Cloning in eukaryotes: transformation and viral transduction

Transformation and transduction of higher eukaryotes

1) DNA transfer methods

2) Non-replicative transformation (transient transfection) vs. Stable transformation (recombination)

3) Utilizing viral machinery for DNA transduction

Guide to readings:

1)37 MC4 Mammalian cell transfection. Short summary of transfection methods.

2)38 MC4 Transfection selection. Selective agents used in stable transformation. Also, some info on lipofection and calcium phosphate transfection methods.

3) *39 MC4 Viral transduction.* The use of viral vectors.

4) *40 MC4 Virus vectors.* Specific viral vectors

Strategies for gene transfer: mammalian cells

- Transfection
  - Biochemical:
    - Cells take up DNA from medium following some kind of chemical treatment
  - Physical:
    - Electroporation
    - Microinjection into nucleus
    - " gene gun" : particles coated with DNA bombarding cells

- Transduction
  - Virus infection is used to transfer nucleic acids into cells

Transfection does not require a vector

- Any kind of DNA can be used (linear DNA, plasmids, etc.)
- The DNA may contain selectable markers, eukaryotic replicons, eukaryote-specific gene expression signals
- Bacterial shuttle vectors are often used during the engineering phase

Transfection of mammalian cells

Chemical:

- Calcium phosphate/DNA co-precipitate
- Liposomes containing DNA
- DEAE-dextran/DNA co-precipitate
- Polybrene (a polycation)

Physical:

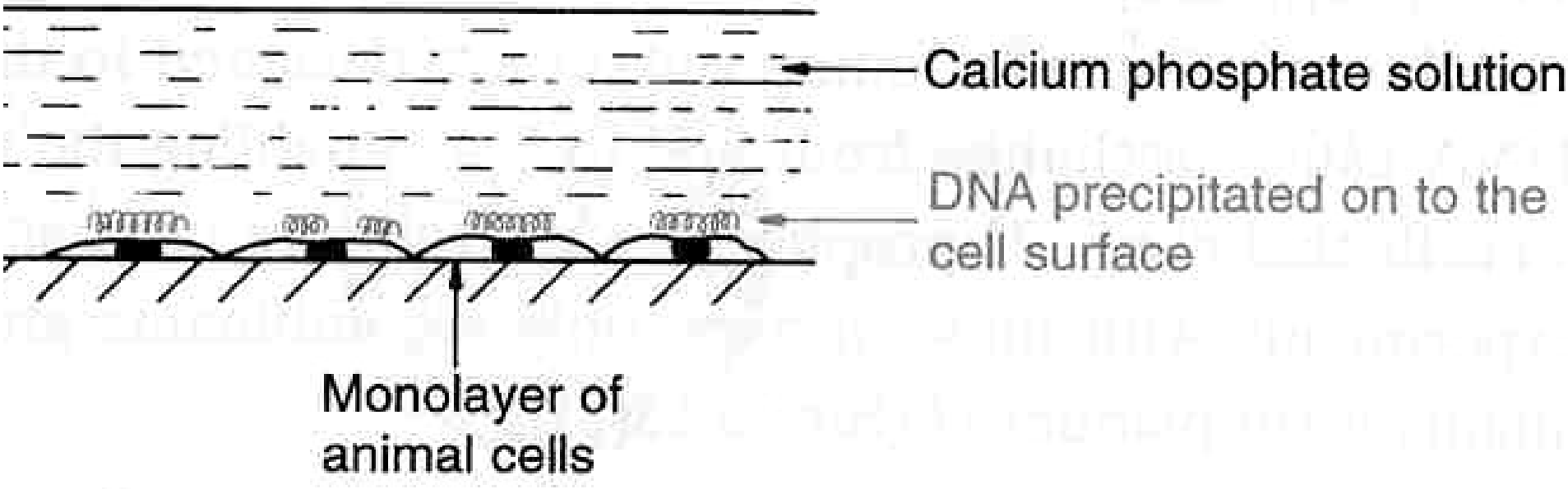
- electroporation
- biolistics
- microinjection

Transfection of mammalian cells:

DNA/Calcium phosphate coprecipitate

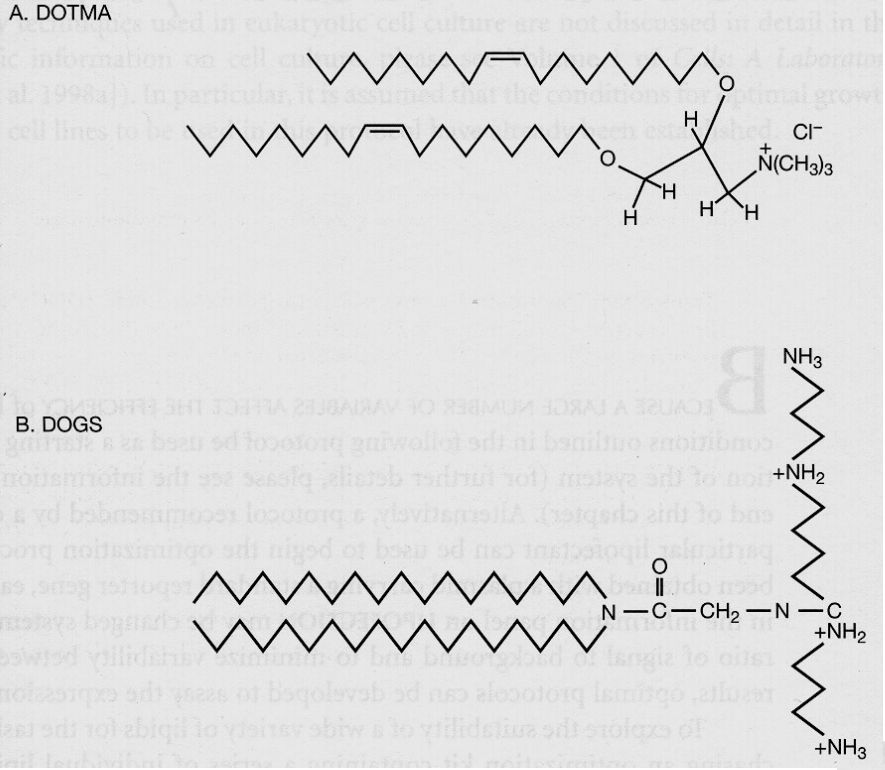
- In cell monolayers, up to 20% of cells take up DNA

- endocytosis of the precipitate?



Lipofection: transfection using liposomes

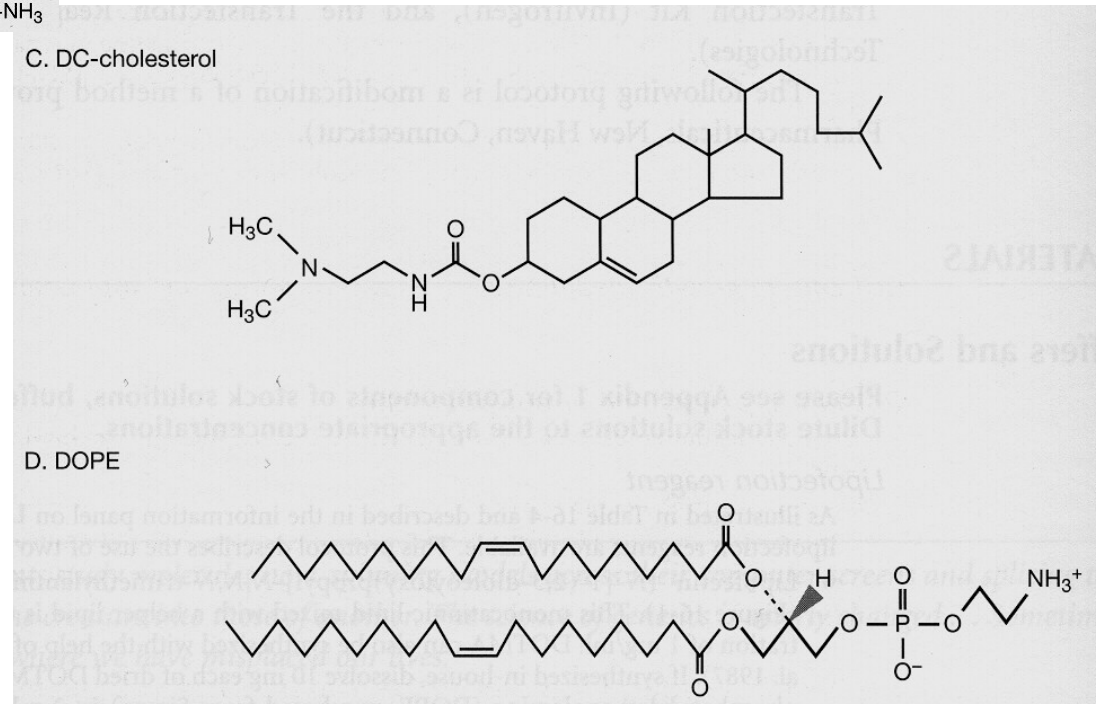
- Cationic/neutral lipid mixtures spontaneously form complexes with DNA
- The lipid vesicles that form are called liposomes
- Liposomes interact with negatively charged cell membranes, and the DNA enters the cell through membrane fusion
- The efficiency is very high: up to 90% of cells in the culture can be transfected



Cationic and neutral lipids create artificial membranes that bind to DNA

The lipids interact with cell membranes, & fusion delivers the DNA

(common example: lipofectamine)

