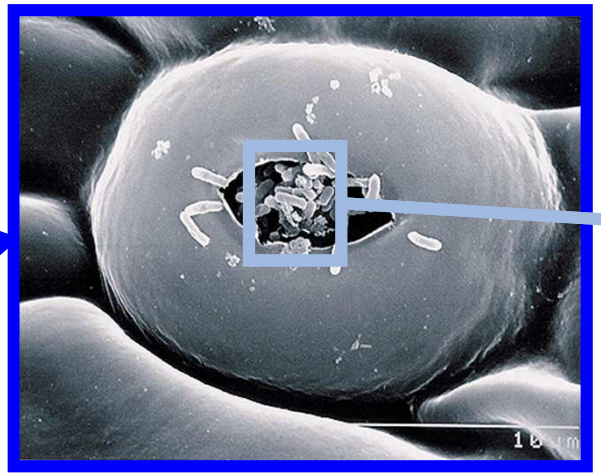
Una tecnologia recente, messa a punto in questo progetto, ha cambiato queste aspettative. Fondendo i multiple engineered zinc-finger DNA-bindingdomains con un non-specific nuclease domain, vengono generate le cosiddette nucleasi ZFNs. Queste possono essere progettate in modo tale da introdurre un DNB (Double Strand Brand) nella

TRANSCRIPTIONAL ACTIVATOR-LIKE EFFECTORS (TALEs)

Origine

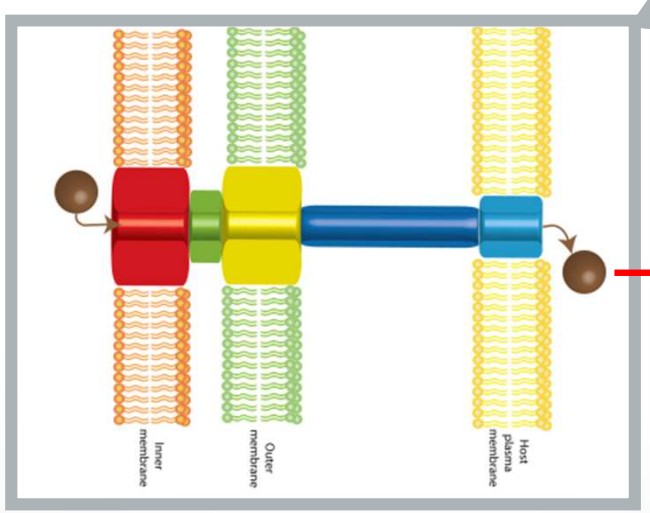


Infezione



Stoma

Xanthomonas
(Gram -)

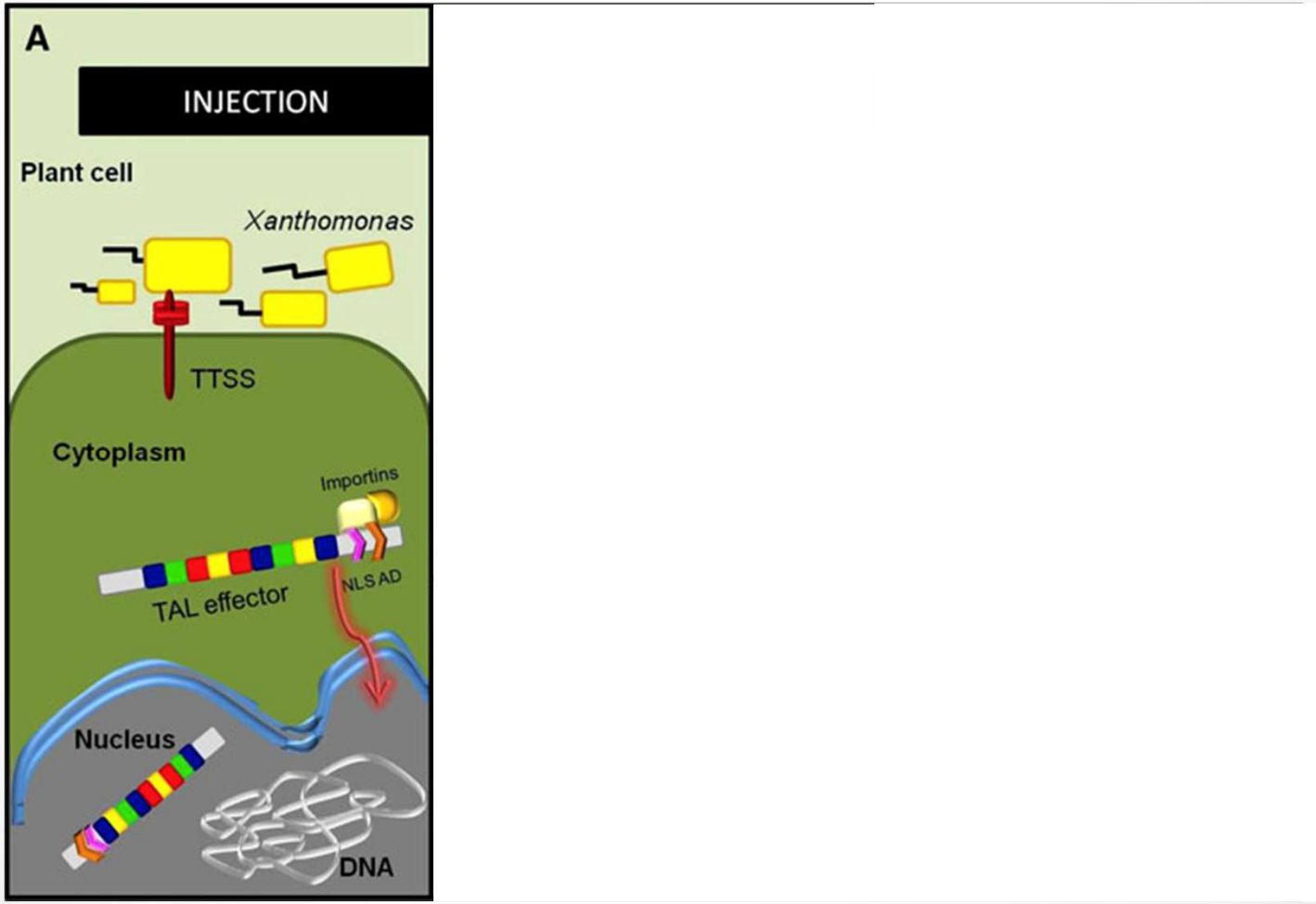


Sistema di secrezione di tipo III

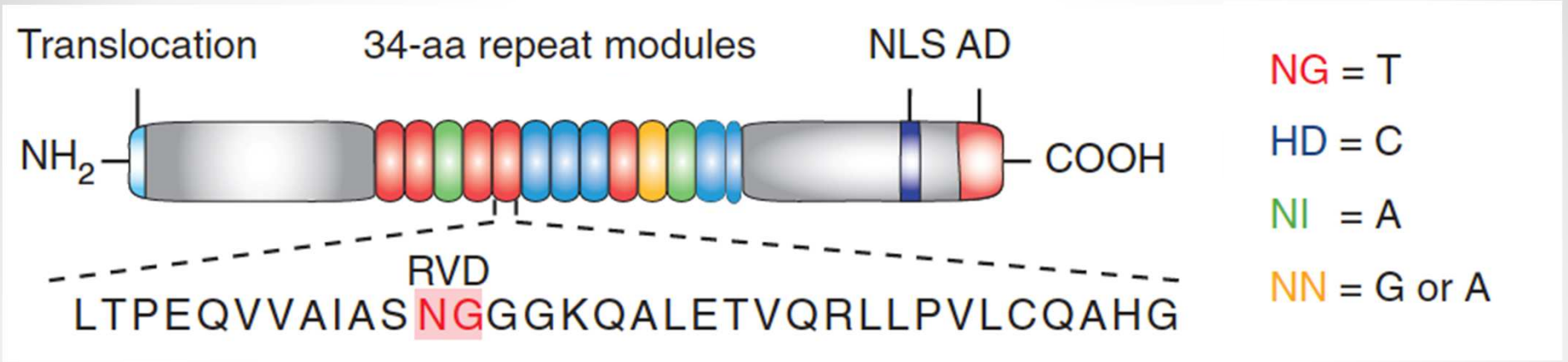
TALE

Funzione

TALE: **T**ranscription **A**ctivator-**L**ike **E**ffector, TALE possono legare e regolare in modo specifico alcuni geni nelle piante durante la patogenesi.



