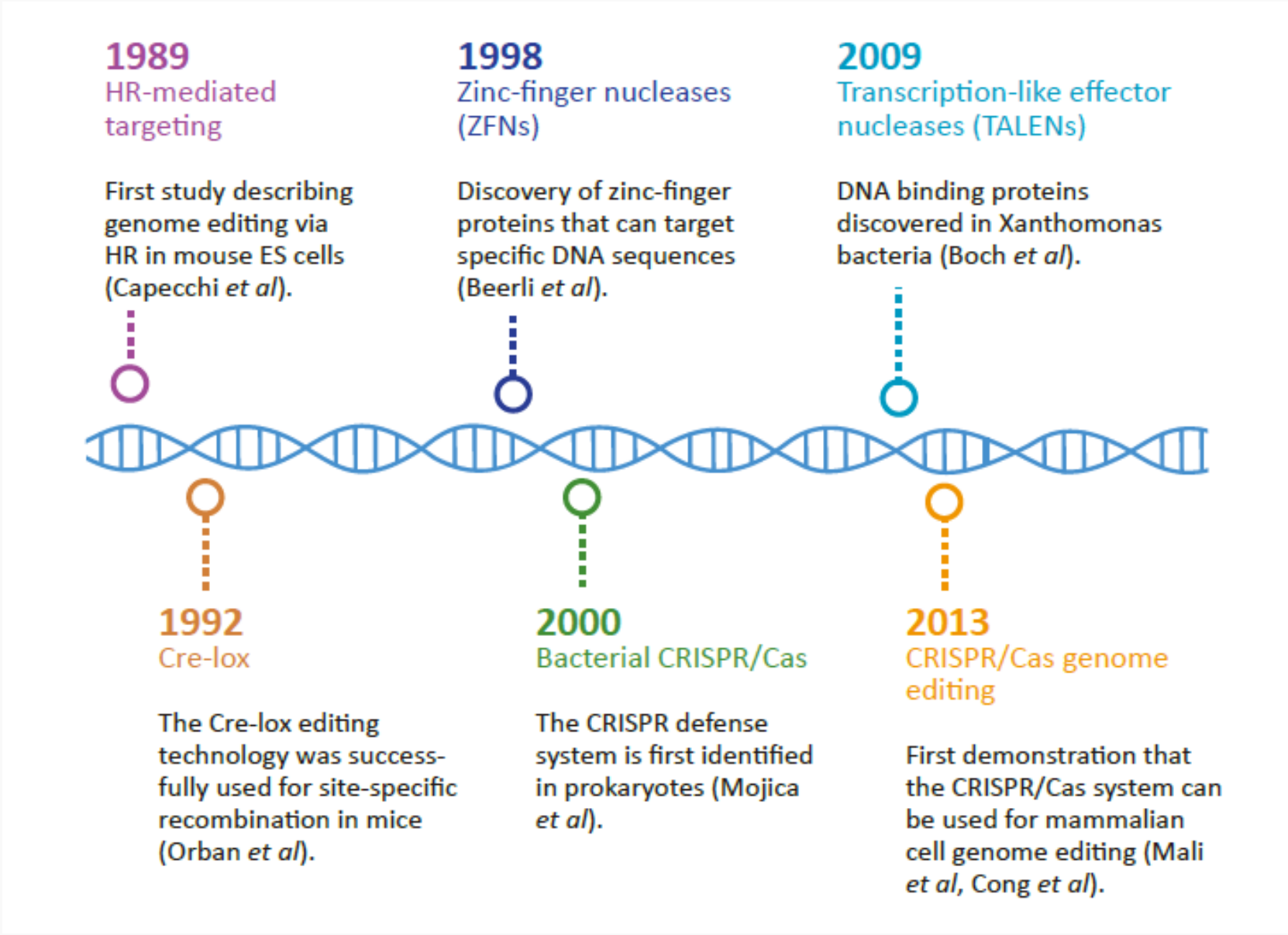
Genome Editing System

UNIT III

Introduction

- Targeted genome editing is a broadly applicable approach for efficiently modifying essentially any sequence of interest in living cells or organisms.
- This technology relies on the use of engineered nucleases, that is, artificial proteins composed of a customizable sequence-specific DNA-binding domain fused to a nuclease that cleaves DNA in a nonsequence- specific manner.
- These targetable nucleases are used to induce double-strand breaks (**DSB**s) into specific DNA sites, which are then repaired by mechanisms that can be exploited to create sequence alterations at the cleavage site.
- Nuclease-mediated genome editing enables genetic studies that were previously difficult or impossible to perform.
- This technology might also provide therapeutic avenues for genetic disorders including monogenic diseases such as sickle cell anemia or cystic fibrosis.

