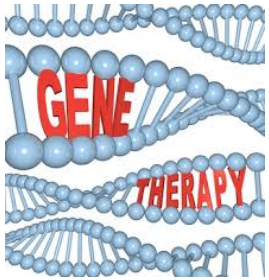
The new frontiers of gene therapy:

CRISPR-Cas9 system



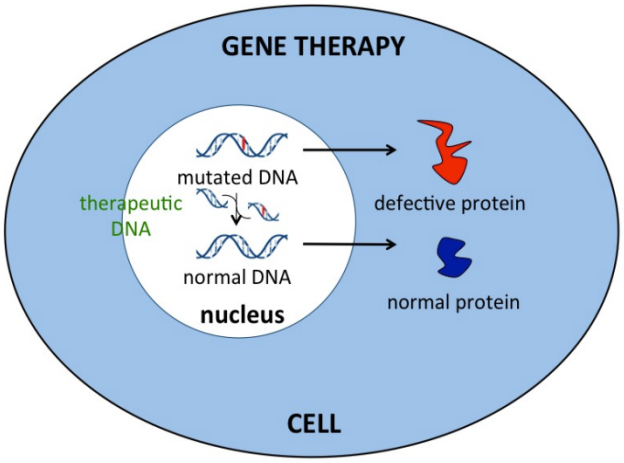
What is gene therapy?

Gene therapy is an experimental technique that uses genes to treat or prevent disease. Researchers are testing several approaches to gene therapy, including:

- Replacing a mutated gene that causes disease with a healthy copy of the gene.

- Inactivating, or "knocking out," a mutated gene that is functioning improperly.

- Introducing a new gene into the body to help fight a disease.



Although gene therapy is a promising treatment option for a number of diseases, the technique remains risky and is still under study to make sure that it will be safe and effective.

Gene therapy is currently only being tested for the treatment of diseases that have no other cures.

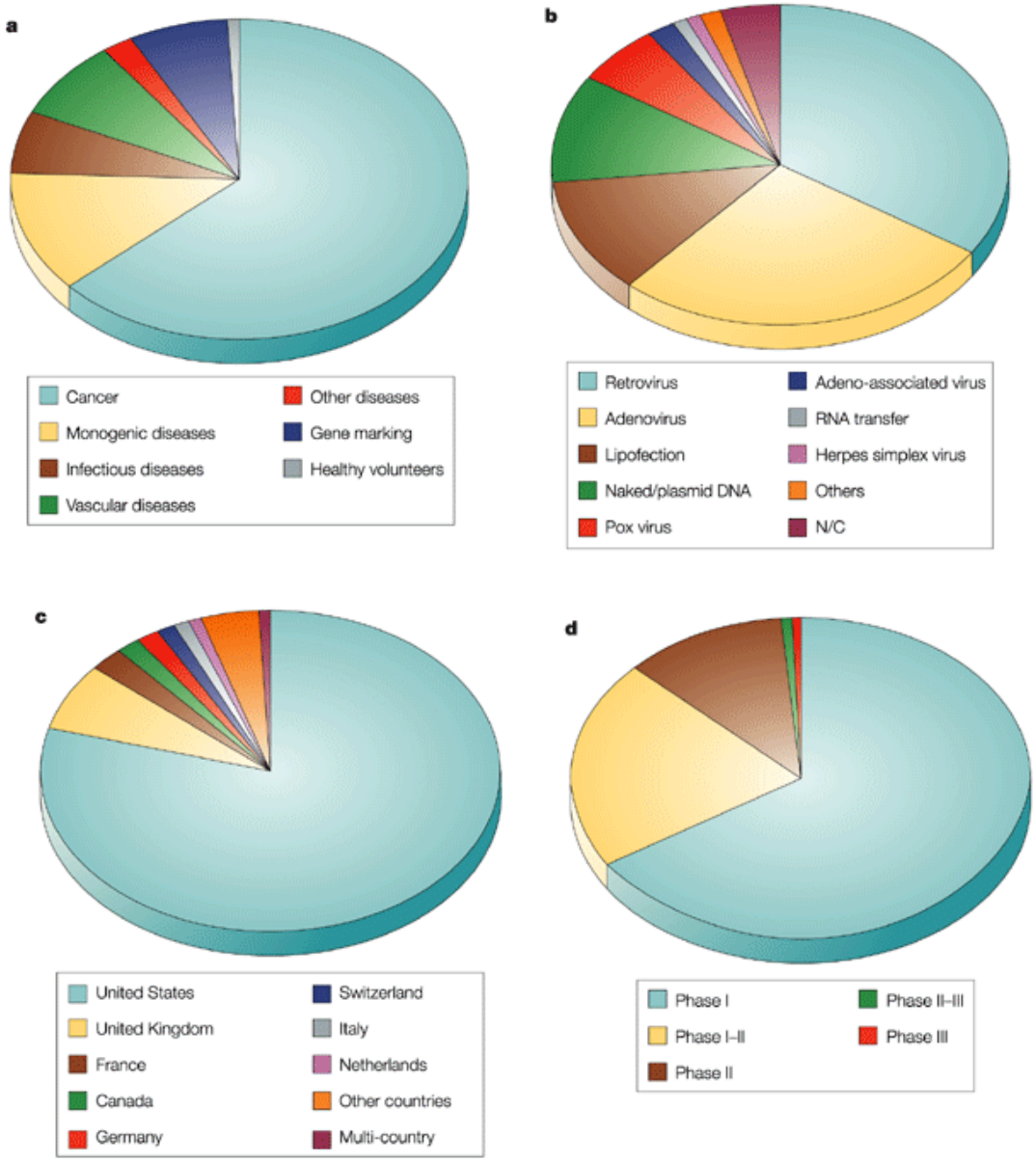
Gene therapy

a)Most gene-therapy clinical trials are designed to treat cancer

b) Retrovirus vectors and adenovirus vectors have, so far, been the most commonly used vectors in gene-transfer trials.

c) Most gene transfer clinical trials are conducted in the United States.

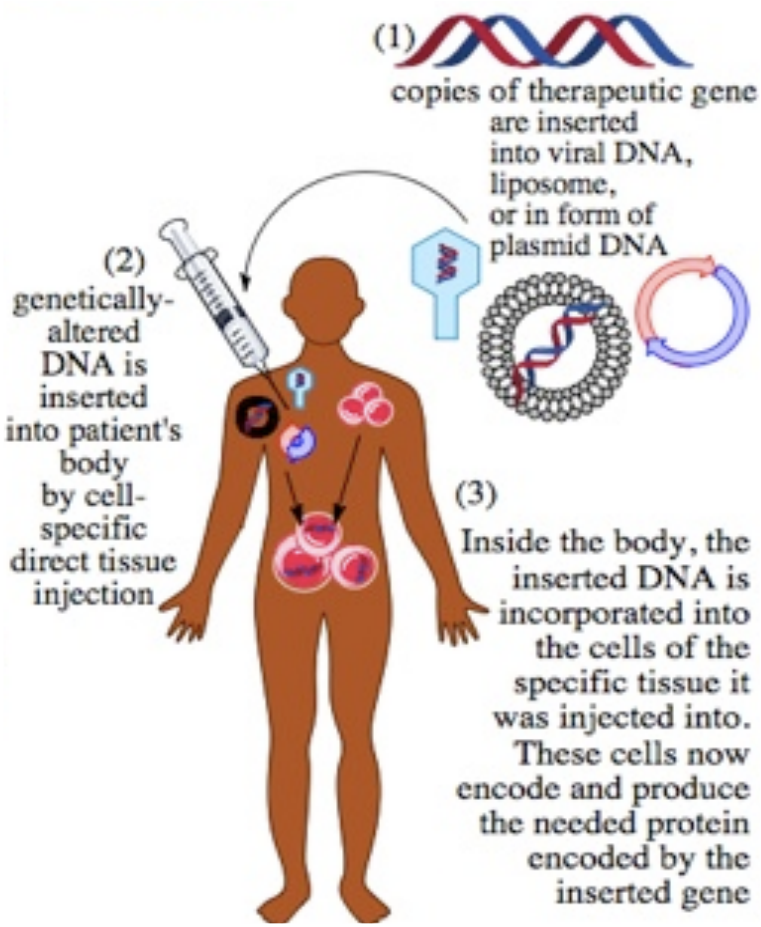
d)Most gene-transfer trials are designed to assess only the safety of a particular gene-therapy approach (Phase I). Few gene therapies are being assessed in Phase II or Phase III efficacy trials.



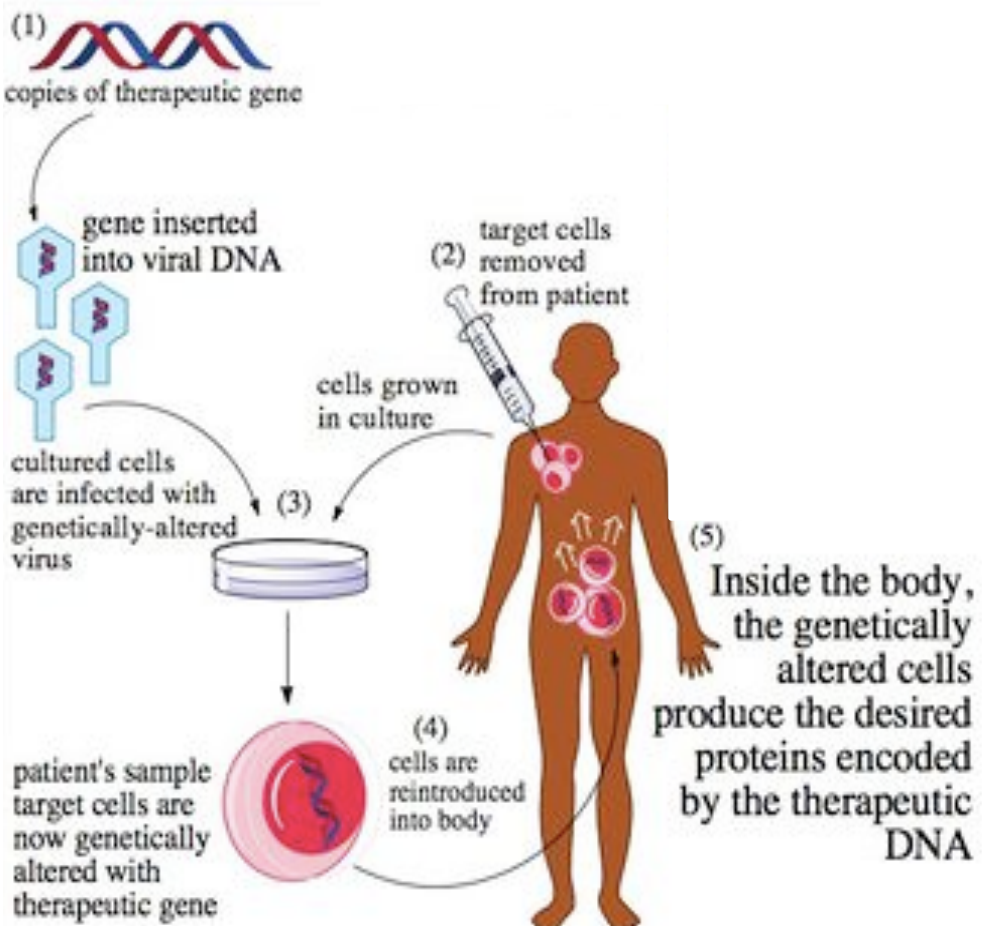
Nature Reviews genetics

Gene-therapy approaches

In vivo



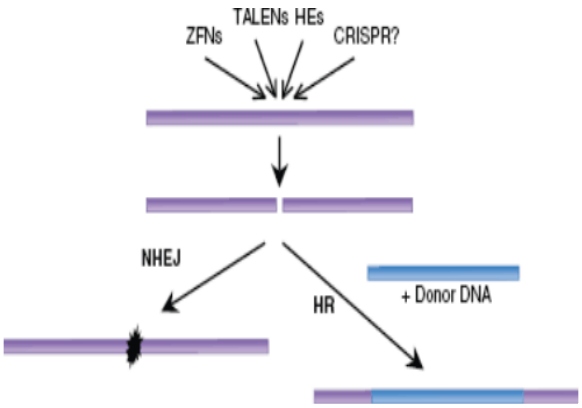
Ex vivo



Genome editing systems



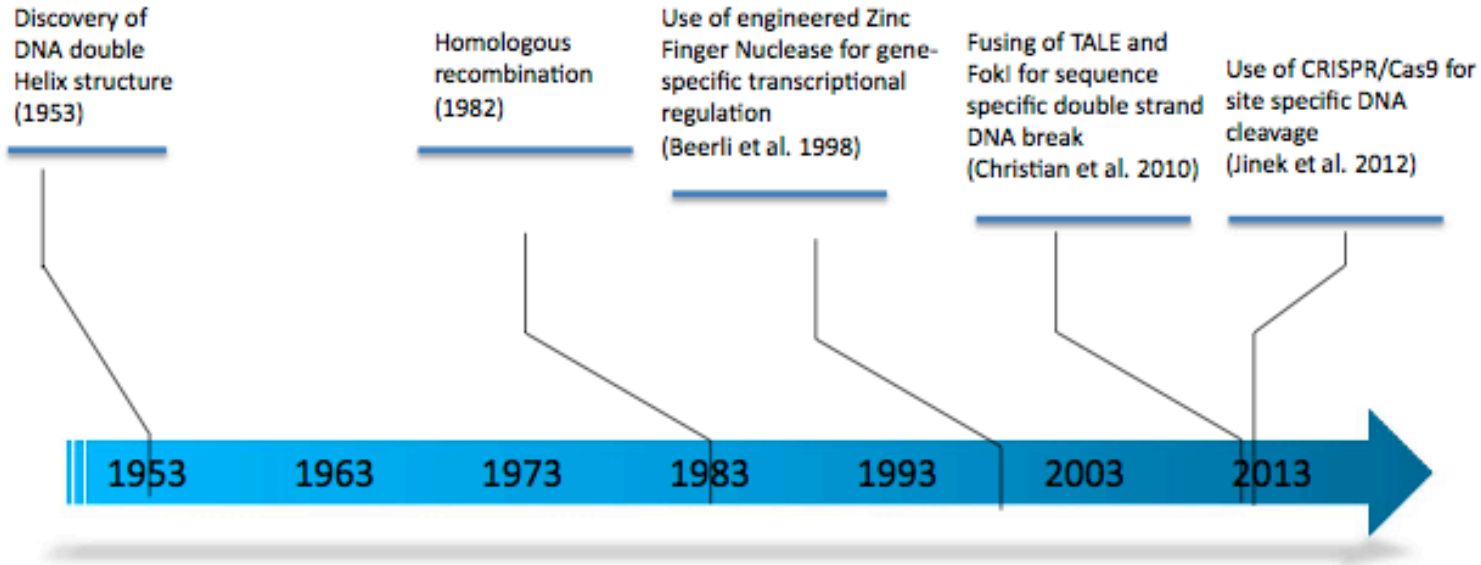
From bacteria to model organisms and human cells, genome editing tools such as zinc-finger nucleases (ZNFs), TALENs, and CRISPR/Cas9 have been successfully used to manipulate the respective genomes with unprecedented precision.



