Generation and Application of Conditional Knock-In Alleles in Zebrafish

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Generation of conditional alleles

- one-step knock-in of loxP sites at an endogenous locus
- application of published protocols
- points for improvement, validation, and emphasis

Application of conditional alleles for cell- and stage-specific knockout

- Cre/lox pitfalls
- using conditional lines with cell-specific CreERT transgenics

Zebrafish genetics

- Long history of forward genetic approaches with mutant screens
- Reverse genetics via targeted knockout now routine with CRISPR
- Phenotypes for 7499 alleles across 4284 genes*

  *-single gene knockouts, source: ZFIN; does NOT include MO phenotypes or CRISPR F0
  - only a handful of these are “conditional alleles”, e.g. temperature sensitive
- conditional gene manipulation more routinely performed via transgenesis
  - typically involves gain-of-function or use of dominant negatives

Addressing cell (or stage) autonomy among most common hurdles for most research groups re: gene function.

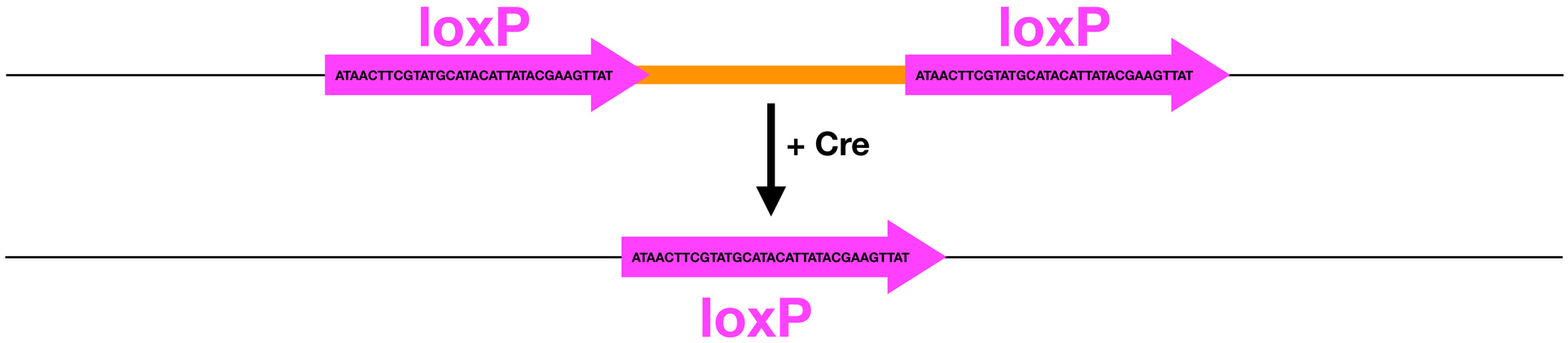
There is a need for standardized methods for conditional gene knockout.

Using Cre-induced recombination for conditional gene knockout

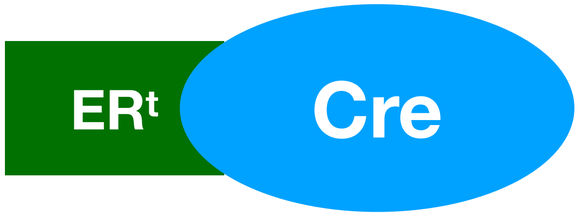
Cre

- **an autonomous recombinase from P1 bacteriophage**

- **Cre catalyzes recombination between two loxP sites oriented as direct repeats in *cis*, deleting the intervening fragment**



Using Cre-induced recombination for conditional gene knockout

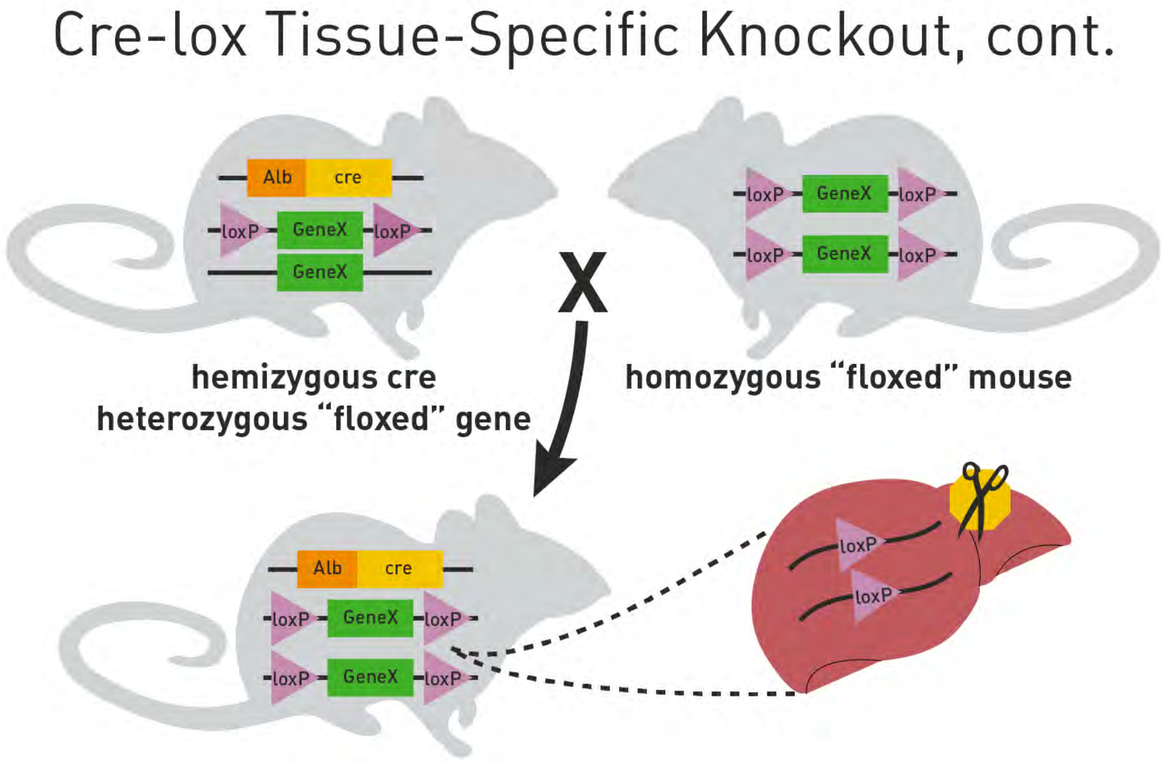


- mutated ligand binding domain from estrogen receptor
- allows inducible Cre activity by adding tamoxifen

- **make germline transgenic driving Cre with cell-specific promoter**

- **introduce loxP sites flanking essential exon(s) in gene of interest**

Using Cre-induced recombination for conditional gene knockout



- Cre/lox revolutionized use of mouse as a genetic model
- Facilitated analysis of gene function in the context of disease processes
- Conditional KO alleles available for nearly every mouse gene

