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Session ID: bio411a





In Person quiz

Codon usage

- A. Does not affect gene expression
- B. Can be ignored for transgenes
- C. Can change mutation frequency
- D. Differs between species

Dominant mutations are always due to a Gain-of-Function of a gene product

- A. True
- B. False

Random insertion of transgenes can be reduced by employing

- A. microinjection
- B. Retrovirus delivery
- C. Transposons
- D. Homologous Recombination



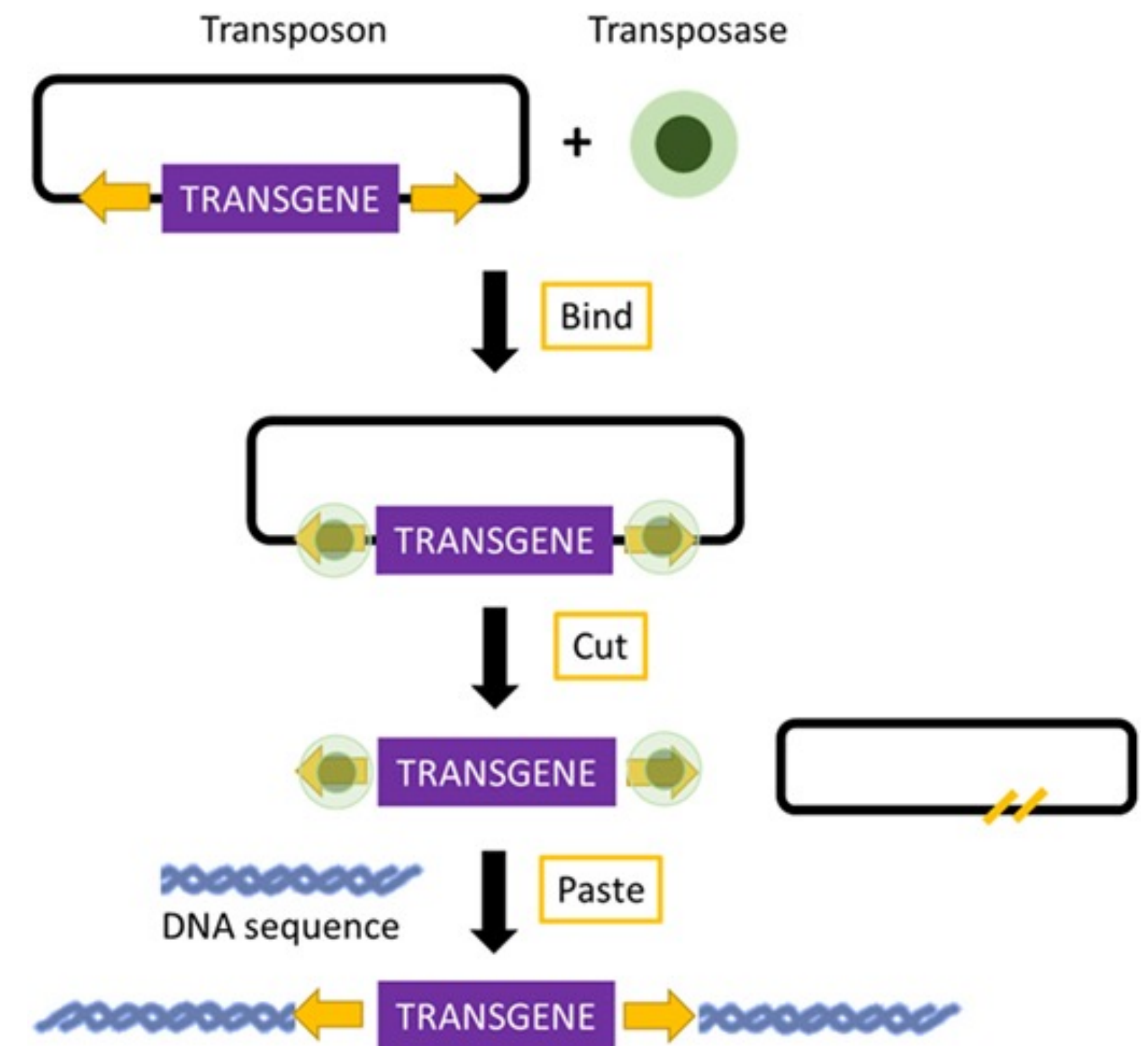
Manipulating Nucleotides II



Genome editing

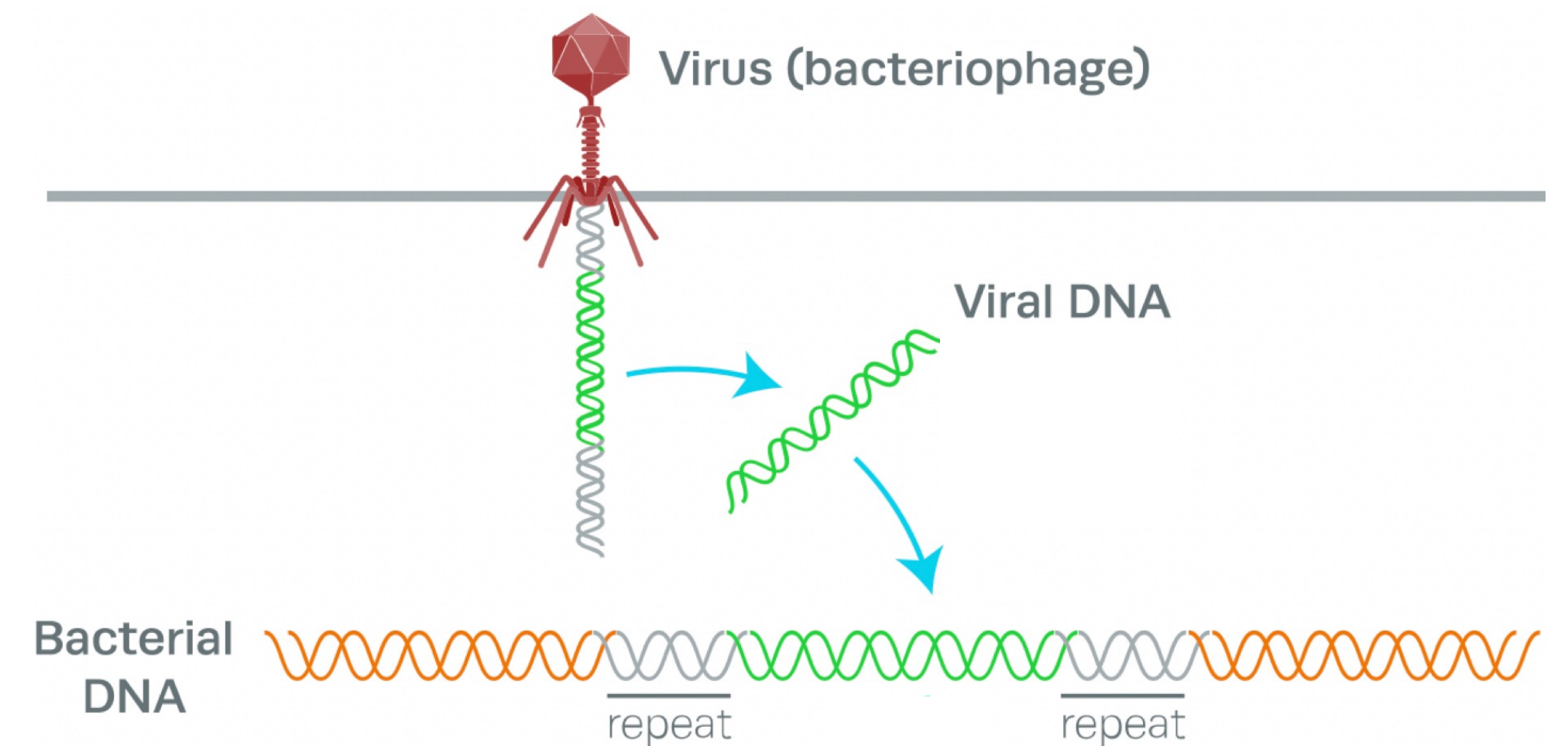
'Forward Genetic' mutagens

- **Forward genetics** – screening for novel mutations based on phenotype
- **'Natural' mutations** selected by breeding
- **Chemical mutagens** e.g. Ethyl methanesulfonate (EMS) causes point mutations
- **Transposons**



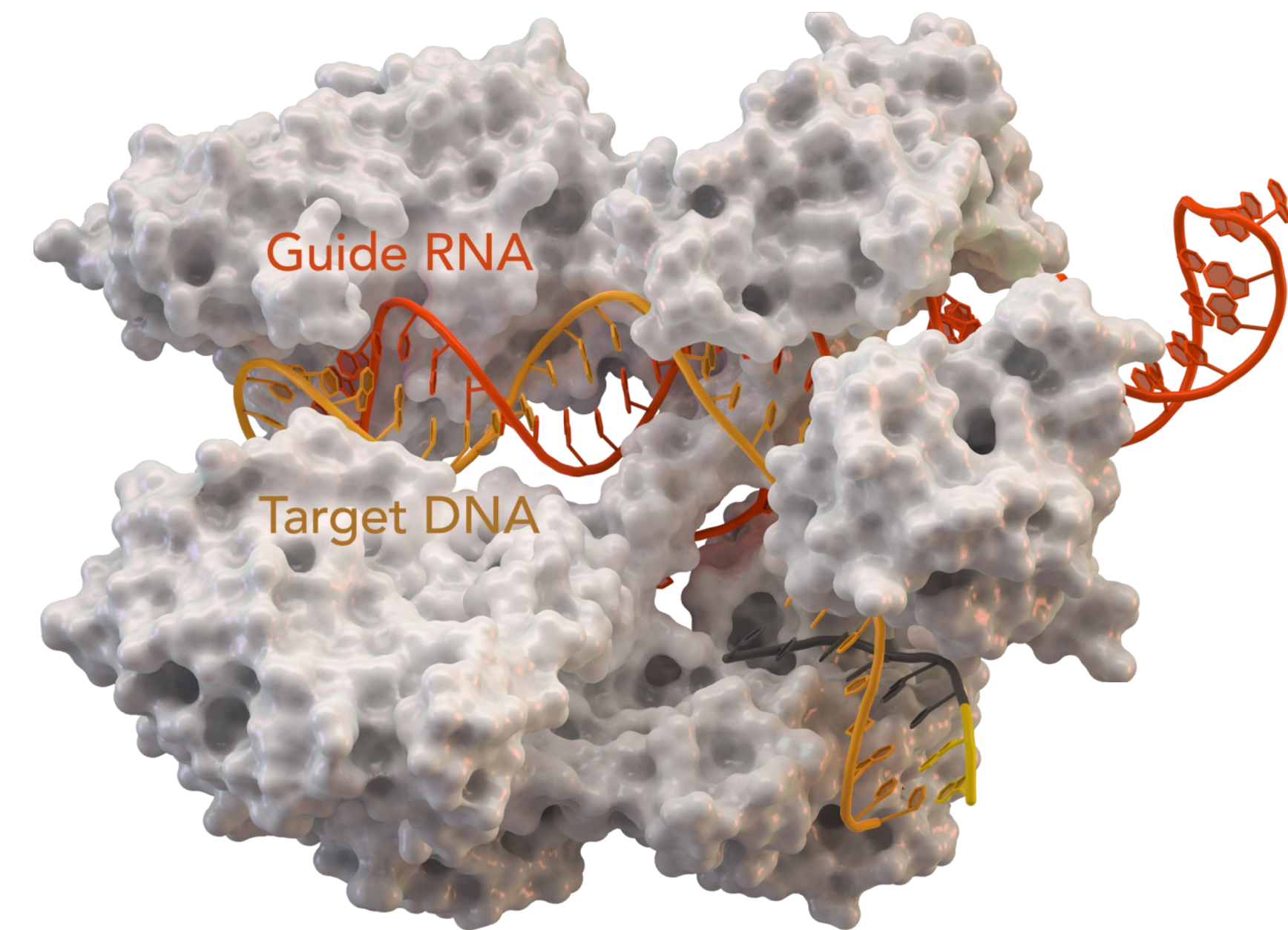
CRISPR

- **CRISPR** (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) are repetitive DNA sequences found in the genomes of prokaryotes including bacteria and archaea.
- CRISPR sequences are derived from **DNA fragments of bacteriophages** that had previously infected the prokaryote. Used to **detect and destroy** subsequent bacteriophages
- Act as antiviral defence system in ~50% of bacteria and ~90% of archaea.



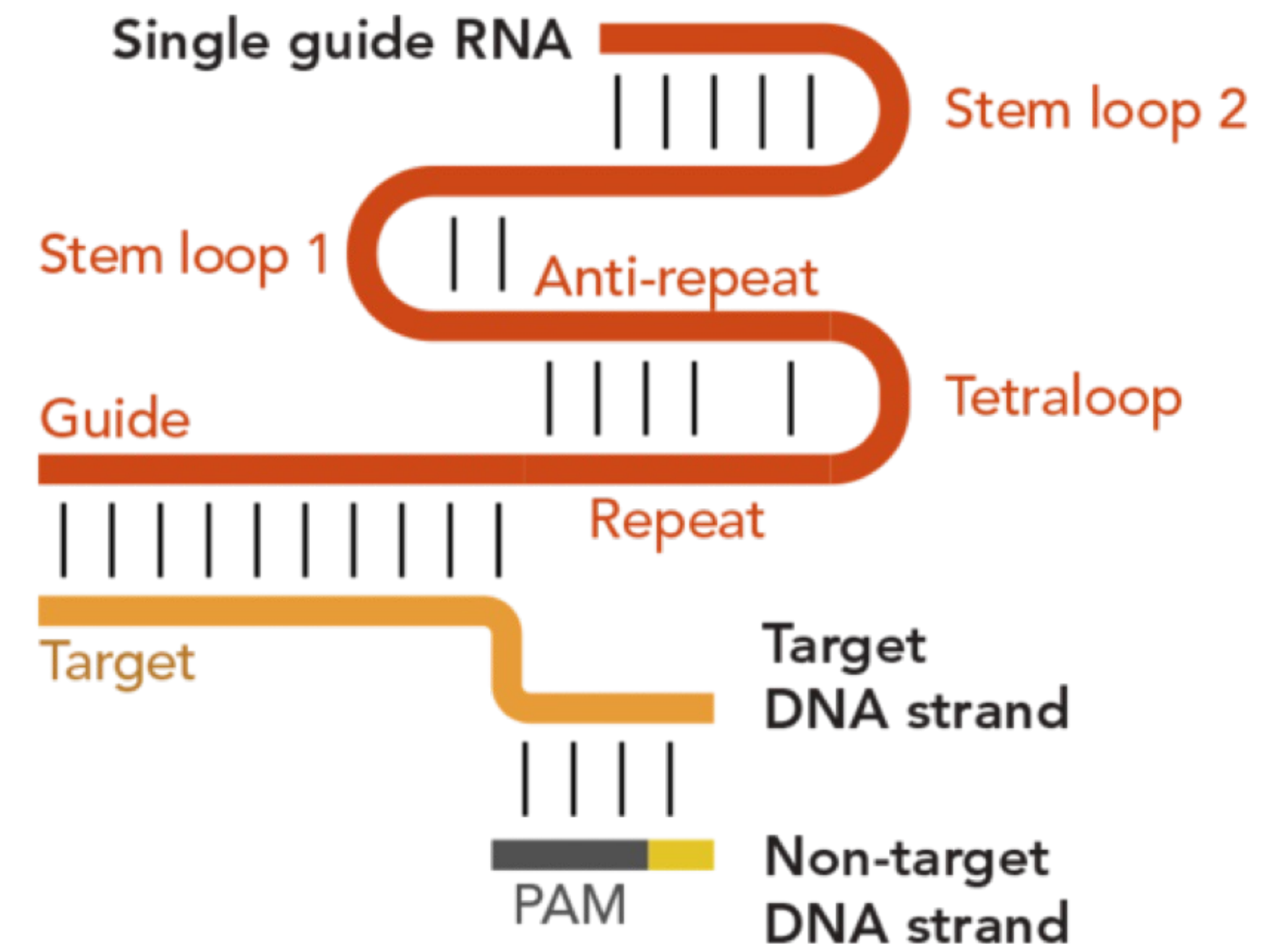
Cas 9

- **Cas9 (CRISPR associated protein 9)** commonly derived from *Streptococcus pyogenes*
- Cas9 is a **dual RNA-guided DNA endonuclease** associated with CRISPR repeat derived mRNAs.
- **mRNA sequences transcribed from bacterial CRISPR regions** are cleaved into fragments by tracrRNAs which also enable interaction with Cas 9.
- The Cas9, tracrRNA, CRISPR mRNA ribonucleoprotein complex **surveils for viral DNA sequences** and, if found, cleaves the viral genome preventing infection.

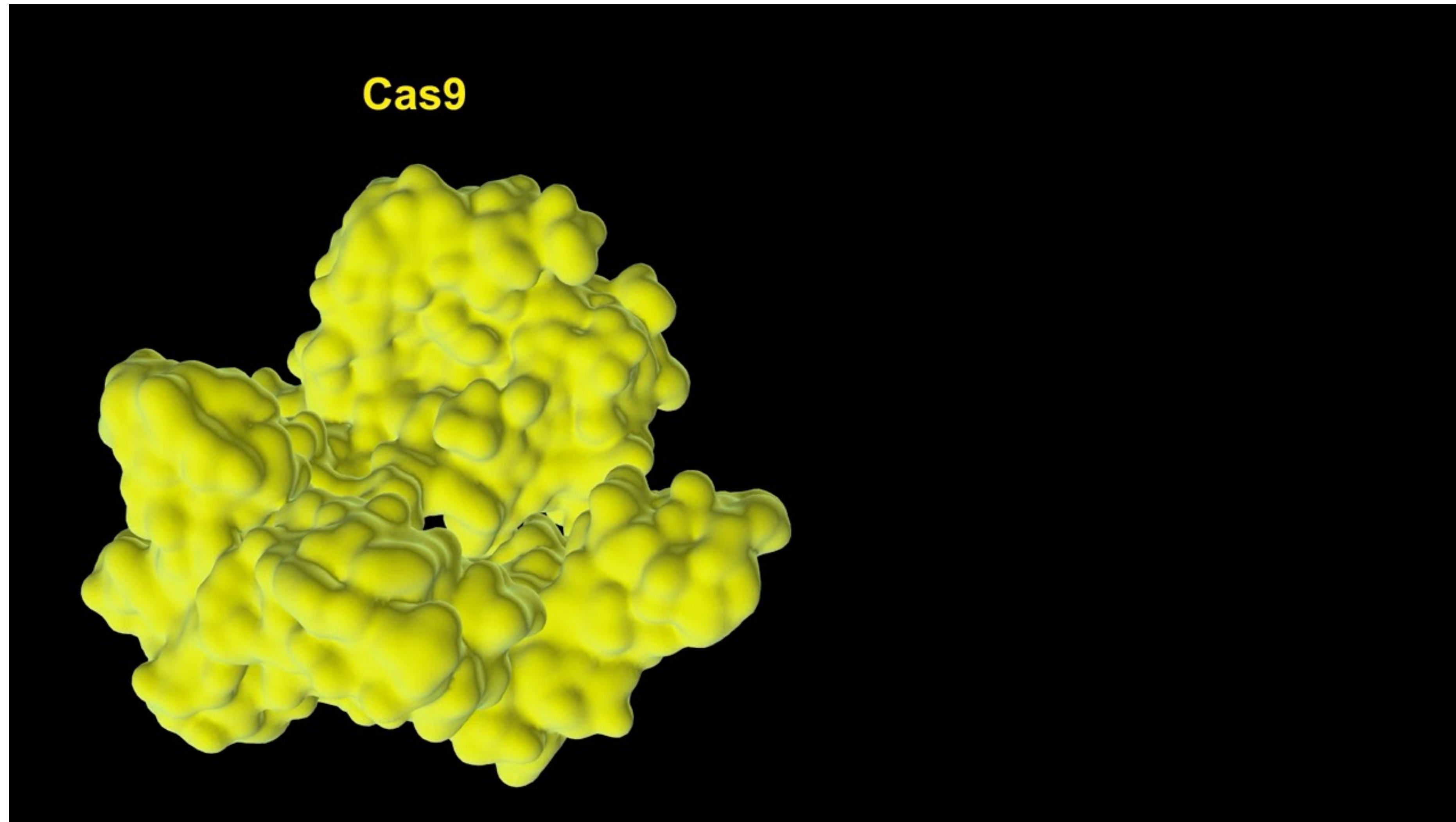


Guide RNA

- **Guide RNA** (gRNA or sgRNA) is a short synthetic RNA
- Composed of a **scaffolding sequence necessary for Cas9 binding** and a user-defined **~20 nucleotide spacer that defines the genomic sequence to be targeted**.
- Includes a **PAM** (protospacer adjacent motif) **sequence enables** binding to Cas9
- Cas9 and the gRNA form a ribonucleoprotein complex inducing Cas9 into an **active DNA-binding conformation**.
- The spacer region of the **gRNA remains accessible to interact with the target DNA**