Editing Options

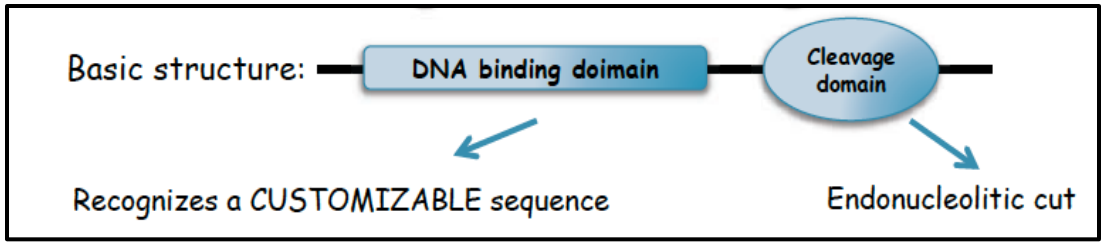
	1 Zinc finger nucleases	2 TALENs	3 CRISPR
What is it?	A protein consisting of a DNA-cutting enzyme and a DNA-grabbing region that can be tailored to recognize different genes.	Also a protein containing a DNA-cutting enzyme and a DNA-grabbing region that can be programmed to recognize different genes, but it is easier to design than zinc finger nucleases.	A DNA-cutting protein guided by an RNA molecule that is able to find the specific gene of interest.
Pros and cons	It was the first programmable genome-editing tool, but it relies on proteins that can be difficult to engineer for new gene targets. Potentially dangerous off-target cuts are also possible.	Though simpler and cheaper to design than zinc finger nucleases, TALEN proteins can still be difficult to produce and deliver. Off-target cuts are a problem.	This technique is affordable and easy to use, and it works for high-throughput, multi-gene experiments. Like the other tools, it can make off-target cuts.

Timeline of genome engineering research

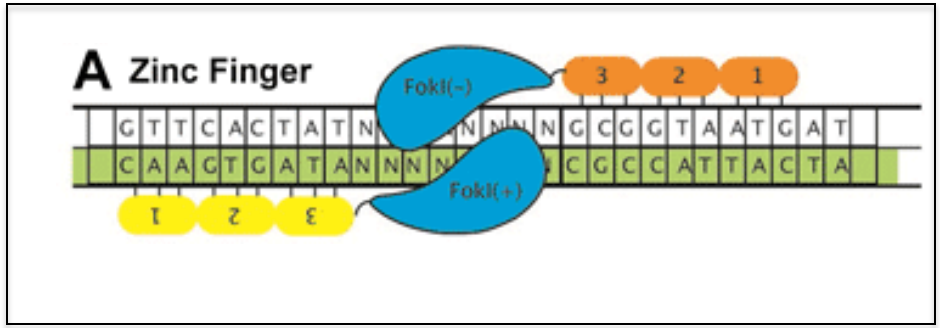


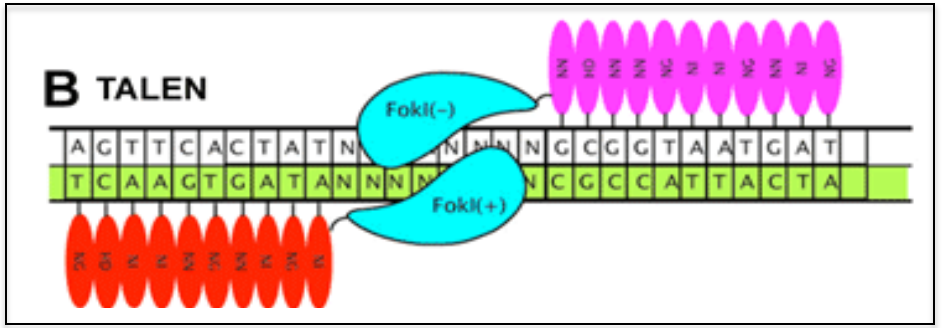
This technology can be used to investigate the function of a gene of interest or to correct gene mutations in cells via genome editing, paving the way for future therapy approaches.

Pellegatti et al. Arch Toxicol 2015

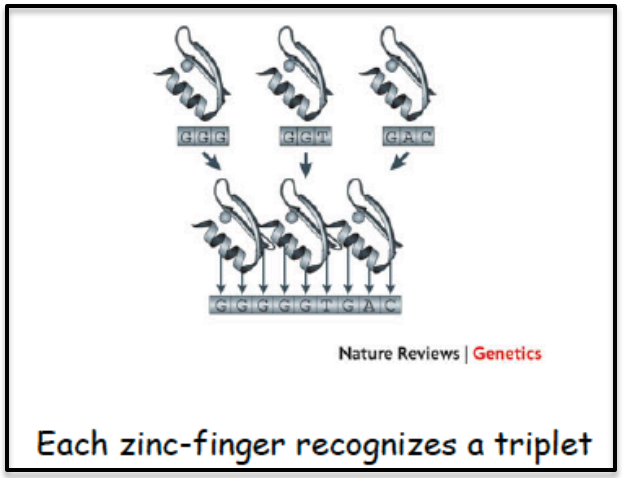


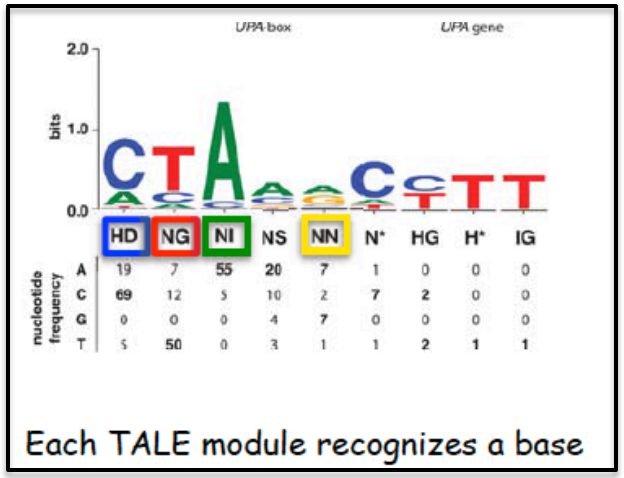
similarities



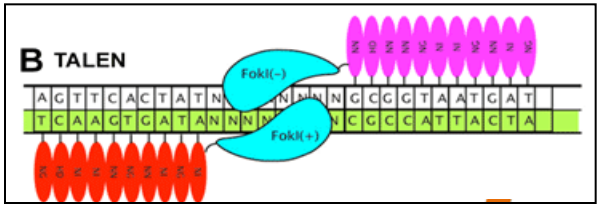


differences



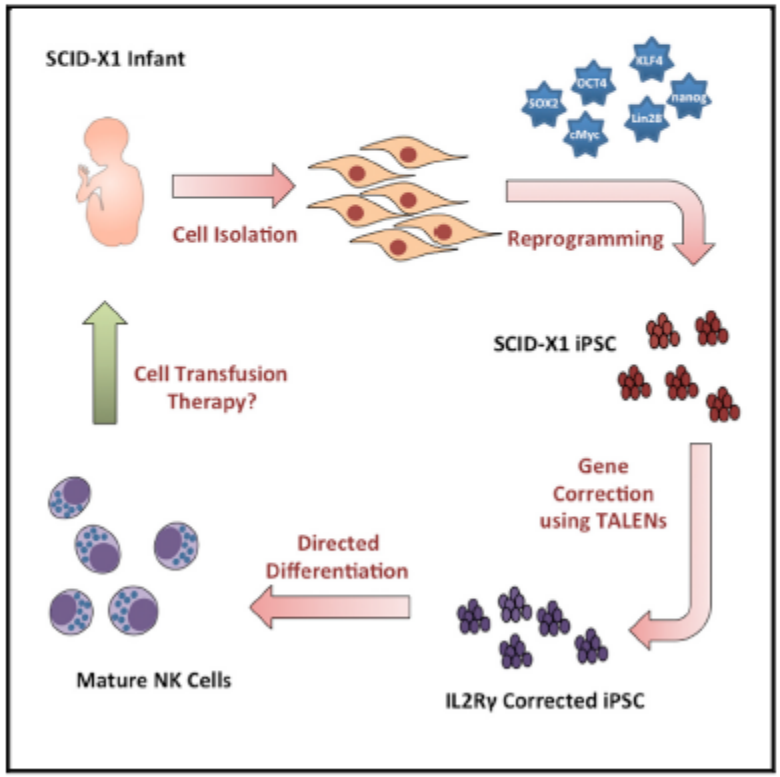


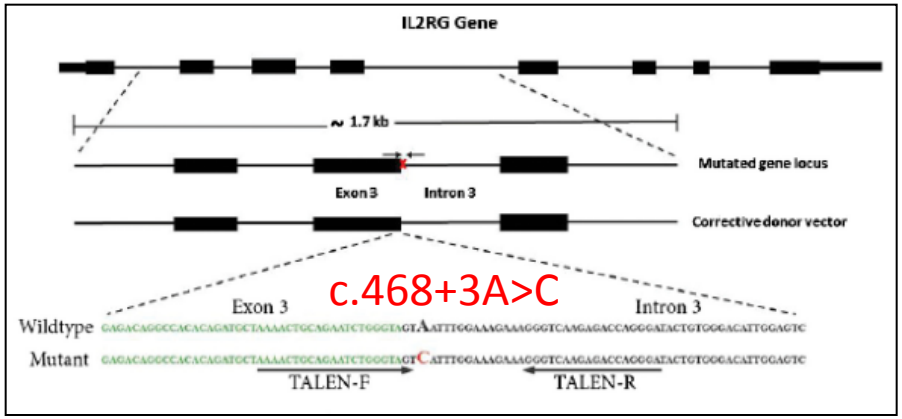
Lymphoid Regeneration from Gene-Corrected SCID-X1 Subject-Derived iPSCs



Tushar Menon,[1,7] Amy L. Firth,[1,7] Deirdre D. Scripture-Adams,[3,4] Zoran Galic,[3,5] Susan J. Qualls,[1] William B. Gilmore,[1] Eugene Ke,[1] Oded Singer,[1] Leif S. Anderson,[1] Alexander R. Bomzin,[1] Ian E. Alexander,[6] Jerome A. Zack,[2,3,4,5] and Inder M. Verma[1,*]

X-linked Severe Combined Immunodeficiency (SCIDX1) is a genetic disease that leaves newborns at high risk of serious infection and a predicted life span of less than 1 year in the absence of a matched bone marrow donor. The disease pathogenesis is due to mutations in the gene encoding the Interleukin-2 receptor gamma chain (IL-2Rg), leading to a lack of functional lymphocytes.

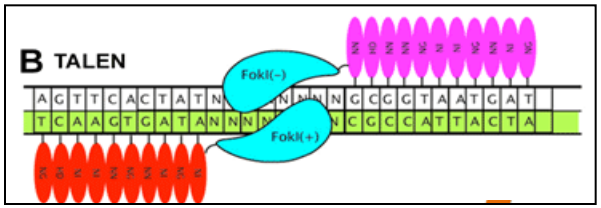


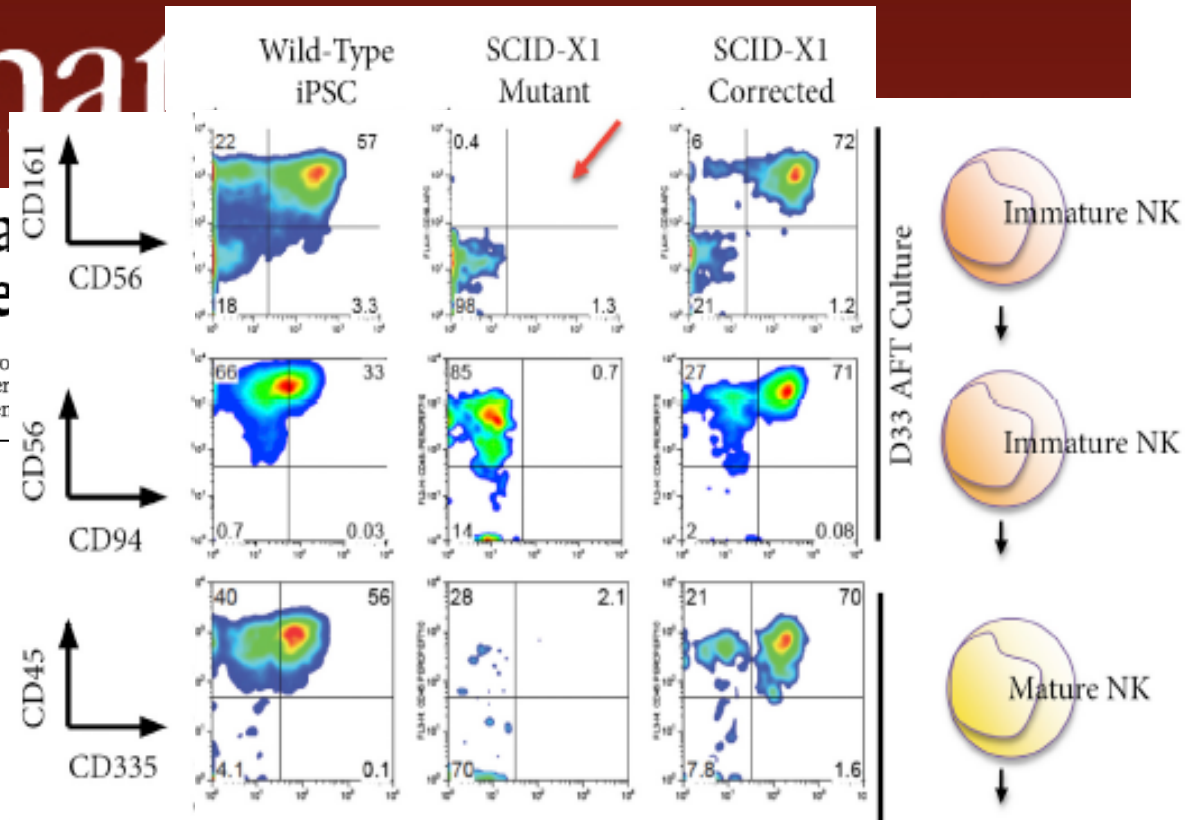


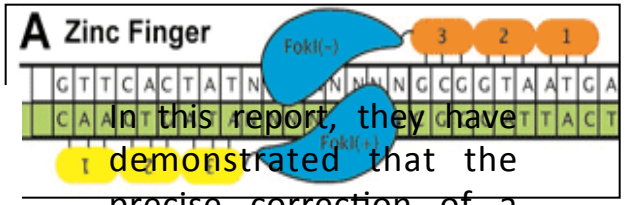
This specific mutation results in a lack of functional NK cells and T cells. The mutation is an A to C substitution in the third base pair of intron 3 of the IL-2Rg gene, leading to aberrant splicing of the IL-2Rg transcript.

Lymphoid Regeneration from Gene-Corrected SCID-X1 Subject-Derived iPSCs

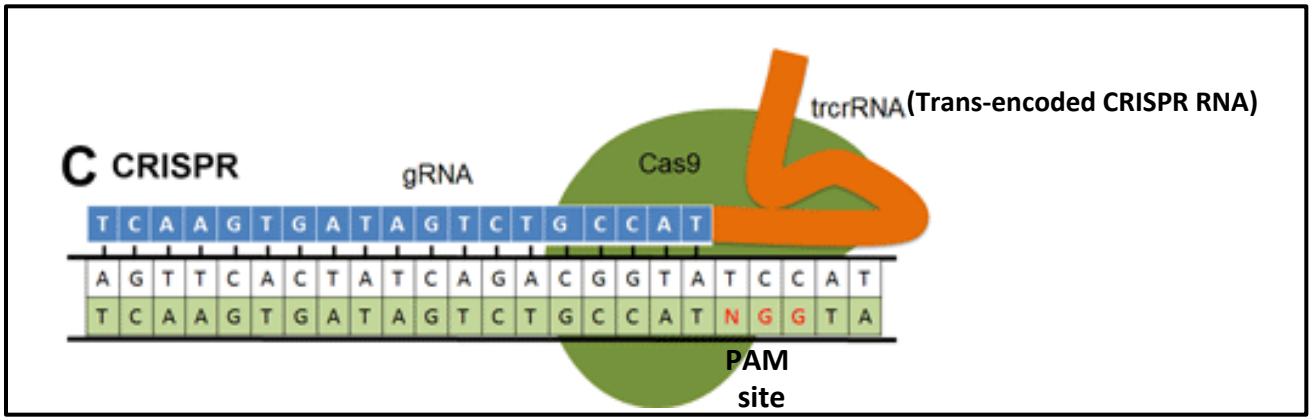
Tushar Menon,[1,7] Amy L. Firth,[1,7] Deirdre D. Scripture-Adams,[3,4] Zoran Galic,[3,5] Susan J. Qualls,[1] William B. Gilmore,[1] Eugene Ke,[1] Oded Singer,[1] Leif S. Anderson,[1] Alexander R. Bomzin,[1] Ian E. Alexander,[6] Jerome A. Zack,[2,3,4,5] and Inder M. Verma[1,*]







In this report, they have demonstrated that the precise correction of a novel splice-site mutation of the IL-2Rg gene at its endogenous genomic locus in subject-derived iPSCs results in the correction of the splicing defect of the IL-2Rg transcript and thus rescues the ability of iPSCs to differentiate into NK cells.



CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

- The CRISPR-Cas9 complex, consisting of the Cas9 protein and the guideRNA (gRNA), is based on the adaptative immune system of Streptococcus Pyogenes .
- Type II system was adapted for genome engineering in many organisms.
- Cas9 protein can introduce ds break or ss nick.
- Provides substrate for error prone repair or HR using recombinant DNA template for custom modification.
- Can also mutate directly by injection into zygote.

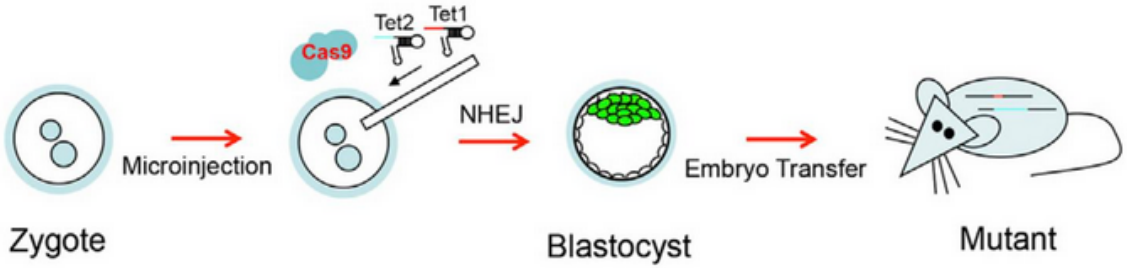
Why is better Crispr-Cas9 system?

The CRISPR/Cas system offers several advantages over the ZNF and TALEN mutagenesis strategies:

1. Target design simplicity. Because the target specificity relies on ribonucleotide complex formation and not protein/DNA recognition, gRNAs can be designed readily and cheaply to target nearly any sequence in the genome specifically.

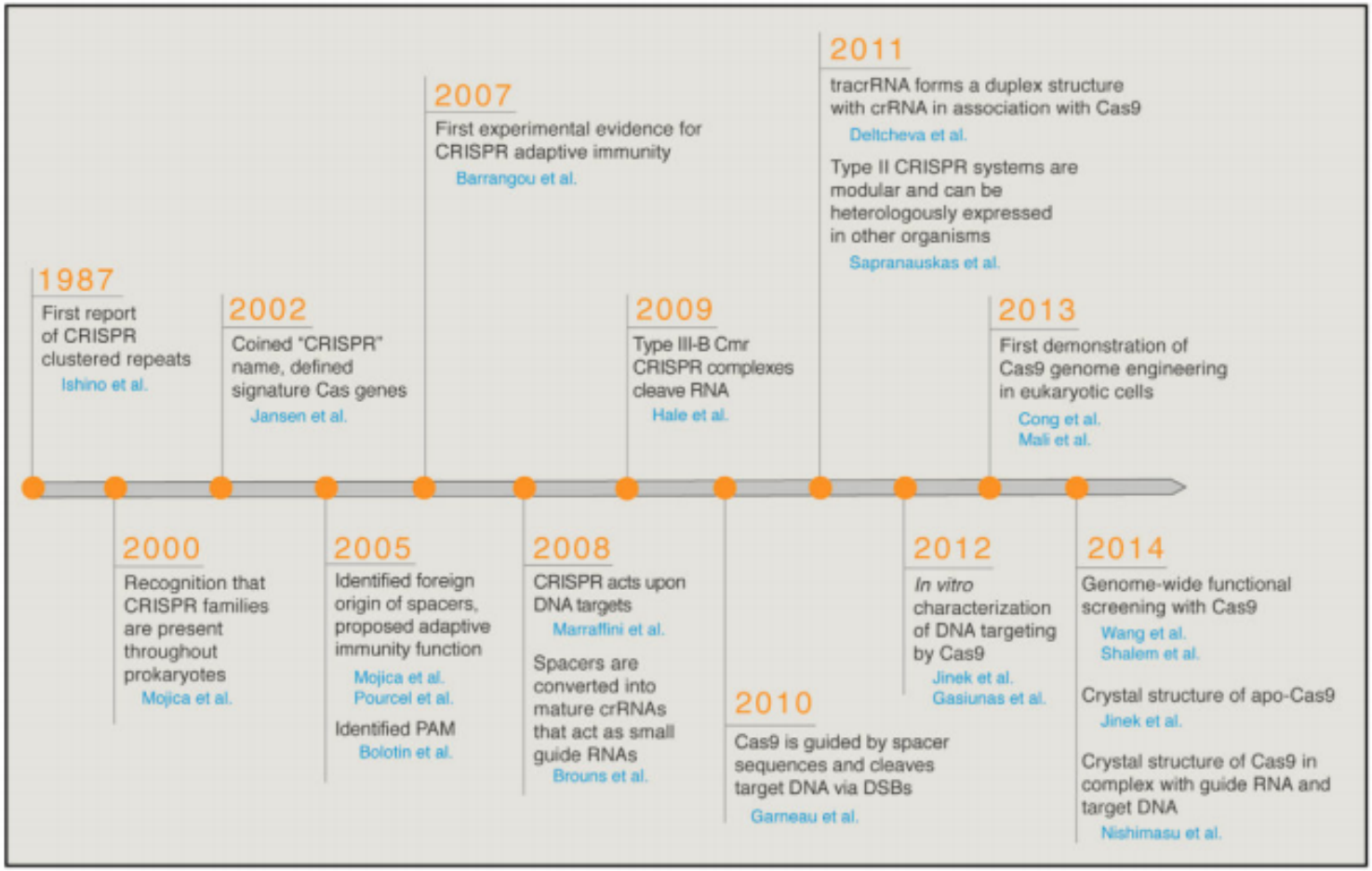
2. Efficiency. The system is super-efficient. Modifications can be introduced by directly injecting RNAs encoding the Cas protein and gRNA into developing mouse embryos. This eliminates the long and laborious processes of transfecting and selecting mouse ES cells that are required to create targeted mutant mice using classical homologous recombination techniques.

3. Multiplexed mutations. Mutations can be introduced in multiple genes at the same time by injecting them with multiple gRNAs. JAX Assistant Professor Dr. Haoyi Wang and his former colleagues in Rudolf Jaenisch's group at the Whitehead Institute recently reported using the CRISPR/Cas system to successfully introducing mutations in five different genes in mouse ES cells simultaneously.



Haoyi Wang et al., Cell 2013

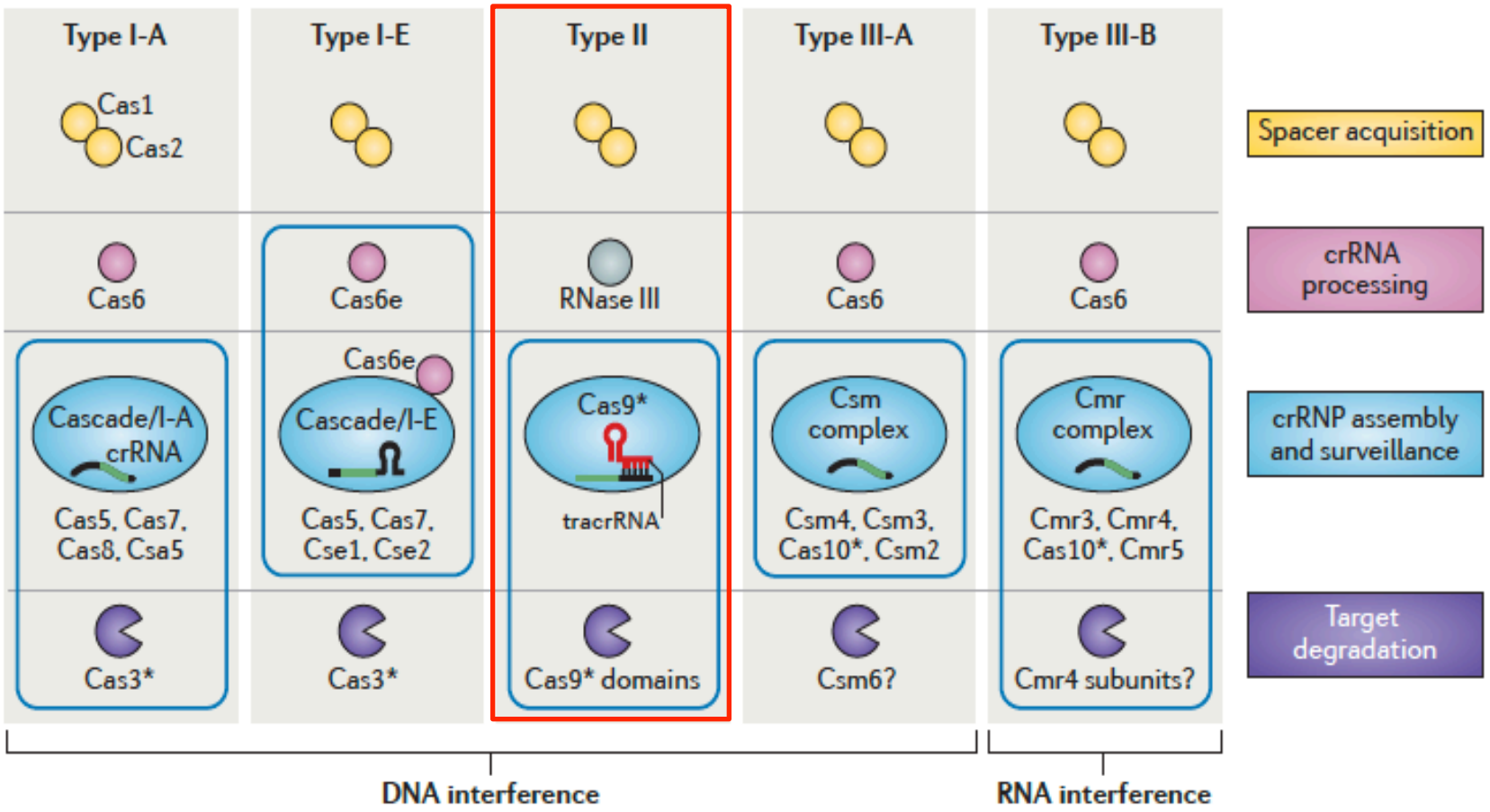
History of the CRISPR-Cas9 system



Patrick D. Hsu et al, Cell 2014

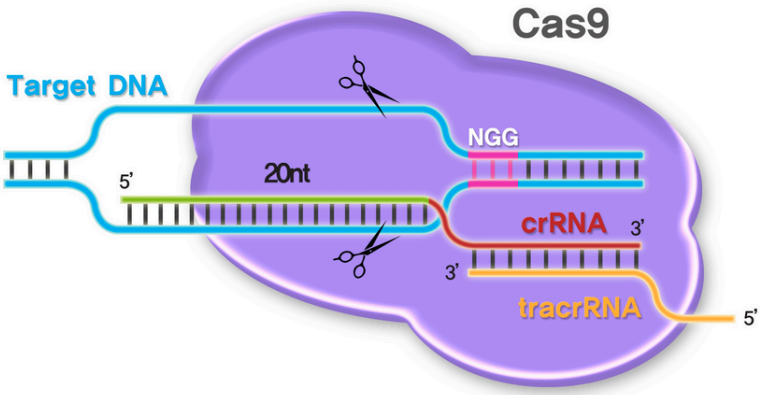
CRISPR-Cas systems

There are at least 11 different CRISPR-Cas systems, which have been grouped into three major types (I-III)



John van der Oost et al. ,Nature Reviewers 2014

The type II system

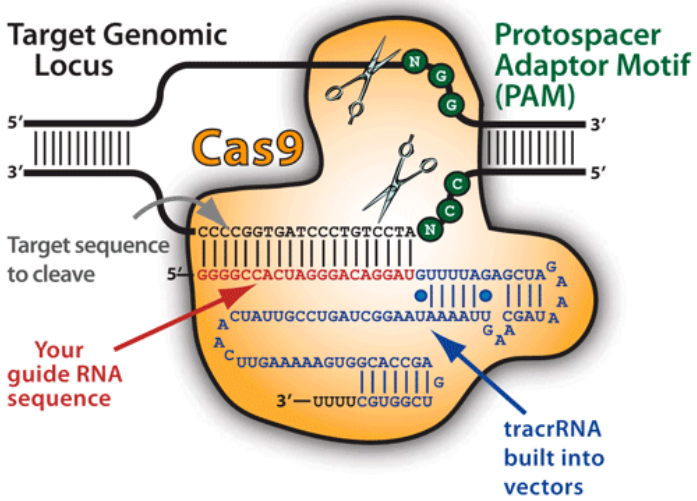


The type II system from *Streptococcus pyogenes* is one of the best characterized consisting of :

-the nuclease Cas9,

-the crRNA array that encodes the guide RNAs (sgRNAs),

-a required auxiliary trans-activating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discete units.



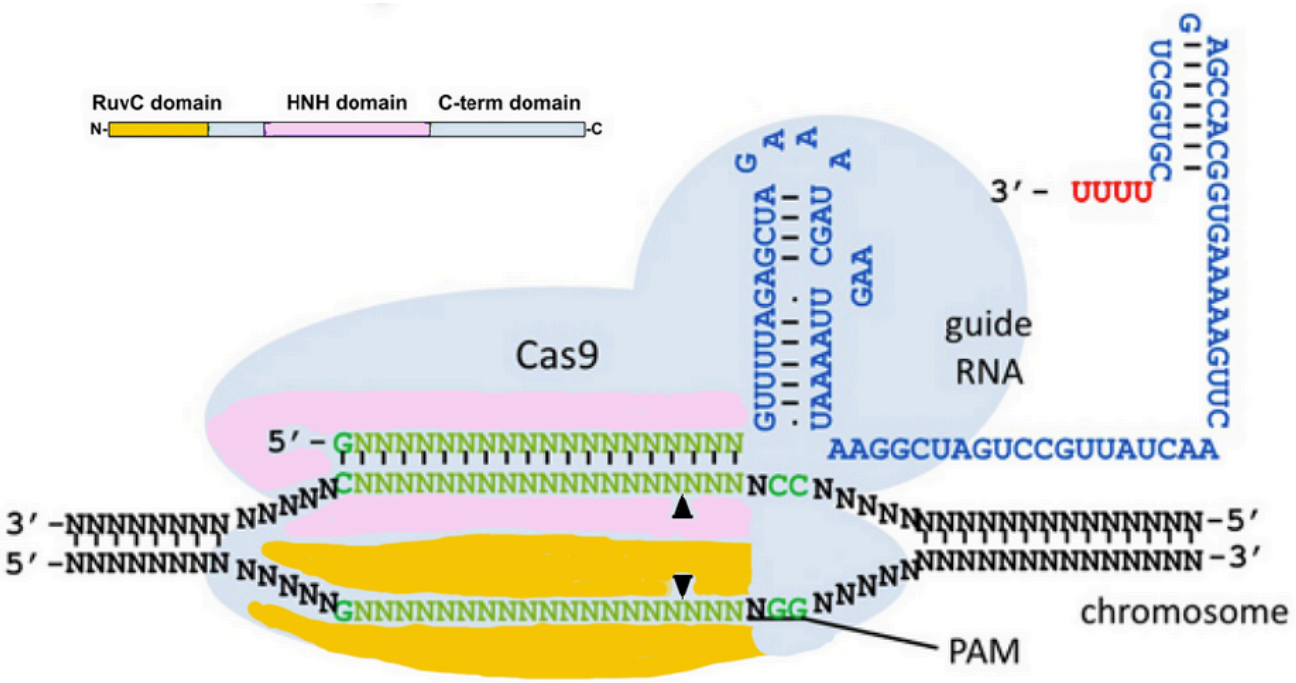
The dual **tracrRNA:crRNA** has been engineered as a sgRNA containing two critical features:

-a DNA complementary guide sequence (tipically 20 nucleotides)

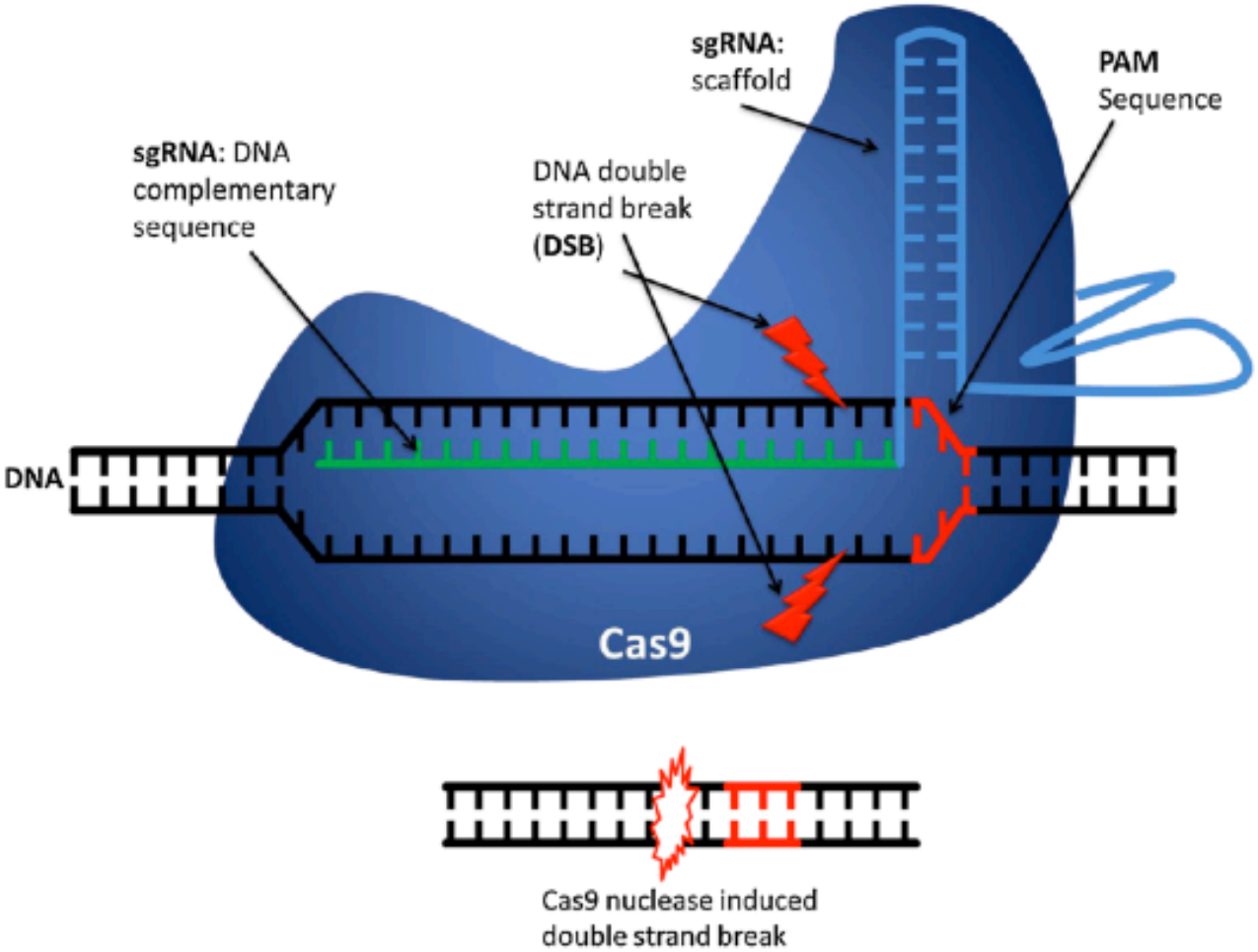
-the double-stranded structure at the 3' side of the guide sequence that bind Cas9

The CRISPR-Cas9 technology uses the Cas9 protein to introduce site-specific DSBs in the target DNA sequence.

- Cas9 nuclease has an RNA binding domain, an α-helical recognition lobe (REC), a nuclease lobe that includes the RuvC and HNH nuclease domains for DNA cleavage and a PAM-interacting site.
- The PAM is a 3 base pair sequence that lies adjacent to the 3' end of the DNA target site and facilitates the target recognition.
- The HNH domain of Cas9 is used to cleave the DNA strand complementary to the crRNA and the RuvC domain is used to cleave the opposite DNA strand

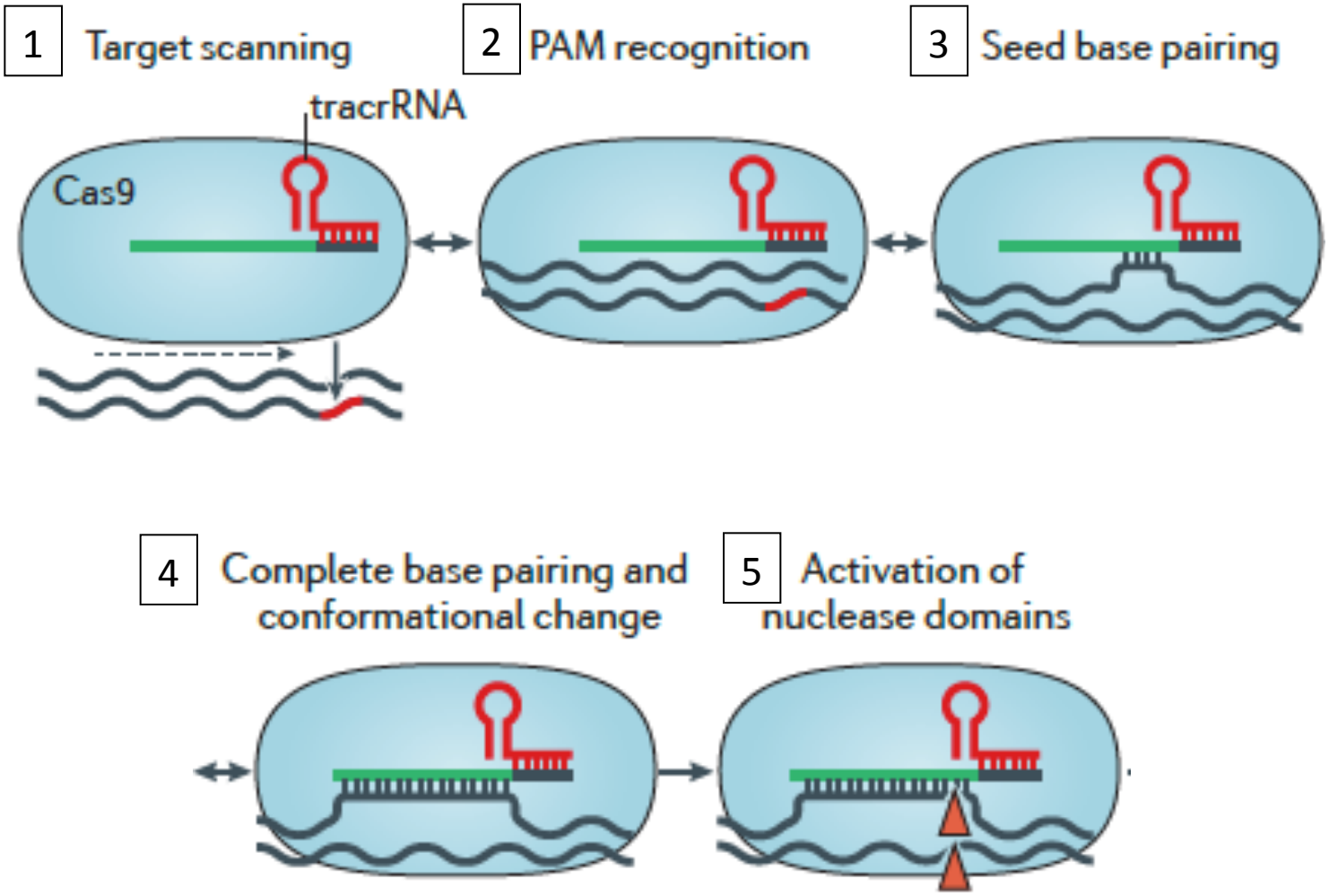


The formation of RNA-DNA heteroduplex is initiated at the PAM site and the binding of the PAM and the presence of a matching target DNA leads to Cas9 nuclease activity.

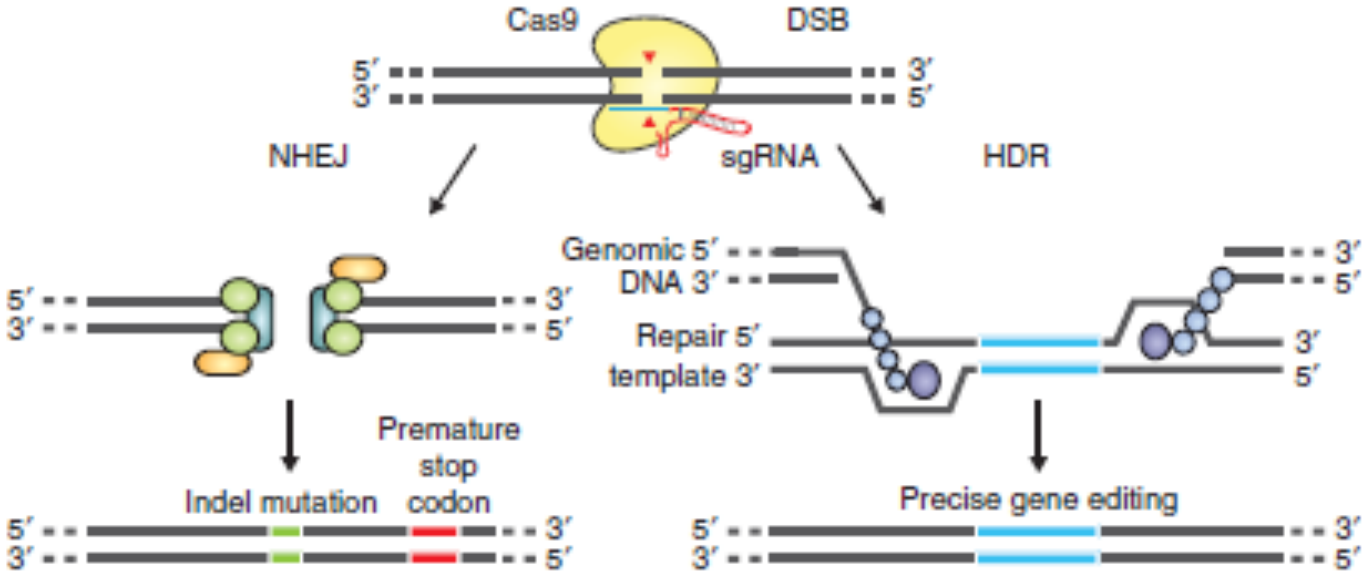


Imaging studies suggest that the complex composed of Cas9 and the RNA-DNA heteroduplex leads to Cas9 initiating DNA strand separation via an unknown mecchanism and subsequently leads to a DSB.

The process:


1
Target scanning
2
PAM recognition
3
Seed base pairing
tracrRNA
Cas9
4
Complete base pairing and conformational change
5
Activation of nuclease domains

Cas9-induced DSBs can be repaired by endogenous DNA repair pathways present in all cell types and organism:

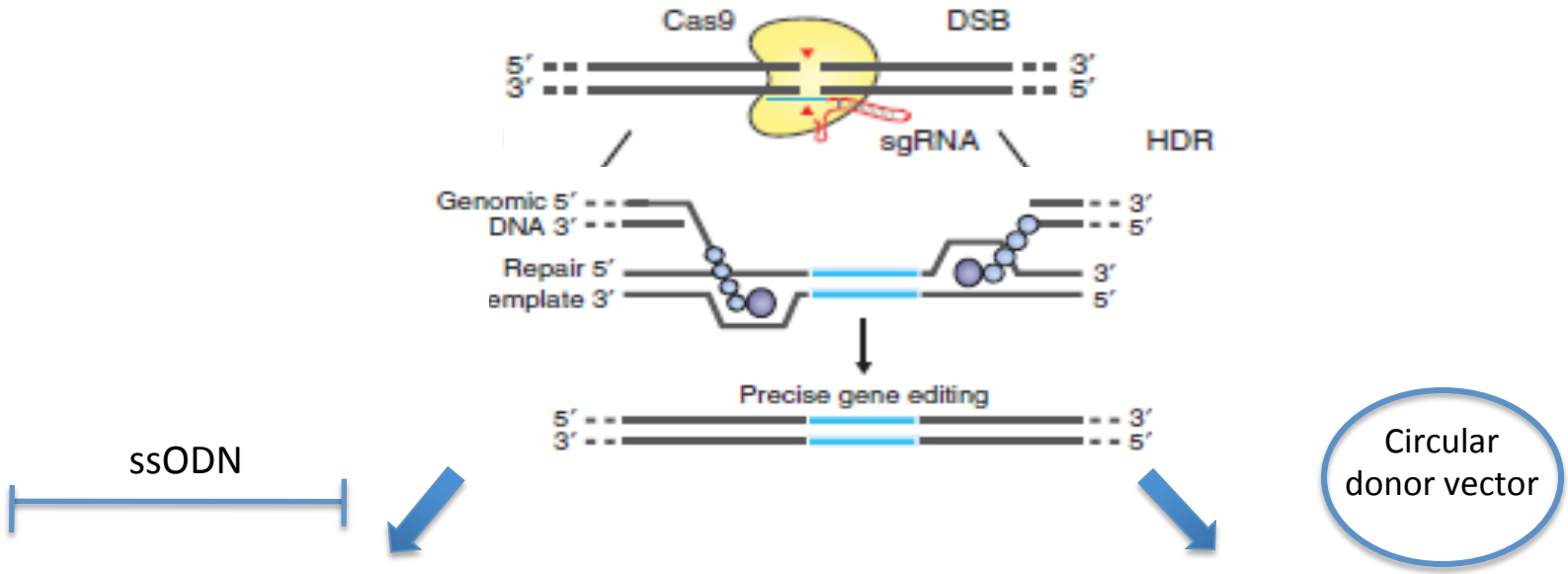


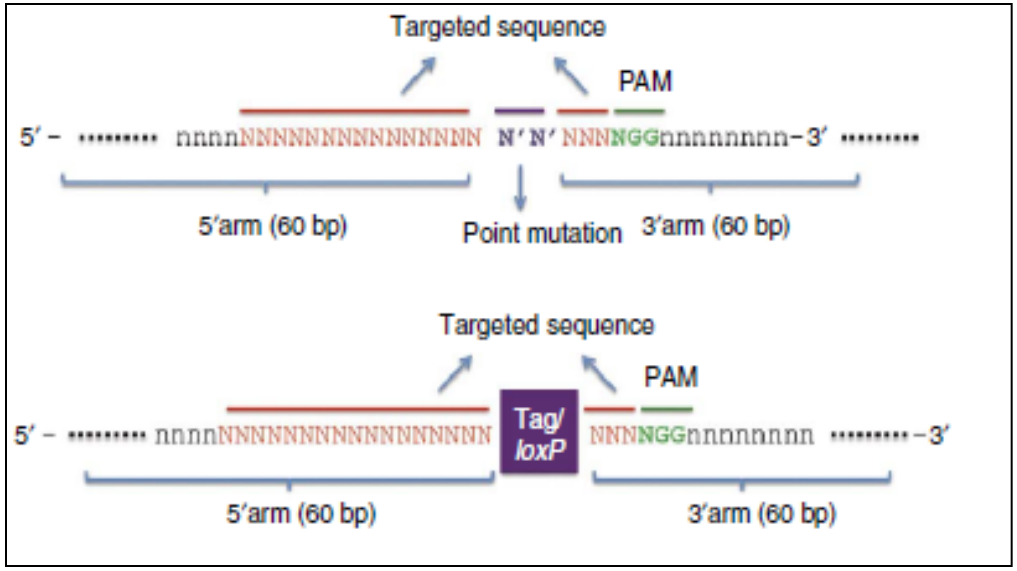
NHEJ (non-homologous end joining) is an error-prone process that involves direct linking of the broken ends and can result in the introduction of insertion/deletion mutations (indels) that, if present in coding exons, can lead to frame-shift and premature stop-codon formation.

