National Cancer Institute

Complex and inducible construct design for mouse models

by Mark Lewandoski

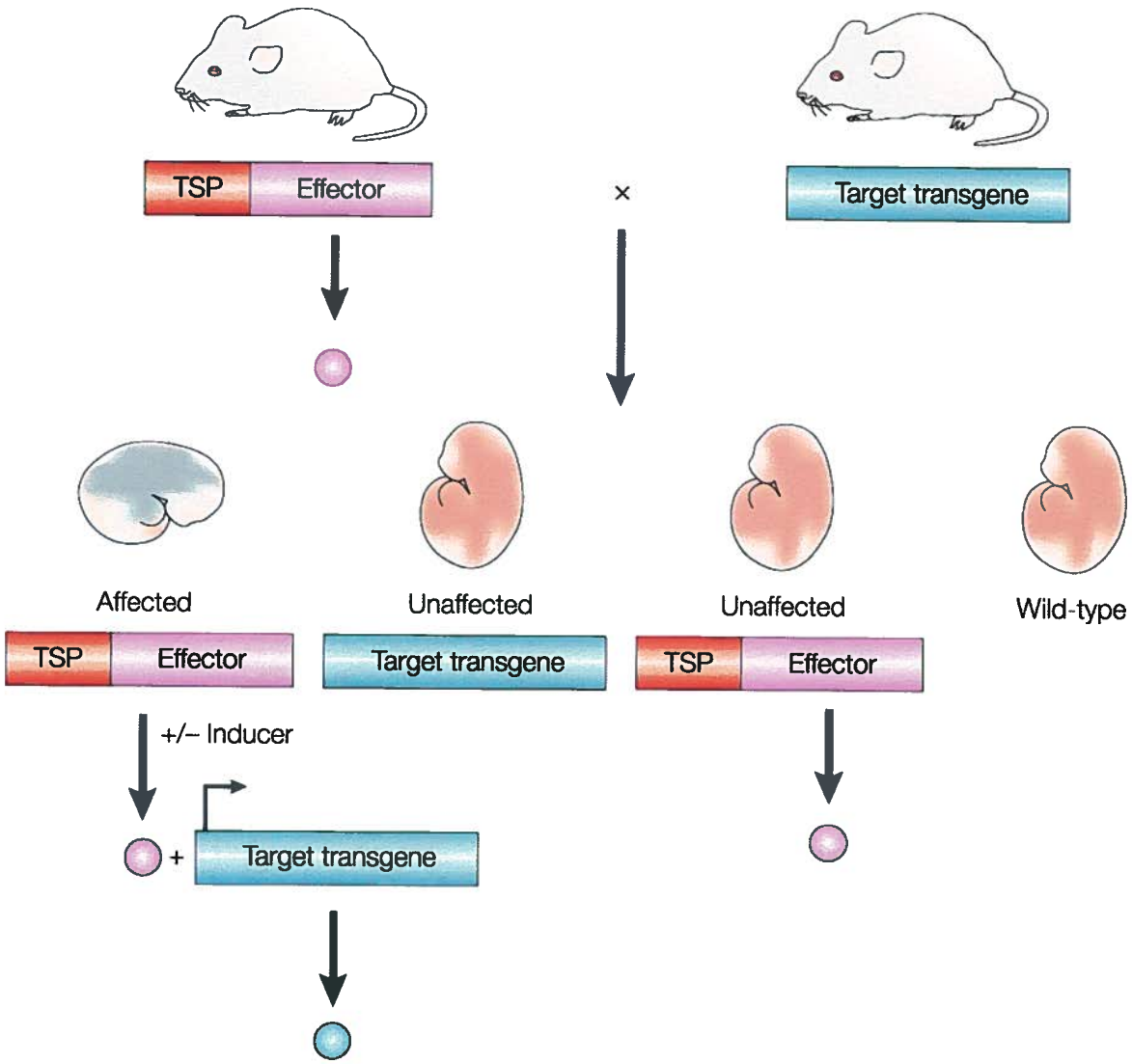
Mouse 101

NCI, NIH November 24, 2009

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

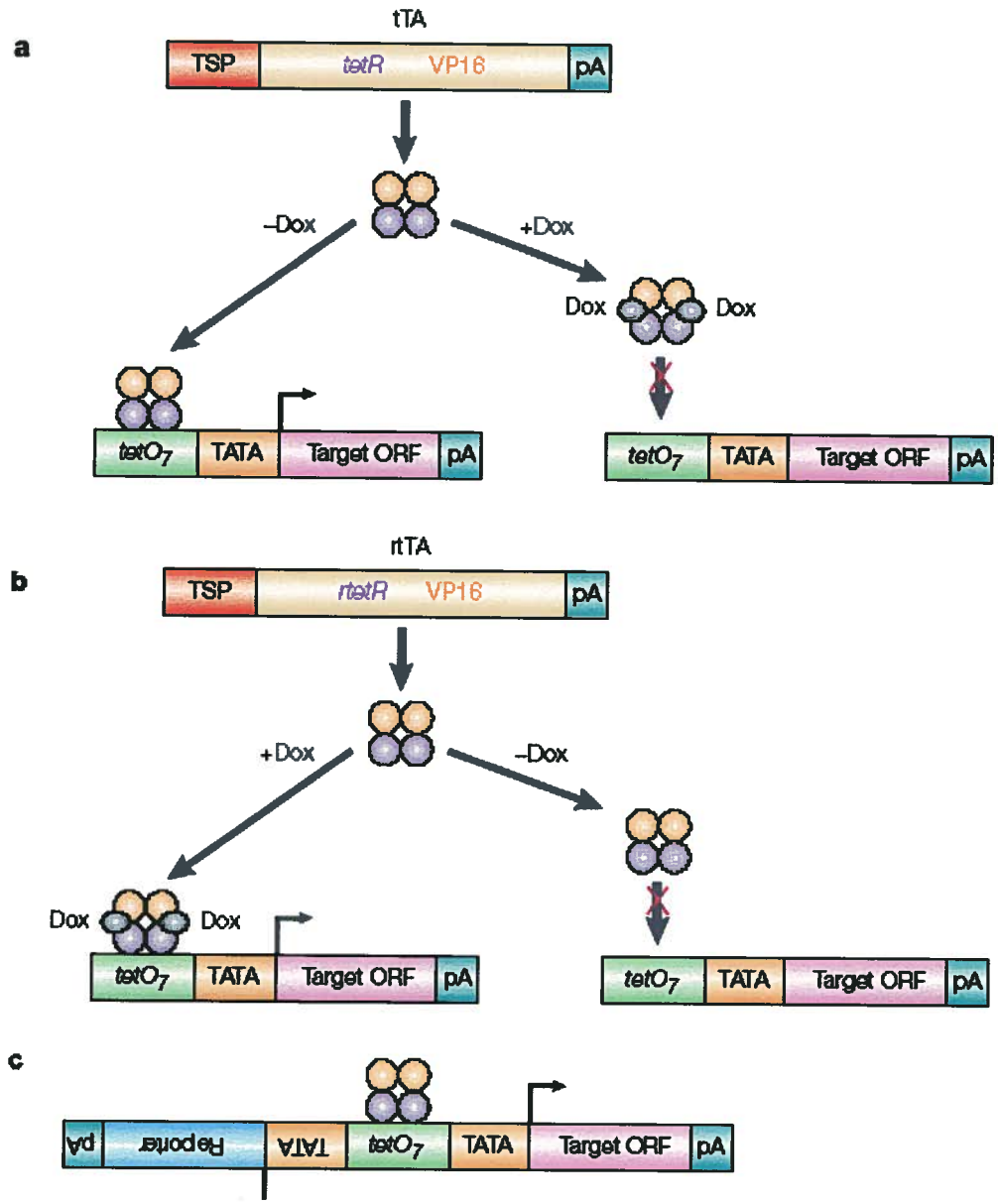
National Institutes of Health

Binary transgenics



Lewandoski, M. (2001) Nature Reviews | Genetics 2: 743

Binary transgenics based on transcriptional transactivation



Lewandoski, M. (2001) Nature Reviews Genetics 2: 743

Binary transgenics based on transcriptional transactivation

In vivo gene regulation using tetracycline-regulatable systems
2009 Stieger K et al Adv Drug Deliv Rev. Jul 2;61(7-8):527-41

Regulatable Gene Expression Systems for Gene Therapy Applications: Progress and Future Challenges
2005 Goverdhana et al *Mol Ther*. 2005 August ; 12(2): 189–211

http://www.zmbh.uni-heidelberg.de/bujard/Homepage.html

Mostly, transcriptional transactivation is used for gain-of-function studies, but see

An Inducible and Reversible Mouse Genetic Rescue System
2008 Zeng et al Plos Genetics 4: e10000069

Binary transgenics can be based on DNA recombination

a

loxP

ATAACTTCGTATAATGTATGCTATACGAAGTTAT

FRT

GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

b

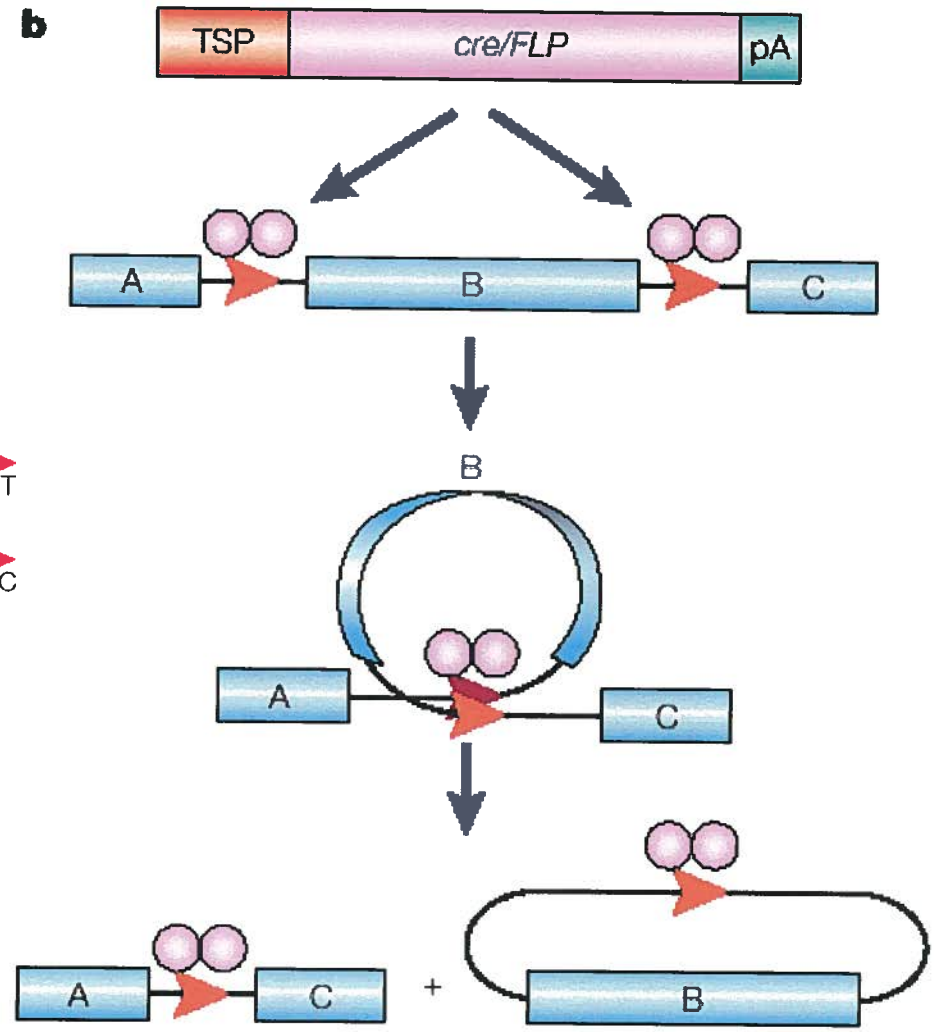


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Table 1 SSRs and some of their derivatives useful for mouse SSR technology

SSR / target site	Properties and application(s)	Reference(s)
A) SSR systems with proven efficiency in cultured mammalian cells as well as in mice		
Cre/loxP	Biological function: DNA excision for dimer reduction of bacteriophage P1 plasmids	Sternberg et al. 1981
	Most efficient and widely used SSR tool *in vitro* and in *vivo*	See text
EGFP-Cre	Fusion with an N-terminal EGFP; facilitates recombinase detection	Le et al. 1999
iCre	Codon-improved version for expression in mammalian cells	Shimshek et al. 2002
Cell-permeable Cre	Fusion with membrane translocation sequences such as the basic HIV-TAT peptide; the efficiency of cell-permeable is not clear Cre proteins in vivo (see the chapter by C. Patsch and F. Edenhofer, this volume)	Jo et al. 2001; Joshi et al. 2002; Peitz et al. 2002
CreLBDs	Various fusions with mutated steroid receptor LBDs; inducible by synthetic ligands of the LBD	See Sect. 4
Flp/*FRT*	Biological function: DNA inversion for amplification of yeast 2-μm plasmid	Vetter et al. 1983
	Removal of selection cassettes and other more specialized transactions	Rodriguez et al. 2000; Schnutgen et al. 2005
Flpe	Mutated version selected in a protein evolution strategy with increased activity	Buchholz et al. 1998; Rodriguez et al. 2000
FlpeER^{T2}	Tamoxifen-inducible version of Flpe, might perform similar to CreER fusions in mice (see Sect. 4)	Hunter et al. 2005
B) SSR systems with proven efficiency in cultured mammalian cells and potential utility in mice		
ΦC31/*att*	Biological function: DNA integration and excision of *Streptomyces* phage ΦC31	Thorpe and Smith 1998
	Potentially useful for stable integration of transgenes	Olivares et al. 2002; Belteki et al. 2003
ΦC31-NLS	A version with a C-terminal nuclear localization signal; displays enhanced efficiency	Andreas et al. 2002
β recombinase/*six*	Biological function: Resolution of plasmid oligomers in Gram-positive bacteria	Rojo and Alonso 1994
	Catalyzes exclusively intramolecular recombination like excision and inversion	Diaz et al. 1999
β-EGFP	Fusion with a C-terminal EGFP; facilitates recombinase detection	Servert et al. 2006
β-AR, β-EGFP-AR	Fusion with the androgen receptor LBD; inducible with mibolerone; also functional as a triple fusion with a central EGFP	Servert et al. 2006
Dre/*rox*	Cre-like recombinase encoded by the P1-related bacteriophage D6	Sauer and McDermott 2004

AR, angrogen receptor; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; LBD, ligand-binding domain; NLS, nuclear localization signal

Feil R (2007) Handb Exp Pharmacol 178:3

Transcriptional transactivation
vs.
Site-specific DNA recombination

<u>Transcriptional transactivation</u>

1) Can be regulated exogenously with an inducer.
2) Reversibility makes it ideal for activation studies (gain of function).
3) Sensitivity of transactivation levels to inducer concentration.

<u>Site-specific DNA recombination</u>

1) Can be regulated exogenously, but many Cre lines do not use such a regulatable Cre variant.
2) Irreversibility makes it ideal for tissue-specific knockouts (loss of function).
3) Irreversibility makes it ideal for cell lineage studies.

International Mouse Knockout Project

The Mouse Knockout Project.
Austin et al (2007) Nature Genetics 36:921

A Mouse for all Reasons.
The International Mouse Consortium (2007) Cell 128:9

http://www.knockoutmouse.org/

http://www.komp.org/

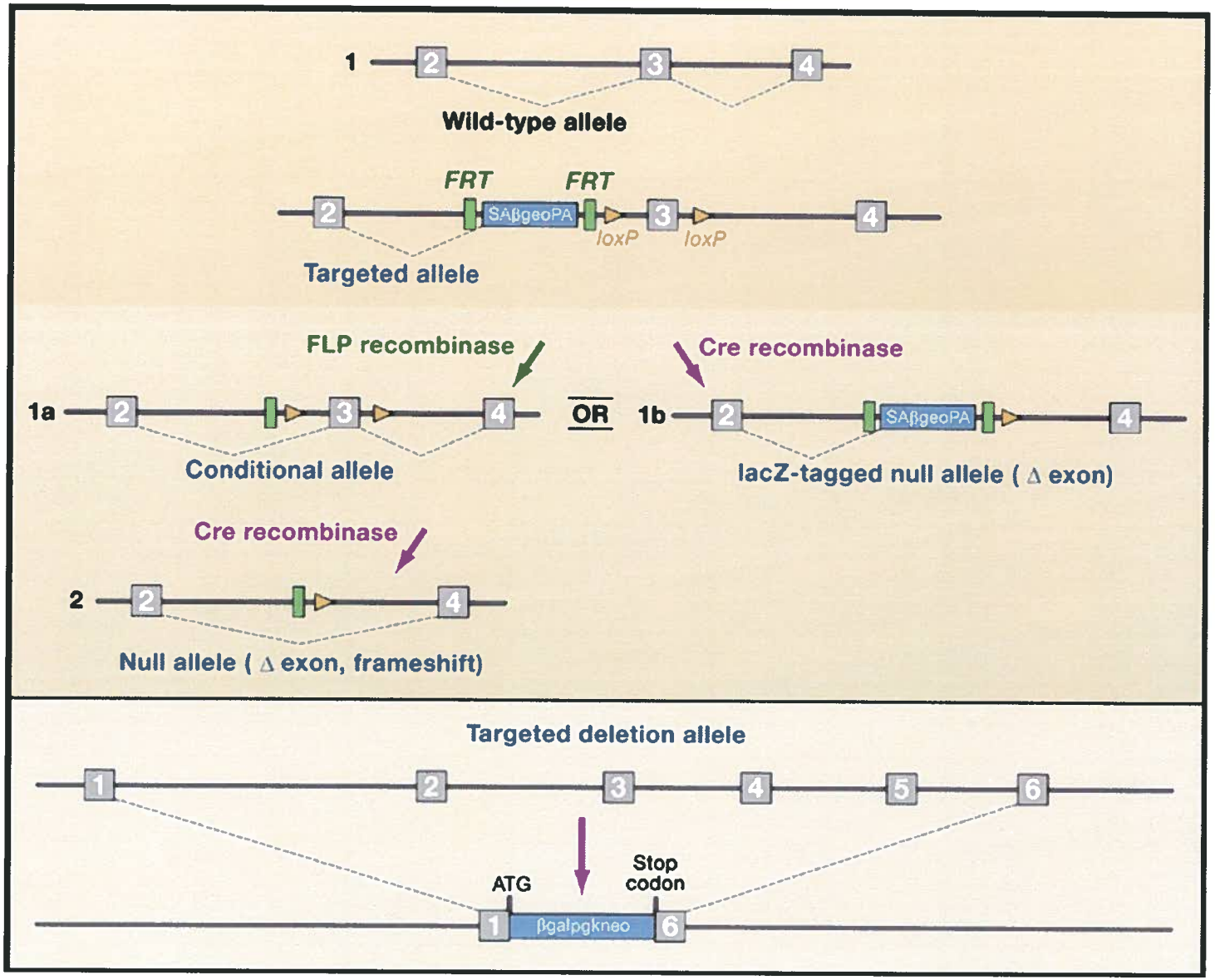
http://www.informatics.jax.org/external/ko/

http://www.mmrrc.org/

http://www.eucomm.org/

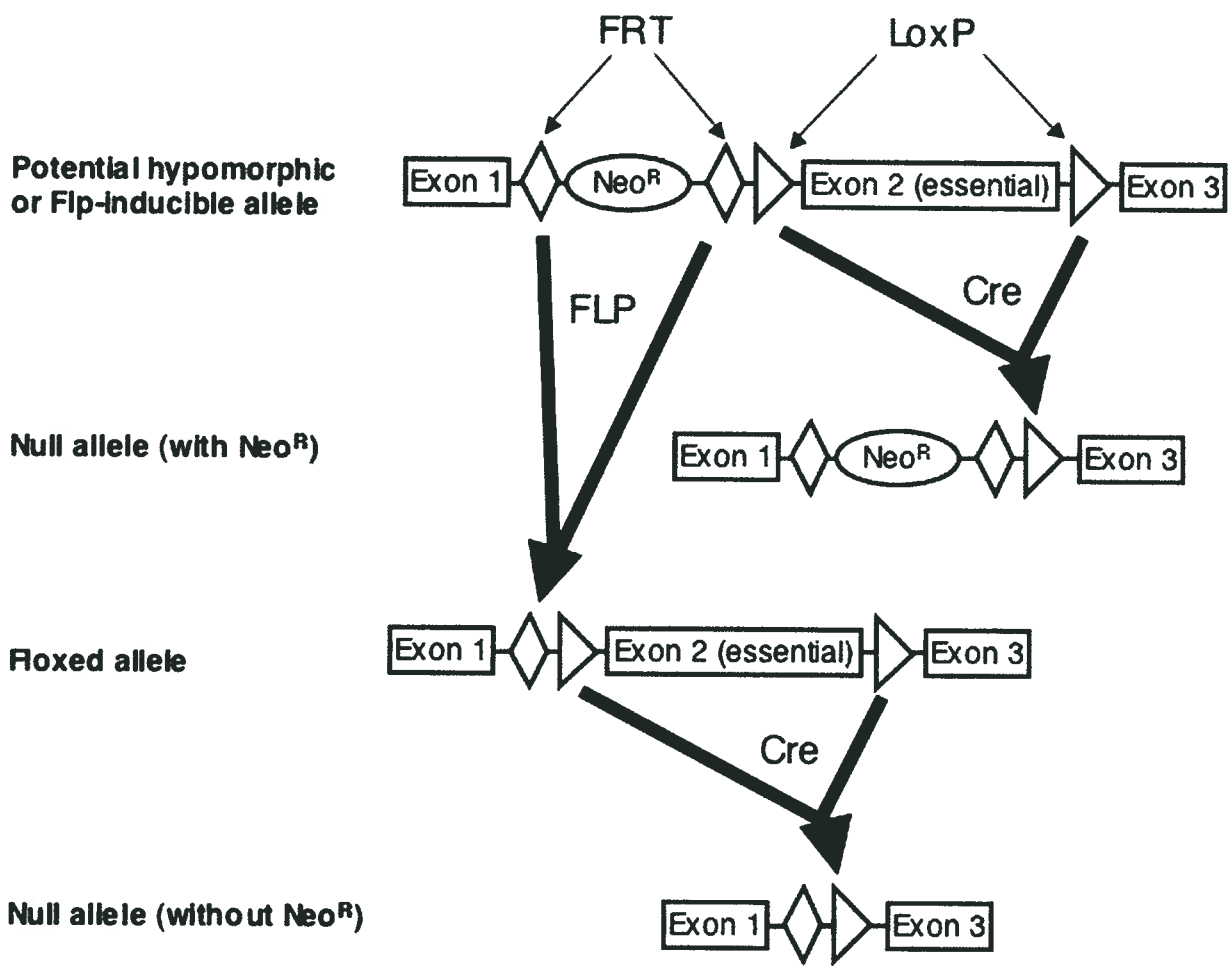
http://norcomm.phenogenomics.ca/index.htm

International Mouse Knockout Project



The International Mouse Consortium (2007) Cell 128:9

Allelogenic strategy

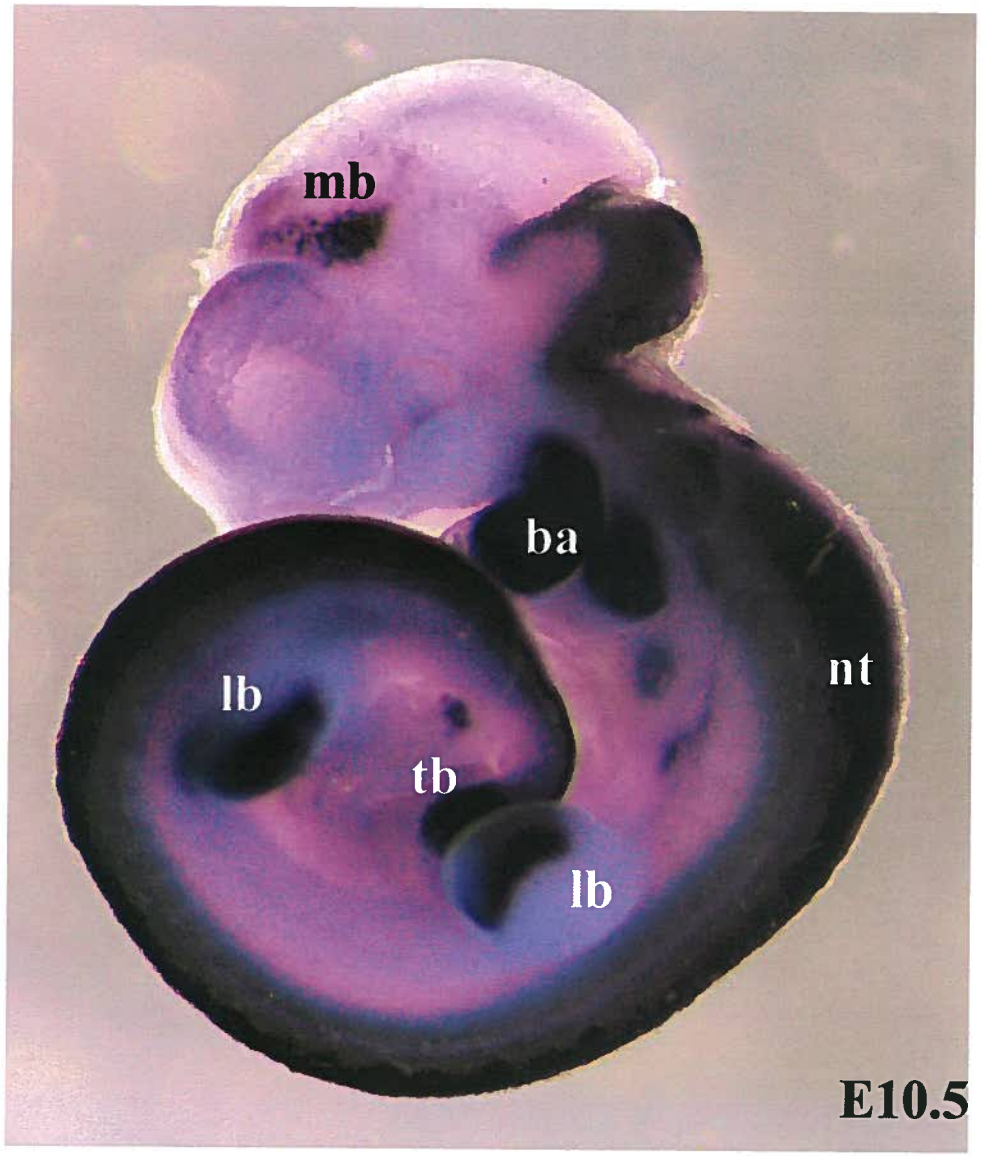


Lewandoski, M. (2007) Handb Exp Pharmacol 178:235

Gbx2

A story about how some of us learned that
the neo cassette can cause
a loss of function (hypomorph) allele.

