Aplicaciones del Sistema CRISPR-Cas9 en Enfermedades Raras

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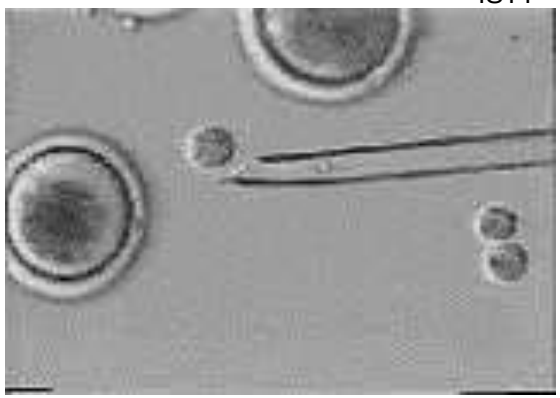
Modifying the Mammalian Genome

Pronuclear microinjection

ES / iPS cells

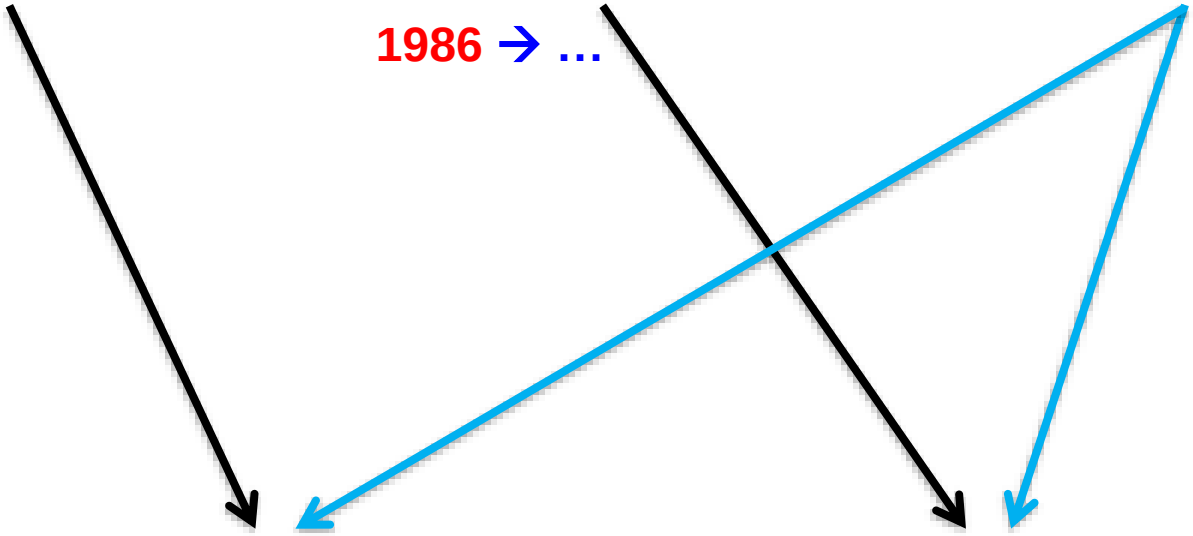


SCNT

1980 → …

1986 → …

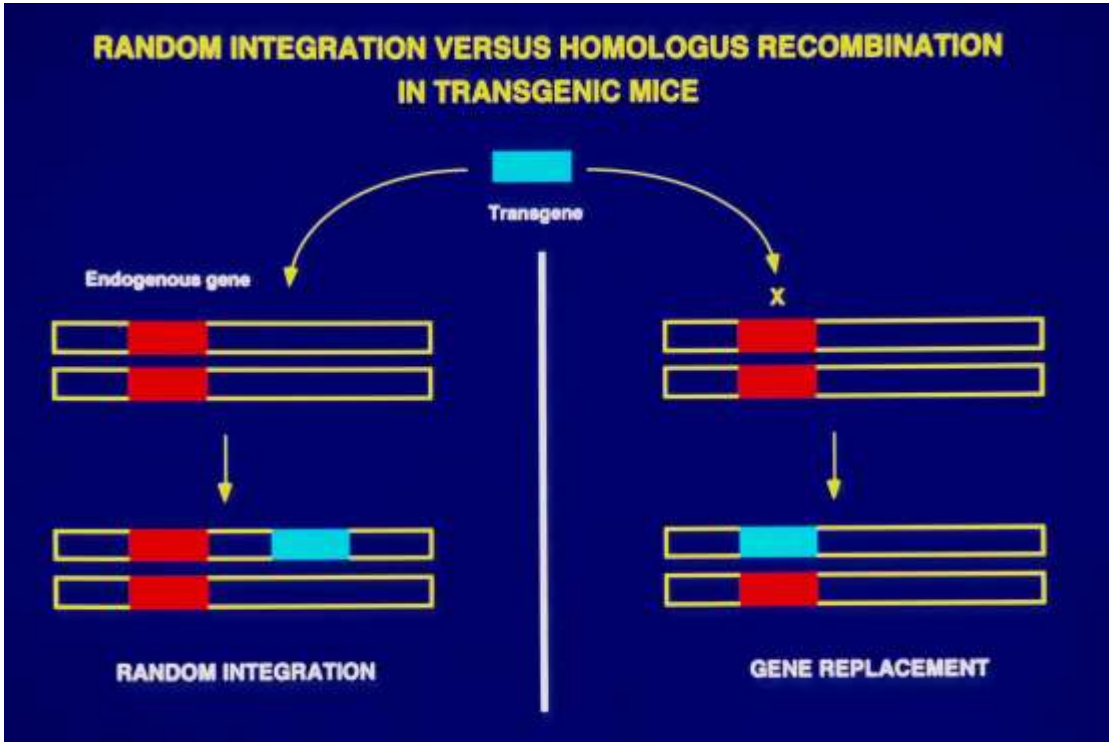
1996 → …

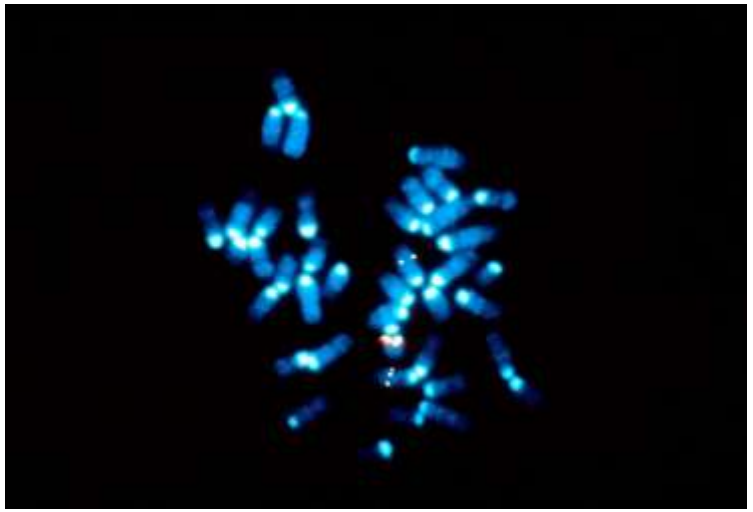


ADDING A GENE

DELETING A GENE

The most relevant and distinctive feature among all gene modifying methods is

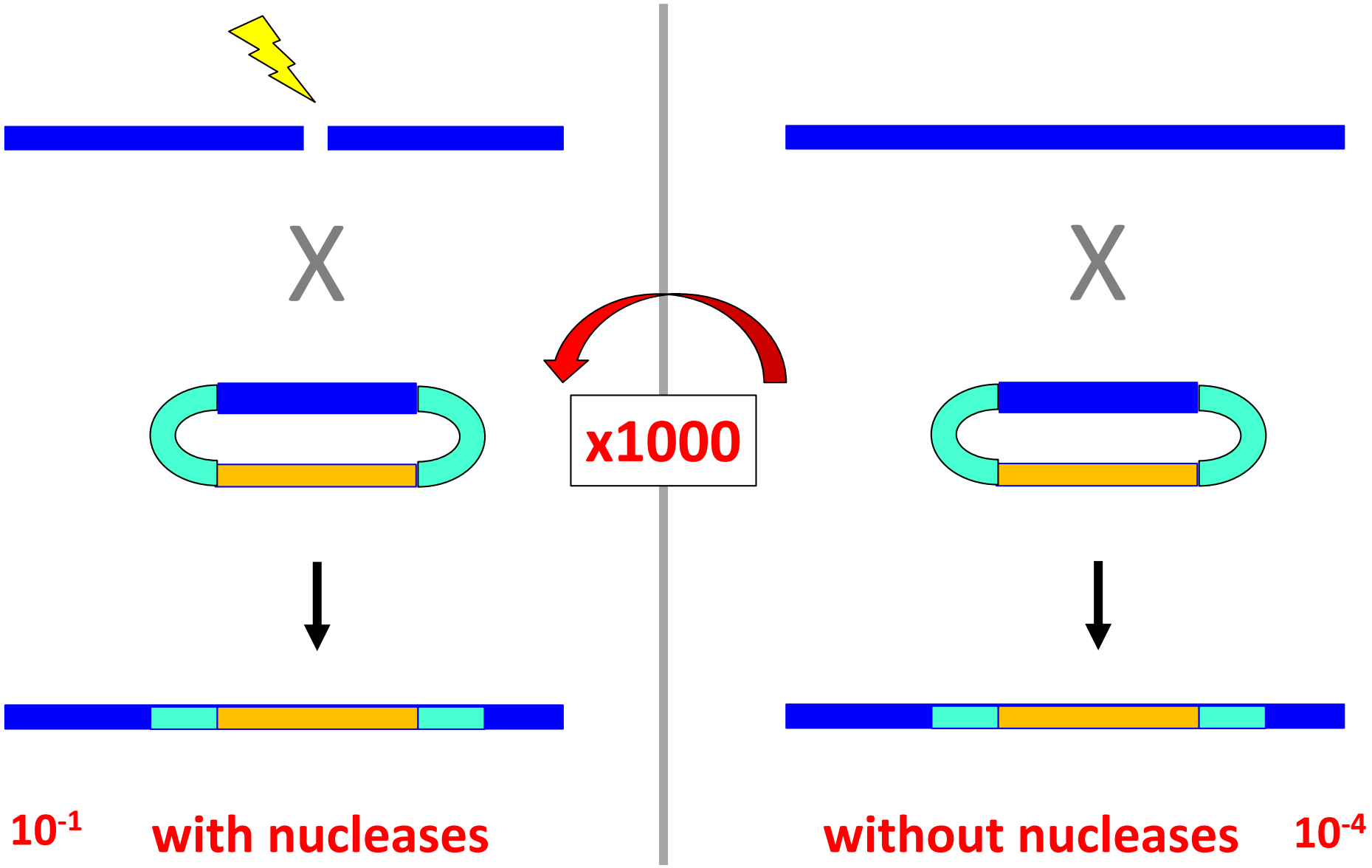


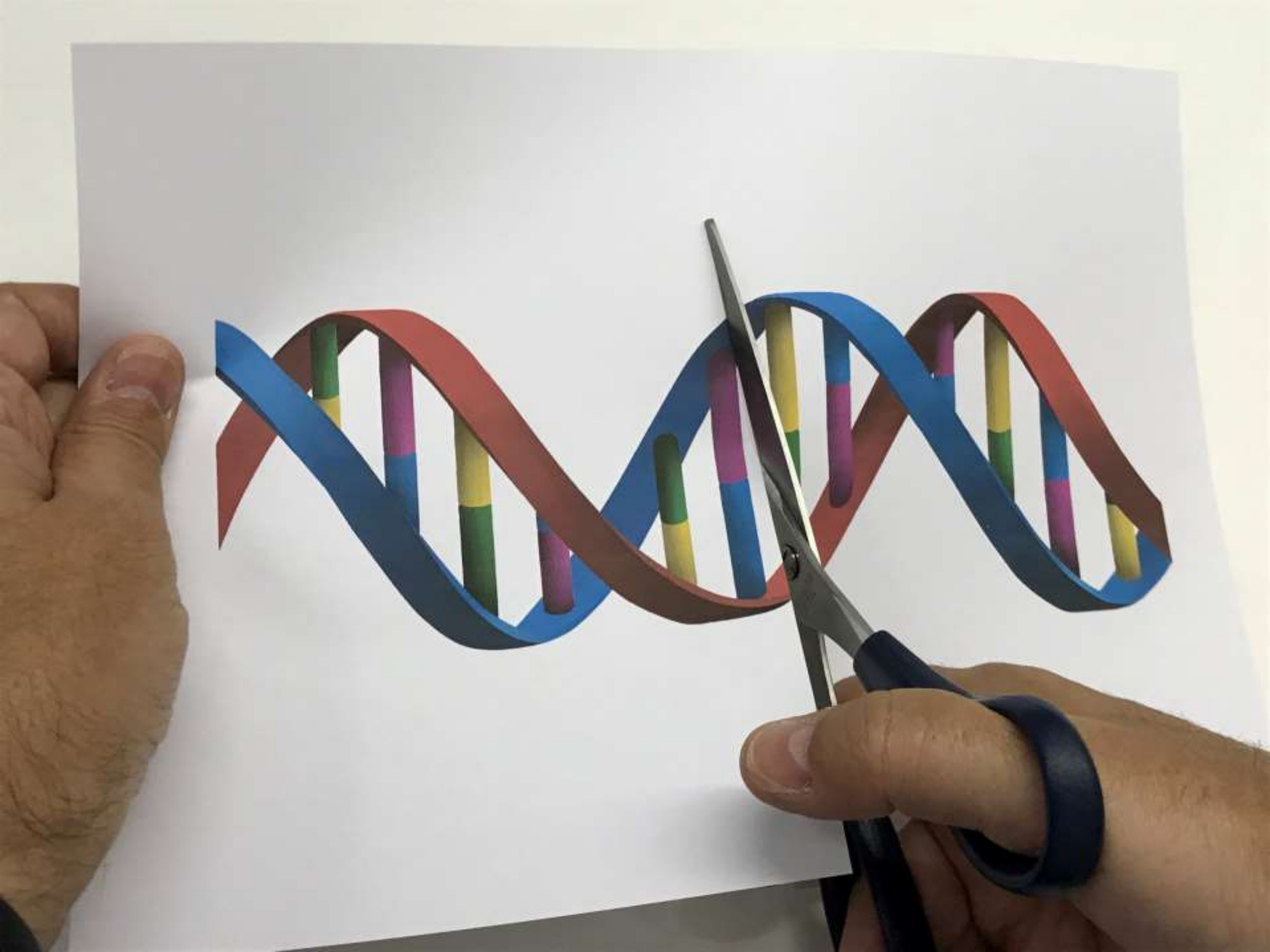


Slides from 1991

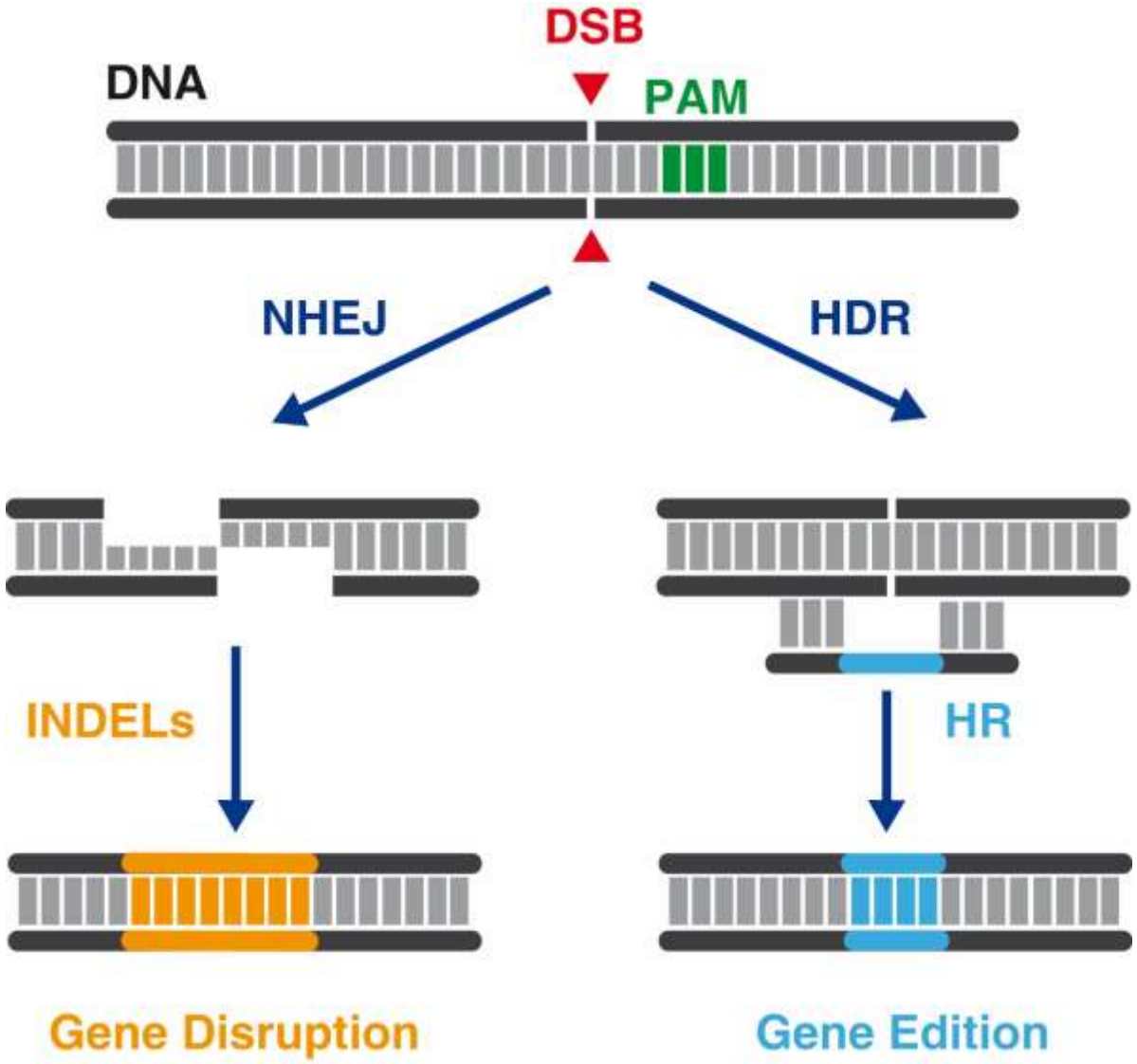
Random versus Targeted genetic modification