Advancements in genome editing



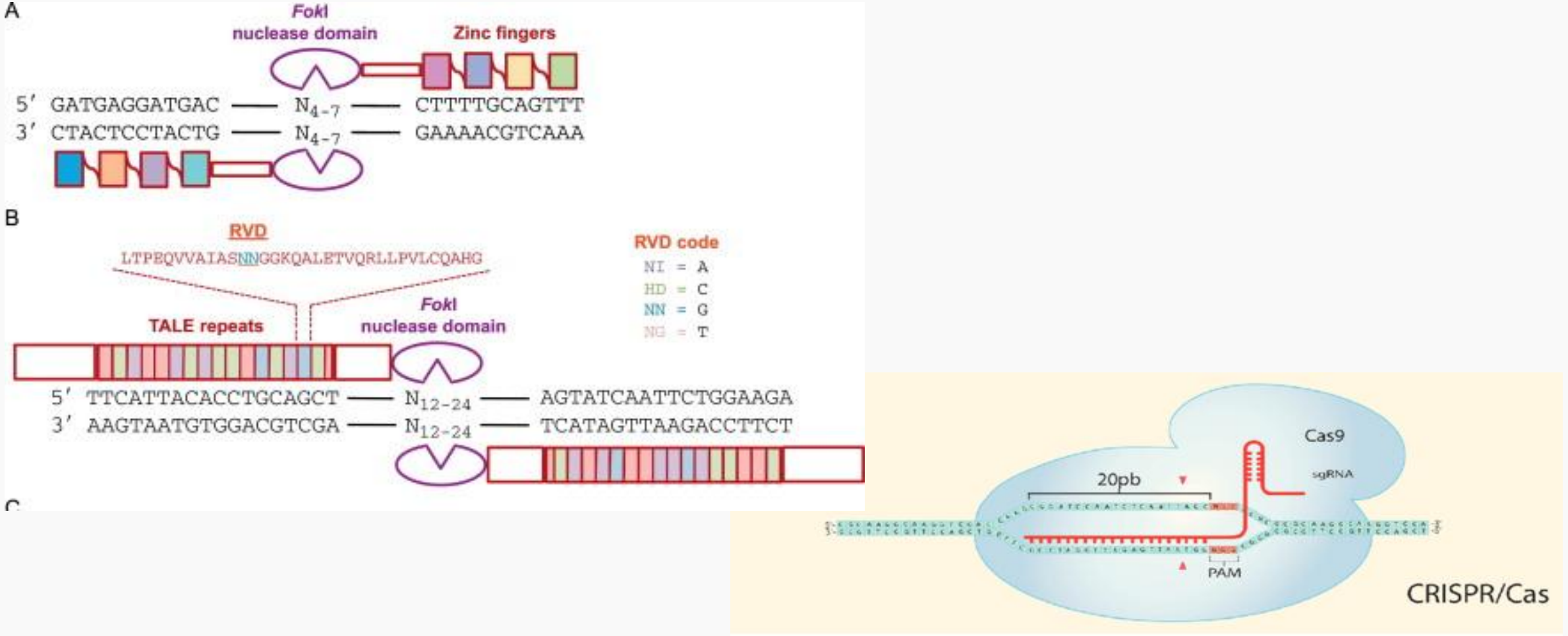
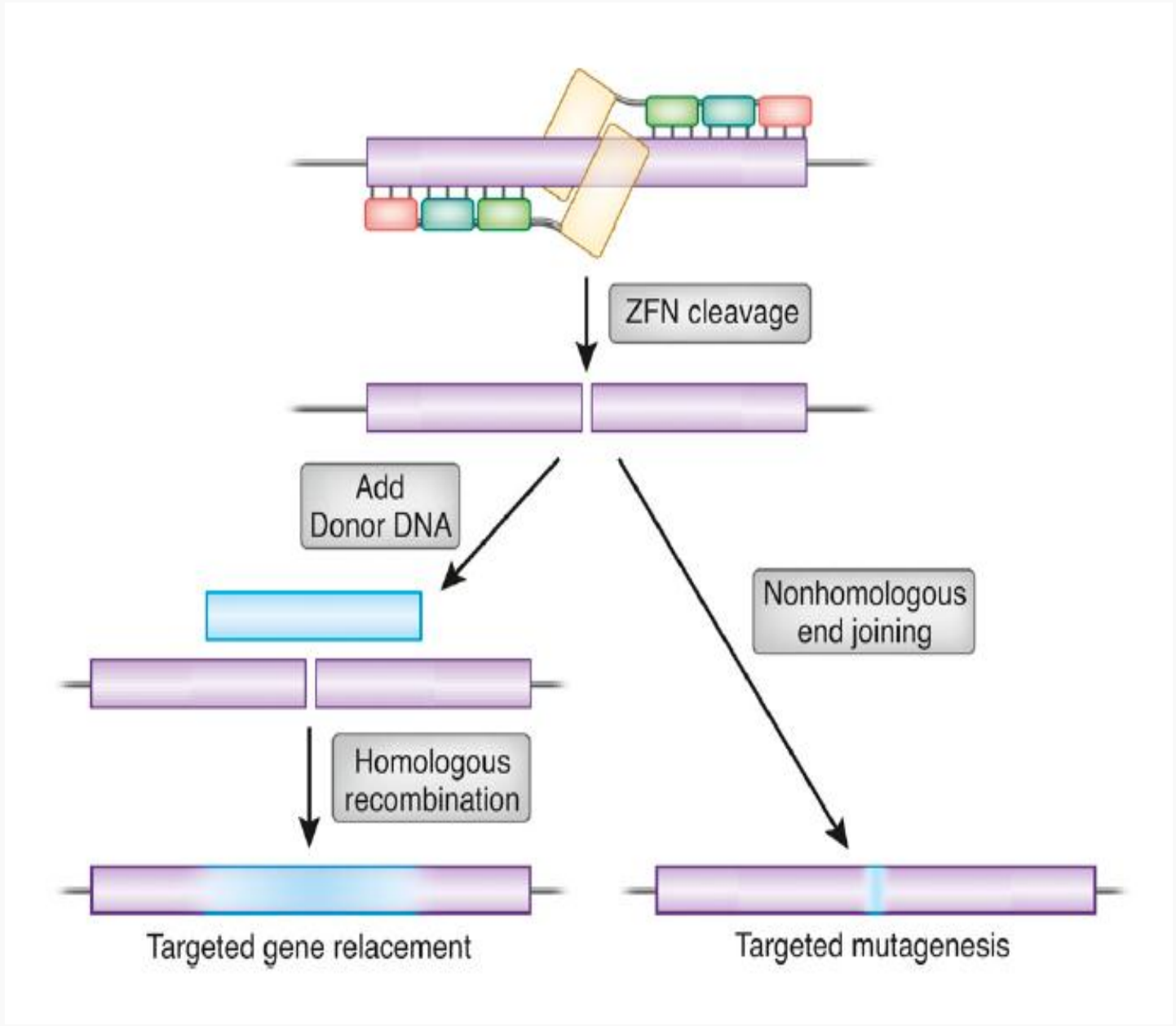
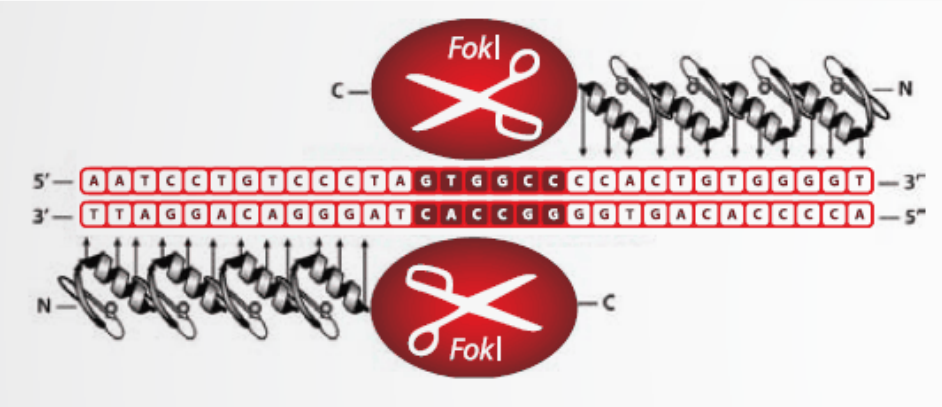


Figure: Editing nucleases. A. ZFNs – two discrete ZFNs recognize and bind to specific sites at opposite DNA strands; assembled *FokI* dimer specifically cleaves target DNA. B. TALENs – two discrete TALENs recognize and bind to specific sites at opposite DNA strands; assembled *FokI* dimer specifically cleaves target DNA. C. In the CRISPR-Cas9 system, the DNA site is recognized by base complementarity between the genomic DNA and sgRNA, associated with tracrRNA, and loaded into Cas9 nuclease, which performs DNA cleavage.

