

**EUROPEAN COURSE ON PATHOLOGY & EMBRYOLOGY
OF GENETICALLY ENGINEERED MICE**

Organized by The D.E.S.V. of Veterinary Pathology

In partnership with:

The French Society of Veterinary Pathology

Veterinary School of Nantes (France)

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GENETIC ENGINEERING: BASIC PRINCIPLES & STRATEGIES

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Definition: Transgenic organism

- Organism in which a segment of DNA has been inserted into the genome *via* genetic engineering

= genetically engineered organism (GEO)

= genetically modified organism (GMO)

inserted sequence = **transgene**

Transgenic Mice

Applications - Why transgenic mice?

– model system for basic research

- gene regulation, cancer biology, development, etc.
- human genetic diseases

– model system for protein production

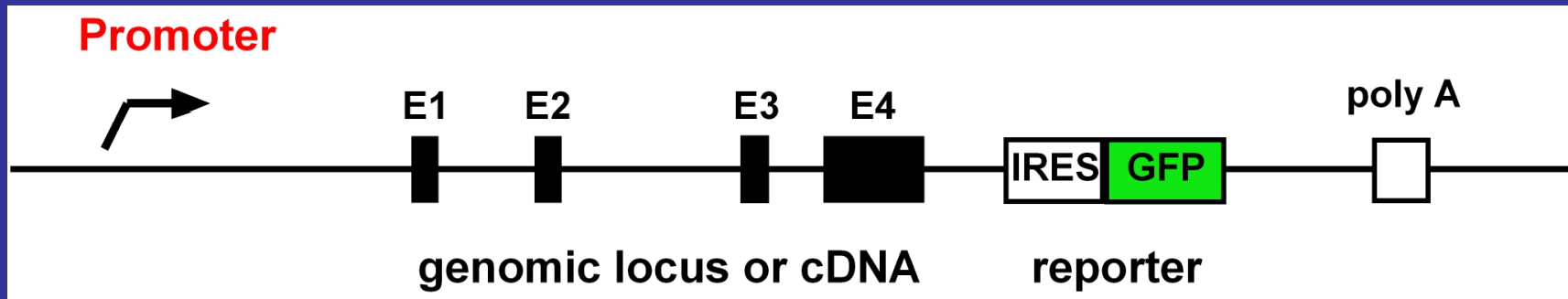
- test in mice first, then try in cattle or other livestock

Transgenic Mice

Methodology

1. **addition** of a new piece of DNA
(“additive transgenesis”)
2. directed **replacement** of endogenous DNA
(gene targeting)

Typical elements of a transgenic vector

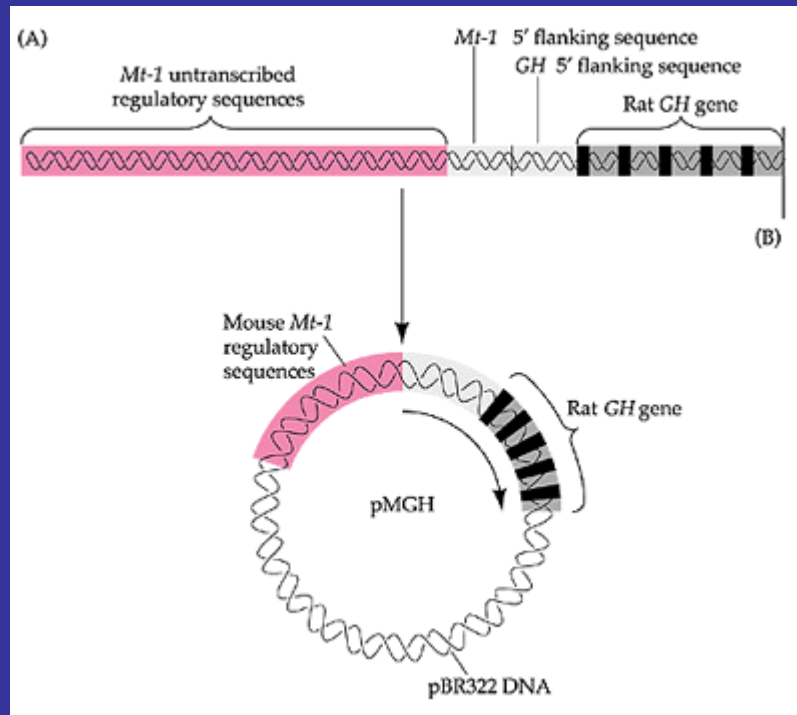


- **promoter**: expression pattern
- **sequence to be expressed**: cDNA, genomic DNA...
- **reporter**: trace transgene expression (optional)
IRES: internal ribosome entry site
- first step: clone the transgenic vector into a plasmid

Transgenic mice: an example

The growth hormone gene has been engineered to be expressed at high levels in animals

The result: BIG ANIMALS



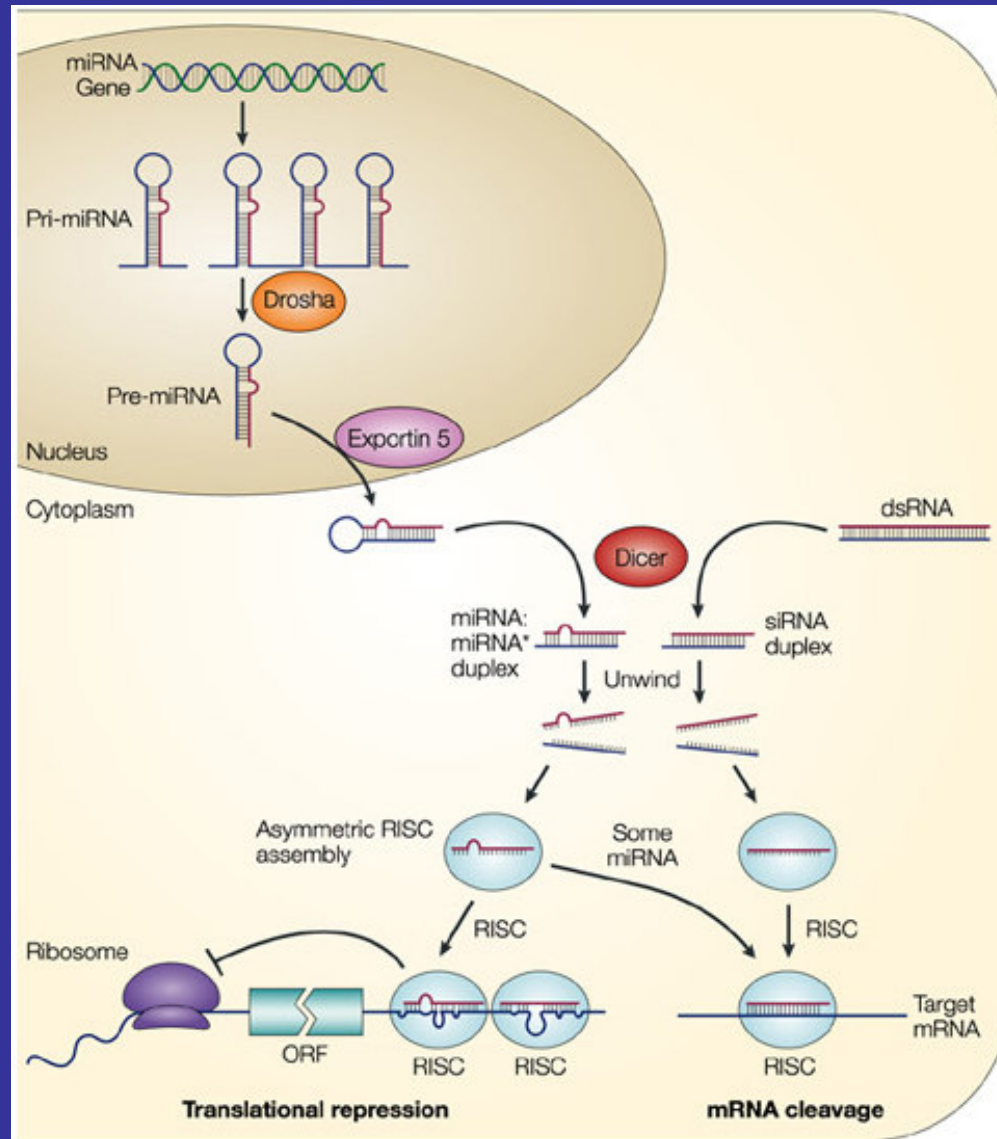
metallothionein promoter
regulated by heavy metals

mice fed with heavy metals
are 2-3 times larger

Some applications of “additive transgenesis”

application	expressed sequence
protein over expression	cDNA, genomic DNA
trace promoter expression	reporter gene
cell ablation	toxin
immortalization	oncogene
protein inactivation	dominant negative mutation
“knock down”	small interfering RNA (siRNA)

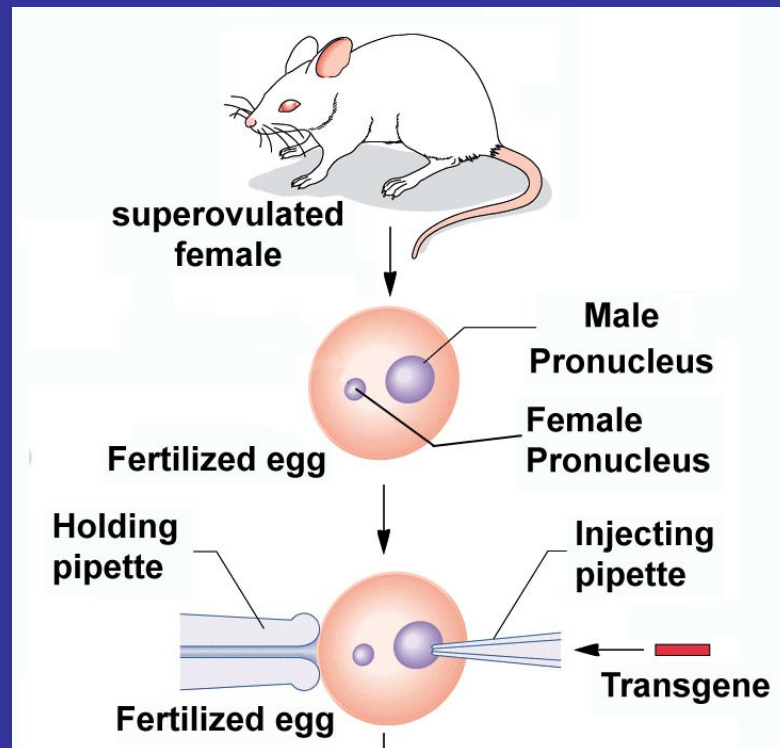
Post-transcriptional suppression by microRNAs and small interfering RNAs