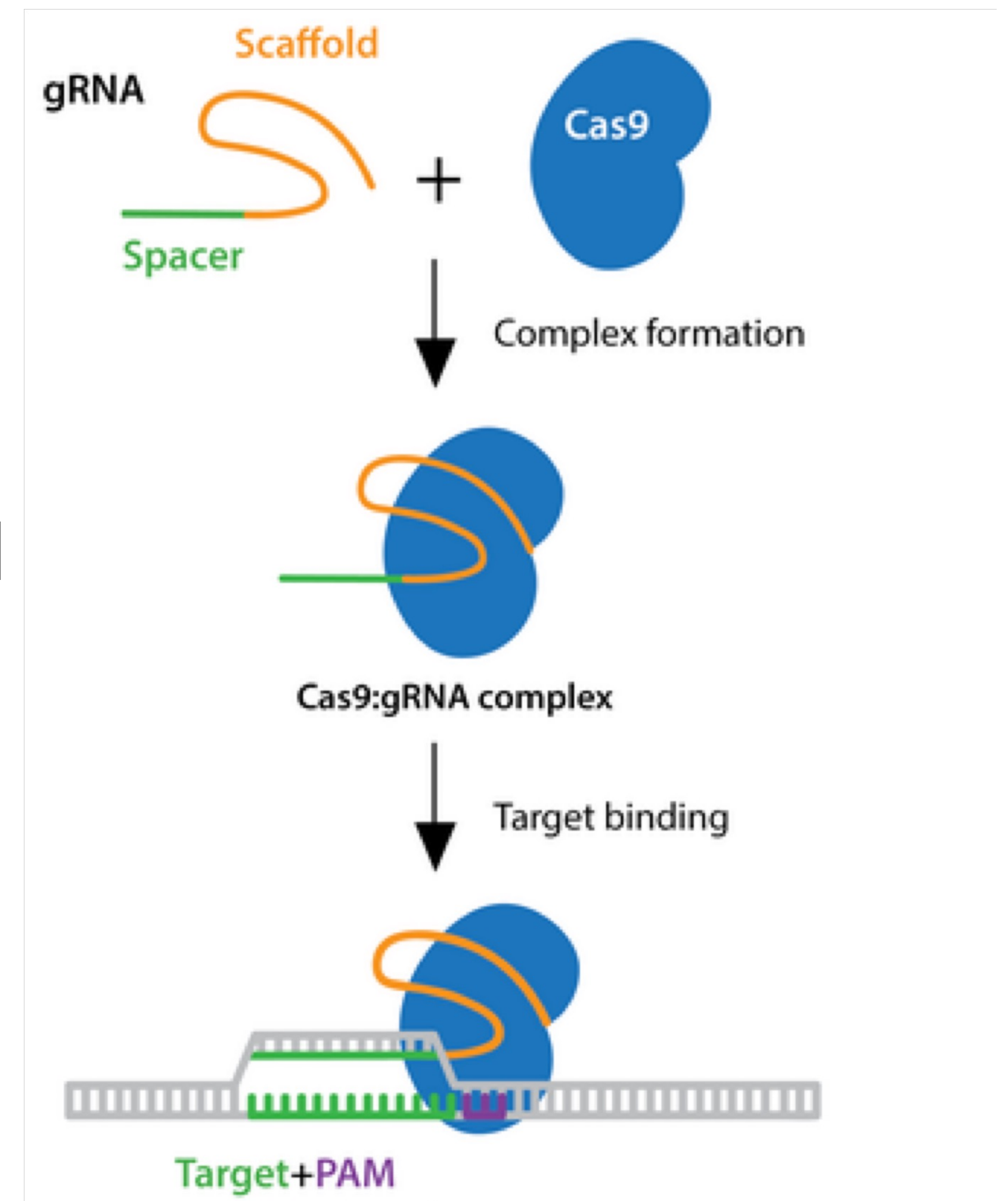


CRISPR/Cas9



Generating mutations with CRISPR/Cas9

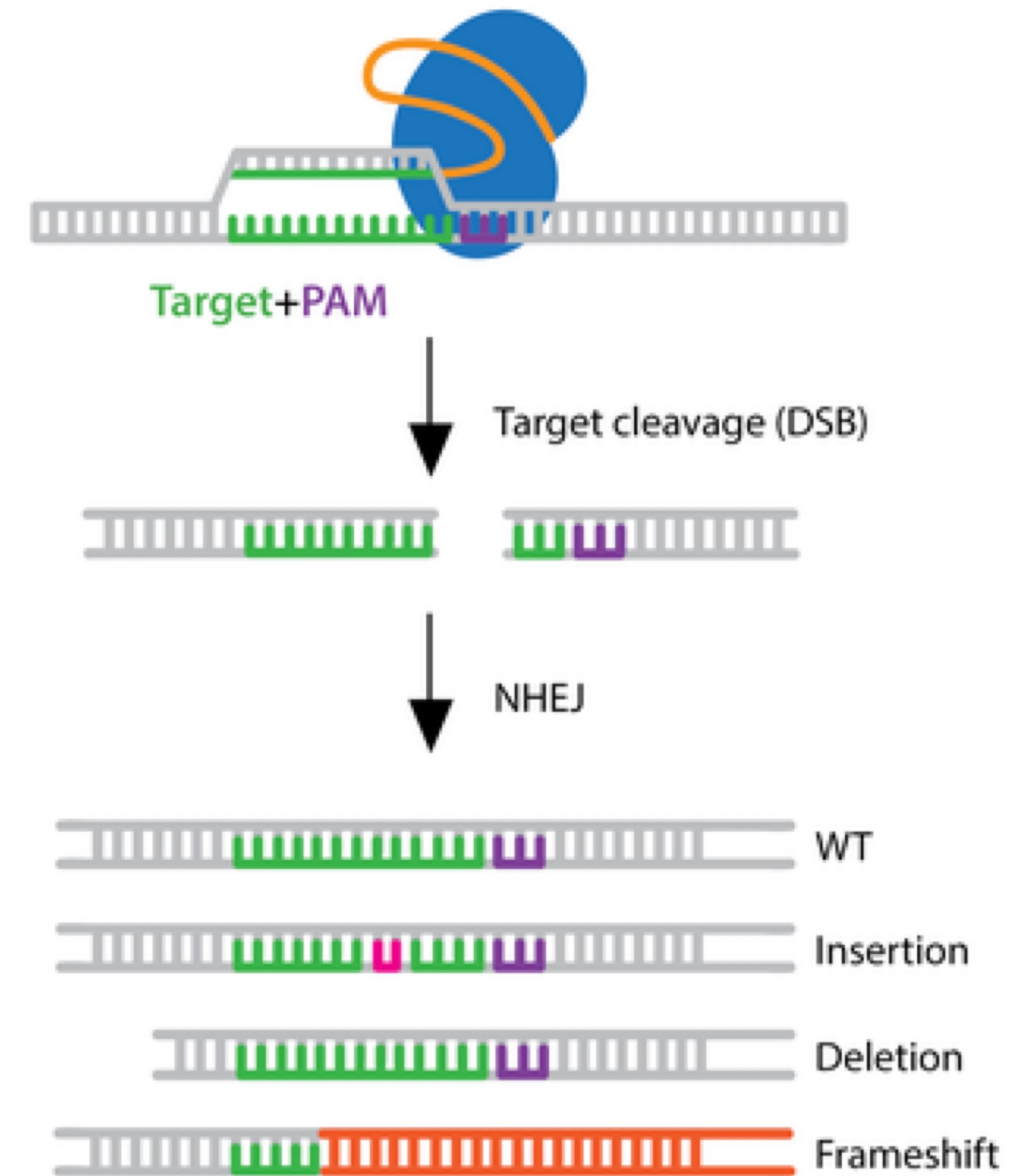
- Cas9 will only cleave the target DNA if the gRNA spacer sequence has **sufficient homology**.
- Once the Cas9-gRNA complex binds a putative DNA target, **the seed sequence** (8-10 bases at the 3' end of the gRNA targeting sequence) begins to anneal to the target DNA. The gRNA will continue to anneal to the target DNA in a 3' to 5' direction.
- Sufficient target DNA binding positions the Cas9 nuclease domains (RuvC & HNH) enabling cleavage of both strands of the target DNA resulting in a **double-strand break** (~3-4 nucleotides upstream of the PAM sequence).



Generating mutations with CRISPR/Cas9

Repair after Double Strand Breaks (DSBs)

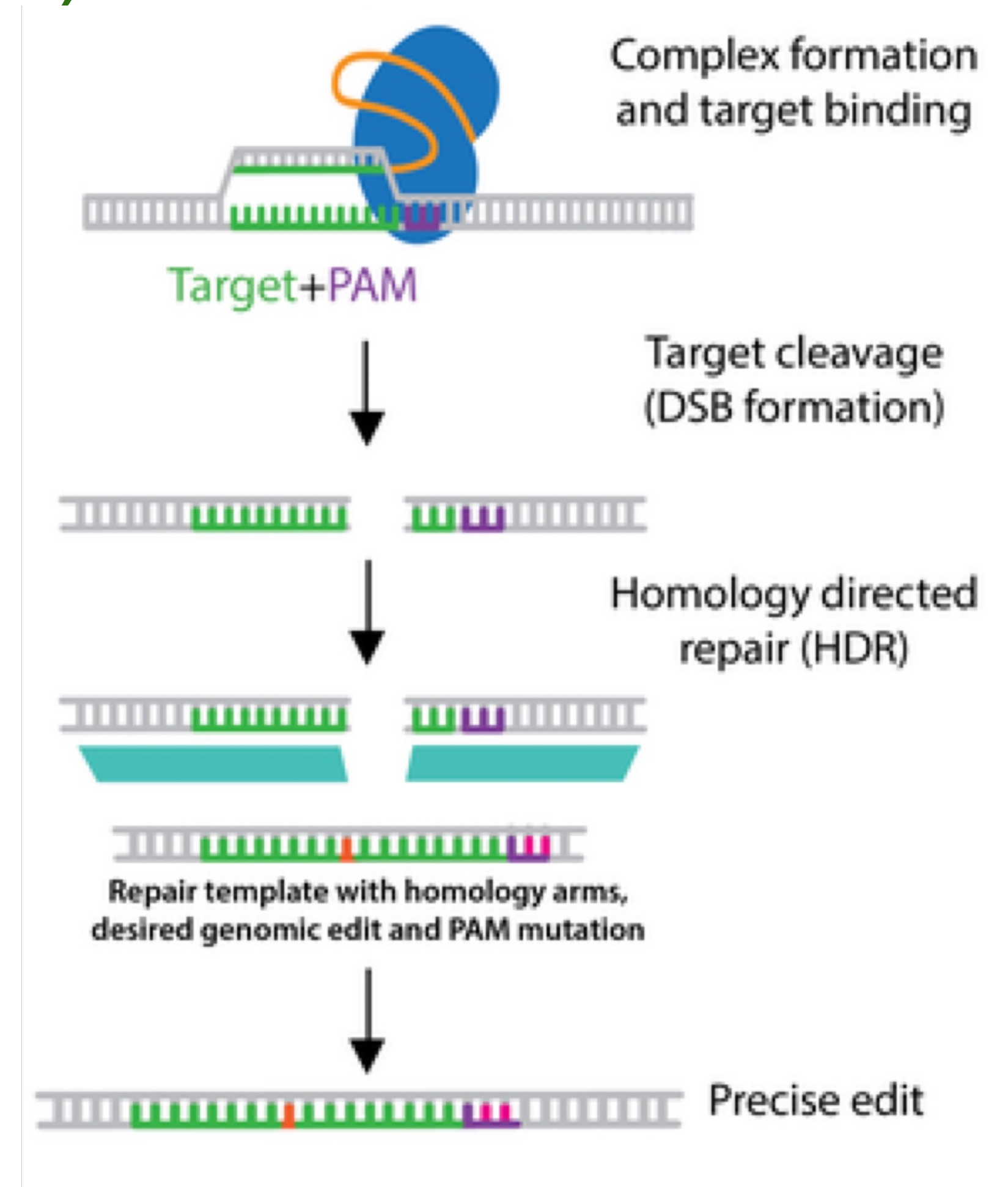
- Double stranded breaks **can be repaired** by one of two general repair pathways
- **Efficient but error-prone** non-homologous end joining (**NHEJ**) pathway
- In many cases, NHEJ gives rise to small **indels** in the target DNA that result in amino acid **deletions, insertions, or frameshift mutations.**



Generating mutations with CRISPR/Cas9

Repair after Double Strand Breaks (DSBs)

- **Less efficient but high-fidelity Homology Directed Repair (HDR) pathway**
- To utilize HDR for gene editing, a **DNA repair template** containing the desired sequence must be delivered together with the gRNA and Cas9
- The repair template contains the desired edit as well as additional homologous sequence upstream and downstream of the target (**homology arms**).
- Efficiency of HDR is low (<10%) but edits can be **very precise**.





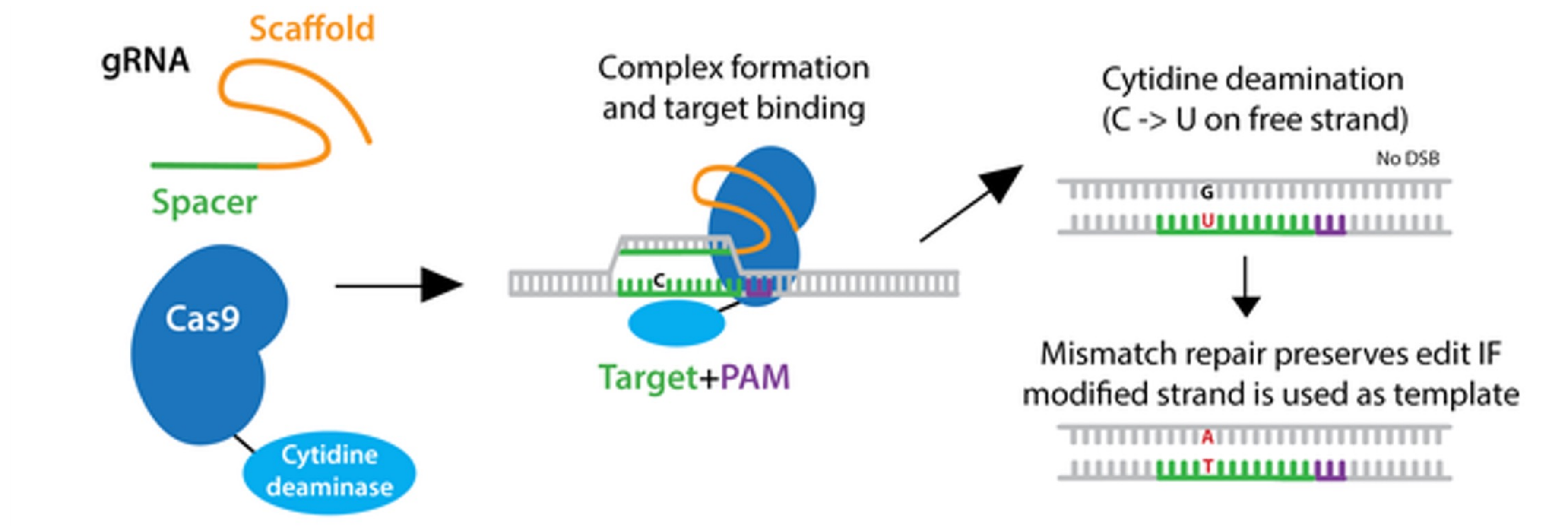
In Person quiz

CRISPR/Cas9 technology can also be used to

- A. Increase gene expression
- B. Introduce point mutations
- C. Alter epigenetic regulation
- D. Target transgenes

Base Editing (prime editing)

Improving upon HDR

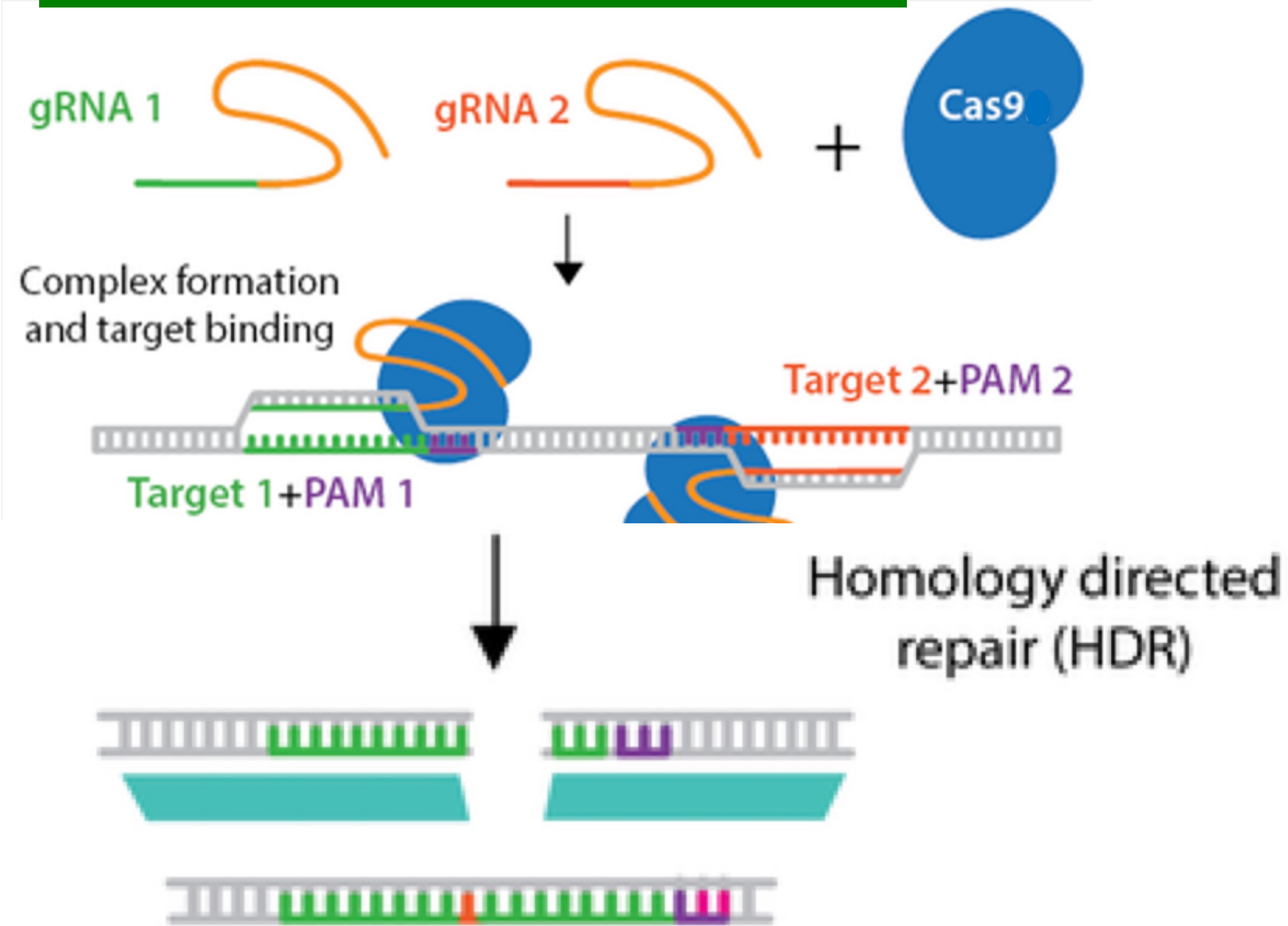


- **Base editors** can be created by fusing catalytically inactive Cas9 (dCas9) to a cytidine deaminase (convert cytosine to uridine - **C to T**) or adenine deaminase (convert adenosine to inosine/guanosine - **A to G**).

