BBS821 – Block 2

Adaptive Immunity

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AS9-2055
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Outline of Block 2

- Basics of mouse genetics (Chan)
- Generation of a T cell (Huseby)
- Generation of a B cell (Gerstein)
- How cell death influences immunity (Chan)
- How to avoid autoimmunity (Kang)
- Mucosal Immune responses (Reboldi)

Mouse Genetics 101

Mus Musculus

Why do immunologists use mice as model organism?

- Complex vertebrate with similarity to human
- Excellent genetic tool (e.g. many naturally occurring mutants in pure genetic background)
- Ease of genetic manipulation (e.g. CRISPR/Cas9 genome editing, RNAi, etc)

Interesting natural mutations

- Scid Prkdc MGI:1857113
- Xid Btk MGI:1857138
- Lpr FAS MGI:1856334
- Gld FAS-L MGI:1856384
- Motheaten Ptpn6 protein tyrosine phosphatase, non-receptor type 6 MGI:1856074
- Beige Lyst lysosomal trafficking regulator MGI:1855969
- Aly Map3k14 mitogen-activated protein kinase kinase kinase 14 MGI:1858522

Resources

- More than 1000 inbred strains
- Hundreds of mutant strains, genetic tools
- Can add a gene- transgenics
- Can delete a gene- knockouts
- Create new mutants with ENU mutagenesis
- Mouse phenome database
- Annotated genome sequence

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

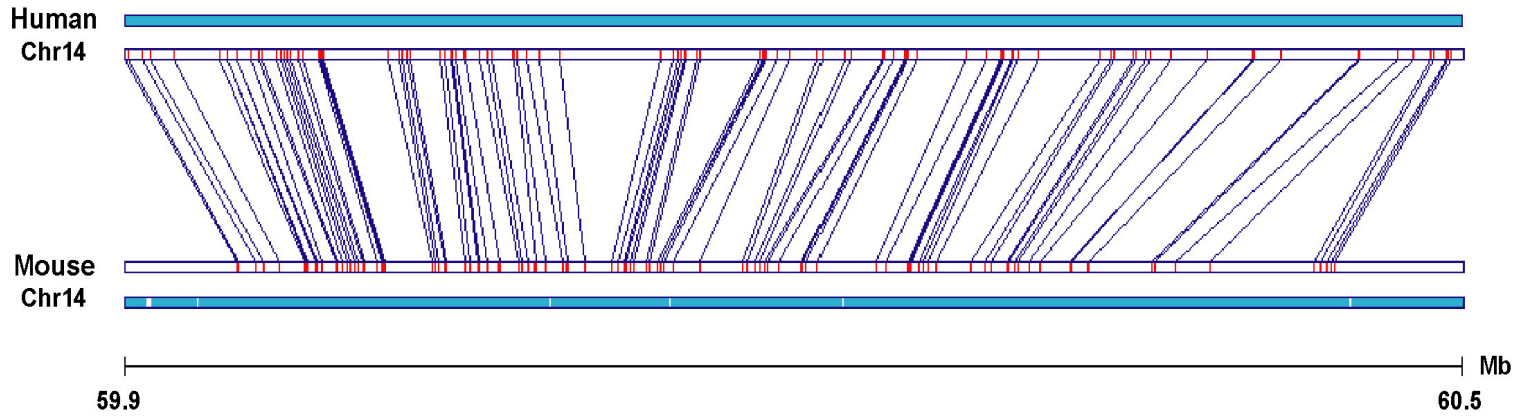
The mouse genome. Guénet JL.
Genome Res. 2005 Dec;15(12):1729-40.

Mouse Characteristics

- **Genome**
  - Number of chromosomes — 19 autosome pairs + XY
  - Diploid DNA content — ~ 6 pg (2.6×10^6 bp)
  - Recombination units — 1600 cM
  - Approx. number of genes — 30,000
- **Reproductive Biology**
  - Gestation time — 20 days
  - Age at weaning — 3 weeks
  - Age at sexual maturity — 7 weeks
  - Life span in lab — 1.5-2.5 years
  - Average litter size — 6-9
  - Litters per female — 4-8

Mouse sequence reveals great similarity with the human genome

Extremely high conservation: 560,000 “anchors”



Mouse-Human Comparison

both genomes 2.5-3 billion bp

> 99% of genes have homologs

> 95% of genome “syntenic”

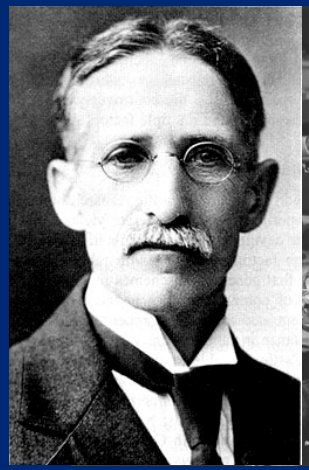
Mark J. Daly www.wi.mit.edu/programs/student/img/**daly**_wsls_0204.pp

Recent mouse history

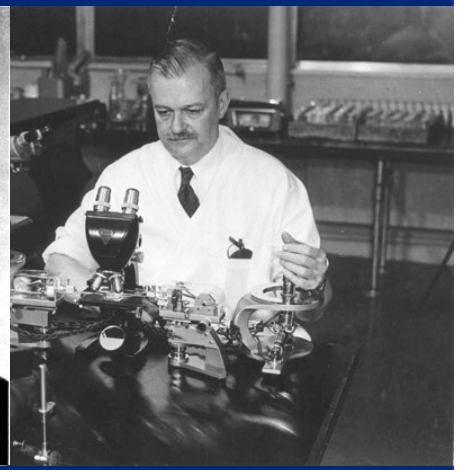
Fancy mouse breeding - Asia, Europe
(last few centuries)

Retired schoolteacher Abbie Lathrop
collects and breeds these mice
Granby, MA – 1900

Castle, Little and others form most commonly used inbred strains from Lathrop stock (1908 on)

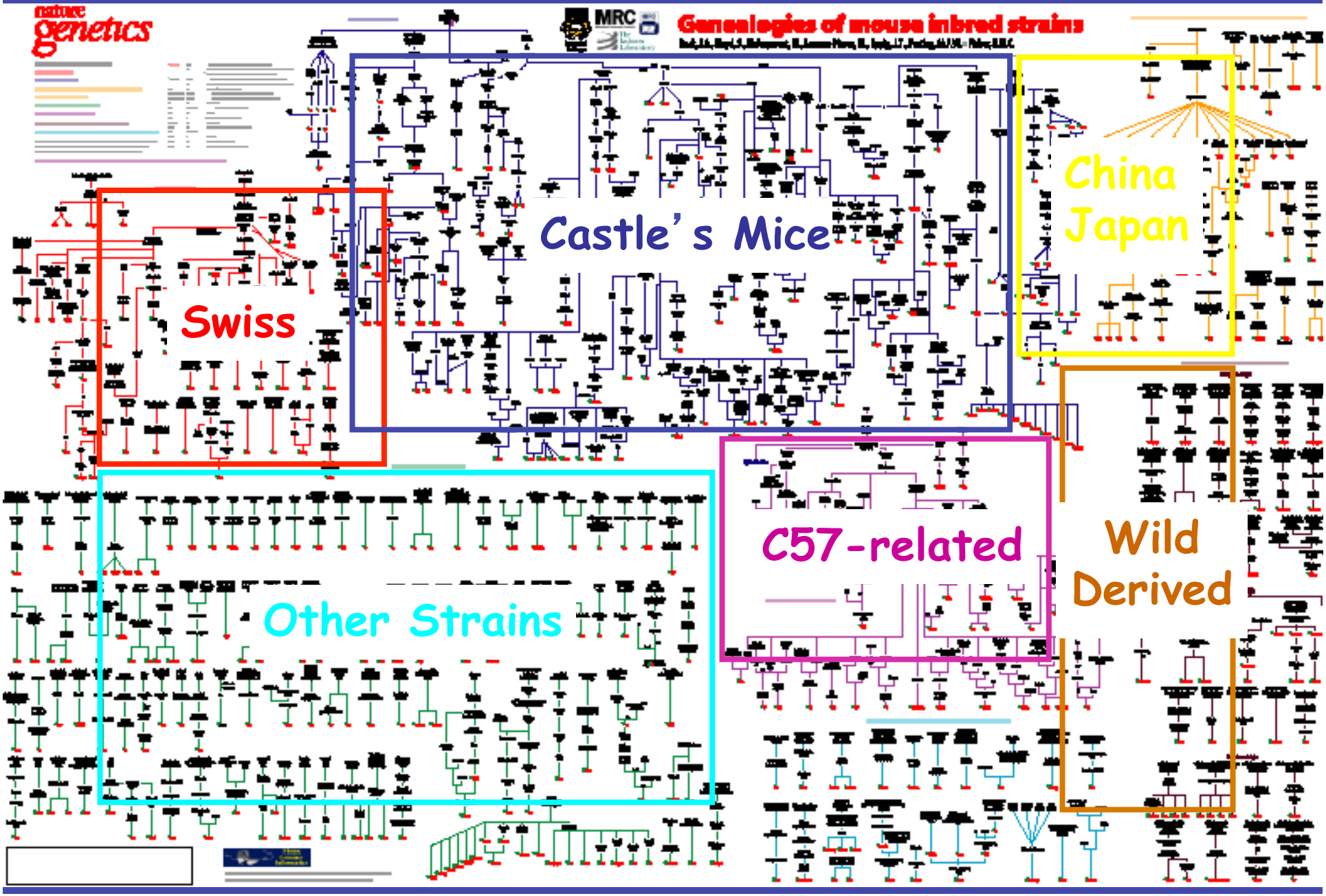


W.E. Castle

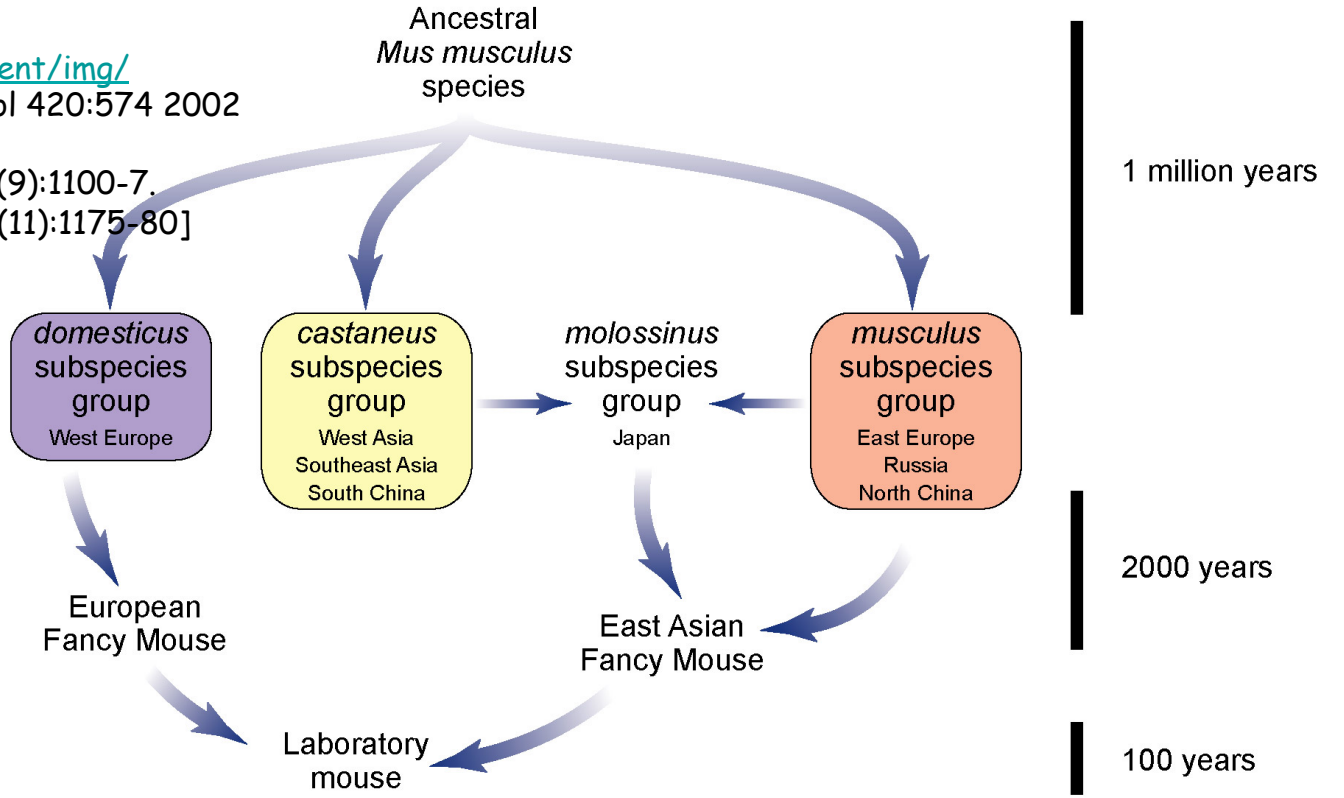


C.C. Little

Mouse Genealogies



Mark J. Daly
www.wi.mit.edu/programs/student/img/daly_wsls_0204.ppt; Nature Vol 420:574 2002
[see also:
Nature Genetics 2007 Sep;39(9):1100-7.
Nature Genetics 2005 Nov;37(11):1175-80]



b

	Strain	Locus 1							Locus 2							Locus 3					
Lab strains	129/SvImJ	TT	GG	TT	GG	GG	TT	GG	CC	TT	DEL	CC	TT	CC	TT	GG	TT	AA	TT	AA	GG
	A/J	AA	GG	CC	GG	AA	CC	TT	TT	TT	T	TT	GG	TT	CC	GG	TT	AA	TT	AA	GG
	AKR/J	AA	GG	CC	GG	AA	CC	TT	TT	TT	T	TT	GG	TT	CC	GG	TT	AA	TT	AA	GG
	BALB/cByJ	AA	GG	CC	GG	AA	CC	TT	TT	TT	T	TT	GG	TT	CC	GG	TT	AA	TT	AA	GG
	C3H/HeJ	TT	GG	TT	GG	GG	TT	GG	CC	TT	DEL	CC	TT	CC	TT	CC	AA	TT	TT	GG	AA
	C57BL/6J	AA	GG	CC	GG	AA	CC	TT	CC	TT	DEL	CC	TT	CC	TT	GG	TT	AA	TT	AA	GG
	DBA/2J	TT	GG	TT	GG	GG	TT	GG	CC	TT	DEL	CC	TT	CC	TT	GG	TT	AA	TT	AA	GG
	FVB/NJ	AA	GG	CC	GG	AA	CC	TT	TT	TT	T	TT	GG	TT	CC	GG	TT	AA	TT	AA	GG
	SJL/J	AA	GG	CC	GG	AA	CC	TT	TT	TT	T	TT	GG	TT	CC	GG	TT	AA	TT	AA	GG
Ancestors	*domesticus*	AA	GG	CC	GG	AA	CC	TT	TT	TT	T	TT	GG	TT	CC	GG	TT	AA	TT	AA	GG
	musculus	TT	GG	TT	GG	GG	TT	GG	CC	TT	DEL	CC	TT	CC	TT	CC	AA	TT	TT	GG	AA
	castaneus	AA	AA	CC	AA	GG	CC	GG	CC	CC	DEL	CC	TT	CC	TT	GG	TT	AA	AA	AA	GG
	molossinus	TT	GG	TT	GG	GG	TT	GG	CC	TT	DEL	CC	TT	CC	TT	CC	AA	TT	TT	GG	AA