Zinc-finger nucleases (ZFNs)

- ZFNs are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain.
- Zinc finger domains can be engineered to target specific desired DNA sequences and this enables zinc-finger nucleases to target unique sequences within complex genomes. By taking advantage of endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms.
- DNA-binding domain: The DNA-binding domains of individual ZFNs typically contain between three and six individual zinc finger repeats and can each recognize between 9 and 18 basepairs.
- DNA-cleavage domain: The non-specific cleavage domain from the type IIs restriction endonuclease FokI is typically used as the cleavage domain in ZFNs. This cleavage domain must dimerize in order to cleave DNA and thus a pair of ZFNs are required to target non-palindromic DNA sites.
- Applications: Zinc finger nucleases are useful to manipulate the genomes of many plants and animals





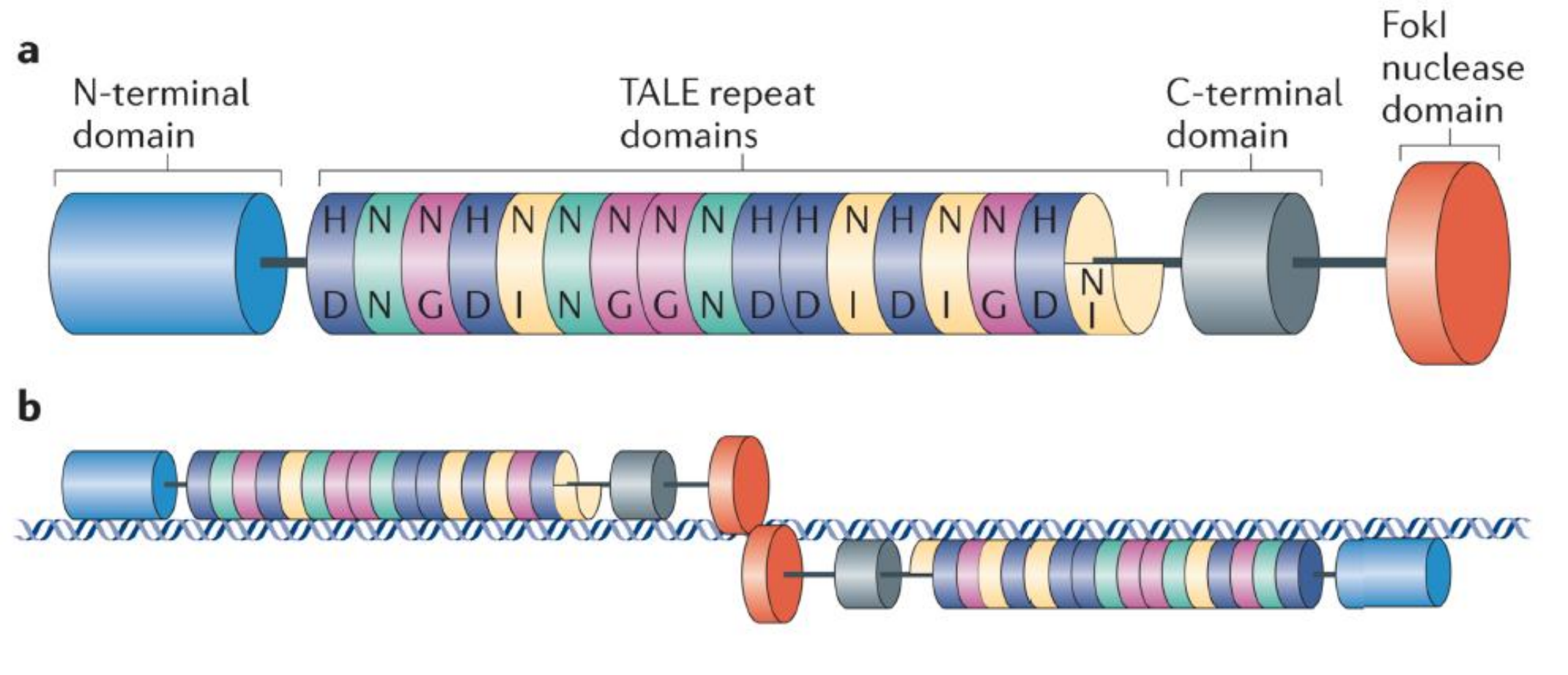
Repair outcomes of a genomic double strand break, illustrated for the case of ZFN cleavage. A pair of three-finger ZFNs is shown at the top in association with a target gene (open box). If a homologous donor DNA is provided (solid box, left), repair can proceed by homologous recombination using the donor as template. The amount of donor sequence ultimately incorporated will typically decline with distance from the original break, as illustrated by the shading. Alternatively, the break can be repaired by non-homologous end joining, leading to mutations at the cleavage site. These may be deletions, insertions, and base substitutions, usually quite localized, but sometimes extending away from the break.

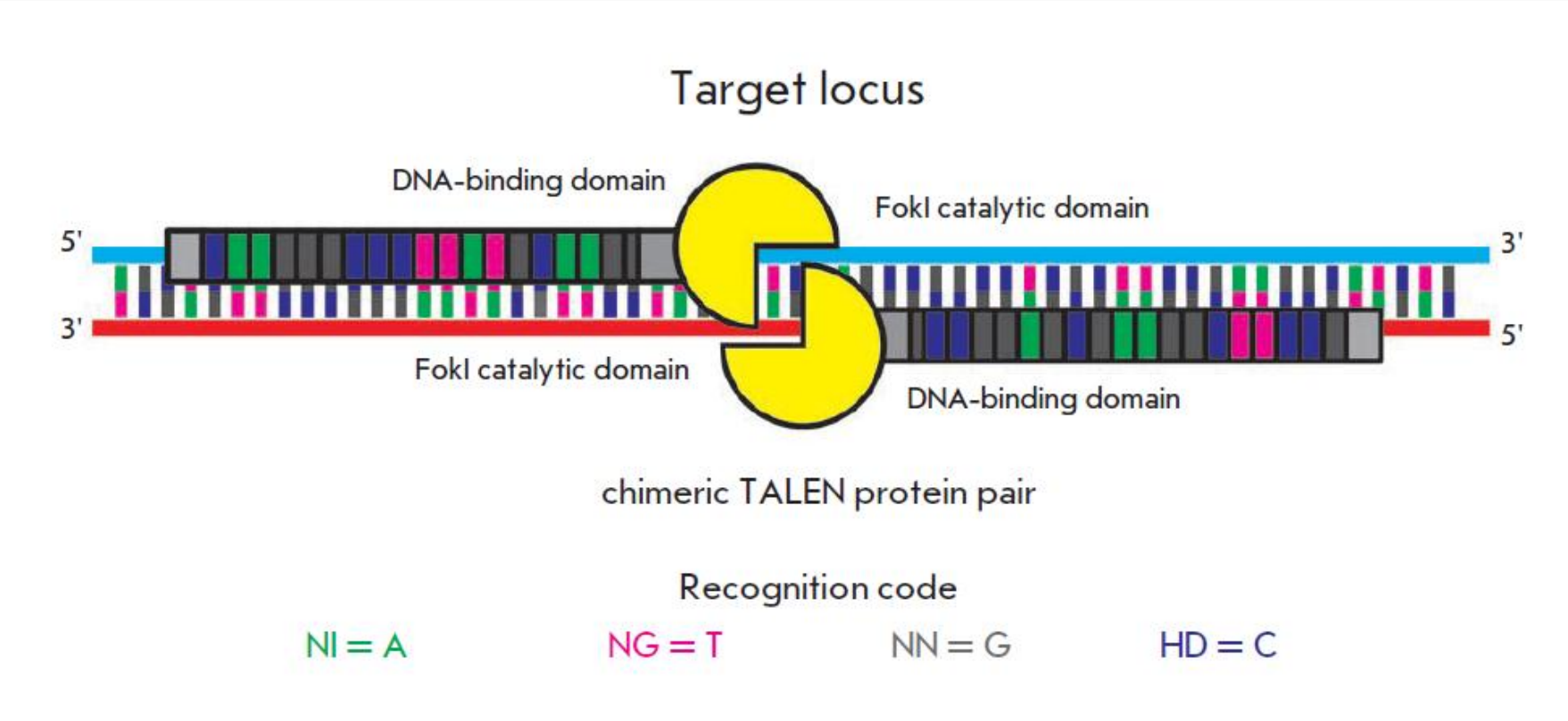
Transcription activator-like effector nucleases (TALENs)

- *Xanthomonas* bacteria are pathogens of crop plants, such as rice, pepper, and tomato; and they cause significant economic damage to agriculture, which was the motivate for their thorough study. The bacteria were found to secrete effector proteins (transcription activator-like effectors, TALEs) to the cytoplasm of plant cells, which affect processes in the plant cell and increase its susceptibility to the pathogen. Further investigation of the effector protein action mechanisms revealed that they are capable of DNA binding and activating the expression of their target genes via mimicking the eukaryotic transcription factors.
- The newly-developed transcription activator-like effector nucleases (TALENs) are restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (a nuclease which cuts DNA strands).
- The restriction enzymes can be introduced into cells, for use in gene editing or for genome editing *in situ*, a technique known as genome editing with engineered nucleases.

