Easi-CRISPR for conditional and insertional alleles

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Types of Genome edits

- **Gene disruption/inactivation**

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- **Subtle changes**

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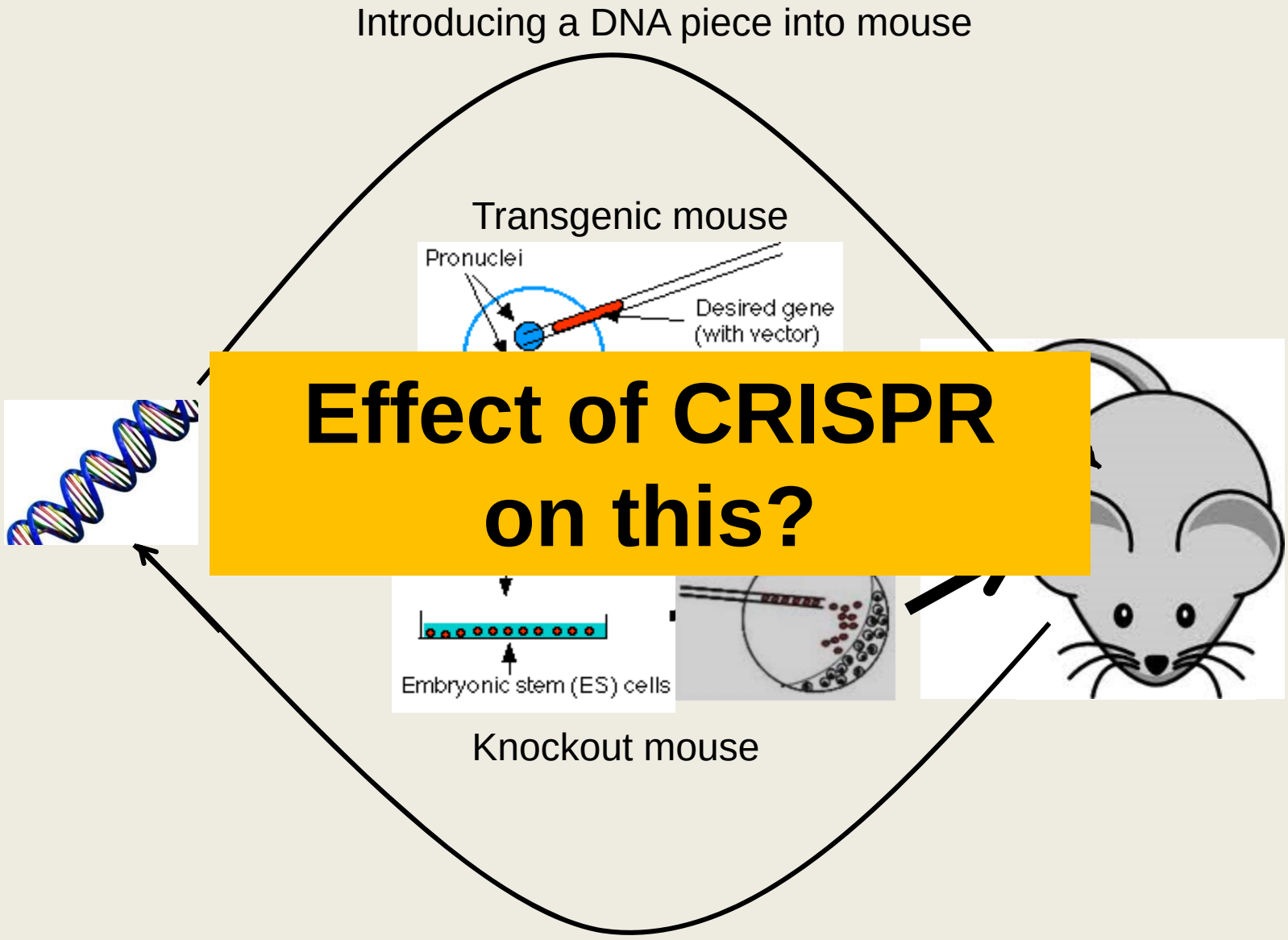
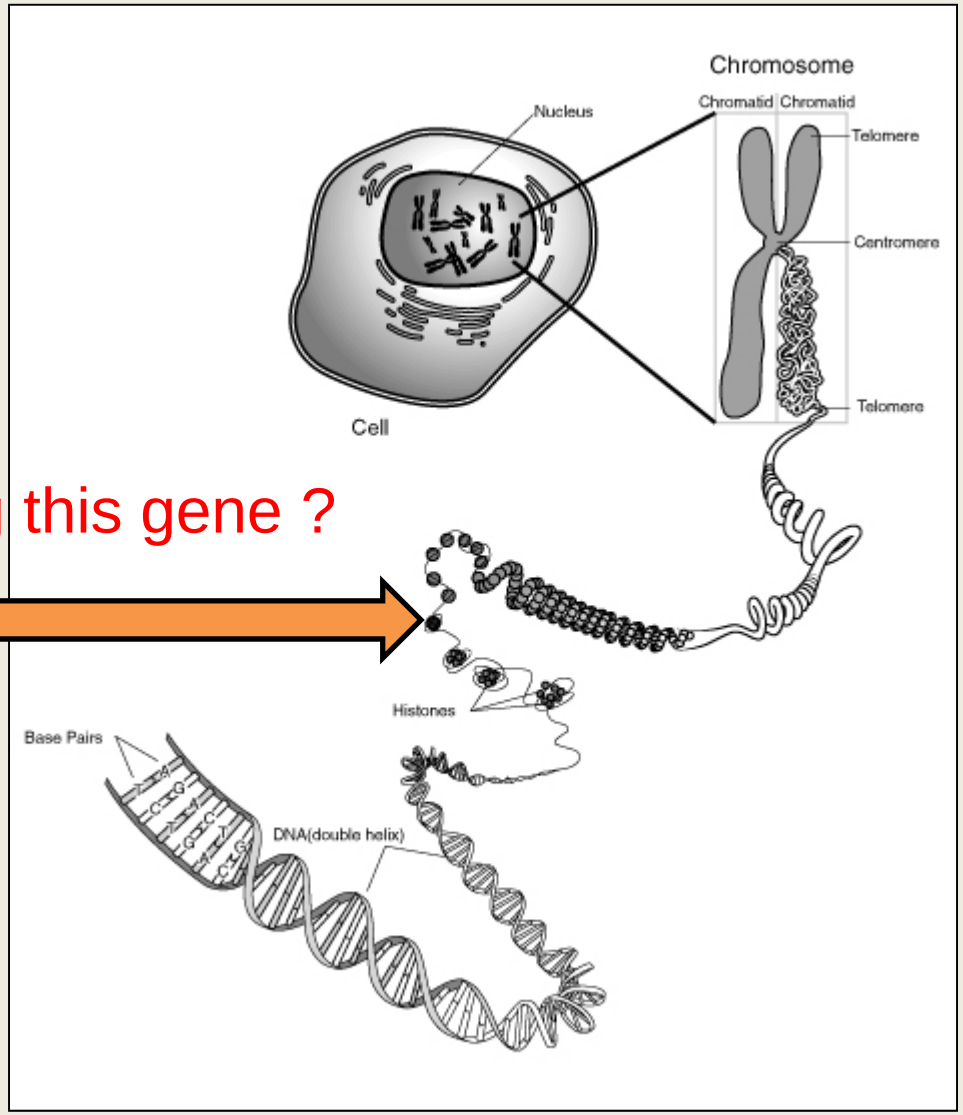
+ donor DNA

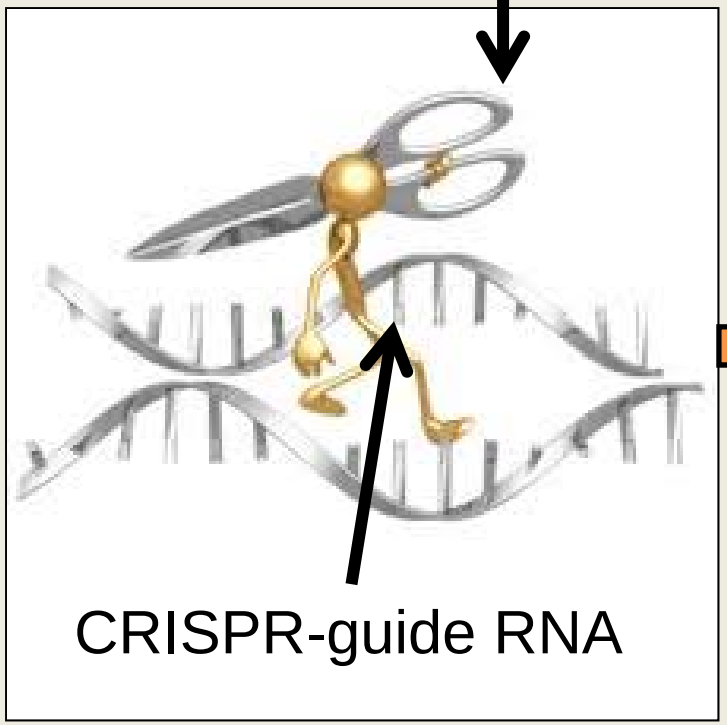

Introducing a DNA piece into mouse
Transgenic mouse
Pronuclei
Desired gene
(with vector)
Effect of CRISPR
on this?
Embryonic stem (ES) cells
Knockout mouse
Deleting a DNA piece from mouse

Targeting this gene ?

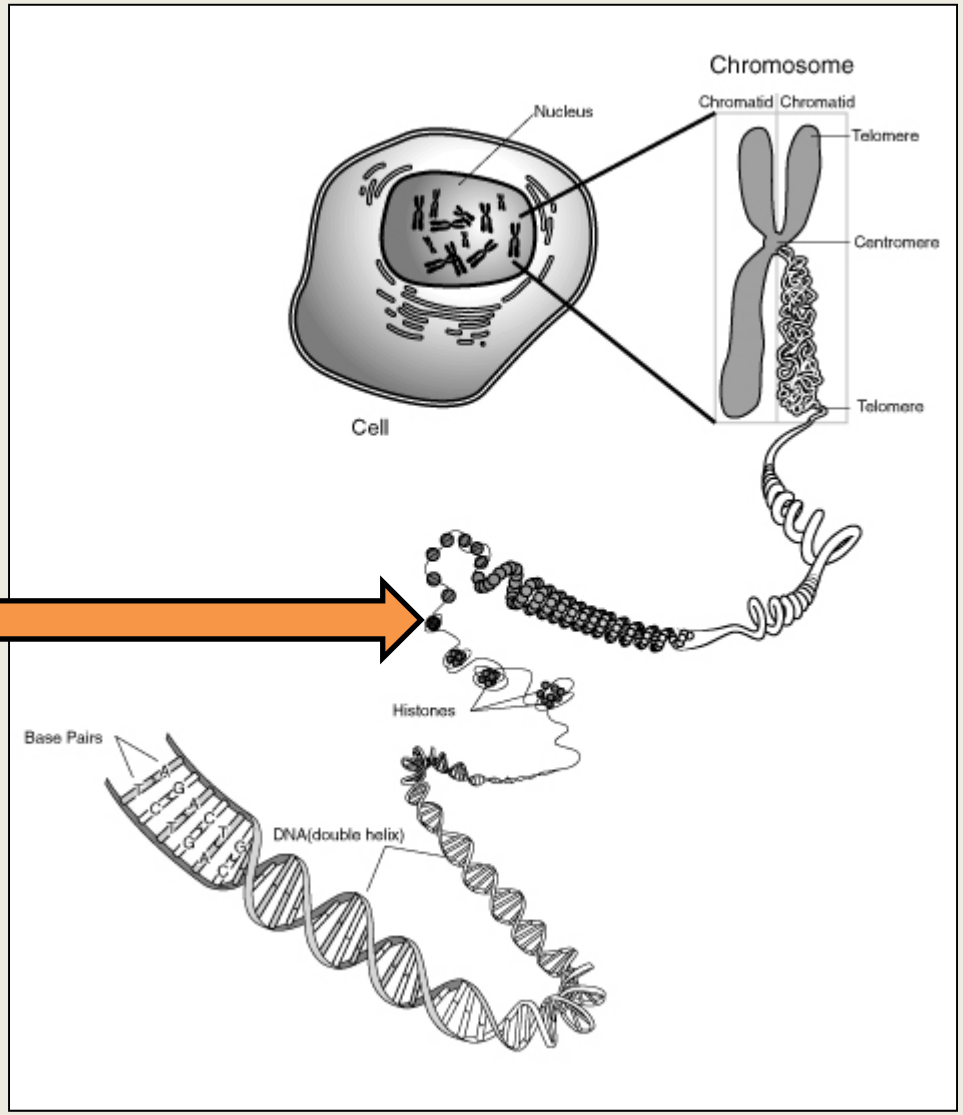


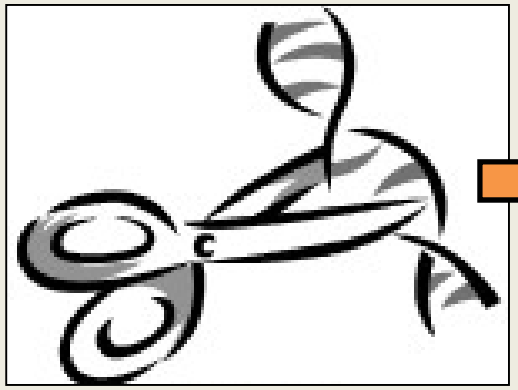
CRISPR/Cas9 system

Cas9 Nuclease

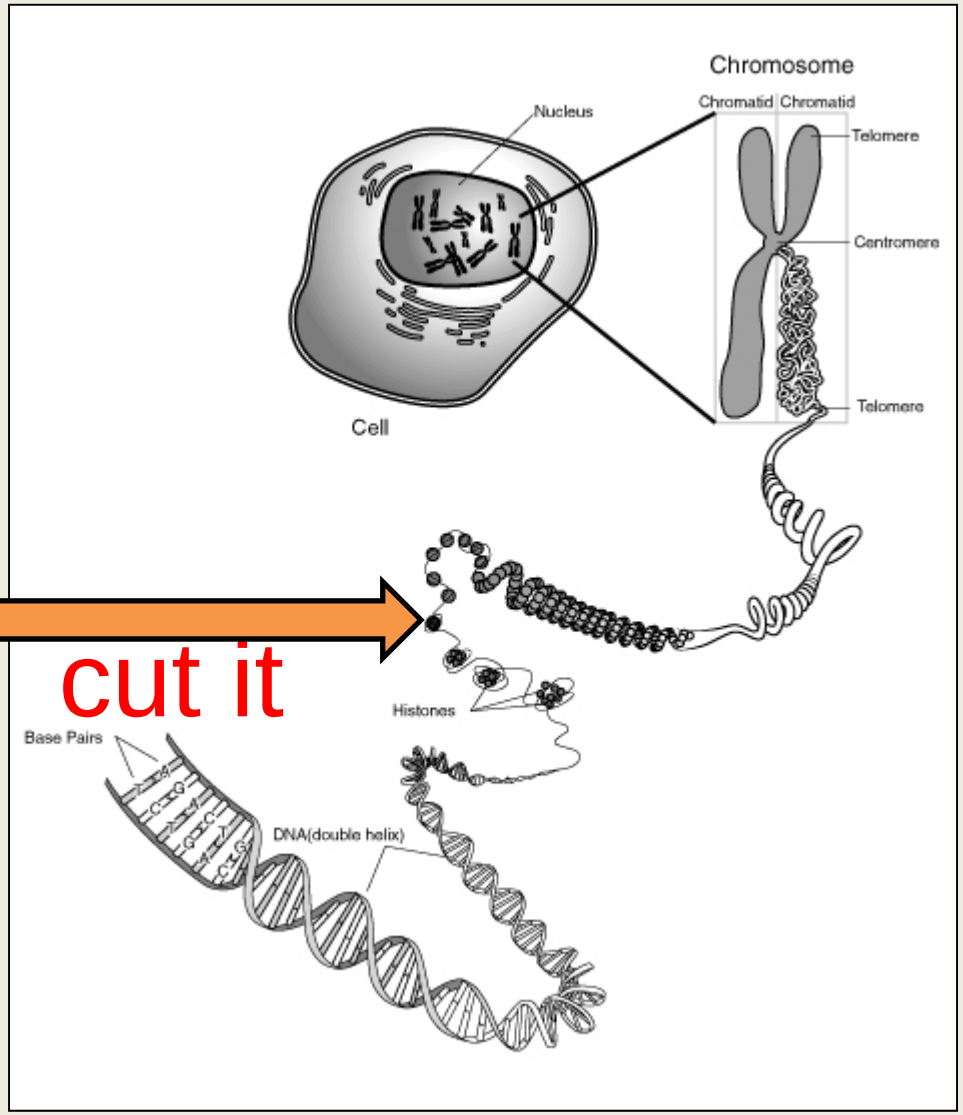


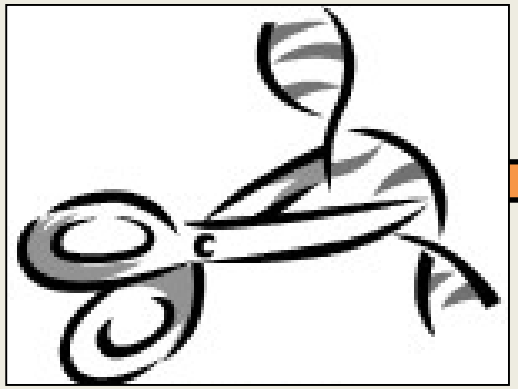
CRISPR-guide RNA

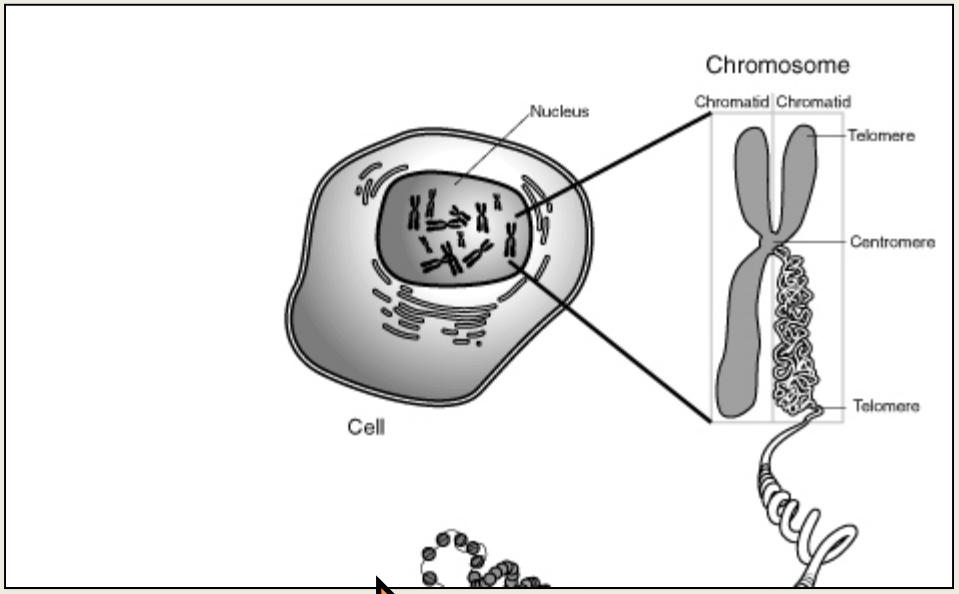




cut it


Chromosome
Nucleus
Chromatid
Chromatid
Telomere
Centromere
Telomere
Cell
Histones
Base Pairs
DNA(double helix)

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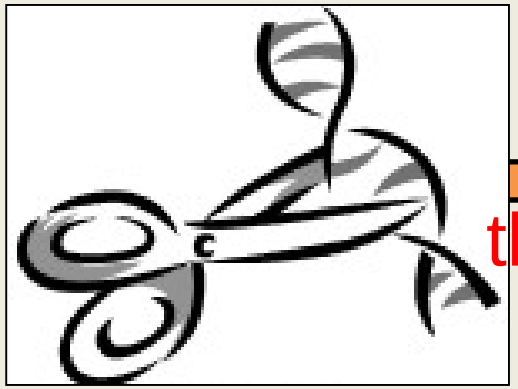
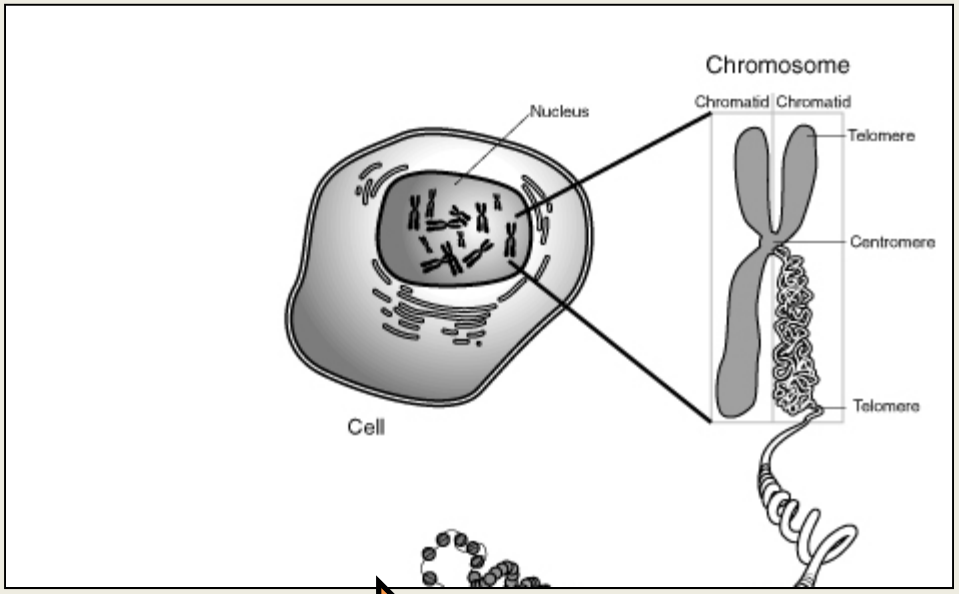
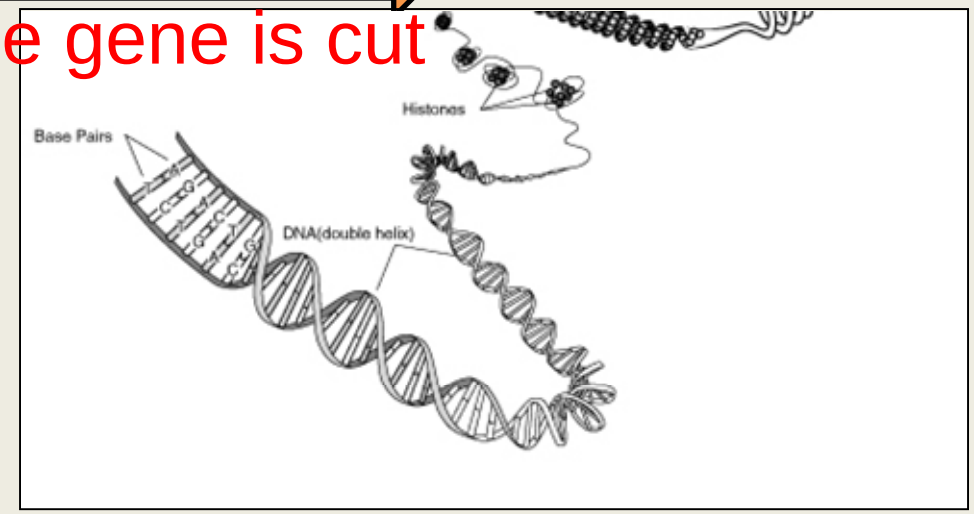
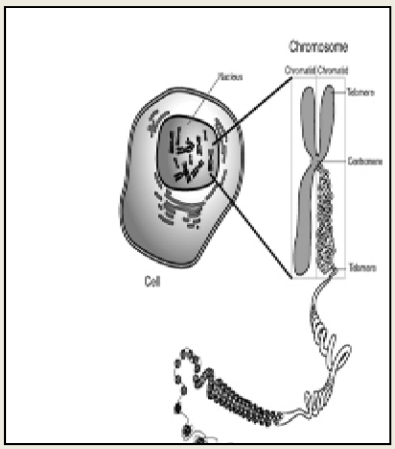




the gene is cut



…what is next?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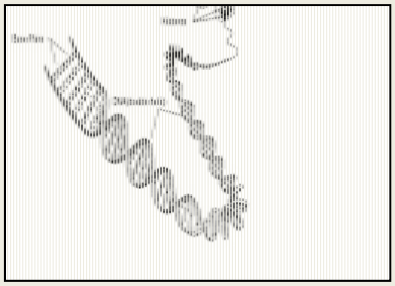
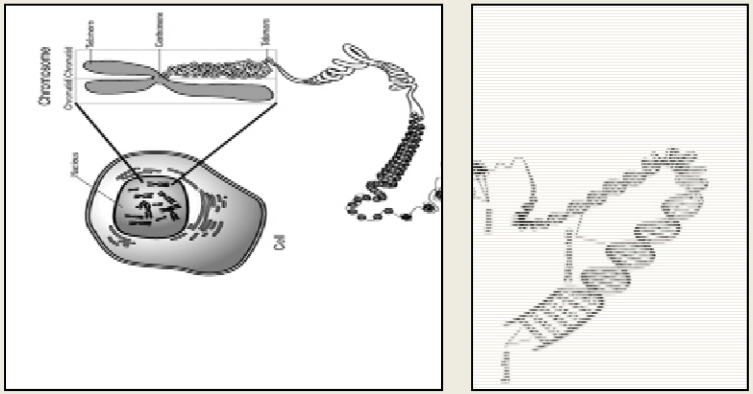
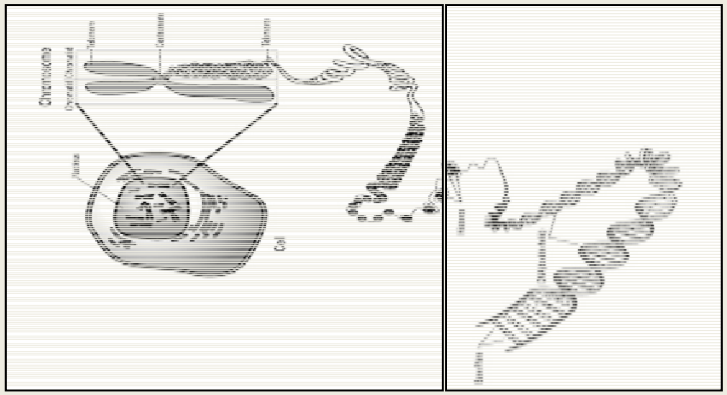




If you do
not do
anything



If you do not do anything

If you add a donor, you can insert the new sequence

Types of Genome edits

- Gene disruption/inactivation
- Subtle changes
- Large cassette insertions
- Gene replacements

The DNA repair system will seal it back quickly…

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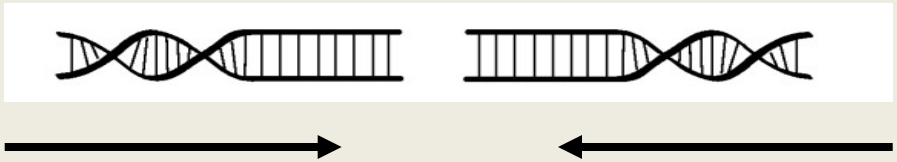

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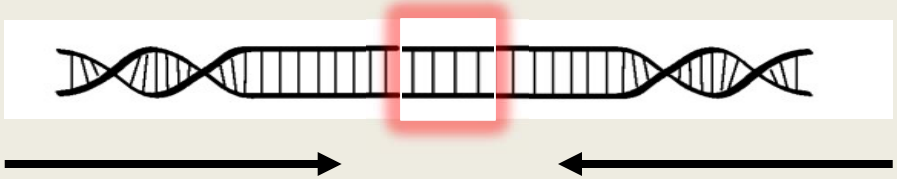

But….joining is usually <u>imprecise</u>

...it disrupts the gene

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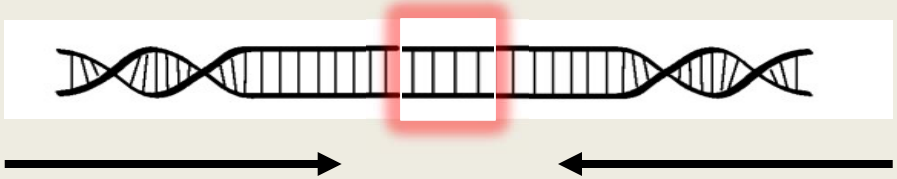

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Non-Homologous End Joining (NHEJ)

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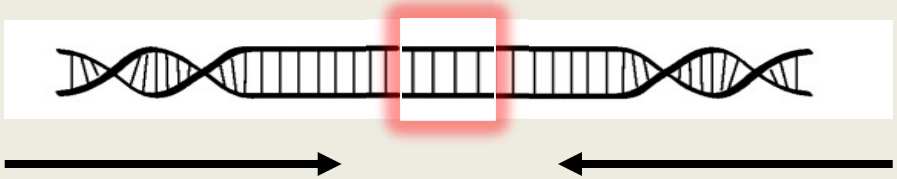

