Czech Centre for Phenogenomics

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MINISTRY OF EDUCATION, YOUTH AND SPORTS

OP Education for Competitiveness

Institute of Molecular Genetics of the ASCR, v. v. i.

INVESTMENTS IN EDUCATION DEVELOPMENT

Talens and CRISPR/Cas9: Generation of genome engineered mouse models using editable nucleases

Silvia Petrezsélyová, PhD & Dominika Fričová, PhD

Czech Centre for Phenogenomics

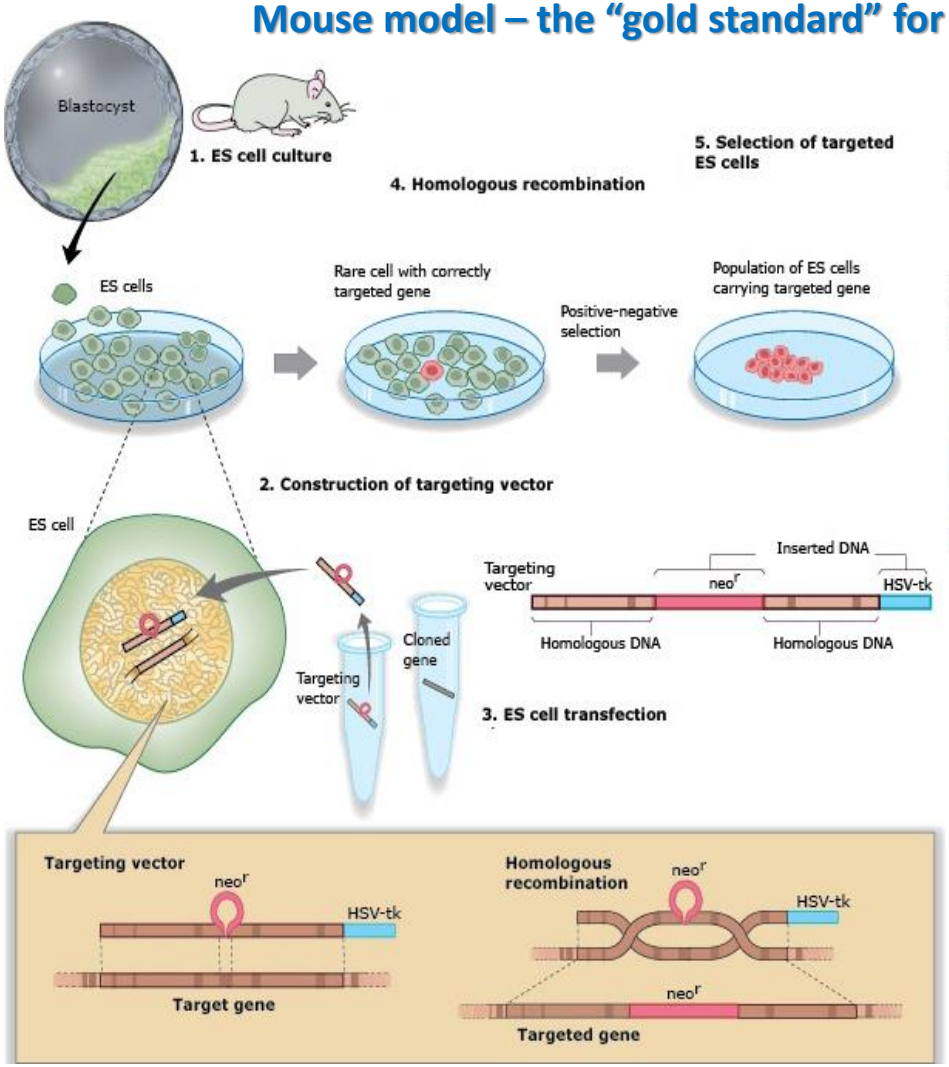
Institute of Molecular Genetics of the ASCR, v.v.i.

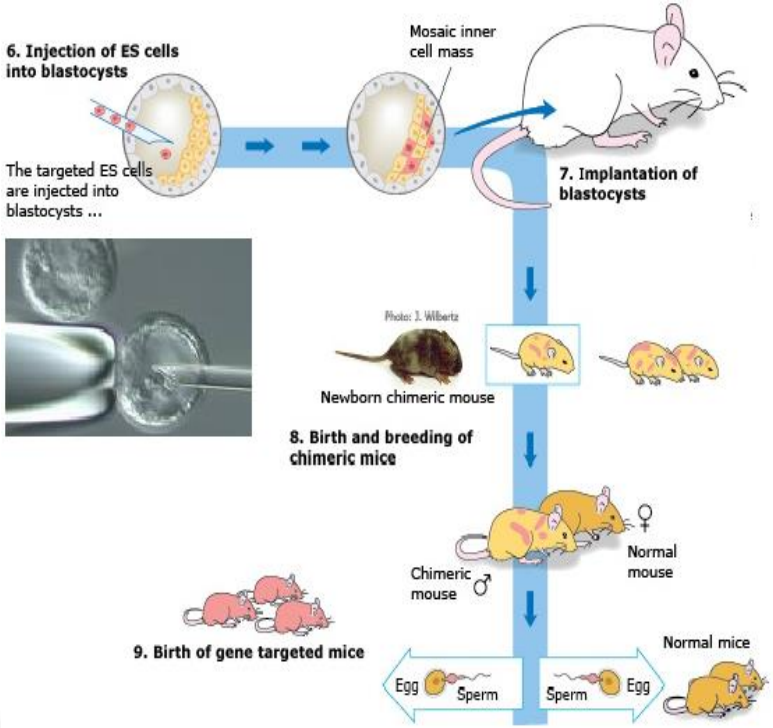
The presentation is supported from the project OP EC CZ.1.07/2.3.00/30.0027 "Founding the Centre of Transgenic Technologies"

IMG

BIOCEV

Mouse model – the "gold standard" for determining gene function in mammals





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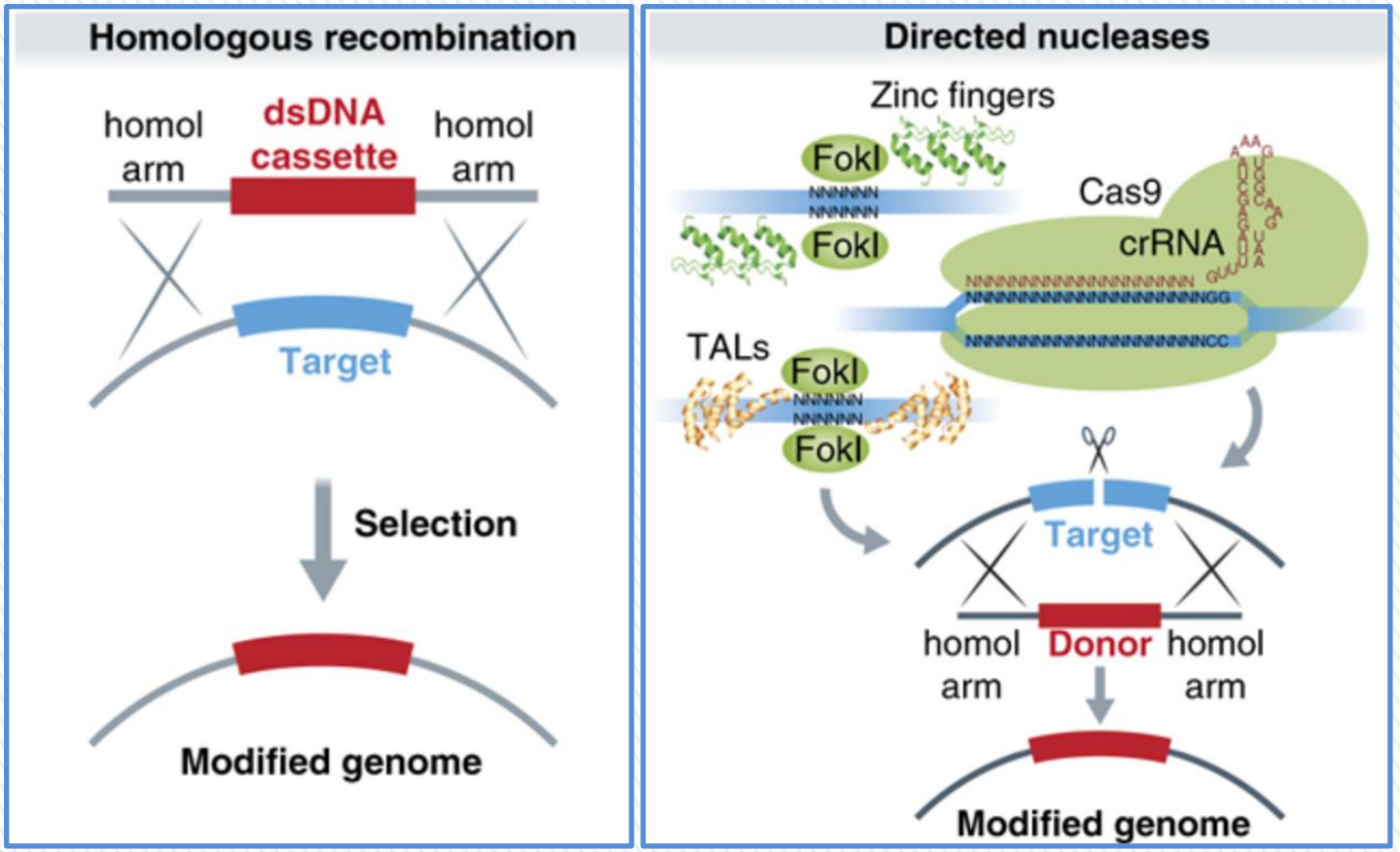

Induction of DSB increases HDR 100 – 1000 x

Esvelt and Wang, *Mol Sys Biol* 2013

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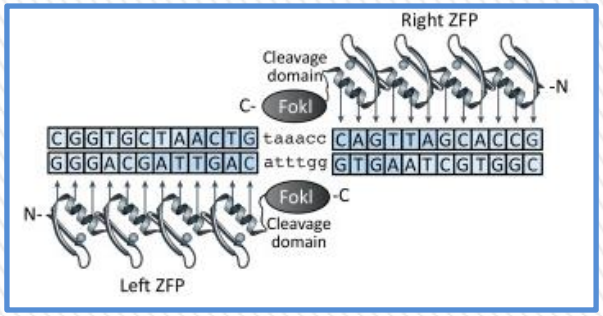
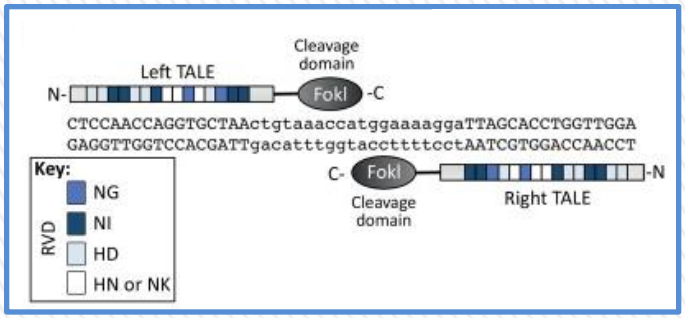
Zinc Finger Nucleases