- **TALE DNA-binding domain:** The DNA binding domain contains a repeated highly conserved 33–34 amino acid sequence with divergent 12th and 13th amino acids. These two positions, referred to as the Repeat Variable Diresidue (RVD), are highly variable and show a strong correlation with specific nucleotide recognition. This straightforward relationship between amino acid sequence and DNA recognition has allowed for the engineering of specific DNA-binding domains by selecting a combination of repeat segments containing the appropriate RVDs.
- **DNA cleavage domain:** The non-specific DNA cleavage domain from the end of the FokI endonuclease can be used to construct hybrid nucleases that are active in a yeast assay. These reagents are also active in plant cells and in animal cells.
- The FokI domain functions as a dimer, requiring two constructs with unique DNA binding domains for sites in the target genome with proper orientation and spacing. Both the number of amino acid residues between the TALE DNA binding domain and the FokI cleavage domain and the number of bases between the two individual TALEN binding sites appear to be important parameters for achieving high levels of activity.
- TALEN has been used to efficiently modify plant genomes, creating economically important food crops with favorable nutritional qualities. TALEN has also been utilized experimentally to correct the genetic errors that underlie disease. For example, it has been used *in vitro* to correct the genetic defects that cause disorders such as sickle cell disease, xeroderma pigmentosum, and epidermolysis bullosa.

Transcription activator-like effector nucleases (TALENs)





A scheme for introducing a double-strand break using chimeric TALEN proteins. One monomer of the DNA-binding protein domain recognizes one nucleotide of a target DNA sequence. Two amino acid residues in the monomer are responsible for binding. The recognition code (single-letter notation is used to designate amino acid residues) is provided. Recognition sites are located on the opposite DNA strands at a distance sufficient for dimerization of the FokI catalytic domains. Dimerized FokI introduces a double-strand break into DNA. Most studies use monomers containing RVDs such as Asn and Ile (NI), Asn and Gly (NG), two Asn (NN), and His and Asp (HD) for binding the nucleotides A, T, G, and C, respectively. Since the NN RVD can bind both G and A, a number of studies was performed to find monomers that will be more specific. It has been shown that the use of NH or NK monomers for more specific binding of guanine reduces the risk of off-target effects (Asn: Asparagine, Ile: Isoleucine, Gly: Glycine, His: Histidine, Asp: Aspartic acid, RVD: Repeat Variable Diresidue).

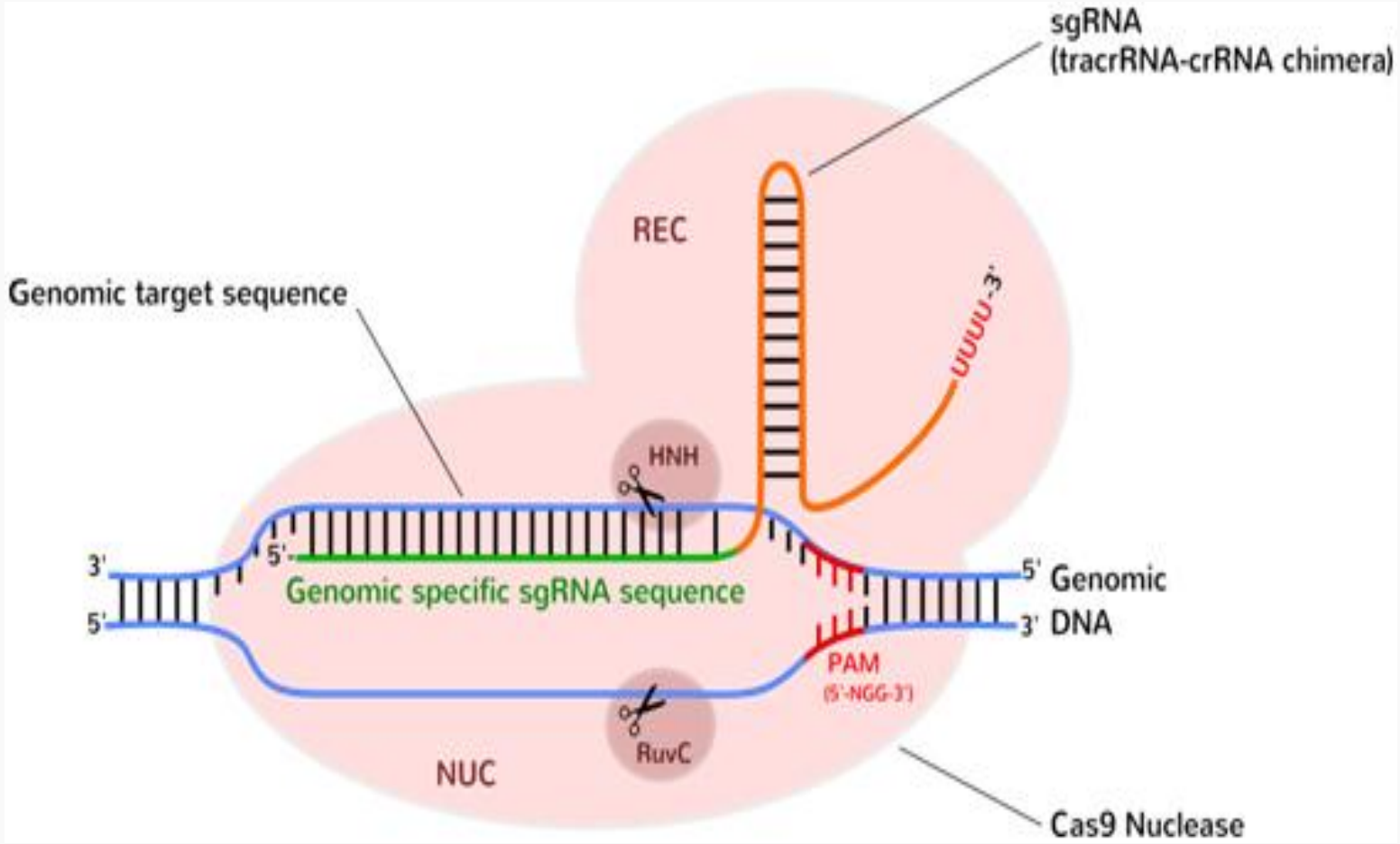
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas-9 system

- CRISPR-Cas9 is a genome editing tool that is creating a buzz in the science world. It is faster, cheaper and more accurate than previous techniques of editing DNA and has a wide range of potential applications.
- The functions of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential in adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic material.
- These repeats were initially discovered in the 1980s in *E. coli*, but their function wasn't confirmed until 2007 by Barrangou and colleagues, who demonstrated that *Streptococcus thermophilus* can acquire resistance against a bacteriophage by integrating a genome fragment of an infectious virus into its CRISPR locus.