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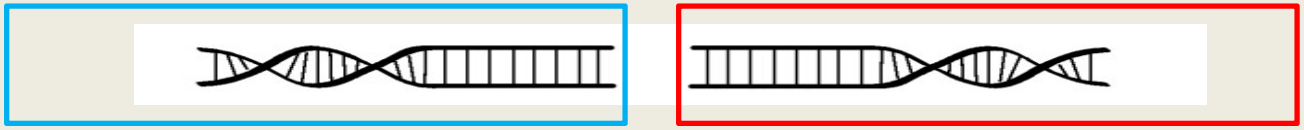

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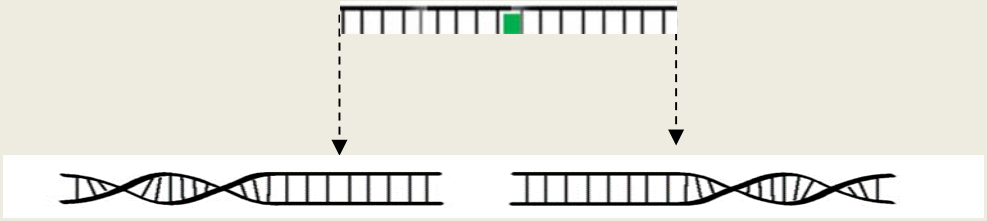

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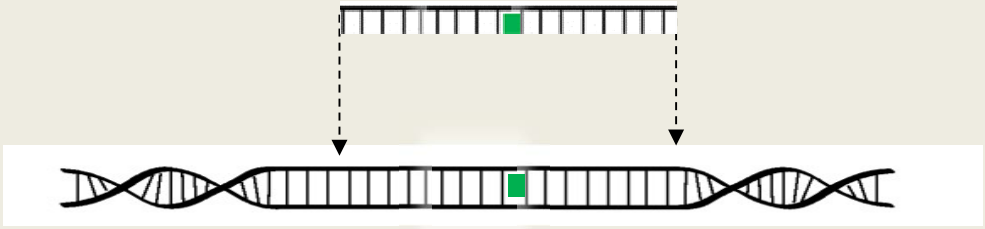

Introduces the change
(only subtle edits)

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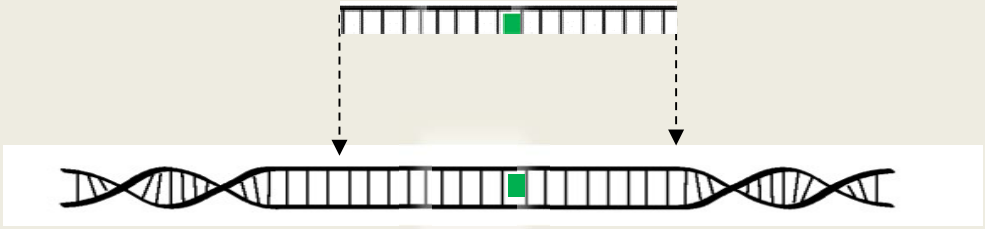

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Homology Directed Repair (HDR)

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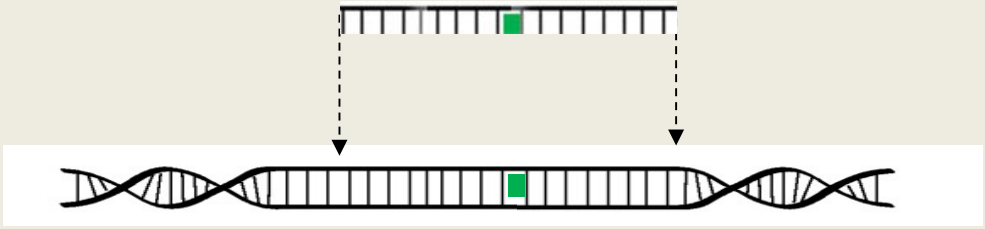

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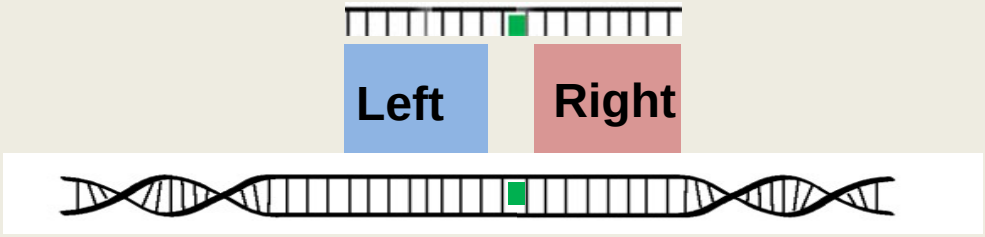

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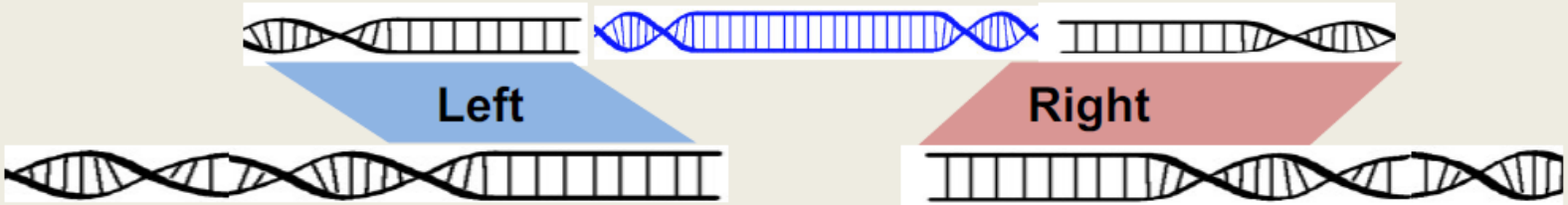

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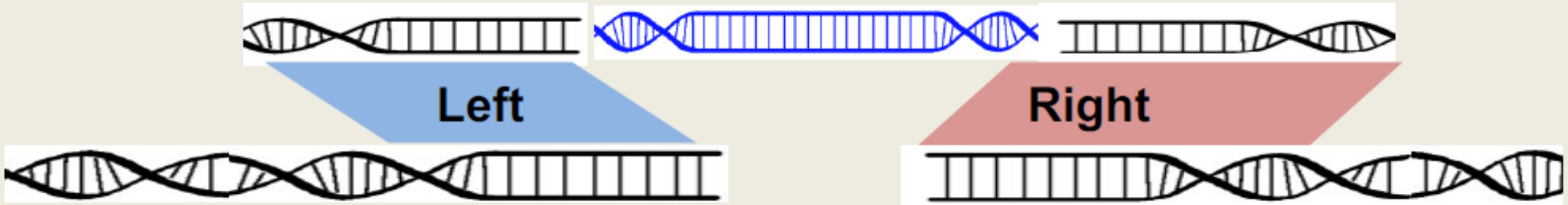

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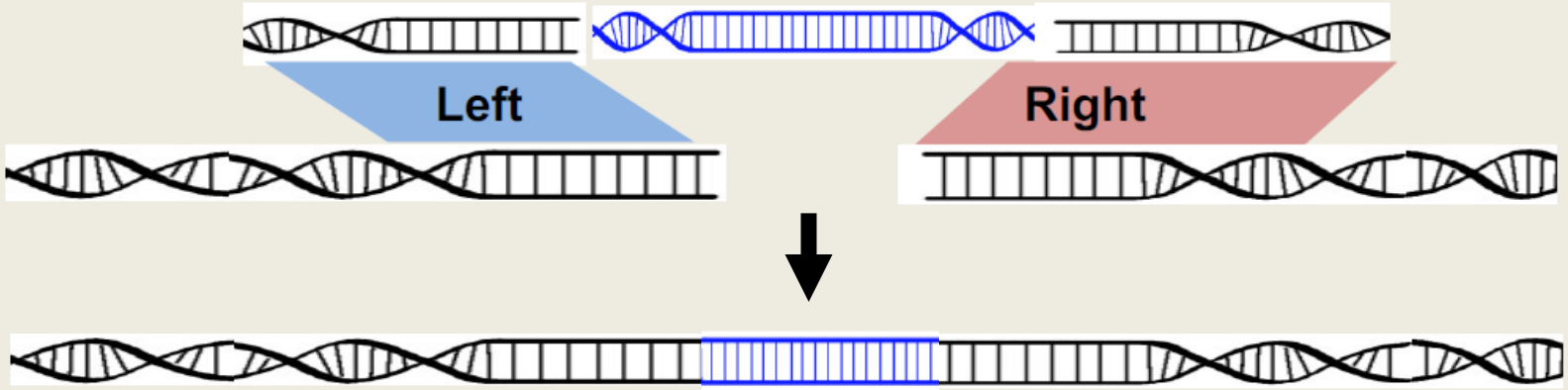
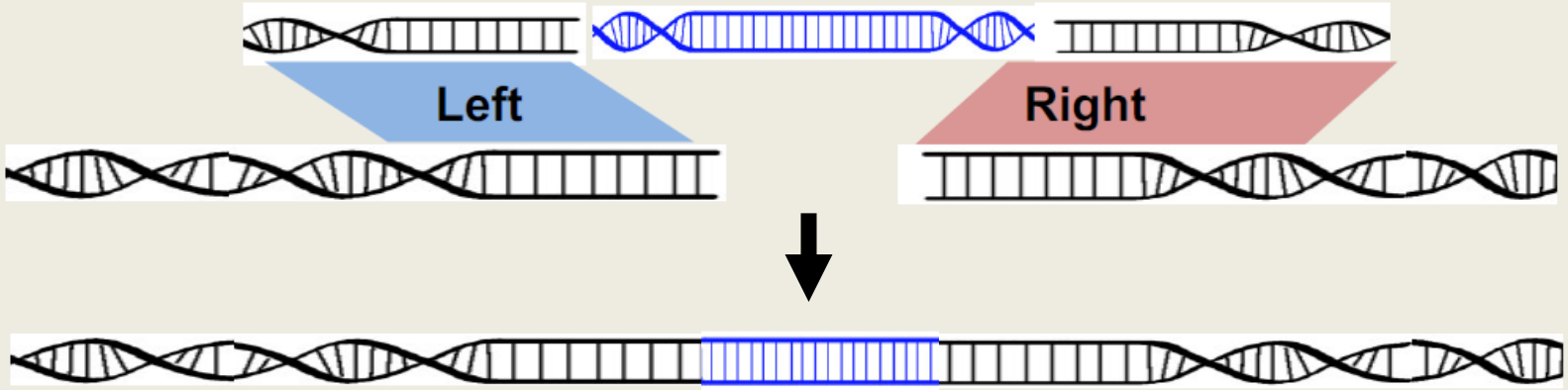


The new DNA cassette gets inserted at the cut site

Provide a longer repair template



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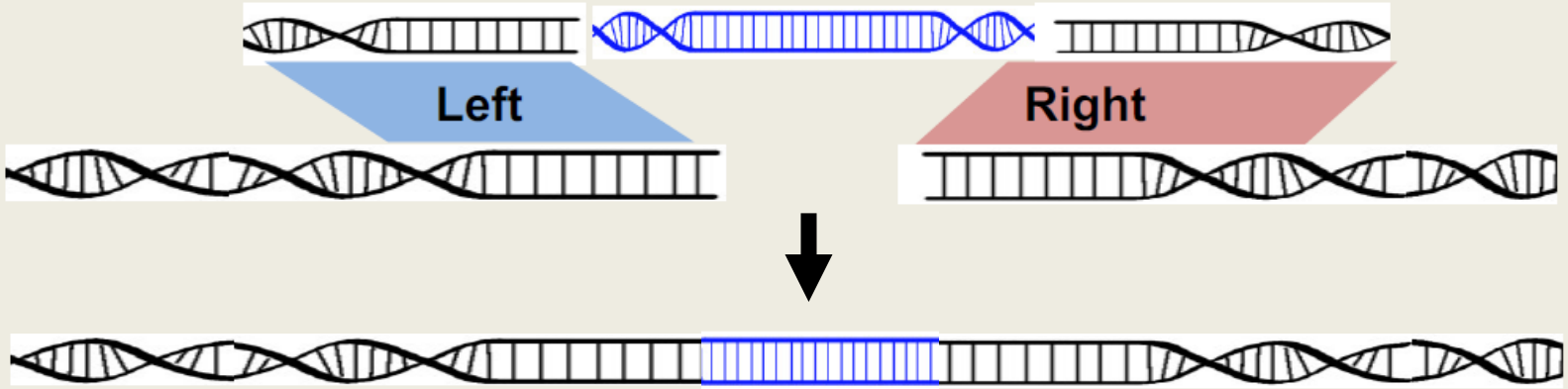
Homologous Recombination (HR)

Provide a **longer** repair template



The new DNA cassette gets inserted at the cut site

Homologous Recombination (HR)

- **Longer homology arms**
- **Usually dsDNA**

Types of Genome edits

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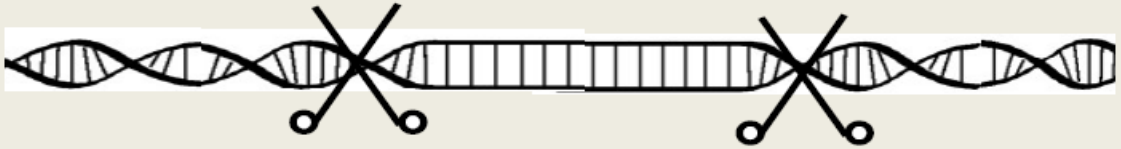
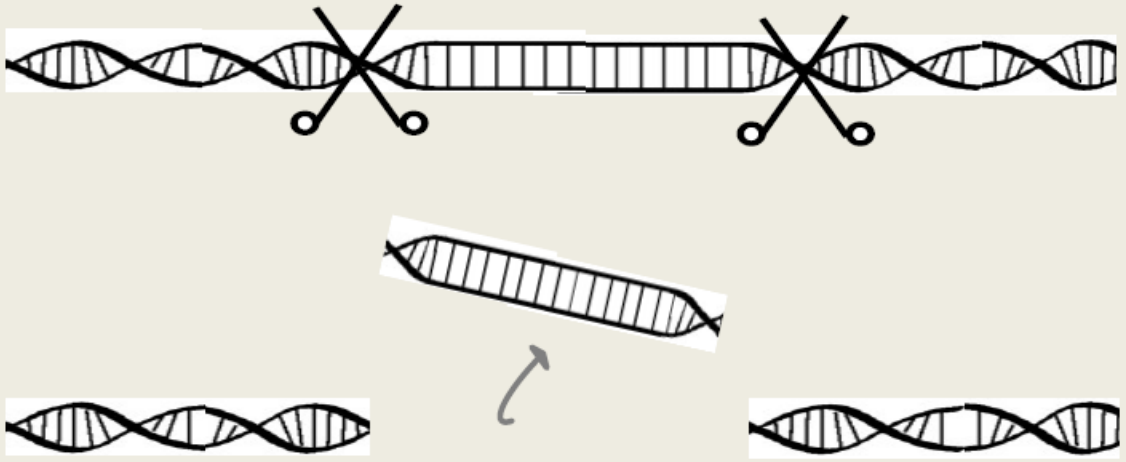
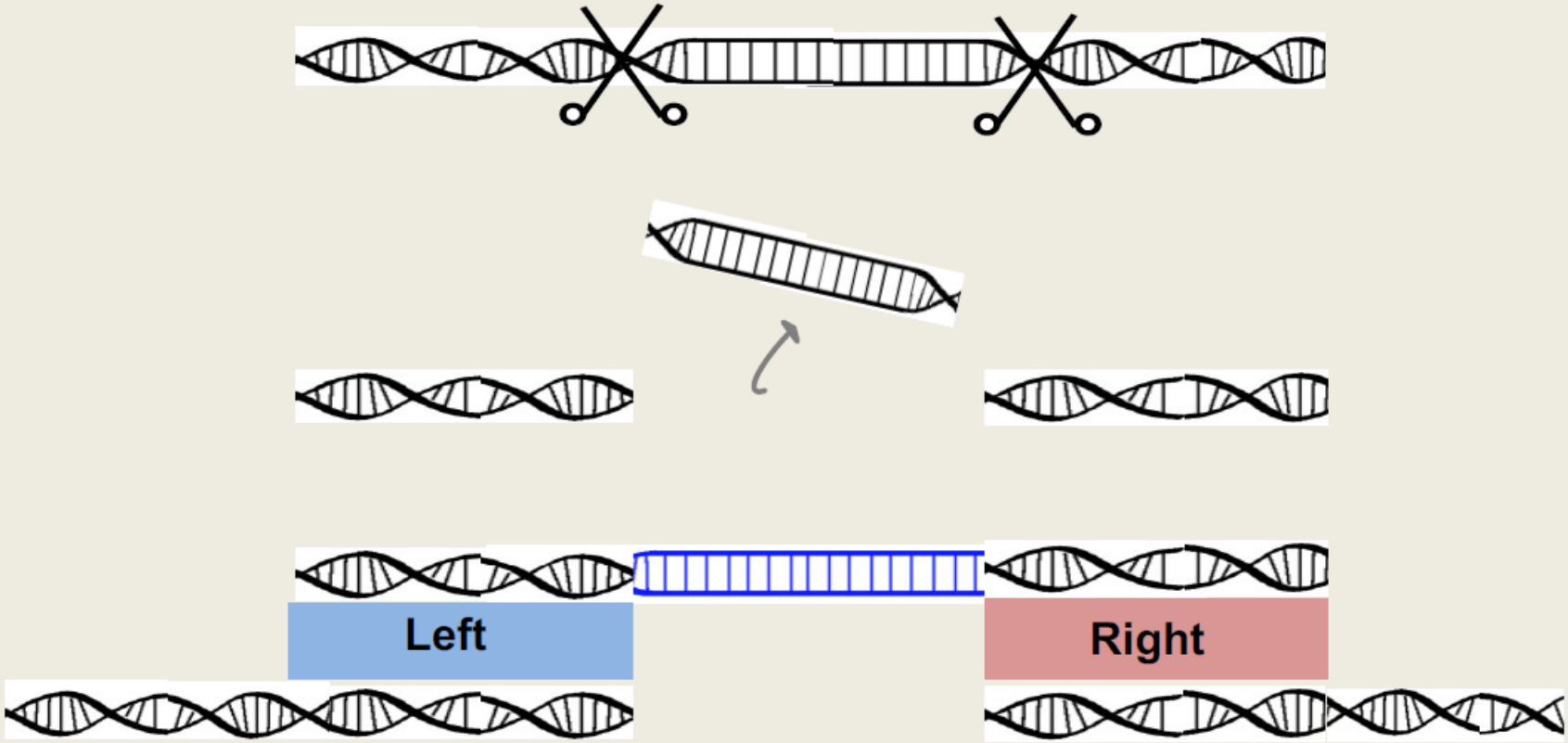
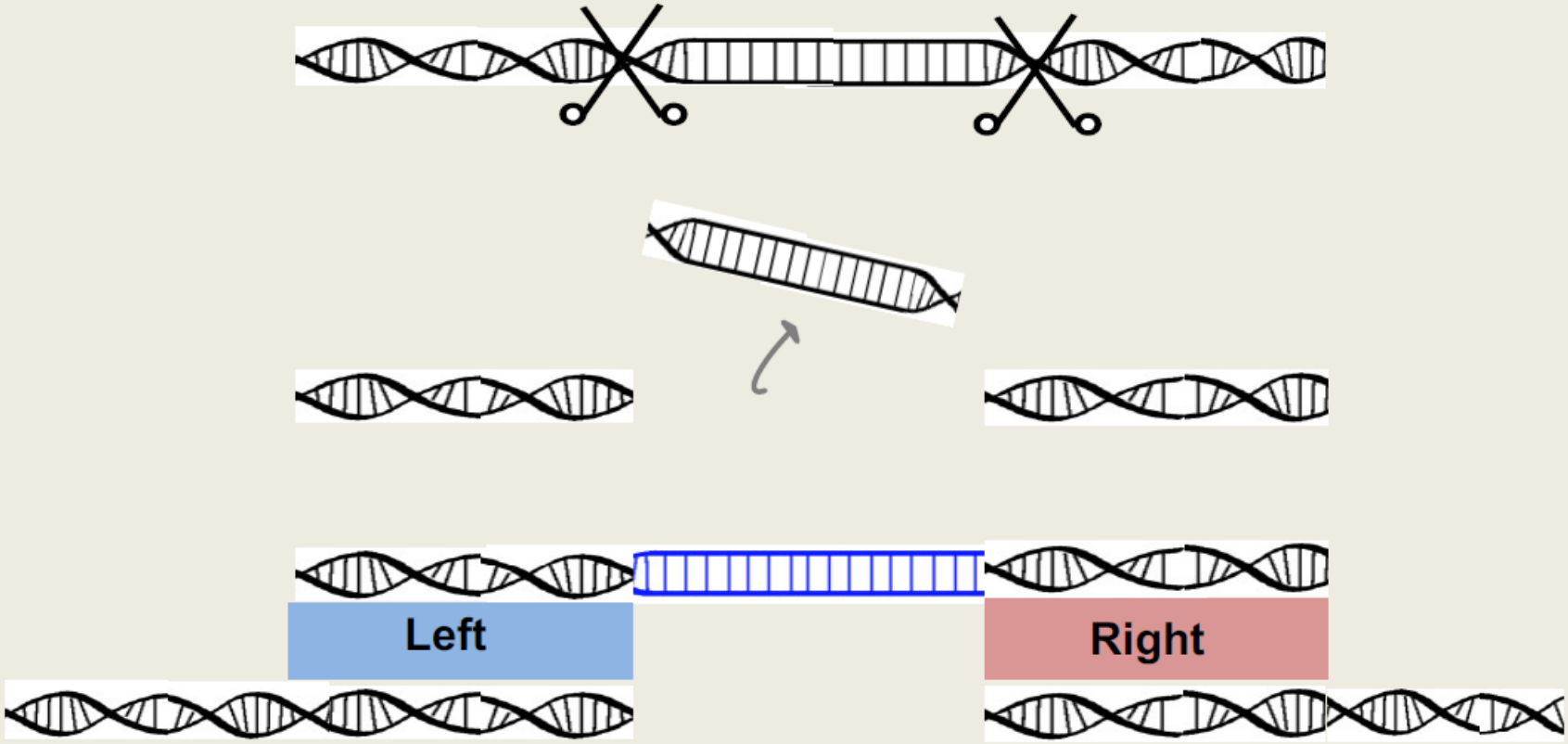
- Subtle changes
- Large cassette insertions
- Gene replacements






Left
Right



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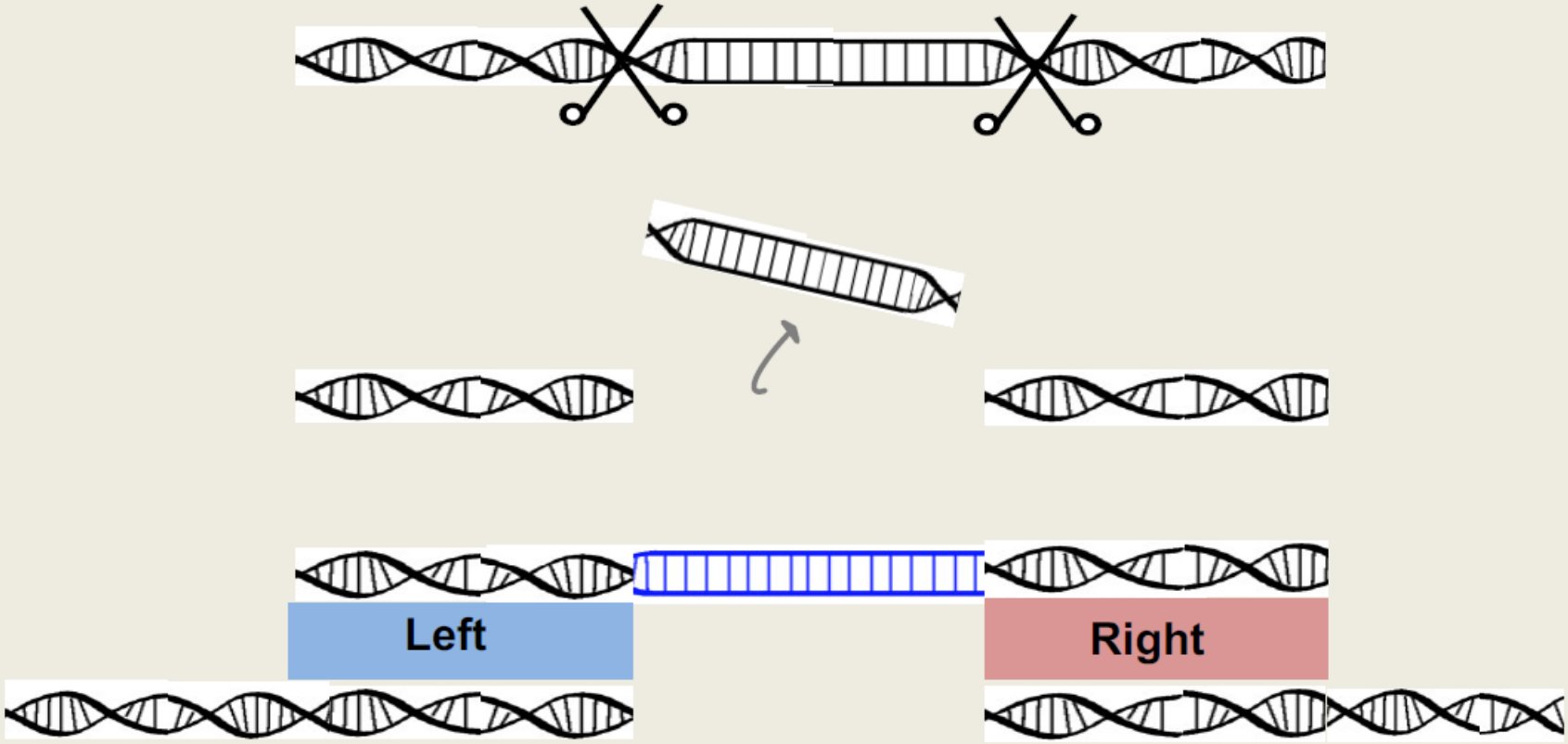

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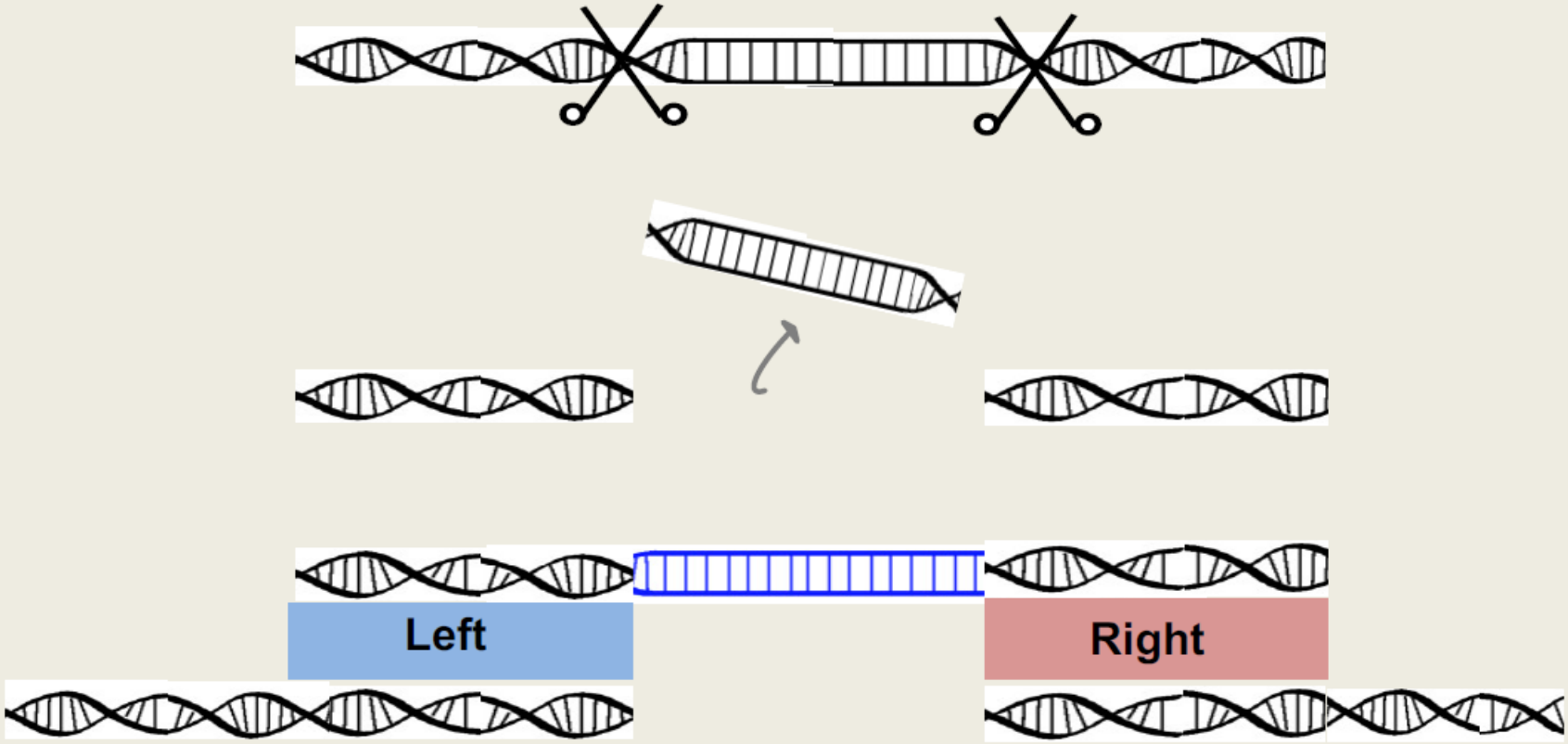

