



Genetic strategies of generating animal models: Non-mammalian models

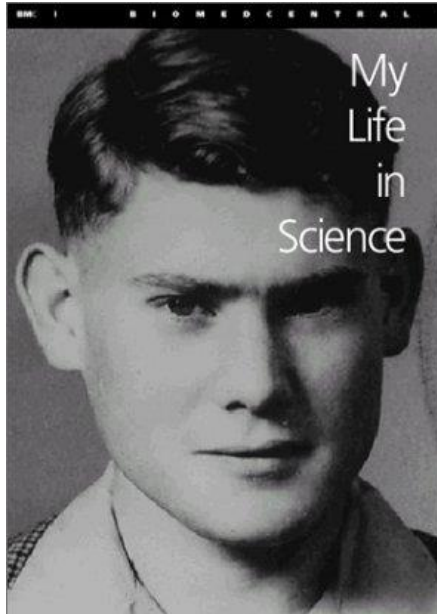
dr Savani Anbalagan

Krogh Principle



"for such a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied."

Sydney Brenner



"choosing the right organism for one's research is as important as finding the right problems to work on"

What is an animal model system?

- non-human species that are studied in order to understand a range of biological phenomena
- with the hope that data, models and theories generated will be applicable to other organisms

Essential characteristics of an animal model organism

- An appropriate model organism for research question
- Easy to rear and work with
- Availability of genome sequence and gene expression profile
- Ability to introduce genetic material
- Ability to develop transgenic animals
- Ability to perform gene knock-down and knock-out
- Ability to perform targeted mutagenesis

Example of non-mammalian animal models

Harpegnathos saltator



Drosophila melanogaster



Caenorhabditis elegans



Danionella

Danio

Nothobranchius furzeri



Xenopus laevis



Ambystoma mexicanum



Lesson for human biology from flies..



T.H. Morgan



H.J. Muller



E.B. Lewis



C. Nüsslein-Volhard



E. Wieschaus



R. Axel



J.A. Hoffmann



J.C. Hall










M. Rosbash



M.W. Young

Comparison of research models

	 2D cell culture	 <i>C. elegans</i>	 <i>D. melanogaster</i>	 <i>D. rerio</i>	 <i>M. musculus</i>	 PDX	 Human organoids
Ease of establishing system	✓/✗	✓	✓	✓	✓	✓	✓
Ease of maintenance	✓	✓	✓	✓	✓	✓	✓
Recapitulation of developmental biology	✗	✓	✓	✓	✓	✗	✓
Duration of experiments	✓	✓	✓	✓	✓	✓	✓
Genetic manipulation	✓	✓	✓	✓	✓	✗	✓
Genome-wide screening	✓	✓	✓	✓	✗	✗	✓
Physiological complexity	✗	✓	✓	✓	✓	✓	✓
Relative cost	✓	✓	✓	✓	✓	✓	✓
Recapitulation of human physiology	✓	✓	✓	✓	✓	✓	✓

✓ Best
✓ Good
✓ Partly suitable
✗ Not suitable

.....

Genetic strategies

- Random mutagenesis
- Targeted genome engineering tools
- RNA perturbation tools*
- Gene expression systems

Random mutagenesis

Types

- Radiation-based
 - X-ray
 - Ultraviolet
 - Gamma-ray
- Chemical-based
 - EMS
 - ENU
- Insertional mutagenesis
 - P-element
 - Tol2-based
 - Retrovirus

Radiation and Chemical-based mutagenesis

Applications

- Gene perturbation

Advantage

- Uniformly saturate the genome with mutations

Disadvantage

- Need to identify the mutated gene using traditional techniques is labor-intensive and time consuming
- However, with whole-genome sequencing (WGS), it is now possible to sequence hundreds of strains,
- but determining which mutations are causative among thousands of polymorphisms remains challenging.

Random mutagenesis

- In 1920s, Hermann Muller's experiment demonstrated that exposure to X-rays, can cause genetic mutations
- Muller exposed *Drosophila* to x-rays, mated the flies, and observed the number of mutant phenotypes in the offspring.
- Muller's experiments with X-rays established that X-rays mutated genes and that egg and sperm cells are especially susceptible to such genetic mutations.

1946 Nobel Prize

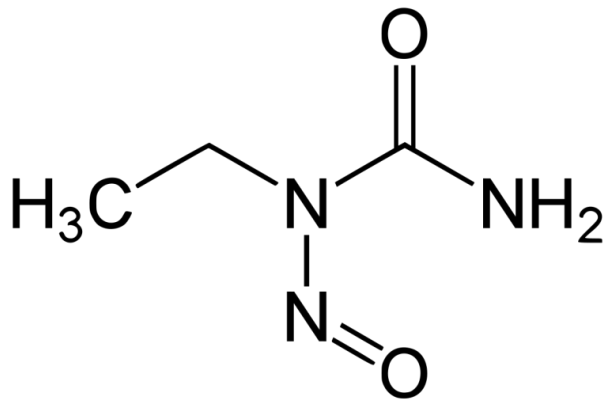


Hermann Joseph Muller

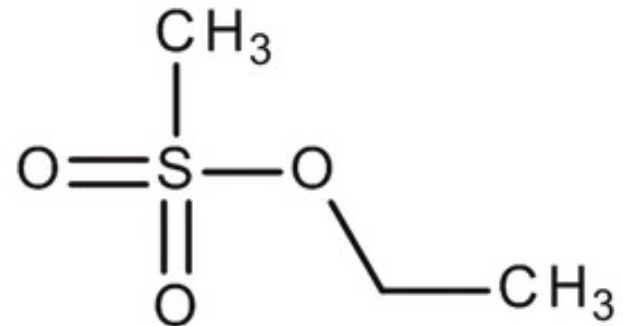
for the discovery of the production of mutations by means of X-ray irradiation

Chemical mutagenesis

- Involves treatment of animals or reproductive cells with alkylating agents
 - N-Ethyl-N-nitrosourea (ENU)
 - Ethyl methanesulfonate (EMS)
- Screen for particular phenotype

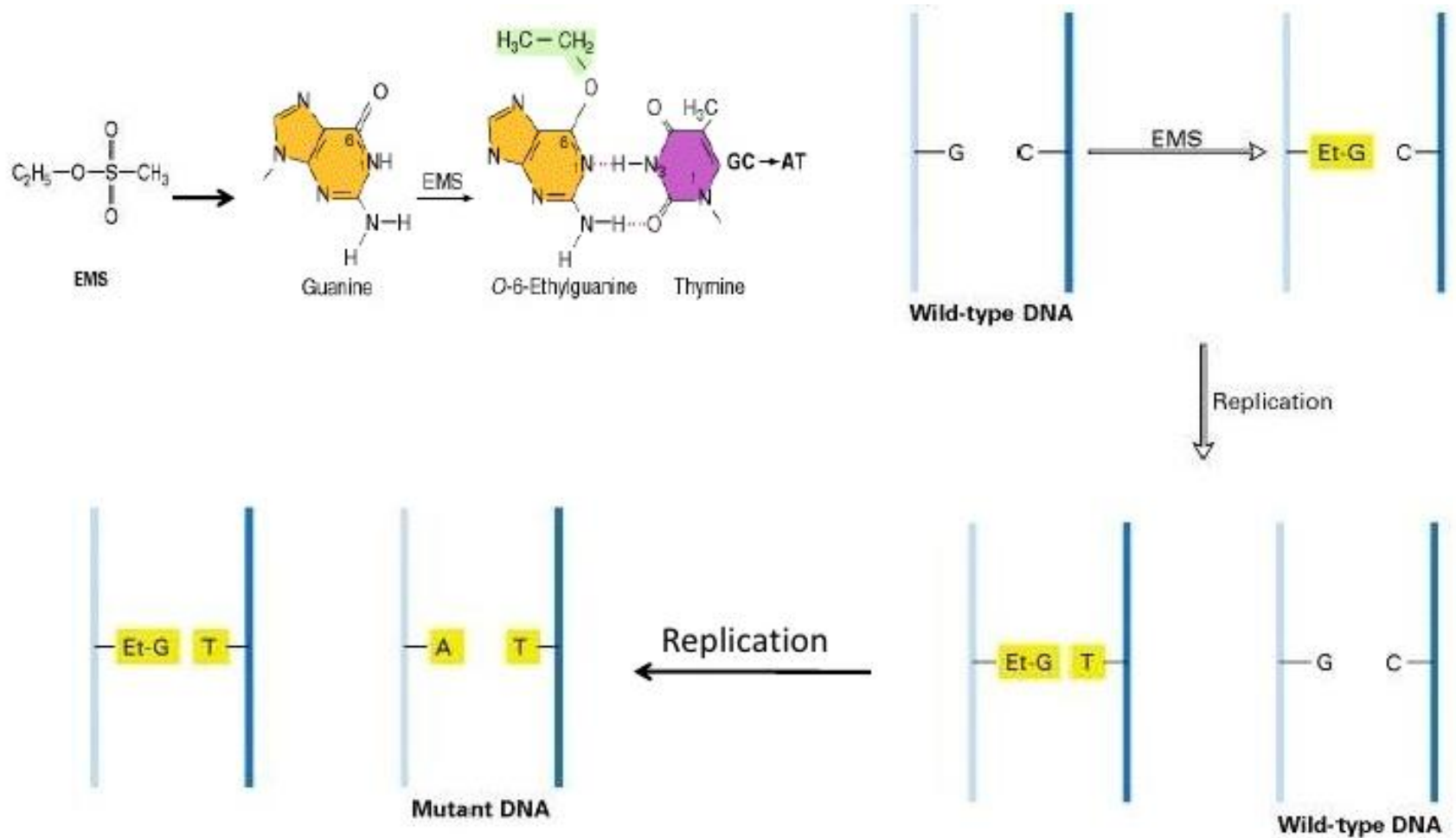


ENU



EMS

Chemical mutagenesis cause DNA base traversions



Chemical mutagenesis in *C. elegans*

- Treat worms with EMS (0.05M) for 4 hours
- Screen offsprings

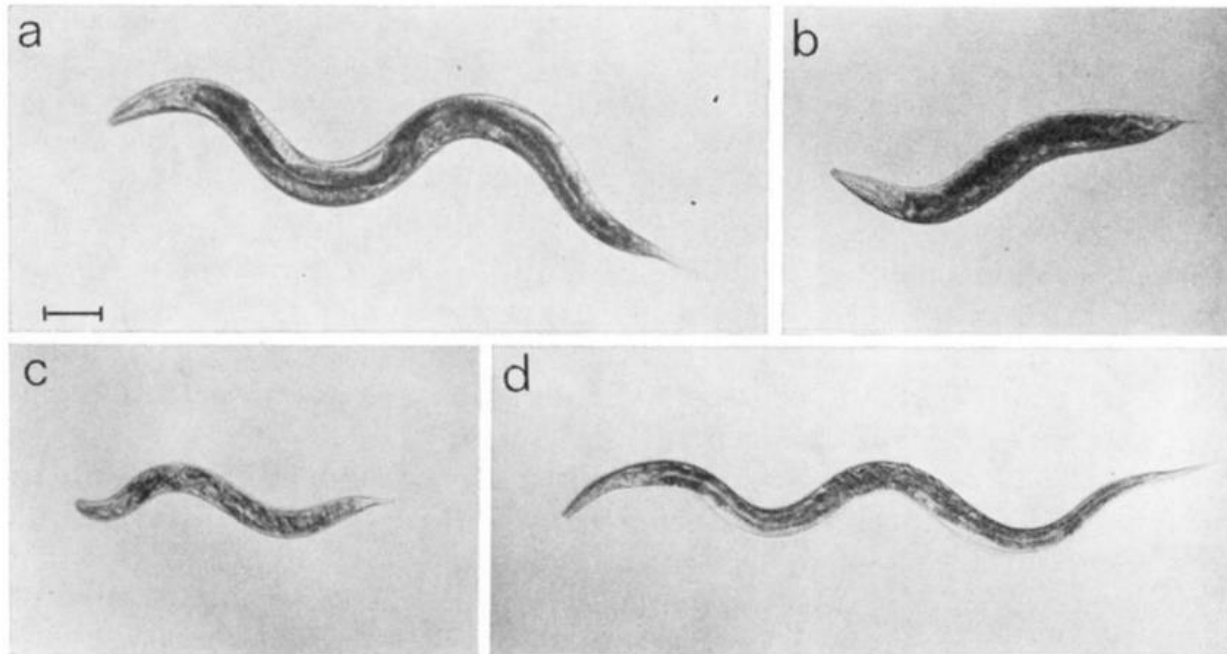
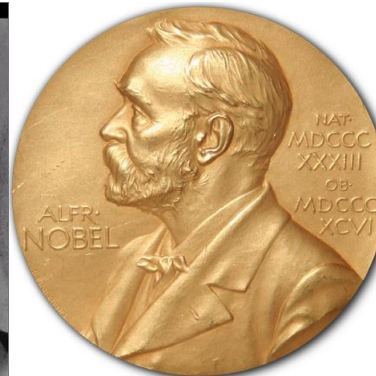
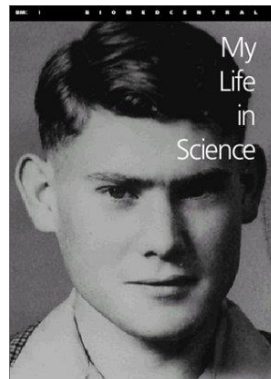


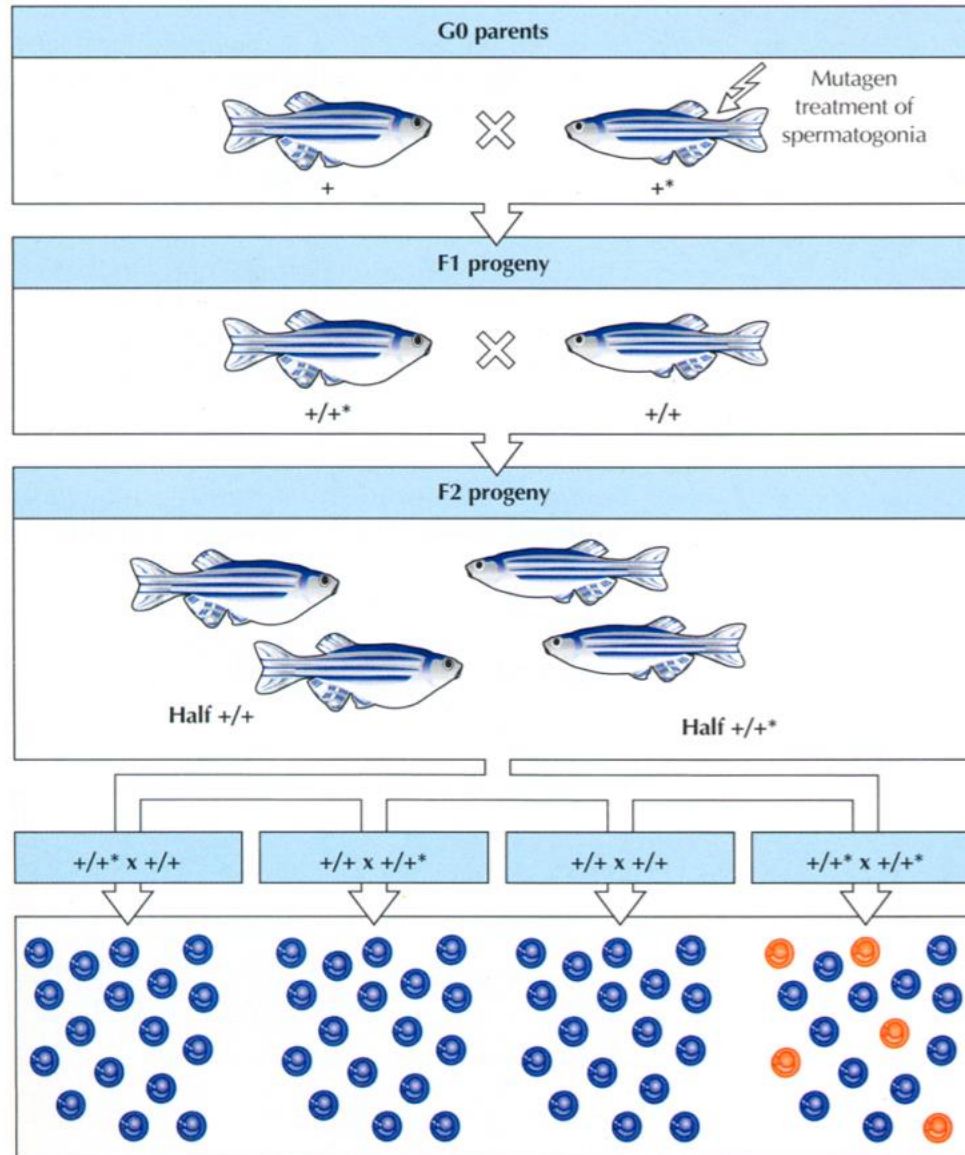
FIGURE 1.—Photomicrographs of *C. elegans* and some of its mutants. a: wild type, b: dumpy (*dyp-1*), c: small (*sma-2*), d: long (*lon-1*). The scale is 0.1 mm.

Chemical mutagenesis in *C. elegans*

Linkage group	Gene	Reference mutant	Number of isolates		Comments
			M	S	
I	<i>bli-3</i>	E767	1		
	<i>unc-35</i>	E259	1		
	<i>unc-56</i>	E403	2		
	<i>unc-11</i>	E47	2		
	<i>unc-40</i>	E271	1		
	<i>unc-57</i>	E406	2		
	<i>unc-38</i>	E264	4	1	Tetramisole-resistant
	<i>unc-63</i>	E384	2		Tetramisole-resistant
	<i>dpy-5</i>	E61	2		
	<i>dpy-14</i>	E188	1		Larvae abnormal
	<i>unc-14</i>	E57	5	1	Small, paralyzed body
	<i>unc-37</i>	E262	1		
	<i>unc-15</i>	E73	1		Paralyzed; defect in body muscle cells
	<i>unc-55</i>	E402	2		
	<i>unc-13</i>	E51	17		Paralyzed; pharyngeal movement irregular
	<i>unc-21</i>	E330	1		
	<i>unc-29</i>	E193	2		Tetramisole-resistant
	<i>unc-54</i>	E190	5		Paralyzed; defect in body muscle cells
	<i>unc-59</i>	E261	1		

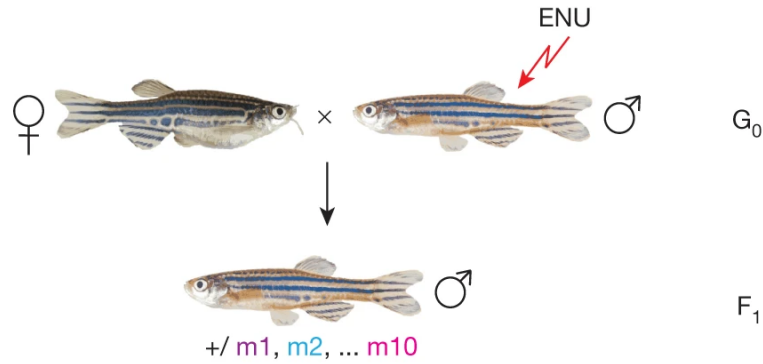


Chemical mutagenesis in zebrafish



Zebrafish mutation project

a



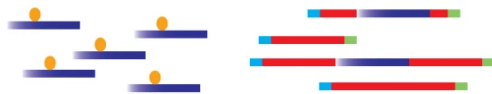
Sperm
archive or
outcross



genomic
DNA

b

Whole-
exome
baits

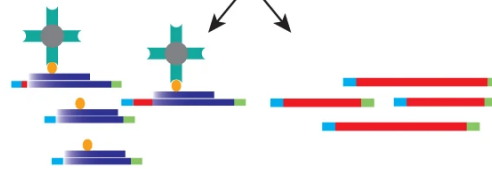


Illumina
library



Exome enrichment
by hybridization

Capture
with streptavidin-
coated beads



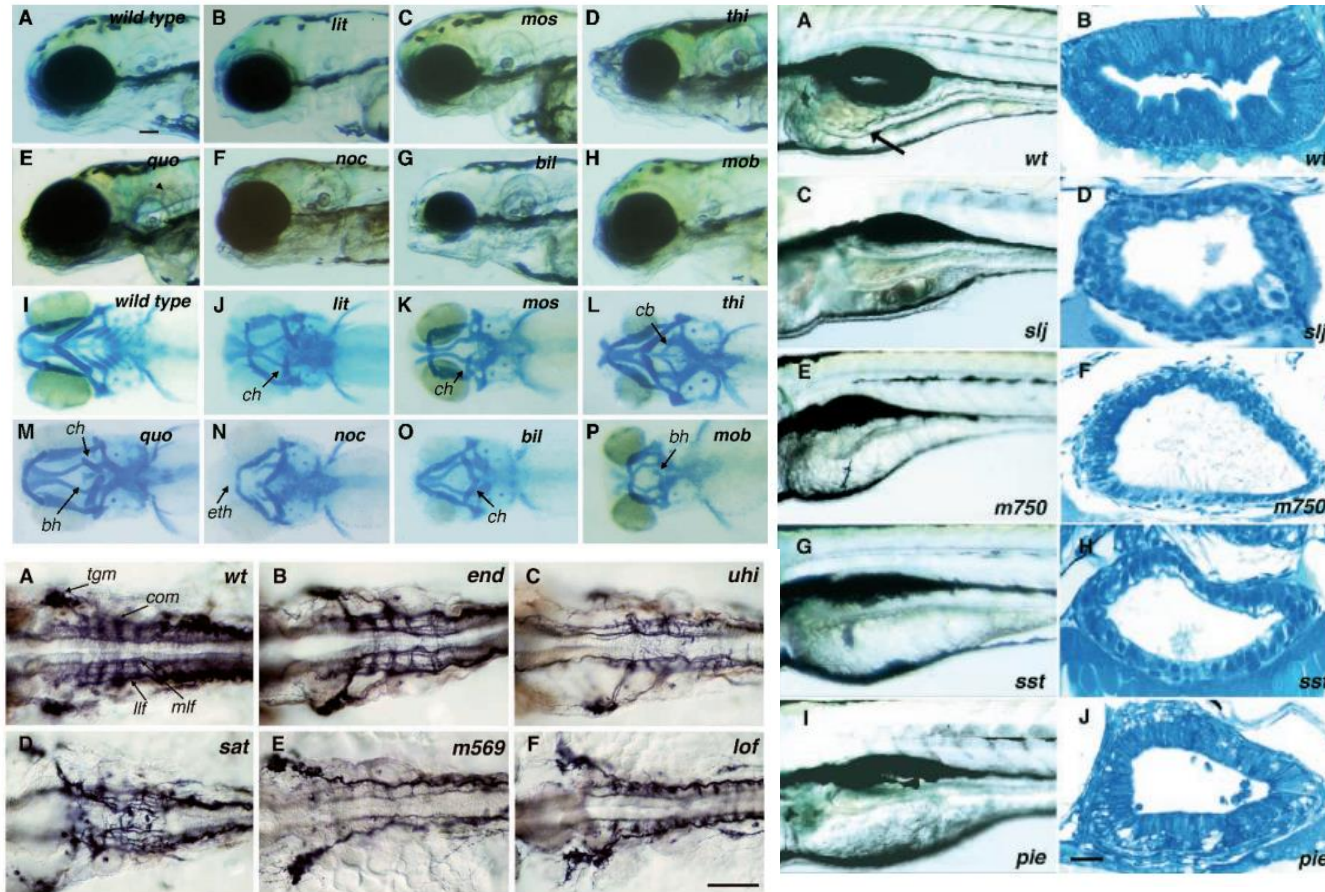
Discard

Digest RNA



Sequence on
Illumina GAII or HiSeq

Chemical mutagenesis in zebrafish



- Zebrafish Mutation Project at the Wellcome Sanger Institute, UK generated a mutant archive of over 40,000 alleles
- Covering 60% of zebrafish protein-coding genes.

Insertional mutagenesis

Types

- P-element transposon-based
- Retrovirus-based

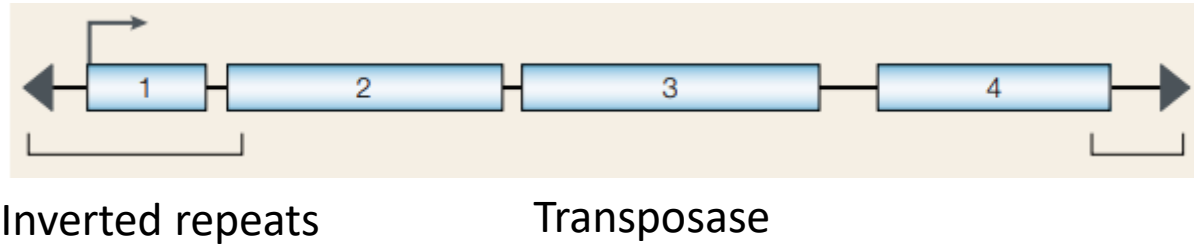
Applications

Knockout

Advantage

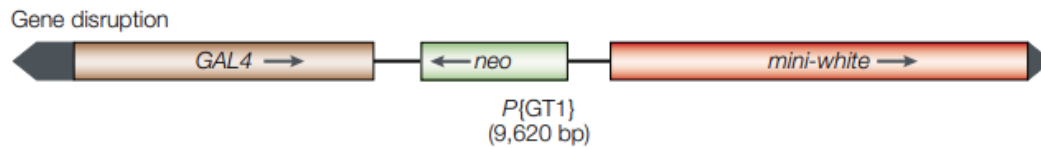
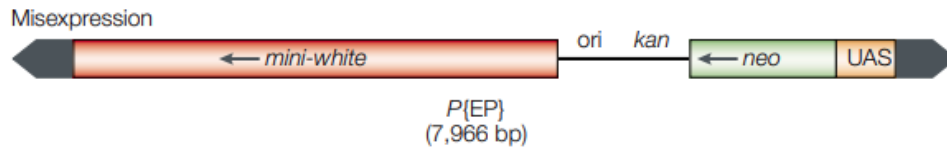
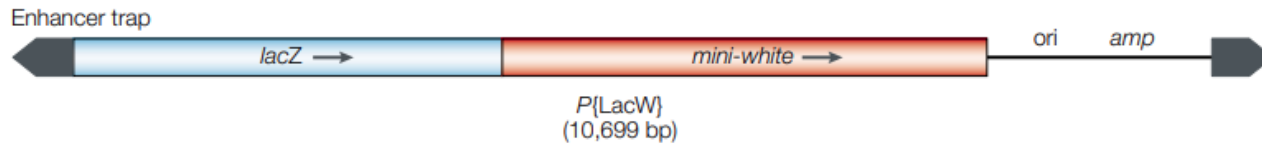
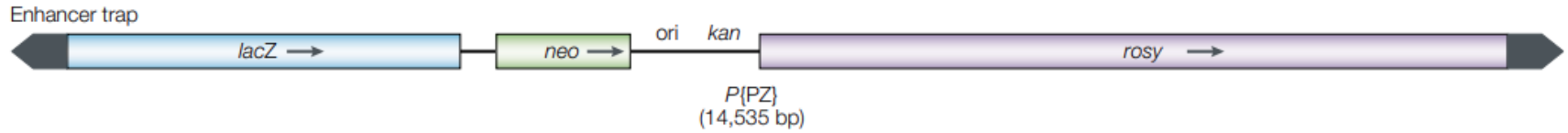
Relative ease of identifying the mutated genes *

P element in Drosophila



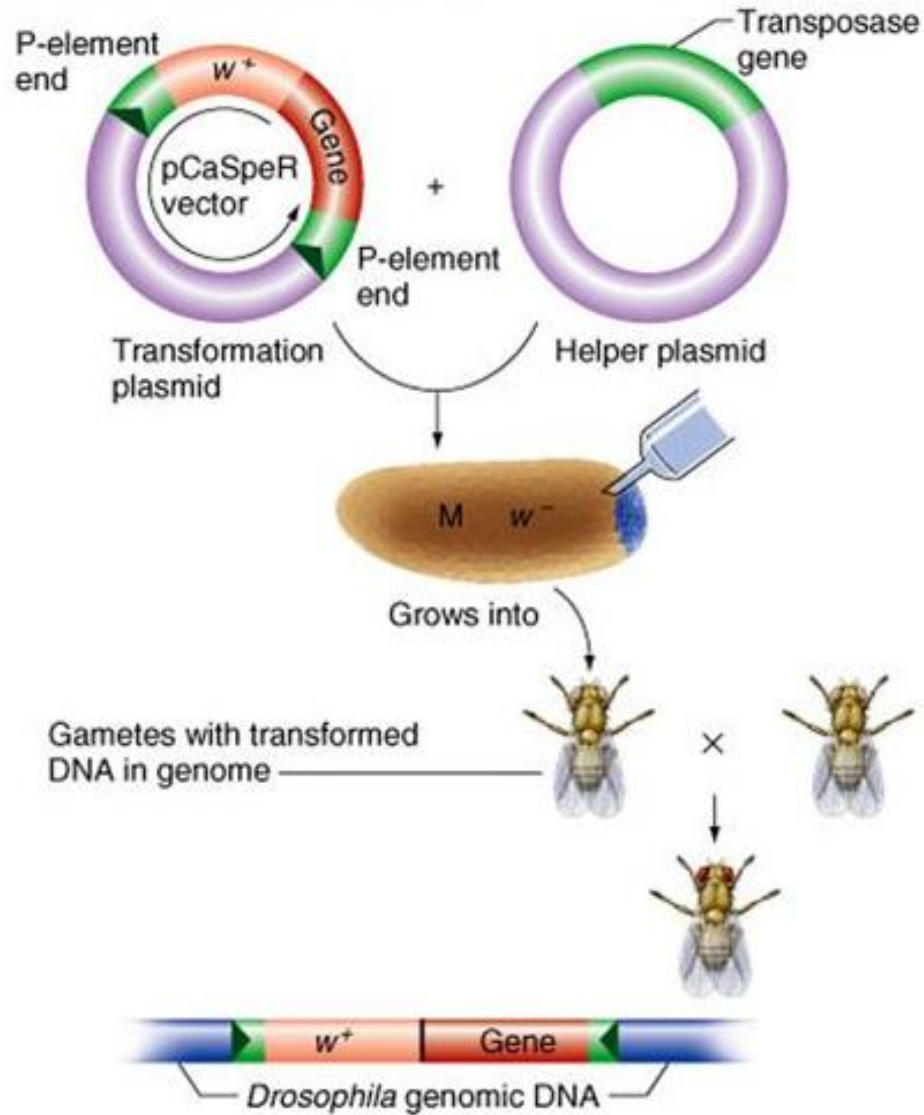
- P elements are cut-and-paste transposons in the genomes of Drosophila
- The transposition of these P elements is catalyzed by an enzyme, the transposase
- This enzyme is naturally produced only in germline tissues.
- 1000s of P-element fly lines have been cataloged with their chromosomal location (FlyBase Consortium, 2002).

