In Person quiz

Manipulating Nucleotides I

What is a mutation?

A mutation is an alteration in the genome of an organism

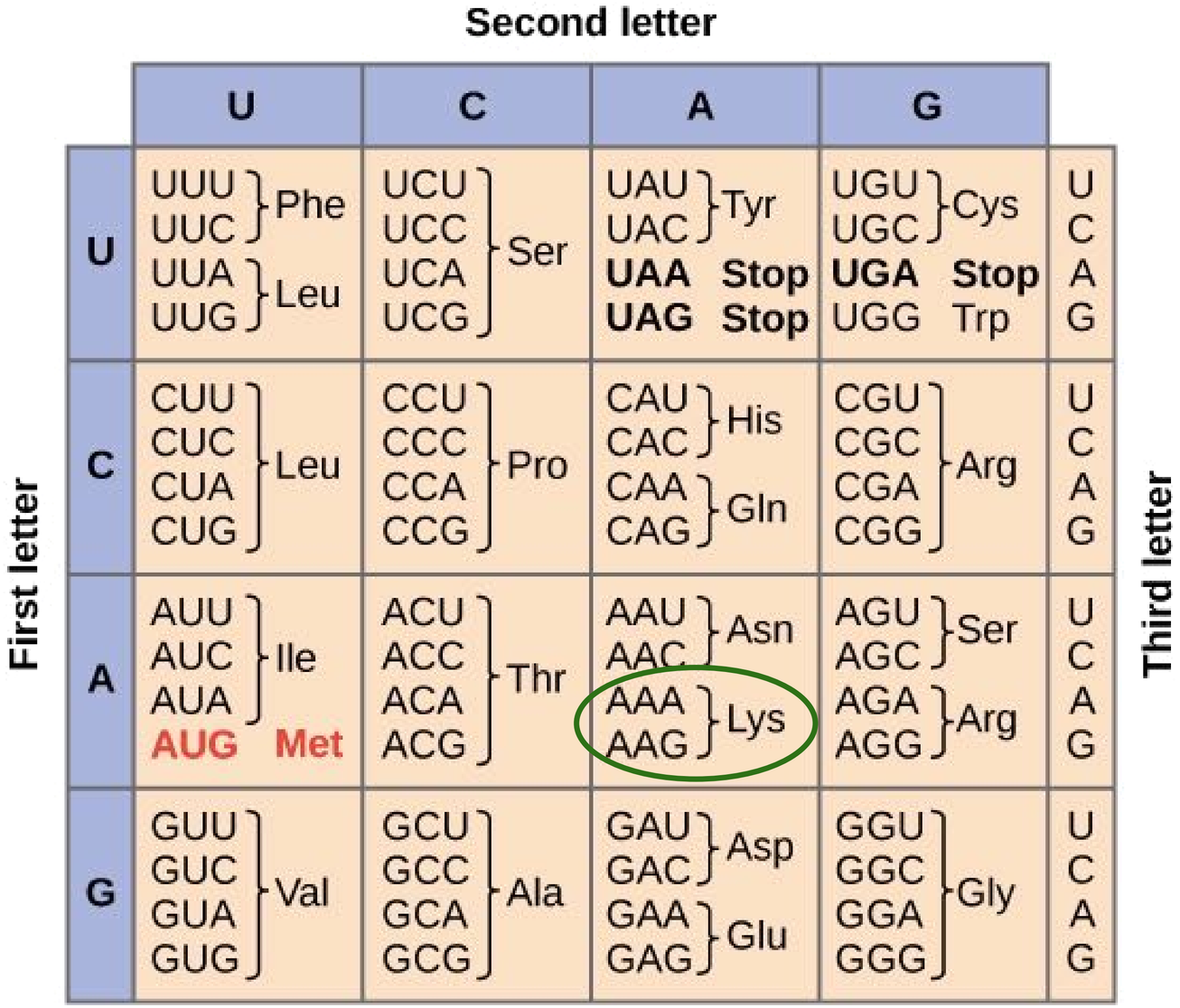
- Chromosomal scale – polyploidy, duplications, inversions, deletions, translocations.

- Nucleotide scale (one or more nucleotides) – insertions, deletions, substitutions.

Effects on gene products

- **Point mutation** – single nucleotide altered
- Effect can be neutral e.g. synonymous substitution

The Genetic code



Synonymous substitution

‘Silent substituion’

GENOME TO FUNCTION
LIFE SCIENCES ENGINEERING

Codon Usage Bias

Some codons are more favoured than others

- Some specific codons are used more often than other **synonymous** codons during translation of genes
- Can vary **within** a species
- Can vary **between** species.
- Use of ‘non-optimal’ codons can **reduce** gene expression
- Selection of the optimal codon usage for the host species during **gene synthesis** is a good idea when moving genes between species

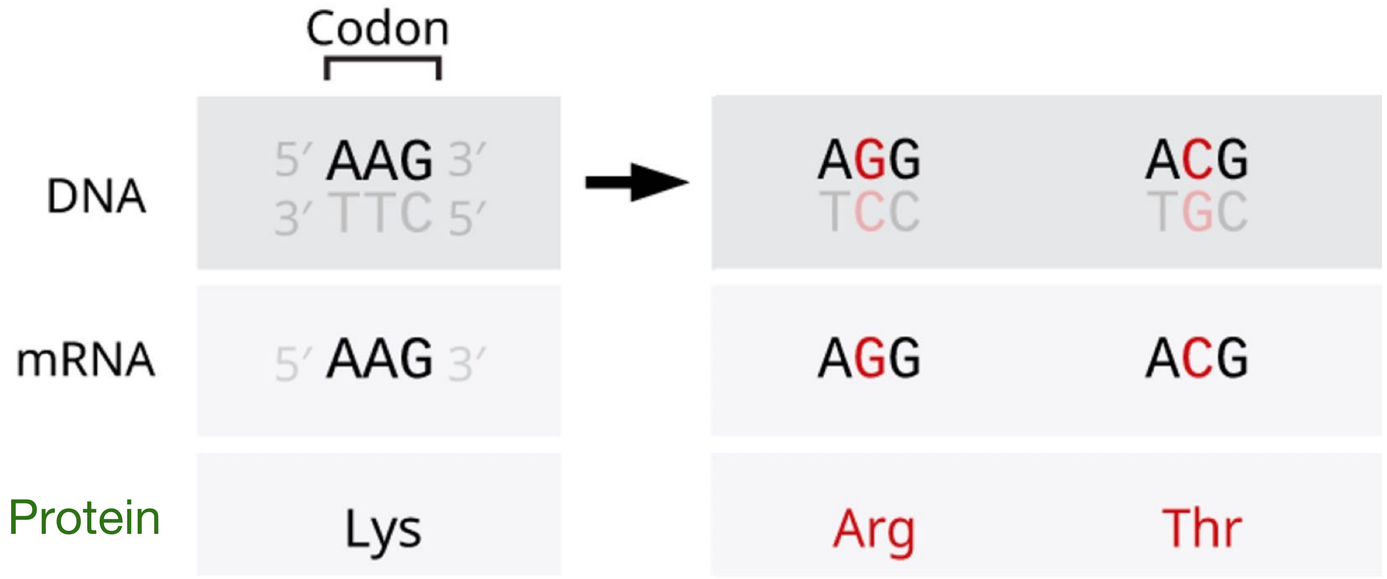
Amino acid	Codon	*A. thaliana*	*S. cerevisiae*	*S. pombe*	*H. Sapiens*
Cys	UGC	+	-	+	+
	UGU	-	+	-	-
Glu	GAA	-	+	-	-
	GAG	+	-	+	+
Phe	UUC	+	+	+	+
	UUU	-	-	-	-
His	CAC	+	+	+	+
	CAU	-	-	-	-
Lys	AAA	-	-	-	-
	AAG	+	+	+	+
Asn	AAC	+	+	+	+
	AAU	-	-	-	-
Gln	CAA	-	-	+	-
	CAG	+	+	-	+
Tyr	UAC	+	+	+	+
	UAU	-	-	-	-

Effects on gene products

- Point mutation – single nucleotide altered
- Effect can be neutral e.g. synonymous substitution
- Change codon to encode a **different amino acid**.

Nonsynonymous mutation

Change amino acid



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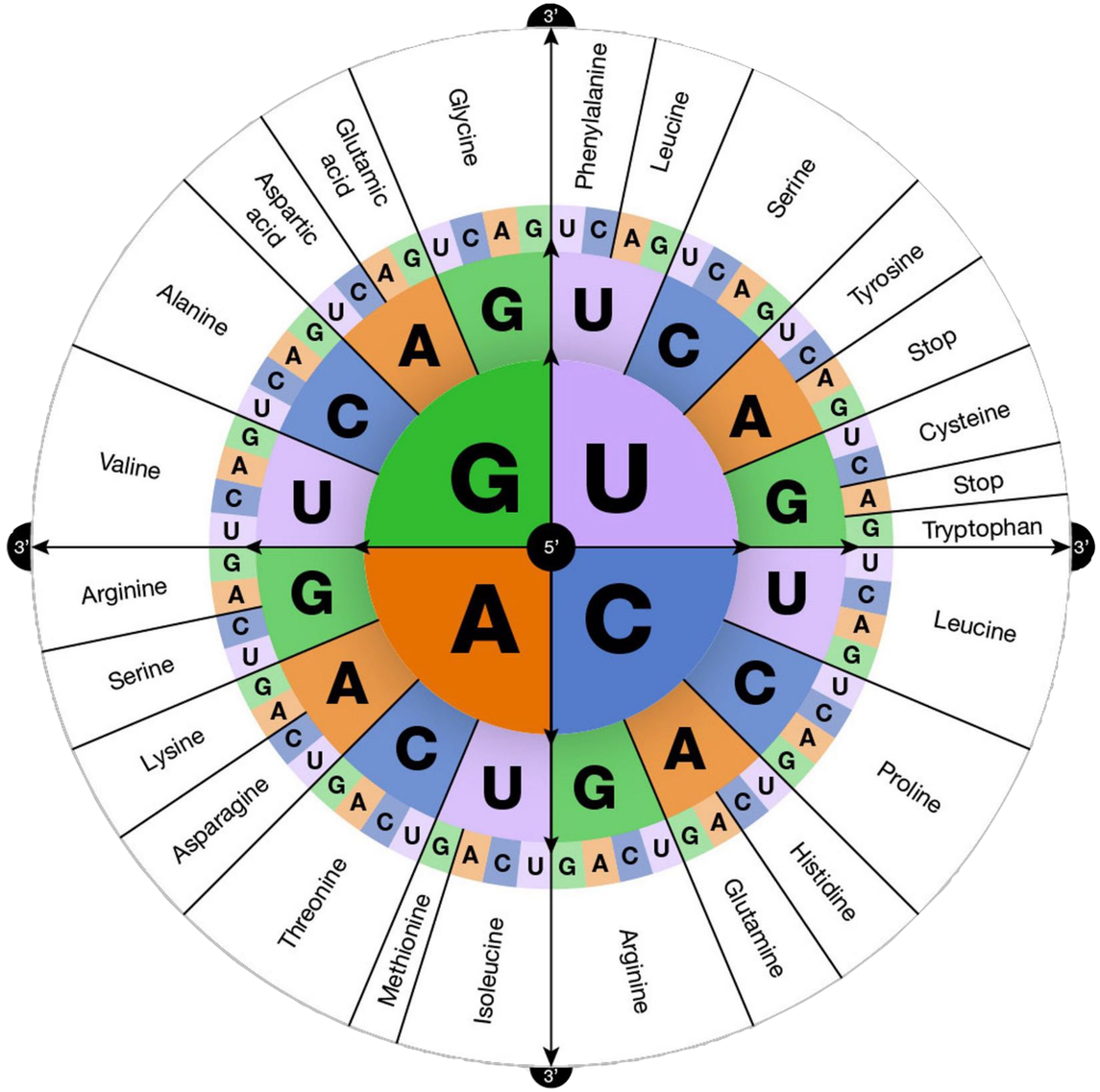
The Genetic code



GENOME TO FUNCTION
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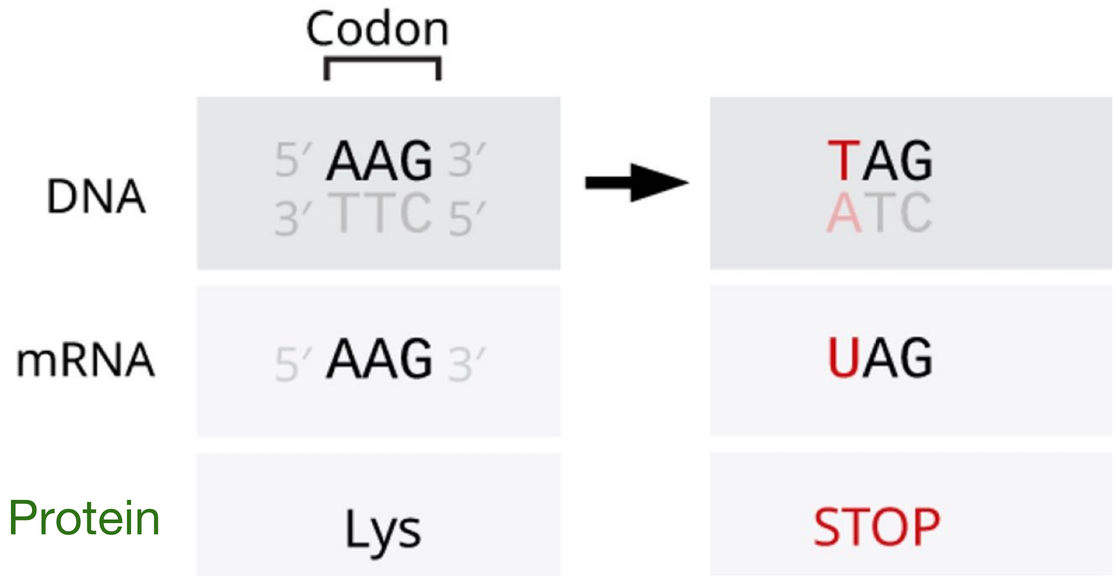
Effects on gene products

- Point mutation – single nucleotide altered
- Effect can be neutral e.g. synonymous substitution
- Change codon to another amino acid.
- Introduce a **new stop codon** i.e. ‘premature stop’ or ‘nonsense’ mutation

Synonymous substitution

‘nonsense mutation’



GENOME TO FUNCTION
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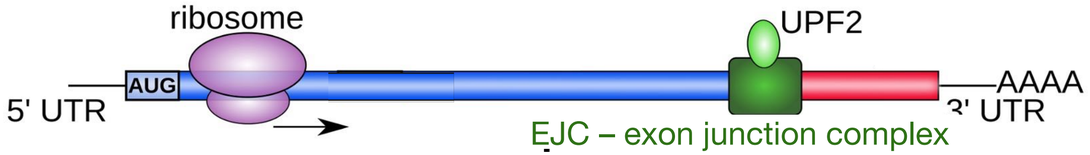
Nonsense-mediated decay

NMD

- NMD is a **surveillance mechanism** for errors in gene expression
- **Degrades mRNA transcripts** that contain premature stop codons
- After splicing, exonic-junction complexes (EJCs) remain 20–24 nucleotides upstream of every exon junction and can bind UPF2 proteins.
- If a ribosome encounters a premature stop codon, **it stalls**, allowing a complex with a downstream EJC/UPF2 promoting **mRNA decay** through the SURF complex.