Gbx2 is expressed in a complex pattern during embryogenesis



Site-specific recombination: Cre/FLP

a

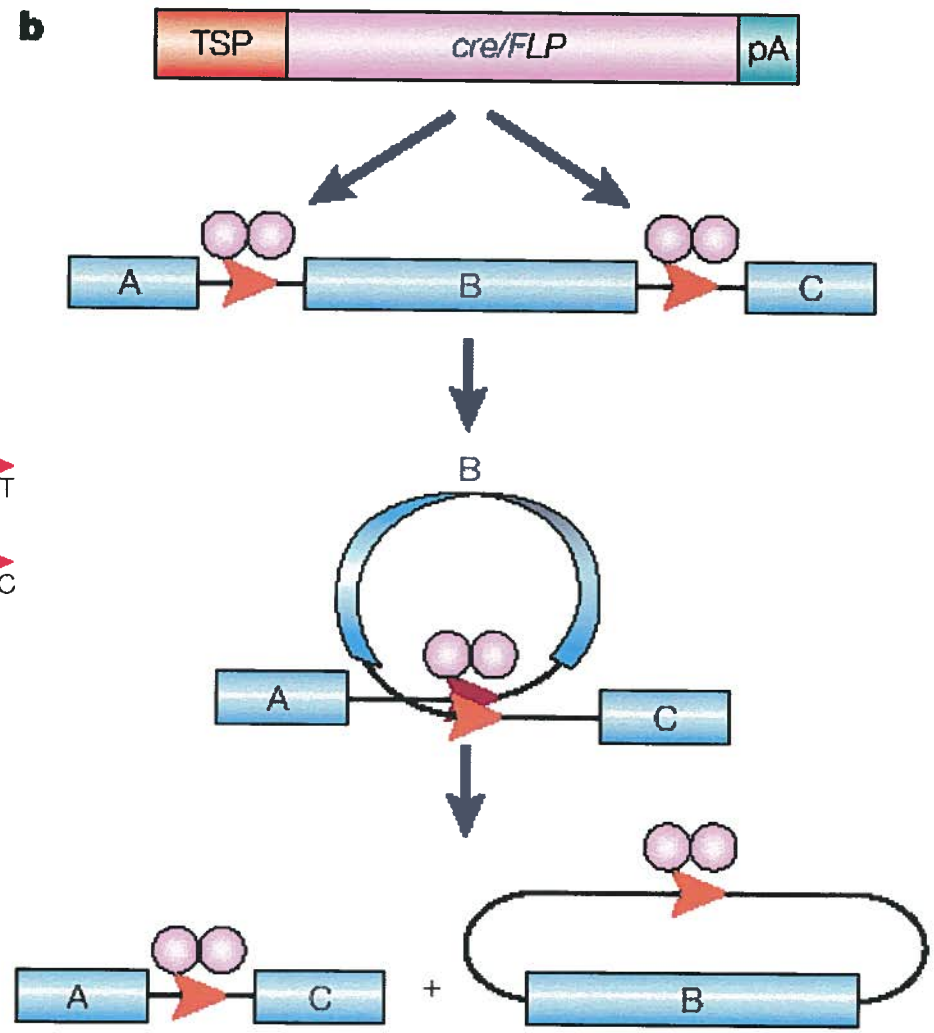
loxP

ATAACTTCGTATAATGTATGCTATACGAAGTTAT

FRT

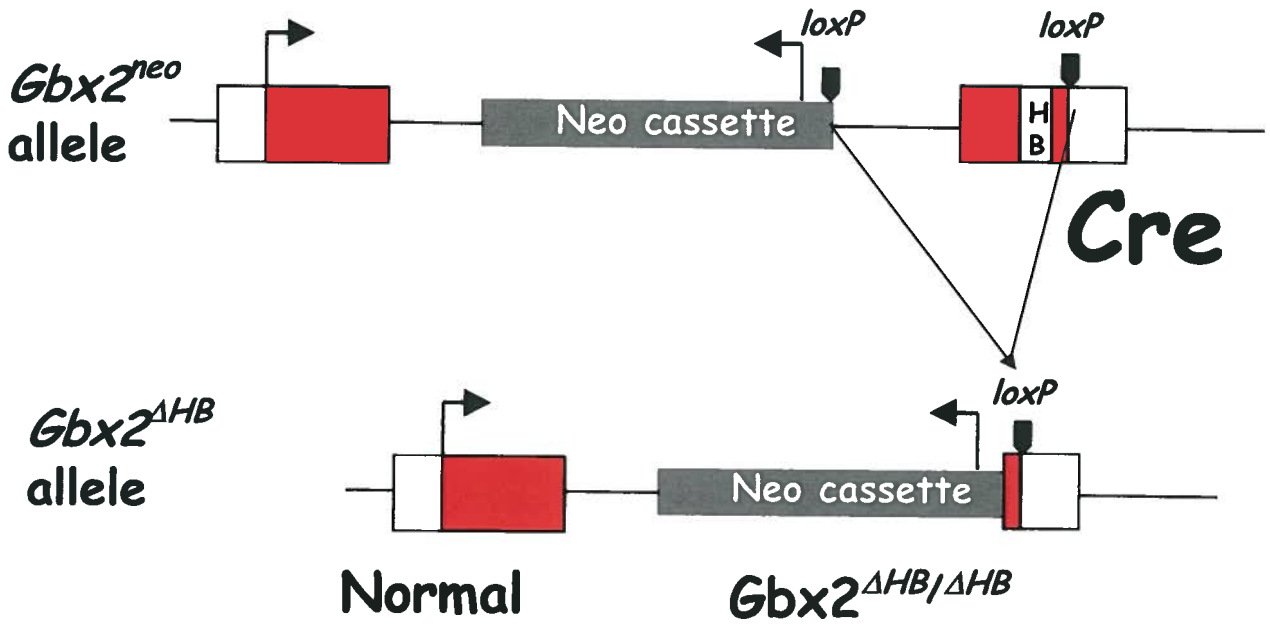
GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

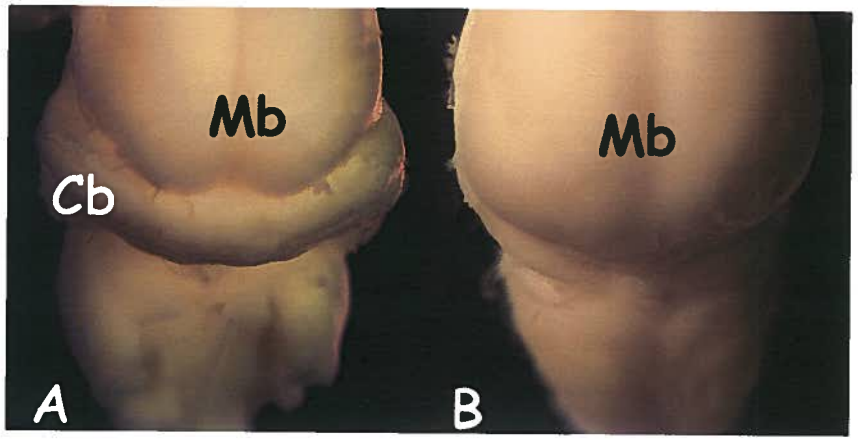
b



Lewandoski, M. (2001) Nature Reviews Genetic 2:743

Gbx2 null homozygotes lack the cerebellum





Wasserman et al (1997) Development 124:2923.

A short summary of Cre and Flp deleters

Cre

Cold Spring Harb Symp Quant Biol (1997) **62** 159-68
Biochem Biophys Res Commun (1997) **237** 318-24
Dev Biol (1999) **212** 307-22
Transgenic Res (1998) **7** 105-12
Transgenic Res. (1999) **8** 53-4.

Flp

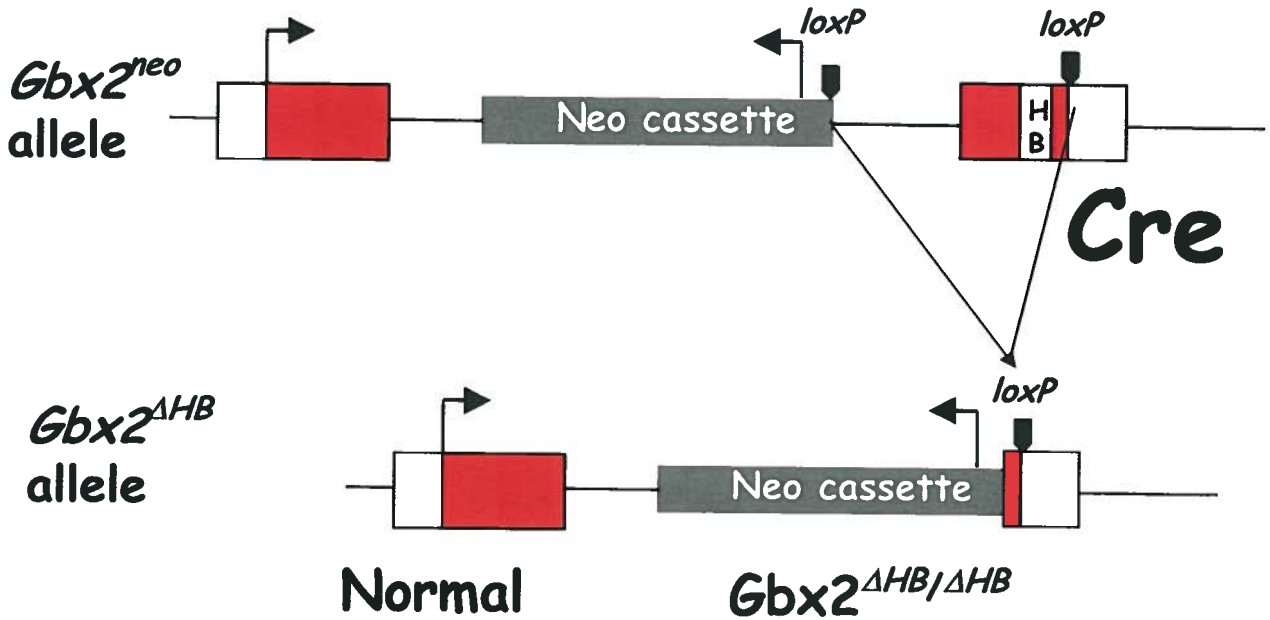
β-actin Flp results in mosaic recombination:
Proc Natl Acad Sci U S A (1996) 93 6191-6

Presumably because Flp is thermolabile at the body temperature of mice:
Nucleic Acids Res (1996) **24** 4256-62

But a thermoresistent Flp, Flpe, was made and...
Nat Biotechnol (1998) **16** 657-62

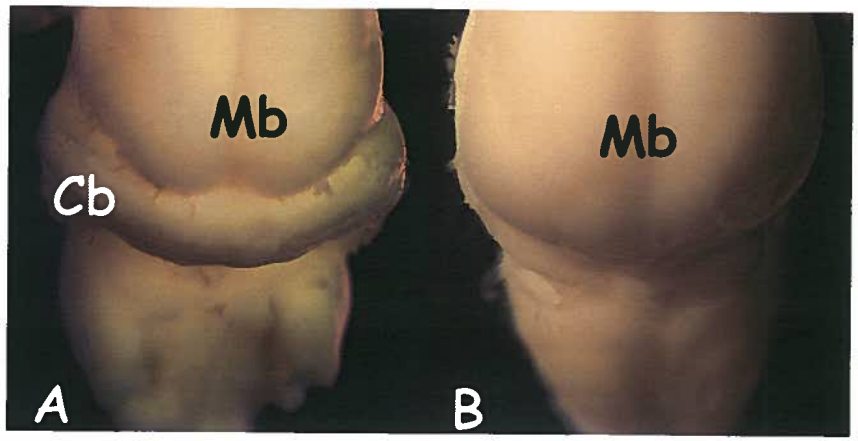
...and β-actin Flpe appears to work as well as Cre:
Nat Genet (2000) **25** 139-40

Gbx2 null homozygotes lack the cerebellum



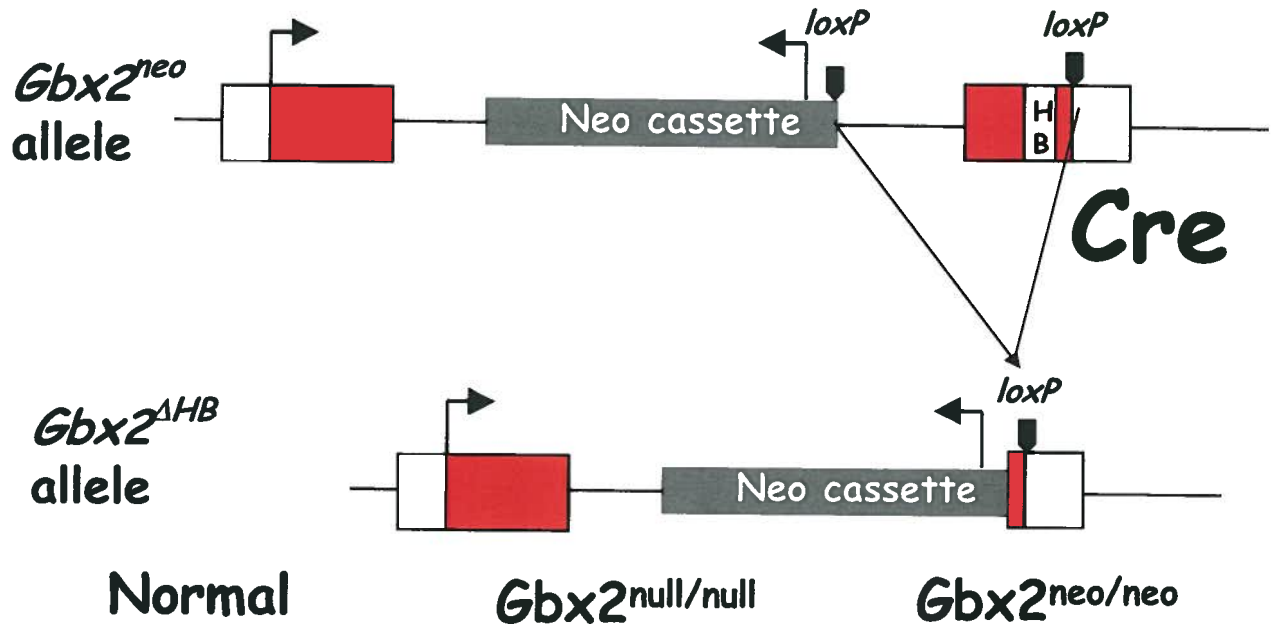
Normal

Gbx2ΔHB/ΔHB

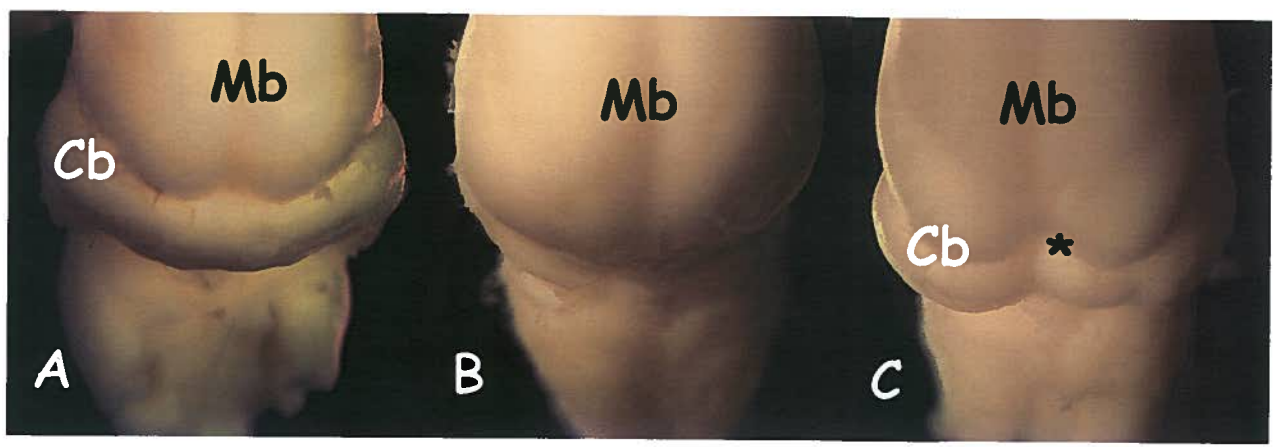


Development (1997) 124:2923.

Gbx2^{neo} allele is a hypomorphic allele

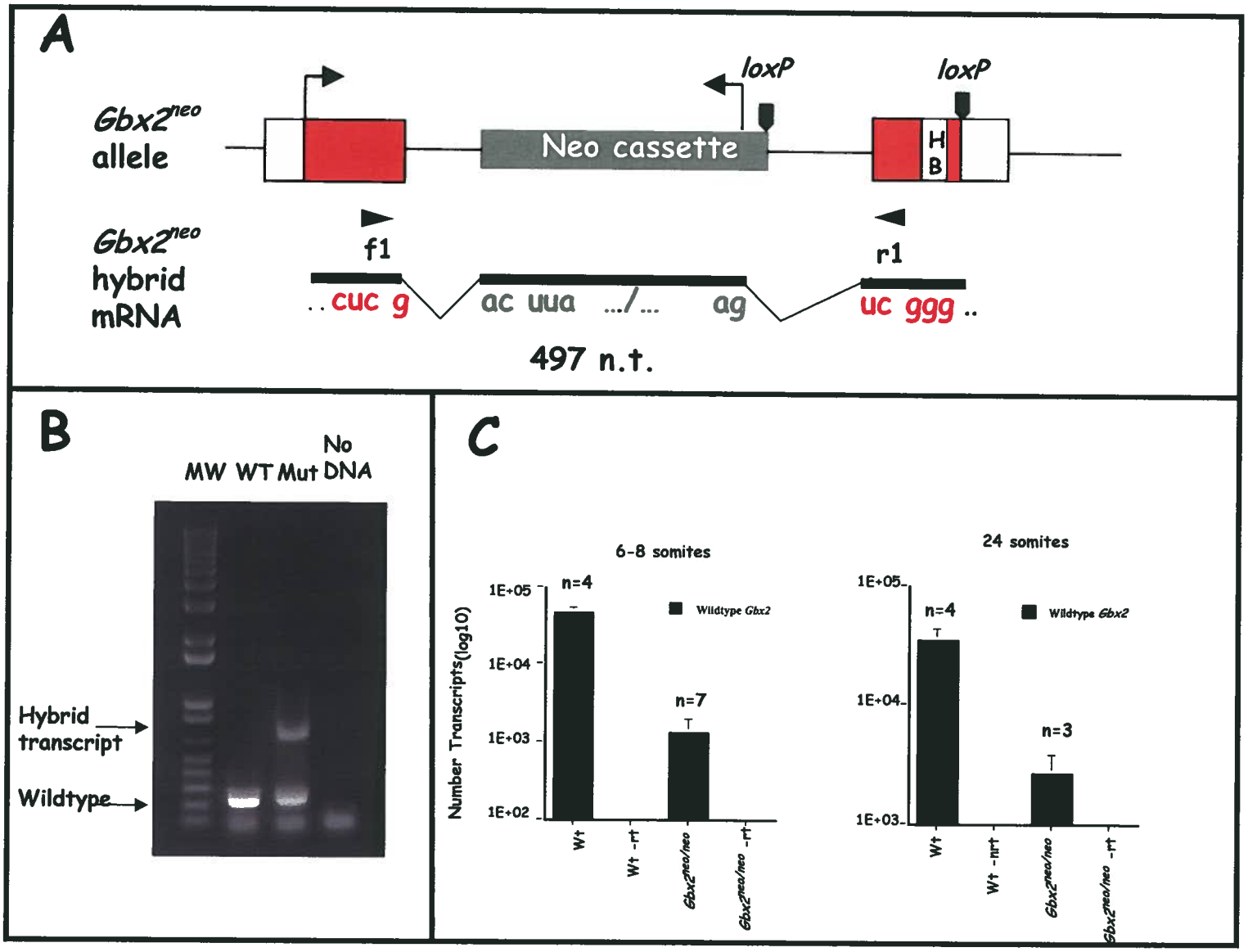


Normal | Gbx2$^{null/null}$ | Gbx2$^{neo/neo}$



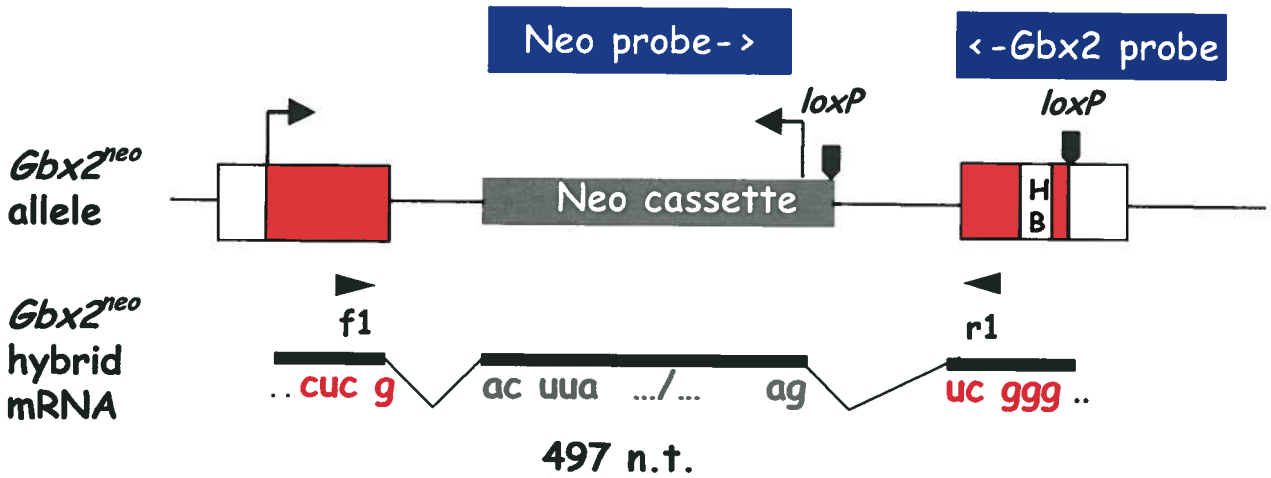
Waters and Lewandoski (2006) Development 133:1991