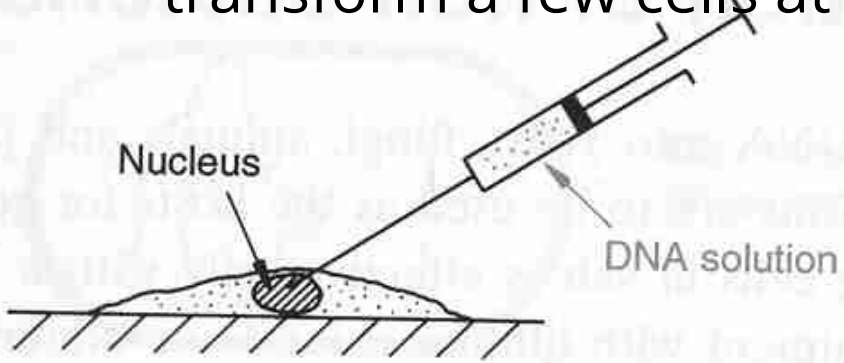


Direct DNA transfer

-- when other, simpler methods are unavailable

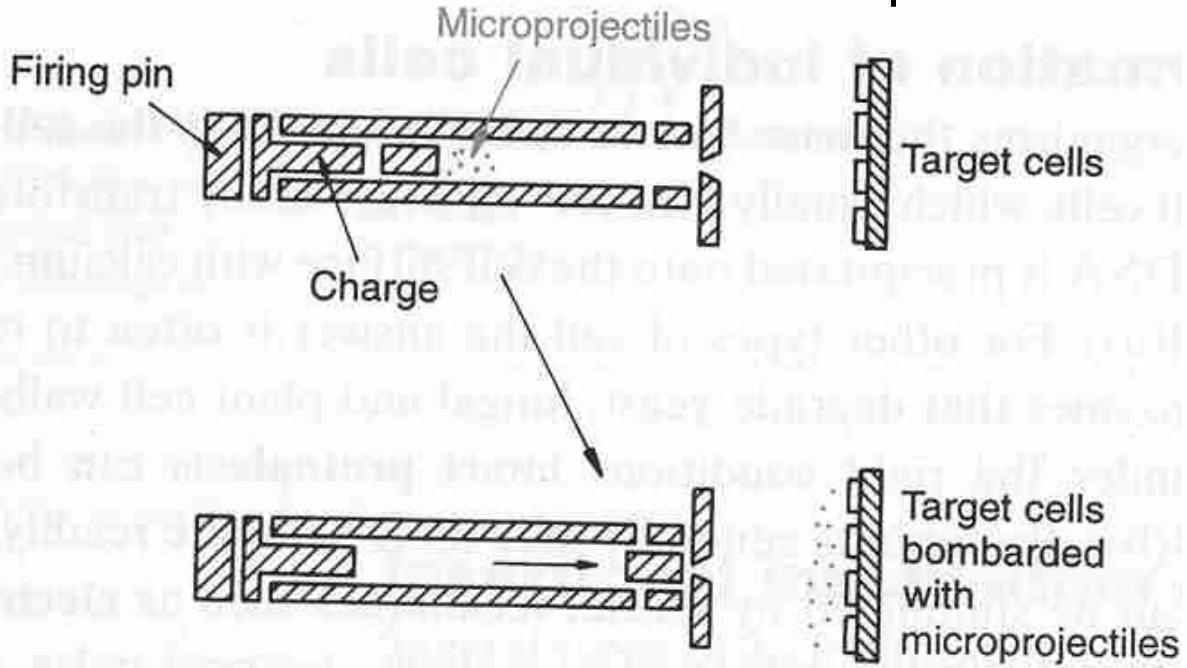
(a) Microinjection

Need large cells, & can only transform a few cells at a time



(b) Transformation with microprojectiles

Works well for tissues, plant cells



Transient transfection

- DNA maintained in nucleus for short time
- Extra-chromosomal, no replicon, no integration
- No selection is required

How is transient transfection used?

- One-time genetic experiments
  - For example:
    - Transfect with a gene and measure phenotypic effect
    - Examine gene regulation of transfected genetic element
- Testing recombinant DNA prior to time-consuming and difficult cell-line construction -- are genes expressed properly?

Stable transfection

- DNA integrates into the genome, thus giving stable, heritable transformation
- Homologous recombination is one possible mechanism, but DNA can also randomly integrate

- Results in formation of a " cell line" that carries and expresses the transgene indefinitely
- Selectable markers assist in capturing these rare events

Stable transfection/transformation

- Mechanism of transport of DNA is not known: “ Some DNA” is transported to the nucleus
- Linear DNA works better than circular plasmids, because non-homologous end-joining (NHEJ) pathways may be activated
- Large, concatameric rDNA structure may eventually integrate, usually by non-homologous insertion
- There is no way to control where in the genome the DNA is integrated
- Up to 1 in 1000 transfected cells may carry the transfected gene in a stable fashion

Viral transduction

- Elements of the viral life cycle can be borrowed
  - attachment to cells, introduction of genetic material
  - Integration into host genome
  - Expression of genes (especially strong promoters)

- Transfer genes to cultured cells or living animals

- Efficiency of transduction makes this an important method for gene therapy

Issues with a viral approach

- Size of DNA that can be cloned is limited

- Some viruses have limited host range (e.g. retroviruses target only dividing cells)

- Pathogenic virus backbones must have harmful aspects removed/ inactivated

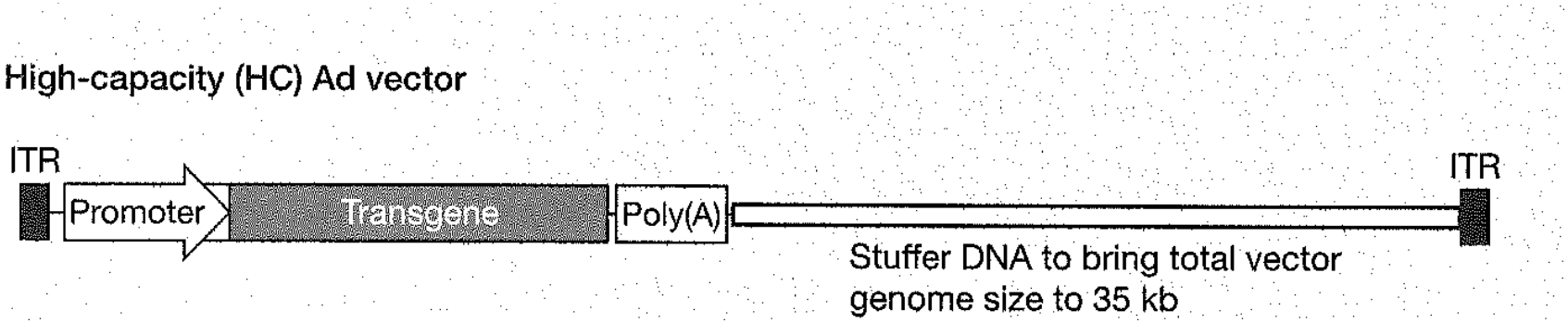
- For gene therapy, patient may have prior immunity to that virus (e.g. adenovirus)

Viral vectors

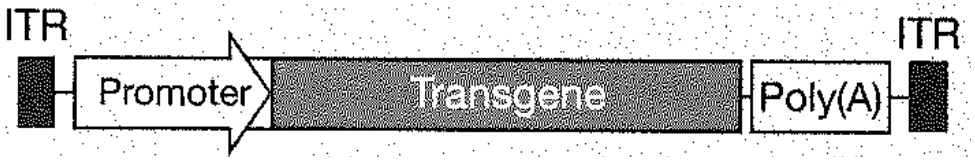
- Adenovirus: double stranded DNA, 37 kb
  - Easy to handle, high titer (>10^9 viral particles/ml)
  - Infects both dividing and non-dividing cells
  - “gutless” adenovirus vectors can carry up to 36 kb DNA

- Adeno-associated virus: single stranded DNA, 4.7 kb
  - Small, single stranded DNA genome (4.7 kb)
  - Integrates in host genome, where it’s stable for years
  - Infects both dividing and non-dividing cells
  - Non-pathogenic

Adenovirus vectors



AAV vectors

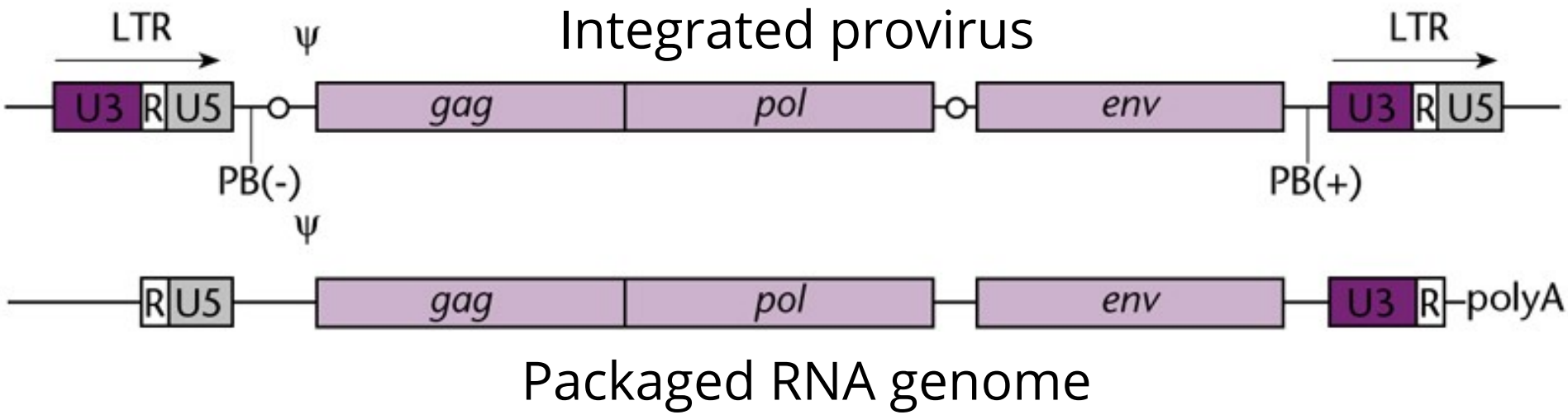


ITR = inverted terminal repeats

Viral vectors: Retroviridae

- Single stranded, RNA genome, 8-10 kb
- Replication intermediate is double stranded DNA
- DNA version of viral genome is integrated into the host genome following infection
- Retrovirus:
  - Gag, pol, and env genes
  - Vectors derived from Moloney murine leukemia virus
  - Up to 6.5 kb DNA can be cloned
  - Only targets dividing cells
- Lentivirus:
  - Additional genes: tat, rev, vpr, vpu, nef, vif
  - Vectors derived from HIV-1 and related viruses. Pathogenicity has been engineered out
  - Infects dividing and non-dividing cells