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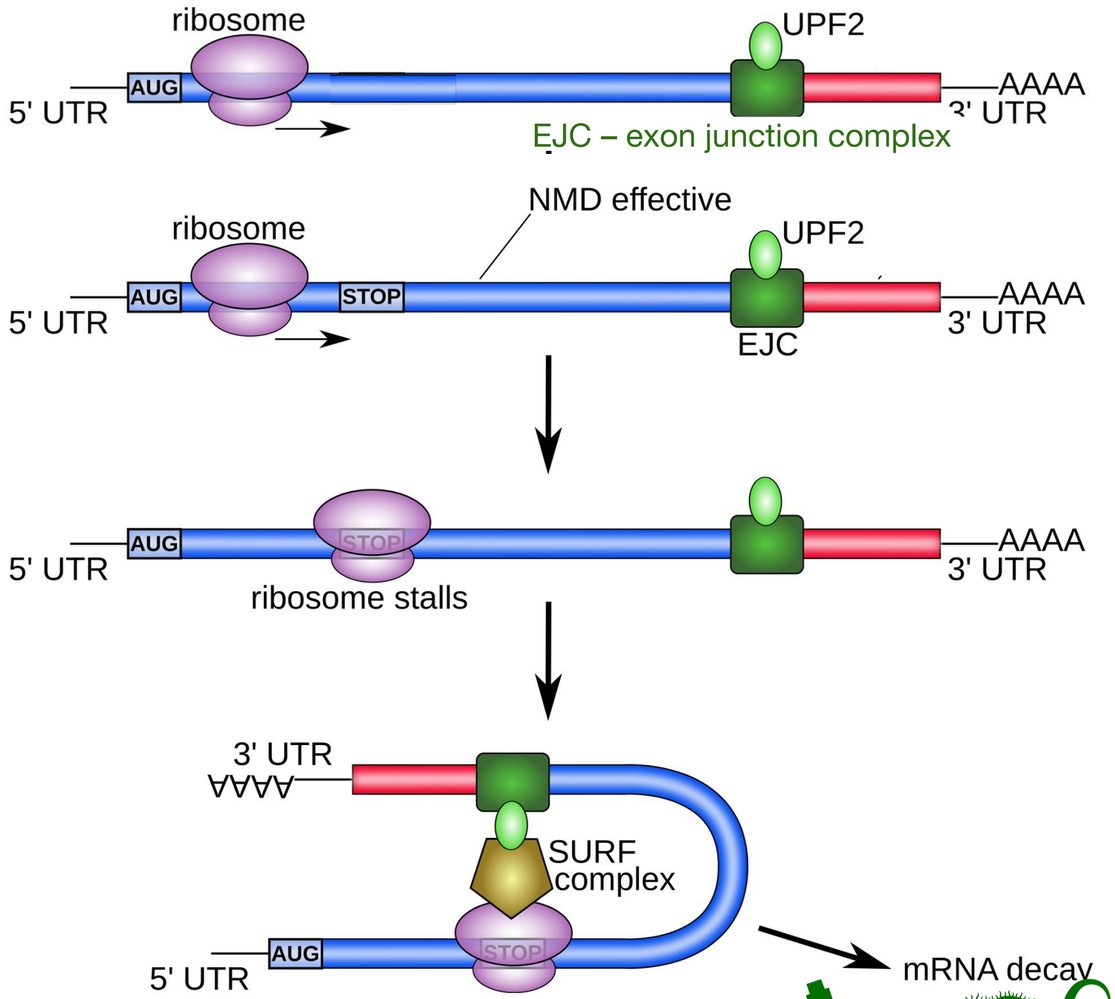

Effects on gene products

- Point mutation – single nucleotide altered
- Effect can be neutral e.g. synonymous substitution
- Change codon to a different amino acid.
- Introduce a new stop codon
- **Frameshift** mutation

Frameshift mutation

- Insertion or deletion of nucleotides that are **not in multiples of 3**
- Changes the reading '**frame**' and thus the protein sequence that will be produced.
- Often results in a premature stop codon.

TAT TGG CTA CTA CAT
Tyr Trp Leu Val His

TAT T**C**G GCT AGT ACA T..
Tyr **Ser Ala Ser Thr**

In Person quiz

‘Natural’ Sources of mutations

Pass the sunblock

- **Spontaneous** mutations – chemical changes to nucleotides
- **Replication** errors
- **DNA repair** errors
- Environmental **mutagens** – **radiation** e.g. Ultraviolet light, ionising radiation e.g. gamma radiation, **chemicals** that cand cause oxidative or other damage.
- Median **somatic** mutation rate per base pair is 2.8×10^{-7} per generation for humans. Order of magnitude **lower in germline cells.**

Effects of mutations

Not usually superpowers

- **Loss-of-function (LOF)** mutations - gene product function reduced (**partial LOF, hypomorph**) or no function (**null allele**, **amorph**). If no protein produced at all, called ‘protein null’.

- Often **recessive** (i.e. both alleles must to be mutant to observe a phenotype) but can be **haploinsufficient** (loss of one allele can cause a phenotype)

- Human disease examples – **Recessive** e.g. Cystic fibrosis. **Haploinsufficient** e.g. DiGeorge syndrome (22q11 deletion)

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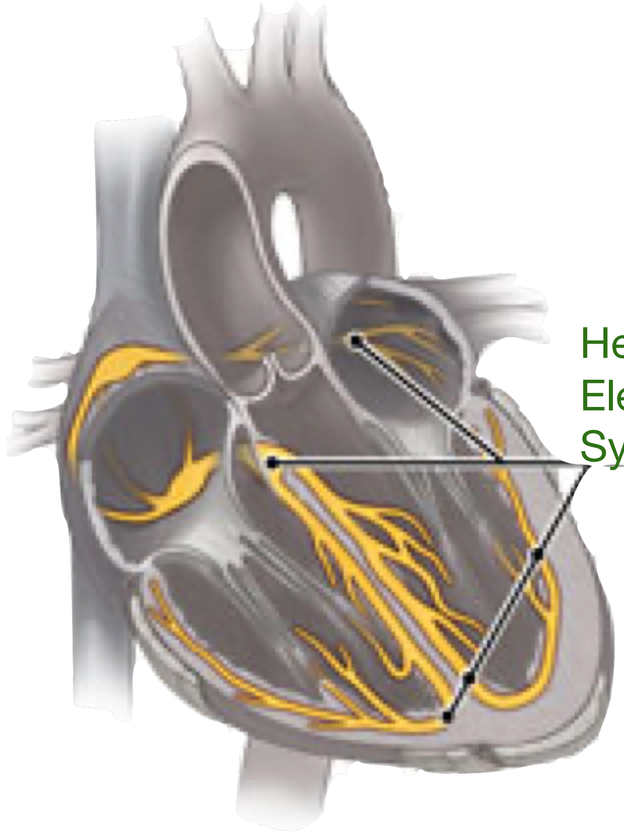
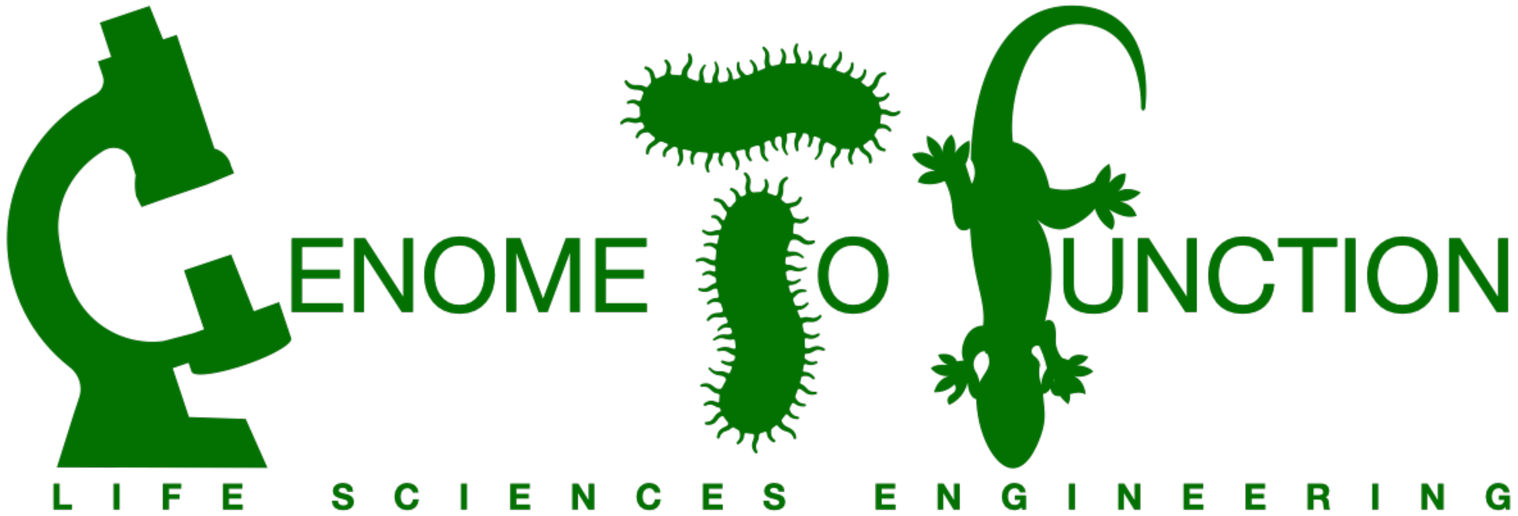
Effects of mutations

- **Gain-of-function (GOF)** mutations (**hypermorphs**).
- Gene product activity is **increased.** Examples include mutations that increase protein expression or increase protein activity.
- GOF mutations often produce **dominant phenotypes** (i.e. one mutated allele is sufficient to produce a phenotype).
- Related are **Neomorphs –** mutations causing **novel gene product functions** (e.g. expression outside of normal tissues, interaction with novel proteins).
- Human disease example - **Brugada syndrome** (GOF mutants in KCNE3) can cause sudden cardiac death in young people



Effects of mutations

- **Dominant Negative (DN)** mutations **(antimorphs)** produce altered gene products that act antagonistically to inhibit to the normal gene product.
- Mutations usually have an altered molecular function (commonly reduced activity)
- DN mutations often produce **dominant phenotypes** (i.e. one mutated allele is sufficient to produce a phenotype).
- Human disease example - **Marfan syndrome** (Dominant negative mutants in FBN1), affects connective tissue.



Genetic Model Organisms

Whistle-stop tour of genetic model organisms

Saccharomyces cerevisiae (Bakers yeast)

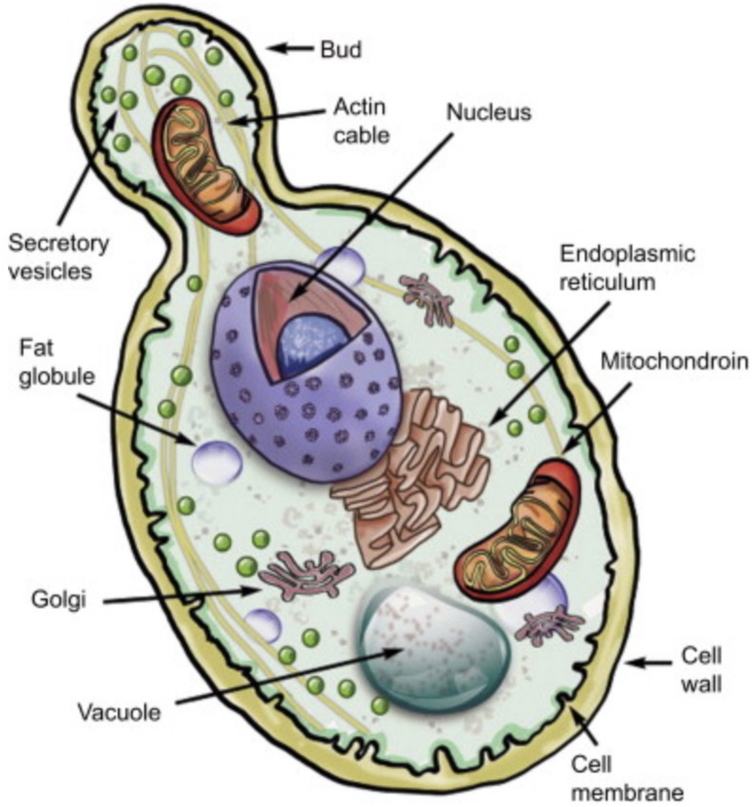
Single cell model organism

Easy to culture, doubling time at 30°C of ~90 minutes

Genome size 12 Mb, 16 chromosomes.

