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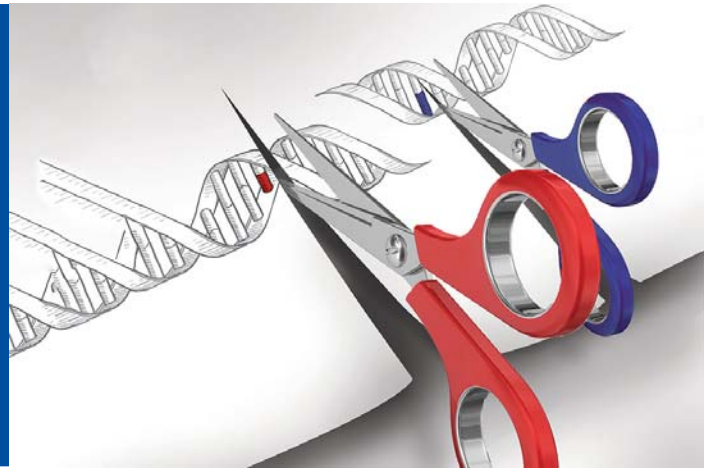
Einführung in die Grundlagen des Genome Editing am Beispiel von Punktmutationen

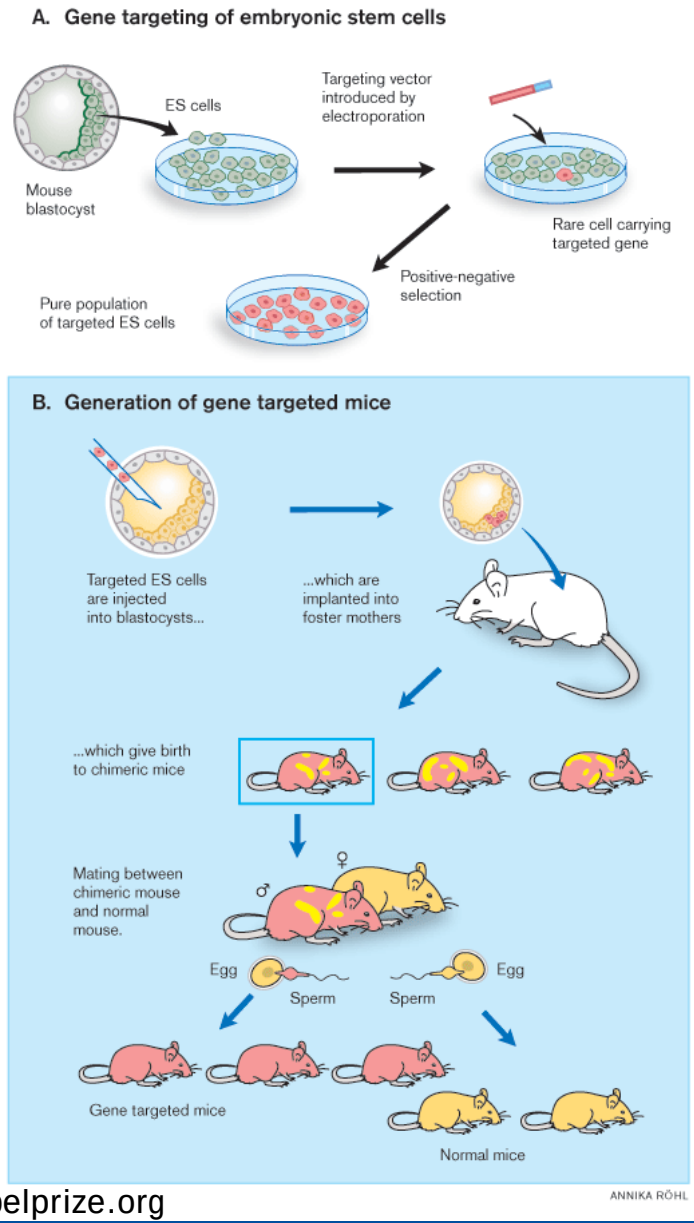
Björn Petersen
Institute of Farm Animal Genetics
Friedrich-Loeffler-Institut (FLI)
Mariensee, Neustadt, Germany



(Illustration by Broad Institute)

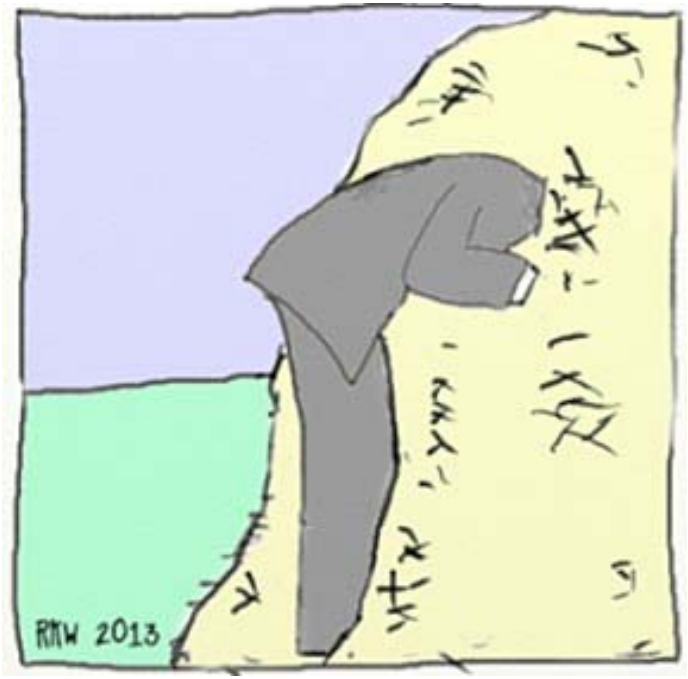
BfR-Symposium, Berlin, 06.12.16

Genome Editing in mice (pre-GE era)



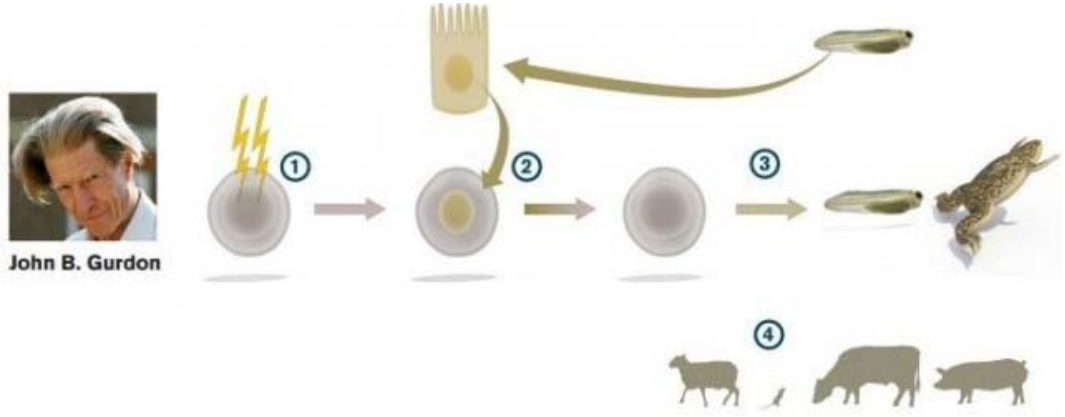
Quelle:http://www.nobelprize.org

Finding the needle in the haystack!!



Dolly breaks a dogma

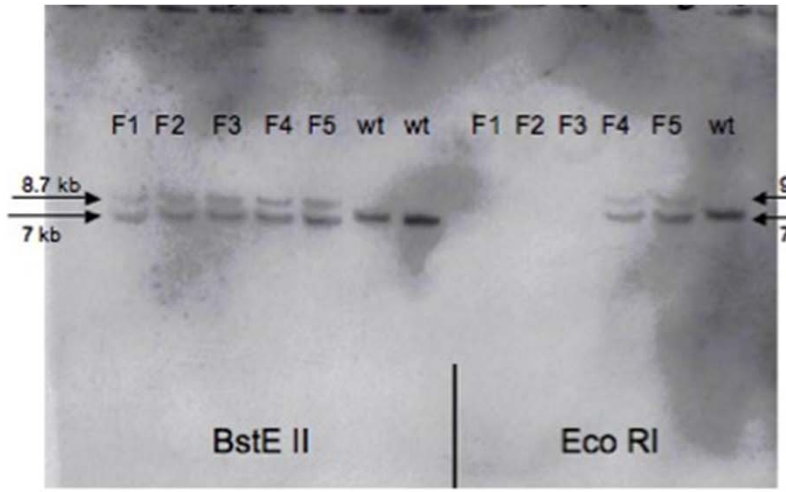
Gurdon 1962

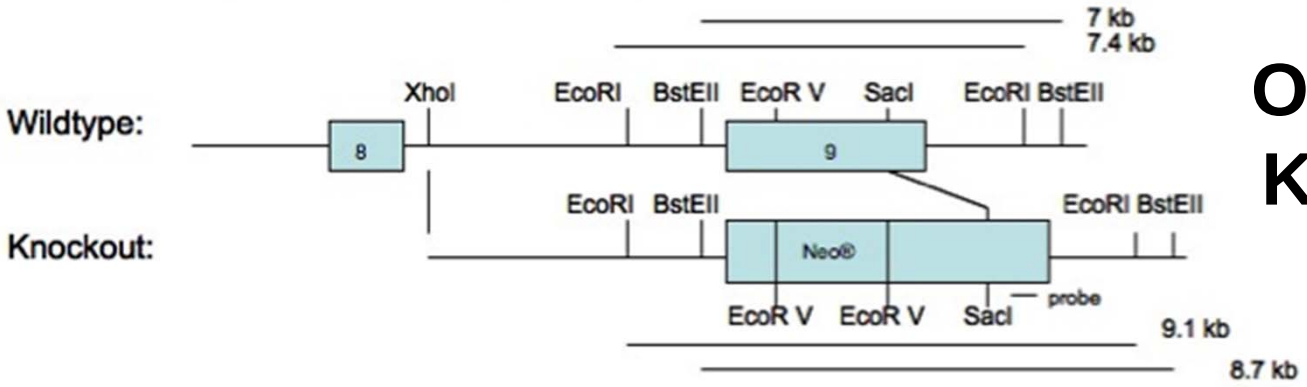


Sir John Gurdon, Nobel laureate 2012
(together with Shinya Yamanaka)

Dolly 1996-2003, with Sir Ian Wilmut

Genome Editing in farm animals (pre-GE era)





Efficiency of successful targeting:
$1x10^{-6}$

Efficiency of pig cloning:
$3\text{-}5x10^{-2}$

Feasible, but very inefficient!!
Only heterozygous KO/KI achieveable

Genome editing tools

[Foto: Gernot Krautberger - Fotolia.com]

Promises of Genome Editors

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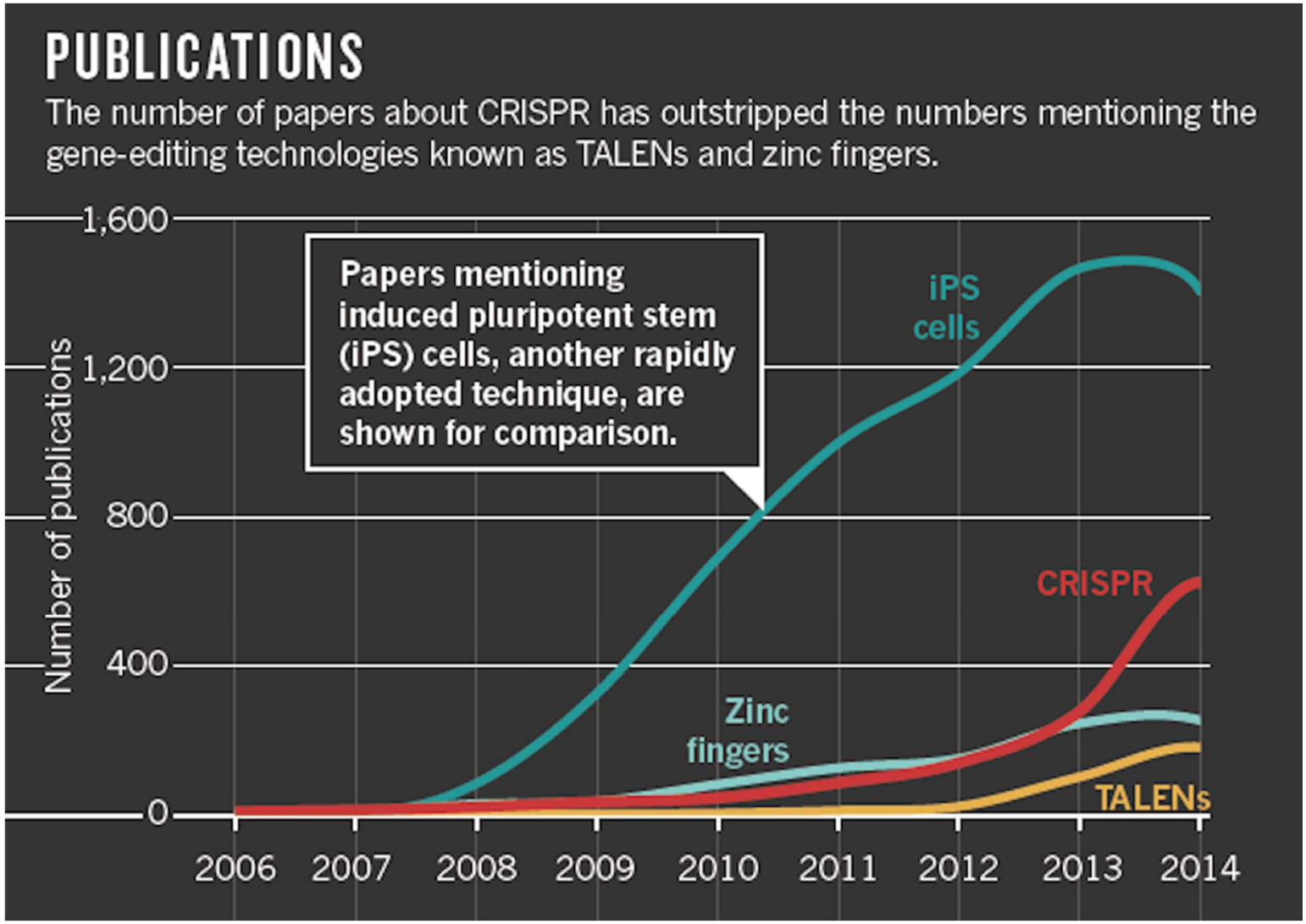
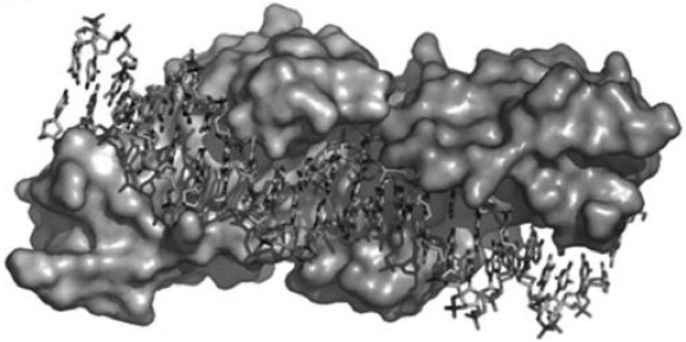
Genome Editing Publications