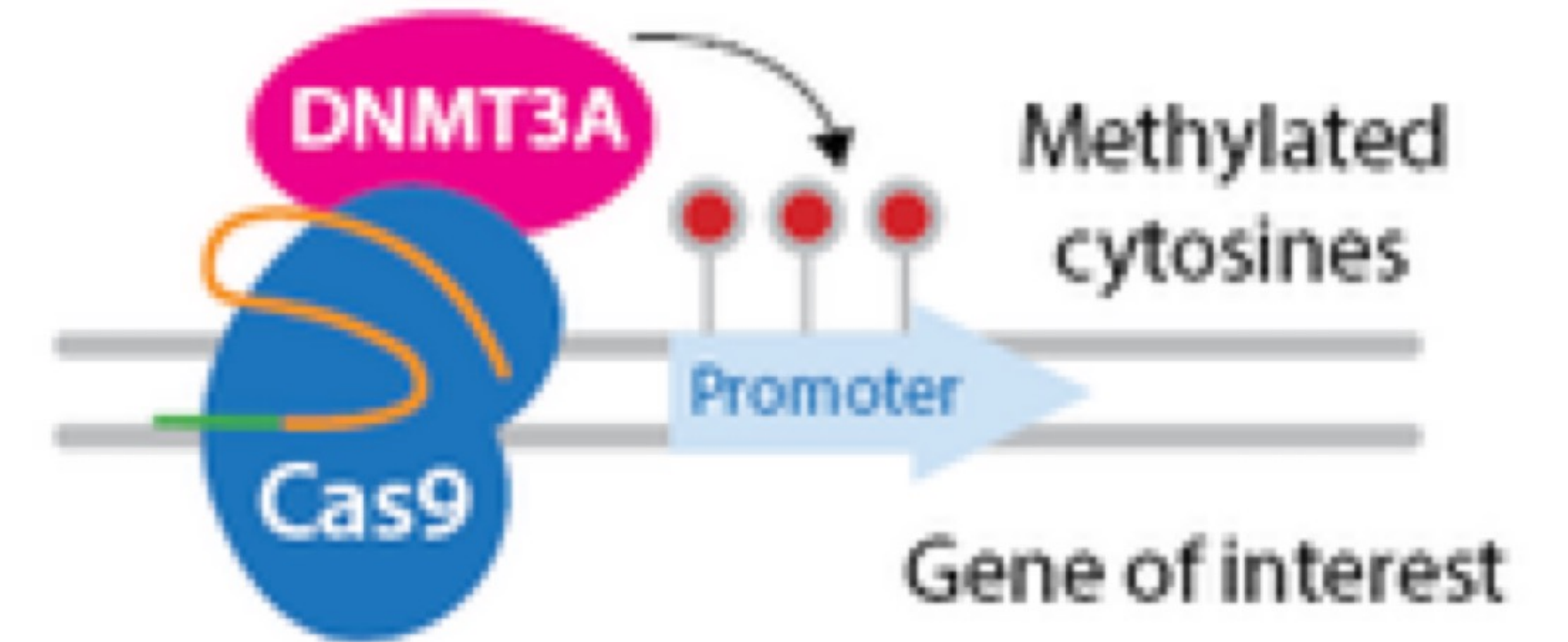
Other uses of CRISPR/Cas9

A flexible gene editing tool

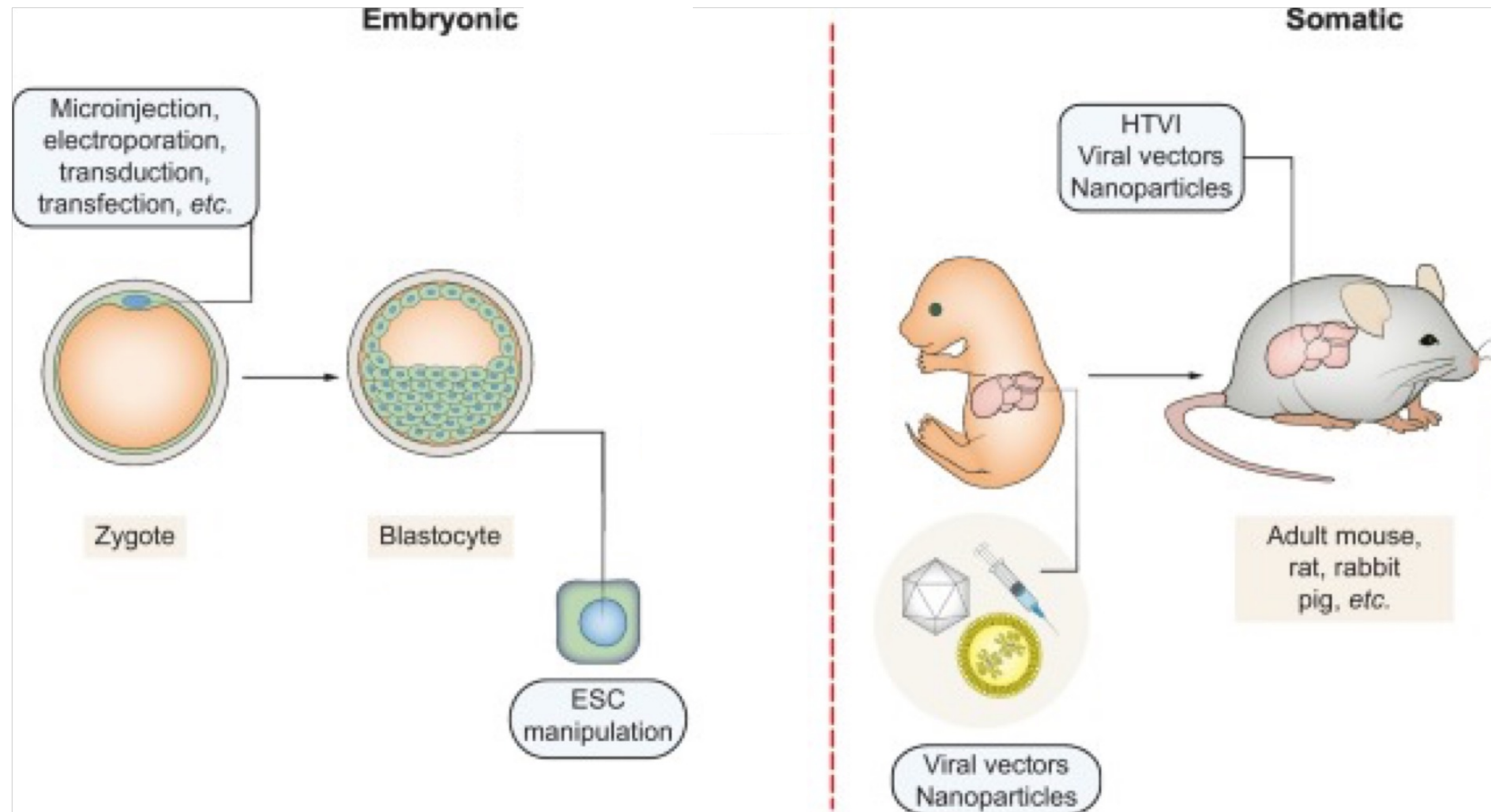
INSERTIONAL MUTAGENESIS



EPIGENETIC MODIFICATION



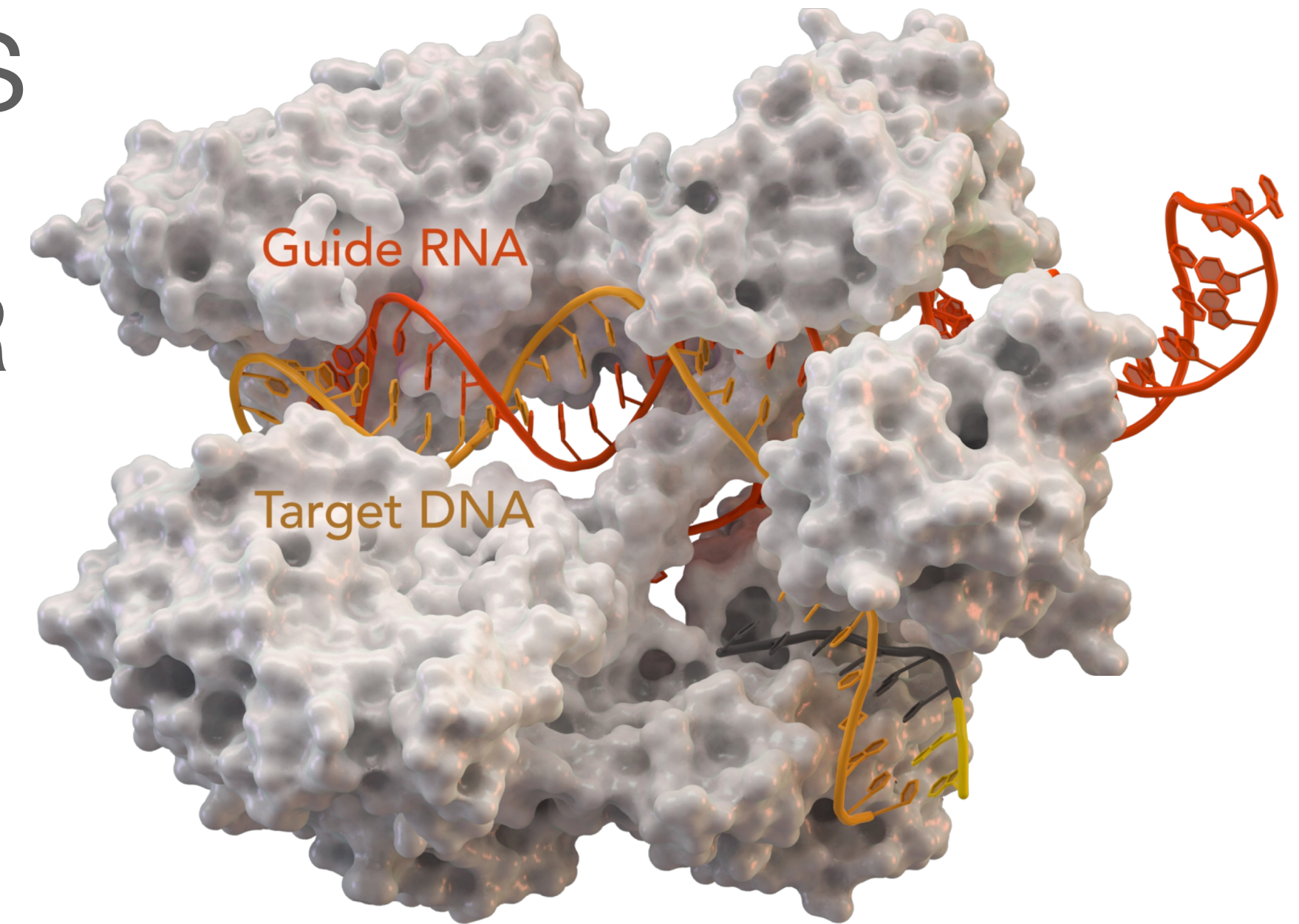
CRISPR/Cas9 germline or somatic

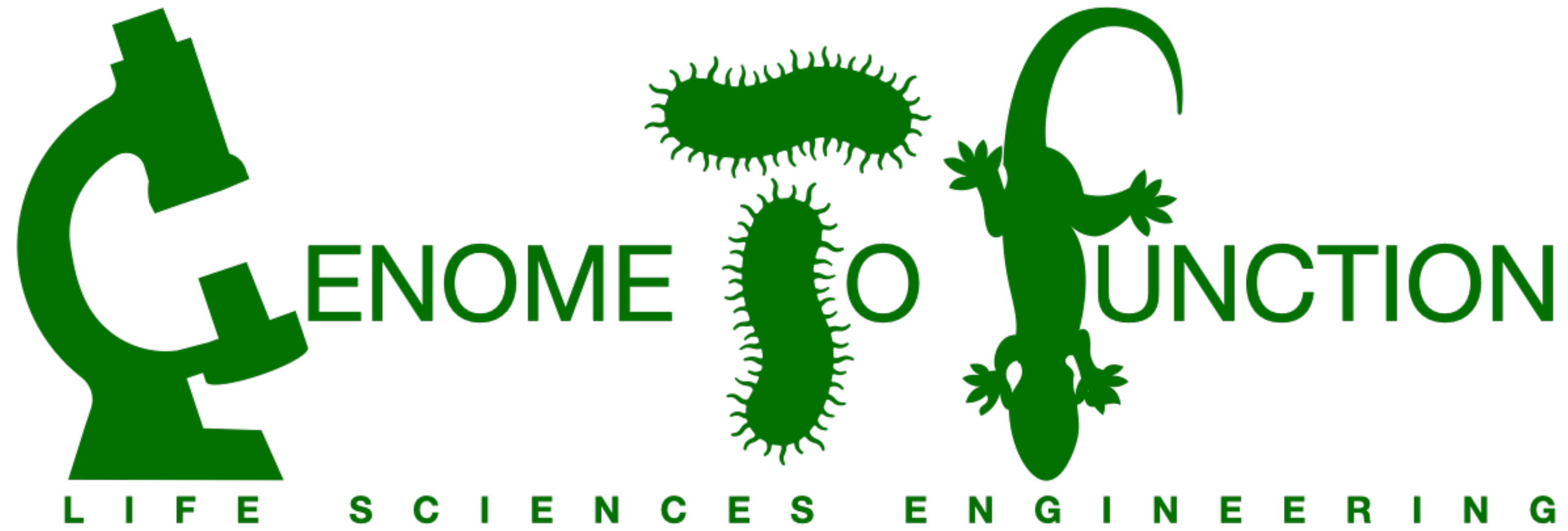


CRISPR/Cas9

Advantages and Disadvantages

- Relatively **easy** vs prior methods e.g. TALENS
- Can be **very precise** when coupled with HDR
- Can cause **off-target effects**
- Efficiency **is variable** based on target
- Immunity against Cas9 may limit human therapeutic use.





Genome editing of Humans

First CRISPR human therapy

Sickle Cell Disease

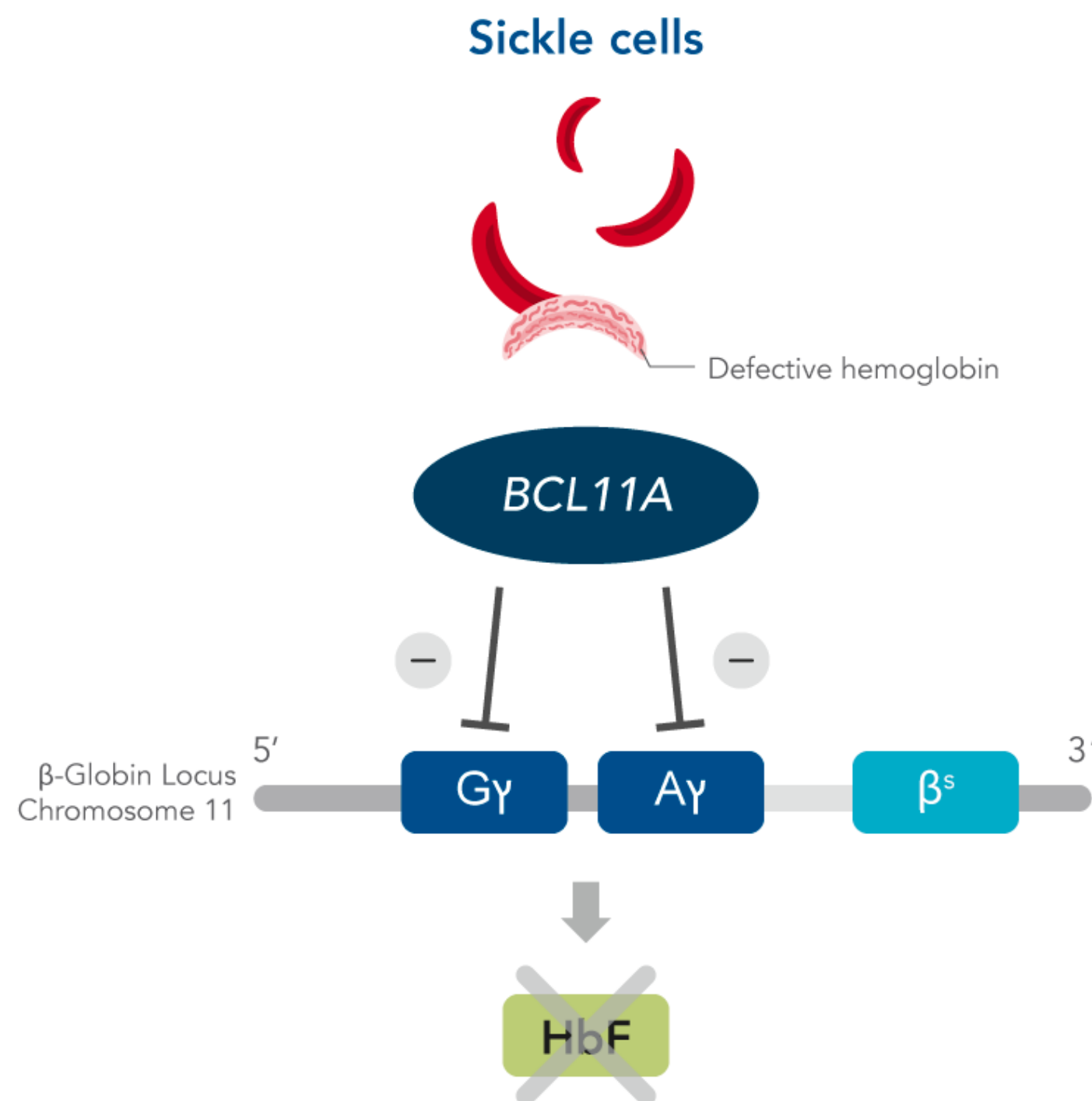
- **Sickle cell disease (SCD)** is one of the most common genetic diseases affecting more than 3 million people worldwide
- SCD is caused by **point mutations** in the beta hemoglobin gene
- A fetal-to-adult hemoglobin (HbF) **switch occurs after birth**
- **BCL11A is a transcription factor** that represses fetal HbF
- **Inhibiting BCL11A increases fetal HbF and represses adult sickle hemoglobin**

First CRISPR human therapy

Sickle Cell Disease ex-vivo treatment

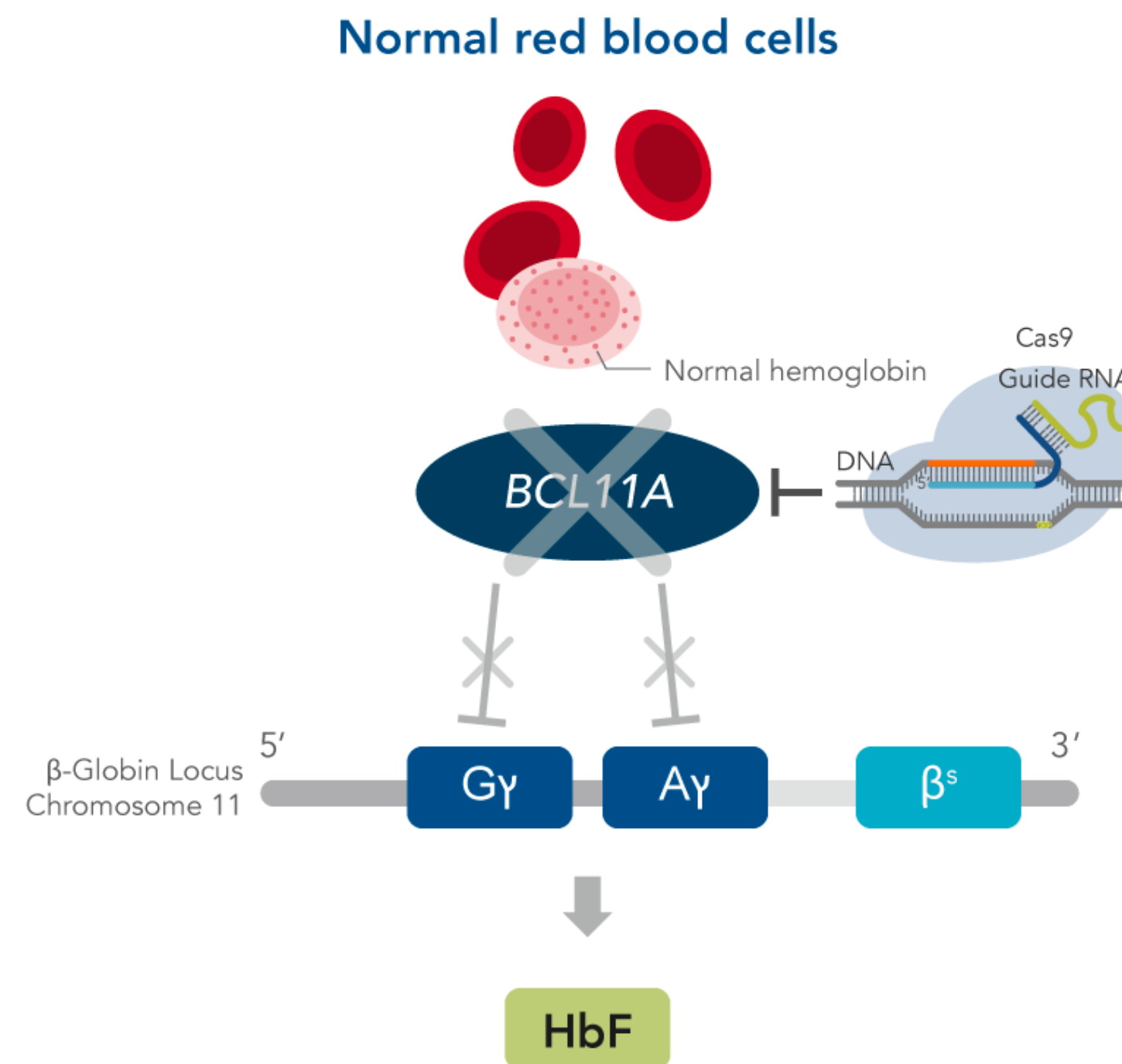
Adult SCD patient

BCL11A blocks gamma globin to repress HbF expression causing sickle hemoglobin

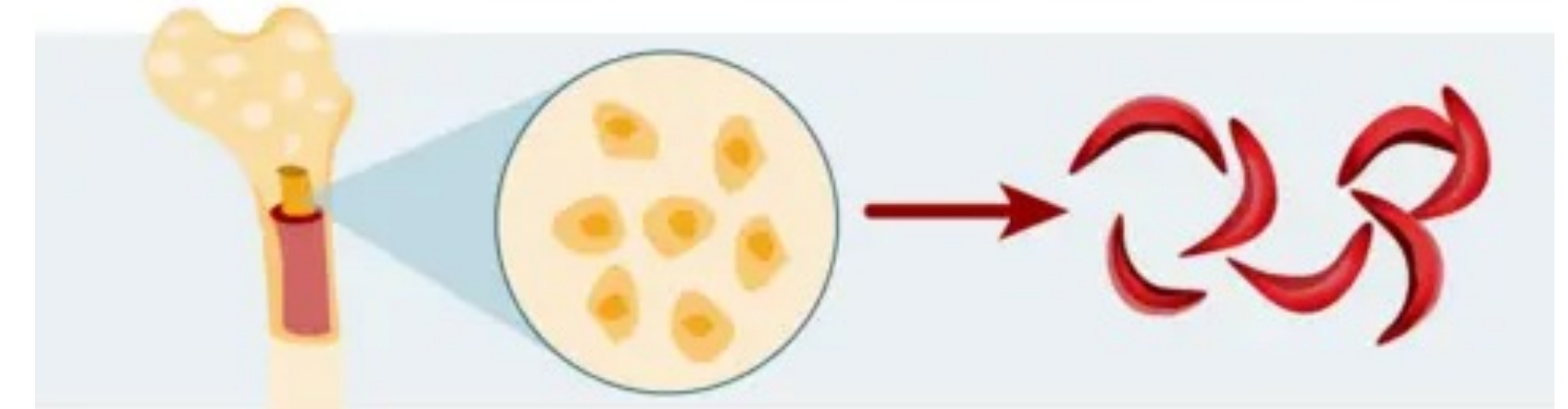


Edited Cells

CRISPR-Cas9 gene editing targets *BCL11A* in erythroid lineage increasing HbF expression and rescuing adult hemoglobin



