PÉCSI TUDOMÁNYEGYETEM
ÁLTALÁNOS ORVOSTUDOMÁNYI KAR
Immunológiai és Biotechnológiai Intézet

Biological therapies

CRISPR-Cas9-based genome engineering

Najbauer József

Lectures 23-24; 2019. 04. 25.

Outline

1. Definitions
2. Applications of genome engineering
3. Mechanisms of microbial CRISPR systems in adaptive immunity
4. Applications of Cas9 as a genome engineering platform
5. Editing the human genome, ethical questions

Abbreviations

CRISPR, **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats

Cas9, **C**RISPR **as**sociated protein **9**

Hsu PD et al, Cell, 2014

Online talk

Online talk about CRISPR by Dr. Jennifer Doudna (University of California, Berkeley)

https://www.ted.com/talks/jennifer_doudna_we_can_now_edit_our_dna_but_let_s_do_it_wisely/discussion

Definition of *CRISPR*

1: a segment of genetic material found in the genomes of prokaryotes (such as some bacteria and archaea) that consists of repeated short sequences of nucleotides interspersed at regular intervals between unique sequences of nucleotides derived from the DNA of pathogens (such as viruses) which had previously infected the bacteria and that functions to protect the bacteria against future infection by the same pathogens

NOTE: The CRISPR segment encodes, via transcription, short RNA sequences that pair with complementary sequences of viral DNA. The pairing is used to guide an enzyme to cleave the viral DNA and prevent further infection.

// CRISPR, he learned, was a strange cluster of DNA sequences that could recognize invading viruses, deploy a special enzyme to chop them into pieces, and use the viral shards that remained to form a rudimentary immune system.— Michael Specter

www.merriam-webster.com/dictionary/CRISPR

Definition of *CRISPR*

2: a gene editing technique in which CRISPR and the RNA segments and enzymes it produces are used to identify and modify specific DNA sequences in the genome of other organisms

// Just a few years after its invention, *CRISPR* gene editing is already having a major impact on biomedical research. It makes it easy to "turn off" genes one at a time, to see what they do. It can introduce specific mutations, to find out why they make cells cancerous or predispose people to diseases. And it can be used to tinker with the genes of plants and animals …— Michael Le Page

// Using *CRISPR*, they have now disabled four rice genes, suggesting that the technique could be used to engineer this crucial food crop.— Elizabeth Pennisi

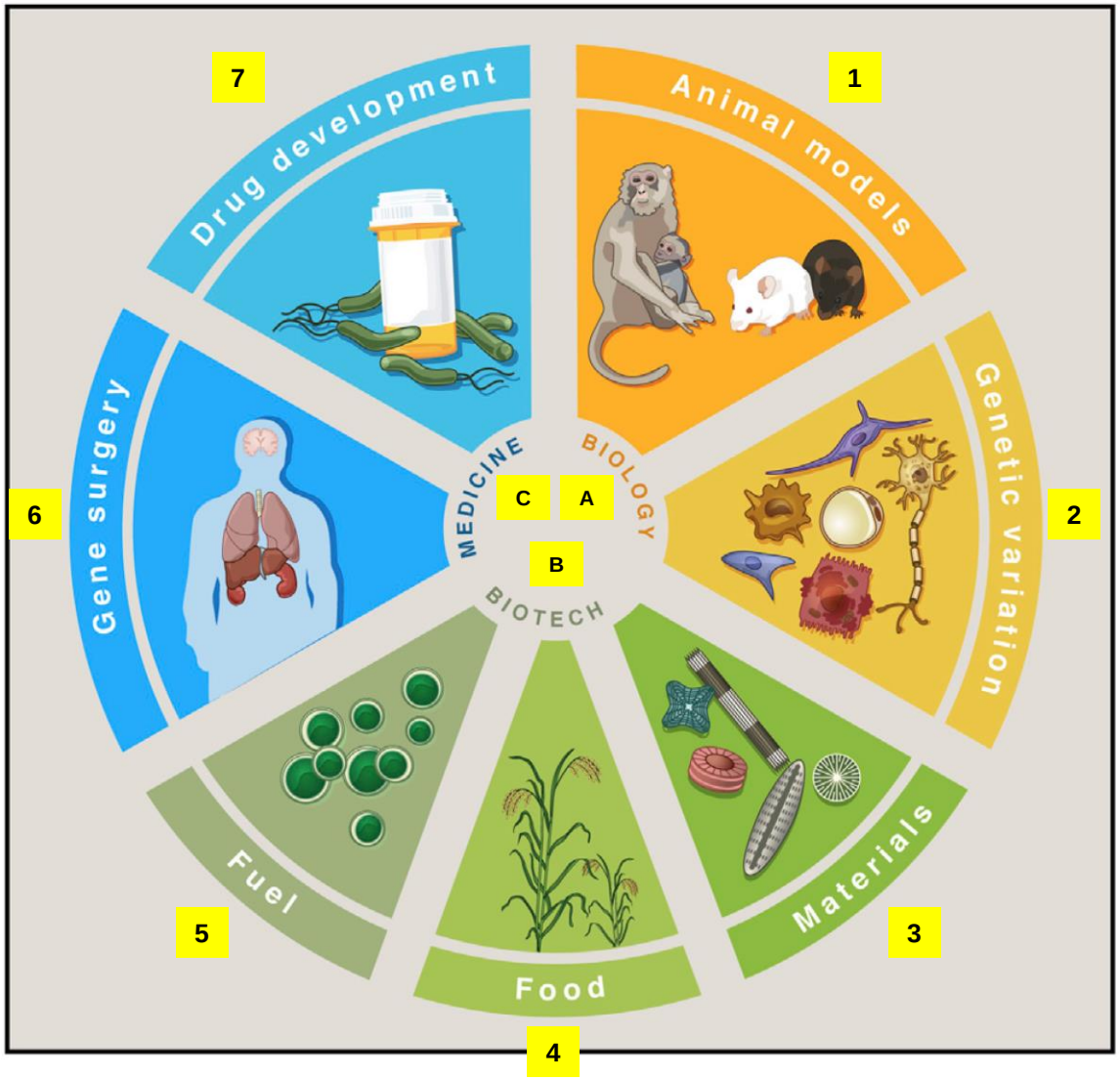
// Scientists hope *Crispr* might also be used for genomic surgery, as it were, to correct errant genes that cause disease.— Andrew Pollack
NOTE: The technique is sometimes called CRISPR-Cas9 , which includes the name of the enzyme that cleaves DNA.

// … an incredibly fast-paced field in which laboratories around the world have used *CRISPR-Cas9* to edit genomes of a wide range of cell types and organisms.— Jennifer A. Doudna and Emmanuelle Charpentier