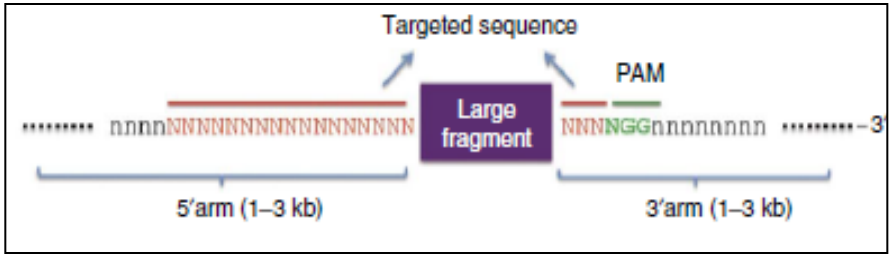
HDR (homology-directed repair) frequently repairs DBSs during the S and G2 phases of cell cycle and uses homologous DNA sequences as templates for repair and, by supplying an exogenous repair template, HDR can be exploited to precisely edit genomic sequence or insert exogenous DNA.

Ran et al., *Nature Protocol* 2013

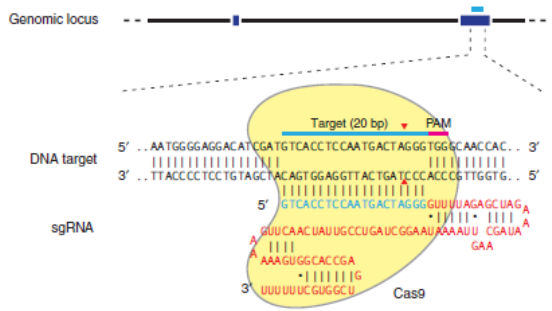
For HDR, we have 2 different strategies based on the recombinant region size :







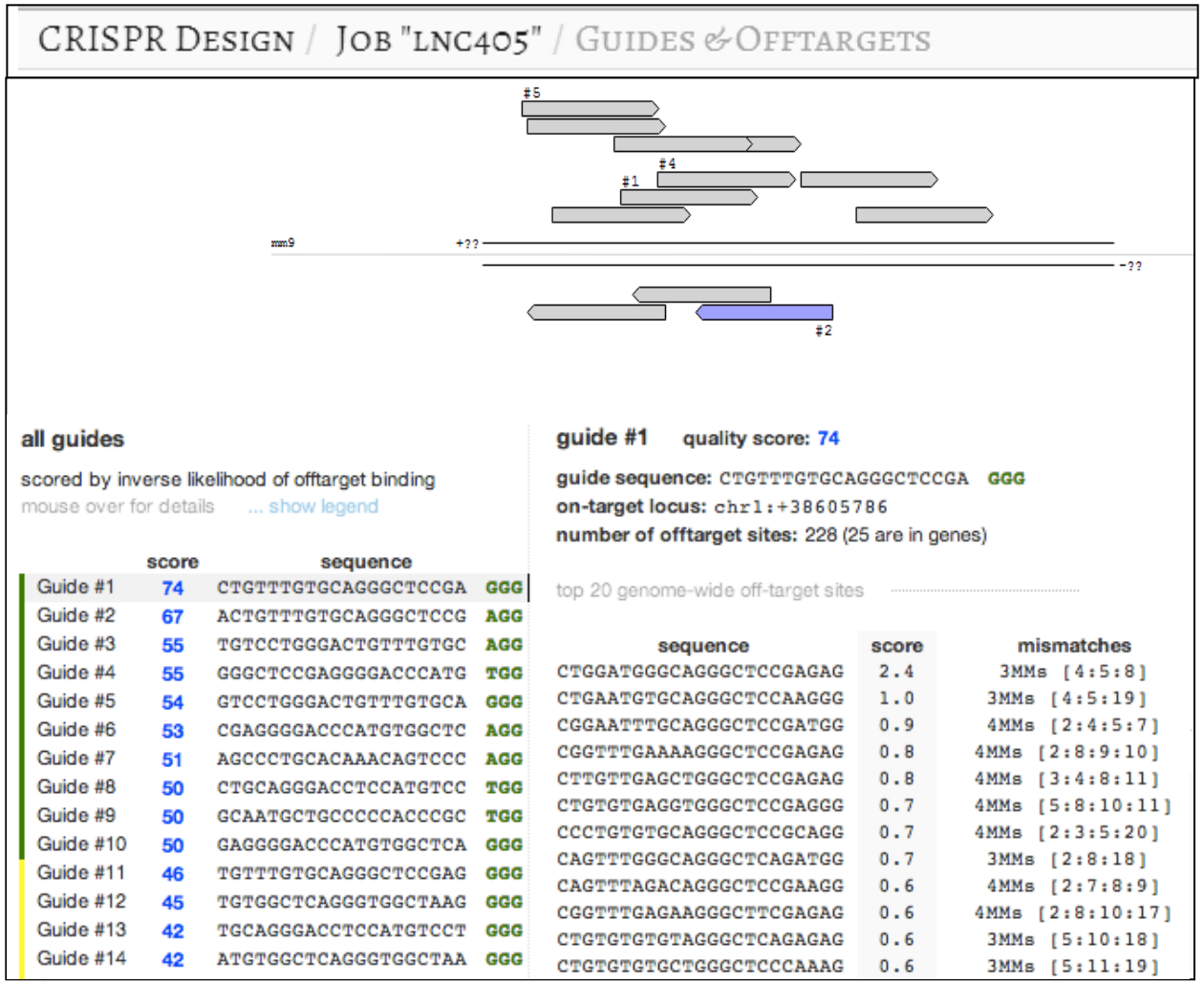
Yang et al., *Nature Protocol* 2014



Design sgRNA

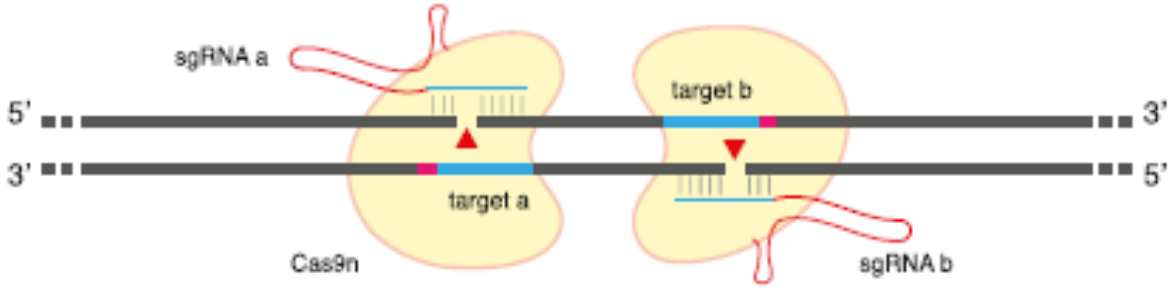
The CRISPR design tool is a web tool crafted to simplify the process of CRISPR guide selection in an input DNA sequence by:

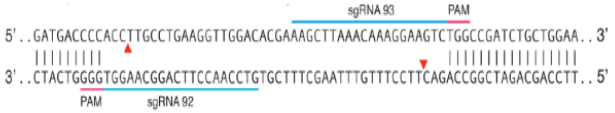
- ✓ discovering possible offtargets genome-wide,
- ✓ highlighting guides with high target specificity,
- ✓ flagging guides with numerous or genic offtargets in target genomes.

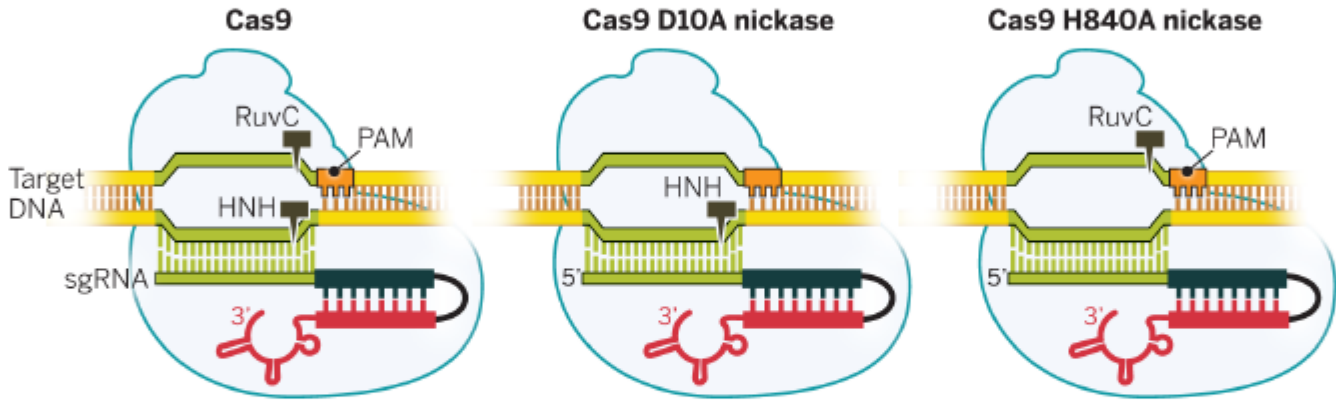


http://crispr.mit.edu/

Double-nicking strategy for minimizing off-target





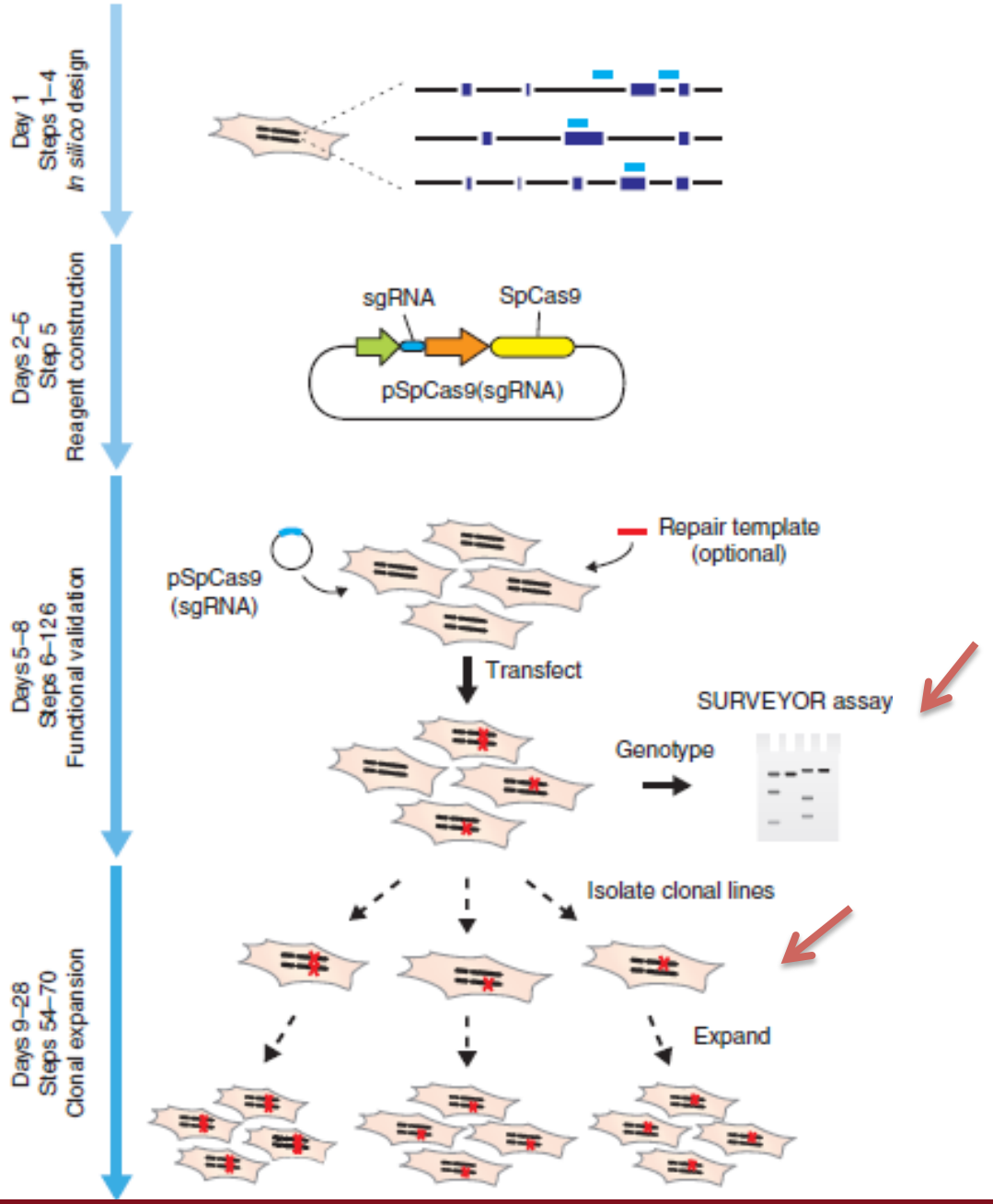


- Cas9 nickase can facilitate targeted DNA double-strand break using two guide RNAs
- Double nicking of DNA reduces off-target mutagenesis by 50- to 1,000-fold
- Multiplex nicking stimulates homology directed repair, microdeletion, and insertion
- Double nicking provides efficient modification of mouse zygotes