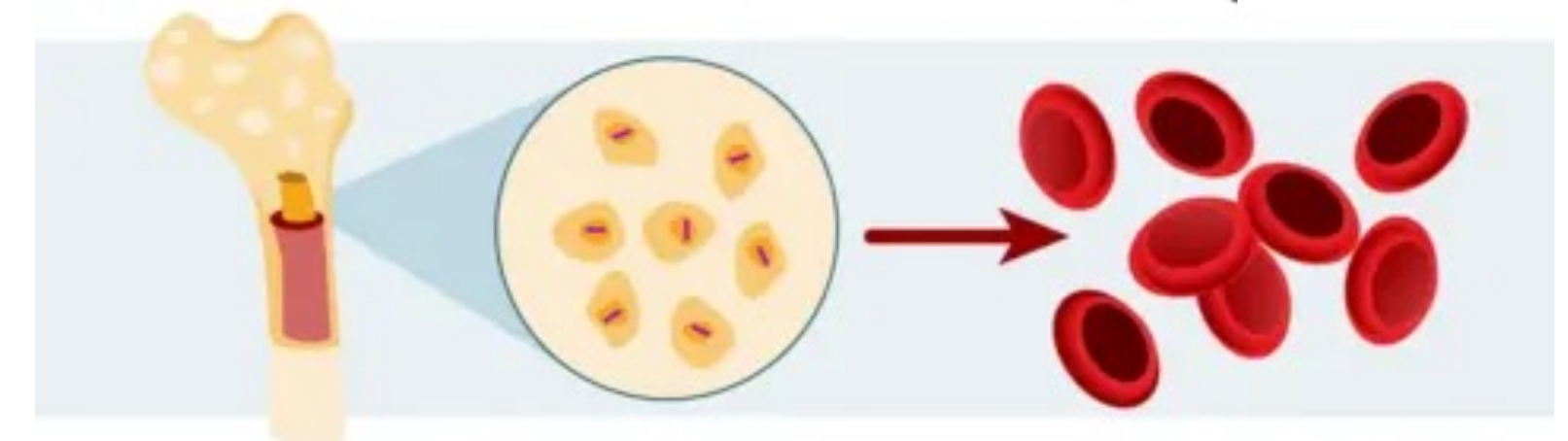
How the treatment works



- 1 stem cells in his bone marrow make diseased haemoglobin that can make red blood cells sickle-shaped



- 2 Stem cells extracted
- 3 Stem cells genetically modified
- 4 Genetically engineered stem cells



- 5 Engineered stem cells make healthy fetal haemoglobin and normal red blood cells

He Jiankui experiment

- Goal was to protect genome edited progeny from HIV infection
- Took sperm and eggs from donor couples, performed *in vitro* fertilisation and edited the genome
- Used CRISPR/Cas9 to produce an indel and thus frameshift of the gene encoding CCR5 (C-C chemokine receptor type 5)
- CCR5 Δ ³² is a natural variant of CCR5 that protects from some but not all modes of HIV infection
- Children were mosaic for the mutation, effects on germline unknown.
- Long term effects on children unknown, researcher jailed for 3 years.





In Person quiz

Human germline gene editing

- A. Is a good idea
- B. Is a bad idea
- C. Has limited use cases
that may be OK



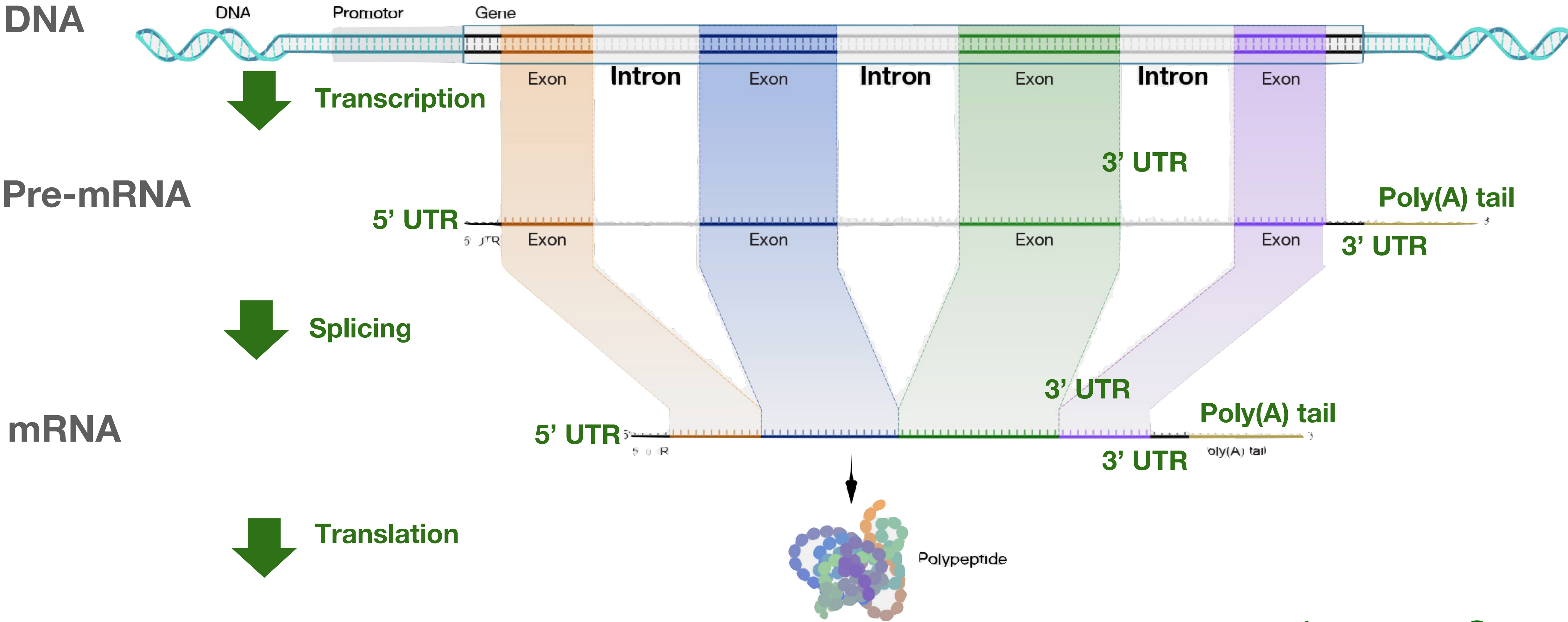
Manipulating RNA

mRNA

messenger RNA

- messenger **R**ibonucleic **A**cid (mRNA) is a single strand of RNA that corresponds to the nucleotide sequence of a gene after introns have been removed.
- mRNAs are read by **ribosomes** to synthesize proteins from protein encoding gene.

mRNA features



5' and 3' UTRs

Untranslated regions of mRNAs

- **Five Prime Untranslated Region (5' UTR)** or leader sequence is the section of mRNA (mRNA) directly 5' (upstream) from the start codon. It is important for efficient protein translation.
- **Three Prime UTR (3'-UTR)** is the region of mRNA that immediately follows the translation stop codon.
- 3'-UTR **often have regulatory functions** that can influence gene expression after transcription (post-transcription regulation)
- 3'-UTRs commonly contain AU-rich elements which can affect the **stability or decay rate of mRNA** transcripts
- 3'-UTR directs the addition of an mRNA **poly(A) tail**. Poly(A) tails (addition of several hundred adenines) are required for **mRNA stability, export and translation**

Non-coding RNAs

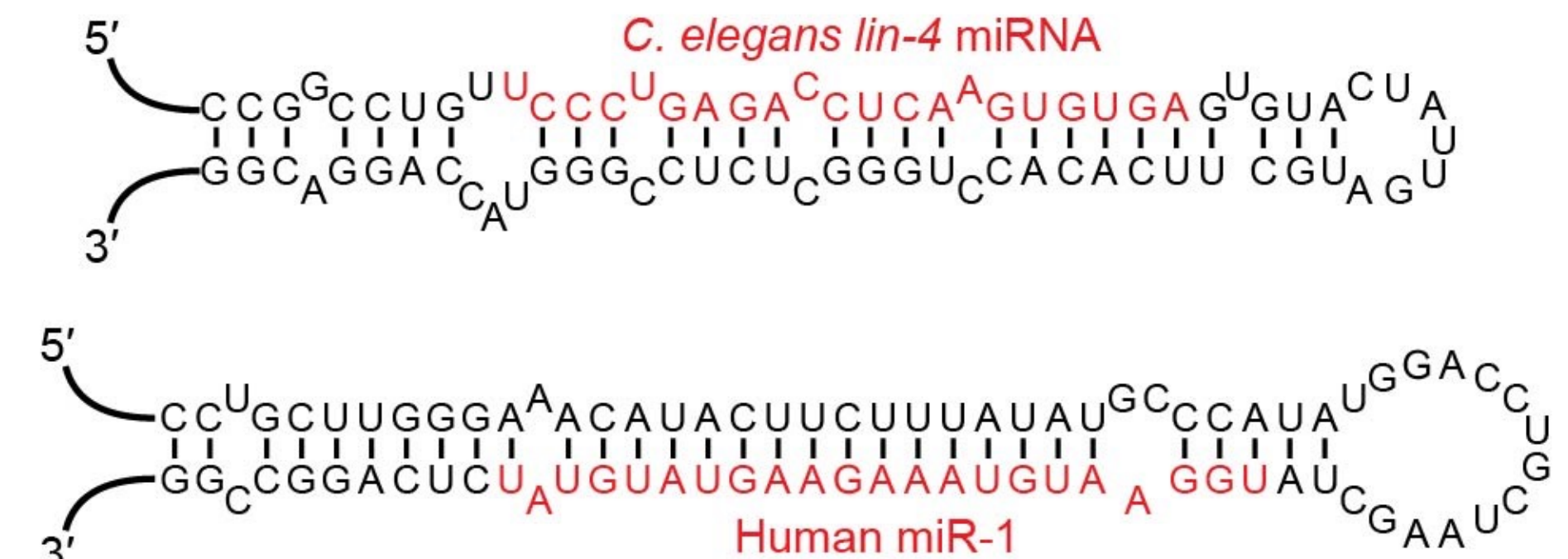
ncRNA

- **‘Housekeeping’ ncRNAs:** e.g. snoRNAs (small nucleolar RNAs), snRNAs (small nuclear RNAs), tRNAs (transfer RNAs), rRNAs (ribosome RNAs) **are involved in protein translation** or other functions like telomere related RNAs.
- **‘Regulatory’ ncRNAs:** Regulate gene expression or cell functions. Include microRNAs (**miRNAs**) and long ncRNAs (lncRNAs) in addition to piwi interaction RNAs (piRNAs), and circular RNAs (circRNAs)

miRNAs

microRNAs

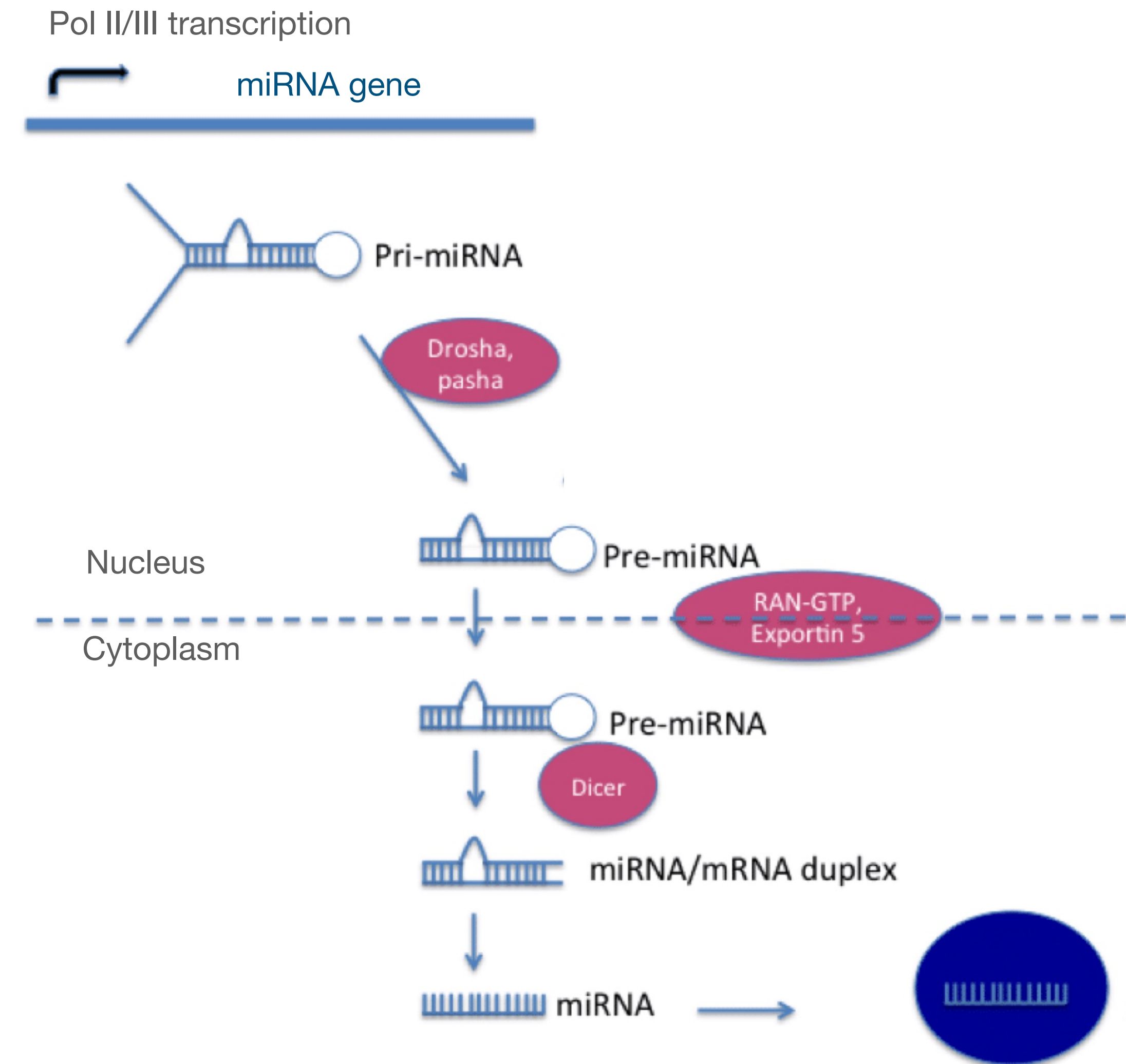
- **miRNAs** are a group of **small** ncRNA molecules ranging from 16 to 27 nt in length that can alter gene expression either during transcription and/or during translation.
- First discovered in *c.elegans*
- miRNAs directly interact with **partially** complementary sequences in the 3' untranslated region (UTR) of genes to alter expression.
- A single miRNAs regulate **can regulate many mRNAs** and thus the expression of many genes.
- The **majority mRNAs in the human genome have predicted miRNA binding sites** in their 3'UTR region
- miRNAs can be expressed **in specific tissues and at specific times.**



miRNA production

miRNAs

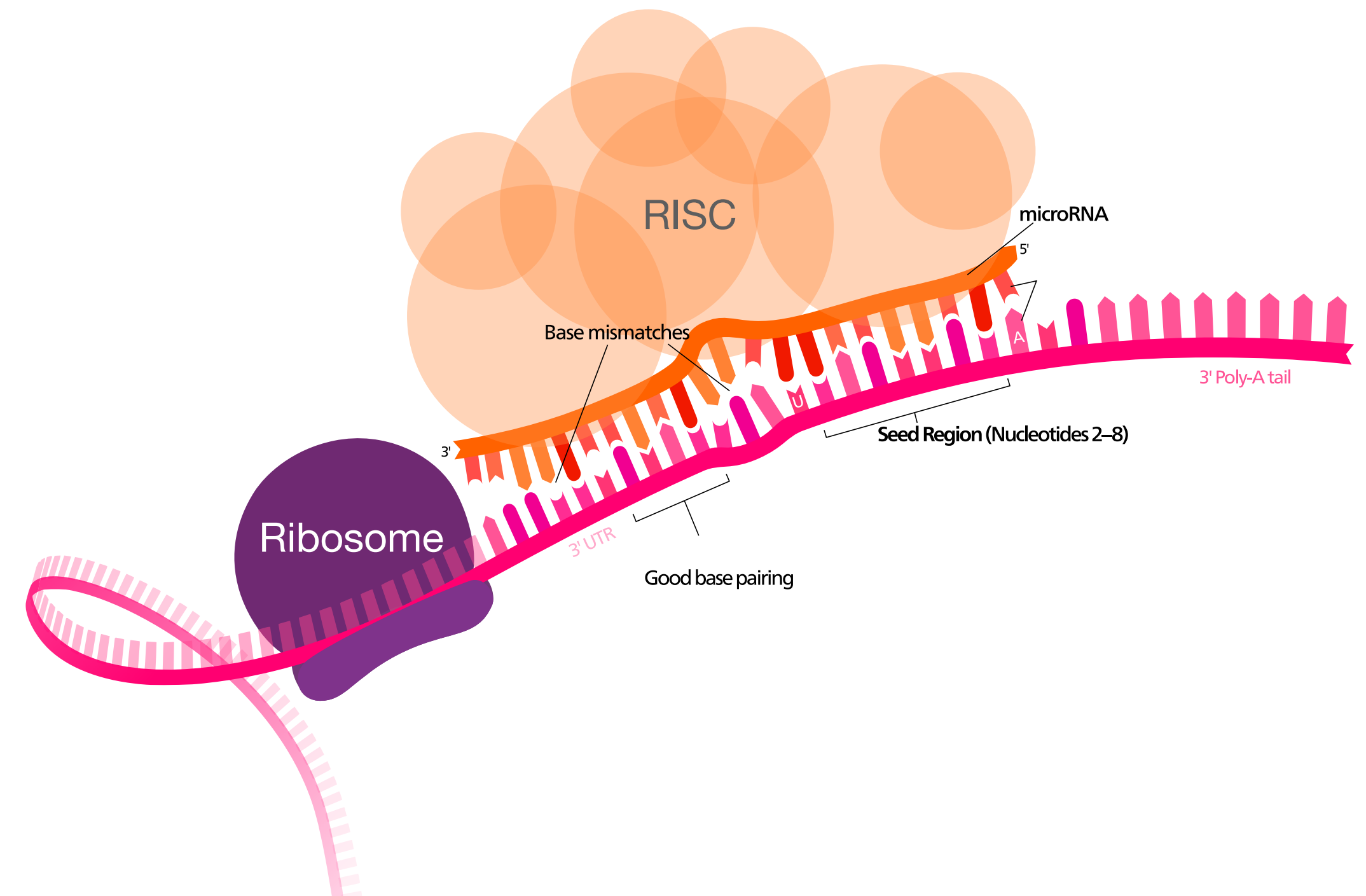
- **miRNAs** are transcribed by miRNA genes by RNA polymerase III or produced from intron sequences using RNA polymerase II as large precursor RNA molecules called **Pri-miRNAs**
- **Pri-miRNAs** are processed in the nucleus by the RNase III enzyme Drosha, and the dsRNA-binding protein, Pasha to make **Pre-miRNAs**. Pre-miRNAs are ~ **70-nucleotides** and have irregular RNA double stranded stem-loop structures.
- **Pre-miRNAs** are exported from the nucleus to the cytoplasm by a complex that includes exportin 5 and Ran-GTP.
- The final 16 to 27 nucleotide **miRNA** is produced from Pre-miRNAs by the RNase III enzyme Dicer
- The miRNA then loaded into the ribonucleotide **RISC (RNA-induced silencing complex)** complex to regulate mRNA expression.



