New Plant Breeding Technologies

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Legal Briefing Paper

The regulatory status of plants resulting from New Breeding Technologies

Produced by the NBT Platform[1]
09 April 2014

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What are the NPBTs?

***RNAi**

***Epigenetic alterations (eg gene silencing)**

Mutagenesis

- random
- **targeted (incl. genome editing):**

  *oligo-directed mutagenesis (ODM)

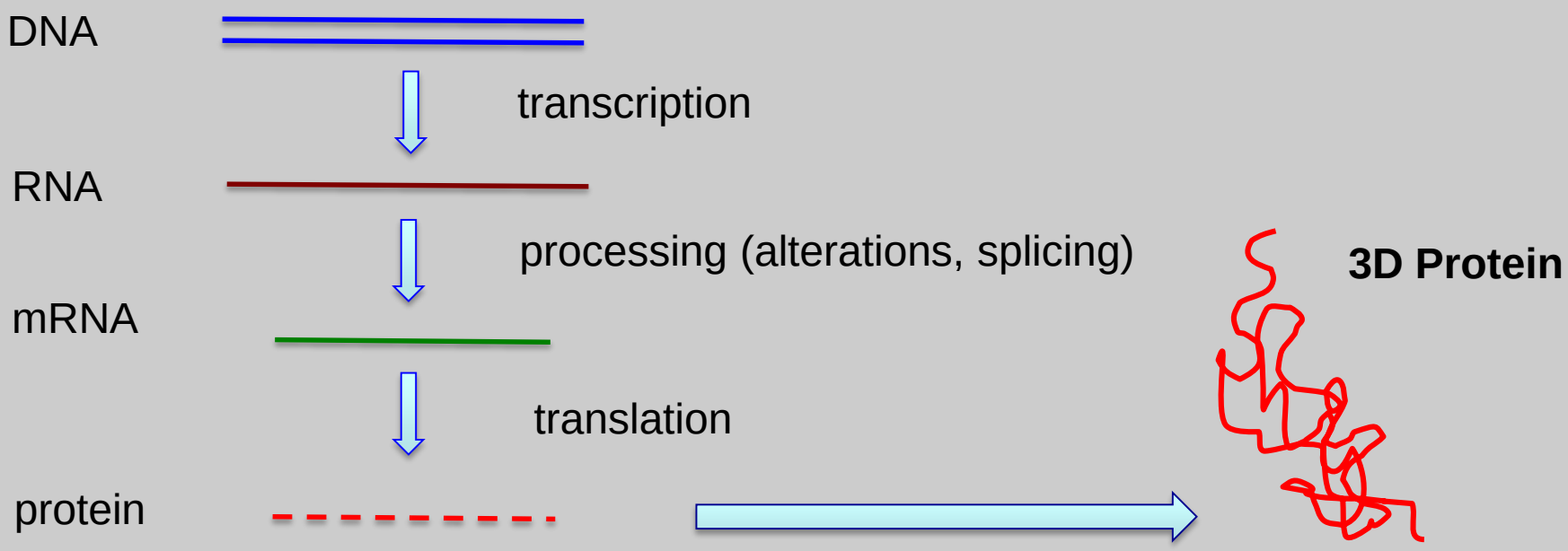
  Nuclease-mediated site directed mutagenesis

  – ***ZFN: zinc-finger nucleases**
  – TALENs: Transcription activator-like nucleases
  – HEs: Meganucleases / Homing endonucleases
  – CRISPR/Cas nucleases clustered regularly interspaced short palindromic repeats (Genomic editing tool) (was bacterial defense system: nucleases attached to RNA)

  SSN: intended modifications by synthetic Site Specific Nucleases

Genes and Genetics:

In many cases, more than one RNA/protein is produced from a given gene.



Epigenetics

epi [greek]: around, over, outside

- **“epigenetics** is the study of heritable changes in gene activity that are *not* caused by changes in the DNA sequence; it also can be used to describe the study of stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable.”
- It can be removed over time (by organism or later generations)
- examples: poplar, petunia

DNA Methylation / epigenetics

RNA-directed DNA methylation (**RdDM**) is an epigenetic process. During RdDM, double-stranded RNAs (dsRNAs) are processed to 21-24 nucleotide small interfering RNAs (siRNAs) and guide methylation of homologous DNA loci.