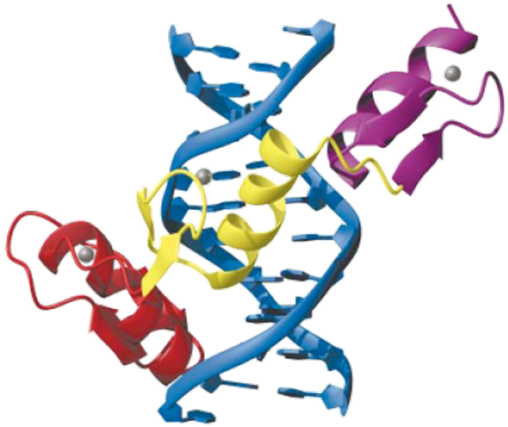


Nature 2015

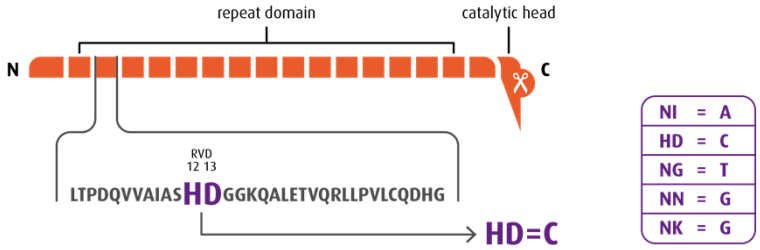
Classes of genome editing tools



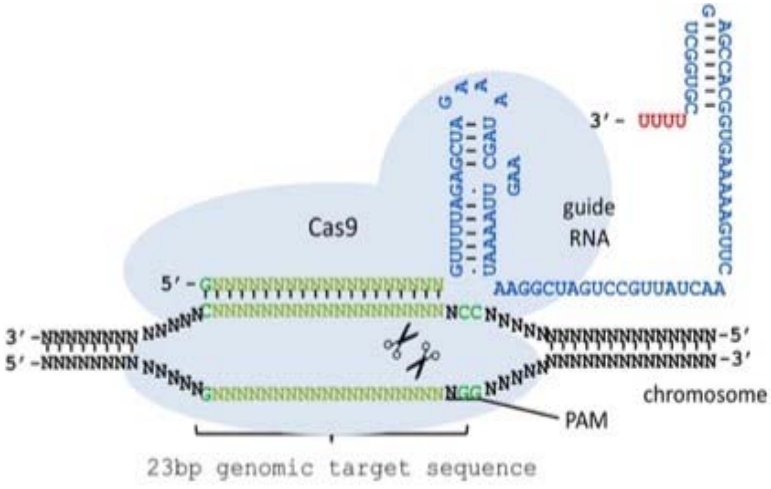
Meganucleases



Zinc-Finger Nucleases

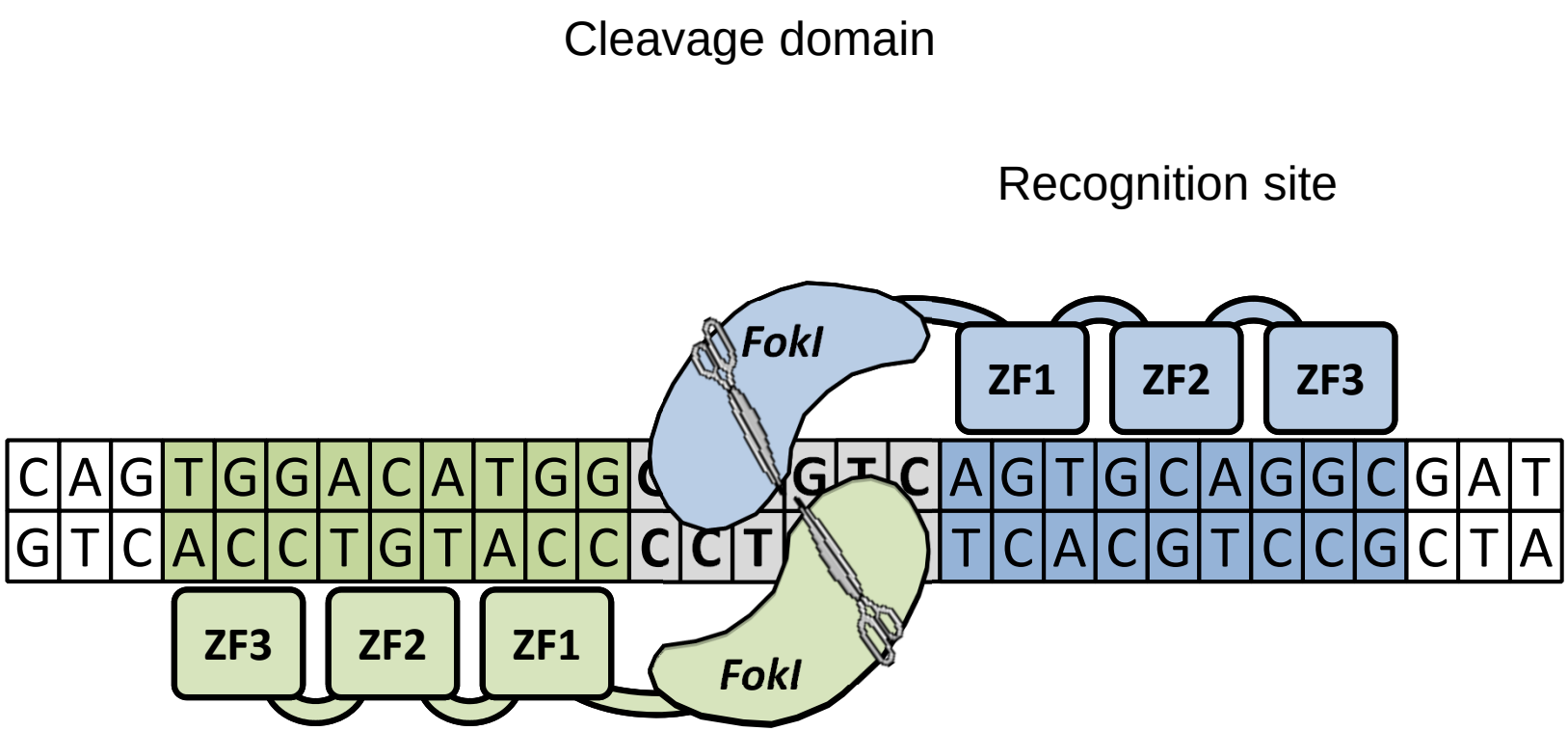


Transcription activator- like effektor nucleases (TALENs)



CRISPR/CAS

General mechanisms of genome editors



Genome editing begins with dsDNA cleavage

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General mechanisms of genome editors

C	A	G	T	G	G	A	C	A	T	G	G	**G**	**G**	**A**
G	T	C	A	C	C	T	G	T	A	C	C	**C**	**C**	**T**

G	**T**	**C**	A	G	T	G	C	A	G	G	C	G	A	T
C	**A**	**G**	T	C	A	C	G	T	C	C	G	C	T	A

HDR

NHEJ

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General mechanisms of genome editors

C	A	G	T	G	G	A	C	A	T	G	G	**G**	**G**	**A**
G	T	C	A	C	C	T	G	T	A	C	C	**C**	**C**	**T**

G	**T**	**C**	A	G	T	G	C	A	G	G	C	G	A	T
C	**A**	**G**	T	C	A	C	G	T	C	C	G	C	T	A

HDR

NHEJ

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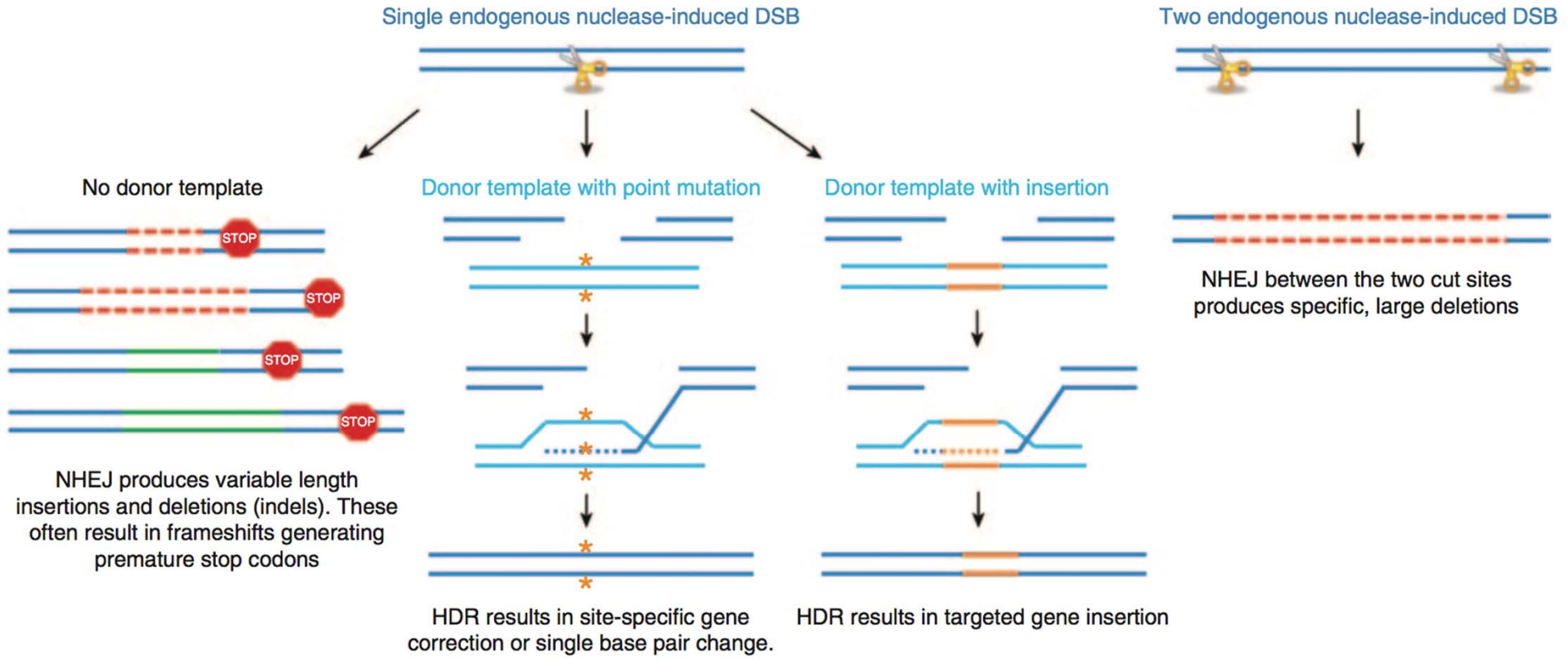
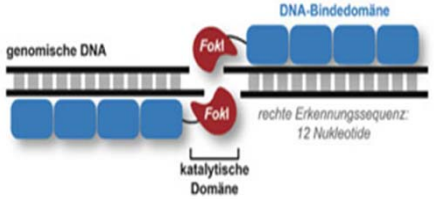
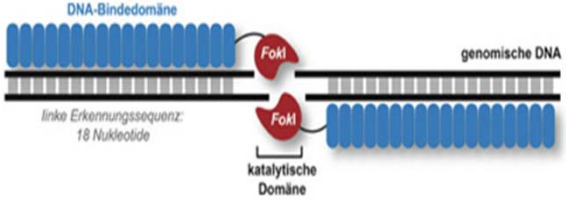
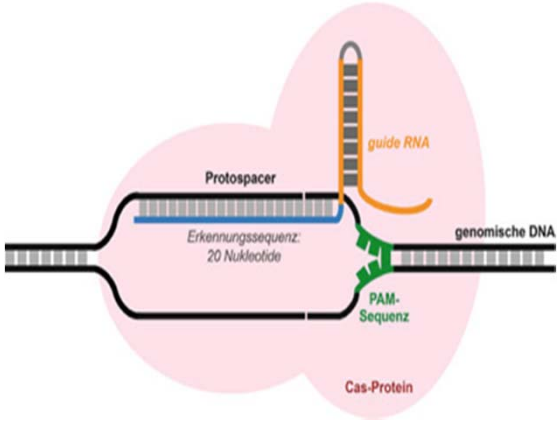
General mechanisms of genome editors



Knockout | Knock-in | Knock-in | Knockout

Modifiziert nach Maeder&Gersbach, Molecular Therapy 2016,(24),3,430-446

Differences between Genome Editors

	Zink-Finger-Nucleases (ZFN)	*TAL-Effektor-Nucleases (TALEN)*	*CRISPR/Cas*
			
Recognition domain:	~24-30 bp	~36 bp	~22 bp
In use since:	seit 2003	seit 2011	seit 2013

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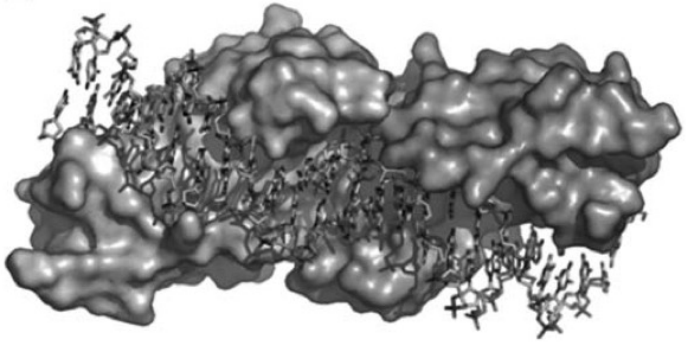
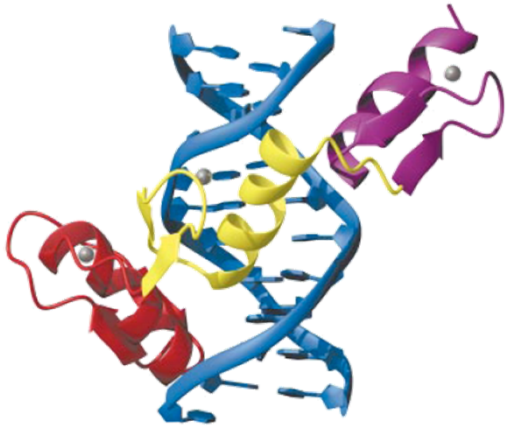
Classes of genome editing tools

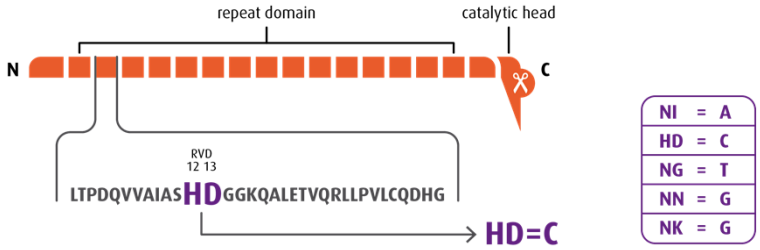


Meganucleases

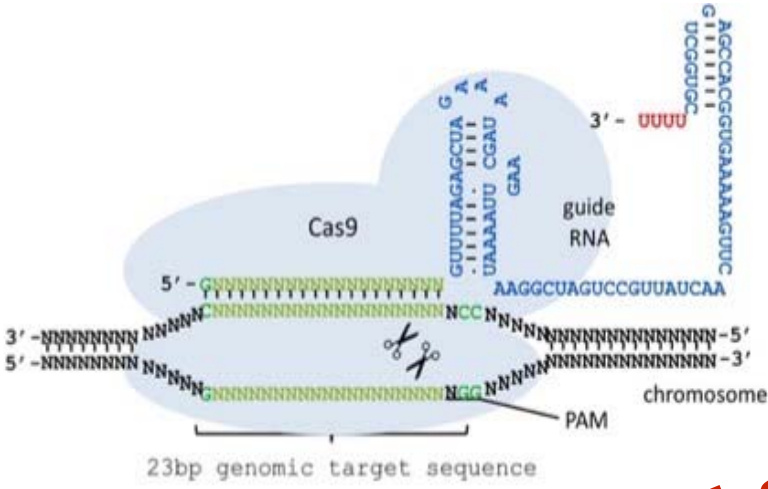
Protein-based



Zinc-Finger Nucleases



Transcription activator- like effektor nucleases (TALENs)