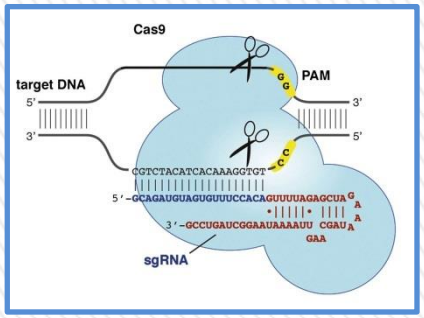
- Cys2-His2 zinc finger domain
- artificial arrays of 3-6 Zinc Fingers (9-18 bp)
- C-terminal fusion with endonuclease (FokI) - ZFN

Transcription Activator-like Effectors Nucleases (TALENs)



- Central Repeat Domain (CRD) responsible for DNA binding
- CRD consists of 34 aa highly homologous repeat modules
- DNA specificity determined by aminoacids 12 and 13 of each repeat
  - repeat variable diresidues (RVDs)

CRISPR/Cas9 system



- Interspaced short palindromic repeats (CRISPR) systems
- CRISPR RNAs (sgRNAs) in complex with CRISPR-associated 9 (Cas9) protein

Gaj *et al*. (2013) Trends in Biotechnology
Jinek *et al*. (2013) Elife

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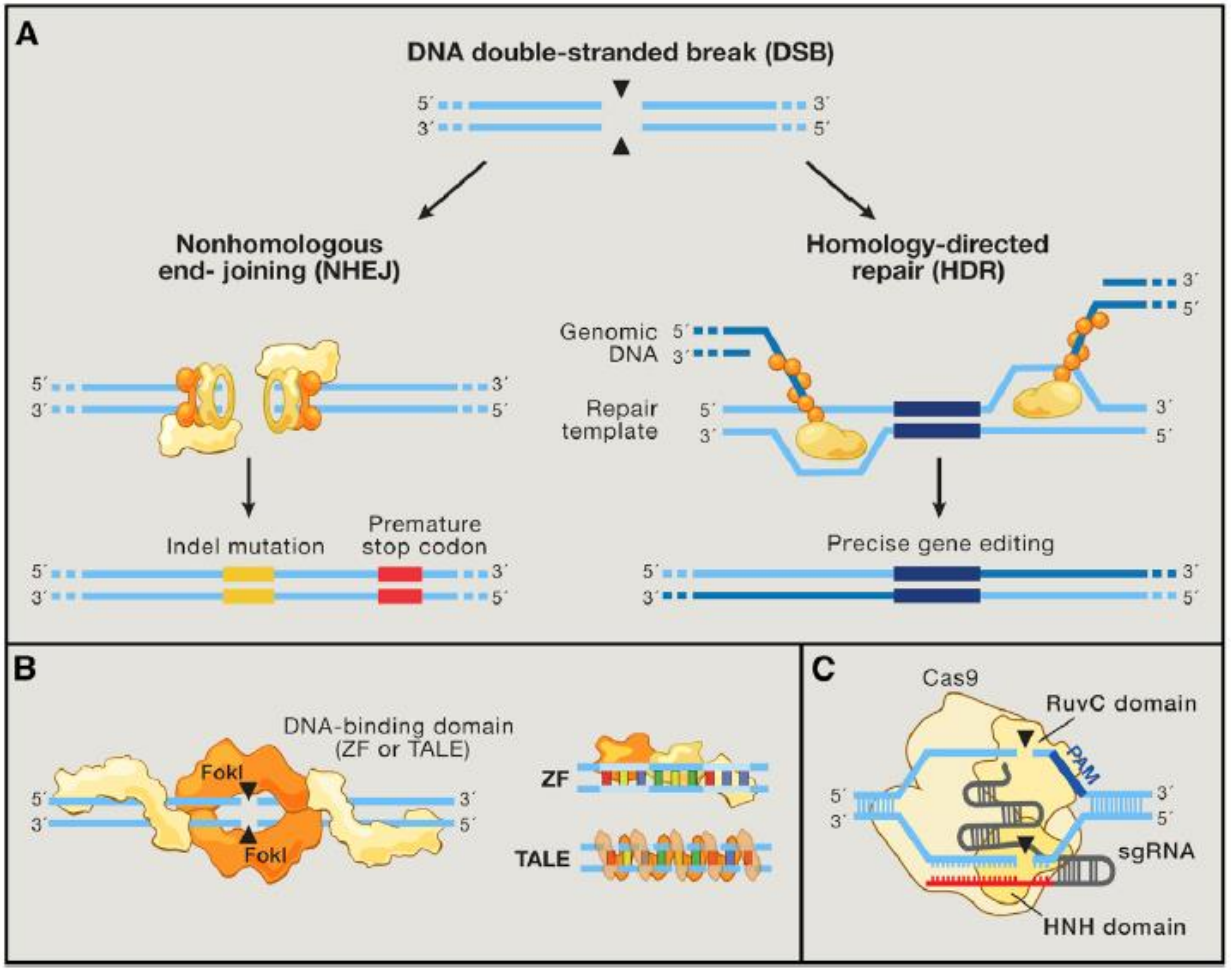
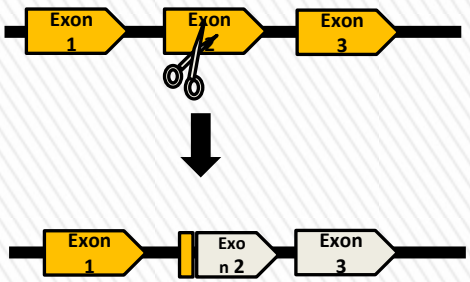


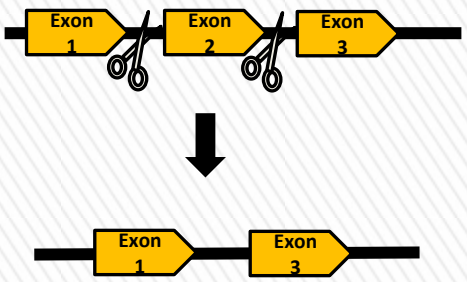
Hsu *et al.*, Cell 2014

possibilities of using programmable nucleases

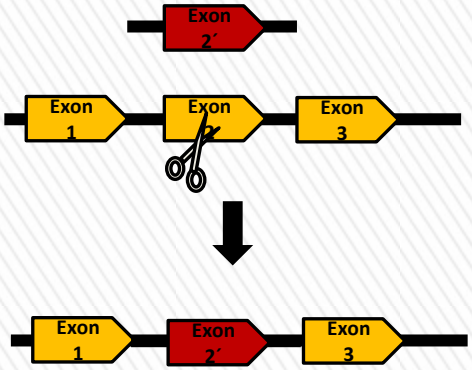
Generation of indel mutations



Excision of DNA fragment



Site-specific integration



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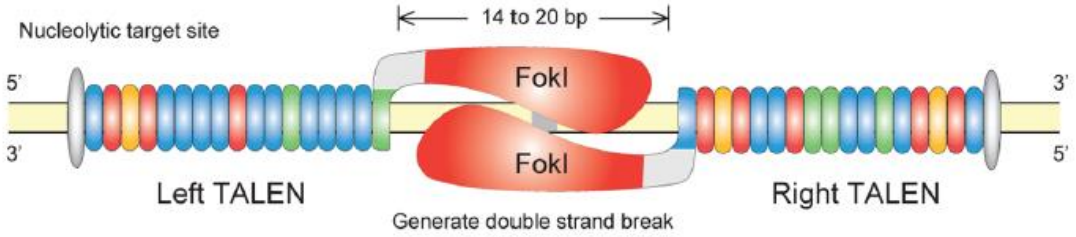
Transcription Activator-like Effector Nucleases (TALENs)



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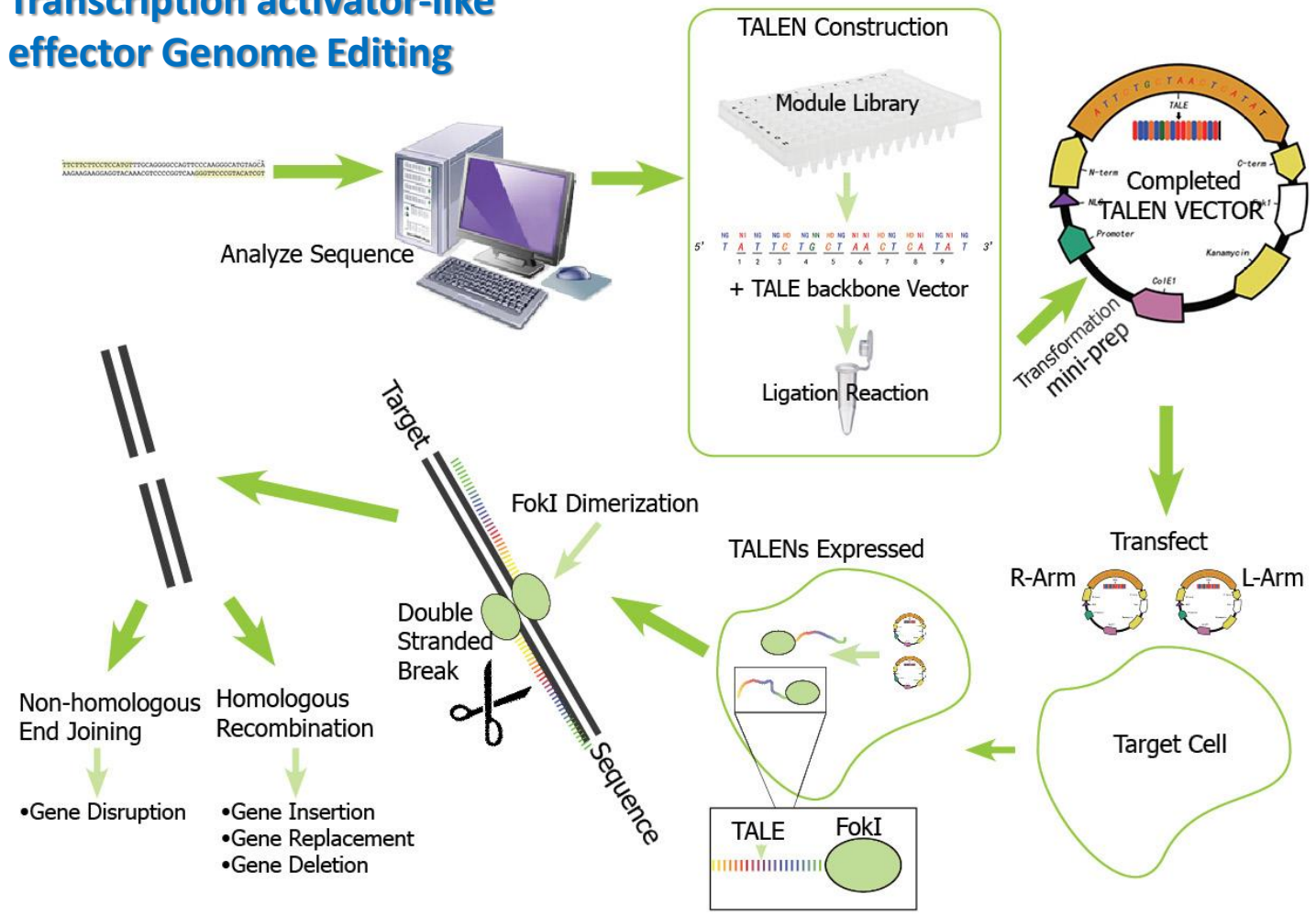
Transcription activator-like effector Genome Editing