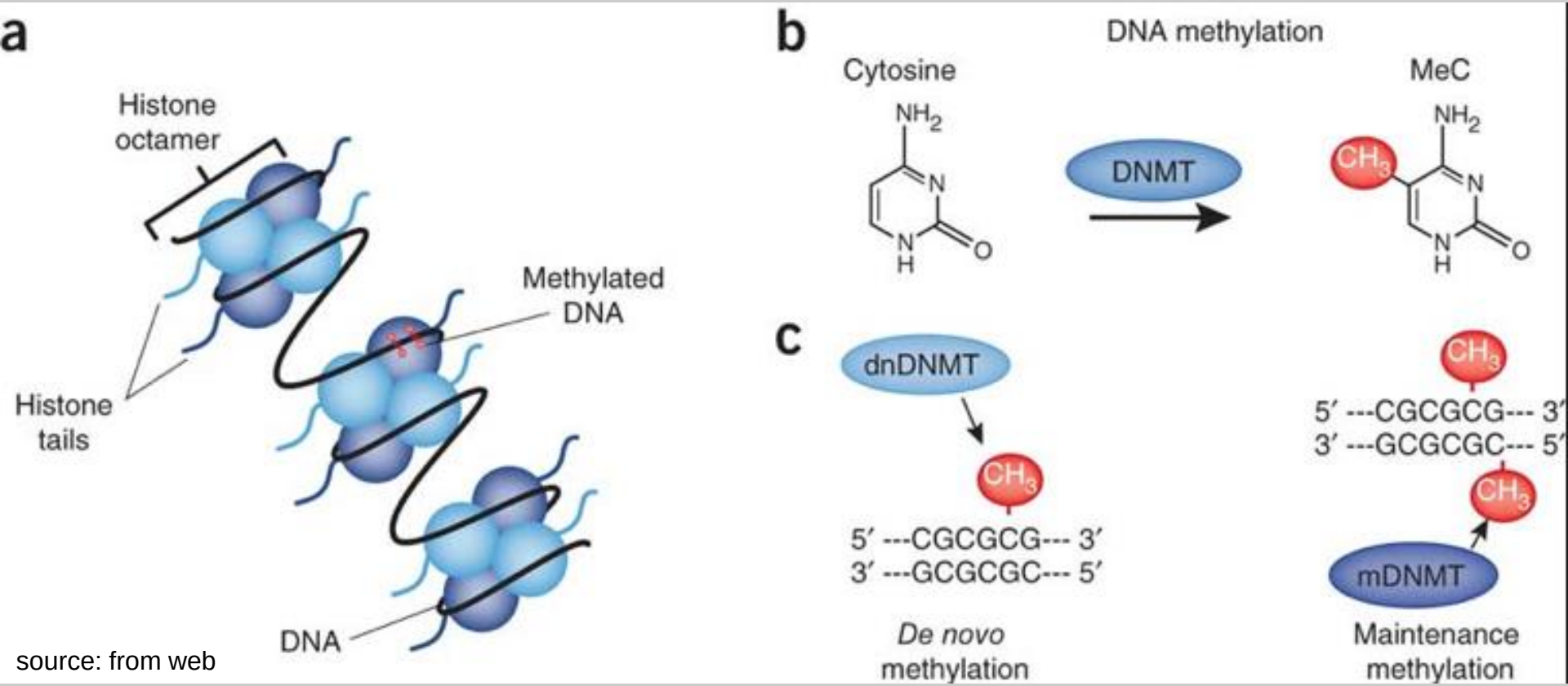
In plants for example, dsRNAs may be generated from three sources:

- Viral replication intermediates
- Products of the endogenous RNA-directed RNA polymerase
- Transcribed inverted repeats

As NBT (new breeding technology):

- add genetic sequence to produce ds RNA *(“insecticide”)*
- add dsRNA or create site directed DNA methylation

