Gene Transfer & Genome-editing Technologies for Gene and Cell Therapy

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- Physical or Mechanical Methods
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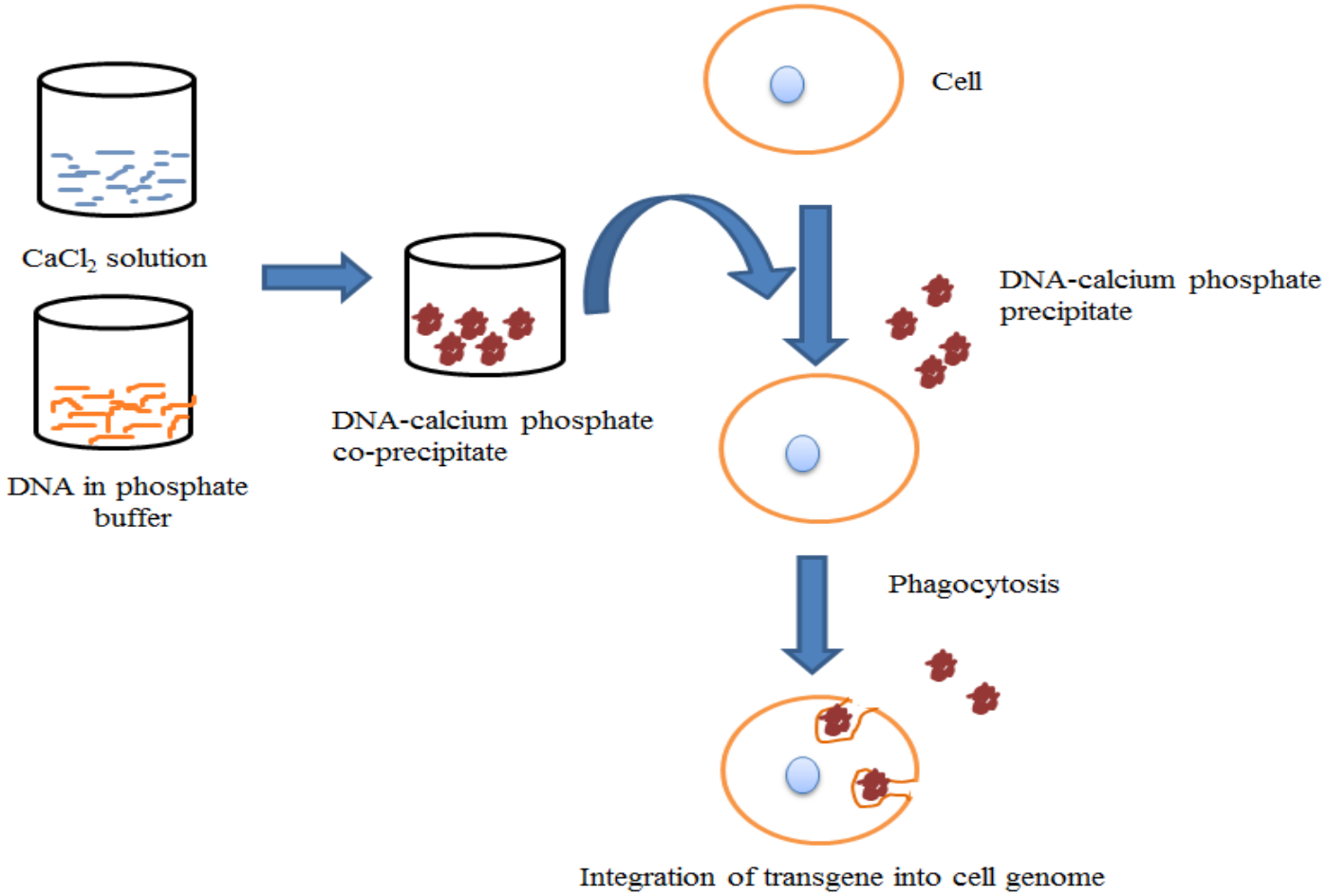
- ZFNs
- TALENs
- CRISPR/Cas9

Chemical Methods: Calcium phosphate transfection



schematic representation of transfection by Calcium Phosphate Precipitation

Chemical Methods: DEAE-Dextran (Diethylaminoethyl Dextran)

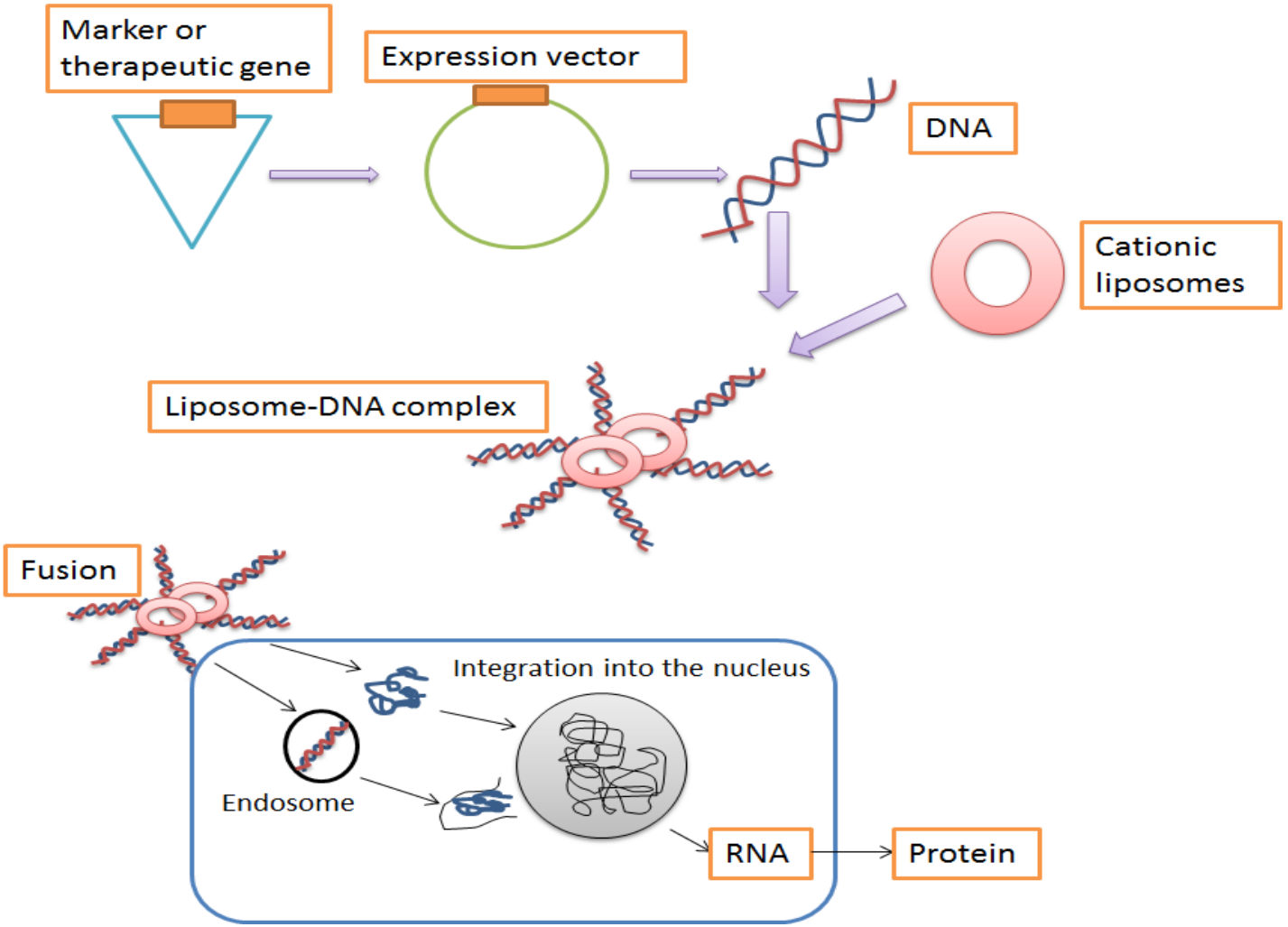
- **Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.**

- **In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex.**

- **DEAE – dextran/ DNA complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.**

- **Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.**

Chemical Methods: Lipofection



Schematic representation of liposome action in gene transfer. *(Source: Pleyer U, Dannowski H. 2002. Delivery of genes via liposomes to corneal endothelial cells. Drug News Perspect, 15(5): 283)*

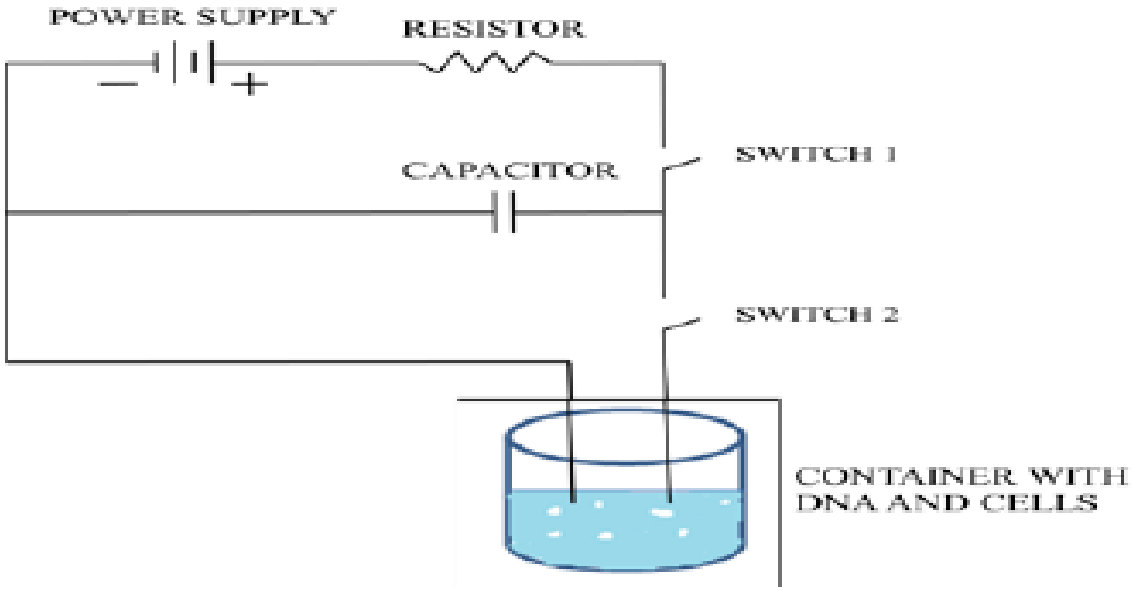
Gene Transfer Techniques: Physical or Mechanical Methods