TALE: Struttura Proteica

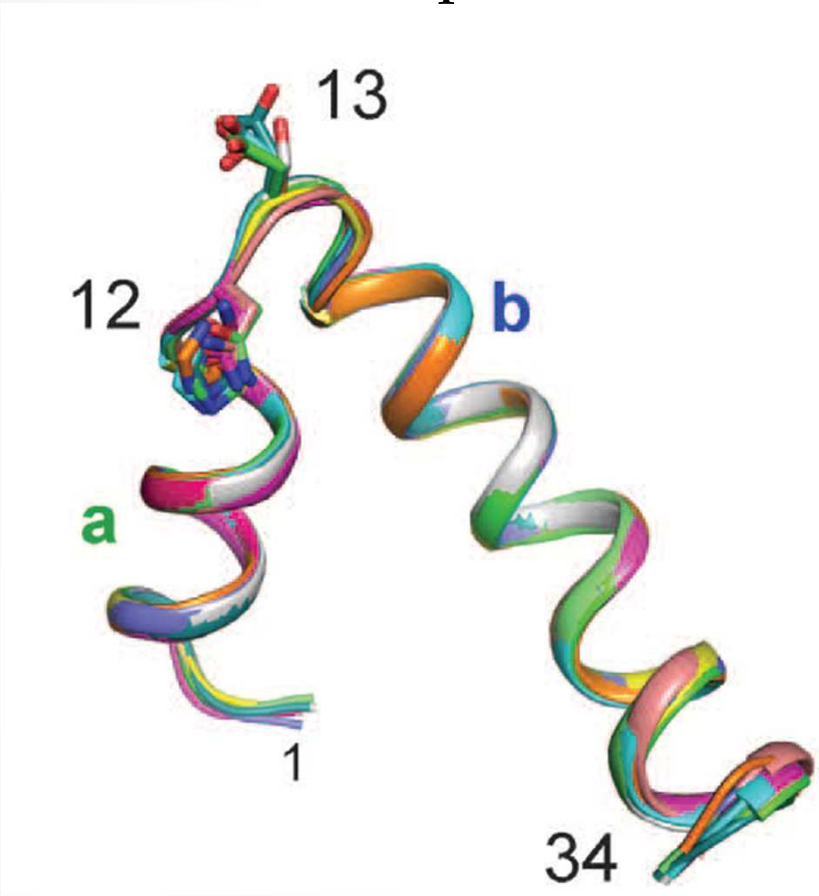


Caratteristiche Principali:

- **Regione Amminoterminale: dominio di traslocazione**
- **Regione centrale composta da tandem repeat di 34 aa (monomeri)**
- **RVD (repeat variable diresidues): in ogni monomero gli aa nelle posizioni 12 e 13 mediano il riconoscimento di uno specifico nucleotide**
- **Regione carbossiterminale: NLS (nuclear localization signal) + dominio effettore**

