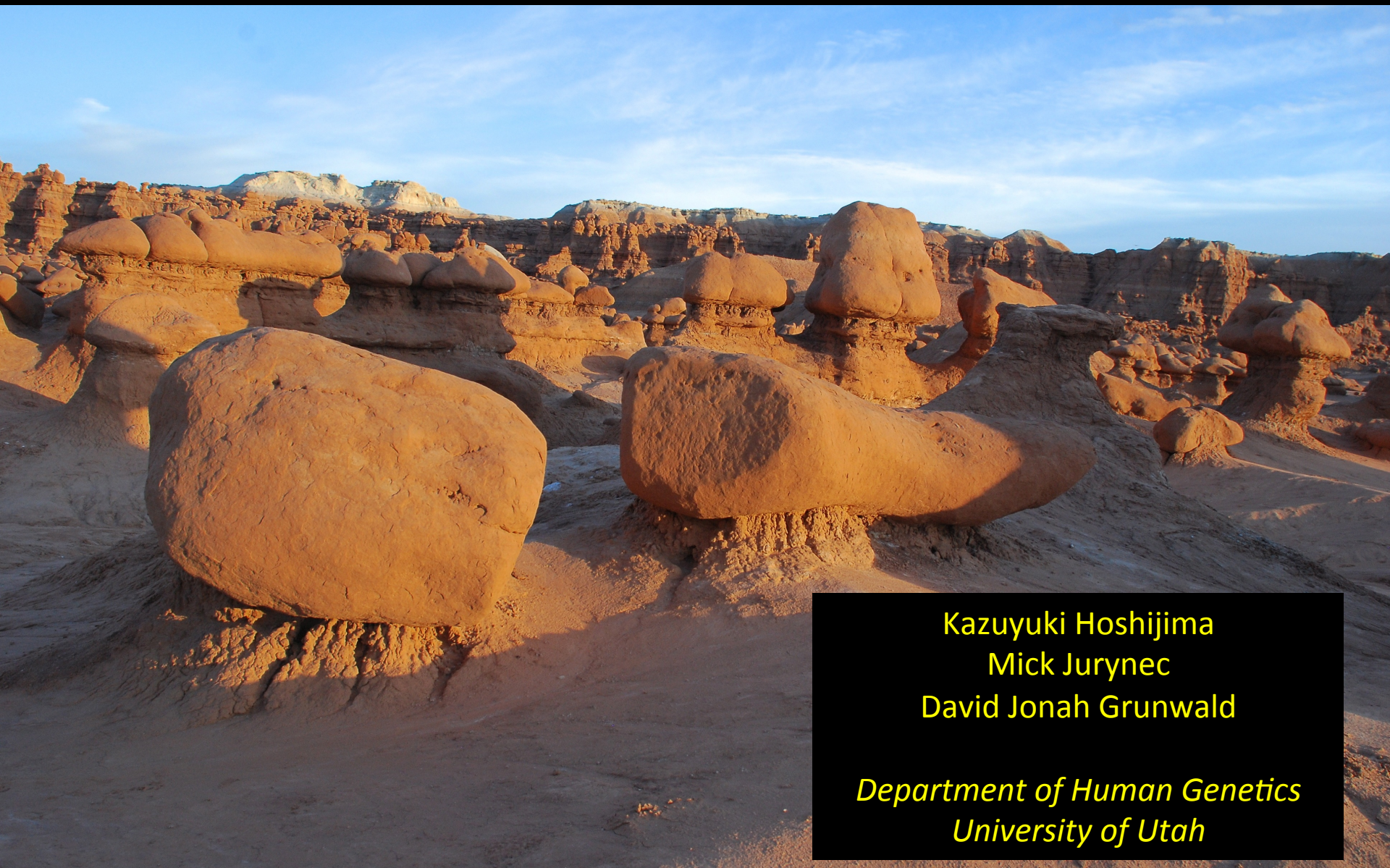
Editing the zebrafish genome



Kazuyuki Hoshijima
Mick Jurynec
David Jonah Grunwald

Department of Human Genetics
University of Utah

Several approaches to genome editing –
your goals will dictate the methods you adopt

Our interests/motivations to develop genome editing

To generate precise disease models (many diseases are due to hypomorphic conditions)

- to understand cellular and biochemical basis

- as assay systems to develop treatments

Test hypotheses about how gene variation might underlie variation in development

- test the functions of naturally occurring coding variants

- test hypotheses of how varying timing/level/duration of a gene's expression might alter patterns of development

To conditionally ablate gene expression

- to study tissue- and temporal-specific gene functions

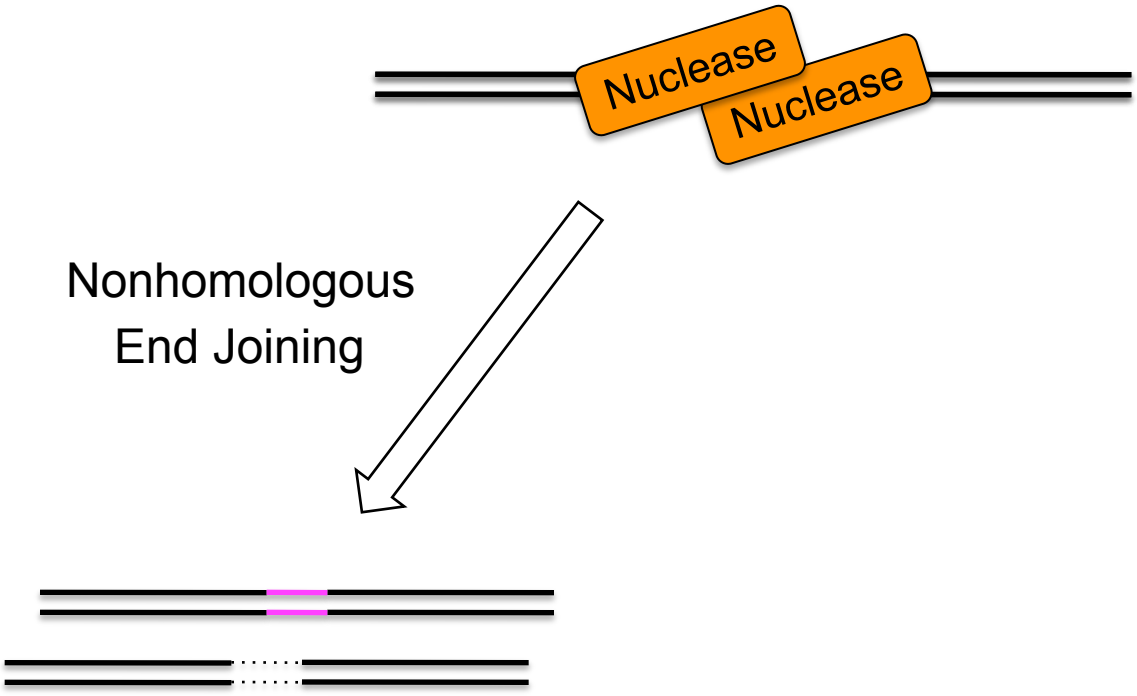
To study subcellular utilization of proteins expressed under physiological conditions

Our goal is to help make genome editing in zebrafish easy to accomplish

Focus of today:

1. *Now possible to replace any host sequence with any desired donor sequence by homologous recombination*
2. *Factors that affect efficiency of editing*
   - *Big picture issues*
   - *Details of our approach*
3. *Factors that simplify the recovery of new alleles*
4. *Problems we have encountered (we think we now understand)*

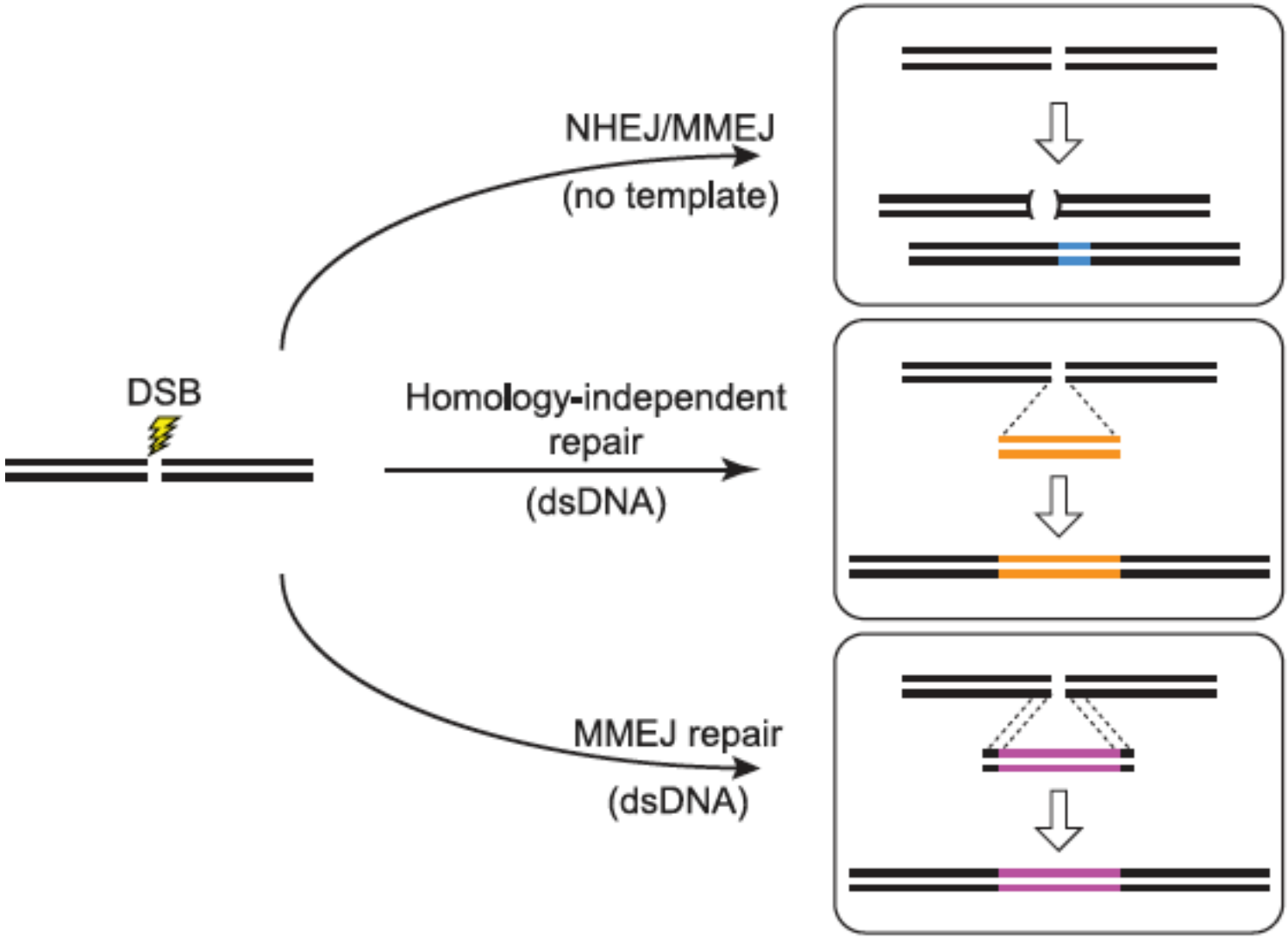
Synthetic (programmable) nucleases can induce targeted DSBs
DSBs stimulate repair events at the site of the DSB



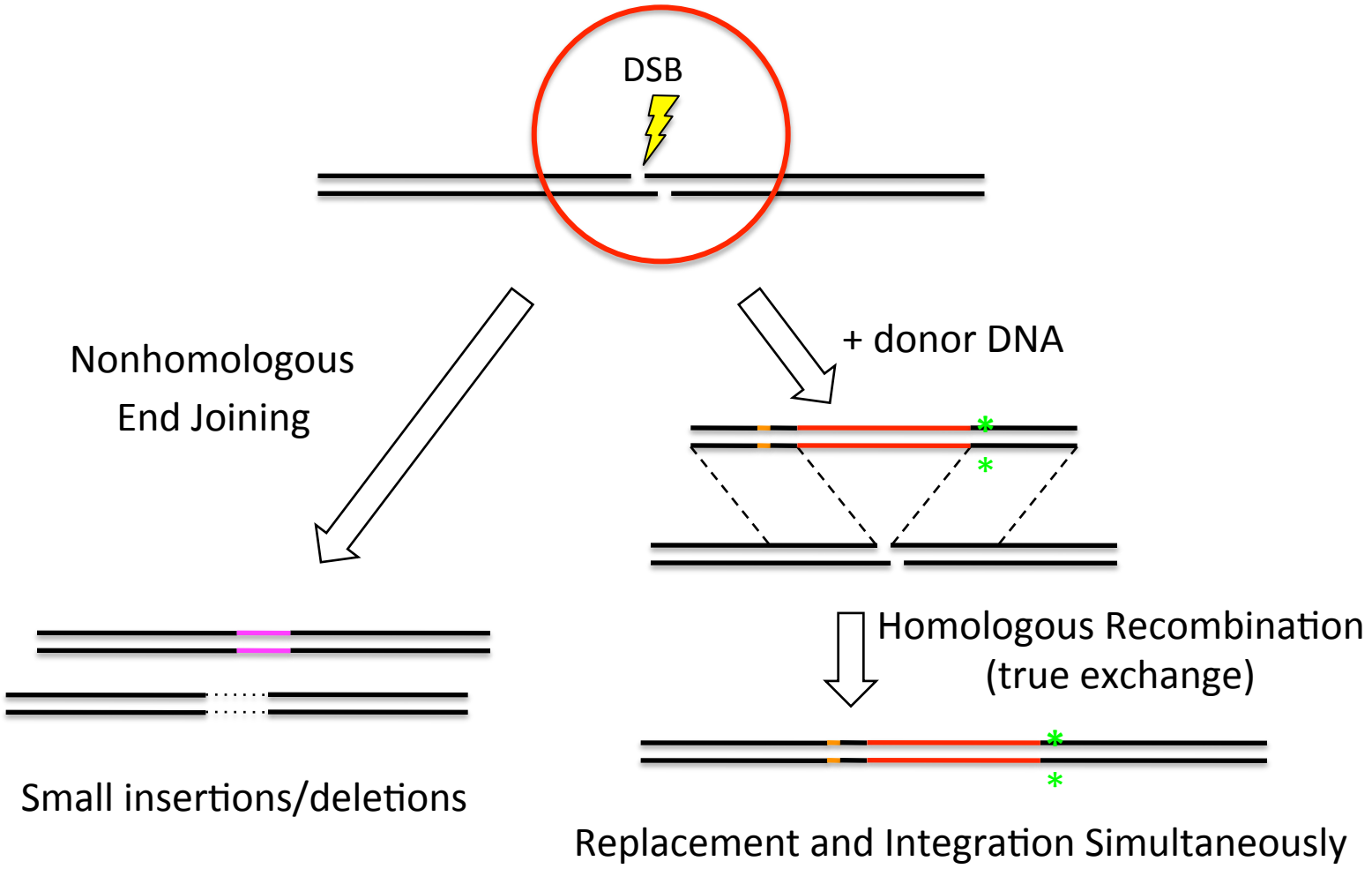
Targeted mutagenesis:

Induction of small deletions/insertions.

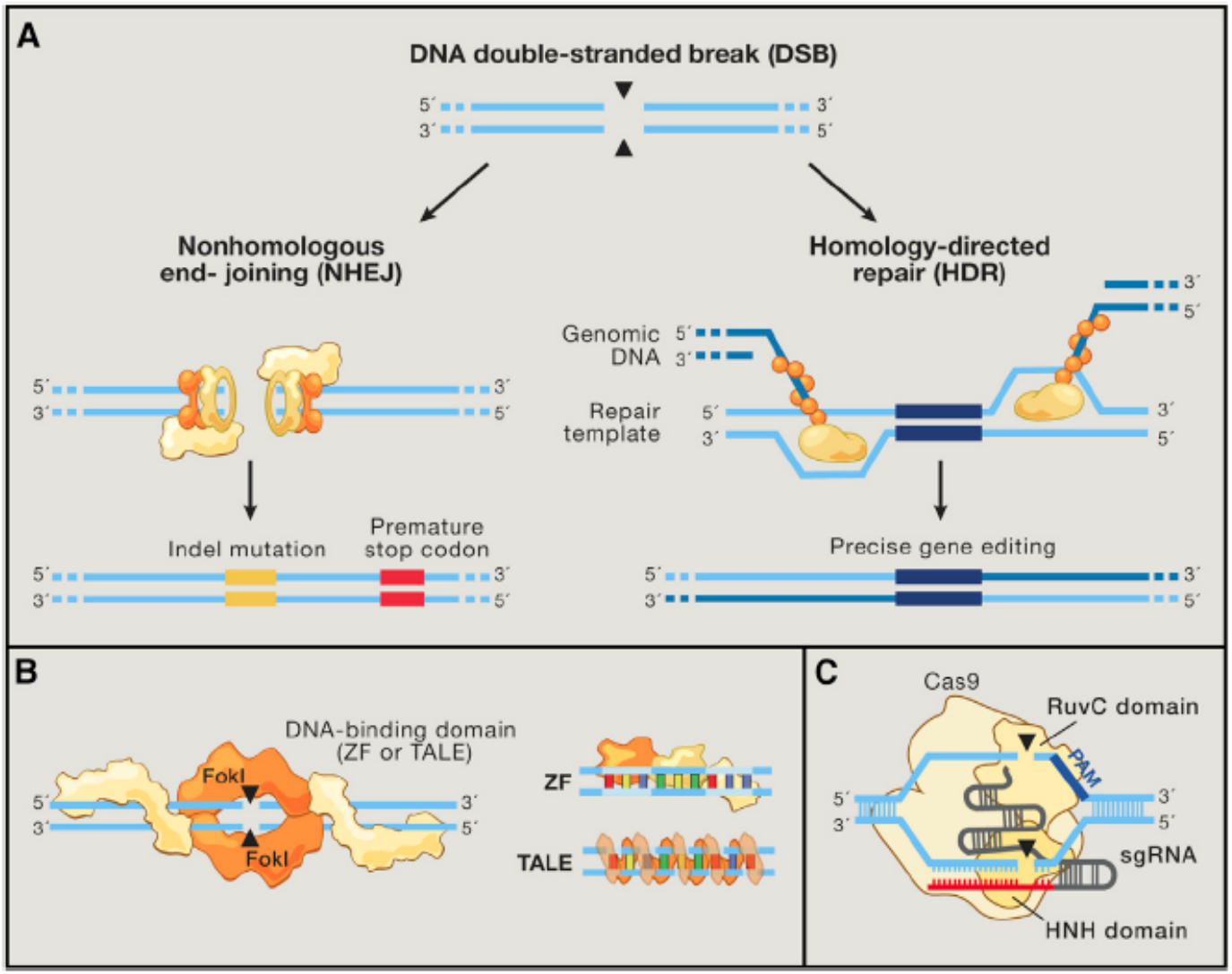
End-joining pathways can be used to insert foreign sequences at a targeted locus donor sequence



Targeted DSBs also stimulate recombination events at the site of the DSB

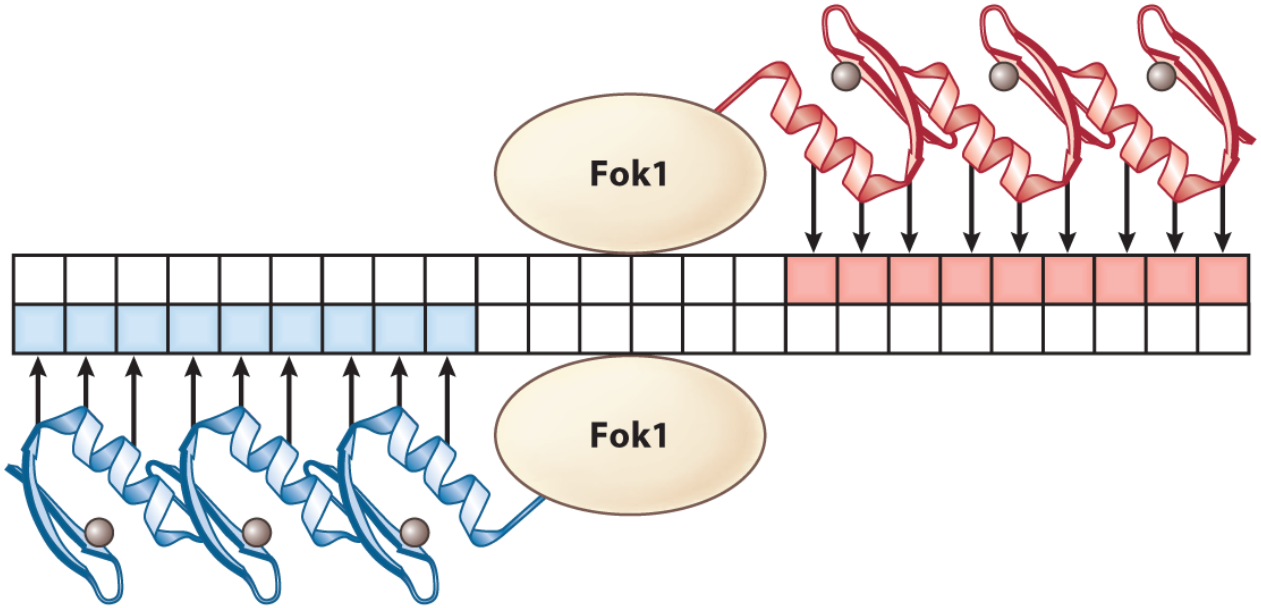


Any type of synthetic (programmable) nuclease can initiate repair and recombination events at the site of induced DSBs



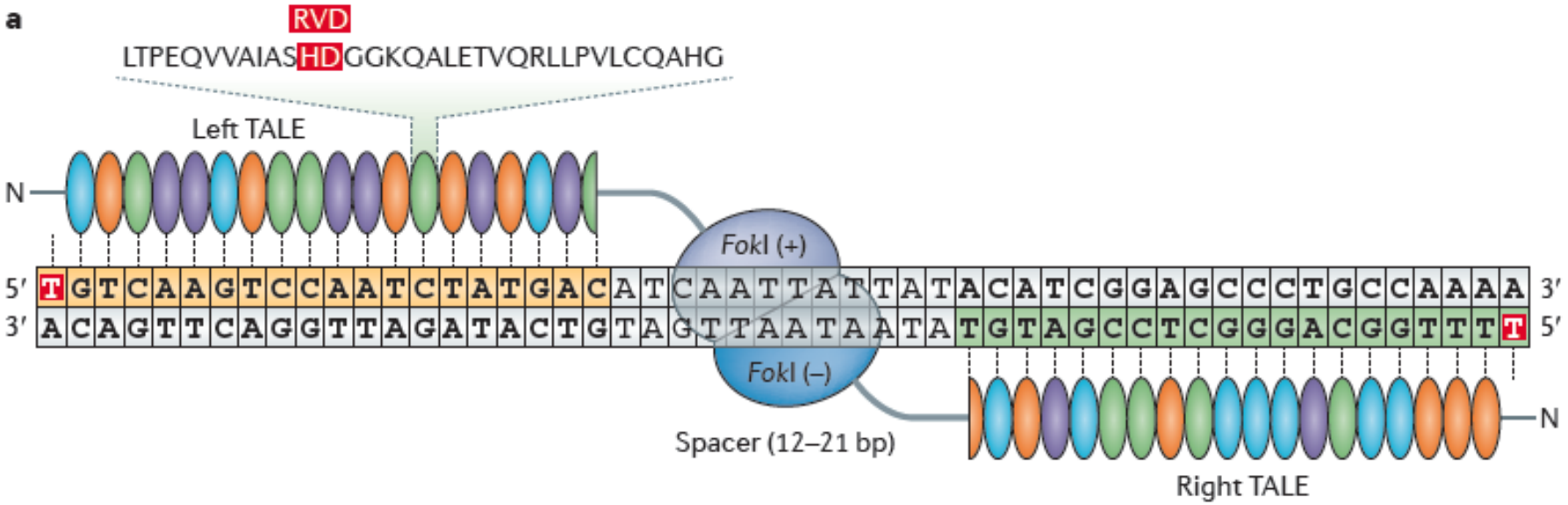
Hsu, Lander, Zhang (2014) Cell 157: 1262

Synthetic / Engineered nucleases built as a fusion between a DNA-binding domain and a non-sequence-specific double strand nuclease domain of Fok1)



Klug (2010) Ann. Rev. Biochem.

