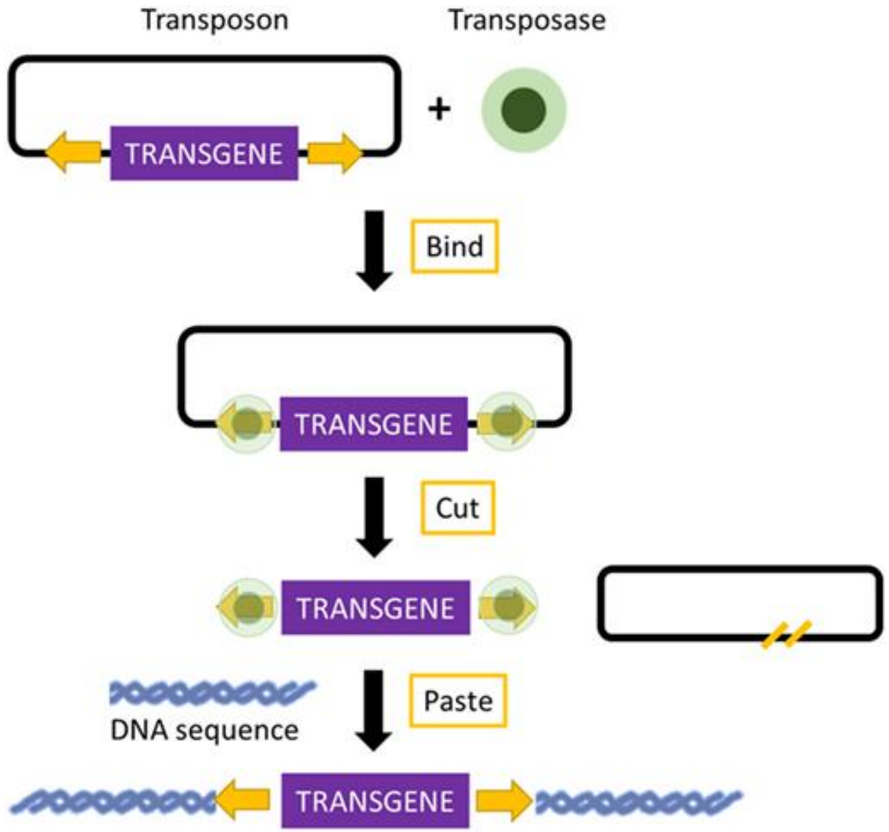
Manipulating Nucleotides II

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**



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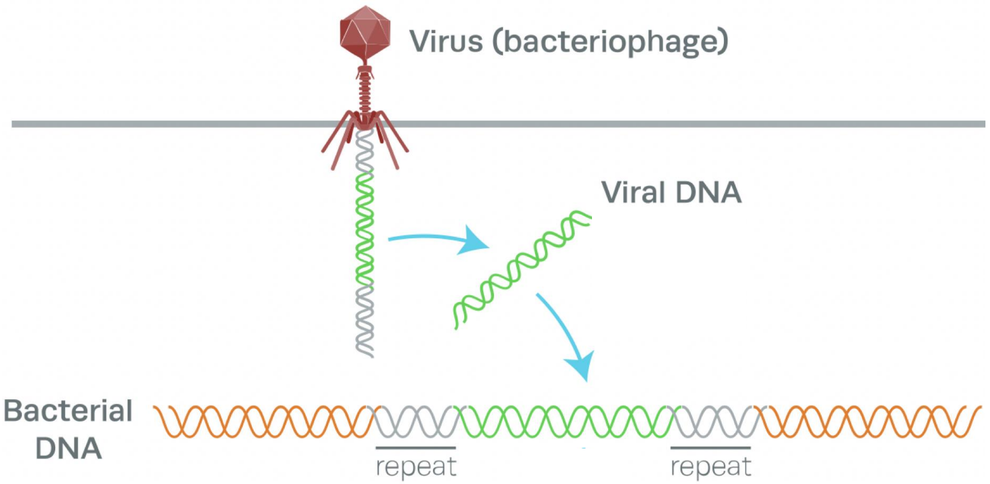
In Person quiz

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.

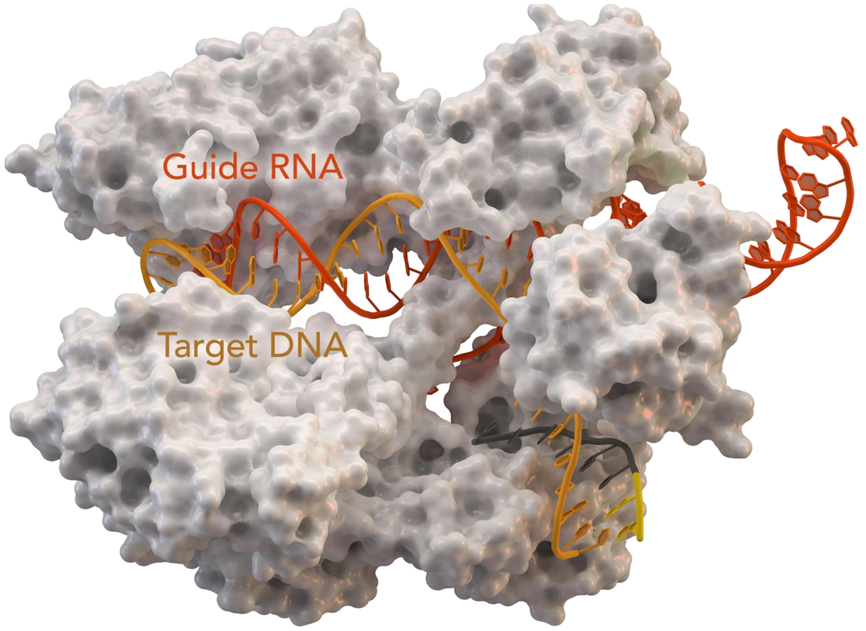
- CRISPR sequences are derived from **DNA fragments of bacteriophages** that had previously infected the prokaryote. Used to **detect and destroy** subsequent bacteriophages

- Act as antiviral defence system in ~50% of bacteria and ~90% of archaea.



Cas 9

- **Cas9 (C**RISPR **as**sociated 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.



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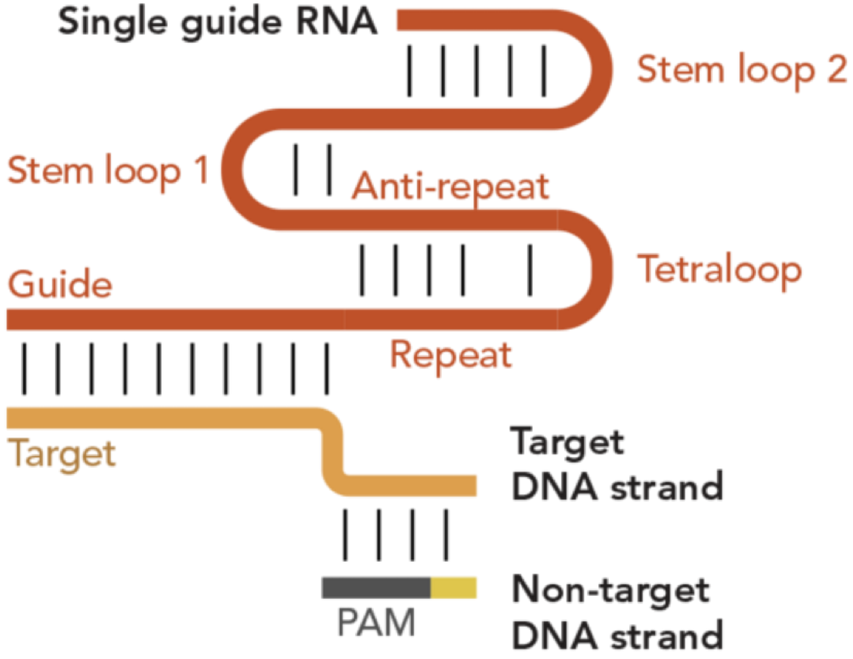
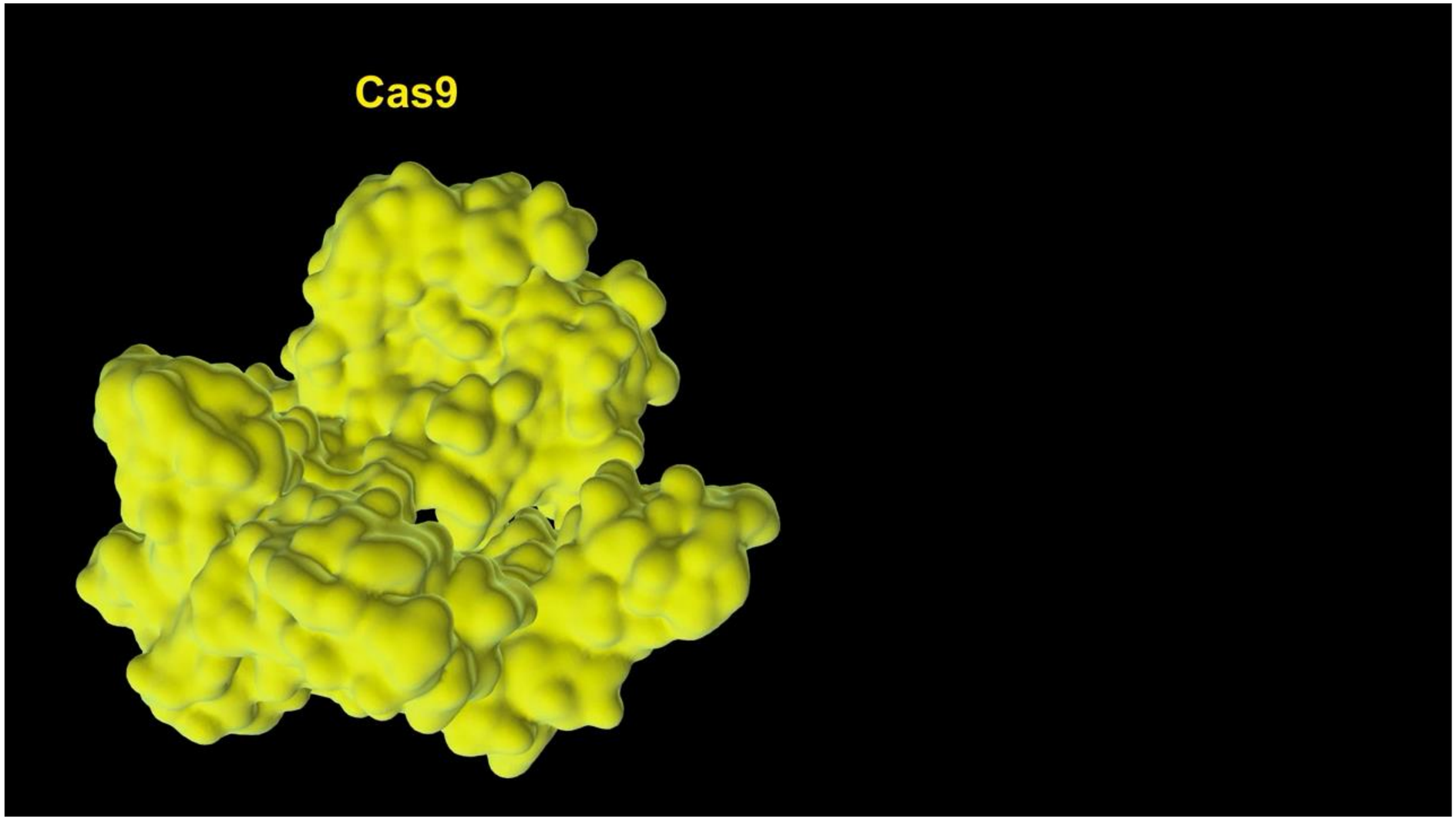
Guide RNA