Gbx2^{neo} is a hypomorphic allele because neo interferes with splicing

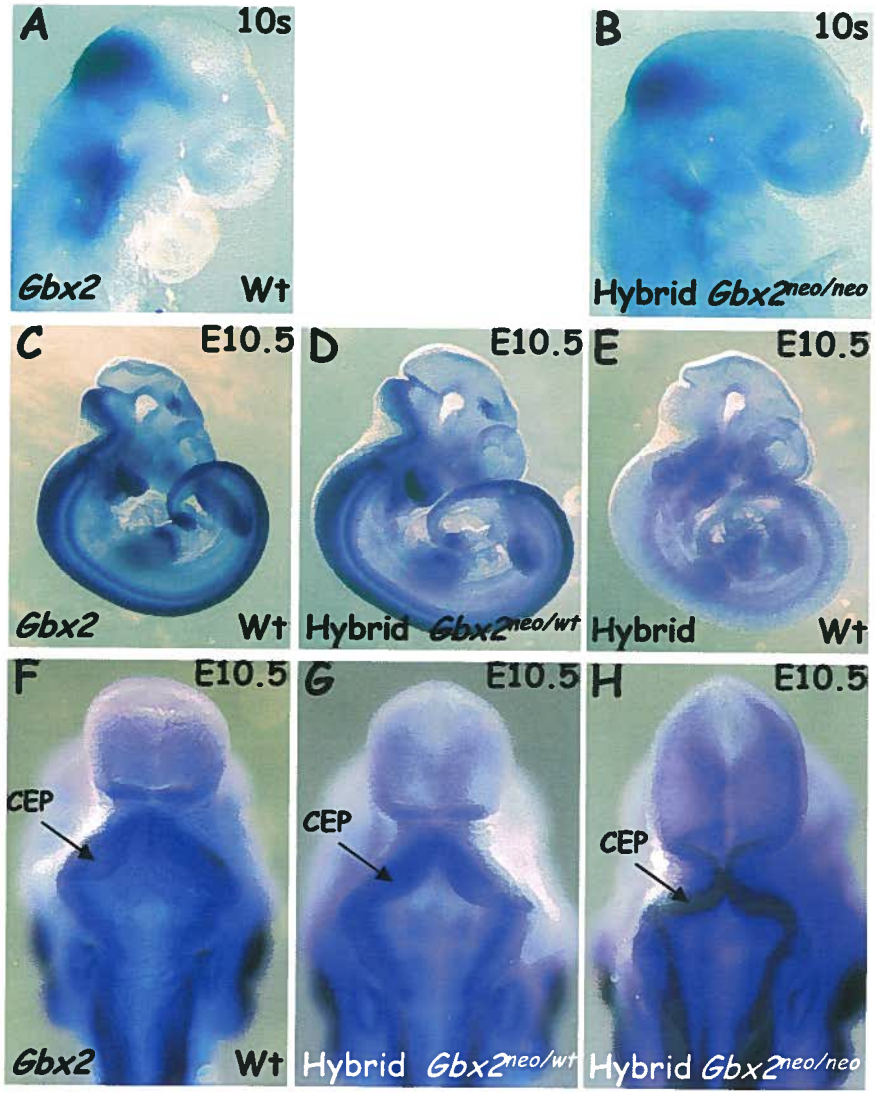


Waters and Lewandoski (2006) Development 133:1991

Because of the orientation of neo in this case, one can make Gbx2- and neo-specific single stranded riboprobes



The Gbx2 hybrid transcript recapitulates wildtype *Gbx2* expression during embryogenesis

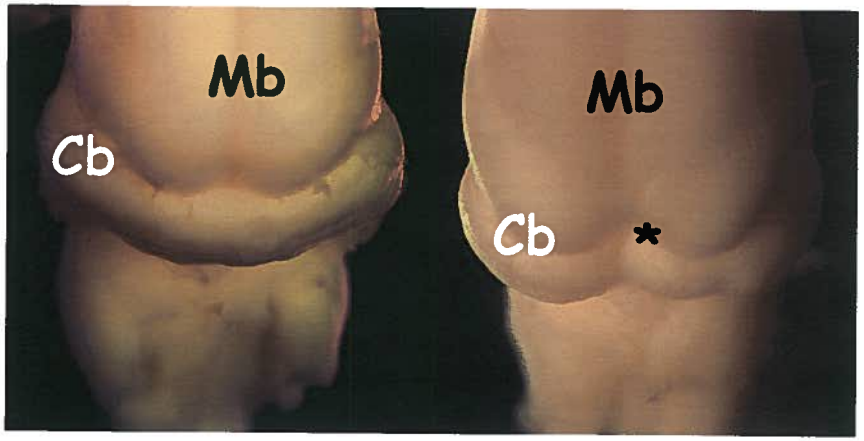


Waters and Lewandoski (2006) Development 133:1991

But if neo causes a hypomorph this may interfere with conditional KOs.

Normal

Gbx2$^{neo/neo}$



$$\frac{\text{MidlineCre}}{+} \quad \frac{\text{Gbx2}^{neo}}{\text{Gbx2}^{neo}}$$

vs

$$\frac{\text{Gbx2}^{neo}}{\text{Gbx2}^{neo}}$$

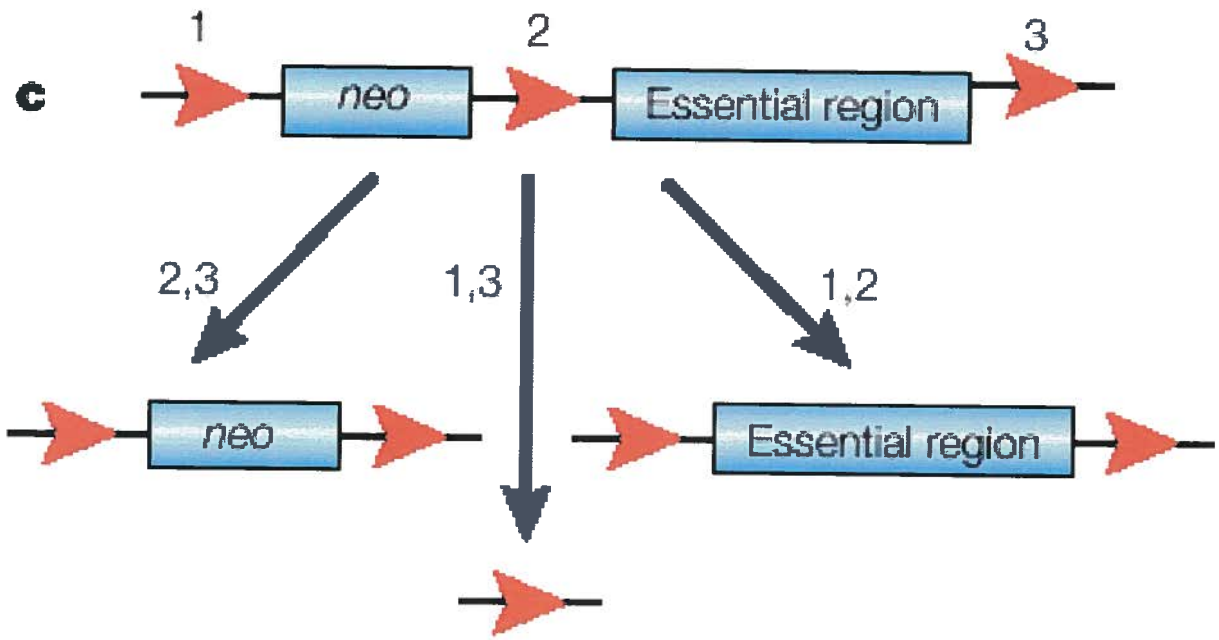
The selection cassette (e.g.neo)
may cause a loss-of function
allele such as a hypomorph...

And thus may interfere with conditional KOs:

But hypomorhs also call tell you
something unique about your system.

So: eat your cake and have it too.

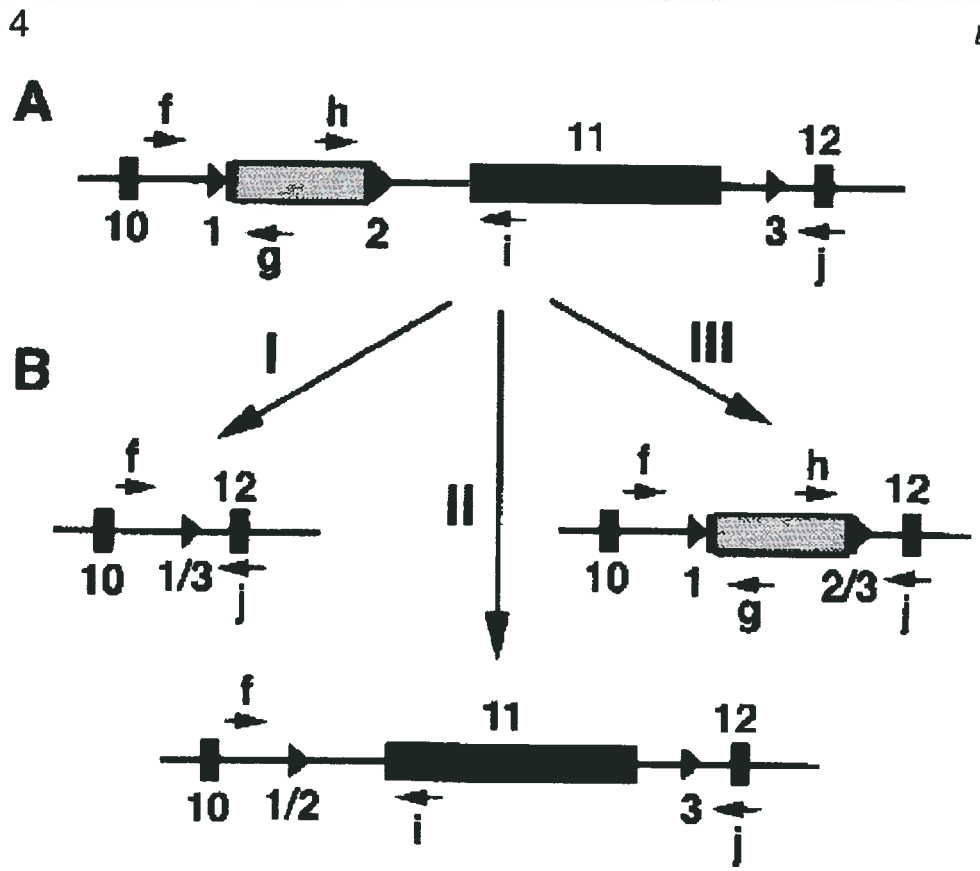
Removal of neo *in vivo* (strategy with 3 loxP sites)



Useful mice and strategy described in
Holzenberger et al (2000) Nucleic Acids Res 28:E92.
Xu et al (2001) Genesis 30:1-6

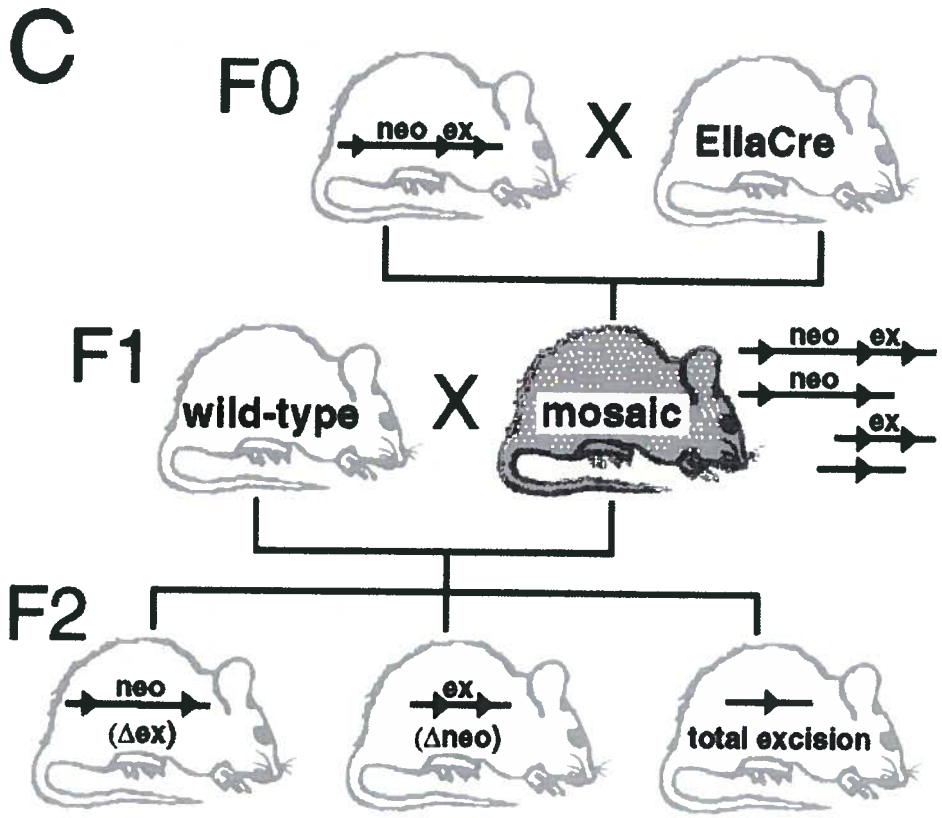
Lewandoski, M. (2001) Nature Reviews Genetic 2:743

Removal of neo *in vivo* (strategy with 3 loxP sites)



Xu et al (2001) Direct Removal in the Mouse of a Floxed Neo Gene From a Three-loxP Conditional Knockout Allele by Two Novel Approaches. Genesis 30:1-6

Removal of neo *in vivo* (strategy with 3 loxP sites)



Holzenberger et al (2000) Cre-mediated germline mosaicism: a method allowing rapid generation of several alleles of a target gene. Nucleic Acids Res 28:E92.

Site-specific recombination: Cre/FLP

a

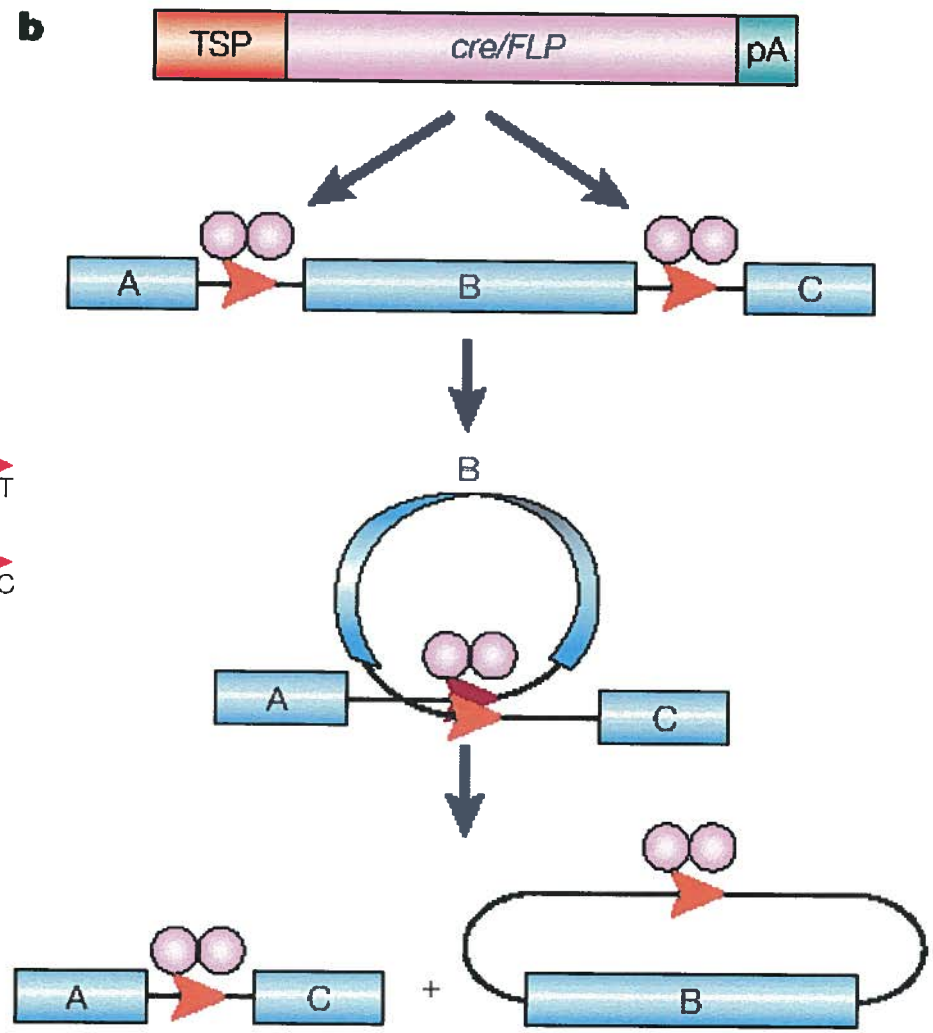
loxP

ATAACTTCGTATAATGTATGCTATACGAAGTTAT

FRT

GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

b

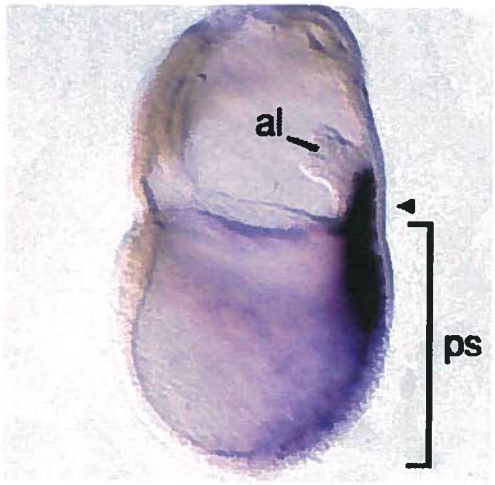


Lewandoski, M. (2001) Nature Reviews Genetic 2:743

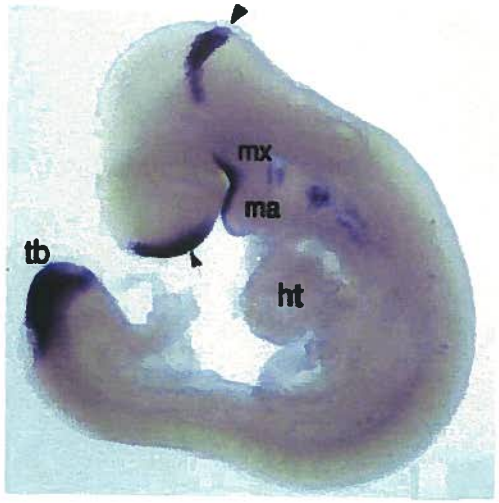
Allelogenic strategy: generating multiple alleles from one targeting event