1. Electroporation
2. Microinjection
3. Gene gun
4. Sonoporation
5. Laser induced
6. Bead transfection

Physical or Mechanical Methods: Electroporation

Applications

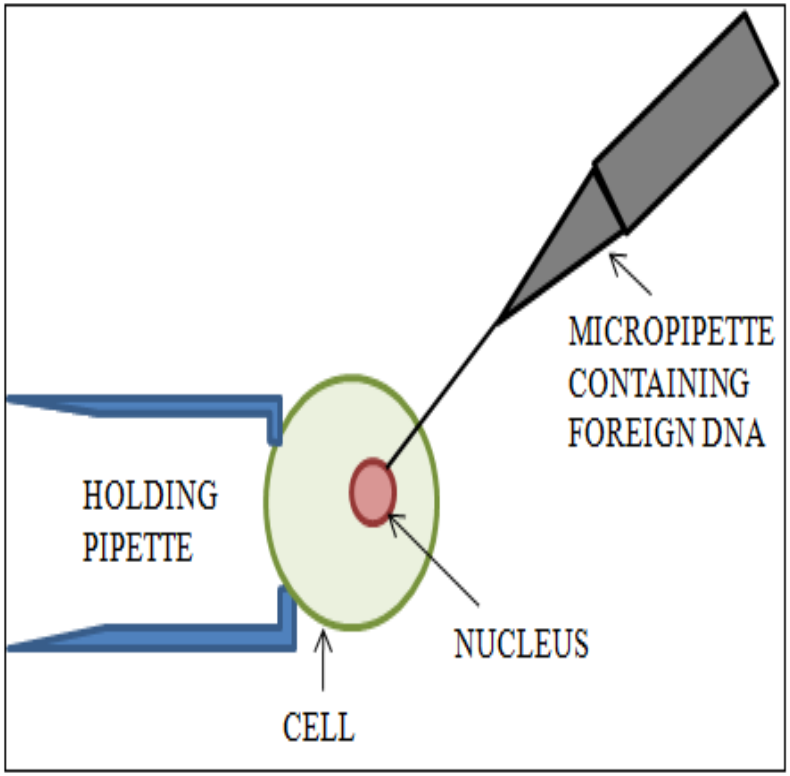
- DNA transfection or transformation
- Direct transfer of plasmids between cells
- Gene transfer to a wide range of tissues



Adapted from http://o]pbs.okstate.edu/~melcher/MG/MGW4/MG431.html

Physical or Mechanical Methods: Microinjection

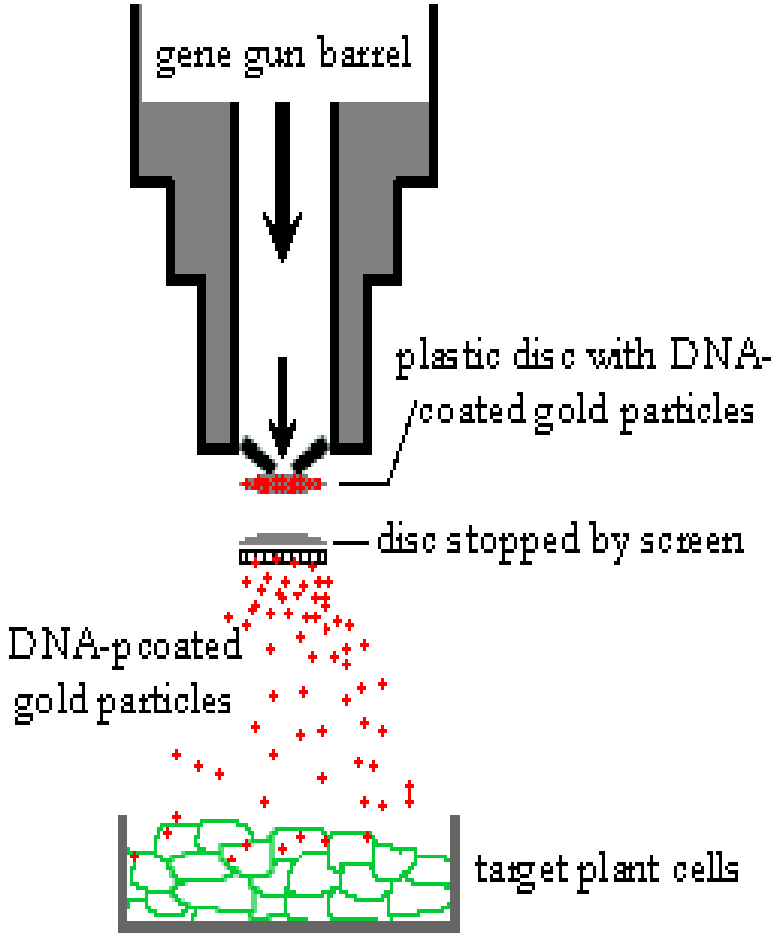
- DNA microijection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century.
- This method is widely used for gene transfection in mammals.
- It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette. The introduced DNA may lead to the over or under expression of certain genes.
- It is used to identify the characteristic function of dominant genes.



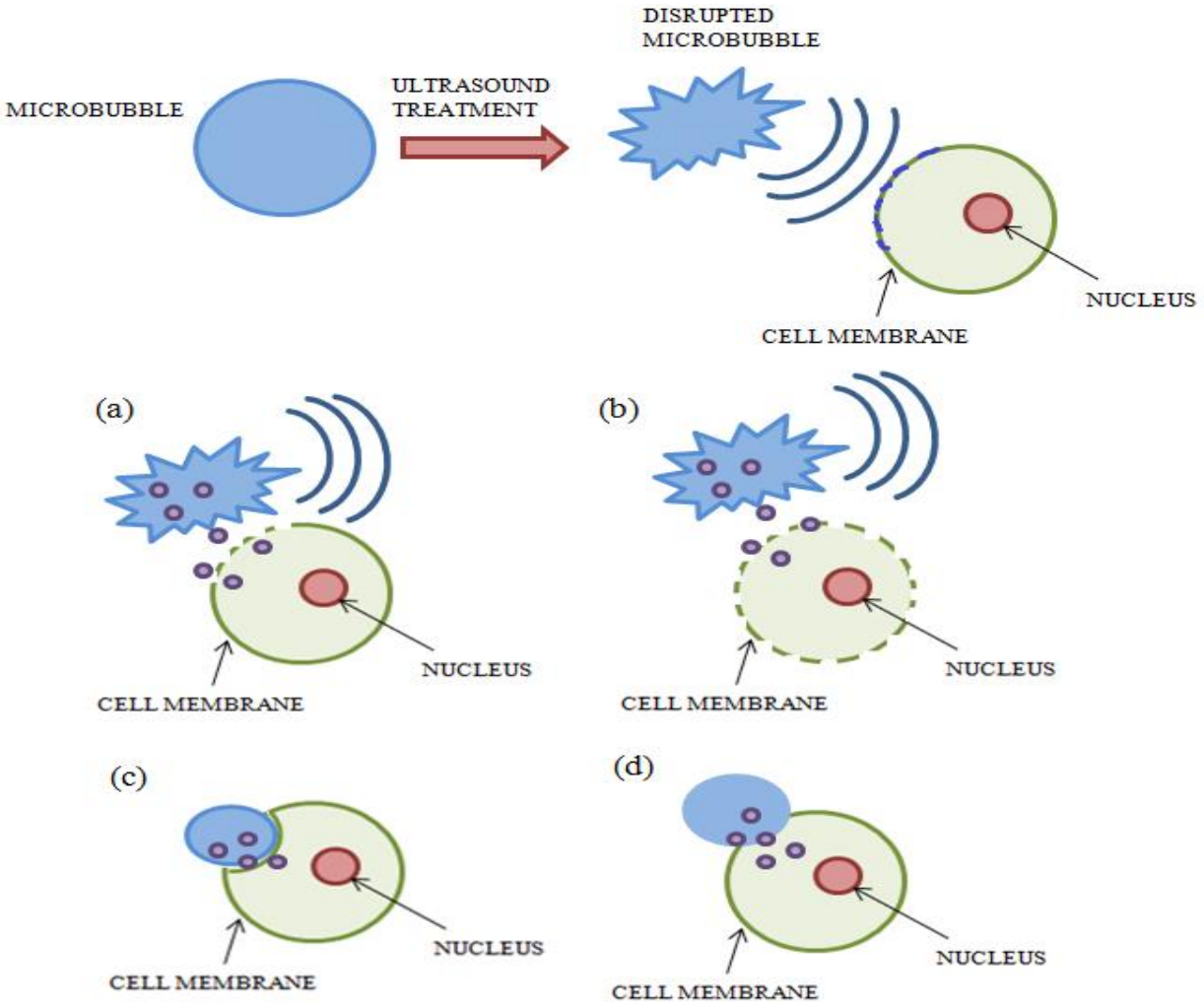
Delivery of DNA into a cell through microinjection

http://www.eplantscience.com/index_files/biotechnology/Genes%20&%20Genetic%20Engineering/Techniques%20of%20Genetic%20Engineering/biotech_microinjection.php)

“Gene gun” Technology



Physical or Mechanical Methods: Sonoporation



(Adapted fromhttp://88proof.com/synthetic_biology/blog/archives/192)

Other Physical or Mechanical Methods

Laser induced transfection

- It involves the use of a brief pulse of focused laser beam.
- In this method, DNA is mixed with the cells present in the culture and then a fine focus of laser beam is passed on the cell surface that forms a small pore sufficient for DNA uptake into the cells. The pore thus formed is transitory and repairs soon.

Bead transfection

- Bead transfection combines the principle of physically producing breaks in the cellular membrane using beads.
- In this method, the adherent cells are incubated for a brief period with glass beads in a solution containing the DNA.
- The efficiency of this rapid technique depends on:

o Concentration of DNA in a solution.

o Timing of the addition of DNA.

o Size and condition of the beads and the buffers utilized.

Gene Transfer Techniques: Biological Methods

Viral System

The vectors, biomolecular carriers of DNA transferring, are one of the most important components of the viral gene transferring system.