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Homologous Recombination (HR)

- **Longer homology arms**
- **Usually dsDNA**

Types of Genome editing in mice

- Gene disruption/inactivation <1%
- Subtle changes ~5%
- Large cassette insertions] 95%
- Gene replacements

Common Types of Genetically Engineered mice

Type	Ease of making (1: easy, 4; very difficult)
Knockout/Gene disruption *(indels)*	1

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Point mutation knock-In	2	

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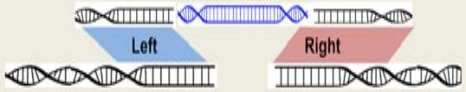
Insertion of new sequences (GFP, Cre, rtTA etc)	3	

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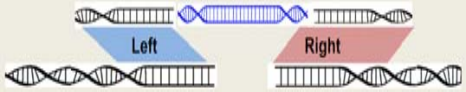
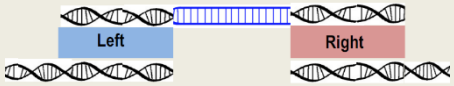
Insertion of new sequences (GFP, Cre, rtTA etc)	3	
Replacement of gene segments (conditional knockout)	4	

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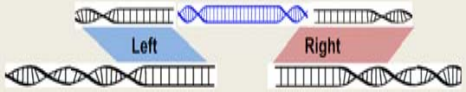
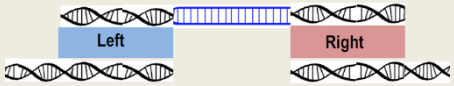
Insertion of new sequences (GFP, Cre, rtTA etc)	3	
Replacement of gene segments (conditional knockout)	4	

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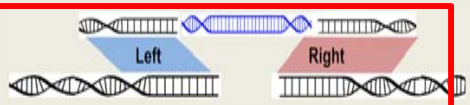
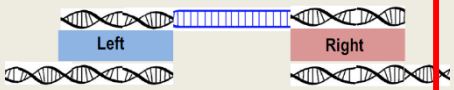
Insertion of new sequences (GFP, Cre, rtTA etc)	3	
Replacement of gene segments (conditional knockout)	4	

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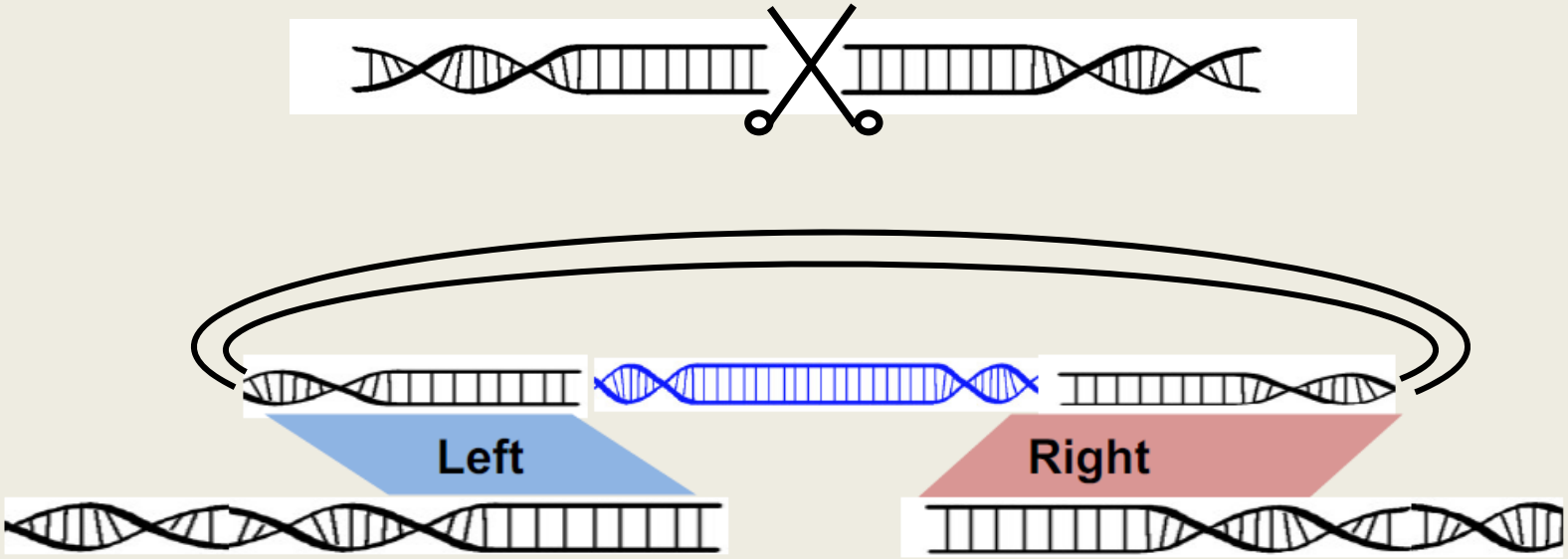
Current Strategies?

Knocking-in



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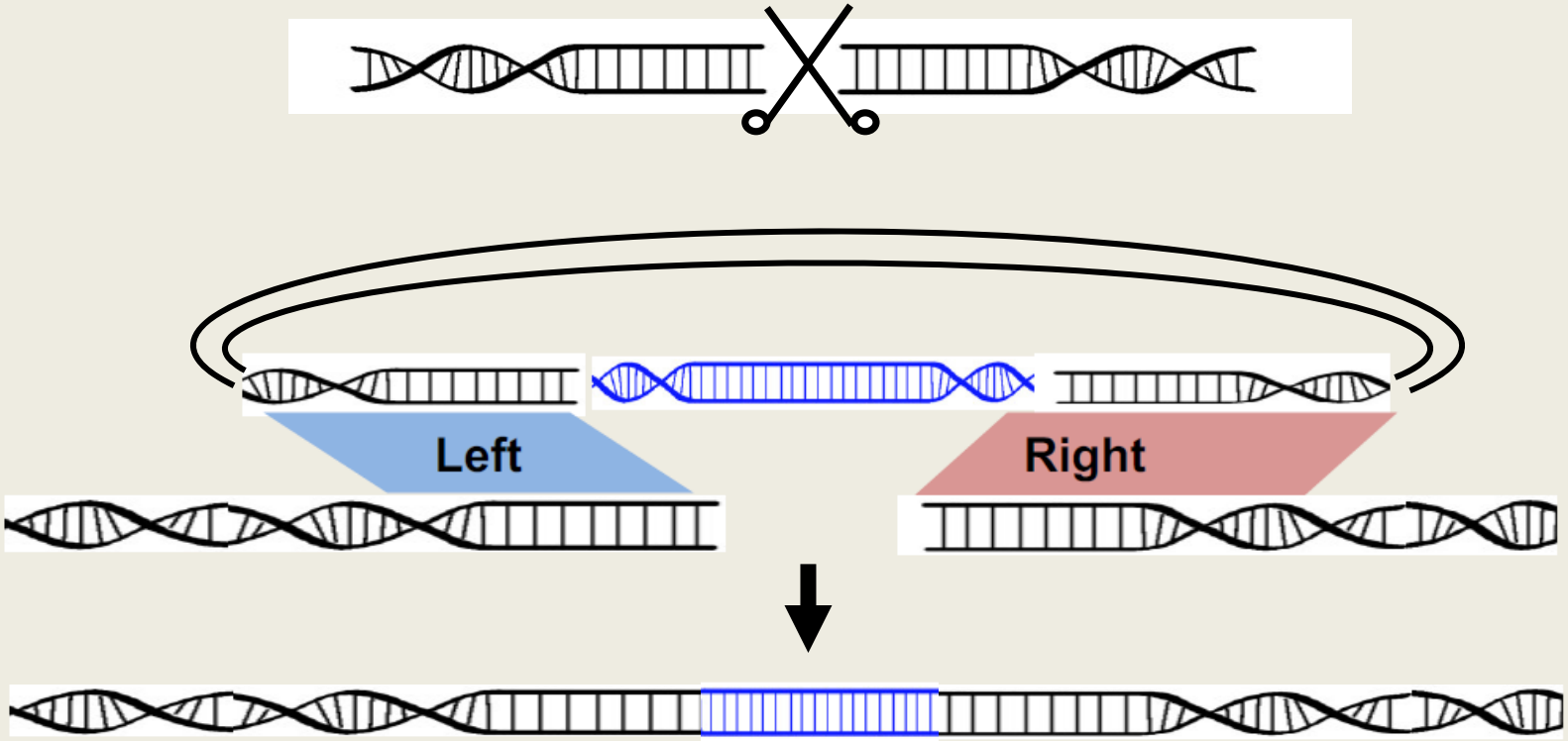

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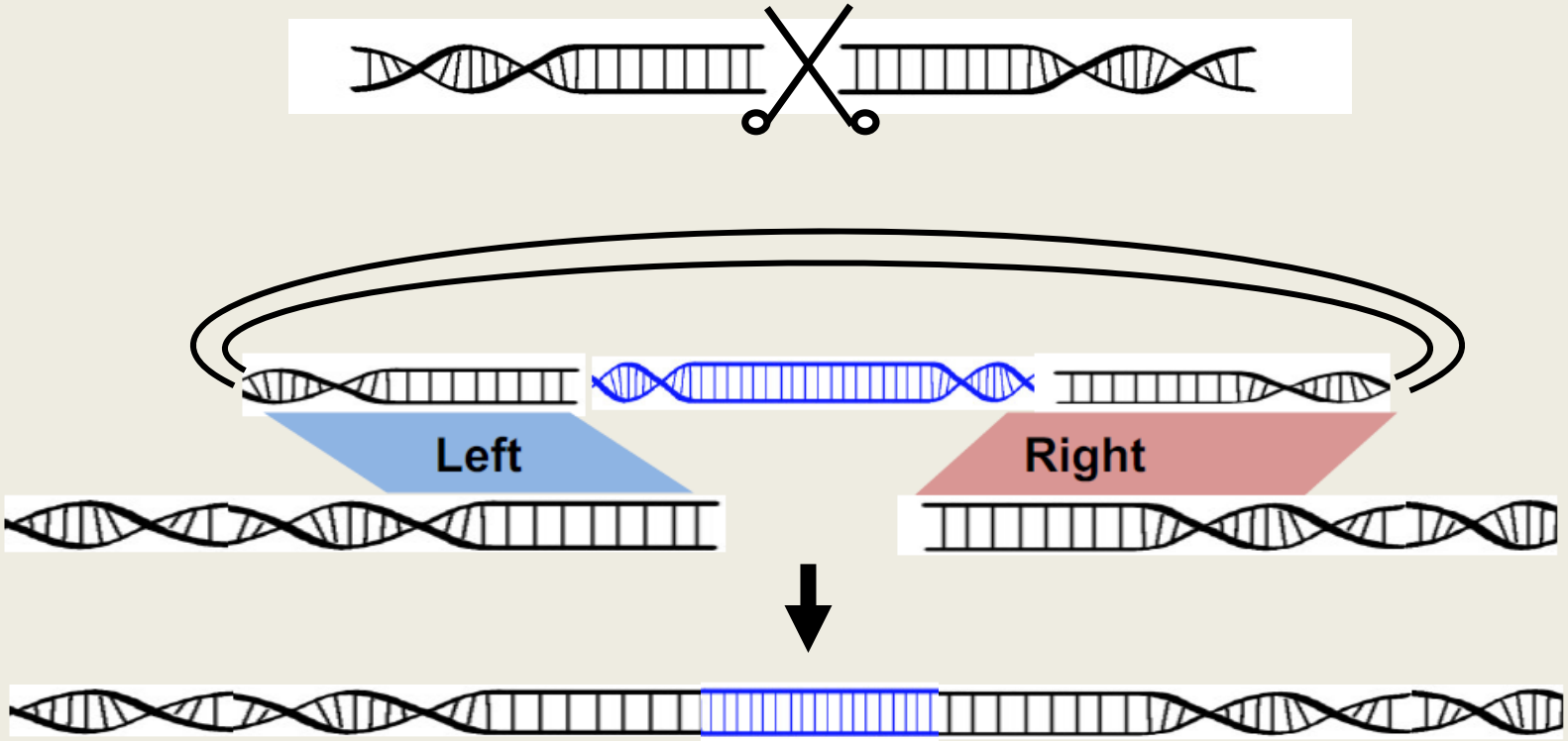
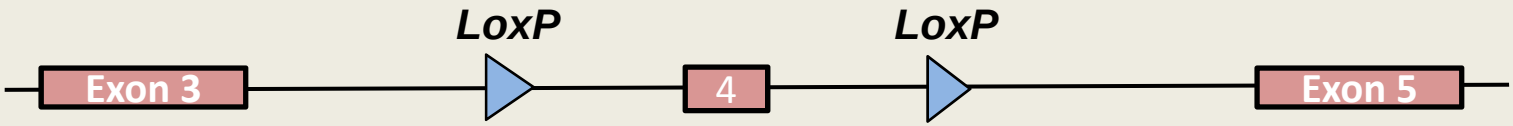


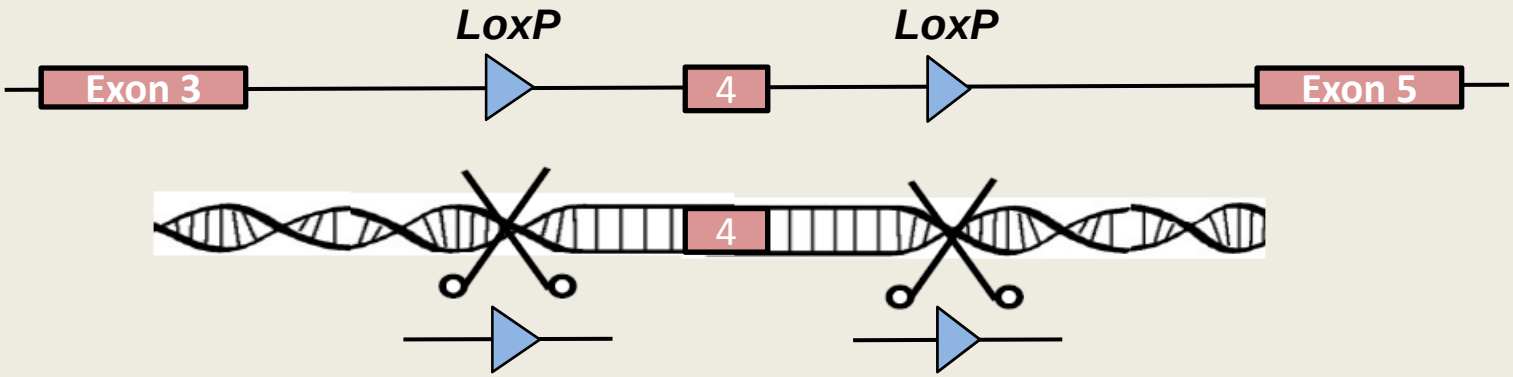
Efficiency?

~1 to 10%

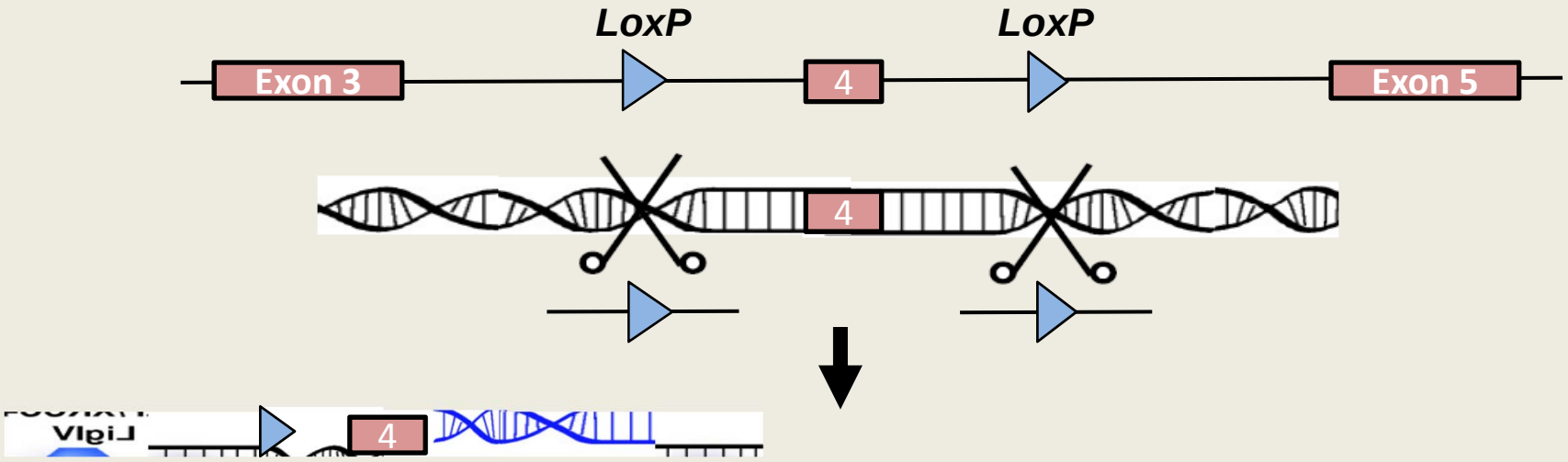
Conditional Knockout



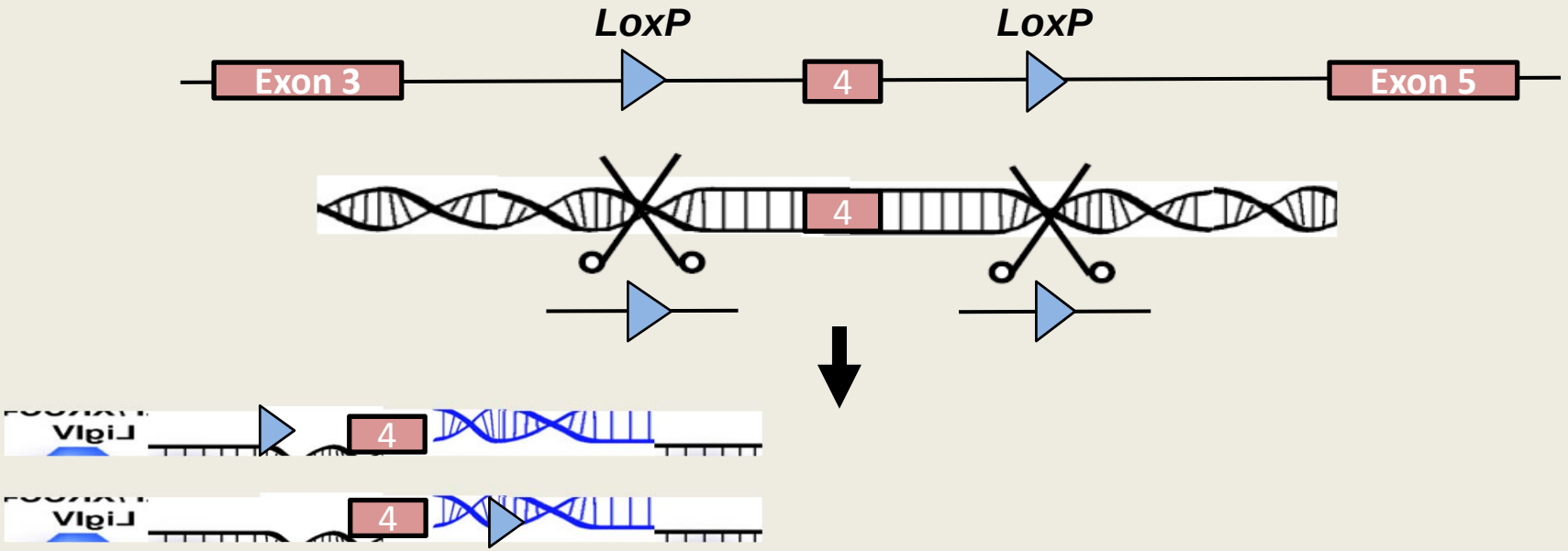
Conditional Knockout



Conditional Knockout



Conditional Knockout



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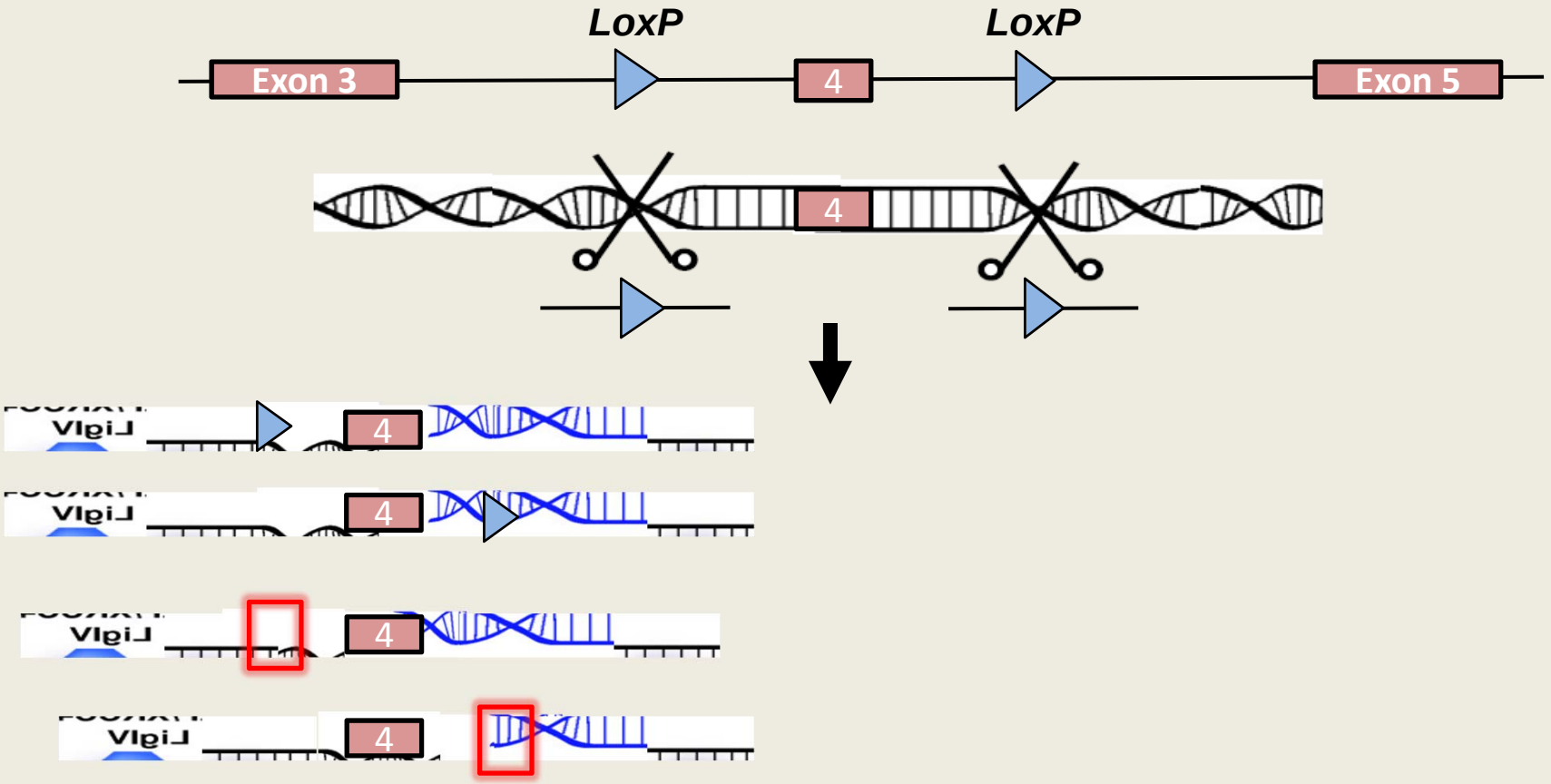
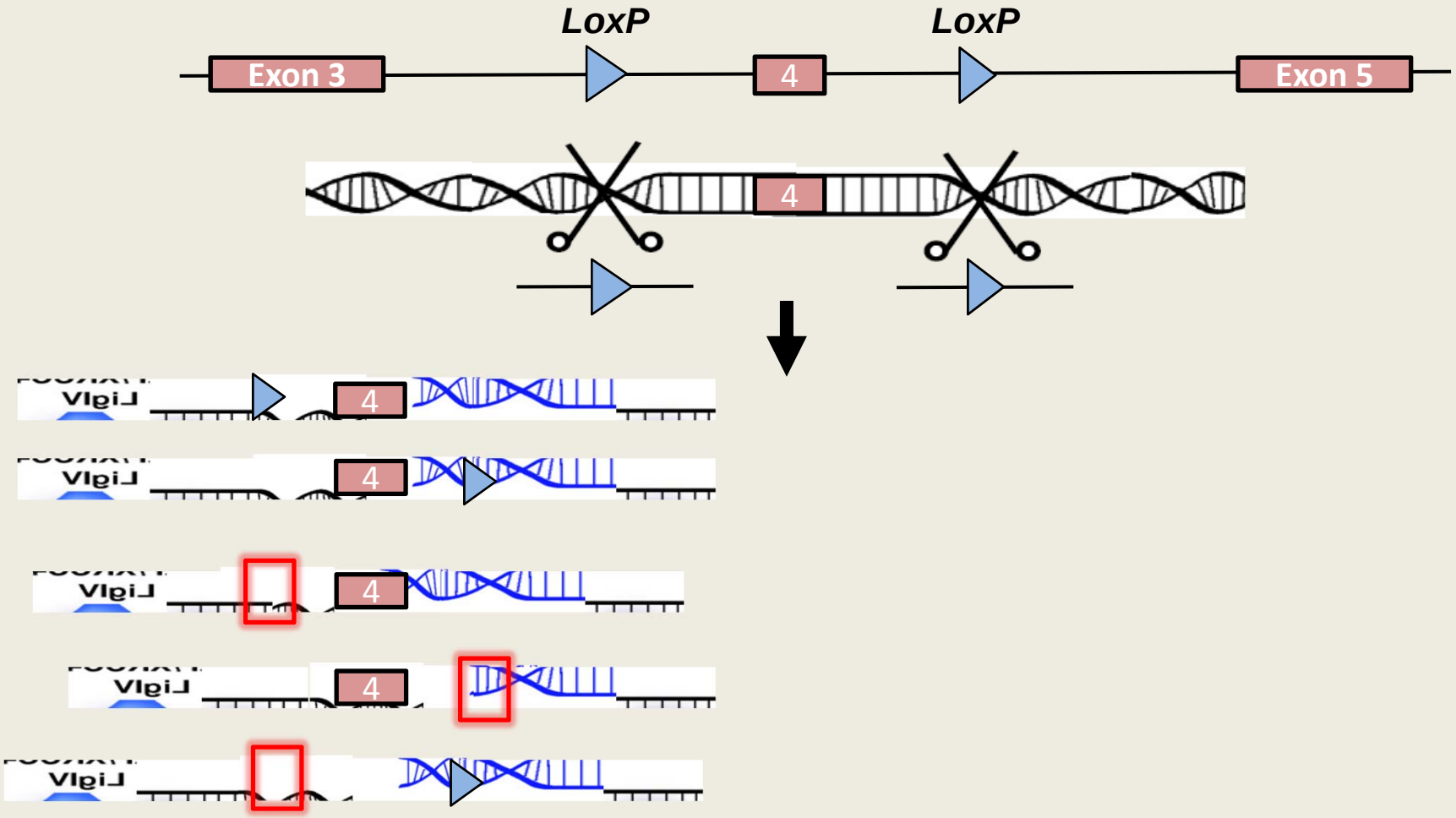
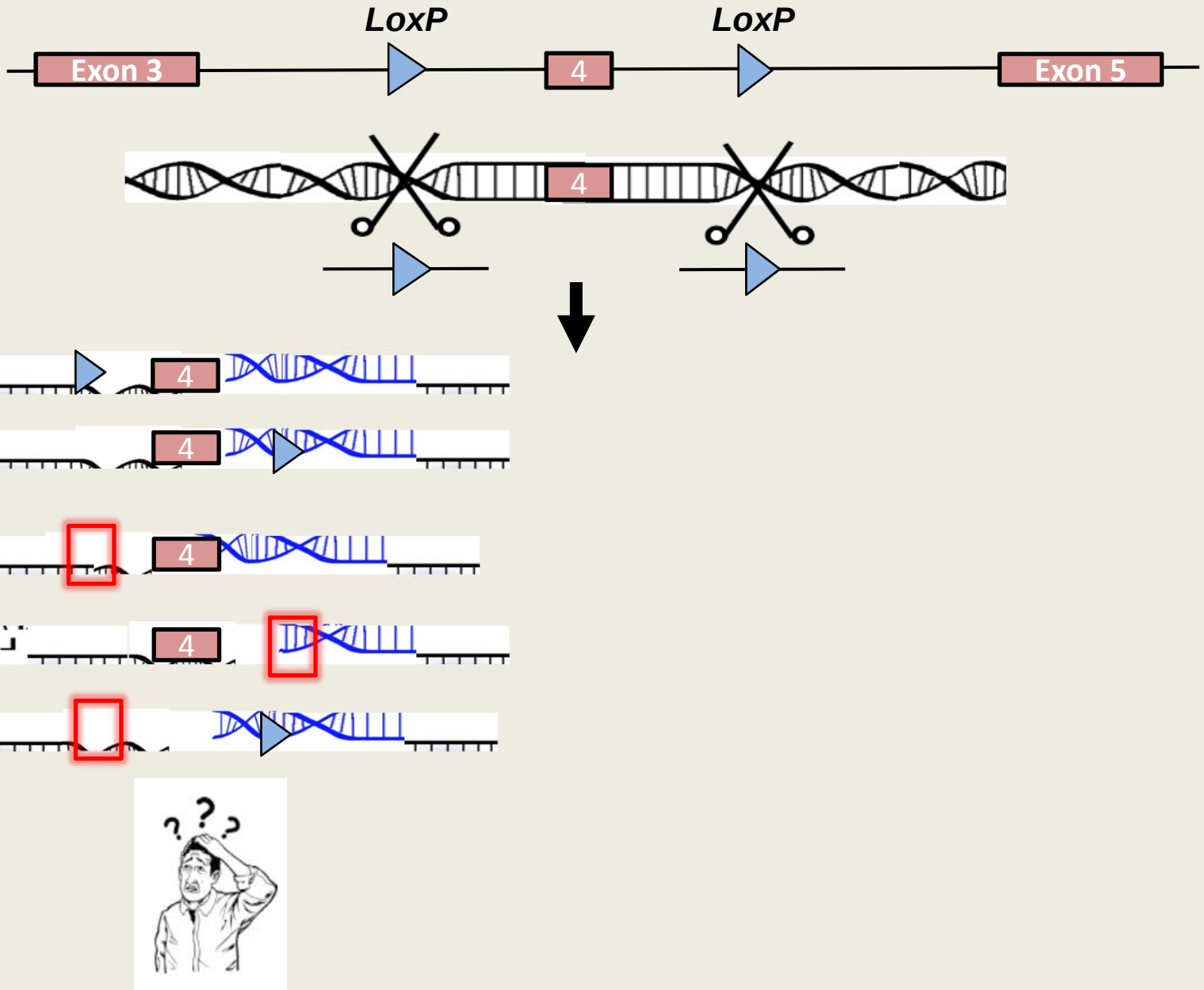