Fgf8 is expressed in a dynamic pattern during embryogenesis

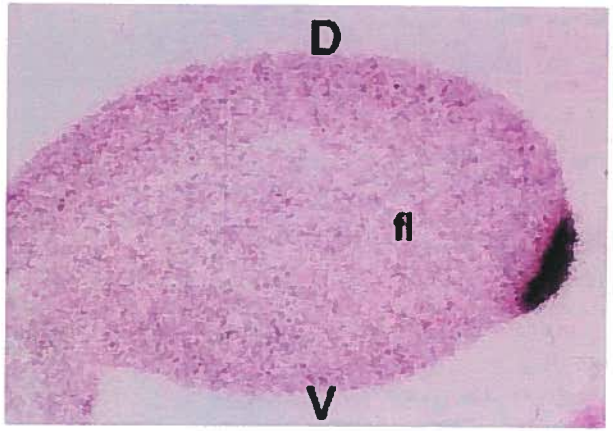
gastrulation

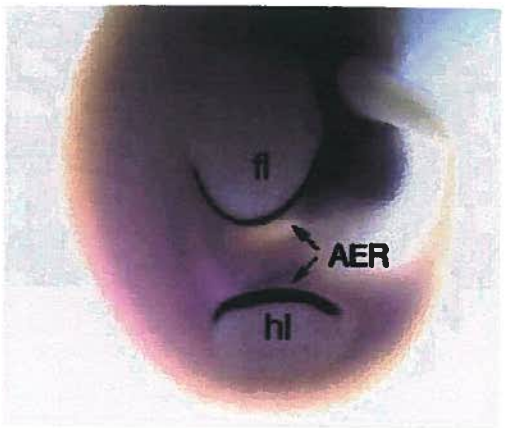


brain

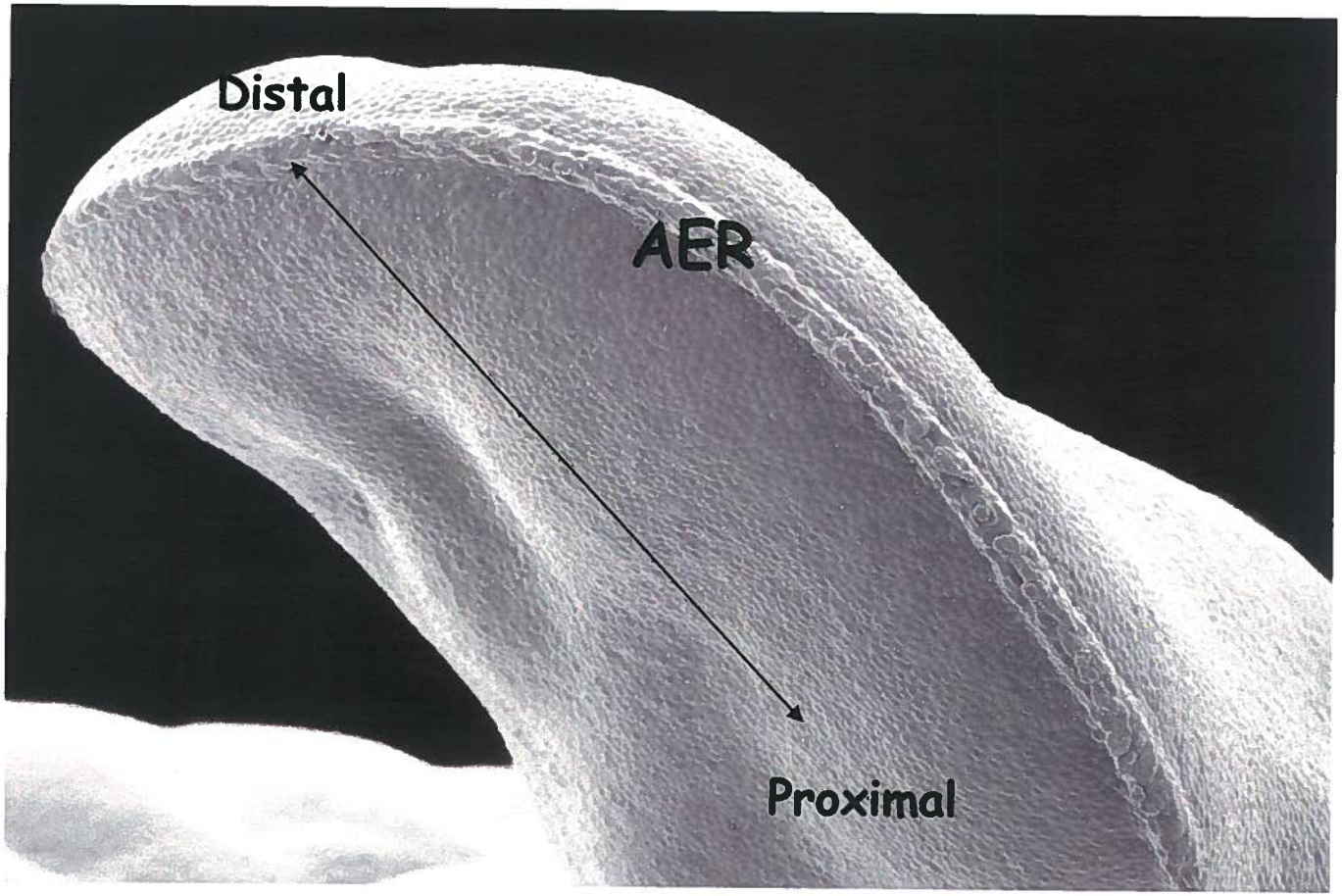


limb

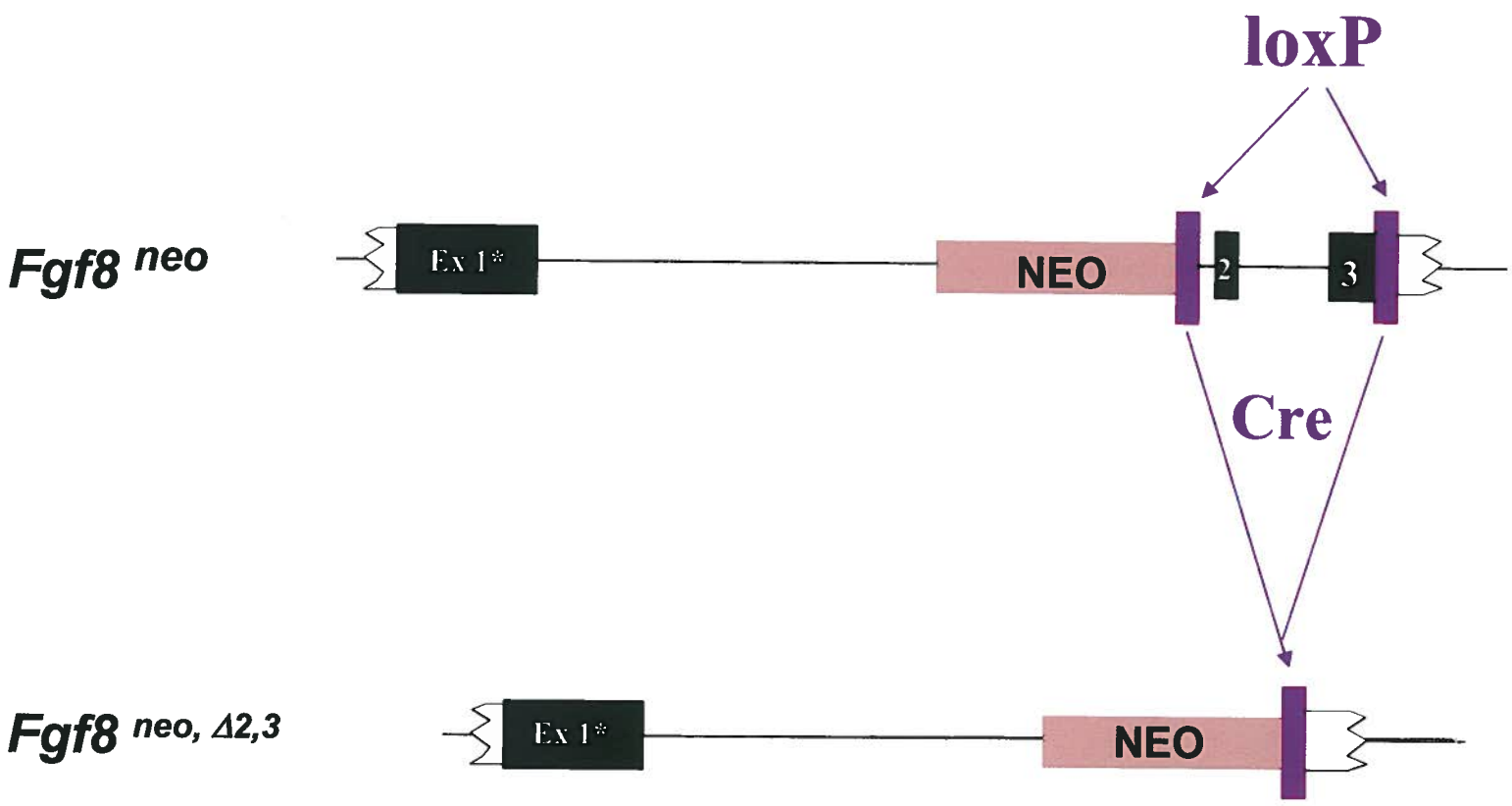




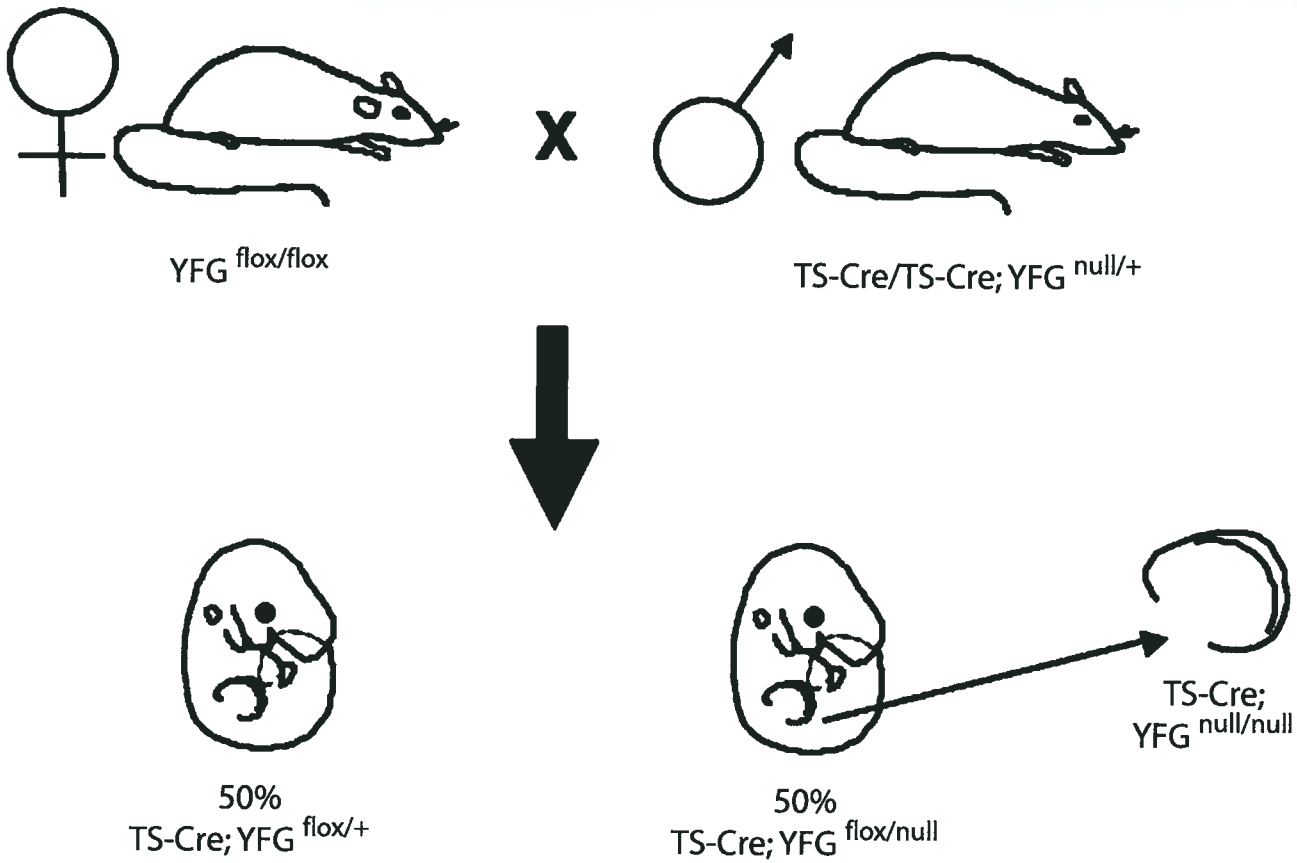
The AER is required for proximal-distal outgrowth



Flox an essential region of *Fgf8* (exons 2 and 3)



Best cross for generation of conditional mutants

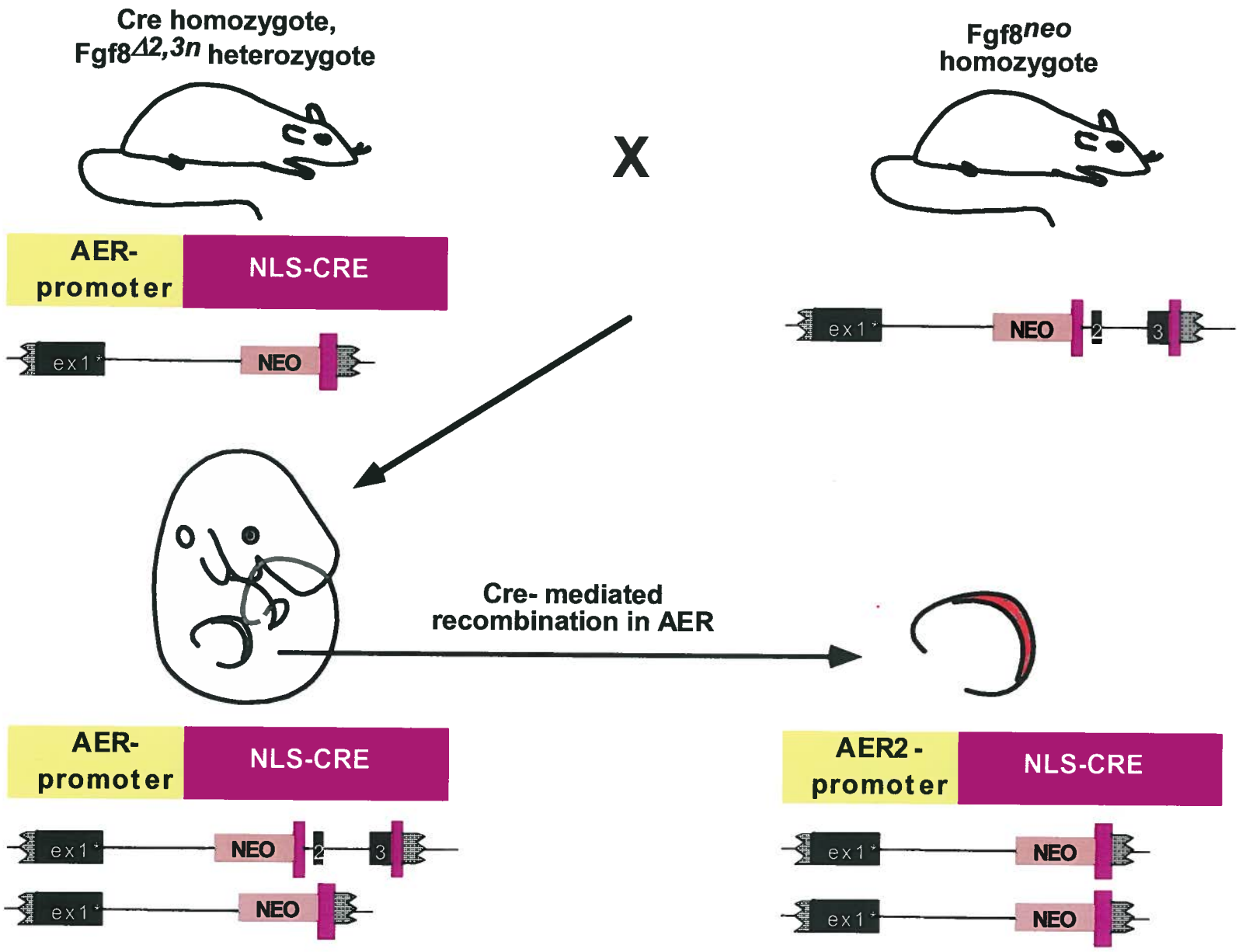


Lewandoski, M. (2007) Handb Exp Pharmacol 178:235

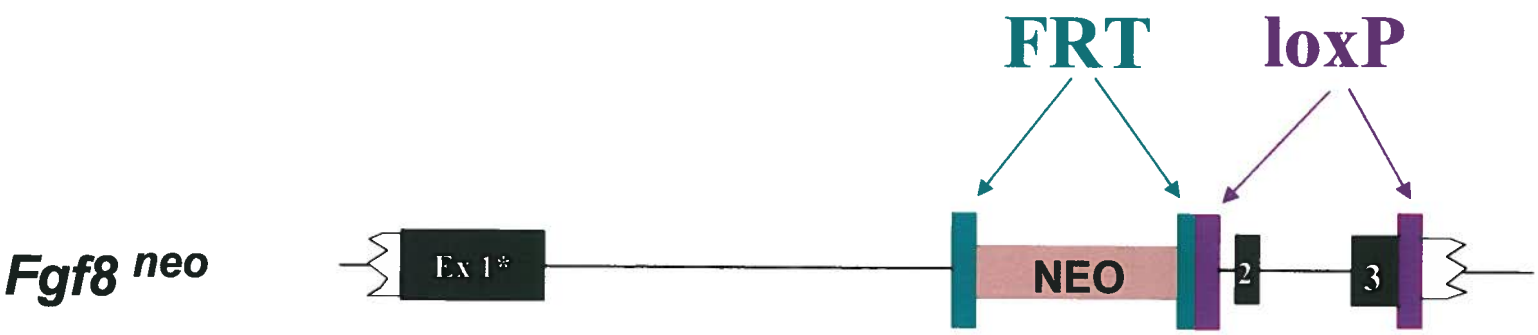
1) Simplest mouse husbandry with 50% mutants and controls.
2) Cre parent is male; avoids complications due to potential germline activity in the Cre+ female (more on this later).

"TS" = Tissue-specific; "YGF" = Your favorite gene.

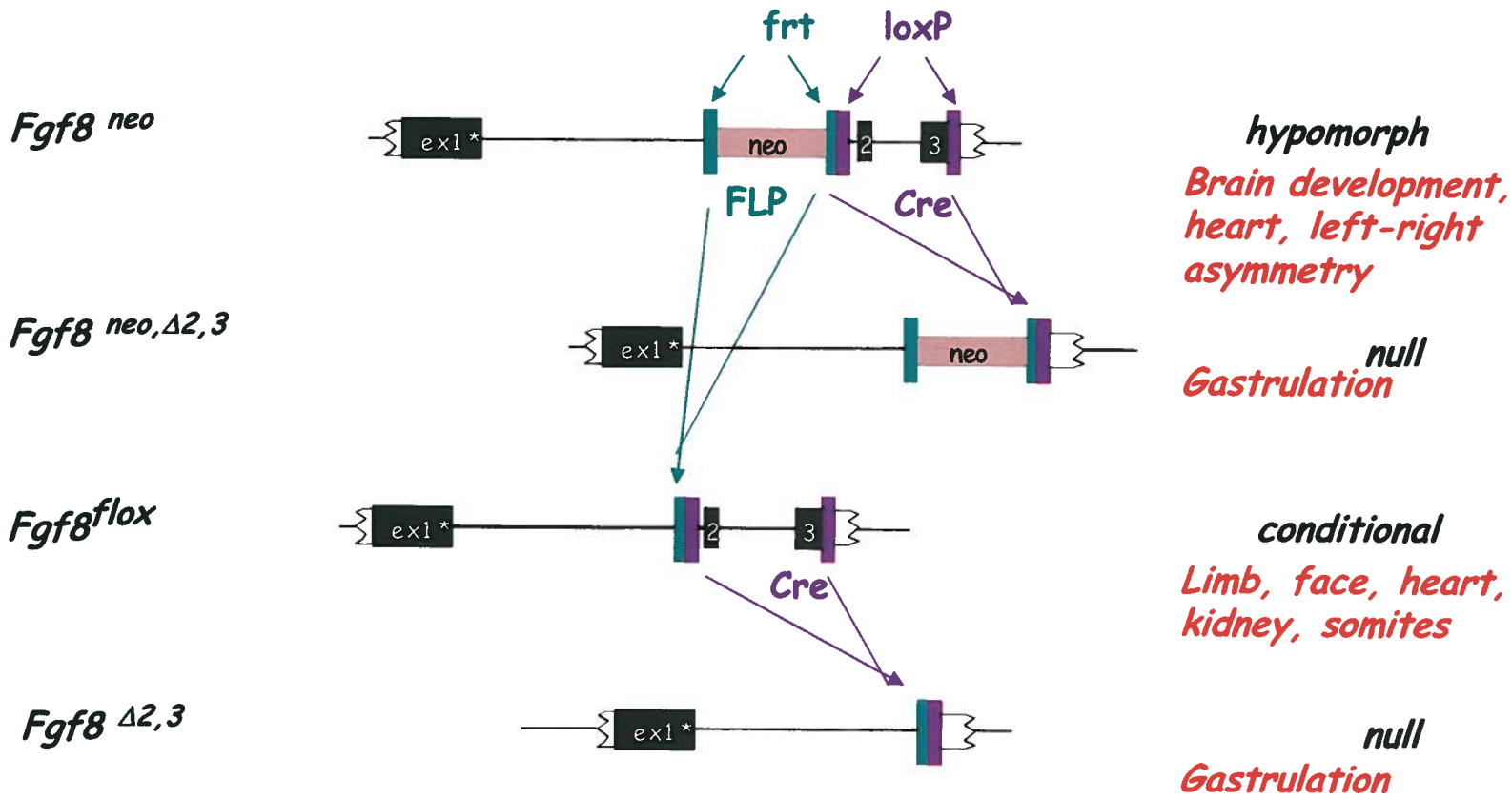
AER-□specific inactivation of *Fgf8*



Flox an essential region of Fgf8 (exons 2 and 3) and "Flrt" neo



Allelogenic Strategy:
An allelic series generated from one targeted mouse line



The neo casette can interfere with expression in any orientation or location

Table 3 | **Loss of target-gene function due to *neo*^{R} cassette**

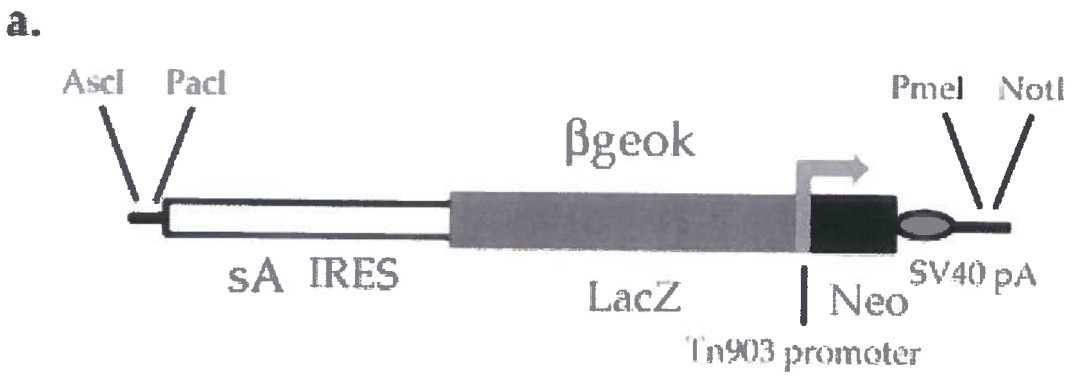
Floxed gene	Location and orientation* of *neo*^{R}	Reference
Fgf8	Intron; same orientation	57
N-Myc	Intron; same orientation	58
Fgfr1	Intron; same orientation	61
Gck	Intron; same orientation	55
Pitx2	Intron; same orientation	62
Pck1	Intron; same orientation	147
Krt1-14	Intron; same orientation	90
Krt1-10	Intron; same orientation	89
Fgfr3	Intron; opposite orientation	148
Hnf4α	Intron; opposite orientation	149
Brca1	Intron; opposite orientation	59
Slc2a4	3' UTR	150
Fgf8	3' UTR	67
Nodal	3' UTR	151
Scap	5' UTR	152
Bcl2l	5' promoter region	153

*Orientation of *neo*^{R} cassette relative to transcription of floxed gene. (*Bcl2l*, B-cell lymphoma; *Brca1*, breast cancer gene 1; *Fgf8*, fibroblast growth factor 8; *Fgfr3*, fibroblast growth factor receptor 3; *Gck*, glucokinase; *Hnf4α*, hepatocyte nuclear factor 4, alpha; *Krt1-x*, keratin complex 1, gene x; *Pck1*, phosphoenolpyruvate carboxykinase; *N-Myc*, neuroblastoma myc-related oncogene; *Pitx2*, pituitary homeobox 2; *ScapP*, Srebp cleavage-activating protein; *Slc2a4*, solute carrier family 2, member 4 (also called *Glut4*); Srebp, sterol regulatory element-binding protein; UTR, untranslated region.)