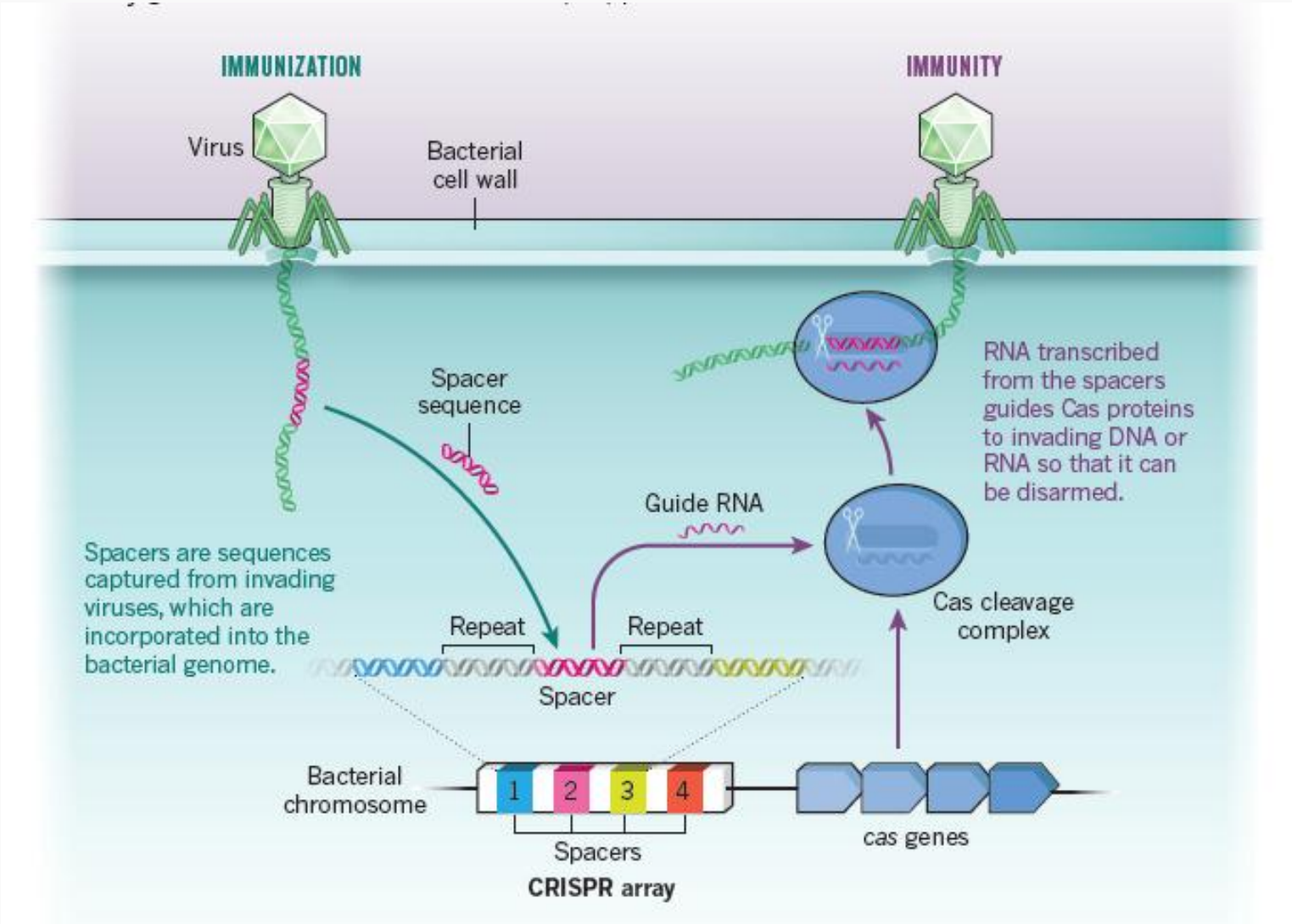
- The CRISPR-Cas9 system consists of two key molecules that introduce a change (mutation) into the DNA (Figure). These are:

  - An enzyme called Cas9. This acts as a pair of ‘molecular scissors’ that can cut the two strands of DNA at a specific location in the genome so that bits of DNA can then be added or removed.
  - A piece of RNA called guide RNA (gRNA). This consists of a small piece of pre-designed RNA sequence (about 20 bases long) located within a longer RNA scaffold. The scaffold part binds to DNA and the pre-designed sequence ‘guides’ Cas9 to the right part of the genome. This makes sure that the Cas9 enzyme cuts at the right point in the genome.