Sequence-based variation map of 8.27 million SNPs in inbred mouse strains

- Nature 30 August 2007 Vol 448:1050
- 15 mouse strains re-sequenced
- http://mouse.perlegen.com/mouse/index.html
- Detailed haplotype map of each strain
- SNP = single nucleotide polymorphism
- Used to characterize allelic variation among mouse strains
- Facilitates allelic variants => phenotypic variation

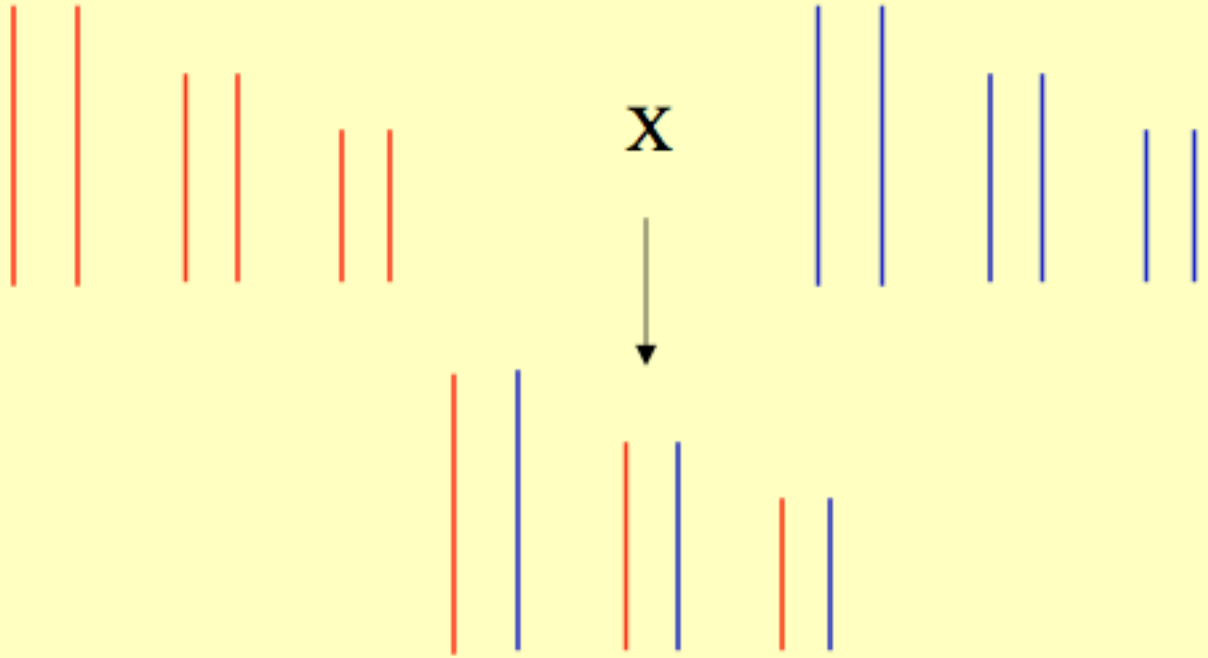
Mating Schemes

	Type of Mating	Offspring		
		+/+	+/-	-/-
Incross (inbreds)	+/+ X +/+	100%		
Intercross (F2)	+/- X +/-	25%	50%	25%
Backcross (congenics)	+/- X +/+	50%	50%	
Outcross (new Strains, F1 hybrids)	+/+ X -/-		100%	

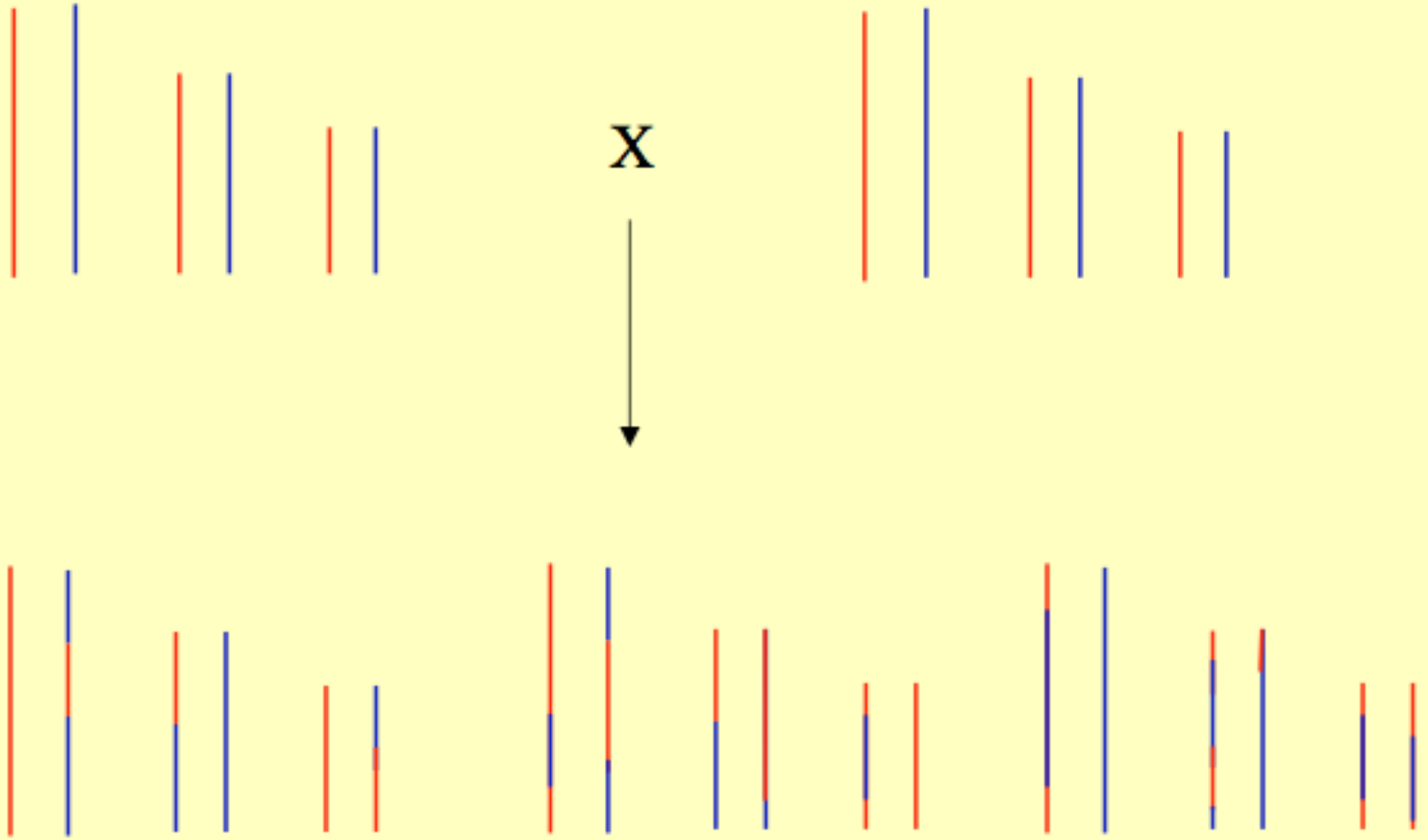
Genetic Intercrossing

- Fundamental basis of all genetic mapping performed in rodents

F1 Intercross

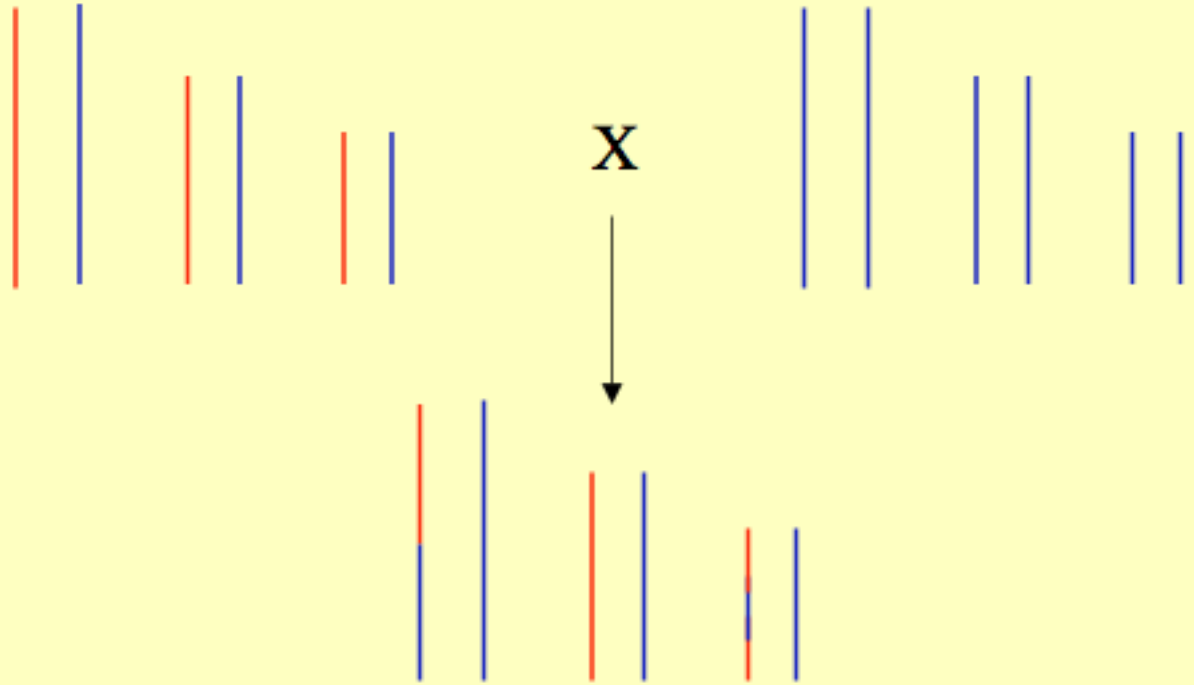


F2 Intercross



Backcross

- Offspring have 1 set of chromosomes from backcross parent and then have mixed genetics for the other chromosome.