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TALEN design guidelines

https://tale-nt.cac.cornell.edu/node/add/talen

Cornell University
TAL Effector Nucleotide Targeter 2.0

- TALEN target site should be preceeded by 5´ T
- TALEN should not have a T at position 1
- TALEN should not have an A at position 2
- Incorporate at least some (~3–4) strong RVDs (e.g., HD or NN)
- Use optimal spacer for your talen backbone (usually 15-17 bp)
- Position strong RVDs to avoid stretches (≥6) of weak RVDs
- For high guanine specificity use NH (to promote TALE activity) or NK
- Use NN for guanine if only a few other strong RVDs are present

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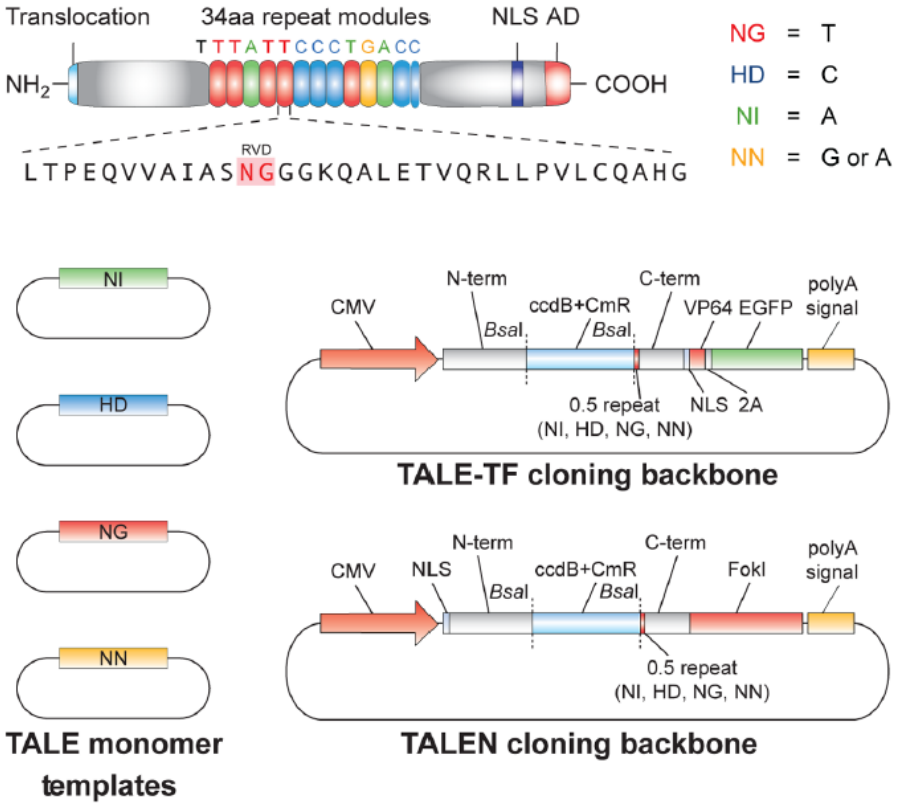
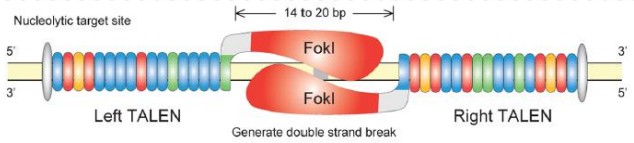
Assembly of TALE nucleases

www.addgene.org Golden Gate TALEN and TAL Effector Kit 2.0 (Cermak *et al.*, 2011)





Sanjana *et al.*, Nat Protoc 2013

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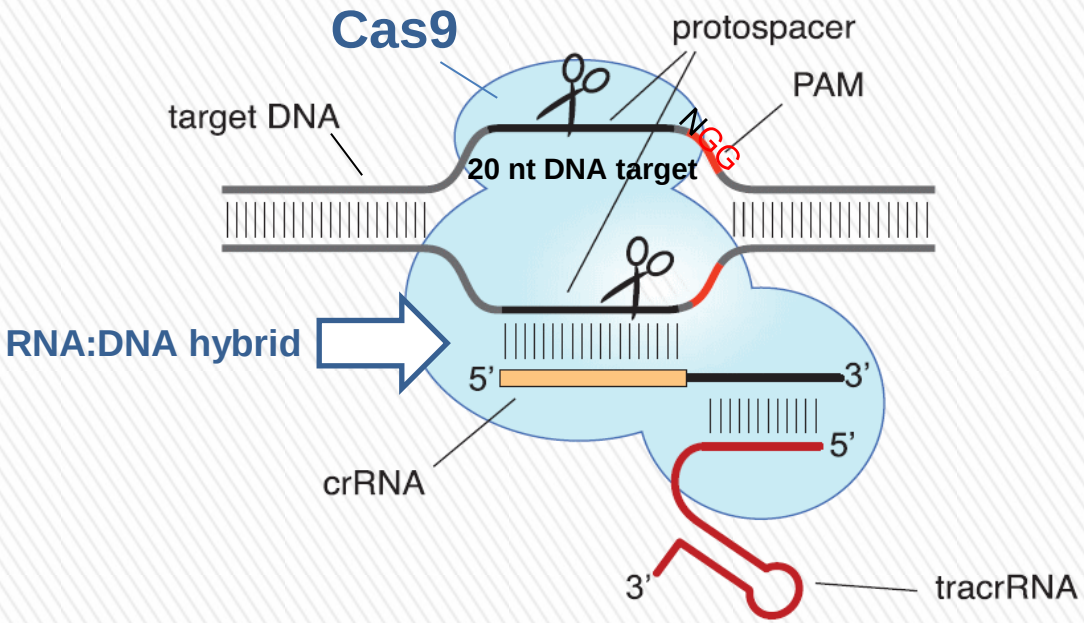
CRISPR/Cas9 Nuclease

CRISPR: **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats
Cas: CRISPR-**as**sociated (protein)



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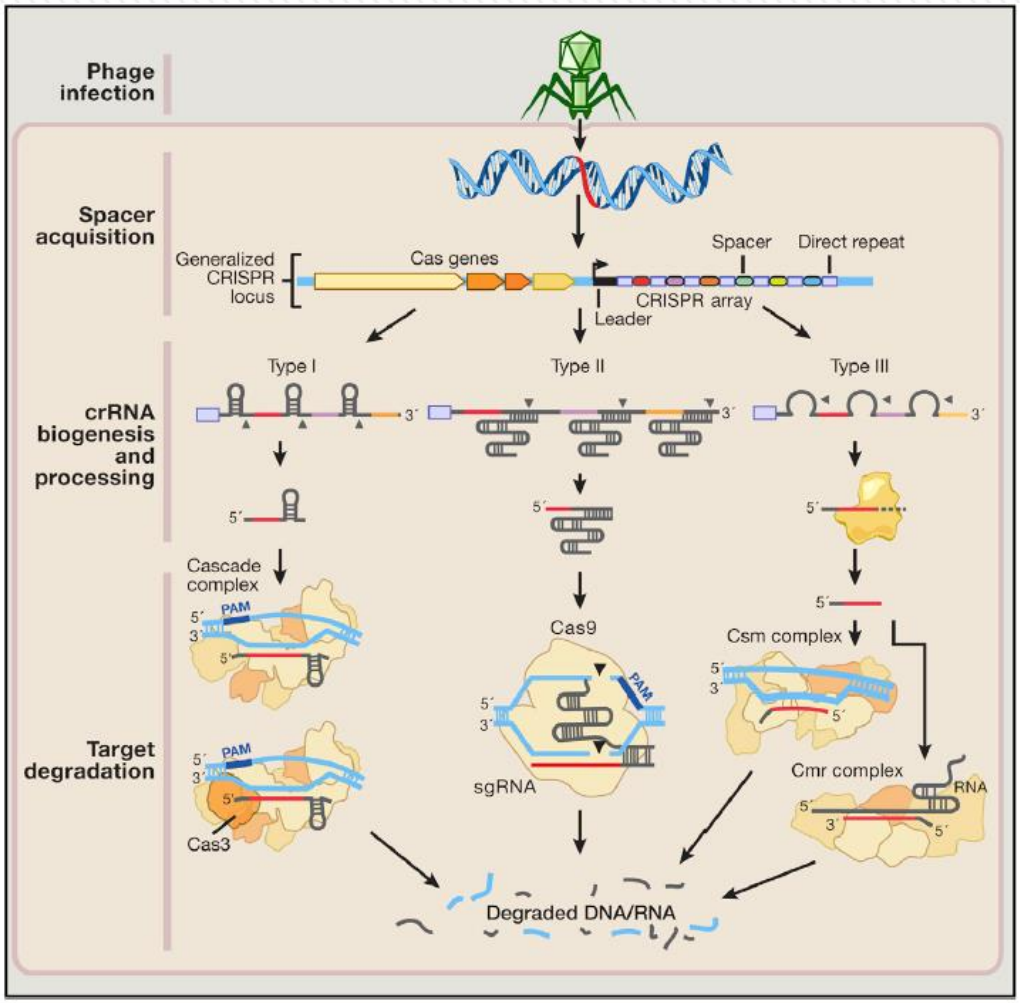
Natural mechanisms of Microbial CRISPR systems in Adaptive Immunity



