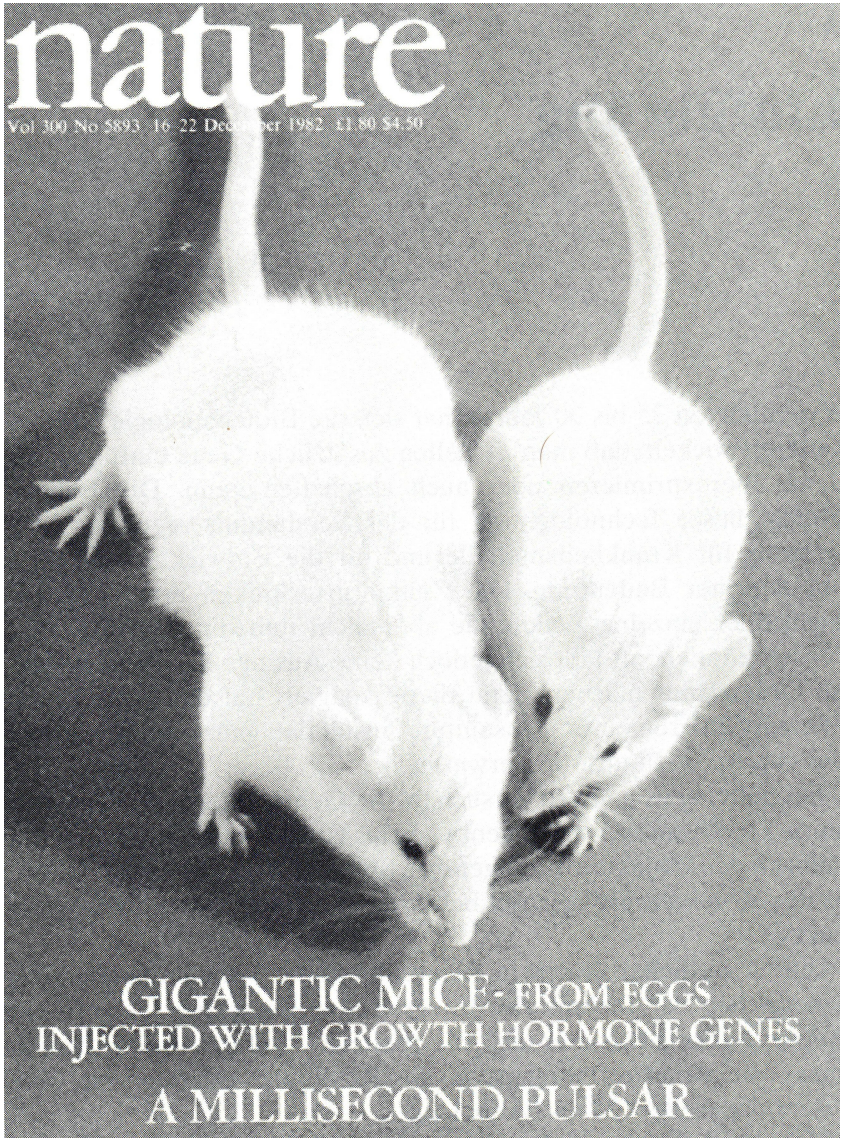
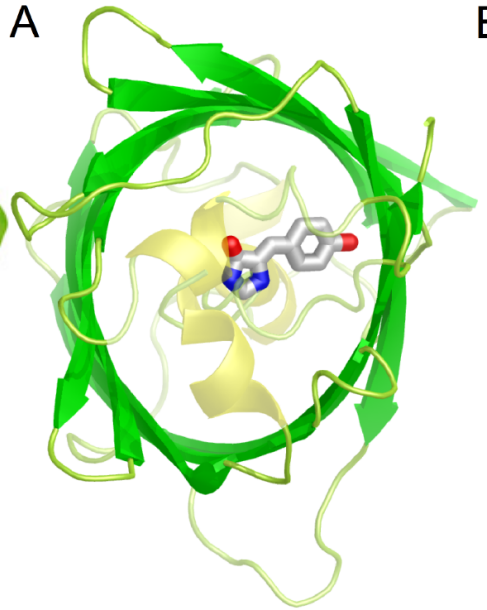
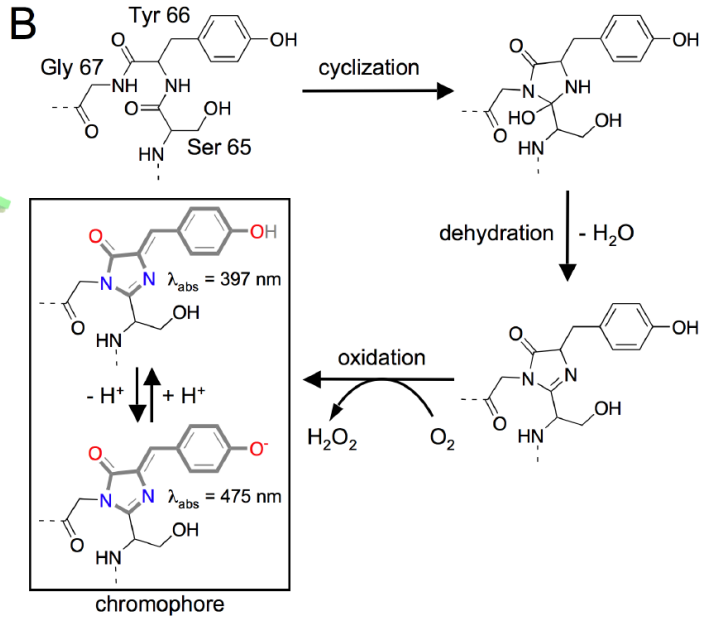
Genetische Modelle in der Immunologie



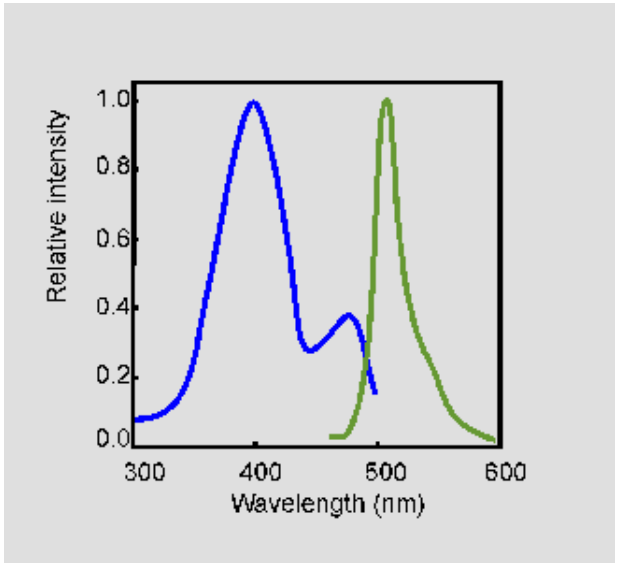
Priv.-Doz. Dr. Michael Stassen

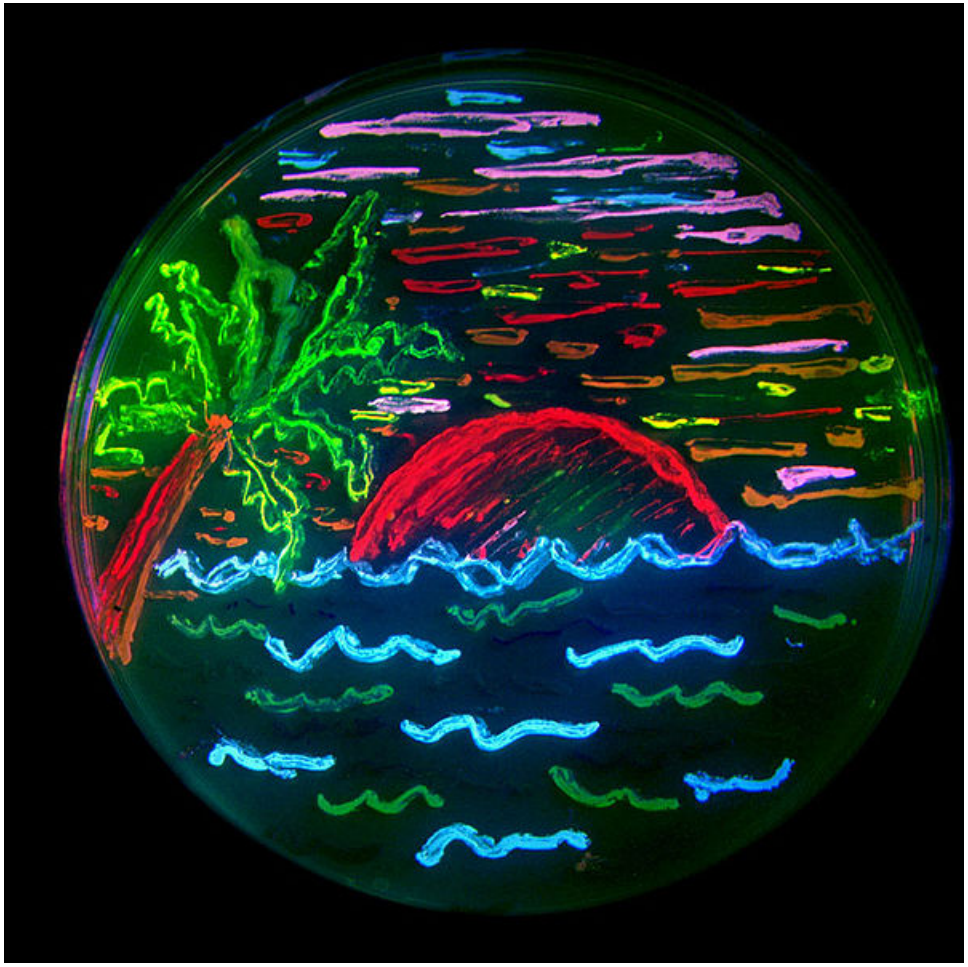
INSTITUTE FOR IMMUNOLOGY MAINZ





(E)GFP: (Enhanced) Green Fluorescent Protein
238 aa Protein aus der Qualle *Aequorea victoria*





GFP-Mutanten und dsRed (Korallen-Spezies)

Reportergene

Vorlesungsinhalt

- Kongene, isogene und Inzuchtstämme der Maus
- Einschleusen von DNA in Zellen *in vitro* und in die Keimbahn *in vivo*
- Herstellung transgener Mäuse
- „Gene targeting“ klassisch: „Knock out“ und „Knock in“
- Konditionale Mutagenese: Das cre / loxP-System
- Gezielte Zell-Ablation: Diphterietoxin / Diphterietoxin-Rezeptor System
- „Gene targeting 2.0“: Das CRISPR/Cas9-System

CRISPR gene-editing tested in a person for the first time
The move by Chinese scientists could spark a biomedical duel between China and the United States.
David Cyranoski
15 November 2016

Maus: Zucht und Genetik

Mausgenom: 1650 cM, 20 Chromosomen (haploid), 20.210 Gene (Mensch: 19.042)

15.178 Gene bei Maus und Mensch haben gemeinsame Vorläufer (orthologe Gene)

(Trennung Nager / Primaten vor etwa 70 Millionen Jahren)

Dies sind rund 75% der Maus- und 80% der menschlichen Gene

Generationszeit etwa 3 Monate

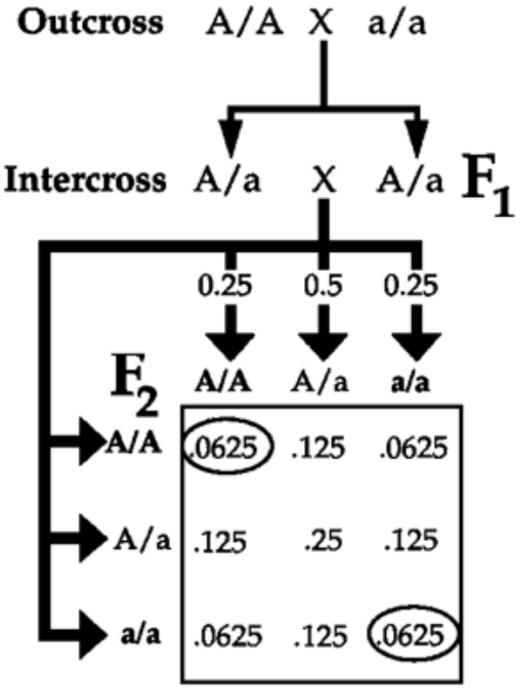
Bis zu 8 Würfe mit bis zu 8 Jungen pro Jahr möglich

Geschlechts-/Zuchtreif mit 6/8 Wochen

Tragzeit 21 Tage

Inzuchtstämme

Mind. 20 Bruder x Schwester Paarungen mit zunehmender Homozygotie (!)
Problem: Zucht-Depression versus Heterosis



98,7%

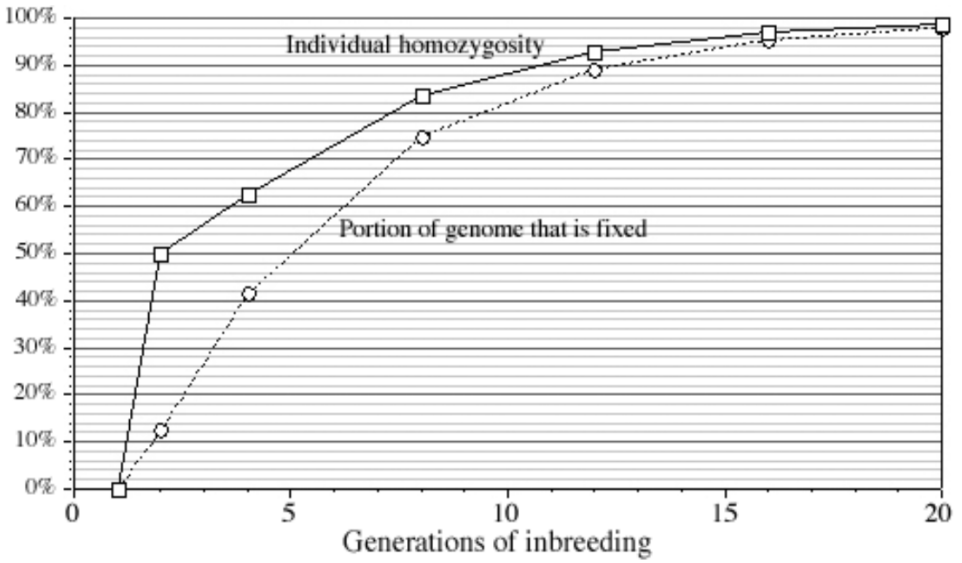


Figure 3.1 Consequences of inbreeding at the F_2 generation. The first cross illustrated is an outcross between animals homozygous for alternative alleles at the *A* locus. The F_1 offspring are all identically heterozygous *A/a*. An intercross between two F_1 animals will produce an F_2 generation with three possible genotypes havingthe probabilities shown. The final box indicates the different combinations of F_2 by F_2 matings that are possible and the probabilities associated with each one. The two mating combinations that cause fixation for one allele are circled. This illustration of the process of inbreeding is based on two simplifying assumptions: (1) genetic homogeneity in each of the original parents used for the outcross, and (2) contrasting alleles at every locus in these original parents. In actuality, inbreeding is often begun with parents that, on the one hand, are not homogeneous but, on the other hand, do share many alleles with each other.

Lee M. Silver *Mouse Genetics*, online available at Mouse Genome Informatics (informatics.jax.org)

Table 3.1 Some important inbred strains.
See also Inbred Strains of Mice and Rats at MGI.

Strain	Color	Major use	Other Charateristics	Origin	Generation[a]
129/Sv-*Sl*/+, c^+p^+ (*129*)	Agouti	Source of most ES cell lines and genetic material used for homologous recombination. Used of studies of embryology and reproduction.	Relatively high testicular teratoma incidence. Relatively small size. Resistant to radiation.	~1930 (Dunn)	F79 (JAX)
BALB/c	Albino	Used in immunological studies and for the production of hybridomas. A new congenic strain BALB/cByJ-Rb(8.12)5Bnr available from JAX is most efficient for hybridoma production.	Docile females. Males of the J substrain only are extremely aggressive. Relatively poor breeders, but variation among sublines. Sensitive to radiation.	1913 (Bagg)	F105 (CR) F180 (JAX) F195 (Taconic)
C57BL/6 (B6)	Black	Standard strain for genetic studies; common backcross partner for congenic construction and mapping panels.	Relatively long lived and hearty. Excellent breeders. Resistant to radiation.	1921 (Little)	F160 (CR) F187 (JAX) F155 (Taconic)
C57BL/10 (B10)	Black	Commonly used in genetic studies performed outside of the US and for the construction of congenics at the *H2* complex.	Common ancestry with B6.	1921 (Little)	F192 (JAX)
CAST/Ei	Agouti	Used in matings with traditional inbred strains to create F_1 hybrids with high levels of heterozygosity for linkage studies. Better intercross reproductive performance than *M. spretus* strains.	Derived from wild animals of the subspecies *M. m. castaneus*. Male F_1 hybrids formed with lab strains are fertile.	1971 (Marshall to Eicher)	F53 (JAX)
C3H	Agouti	Used commonly in genetic studies.	High mammary tumor incidence. Large adults.	1920 (Strong)	F167 (CR) F139 (JAX) F160 (Taconic)
DBA/2	Dilute	Used in crosses with B6		1909	F164 (CR)

Kennzeichnung von Inzuchtstämmen und Substämmen

Die Individuen eines Inzuchtstammes sind alle gleichen Ursprungs, aber die Zuchtkolonien verschiedener Anbieter sind teilweise seit Jahrzehnten voneinander getrennt, was eine langsame genetische Drift erlaubt (Kontamination, restl. Heterozygosität, Mutation).

Tiere sollten immer vom selben Anbieter stammen !

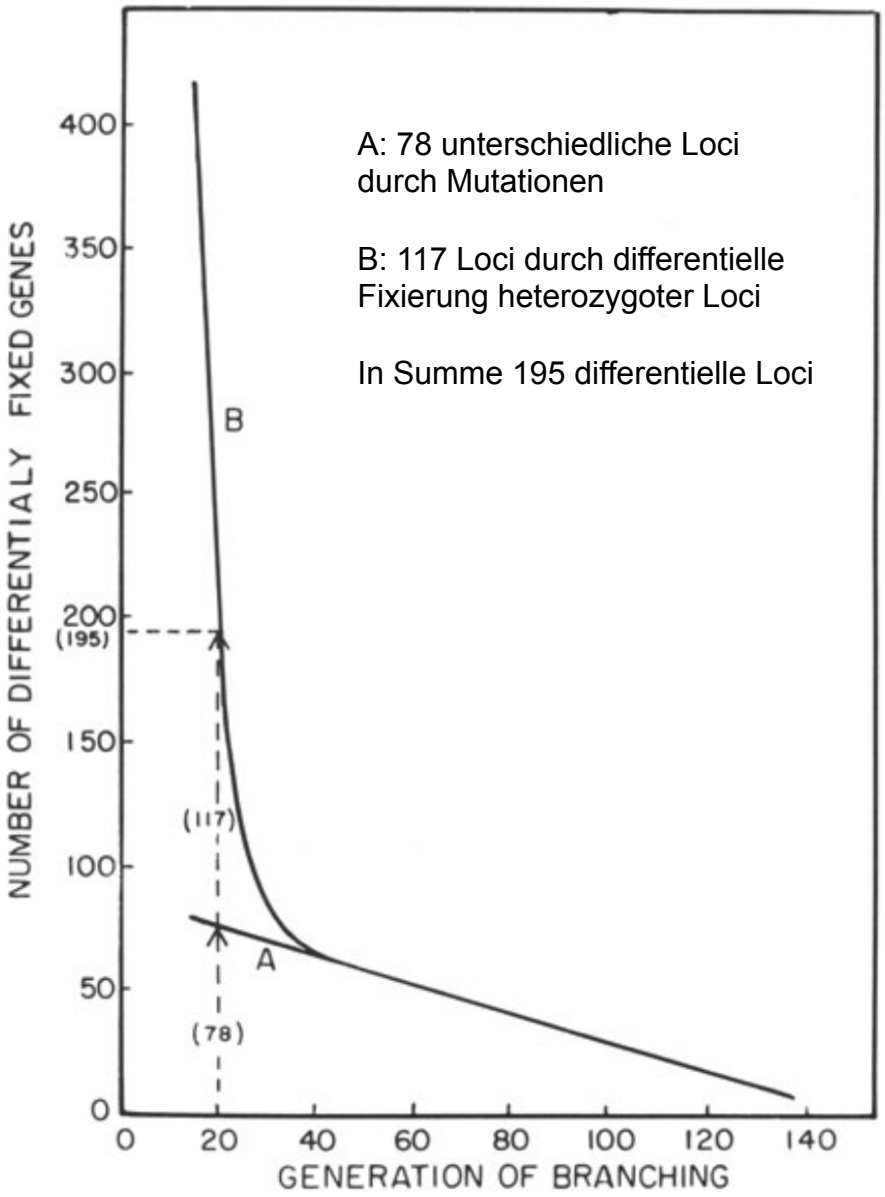
C57BL/6	C57BL/6J	C57BL/6NCrlBR	C57BL/6NTacfBR

JAX, Taconic Farms, Charles River Laboratories

N = Abstammung vom NIH

BR = Barrier Facility

Individuen eines Inzuchtstammes werden ab F20 getrennt und für weitere 134 Generationen weiter gezüchtet (F154)