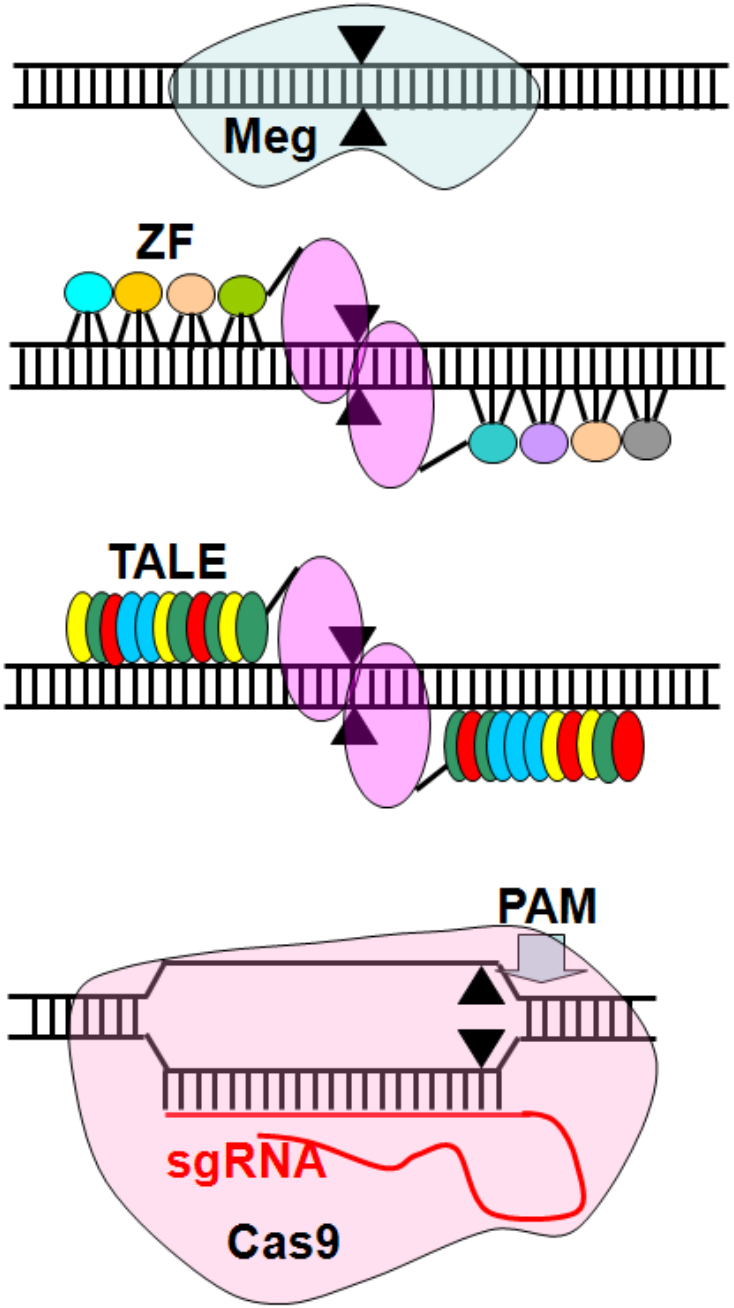
Homologous Recombination and Nucleases





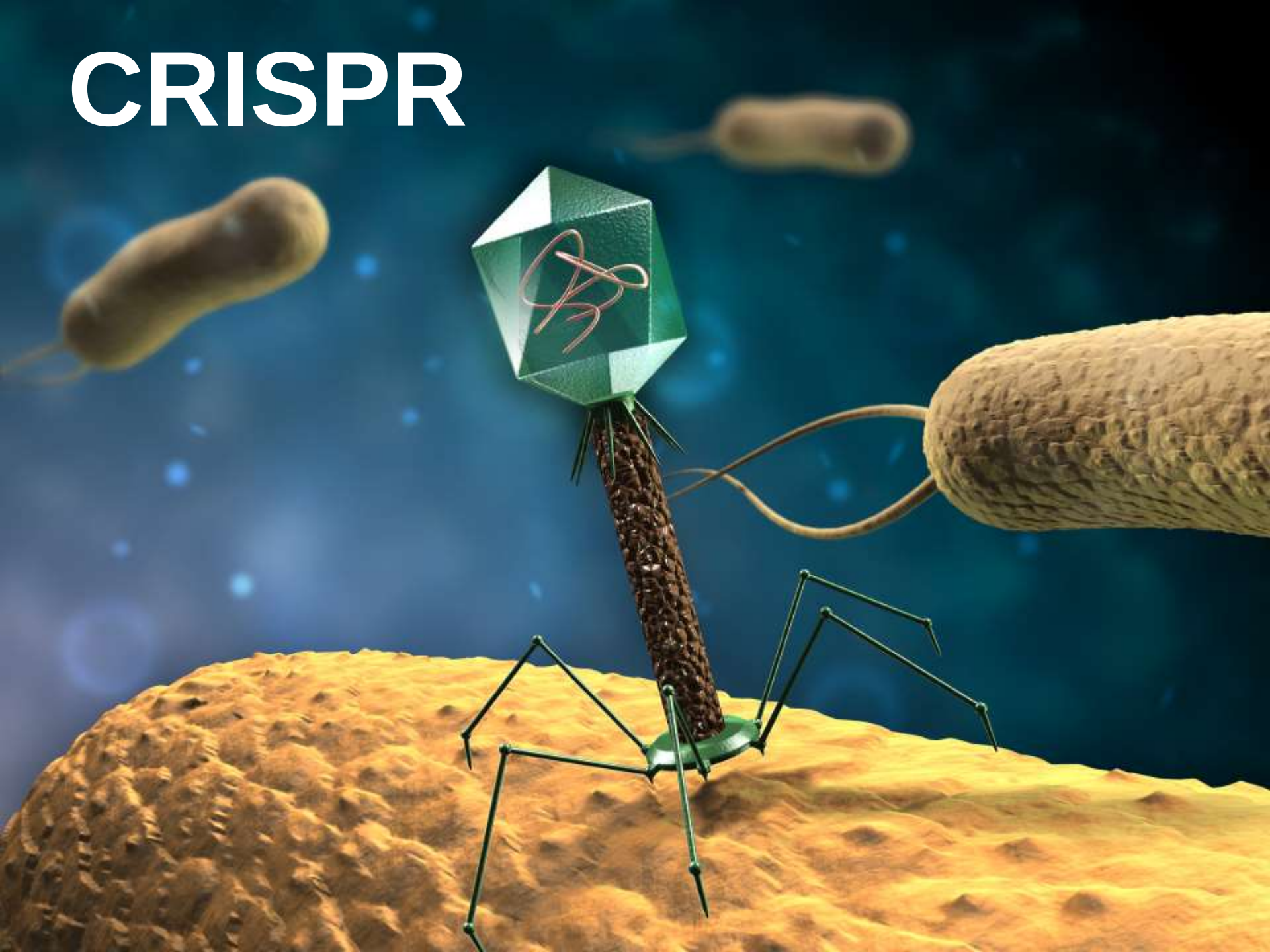
Fixing the DSB: NHEJ vs HDR





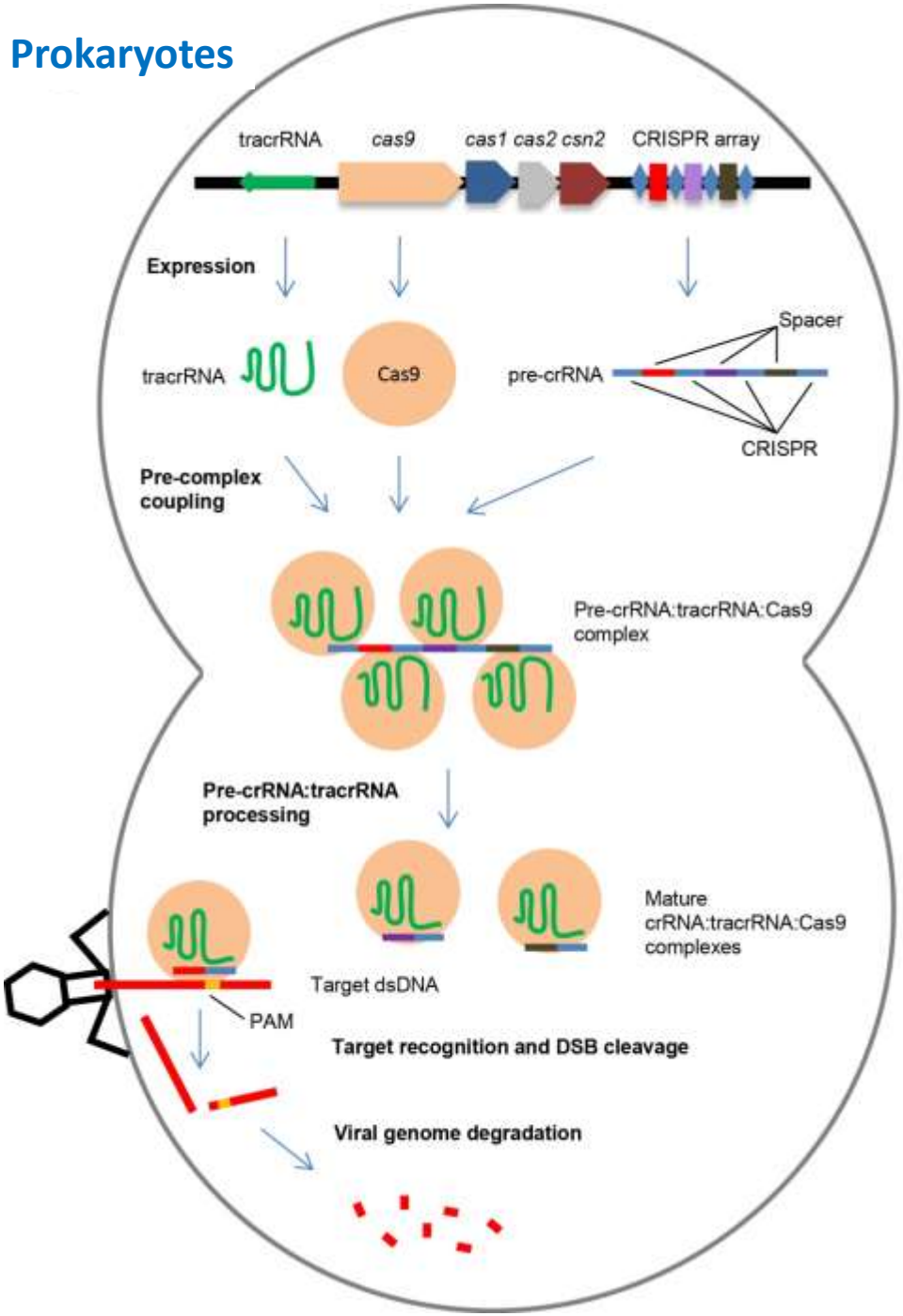
Meganuclease	20-40 bp/Enzyme
ZFN	3 bp/Finger
TALEN	1 bp/Module
CRISPR-Cas	1 bp/Base

CRISPR

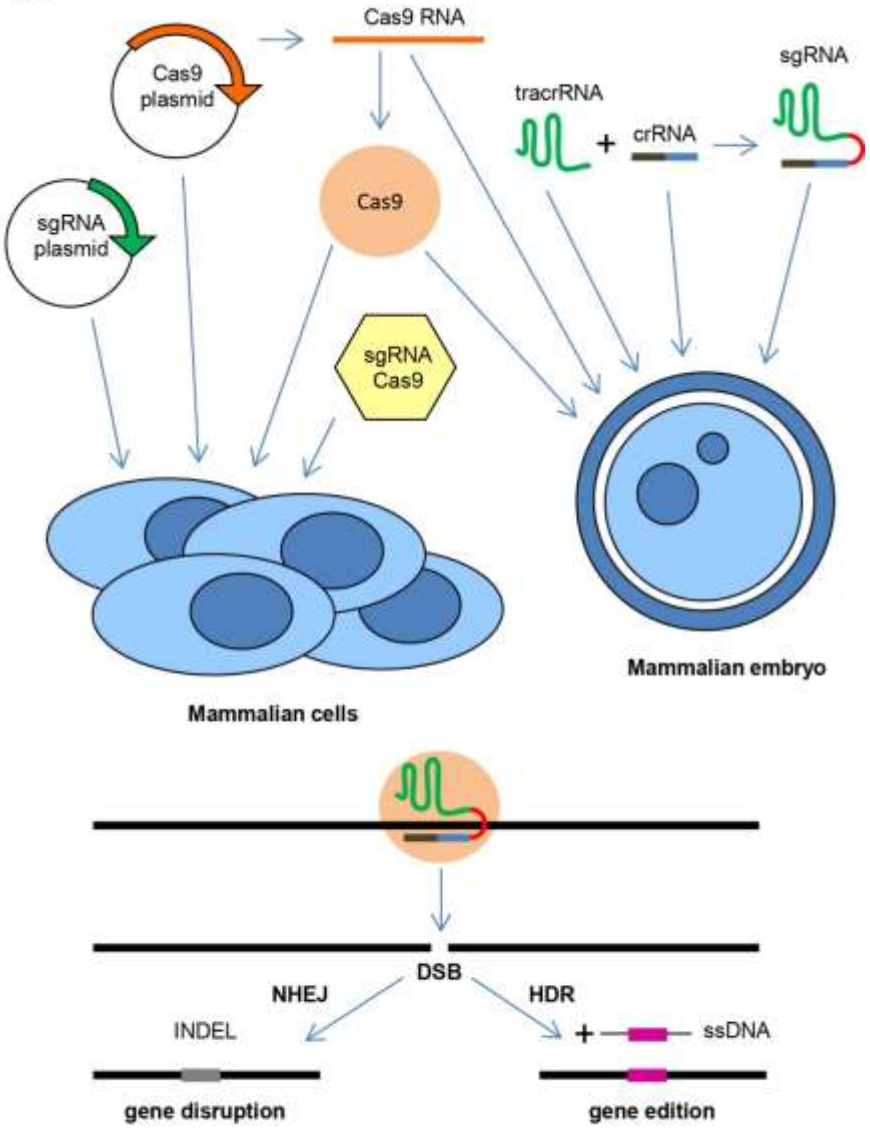


Francisco Juan Martínez Mojica

Prokaryotes



Eukaryotes



Mojica and Montoliu (Trends in Microbiology 2016)

The CRISPR-Cas pioneers

Francisco Mojica
University of Alicante, Spain

Rodolphe Barrangou
North Carolina State Univ, Raleigh, USA

Philippe Horvath
DuPont Nutrition and Health, France

Luciano Marraffini
The Rockefeller Univ, New York, USA

John van der Oost
Wageningen University, The Netherlands

Emmanuelle Charpentier
MPI for Infect. Biol., Berlin, Germany

Jennifer Doudna
Univ California Berkeley, CA, USA

Virginijus Siksnys
Vilnius University, Lithuania

Feng Zhang
BROAD-MIT, Cambridge, MA, USA

George Church
Harvard Med School, Boston, MA, USA

Rudolf Jaenisch
Whitehead Inst, Cambridge, MA, USA

J. Keith Joung
Mass Gen Hosp, Charlestown, MA, USA

The CRISPR-Cas System: targeting nucleases to specific DNA sequences a binary system: Cas9 protein and sgRNA

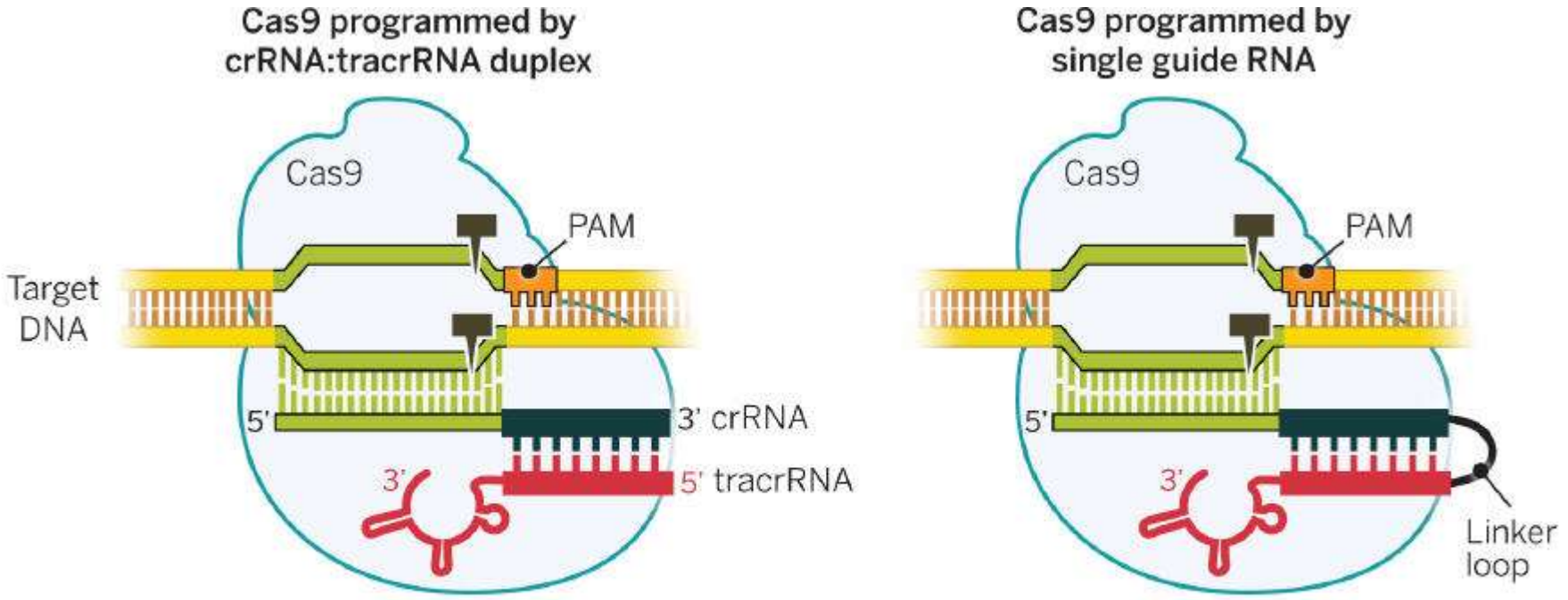
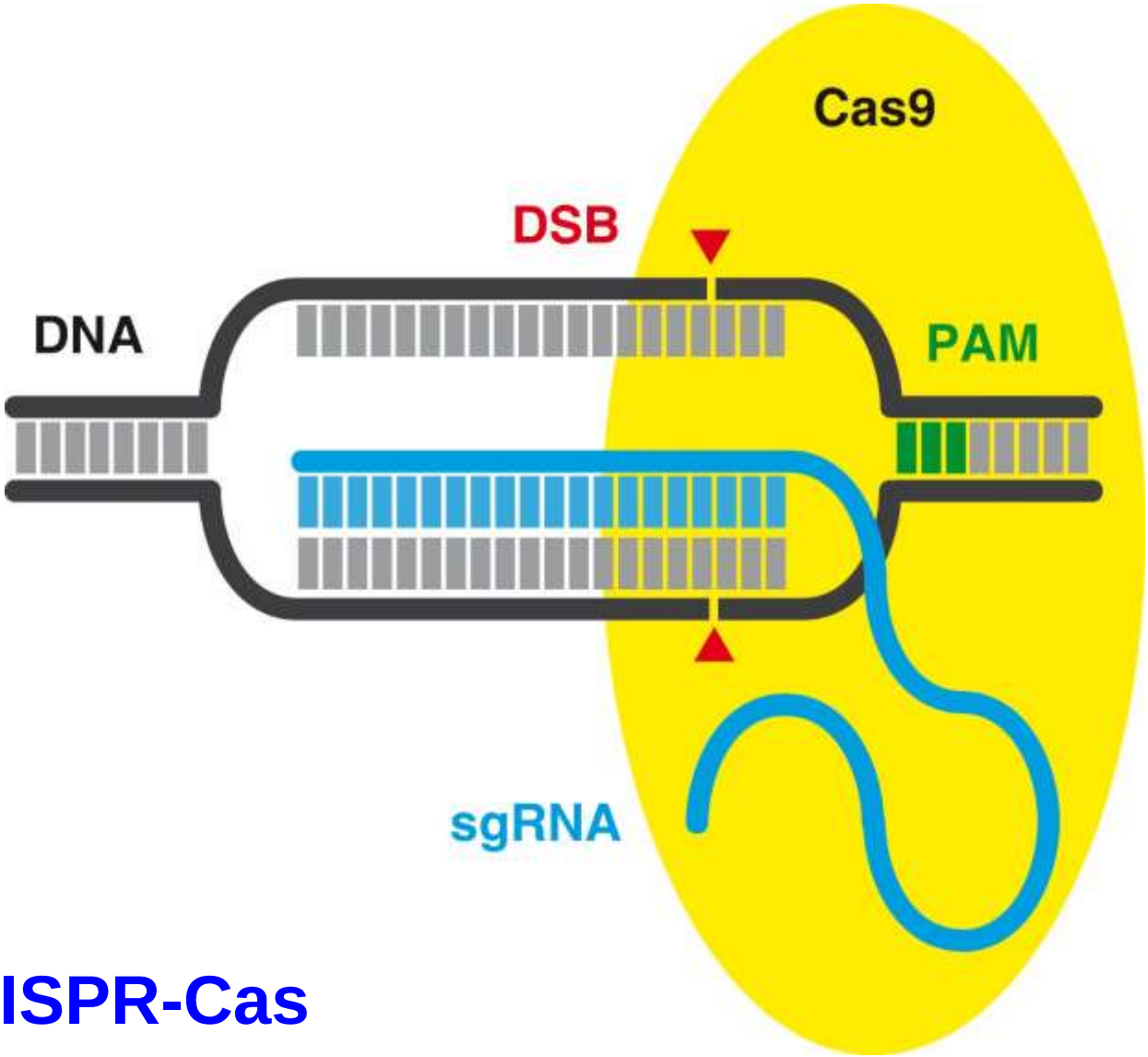


Fig. 3. Evolution and structure of Cas9. The structure of *S. pyogenes* Cas9 in the unliganded and RNA-DNA–bound forms [from (*77*, *81*)].

