# Mutagenesis in mice and rats using mobile DNA technology

**David A. Largaespada**
University of Minnesota

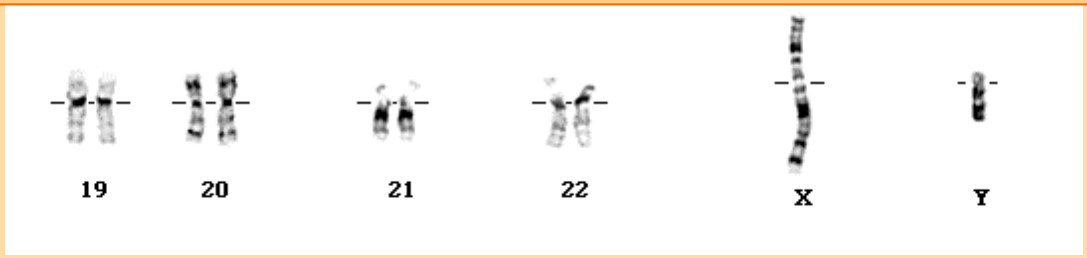
# Disclosures

• I am a consultant, co-founder, and co-owner of NeoClone Biotechnology, Inc., an antibody company. This presentation does not relate to NeoClone's business area.

• I am a consultant, co-founder, and co-owner of Discovery Genomics, Inc. (DGI), a company developing *Sleeping Beauty* (SB) for human gene therapy. This work did not involve DGI personnel or funds and is not directly related to DGI's business area.

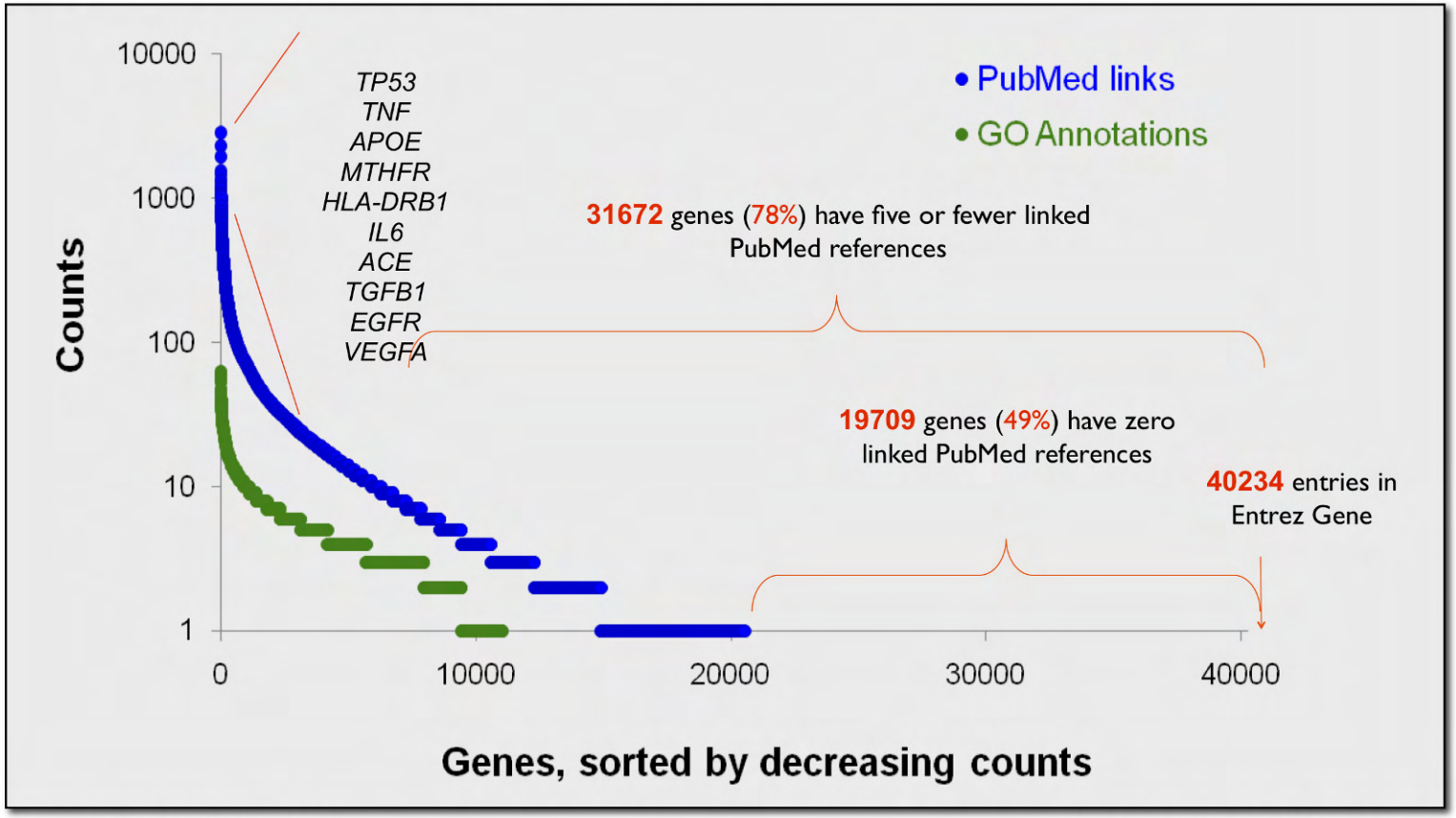
# The human genome

**How many genes are there?**
**What do they do?**
**Which are critical in disease?**
**What does the functional landscape of the mammalian genome look like?**



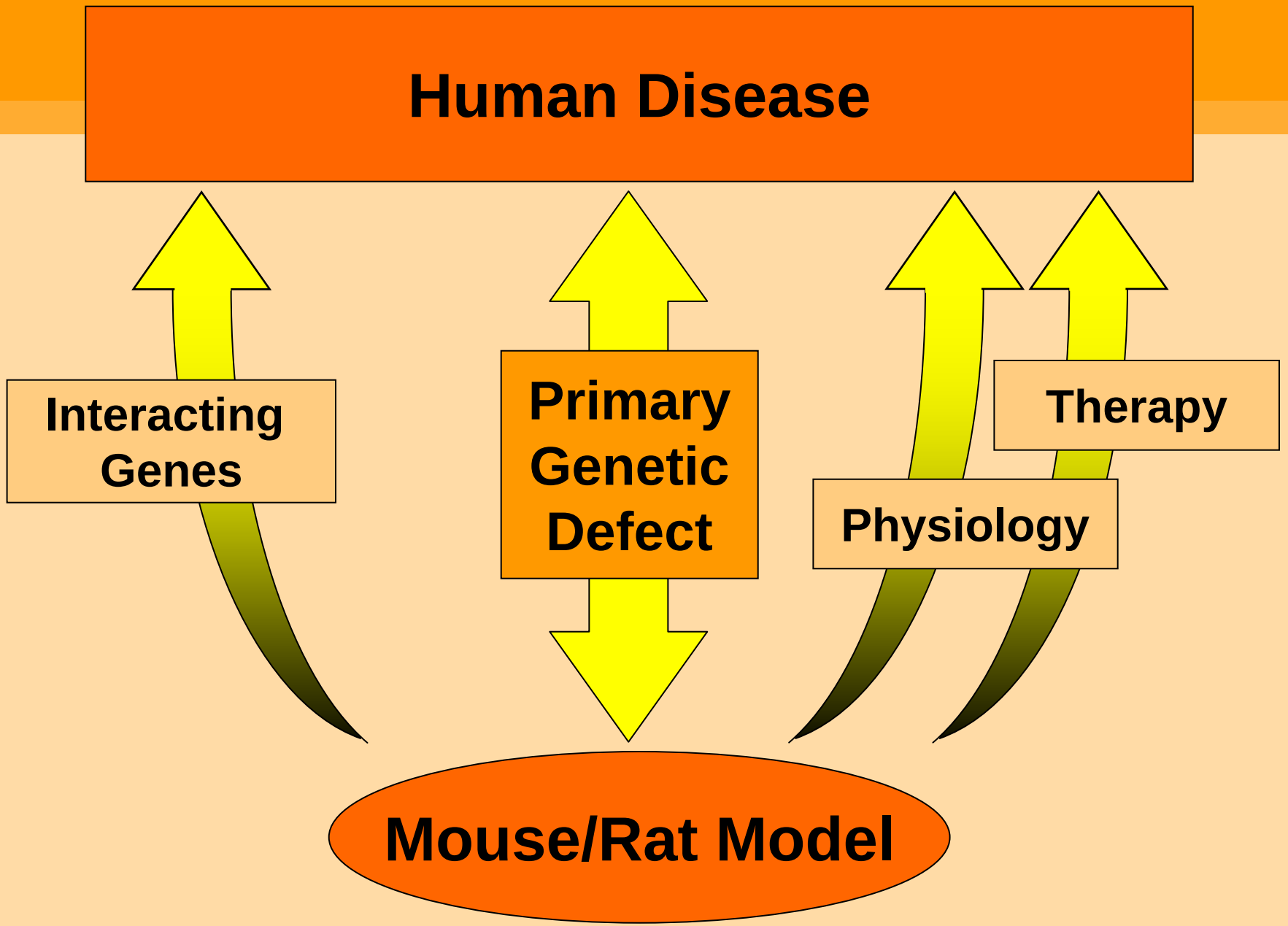
# Well Studied Genes Continue to Get all the Attention

- How do we efficiently annotate the function of all the genes in the mammalian genome?
- Goal: "Genome-wide functional genomics"

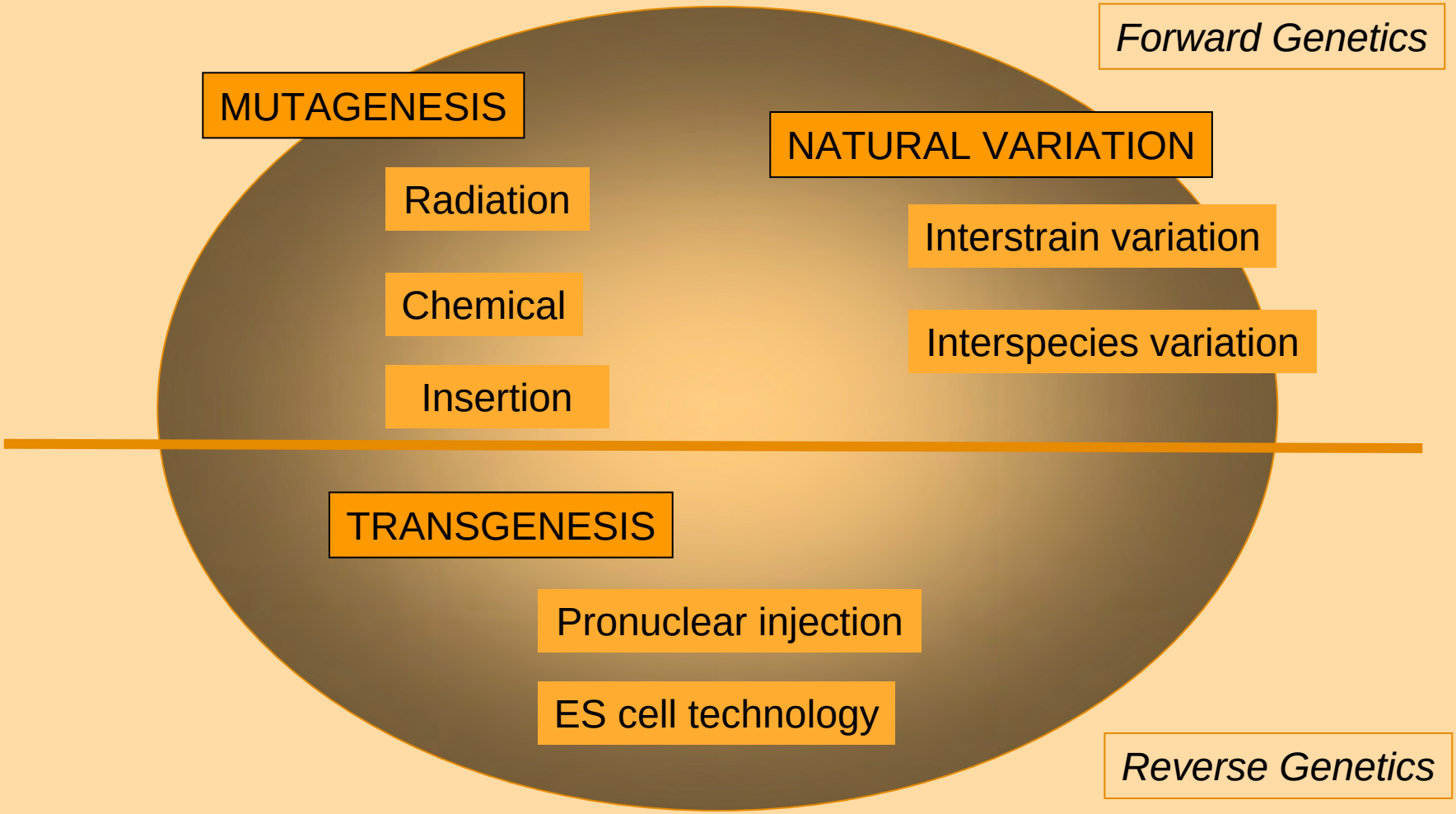



*Huss et al., NAR, 2009*

*Slide from Dr. Andrew Su*


Human Disease
Interacting Genes
Primary Genetic Defect
Physiology
Therapy
Mouse/Rat Model

# Sources of genetic variation



# Forward versus reverse genetics

*Reverse Genetics*


GENE

Transgenesis

PHENOTYPE

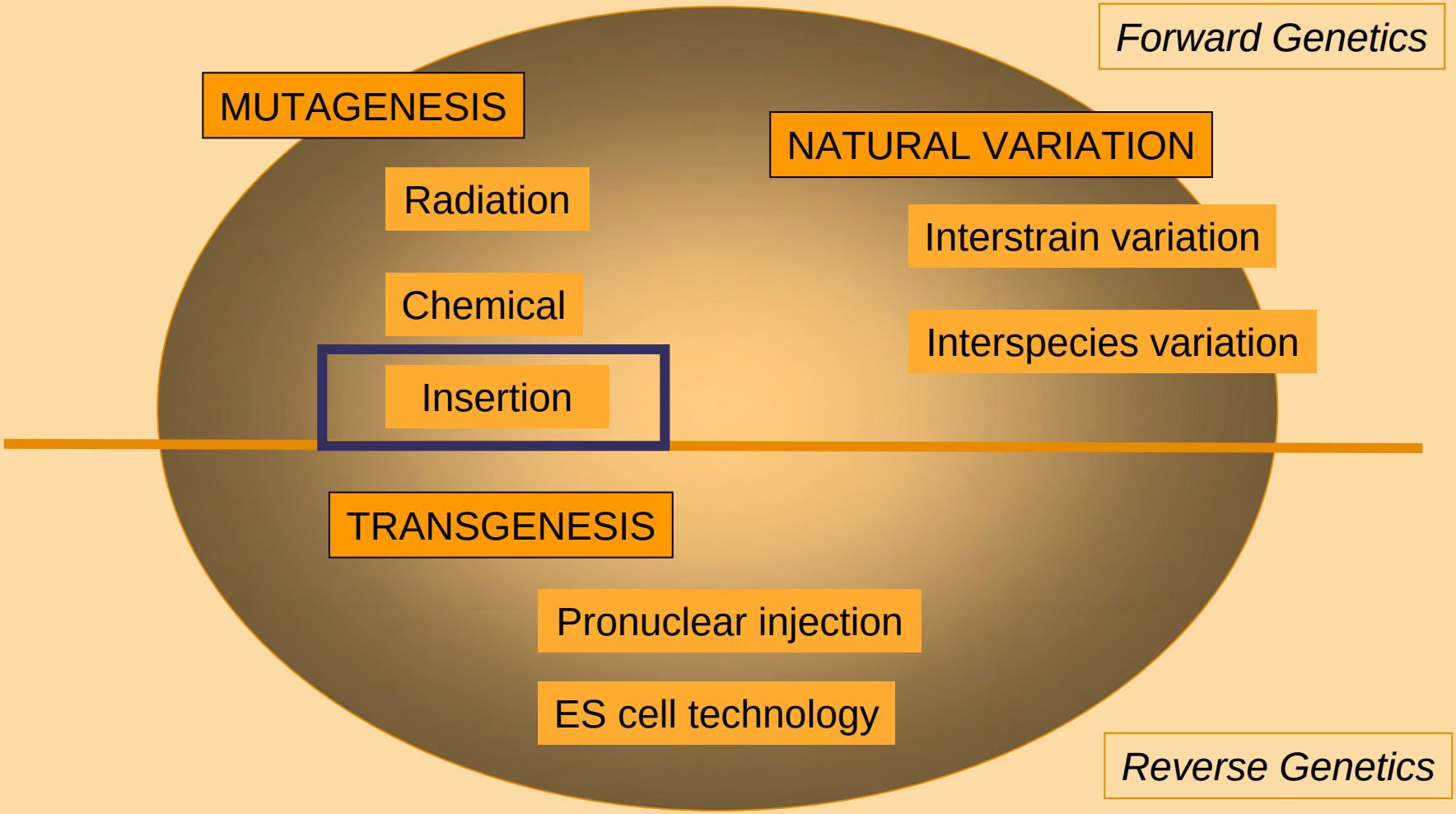
*Forward Genetics*

Mutagenesis, Natural variation

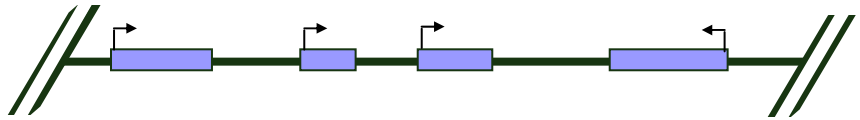
PHENOTYPE


GENE

# Sources of genetic variation



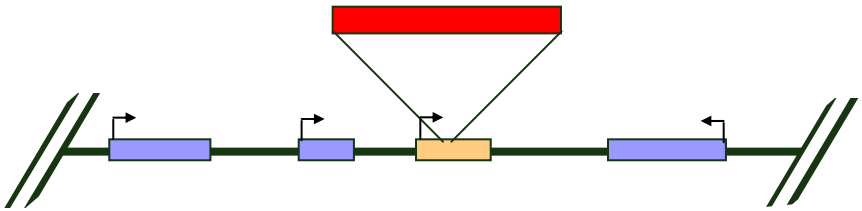
# Insertional mutagenesis can simplify gene identification



Wild Type

*genome size: 3 x $10^9$bp*

*# of genes: 25,000+?*

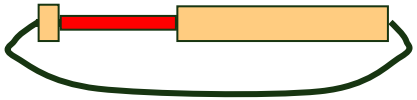


Mutant

*clone size: <$10^3$bp*

*# of genes: 1*

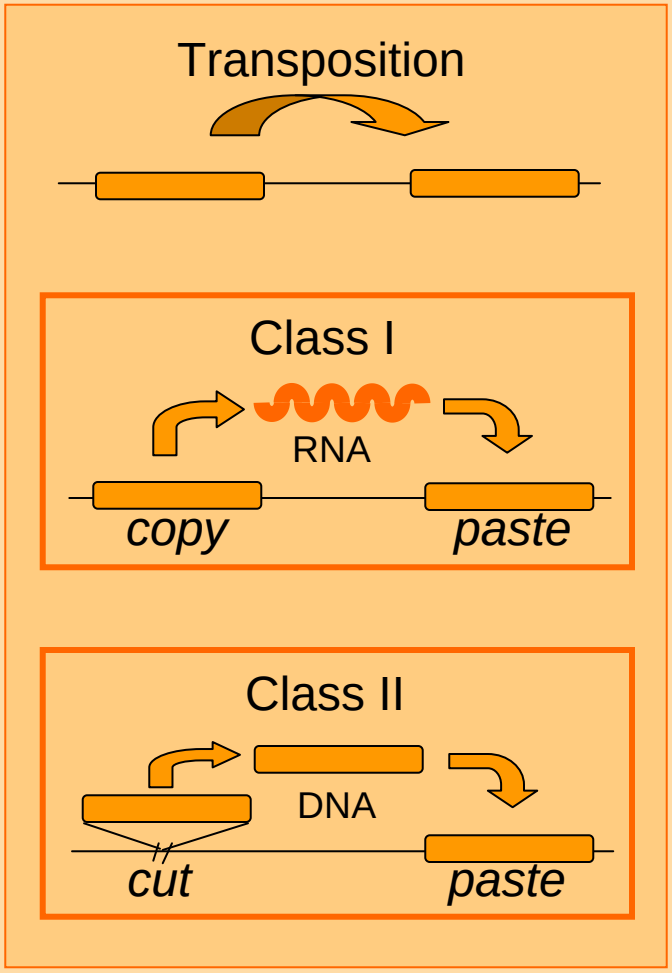
*Molecular Cloning*



Naturally occurring systems of mobile DNA are ideal for this purpose

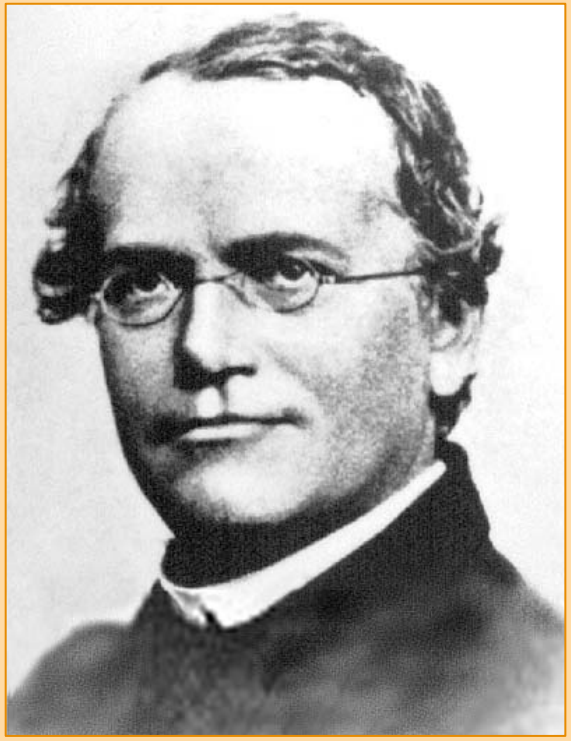
# Transposable elements

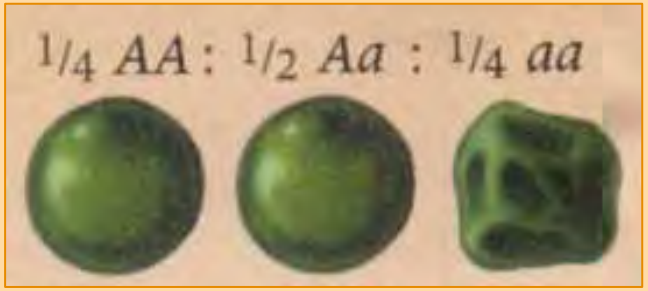
- Sequences of DNA that move around to different positions within genome of a single cell
- “Jumping genes” or “mobile genetic elements”
- Class I or retrotransposons and Class II or DNA transposons
- Encode proteins needed for transposition reaction, e.g. transposases



