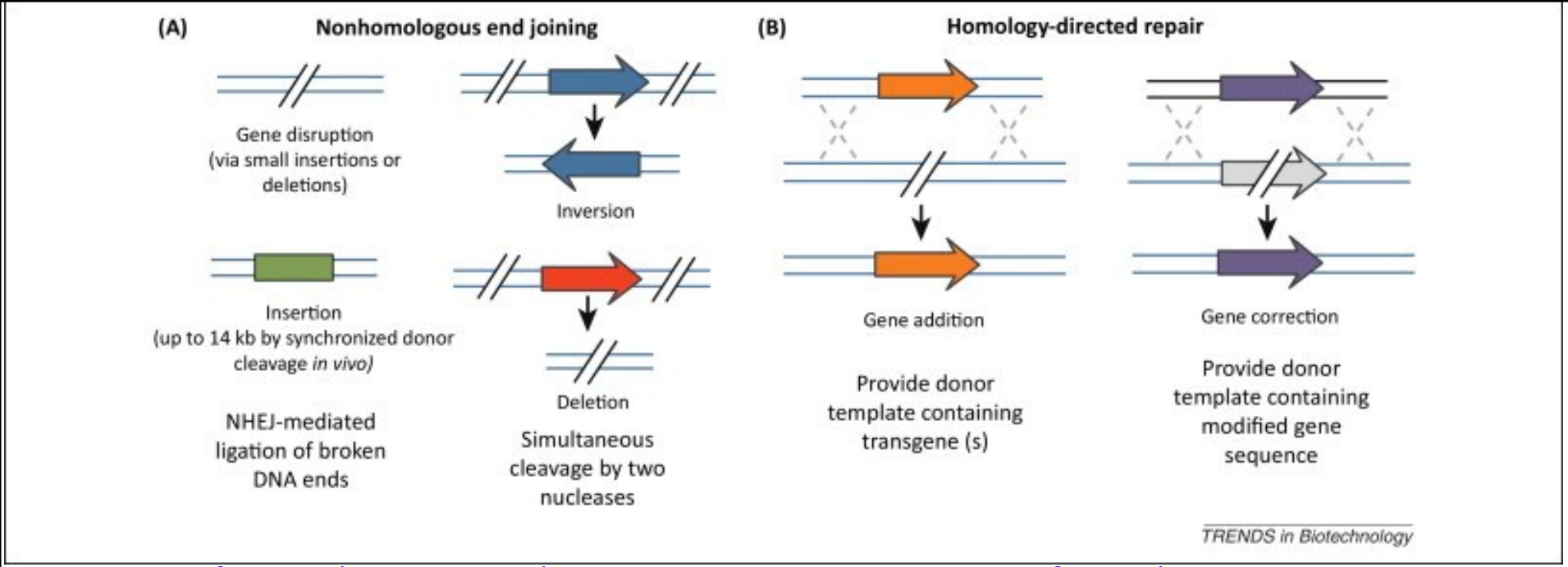
Several genome-editing

- zinc-finger nucleases (ZFNs)
- transcription activator-like effector nucleases (TALENs)
- Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) system,

Used to produce knockout/knockin/conditional alleles

- Rat models by generating DNA double-strand breaks (DSBs) followed by non-homologous end joining (NHEJ)-mediated repair

Cell Research (2014) 24:122-125



Overview of possible genome editing outcomes using site-specific nucleases.
Nuclease-induced DNA double-strand breaks (DSBs) can be repaired by homology-directed repair (HDR) or error-prone nonhomologous end joining (NHEJ).

(A) In the presence of donor plasmid with extended homology arms, HDR can lead to the introduction of single or multiple transgenes to correct or replace existing genes.

(B) In the absence of donor plasmid, NHEJ-mediated repair yields small insertion or deletion mutations at the target that cause gene disruption.

In the presence of double-stranded oligonucleotides or *in vivo* linearized donor plasmid, DNA fragments up to 14 kb have been inserted via NHEJ-mediated ligation.
Simultaneous induction of two DSBs can lead to deletions, inversions and translocations of the intervening segment.

Trends in Biotechnology
Volume 31, Issue 7, July 2013, Pages 397-405

ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering

ZFNs

- zinc-finger nucleases are fusions of the nonspecific DNA cleavage domain from the *FokI* restriction endonuclease with zinc-finger proteins. ZFN dimers induce targeted DNA DSBs that stimulate DNA damage response pathways. The binding specificity of the designed zinc-finger domain directs the ZFN to a specific genomic site.

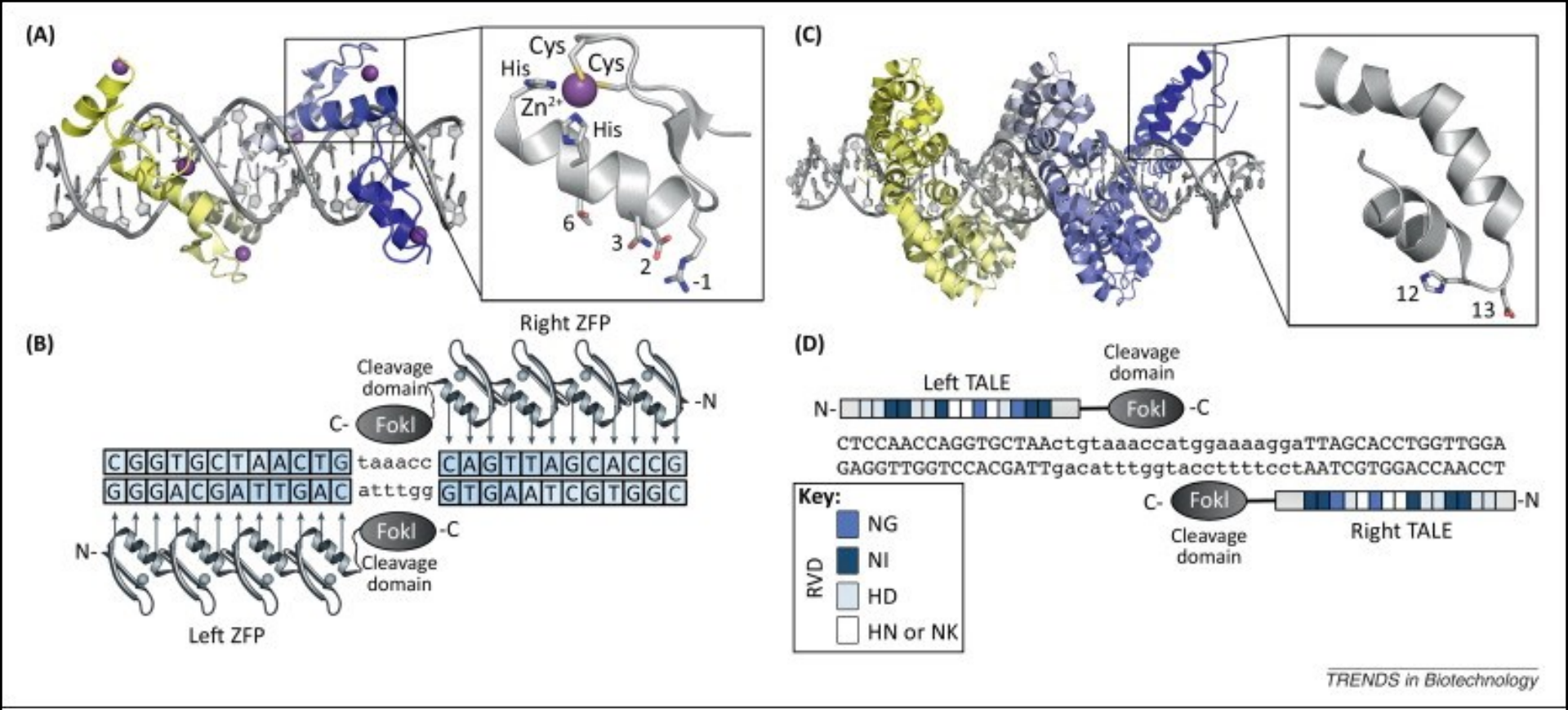
ZFNickases

- zinc-finger nickases are ZFNs that contain inactivating mutations in one of the two *FokI* cleavage domains. ZFNickases make only single-strand DNA breaks and induce HDR without activating the mutagenic NHEJ pathway.

TALENs

- transcription activator-like effector nucleases are fusions of the *FokI* cleavage domain and DNA-binding domains derived from TALE proteins. TALEs contain multiple 33–35-amino-acid repeat domains that each recognizes a single base pair. Like ZFNs, TALENs induce targeted DSBs that activate DNA damage response pathways and enable custom alterations.

Trends in Biotechnology
Volume 31, Issue 7, July 2013, Pages 397–405



The 'modular assembly' approach involves the use of a preselected library of zinc-finger modules or selection-based approaches, such as oligomerized pool engineering (OPEN)

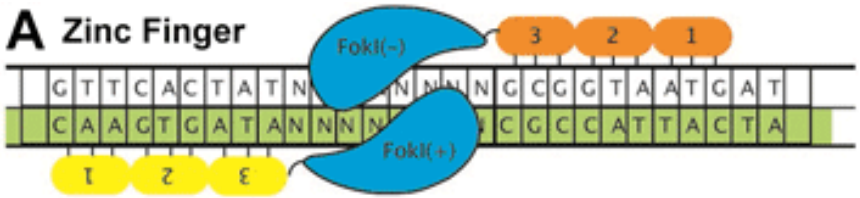
Broadly, zinc-finger protein technology enables targeting of virtually any sequence.

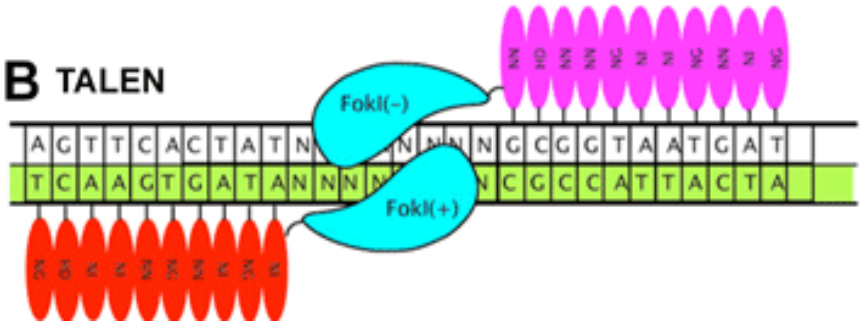
TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair

Trends in Biotechnology
Volume 31, Issue 7, July 2013, Pages 397–405

Genetic manipulation in mouse;

ZFN, TALEN and CrispR/cas systems;





- Cys_2-His_2 zinc finger domain contacts 3bp of sequence in major groove with varying levels of selectivity.
- Can use as modular component to get sequence specific targeting of FokI restriction endonuclease monomer. Cleavage requires targeting second monomer to other strand to generate functional FokI dimer.
- Provides substrate for error prone repair or HR using recombinant DNA template for custom modification.

- TALE effector proteins secreted by Xanthomonas bacteria in order to activate host plant gene expression that aids infection.
- Modular composition of sequence specific binding domains comprising 33-34 amino acids with positions 12 and 13 being highly variable.
- Can be used to construct designer Transcription Activator Like Effector Nuclease (TALEN) to introduce DNA breaks at defined target sequence.
- Provides substrate for error prone repair or HR using recombinant DNA template for custom modification.

Table 1
Generalized comparison of various genome engineering tools.

Nuclease platform	MN	ZFN	TALEN	Targetron	CRISPR/Cas
Source	Organellar DNA, Bacteria, Phages	Bacteria, Eukaryotes	Bacteria (*Xanthamonas* sp.)	Organellar DNA, Bacteria, Phages	Bacteria (*Streptococcus* sp.)[a]
Number of component(s)	1	2	2	2	1–2 (depends)[b]
Availability of core components[c]	Restricted	Available	Available	Restricted	Available
Type of recognition	Protein-DNA	Protein-DNA	Protein-DNA	RNA-DNA	RNA-DNA
Recognition site (bp)	18–44[d]	18–36	24–40	14–15	17–23
Double-stranded break pattern	Staggered cut (4 nt, 3′ overhang)	Staggered cut (4–5 nt, 5′ overhang)	Staggered cut (Heterogeneous overhangs)	Staggered cut[e]	SpCas9 creates blunt ends; Cpf1 creates staggered cut (5′ overhang)
Function	Nuclease, Nickase	Nuclease, Nickase	Nuclease, Nickase	Site-specific bacterial gene disruption[f]	Nuclease, Nickase
Best suited for	Gene editing	Gene knockout, Transcriptional regulation	Gene knockout, Transcriptional regulation	Gene knockout	Gene knockout, Transcriptional regulation, Base editing
Ease of design	Difficult	Difficult; Design of new ZFNs is much easier than MNs	Moderate	Moderate	Easy
Dimerization required	No	Yes	Yes	No	No
Ease of generating large scale libraries	Laborious	Laborious	Moderately laborious	Unknown	Easy
Specificity	High	Low–Moderate	Moderate	Moderate	Low–Moderate[g]
Multiplexing	Low	Low	Moderately high	Low	High
Gene drive	Possible	Unknown	Unknown	Unknown	Possible
Improved/other versions	MegaTEV, MegaTAL	AZP-SNase	Tev-mTALEN	Thermotargetron	Cpf1, eSpCas9
Cost (USD)[h]	4000–5000	5–10,000	<1000	450–1500	<100
Targeting constraints	Chromatin compaction	Non-guanosine rich sequence hard to target	5′ targeted base must be thymine for each TALEN monomer	Entry of RNP complex in nucleus difficult	PAM sequence must follow target site
Efficiency/Inefficiency	Small size of MN allows use in a variety of viral vectors	Small size of ZFN expression cassettes allows use in a variety of viral vectors	Large size of each TALEN makes it difficult to pack in viral vectors	Large size of ribonucleoprotein complex makes it difficult for entry into nucleus	Commonly used Cas9 from *S. pyogenes* is large, impose packaging problems in viral vectors [i]
Methylation sensitive	Yes	Yes	Yes	Unknown	No
First use in human cells	1994	2003	2011	2015	2013
Immunogenicity	Unknown	Low	Unknown	Unknown	Unknown
Vector packaging[j]	Multiple	Multiple	Few	Multiple	Multiple
Size of mRNA transcripts	Short	Short	Long	Short	Long
Mode of ex vivo delivery in animal cells	Electroporation, Viral transduction, Direct injection into zygotes	Electroporation, Lipofection, Viral transduction, Direct injection into zygotes	Electroporation, Lipofection, Viral transduction, Direct injection into zygotes	Electroporation, Lipofection	Electroporation, Lipofection, Viral transduction, Direct injection into zygotes

Glossary

Genome Editing Glossary

Cas = CRISPR-associated genes

Cas9, Csn1 = a CRISPR-associated protein containing two nuclease domains, that is programmed by small RNAs to cleave DNA

crRNA = CRISPR RNA

dCAS9 = nuclease-deficient Cas9

DSB = Double-Stranded Break

gRNA = guide RNA

HDR = Homology-Directed Repair

HNH = an endonuclease domain named for characteristic histidine and asparagine residues

Indel = insertion and/or deletion

NHEJ = Non-Homologous End Joining

PAM = Protospacer-Adjacent Motif

RuvC = an endonuclease domain named for an *E. coli* protein involved in DNA repair

sgRNA = single guide RNA

tracrRNA, trRNA = trans-activating crRNA

TALEN = Transcription-Activator Like Effector Nuclease

ZFN = Zinc-Finger Nuclease

https://www.neb.com/tools-and-resources/feature-articles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology

ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering

CRISPR/Cas (CRISPR associated) systems

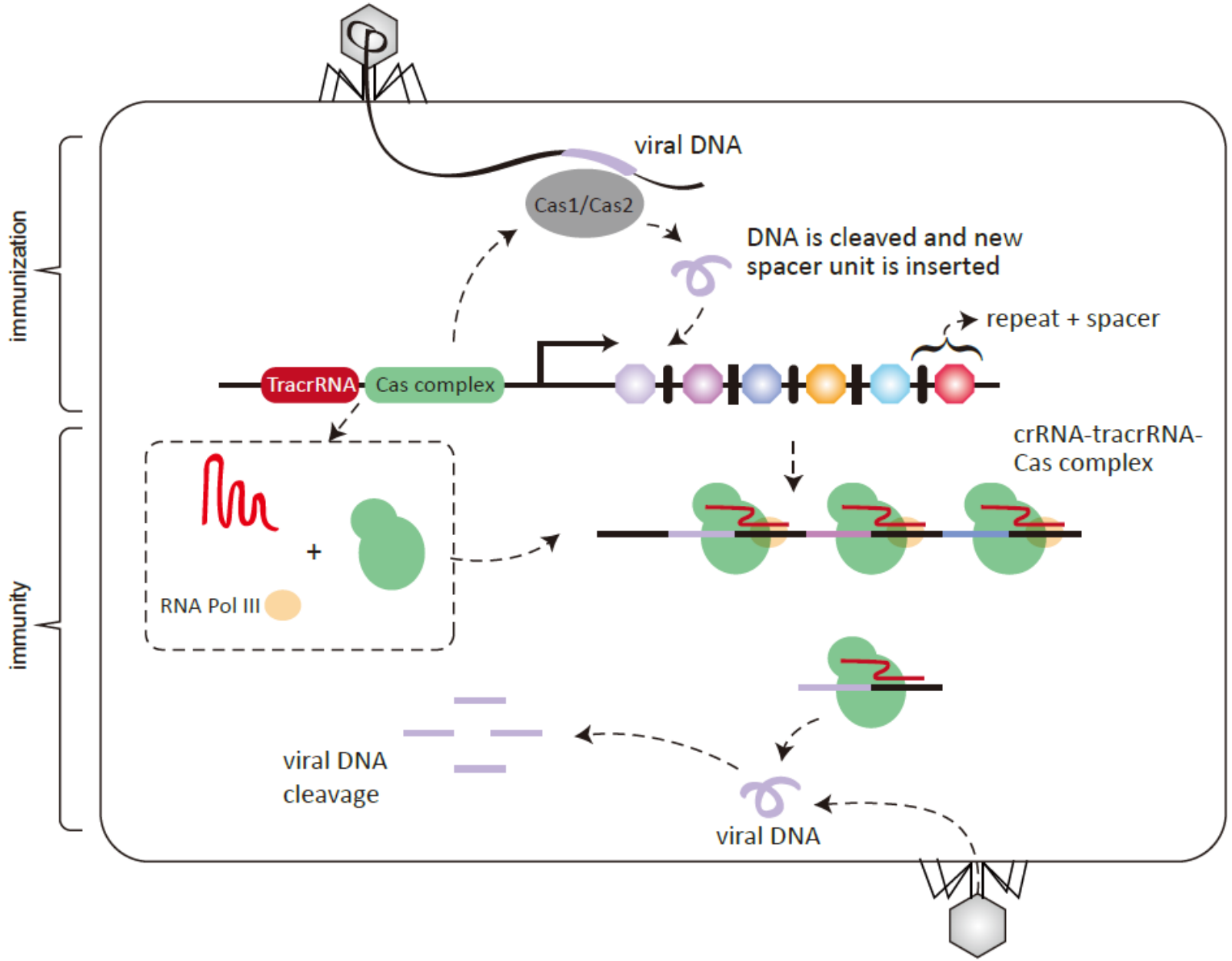
- Clustered Regulatory Interspaced Short Palindromic Repeats are loci that contain multiple short direct repeats, and provide acquired immunity to bacteria and archaea.
  CRISPR systems rely on crRNA and tracrRNA for sequence-specific silencing of invading foreign DNA.
  Three types of CRISPR/Cas systems exist:
  in type II systems, Cas9 serves as an RNA-guided DNA endonuclease that cleaves DNA upon crRNA–tracrRNA target recognition.

crRNA

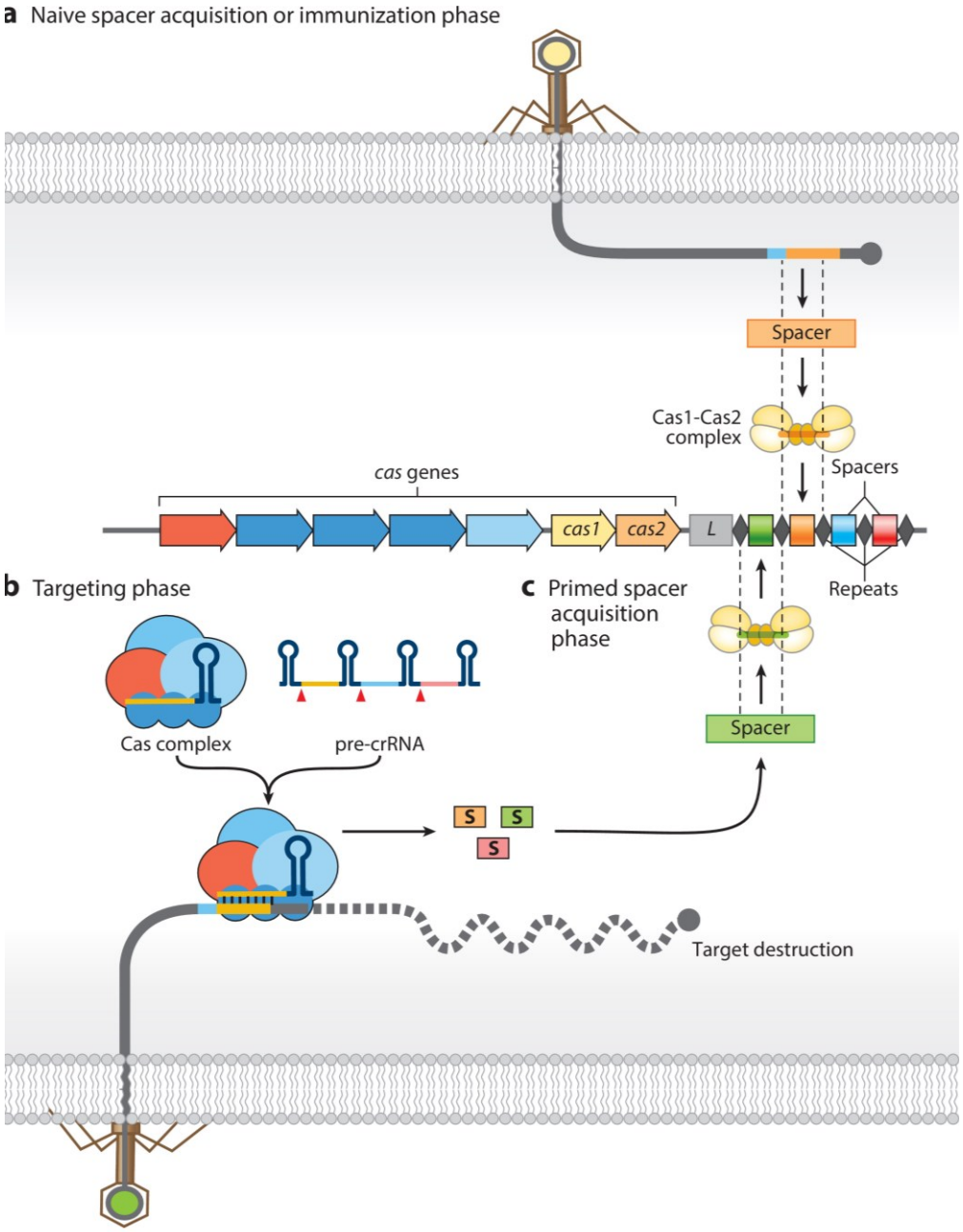
- CRISPR RNA base pairs with tracrRNA to form a two-RNA structure that guides the Cas9 endonuclease to complementary DNA sites for cleavage.