P-element in Drosophila

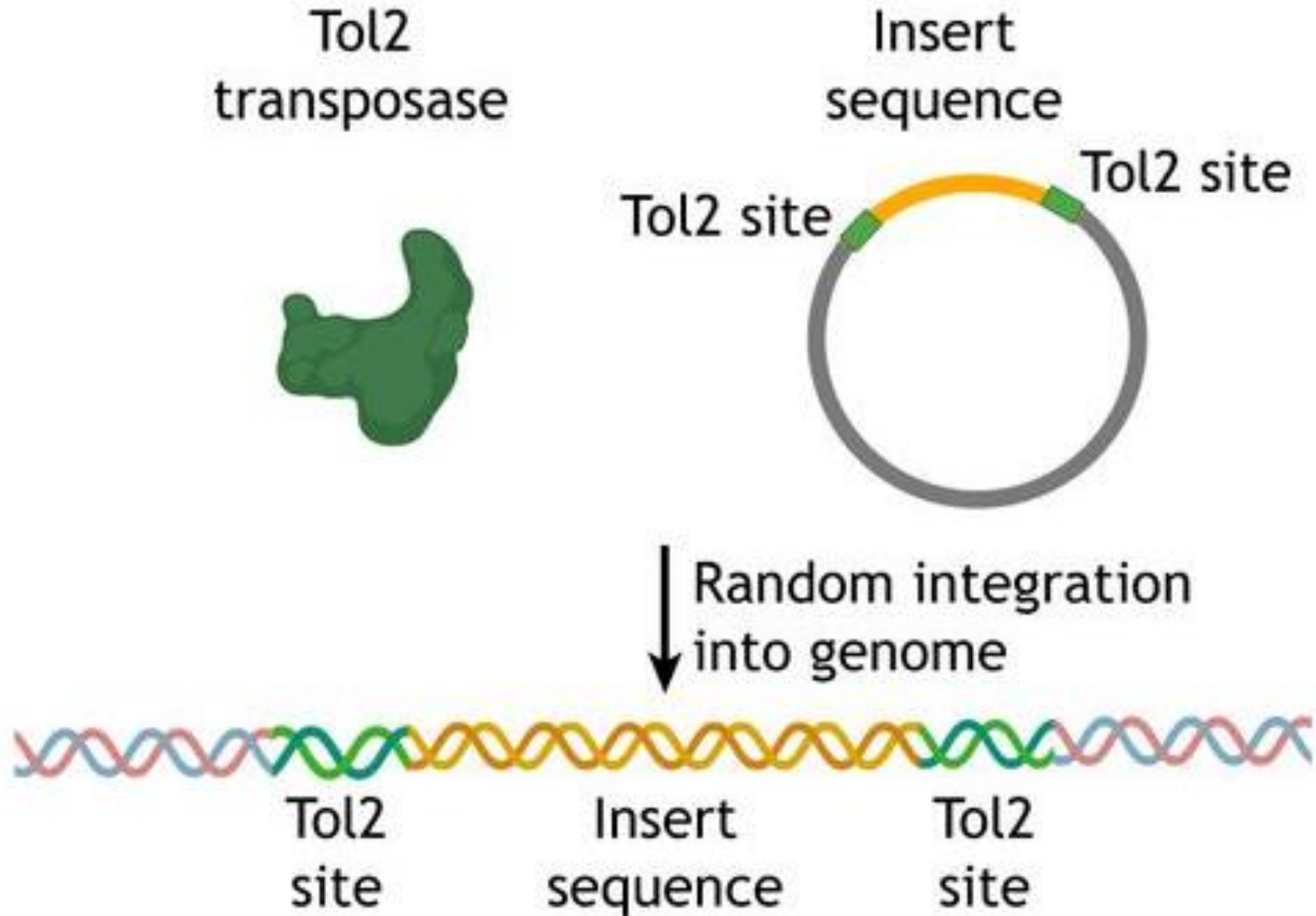


P-element in *Drosophila*

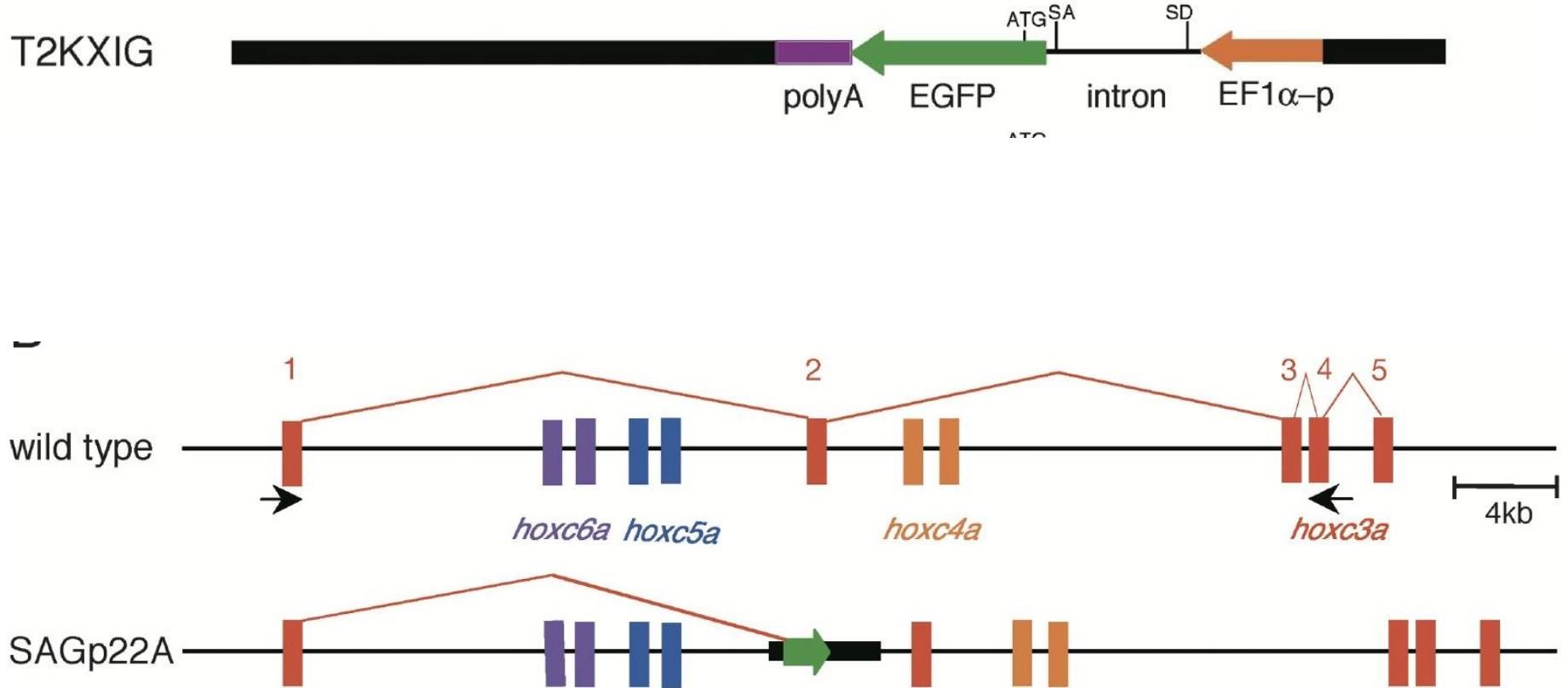
(a) Transformation of *Drosophila*



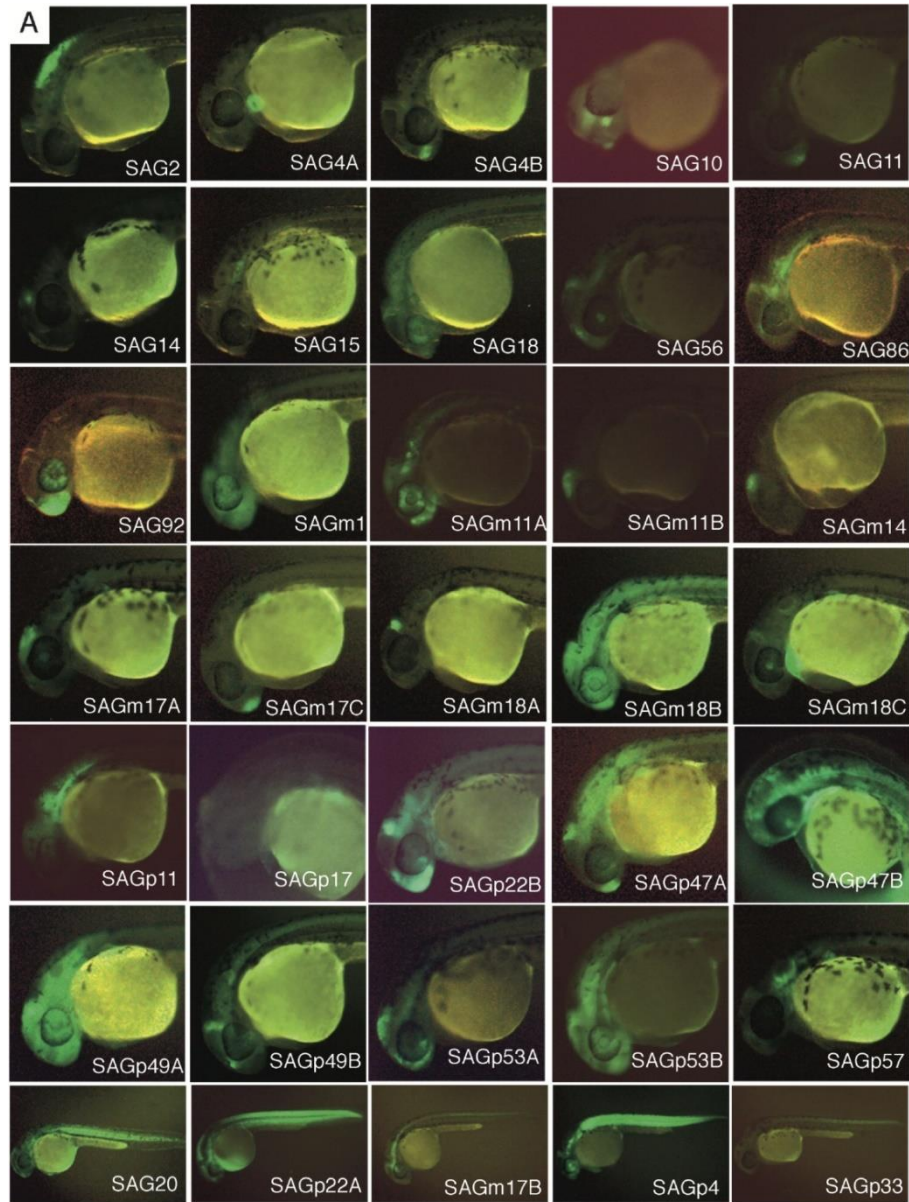
Tol2 in zebrafish



Tol2-mediated Gene trap

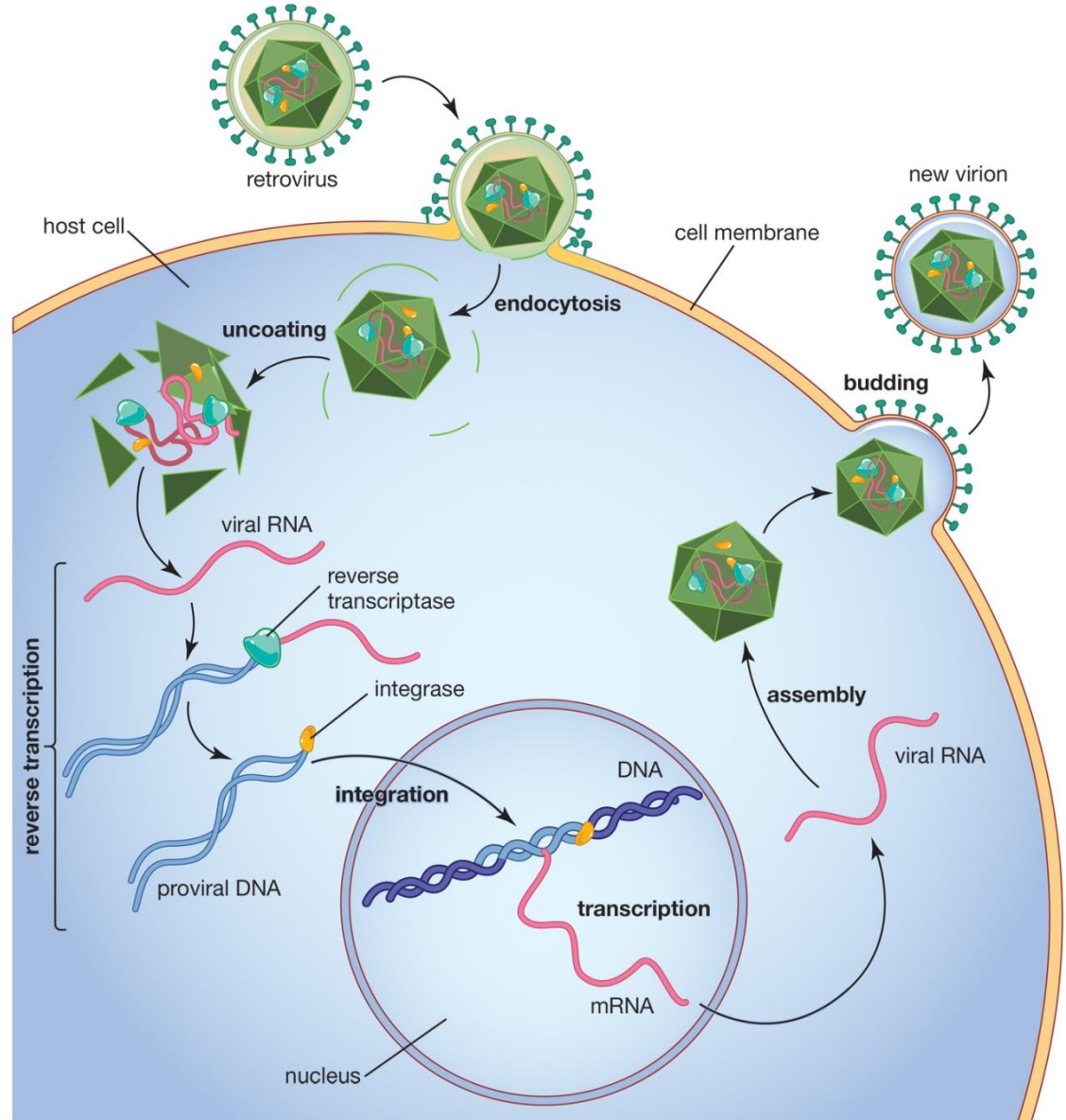


Tol2-mediated Gene trap



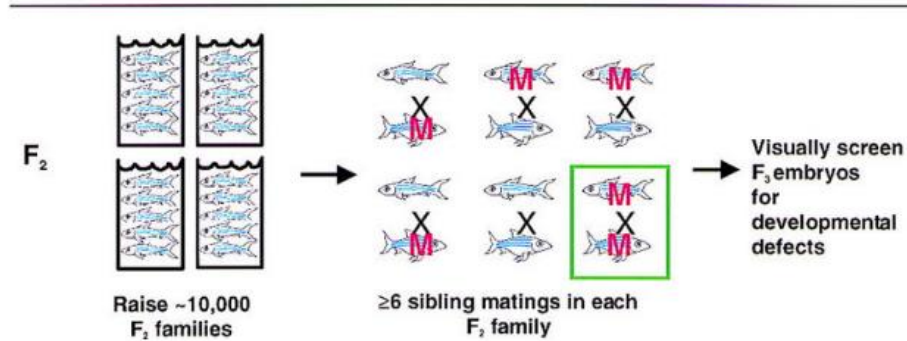
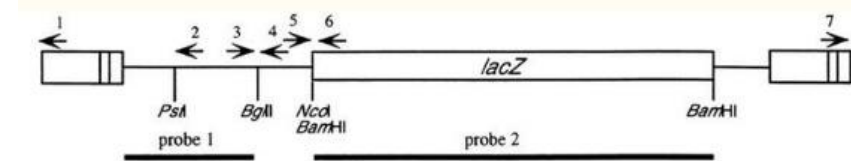
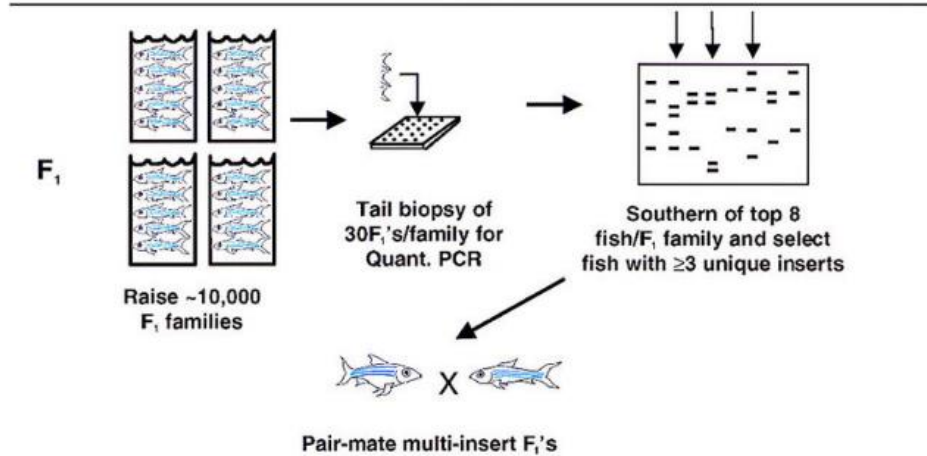
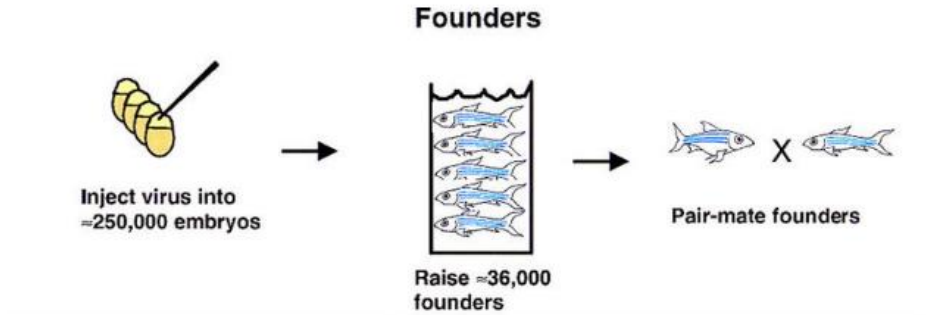
Retrovirus-based mutagenesis in zebrafish

- Murine leukaemia virus/vesicular stomatitis virus
- Can integrate into many different sites in the chromosome

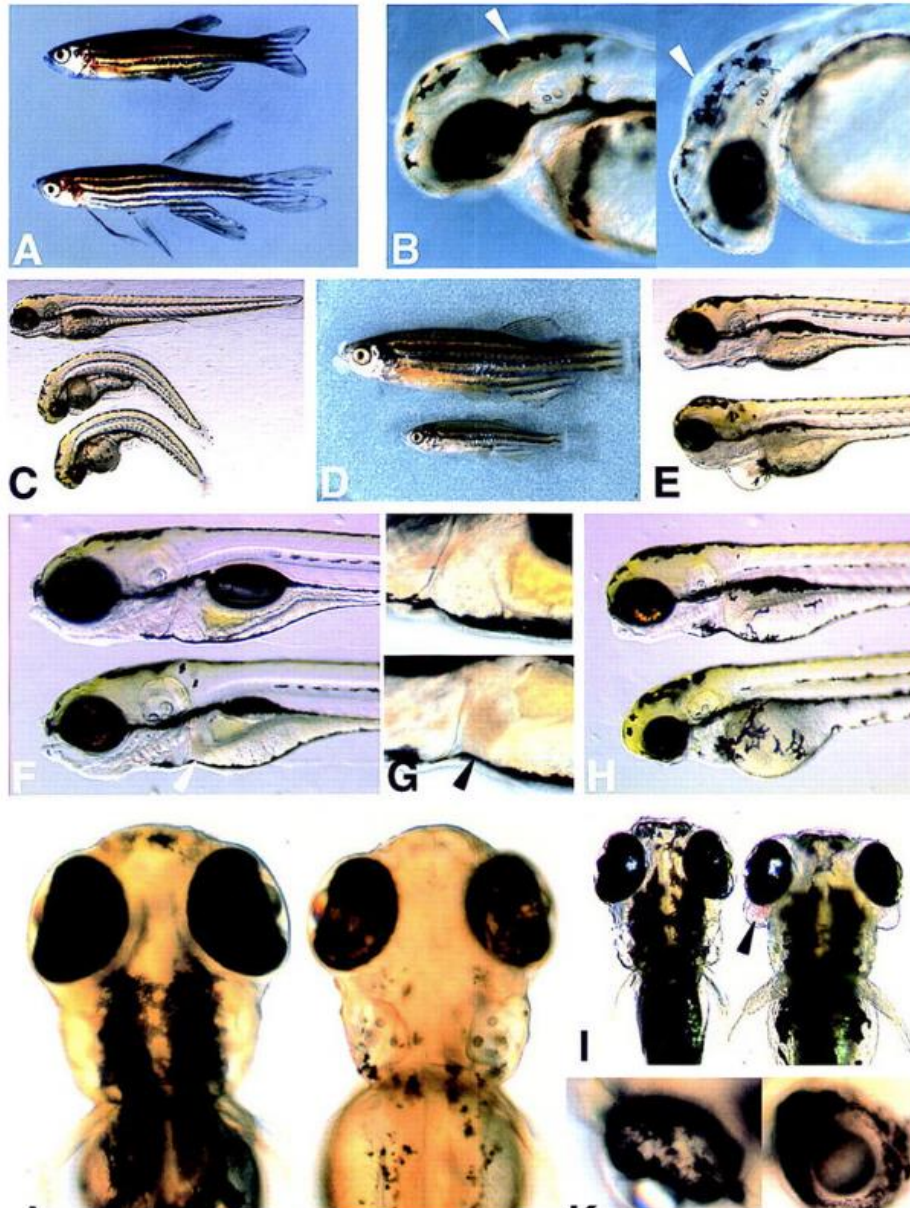


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Retrovirus-based mutagenesis in zebrafish



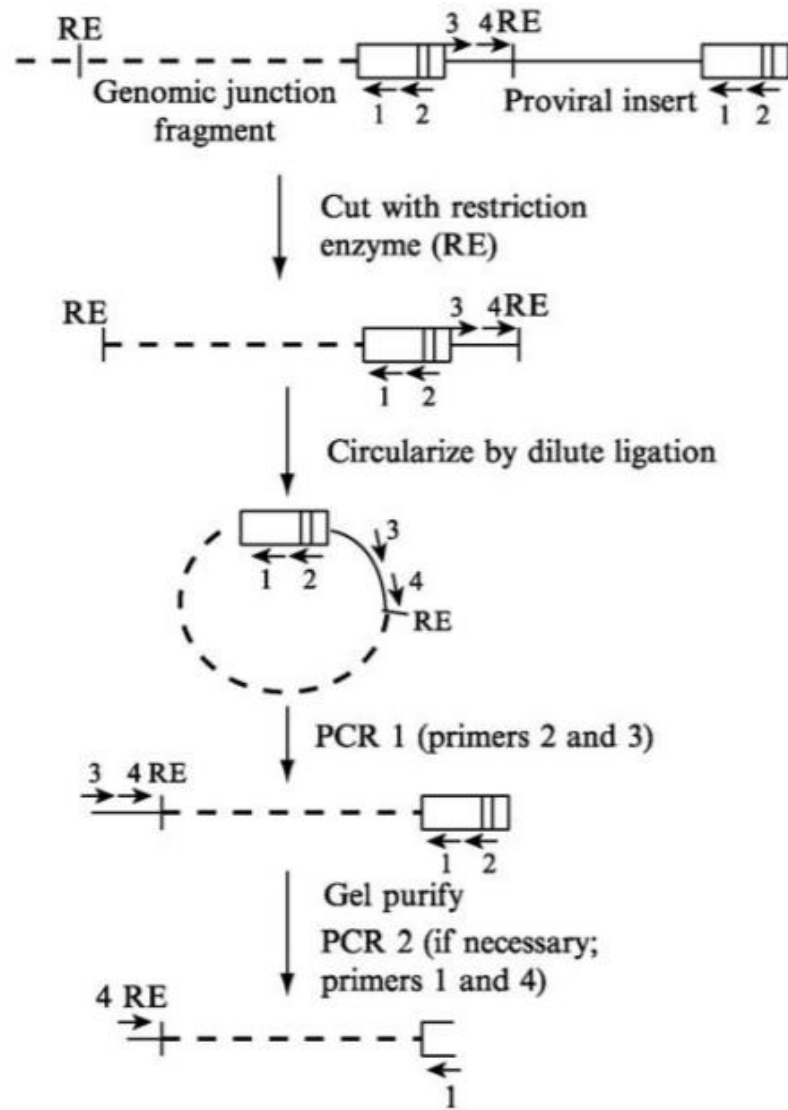
Retrovirus-based mutagenesis in zebrafish



Retrovirus-based mutagenesis in zebrafish

- Advantage:
 - insertion provides a molecular tag that can be used to identify the disrupted gene.

Inverse PCR to identify disrupted gene



Chemical vs insertional mutagenesis in zebrafish

Steps	Chemical mutagenesis	Insertional mutagenesis
Founders population	Expose five-to-ten male fish to ENU	Inject virus into embryos; raise ~1200 founders capable of transmitting ~30 000 inserts Time: this should take two people two months
Generate F1	Raise 250-300 F1 fish from outcrossed founders	Raise ~15 000 F1 fish from 500 founder crosses; select 1500 F1 fish that each have five-to-ten inserts Time: this should take two people one year
Generate F2	Raise 100 F2 families from F1 crosses	Raise 700 F2 families from F1 crosses
Screen F3	Screen 600 crosses (approximately six crosses per F2 family) Time: this should take four people one month	Screen 4200 crosses (approximately six crosses per F2 family) Time: this should take four people seven months
Cloning mutated gene	One person will need six-to-twelve months per mutant; 50-100 researcher-years for 100 mutants	One person will need three-to-four weeks per mutant (can do many simultaneously); one researcher-year for 100 mutants

Targeted genome editing

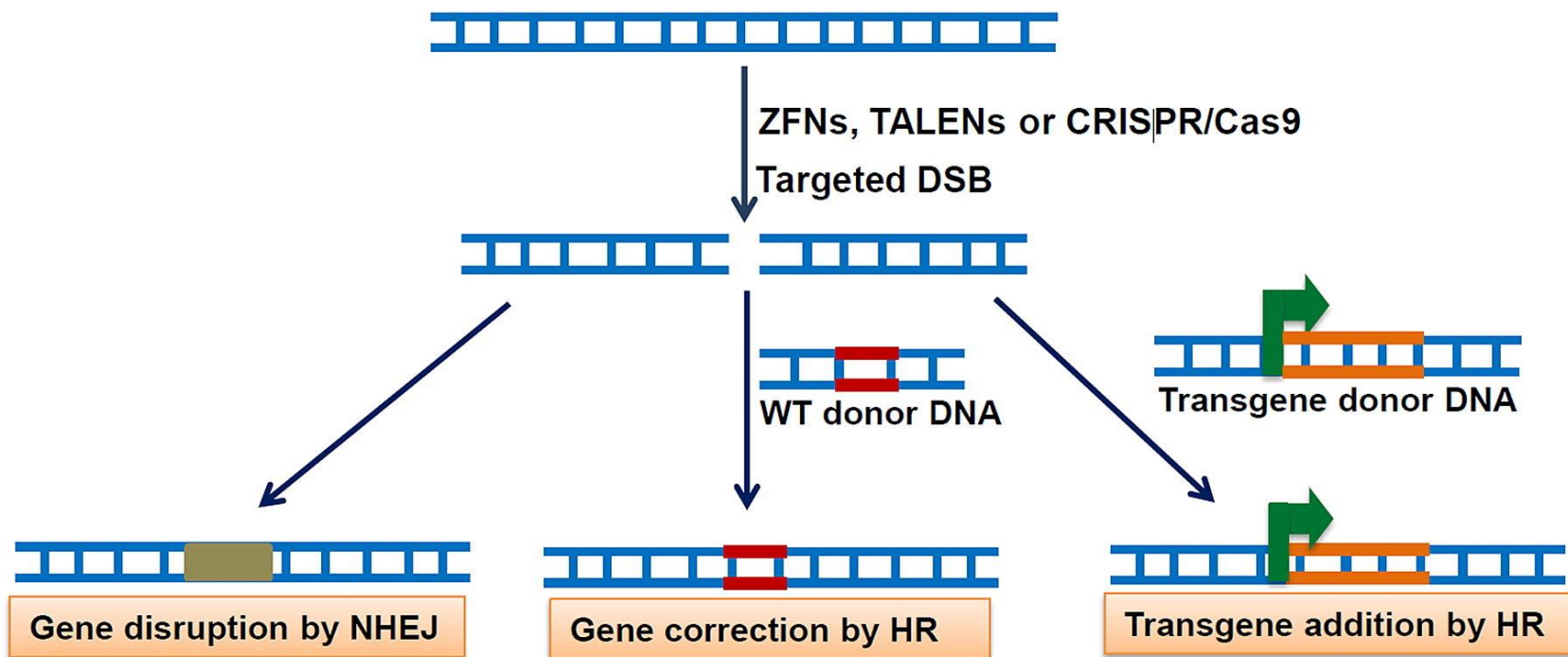
- ZFN
- TALEN “Class of programmable nuclease”
- CRISPR

How it is done?

- Inject into embryos or eggs
 - ZFN – mRNA or
 - TALEN – mRNA or
 - CRISPR – Cas9 mRNA or protein and gRNA targeting specific DNA sequence

Targeted genome editing

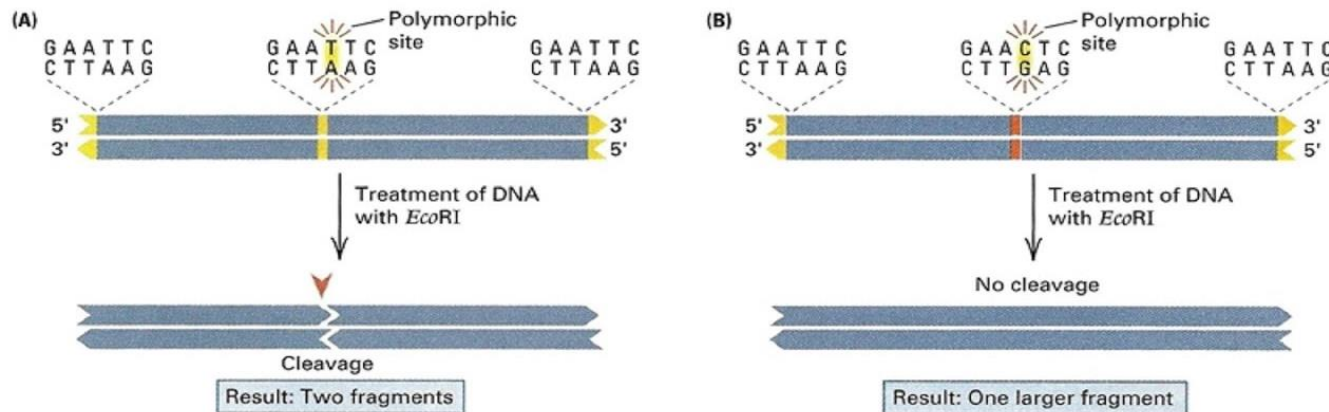
- Genome editing with programmable nucleases depends on cellular responses to a targeted double-strand break (DSB).



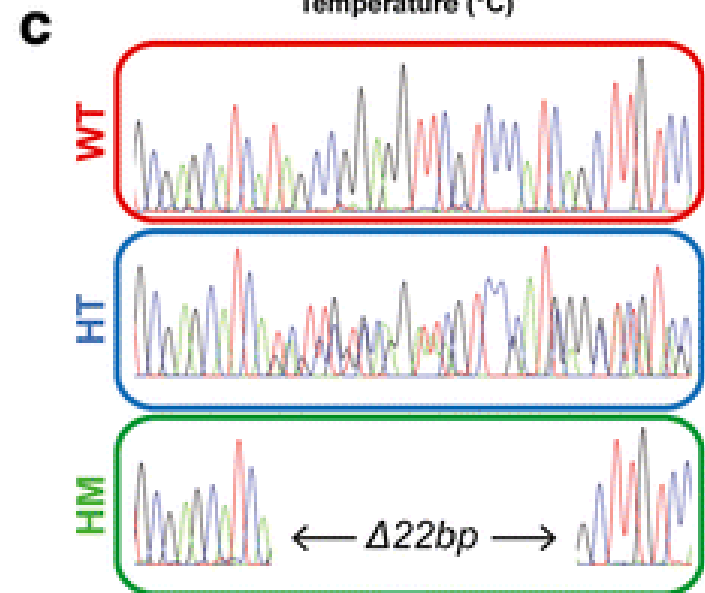
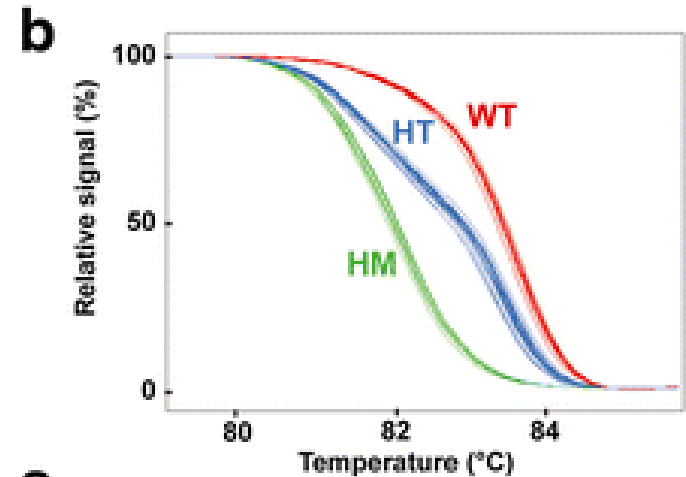
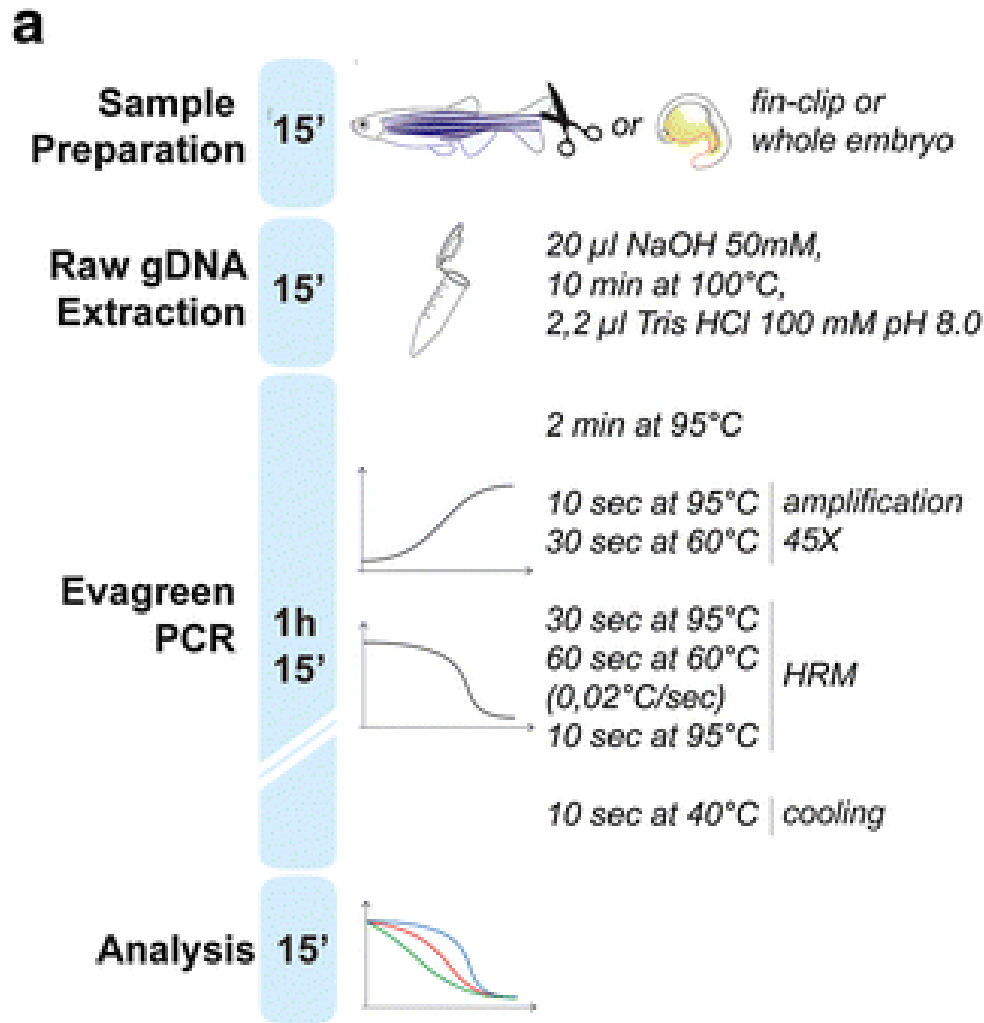
How to detect mutation?

- RFLP (Restriction fragment length polymorphism)
- HRM
- T7 endonuclease assay
- DNA sequencing

Restriction fragment length polymorphism

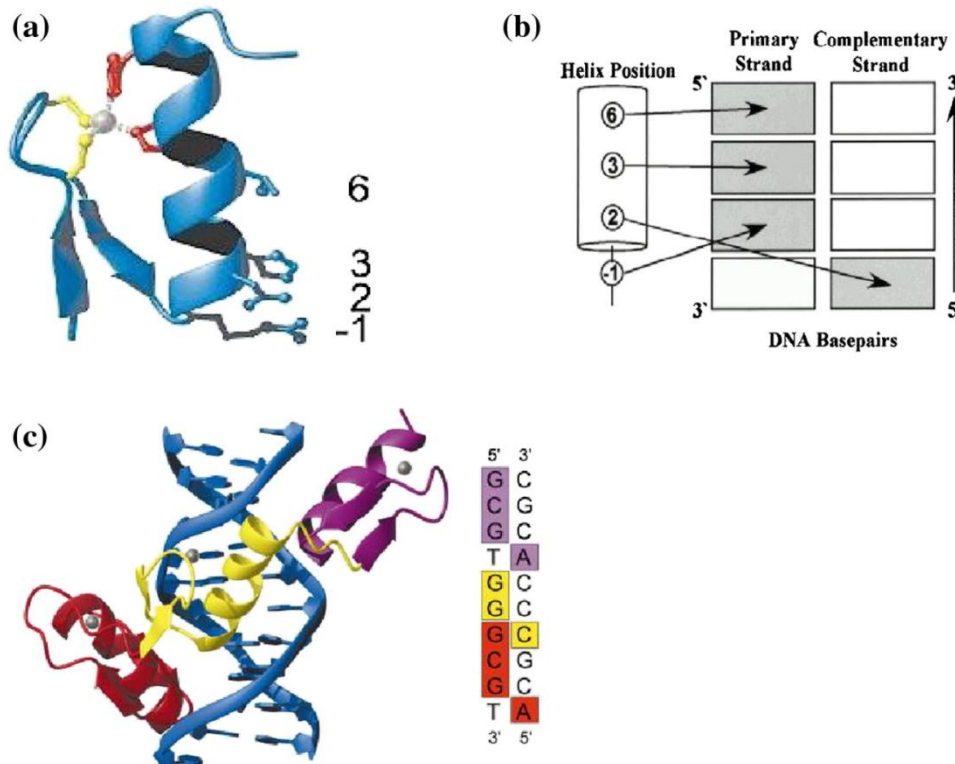


High resolution melting analysis

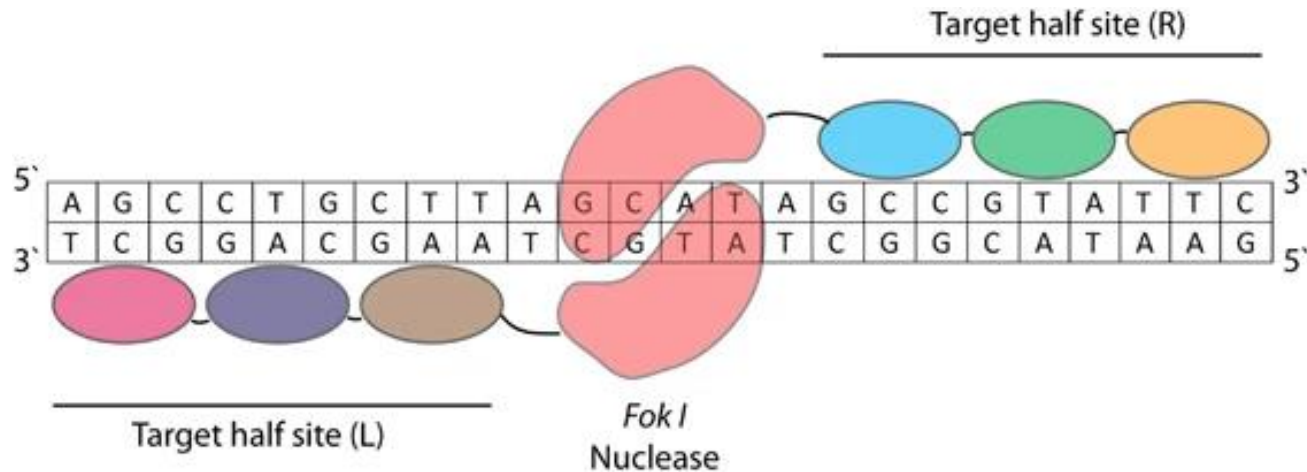


Zinc-finger nuclease

- Zinc finger - small protein structural motif
- most abundant DNA recognition domain in eukarya



Zinc-finger nuclease



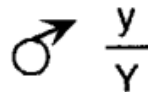
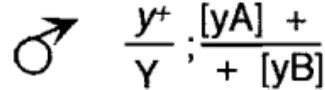
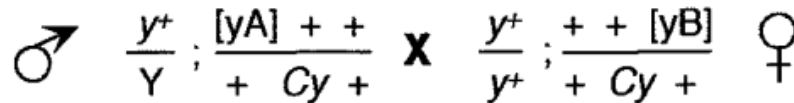
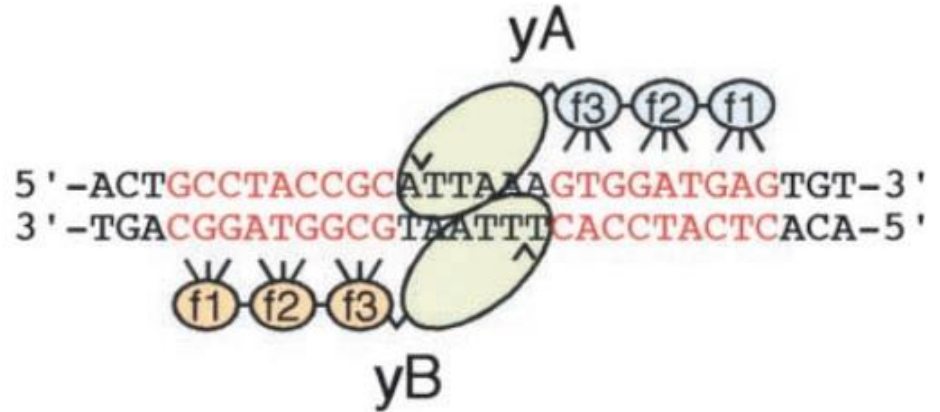
- 3 and 6 individual zinc finger motifs and bind target sites ranging from 9 basepairs to 18 basepairs in length
- Engineered zinc finger arrays are fused to a DNA cleavage domain of FokI nuclease to generate zinc finger nucleases.