# Transposons at the beginning of modern genetics

- Mendel's wrinkled pea mutation
- Transposon insertion into a gene encoding a starch branching enzyme



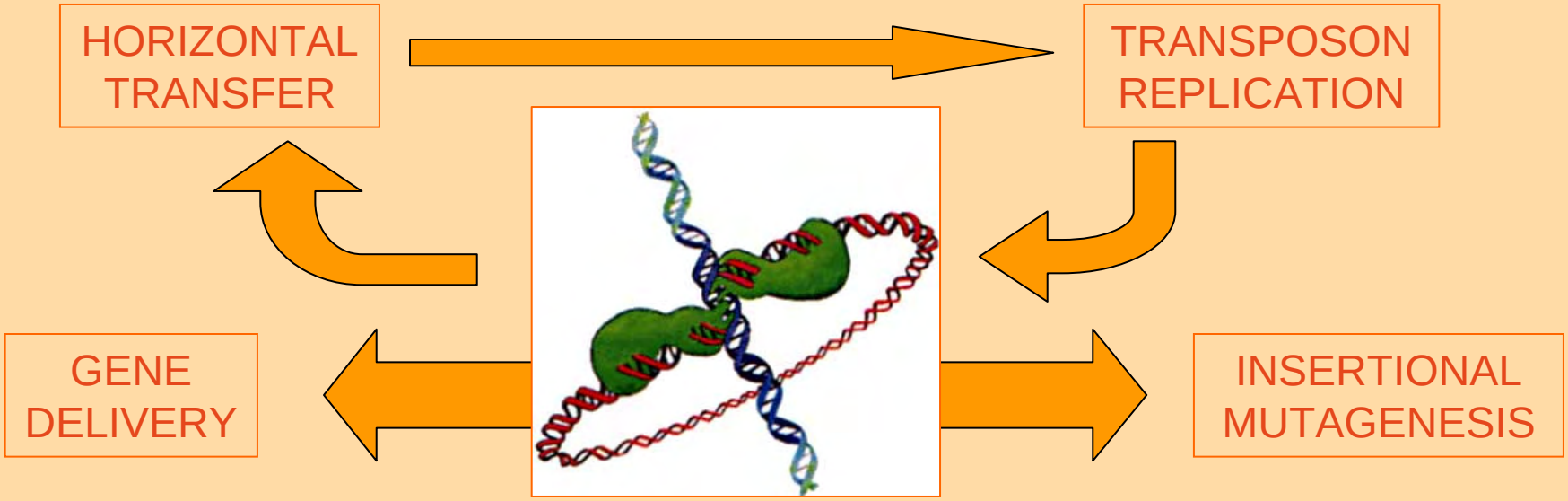



*Bhattacharra et al , Cell, 1990.*

# Barbara McClintock discovered transposable elements

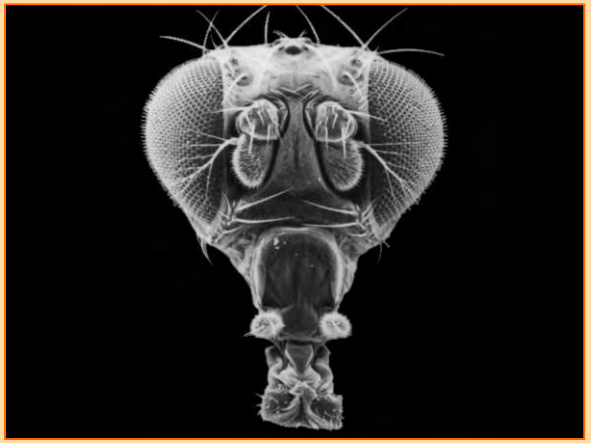
- Definitive proof in 1952
- Showed linkage arrangement of genes could change in Maize
- Nobel Prize in 1983

# The properties of transposable elements make them useful for genetics



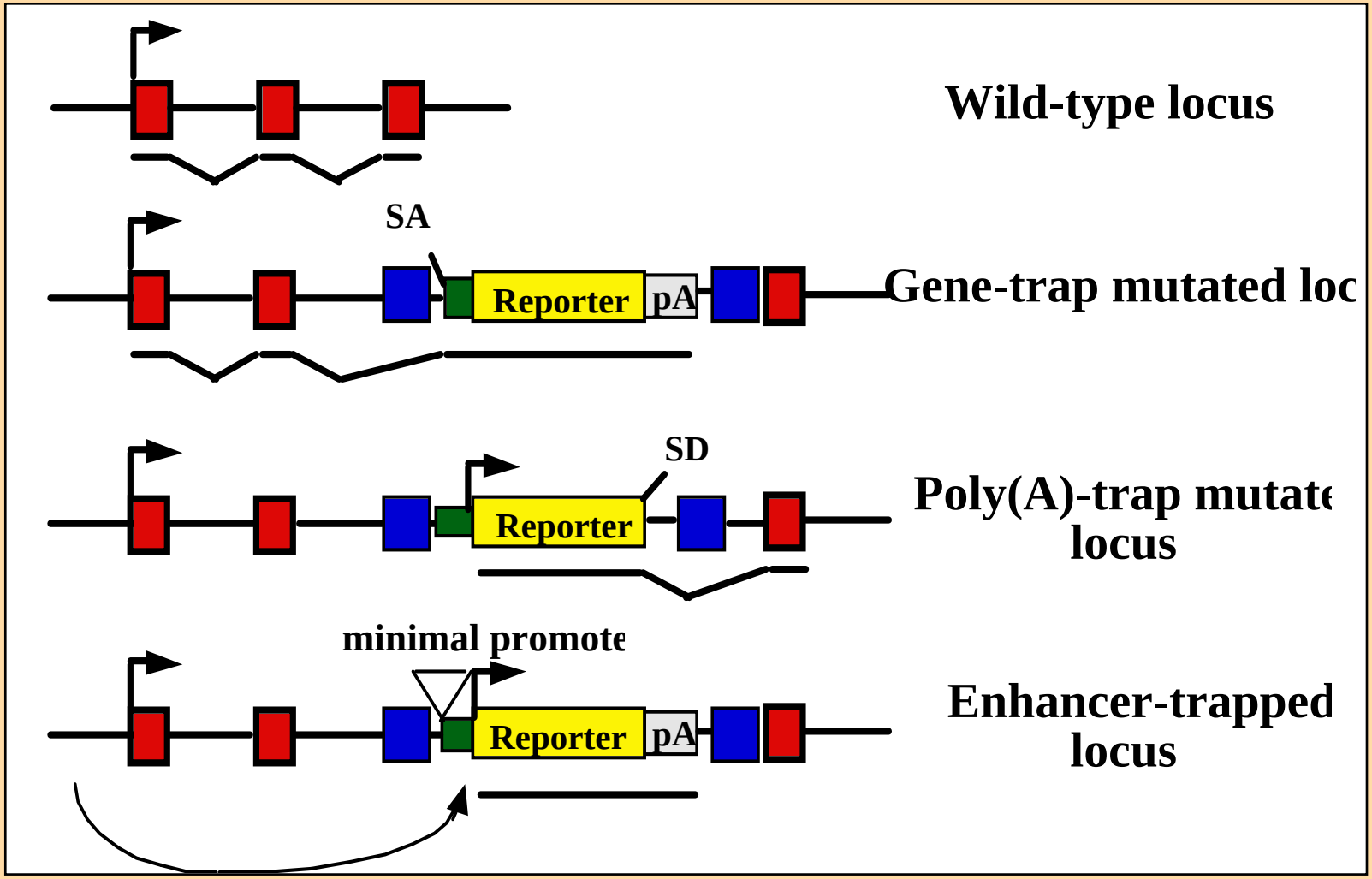
# Transposable elements become useful tools for geneticists

- 1982: The P element used to genetically transform *Drosophila*
- 1989: P element "enhancer traps" reveal patterned gene expression in Drosophila



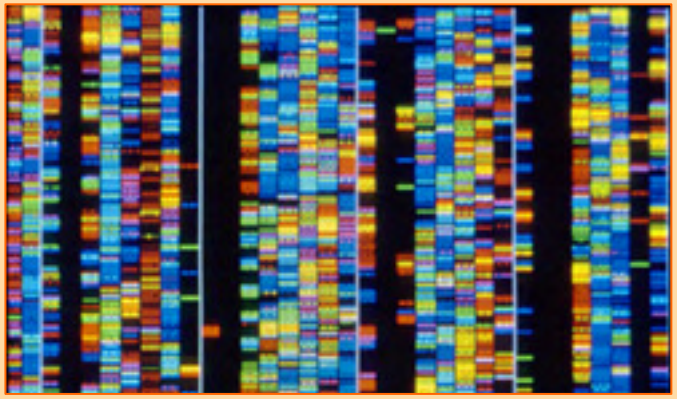
# Transposable elements are probes for the functional parts of a gene

Reporter gene vectors sensitive to insertion site context



# Transposable elements have entered mainstream basic and applied science

- Now that thousands of elements have been identified the possibilities for their use have expanded



- Biotechnology companies founded on transposable elements

EXELIXIS™


Minos BioSystems

DGI™
Discovery Genomics, Inc.

# Transposons for genetics/genomics

- Comprehensive insertion mutation libraries established in many species: *Drosophila*, plants, bacteria, fungi
- Complement chemical mutagenesis approaches
- Can be used to create useful “tagged” libraries (e.g. GFP-tagged ORFs)
- Fundamental platform for functional genomics
- Vertebrate genetics/genomics?

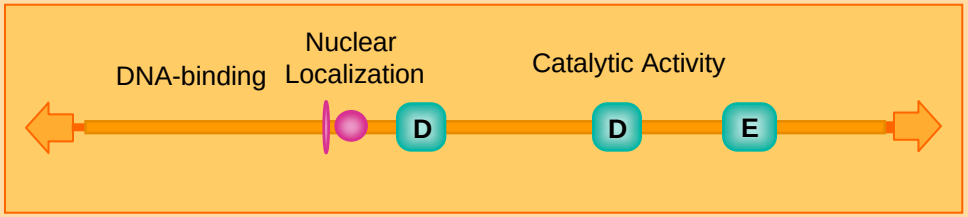
# *Sleeping Beauty* (SB)

•A synthetic “cut-and-paste” DNA transposon system

•Derived from inactive Tc1/mariner family transposon elements in fish

-Tc1/mariner elements widespread - animals, plants, fungi, ciliates and bacteria but all vertebrate elements dead

-Inverted terminal repeats, internal transposase with paired-like DNA binding and DDE catalytic domains



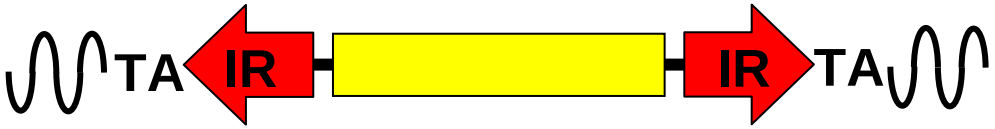

•Created in Dr. Perry Hackett’s lab by Dr. Zoltan Ivics and Dr. Zsuzsanna Izsvak - creating a consensus transposase sequence - reverse evolutionary approach

*Ivics et al., Cell, 1997*

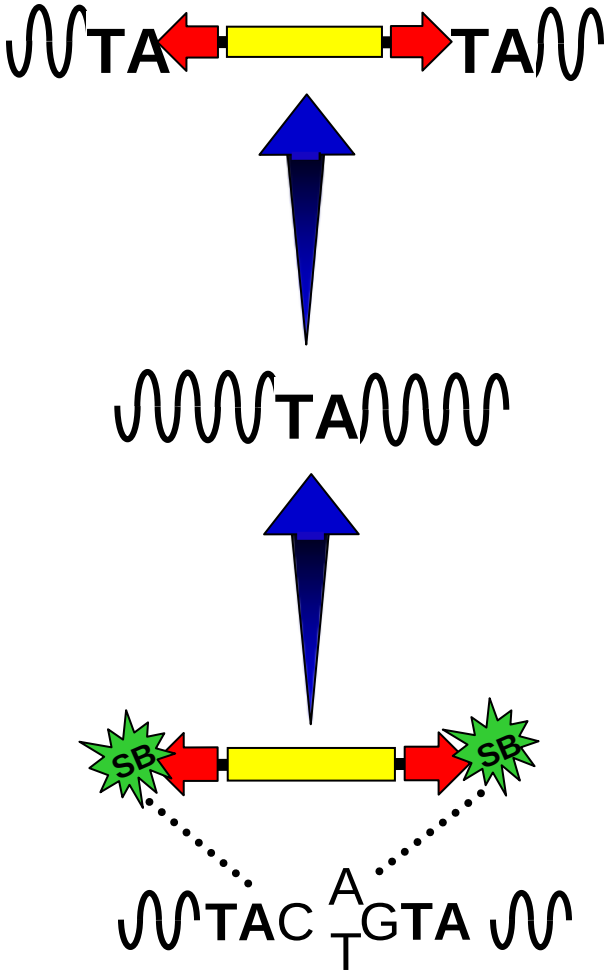
# *Sleeping Beauty* is a two part system: The transposon and the transposase

**1. Transposase protein**

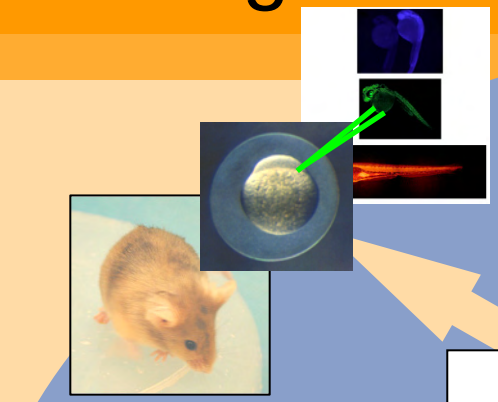

**2. Transposon vector DNA**

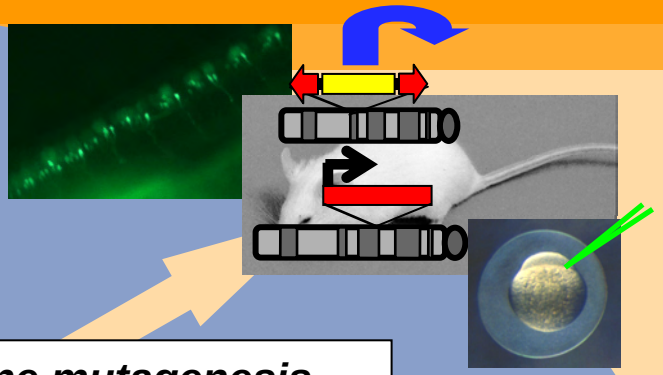



**3. A "cut and paste" reaction**



# SB: A versatile tool for applied and basic biological research





Germline

Germline transgenesis

Germline mutagenesis

Gene Transfer

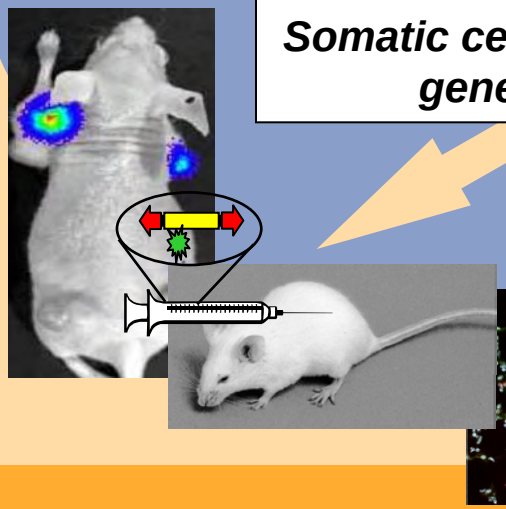
SB

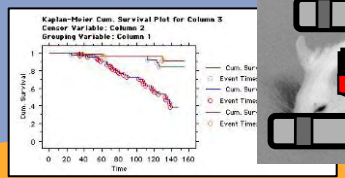
Insertional Mutagenesis

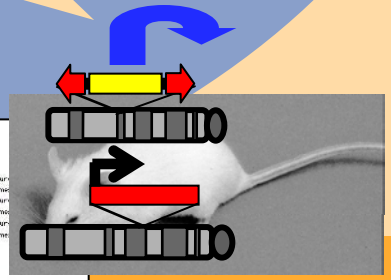
Somatic cell transgenesis, gene therapy

Somatic cell mutagenesis

Somatic

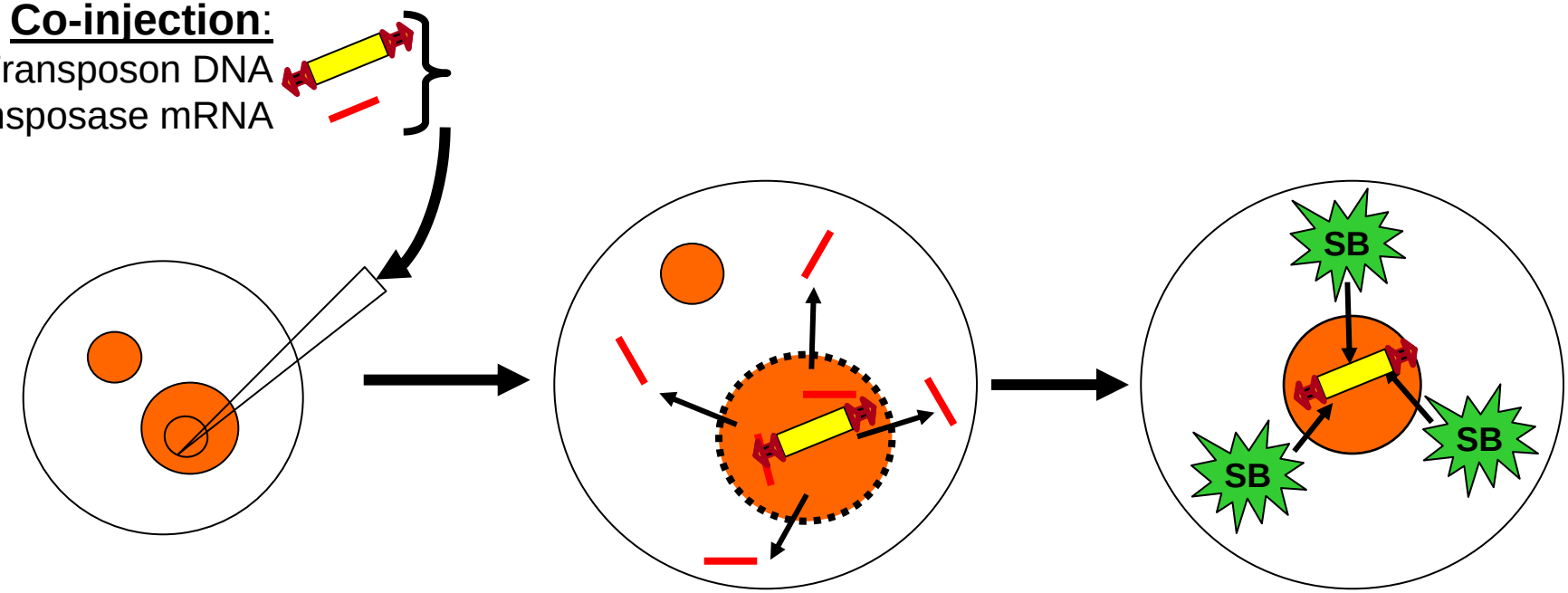






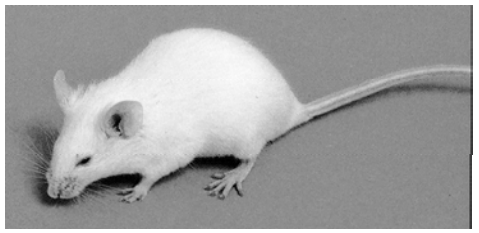
# Germline transgenesis by transposition