TALENs: TALE-derived nucleases



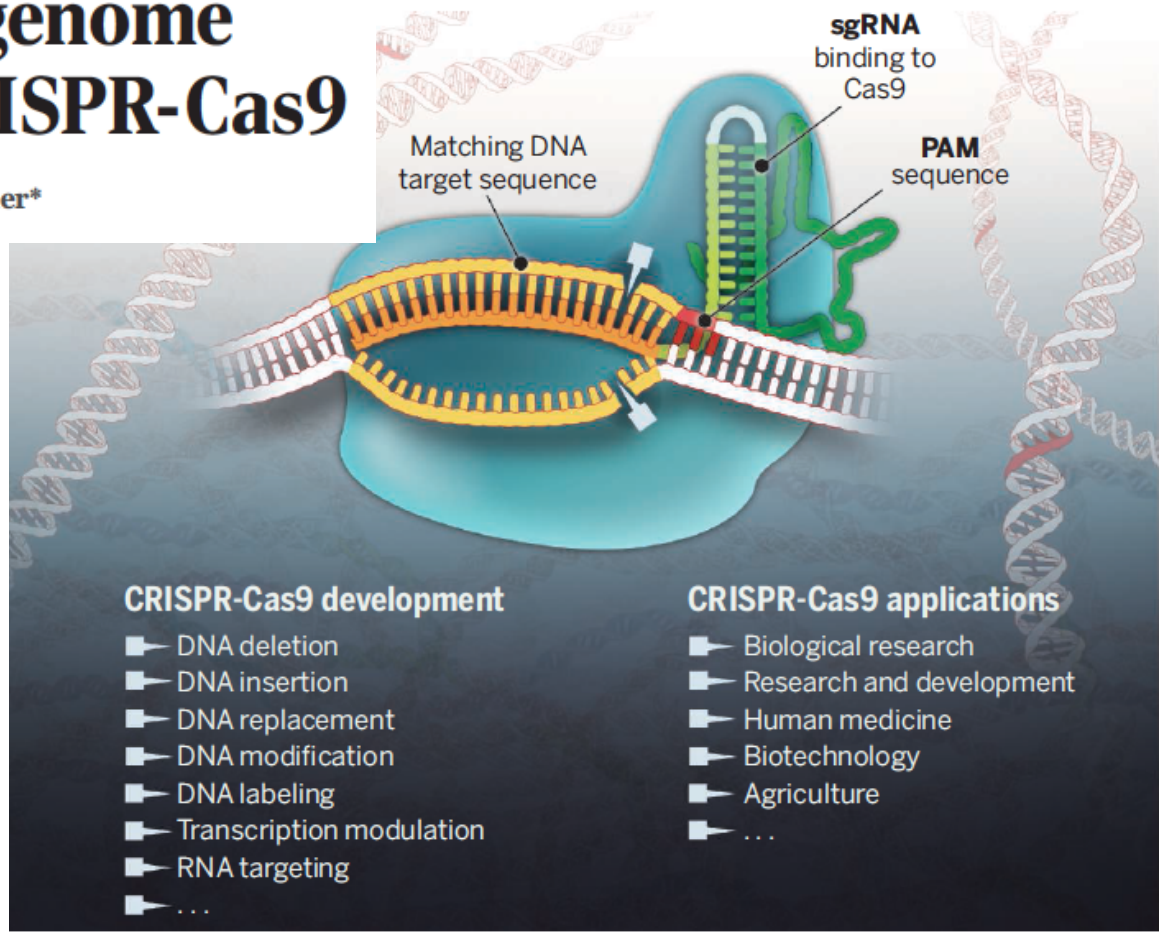
Issues: i) Assembly of gene encoding TALEN; ii) Expression following mRNA production

RNA-guided Nucleases (RGN): A True Revolution in Genome Engineering

GENOME EDITING

The new frontier of genome engineering with CRISPR-Cas9

Jennifer A. Doudna* and Emmanuelle Charpentier*

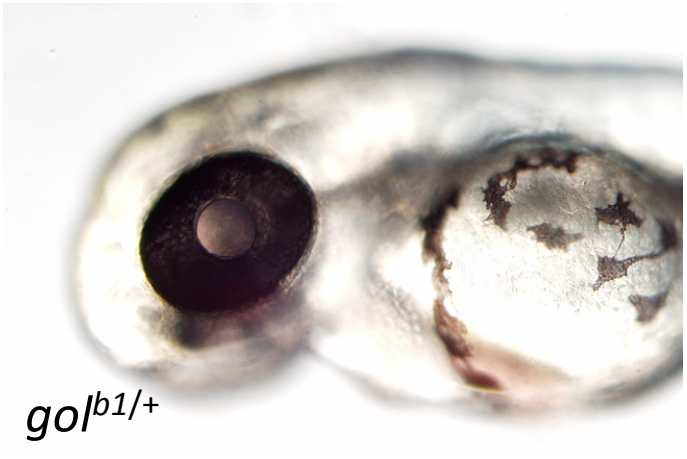


CRISPR and Cas9 proteins constitute an adaptive immunity system, found in ~40% bacterial genomes and most archaeal genomes

Doudna and Charpentier (2014) Science *346*,

In our approach, we generate DSBs in a mosaic fashion in growing embryos: Greater than 50% of targeted loci experience DSBs

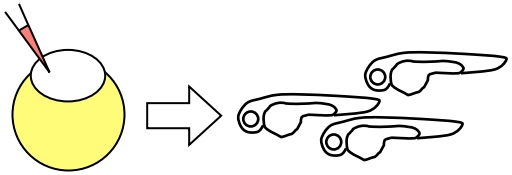
- WT embryos have pigment in eyes and on the skin at 48hpf
- *gol* mutations are recessive and don't have pigment at 48hpf



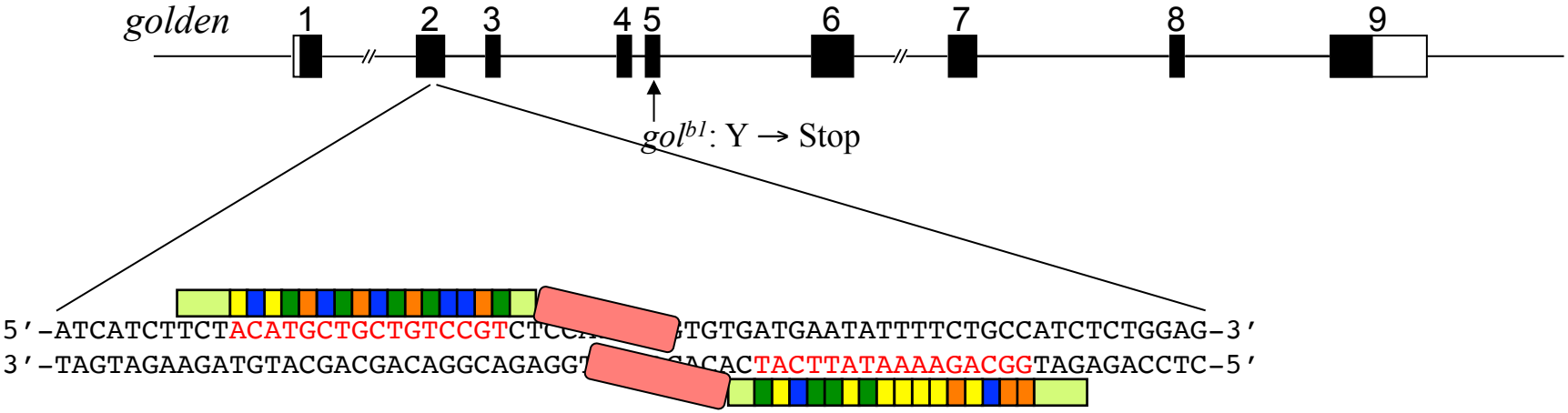
Dahlem et al (2012) PLoS Genetics

In our approach, we generate DSBs in a mosaic fashion in growing embryos:

Inject [CRISPR/Cas9 RNA] or TALEN-RNAs into *gol*$^{b1/+}$ zebrafish eggs

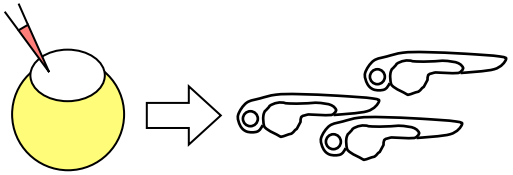


Analyze 48hpf G0 embryos for *golden* cells



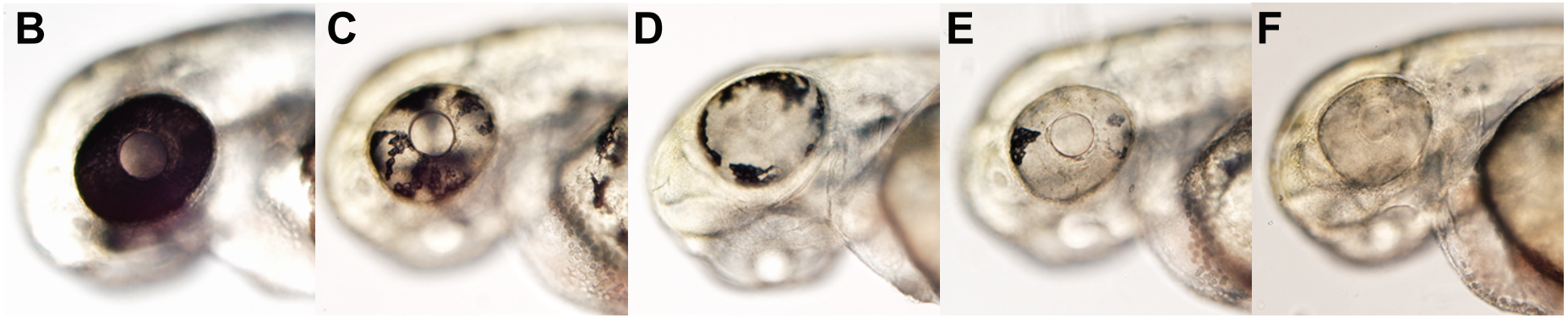
Mutations are induced in a mosaic fashion: Greater than 50% of targeted loci experience DSBs

Inject [CRISPR/Cas9 RNA] or TALEN-RNAs into *gol*$^{b1/+}$ zebrafish eggs

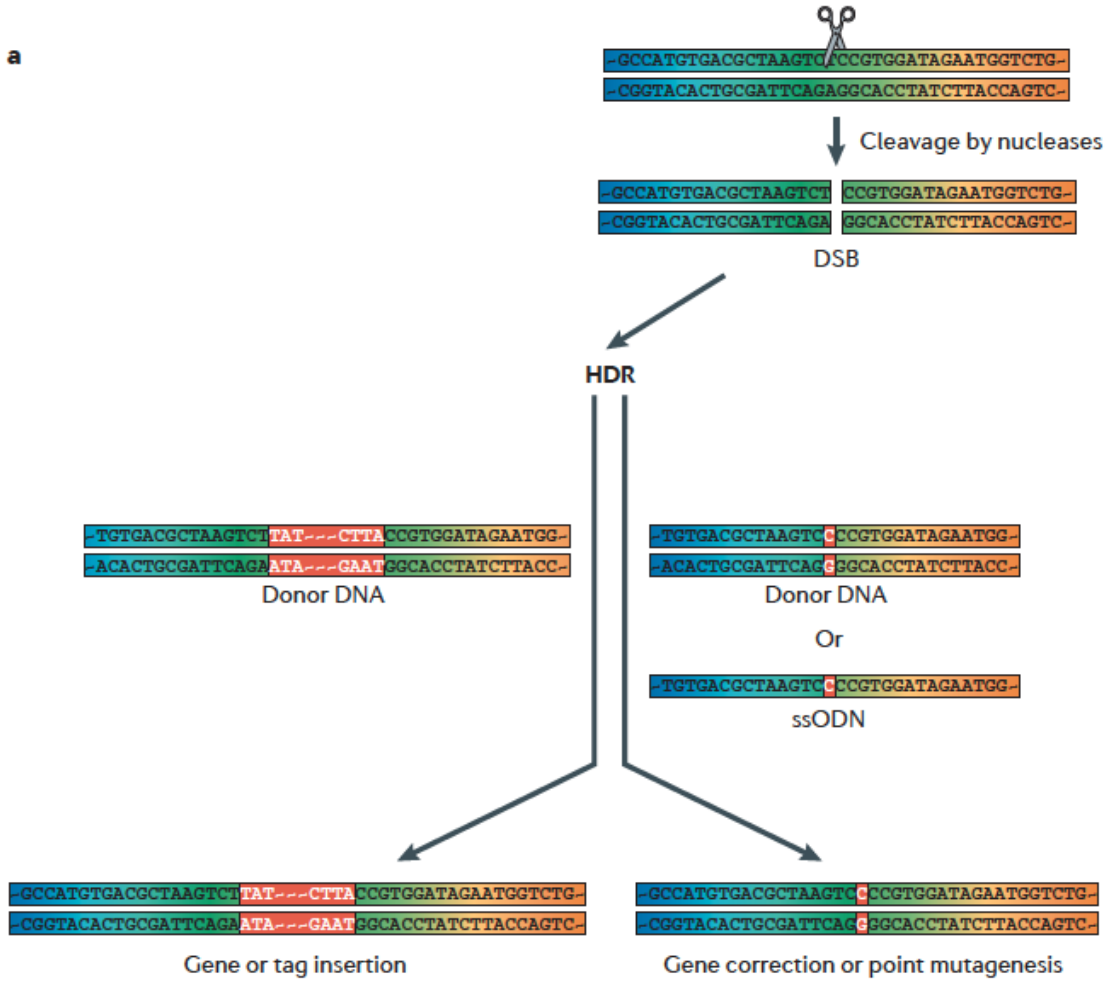


Analyze 48hpf G0 embryos for *golden* cells

gol$^{b1/+}$ control | *gol-ex2* TALEN-injected *gol*$^{b1/+}$

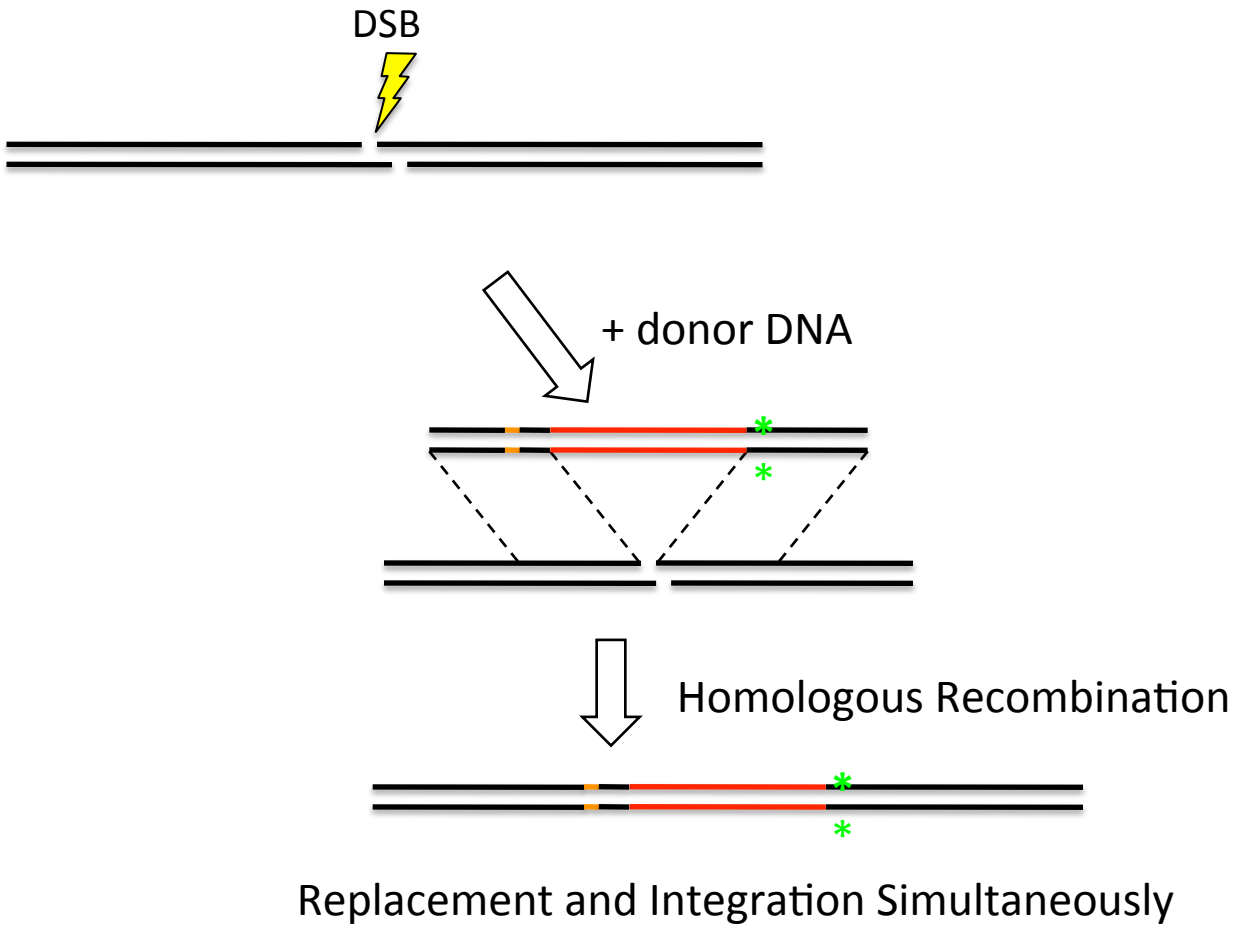


Short ssODN donors can be used as templates for Homology-Directed Repair – this affects the kinds of edited alleles that can be generated

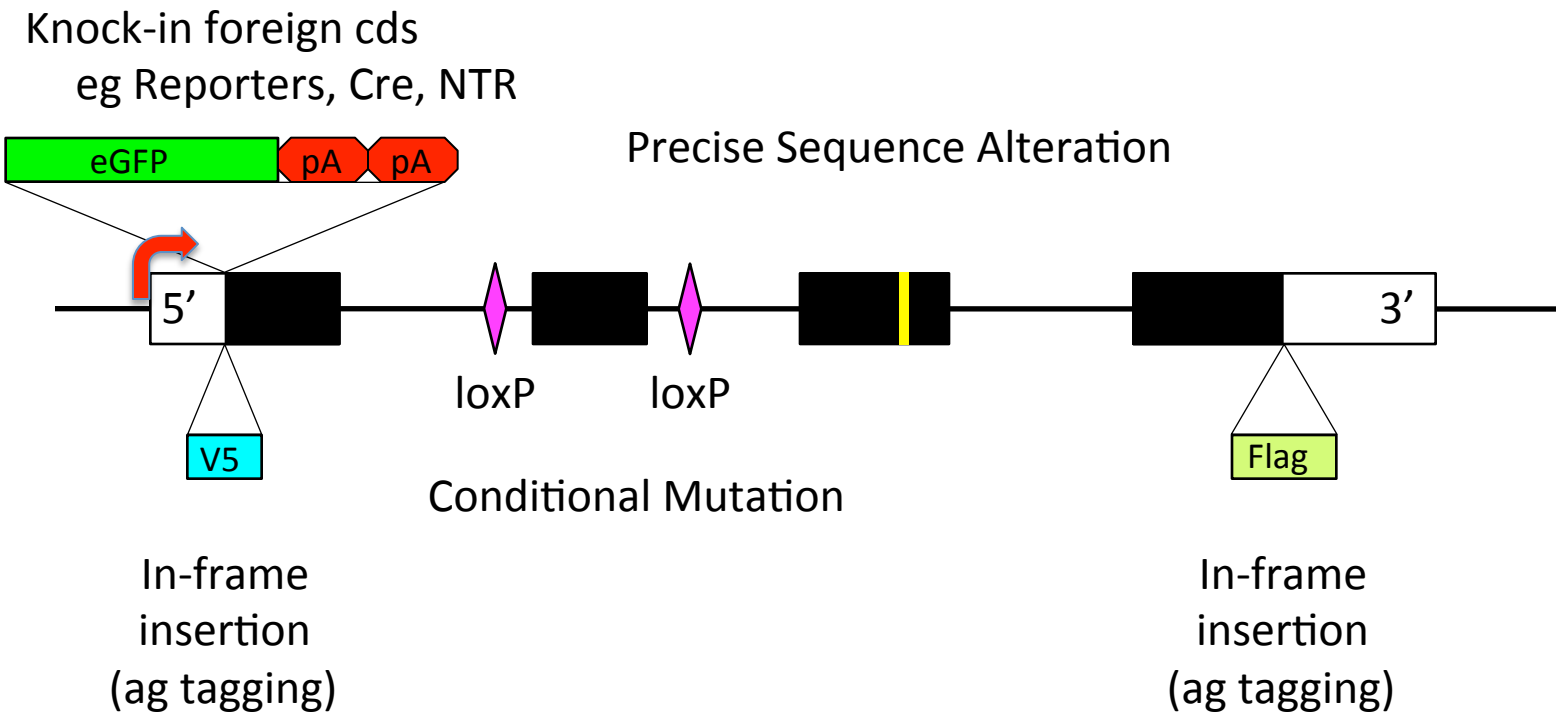


Kim and Kim, Nature Reviews Genetic 15:321-334, 2014

Long dsDNA donors can produce a range of modifications



Take-home lessons:
1. Now possible to replace any host sequence with any desired donor sequence



Take-home lessons:
2. Approaches that improve the efficiency of generating edited alleles

Factors that affect efficiency include:

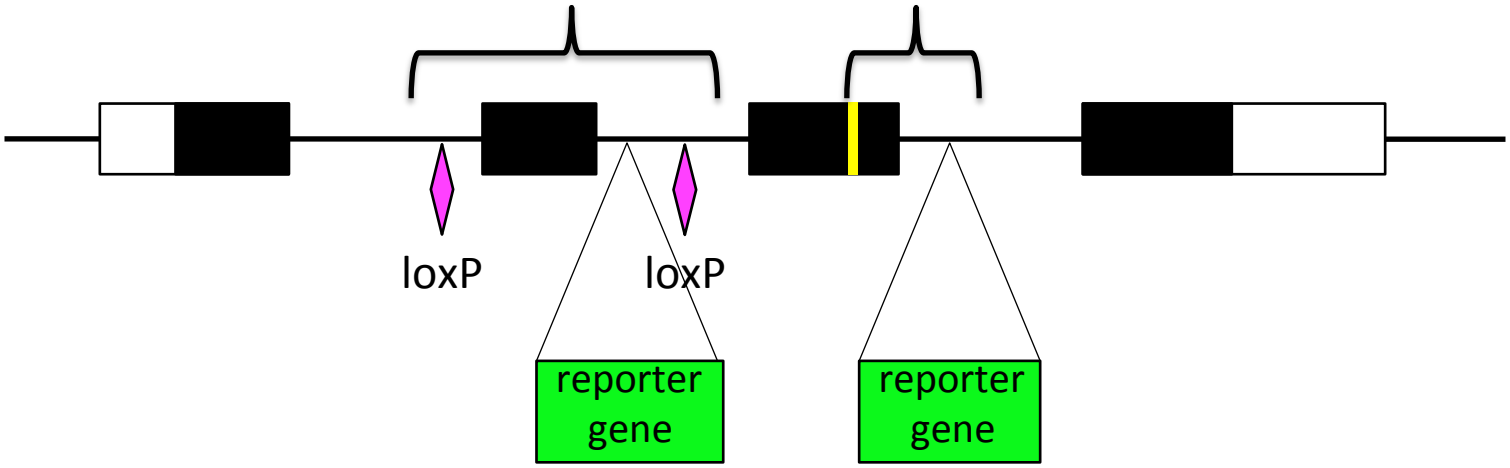
-- Optimizing the induction of DSBs

-- Template presentation

-- Homology arms

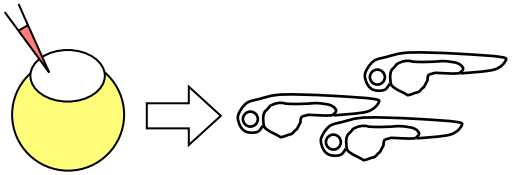
Take-home lessons:

3. By tagging edited alleles with reporter genes (which can later be excised), we improve the recovery of edited alleles by orders of magnitude

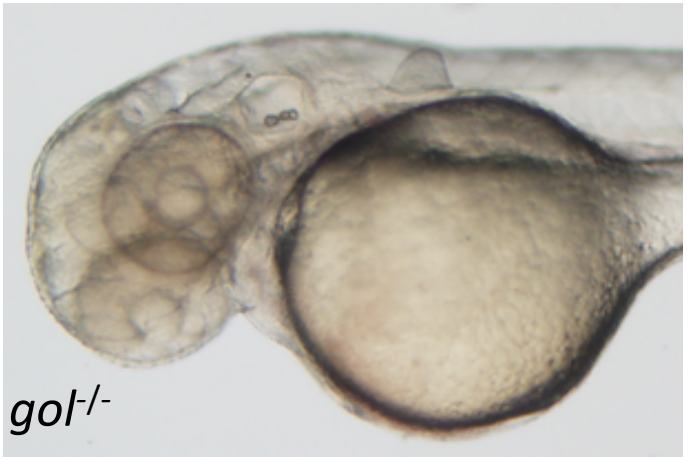


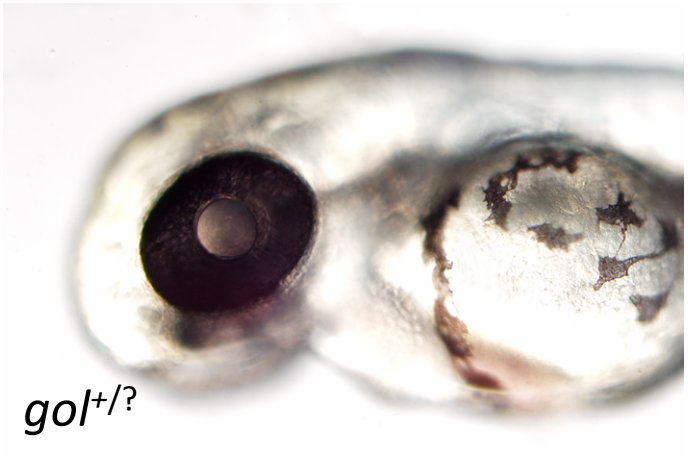
Gene editing at *golden*

Inject TALEN-RNAs +
WT donor DNA
into *gol*$^{-/-}$ zebrafish eggs

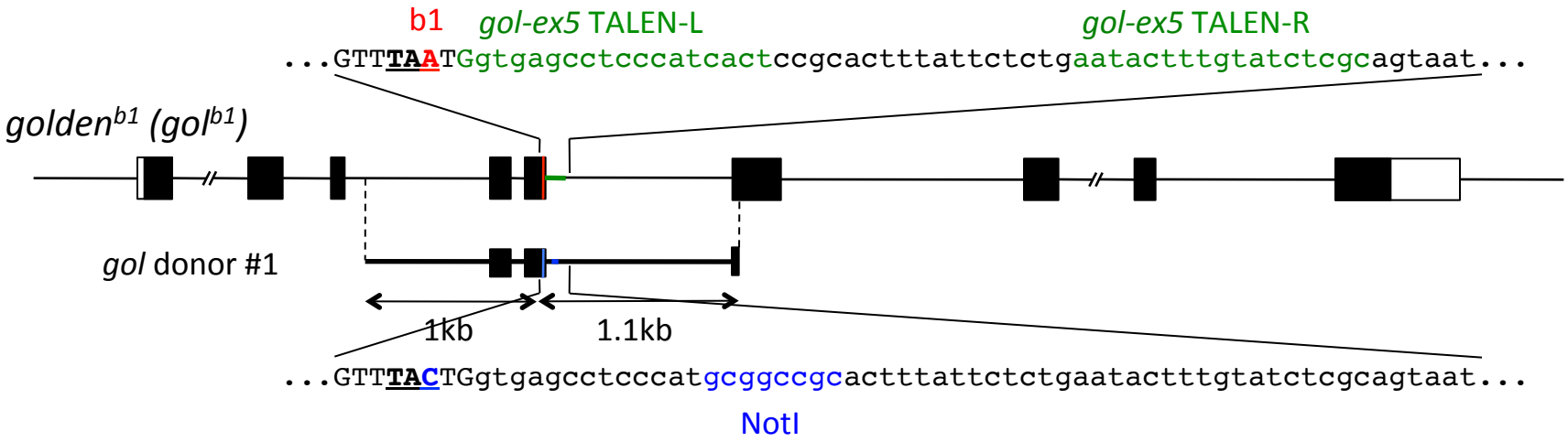


Analyze 48hpf G0 embryos
for *pigmented* cells





Targeting to convert the golden^{b1} mutation to wildtype



Pigment cells appeared in 95% of donor & TALEN-injected embryos (n = 368)

Edited golden$^{b1>+}$ alleles are readily induced

Induction of edited alleles occurs during growth of the embryo – meaning both the soma and the germ line of F0's are mosaic

The editing events are usually perfect

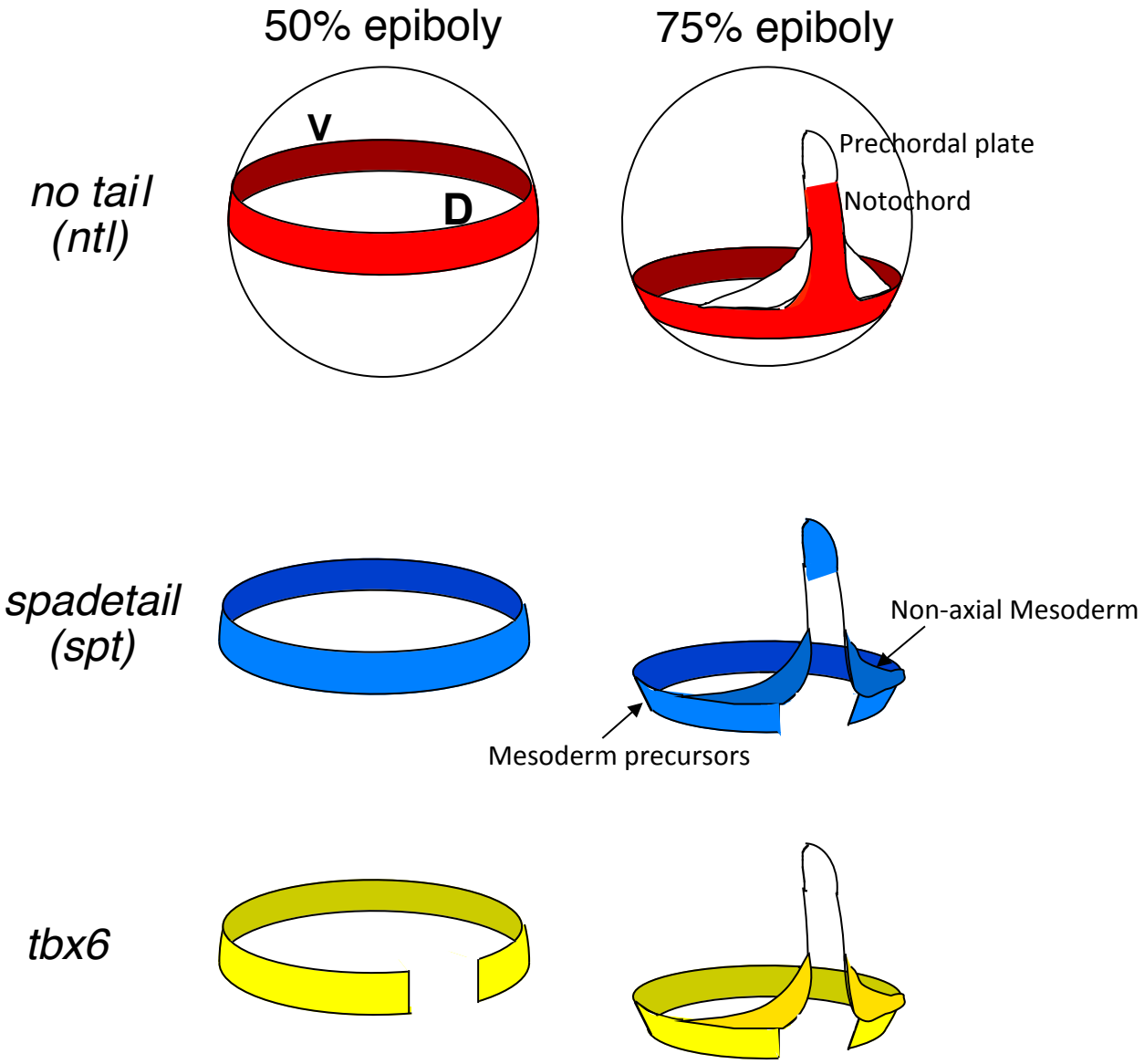
About 1 in 6 F0's transmit an edited golden allele

On average, 2.5 – 5% of germ line carries an edited allele

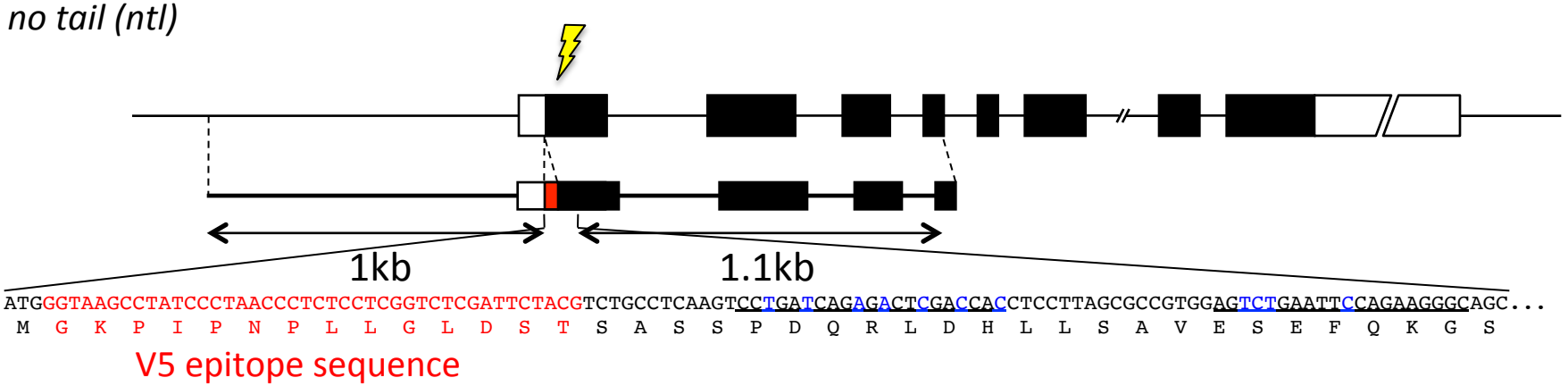
Hoshijima et al (2016) Dev. Cell

One application: in-frame introduction of sequences that encode an antigen tag

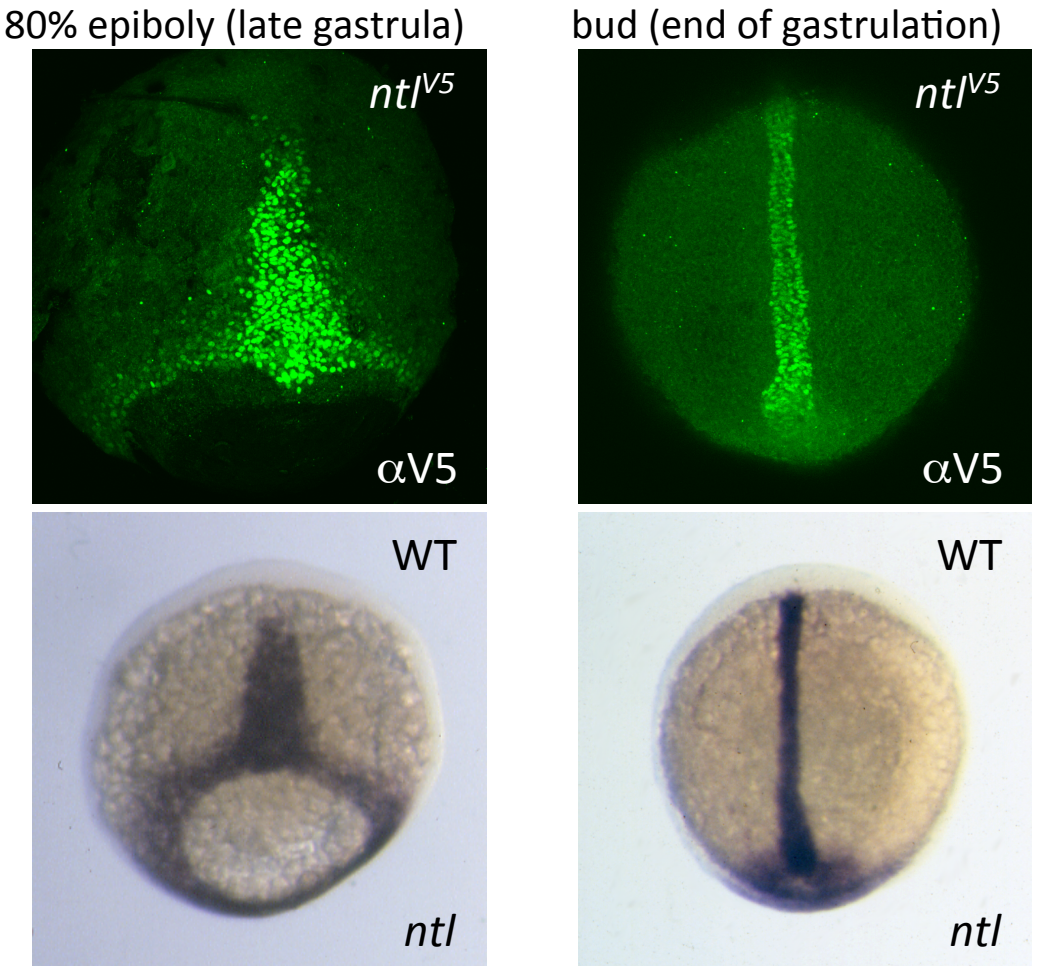
Multiple T-box genes are expressed in nested and overlapping domains in developmental fields



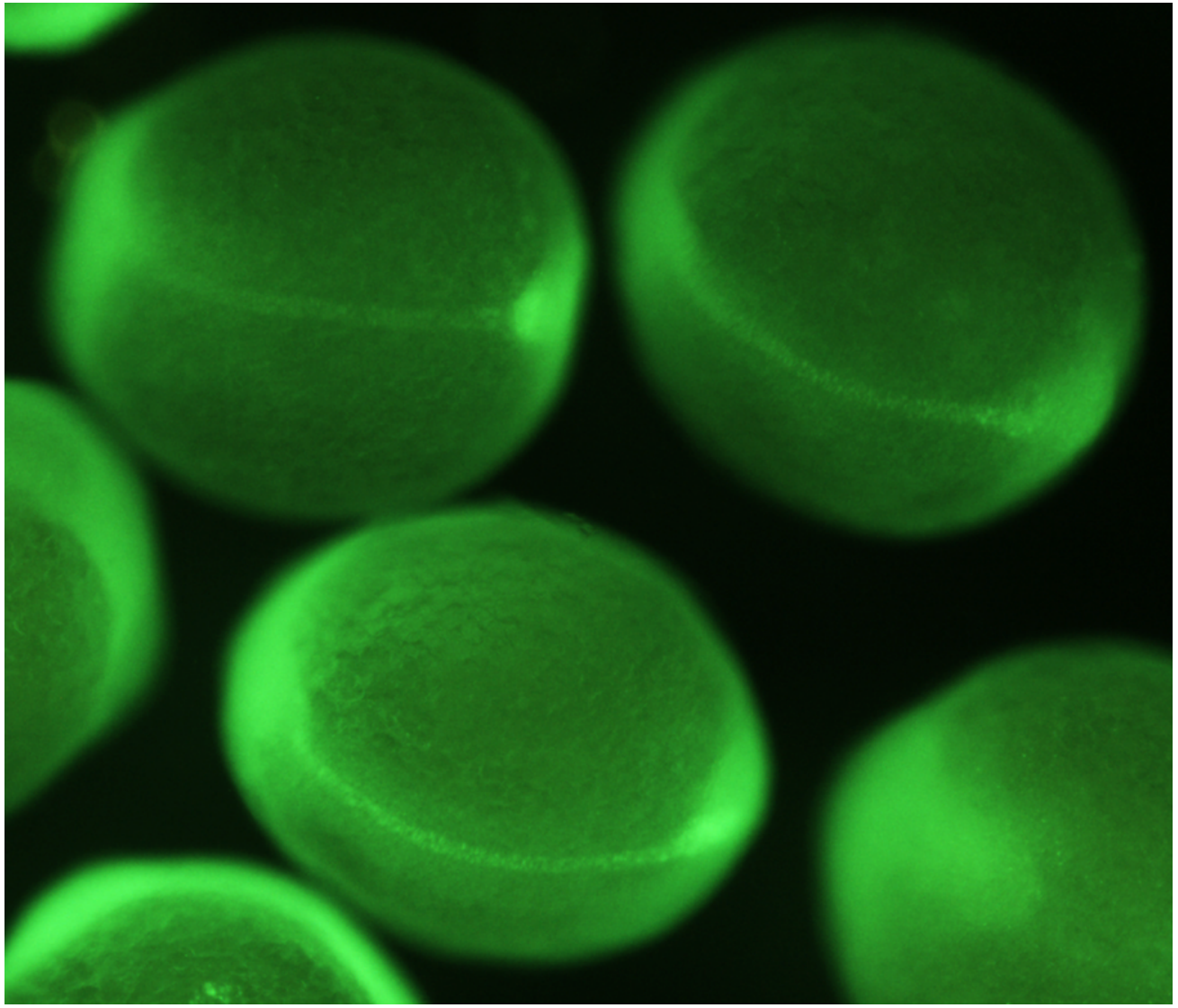
In-frame integration of an epitope tag sequence at the *no tail locus*



Epitope-tagged No Tail recapitulates *no tail* expression



The epitope-tagged No Tail recapitulates No Tail function and thus is a suitable substrate for biochemical studies



Take-home lessons:
2. Approaches that affect the efficiency of generating edited alleles

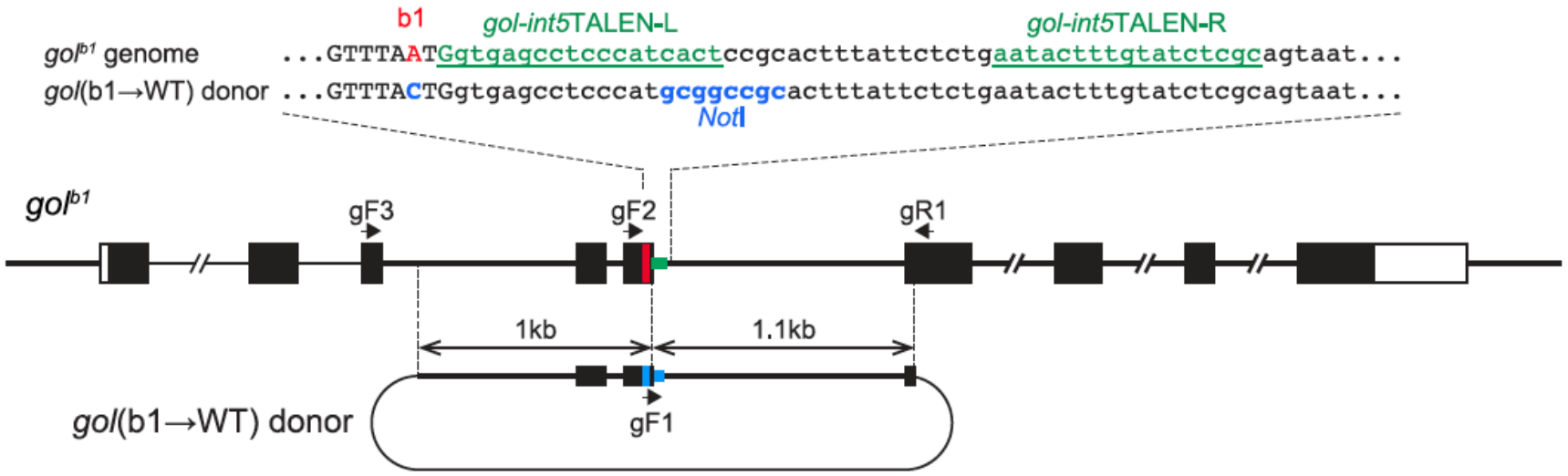
Factors that affect efficiency include:

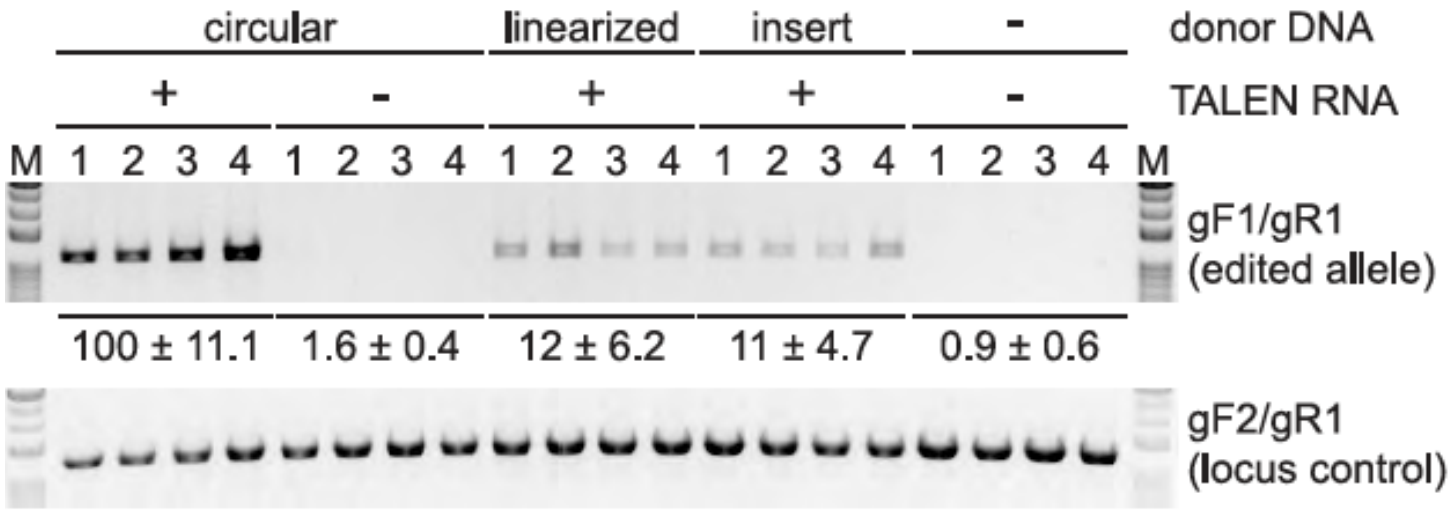
-- Template presentation

-- Optimizing the induction of DSBs

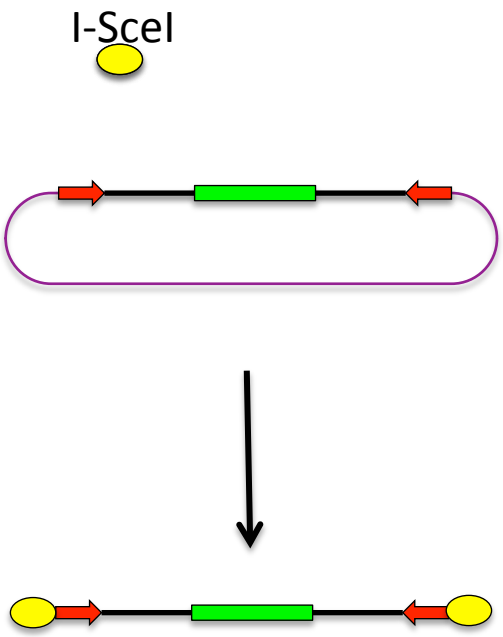
-- Homology arms

Configuration of template affects editing frequency

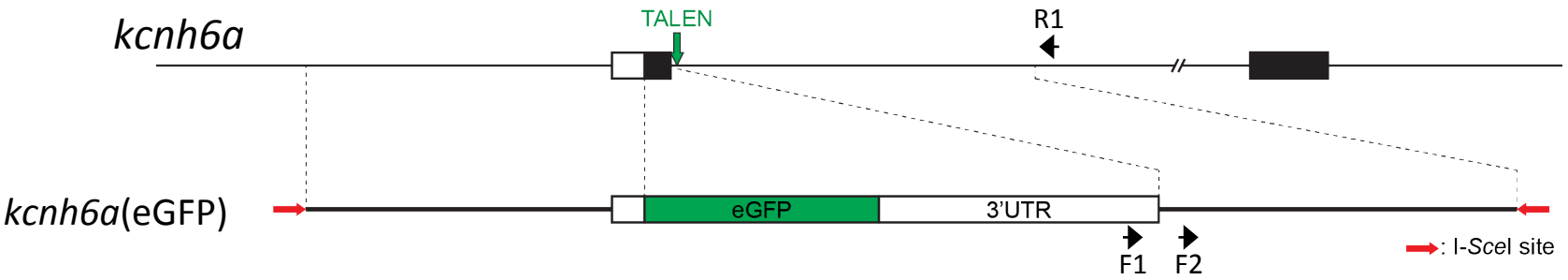


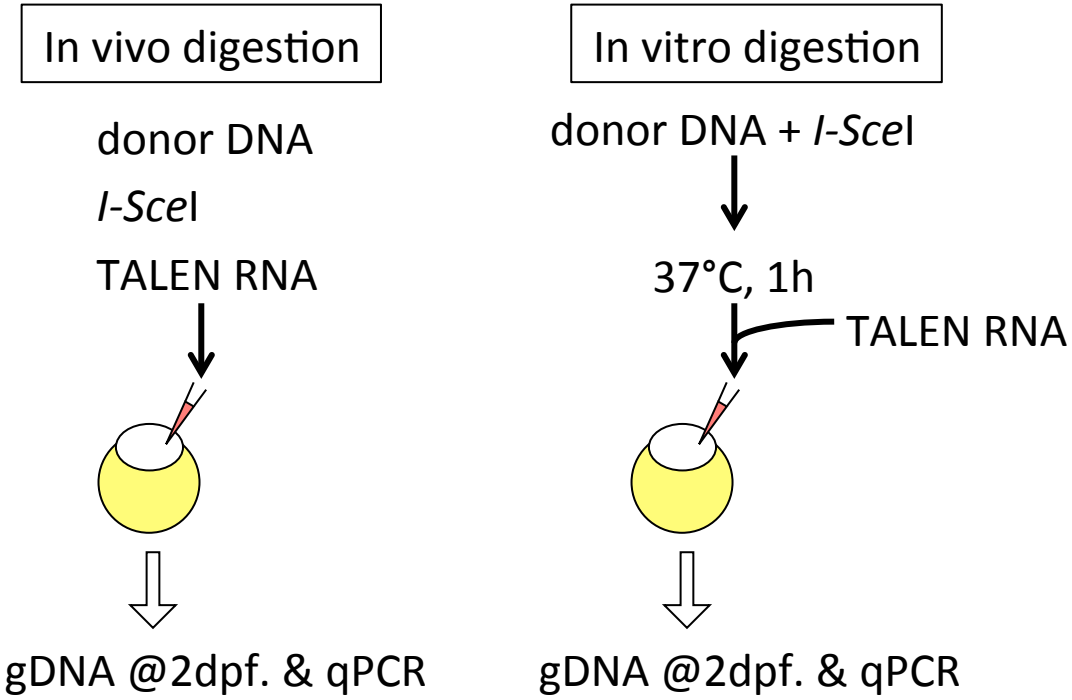


Current strategy for gene targeting – generating linearized donors with protected ends in vivo



Donor digestion with *I-SceI* increases gene editing efficiency





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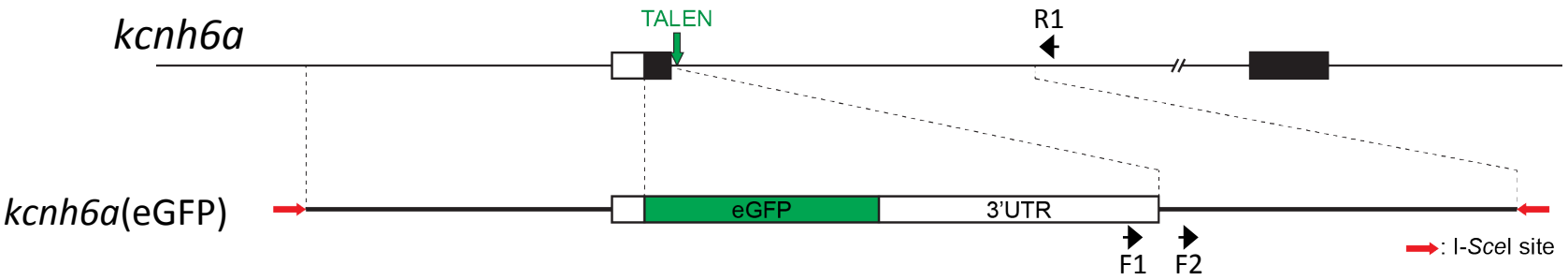
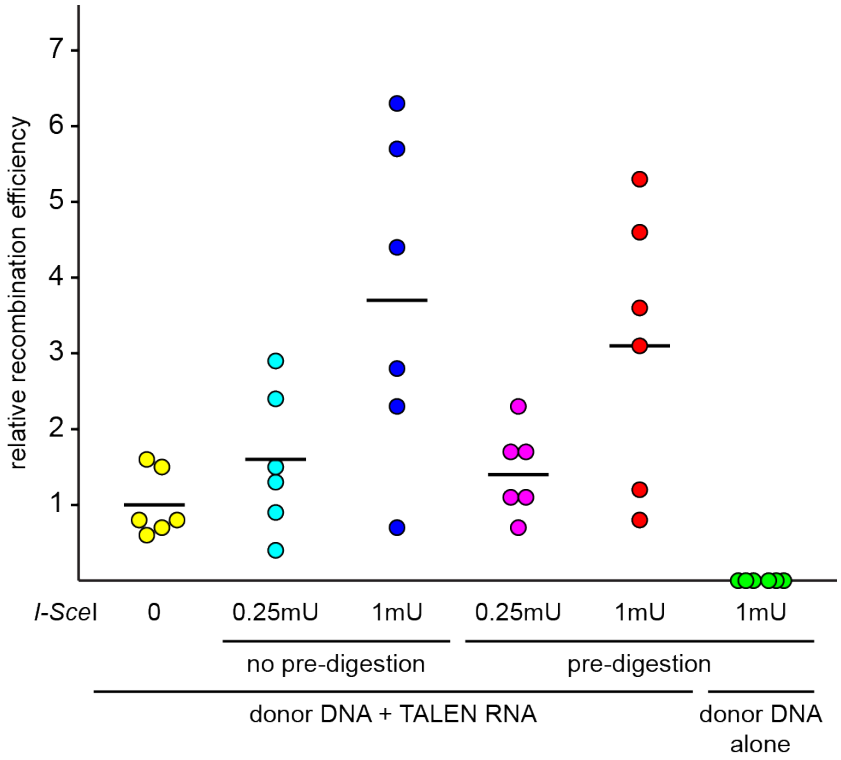
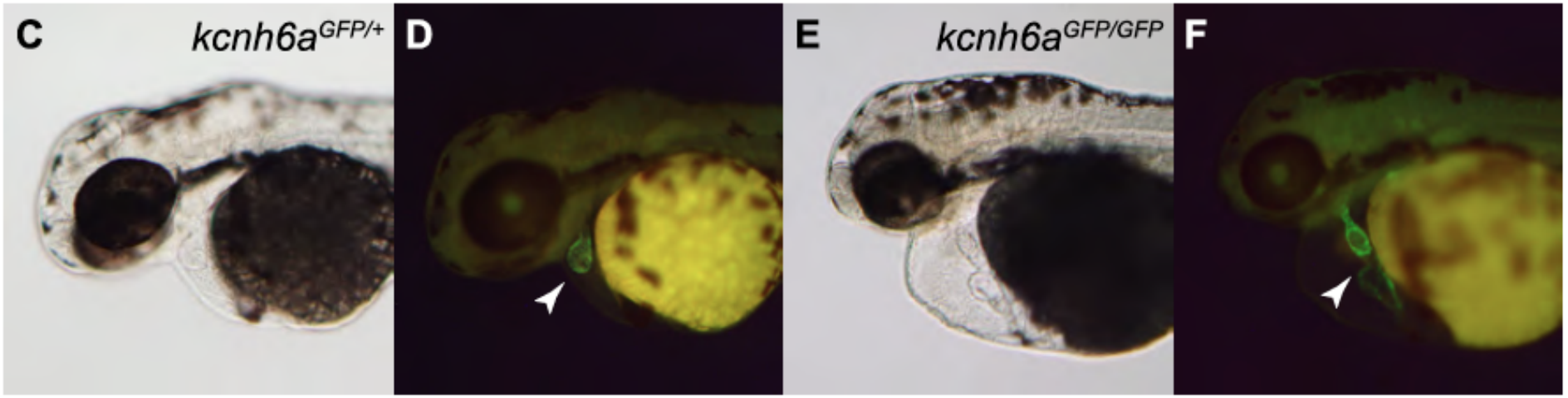
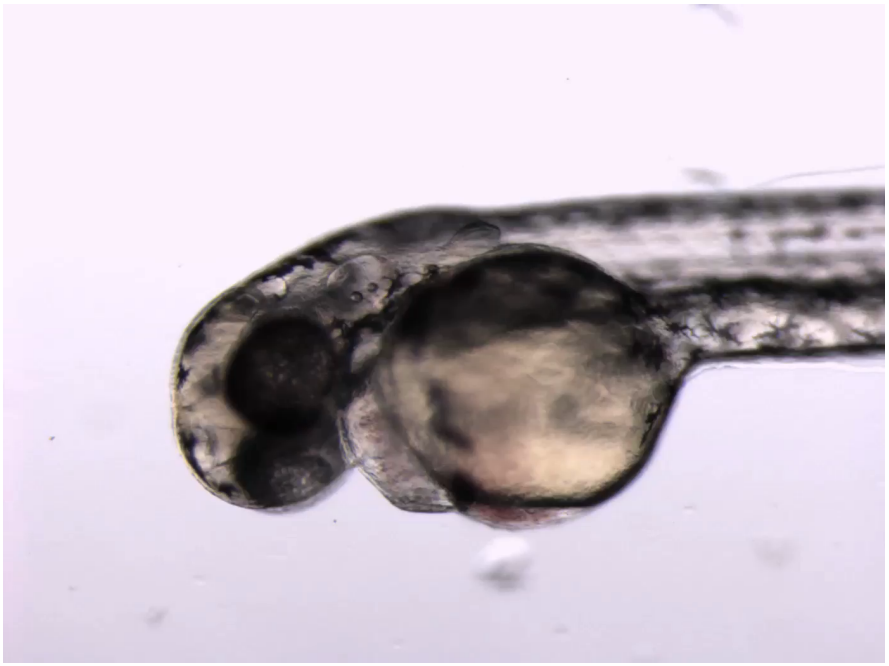


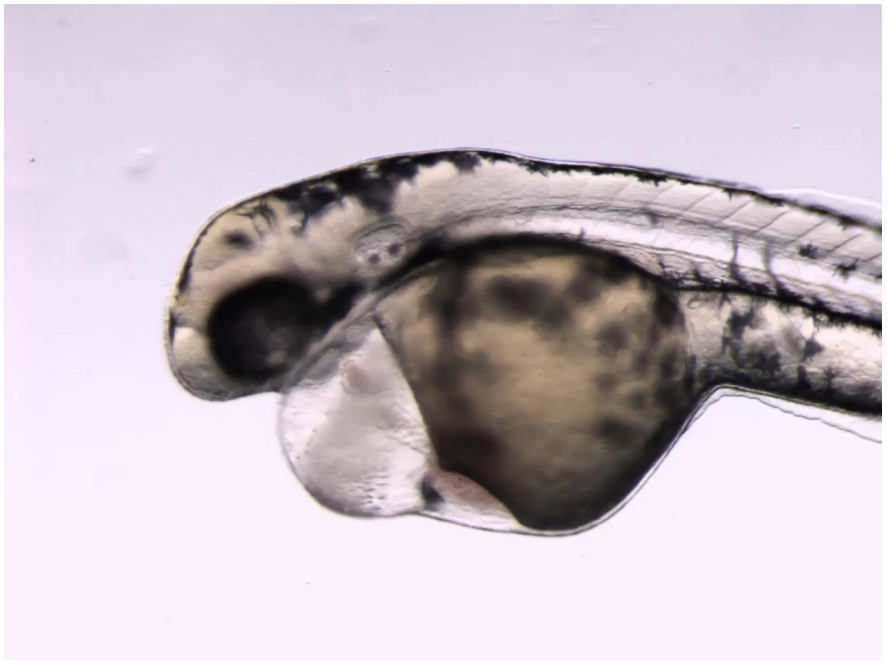


With I-SceI-*cleaved templates, 2 of 14 F0's (14%) transmitted edited alleles*

Knock-In/Knock-Out Alleles of *kcnh6*

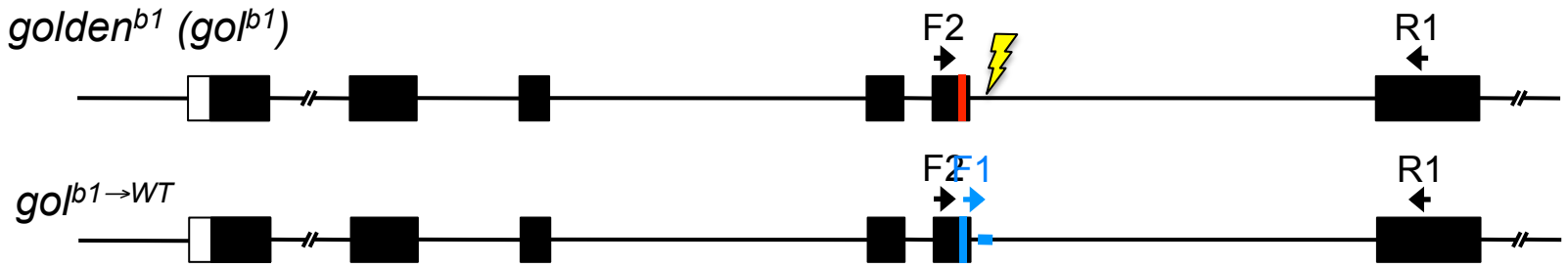


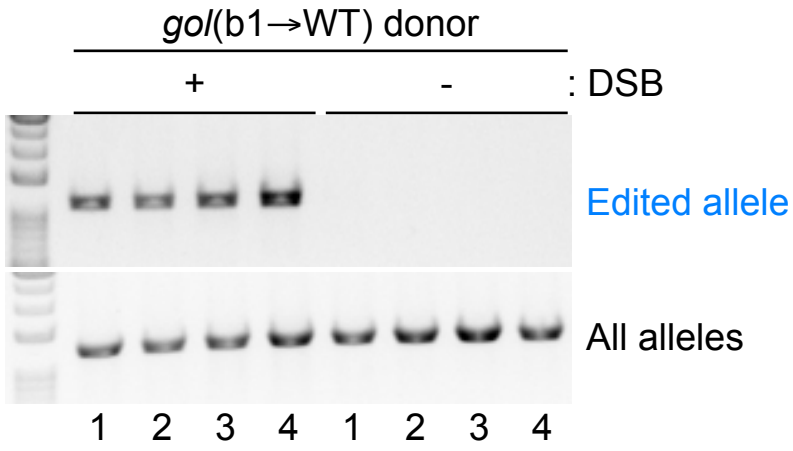




Take-home lesson 2a:
Delivery and presentation of template is important
– we don't yet know what is optimal but we think stabilizing donor DNA will help

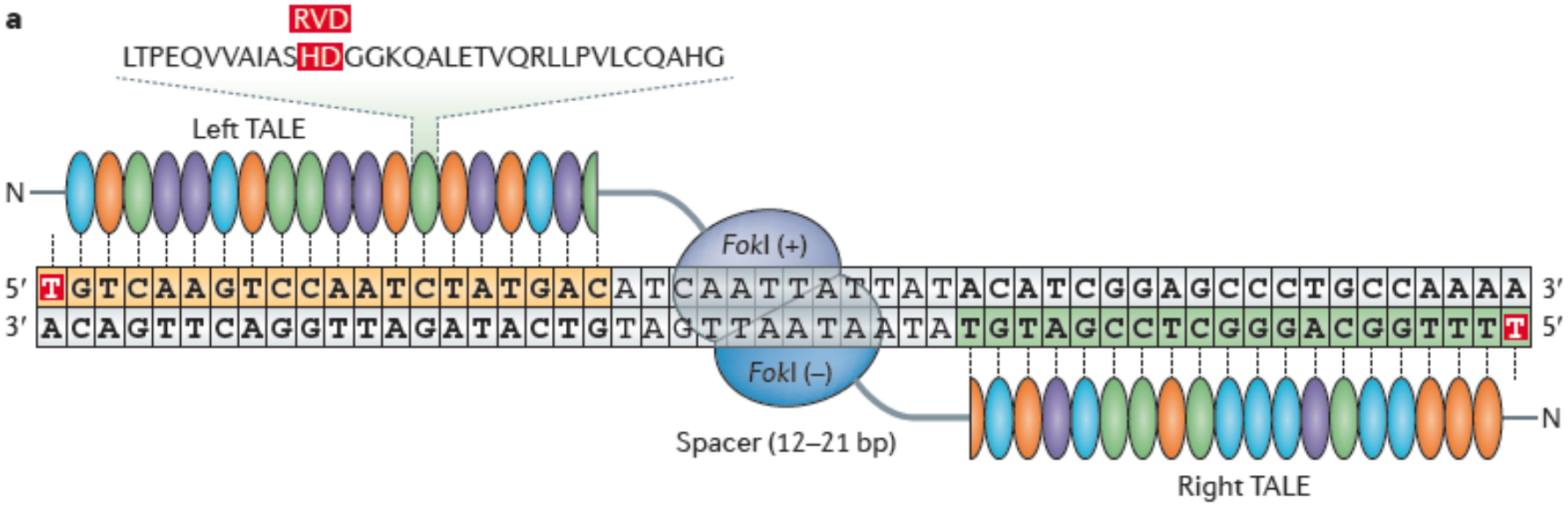
Take-home lesson 2b: Genome editing via HR is completely dependent on the induction of DSBs



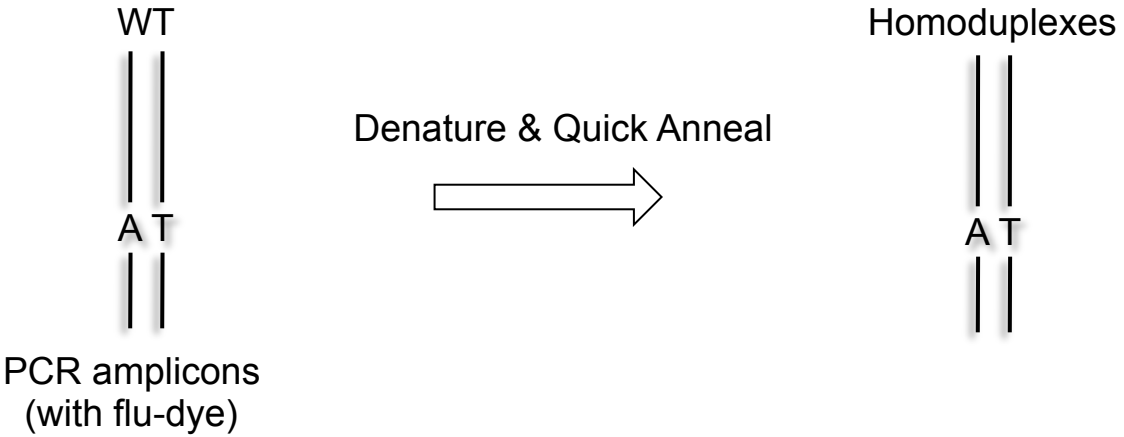


Hoshijima et al (2016) Dev. Cell

One can alter cutting efficiency of a TALEN by using different Fok1 domains



Detection of induced sequence variants by heteroduplex formation followed by High Resolution Melt Analysis