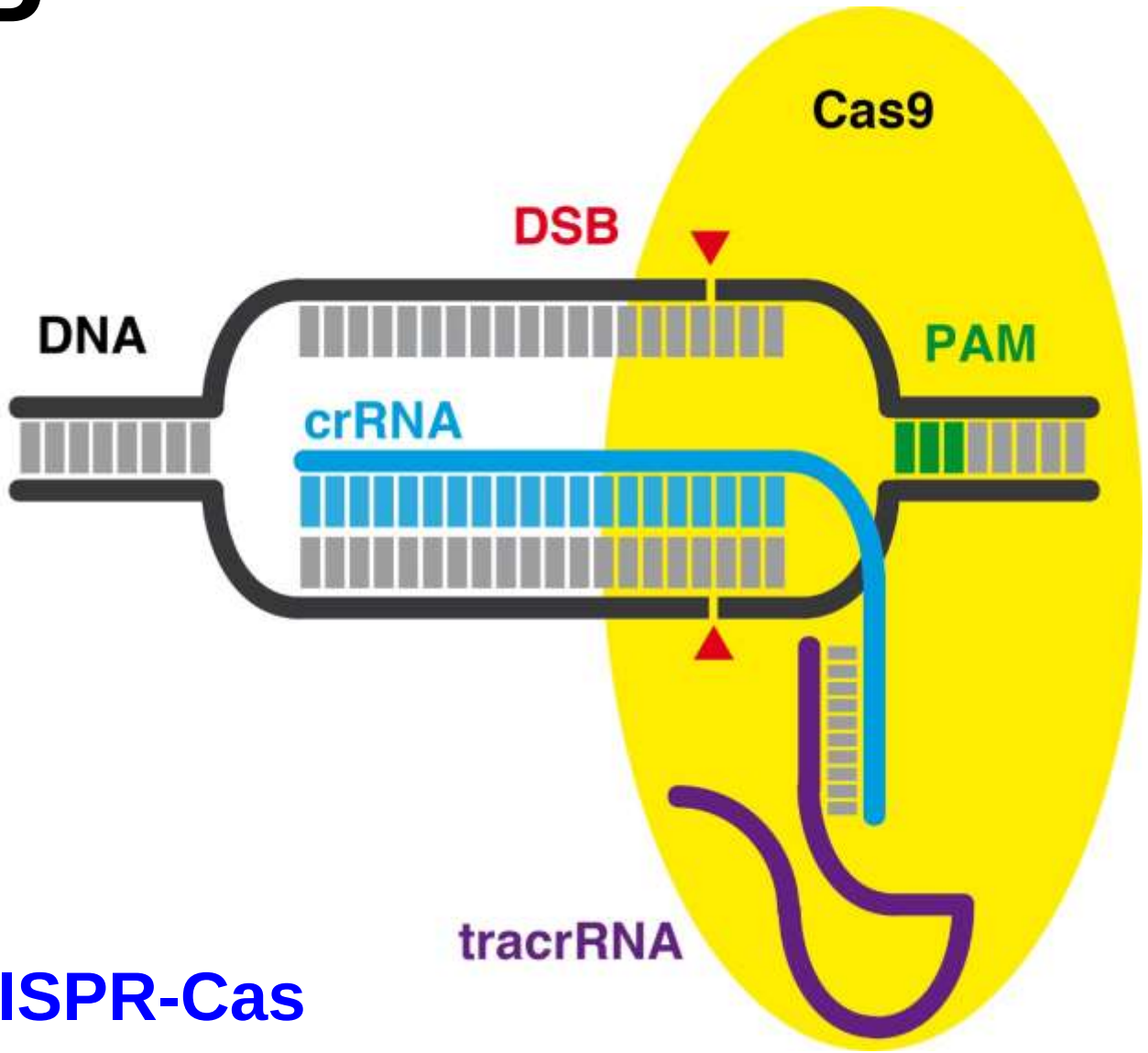
Jennifer Doudna
Emmanuelle Charpentier

Doudna & Charpentier (2014) Science

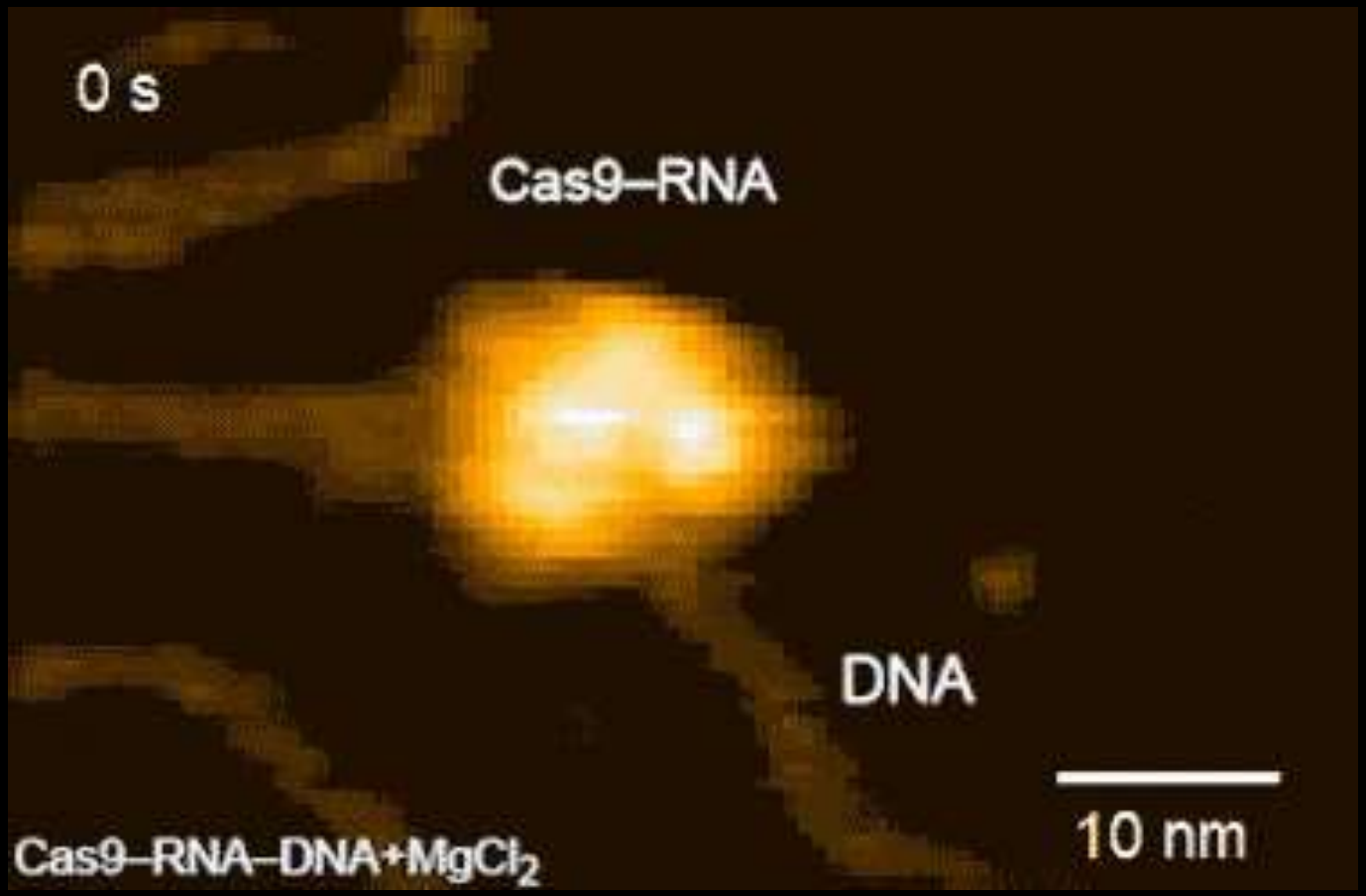


The CRISPR-Cas system

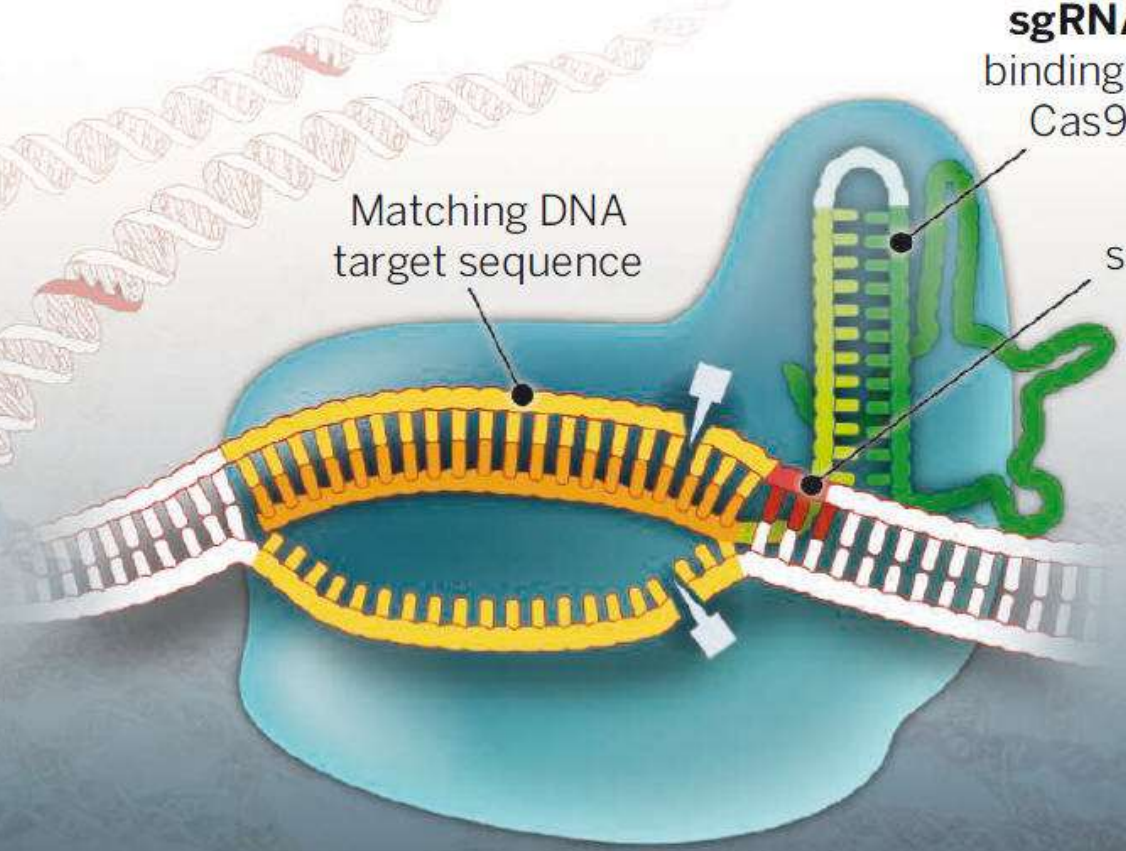
RNP



The CRISPR-Cas system



Hiroshi Nishimasu and Osamu Nureki (University of Tokyo)
Shibata et al. Nature Comm. 2017

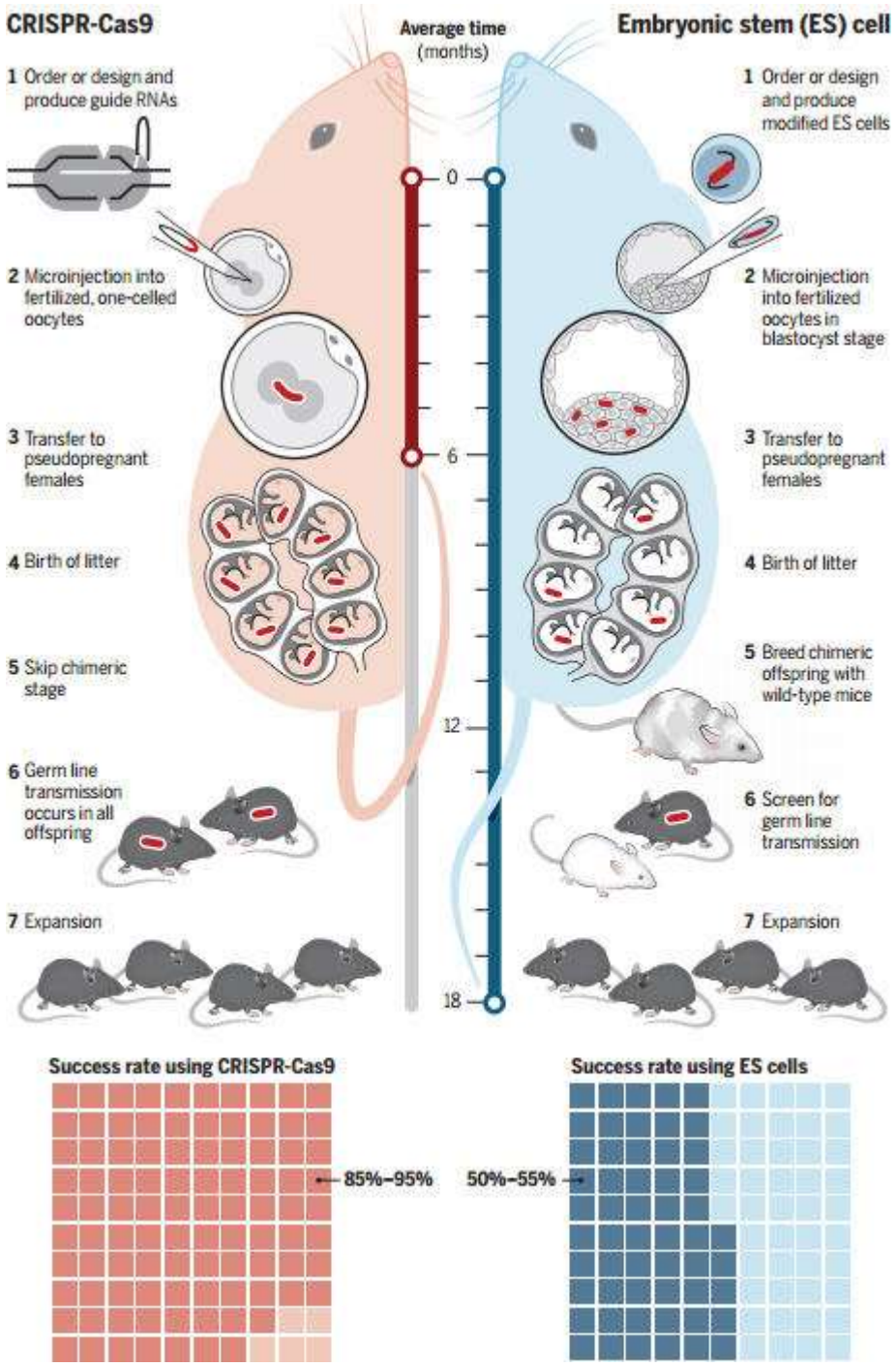


CRISPR-Cas9 development

- DNA deletion
- DNA insertion
- DNA replacement
- DNA modification
- DNA labeling
- Transcription modulation
- RNA targeting
- . . .

CRISPR-Cas9 applications

- Biological research
- Research and development
- Human medicine
- Biotechnology
- Agriculture
- . . .