Lewandoski, M. (2001) Nat Rev Genetics 2:743

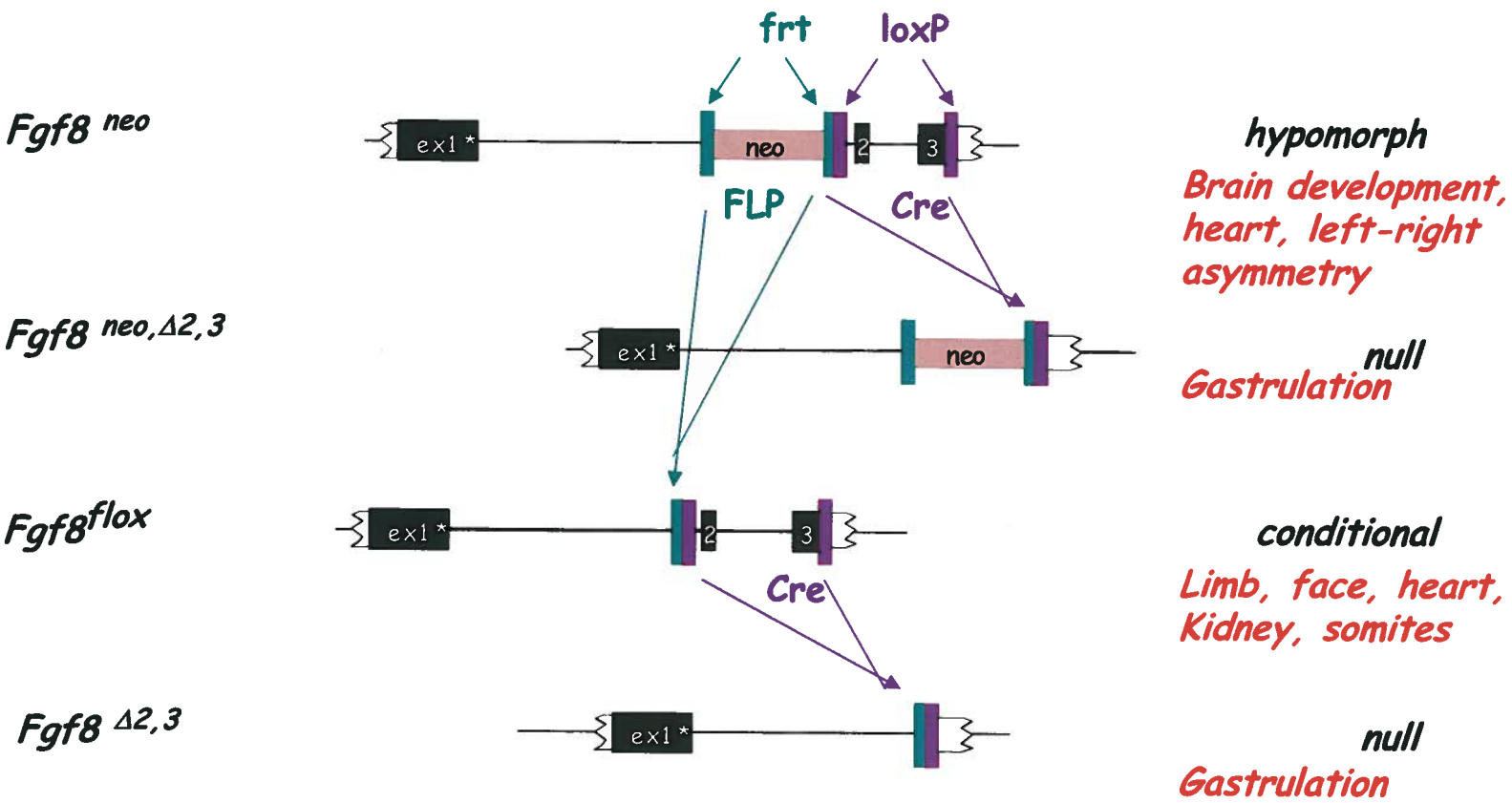
Will the selection cassette (e.g. neo) always interfere with gene expression?

No…but cassettes exist to ensure that it will:

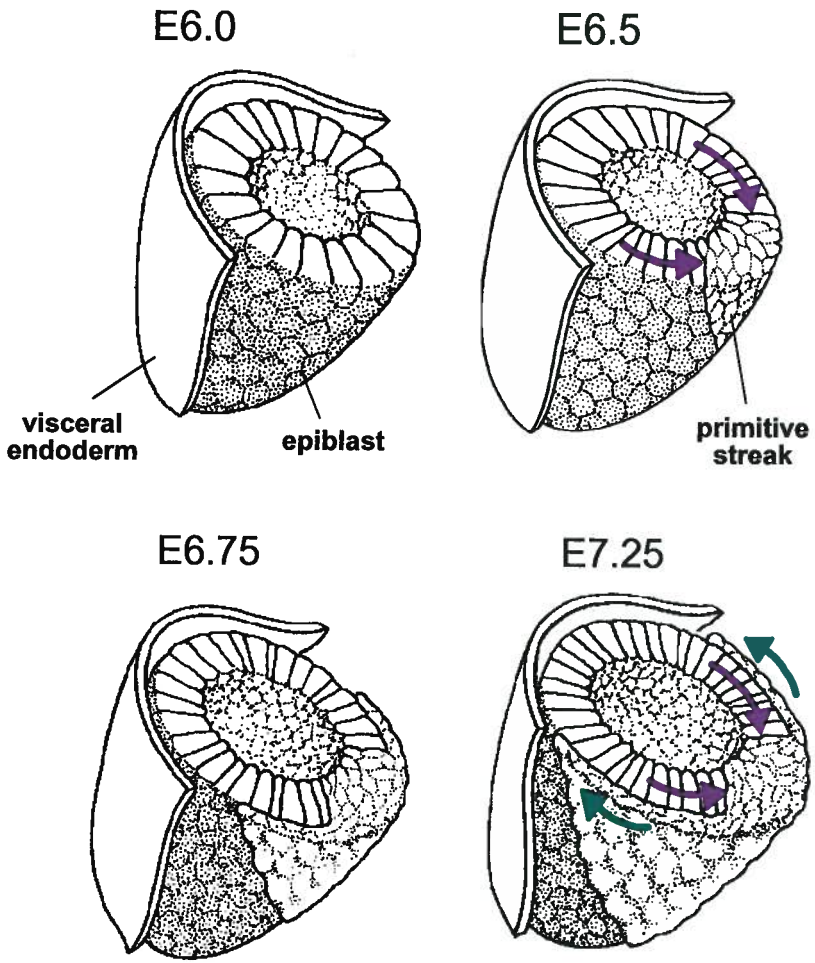


A Reliable lacZ Expression Reporter Cassette for Multipurpose, Knockout-First Allele (2004) Testa et al Genesis 38: 151

Let's examine some of these phenotypes in more detail

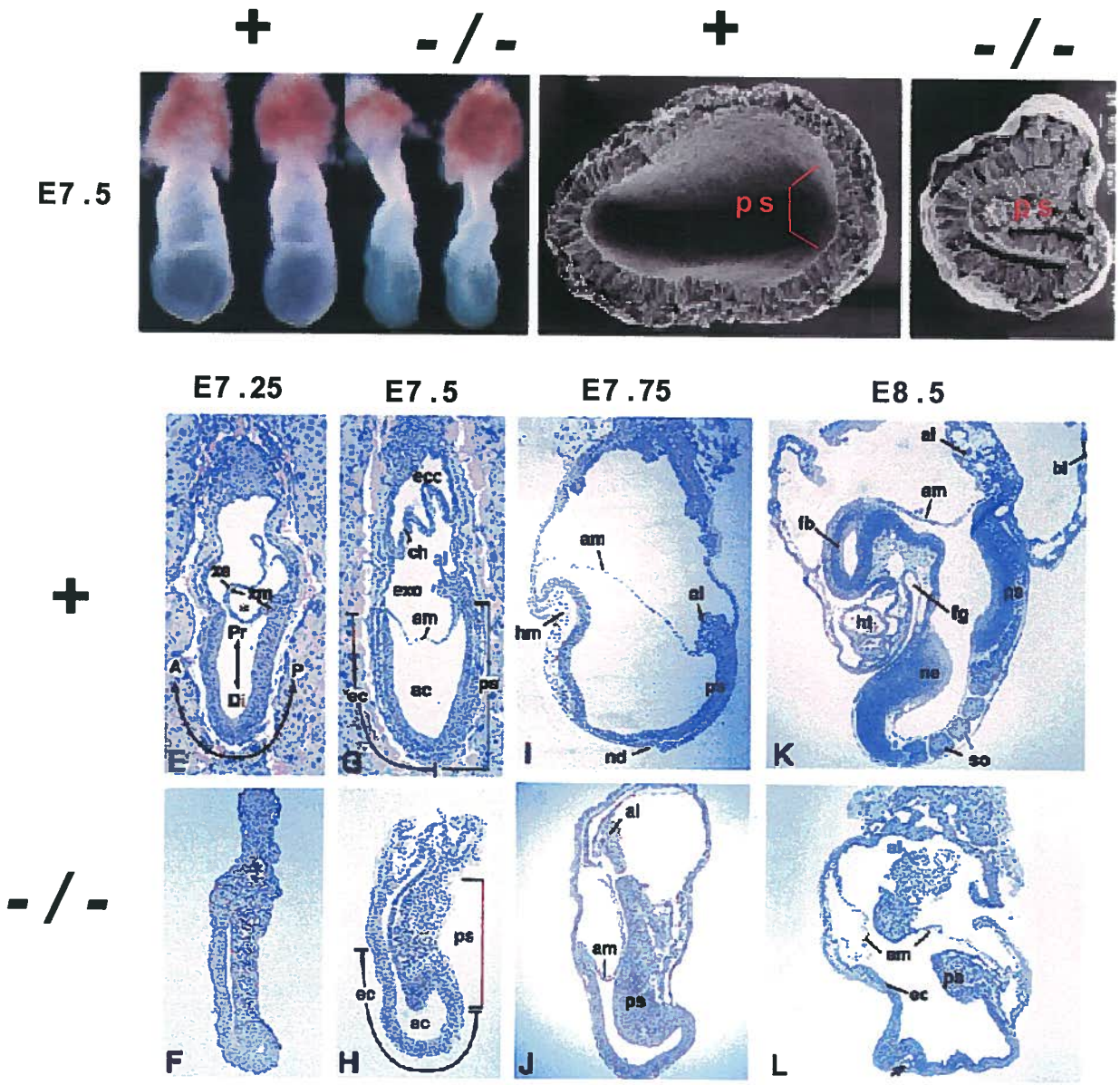


What is gastrulation?



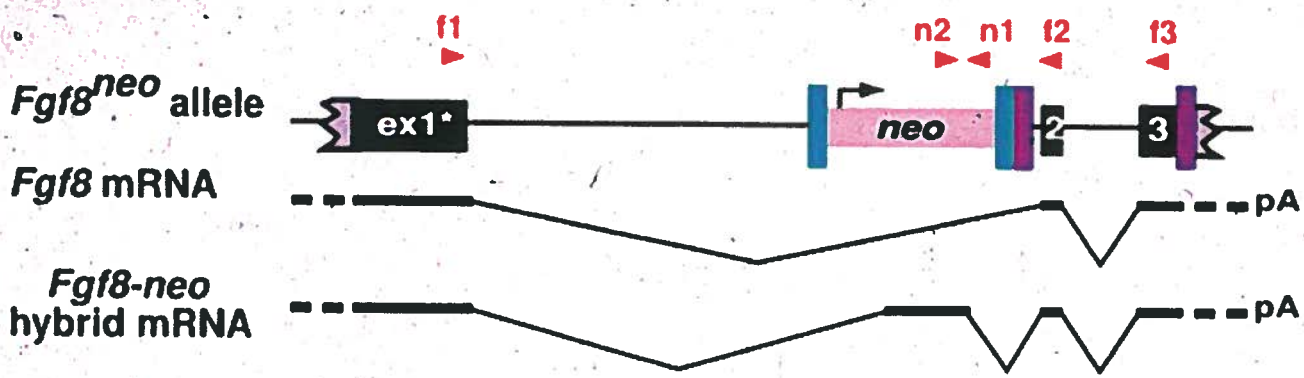
1) Mesoderm induction
2) Cell migration
3) Axis determination

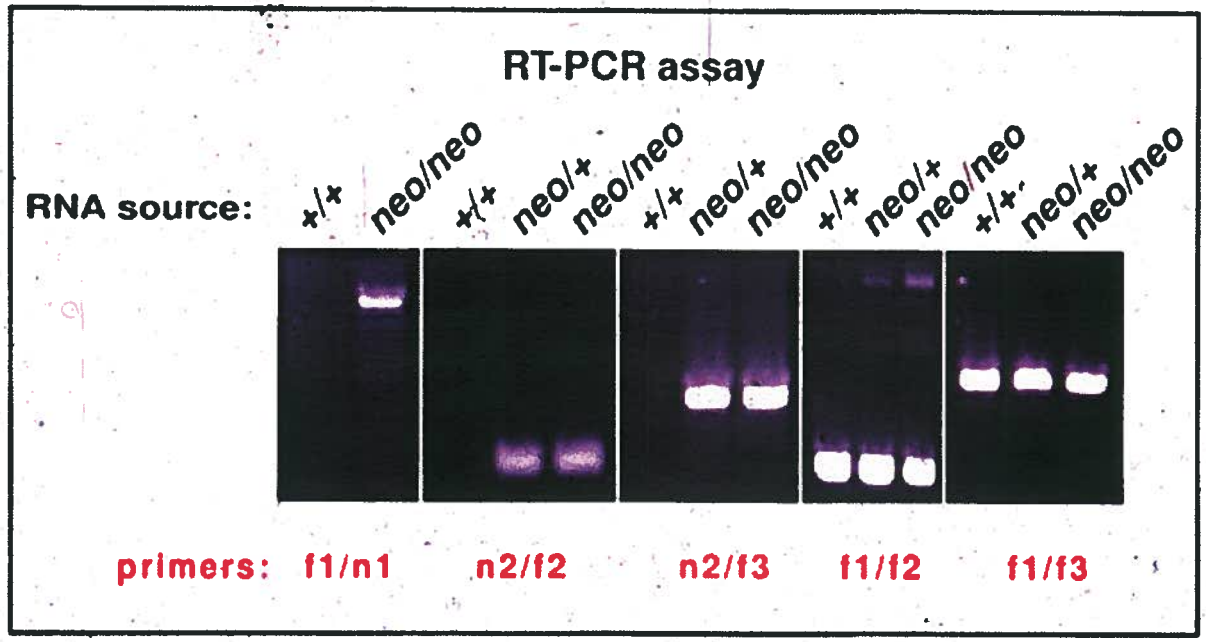
Fgf8 null embryos reveal a role for FGF8 in cell migration



Sun et al (1999) Genes & Development 13:1834

The neo cassette interferes with splicing in the *Fgf8* hypomorph allele

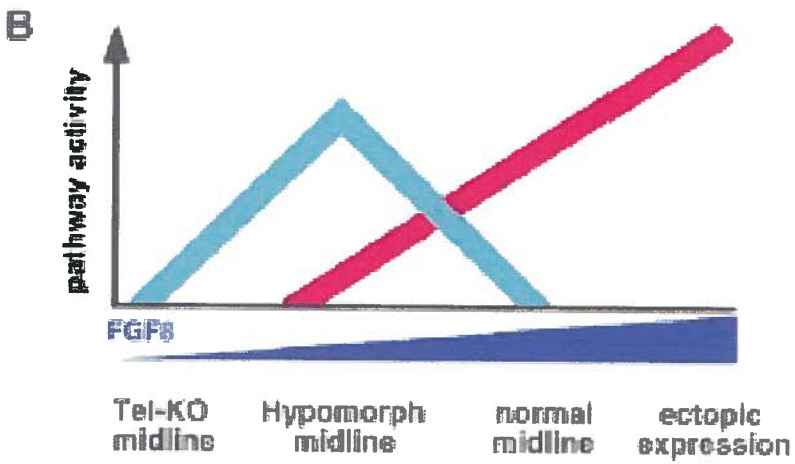




Meyers et al (1998) Nature Genetics 18:136

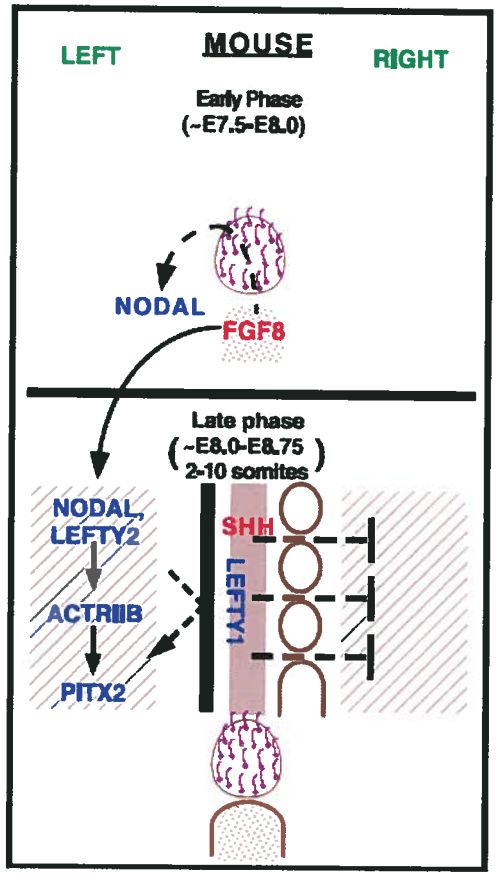
Fgf8 hypomorhic allele has been important in studies of…

…brain development



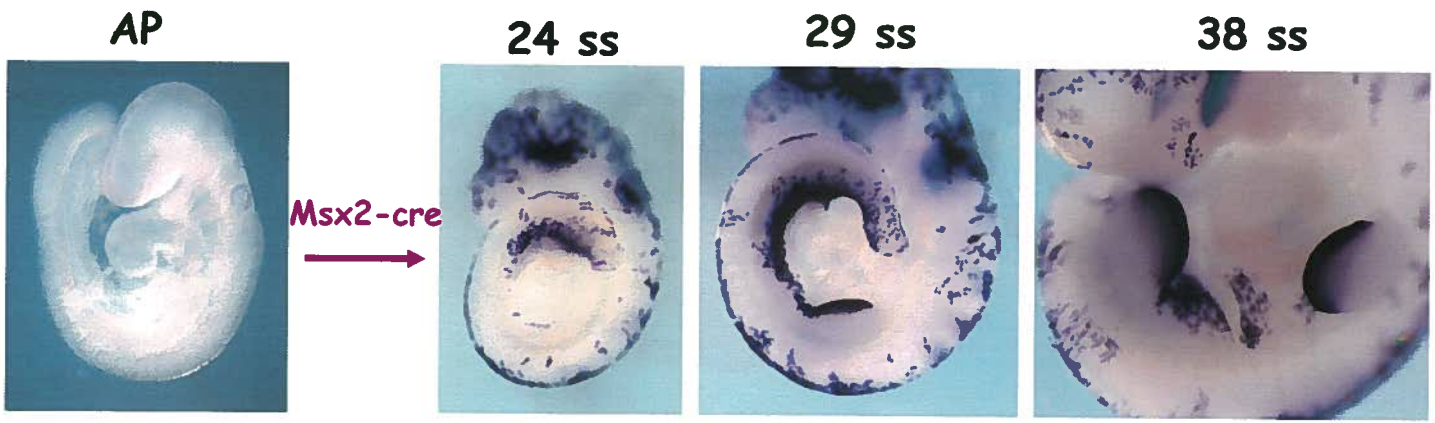
Storm et al (2003) Dosage of Fgf8 determines whether cell survival is positively or negatively regulated in the developing forebrain PNAS. 100:1757.

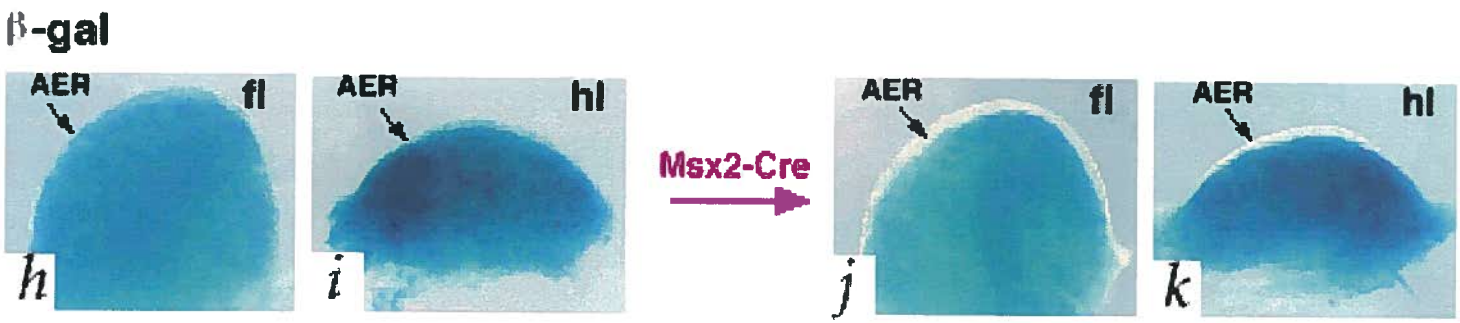
…left/right axis determination



Meyers EN, Martin GR (1999) Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. Science. 285:403-6.

Msx2-cre is expressed in the AER





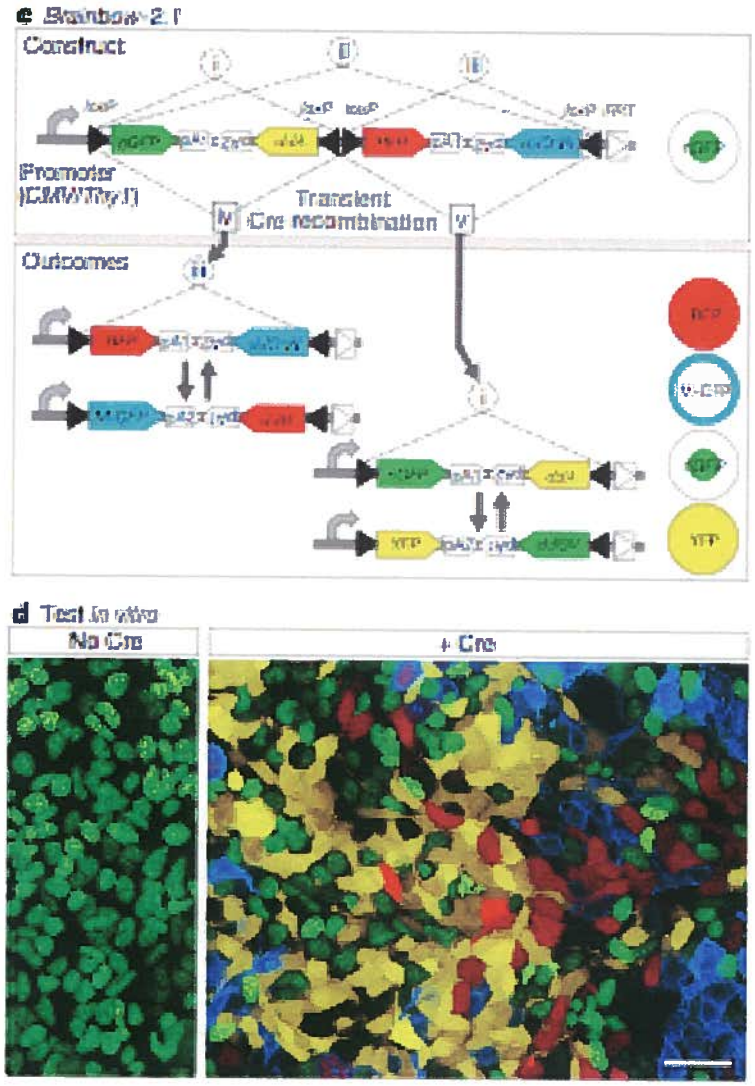
* Lobe, C. G. et al (1999) Dev. Biol. 208: 281

Other available Cre reporters

Alkaline phosphatase (Z/AP)
β-galactosidase (R26R)
Enhanced green fluorescent protein
Enhanced yellow fluorescent protein
Enhanced cyan fluorescent protein
luciferase