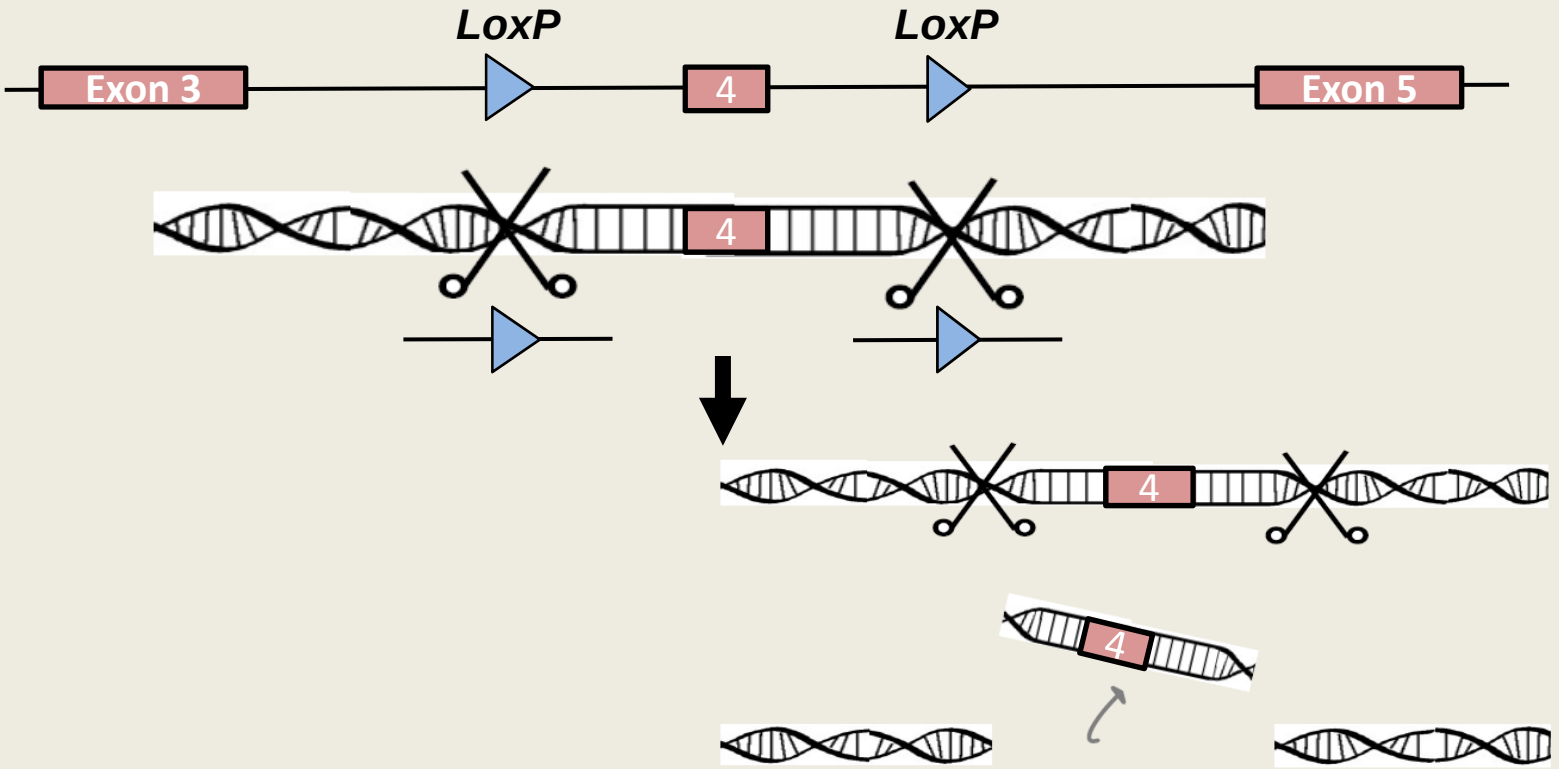
Conditional Knockout



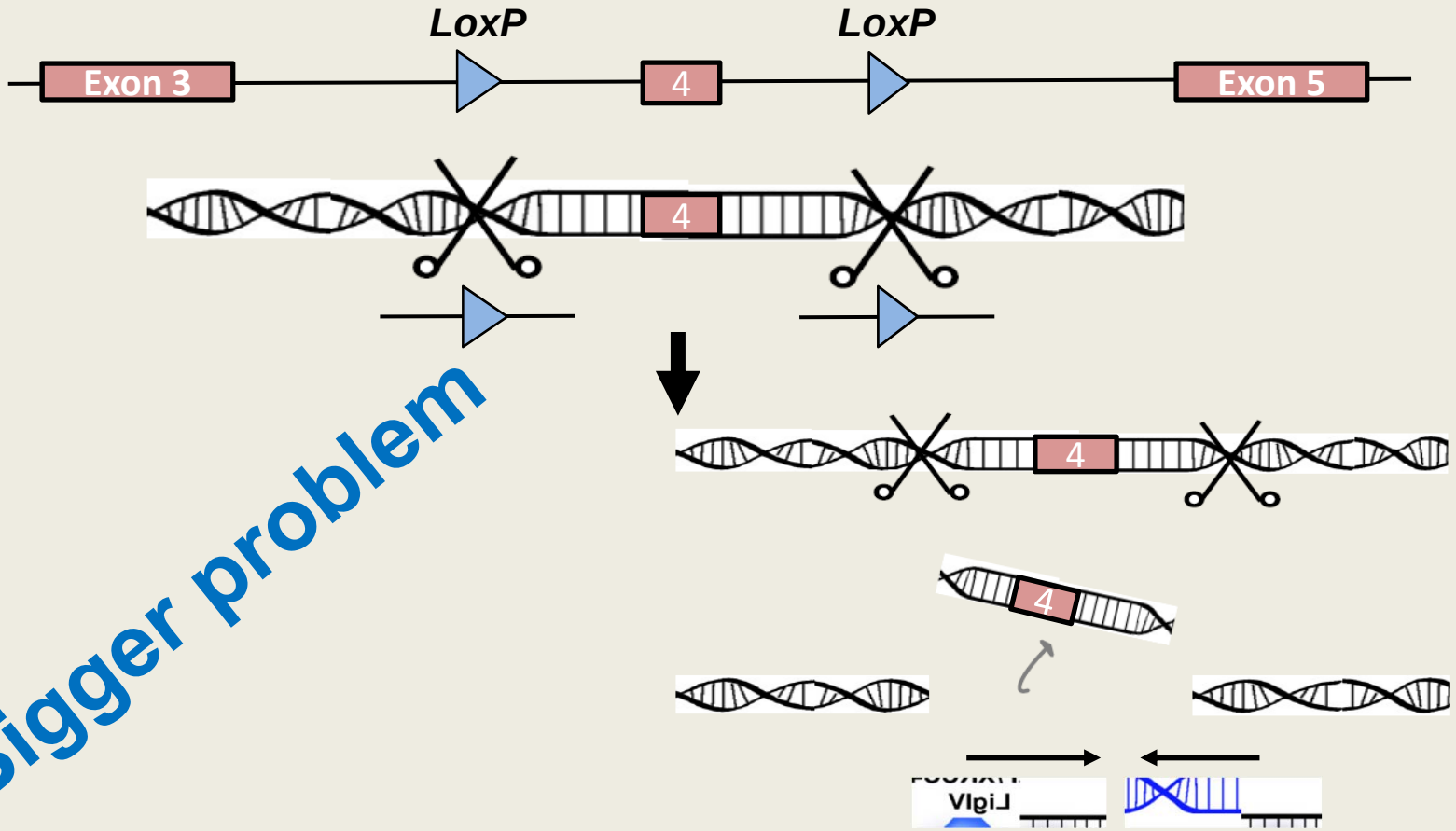
Conditional Knockout



Conditional Knockout

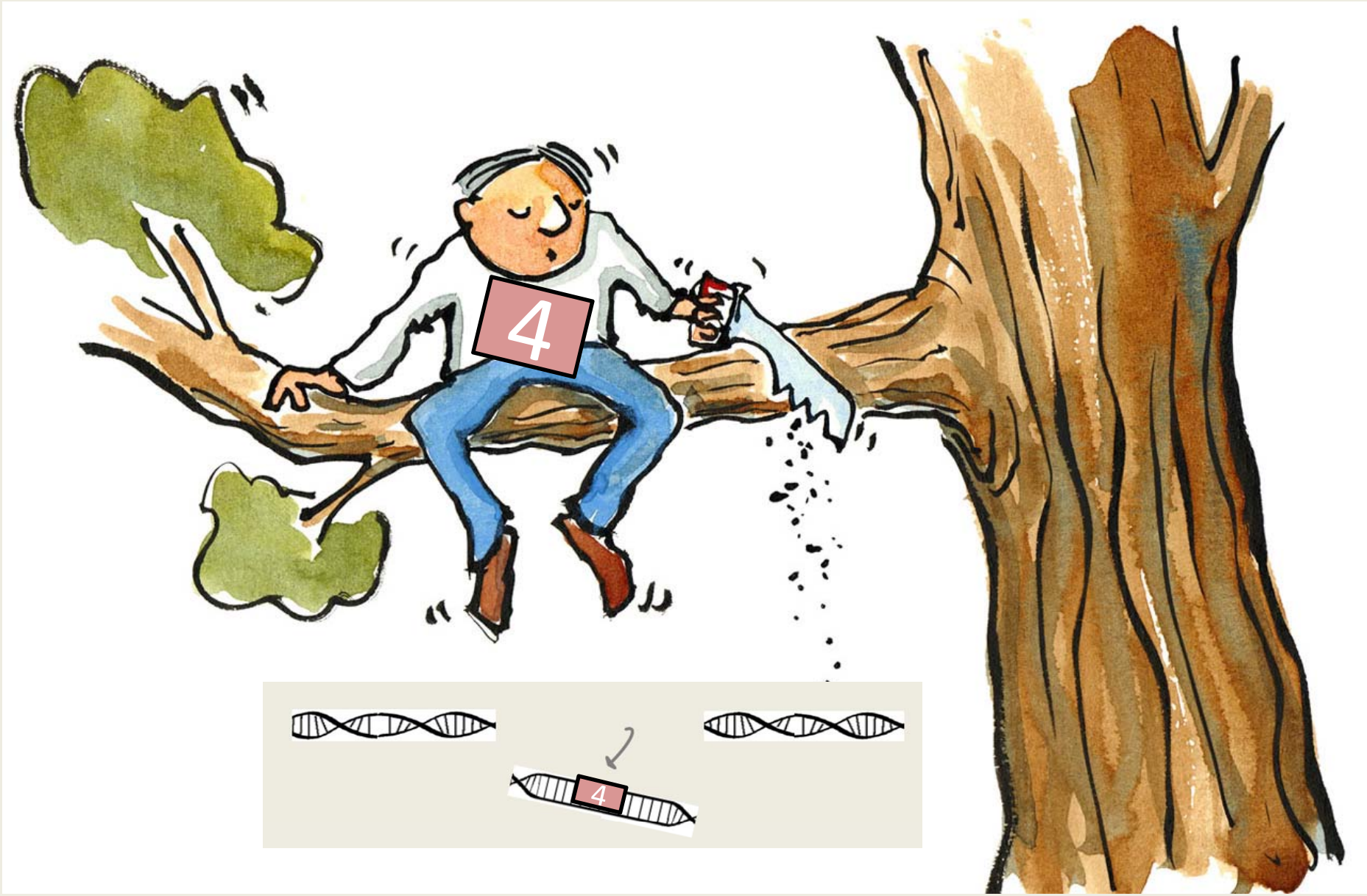


Conditional Knockout

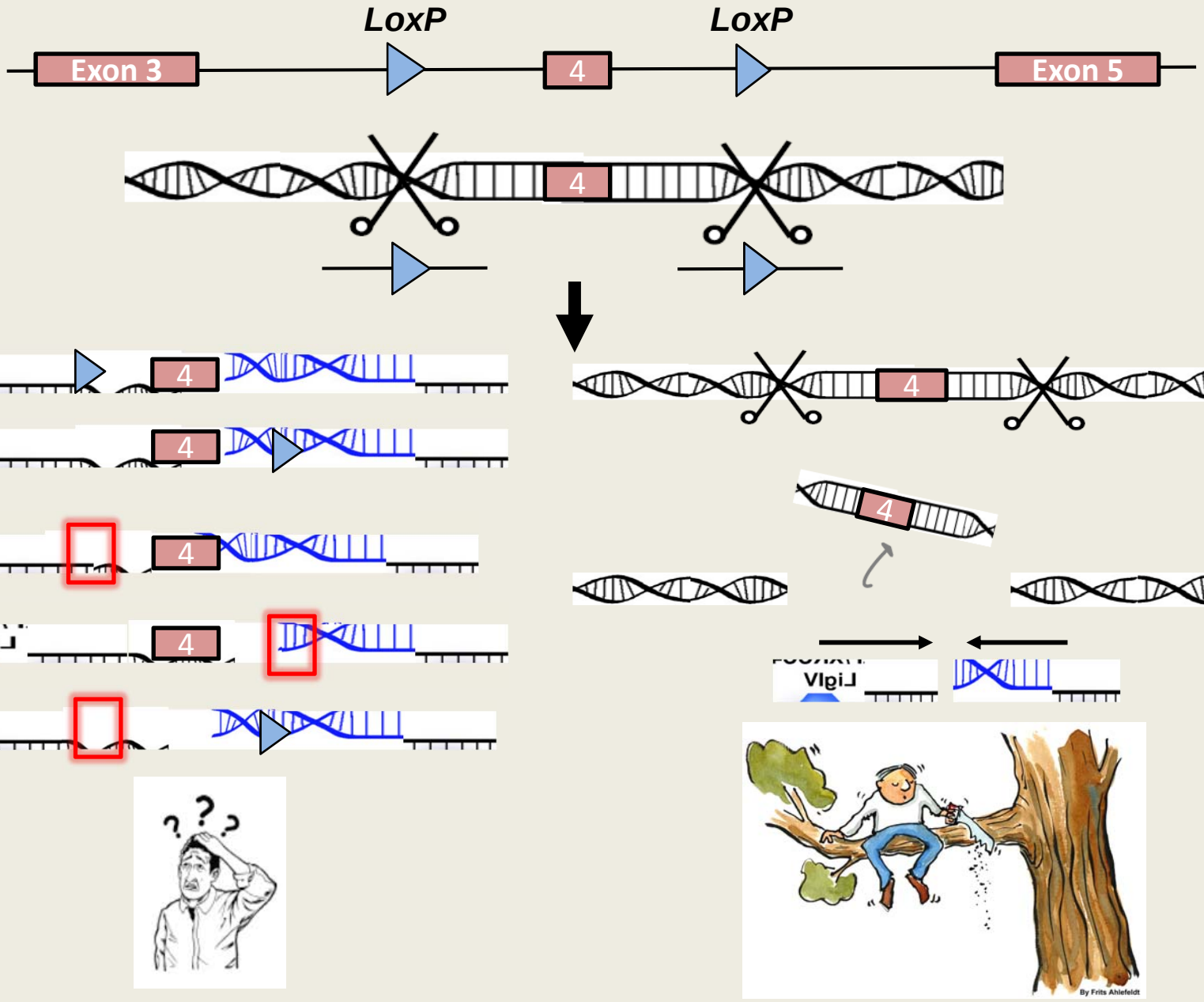


Bigger problem

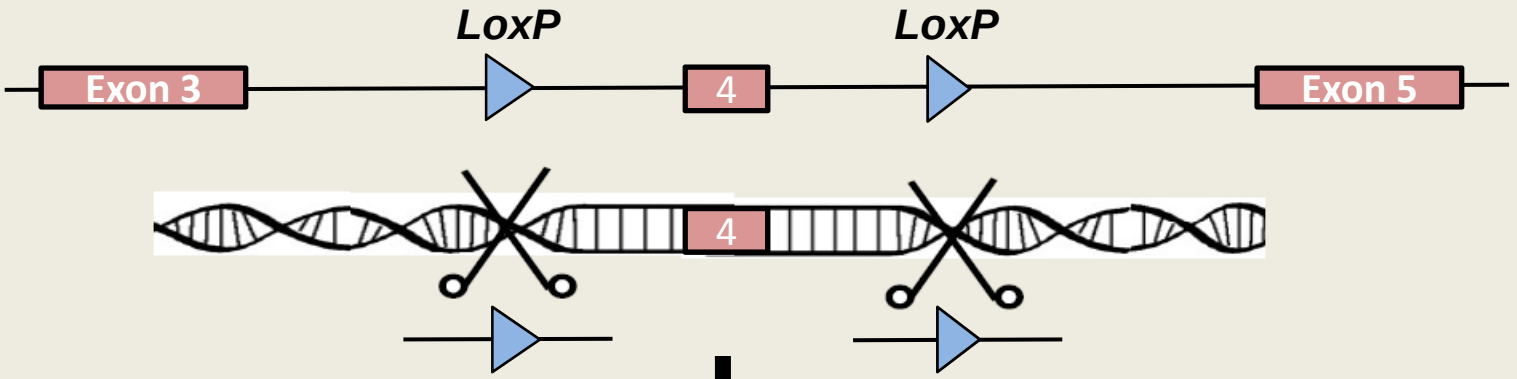
Conditional Knockout

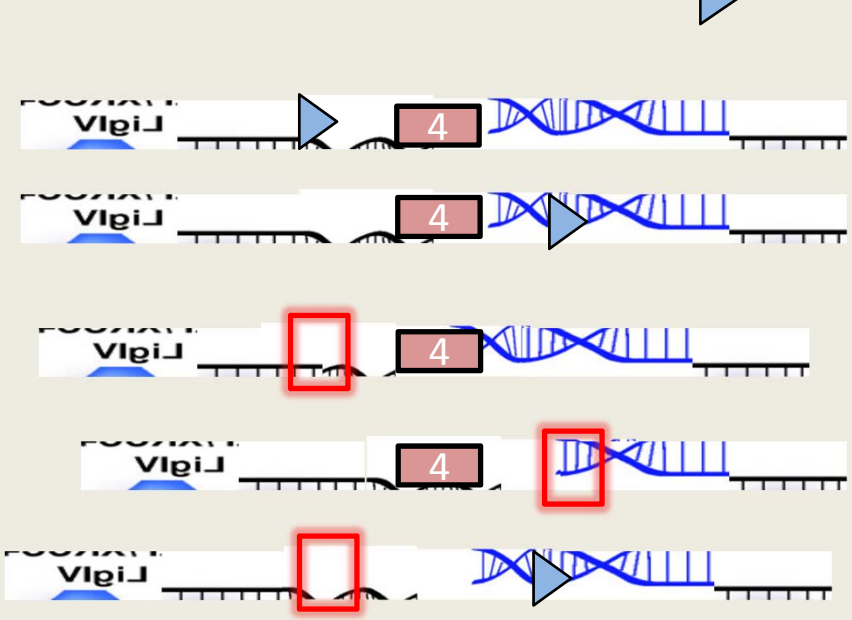


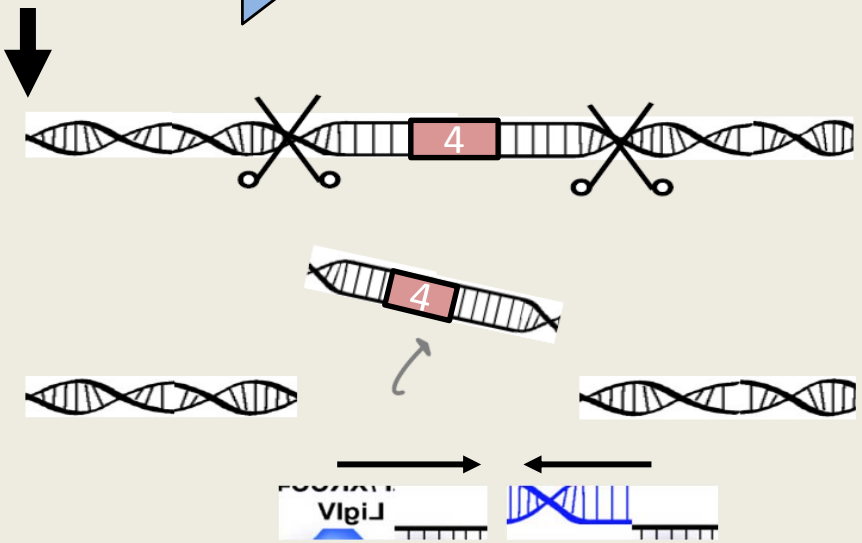
Conditional Knockout

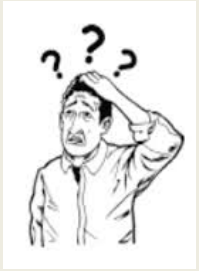


Conditional Knockout



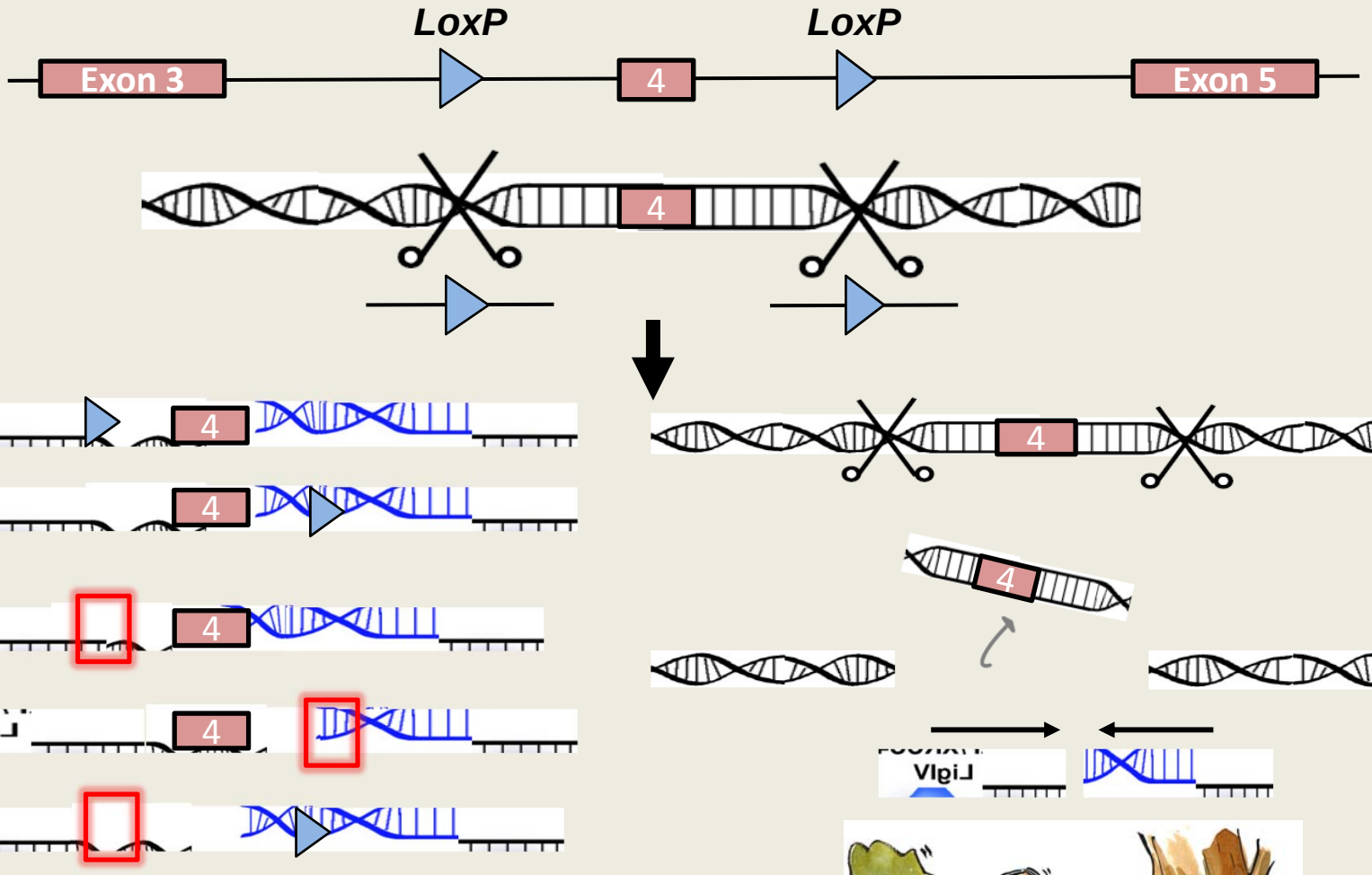




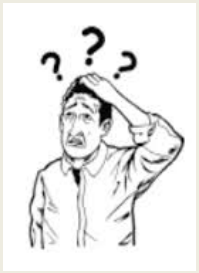


~40-50%

Conditional Knockout



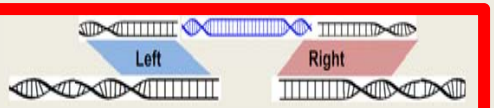
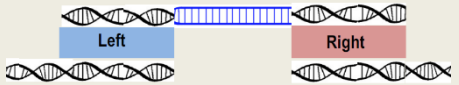
~1-10% correct (if lucky!)