Hsu *et al.*, Cell 2014

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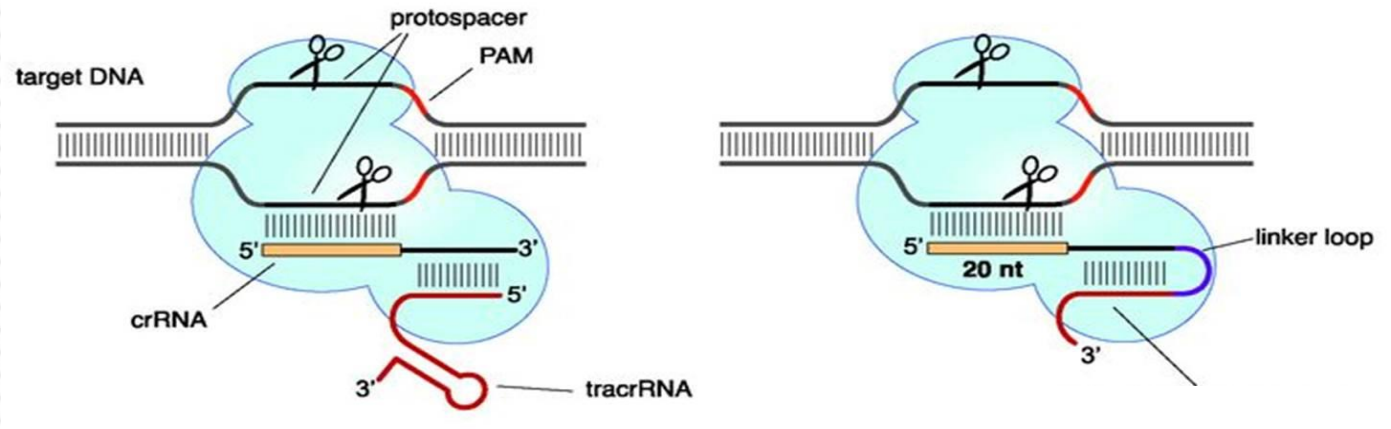
Natural configuration
Cas9 & crRNA:tracrRNA duplex

Simplified configuration
Cas9 & single guiding (sg) RNA



Programming of Cas9 to a specific target sequence

Any 20 nt sequence, located upstream of a NGG (PAM) motif, can be adressed

➢ **guides Cas9 to introduce a DSB 3 bp upstream of PAM**

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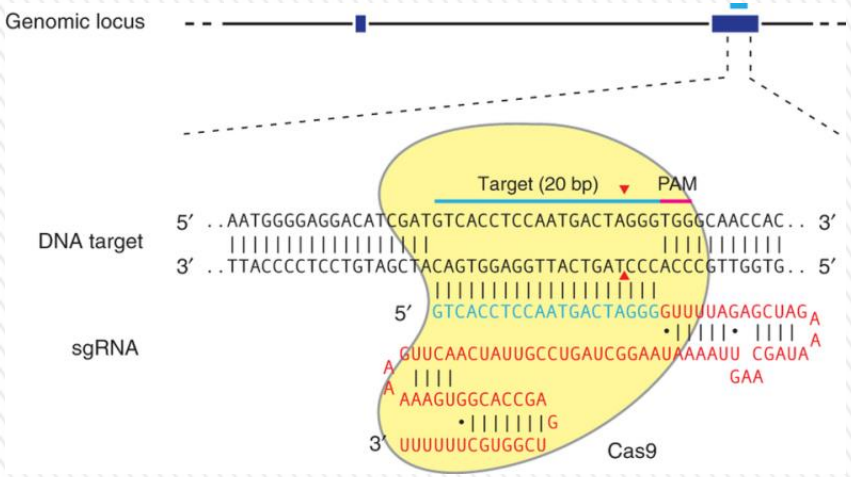
The Crispr/Cas9 system

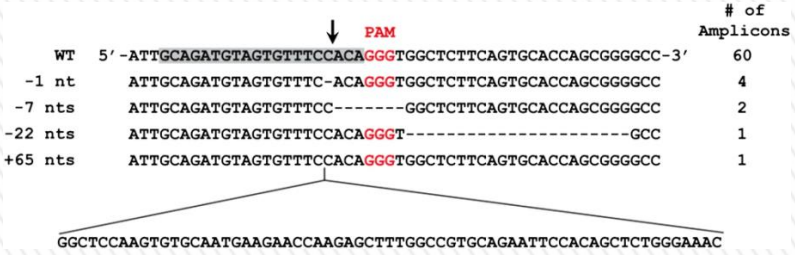
http://crispr.mit.edu/

targeting

Cas9 nuclease is guided by small crRNAs to recognize and cut target DNA ("protospacer") sequences, next to an NGG motif (PAM – protospacer adjacent motif)

Functions efficiently for gene editing in mammalian cells (Mali et al., Cong et al., Science 2013).





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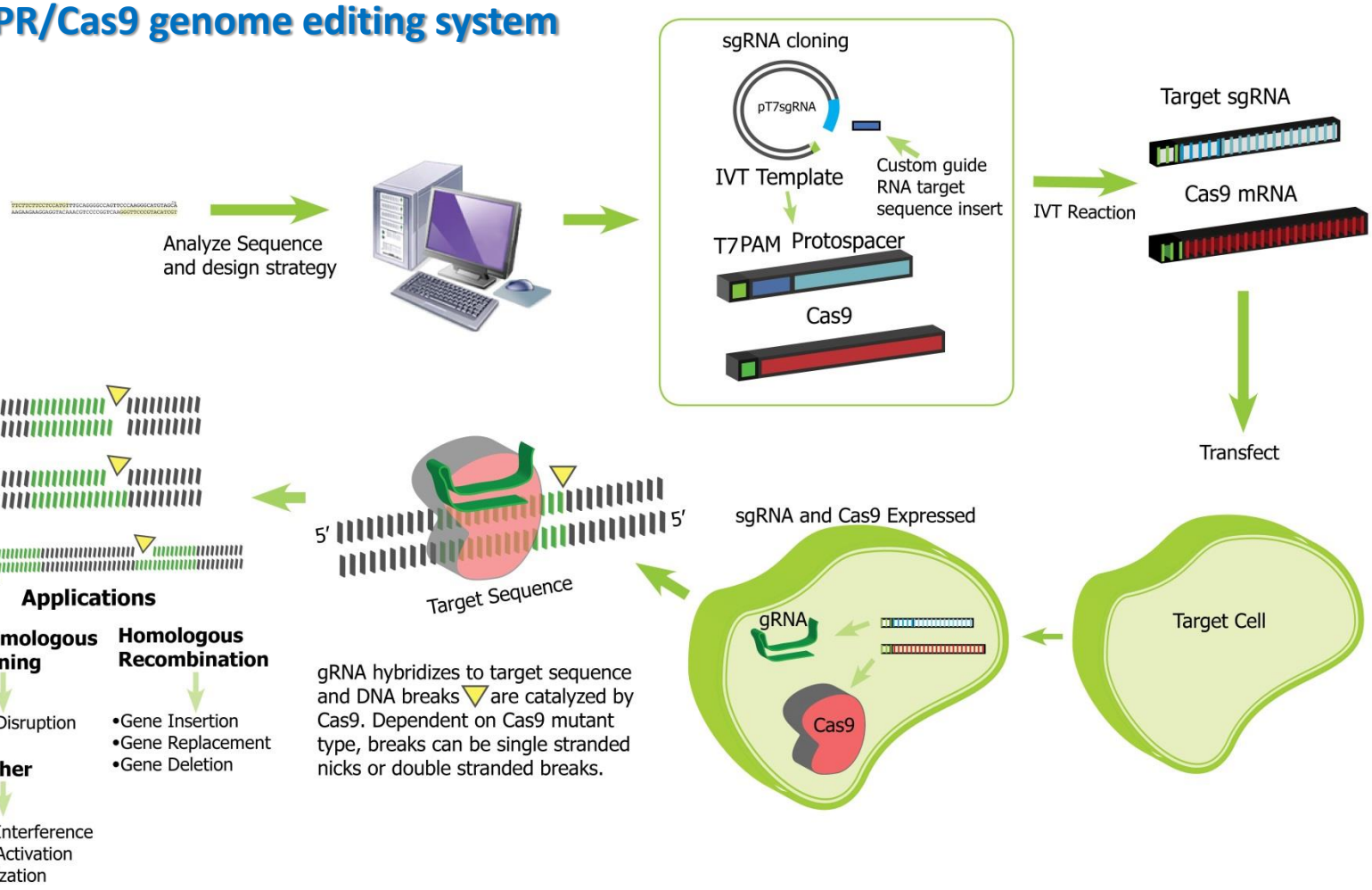
CRISPR/Cas9 genome editing system



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Generation of specific Crispr/Cas9:

Oligo annealing and cloning into backbone vectors:

easy task

1. Digest 1ug of pX260 or pX330 with *Bbs*I for 30 min at 37C:

1 ug	pX260 or pX330
1 ul	FastDigest *Bbs*I (Fermentas)
1 ul	FastAP (Fermentas)
2 ul	10X FastDigest Buffer
X ul	ddH_2O
20 ul	total

2. Gel purify digested pX260 or pX330 using QIAquick Gel Extraction Kit and elute in EB.

3. Phosphorylate and anneal each pair of oligos:

1 ul	oligo 1 (100μM)
1 ul	oligo 2 (100μM)
1 ul	10X T4 Ligation Buffer (NEB)
6.5 ul	ddH_2O
0.5 ul	T4 PNK (NEB)
10 ul	total

Anneal in a thermocycler using the following parameters:

37°C	30 min
95°C	5 min and then ramp down to 25°C at 5°C/min

4. Set up ligation reaction and incubate at room temperature for 10 min:

X ul	*Bbs*I digested pX260 or pX330 from **step 2** (50ng)
1 ul	phosphorylated and annealed oligo duplex from **step 3** (1:250 dilution)
5 ul	2X Quickligation Buffer (NEB)
X ul	ddH_2O
10 ul	subtotal
1 ul	Quick Ligase (NEB)
11 ul	total

5. (optional but highly recommended) Treat ligation reaction with PlasmidSafe exonuclease to prevent unwanted recombination products:

11 ul	ligation reaction from **step 4**
1.5 ul	10X PlasmidSafe Buffer
1.5 ul	10mM ATP
1 ul	PlasmidSafe exonuclease
15 ul	total

Incubate reaction at 37C for 30 min.