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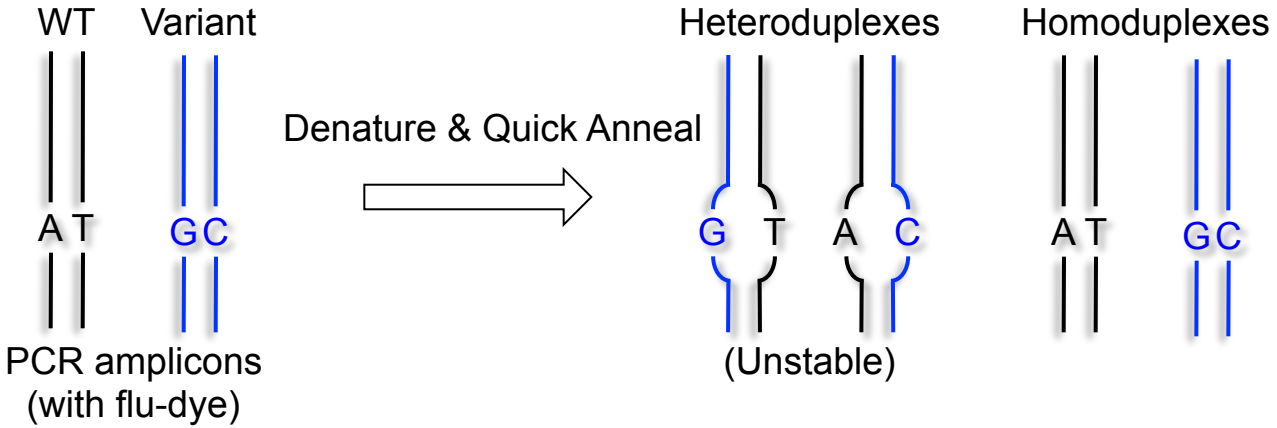
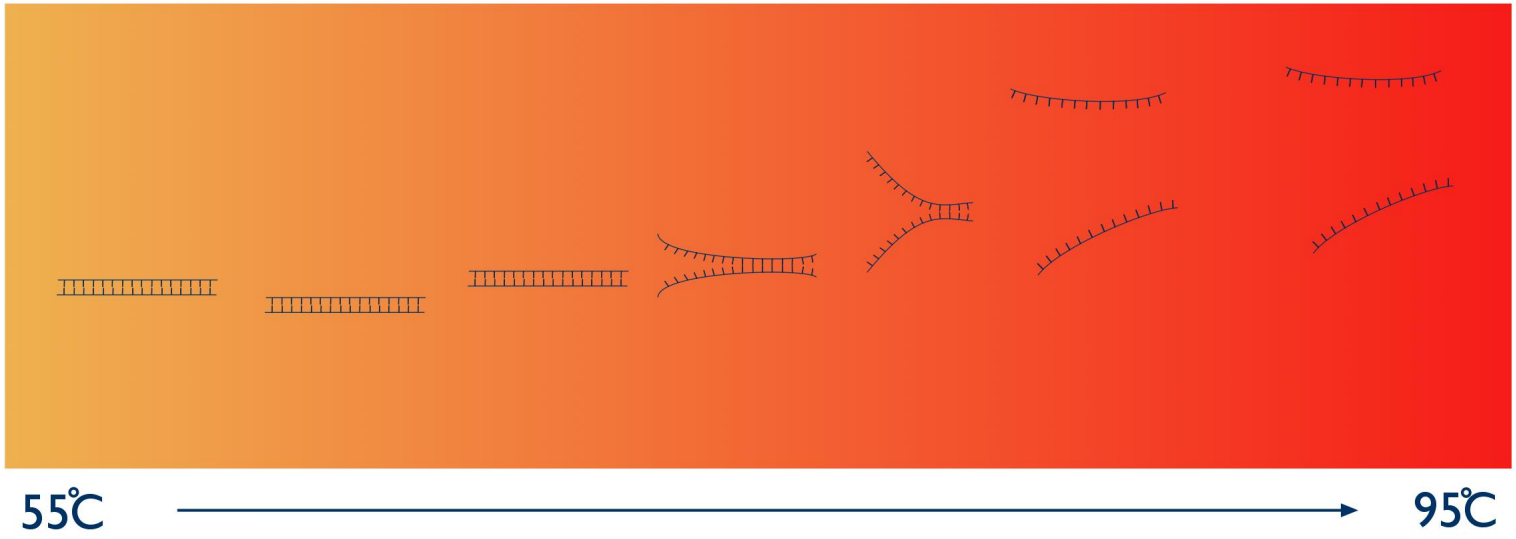
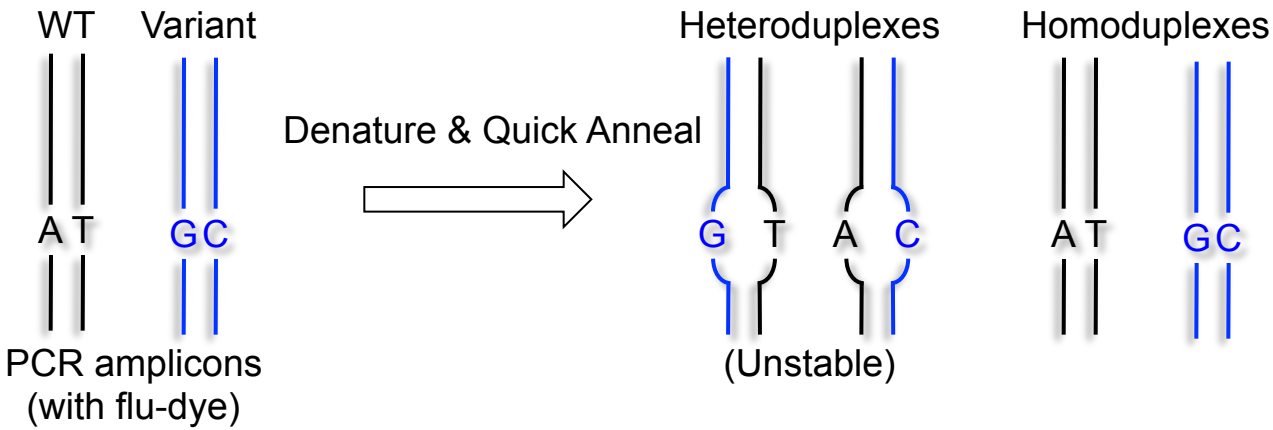
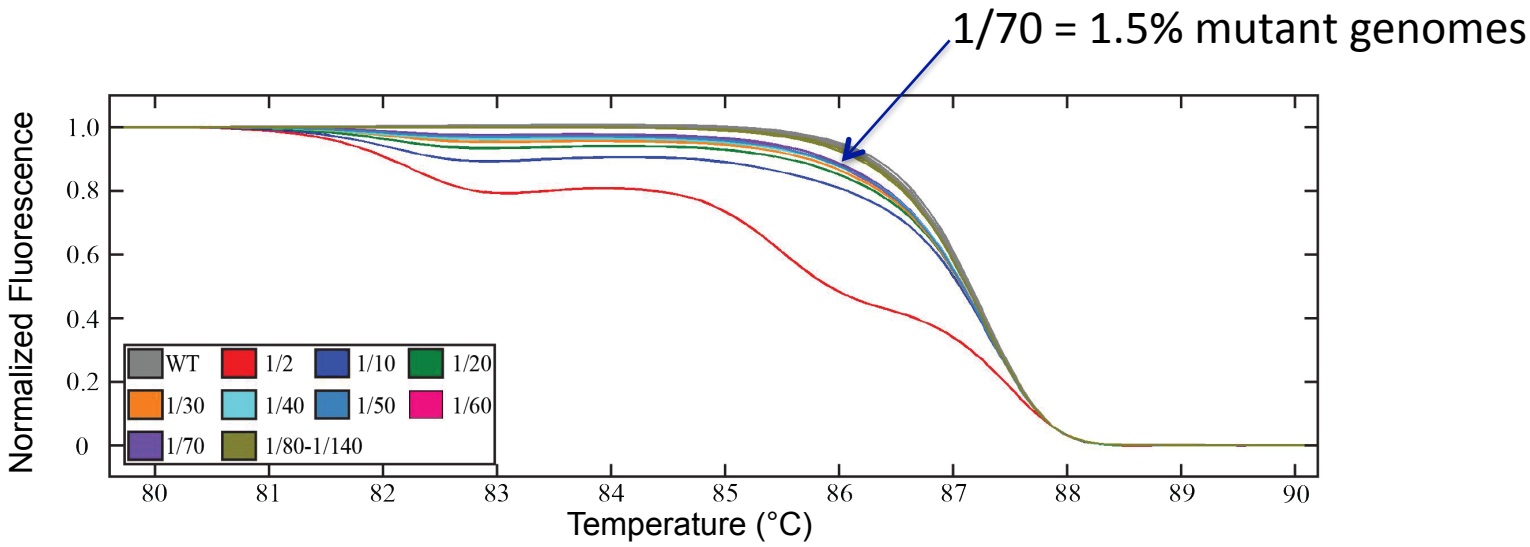




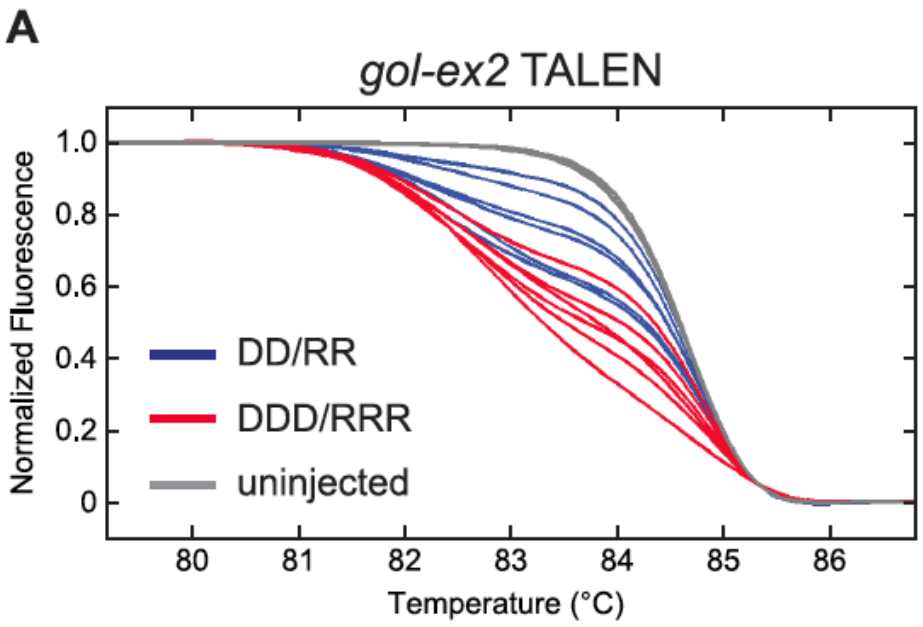
Detection of induced sequence variants by heteroduplex formation followed by High Resolution Melt Analysis

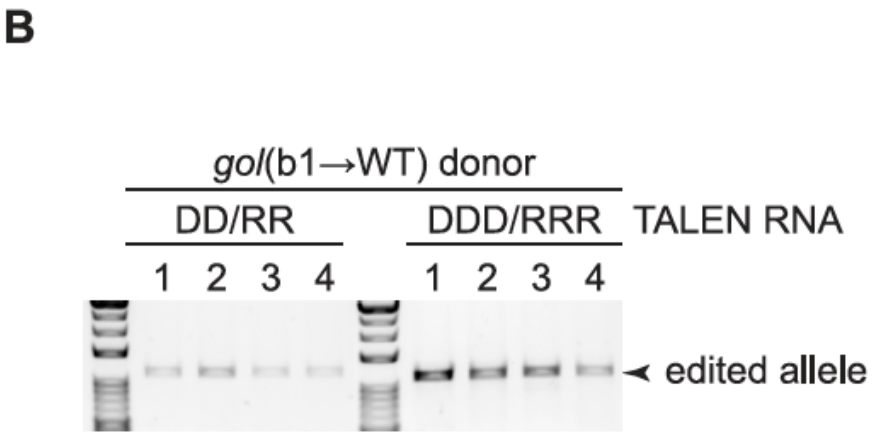
detects even small differences in DNA sequence – even in mixtures





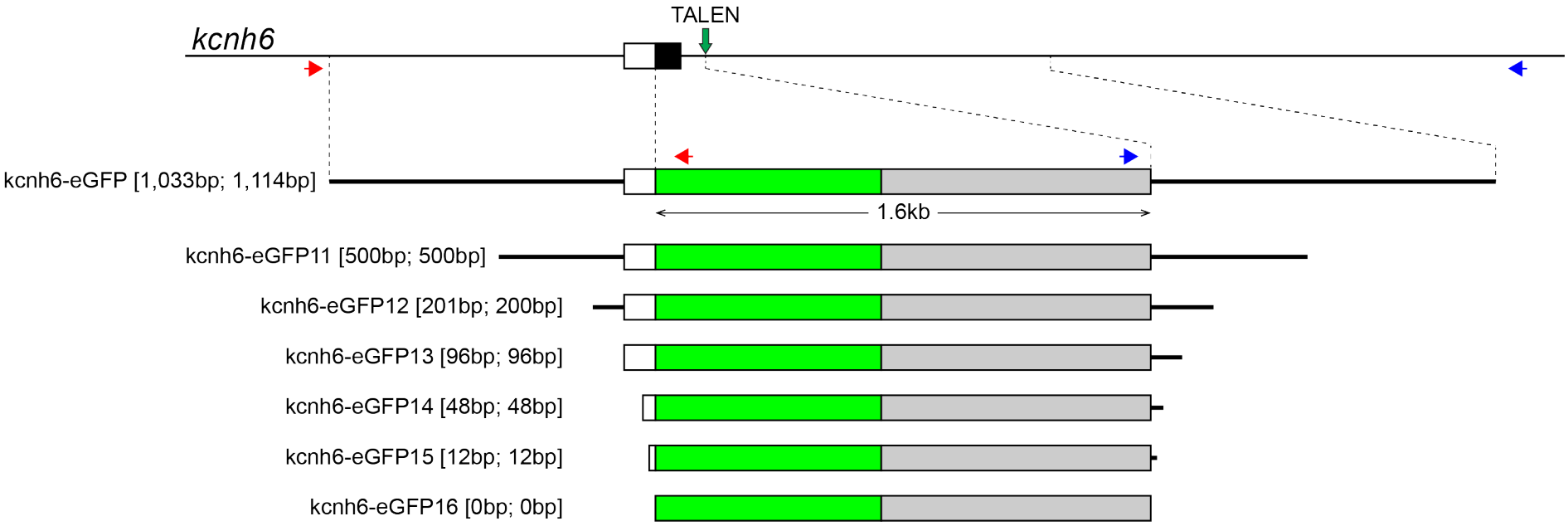
Induction of DSBs (as measured by induced mutations) correlates with ability to stimulate HR



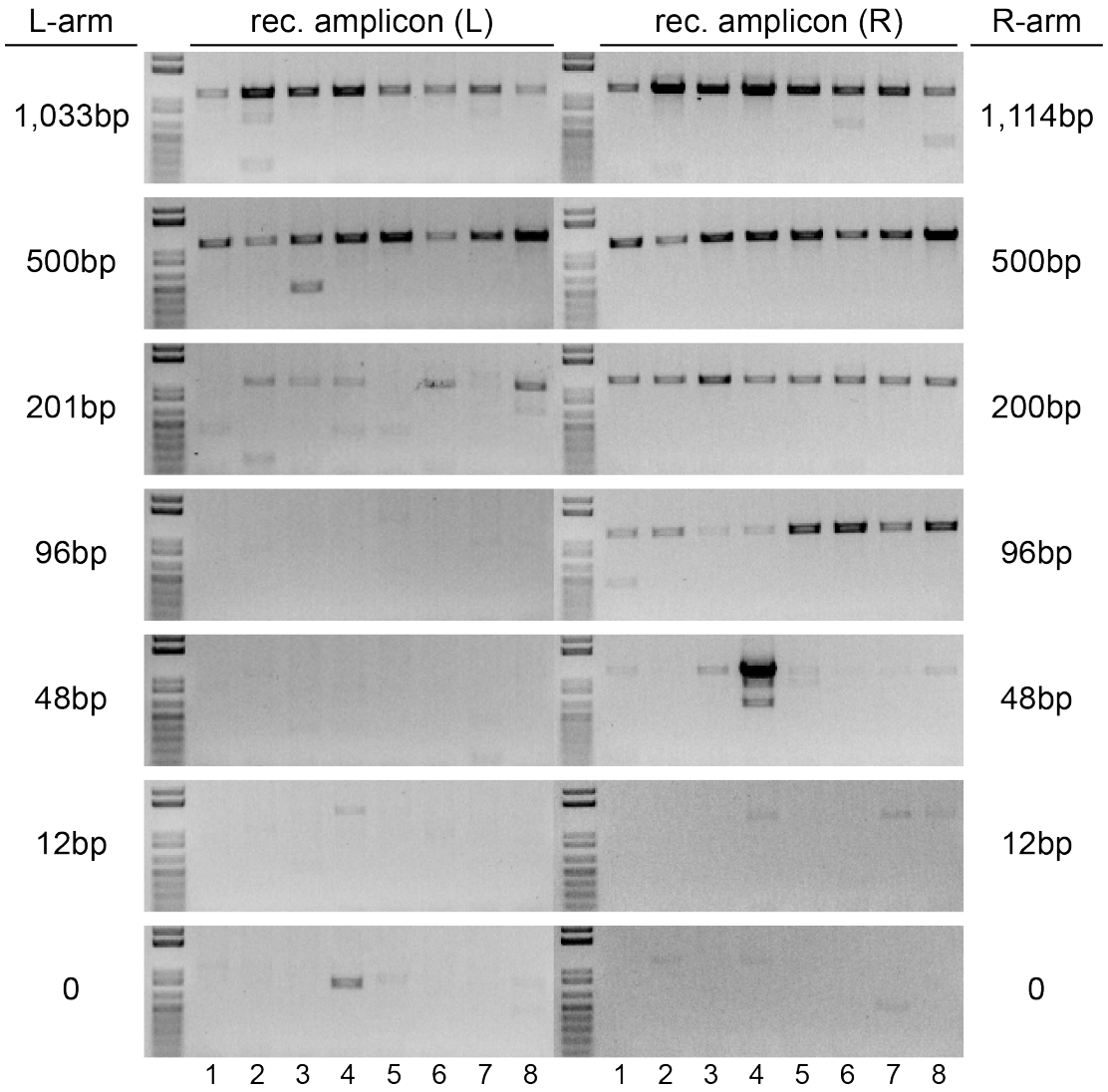


Take Home Message:
We need to consider that the induction of DSBs stimulates HR
The optimal method/timing for inducing is not yet known

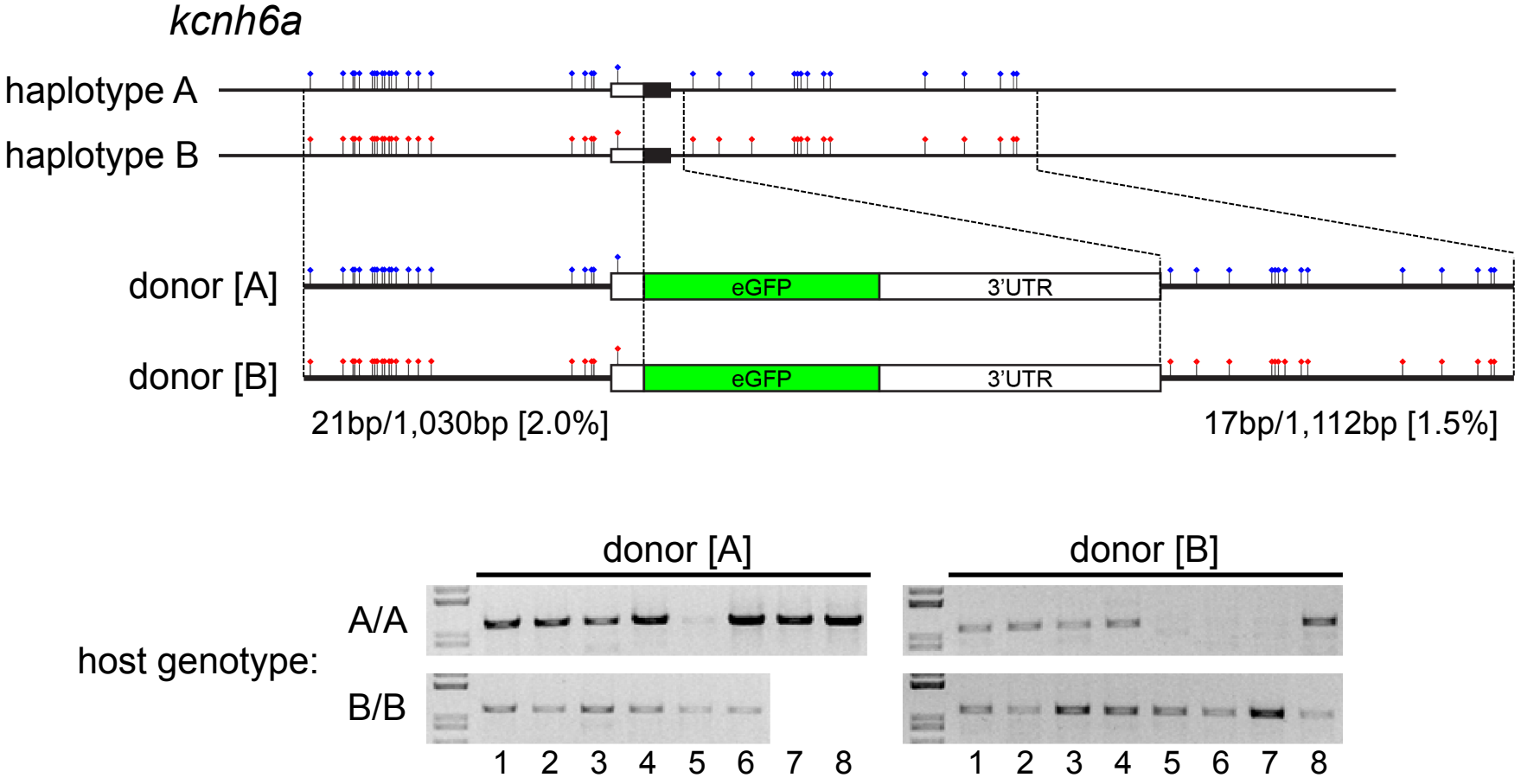
Take-home lesson 2c:
The length of homology arms affect recombination efficiency



Take-home lesson 2c:
The length of homology arms affect recombination efficiency



Take-home lesson 2d:
Homology with targeted locus can affect recombination efficiency

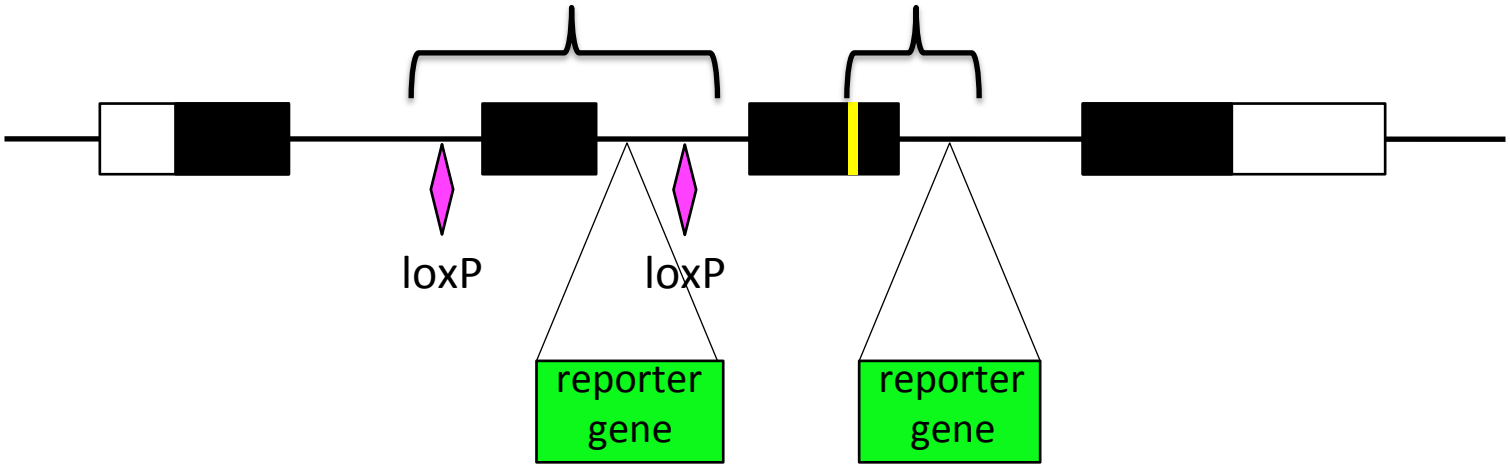


Take-home lesson 3:

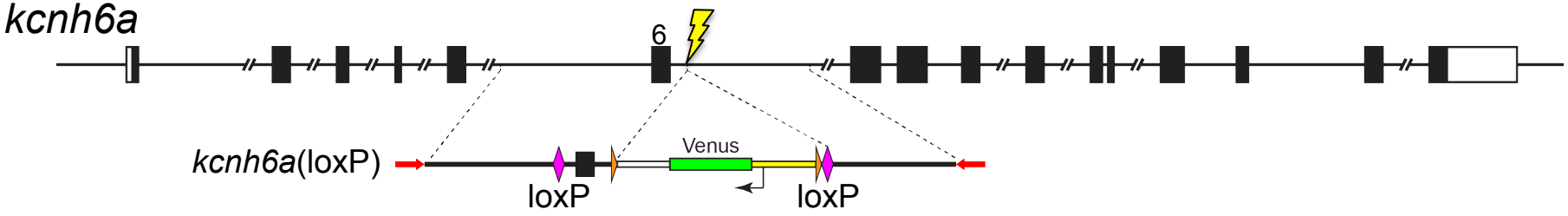
Recovery is a big problem:

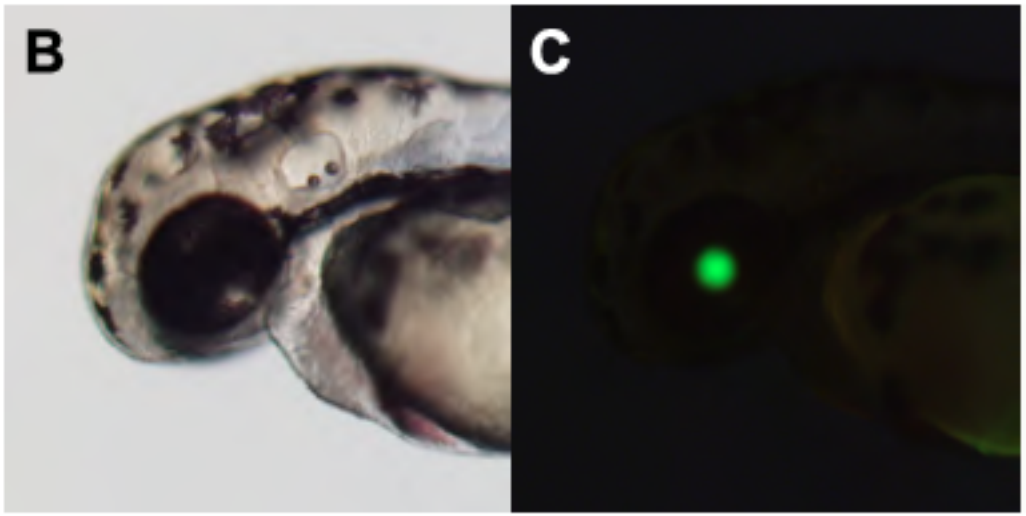
most editing events not associated with a visible phenotype in the heterozygous state in the F1 generation

By tagging edited alleles with reporter genes (which can later be excised), we improve the recovery of edited alleles by orders of magnitude



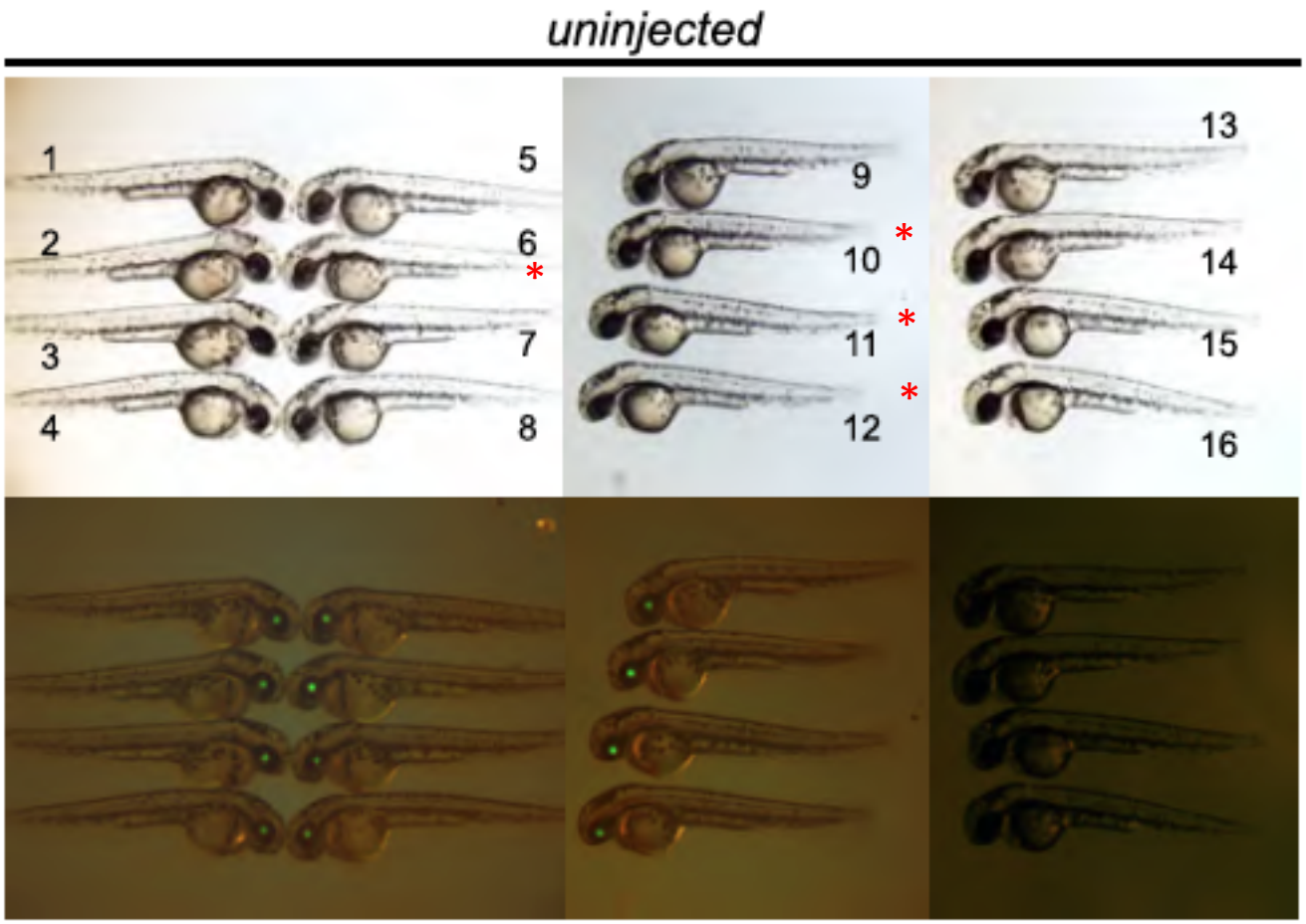
Conditional Alleles of *kcnh6*





Among the GFP+ F1 progeny, 50% carried precisely edited alleles.

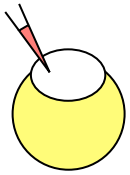
Conditional alleles of *kcnh6 are fully viable*



* = kcnh6$^{lox/lox}$

Conditional alleles are converted to null alleles upon expression of Cre recombinase

Cre mRNA

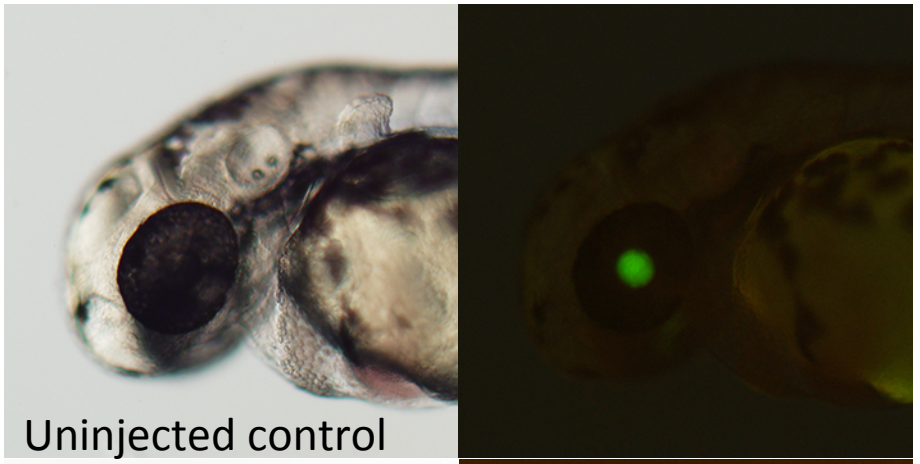


embryos from
kcnh6a$^{loxP/+}$ x *kcnh6a*$^{loxP/+}$

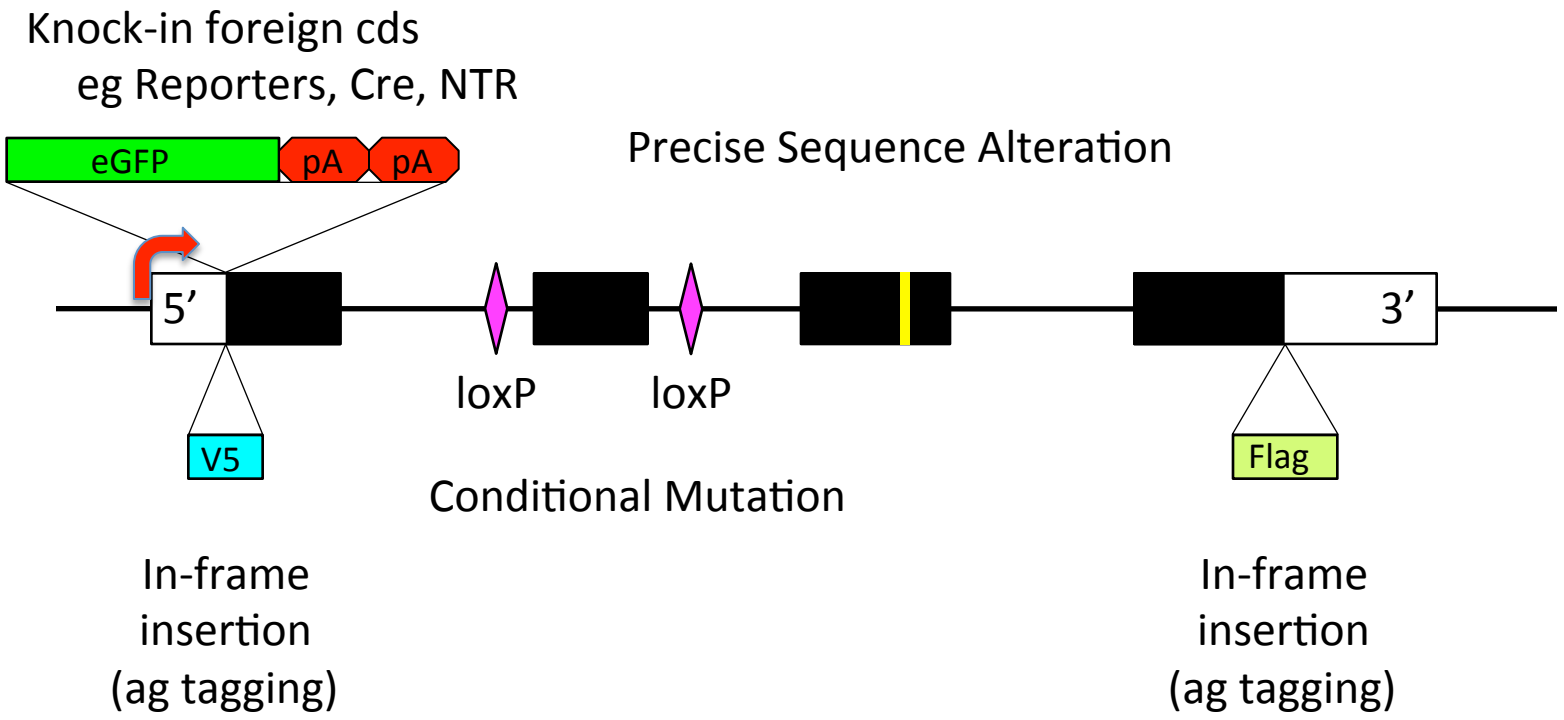
analyze @2dpf.;

heart formation

GFP expression in the lens



Three take-home lessons:
Now possible to replace any host sequence with any desired donor sequence



Three take-home lessons:
Approaches that improve the efficiency of generating edited alleles

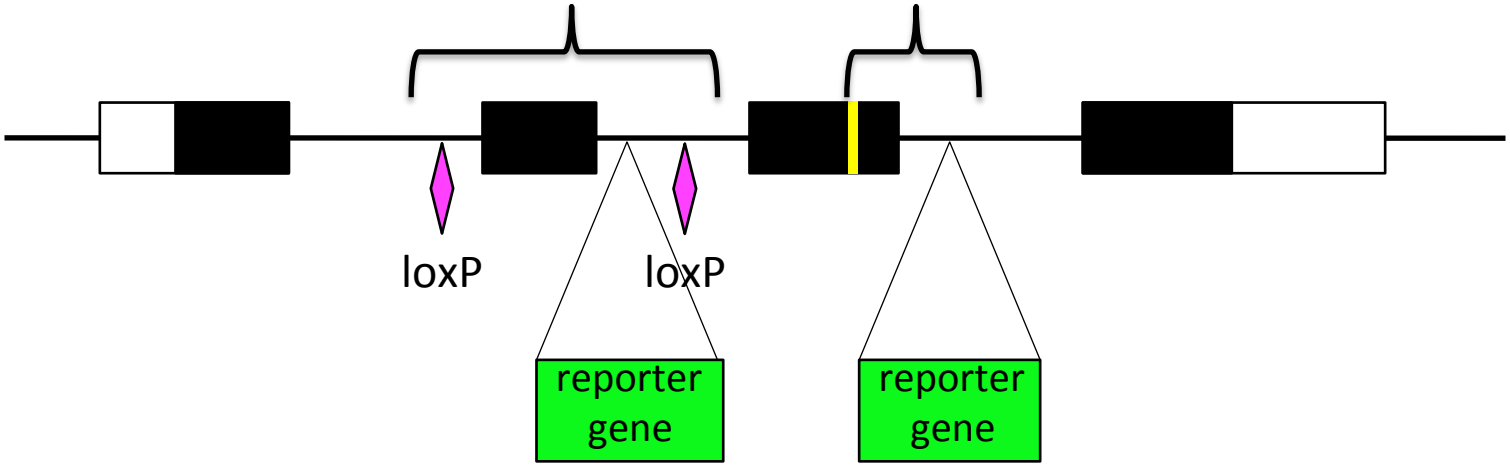
Factors that affect efficiency include:

-- Template presentation

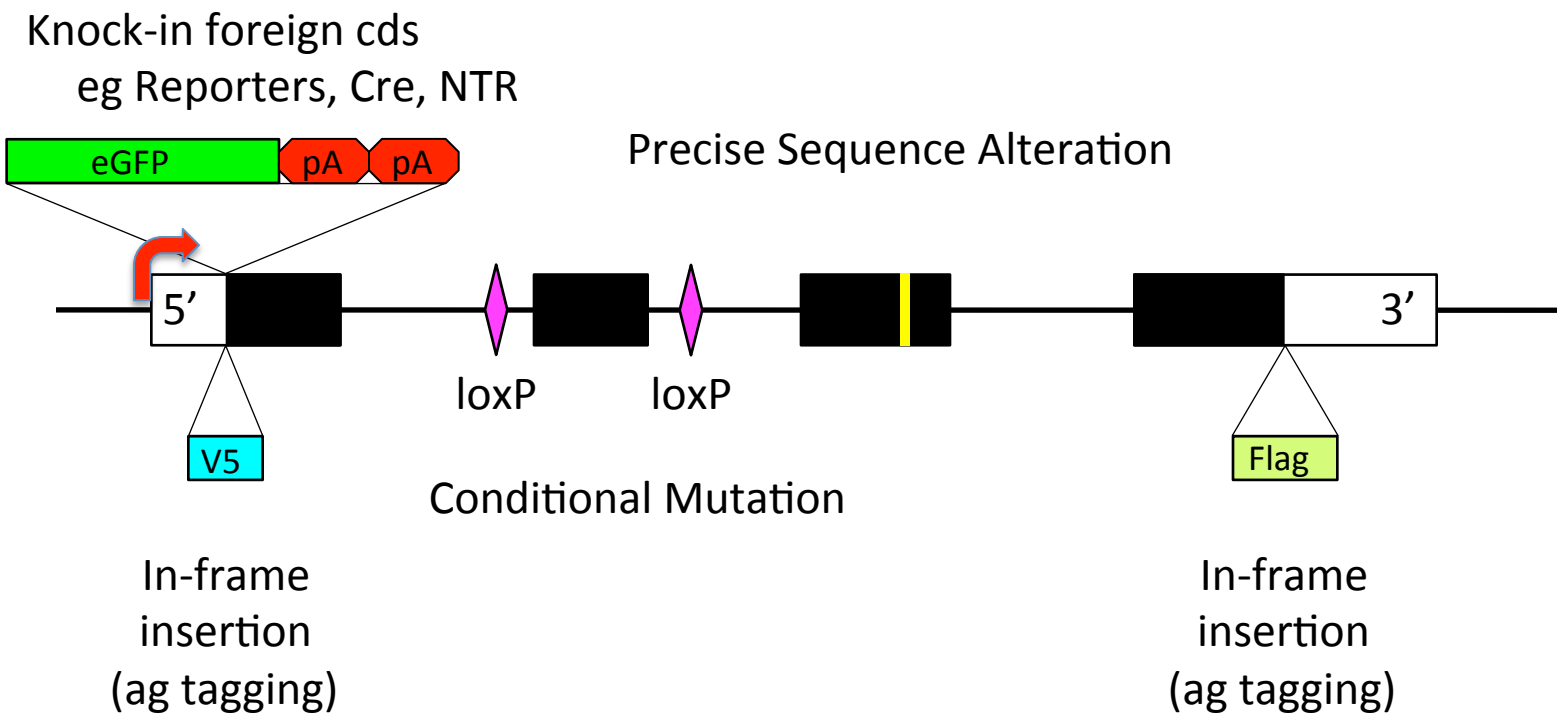
-- Optimizing the induction of DSBs

-- Effects of homology arms

Three take-home lessons:
By tagging edited alleles with reporter genes (which can later be excised), we improve the recovery of edited alleles by orders of magnitude



Fantastic???


Knock-in foreign cds
eg Reporters, Cre, NTR
eGFP
pA
pA
Precise Sequence Alteration
5’
3’
loxP
loxP
V5
Flag
Conditional Mutation
In-frame
insertion
(ag tagging)
In-frame
insertion
(ag tagging)

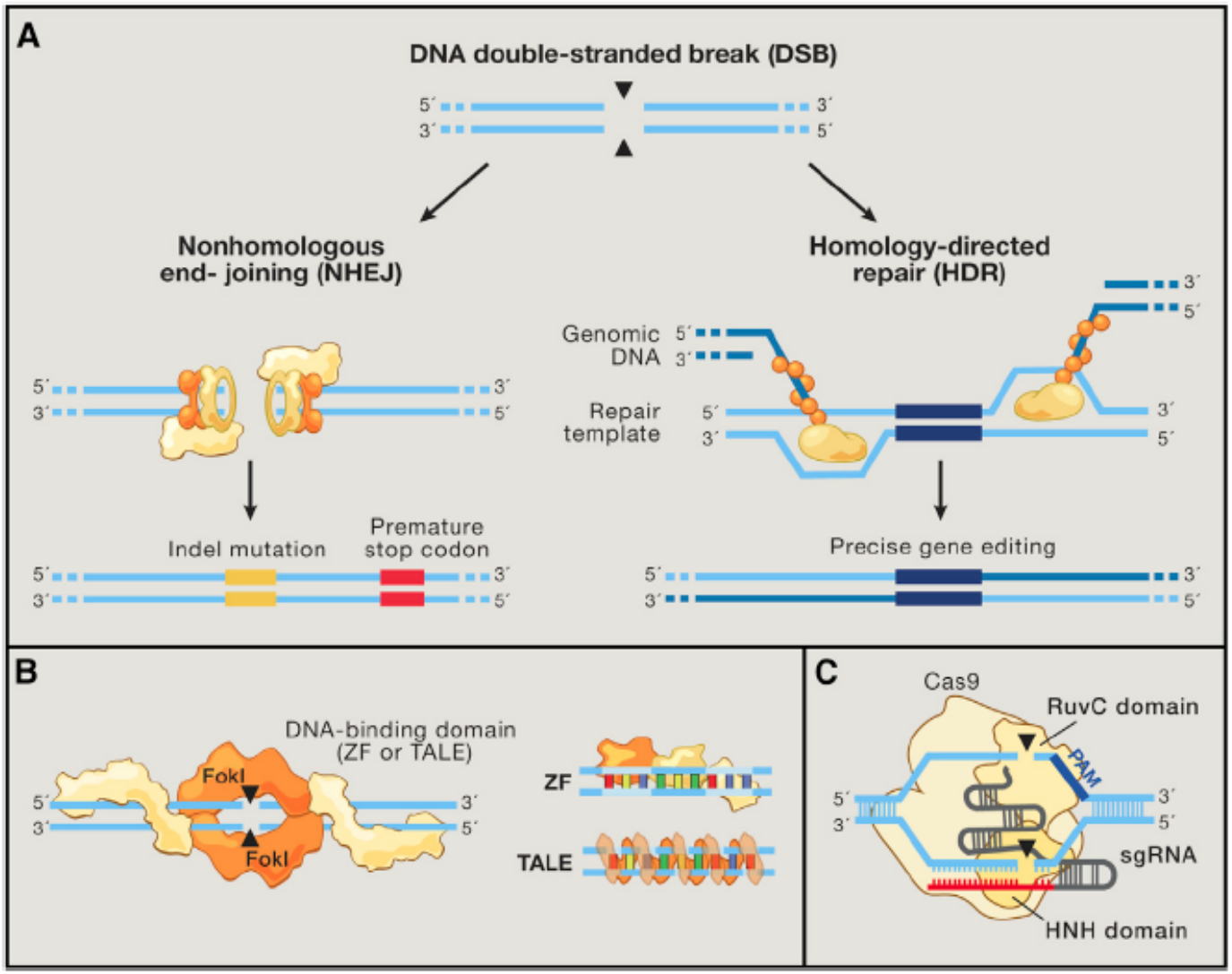
Never get too cocky – or make sure you have honest friends

cryptic smile

leaving to be
a cowboy

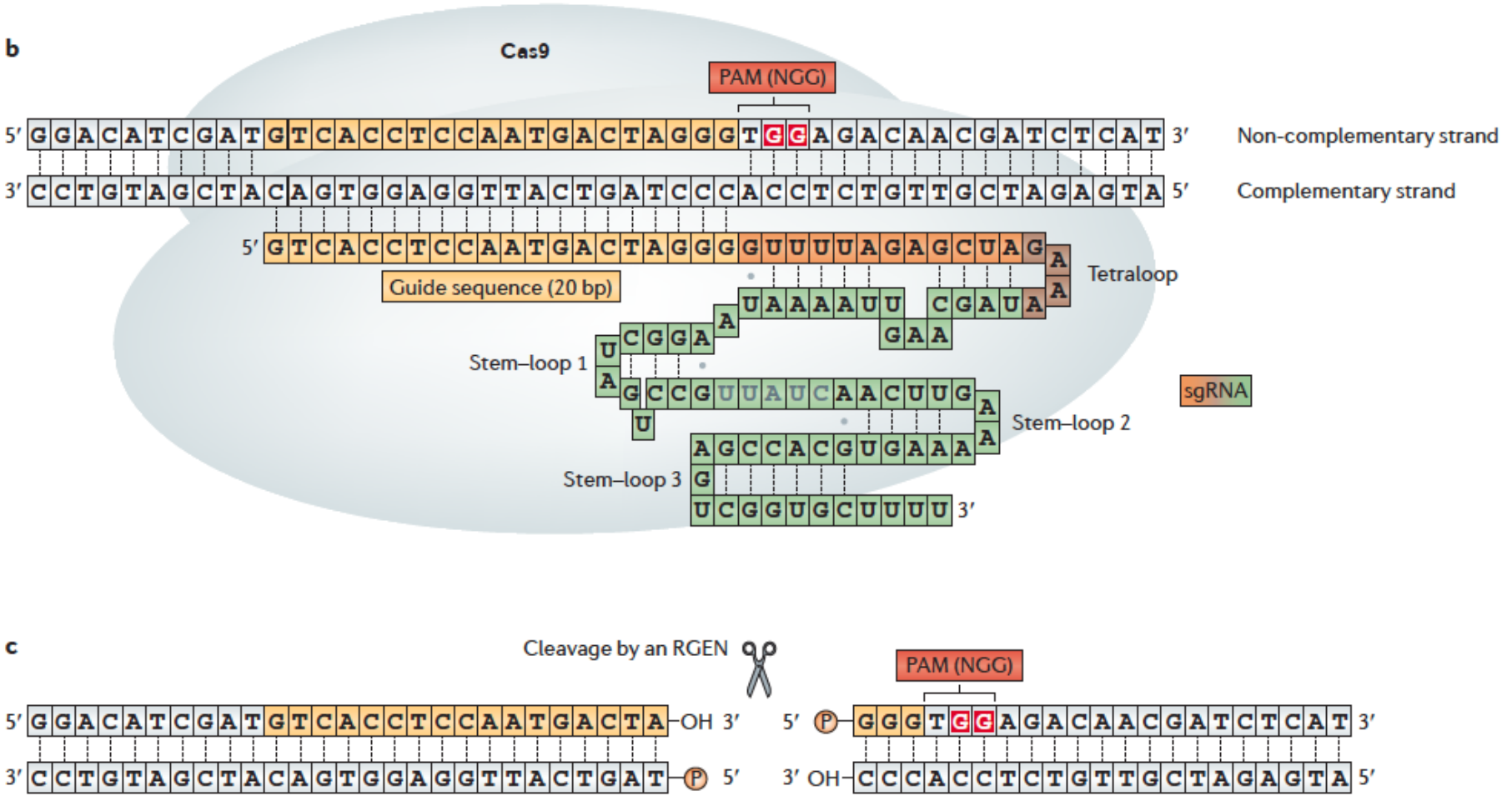
even the
fauna have
their doubts

We were misled:
Any type of synthetic (programmable) nuclease can initiate repair and recombination events at the site of induced DSBs