Trends in Biotechnology
Volume 31, Issue 7, July 2013, Pages 397–405

Figure 1: Mechanism of CRISPR-mediated immunity in bacteria



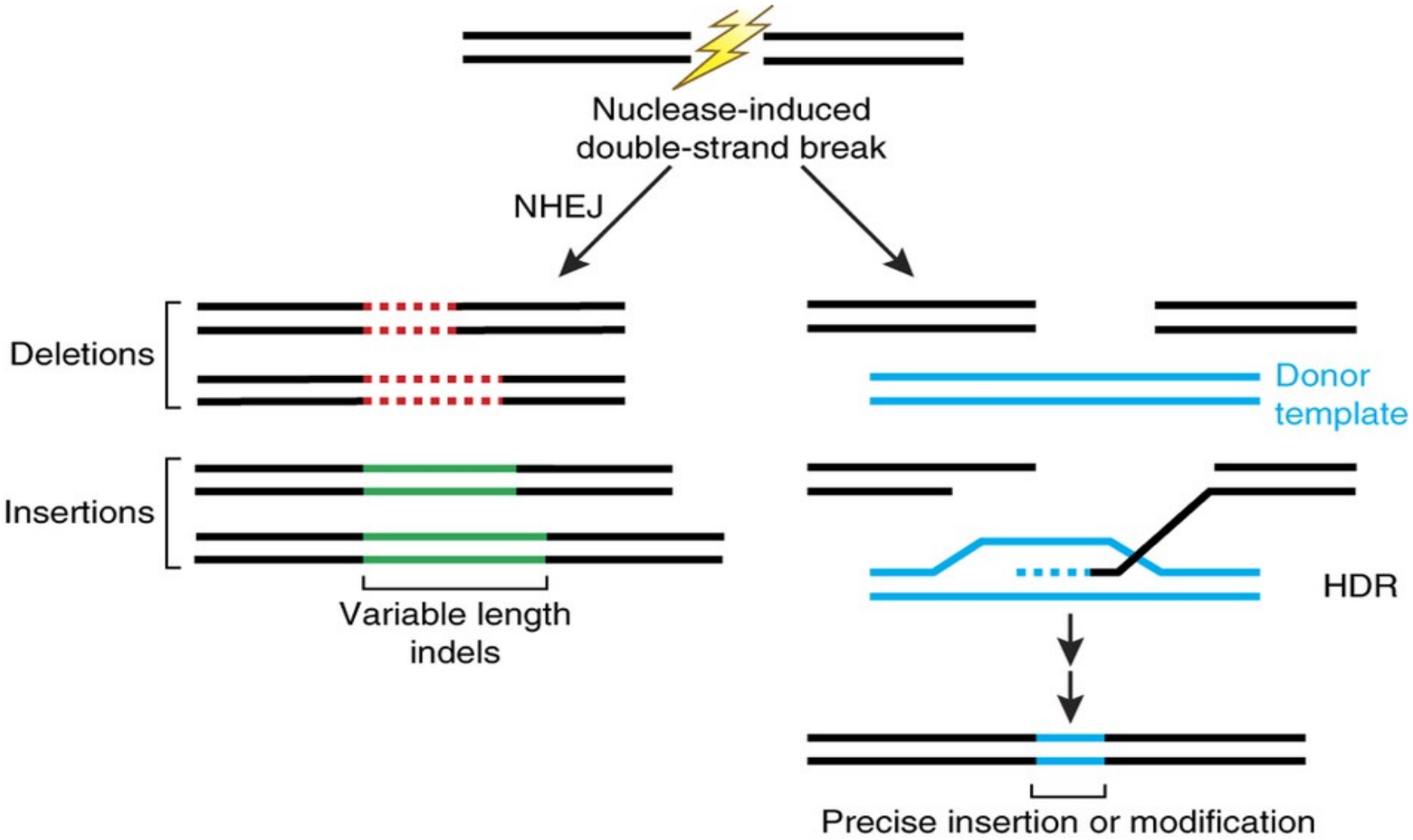
Molecular Mechanisms of CRISPR-Cas Immunity in Bacteria



Naïve spacer acquisition or immunization

Targeting phase

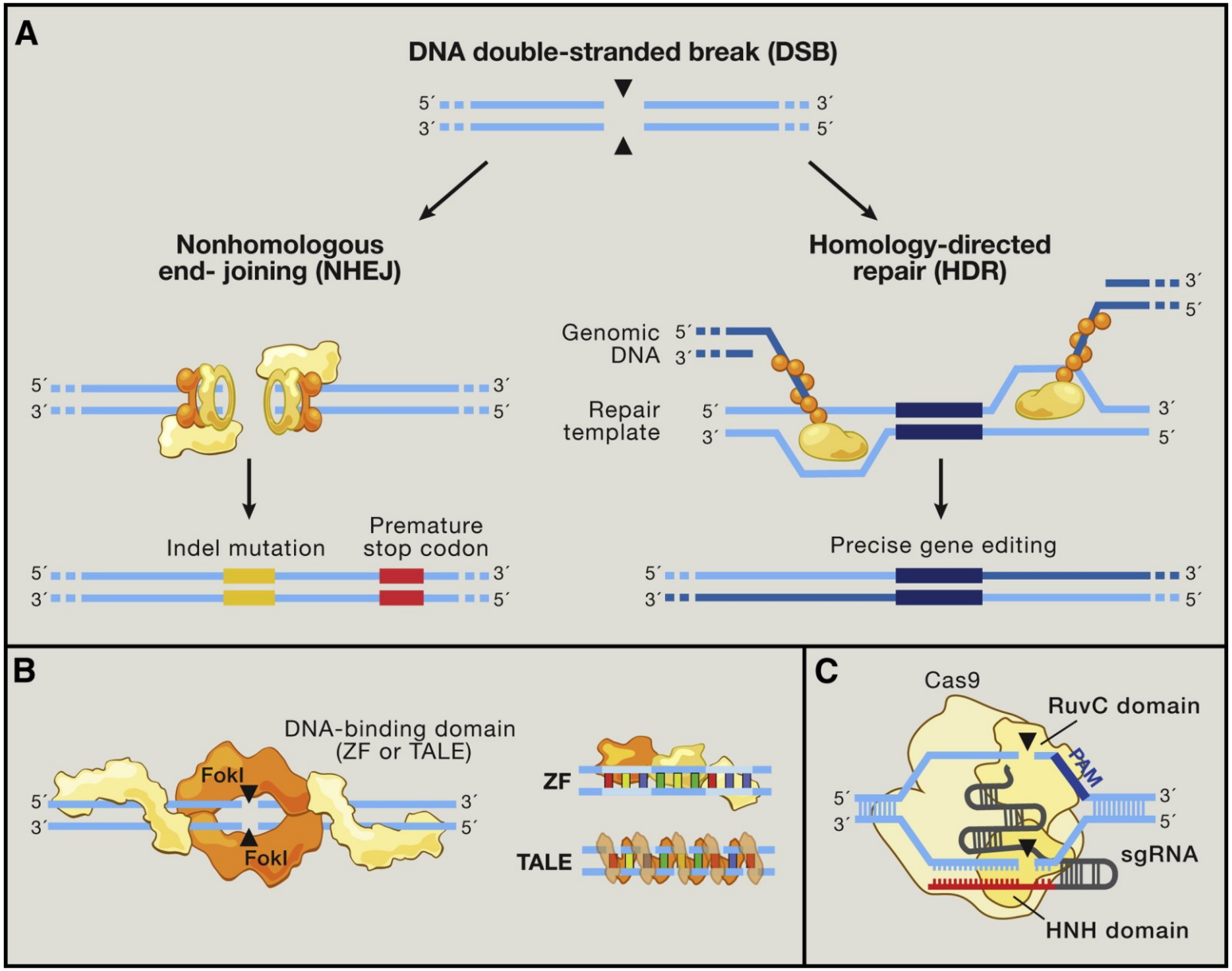
Nussenzweig, Philip M, and Luciano A Marraffini. "Molecular Mechanisms of CRISPR-Cas Immunity in Bacteria." *Annual review of genetics* vol. 54 (2020): 93-120. doi:10.1146/annurev-genet-022120-112523



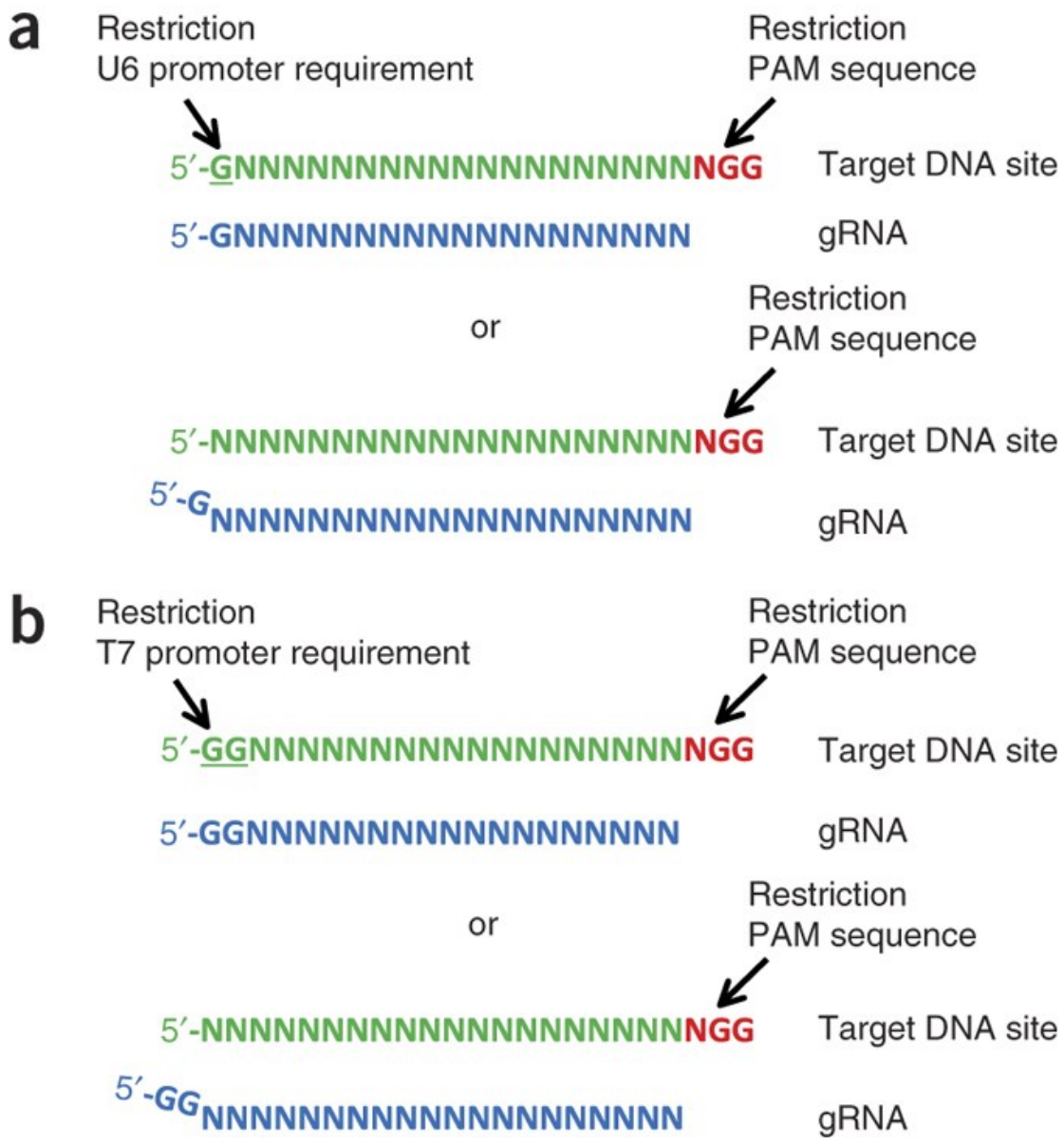
Nuclease-induced double-strand breaks (DSBs) can be repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR) pathways.
Imprecise NHEJ-mediated repair can produce insertion and/or deletion mutations of variable length at the site of the DSB.
HDR-mediated repair can introduce precise point mutations or insertions from a single-stranded or double-stranded DNA donor template.

Nature Biotechnology 32, 347–355 (2014) doi:10.1038/nbt.2842

Figure 2



Cell 2014 157, 1262-1278DOI: (10.1016/j.cell.2014.05.010)



(**a**) Ranges of potential target sites for gRNAs expressed from a U6 promoter. Target DNA sequence restrictions are imposed by the requirement for a *G* at the first 5′nucleotide of the gRNA (blue letters) targeted to the DNA site (required for efficient expression from a U6 promoter) and by the need for an NGG (the PAM sequence; red letters) adjacent to the complementarity region of target site (green letters) (top panel). One strategy to avoid the requirement for a 5′G in the target site is to append an extra *G* to the 5′ end of the gRNA (bottom panel).

(**b**) Ranges of potential target sites for gRNAs expressed from a T7 promoter. Target DNA sequence restrictions are imposed by the requirement for a *GG* at the first two nucleotides of the gRNA targeted to the DNA site (required for efficient expression from the T7 promoter) and by the need for an NGG adjacent to the complementarity region target site (top panel). One strategy to avoid the requirement for a *GG* dinucleotide in the target sequence is to append an extra *GG* dinucleotide to the 5′end of the gRNA (bottom panel).

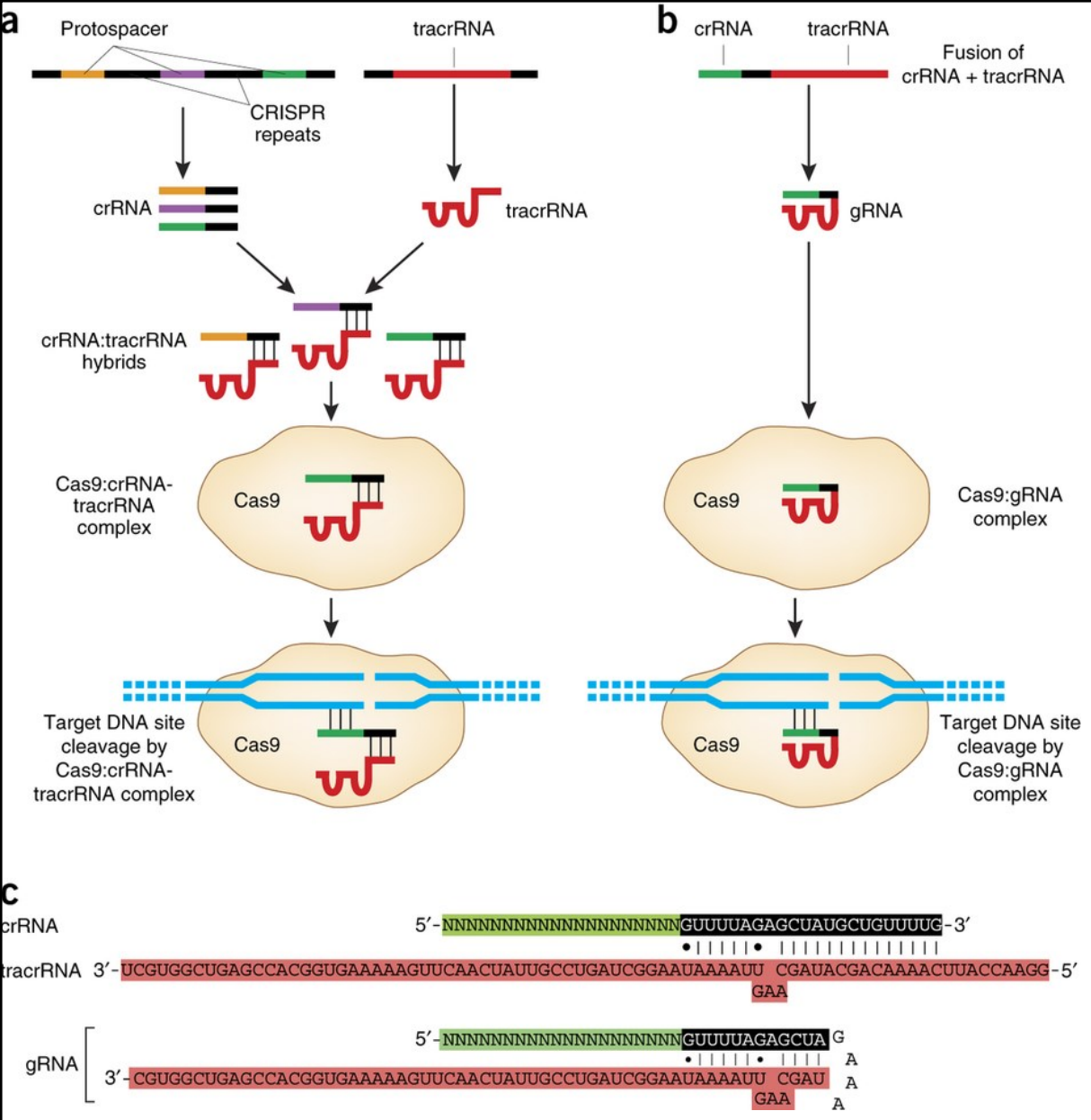
Nature Biotechnology 32, 347–355 (2014) doi:10.1038/nbt.2842

Cas9-structure

An updated evolutionary classification of CRISPR–Cas systems

- CRISPR–Cas systems provide archaea and bacteria with adaptive immunity against viruses and plasmids.
- CRISPR–Cas genomic loci show extreme diversity in sequence and gene arrangement.
- Developed a computational approach for CRISPR–Cas classification, combining comparisons of Cas protein sequences and locus architectures.
- Two classes, five types and 16 subtypes of CRISPR–Cas systems were identified based on this approach.
- An automated classifier was developed for assigning CRISPR–Cas loci from sequenced genomes to specific subtypes.
- The evolution of CRISPR–Cas systems is marked by extensive horizontal transfer and recombination of functional modules.

Nature Reviews Microbiology **volume 13**, pages 722–736 (2015)



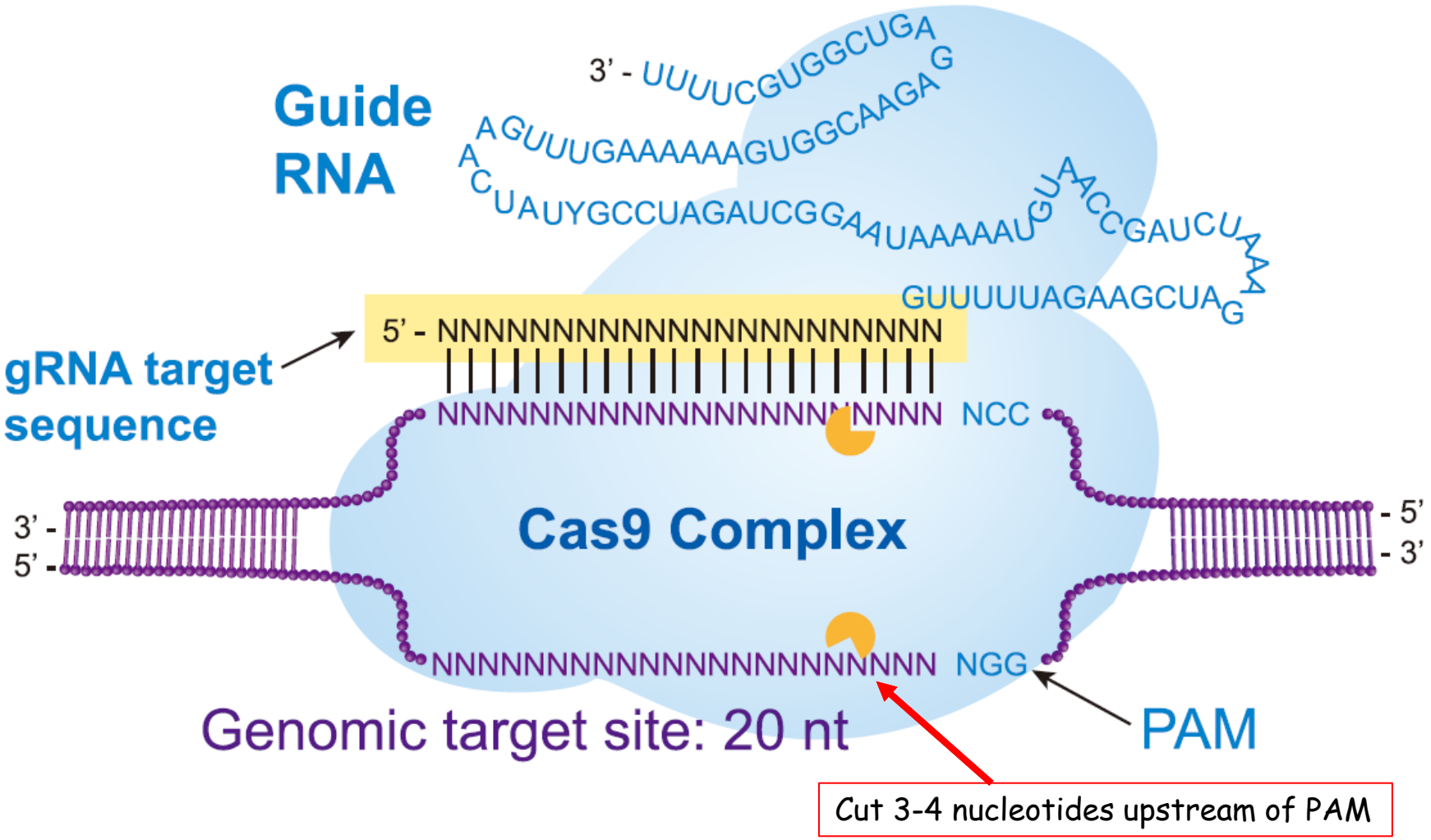
(**a**) Naturally occurring CRISPR systems incorporate foreign DNA sequences into CRISPR arrays, which then produce crRNAs bearing "protospacer" regions that are complementary to the foreign DNA site. crRNAs hybridize to tracrRNAs (also encoded by the CRISPR system) and this pair of RNAs can associate with the Cas9 nuclease. crRNA-tracrRNA:Cas9 complexes recognize and cleave foreign DNAs bearing the protospacer sequences.

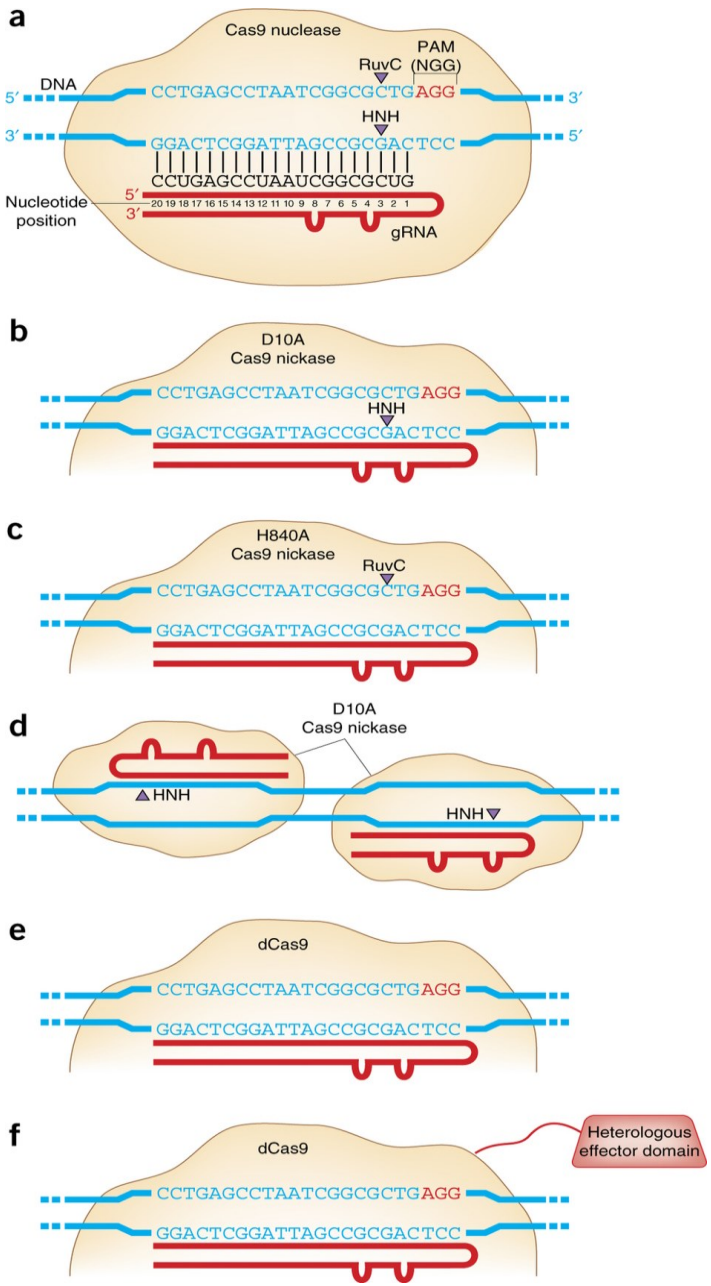
(**b**) The most widely used engineered CRISPR-Cas system utilizes a fusion between a crRNA and part of the tracrRNA sequence. This single gRNA complexes with Cas9 to mediate cleavage of target DNA sites that are complementary to the 5′20 nt of the gRNA and that lie next to a PAM sequence.

(**c**) Example sequences of a crRNA-tracrRNA hybrid and a gRNA.

Nature Biotechnology 32, 347–355 (2014) doi:10.1038/nbt.2842

Overview of CRISPR/Cas9 Technology





(a) Cas9 nuclease creates double-strand breaks at DNA target sites with complementarity to the 5′ end of a gRNA. Cas9 contains RuvC and HNH nuclease domains (arrowheads).

(b) Cas9 nickase created by mutation of the RuvC nuclease domain with a D10A mutation. This nickase cleaves only the DNA strand that is complementary to and recognized by the gRNA.

(c) Cas9 nickase created by mutation of the HNH nuclease domain with a H840A mutation. This nickase cleaves only the DNA strand that does not interact with the gRNA.

(d) Paired nickase strategy for improving Cas9 specificity. Two D10A Cas9 nickases are directed by a pair of appropriately oriented gRNAs. This leads to induction of two nicks that, if introduced simultaneously, would be expected to generate a 5′ overhang.

(e) Catalytically inactive or 'dead' Cas9 (dCas9) (e.g., with mutations in both the RuvC and HNH domains). This can be recruited by a gRNA without cleaving the target DNA site.

(f) Catalytically inactive dCas9 can be fused to a heterologous effector domain.

