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New concepts of gene engineering in mammalian cells

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RG Functional Genomics

1/11/2021

Myself

- Head of Functional Genomics research group at CEITEC MU
- Studying hematological malignancies (B-cell lymphoma, CLL, AML)
- Research aim: identify novel options of targeted therapy tailored to individual mutations or genetic background of patients
- Focus on immunotherapy with monoclonal antibodies or genetically modified T cells
- Approaches include drug screening, genome-wide CRISPR/Cas9 screening, CRISPR/Cas9-mediated gene modifications, molecular and cell biology techniques

- Enthusiastic students always welcome! ;-)

Gene engineering

- Direct manipulation of organisms´s genes to produce improved or novel organisms
- Changes to genetic makeup, transfer of genes within and across species
- Inserting, removing or altering genes –> genetically modified organisms (GMO)

Genetically modified organisms (GMOs)

- Transgenic – genetic material inserted from another species
- Cisgenic – genetic material inserted from the same species
- Knockout – genetic material was removed
- Knock-in – genetic material replaced

Application of genetic engineering

- Gene mutation
- Gene therapy
- Creating chromosome rearrangement
- Study gene function
- Transgenic animals
- Endogenous gene labeling
- Transgene addition

History of gene engineering

- 1972 – first recombinant DNA by Paul Berg (SV40 monkey virus + lambda virus)
- 1973 – first transgenic organism – bacterium by H. Boyer and S. Cohen (insertion of resistence genes)
- 1974 – first GMO animal – transgenic mouse by R. Jaenisch (retroviral DNA into embryo)
- 1976 – first gene engineering company Genentech established – production of human proteins in E.coli
- 1978 – genetically engineered human insulin
- 1982 – insulin-producing bacteria commercialised (FDA approval)
- 1987 – first GMO released into environment – ice-minus *P. syringae* to protect strawberry and potato fields from frost
- 1986 – first GMO plant – tobacco resistant to herbicides

History of gene engineering

- 1992 – first commercial transgenic plants (China) – virus-resistant tobacco
- 1994 – first commercial GMO plant in EU - tobacco resistant to herbicides
- 1994 – genetically modified food – Flavr Savr tomato
- 1995 – approval of pesticide-producing crop (Bt Potato)
- 2003 – first GMO pet sold in US – GloFish
- 2010 – first synthetic genome introduced into empty bacterial cell (J.Craig Venter institute) -> Mycoplasma laboratorium could replicate and produce proteins
- 2016 – salmon modified with growth hormon sold

GloFish

Six brilliant colors. Five types of tropical fish.
Barb
Danio
Tetra
Long-Fin Tetra
Shark
GloFish
Experience the Glo!
Sunburst® Orange
Electric® Green
Cosmic® Blue
Galactic® Purple
Moonrise® Pink
Starfire® Red

Awards for gene engineering milestones

- 1978 – Nobel prize for Physiology or Medicine to W. Arber, D. Nathans, H. O. Smith for their discovery and characterization of **restriction enzymes**, which led to development of recombinant DNA technology
- 1993 – Nobel prize in Chemistry to K. Mullis for his invention of **PCR method** and to M. Smith for his contributions to the establishment of oligonucleotide-based, **site-directed mutagenesis**
- 2007 – Nobel prize for Physiology or Medicine to M. Capecchi, M. Evans, O. Smithies for their discovery of how **homologous recombination** can be used to introduce genetic modifications in mice through embryonic stem cells
- 2020 – Nobel prize in Chemistry to J. Doudna, E. Charpentier for development of **CRISPR/Cas9 method** for genome editing

Approaches in gene engineering

1) **Selective breeding** (plants, animals)

- historical approach (Romans)
- selection of males and females to produce desired characteristics

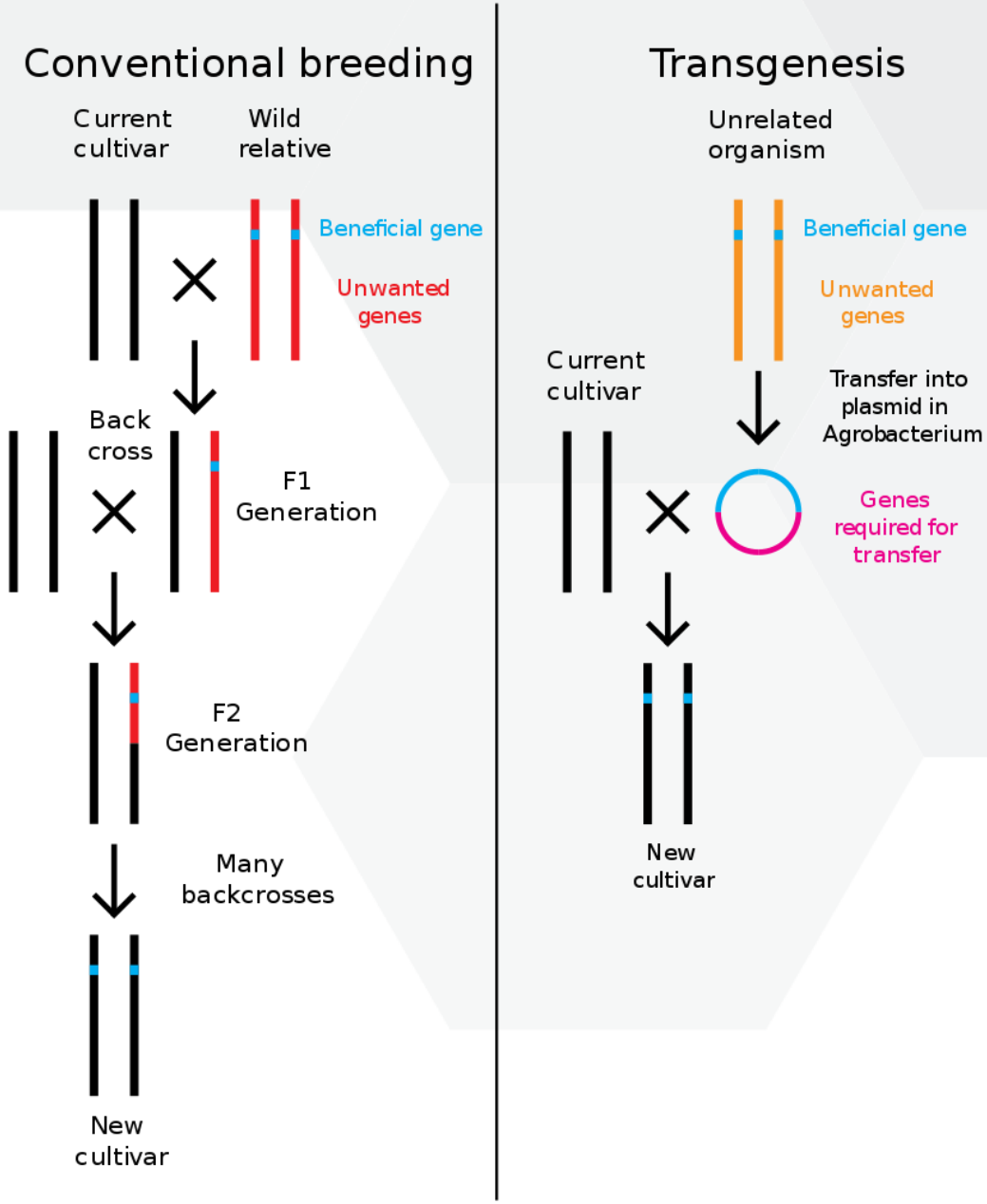
  (higher yield, growth rate, survival rate, meat quality, resistence)

Traditional breeding

- Multiple crosses and selection for desired phenotype

Gene engineering

- Direct transfer of desired gene – faster, suitable for any gene from any organism, no co-occurrence of undesired gene



Approaches in gene engineering

2) Random mutagenesis

- exposure to chemicals or radiation
- Atomic gardening since 1950s
- Development of over 2000 new plant varieties (e.g. peppermint, grapefruit)

Approaches in gene engineering

3) Molecular cloning

- Isolate and copy genetic material using recombinant DNA methods (or artificially synthesize DNA)
- Clone gene into a suitable vector
- Insert vector containing gene of interest into the target cells

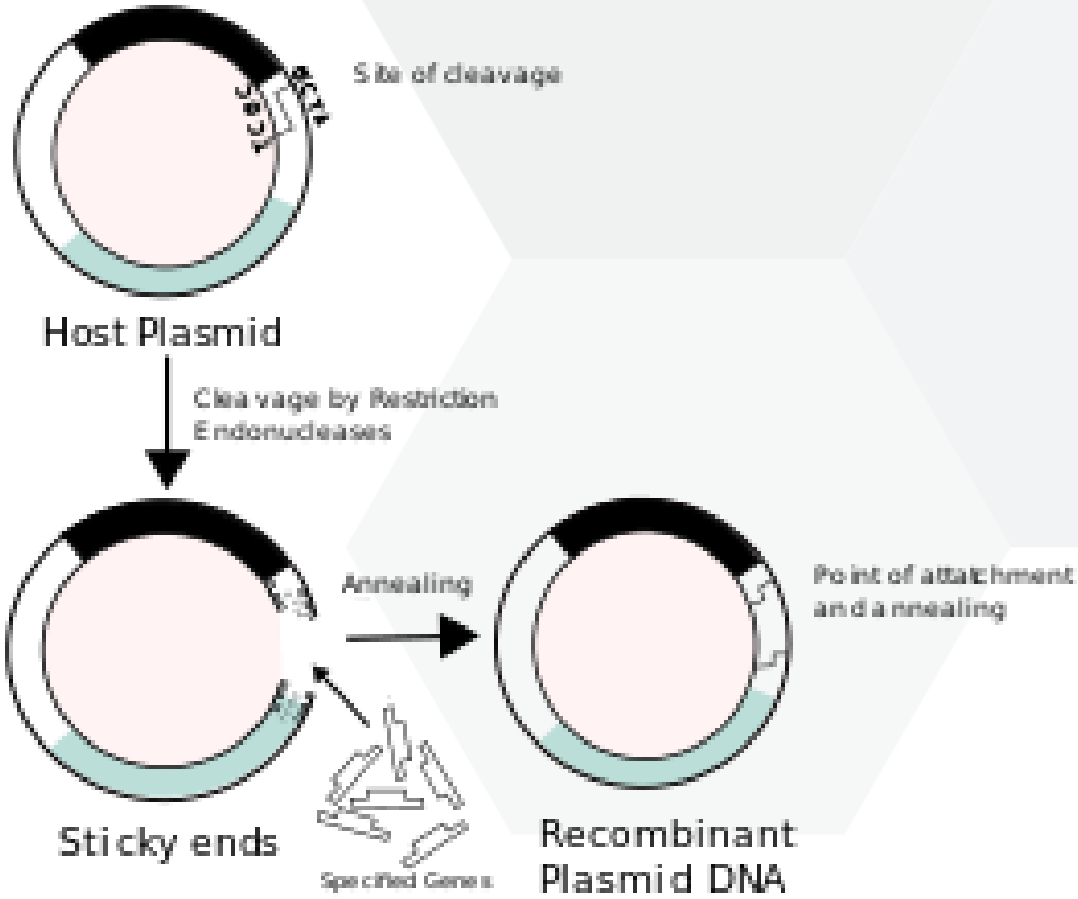
- Insertion random or targeted to a specific part

Steps of molecular cloning

1) Choice of host organism and cloning vector
2) Preparation of vector DNA
3) Preparation of DNA to be cloned
4) Creation of recombinant DNA
5) Introduction of recombinant DNA into host organism
6) Selection of organisms containing recombinant DNA
7) Screening for clones with desired DNA inserts and biological properties

Creating a recombinant DNA carrying gene of interest

- Recombinant DNA cloning: PCR, restriction digest, ligation
- Use of **restriction enzymes**
- Include promoter, terminator and selection marker into the construct