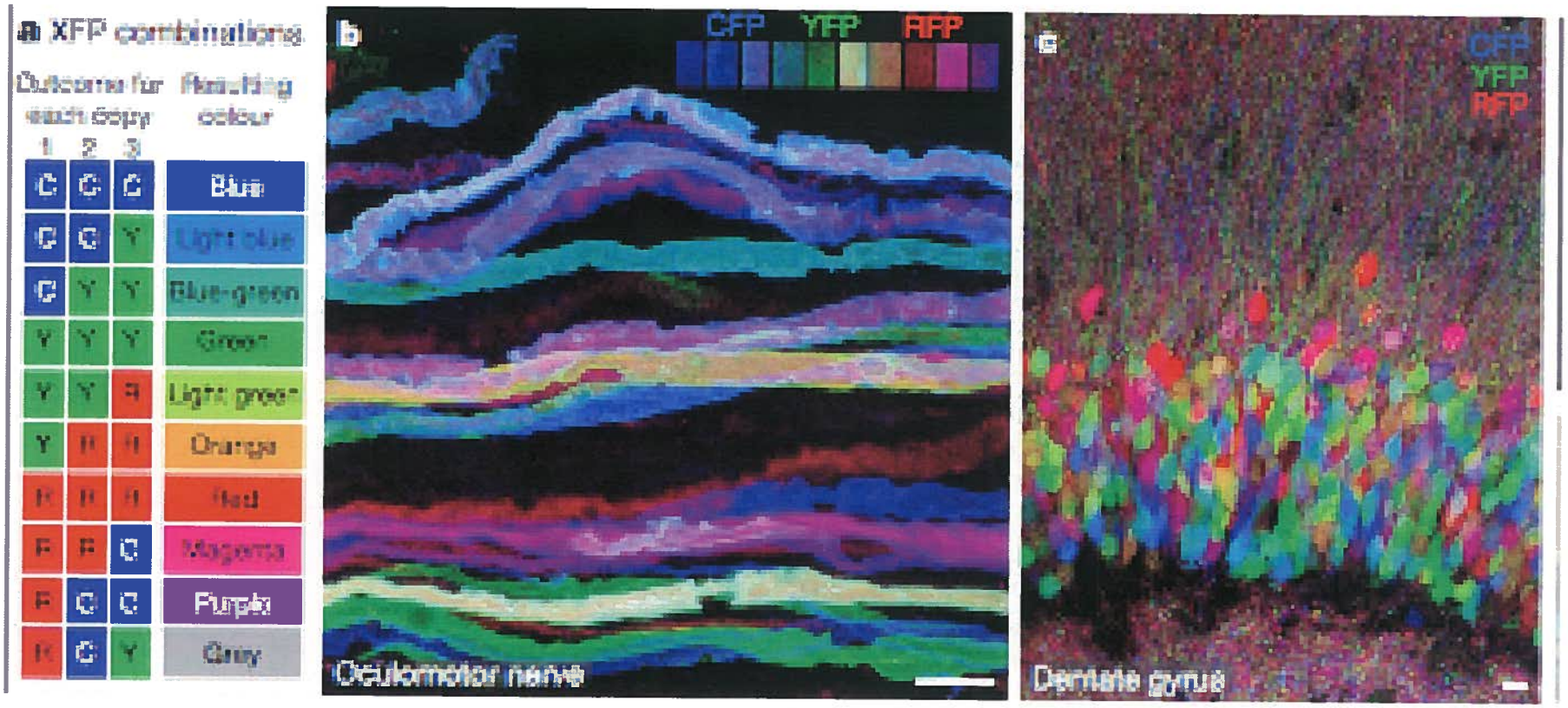
For details see
Lewandoski, M. (2001) Nat Rev Genetics 2:743
Lewandoski, M. (2007) Handb Exp Pharmacol 178:235
Woolfenden S (2009) Genesis 47: 659
Levit et al (2007) Nature 450:56

"Brainbow"



Transgenic strategies for combinational expression of fluorescent proteins in the nervous system. Levit et al (2007) Nature 450:56

"Brainbow"



Transgenic strategies for combinational expression of fluorescent proteins in the nervous system. Levit et al (2007) Nature 450:56

Inactivation of *Fgf8* in the AER

♀ $\frac{Fgf8^{flox}}{Fgf8^{flox}}$

♂ $\frac{Fgf8^{\Delta 2,3}}{Fgf8^{+}}$ $\frac{Msx2\text{-}cre}{Msx2\text{-}cre}$

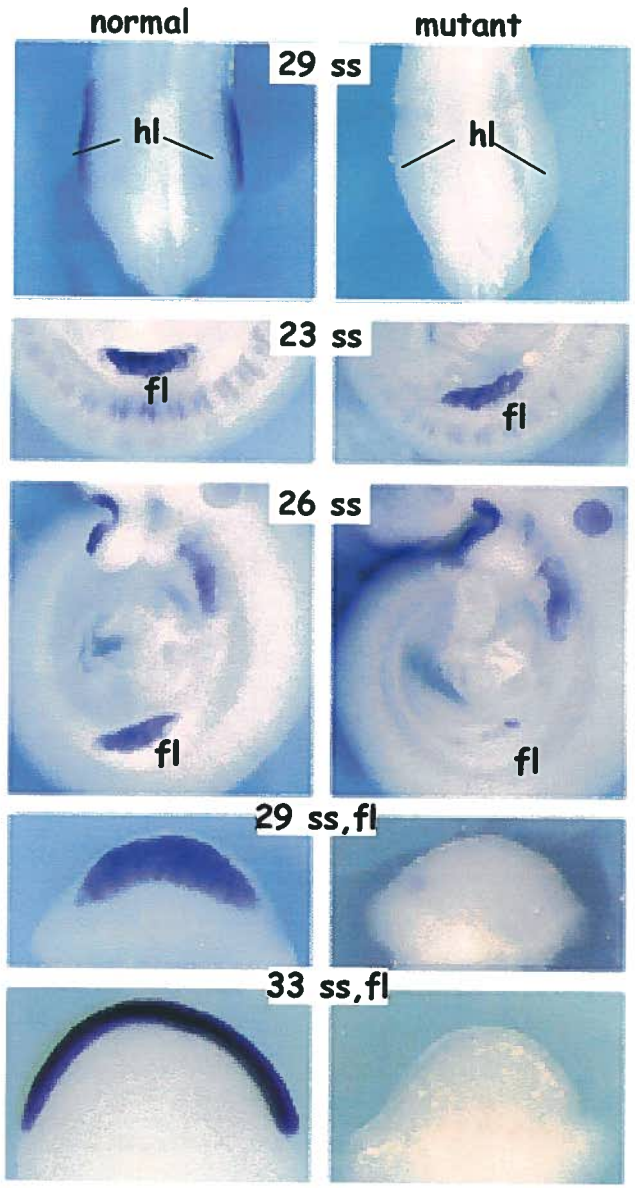
↓

$Msx2\text{-}cre\ \frac{Fgf8^{+}}{Fgf8^{flox}}$

$Msx2\text{-}cre\ \frac{Fgf8^{\Delta 2,3}}{Fgf8^{flox}}$

50% normal

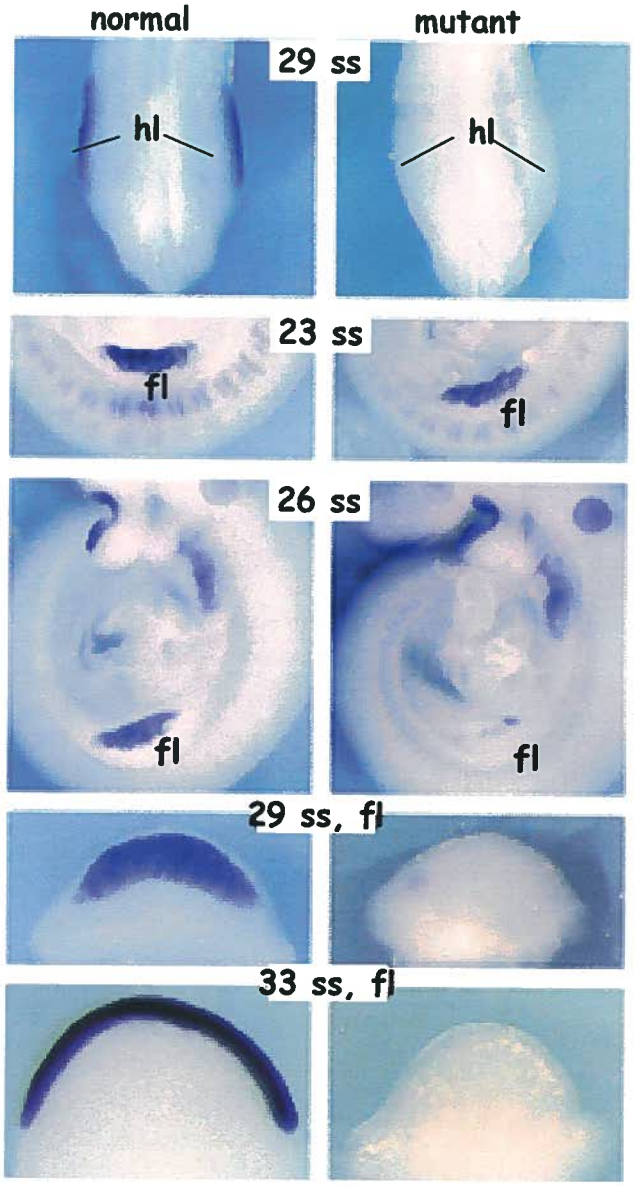
50% mutant



Lewandoski, M. et al (2000) Nature Gen. 26: 460

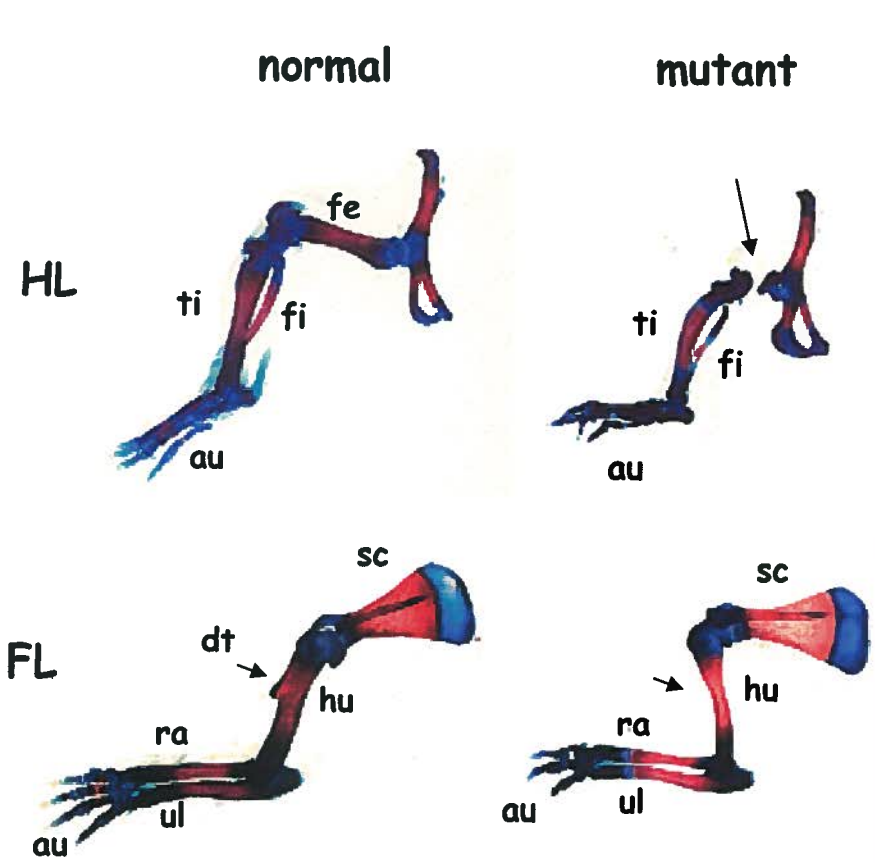
Inactivation of *Fgf8* in the AER

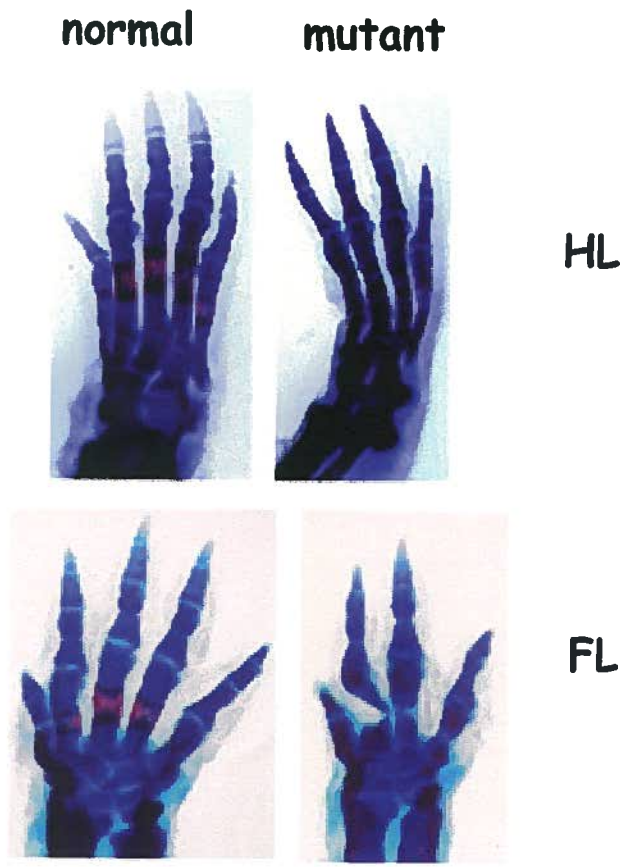
Fgf8^{flox} *Conditional allele*



Lewandoski, M. et al (2000) Nature Gen. 26: 460

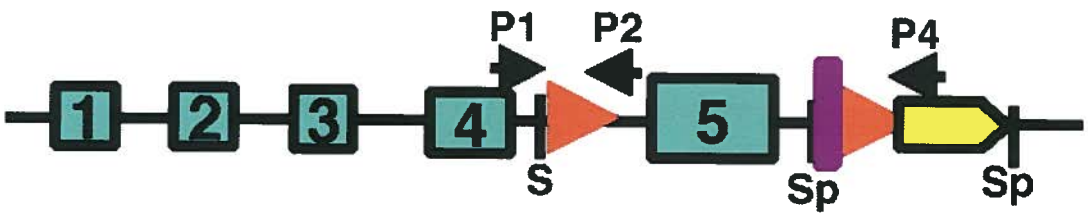
Inactivation of Fgf8 in the AER



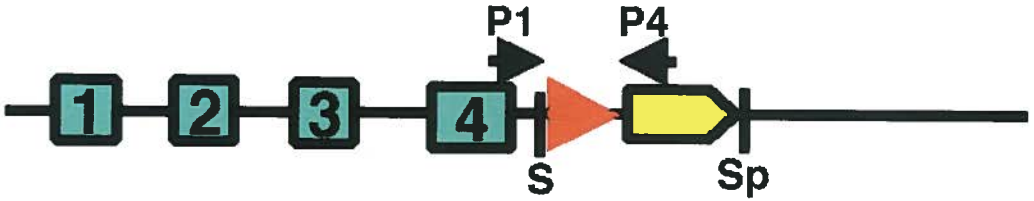


Lewandoski, M. et al (2000) Nature Gen. 26: 460

One could design the target allele so recombination BOTH knocks out the gene & activates a reporter...



Fgf8^{C} conditional allele

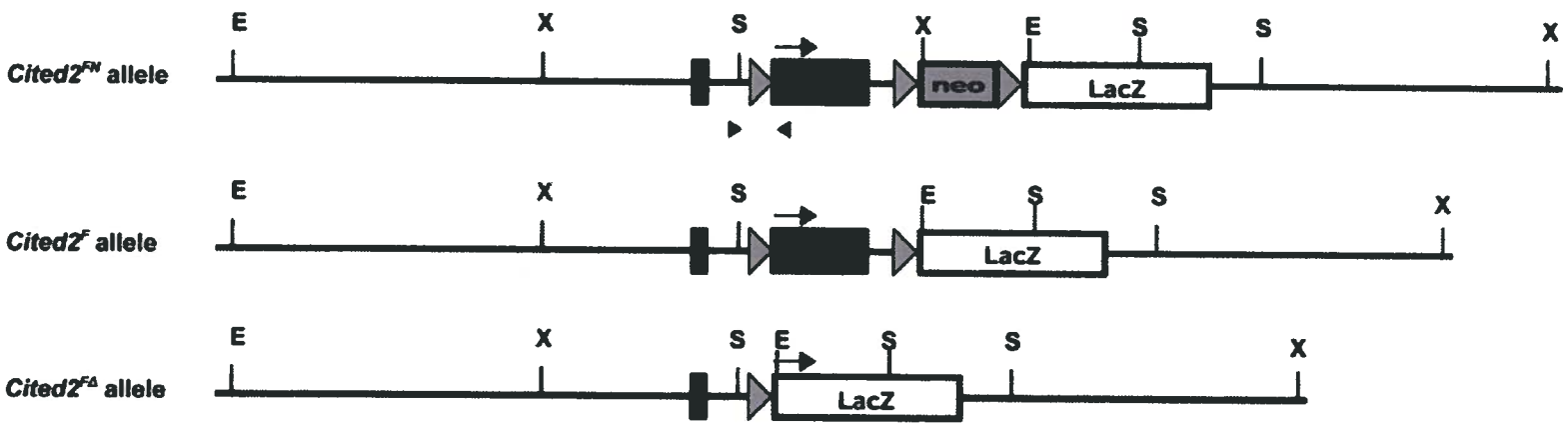


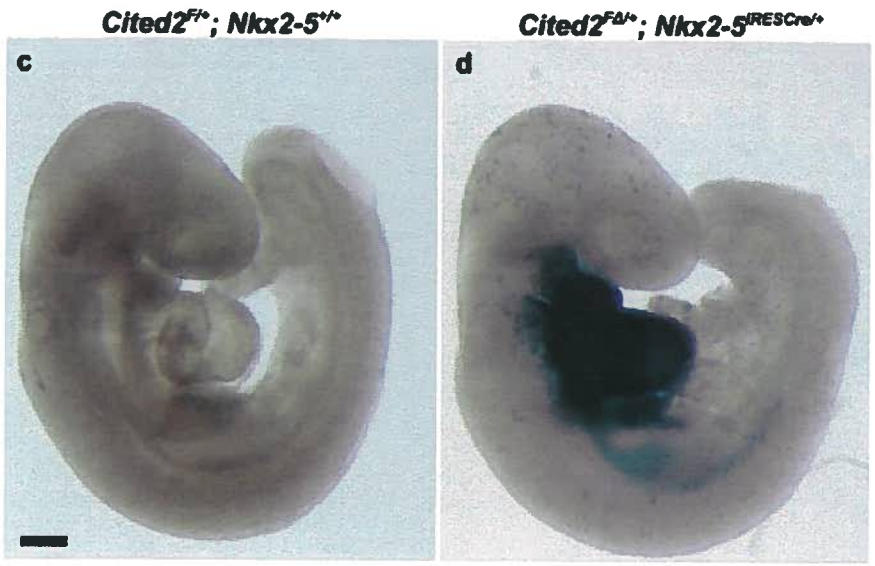
Cre-recombined *Fgf8*^{CR}

... but the reporter may generate a hypomorph.

Moon and Capecchi (2000) Nature Genet. 26: 455

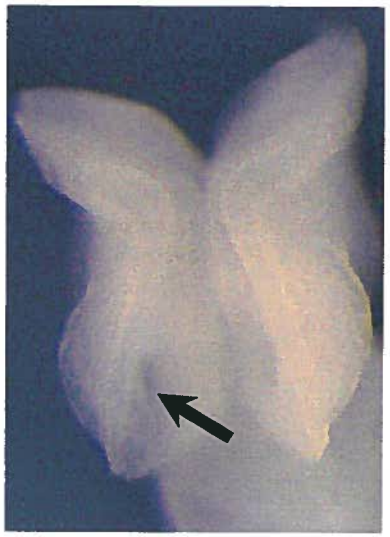
One could design the target allele so recombination BOTH knocks out the gene & activates a reporter...