www.merriam-webster.com/dictionary/CRISPR

Applications of genome engineering

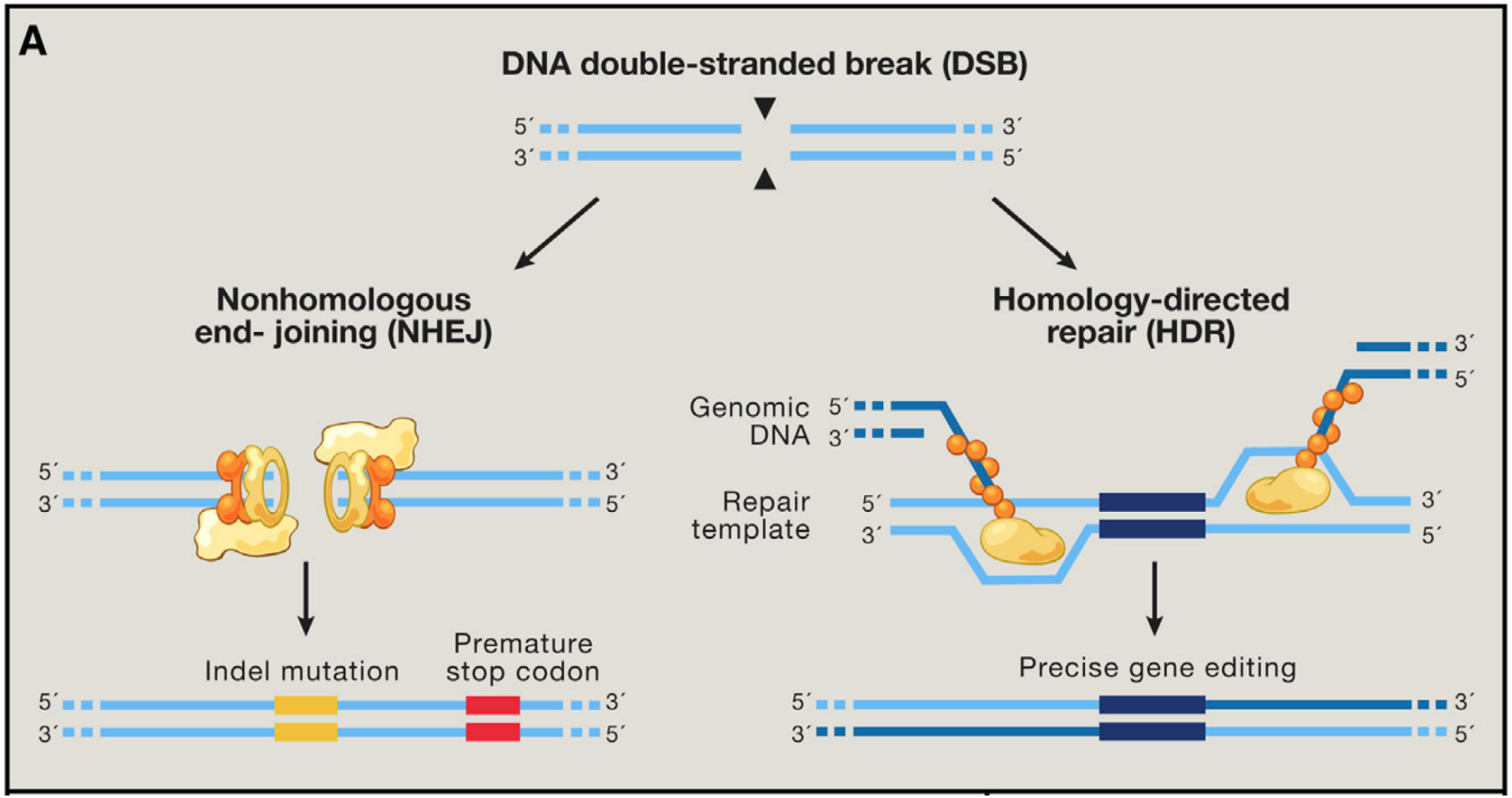
Genetic and epigenetic control of cells with genome engineering technologies is enabling a broad range of applications from basic biology to biotechnology and medicine.



Hsu PD et al, Cell, 2014

- Causal genetic mutations or epigenetic variants associated with altered biological function or disease phenotypes can now be rapidly and efficiently recapitulated in animal or cellular models (1, 2).
- Manipulating biological circuits could facilitate the generation of useful synthetic materials, such as algae-derived, silica-based diatoms for oral drug delivery (3).
- Precise genetic engineering of important agricultural crops could confer resistance to environmental deprivation or pathogenic infection, improving food security while avoiding the introduction of foreign DNA (4).
- Sustainable, cost-effective biofuels are attractive sources for renewable energy, which could be achieved by creating efficient metabolic pathways for ethanol production in algae or corn (5).
- Direct in vivo correction of genetic or epigenetic defects in somatic tissue would be permanent genetic solutions that address the root cause of genetically encoded disorders (6).
- Engineering cells to optimize high yield generation of drug precursors in bacterial factories could significantly reduce the cost and accessibility of useful therapeutics (7).

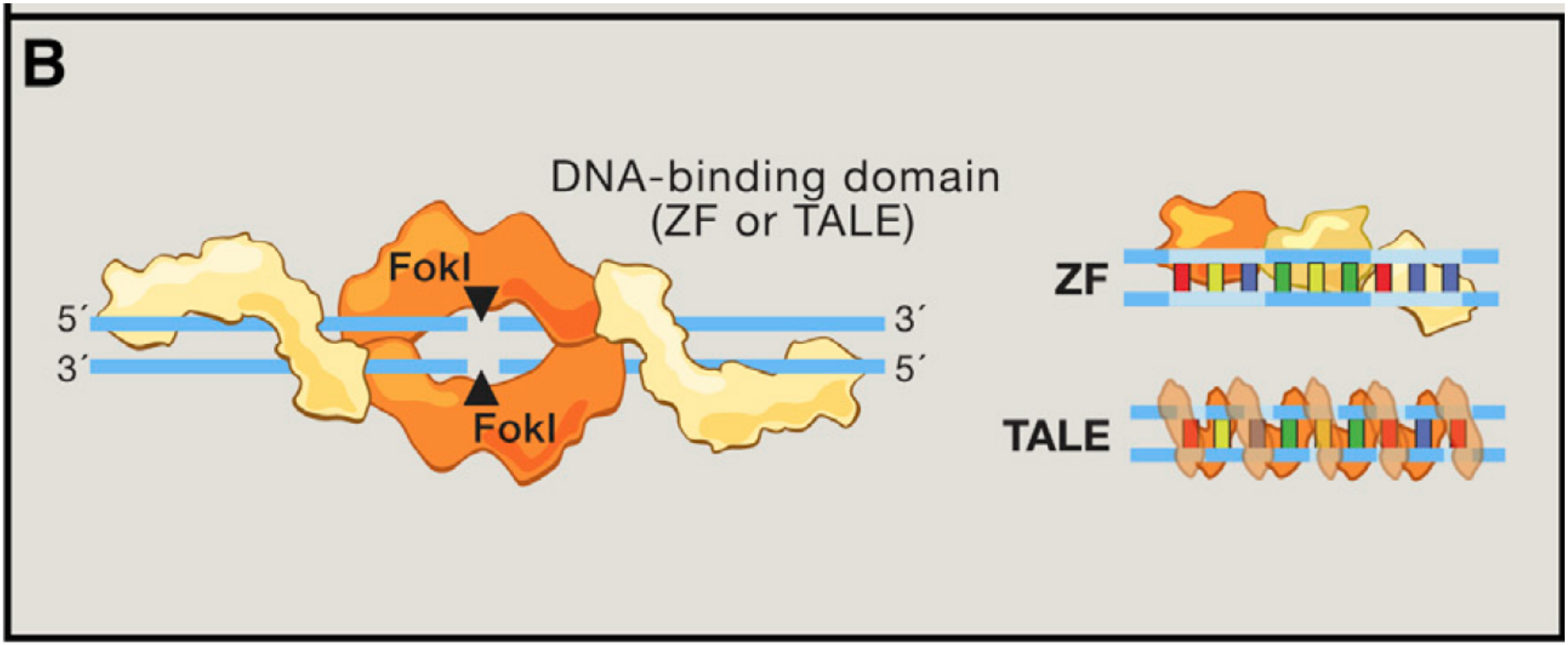
Genome editing technologies exploit endogenous DNA repair machinery (1)



Hsu PD et al, Cell, 2014

(A) DNA double-strand breaks (DSBs) are typically repaired by nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). In the error-prone NHEJ pathway, Ku heterodimers bind to DSB ends and serve as a molecular scaffold for associated repair proteins. Indels are introduced when the complementary strands undergo end resection and misaligned repair due to microhomology, eventually leading to frameshift mutations and gene knockout. Alternatively, Rad51 proteins may bind DSB ends during the initial phase of HDR, recruiting accessory factors that direct genomic recombination with homology arms on an exogenous repair template. Bypassing the matching sister chromatid facilitates the introduction of precise gene modifications.