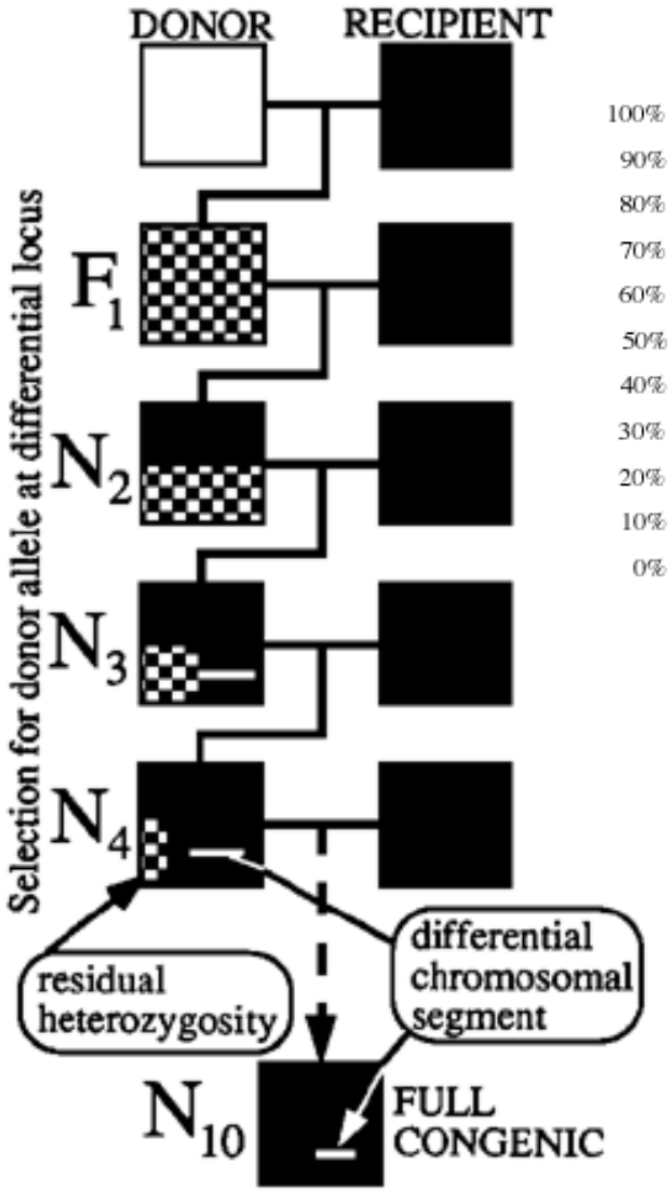


Koisogene und kongene Stämme

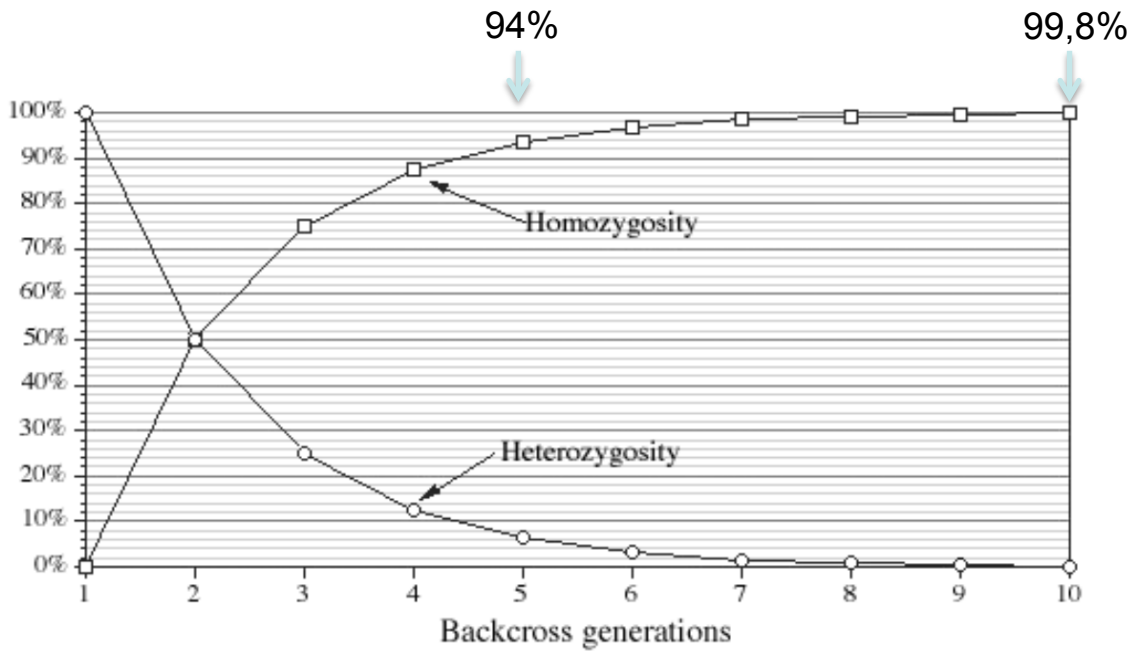
Koisogene Mäuse unterscheiden sich nur in einem genetischen Lokus (spontane Mutation, Transgen, knock in, knock out etc.) auf dem Hintergrund eines definierten Inzuchtstammes

Ein kongener Stamm wird erzeugt, indem ein spezifischer genetischer Lokus (Differentiallokus) von einem Donorstamm auf einen Rezipientenstamm übertragen wird

Zuchtschema zur Herstellung kongener Stämme



Lee M. Silver *Mouse Genetics*, online available at Mouse Genome Informatics (informatics.jax.org)



Cave: Den Differentiallokus flankierende Bereiche Bilden ein differentielles chromosomales Segment (Kopplungsgruppe) aus dem Donorstamm !

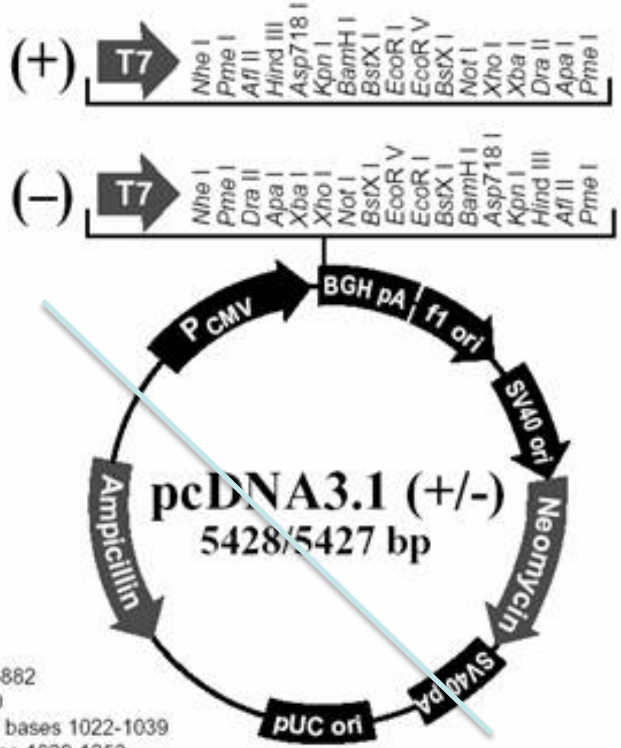
Für dessen Größe gilt:

Ln (cM) = 200/N (für N > 5)

Für N = 10 ist Ln = 20 cM
(ca. 20 – 40 MBp; 1% des Genoms!)

1. Klonierung in Expressionsvektor

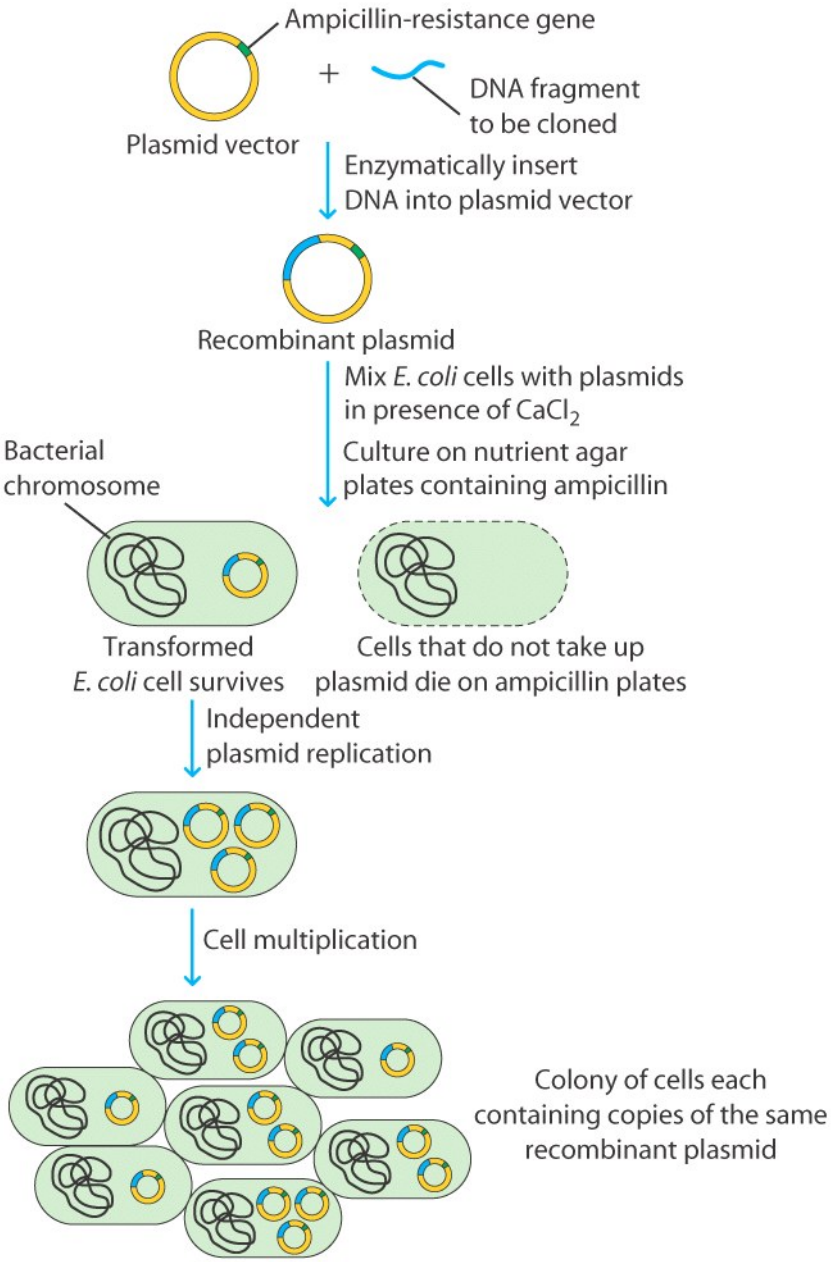
Invitrogen
life technologies



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

2. Vermehrung in Prokaryoten



3. Transfektion in eukaryotische Zellen

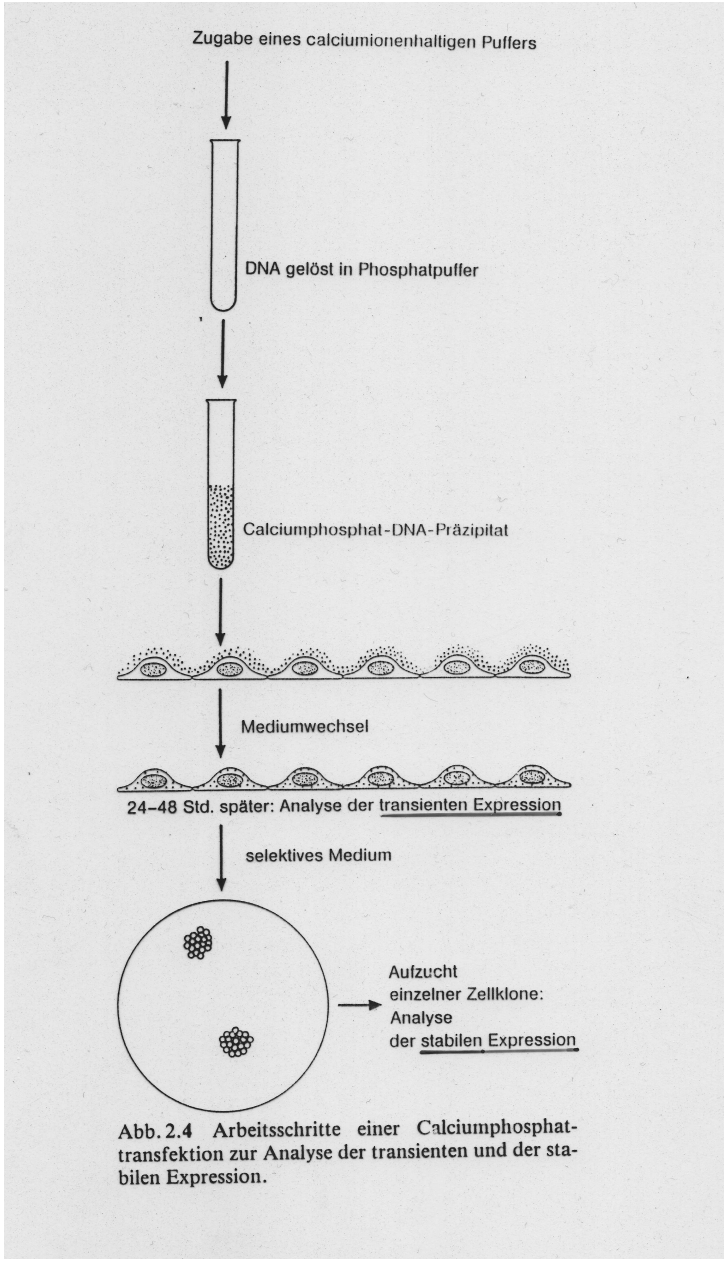


Abb. 2.4 Arbeitsschritte einer Calciumphosphat-transfektion zur Analyse der transienten und der stabilen Expression.

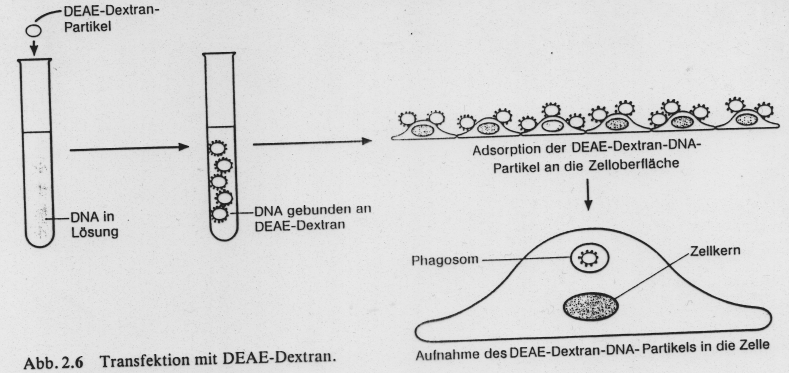


Abb. 2.6 Transfektion mit DEAE-Dextran.

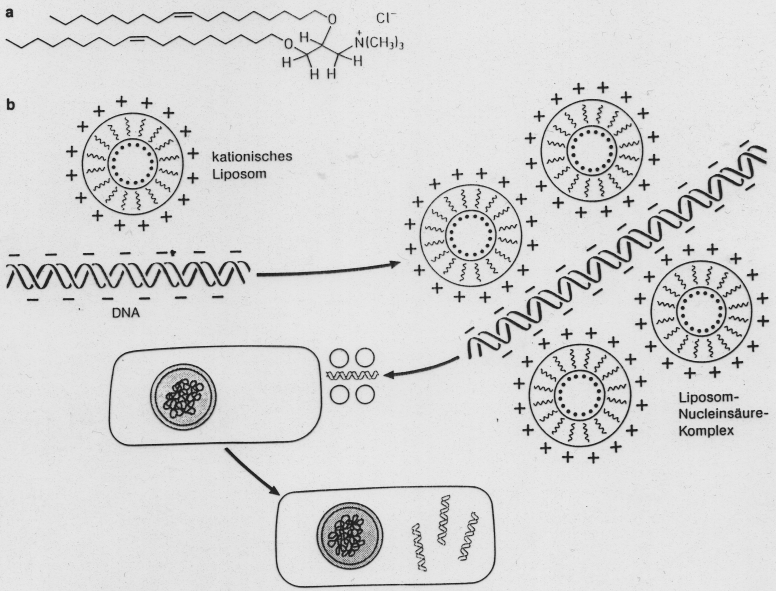


Abb. 2.8 Transfektion mit kationischen Liposomen. **a** Struktur des kationischen Lipids DOMTA. **b** Schematische Darstellung der durch kationische Liposomen vermittelten Transfektion.

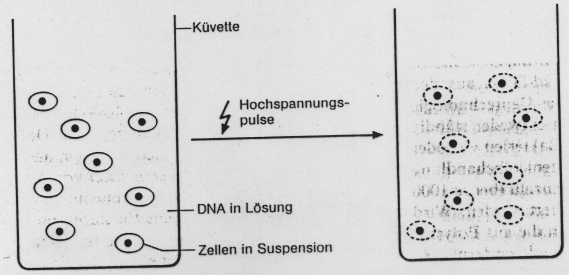
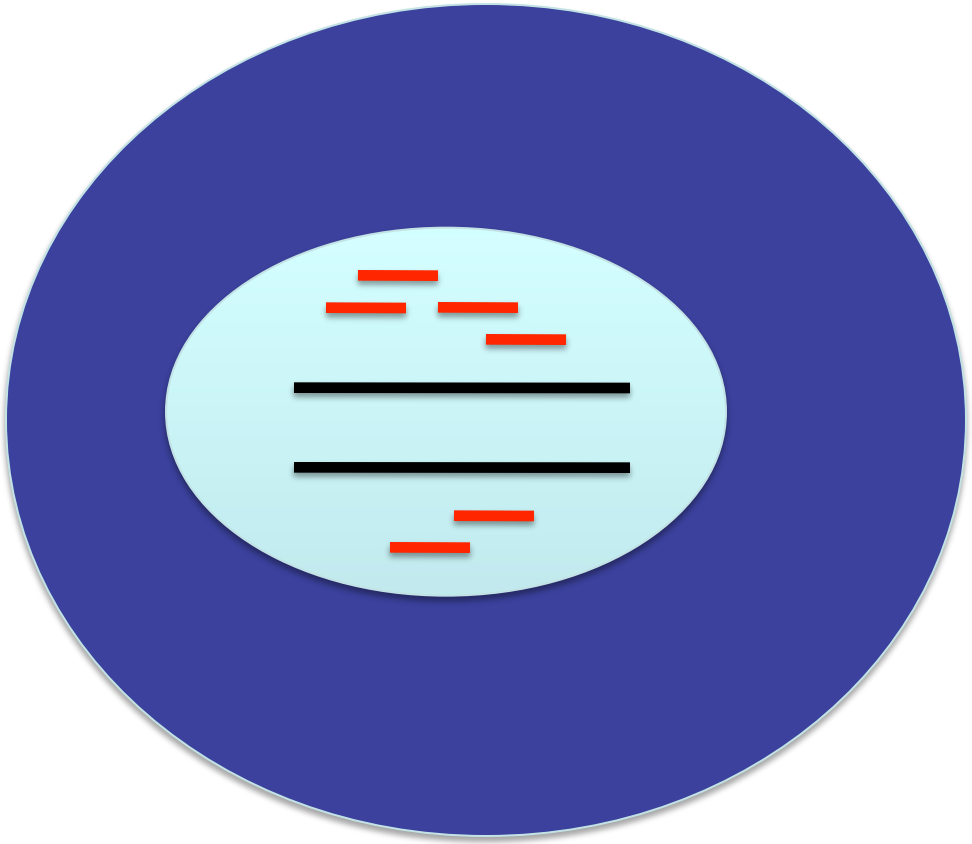


Abb. 2.11 DNA-Transfer durch Elektroporation.

Transiente Transfektion

Zelluläre DNA

Transfizierte DNA

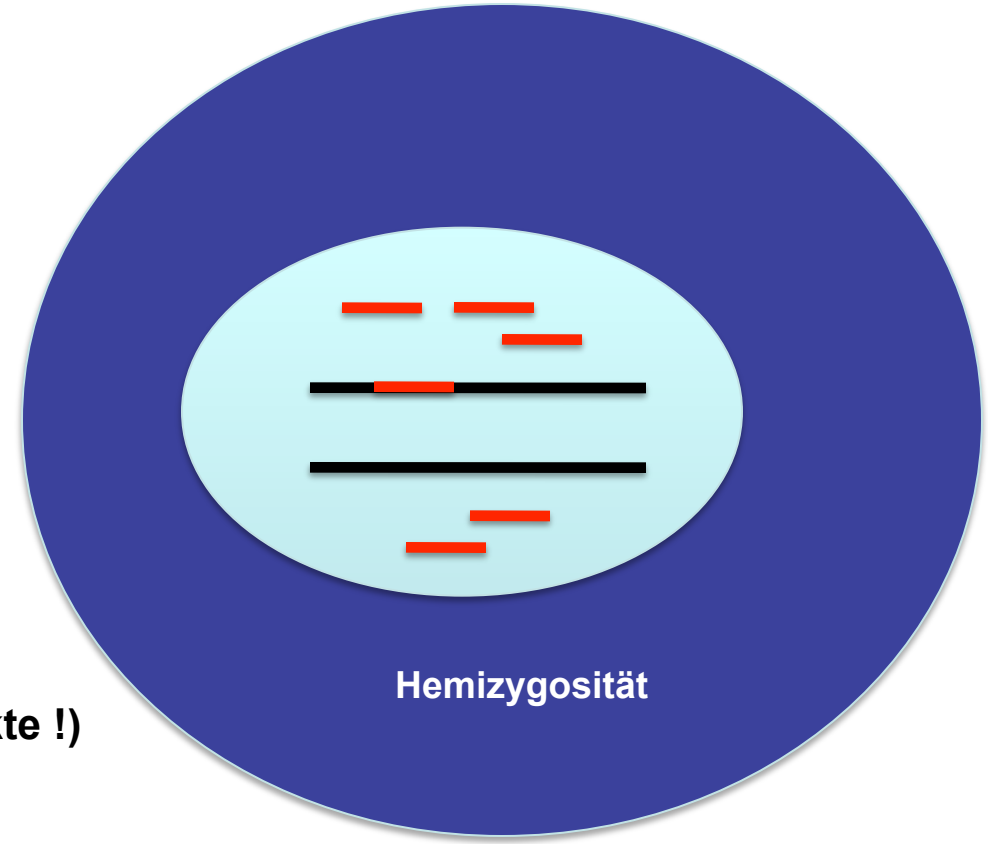


Hohe Effizienzen möglich
Nicht stabil
Abbau der transfizierten DNA

Stabile Transfektion durch Integration

Zelluläre DNA

Transfizierte DNA



Seltenes Ereignis
Einbau randomisiert (Positionseffekte !)
Konkatemere (Gen-Dosis-Effekte !)