Gene Editing
CRISPR vs ES cells

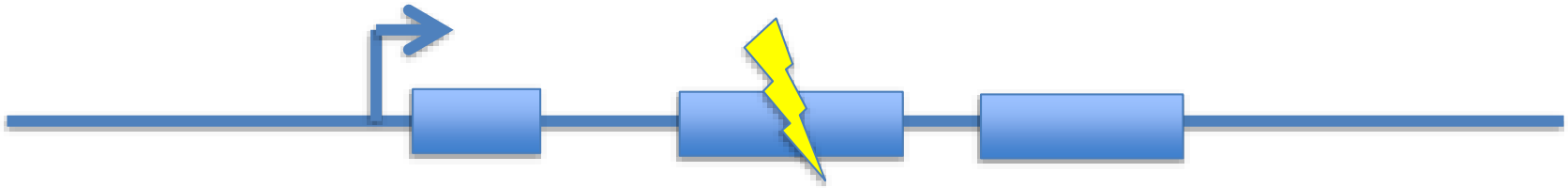
- **Generation** of target mutations is **faster** with CRISPR (x3)
- **Generation** of target mutations is **more efficient** with CRISPR

Cohen, Science (2016)

Types of CRISPR-Cas-induced genome editions

- INDELs: small insertions and deletions
- KOs: disrupting ORF
- Gene editions: point mutations
- KIs: introducing specific DNA segments
- Large deletions
- Inversions
- …

Disrupting a gene: KO



- The easiest approach
- almost trivial nowadays
- Many similar alleles will be generated
- Efficient protocol

We use the Tyrosinase gene (*Tyr*) in mice as experimental model to study mammalian gene regulation and …

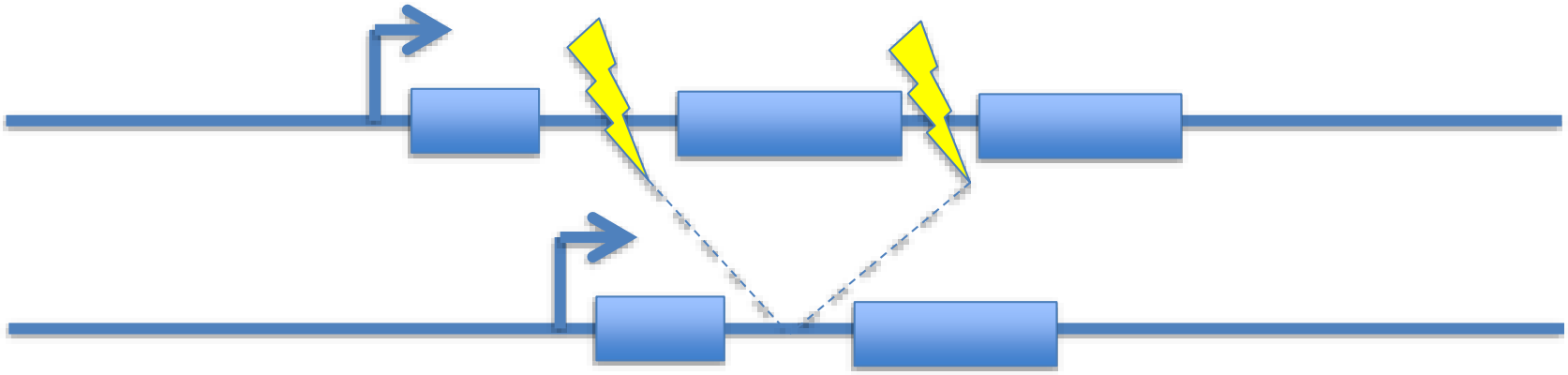
…as animal models of OCA1 albinism, a human rare disease

There are at least 20 genes associated with albinism

Mouse	Human	Albinism	Mutations (HGMD)	
Tyr	*TYR*	OCA1	303	melanocytes
Oca2	*OCA2*	OCA2	154	
Tyrp1	*TYRP1*	OCA3	16	
Slc45a2	*SLC45A2*	OCA4	78	
??	*4q24*	OCA5	1	
slc24A5	*SLC24A5*	OCA6	2	
Lrmda	*LRMDA*	OCA7	6	
Gpr143	*GPR143*	OA1	114	
Sl38a8	*SLC38A8*	FHONDA	8	
Lyst	*LYST*	CHS1	53	Melanosomes/lysosomes
Hps1	*HPS1*	HPS1	31	
Ap3b1	*AP3B1*	HPS2	20	
Hps3	*HPS3*	HPS3	7	
Hps4	*HPS4*	HPS4	13	
Hps5	*HPS5*	HPS5	11	
Hps6	*HPS6*	HPS6	9	
Dtnbp1	*DTNBP1*	HPS7	2	
Bloc1s3	*BLOC1S3*	HPS8	2	
Bloc1s6	*BLOC1S6*	HPS9	2	
Ap3d1	*AP3D1*	HPS10	1	

Montoliu et al. PCMR 2014; Montoliu & Marks PCMR 2016

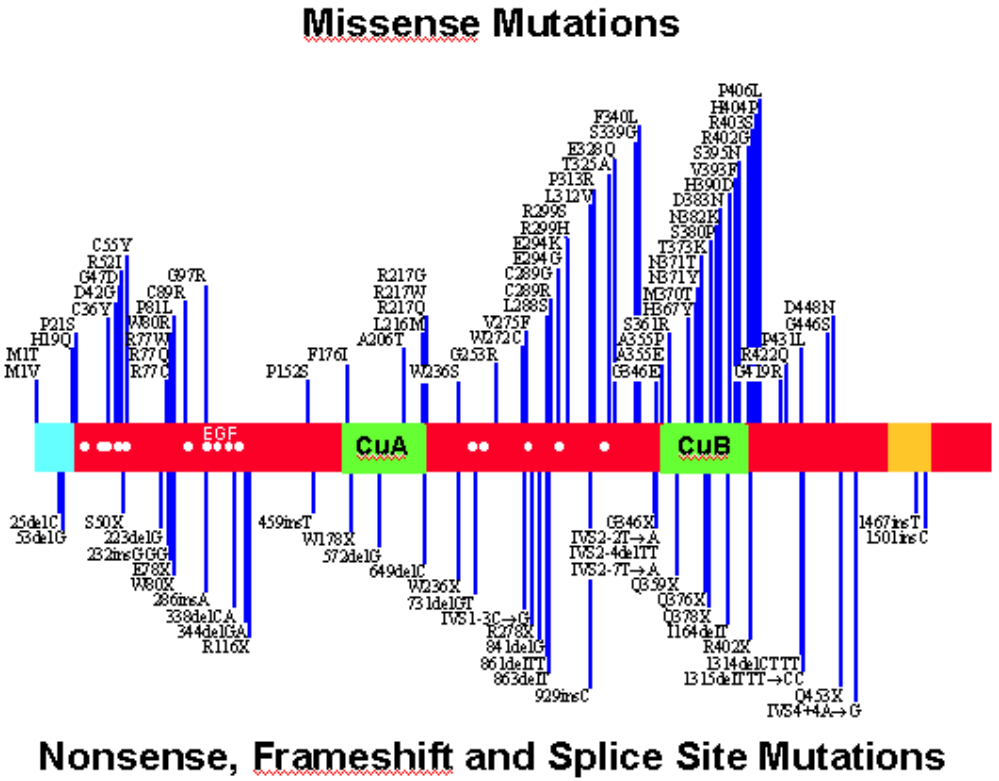
Deletions



- Relatively straight forward, many alleles will be generated
- INDELs at both targeting sites will be **also** generated
- Consider **inversions** can also be generated
- Efficient protocol

Relevance of regulatory elements at the endogenous *Tyr* locus

Mutations in regulatory elements could possibly account for OCAI individuals (~30%) in which no other alterations are found within the tyrosinase locus



Oculocutaneous albinism type I

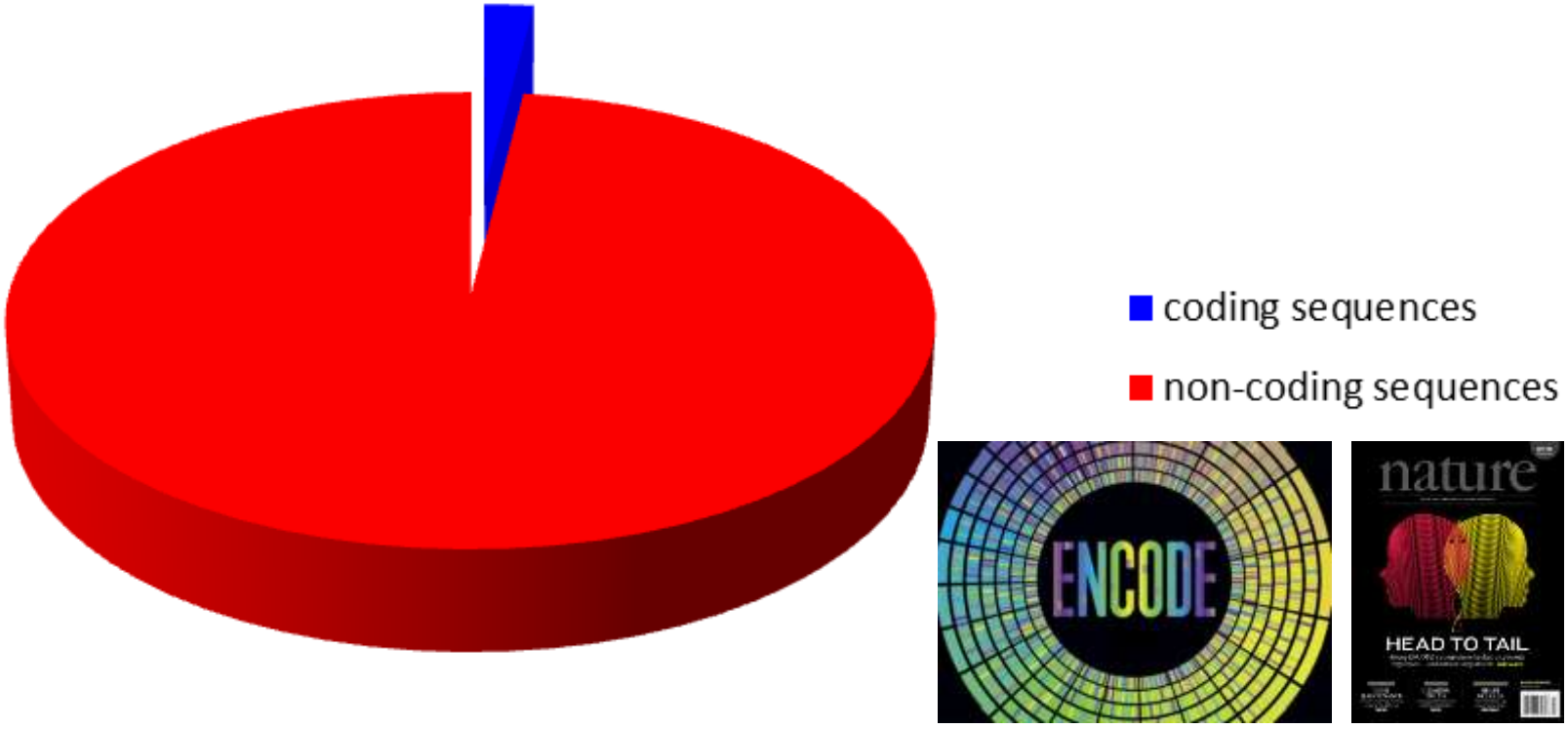
ciberer isciii

Montoliu et al. PCMR 2014; Montoliu & Marks PCMR 2016

The non-coding genome

DNA coding sequences represent 2% genome

DNA non-coding sequences represent 98% genome

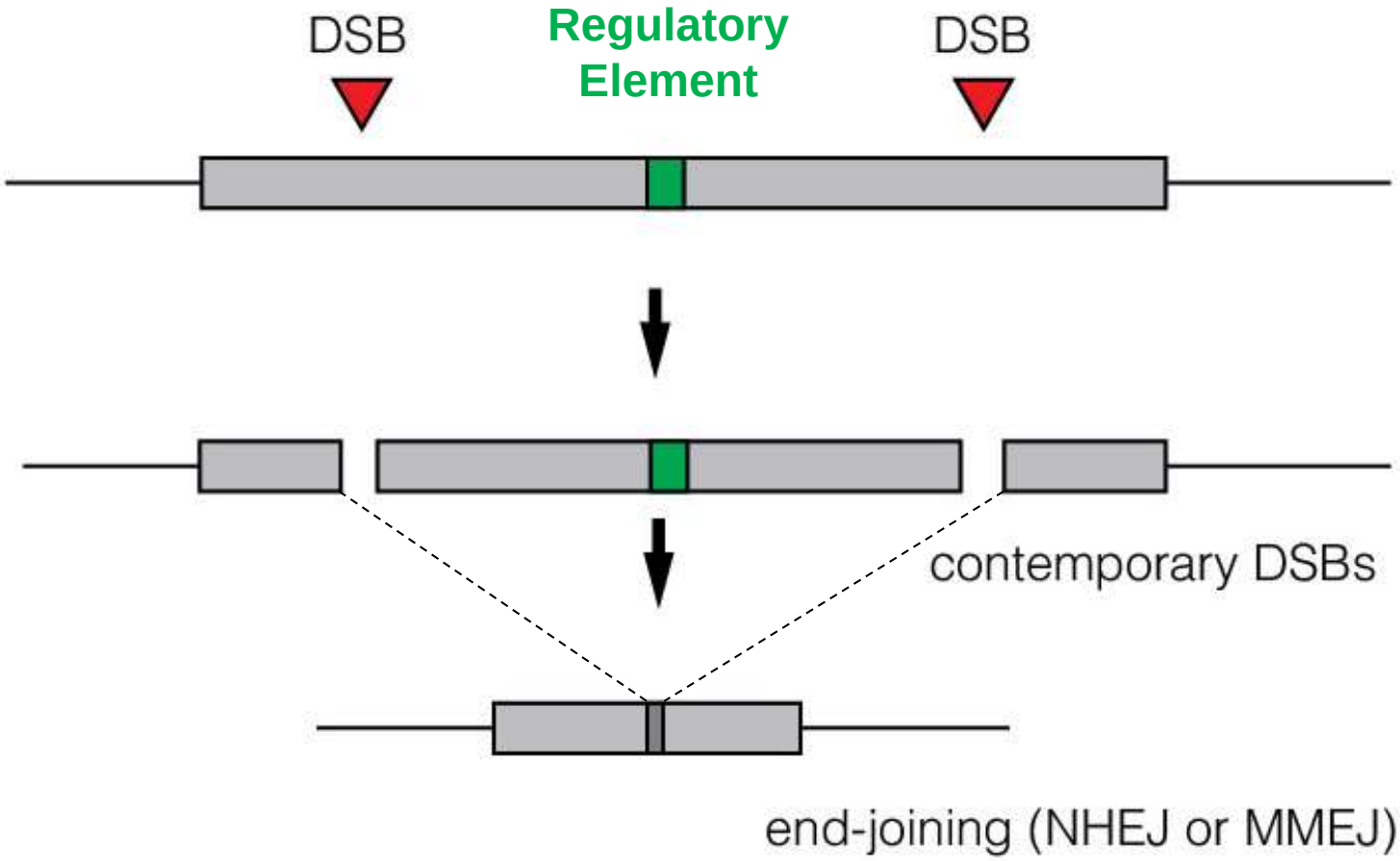


DNA non-coding sequences contain mainly:

DNA repetitive elements, mobile elements and

DNA regulatory elements

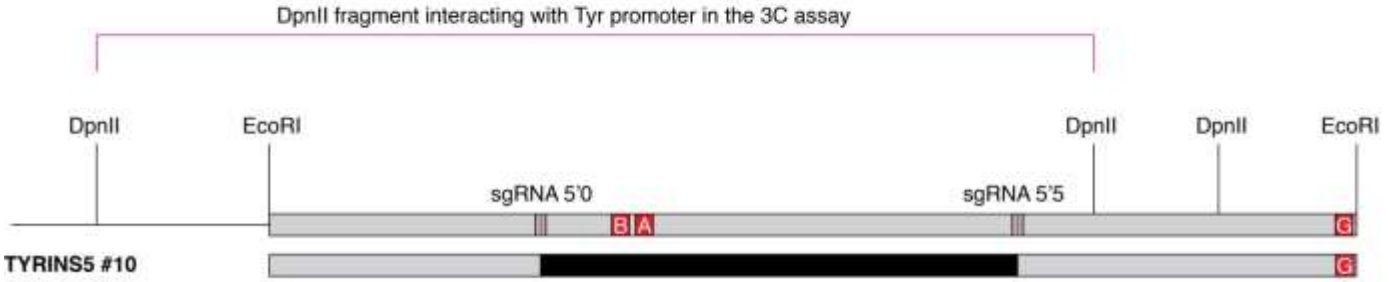
Alternative: using CRISPR-Cas9 genome editing to target *Tyr* regulatory elements



CRISPR-Cas9 genome editing

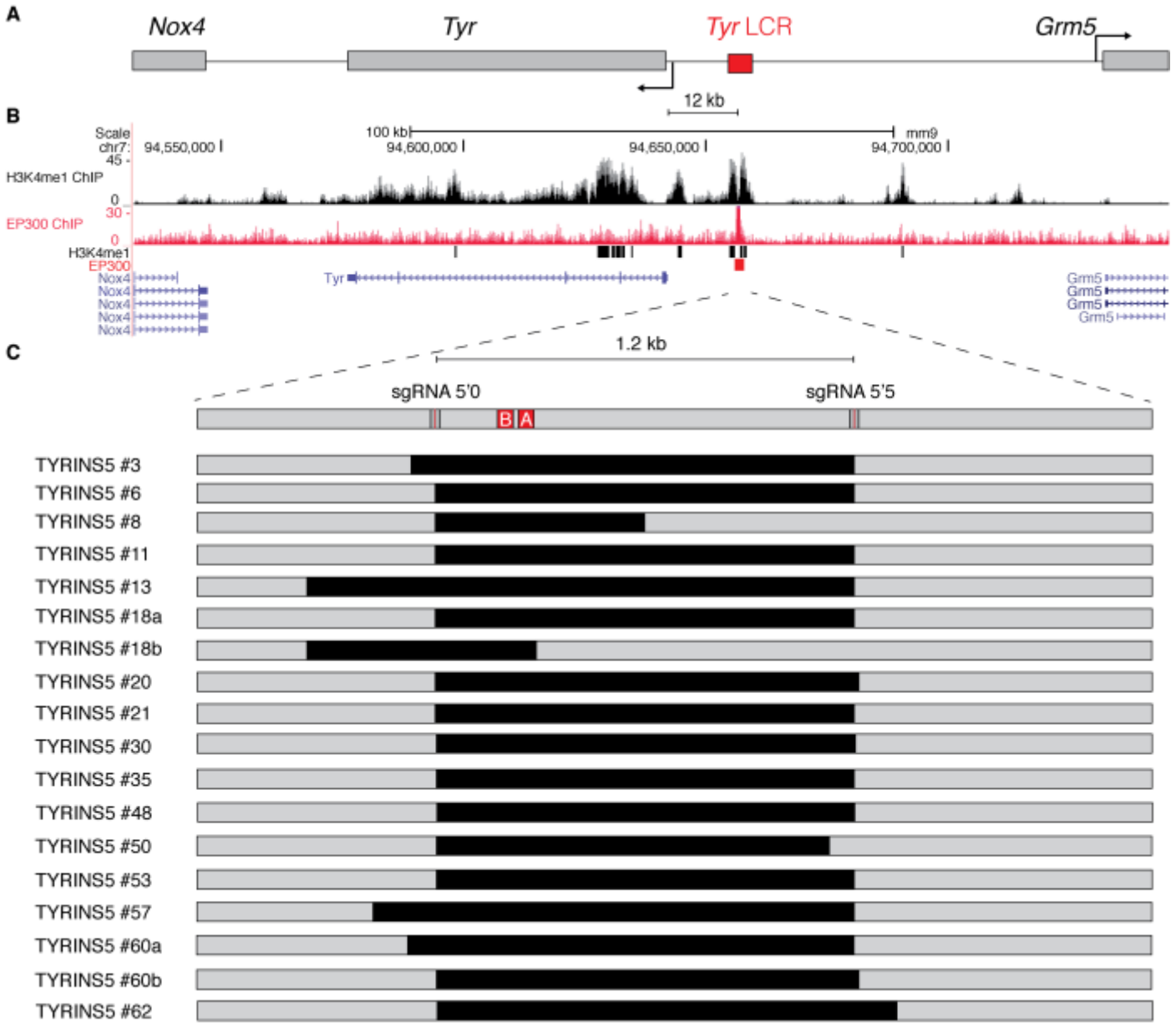
Deleting *Tyr* 5’ boundary with CRISPRs *in vivo*