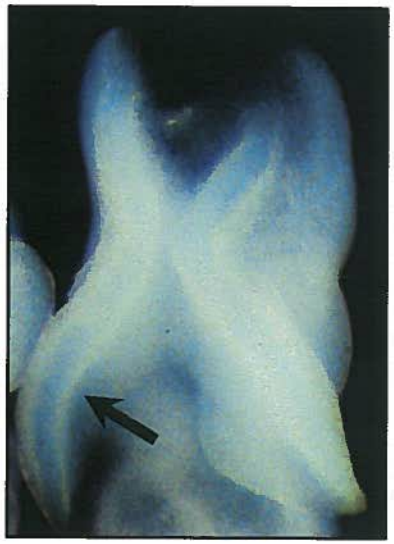


Preis et al (2006) Genesis 44: 579

Rx Knockout Leads to Defects in optic pit formation

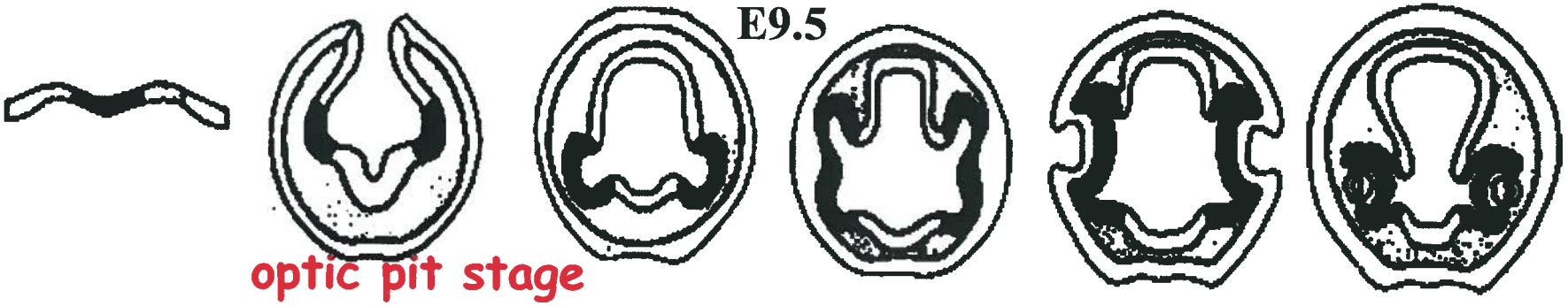


normal

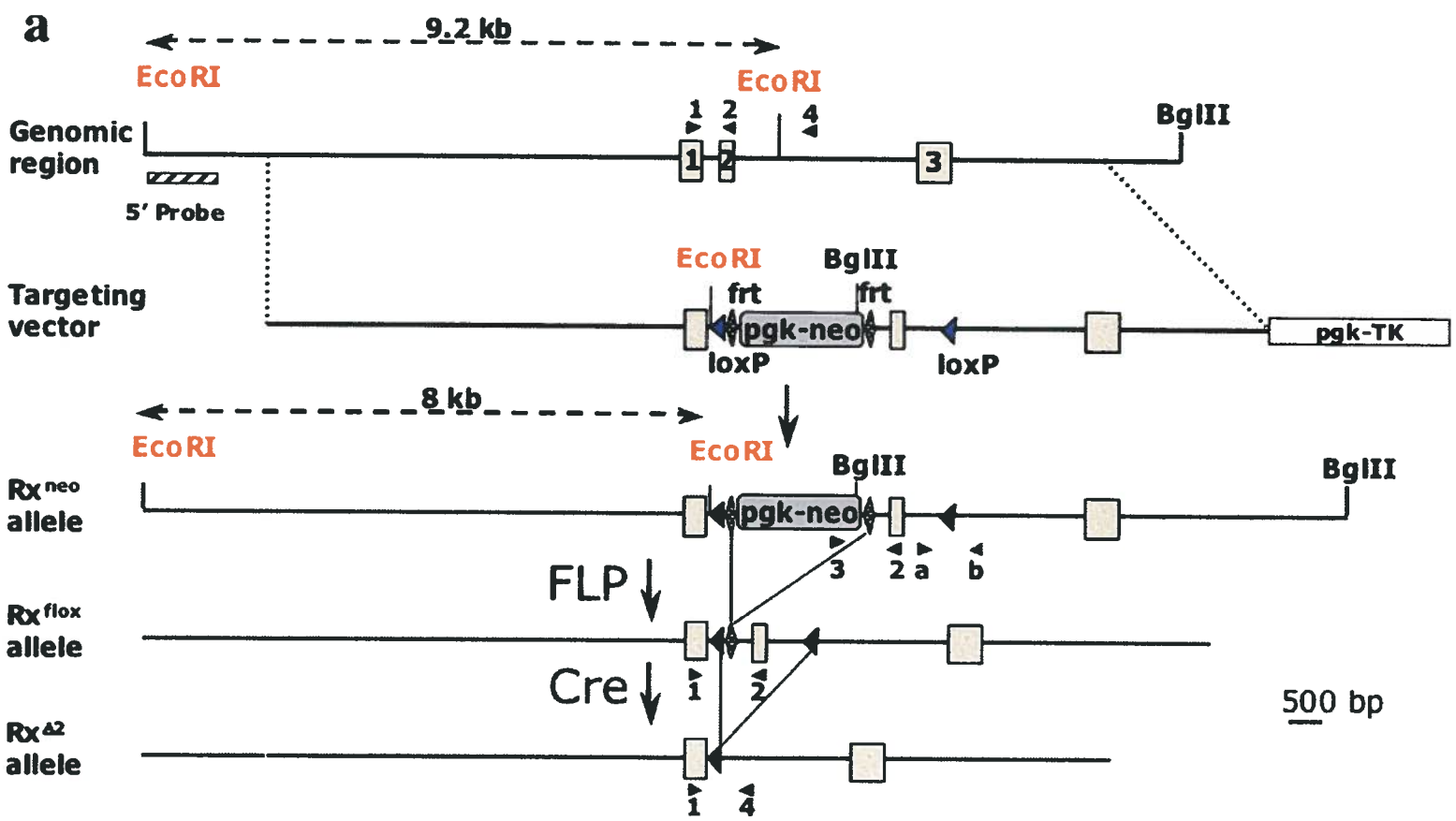


Null

Null

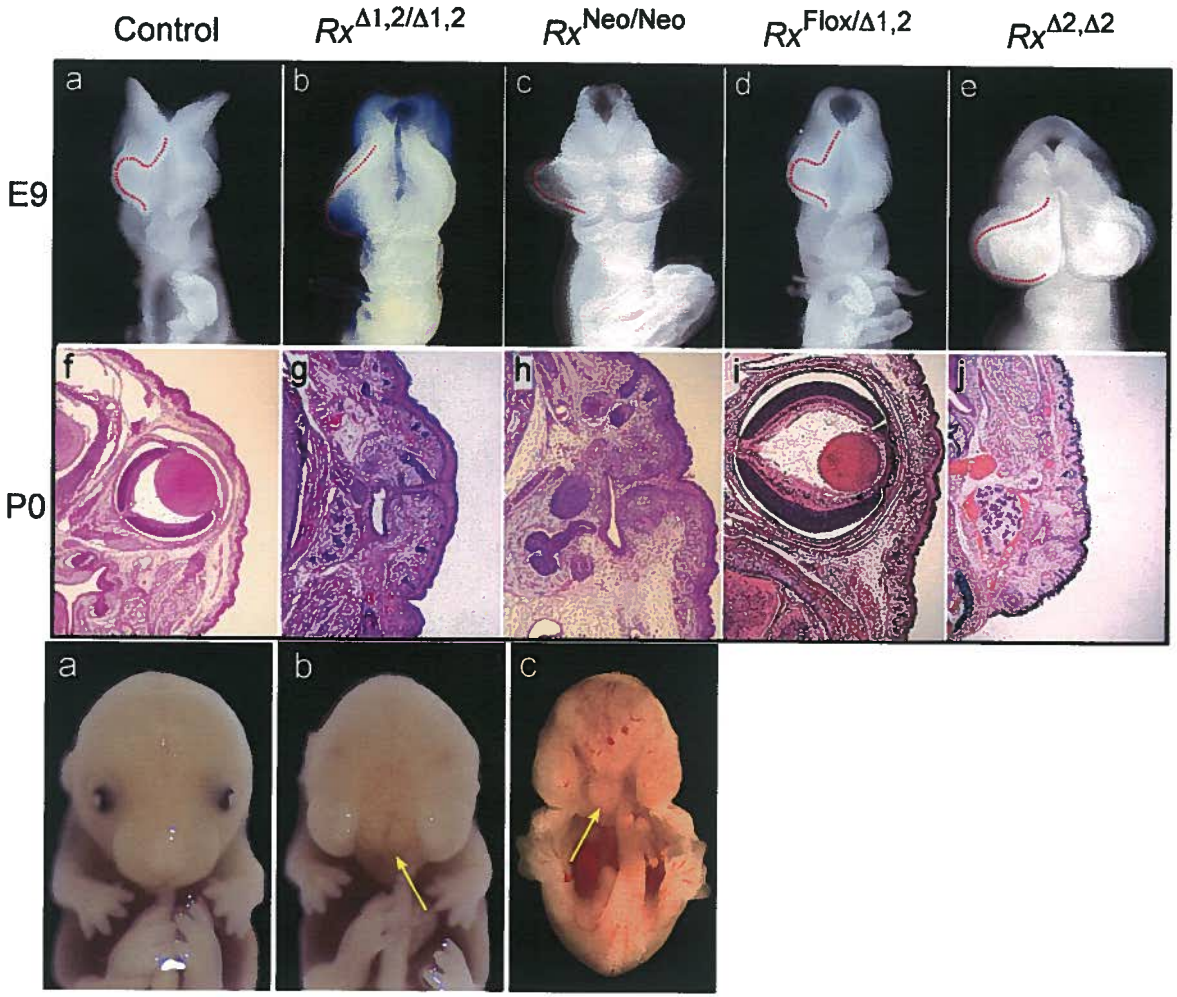


An allelic series at the *Rx* locus



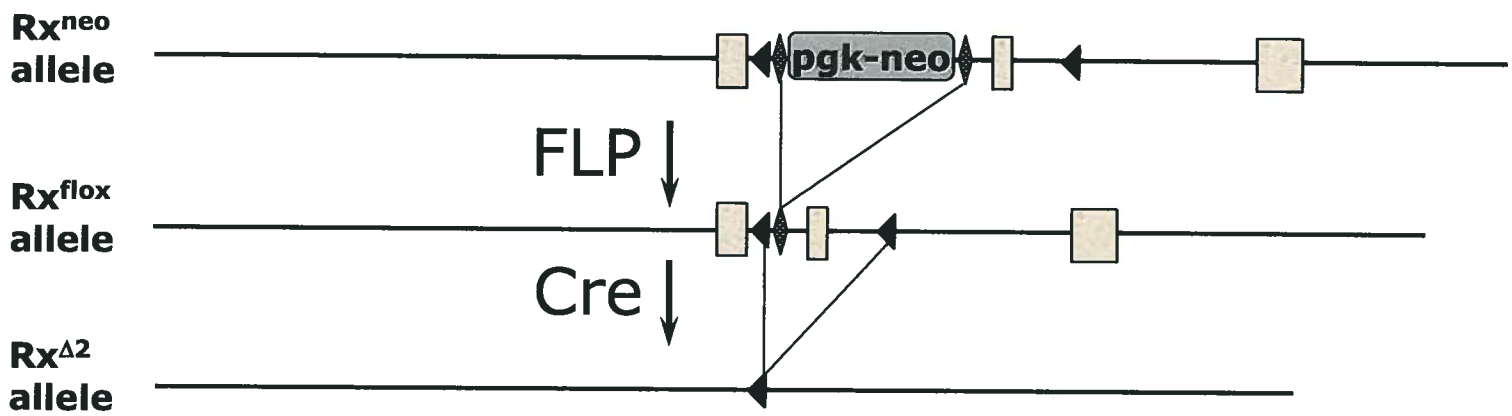
Voronina V et al (2006) Genesis 41:160

The *Rx*^{neo} allele is equivalent to a null allele



Rx^{neo} can be used as a FLP-activatable allele

TspFLP ;$Rx^{neo/\Delta 2}$



Rx^{flox} can be used as a Cre-inactivatable allele

TspCre ;$Rx^{flox/\Delta 2}$

Some advice about making mice carrying Cre and floxed alleles

Some limitations of Cre/loxP approaches

1) Not all floxed regions in the genome are equally recombinable

Vooijs, M.et al. A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. EMBO Rep. 2 :292 (2001).

2) High Cre activity can be detrimental

Schmidt, E. E. et al. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc. Natl Acad. Sci. USA 97, 13702 (2000).

Loonstra, A. et al. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc.Natl Acad. Sci. USA 98, 9209–9214 (2001).

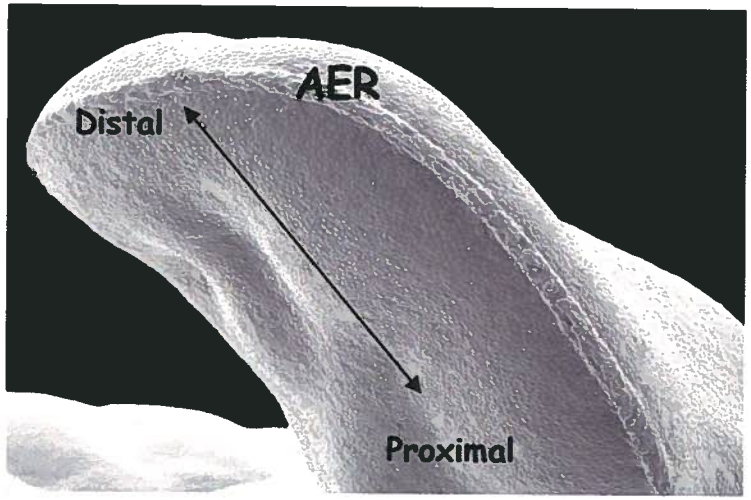
Silver, D. P. & Livingston, D. M. Self-excising retroviral vectors encoding the Cre recombinase overcome the Cre-mediated cellular toxicity. Molecular Cell 8, 233-243 (2001).

Pfieffer A. et al. Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting *in vivo* Proc.Natl Acad. Sci. USA 98, 11450–11455 (2001).

Naiche, L.A. & Papaioannou. V.E. Cre activity causes widespread apoptosis and lethal anemia during embryonic development Genesis 45, 768 (2007)

Carefully characterize a new Cre line…

… because different Cre lines with similar but non-identical activity may be useful in quickly changing biological contexts



Earlier AER-specific inactivation of Bmpr1a:
"BMPs required for AER formation"

Ahn et al (2001□□) Development 128: 4419

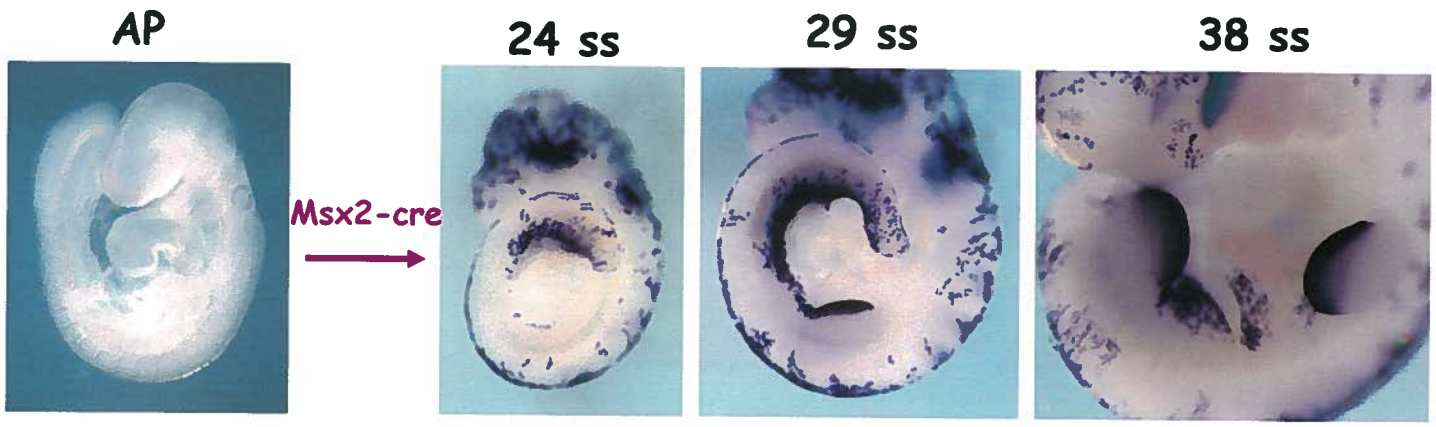
Latter AER-specific inactivation of Bmpr1a:
"BMPs required for AER loss"

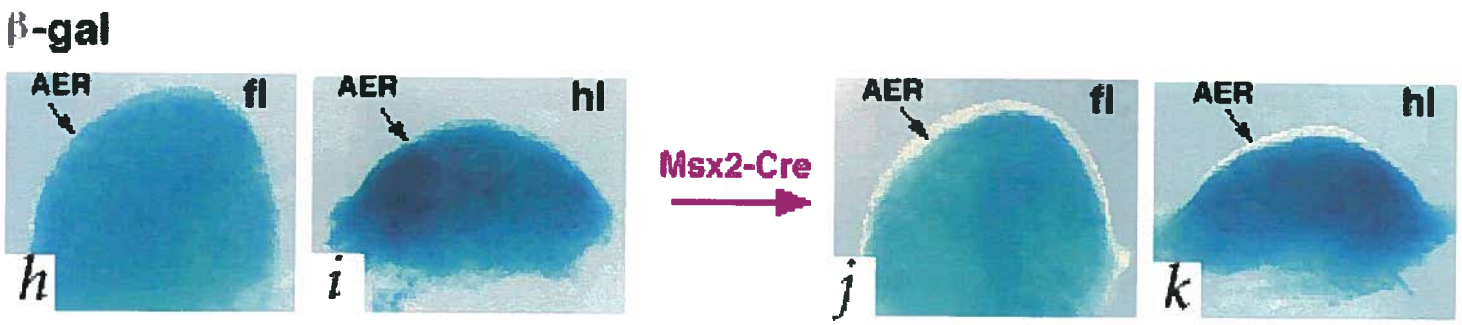
Pajni-Underwood et al (2007) Development 134: 2359

Carefully characterize a new Cre line…

… because unexpected activity may open doors to collaborations

Msx2-cre is one of 13 published lines expressed in the limb bud, not all intentionally generated for that purpose.

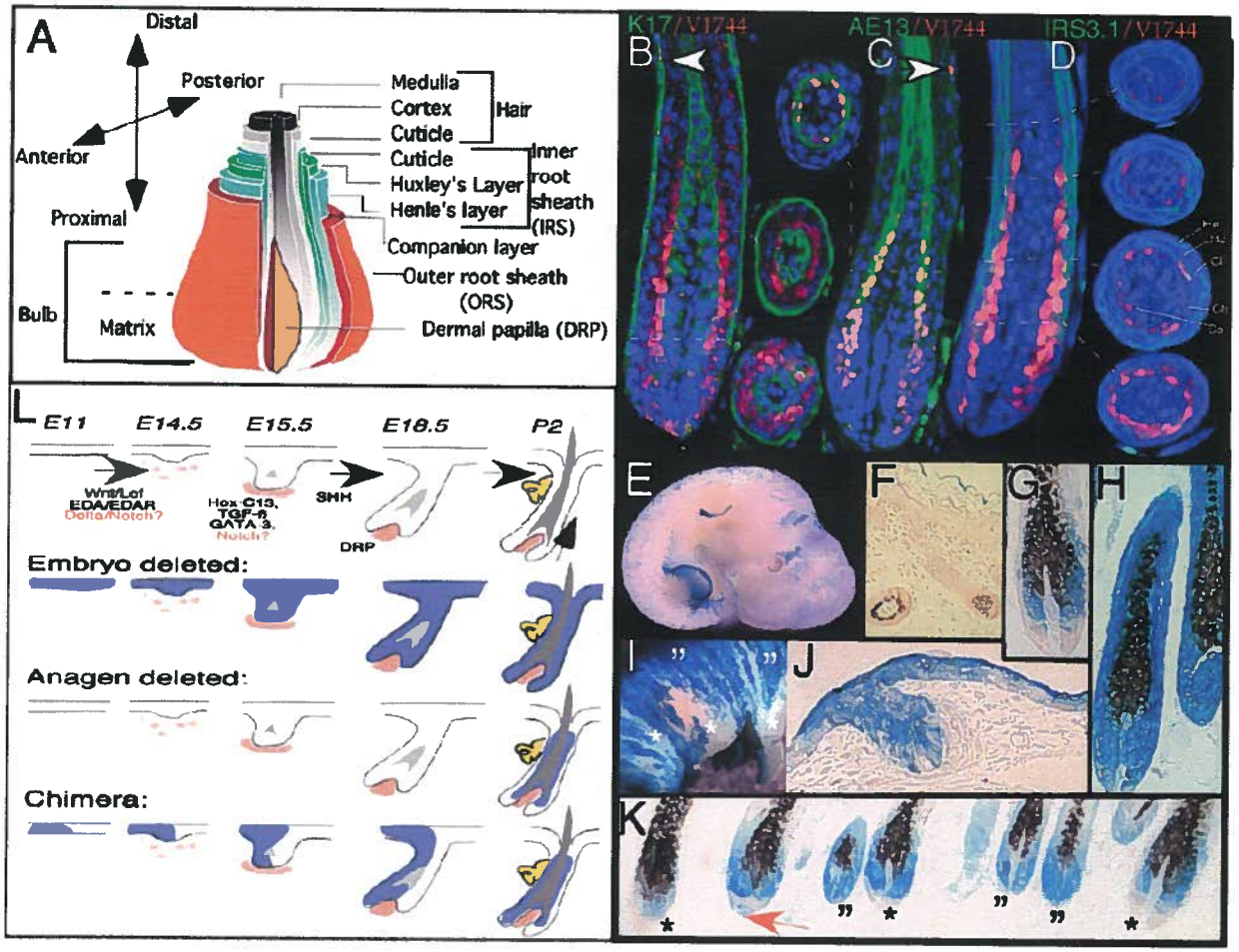




Carefully characterize a new Cre line…

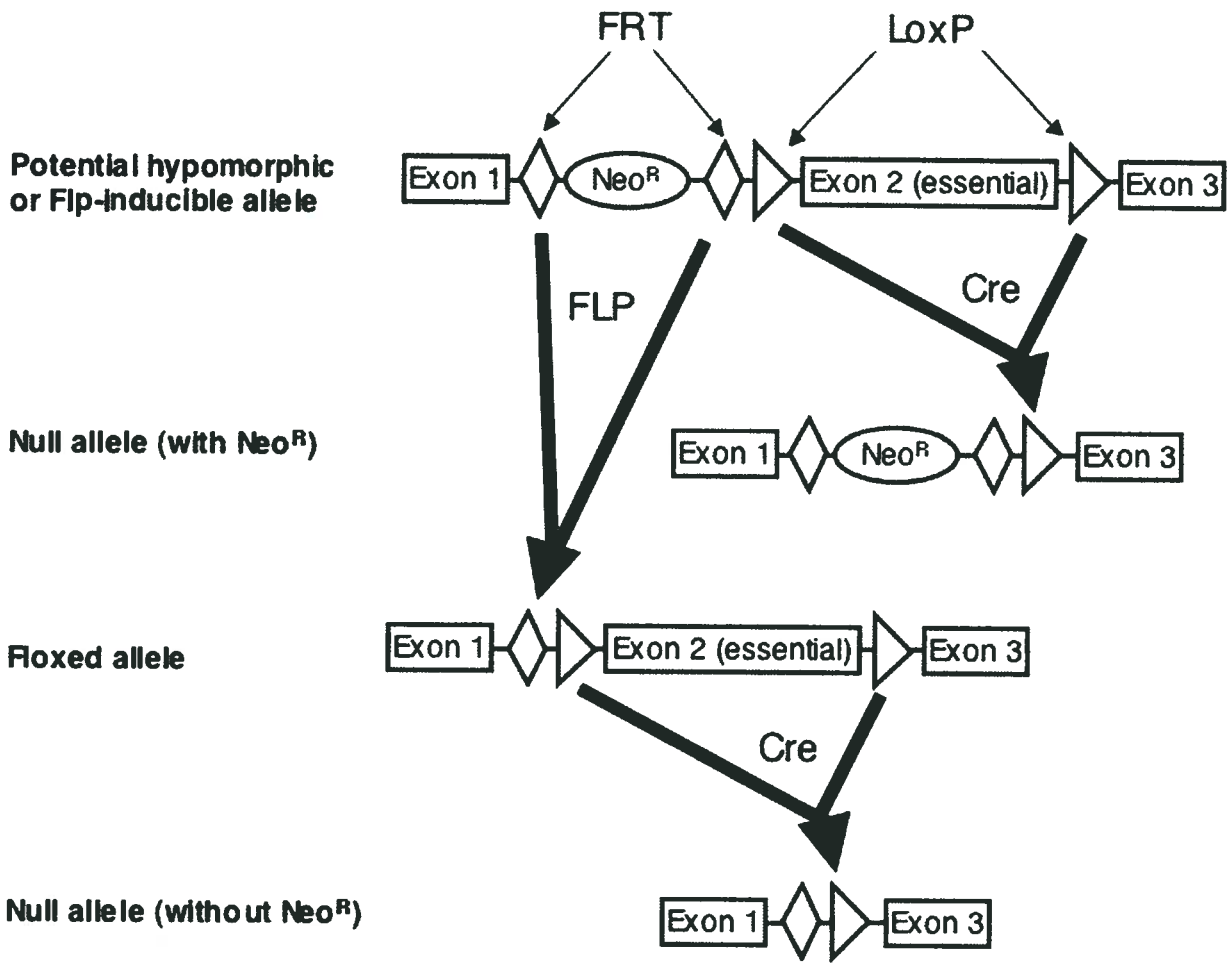
… because unexpected activity may open doors to collaborations

Msx2-cre is active in the hair follicle.