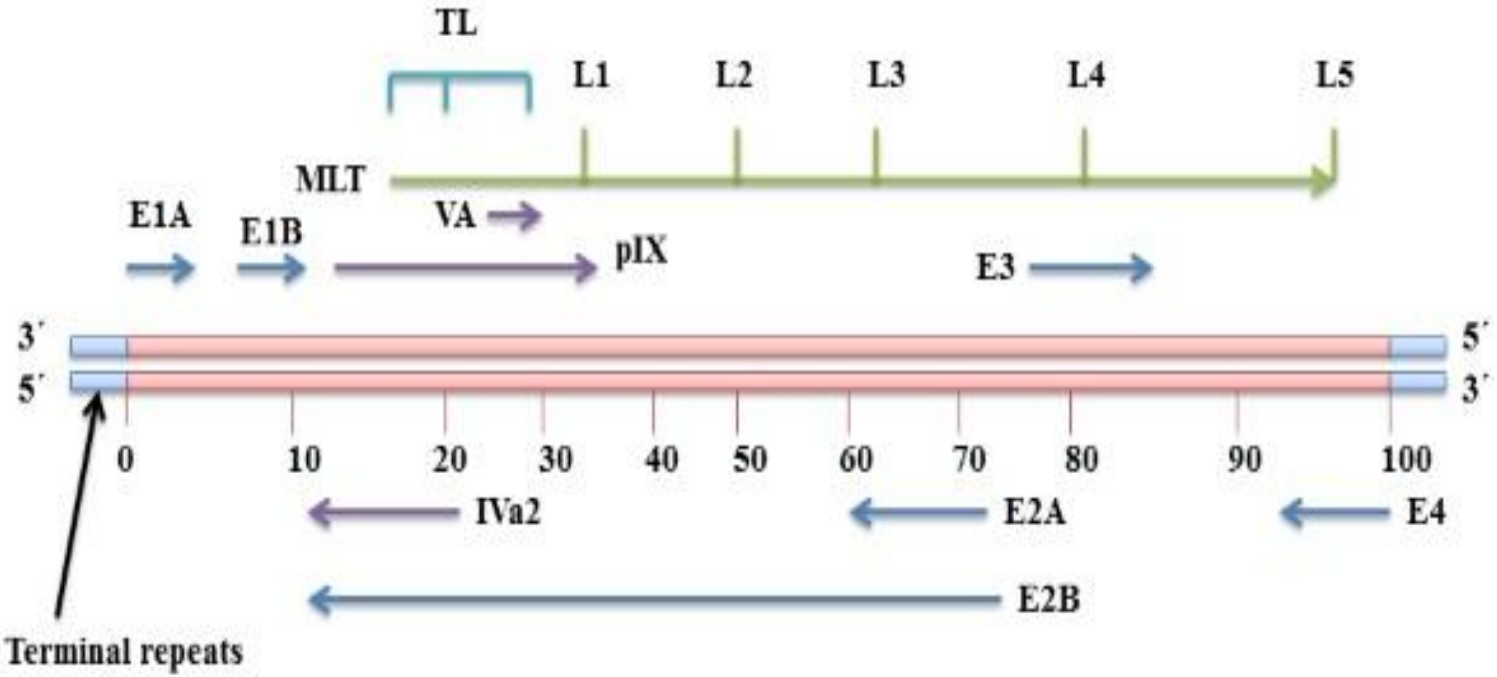
Viral Vectors:

1. Adenovirus
2. Adeno- associated virus (AAV)
3. Herpes virus
4. Retrovirus
5. Lentivirus

Adenoviruses

- Adenoviruses are medium-sized (90-100 nm), non-enveloped,
- viruses containing linear, double-stranded DNA of approximately 36 kb.
- 57 immunologically distinct types (7 subgenera) of adenoviruses cause human infections.
- They are unusually stable to physical or chemical agents and adverse pH conditions for long-term survival outside the body.
- There are six early-transcription units, most of which are essential for viral replication, and a major late transcript that encodes capsid.
- They result in transient expression in dividing cells as they do not integrate efficiently into the genome, but prolonged expression can be achieved in post-mitotic cells, like neurons.
- Adenoviruses are mostly attractive as gene therapy vectors, because the virions are taken up efficiently by cells *in vivo*. Adenovirus-derived vaccines have been used in humans with no reported side-effects.

Wild Type Adenovirus Genome



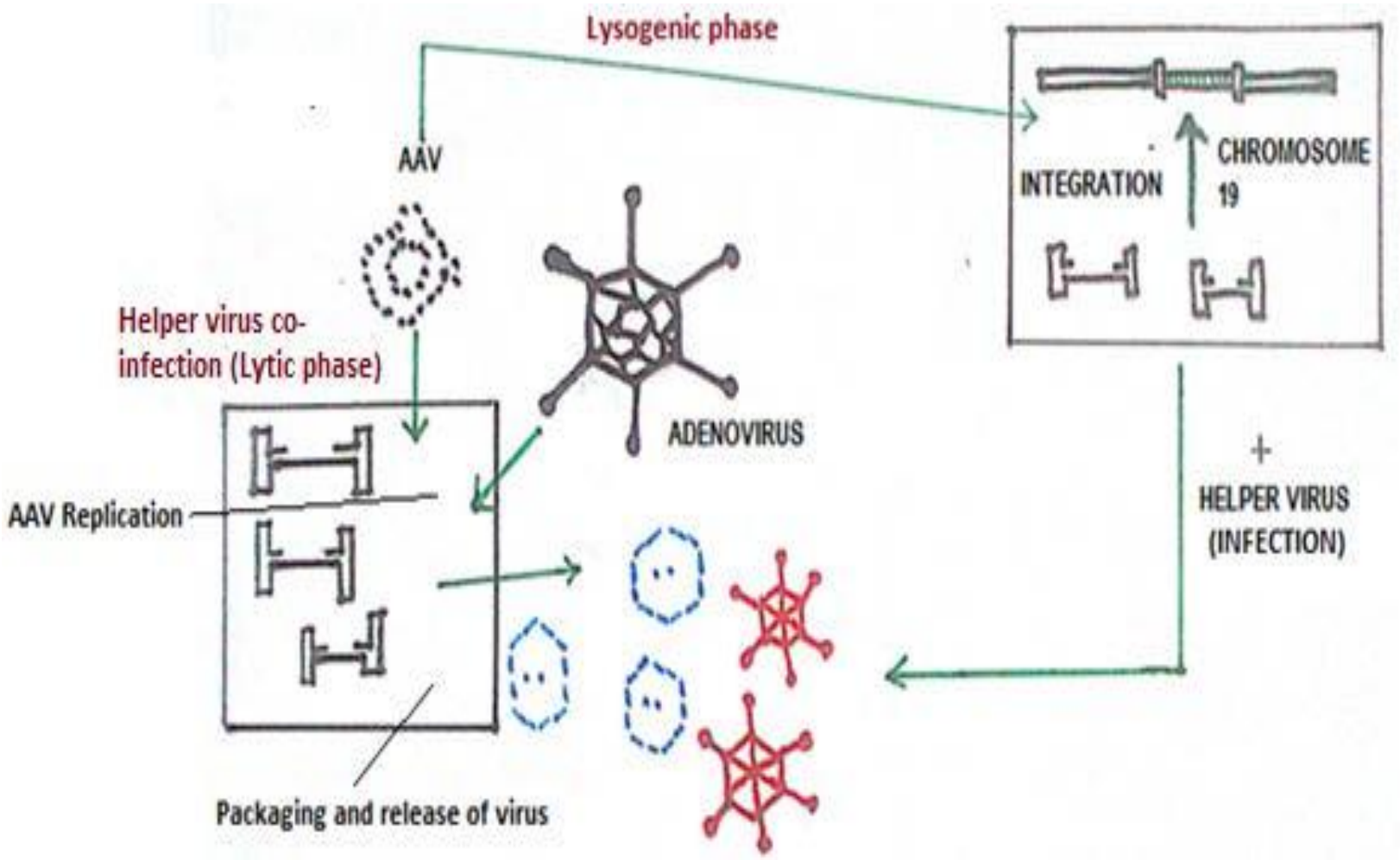
Adeno-associated virus (AAV)

- It is a small, non-enveloped virus packaging a linear single stranded DNA belonging to Parvovirus family.

- It is naturally replication defective thus requiring a helper virus (usually adenovirus or herpes virus) for productive infection.

- In human cells, the provirus integrates predominantly into a 4-kb region (AAVS1) on chromosome19.

AAV life cycle



Herpes virus vectors

- The herpes viruses are linear ds-DNA viruses of approximately 150 kb size *e.g. EBV and the HSVs.*

- Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional). With the help of two viral glycoproteins the virus binds to cells.

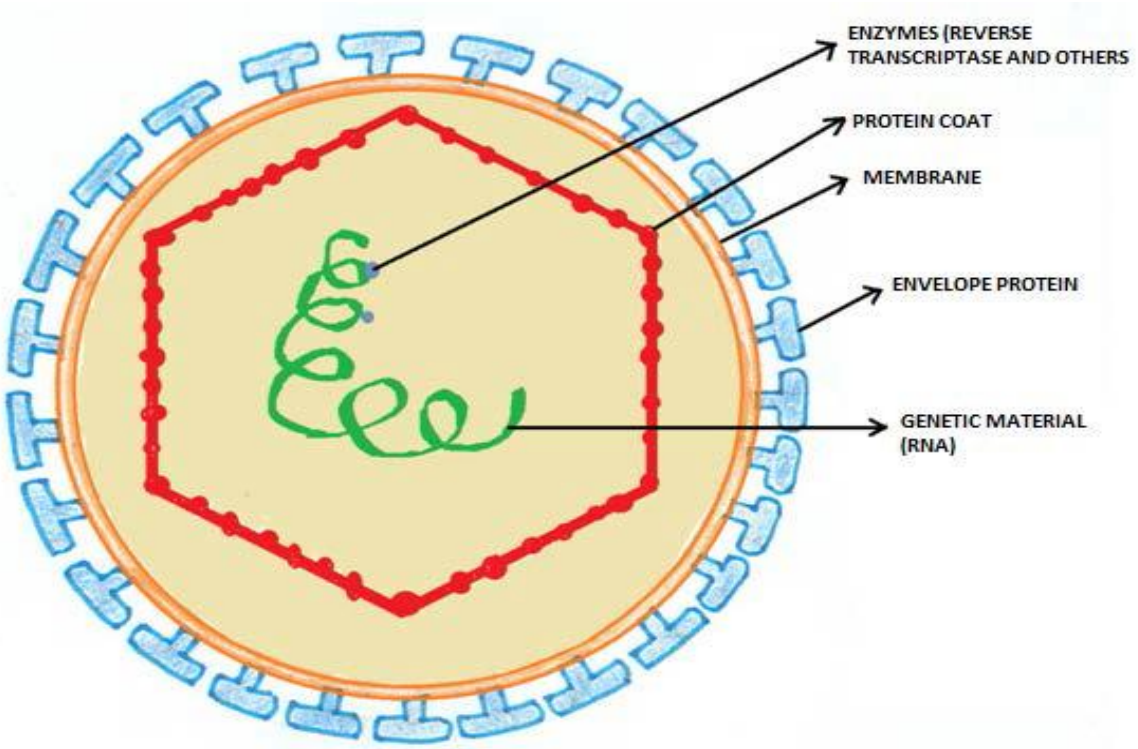
- Unlike EBV as a replicon vector HSV-I have been developed as a transduction vector for purpose of gene transfer and can efficiently transduce a wide range of cell types.