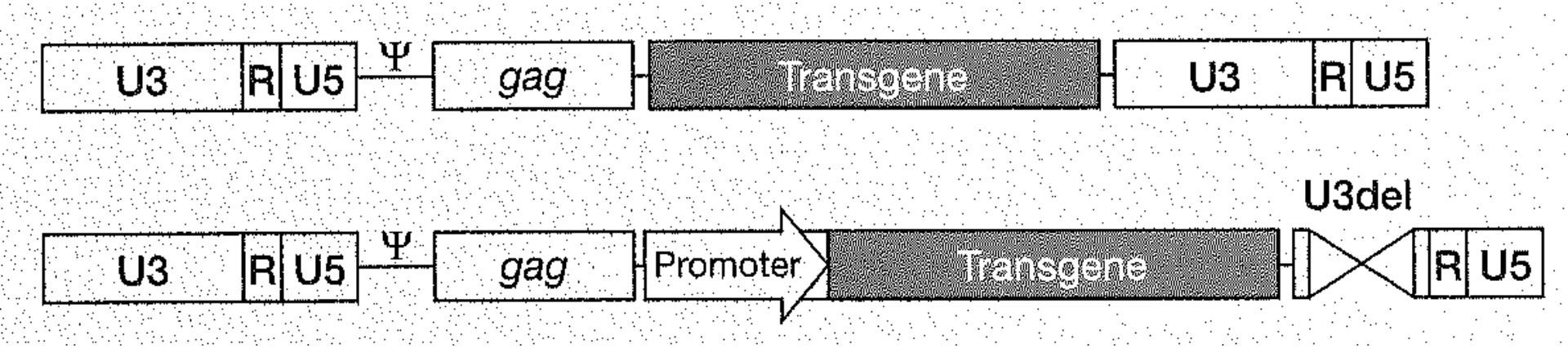
Moloney Murine Leukemia Virus (MMLV) an oncoretroviral vector



U5: unique 5’ region (poly adenylation signal)
U3: unique 3’ region (strong promoter)

For more info: https://www.addgene.org/viral-vectors/retrovirus/retro-guide/

Engineered retroviral vectors

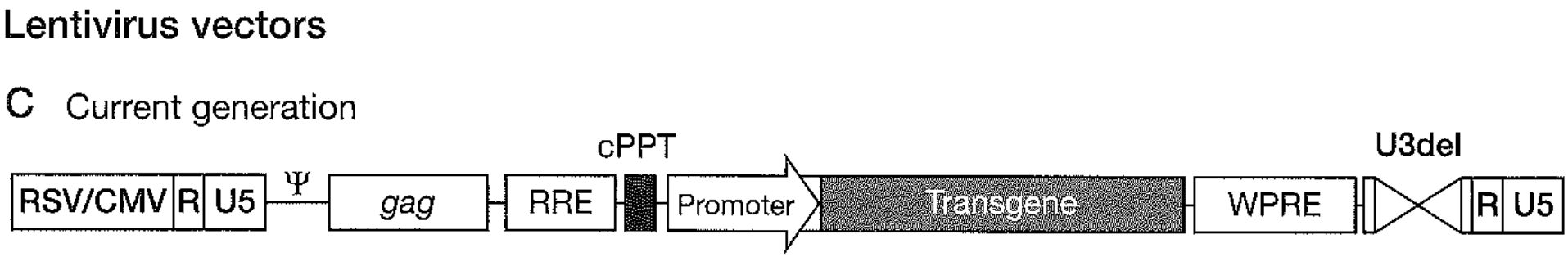


Typical retroviral vector: LTR (promoter), packaging signal (psi), promoter, transgene, LTR
U3 deletion causes 'self-inactivation'
8 kb max transgene size

LTRs are retained for:

- transcription activity
- packaging
- insertion into genome

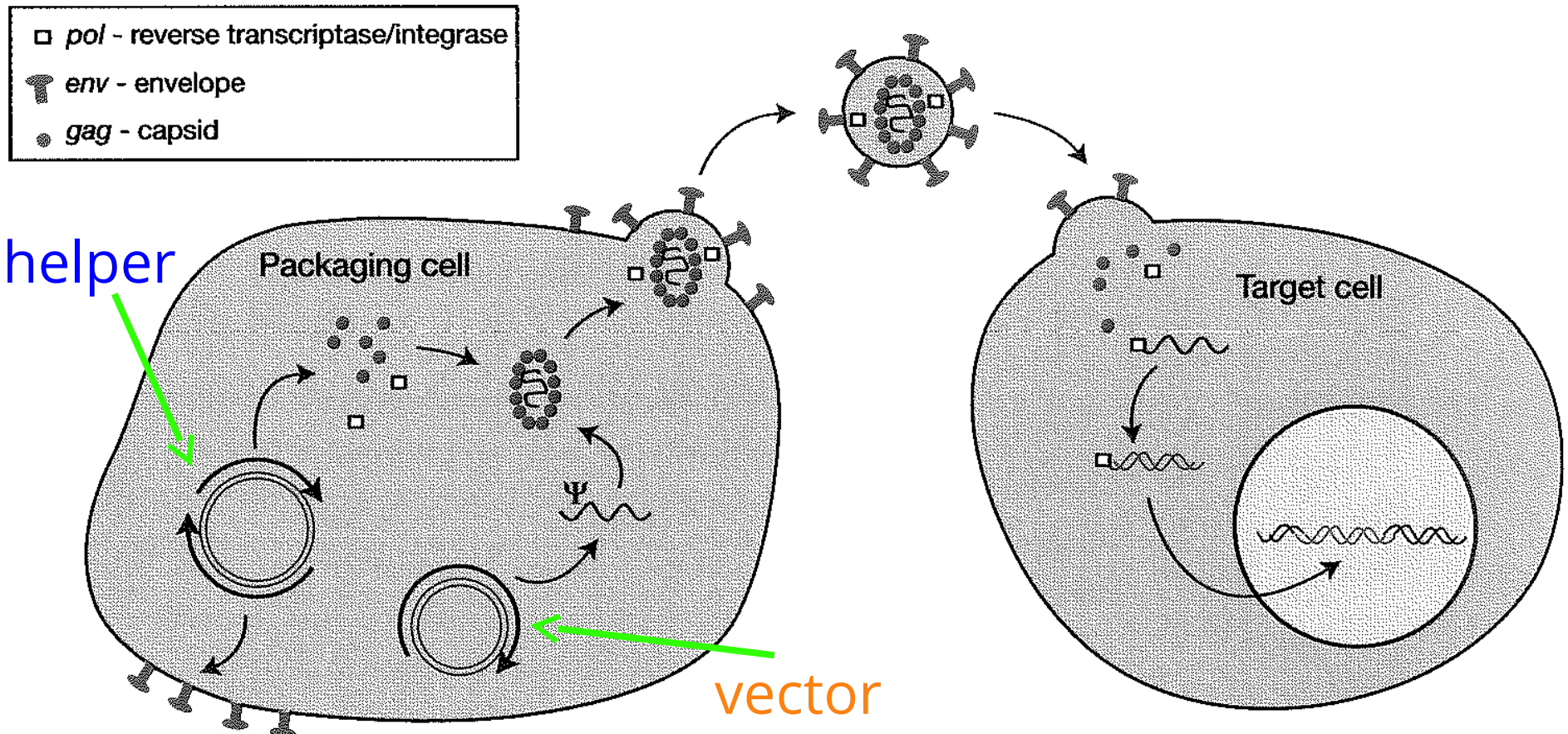
Essential lentiviral vector features



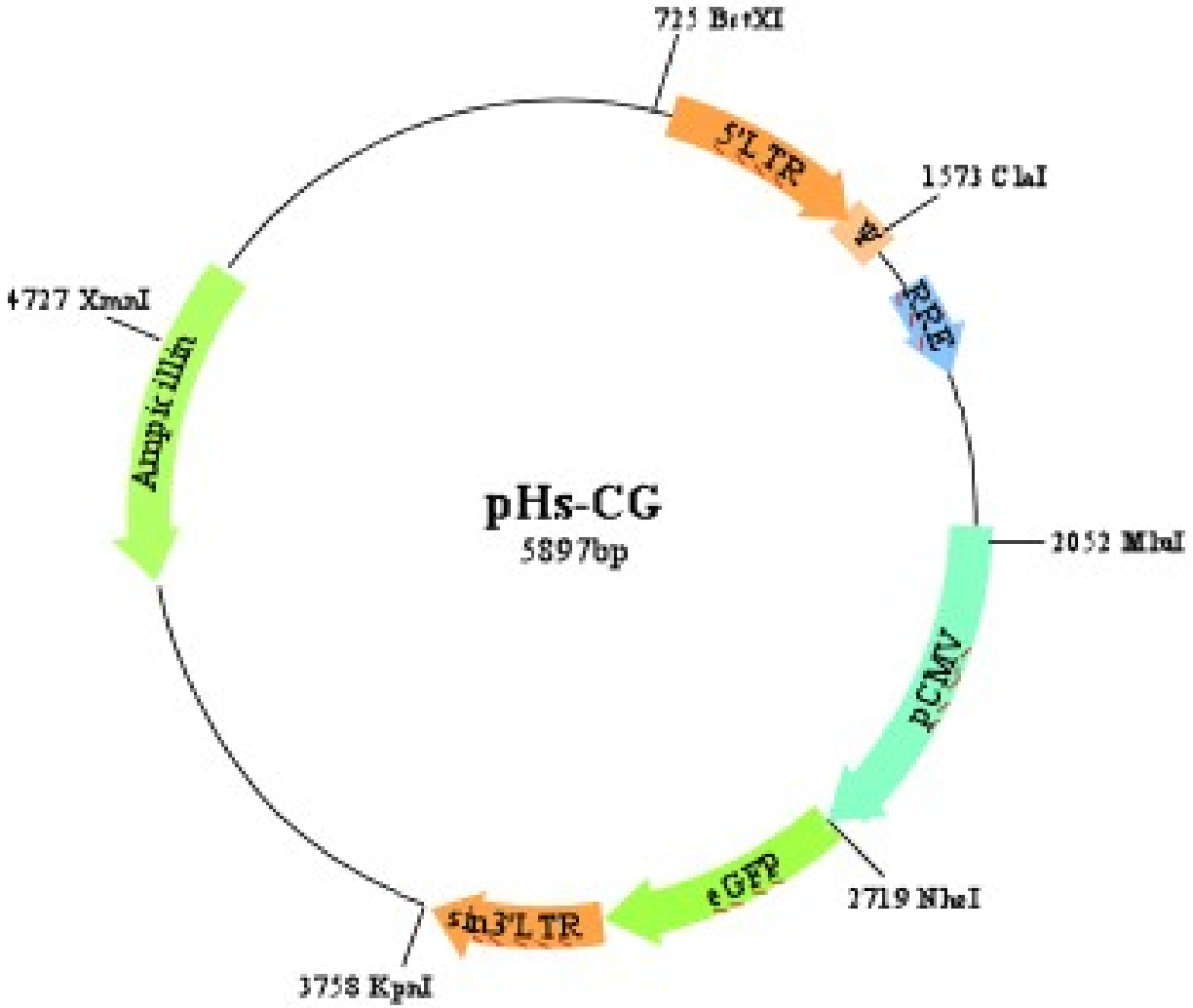
- RSV/CMV: enhancer sequences
- U5, R: Reverse transcriptase sites
- psi: packaging signal
- RRE: rev responsive element, gets RNA to the cytoplasm
- cPPT: poly purine tract for cDNA synthesis
- WPRE: woodchuck post transcriptional regulatory element (increases nuclear export)

Safety features of lentiviral vectors

- Most viral genes are deleted
- Gag, pol, and env genes are expressed in the production cell, not the vector itself
- Self-inactivating (sin): no functional U3 promoter, prevents formation of replication competent virus



An example of a lentiviral vector



Gfp marker allows identification of infected cells

Basic HIV-1 based sin vector with the GFP marker driven by pCMV.

https://web.stanford.edu/group/nolan/_OldWebsite/retroviral_systems/helix.html
https://web.stanford.edu/group/nolan/_OldWebsite/retroviral_systems/retsys.html

Cloning in eukaryotes

Transformation and transduction of higher eukaryotes

1) DNA transfer methods: biochemical vs. physical treatments

2) Non-replicative transformation: (transient transfection) vs. stable transformation (recombination)

3) Utilizing viral machinery for DNA transduction: adenovirus, AAV, and retroviruses

Gene transfer to higher eukaryotes: selection and control

1) Positive / negative selection, reporter genes

2) Gene targeting by recombination

3) Use of cre/lox site-specific recombination

4) Controlled gene expression (examples: tetracycline and light)

Readings guide:

1) *Capecchi 2005*. Retrospective by Mario Capecchi, who made key discoveries in mammalian genome engineering

2) *Brainbow 2007*. A cre/lox based system for giving color to neurons

3) *41 MC4 Reporter genes.* Review of reporters, also overview of the TetR system

4) *CRY2 optogenetics 2010.* Control of gene expression w/ light

Positive selection for transformation (part I)
"Dominant" selectable markers

- Neo: aminoglycoside phosphotransferase confers survival in presence of aminoglycoside antibiotics, e.g. G418 (similar to neomycin, kanamycin)

- Pac: puromycin N-acetyltransferase, confers resistance to puromycin

- Ble: glycopeptide binding protein, confers resistance to bleomycin, Zeocin

- These selectable markers do not require a specific genotype in the transfected cell-line

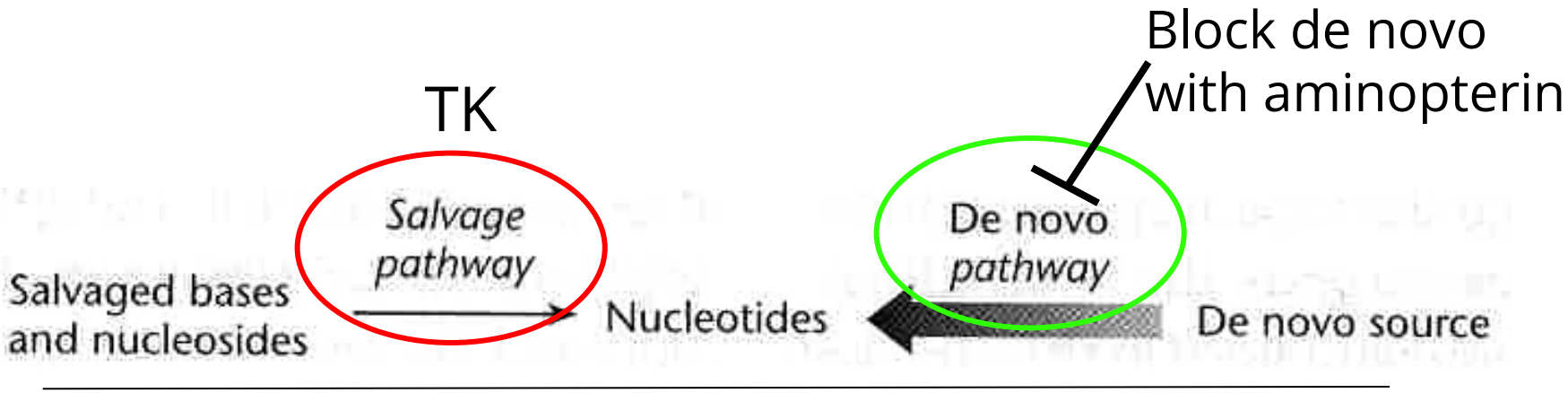
Positive selection for transformation (Part II)
Endogenous markers

- Confer a property that is normally present in cells
  - thymidine kinase (TK) (required for salvage pathway of nucleotide biosynthesis)

- These markers may only be used with cell lines that already contain mutations in the marker genes

Positive selection for transformation:

The Thymidine Kinase (TK) gene functions in the salvage pathway in production of nucleotides



TK is only essential is the cell is forced to use the salvage pathway (the de novo pathway is shut off)

Counter-selectable markers

You can select *AGAINST* thymidine kinase (TK):

Add a nucleotide analogue that is TOXIC if the TK protein is present

examples: 5-bromo-deoxyuridine, ganciclovir, AZT

Cells that express TK die in the presence of these compounds, Cells without the Tk gene survive

This allows SELECTION for the loss of a specific piece of DNA (the term is ‘counter selection’)

Other toxic genes may be used: e.g. diptheria toxin gene [dipA] is sometimes used in counter-selection)

Eukaryotic cell transformation

1) Getting DNA in: several possible methods

2) Transient transformation: no selection

3) Stable transformation: selection is required
   (also, counter-selection can be useful)

Gene targeting (genome engineering)

- Homozygous, null mutants (“ knock-out”): what is the effect on the organism?
- Exchange of one gene for another (gene “ knock-in”)
- Correction of mutated genes: gene therapy for diseases with a genetic origin