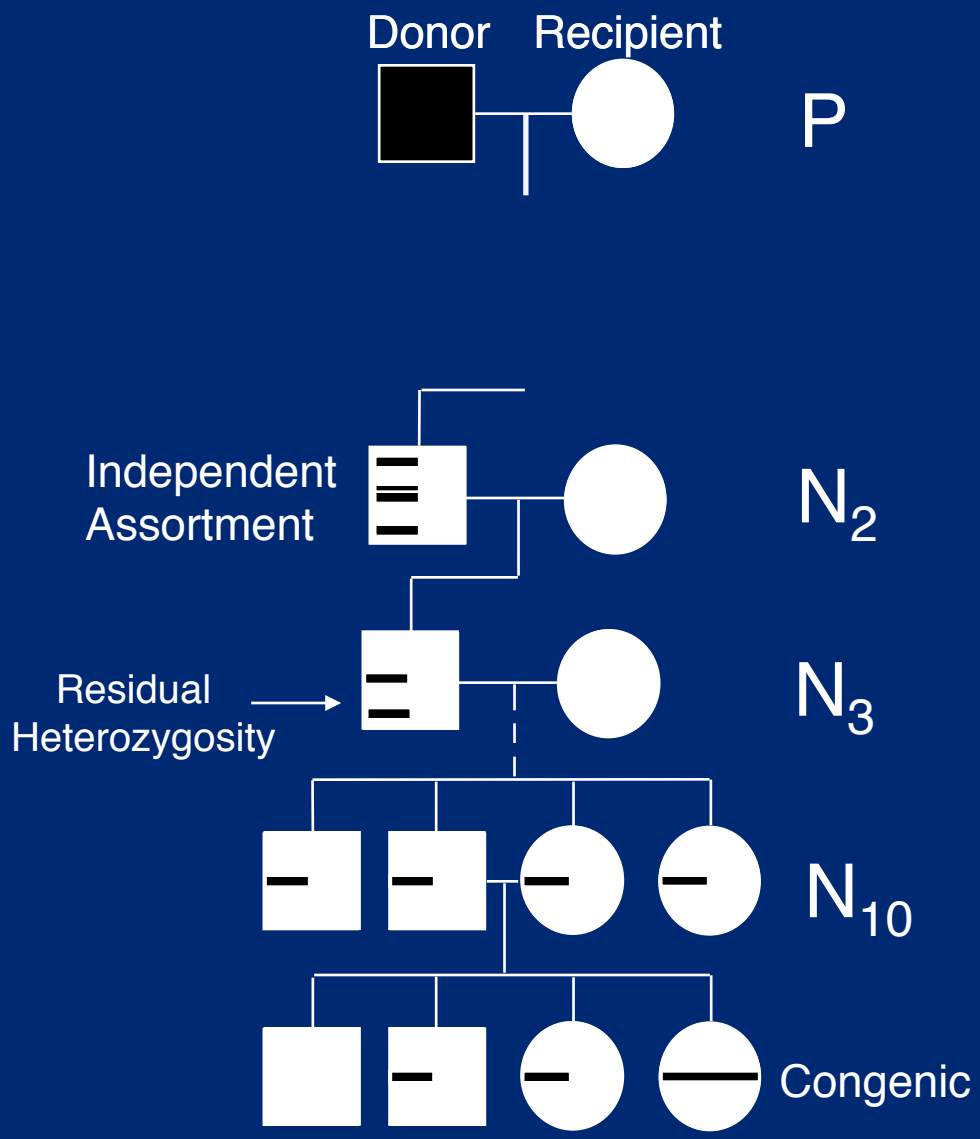
Use of crosses

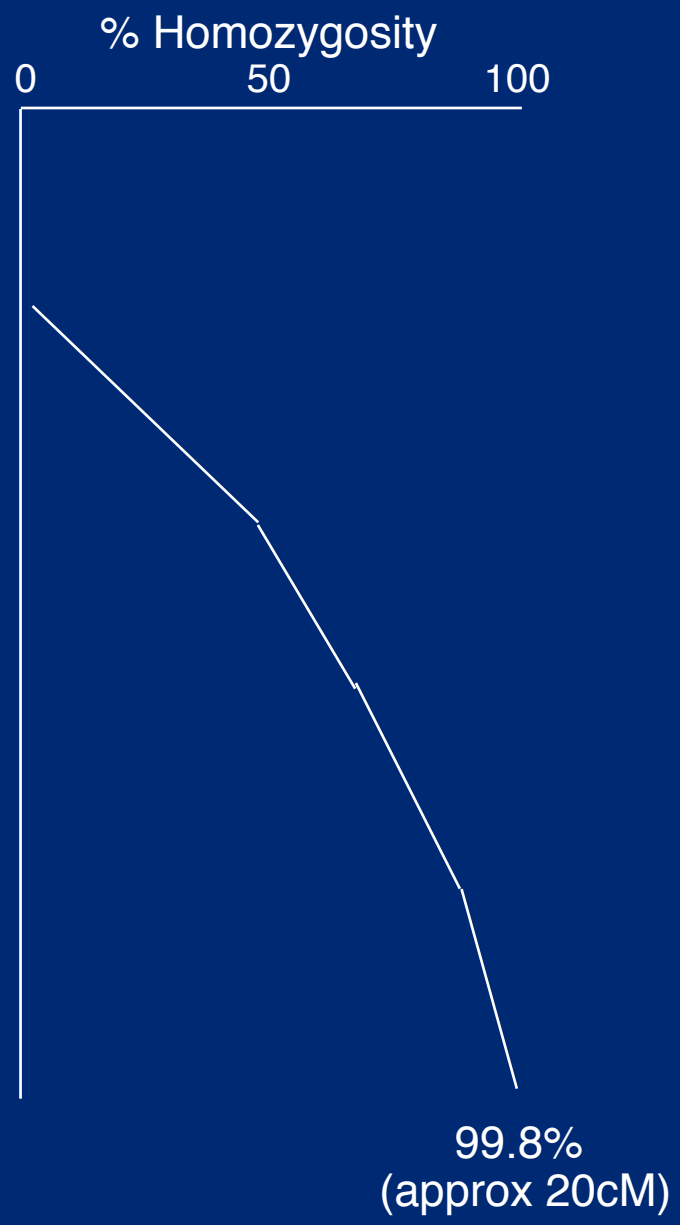
- Can assess if trait is dominant vs recessive.
- Can assess if trait is single vs multiple genes.
- Can assess if the penetrance of a phenotype is affected by the genetic background.
- Genetic mapping

Congenic Strains

- Inbred mice which differ with respect to only one chromosomal region.
- Can transfer specific chromosomal region for one strain onto the genetic background of another strain.
- Start with F1
- Successive backcross to one parental strain (strain to which you are transferring the chromosomal region) until only the region of interest remains.
- Inbreeding >20 generations.

Making a Congenic Strain





Uses of congenic mice

- Compare same gene on different backgrounds
  - *xid*
- Compare mice with same background with different alleles of the same gene
  - H-2 haplotypes
  - Ly5.1 vs Ly5.2 (Ly5 = CD45)

Speed congenics

- Wakeland E, Morel L, Achey K, Yui M, Longmate J.Immunol Today. 1997 Oct;18(10):472-7.
- JAX Communications #6 Nov 2001, www.jax.org/jaxmice/services/speedcongenic

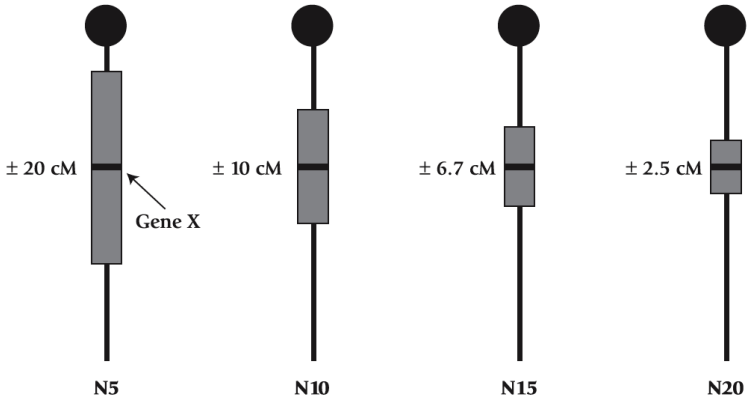


Figure 1b. Average length of surrounding linked DNA with increasing generations of backcrossing.

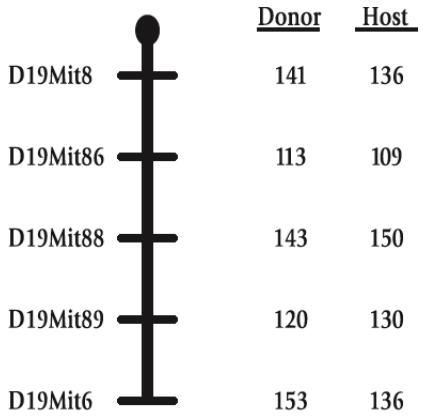


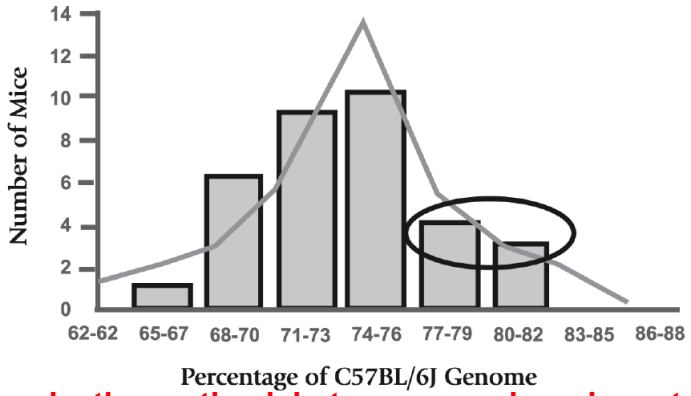
Figure 2. Chr 19 DMit marker product sizes for donor: 129P3/J and host: C57BL/6J

First generation:

6000 DNA microsatellite markers (data is accessible through the Mouse Genome Database at *www.informatics.jax.org*). Microsatellite markers are dinucleotide repeats present in noncoding regions of the genome. Inbred strains often differ from each other in the number of dinucleotide repeats that are amplified by many of the microsatellite marker primers. These marker differences are called simple sequence length polymorphisms (SSLPs).

Current:

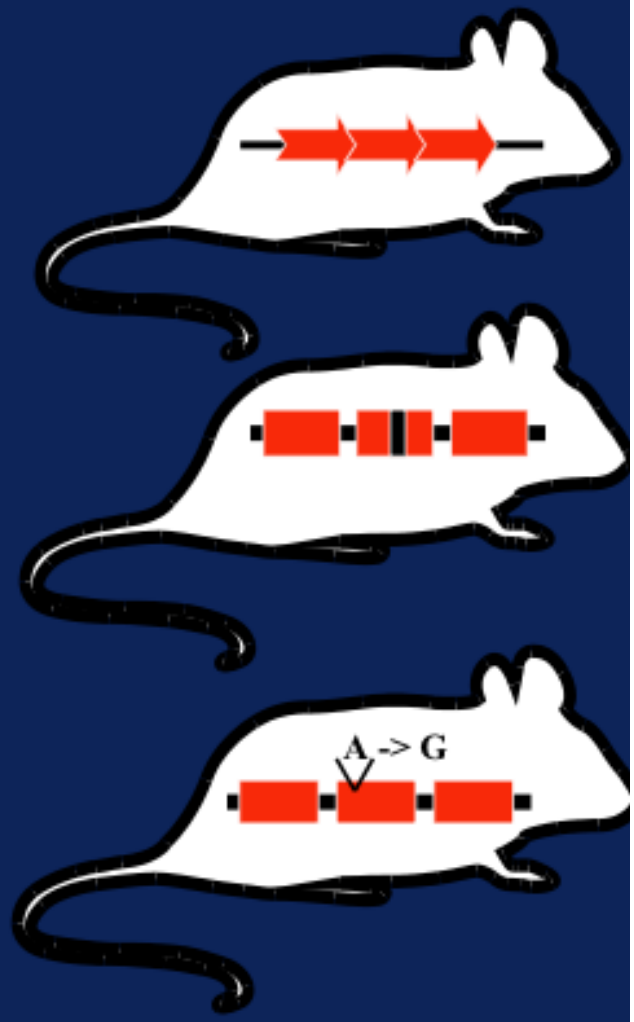
use markers from an established and validated database of 2,199 single nucleotide polymorphism (SNP) markers



By selecting optimal, heterozygous breeders at each backcross generation, it is possible to reach 99% recipient strain genomic identities after five generations (N5) (~12-16 months).

Genetic Engineering Technologies

- **Transgenesis**
  - Adding new genetic material
- **Homologous Recombination**
  - Targeting a specific gene using ES cells
- **Random Mutagenesis**
  - Altering genetic material via chemicals or irradiation



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Generation of transgenic mice

- Pronuclear microinjection
  - Random insertion
  - Integration of multiple copies
- Tissue specific expression
- Controlled, inducible expression
  - E.g. ER/tamoxifen, Tet-On/Off
- Usage
  - Study of gene function
  - Modeling diseases (introduction of diseas-associated allele)

Basic Transgenic Construct

Promoter
(to direct
tissue-specific
expression)

Intron
(to improve
transcript
expression)

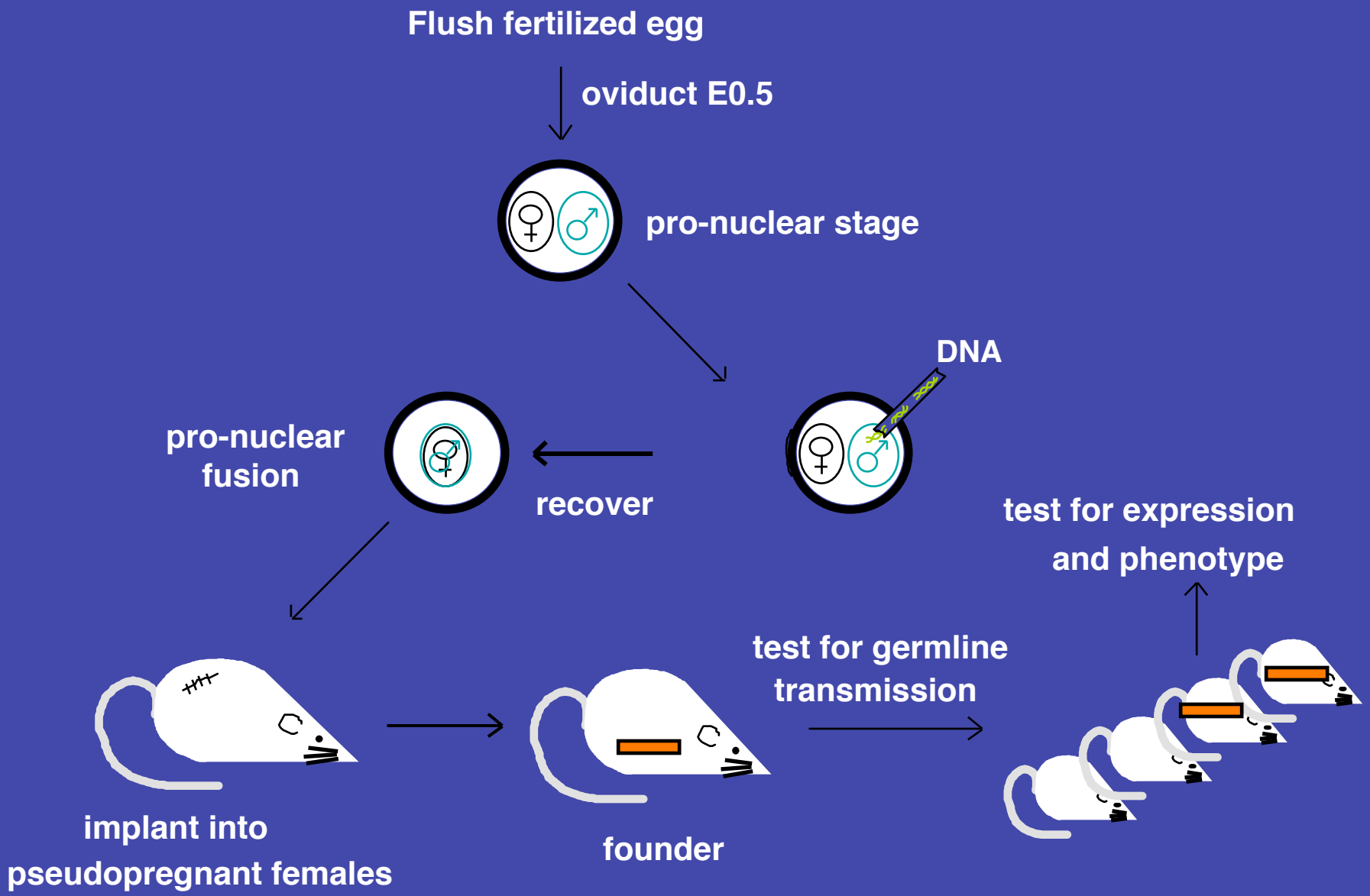
PolyA
(stabilize mRNA)