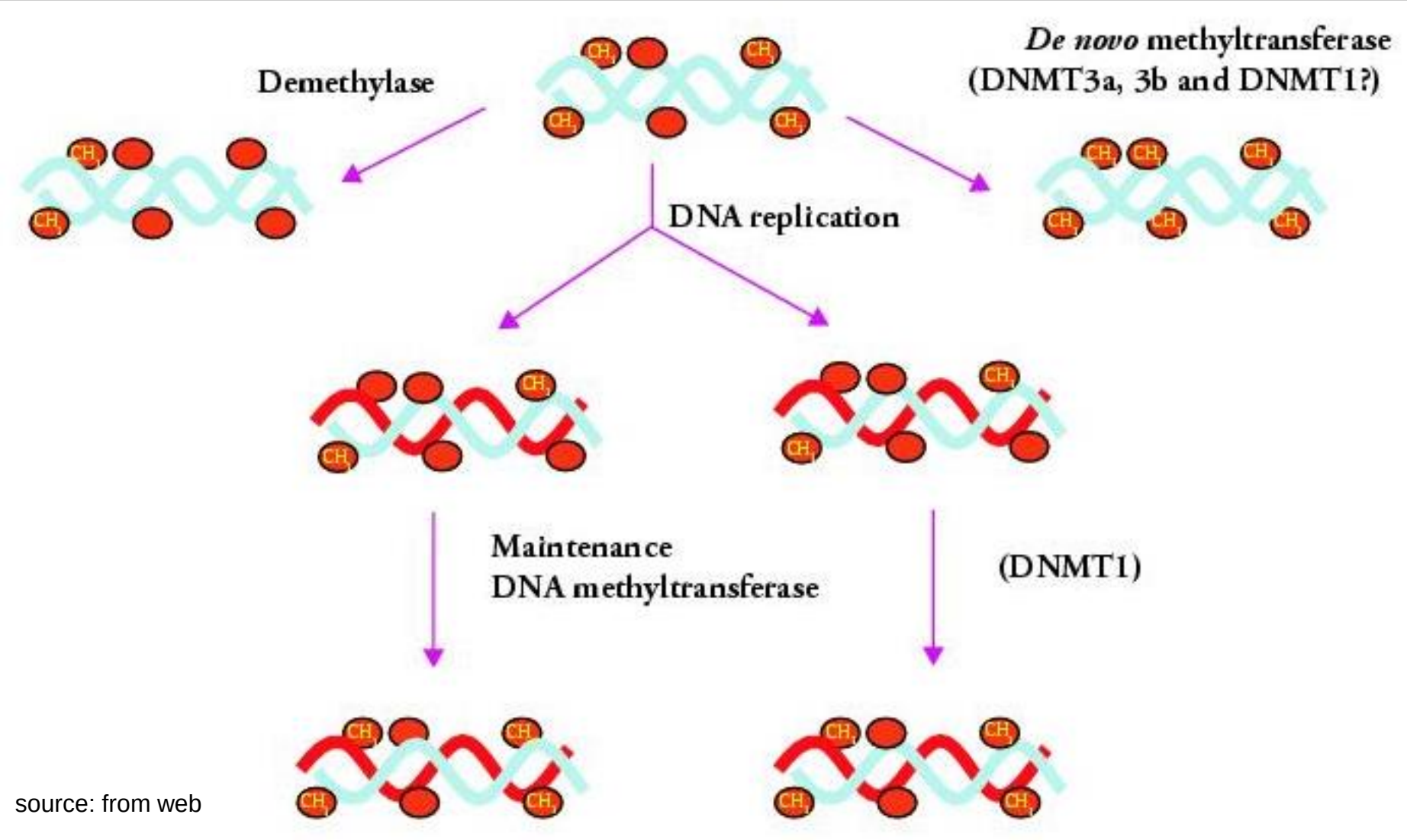
DNA Methylation / epigenetics



source: from web

DNA methylation results in gene silencing through blocking of transcription

DNA Methylation / epigenetics



source: from web

DNA methylation results in gene silencing **and is inheritable**

RNA-dependent DNA methylation

as a New Plant Breeding Technology:

GMO?

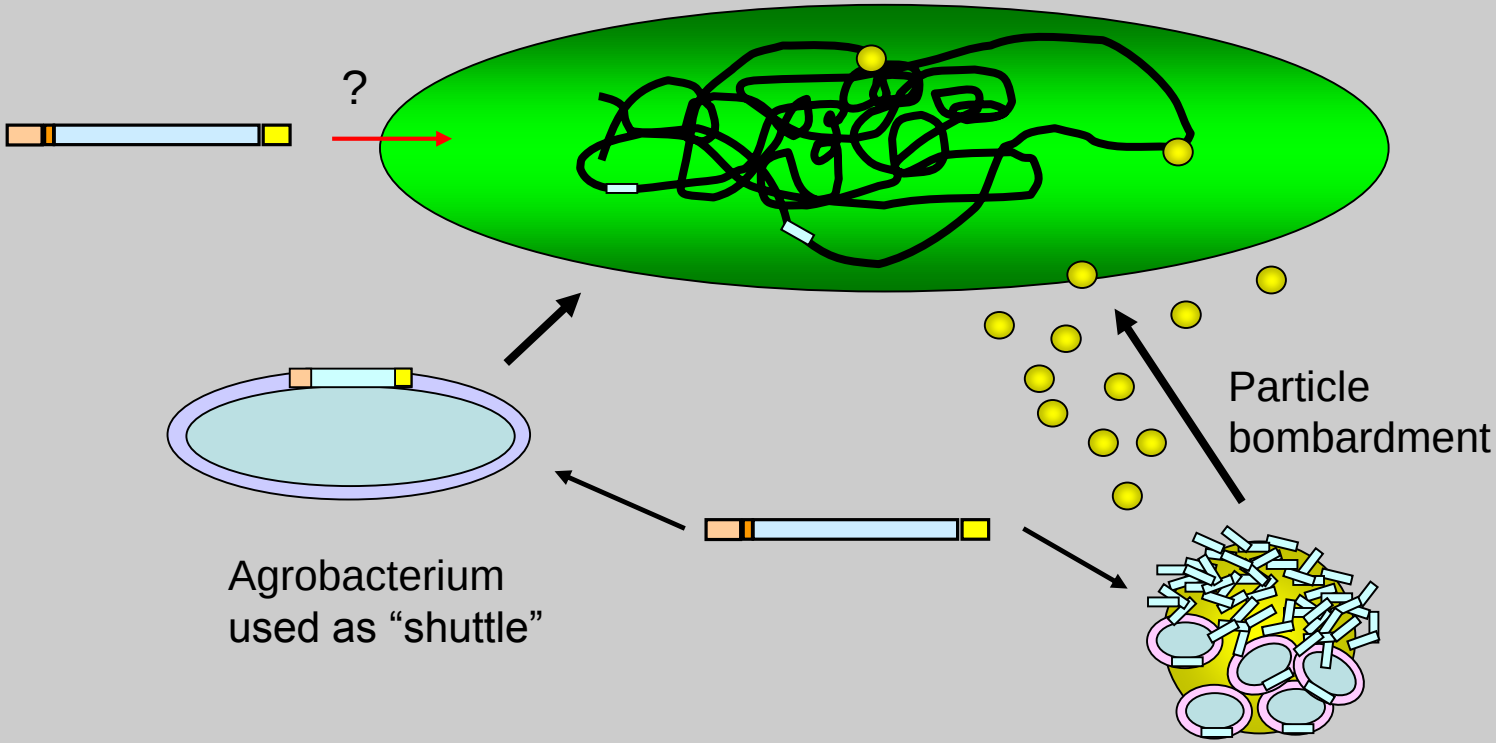
Synthetic Biology?

regulatory invisibility?

and what about the risks??

- eg disruption of regulatory pathways?
- unintended epigenetic changes (organism to ecosystem)