- **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**



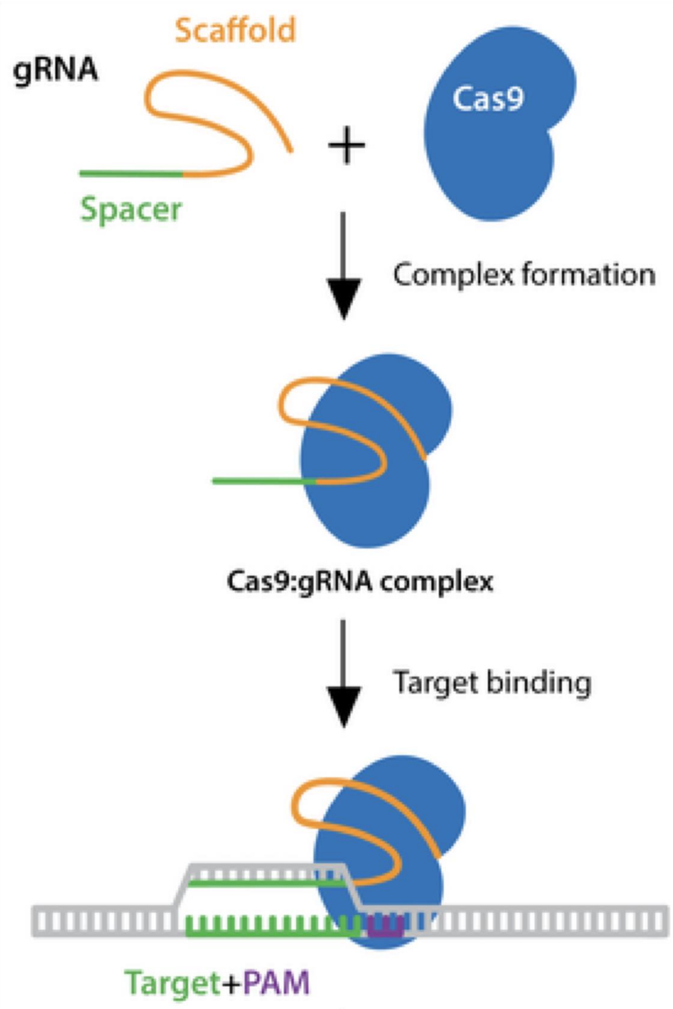
CRISPR/Cas9



Adapted from - https://vimeo.com/112757040

Generating mutations with CRISPR/Cas9

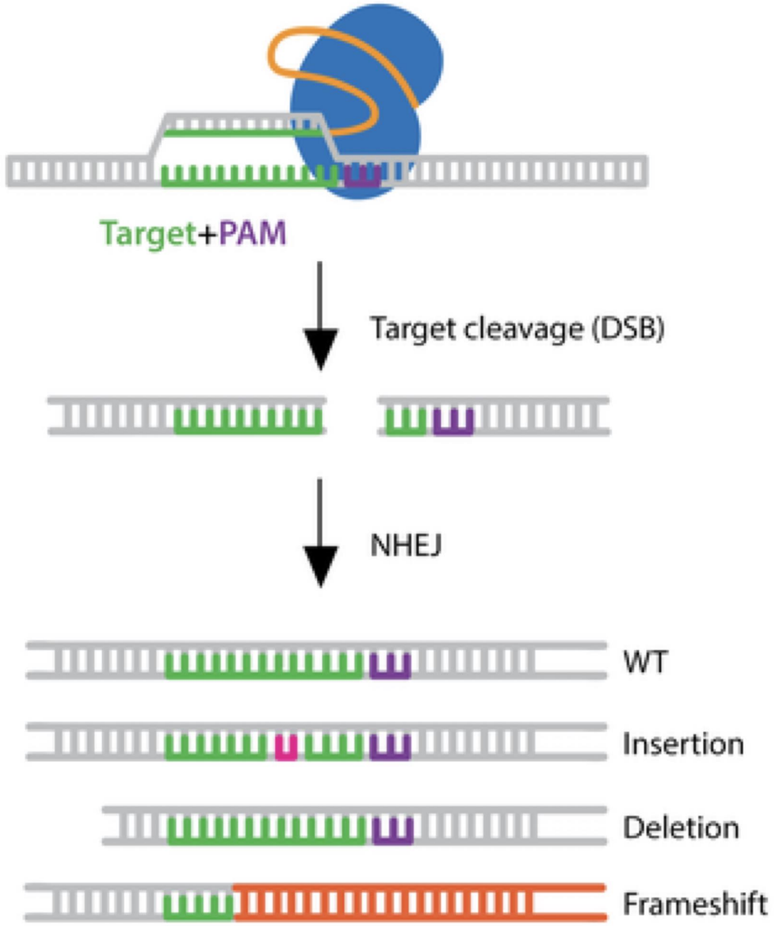
- Cas9 will only cleave the target DNA if the gRNA spacer sequence has **sufficient homology**.
- Once the Cas9-gRNA complex binds a putative DNA target, **the seed sequence** (8-10 bases at the 3′ end of the gRNA targeting sequence) begins to anneal to the target DNA. The gRNA will continue to anneal 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)

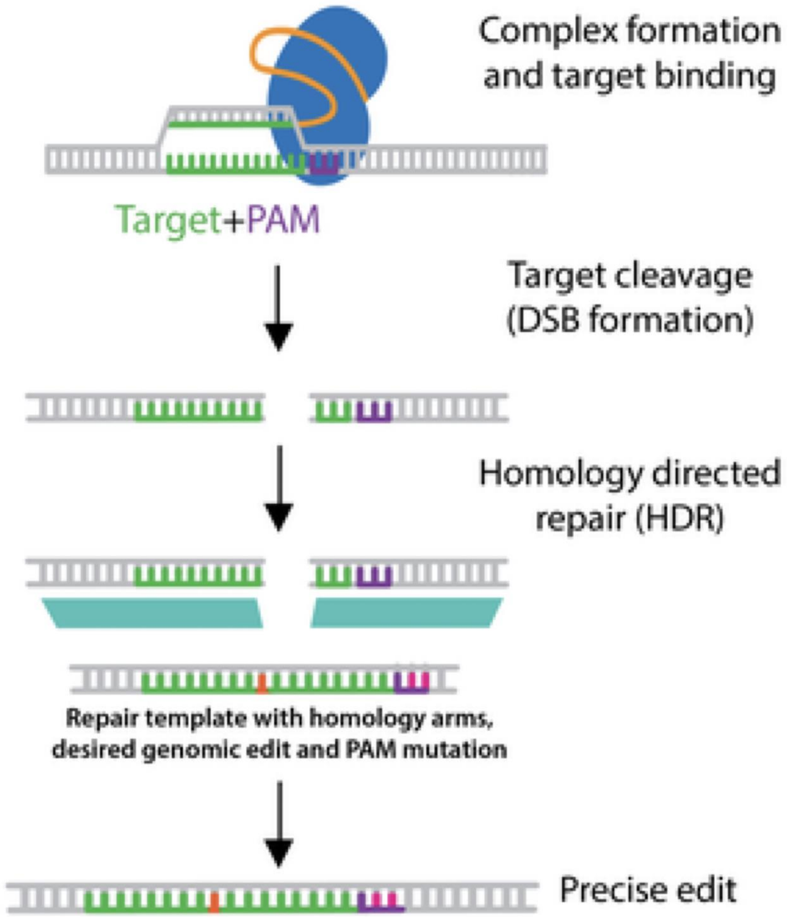
- Double stranded breaks **can be repaired** by one of two general repair pathways
- **Efficient but error-prone** non-homologous end joining (**NHEJ**) pathway
- In many cases, NHEJ gives rise to small **indels** in the target DNA that result in amino acid **deletions, insertions, or frameshift mutations.**



Generating mutations with CRISPR/Cas9

Repair after Double Strand Breaks (DSBs)

- **Less efficient but high-fidelity H**omology **D**irected **R**epair (**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**.



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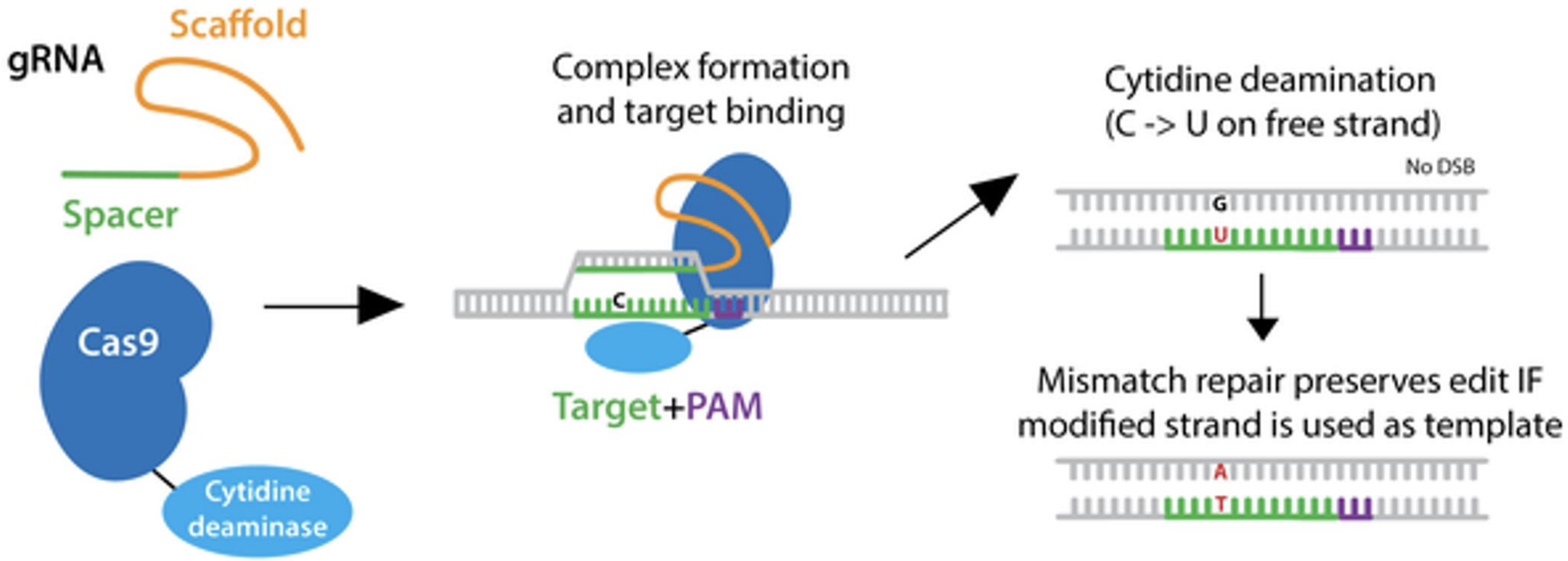
Base Editing (prime editing)

Improving upon HDR

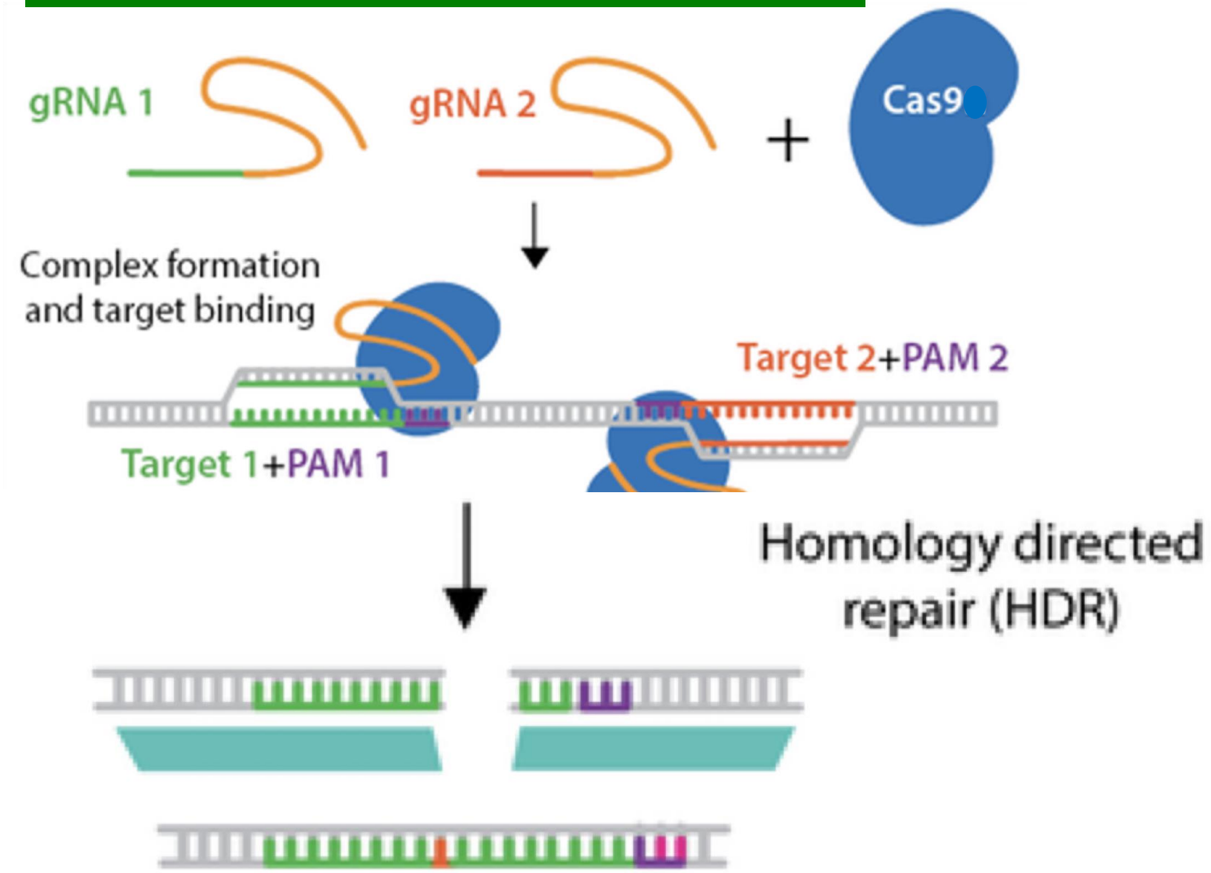


- **Base editors** can be created by fusing catalytically inactive Cas9 (dCas9) to a cytidine deaminase (convert cytidine to uridine - **C to T**) or adenine deaminase (convert adenosine to inosine/guanosine - **A to G**).