Ran et al., *Cell* 2013

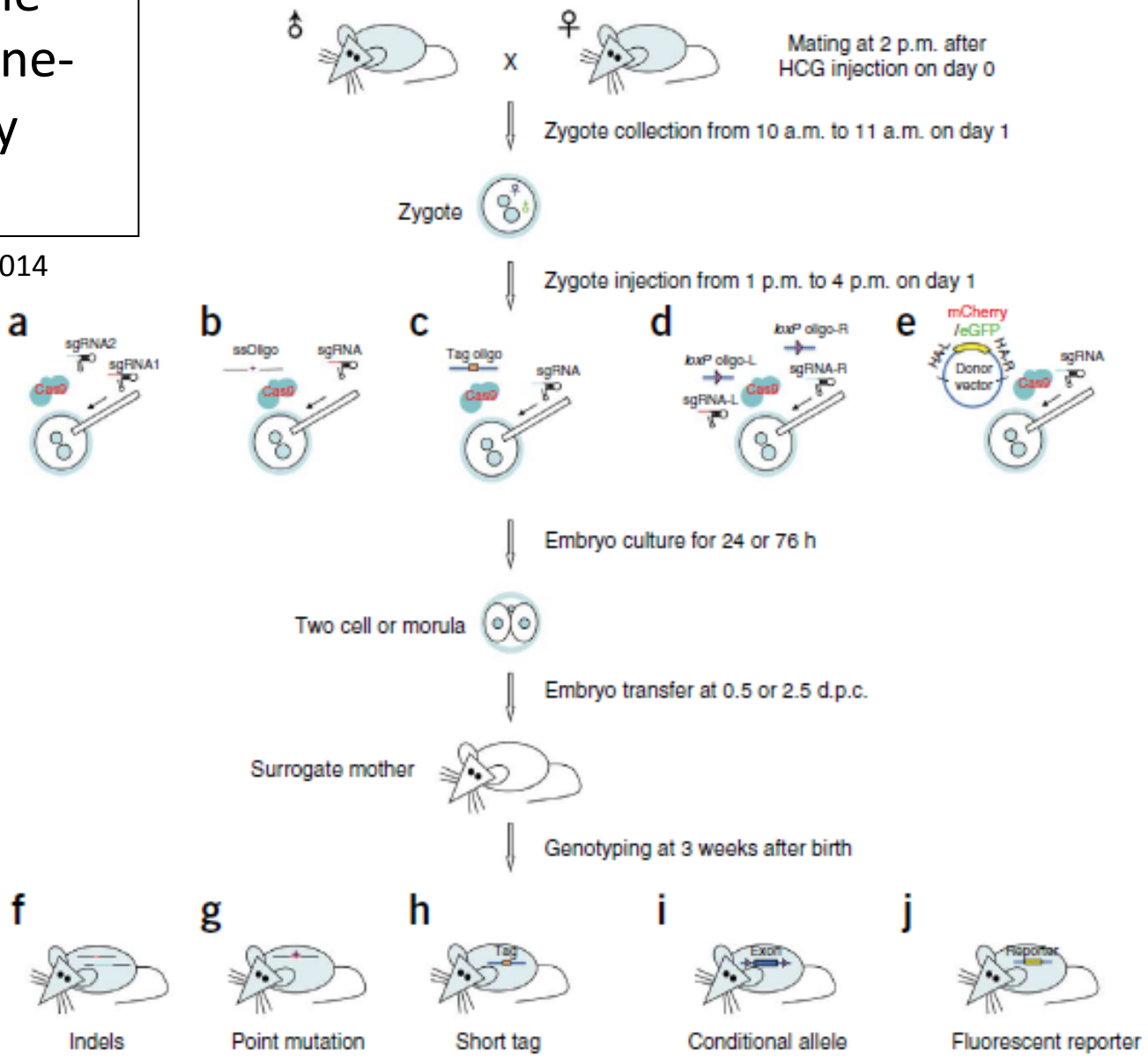
Procedure for the generation of gene-modified cell line by CRISPR-Cas9

Ran et al., *Nature Protocol* 2013

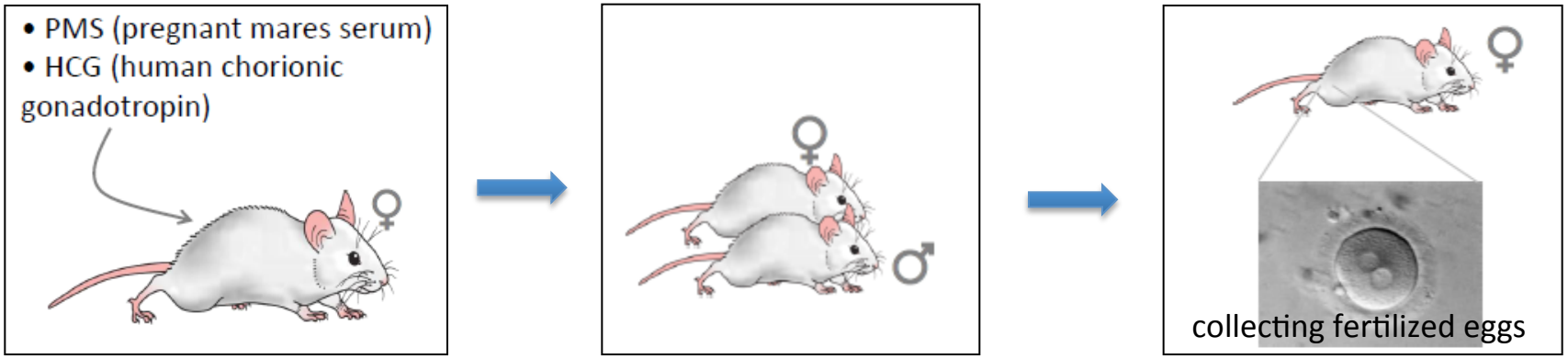


Procedure for the generation of gene-modified mice by CRISPR-Cas9

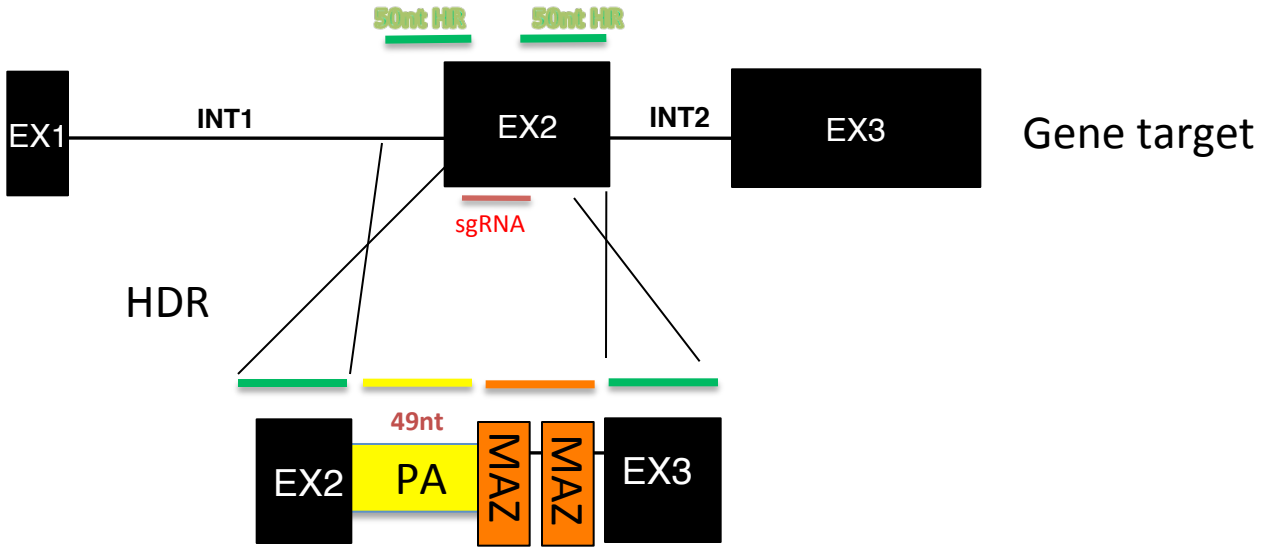
Yang et al., Nature protocol 2014



in detail …*in vivo*



1°example HDR: poliA insertion

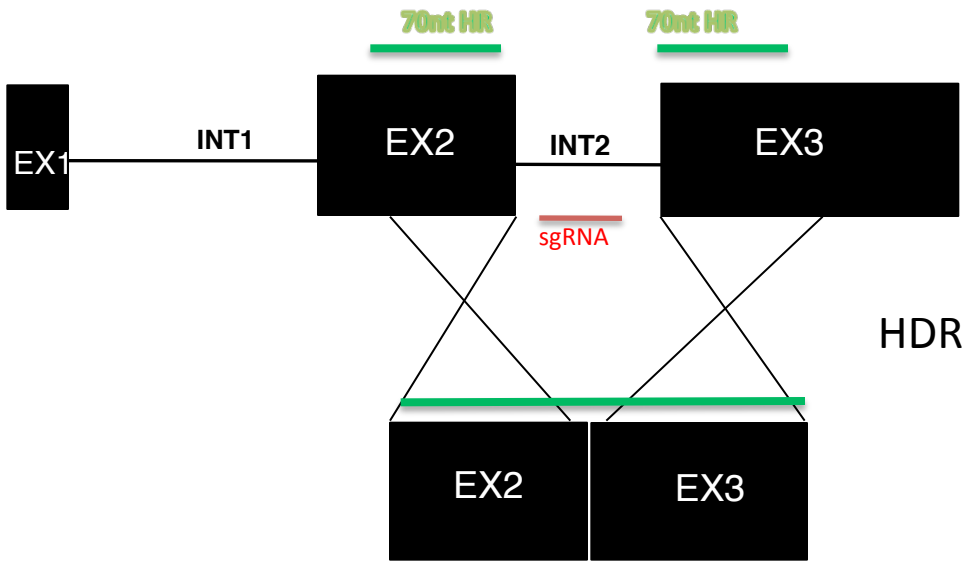


Single-stranded oligodeoxynucleotide (ssODN) as a template for HDR-mediated gene repair

200nt

tggacactggctggagGAATGAGGCCTGGAAGCAGGAAGCAAAAGATCAGAATAAAAGATCTTTATTTT
CATTAGATCTGTGTGTTGGTTTTTTGTGTGCTTGATCGGGGGGAGGGGGagcttcacgtacaatcgagtc
GGGGGAGGGGG CTCGGAGAGAAGGAAGAGTGCTGCCCCAGACACAGCGGgtaagagccag

2°example HDR: intron deletion



Gene target

Another strategy?

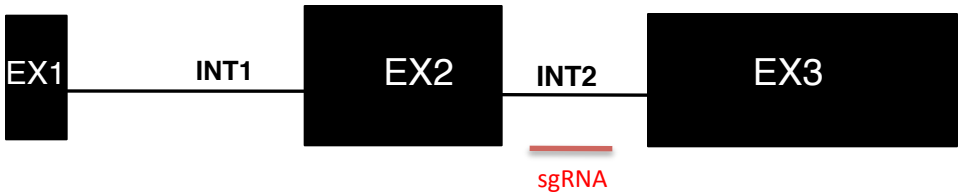
Single-stranded oligodeoxynucleotide (ssODN) as a template for HDR-mediated gene repair

140nt

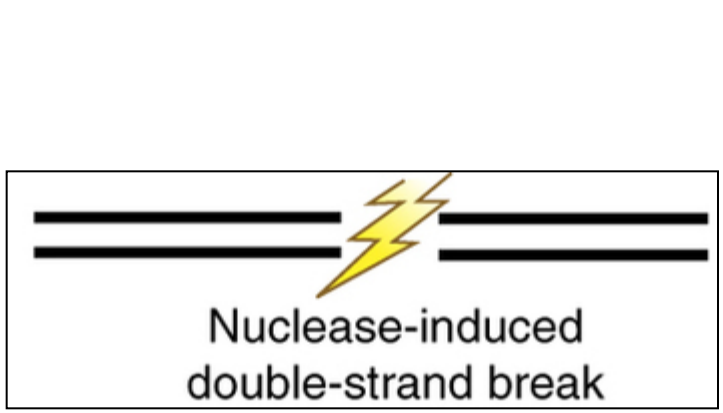
ssODN: 70nt (ex2)+70nt (ex3)

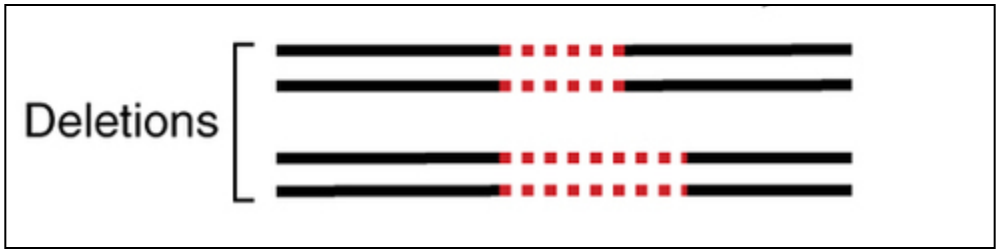
atgaggcctggaagcaggaagcaaaagatcagctcggagagaaggaagagtgctgcccca
gacacagcgggtgccccatcttcttcaggcatcagcaatgaaagacctcggctgacttca
gttttccagatgaaaacatc

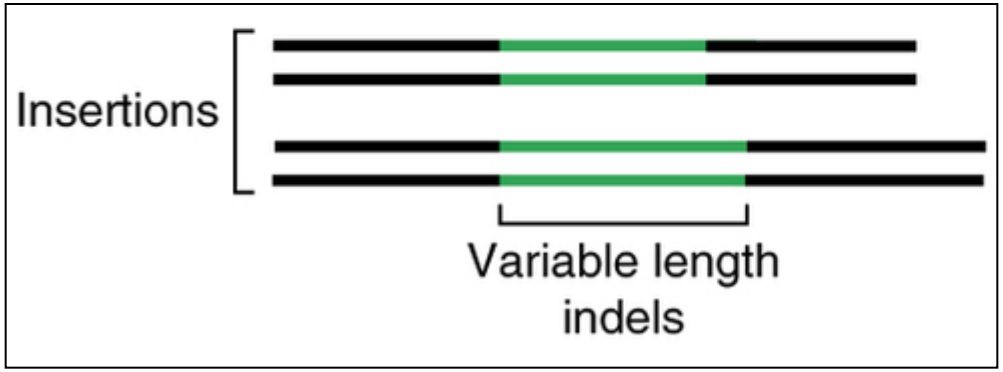
Example NHEJ



Gene target

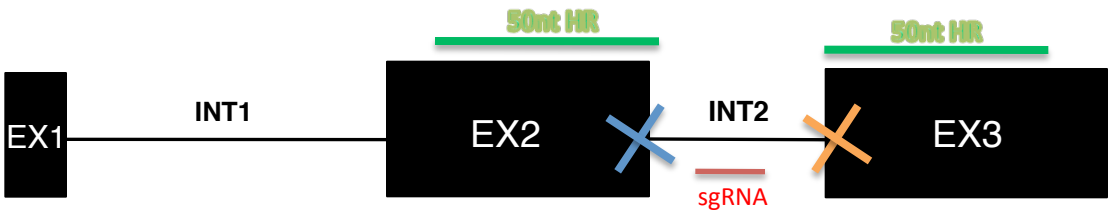






This system is not accurate

3°example HDR: splice site mutation



5' SPLICE SITE mutation:

wt GCGGGTAA
mut CCGCGGAA (SACII)

3' SPLICE SITE mutation

wt : AG

mut : AC

Single-stranded oligodeoxynucleotide (ssODN) as a template for HDR-mediated gene repair