~40-50%

~40-50%

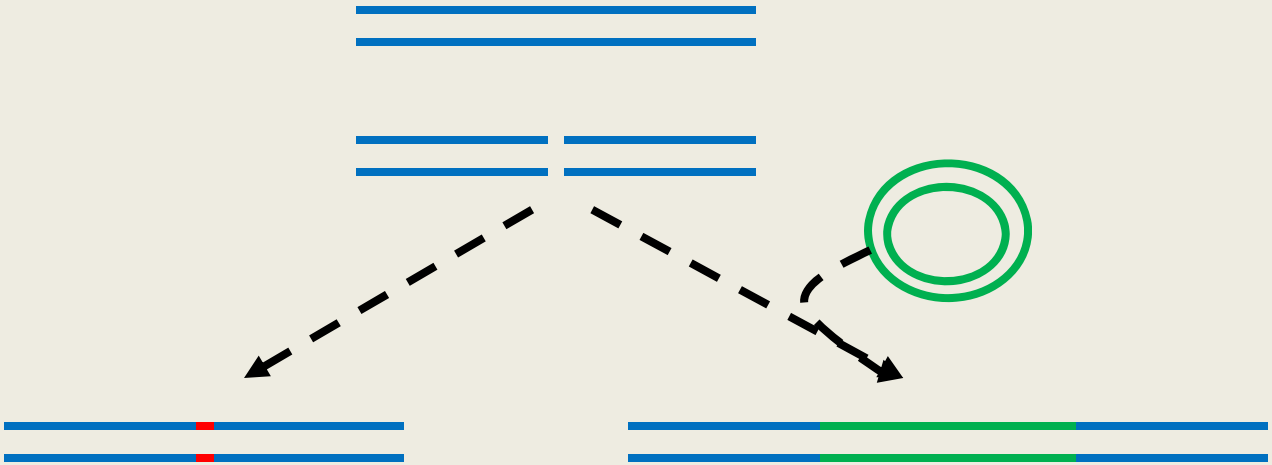
Common Types of Genetically Engineered mice

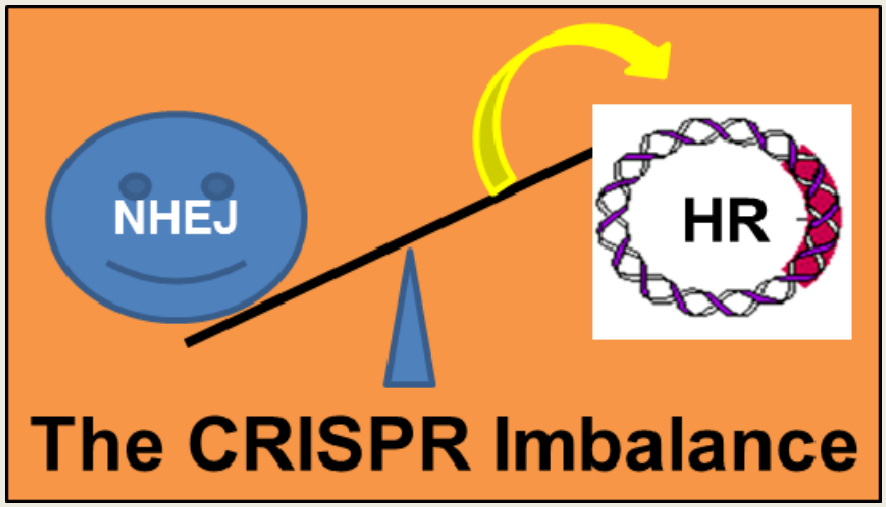
Type		Ease of making (1: easy, 4; very difficult)	
Knockout/Gene disruption *(indels)*	5%	1	
Point mutation knock-In		2	
Insertion of new sequences (GFP, Cre, rtTA etc)	95%	3	
Replacement of gene segments (conditional knockout)		4	

Common Types of Genetically Engineered mice

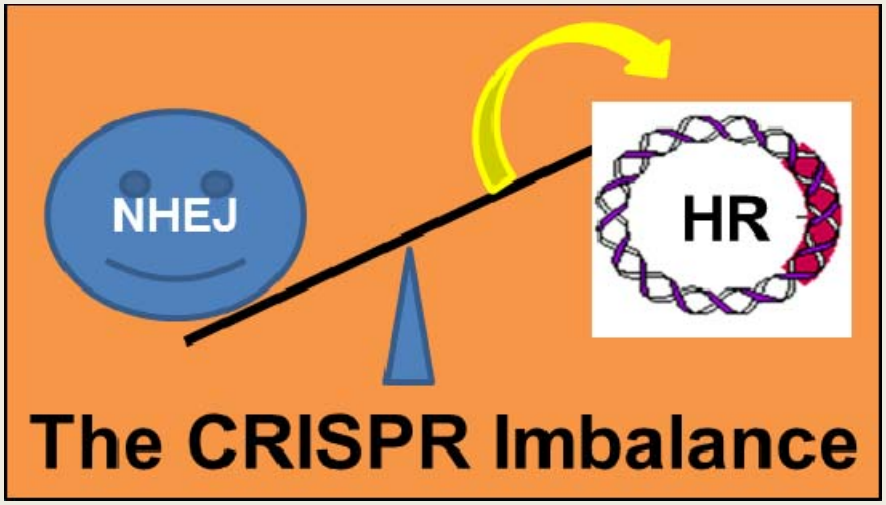
Type	Ease of making (1: easy, 4; very difficult)	
Knockout/Gene disruption *(indels)*	1	
Point mutation knock-In	2	

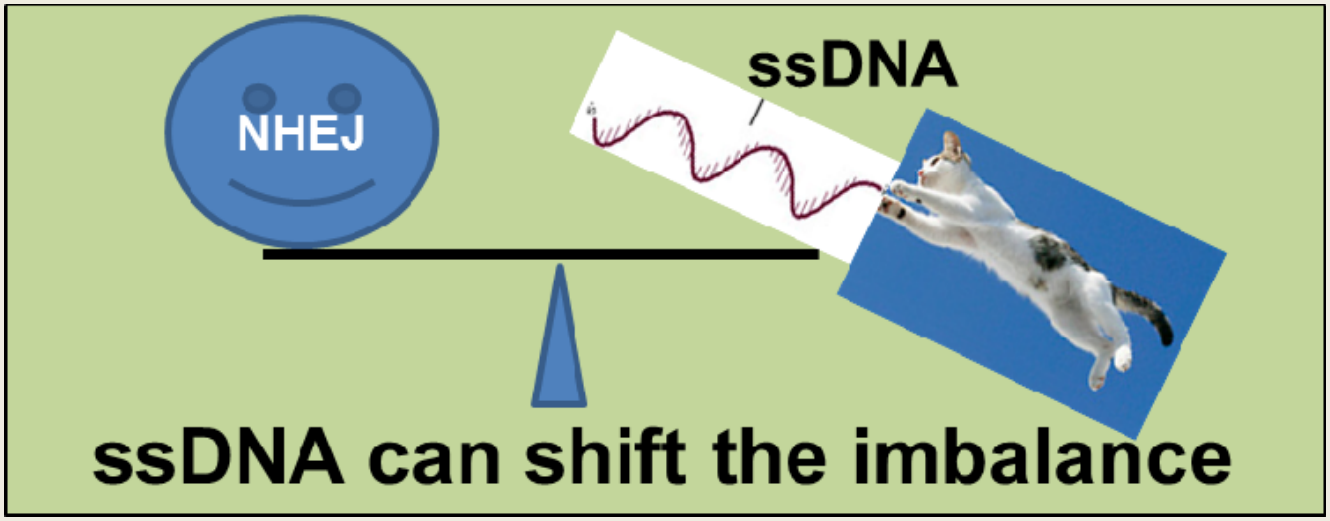
***Easi*-CRISPR**





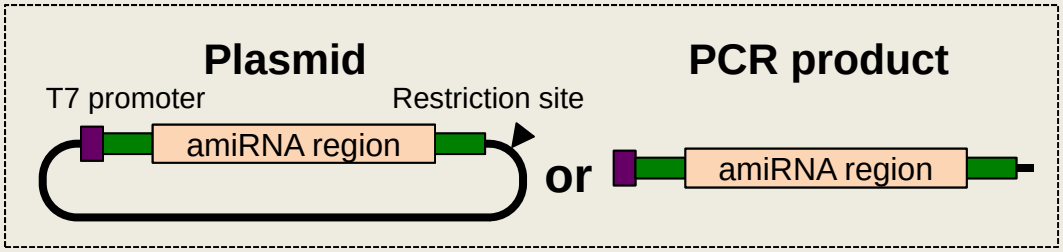
Can we use longer single stranded DNA templates to increase *HR?*


NHEJ
HR
The CRISPR Imbalance

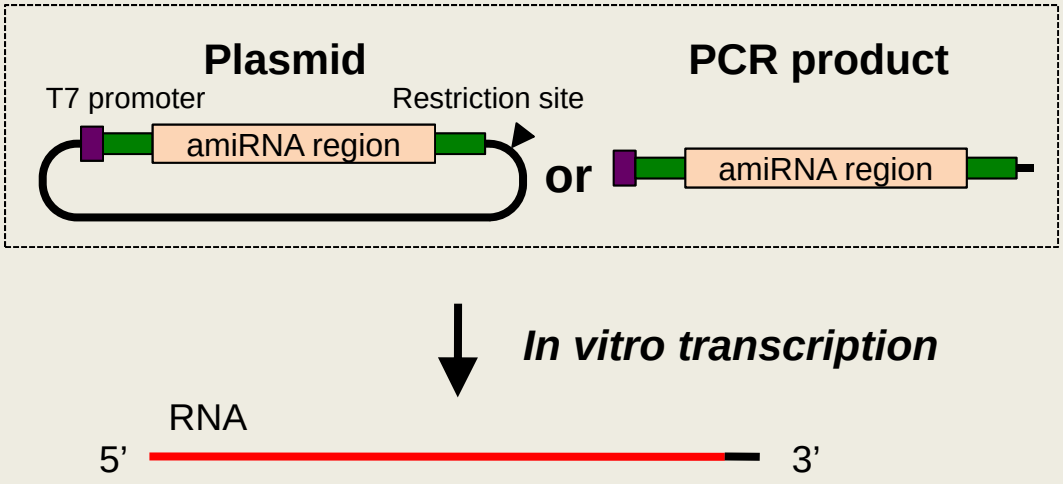

NHEJ
ssDNA
ssDNA can shift the imbalance

Easi-CRISPR:
Efficient additions with ssDNA inserts- CRISPR

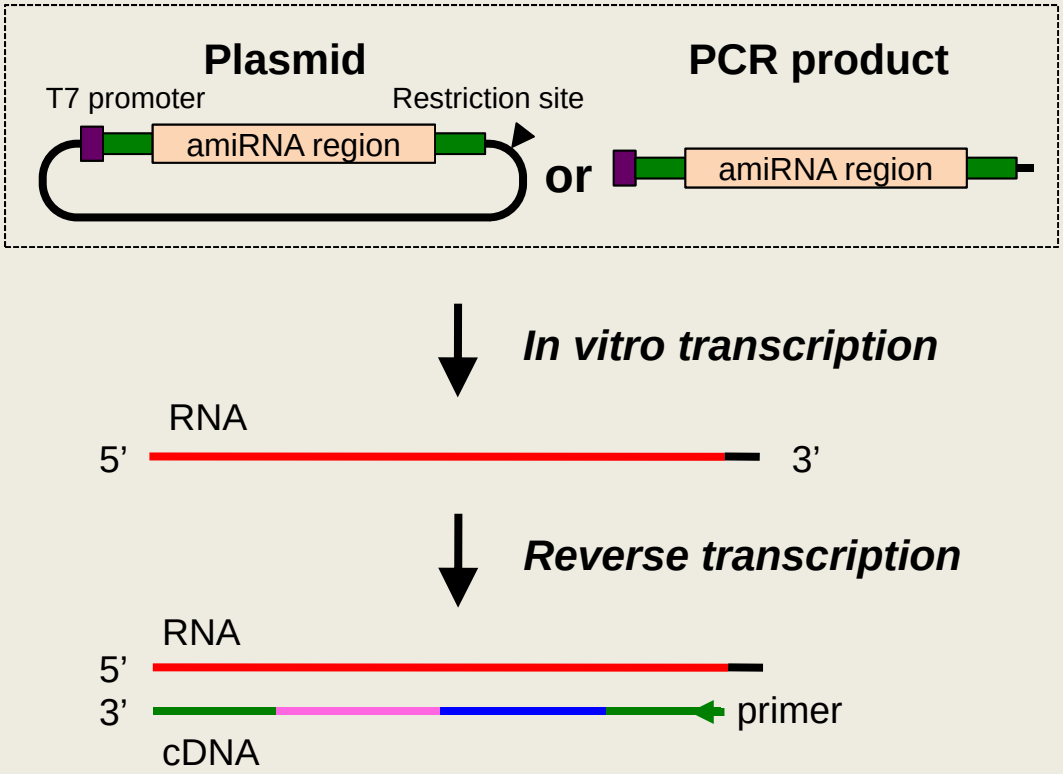
IvTRT: In vitro Transcription and Reverse Transcription



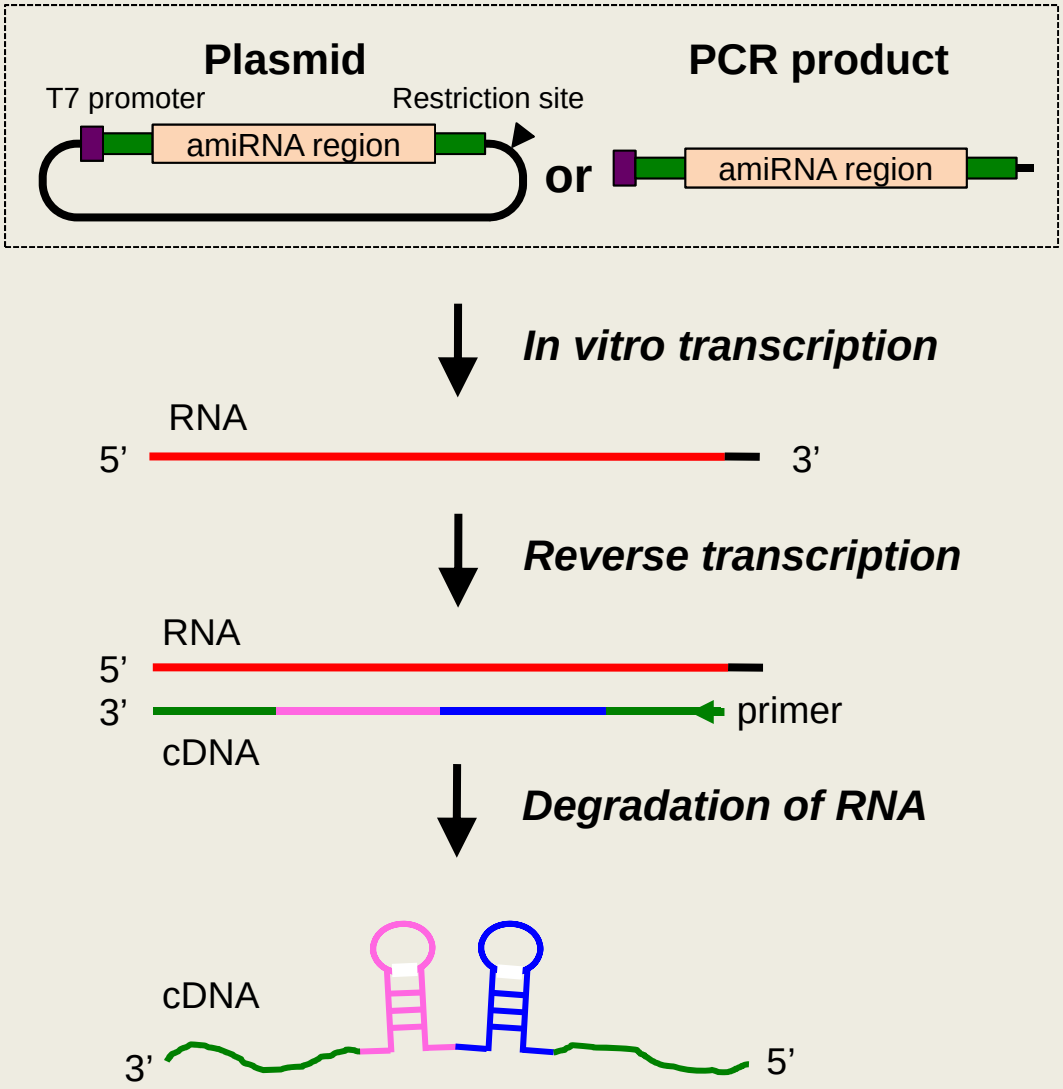
IvTRT: In vitro Transcription and Reverse Transcription



IvTRT: In vitro Transcription and Reverse Transcription

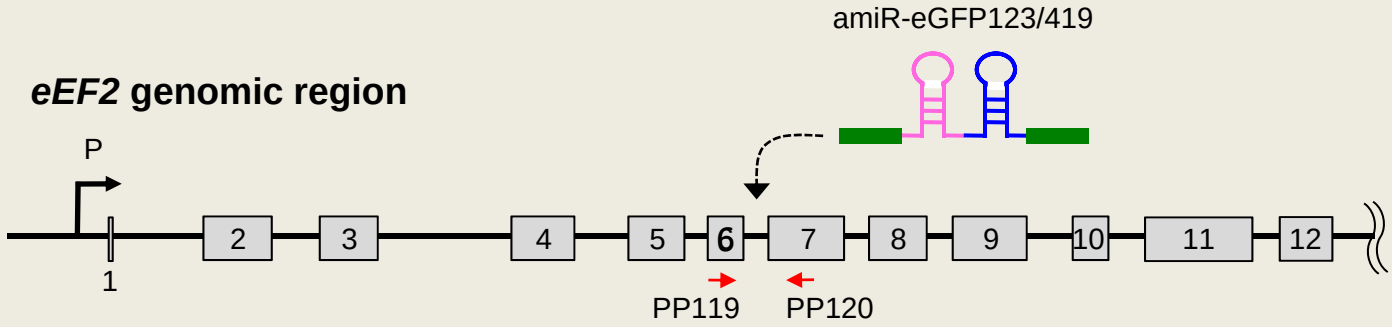


IvTRT: In vitro Transcription and Reverse Transcription

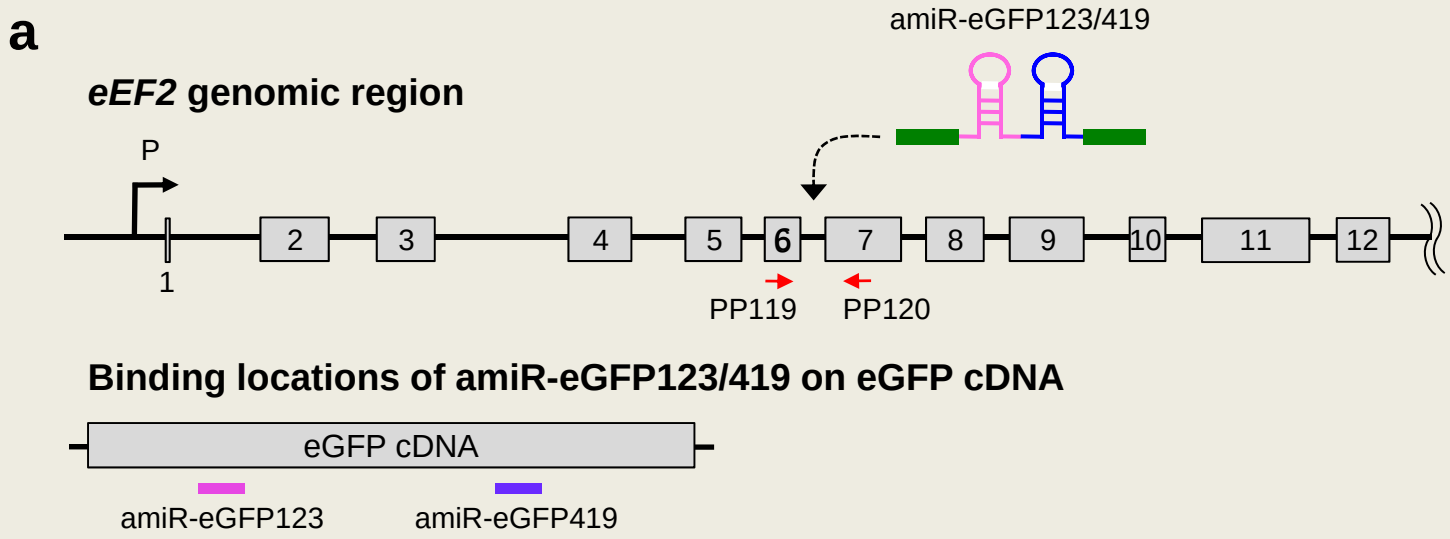


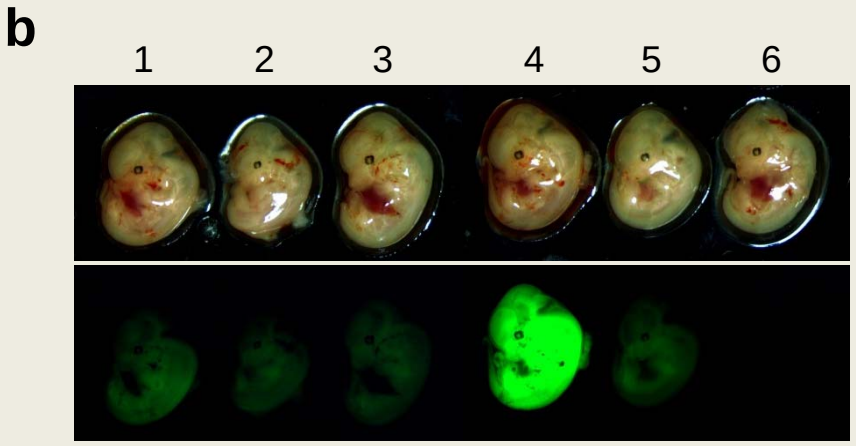
(Figure from Miura *et al.,* 2015 *Scientific Reports* **5**, Article number: 12799)

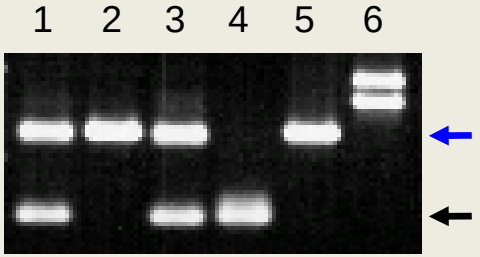
a



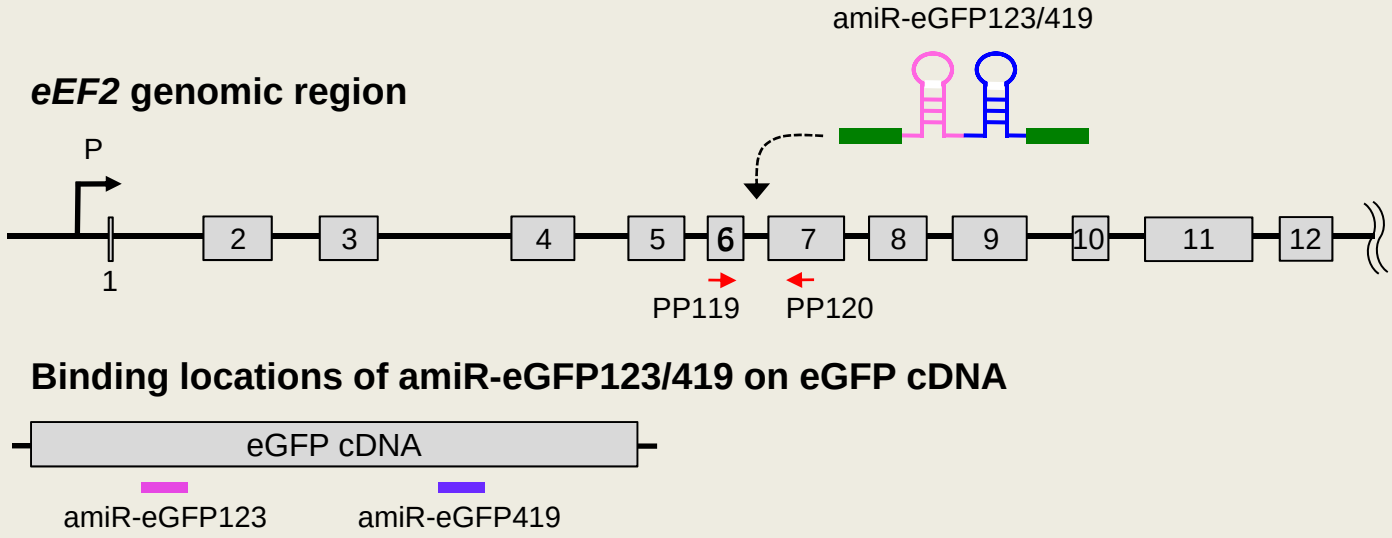
Binding locations of amiR-eGFP123/419 on eGFP cDNA


a
amiR-eGFP123/419
eEF2 genomic region
P
1
2
3
4
5
6
7
8
9
10
11
12
PP119
PP120
Binding locations of amiR-eGFP123/419 on eGFP cDNA
eGFP cDNA
amiR-eGFP123
amiR-eGFP419

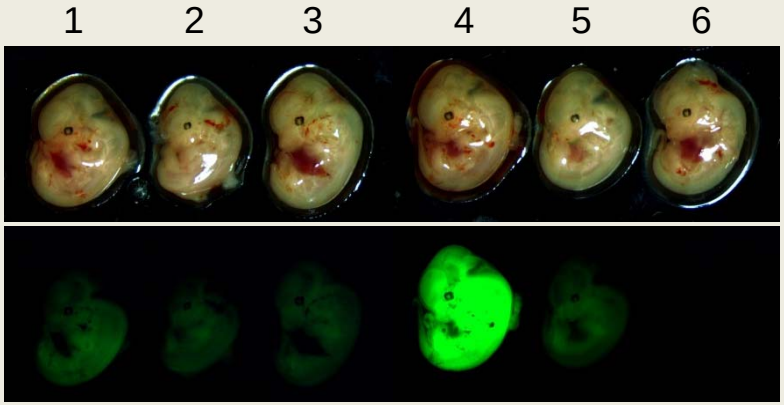

b
1
2
3
4
5
6


c
1
2
3
4
5
6

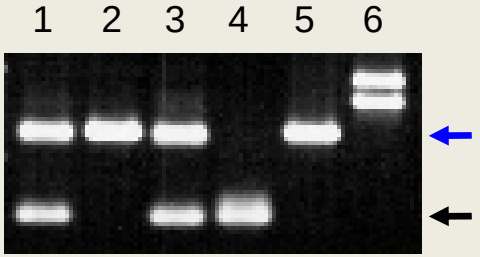
a



b



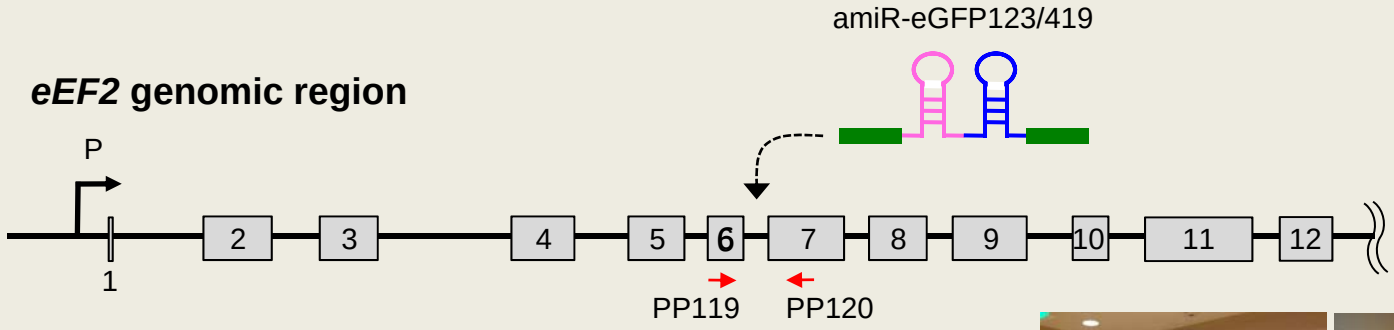
c



d

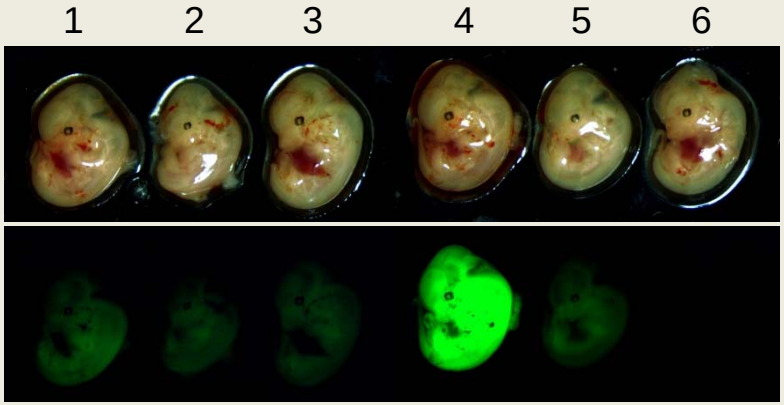
Exp. No.	CRISPR target site	Target for knockdown	Embryos injected	Embryos recovered	Embryos with targeted insertion (%)
2	6th intron of *eEF2* (TS2)	eGFP	56	6	5 (83.3%)

a

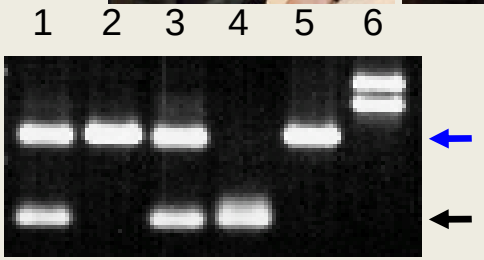


Binding locations of amiR-eGFP123/419 on eGFP cDNA

b



c



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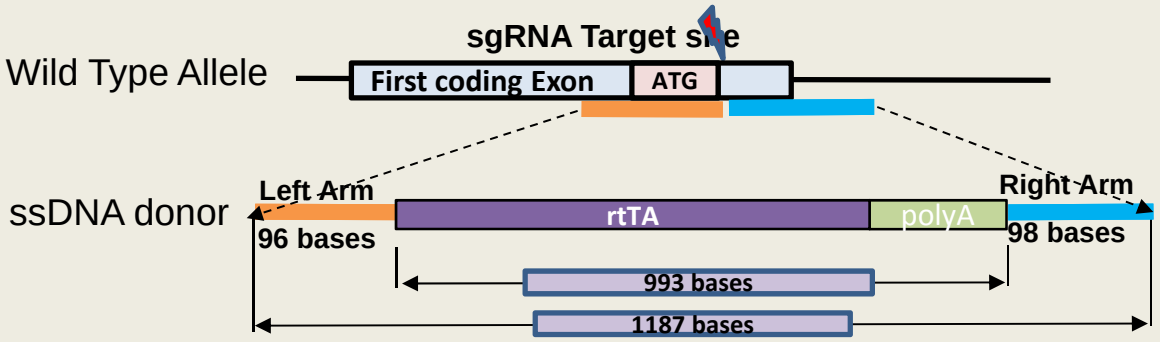
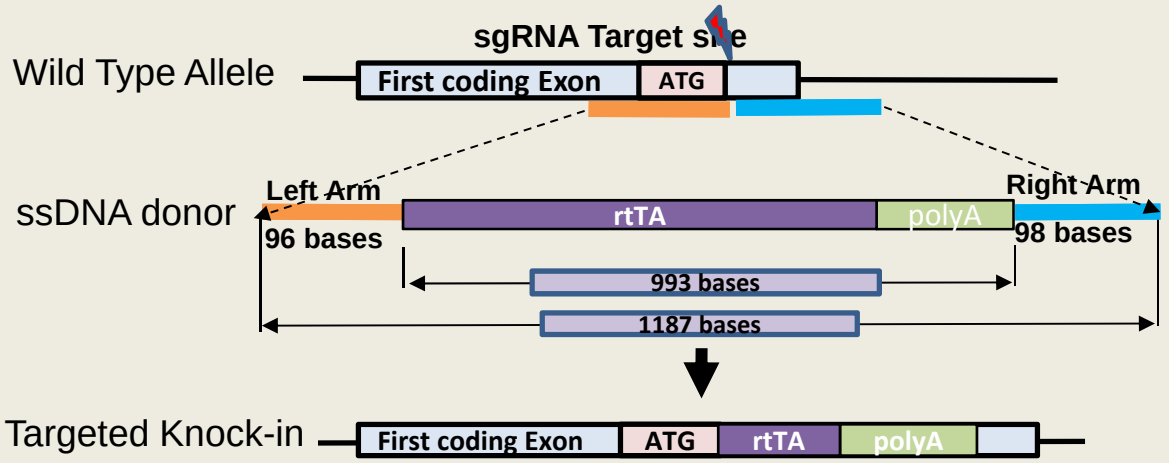
http://www.nature.com/articles/srep12799