Other uses of CRISPR/Cas9

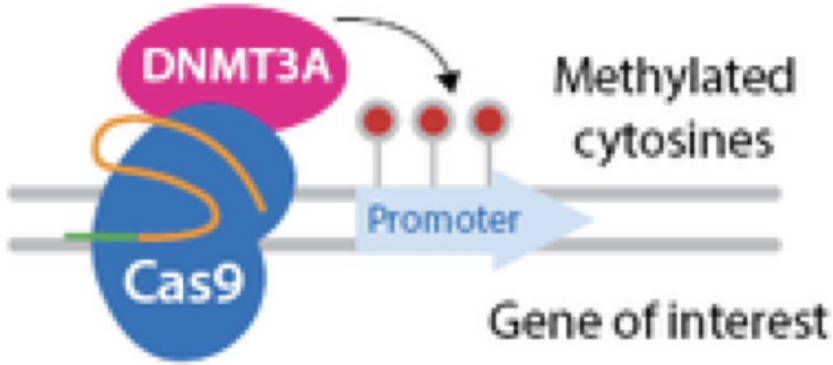
A flexible gene editing tool

INSERTIONAL MUTAGENESIS



Repair template with DNA sequence to be inserted, homology arms and PAM sequence

EPIGENETIC MODIFICATION



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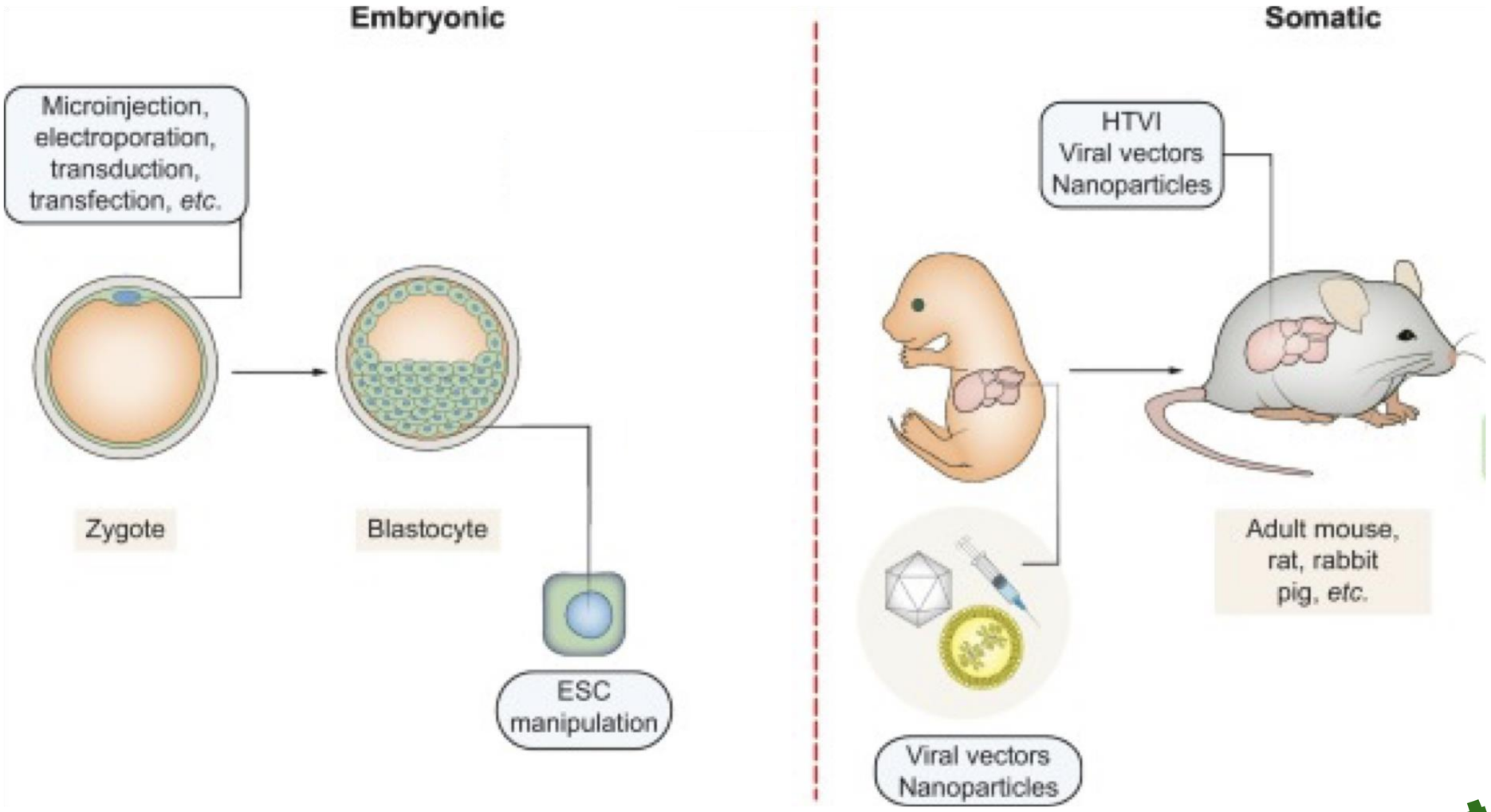
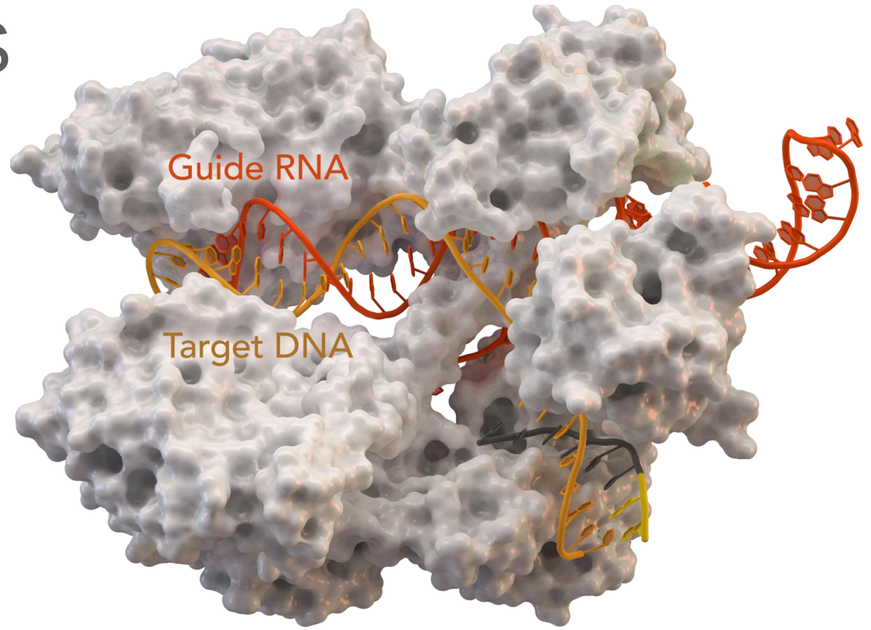
CRISPR/Cas9 germline or somatic

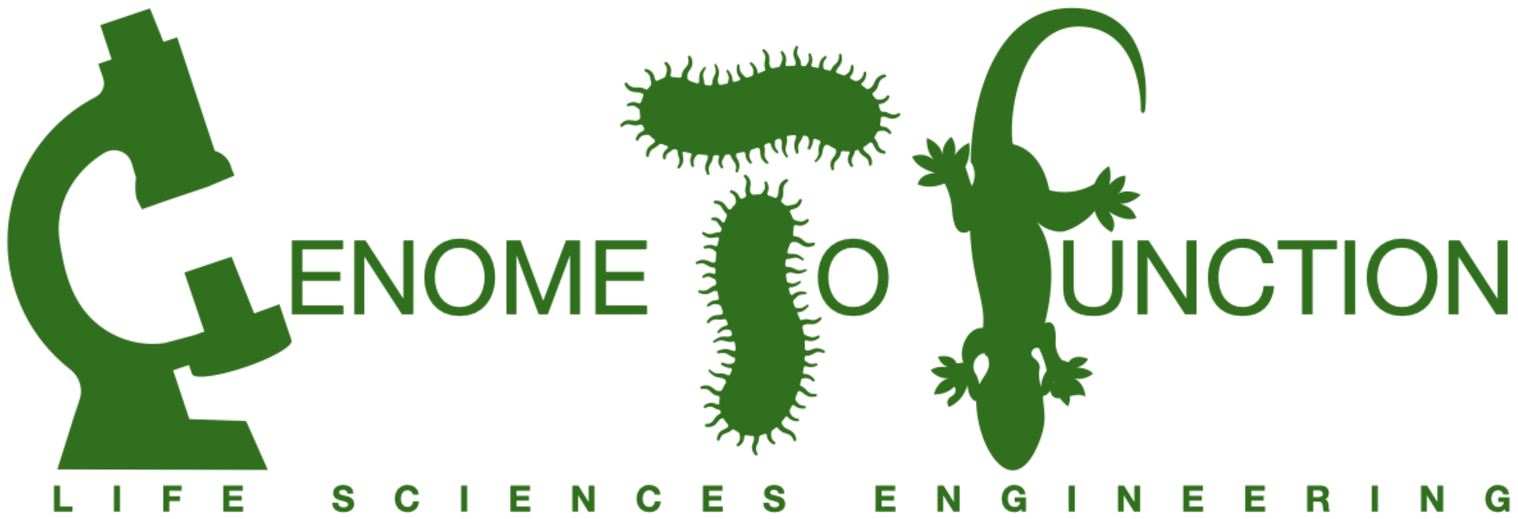


CRISPR/Cas9

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

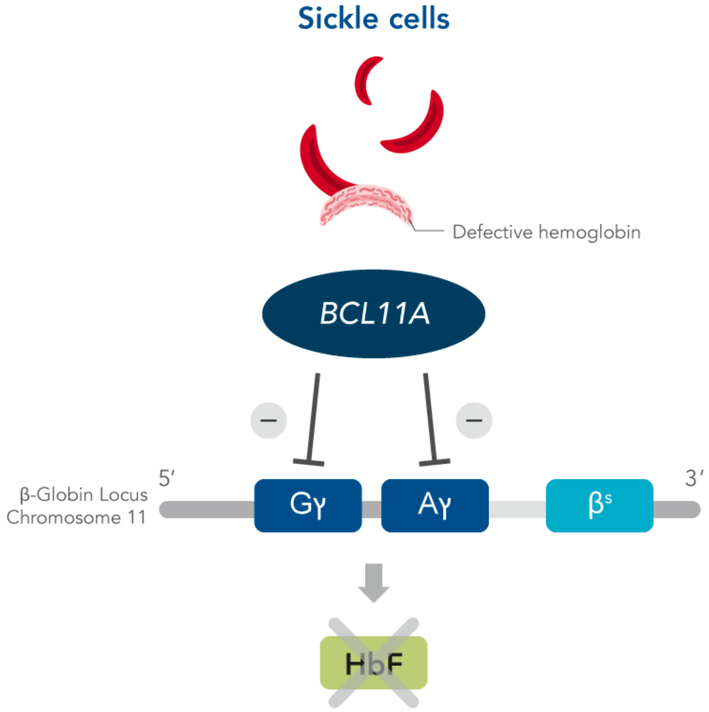
- **Sickle cell disease (SCD)** is one the the most common genetic diseases affecting more than 3 million people worldwide
- SCD is caused by **point mutations** in the beta hemoglobin gene
- A fetal-to-adult hemoglobin (HbF) **switch occurs after birth**
- **BCL11A is a transcription factor** that represses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

First CRISPR human therapy

Sickle Cell Disease ex-vivo treatment

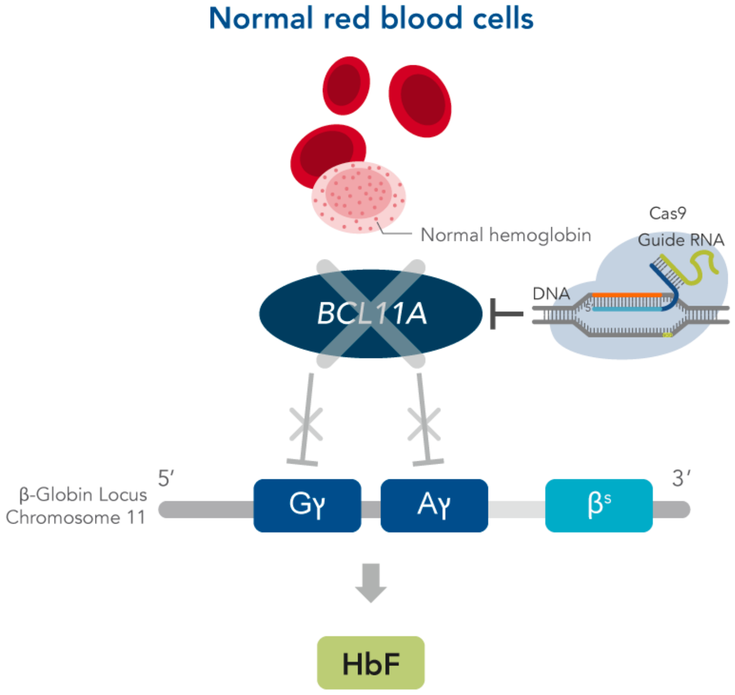
Adult SCD patient

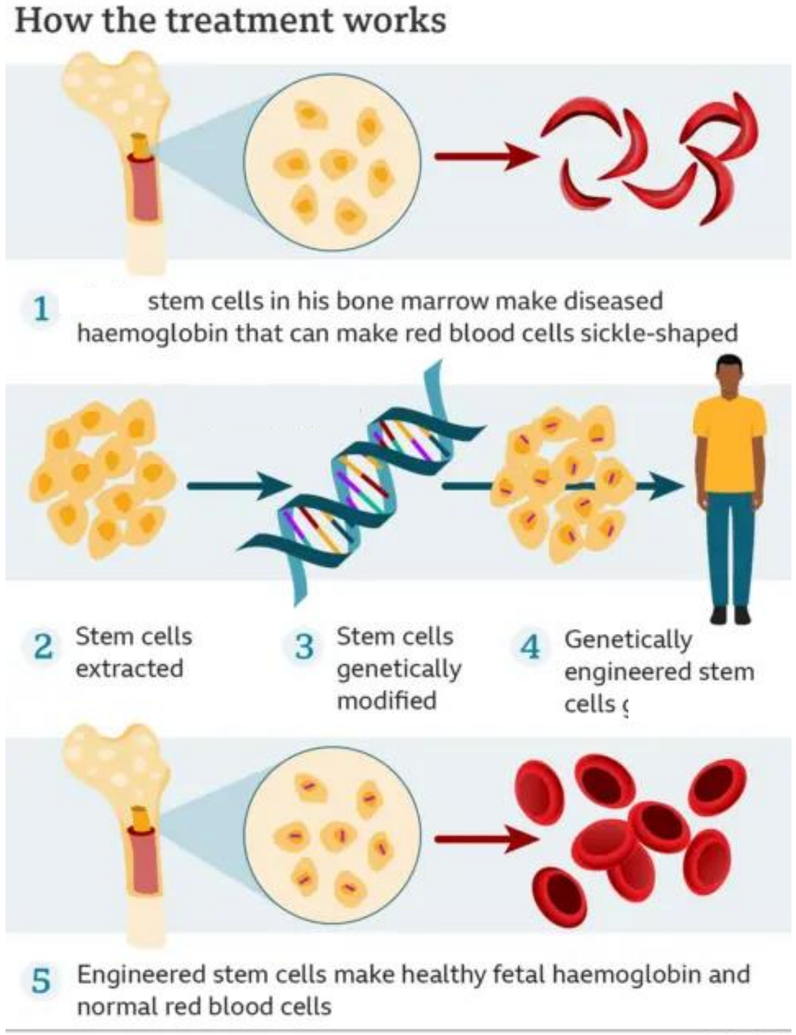
BCL11A blocks gamma globin to repress HbF expression causing sickle hemoglobin



Edited Cells

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





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)
- $CCR5^{\Delta 32}$ is an 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.