Restriction enzymes

- DNA endonucleases
- Found in bacteria and archaea, defense against invading viruses
- More than 3600 restriction enzymes known, 250 different specificities, 800 commercially available
- Cutting DNA at/near precise recognition sites (restriction sites):

  = defined 4-8 bp sequences specific for each enzyme, often palindromic

EcoRI: (sticky ends)

SmaI: (blunt ends)

Means of delivering transgenes

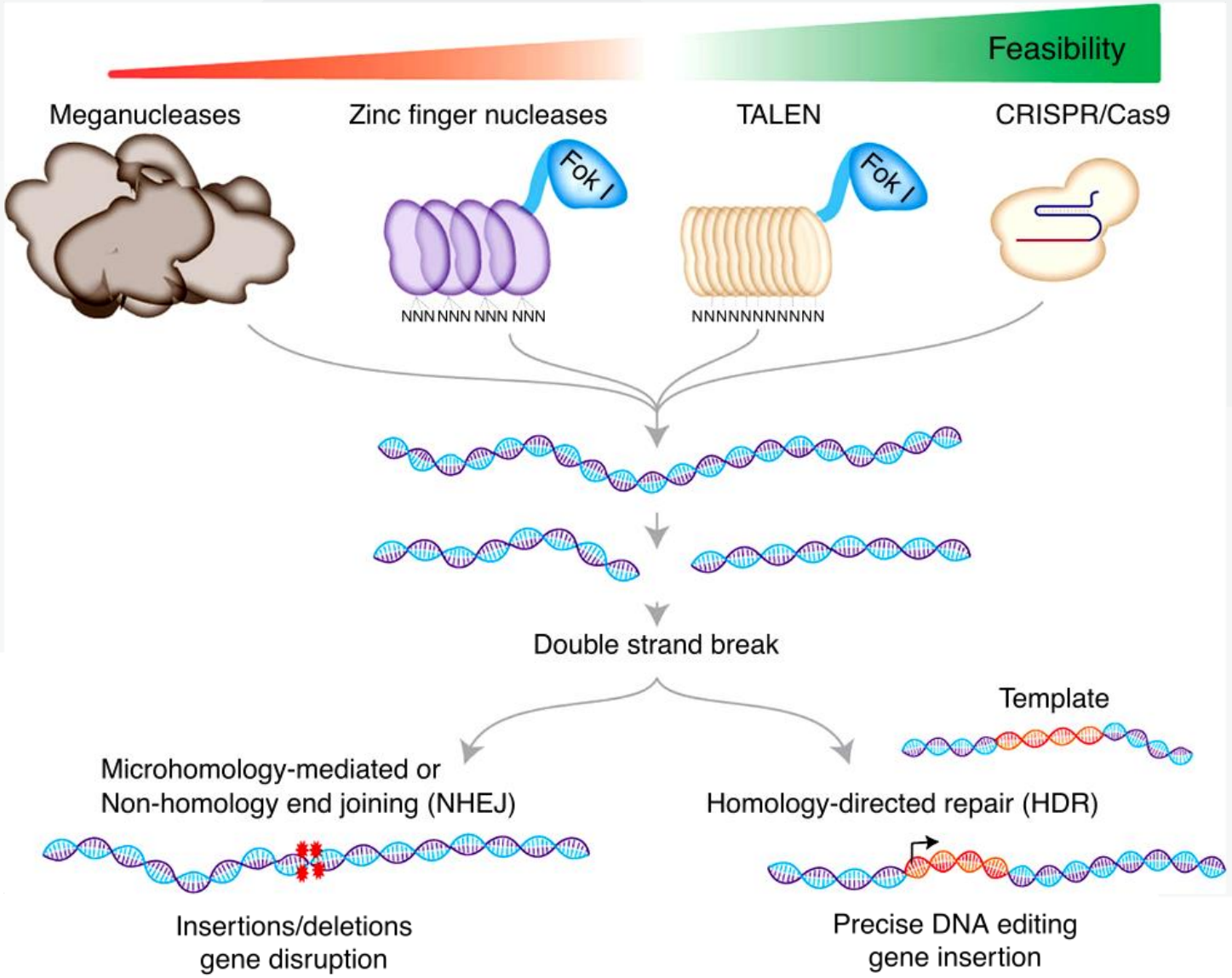
- Transformation (bacteria) – electroporation, chemical transformation with heat shock
- Agrobacterium-mediated recombination (plants)
- Transfection (animal cells) – electroporation, calcium phosphate, lipofection
- Microinjection (embryos, eggs)
- Microencapsulation
- Viral infection – retroviruses, lentiviruses, adenoviruses

Approaches in gene engineering

4) Genome editing technologies – 2011 Method of the year

Targeted modifications, using artificially engineered nucleases inducing double-strand breaks at desired locations

- Meganucleases
- ZFN
- TALENs
- CRISPR/Cas9 – 2015 Breakthrough of the year


Feasibility
Meganucleases
Zinc finger nucleases
TALEN
CRISPR/Cas9
Fok I
Fok I
NNN NNN NNN NNN
NNNNNNNNNNN
Double strand break
Template
Microhomology-mediated or
Non-homology end joining (NHEJ)
Homology-directed repair (HDR)
Insertions/deletions
gene disruption
Precise DNA editing
gene insertion

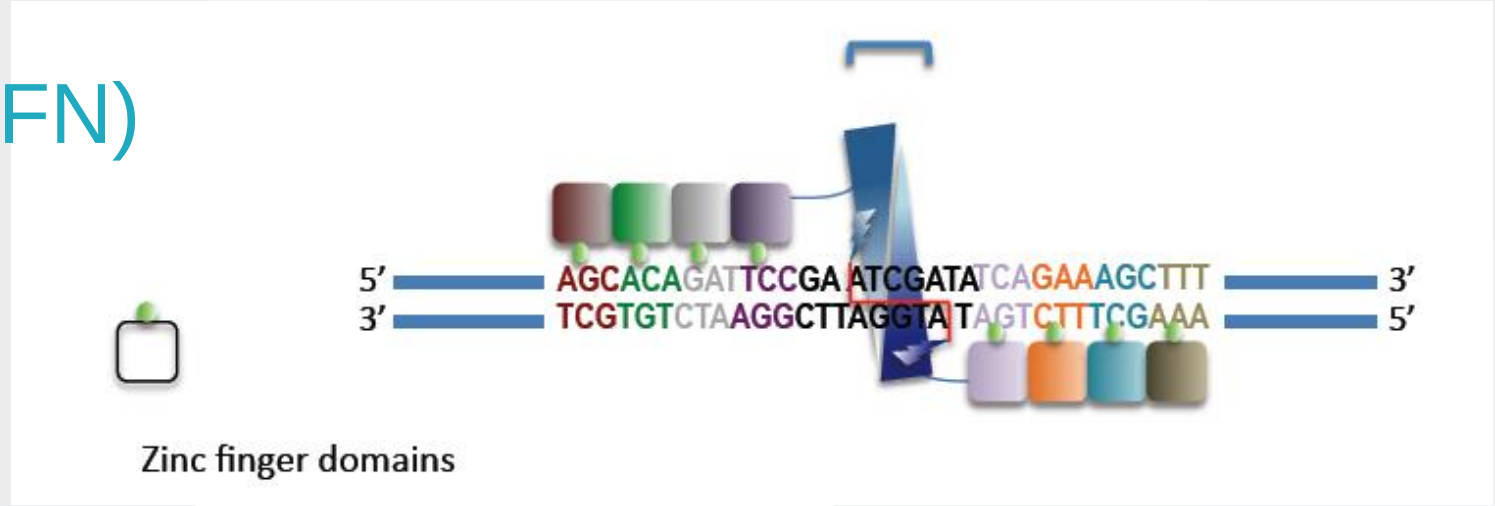
Meganucleases

- First used in 1988
- Endodeoxyribonucleases similar to restriction enzymes
- Long recognition sites (14-40 bp) -> more specific, less toxic (less targets within genome)
- Most studied are LAGLIDADG family
- Difficult to target an exact sequence -> mutagenesis/screening to create variants
- Large bank created containing tens of thousands protein units for combinations

Meganucleases

- Construction of sequence specific enyzmes for all possible sequences is costly and time-consuming
- Off-target binding still too high, but small size is of advantage
- Industrial-scale production of 2 meganucleases to cleave human XPC gene (mutations cause Xeroderma pigmentosum predisposing to skin cancer and burns after UV exposure)

Zinc-finger nucleases (ZFN)



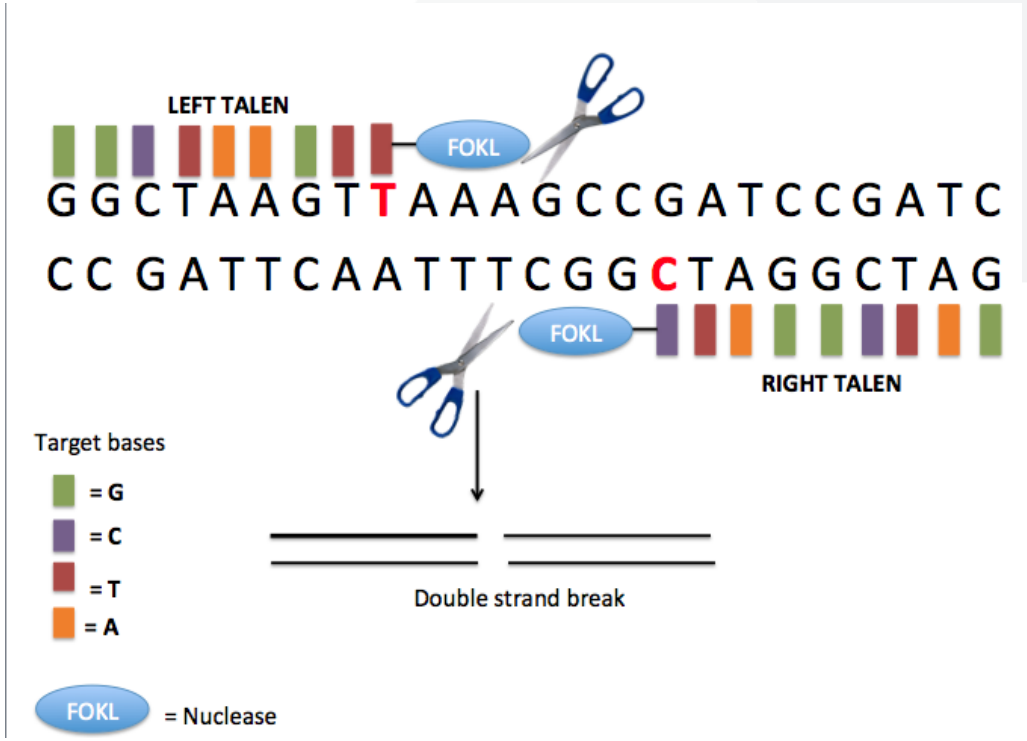
- First use in 1996
- Cleave DNA at target sites
- Based on non-specific DNA cutting catalytic domain linked to specific sequence-recognizing peptides
- Fusion of FokI nuclease domain (separated from its recognition domain) with the Zinc-finger domains , usually 3-6 Zinc-fingers (each having 3nt recognition site)
- Works as a heterodimer – recognize two DNA sequences few nt apart -> dramatic increase in specificity

Zinc-finger nucleases (ZFN)

- Greater specificity than meganucleases, but still relatively high potential for binding to non-specific sequences
- Used for genetic engineering of stem cells
- Modification of immune cells for therapy
- Genetically modified T cells in clinical trials for therapy of brain tumor and HIV