CRISPR/CAS

RNA-based

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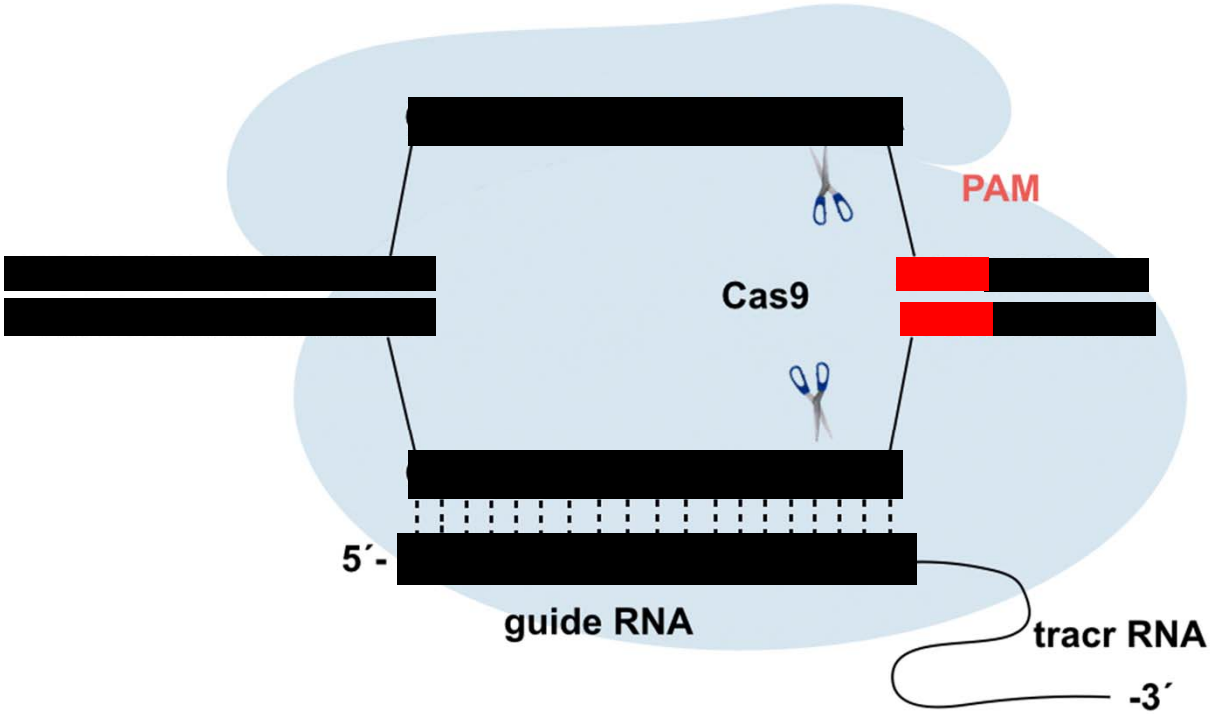
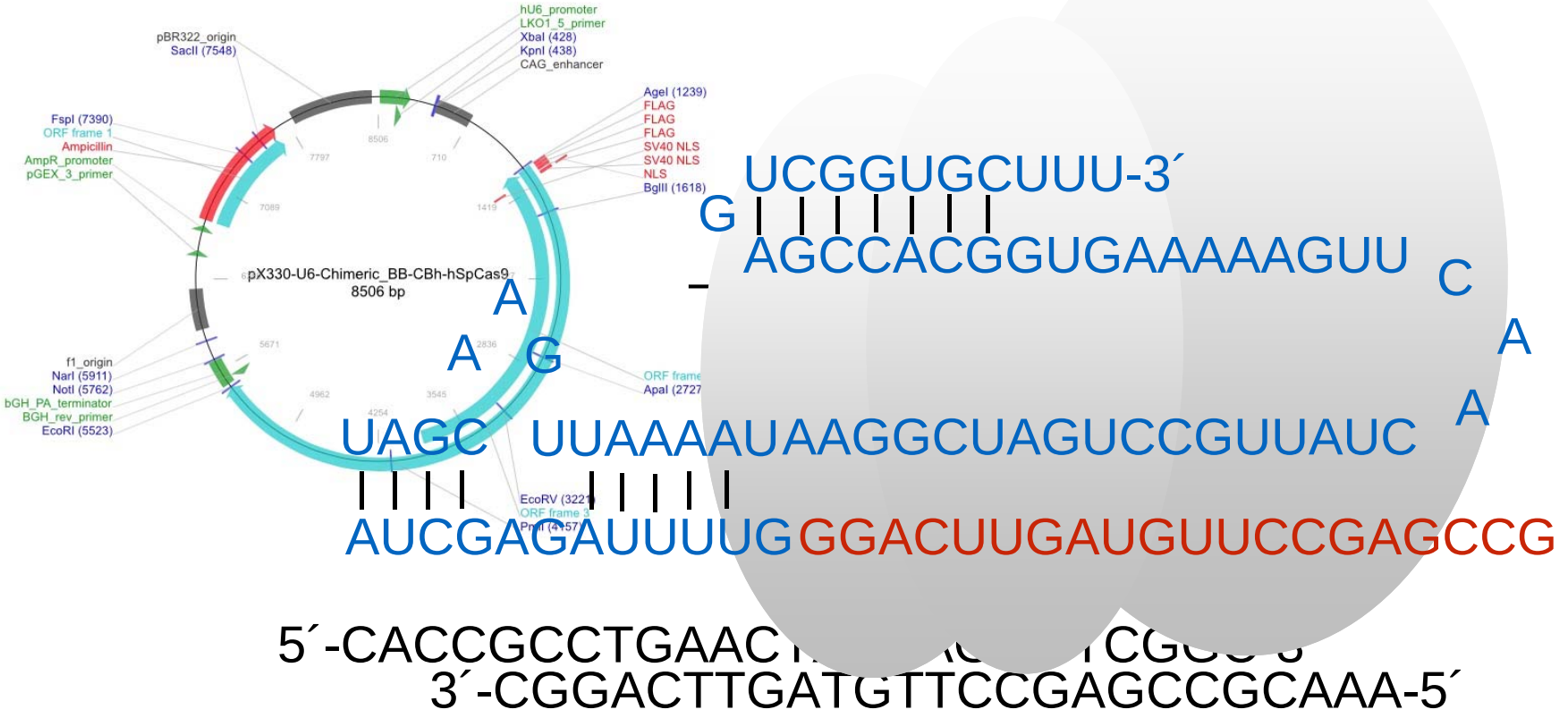
Genome Editing by CRISPR/Cas



CRISPR/Cas in action



TARGETED LOCUS: **20nt** **PAM**

ATTGCAGGTTGCATTAGCCCTGAACTACAAGGCTCGGCAGGTCACCCAA

TAACGTCCAACGTAATCGGACTTGATGTTCCGAGCCGTCCAGTGGGTT

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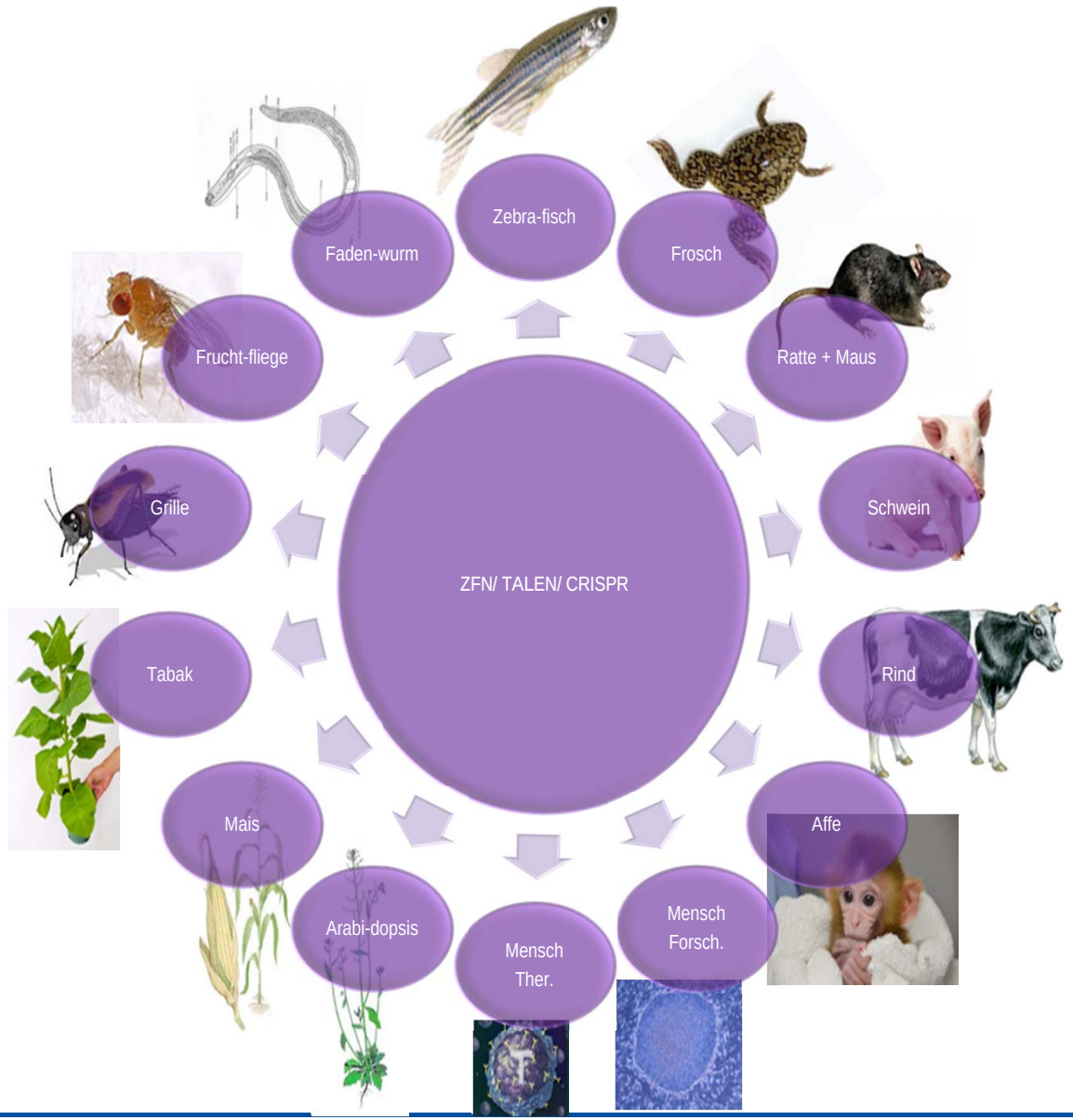
Universal application of Genome Editors



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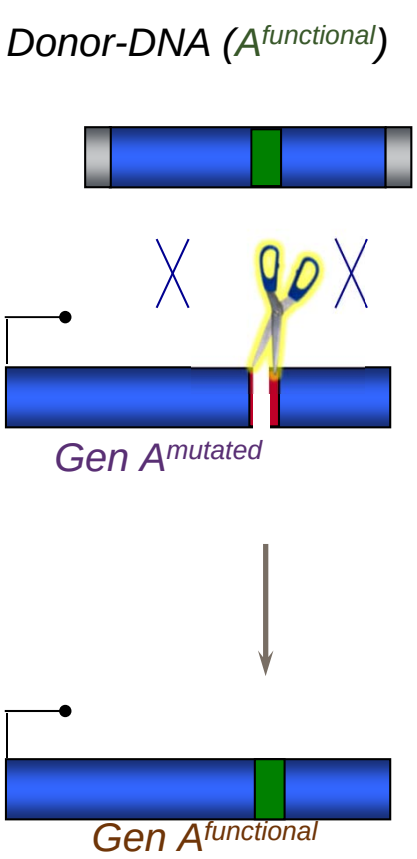
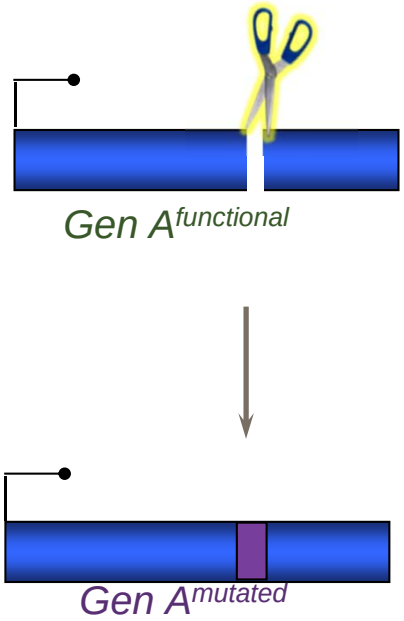
Genome Editing

Advantage: Gene correction and Gene inactivation

Gene-correction



Gene-Knockout



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Genome Editing

- Use of Genome Editors to correct a genetic information

Es kann der Dümmste nicht in Frieden leben, wenn es dem bösen Nachbar nicht gefällt.

Es kann de mste nicht in Frieden leben, wenn es dem bösen Nachbar nicht gefällt.

kann der Frömmste nicht in Frieden

Es kann der Frömmste nicht in Frieden leben, wenn es dem bösen Nachbar nicht gefällt.

(Friedrich Schiller, Wilhelm Tell)

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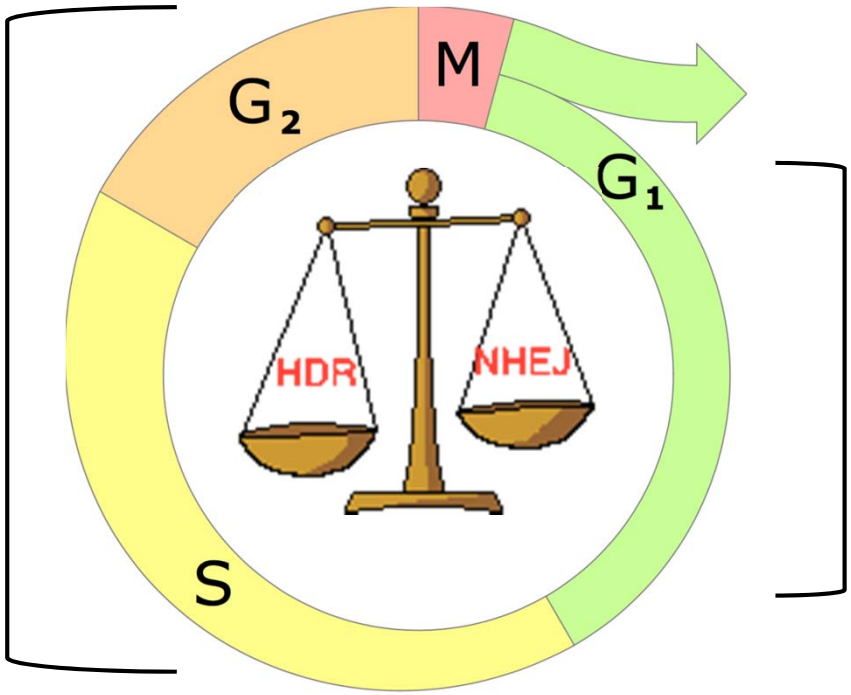
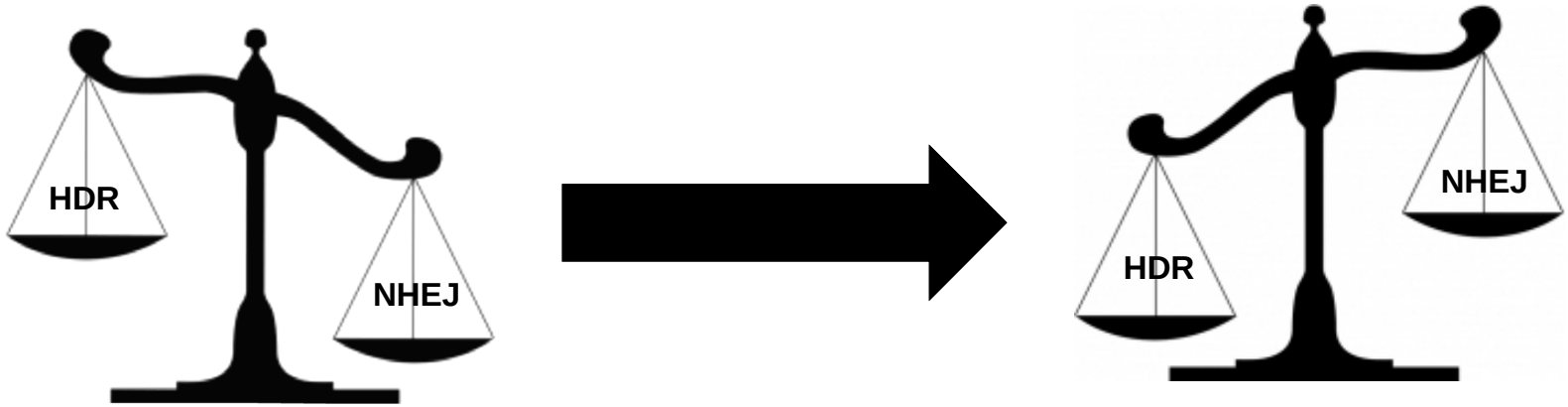
HDR
(10%)



NHEJ
(90%)