CRE LOX BREEDING FOR BEGINNERS, PART 1,
Kelmenson, P., The Jackson Laboratory
https://www.jax.org/news-and-insights/jax-blog/2011/september/cre-lox-breeding

Cre/lox recombination works in zebrafish

- PubMed - zebrafish & Cre >250 articles
- Cre lines in ZFIN: 290 alleles using 189 different regulatory elements (promoter-driven, enhancer-, or gene-traps), 95 are CreERT
- loxP lines: 1092 alleles across 489 transgenes
  - **~5 are “traditional” conditional knockout alleles, i.e. endogenous exons flanked by loxP sites introduced via homologous recombination**

Generation of loxP knock-in alleles is feasible in zebrafish

CellPress

Developmental Cell
Technology

Precise Editing of the Zebrafish Genome Made Simple and Efficient

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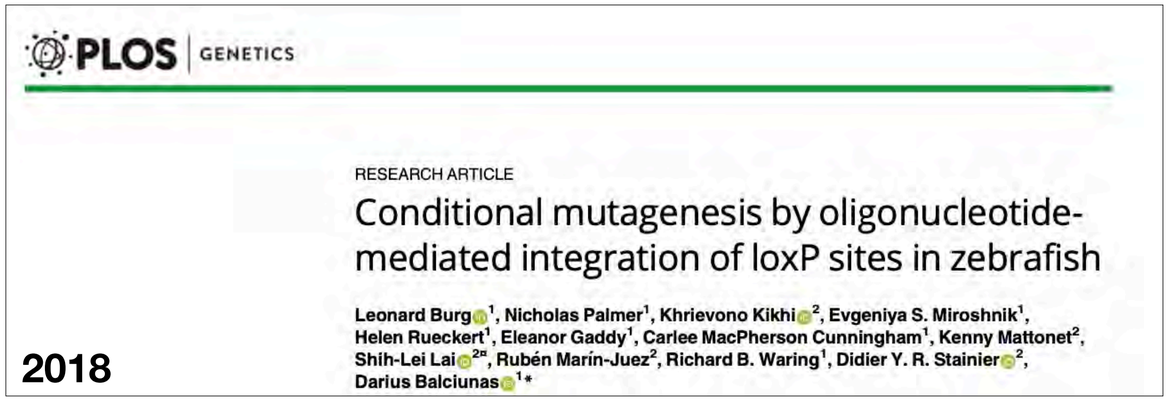
*Correspondence: grunwald@genetics.utah.edu
http://dx.doi.org/10.1016/j.devcel.2016.02.015

2015



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RESEARCH ARTICLE

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2018

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TOOLS AND RESOURCES

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2019

Masahiro Shin, Ph.D.

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We started by using the Hoshijima protocol