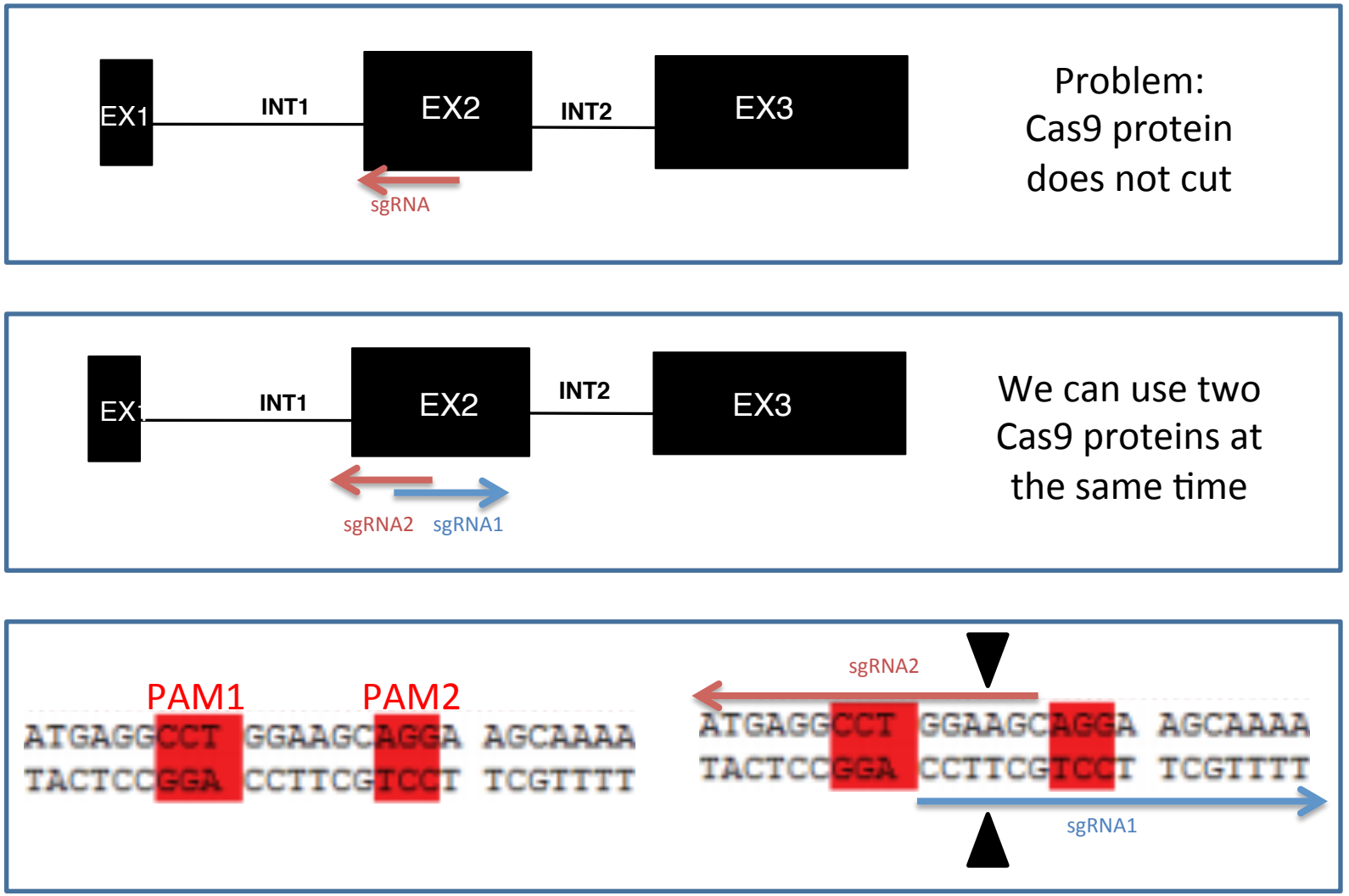
200nt

50nt HR: caaaagatcagctcggagagaaGgaagagtgctgccccagacacaCcgC

INT2:GGAAGAGCCAGAACTGTGTGCAATCCTGGGCTGGCCAGCGATGGGCTCTGGGACTCTTGTGAGA
TTGATGAAGGCTGATGAGGCTGCTCTGTCCCCTGCCAC

50nt HR: gtgccccatcttcttcaggcatcagcaatgaaagacctcggctgacttc

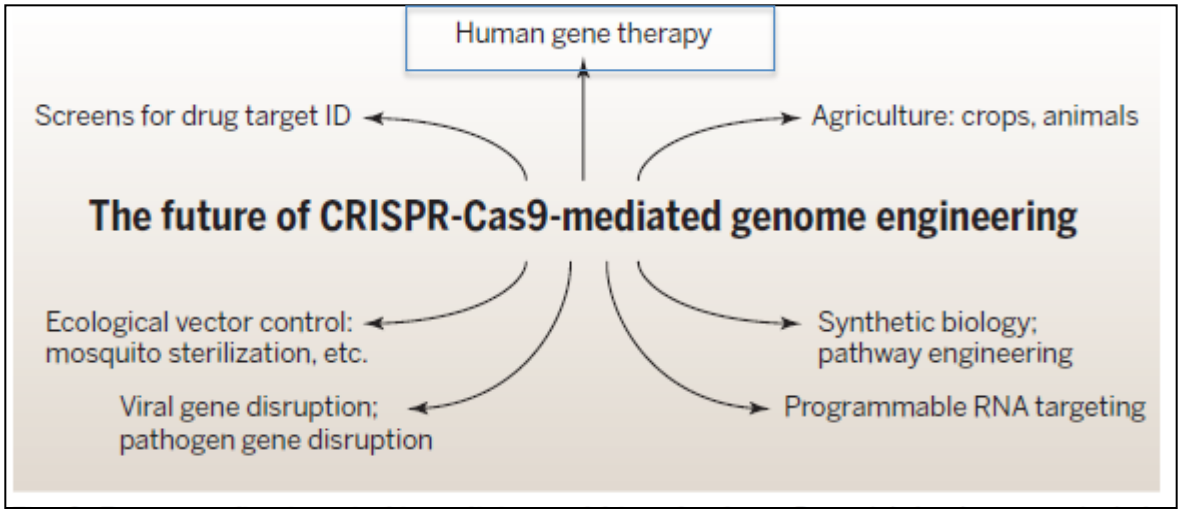
4°example: insertion with 2 Cas9 proteins



Examples of cell types and organism that have been engineered using Cas9

Biology		Biotechnology		Biomedicine
Cell lines	**Model organisms**	**Crop plants**	**Fungi**	Organoids
HEK293	Mice	Rice	*Kluyveromyces*	hESCs
U2OS	Rats	Wheat	*Chlamydomonas*	iPSCs
K562	Fruit flies	Sorghum		
	Nematodes	Tobacco		
	Arabidopsis			
	Salamanders			
	Frogs			
	Monkeys			

Future applications in biomedicine and biotechnology



Doudna and Charpentier, Science 2014

Examples of TALENs and CRISPR/Cas9-mediated genome editing in human cells

Niu et al.,Mol Biotechnol 2014

Type of modification	Cell type	Genes	Nucleases	Efficiency (%)	Method of transfection	Refs.
Gene addition	HUES62	EMX1	CRISPR	0.40	Nucleofector	[45]
	hESCs	AKT2	TALEN	1.60	FuGENE 6	[5]
	hESCs	AKT2	CRISPR	11	FuGENE 6	[36]
	K562	CCR5	TALEN	16	Nucleofector	[8]
	293T	EMX1	CRISPR	0.18–27	Lipofectamine 2000	[28]
	293T	EMX1	CRISPR	0.46–0.7	Lipofectamine 2000	[33]
	293FT	EMX1	CRISPR	1–20	Lipofectamine 2000	[43]
	HUES9	EMX1	CRISPR	1.6–6.1	Nucleofector	[43]
	293T	EMX1	CRISPR	1.7–17	Lipofectamine 2000	[45]
	HUES9	EMX1	CRISPR	2.2–6	Nucleofector	[28]
Gene correction	iPSCs	OCT4	TALEN	0–100	Nucleofector	[9]
	iPSCs	PITX3	TALEN	1–23	Nucleofector	[9]
	hESCs	PITX3	TALEN	13–23	Nucleofector	[9]
	293T	HPRT1	TALEN	2.2–8.8	Lipofectamine LTX	[60]
	293T	AAVS1	CRISPR	3–8	Lipofectamine 2000	[23]
	iPSCs	AAVS1	TALEN	43–66	Nucleofector	[9]
	hESCs	AAVS1	TALEN	47–77	Nucleofector	[9]
	hESCs	OCT4	TALEN	6–97	Nucleofector	[9]
Gene disruption	HuH-7	APOB	TALEN	7.10	FuGENE 6	[5]
	iPSCs	CIITA	TALEN	12.70	FuGENE 6	[36]
	293T	IL2RG	TALEN	14	Polyethylenimin (PEI)	[4]
	293T	CCR5	TALEN	17	Polyethylenimin (PEI)	[4]
	KBM-7	AAVS1	CRISPR	72	Lentiviral vectors	[41]
	293T	ATM, APC	TALEN	0.3–19.8	Lipofectamine LTX	[40]
	hESCs	AKT2, ANGPTL3, ATGL, CELSR2, C6orf106, GLUT4, LINC00116, PLIN1, SORT1, TRIB1	TALEN	1.6–33.5	FuGENE 6	[5]
	293T	EMX1	CRISPR	1.9–29	Lipofectamine 2000	[33]
	293T	AAVS1	CRISPR	10–25	Lipofectamine 2000	[23]
	K562	AAVS1	CRISPR	13–38	Nucleofector	[23]
	293FT	PVALB	CRISPR	18.3–49.9	Lipofectamine 2000	[43]
	293T	CCR5	CRISPR	2–18	Lipofectamine 2000	[32]
	iPSCs	AAVS1	CRISPR	2–4	Nucleofector	[23]
	iPSCs	CFTR, CIITA, NLRC5, PLIN1, TTN	TALEN	2.1–12.7	FuGENE 6	[5]
	293T	EMX1	CRISPR	24.1–40	Lipofectamine 2000	[45]
	K562	NTF3	TALEN	3–9	Nucleofector	[8]
	293T	EMX1	TALEN	3.6–4.5	Lipofectamine 2000	[33]
	293FT	EMX1	CRISPR	4.9–51.9	Lipofectamine 2000	[43]
	K562	CCR5	TALEN	5–21	Nucleofector	[8]
	K562	C4BPB	CRISPR	5–33	Nucleofector,	[32]
	hESCs	AKT2, CELSR2, GLUT4, LINC00116, SORT1, LDLR	CRISPR	51.1–78.7	FuGENE 6	[36]
	293T	PVALB	CRISPR	7.3–15	Lipofectamine 2000	[33]

CRISPR-Cas9 revolution

Mol Biotechnol. 2014 Aug;56(8):681-8. doi: 10.1007/s12033-014-9771-z.

Applications of TALENs and CRISPR/Cas9 in human cells and their potentials for gene therapy.

Niu J[1], Zhang B, Chen H.

J Med Genet. 2015 Feb 24. pii: jmedgenet-2014-102968. doi: 10.1136/jmedgenet-2014-102968. [Epub ahead of print]

CRISPR-Cas9: a new and promising player in gene therapy.

Xiao-Jie L[1], Hui-Ying X[2], Zun-Ping K[3], Jin-Lian C[4], Li-Juan J[5].

Expert Opin Biol Ther. 2015 Mar;15(3):311-4. doi: 10.1517/14712598.2015.994501. Epub 2014 Dec 23.

Unraveling the potential of CRISPR-Cas9 for gene therapy.

Barrangou R[1], May AP.

Arch Toxicol. 2015 Apr 1. [Epub ahead of print]

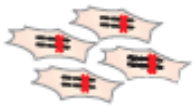
Application of CRISPR/Cas9 genome editing to the study and treatment of disease.

Pellagatti A[1], Dolatshad H, Valletta S, Boultwood J.

2014

Today

Gene terapy with Crispr-Cas9 system

 *In vivo* animal studies	 Cell-based studies
Crygc-associated cataract: 1bp deletion in exon3	HIV-1 resistance: editing CCR5
Fah mutation-related tyrosinemia in hepatocytes: point mutation in exon 8	β-thalassemia: correction of human hemoglobin β-associated β-thalassamia mutations
Reduction cholesterol levels: *PCSH9* knockout mice	Cystic fibrisis transmembrane conductor receptor (CFTR): CFTR exon 11
Duchenne's muscular dystrophy (DMD): *dmd* dystrophin gene correction	Duchenne's muscular dystrophy (DMD): dmd dystrophin gene correction

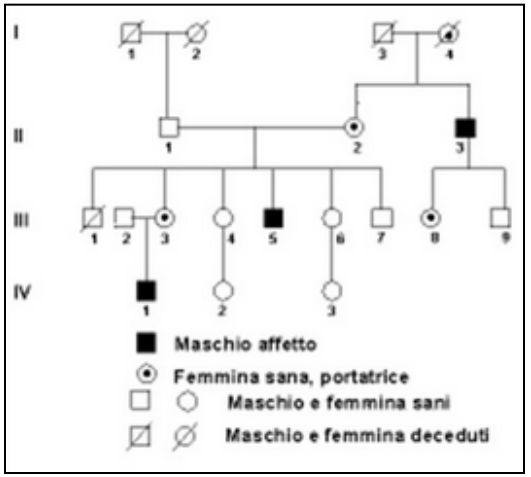
Pellagatti et al.,Arch Toxicol 2015

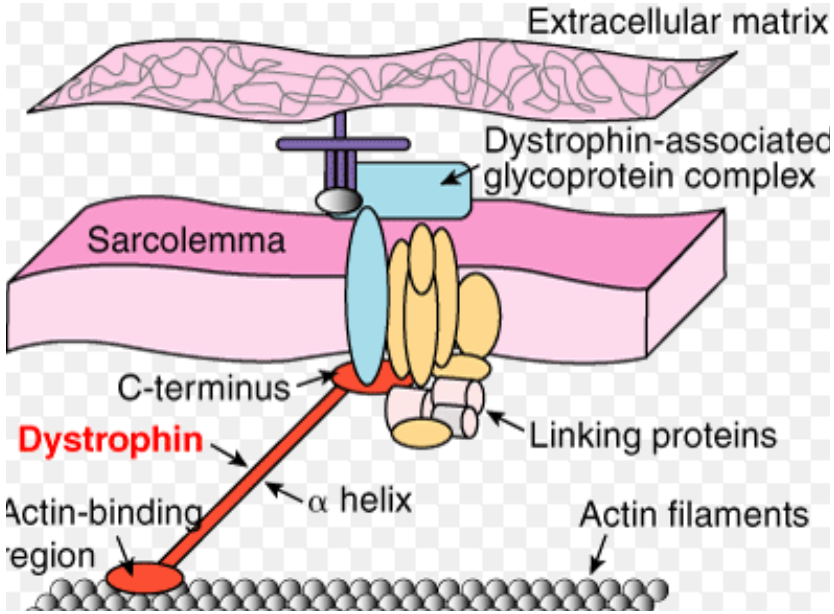
: : Distrofia muscolare di Duchenne

ORPHA98896

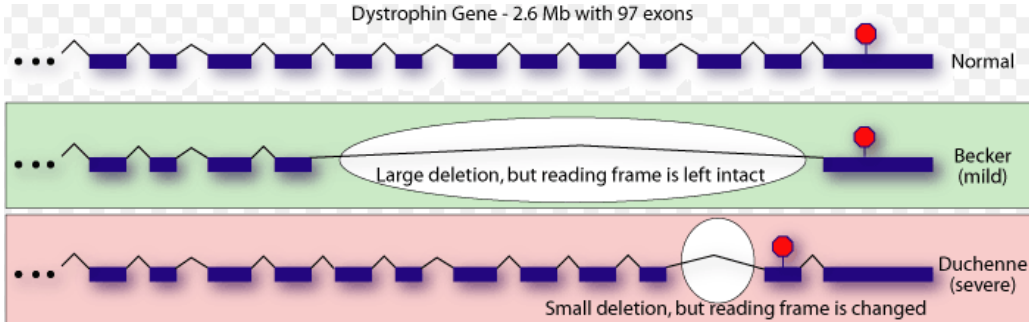
orphanet

Sinonimo/i :	DMD Distrofinopatia grave, tipo Duchenne
Prevalenza:	1-9 / 100 000
Trasmissione :	Recessiva legata all'X
Età di esordio :	Infanzia





The X-linked muscle-wasting disease Duchenne muscular dystrophy is caused by mutations in the **gene** encoding **dystrophin**.