Nature Biotechnology 32, 347–355 (2014) doi:10.1038/nbt.2842

Figure 7. CRISPR/Cas9 *In Vivo* Applications

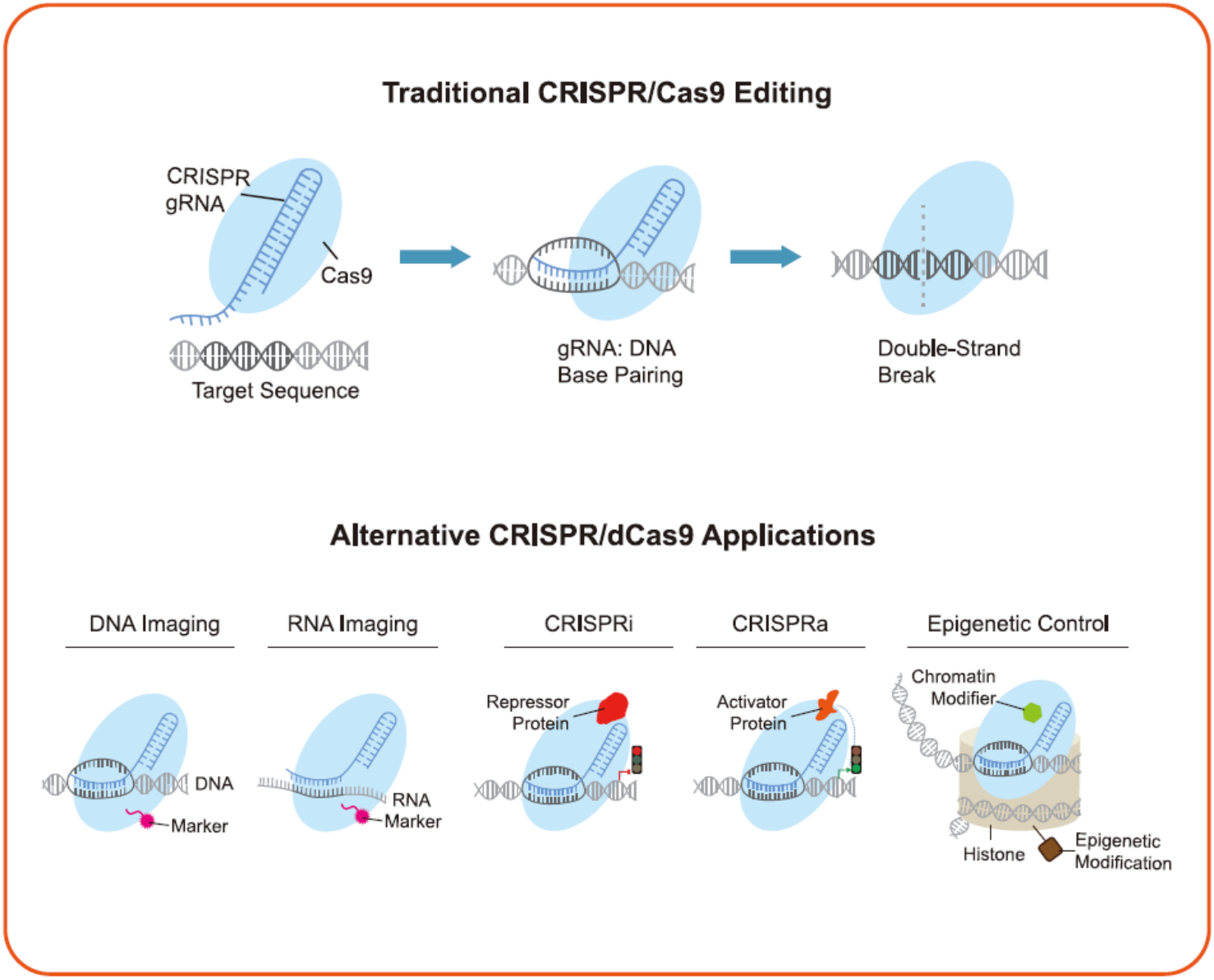
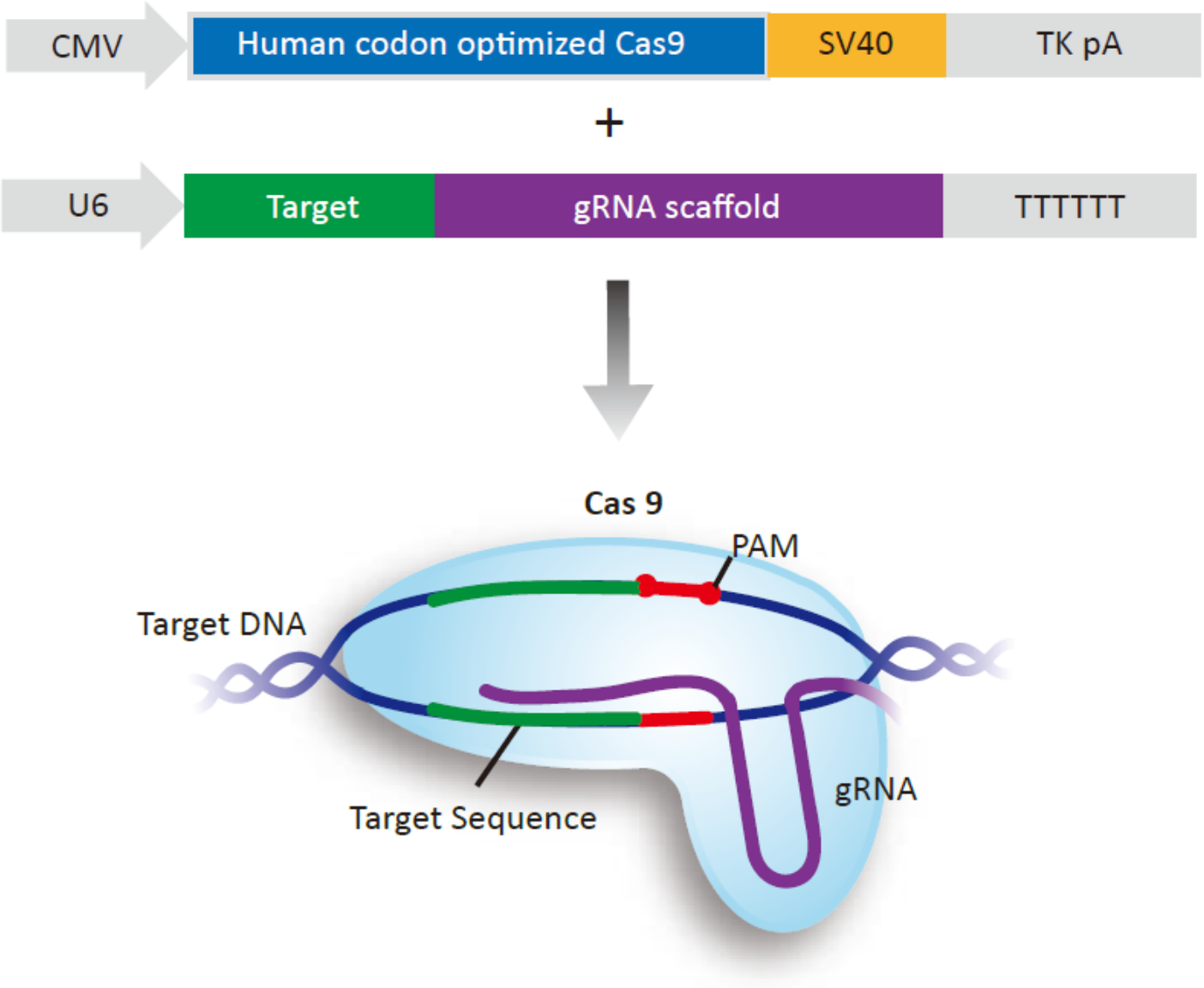
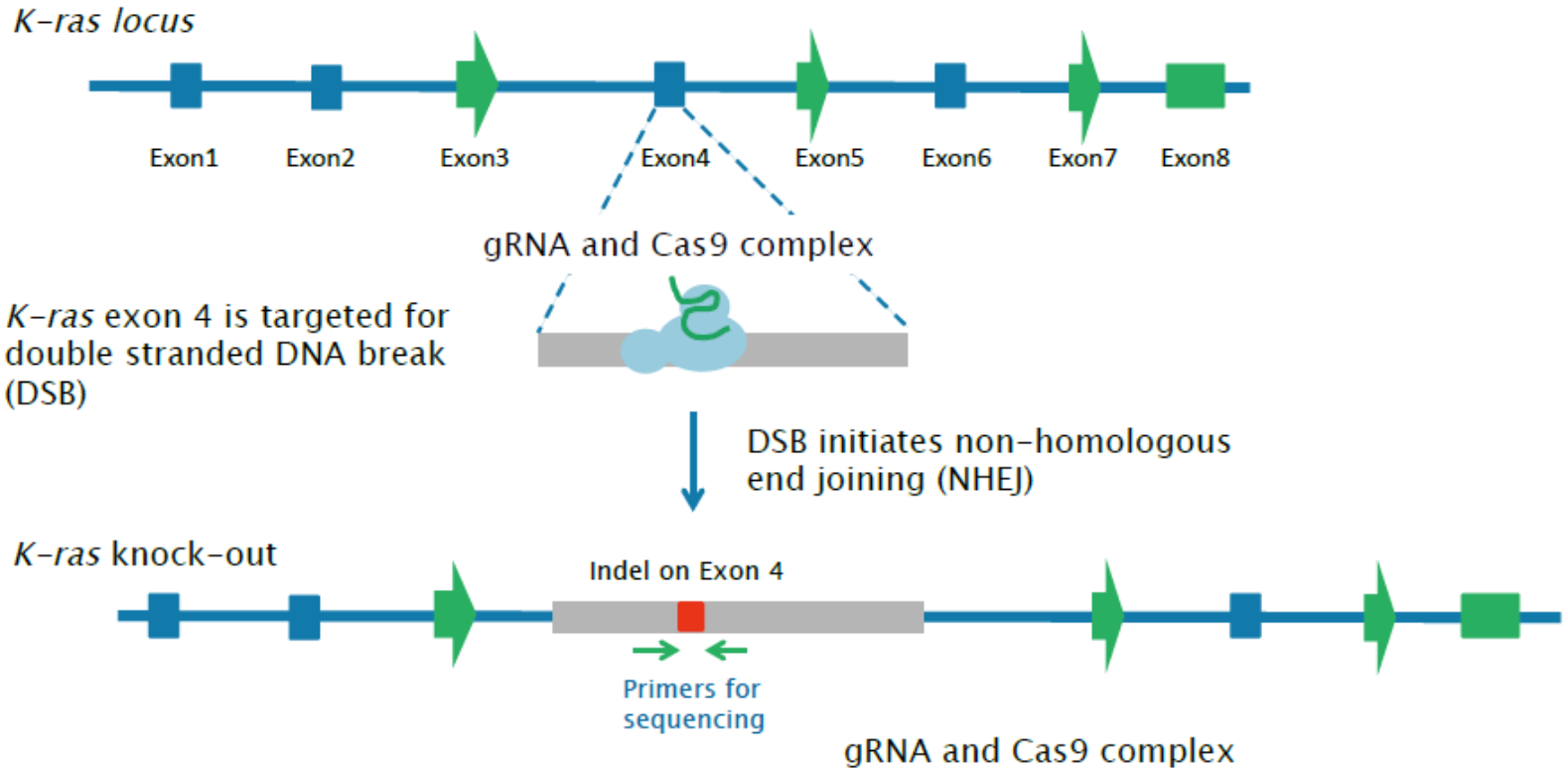


Figure 4: CRISPR/Cas system for genome editing in mammalian cells



i.e. Knock-out

Figure 7: Knock-out targeting strategy for K-Ras

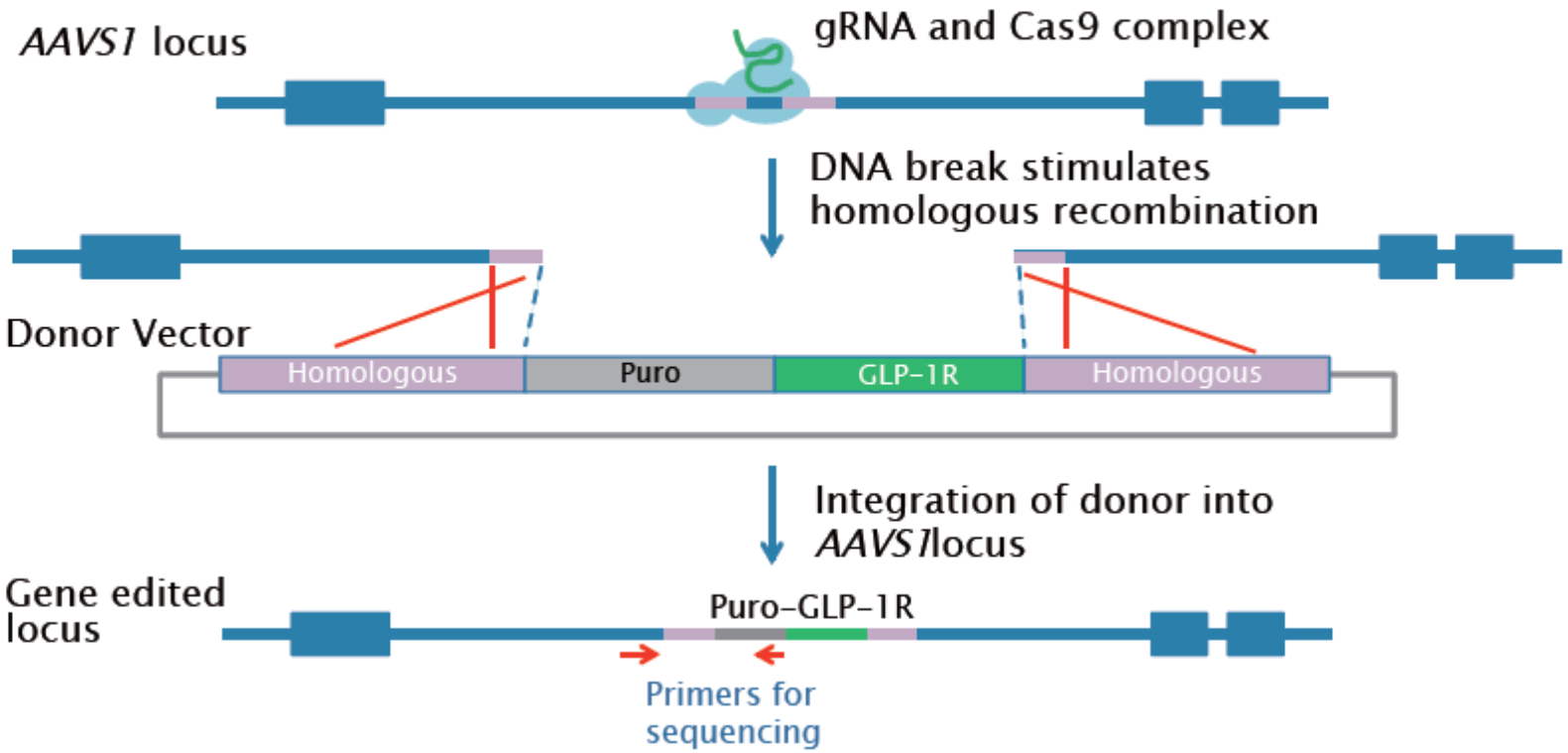


This gene, a **Kirsten ras oncogene homolog** from the mammalian ras gene family, encodes a protein that is a member of the small GTPase superfamily.

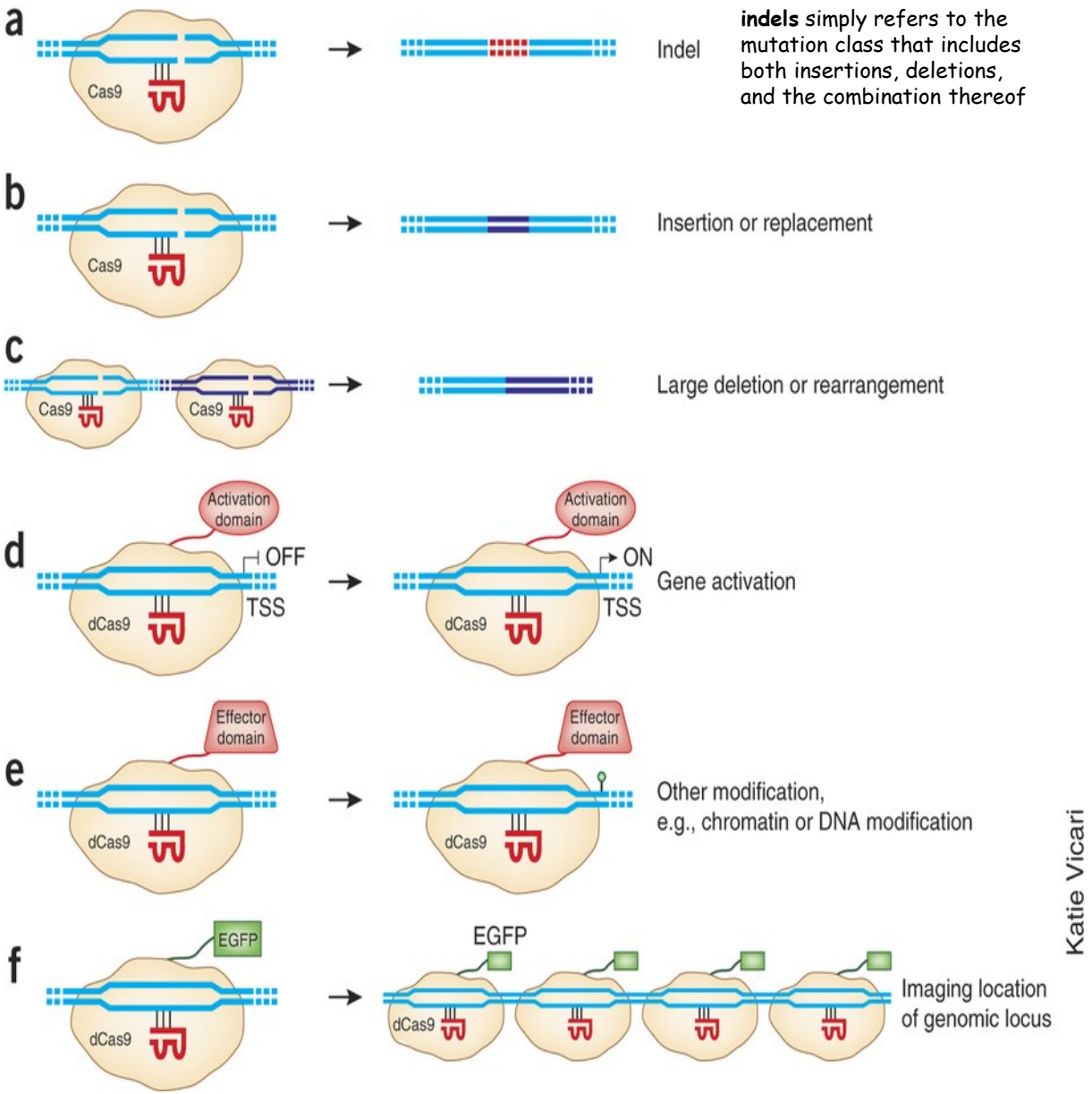
A single amino acid substitution is responsible for an activating mutation. The transforming protein that results is **implicated in various malignancies**, including lung adenocarcinoma, mucinous adenoma, ductal carcinoma of the pancreas and colorectal carcinoma. Alternative splicing leads to variants encoding two isoforms that differ in the C-terminal region.

i.e. Knock-in

Figure 9: Integration of GLP-1R into HEK 293T cells



This gene encodes a 7-transmembrane protein that functions as a **receptor for glucagon-like peptide 1 (GLP-1) hormone**, which stimulates glucose-induced insulin secretion.
This receptor, which functions at the cell surface, becomes internalized in response to GLP-1 and GLP-1 analogs, and it plays an **important role in the signaling cascades leading to insulin secretion**. It also displays neuroprotective effects in animal models. Polymorphisms in this gene are associated with diabetes.



(**a**,**b**) gRNA-directed Cas9 nuclease can induce indel mutations (**a**) or specific sequence replacement or insertion (**b**).

(**c**) Pairs of gRNA-directed Cas9 nucleases can stimulate large deletions or genomic rearrangements (e.g., inversions or translocations).

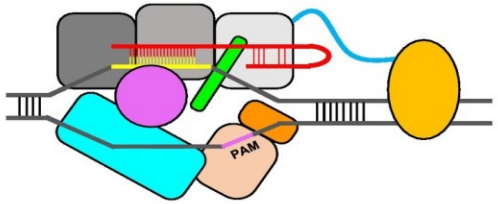
(**d**–**f**) gRNA-directed dCas9 can be fused to activation domains (**d**) to mediate upregulation of specific endogenous genes, heterologous effector domains (**e**) to alter histone modifications or DNA methylation, or fluorescent proteins (**f**) to enable imaging of specific genomic loci. TSS, transcription start site.

Nature Biotechnology 32, 347–355 (2014) doi:10.1038/nbt.2842

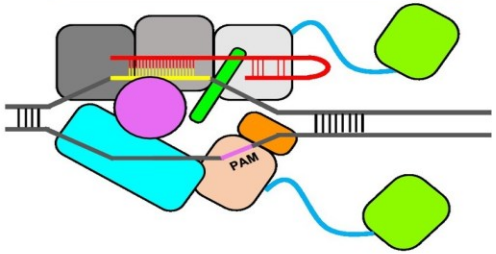
(A)

Introducing exogenous protein domains for multiplex functions

End-to-end fused domains



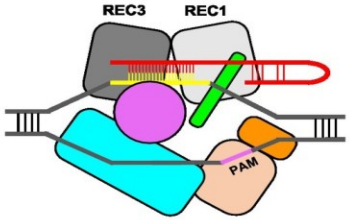
Insertion of active domains



(B)

Deletion of protein domains for smaller CRISPR nucleases

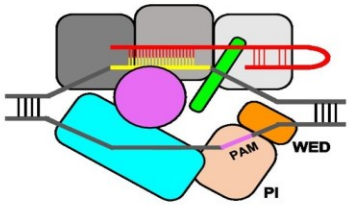
Deletion of domains



(C)

Protein engineering of Cas protein domains for altered PAM specificity

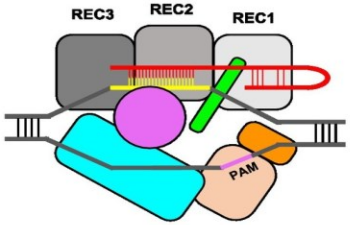
PI and WED domains



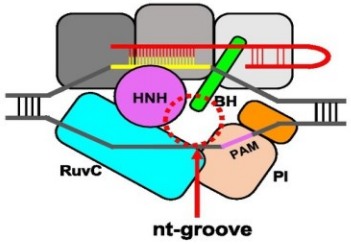
(D)

Protein engineering of Cas protein domains for on-target specificity

REC domains



nt-groove domains



Trends in Biotechnology

Trends in Biotechnology 2021 39262-273DOI: (10.1016/j.tibtech.2020.07.005)

PAM sequences

Cas Variant	PAM Sequence
SpCas9	NGG
SpCas9 VRER Variant	NGCG
SpCas9 EQR Variant	NGAG
SpCas9 VQR Variant	NGAN or NGNG
SaCas9	NNGRRT
Cpf1	TTN

SpCas9, the most widely used and well-characterized Cas9 homolog[1], recognizes an NGG PAM immediately 3′ of the target DNA sequence[3].

[Miller, S.M., Wang, T., Randolph, P.B. *et al.* Continuous evolution of SpCas9 variants compatible with non-G PAMs. *Nat Biotechnol* **38**, 471–481 (2020)]recognises

CRISPR-Cas applications

Genetic Modification	Application	Nuclease Activity	gRNA
Knock-out	Permanently remove gene function	Cas or Cas9n	gRNA targeting 5′ exon or essential protein domains
Knock-in	Generate a specific sequence change	Cas or Cas9n	gRNA targeting region of interest
Interference	Reduce gene expression	dCas-repressor	gRNA targeting gene promoter elements
Activation	Increase gene expression	dCas-activator	gRNA targeting gene promoter elements

Compared to Cas9-mediated genome editing methods, Cas9n causes less damage and toxicity to the host [26]. At the same time, the single-strand nick created by Cas9n is highly suitable for repair and thus improves the genome manipulation efficiency [27, 28]. CRISPR/Cas9n assisted genome editing tools have recently been developed for a number of bacteria, including *E.coli* [29], *Lactobacillus reuteri* [30], *Clostridium* sp. [31] and *Bacillus licheniformis* [32].