6604 protein coding genes, ~2000 non-coding genes,

Mutants in every gene available



GENOME TO FUNCTION
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Whistle-stop tour of genetic model organisms

Caenorhabditis elegans (nematodes)

1000 somatic cells in adults plus 1000-2,000 germ cells

Generation time 3 days at 25°C

Genome size 100 Mb, 12 chromosomes

20,470 protein coding genes, ~1300 non-coding genes, mutants in most genes available

Whistle-stop tour of genetic model organisms

Drosophila melanogaster (fruit flies)

>million cells

Generation time 10 days at 25°C

Genome size 180 Mb, 4 chromosomes

13,968 protein coding genes, 4,044 non-coding genes, mutants in most genes available

https://drosophila.epfl.ch

Whistle-stop tour of genetic model organisms

Danio rerio (Zebrafish)

millions of cells

Generation time 3 months

Genome size 1.4 Gb, 25 chromosomes

25,545 protein coding genes, 6,599 non-coding genes, mutants in some genes available

Whistle-stop tour of genetic model organisms

Mus Musculus (mice)

Millions of cells

Generation time 10 weeks

Genome size 2.6 Gb, 20 chromosomes

22,213 protein coding genes, 17,398 non-coding genes, mutants in some genes available

Whistle-stop tour of genetic model organisms

Arabidopsis thaliana (Thale cress)

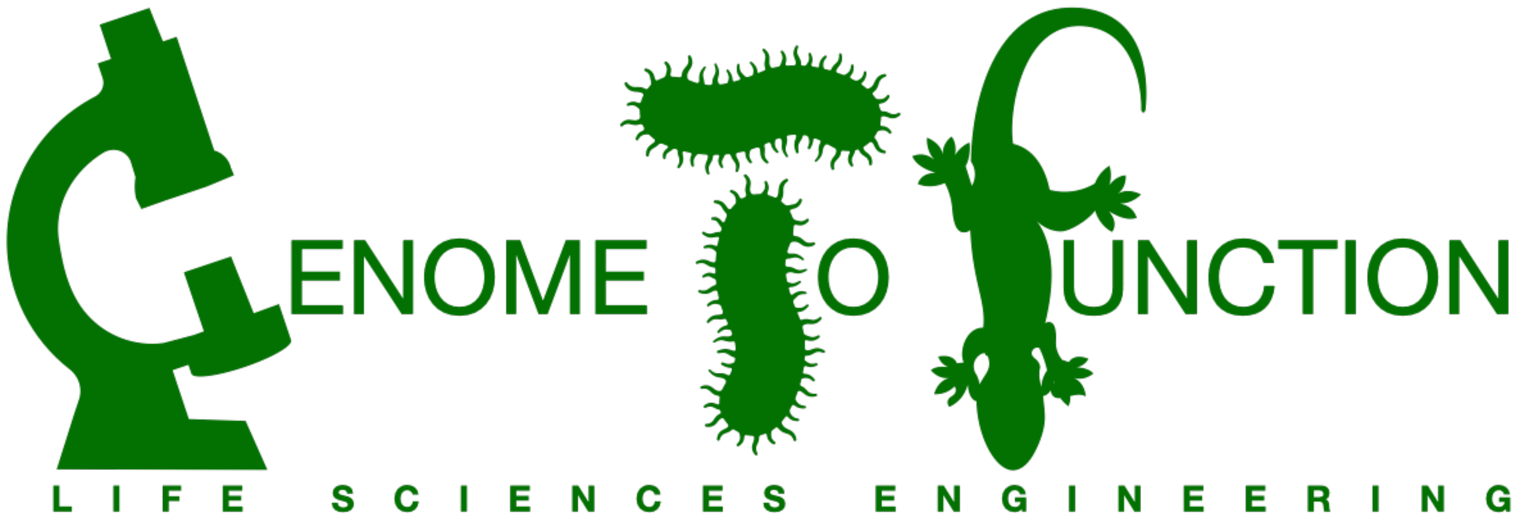
millions of cells

Generation time 6 weeks

Genome size 1.35 Gb, 5 chromosomes

27,655 protein coding genes, 5,178 non-coding genes, mutants in some genes available

In Person quiz



Transgenesis

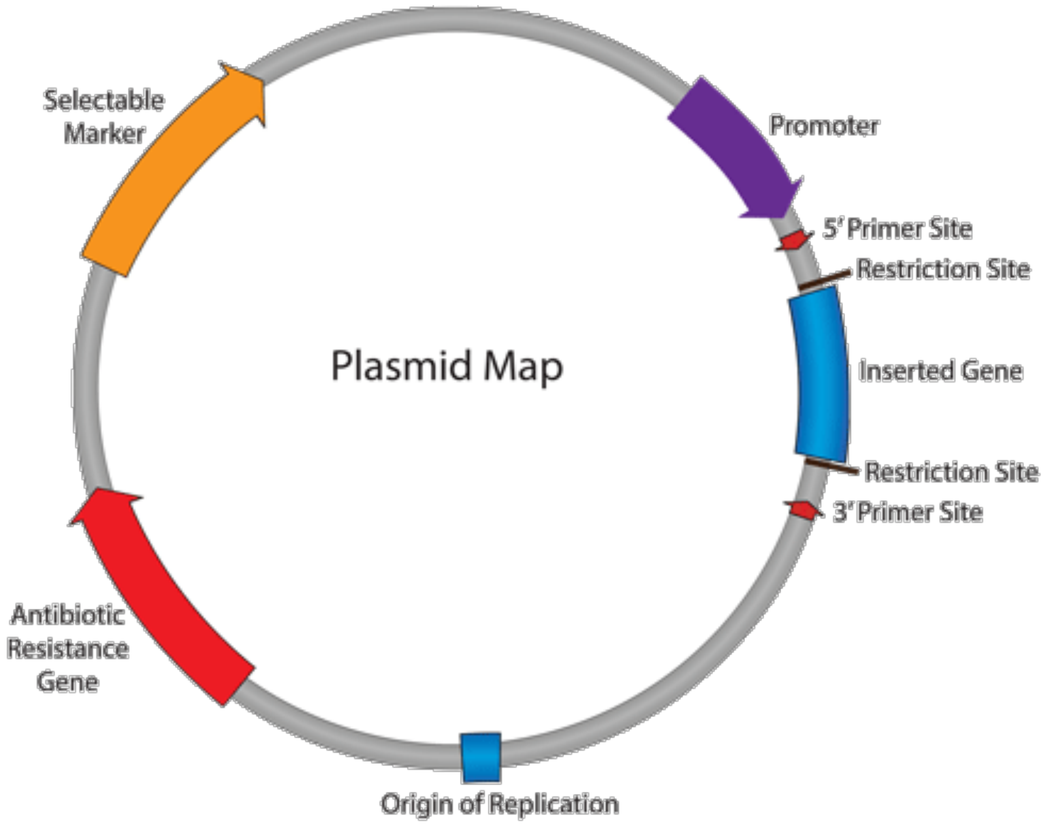
What is Trangenesis?

- The process of introduction of a transgene into an organism
- A transgene is any exogenous genetic sequence either derived from the same species (e.g. an extra copy of a gene), a different species or an artificial sequence.
- Most transgenic animals are generated for research but transgenic animals (GMO – genetically modified organisms) are used in agriculture.

Transgene Donor constructs

Plasmids

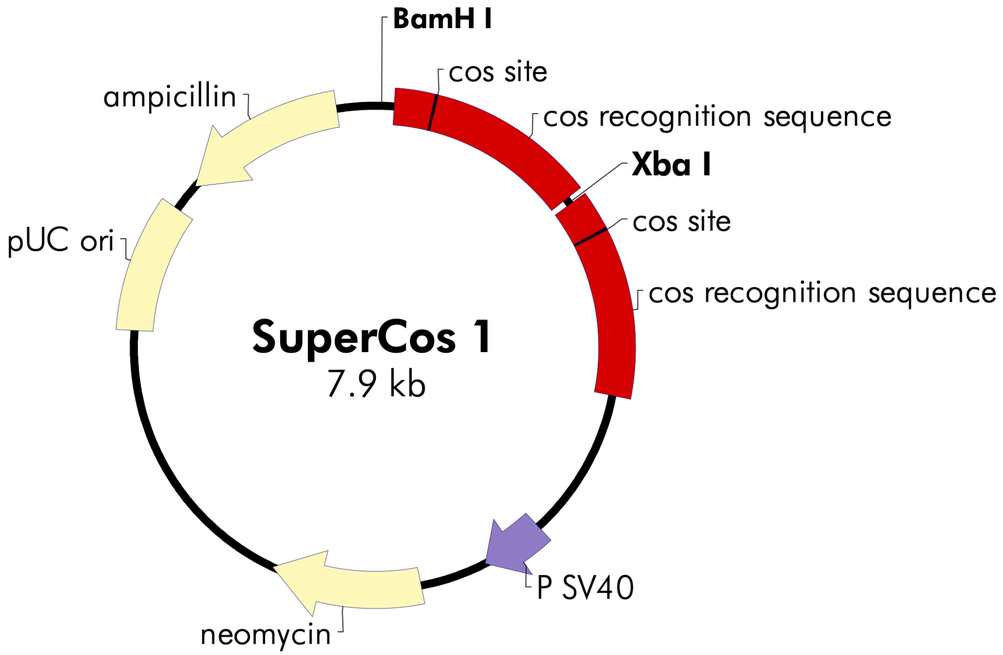
- Easy to work with - Convenient size (generally 1,000-20,000 bp), grow in bacteria (often e.coli).
- Self-replicating - endless number of copies.
- Stable – store in freezer or even dried.
- Useful for lots of things, not species limited, many types of sequences proteins, RNA’s etc. etc.
- Larger DNA sequences are difficult.



Transgene Donor constructs

Cosmids

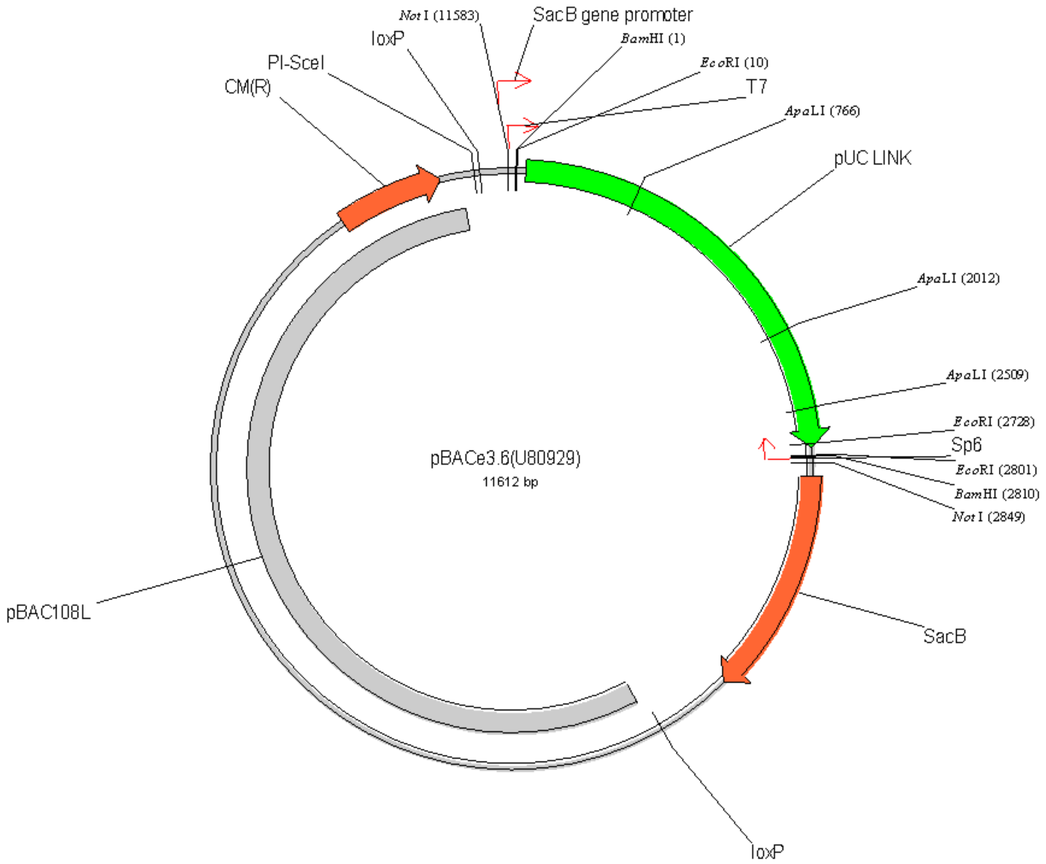
- **Cos** sequence containing plas**mid**
- Can replicate like a plasmid but unlike plasmids can be packaged in phages.
- Can accommodate larger DNA fragments ranging in size from 30 to 45 kb.
- DNA fragments have to be introduced by restriction digestion.
- Most useful for larger genomic DNA fragments, less so for engineered constructs.