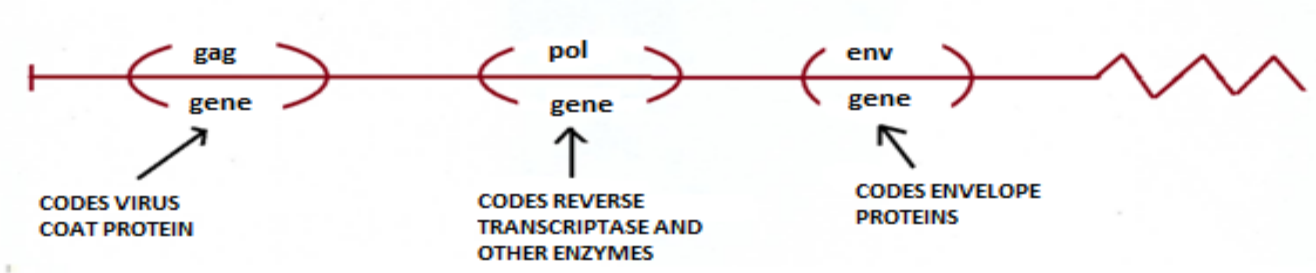
- HSV virus is remarkably neurotropic and thus HSV vectors are particularly suitable for gene therapy in the nervous system.

Retroviral vectors

- Retroviruses are RNA viruses that replicate via a ds-DNA intermediate.
- The infection cycle begins with the interaction between viral envelope and the host cell's plasma membrane, delivering the particle into the cell.
- The capsid contains two copies of the RNA genome, as well as revers transcriptase/integrase.
- After infection, the RNA genome is reverse transcribed to produce a cDNA copy, a DNA intermediate, which integrates into the genome randomly.

Structure of a Retrovirus vector





Lentivirus

- They are subclass of retroviruses.
- They are more efficient and advantageous for gene transfer than other vectors due to following reasons:

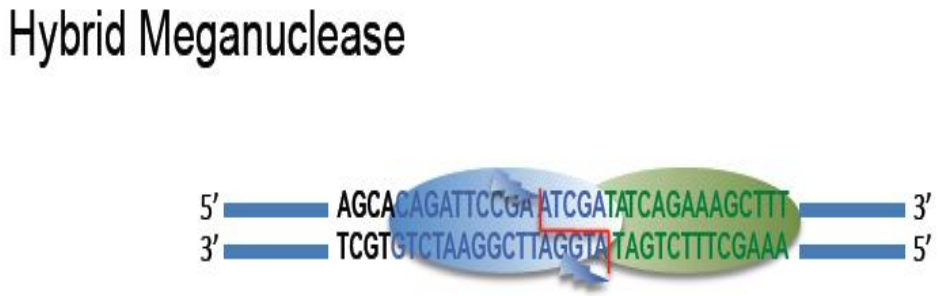
- Unlike retroviruses which can infect only dividing cells, lentiviruses can be used as vectors due to their ability to infect both **dividing and non-dividing cells.**
- **Low immunogenicity**
- **Stable, long term transgene expression**

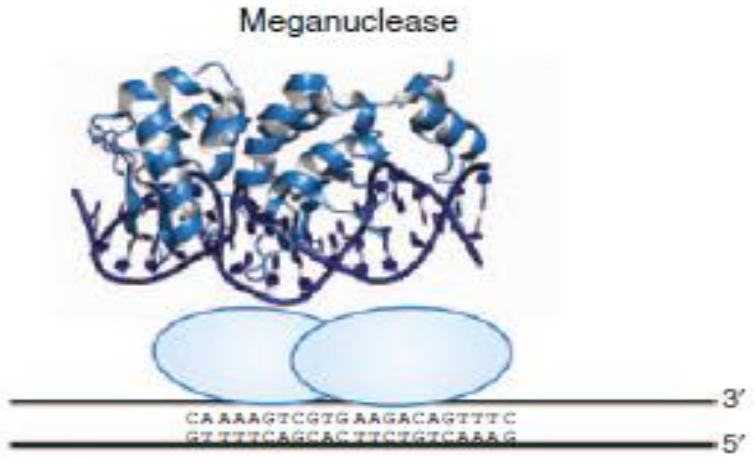
Gene Editing Methods

- Meganucleases
- ZFNs
- TALENs
- CRISPR/Cas9

Gene Editing Methods: Meganucleases

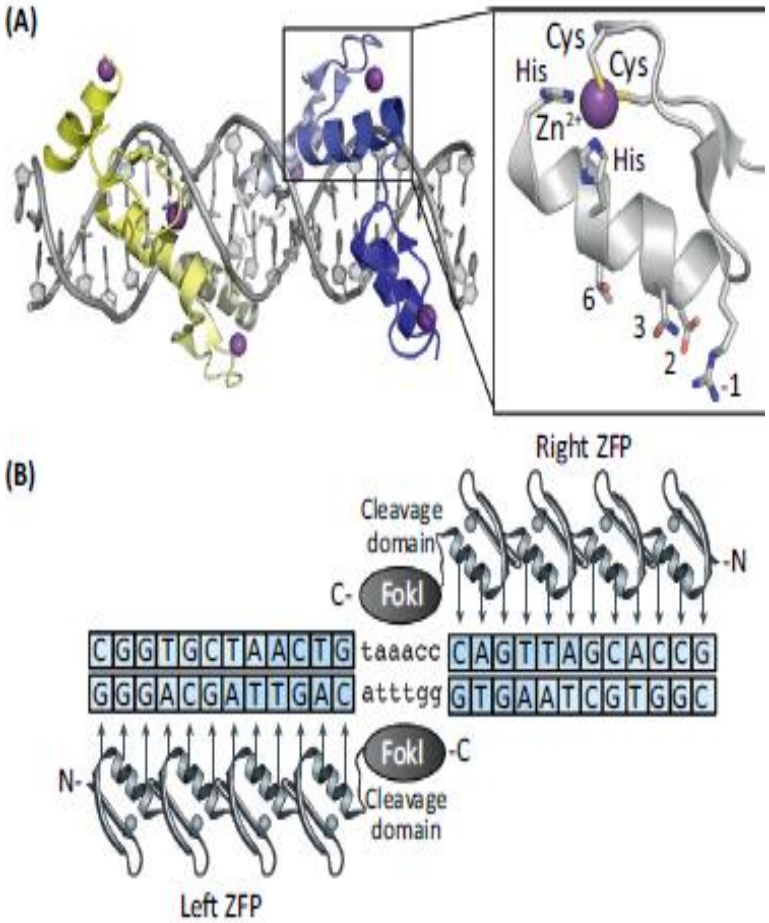
- Meganucleases are the smallest class of engineered nucleases, making them potentially amenable to all standard gene delivery methods.
- Many studies show promise for the use of meganucleases in genome editing
- The DNA-binding and cleavage domains of homing endonucleases are difficult to separate, and the relative difficulty of engineering proteins with novel specificities has traditionally limited the use of this platform
- Multiple meganuclease monomers could be readily packaged into single viral vectors to simultaneously create multiple DSBs





Gene Editing Methods: Zinc finger nucleases (ZFNs)

- ZF proteins are the one of most abundant class of transcription factors in human.
- The Cys_2-His_2 zinc finger domain is one of the most common DNA binding domain in the human genome.
- In the presence of Zn atom, the zinc finger domain forms a compact ββα structure.
- The α- helical protein of each finger making contact with 3 or 4 bp in the major groove of the DNA.
- In ZFNs a three finger protein binds a 9bp target site.
- In ZFNs the DNA binding domain and the cleavage domain of the Fok1 restriction endonuclease function independently of each other.
- Because of the FoK1 nuclease function as a dimer, two ZFNs binding opposite strand of DNA are required for induction of a DSB.
- ZFNs- induced DSBs could be used to modify the genome through either NHEJ or HDR.
- This technology has been used to successfully modify genes in human somatic and pluripotent stem cells.



TALENs - transcription activator-like effector nucleases

- Like ZFNs, TALEs were fused to the catalytic domain of the FokI endonuclease and shown to function as dimmers to cleave their intended DNA target site.

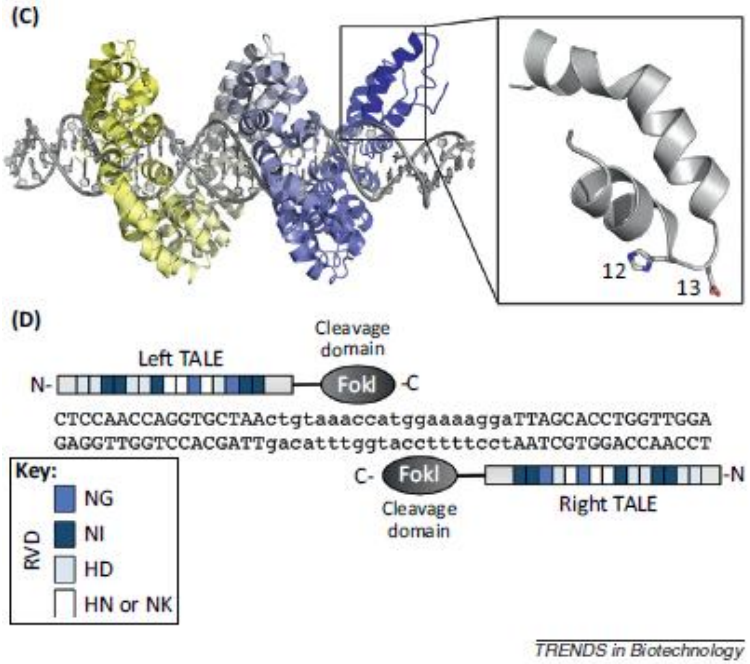
- Also similar to ZFNs, TALENs have been shown to efficiently induce both NHEJ and HDR in human somatic and pluripotent stem cells.