Transformation

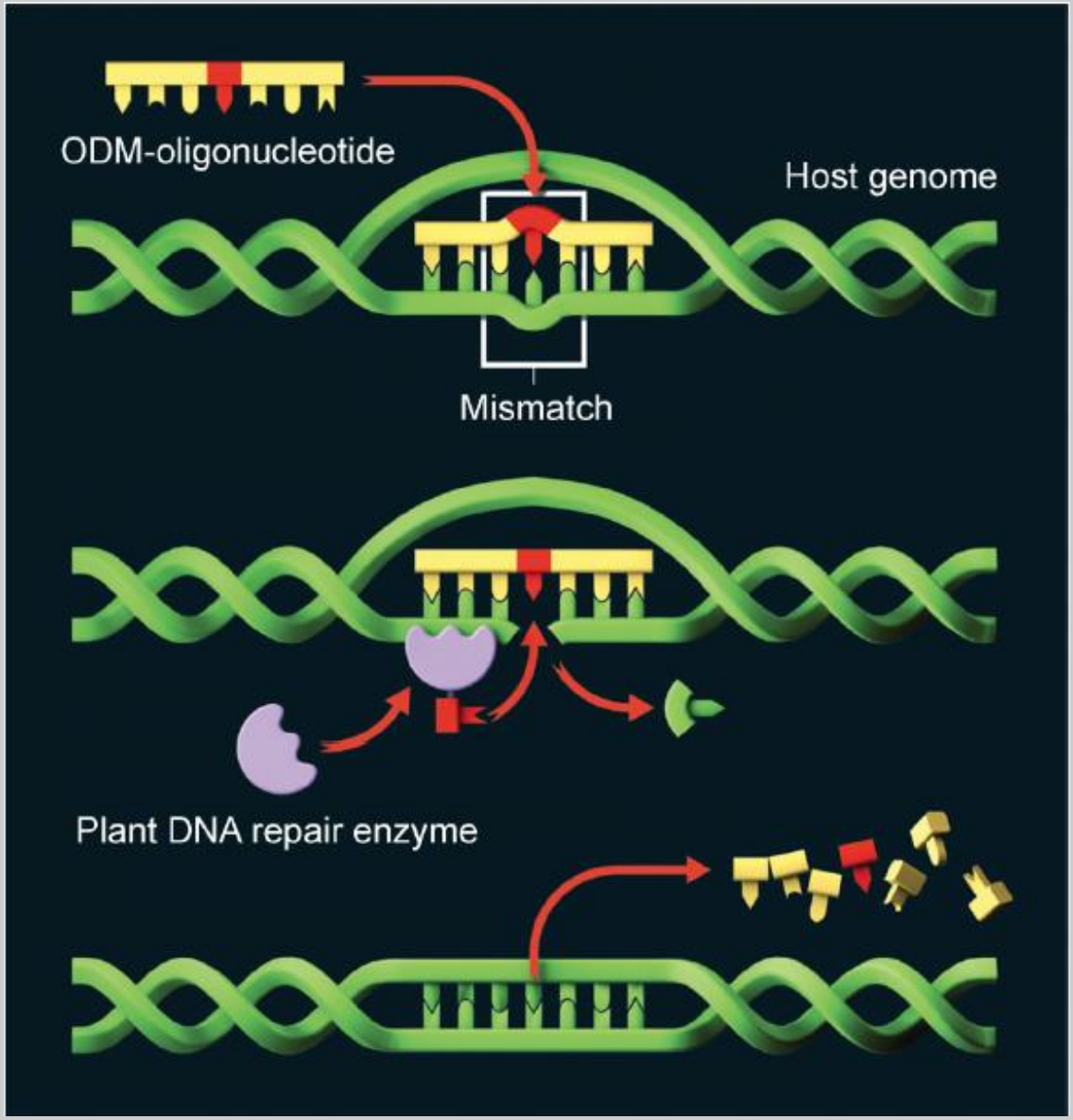


No control of where the gene will insert itself:

- Random integration
- Imprecision (incl. superfluous DNA)
- 100 -1000s of Mutations (Sala et al. 2000, Wang et al. 1996, Labra et al 2001)

Genome Editing Techniques

the concept of precision



oligo-directed mutagenesis (ODM)

mismatches of
1-4 nucleotides:

eg herbicide
tolerance,
& loss of function
(knock-outs)

Schematic diagram of ODM (adaped from http://cibus.com/) in:
Eckerstorfer et al. 2014, report rep-0477, umweltbundesamt, Vienna

ODM – risk relevant issues

- **Off-target mutations** in genomic elements sharing homologous sequences and unintended integration of whole or partial ODM oligonucleotide sequences.
- **Expression of fusion-proteins** for some types of knock-out mutations.
- **Unintended modification** due to transfection and regeneration methods (as with other GM transformations).
- Unintended effects of ODM oligonucleotides in **cellular regulation pathways** for gene expression (e.g. RNAi).

taken from: Eckerstorfer et al, report rep-0477, umweltbundesamt, Vienna 2014

Nuclease-mediated site-directed mutagenesis

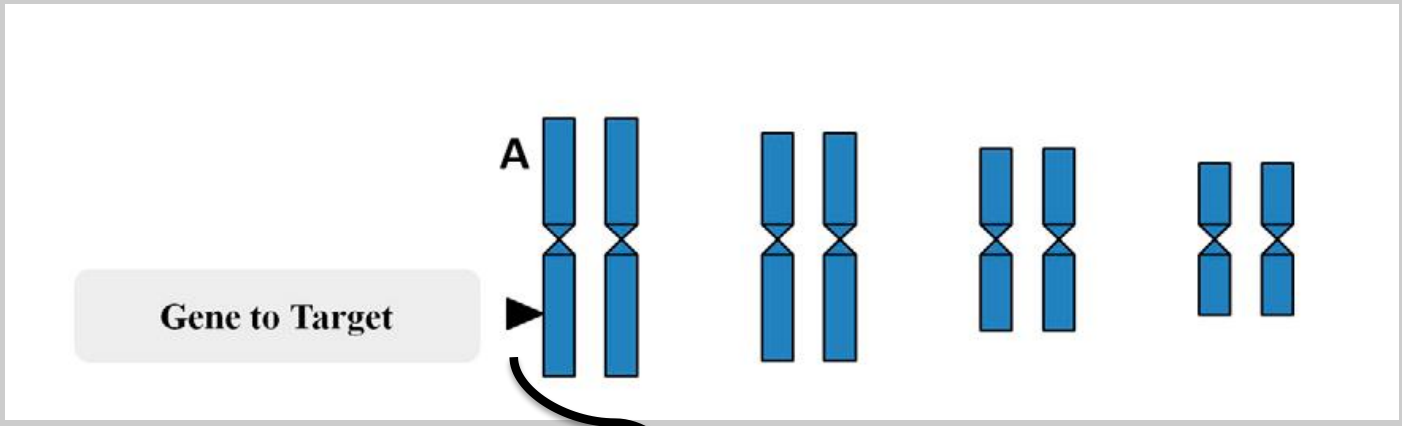
- **nucleases:** enzymes that cut nucleic acids (molecular scissors)
- ***SSN: synthetic site specific nucleases***