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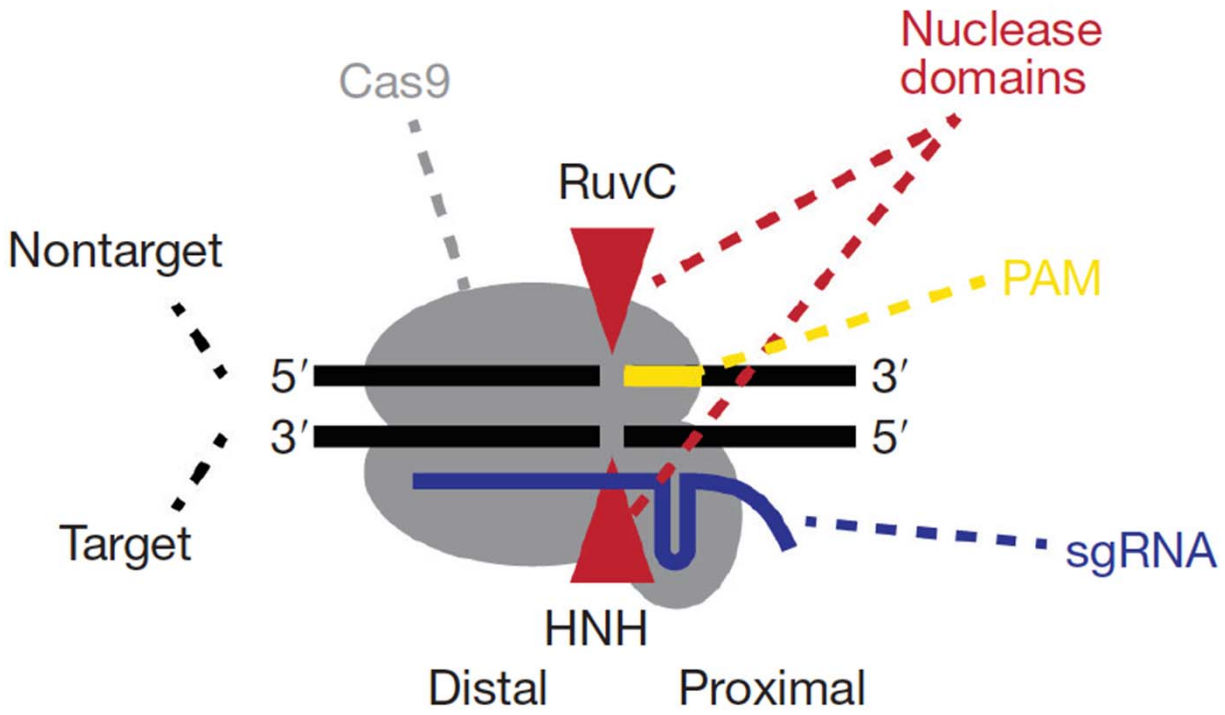
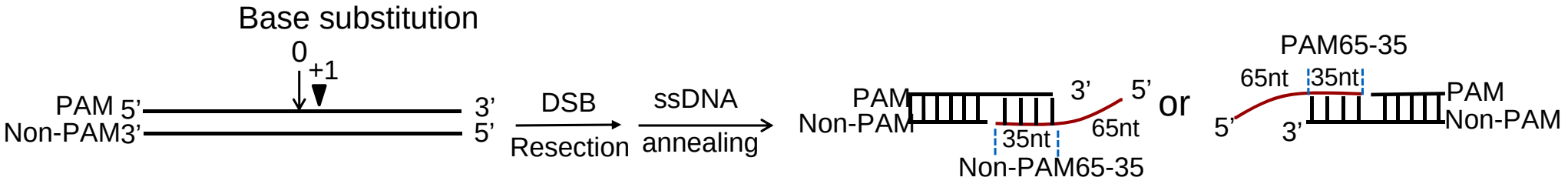


Richardson et al., Nature Biotechnol (2016), Vol 34 (3), 339-44

Asymmetric donor design facilitates HDR

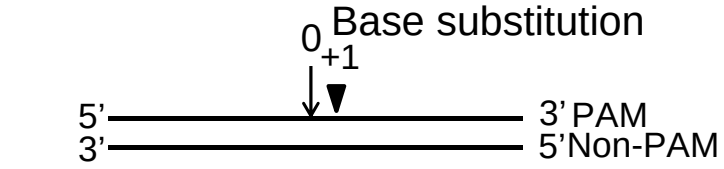
Asymmetric PAM or Non-PAM strand ssDNA annealing



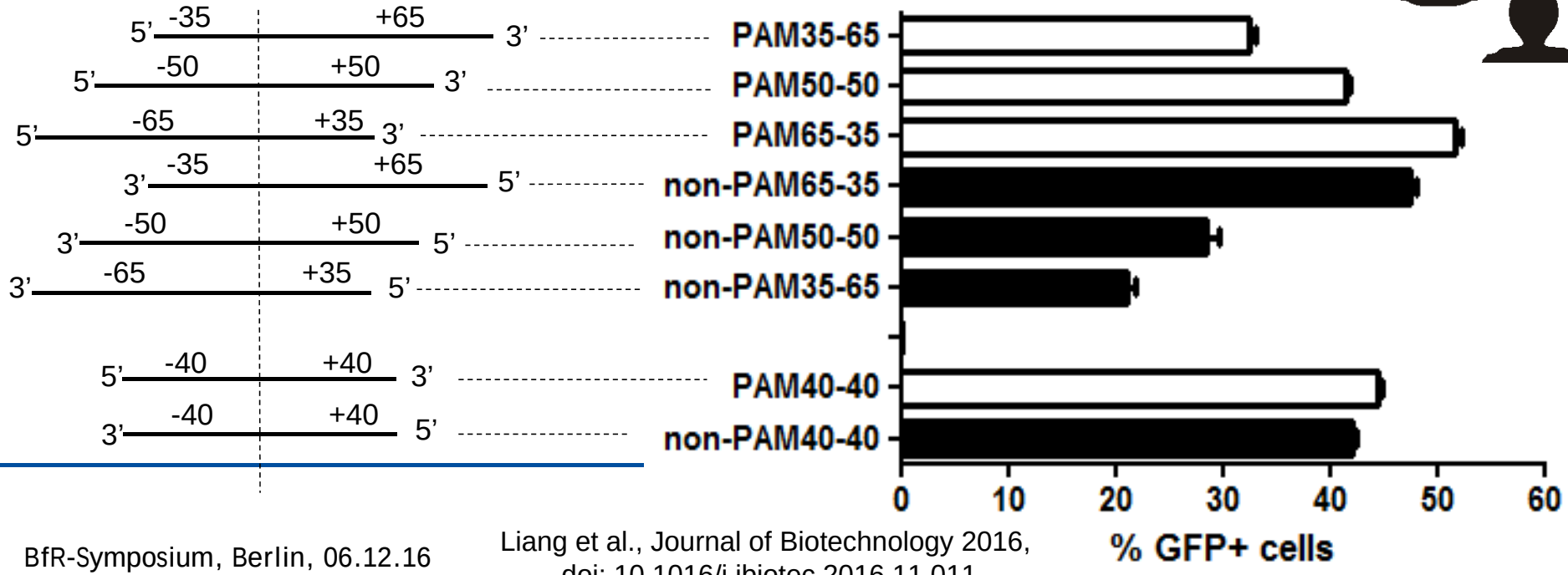
PAM ssDNA is defined as the PAM-containing strand

(a) (b)

Asymmetric donor design

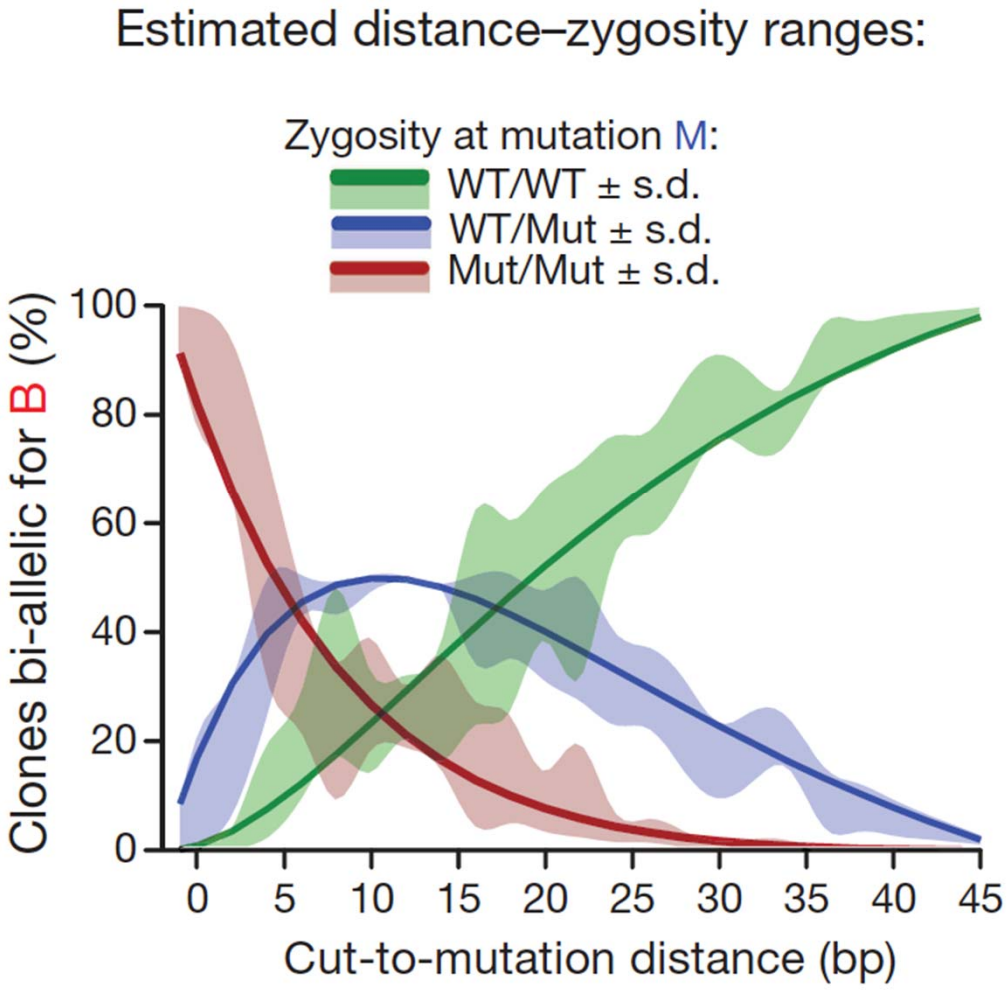


HDR efficiencies



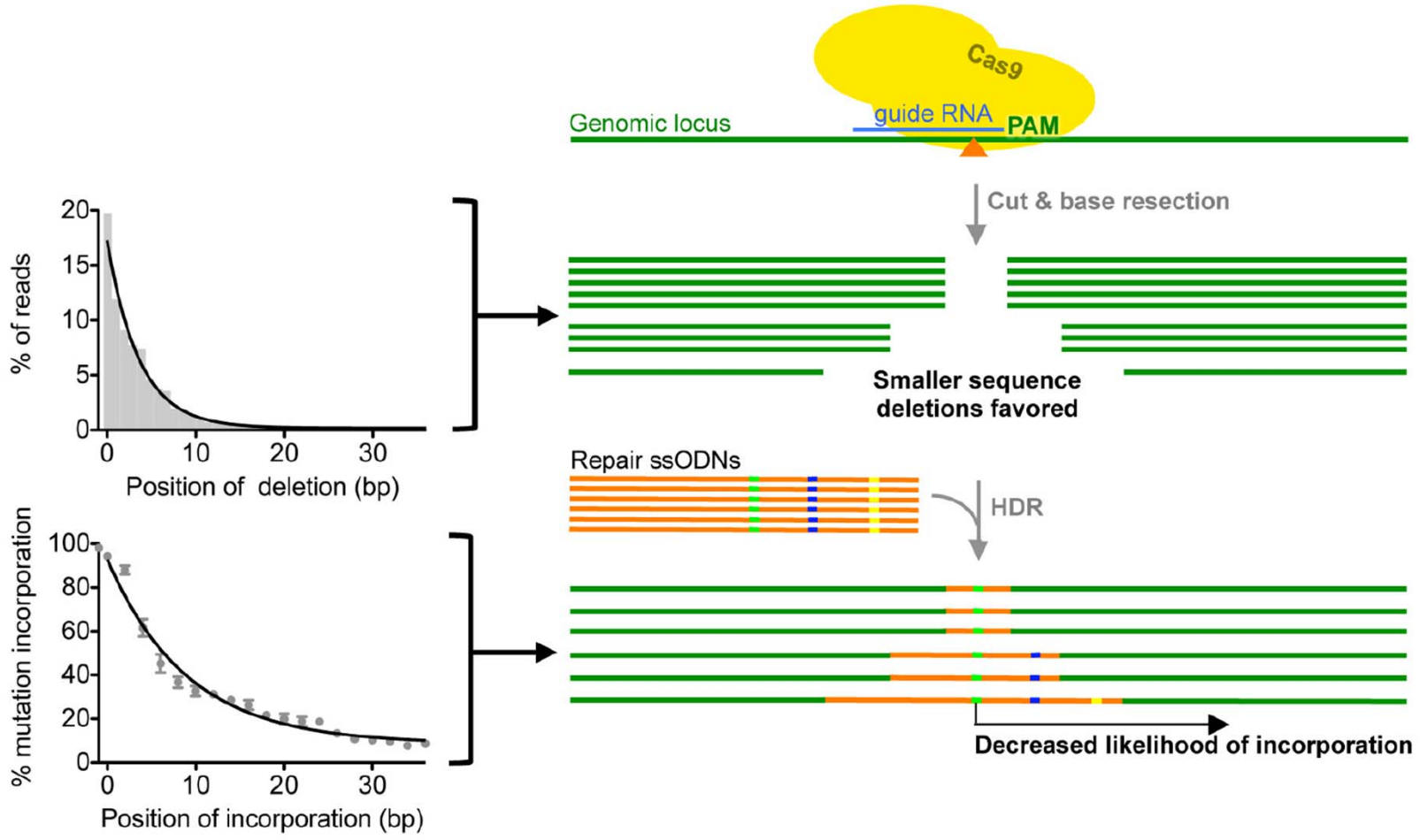
Liang et al., Journal of Biotechnology 2016,
doi: 10.1016/j.jbiotec.2016.11.011

Correlation between cut-to-mutation distance



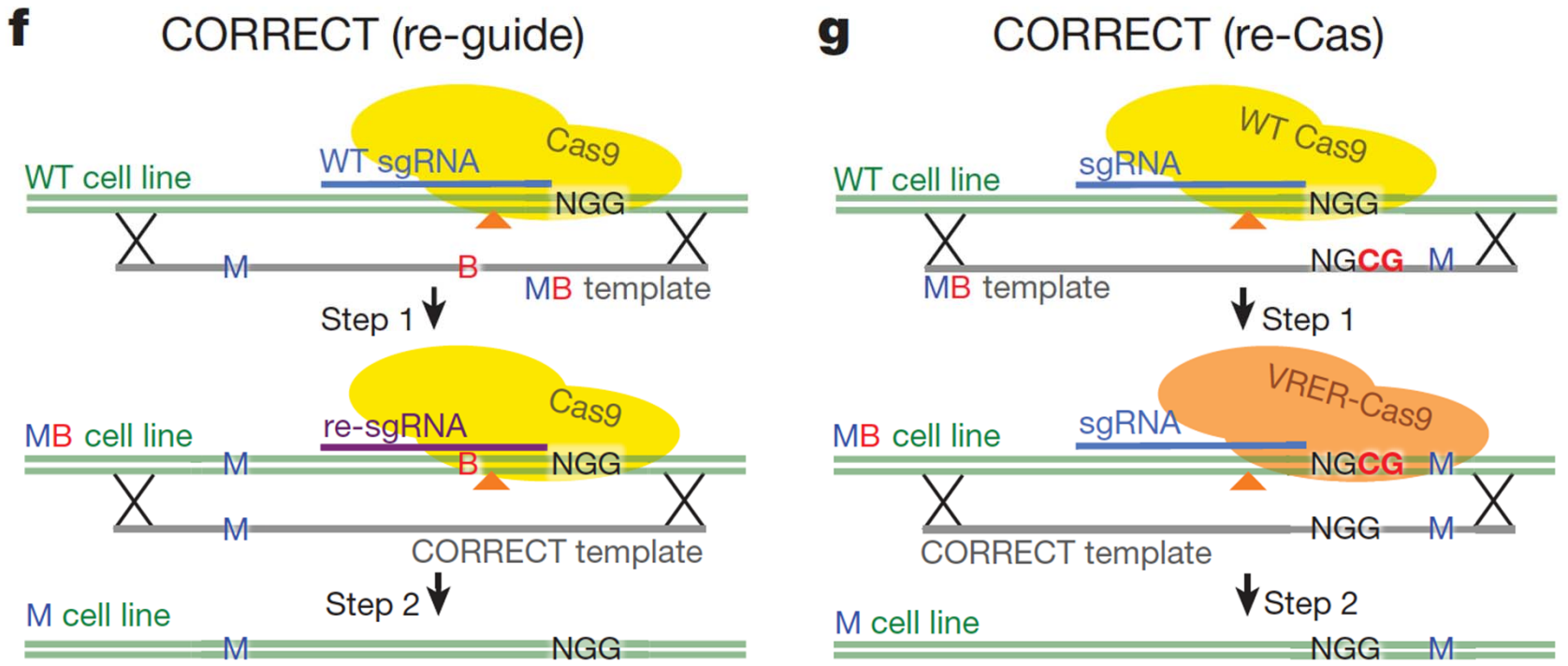
Paquet et al., Nature (2016), Vol 533, 125-29