ZFN in Drosophila

wt



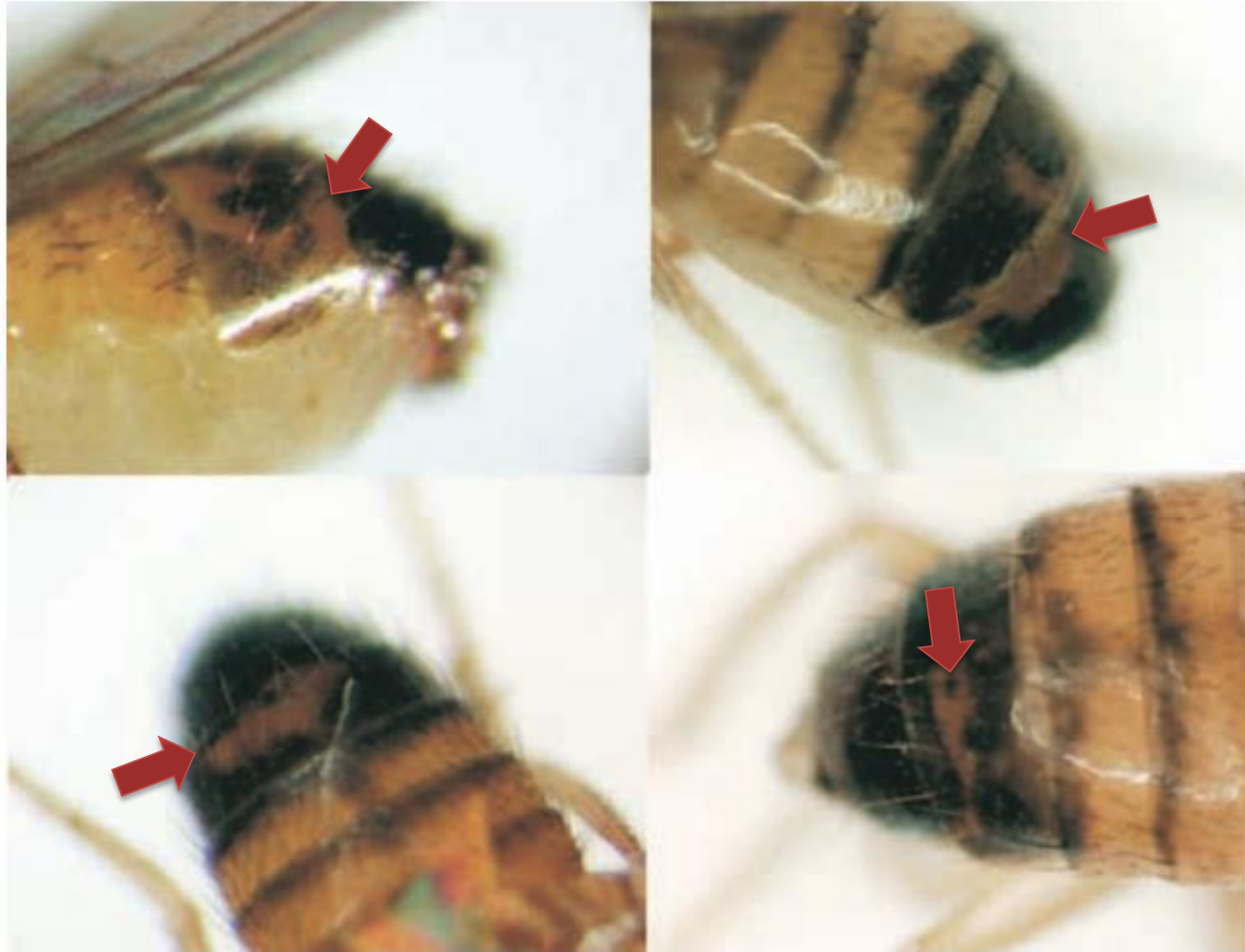
y1



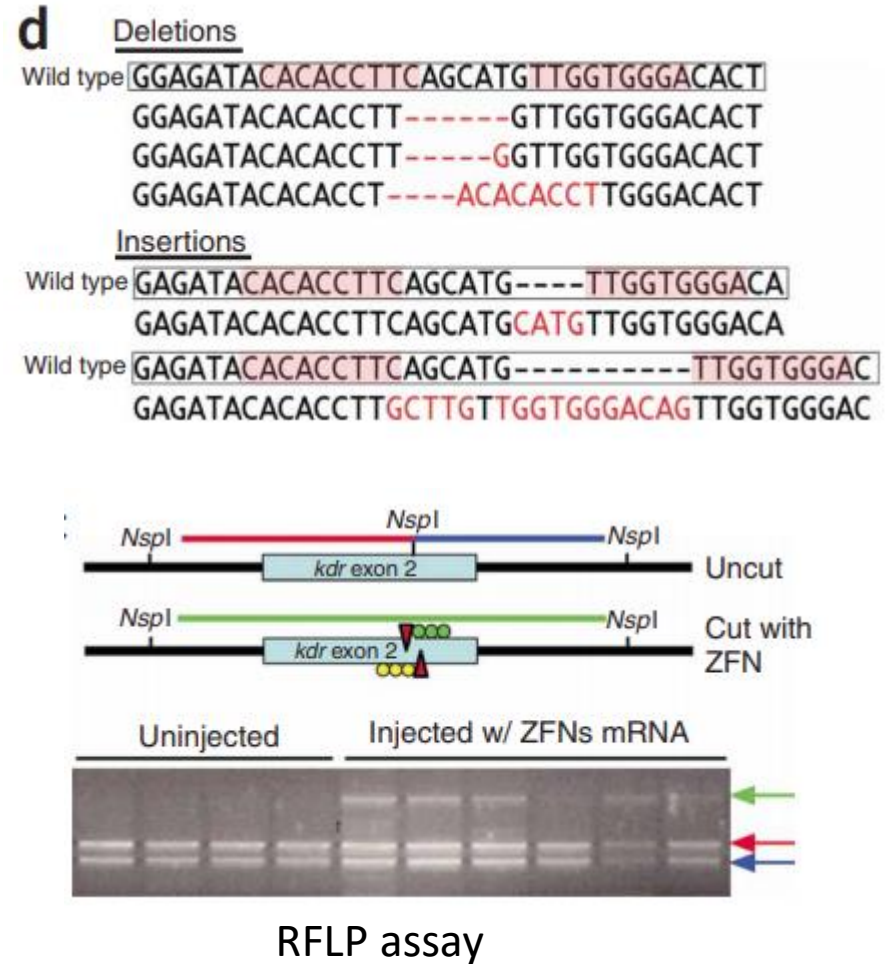
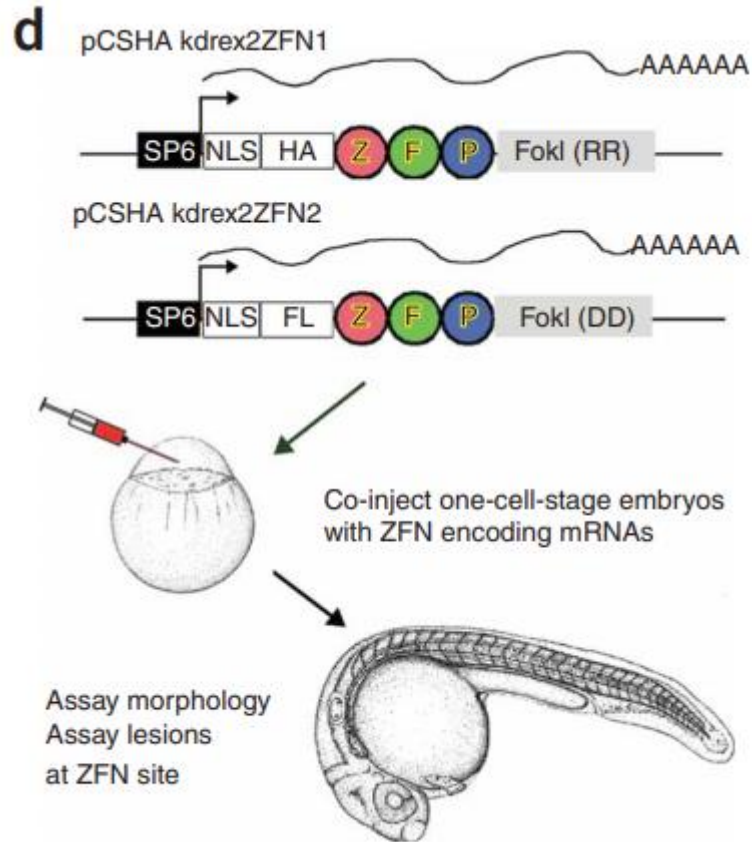
1. Heat shock larvae
2. Examine males for y clones
3. Test cross males to C(1)DX females

1. Screen males for yellow body
2. Isolate DNA; PCR; sequence

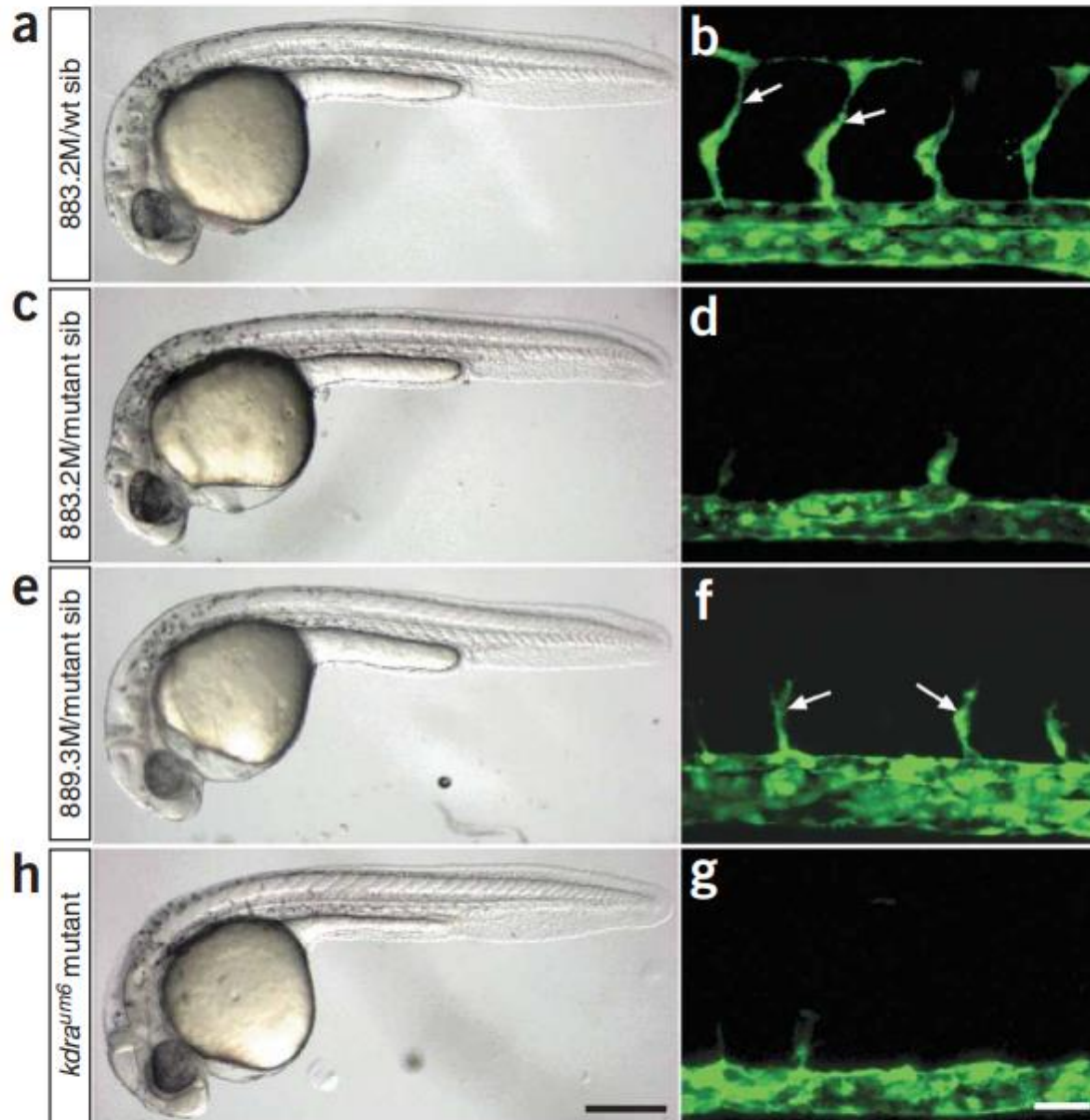
ZFN in *Drosophila*



Zinc-finger nuclease in zebrafish



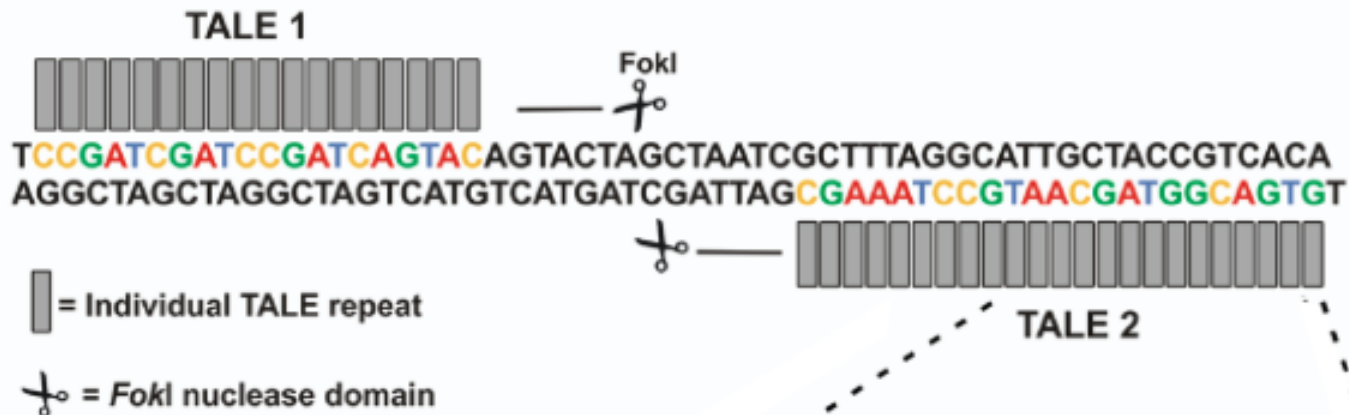
Zinc-finger nuclease in zebrafish



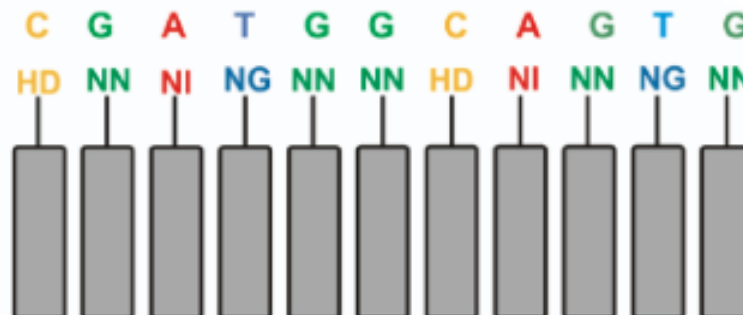
TALEN

- Transcription activator-like (TAL) effector nucleases (TALEN)
- made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (nuclease)
- TAL effector?
 - proteins secreted by *Xanthomonas* bacteria when they infect plants
 - here they enter the nucleus, bind to effector-specific promoter sequences, and activate the expression of individual plant genes, which can either benefit the bacterium or trigger host defenses

TALEN



Nucleotide - RVD (Repeat Variable Diresidues) Recognition Code	
T	NG
A	NI
C	HD
G	NN



TALEN

Applications

Knock-out

Knock-in

Advantages

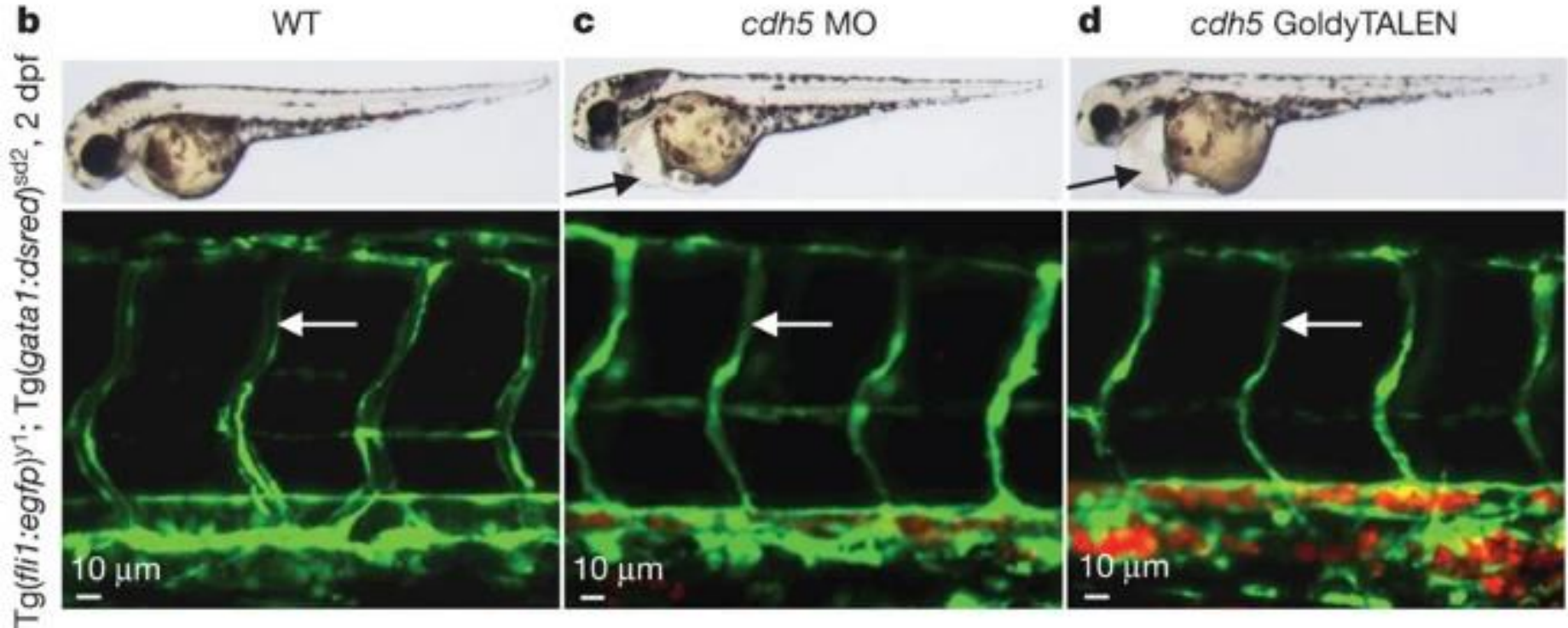
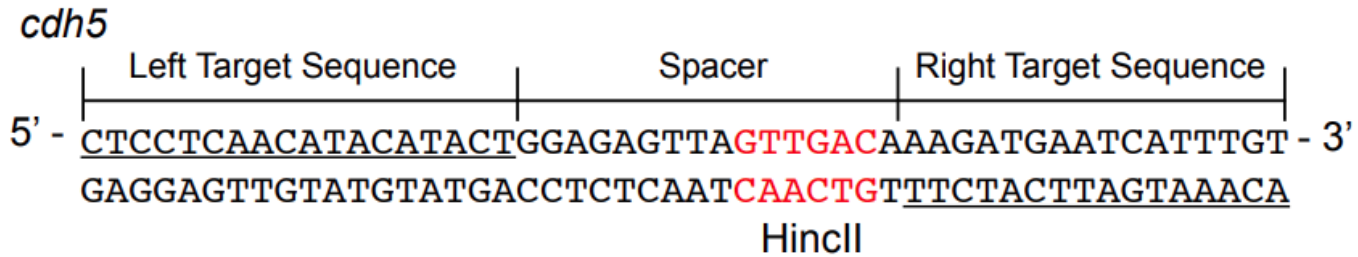
Target almost any region

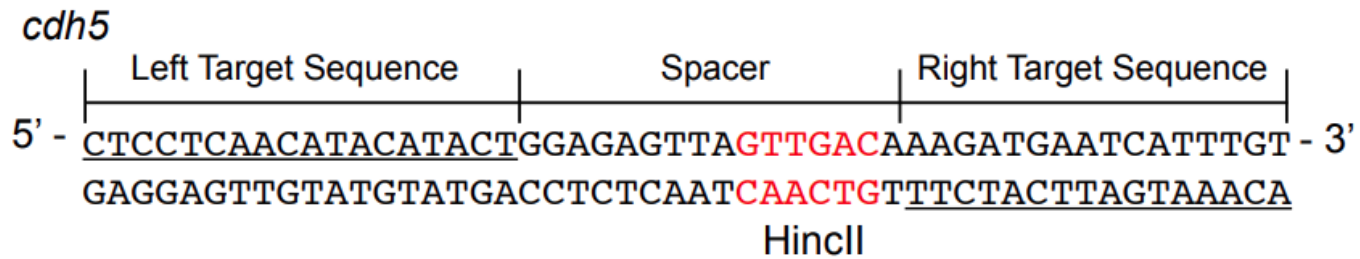
Relatively more precise

Disadvantages

Relatively complex to construct

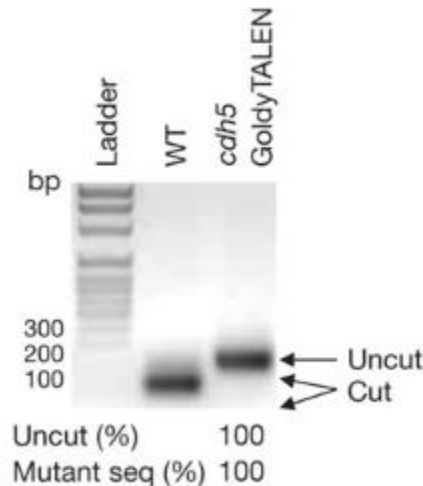
TALEN in zebrafish





d *cdh5* GoldyTALEN targeted somatic mutations n=10

- 5 - CTCCTCAACATACATACTGGAGAGTTAGTTGACAAAGATGAATCATTGT - 3' (WT)
- 5' - CTCCTCAACATACATACTGGAGAG:TA::TGACAAAGATGAATCATTGT - 3'
- 5' - CTCCTCAACATACATACTGGAGAGTATGTACAAAAGATGAATCATTGTGAC - 3'
- 5' - CTCCTCAACATACATACTGGAGAG:::::::::AAAGATGAATCATTGT - 3'
- 5' - CTCCTCAACATACATACTGGAGAG:::::::::ACAAAGATGAATCATTGT - 3' (2x)
- 5' - CTCCTCAACATACATACTGGAGAG::A::TGACAAAGATGAATCATTGT - 3'
- 5' - CTCCTCAACATACATACTGGAGAGTTAGTTGACAAAGATGAATCATTGT - 3'
- 5' - CTCCTCAACATACATACTGGA:::::::::GACAAAGATGAATCATTGT - 3'
- 5' - CTCCTCAACATACATACTGGAGA:::::::::GACAAAGATGAATCATTGT - 3'
- 5' - CTCCTCAACATACATACT:::::::::GACAAAGATGAATCATTGT - 3'

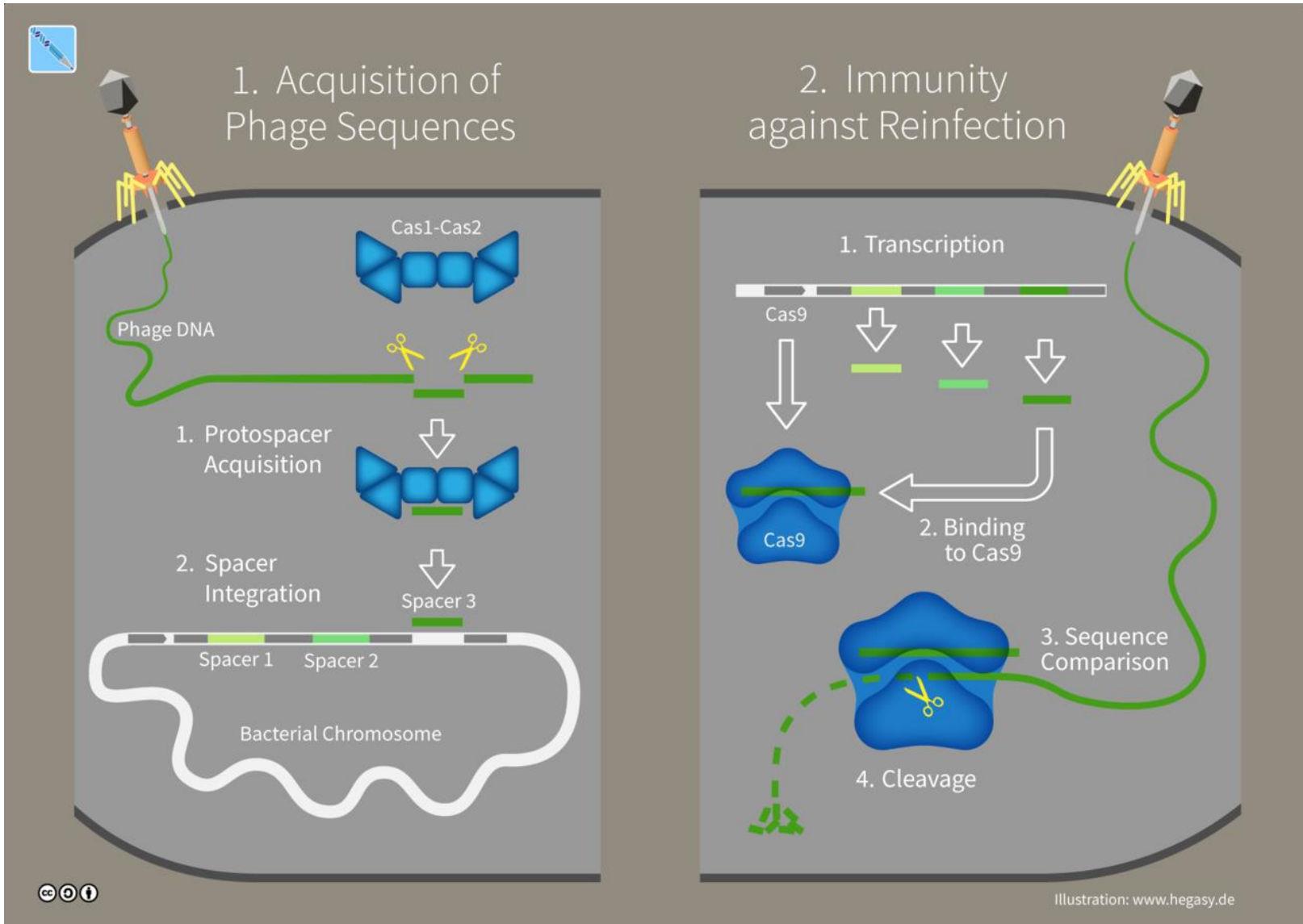


CRISPR

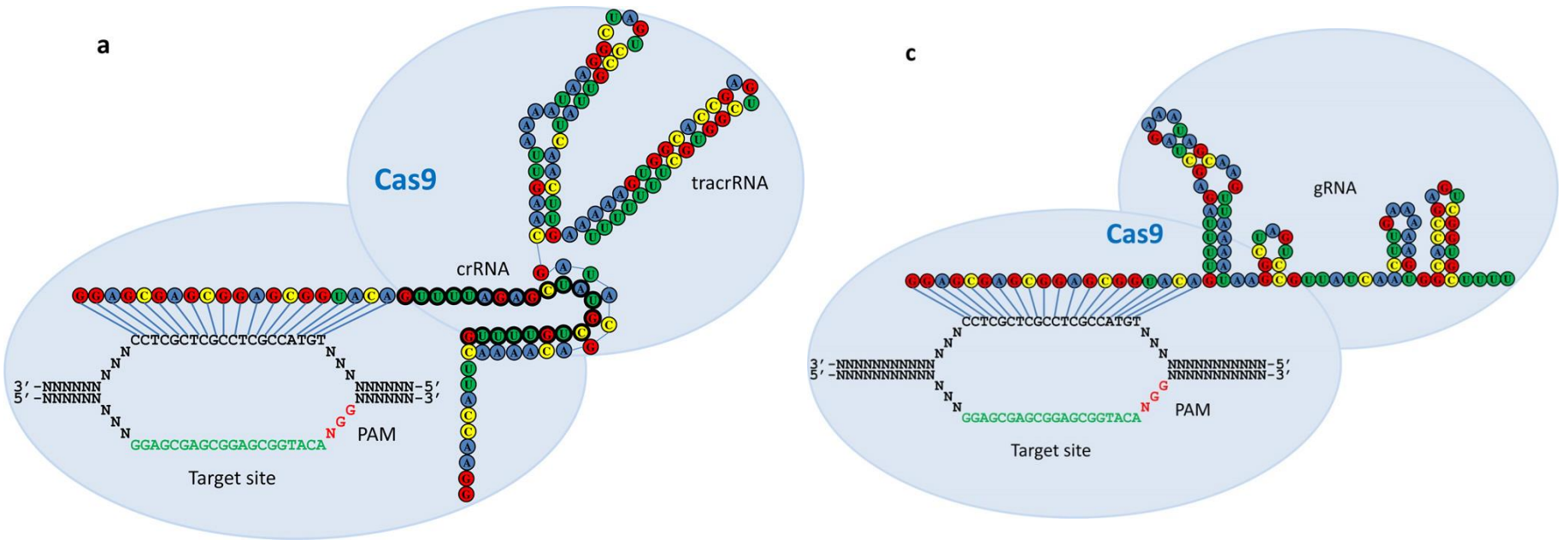
- clustered regularly interspaced short palindromic repeats (CRISPR)
- Bacterial defense mechanism against foreign DNA and viruses



CRISPR



CRISPR



CRISPR

Applications

Knockout

Knock-in

Knockdown

Lineage tracing

Advantages

Easy to construct

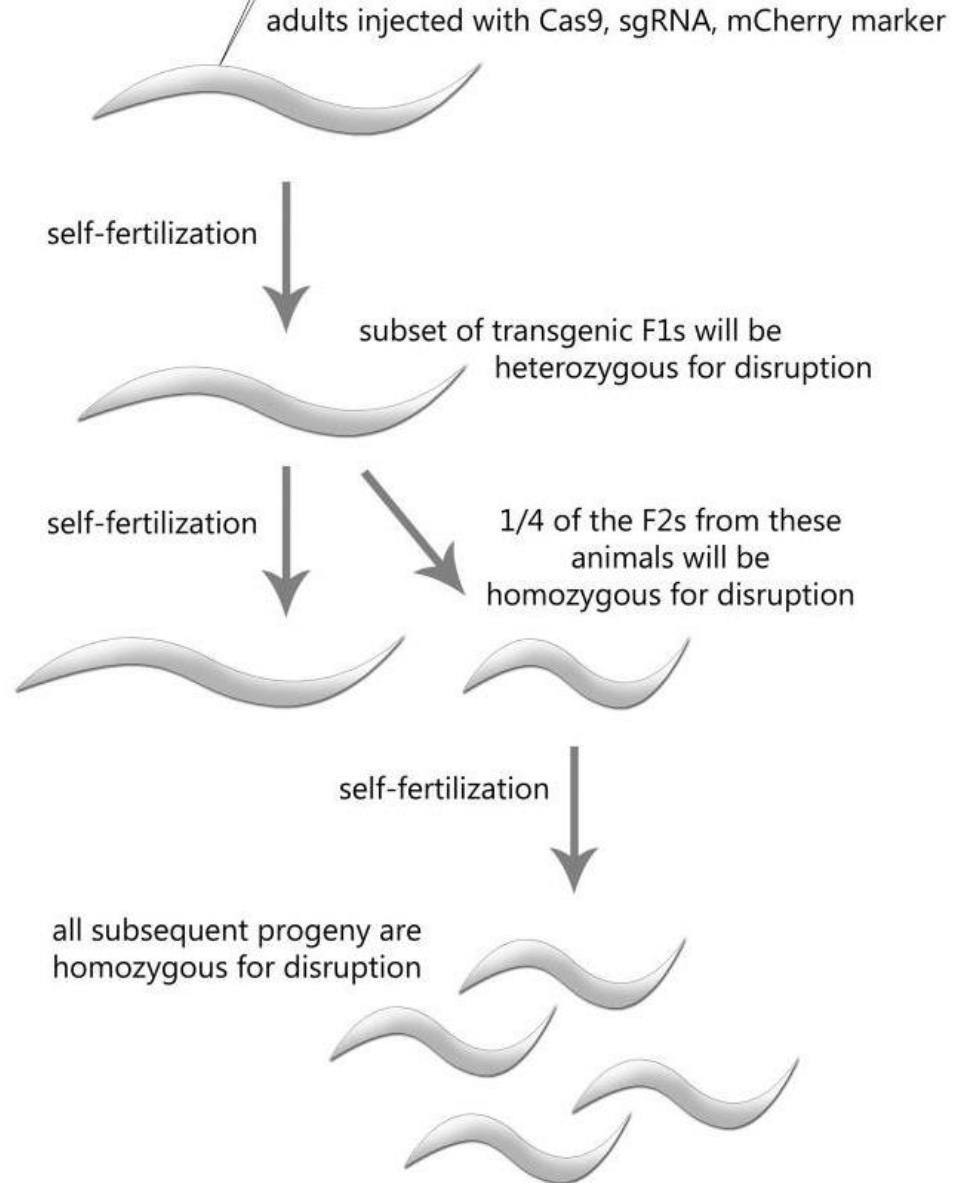
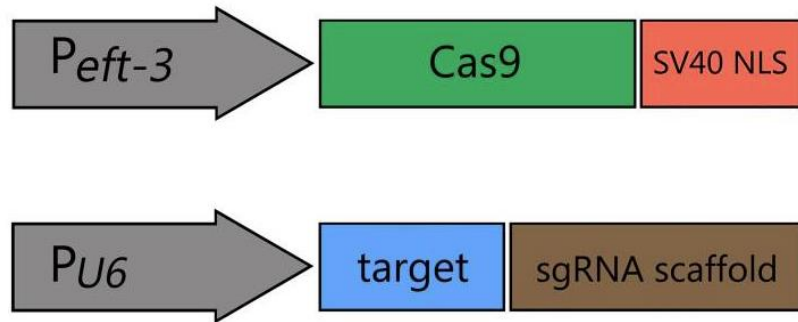
Relatively economic