Selektion durch Resistenzgen, Züchtung von Zellklonen

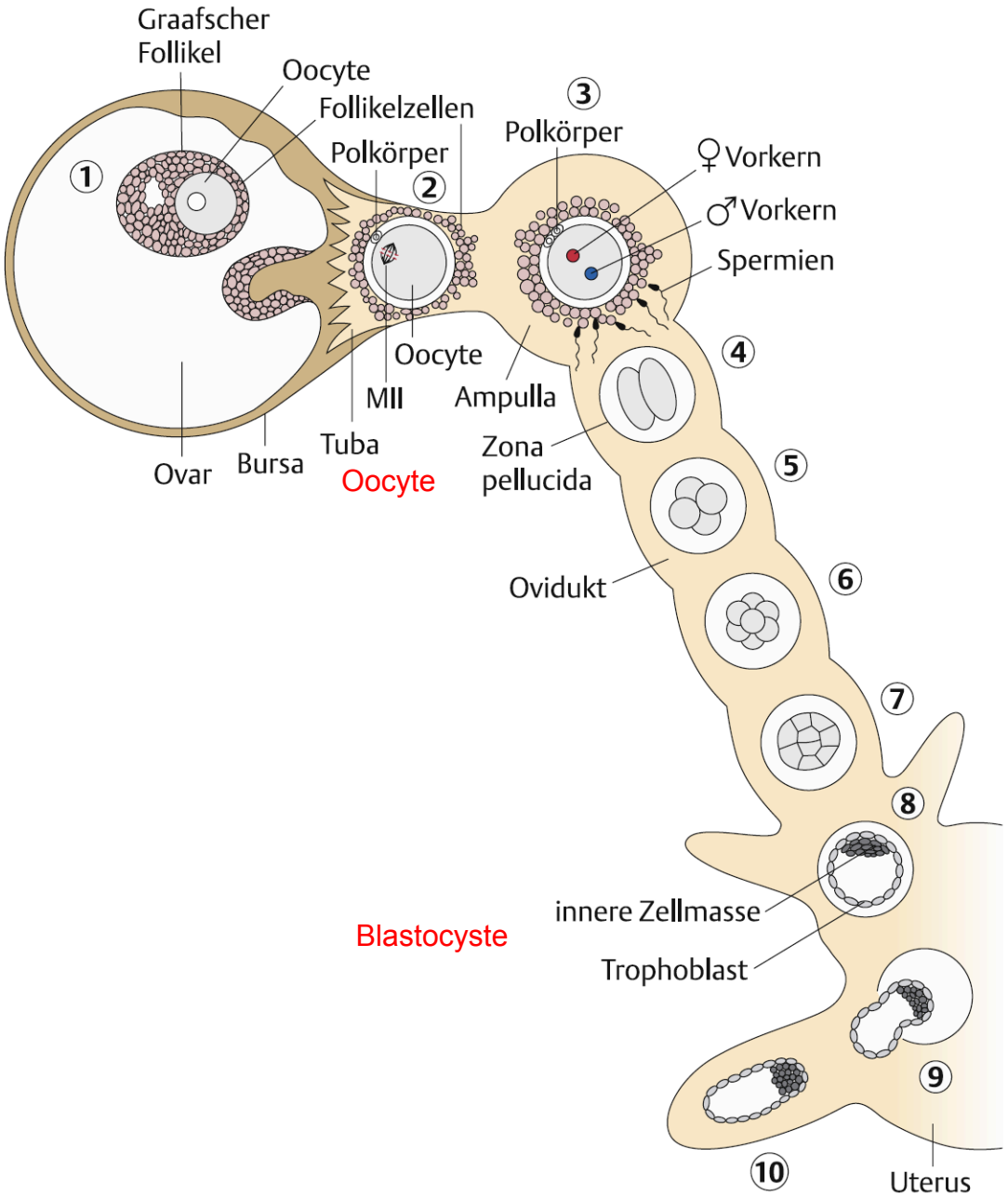
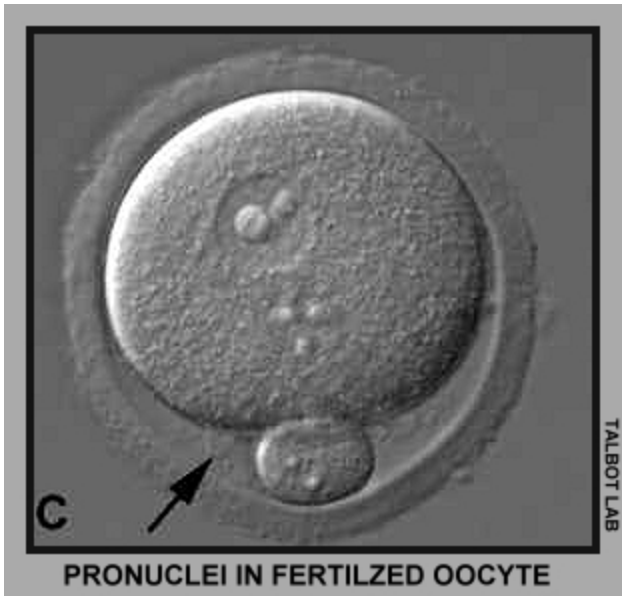
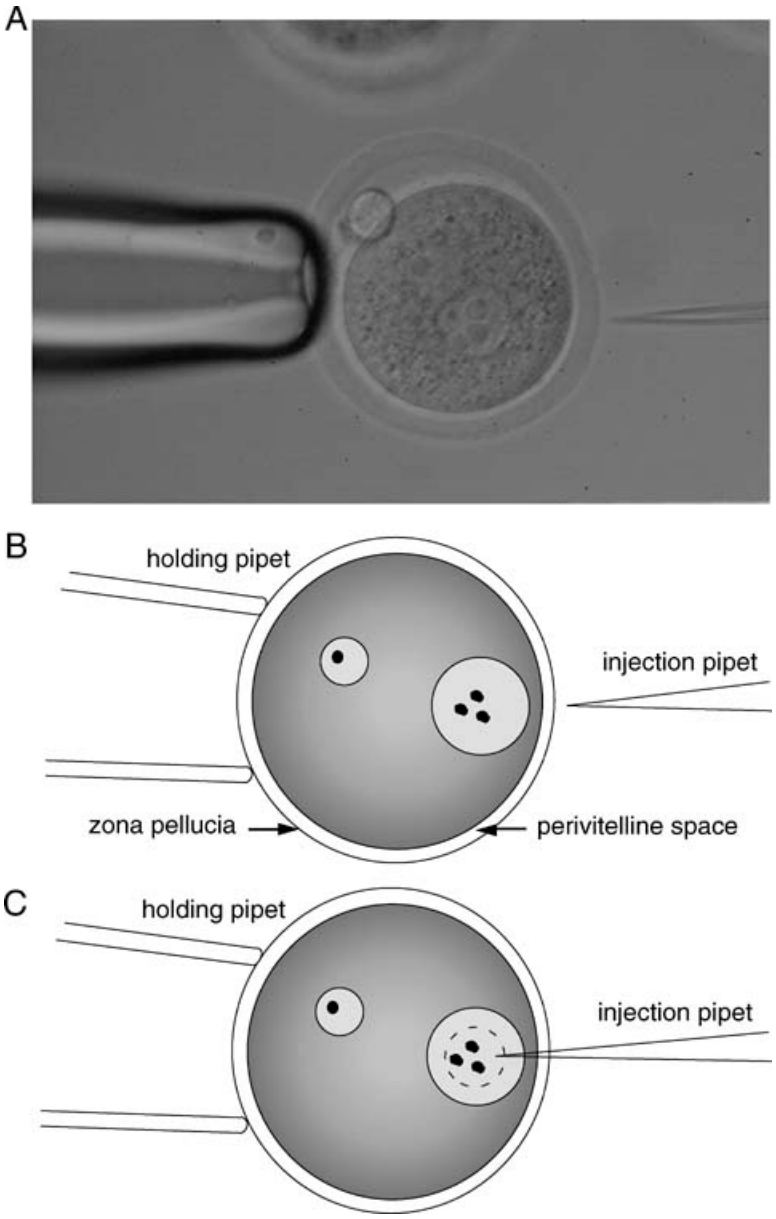
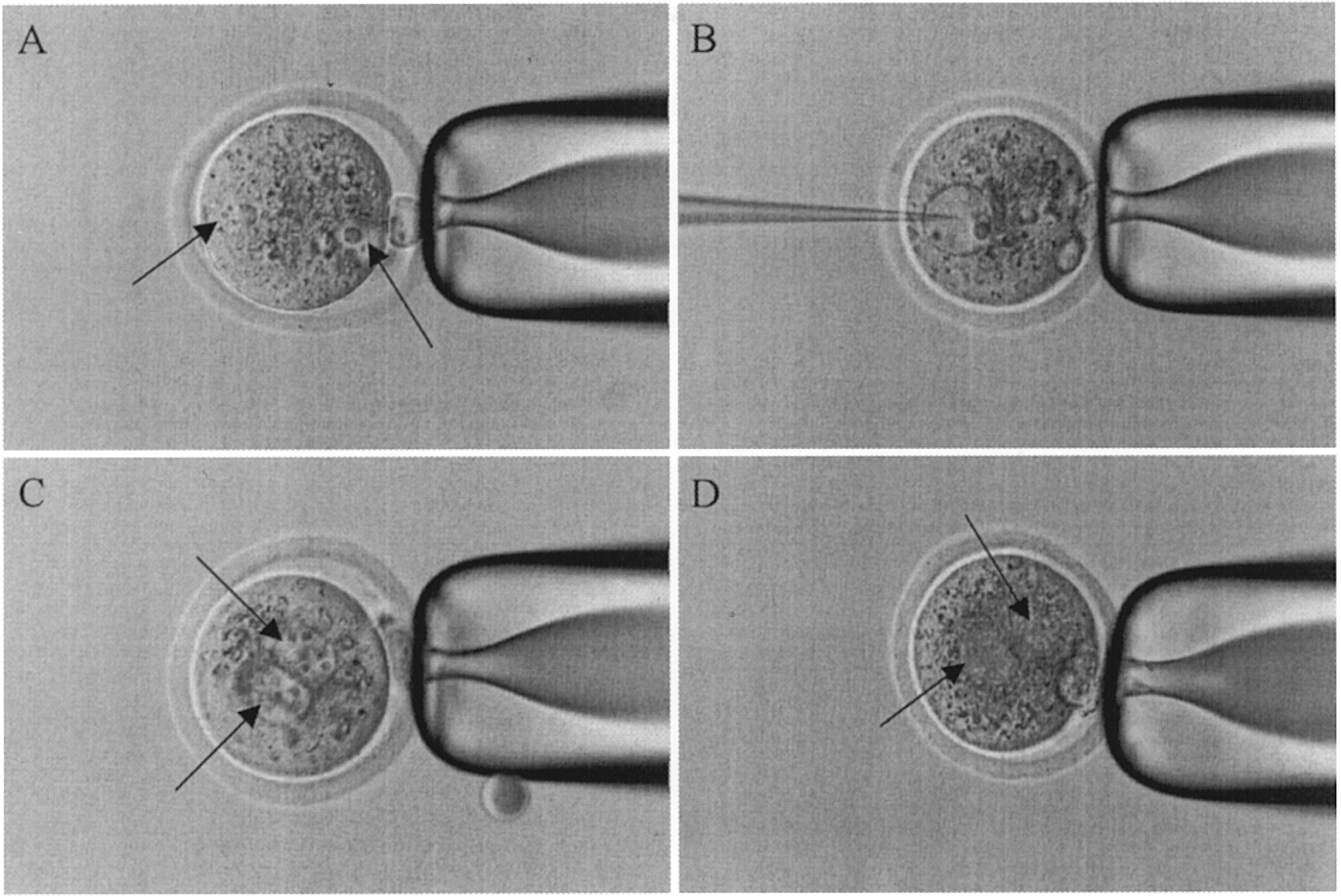


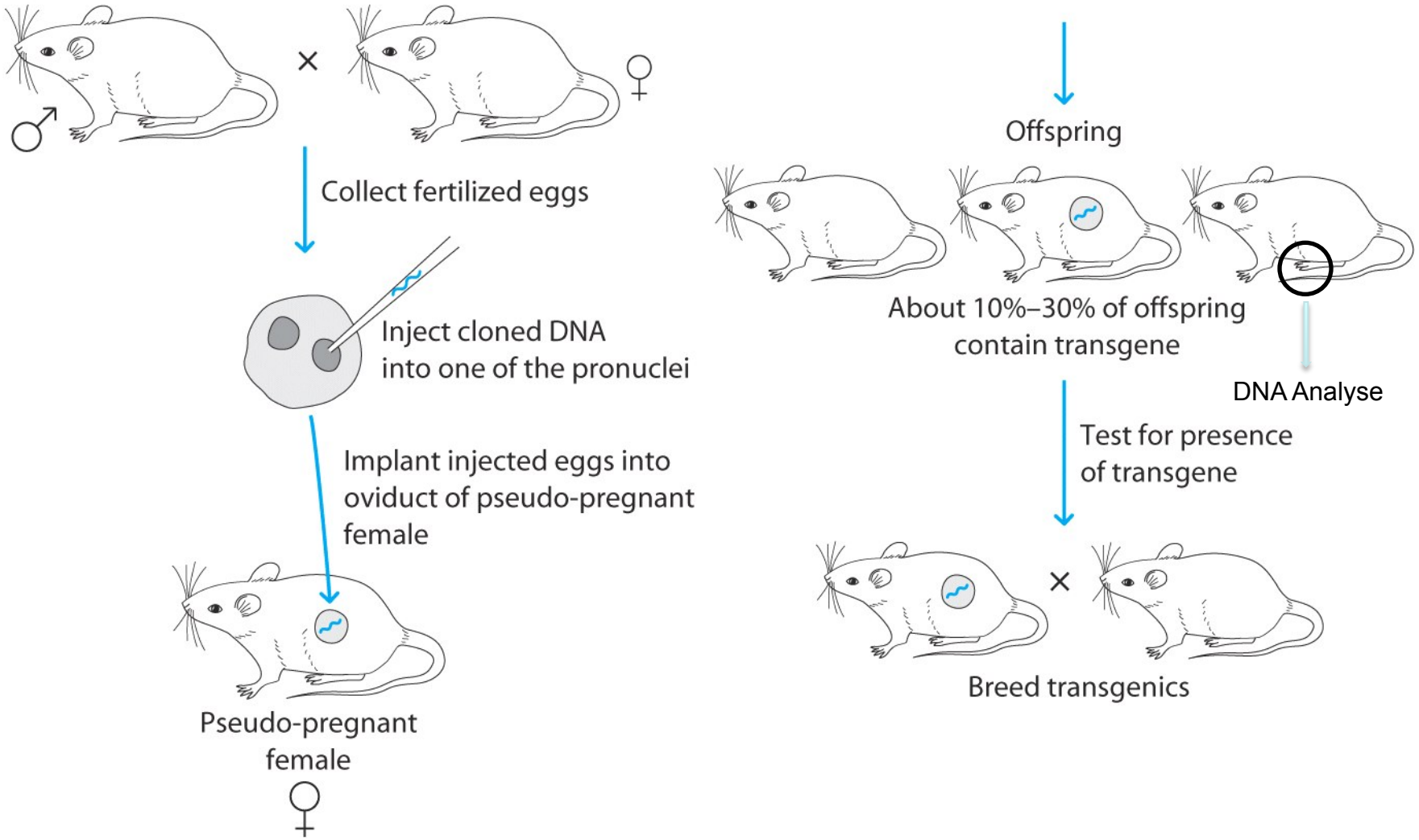
Abb. 3.17 Frühe Embryonalentwicklung der Maus bis zur Implantation. 1 Graafscher Follikel mit Oocyte. **2** Ovulation. **3** Befruchtung. **4** Furchung (2-Zeller). **5** 4-Zellstadium. **6** Morula. **7** Kompaktion der Morula. **8** Blastocyste. **9** Schlüpfen. **10** Implantation.




A
B
holding pipet
injection pipet
zona pellucia
perivitelline space
C
holding pipet
injection pipet


A
B
C
D

Transgenese der Maus durch Mikroinjektion



Richard A. Goldsby, *Immunology,* Fifth Edition

Tabelle 15.4: Ausbeuten bei der Herstellung transgener Rinder (nach [38])

Schritt	Gesamtzahl	Ausbeute
Eizellen	2 470	100%
reife Eizellen	2 297	93%
befruchtete Eizellen	1 358	55%
injizierte Eizellen	1 154	47%
die Injektion überlebende Eizellen	981	40%
injizierte Zellen, die sich teilen	687	28%
transferierte Blastocysten	129	5%
Embryonen, aus denen eine Trächtigkeit resultierte	21	(0.85%)
erhaltene Tiere	16	0.65%
transgene Nachkommen	2	0.08%
Nachkommen, die das Transgen komplett integriert haben	1	0.04%

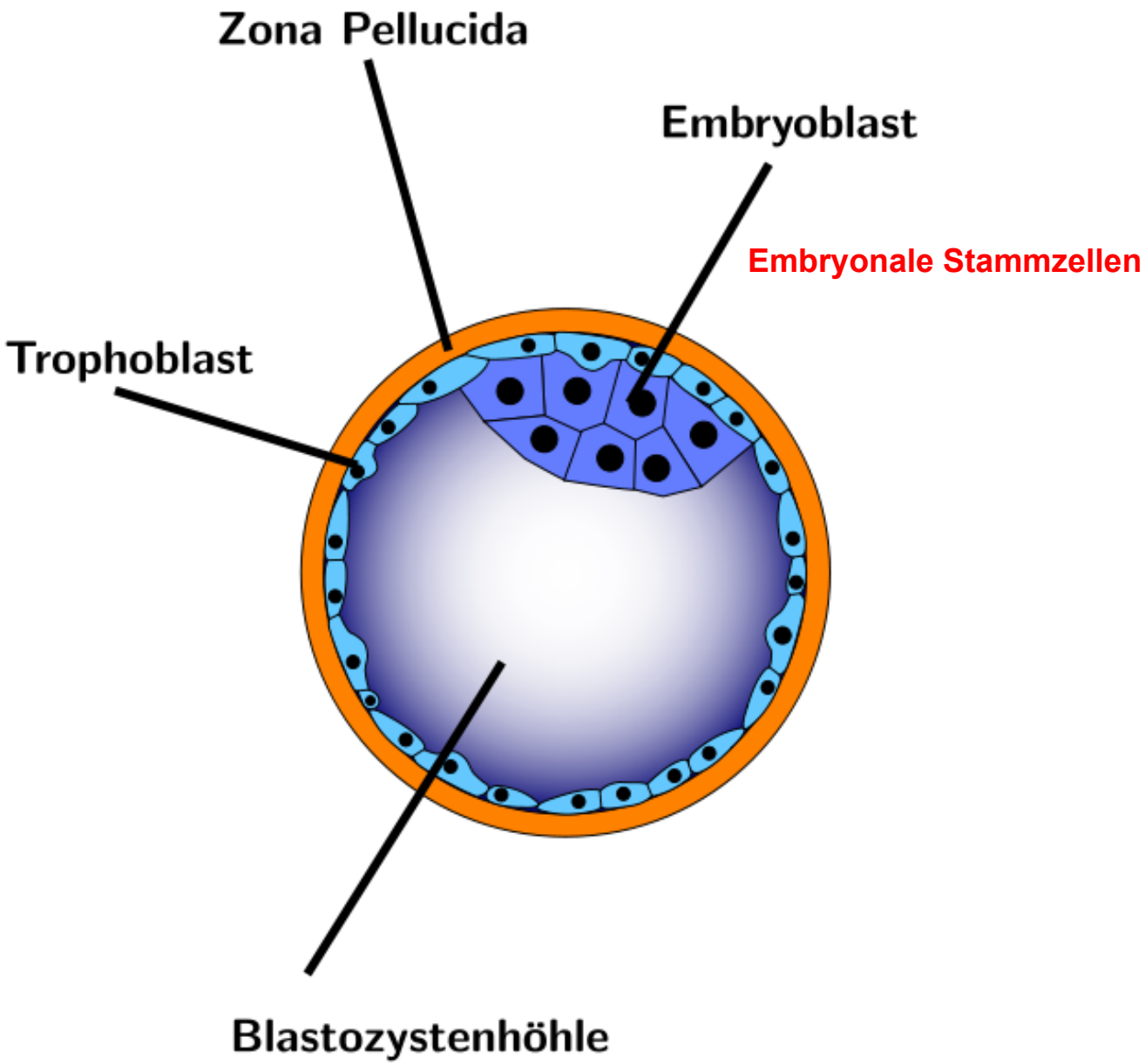
TABLE 23-7 Comparison of transgenic and knockout mice