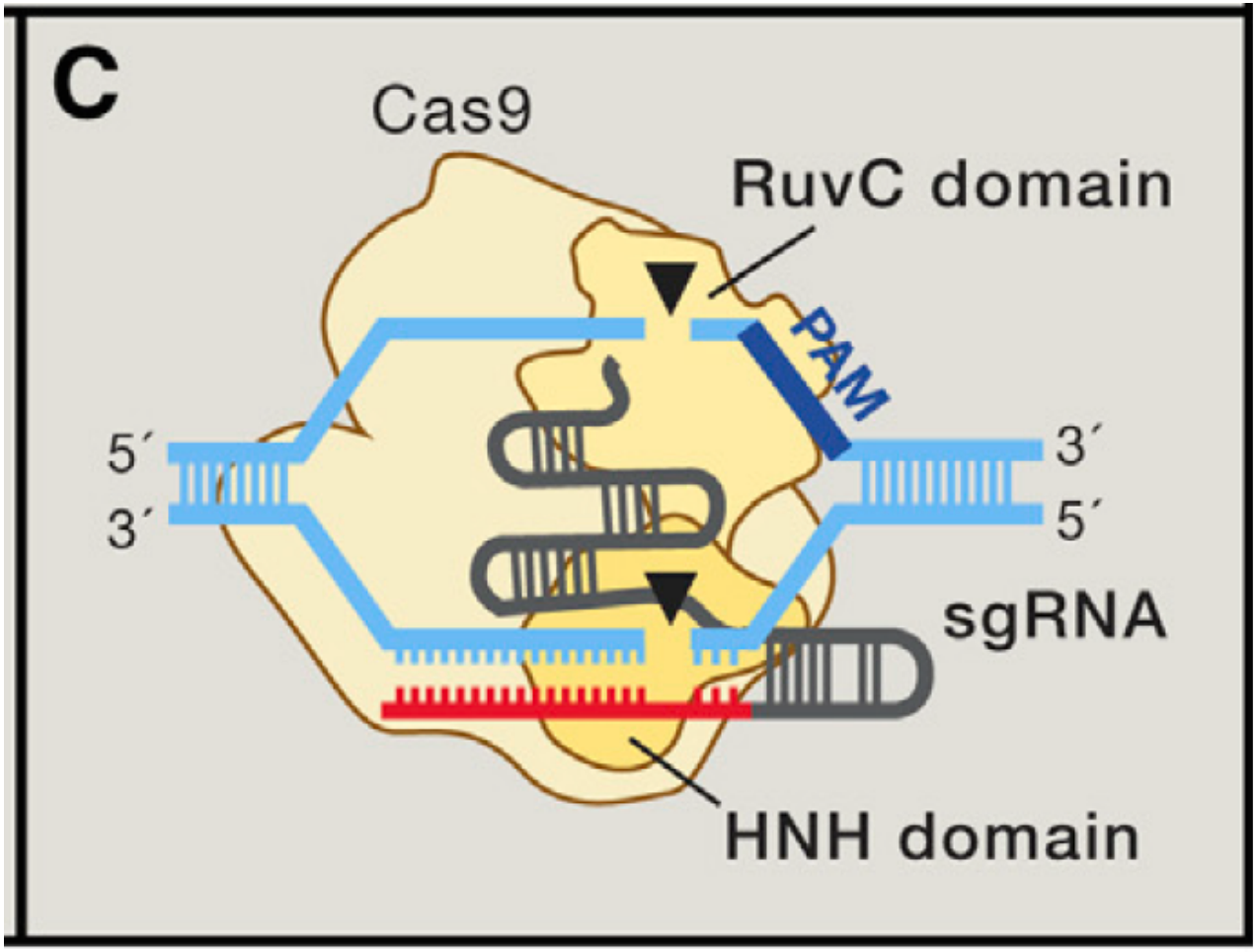
Genome editing technologies exploit endogenous DNA repair machinery (2)



(B) Zinc finger (ZF) proteins and transcription activator-like effectors (TALEs) are naturally occurring DNA-binding domains that can be modularly assembled to target specific sequences. ZF and TALE domains each recognize 3 and 1 bp of DNA, respectively. Such DNA-binding proteins can be fused to the FokI endonuclease to generate programmable site-specific nucleases.

Hsu PD et al, Cell, 2014

Genome editing technologies exploit endogenous DNA repair machinery (3)



Cas9, CRISPR associated protein 9
HNH, Nuclease domain
PAM, Protospacer-adjacent motif
RuvC, Nuclease domain
sgRNA, single guide RNA

(C) The Cas9 nuclease from the microbial CRISPR adaptive immune system is localized to specific DNA sequences via the guide sequence on its guide RNA (red), directly base-pairing with the DNA target. Binding of a protospacer-adjacent motif (PAM, blue) downstream of the target locus helps to direct Cas9-mediated DSBs.

Hsu PD et al, Cell, 2014

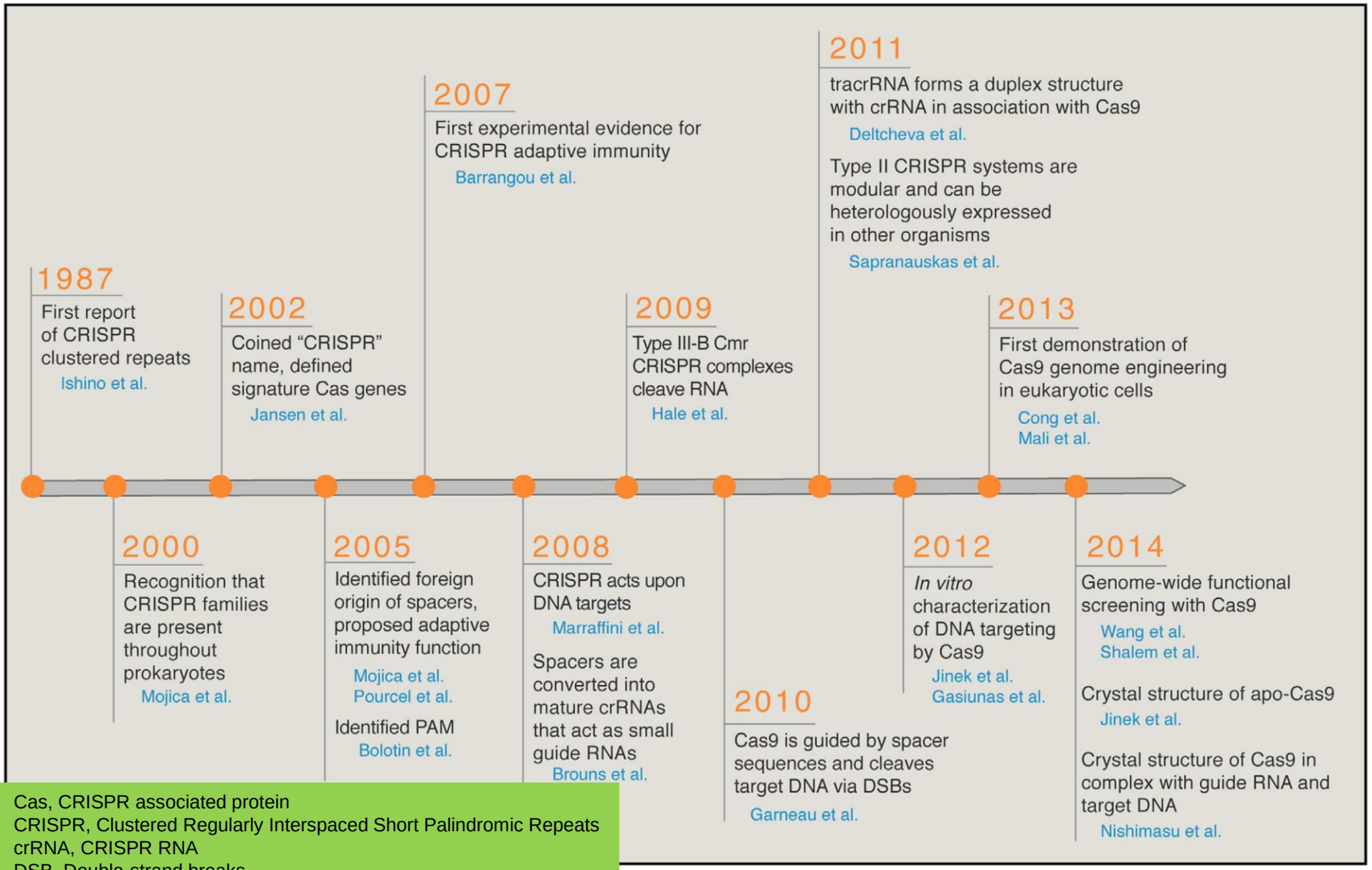
Comparison of approaches for gene knockdown and knockout