Gene replacement vectors: homologous recombination

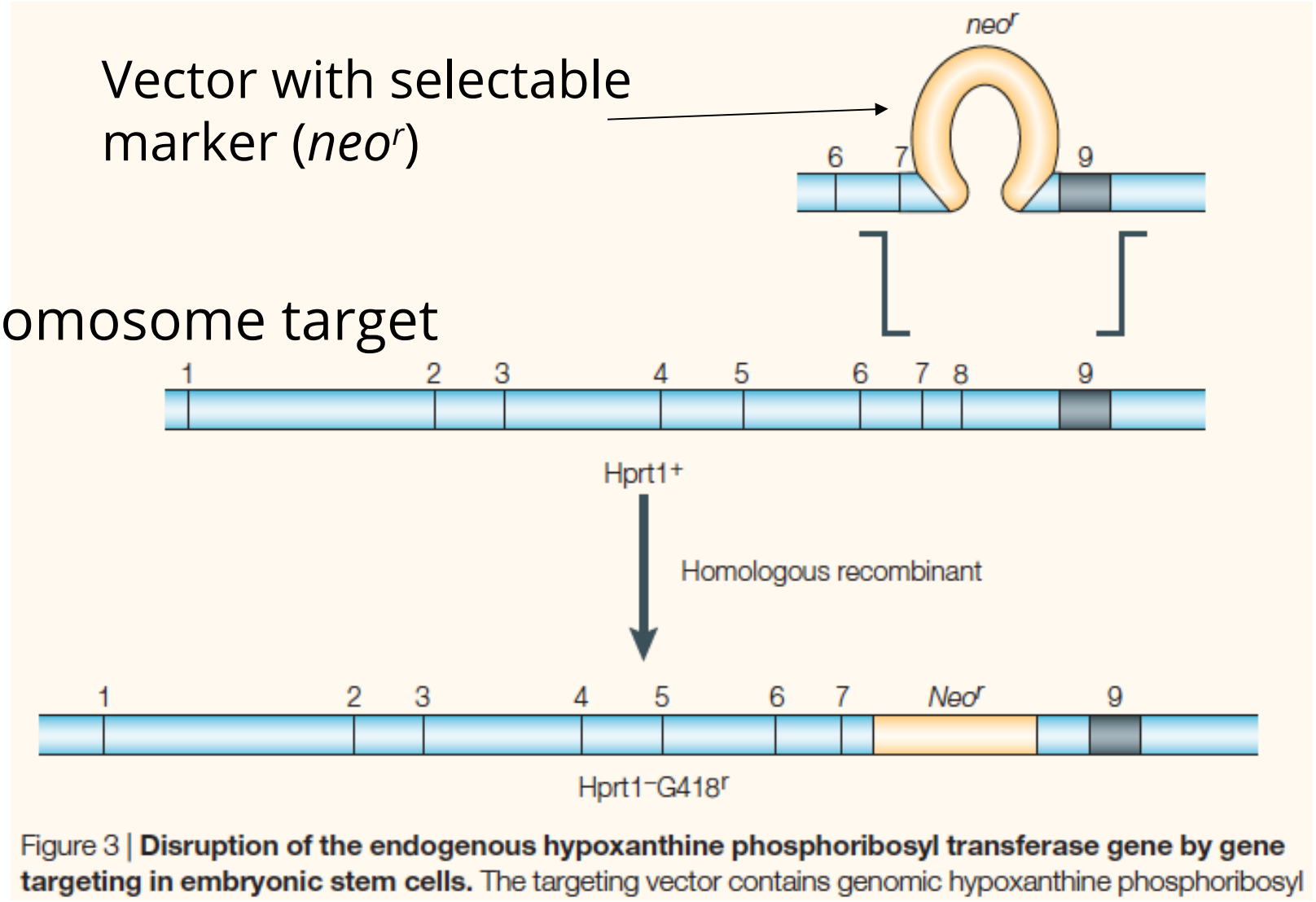


Figure 3 | **Disruption of the endogenous hypoxanthine phosphoribosyl transferase gene by gene targeting in embryonic stem cells.** The targeting vector contains genomic hypoxanthine phosphoribosyl

HPRT gene disruption strategy

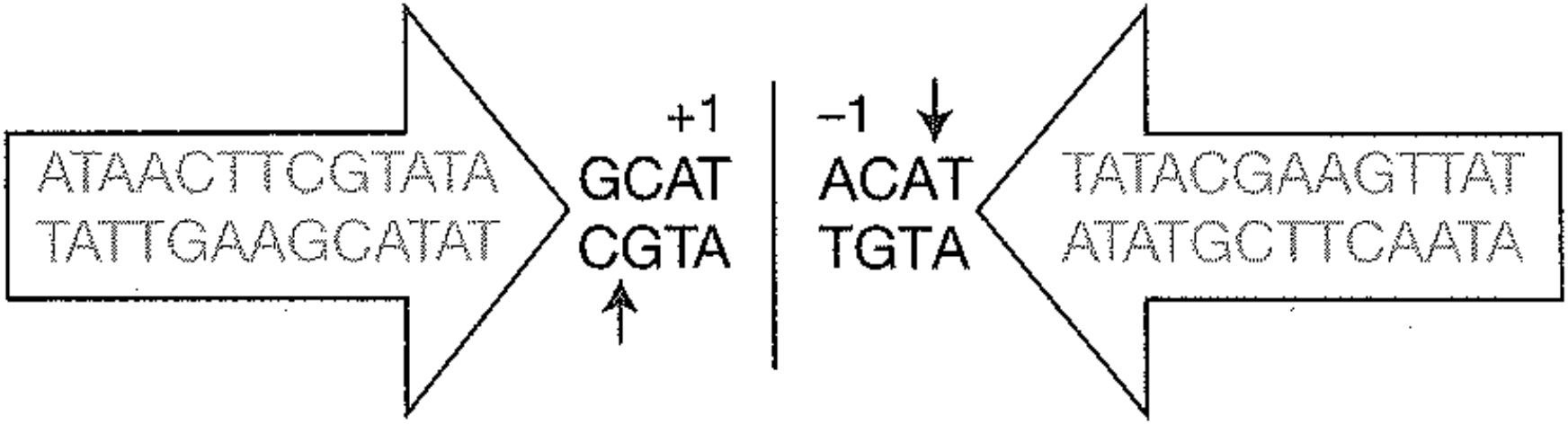
Homologous recombination

- Ubiquitous process
- Requires regions of homology between recombining DNAs

Site-specific recombination

- Specialized machinery governs process
- Recombination occurs at short, specific recognition sites

Cre and Lox:
a site-specific recombination system

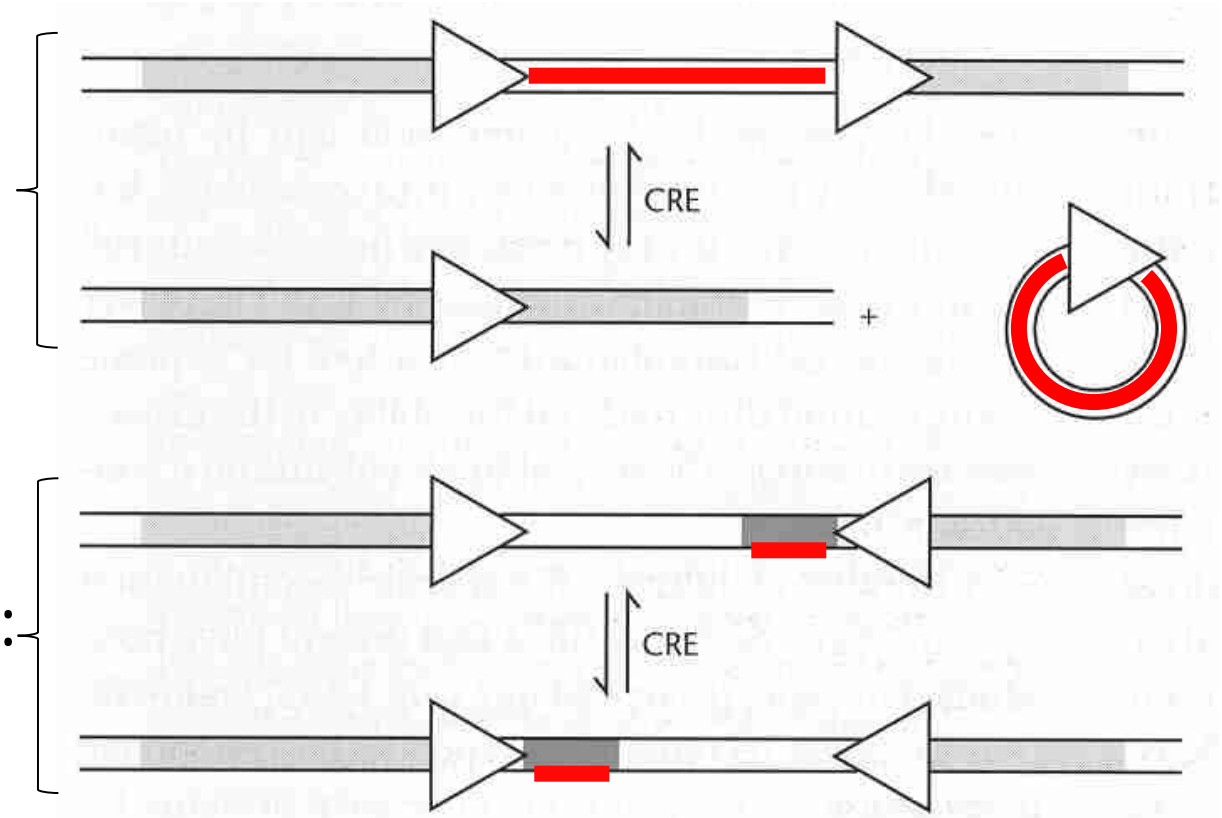


A single loxP site includes two 13 bp inverted repeats separated by an asymmetric linker

Cre-Lox (site-specific) recombination

- Cre is a protein that catalyzes the recombination process (recombinase)
- LoxP sites: DNA sequences recognized by the Cre recombinase