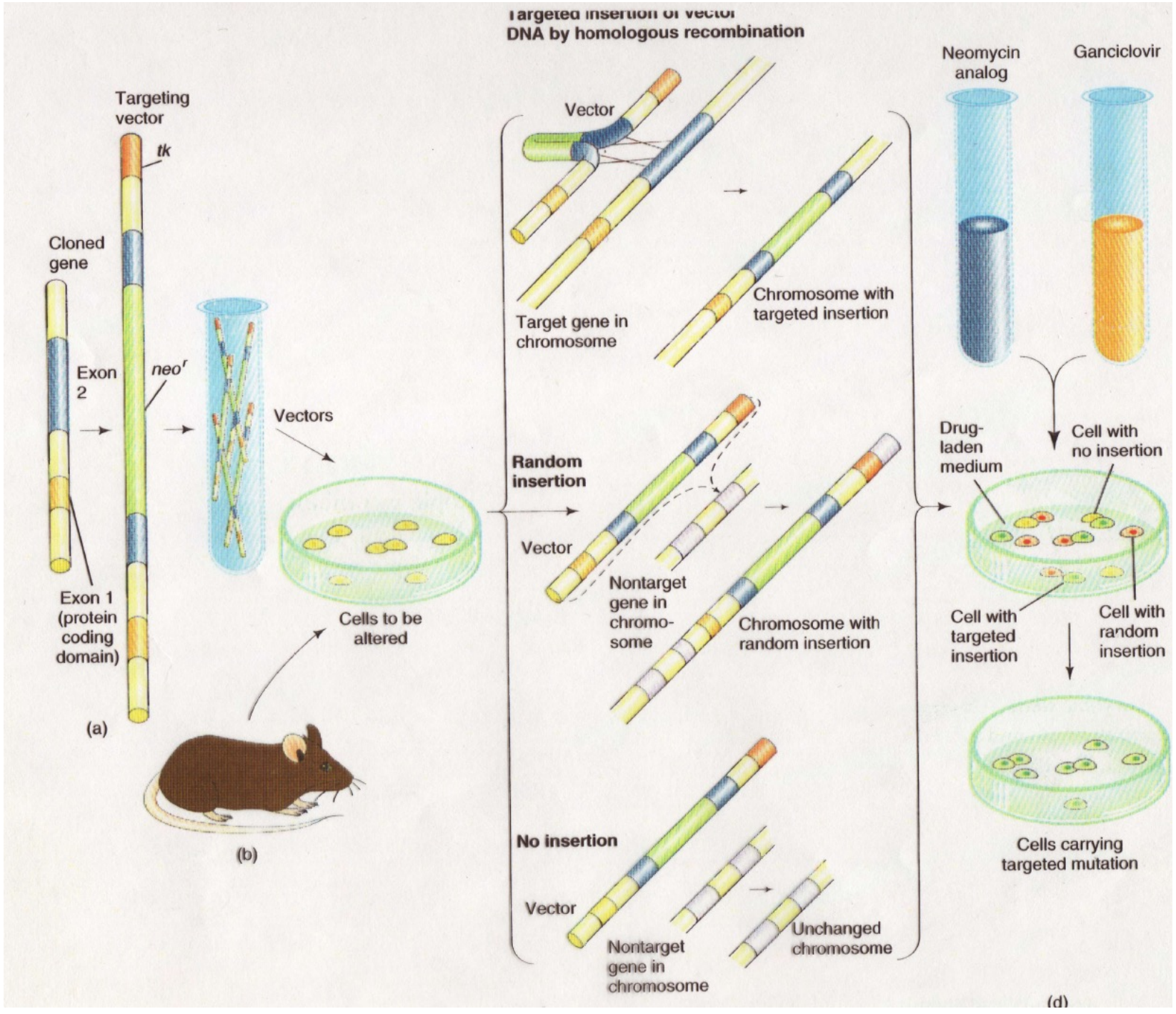
Characteristic	Transgenic mice	Knockout mice
Cells receiving DNA	Zygote	Embryonic stem (ES) cells
DNA constructs used	Natural gene or cDNA	Mutated gene
Means of delivery	Microinjection into zygote and implantation into foster mother	Transfer of ES cells to blastocyst and implantation into foster mother
Outcome	Gain of a gene	Loss of gene

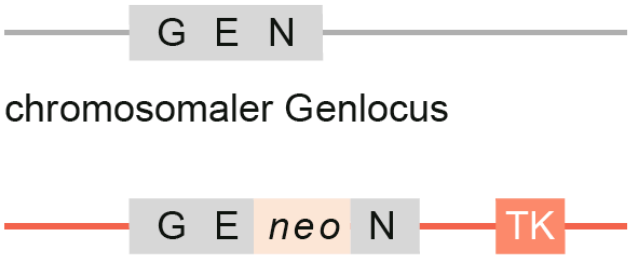
➡ Ungerichtete Integration des Transgens

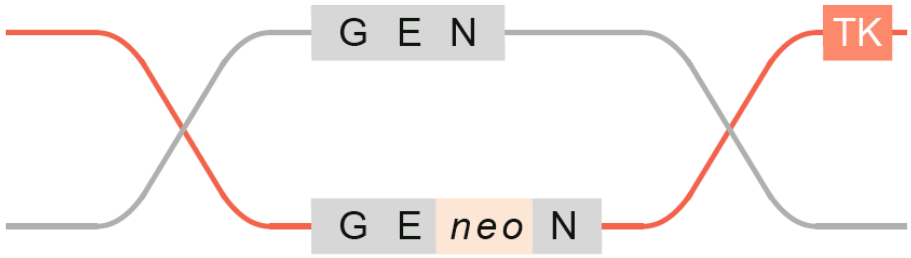
➡ Gerichtete Integration durch Homologe Rekombination

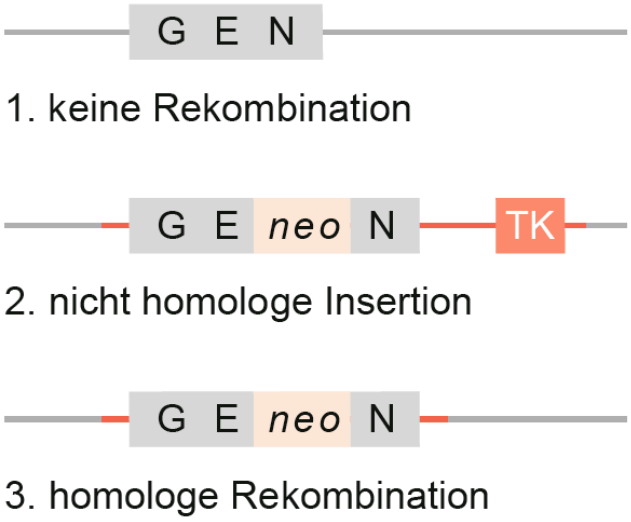
Richard A. Goldsby, *Immunology,* Fifth Edition


Zona Pellucida
Embryoblast
Embryonale Stammzellen
Trophoblast
Blastozystenhöhle

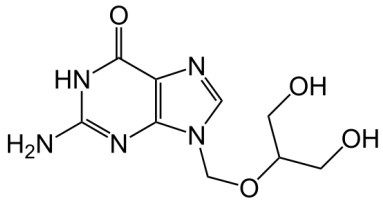

Targeted insertion of vector DNA by homologous recombination
Targeting vector
tk
Cloned gene
Exon 2
neo^r
Exon 1 (protein coding domain)
(a)
Vectors
Cells to be altered
(b)
Vector
Target gene in chromosome
Chromosome with targeted insertion
Random insertion
Vector
Nontarget gene in chromo-some
Chromosome with random insertion
No insertion
Vector
Nontarget gene in chromosome
Unchanged chromosome
Neomycin analog
Ganciclovir
Drug-laden medium
Cell with no insertion
Cell with targeted insertion
Cell with random insertion
Cells carrying targeted mutation
(d)



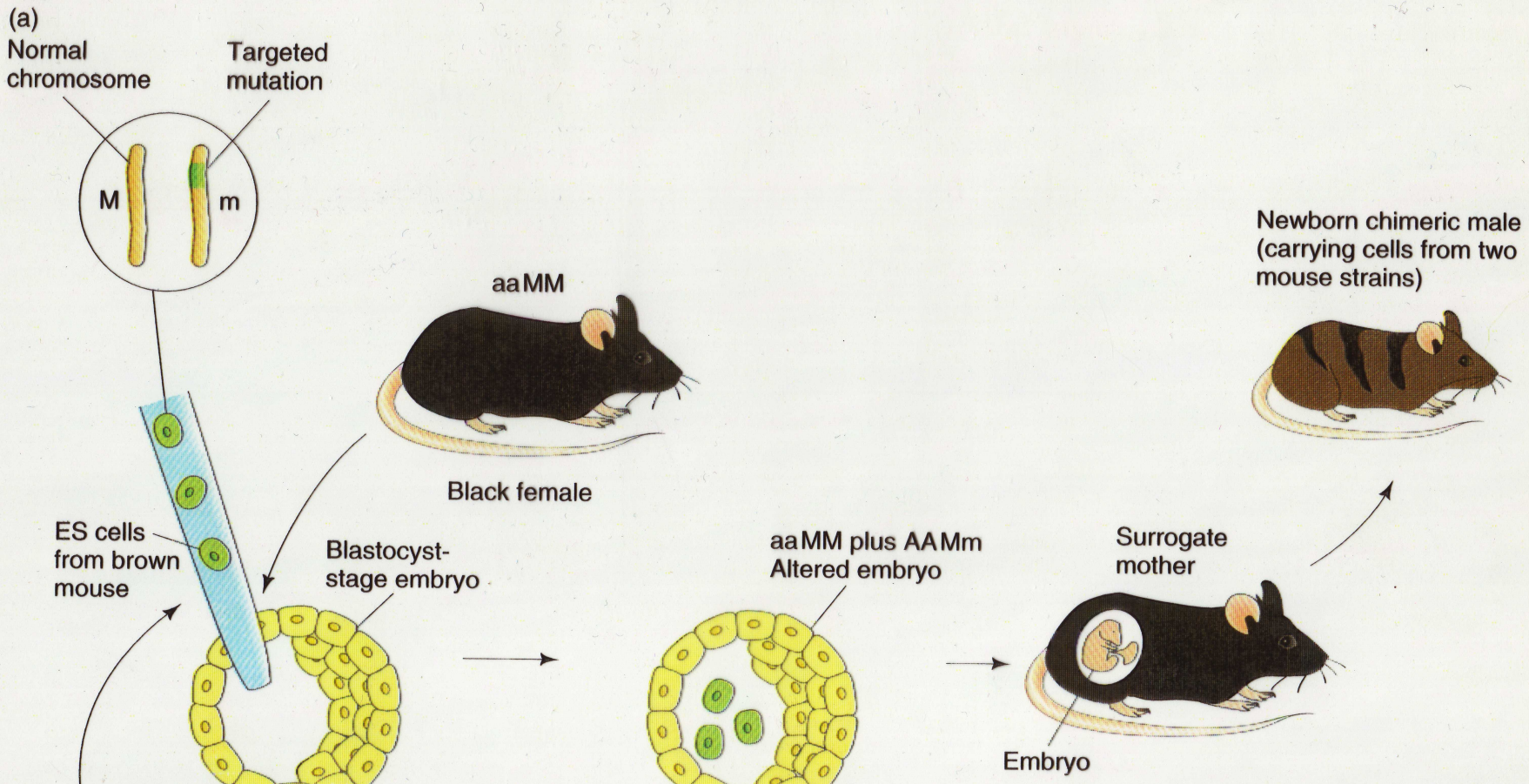




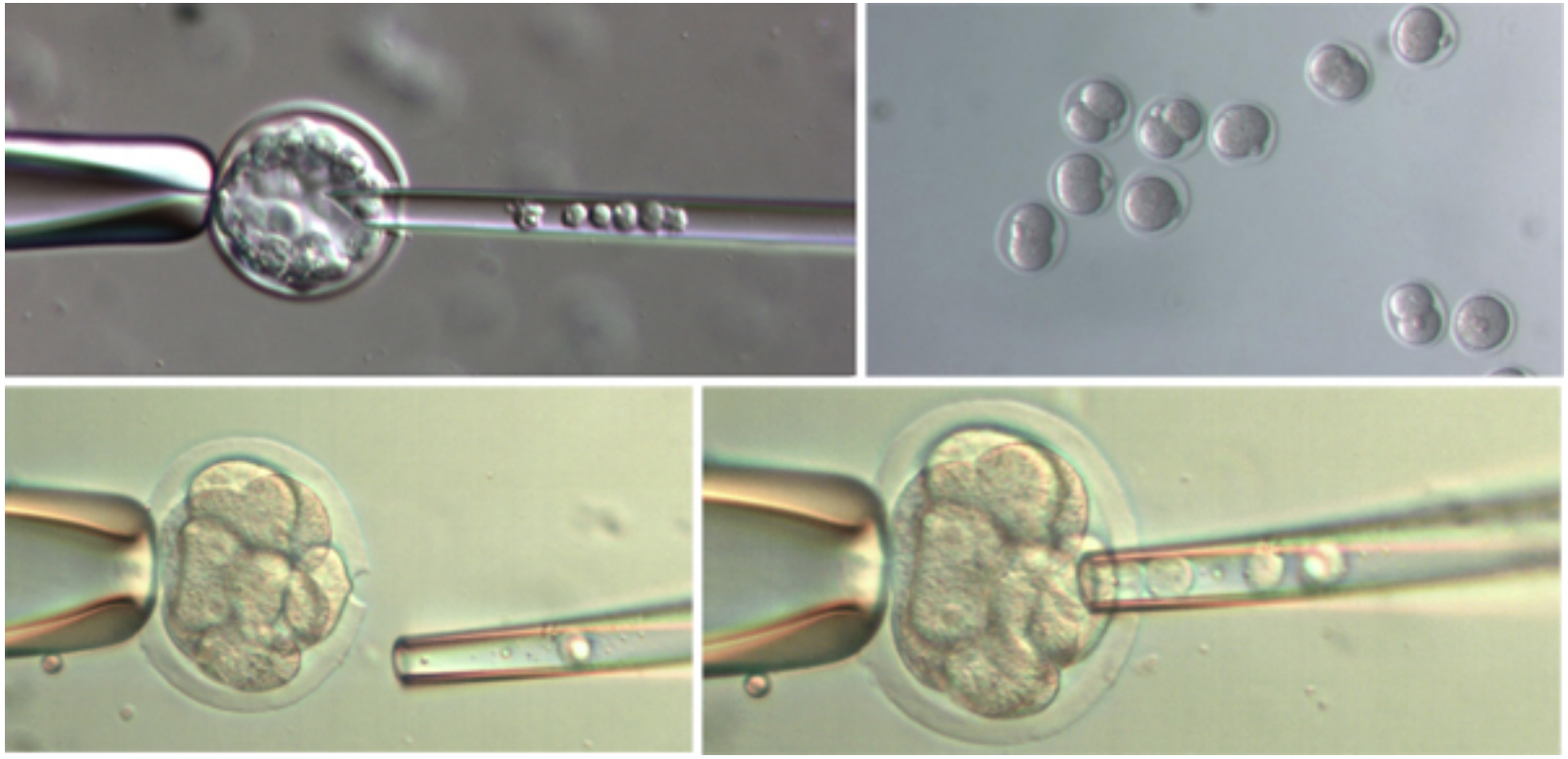
Selektion	
Neomycin	Ganciclovir
stirbt	
überlebt	stirbt
überlebt	überlebt

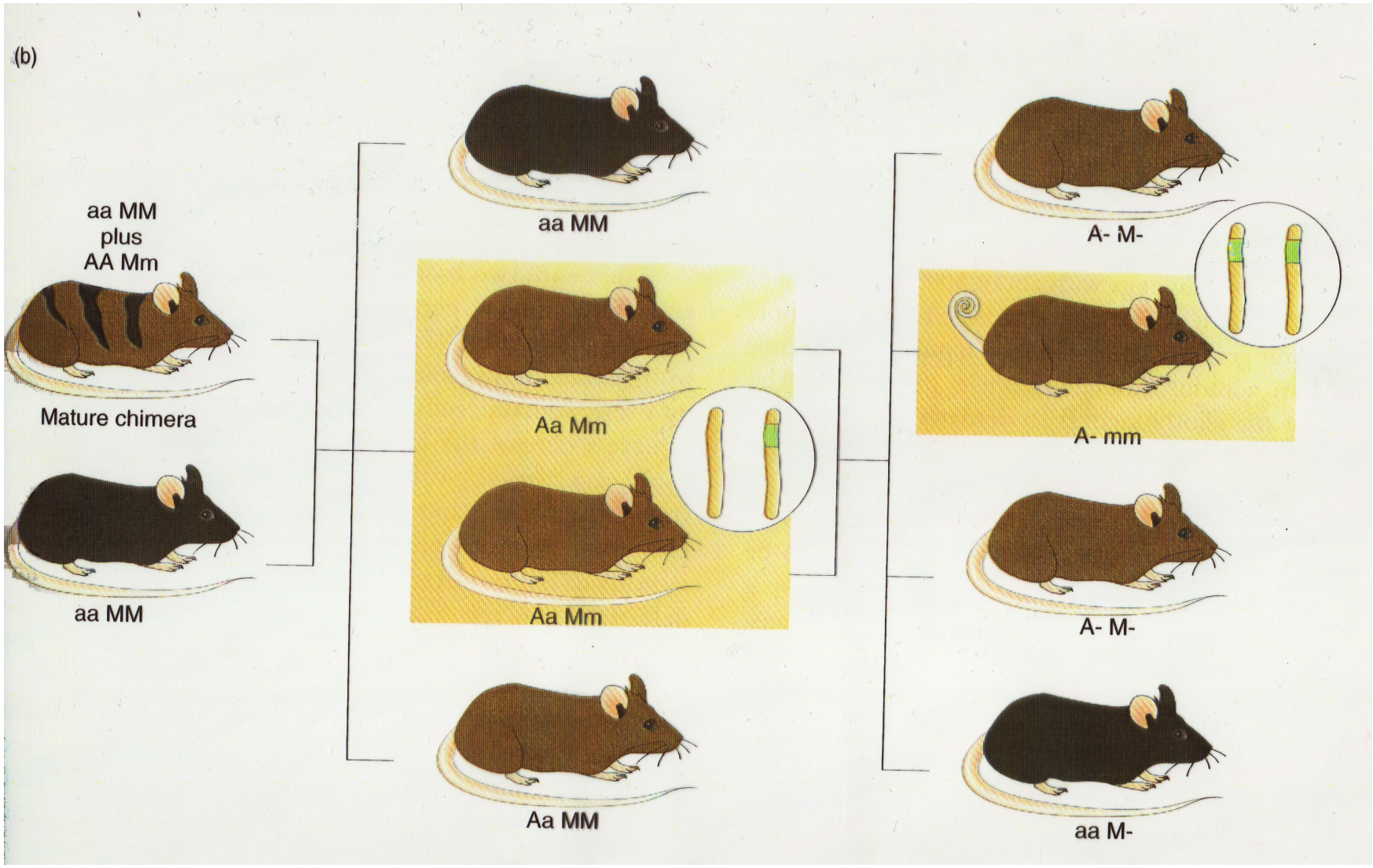


Schütt & Bröker, „Grundwissen Immunologie“, 3. Aufl. 2011

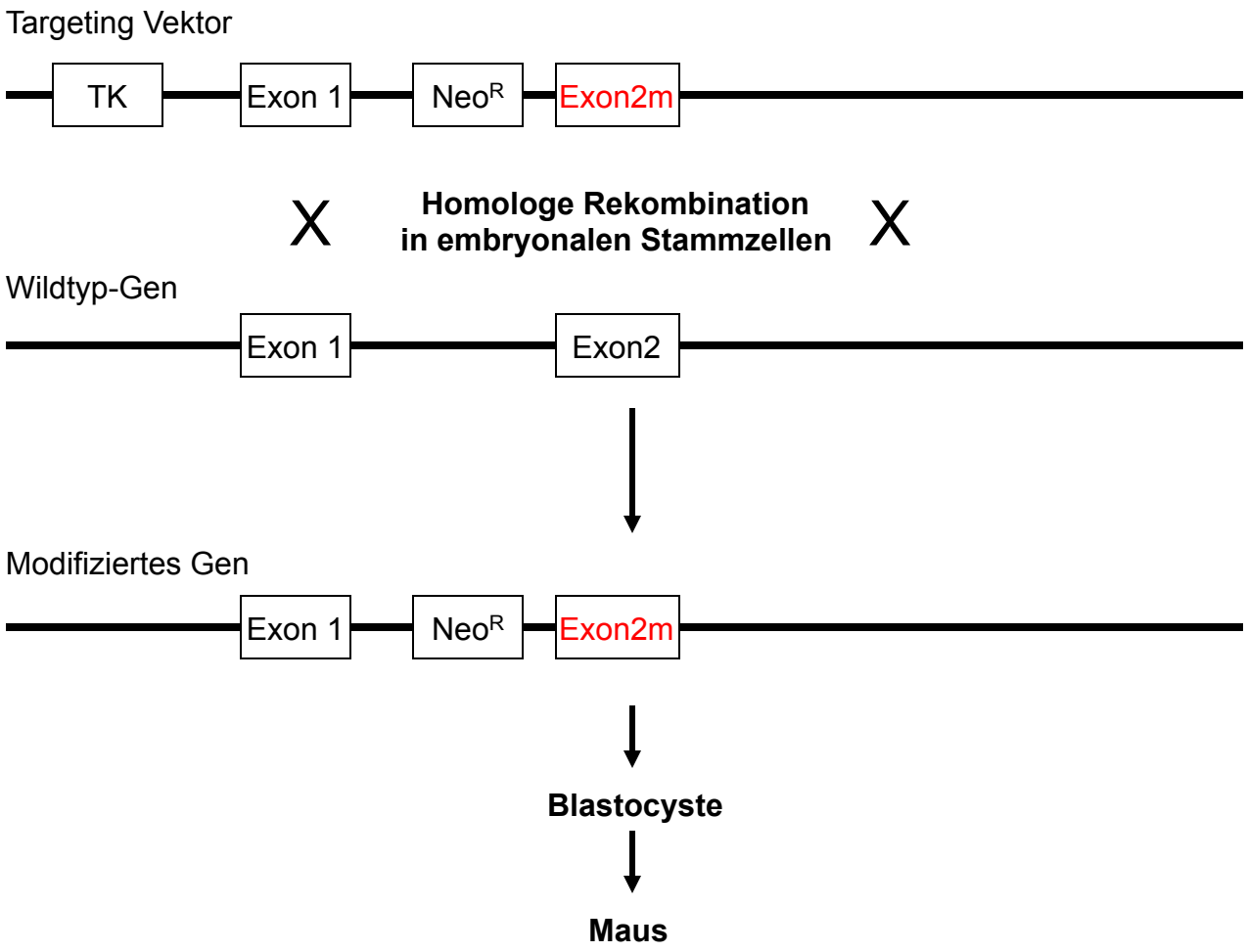


defective portion of the gene into nonhomologous (ec
sites is much more frequent than into homozygous
(Figure 15-29c), selections for site-specific recombinan
against ectopic recombinants can be used, as shown i
ure 15-29d. Second, the ES cells that contain one co
the disrupted gene of interest are injected into an earl
bryo (Figure 15-30). The resulting progeny are chi