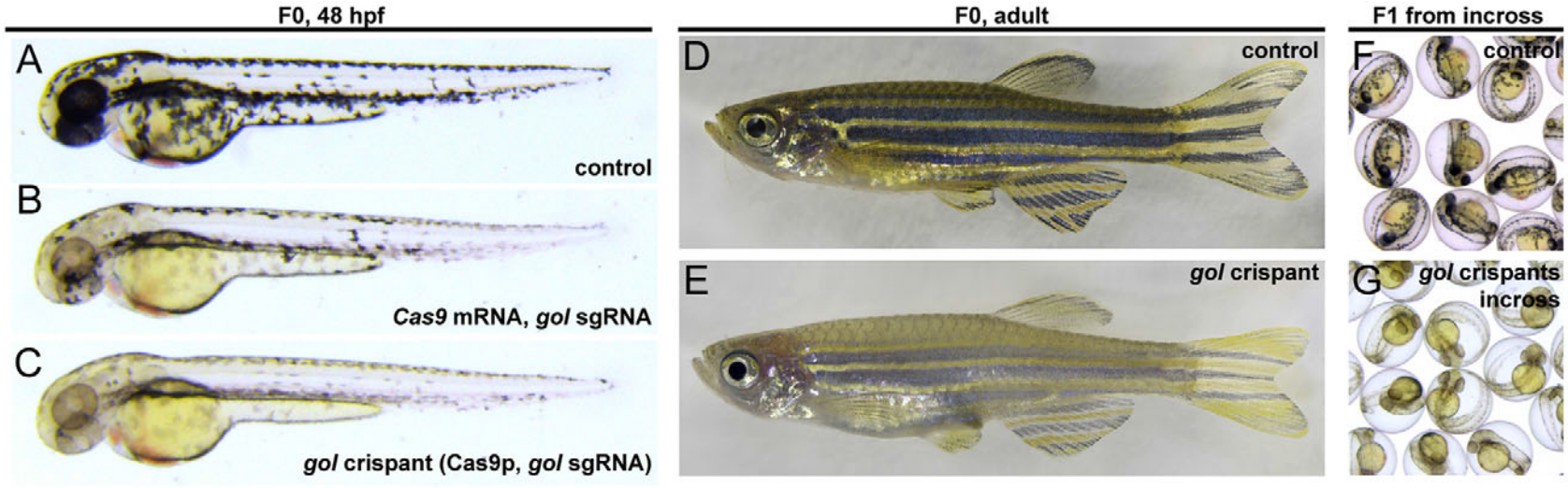
Hsu, Lander, Zhang (2014) Cell 157: 1262

Simplicity sgRNA/Cas9 system



Kim and Kim, Nature Reviews Genetic 15:321-334, 2014

Maximizing CRISPR/Cas9 activity with pre-formed RNPs

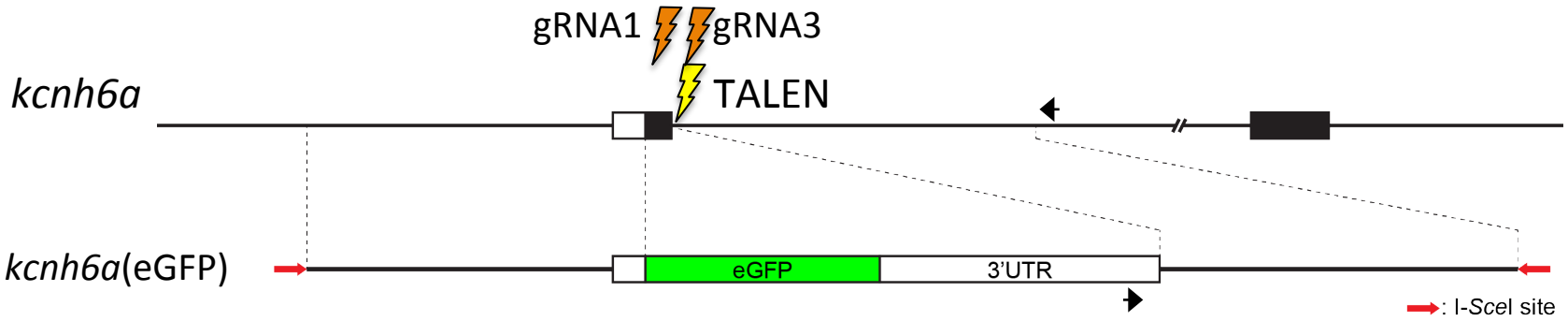


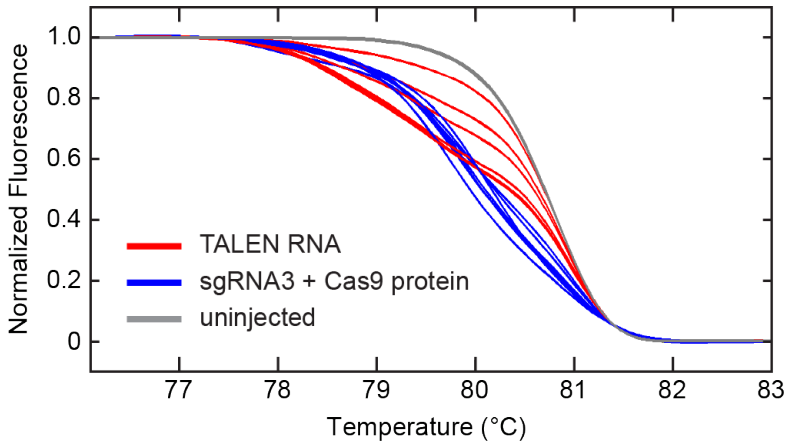
Deep sequencing of individual "crispant" embryos reveals:

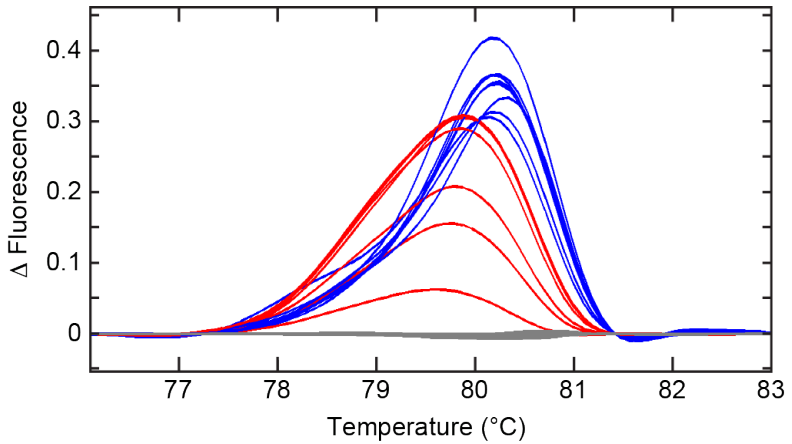
1) *Often close to 100% of loci have induced mutations*
2) *Relatively few independent alleles indicating early mutagenesis*

Burger *et al.* (2016) Development 143: 2025

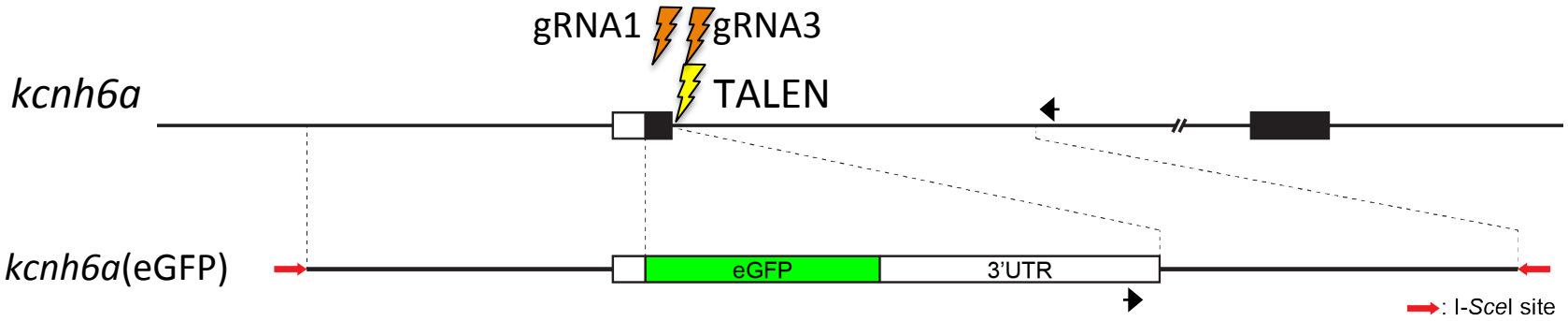
Cas9 RNPs cut effectively but poorly stimulate HR

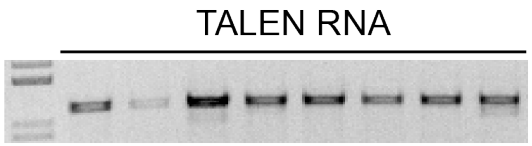




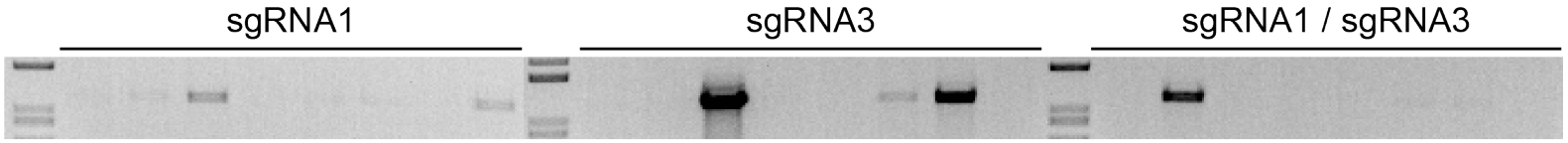


Cas9 RNPs cut effectively but poorly stimulate HR

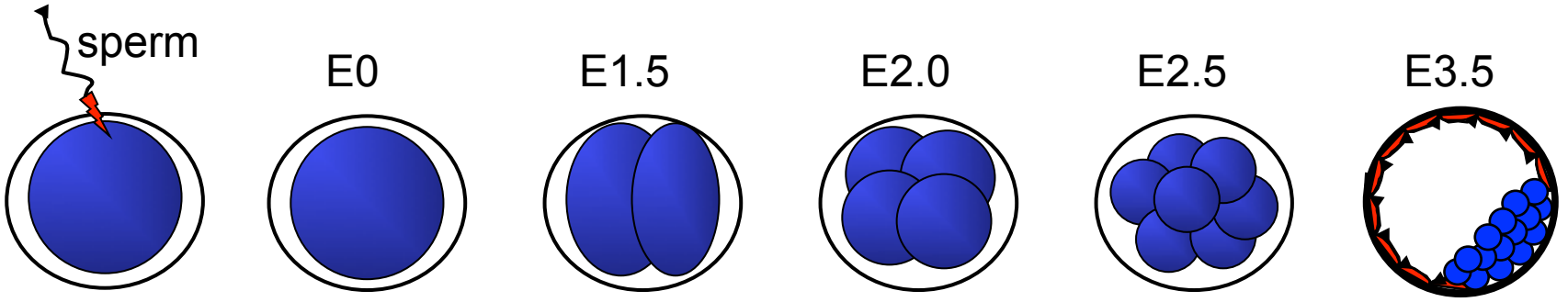


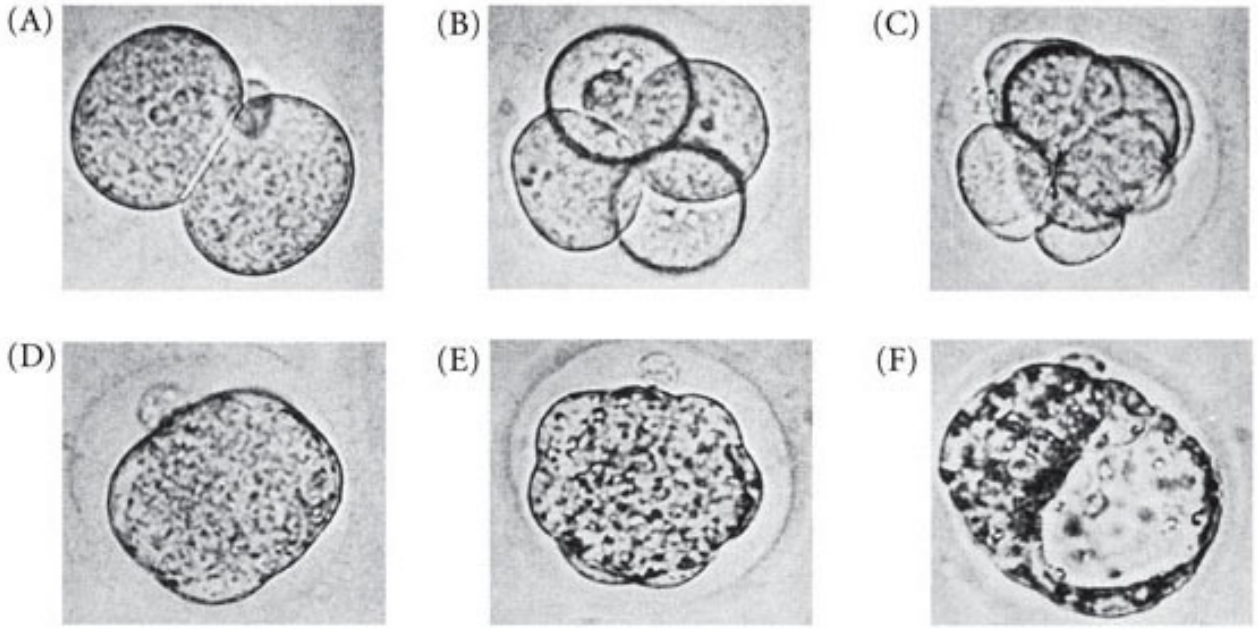


Edited alleles



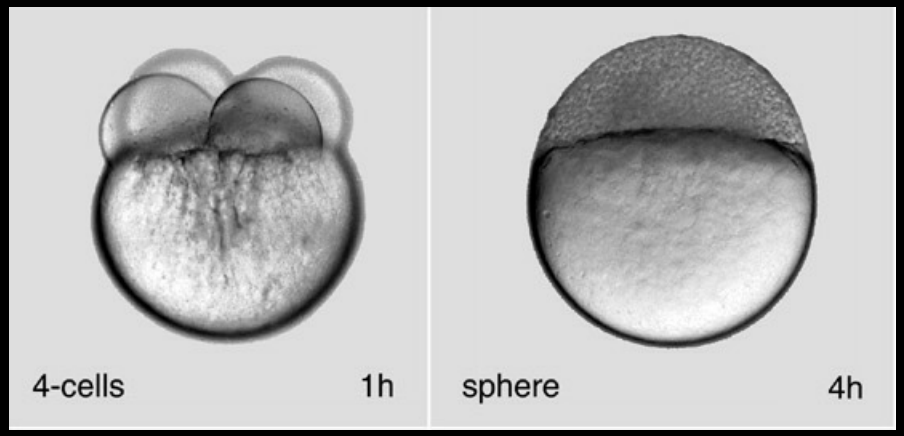
Pay attention to the biology of your system/organism

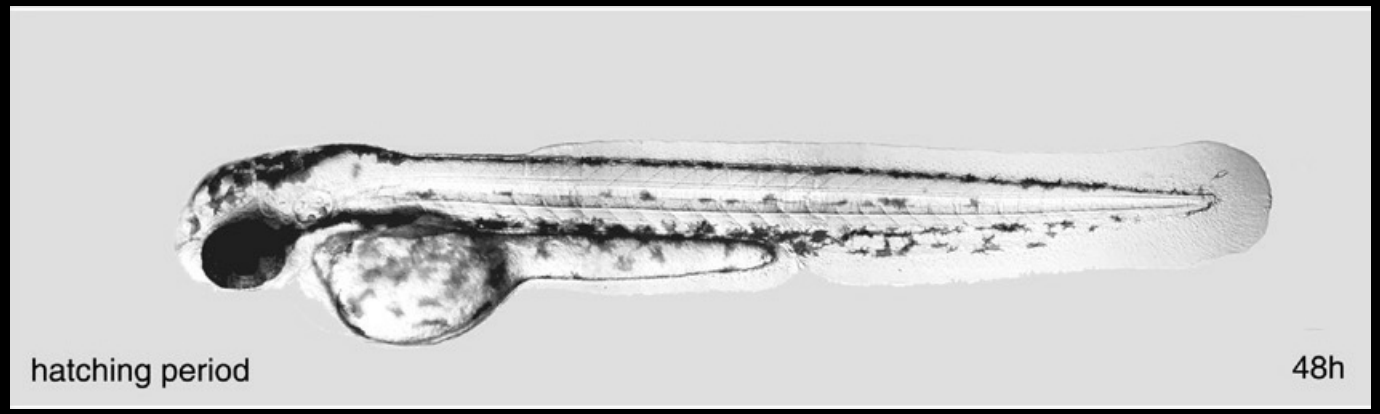




(1-cell => 64-128-cell stage, >3.5 dpf)

Pay attention to the biology of your system/organism





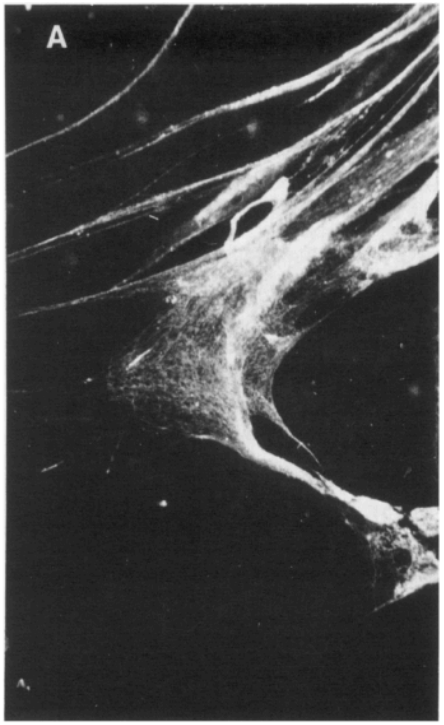
Zebrafish, Xenopus, Drosophila have an extensive early cleavage period

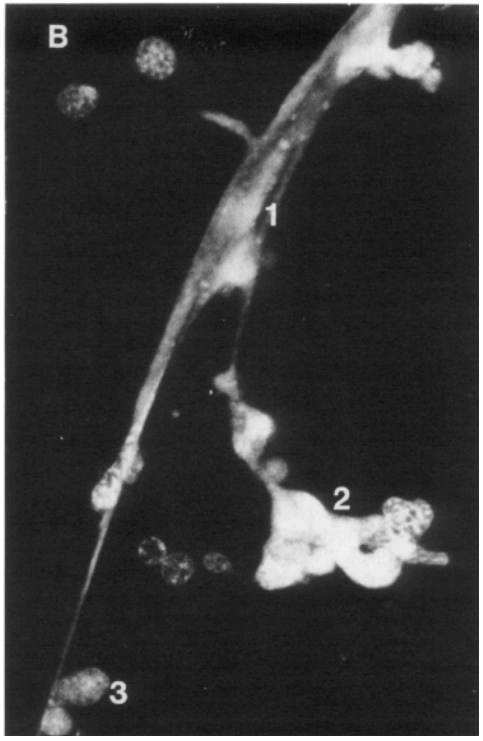
Zebrafish, Xenopus, Drosophila eggs are packed with maternal stores to support cellular division during cleavage

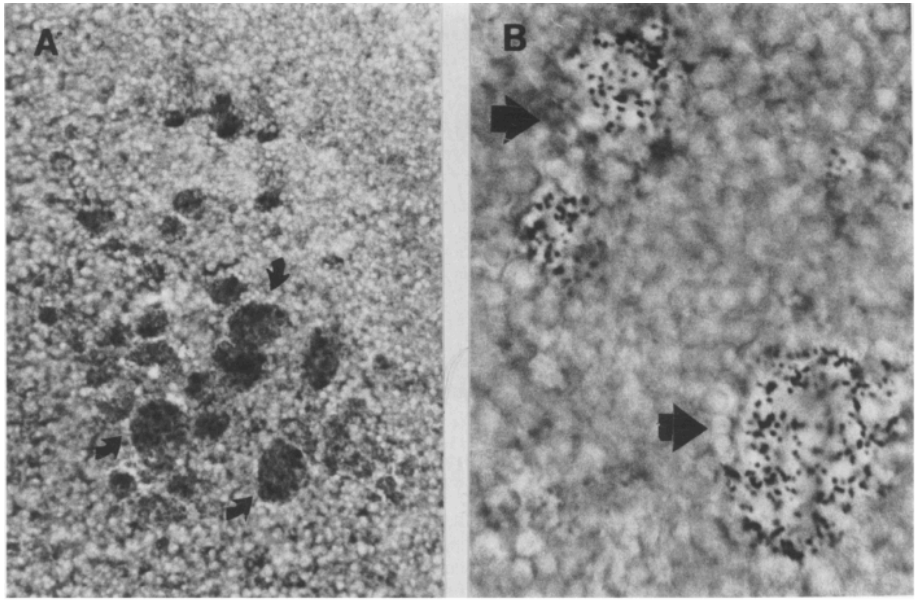
Cell, Vol. 34, 13–23, August 1983, Copyright © 1983 by MIT

Spontaneous Formation of Nucleus-Like Structures around Bacteriophage DNA Microinjected into Xenopus Eggs