6. Transformation

CRISPR GENOME ENGINEERING TOOLBOX ZhangLab

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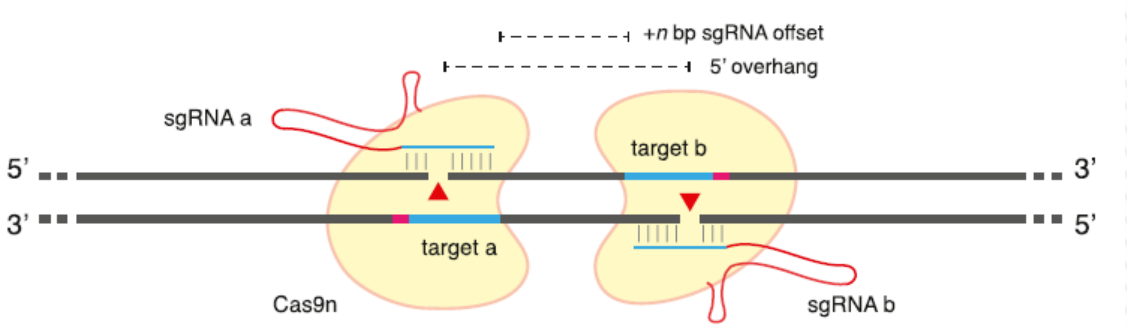
The Crispr/Cas9 system

increasing specificity

(low off-target activity)

Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity

F. Ann Ran,[1,2,3,4,5,11] Patrick D. Hsu,[1,2,3,4,5,11] Chie-Yu Lin,[1,2,3,4,6] Jonathan S. Gootenberg,[1,2,3,4] Silvana Konermann,[1,2,3,4] Alexandro E. Trevino,[1] David A. Scott,[1,2,3,4] Azusa Inoue,[7,8,9,10] Shogo Matoba,[7,8,9,10] Yi Zhang,[7,8,9,10] and Feng Zhang[1,2,3,4,*]



Offset from 4bp to 20bp

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Programmable nucleases:

cell culture / *in vitro* validation

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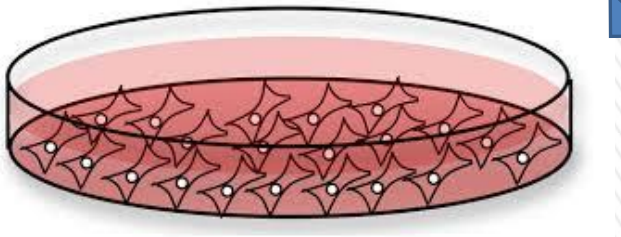
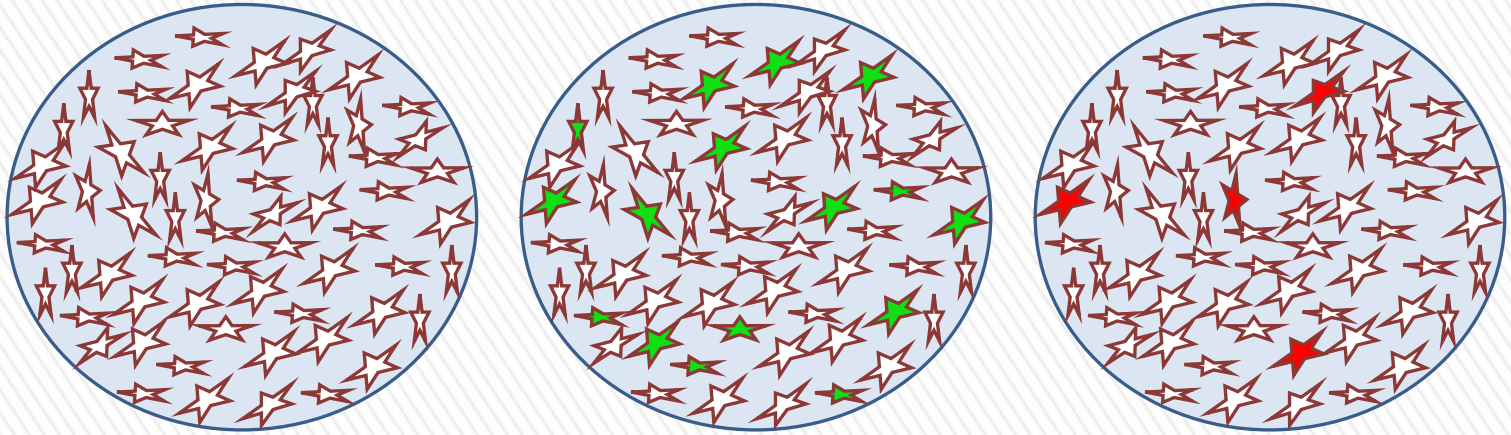
1) Design and generation of TALEN/CRISPR plasmids

TALEN/CRISPR plasmid DNA

2) Transfection of cells



3) Selection of clones



Transfected cells (15 %)

Targeted cells (1.5 %)

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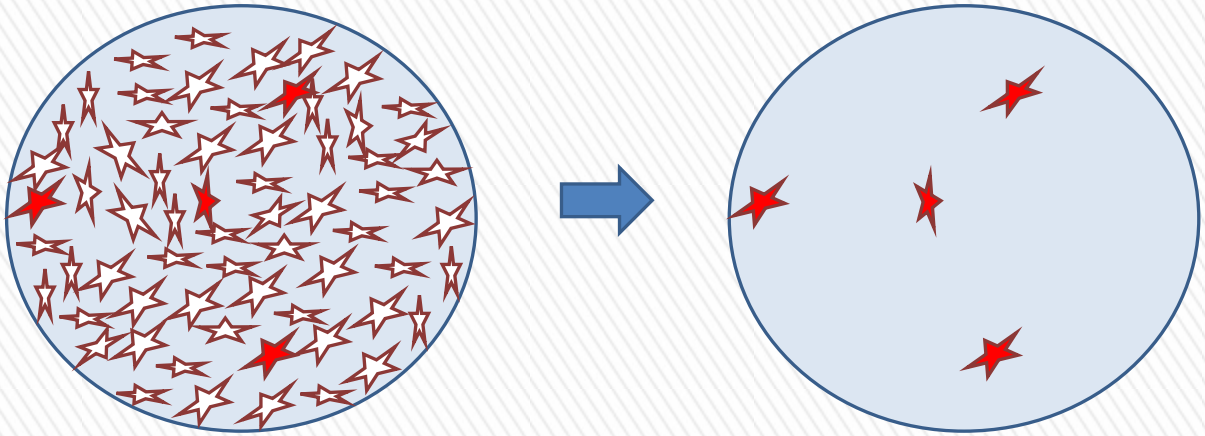
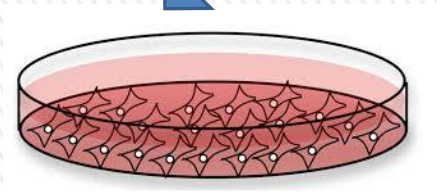
Surrogate plasmids for selection of targeted cells



TALEN/CRISPR plasmid DNA

+ Activity reporter plasmid (Blasticidin resistance)



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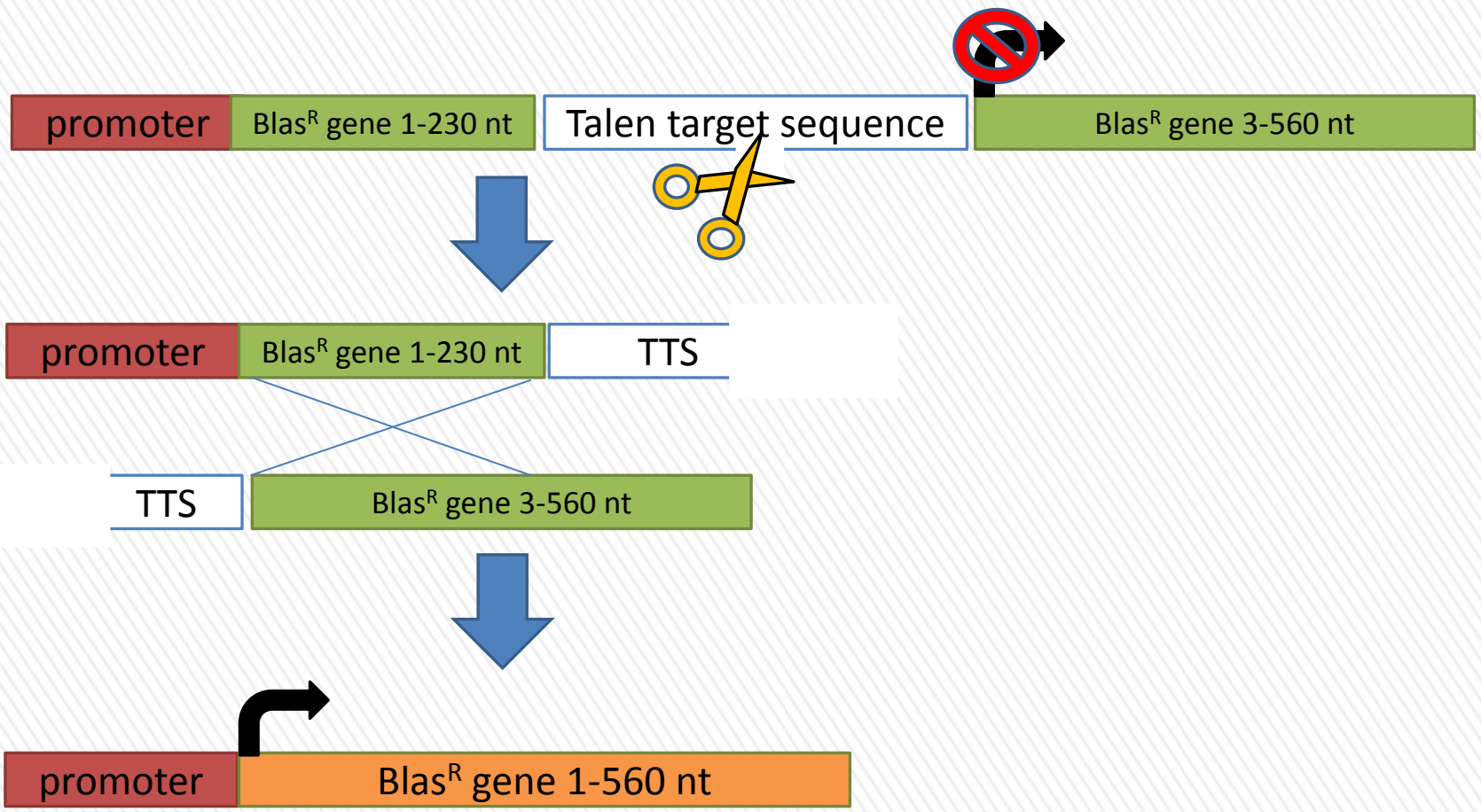