(b)
aa MM
plus
AA Mm
Mature chimera
aa MM
aa MM
Aa Mm
Aa Mm
Aa MM
A- M-
A- mm
A- M-
aa M-

„Knock in“: Gezieltes Modifizieren von Genen durch homologe Rekombination



Cytokine	T-cell source	Effects on					Effect of gene knockout
		B cells	T cells	Macrophages	Hematopoietic cells	Other somatic cells	
Interleukin-2 (IL-2)	Naive, T_H1, some CD8	Stimulates growth and J-chain synthesis	Growth	–	Stimulates NK cell growth	–	↓ T-cell responses IBD
Interferon-γ (IFN-γ)	T_H1, CTL	Differentiation IgG2a synthesis (mouse)	Inhibits T_H2 cell growth	Activation, ↑ MHC class I and class II	Activates NK cells	Antiviral ↑ MHC class I and class II	Susceptible to mycobacteria, some viruses
Lymphotoxin (LT, TNF-β)	T_H1, some CTL	Inhibits	Kills	Activates, induces NO production	Activates neutrophils	Kills fibroblasts and tumor cells	Absence of lymph nodes Disorganized spleen
Interleukin-4 (IL-4)	T_H2	Activation, growth IgG1, IgE ↑ MHC class II induction	Growth, survival	Inhibits macrophage activation	↑ Growth of mast cells	–	No T_H2
Interleukin-5 (IL-5)	T_H2	Mouse: Differentiation IgA synthesis	–	–	↑ Eosinophil growth and differentiation	–	Reduced eosinophilia
Interleukin-10 (IL-10)	T_H2, (human: some T_H1)	↑ MHC class II	Inhibits T_H1	Inhibits cytokine release	Co-stimulates mast cell growth	–	IBD
Interleukin-3 (IL-3)	T_H1, T_H2, some CTL	–	–	–	Growth factor for progenitor hematopoietic cells (multi-CSF)	–	–
Tumor necrosis factor-α (TNF-α)	T_H1, some T_H2, some CTL	–	–	Activates, induces NO production	–	Activates microvascular endothelium	Resistance to Gram –ve sepsis
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	T_H1, some T_H2, some CTL	Differentiation	Inhibits growth ?	Activation Differentiation to dendritic cells	↑ Production of granulocytes and macrophages (myelopoiesis) and dendritic cells	–	–
Transforming growth factor-β (TGF-β)	CD4 T cells	Inhibits growth IgA switch factor	Inhibits growth, promotes survival	Inhibits activation	Activates neutrophils	Inhibits/ stimulates cell growth	Death at ~10 weeks

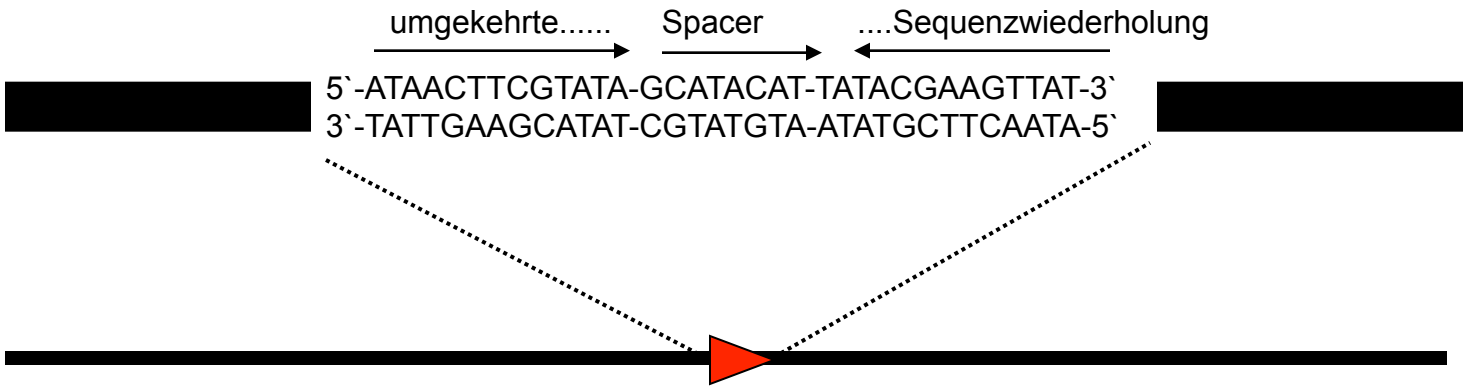
Figure 8-32 Immunobiology, 6/e. (© Garland Science 2005)

Cytokine	T-cell source	Effects on					Effect of gene knockout
		B cells	T cells	Macrophages	Hematopoietic cells	Other somatic cells	
Interleukin-2 (IL-2)	Naive, T_H1, some CD8	Stimulates growth and J-chain synthesis	Growth	–	Stimulates NK cell growth	–	↓ T-cell responses IBD
Interferon-γ (IFN-γ)	T_H1, CTL	Differentiation IgG2a synthesis (mouse)	Inhibits T_H2 cell growth	Activation, ↑ MHC class I and class II	Activates NK cells	Antiviral ↑ MHC class I and class II	Susceptible to mycobacteria, some viruses
Lymphotoxin (LT, TNF-β)	T_H1, some CTL	Inhibits	Kills	Activates, induces NO production	Activates neutrophils	Kills fibroblasts and tumor cells	Absence of lymph nodes Disorganized spleen
Interleukin-4 (IL-4)	T_H2	Activation, growth IgG1, IgE ↑ MHC class II induction	Growth, survival	Inhibits macrophage activation	↑ Growth of mast cells	–	No T_H2
Interleukin-5 (IL-5)	T_H2	Mouse: Differentiation IgA synthesis	–	–	↑ Eosinophil growth and differentiation	–	Reduced eosinophilia
Interleukin-10 (IL-10)	T_H2, (human: some T_H1)	↑ MHC class II	Inhibits T_H1	Inhibits cytokine release	Co-stimulates mast cell growth	–	IBD
Interleukin-3 (IL-3)	T_H1, T_H2, some CTL	–	–	–	Growth factor for progenitor hematopoietic cells (multi-CSF)	–	–
Tumor necrosis factor-α (TNF-α)	T_H1, some T_H2, some CTL	–	–	Activates, induces NO production	–	Activates microvascular endothelium	Resistance to Gram –ve sepsis
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	T_H1, some T_H2, some CTL	Differentiation	Inhibits growth ?	Activation Differentiation to dendritic cells	↑ Production of granulocytes and macrophages (myelopoiesis) and dendritic cells	–	
Transforming growth factor-β (TGF-β)	CD4 T cells	Inhibits growth IgA switch factor	Inhibits growth, promotes survival	Inhibits activation	Activates neutrophils	Inhibits stimulates cell growth	Death at ~10 weeks

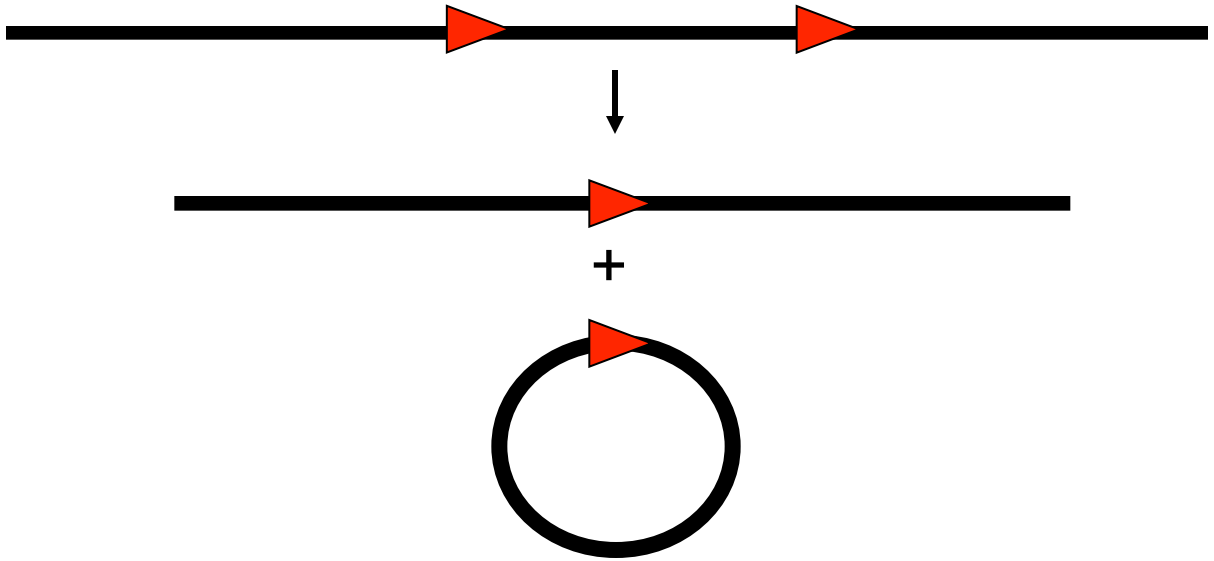
Figure 8-32 Immunobiology, 6/e. (© Garland Science 2005)

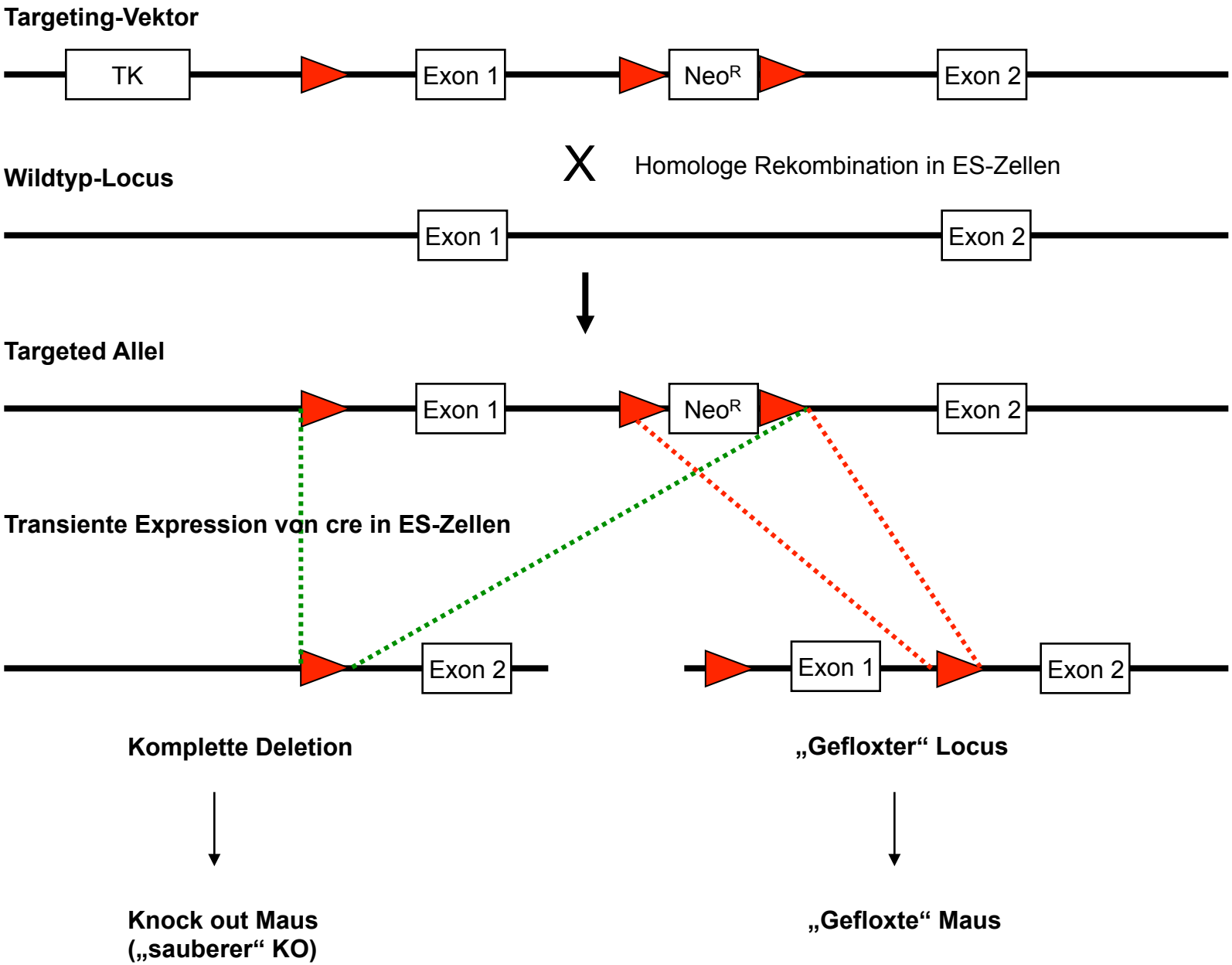
Konditionale Mutagenese: Das Cre-lox System

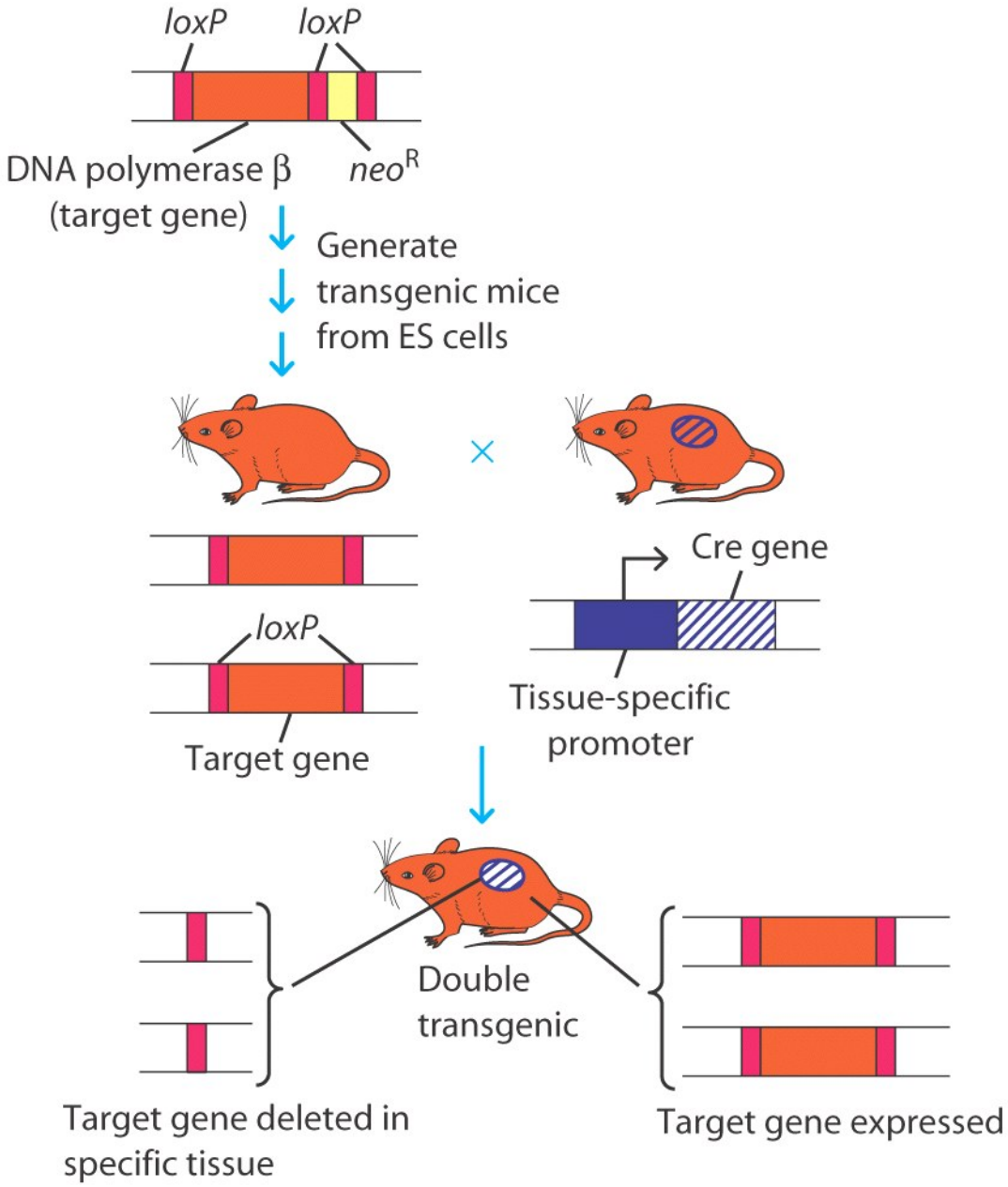
Struktur eines loxP-Elementes (locus of crossing over)



Cre-vermittelte Deletion (causes recombination)

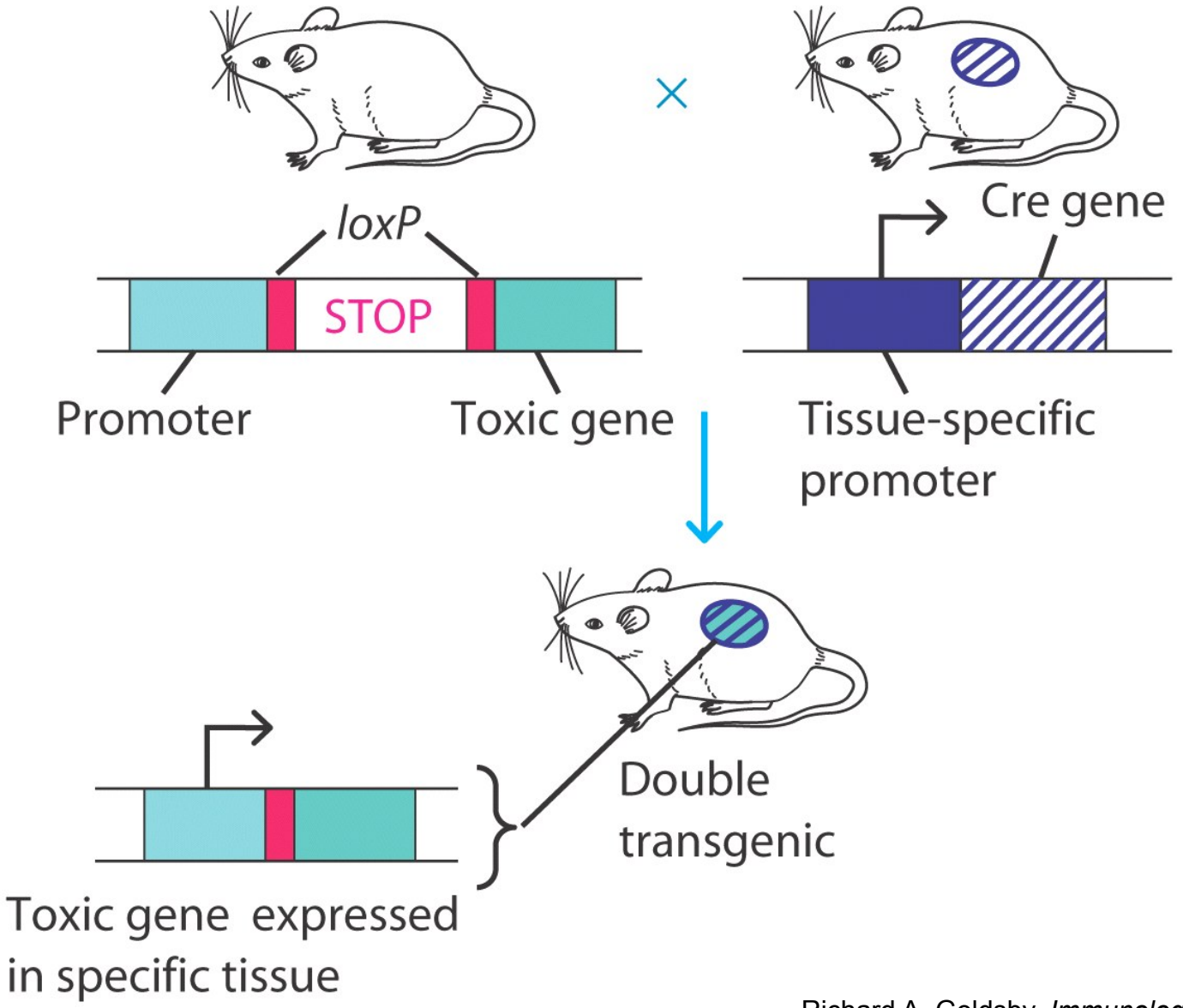



Targeting-Vektor
TK
Exon 1
NeoR
Exon 2
X
Homologe Rekombination in ES-Zellen
Wildtyp-Locus
Exon 1
Exon 2
Targeted Allel
Exon 1
NeoR
Exon 2
Transiente Expression von cre in ES-Zellen
Exon 2
Exon 1
Exon 2
Komplette Deletion
„Gefloxter“ Locus
Knock out Maus
(„sauberer“ KO)
„Gefloxte“ Maus