  introduce double strand breaks at specific DNA sites

  this triggers different DNA repair mechanisms

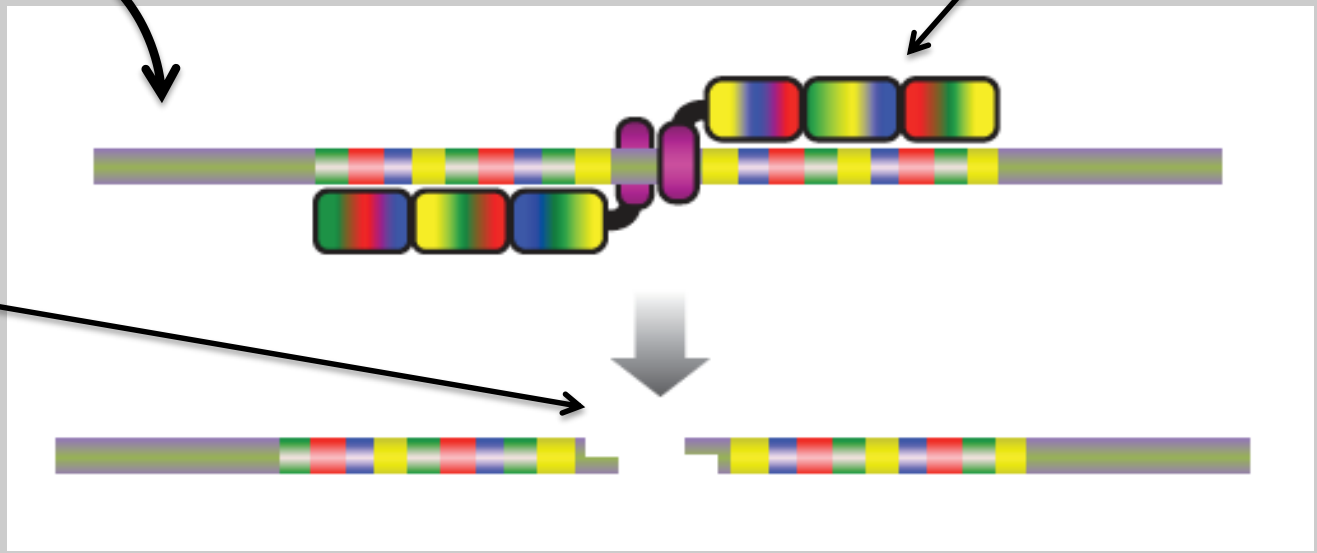
  - non-homologous end joining (NHEJ)
  - homologous recombination (HR)
- **contains two functions:**
  - specific DNA recognition & binding site to direct nucl.
  - enzyme domain that cuts DNA