Transgene Donor constructs

BACs & YACs

- **B**acterial **A**rtificial **C**hromosome
- Artificial circular chromosomes based on E.coli F-plasmids
- Can accommodate larger DNA fragments ranging in size up to 300 kb.
- YACs are similar but grow in Yeast not bacteria. Can accommodate up to 2000 Kb
- DNA fragments have to be introduced by restriction digestion.
- Useful for large genomic DNA fragments, often used as donors for mouse genetic engineering
- Not useful for small constructs



GENOME TO FUNCTION
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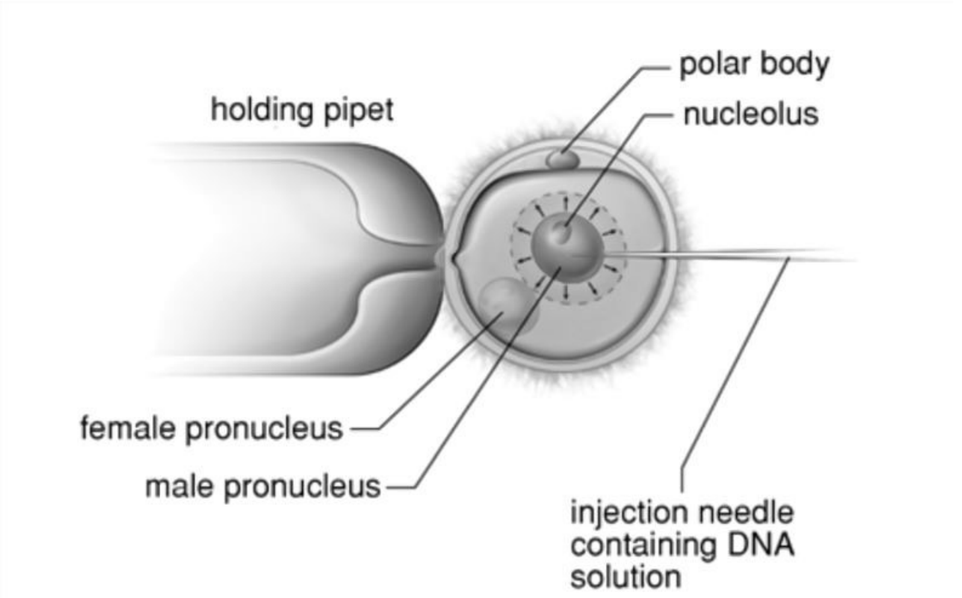
Transgenic Techniques - mammals

DNA microinjection

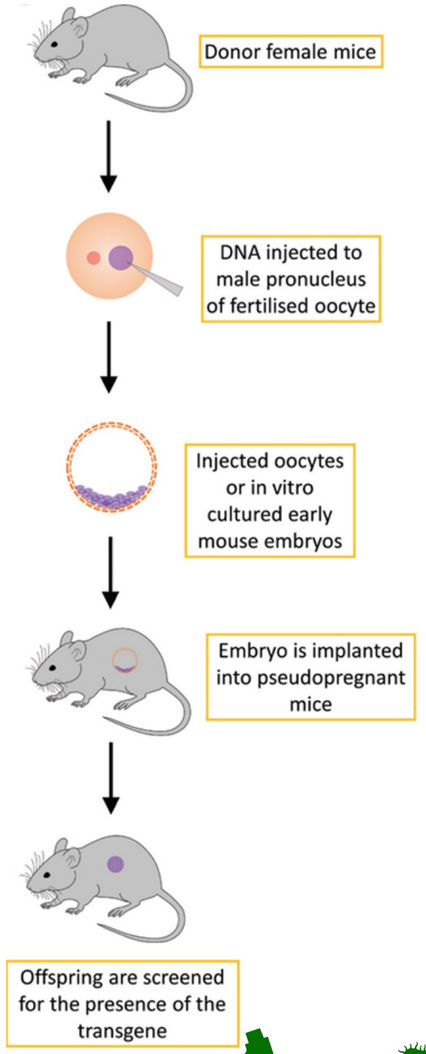
- A plasmid, cosmid or BAC is microinjected
- DNA is introduced into the pronucleus of a developing zygote
- Eggs that survive the injections are transferred into the oviduct of a foster mother.



Transgenic Techniques - mammals

DNA microinjection

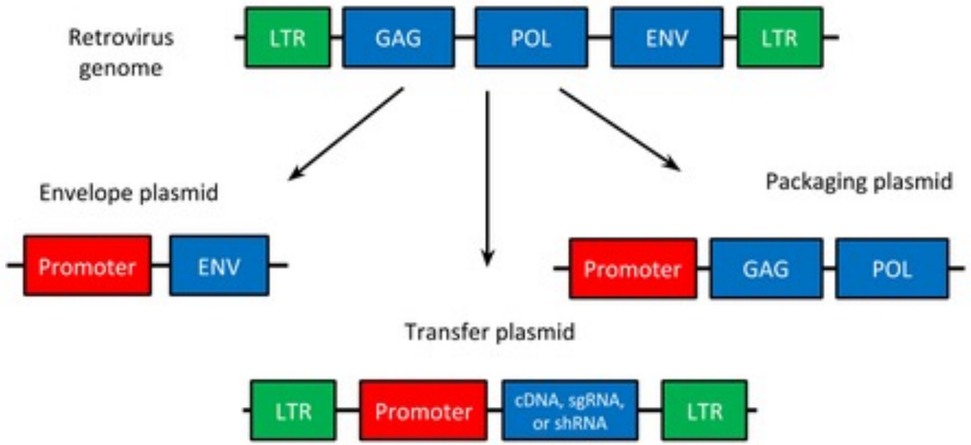
- Widely used
- The amount of DNA delivered per cell is not limited
- Broadly useful across mammalian hosts
- Low success rate
- Mosaic founders
- **Random integration**



Transgenic Techniques - mammals

DNA microinjection with retrovirus

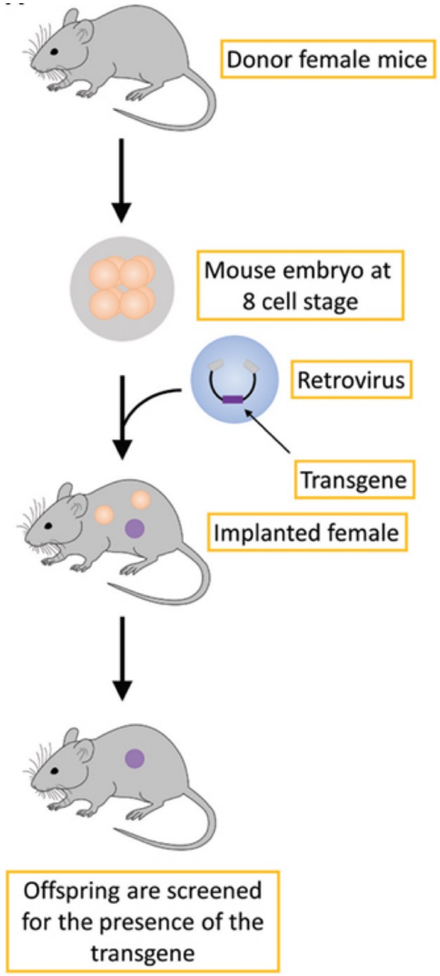
- Commonly use γ-retroviruses (gamma-retroviruses)
- Mostly derived from MoMLV (Moloney Murine Leukemia Virus) or MSCV (Murine Stem Cell Virus) sequences
- Sequences between and including the LTRs is integrated into the host genome upon viral transduction



Transgenic Techniques - mammals

DNA microinjection with retrovirus

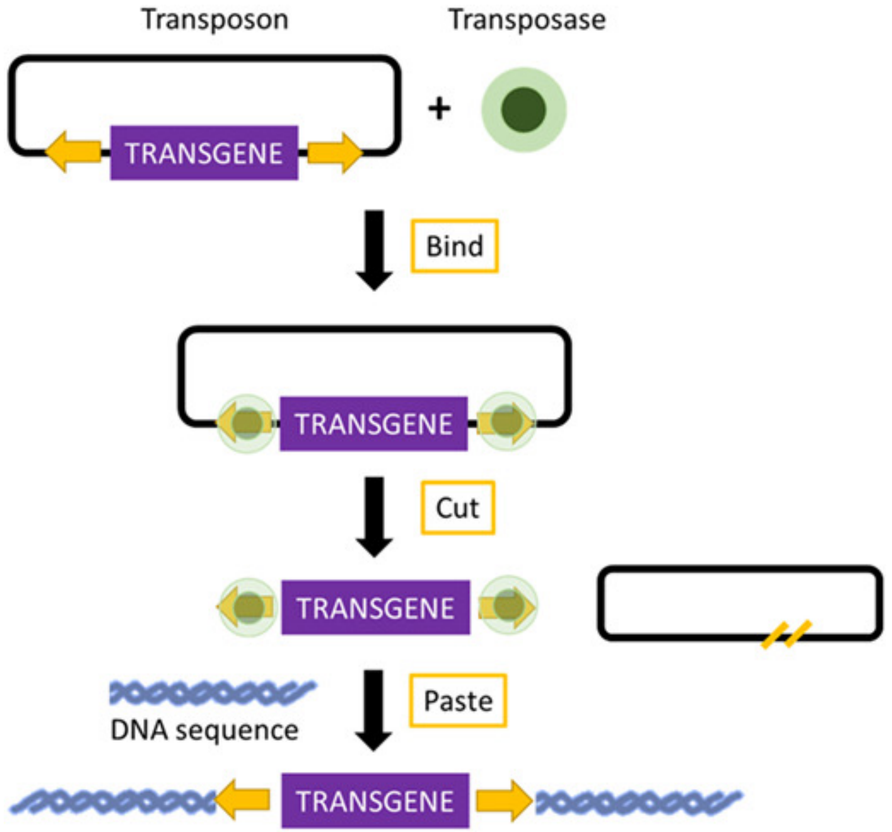
- Virus integration is very efficient
- The amount of DNA delivered per cell is **limited** by virus size max 8.5Kb, but ~3Kb is better
- **Random** integration
- Transgene can be silenced by DNA methylation



Transgenic Techniques - transposons

Transposons

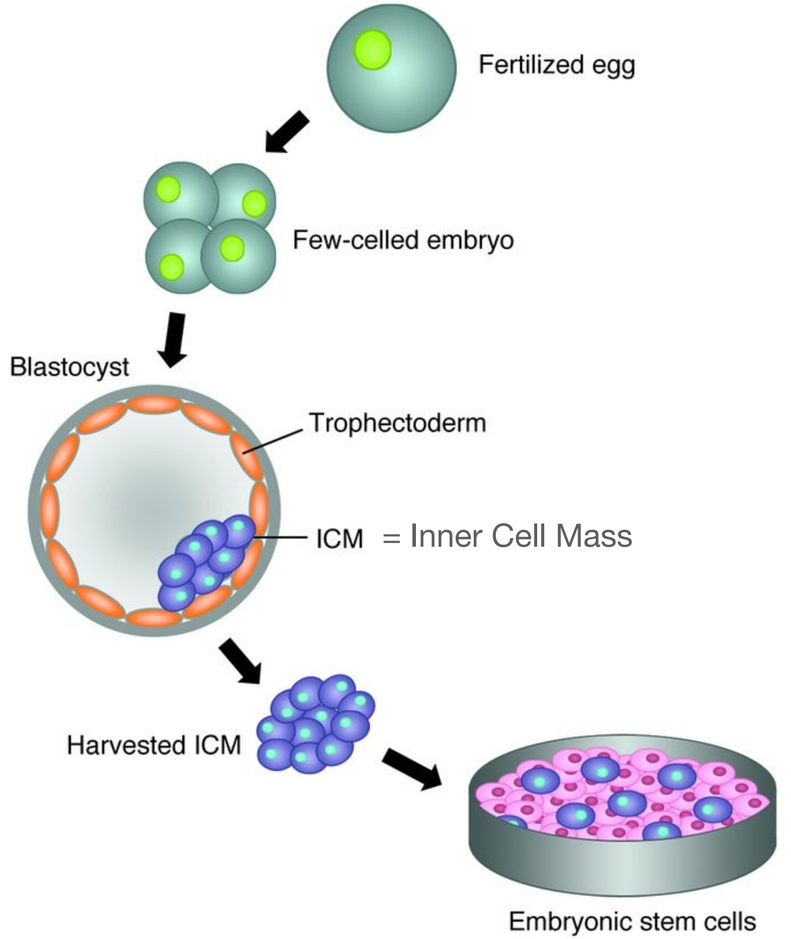
- Transposons, ‘jumping genes’, are genetic elements that can translocate within the genome – Selfish genetic elements
- Transposition process requires sequences at the ends of the transposon and a specific transposase protein
- Can be engineered to replace internal sequences with transgenes
- Have specific sequence preferences e.g. PiggyBaC - TTAA
- Random insertion



Transgenic Techniques - mammals

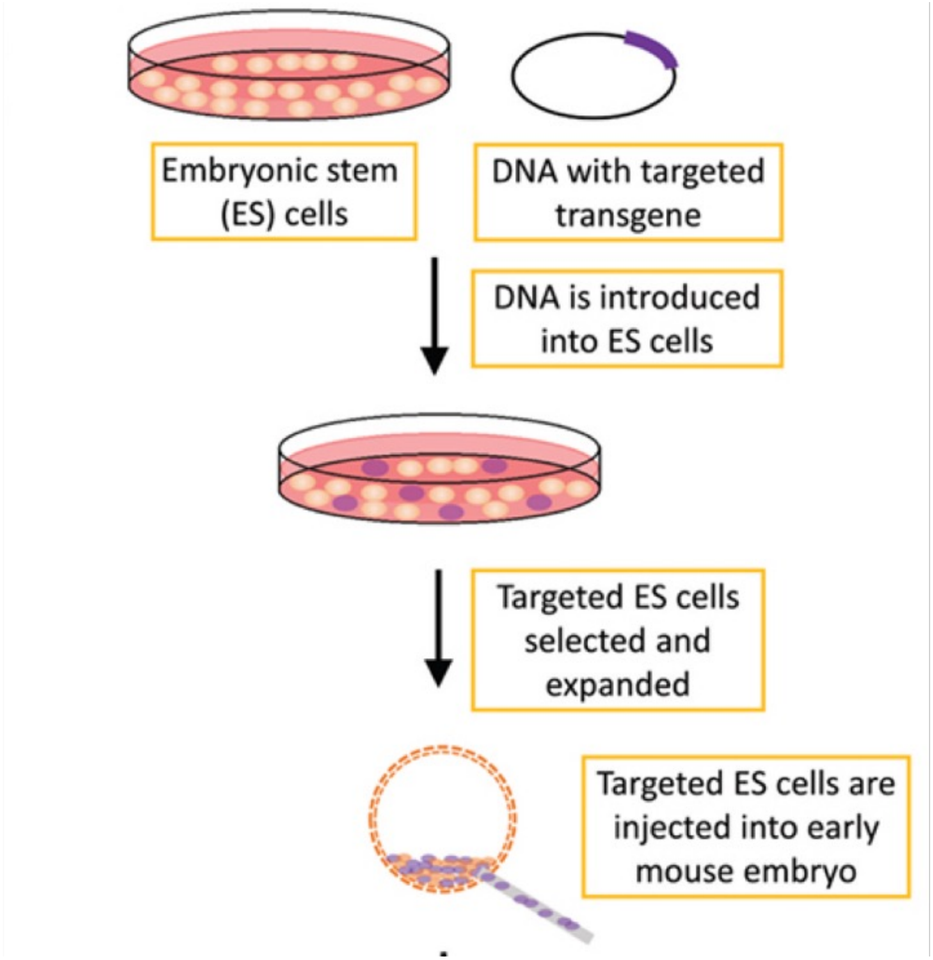
Embryonic Stem Cells (ES cells)