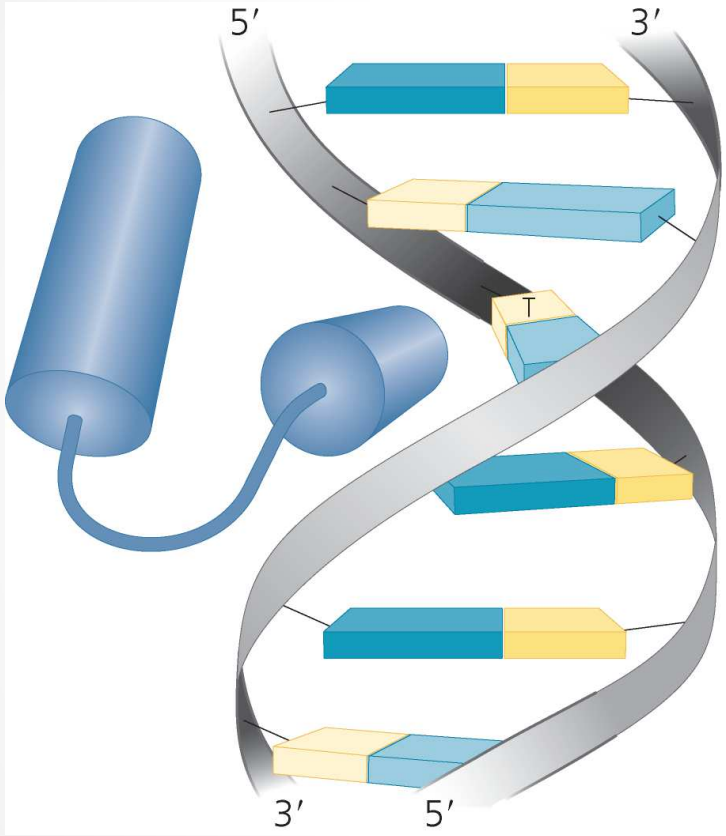
Struttura Proteica

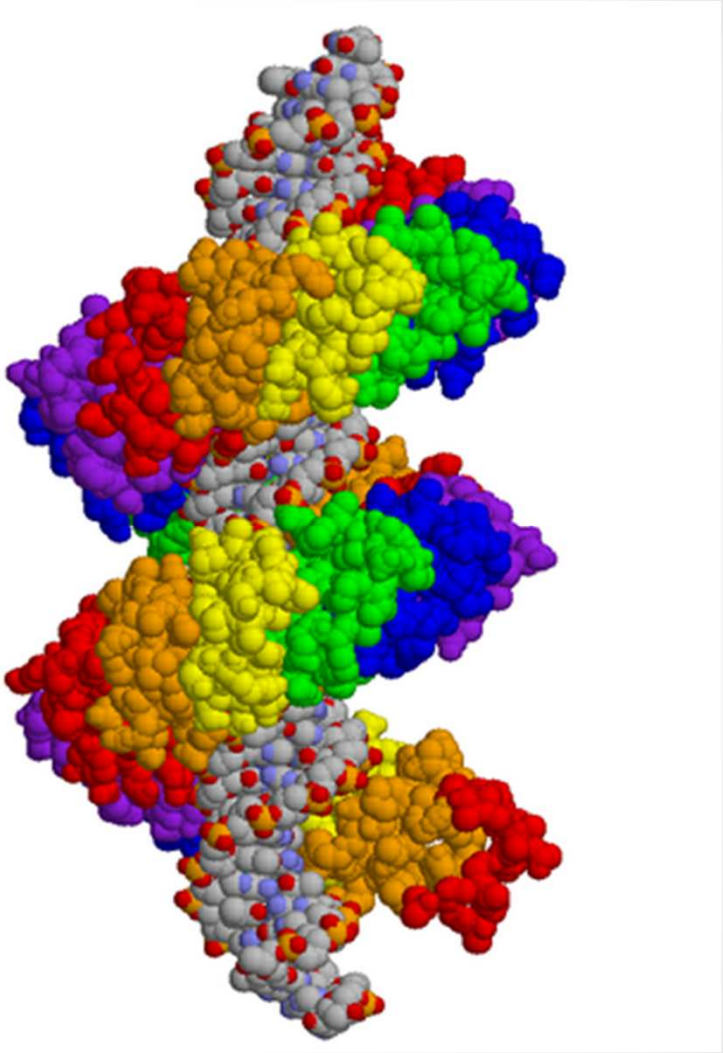
Helix-loop-helix



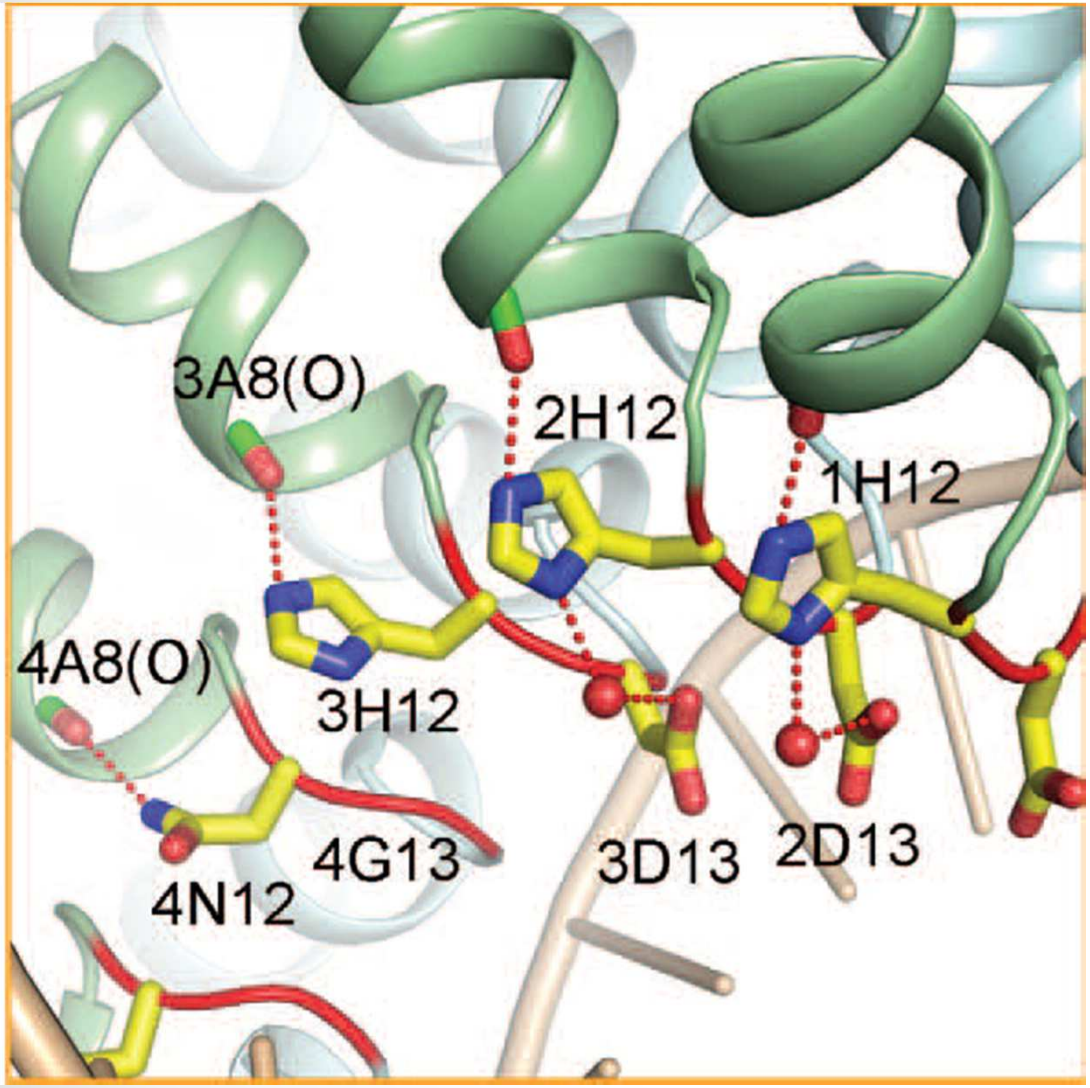
Struttura terziaria di un monomero

Pinza


5′
3′
T
3′
5′



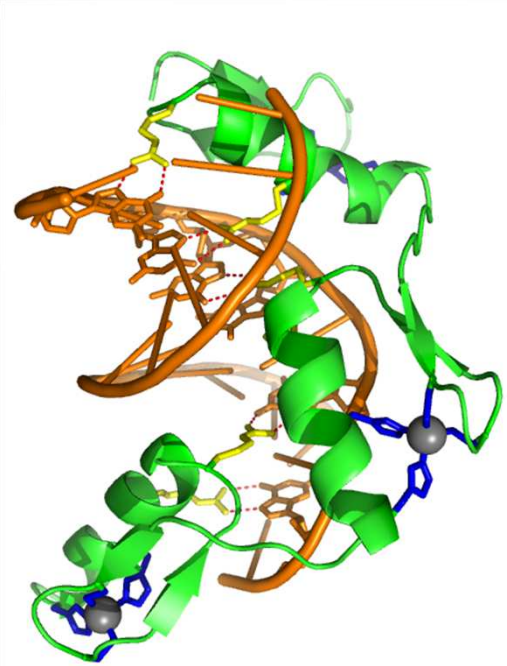
INTERAZIONE TALE-DNA

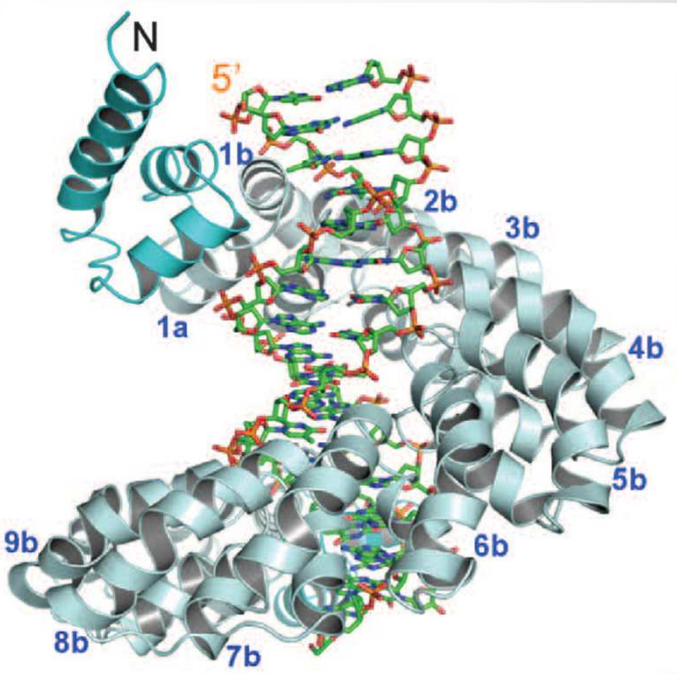


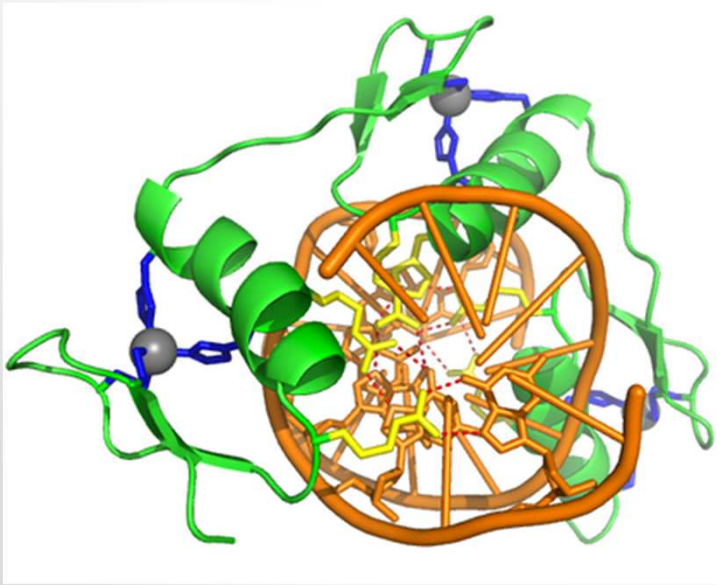
L'aminoacido in posizione 12 stabilizza il loop interagendo con l'alfa-elica minore (alanina in posizione 8).

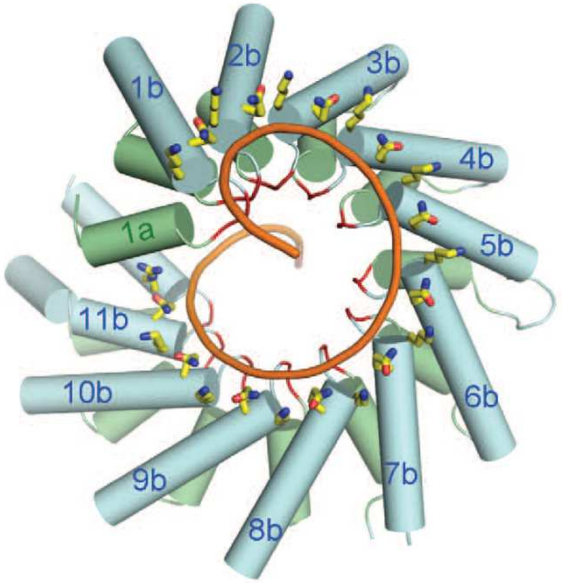
L'interazione TALE/DNA avviene ad esclusivo carico dell'aminoacido in posizione 13 che interagisce direttamente con il nucleotide formando **legami idrogeno o interazioni di van der Waals**.

ZFPs Versus TALEs









ZFPs Versus TALEs

ZFPs

Monomero: 28-30 aa
Target: NNN

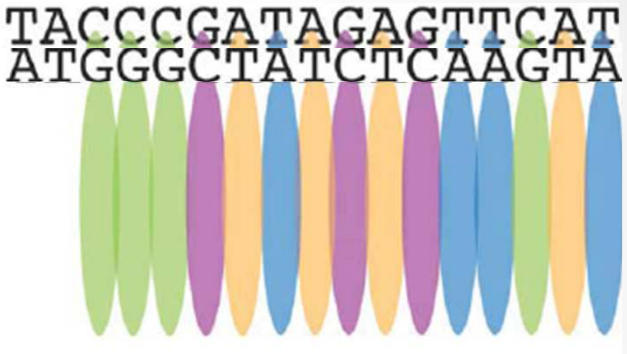
Totale per il riconoscimento di 15 nucleotidi: 150 aa

Design complicato: non esistono ZFP specifiche per tutte le triplette

Tecnologia brevettata:

Sangamo BioSciences, Inc.