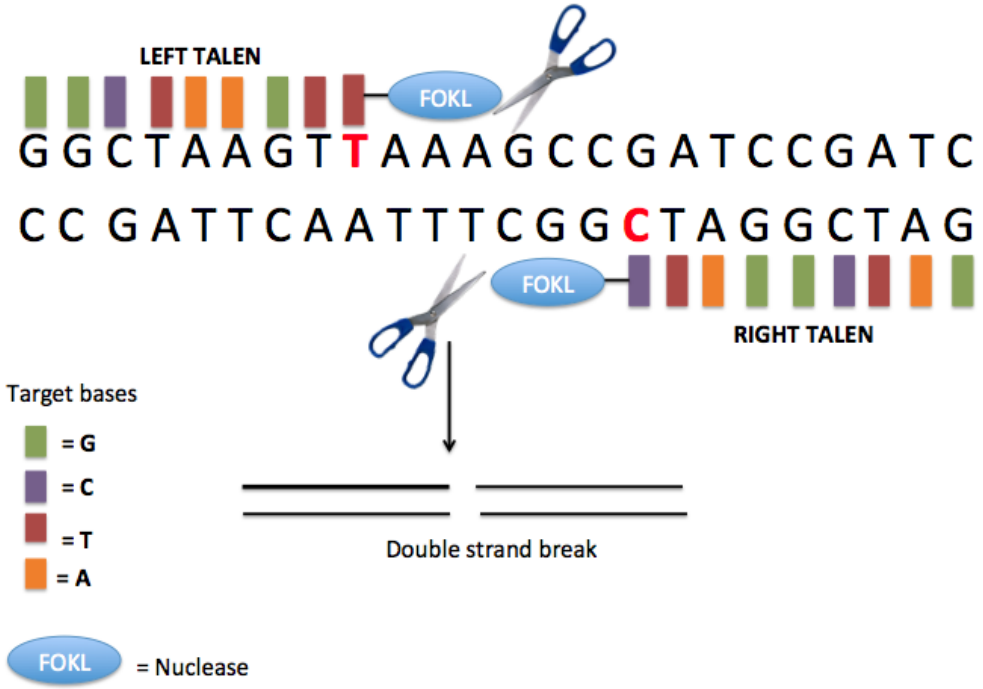
TALENs (Transcription activator-like effector nucleases)

- Artificial restriction enzymes
- Fusion of FokI nuclease domain with TALE sequence-recognizing domains
- TALE originate from bacteria *Xanthomonas*



TALENs (Transcription activator-like effector nucleases)

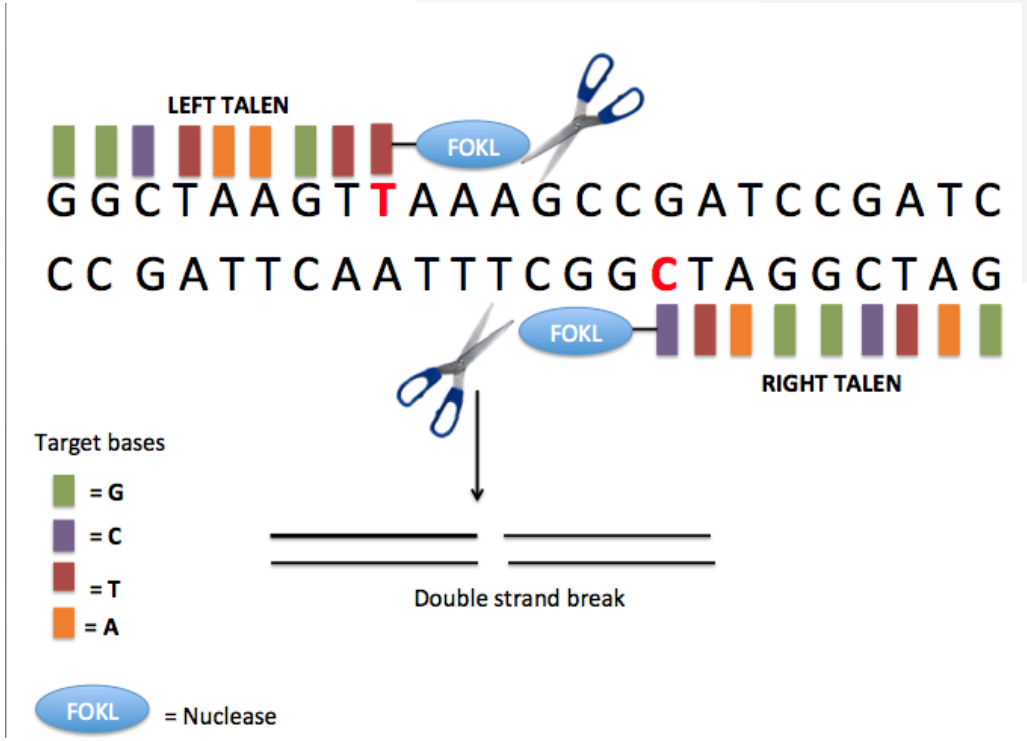
- TAL effectors consist of repeated domains, each containing highly conserved sequence of 34 aminoacids
- Each domain recognize a single nucleotide within target site -> highly modular
- Two TALENs designed within 6-bp range of any single nucleotide



TALENs (Transcription activator-like effector nucleases)

Compared to ZFNs:

- Higher DNA binding specificity
- Lower off-target effects
- Easier construction of DNA-binding domains

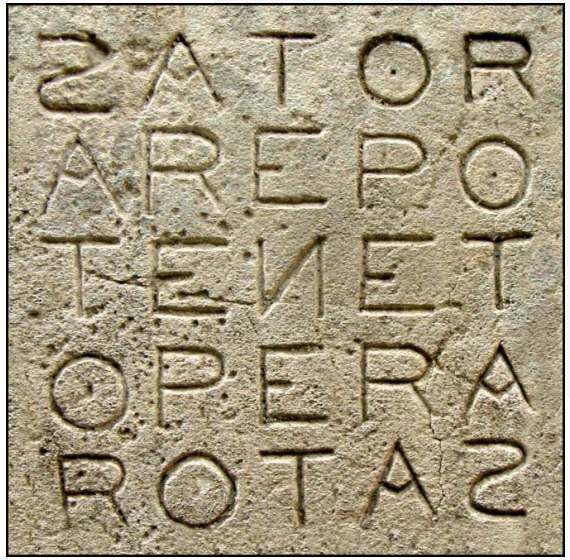


CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) technology

Palindromes

- from greek *palin* (*πάλιν*; „again") and *dromos* (*δρόμος;* „direction")
- Sequence of letters, numbers, words or phrases that can be read the same backward as forward

- **Examples:**
  madam
  racecar
  Jelenovi pivo nelej.
  Jamesi, ti co míří moci, ti se maj.
  Maloval hlavolam.
  123454321

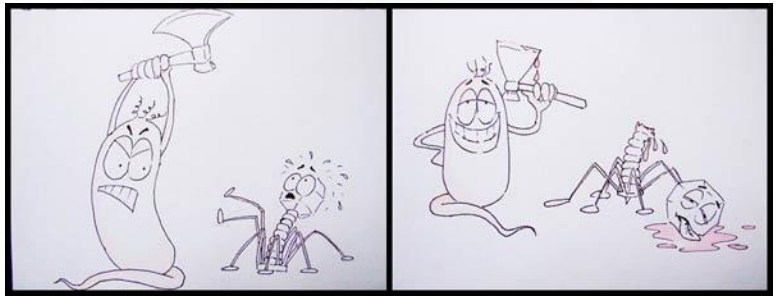


Pompeie, 79AD

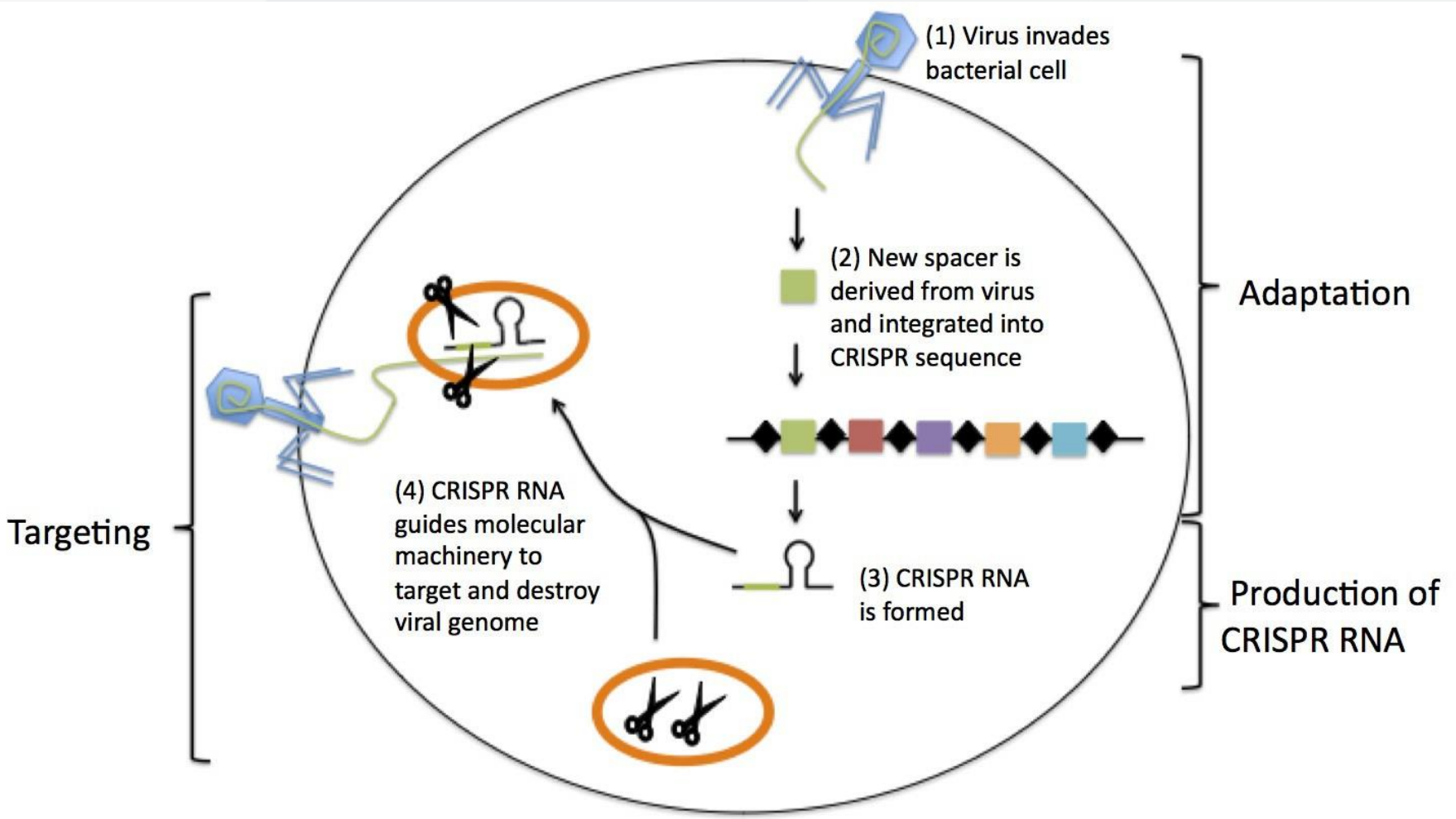
CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) technology

- Adaptive immune system of prokaryotes
- CRISPR array consists of parts of prokaryotic DNA containing short nucleotide repeats (28-37 bp)
- Each repeat is separated by spacer (32-38 bp) – generated from previous encounters with viruses
- CRISPR array contains up to 50 units of repeat-spacer sequence
- Ensures resistence against invading genetic elements