CRISPR)/Cas-9 system

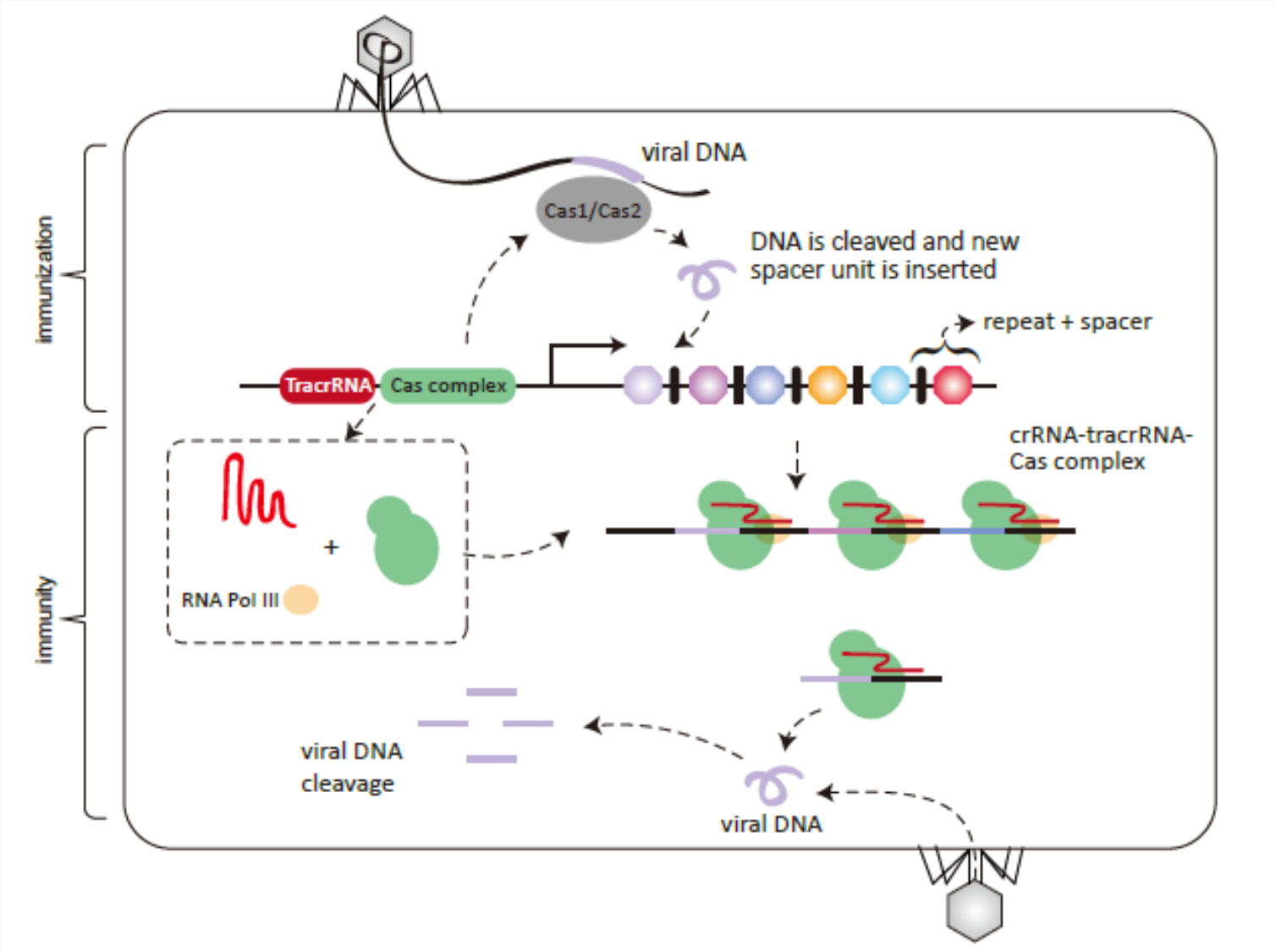


The guide RNA is designed to find and bind to a specific sequence in the DNA. The guide RNA has RNA bases that are complementary to those of the target DNA sequence in the genome. This means that, at least in theory, the guide RNA will only bind to the target sequence and no other regions of the genome. The Cas9 follows the guide RNA to the same location in the DNA sequence and makes a cut across both strands of the DNA.



About 90% of known archaea and one-third of bacteria have some form of CRISPR–Cas immunity. This is controlled by a cluster of short DNA repeats separated by ‘spacer’ sequences and a series of nearby genes that encode CRISPR-associated (Cas) proteins.

Mechanism of CRISPR-mediated immunity in bacteria



CRISPR-based immunity is composed of two main phases: immunization and immunity. In the immunization phase, Cas proteins (Cas1/Cas2) form a complex that cleaves the foreign, viral DNA (Jiang *et al*., 2015). This foreign DNA is then incorporated into the bacterial CRISPR loci as repeat-spacer units. In the immunity phase, following re-infection, the repeat spacer units are transcribed to form pre-CRISPR RNA (pre-crRNA). The Cas9 endonuclease and trans-activating crRNA (tracrRNA, which helps guide Cas9 to crRNA) then bind to the pre-crRNA. A mature crRNA-Cas9-tracrRNA complex is formed following cleavage by RNA polymerase. This crRNA-Cas9-tracrRNA complex is essential to target and destroy the foreign DNA.

Target specificity, mechanism of action, and experimental design for commonly used editing nucleases.

Feature	ZFNs	TALENs	CRISPR-Cas9
Length of recognized DNA target	9–18 bp	30–40 bp	22 bp + PAM sequence
Mechanism of target DNA recognition	DNA–protein interaction	DNA–protein interaction	DNA–RNA interaction via Watson-Crick base pairing
Mechanism of DNA cleavage and repair	Double-strand break induced by FokI	Double-strand break induced by FokI	Single- or double-strand break induced by Cas9
Design	Challenging. Available libraries of zinc finger motifs with pre-defined target specificity, but zinc finger motifs assembled in arrays can affect specificity of neighboring zinc finger motifs, making the design challenging.	Easy. TALE motifs with target specificities are well defined.	Easy. SgRNA design based on complementarity with the target DNA.
Cloning	Requires engineering linkages between zinc finger motifs.	TALEs do not require linkages. Cloning of separate TALE motifs can be done using Golden Gate assembly[5].	Expression vectors for Cas9 available. SgRNA can be delivered to cells as a DNA expression vector or directly as an RNA molecule or pre-loaded Cas9-RNA complex.

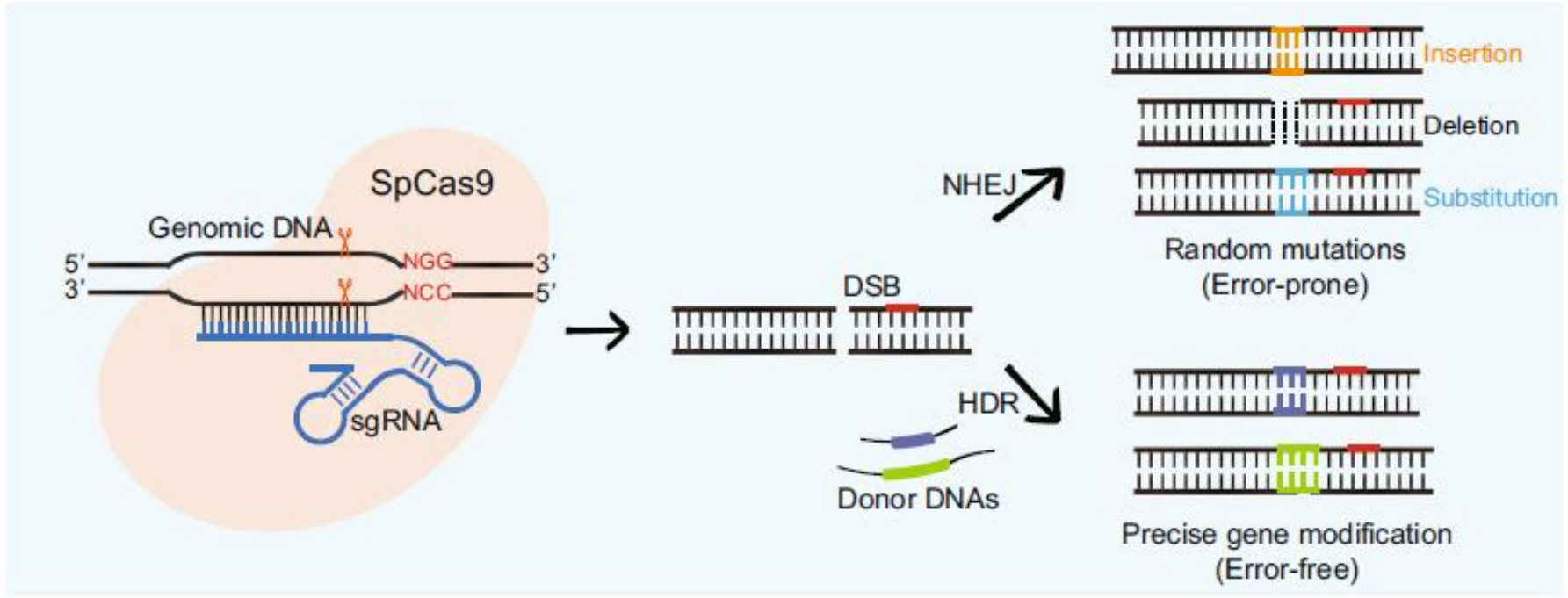


Fig. 1 The mechanism of CRISPR-Cas9-mediated genome engineering in plants. The sgRNA directs the SpCas9 protein to bind genomic DNA through a 20-nucleotide sequence and further guides it to introduce a DSB. This DSB causes random mutations when repaired by the error-prone NHEJ pathway or precise gene modification when repaired by the error-free HDR pathway. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated; DSB, double-strand break; HDR, homology-directed repair; NHEJ, non-homologous end-joining; sgRNA, single-guide RNA