Kasparek- *unpublished data*

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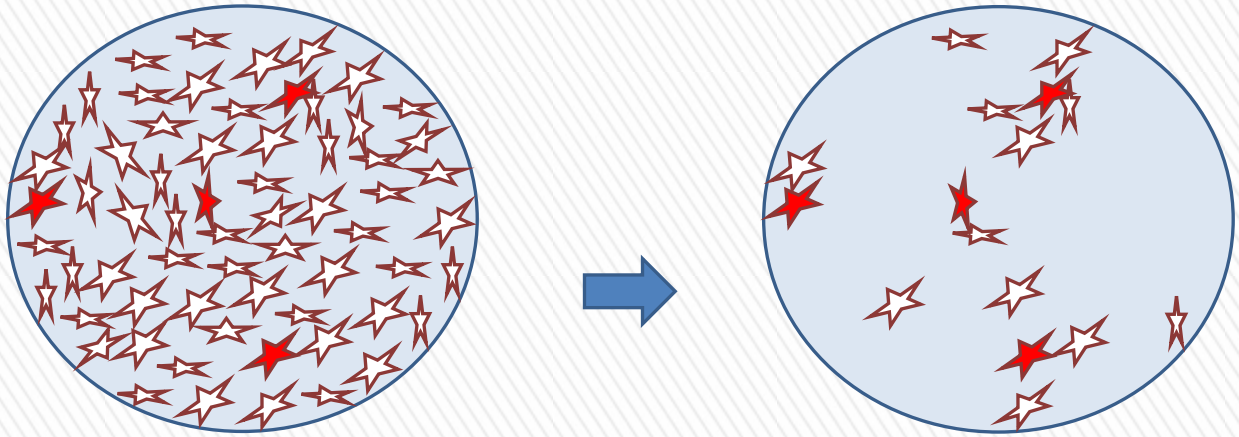
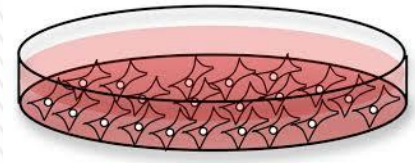


TALEN/CRISPR plasmid DNA
+ Activity reporter plasmid (Blasticidin resistance)
+ targeting construct



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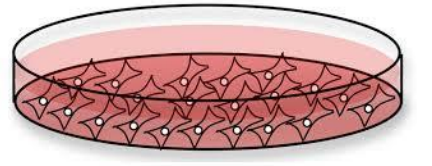
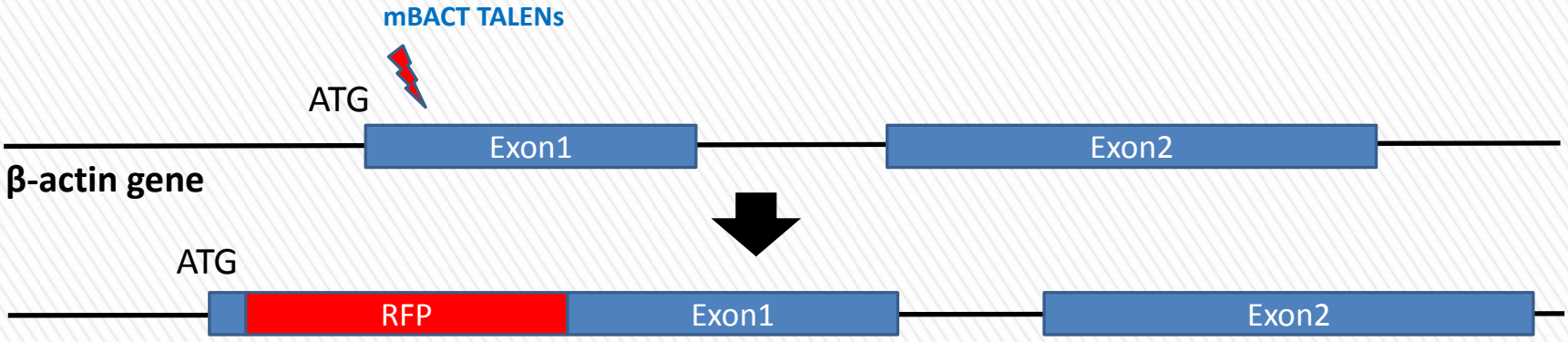


Targeting vector



Kasparek- *unpublished data*

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TALEN/CRISPR plasmid DNA
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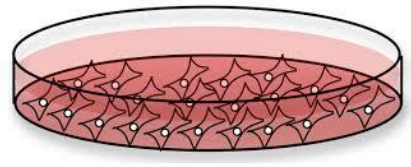
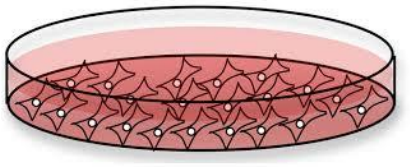
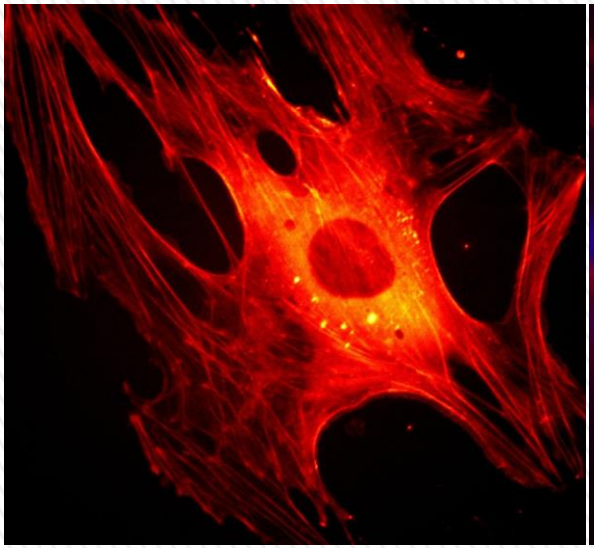
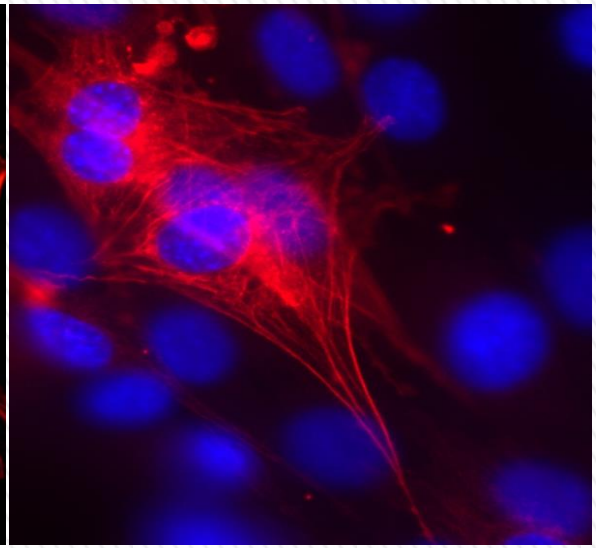


selection on Blas (8 days)



FACS





Kasparek- ***unpublished data***

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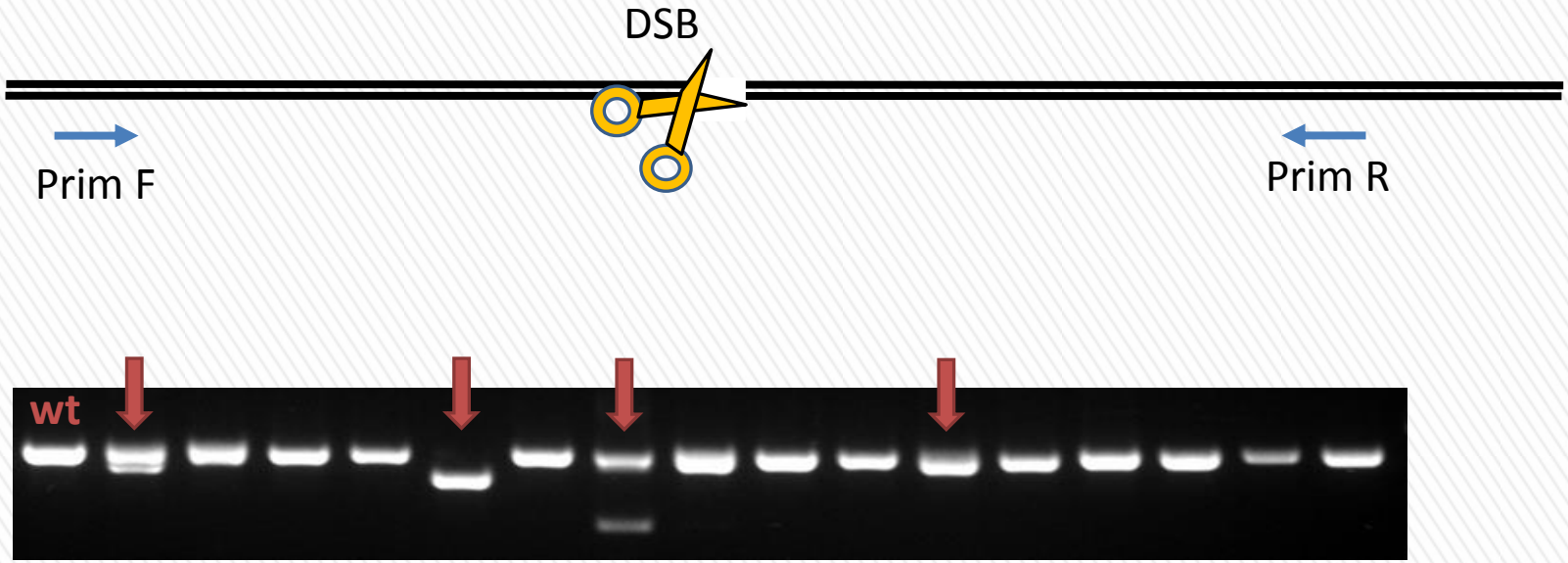
Verification of TALEN activity (PCR)