miRNA gene regulation

RISC: RNA-induced silencing complex

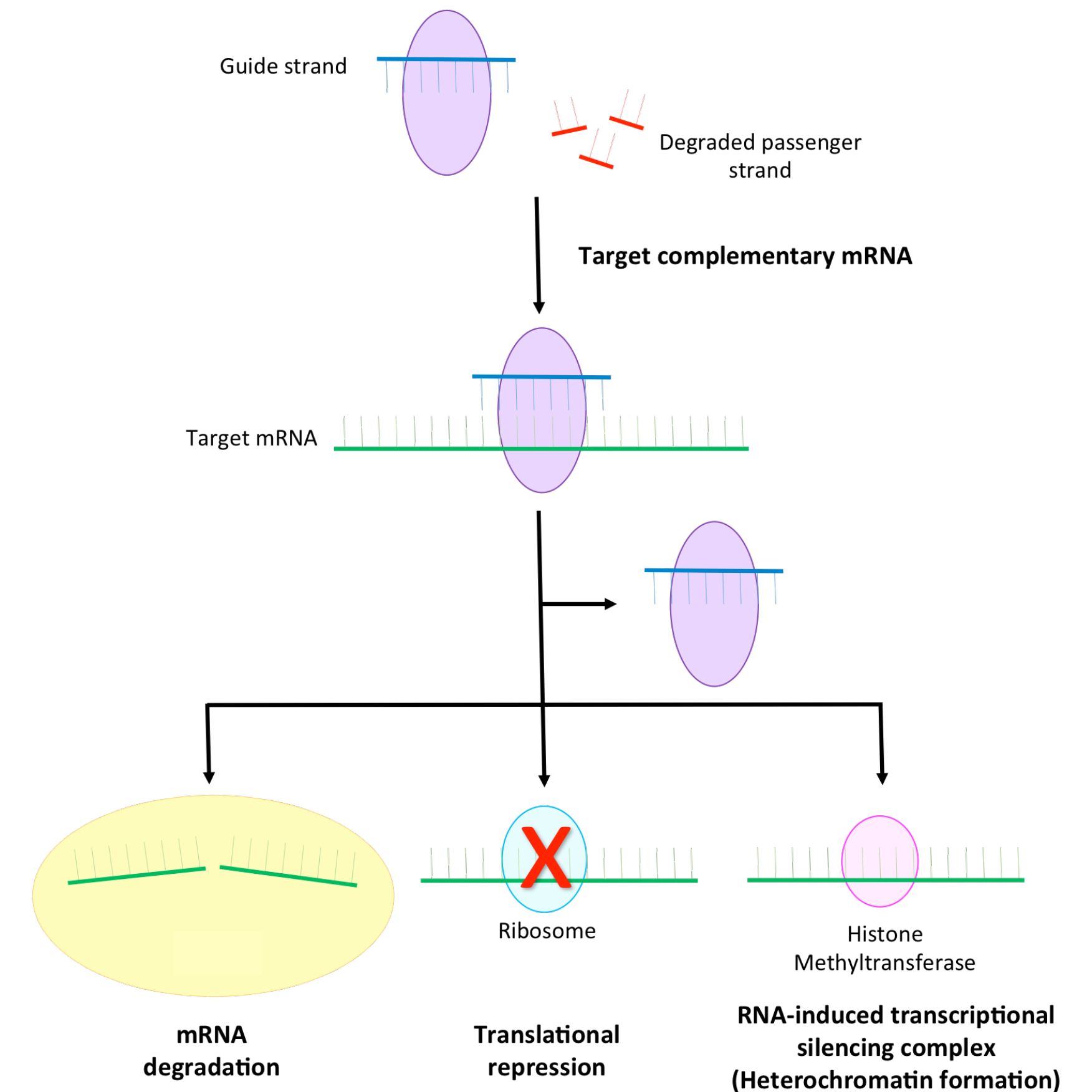
- **RISC complex:** Key proteins include Ago2, SND1, AEG-1 are essential for function
- RISC utilises the guide strand of the miRNA to target regions (**usually in the 3'UTR**) of complementary mRNA transcripts.
- Base pairing needs to be fully complementary in a seed region **but mismatches or imperfect pairing are common** in other regions



miRNA gene regulation

RISC: RNA-induced silencing complex

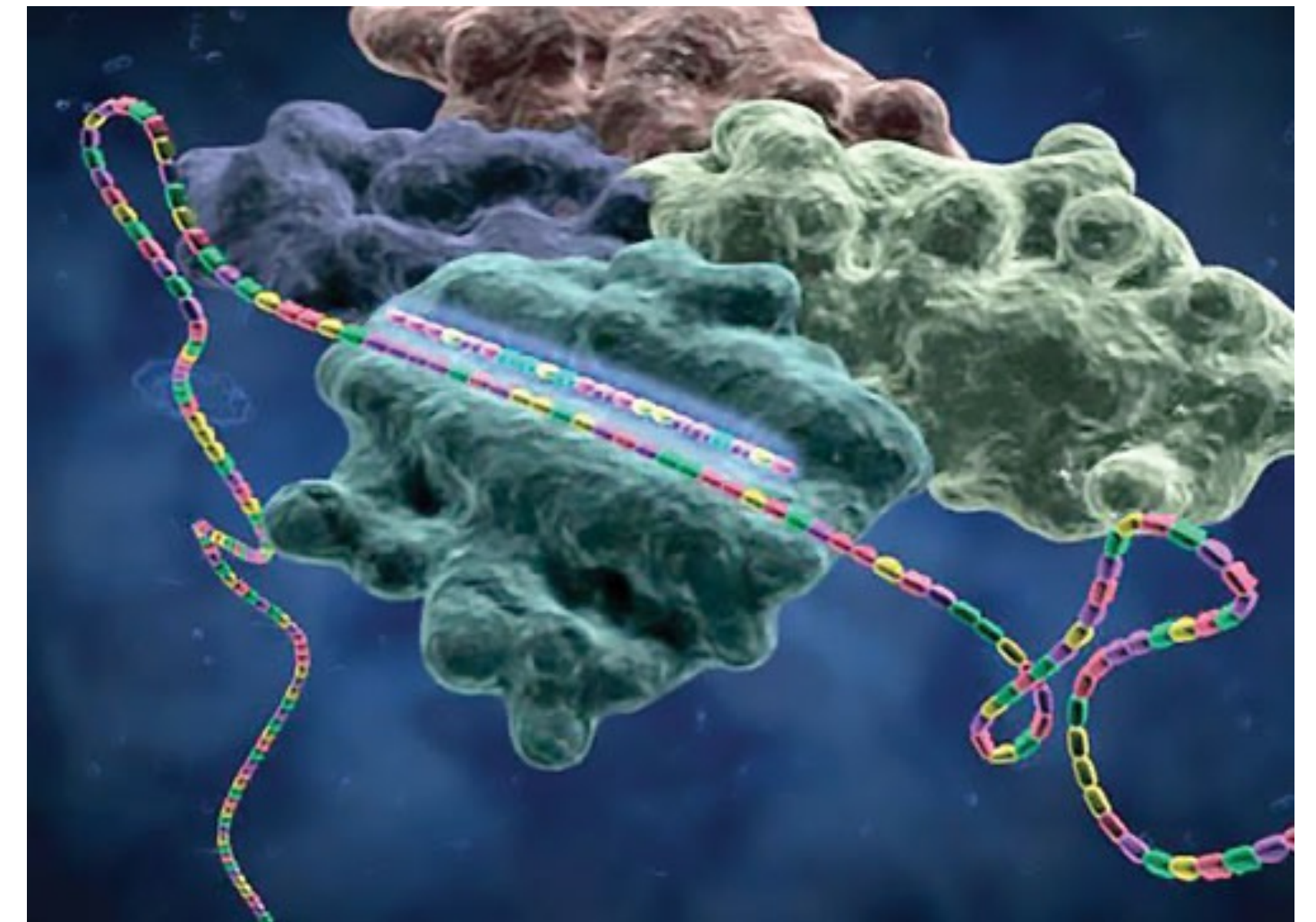
- **RISC complex:** Key proteins include Ago2, SND1, AEG-1 are essential for function
- RISC utilises the guide strand of the miRNA to target regions (usually in the 3'UTR) of **complementary mRNA transcripts.**
- Base pairing needs to be fully complementary in a seed region **but mismatches or imperfect pairing are common** in other regions
- 2 primary modes of mRNA silencing - **mRNA degradation** or **translational inhibition.**
- Less well understood is miRNA dependent **epigenetic regulation**



RNA interference

RNAi

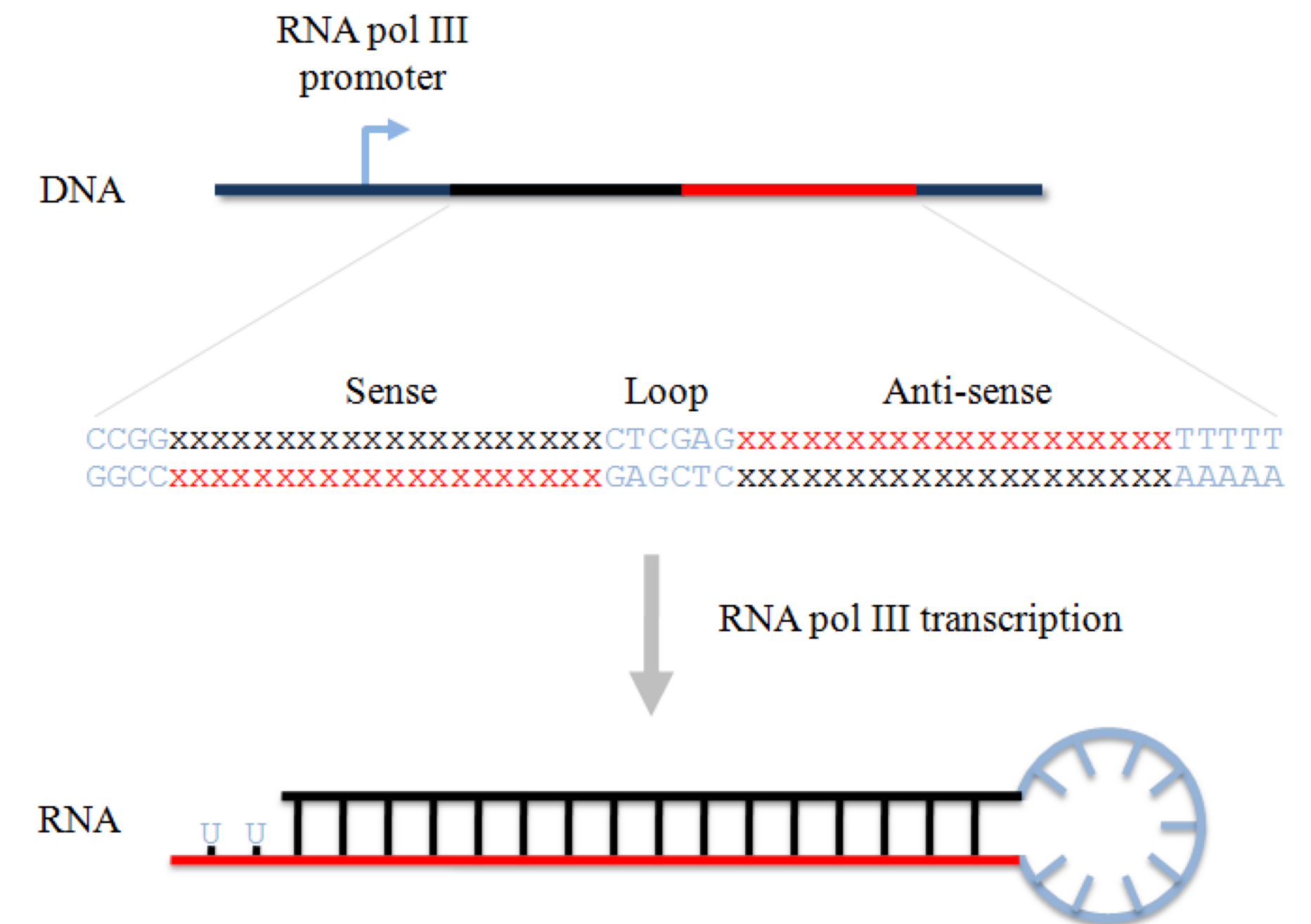
- **RNA interference (RNAi)** is a method to inhibit gene expression with RNA molecules
- short RNA molecules are created that are **perfectly complementary** to endogenous target mRNAs
- Binding of the introduced short RNA to the target mRNA **inhibits the target mRNA** expression via similar mechanisms to endogenous miRNAs i.e. through the RISC complex
- The target sequence does **NOT** have to be in the 3'UTR of the mRNA



Short Hairpin RNAs

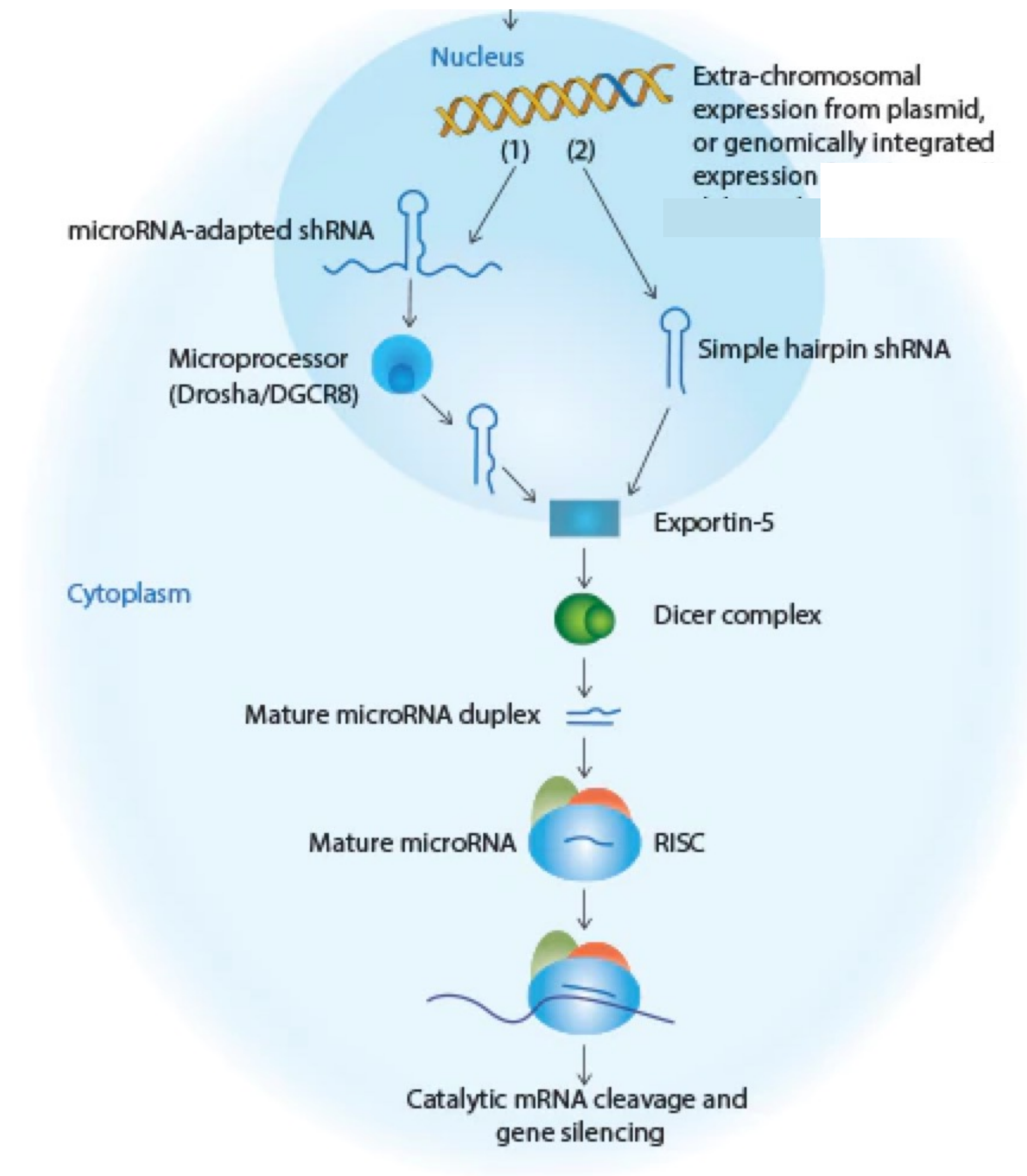
shRNAs

- **Small hairpin RNAs (shRNAs)** are sequences of RNA, ~80 base pairs in length, that include regions designed to create a hairpin structure.
- shRNA molecules **are processed similarly to endogenous miRNA** molecules
- **ShRNA encoding genes** be introduced into cells on plasmids or via transgenes.
- **Libraries** of shRNA genes can be created to screen the entire genome
- Some shRNAs are **more effective than others** so often several shRNAs targeting a single gene are tested.



Short Hairpin RNAs

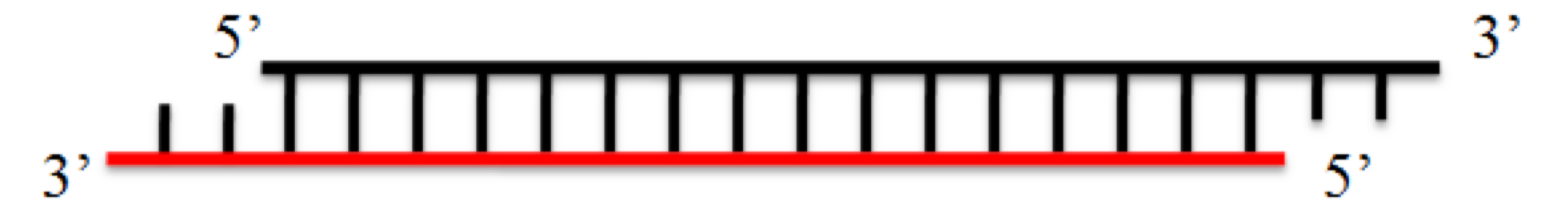
shRNAs



Short interference RNA

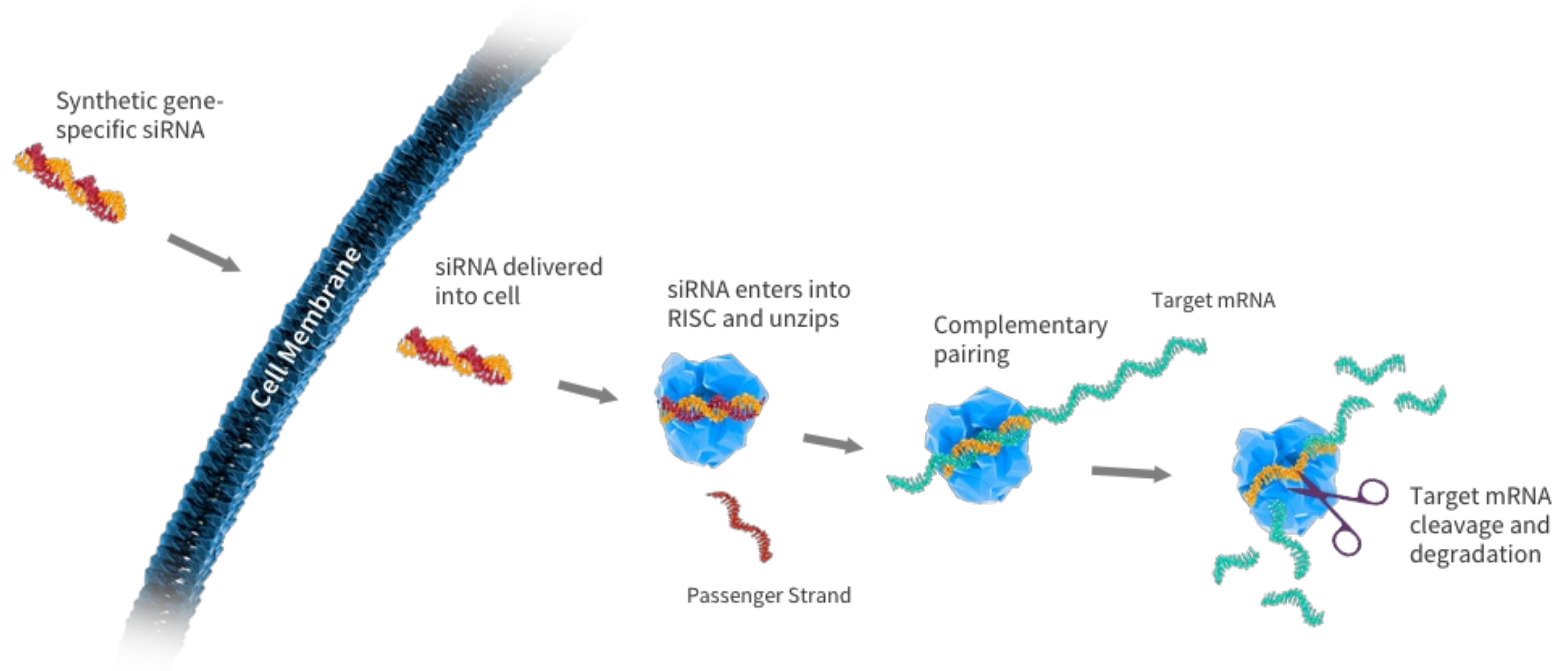
siRNAs

- **Small interfering RNA (siRNA)** is a double stranded RNA with 2 nucleotide 3' end overhangs
- 3' overhangs **help activate RNAi**
- siRNAs can be **directly** introduced into cells in tissue culture
- Similarly to shRNAs they will be **loaded in the RISC complex**
- siRNAs between **19–29 nucleotides** are the most effective.
- Ideally employ multiple siRNAs with **target sequences at different positions** in the mRNA to be inhibited.
- **Libraries** of siRNAs allow the entire genome to be screened in cell culture.