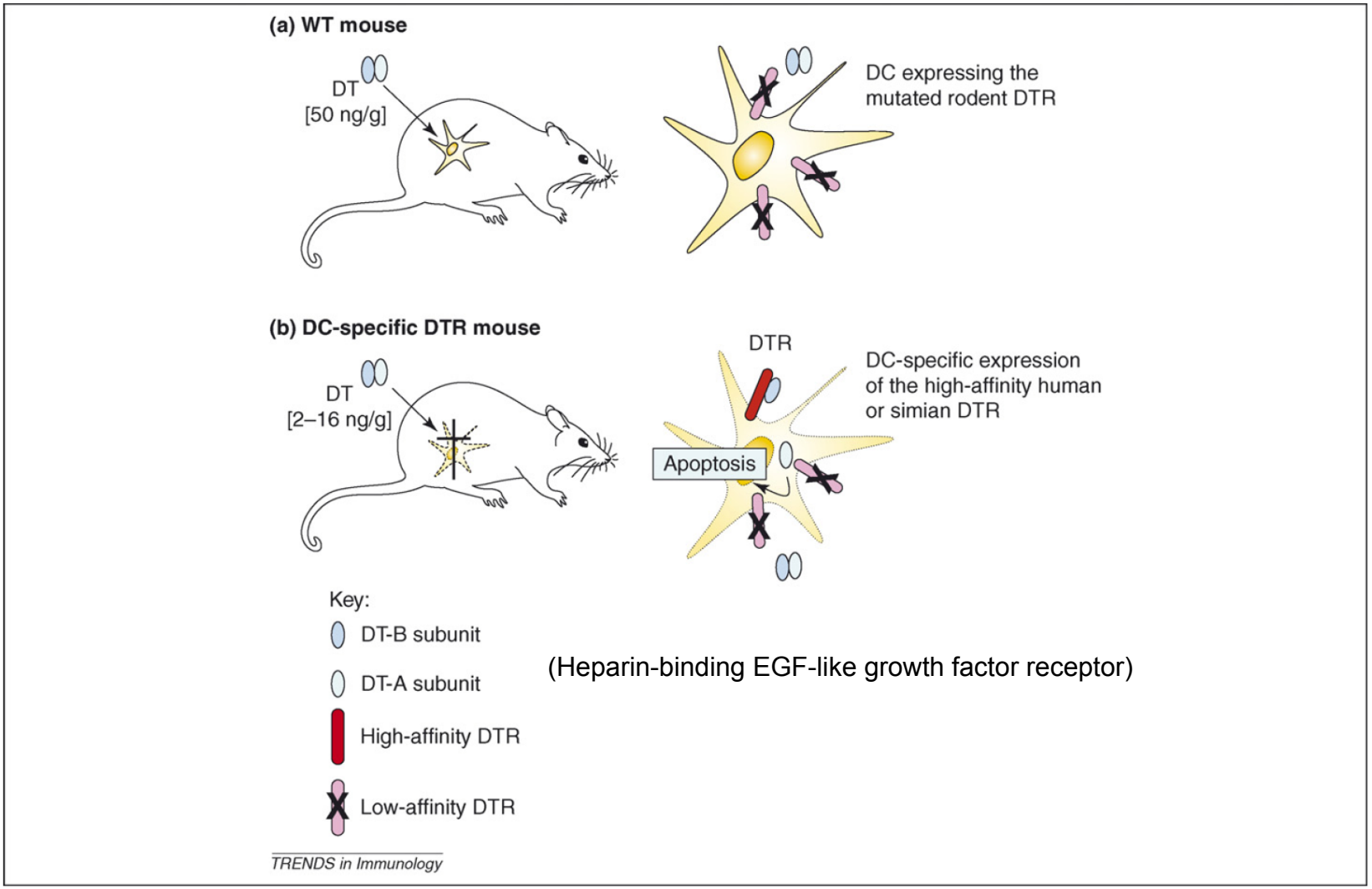
Richard A. Goldsby, *Immunology,* Fifth Edition

Gezielte Ablation von Zellen durch das Cre-lox System: Expression des Diphteria-Toxins



Richard A. Goldsby, *Immunology,* Fifth Edition

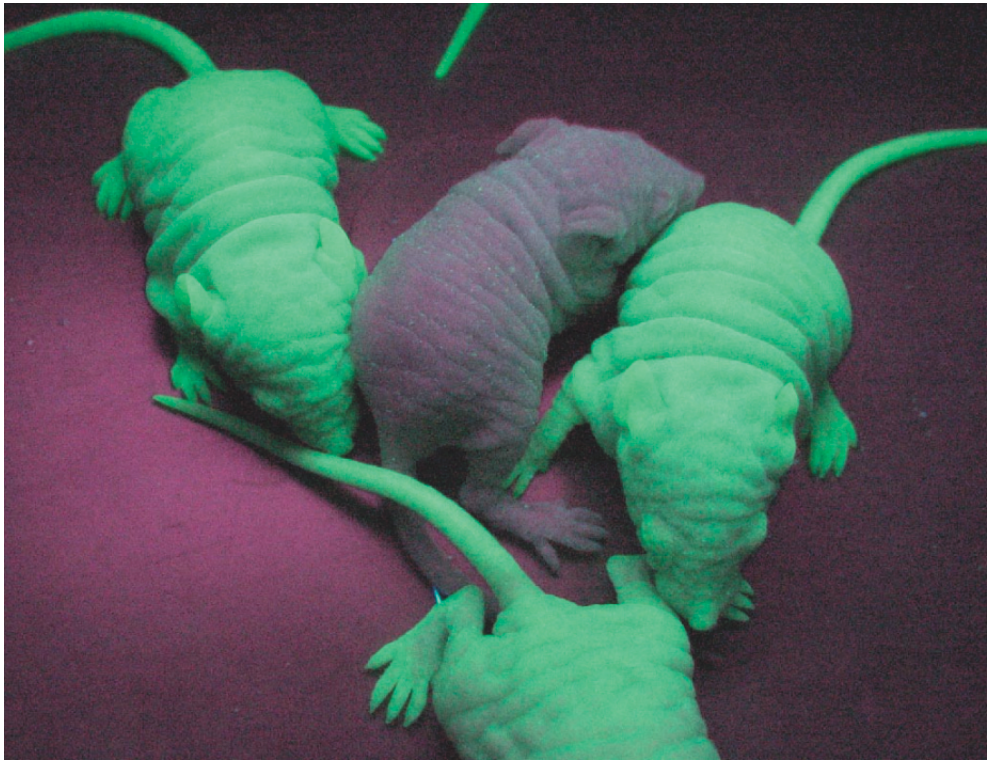
Gezielte Ablation von Zellen mit Hilfe des Diphteria-Toxin Rezeptors



(Heparin-binding EGF-like growth factor receptor)

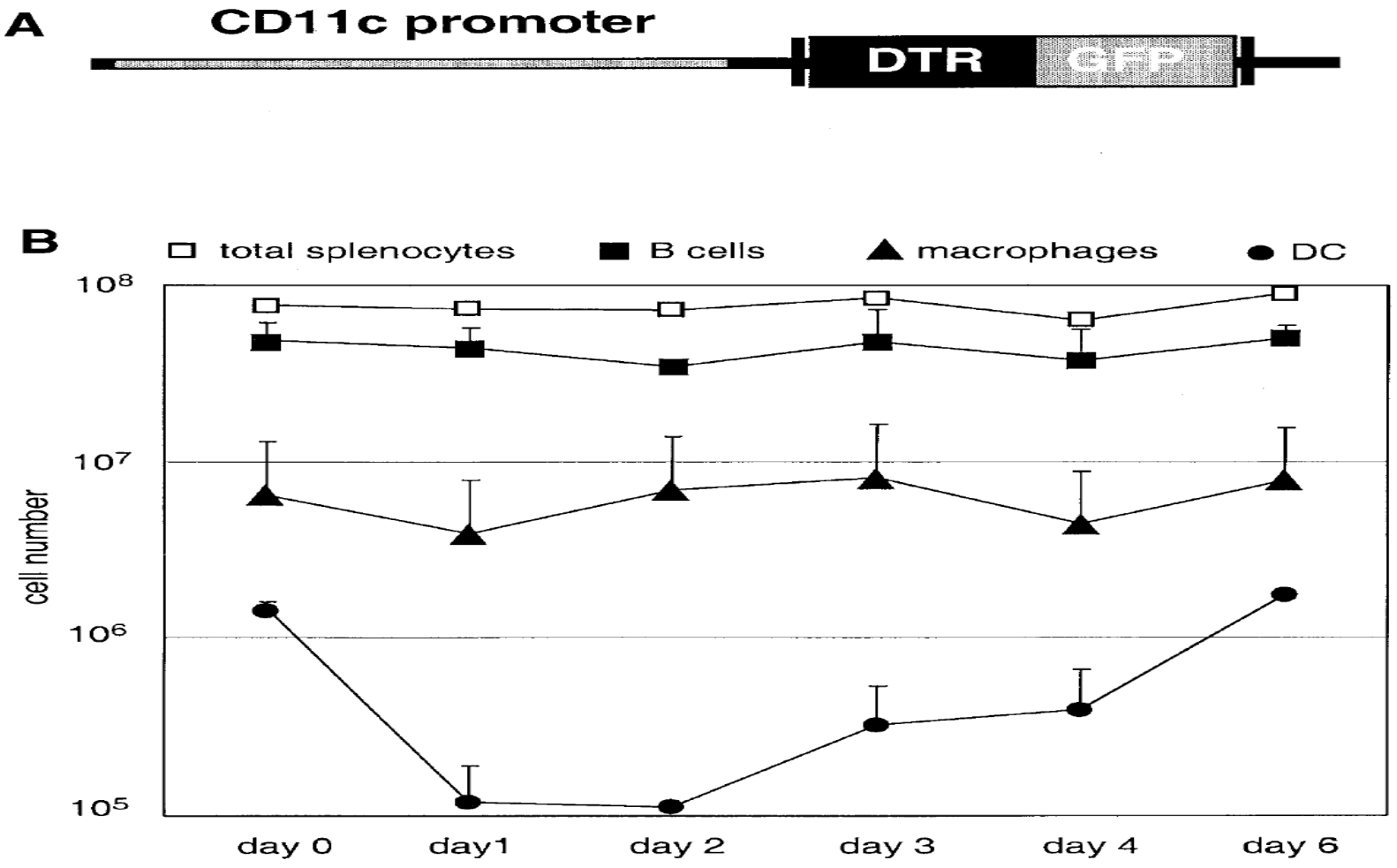
Figure 1. Depletion of DC from mice engineered to express a high-affinity DTR. (**a**) Mice are more than 1000 times more resistant to DT than humans or monkeys, principally owing to three amino acid changes in the DTR that prevent binding of the DT-B subunit to its receptor. (**b**) Expression of a high-affinity DTR by murine DC renders them uniquely sensitive to killing by low doses of DT. Binding of the B subunit to the DTR facilitates entry of the A subunit into the cell. Once internalised, the A subunit efficiently inhibits protein translation and induces apoptotic cell death.

Bennett & Clausen, *Trends Immunol*. 2007



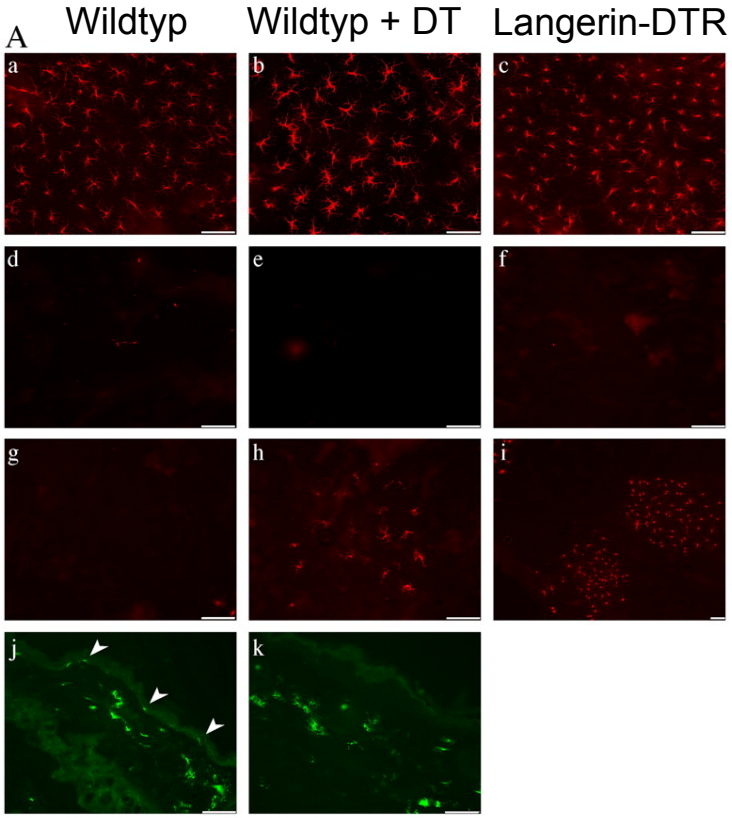
(E)GFP: (Enhanced) Green Fluorescent Protein
238 aa Protein aus der Qualle *Aequorea victoria*
als REPORTERGEN

Einbringen durch ungerichtete Transgenese oder gerichtetes „knock in“



Jung et al., *Immunity*, 2002

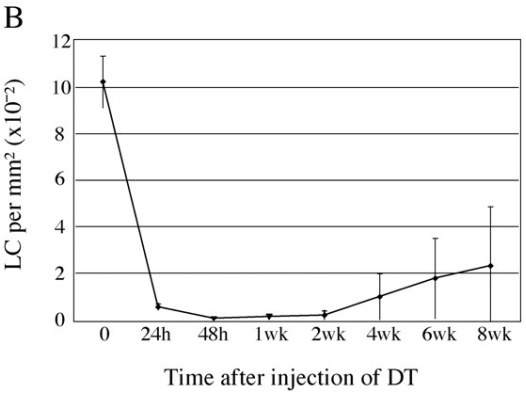
„Knock-in“ des Diphteria Toxin Rezeptors (DTR) in den Langerin-Locus (Langerin-DTR)

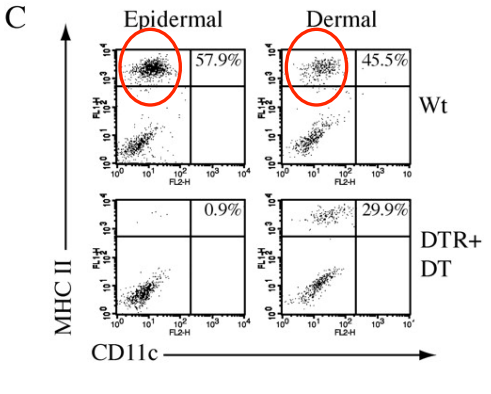


MHC II Färbung (a-c)

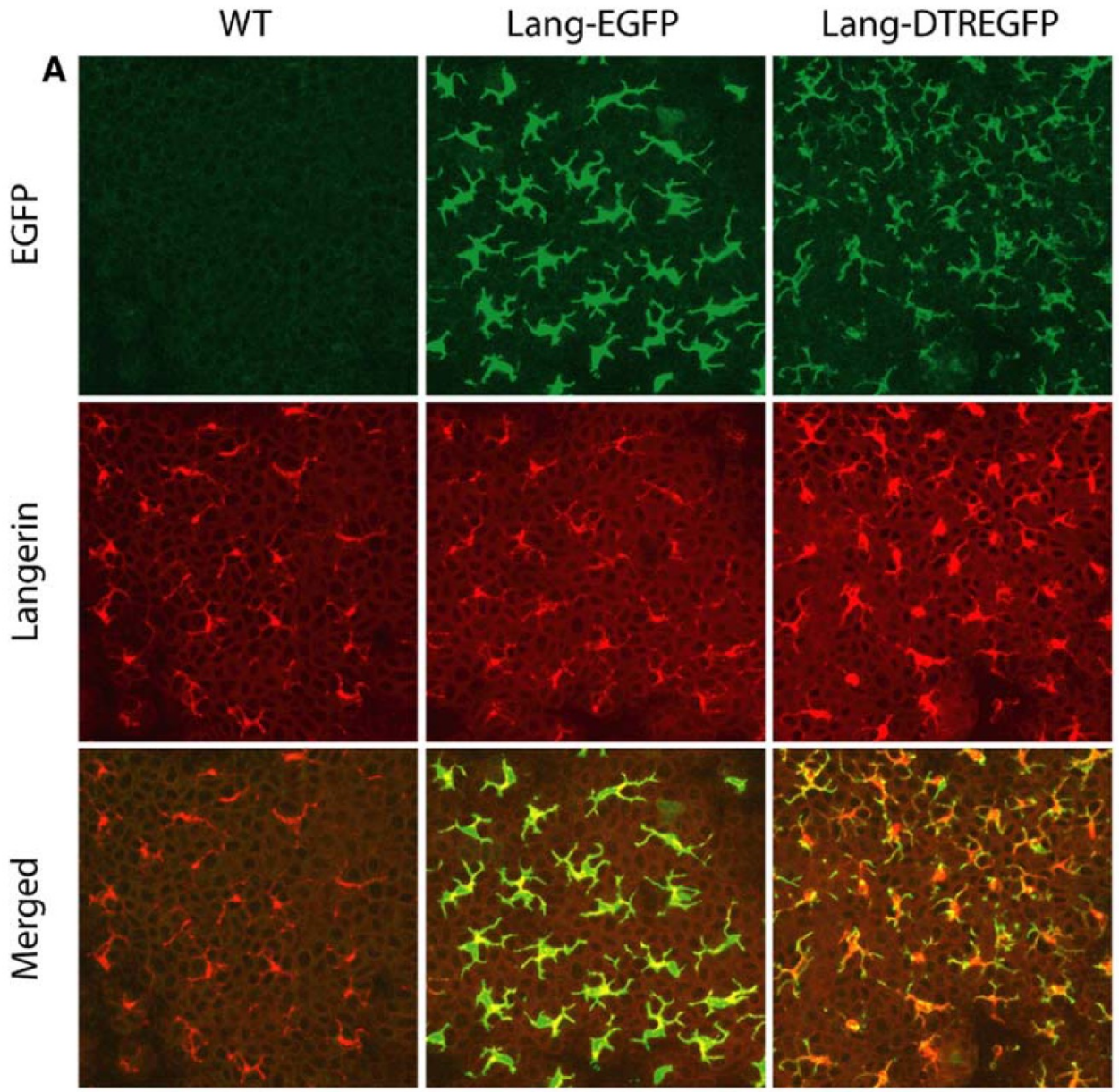
Langerin-DTR Mäuse nach DT Injektion:

d: nach 24 h
e: nach 48 h
f: nach 2 Wochen
g-i: nach 4 Wochen





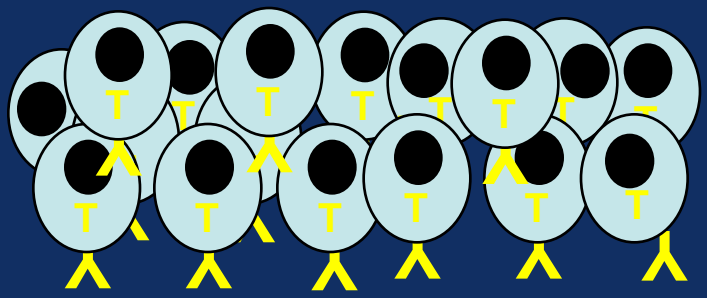
Bennet et al. *J Cell Biol,* 2005



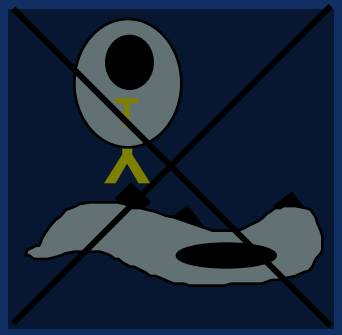
Bennet et al. *J Cell Biol,* 2005

Thymic education: die young

Randomly generated TCR repertoire ensures diversity



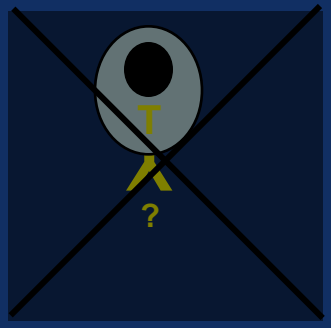
Very high avidity to self



Harmful

Negatively selected

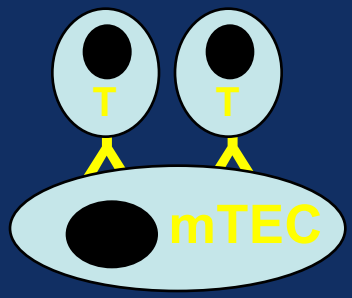
No avidity to self



Useless

Neglected

Intermediate avidity to self



Useful

Positively selected

Is central tolerance sufficient ?

No, not all self-antigens are expressed in the thymus during development.

No, a single TCR may bind to a variety of different peptide/MHC complexes ("Promiscuity" of the TCR)

Many potentially auto-reactive T cells escape central tolerance

How can the immune system prevent the activation of these potentially self-reactive T cells in the periphery?

The "third function" of the thymus

- Negative selection of autoreactive T cells
- Positive selection of peripheral T effector cells
- Positive selection of $CD4^+CD25^+$ Tregs

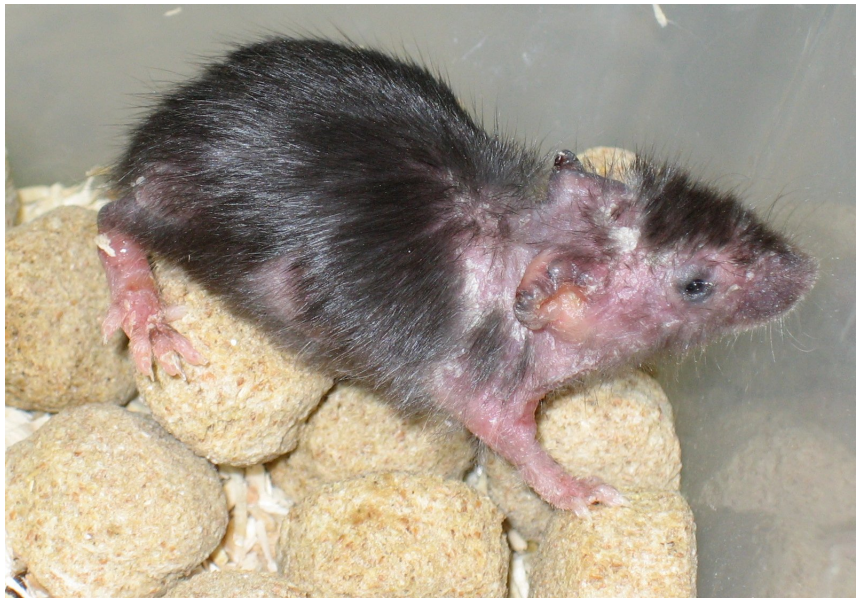
FoxP3 ("scurfin")

- Transcription factor of the forkhead family
- Loss of function mutation gives rise to the scurfy phenotype:

  X-linked recessive lymphoproliferative disease
  Hyperresponsive $CD4^+$ T cells
  over-expression of various cytokines (IL-2, IL-4, etc.)