Founder mosaic mice with clear coat colour pigmentation phenotype carrying BIALLELIC deletion of *Tyr* 5’ boundary

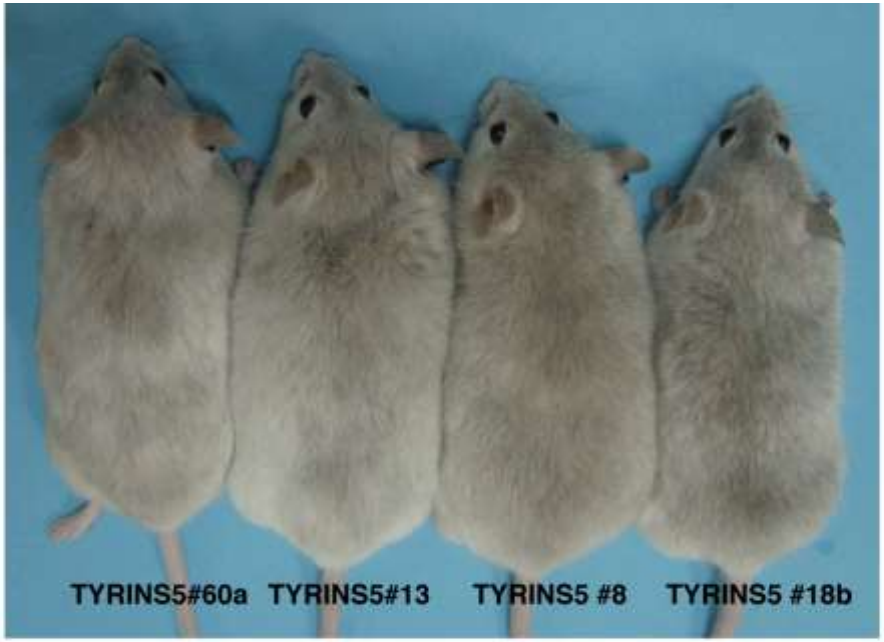



```
reference:    CAAGAATTAAGTGTGACAGTGCAAGATAACAGGAAAATAA......1170bp........CTCTAGGCCAAAATTGCCTAGTTTTATCACTACAAAACTT
TYRINS5#18_1: CAAGAATTAAGTGTGACAGTGCAAGAT-------------......1170bp.....-------------TTGCCTAGTTTTATCACTACAAAACTT
TYRINS5#18_1: CAAGAATTAAGTGTGACAGTGCAAGAT-------------......1170bp.....-------------TTGCCTAGTTTTATCACTACAAAACTT
```

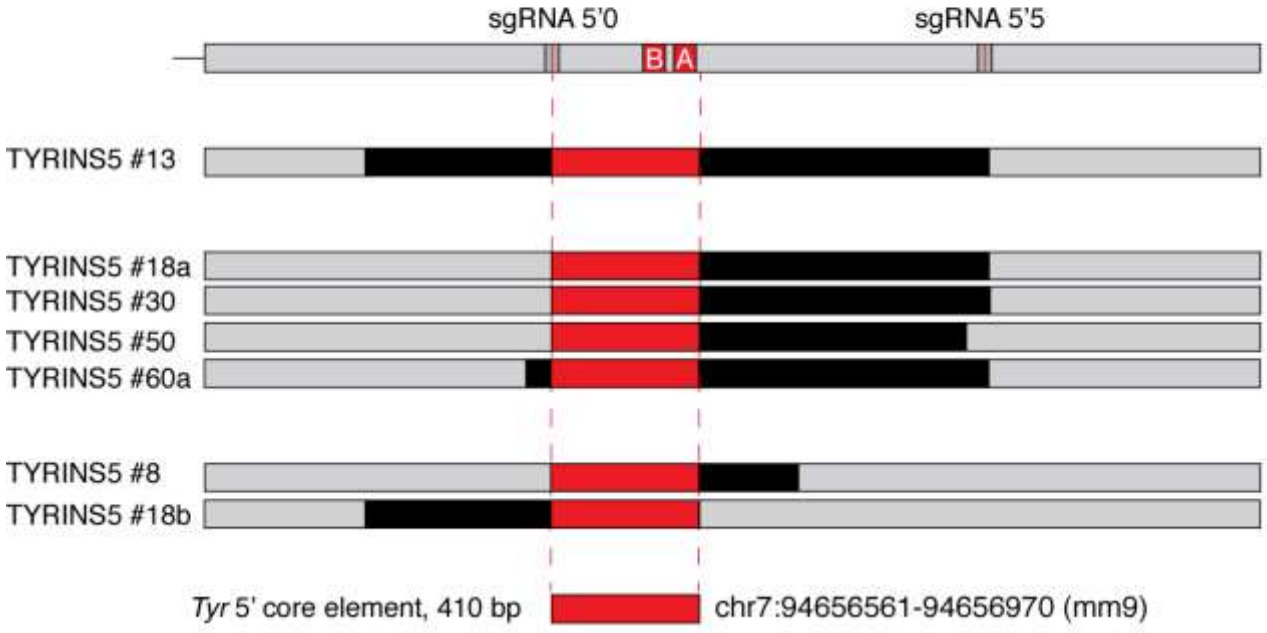
Many mutant deletion alleles generated upon targeting the 5' *Tyr* Boundary by CRISPR-Cas9



Seruggia et al. 2015 NAR



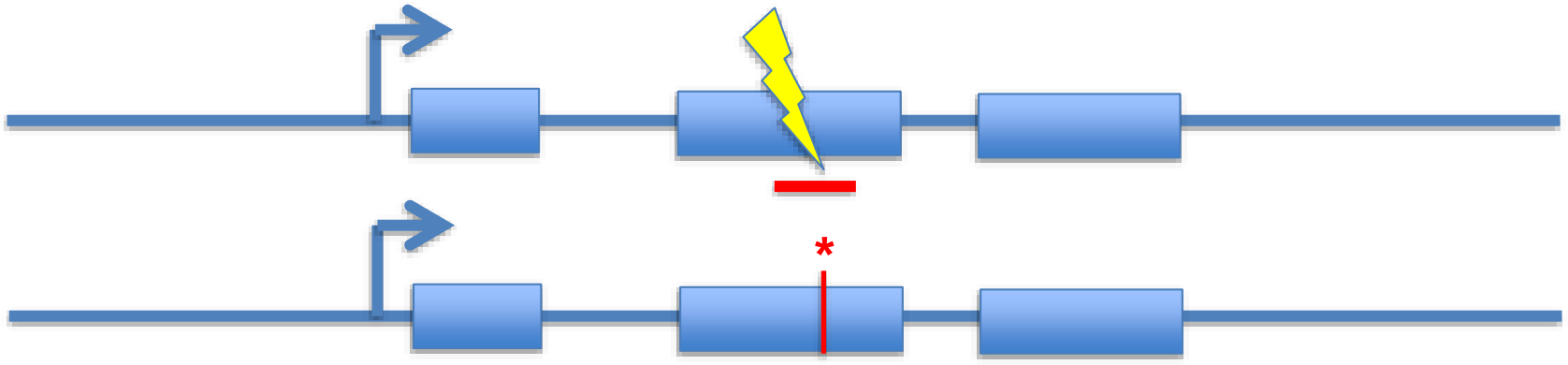
Comparing different *Tyr* 5' Boundary targeted alleles with similar phenotypes reveals the location of the functionally relevant endogenous regulatory DNA sequences



Genetic analysis now possible in mice!

Seruggia et al. 2015 NAR

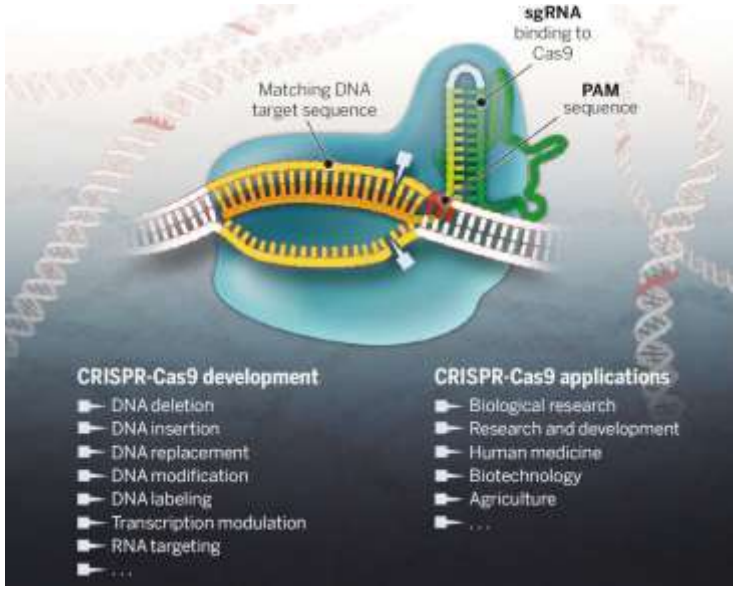
Point mutations



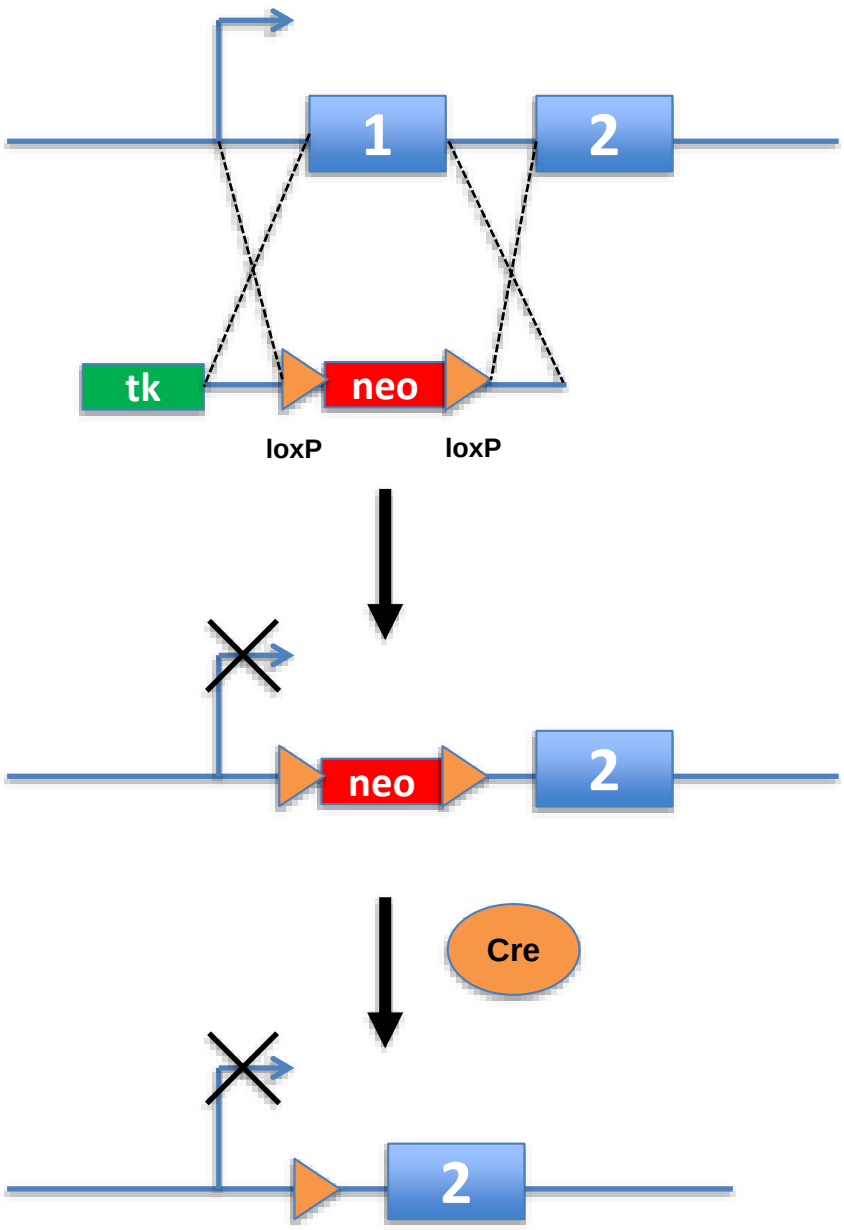
- Relatively straight forward, but can be challenging, many alleles will be generated
- INDELs will mostly be **also** generated
- Relatively doable protocol (however, if expected mutation not found, the number of embryos/animals to be used rapidly increases)
- Optimally: must have screening protocols in place (silent mutations/RFLPs/sequencing…)

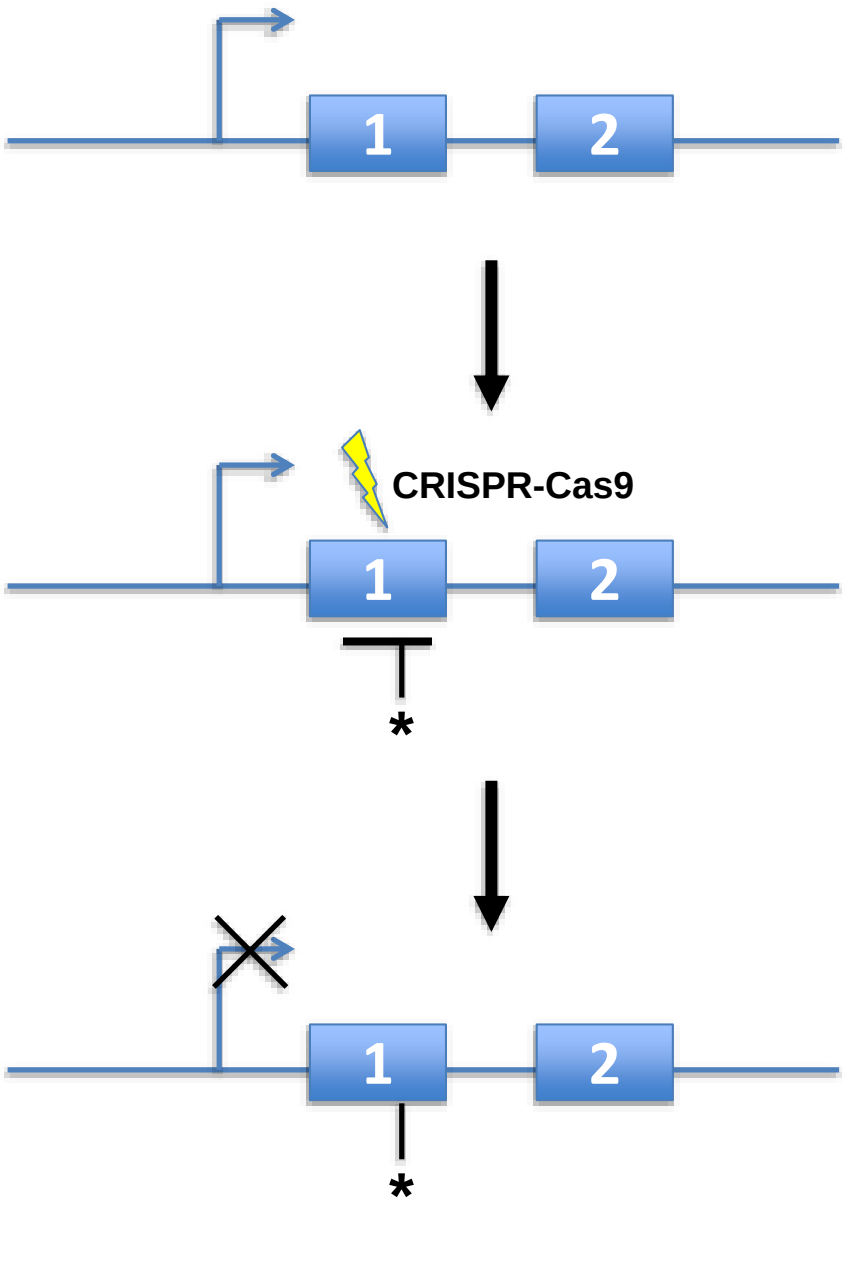
AVATAR CRISPR mice

- Easier approach to reproduce human mutations in animal models



“Classical” versus CRISPR-mediated mutagenesis





El Málaga juega en primera división

El Málaga juega en primera división

El Málag juega en primera división

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El museo Picasso está en Málaga

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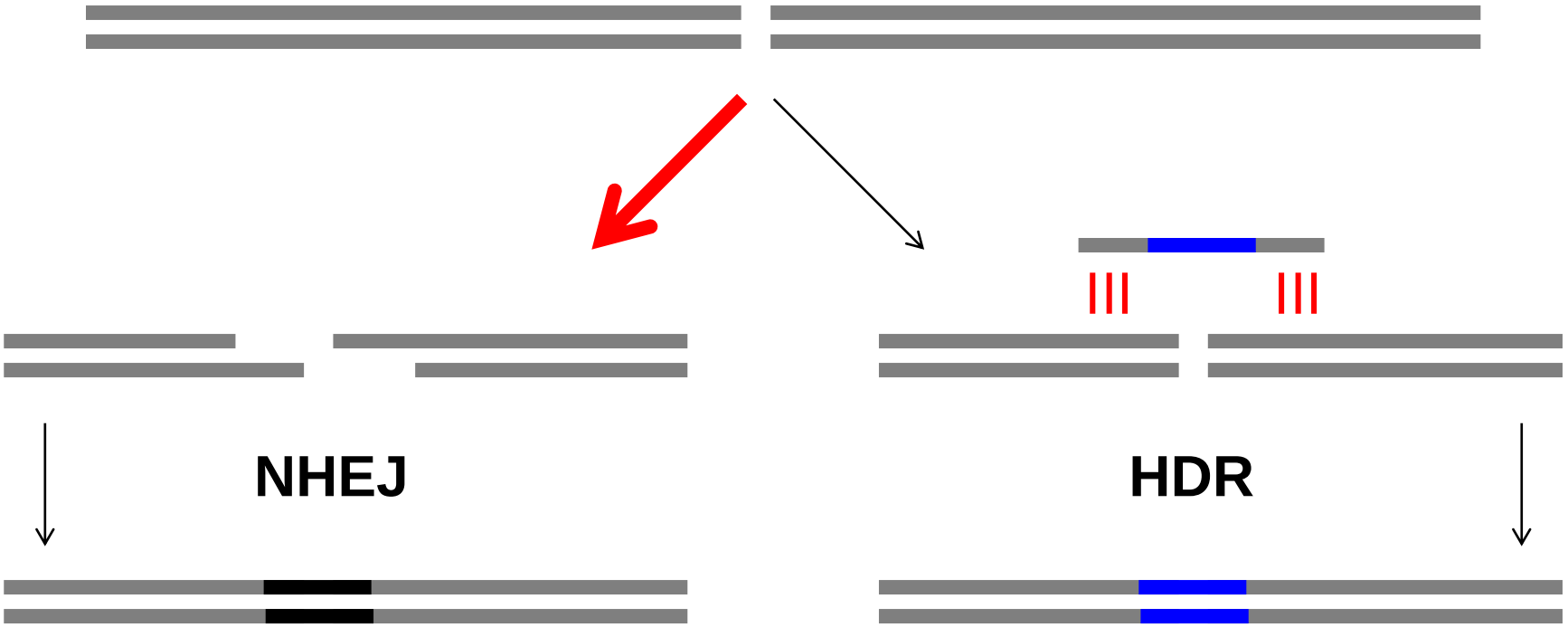
El museo Picasso está en Málaga