Short interference RNA

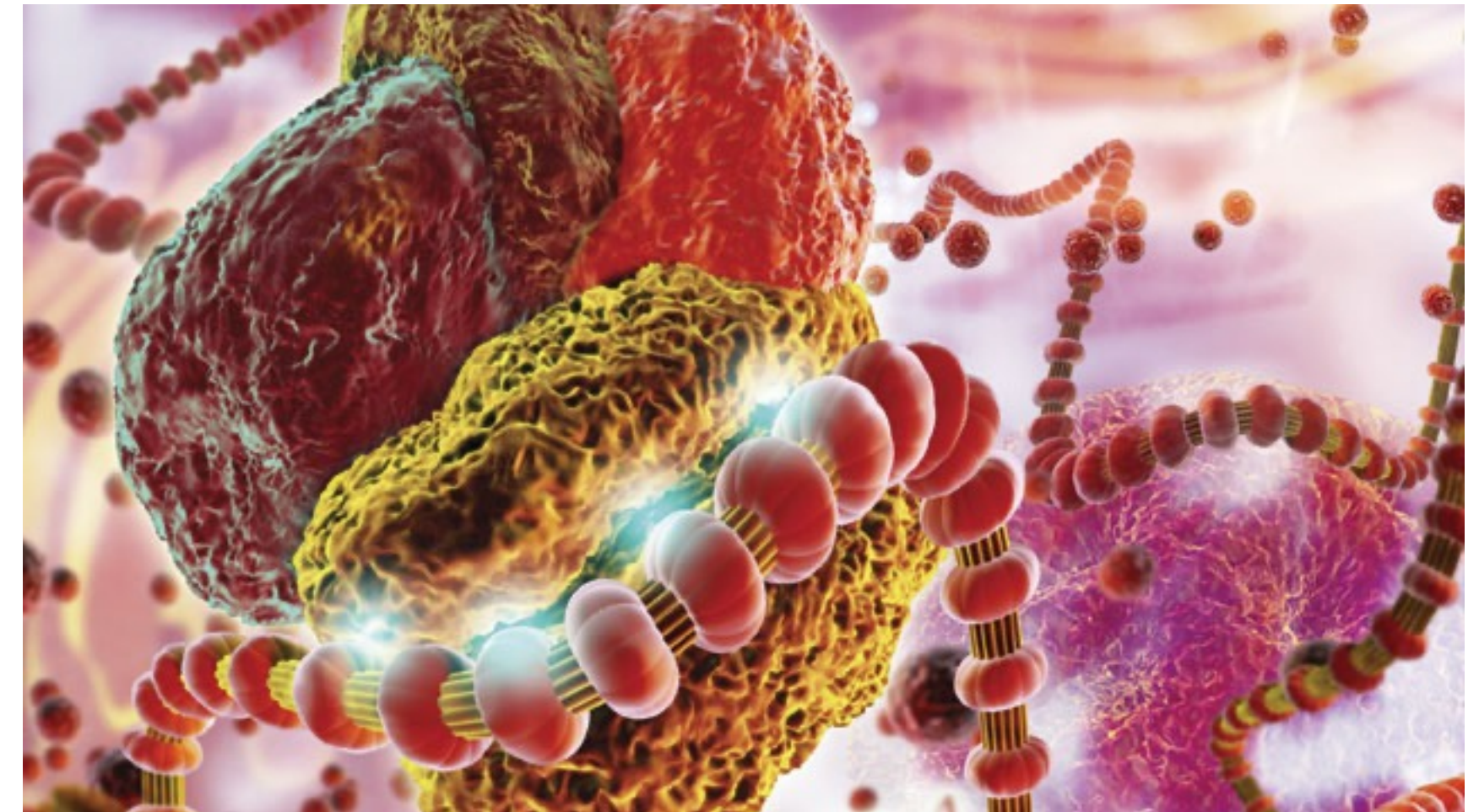
siRNAs



RNA interference

Problems

- **Off target** mRNA inhibition. One reason to use multiple RNAi constructs targeting the same mRNA. siRNAs may have a higher incidence of off target effects than shRNAs
- Cell exposure to siRNA and shRNAs may change cell functions. **Need to control** with a scrambled RNAi construct
- Some siRNA and shRNA constructs **don't work** perhaps because of 3D structure of target mRNA.



Antisense Oligonucleotides

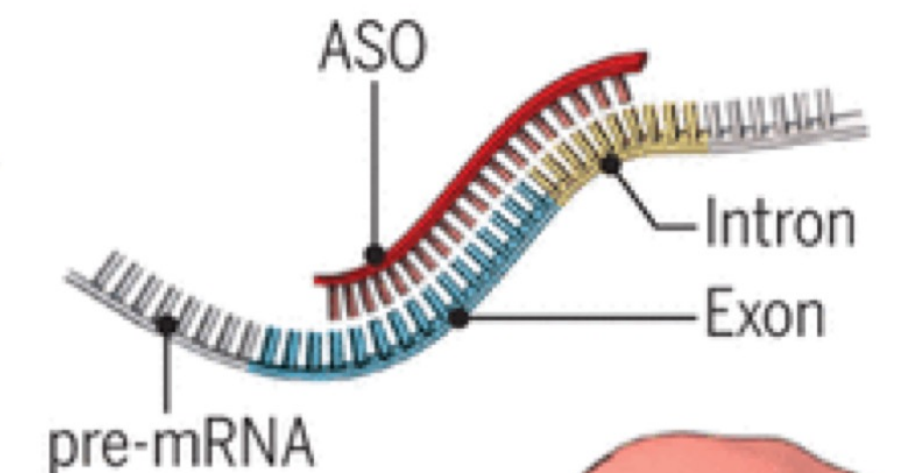
ASOs

- **Antisense oligonucleotides (ASOs)** are short, synthetic, single-stranded oligodeoxynucleotides that can alter mRNAs
- Can be used to **reduce, restore, or modify protein expression**
- **Chemically modified** oligodeoxynucleotides designed to increase hybridization affinity to their target RNA, increased resistance to nuclease degradation and reduce immunostimulatory activity
- **cell uptake is relatively poor** (high concentrations needed) and ASOs do not cross the blood-brain barrier in humans

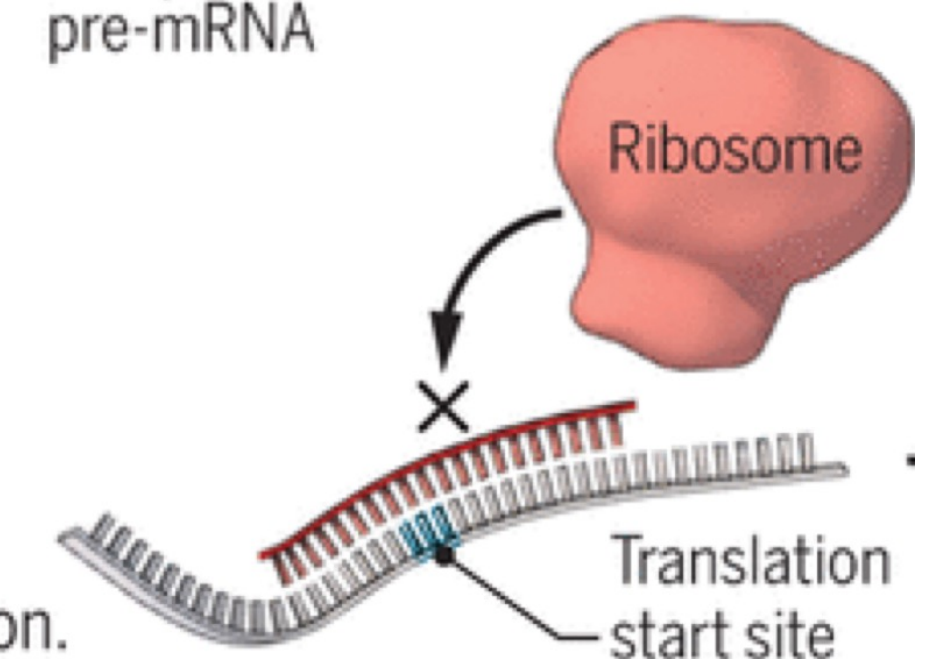
Target mutations
ASOs can target RNA transcripts that produce disease-causing proteins.



Target splice sites
Unique sequences at splice sites in pre-mRNAs can allow ASOs to modulate RNA splicing.

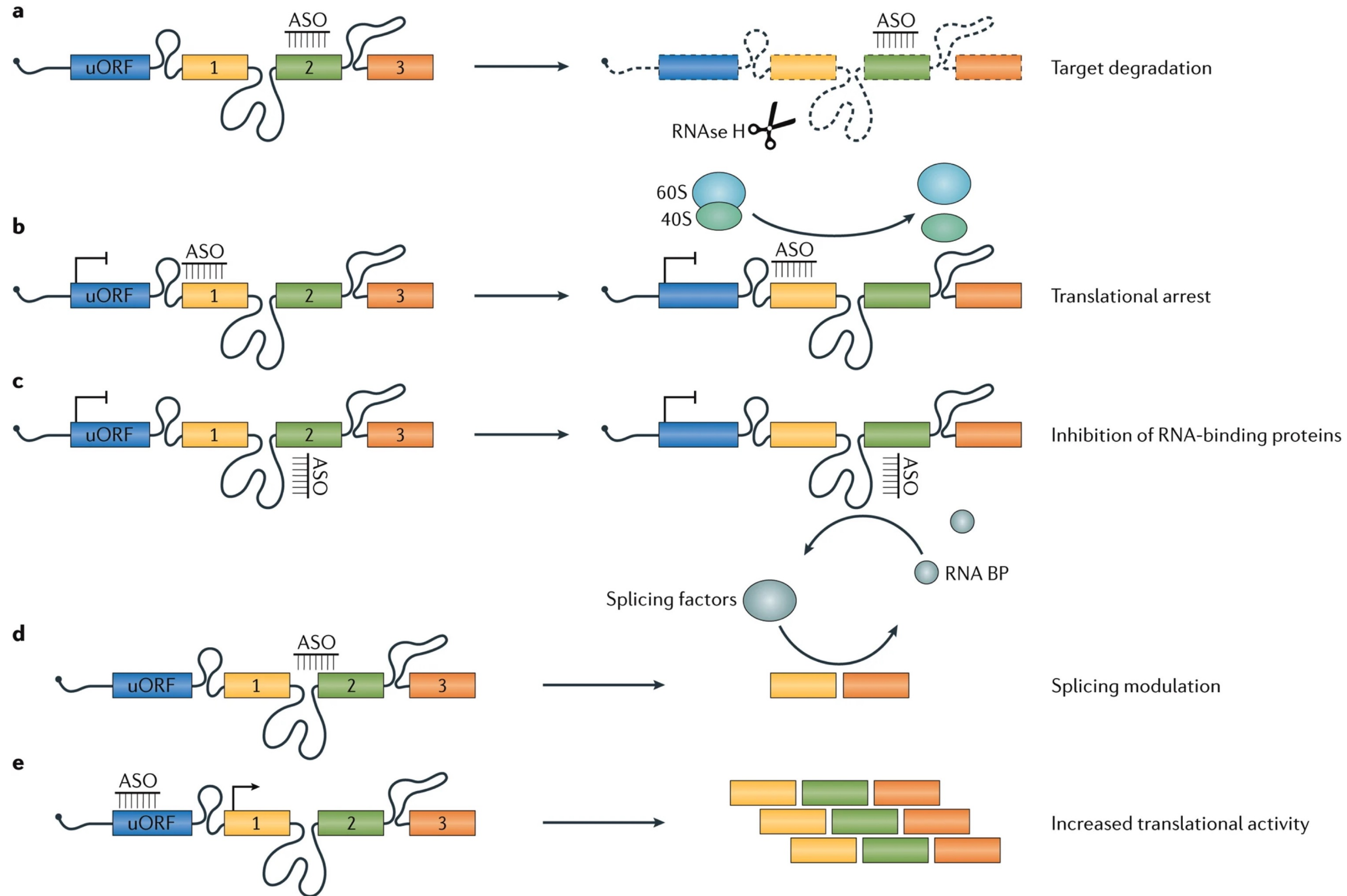


Target translation start sites
ASOs can selectively target translation start sites in mRNAs, which prevents protein translation.



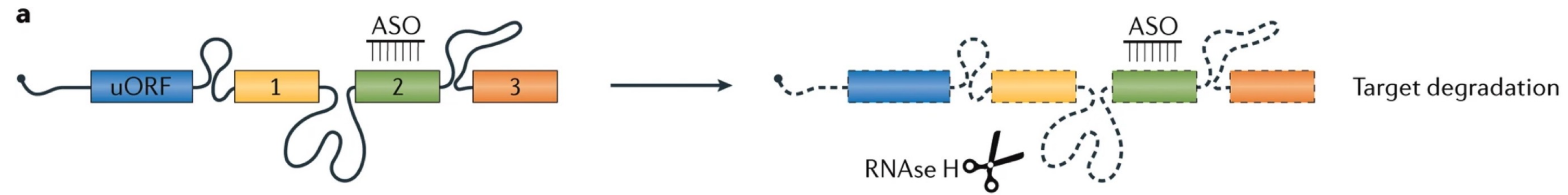
Antisense Oligonucleotides

ASOs



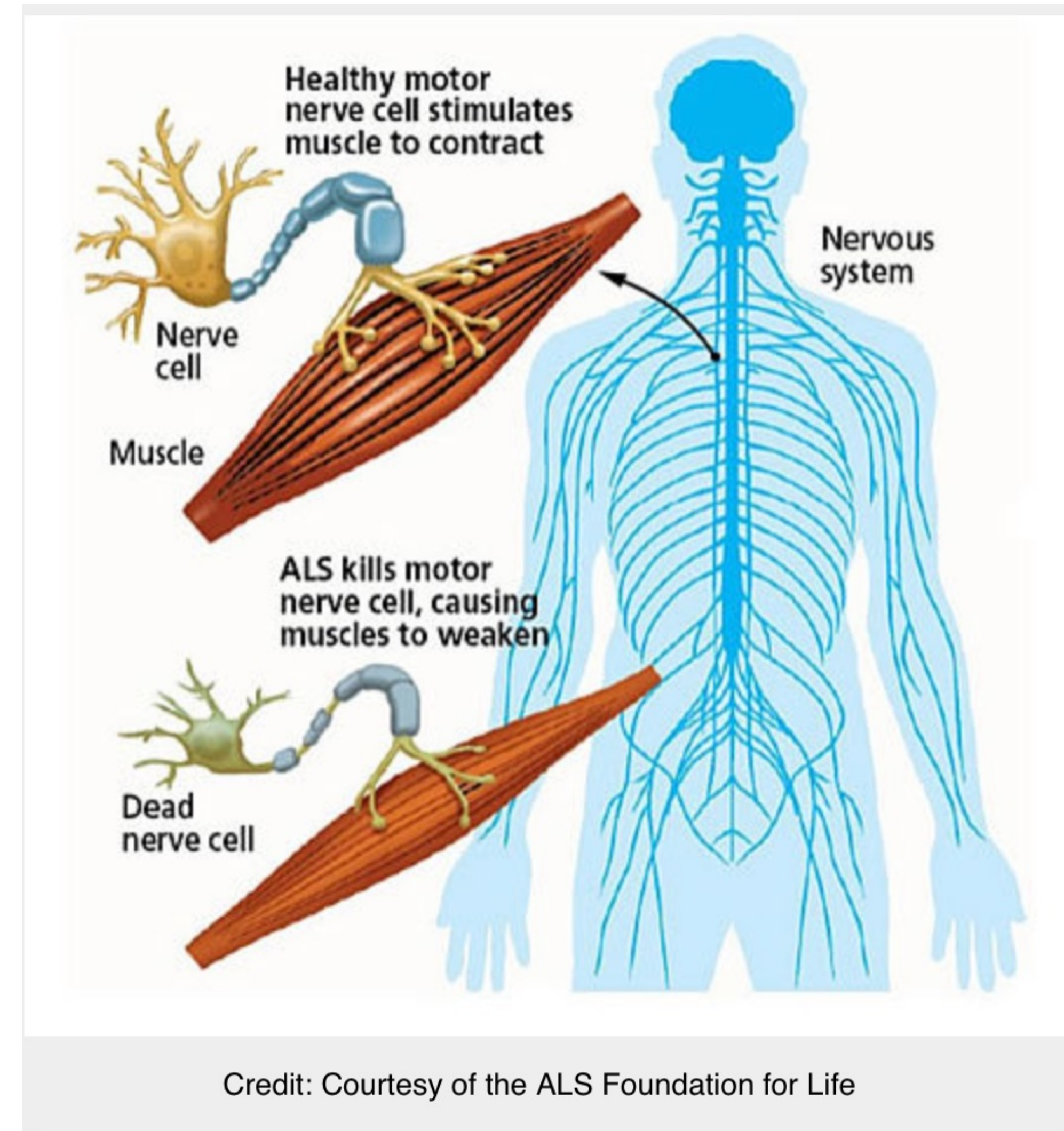
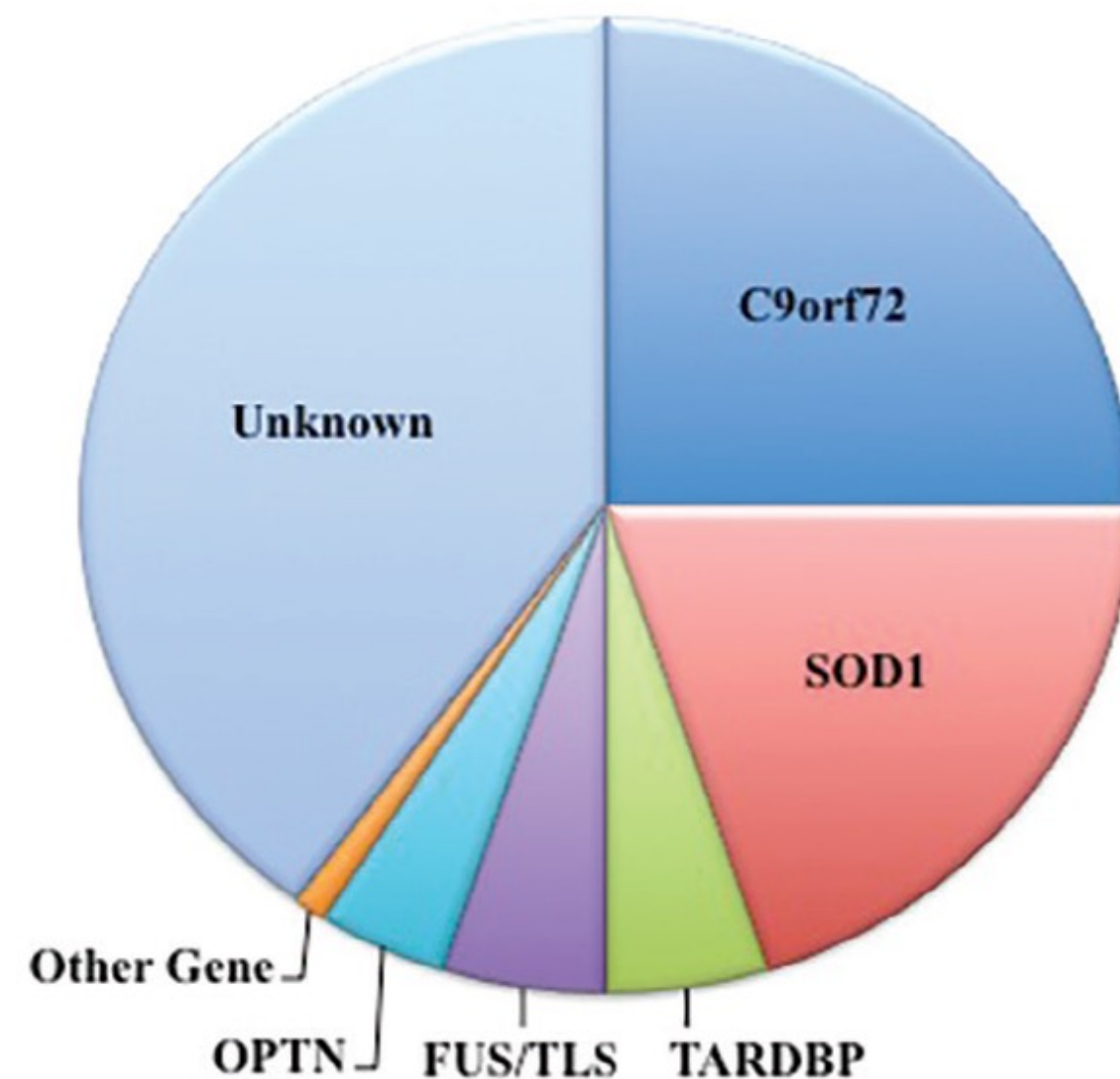
Antisense Oligonucleotides