Delivering transgenic vectors

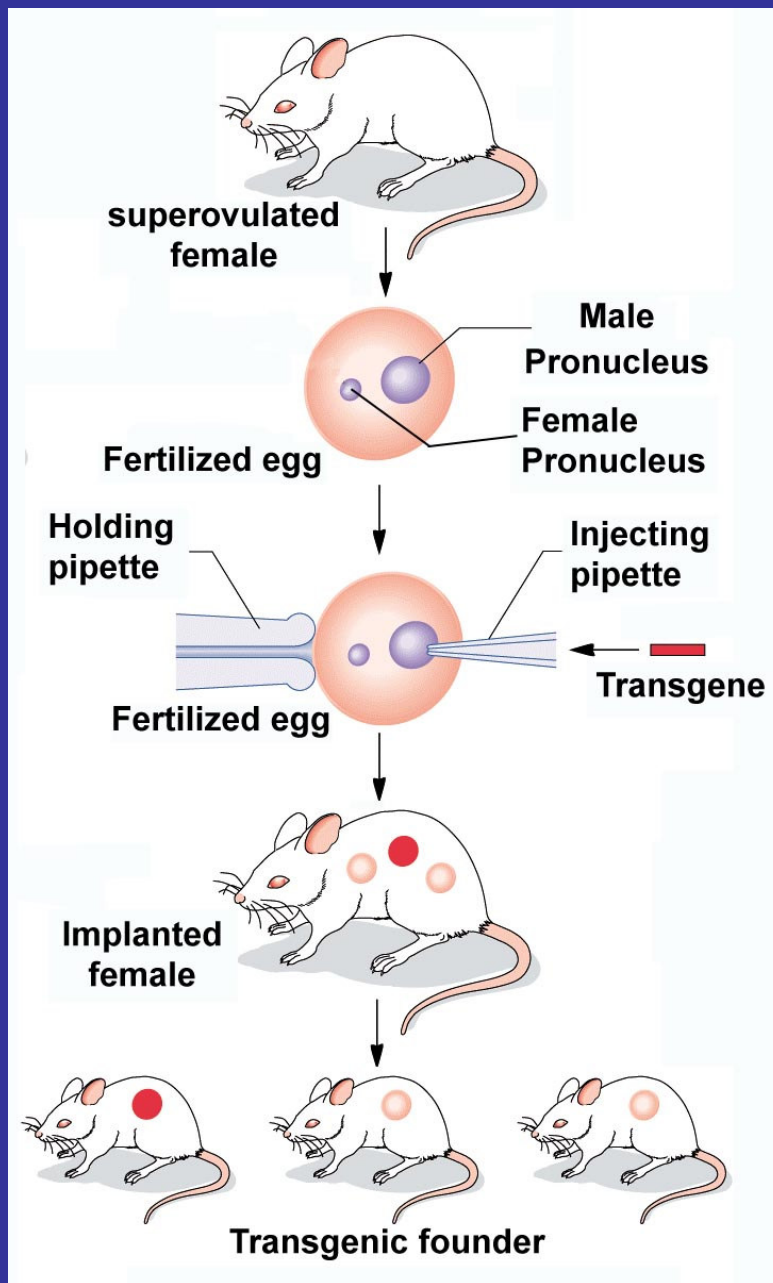
- Methodology
 - DNA Microinjection (1980-81)
 - Retroviruses (1976)
 - Lentiviruses (2002)

DNA microinjection



DNA microinjection





transgene identified by PCR
or Southern blotting

Transgene integration

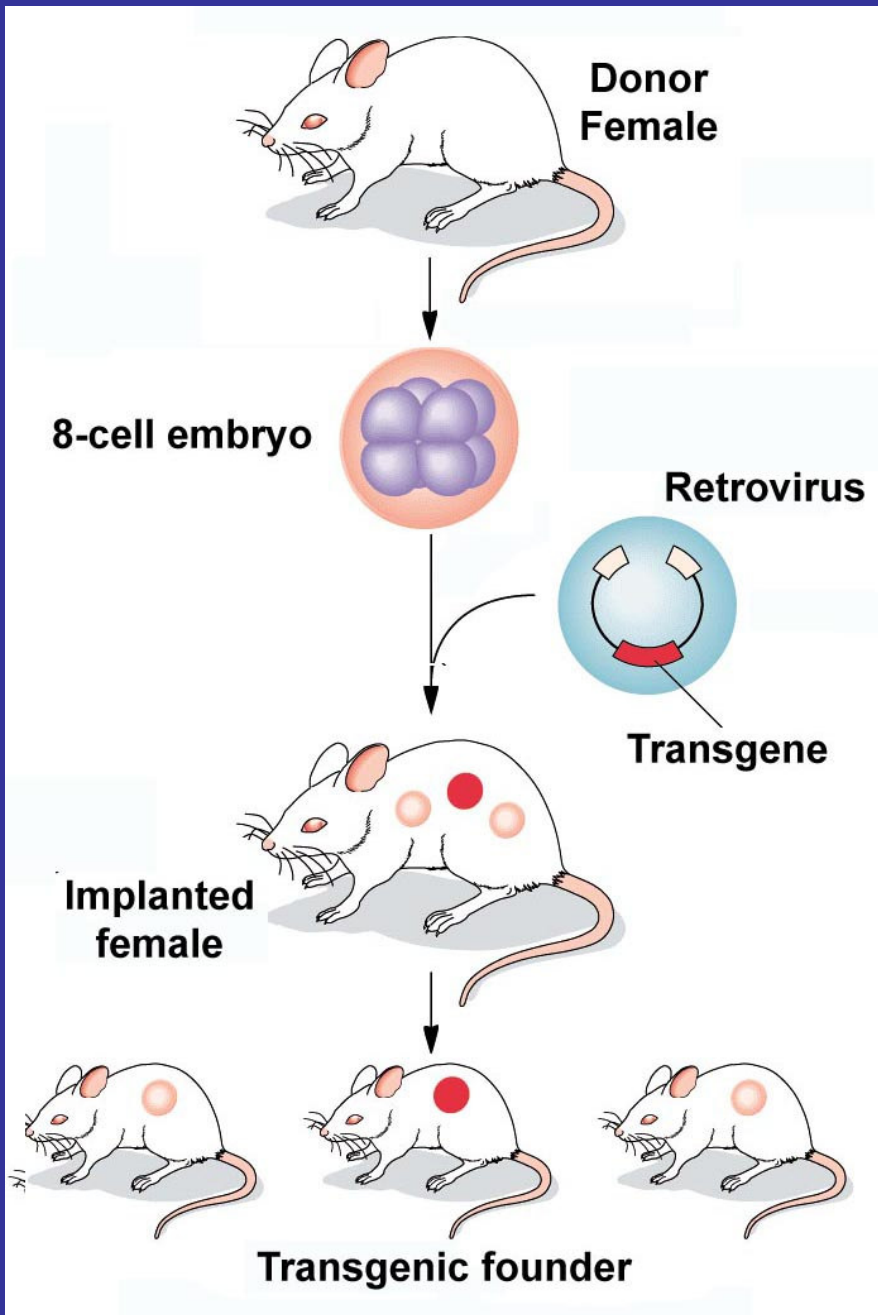
- **Stable**: transgene present in all cells
- **Random**: non-homologous recombination
mutagenesis
transgene expression level
- **n copies**: tandem integration

Transgenesis with High Capacity Vectors

- Most transgenic vectors are plasmid-derived
 - transgene size is limited
- Vectors for large inserts
 - BACs (bacterial artificial chromosome), MACs (mammalian), YACs (yeast)...
 - 100 to >1,000 kb of DNA
 - microinjection of artificial chromosomes
 - creation of transgenic mice making human antibodies
 - ~1,000 kb DNA segment containing human immunoglobulin locus

DNA microinjection

- Inefficient process (only ~5% of injected eggs develop into transgenic mice)
- Technically demanding and costly
- Screen transgenic founders for transgene expression
- Still routinely used (preferred method for creation of “transgenic” mice)
- Can be used for many animals



Transgenic Mice using Retroviruses

- 8 cell embryos from donor
- Infect with retroviral vector
- Reimplant in surrogate
- Screen pups for transgene
- Silencing of the provirus during development