Genome editing

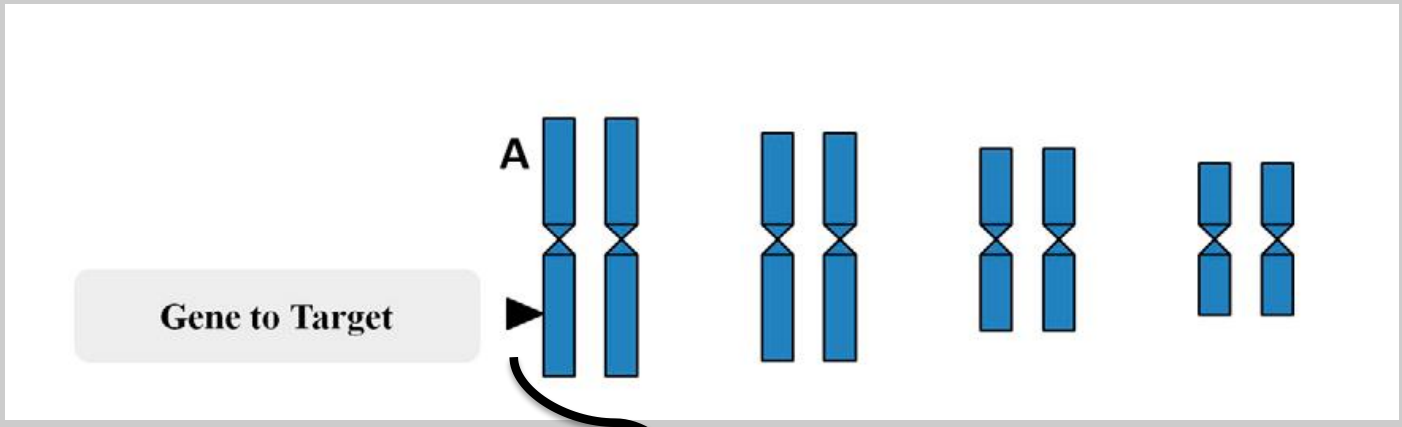


Special enzyme

Targeted ‘break’ of both strands of DNA in a chromsome

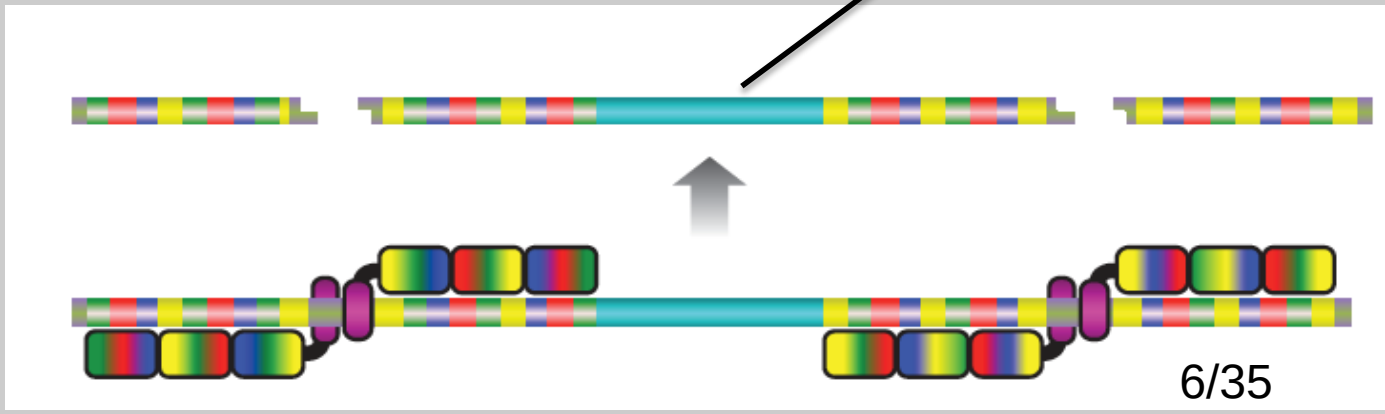


Genome editing

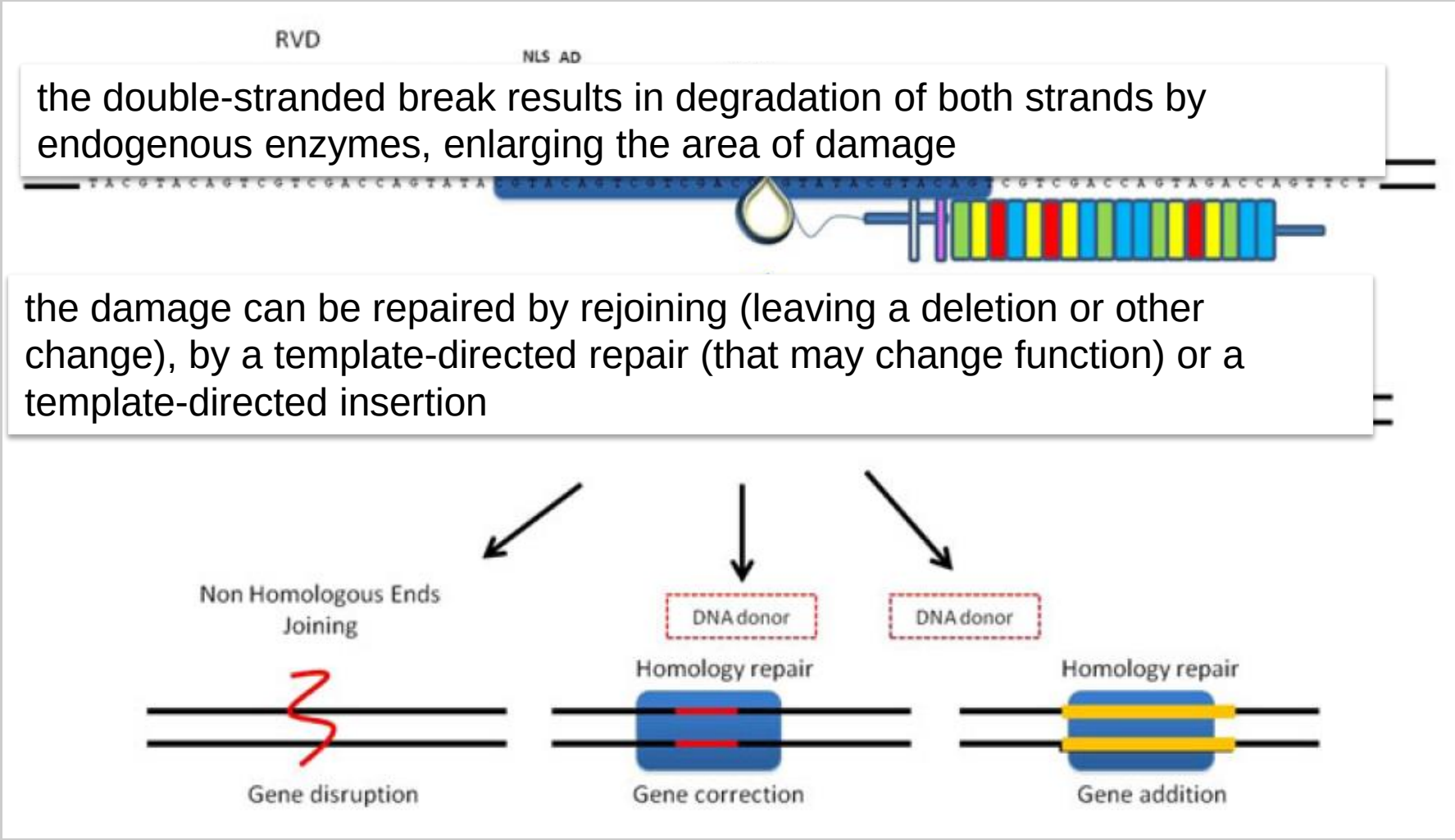


Material ‘deleted’