**Co-injection**:
Transposon DNA
Transposase mRNA




- 50% transgenesis rate
- 3 transposase-mediated insertions/mouse

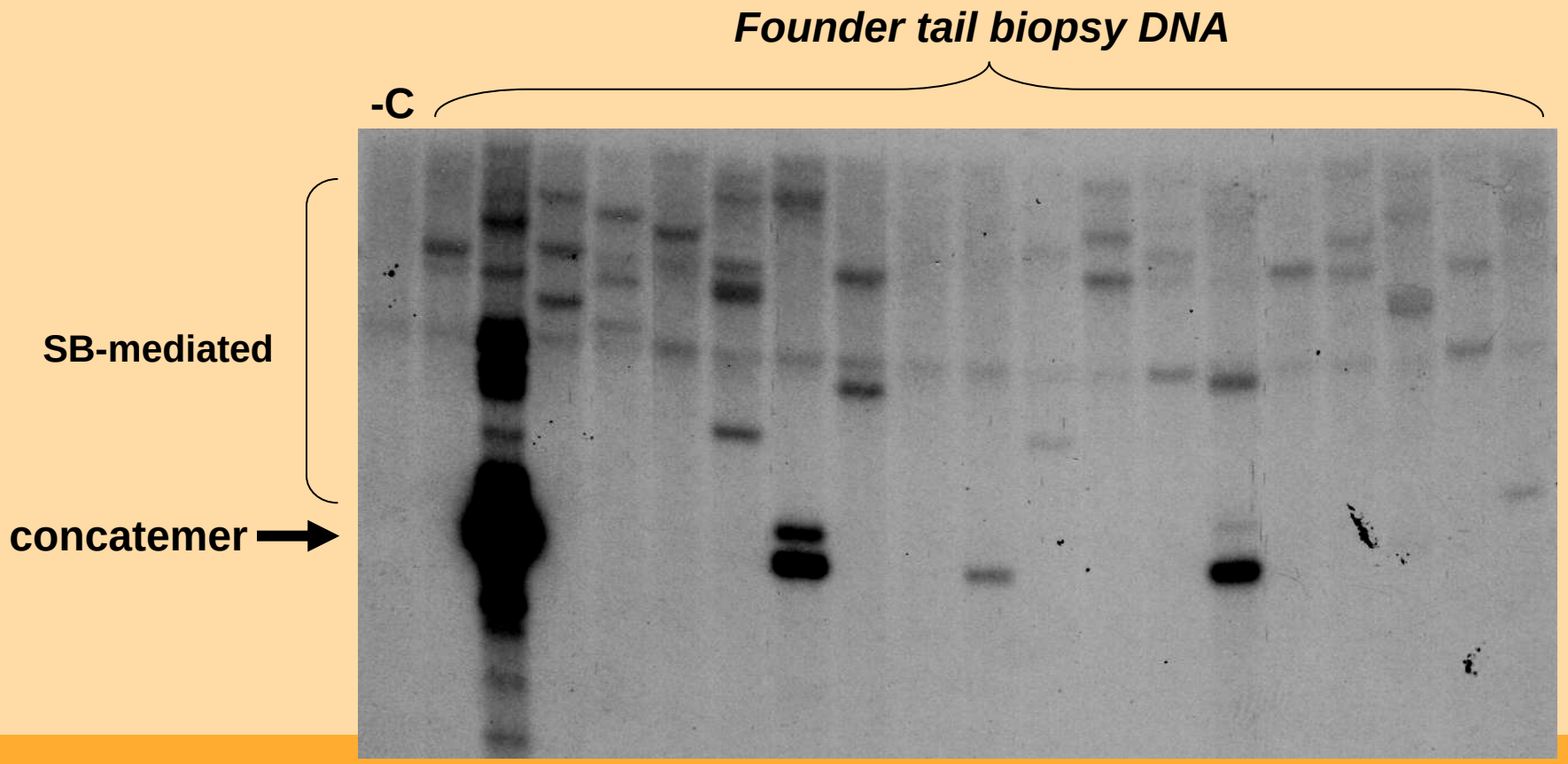
Analyze offspring by:
- Southern
- PCR



***Dupuy et al., PNAS, 2002.***

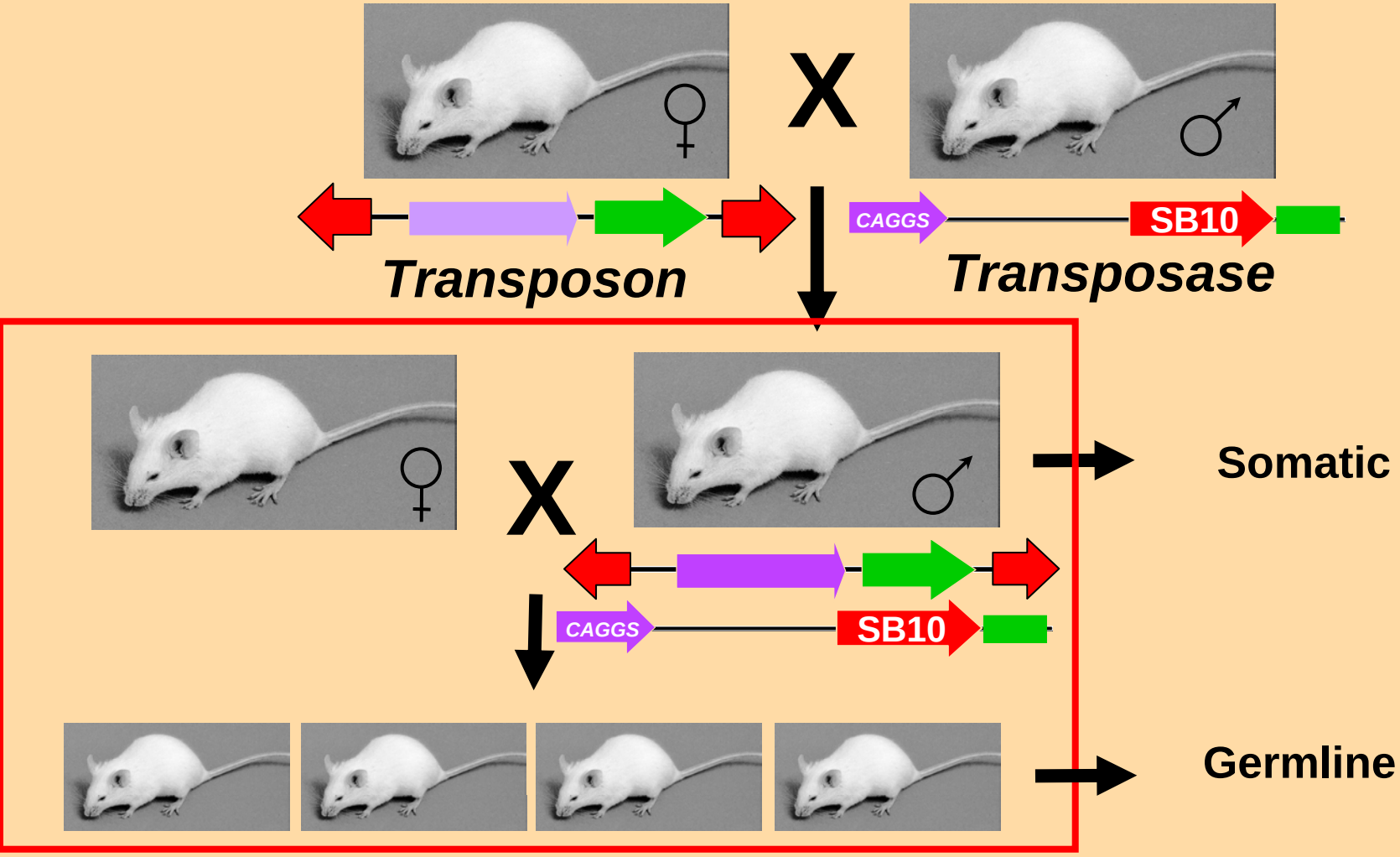
# Improved germline transgenesis using SB

- Optimized *SB11* transposase mRNA created
- Methylated transposon vector DNA
- Experiment repeated: ~<u>90% of offspring transgenic</u>

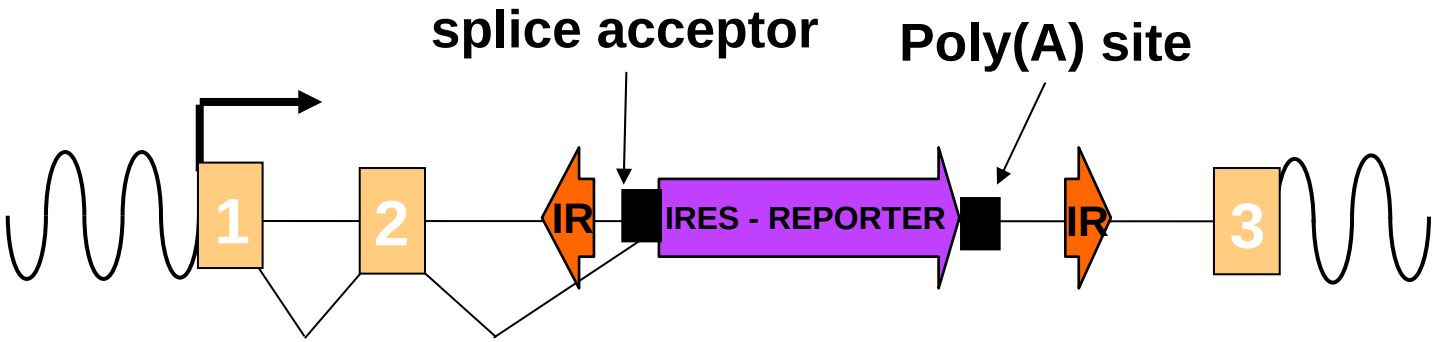



*Carlson et al., Transgenic Research, 2010.*

# Mutagenesis with *Sleeping Beauty:* Inducing transposition of chromosomally-resident transposon vectors



# Gene trap transposons are designed to disrupt genes





**Truncated protein**
**from gene X and expression of reporter**

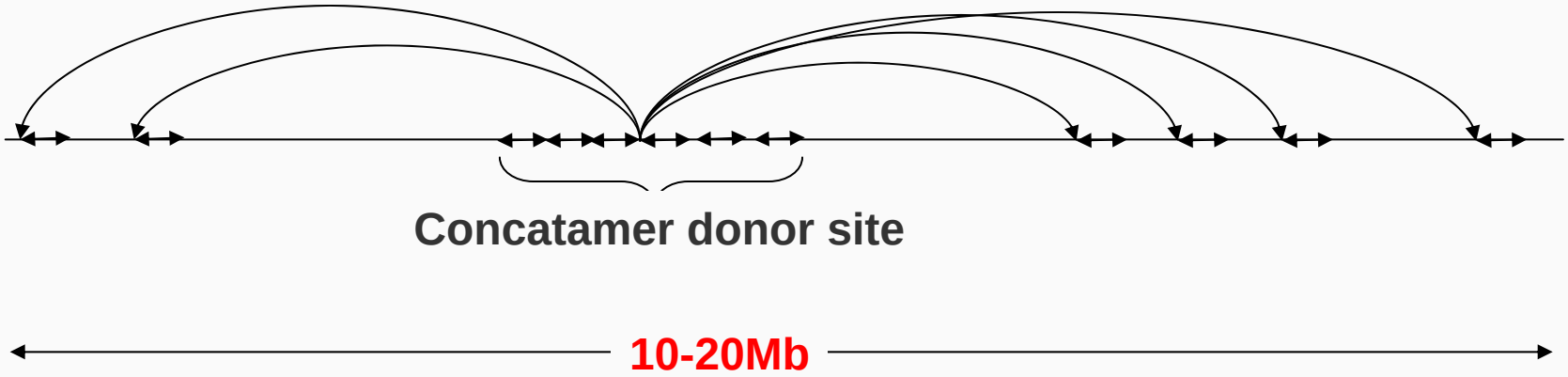
# SB can efficiently induce germline mutations

- Up to three insertions per gamete
- Roughly1/4 transposition events land in known or predicted transcription unit
- No strong tendency toward or away from genes
- No strong tendency to land in promoters of genes
- Transposons can hop into and mutate genes (some are nulls, some hypomorphs, some silent)
- Mutations can be reverted by remobilization
- Transposons land near donor locus 50% of the time - i.e “local hopping”

***Dupuy et al., Genesis, 2001.***
***Carlson et al., Genetics, 2003.***

| Chr | position | mouse gene hit | NRAA definition or Panther best hit |
|---|---|---|---|
| 8 | 5745814 | EST | No known function |
| 7 | unknown | mCG67976 (-) | Krueppel-related C2H2-type zinc-finger |
| 9 | 14154737 | mCG1034501 (+) | No known function |
| 14 | 32352513 | mCG52624 (-) | No known function |
| 9 | 4309928 | EST | No known function |
| 9 | 34541781 | mCG127192 (-) | No known function |
| 3 | unknown | mCG9496 (-) | Guanylate cyclase alpha 2 subunit |
| 7 | 88114871 | mCG1028279 (-) | Reverse Transcriptase-related |
| 3 | unknown | mCG1044682 (+) | No known function |
| | | mCG59825 (-) | No known function |
| 12 | unknown | mCG1039718 (+) | No known function |
| 17 | 80326798 | mCG12054 (+) | No known function |
| 6 | 93423488 | mCG127714 (+) | Mitochondrial carrier protein-related |
| 8 | 1931386 | mCG1814 (-) | Shc SH2-binding protein 1 |
| 9 | 95742434 | mCG1032876 (+) | No known function |
| 9 | 60,950,446 | mCG7690 (+) | Similar to Carbonic anhydrase XII [*Hs* ] |
| 1 | 116,266,773 | mCG132548 (+) | Similar to Caspr 5 protein isoform 1 [*Hs* ] |
| 1 | 32,392,298 | | |
| 1 | 41,557,972 | mCG121449 (+) | Family not named |
| | | mCG121450 (-) | Similar to Eukaryotic initiation factor 4B [Hs] |
| 1 | 41,987,901 | | |
| 1 | 43,571,384 | mCG116075 (-) | Similar to axonemal dynein heavy chain 7 [*Hs* ] |
| 1 | 62,781,818 | mCG123134 (-) | Parathyroid hormone receptor (Panther) |
| 13 | 61,342,598 | mCG121043 (-) | Kruppel-related C2H2-type zinc-finger protein (Panther) |
| 10 | 121,530,761 | mCG49173 (-) | Glyceraldehyde-3-phosphate dehydrogenase |
| 9 | 59,566,450 | mCG1034698 (-) | unnamed protein product (pseudogene) |
| 9 | 60,485,914 | mCG7689 (-) | Death-associated kinase 2 |
| | unknown | | |
| 10 | 88,689,166 | mCG1039017 (+) | no description |
| 1 | 138,297,839 | | |
| 16 | 7,487,380 | mCG1038218 (-) | no description |

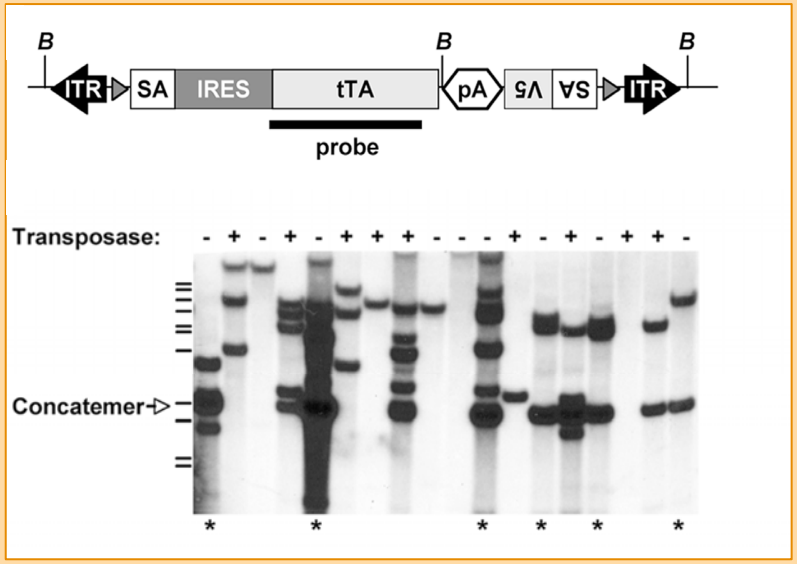
# Local hopping could allow regional saturation mutagenesis



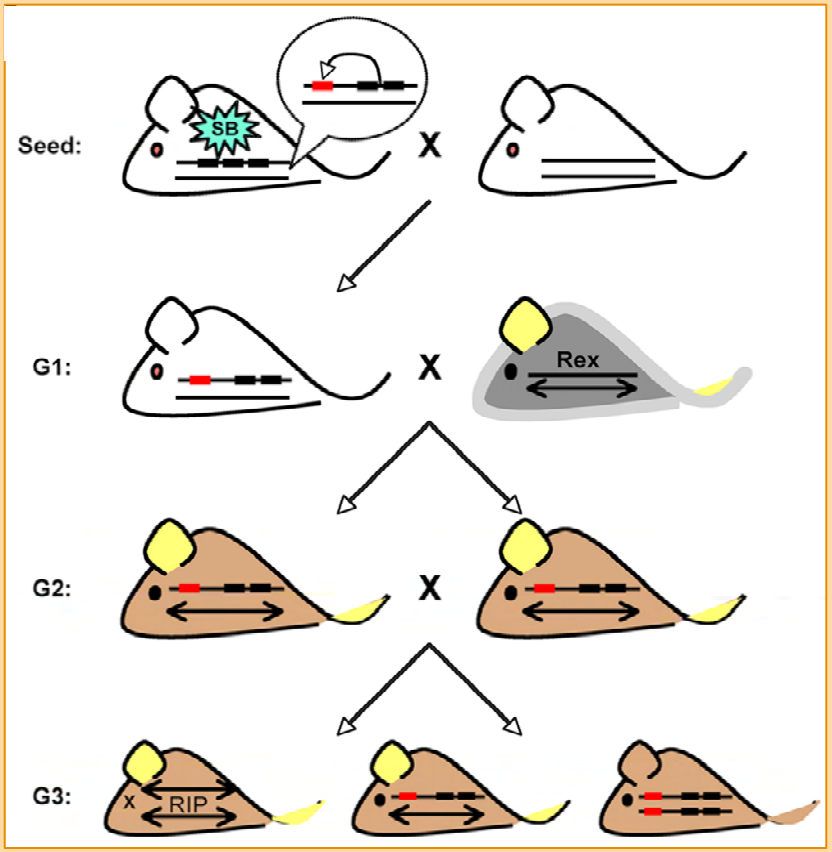

- Generate concatemer donor sites in gene-rich regions of high interest
- Need high hopping rate, concatemer donor homozygous viable, with no phenotype
- Selected a transposon transgenic line on chr. 11 in the *Trp53-Wnt3* interval for this project
- Kile et al. (2003 *Nature*) had performed ENU based screen in this interval
- Keng et al. (2005 *Nature Methods)* suggested SB can be used for regional mutagenesis