Liu, D., Huang, C., Guo, J. *et al.* Development and characterization of a CRISPR/Cas9n-based multiplex genome editing system for *Bacillus subtilis*. *Biotechnol Biofuels* **12**, 197 (2019). https://doi.org/10.1186/s13068-019-1537-1

CRISPR/Cas9 is a powerful technique for gene editing

Two significant challenges remain:

- obtaining efficient delivery of Cas9 and sgRNA to a broad range of cell types,

And

- leaving no additional footprint (i.e., persistent and elevated expression of Cas9 in target cells) that could lead to off-target effects.

https://www.takarabio.com/products/gene-function/gene-editing/crispr-cas9/in-vitro-transcription-and-screening-kits

CRISPR interference system delivery

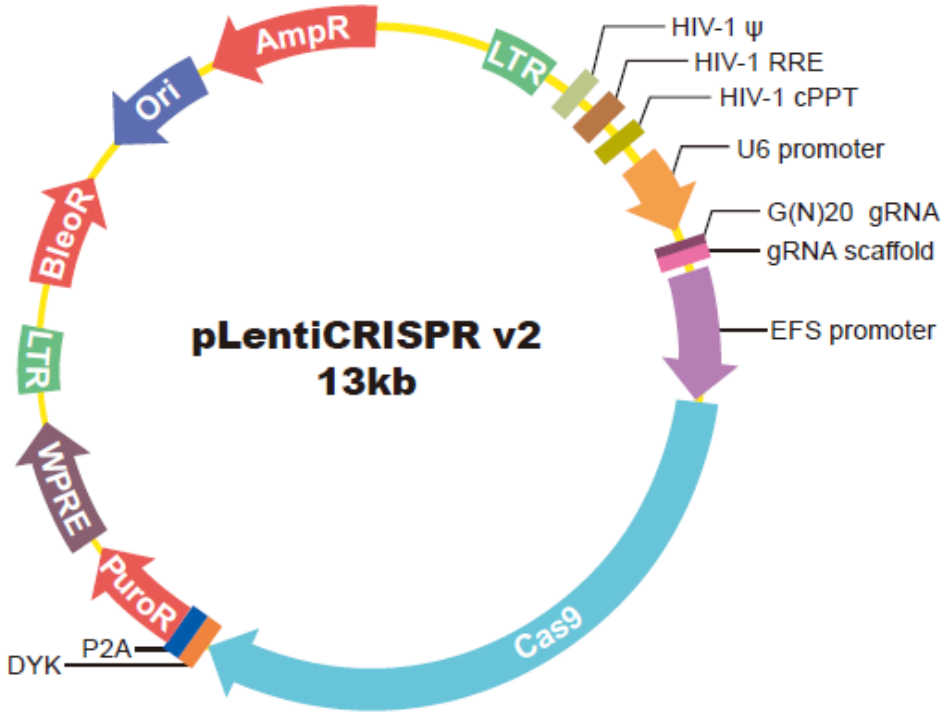
Expression System	Components	Application
Plasmid Vector	Constitutive or inducible Cas9 Constitutive or inducible gRNA Reporter/selection marker	Expression of Cas9 and gRNA
Lentiviral Vector	Constitutive or inducible Cas9 Constitutive or inducible gRNA Reporter/selection marker	Expression of Cas9 and gRNA For infection of difficult-to-transfect cell types
AAV Vector	Constitutive or inducible Cas9 Constitutive or inducible gRNA Reporter/selection marker	Transient or stable expression of SaCas9 and/or gRNA For non-toxic infection of dividing and non-dividing cells
Cas9 mRNA and gRNA	Transcription reactions *in vitro* to generate Cas9 mRNA and gRNA Delivery via microinjection or electroporation	Transient expression of CRISPR gene editing components
crRNA/Cas9 Ribonucleoprotein Complexes	Purified Cas9 protein and *in vitro* transcribed gRNA Delivery via microinjection or electroporation	Transient expression of CRISPR gene editing components

Different Hosts

Host	PAM Sequence
Mammalian Cells	Lipofection-based transfection of DNA plasmids Electroporation of DNA plasmids or RNP Lenti or AAV virus-based transfection of DNA plasmids
Bacteria	Transformation of plasmids into competent cells
Yeast	Electroporation of plasmids and galactose induction of Cas9
Mouse: **Germline Mutations**	Direct injection into embryos Electroporation into zygotes
Mouse: **Somatic Mutations**	Direct injection of AAV into tissue of interest
Danio rerio	Direction injection into one-cell embryos
Drosophila melanogaster	Direct injection into embryo germline
Danio rerio	Direction injection into one-cell embryos
Caenorhabditis elegans	Direct injection into hermaphrodite germline
Plants	*Agrobacterium*-mediated transformation of gRNA/Cas9 vector

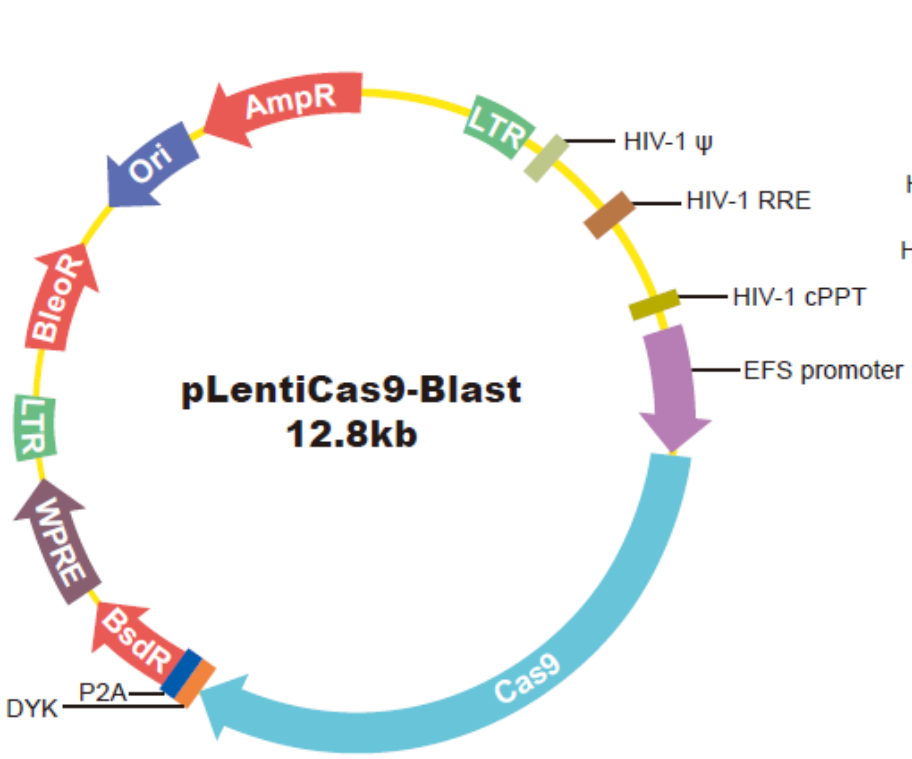
Optimazed lentiviral vectors for CRISPR genome editing in mammalian

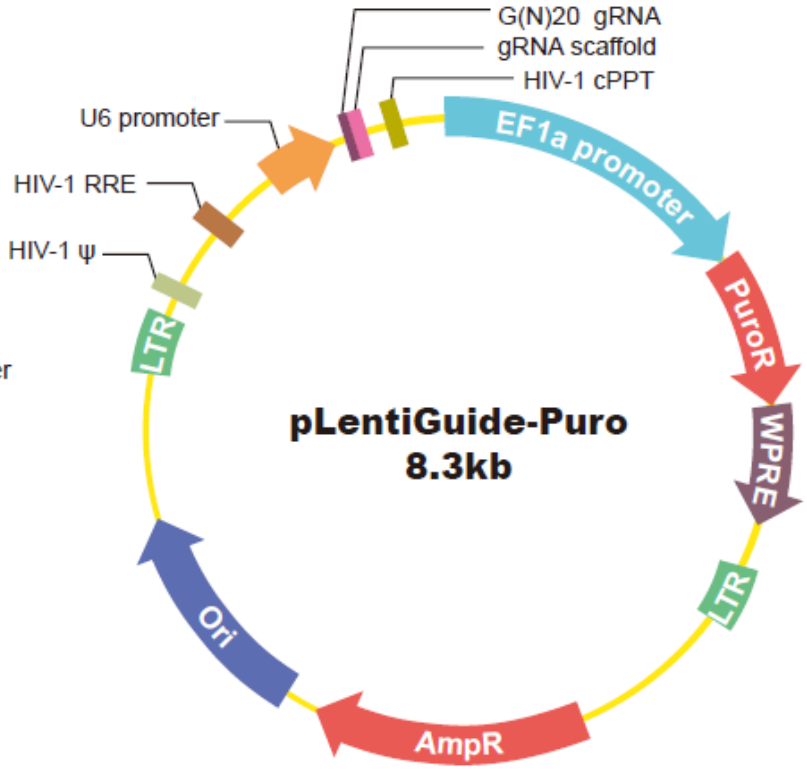
Option 1: An all-in-one vector, pLentiCRISPRv2, enables CRISPR editing in any cell type of interest without generating stable Cas9-expressing cell line first.



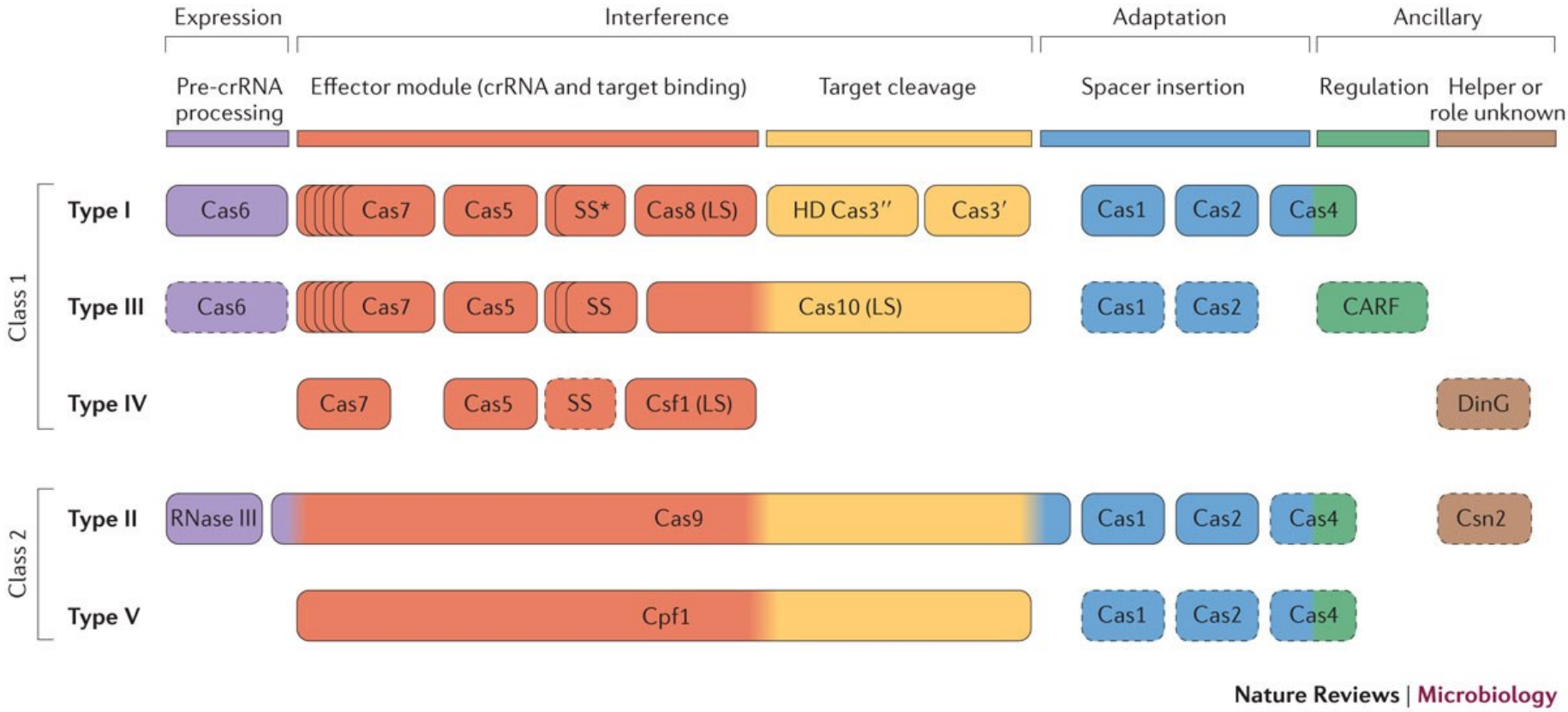
Optimazed lentiviral vectors for CRISPR genome editing in mammalian

Option 2: A two-vector system; sequential transduction with, and selection for, pLenti-Cas9-Blast followed by pLentiGuide-Puro, shows 10-fold higher efficiency compared to pLentiCRISPRv2.





An updated evolutionary classification of CRISPR–Cas systems



Nature Reviews Microbiology **volume 13**, pages 722–736 (2015)

An updated evolutionary classification of CRISPR–Cas systems

Class 1 CRISPR–Cas systems

- Class 1 CRISPR–Cas systems are defined by the presence of a multisubunit crRNA–effector complex. The class includes type I and type III CRISPR–Cas systems, as well as the putative new type IV.

Class 2 CRISPR–Cas systems

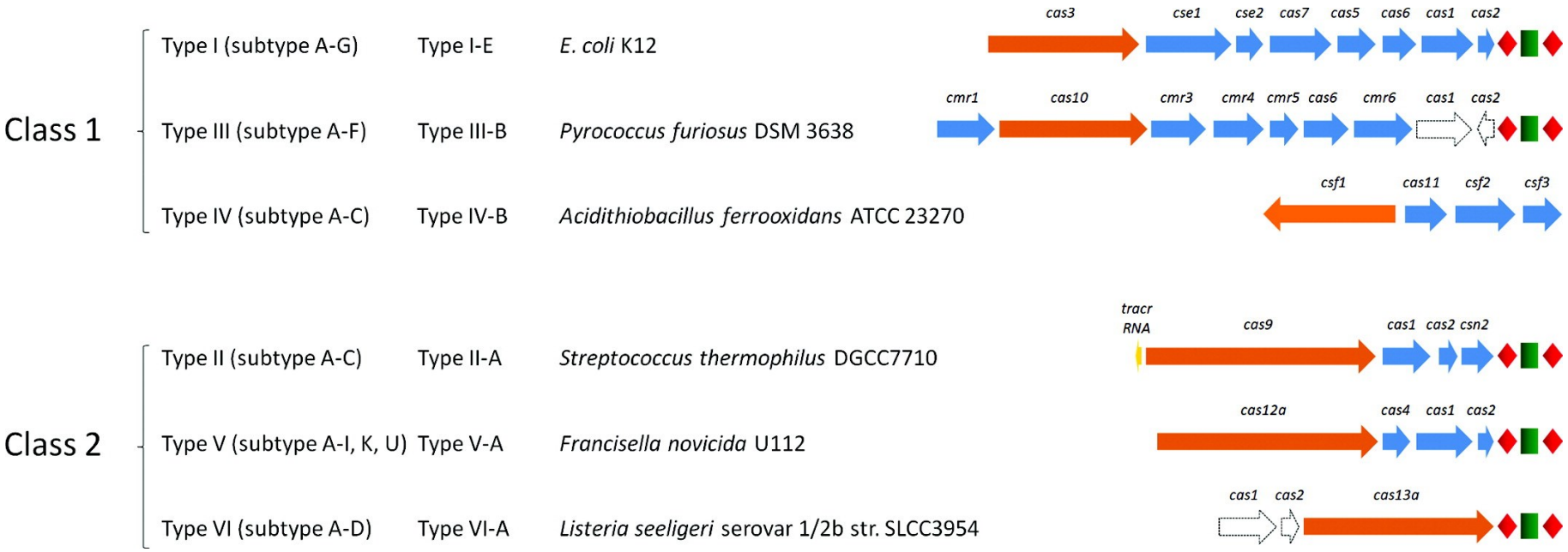
- Class 2 CRISPR–Cas systems are defined by the presence of a single subunit crRNA–effector module. This class includes type II (e.g cas9) CRISPR–Cas systems as well as type V and type VI (e.g. cas13).

Types I, II and V target → double-stranded DNA

Types III and VI target → single-stranded RNA.

-***Nature Reviews Microbiology*** volume 13, pages 722–736 (2015)
-**Viruses**. 2019 Feb; 11(2): 120. Published online 2019 Jan 29. doi: 10.3390/v11020120

Classification of CRISPR-Cas systems



The signature genes are highlighted in orange.
The other genes are blue.
The **Type III-B** and **Type VI-A** systems **do not possess their own cas1 and cas2 genes** but use those of other systems and these genes are therefore presented in white with dotted lines.
The CRISPR locus is illustrated in the form of red diamonds (repeats) and green squares (spacers).

Can. J. Microbiol. **67**: 1–12 (2021) dx.doi.org/10.1139/cjm-2020-0212

Table 1. Overview of diverse CRISPR/Cas systems.

Type/class	Effector	Nuclease domains	Target	Subtypes	Ref
I/1	Cas3	HD	DNA	A, B, C, D, E, F, G	[9,10]
II/2	Cas9	RuvC, HNH	DNA	A, B, C	[[11], [12], [13]]
III/1	Csm3, Cmr4	Autocatalytic?	RNA	A (Csm), B (Cmr), C,	[14,15]
	Csm6, Csx1	HEPN			
	Cas10	HD	DNA	D	
IV/1	Csf1	?	DNA?	A, B	[16,17]
V/2	Cas12	RuvC	DNA	A (Cpf1), B (C2c1), C (C2c3), D (Cas Y), E (Cas X)	[13]
VI/2	Cas13	HEPN × 2	RNA	A (Cas13a), B, C, D	[13]

Xiangrong Song, Chao Liu, Ning Wang, Hai Huang, Siyan He, Changyang Gong, Yuquan Wei,
Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy,
Advanced Drug Delivery Reviews,Volume 168, 2021, Pages 158-180, https://doi.org/10.1016/j.addr.2020.04.010

CRISPR/Cas9 is a powerful technique for gene editing

Significant challenges remain:

Knock-out mutations can usually be obtained with high efficiency,

But

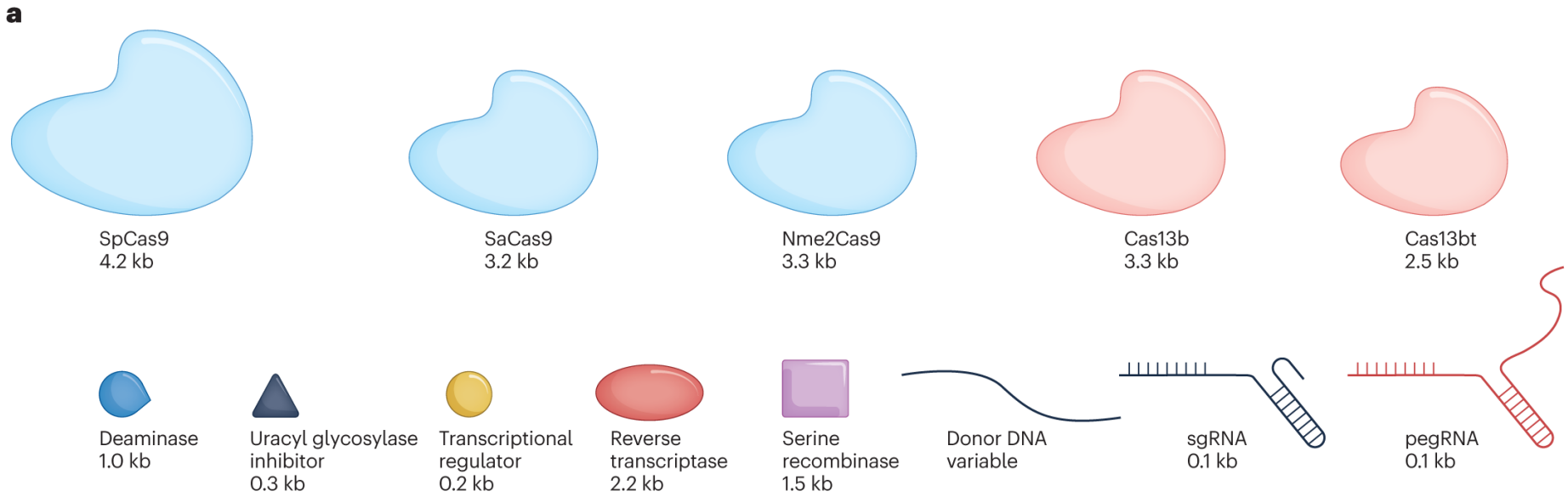
Knock-in of longer sequences (more than 200 bp) via homology directed repair is more difficult to achieve.

Although single-stranded DNA (ssDNA) donor templates have recently been shown to have several advantages over double-stranded DNA, the usefulness of long ssDNA templates is limited due to the difficulty and high cost of producing them.
(Takara Bio has recently developed a simple and economical method for generating long ssDNA donor templates up to 5 kb.)

https://www.takarabio.com/products/gene-function/gene-editing/crispr-cas9/in-vitro-transcription-and-screening-kits
https://www.takarabio.com/products/gene-function/gene-editing/crispr-cas9/long-ssdna-for-knockins

The size of commonly used gene-editing cassettes.

From: Drug delivery systems for CRISPR-based genome editors

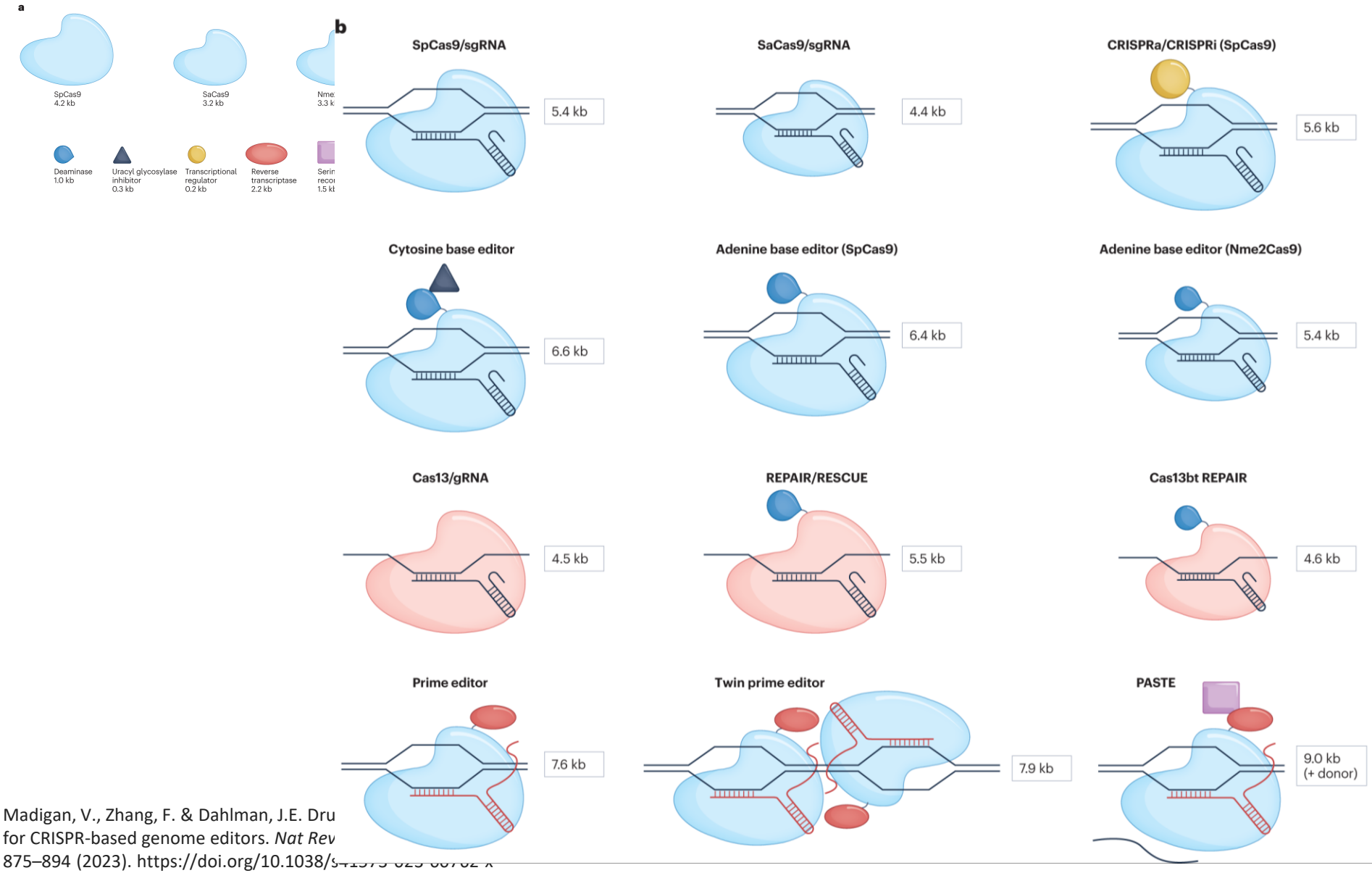


Streptococcus pyogenes Cas9 (SpCas9)
Staphylococcus aureus Cas9 (SaCas9)
Neisseria meningitidis Cas9 (NmeCas9)
type VI Cas13b, Cas13bt (RNA nuclease)

Madigan, V., Zhang, F. & Dahlman, J.E. Drug delivery systems for CRISPR-based genome editors. *Nat Rev Drug Discov* **22**, 875–894 (2023). https://doi.org/10.1038/s41573-023-00762-x

The size of commonly used gene-editing cassettes.

From: Drug delivery systems for CRISPR-based genome editors



Madigan, V., Zhang, F. & Dahlman, J.E. Dru
for CRISPR-based genome editors. *Nat Rev*
875–894 (2023). https://doi.org/10.1038/s

Table 1 Selected examples of AAV-based and LNP-based delivery systems used in clinical trials

From: Drug delivery systems for CRISPR-based genome editors

Disease indication	Therapeutic approach	Delivery vehicle	Phase	Clinical trial
Leber congenital amaurosis type 2	RPE65	AAV2	Approved	NCT00999609
Spinal muscular atrophy type 1	SMN	AAV9	Approved	NCT03306277
Haemophilia B	Factor IX	rAAV Spark 100; AAV5	III; approved	NCT03861273; NCT03569891
Duchenne muscular dystrophy	Mini-dystrophin	AAV9; AAVrh74	I/II, III; approved	NCT03368742, NCT04281485; NCT05096221
Haemophilia A	Factor VIII	Engineered AAV LK03; AAV5	I/II; approved	NCT03003533; NCT04323098
Leber congenital amaurosis 10	SaCas9 with guide RNAs targeting the *CEP290* mutation	AAV5	I/II	NCT03872479
Wet age-related macular degeneration	Aflibercept and an anti-VEGFC RNAi	Engineered AAV 4D-150	I/II	NCT05197270

Madigan, V., Zhang, F. & Dahlman, J.E. Drug delivery systems for CRISPR-based genome editors. *Nat Rev Drug Discov* **22**, 875–894 (2023). https://doi.org/10.1038/s41573-023-00762-x

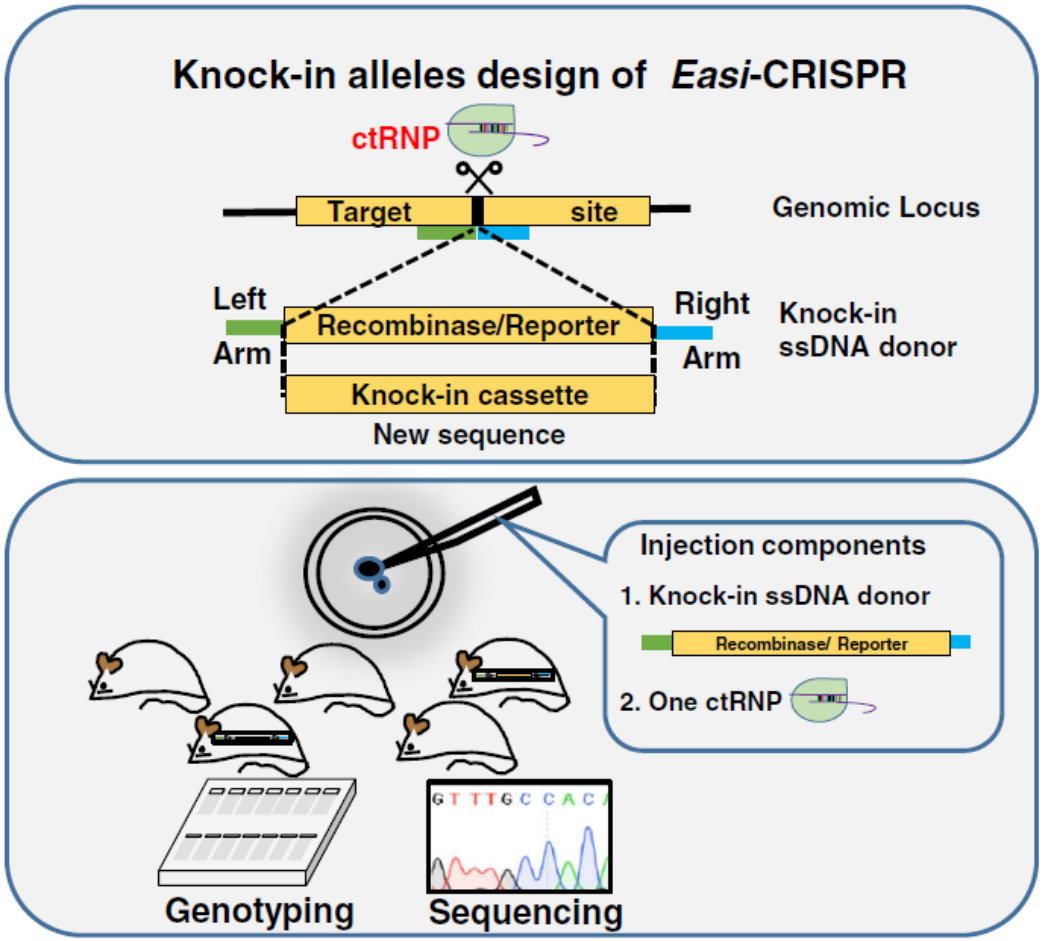
Table 1 Selected examples of AAV-based and LNP-based delivery systems used in clinical trials