Two separate breaks result in large deletion of material



alternative endings



Special enzymes

The special enzymes are called:

1. CRISPR/Cas
2. ZFN (zinc finger nucleases)
3. Tales/Talens (transcription activator–like effector nucleases)
4. Meganucleases

CRISPR/Cas-nuclease

clustered regularly interspaced short palindromic repeats

CRISPR/Cas nucleases are synthetic nuclease complexes, *developed from the bacterial nuclease Cas9* (CRISPR associated 9), *which is a component of the adaptive immunity system in bacteria aimed to recognize and destruct foreign DNA*, e.g. phage DNA or plasmid DNA.

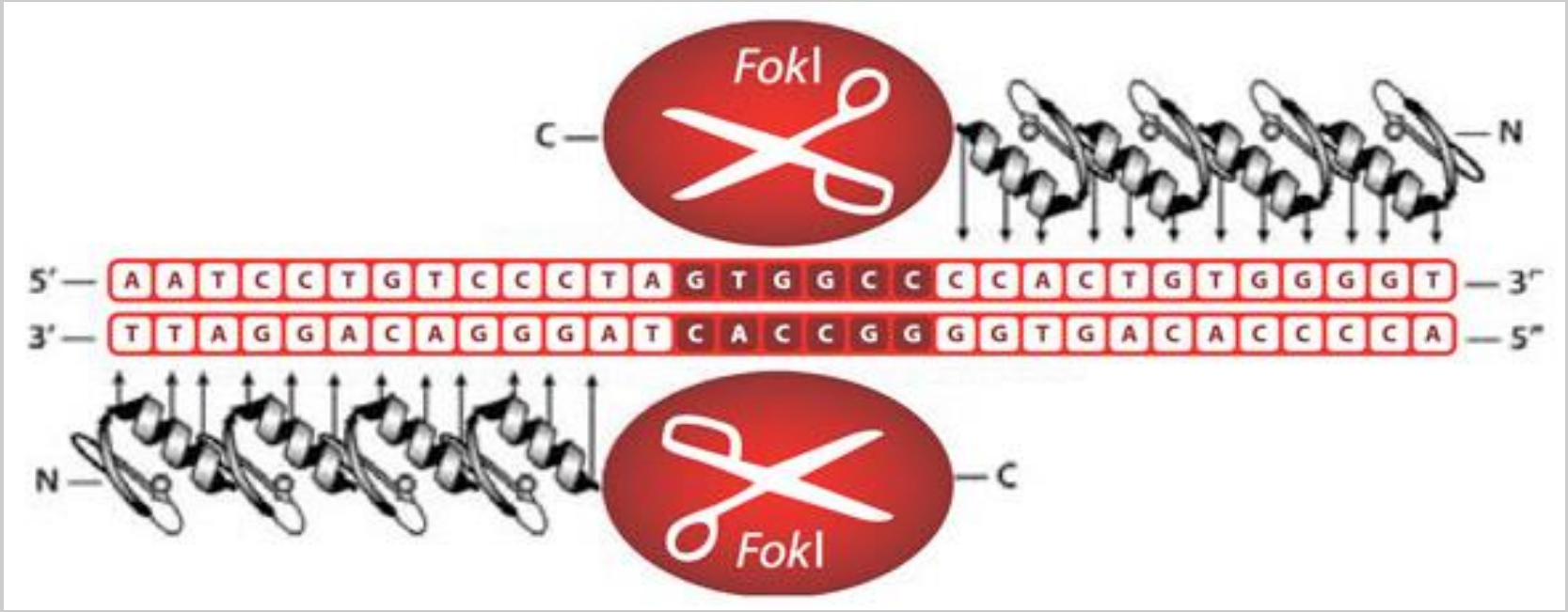
CRISPR/Cas nucleases are guided to a particular genomic DNA sequence by **guide RNAs attached to the nuclease enzyme**.

The enzyme also accepts specifically designed **synthetic guide RNAs** modeled on the Cas9 guide RNA. These synthetic guide RNAs direct the nuclease activity to intended target sequences in the crop genome, which are complementary to the synthetic recognition sequence of the guide RNA. By this way a multitude of different target sequences and thus different genome sites can be targeted. [Eckerstorfer et al. 2014]

What makes them special?

Hybrid proteins (chimeras) of two functional domains. Domain that-

1. binds specifically to a particular sequence of nucleotides;
2. hydrolyses (breaks) a DNA strand.



TALENs

Transcription activator-like nucleases

TALENs are dimeric composed of a nuclease domain fused to a DNA-binding domain.

Similar to ZFNs, FokI is usually used as a nuclease domain.

However the DNA-binding domain of TALENs is more flexible because it consists of modules recognizing single nucleotides in a DNA sequence. The DNA-binding domain then consists of an array of up to 30 modules, which are specific for a particular nucleotide sequence of 30 nucleotides. Due to their longer DNA recognition sites TALENs are more specific for particular genomic locations and thus cause fewer unwanted off-target effects than ZFNs.

taken from: Eckerstorfer et al, report rep-0477, umweltbundesamt, Vienna 2014

Risk issues

1. socioeconomic issues of ownership and application of strict intellectual property rights instruments
2. unintended and off-target effects

   off target esp. present in ZFNs and also in CRISP/Cas systems

   Even if targeting is specific, the outcome of repair at the double strand breaks induced by SSNs can be very diverse (e.g. point mutations, sequence/gene deletion, integration of non-native sequences, inversions/ translocations of chromosomal sections).