TALEs



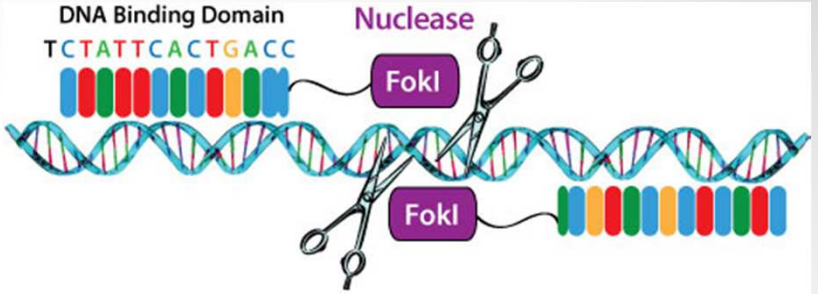
Monomero: 34 aa
Target: N

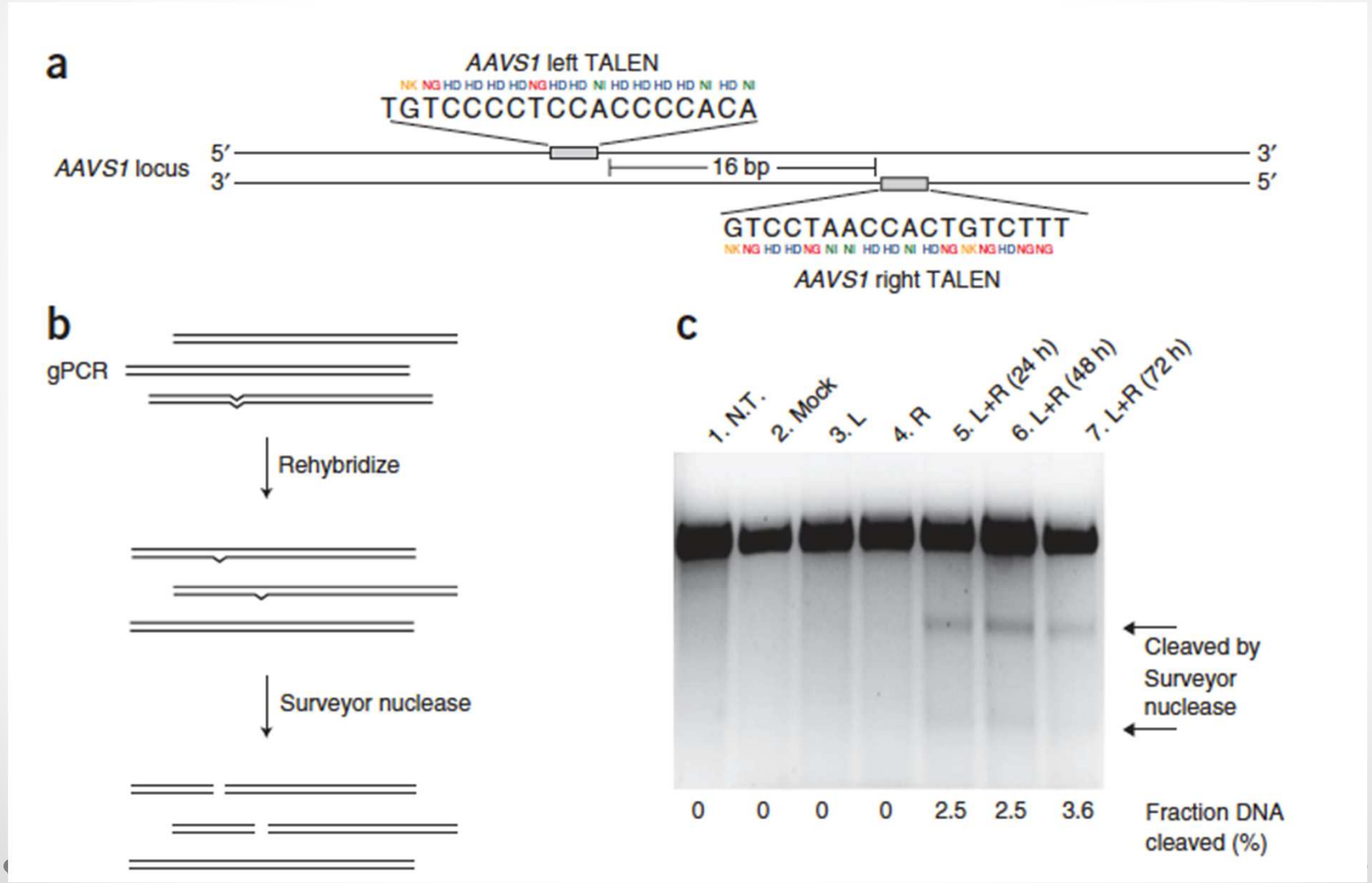
Totale per il riconoscimento di 15 nucleotidi: 510 aa

Design semplice: il nucleotide prima della sequenza target riconosciuta deve essere una Timina

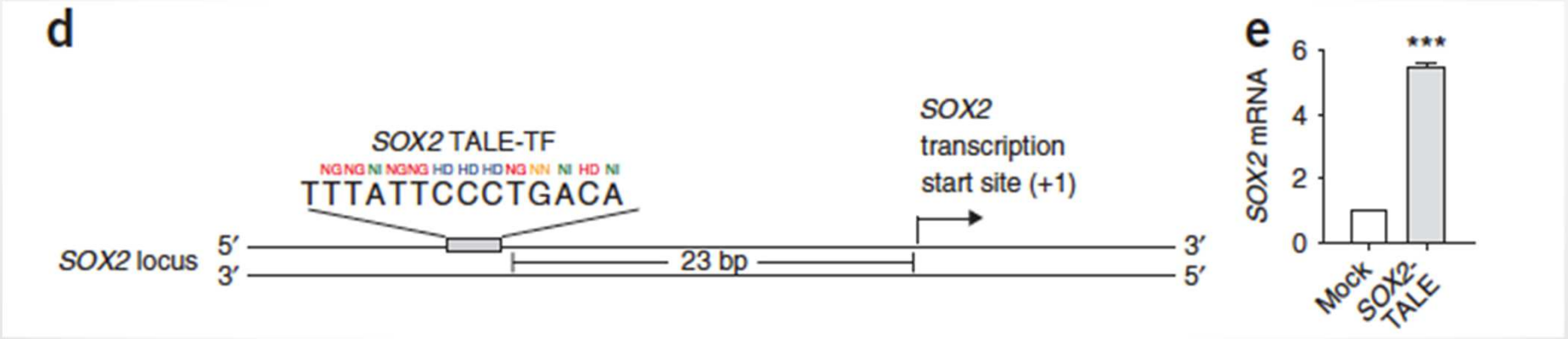
open source

TALEN: TALE-Nuclease





TALE-TF: TALE-Transcriprion Factor



CRISPRs/Cas9 SYSTEM

Storia

- 1987: prima osservazione di sequenze clusterizzate ripetute e palindromiche nel genoma batterico

- 1997: regioni simili identificate nel 90% degli Archea e 40% dei batteri e denominate CRISPR

Clustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats

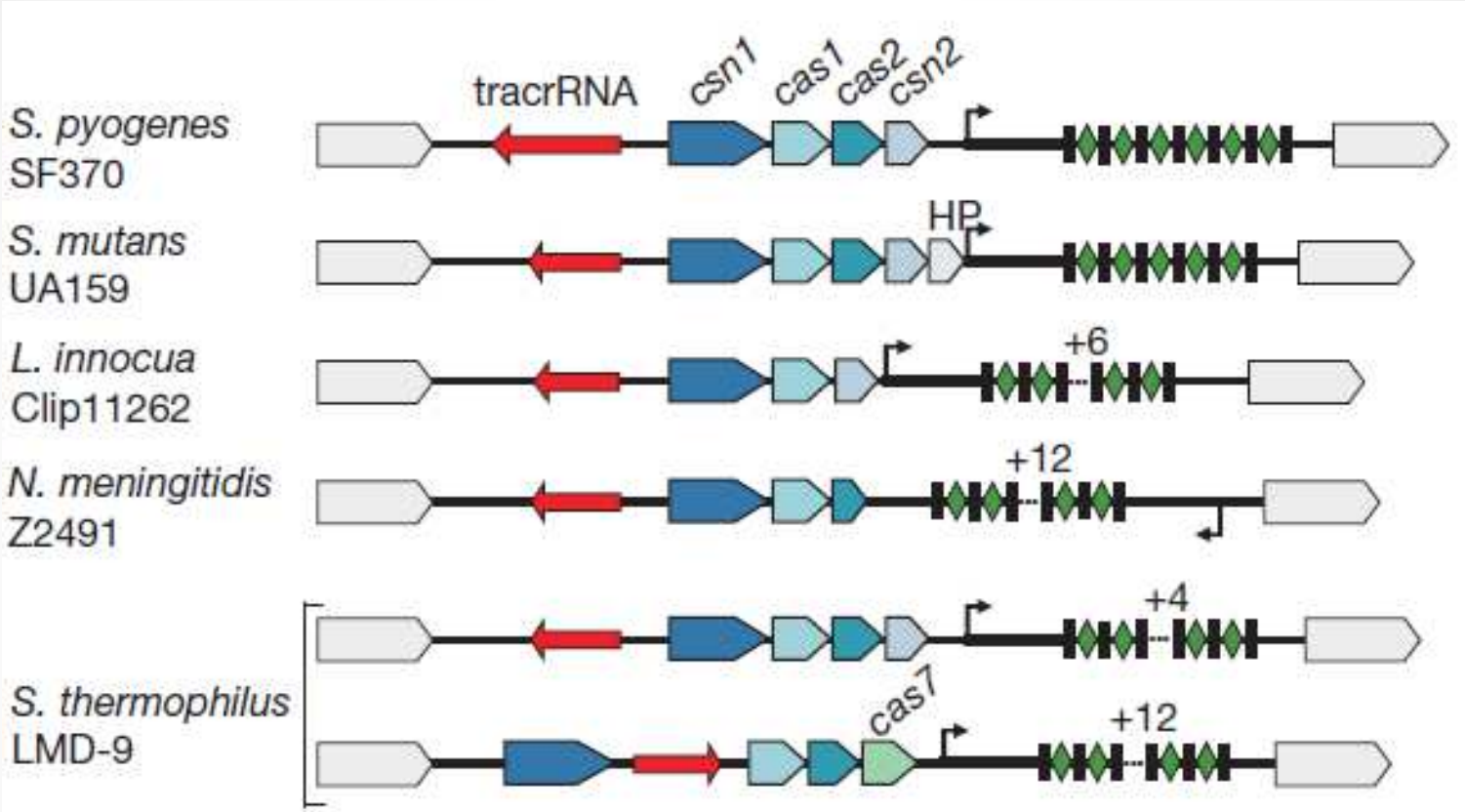
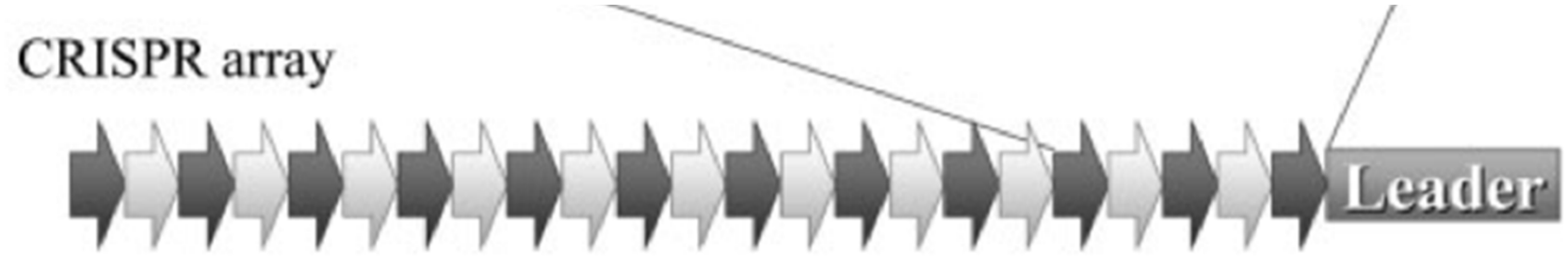