ASOs

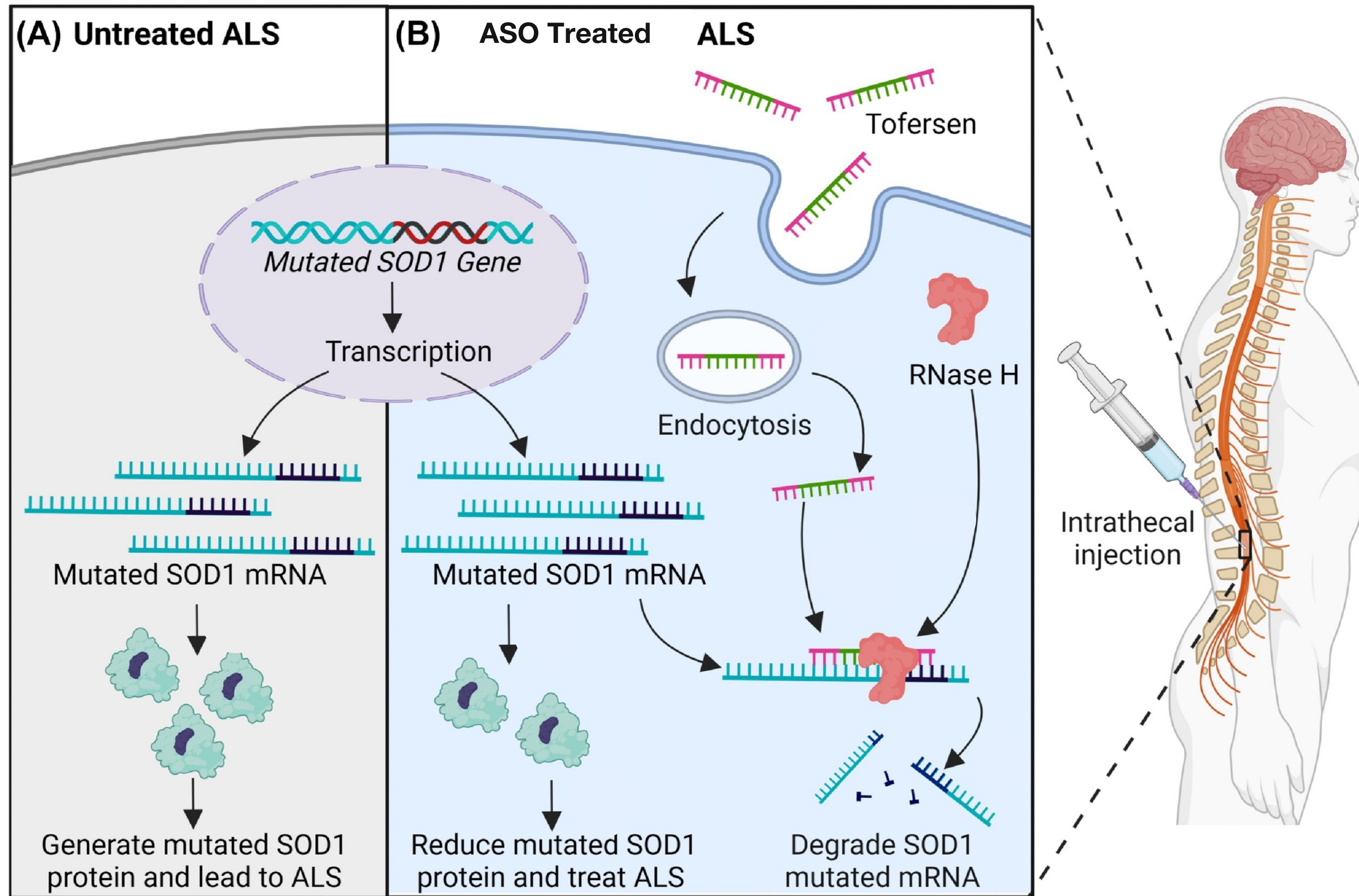


Amyotrophic lateral sclerosis

- Degeneration of motor neurons
- Muscular weakness, spasticity, paralysis
- Death after 3-5 years
- Treatments can only prolong by months
- 90% sporadic cases – cause unknown
- 10% familial mutations

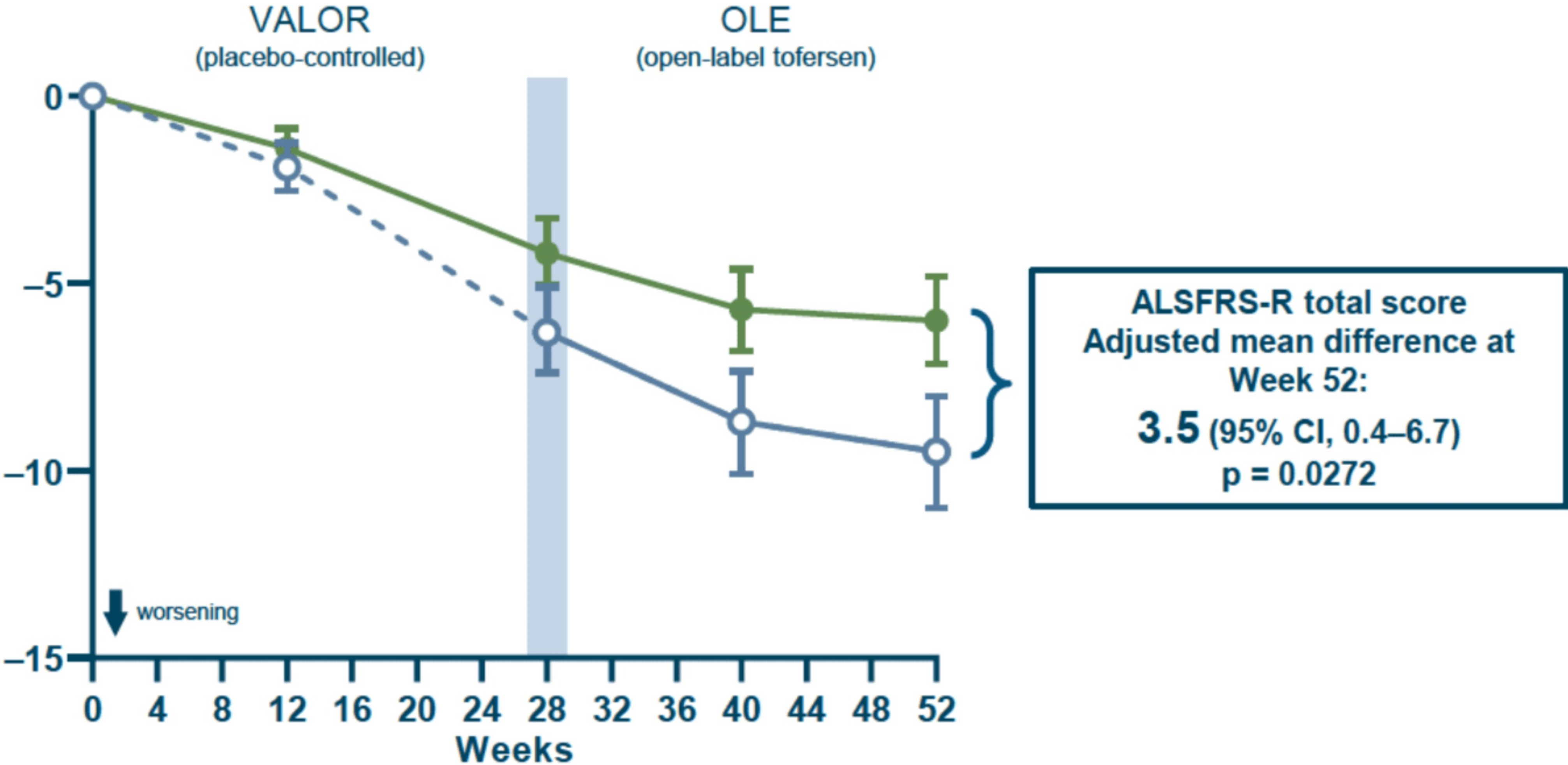


SOD1 ASO



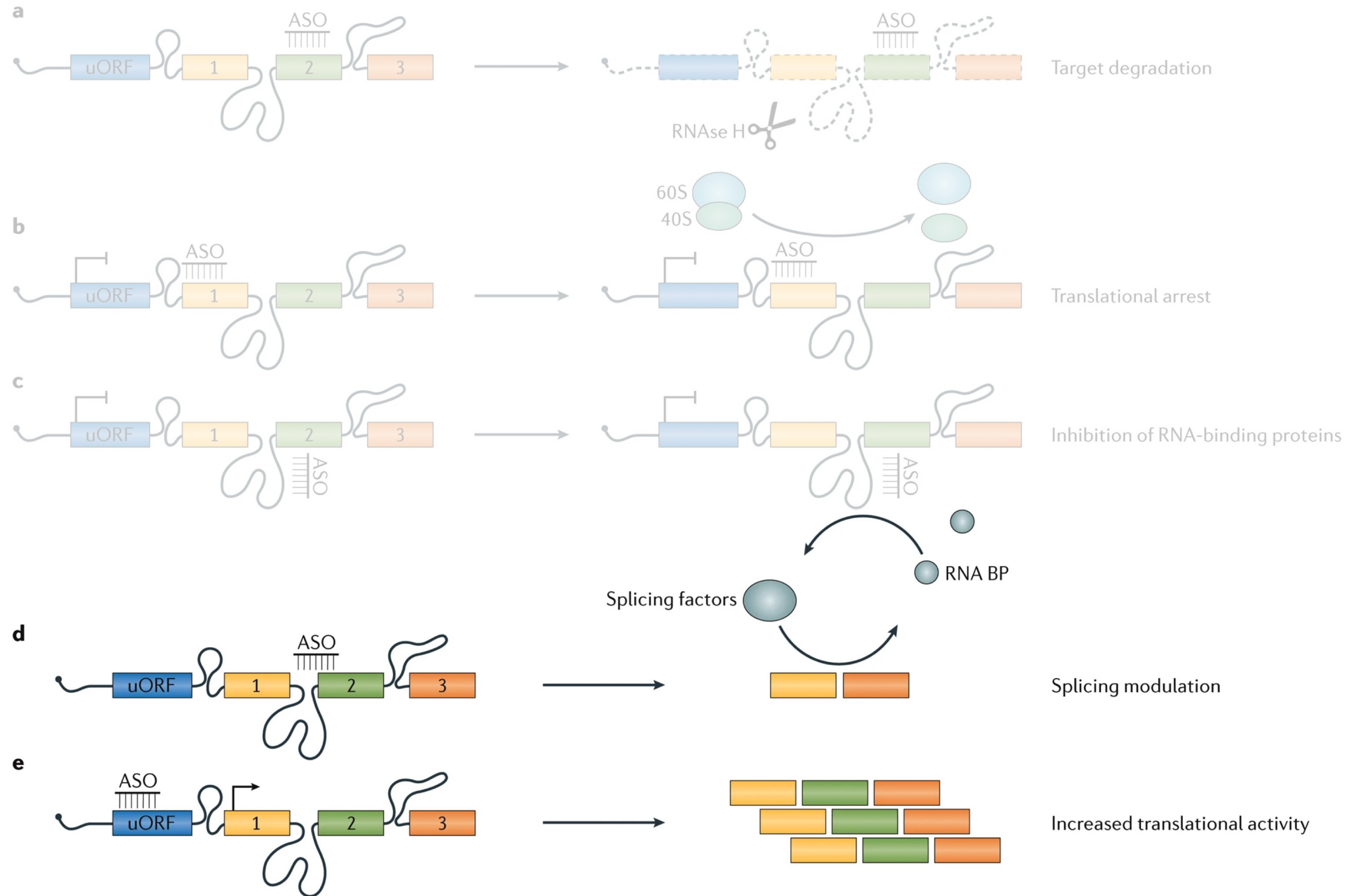
Trends in Pharmacological Sciences

SOD1 ASO



Antisense Oligonucleotides

ASOs



Spinal Muscular Atrophy (SMA)



SMA Type I

Severe form
Never sit
Limited life expectancy
Respiratory failure

Birth Prevalence 60%



SMA Type II

Intermediate form
Sitting or standing
Life expectancy shortened
Skeletal deformities

Birth Prevalence 27%

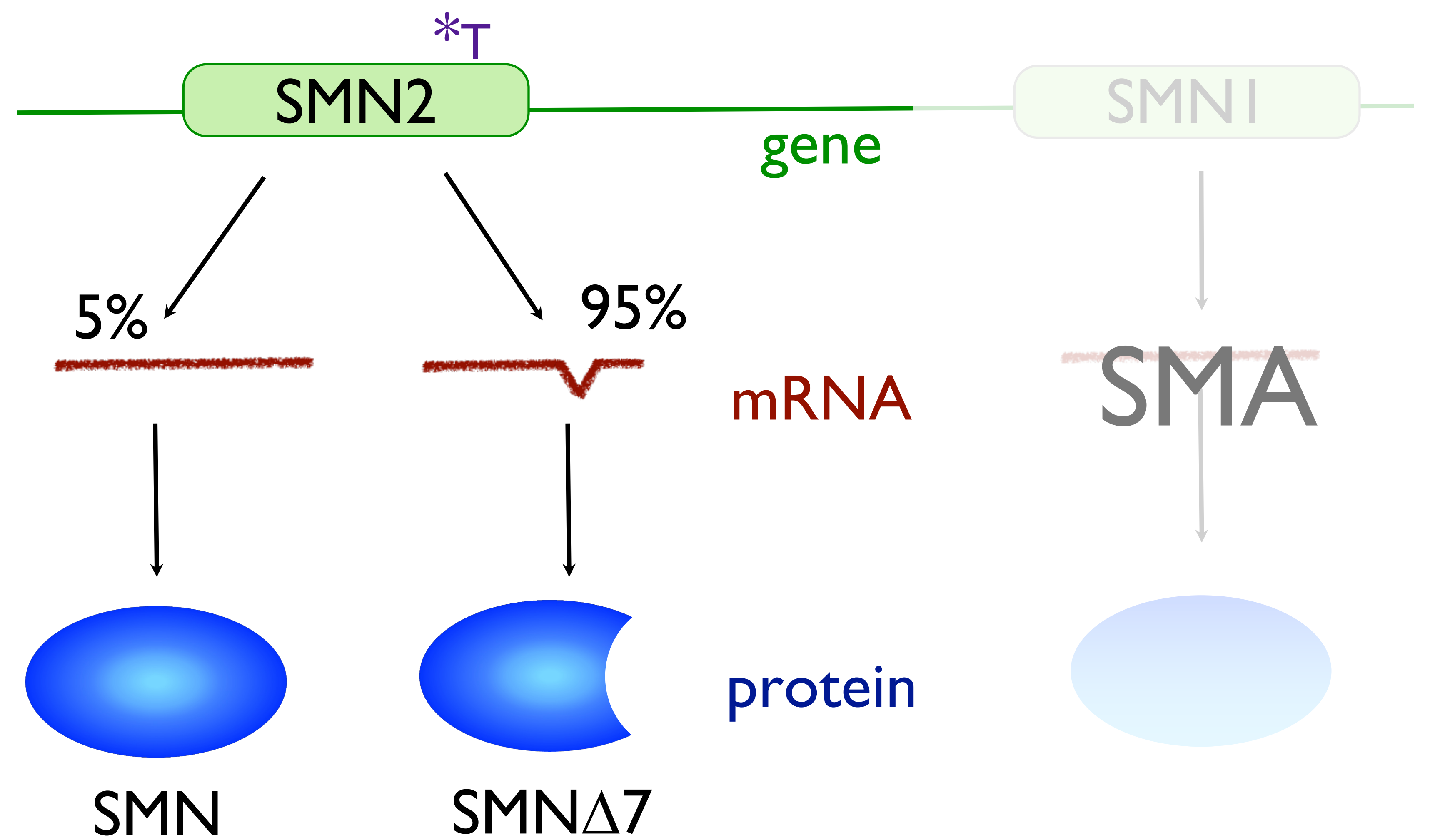


SMA Type III

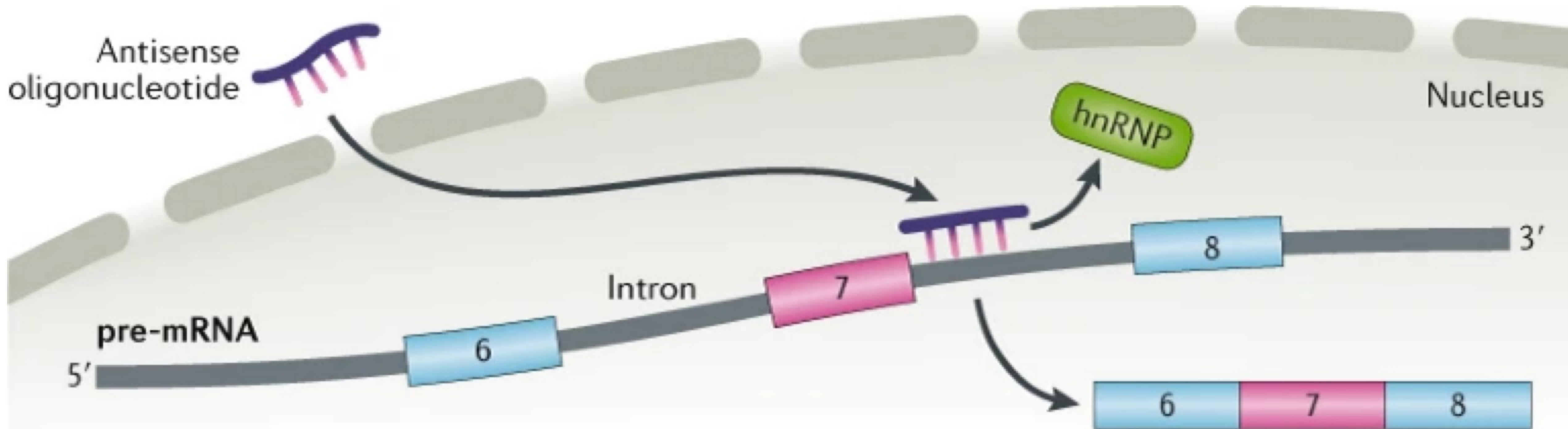
Mild form
Walkers at some point
Life expectancy (nearly) normal
Proximal weakness prominent