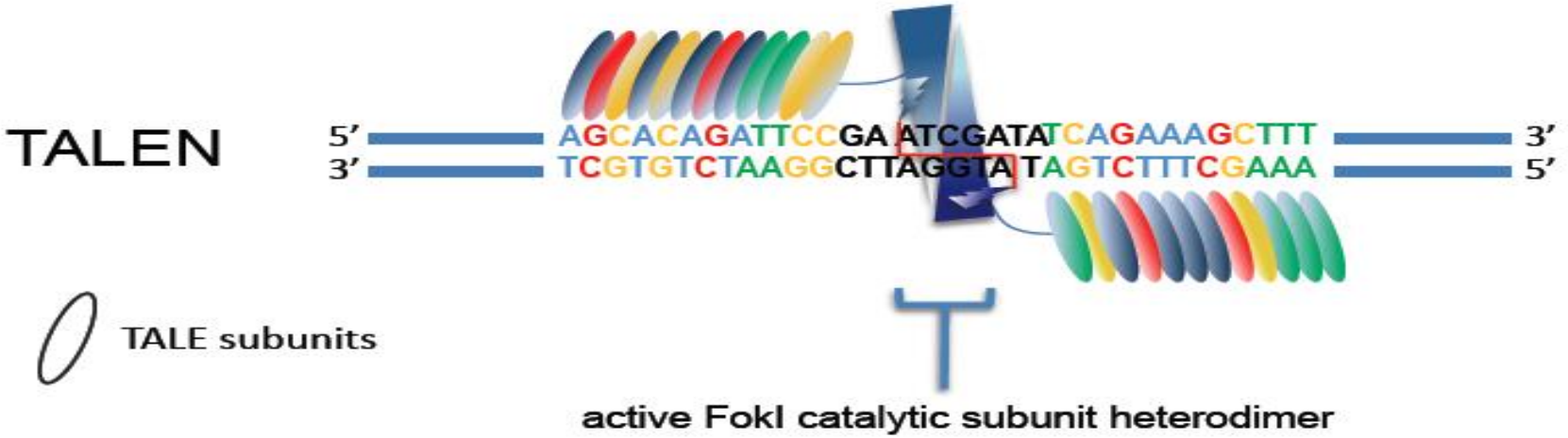
- The large size and repetitive nature of TALE arrays presents a hurdle for in vivo delivery of these proteins.

- As opposed to a 30 amino acid zinc finger, which binds three bases of DNA, TALENs require 34 amino acids to specify a single base pair and this size difference can prohibit delivery of both TALEN monomers in a single viral vector with limited packaging capacity.

- TALENs delivered by lentivirus have been shown to be susceptible to re-arrangements, although this phenomenon may be mitigated by codon diversification between the repeats.

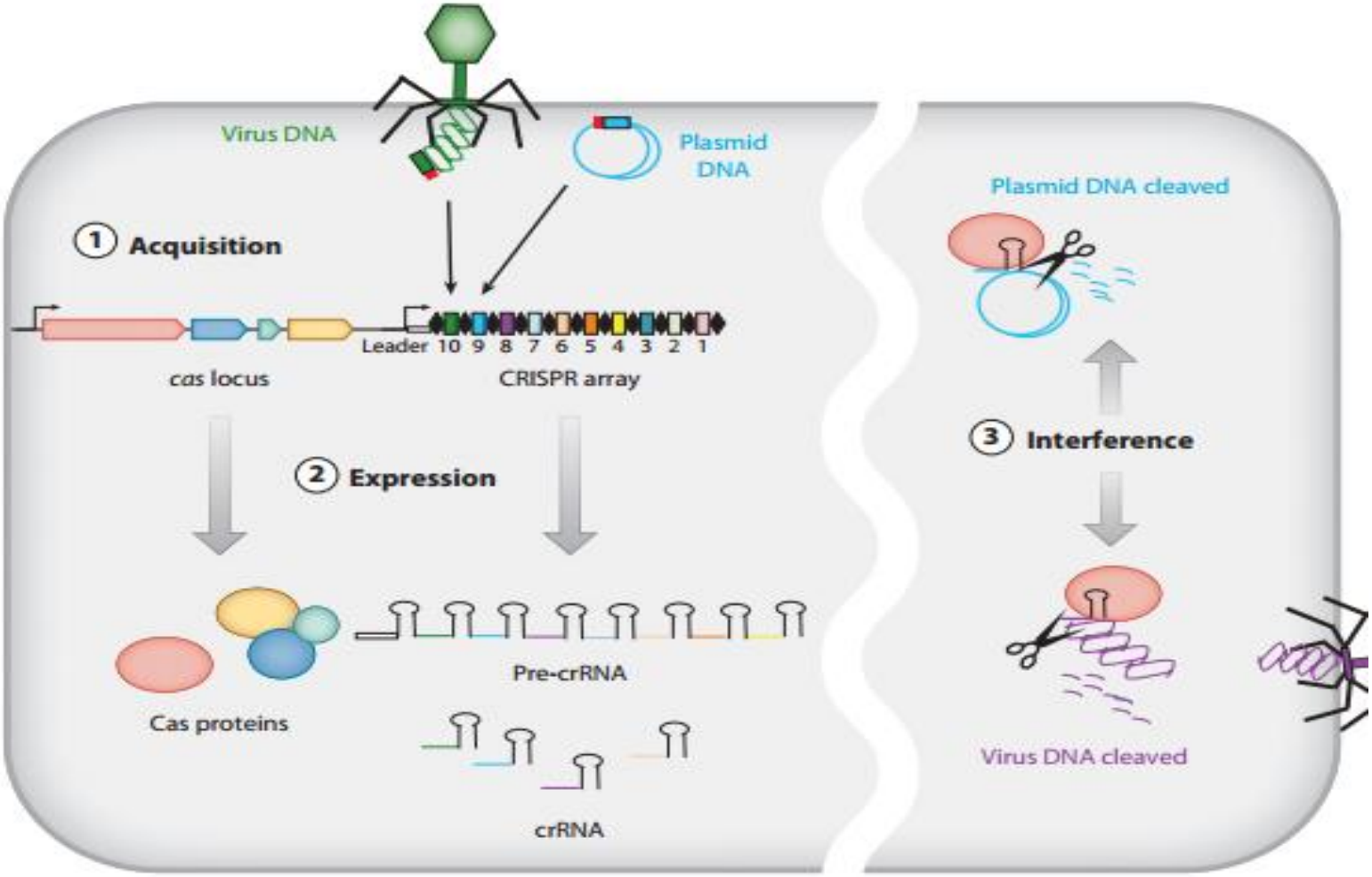
- Adenoviral systems have also been used to successfully deliver TALENs.


(C)
12
13
(D)
Left TALE
Cleavage domain
N-
FokI
-C
CTCCAACCAGGTGCTAActgtaaaccatggaaaaggaTTAGCACCTGGTTGGA
GAGGTTGGTCCACGATTgacatttggtacctttcctAATCGTGGACCAACCT
Key:
RVD
NG
NI
HD
HN or NK
C-
FokI
-N
Cleavage domain
Right TALE
TRENDS in Biotechnology


TALEN
5′
3′
AGCACAGATTCCGA ATCGATA TCAGAAAGCTTT
TCGTGTCTAAGGCTTAGGTA TAGTCTTTCGAAA
3′
5′
TALE subunits
active FokI catalytic subunit heterodimer

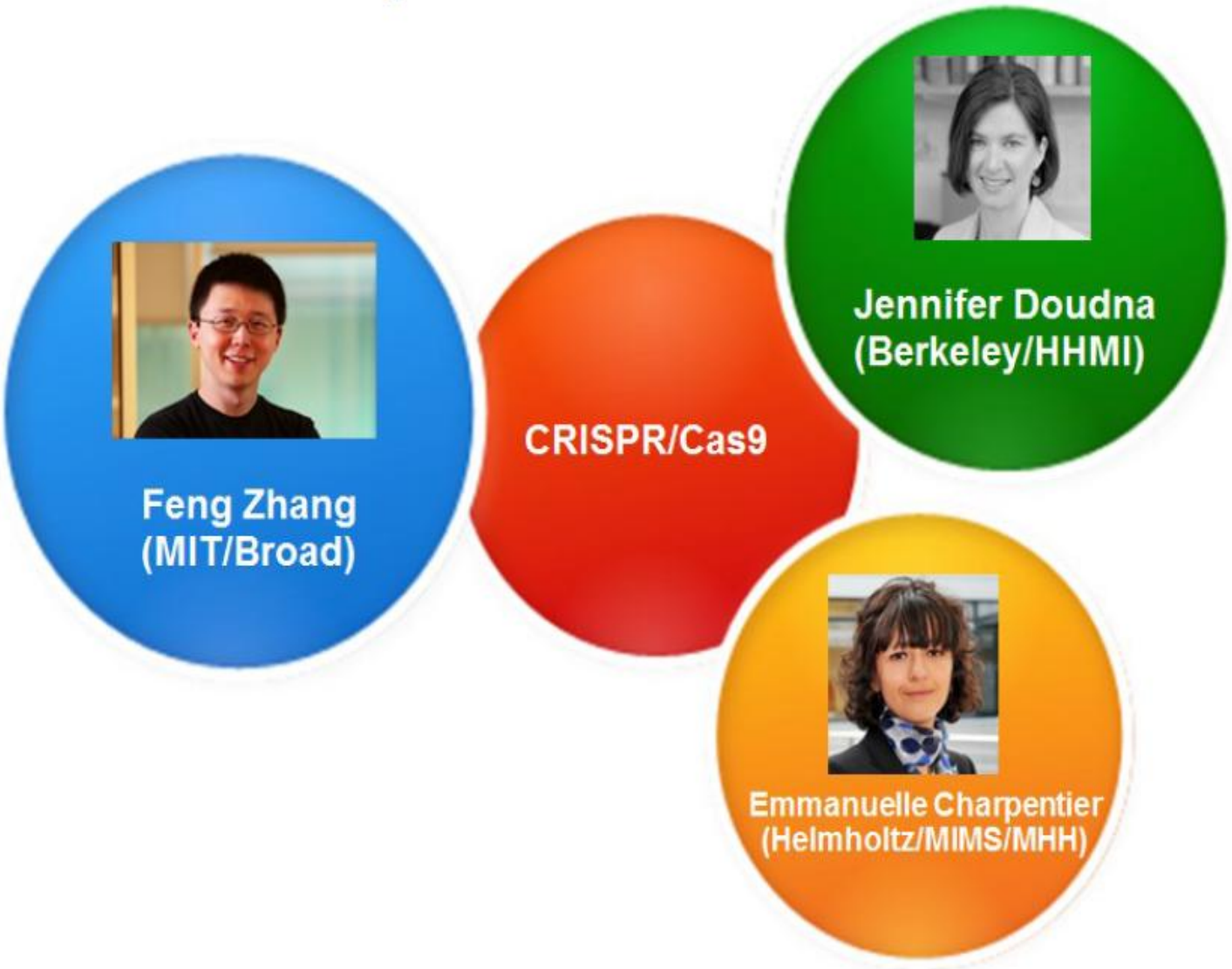
CRISPR/Cas

Clustered Regularly Interspaced Short Palindromic Repeats



Figures: Bhaya et al., Annu. Rev. Genet. 2011. 45:273-97 and Horvath: Science (2010) Vol. 327; 167-170: CRISPR/Cas, the Immune System of Bacteria and Archaea Text: Singh et al: A Mouse Geneticist's Practical Guide to CRISPR Applications; Genetics. 2015 Jan; 199(1): 1–15.