Correlation between cut-to-mutation distance

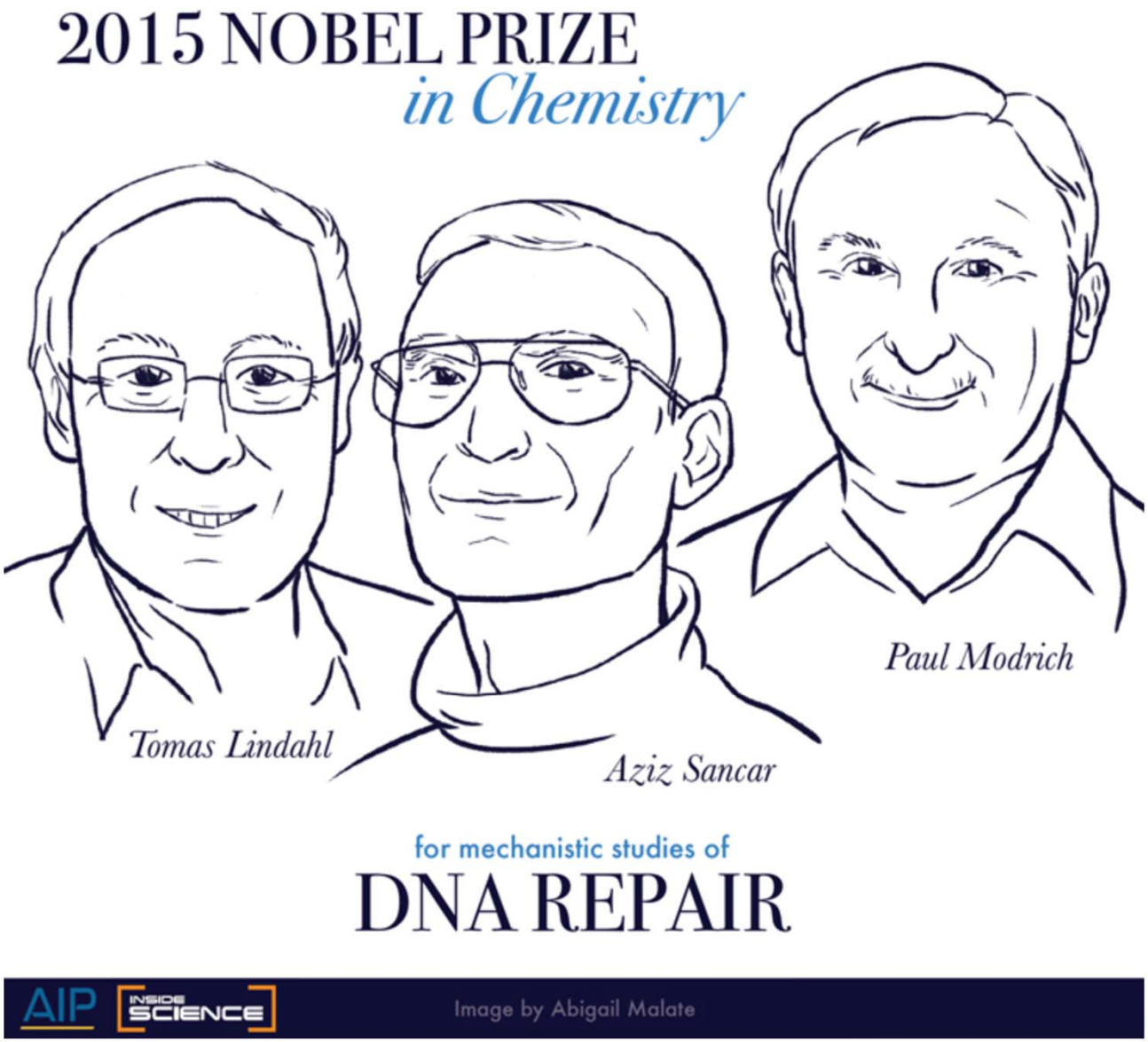


Paquet et al., Nature (2016), Vol 533, 125-29

Ways to a “Correct“ HDR



Paquet et al., Nature (2016), Vol 533, 125-29



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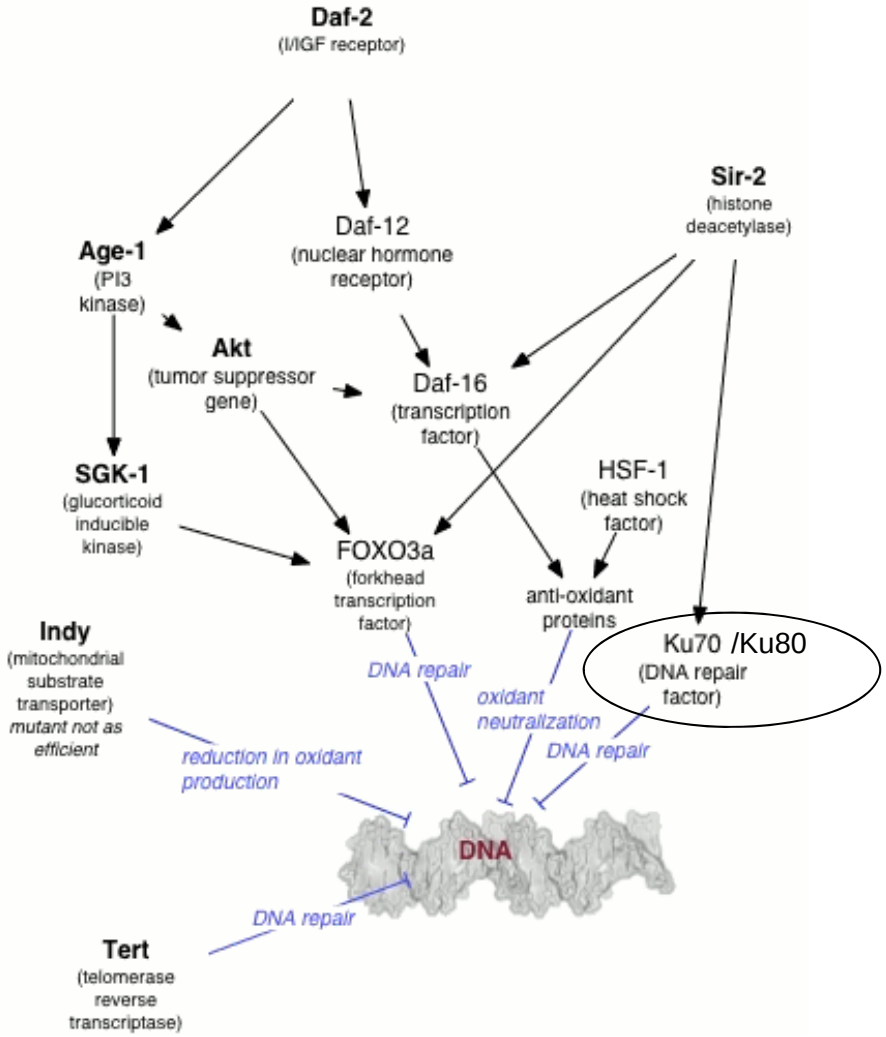
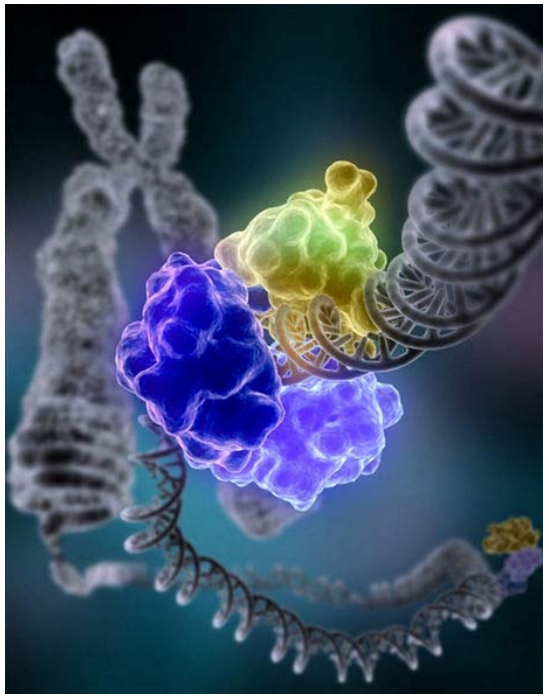
Inhibition of key molecules of DNA repair by NHEJ



SCR-7, Ad4 proteins E1B55K, E4orf6

⊣

DNA Ligase IV

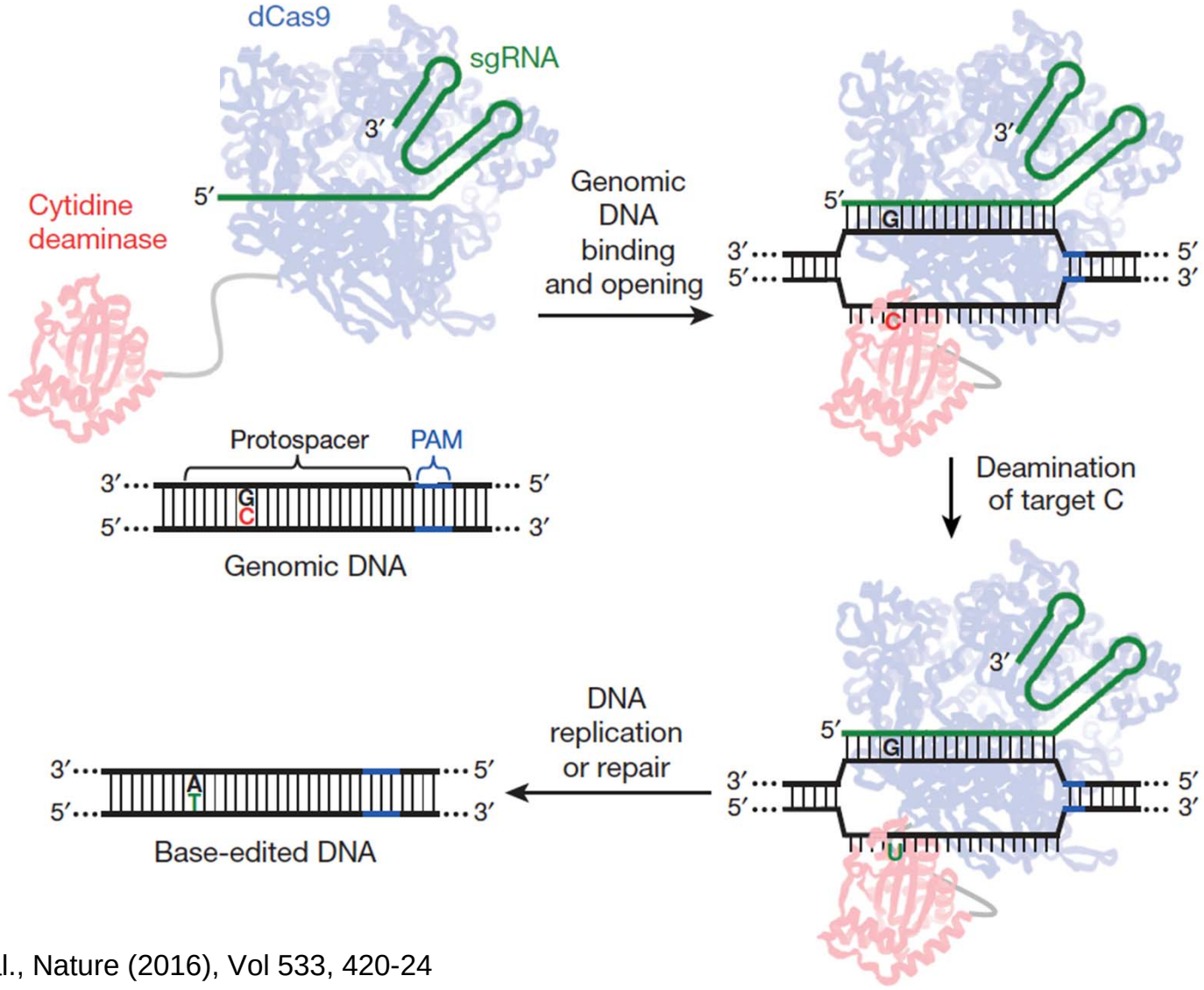


- Suppression of KU70 and DNA ligase IV promotes HDR efficiency 4-5-fold
- Co-expression of AD4 proteins improved HDR up to 8-fold

(Chu et al. 2015, Nature biotechnol. 33 (5), 543-48)

Image credits:
David Gohara, Saint Louis University, and Tom Ellenberger, Washington University School of Medicine in St. Louis

Programmable Editing without DNA cleavage



Komor et al., Nature (2016), Vol 533, 420-24

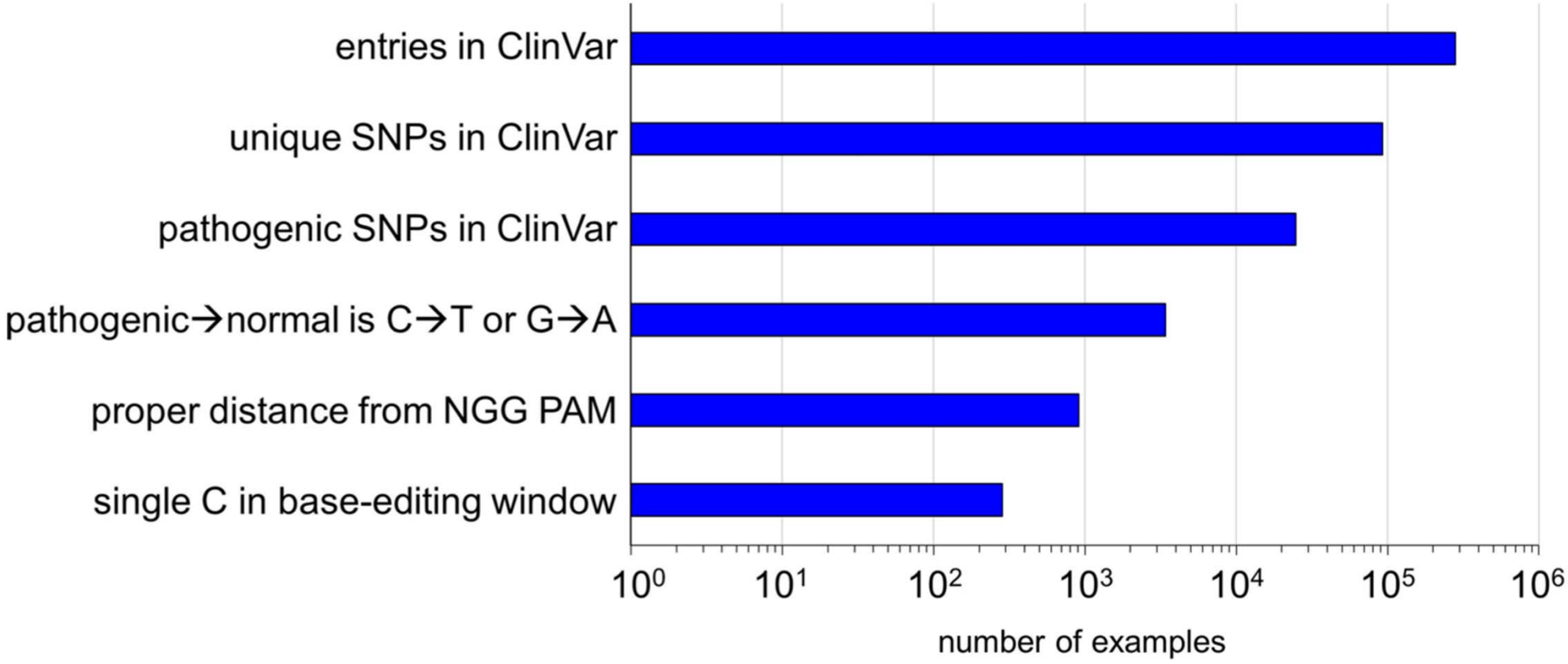
Programmable Editing without DNA cleavage

protospacer and PAM sequence: 5'-TTCCCCCCCCCGATTTATTTATGG-3'

sequence	% of total reads
...CCCCCCCC...	62.4
...TTTTTTCC...	18.2
...TTTTTTTC...	13.4
...TTTTTTTT...	3.3
...TCCCCCCC...	0.8
...CCCCTTCC...	0.3
...CCCTTTCC...	0.3
...TTTTTCCC...	0.3
...CCCCTCCC...	0.3