	1	2	3	4	5
	Approach				
	RNAi	**ASO**	**ZFN**	**TALEN**	**CRISPR–Cas**
Molecular target	RNA	RNA	DNA	DNA	DNA
Result of targeting	Reversible knockdown	Reversible knockdown	Irreversible knockout	Irreversible knockout	Irreversible knockout
Ease of generating target specificity	Easy: simple oligo synthesis and cloning steps and limited chemical modifications to enhance RNA degradation	Easy: simple oligo synthesis and cloning steps; often chemical modification is required to enhance RNA binding and ASO stability	Difficult: substantial cloning and protein engineering required	Moderate: substantial cloning steps required	Easy; simple oligo synthesis and cloning steps
Off-target activity	High	High	Moderate	Low	Low
Ease of multi-plexing	High	High	Low	Moderate	High
Transcriptional and epigenetic control	Direct control not possible	Direct control not possible; TSOs can interfere with protein translation	DNA-binding ZF domains can be fused to new functional domains	DNA binding domains can be fused to new functional domains	Enzymatically inactive dCas9 can be fused to new functional domains
Ease of delivery into the mammalian CNS	High: delivered by nanoparticles, bioconjugates, cell-penetrating peptides or viral vectors	High: delivered by nanoparticles, bioconjugates, cell-penetrating peptides or viral vectors	Moderate: delivered by viral vectors	Moderate: delivered by viral vectors but large size makes packaging into viral vectors challenging	Moderate: delivered by electroporation, PEI-mediated transfection, nanoparticles and viral vectors
Ease of generating large-scale libraries	High: simple oligo synthesis and cloning required	High: simple oligo synthesis and cloning required	Low: complex protein engineering required for each gene	Moderate: technically challenging cloning steps	High: simple oligo synthesis and cloning required
Costs	Low	Low	High	Moderate	Low

ASO, DNA antisense oligonucleotide; Cas, CRISPR-associated protein; CRISPR, clustered regularly interspaced short palindromic repeat; dCas9, dead Cas9; PEI, polyethylenimine; RNAi, RNA interference; TALEN, transcription activator-like effector nuclease; TSOs, translation-suppressing oligonucleotides; ZF, zinc-finger; ZFN, ZF nuclease.

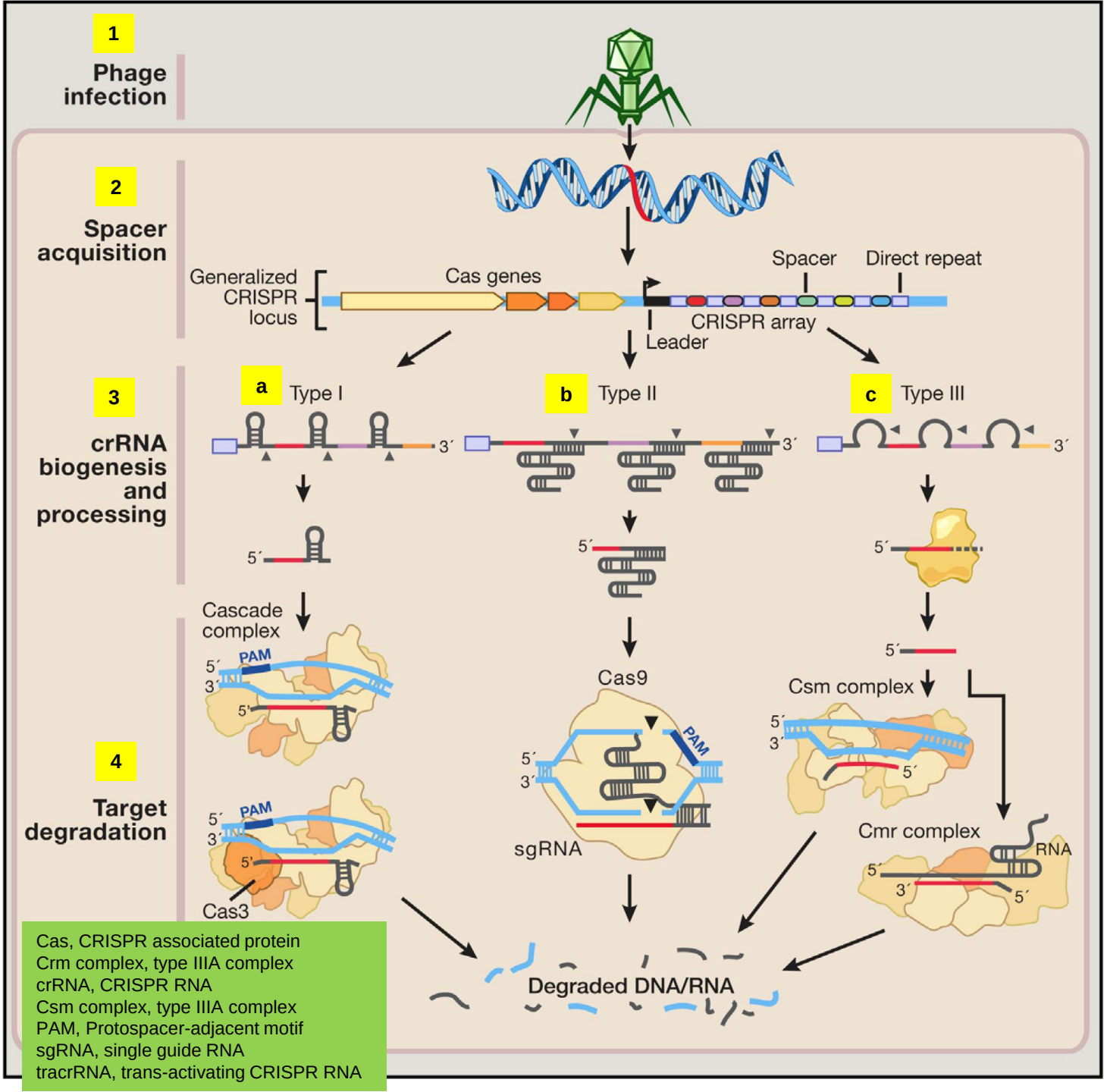
Heidenreich and Zhang, Nature Rev Neurosci, 2016

Key studies characterizing and engineering CRISPR systems



Cas, CRISPR associated protein
CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats
crRNA, CRISPR RNA
DSB, Double-strand breaks
PAM, Protospacer-adjacent motif
tracrRNA, trans-activating CRISPR RNA

Hsu PD et al, Cell, 2014



Cas, CRISPR associated protein
Crm complex, type IIIA complex
crRNA, CRISPR RNA
Csm complex, type IIIA complex
PAM, Protospacer-adjacent motif
sgRNA, single guide RNA
tracrRNA, trans-activating CRISPR RNA

Natural mechanisms of microbial CRISPR systems in adaptive immunity

1. Invasion of the cell by foreign genetic elements from bacteriophages or plasmids.

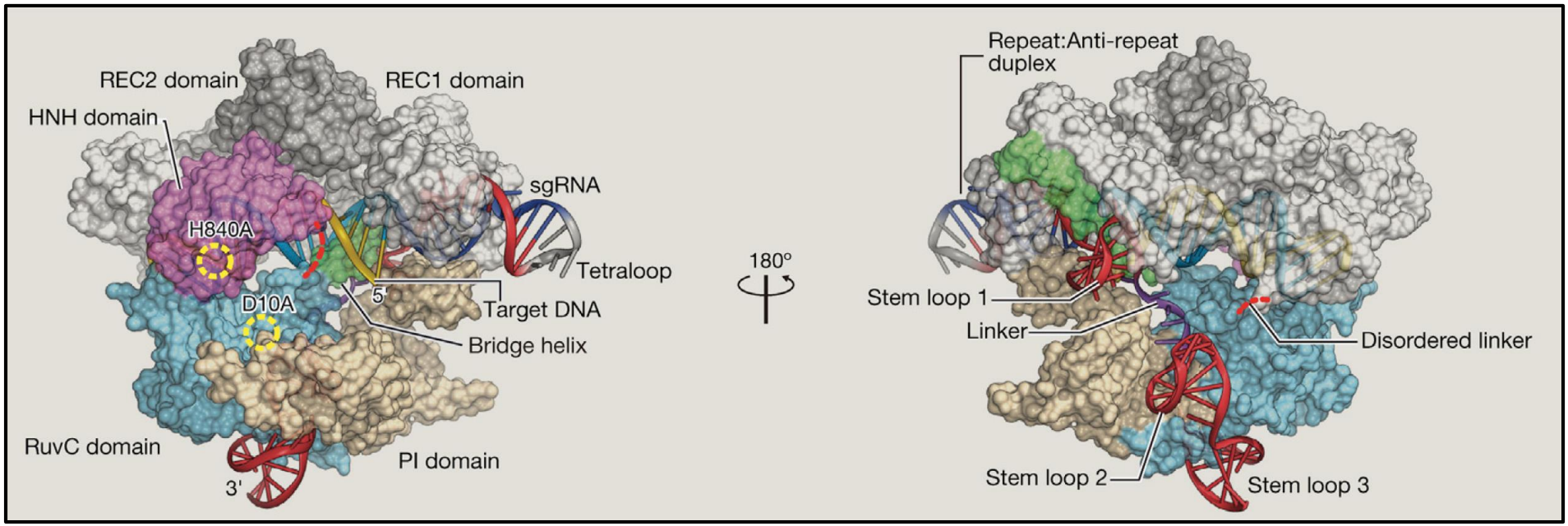
2. Certain Cas enzymes acquire spacers from the exogenous protospacer sequences and install them into the CRISPR locus within the prokaryotic genome.