# Three-generation forward genetic screen for recessive lethal, visible, and behavioral mutants

•The GT3A donor on Chr. 11 hops at a high rate




•The breeding scheme



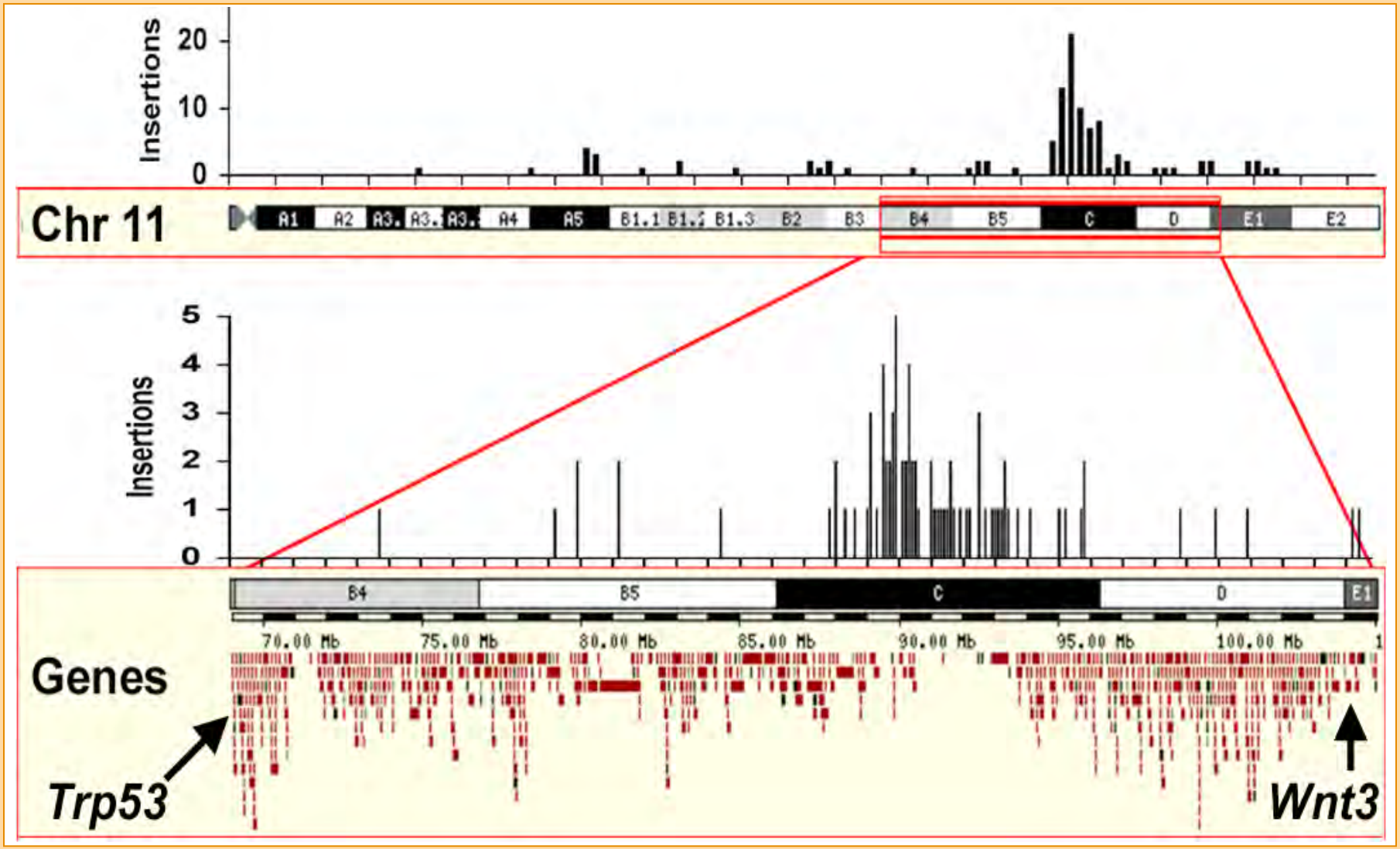

•Also cryopreserved sperm, cloned transposon insertions

***Geurts et al., PLoS Genetics, 2006.***

# Results of 50+ pedigrees

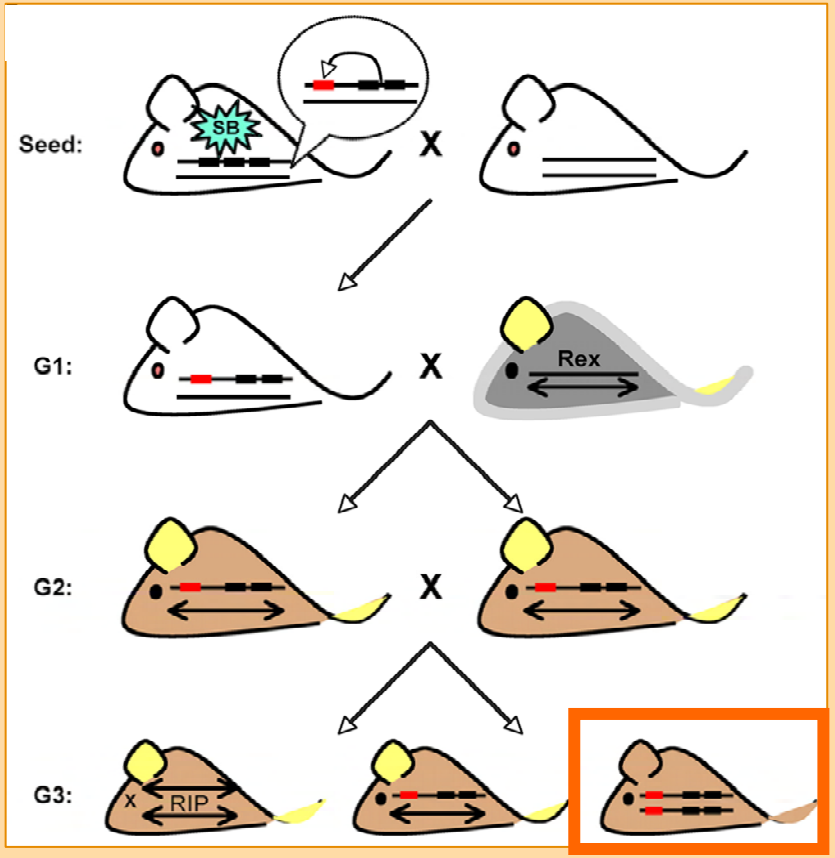
- Cloned hundreds of germline transposon insertions from G2 or G3 animals
- 50% inserted into chromosome 11 sequences
- Of those, about 70% landed in a known or predicted transcription unit

# The distribution of gene-trap transposon insertions on chromosome 11 show localized mutagenesis in pedigrees



# Results of 50+ pedigrees

- Phenotypes sought in G3 mice
- Roughly 50% of pedigrees carry a pre-natal lethal mutation
- Some viable mutants also obtained



# Insertion mutations present in pedigrees

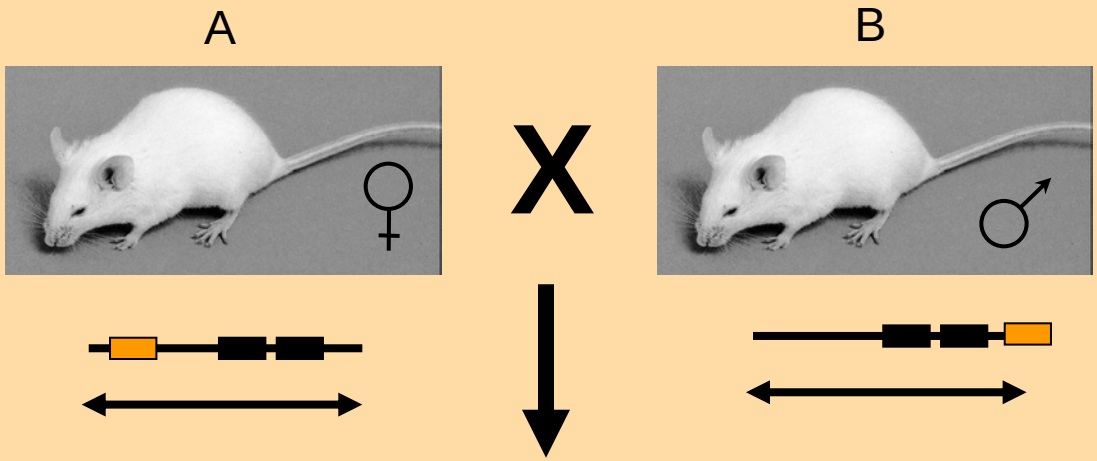
**Table 1.** Pedigree Phenotype and Cumulative Insertion Data

| GT3A; CAGGS-SB10 | Number | Pedigree Designation | Chromosome-11 Insertions[a] | In Genes[b] |
|---|---|---|---|---|
| Recessive prenatal lethal | 19 | M, Q, V, W, Z, AG, AL, AO, AP, AS, AU, AX, AY, BA, BB, BC, BH, BL, BM | 50 | 32 |
| Behavioral | 1 | BG | 4 | 3 |
| Skeletal | 1 | BM[c] | 2 | 1 |
| No phenotype | 9 | T, AD, AM, AQ, AT, AV, AZ, BI, BK | 19 | 10 |
| GT3A; RosaSB11 | | | | |
| Recessive prenatal lethal | 2 | CD, CQ | 4 | 3 |
| No phenotype | 6 | CE, CF, CG, CH, CJ, CL | 1 | 1 |

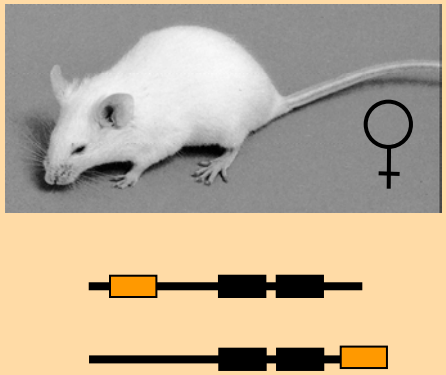
[a]Number of independent insertions identified.
[b]Includes insertions in known or predicted genes and mapped expressed sequence tags.
[c]This pedigree also displays recessive early embryonic lethality.
DOI: 10.1371/journal.pgen.0020156.t001

- Complementation testing performed on many of the lethal mutants

# Complementation testing




- *Can we obtain mice carrying a copy of each mutagenized chromosome 11 - one from pedigree A plus one from pedigree B?*



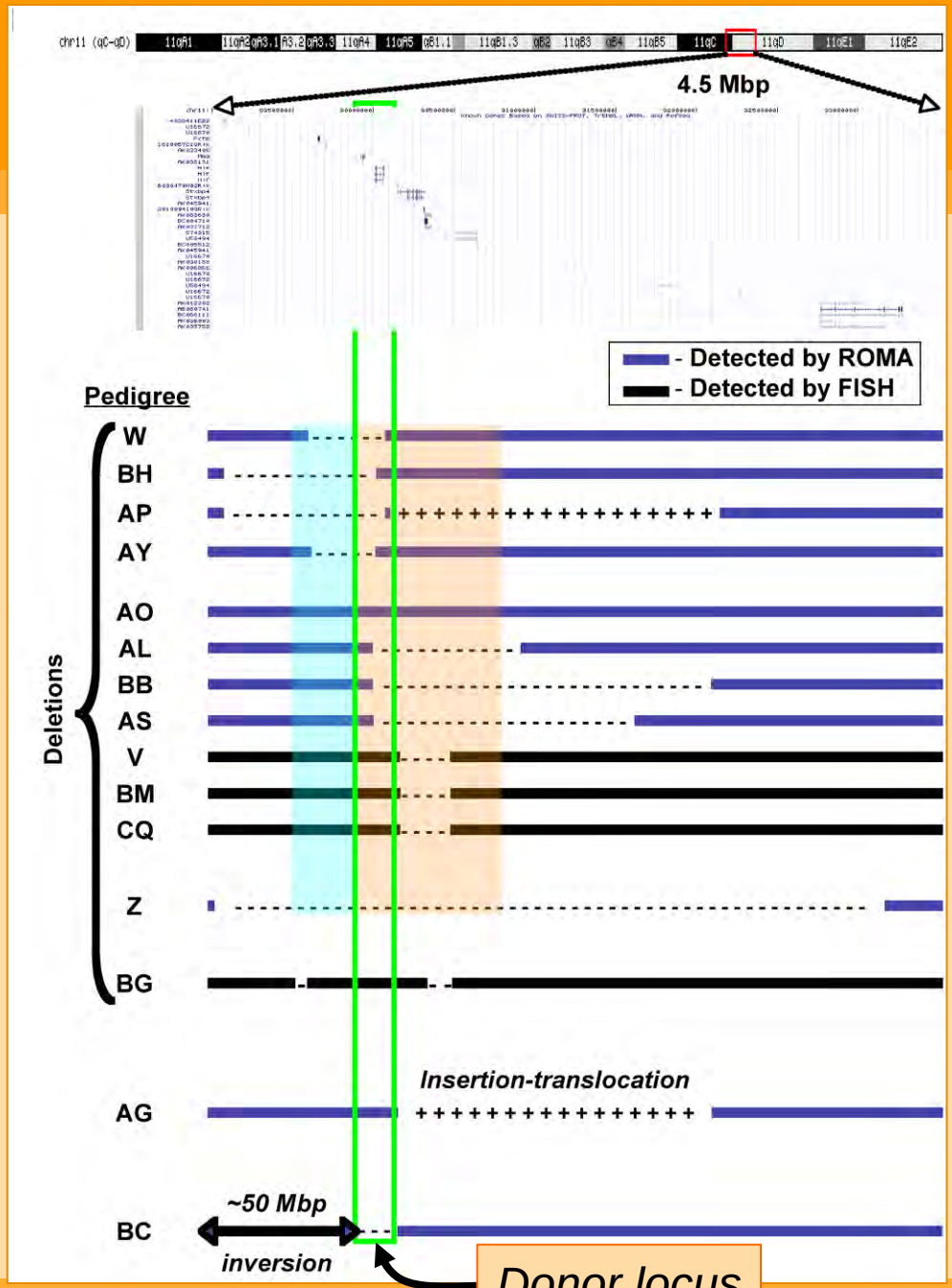
We found six complementation groups in 19 pedigrees

- Two major complementation groups (I and II)
- Indicating common mutations occurring repeatedly

| | GROUP I | | | | | | | | | GROUP II | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | V | AL | AO | AU | BB | CQ | BM | AS | AG | AX | BC | Z | BL | W | AP | AY | BH | CD | |
| M | - | - | | - | - | - | | | | + | + | | - | - | + | | + | + | + | M |
| V | | - | | | - | - | - | - | - | | + | + | - | | + | + | + | + | + | V |
| AL | | | - | | - | | | - | | + | + | + | | | + | + | + | + | | AL |
| AO | | | | - | - | - | | | - | + | + | + | | | + | + | + | + | | AO |
| AU | | | | | - | - | - | | | + | | + | | | + | + | + | + | | AU |
| BB | | | | | | - | - | | - | + | + | + | - | - | + | + | + | + | + | BB |
| CQ | | | | | | | - | - | | | | | | | + | + | + | + | | CQ |
| BM | | | | | | | | - | | | | | | | | | | + | | BM |
| AS | | | | | | | | | - | + | - | + | | - | + | + | + | + | | AS |
| AG | | | | | | | | | | - | + | + | | + | + | + | + | | | AG |
| AX | | | | | | | | | | | - | | + | | + | | | + | | AX |
| BC | | | | | | | | | | | | - | + | | + | + | | + | | BC |
| Z | | | | | | | | | | | | | - | | - | - | - | - | | Z |
| BL | | | | | | | | | | | | | | - | - | | | - | | BL |
| W | | | | | | | | | | | | | | | - | | - | - | - | W |
| AP | | | | | | | | | | | | | | | | - | - | - | | AP |
| AY | | | | | | | | | | | | | | | | | - | - | | AY |
| BH | | | | | | | | | | | | | | | | | | - | | BH |
| CD | | | | | | | | | | | | | | | | | | | - | CD |

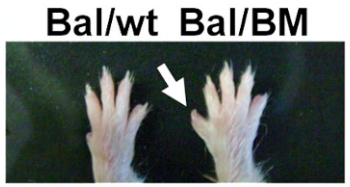
# Most lethal pedigrees carry deletions/rearrangements

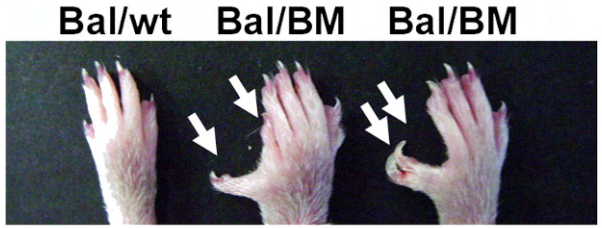
- FISH or CGH used to define sequences lost in pedigrees carrying lethal mutations
- Complementation group I pedigrees - deletions extending telomeric from the donor site
- Complementation group II pedigrees - deletions extending centromeric from the donor site
- Deletions are 100's of kb in size, up to 4.3 Mbp (pedigree Z)
- In pedigree AG, donor site plus several hundred kb has jumped to chromosome 5 and in pedigree BC, a 50 Mbp inversion

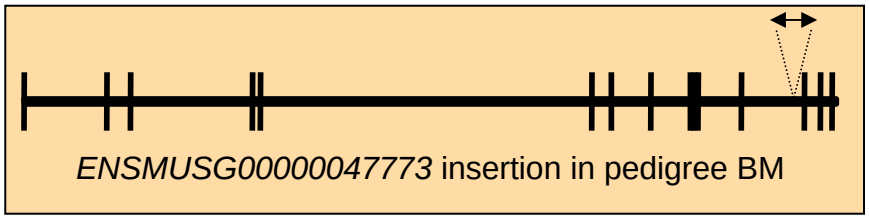


# Viable phenotypes are recovered in forward transposon-based screen

- Dominant polydactyly (extra digits) or polysyndactyly (extra, fused digits)

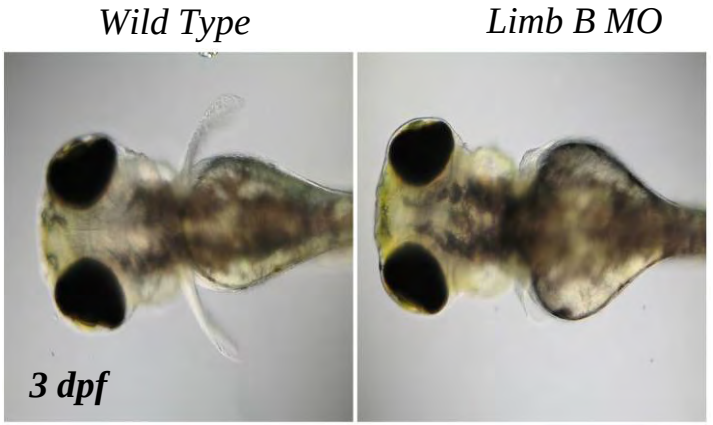



- Pioneer gene, ankyrin-repeats and NLS, well-conserved

- Two zebrafish versions of this gene (LimbA and limbB)
- Morpholino (MO) knockdown causes absence of pectoral fins - other hedgehog LOF phenotypes



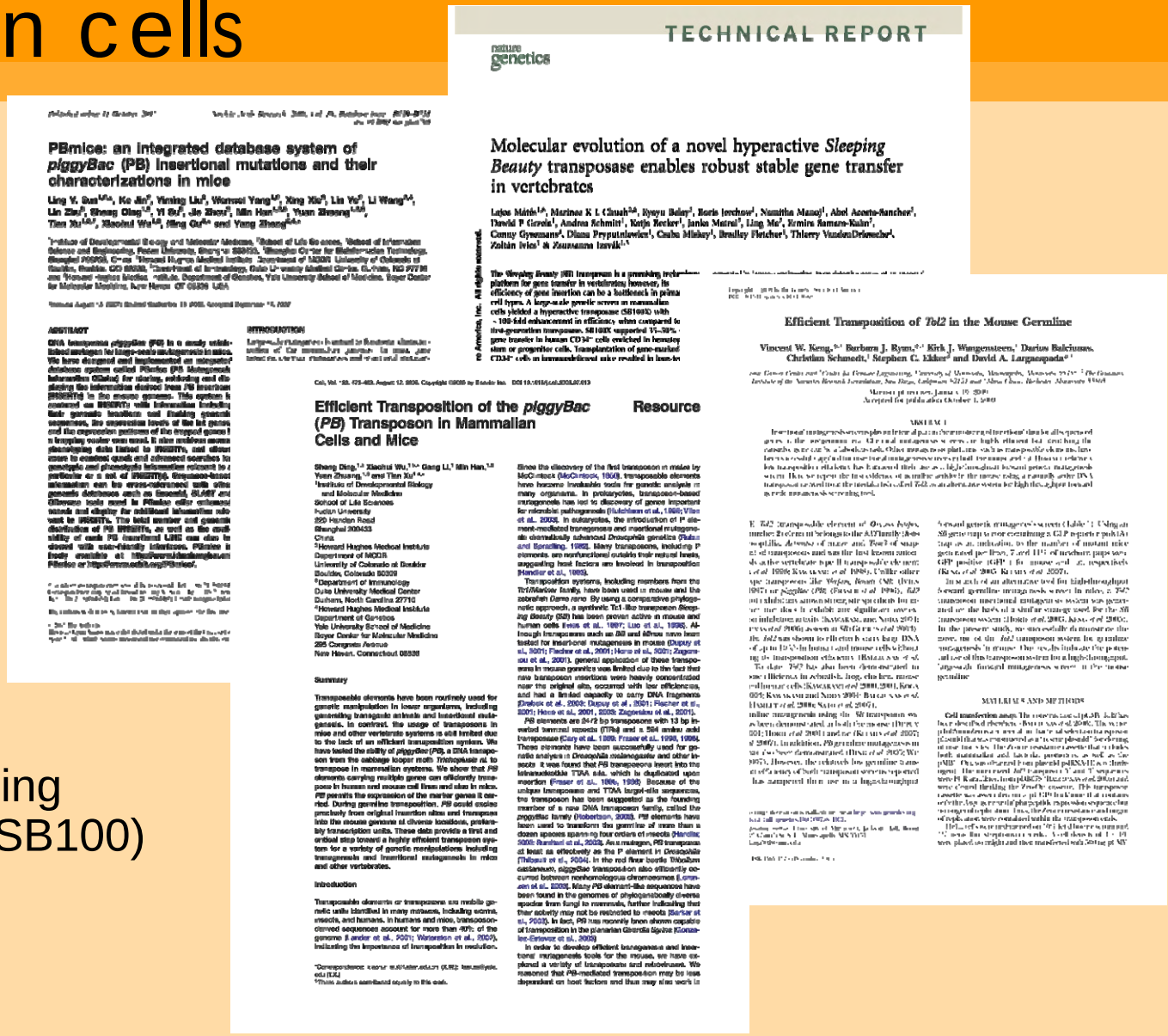

- BM mutation in mice may be a GOF mutation

# Germline mutagenesis conclusions

- Local mutagenesis with SB - very high yield of mutants
- Genes required for viability, limb development, and behavior identified
- But, complementation testing and FISH/CGH analyses show SB can induce chromosomal deletions and these are responsible for most of the lethal mutants. (Roughly 40% of pedigrees)
- Local saturation mutagenesis of the germline requires measures to eliminate recurrent chromosomal deletions from screen
  - Alternatively, do genome-wide project after improving transposition rate
- It may be more practical to generate germline insertion mutations by transposase mRNA + transposon DNA pronuclear injections.