Komor et al., Nature (2016), Vol 533, 420-24

Genetic variants from ClinVar, that could be corrected by Base editing



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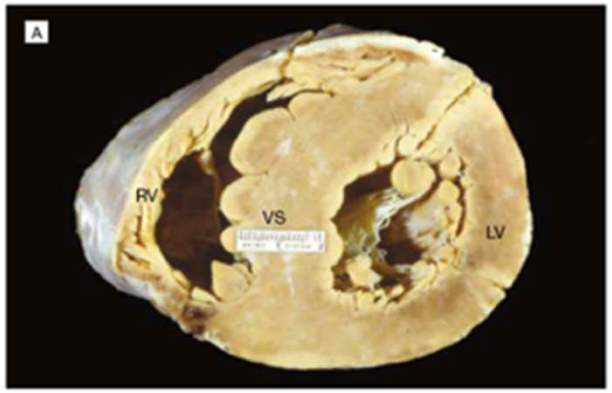
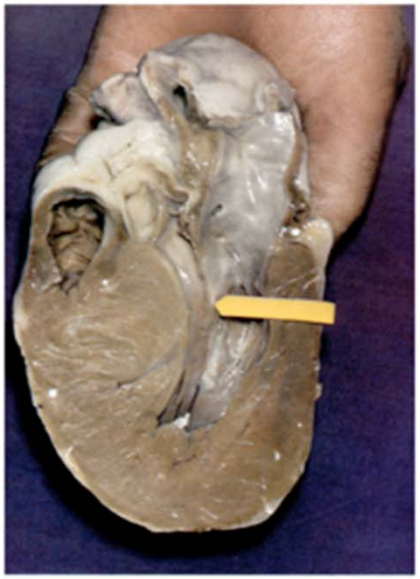
Familial Hypertrophic Cardiomyopathy (FHC)

FHC: - Prevalence 1:500, most frequent disease of the myocard
- frequent cause of sudden death, particularly in young adults including athletes
- inherited as a mendelian autosomal dominant trait, caused by mutations in any 1 of 10 genes



from Maron, BJ, JAMA 2002

Hypertrophy of LV and Septum



Adapted from G. Farrer-Brown, Farbatlas der Herzkrankheiten, 1980

Typical symptoms:

- asymmetric left ventricular hypertrophy
- cardiac arrhythmias
- fainting
- ventricular fibrillation

→ frequent cause of sudden death, particularly in young athletes

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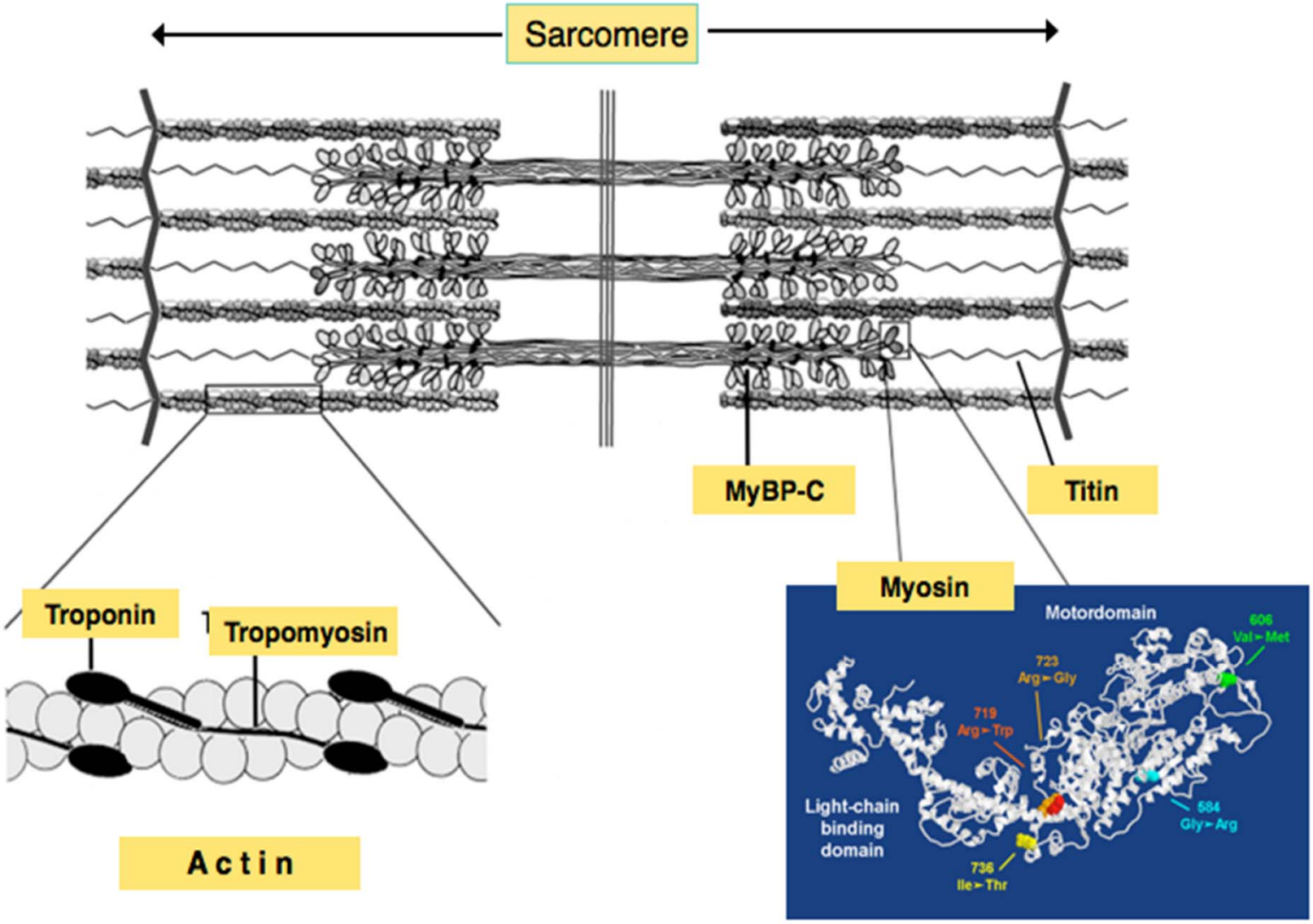
FHC is mainly caused by mutations in genes encoding proteins of the cardiac sarcomere



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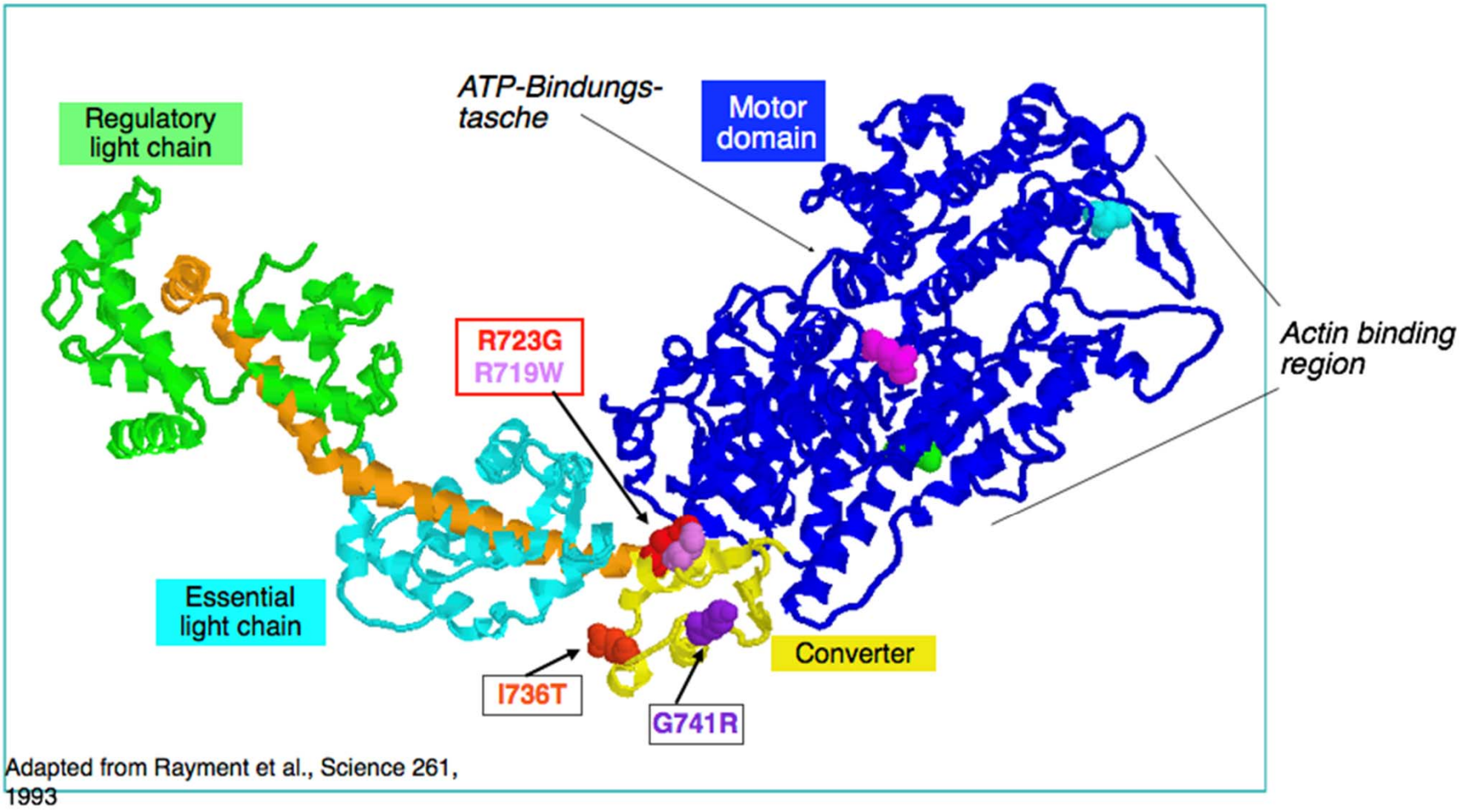
Myosin head domain with some mutations studied in biopsies from FHC patients



Known proteins with mutations and the frequency of the mutations

HCM Disease Genes and Mutation Frequency*

Gene	Protein	Number of mutations
MYH7[a]	β-Myosin heavy chain	193
MYBPC3[a]	Cardiac myosin binding protein C	138
TNNT2[a]	Cardiac troponin T	33
TNNI3[a]	Cardiac troponin I	32
CSRP3[a]	Cardiac muscle Lim protein	12
TPM1[a]	α-Tropomyosin	11
MYL2[a]	Regulatory myosin light chain	10
ACTC[a]	Cardiac actin	7
MYL3[a]	Essential myosin light chain	5
PRKAG2[a]	AMP-activated protein kinase	4
PLN[a]	Phospholamban	2
TNNC1[a]	Cardiac troponin C	1
TTN	Titin	2
MYH6	α-myosin heavy chain	2
TCAP	Telethonin	2
CAV3	Caveolin-3	1
Total		455

= 42% of the genetically characterized cases

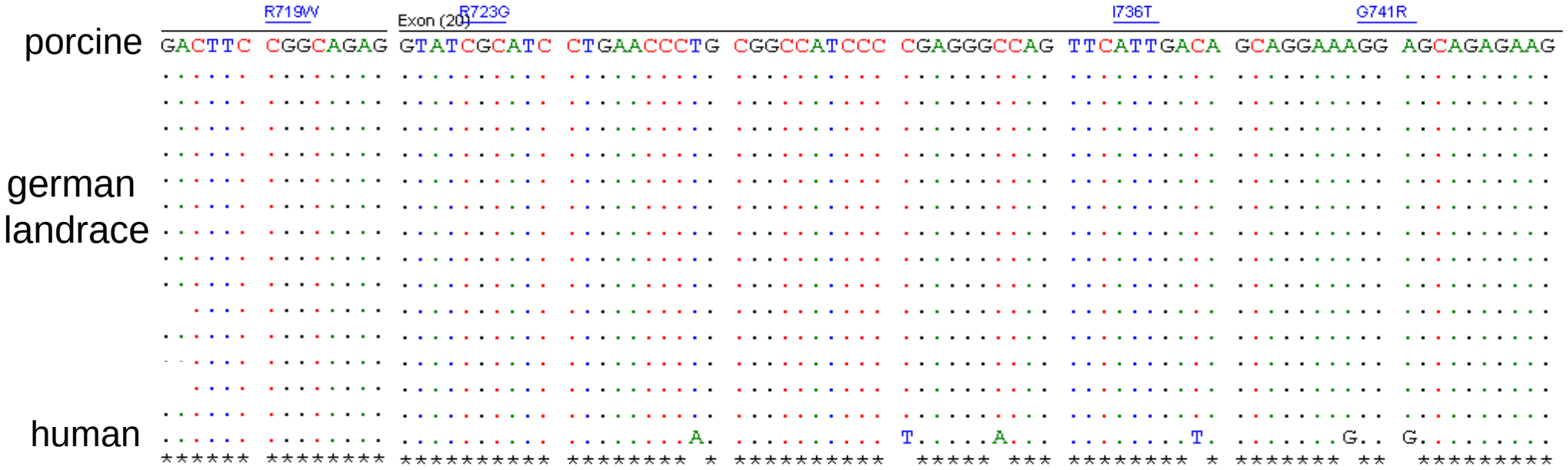
*According to HGMD (www.hgmd.cf.ac.uk/ac/index.php).
[a]Gene sequences placed on the Geneva HCM resequencing chip.