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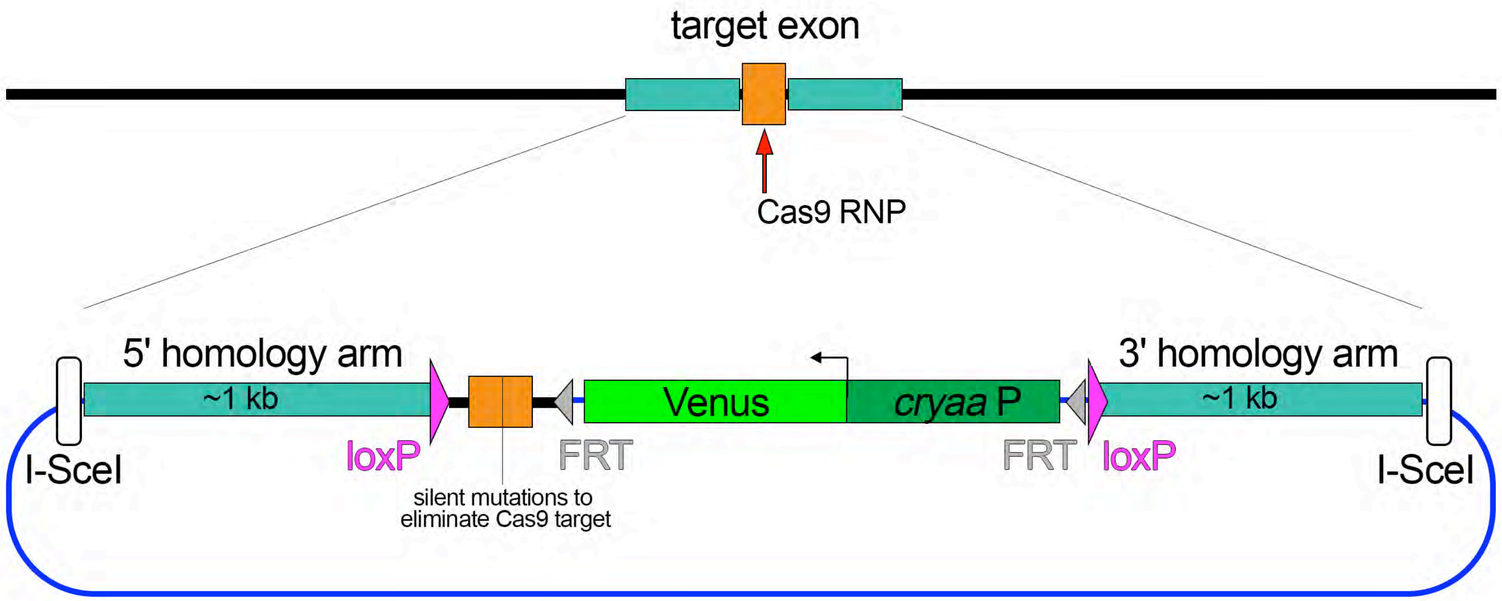
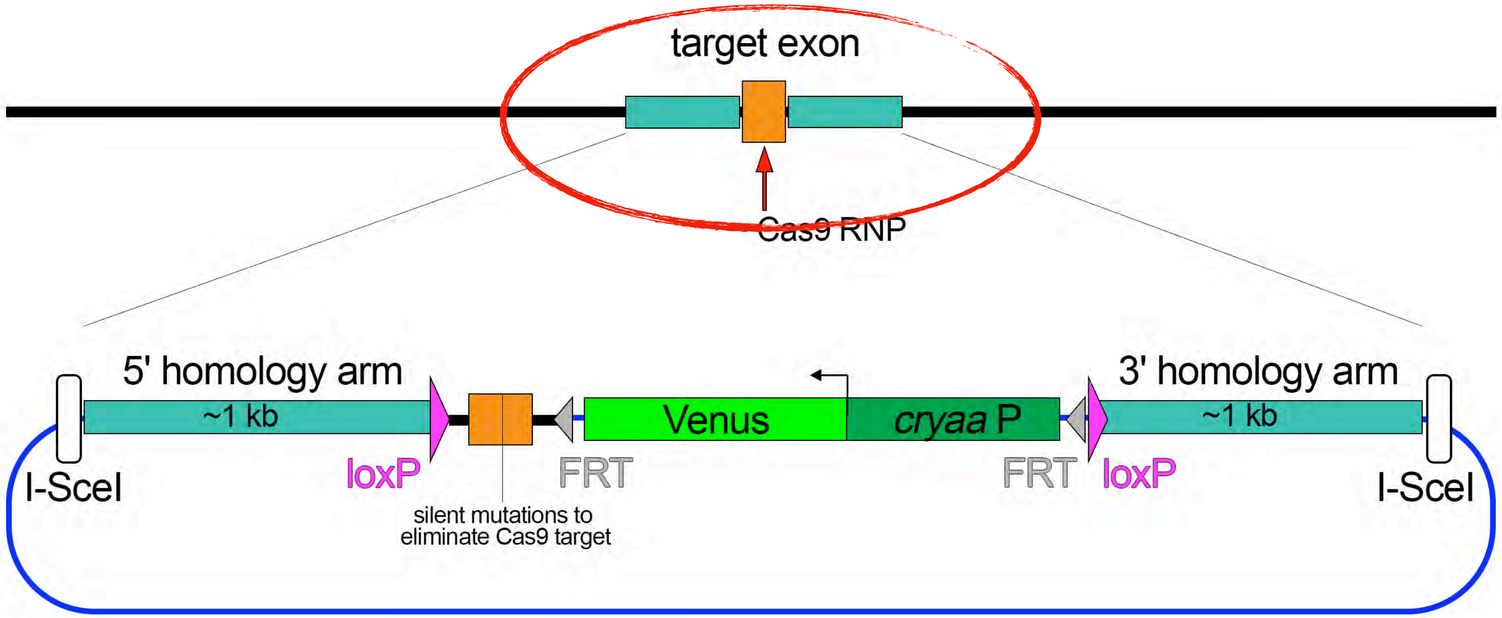
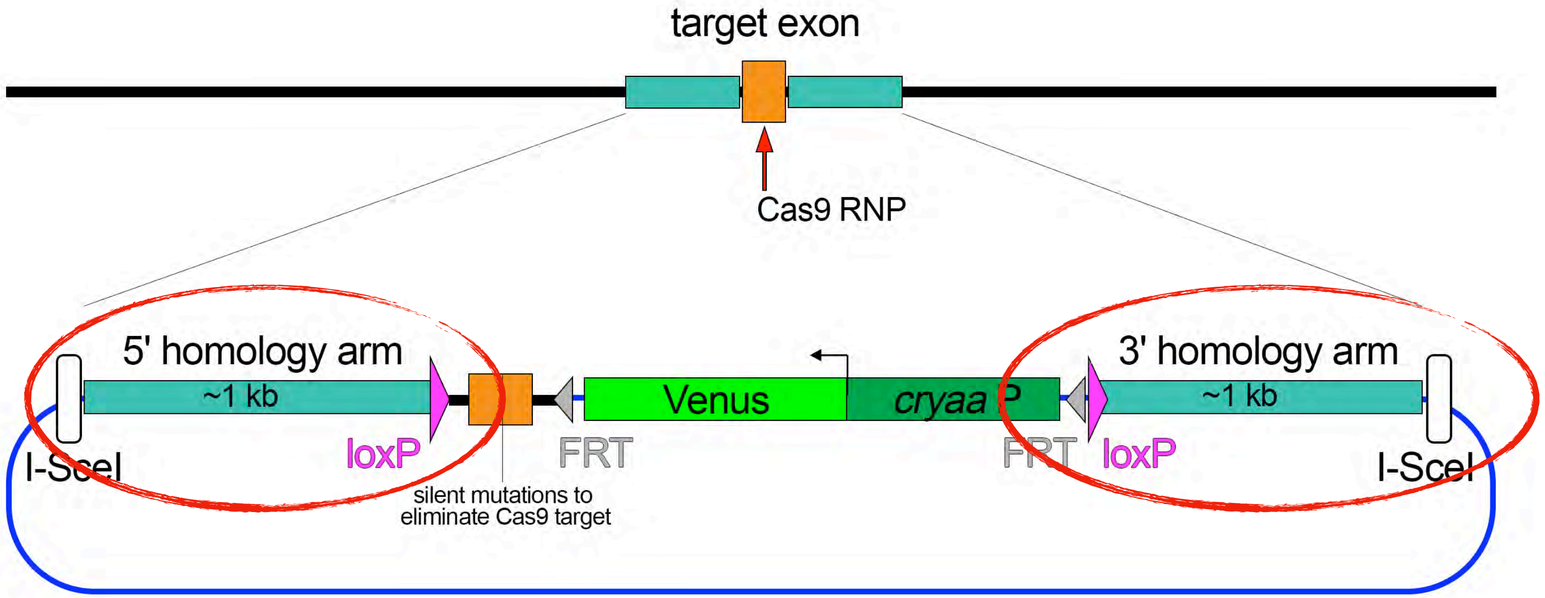




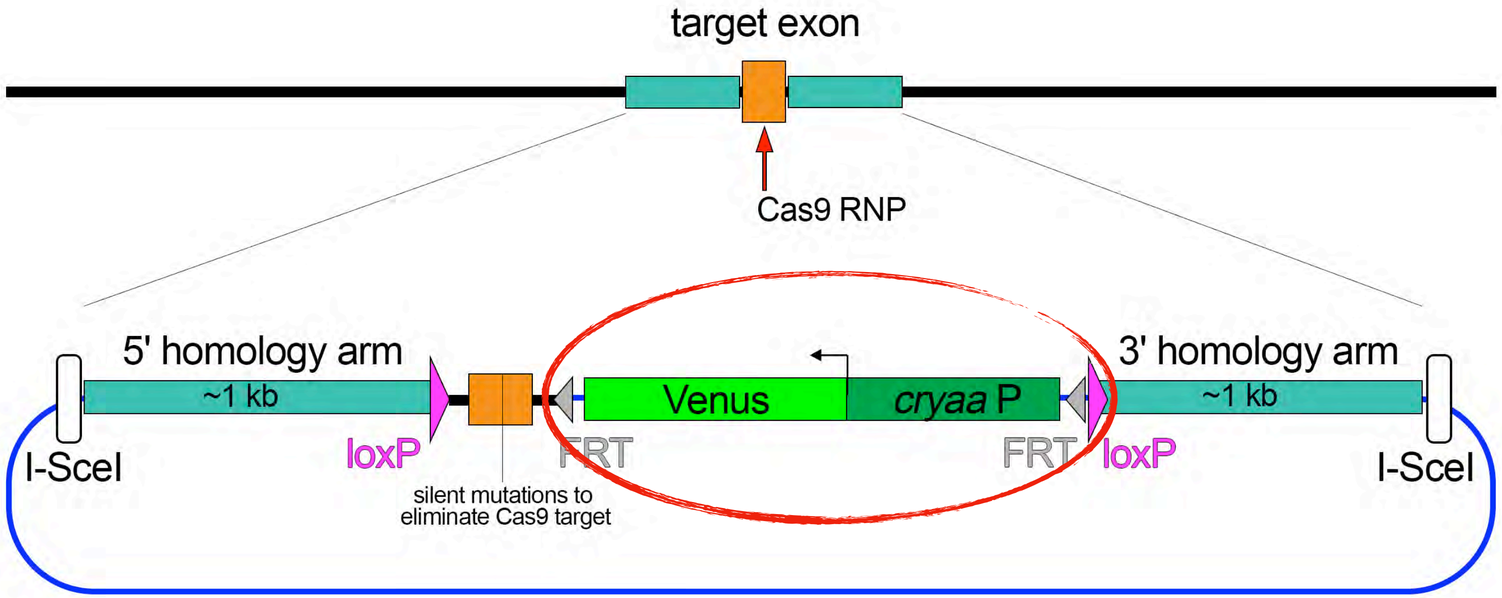
Target site considerations:

- exons where indels give a strong phenotype
- exons encoding signaling domains known to be essential for function
- are flanking exons in frame following knockout?
- look at conditional alleles for mouse orthologs
- don’t put loxP sites too far apart; deletion efficiency is inversely proportional to distance between loxP sites



Homology arms:

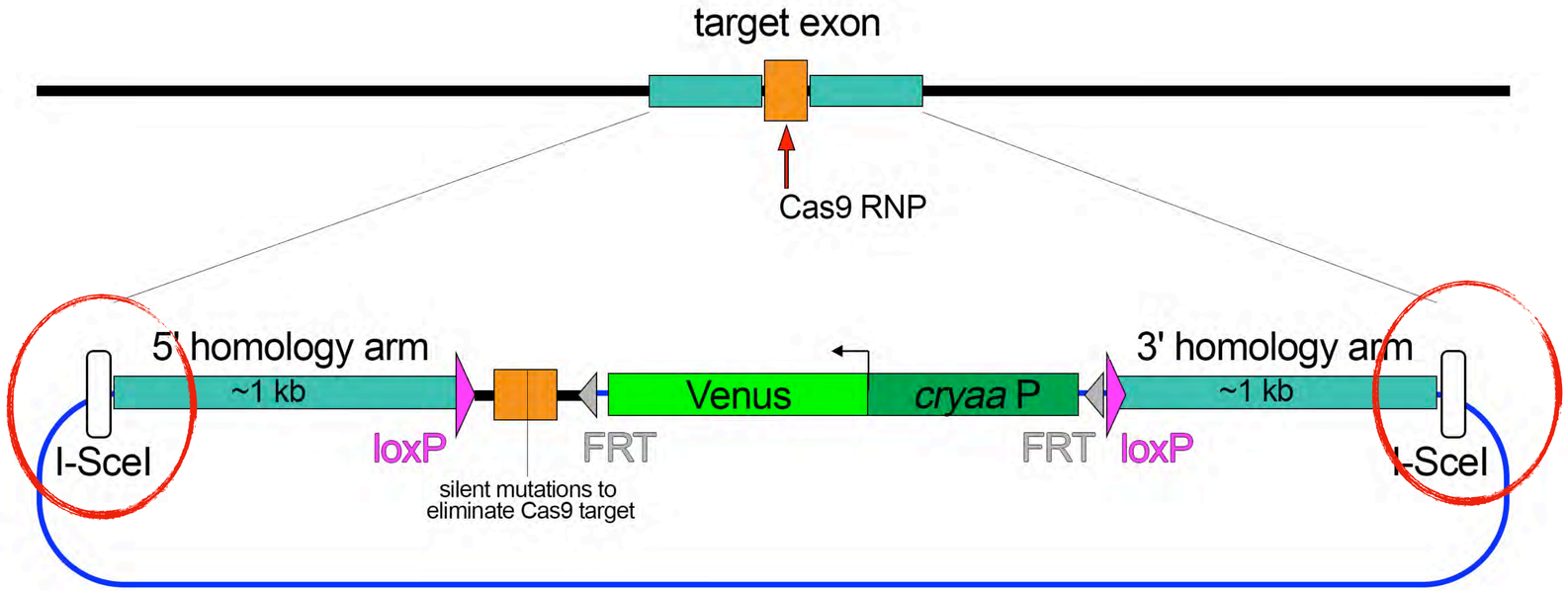
- ~1kb
  - we have used 400 bp with success
- **sequence needs to match what is in the fish you are injecting**
  - **yes, this is a pain**
  - **knowing the sequence is also important for ID'ing CRISPR sites, designing primers, digests for Southern blots, etc. .**
  - **sequencing is the very first step for us**



Heterologous marker

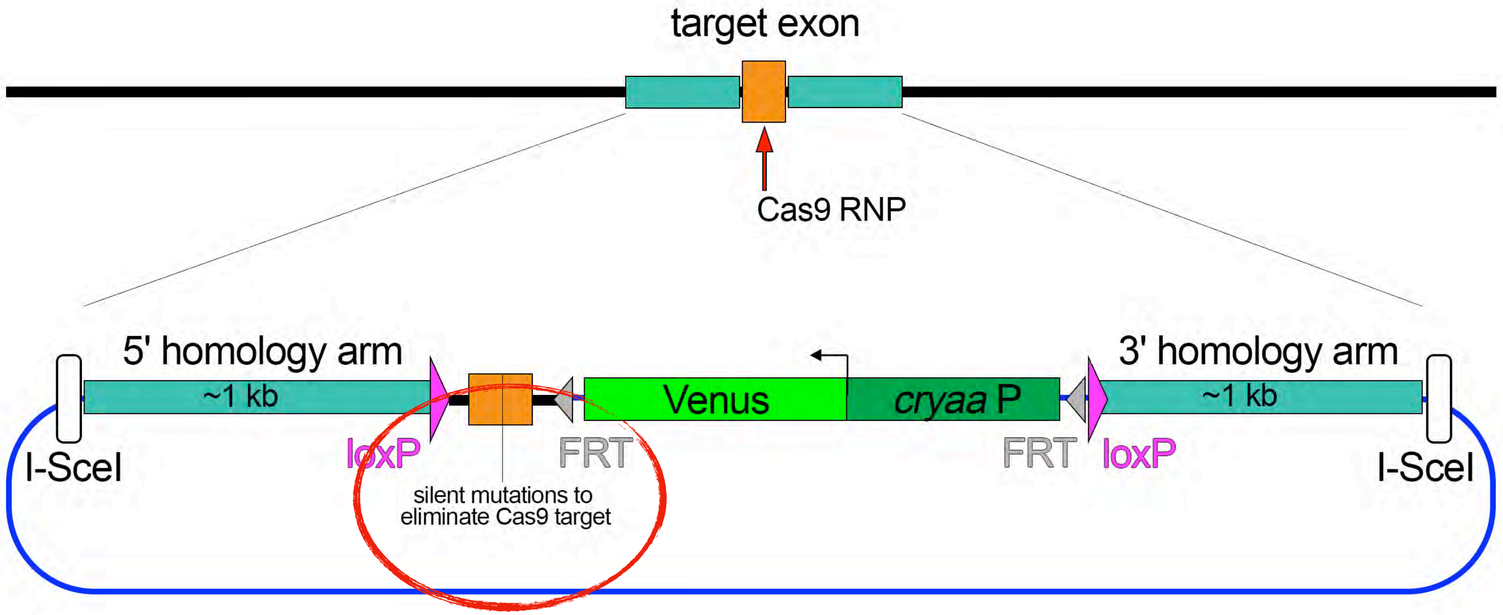
- we are using *cryaa:Venus*, but can be anything that won't interfere with downstream analysis
    - consider what CreERT marker will be used; being able to easily identify *gene*^{loxp};CreERT embryos or larvae is very helpful
- definitely useful for post-injection and founder identification
- is flanked by FRT sites for removal, if needed*