Main Actors in CRISPR/CAS9 patent war*



Timeline

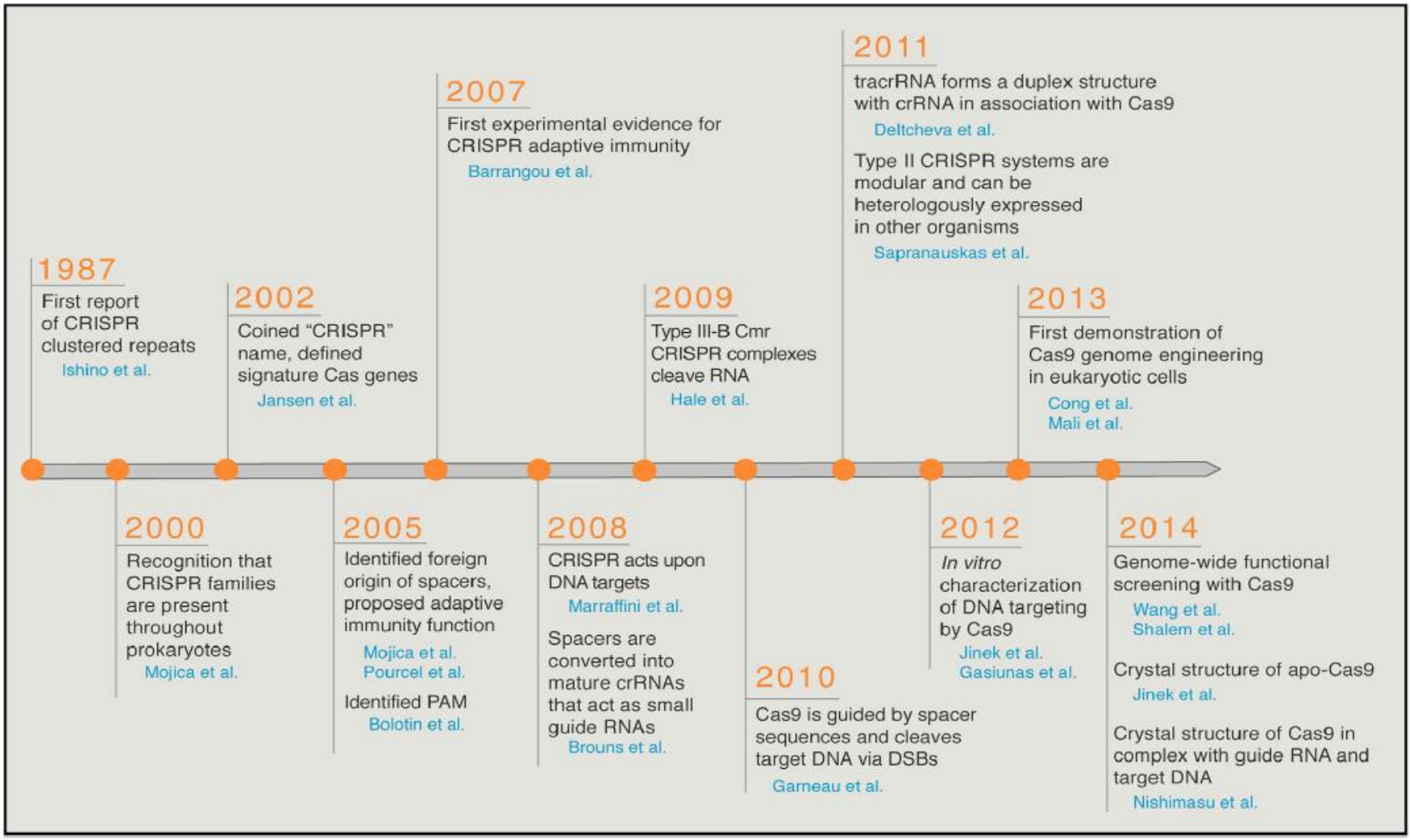
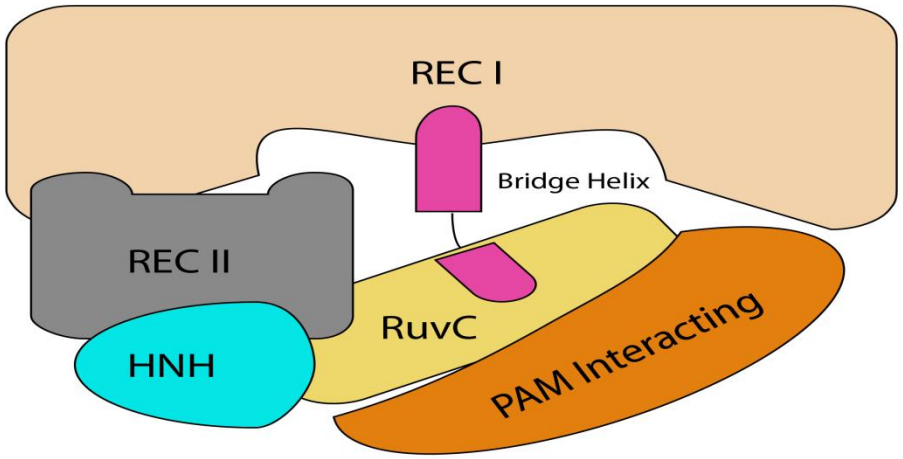
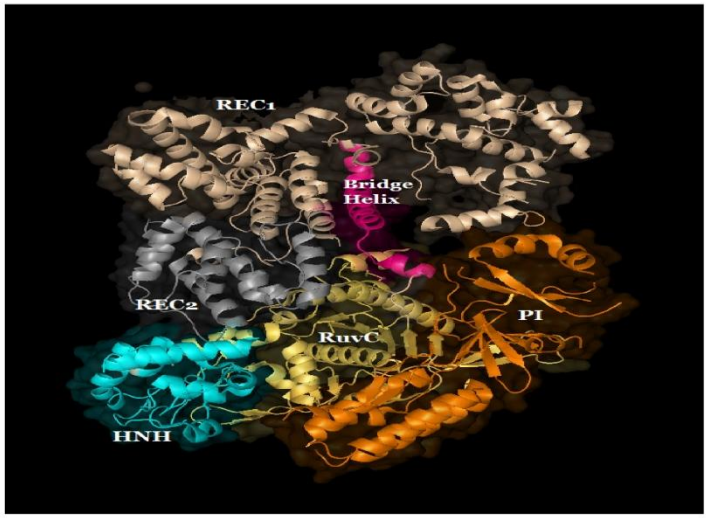
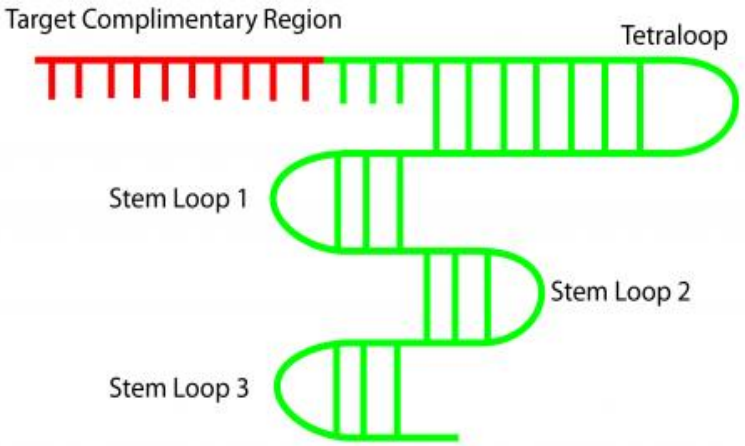
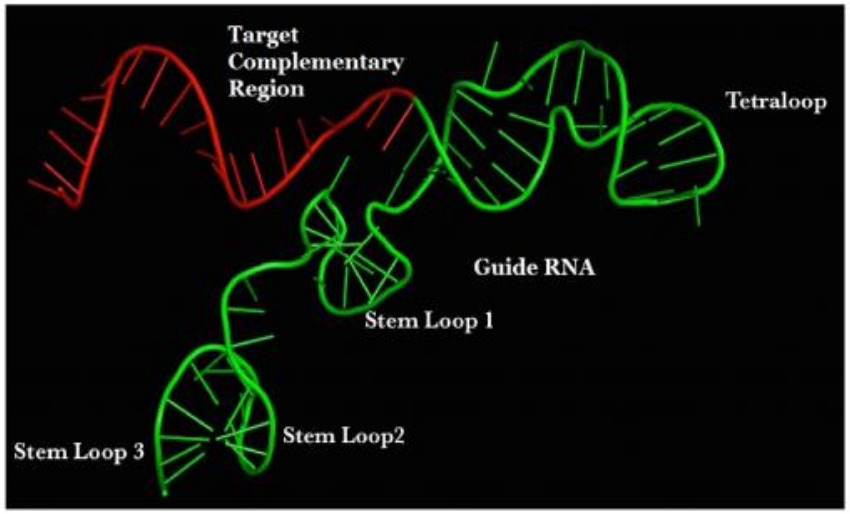


Figure: Hsu, Lander, Zhang: Development and Applications of CRISPR-Cas9 for Genome Engineering; Cell 157, June 5, 2014