Fast

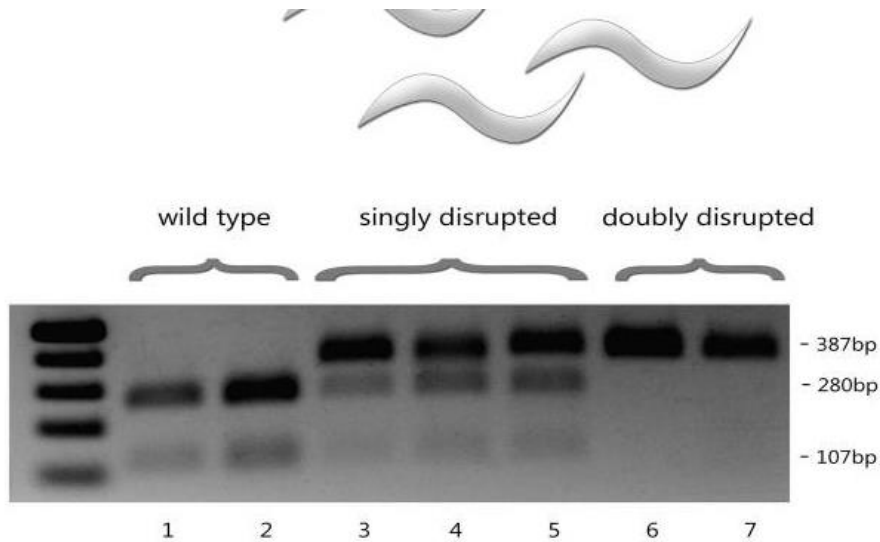
Disadvantages

Off-target targeting

CRISPR in *C. elegans*



CRISPR in *C. elegans*

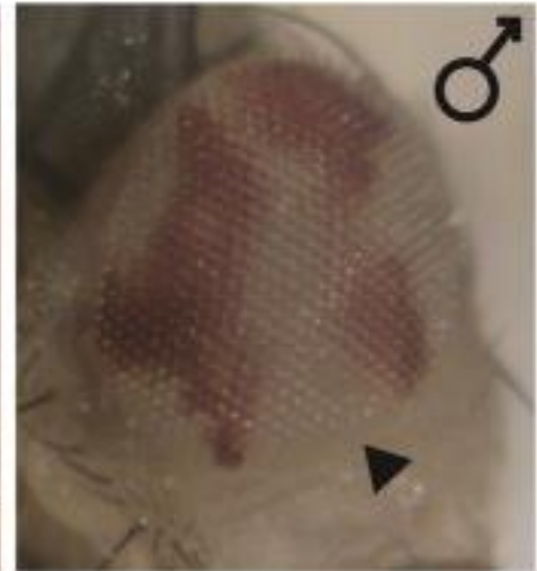
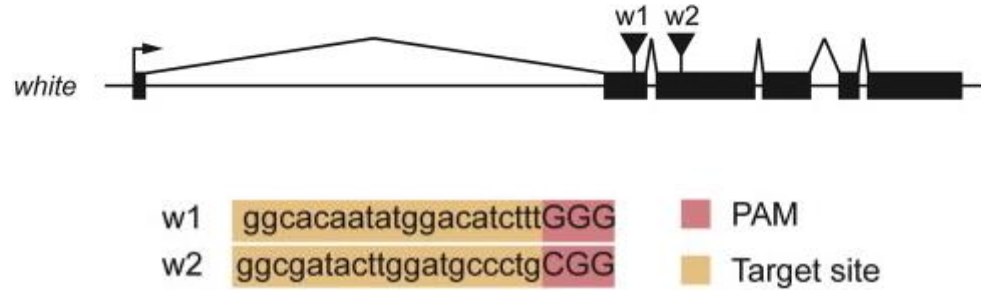


Mutations in *Y61A9LA.1*

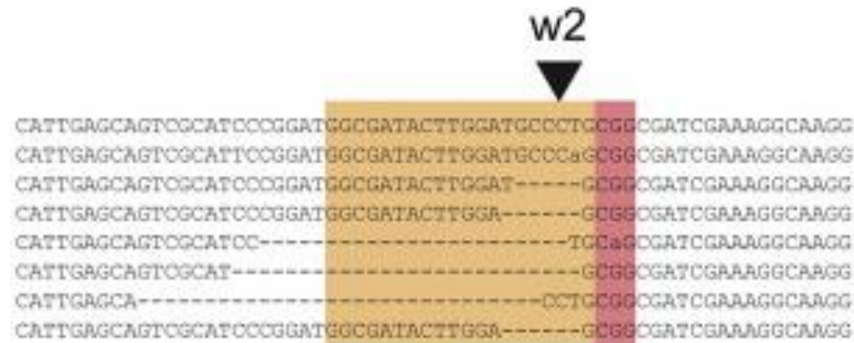
TGGATGTGTAGTCAATT**CGG**CAGGAAGCATACTGCCCTG Wild Type

TGGATGTGTA----- TCGGCAGGAAGCATACTGCCCTG -5
 TGGATGTGTAGTC**ttt**TT**CGG**CAGGAAGCATACTGCCCTG +1 (-2, +3)
 TGGATGTGTAGT---- T**CGG**CAGGAAGCATACTGCCCTG -4
 TGGATGTGTAGTC**att**TT**CGG**CAGGAAGCATACTGCCCTG +1 (-1, +2)
 TGGATGTGTAGT**att**ATT**CGG**CAGGAAGCATACTGCCCTG +1 (-2, +3)
 TGGATGTGTAG ----- AAGCATACTGCCCTG -13
 TGGATGTGTAGTC **gatggatgtgtagtc** AATT**CGG**CAGGAA +15
 TGGATGTGTAGTCA - TT**CGG**CAGGAAGCATACTGCCCTG -1
 TGGATGTGTAGT**Ctcggcatgtg**ATT**CGG**CAGGAAGCATACTGCCCTG +9 (-1, +10)
 TGGATGTGTAGT---- T**CGG**CAGGAAGCATACTGCCCTG -4
 TGGATGTGTA----- TT**CGG**CAGGAAGCATACTGCCCTG -5

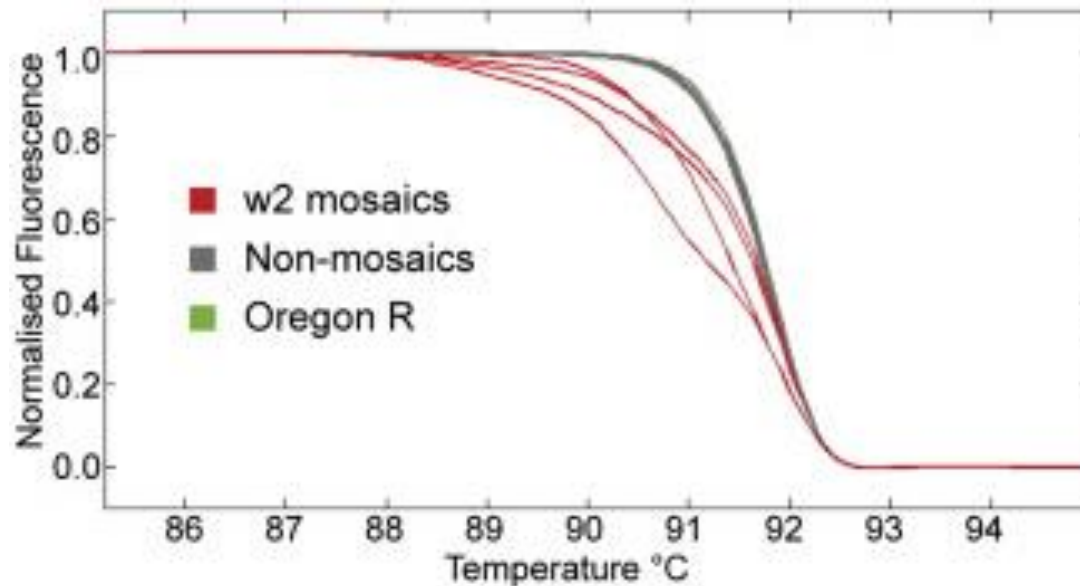
CRISPR in Drosophila



CRISPR in Drosophila

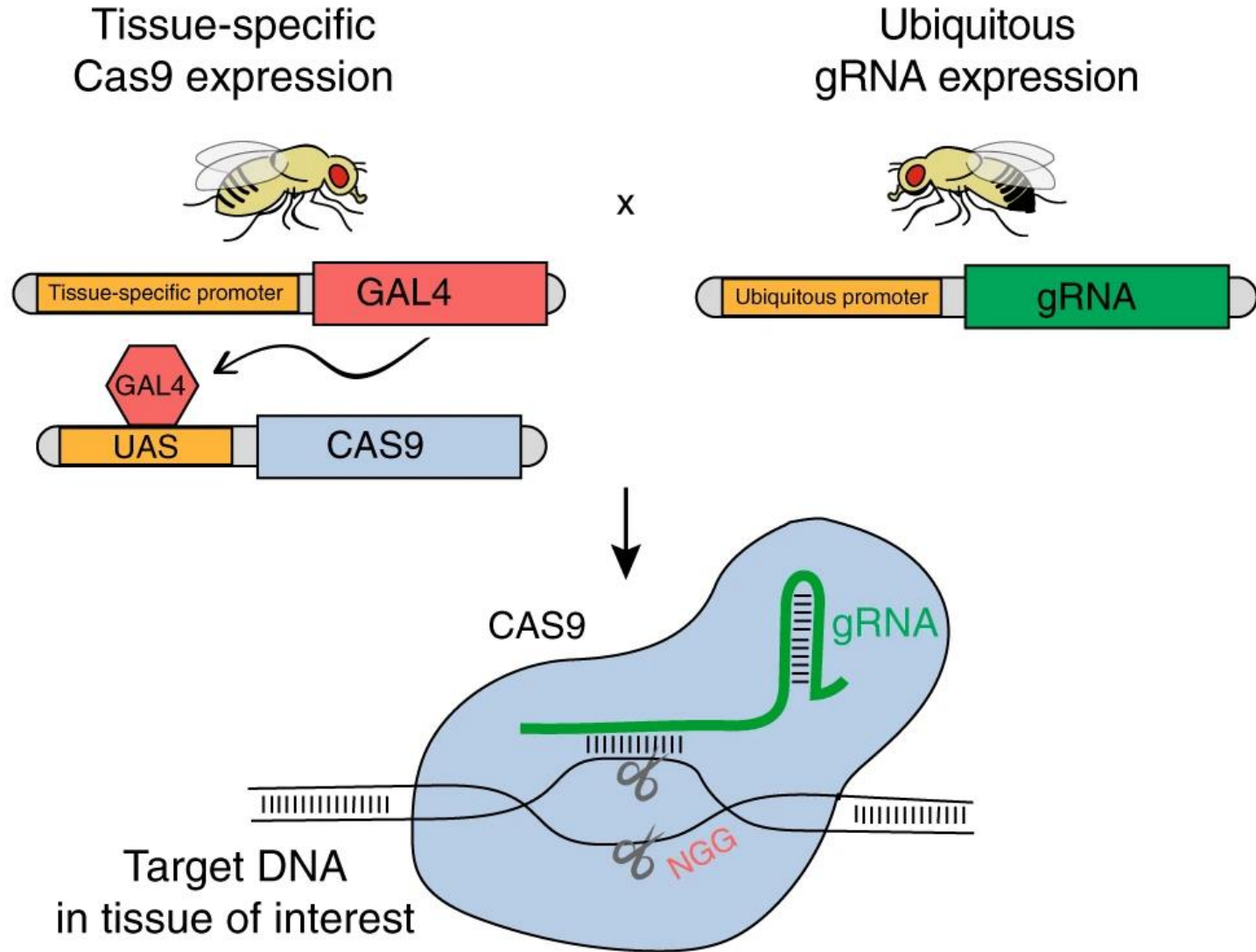


HRM analysis

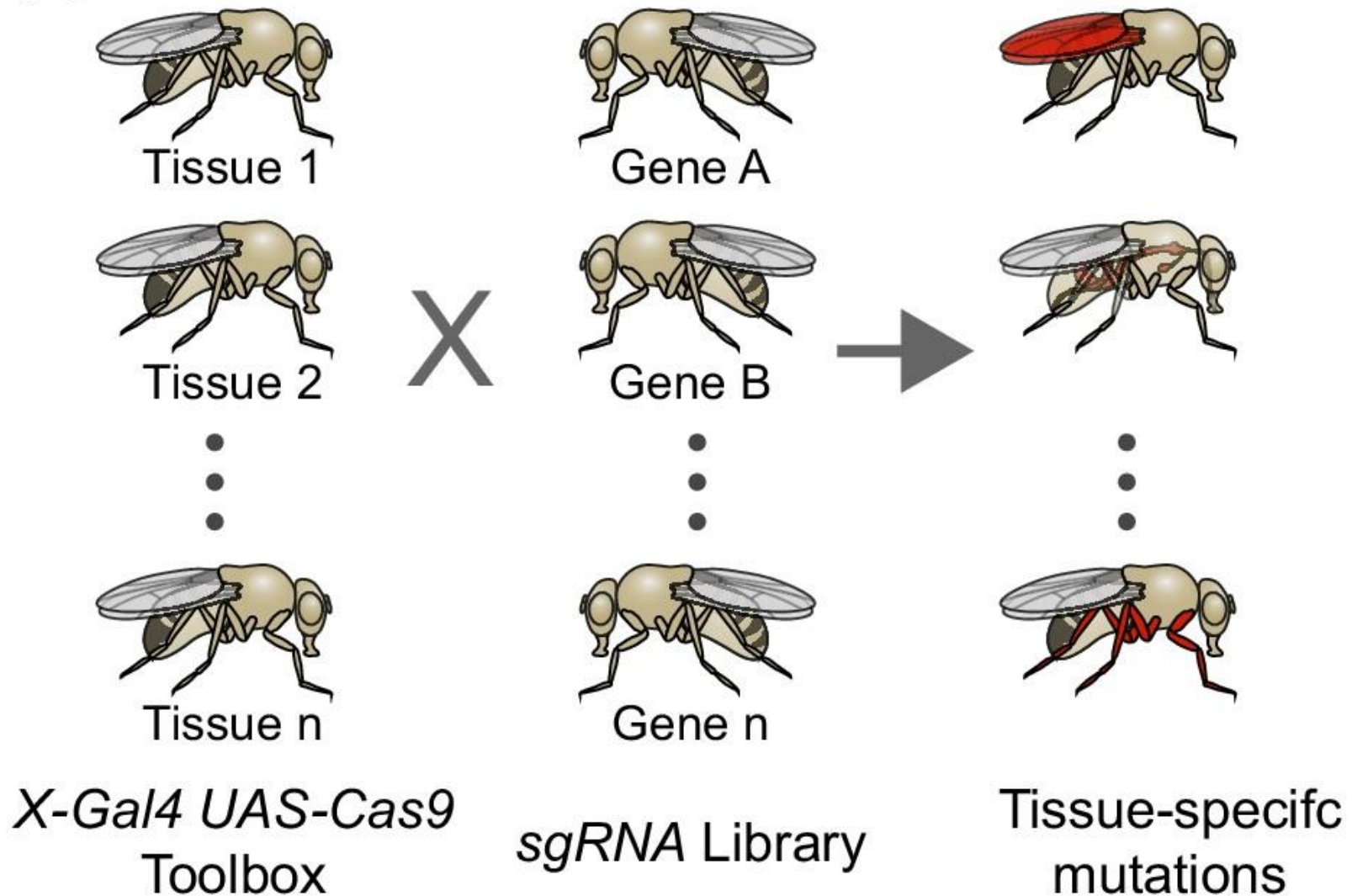


CRISPR screen in Drosophila

a

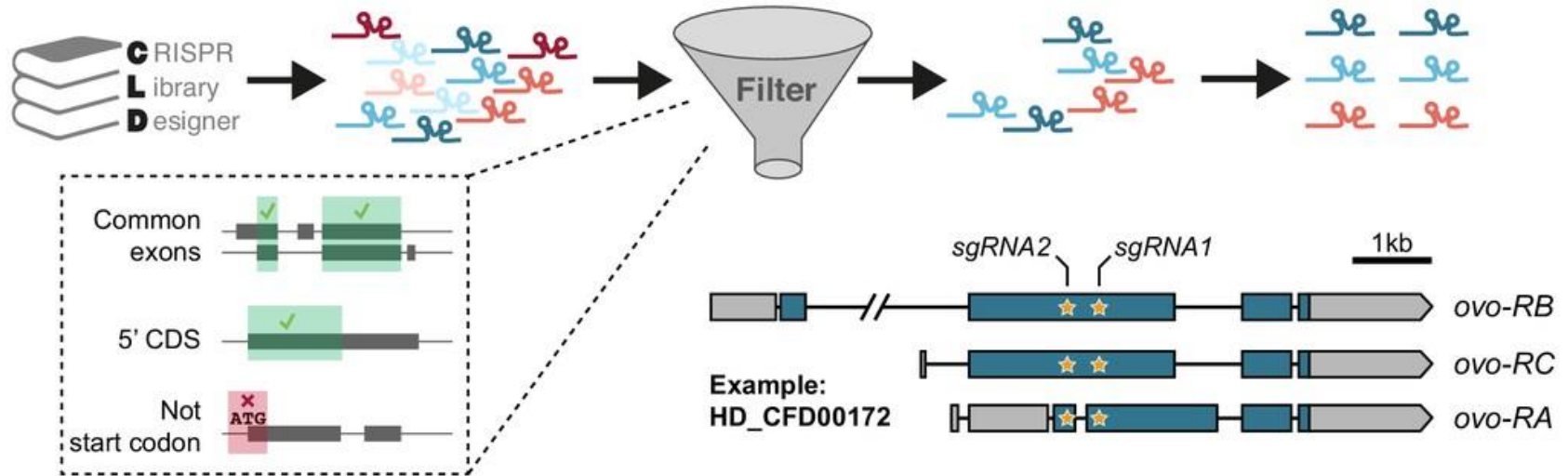


Large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila*

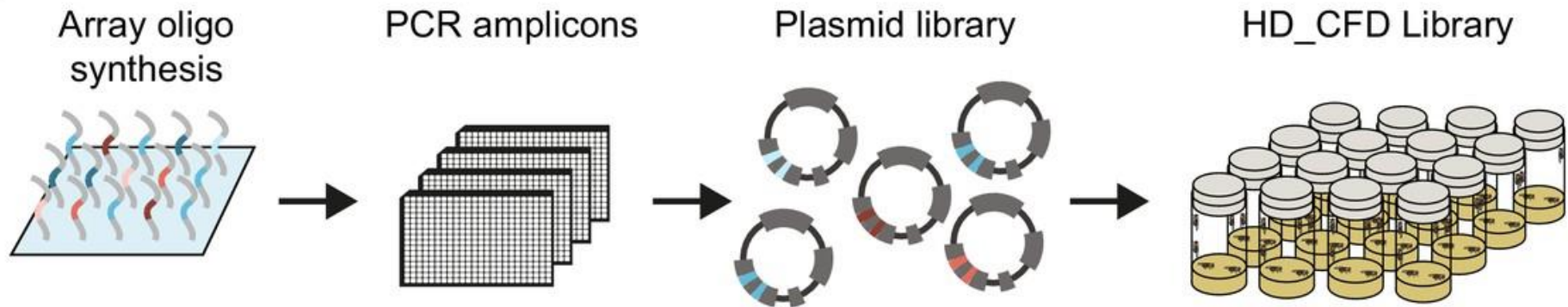


Large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila*

A



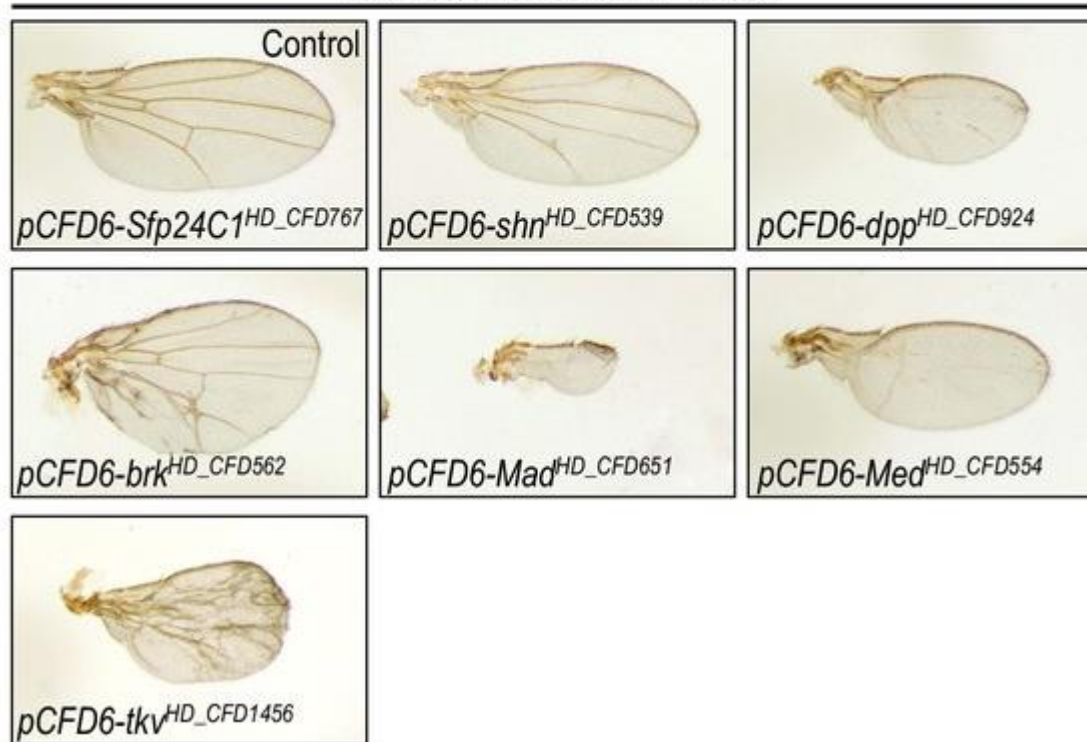
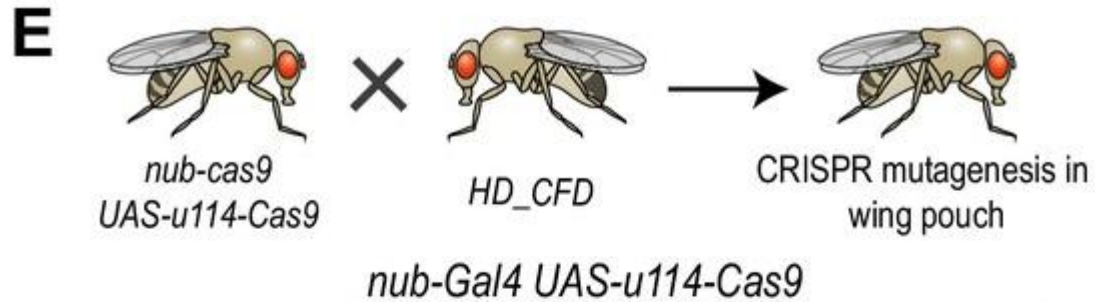
B



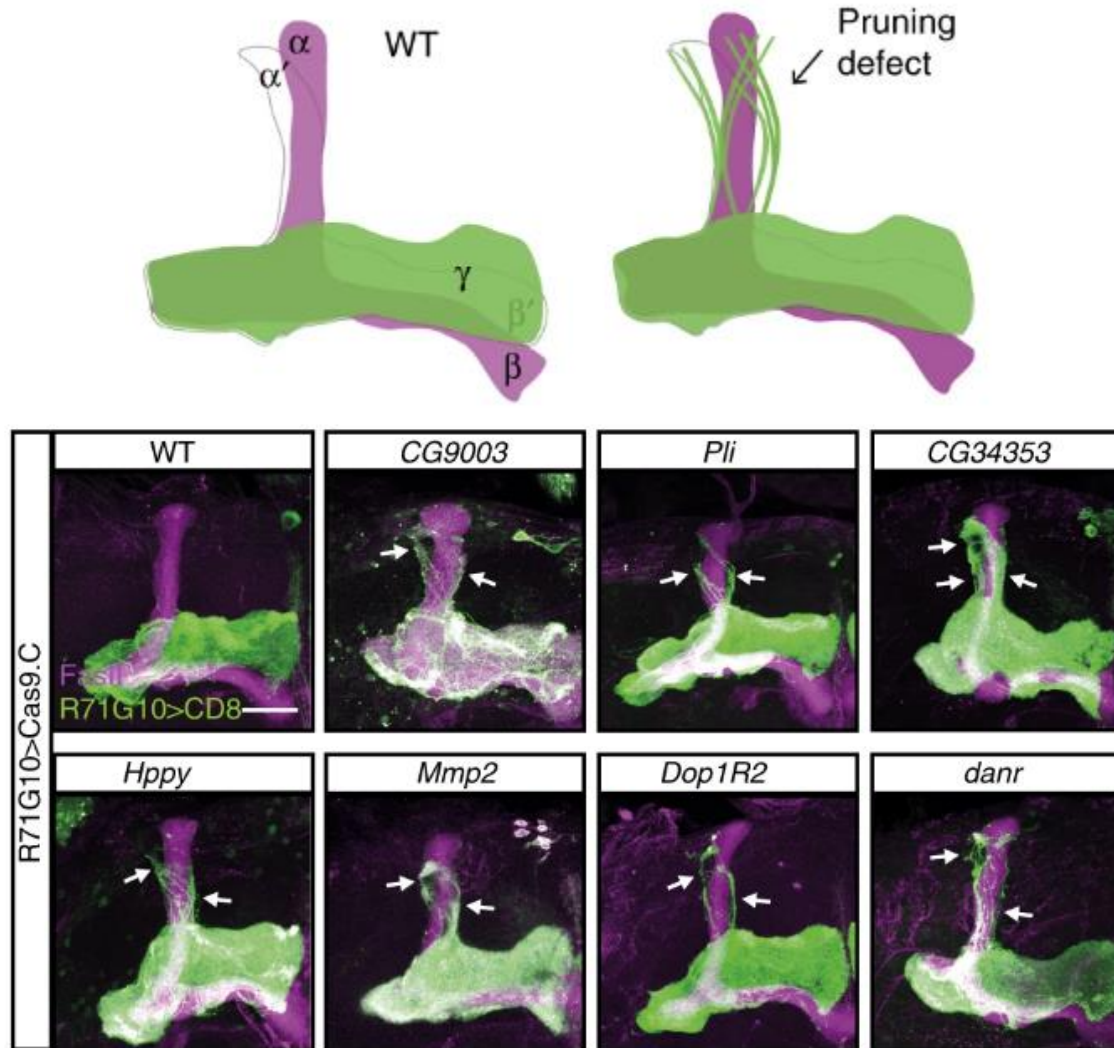
Large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila*

- Large-scale transgenic sgRNA library, - 'Heidelberg CRISPR Fly Design Library' (short HD_CFD library).
- 2622 plasmids and 1739 fly stocks targeting 1513 unique genes.
- Fly lines so far available for
 - 545/754 (72%) transcription factors,
 - 199/230 (87%) protein kinases and
 - 141/207 (68%) phosphatases

Large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila*



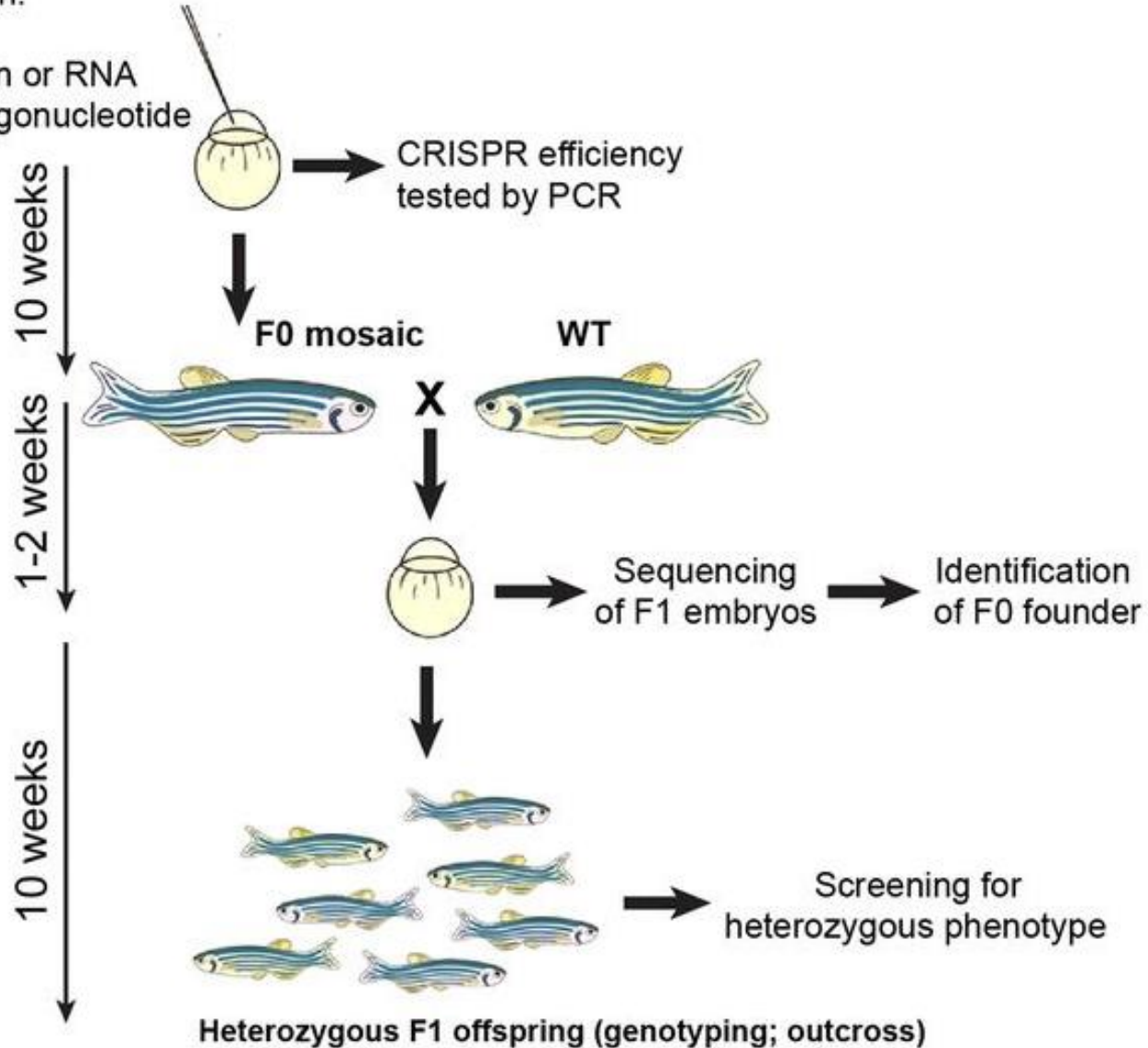
Large-scale resource for tissue-specific CRISPR mutagenesis in *Drosophila*



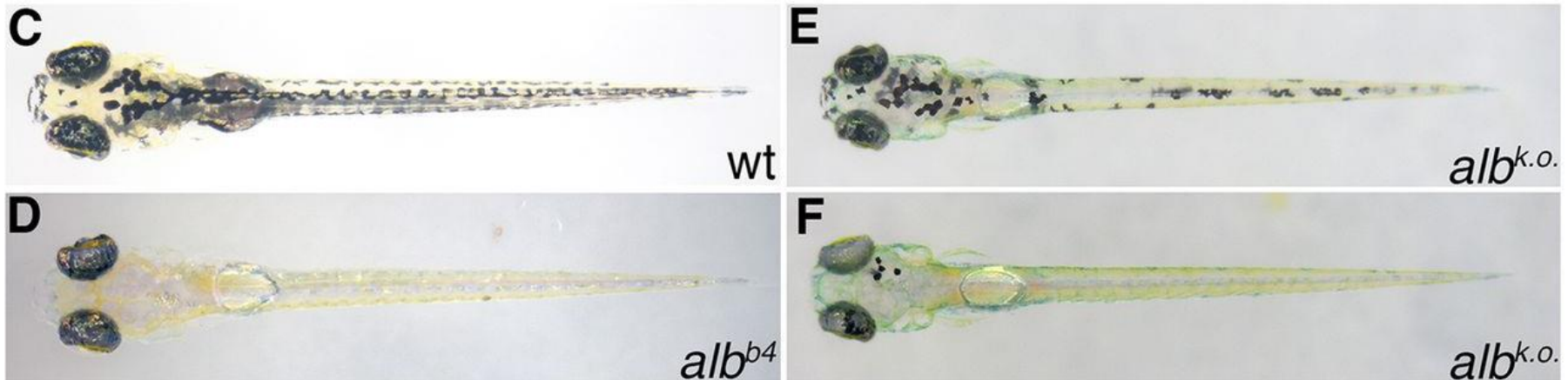
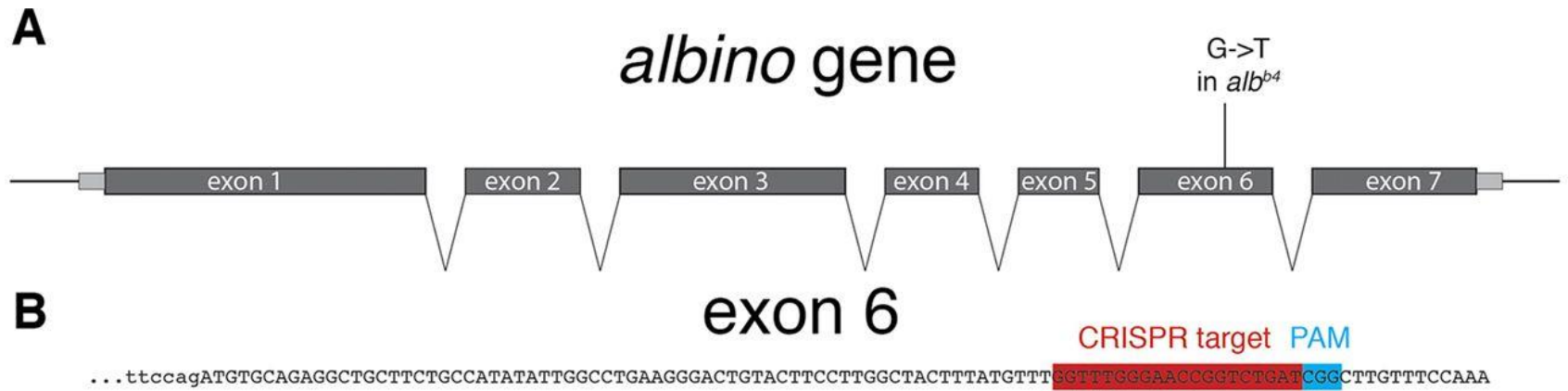
CRISPR in Zebrafish

Microinjection:

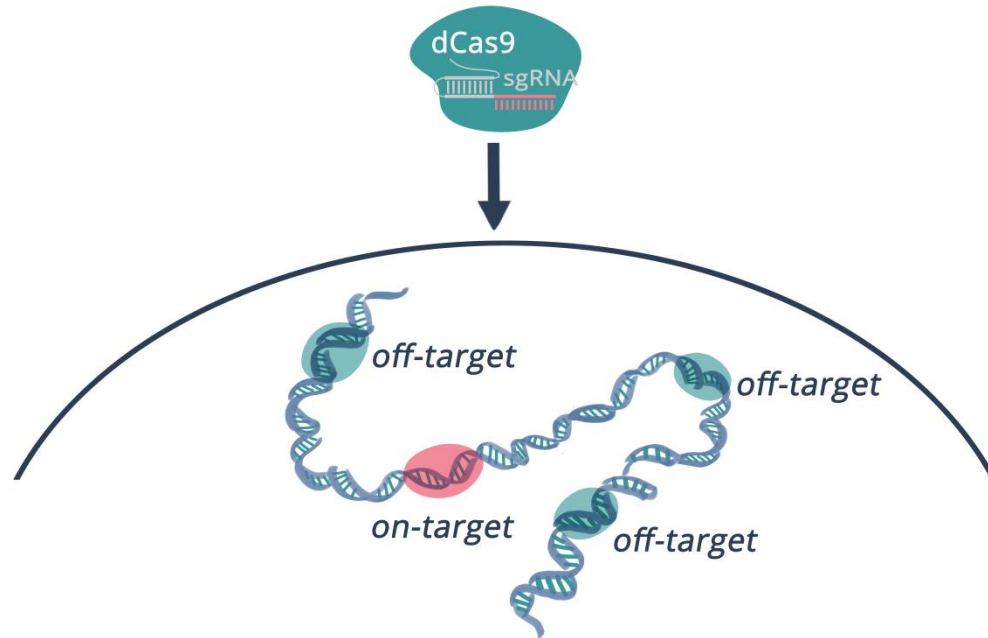
- sgRNA
- Cas9 protein or RNA
- template oligonucleotide



CRISPR in Zebrafish



Off-target



target site	gRNA motif (mismatches in red)	PAM	reads total	reads indel	% indel
C9t3 target site	GGACAGGCTGAAGACATGAT	AGG	118682	59891	50.5
off-target site 1	CGCTC GGCTGAAGACATGAT	CGG	89957	1001	1.1
off-target site 2	TAGATGC CTGAAGACATGAT	TGG	86563	1915	2.2
off-target site 3	TAGATGC CTGAAGACATGAT	TGG	440259	10336	2.3
off-target site 4	TAGCTGC CTGAAGACATGAT	TGG	68905	1729	2.5

Large-scale CRISPR mutagenesis in zebrafish

Table 1. Selection of target genes on zebrafish Chromosome 1

Gene classification	Gene counts ^a	Selected for targeting
Pseudogene	7	0
Coding gene	1202	1202
Noncoding gene: housekeeping	99	21 ^b
Noncoding gene: miRNA	31	31
Noncoding gene: lincRNA	6	6
Noncoding gene: other	73	73
Sum	1418	1333

^aBased on Zv9 release 60, 2013-01.