Construct can be up to 50 kb; larger constructs possible for BAC transgene

Useful for:

- Studying gene expression profiles
- Over-expression
- Dominant negative
- Complementation

Steps in the construction of transgenic mice



Basic Transgenic Mouse Considerations

Important Aspects:

- Stable integration occurs in 10-40% of mice
- Most integration at the 1-cell stage, so all cells receive the transgene
- In 20-30% of cases, integration may occur at later stages, resulting in mosaicism

More Important Aspects to remember for transgenic mice:

- Number of copies per cell can be in the hundreds, so different strains/lines will have different levels of expression - choose a variety of lines

- Many transgenes show appropriate expression patterns, relatively independent of the site of integration

- Local chromatin structure and regulatory elements can influence transgene expression

- Transgenes can disrupt endogenous genes at the site of integration

Limitations to transgenics

- Position effects
- Multiple founder lines must be compared
- Expression levels may not correlate with copy number
- Rearrangements can complicate analyses
- Regulatory sequences for your gene of interest may not be located on the transgene
  - Solution: Targeting the Rosa26 locus
  - Allows ubiquitous and constitutive expression of transgene without disrupting endogenous gene function

Uses of transgenic mice

- Gain of function
  - Monoclonal Ig or TCR repertoire
  - Test cis-acting sequences for tissue-specificity
  - recombination substrates for V(D)J, switch
  - Test the ability of cloned genes to complement
  - Make cell lineage markers using reporters

Uses of transgenics, II

- Loss of function
  - Insertional mutagenesis (gene trap)
    - Consortium formed that uses lacZ gene trap (KOMP, EUCOMM, etc.)
  - Transgene marks the disrupted gene for cloning
  - Antisense or RNAi to ablate gene expression
  - Dominant-negative
  - Cell lineage ablation (e.g. TK gene + gancyclovir)

BAC transgenes

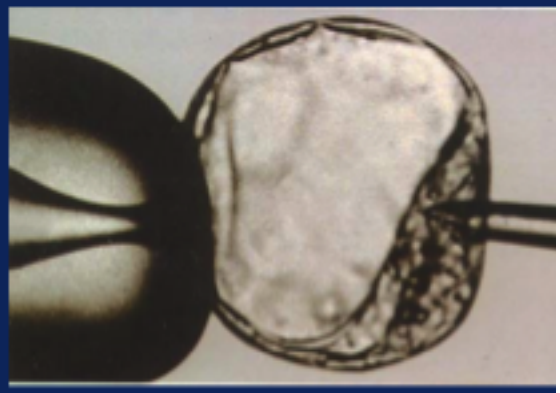
- Bacterial artificial chromosomes
- Up to 2 MB insertions
- Allows study of transgenes with endogenous elements that control their expression
- Position effect is less of an issue
- Tend to be copy# independent
- E.g. “humanized” mice (Ig loci)

See Sparwasser and Eberl review
Immunology. 2007 Jul;121(3):308-13

Knock-out mice - gene targeting via homologous recombi

- **Targeted mutations**
  - null mutation ("knockout")
  - specific alterations in gene
- **Process**
  - *in vitro* gene targeting in ES cell lines
  - Blastocyst injection
  - Production of mice carrying mutation (chimeras)
  - Generation of strains
- **Uses**
  - Disease Models
  - Drug Discovery
  - Conditional Mutagenesis

Blastocyst Injection



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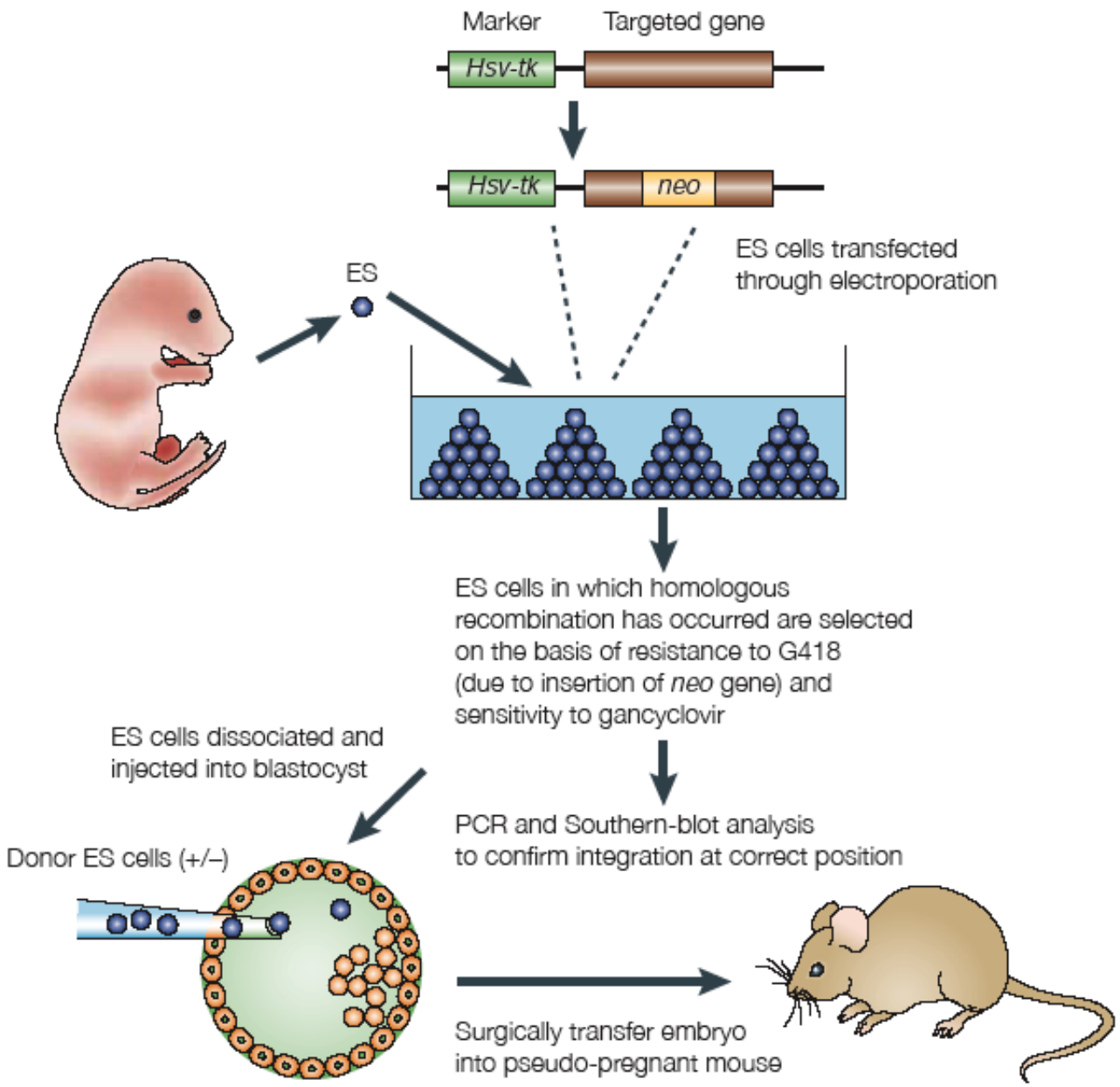
Classical Knockout Construction

Important Aspects:

- Length of homology on either side of the disrupted locus is important, ~5-10 kb required
- Source of the targeted gene sequence is also important

  129/Sv vs. C56BL/6N
- The local chromatin structure may influence targeting
- Clones are screened by Southern hybridization and PCR
- It is important to remove the selection markers (e.g. Neomycin resistance gene)

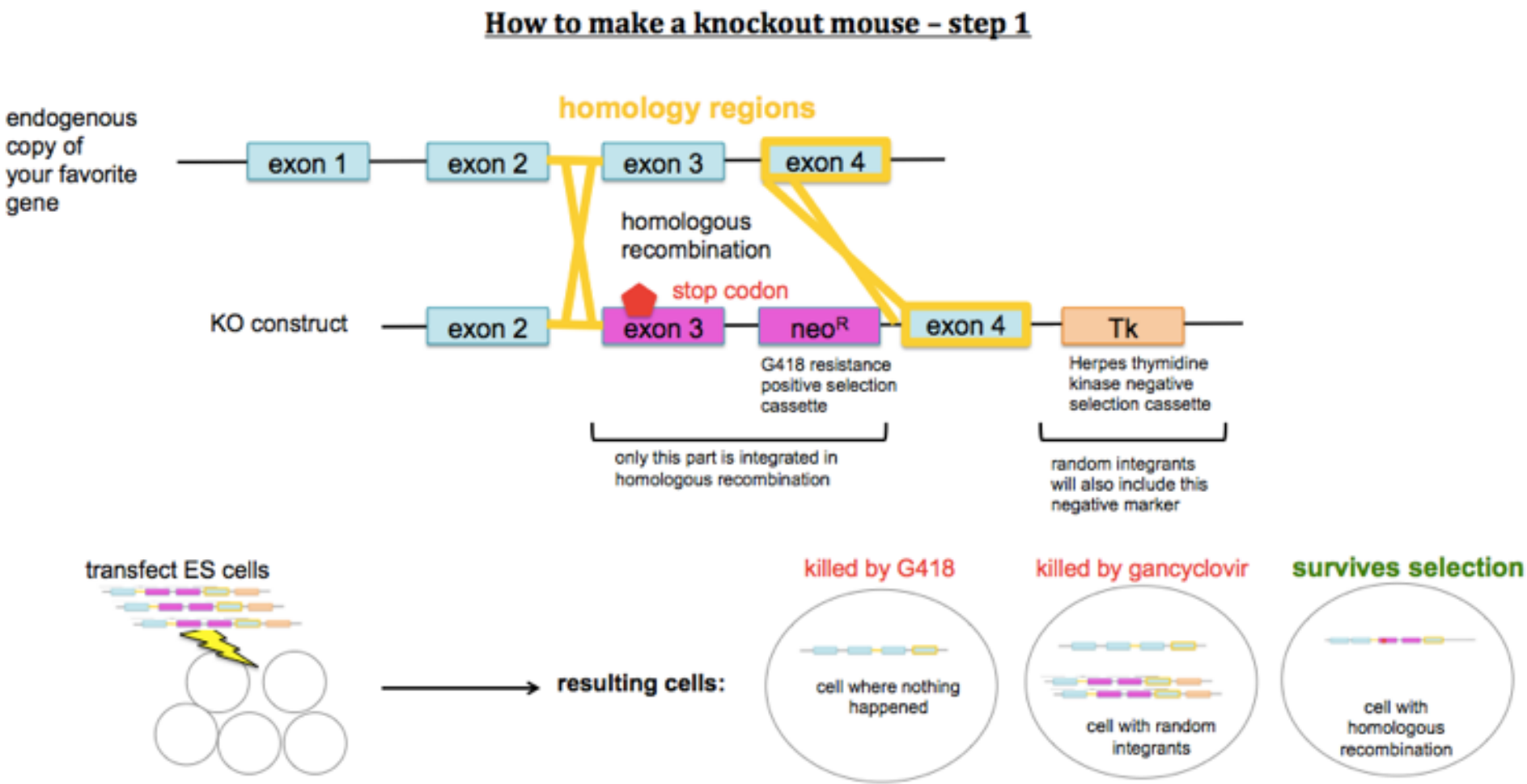
Overview of procedures for the production of knock-out mice via targeting



Positive Negative Selection

- An additional marker gene cassette is placed at one end of the construct, outside the homology region
- HSV*tk* is the most commonly used, conferring sensitivity to gancyclovir
- Two possible integration outcomes:
  - RANDOM INTEGRATION - retention of positive selection cassette and *tk*, resulting in lethality in the presence of gancyclovir
  - HOMOLOGOUS INTEGRATION - positive selection cassette will integrate into the target locus, while the *tk* is lost because of lack of homology