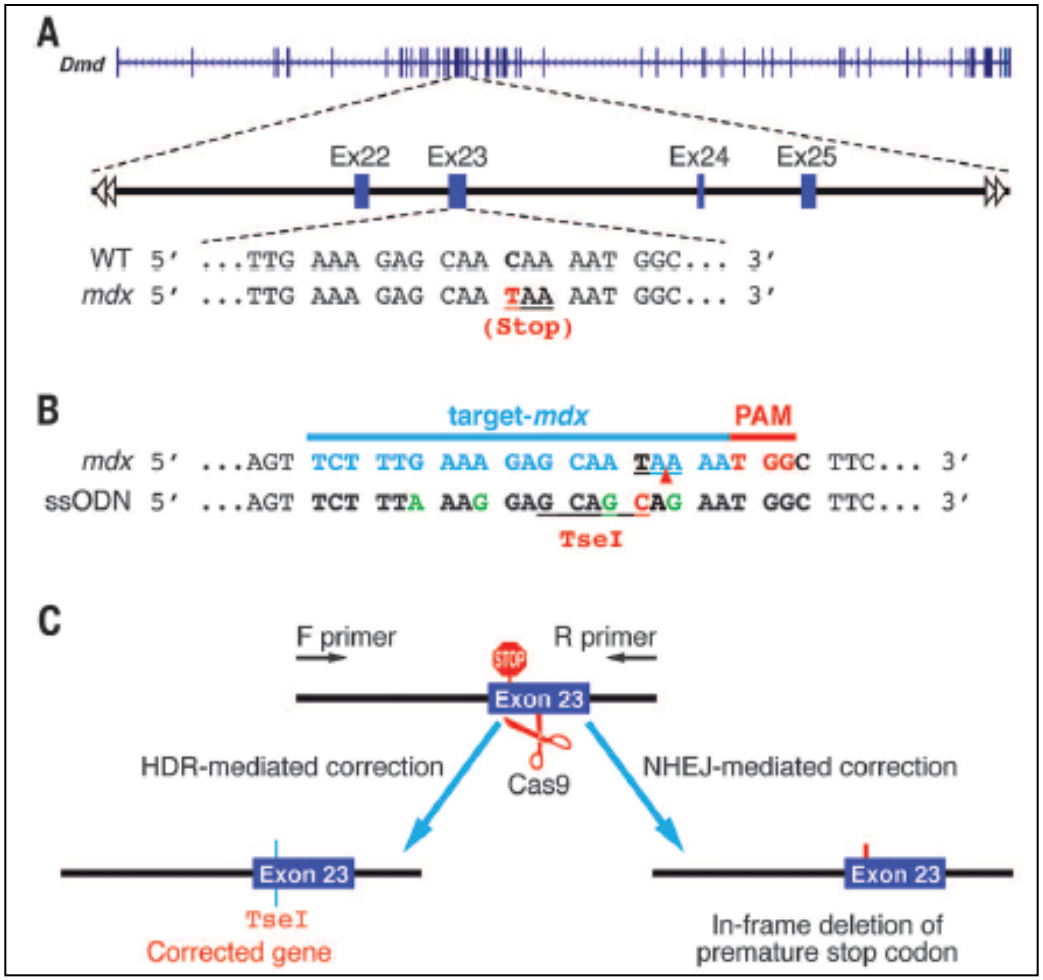


Science
AAAS

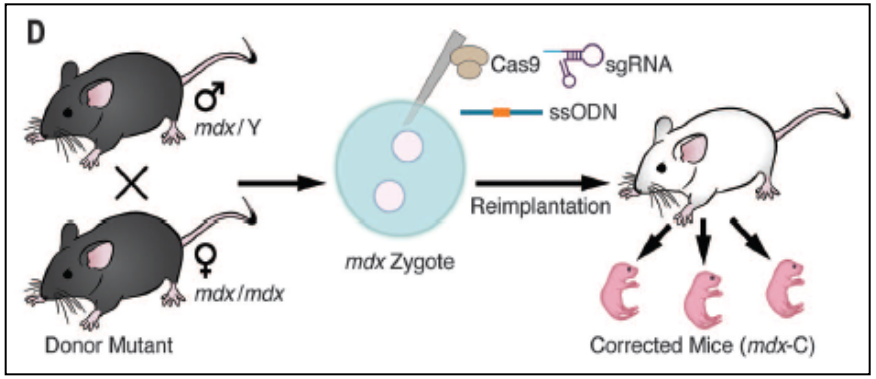
Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA

Chengzu Long *et al.*

Science **345**, 1184 (2014);
DOI: 10.1126/science.1254445



In vivo animal studies

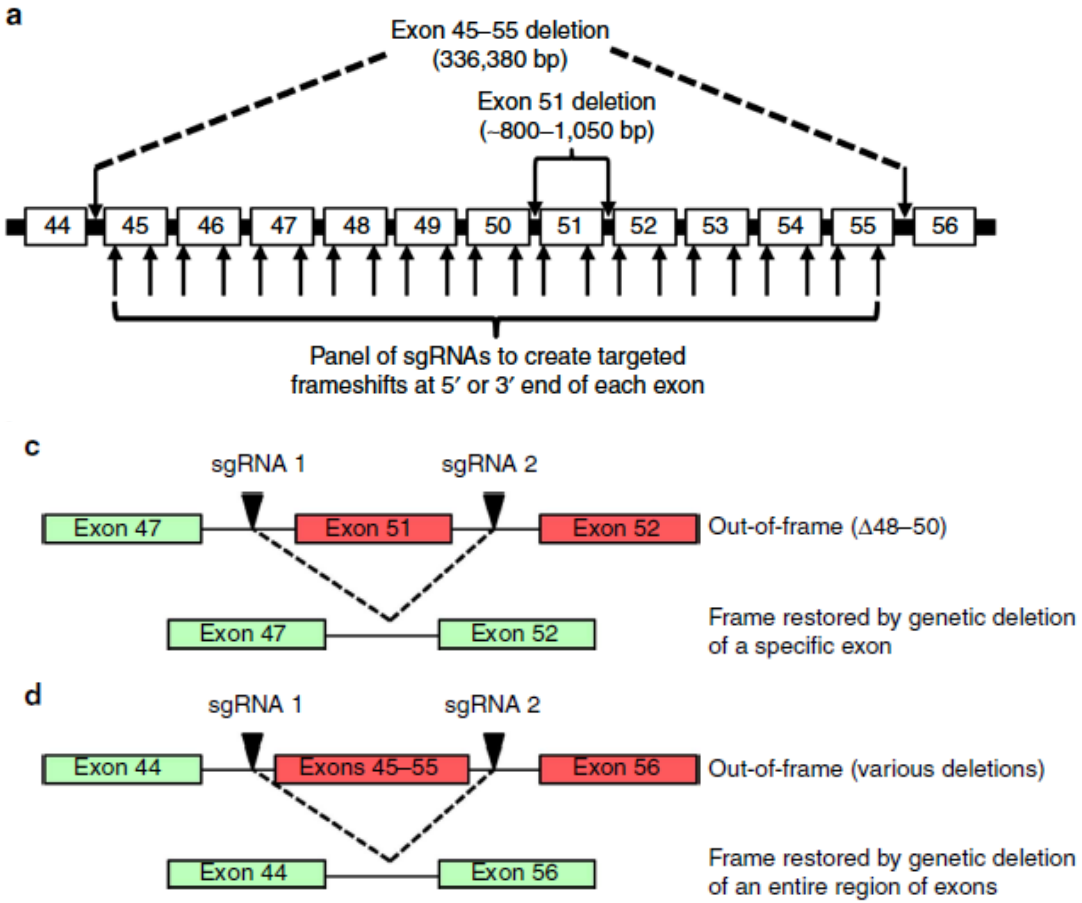


Our results show that CRISPR/Cas9-mediated genomic editing is capable of correcting the primary genetic lesion responsible for muscular dystrophy and preventing development of characteristic features of this disease in *mdx* mice.

Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy

David G. Ousterout, Ami M. Kabadi, Pratiksha I. Thakore, William H. Majoros, Timothy E. Reddy & Charles A. Gersbach

Received 16 November 2014 | Accepted 08 January 2015 | Published 18 February 2015



Cell-based studies

Following gene editing in DMD Patient myoblast, dystrophin expression is restored *in vitro*. Human dystrophin is also detected *in vivo* after transplatation of genetically corrected patient cells into immunodeficient mice.

Importantly, the unique multiplex gene-editing capabilities of CRISPR-Cas9 system facilitate the generation of a single large deletion that can correct up to 62% of DMD mutations.

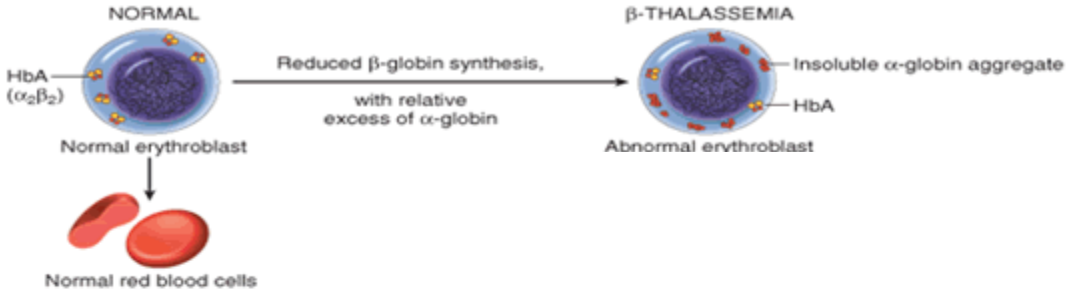
World's first genetic modification of human embryos reported:

Protein & Cell

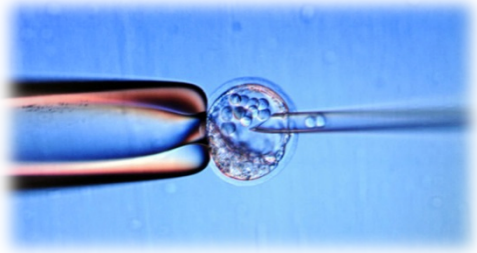
CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

Puping Liang, Yanwen Xu, Xiya Zhang, Chenhui Ding, Rui Huang, Zhen Zhang, Jie Lv, Xiaowei Xie, Yuxi Chen, Yujing Li, Ying Sun, Yaofu Bai, Zhou Songyang, Wenbin Ma, Canquan Zhou✉, Junjiu Huang✉

Guangdong Province Key Laboratory of Reproductive Medicine, the First Affiliated Hospital, and Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China
✉ Correspondence: hjunjiu@mail.sysu.edu.cn (J. Huang), zhoucanquan@hotmail.com (C. Zhou)
Received March 30, 2015 Accepted April 1, 2015



Cell-based studies



Embryo n°

71	→	54	→	28 Successful clevage	→	4 HDR

"Taken together, our work highlights the pressing need to further improve the fidelity and specificity of the CRISPR/Cas9 platform, a prerequisite for any clinical applications of CRSIPR/Cas9-mediated editing."

CRISPR germline engineering—the community speaks

Katrine S Bosley, Michael Botchan, Annelien L Bredenoord, Dana Carroll, R Alta Charo, Emmanuelle Charpentier, Ron Cohen, Jacob Corn, Jennifer Doudna, Guoping Feng, Henry T Greely, Rosario Isasi, Weihzi Ji, Jin-Soo Kim, Bartha Knoppers, Edward Lanphier, Jinsong Li, Robin Lovell-Badge, G Steven Martin, Jonathan Moreno, Luigi Naldini, Martin Pera, Anthony CF Perry, J Craig Venter, Feng Zhang & Qi Zhou

nature biotechnology

Nature Biotechnology **asks selected members of the international community to comment on the ethical issues raised by the prospect of CRISPR-Cas9 engineering of the human germline.**

YES

I think that human germline engineering <u>is inevitable</u>, and there will be basically no effective way to regulate or control the use of gene editing technology in human reproduction.

J. Craig Venter

MAYBE

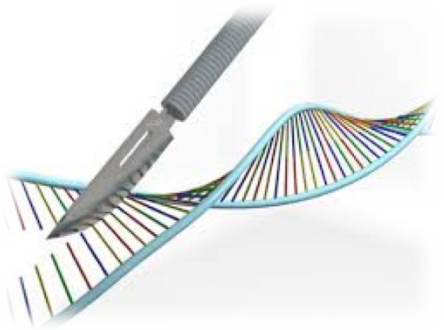
Annelien Bredenoord: I am inclined to say that inheritable genetic modification is on the horizon, but perhaps the first application of germline modification would be mitochondrial donation (also known as <u>*mitochondrial gene therapy*</u>), which does not involve gene-editing techniques. <u>*Recently, the UK Parliament legalized this technique aimed at preventing the transmission of mitochondrial DNA mutations from mother to child.*</u>

NO

urrent methods for ene editing are efficient in primary ells and require election of a small action of the treated ells bearing the esired edit. This is not asily applicable to rmline engineering, pecially in humans.

Luigi Naldini

Conclusions:

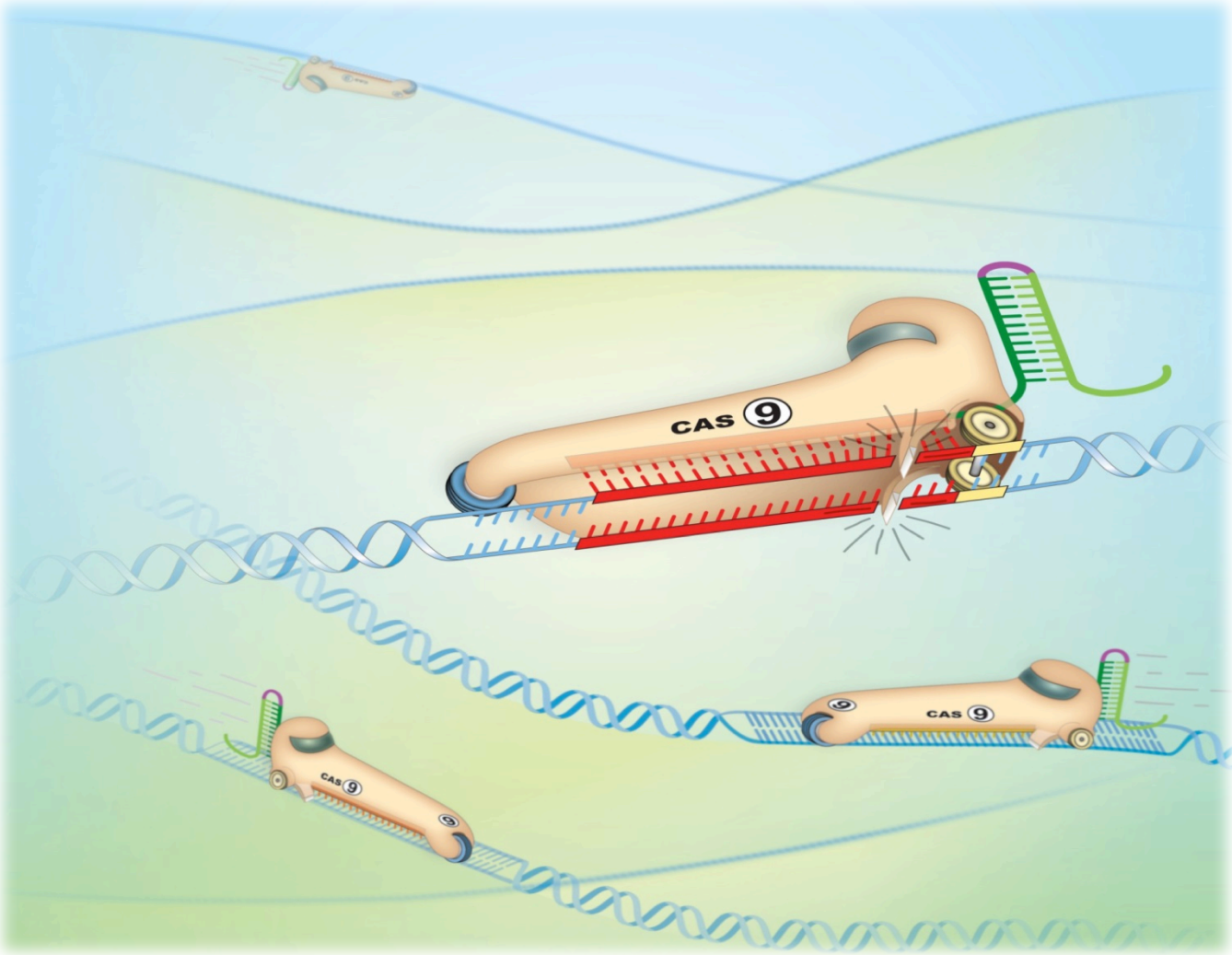


-Crispr-Cas9 technology can be used to investigate the function of a gene of interest or to correct gene mutations in cells via genome editing, paving the way for future therapy approaches.

- In the last 2 years, over 600 articles have been published on CRISPR-Cas9 applications.

-Further investigation of the molecular mechanisms of CRISPR/Cas9-mediated gene editing in human model is sorely needed.

- In particular, off-target effect of CRISPR/Cas9 should be investigated thoroughly before any clinical application.



…the end