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RESEARCH ARTICLE

A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

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Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

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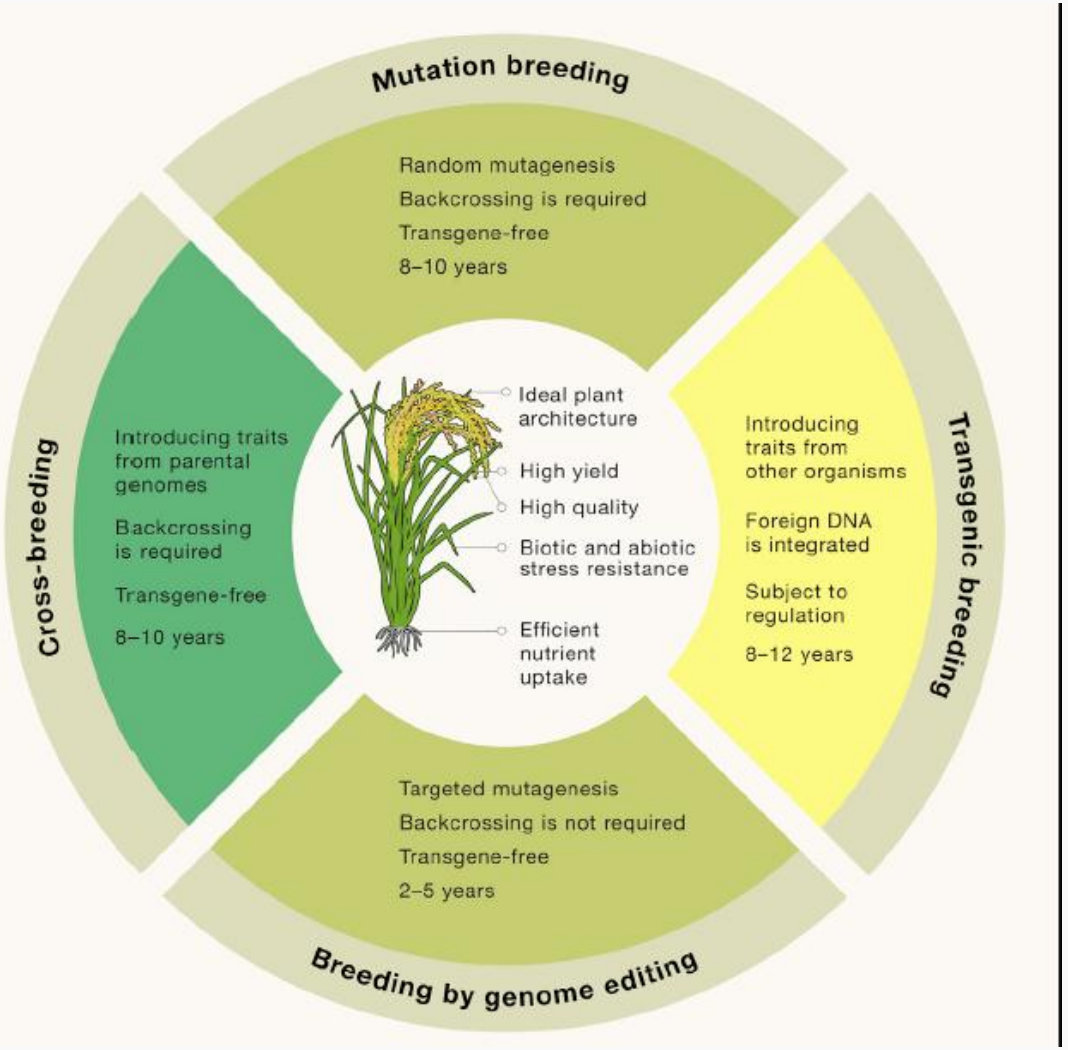
Applications



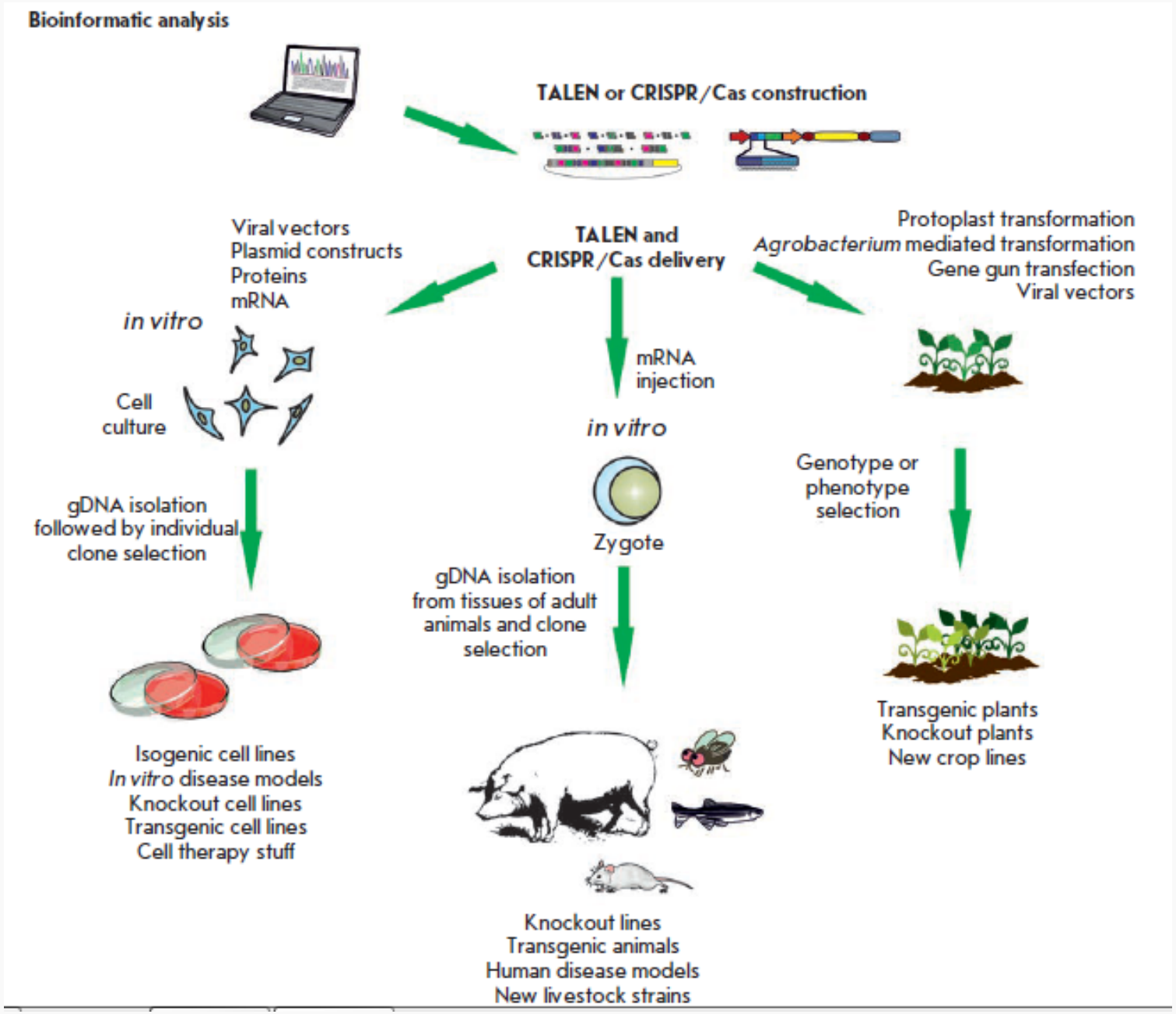
Applications as a Genome-editing and Genome Targeting Tool