Gene Replacement Vector

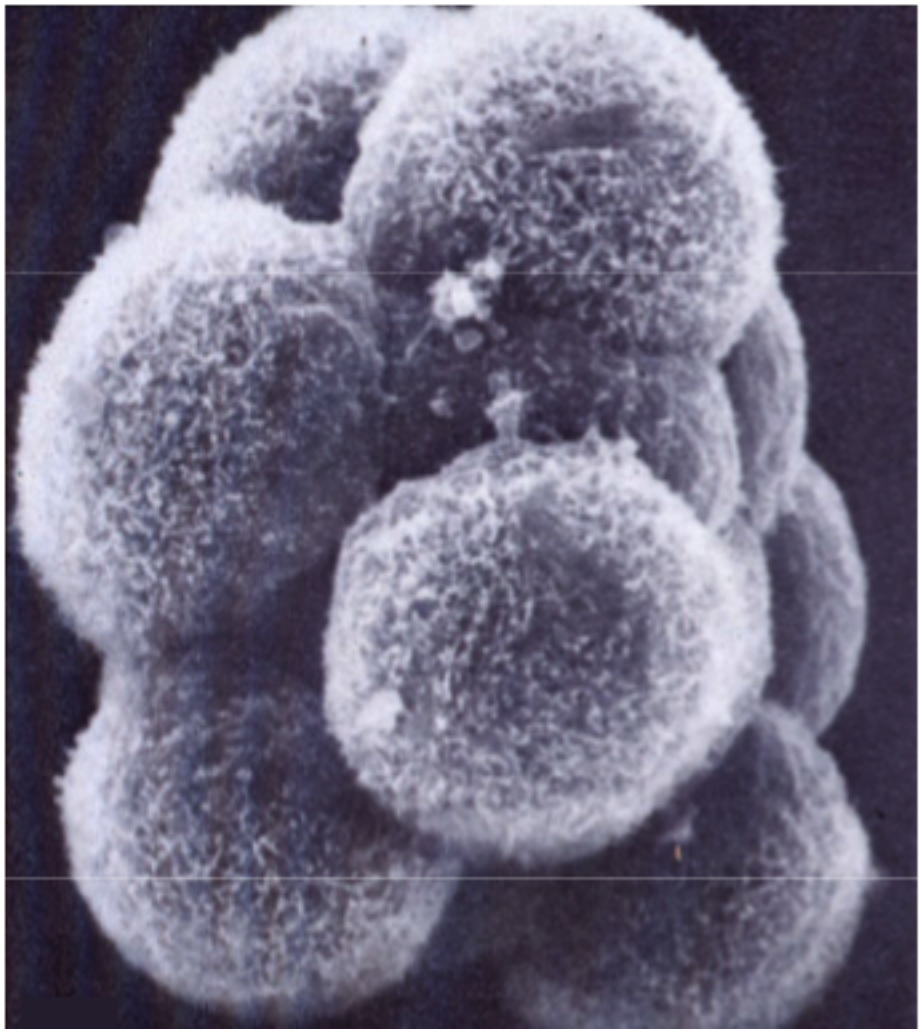


Positive selection of ES cell clones

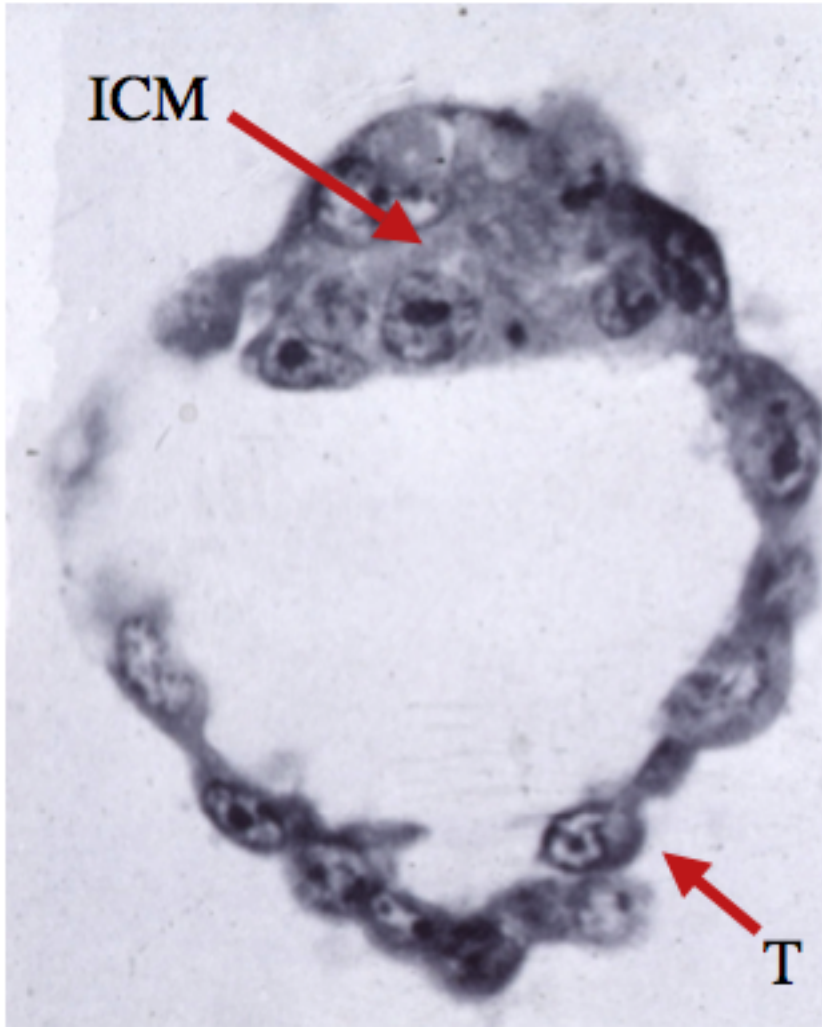
Drug Resistance Marker Genes for Positive Selection :

- The most commonly used markers are neomycin phosphotransferase (*neo*) and hygromycin B phosphoglycerate (*hyg*)
- Confers resistance to the neomycin analog, G418, or hygromycin, respectively
- Use of Reporter gene cassettes: GFP, LacZ and, βGeo

Morula (8-16 cell)



Blastocyst (32-64 cell)



Embryonic stem cells

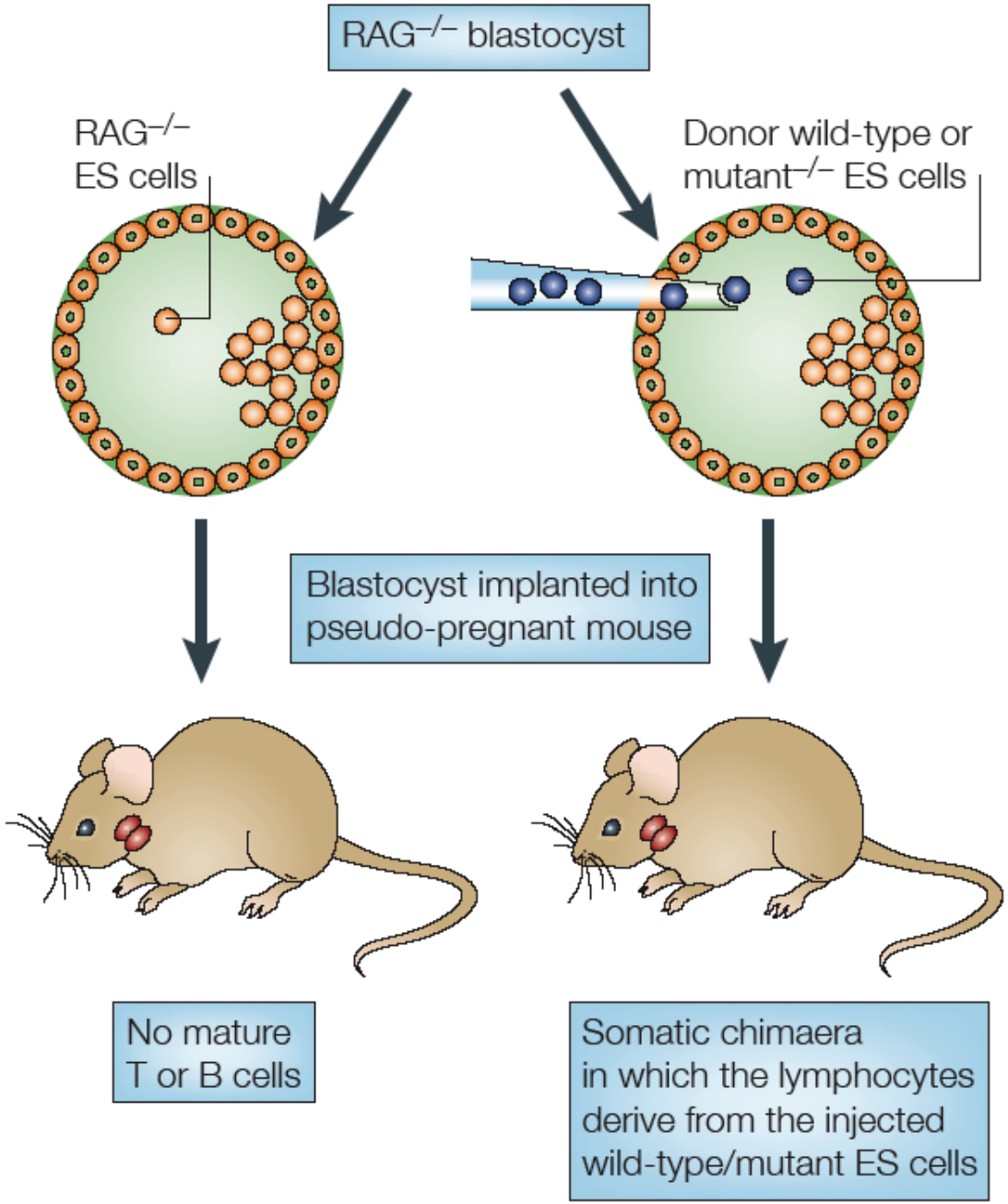
- ES cells are derived from blastocysts
- Retain toti-potency
  - can contribute to all tissues, including germ line
- Can differentiate *in vitro* into hematopoietic cells
  - Useful if mutation is embryonic lethal

Strategies to overcome embryonic lethality

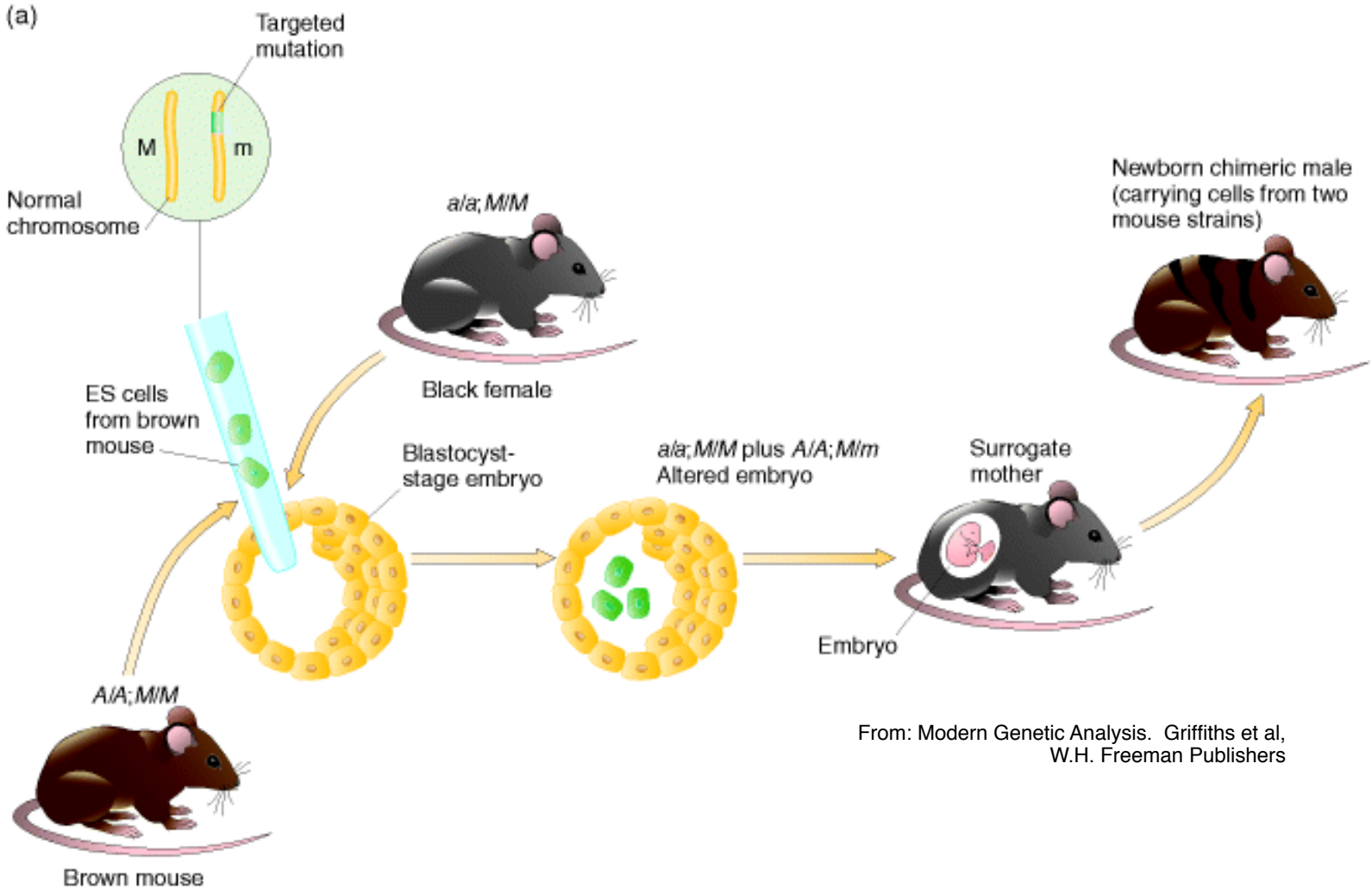
- In vitro differentiation of ES cells into different hematopoietic cells
- Generation of fetal liver cells from e13 embryos
  - Transfer to sub-lethally irradiated host to regenerate the hematopoietic system
- RAG2-/- complementation
  - Immune system develops from KO mouse

RAG2-/- complementation

- Rag2 is required for V(D)J recombination
- Rag2-/- mice: complete block in B and T
- Transfer ES cell to rag2-/- blastocyst
- Fast, analyze chimeras
- To assess degree of chimerism
  - Use C57Bl/6 rag2-/-: Ly9.2, H-2^b
  - ES from 129 are Ly9.1, H-2^k
  - Use FACS to determine the fraction of ES-derived cells

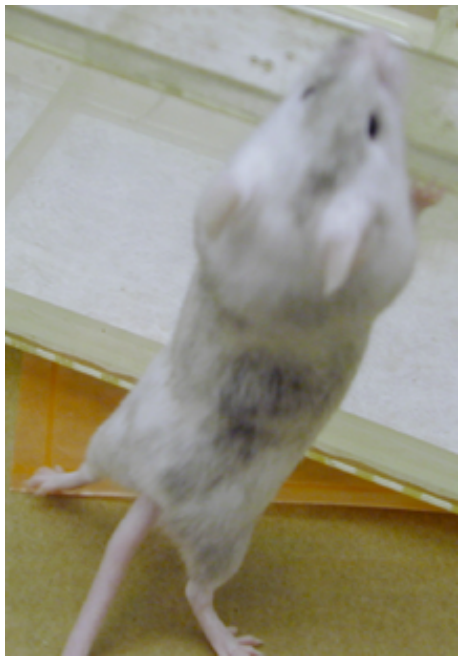

RAG−/− blastocyst
RAG−/− ES cells
Donor wild-type or mutant−/− ES cells
Blastocyst implanted into pseudo-pregnant mouse
No mature T or B cells
Somatic chimaera in which the lymphocytes derive from the injected wild-type/mutant ES cells