3. The CRISPR array is a noncoding RNA transcript that is enzymatically maturated through distinct pathways that are unique to each type of CRISPR system.

4. Maturated crRNAs from type I and III CRISPR systems are loaded onto effector protein complexes for target recognition and degradation. In type II systems, crRNA-tracrRNA hybrids complex with Cas9 to mediate interference.

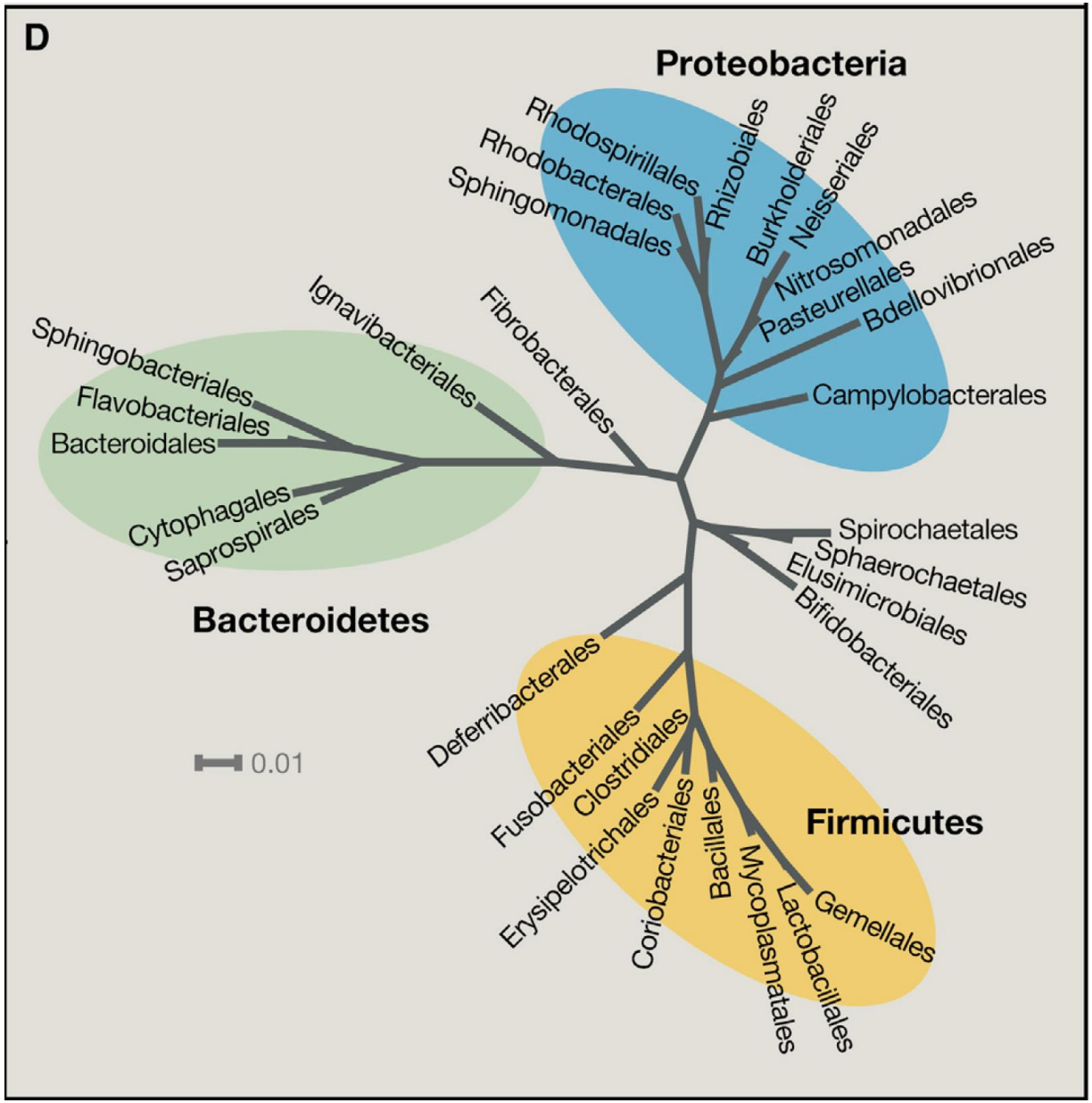
Hsu PD et al, Cell, 2014

Crystal structure of *Streptococcus pyogenes* Cas9 in complex with guide RNA and target DNA



Hsu PD et al, Cell, 2014

Phylogenetic tree displaying the microbial origin of Cas9 nucleases from the type II CRISPR immune system



Taxonomic information was derived from greengenes 16S rRNA gene sequence alignment, and the tree was visualized using the Interactive Tree of Life tool (iTol).

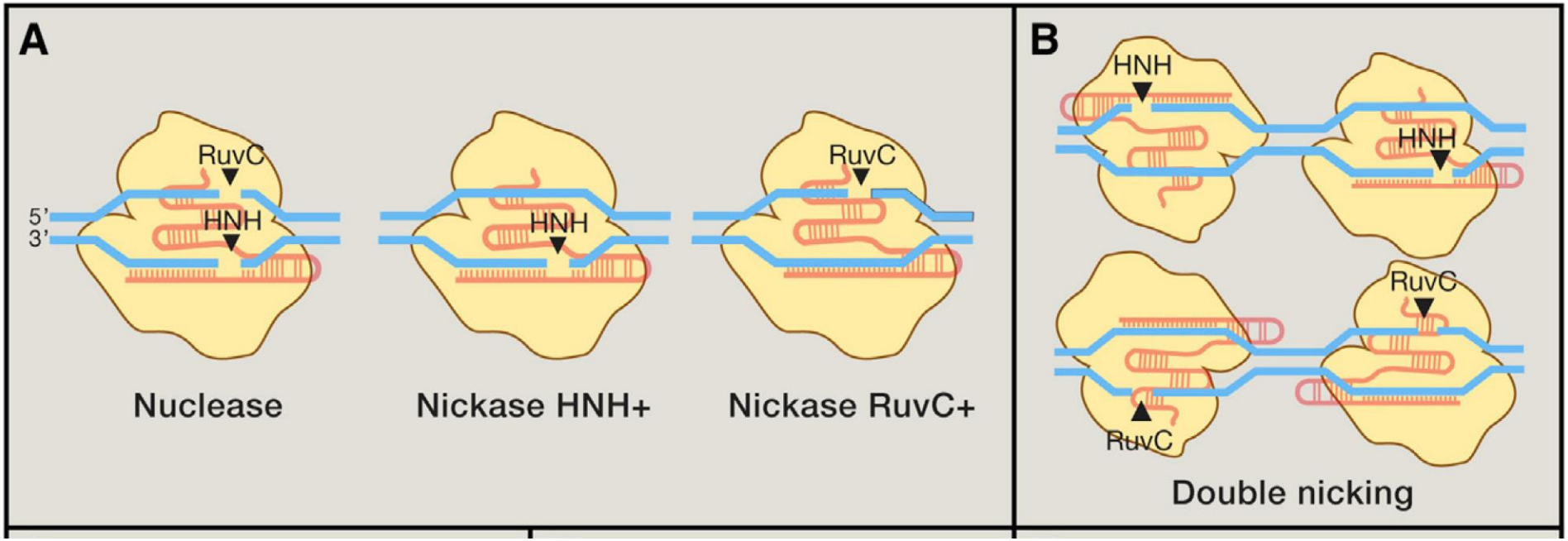
Bacteroidetes, this phylum is composed of three large classes of Gram-negative, nonsporeforming, anaerobic or aerobic, and rod-shaped bacteria that are widely distributed in the environment, including in soil, sediments, and sea water, as well as in the guts and on the skin of animals.

Firmicutes, (Latin: *firmus*, strong, and *cutis*, skin, referring to the cell wall) are a phylum of bacteria, most of which have gram-positive cell wall structure.

Proteobacteria, a major phylum of gram-negative bacteria. They include a wide variety of pathogens, such as *Escherichia, Salmonella, Vibrio, Helicobacter, Yersinia, Legionellales* and many other notable genera. Others are free-living (non-parasitic) and include many of the bacteria responsible for nitrogen fixation.