  Autoimmune pathologies (diabetes, thyroiditis etc.)

  Severe infections

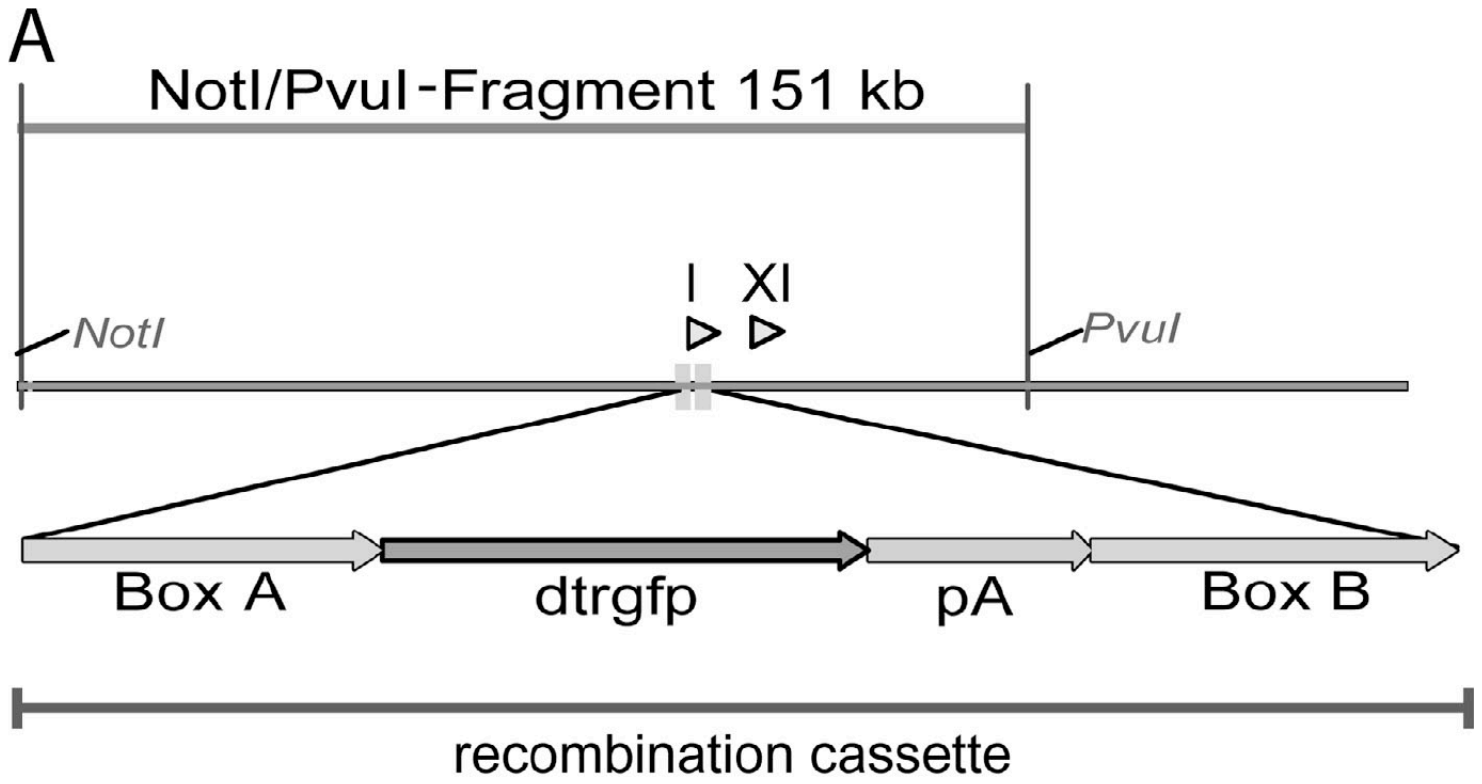


- Human equivalent: IPEX
  (Immunodysregulation, polyendocrinopathy and enteropathy, X-linked)

DEREG Mäuse: Depletion of regulatory T cells

Exprimieren ein Diphteria Toxin Rezeptor-enhanced green fluorescent protein Fusionsprotein
Unter Kontrolle des *foxp3* Gens

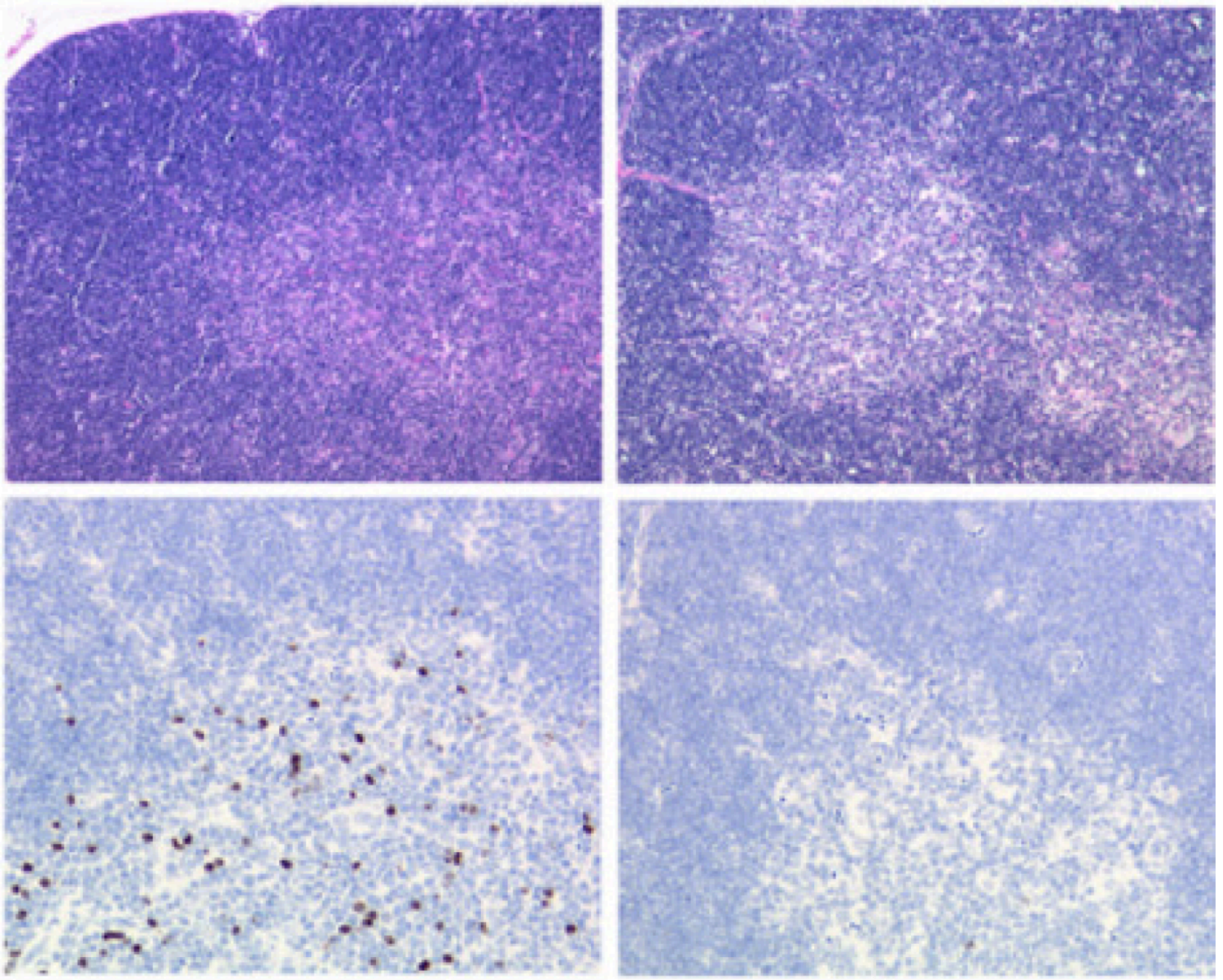
Strategie: Generierung eines modifizierten „bacterial artificial chromosome“ und
Injektion des BAC in den Pronucleus als Transgen



Box A, B: jeweils 1 kb homologer DNA

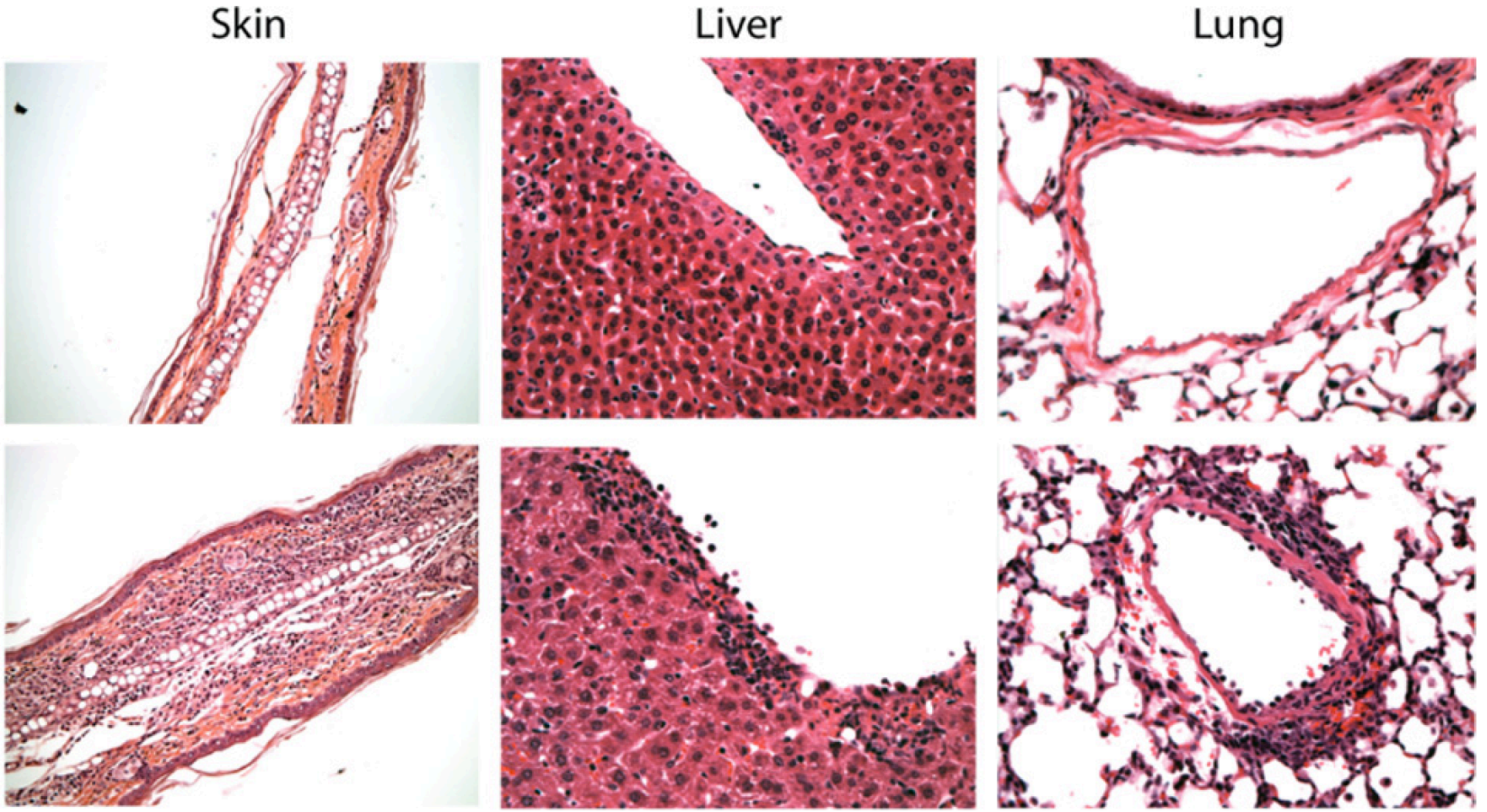
Lahl et al. J Exp Med (2005) 204:57

Depletion der regulatorischen T-Zellen durch Gabe des Diphteritoxins in DEREG-Mäuse...



Kim et al. J Immunol (2009) 183:7631

...führt zur Entwicklung autoaggressiver Phänomene



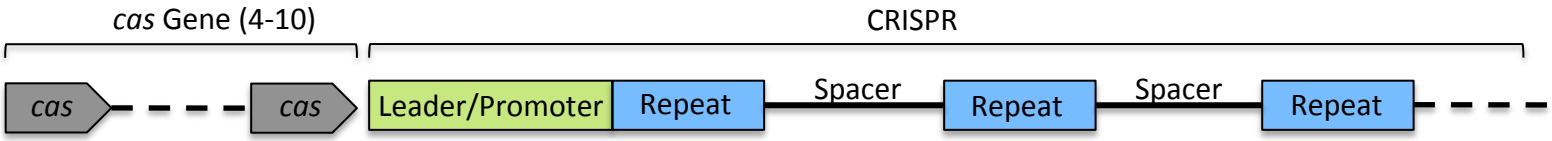
Kim et al. J Immunol (2009) 183:7631

CRISPR/Cas: Immunität von Archaeen und Bakterien gegen fremde Nukleinsäuren

CRISPR: clustered regularly interspaced short palindromic repeats

Cas: CRISPR-associated (genes, proteins)

Finden sich in Archaeen (90%) und Bakterien (40%)
Entdeckt in E. coli (1987)



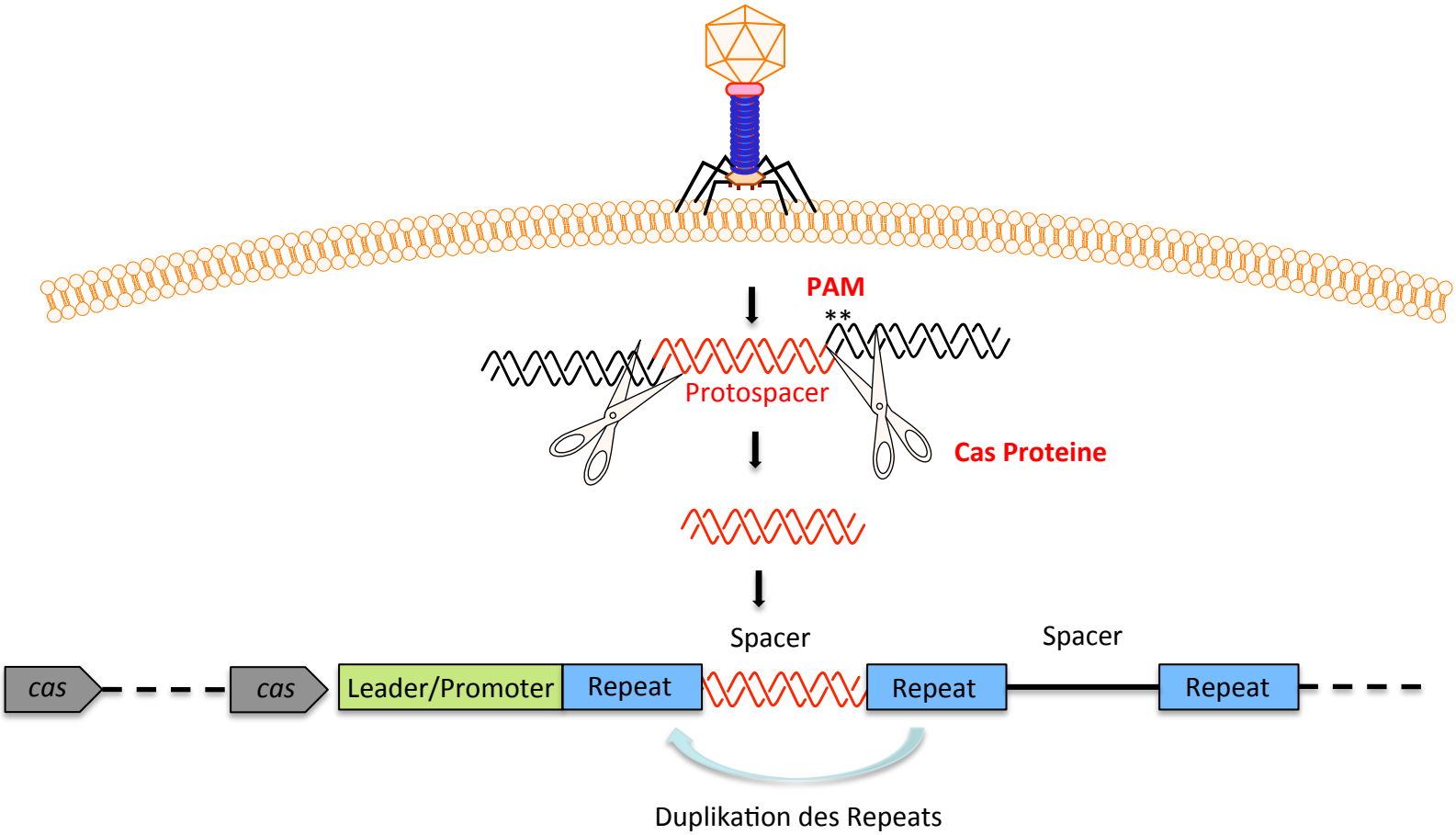
Cas: Große heterogene Gengruppe, deren Produkte Helikase u./o. Nuklease-Aktivität aufweisen

Repeats: 20-50 bp, innerhalb eines CRISPR-Locus konserviert, ansonsten heterogen

Spacer: Kurzes DNA-Fragment, das aus Viren oder Plasmiden stammt (Protospacer)

CRISPR/Cas-System verleiht Immunität gegen fremde Nukleinsäuren !

Einbau fremder DNA-Fragmente in den CRISPR-Locus



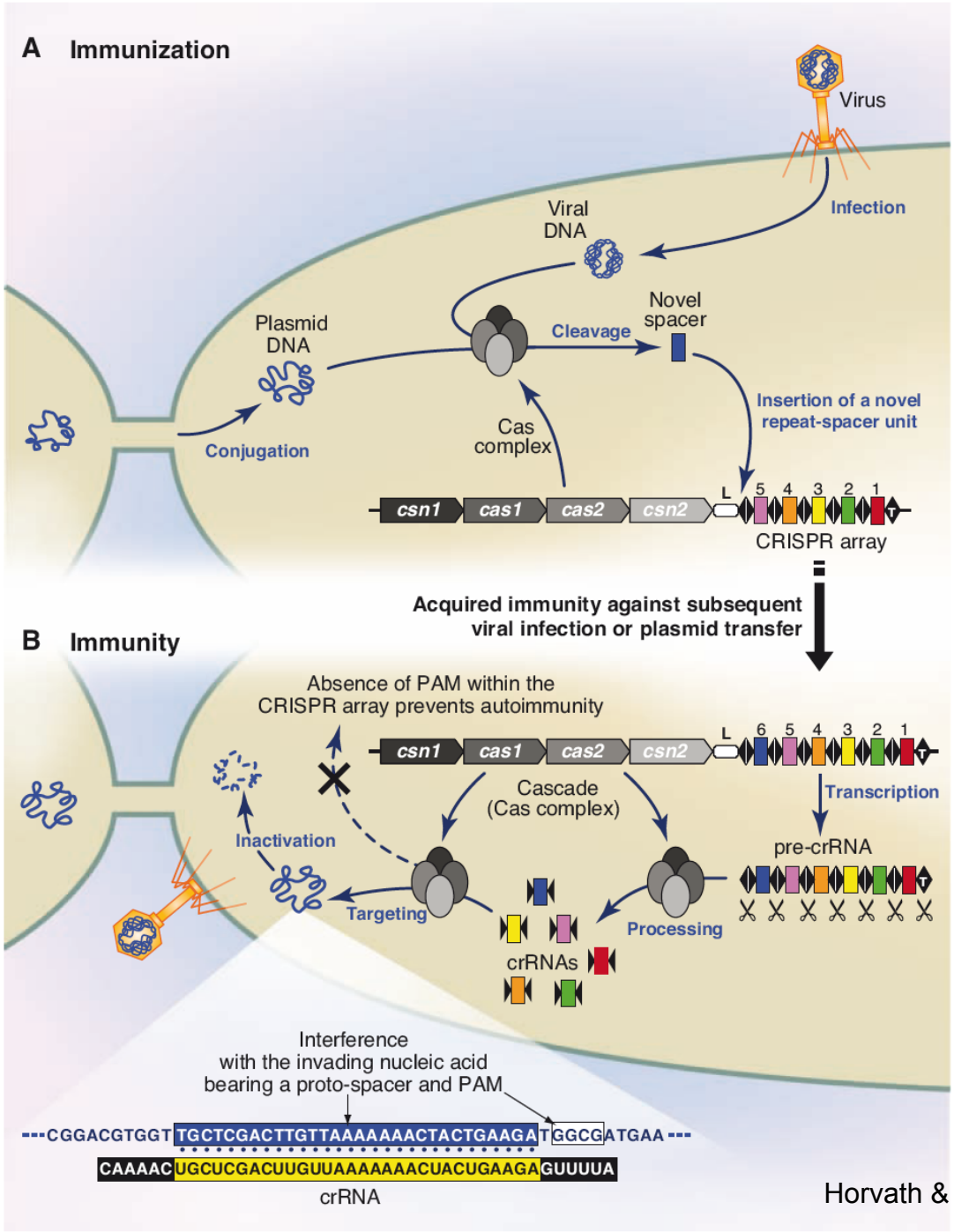
Protospacer werden nicht zufällig ausgewählt, sondern flankiert von PAM:

Protospacer adjacent motifs

Table 2. CRISPR-Cas Systems Developed for Genome Editing and Their Cognate PAM Requirements

Species	PAM	References
Streptococcus pyogenes	NGG	Hsu et al., 2013
Streptococcus mutans	NGG	van der Ploeg, 2009
Streptococcus thermophilus (CRISPR3)	NGGNG	Deveau et al., 2008; Fonfara et al., 2014; Horvath et al., 2008
Streptococcus thermophilus (CRISPR1)	NNAAAAW	Fonfara et al., 2014
Campylobacter jejuni	NNNNACA	Fonfara et al., 2014
Neisseria meningitidis	NNNNGATT	Hou et al., 2013; Zhang et al., 2013
Pasteurella multocida	GNNNCNNA	Fonfara et al., 2014
Francisella novicida	NG	Fonfara et al., 2014
Treponema denticola	NAAAAN	Esvelt et al., 2013

Pelletier et al. *Immunity* (2015)

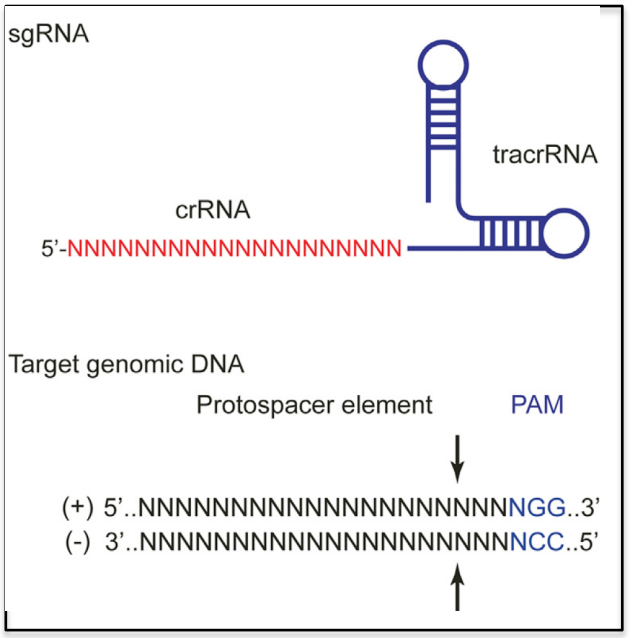


Horvath & Barrangou *Science* (2010)

Genmanipulationen durch „RNA-guided DNA targeting“ mit Cas9

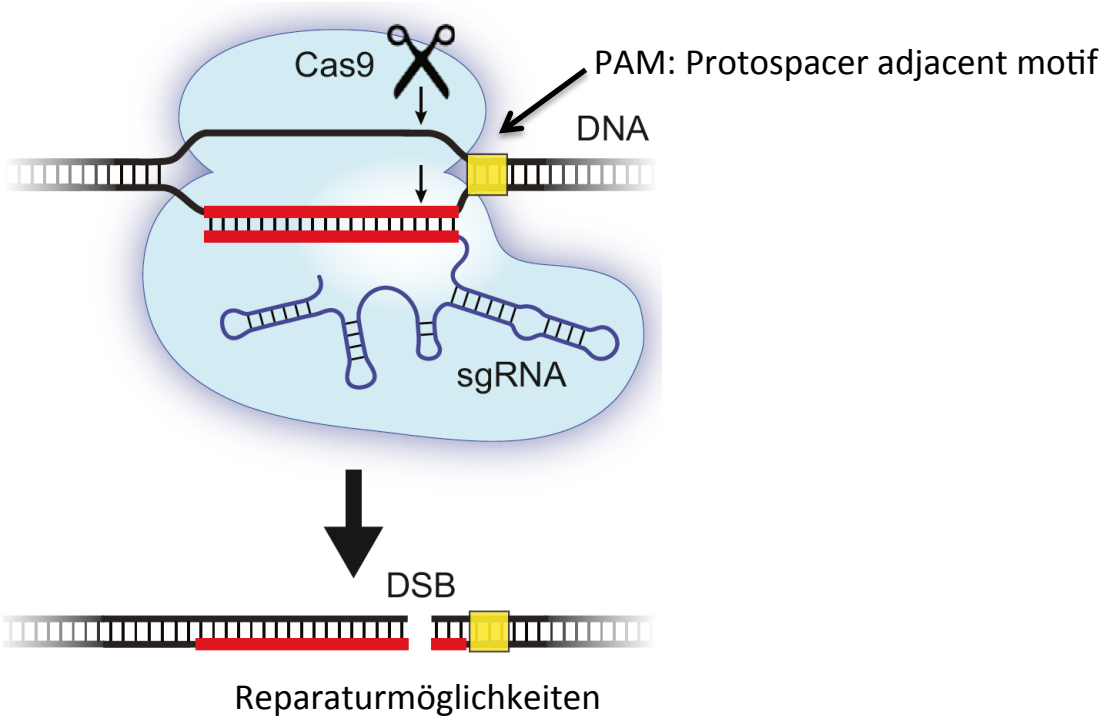
- Cas9 Endonuklease erzeugt Doppelstrangbrüche in DNA-Zielsequenzen
- Zielsequenzen werden durch einzelsträngige guide RNA (sgRNA) identifiziert
- sgRNA besteht aus crRNA und tracrRNA
- 5‘-Ende der crRNA macht spez. Basenpaarungen mit DNA-Zielsequenz
- 3‘-Ende der tracrRNA rekrutiert Cas9

Cas9 dient der Erkennung und Spaltung der Zielsequenz



Pelletier et al. *Immunity* (2015)

Zelleigene Reparatursysteme werden zur Mutagenese benutzt



„Non-homologous end-joining“

In Abwesenheit geeigneter Matrizen
Fehleranfällig; oft kleine Insertionen oder Deletionen („Indels“)

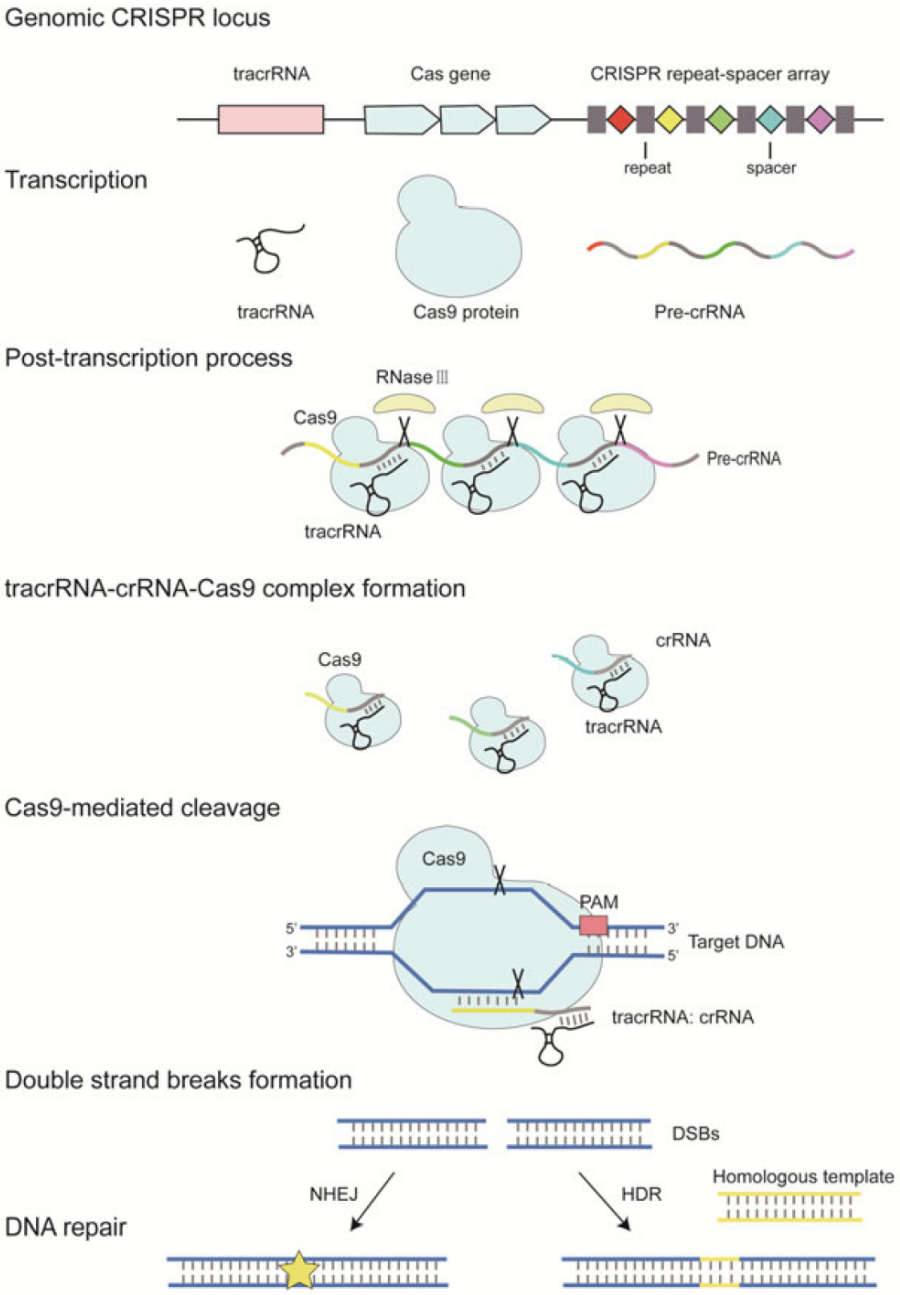
→ Einbau zufälliger Mutationen, STOP-Codons

„homology-directed repair“

Einbau neuer Sequenzen mit homologen Enden zur Zielsequenz

→ Punktmutationen, Deletionen, Insertionen, konditionale Allele

Sternberg & Douda (2015*) Mol Cell* (modifiziert)



PAM: protospacer adjacent motifs
DSB: double strand break
NHEJ: non-homologous end joining
HDR: homology-directed repair

Peng et al. FEBS J 2015

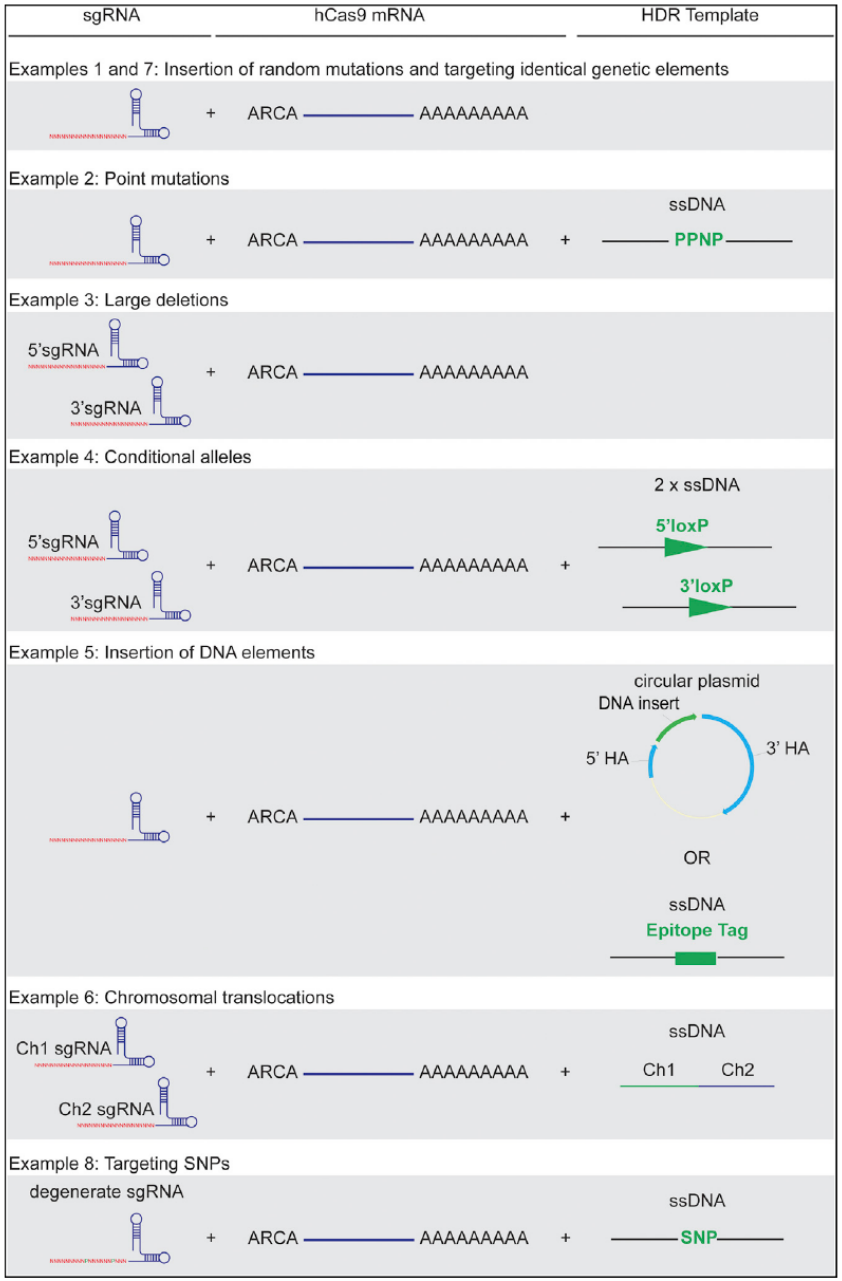


Figure 2. Targeting Strategies Used to Introduce Various Types of Mutations by CRIPSR-Cas9 Technology

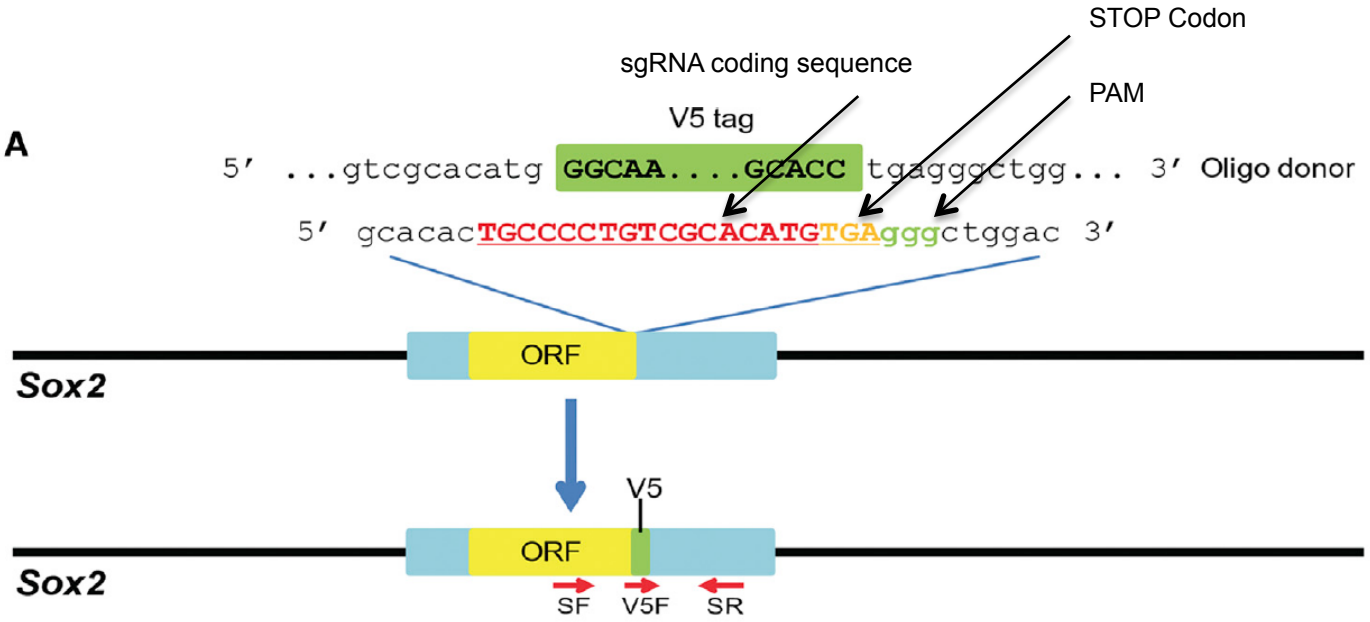
Examples 1 and 7 (insertion of random mutations and targeting identical genetic elements) make use of a single sgRNA together with Cas9 to target a single copy or multiple copy number genes. Resolution of the DSB(s) by NHEJ will result in the generation of several genetic modifications, including nonsense, missense, or various deletions. Example 2 (point mutations) makes use of a sgRNA together with Cas9 and an ssDNA oligonucleotide to facilitate HDR. Resolution of the DSB by HDR will result in the introduction of the desired mutation (P). Example 3 (large deletions) makes use of two sgRNAs flanking a region of interest. Resolution of the DSBs by NHEJ will result in the deletion of the region of interest. Example 4 (conditional alleles) also makes use of two sgRNAs flanking a region of interest, Cas9 and Cas2 ssDNA oligonucleotides, to facilitate HDR. Resolution of DSBs by HDR will introduce *loxP* sites at the desired locations. Example 5 (insertion of DNA elements) makes use of one sgRNA, Cas9, and a circular plasmid or ssDNA oligonucleotide. Resolution of DSBs by homologous recombination will introduce the DNA elements. Example 6 (chromosomal translocations) makes use of two sgRNAs designed to introduce DSBs on separate chromosomes (Ch1 and Ch2) together with Cas9 and a single ssDNA with sequence complementarity flanking the translocation break. Example 8 (targeting SNPs) makes use of a single degenerate sgRNA containing one or two mismatches with its target sequence. Resolution of the DSB by HDR will introduce a single-nucleotide mutation. The newly form sites, now having an extra mismatch, might no longer be recognized by the degenerate sgRNA-Cas9 ribonucleoprotein complex and cleaved again, allowing the insertion of a SNP.

Pelletier et al. *Immunity* (2015)

Anwendungen des CRISPR/Cas-Systems

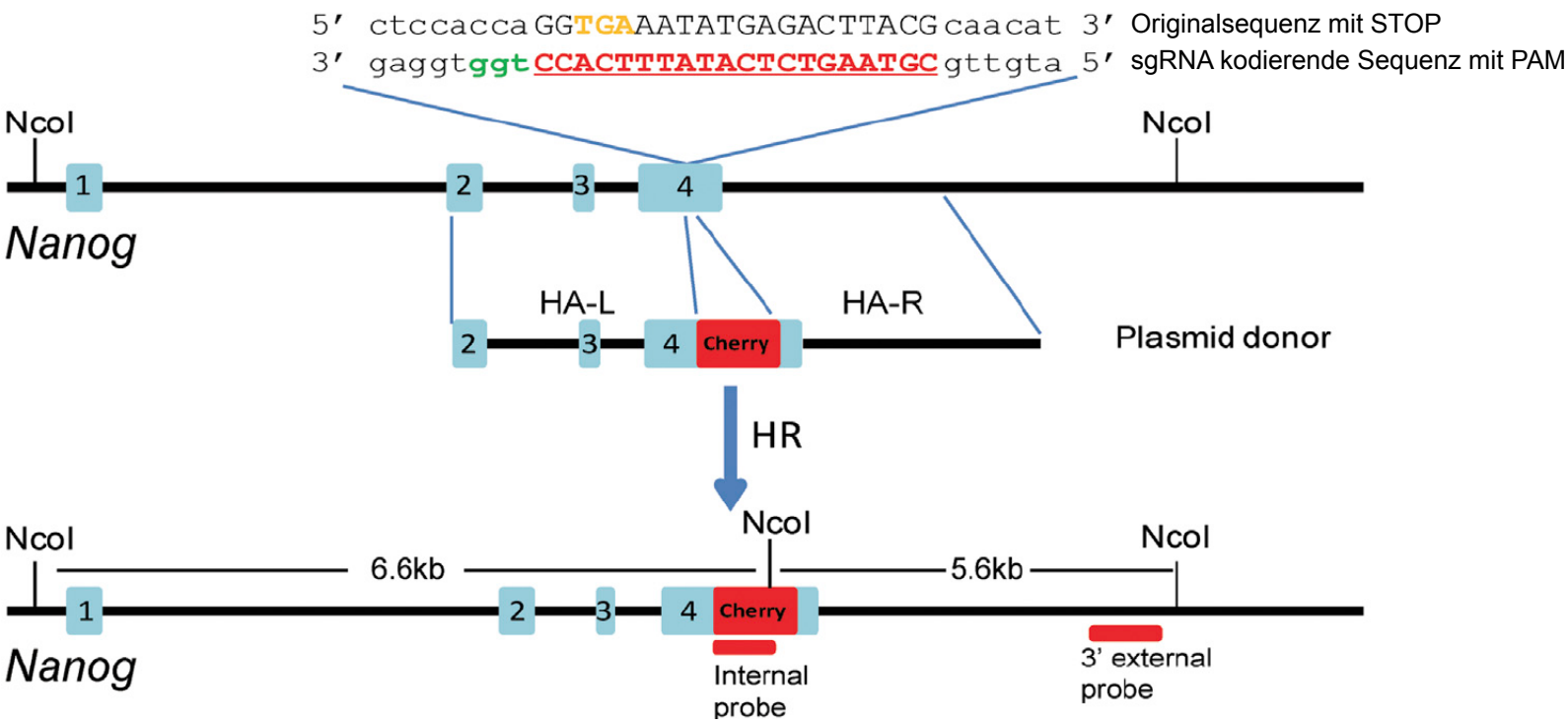
Beispiel 1: Einbau eines synthetischen Epitops („tag“) in endogenes Gen

- sgRNA ist homolog zum Bereich des Stop-Codons des Zielgens
- ssDNA-Oligo mit 42b V5 tag und 60 homologen Basen auf jeder Seite fusioniert mit letztem Codon
- Injektion von sgRNA, Cas9mRNA und Oligo in Zygoten



Yang et al. (2013) *Cell*

Beispiel 2: Knock in eines Reportergens



Yang et al. (2013) *Cell*