Direct repeats: Deletion of intervening sequences



Inverted repeats: inversion

Cre-mediated conditional deletion

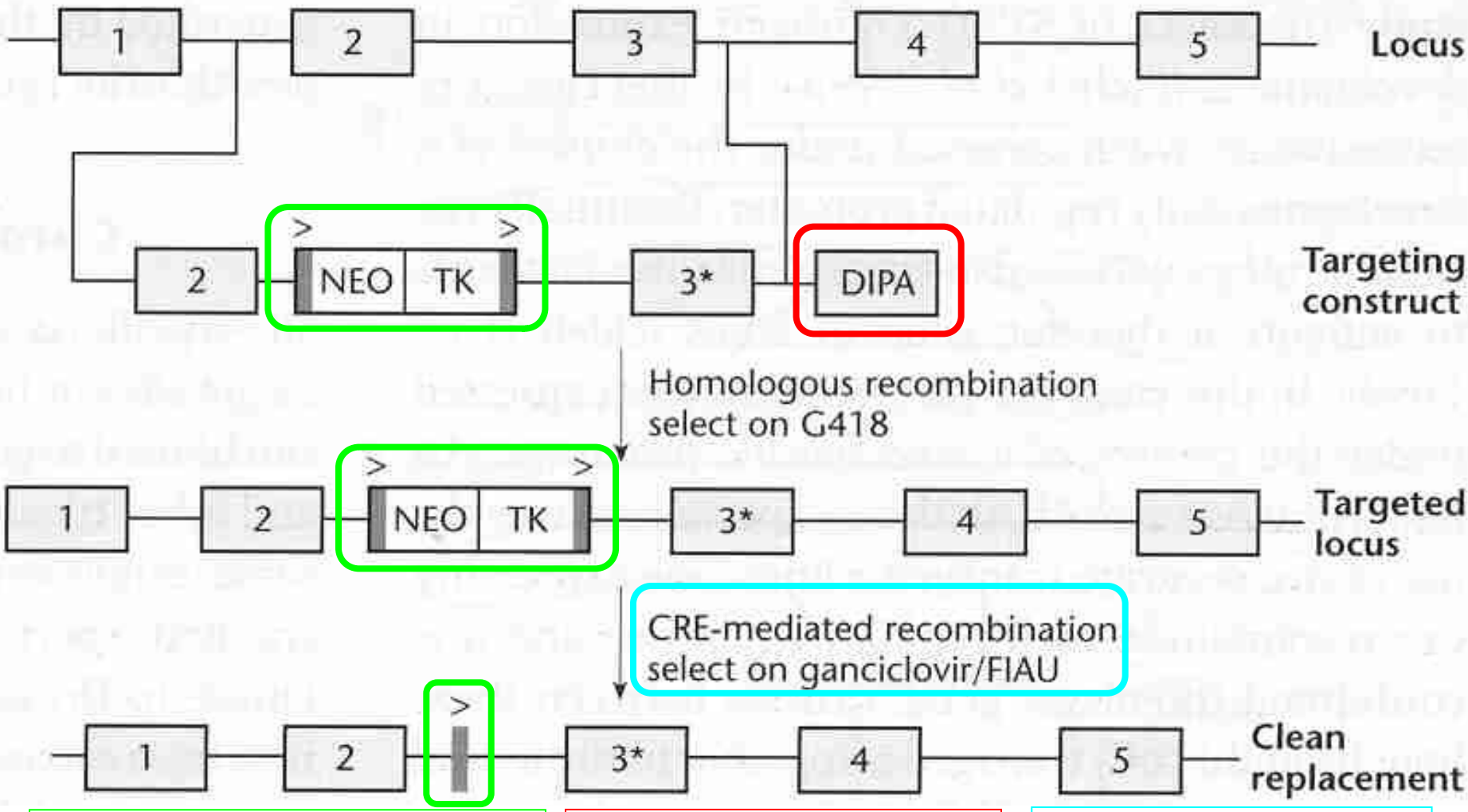
- Surround gene of interest with lox sites (gene is “ floxed”, flanked by lox sites)
- Place Cre gene under inducible control
- Induce Cre at appropriate time or in appropriate place
- Gene of interest can be deleted whenever necessary, e.g. deletions that are lethal in embryo stage can be made once animal has made it to adulthood

Considerations in homologous recombination strategies

Random insertion of DNA often occurs--how to get around this problem?

1) Add a negative selection gene to the DNA outside of the region of homology (ensure that the cells containing this gene via non-specific integration will die)

2) Screen transformants by PCR for correct position of recombinant DNA insertion

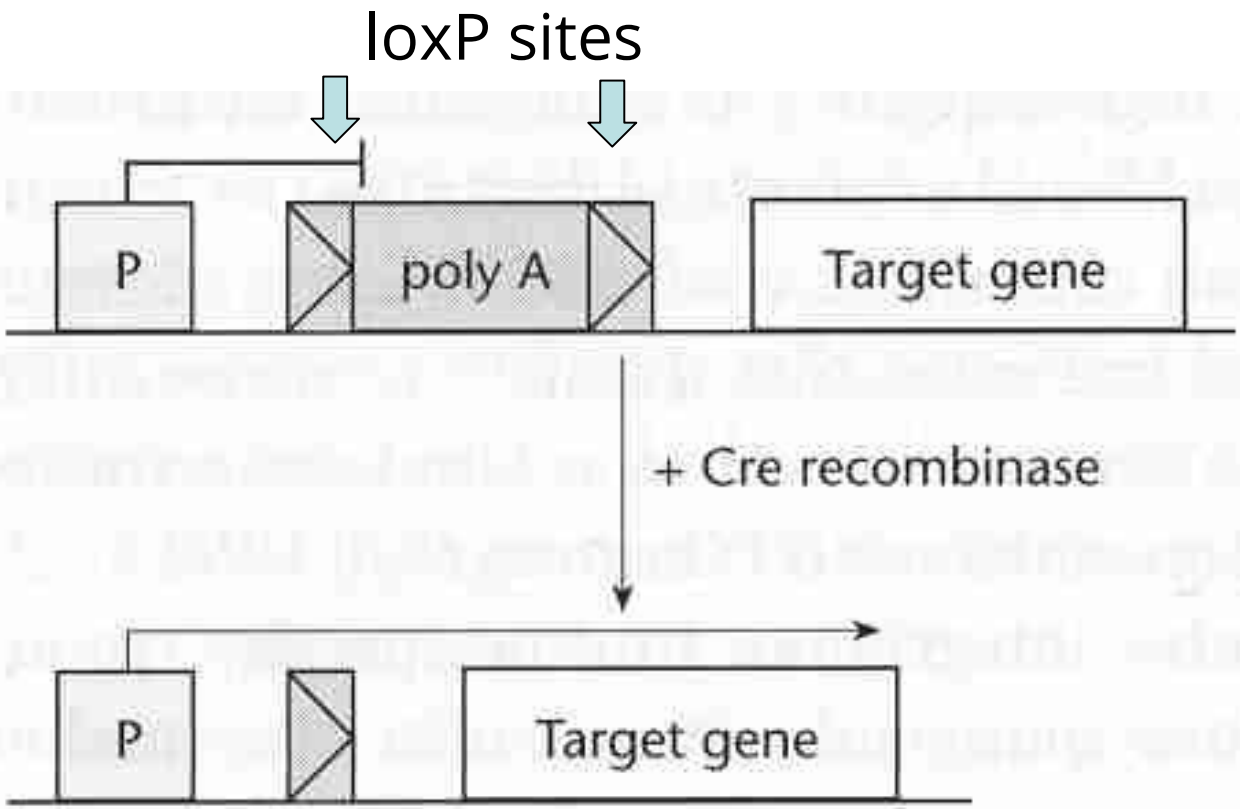


Selection and counter-selection markers flanked by loxP sites

Diptheria toxin: Prevents non-homologous recombination

Cre expression induced by transient transfection

Cre recombinase-dependent activation of gene expression



Transcription blocked by terminator sequence

Terminator sequence removed, gene is expressed

Conditional expression of Cre defines gene activation

Another use for cre-lox recombination: Mapping neurons in brains

It can be difficult to track the paths and connection of neurons in brain tissue

This makes it difficult to understand neuronal network architecture

The solution? Give give neurons different colors with various fluorescent proteins

How can different neurons be given different coloration?

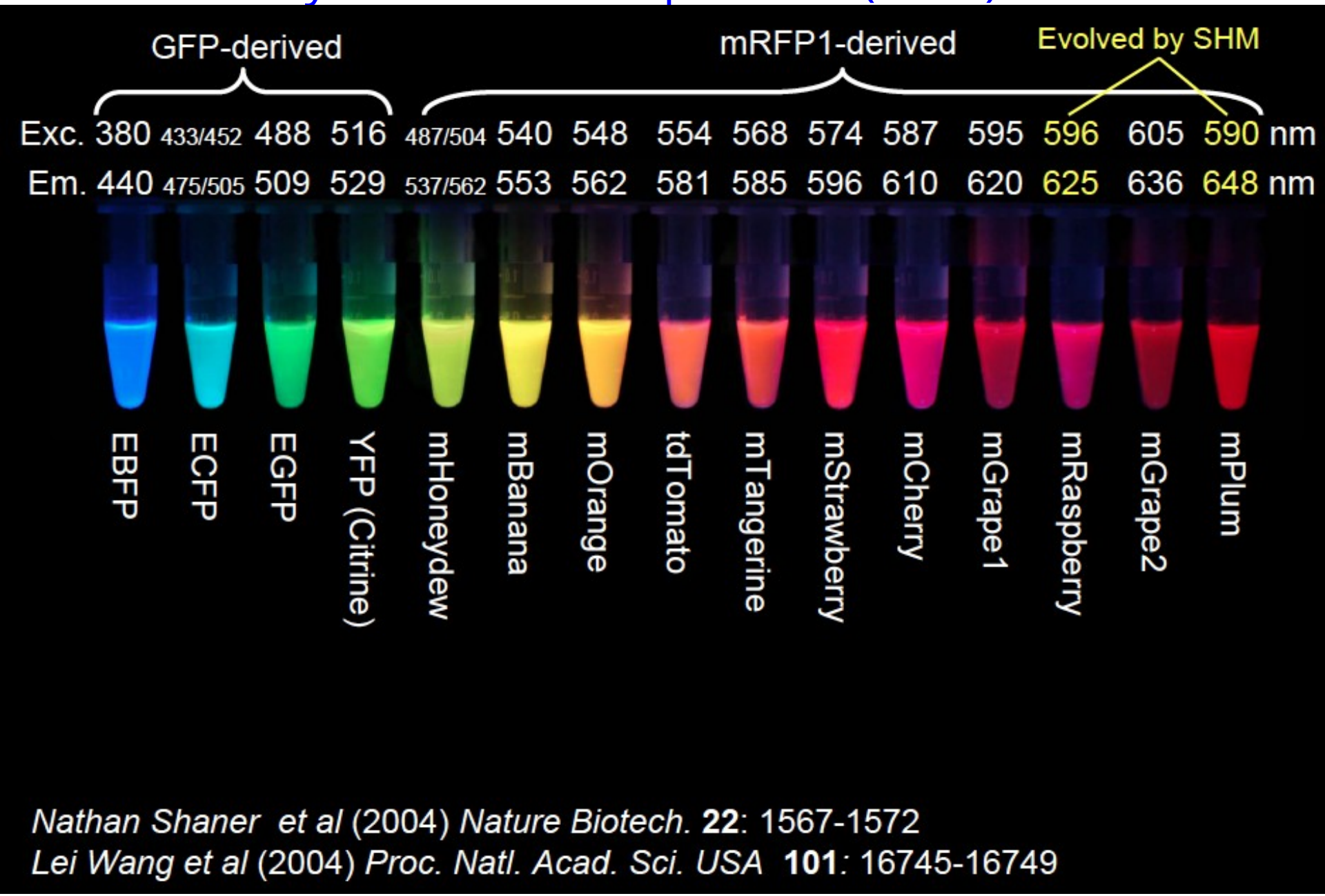
Reporter genes

- Is the DNA construct present in the transformed cell?
- Is the gene being expressed?
- Where and when is the gene expressed?