PCR amplification, DNA electrophoresis to analyze variability of PCR products



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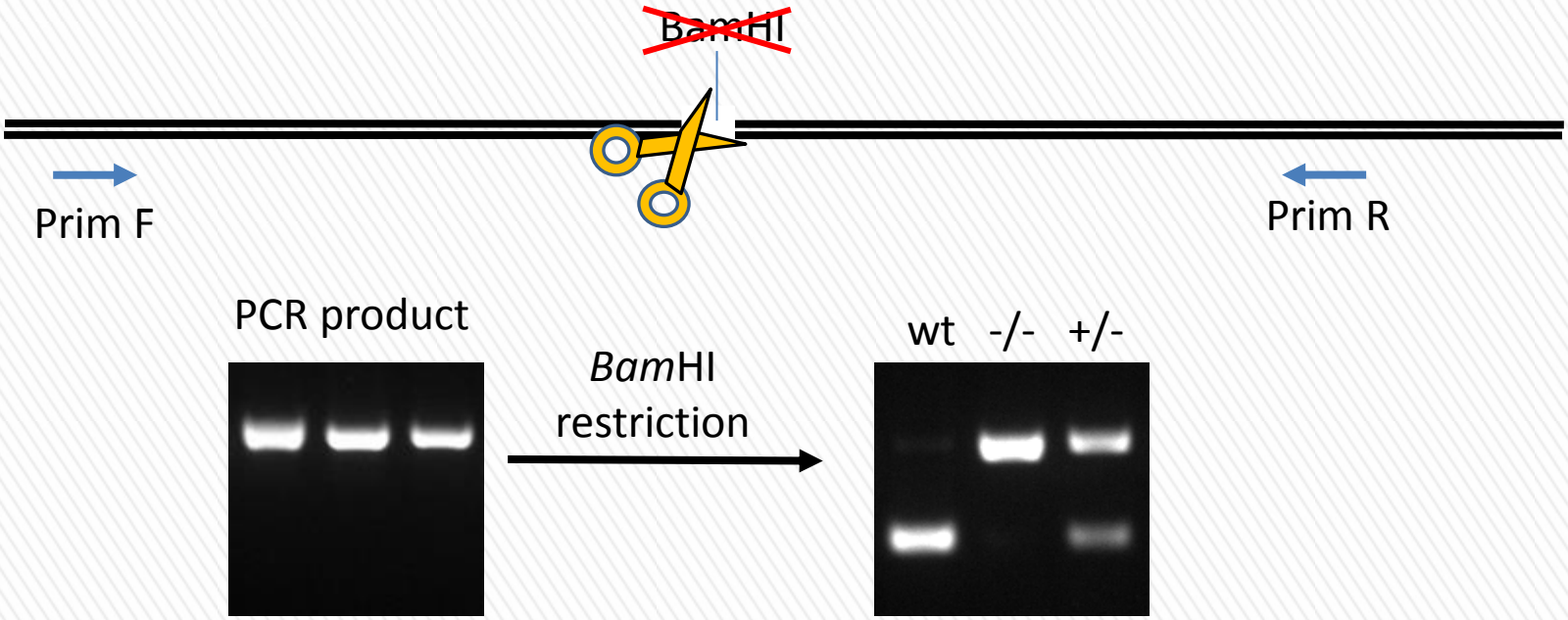
Verification of TALEN activity (RFLP)

Restriction Fragment Length Polymorphism

- mutation of endogenous restriction site by TALENs
- digestion of PCR product by restriction enzyme



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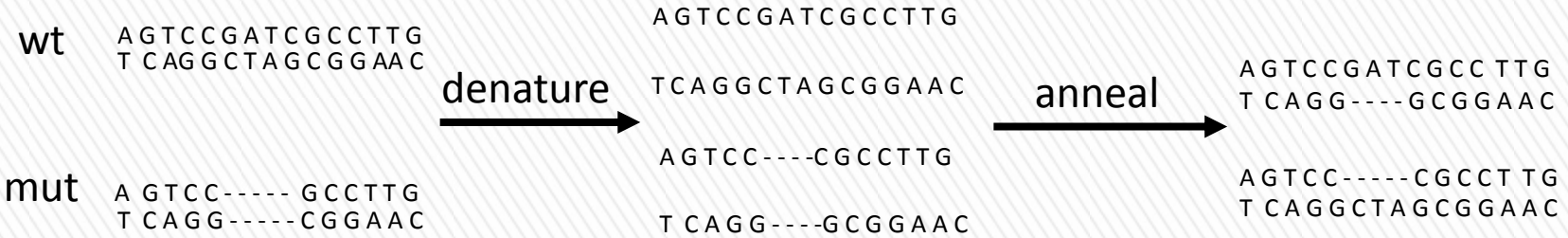
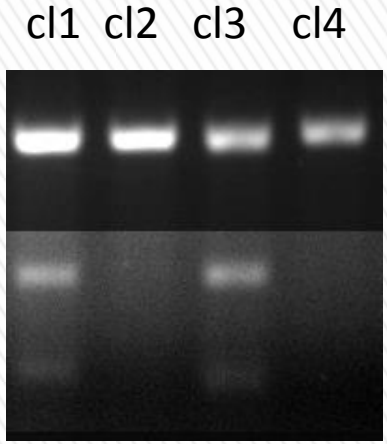
Verification of TALEN activity (T7 endonuclease)

mismatch-specific DNA endonuclease detection of short indels



T7 endonuclease cleavage site



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Programmable nucleases:

in vivo model

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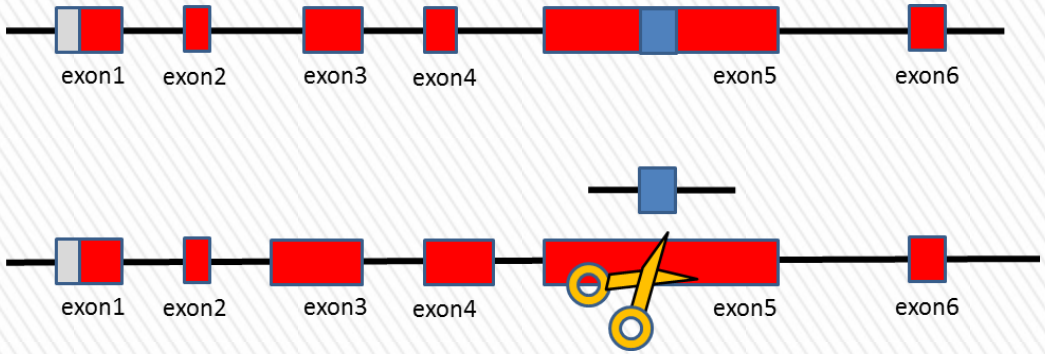
Examples - Knock-out mutant

Netherton syndrome

- disorder that affects the skin, hair, and immune system - autosomal recessive (1:200 000)
- chronic skin inflammation in epidermis, abnormal desquamation, disrupted epidermal barrier
- Point mutation in exon 5 Spink 5 gene



Kasparek- ***unpublished data***

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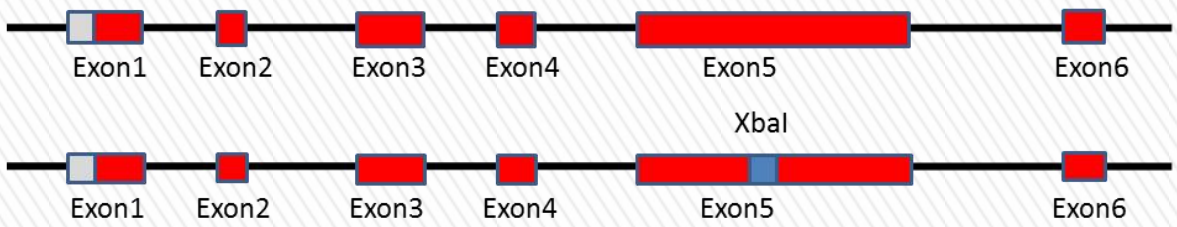
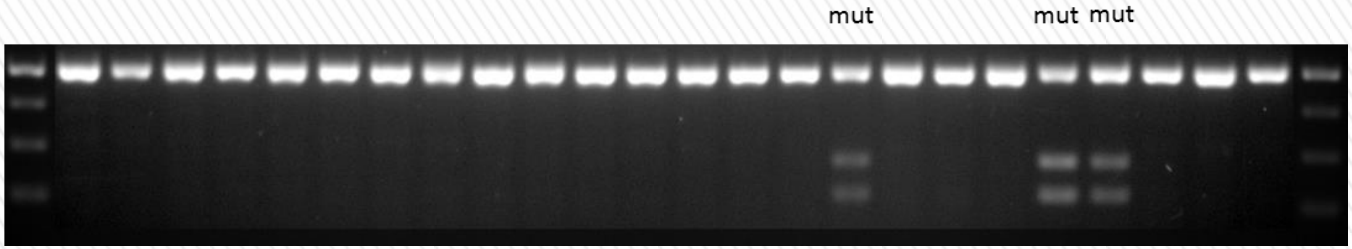
Examples - Knock-out mutant



Spink5 PCR - XbaI



Kasparek- *unpublished data*

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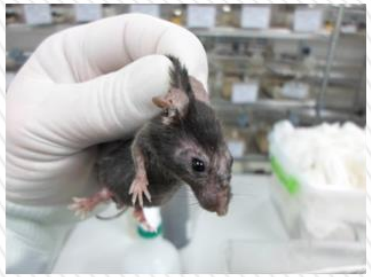
Examples - Knock-out mutant