El museo Picassa está en Málaga

Treatments

Improving Gene Edition



INDELs – Gene disruption

Gene repair / edit

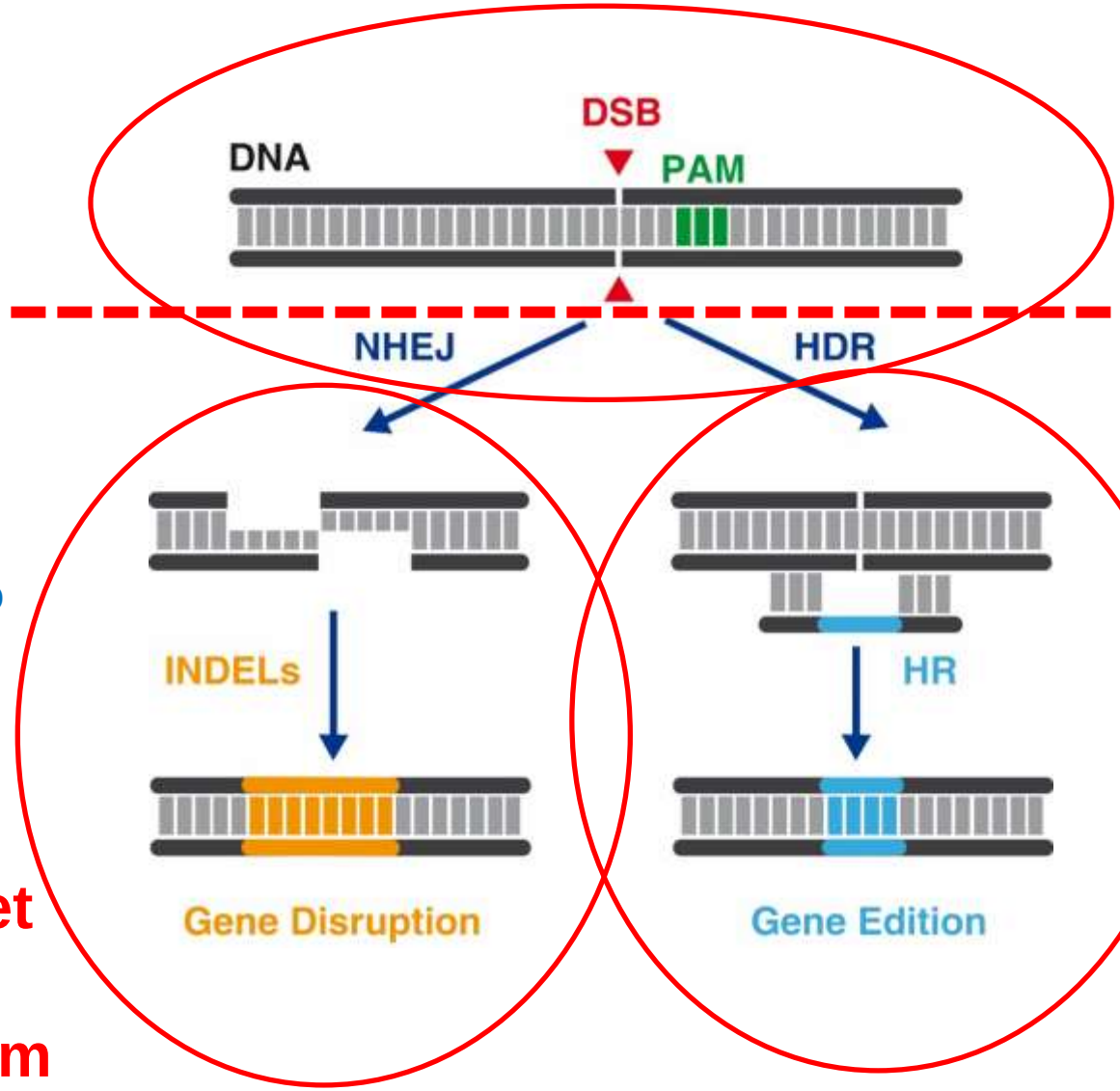
Current limitations of CRISPR

- **On-target uncertainty**: many alleles are generated through NHEJ
- Most/all founder edited-organisms are **mosaic**
- Error-prone NHEJ is the default repairing pathway
- Donor template-specific HDR is not the preferred repairing pathway
- **Off-targets**: similar target sequences can be altered
- Reaching a significant number of target cells (viral & non-viral delivery systems)

Current limitations of genome editing

Off-target effects

Related to CRISPR



Unrelated to CRISPR

On-target effects Mosaicism

HDR is not the preferred repairing pathway

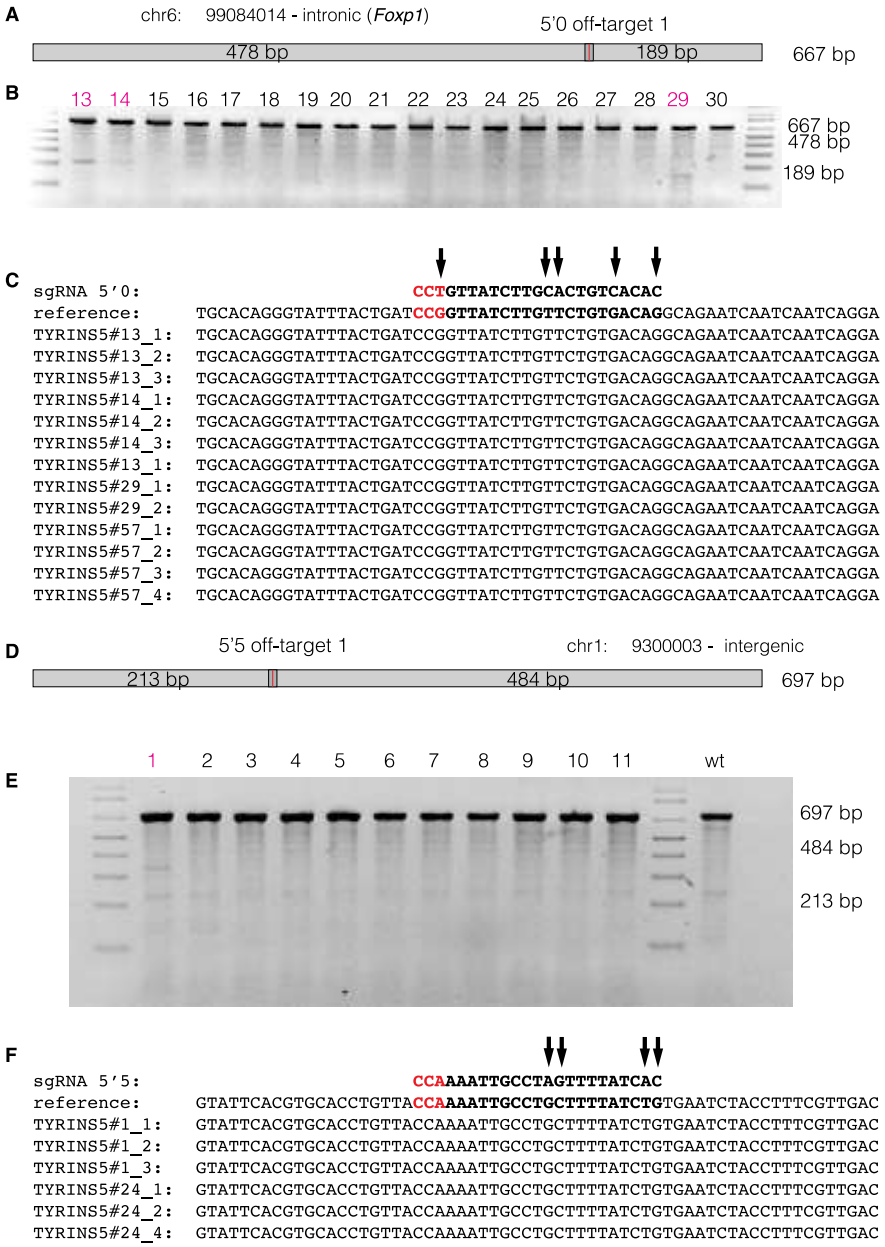
Off-targets: we can deal with them

- Off-targets depend mainly on the selected guide RNA and, to a lesser extent, on the Cas (different Cas have different properties)
- New algorithms developed for selecting optimal guide RNAs (Breaking-Cas, CRISPOR, Crispr-GOLD…)
- Can be reduced to an acceptable minimum by reducing the amount (Cas protein, not RNA or DNA) and the time of action of Cas nucleases (inhibitors)

Improved design of RNA guides for optimized CRISPR experiments

Oliveros *et al.* Nucleic Acids Res. 2016

What about off-targets?



Mostly observed in vitro

We have not found off-target sites with altered sequences in genome-edited mice

Off-target mutations are rare in Cas9-modified mice

Vivek Iyer, Bin Shen, Wensheng Zhang, Alex Hodgkins, Thomas Keane, Xingxu Huang & William C Skarnes

Affiliations | Corresponding authors

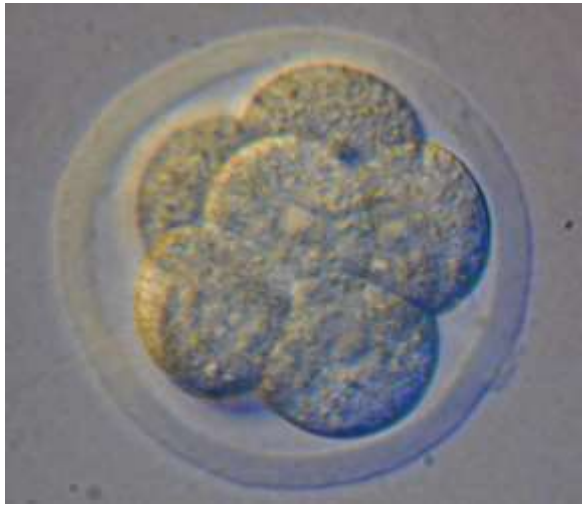
Nature Methods **12**, 479 (2015) | doi:10.1038/nmeth.3408
Published online 28 May 2015

Confirmed by NGS

Seruggia et al. 2015 NAR

On-targets: the real problem

- Founder animals are nearly always complex mosaic
- Many different alleles can be present
- Not all of them might transmit through germline