- ES cells are derived from cells of the early embryos (late blastocyst stage)
- Have the capacity to self-renew indefinitely.
- Pluripotent i.e. can differentiate into all cell types in the body including germ cells



Transgenic Techniques - mammals

Introducing transgenes using ES cells



Transgenic Techniques - mammals

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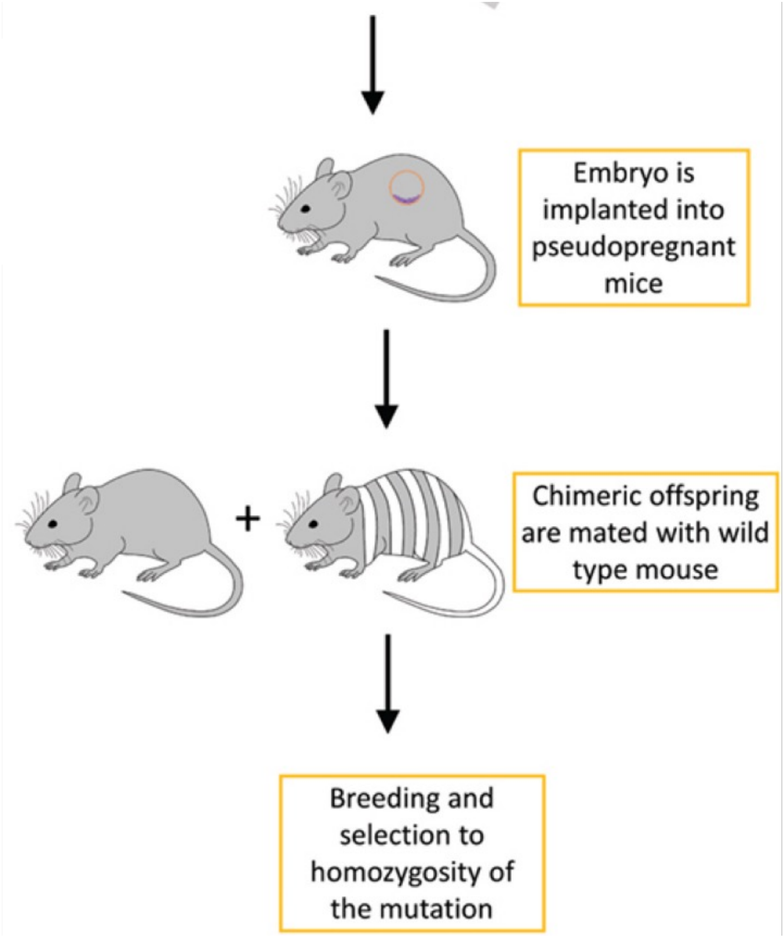
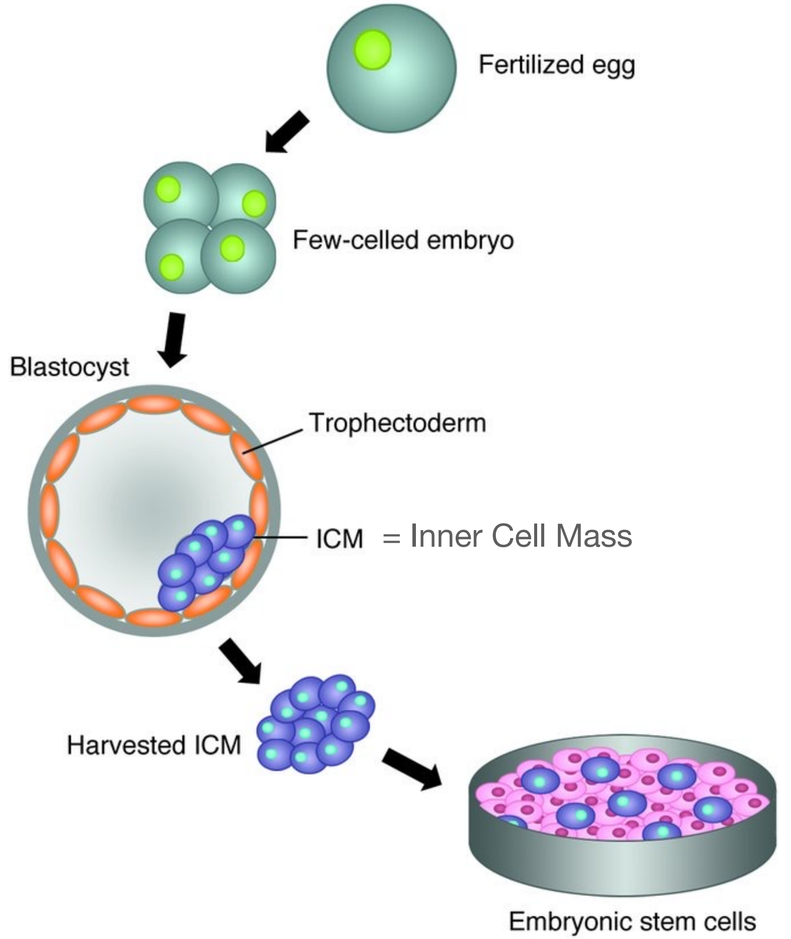


Transgenic Techniques - mammals

Introducing transgenes using ES cells

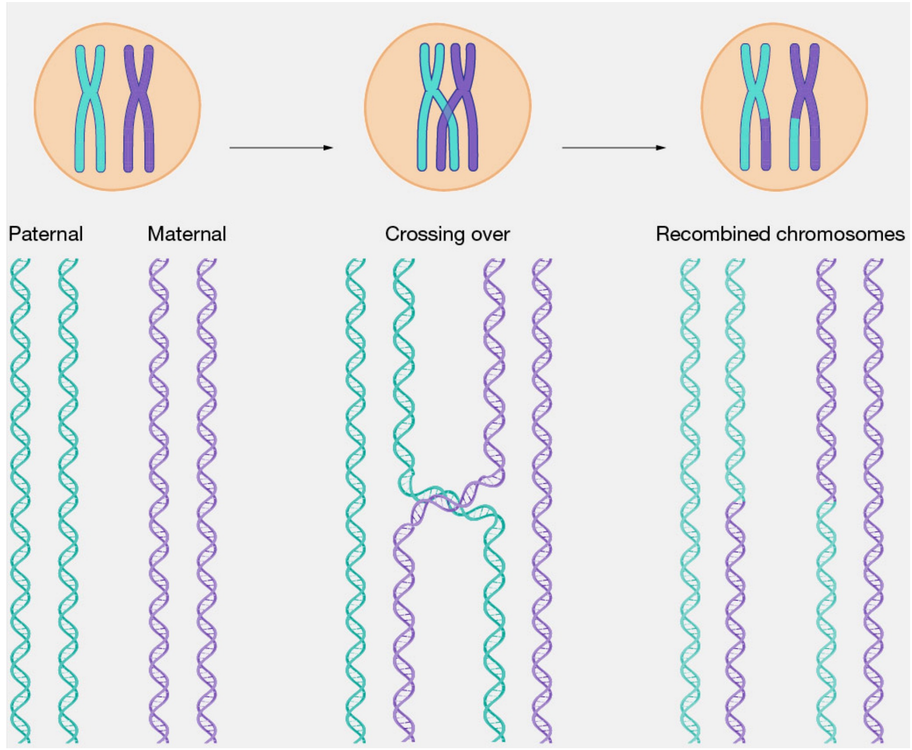
- Efficient through use of cell selectable markers
- Transgene location can be confirmed in ES cells
- Takes longer to have completely transgenic animals.



Transgenic Techniques - mammals

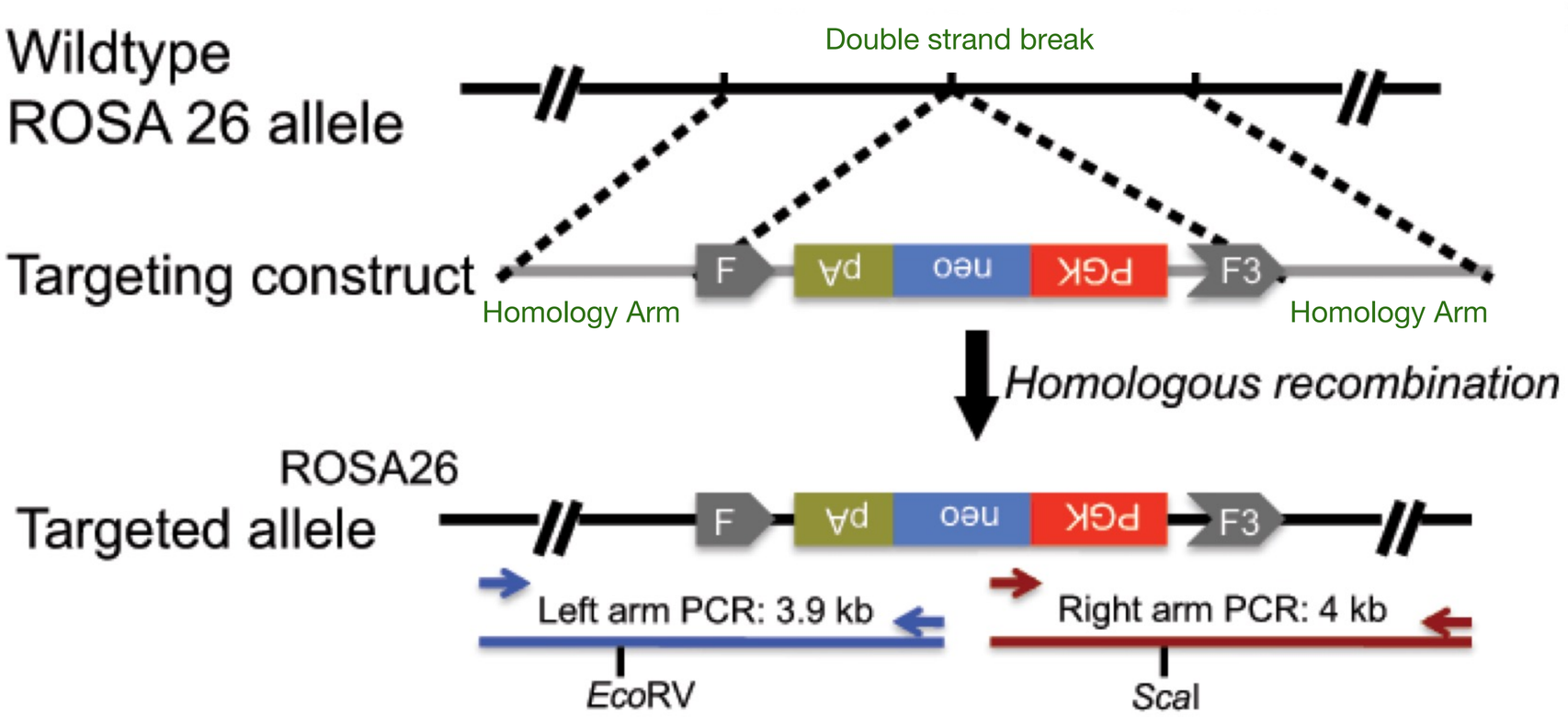
Targeting transgenes to specific locations

- Homologous recombination is a series of processes that enable the repair of DNA and allow interstrand crosslinks.
- essential to exploit the redundancy of genetic information that exists in the form of sister chromatids or homologous chromosomes
- Very important when both strands of the DNA double helix are compromised (double-strand breaks).
- Used during DNA replication somatic cells and during meiosis.



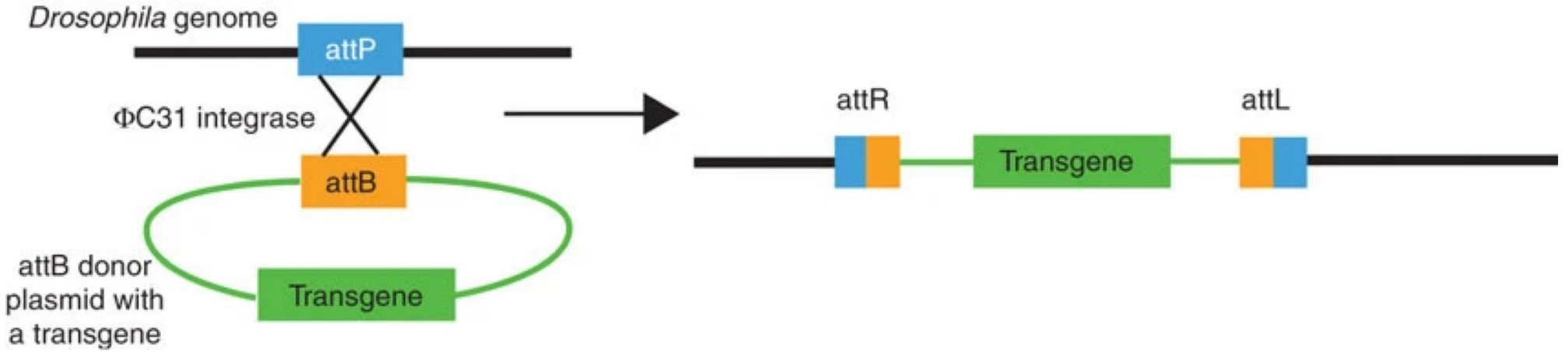
Transgenic Techniques - mammals

Targeting transgenes to specific locations



Transgenic Techniques - recombinase

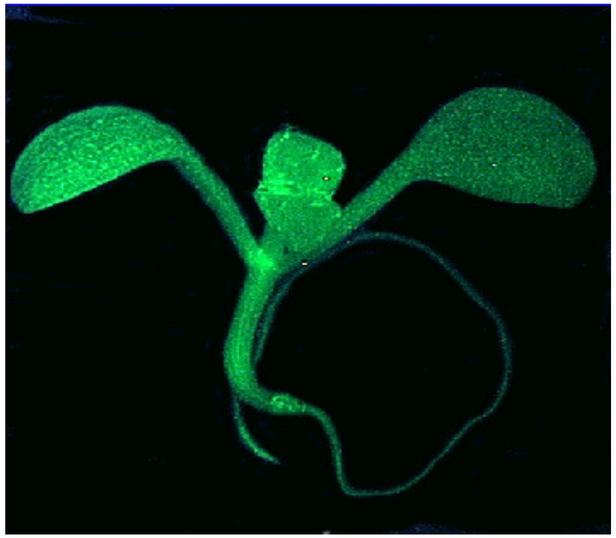
Drosophila - Targeting transgenes phiC31



- A preplaced (with transposon) attP sequence (attP) acts as the recipient site in the Drosophila genome. These sequences are derived from phages.
- An attB plasmid containing both a transgene and donor sequence (attB) is injected togthere with ϕC31 integrase mRNA into attP-containing recipient embryos
- This results in the site-specific insertion of the transgene into the attP site.