# Other transposon systems function in mammalian cells

- Tol2
- PiggyBac
- Minos
- Frog Prince
- Hyperactive transposases being developed (e.g. SB100)



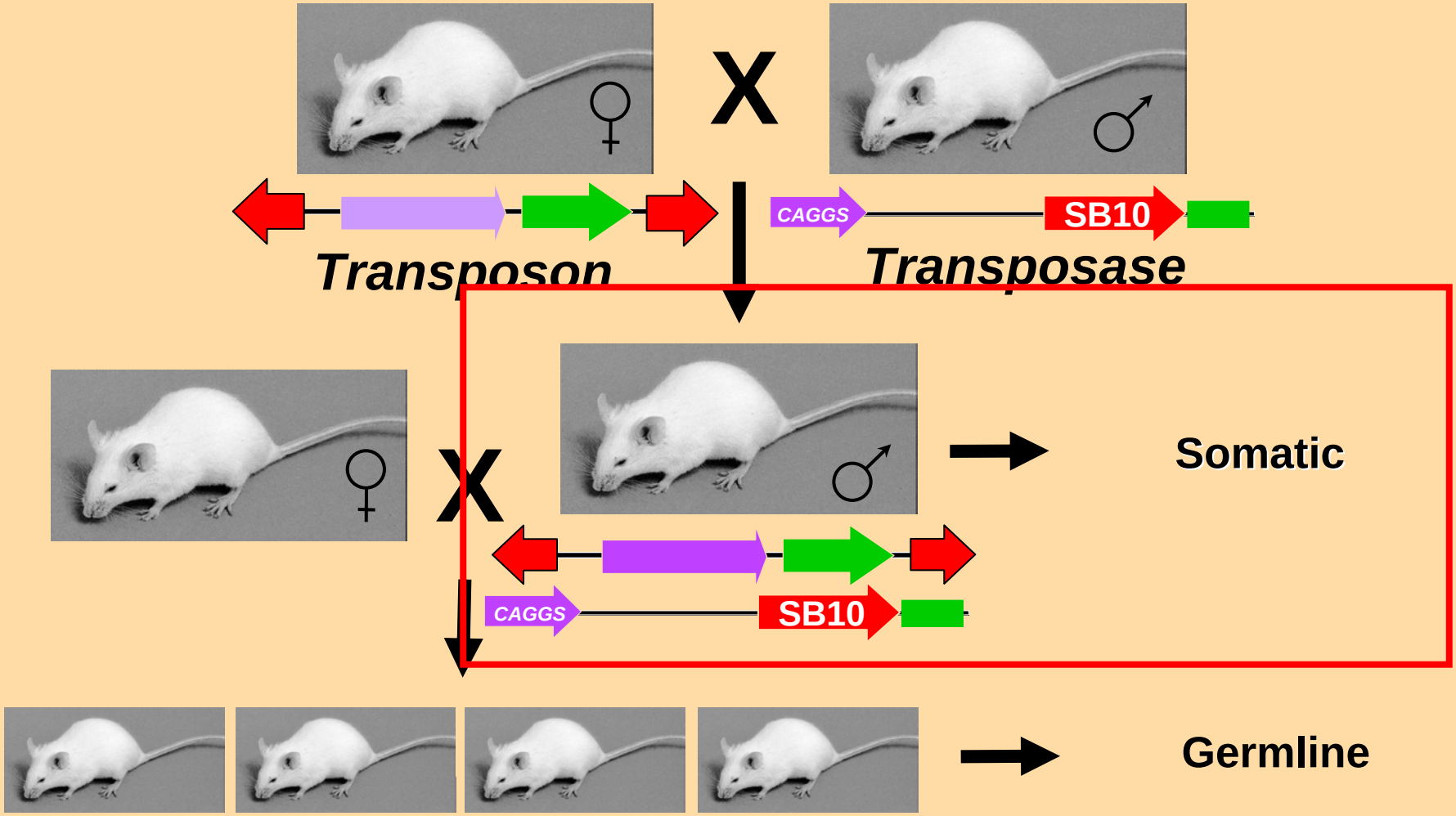
PBmice: an integrated database system of *piggyBac* (PB) insertional mutations and their characterizations in mice

Molecular evolution of a novel hyperactive *Sleeping Beauty* transposase enables robust stable gene transfer in vertebrates

Efficient Transposition of the *piggyBac* (*PB*) Transposon in Mammalian Cells and Mice

Efficient Transposition of *Tol2* in the Mouse Germline

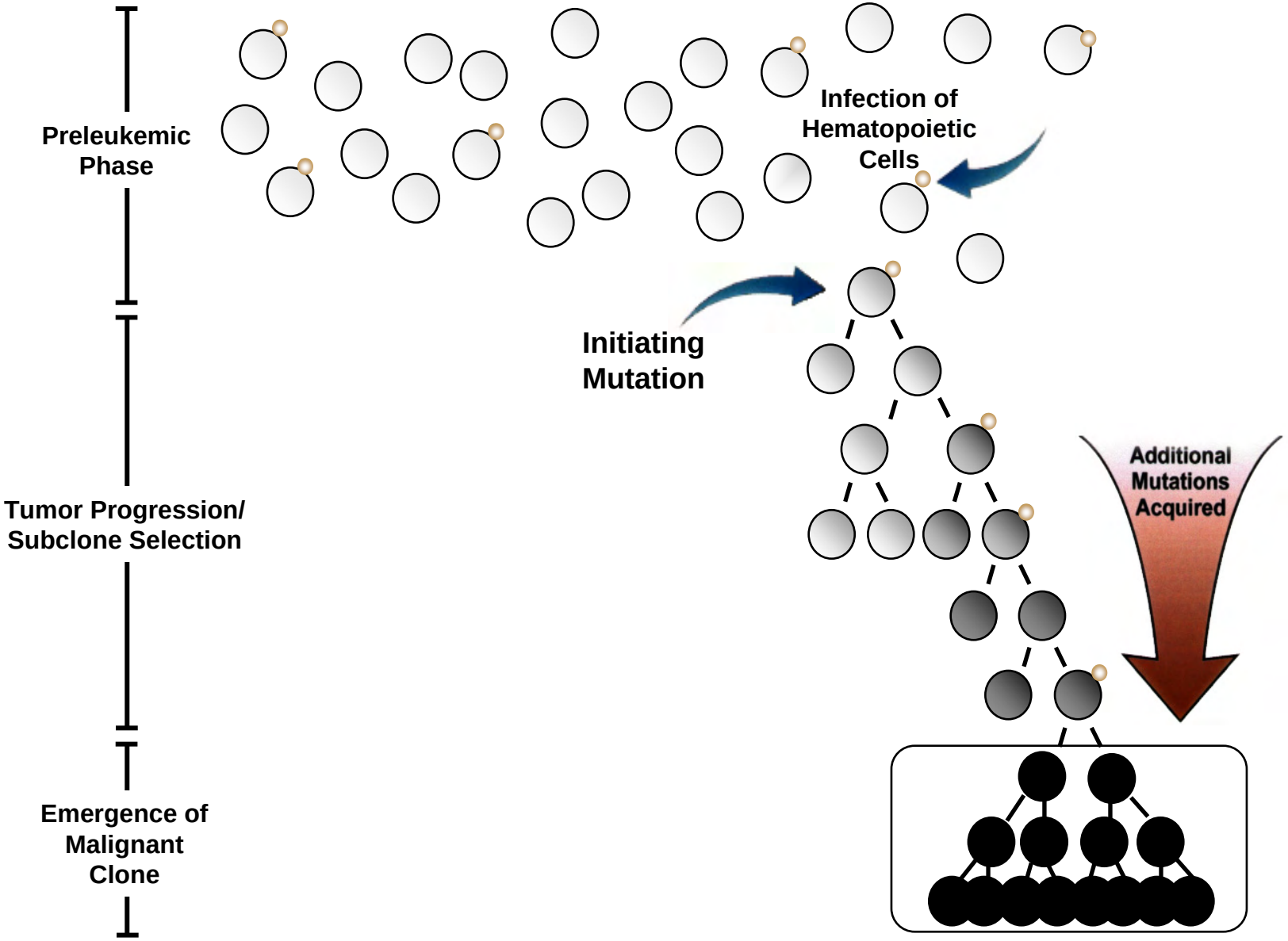
# Mutagenesis with *Sleeping Beauty:* Inducing transposition of chromosomally-resident transposon vectors



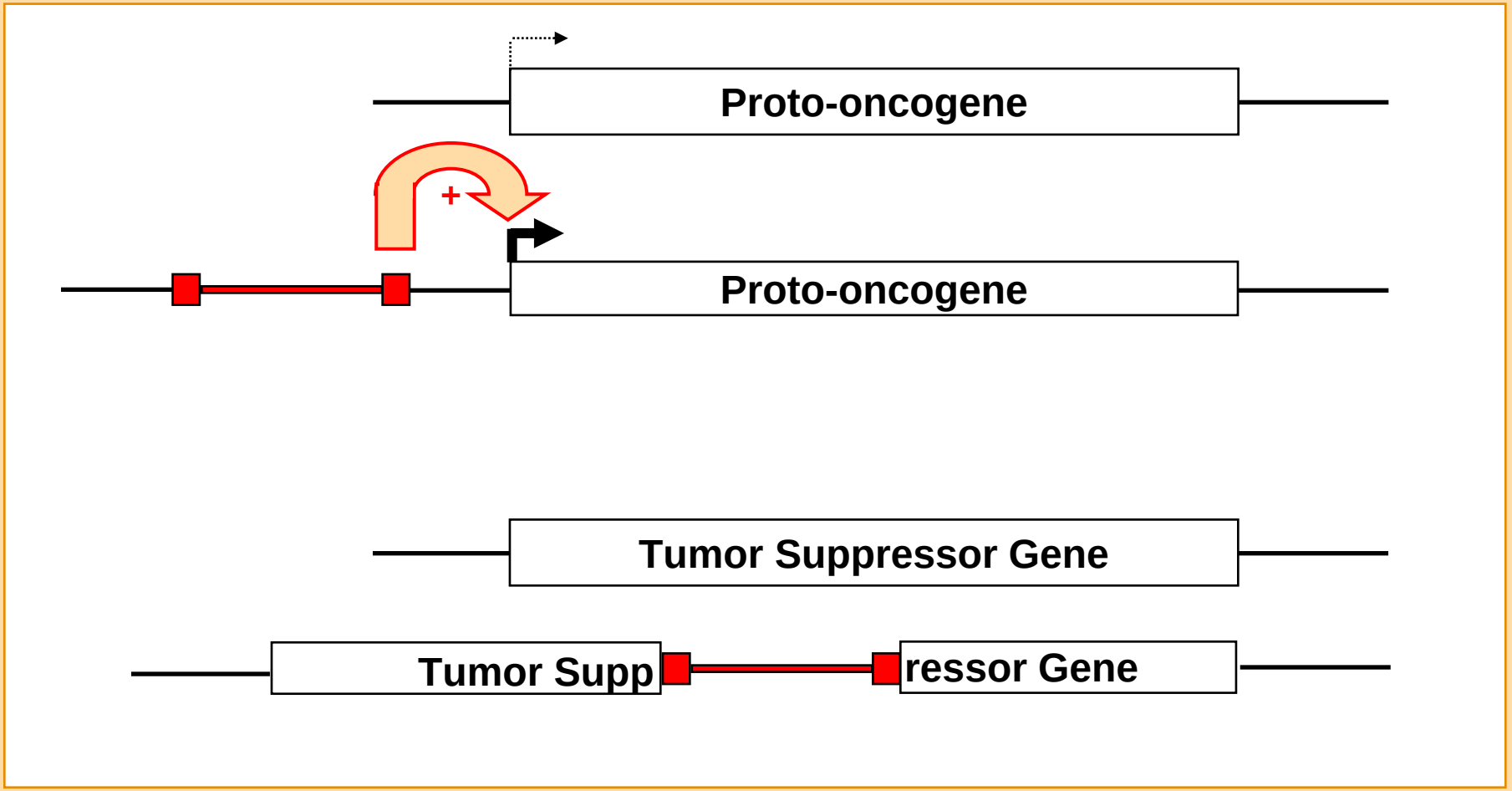
# SB transposes efficiently in mouse somatic cells

- Transposon excision marks about 1 in 15 cells in all tissues
- Transposon re-insertion sites cloned from somatic cells - local hopping observed but insertions occur throughout genome
- Most transposon re-insertion events are rare within a given tissue (1 in 10,000 cells)
- Complexity of re-insertions is high and many, many insertion mutations probably occur in any tissue

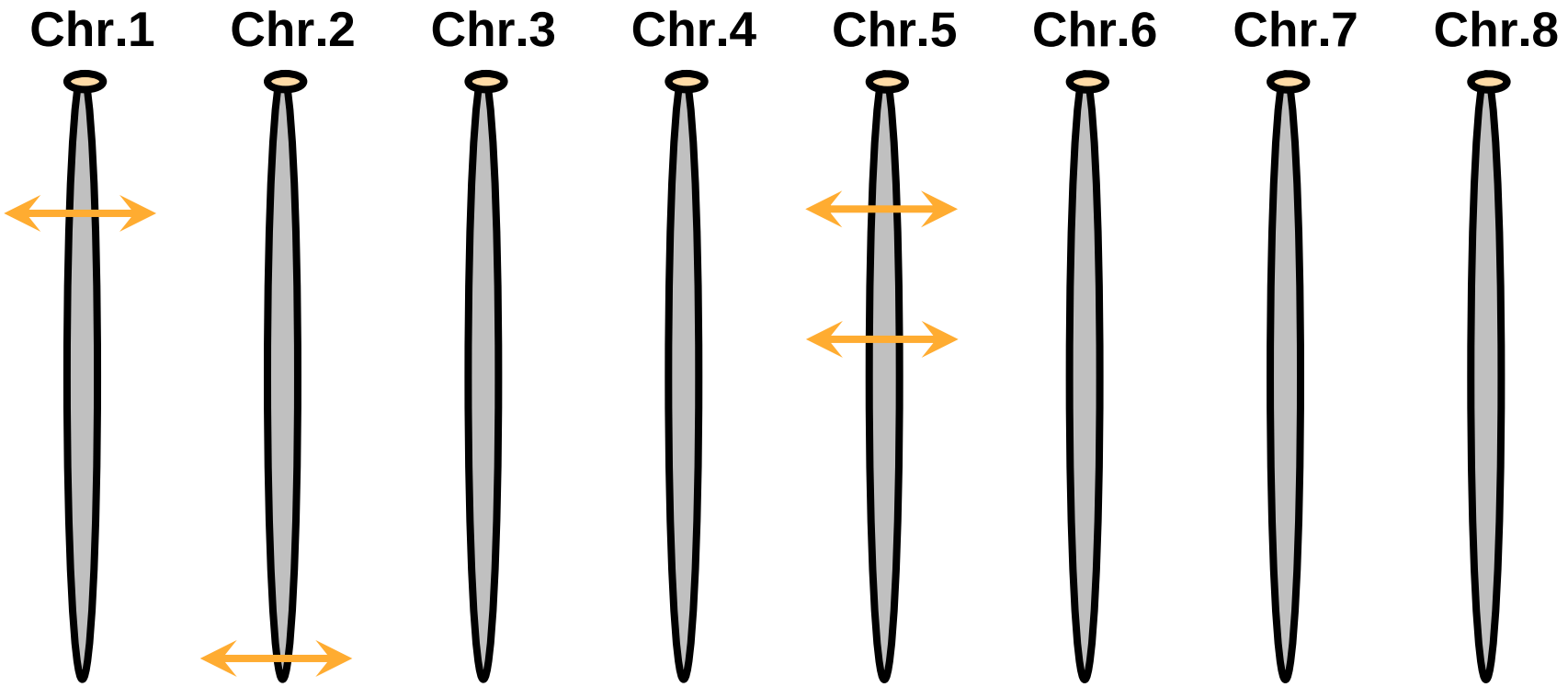
# Retrovirally induced leukemia



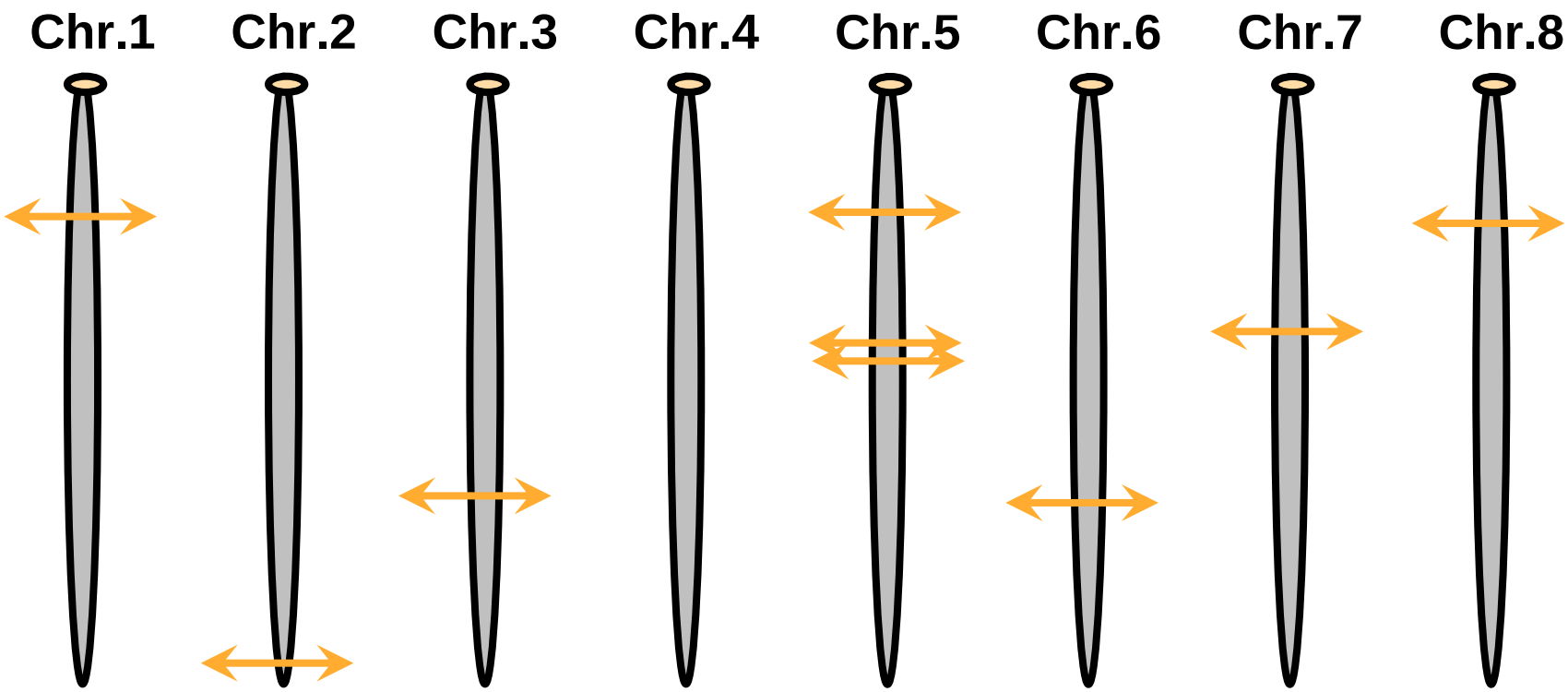
# Could we mimic retroviral insertional mutagenesis?