OR

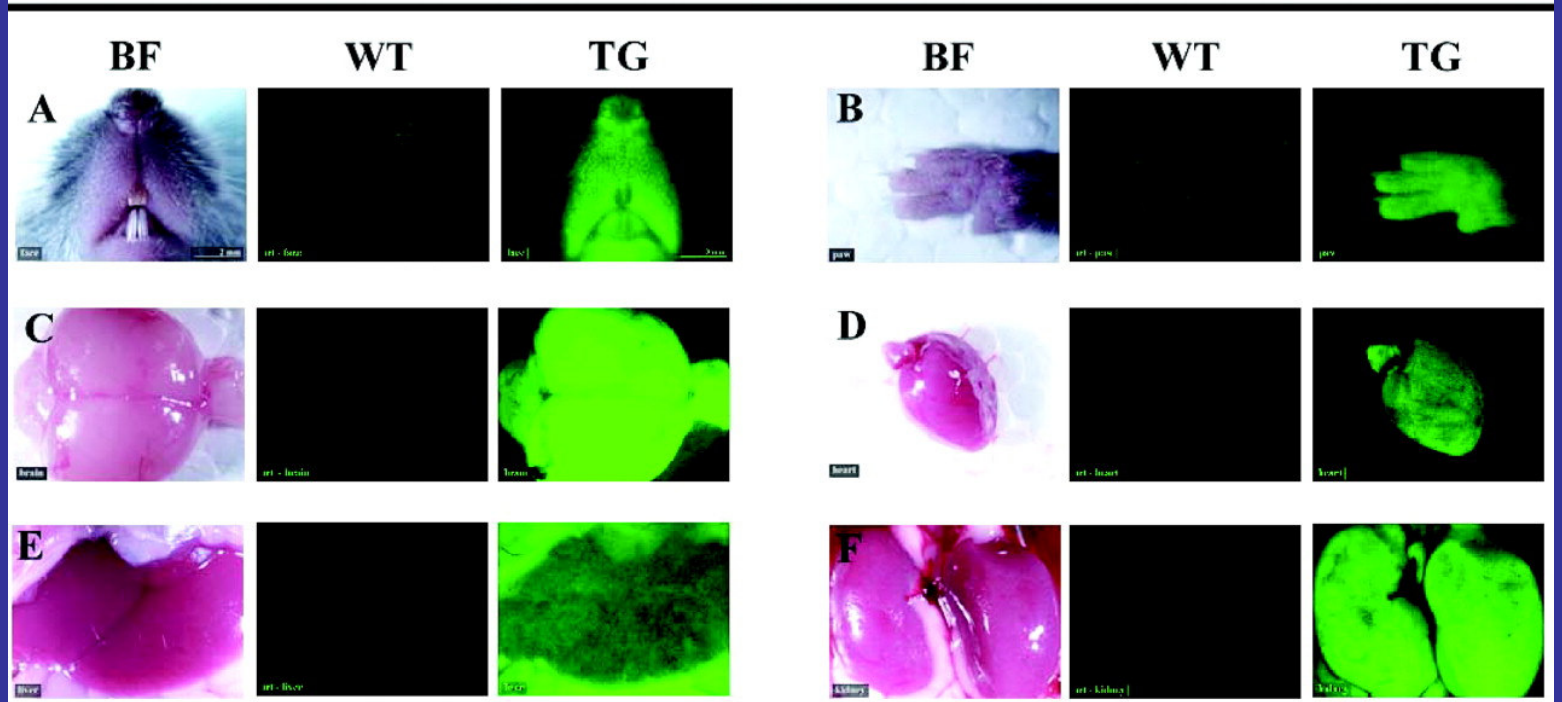
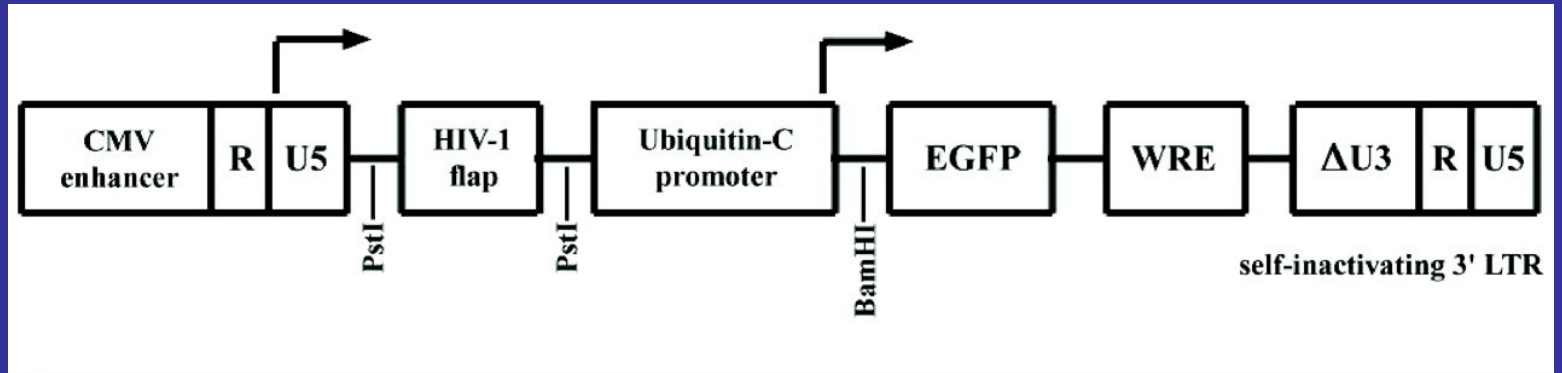
- Low transgene expression

Transgenic Mice using Lentiviruses

- lentiviruses are a class of retroviruses (e.g. HIV)
- cause chronic illnesses in host organisms
- are able to infect dividing and nondividing cells
- might be immune to developmental silencing

Transgenic Mice using Lentiviruses

HIV1
derived
vector

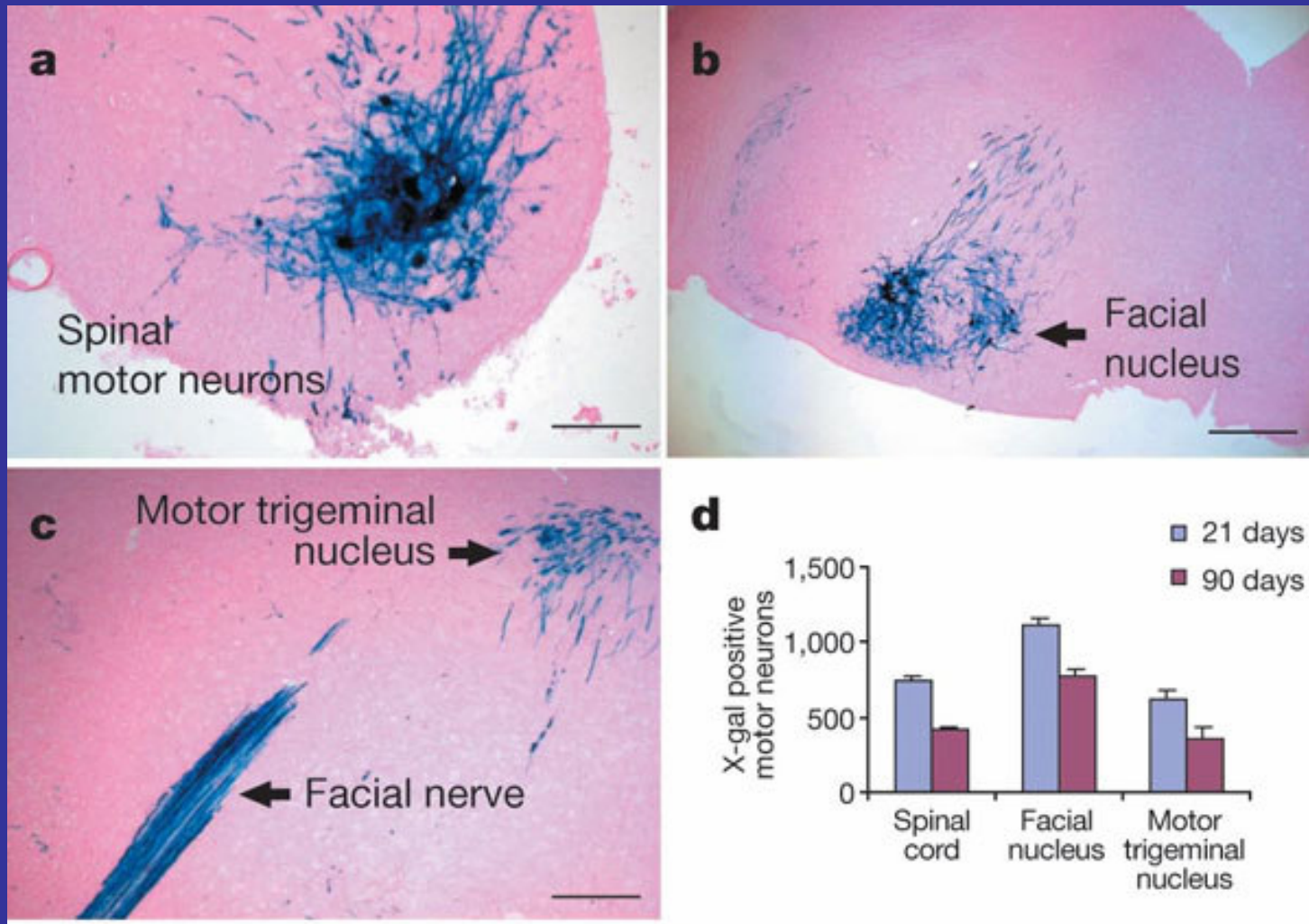


(Lois et al,
Science 2002)

Lentiviruses vs DNA microinjection

- **Lentiviruses:**
 - less invasive and technically less demanding
(delivering by co-incubation with denuded embryos)
 - more efficient (?)
 - more cost-effective (?)
- **DNA microinjection:**
 - bigger transgenes
 - higher copy number – higher expression
- **Lentiviral-mediated somatic transgenesis**
 - deliver a transgene to specific cells

Lentiviral-mediated somatic transgenesis



(Azzouz et al, Science 2004)

“Additive transgenesis”

- addition of a piece of DNA
- random integration *via* non-homologous recombination
- selection is not possible

Transgenic Mice

Methodology

1. **addition** of a new piece of DNA
("additive transgenesis")
2. directed **replacement** of endogenous DNA
("gene targeting")

Embryonic Stem Cell Method for Transgenic Mice

- Uses embryonic stem (ES) cells rather than eggs
- Transfection by electroporation, liposomes, etc.
- Transfected cells reimplanted into new blastocyst and allowed to develop

Embryonic Stem (ES) cells

Can be cultured, manipulated and then reinjected into blastocysts, where they can go on to contribute to all parts of embryo
(isolated for the first time in 1981)