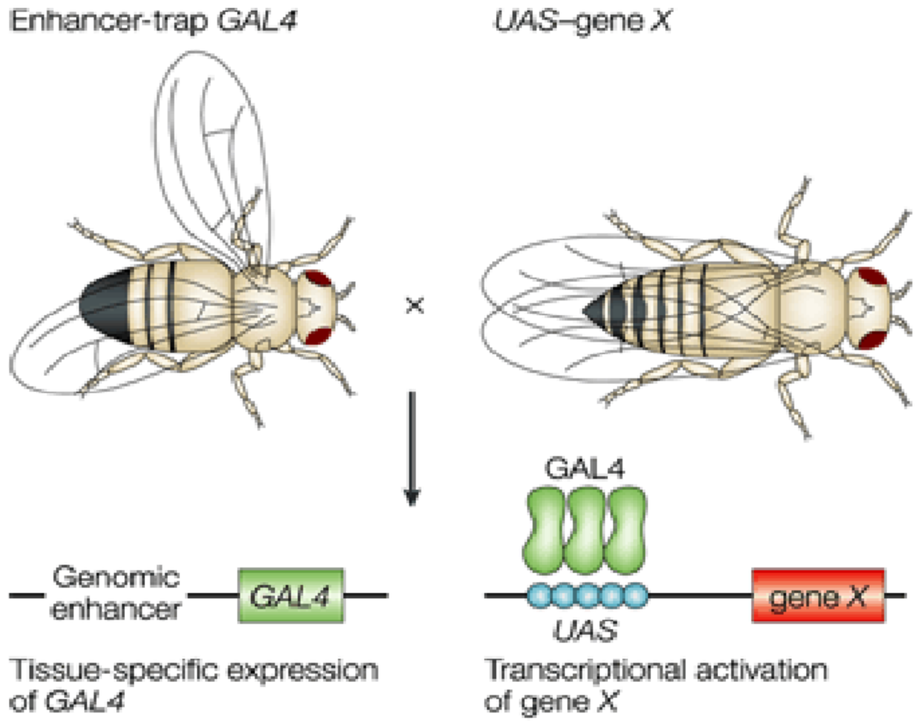
How do I know my animal is transgenic?

- Molecular confirmation e.g. PCR, commonly used in mice
- Selectable marker e.g. GFP
- Rescue of a mutant phenotype e.g. coat colour, eye colour



Controlling transgene expression - space

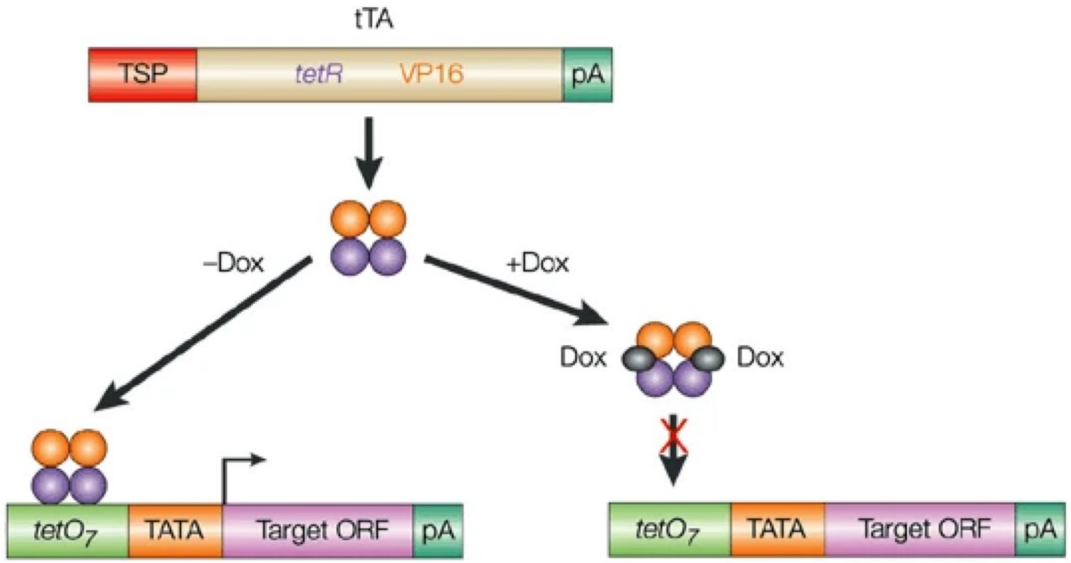
- Binary gene expression system
- Yeast derived Gal4 transcription factor is expressed under control on tissue specific enhancer sequence
- Transgene has Gal4 binding UAS (upstream activating sequence) before gene to be expressed. Without Gal4 – no expression
- In tissues that express Gal4, gene is expressed. Many UAS regulated genes can be expressed simultaneously.



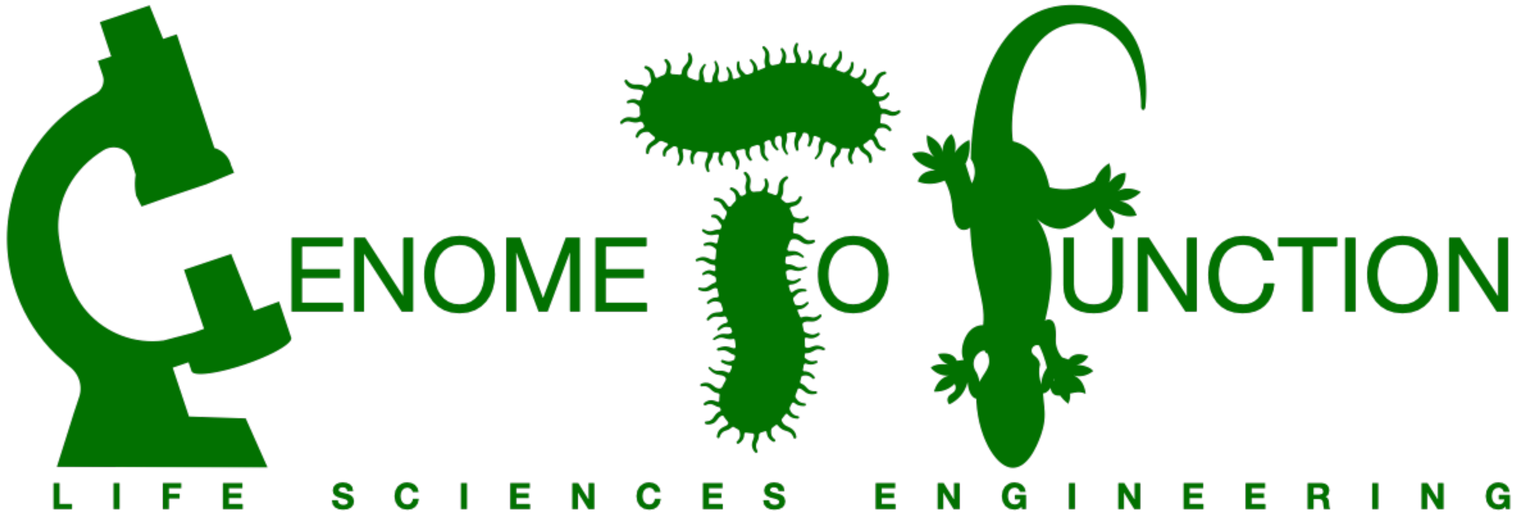
GENOME TO FUNCTION
LIFE SCIENCES ENGINEERING

Controlling transgene expression - time

- **Tet-Off**
- Tetracycline-controlled transactivator (tTA) of transcription regulates gene expression.
- In the absence of the drug doxycycline (Dox), tTA dimers specifically bind to tetO sequences, activating transcription of the target transgene
- When Dox is provided , tTA undergoes a conformational change and cannot bind tetO sequences.
- Tet-On and other variants available



In Person quiz



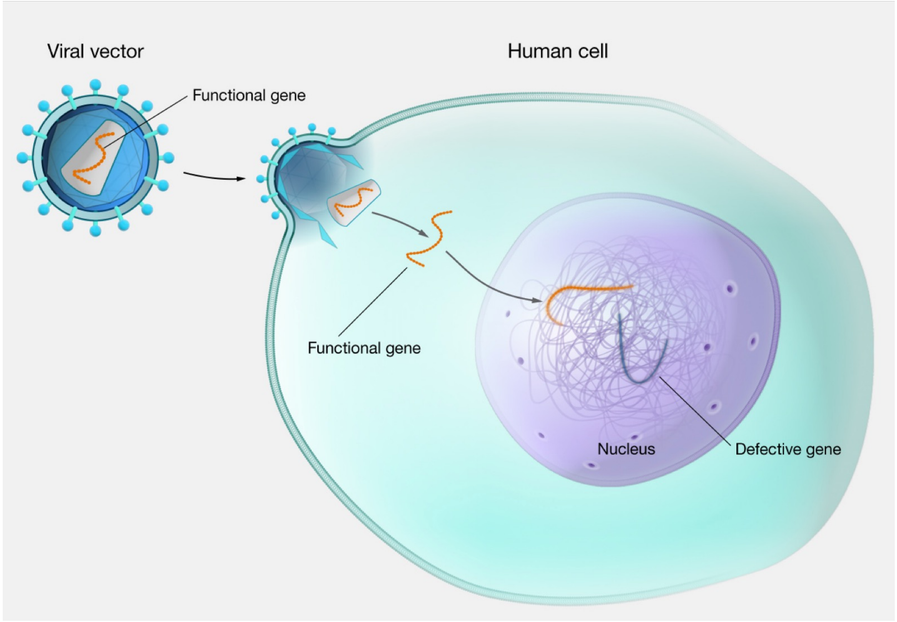
Gene Therapy

Gene Therapy

Treatng humans with transgenes

- Gene therapy uses genetic technology to treat, prevent or cure a disease or medical disorder.

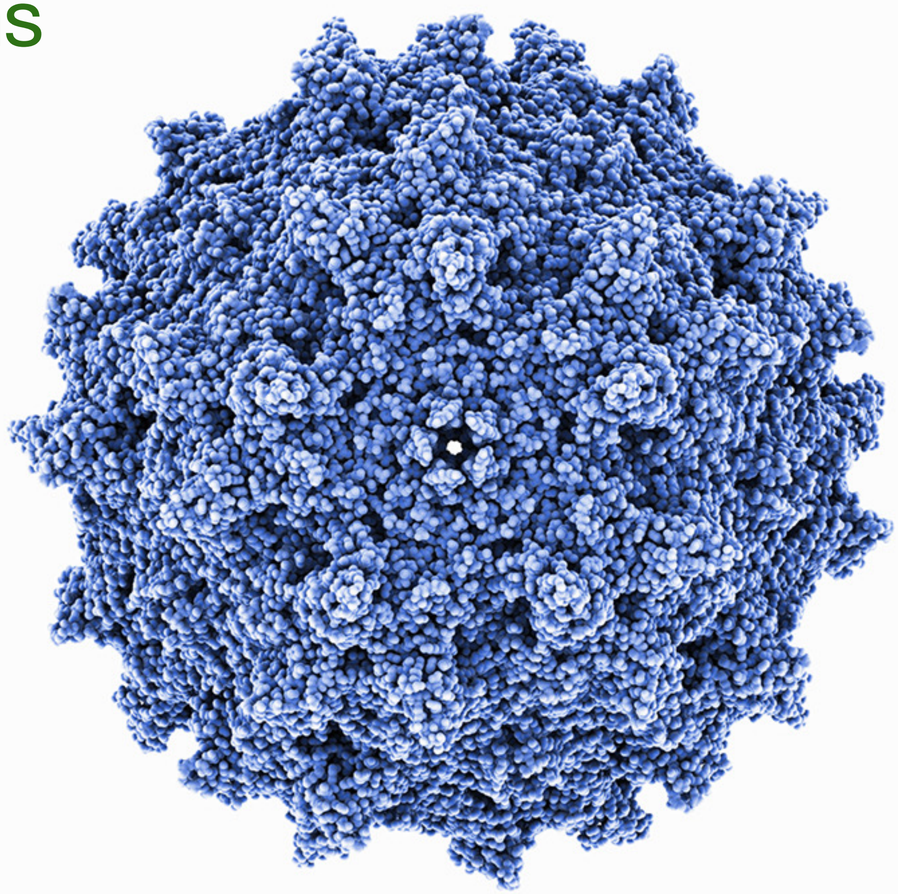
- Commonly utilises additional new copies of a gene to compensate for a defective or absent gene in a patient