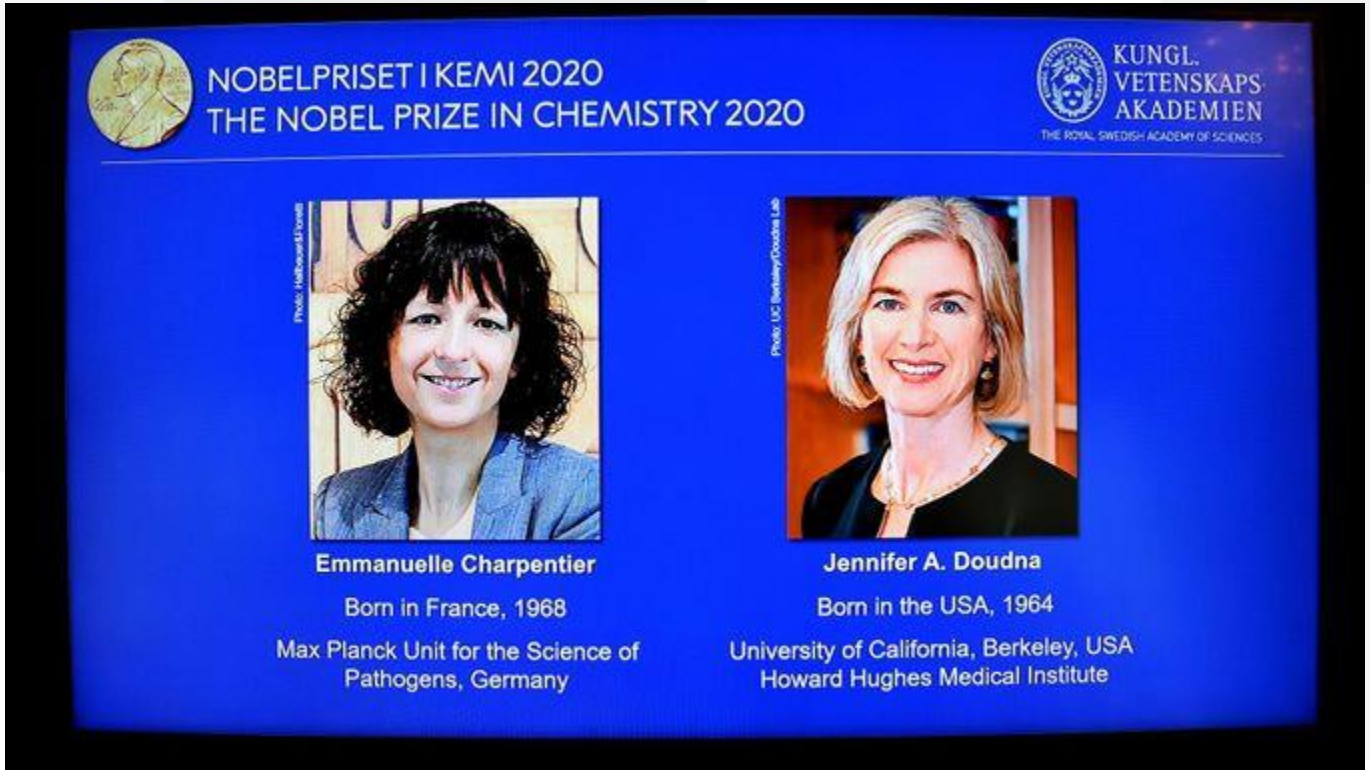
CRISPR array:



CRISPR/Cas system in prokaryotes



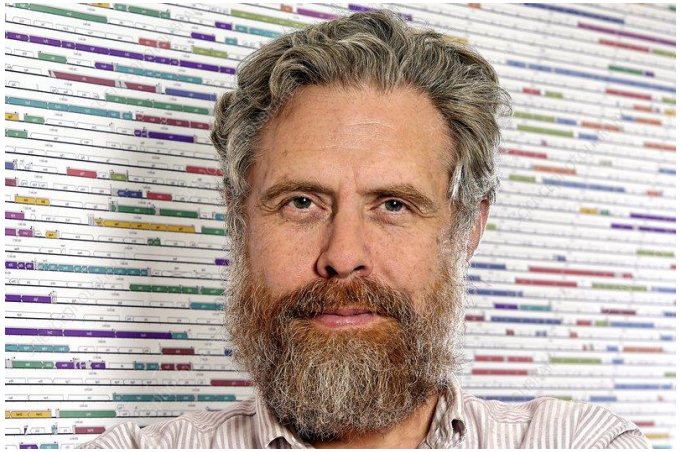
Nobel Prize in Chemistry 2020



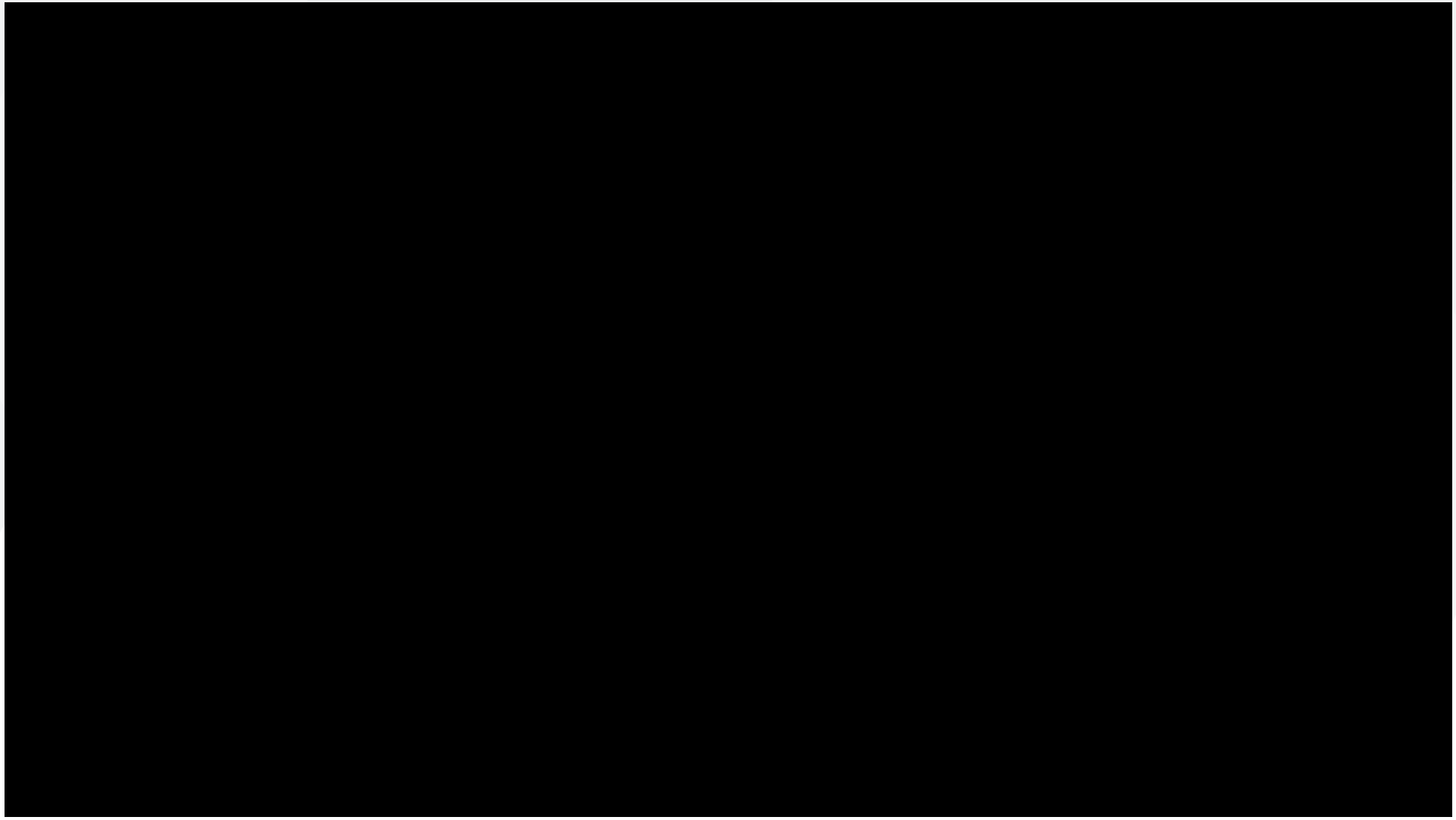
The use of CRISPR/Cas for editing in human cells

Feng Zhang
Broad Institute of MIT and Harvard,
McGovern Institute at MIT

George Church
Harvard University and MIT



CRISPR/Cas system

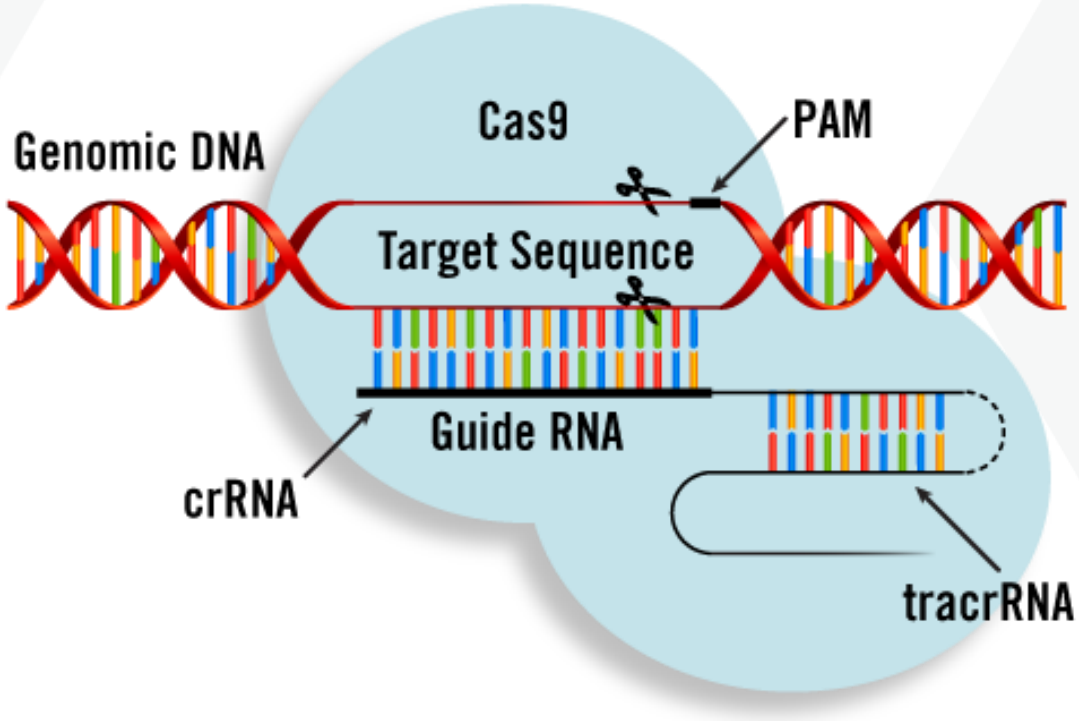


CRISPR/Cas system composition

- CRISPR RNA (crRNA) – specific to target sequence
- Transactivating CRISPR RNA (tracrRNA) – binding Cas protein

} Single-guide RNA (sgRNA)