  *turns out FLP is mildly toxic and not very efficient, so CRISPR might be better option here



I-SceI sites

- to release and linearize the HR template
- linear works better than circular/“un-released” template
- still not clear if it works better to pre-digest or not



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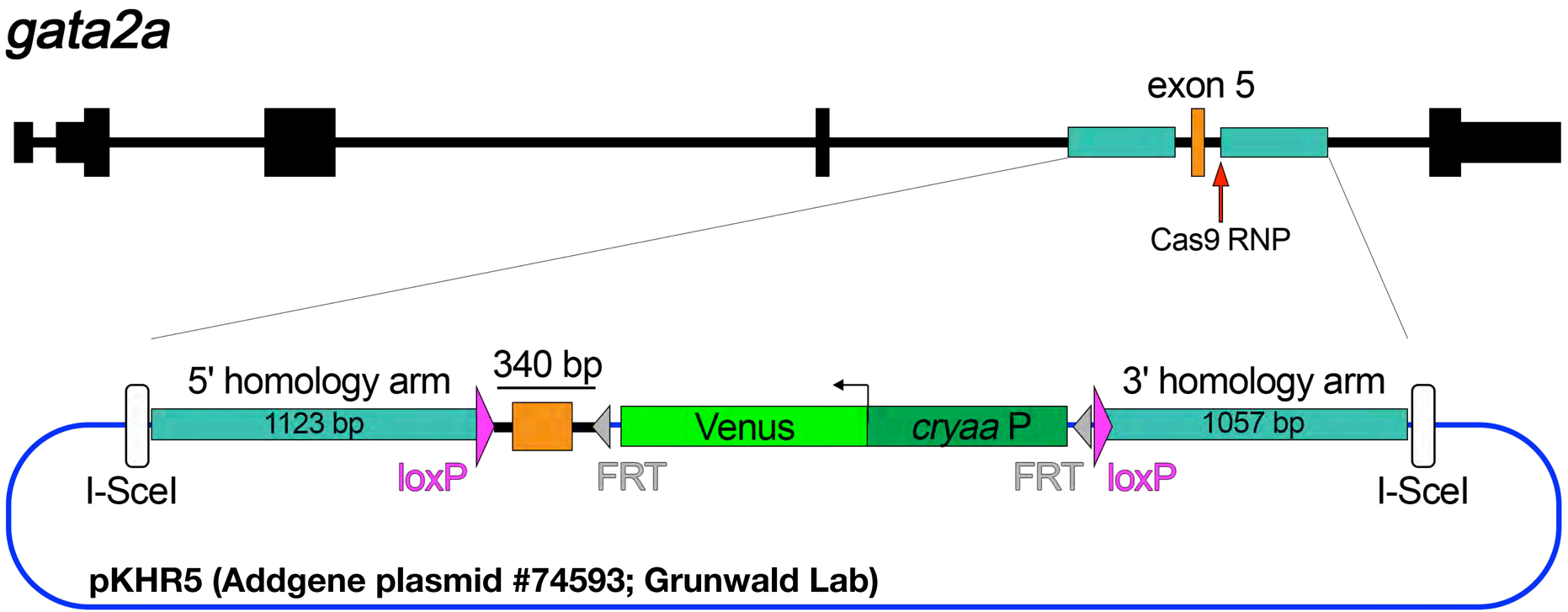
Our first target: ***gata2a***

- encodes a zinc finger transcription factors that bind a GATA consensus motif
- mouse and human orthologs required for hematopoiesis and lymphatic valve development

- ***gata2a^{um27}***
  - indel introduced in exon 4 using ZFNs
  - mutant embryos exhibit:
    - partial loss of trunk circulation at 48 hpf
    - loss of lymphatic valve (4 dpf) leading to lymphedema (5-7dpf)
      - this phenotype is confounded by earlier circulation defect, loss of swim bladder, and other possible non-autonomous phenotypes