Hsu PD et al, Cell, 2014

Applications of Cas9 as a genome engineering platform (1)

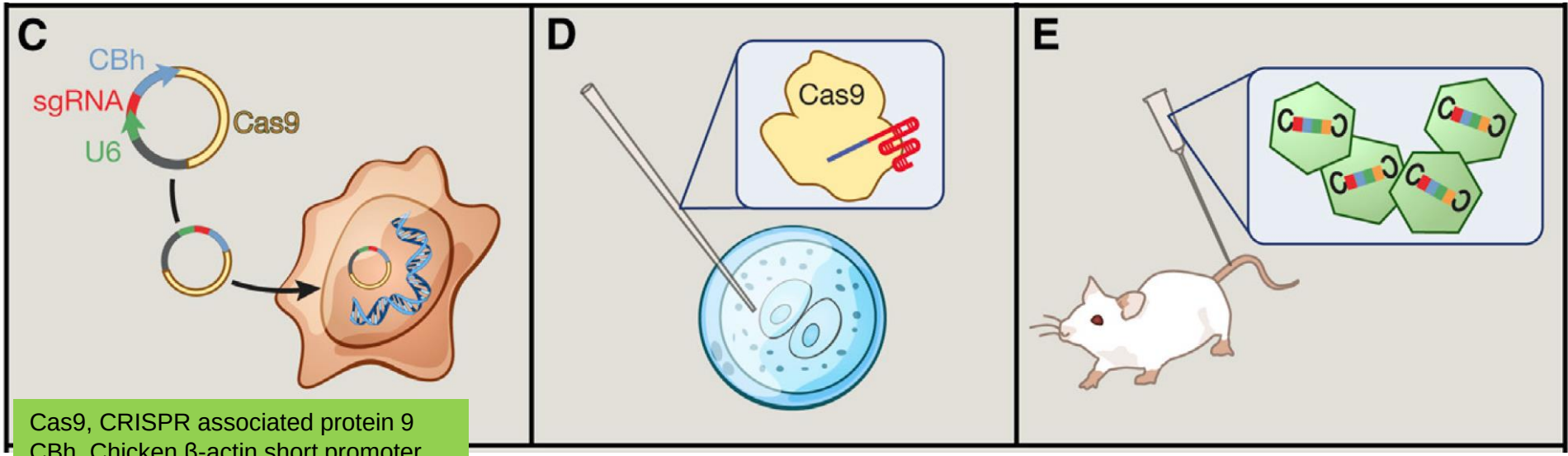


(A) The Cas9 nuclease cleaves DNA via its RuvC and HNH nuclease domains, each of which nicks a DNA strand to generate blunt-end DSBs. Either catalytic domain can be inactivated to generate nickase mutants that cause single-strand DNA breaks.

(B) Two Cas9 nickase complexes with appropriately spaced target sites can mimic targeted DSBs via cooperative nicks, doubling the length of target recognition without sacrificing cleavage efficiency.

Hsu PD et al, Cell, 2014

Applications of Cas9 as a genome engineering platform (2)



Cas9, CRISPR associated protein 9
CBh, Chicken β-actin short promoter
sgRNA, Short guide RNA
U6, RNA polymerase III promoter

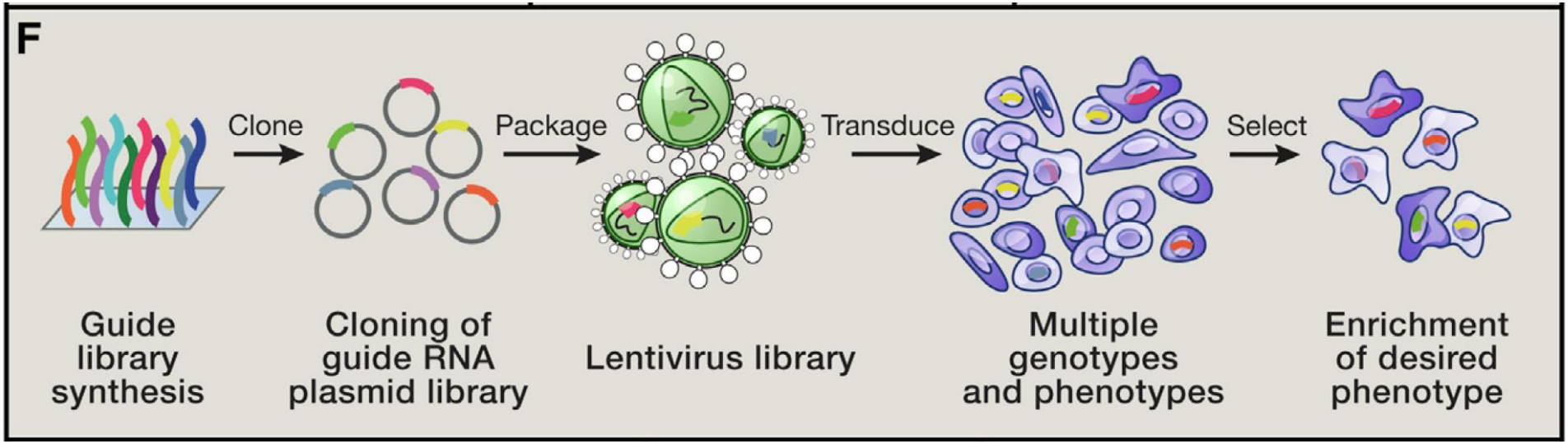
(C) Expression plasmids encoding the Cas9 gene and a short sgRNA cassette driven by the U6 RNA polymerase III promoter can be directly transfected into cell lines of interest.

(D) Purified Cas9 protein and in vitro transcribed sgRNA can be microinjected into fertilized zygotes for rapid generation of transgenic animal models.

(E) For somatic genetic modification, high-titer viral vectors encoding CRISPR reagents can be transduced into tissues or cells of interest.

Hsu PD et al, Cell, 2014

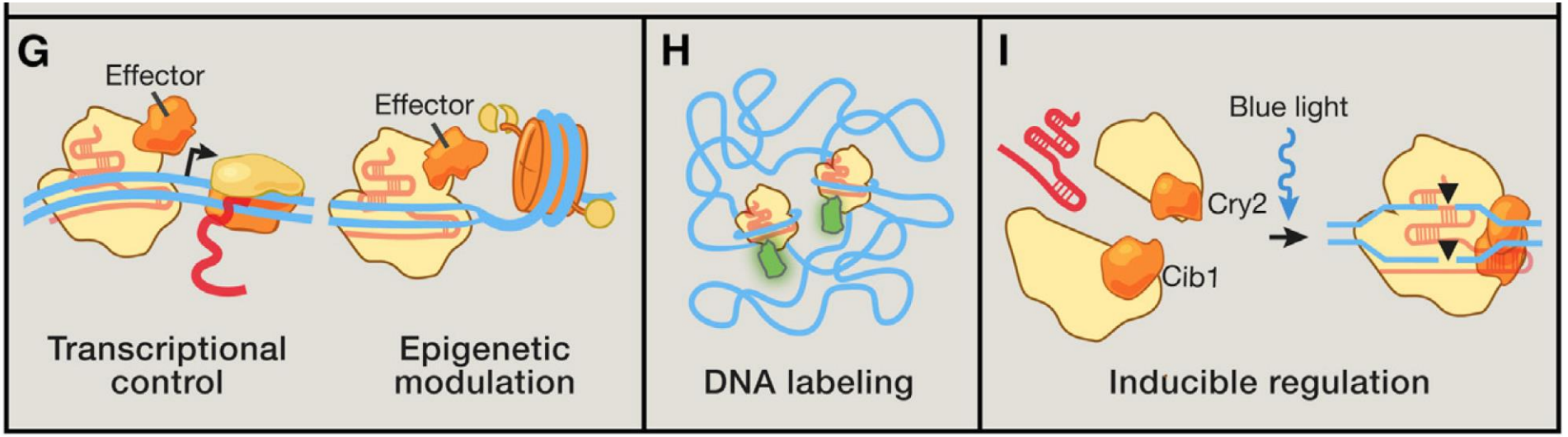
Applications of Cas9 as a genome engineering platform (3)



(F) Genome-scale functional screening can be facilitated by mass synthesis and delivery of guide RNA libraries.