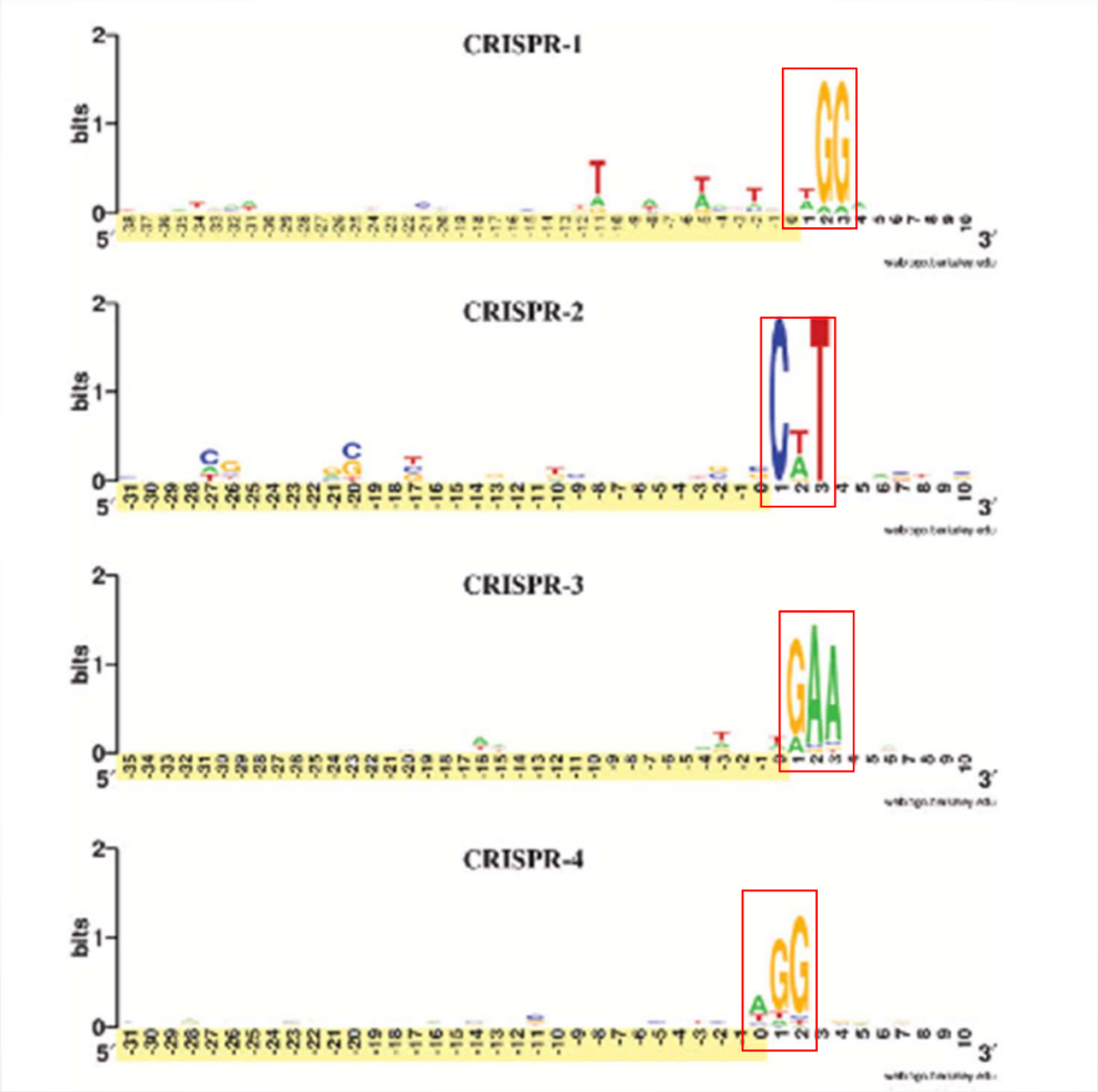
Figure 6 | tracrRNA-mediated crRNA maturation is conserved among different bacterial species. tracrRNA-mediated crRNA maturation is inherent to the type II (Nmeni/CASS4) CRISPR/Cas systems. Type II (Nmeni/CASS4) loci from *S. pyogenes* SF370, *S. mutans* UA159, *L. innocua* Clip11262, *N. meningitidis* Z2491 and *S. thermophilus* LMD-9 (Nmeni/CASS4a); red, tracrRNA; rectangles, repeats; diamonds, spacers.

Storia

- 1987: prima osservazione di sequenze clusterizzate ripetute e palindromiche nel genoma batterico
- 1997: regioni simili identificate nel 90% degli Archea e 40% dei batteri e denominate CRISPR
- 2005: bioinformatici identificano in queste regioni piccoli frammenti corrispondenti a genomi fagici


CRISPR array
Leader

Proto-spacer adjacent motifs (PAMs)