From: Drug delivery systems for CRISPR-based genome editors

Disease indication	Therapeutic approach	Delivery vehicle	Phase	Clinical trial
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator with a deletion in the regulatory domain	Engineered AAV 4D-A101	I/II	NCT05248230
HIV	SaCas9 with guide RNAs targeting the HIV genome	AAV9	I	NCT05144386
Vaccination	SARS-CoV-2 spike protein	LNP	Approved	NCT04368728, NCT04470427
Transthyretin amyloidosis	RNAi targeting hepatic transthyretin protein synthesis	LNP	Approved	NCT01960348
Hereditary angioedema	SpCas9 with guide RNA targeting the gene encoding kallikrein B1	LNP	I/II	NCT05120830
Transthyretin amyloidosis	SpCas9 with guide RNA targeting the gene encoding transthyretin	LNP	I	NCT04601051
Heterozygous familial hypercholesterolaemia	Adenine base editor with guide RNA targeting the gene encoding PCSK9	LNP	I	NCT05398029

Madigan, V., Zhang, F. & Dahlman, J.E. Drug delivery systems for CRISPR-based genome editors. *Nat Rev Drug Discov* **22**, 875–894 (2023). https://doi.org/10.1038/s41573-023-00762-x

Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins



Quadros et al. *Genome Biology* (2017) 18:92 DOI 10.1186/s13059-017-1220-4
Miura et al (2018) *Nature Protocols* volume 13, pages 195–215 (2018)
"*Easi*-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors"

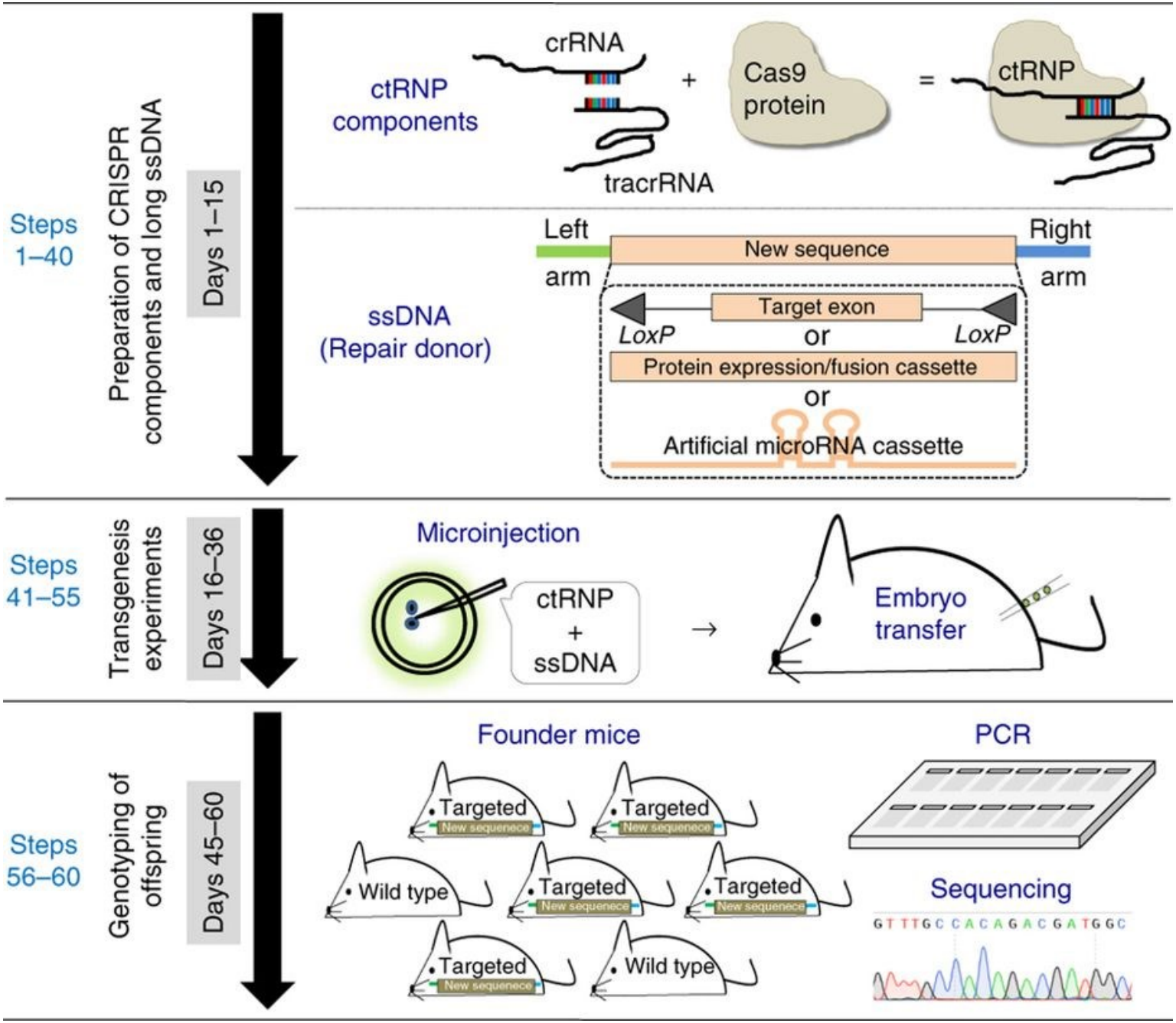


Fig1 from Miura et al (2018) *Nature Protocols* volume 13, pages 195–215 (2018)

"Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors"

New CRISPR/Cas13 as a Tool for RNA Interference

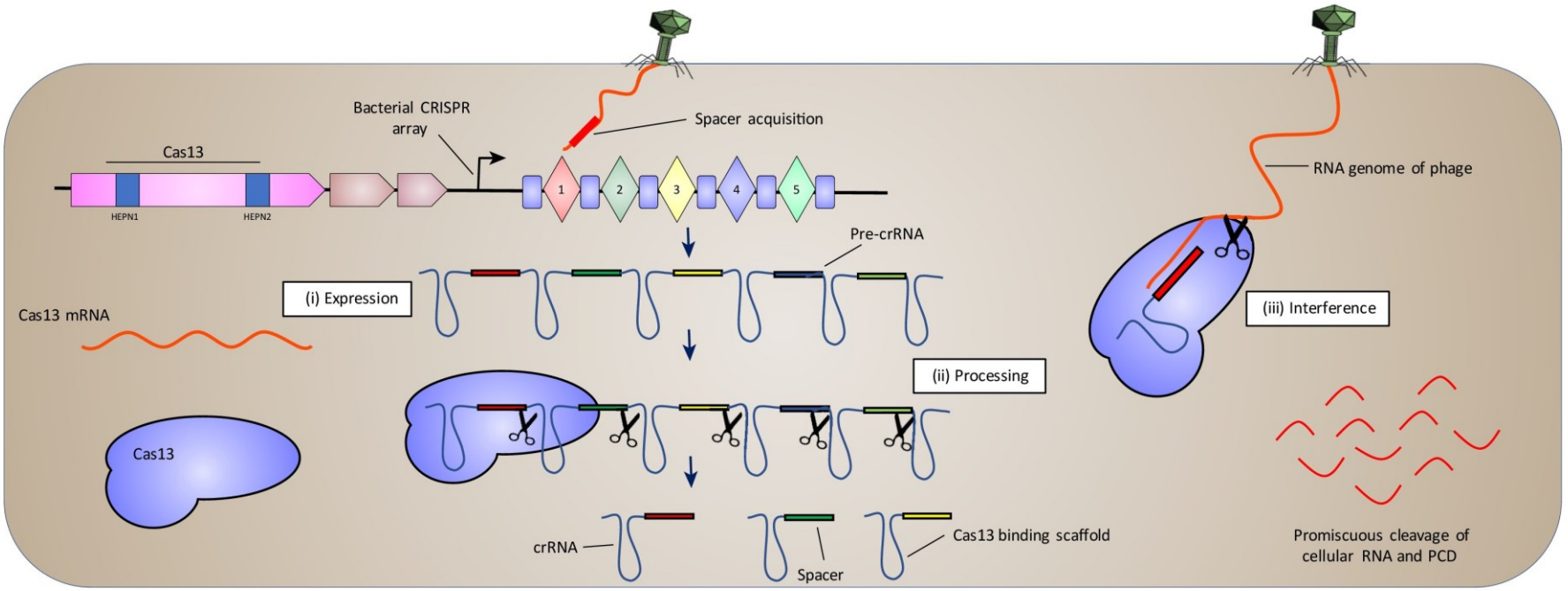
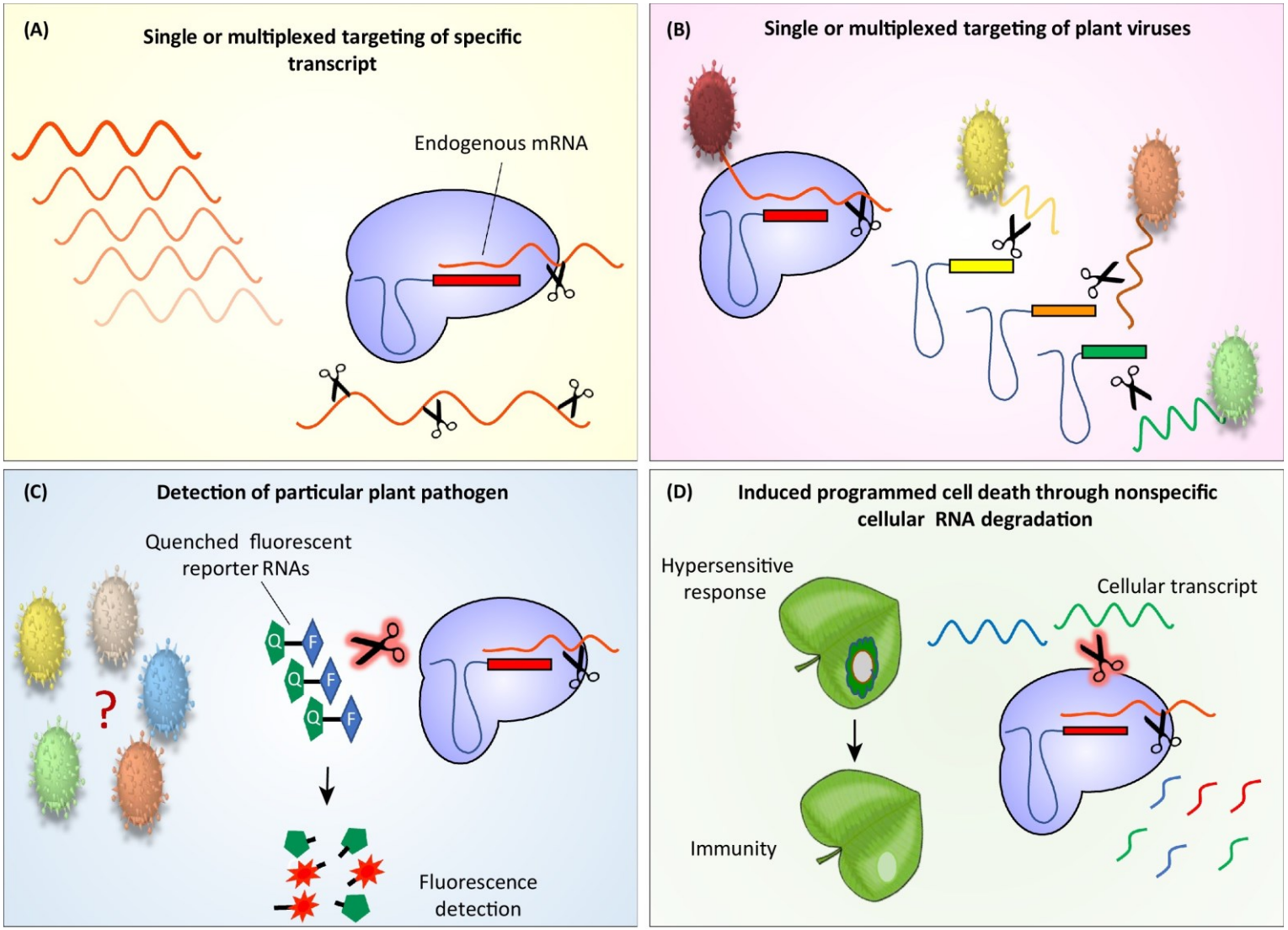


Figure I. Steps in the Acquisition of Antiviral Immunity.

Trends in Plant Science

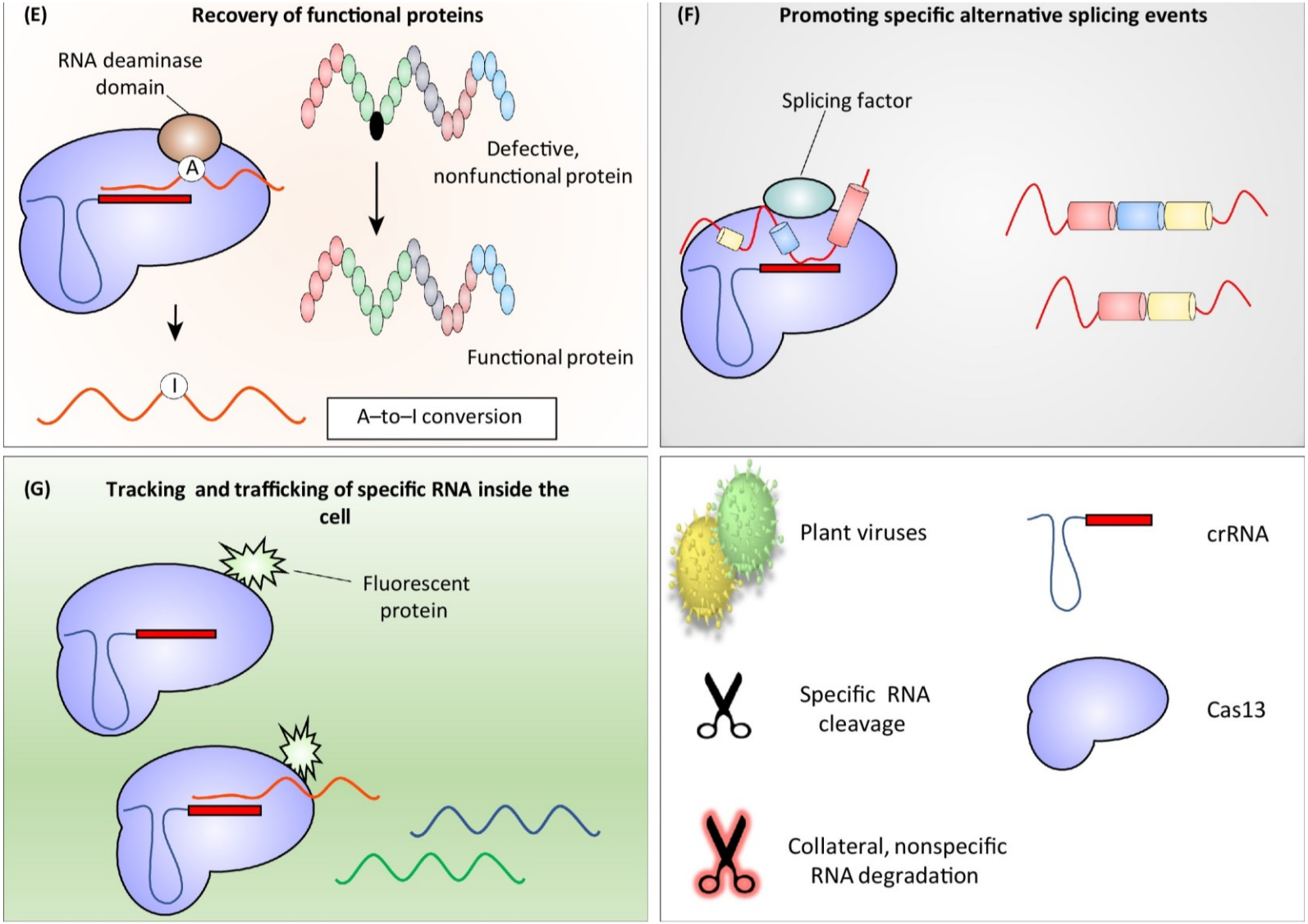
https://doi.org/10.1016/j.tplants.2018.03.003

Potential Applications of CRISPR/Cas13 in Plant Biotechnology.



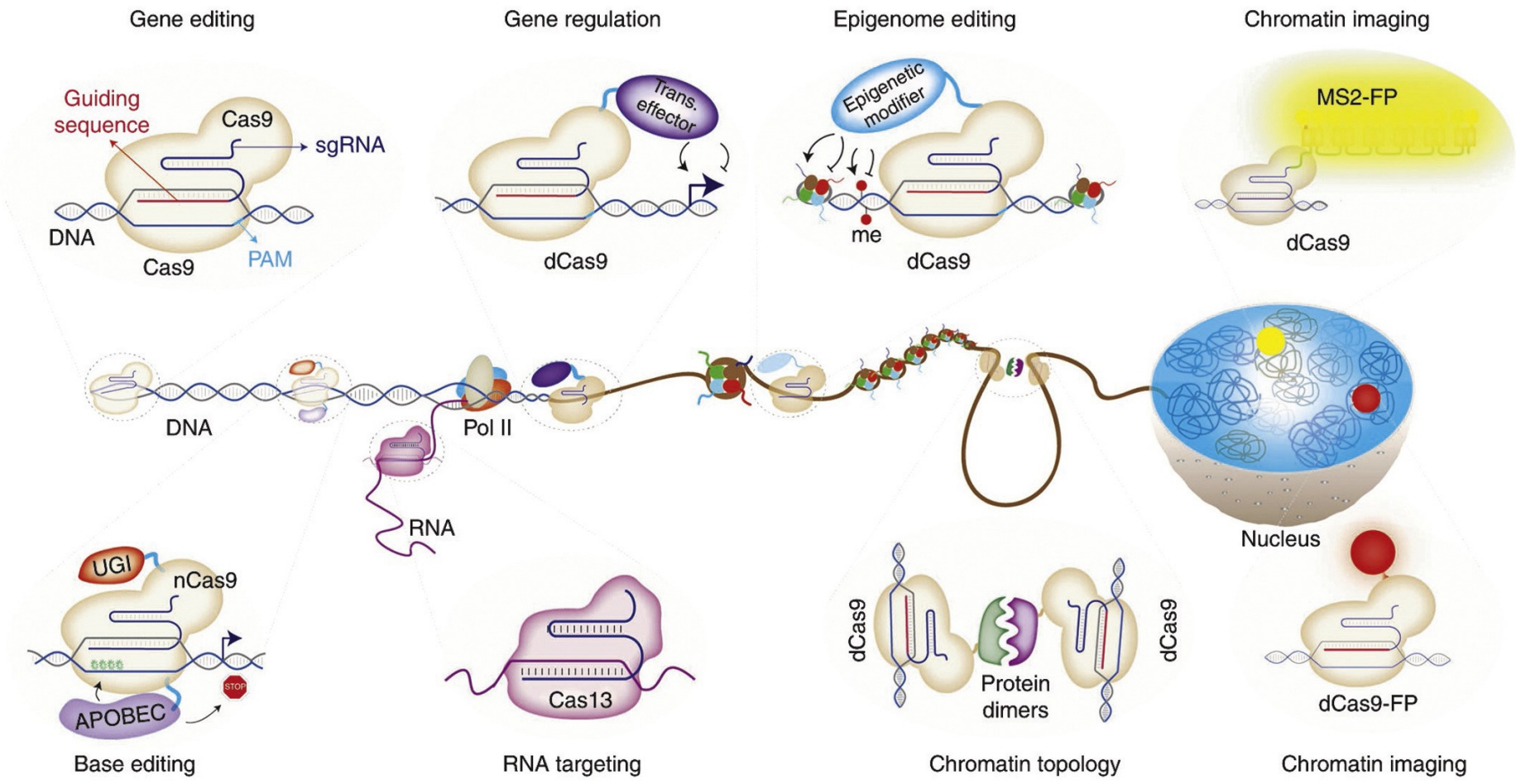
https://doi.org/10.1016/j.tplants.2018.03.003

Potential Applications of CRISPR/Cas13 in Plant Biotechnology.



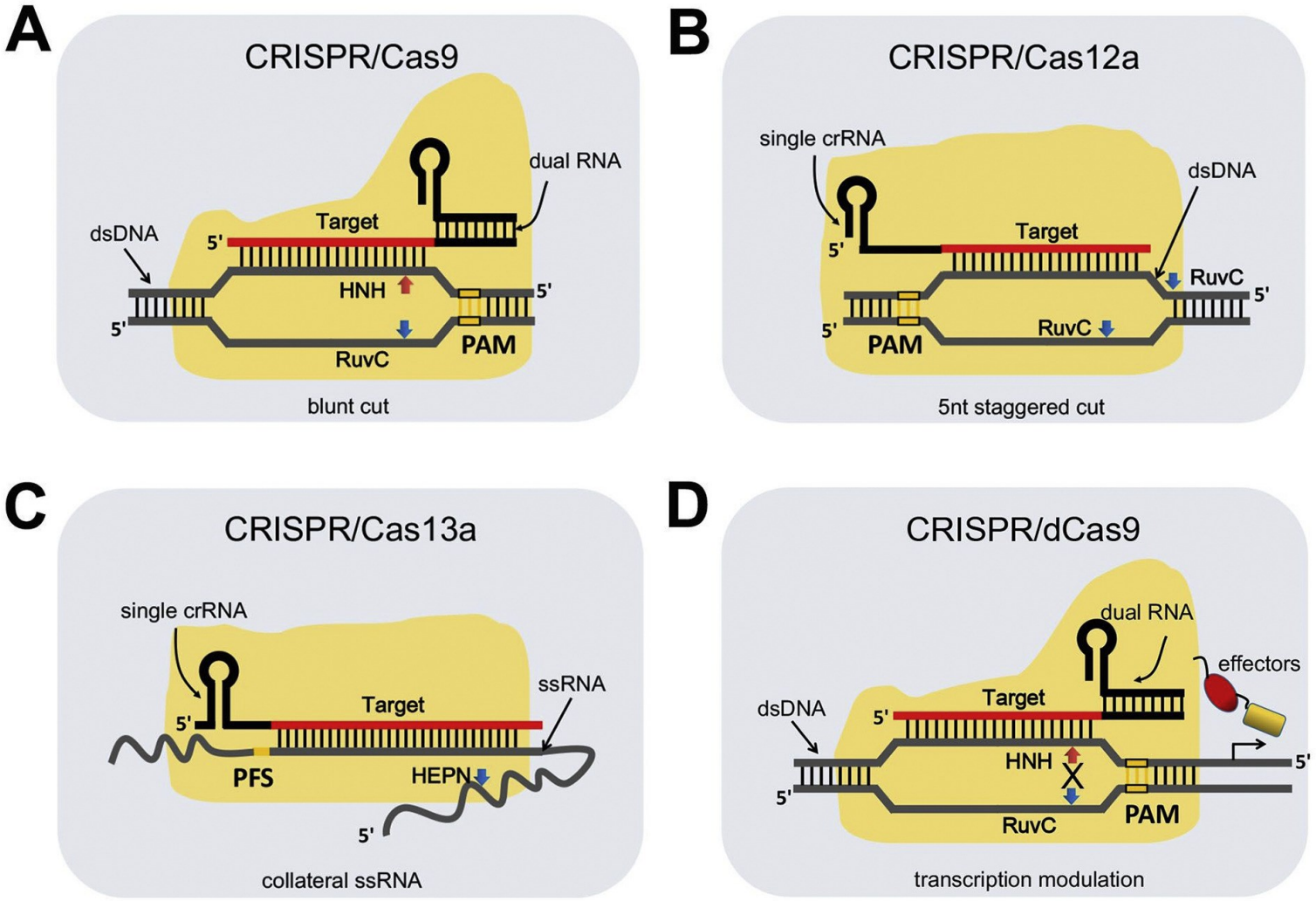
https://doi.org/10.1016/j.tplants.2018.03.003

Summary of CRISPR-Cas-based technologies.