Pluripotent stem cells derived from the inner cell mass of the blastocyst

Early development of a mouse embryo

fertilized egg



blastocyst

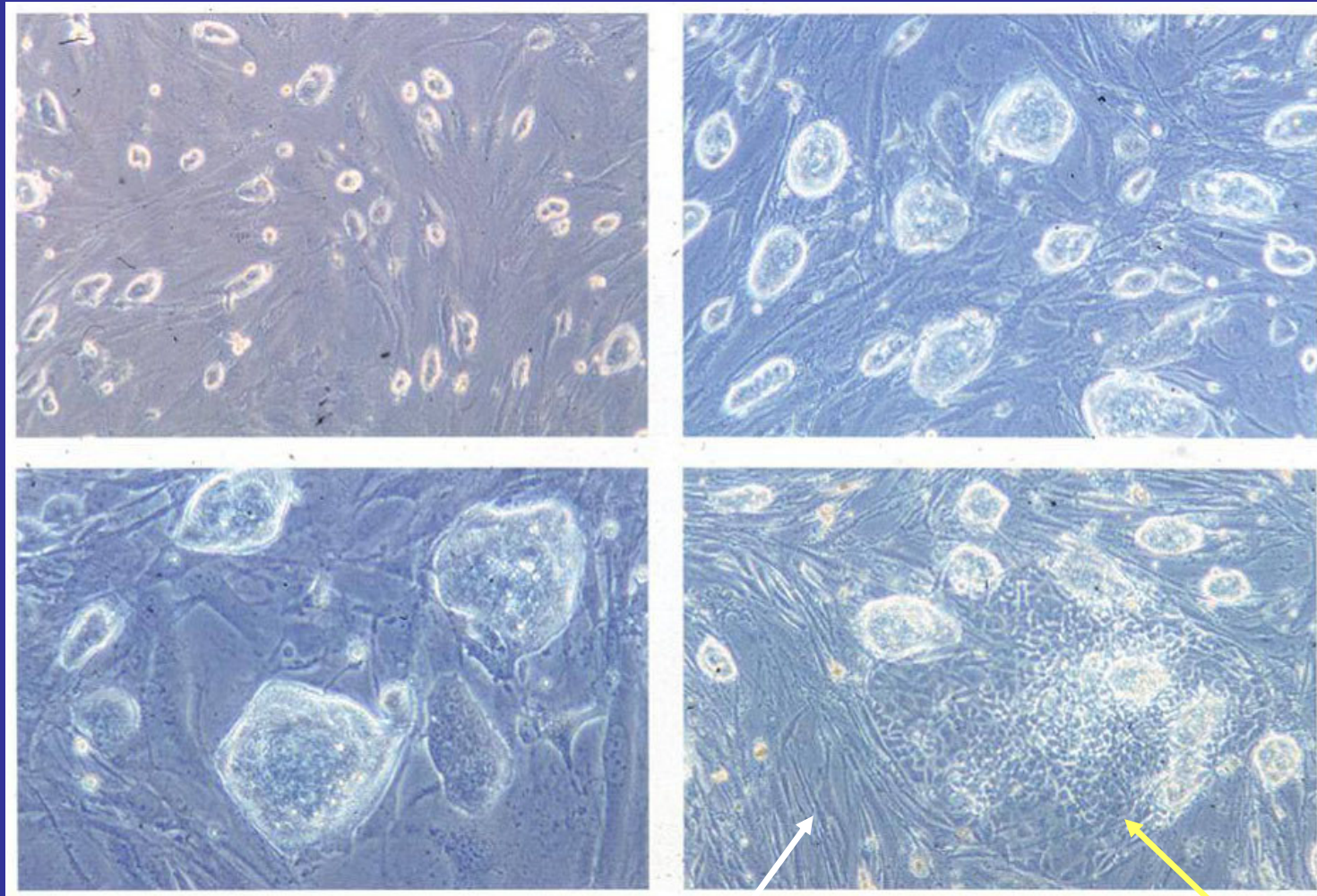
inner cell mass (ICM)



trophectoderm



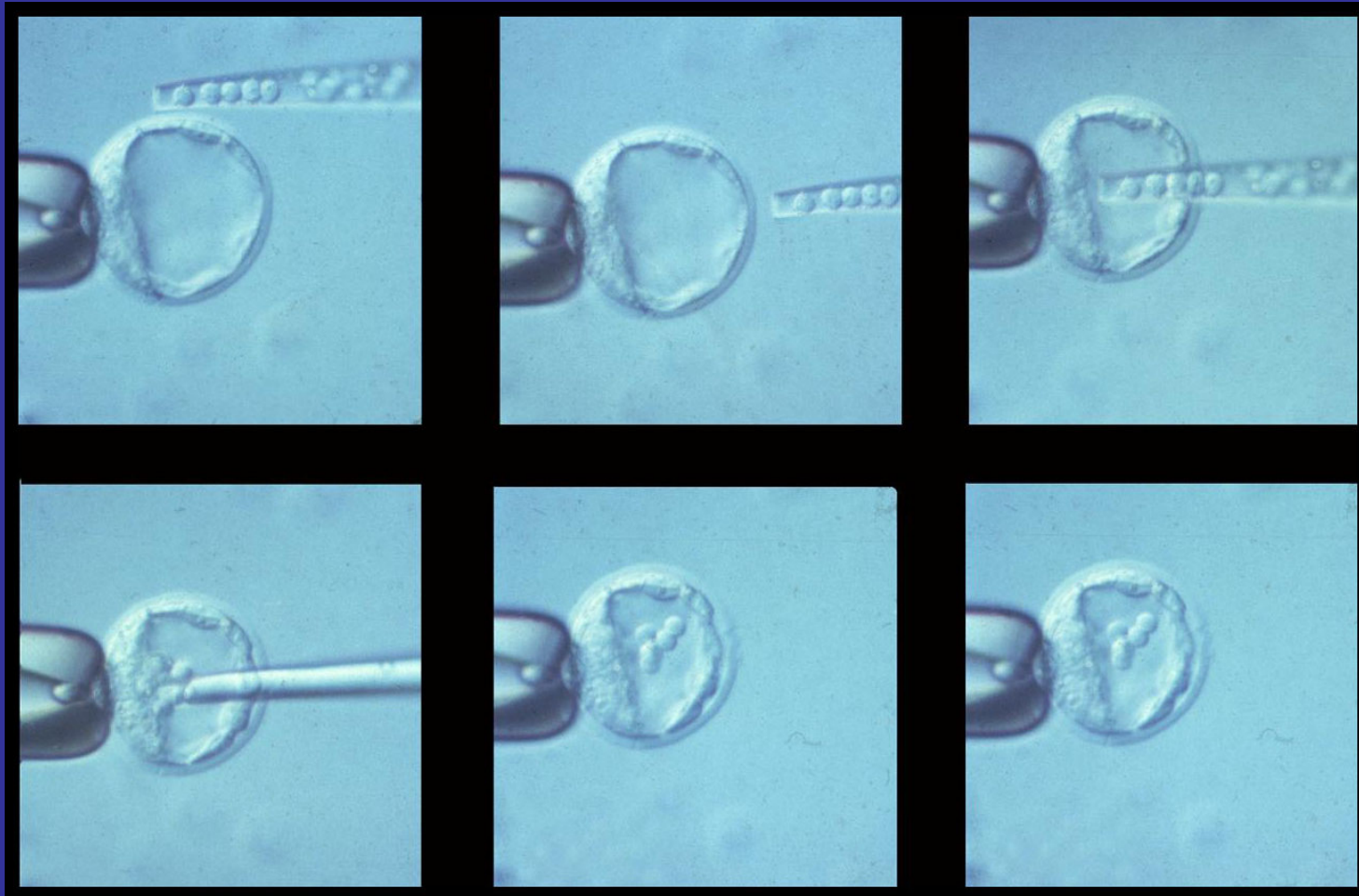
Culture of ES cells



feeder cells

ES cells

Injection of ES cells into a blastocyst

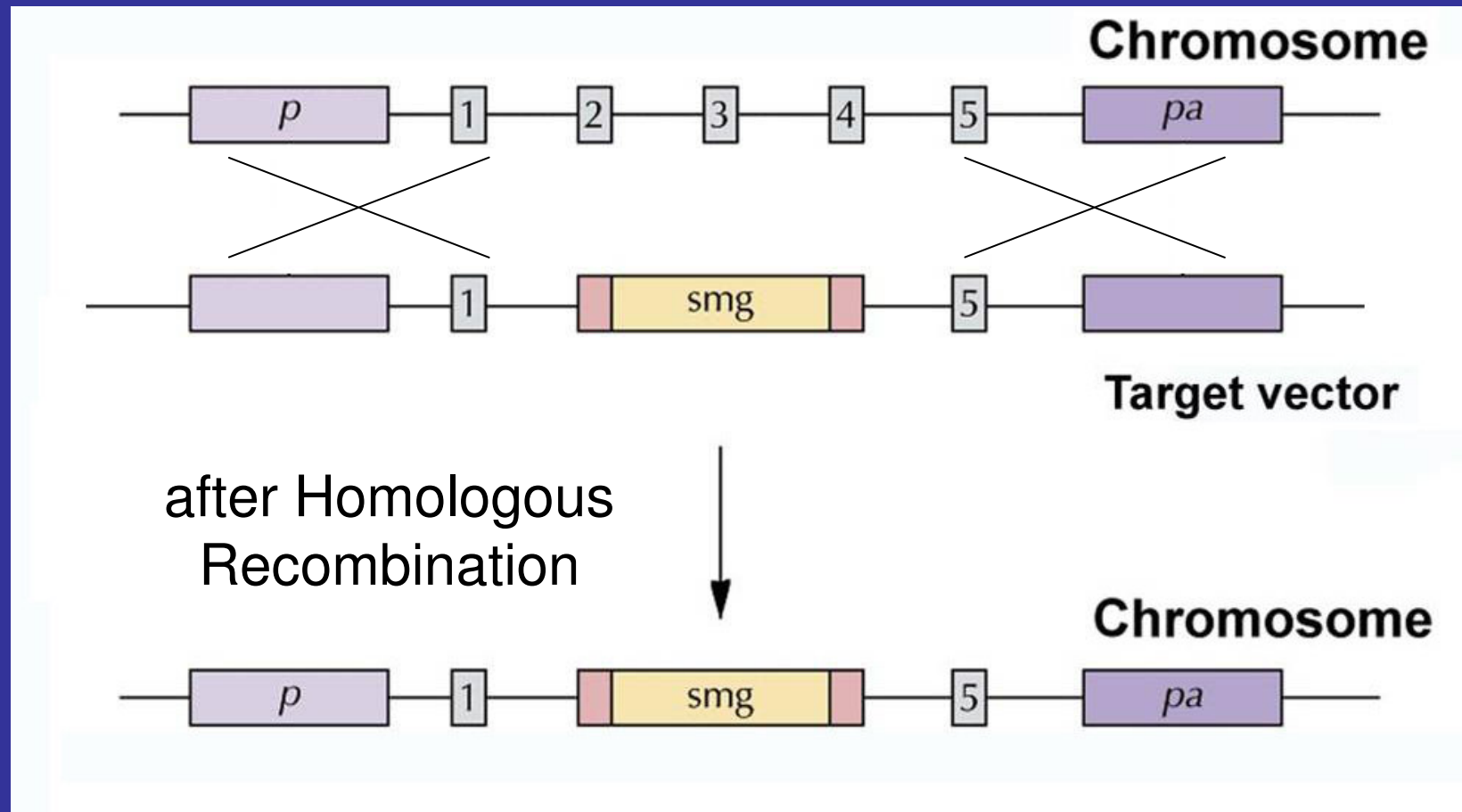


chimeric mice

Embryonic Stem Cell Method for Transgenic Mice

- Main benefit: **selection** is possible
 - selection for integration of the DNA to a specific site
 - homologous recombination:
 - a process by which one DNA segment can replace another DNA segment that has a similar sequence
 - occurs naturally during meiosis
 - works better in some organisms (e.g. yeast) than others (e.g. mammals)