Manipulating RNA

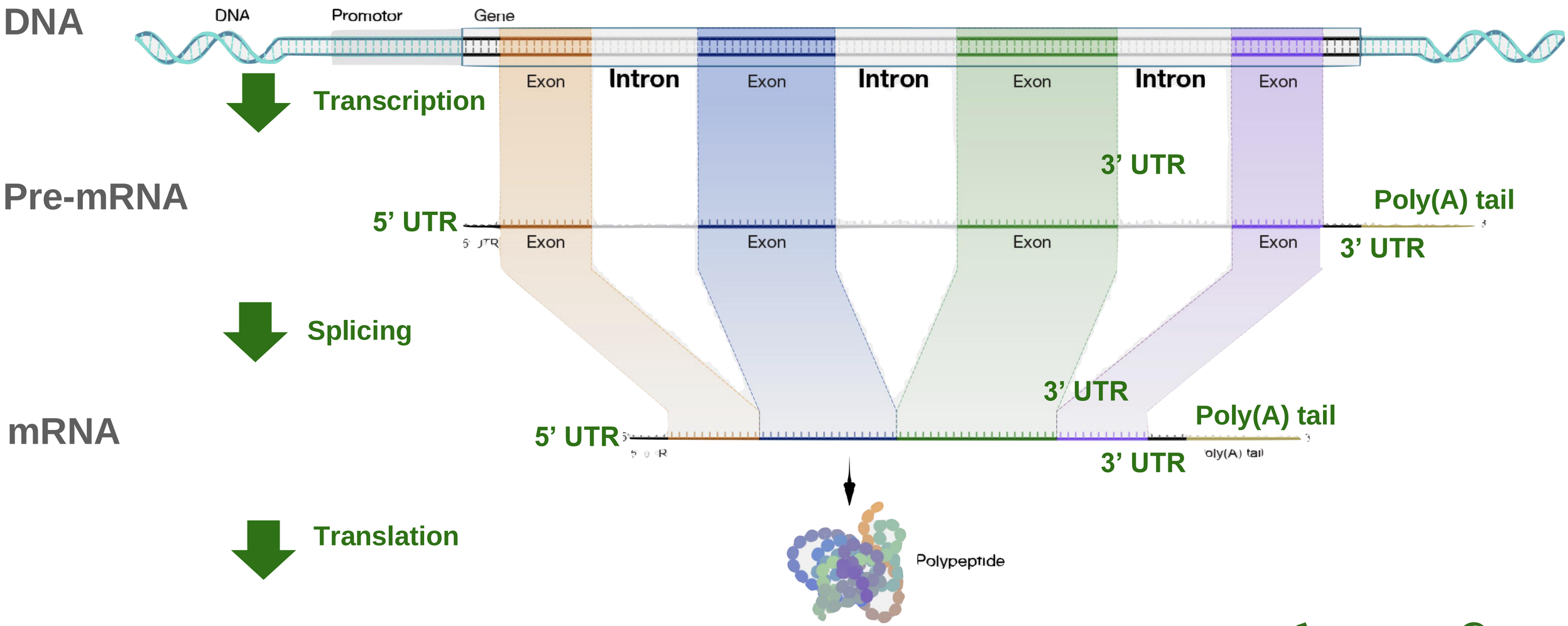
mRNA

messenger RNA

- **m**essenger **R**ibo**n**ucleic **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

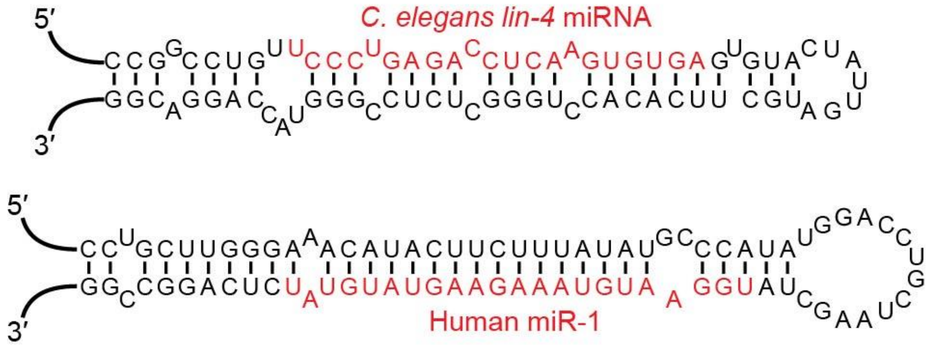
- **‘Housekeeping’ ncRNAs:** e.g. snoRNAs (small nucleolar RNAs), snRNAs (small nuclear RNAs), tRNAs (transfer RNAs), rRNAs (ribosome RNAs) **are involved in protein translation** or other functions like telomere related RNAs.

- ‘**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

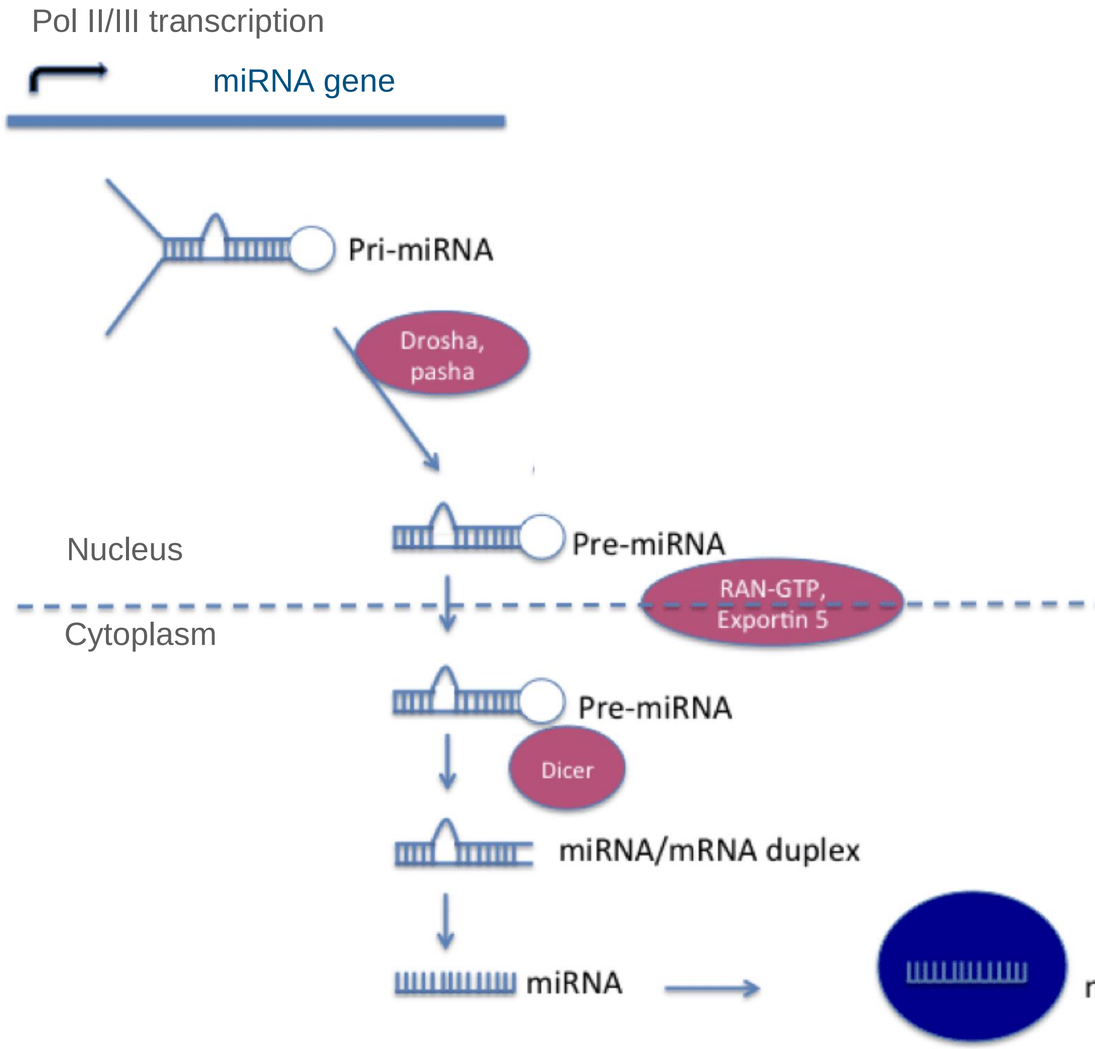
- **miRNAs** are a group of small ncRNA molecules ranging from 16 to 27 nt in length that can alter gene expression either during transcription and/or during translation.
- First discovered in *c.elegans*
- miRNAs directly interact with **partially** complementary sequences in the 3′ untranslated region (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

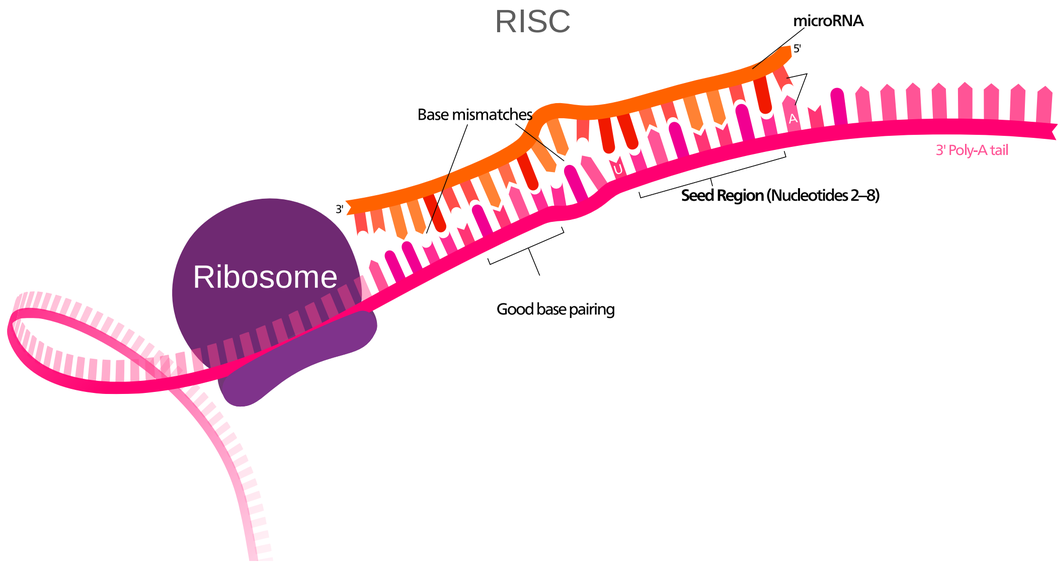
- **miRNAs** are transcribed by miRNA genes by RNA polymerase III **or produced from intron sequences** using RNA polymerase II as large precursor RNA molecules called **Pri-miRNAs**
- **Pri-miRNAs** are processed in the nucleus by the RNase III enzyme Drosha, and the dsRNA-binding protein, Pasha to 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