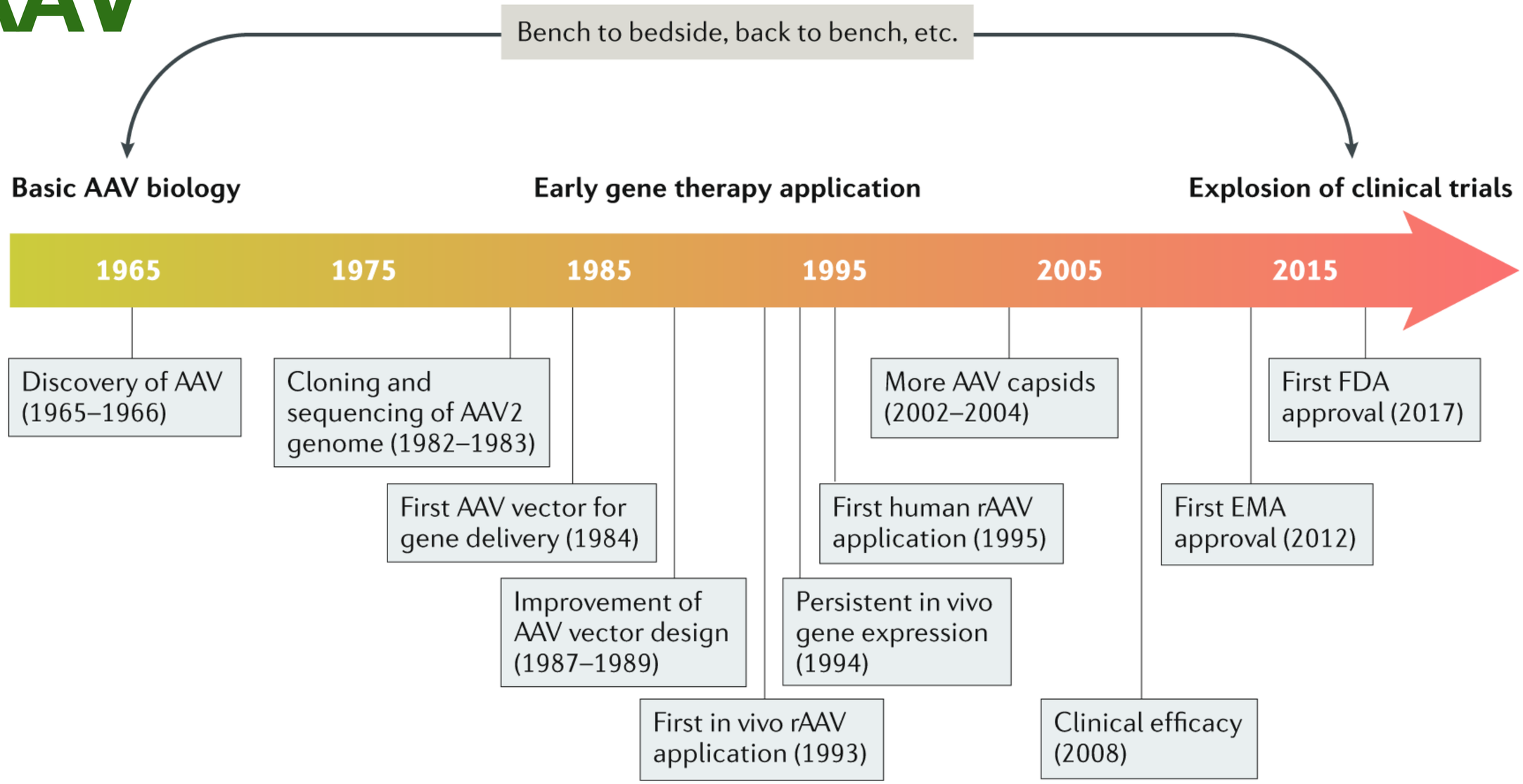
AAV

Targeting transgenes to specific locations

- Adeno-associated viruses (AAV) viruses that infect humans and some other primates.
- Are not thought to cause disease but can elicit a mild immune response.
- Single-stranded DNA genome of 4.8 Kb
- **Does not integrate into genome**



AAV



GENOME TO FUNCTION
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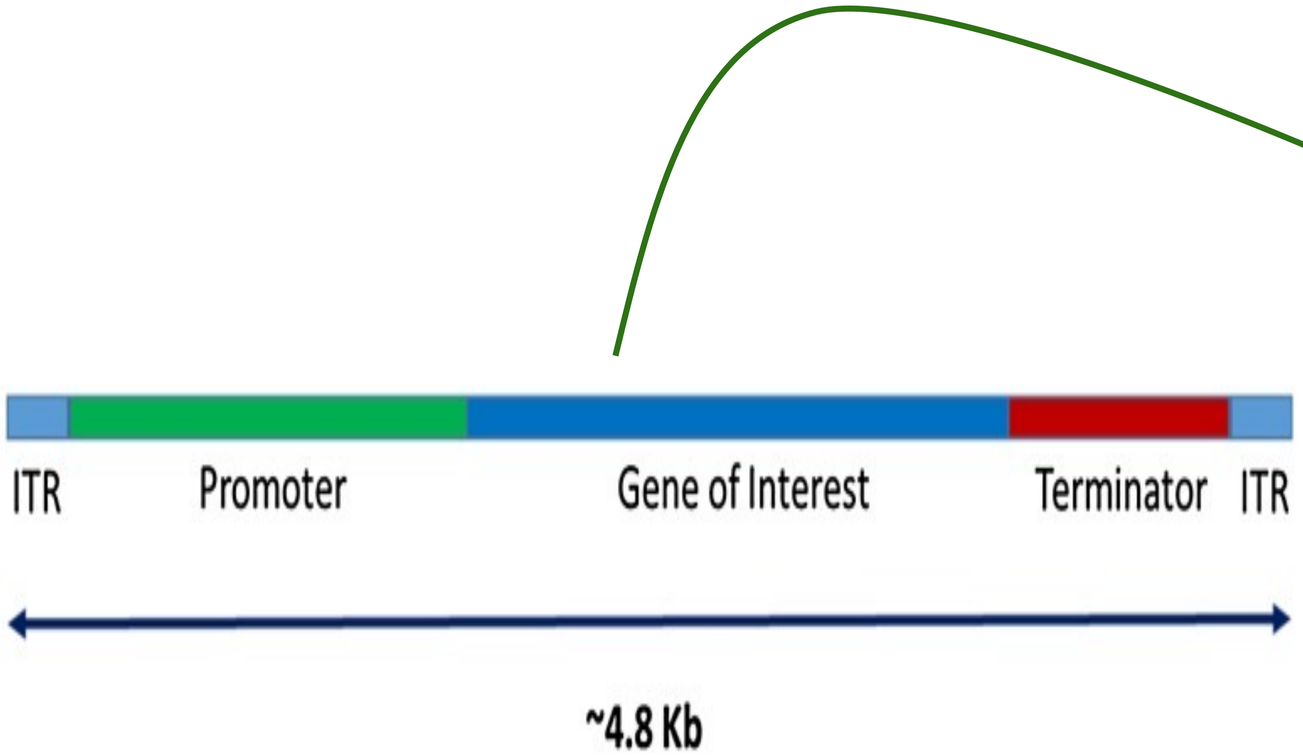
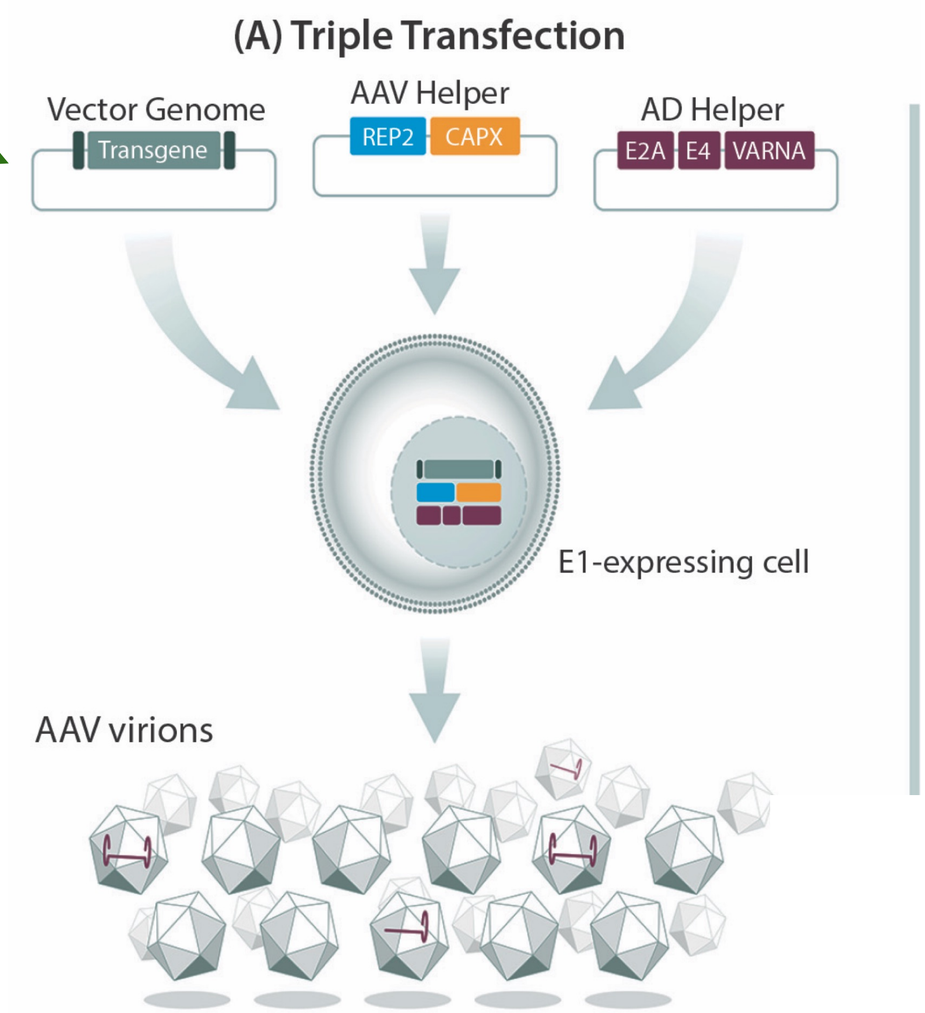
AAV





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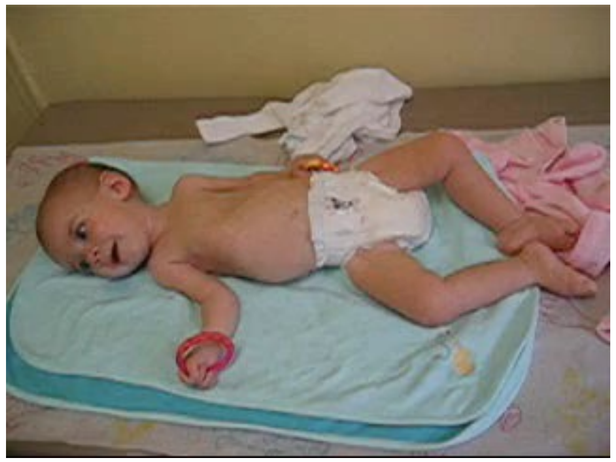
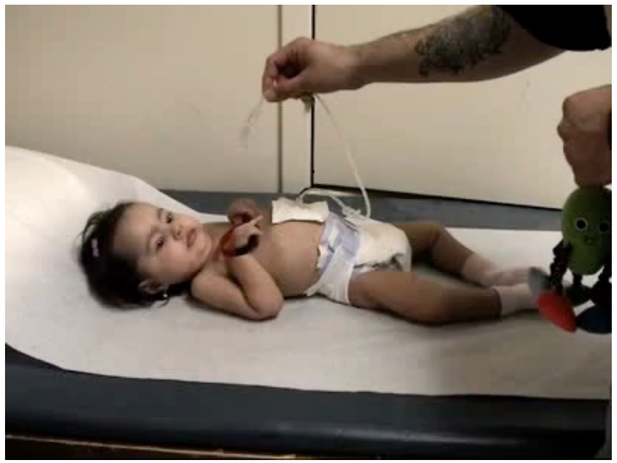
Spinal Muscular Atrophy (SMA)



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%

GENOME TO FUNCTION
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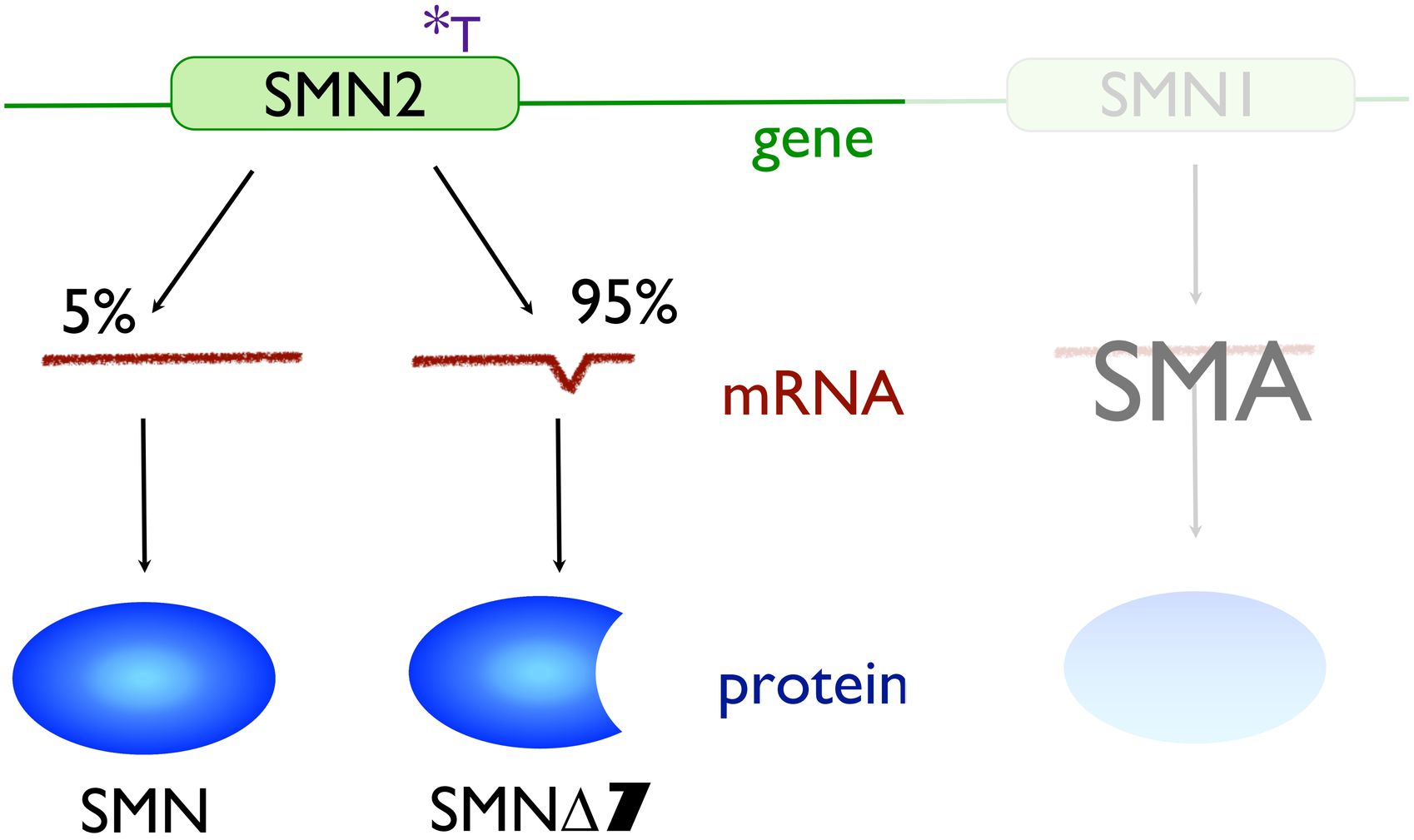
Reduction of SMN (Survival of Motor Neuron)causes SMA