Hsu PD et al, Cell, 2014

Applications of Cas9 as a genome engineering platform (4)



Cib1, Cryptochrome-interacting basic-helix-loop-helix protein 1
Cry2, Cryptochrome 2 (photolyase-like blue-light receptor that mediates light responses in plants and animals)

(G) Catalytically dead Cas9 (dCas9) can be converted into a general DNA-binding domain and fused to functional effectors such as transcriptional activators or epigenetic enzymes. The modularity of targeting and flexible choice of functional domains enable rapid expansion of the Cas9 toolbox.

(H) Cas9 coupled to fluorescent reporters facilitates live imaging of DNA loci for illuminating the dynamics of genome architecture.

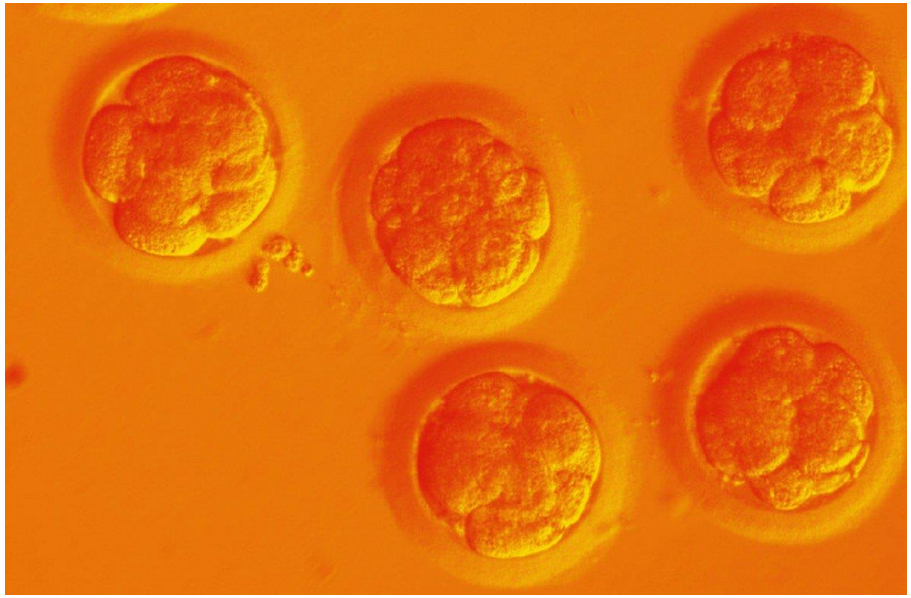
(I) Reconstituting split fragments of Cas9 via chemical or optical induction of heterodimer domains, such as the cib1/cry2 system from Arabidopsis, confers temporal control of dynamic cellular processes.

Hsu PD et al, Cell, 2014

GENE EDITING OF HUMANS

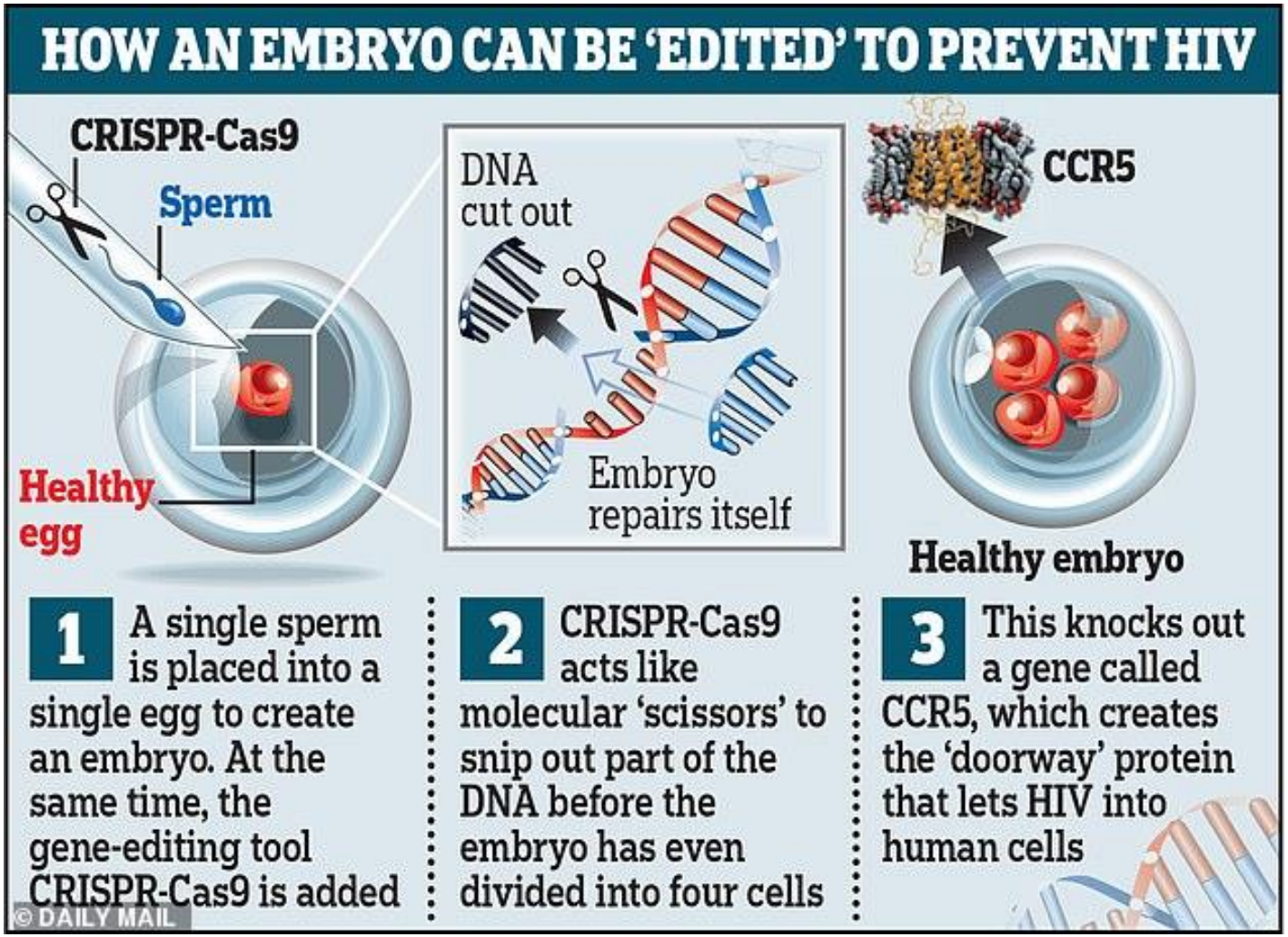
He Jiankui speaks during the Human Genome Editing Conference in Hong Kong on November 28, 2018

EXCLUSIVE: Chinese scientists are creating CRISPR babies



https://www.technologyreview.com/

A 'monstrous' experiment or a huge leap for science? Experts condemn 'irresponsible' Chinese scientists who claim to have edited the DNA of twins to give them immunity to HIV



https://www.dailymail.co.uk/sciencetech/article-6429665/Experts-condemn-irresponsible-experiment-saw-scientists-edit-twins-DNA.html

TIMELINE
HUMAN GENE EDITING

March 2015: Chinese researchers become the first to edit genes in a human embryo.
June 2016: He Jiankui launches a project to edit genes in human embryos, with the goal of a live birth.
March 2017: He starts recruiting couples (each with an HIV-positive father) for the experiments.
Early November 2018: Gene-edited twin girls are reportedly born, and a second pregnancy with a third gene-edited embryo is established.
25–26 November 2018: The *MIT Technology Review* reveals the existence of the research programme; the Associated Press quickly goes public with the story of the girls' birth.
28 November 2018: He offers details about his work at a gene-editing summit in Hong Kong and is roundly criticized.
November–December 2018: China's National Health Commission orders an investigation into He's work.
January 2019: He is censured by the Guangdong health ministry and fired from his university.
18 March 2019: A World Health Organization committee will meet to set guidelines for human gene editing.
August 2019: Third gene-edited baby expected.

Cyranoski D, Nature, 2019

WHAT'S NEXT FOR CRISPR BABIES?

Following last year's bombshell revelation, investigations mount and debates swirl about the future for gene-edited humans.

Here are the four most pressing questions.

WHAT WILL HAPPEN TO HE — AND THE CHILDREN?

WHAT ABOUT THE OTHER SCIENTISTS IMPLICATED?

HOW COULD HE'S ACTIONS AFFECT OTHER RESEARCH?

WHERE WILL THE NEXT CRISPR BABIES BE BORN?