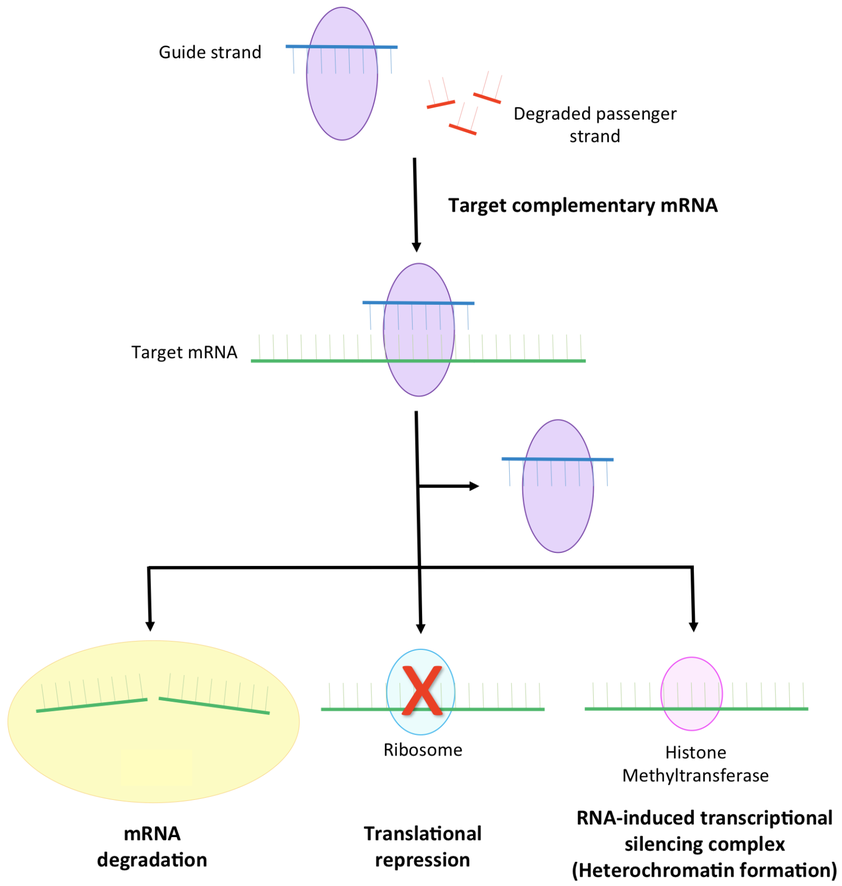
- **RISC complex:** Key proteins include Ago2, SND1, AEG-1 are essential for function
- RISC utilises the guide strand of the miRNA to target regions (**usually in the 3'UTR**) of complementary mRNA transcripts.
- Base pairing needs to be fully complementary in a seed region **but mismatches or imperfect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



miRNA gene regulation

RISC: RNA-induced silencing complex

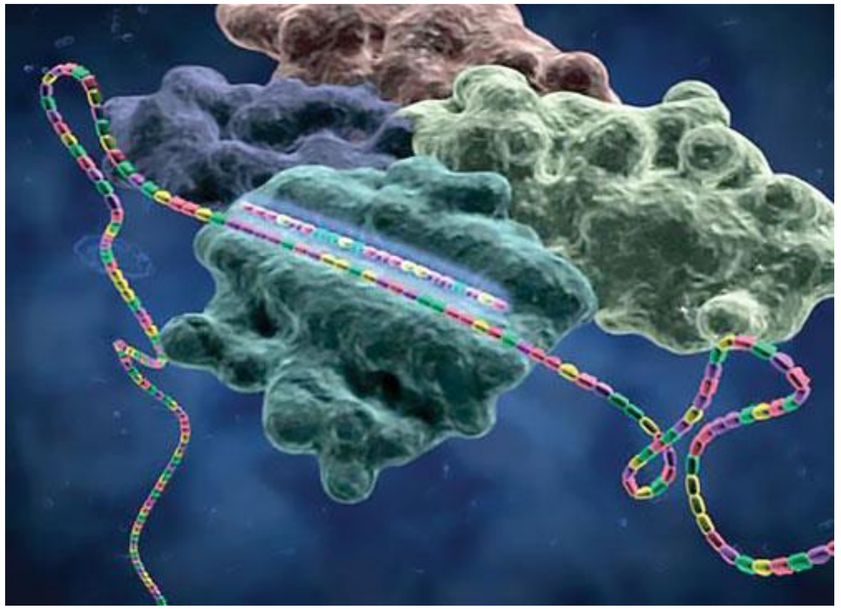
- **RISC complex:** Key proteins include Ago2, SND1, AEG-1 are essential for function
- RISC utilises the guide strand of the miRNA to target regions (usually in the 3'UTR) of **complementary mRNA transcripts**.
- Base pairing needs to be fully complementary in a seed region **but mismatches or imperfect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

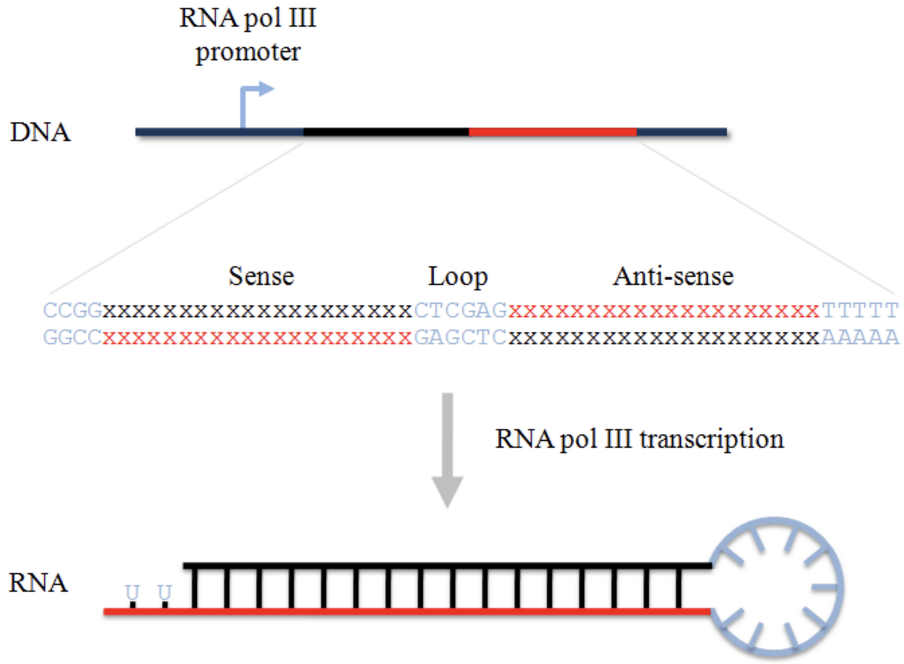
- **RNA interference (RNAi)** is a method to inhibit gene expression with RNA molecules
- short RNA molecules are created that are **perfectly complementary** to endogenous target mRNAs
- Binding of the introduced short RNA to the target mRNA **inhibits the target mRNA** expression via similar mechanisms 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

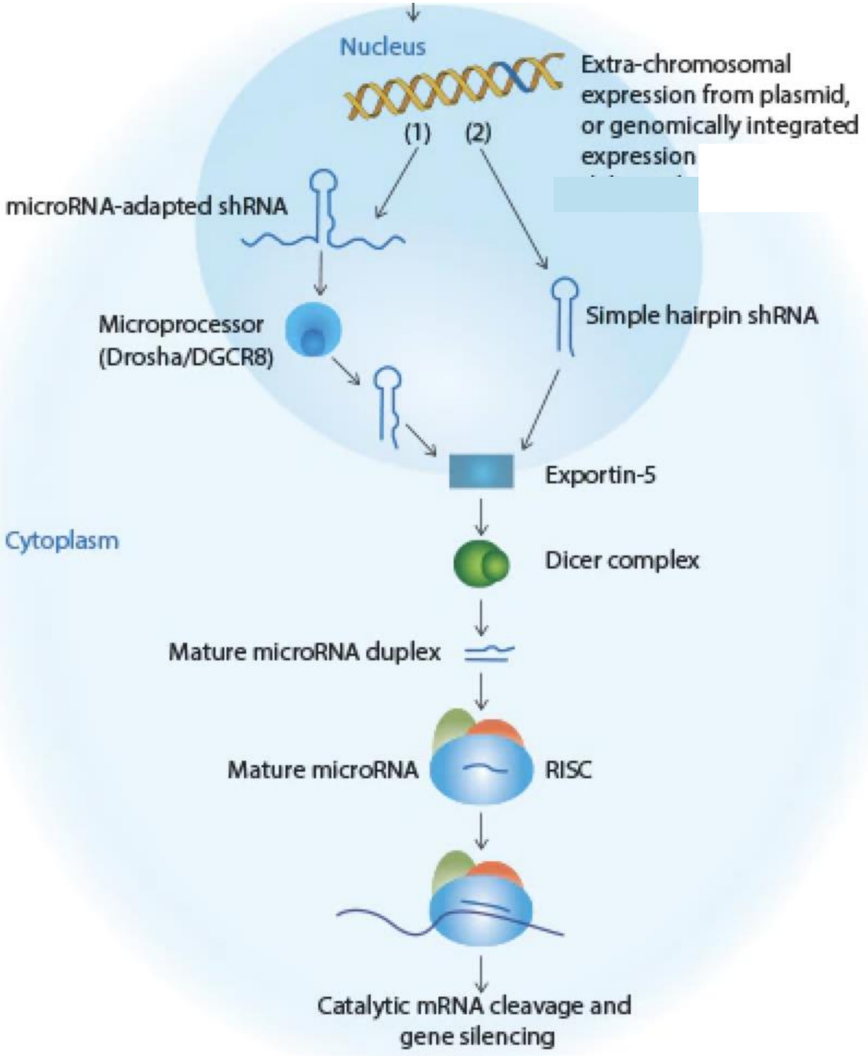
shRNAs

- **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



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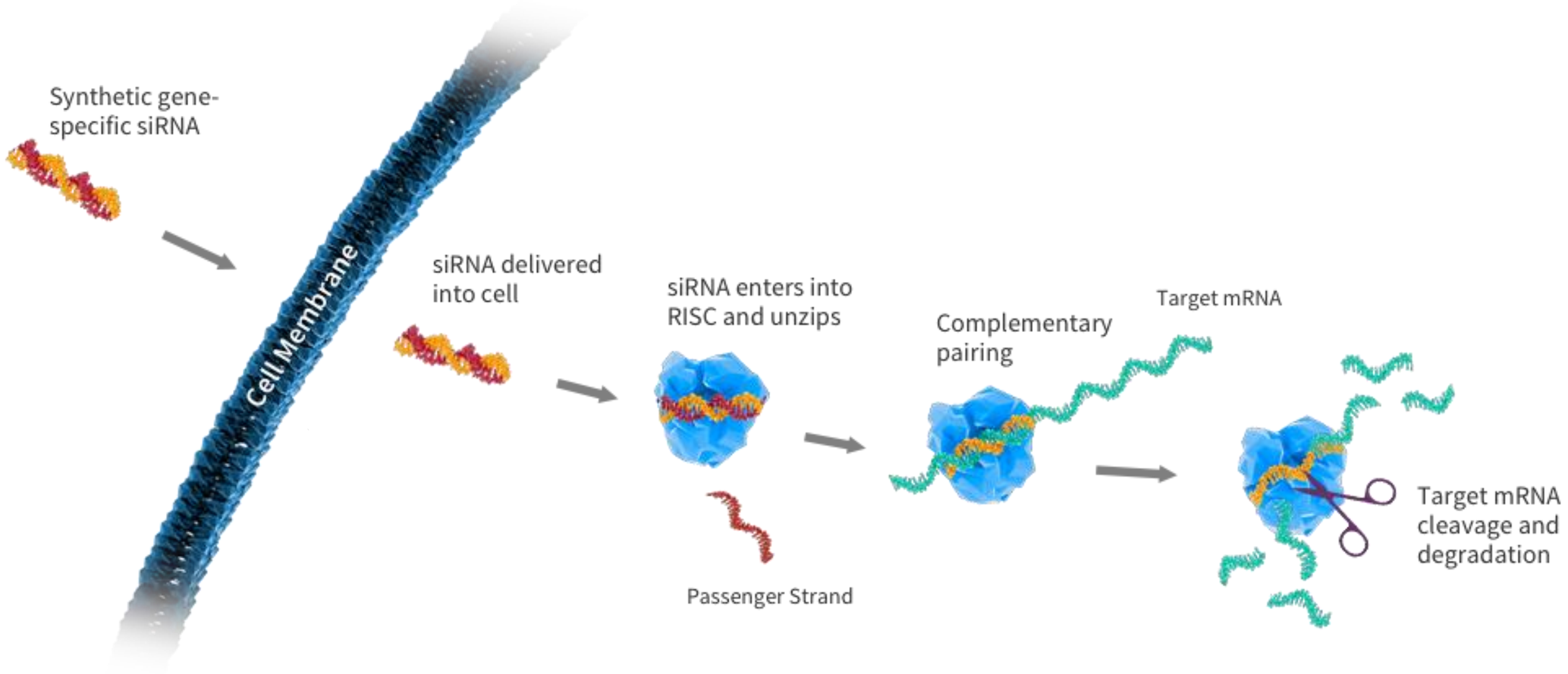
Short interference RNA

siRNAs

- **Small interfering RNA (siRNA)** is a double stranded RNA with 2 nucleotide 3’ end overhangs
- 3’ overhangs **help activate RNAi**
- siRNAs an 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 emplaoy multiple siRNAs with **target sequences at different positions** in the mRNA to be inhibited.
- **Libraries** of siRNAs allow to the entire genome to be screened in cell culture.