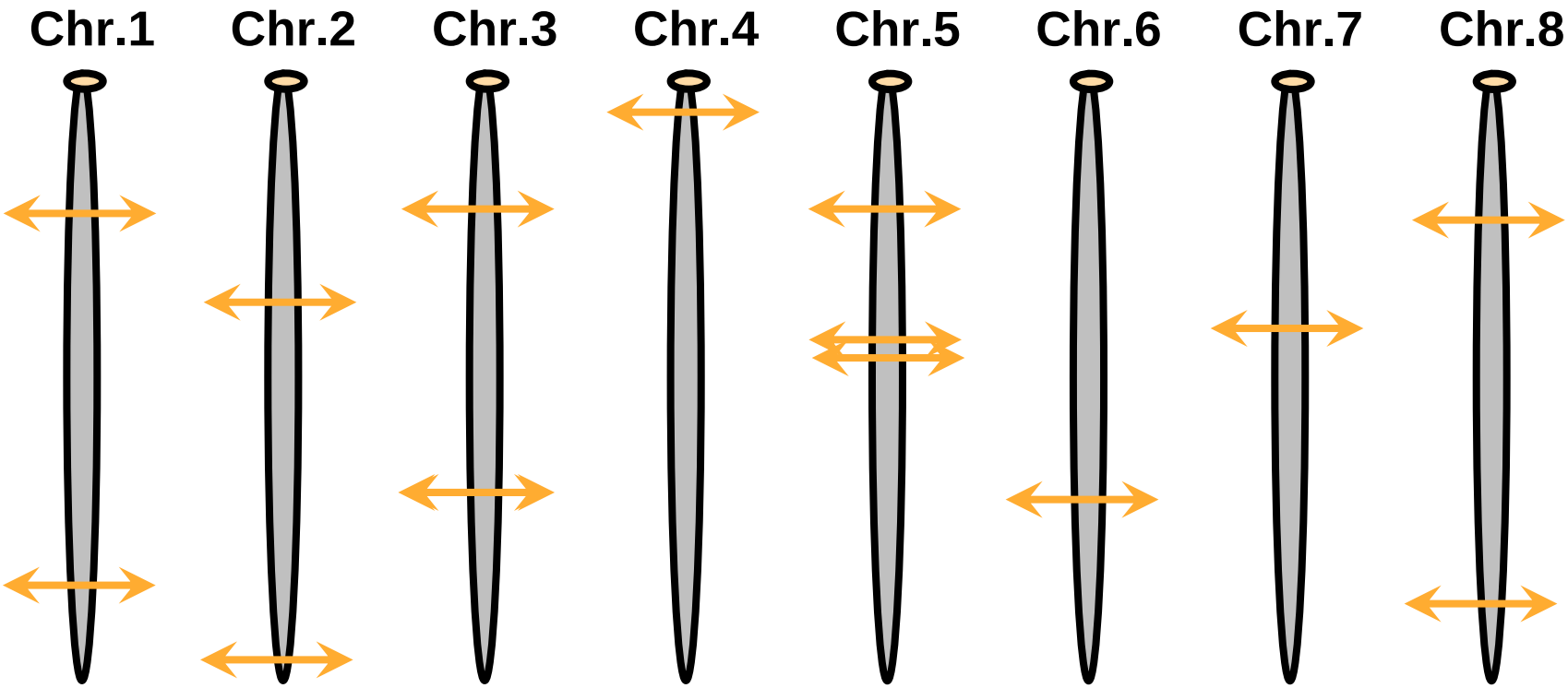
# Identifying leukemogenic insertions



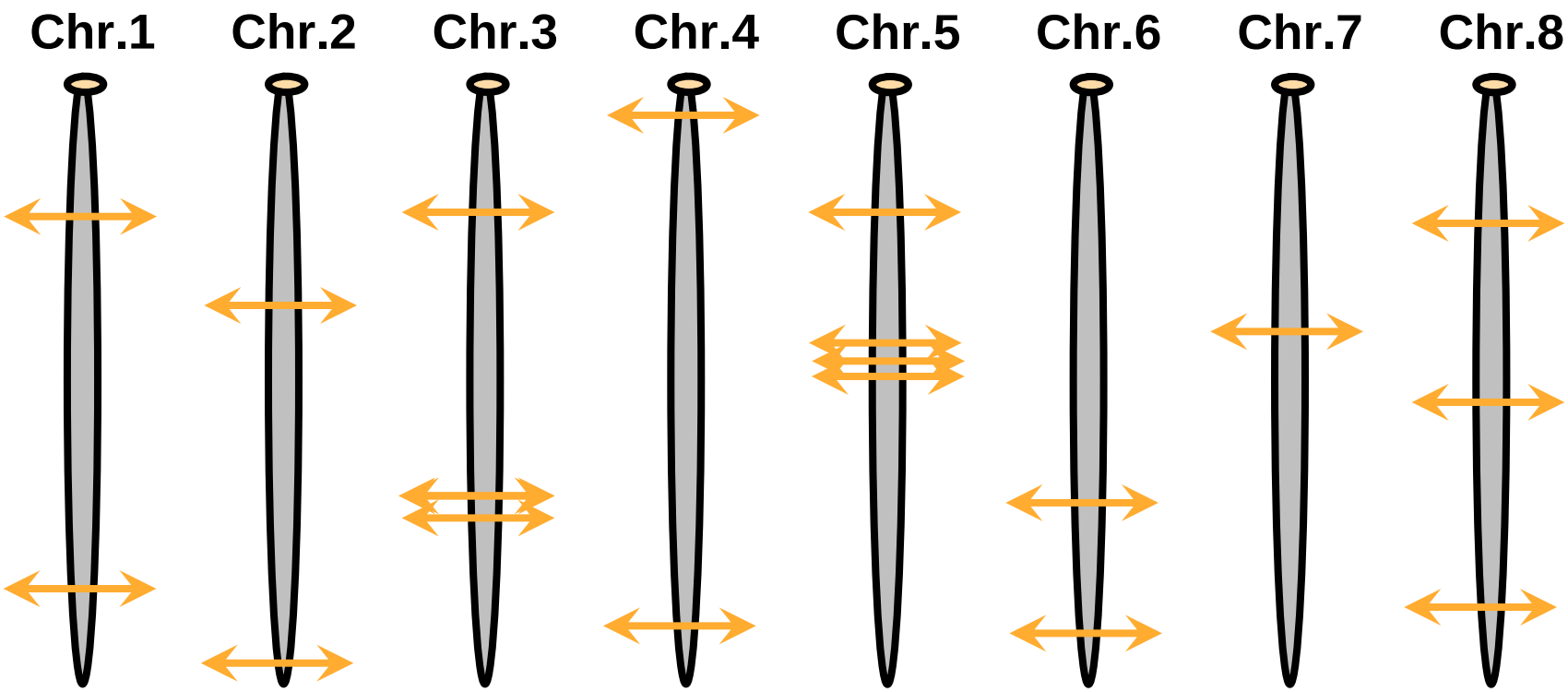
# Identifying leukemogenic insertions



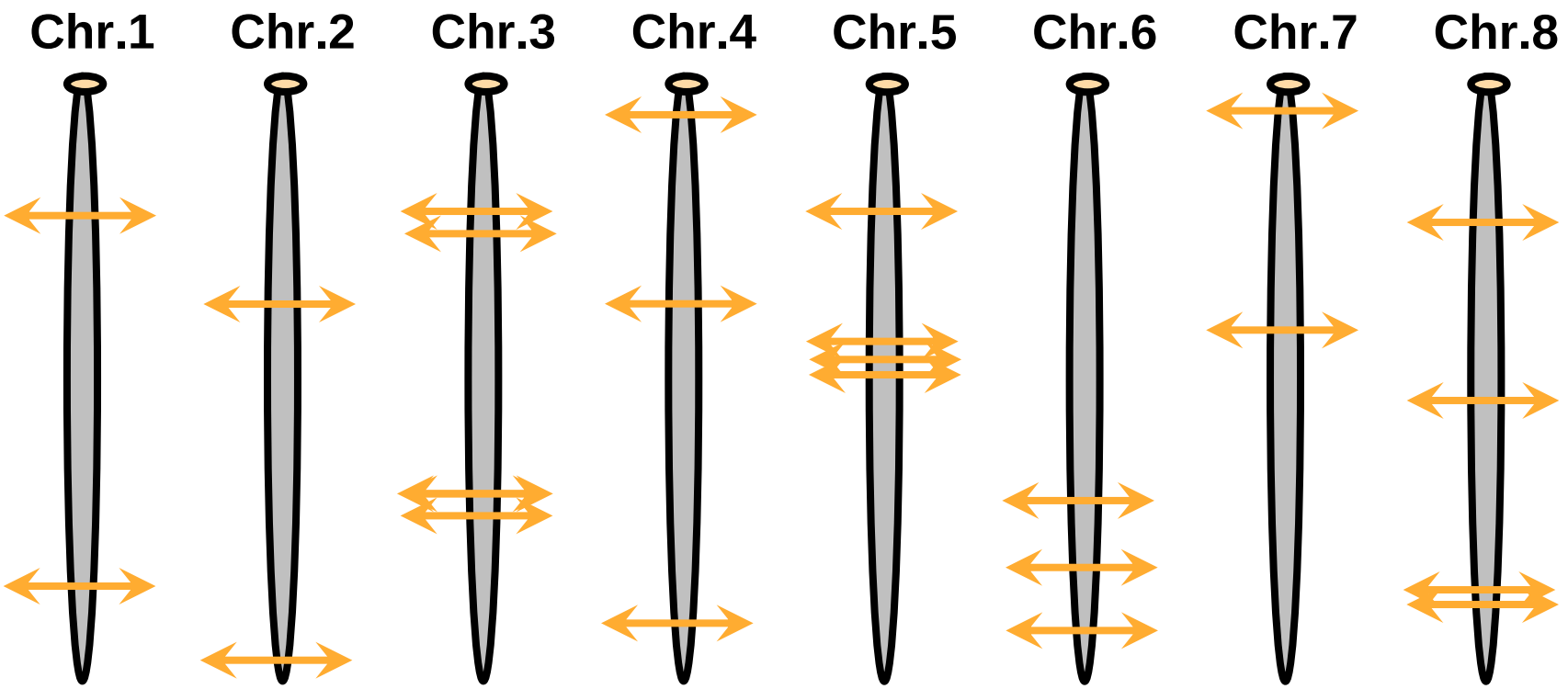
# Identifying leukemogenic insertions



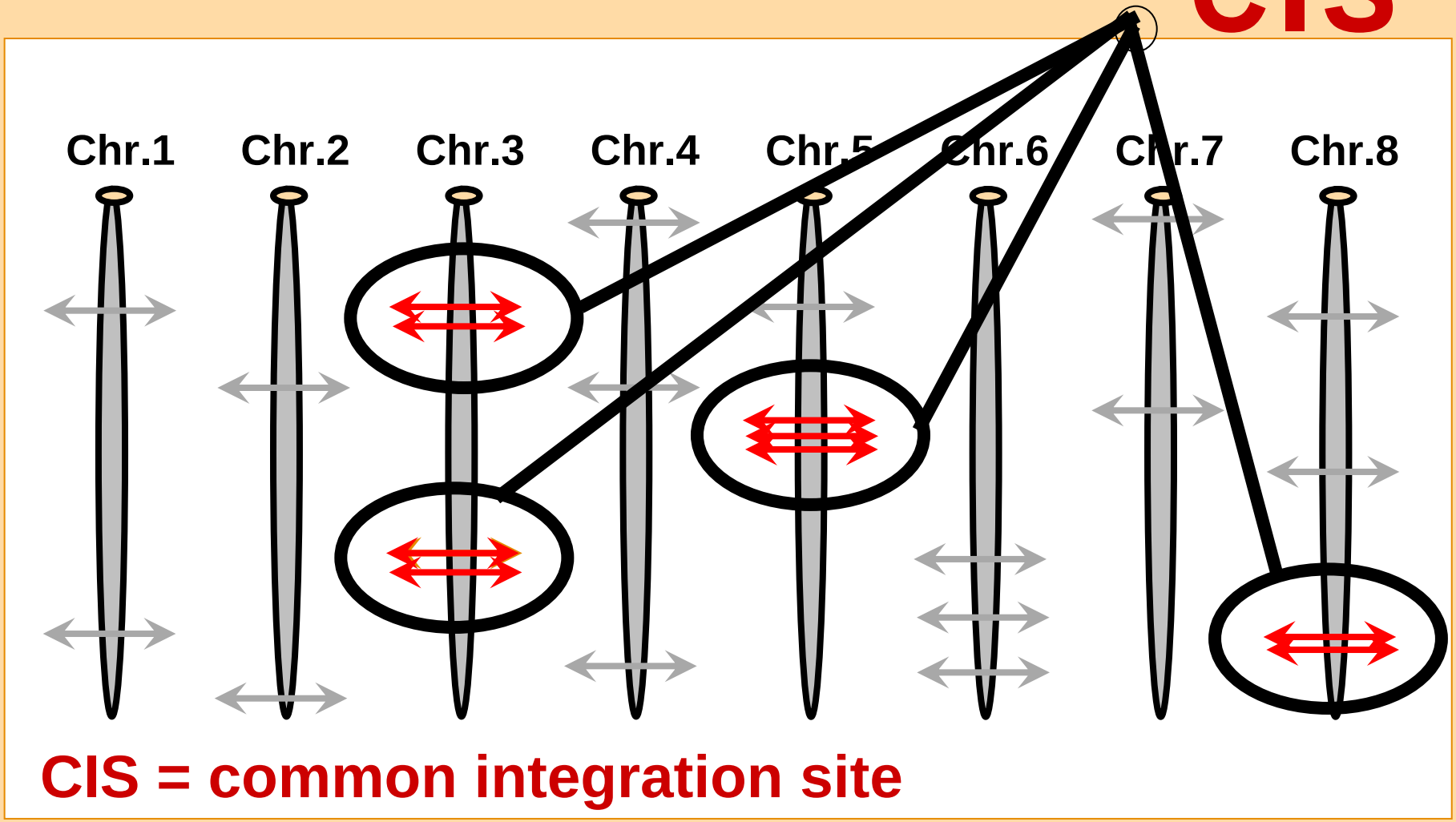
# Identifying leukemogenic insertions



# Identifying leukemogenic insertions

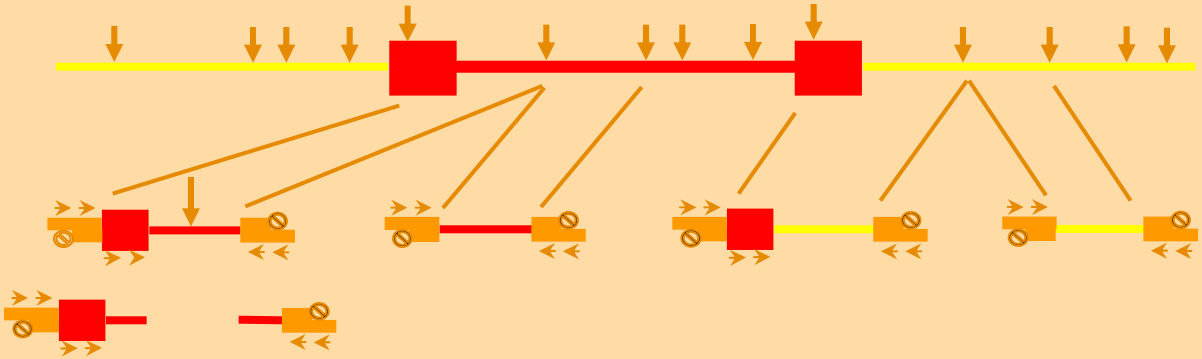


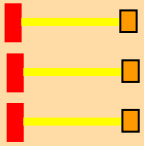
# Identifying leukemogenic insertions



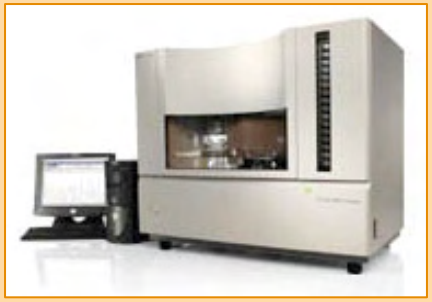

**CIS = common integration site**

# Shotgun cloning strategy using ligation-mediated PCR



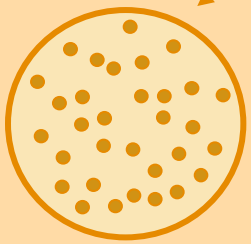


NextGen Sequencing



Shotgun clone

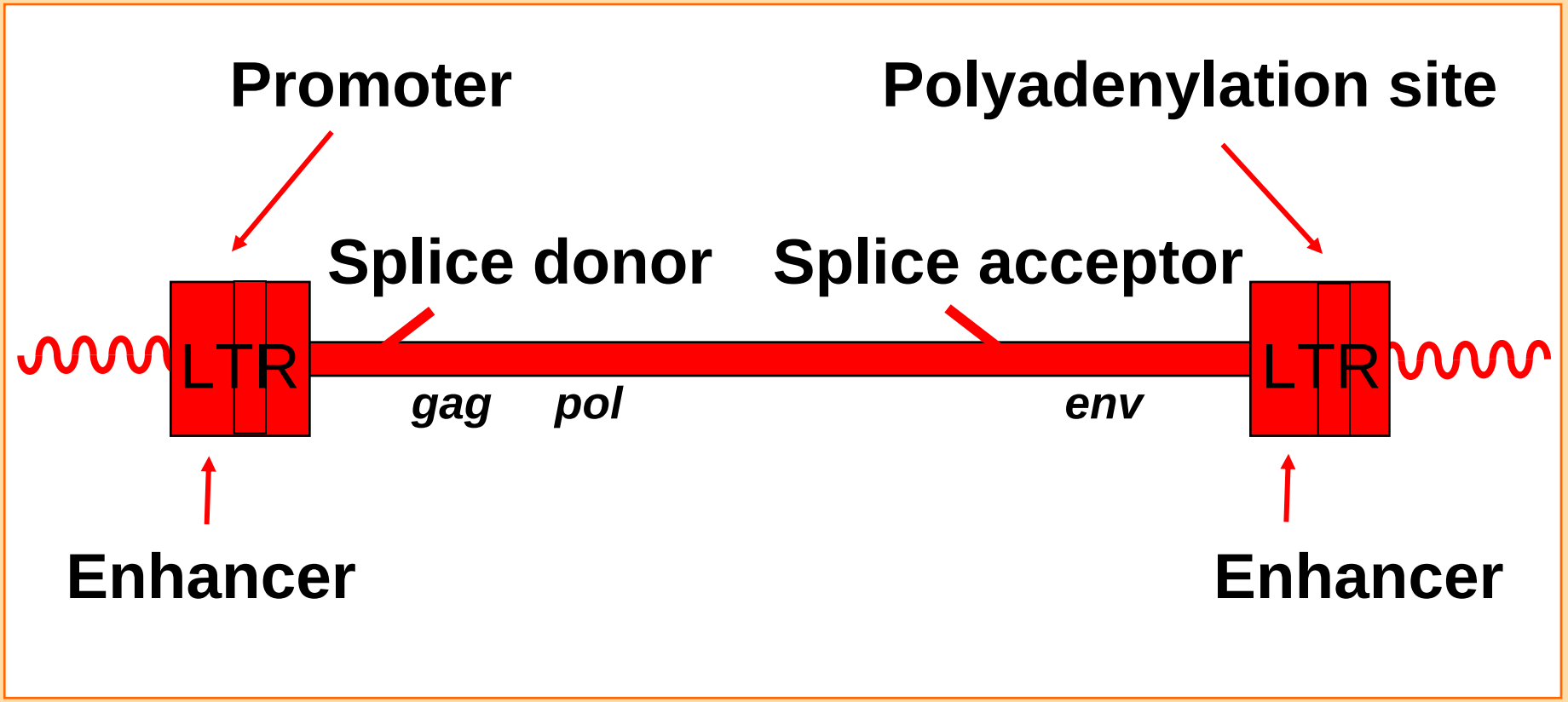
Plate plasmid library



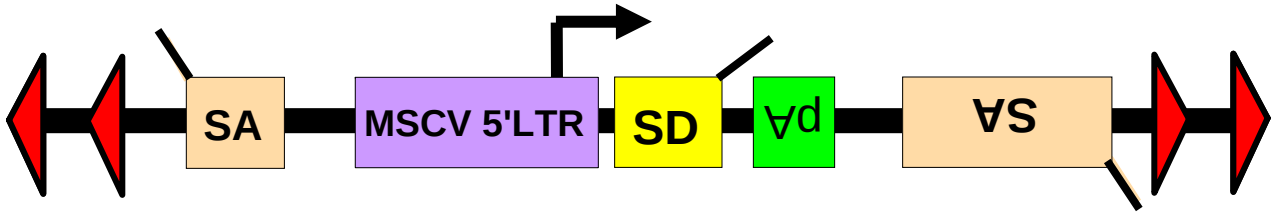
Pick colonies

Sequence colonies

# Proviruses are potent somatic mutagens



# Mimicking retroviral mutagenesis using an "insertionally oncogenic" SB transposon



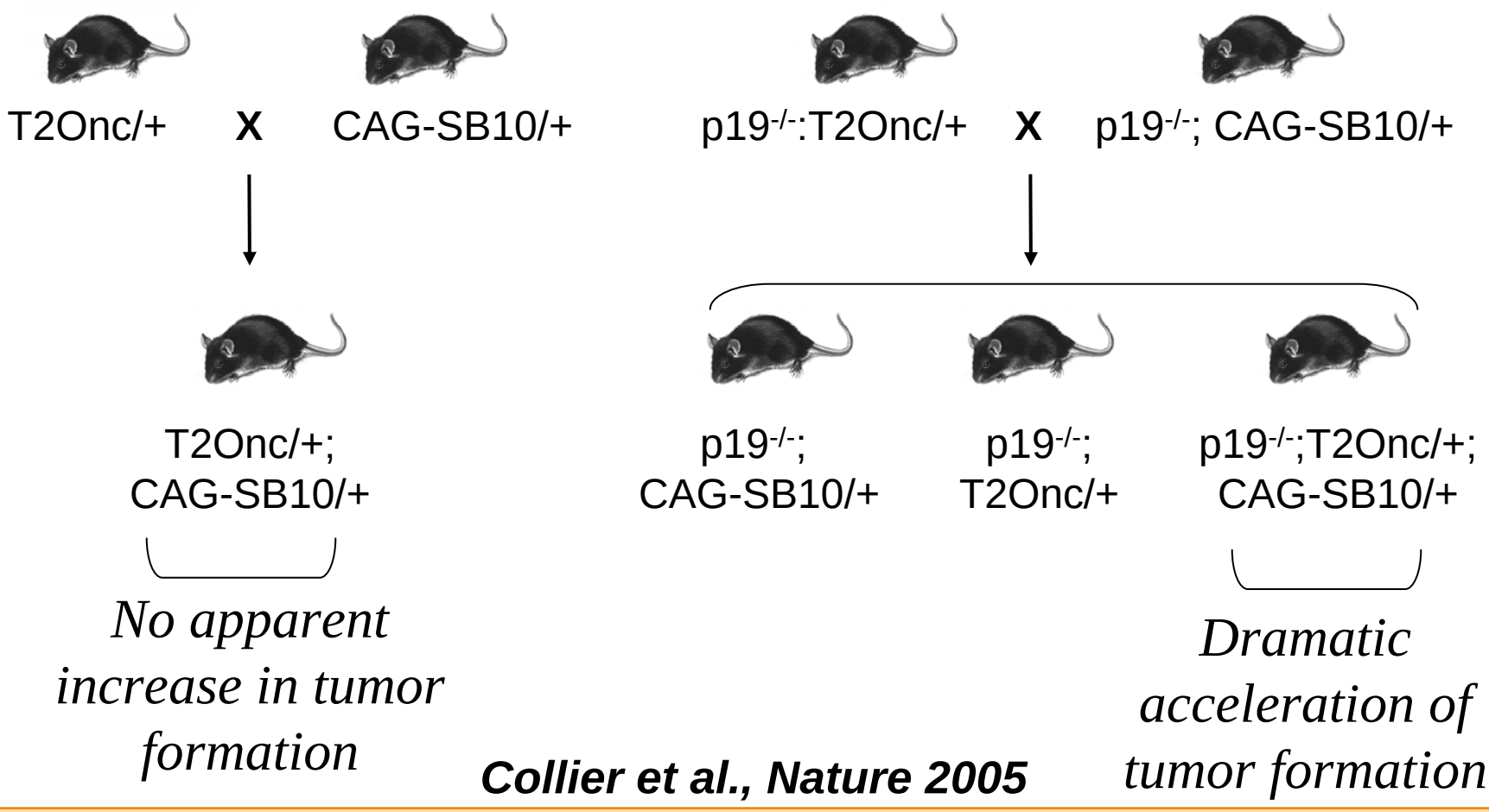

***T2/Onc***

**<u>Types of mutations that can be induced:</u>**

- C-terminal truncations (both orientations)
- N-terminal truncations
- Promoter/enhancer insertions