^bSeventy-eight rRNA genes were excluded from our project.

Large-scale CRISPR mutagenesis in zebrafish



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Search: ZKO number: Ensembl ID: Gene name:

ZKO number	Ensembl ID	Gene name	Availability
ZKO1	ENSDARG00000076045	zgc:163025	2013/12/24
ZKO2	ENSDARG00000034862	f7	2013/12/24
ZKO6	ENSDARG00000013802	pcid2	2013/12/24
ZKO8	ENSDARG00000041589	adprhl1	2013/12/17
ZKO9	ENSDARG00000041592	dcun1d2	2013/12/24
ZKO10	ENSDARG00000075108	tmco3	2013/12/24
ZKO12	ENSDARG00000058803	grk1a	2013/12/24
ZKO13	ENSDARG00000063385	cenpe	2013/12/18
ZKO14	ENSDARG00000007804	gas6	2013/12/24
ZKO16	ENSDARG00000063371	rasa3	2013/12/24

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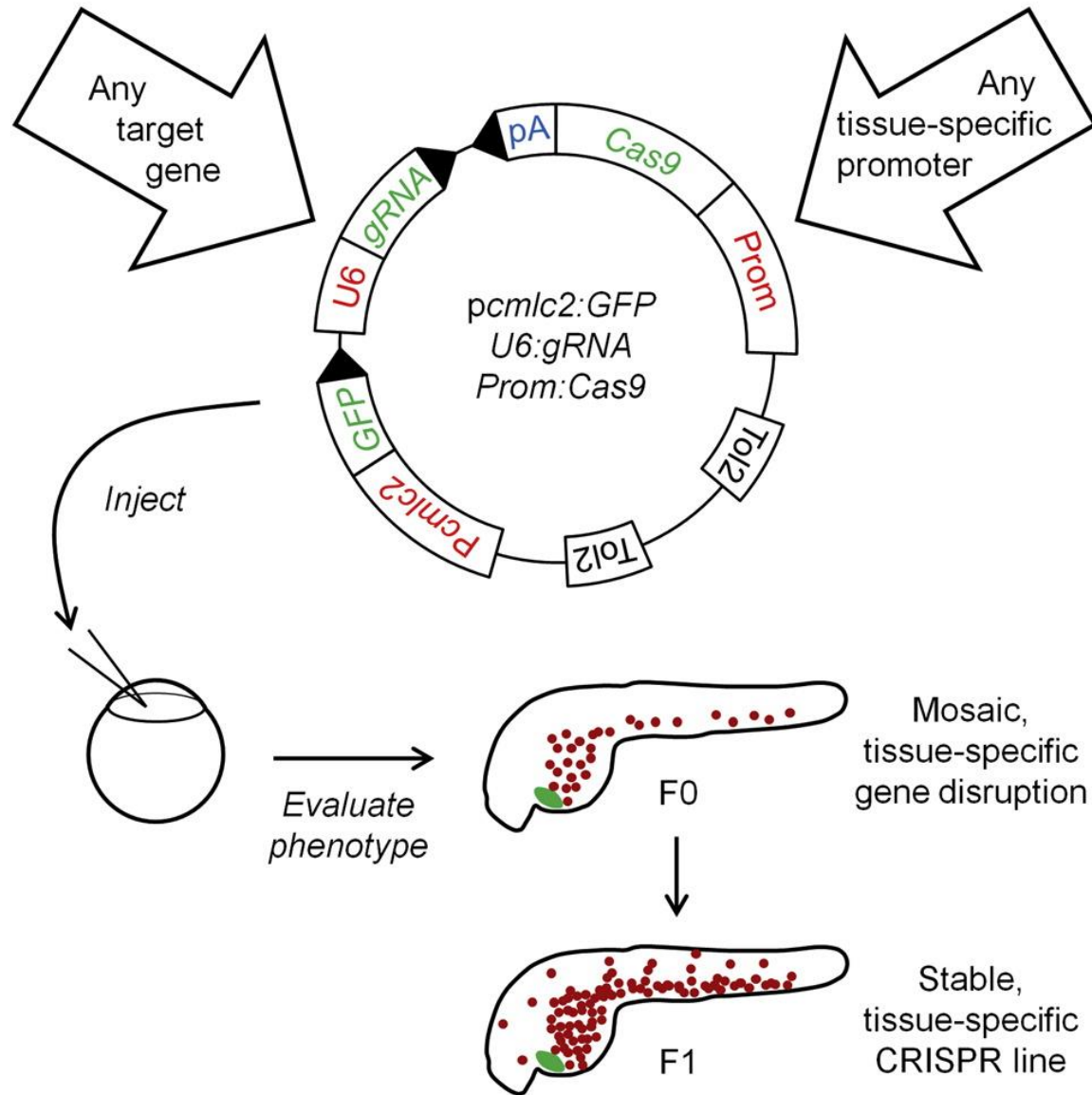
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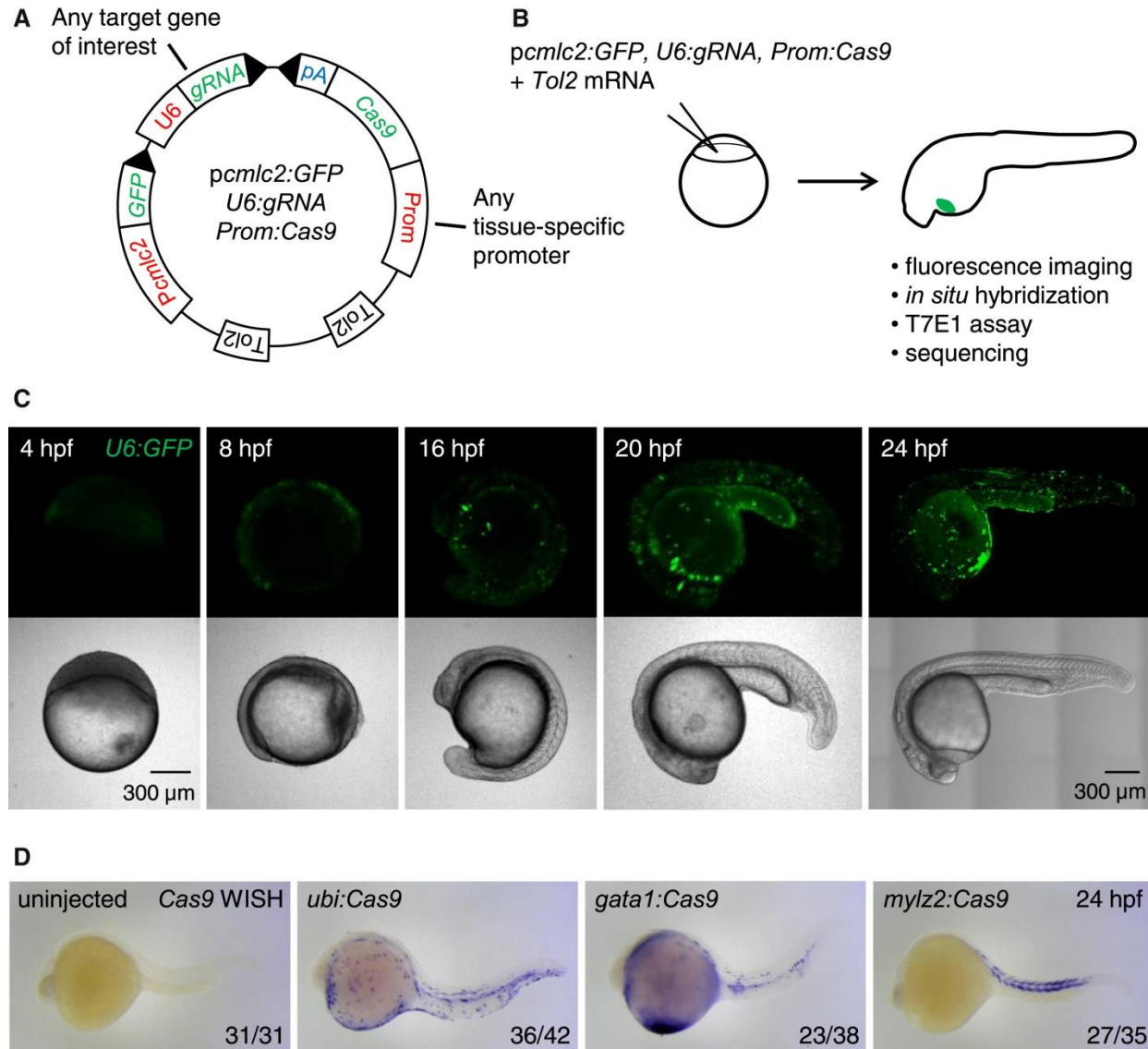
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Tissue-specific CRISPR in zebrafish



Tissue-specific CRISPR in zebrafish



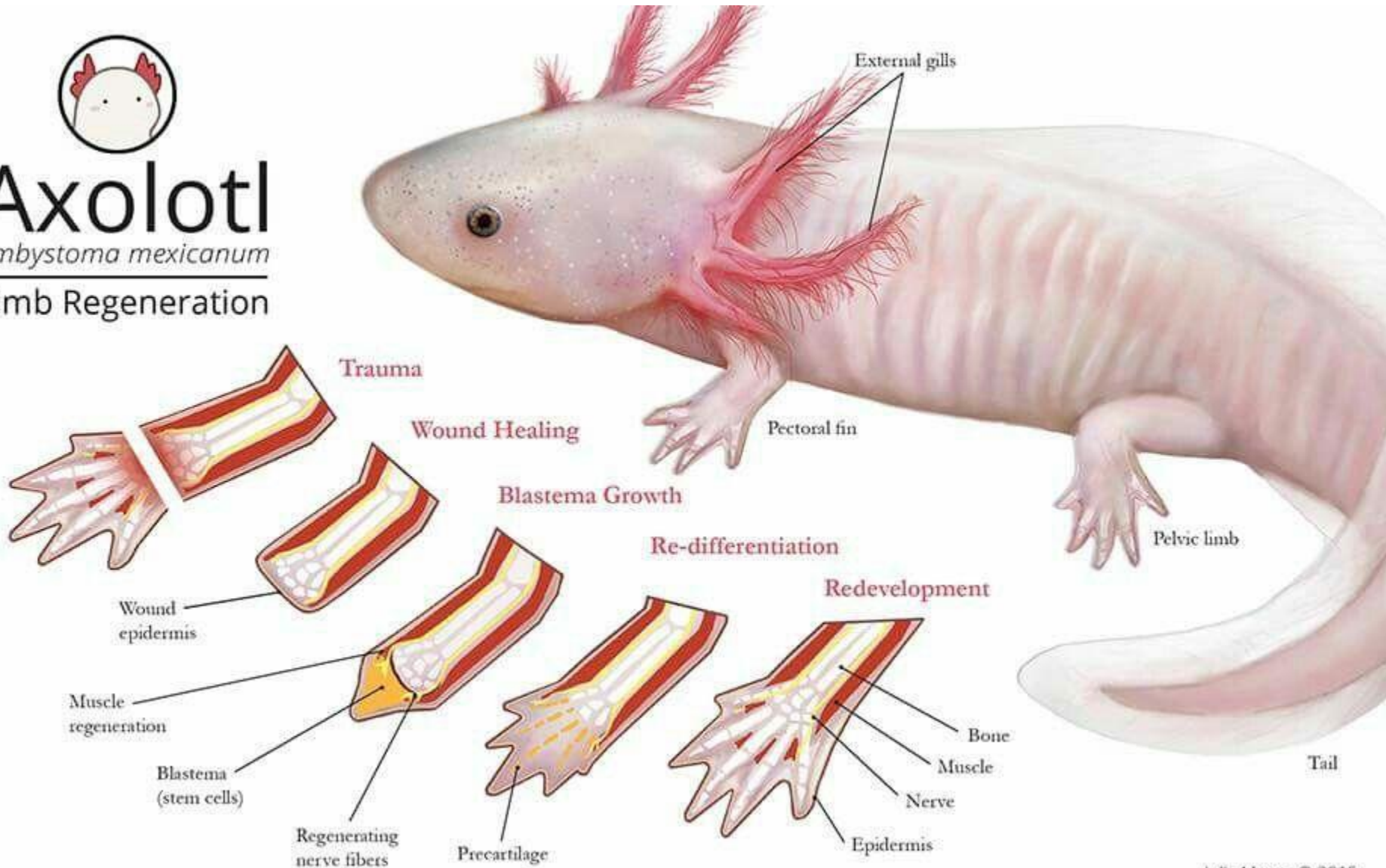
Axolotl



Axolotl

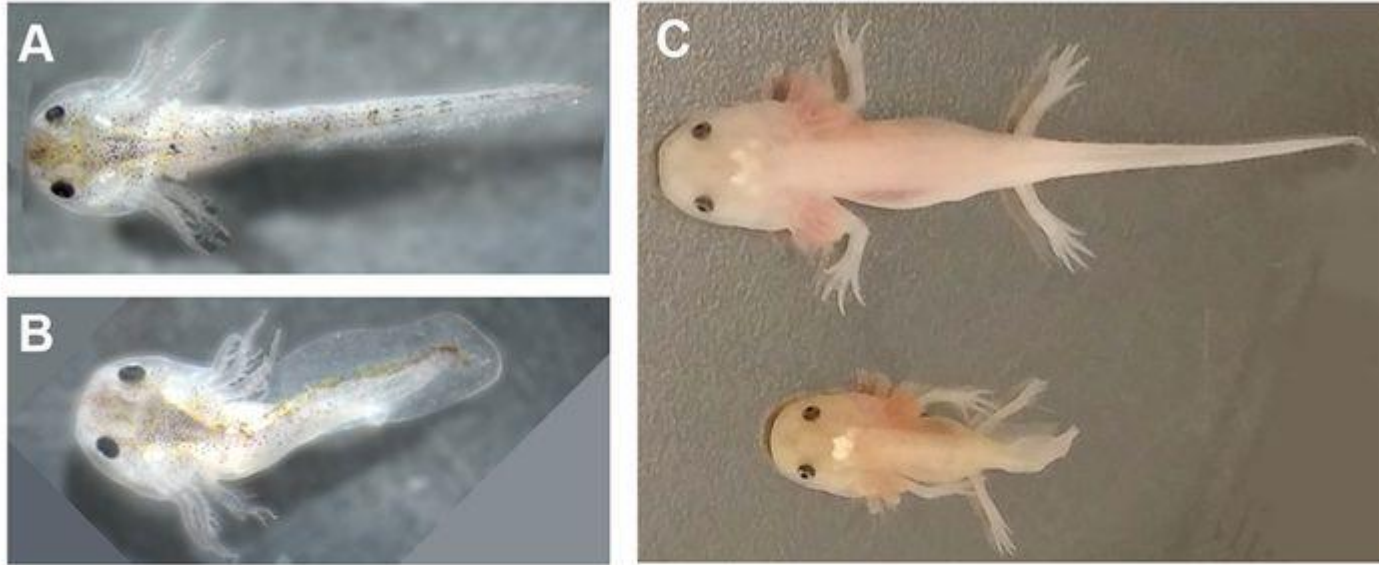
Ambystoma mexicanum

Limb Regeneration



Julia Moore © 2015

CRISPR in Axolotl

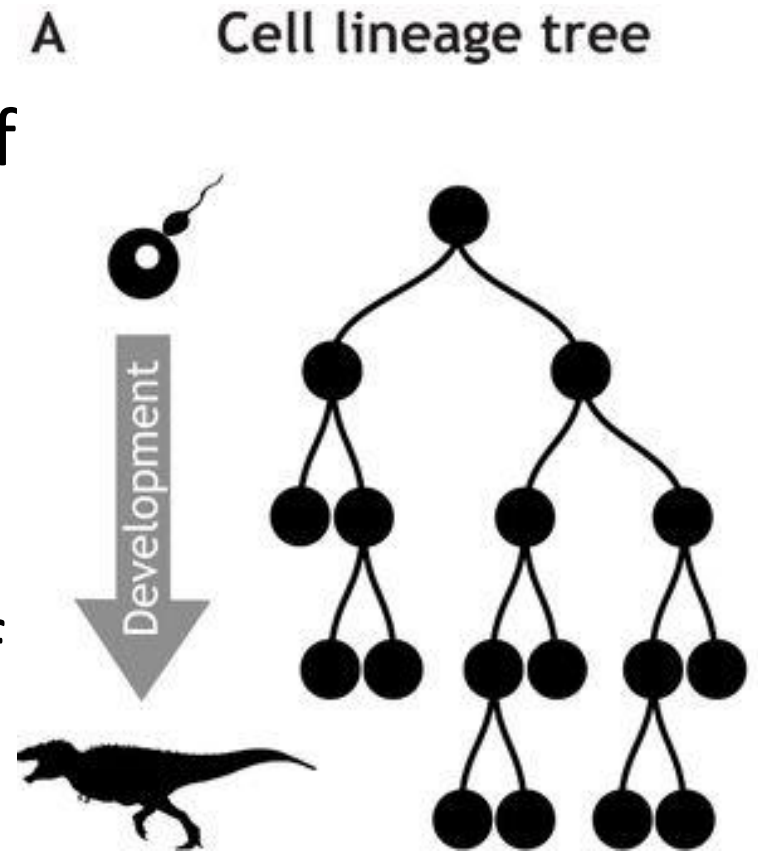


D *brachyury*, whole larva, day 21, 20 of 20 sequences mutant

ACGCTTACCCCCATAGCTGTTTCTCAGTCAGGAGGAATGCCTTCCGGTTTGGGGTCCCAGTAC	WT	X 0
ACGCTTACCCCCATAGCTGTTTCTCAGTCAGGA-----GGTCCCAGTAC	(-19)	
ACGCTTACCCCCATAGCTGTTTCTCAGTCAG-----TTTGGGGTCCCAGTAC	(-16)	X 6
ACGCTTACCCCCATAGCTGTTTCTCAGTCAGCCA-----GTTTGGGGTCCCAGTAC	(-15, +3)	
ACGCTTACCCCCATAGCTGTTTCTCAGTCAGGAGGAATGCCTT-----GGGGTCCCAGTAC	(-7)	
ACGCTTACCCCCATAGCTGTTTCTCAGTCAGGAGGAATGCCTT-----TGGGGTCCCAGTAC	(-6)	
ACGCTTACCCCCATAGCTGTTTCTCAGTCAGGAGGAATGCCTTCCGG-----GGTCCCAGTAC	(-5)	X 3
ACGCTTACCCCCATAGCTGTTTCTCAGTCAGGAGGAATGCCTTCCGG---GGGGTCCCAGTAC	(-3)	X 7

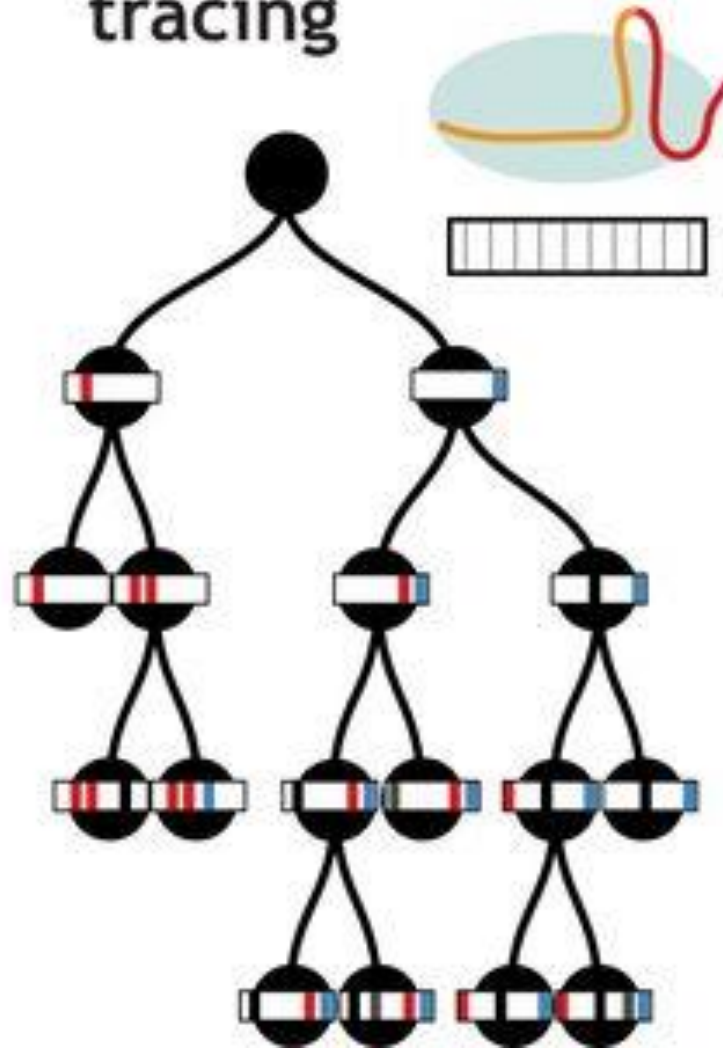
CRISPR in lineage analysis

- Lineage tracing is the identification of all progeny of a single cell.
- Lineage tracing is an essential tool for studying stem cell properties in adult tissues.
- Provides a powerful means of understanding tissue development, homeostasis, and disease

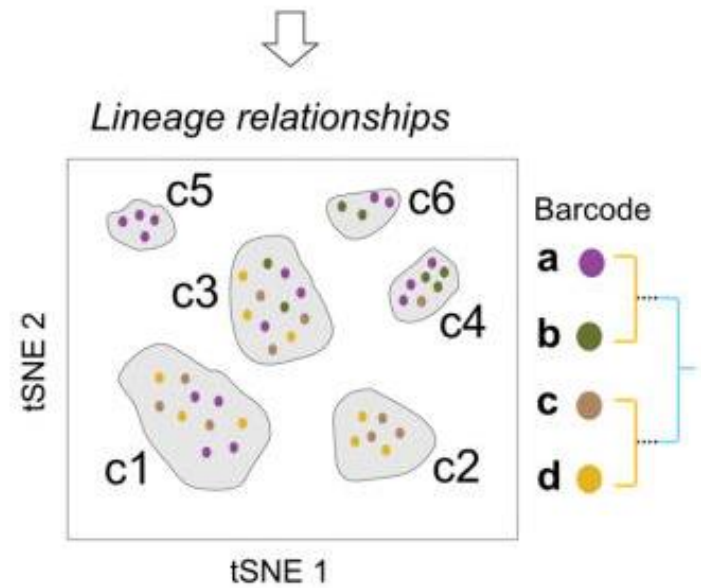
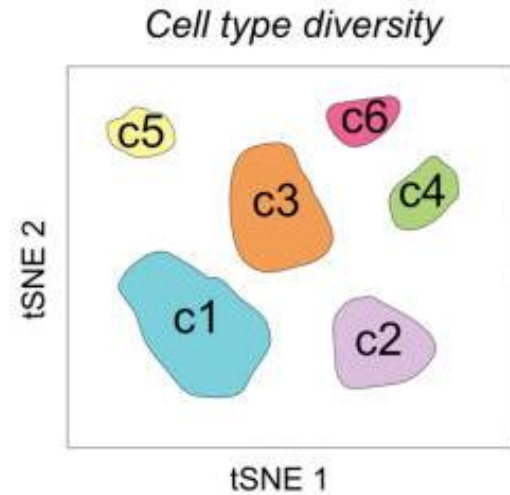
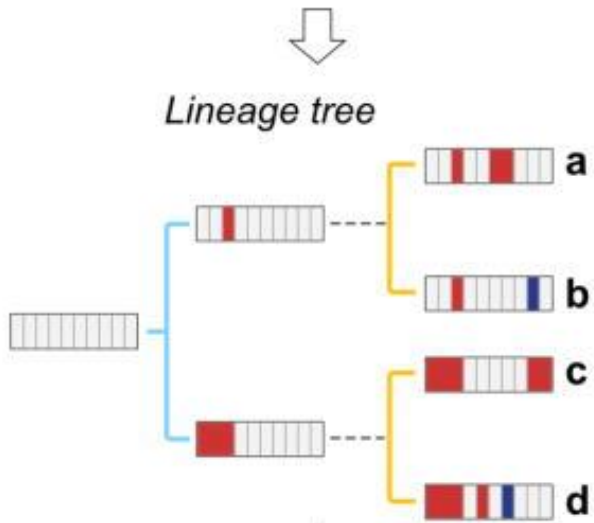
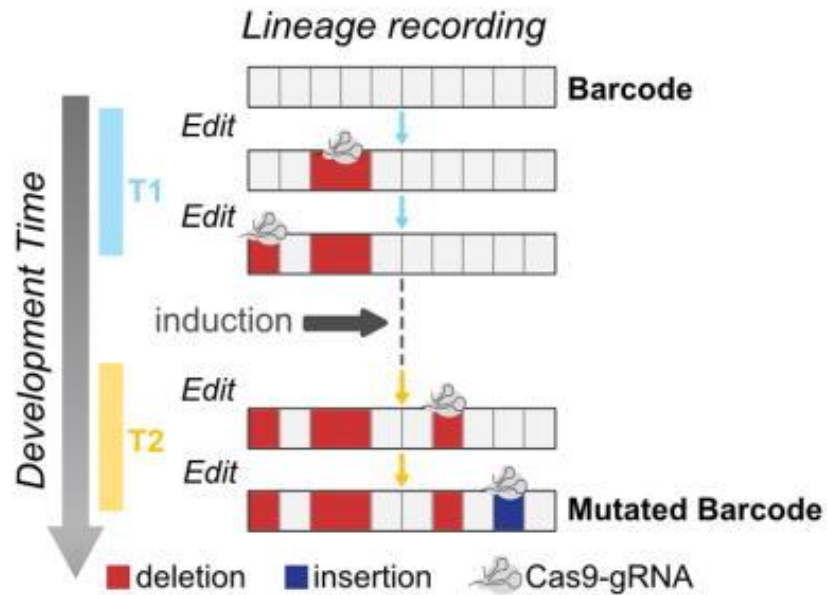


CRISPR in lineage analysis

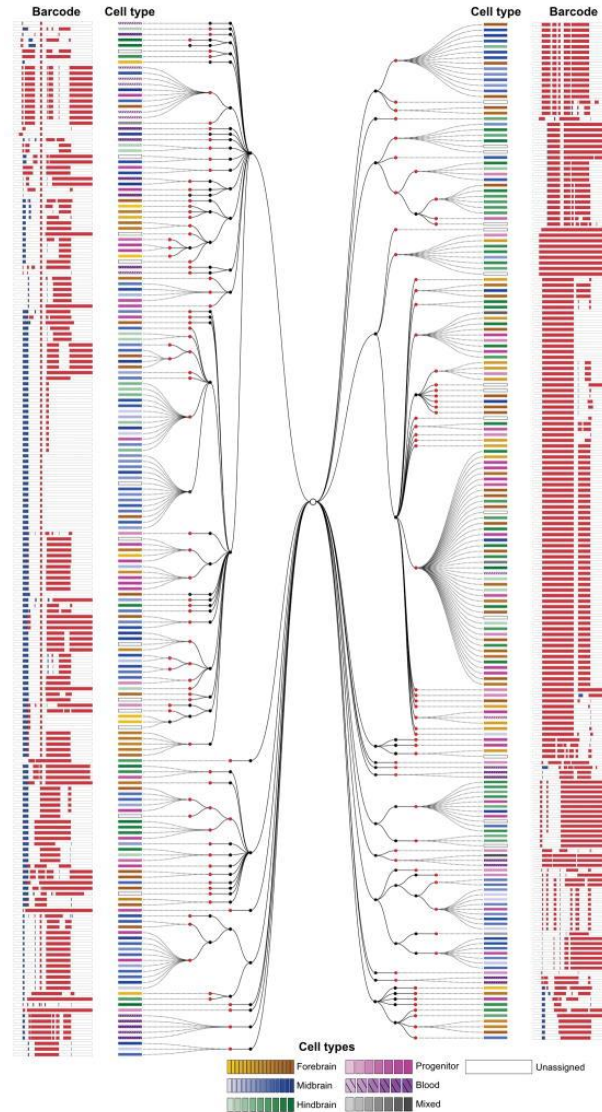
E Dynamic lineage tracing



CRISPR in lineage analysis



CRISPR in lineage analysis



RNA perturbation

RNA perturbation:

Technique to disrupt or degrade target gene mRNA

Major RNA perturbation techniques*

- RNAi
- Morpholino
- CRISPRi

RNA interference (RNAi)



Photo: L. Cicero
Andrew Z. Fire

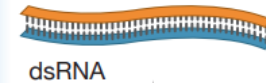


Photo: J. Mottern
Craig C. Mello



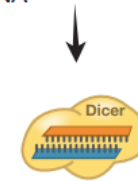
3. The RNAi mechanism

RNA interference (RNAi) is an important biological mechanism in the regulation of gene expression.

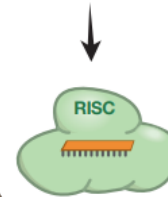


dsRNA

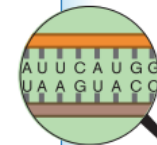
Double-stranded RNA (dsRNA) binds to the protein Dicer ...



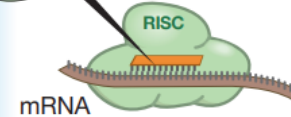
... which cleaves dsRNA into smaller fragments.



One of the RNA strands is loaded into a RISC complex...

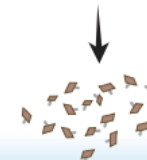
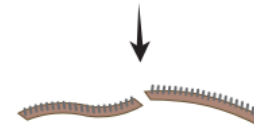


...and links the complex to the mRNA strand by basepairing.



mRNA

mRNA is cleaved and destroyed. No protein can be synthesized.



RNAi

Applications

- Knockdown

Advantages

- Easy to construct
- Fast
- Large-scale libraries exist (*Drosophila* and *C. elegans*)

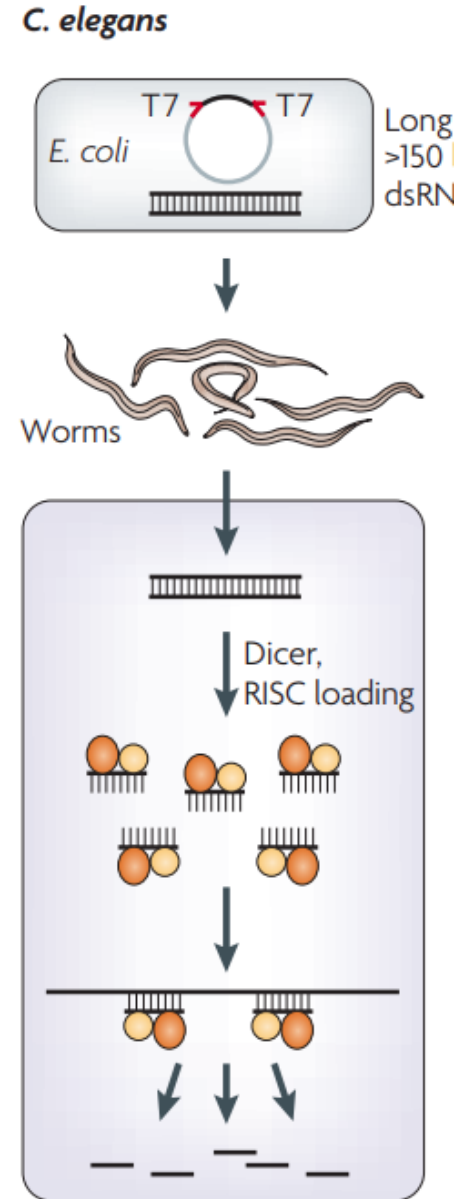
Disadvantages

- Nonspecific
- Variability in level of knockdown
- Not every gene is susceptible to RNAi — some tissues are resistant and genes encoding proteins with long half-lives are hard to knock down effectively

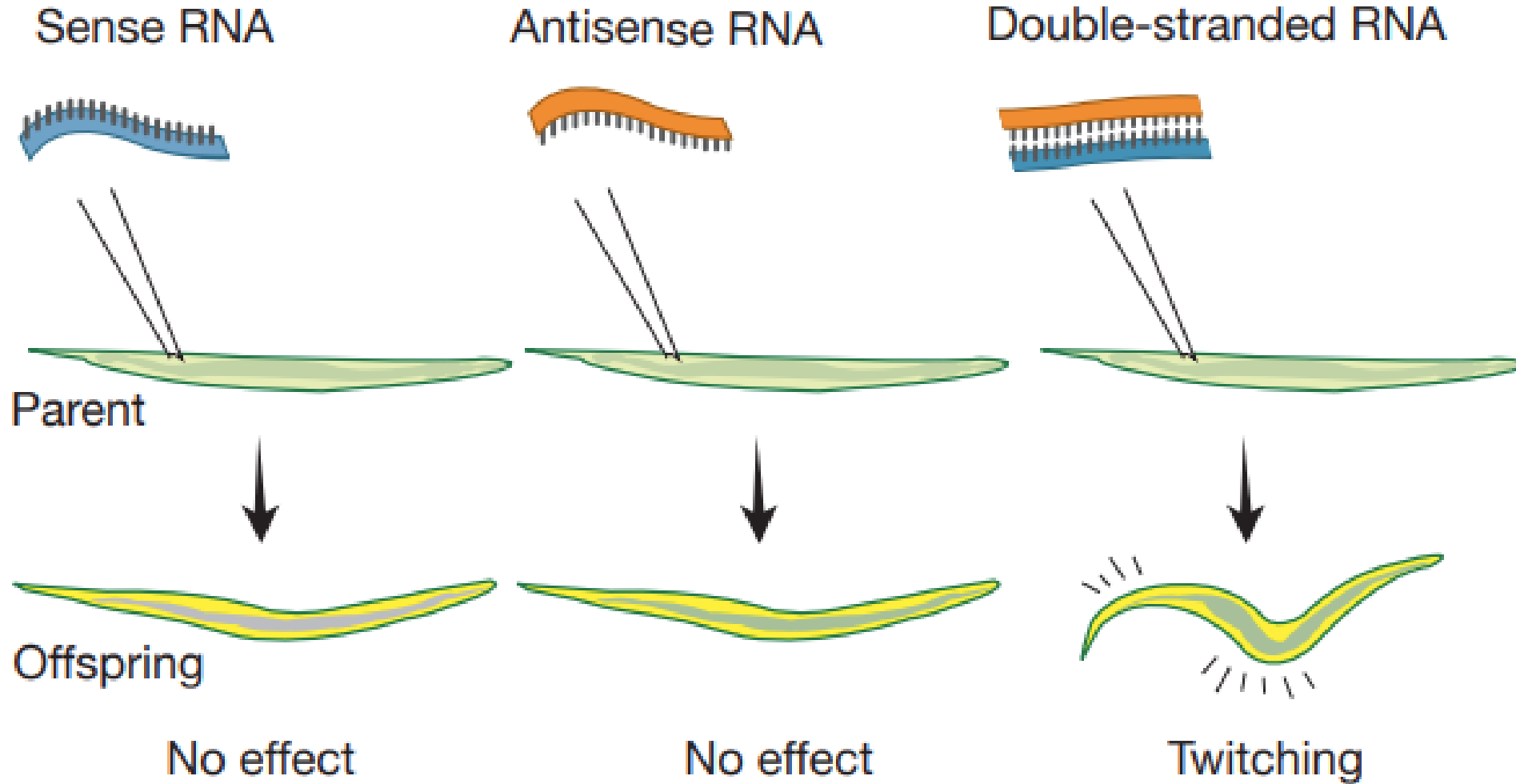
RNAi

How it is done?