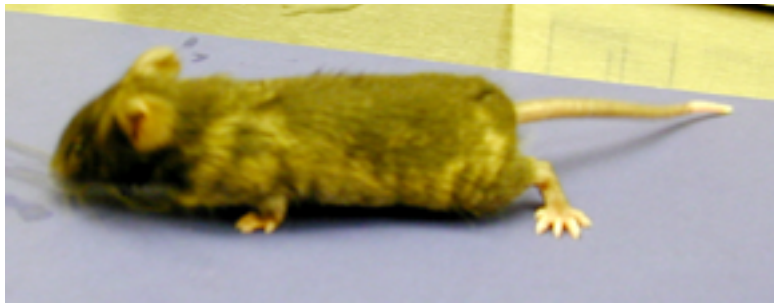
Basic Principles of Gene Knockouts

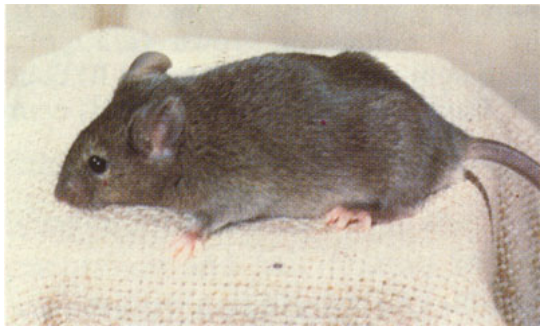


From: Modern Genetic Analysis. Griffiths et al, W.H. Freeman Publishers

Coat Color Chimeras:

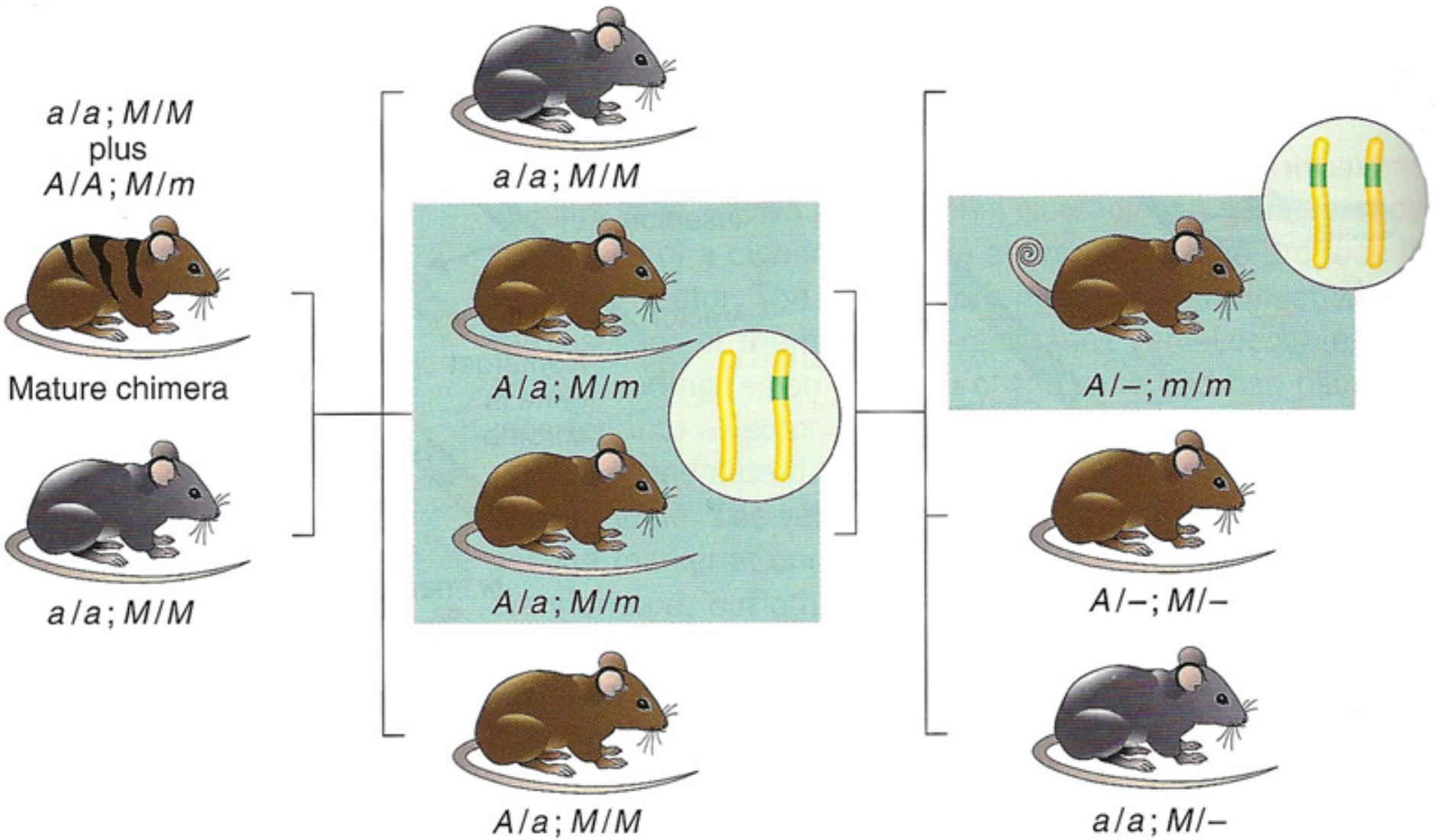






Agouti mouse

Breeding chimeric mice to recover knockout



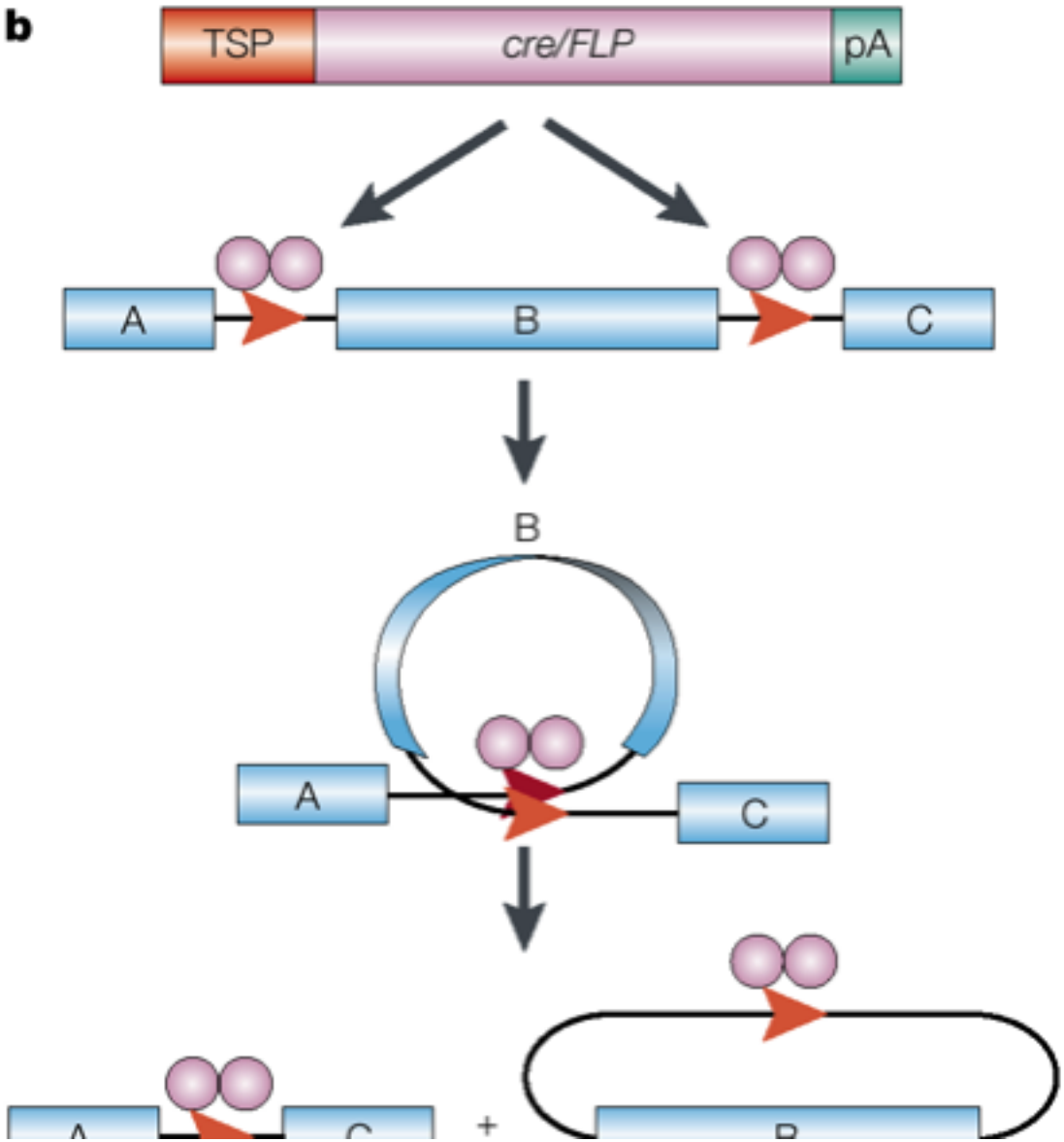
Using recombinases for designer KOs

- Tissue-specific KO's
- Other strategies for conditional KO's
- Regulate timing in development
  - Can overcome embryonic lethality
- Can be used to manipulate transgenes also
  - Tissue-specific expression