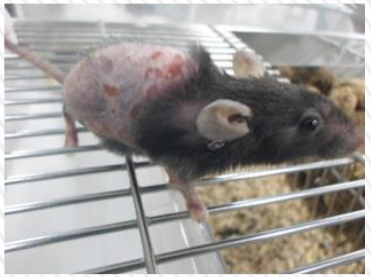
2 days

9 days

18 weeks



26 weeks



Kasparek- ***unpublished data***

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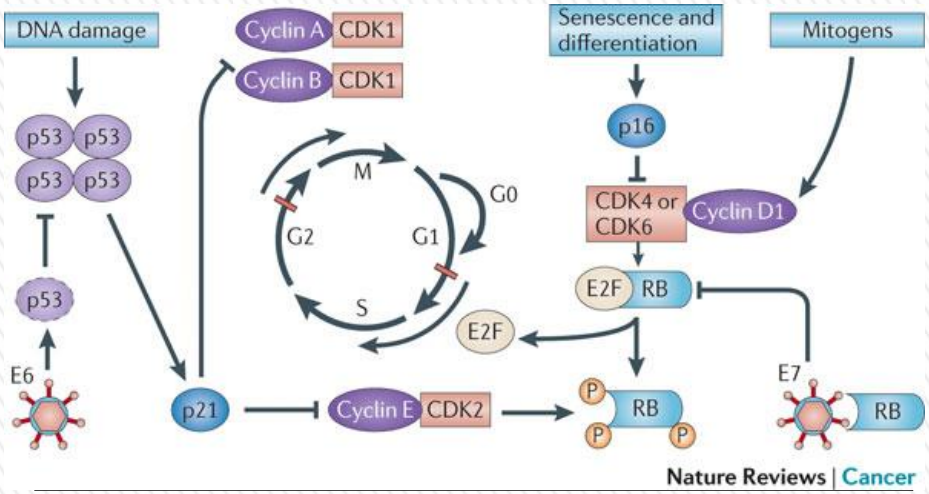
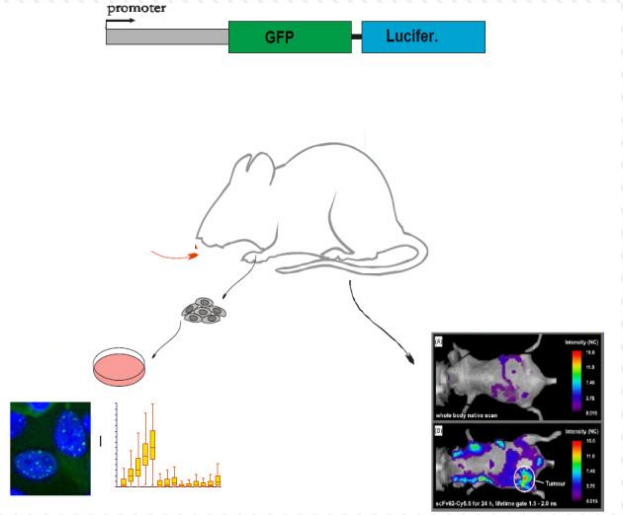
Examples - Reporter mice

p21 promoter reporter mice for study of aging

p21$^{WAF1/CIP1}$ - cyclin-dependent kinase inhibitor 1
Function : regulator of cell cycle progression at G1 and S phase





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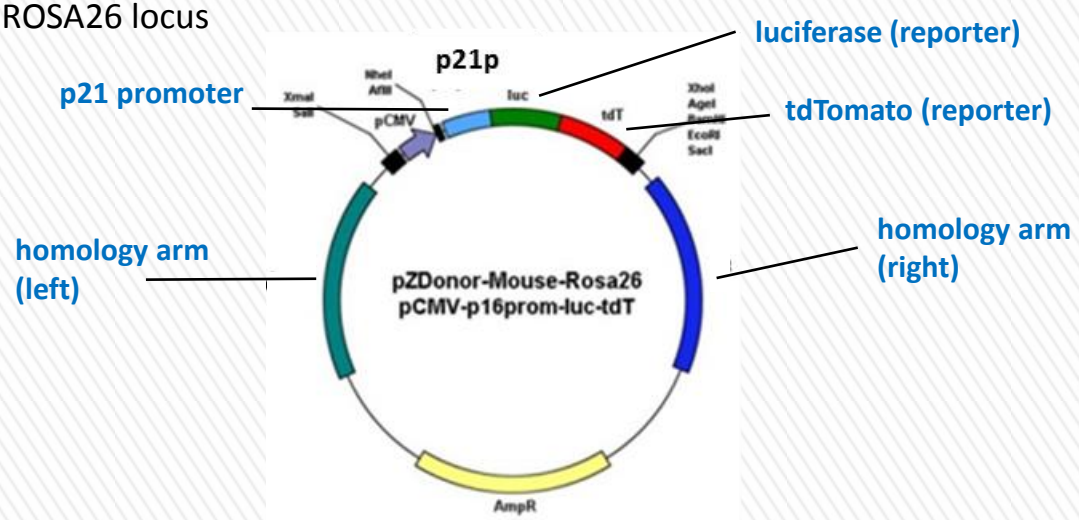
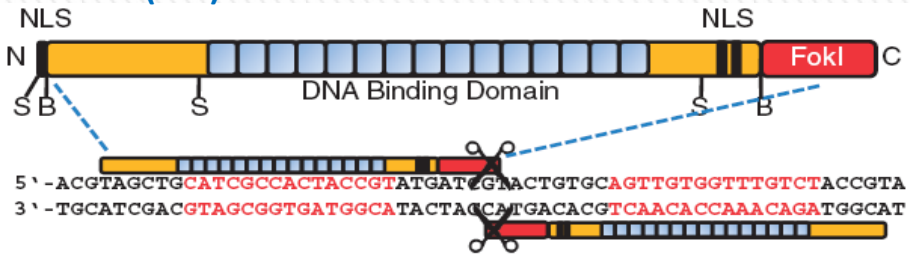
Examples - Reporter mice

Where and how to insert p21 reporter construct?

ROSA26 is a locus used for constitutive, ubiquitous gene expression, over 130 knock-in lines have been created based on the ROSA26 locus

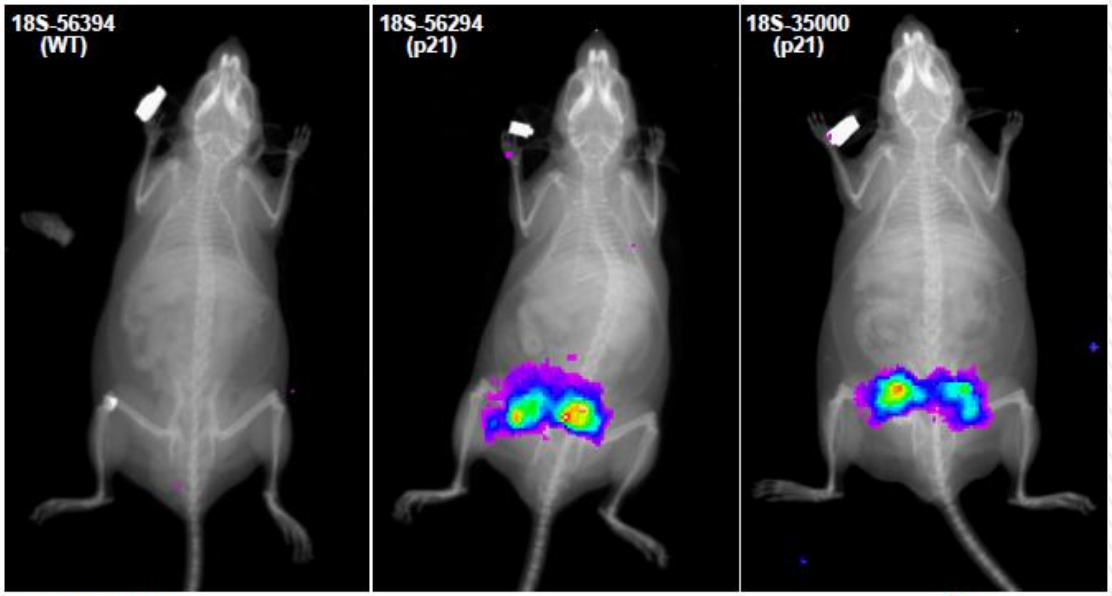


TALENs - transcription activator-like (TAL) effector nucleases



Cermak *et al.*, Nucleic Acids Research, 2011, 1-

Examples-
Reporter mice



exposure time 5 minutes
bining 8x8
FOV 15 cm
Luciferin IP injection 200ul stock solution per mouse, directly after anestesia
Imaging 10 minutes after Luciferin IP injection

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Conclusions

- both TALEN and CRISPR/Cas9 technology represent very efficient and elegant techniques for gene targeting (knock-out as well as knock-in)
- specific gene knock-out and knock-in is powerful tool for understanding of its role (models of disease)
- possibility of construction of *in vivo* (animals) and *in vitro* (cell culture) models using programmable nucleases

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OP Education for Competitiveness

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IMG BIOCEV

1. Which of the following molecular tools DOES NOT belong to genome editing tools?

a) Zinc finger nucleases b) Tale nucleases **c) RNA ribozymes**

2. What type of direct interaction at the level of DNA is it in case of Crispr/Cas9 system?

a) protein-DNA **b) RNA-DNA** c) DNA-DNA

3. In which process/feature Cas9 nickase and wild-type Cas9 differ from each other?

a) wild-type Cas9 creates double stranded DNA breaks, Cas9 nickase creates single stranded DNA breaks

b) were isolated from different organisms

c) use different cofactors

4. How many of guideRNAs are necessary to create double stranded DNA breaks using the wild-type Cas9?

a) 1 guideRNA

b) 2 guideRNA

c) formation of double stranded DNA breaks does not depend on the number of guideRNAs used but on the activity of Cas9

5. What type of DNA repair in cell is used for targeting of a construct into a defined genomic site to generate reporter models?

a) homologous recombination (HR)

b) non-homologous end joining (NHEJ)

c) base excision repair (BER)

6. A targeting construct that we plan to use for insertion of a reporter gene into a specific genomic site must contain: 1. reporter gene 2. selection marker and 3. .....

a) ampicilin

b) homologous arms

c) gen encoding fluorescent protein

7. Which mouse locus is often used for knock-in (insertion of a foreign DNA) strategy?

a) ORF b) GAL1 **c) Rosa26**

8. Which of the following genetic elements can be used as reporter gene?

a) promoter

b) gene encoding fluorescent protein or enzyme, the activity of which can be easily measured (e.g. luciferase)

c) gene encoding transcriptional factor

9. Which of the following methods can be used to verify gene insertion into a specific genomic site?

a) luciferase assay

b) PCR, sequencing

c) fluorescence microscopy

10. What is off-target?

a) a process, which leads to „turning off“ of genes that have been modified

b) targeting of several genes by use of programmable nucleases

c) undesired/unplanned/non/specific change in a gene other than that for which programable nuclease have been targeted