- Injection of dsRNA or
- Soaking with dsRNA or
- Feeding engineered bacterial strains to express dsRNA



RNAi

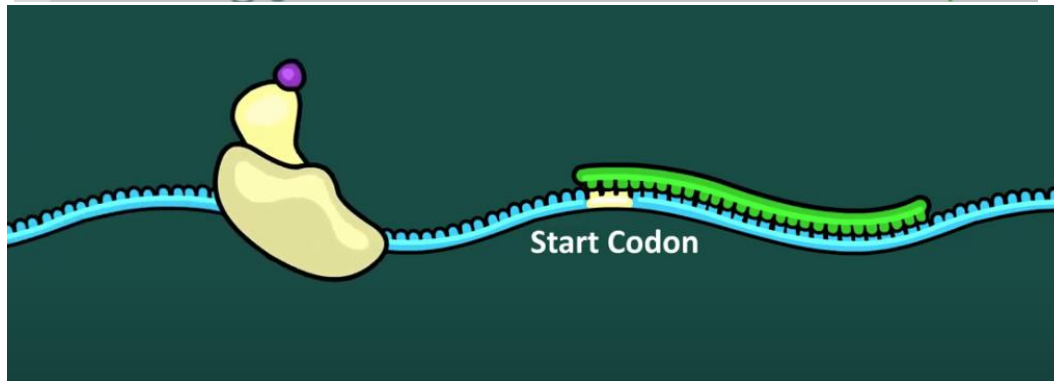
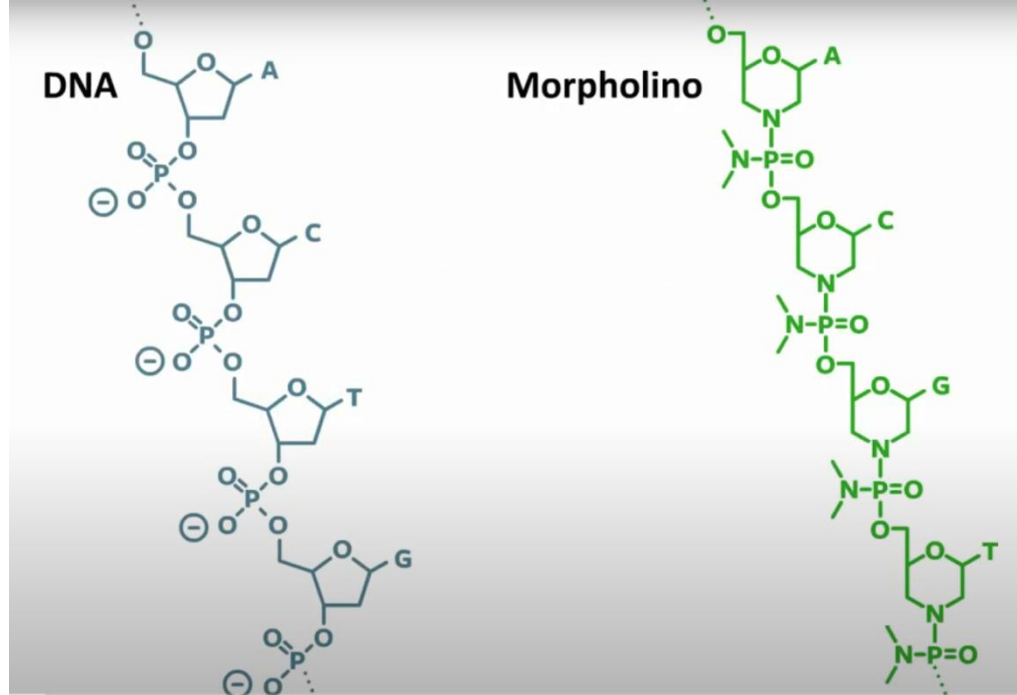


RNAi

Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F ₁ phenotype
<i>unc-22</i>				<i>unc-22</i> -null mutants: strong twitchers ²⁴
<i>unc-22A</i> *	Exon 21-22	742	Sense Antisense Sense + antisense	Wild type Wild type Strong twitchers (100%)
<i>unc-22B</i>	Exon 27	1,033	Sense Antisense Sense + antisense	Wild type Wild type Strong twitchers (100%)
<i>unc-22C</i>	Exon 21-22†	785	Sense + antisense	Strong twitchers (100%)

Morpholino



Morpholino

- 25-bp long
- Antisense oligonucleotides
- bind to target sequence within an RNA and inhibit RNA-interacting molecules
- Block translation or splicing
- Largely used in zebrafish
- Outdated? (depends)

Morpholino

Applications

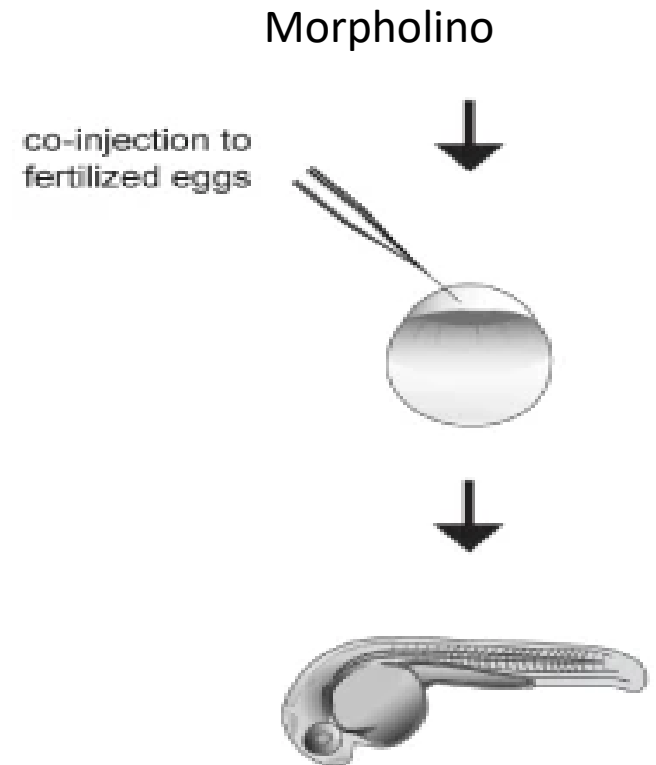
- Knockdown

Advantages

- Efficient knockdown
- Fast

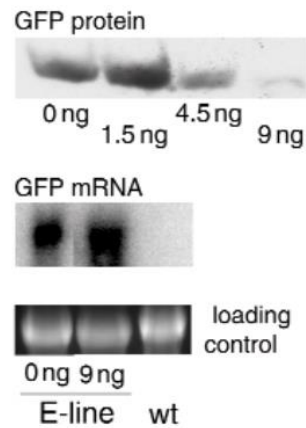
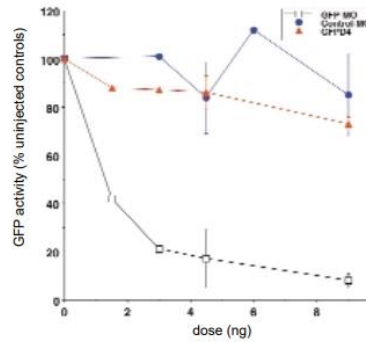
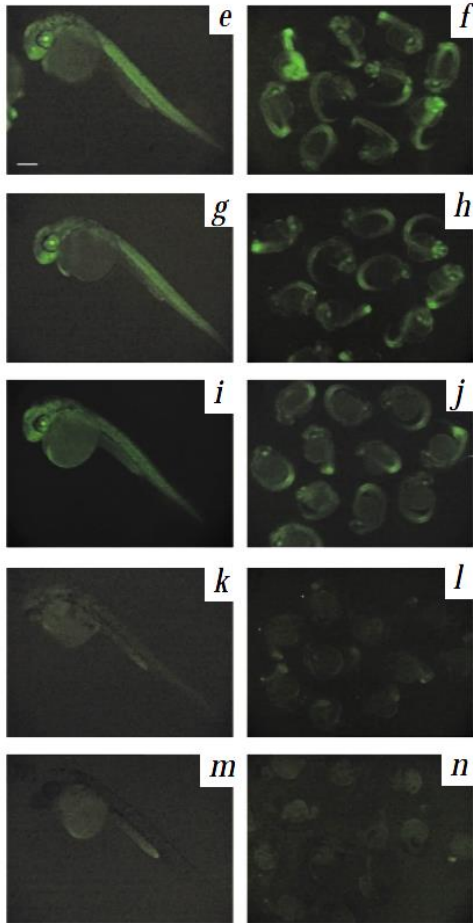
Disadvantages

- Expensive
- Nonspecific
- Only at early stages (upto 3-dpf)

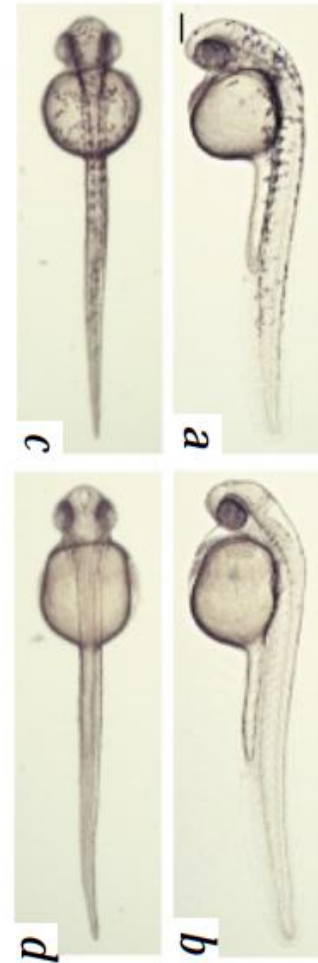


Morpholino

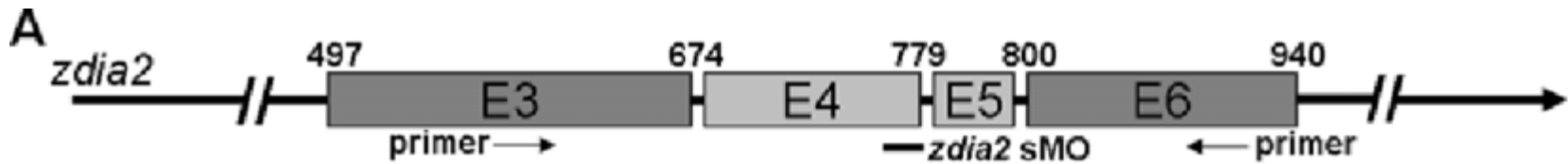
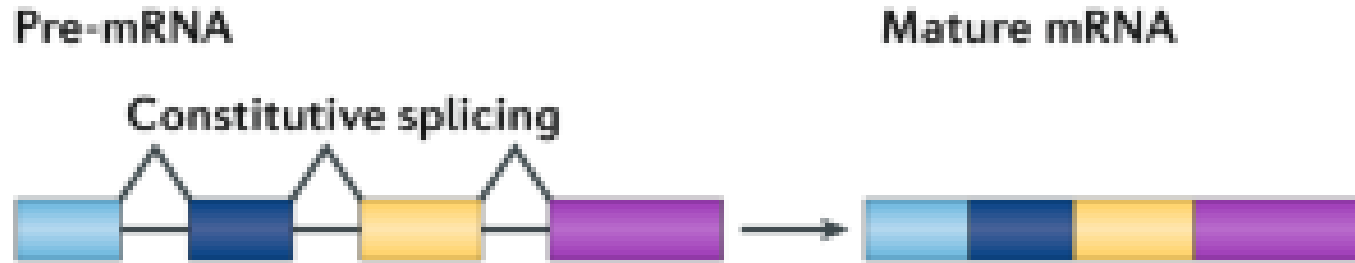
anti-GFP morpholino



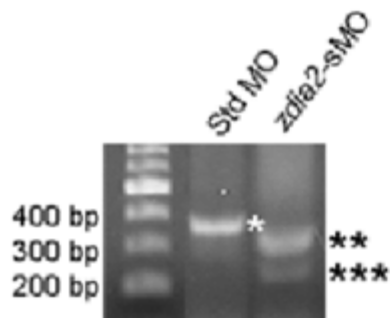
nacre morpholino



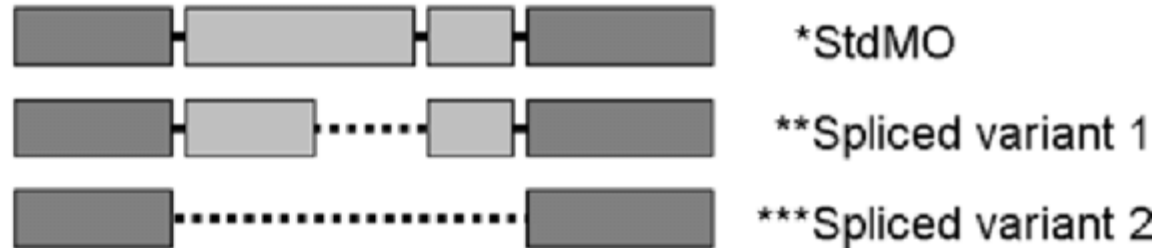
Splice-blocking morpholino



B

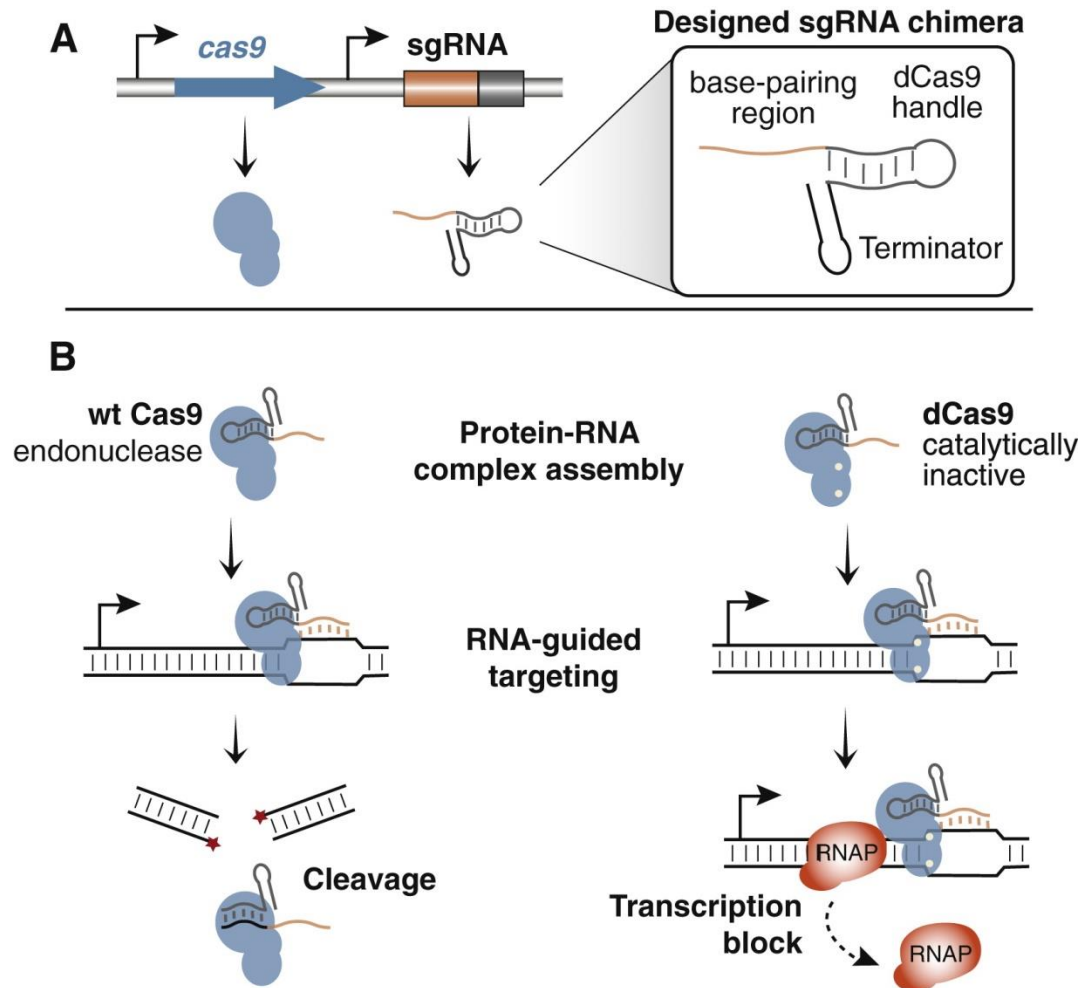


C



CRISPR interference (CRISPRi)

- Catalytically “dead” Cas9 + guide RNA



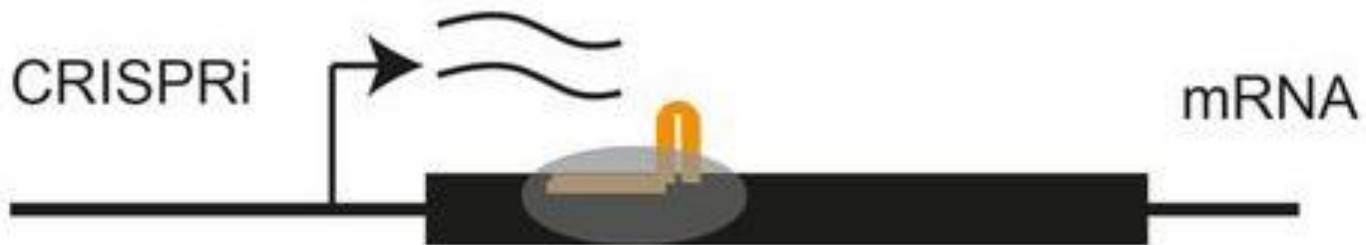
Qi LS et al., *Cell*, 2013

Larson MH et al., *Nat. Prot.* 2013

CRISPRi



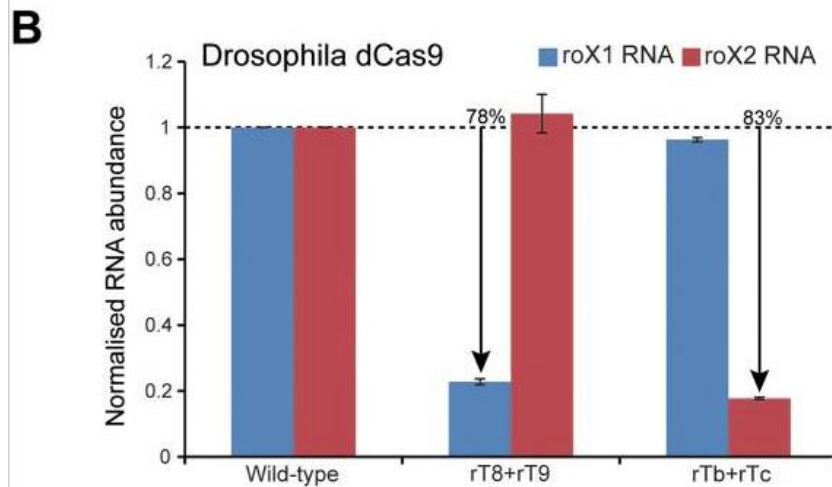
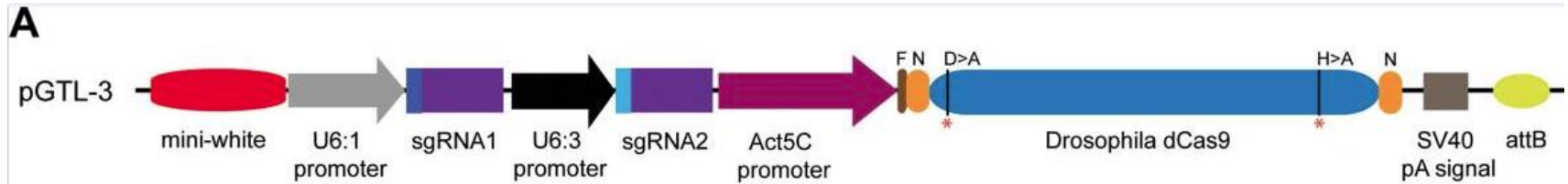
Transcriptional repression



CRISPRi

- CRISPR mutants can exhibit genetic compensation – other genes play a backup role
- CRISPRi do not exhibit genetic compensation

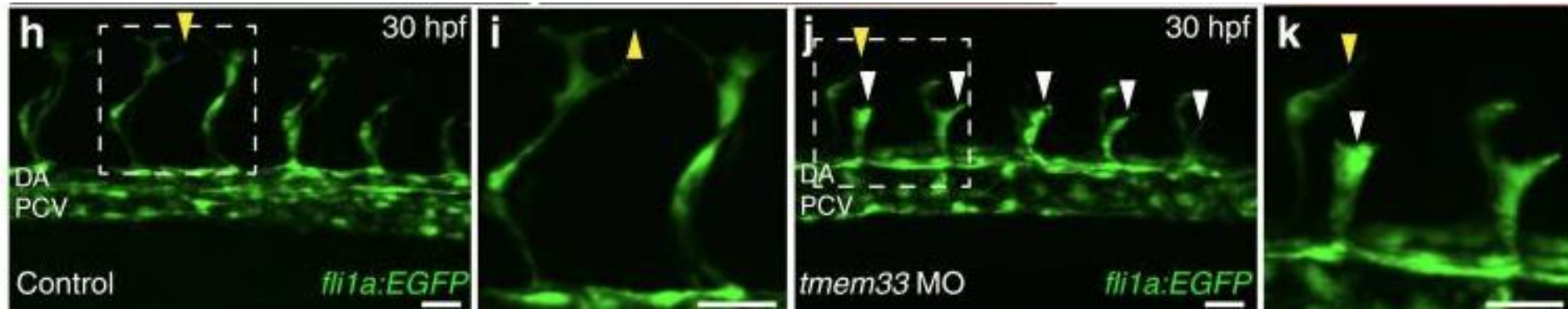
CRISPRi in Drosophila



C

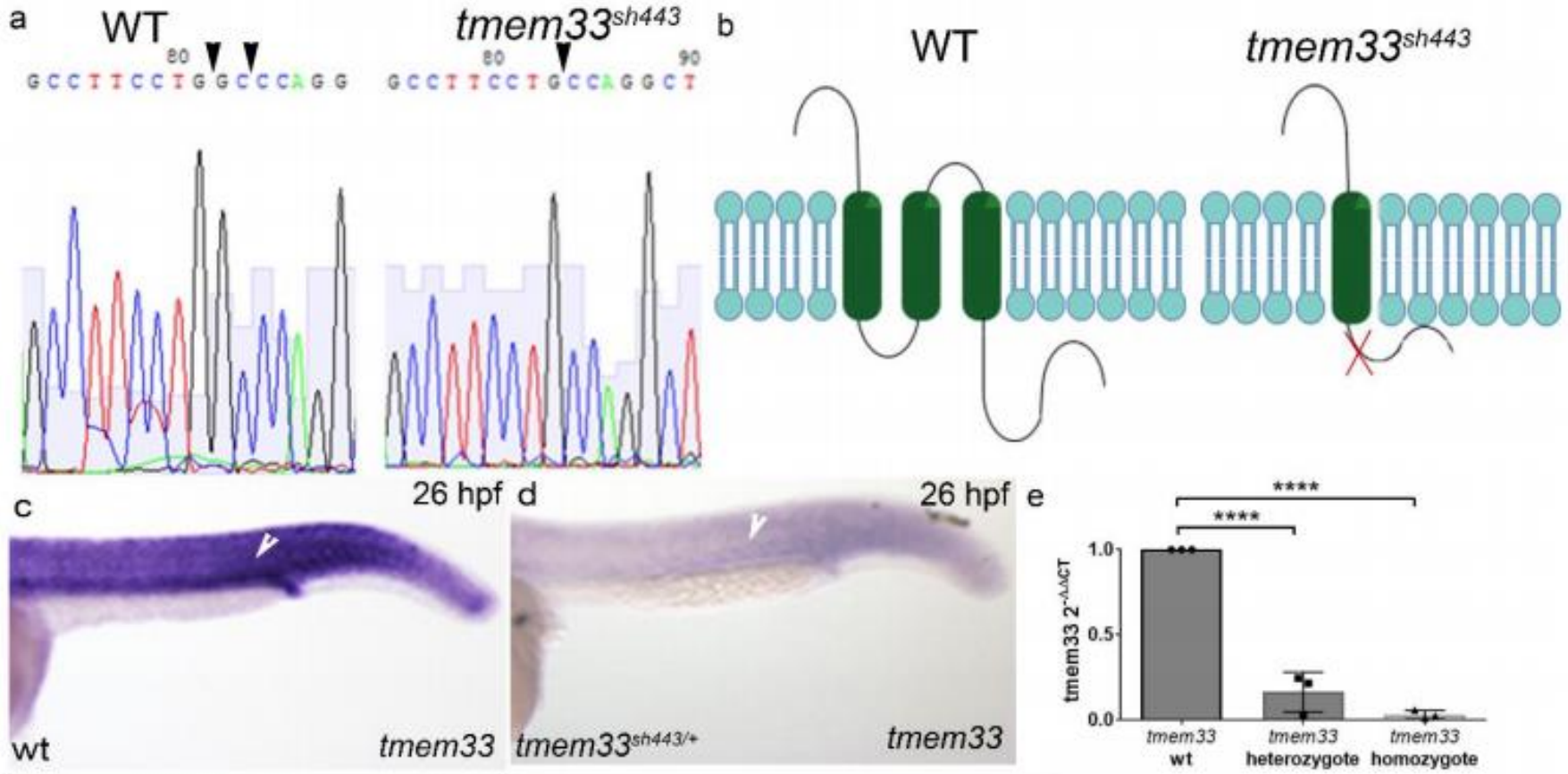
Genotype	Adult males	Adult females	% Adult males
<i>y,w</i>	901	828	52.1
<i>w;; rT8+rT9-Actin:dCas9</i> <i>rTb+rTc-Actin:dCas9</i>	40	484	7.6

CRISPRi in Zebrafish



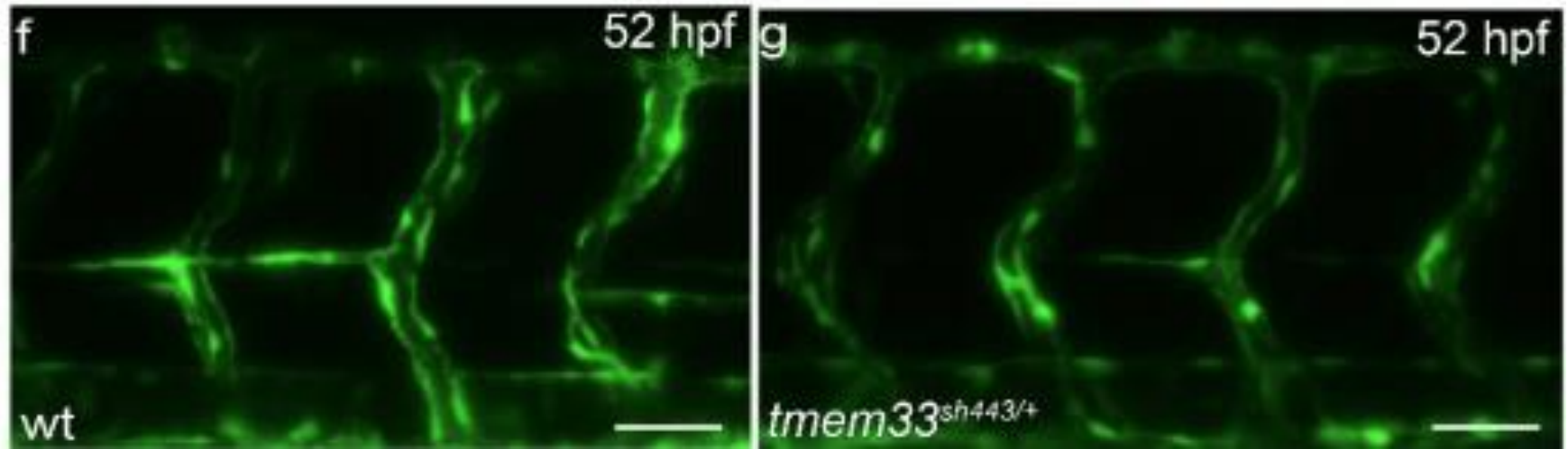
Morpholino shows a vascular phenotype

CRISPRi in Zebrafish



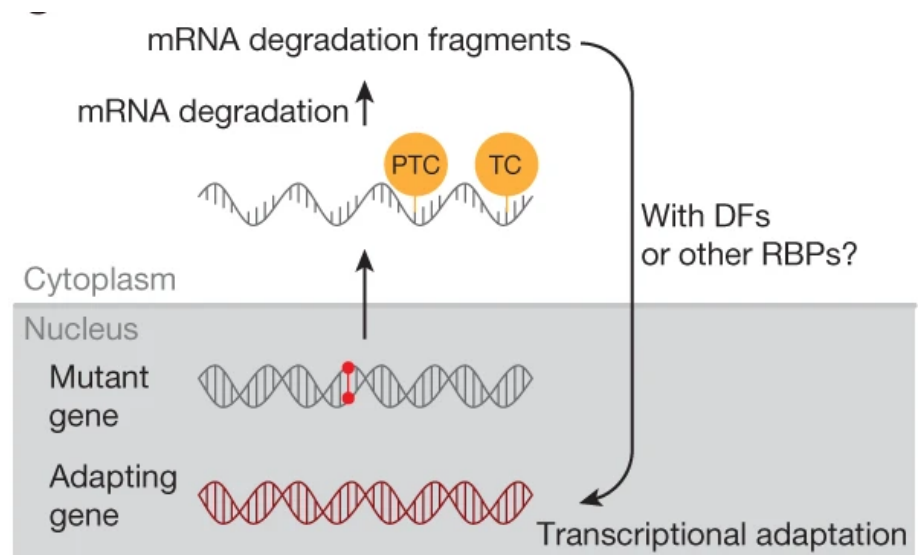
CRISPR mutants lack mRNA expression of targeted gene

CRISPRi in Zebrafish



CRISPR mutants do not show a vascular phenotype

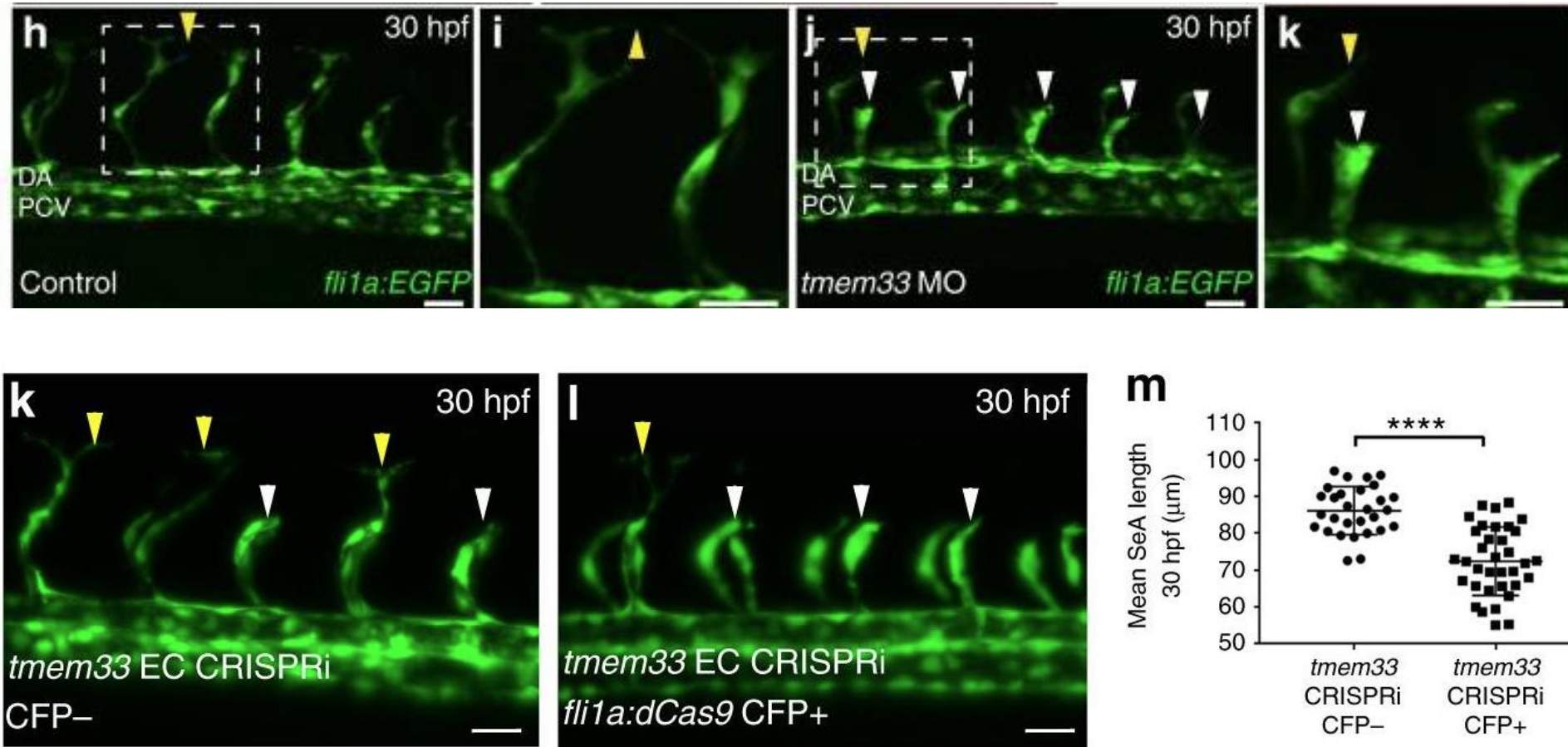
Genetic compensation



Savage A M et al., *Nat Comm.* 2019

El-Brolosy MA et al., *Nature* 2019

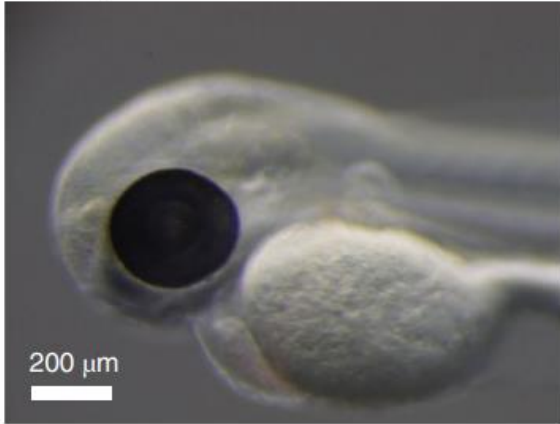
CRISPRi in Zebrafish



Like morpholino, CRISPRi exhibits vascular phenotype

CRISPR in *Danio rerio*

f WT, 3 dpf



gRNAs 1 + 2 + CRISPR-Cas9, 3 dpf



gRNAs 1 + 2 + CRISPR-Cas9, 3 dpf



g WT, 2 mpf



CRISPR *tyr*(-) F1, 2 mpf



Other genetic tools

Gene expression tools

- Tol2*
- Gal4-UAS
- Heat-shock system
- Tetracycline-transactivator system

Gene perturbation tools

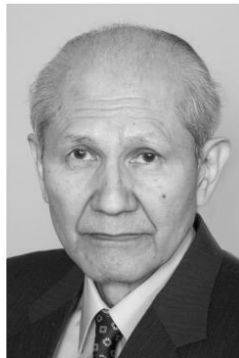
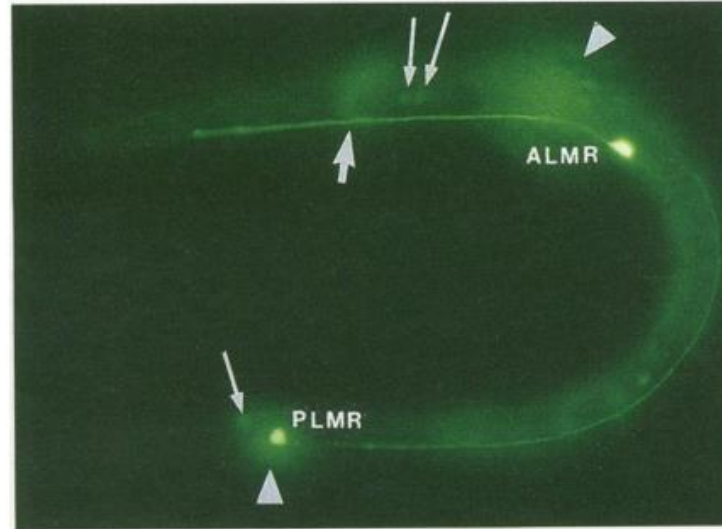
- Cre-Lox
- FLP-FRT
- ...

Applications

- To generate transgenic animals
- To label cell or tissue of interest
- To conditionally induce gene expression or knockout gene

Genome integration of foreign DNA

Fig. 3. Expression of GFP in a first-stage *C. elegans* larva. Two touch receptor neurons (ALMR and PLMR) are labeled at their strongly fluorescing cell bodies. Processes can be seen projecting from both of these cell bodies. Halos produced from the out-of-focus homologs of these cells on the other side of the animal are indicated by arrowheads. The thick arrow points to the nerve ring branch from the ALMR cell (out of focus); thin arrows point to weakly fluorescing cell bodies. The background fluorescence is the result of the animal's autofluorescence.