Homologous recombination

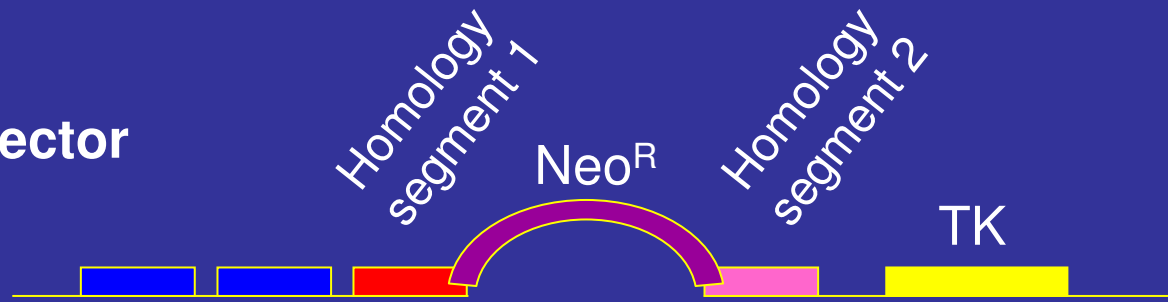


very rare event in mammalian cells

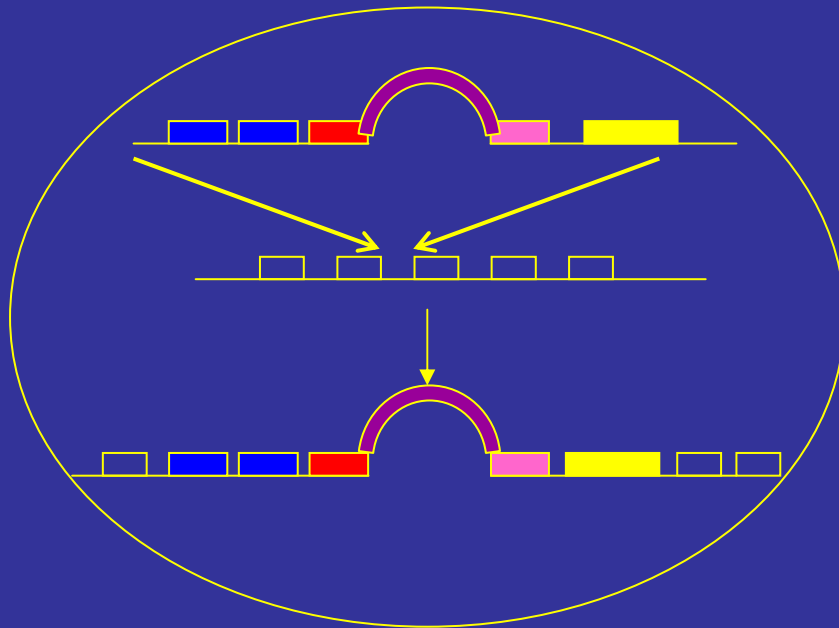
Positive and Negative Selection

- Positive Selection for integration
 - kills all non-transfected cells
 - G418 kills all cells except those containing NeoR gene
- Negative Selection for NON-specific integration
 - kills cells modified by non-homologous recombination
 - Ganciclovir is converted to toxic compounds by thymidine kinase gene

Targeting vector



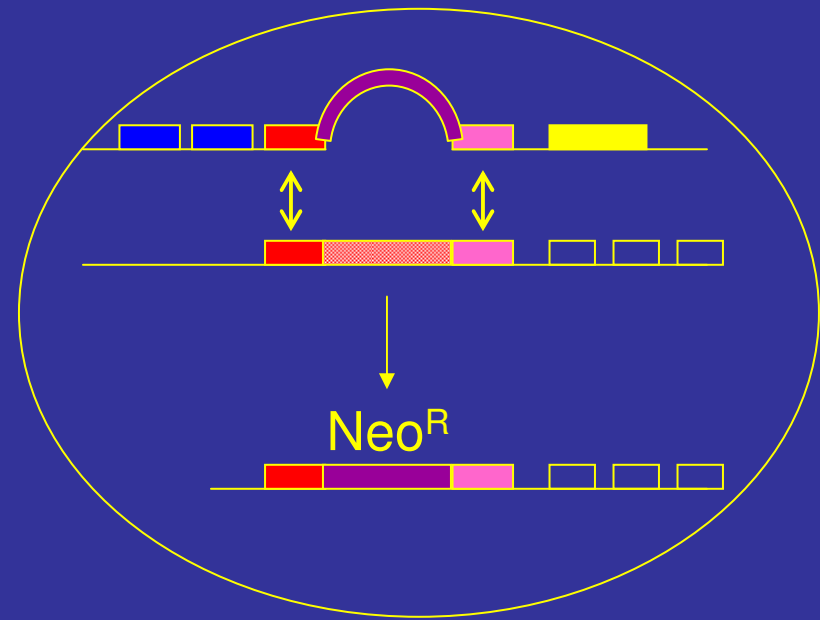
Random
integration



Neo^R+ $TK+$

TK will convert gancyclovir into a toxic drug
and kill the cell

Homologous
recombination

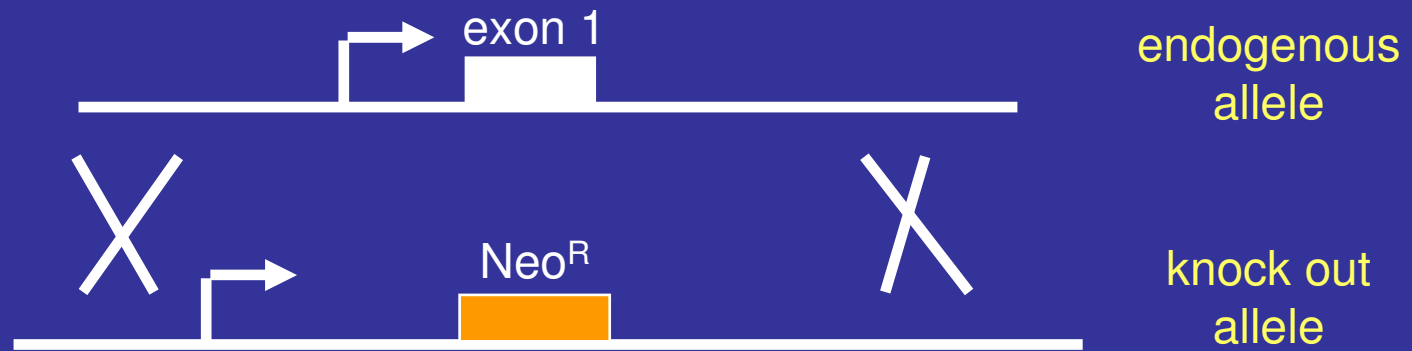


Neo^R+ $TK-$

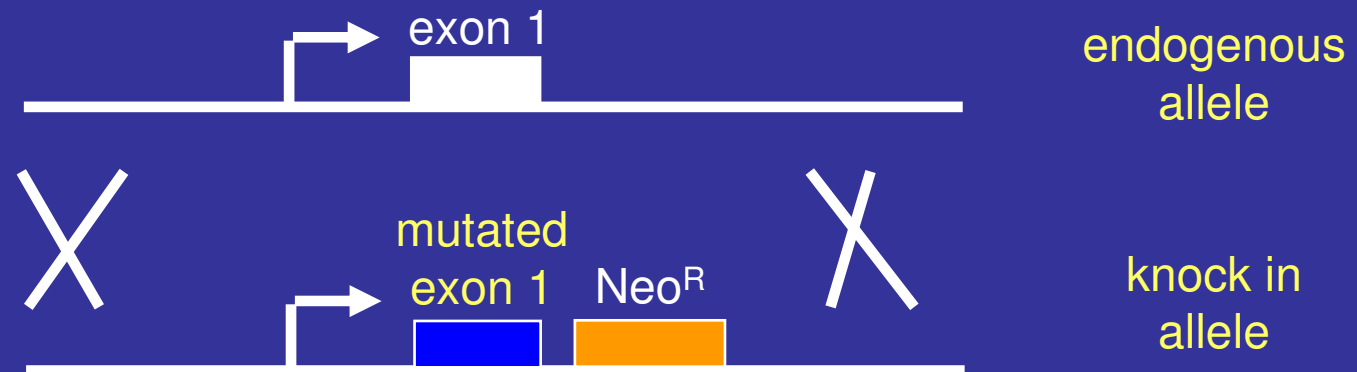
not sufficient: verify ES clones by
Southern blotting

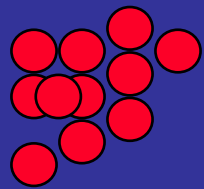
“Knock outs” and “Knock ins”

- Targeted insertion can be used to:
 - disrupt a specific gene = **knock out**



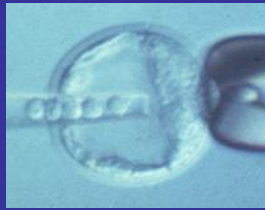
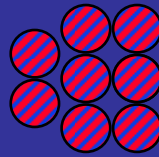
- modify or replace a gene = **knock in**





ES cells

Electroporation
of targeting vector



Selection of targeted clones

Injection into blastocyst

Reimplantation

Transgenic chimeras



first strain: 1989

Wild type



X



heterozygous mice

homozygous mice

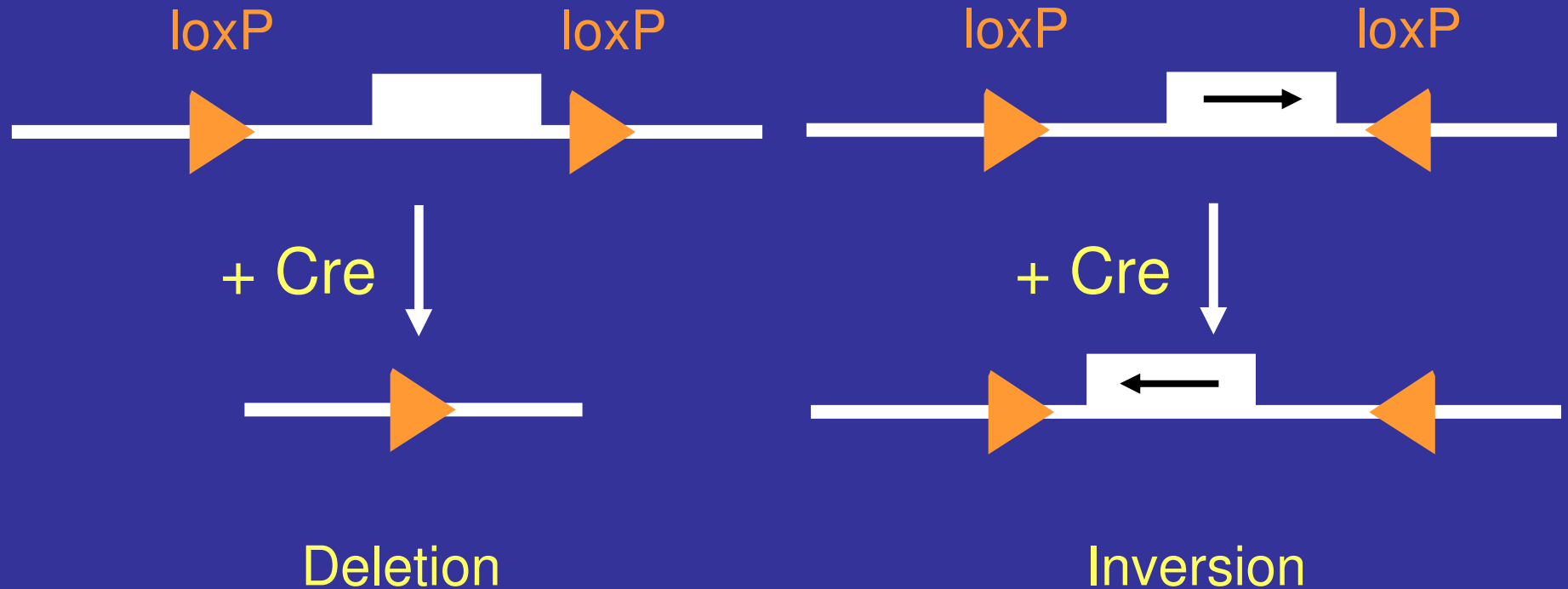


Conditional genome modifications

- spatially- and/or temporally-regulated
- applications:
 - lethality of a given mutation: study later roles of a gene
 - complex phenotypes: study specific roles of a gene
- tools:
 - the Cre/loxP system
 - Tet-On and Tet-Off systems