Cyranoski D, Nature, 2019

China's CRISPR twins might have had their brains inadvertently enhanced

https://www.technologyreview.com/s/612997/the-crispr-twins-had-their-brains-altered/

- The twins, called Lulu and Nana, reportedly had their genes modified before birth by a Chinese scientific team using the new editing tool CRISPR. The goal was to make the girls immune to infection by HIV, the virus that causes AIDS.

- Now, new research shows that the same alteration introduced into the girls' DNA, deletion of a gene called CCR5, not only makes mice smarter but also improves human brain recovery after stroke, and could be linked to greater success in school.

CRISPR Ethics: Moral Considerations for Applications of a Powerful Tool

Carolyn Brokowski [1] and Mazhar Adli [2]

1 - Department of Emergency Medicine, *Yale School of Medicine, 464 Congress Avenue, New Haven, CT 06519-1362, USA*
2 - Department of Biochemistry and Molecular Genetics, *University of Virginia School of Medicine, 1340 Jefferson Park Avenue, Charlottesville, VA 22908, USA*

Correspondence to Mazhar Adli: *adli@virginia.edu*
https://doi.org/10.1016/j.jmb.2018.05.044
Edited by Prashant Mali

Abstract

With the emergence of CRISPR technology, targeted editing of a wide variety of genomes is no longer an abstract hypothetical, but occurs regularly. As application areas of CRISPR are exceeding beyond research and biomedical therapies, new and existing ethical concerns abound throughout the global community about the appropriate scope of the systems' use. Here we review fundamental ethical issues including the following: 1) the extent to which CRISPR use should be permitted; 2) access to CRISPR applications; 3) whether a regulatory framework(s) for clinical research involving human subjects might accommodate all types of human genome editing, including editing of the germline; and 4) whether international regulations governing inappropriate CRISPR utilization should be crafted and publicized. We conclude that moral decision making should evolve as the science of genomic engineering advances and hold that it would be reasonable for national and supranational legislatures to consider evidence-based regulation of certain CRISPR applications for the betterment of human health and progress.

Risk-benefit considerations in CRISPR technology (1)

	A Benefit(s)	B Risk(s)/Harm(s)
Basic and pre-clinical research 1	• 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-editing approaches (base editing and RNA targeting) • Knowledge advancement	• Experimentation involving human embryos is controversial and illegal in some countries • Potential for privacy and confidentiality breaches
Translational and clinical medicine 2	• Immunotherapy • Organoids • Novel drug targets • Artificial intelligence • Modification of pathological genes • Novel therapeutics and fertility applications • 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

Brokowski & Adli, J Mol Biol, 2018

Risk-benefit considerations in CRISPR technology (2)

	A Benefit(s)	B Risk(s)/Harm(s)
Non-therapeutic applications 3	• 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
Access to CRISPR technology 4	• Inexpensive (technology itself) • Widely available • Profit, economic growth • Innovation	• Price gouging • Prohibitively expensive applications
Regulations for clinical research involving human subjects 5	• 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
National and international regulations, law, and policy 6	• Prevention against misuses of technology • Safeguard against risky, potentially harmful conditions	• 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

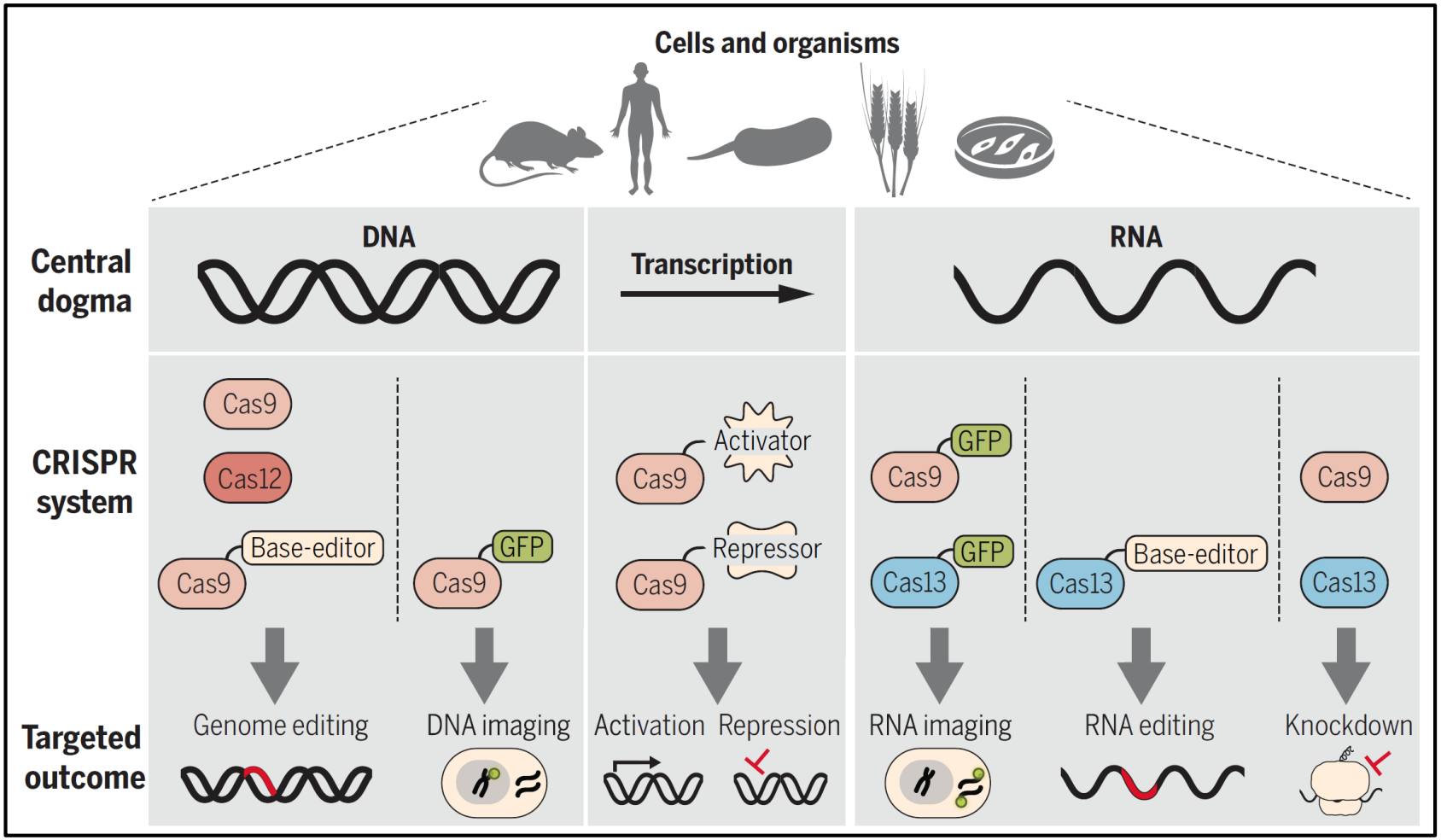
Brokowski & Adli, J Mol Biol, 2018

SUMMARY
CRISPR-Cas and the future of genetic engineering

- The diversity, modularity, and efficacy of CRISPR-Cas systems are driving a biotechnological revolution.
- RNA-guided Cas enzymes have been adopted as tools to manipulate the genomes of cultured cells, animals, and plants, accelerating the pace of fundamental research and enabling clinical and agricultural breakthroughs.
- The basic mechanisms set the CRISPR-Cas toolkit apart from other programmable gene-editing technologies, highlighting the diverse and naturally evolved systems now functionalized as biotechnologies.
- There is a rapidly evolving landscape of CRISPR-Cas applications, from gene editing to transcriptional regulation, imaging, and diagnostics.
- Continuing functional dissection and an expanding landscape of applications position CRISPR-Cas tools at the cutting edge of nucleic acid manipulation that is rewriting biology.

Knott & Doudna, Science, 2018

CRISPR-Cas systems allow genetic manipulation across the central dogma



Knott & Doudna, Science, 2018

From left to right, Cas9 and Cas12a are used for inducing dsDNA breaks for genome editing. nCas9 (nickase Cas9) can be fused to base editors to modify nucleotides in dsDNA for genome editing without introducing a dsDNA break. dCas9 (dead Cas9) can be fused to transcriptional activators, repressors, or epigenetic modifiers to regulate transcription. Cas9 and Cas13a can be used for targeted RNA interference. Cas13a fused to base editors can be used to modify nucleotides in RNA. dCas9 or dCas13a can be fused to green fluorescent protein (GFP) to visualize DNA or RNA.