© The Nobel Foundation. Photo: U. Montan

Osamu Shimomura

Prize share: 1/3



© The Nobel Foundation. Photo: U. Montan

Martin Chalfie

Prize share: 1/3



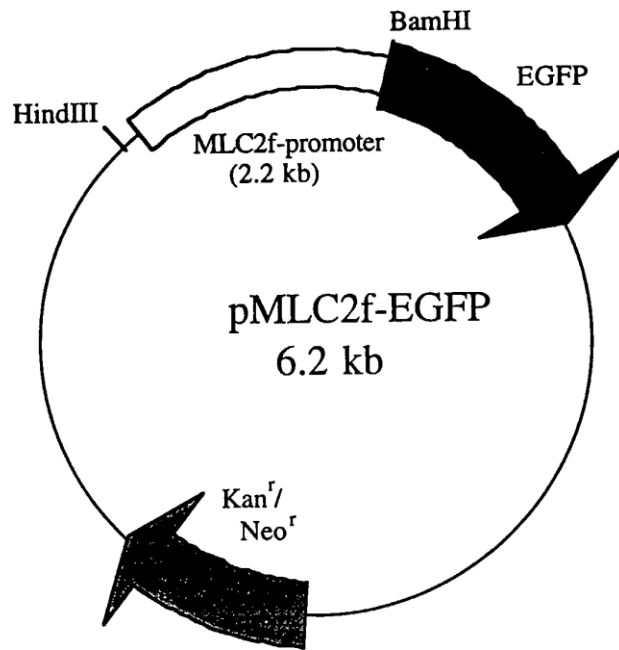
© The Nobel Foundation. Photo: U. Montan

Roger Y. Tsien

Prize share: 1/3

The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien "for the discovery and development of the green fluorescent protein, GFP."

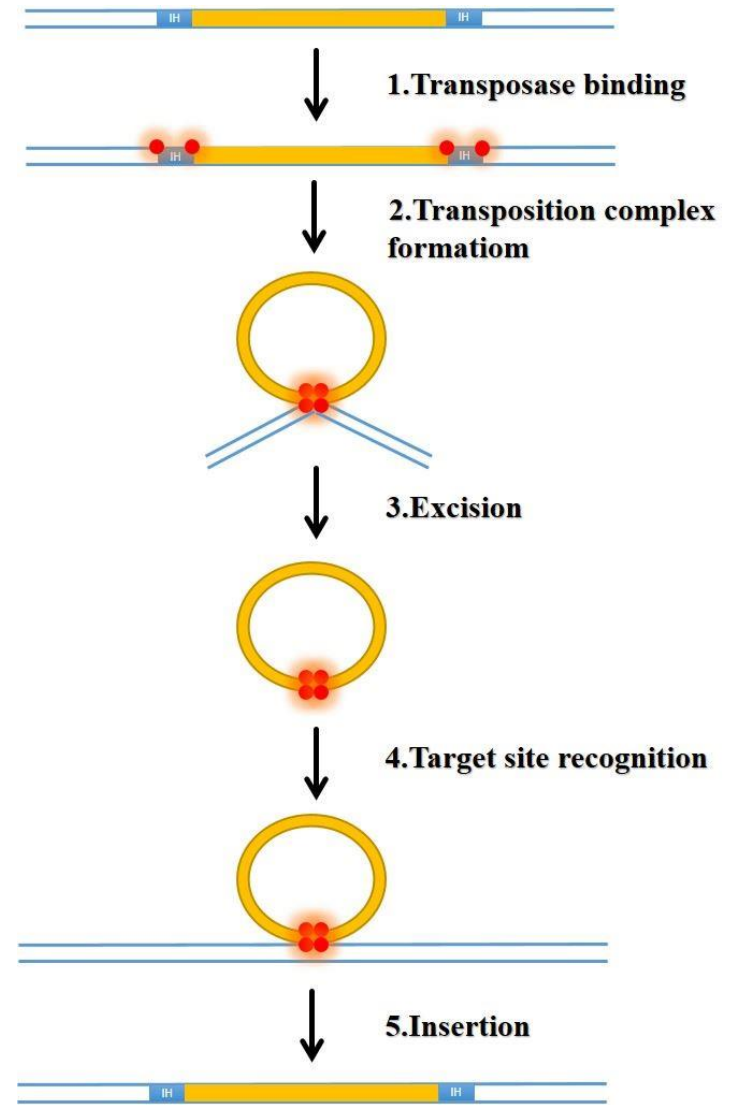
Genome integration of foreign DNA



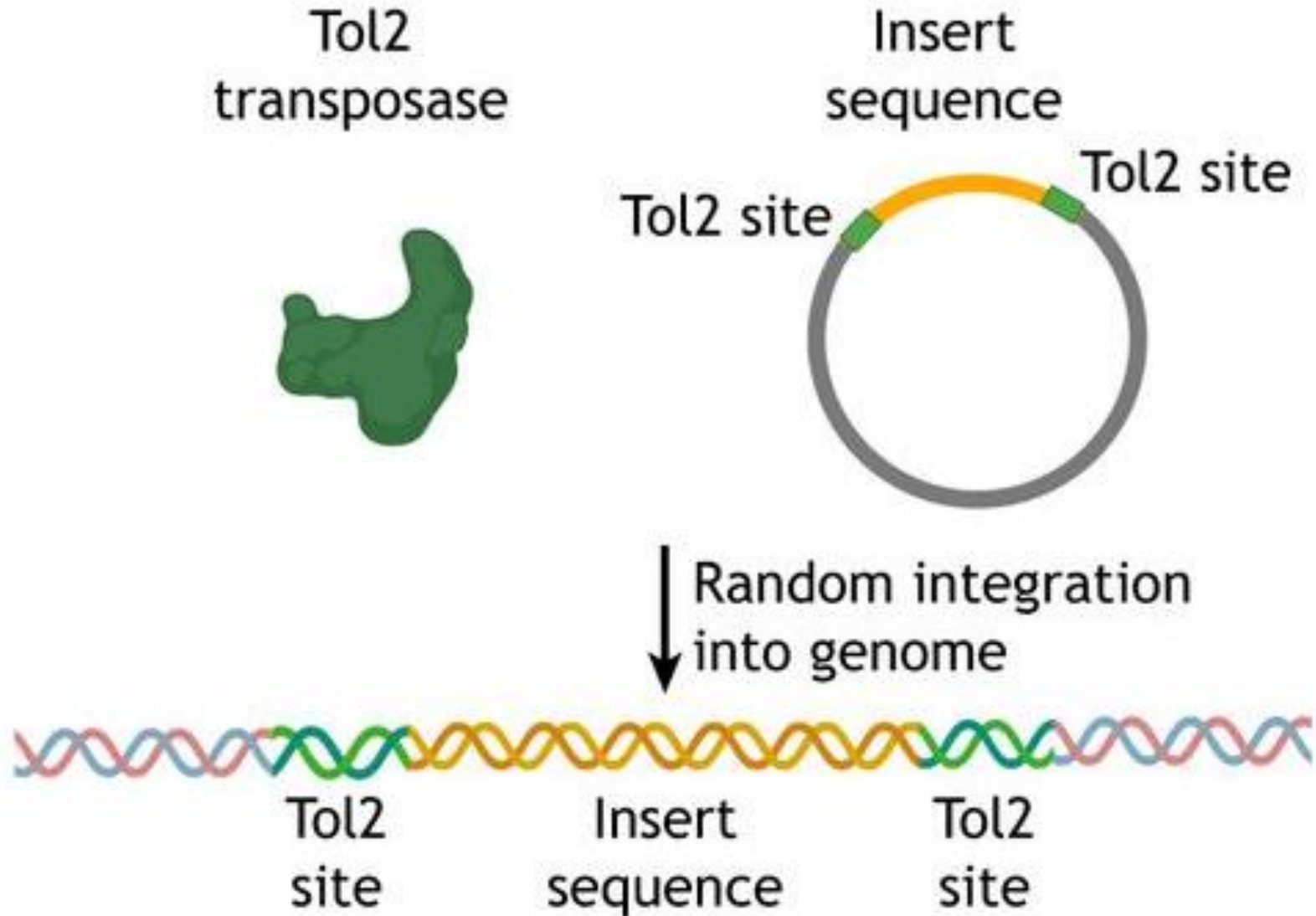
- Introduction of foreign DNA into zebrafish genome occurs at low percentage (approx. 25%).
- Need for better techniques to efficiently introduce foreign DNA

Tol2 in zebrafish

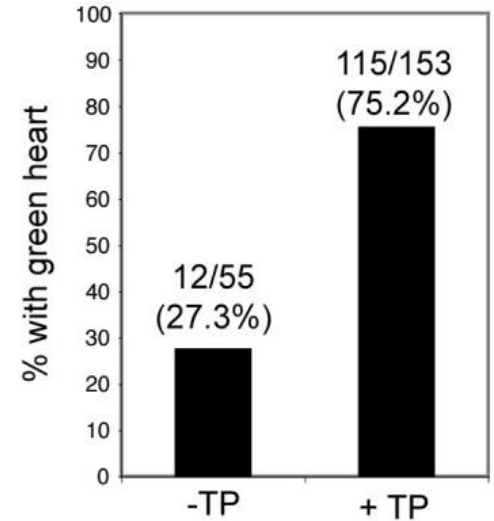
- Autonomous transposon from Medaka fish (*Oryzias latipes*)
- **Components:**
 - Transposase enzyme
 - Tol2 *cis* sequence (150bp and 200bp) with 12bp terminal inverted repeats
- **Application:**
 - Spatial expression of transgene



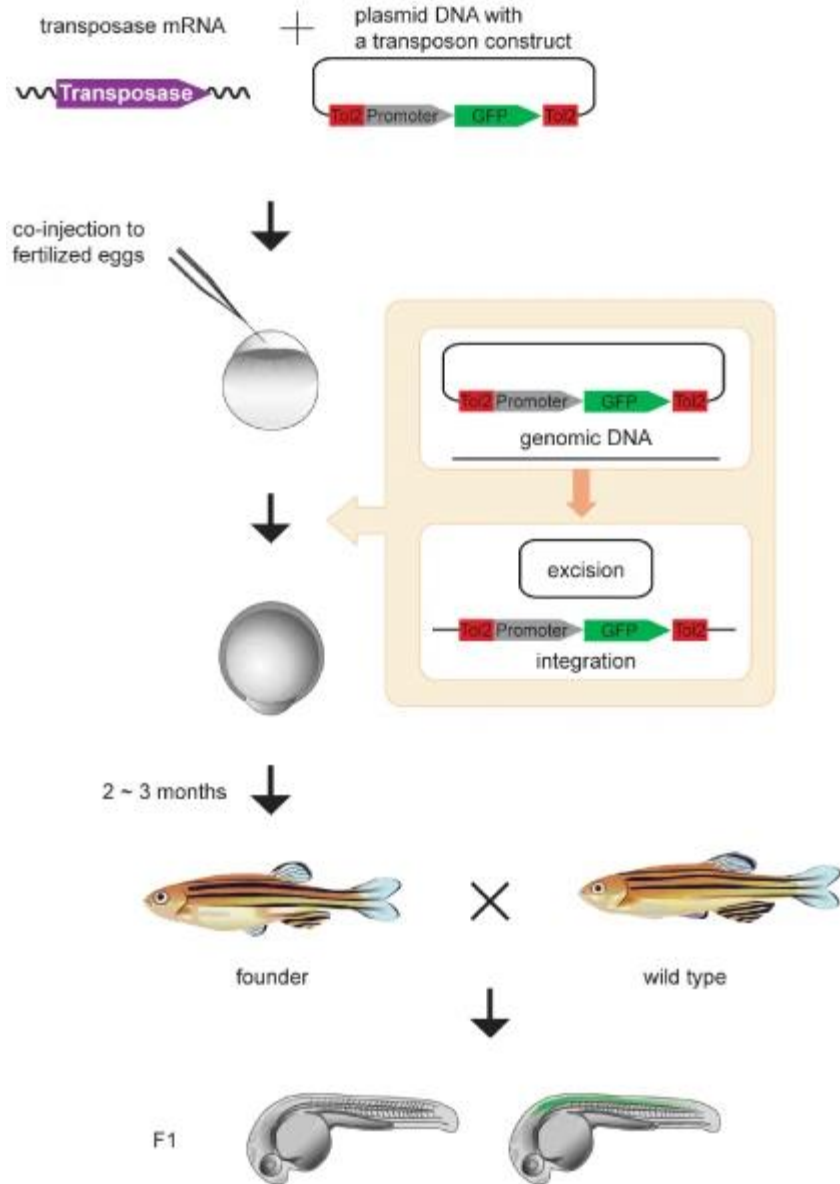
Tol2 in zebrafish



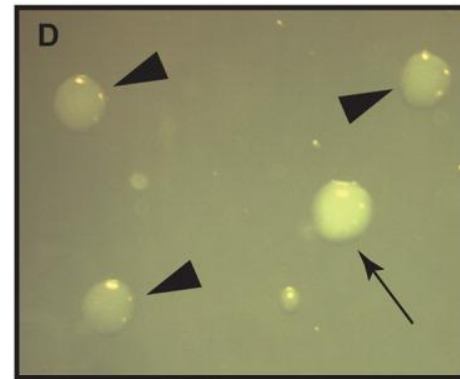
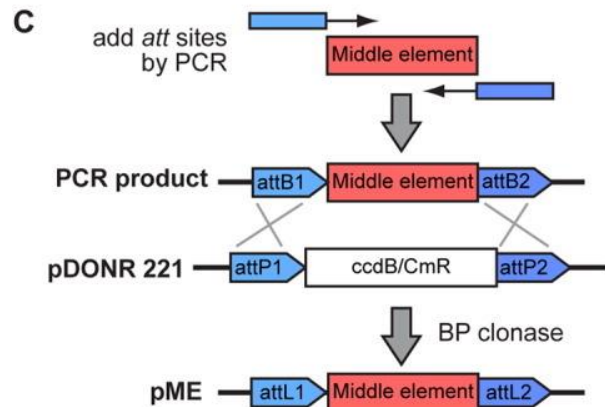
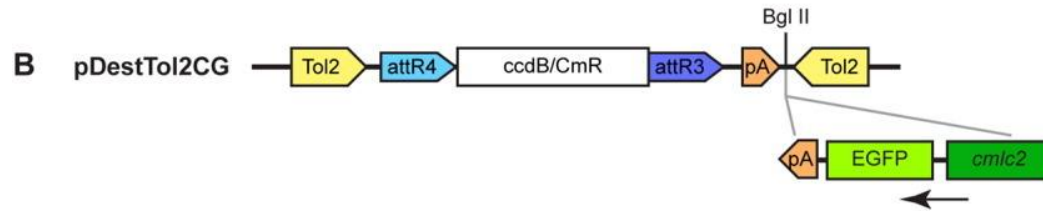
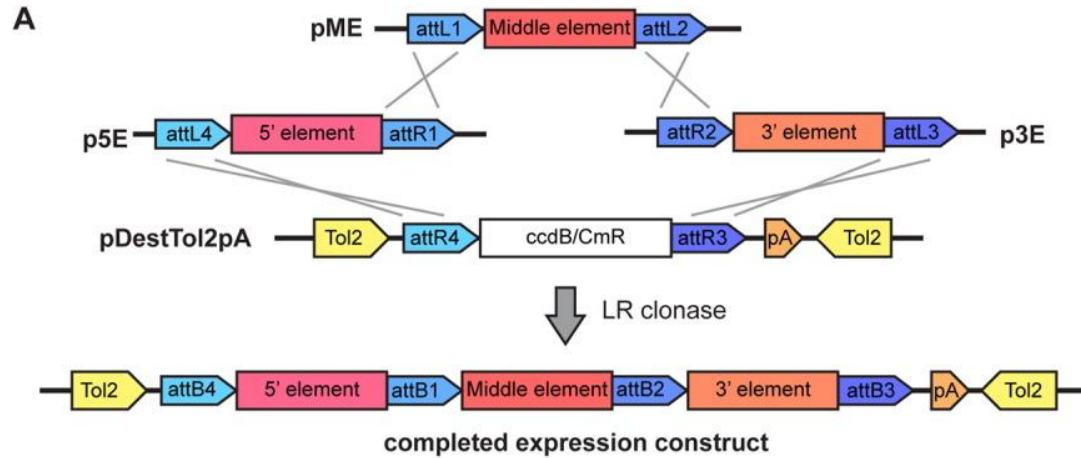
Tol2 in Zebrafish



Tol2 in Zebrafish



Tol2 in Zebrafish



Tol2 in Zebrafish

TABLE 1. Components of the Tol2kit^a

Name	Description	Test	Figure
5' entry clones			
p5E- <i>bactin2</i>	5.3-kb beta-actin promoter (ubiquitous)	F0, F1	2
p5E- <i>h2afx</i>	1-kb H2A-X promoter (quasi-ubiquitous)	F0	2b
p5E-CMV/SP6	1-kb CMV/SP6 cassette from pCS2+	F0	
p5E- <i>hsp70</i>	1.5-kb <i>hsp70</i> promoter for heat-shock induction	F0, F1	4
p5E-UAS	10x UAS element and basal promoter for Gal4 response	F0	
p5E-MCS	Multicloning site from pBluescript	F0	
p5E-Fse-Asc	Restriction sites for 8-cutters <i>FseI</i> and <i>AscI</i>	F0	
Middle entry clones			
pME-EGFP	EGFP	F0	
pME-EGFPCAAX	Membrane-localized (prenylated) EGFP; fused to the last 21 amino acids of H-ras	F0, F1	3
pME-nlsEGFP	Nuclear-localized EGFP	F0	2f
pME-mCherry	Monomeric red fluorophore mCherry	F0, F1	2c
pME-mCherryCAAX	Membrane-localized (prenylated) mCherry	F0	2e, 4
pME-nlsmCherry	Nuclear-localized mCherry	F0	2abd
pME-H2AmCherry	mCherry fused to the zebrafish histone H2A.F/Z	F0, F1	
pME-Gal4VP16	Gal4 DNA binding domain fused to the VP16 transactivation domain	F0	
3' entry clones			
p3E-polyA	SV40 late poly A signal sequence from pCS2+	F0, F1	3, 4
p3E-MTpA	6x myc tag for protein fusions, plus SV40 late polyA		
p3E-EGFPpA	EGFP for protein fusions, plus SV40 late polyA	F0, F1	
p3E-mCherrypA	mCherry for protein fusions, plus SV40 late polyA	F0, F1	
p3E-IRES-EGFPpA	EMCV IRES driving EGFP plus SV40 late polyA	F0	2d
p3E-IRES-EGFPCAAXpA	EMCV IRES driving EGFPCAAX (prenylated EGFP) plus SV40 late polyA	F0, F1	2abcf
p3E-IRES-nlsEGFPpA	EMCV IRES driving nlsEGFP (nuclear EGFP) plus SV40 late polyA	F0	2e
Destination vectors			
pDestTol2pA/pDestTol2pA2	attR4-R3 gate with SV40 polyA flanked by Tol2 inverted repeats	F0, F1	3
pDestTol2CG/pDestTol2CG2	pDestTol2pA/pDestTol2pA2 with <i>cmc2</i> :EGFP transgenesis marker	F0, F1	4
Other			
pCS2FA-transposase	For in vitro transcription of capped Tol2 transposase RNA		3, 4

^aEGFP, enhanced green fluorescent protein; F0, yields appropriate expression in transient transgenics; F1, yields appropriate expression in stable transgenics.

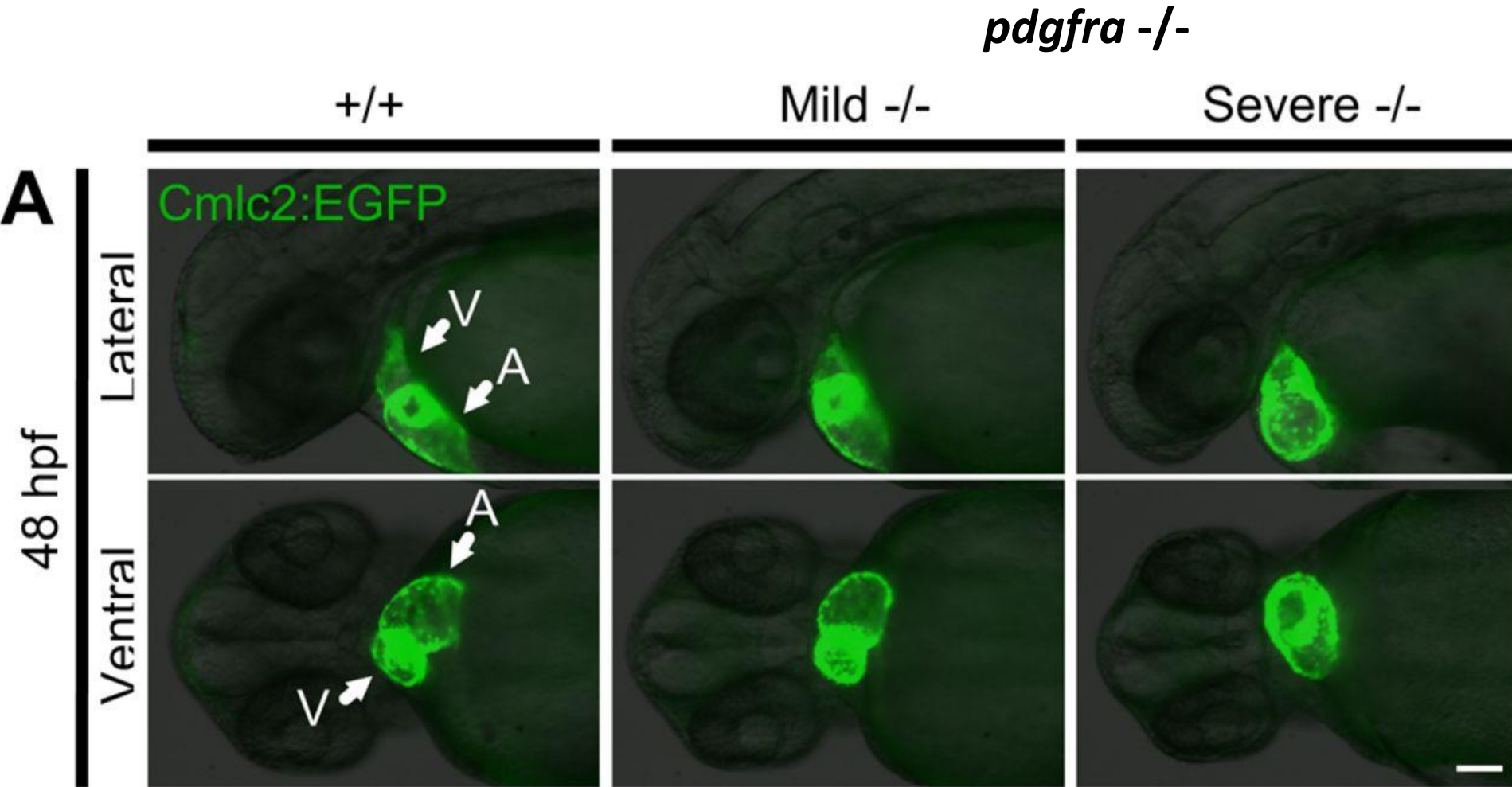
Tol2 in Zebrafish

- Using specific promoters, it is possible to achieve spatially restricted gene expression
- Allows spatial expression of transgene
 - *flk* promoter – vasculature
 - *oxt* promoter – oxytocin neurons
 - *cmlc2* promoter - heart
- In combination with other techniques, allows temporal control of transgene

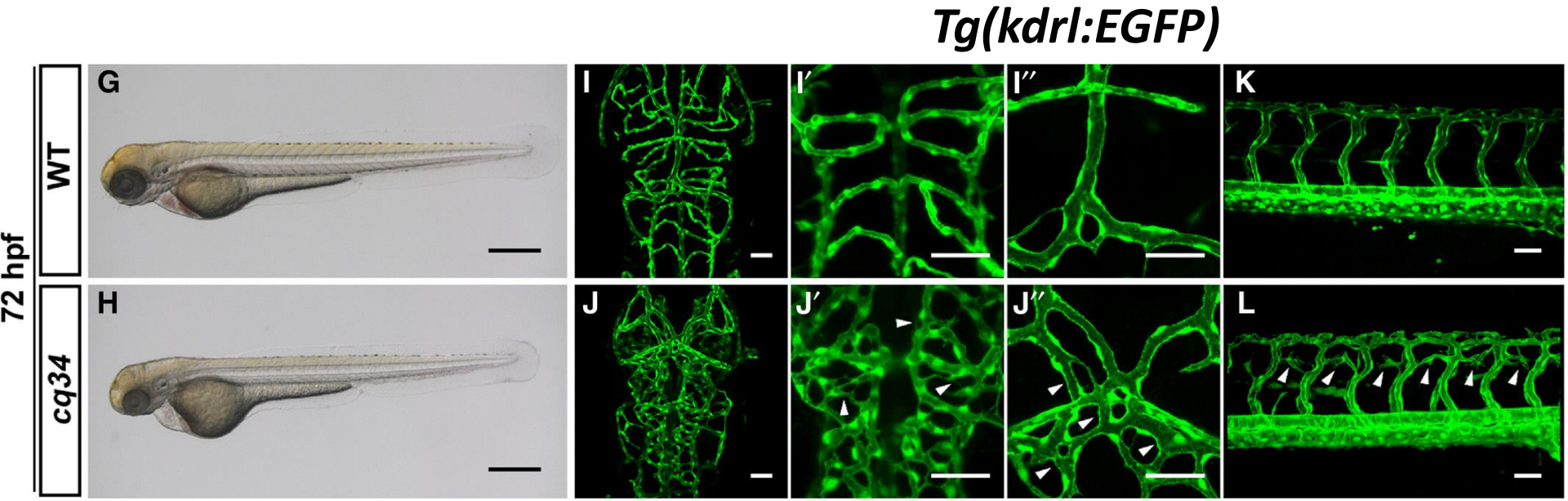
Tol2 in Zebrafish



Tol2 in Zebrafish



Tol2 in Zebrafish



Tol2 in Zebrafish

4 dpf

Tg(fabp10:dsRed); Tg(ptf1a:GFP)

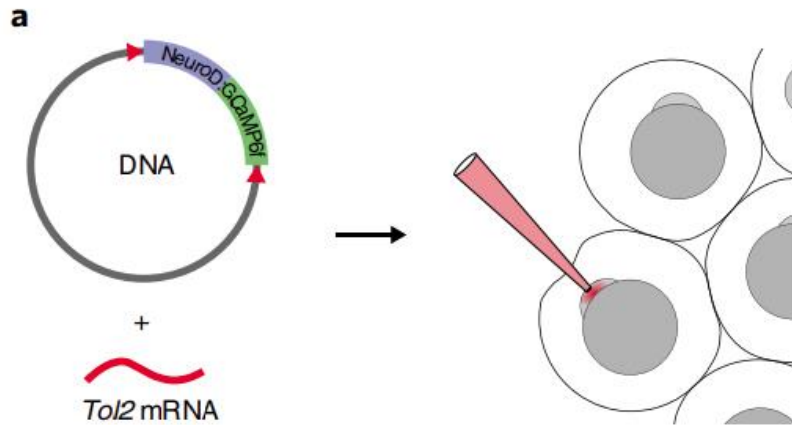


Tg(ins:Kaede)

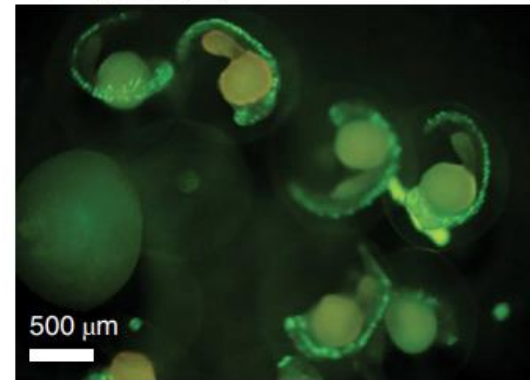


Kamel and Ninov, *Curr Opin Pharmacol.*, 2017

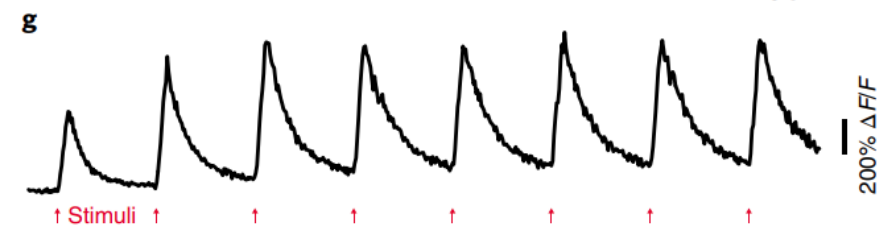
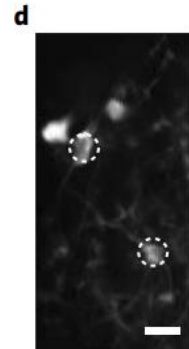
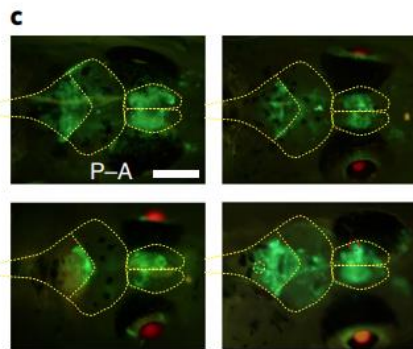
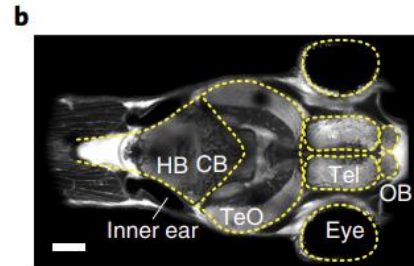
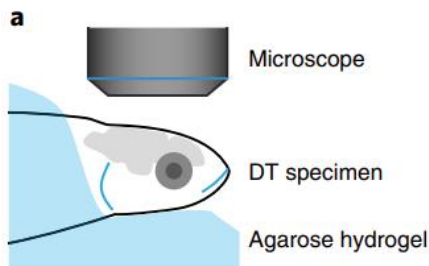
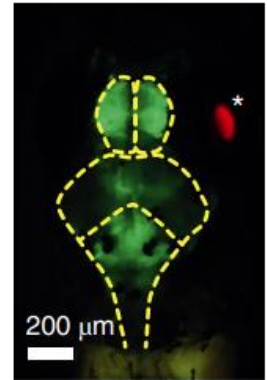
Tol2 in *Danio rerio*



b Tol2 injected (F0)

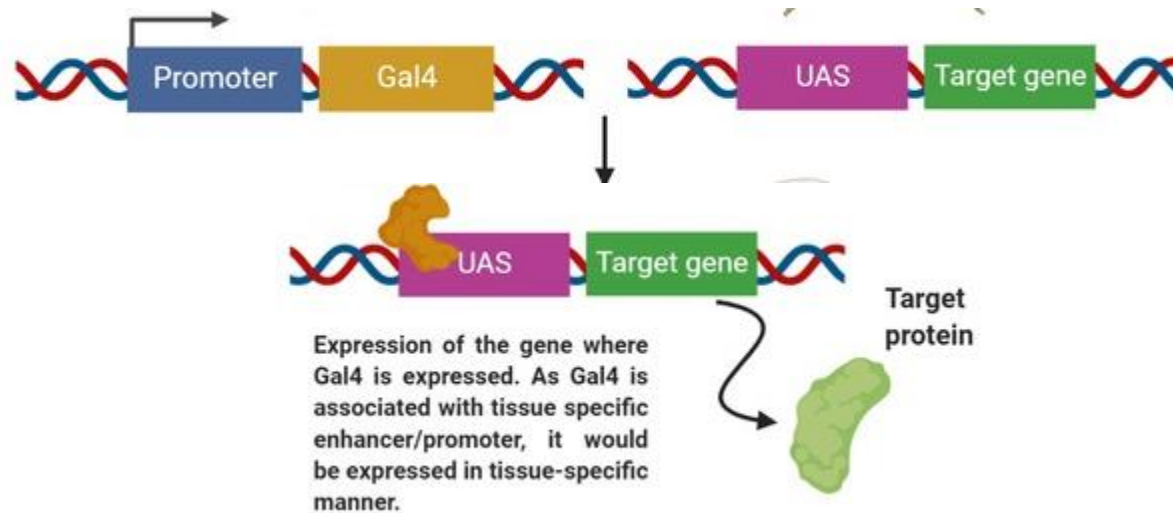


c F1



Gal4-UAS system

- Yeast Gal4 – transcriptional activator
- UAS - Gal4 responsive Upstream Activator Sequence



Gal4-UAS system

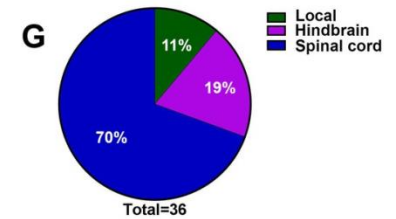
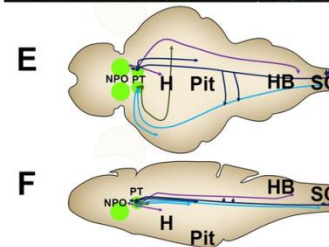
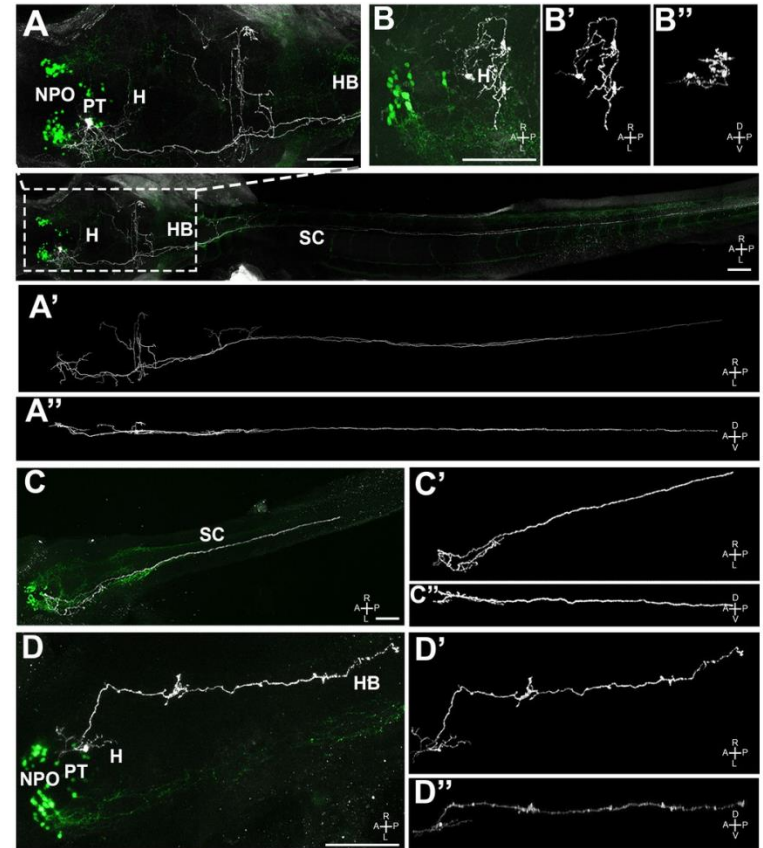
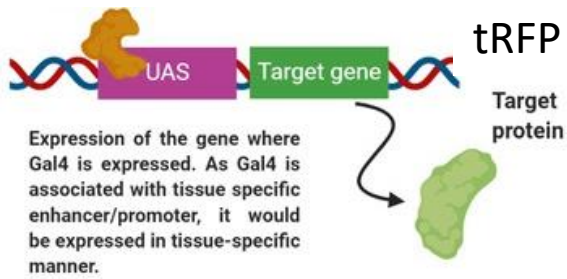
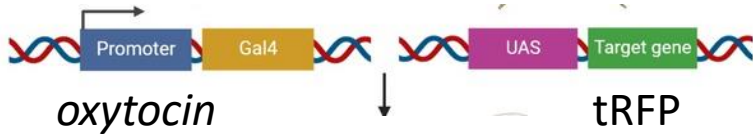
Advantage:

- Allows spatially restricted expression of transgene
- 1000s of Drosophila GAL4 lines exist