The Cre/loxP system

- Cre recombinase of the P1 phage
- LoxP sites (34 bp)
- stable, heritable recombination



Designing conditional alleles

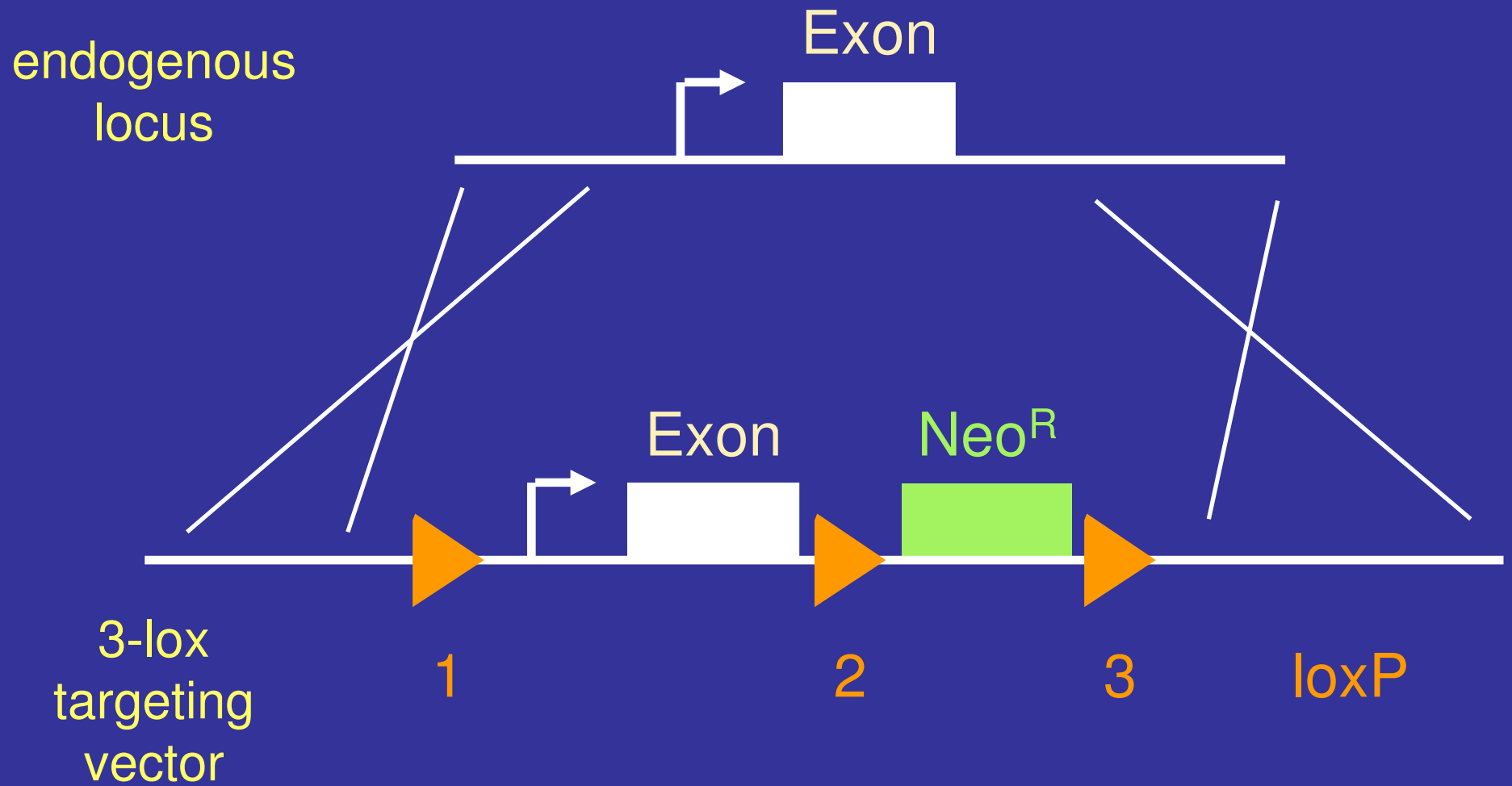
conditional
loss-of-function
allele
(floxed allele)



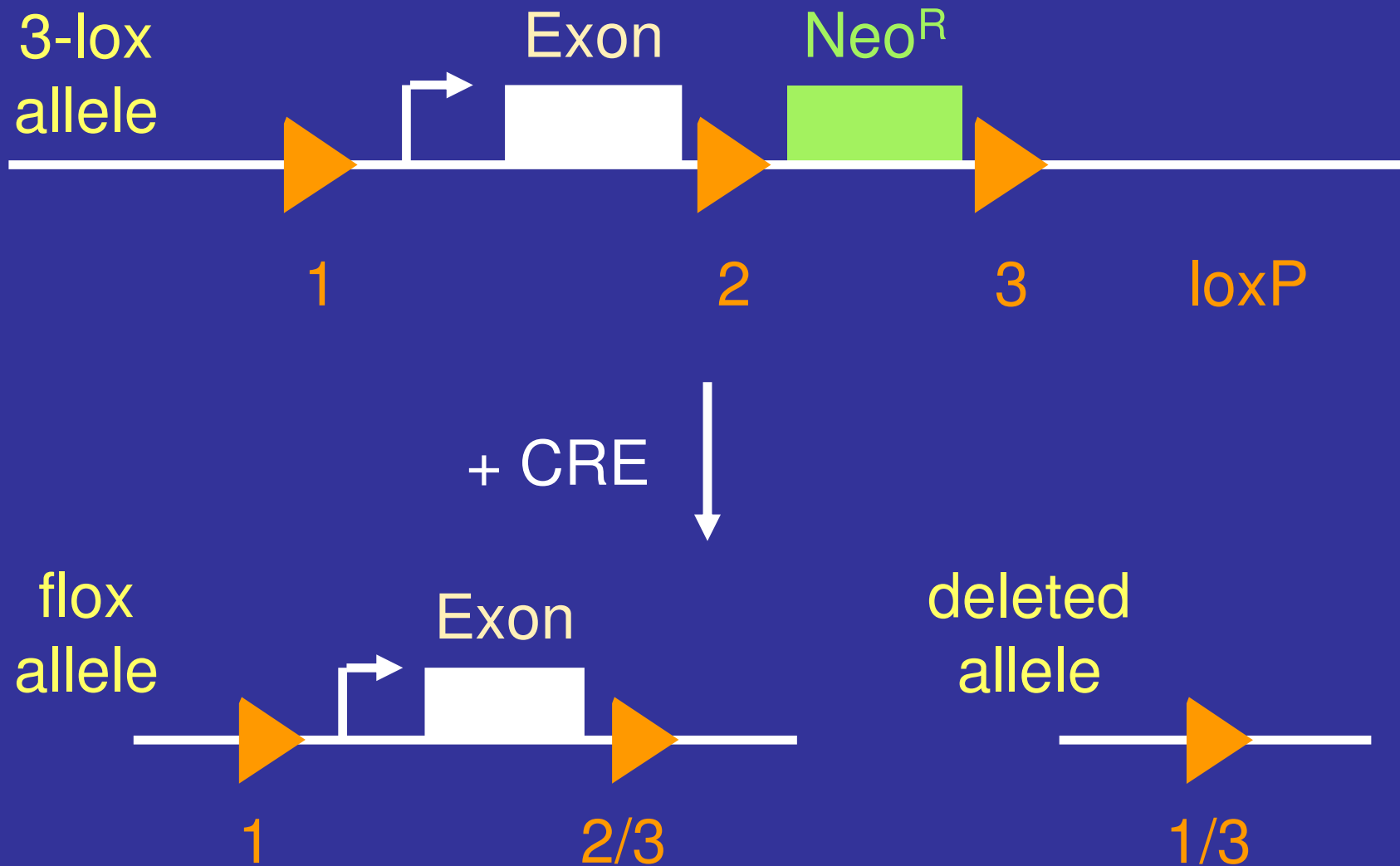
conditional
gain-of-function
allele
(lox-STOP-lox
allele)