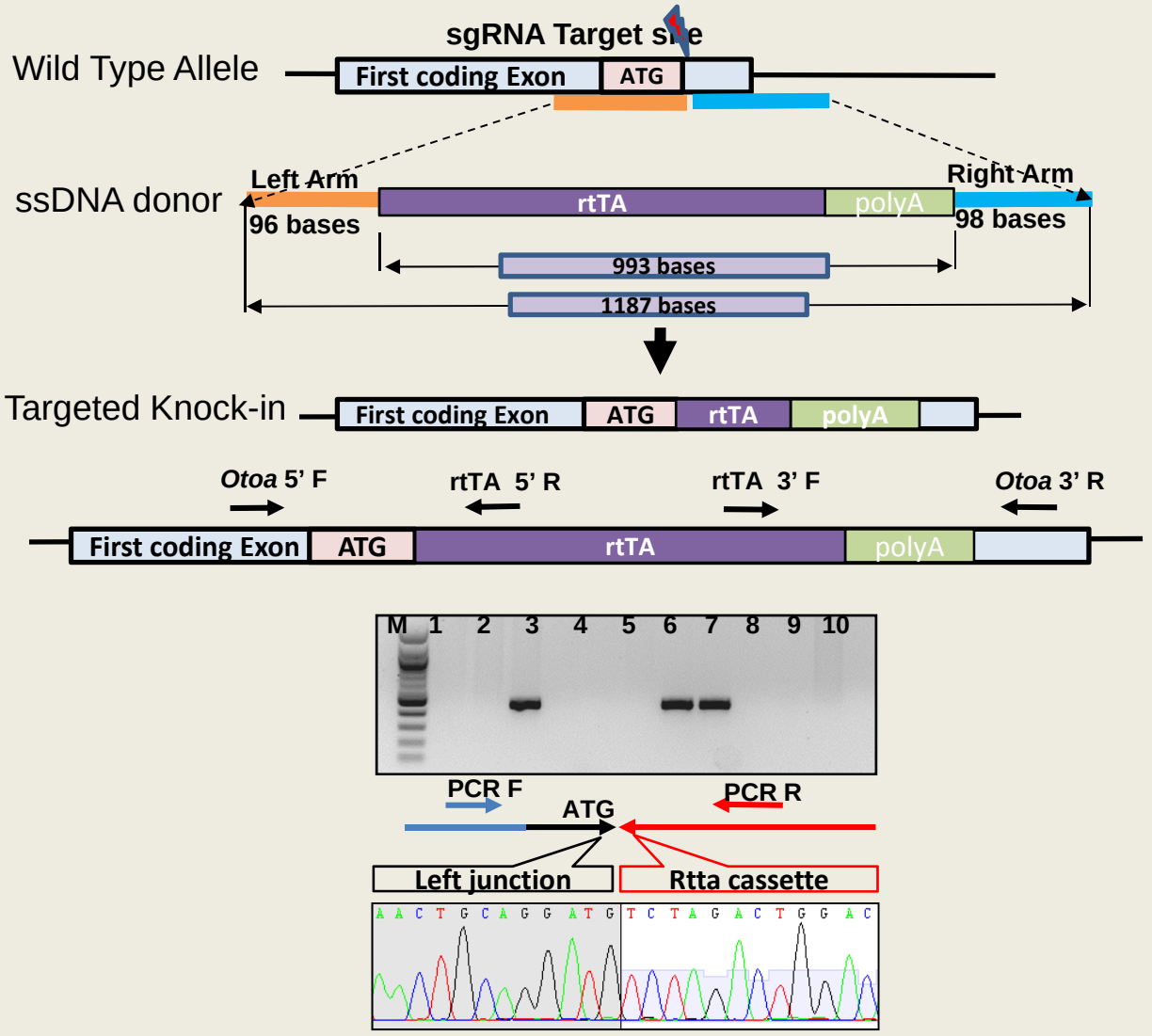
Insertion of ~1 kb new sequence at the Cas9 cut sites



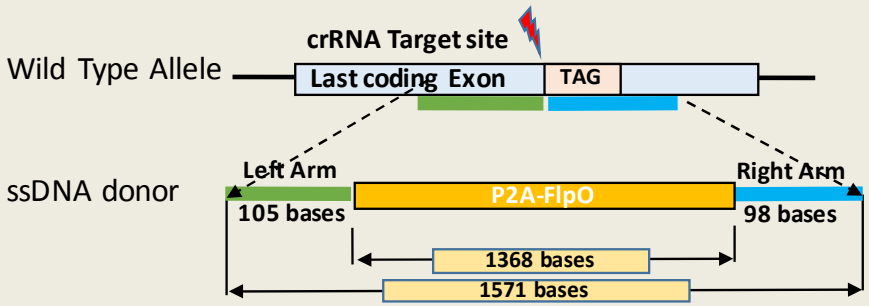
Insertion of ~1 kb new sequence at the Cas9 cut sites



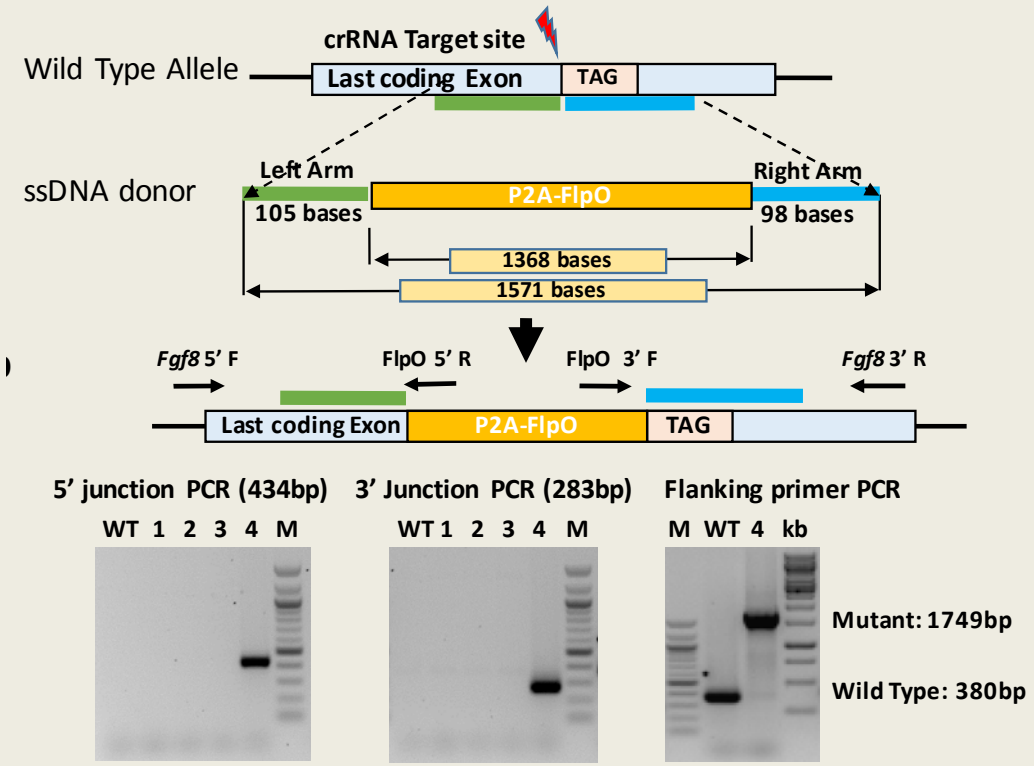
Insertion of ~1 kb new sequence at the Cas9 cut sites



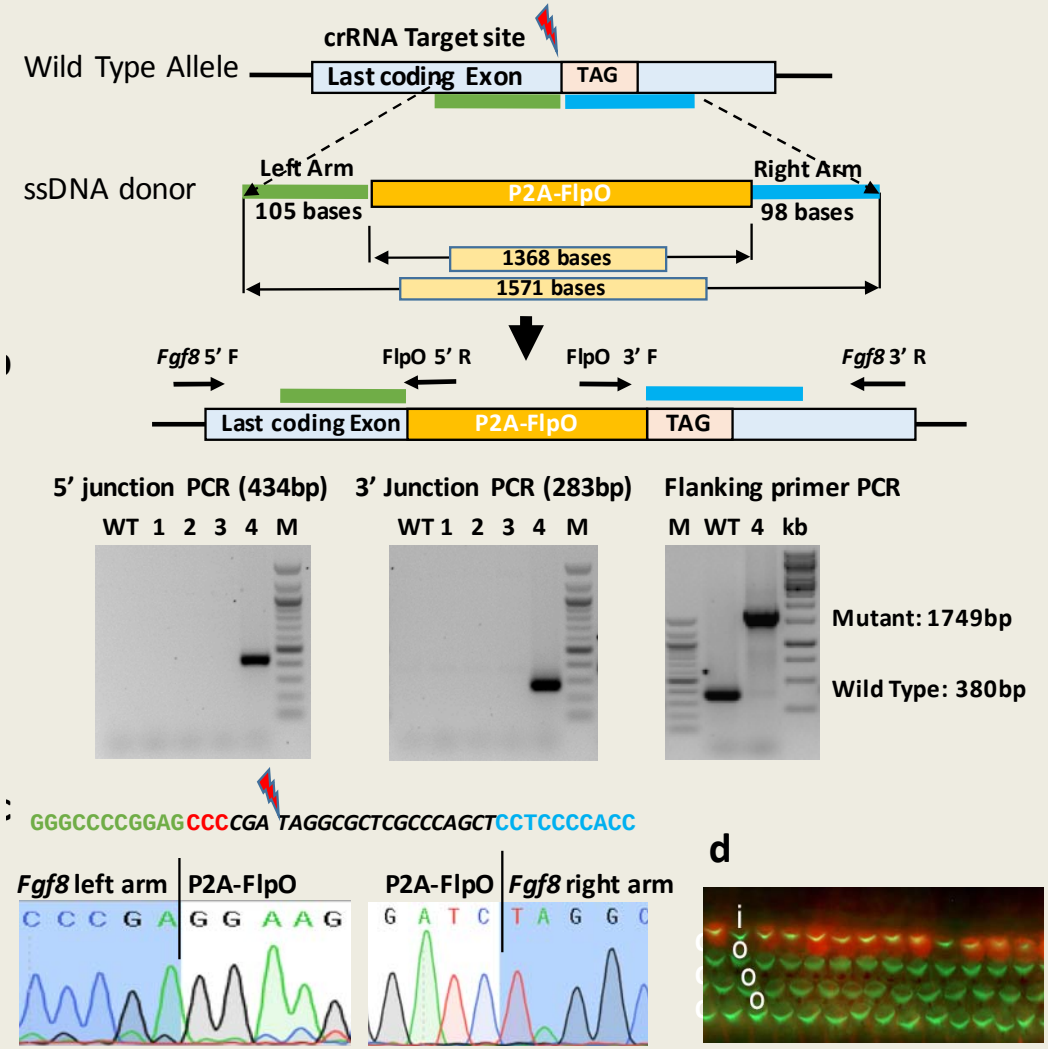
Insertion of ~1.5 kb new sequence at the Cas9 cut sites



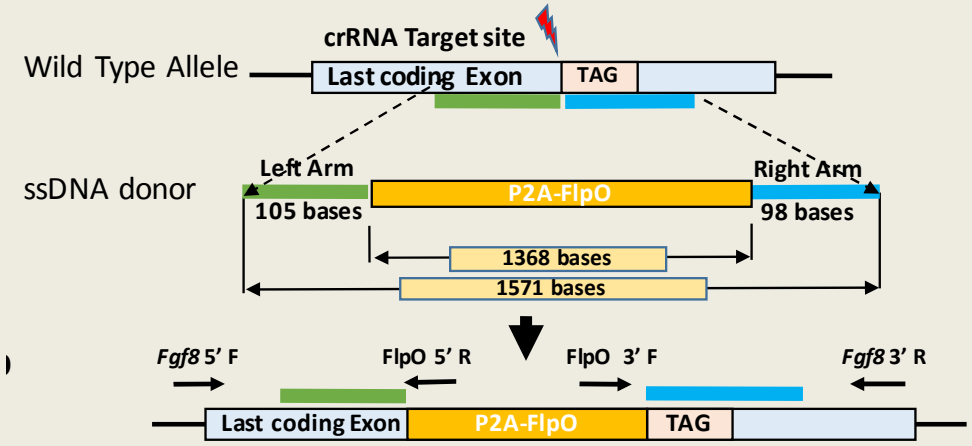
Insertion of ~1.5 kb new sequence at the Cas9 cut sites

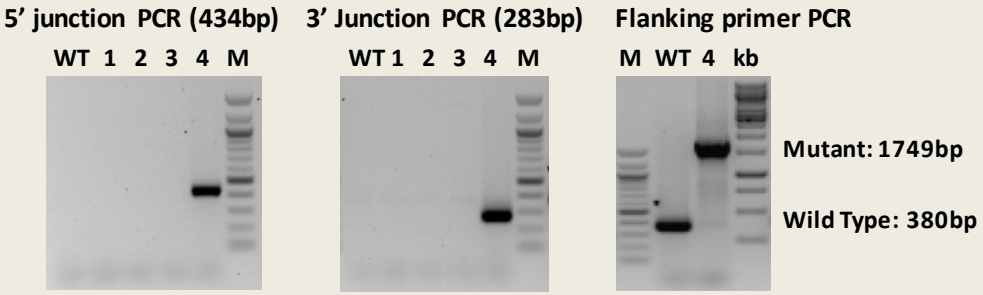


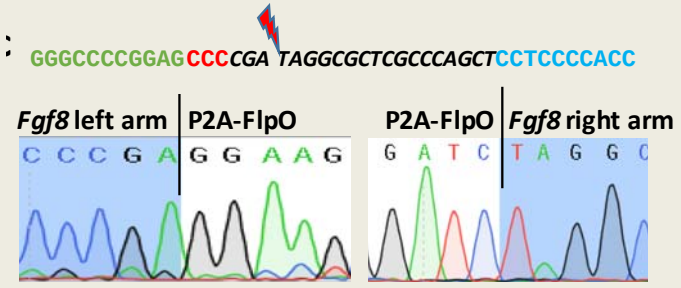
Insertion of ~1.5 kb new sequence at the Cas9 cut sites

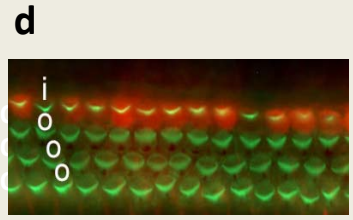


Insertion of ~1.5 kb new sequence at the Cas9 cut sites





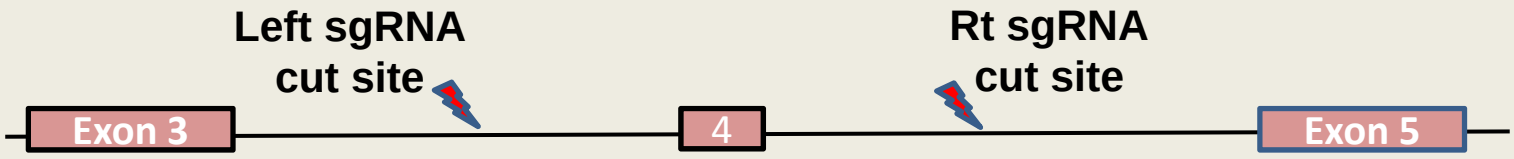




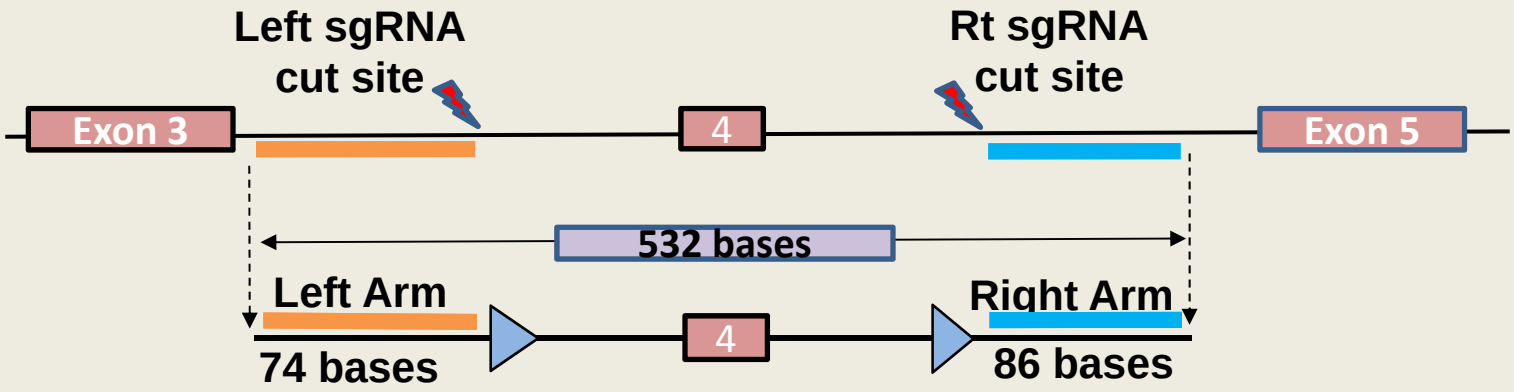
Suzanne Mansour, University of Utah

***Gene*-insertion cassette**	**ssDNA length Left Arm-Cassette-Right Arm (bases)**	**Zygotes injected**	**Zygotes transferred**	**Live-born animals**	**Targeted animals (%)**
Fgf8-P2A-FlpO	105 + 1368 + 98	22	13	4	1 (25%)
Slc26a5-P2A-FlpO	99 + 1368 + 72	28	22	3	1 (33%)
Mafb-P2A-FlpO	85 + 1368 + 96	58	53	8	2 (25%)
Otoa-rtTA	96 + 1220 + 98	19	18	2	1 (50%)
Mmp9-T2A-mCitrine	60 + 782 + 60	52	50	12	8 (67%)
Mmp13-T2A-mCherry	60 + 779 + 60	55	52	10	4 (40%)

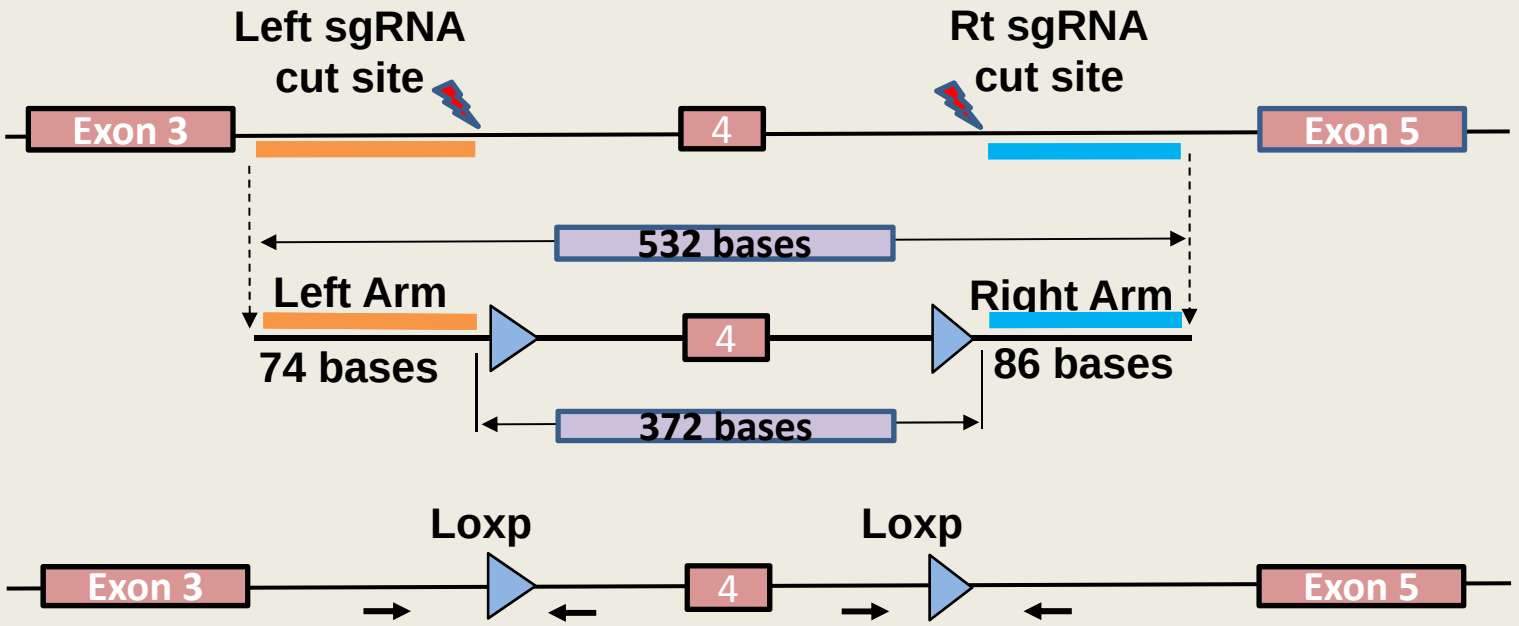
Generation of conditional KO (floxed) models



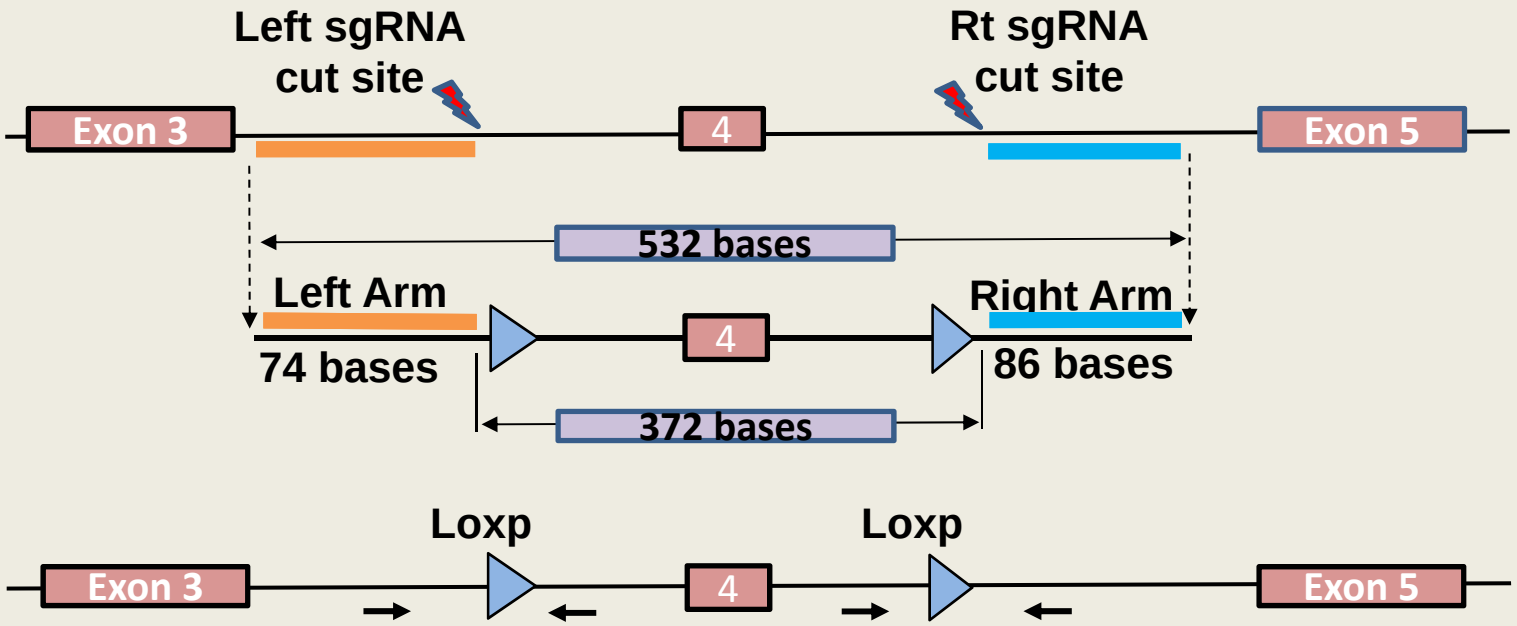
Generation of conditional KO (floxed) models

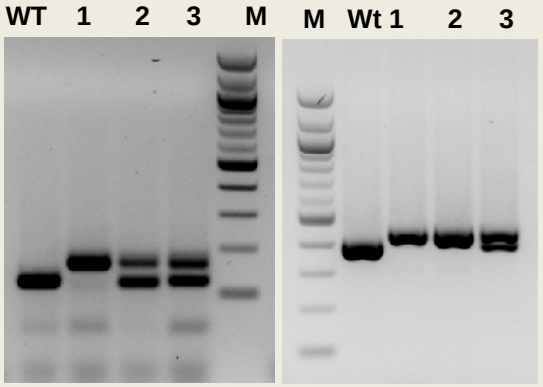


Generation of conditional KO (floxed) models



Generation of conditional KO (floxed) models





5' PCR
Wild Type: 126bp;
Mutant: 166bp;

3' PCR
Wild Type: 397bp;
Mutant: 431bp;

Efficiency 100% (3/3)

***Gene*-insertion cassette**	**ssDNA length Left Arm-Cassette-Right Arm (bases)**	**Zygotes injected**	**Zygotes transferred**	**Newborn pups**	**Targeted pups (%)**
Pitx1-exon 2 floxing	93 + 862 + 91	85	76	10	4 (40%)
Hal-exon 9 floxing	83 + 517 + 85	61	56	7	5 (71%)
Paf1- exon 4 floxing	74 + 372 + 86	91	82	3	3(100%)
Ppp2r5a-exon 3 floxing	95 + 619 + 84	34	33	3	3 (100%)
Ambra1-exon 4 floxing	96+589+103	67	63	8	6 (75%)
Syt1 Exon 6 floxing	75 + 635 + 75	29	26	8	1 (12.5%)