Gain of function or loss of function mutations

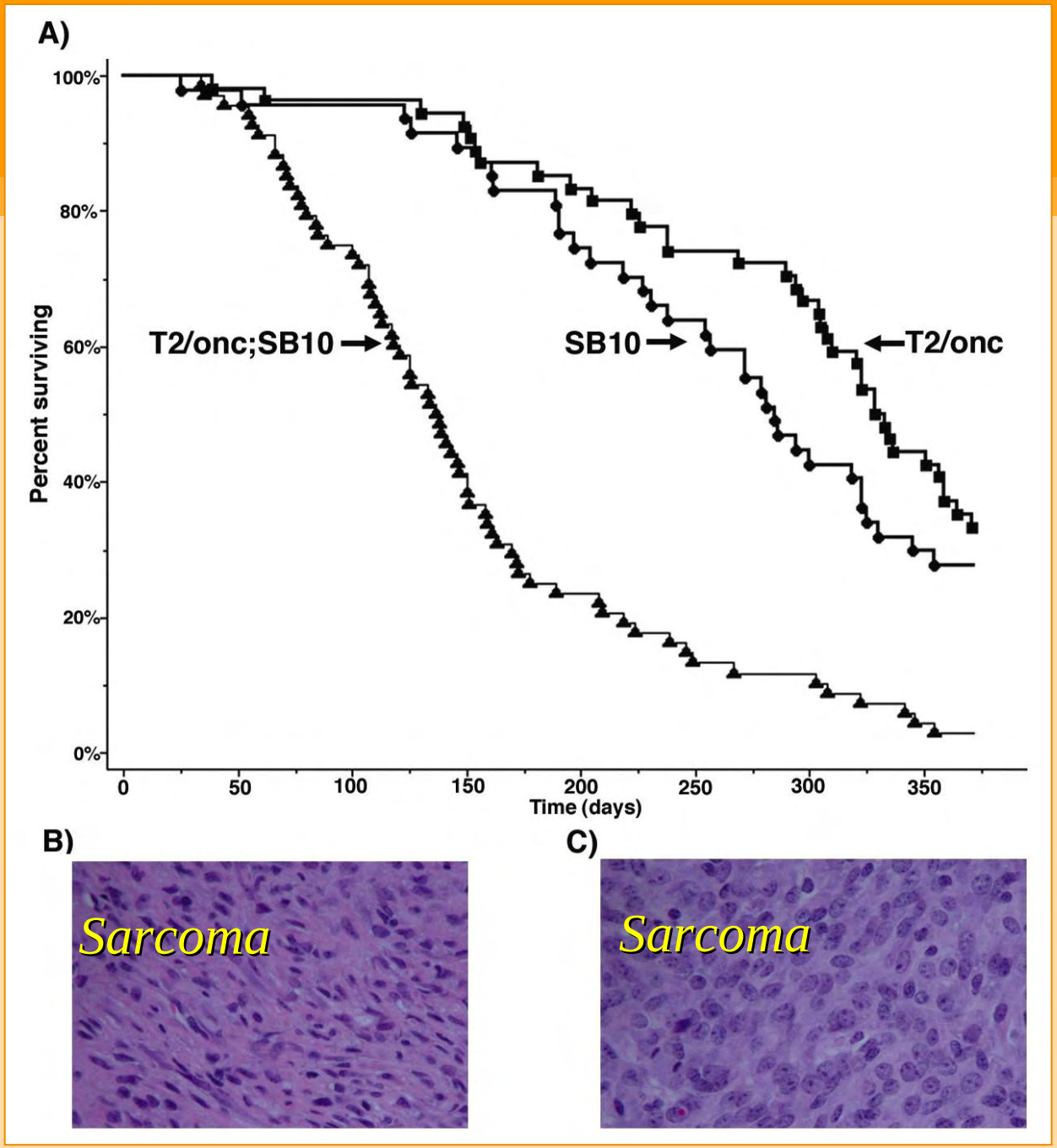
# CAGGS-SB10 plus T2/Onc transgenes do not cause highly-penetrant tumor formation on a wild-type background, but accelerate cancer in *p19Arf-/-* mice




***Collier et al., Nature 2005***

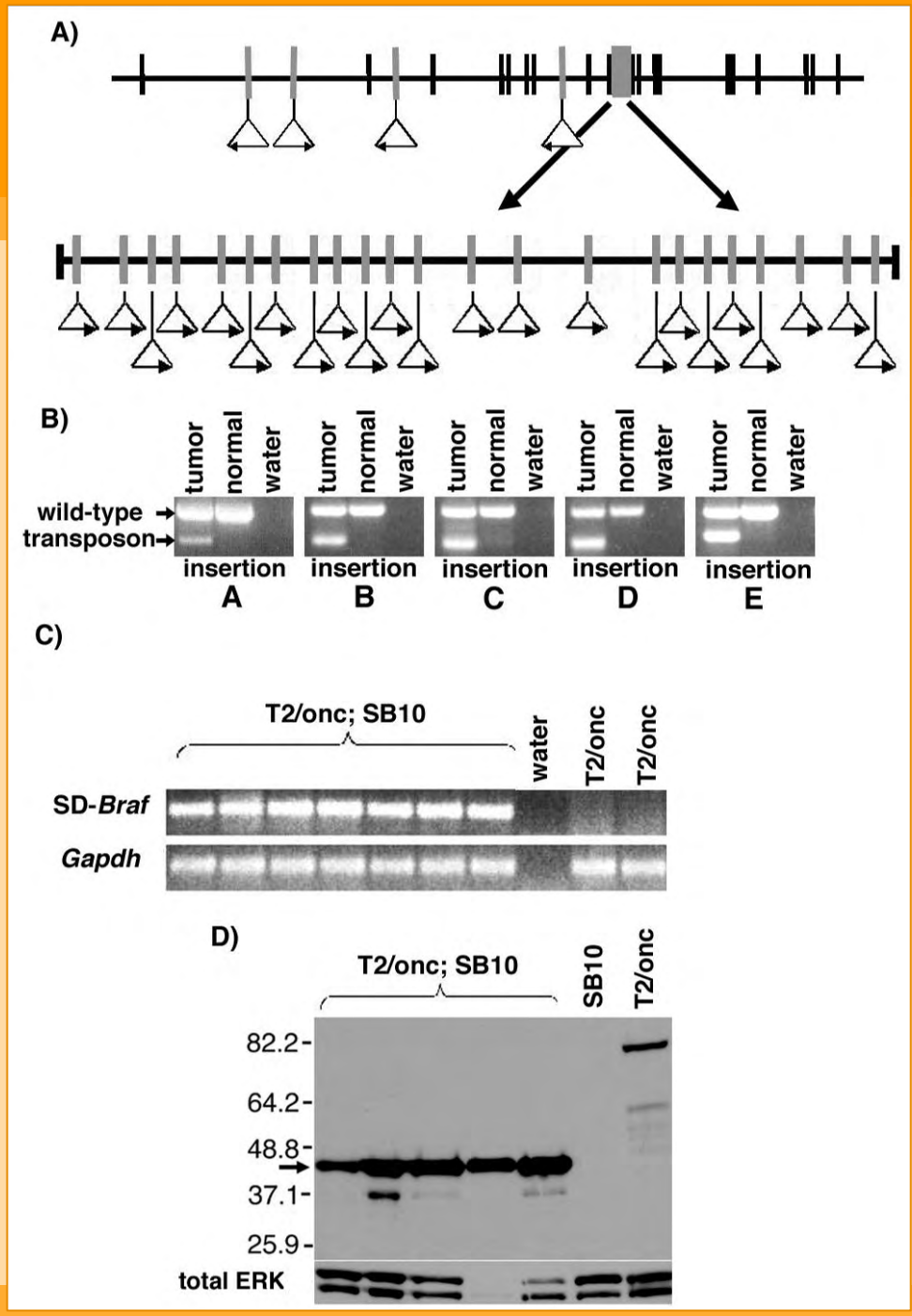
# CAGGS-SB10 plus T2/Onc accelerates tumor formation on the *p19Arf*$^{-/-}$ background

- Results similar with two independent T2/Onc lines (one on Chr. 1 and one on Chr 15)
- Primarily sarcomas



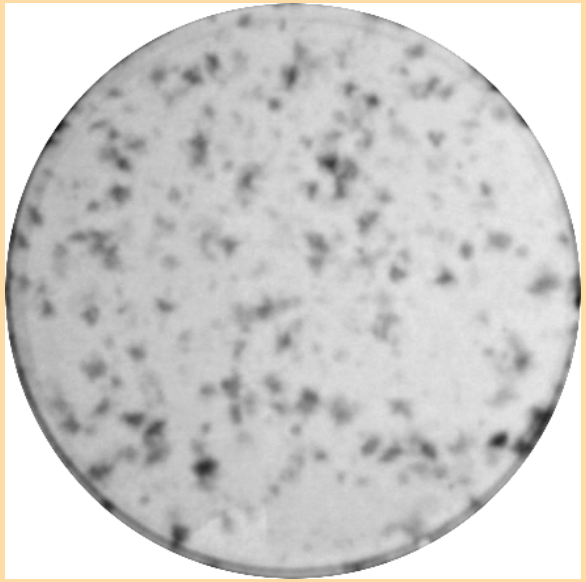
# *Braf* gene T2/Onc insertions

- Tumor specific
- Produce chimeric transcript
- C-terminal “kinase domain only” peptide