Nach: Fokstuen et al., Hum Mutat 29, 2008

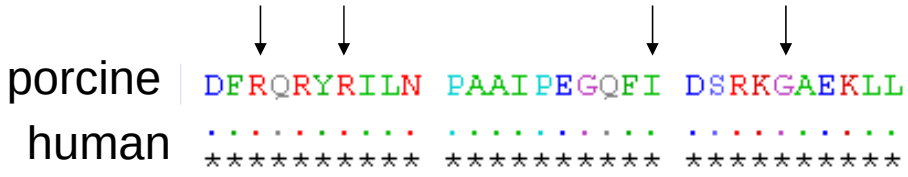
Sequence analysis of porcine MYH7 gene

Codons 717- 744

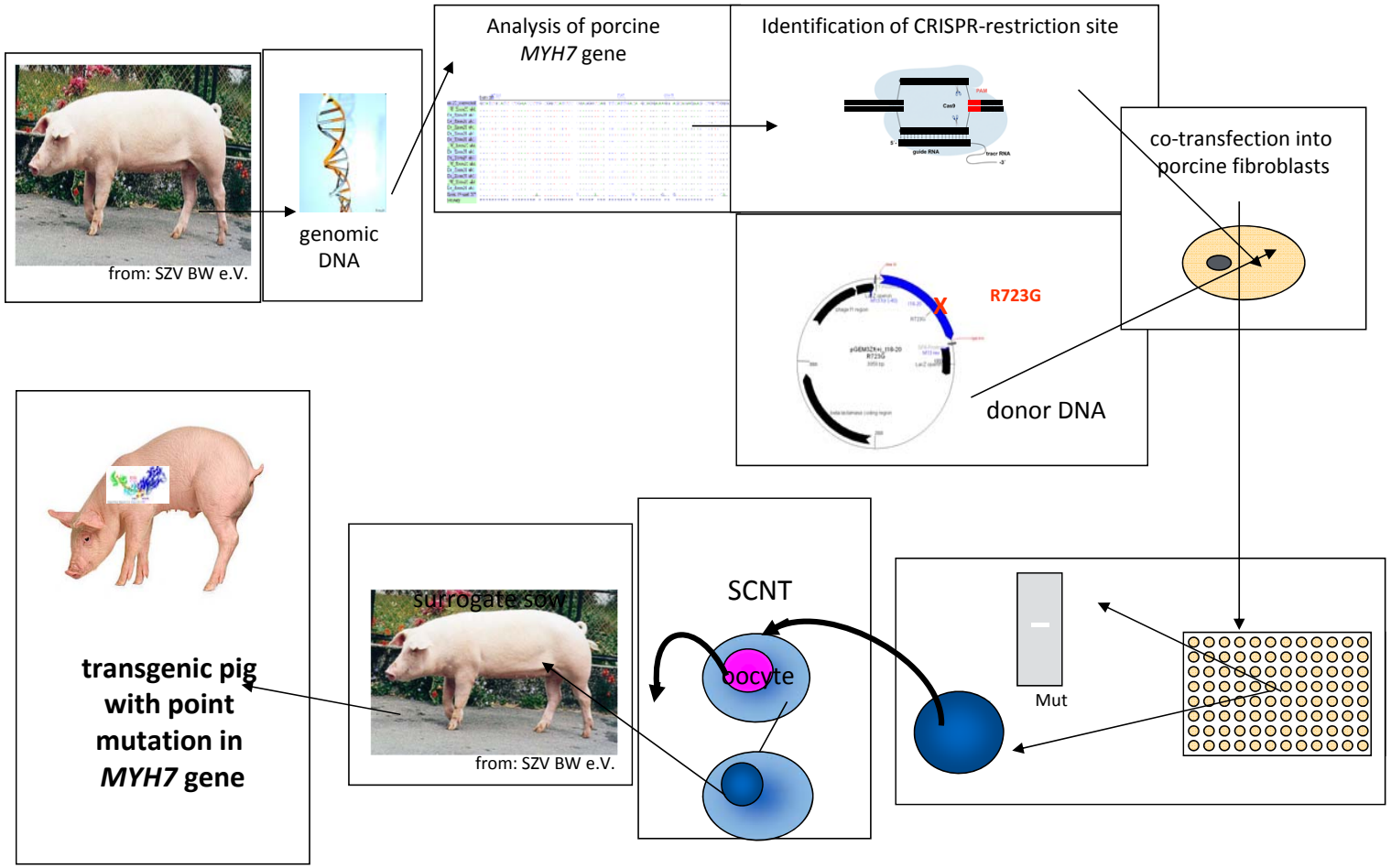
DNA-Alignment



Amino Acid-Alignment



The project:
Generation of transgenic pigs that carry FHC-related *MYH7*-mutations



Codon 723: Wildtyp: CGC
R723G: GGC

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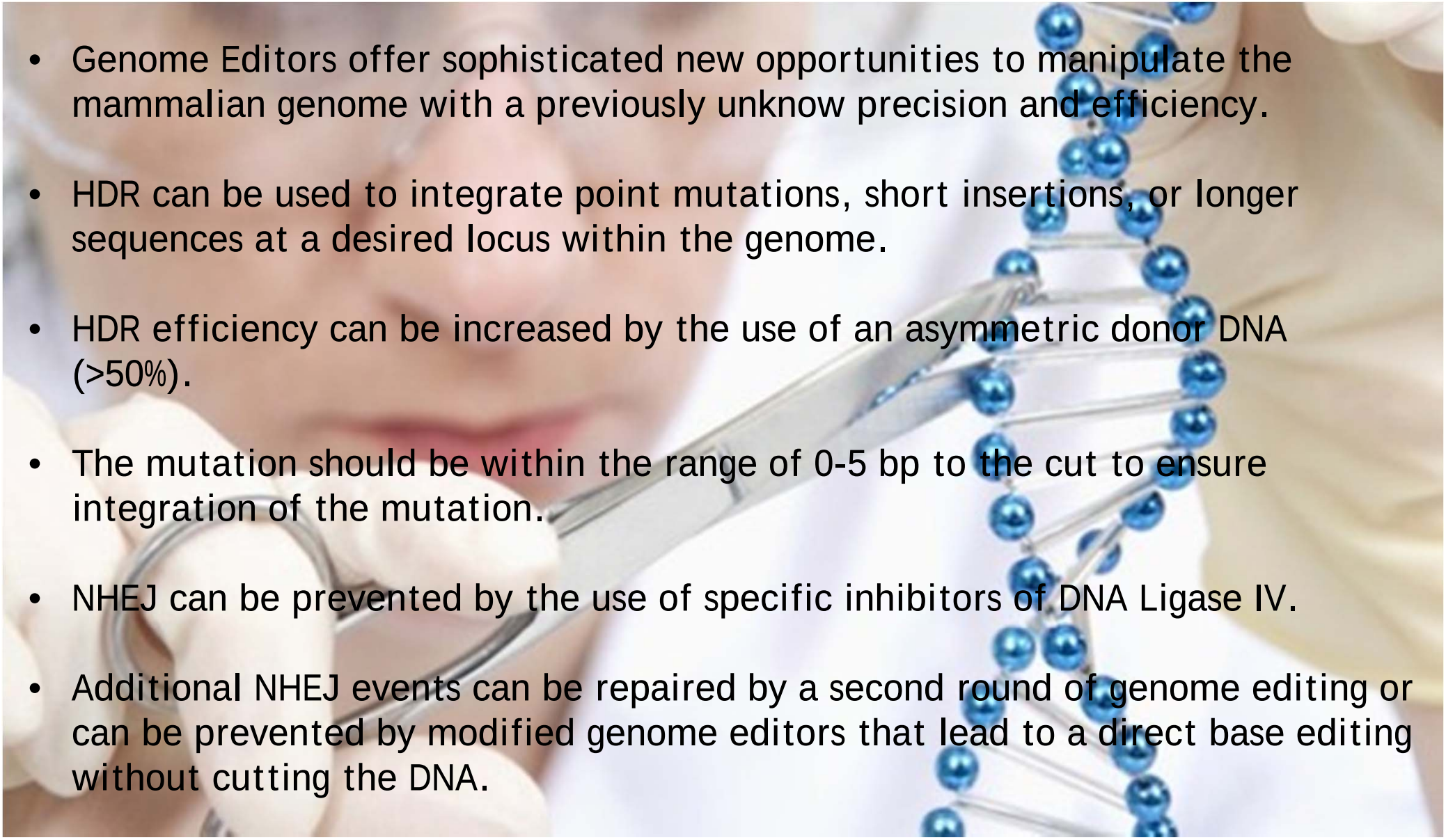
Summary and Conclusions



- Genome Editors offer sophisticated new opportunities to manipulate the mammalian genome with a previously unknow precision and efficiency.
- HDR can be used to integrate point mutations, short insertions, or longer sequences at a desired locus within the genome.
- HDR efficiency can be increased by the use of an asymmetric donor DNA (>50%).
- The mutation should be within the range of 0-5 bp to the cut to ensure integration of the mutation.
- NHEJ can be prevented by the use of specific inhibitors of DNA Ligase IV.
- Additional NHEJ events can be repaired by a second round of genome editing or can be prevented by modified genome editors that lead to a direct base editing without cutting the DNA.

Biology´s IT toolbox

- DNA structure/sequencing
- Restriction enzymes
- PCR
- Genome editing

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Hendrik Sake
Eva Mall
SCNT Team
SVA Team

Thank you for your attention

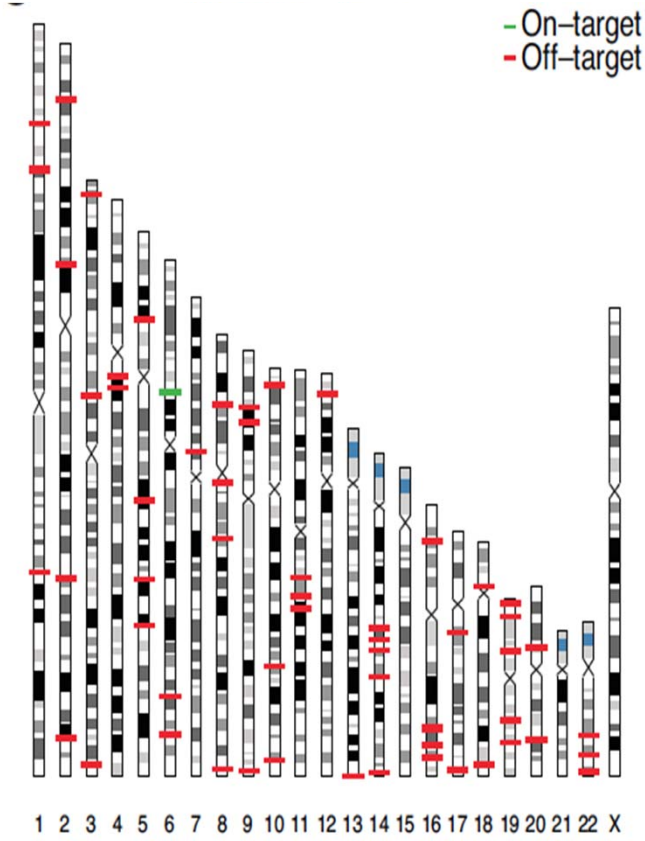
„Success is the ability to go from one failure to another with no loss of enthusiasm“
(Winston Churchill)

Specificity of Genome Editors

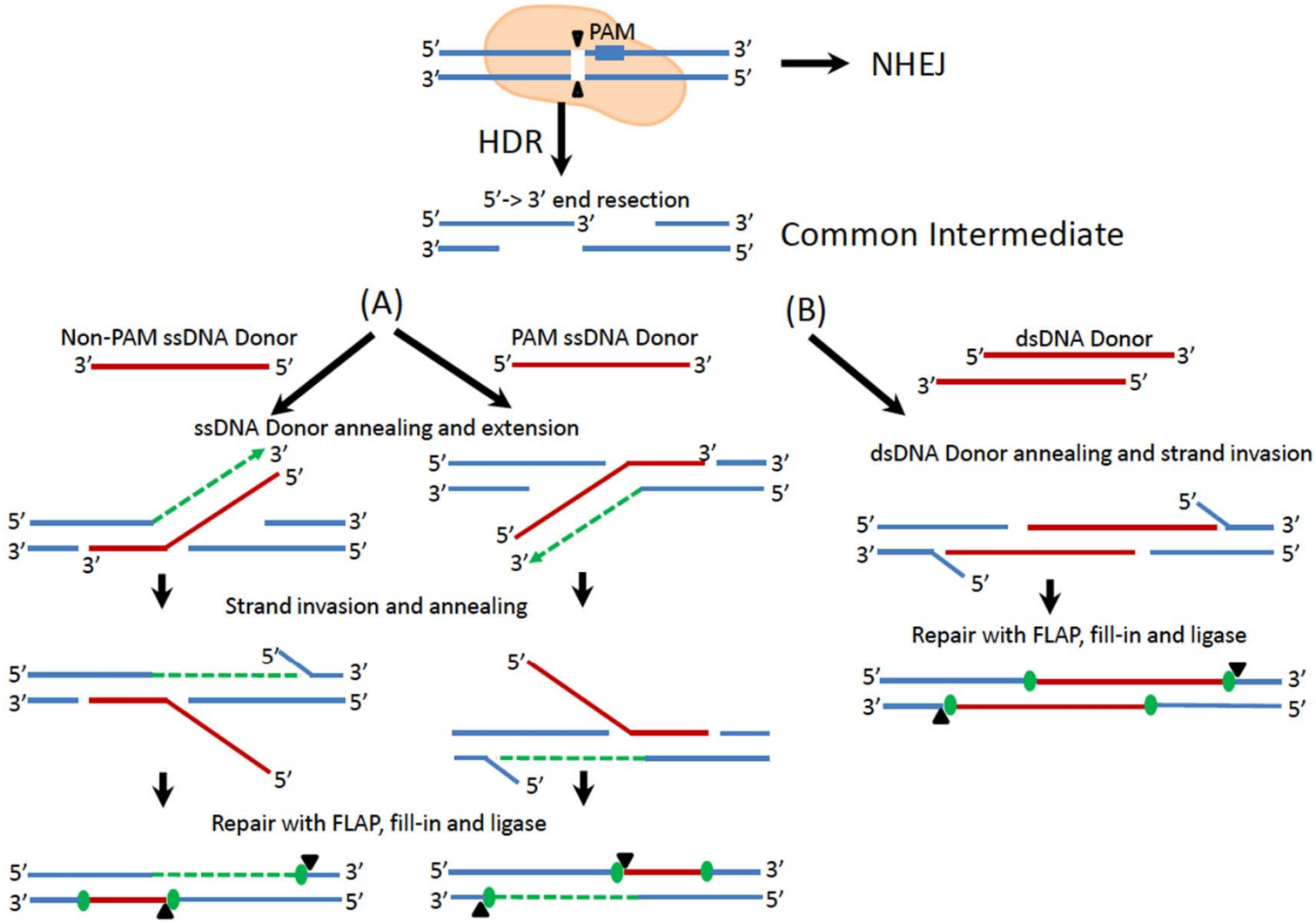
Activity and Specificity (Precision)

- **High Activity** at targeted locus
  → *On-Target*
- **No Activity** at non-targeted loci
  → *Off-Target*