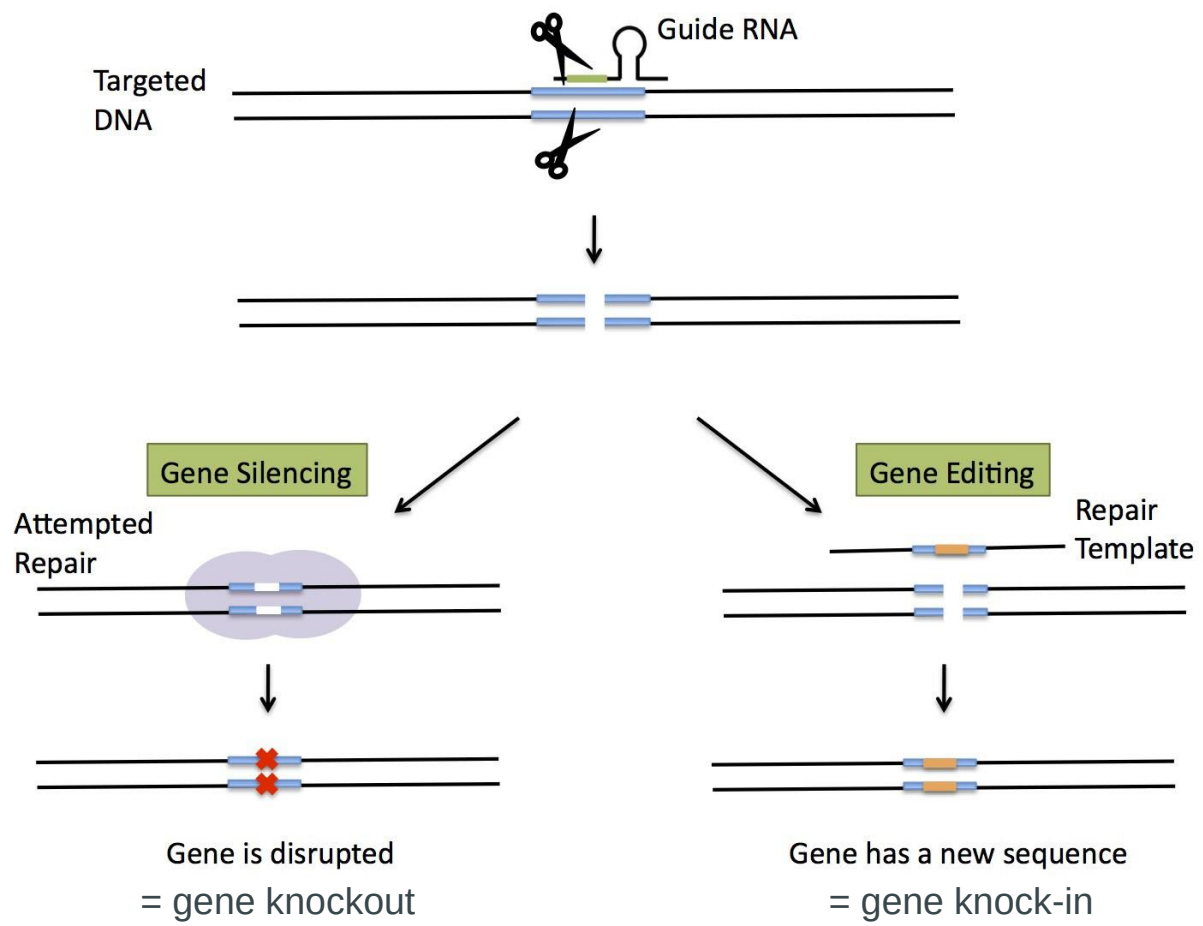
- PAM motif – 3 nt motif (NGG) at the end of target sequence
- Cas endonuclease



CRISPR/Cas variants

- 3 types of CRISPR system, only type II used for gene editing – the only system using single large Cas protein
- 93 cas genes, grouped into 35 families
- Mainly Cas9 endonuclease exploited
- Cas12a – smaller, simpler, requires only crRNA (no tracrRNA), T-rich PAM motifs (alternative targeting sites to Cas9), causing staggered cut (not blunt-end)
- Cas13 – RNA-guided RNA endonuclease, cutting RNA instead of DNA (no changes to genome)
- CasMINI – twice smaller than Cas9

The classical use of CRISPR / Cas9 system

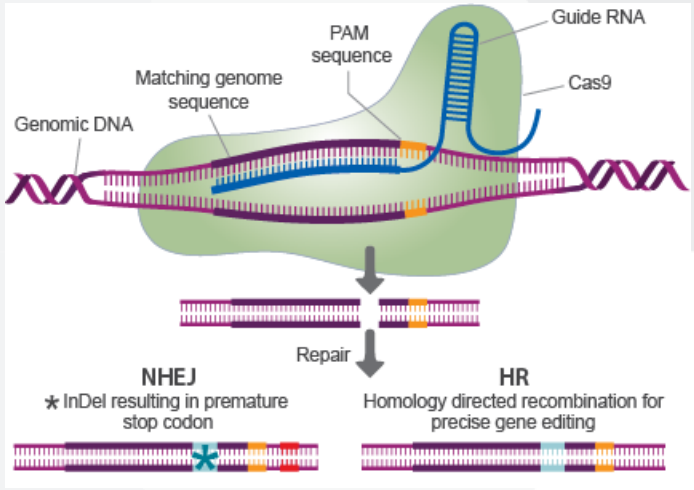


Cas9 = enzyme inducing dsDNA breaks

CRISPR guide RNA = guiding Cas9 to the specific site within the gene of interest

Mali et al., Science 2013
Cong et al., Science 2013

Generation of knockout cells via Non-homologous end joining



dsDNA break

CGACCACAGTCTGATCTGATCTGATT
GCTGGTGTCAGACTAGACTAGACTAA

Attempted repair (insertion, deletion)

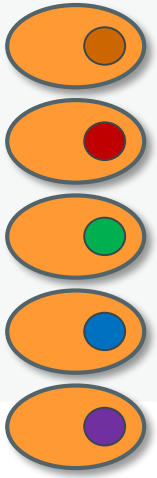
CGACC GCTGG	NACAGTCTGATCTGATT NTGTCAGACTAGACTAA	+1N
CGACC GCTGG	NNACAGTCTGATCTGATT NNTGTCAGACTAGACTAA	+2N
CGACC GCTGG	NNNACAGTCTGATCTGATT NNNTGTCAGACTAGACTAA	+3N

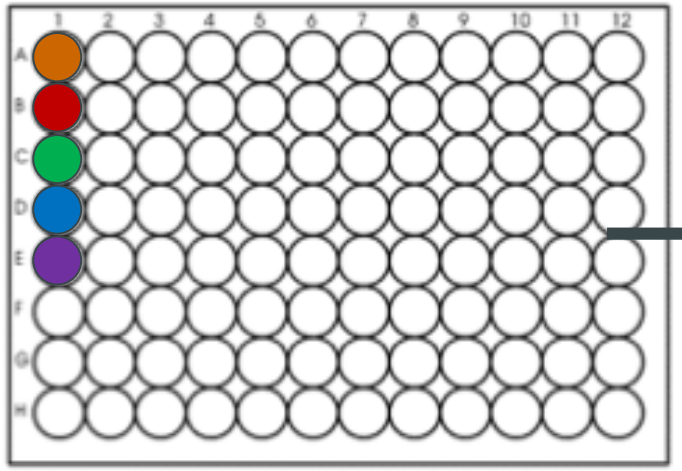
* Stop codon

- target early consecutive exons to generate full knockout

Isolation of knockout cell line in practice

Isolation of knockout cell clones:



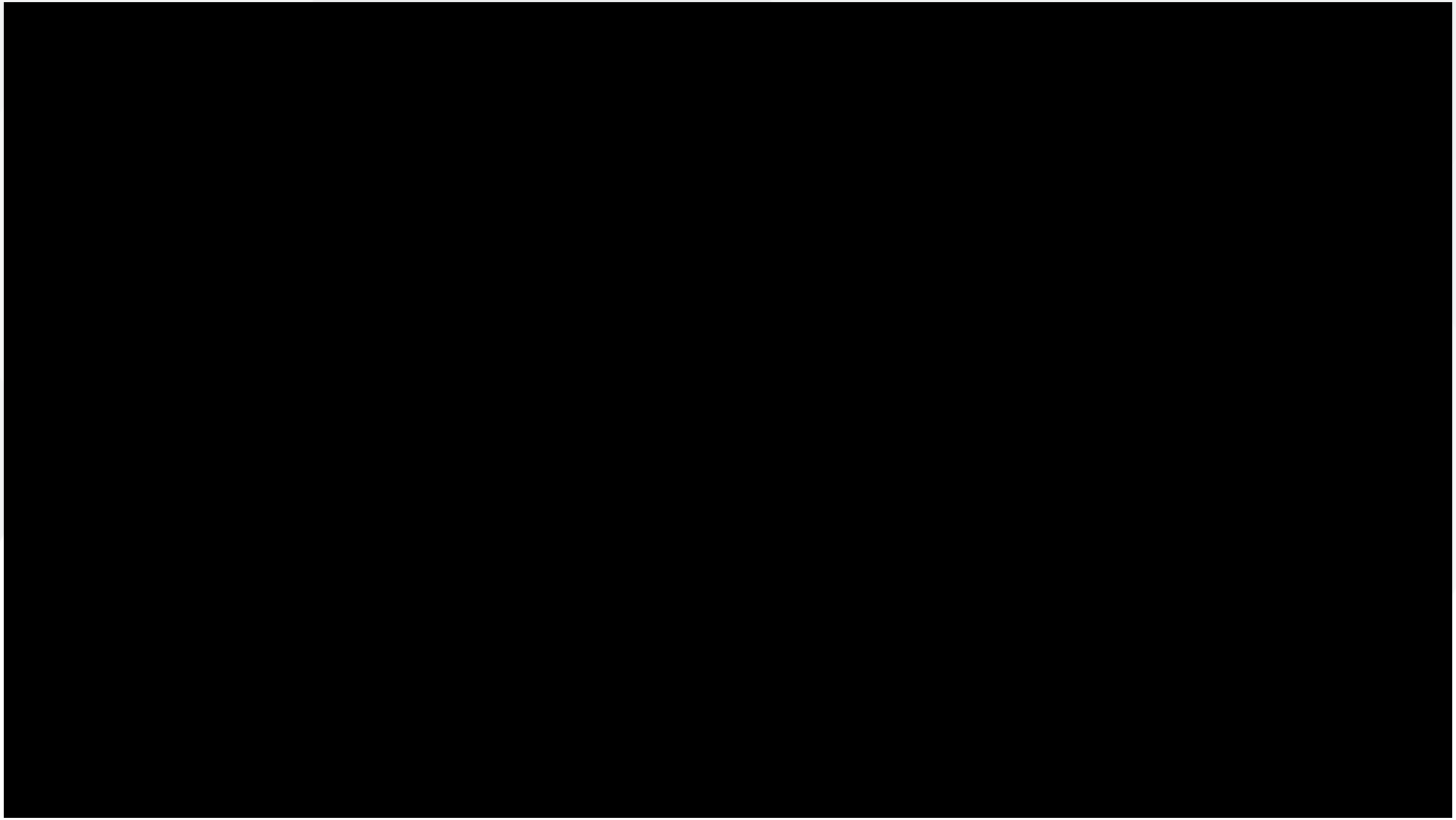


Sequence individual clones

Pick clones with editing in both alleles

Expand the clones

Other applications of CRISPR/Cas9 technology



Other applications of CRISPR/Cas9 technology

Use of catalytically dead Cas9 (dCas9) – 2 point mutations, no cutting

- CRISPR activation:

  dCas9 fused to transcriptional activators (VP64, VPR, SAM, SunTag) that recruit other co-factors and RNA polymerase

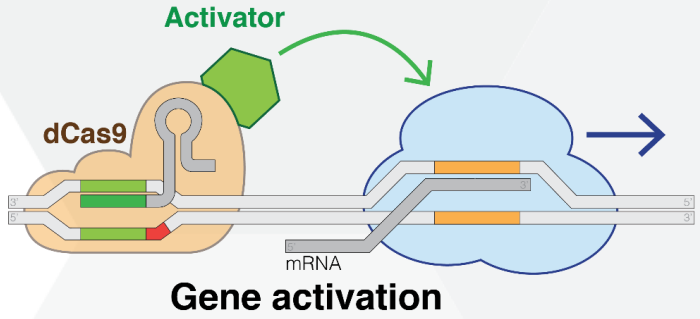
- CRISPR interference:

  sgRNA complementary to promotor

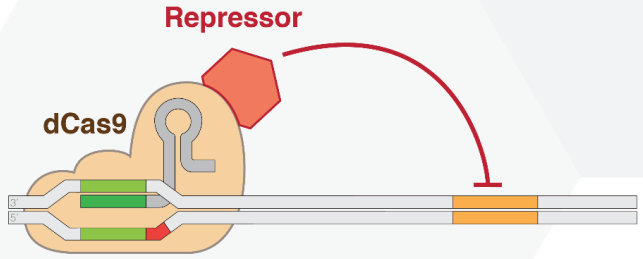
  dCas9 fused to repressory KRAB domain

- Chromatin visualisation to study chromatin architecture:

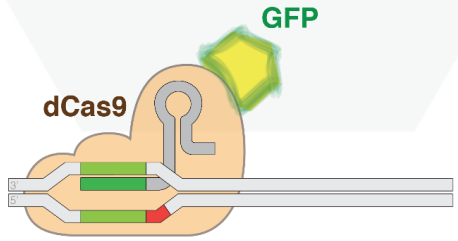
  dCas9 fused to GFP



Gene activation



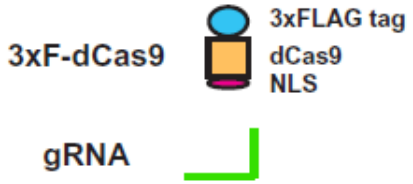
Gene repression

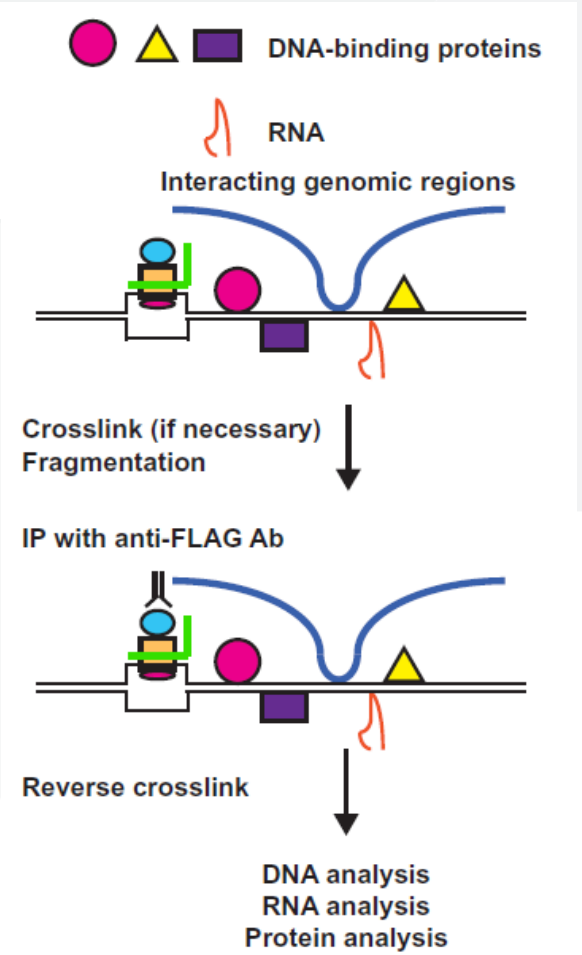


Visualisation

Other applications of CRISPR/Cas9 technology

- Chromatin proteomics – engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR
  - to identify specific molecules bound to desired locus
  - dCas9 fused to specific tag





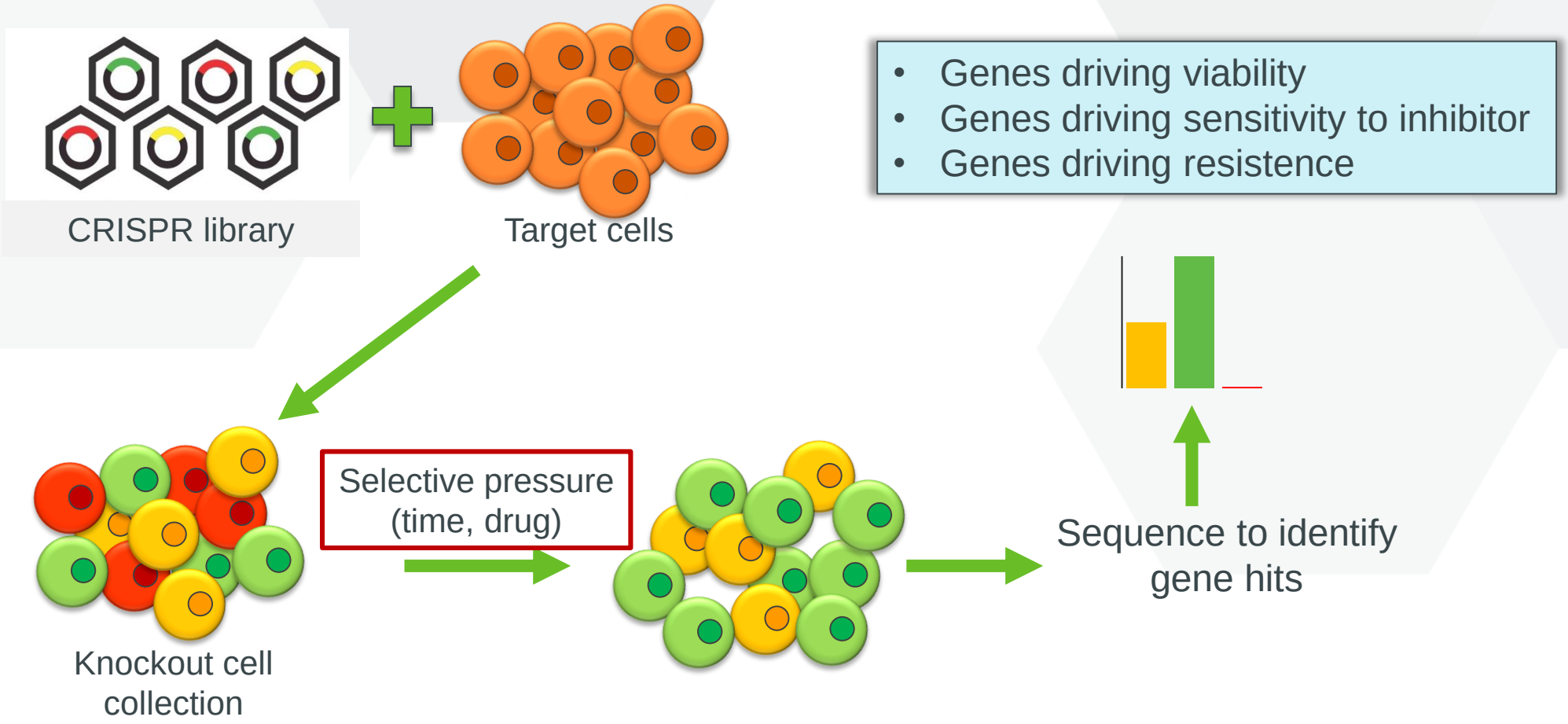
- Detection of gene transcriptional activity
  – dCas9 fused with luciferase reporter

- Epigenetic modifications – fusion with chromatin remodelling enzymes (methyltransferases, demethylases, etc.)

- Genome-wide screening

Identification of essential vulnerability nodes through whole-genome knockout screen

Human CRISPR knockout library (Brunello) targeting 19,114 human genes



Risks of CRISPR-Cas9 technology

Off-target effects

- knock-out of undesired genes
- mutagenesis
- Cancerogenesis

Pros and Cons of gene editing technologies

- **Meganucleases** – least efficient, only approx. 1 target every 1000 nts
- **ZFNs** – 1 target in every 140 nts, more cytotoxicity than TALENs and CRISPR
- **TALENs** – the most precise, higher efficiency than MNs and ZFNs, specific expertise in molecular biology and protein engineering required
- **CRISPR** – quickest and cheapest, requires the least expertise (RNA instead of proteins)

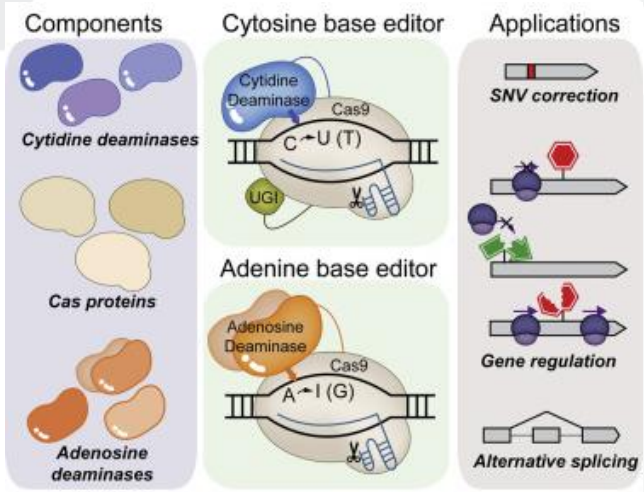
MNs and ZFNs: their DNA-binding elements affect each other -> unpredictable outcome -> high expertise required, lengthy and costly validations

ZFNs and TALENs: construction and testing of proteins created for each sequence

TALENs and CRISPR – greatest efficiency, fewer off-targets

New alternatives

- MegaTAL – combination of TALENs DNA binding domain and meganucleases
- Base editors – no double-strand break



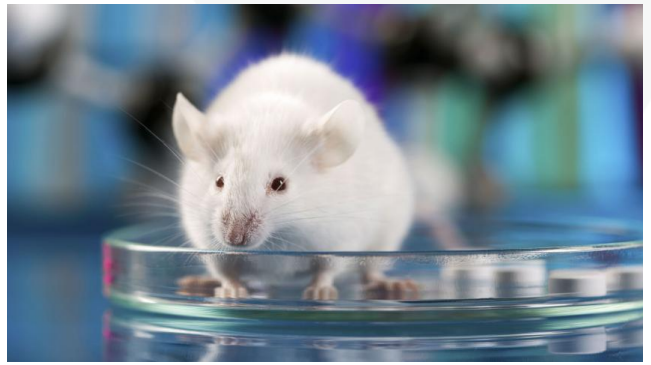
- OMEGA (obligate mobile element-guided activity) endonuclease proteins found in transposons as CRISPR alternatives

Application of genetic engineering

- Research – study gene function and expression, generate animal models of human diseases
- Agriculture – GM crops, tomatoes, GM animals
- Medicine – produce hormones and vaccines, gene therapy
- Industrial biotechnology – produce enzymes for laundry detergents, cheeses and other products, Yeast producing plastic (no dependence on petrol)

Gene engineering in research

- Targeted gene knockouts *in vitro* and *in vivo*
- Replacement of mutated gene with the healthy one or with newly designed variant
- Introducing gene mutation to study its function
- Search for synthetically lethal genes
- Generation of model organisms



Gene engineering in agriculture

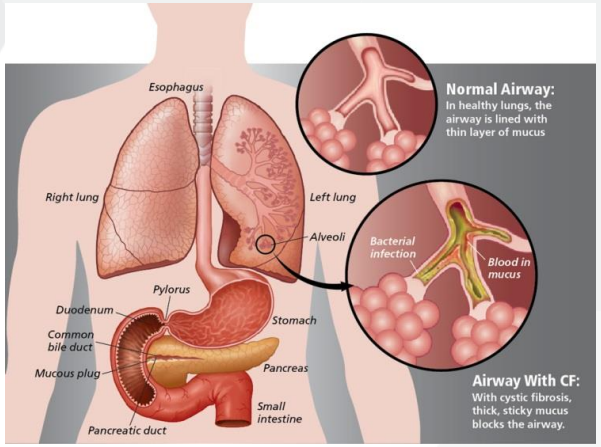
- GM crops, tomatoes, potatoes, bananas, soybean, GM animals
- More muscles in kettle, pigs, etc.
- Resistant crops, tomatoes
- Gene editing in animals (cows, pigs, salmon) – improve growth, disease resistence, controlled reproduction, color
- Ensuring food security by increasing yield, nutritional value, tolerance to environmental stresses

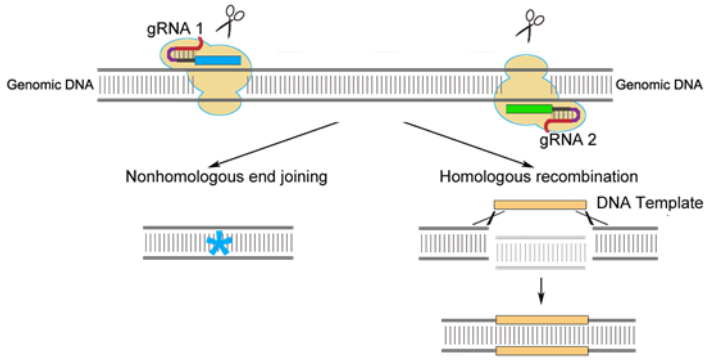
Gene engineering in medicine

- Fix severe genetic disorders by replacing defective gene
- Tested for correcting aberrations causing Down syndrome, spina bifida, anencephaly, Turner, Klinefelter and Hunter syndrome
- Production of drugs, vaccines and other products from engineered organisms (Hepatitis B vaccine, insulin, recombinant factor VIII, tissue plasminogen activator)
- Spare organs from transgenic animals (pigs)