Storia

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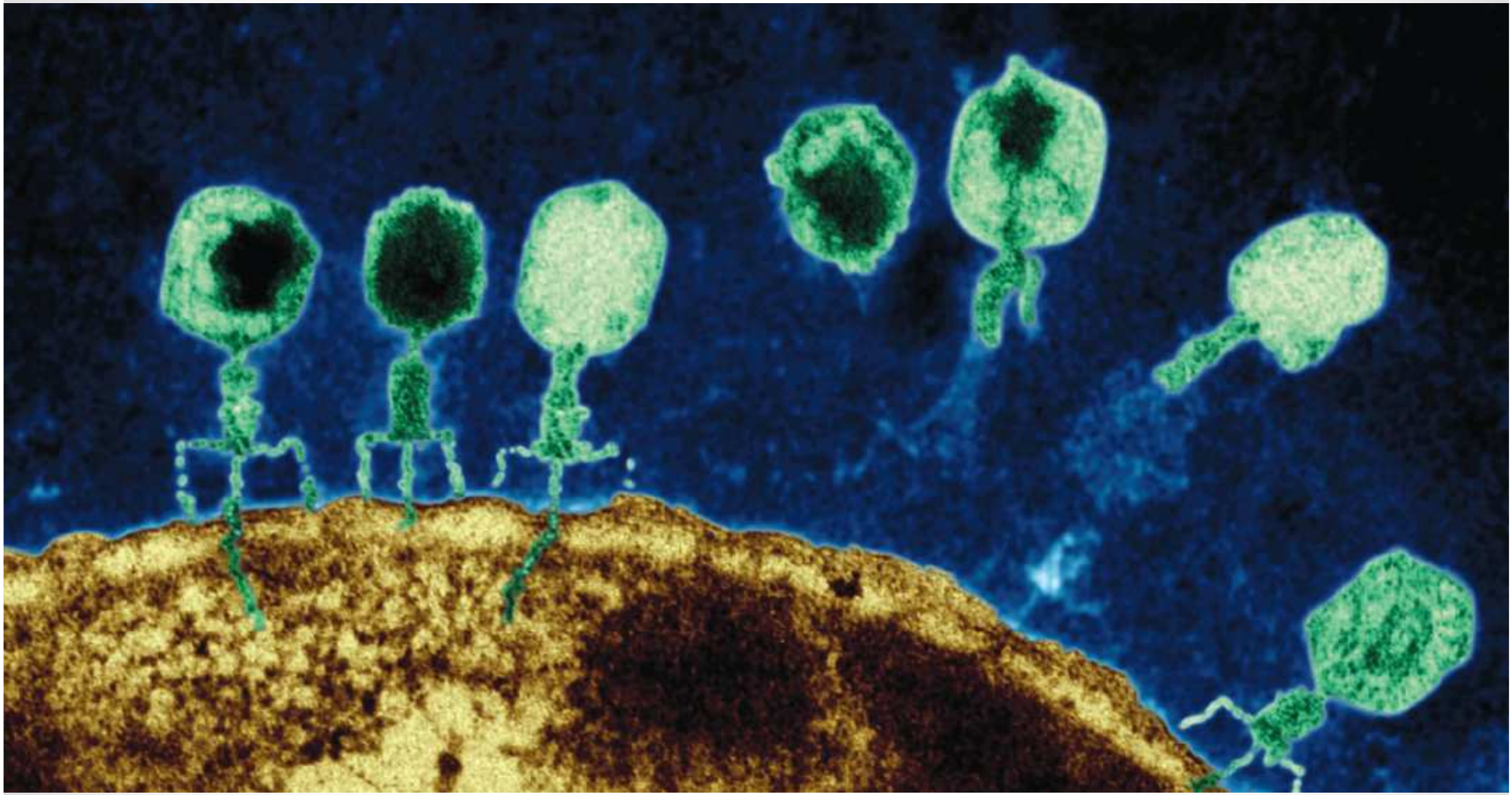
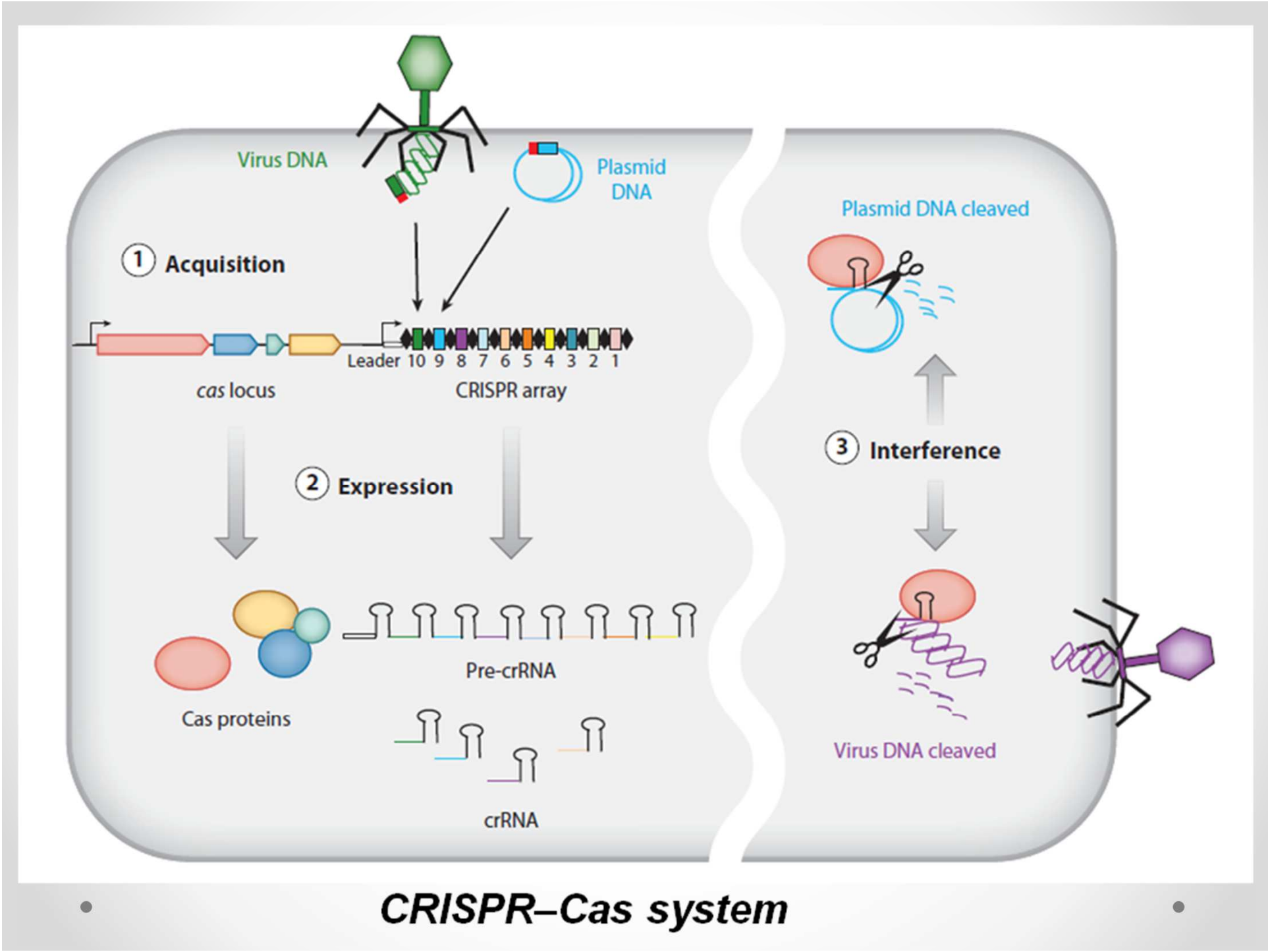
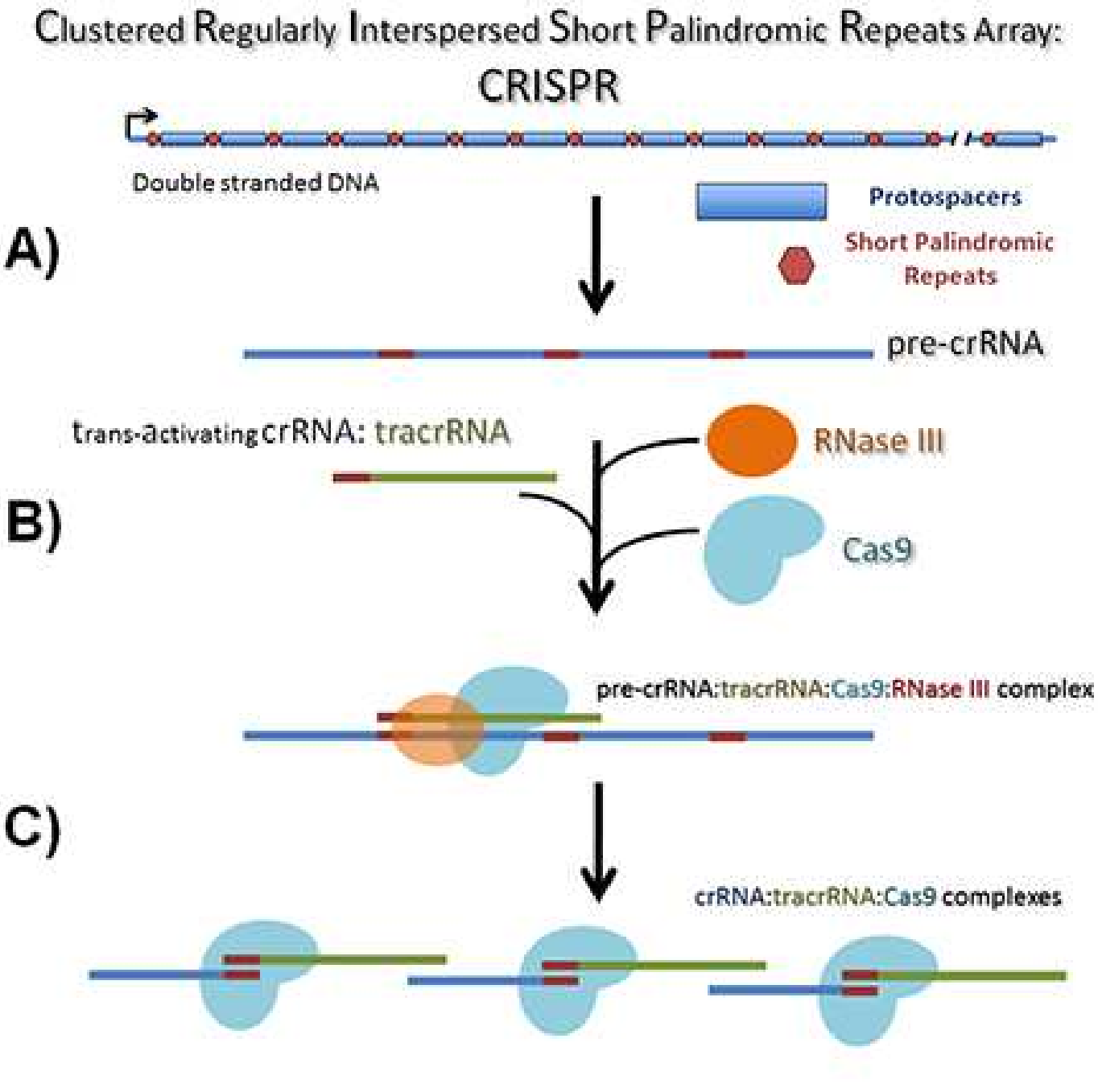
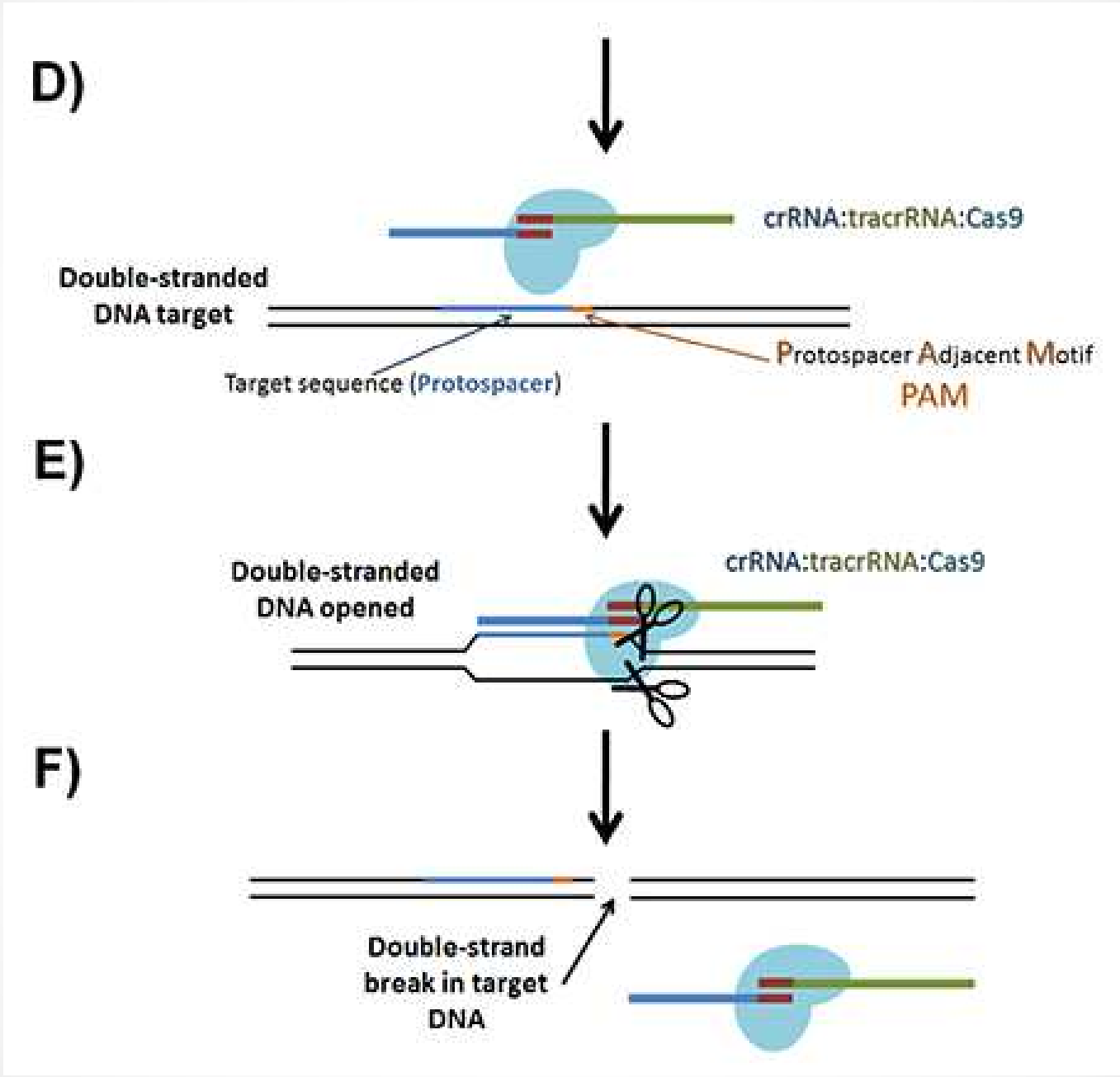
- 1997: regioni simili identificate nel 90% degli Archea e 40% dei batteri e denominate CRISPR
- 2005: bioinformatici identificano in queste regioni piccoli frammenti corrispondenti a genomi fagici
- 2007: ricercatori dell'industria casearia modificano la resistenza di S. thermophilus ai fagi aggiungendo o rimuovendo sequenze presenti nelle regioni CRISPR