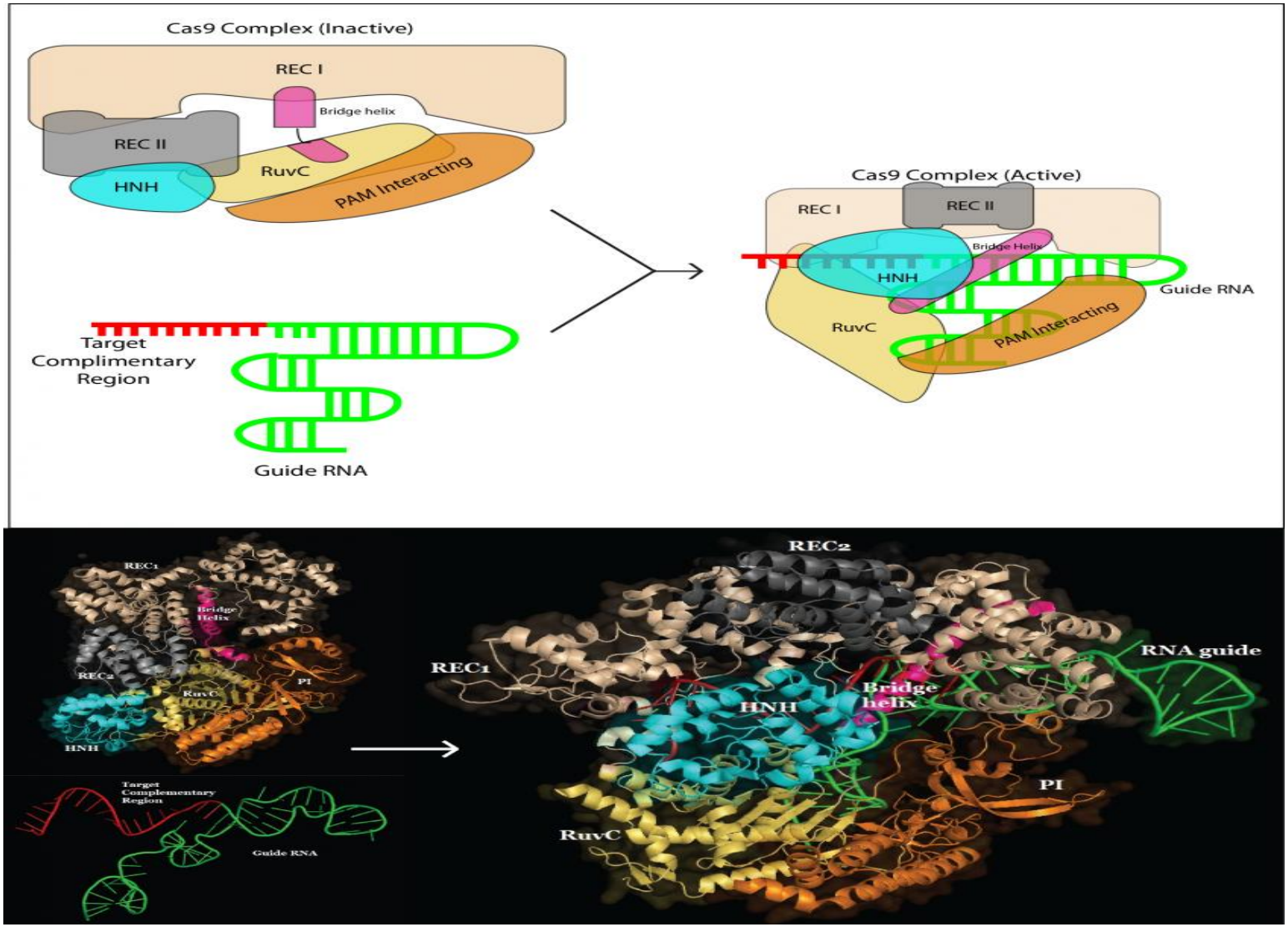




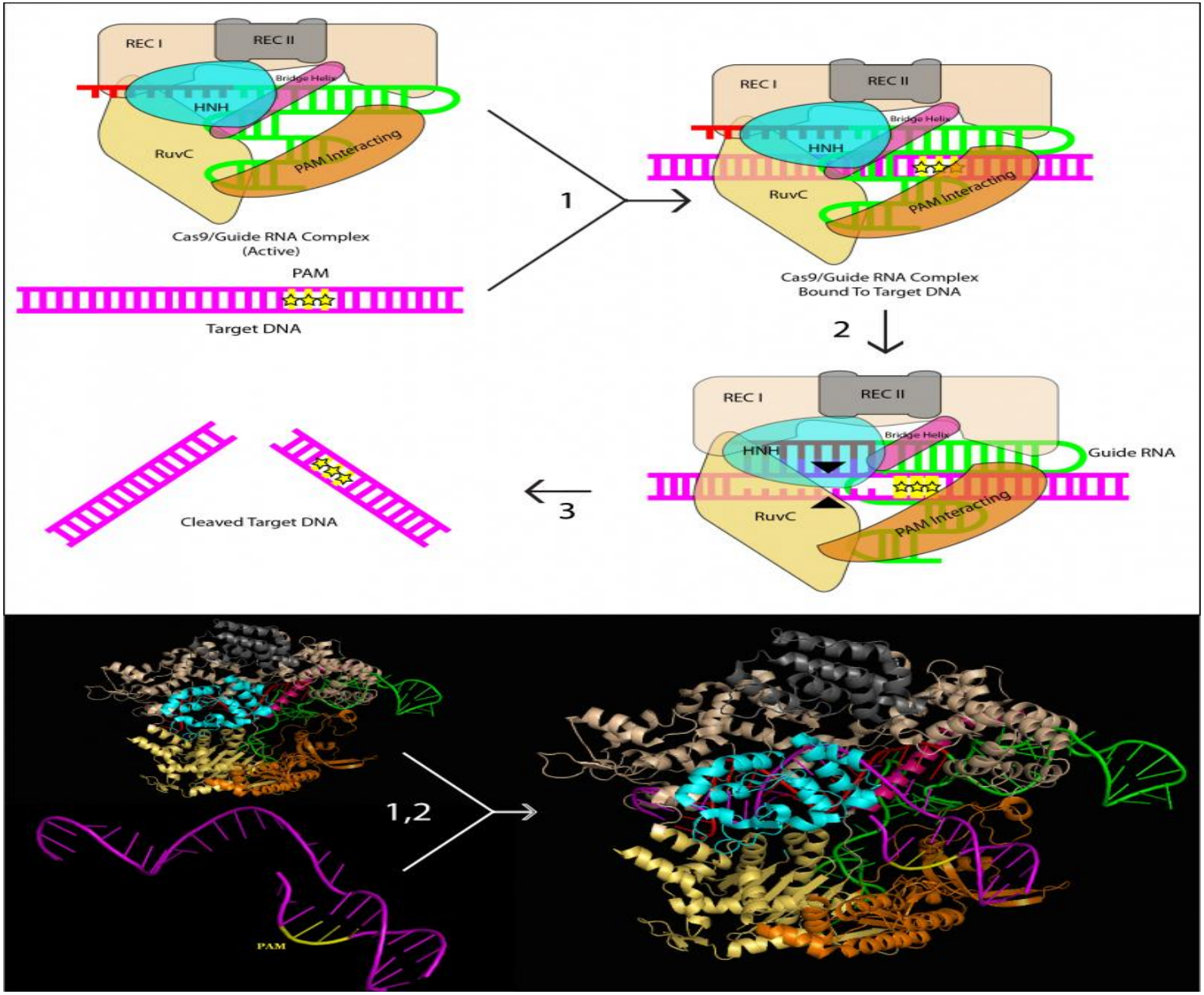


Nature. 2014 Sep 25;513(7519):569-73. doi: 10.1038/nature13579. Epub 2014 Jul 27

Activation of Cas9 protein by guide RNA binding

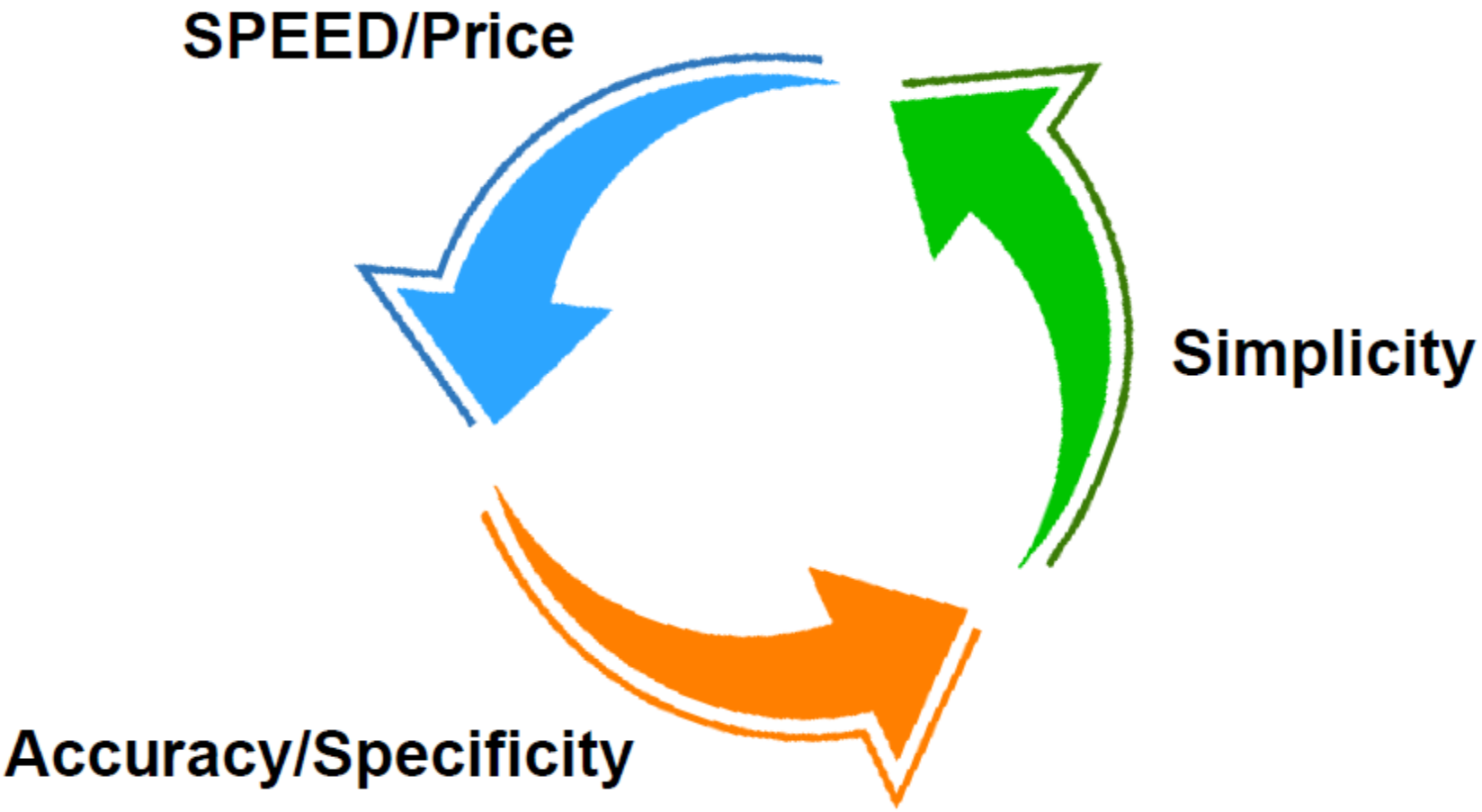


Nature. 2014 Sep 25;513(7519):569-73. doi: 10.1038/nature13579. Epub 2014 Jul 27



Nature. 2014 Sep 25;513(7519):569-73. doi: 10.1038/nature13579. Epub 2014 Jul 27

Why is CRISPR/Cas9 better?



limitations and complications

- **Off-site effects**

Mutation introduced at non-specific loci with similar, but not identical, homology to the target sites are one of the most important complication of these technologies. These can be difficult to identify and require scanning the genome for mutations at sites with sequence similarity to the gRNA target sequence.