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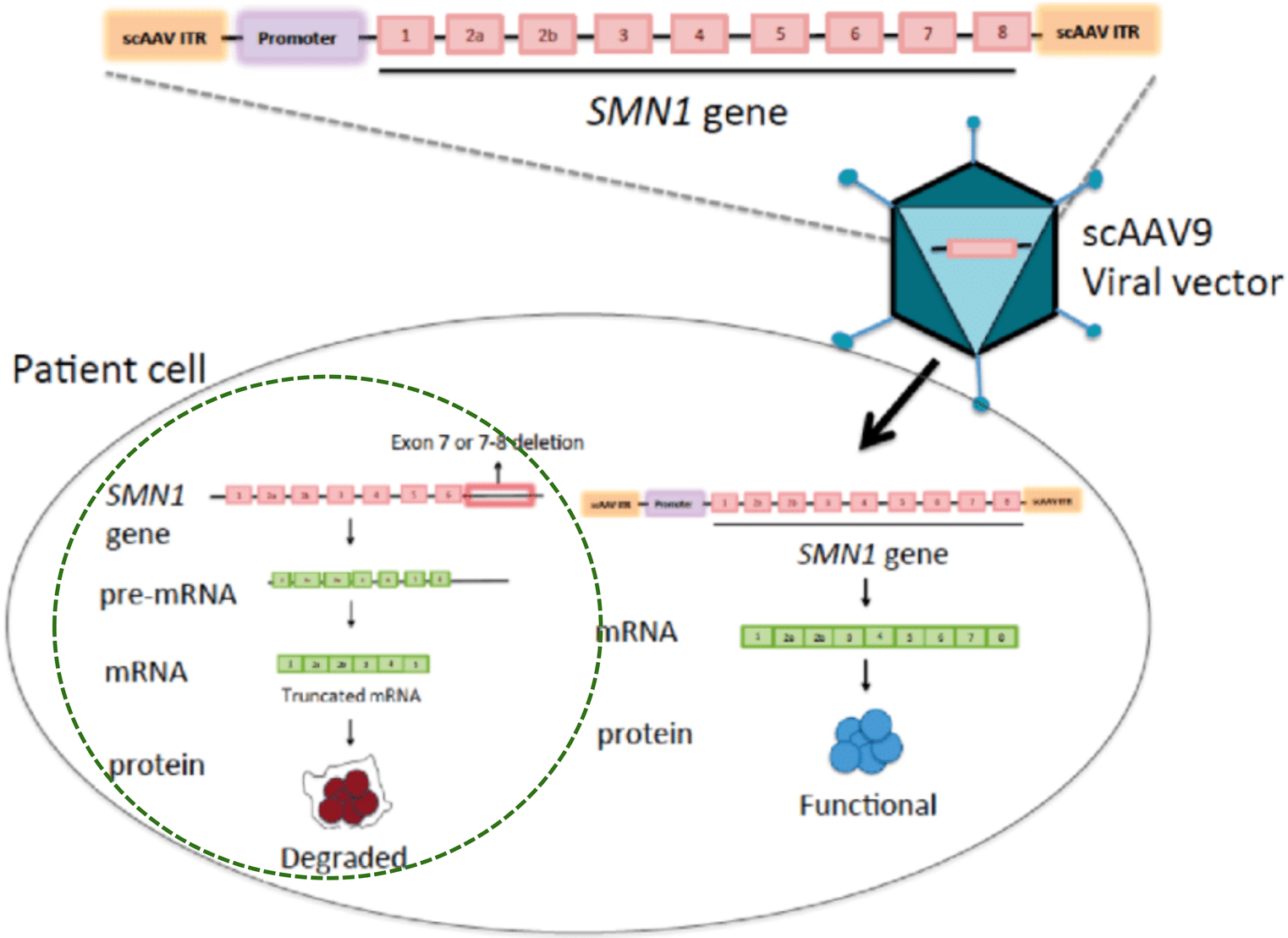
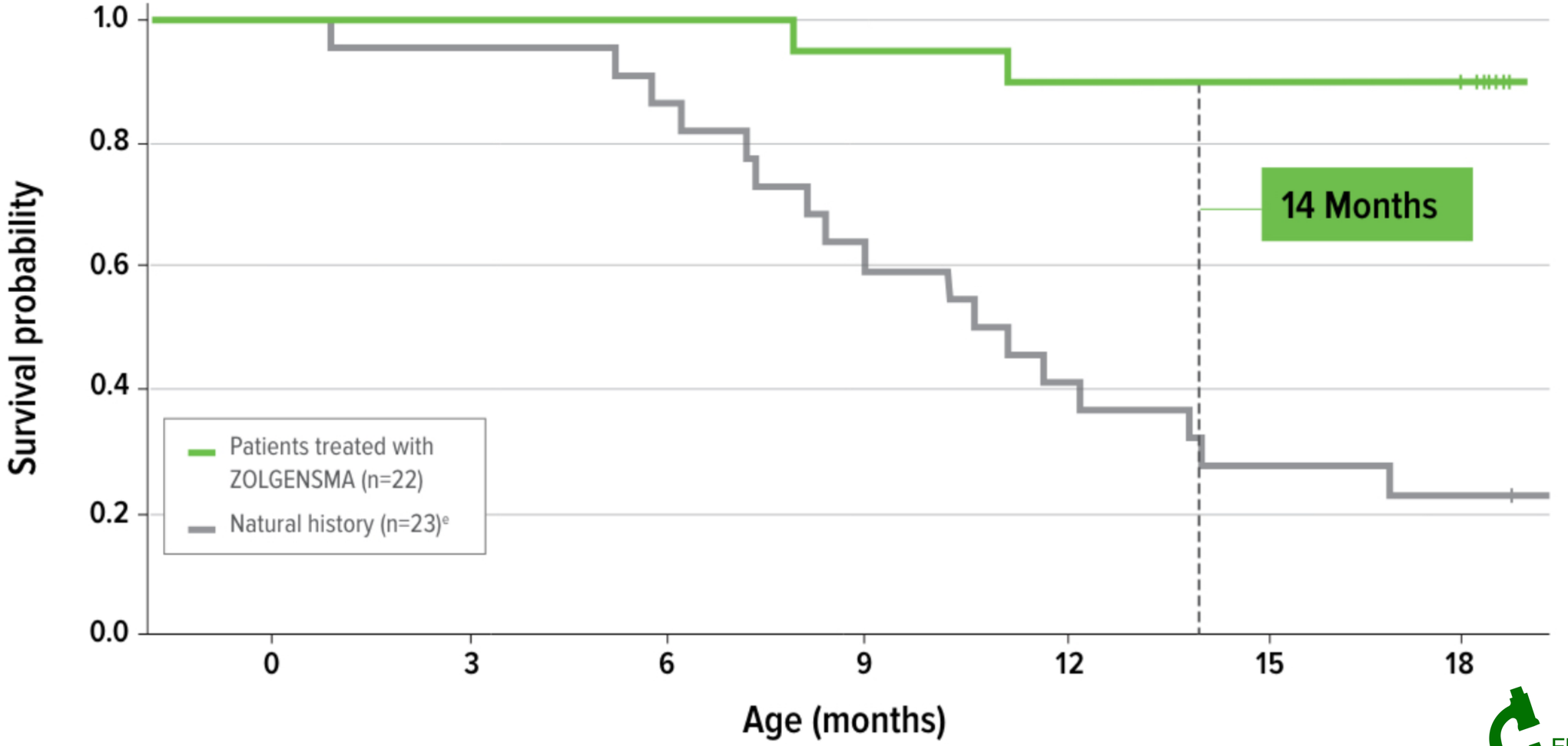
Gene Therapy for SMA



Gene Therapy for SMA



GENOME TO FUNCTION
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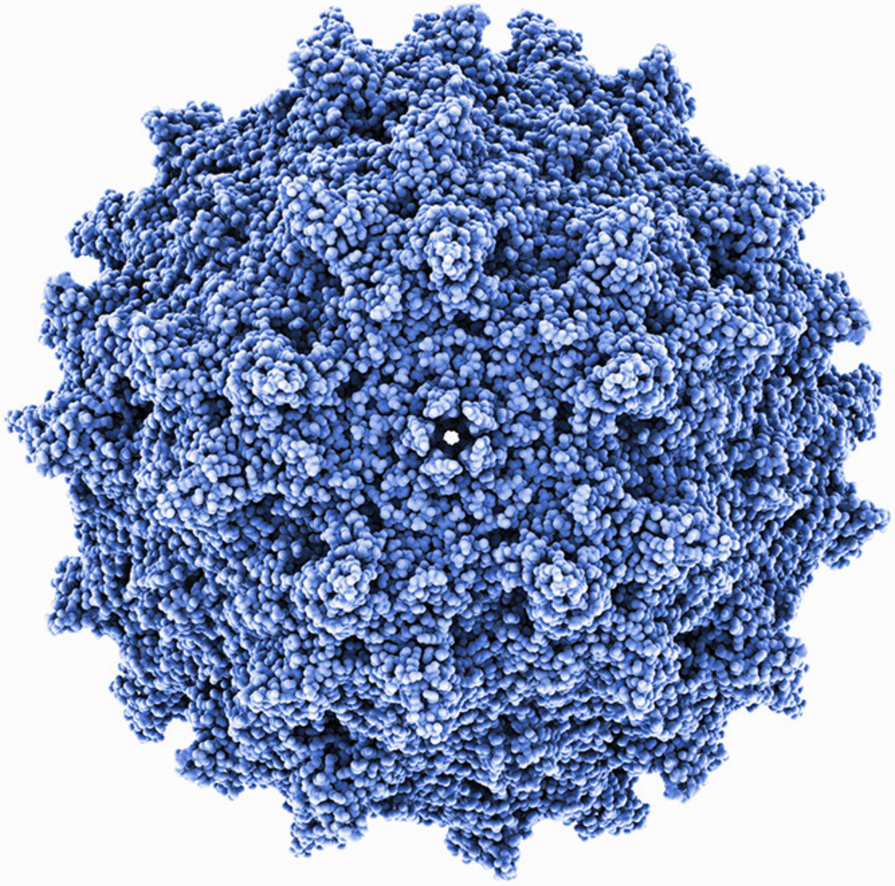
AAV gene therapy

Advantages

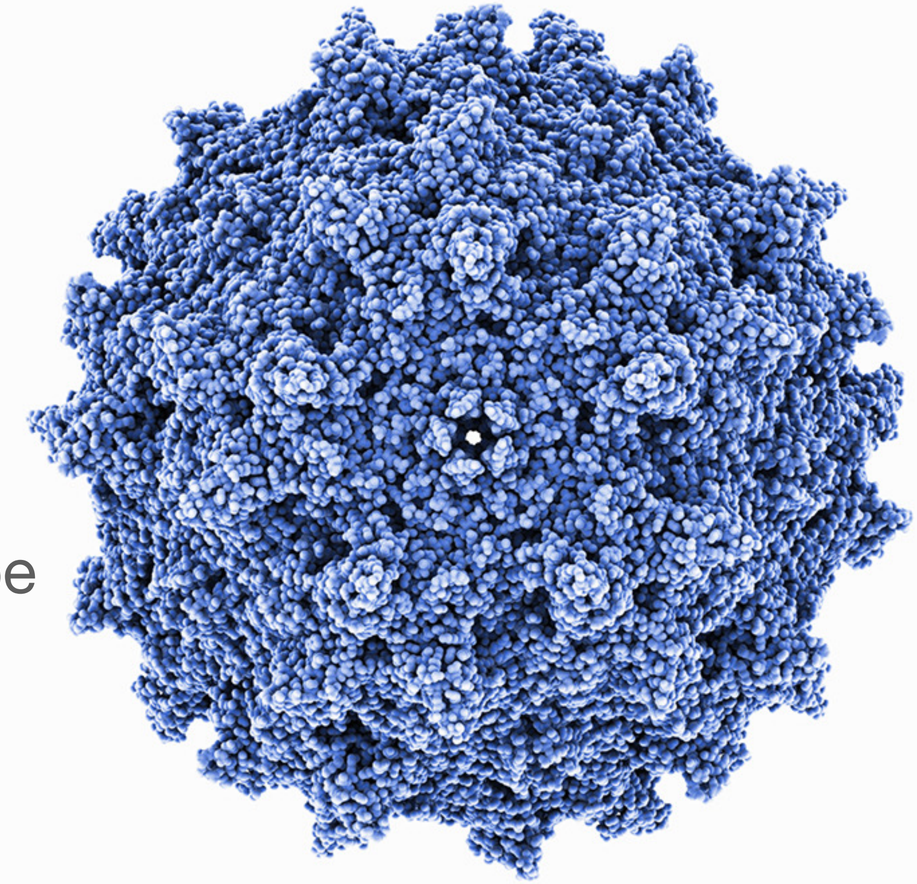
- Single administration
- Does not integrate – could cause mutation.
- Different types of manipulations can be achieved e.g. overexpression or reduction
- Can target cells hard to access with proteins e.g. brain
- May be faster to move from animal model to therapy – i.e. no slow drug development



AAV gene therapy

Disadvantages

- Small insert size
- Viral vectors have tropism for some cells.
- Hard to target large numbers of cells (e.g. entire brain in adults)
- Expression levels and pattern of gene may not be correct
- Non-integrating virus like AAV will be lost eventually to cell division.
- Expensive – SMA treatment was originally $2M!



Thank You & Questions