Short interference RNA

siRNAs



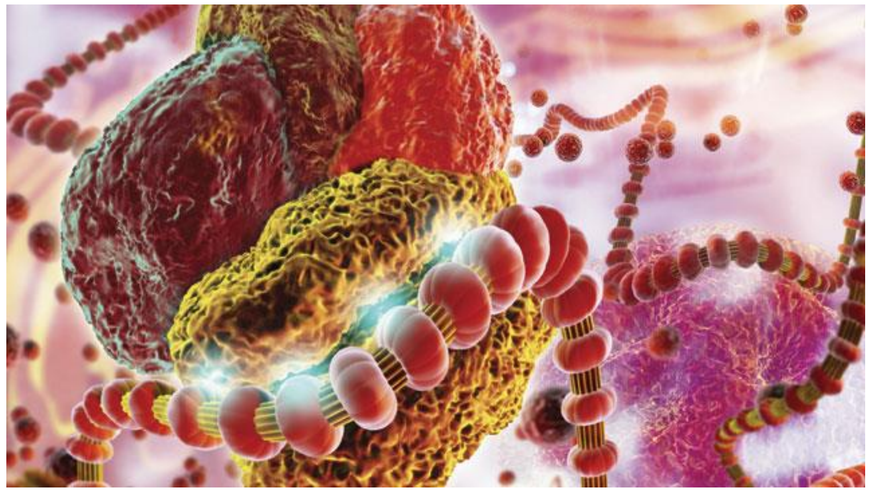
RNA interference

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.



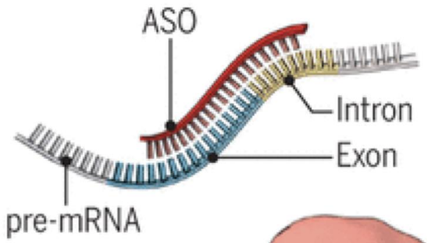
Antisense Oligonucleotides

ASOs

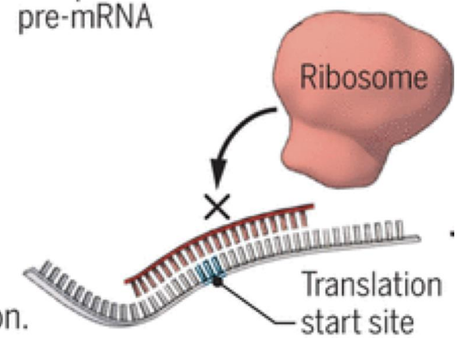
- **Antisense oligonucleotides (ASOs)** are short, synthetic, single-stranded oligodeoxynucleotides that can alter mRNAs
- Can be used to **reduce, restore, or modify protein expression**
- **Chemically modified** oligodeoxynucleotides designed to increase hybridization affinity to their target RNA, 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.



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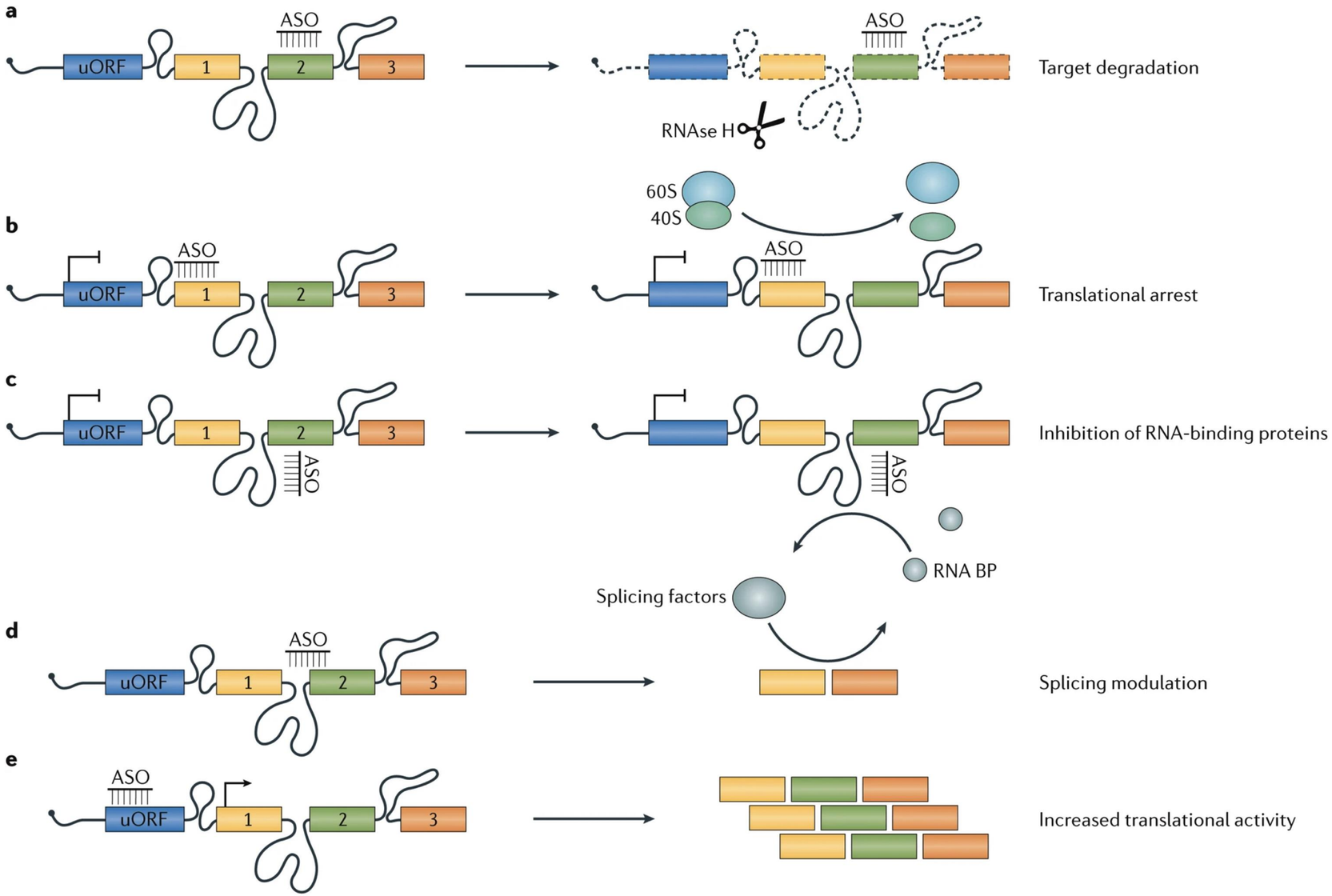
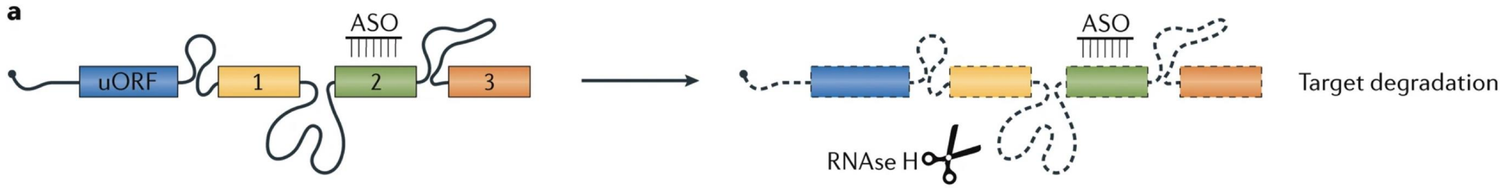
Antisense Oligonucleotides