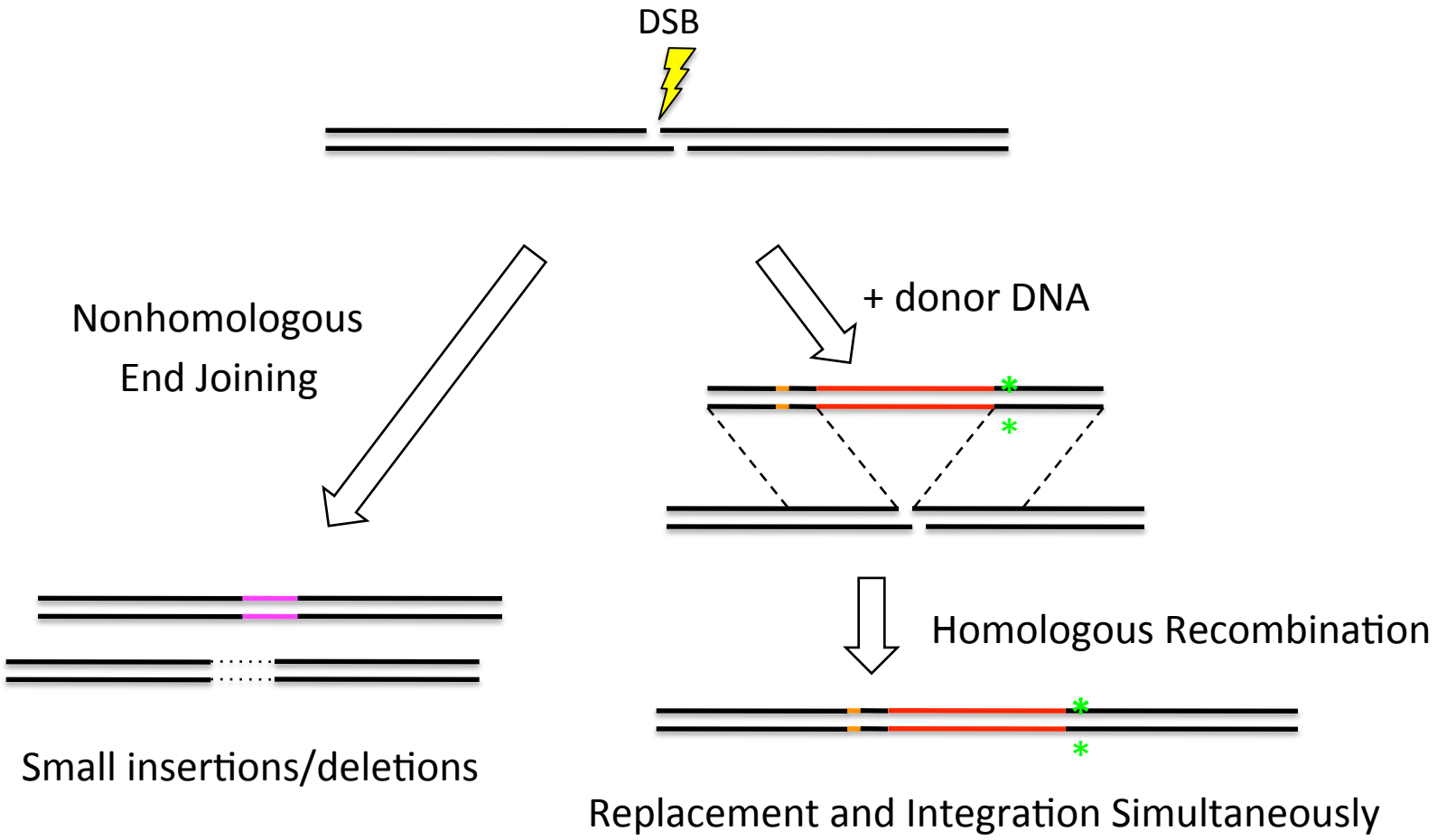
Douglass J. Forbes, Marc W. Kirschner, and John W. Newport
Department of Biochemistry and Biophysics
School of Medicine
University of California
San Francisco, California 94143



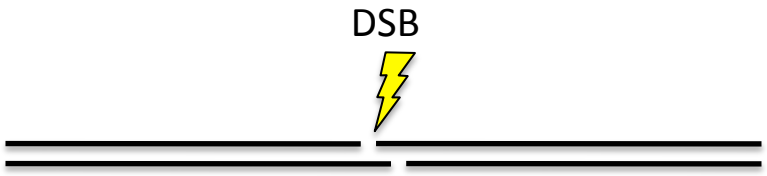




Targeted DSBs also stimulate recombination events at the site of the DSB

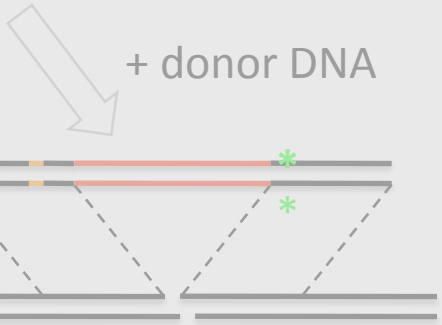


Targeted DSBs also stimulate recombination events at the site of the DSB

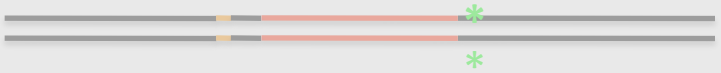


Nonhomologous End Joining

Small insertions/deletions



Homologous Recombination

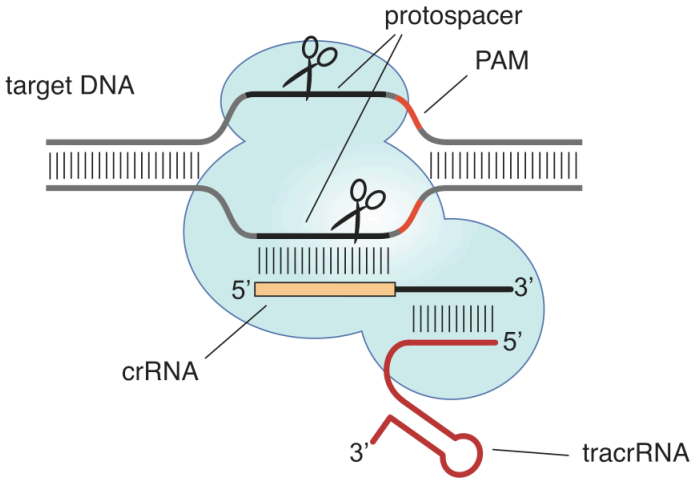


Replacement and Integration Simultaneously

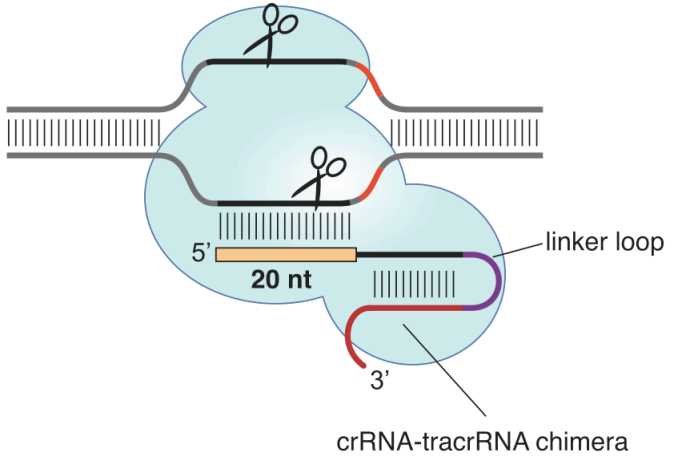
If this model is correct, then increasing the efficiency of DNA cleavage should reduce the incidence of recombination

Use of dual crRNA/tracrRNA gRNA improves the activity of Cas9 RNPs

Cas9 programmed by crRNA:tracrRNA duplex



Cas9 programmed by single chimeric RNA



Jinek *et al. (2012)* Science 337: 816

crRNA (42nt: target-specific)

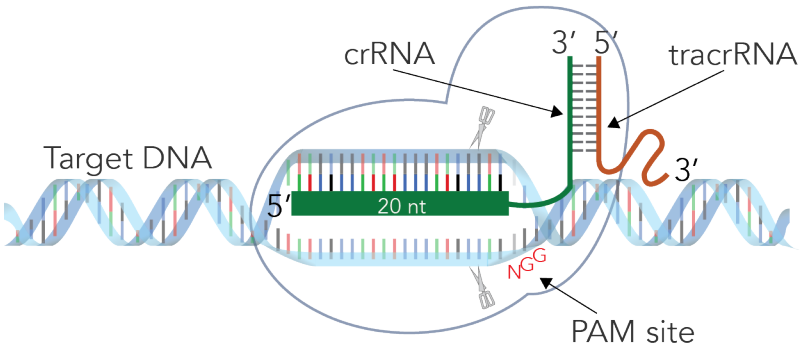
tracrRNA (69nt: non-specific)

sgRNA (102nt)

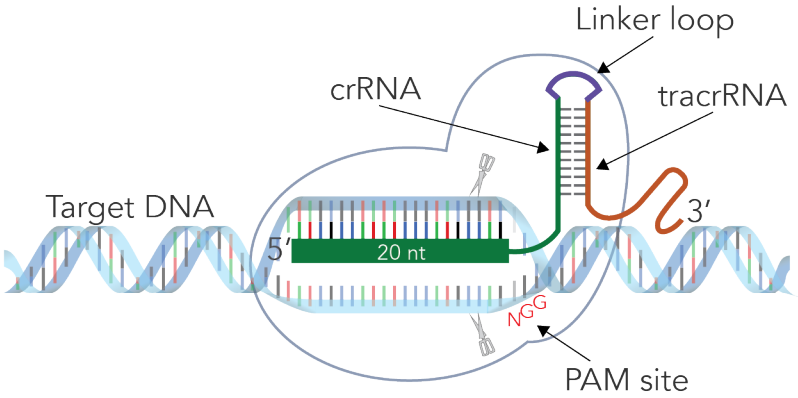
IDT provides artificially synthesized crRNA and tracrRNA:

- with modification to avoid RNase attack *in vivo*.

Dual crisprRNAs are more active than sgRNAs

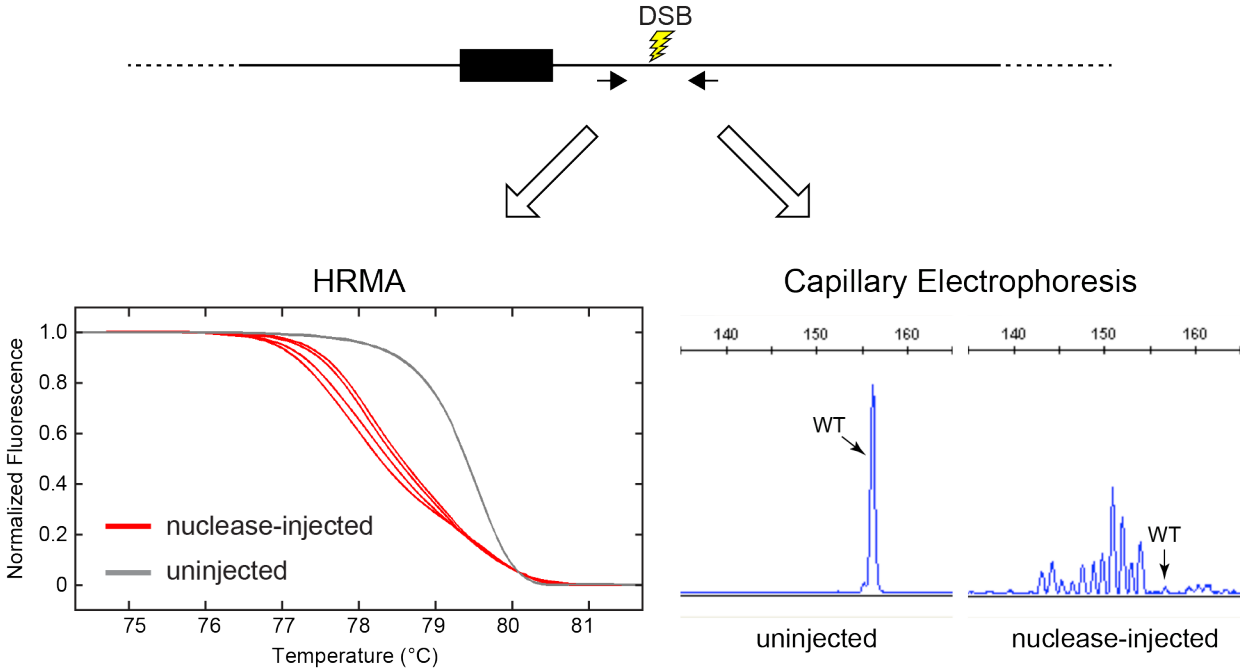


A. 2-part crRNA:tracrRNA complex



B. Single fusion sgRNA trigger

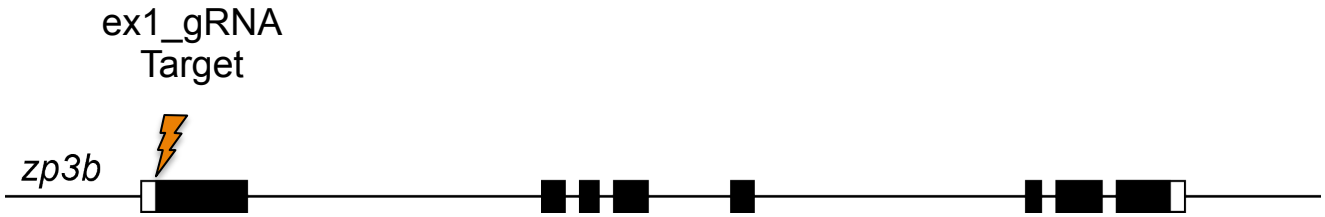
We use two methods to measure mutation induction



$$\text{Indel mutation efficiency (\%)} = \frac{\text{total fluorescence of peaks} - \text{WT peaks' fluorescence}}{\text{total fluorescence of peaks}} \times 100$$

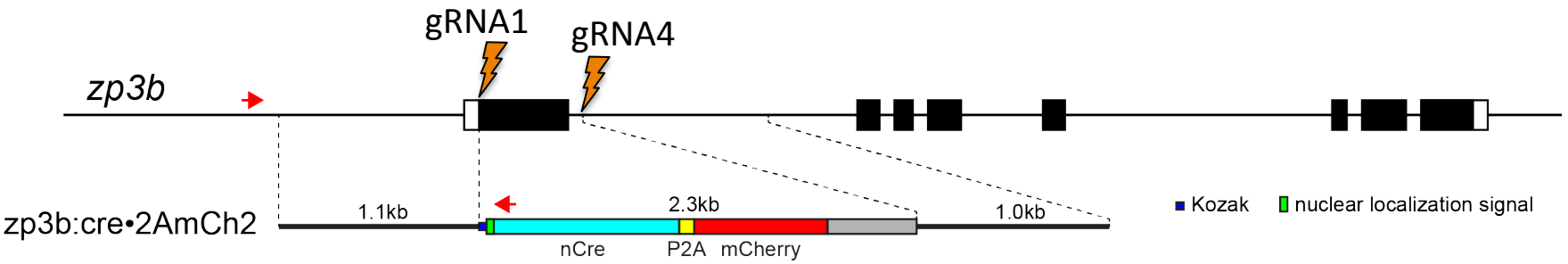
Carrington et al (2015) NAR

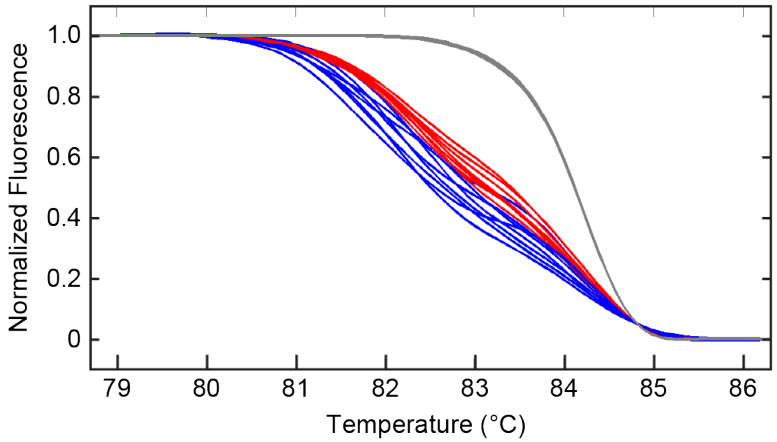
Comparing mutation induction at the *zp3b* locus

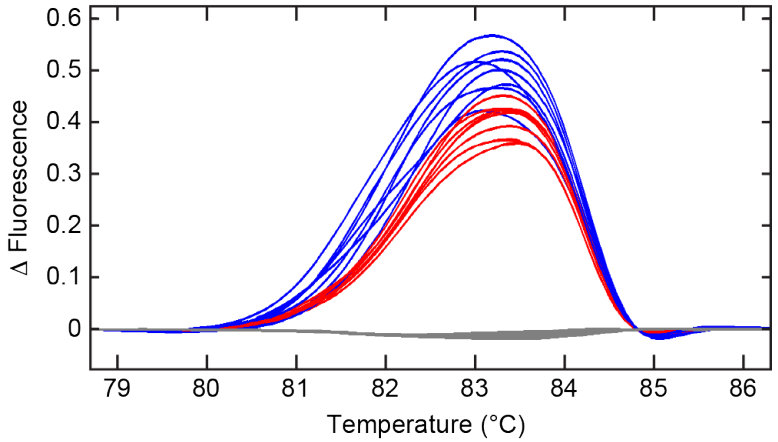


		Dual guide RNA	
embryo#	sgRNA	cr/tracr (min.)	cr/tracr (med.)
#1	40.3	98.4	92.4
#2	23.7	98.8	99.6
#3	29.7	89.5	97.0
#4	31.7	88.3	99.3
#5	39.9	90.2	85.5
#6	31.2	99.5	96.3
#7	41.0	84.3	97.1
#8	45.8	97.8	97.0
average	35.4 ± 6.9	93.4 ± 5.5	95.5 ± 4.3

Use of dual crRNA/tracrRNA gRNA improves the activity of Cas9 RNPs

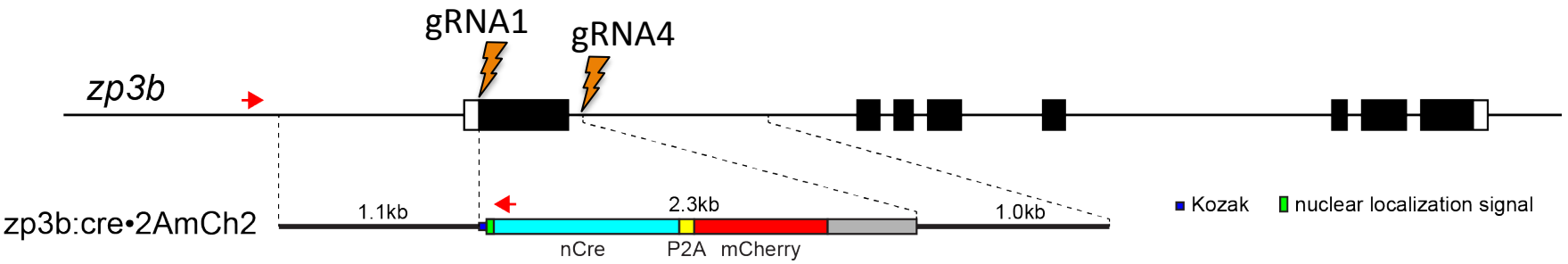


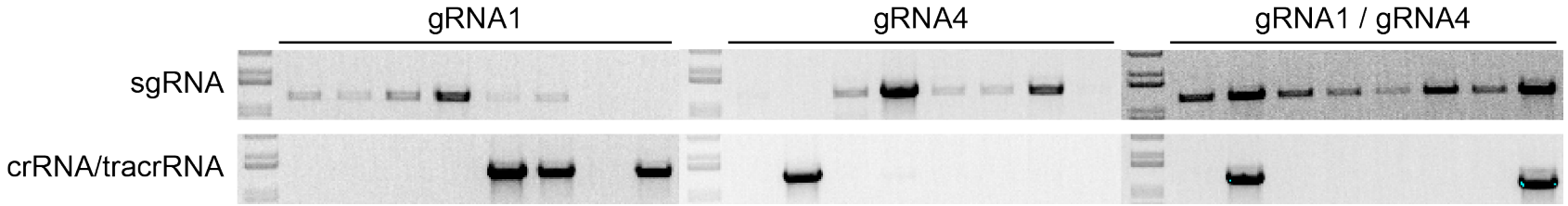




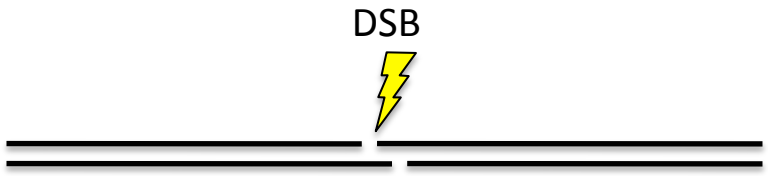
sgRNA1 + Cas9 protein
crRNA1/tracrRNA + Cas9 protein
uninjected

Improved Cas9 RNP activity results in diminished gene editing



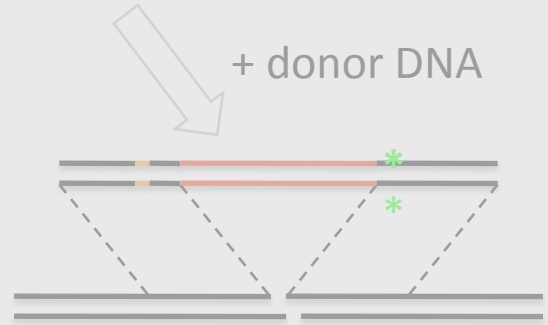


Targeted DSBs also stimulate recombination events at the site of the DSB



Nonhomologous End Joining

Small insertions/deletions

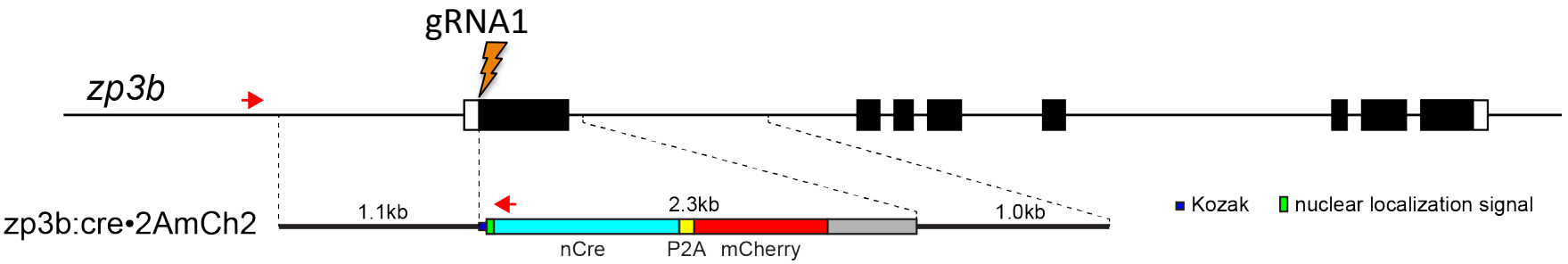


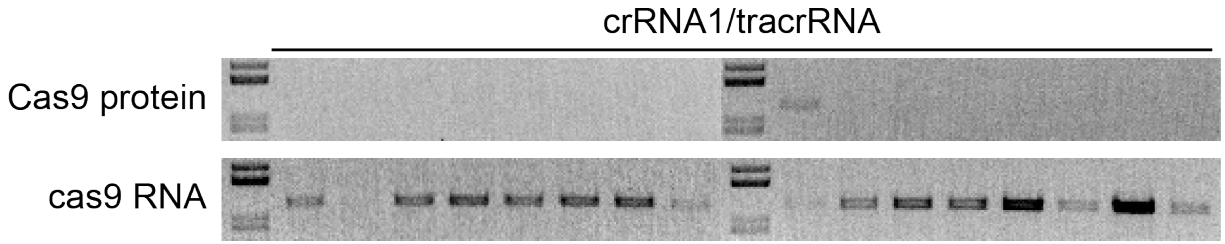
Homologous Recombination

Replacement and Integration Simultaneously

If this model is correct, delaying cutting until there are many cells (genomes) should improve recombination

Delaying Cas9 activity improves gene editing





Edited alleles

Things to consider

How & when to induce DSBs
How to optimize delivery of template for HDR
How to detect your edited allele
Remember the biology of the system

Genome Editing Team

Kazuyuki Hoshijima

Mick Jurynec