Easi-CRISPR:
Efficient additions with ssDNA inserts- CRISPR

Quadros *et al. Genome Biology* (2017) 18:92
DOI 10.1186/s13059-017-1220-4

Genome Biology

RESEARCH Open Access

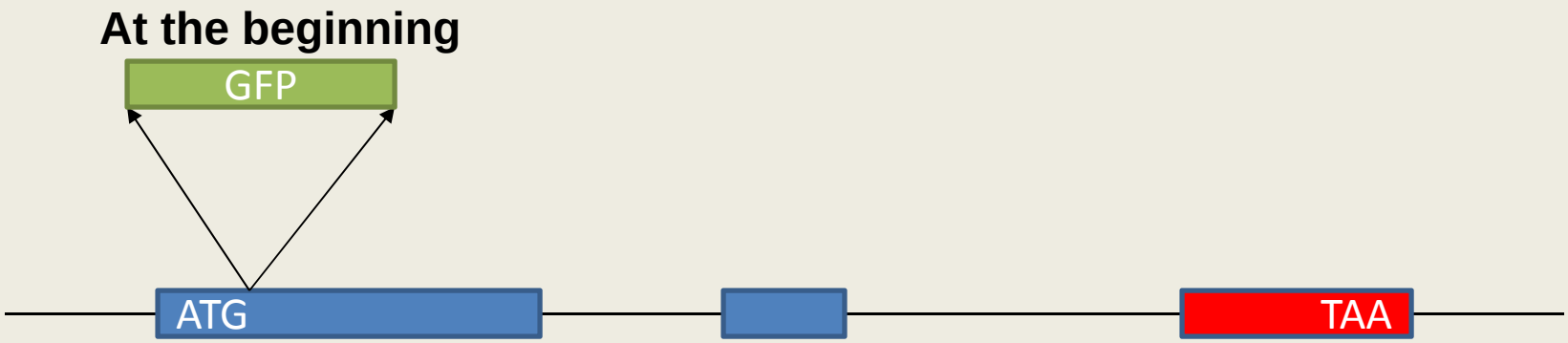
Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins

Rolen M. Quadros[1†], Hiromi Miura[2,3†], Donald W. Harms[1], Hisako Akatsuka[2,4], Takehito Sato[4], Tomomi Aida[5,6,7], Ronald Redder[8], Guy P. Richardson[9], Yutaka Inagaki[3,10,11], Daisuke Sakai[10,12], Shannon M. Buckley[13,15], Parthasarathy Seshacharyulu[14], Surinder K. Batra[14,15], Mark A. Behlke[16], Sarah A. Zeiner[16], Ashley M. Jacobi[16], Yayoi Izu[17], Wallace B. Thoreson[18], Lisa D. Urness[19], Suzanne L. Mansour[19*], Masato Ohtsuka[2,3,10*] and Channabasavaiah B. Gurumurthy[1,20*]

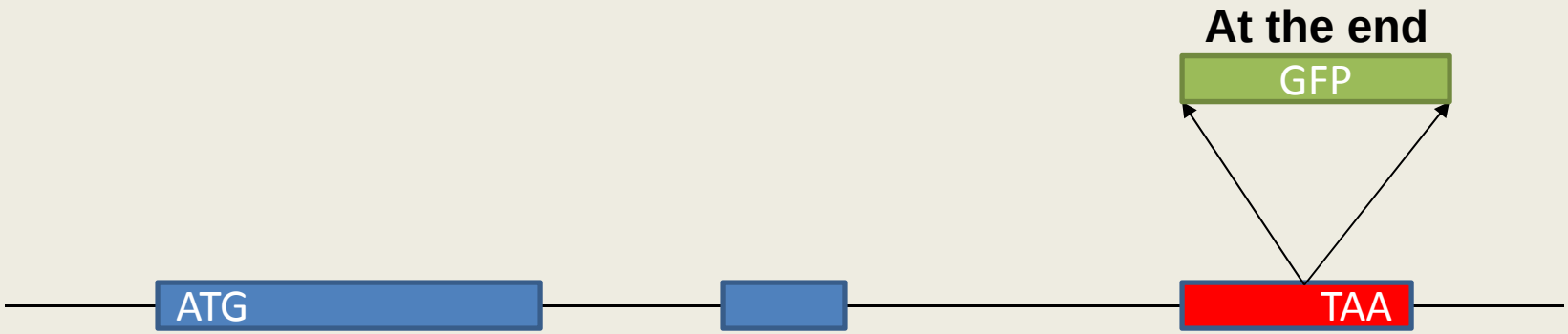
https://genomebiology.biomedcentral.com/articles/10.1186/s13059-017-1220-4

Easi-CRISPR Summary

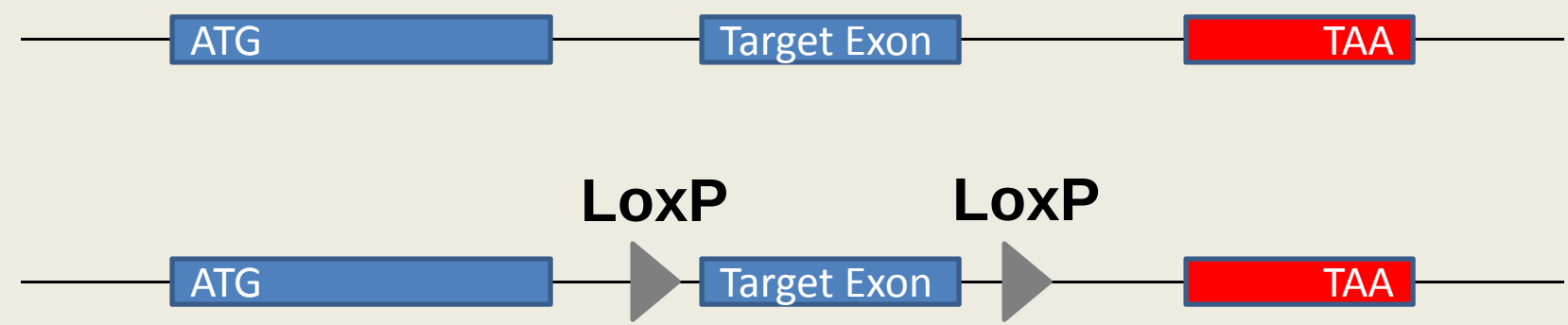
Easi-CRISPR can fuse expression cassettes (at the beginning, or at the end)



Easi-CRISPR can fuse expression cassettes (at the beginning, or at the end)

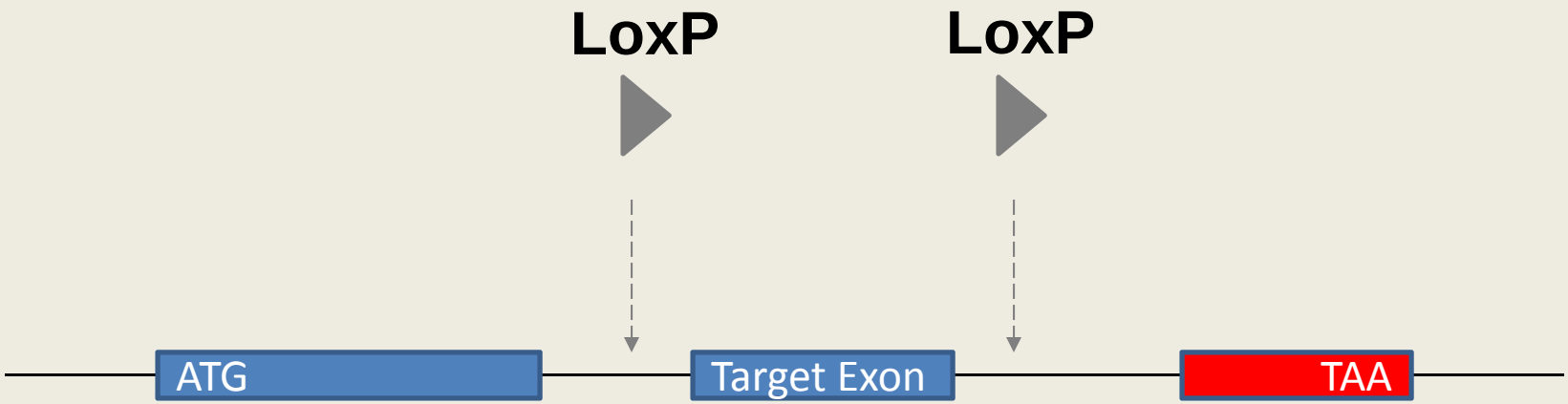


Creating conditional knockout models

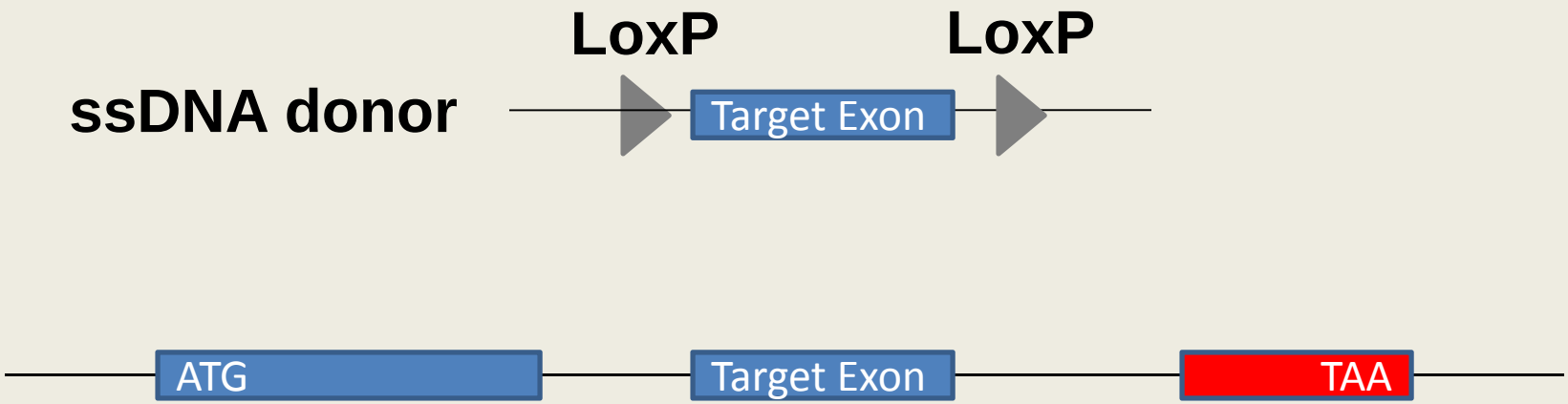


Conditional KO

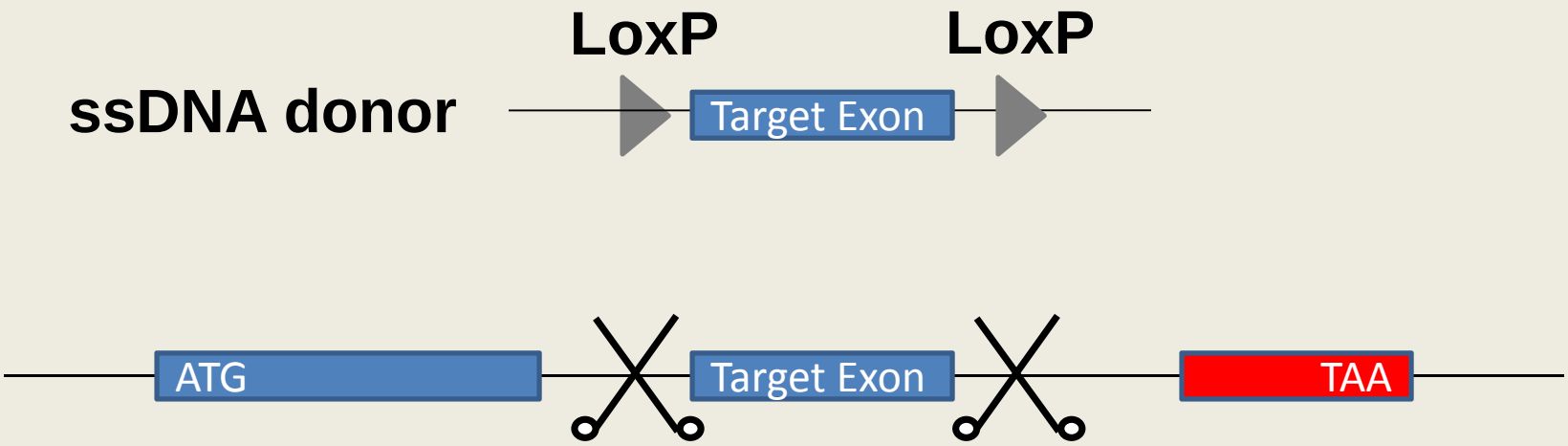
Creating conditional knockout models



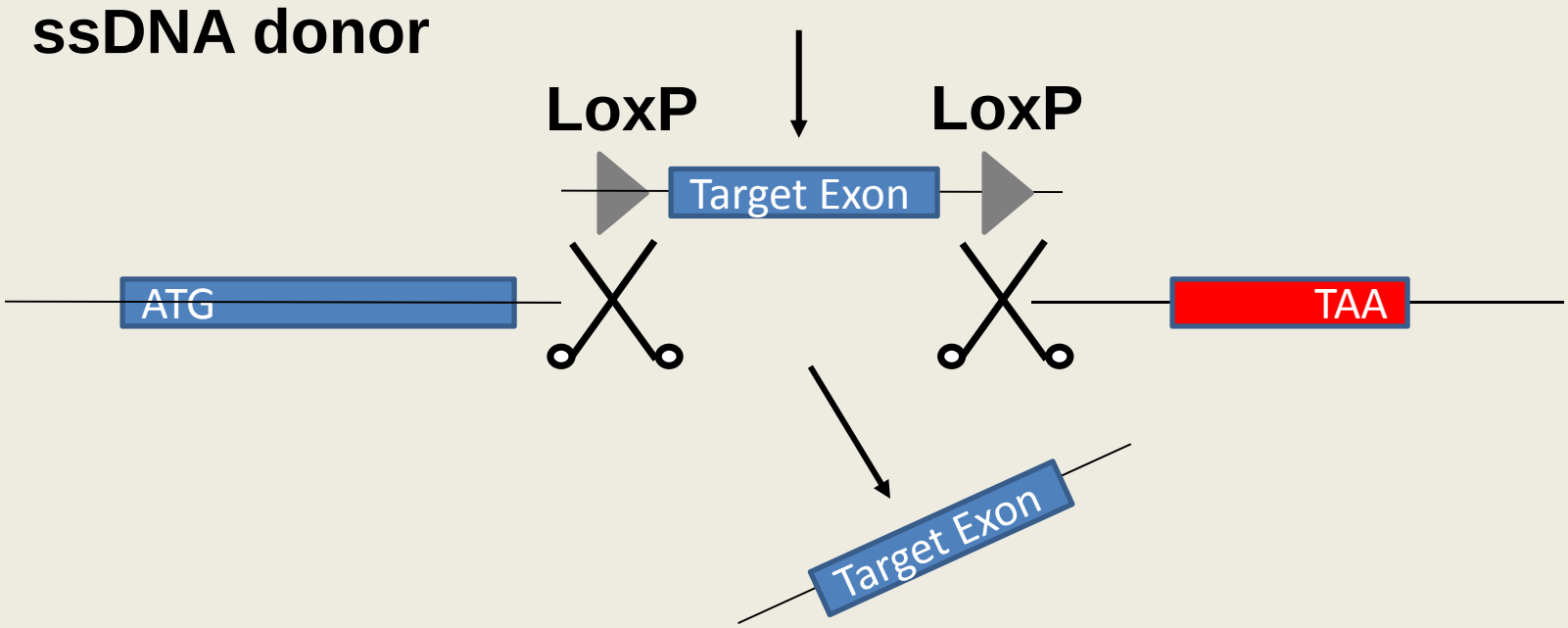
Creating conditional knockout models



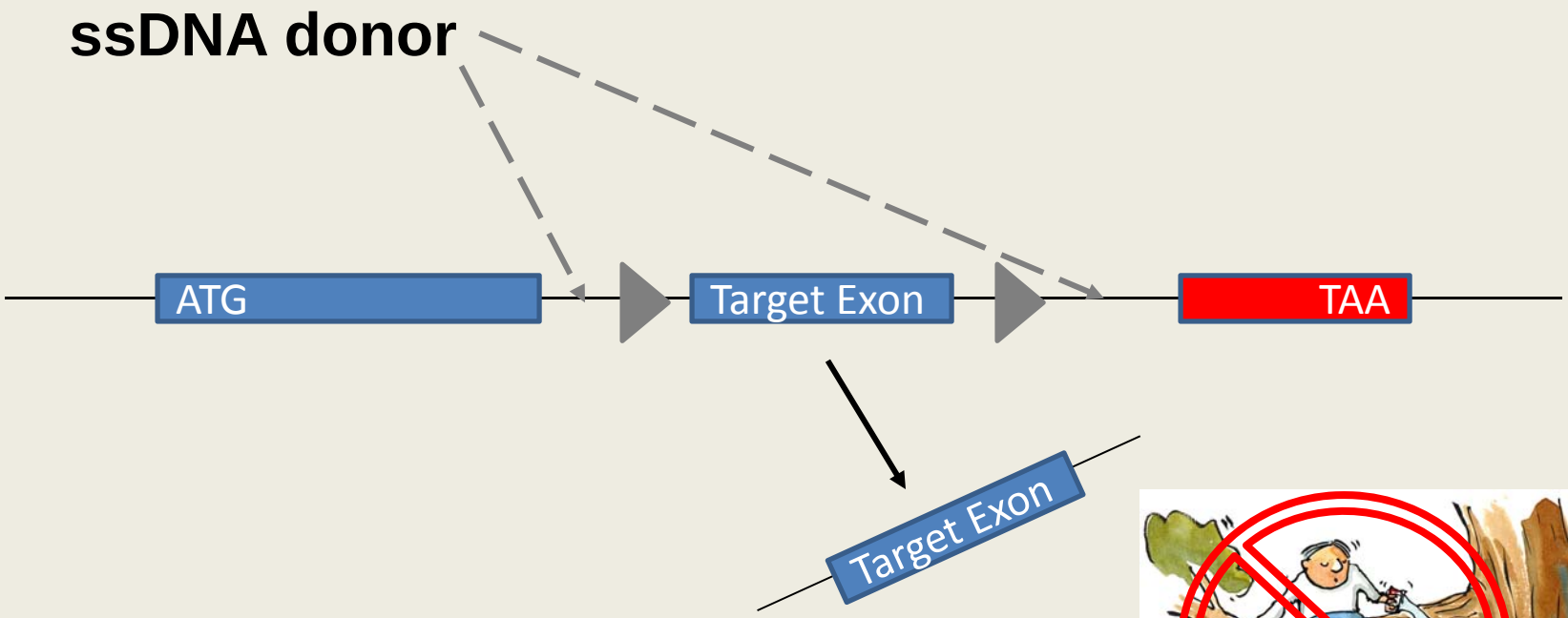
Creating conditional knockout models



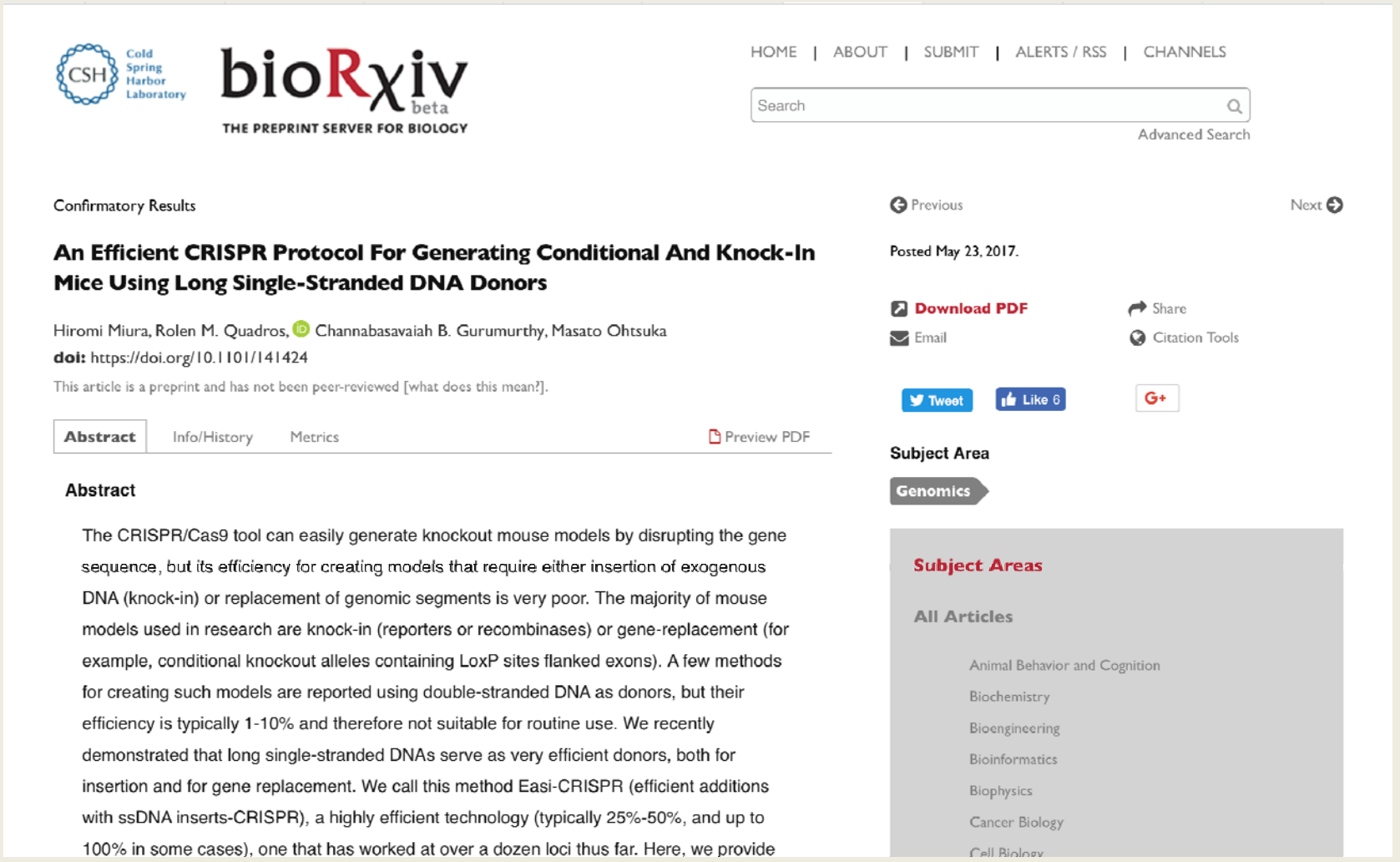
Creating conditional knockout models



Creating conditional knockout models



Easi-CRISPR Protocol?



http://www.biorxiv.org/content/early/2017/05/23/141424

Knock-in design

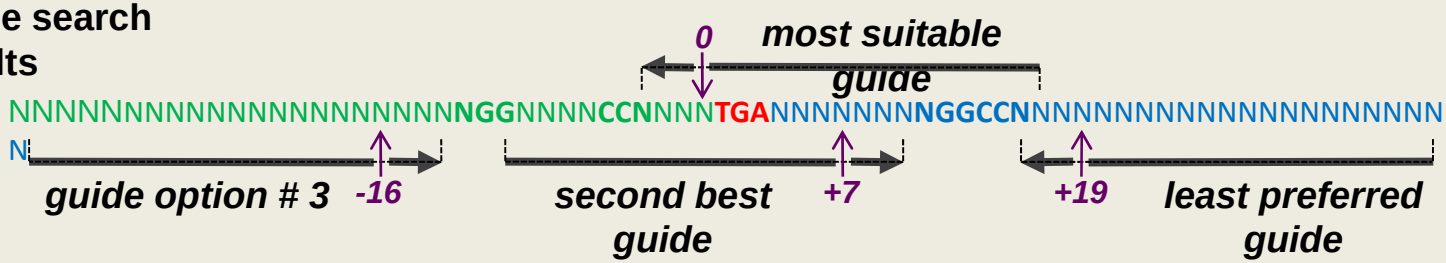
a **Genomic locus**

~50-80 base sequence used as an *in put* for searching guides

Knock-in design

a **Genomic locus**

b **Guide search results**

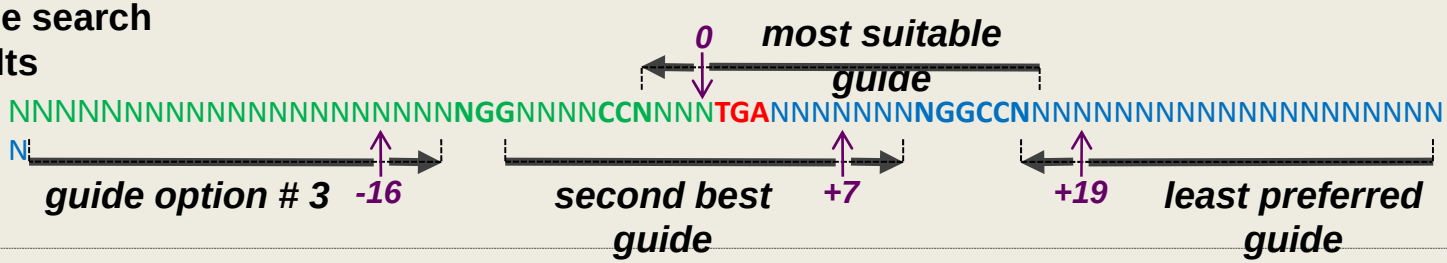


Knock-in design

a **Genomic locus**

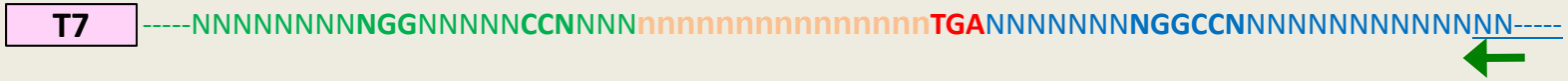
~50-80 base sequence used as an *in put* for searching guides

b **Guide search results**



c **Schematic of donor DNA**

Sequence of ssDNA donor



Knock-in design

a **Genomic locus**

NNNNNNNNNNNNNNNNNNNNNNNNNGGNNNNNCCNNNNTGANNNNNNNNGGCCNNNNNNNNNNNNNNNNNNNNNNNNN

~50-80 base sequence used as an *in put* for searching guides

b **Guide search results**

0 *most suitable guide*

NNNNNNNNNNNNNNNNNNNNNNNNNGGNNNNNCCNNNNTGANNNNNNNNGGCCNNNNNNNNNNNNNNNNNNNNNNNNN

guide option # 3 *-16* *second best guide* *+7* *+19* *least preferred guide*

c **Schematic of donor DNA**

Sequence of ssDNA donor

T7 -----NNNNNNNNNGGNNNNNCCNNNNnnnnnnnnnnnnnnnnnTGANNNNNNNNGGCCNNNNNNNNNNNNNNN-----

d **Knock-in locus**

-----NNNNNNNNNGGNNNNNCCNNNNnnnnnnnnnnnnnnnnnTGANNNNNNNNGGCCNNNNNNNNNNNNNNN-----

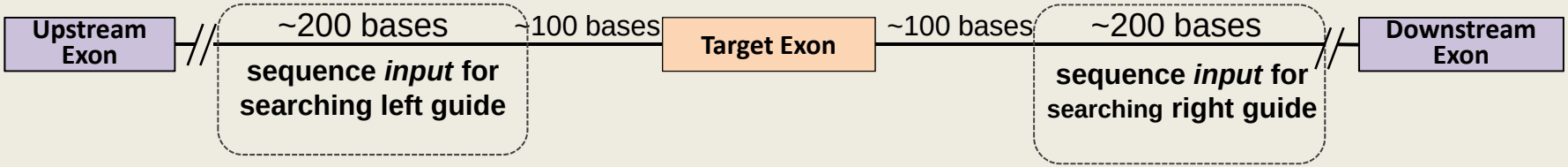
Upstream arm (~55-100 bases) | New sequence | Downstream arm (~55-100 bases)