Birth Prevalence 12%

Reduction of SMN (Survival of Motor Neuron) causes SMA

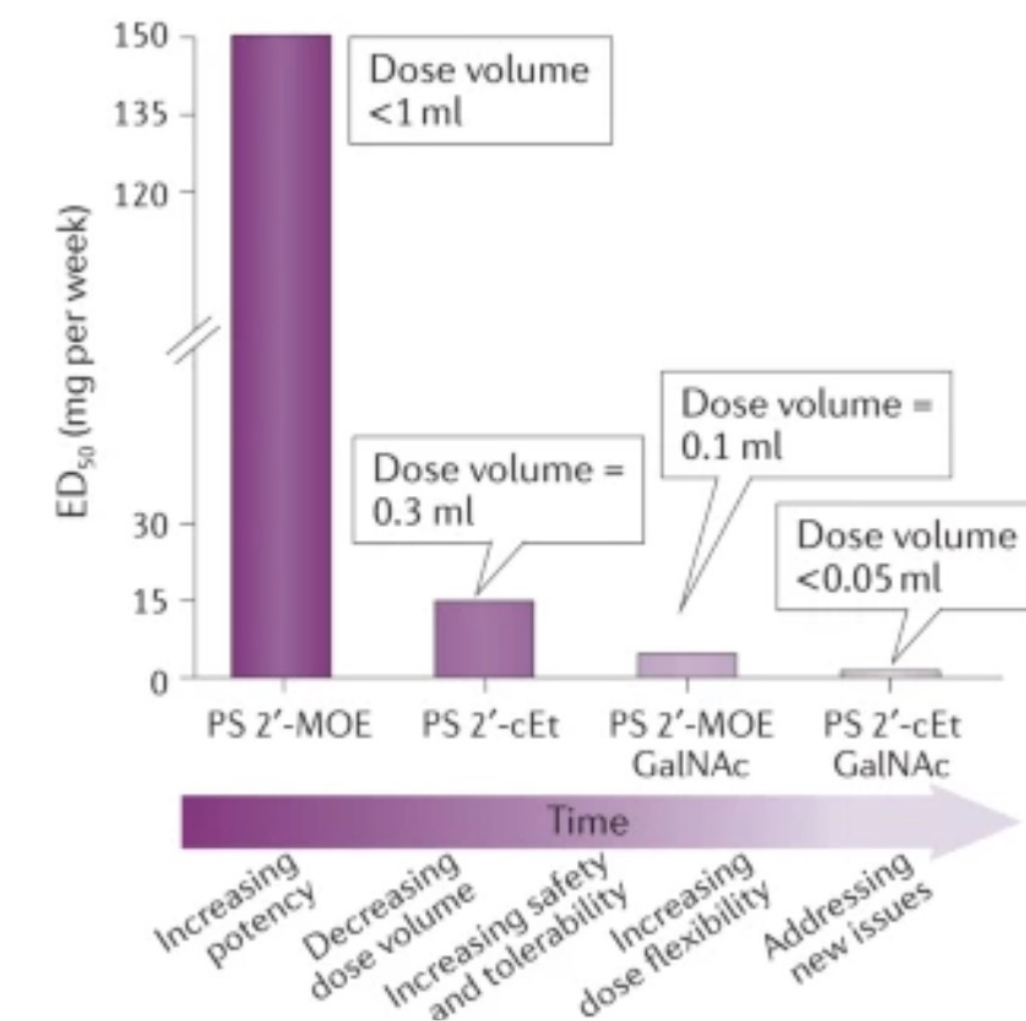
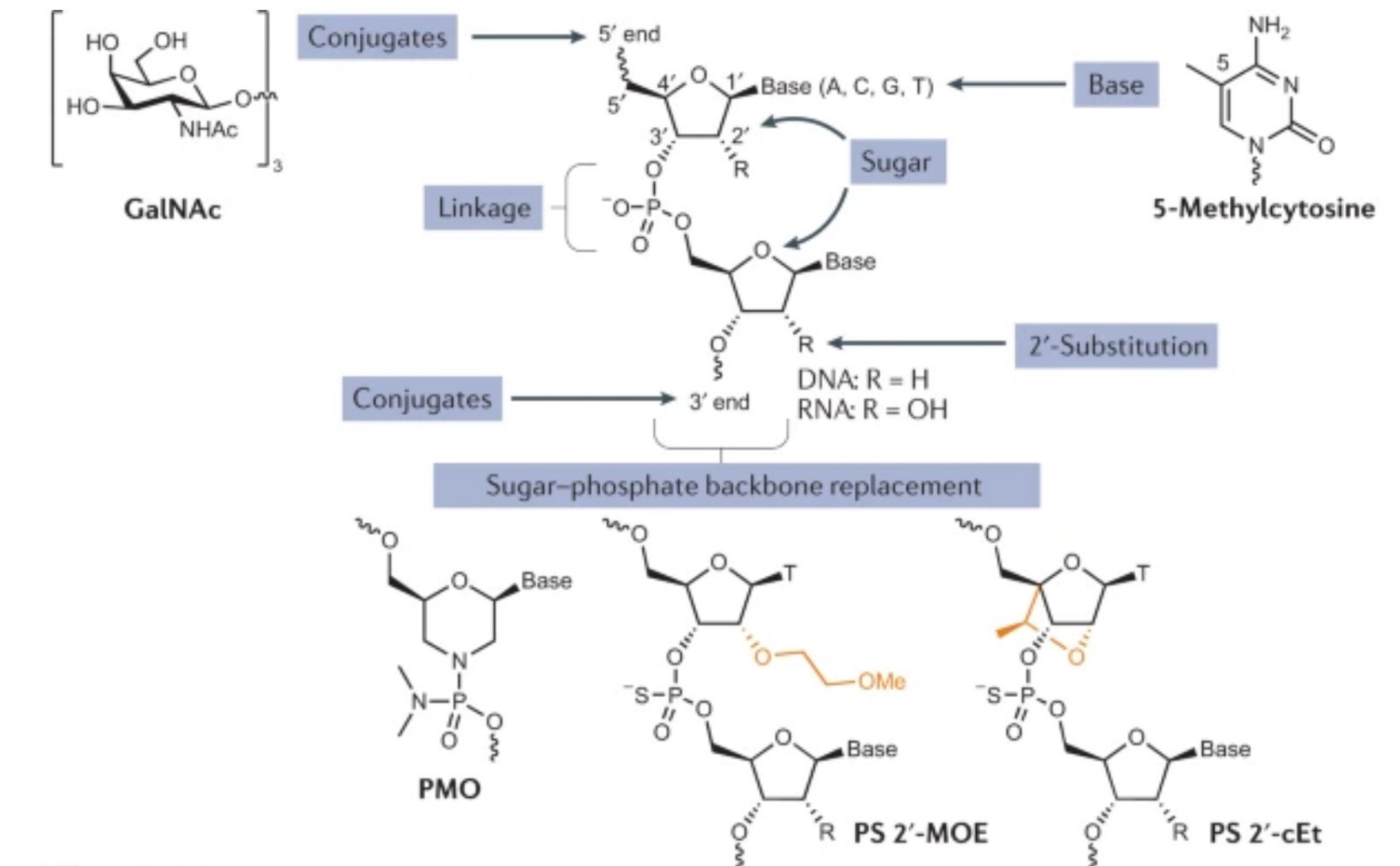


SMA ASO treatment



Antisense Oligonucleotides

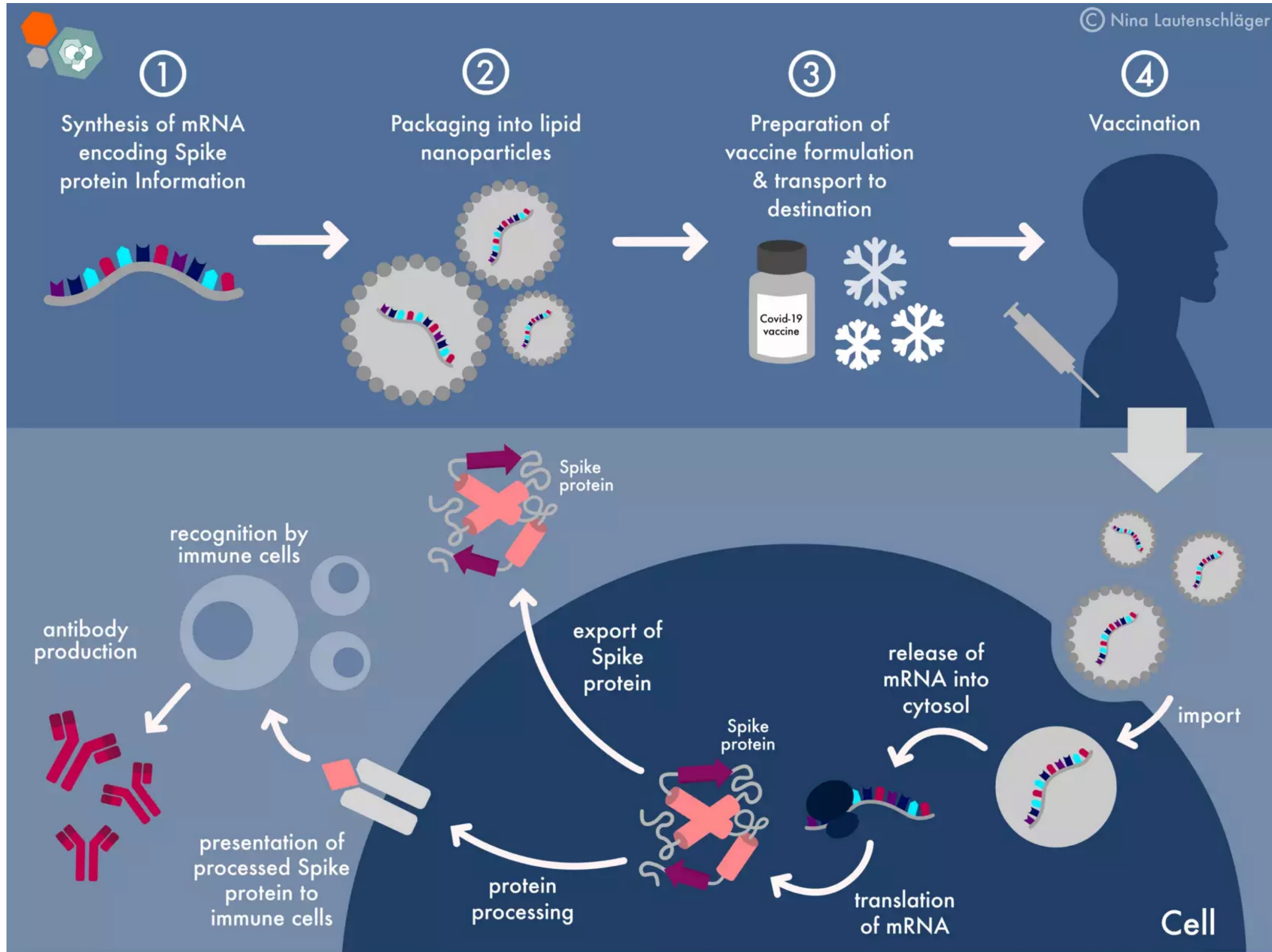
- **Chemical modifications improving** increase hybridization affinity to their target RNA, increased resistance to nuclease degradation and reduce immunostimulatory activity
- **Reduce dosage volume** safer easier administration
- **Repeated administrations** required, can cause complications, unlike gene therapy
- Unlike gene therapy **treatment can be halted**



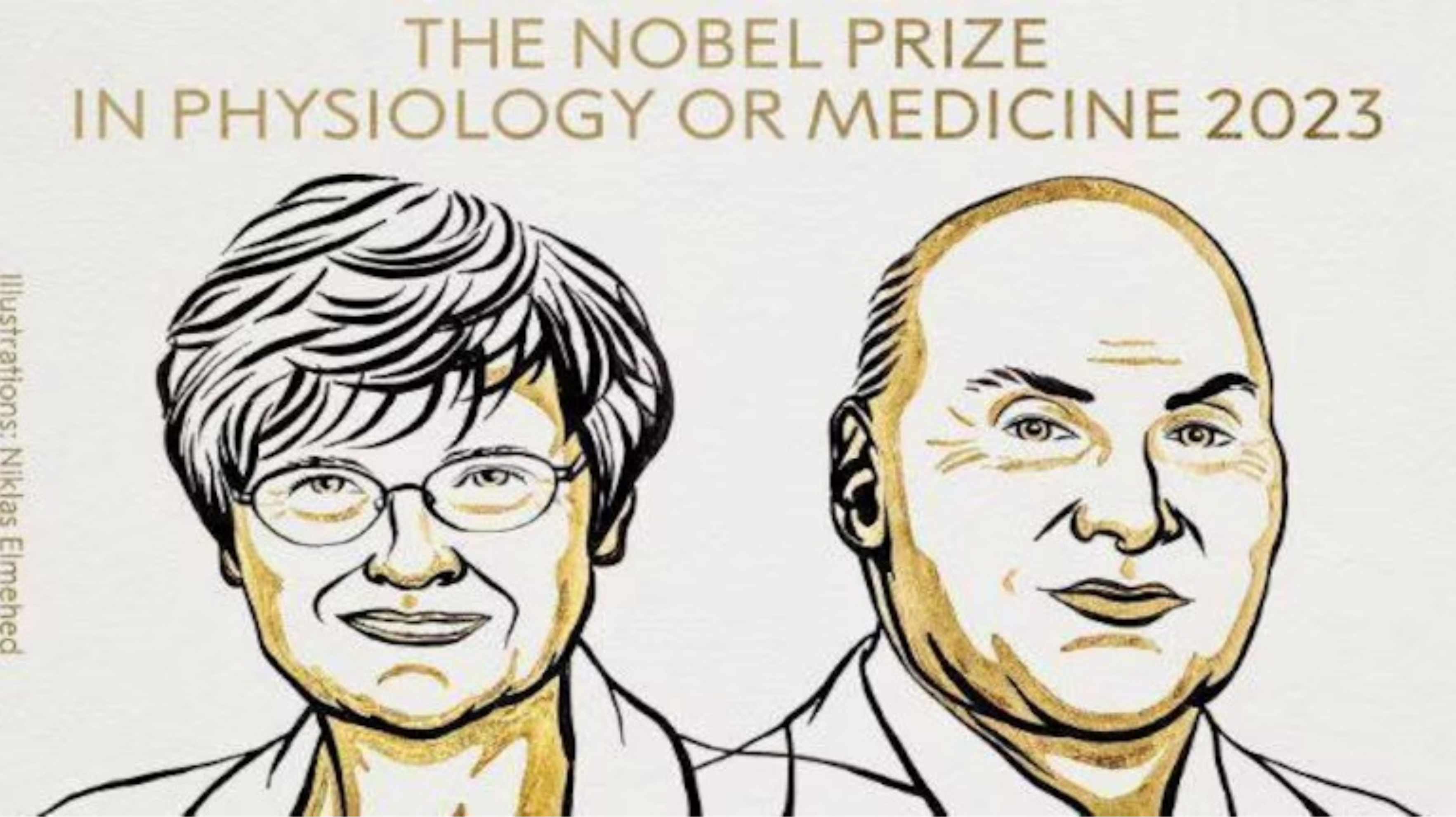
Antisense Oligonucleotides

Drug	Chemistry ^a	Target (organ)	Dose (route)	Indication (approval year, agency)	Key observations	
					Efficacy	Safety
Fomivirsen	2'-H	CMV IE2 (eye)	330 µg per eye once every 4 weeks (ITV)	CMV retinitis (FDA, 1998; EMA, 1999)	Inhibited the progression of CMV retinitis in patients with AIDS	Mild ocular inflammation
Mipomersen	2'-MOE	ApoB-100 (liver)	200 mg once weekly (SC)	HoFH (FDA, 2013)	Reduced apoB-100, LDL-C and VLDL	ALT increases, ISRs
Eteplirsen	PMO	Dystrophin exon 51 (muscle)	30 mg kg ⁻¹ once weekly (IV)	DMD (FDA, 2016)	Increased dystrophin production in skeletal muscle; the interpretation of the clinical significance of this effect is controversial	Generally well tolerated
Nusinersen	2'-MOE, fully modified	SMN2 intron 7 (CNS)	12 mg once every 4 months (IT)	SMA (FDA, 2016; EMA, 2017)	Corrected the SMN2 splicing defect; the two phase III studies were stopped early owing to demonstration of significant clinical benefit	No drug-related adverse events
Inotersen	2'-MOE	TTR (liver)	300 mg once weekly (SC)	Hereditary ATTR (FDA, 2018; EMA, 2018)	Reduced progression of neuropathic disease and improved quality of life	Decreased platelets, ISRs, renal dysfunction
Golodirsen	PMO	Dystrophin exon 53 (muscle)	30 mg kg ⁻¹ once weekly (IV)	DMD (FDA, 2019)	Increased dystrophin production in skeletal muscle	Hypersensitivity reactions and renal toxicity
Volanesorsen	2'-MOE	ApoC-III (liver)	300 mg once weekly (SC)	FCS (EMA, 2019)	Reduction of triglycerides (mean reduction of 1,712 mg dl ⁻¹), reduced abdominal pain and pancreatitis	Decreased platelets, ISRs
Viltolarsen	PMO	Dystrophin exon 53 (muscle)	80 mg kg ⁻¹ once weekly (IV)	DMD (MHLW, 2020; FDA, 2020)	Increased dystrophin production in skeletal muscle and clinical improvement in the 6-minute walk test	Generally well tolerated
Casimersen	PMO	Dystrophin exon 45 (muscle)	30 mg kg ⁻¹ once weekly (IV)	DMD (FDA, 2021)	Increased dystrophin production	Generally well tolerated

mRNA Vaccines



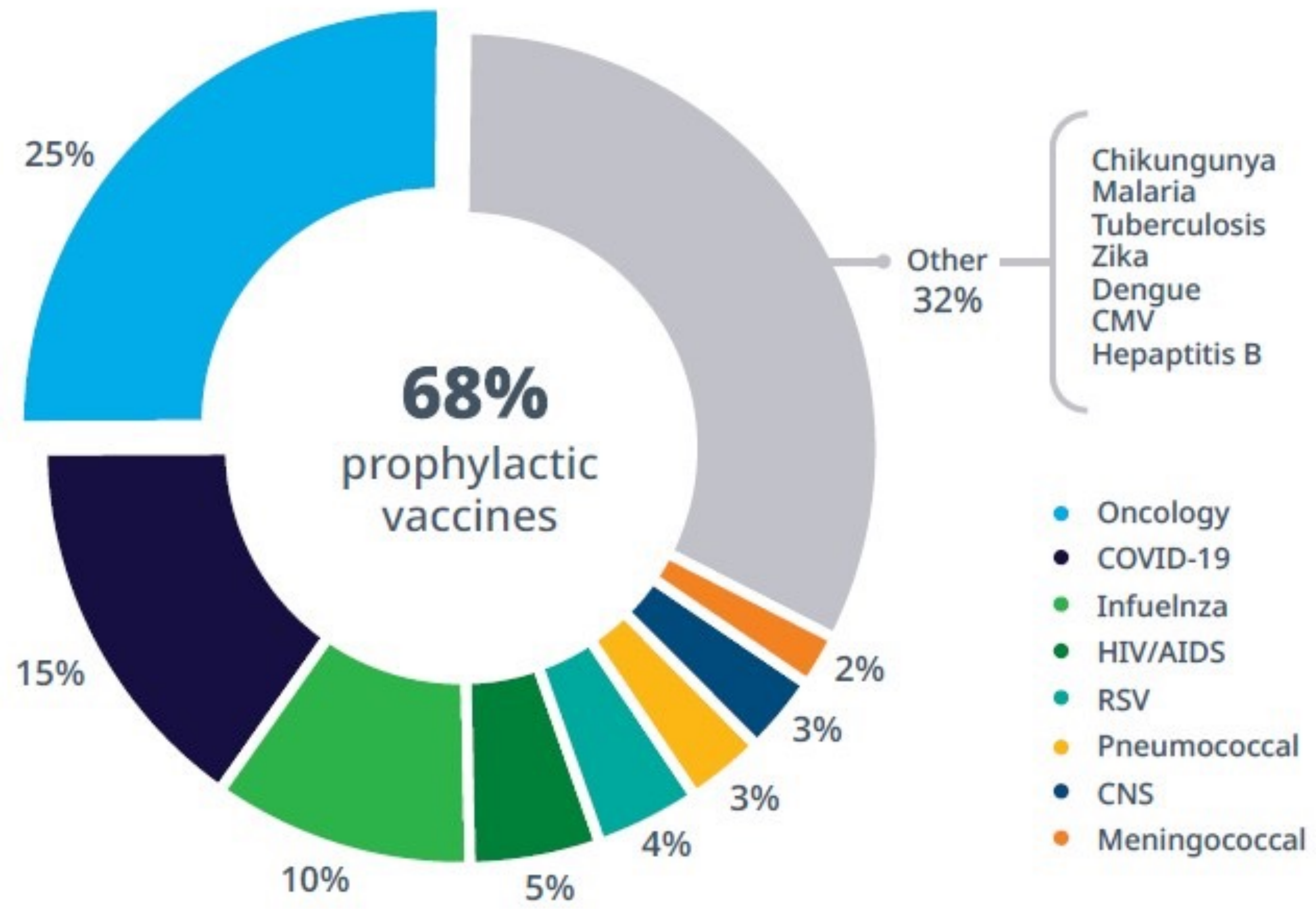
mRNA Vaccines

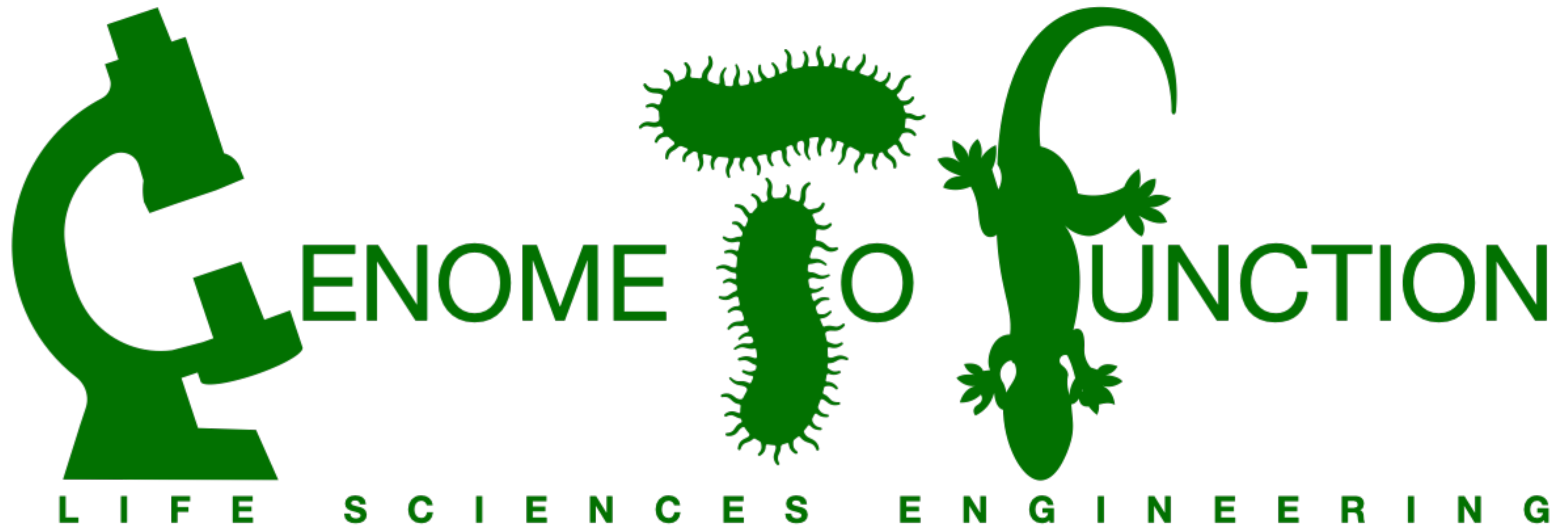


Katalin Karikó Drew Weissman

mRNA Vaccines future

Vaccines pipeline, all assets in phase 1-3 by indication (n=277)





Thank You & Questions