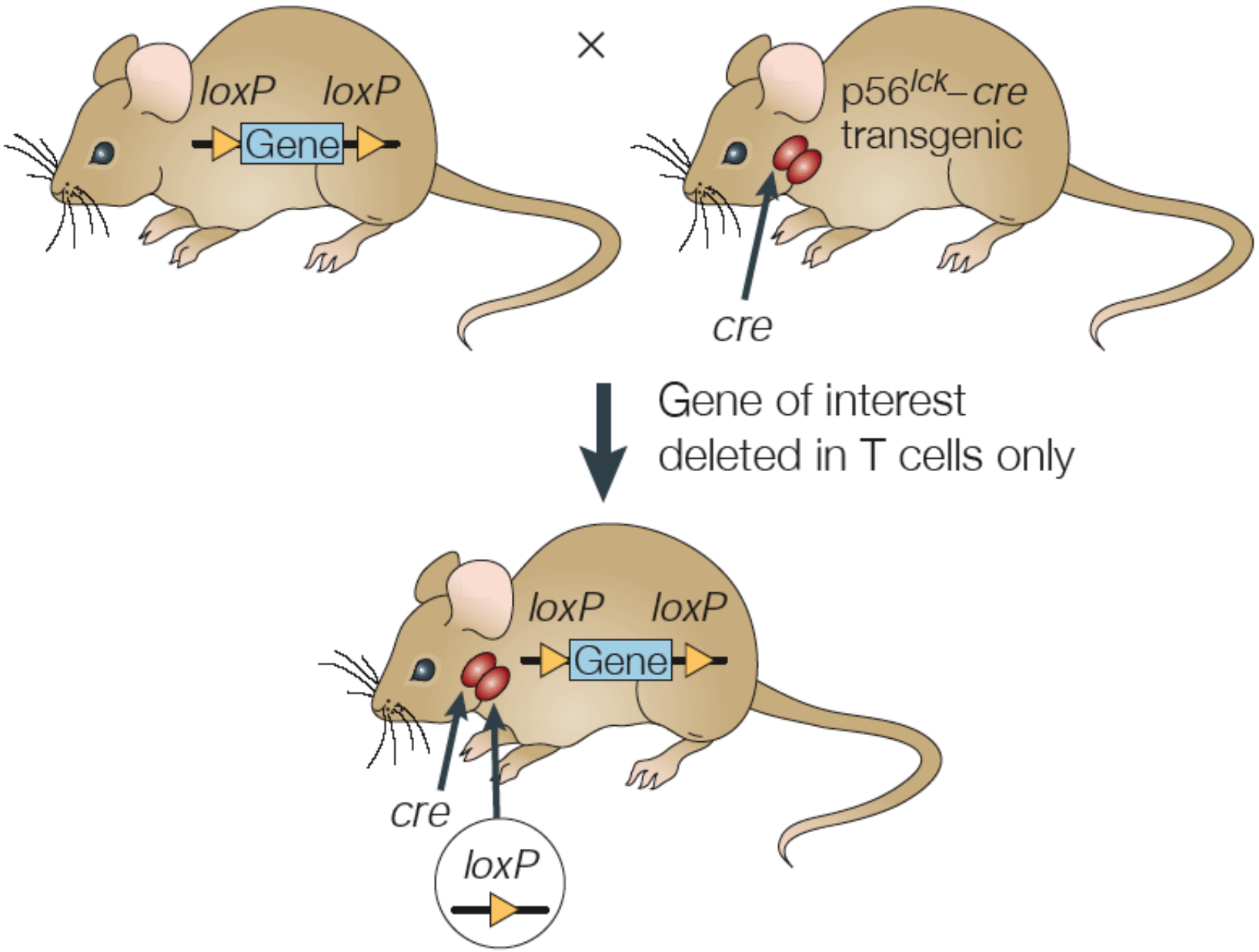
DNA Recombinases

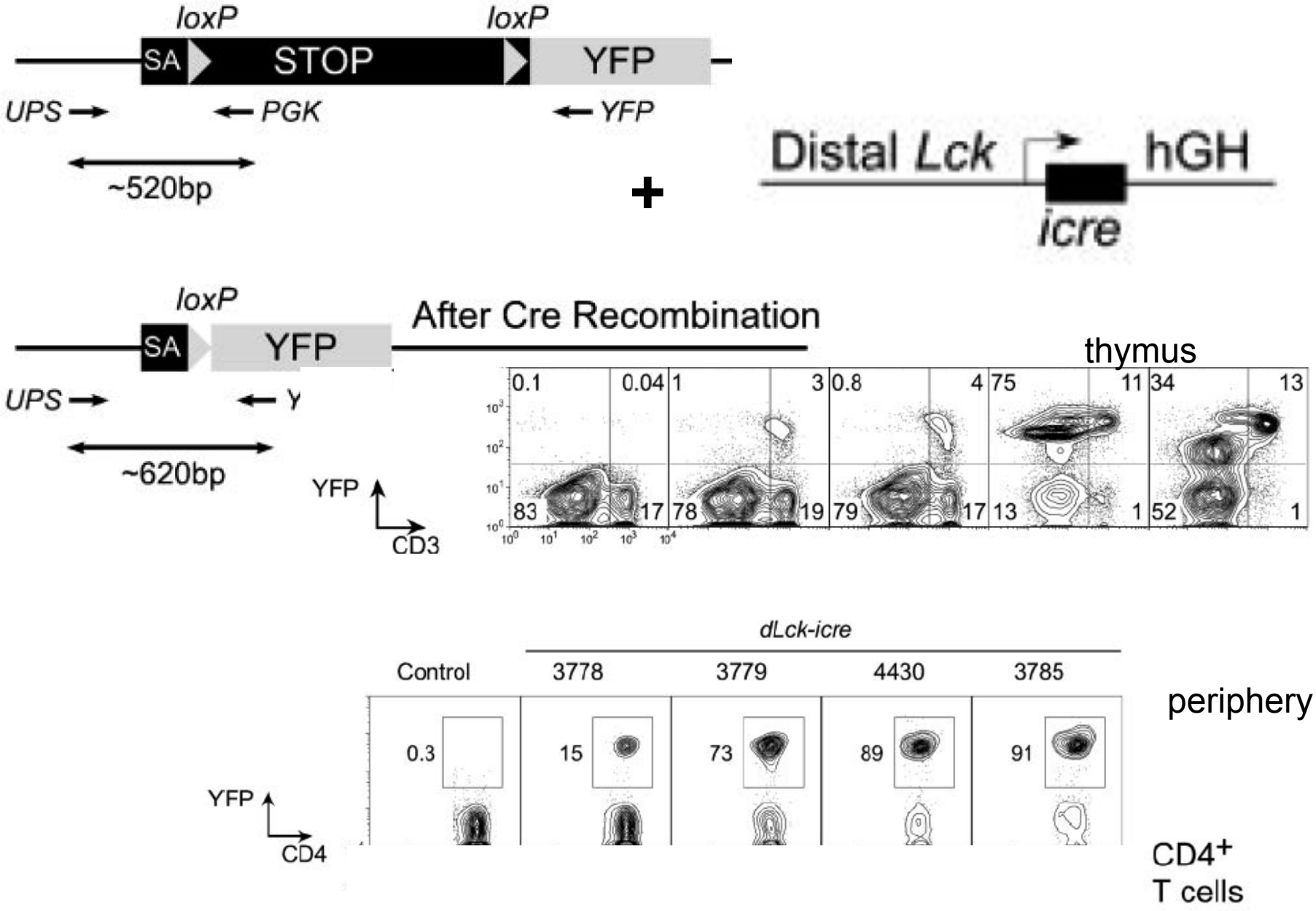
- No Co-factors required
  - 34 base pair recognition Sequence
  - directionality
- Cre
  - Bacteriophage P1
  - Recognizes LoxP site
- Flp
  - S. cerevisiae
  - Recognizes Frt site

Cre/Flp

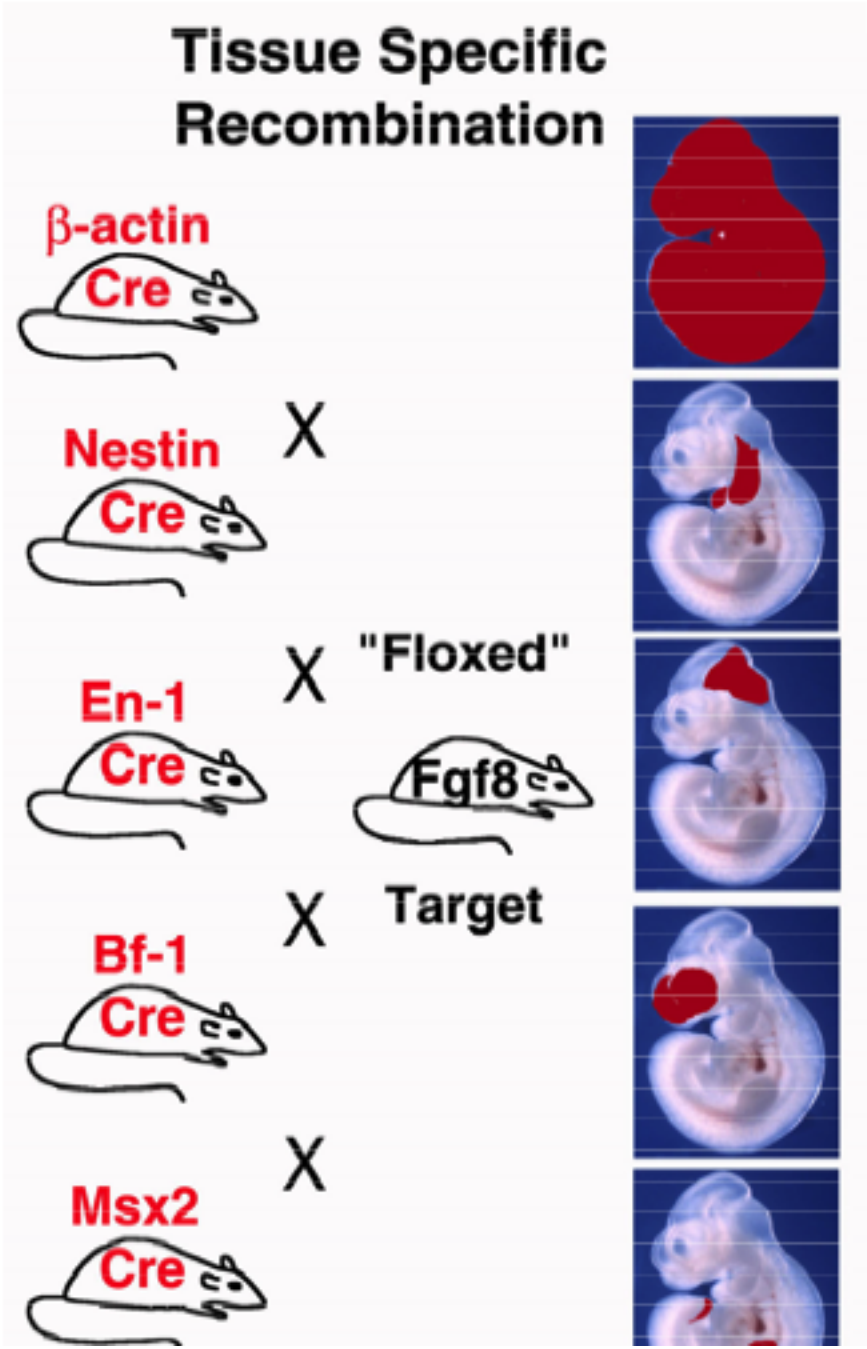


Tissue-specific deletion using lck-cre transgene





J Immunol. 2005 Jun 1;174(11):6725-31.



Fgf8 Expression

Vagaries of conditional gene targeting

Schmidt-Supprian & Rajewsky NI 8:665 2007

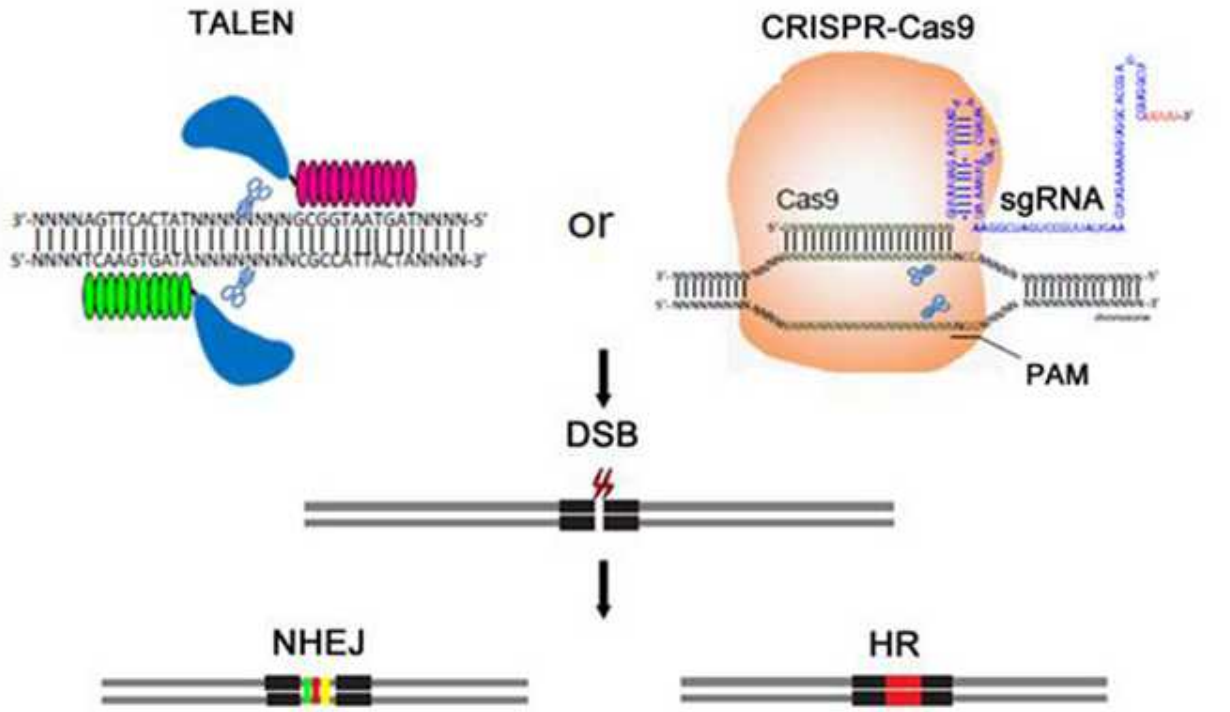
- Fieldity of Cre expression
- Efficiency of cre-mediated deletion
- Cre toxicity
- need Cre Tg control

Creation and use of a cre recombinase transgenic database.

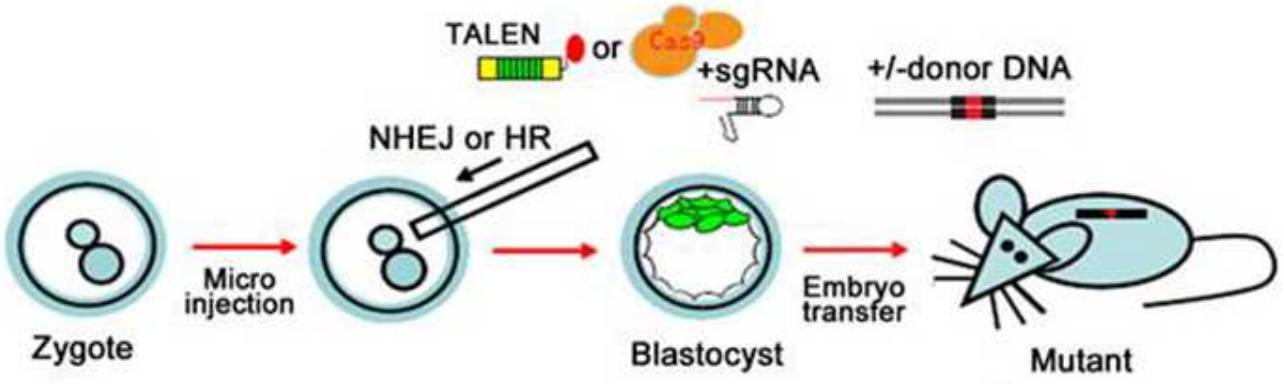
Nagy A, Mar L, Watts G.

Methods Mol Biol. 2009;530:365-78. Review.

Overview of genome editing by TALEN and CRISPR-Cas9



One-step generation of mice with genome modifications



Random Mutagenesis: create a library of mutant alleles

ENU mutagenesis centres
www.mouse-genome.bcm.tmc.edu
www.mgu.har.mrc.ac.uk/mutabase
www.jax.org/nmf
www.gsf.de/ieg/groups/enu-mouse.html
http://jcsmr.anu.edu.au/group_pages/mgc/MedGenCen.html
http://cmhd.mshri.on.ca
http://www.**tnmouse.org**

See also
Peters LL etal Nature Genetics 8: 58-69. 2007.
Acevedo-Arozena etal Ann. Rev Genomics Hum Genetics 2008 9:49-69

Means of inducing mutations in mice

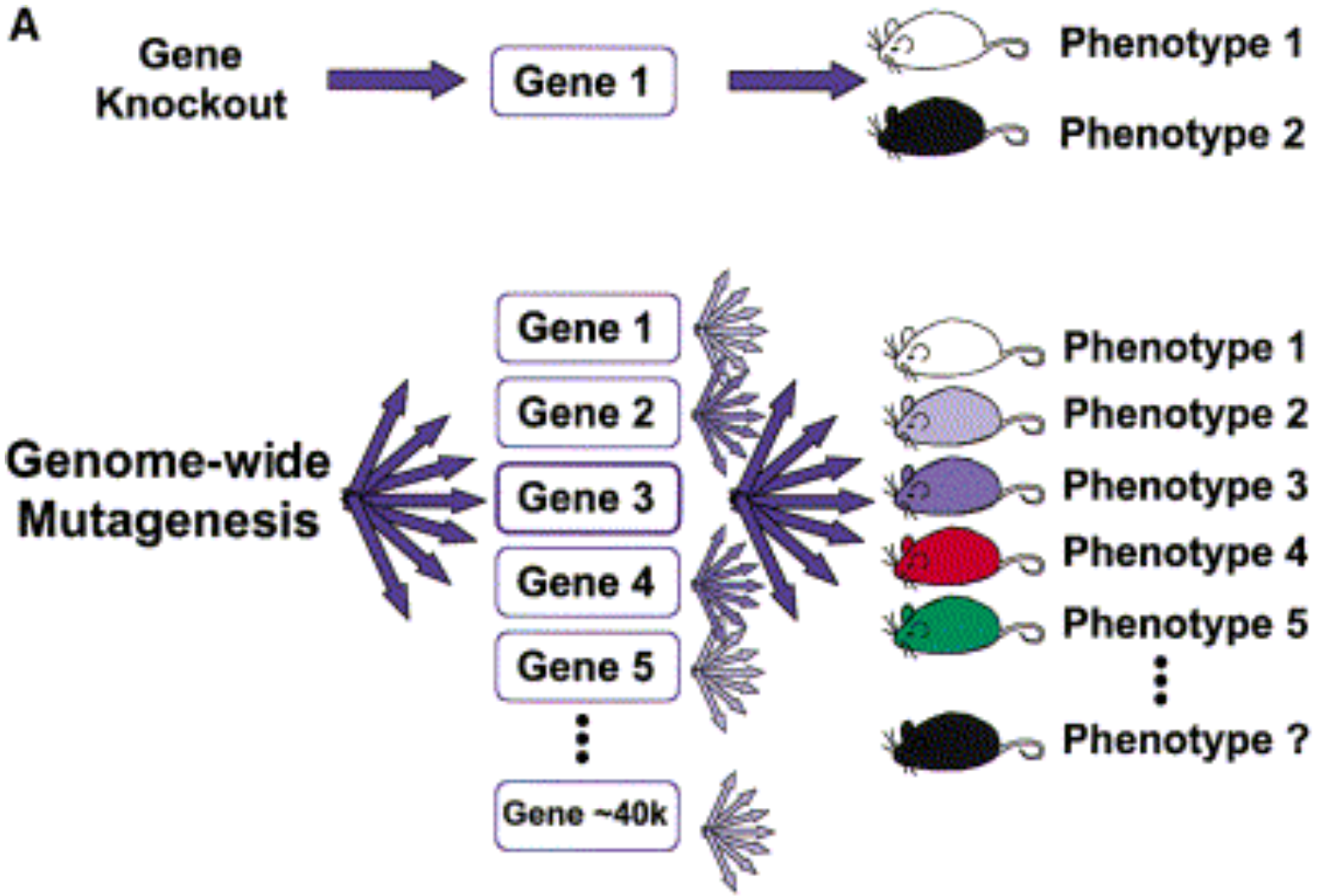
Agent	Dose	Target	Mutation rate	Type of mutation
X-rays	4-6 Gys	spermatogoneal cells	50 x10^{-5}	Large lesion, others
		oocytes	19 x10^{-5}	
Chlorambucil	10 mg/kg	postmeiotic cells	127 x10^{-5}	Large lesion, others
Procarbazine	600 mg/kg	spermatogoneal cells	5 x10^{-5}	Large lesion, others
ENU	**3X100 mg/kg**	**spermatogoneal cells**	**150 x10^{-5}**	**Base subst., fine lesions**
none			0.5-1 x10^{-5}	varies

ENU can transfer its ethyl group to oxygen or nitrogen radicals in DNA, resulting in Miss-pairing and base-pair substitution if not repaired.