Inserting loxP sites



Cre recombination in ES cells



Delivering the Cre recombinase

- from “inside”: genetic means

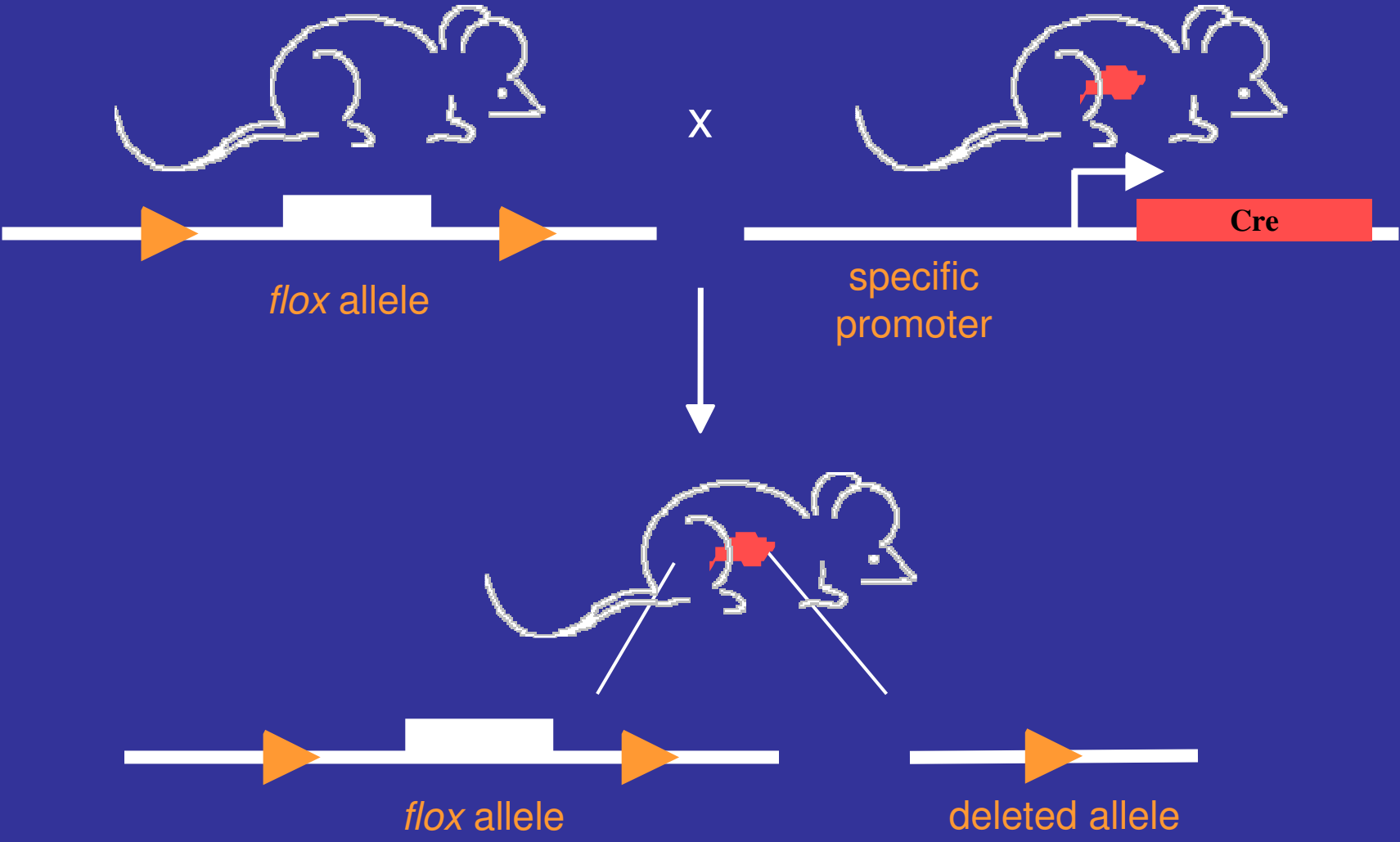
Cre-expressing “transgenic” or knock in mice

- from “outside”: transfection

in vitro: primary cells from flox mice + Cre
expressing vector

in vivo: injection of a Cre-expressing vector

Conditional gene inactivation: the Cre/loxP system



Regulatory sequences for tissue-specific expression of the Cre

Brain:

Nestin
CaMKII-alpha
Myelin basic protein

Liver:

Albumin
 α -Fetoprotein
Transthyretin

Heart:

Muscle creatinine kinase

Ubiquitous:

CMV

Pancreas:

Insulin
Pdx1

Bone:

Osteocalcin (OB)
Lysozyme M (OC)

Chondrocytes:

Col2a1

Gut:

Keratin 5
Villin

Prostate:

Rat probasin

Retina:

Six3

Oocytes:

Zona pellucida protein 3

Epiblast:

Mox2

Mesoderm:

HoxB6

and many more...

Spatial and temporal regulation of the Cre

- constitutive Cre
 - constitutive promoter + Cre
- inducible Cre
 - inducible promoter + Cre: e.g. Mx-Cre
 - constitutive promoter + Cre-ER fusion

Inducible Cre activity by tamoxifen

- special Cre used = Cre-ER fusion protein
 - ER = estrogen receptor
 - the ER is mutated: does not bind estrogen
binds tamoxifen
- tamoxifen
 - a fake estrogen
 - used as anti-estrogen to treat breast cancer
 - activates the nuclear translocation of mutated ER

Cre-ER is activated by tamoxifen

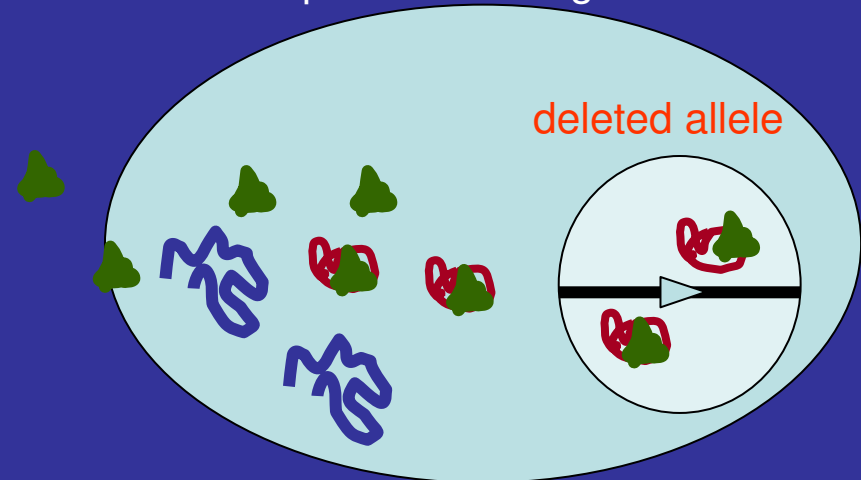
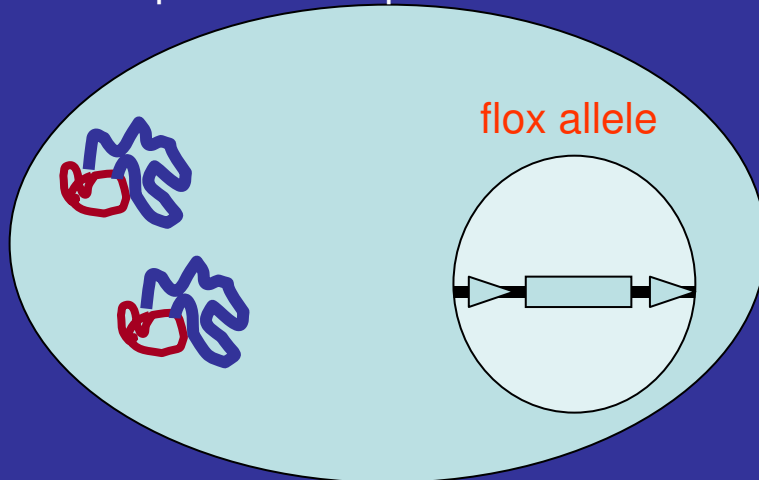
NO TAMOXIFEN