ASOs



Antisense Oligonucleotides

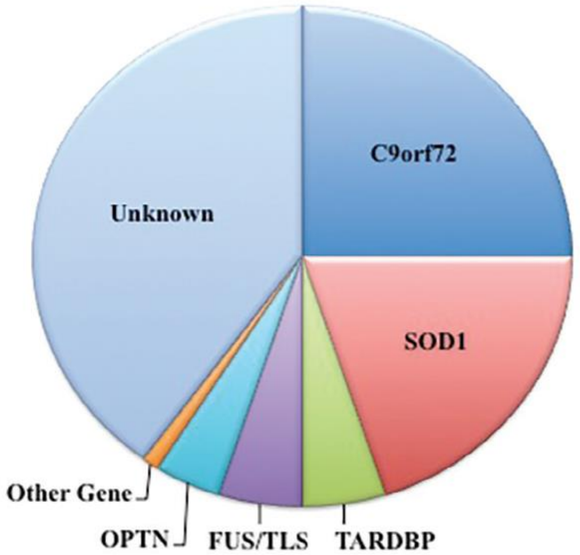
ASOs

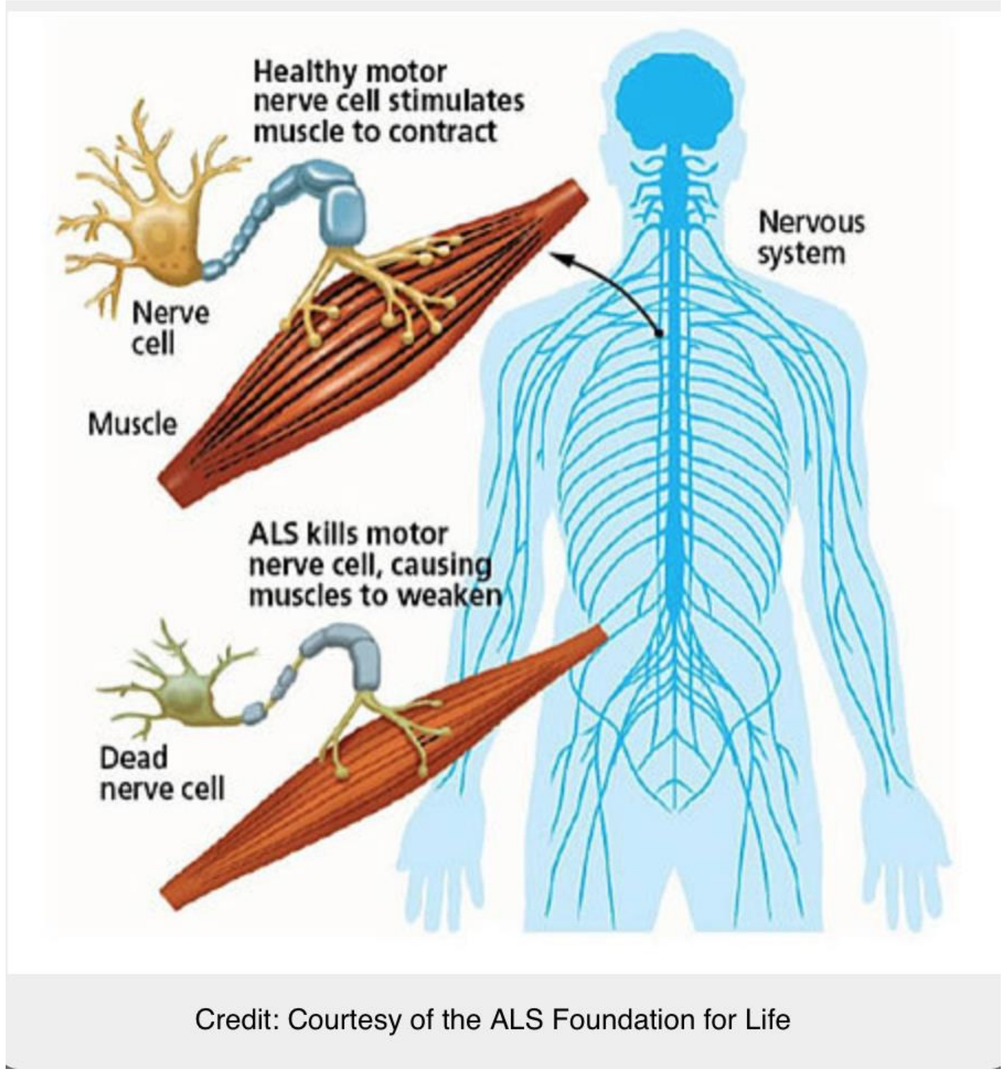


Amyotrophic lateral sclerosis

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

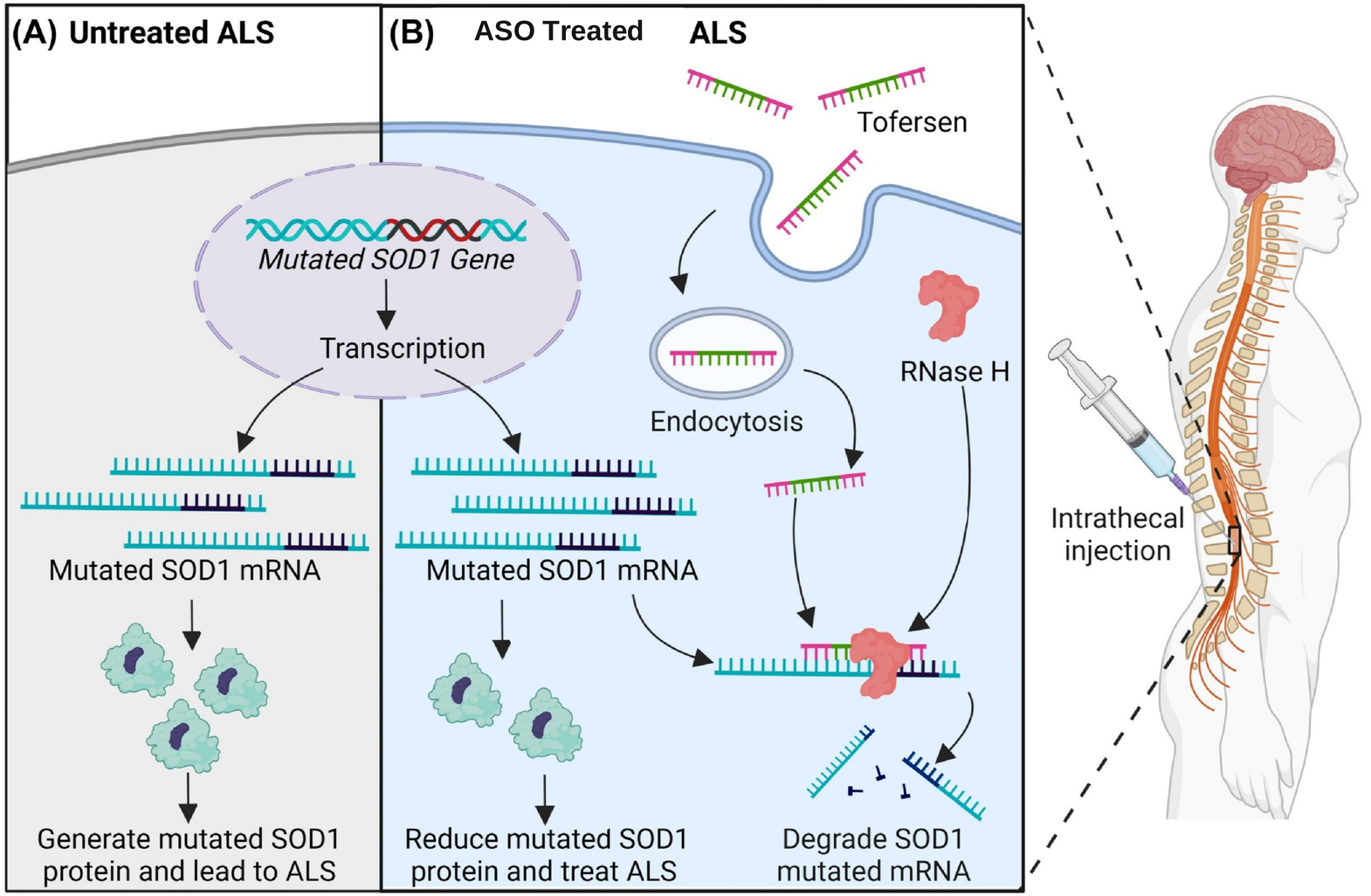
- 10% familial mutations





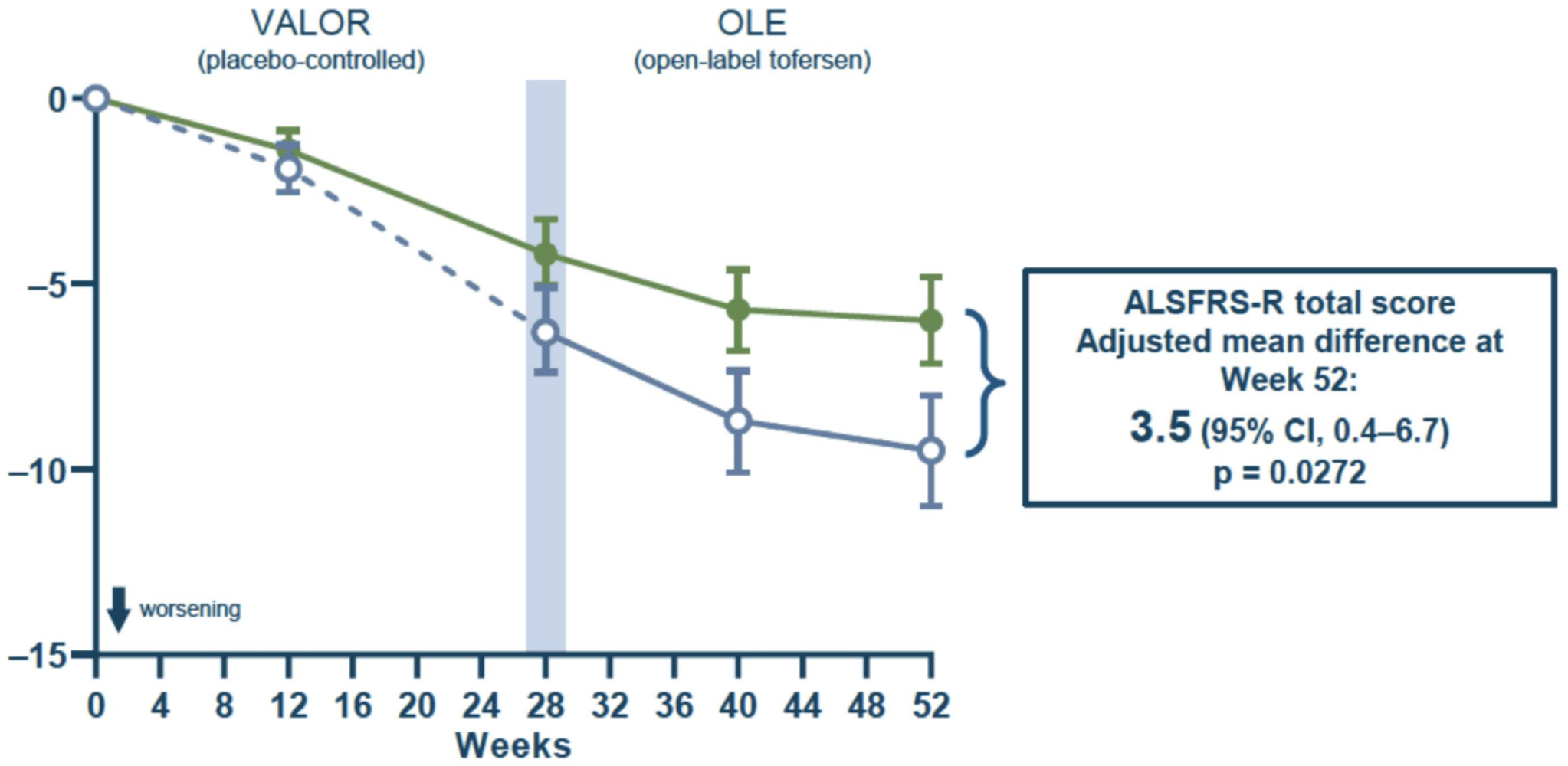
Credit: Courtesy of the ALS Foundation for Life

SOD1 ASO



Trends in Pharmacological Sciences

SOD1 ASO



https://biopharma.media/

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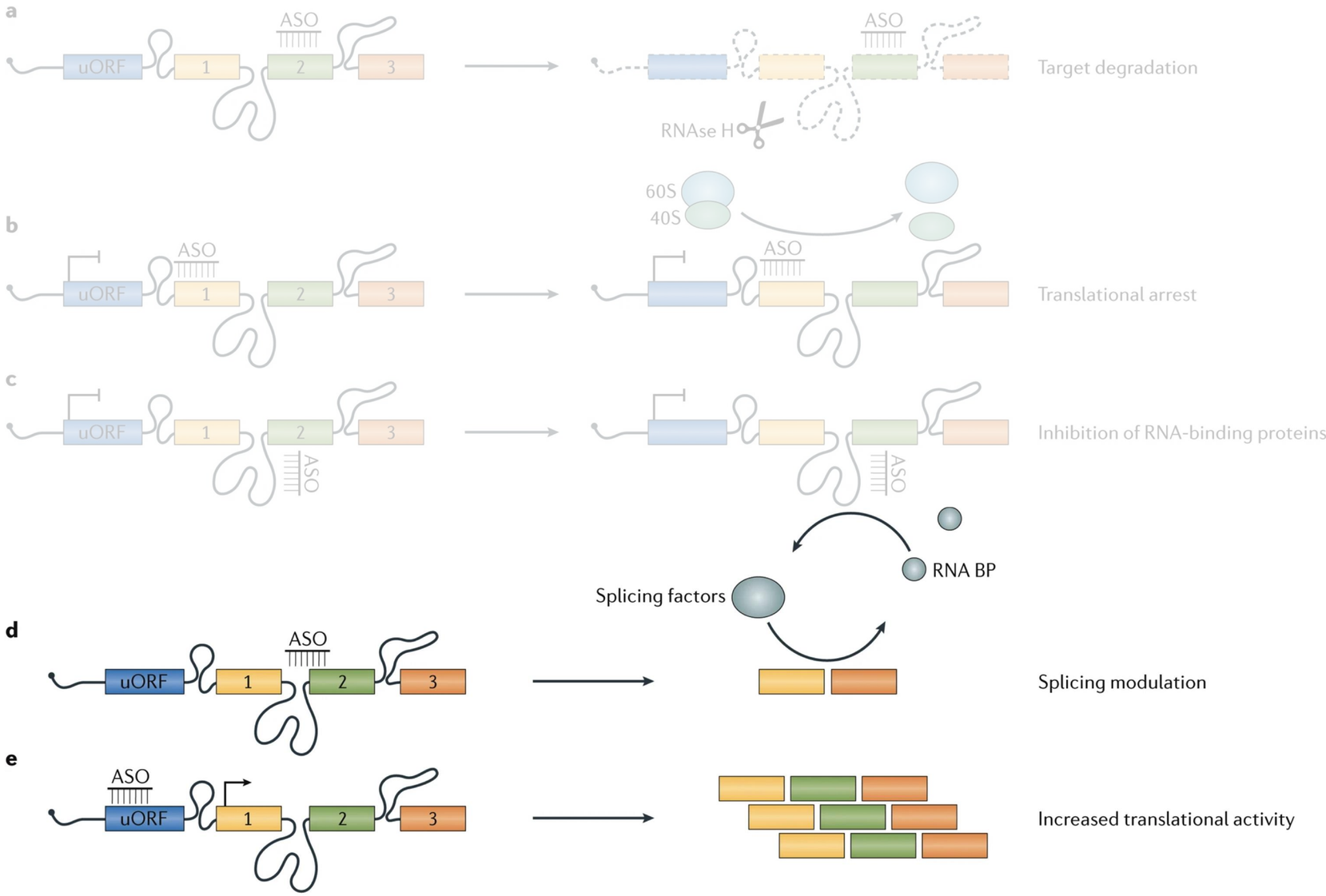
Antisense Oligonucleotides

ASOs

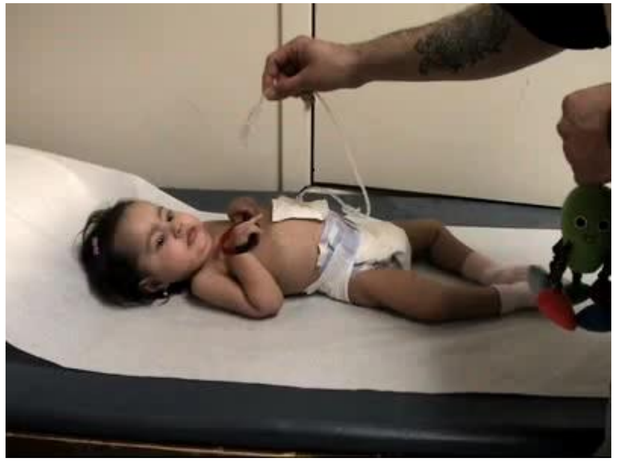


Spinal Muscular Atrophy (SMA)