- **Mosaicism**

Mice with a mutant allele in only some of their cells can be produced , because the nucleases may not necessarily cut the DNA at the one cell stage of embryonic development.

- **Multiple alleles**

Healing of the nuclease cleavage site by non-homologous end joining can produce cohorts of mice with different mutations from the same targeting constructs, requiring genome sequencing to verify the nature and position of the specific mutation.

Endogenous DNA repair mechanisms

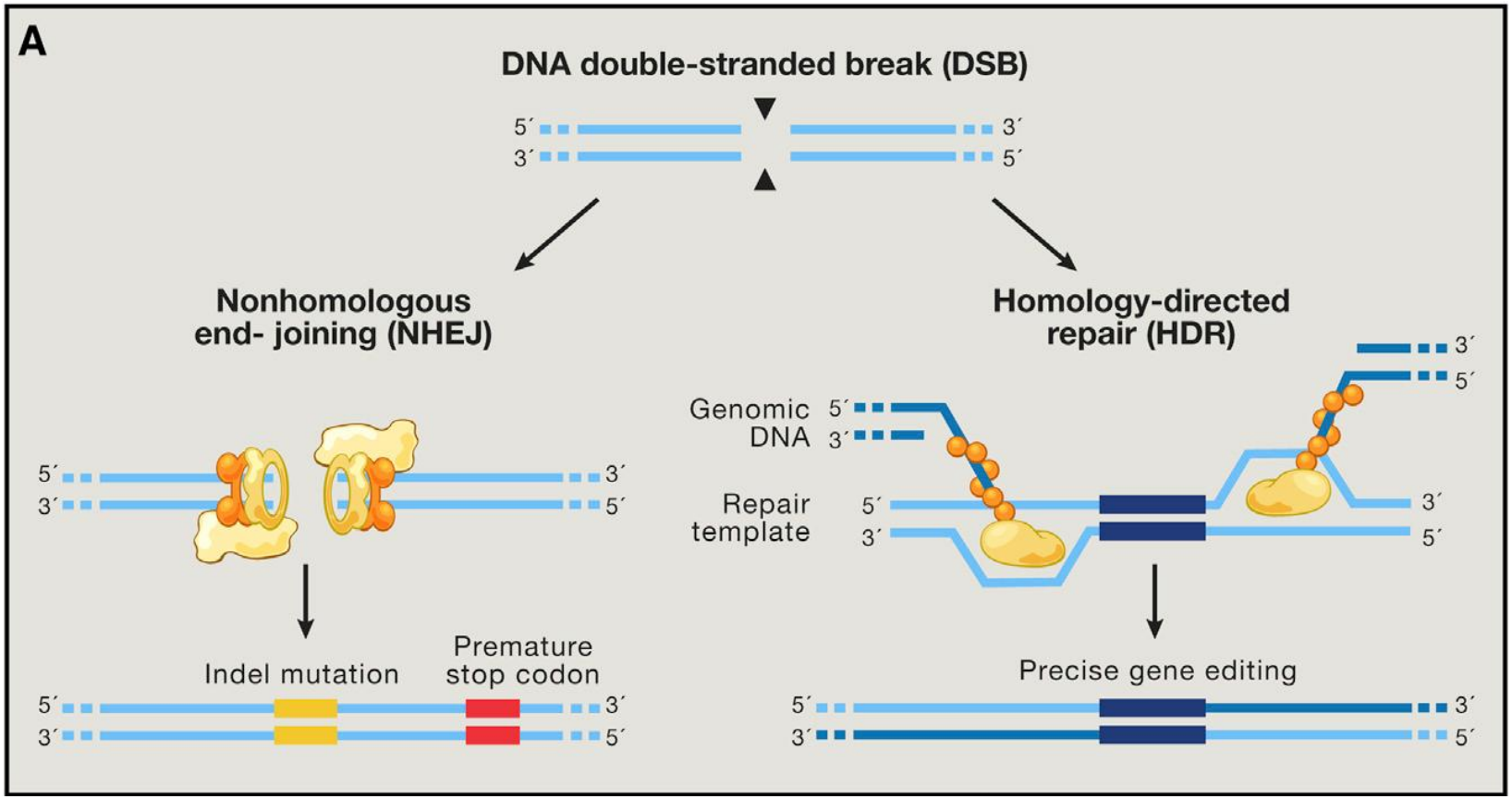


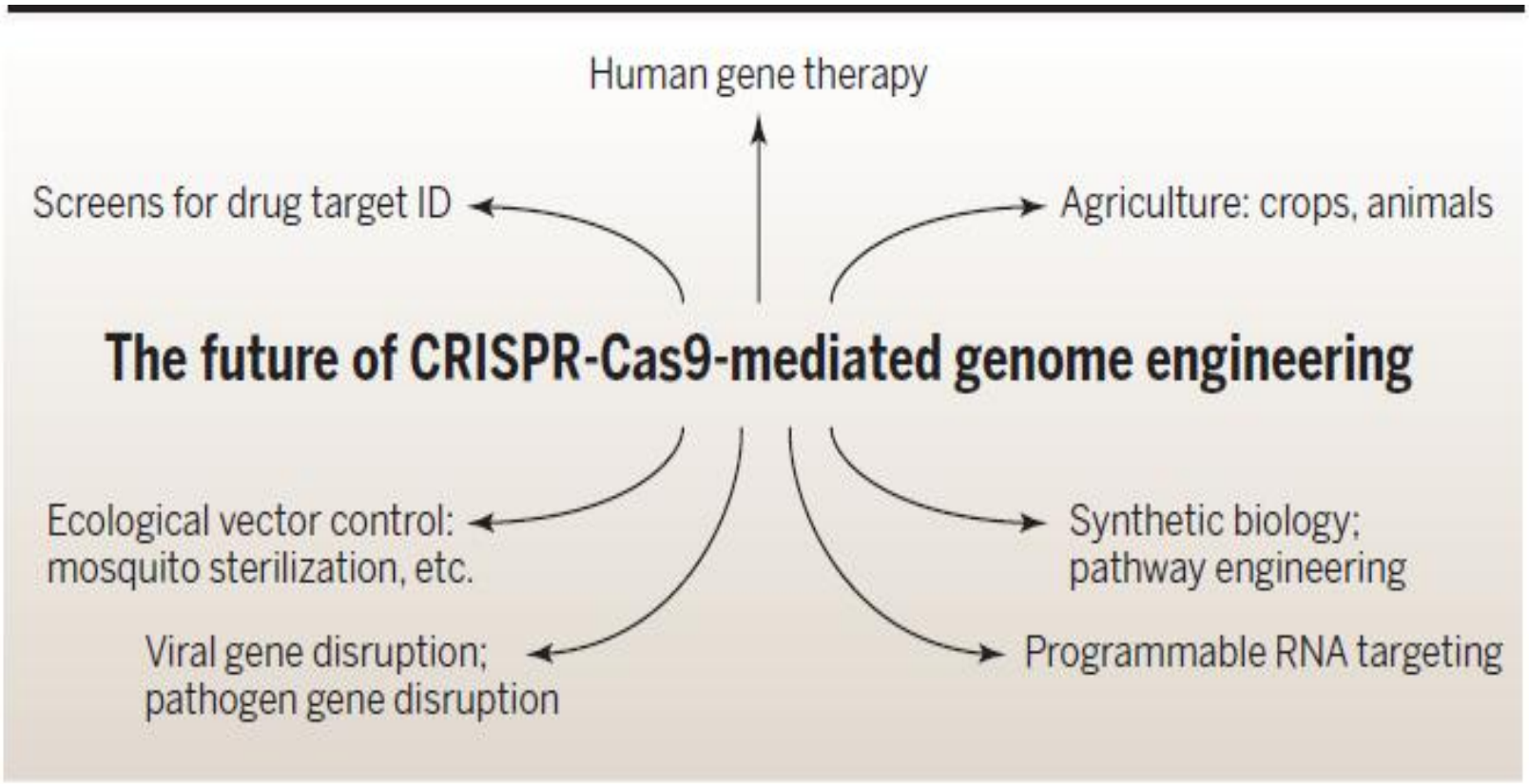
Figure: Hsu, Lander, Zhang: Development and Applications of CRISPR-Cas9 for Genome Engineering; Cell 157, June 5, 2014

Examples of applications of genome editing to therapeutic models

Disease Type	Nuclease Platform Employed	Therapeutic Strategy	References
Hemophilia B	ZFN	HDR-mediated insertion of correct gene sequence	48
HIV	ZFN and CRISPR	NHEJ-mediated inactivation of CCR5	46,69,70,131
DMD	CRISPR and TALEN	NHEJ-mediated removal of stop codon, and HDR-mediated gene correction	132,133
HBV	TALEN and CRISPR	NHEJ-mediated depletion of viral DNA	65,66
SCID	ZFN	HDR-mediated insertion of correct gene sequence	47
Cataract	CRISPR	HDR-mediated correction of mutation in mouse zygote	134
Cystic fibrosis	CRISPR	HDR-mediated correction of CFTR in intestinal stem cell organoid	135
Hereditary tyrosinemia	CRISPR	HDR-mediated correction of mutation in liver	49

Nat Med. 2015 Feb;21(2):121-31. doi: 10.1038/nm.3793

The future of CRISPR/Cas9 system



Federal panel approves first use of CRISPR in humans

Thank you!