One 8-cell embryo = 16 possible alleles

Multiple alleles present in CRISPR founder gene-edited mice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Reference:  AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCACGGTAGGGTTGATTTCAGGAAATGTAA
B9040.1     AACATTGGAGGAGCTGC ACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAG----------GTAGGGTTGATTTCAGGAAATGTAA
B9040.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGGTAGGTAGG-TTGATTTCAGGAAATGTAA
B9040.3     AACACTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAG----------GTAGGGTTGATTTCAGGAAATGTAA
B9040.4     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGC-----AGGGTTGATTTCAGGAAATGTAA
B9040.5     CAAGCTCCTGCCCCACTTTCAAAGCTGTACTGAACTGCAGTTTCTTCTCCACCCAGATTCCTGCAAGACCTTGCACCGGGG----------(437bp)----------
B9040.6     ----------------------------------------(561bp)----------------------------------------------------------------
B9041.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCTGTTACCTAGGGTTGATTTCAGGAAATG
B9041.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAAC--------AGGGTTGATTTCAGGAAATGTAA
B9041.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACA----GGTAGGGTTGATTTCAGGAAGTGTAA
B9041.4     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACG----GGTAGGGTTGATTTCAGGAAATGTAA
B9041.5     AACATTGGAGGAGCTGCCACTGCTATTTGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACA----GGTAGGGTTGATTTCAGGAAATGTAA
B9042.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCACGGTAGGGTTGATTTCAGGAAATGTAA
B9042.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAAT--------------GGTAGGGTTGATTTCAGGAAATGTAA
B9042.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAA-TGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCACGGTAGGGTTGATTTCAGGAAATGTAA
B9042.4     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAAT--------------GGTAGGGTTGATTTCAGGAAGTGTAA
B9043.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAAT--------------GGTAGGGTTGATTTCAGGAAGTGTAA
B9043.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCACACTGGTAGGGTTGATTTCAGGAAGTG
B9043.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTCCCAATCAGGAGTTGAGAAAAAT--------------GGTAGGGTTGATTTCAGGAAGTGTAA
B9043.4     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGATAGACATGTCGAGGA------(134bp)--------------------
B9044.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAAT--------------GGTAGGGTTGATTTCAGGAAATGTAA
B9044.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCGATCAGGAGTTGAGAAAAATGGTAGGTAAC--------AGGGTTGATTTCAGGAAATGTAA
B9045.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTTGTTTAT------------------------------CAAGGTAGGGTTGATTTCAGGAAATGTAA
B9045.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAAT--------------------GGTTGATTTCAGGAAATGTAA
B9045.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTTGTTTAT------------------------------CAAGGTAGGGTTGATTTCGGGAAATGTAA
B9046.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCACCTAGGTAGGGTTGATTTCAGGAAATG
B9046.2     AACATTG--------------------------------(87bp)----------------------------------------------------TAGGGTTGATTTCAGGAAATGTAA
B9046.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCAATGGTAGGGTTGATTTCAGGAAATGTA
B9046.4     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCACCTAGGTAGGGTTGATTACAGGAAATG
B9060.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACA----GGTAGGGTTGATTTCAGGAAATGTAA
B9060.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAGATGGTAGGTAACA----GGTAGGGTTGATTTCAGGAAATGTAA
B9060.2     GACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACA----GGTAGGGTTGATTTCAGGAAATGTAA
B9060.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCT-------GTTGATTTCAGGAAATGTAA
B9060.4     AACATCGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACA----GGTAGGGTTGATTTCAGGAAATGTAA
B9064.1     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCAAAAATGGTAGGTAGGGTTGATTTCAGG
B9064.2     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTA----------GGTAGGGTTGATTTCAGGAAATGTAA
B9064.3     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAGTGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGCAAAAATGGTAGGTAGGGTTGATTTCAGG
B9064.4     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCAGGAGTTGAGAAAAATGGTAGGTAACAGC-----AGGGTTGATTTCAGGAAATGTAA
B9064.5     AACATTGGAGGAGCTGCCACTGCTATTGGGGACCCACCAAATGTTATCATTGTTTCCAATCA----------------------------GGTAGGGTTGATTTCAGGAAATGTAA
```

CRISPR-Cas is the future

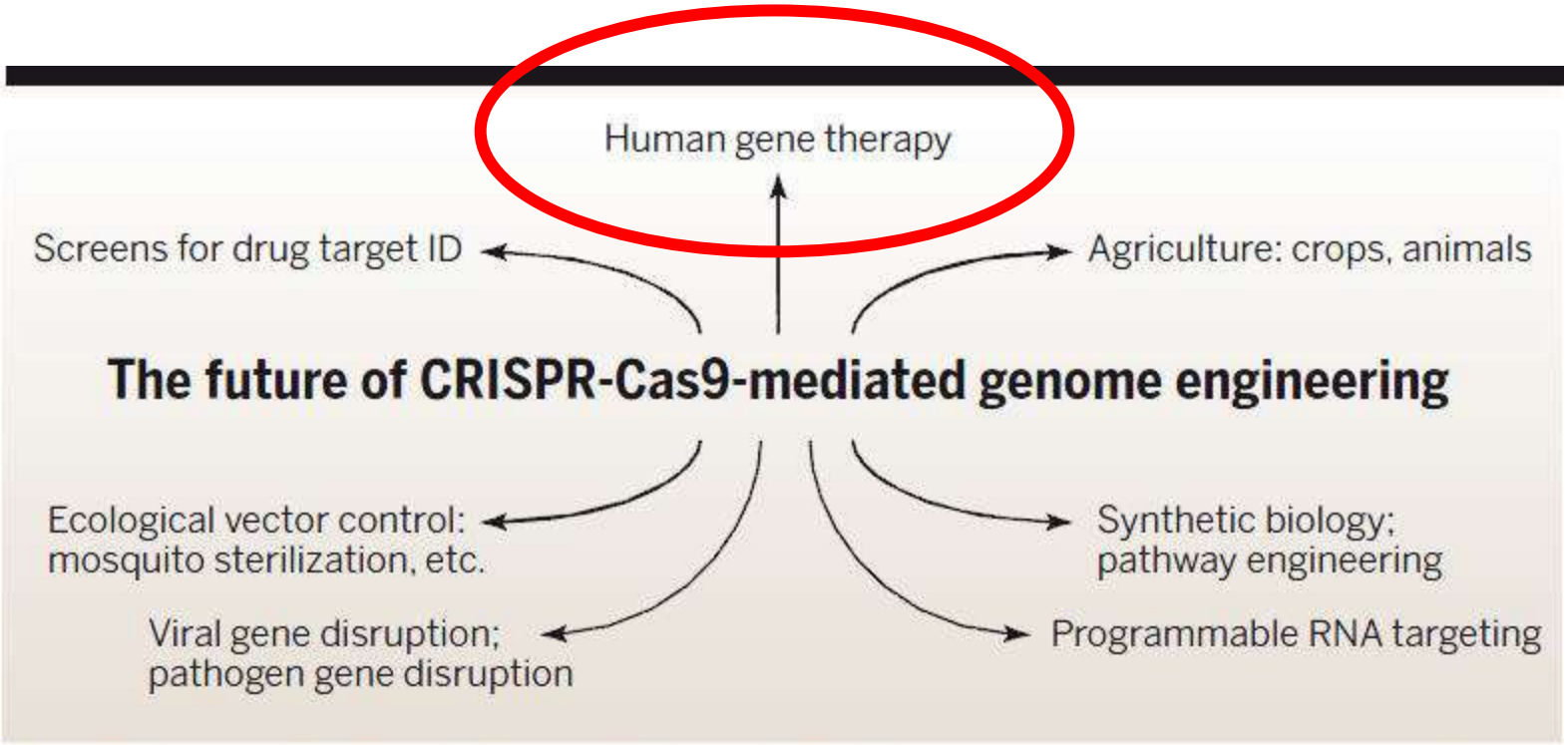
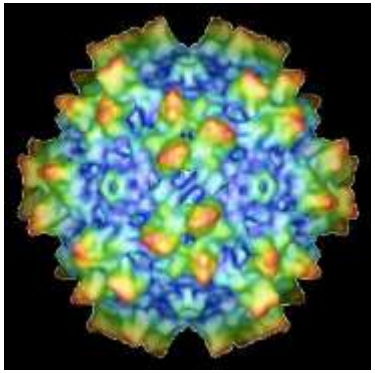