Fusion proteins: track position, expression of genes by adding a reporter tag to it

- Common reporters:
  - Fluorescent proteins: detect with UV illumination
  - Beta galactosidase: detect with chromophore
  - Luciferase: emits light

Wide variety of fluorescent proteins (2004)

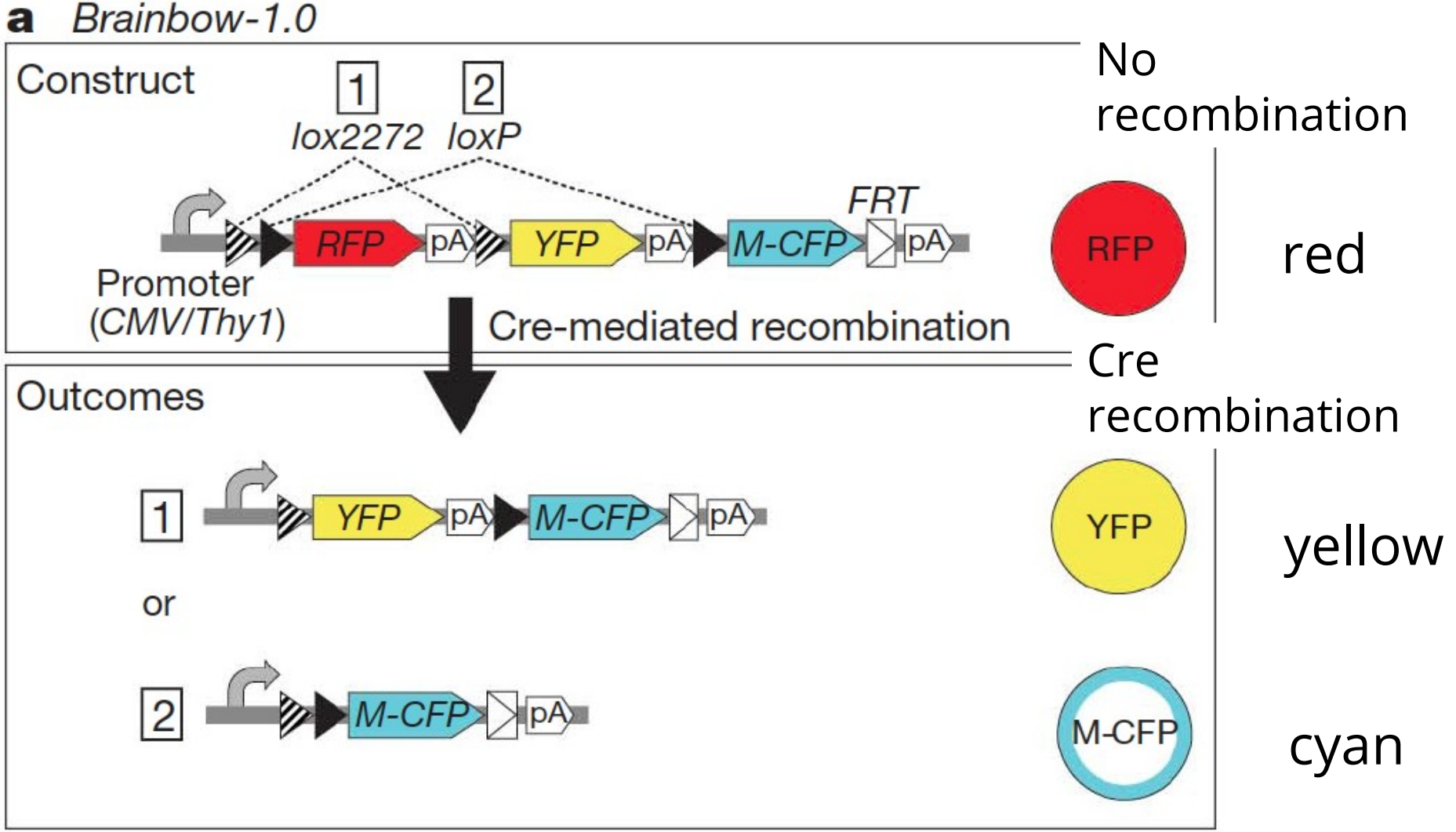


Nathan Shaner et al (2004) *Nature Biotech.* **22**: 1567-1572
Lei Wang et al (2004) *Proc. Natl. Acad. Sci. USA* **101**: 16745-16749

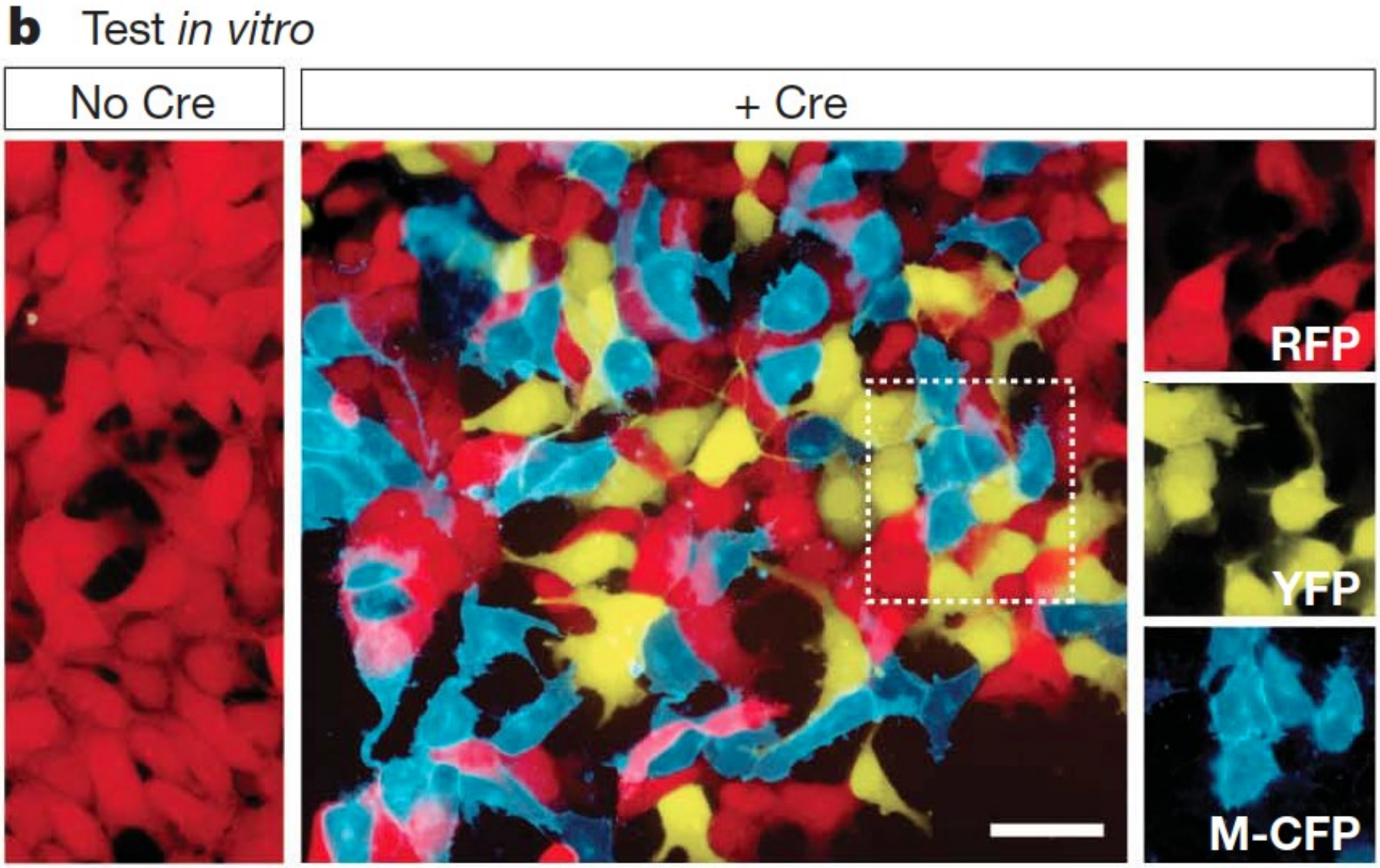
http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/tsien-slides.pdf

Cre-Lox is at the heart of the 'brainbow' technique

- Two incompatible lox sites (P and 2272)
- Induce cre expression in a cell, one lox site is chosen
- Three possible colors



HEK cells, transformed with DNA construct, with and without Cre induction



What if there are three constructs in the same cell?

Brainbow-1 under control of the Thy1 gene (expression in neurons)

a XFP combinations

Outcome for each copy 1	2	3	Resulting colour
C	C	C	Blue
C	C	Y	Light blue
C	Y	Y	Blue-green
Y	Y	Y	Green
Y	Y	R	Light green
Y	R	R	Orange
R	R	R	Red
R	R	C	Magenta
R	C	C	Purple
R	C	Y	Grey

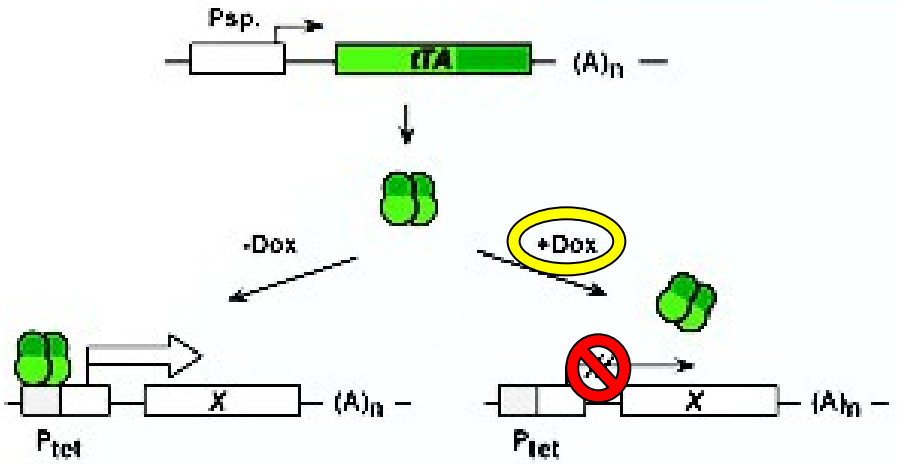
10 colors?