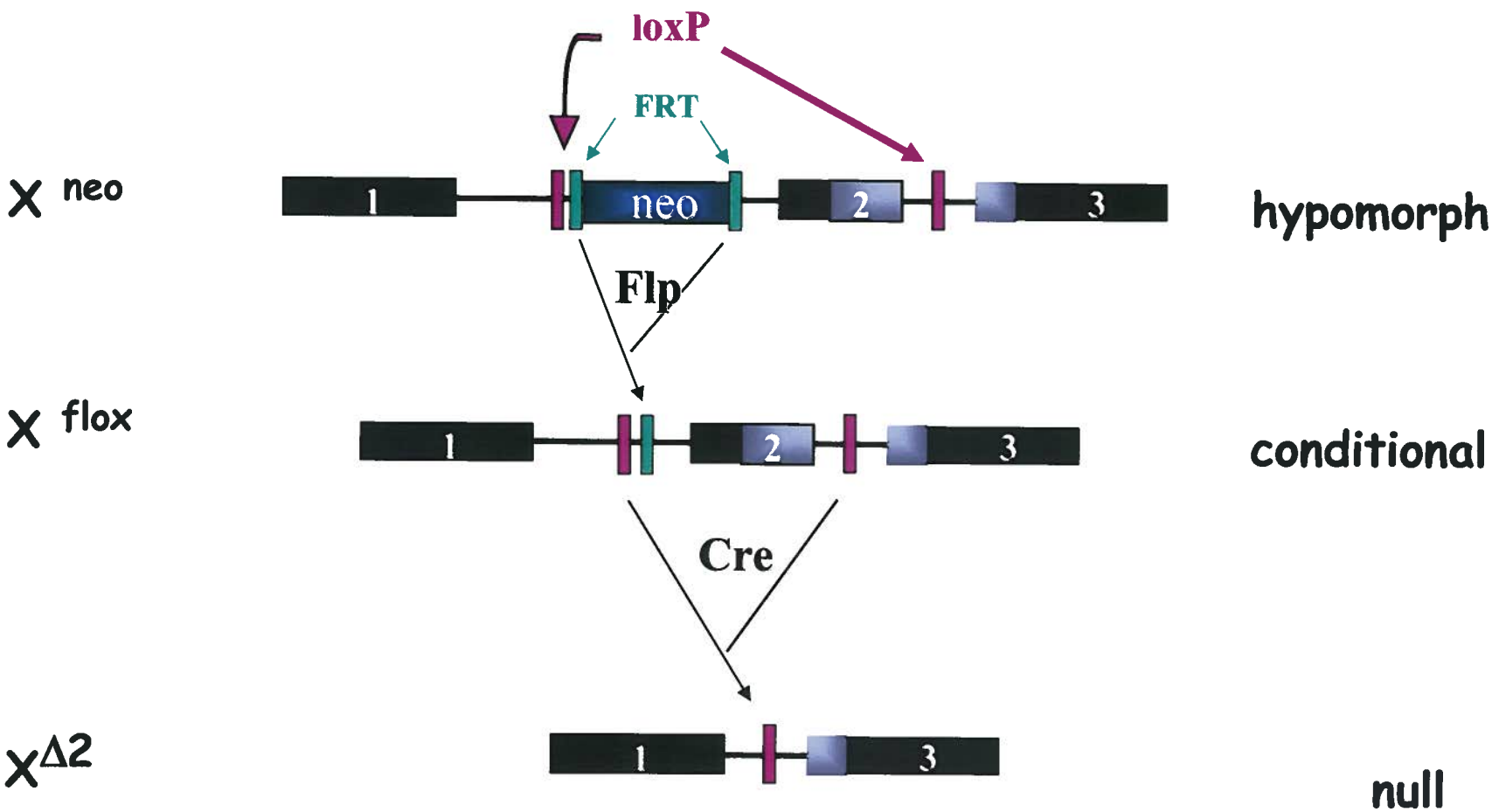
Pan et al (2004□□) Dev Cell 7: 731

Allelogenic strategy



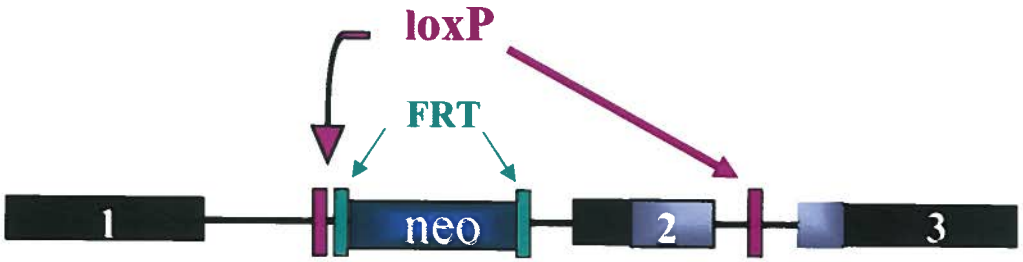
Lewandoski, M. (2007) Handb Exp Pharmacol 178:235

Placement of flrted neo between loxP sites limits your options



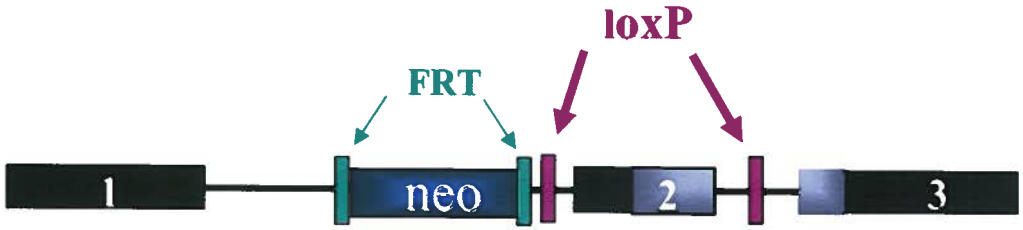
Placement of flrted neo between loxP sites limits your options

Not preferred



Null allele

Preferred



Null (neo) allele

Null allele

x

BAC(wt); $YFG^{null/+}$

$YFG^{null/+}$ or $YFG^{nullneo/+}$

Genotype progeny with normal phenotype

BAC(wt); $YFG^{null/null}$

$YFG^{+/+}$

$YFG^{null/+}$

No (non quantitative) PCR can distinguish between these three genotypes

BAC(wt); $YFG^{nullneo/null}$

$YFG^{+/+}$

$YFG^{null/+}$

$YFG^{nullneo/+}$

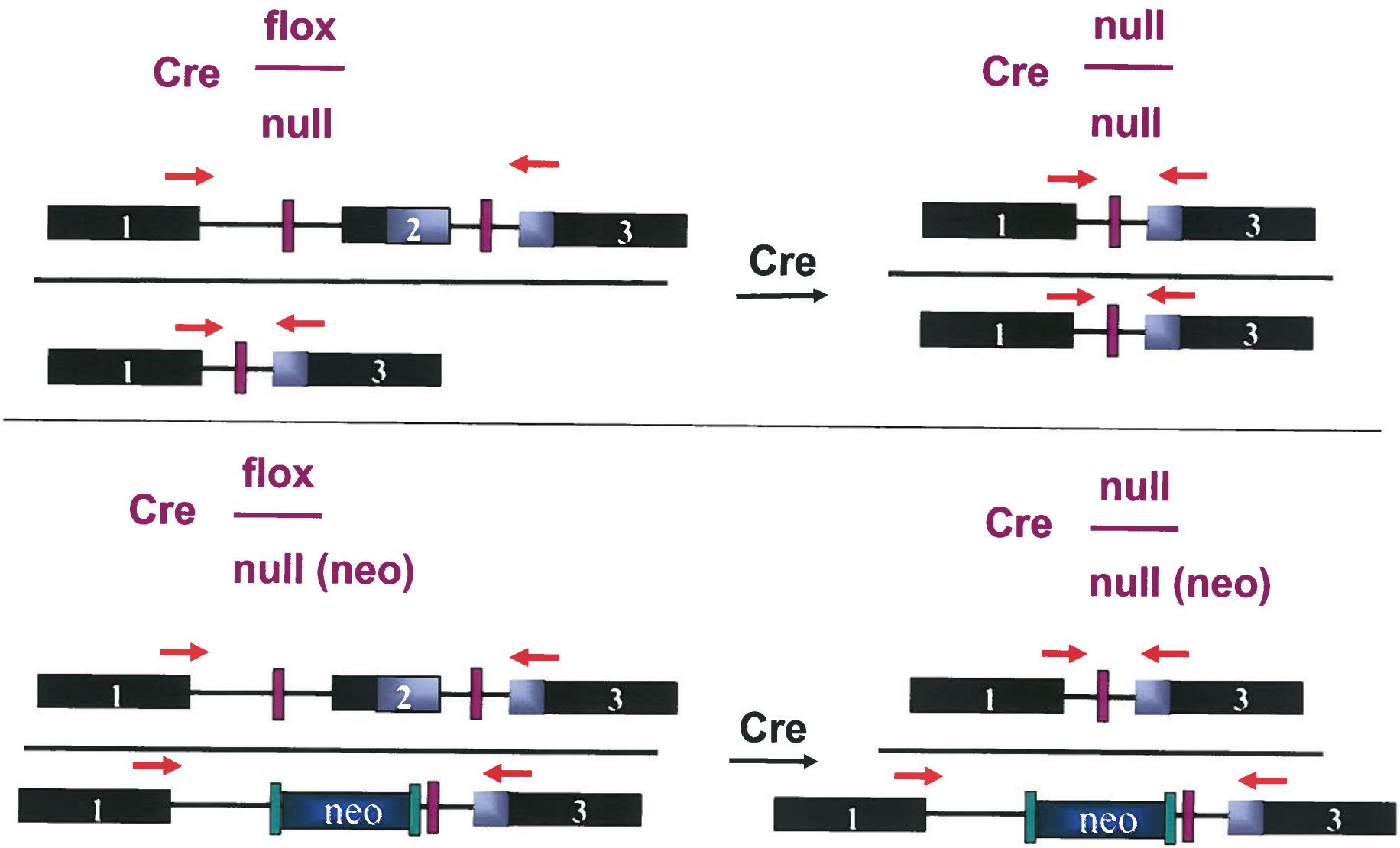
PCR can easily distinguish between these four genotypes

You may need a PCR assay to monitor Cre-mediated recombination

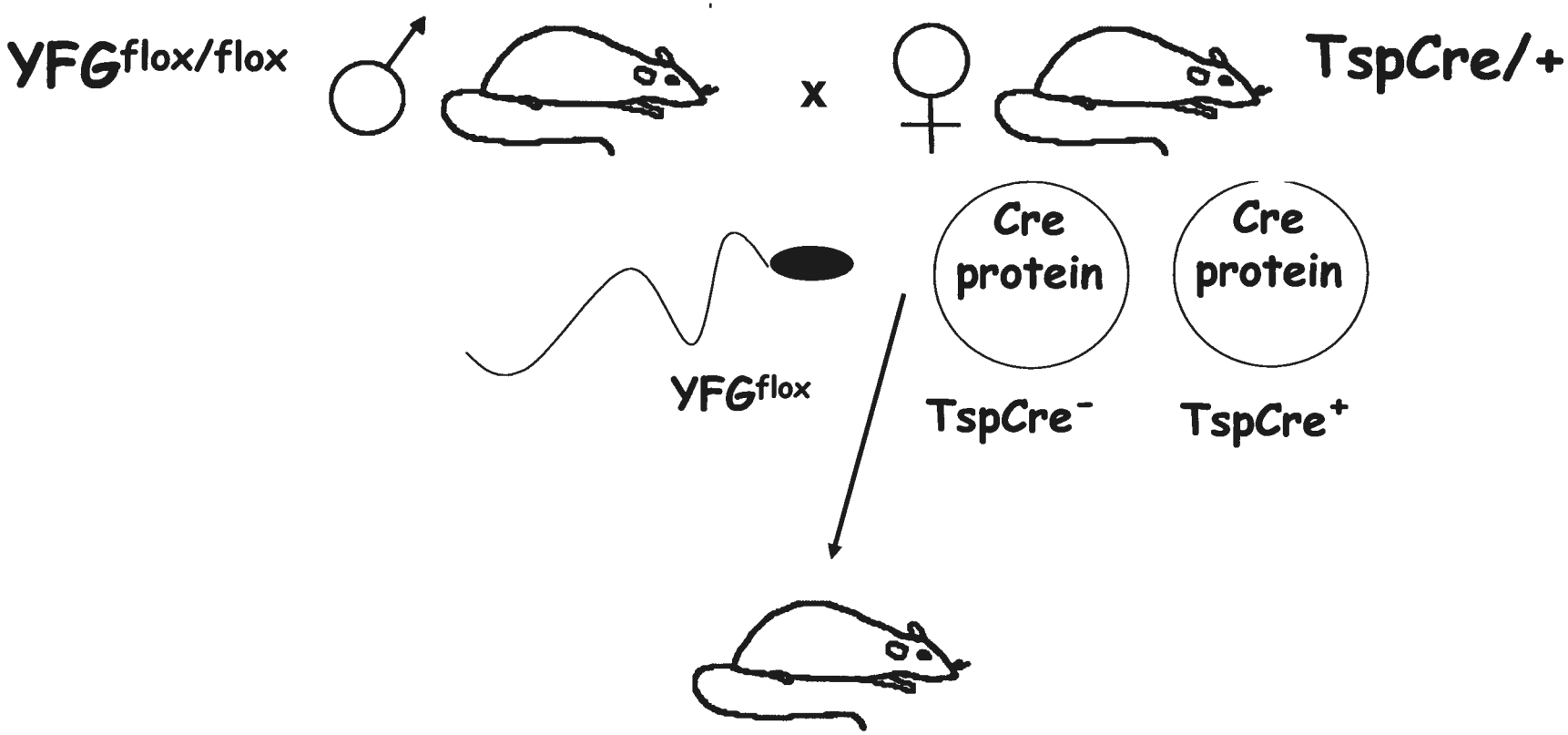
TSPCre; YFG$^{flox/null}$ ⟶ TSPCre; YFG$^{null/null}$

TSPCre; YFG$^{flox/nullneo}$ ⟶ TSPCre; YFG$^{null/nullneo}$

Placement of flrted neo between loxP sites limits your options



Test your Cre line through *paternal* not *maternal* inhertance of the Cre transgene or allele: because sometime Cre can act as an maternal effect gene as follows:



YFG$^{rec/+}$ (Cre-);
Recombination looks complete & non-specific

Site-specific recombination: Cre/FLP

a

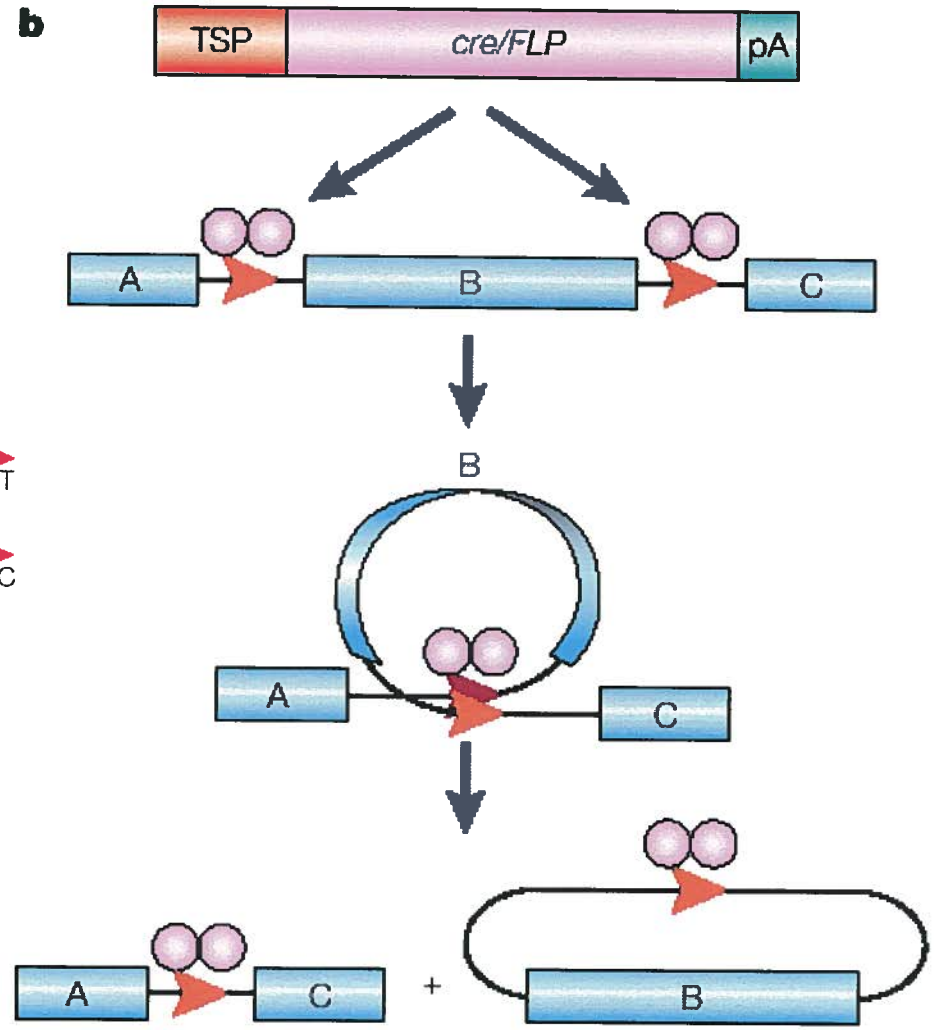
loxP

ATAACTTCGTATAATGTATGCTATACGAAGTTAT

FRT

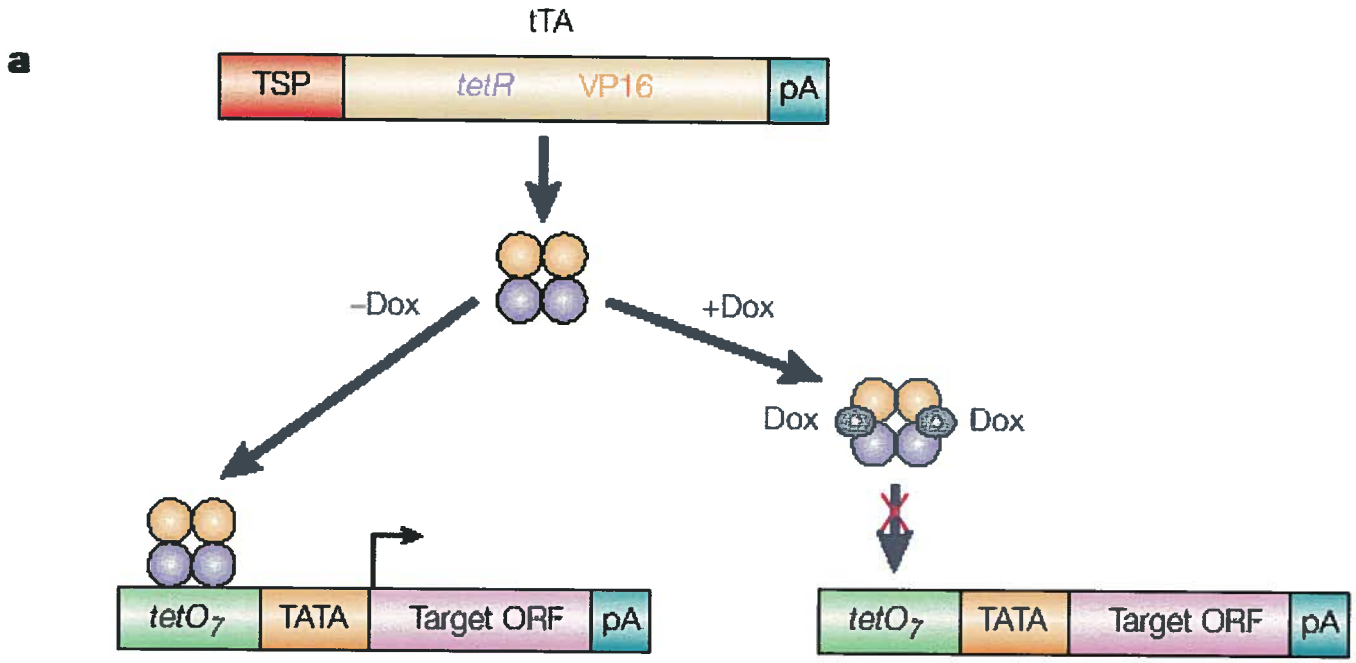
GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC

b



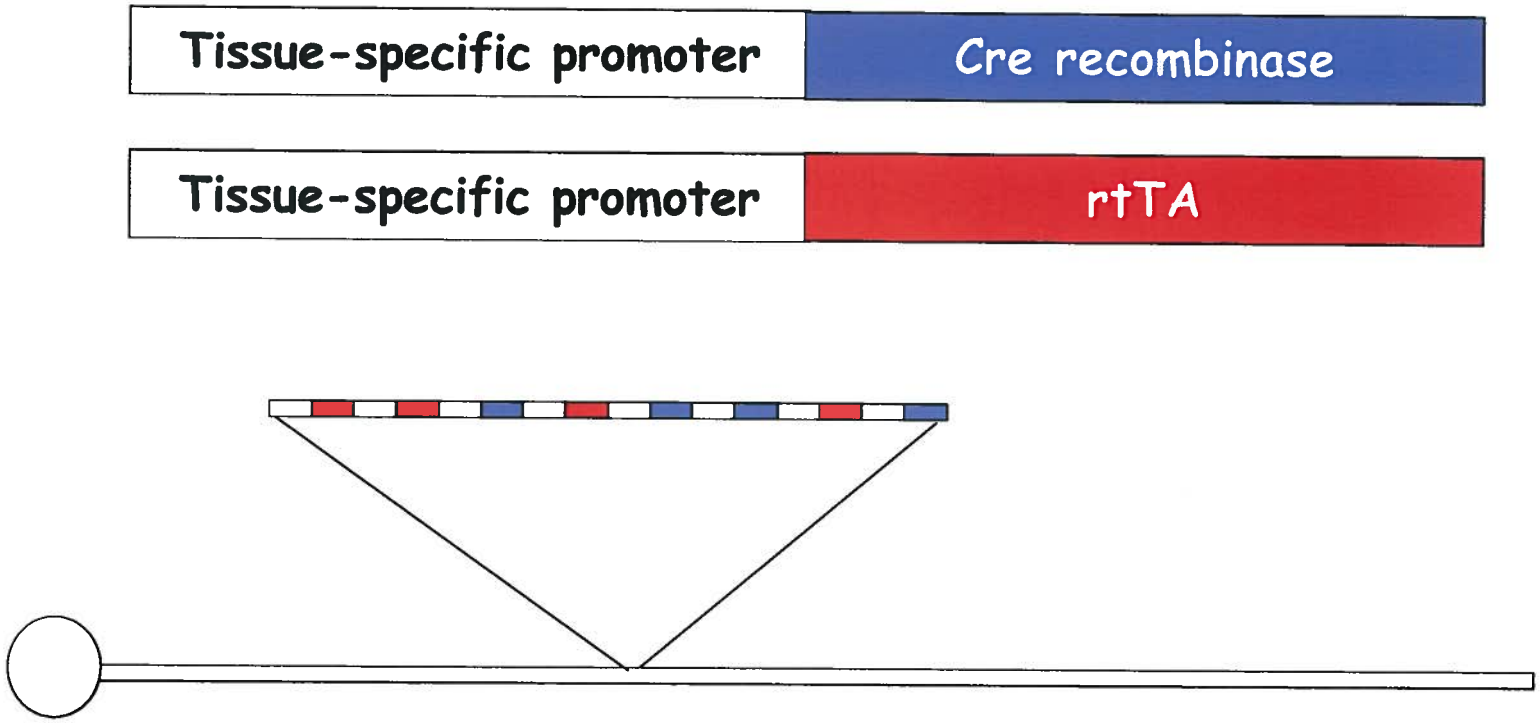
Lewandoski, M. (2001) Nature Reviews Genetic 2:743

The tetracycline-responsive regulatory system for transcriptional transactivation: "tet-off"



Lewandoski, M. (2001) Nature Reviews Genetic 2:743

Co-injection of two transgenes to generate a dual function trangenic effector line



“Cheating Mendel”

germline-cre ***Flox allele***

Null allele

"Cheating Mendel"

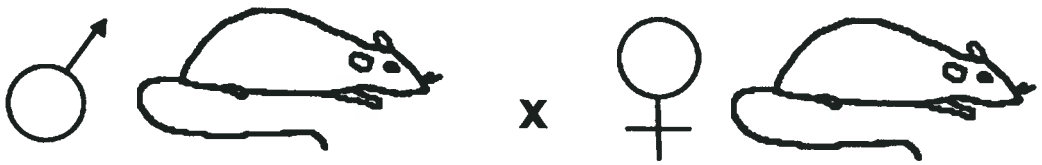
x

→ **50 % homozygous mutants**

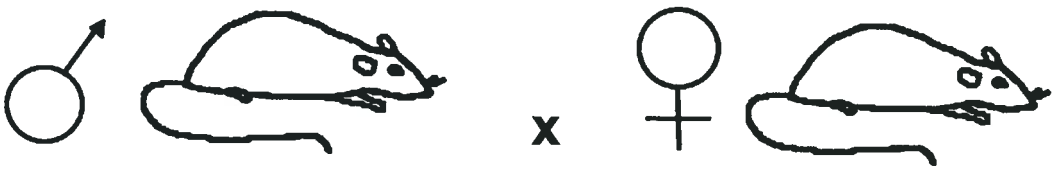
germline-cre *Flox allele* / *Null allele*

WT allele / *Null allele*

"Cheating Mendel"



germline-cre *Flox allele* / *Null allele* x *WT allele* / *Null allele* → 50 % homozygous mutants



germline-cre *Flox allele* / *Null allele* x germline-cre *Flox allele* / *Null allele* → 100 % homozygous mutants

Acknowledgements

Sam Waters, Assistant Professor at University of Missouri
Vera Voronina, Scientist at Regeneron Pharmaceuticals