Strategy for generating a *gata2a* conditional allele

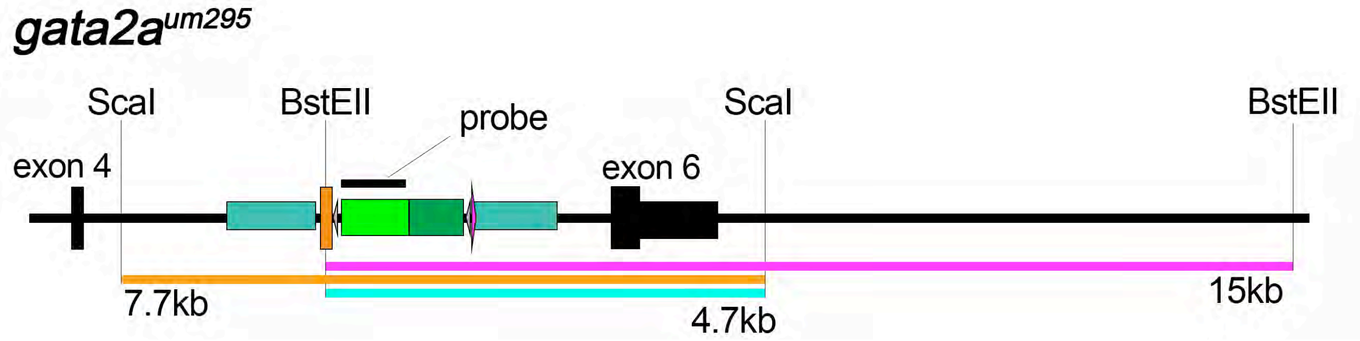


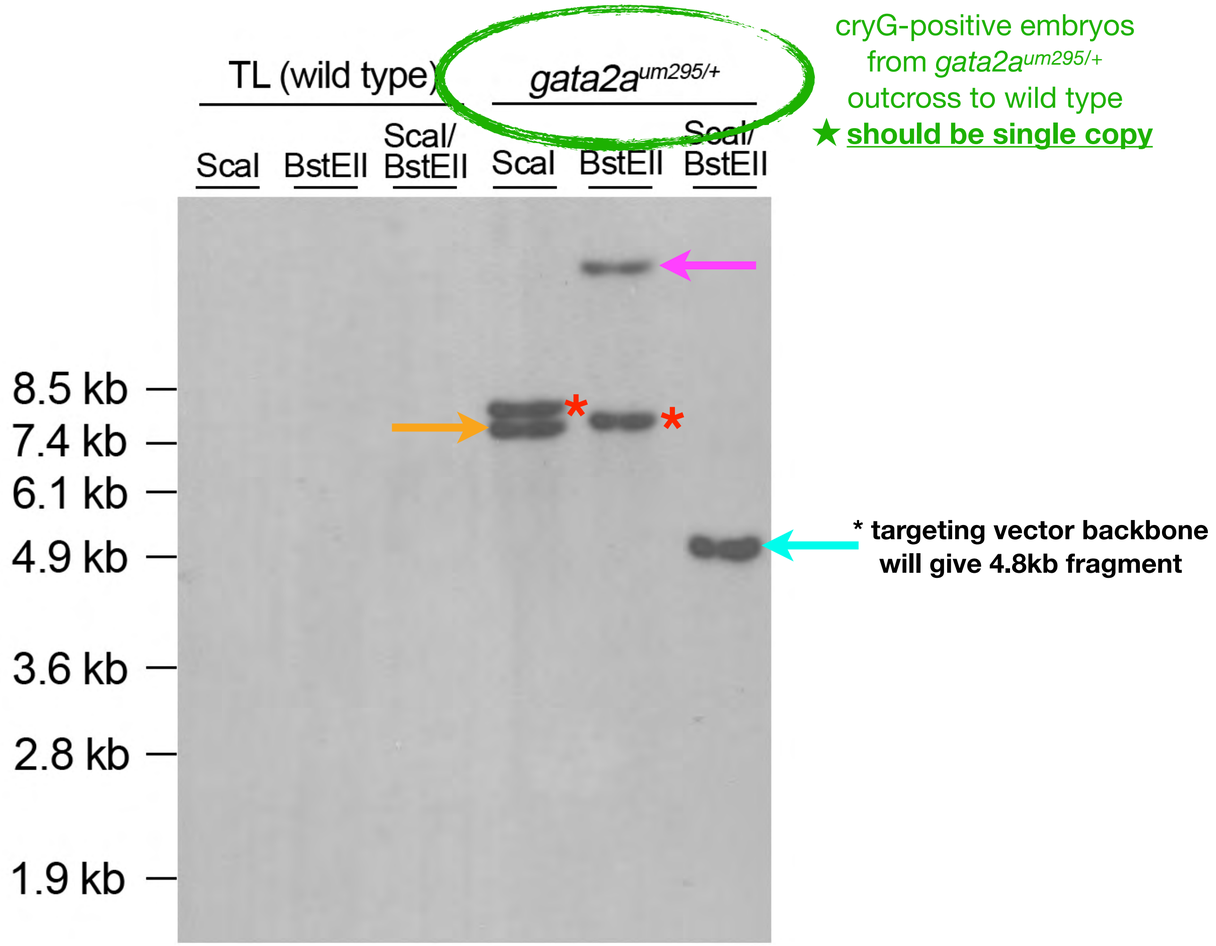
co-inject into wild type (per embryo):
50 pg targeting vector
~6-8 fmol Cas9/sgRNA RNP
$1x10^{-3}$ U I-SceI

- **verify KI in individual embryos**
- **grow up cryG+ embryos**

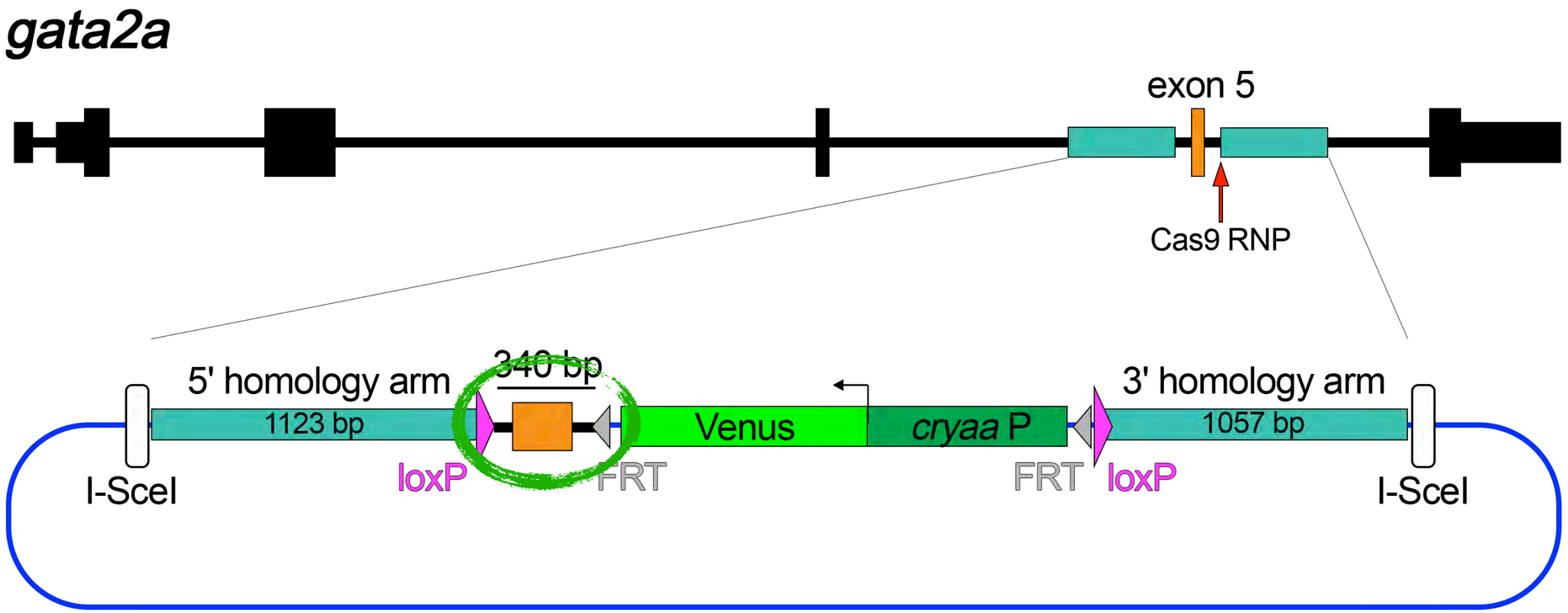
Identification and molecular characterization of germline founders

- total fish screened by outx: 115
- total **cryVenus+** clutches: 12
- total PCR positive: 2
  . . . except only 3' KI detected!!!
- further characterization by Southern analysis
- multiple inserts in *um295*
- molecular analysis and genetics suggest that off-target insertion is linked to *gata2a*





Exon 5 sequence likely sufficient to stimulate HR-mediated knock-in



Next steps with *gata2a*^{um295}

- confirmed (by Southern) that loxP sites from linked off-target integration do not cause multi-locus deletion
- introduce 5’ loxP at target site to generate functional conditional *gata2a* allele (via Cas12a RNP/oligo)
- confirmed phenotype in *cre* mRNA-injected *gata2a*$^{loxp/um27}$ embryos