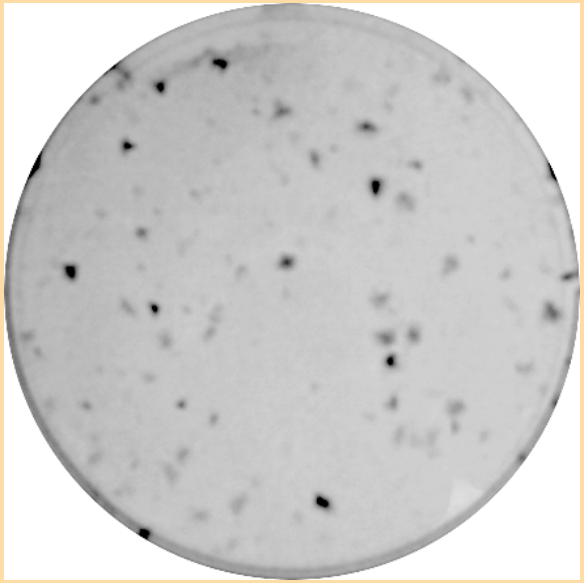


# The cloned T2/Onc - *Braf* fusion transforms NIH 3T3 cells

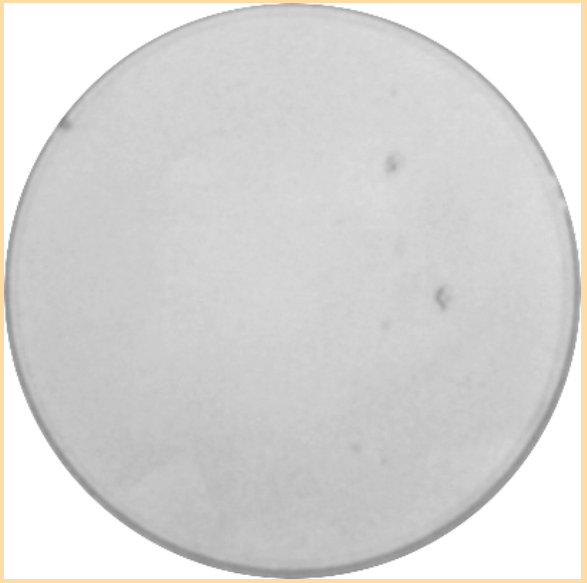
***NRAS***

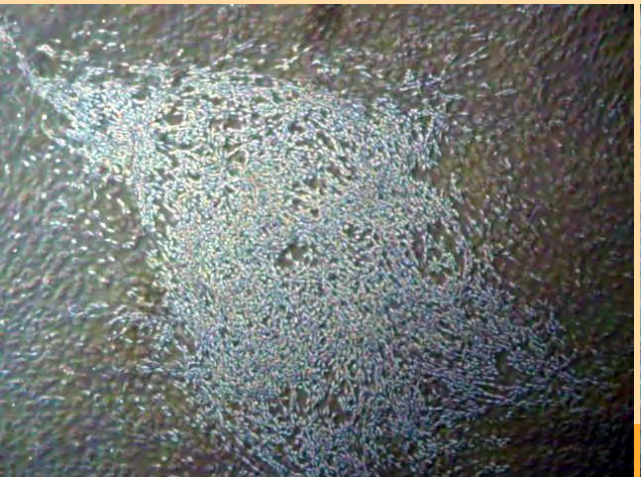


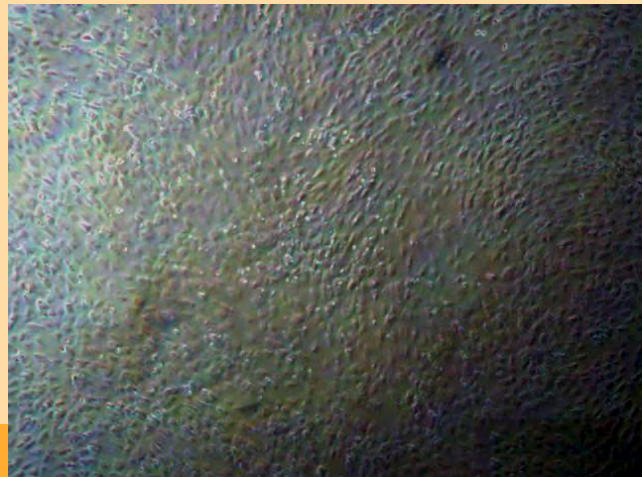
***T-Braf* (Fwd)**



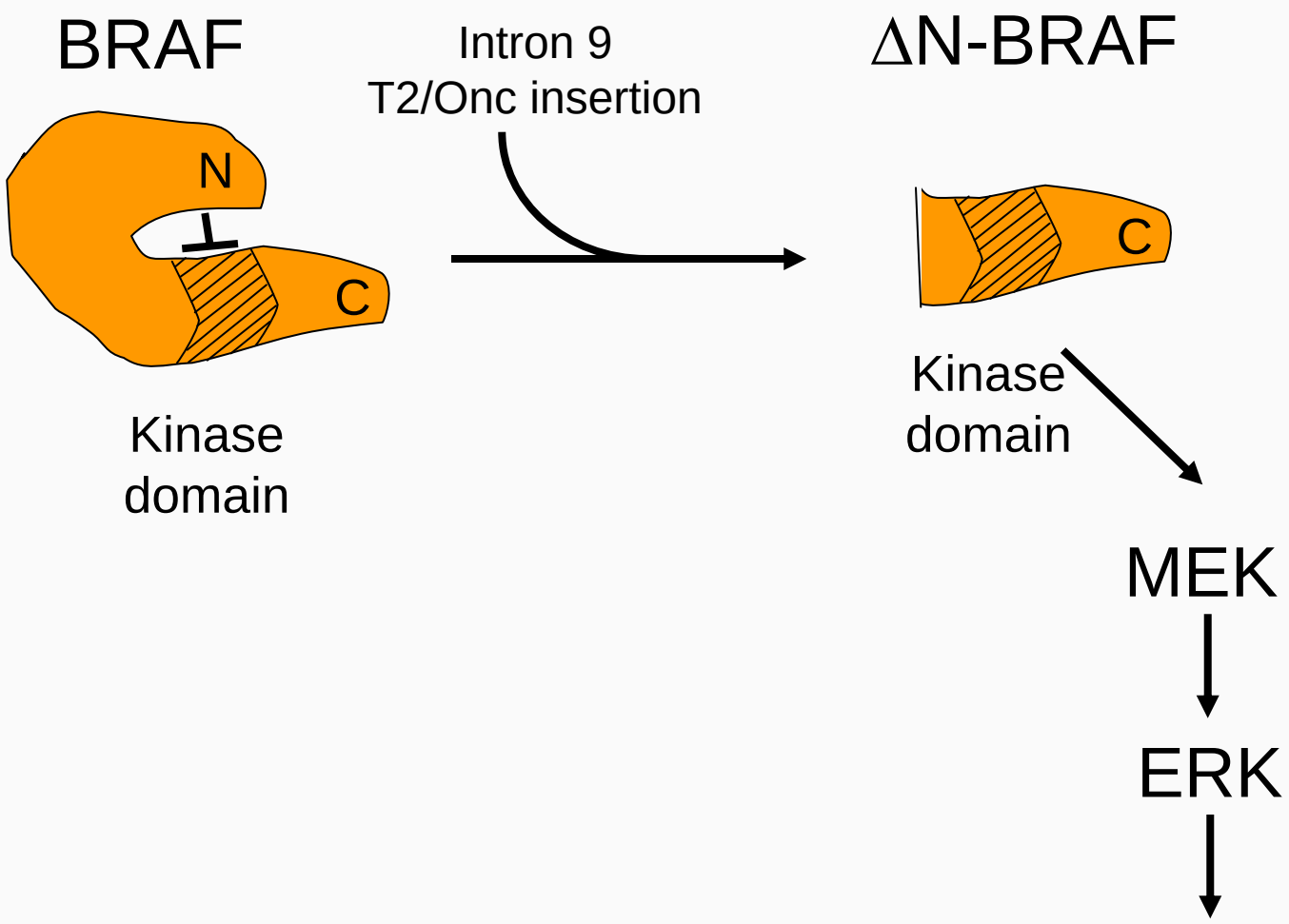
***T-Braf* (Rev)**







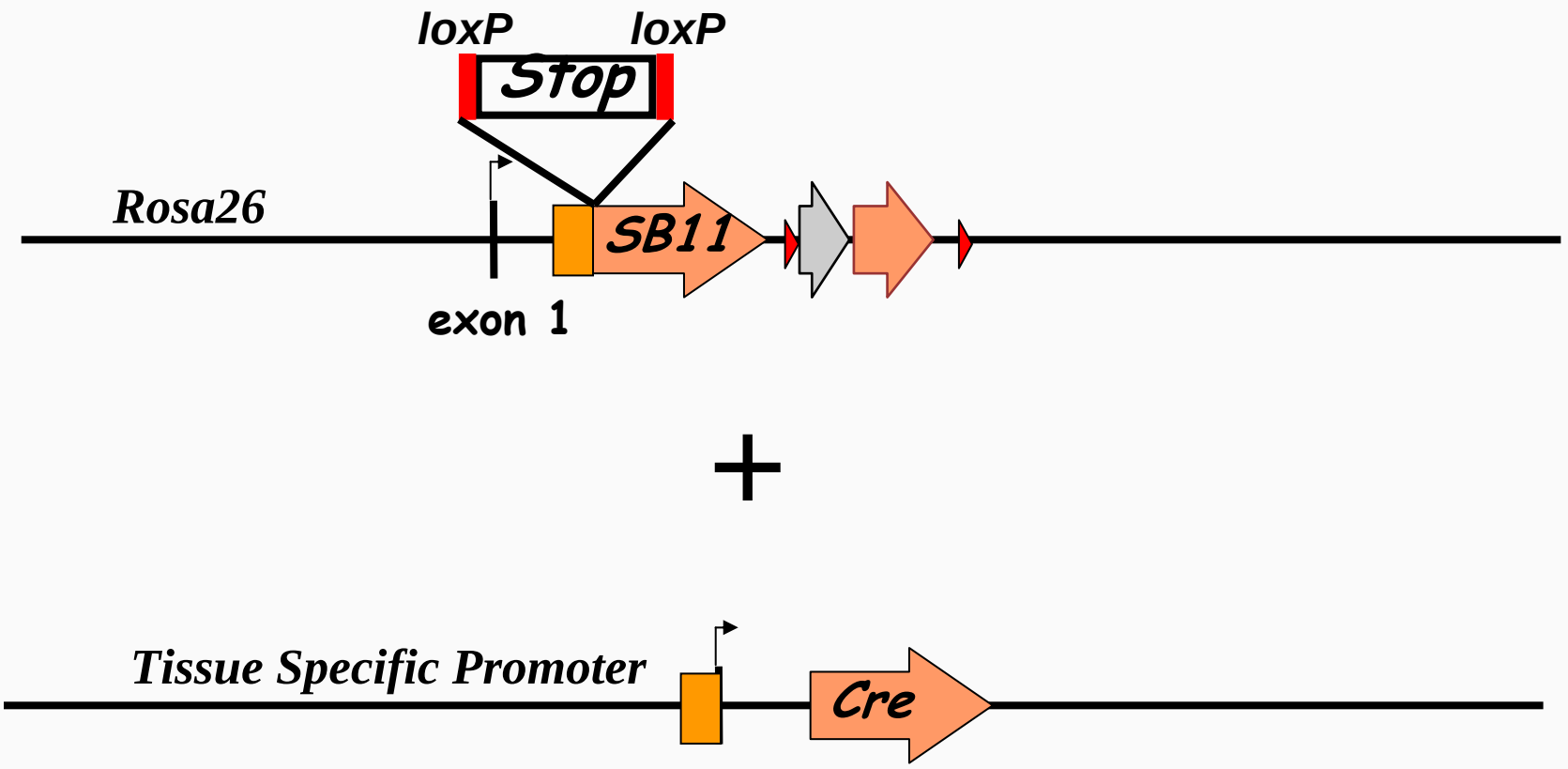
# T2/Onc activation of *Braf*



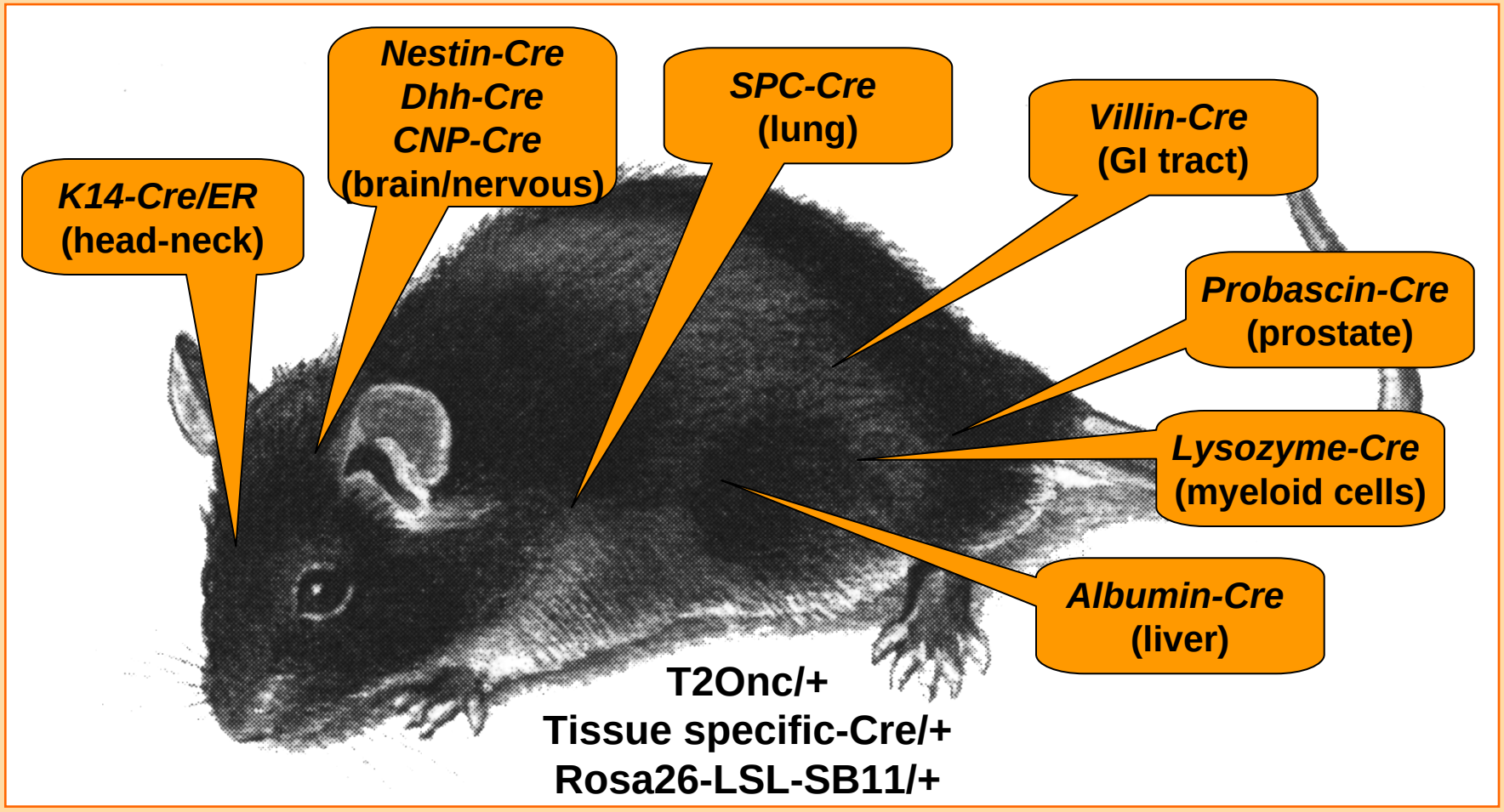
# Can SB somatic mutagenesis cause cancer in other tissues?

- Common epithelial derived cancers?
- Copeland/Jenkins lab generated knock-in allele to express SB11 transposase from the *Rosa26* locus using a loxP-flanked “STOP” cassette

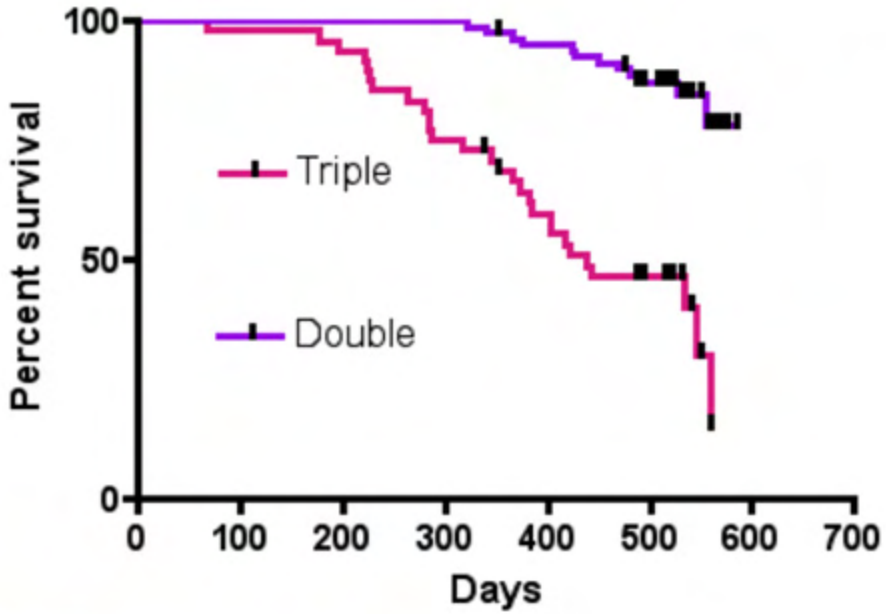
# A conditional Rosa26-SB11 transposase knockin allele



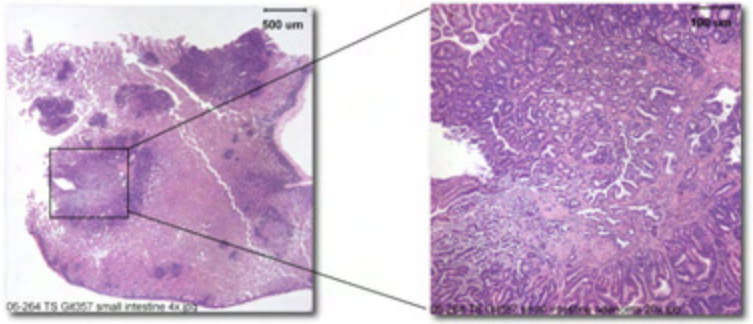
# Tissue specific transposon SB mutagenesis



# Accelerated Death in Triple Versus Double Transgenics (Villin-Cre)




- Triple transgenic mice developed GI tract tumors
- All had small intestine tumors (adenomas and some adenocarcinomas)
- 25% also had tumor(s) in colon



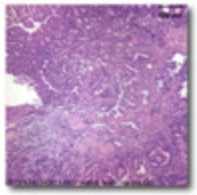
Villin-Cre + T2/Onc
Rosa26-LSL-SB11
= Triple

T2/Onc +
Rosa26-LSL-SB11, etc
= Double

*Tim Starr*

Starr et al., *Science*, 2009

# Pipeline for Genetic Analysis of GI Tract Tumors



DNA prepared from SB-induced tumors - 136 GI tract tumors

linker-mediated PCR to amplify T2/Onc junction fragments - secondary primers bar-coded for each tumor

454 pyrosequencing

~16,500 unique T2Onc insertions

Bioinformatic distillation

77 common transposon insertion sites

# Mutation of Human CRC Genes in SB-Induced GI Tract Tumors

| Gene | Notes |
|---|---|
| *Apc* | TSG mutated in >80% colorectal cancer (CRC) |
| *Bmpr1a* | TSG mutated in CRC, encodes BMP receptor |
| *Smad4* | TSG mutated in CRC, TGFb signal transducer |
| *Fbxw7* | TSG mutated in CRC, F-box protein |
| *Cdk8* | Oncogene amplified in CRC, Cyclin dependent kinase 8 |
| *Pten* | TSG mutated in CRC, PTEN |
| *Dcc* | TSG mutated in CRC, “deleted in colon cancer” |
| *Nsd1* | TSG mutated in Sotos syndrome |
| *Notch1* | Oncogene activated in cancer |
| *Pi3kr1* | Oncogene activated in cancer, p85alpha PI3K subunit |
| *Tcf12* | Transcription factor, mutated in chondrosarcoma |

*Mutated in human colorectal cancer*
*Mutated in other human cancers*

# CIS-Associated Genes are Altered in Human Cancer

**Of 77 candidate GI tract cancer genes:**

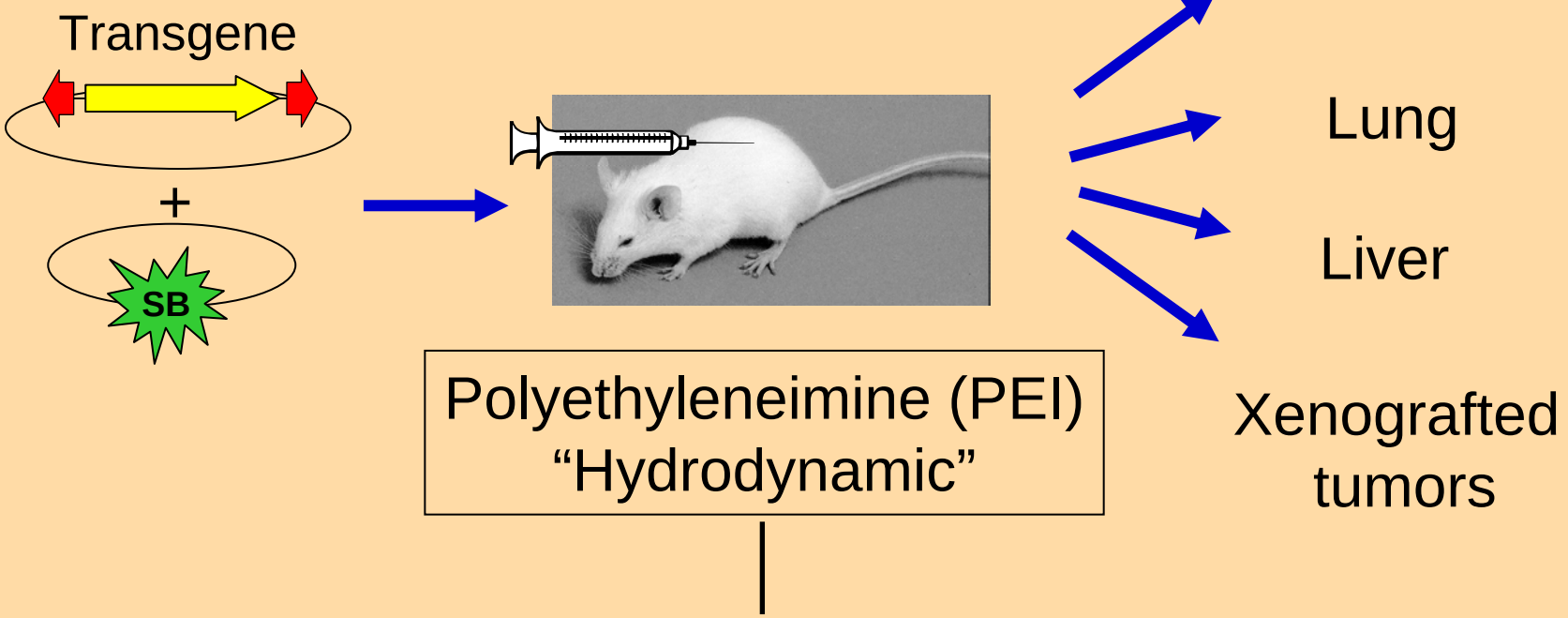
- 47% of those examined have one or more non-silent somatic mutations in human tumor (COSMIC database) [$p<0.05$]
- Significant number show copy loss or gene amplification in a study of ~150 human colorectal cancer by array comparative genome hybridization (Scott Powers at CSHL) [$p<0.05$]
- 14% have somatic mutations in human colorectal cancer (Wood et al, 2008) and 5% were CAN genes suggested to be drivers for human colorectal cancers [$p<0.005$]

| SB Screen | Re-seq Screen |
|---|---|
| Smad4 | **SMAD4** |
| Uhrf2 | **UHRF2** |
| Apc | **APC** |
| Cntn4 | **CNTN4** |
| Cutl1 | **CUTL1** |
| Fbxw7 | **FBXW7** |
| Pten | **PTEN** |
| Add3 | ADD3 |
| Ankrd11 | ANKRD11 |
| Dcc | DCC |
| Dnahc1 | DNAH1 |
| Dstn | DSTN |
| Gpbp1 | GPBP1 |
| Ppp1r12a | PPP1R12A |
| Rreb1 | RREB1 |
| Wac | WAC |

# Comparative Genomics: CIS Genes Likely to be Drivers of CRC

| Gene Symbol | Mutated in human cancer* | Amplified or deleted in human CRC† | Aberrantly expressed in human CRC‡ | Known human cancer gene§ |
|---|---|---|---|---|
| *ANKRD11* | x | x | x | |
| *APC* | x | | x | x |
| *BMPR1A* | x | x | x | x |
| *DSTN* | x | x | x | |
| *EVI1* | x | | x | x |
| *FBXW7* | x | | | x |
| *GPBP1* | x | x | x | |
| *NOTCH1* | x | x | x | x |
| *NSD1* | x | | x | x |
| *PPP1R12A* | x | | x | |
| *PTEN* | x | x | x | x |
| *RREB1* | x | x | x | |
| *SMAD4* | x | x | x | x |
| *TCF12* | x | x | x | x |
| *TNPO3* | x | x | x | |

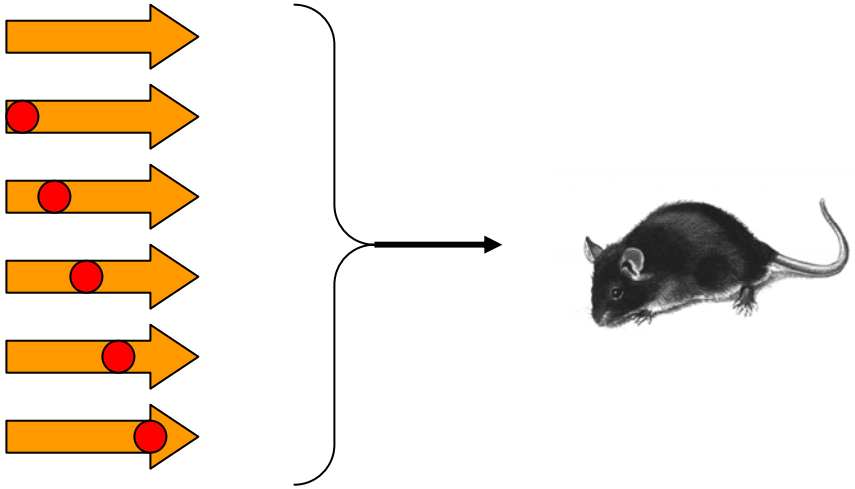
# Somatic cell gene transfer: *SB* can be used for long-term gene transfer and expression in many settings



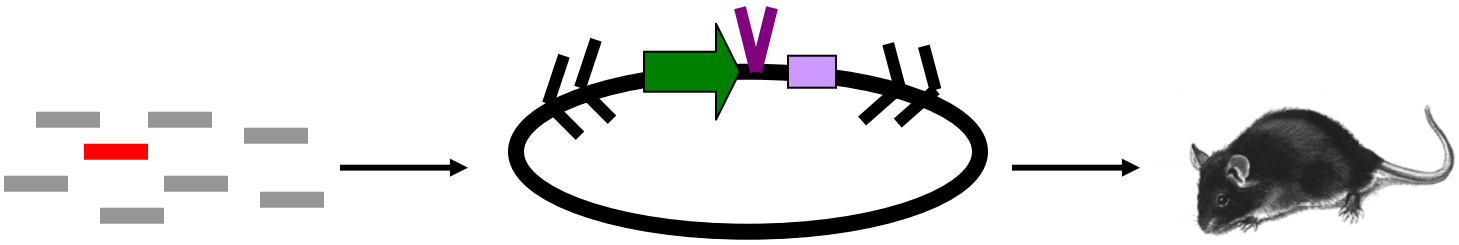

***Yant et al., 2000; Beleur et al., 2003; Ohlfest et al., 2004 and others.***

# Can SB be used to deliver activated oncogenes to soma for creating cancer models?

*Testing of mutant oncogenes:*



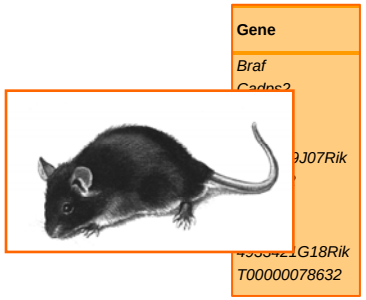
*Mini-library screening:*



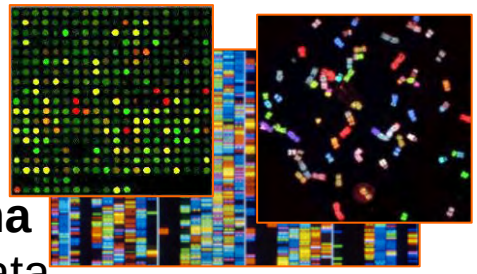

*cDNA library*

*Transposon Expression Vector*

# Ultimately what do we want to do with these cancer models?




**Mouse glioma**
-common insertion site gene list



**Human glioma**
-microarray data
-exon resequencing
-array CGH
-recurrent chromosomal aberrations

Cellular transformation assays
Mouse transgenesis

Target identification
Prognostic and diagnosis

# Conclusions and future directions

- Chromosomally resident SB vectors transpose in mouse soma
- T2/Onc SB vector + SB transposase transgenes can accelerate or initiate tumor formation in cancer predisposed (*Arf-/-*) or wild-type mice
- SB-induced tumor development due to insertional mutagenesis, allows identification of common sites of transposon insertion and associated cancer genes - lymphoma, sarcoma, brain tumors, carcinomas also
- SB can integrate transposons containing oncogenes into genomes of somatic cells to model tumorigenesis in vivo

# Acknowledgments

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Rachel Saplis
Won-Il Kim
Bin Yin
Micki Diers

**Nancy Jenkins**
**Neal Copeland**
Adam Dupuy
Keiko Akagi

**Martine Roussel/**
**Charles Sherr**

**Beckman Center for Transposon Research**

**Paul Marker**

**Scott Fahrenkrug**
Dan Carlson

**Scott McIvor**
Andy Wilber
Joel Franzen

**Perry Hackett**
Karl Clark
Jason Bell

**Ekker Lab**
Darius Balciunas

**Production of transgenic mice**
Sandra Horn
Cesilie Granum

**Bioinformatics**
Kevin Roberg Perez
Anne Lamblin
Rod Stagg

**Histopathology**
Nicole Kirchoff
Cathy Carlson

**Cytogenetics**
LeAnn Oseth
Betsey Hirsch

**Sequencing**
David Adams
Allan Bradley
Tony Cox