- This technique already been successfully used to target important genes in many cell lines and organisms, including human, bacteria, zebrafish, *C. elegans*, plants, *Xenopus tropicalis*, yeast, *Drosophila*, monkeys, rabbits, pigs, rats and mice.
- A recent exciting development is the use of the dCas9 version of the CRISPR/Cas9 system to target protein domains for transcriptional regulation, epigenetic modification, and microscopic visualization of specific genome loci.
- CRISPR/Cas9 enables rapid genome-wide interrogation of gene function by generating large gRNA libraries for genomic screening.
- The future of CRISPR/Cas9: The rapid progress in developing Cas9 into a set of tools for cell and molecular biology research has been remarkable, likely due to the simplicity, high efficiency and versatility of the system. Of the designer nuclease systems currently available for precision genome engineering, the CRISPR/Cas system is by far the most user friendly.

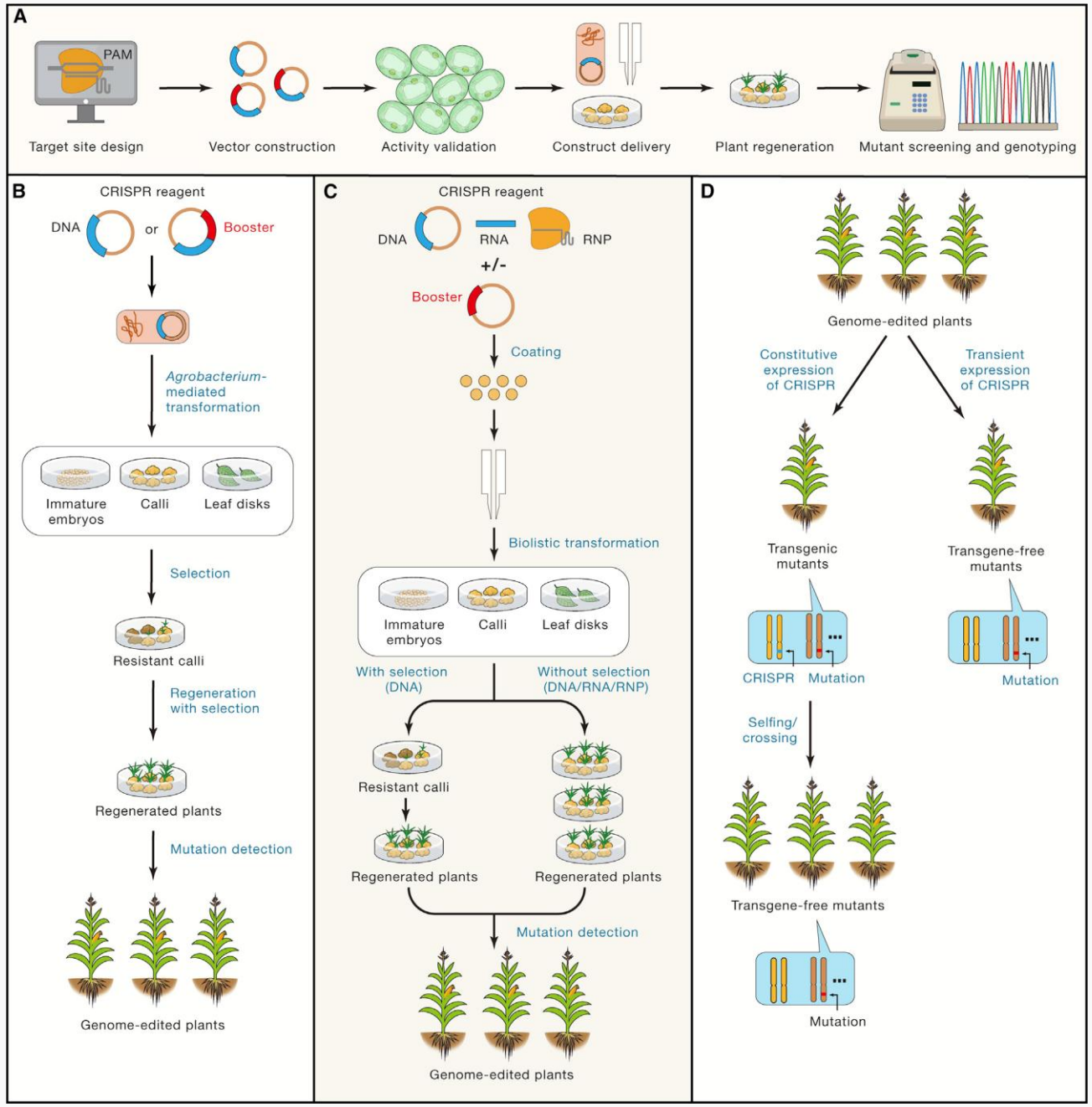
Genomic engineering using talens and crispr/cas9

The general strategy in genomic engineering using site-specific nucleases comprises four main stages (*Figure*):

1. Selection of a target nucleotide sequence in the genome;
2. Generation of a nuclease construct directed at the selected target;
3. Delivery of this construct to the cell nucleus; and
4. Analysis of produced mutations.



A general scheme of the strategy for using the TALEN and CRISPR/Cas systems in genomic engineer



General procedure for plant genome editing
(A) Schematic illustration of the six major steps in plant genome editing.
(B) Genome-edited plants generated by Agrobacterium-mediated delivery of CRISPR DNA.
(C) Conventional and transient expression methods for particle bombardment-mediated genome editing by delivery of CRISPR DNA, RNA, or RNP.
(D) Two strategies used to obtain transgene-free mutants.

Applications: Crop improvement

Crop species	Gene editor	Target gene	DNA repair type	Target trait
Rice	TALENs	OsSWEET14	NHEJ	Bacterial blight resistance
Wheat	TALENs	TaMLO	NHEJ	Powdery mildew resistance
Wheat	CRISPR/Cas9	GW2	NHEJ	Increased grain weight and protein content
Orange	CRISPR/Cas9	CsLOB1 promoter NHEJ Citrus canker resistance	NHEJ	Citrus canker resistance
Cucumber	CRISPR/Cas9	eIF4E NHEJ Virus resistance	NHEJ	Virus resistance
Rice	CRISPR/Cas9	ALS	HR	Herbicide résistance
Maize	CRISPR/Cas9	ARGOS8	HR	Drought stress tolerance

CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes

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Review

Genome engineering for crop improvement and future agriculture

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REVIEW ARTICLE OPEN

CRISPR/Cas9 – An evolving biological tool kit for cancer biology and oncology

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Development and Applications of CRISPR-Cas9 for Genome Engineering

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TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery

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