The end of hereditary diseases

- Cystic fibrosis
- Hemophilia A, B
- Spinal muscular dystrophy
- Huntington´s disease
- Neurodegenerative diseases
- Genetic predispositions to cancer





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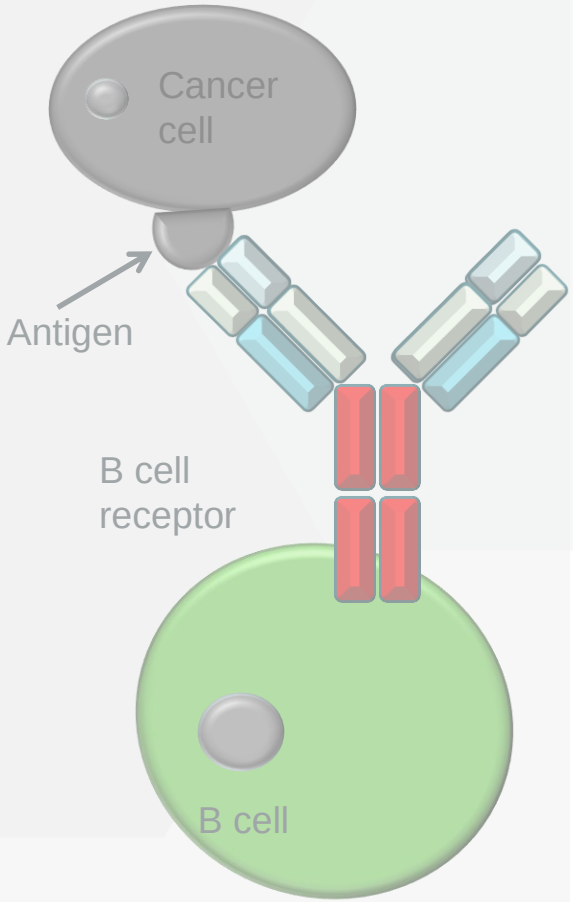
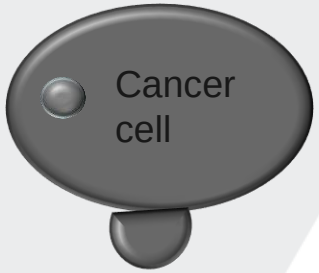
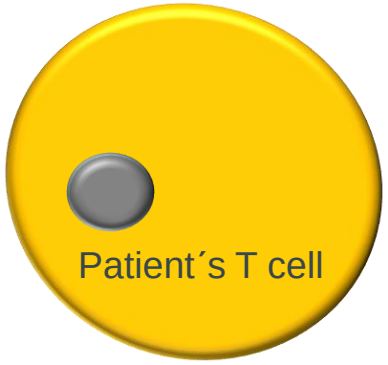
Gene engineering in medicine

- Modify genes associated with sterility in Anopheles mosquito transmitting malaria, eradicate other vector borne diseases like yellow fever, dengue, Zika
- Eliminate pathogen bacteria
- Target human viruses like HIV, Herpes, Hepatitis B virus – either target virus or human receptor proteins
- Curing cancer
- Generation of „off-the-shelf“ CAR-T cell therapy to cure cancer

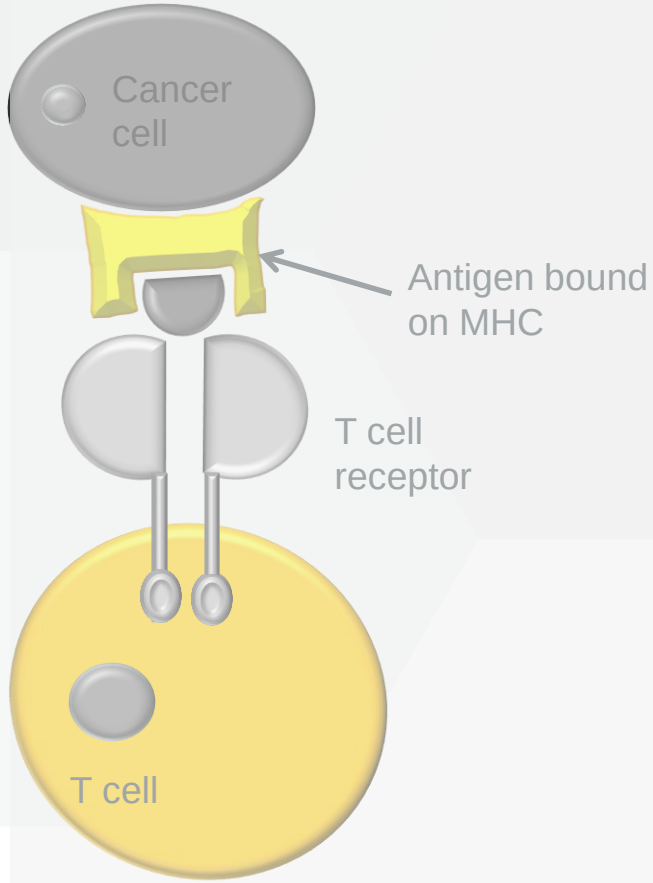
Chimeric Antigen Receptor (CAR): principle





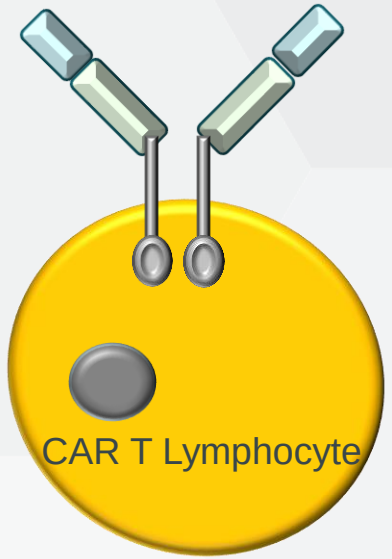


CAR T Lymphocyte



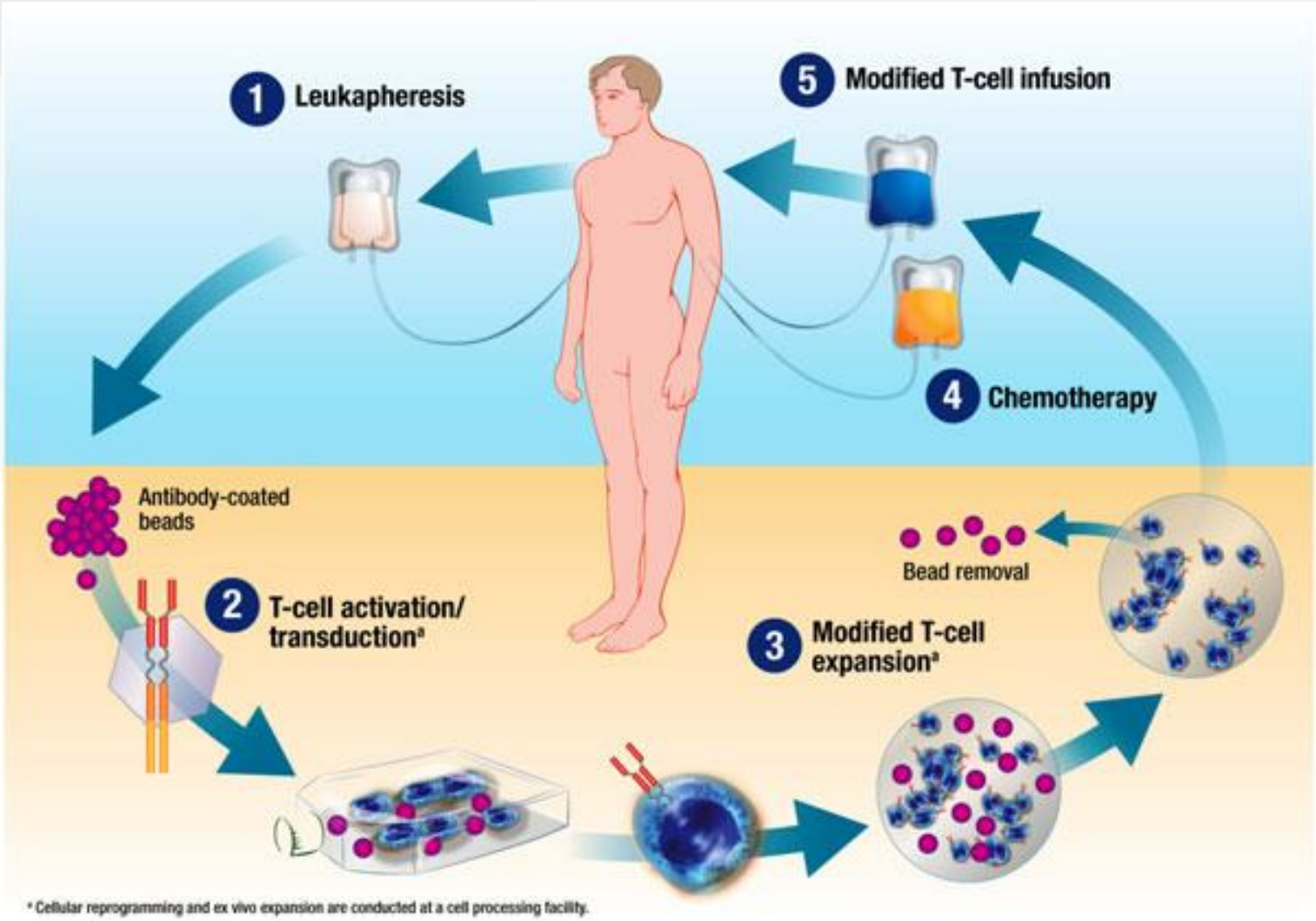
Chimeric Antigen Receptor T cells

- Reprogrammed to specifically target and kill tumor cells
- Potentially able to permanently cure cancer
- Approved for ALL, B-cell lymphoma and multiple myeloma
- Many different target antigens under study
- Being tested for most solid cancers