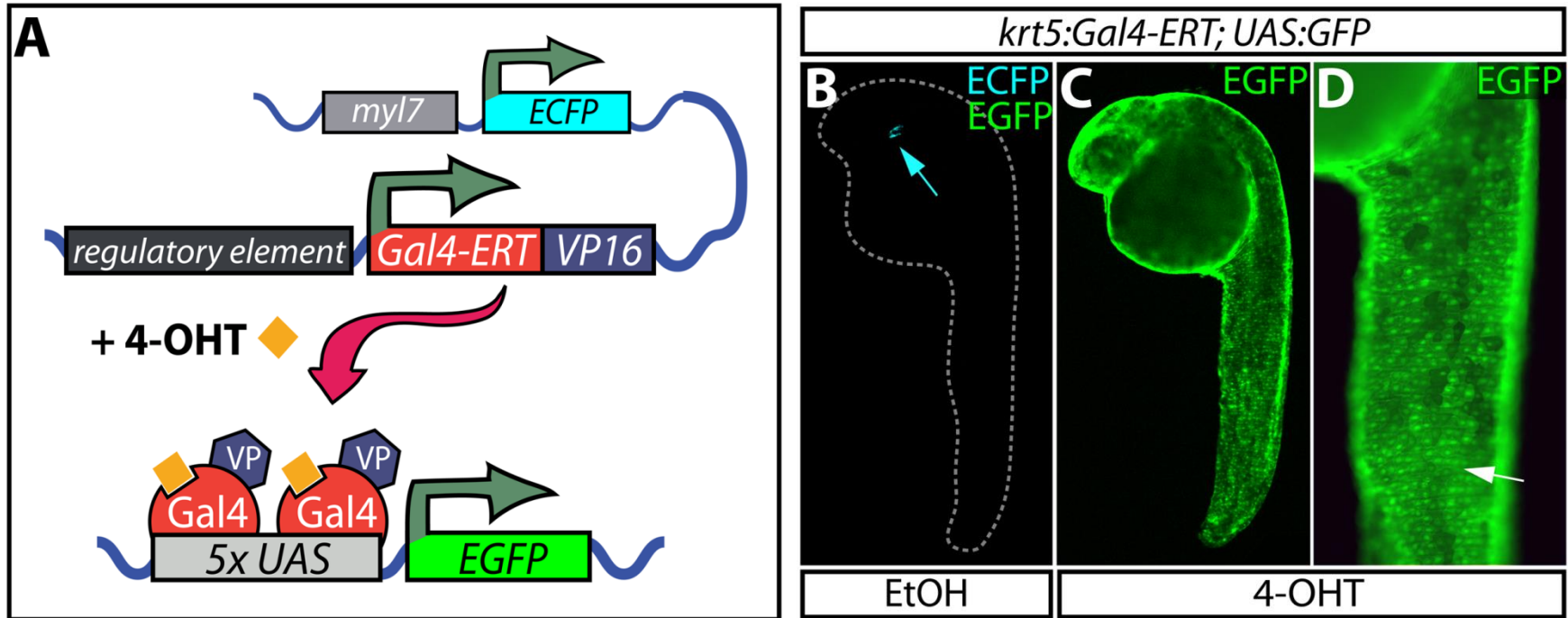
Drawback:

Only spatially control and not temporal control of gene expression*

Gal4-UAS to label single neurons in zebrafish brain



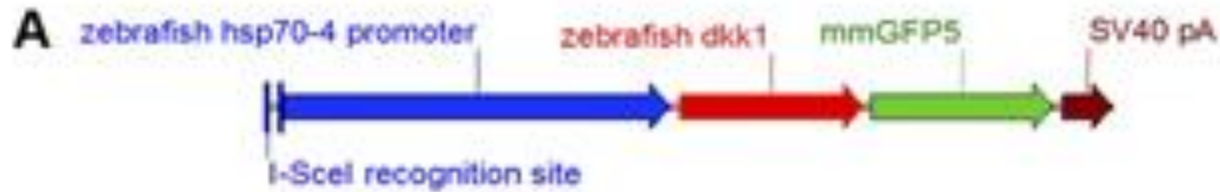
Gal4-ERT-UAS system



Advantage:

- Allows spatially and temporal control of transgene expression

Heat-shock system



Applications

- Temporal control of transgene expression

Heat-shock system

Drawbacks:

- heat-shock promoters produce a low level of basal transcription even under non-heat-shock conditions.
- Phenotypic effects can be produced with the expression of certain genes, such as toxin genes, even without an elevation in temperature.
- Heat shock procedure can itself produce undesired effects on the animals depending on the timing of the heat shock and the inducing temperature.
- Transgene expression is induced in essentially all cells in the organism,

Cre-Lox system

- The Cre-Lox system is a site-specific recombination method.
- Derived from bacteriophage P1.
- Cre recombinase recognizes LoxP sites and depending upon LoxP orientation, it can create inversions or deletions or translocations

Applications

- Generate conditional knockouts
- spatial and temporal control (combination with heatshock or tamoxifen) of gene expression
- Useful to study genes whose knockouts are lethal

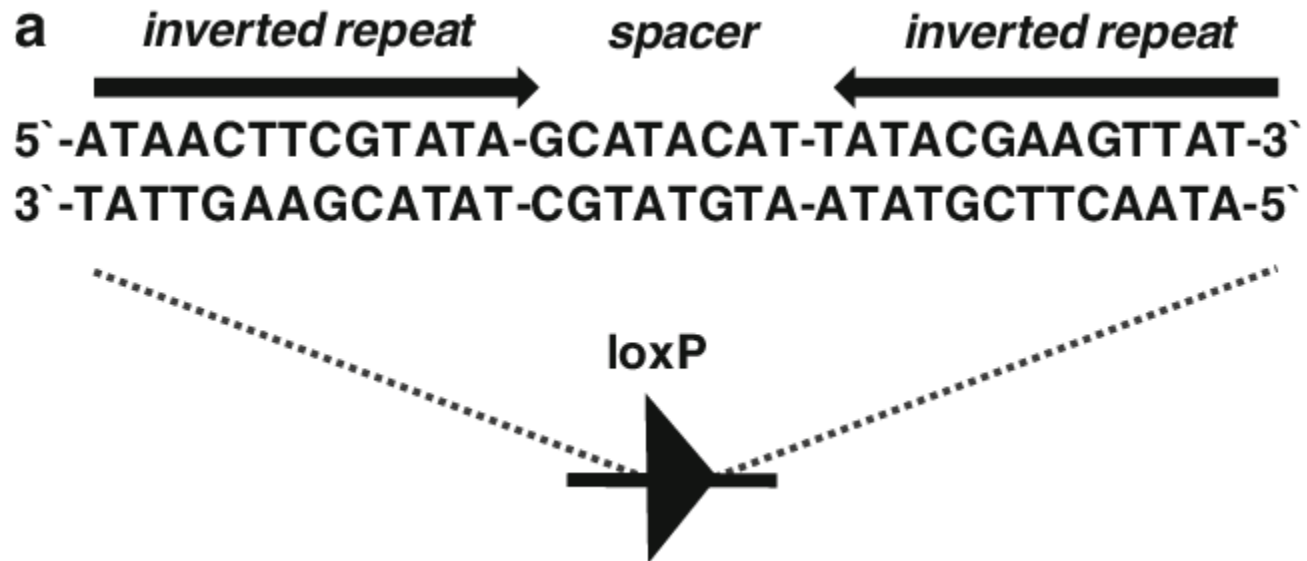
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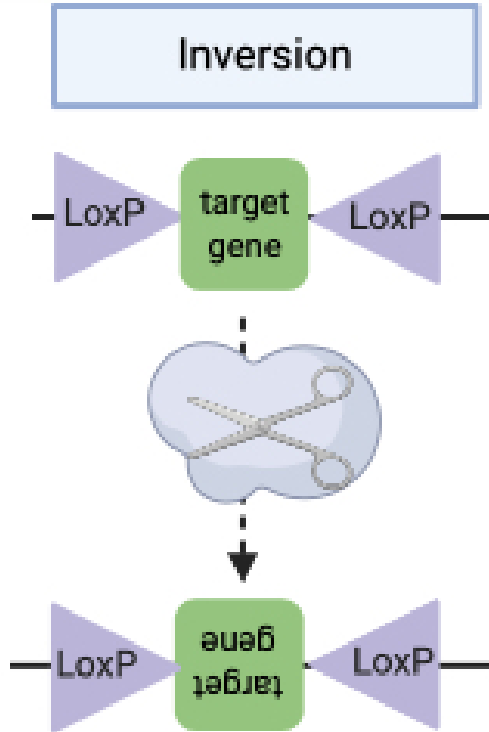
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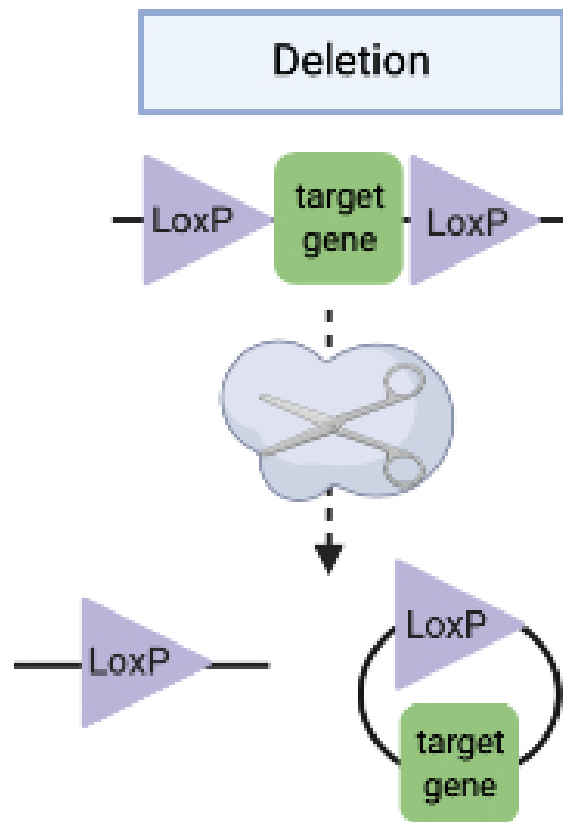
Cre-Lox system



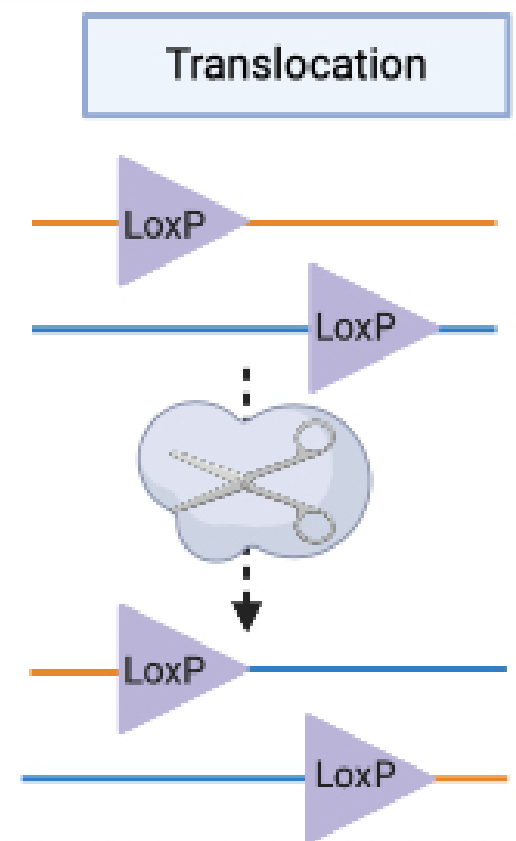
Cre-Lox system



When loxP sites on the same strand are in opposite orientations, recombination leads to inversion



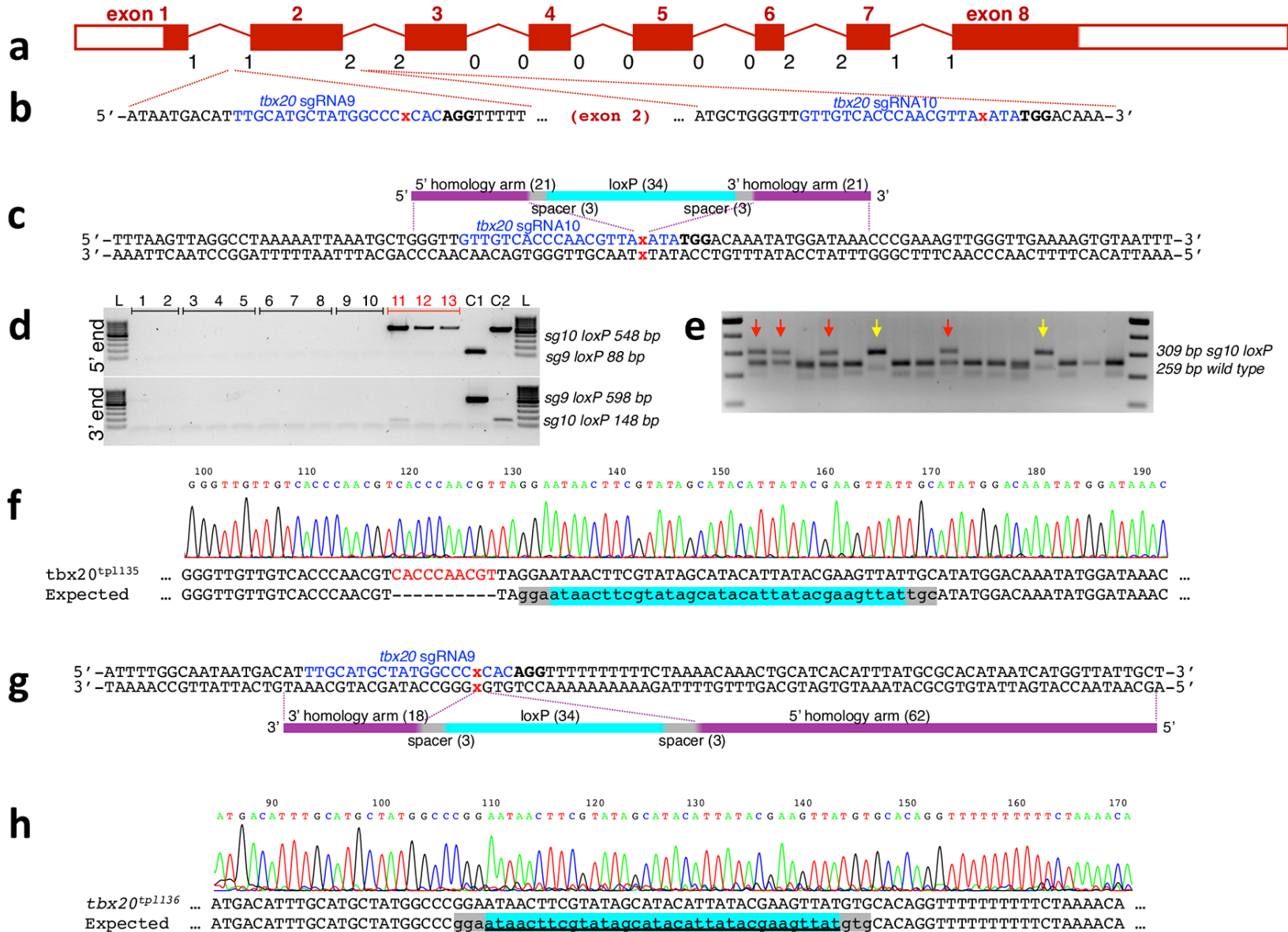
When loxP sites face the same direction, the sequence in between is excised as a circular fragment



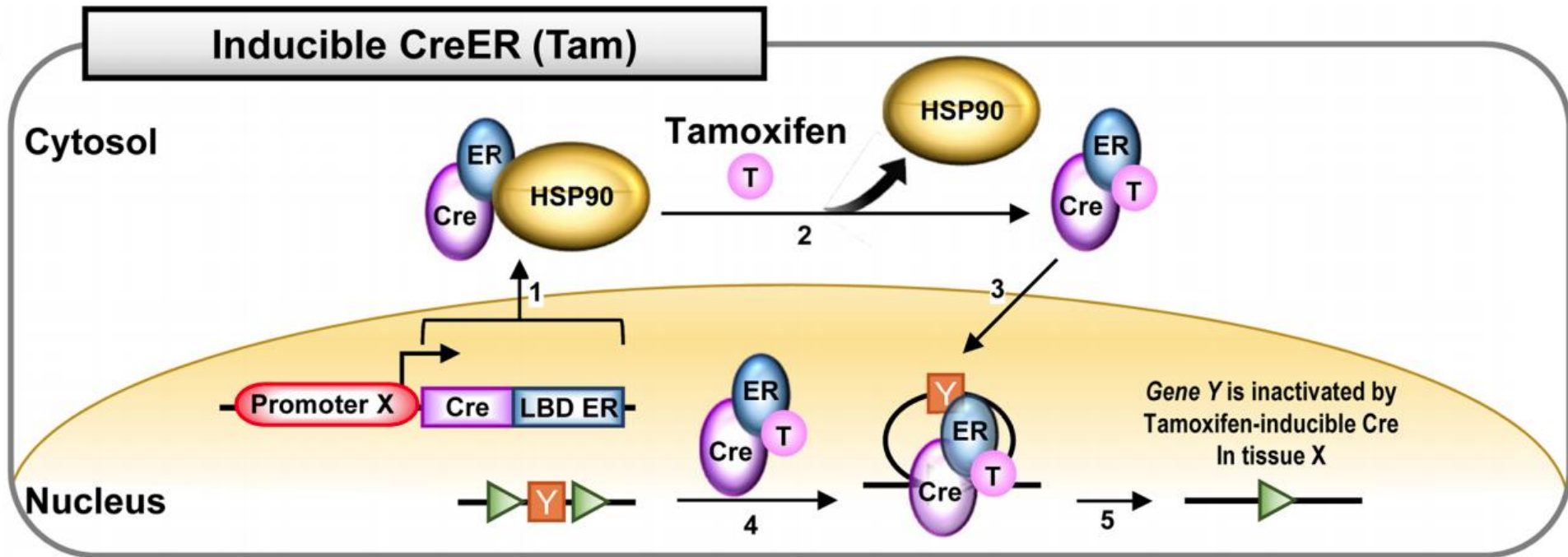
When loxP sites are on different strands, a translocation event is generated

- The gene flanked by LoxP sites is called a floxed gene

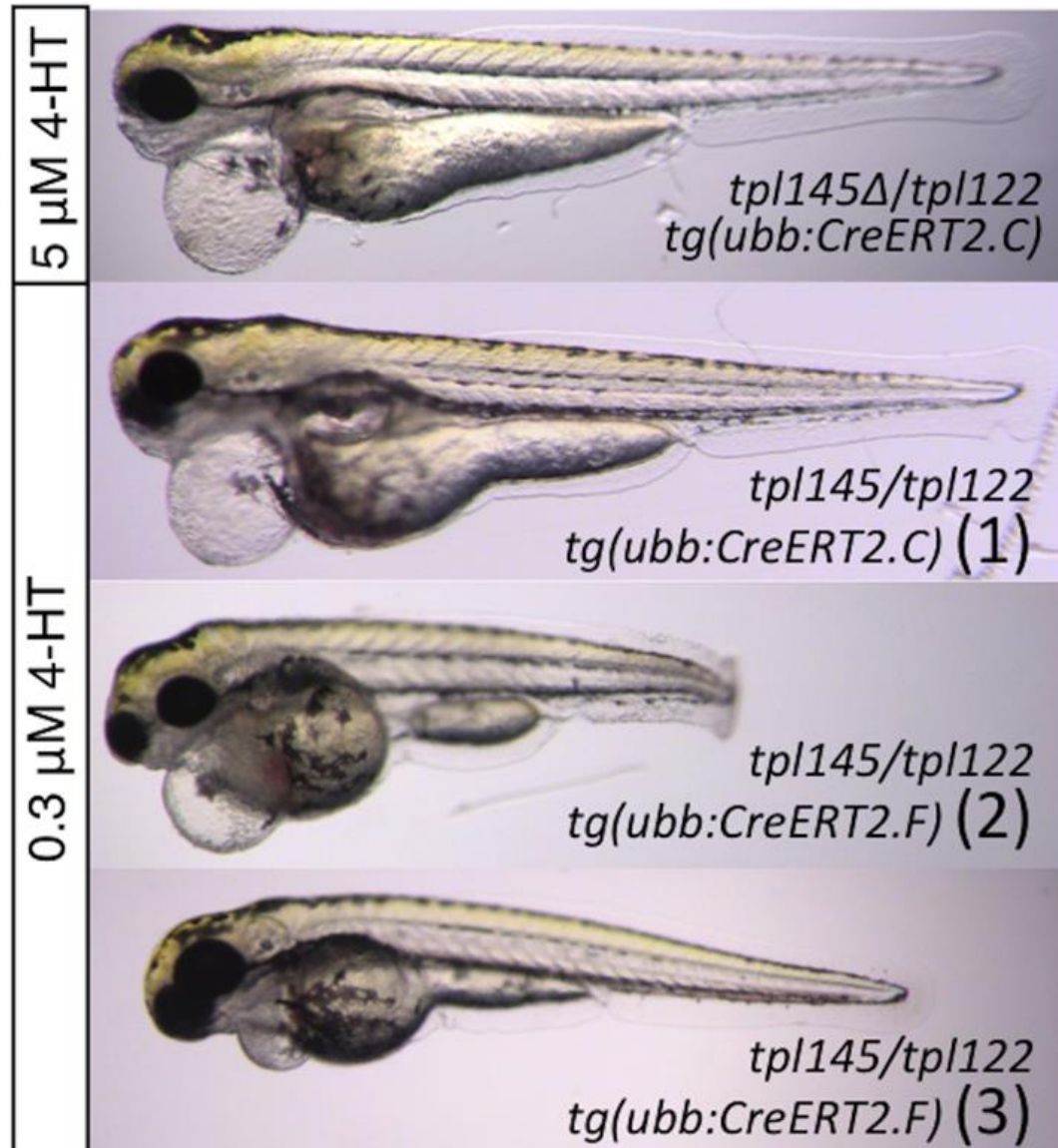
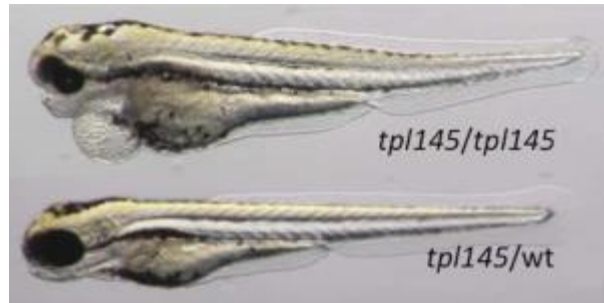
Cre-Lox system in zebrafish



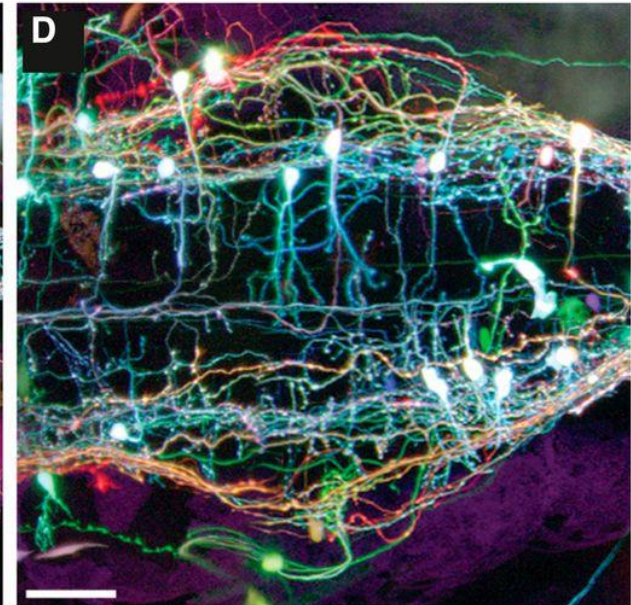
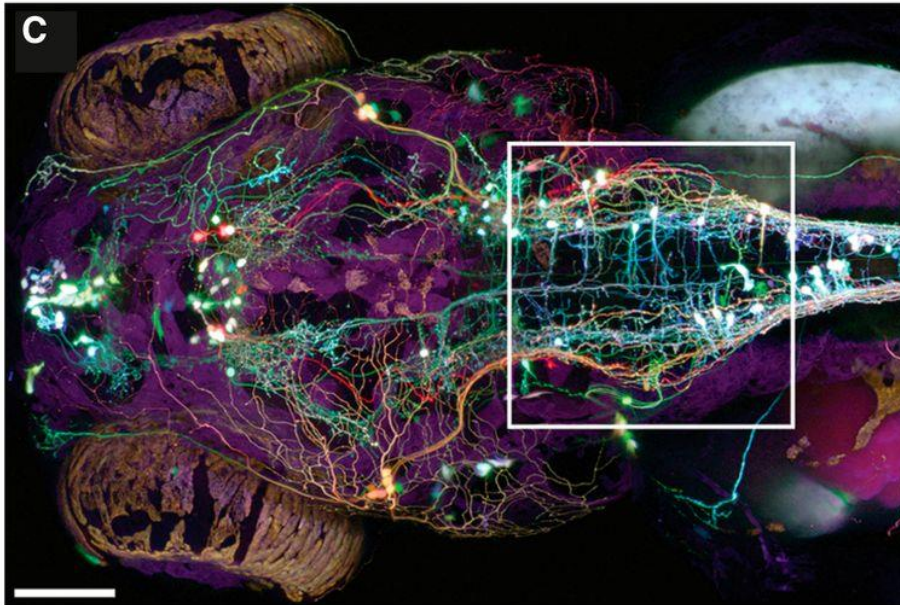
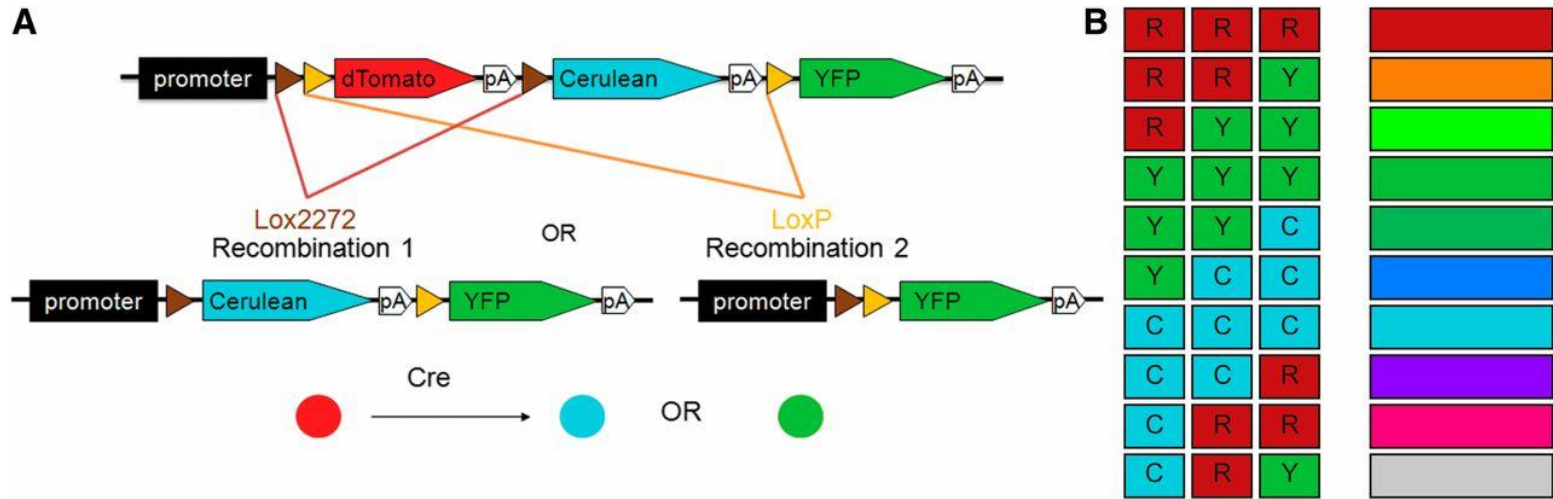
Inducible Cre-ER system



Inducible Cre-ER system in zebrafish



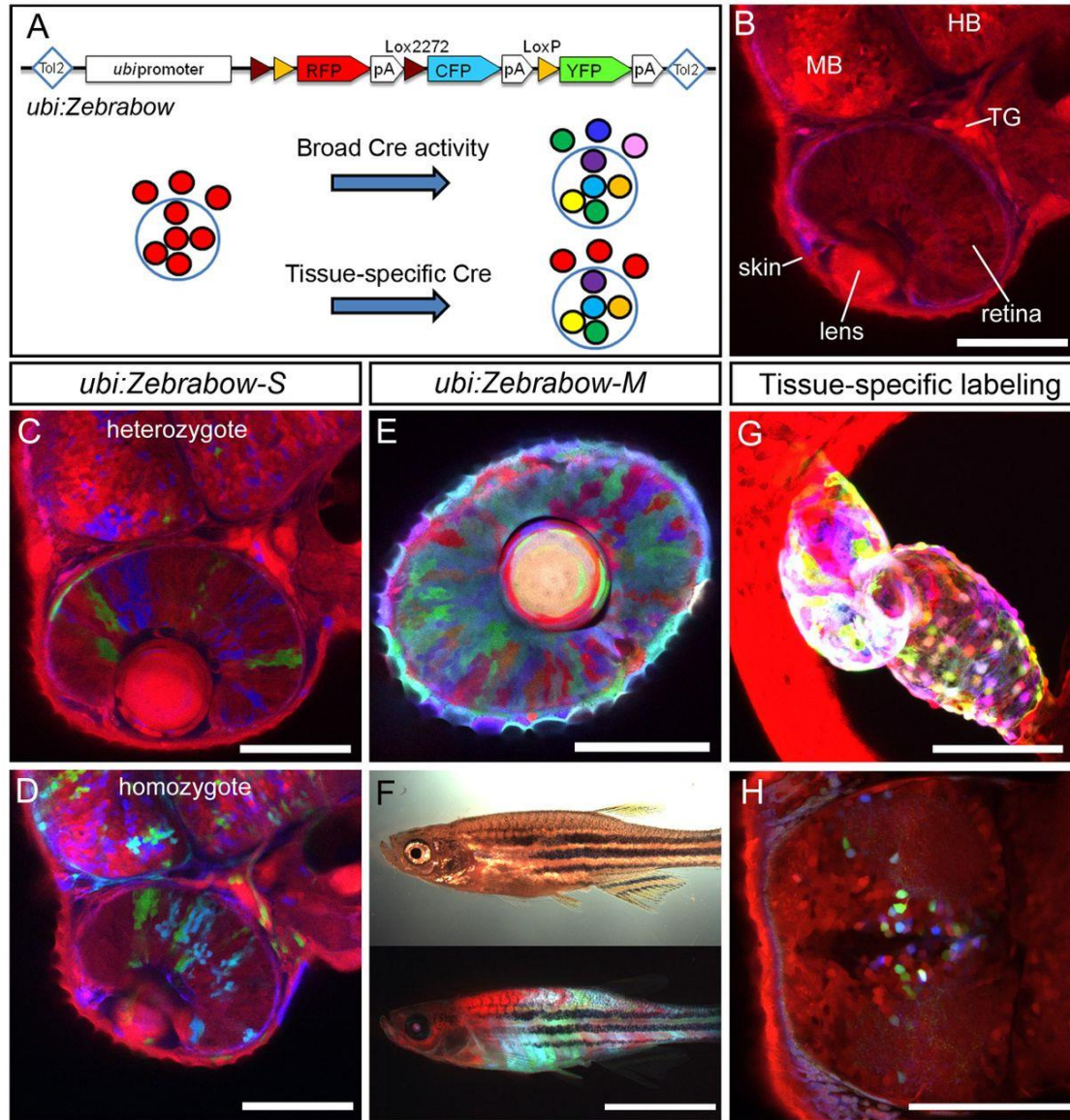
Cre-Lox in Brainbow zebrafish



Lichtman *et al.* (2008)

Pan *et al.* (2011).

Cre-Lox in zebrafish

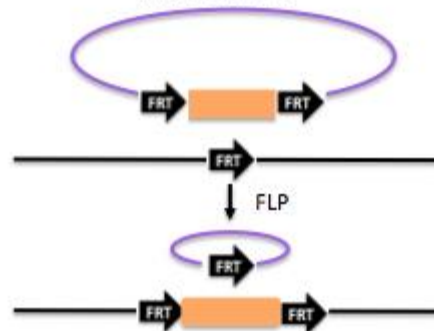


FLP-FRT system

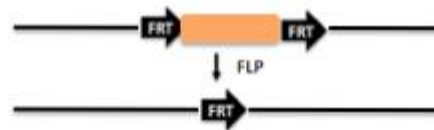
- Derived from yeast 2 μ m plasmid
- Involves the recombination of sequences between short flippase recognition target (FRT) sites by the recombinase flippase (Flp)

FLP-FRT system

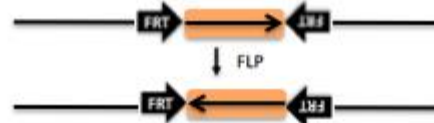
1: Insertion



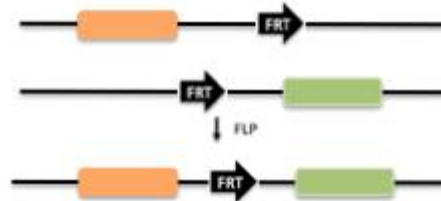
2: Excision



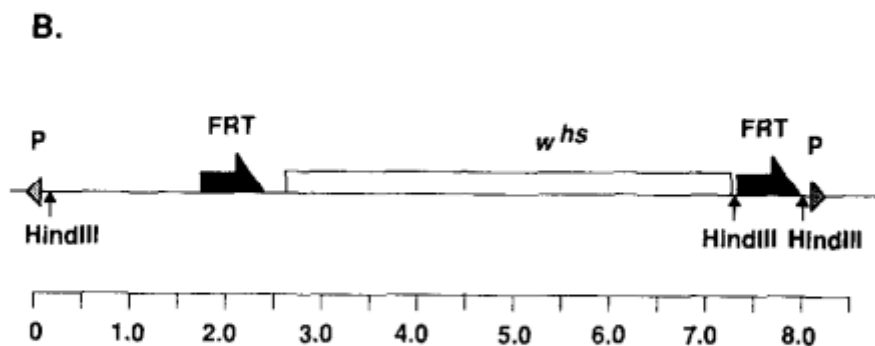
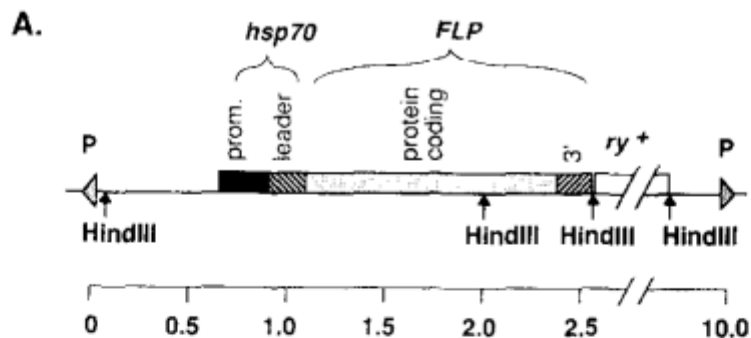
3: Inversion



4: Translocation



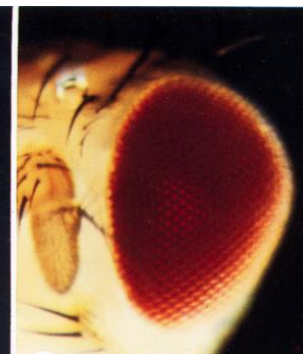
FLP-FRT system in Drosophila



w1118/Y



w1118/Y;
P[>w^{hs}>]/+



w1118/Y;
P[>w^{hs}>]/
P[>w^{hs}>]

Figure 2. Schematic Diagram of FLP and FRT Constructs

(A) *P[ry⁺, hsFLP]*. The *hsp70-FLP* fusion gene (*hsFLP*) is diagrammed. This was cloned into a P element vector which also carried *ry⁺* as a marker for germline transformation.

(B) *P[>w^{hs}>]*. The FRT-flanked *w^{hs}* gene is diagrammed. In both (A) and (B), the P element terminal inverted repeats are indicated by shaded arrowheads. The coordinates beneath each diagram indicate approximate distance in kilobase pairs from the leftmost P element end.

Lessons from mice studies

- Majority of CRE strains exhibit some degree of unreported recombinase activity.
- Strains exhibit
 - frequent mosaicism,
 - inconsistent deletion activity and
 - parent-of-origin effects.
- It is necessary to characterize CRE strains robustly.



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