First round problems with single-step conditional allele generation

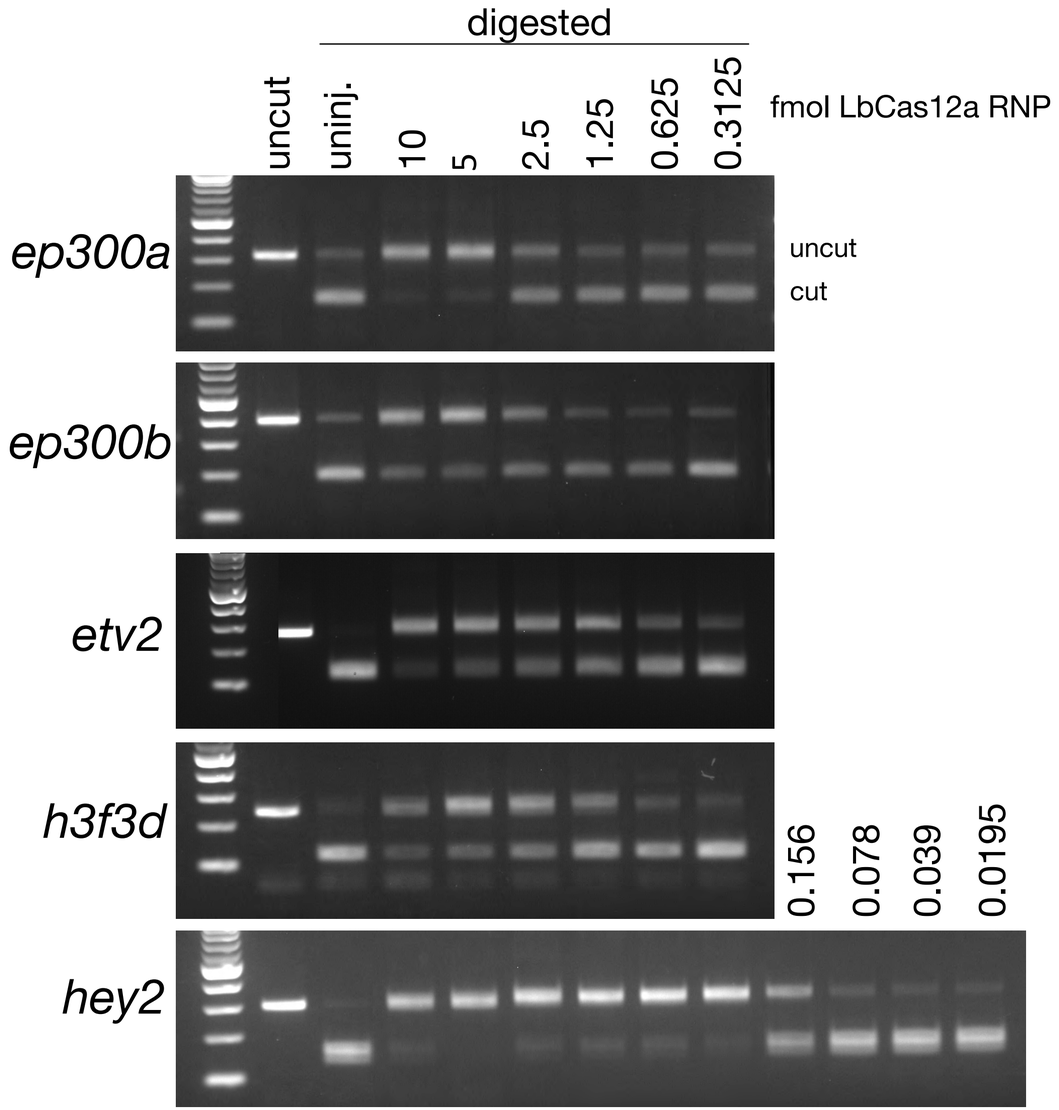
Problem: Partial targeted knock-in, likely due to sufficient upstream homology between Cas9 target and 5’ loxP site
Solution: use **two** CRISPR RNPs to force homology-directed repair outside of loxP sites; more carefully confirm integration on both sides following injection

Problem: off-target insertion linked to knock-in allele
Solution: use marker outside of homology arms to detect off-target/non-homologous integration

Additional improvements:
use Cas12a RNPs instead of Cas9

optimize CRISPR RNP dose to avoid “over-cutting” site

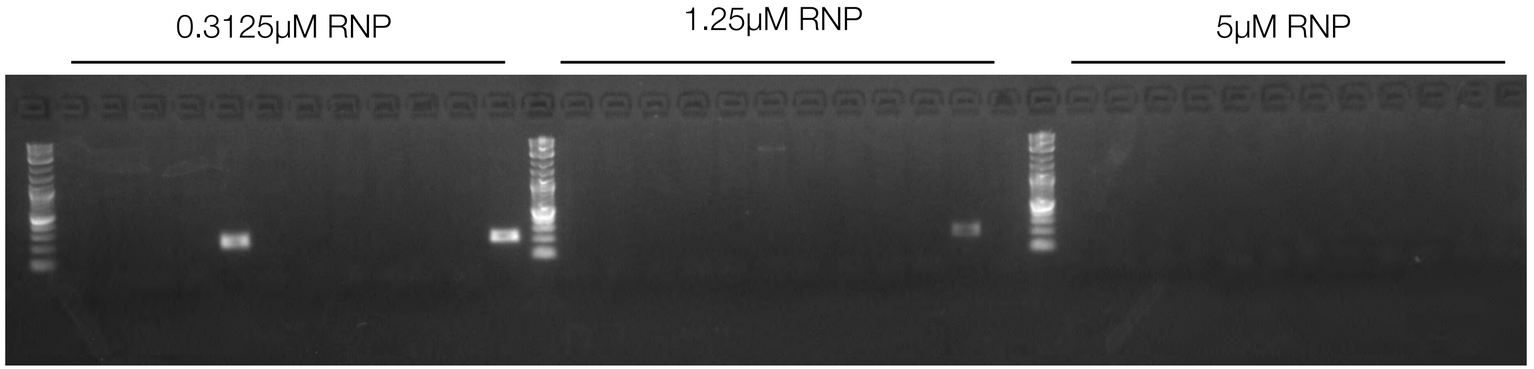
Optimizing CRISPR RNP dose for targeted knock-in