Fig. 6. Future applications in biomedicine and biotechnology. Potential developments include establishment of screens for target identification, human gene therapy by gene repair and gene disruption, gene disruption of viral sequences, and programmable RNA targeting.

Doudna & Charpentier (2014) *Science*

CRISPR tools and somatic gene therapy of human rare diseases

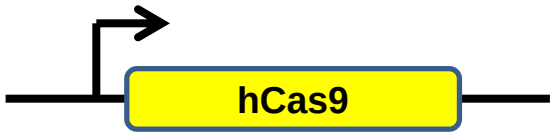


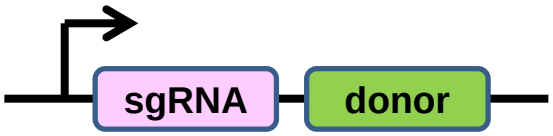
Adeno Associated Virus
AAV

AAV 1

AAV 2

Different serotypes with diverse tropism to different cellular types
Cargo ~4.7 kb





Increasing number of animal models of rare monogenic diseases corrected via CRISPR

Preclinical animal models

- Duchenne muscular distrophy (DMD)
- Ornithine transcarbamylase (OTC) deficiency
- Hereditary tyrosinemia I (FAH deficiency)
- Congenital cataract (CRYGC)
- Chronic granulomatous disease (CGD)
- Retinitis pigmentosa (RP)
- Leber congenital amaurosis (LCA)
- Hungtinton Disease (HD)
- …
- Also **many iPS cells models** correcting gene mutations via CRISPR strategies

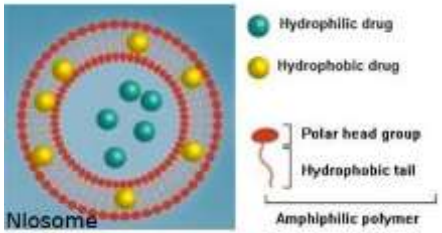
CRISPR-mediated gene therapy of a human rare disease: chronic granulomatous disease (CGD) in human iPS cells

Challenge: multiple alleles are generated

Flyn et al. (2015) Experimental Hematology

Challenges for CRISPR-mediated gene therapy in patients

- Immune response against Cas9
- Immune response against AAV
- Finding the right AAV serotype (preferential)
- Non-viral delivery systems (nanoparticles, liposomes) → the future



Niosomes
J.L. Pedraz (UPV/EHU)

- Reaching a significant number of target cells
- On-target allelic multiplicity (indetermination)
- Off-targets
- HDR (dividing cells) versus NHEJ (quiescent cells/neurons) →HITI (homology-independent targeted integration)

Cas9: Bang Wong, Broad Institute of Harvard and MIT, Cambridge, MA

- Cas9 antibodies found in human serum
- Anti-Cas9 T lymphocites found in human blood
- 79% individuals have antibodies against SaCas9
- 65% individuals have antibodies against SpCas9
- 46% individuals have anti-Cas9 T cells
- Immunosupression or alternative Cas proteins

Cold Spring Harbor Laboratory

bioRxiv beta

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5 Jan 2018

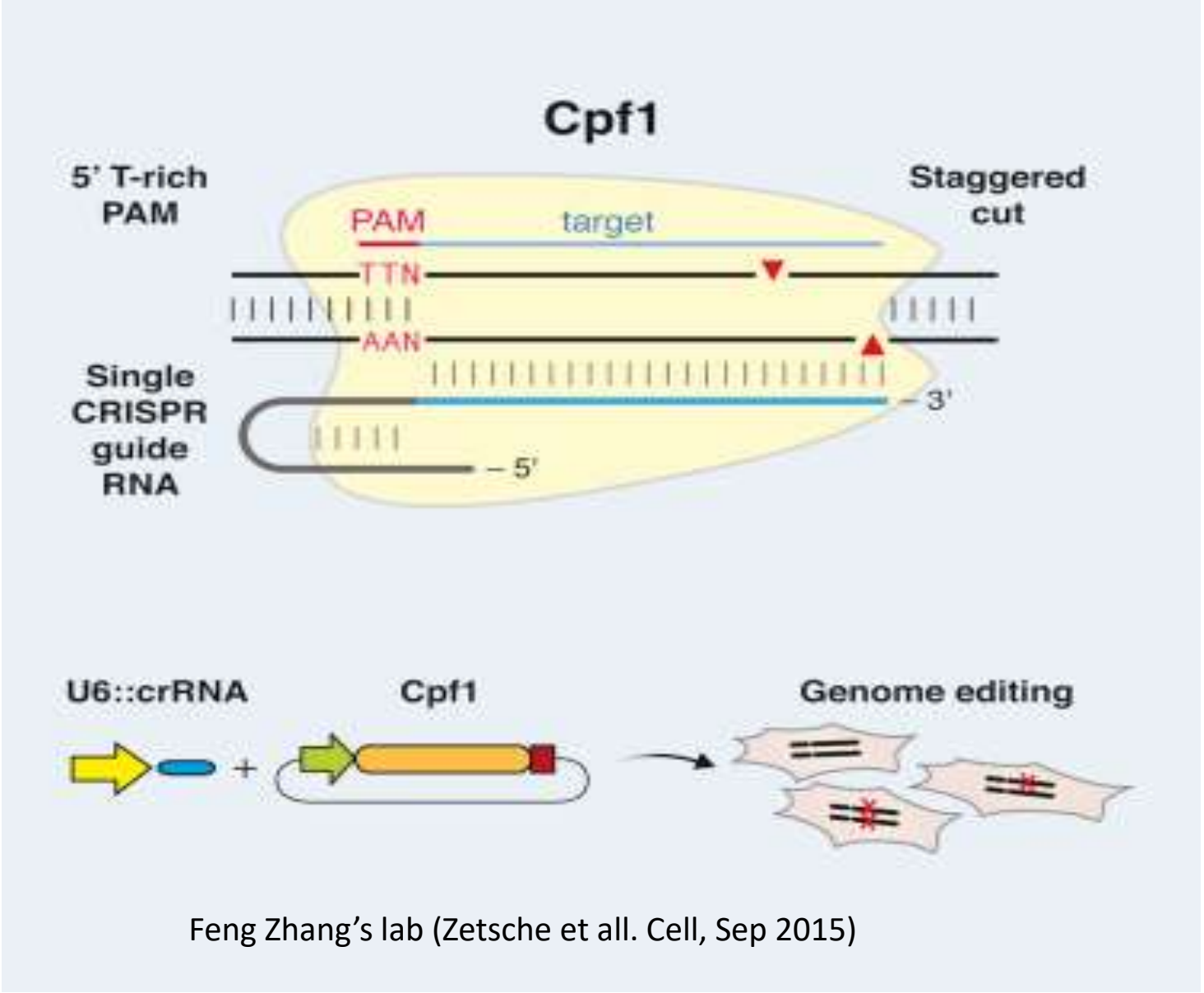
New Results

Identification of Pre-Existing Adaptive Immunity to Cas9 Proteins in Humans

Carsten Trevor Charlesworth, Priyanka S Deshpande, Daniel P Dever, Beruh Dejene, Natalia Gomez-Ospina, Sruthi Mantri, Mara Pavel-Dinu, Joab Camarena, Kenneth I Weinberg, Matthew H Porteus

doi: https://doi.org/10.1101/243345

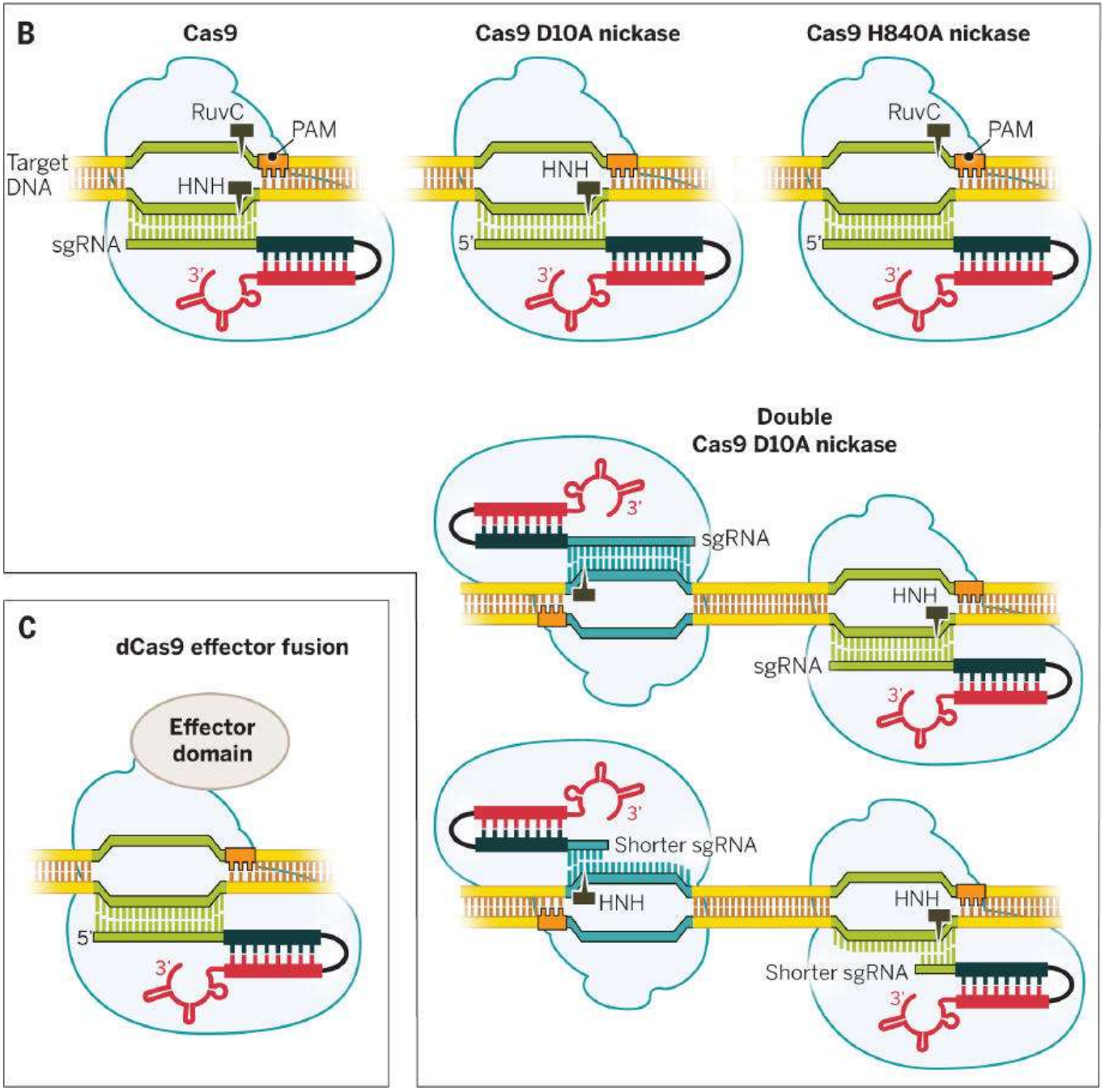
Alternative Cas-like proteins



Feng Zhang's lab (Zetsche et all. Cell, Sep 2015)

Cas12a

Francisella tularensis

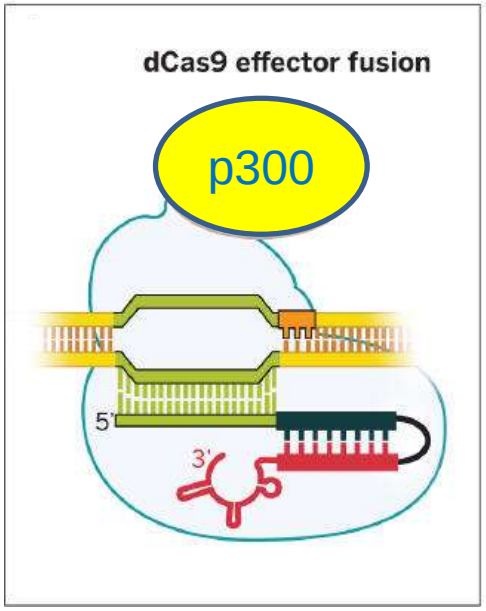


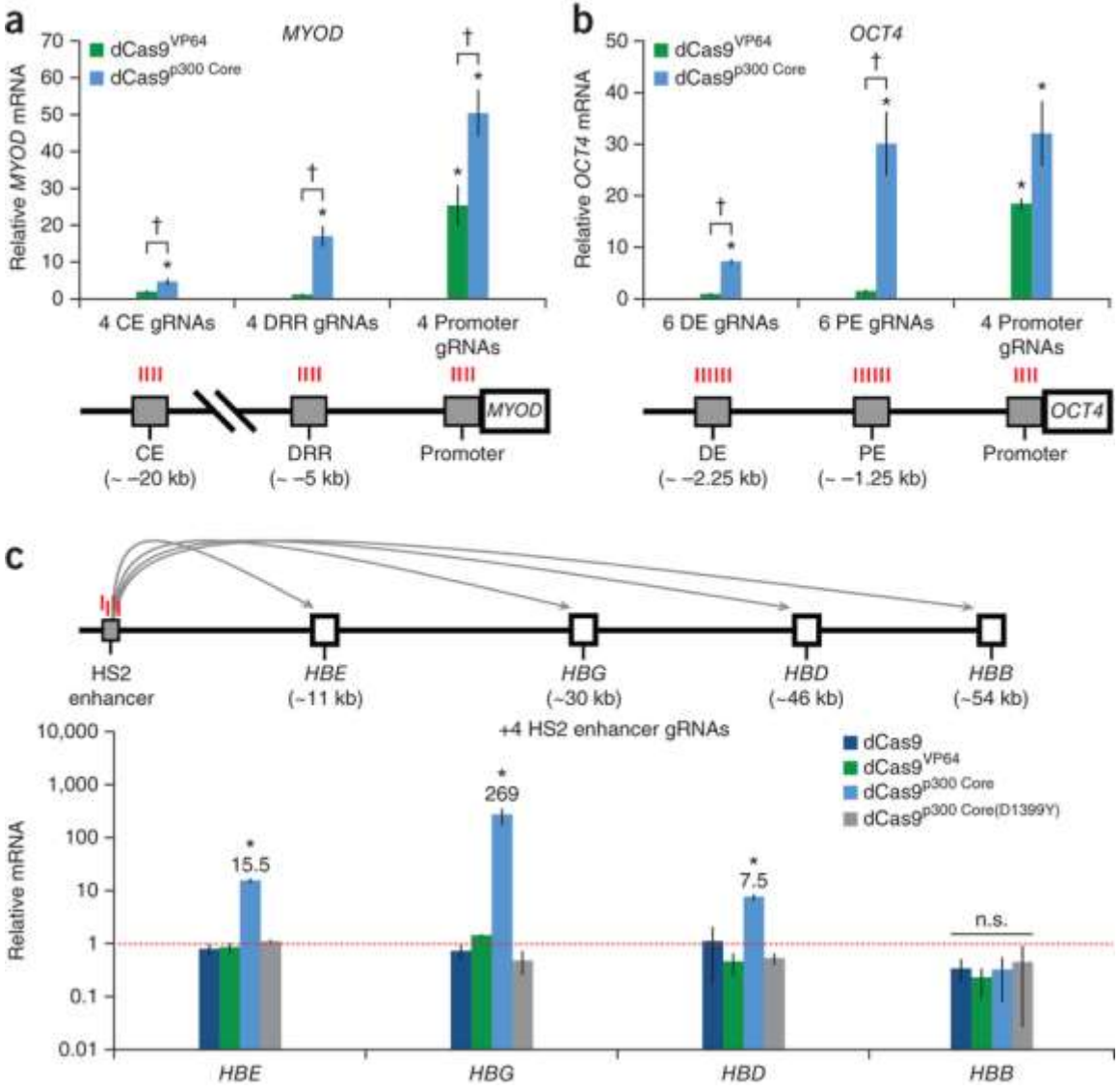
Doudna & Charpentier (2014) Science

Targeting acetylation of Histone H3 Lysine 27 in the genome

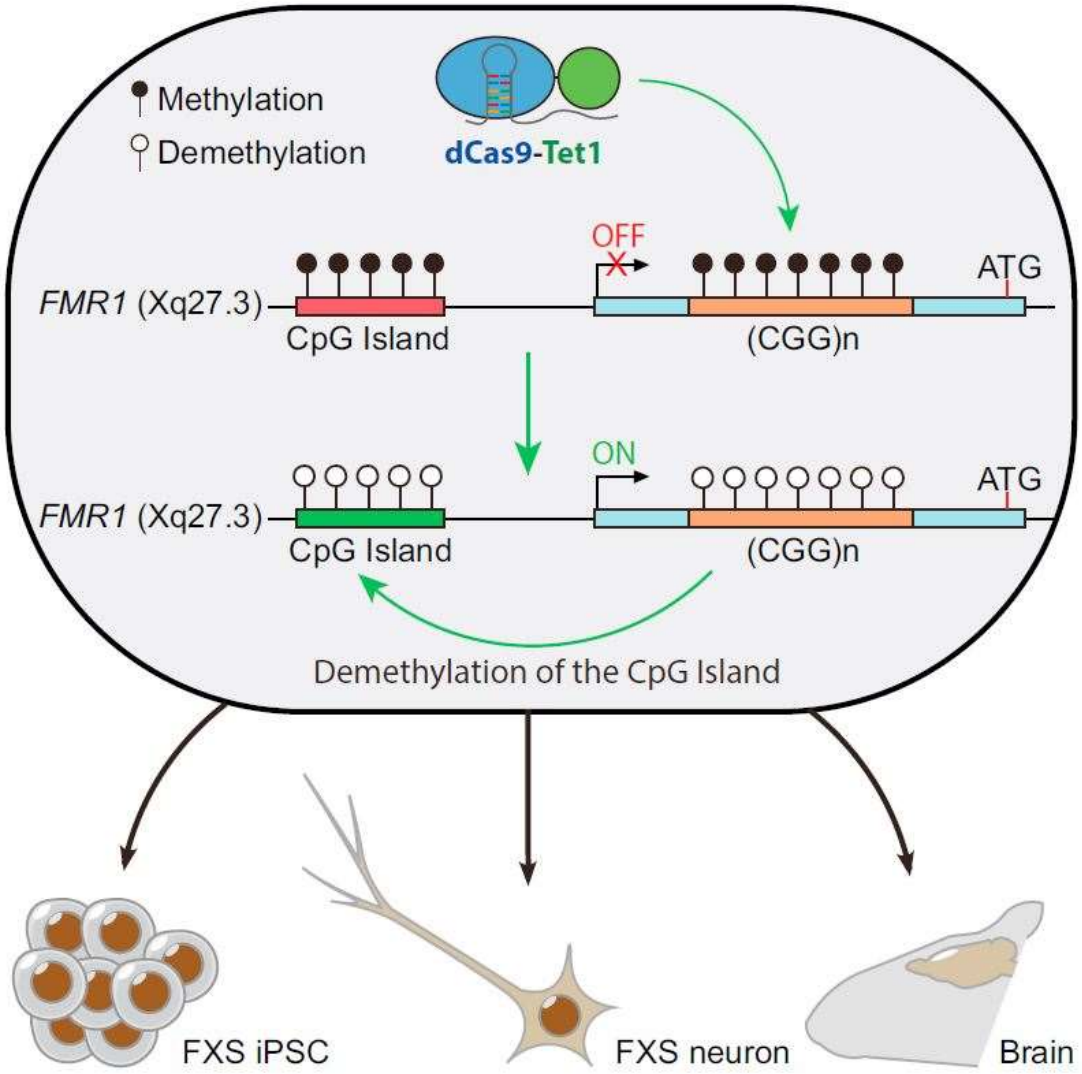
Activating gene expression by CRISPR-dCas9/p300

Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers
Hilton *et al.*
Nature Biotechnology 33, 510–517 (2015)

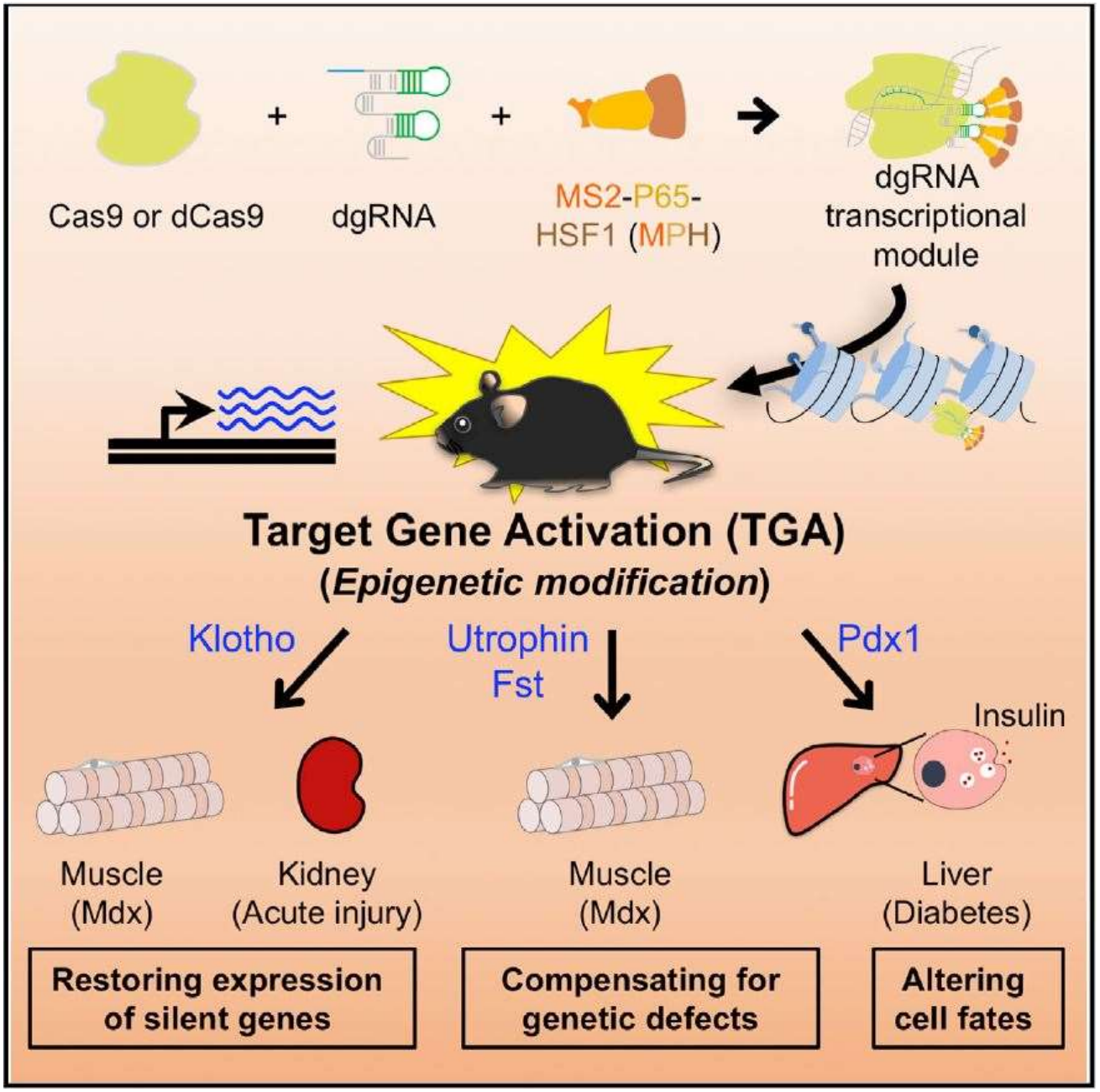




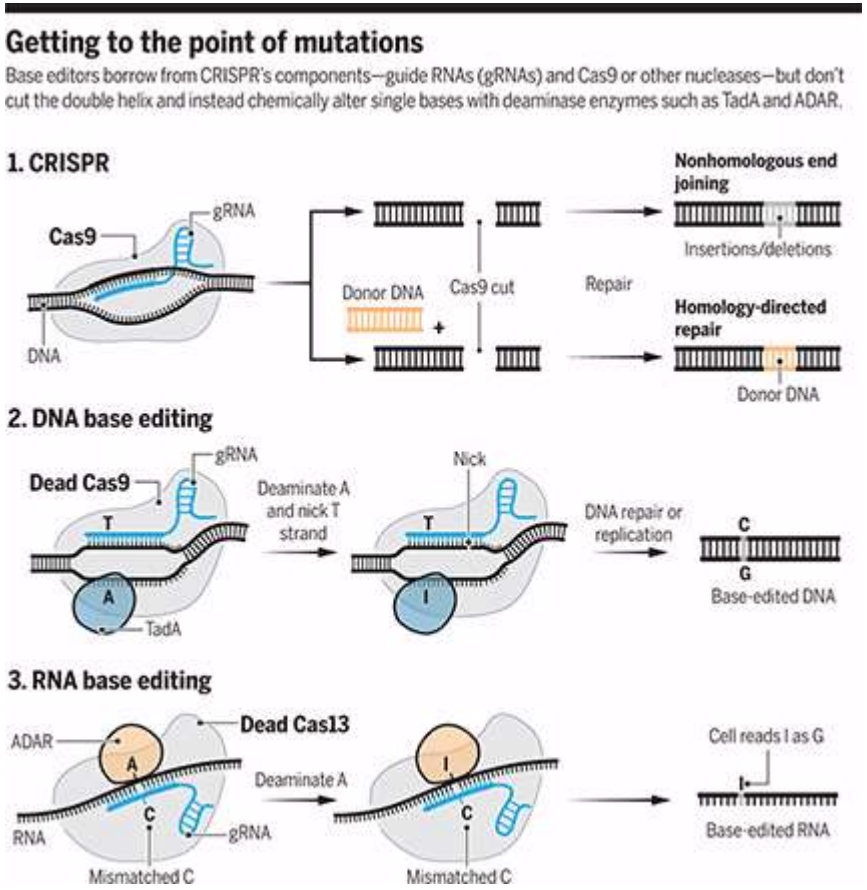
CRISPR-Cas rescues Fragile X syndrome



Liu et al. Cell 2018



Liao et al. Cell 2017



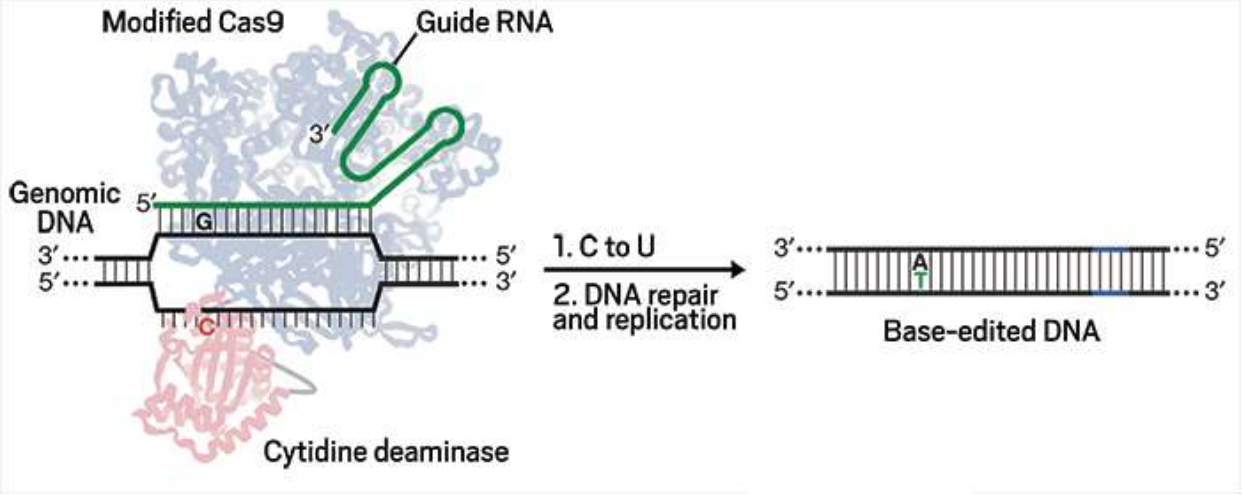
C → U → T

A → I → G

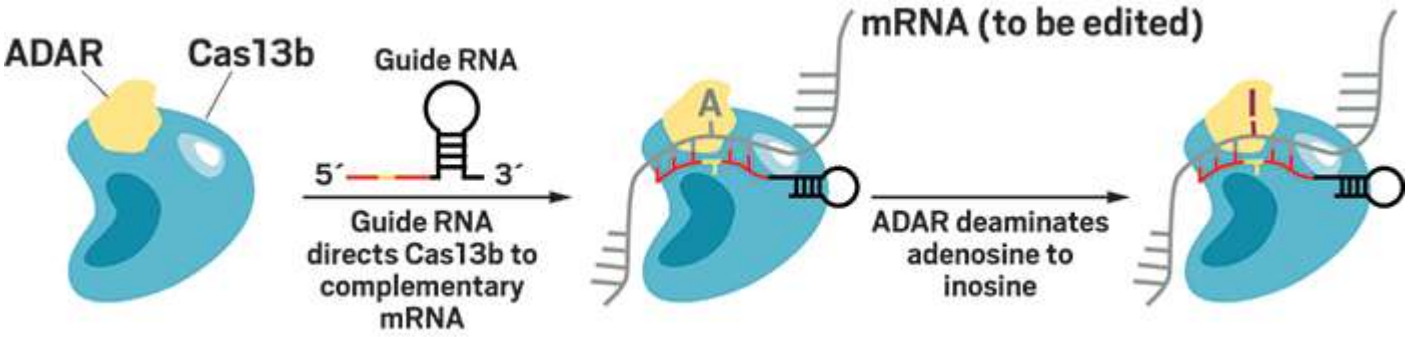
Kim et al. Nat Biotech. 2017

David Liu Lab

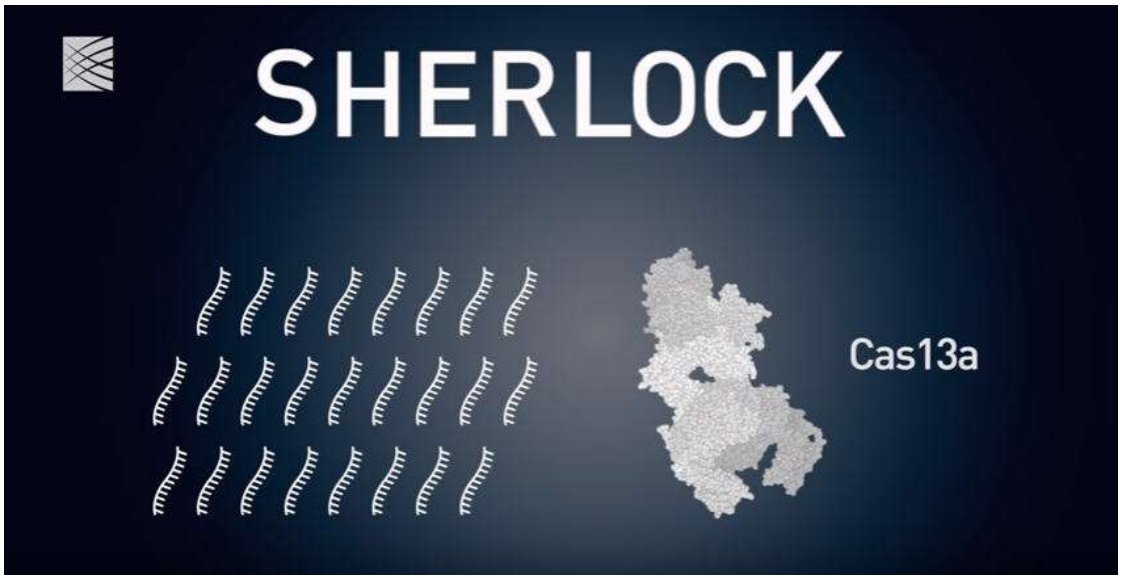
CRISPR-derived BASE EDITORS



CRISPR 2.0 (editing RNA)



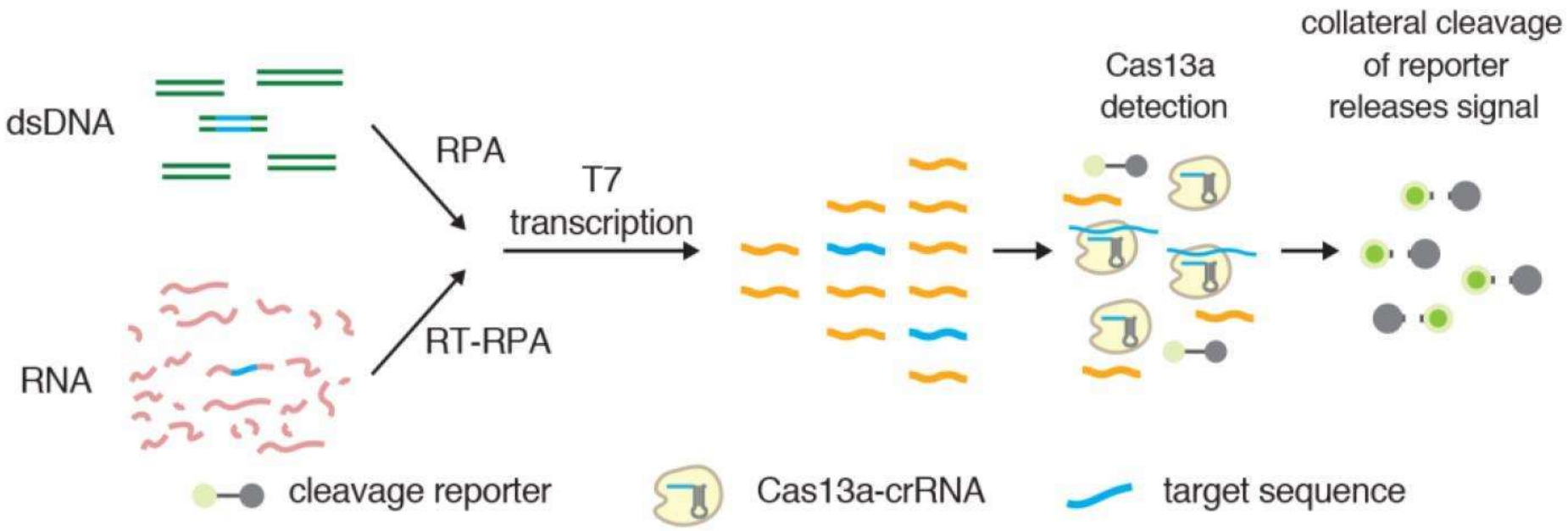
Feng Zhang's Lab Nature 2017



CRISPR-Cas as a diagnostic tool 1

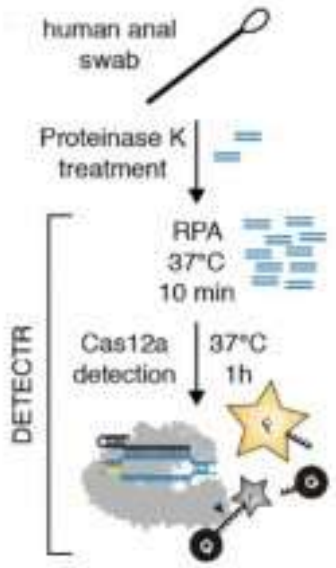
Feng Zhang Lab

Gootenberg et al. Science 2017



CRISPR-Cas as a diagnostic tool - 2

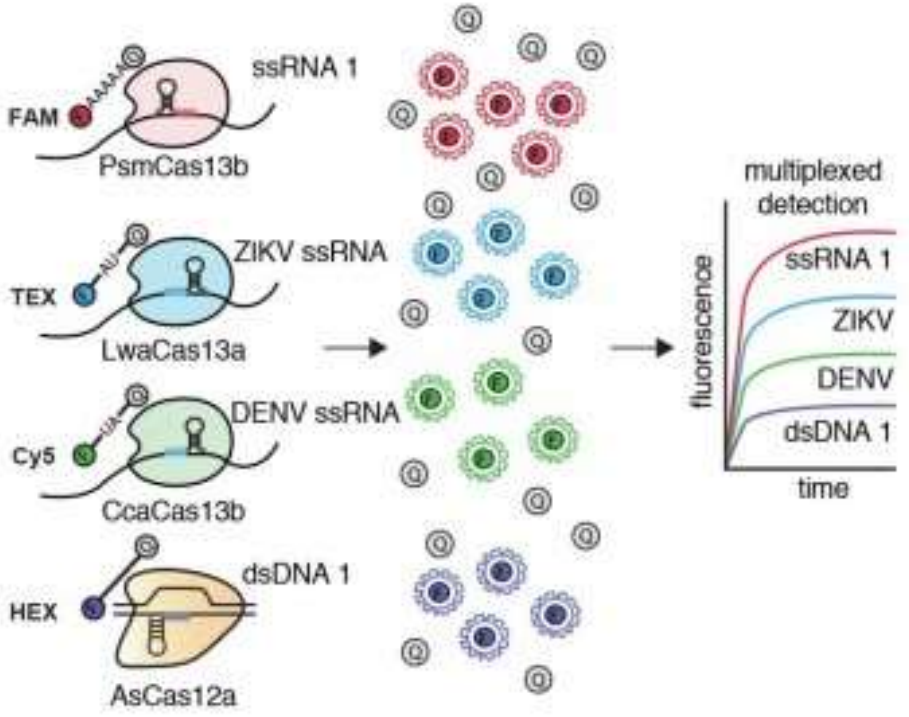
Doudna Lab



J. S. Chen et al., Science 10.1126/science.aar6245 (2018)

DETECTR

Zhang Lab



SHERLOCKv2

J. S. Gootenberg et al., Science 10.1126/science.aaq0179 (2018)


CRISPR
EVERYWHERE

http://www.cnb.csic.es/~montoliu/

Fotos: Ana Ytyrralde

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Pawel Pelczar (Univ. Zürich / Univ. Basel) Transgenic Unit
Belén Pintado (CNB-CSIC, Madrid) Transgenic Unit
Juan Carlos Oliveros (CNB-CSIC, Madrid) Bioinformatics

The CRISPR web page at CNB

www.cnb.csic.es/~montoliu/CRISPR/

Google for CNB + CRISPR

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www.albinismo.es