Floxing design

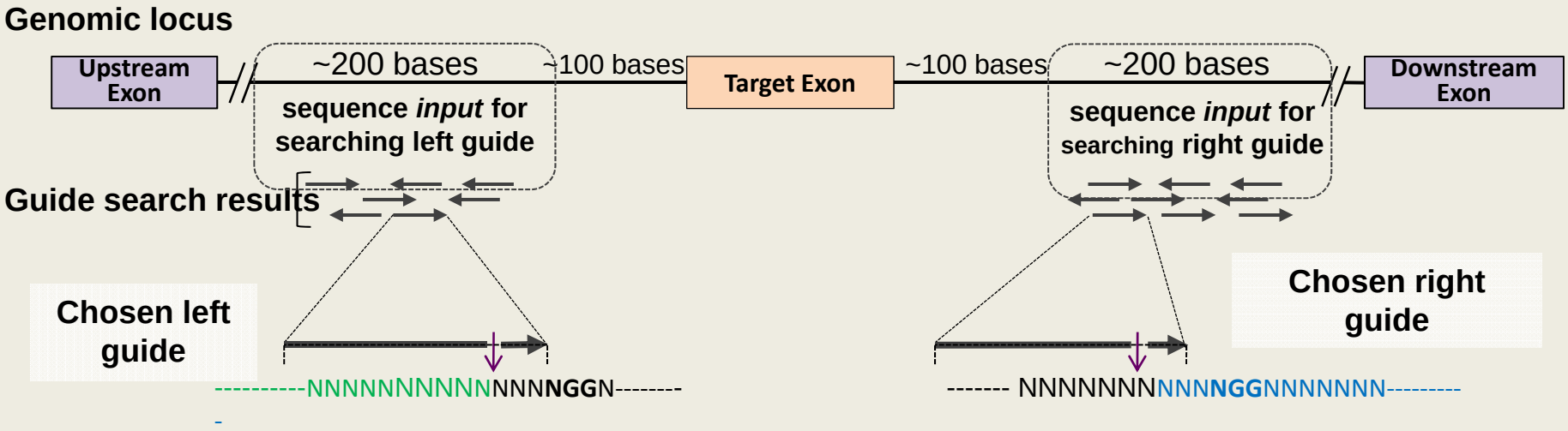
Genomic locus

Floxing design

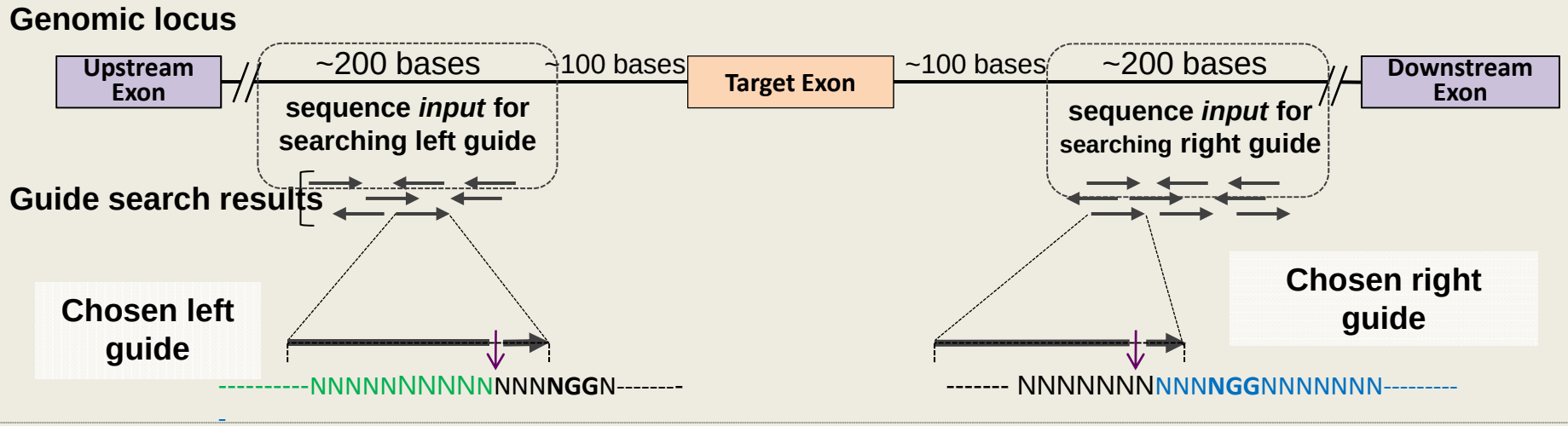
Genomic locus

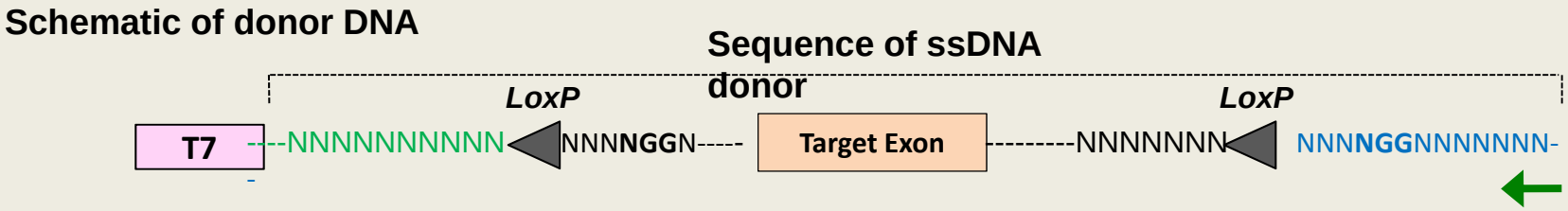


Floxing design

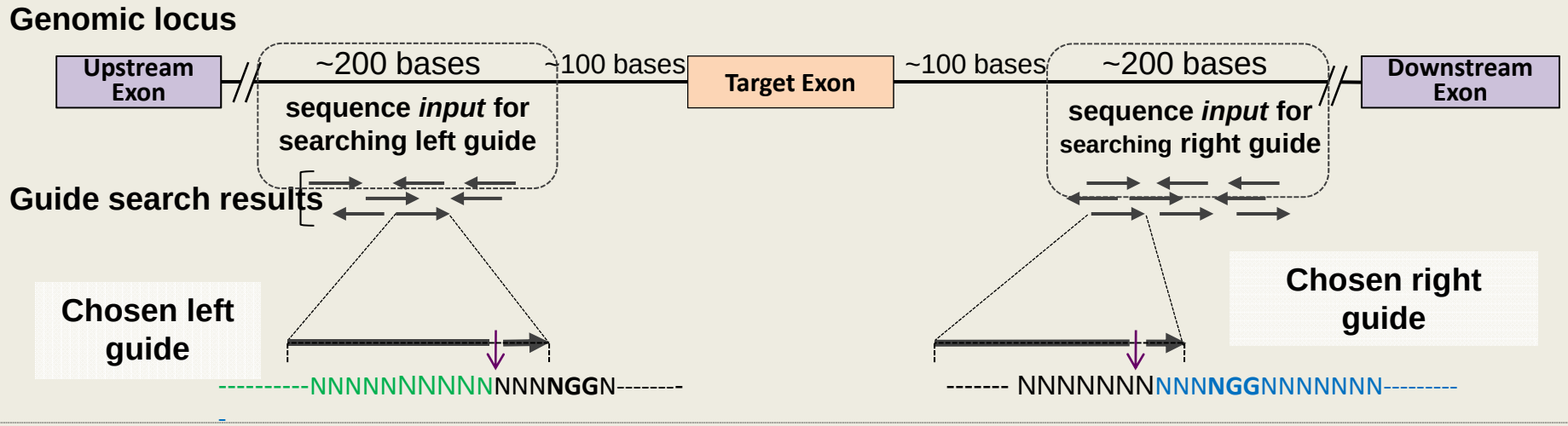


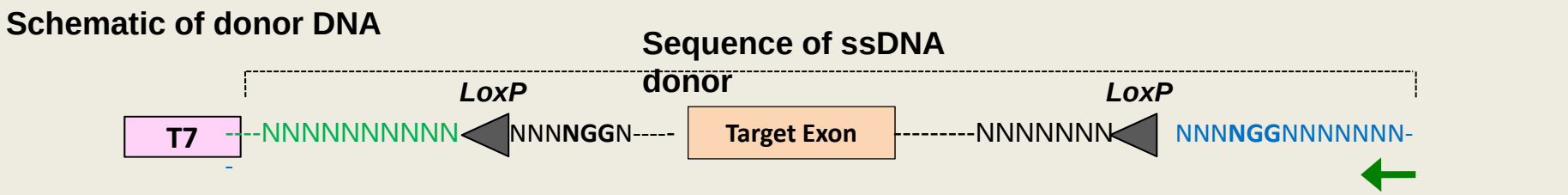
Floxing design

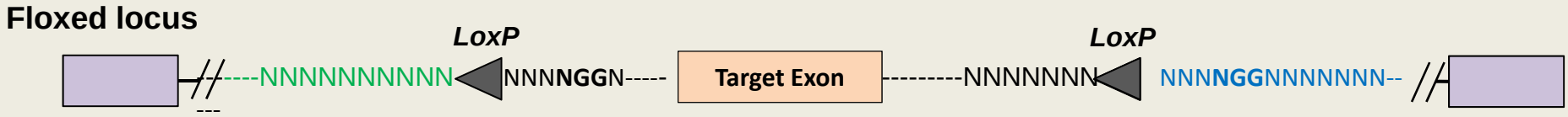


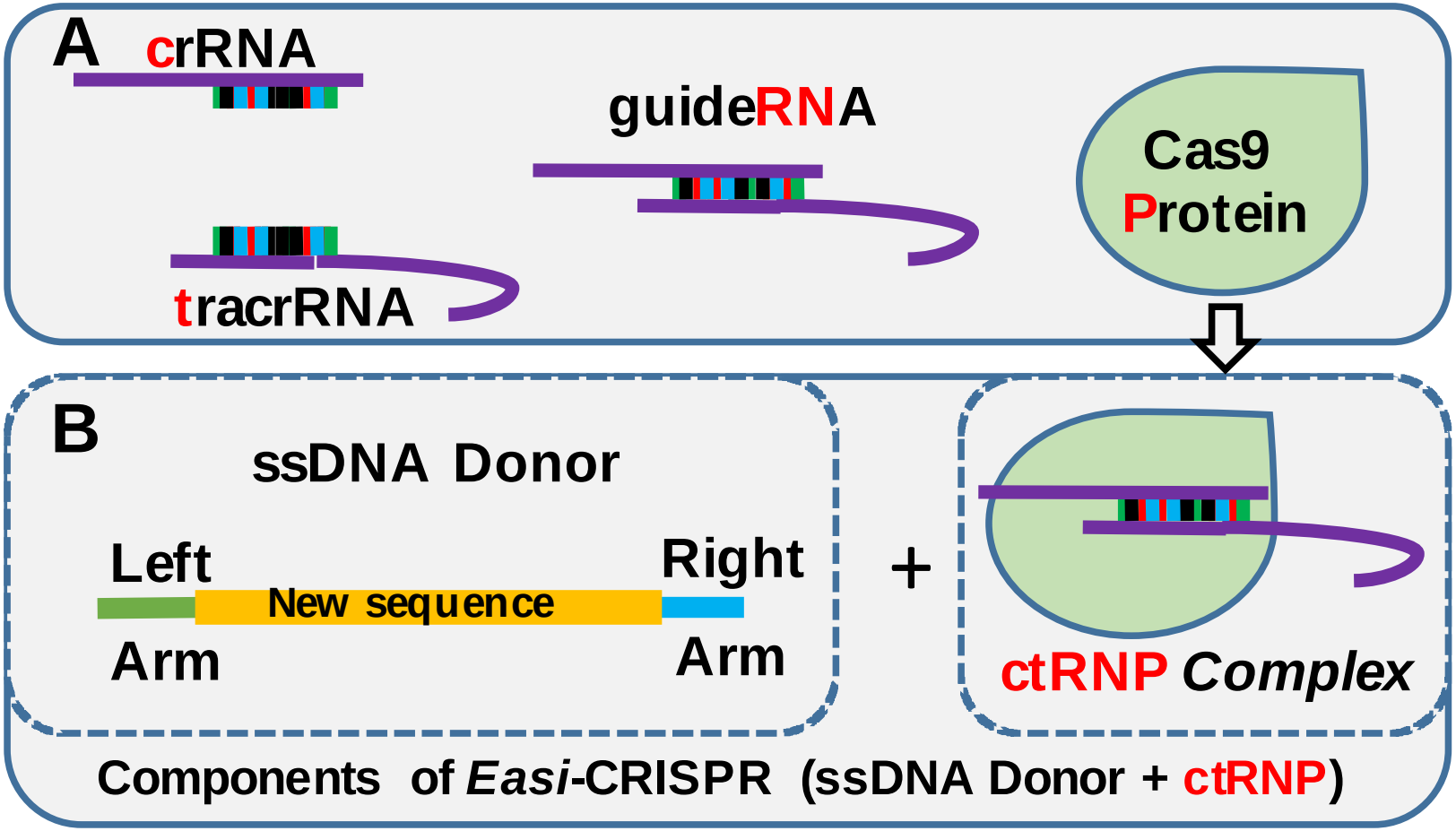


Floxing design

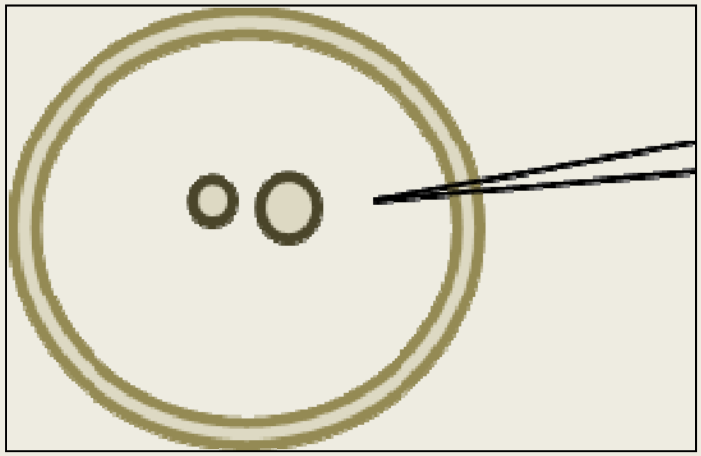







A
crRNA
guideRNA
tracrRNA
Cas9
Protein
B
ssDNA Donor
Left
Arm
New sequence
Right
Arm
+
ctRNP Complex
Components of Easi-CRISPR (ssDNA Donor + ctRNP)

Microinjection

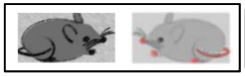


What to inject?	**Guide RNA: Cas9 protein: Donor DNA**
How much to inject?	**10:10:10 (ng/ul)**
Where to inject?	**Primarily Pronucleus**

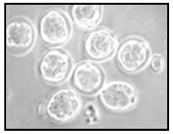
Can *Easi*-CRISPR be done *in situ*?

(without handling the zygotes *ex vivo*)

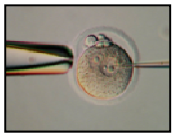
Traditional Gene editing steps



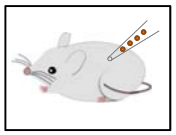
Mate mice



Isolate fertilized eggs

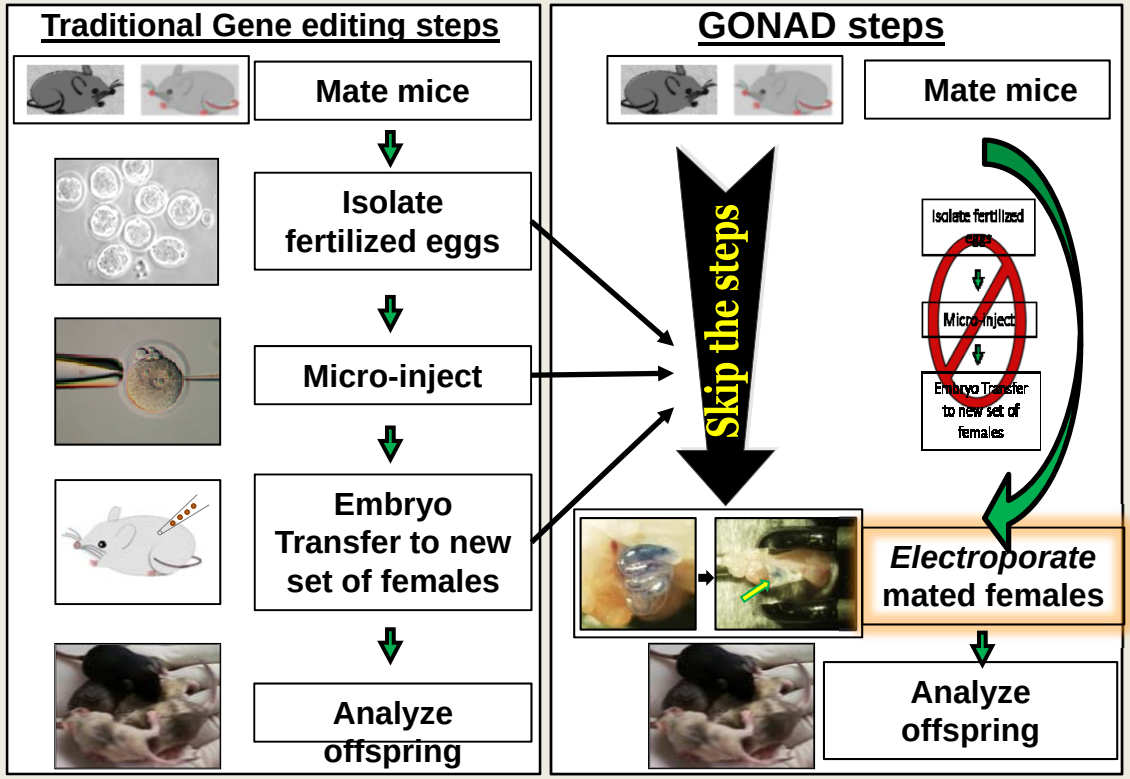


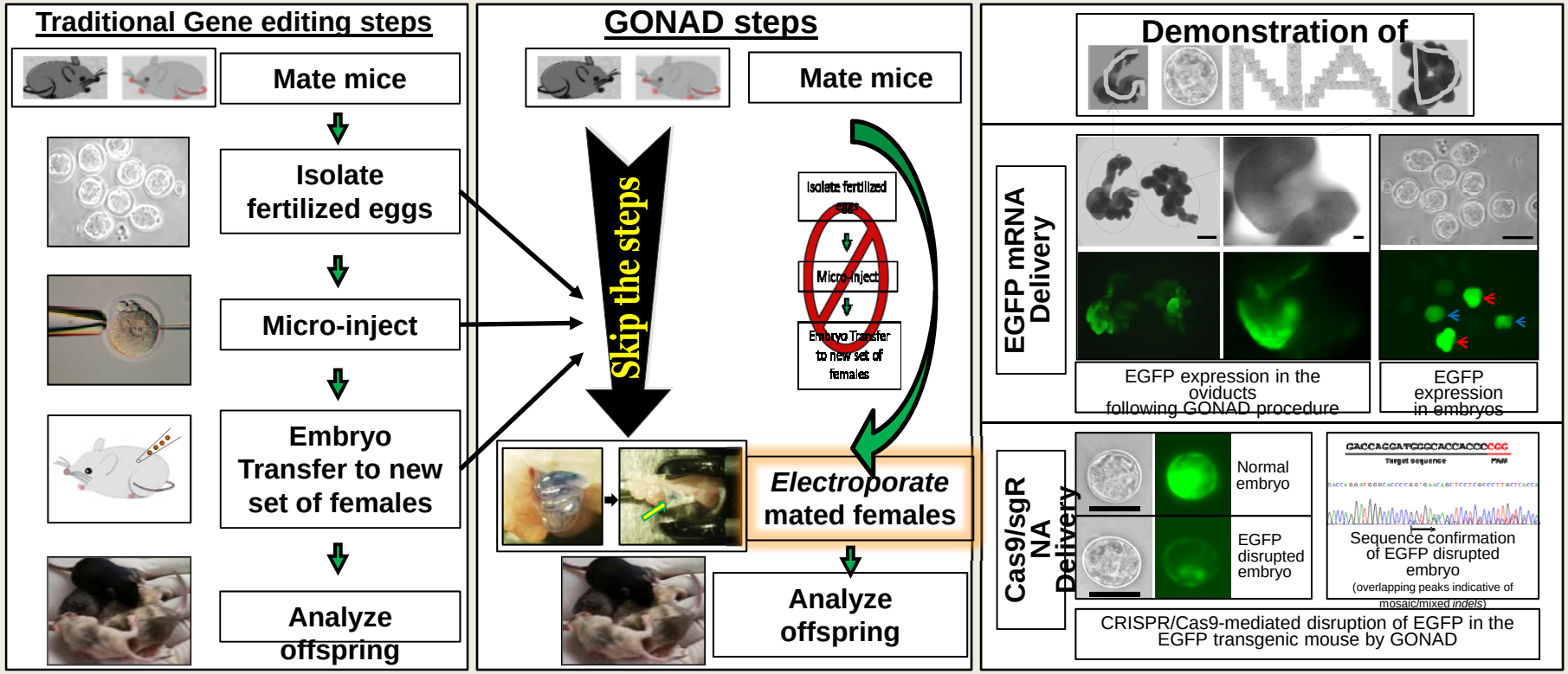
Micro-inject



Embryo Transfer to new set of females

Analyze offspring


Traditional Gene editing steps
Mate mice
Isolate fertilized eggs
Micro-inject
Embryo Transfer to new set of females
Analyze offspring
GONAD steps
Mate mice
Skip the steps
Isolate fertilized eggs
Micro-inject
Embryo Transfer to new set of females
Electroporate mated females
Analyze offspring


Traditional Gene editing steps
Mate mice
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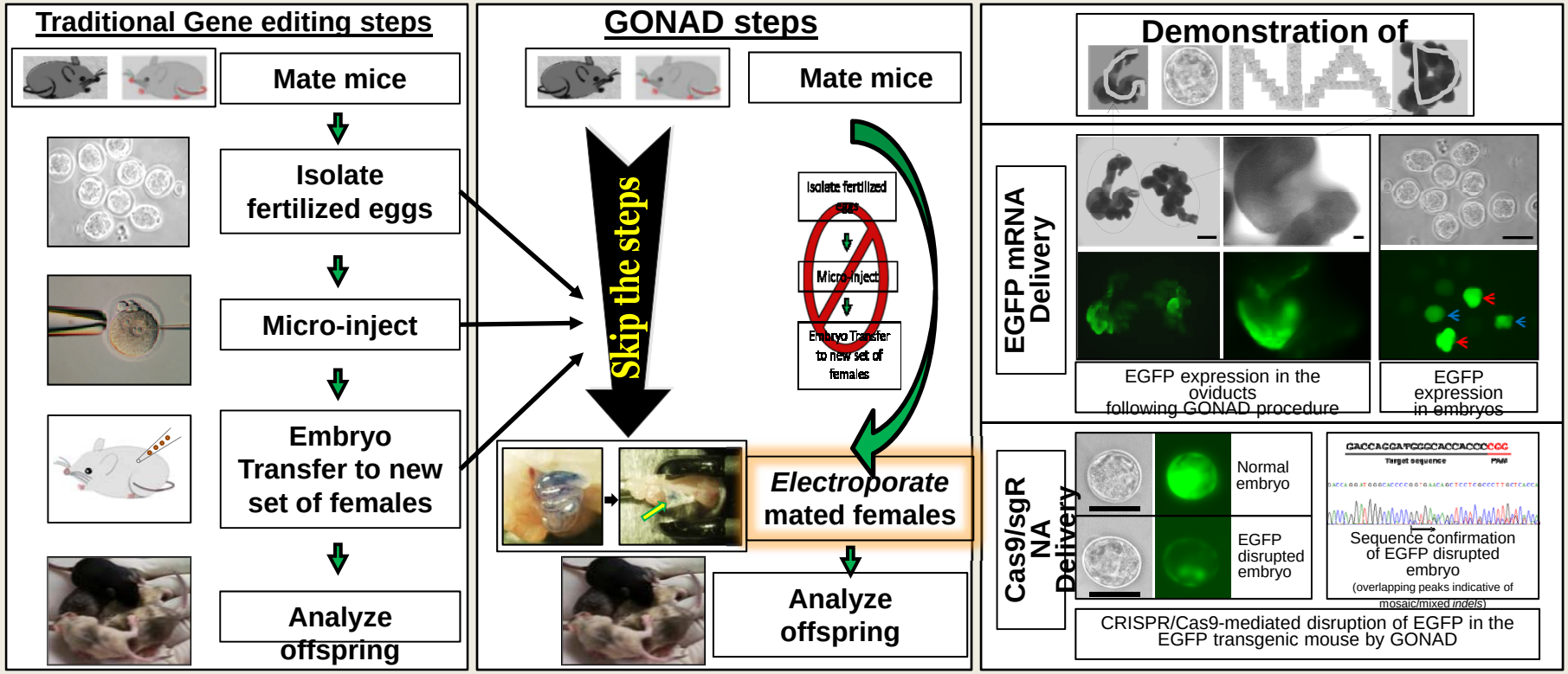
GONAD steps
Mate mice
Skip the steps
Isolate fertilized eggs
Micro-inject
Embryo Transfer to new set of females
Electroporate mated females
Analyze offspring
Demonstration of GONAD
EGFP mRNA Delivery
EGFP expression in the oviducts following GONAD procedure
EGFP expression in embryos
Cas9/sgRNA Delivery
Normal embryo
EGFP disrupted embryo
Target sequence
PAM
Sequence confirmation of EGFP disrupted embryo
(overlapping peaks indicative of mosaic/mixed indel(s))
CRISPR/Cas9-mediated disruption of EGFP in the EGFP transgenic mouse by GONAD



GONAD: *G*enome-editing via *O*viductal *N*ucleic *A*cids *D*elivery system, a novel *microinjection-independent* genome editing method

https://www.nature.com/articles/srep11406

http://www.biorxiv.org/content/early/2017/08/04/172718

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***In situ* genome editing method suitable for routine generation of germline modified animal models**

Masato Ohtsuka, Hiromi Miura, Naomi Arifin, Shingo Nakamura, Kenta Wada, Channabasavaiah Gurumurthy, Masahiro Sato

doi: https://doi.org/10.1101/172718

Posted August 4, 2017.

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Subject Area

Genomics

Abstract

Animal genome engineering experimental procedures involve three major steps: isolation of zygotes from pregnant females; microinjection of zygotes, and; transfer of injected zygotes into recipient females, that have been practiced for over three decades. The laboratory set ups intending to performing these procedures require to have sophisticated equipment as well as highly skilled technical personnel. Because of these reasons, animal transgenesis experiments are typically performed at centralized core facilities in most research organizations. We recently

Subject Areas

All Articles

Animal Behavior and Cognition

Biochemistry

http://www.biorxiv.org/content/early/2017/08/04/172718

Easi-CRISPR + GONAD = a total solution

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All technology developers….

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Hiromi Miura
Tokai University, Japan


Masahiro Sato
Kagoshima University

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Institutional funds,
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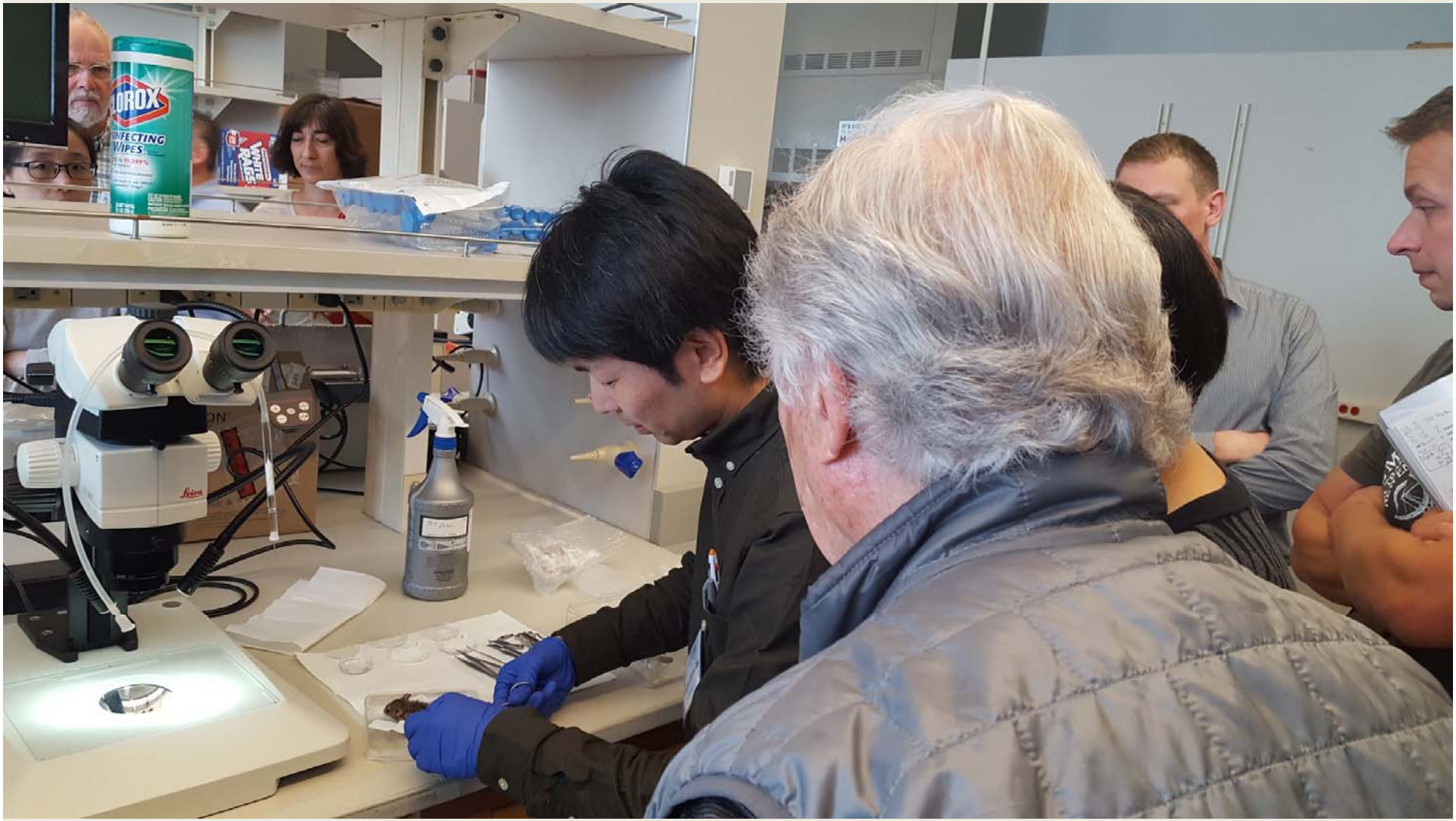
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DISINFECTING
WIPES

Thank You!

cgurumurthy@unmc.edu