TAMOXIFEN ADDED

Cre-ER is kept in the cytoplasm
in a complex with Hsp90

Cre-ER-Tamoxifen
dissociates from Hsp90

Cre-ER-Tamoxifen
goes to the nucleus

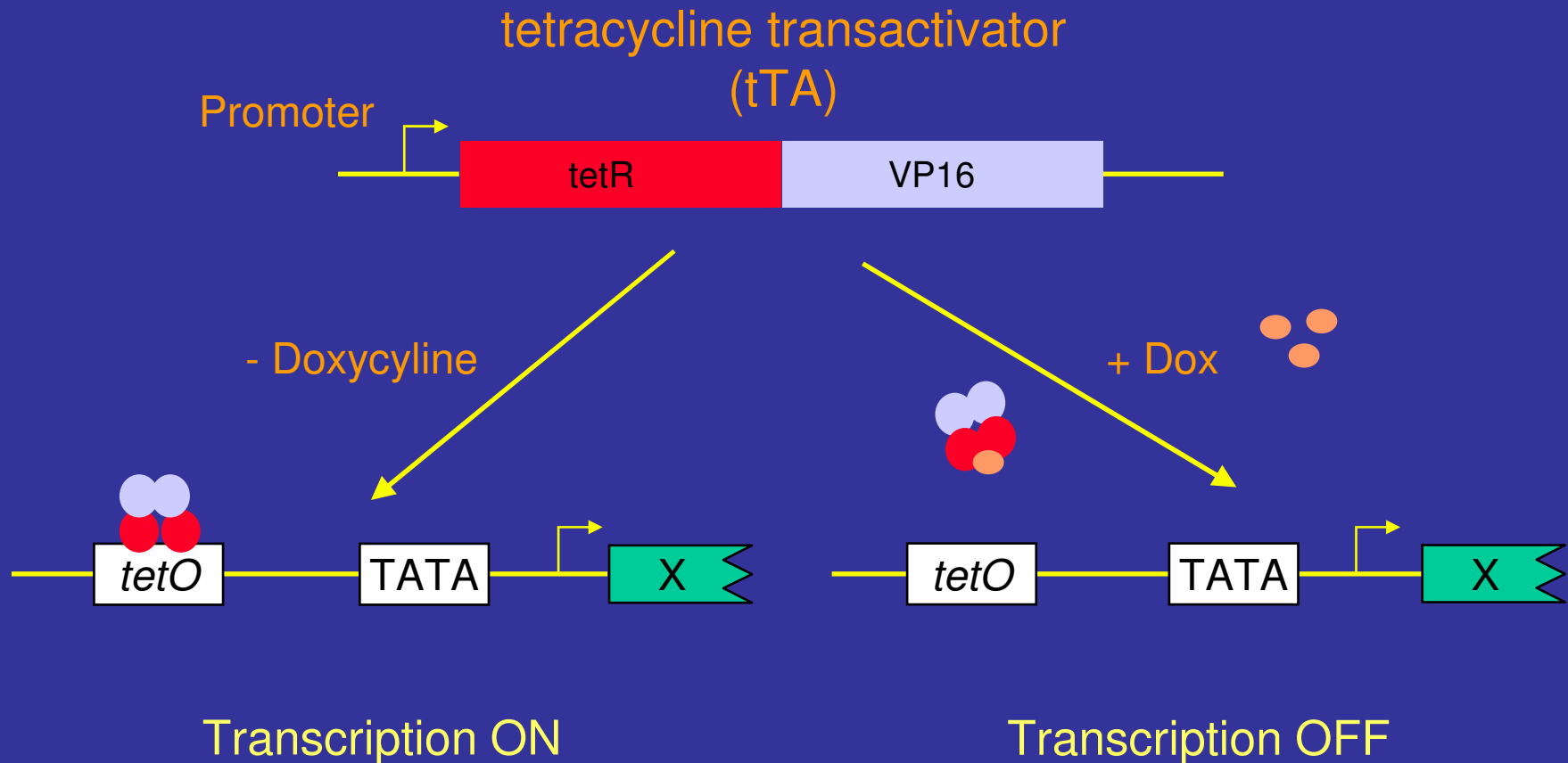


GENE IS ACTIVE

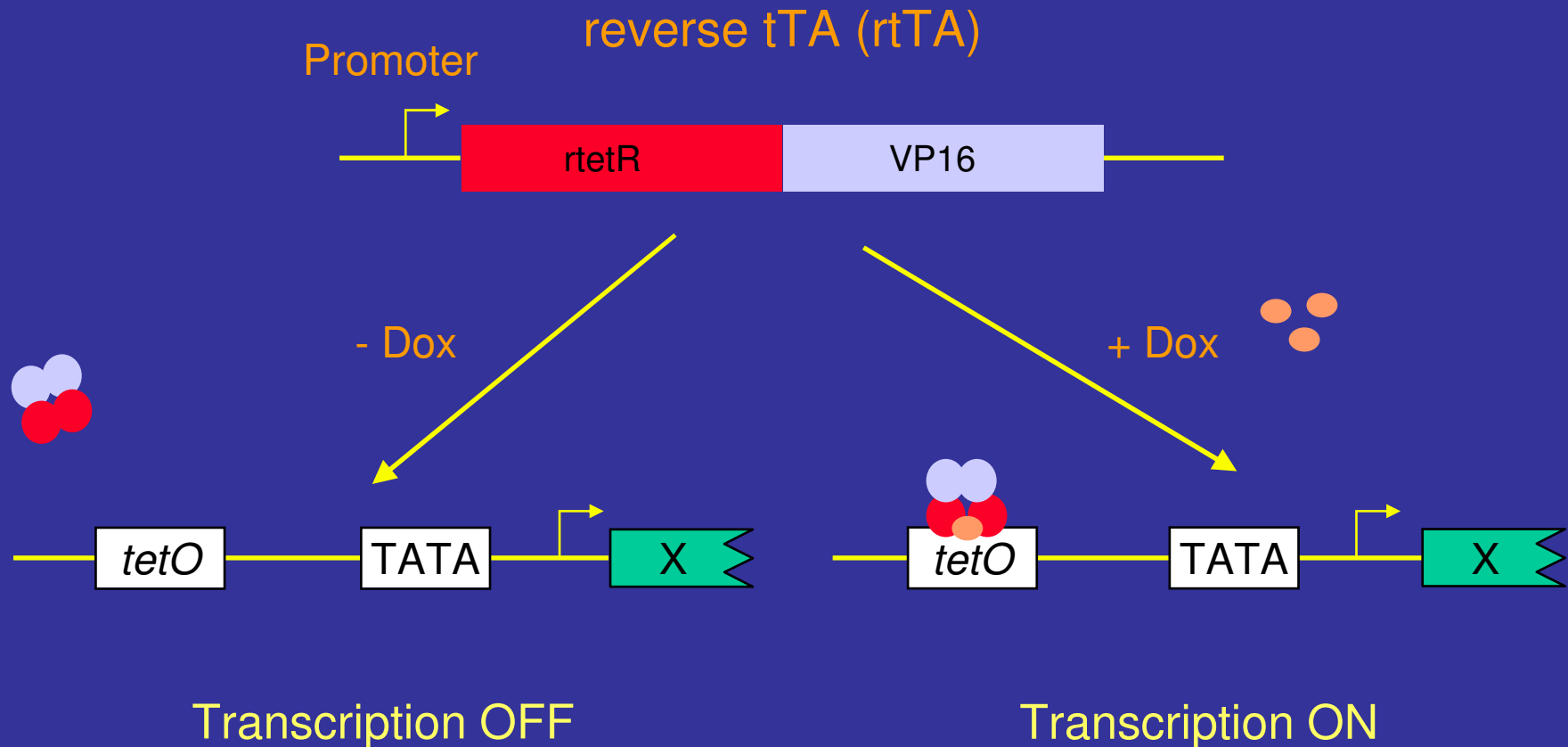
GENE IS INACTIVATED

control of the Cre **both** from promoter and tamoxifen addition

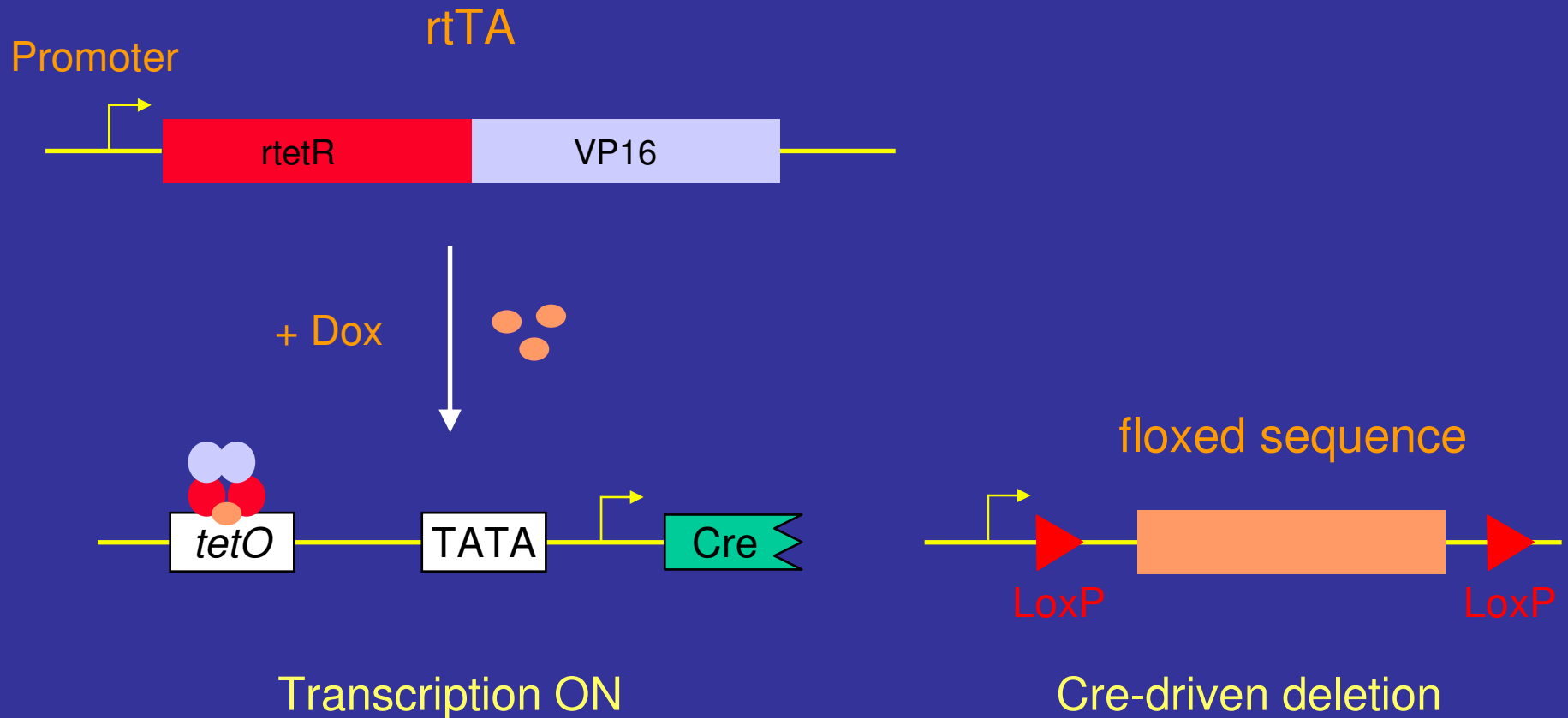
Transcriptional induction by tetracyclins: the Tet-off system



Transcriptional induction by tetracyclins: the Tet-on system

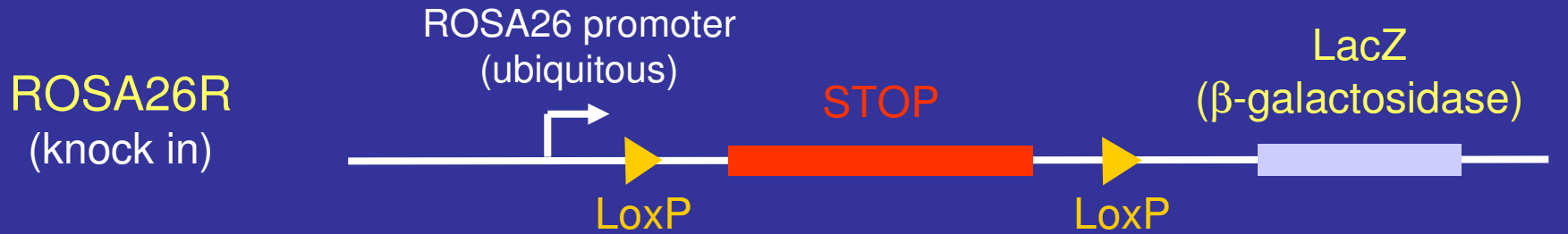


Spatial and temporal control: the Tet-Cre system



Target gene disrupted only in the presence of Dox (at a chosen time) and only in cells where the promoter is active

Following Cre-driven recombination with reporter strains



non-recombined cells: β -gal negative
Cre-recombined cells: β -gal positive



non-recombined cells: β -gal positive
Cre-recombined cells: AP positive

Genetic engineering in mice

- “additive transgenesis”
 - selection is impossible
 - random integration
 - non homologous recombination
- gene targeting
 - selection is possible
 - site-specific integration
 - homologous recombination



For more information...

- general

- Transgenic Animals: generation and use, edited by Louis-Marie Houdebine, Harwood Academic Publishers, 1997.

- Special issue of the *International Journal of Developmental Biology* (vol. 42(7), 1998) on Stem Cells and Transgenesis.

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- Pfeifer A, Lentiviral transgenesis, *Transgenic Res*, 2004, 13:513-22.

- ES

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- Babinet C, Cohen-Tannoudji M, Genome engineering via homologous recombination in mouse embryonic stem (ES) cells: an amazingly versatile tool for the study of mammalian biology, *An Acad Bras Cienc*, 2001, 73:365-83.

- Cre/loxP

- Tronche F, When reverse genetics meets physiology: the use of site-specific recombinases in mice, *FEBS Letters*, 2002, 529:116-121.