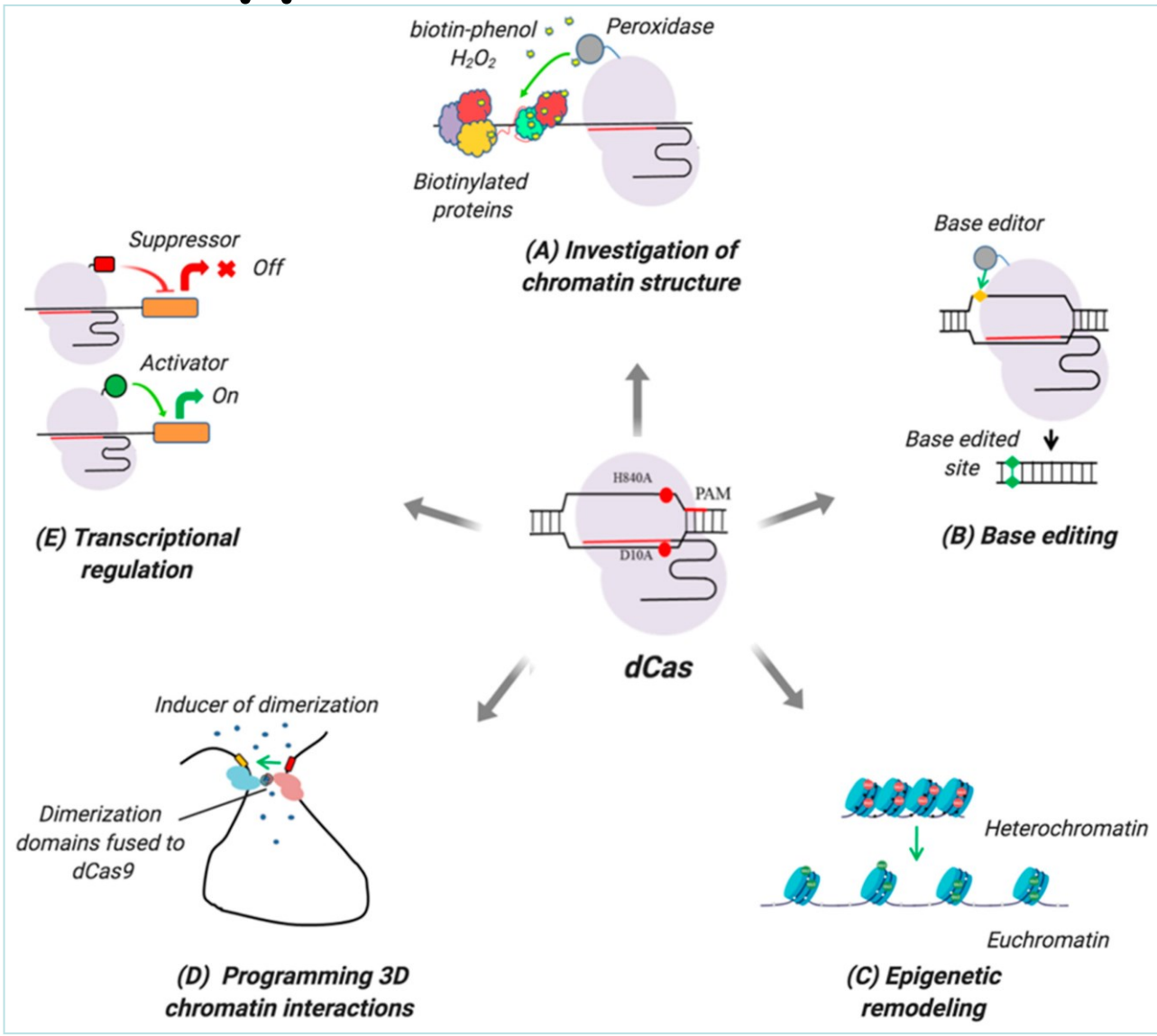
Cong-Fei Xu, Guo-Jun Chen, Ying-Li Luo, Yue Zhang, Gui Zhao, Zi-Dong Lu, Anna Czarna, Zhen Gu, Jun Wang,
Rational designs of in vivo CRISPR-Cas delivery systems,
Advanced Drug Delivery Reviews, Volume 168,2021,Pages 3-29, https://doi.org/10.1016/j.addr.2019.11.005

Schematic diagram of frequently used CRISPR/Cas systems in cancer therapy.

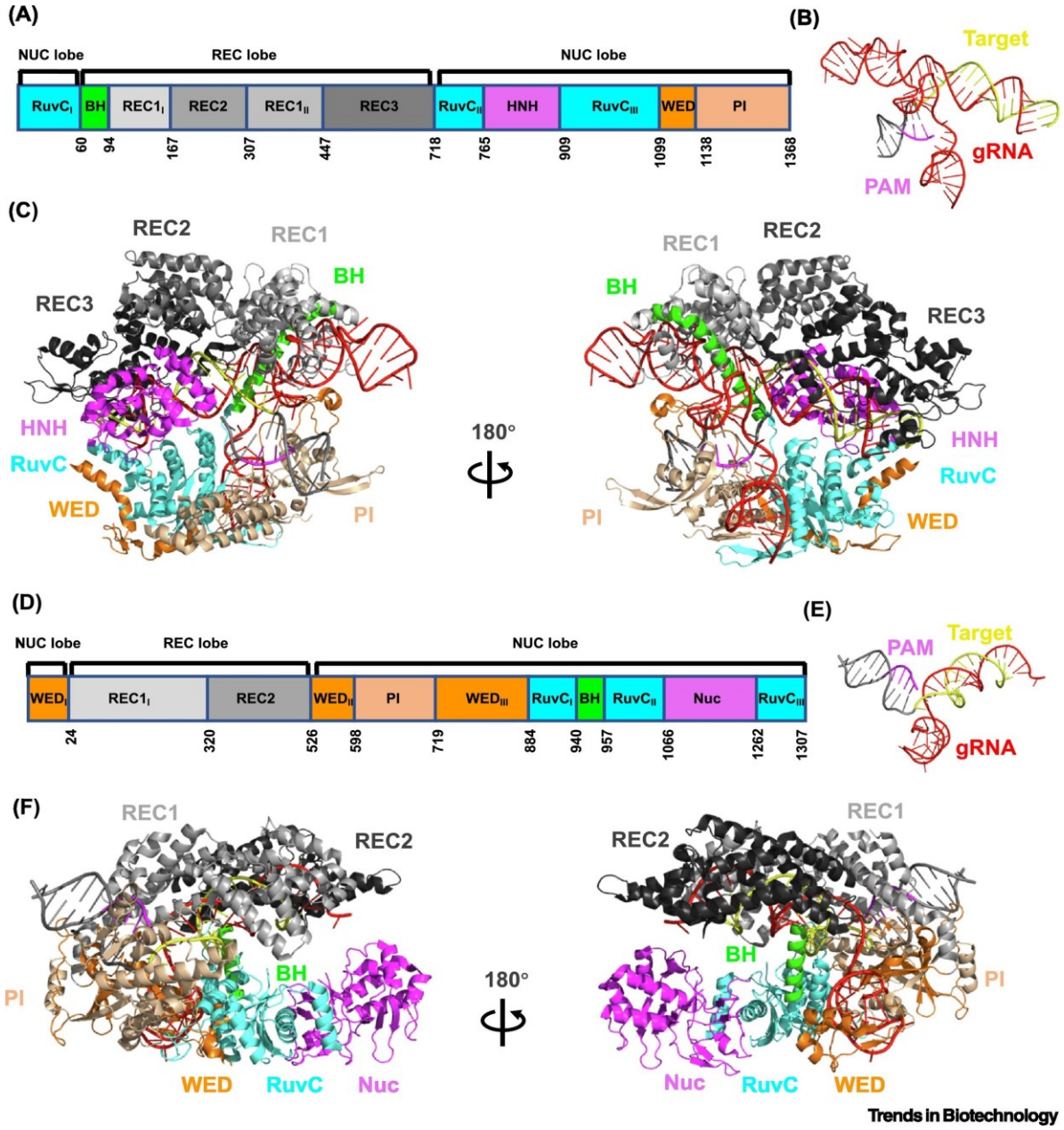


Xiangrong Song, Chao Liu, Ning Wang, Hai Huang, Siyan He, Changyang Gong, Yuquan Wei,
Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy,
Advanced Drug Delivery Reviews,Volume 168, 2021, Pages 158-180, https://doi.org/10.1016/j.addr.2020.04.010

Dead Cas Systems: Types, Principles, and Applications



Int. J. Mol. Sci. **2019**, *20*(23), 6041; https://doi.org/10.3390/ijms20236041



Cas9 from *Streptococcus pyogenes* (SpCas9) consists of nuclease (NUC) and recognition (REC) lobes. The REC lobe can be separated into four regions, the bridge helix (BH) as well as REC1, REC2, and REC3 domains [46]. The NUC lobe can be divided into RuvC, HNH, and protospacer-adjacent motif (PAM)-interacting (PI) domains [46].

Cas12a from *Acidaminococcus* sp. (AsCas12a) consists of NUC and REC lobes. The REC lobe can be separated into two regions, REC1 and REC2 domains [51]. The NUC lobe can be divided into WED, PI, RuvC, BH, and Nuc domains

Trends in Biotechnology 2021 39262-273DOI: (10.1016/j.tibtech.2020.07.005)

Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage

Nicole M. Gaudelli, Alexis C. Komor, Holly A. Rees, Michael S. Packer, Ahmed H. Badran, David I. Bryson & David R. Liu

Nature http://dx.doi.org/10.1038/nature24644 (**2017**).

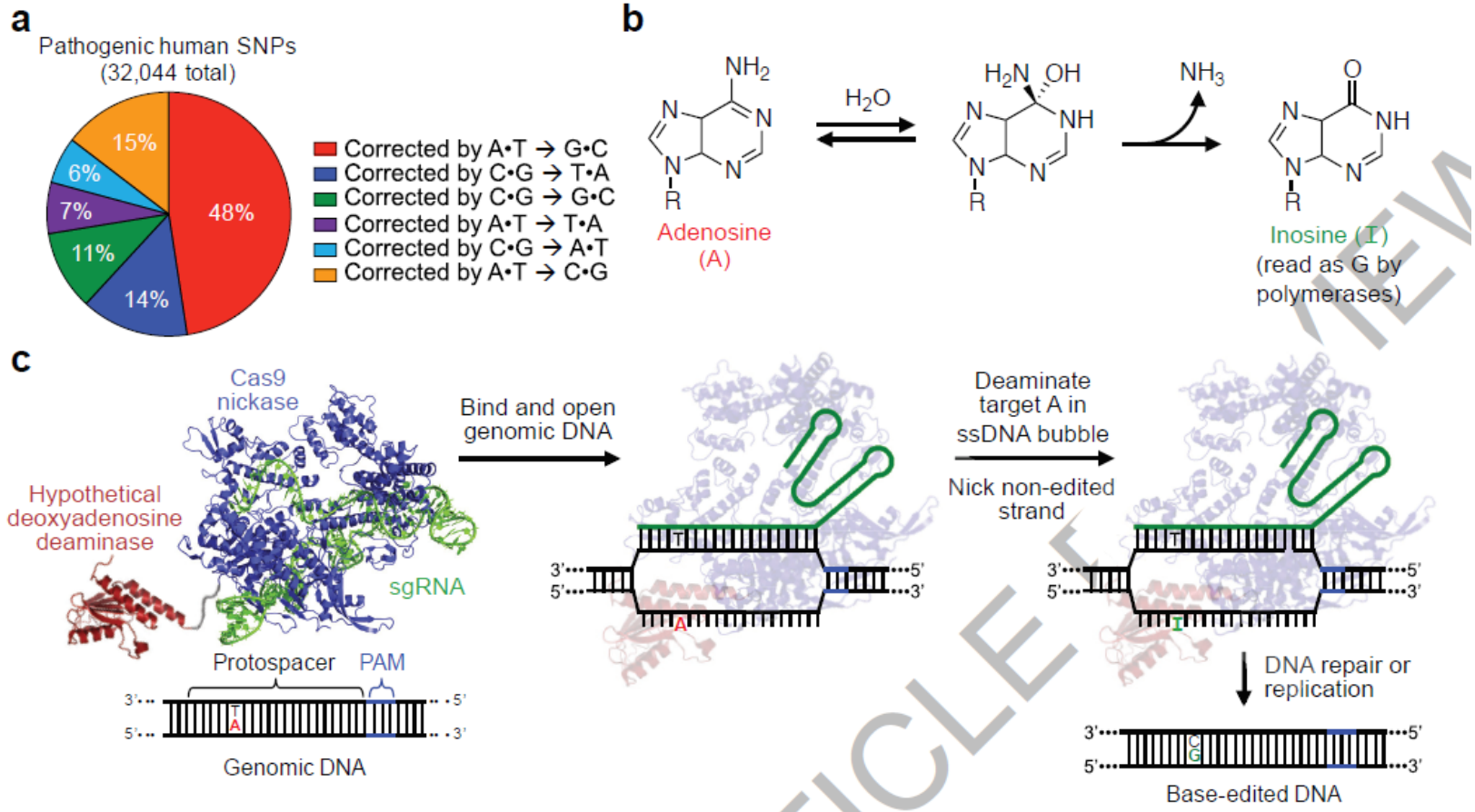


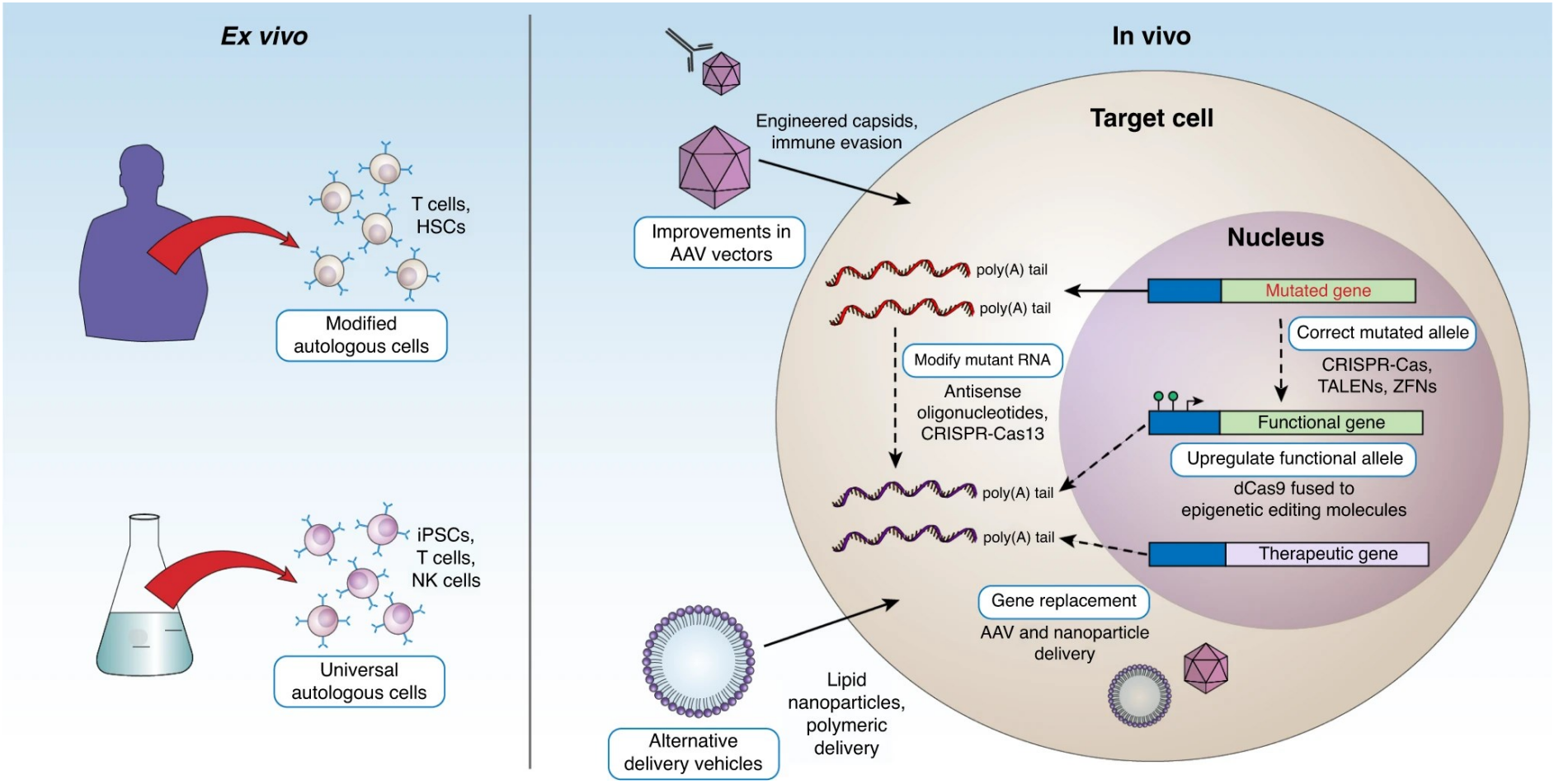
Figure 1 | Scope and overview of base editing by an A•T to G•C base editor (ABE). a, Base pair changes required to correct pathogenic human SNPs in the ClinVar database. **b,** The deamination of adenosine (A) forms inosine (I), which is read as guanosine (G) by polymerase enzymes. **c,** ABE-mediated A•T to G•C base editing strategy. ABEs contain a hypothetical deoxyadenosine deaminase, which is not known to exist in nature, and a catalytically impaired Cas9. They bind target DNA in a guide RNA-programmed manner, exposing a small bubble of single-stranded DNA. The hypothetical deoxyadenosine deaminase domain catalyzes A to I formation within this bubble. Following DNA repair or replication, the original A•T base pair is replaced with a G•C base pair at the target site.

Applicazioni Editing

Tabella 19.1 Applicazione dell'editing genomico mediante tecnica CRISPR/Cas9.

Malattia	Gene	Modello
Studio di meccanismi patologici		
Cancro al polmone	*KRAS, p53, LKB1*	Animale
Cancro al fegato	*Pten, p53*	Animale
Trattamento di malattie genetiche		
Tirosinemia	*FAH*	Animale
Deficit di ornitina transcarbamilasi	*OCT*	Animale
Distrofia muscolare di Duchenne	*DMD*	Animale
Malattia di Huntington	*HTT*	Animale
Atassia di Friedreich	*FXN*	Animale
Fibrosi cistica	*CFTR*	Organoidi
Trattamento di infezioni virali		
HIV	*TAT* e *REV*	Cellulare (T $CD4^+$)
Epatite B	Regioni del genoma di HBV	Cellulare (HepG2)
Papilloma virus	*E6* ed *E7*	Cellulare (HeLa)
Trattamento di patologie tumorali		
Leucemia a cellule B Leucemia linfoblastica acuta a cellule T Mieloma multiplo Liposarcoma Cancro dell'esofago Cancro del polmone non a piccole cellule	*TCR, PD1, CD7, CD19*	Trial clinici, studi di fase I (editing *ex vivo* di cellule T)

Schematic of *ex vivo* and *in vivo* strategies for treating genetic diseases.



NATURE COMMUNICATIONS | (2020) 11:5820 | https://doi.org/10.1038/s41467-020-19505-2 |

Cronologia degli sviluppi scientifici e del Human Genome Editing

2020/12/09

CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia
Frangoul, H. et al.
N. Engl J. Med. https://doi.org/10.1056/NEJMoa2031054 (2020).

[invece → di seguito un Follow-Up di pazienti trattati con

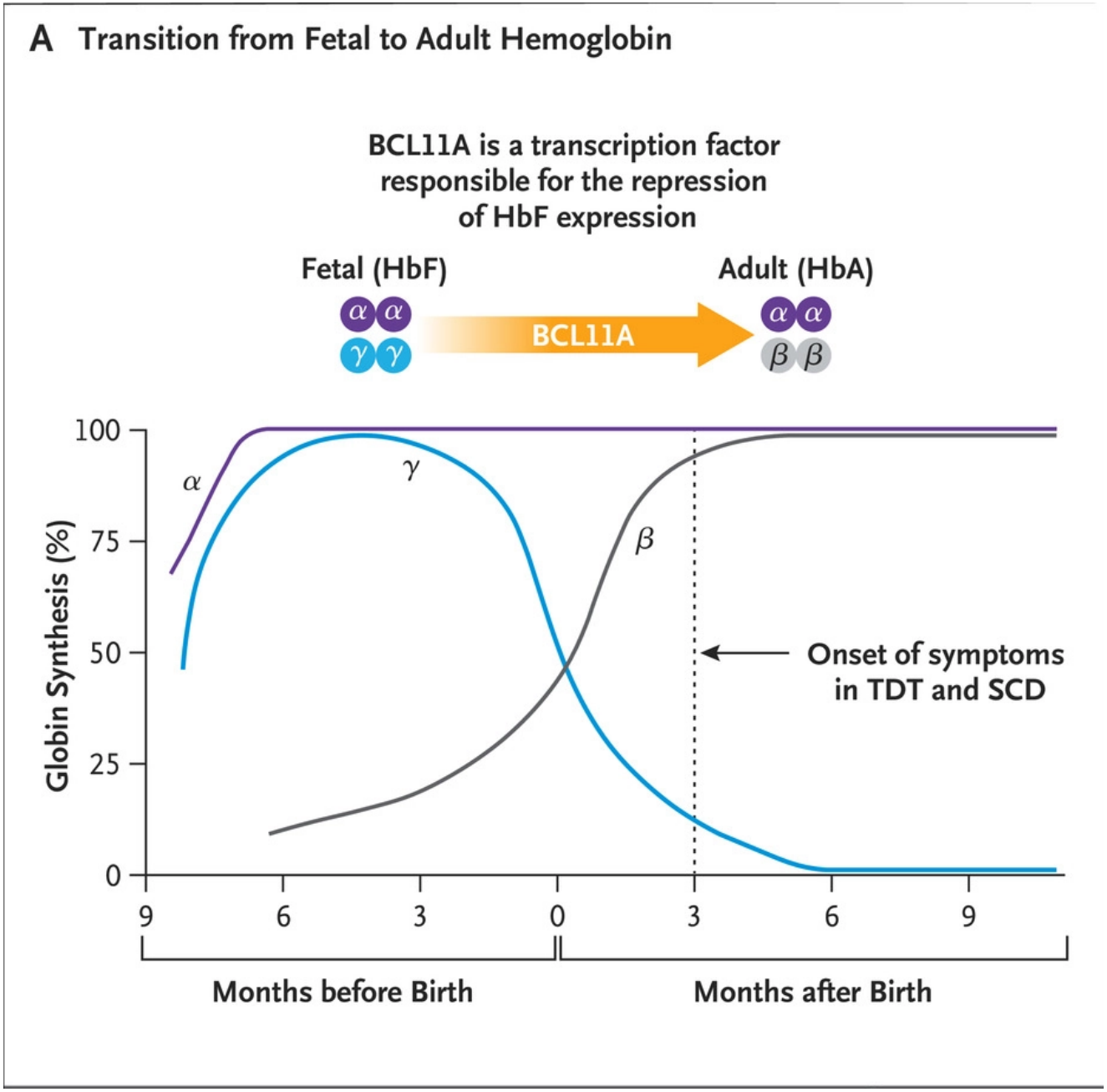
BCH-BB694 lentiviral vector, which encodes a **short hairpin RNA (shRNA)** targeting ***BCL11A* mRNA** embedded in a microRNA (shmiR), allowing erythroid lineage–specific knockdown].

Post-Transcriptional Genetic Silencing of BCL11A to Treat Sickle Cell Disease
Esrick, E. B. et al. N.
N. Engl J. Med. https://doi.org/10.1056/NEJMoa2029392 (2020).

CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia

N. Engl J. Med. https://doi.org/10.1056/NEJMoa2031054 (2020).

- Elevated levels of **fetal hemoglobin (consisting of two alpha and two gamma chains)** are associated with improved morbidity and mortality in patients with TDT and SCD.
- The production of fetal hemoglobin is developmentally regulated so that the level of γ-globin that is produced in utero decreases postnatally as **the production of β-globin and adult hemoglobin (consisting of two alpha and two beta chains) increases.**
- **Neonates and infants with TDT or SCD are typically asymptomatic while their fetal hemoglobin levels remain high** and become symptomatic during the first year of life when the synthesis of fetal hemoglobin declines·
- Patients with TDT or SCD who **co-inherit hereditary persistence of fetal hemoglobin**, in which fetal expression continues into adulthood, **have little or no disease**.



N. Engl J. Med. https://doi.org/10.1056/NEJMoa2031054 (2020).

CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia

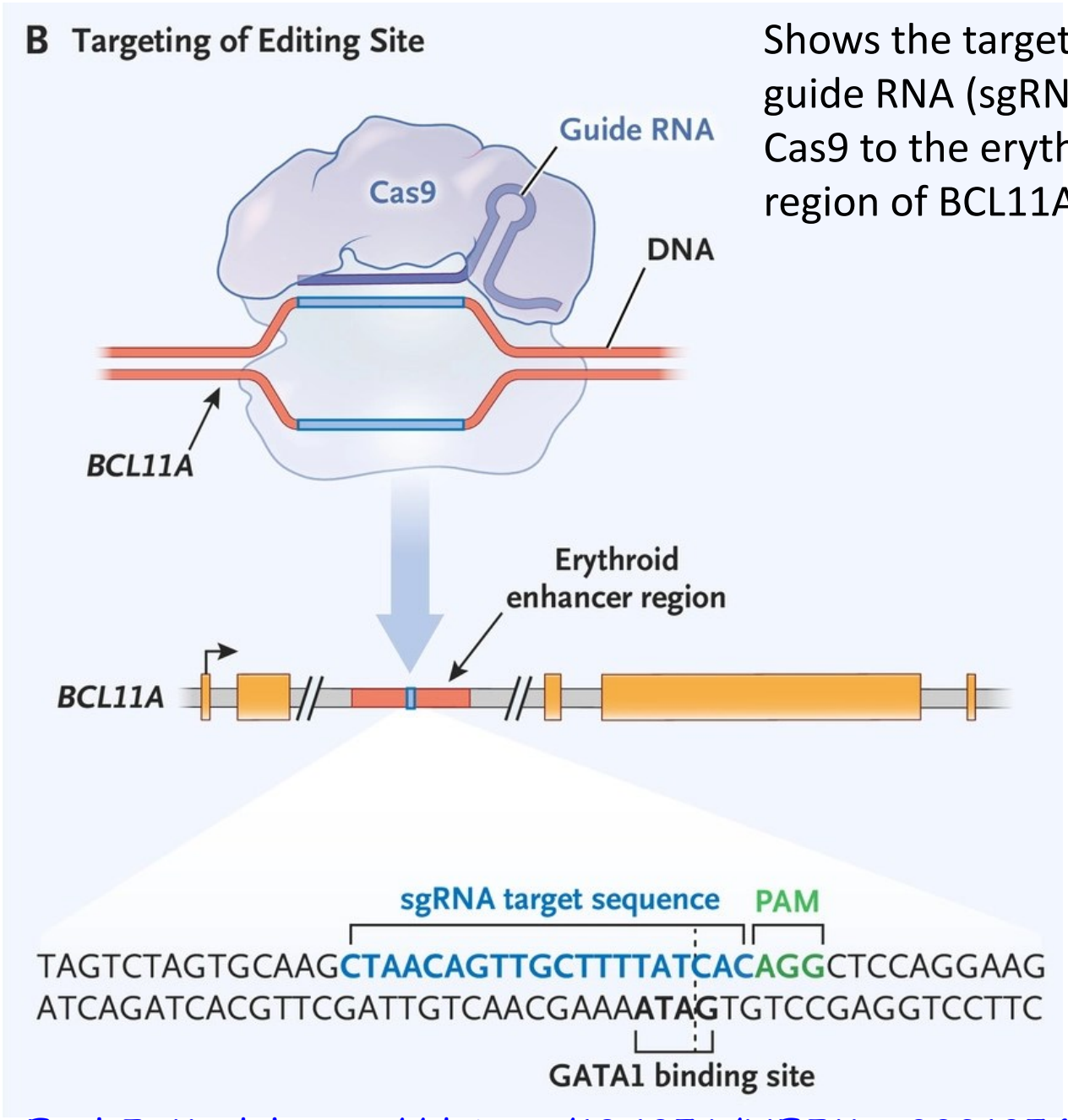
N. Engl J. Med. https://doi.org/10.1056/NEJMoa2031054 (2020).