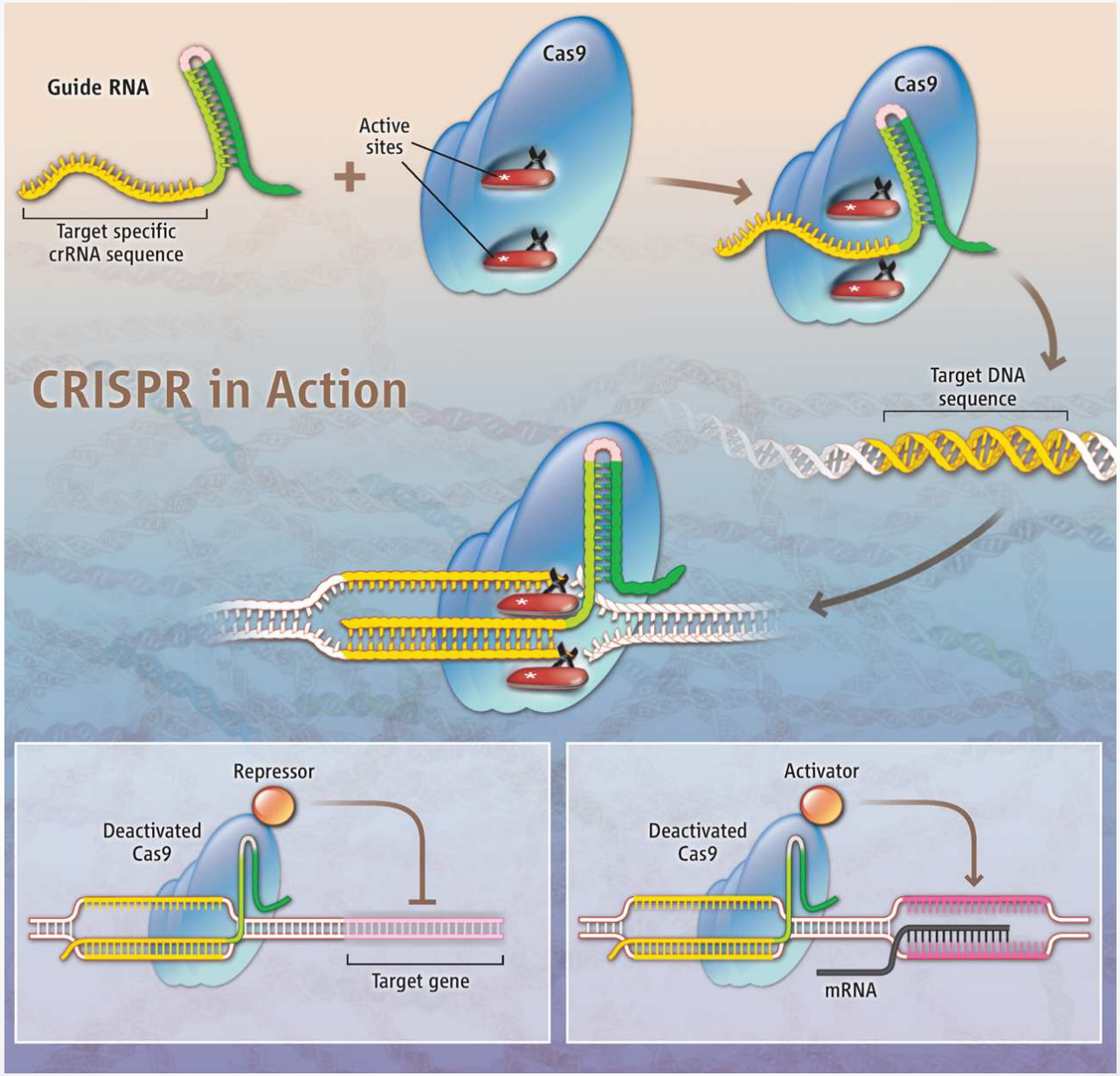


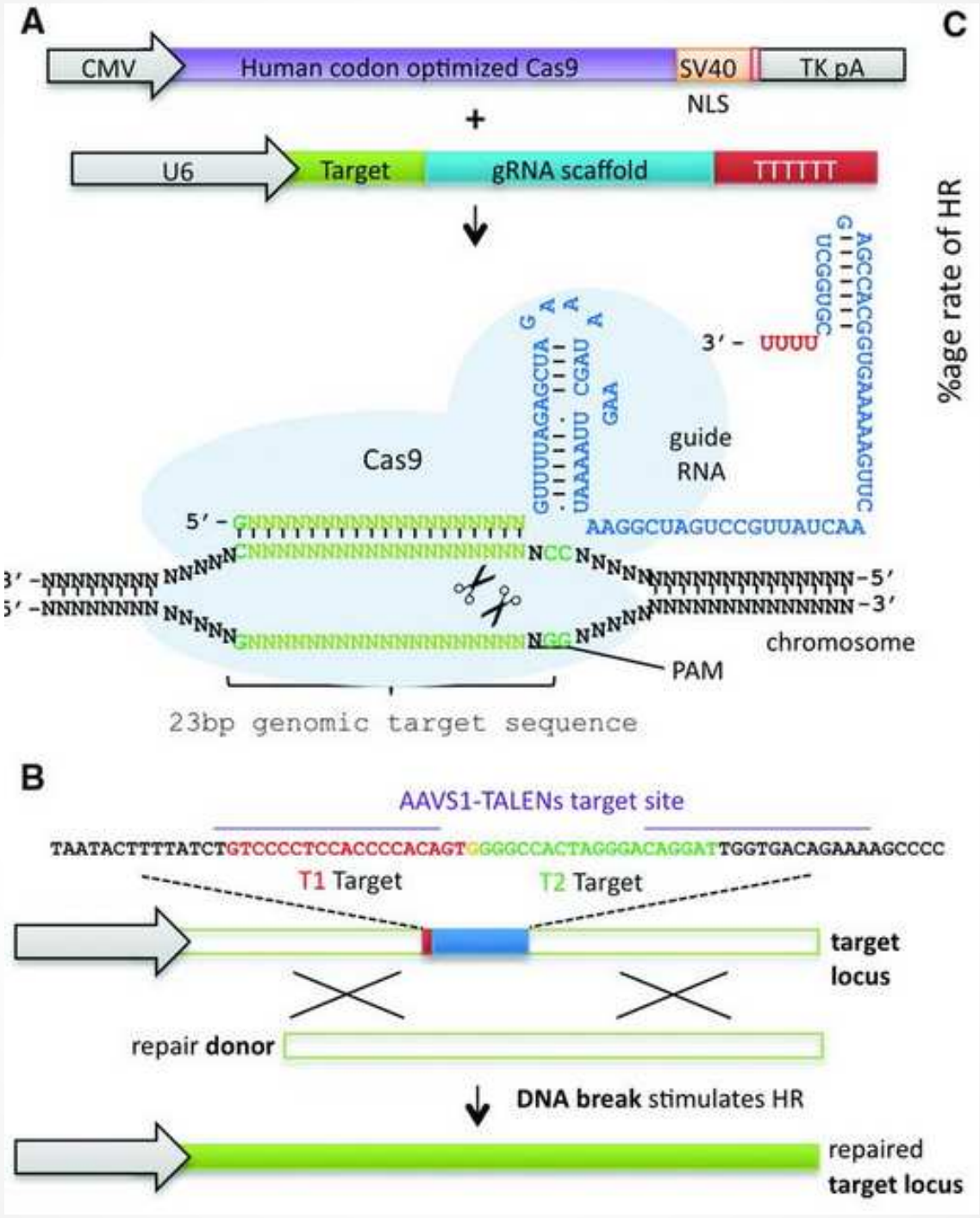
CRISPR–Cas system

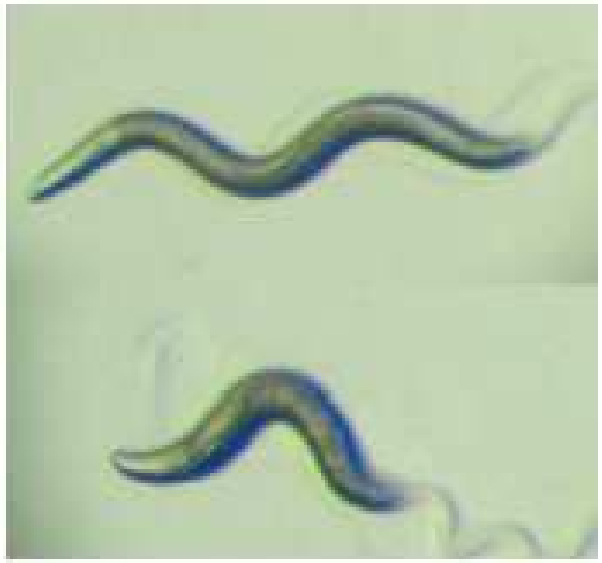

Clustered Regularly Interspersed Short Palindromic Repeats Array:
CRISPR
Double stranded DNA
Protospacers
Short Palindromic Repeats
A)
pre-crRNA
trans-activating crRNA: tracrRNA
RNase III
B)
Cas9
pre-crRNA:tracrRNA:Cas9:RNase III complex
C)
crRNA:tracrRNA:Cas9 complexes

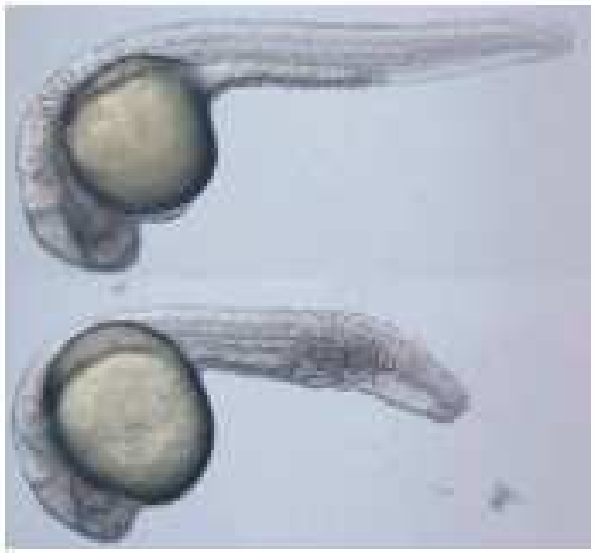

D)
crRNA:tracrRNA:Cas9
Double-stranded
DNA target
Protospacer Adjacent Motif
PAM
Target sequence (Protospacer)
E)
Double-stranded
DNA opened
crRNA:tracrRNA:Cas9
F)
Double-strand
break in target
DNA

RNA-GUIDED GENOME EDITING

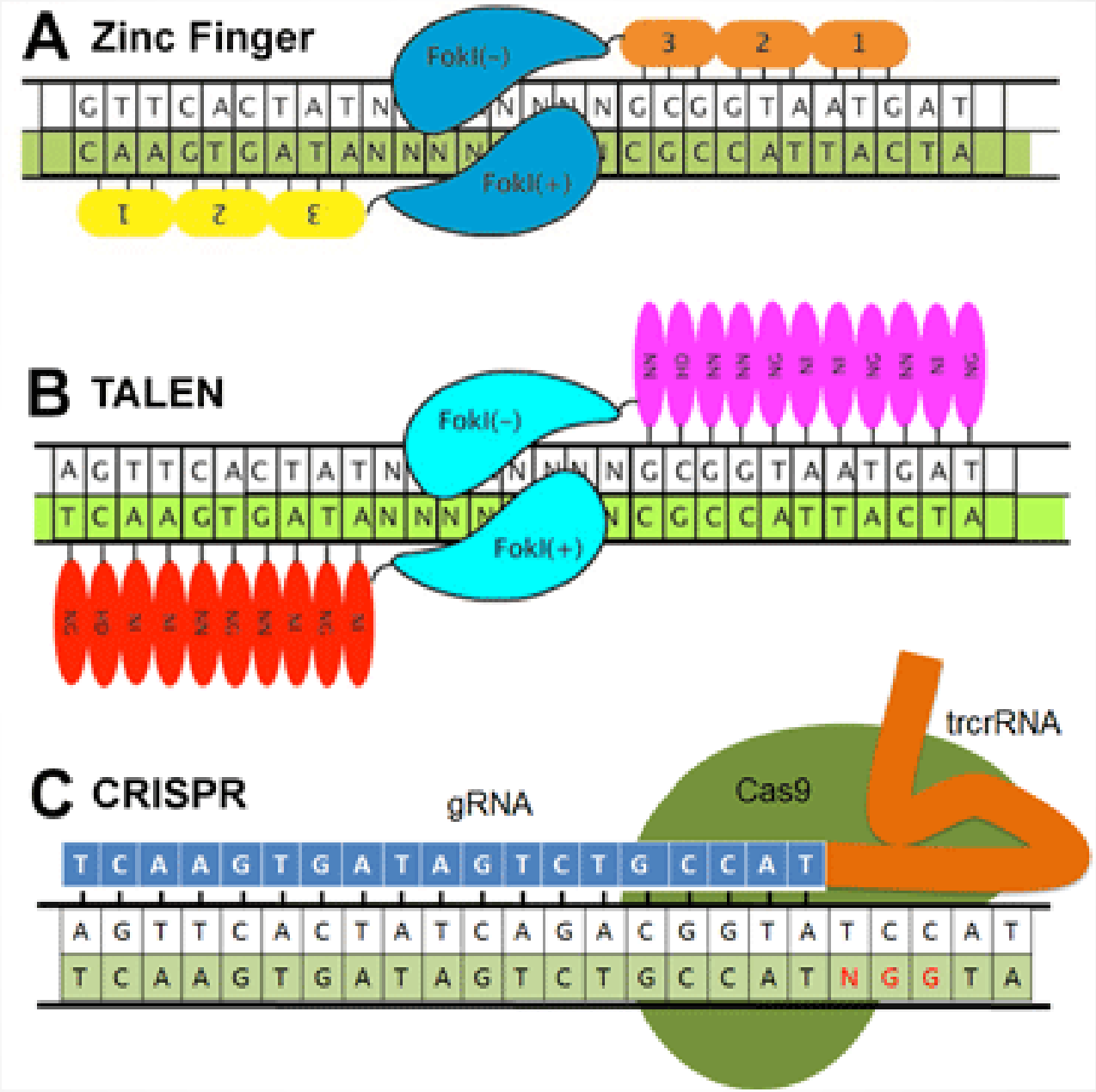



A
CMV
Human codon optimized Cas9
SV40
NLS
TK pA
+
U6
Target
gRNA scaffold
TTTTTT
Cas9
guide
RNA
5′ –
3′ –
UUUU
AAGGCUAGUCCGUUAUCAA
chromosome
PAM
23bp genomic target sequence
C
%age rate of HR
B
AAVS1-TALENs target site
TAATACTTTTATCTGTCCCCTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGTGACAGAAAAGCCCC
T1 Target
T2 Target
target
locus
repair donor
DNA break stimulates HR
repaired
target locus





Precise cuts. In just 8 months, CRISPR modifications of DNA resulted in dumpier nematodes (*top*, bottom), zebrafish embryos with an excess of ventral tissue (*middle*, bottom), and fruit flies with dark eyes (*bottom*, right), demonstrating its broad utility for editing genes in animals.


A Zinc Finger
FokI(−)
FokI(+)
3
2
1
B TALEN
FokI(−)
FokI(+)
C CRISPR
gRNA
Cas9
trcrRNA

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