Activity Profile
CRISPR/Cas9-Nuclease targeting *VEGF-A*

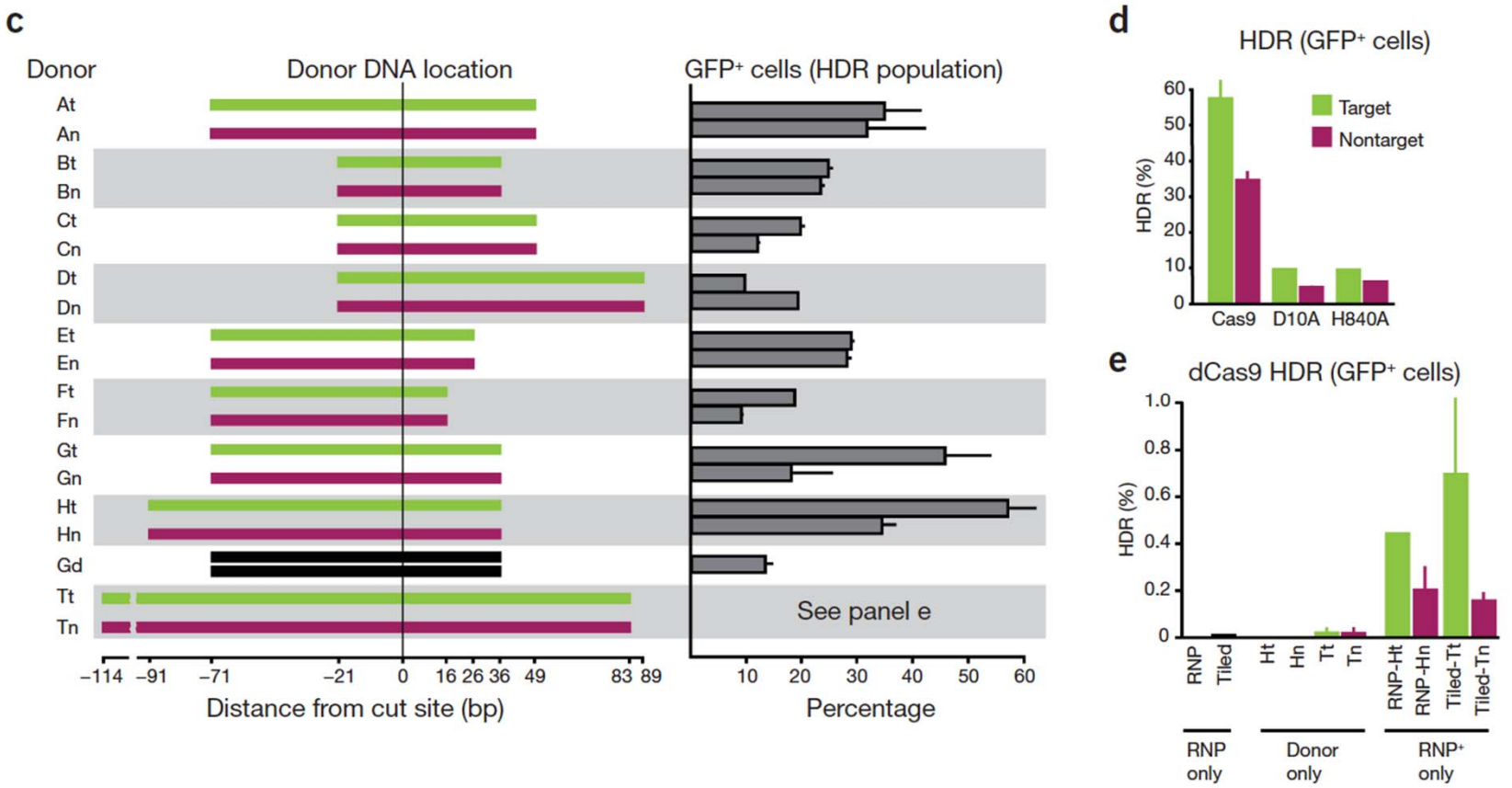


Quelle: Tsai et al., Nature Biotechnology 2015



Liang et al., Journal of Biotechnology 2016,
doi: 10.1016/j.jbiotec.2016.11.011

Asymmetric donor design facilitates HDR



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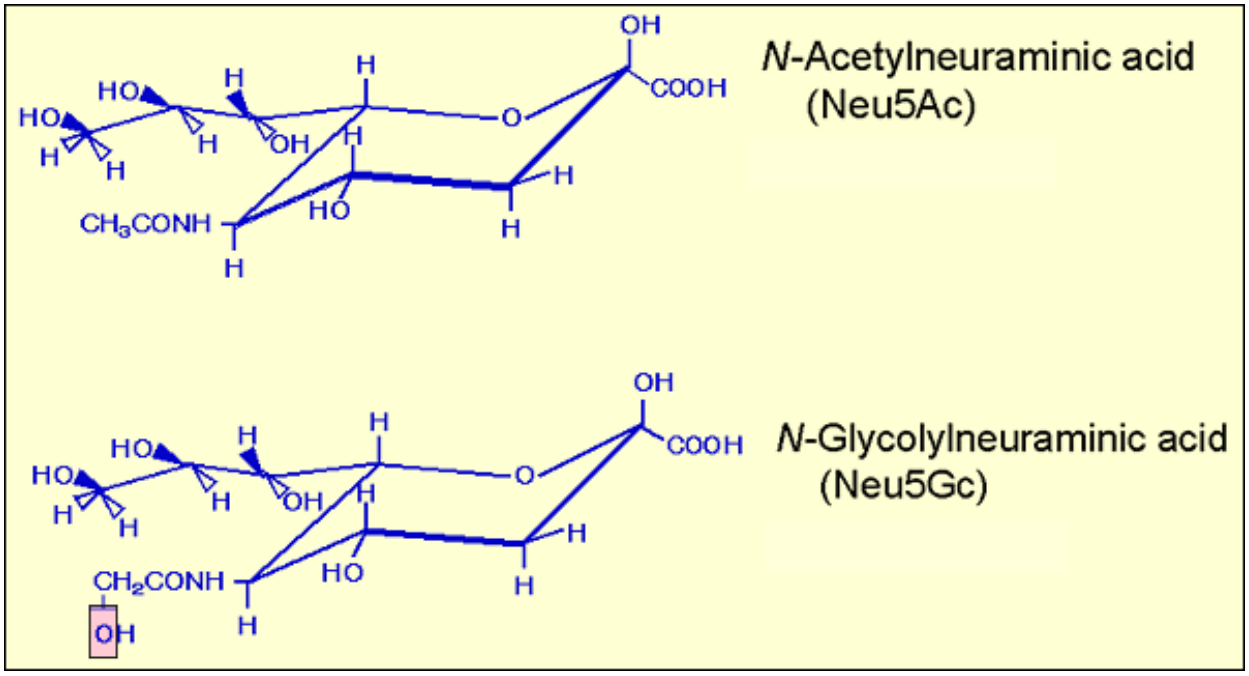
Target 2: CMAH (Neu5GC epitopes)



human

all other mammals

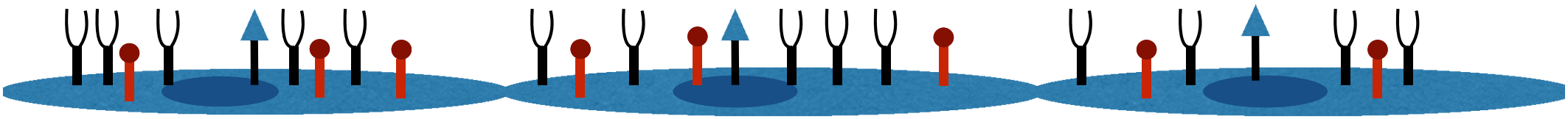
- Humans can´t produce Neu5GC
- Uptake by animal products
- Preformed antibodies
- Necessary to knockout the porcine CMAH

Target epitopes on the pig endothelium

Alpha1,3Gal:
-major antigen
-1% of all preformed antibodies
-Locus:GGTA1

Neu5GC:
-not present in humans
-role in xenotransplantation to be determined
-Locus:CMAH cytidine monophosphate-N-acetylneuraminic acid hydroxylase

B4GalNT2:
-not present on human endothelium
-5% lack functional gene
-role in xenotransplantation to be determined
-Locus:B4GalNT2
beta-1,4-N-acetyl- galactosaminyl transferase 2



pig endothelium

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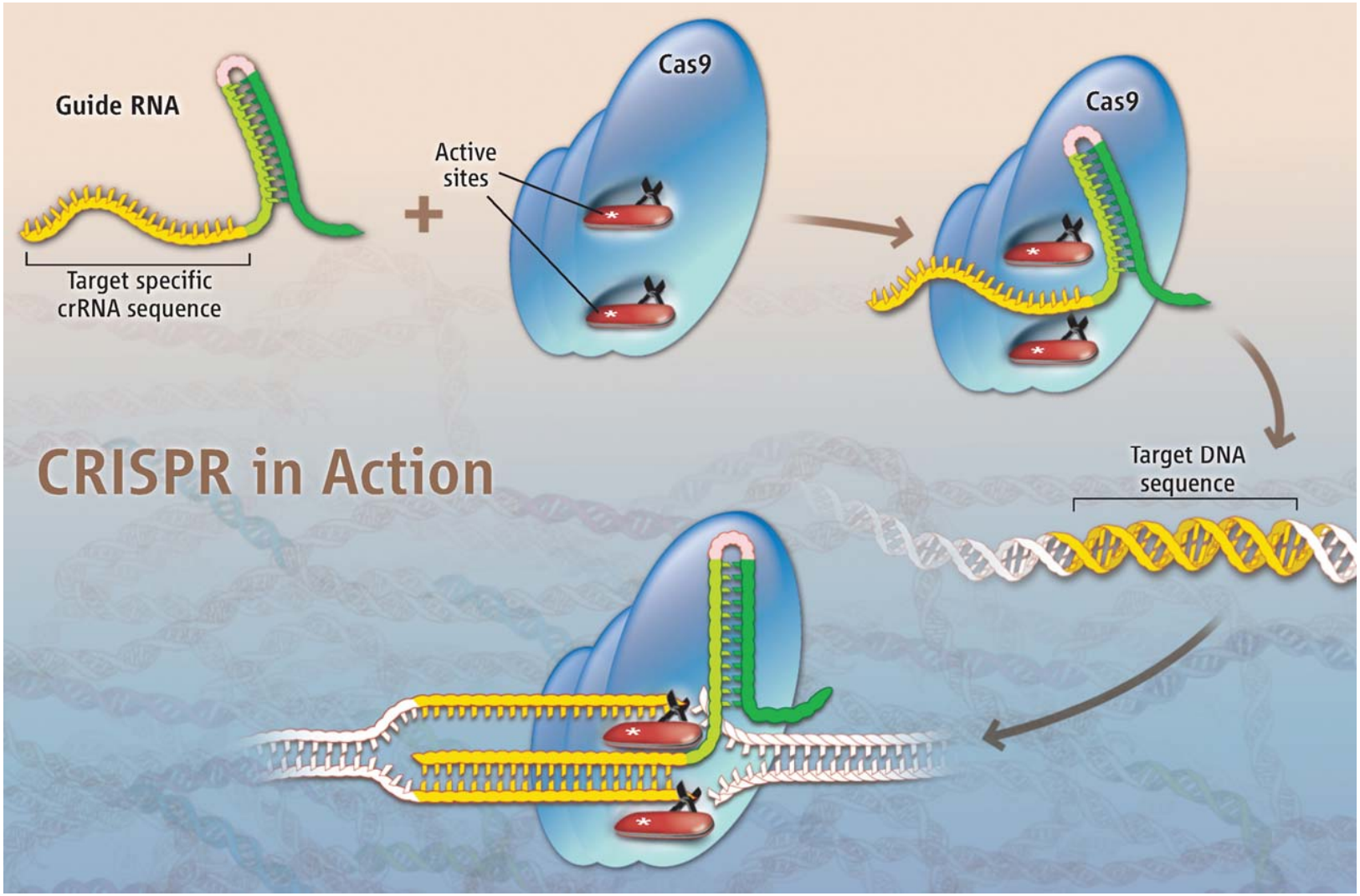
CRISPR/Cas in action



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Emmanuelle Charpentier

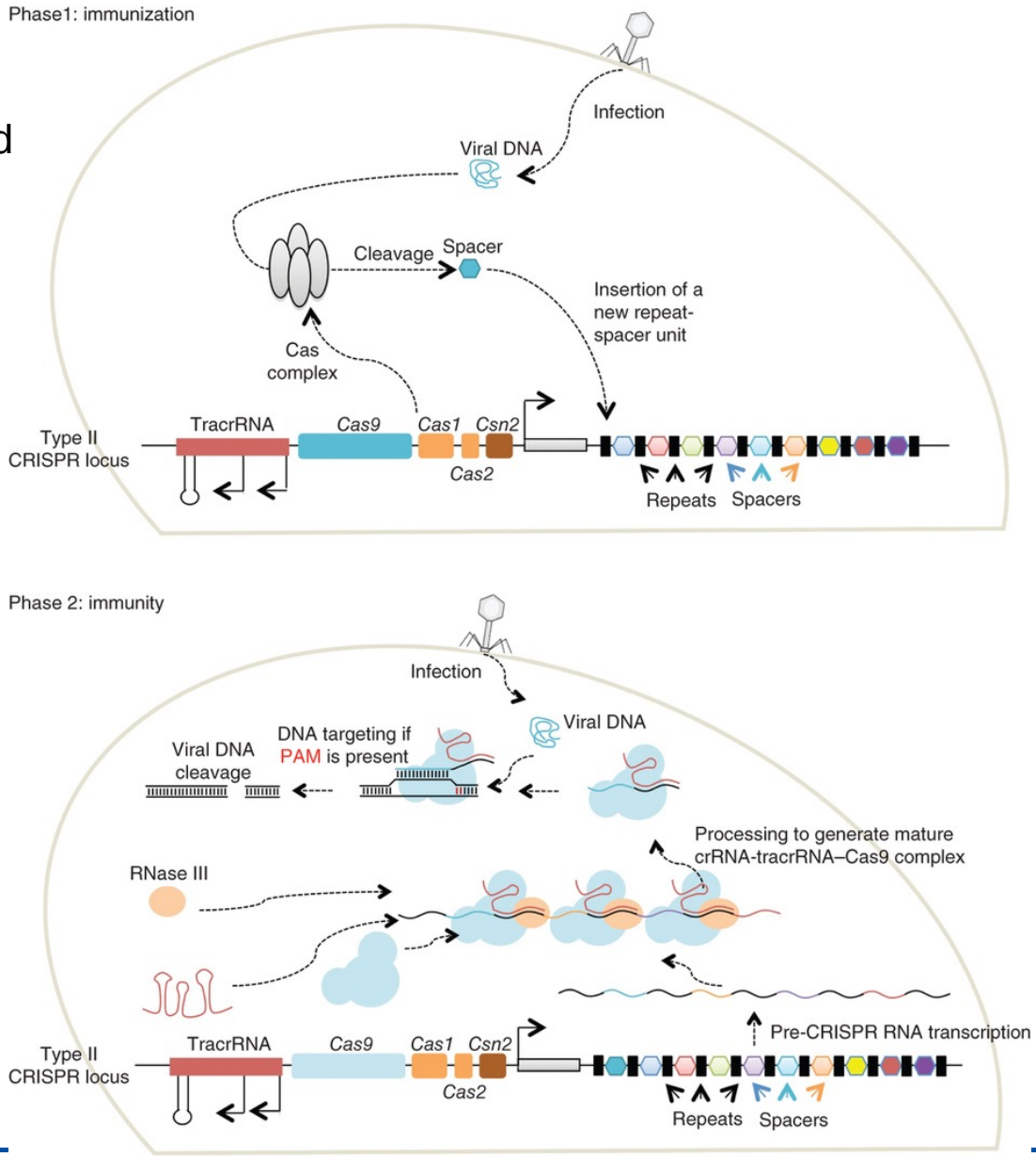
Max Planck Institute for Infection Biology, Director, Berlin,Germany

Jennifer Doudna

Doudna Lab
University of California
Berkeley, USA

CRISPRs: Hallmarks of acquired immunity in bacteria

CRISPR:
Clusters of regularly interspaced short palindromic repeats



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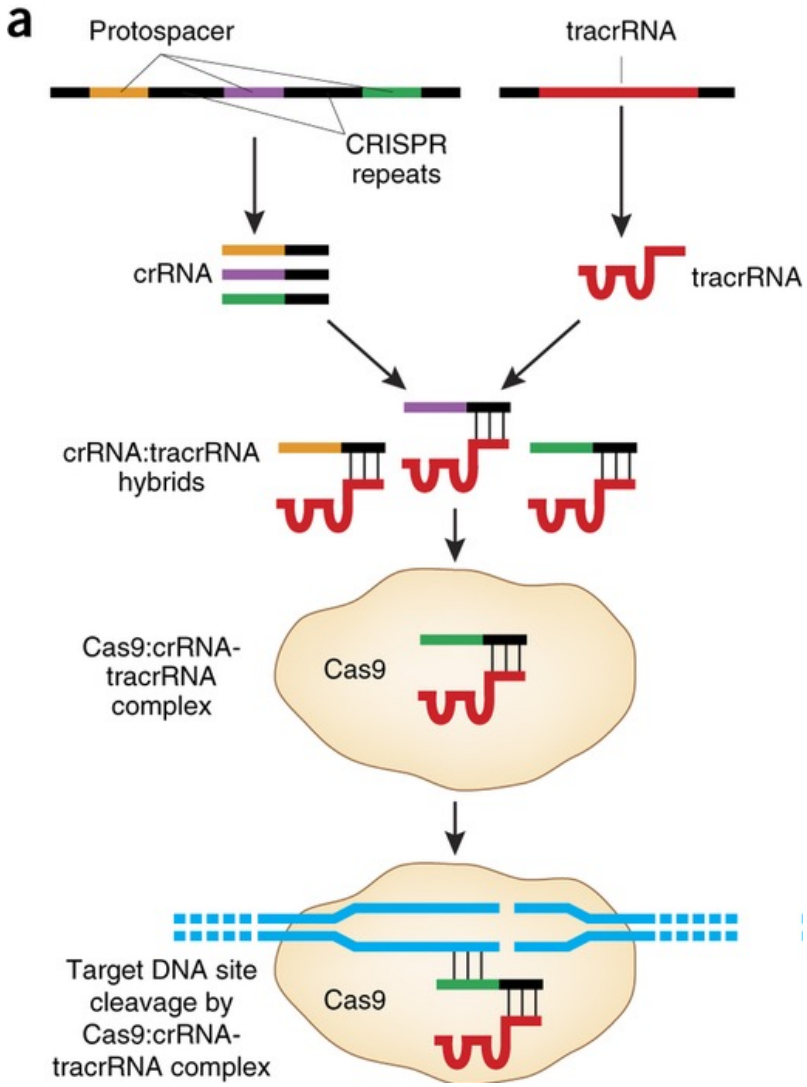
a
Protospacer
tracrRNA
CRISPR
repeats
crRNA
tracrRNA
crRNA:tracrRNA
hybrids
Cas9:crRNA-
tracrRNA
complex
Cas9
Target DNA site
cleavage by
Cas9:crRNA-
tracrRNA complex
Cas9