- Genomewide association studies have identified single-nucleotide polymorphisms **(SNPs) associated with increased expression of fetal hemoglobin in adults**

- Some of these SNPs are **located in the BCL11A locus** on chromosome 2 and are associated with a lower severity of both TDT and SCD.

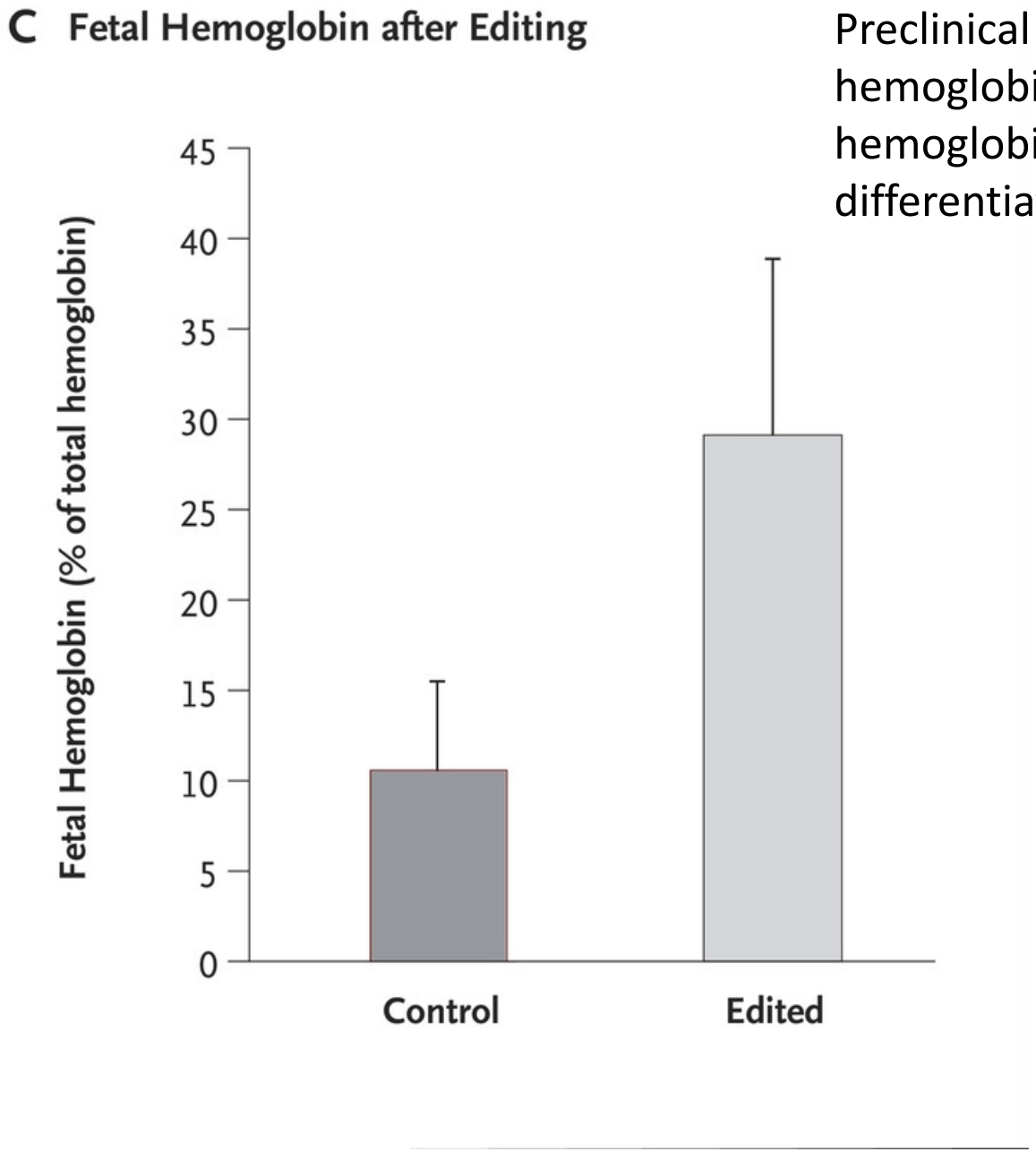
- **BCL11A is a zinc finger–containing transcription factor** that **represses γ-globin expression** and fetal hemoglobin in erythroid cells;

- The **SNPs** that are associated with fetal hemoglobin are in an erythroid-specific enhancer, **down-regulate BCL11A** expression, and **increase the expression of fetal hemoglobin.**



Shows the target editing site of the single guide RNA (sgRNA) that directs CRISPR-Cas9 to the erythroid-specific enhancer region of BCL11A.

N. Engl J. Med. https://doi.org/10.1056/NEJMoa2031054 (2020).



Preclinical data regarding fetal hemoglobin as a percentage of total hemoglobin after editing and the differentiation of erythroid cells

N. Engl J. Med. https://doi.org/10.1056/NEJMoa2031054 (2020).

Table 2. Engineered or new Cas9 variants that have been applied for HBV treatment.

Category	Advantages	Disadvantages
SpCas9-BE (2016)	•It inactivates HBV genomes by introduction of premature stop codons without inducing DSBs. •It avoids DSBs in the integrated HBV DNA of host genome.	•It has a smaller pool of candidate protospacer sequences due to the requirements for target base-editing sites and PAM. •It has larger gene size.
SpCas9 nickase (2013)	•It enhances the specificity of target cleavage by producing two nicks on two. opposite strands of DNA with a pair of gRNAs.	•It has larger gene size. •Two gRNAs are required to cleave one site.
SaCas9 (2015)	•It has smaller Cas9 size, so it fits into the. AAV vectors.	•It has a smaller pool of candidate gRNAs. due to the requirement of the longer 5 ′NNGRRT-3 ′PAM .
Cas9 with less restriction of PAM (2015)	•It loosens the restriction of PAM. •It can broaden the pools of candidate gRNAs targeting the conserved HBV sequences, particularly for Cas9-BE.	•The efficacy of Cas9 variants may be lower than wild-type Cas9.

Yang Y-C, Yang H-C. Recent Progress and Future Prospective in HBV Cure by CRISPR/Cas. *Viruses*. 2022; 14(1):4. https://doi.org/10.3390/v14010004

Table 3. Viral and nonviral delivery vectors for studying the effect of CRISPR-Cas9-mediated gene editing on HBV genome.

Category	Advantages	Disadvantages
Delivery of Cas9 by viral vector		
AAV	•It has been approved for clinical use in genetic diseases. •It has low pathogenicity and immunogenicity, wide range of cell tropism and long-term gene expression.	•It has limitation of cargo capacity. •The risk of DNA integration into host genome. •Long-term Cas9 gene expression may lead to a higher risk of off-target effect.
Adenovirus	•It has larger cargo capacity than AAV vectors. •It has high transduction efficiency and a wide range of cell tropism.	•It has a risk of integration of viral DNA into host genome. •It induces the inflammatory response.
Delivery of Cas9 by nonviral vector		
HDI with Cas9-expressing plasmid	•It is convenient in mouse models.	•It is not practical in clinical setting. •The in vivo delivery efficacy is low.
RNP or mRNA/LLN (lipid-like nanoparticles)	•It is convenient and efficient for delivery of Cas9. •It has lower cytotoxicity and immunogenicity, and no risk of DNA integration to host genome. •Its transient expression of Cas9 results in lower off-target risk. •It has larger cargo capacity than the AAV vectors.	•The cost of production is higher than that of viral vectors.

Yang Y-C, Yang H-C. Recent Progress and Future Prospective in HBV Cure by CRISPR/Cas. *Viruses*. 2022; 14(1):4. https://doi.org/10.3390/v14010004

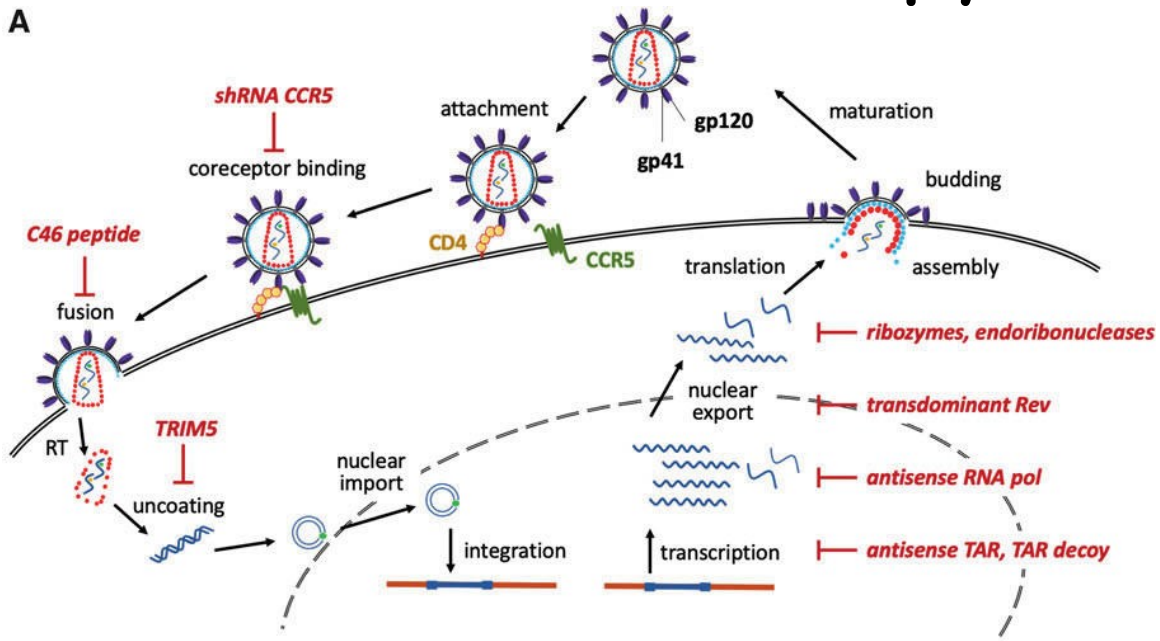
Cronologia degli sviluppi scientifici e del Human Genome Editing

- **Elimination of HIV-1 Genomes from Human T-lymphoid Cells by CRISPR/Cas9 Gene Editing**
  Rafal Kaminski et al. *Scientific Reports* **6**, Article number: 22555 (2016)
  doi:10.1038/srep22555

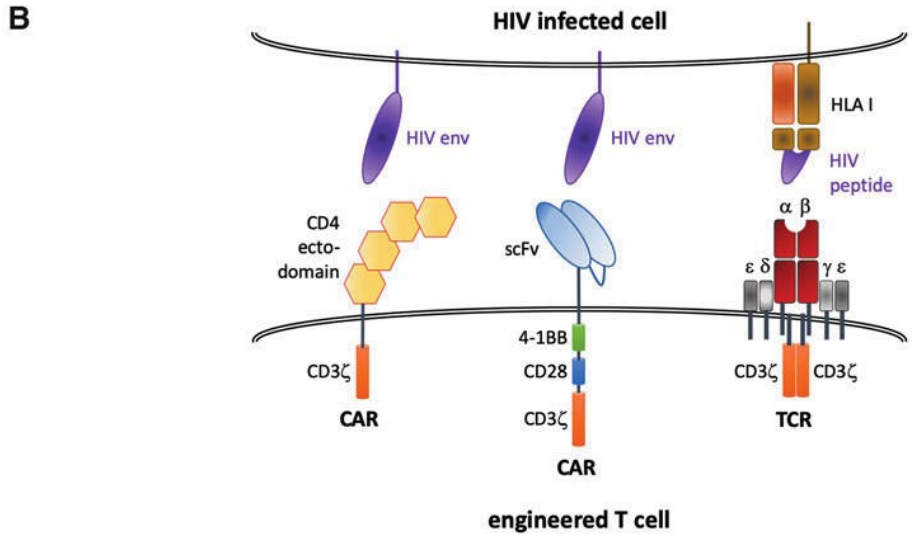
- **Sequential LASER ART and CRISPR Treatments Eliminate HIV-1 in a Subset of Infected Humanized Mice.**
  Dash PK, Kaminski R, et al.
  Nat Commun. 2019 Jul 2;10(1):2753. - doi: 10.1038/s41467-019-10366-y.

- **Efficient Gene Editing of Human Induced Pluripotent Stem Cells Using CRISPR/Cas9**
  Yumlu S., Bashir S., Stumm J., Kühn R. (**2019**) Luo Y. (eds) **CRISPR Gene Editing. Methods in Molecular Biology**, vol 1961. Humana Press, New York, NY - DOI: 10.1007/978-1-4939-9170-9_10

HIV Gene Therapy: An Update



Defensive strategies

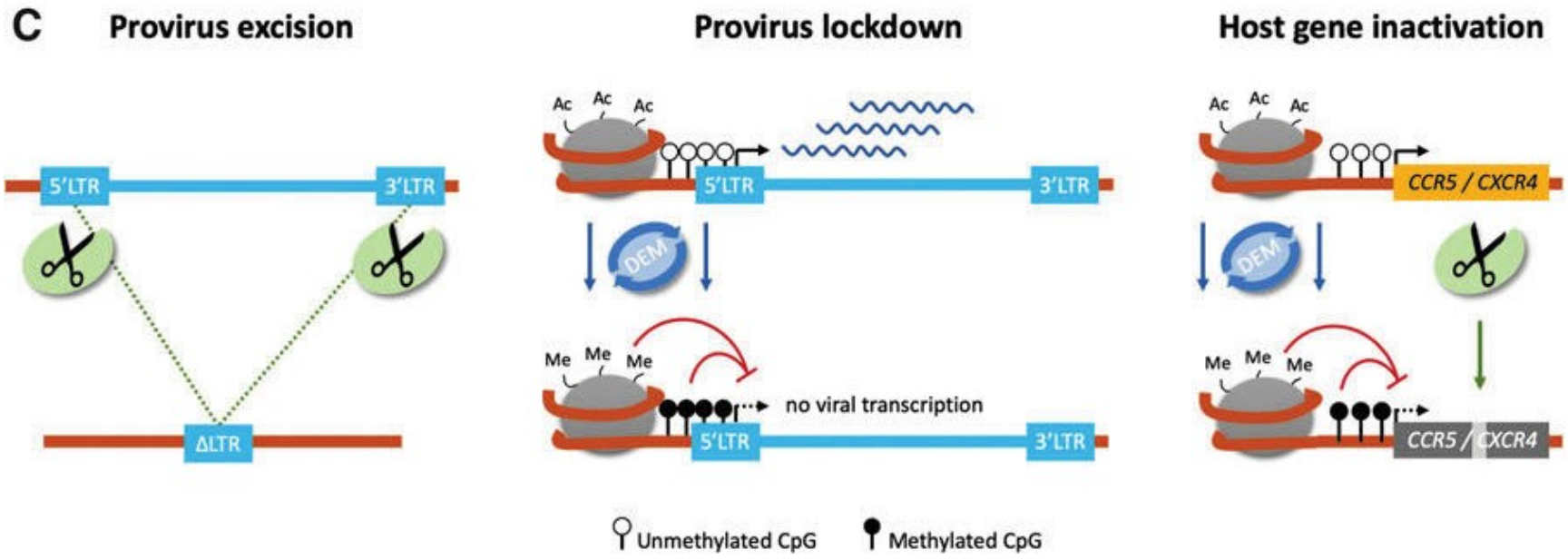


Offensive strategies

Cornu TI, Mussolino C, Müller MC, Wehr C, Kern WV, Cathomen T. HIV Gene Therapy: An Update. Hum Gene Ther. 2021 Jan;32(1-2):52-65. doi: 10.1089/hum.2020.159. PMID: 33349126.

HIV Gene Therapy: An Update

HIV Gene Therapy: An Update

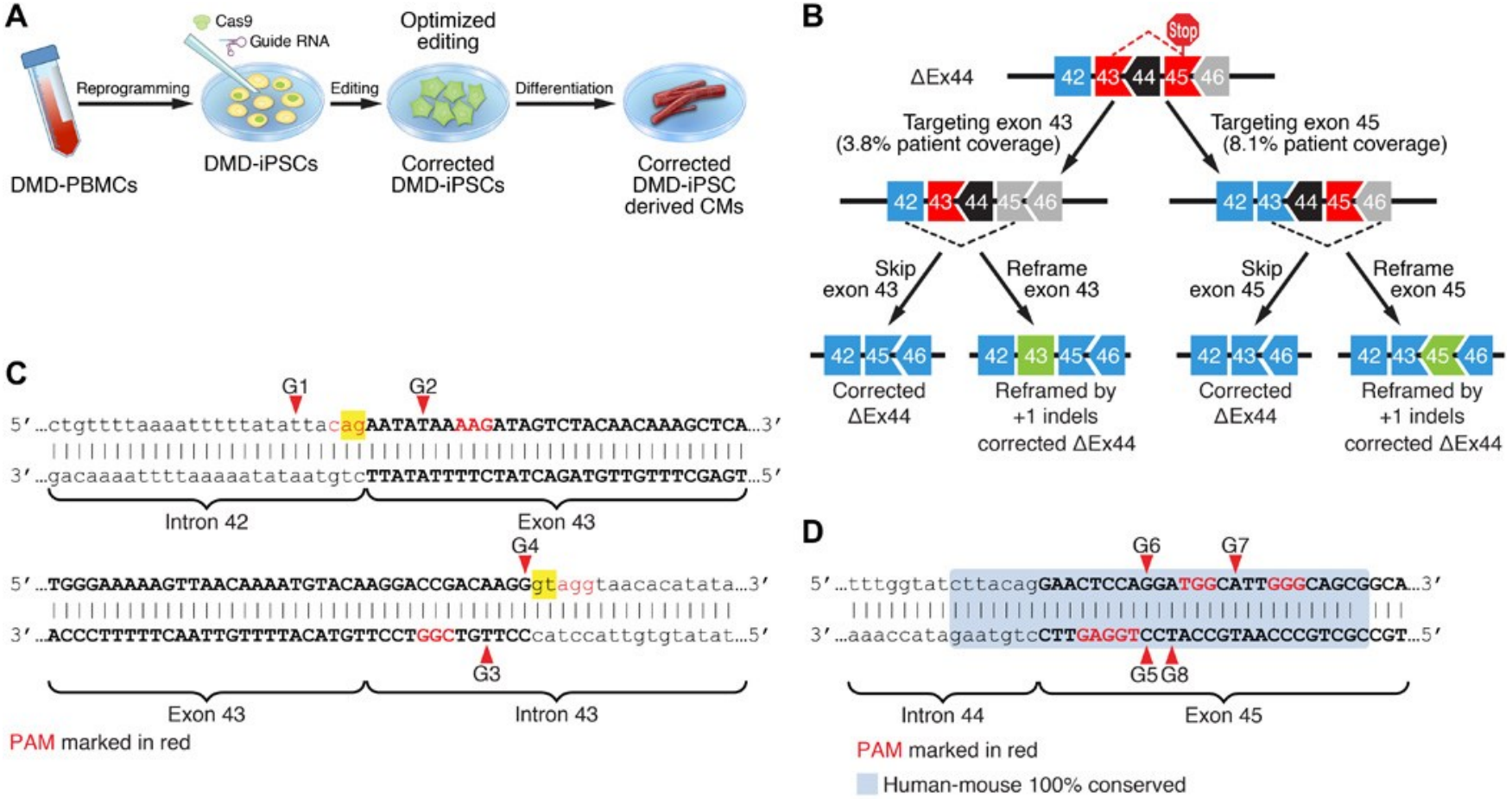


Targeted strategies. The HIV provirus can either be removed or inactivated using genome editing or epigenetically silenced using epigenome editing.

Cornu TI, Mussolino C, Müller MC, Wehr C, Kern WV, Cathomen T. HIV Gene Therapy: An Update. Hum Gene Ther. 2021 Jan;32(1-2):52-65. doi: 10.1089/hum.2020.159. PMID: 33349126.

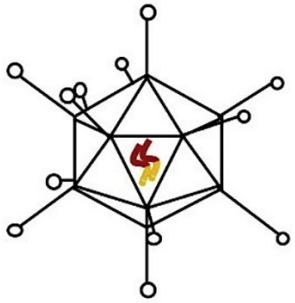
CRISPR-Cas9 corrects Duchenne muscular dystrophy exon 44 deletion mutations in mice and human cells

Deletion of exon 44 (black) results in splicing of exons 43 to 45, generating an **out-of-frame stop mutation** of dystrophin. Disruption of the splice junction of exon 43 or exon 45 results in **splicing of exons 42 to 45 or exons 43 to 46, respectively, and restores the protein reading frame**. The protein reading frame can also be restored by reframing exon 43 or 45 (green)



Min YL, Li H, Rodriguez-Caycedo C, Mireault AA, Huang J, Shelton JM, McAnally JR, Amoasii L, Mammen PPA, Bassel-Duby R, Olson EN. CRISPR-Cas9 corrects Duchenne muscular dystrophy exon 44 deletion mutations in mice and human cells. Sci Adv. 2019 Mar 6;5(3):eaav4324. doi: 10.1126/sciadv.aav4324

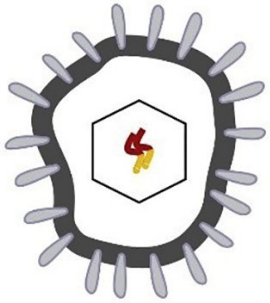
Potential CRISPR/Cas-loaded viral vectors for tumor therapy



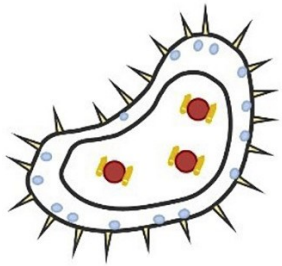
Adenovirus/Adeno-associated viral vector

Retroviral/Lentiviral vector

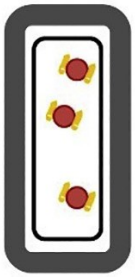
Ad and lentivirus have displayed powerful delivery of CRISPR/Cas systems to tumor cells *in vivo*