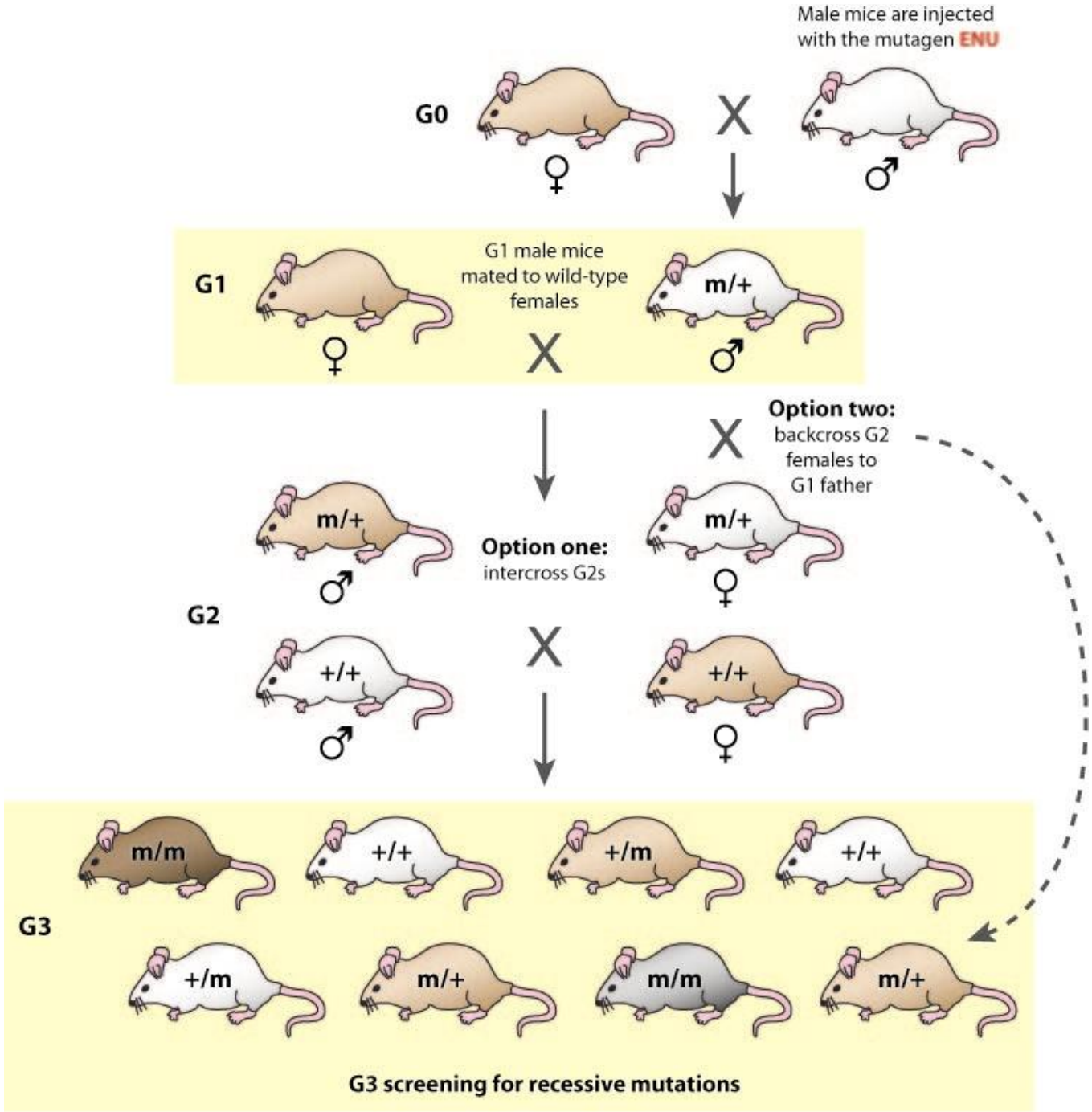
The highest mutation rates occur in pre-meiotic spermatogonial stem cells, with single locus mutation frequencies equivalent to obtaining a mutation in a single gene of choice in one out of every 175-655 gametes screened.

At this mutation rate, there are multiple mutations per gamete.

Multiple hits per gamete can reveal gene interactions

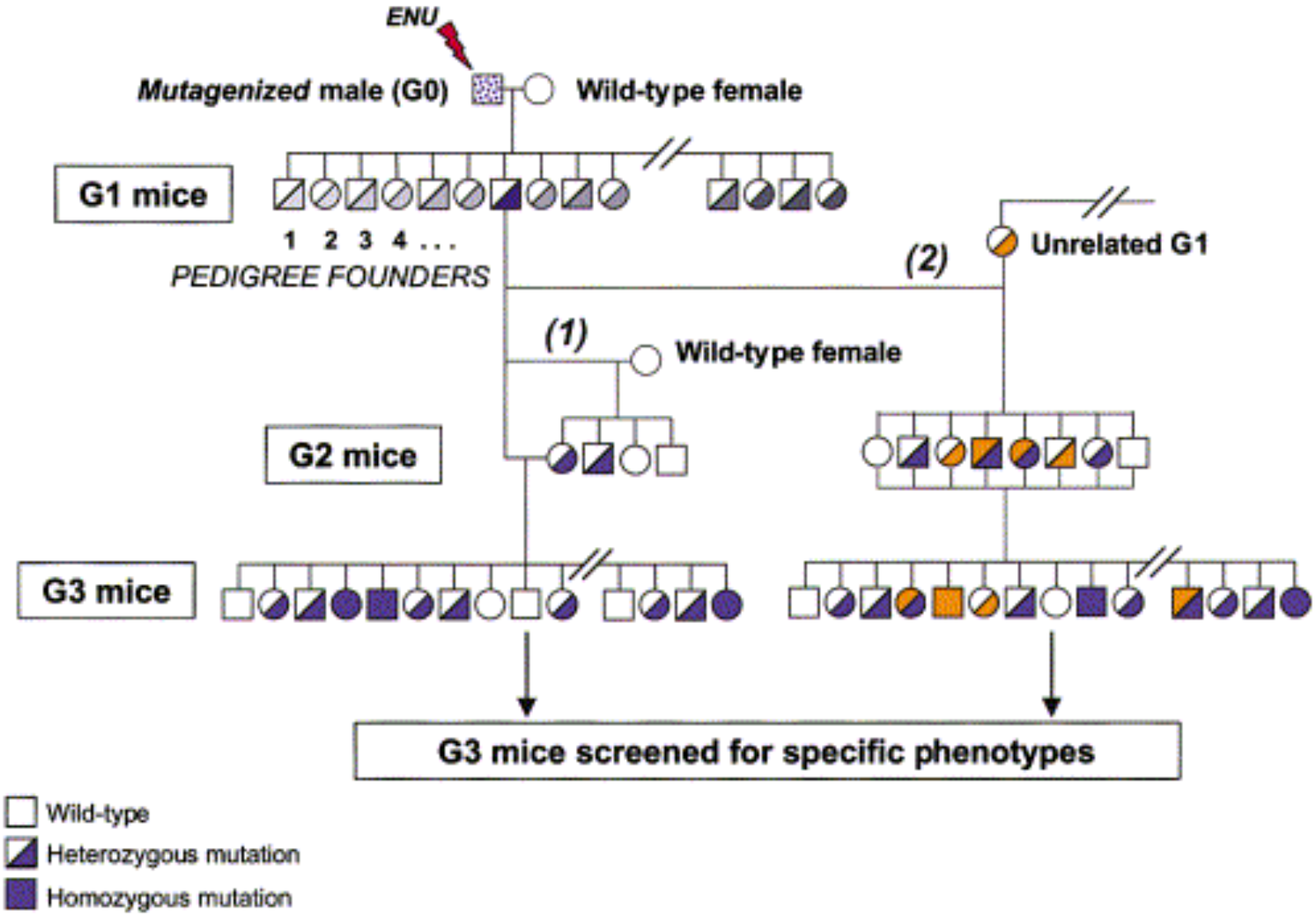


Nelms & Goodnow CC Immunity 15:409-418 2001



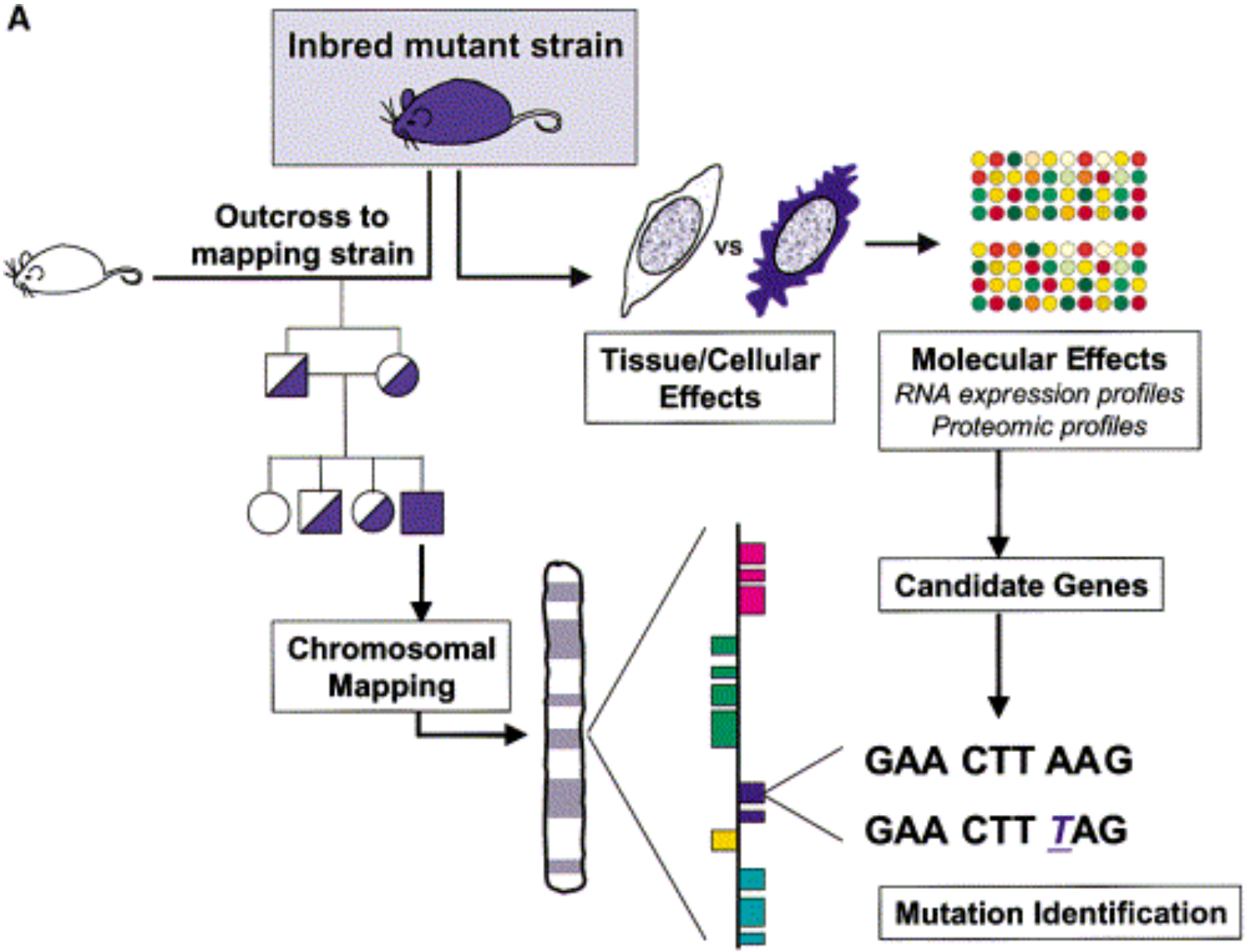
Acevedo-Arozena A., et al. 2008

GENERALIZED MATING SCHEME FOR ENU-INDUCED MUTANTS



Nelms & Goodnow CC Immunity 15:409-418 2001

OVER-VIEW OF STRATEGY FOR SCREENING AND GENE MAPPING



Nelms & Goodnow CC Immunity 15:409-418 2001

The forward genetic dissection of afferent innate immunity.

Beutler B, Moresco EM.

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ENU mutagenesis in mice.

Georgel P, Du X, Hoebe K, Beutler B.

Methods Mol Biol. 2008;415:1-16.

Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B.

Science. 1998 Dec 11;282(5396):2085-8.