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

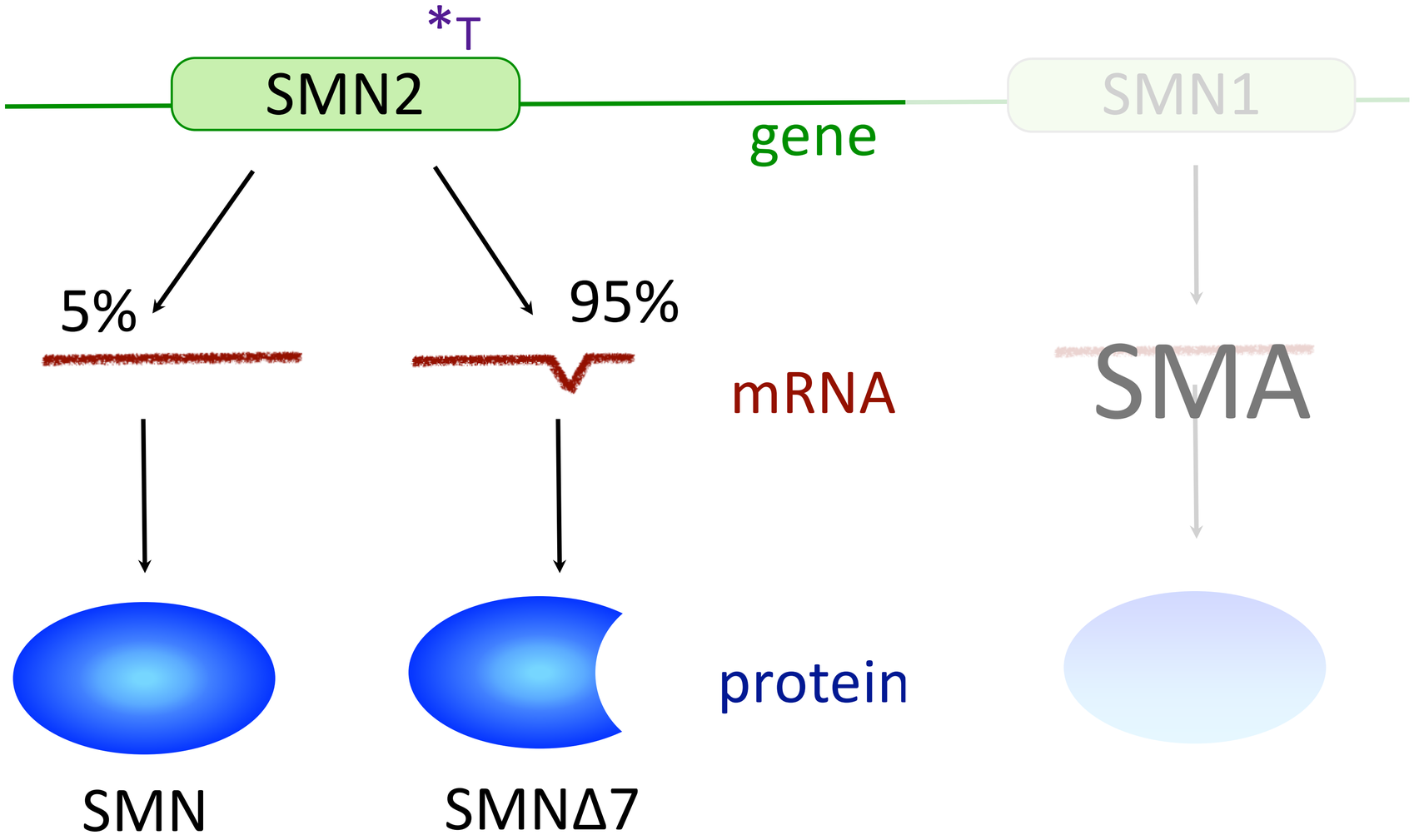
Birth Prevalence 27%

SMA Type III

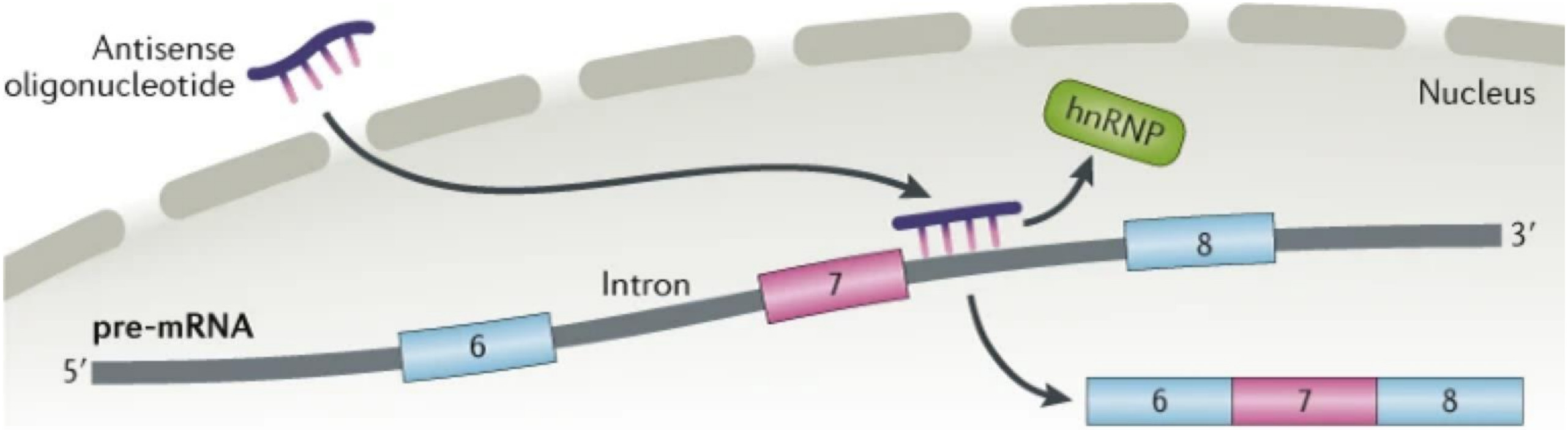
Mild form
Walkers at some point
Life expectancy (nearly) normal
Proximal weakness prominent

Birth Prevalence 12%

Reduction of SMN (Survival of Motor Neuron)causes SMA

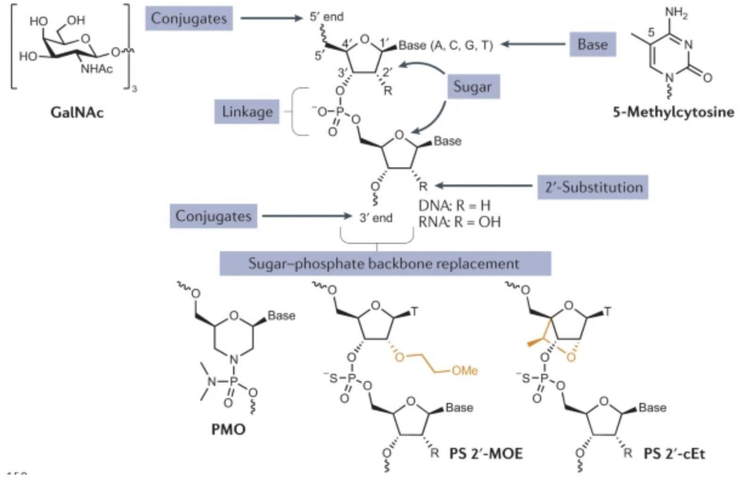


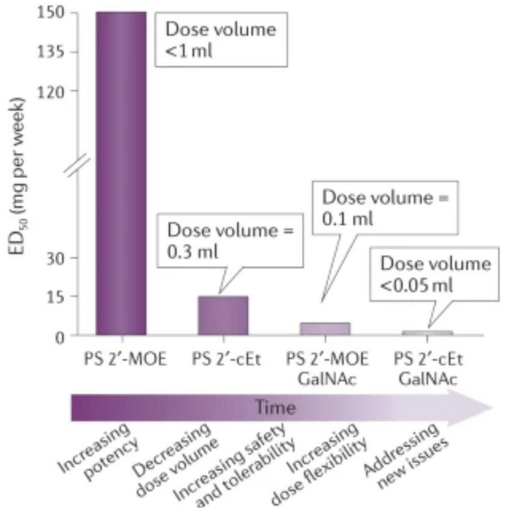
SMA ASO treatment



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**





GENOME TO FUNCTION
LIFE SCIENCES ENGINEERING

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

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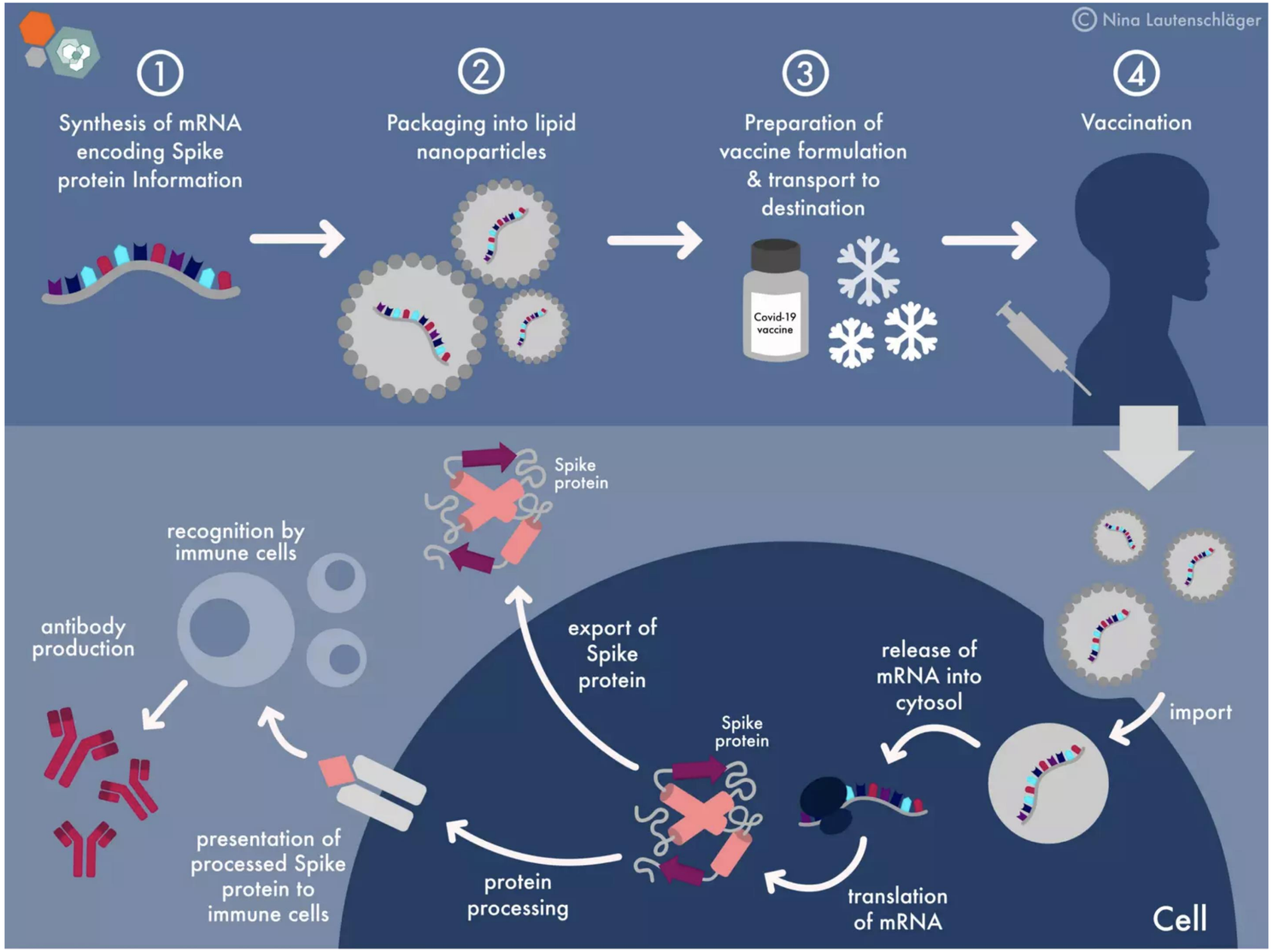
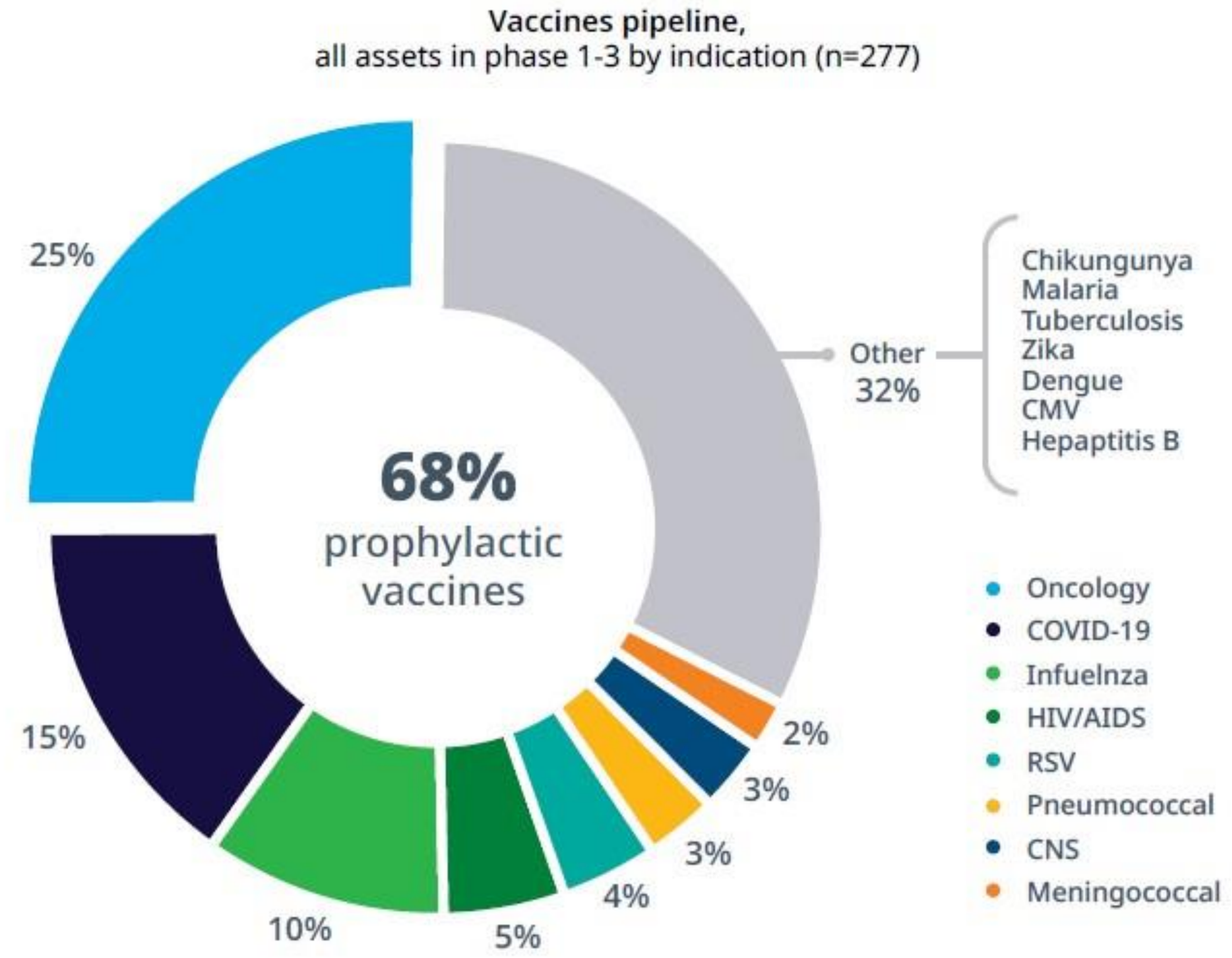
mRNA Vaccines



mRNA Vaccines

Katalin Karikó Drew Weissman

mRNA Vaccines future



https://www.iqvia.com/blogs/2024/04/advances-in-vaccine-technology)

Thank You & Questions