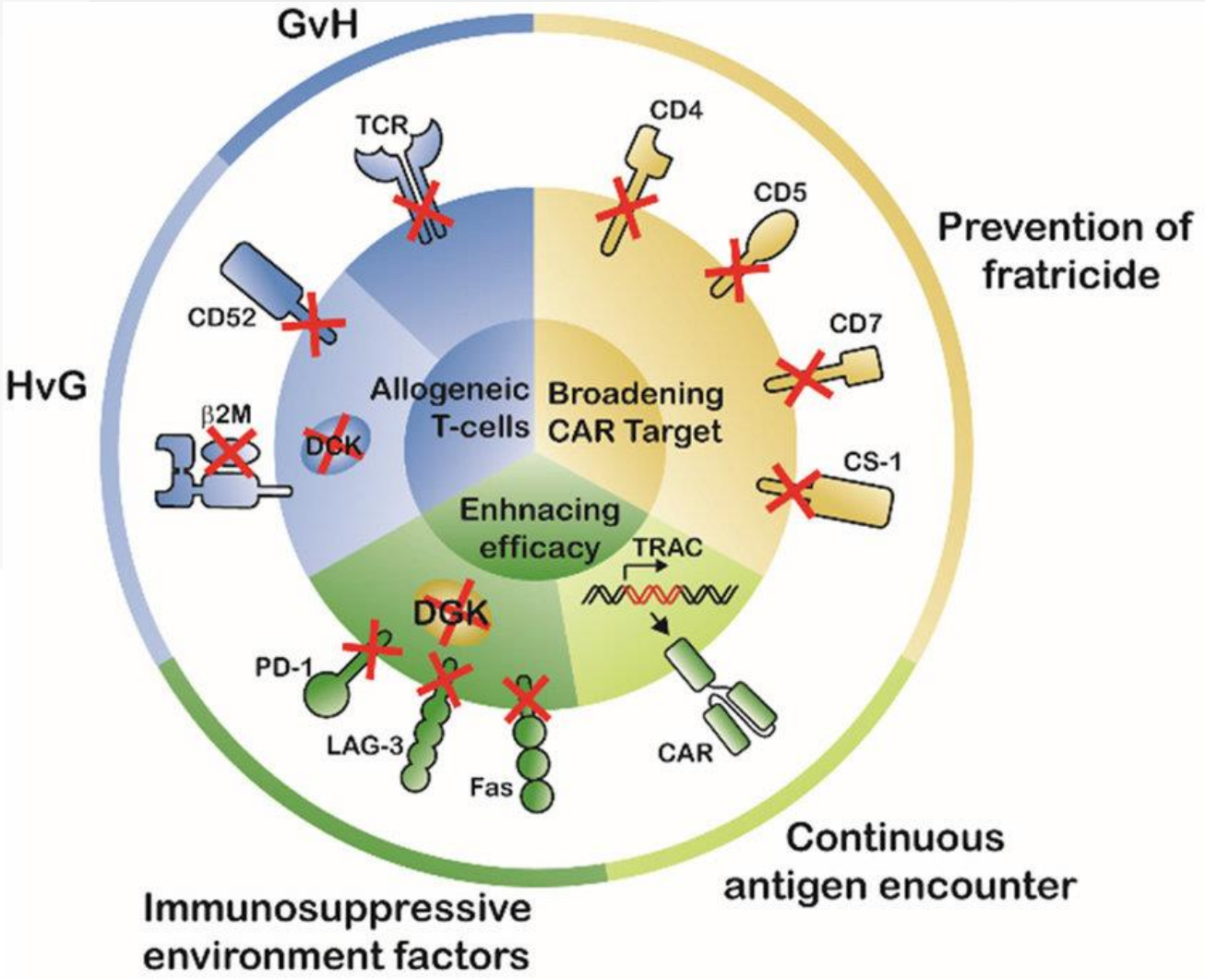
Generation of CAR-T cells

- 10 – 14 days (*ex vivo*)
- Autologous cells
- Very expensive
  (10 Mil. CZK)



Off-the-shelf and Enhanced CAR-T cells

Use of TALENs
or CRISPR/Cas9:



Current advances in gene editing

- 2020 – US trial using CRISPR editing on 3 cancer patients
- 2020 – Sicilian Rouge High GABA – tomato with higher production of GABA (promoting relaxation) approved in Japan
- 2021 – England plans to remove restrictions on gene-edited plants and animals
- April 2021 – EC finds „strong indications“ that current regulations are not appropriate for gene editing

What are the borders of gene editing?

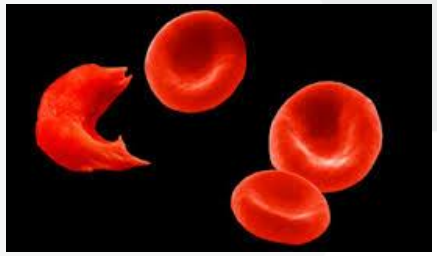
„With great power, comes great responsibility.“

Ethics of gene editing

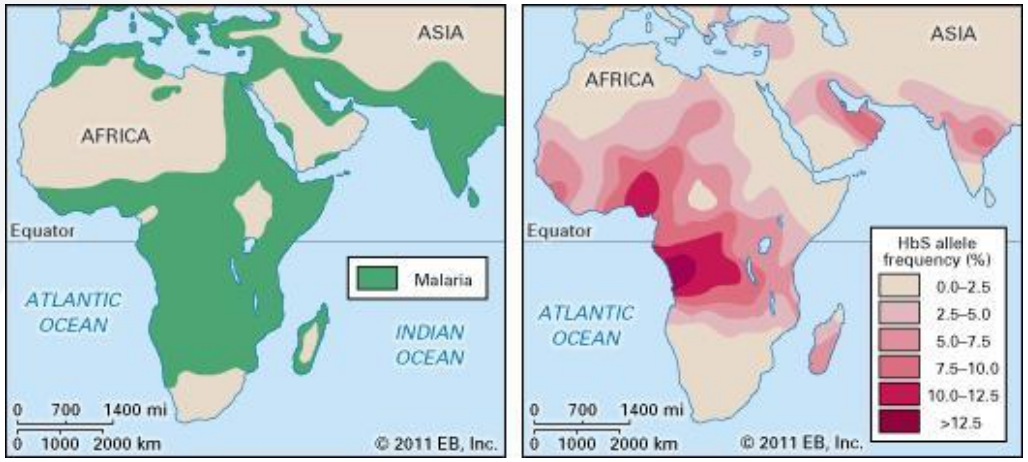
- Can we / shall we alter human genome postnatally/prenatally?
- What are the long-term consequences of these changes?
- How to legally manage these alterations?
- Who has the right to hold the patent on these modified genes?

Disease is not always bad – the importance of genetic variability

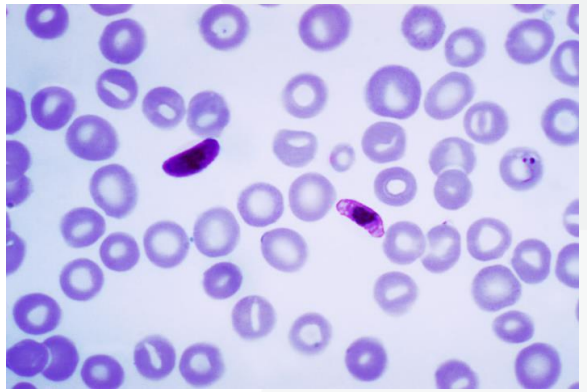
Sickle-cell disease



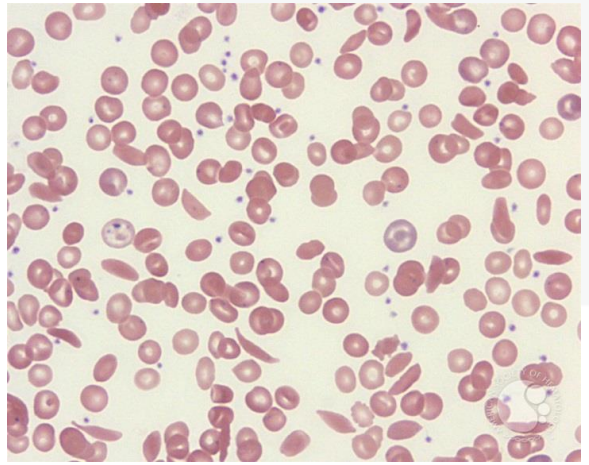
Correlation between sickle-cell disease and malaria



P. falciparum



Blood smear of sickle-cell disease



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Threats of genome editing

- US Director of National Intelligence James R.Clapper:

Genome editing as a potential **weapon of mass destruction**.

Countries with regulatory or ethical standards „different from Western countries“ might create harmful biological agents or products

Biological weapons – vaccine-resistant smallpox viruses, killer mosquitoes, etc.

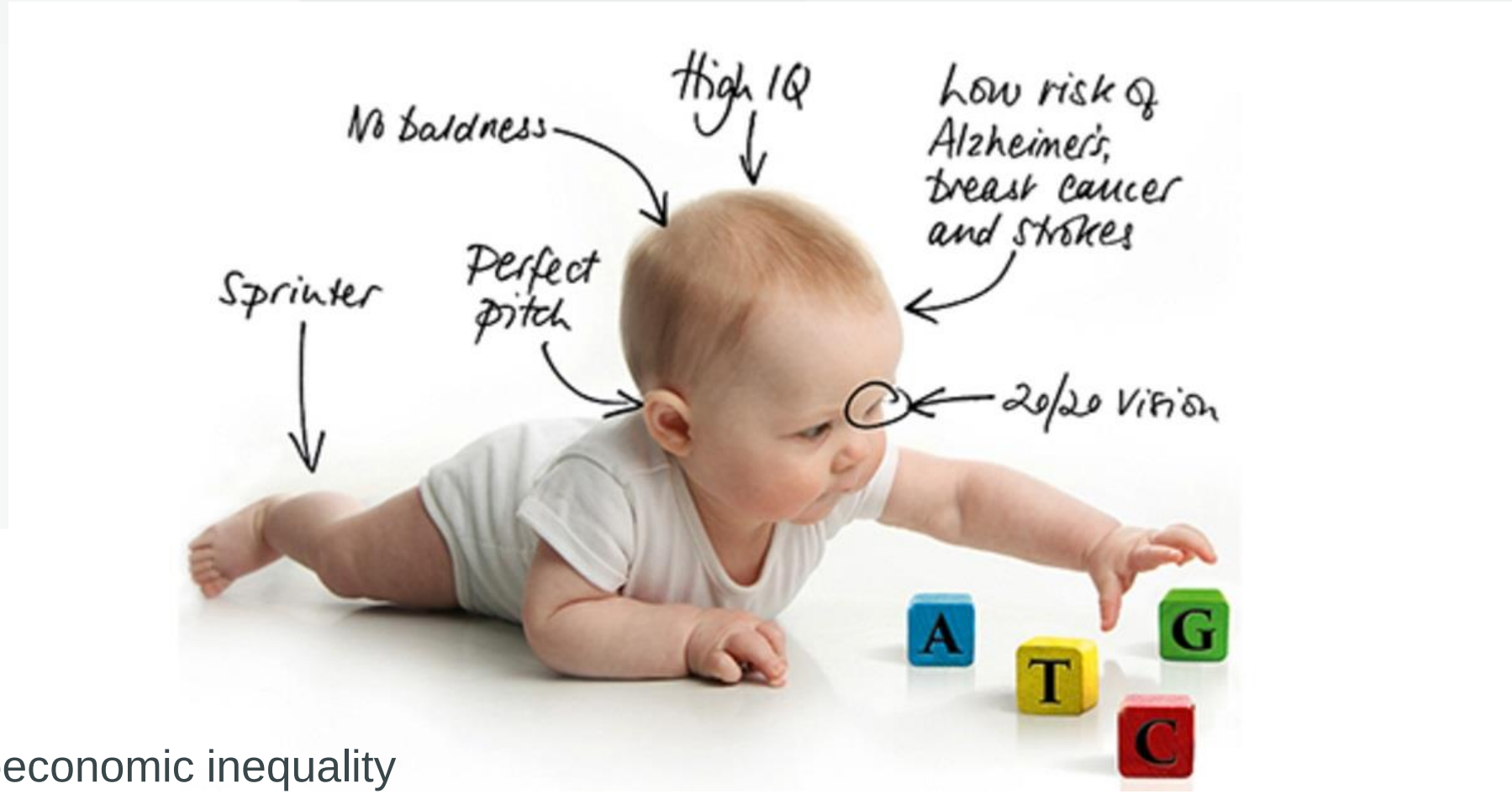
- Ecological risks of gene drive

Where do we stop?

Super-humans

Genetically enhanced super-soldiers

Designer babies



- Socioeconomic inequality
- Commodification of human life

Lulu and Nana

- 28. 11. 2018

  Second International Summit on Human Genome Editing (Hong Kong):

Chinese scientist He Jiankui claimed to have edited the genomes of twin girls to make them less susceptible to HIV

- disabled CCR5 gene – receptor for HIV

  BUT: potentially increased susceptibility to West Nile virus or influenza

  CCR5 may play a role in intelligence!

- Too premature, too risky (high potential for introducing mutation with harmful effects), little benefit

Consequences

- Fired by his University
- 3 years in prison for „illegal medical practice“
- 3 million yuan fine (US$ 430,000)
- Banned from working with human reproductive technology ever
- Banned from applying for a research grant

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