Epstein-Barr viral vector



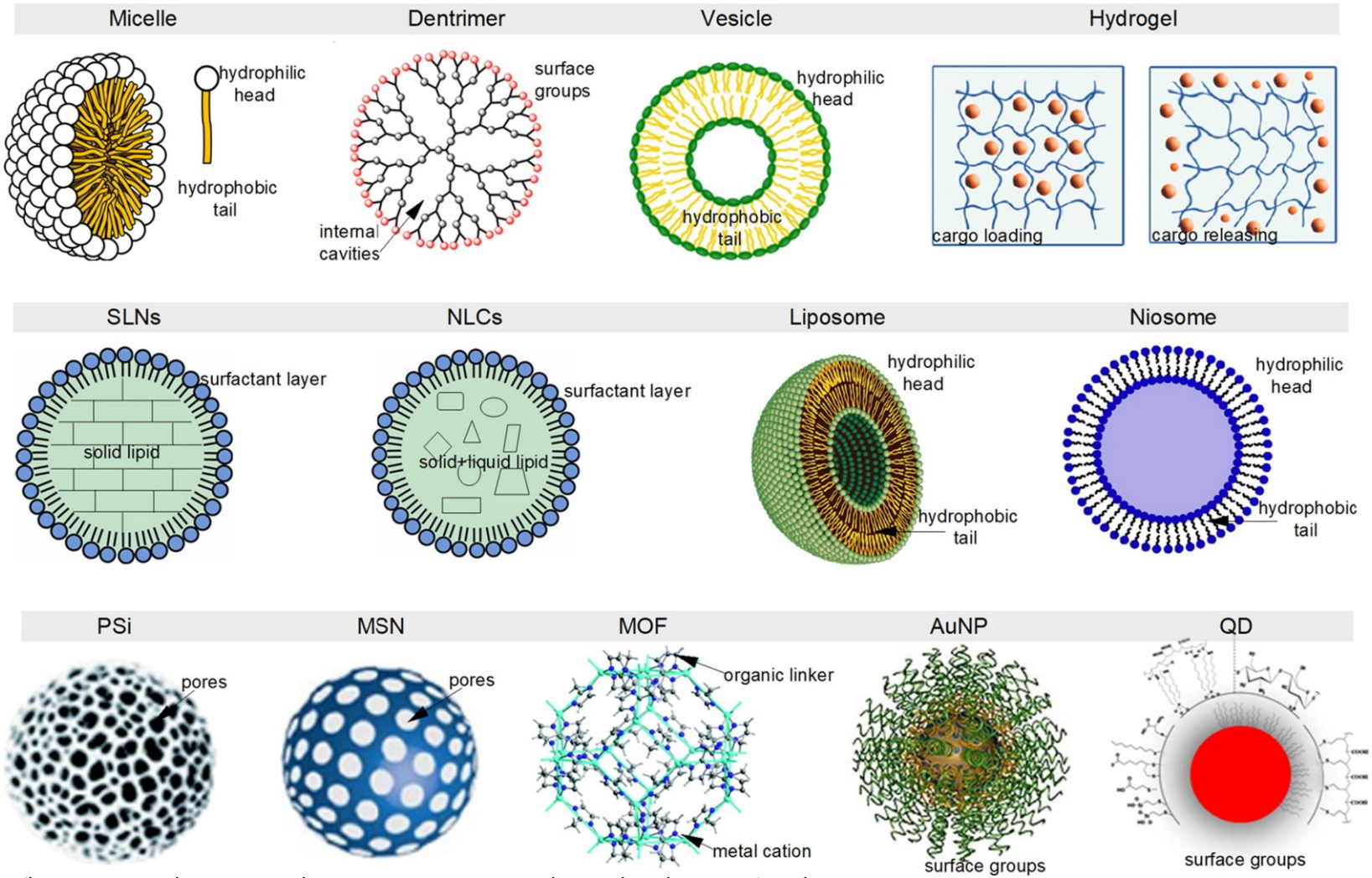
Sendai viral vector



Baculoviral vector

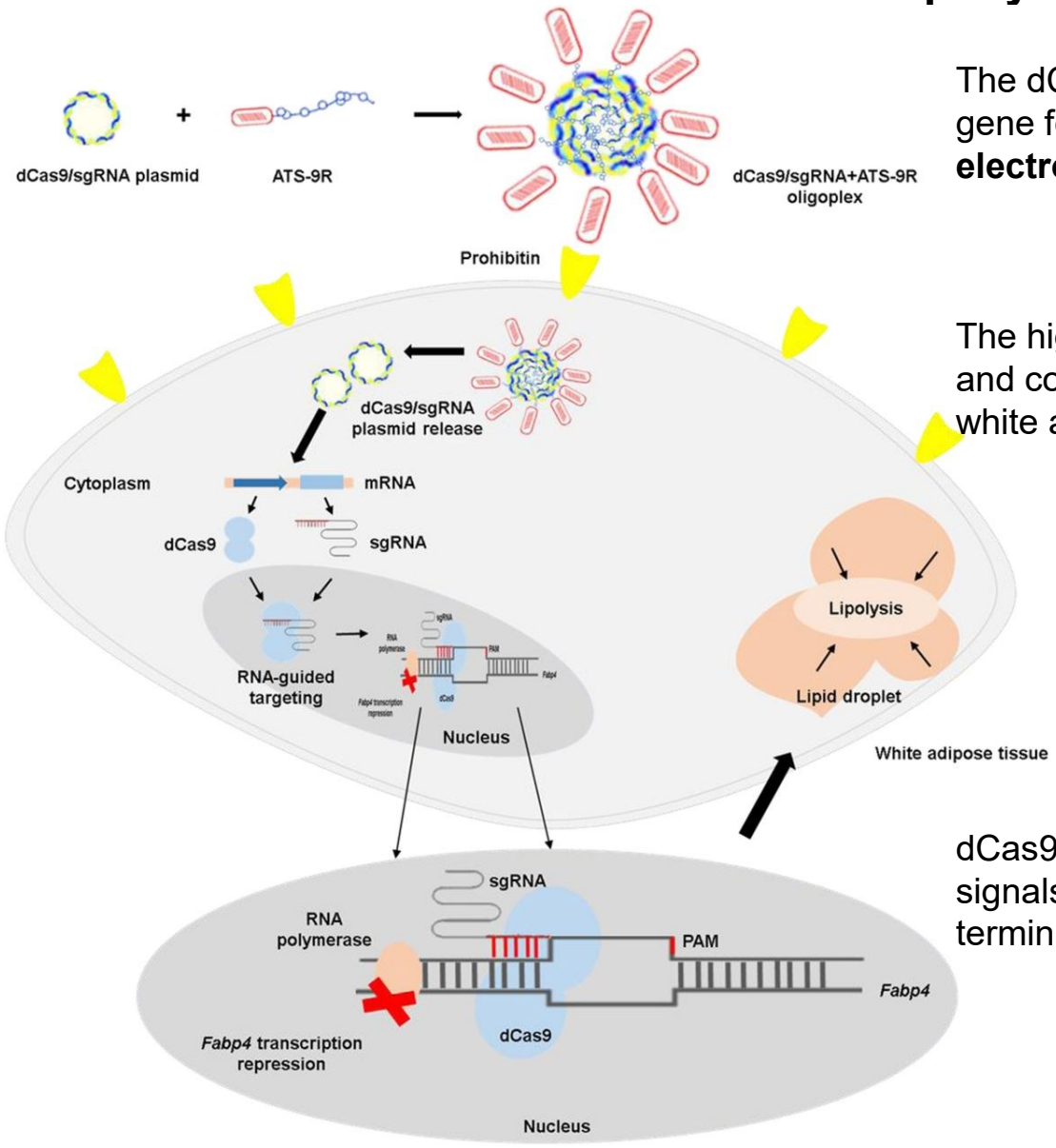
Xiangrong Song, Chao Liu, Ning Wang, Hai Huang, Siyan He, Changyang Gong, Yuquan Wei,
Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy,
Advanced Drug Delivery Reviews,Volume 168, 2021, Pages 158-180, https://doi.org/10.1016/j.addr.2020.04.010

Structural illustration of different nanocarriers for anti-cancer drugs delivery.



Xiaoyu Xu, Chang Liu, Yonghui Wang, Oliver Koivisto, Junnian Zhou, Yilai Shu, Hongbo Zhang, Nanotechnology-based delivery of CRISPR/Cas9 for cancer treatment, Advanced Drug Delivery Reviews, Volume 176, 2021, 113891, https://doi.org/10.1016/j.addr.2021.113891.

Schematic illustration representing "NONVIRAL" CRISPR interference system delivery to white adipocytes.



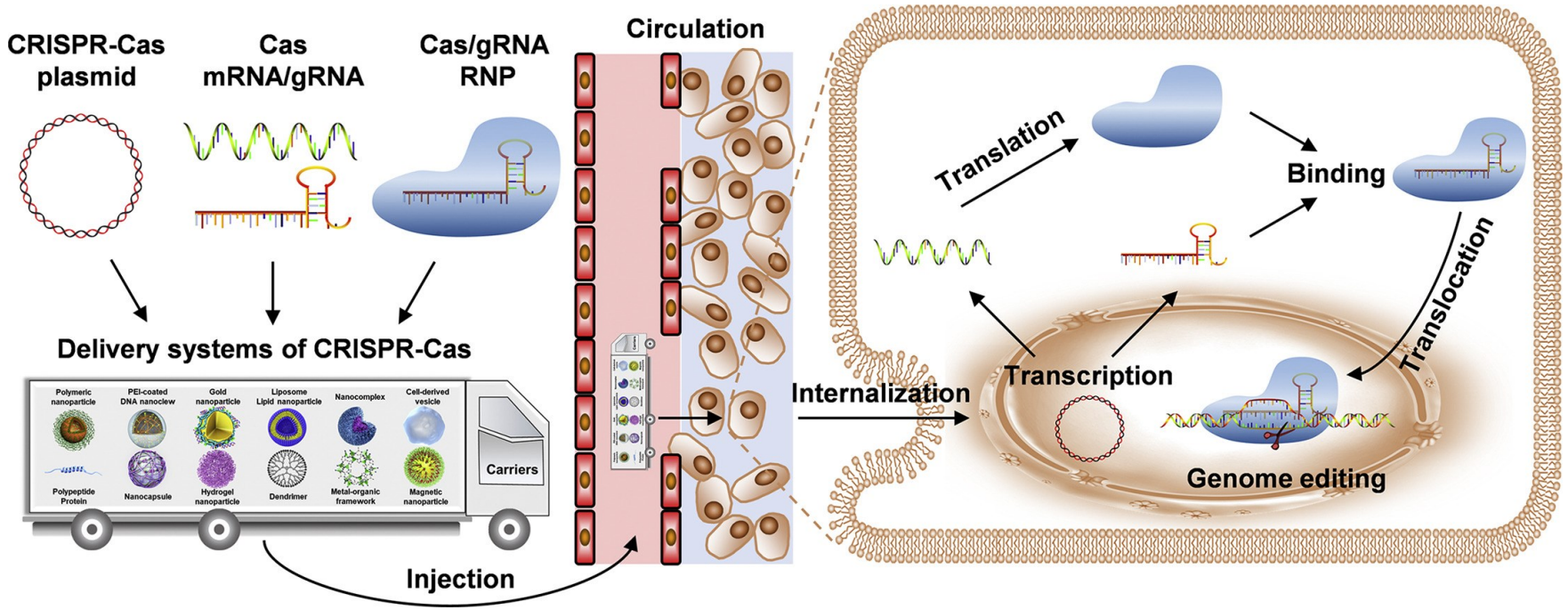
The dCas9 plasmid and sgRNA against *Fabp4* gene formed complexes with **ATS-9R peptide via electrostatic interaction**.

The highly cationic ATS-9R functions as a targeting and condensing peptide to deliver dCas9/sgRNA to white adipose tissues

dCas9 plasmid contains nuclear localization signals (NLSs) on both the N-terminus and C-terminus for delivery to the nucleus.

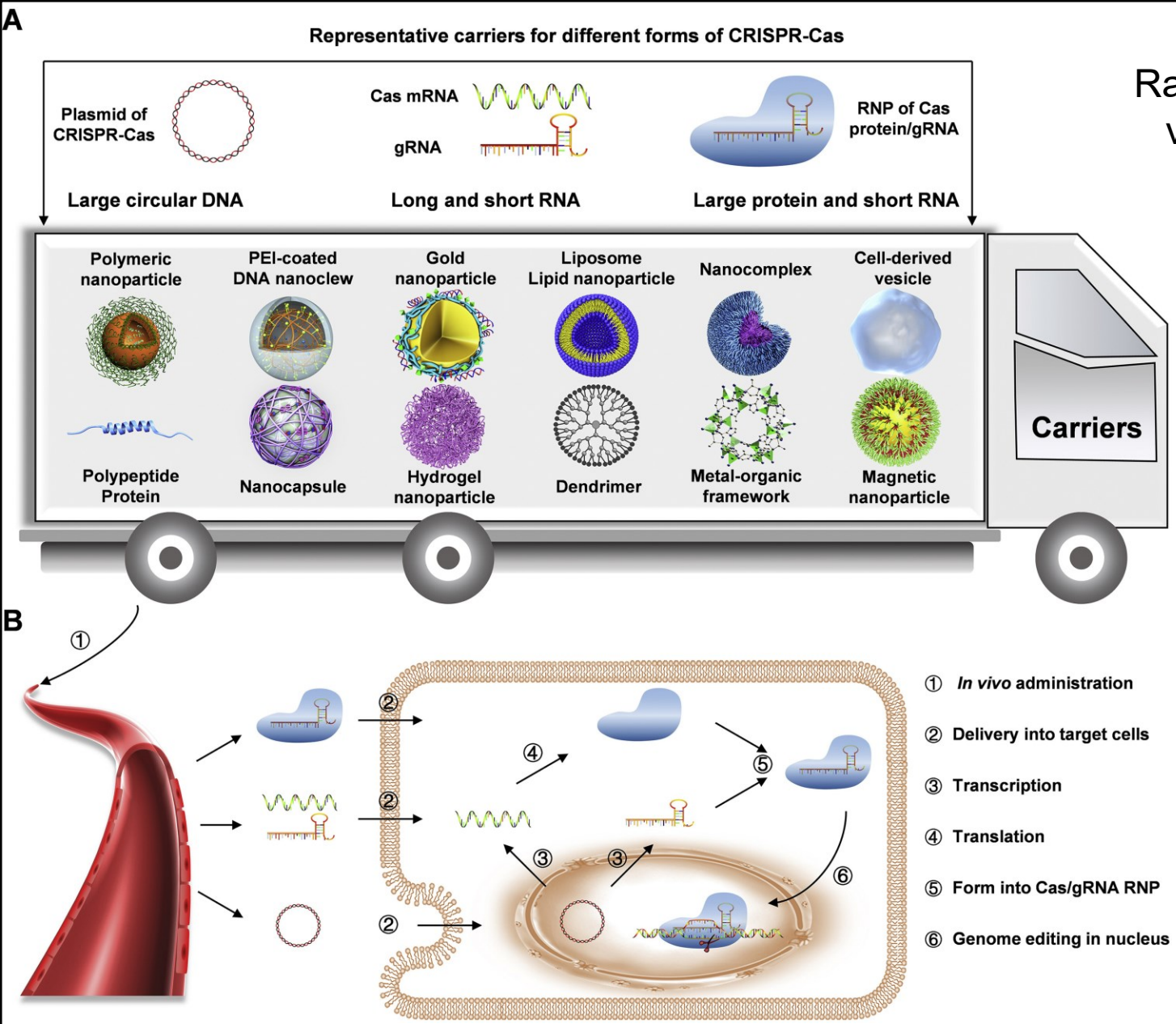
Jee Young Chung et al. Genome Res. 2019;29:1442-1452

Rational designs of in vivo CRISPR-Cas delivery systems



Cong-Fei Xu, Guo-Jun Chen, Ying-Li Luo, Yue Zhang, Gui Zhao, Zi-Dong Lu, Anna Czarna, Zhen Gu, Jun Wang, Rational designs of in vivo CRISPR-Cas delivery systems, Advanced Drug Delivery Reviews, Volume 168,2021,Pages 3-29, https://doi.org/10.1016/j.addr.2019.11.005

Rational designs of in vivo CRISPR-Cas delivery systems



Cong-Fei Xu, Guo-Jun Chen, Ying-Li Luo, Yue Zhang, Gui Zhao, Zi-Dong Lu, Anna Czarna, Zhen Gu, Jun Wang,
Rational designs of in vivo CRISPR-Cas delivery systems,
Advanced Drug Delivery Reviews, Volume 168,2021,Pages 3-29, https://doi.org/10.1016/j.addr.2019.11.005

Risk-benefit considerations in CRISPR technology

	Benefit(s)	Risk(s)/Harm(s)
Basic and pre-clinical research	•New model organisms and cell lines •Increased gene-editing efficiency •High-throughput screens •Novel drug targets •Access to totipotent cells •Identification of novel signaling,regulatory, and developmental pathways •Development of novel gene-editingapproaches (base editing and RNA targeting) •Knowledge advancement	•Experimentation involving human embryos is controversial and illegal in some countries •Potential for privacy and confidentiality breaches

CRISPR Ethics: Moral Considerations forApplications of a Powerful Tool
https://doi.org/10.1016/j.jmb.2018.05.044

Risk-benefit considerations in CRISPR technology

	Benefit(s)	Risk(s)/Harm(s)
Translational and clinical medicine	•Immunotherapy •Organoids •Novel drug targets •Artificial intelligence •Modification of pathological genes •Novel therapeutics and fertilityapplications •Procreative liberty •Ability to“fix”single base changes •Knowledge advancement •Potential for equitable access	•Serious injury, disability, and/or death to research participant(s) and/or offspring •Blurry distinction between therapeutic and enhancement applications, leading to potential subtle or obvious exacerbation of inequalities •Misapplications •Eugenics •Potential for inequitable access and exacerbation of inequalities

CRISPR Ethics: Moral Considerations for Applications of a Powerful Tool
https://doi.org/10.1016/j.jmb.2018.05.044

Risk-benefit considerations in CRISPR technology

	Benefit(s)	Risk(s)/Harm(s)
Non-therapeutic applications	•Enhancement to augment select faulty or normal human characteristics •Fortification of crops and livestock •Successful control of pests, invasive species, and reservoirs (gene drives) •Disease/infection control (e.g., malaria, dengue fever, Lyme and Chagas disease, schistosomiasis) •Ecosystem alteration to protect endangered species (gene drives) •Safety •Crop cultivation •Knowledge advancement	•Eugenics •Exacerbation of racism and inequality •Theoretical risk for damage to ecosystems •Theoretical risk of misuse

CRISPR Ethics: Moral Considerations forApplications of a Powerful Tool
https://doi.org/10.1016/j.jmb.2018.05.044

Risk-benefit considerations in CRISPR technology

	Benefit(s)	Risk(s)/Harm(s)
Access to CRISPR technology	•Inexpensive (technology itself) •Widely available •Profit, economic growth •Innovation	•Price gouging •Prohibitively expensive application
Regulations for clinical research involving human subjects	•Established framework in some countries to manage research risks •Legal mechanisms for redress already exist, depending on location	•Lack of appropriate supervisory infrastructure, oversight, and/or regulatory framework in many nations •Unclear how to supervise the research even in some countries with regulatory oversight •Over-regulation might hinder progress

CRISPR Ethics: Moral Considerations forApplications of a Powerful Tool
https://doi.org/10.1016/j.jmb.2018.05.044

Risk-benefit considerations in CRISPR technology

	Benefit(s)	Risk(s)/Harm(s)
National and international regulations, law, and policy	•Prevention against misuses of technology •Safeguard against risky, potentially harmful condition	•Potential to encroach on individual, scientific, and societal autonomy •Limit discovery and progress •Difficult enforcement •Lack of uniformity may create inconsistencies in applications of laws/regulations

CRISPR Ethics: Moral Considerations forApplications of a Powerful Tool
https://doi.org/10.1016/j.jmb.2018.05.044

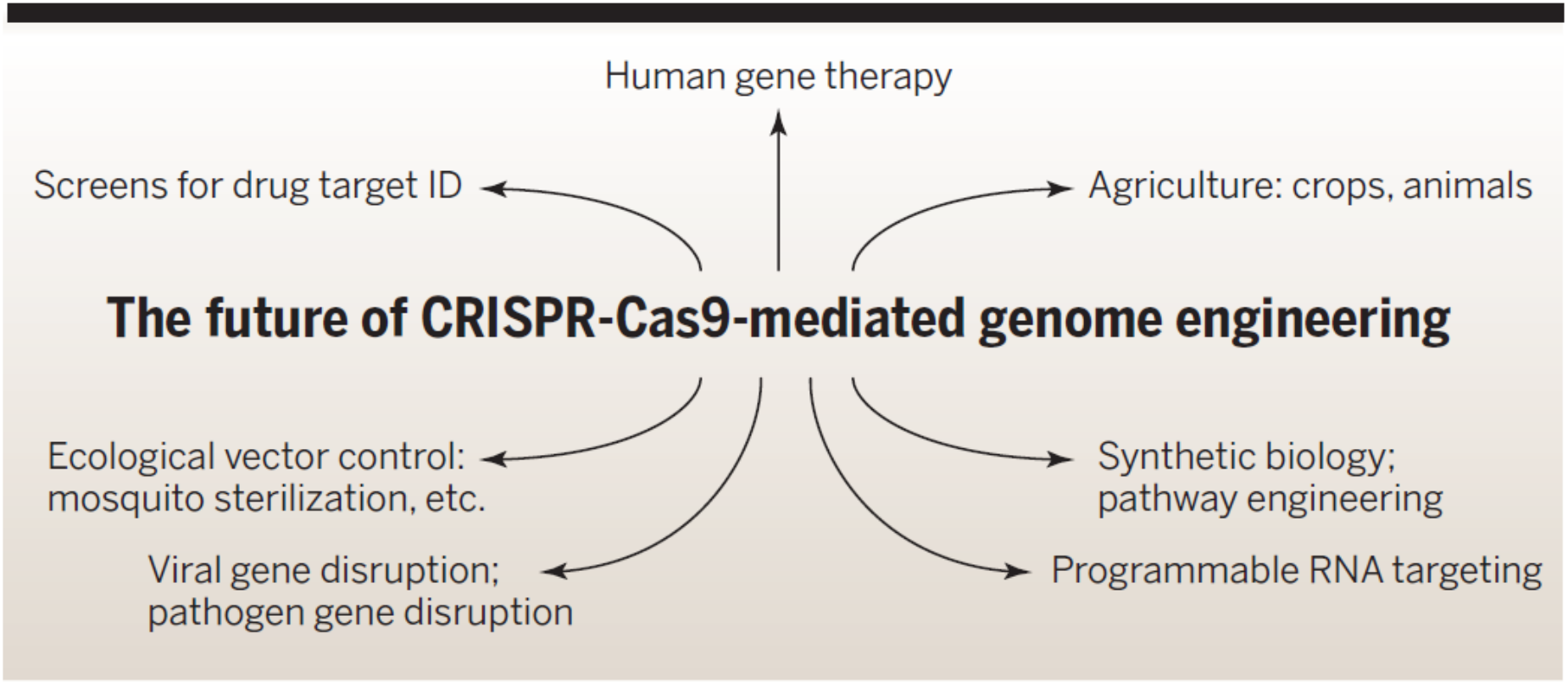
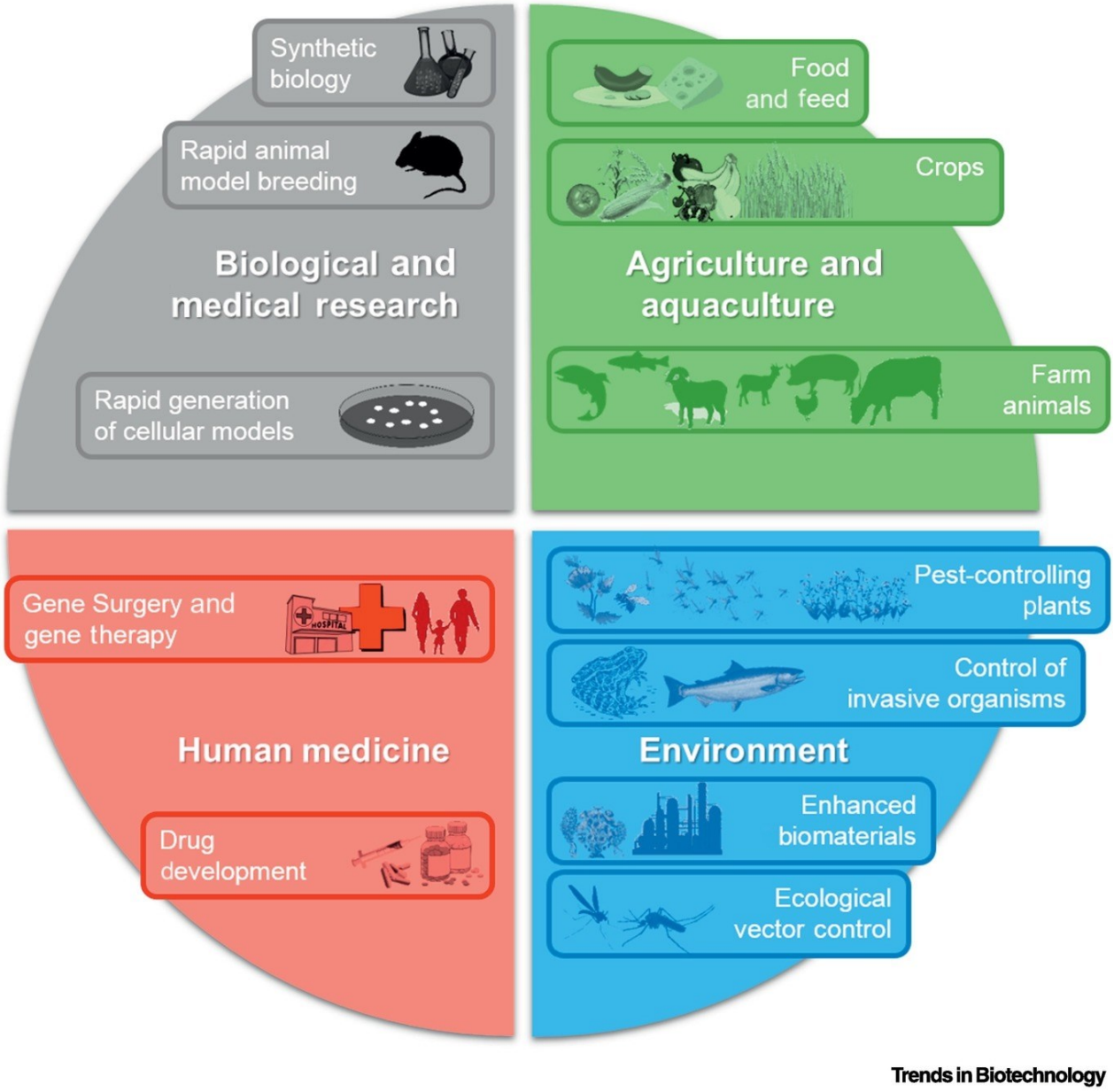


Fig. 6. Future applications in biomedicine and biotechnology. Potential developments include establishment of screens for target identification, human gene therapy by gene repair and gene disruption, gene disruption of viral sequences, and programmable RNA targeting.

Science 28 Nov 2014: Vol. 346, Issue 6213, DOI: 10.1126/science.1258096

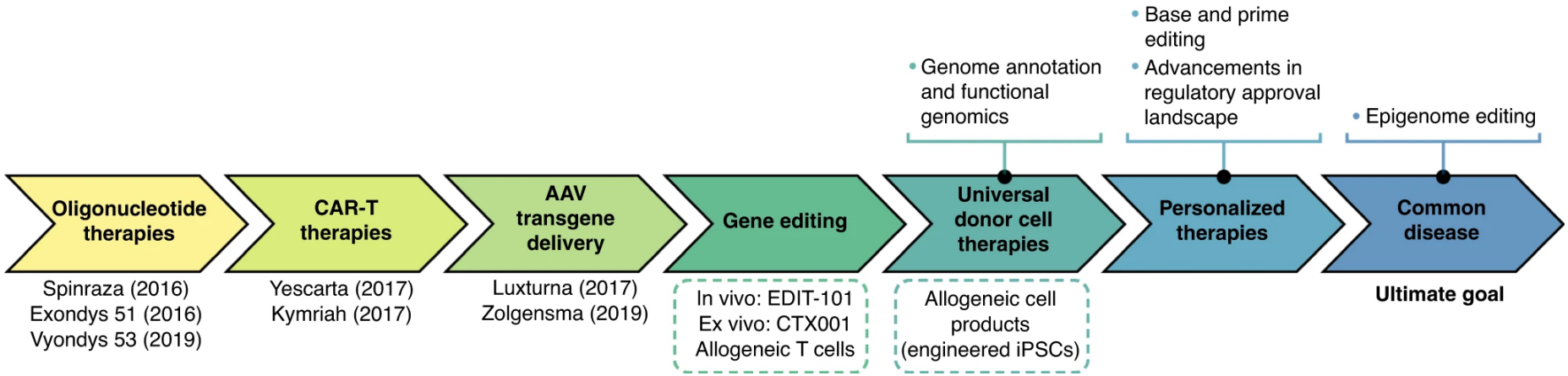
What is CRISPR? Animation
https://www.youtube.com/watch?v=O3e2_Ctty_M

Current Research Applications of Genome Editing in Different Sectors of the Life Sciences.



Trends in Biotechnology, 2019, 37(10), Pages 1029-1032.

Timeline depicting milestones towards gene therapies for common disease.



NATURE COMMUNICATIONS | (2020) 11:5820 | https://doi.org/10.1038/s41467-020-19505-2 |