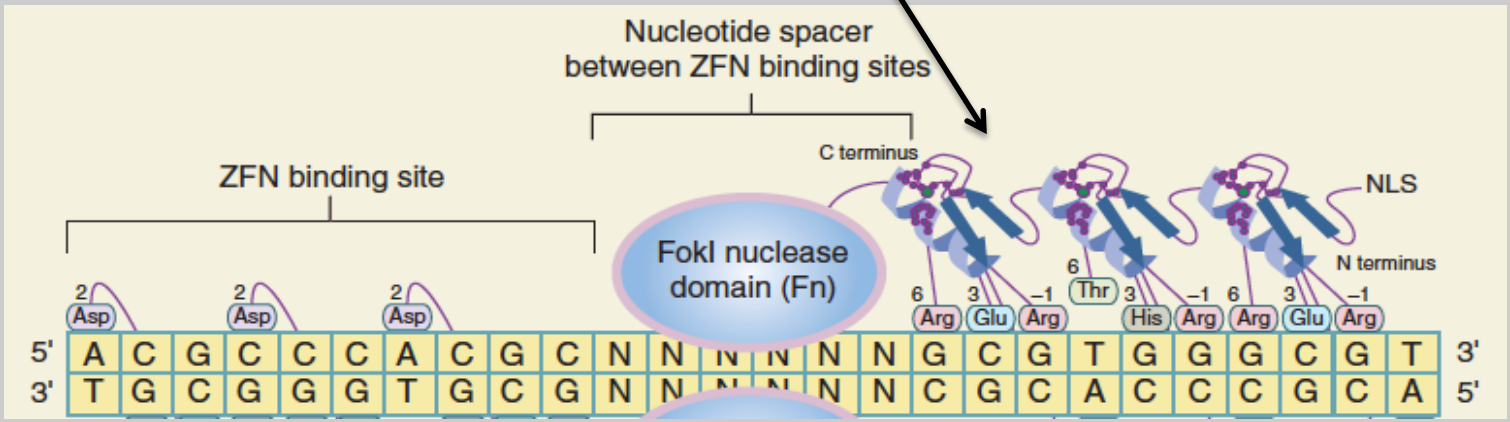
"Using the method presented here we identified hundreds of thousands of sequences that can be cleaved by two active, dimeric ZFNs, including many that are present and can be cut in the genome of human cells." - Nature Methods | VOL.8 NO.9 | SEPTEMBER 2011 | 765

What makes these products GMOs?

1. The hybrid proteins are products of in vitro techniques.

ZFNs, Tales/Talens and meganucleases are the product of *in vitro* nucleic acid techniques and they create a novel combination of genetic material in the organism.

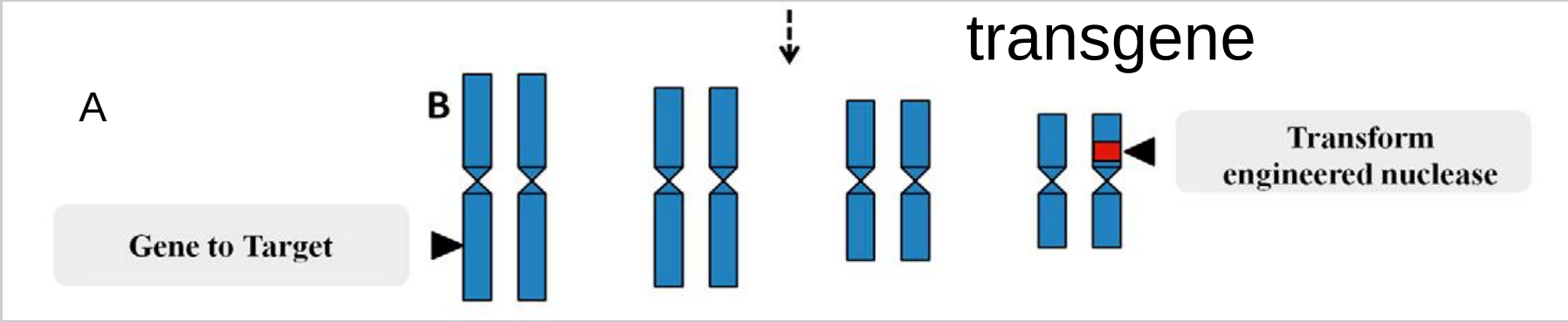
does not exist in nature
is the product of recombinant DNA techniques



from JH

What makes these products GMOs?

2. They may work in conjunction with another nucleic acid (either RNA or DNA) inside the cell.



B

C

Microinject ZFN mRNA pair into pronucleus of fertilized 1 - cell embryos

(or ‘transient’ DNA for ZFN pair)

+ DNA for repair/insertion

from JH

consensus?

NO

e.g., New Zealand High Court ruled in 2014 that these techniques are unambiguously genetic engineering.

“There is a general agreement that the resulting organism from the use of ZNF-1/ZFN-2 is a GMO...” –EFSA

However, EFSA experts have majority view that the use of these techniques should be excluded by directive from producing GMOs.

from JH

The idea of Precision

The idea of precision is based on that you know what you are doing – and this is not the case.

Knowledge & precision at the level of nucleotides is only the bottom layer.

What is missing is the contextualisation into

- the genome
- the epigenetic landscape
- the organism
- the ecosystems
- the socio-economic conditions that differ around the world

Precision around nucleotides gives a false sense of predictability and safety – there is no data to support such extrapolations.