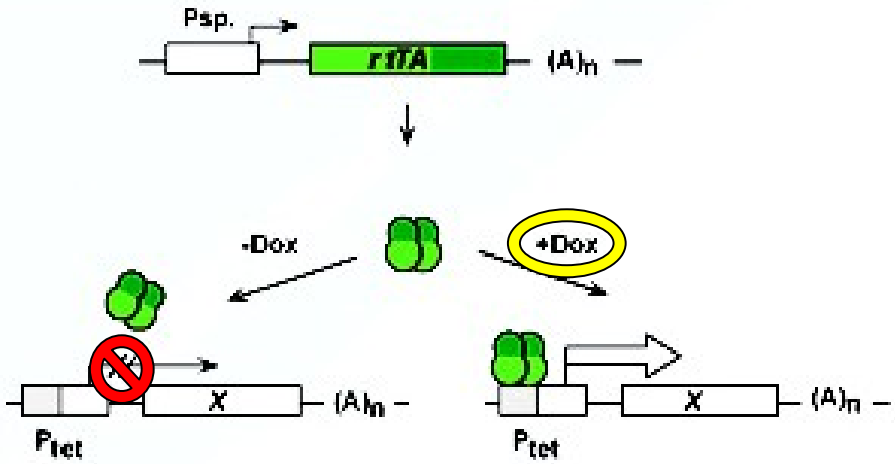
In transgenic mouse, Cre induced by tamoxifen

Recombination leads to numerous combinations of fluorescent proteins; about 100 distinct hues detectable

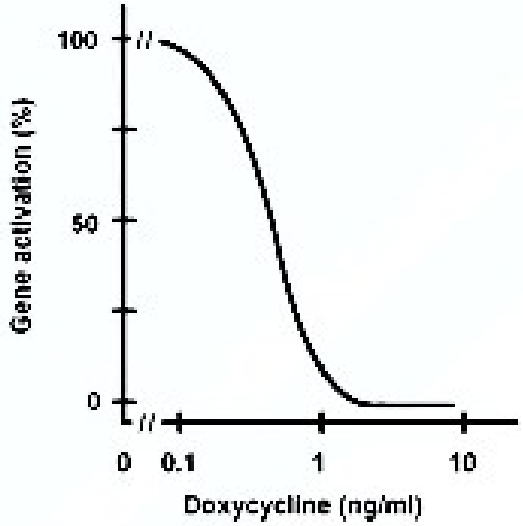
Control of gene expression: the Tet system

tTA = tet TransActivator: tet repressor fused to the VP16 transactivator. Doxycycline either prevents or allows tTA binding

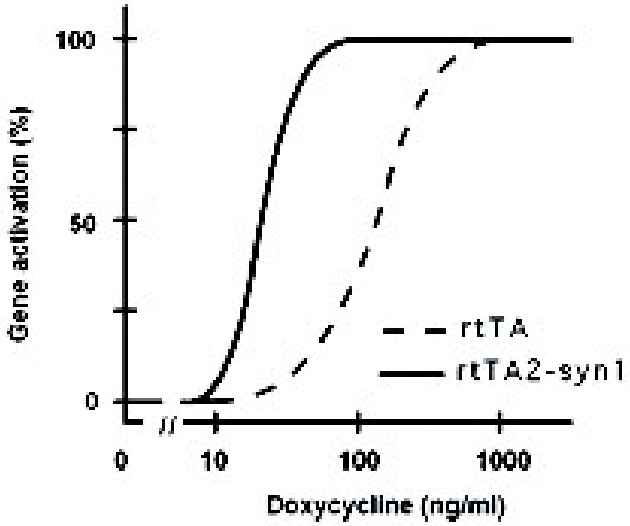




Induce shut-down



Induce activation



https://www.tetsystems.com/science-technology/scientific-figures#c22

Inhibiting a gene (without knock out)

- Antisense RNA transgenes: synthesize complement to mRNA, prevent expression of that gene

- RNA interference (RNAi): short double-stranded RNAs (siRNAs) silence gene of interest--can be made by transgenes or injected, or by soaking in a solution of dsRNA (C. elegans)

- Intracellular antibody inhibition: transgene expresses antibody protein, antibody binds protein of interest, inhibits expression

Optogenetics: controlling protein activity with light

A number of light-responsive proteins are known, and have been used for controlling cellular processes

Two examples:

•Prokaryotic opsins: in response to illumination, protons or other ions are pumped across a membrane

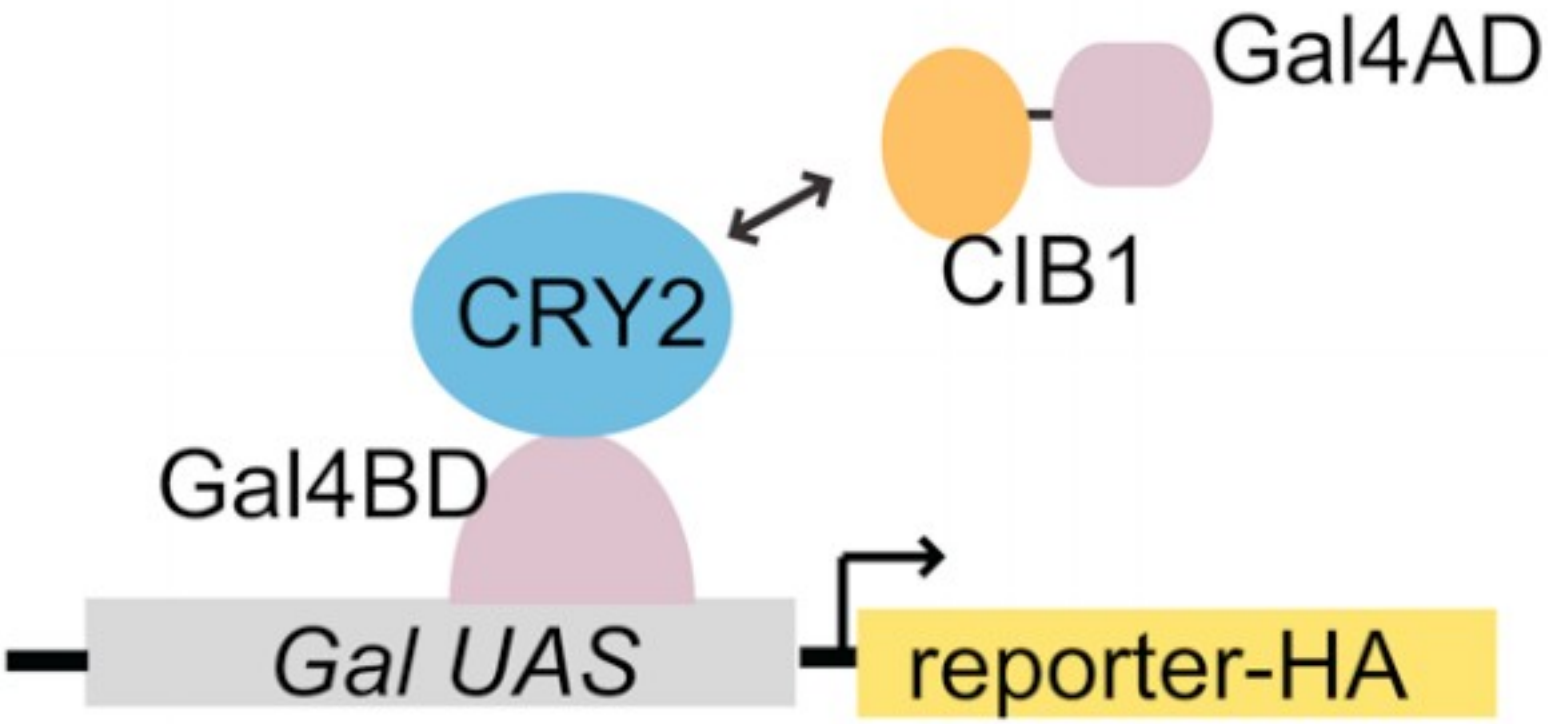
- Allows neurons to be turned on and off with millisecond precision
- https://www.youtube.com/watch?v=I64X7vHSHOE

-

•Plant cryptochrome 2 (CRY2) interacts with a partner protein (CIB1) following blue light illumination

Optogenetics: controlling gene expression with light

Illuminate with blue light, CRY2 interacts with CIB1, and the reporter is expressed



Nat Methods. 2010 December ; 7(12): 973–975. doi:10.1038/nmeth.1524.

Rapid blue light induction of protein interactions in living cells

Matthew J. Kennedy[1,4], **Robert M. Hughes**[2,4], **Leslie A. Peteya**[2], **Joel W. Schwartz**[1], **Michael D. Ehlers**[1,3], and **Chandra L. Tucker**[2,*]

Summary:

1) Selection methods

2) Homologous recombination can be used to manipulate the genome with positive selection as well as negative selection

3) Site specific recombination (cre/lox) allows controlled removal of specific sequences: deletion of a gene, or activation of some other genetic program

4) Controlling gene expression