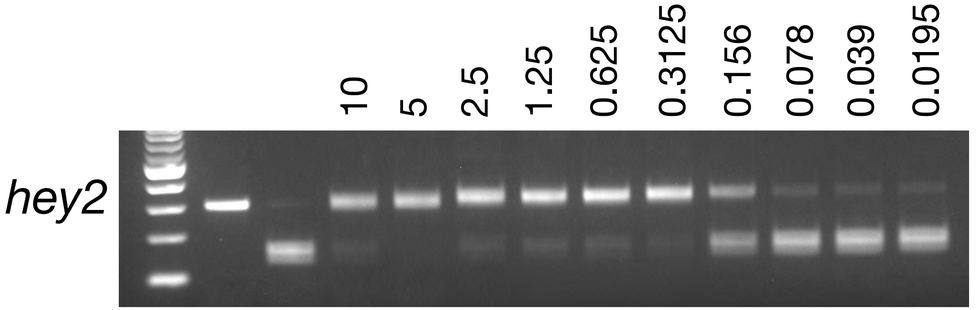
Sarah Oikemus

Optimizing CRISPR RNP dose for targeted knock-in

+ 10pg pre-digested (Isce1) avitag donor template



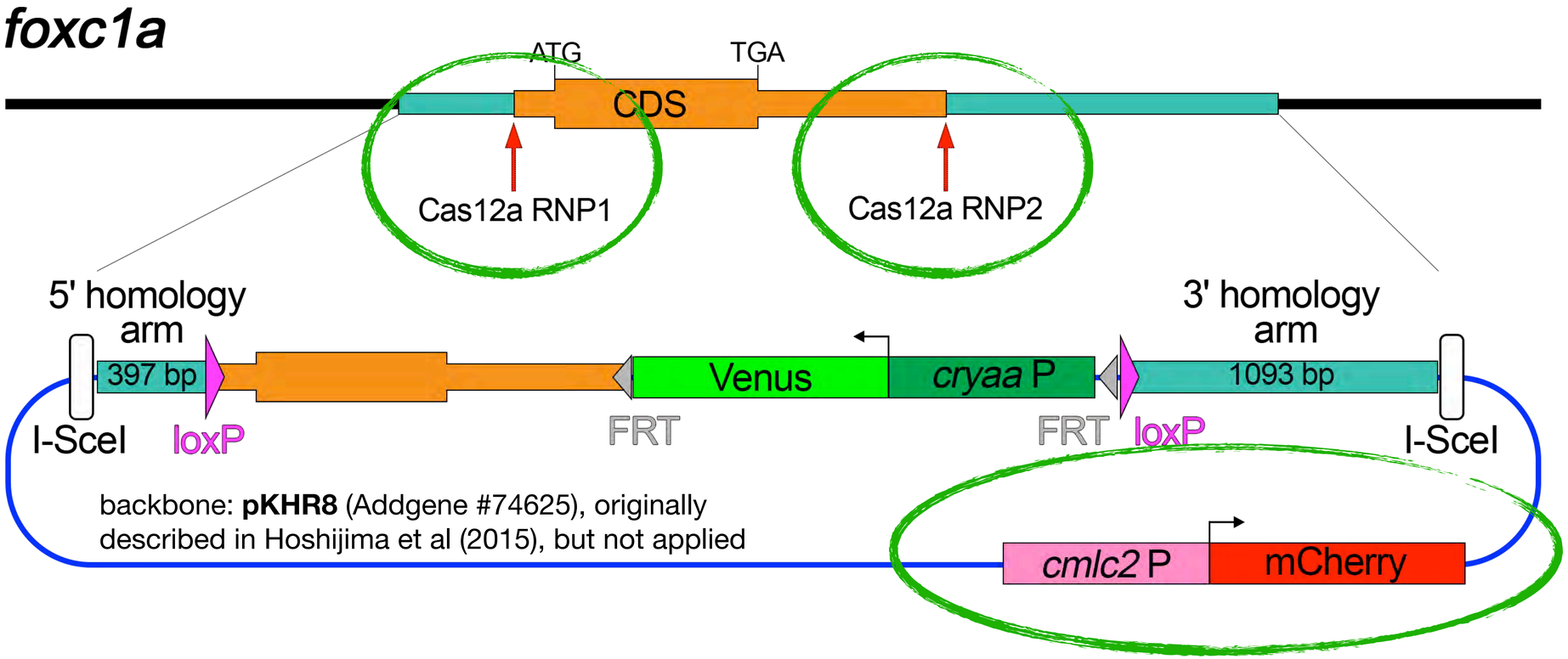
PCR across 5’ junction



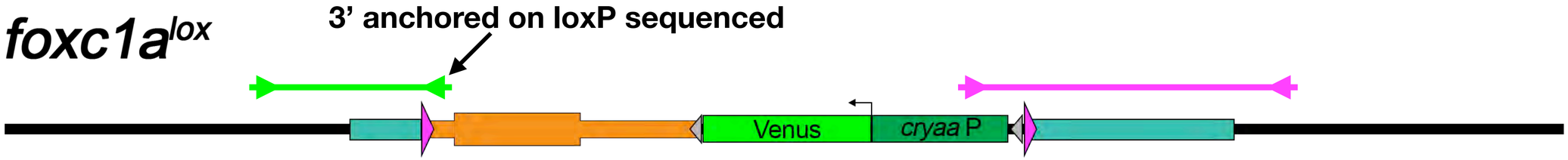
Applying potential improvements: ***foxc1a***

- encodes a forkhead transcription factor
- mouse and human orthologs required for development of multiple tissues, including lymphatic vessels; *FOXC1/2* orthologs mutated in multiple human syndromes
- *foxc1a*^{p162} (Granato lab)
  - nonsense mutation (single exon gene)
  - mutant embryos exhibit:
    - defects in multiple tissues including loss of trunk circulation
    - loss of lymphatic valve (4 dpf) leading to lymphedema (5-7dpf)
      - required mRNA rescue to alleviate early defects

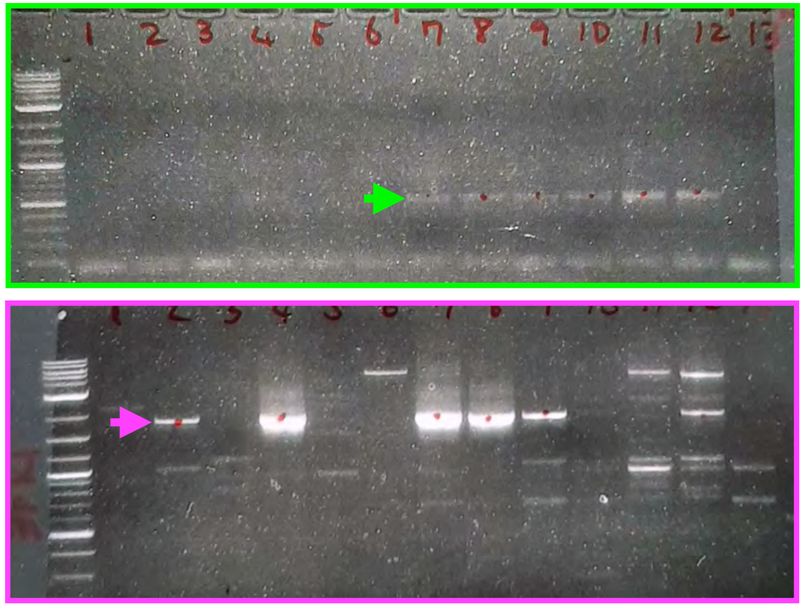
Targeting strategy to generate *foxc1a*^{loxp}



Screen individual embryos for 5’ and 3’ knock-in



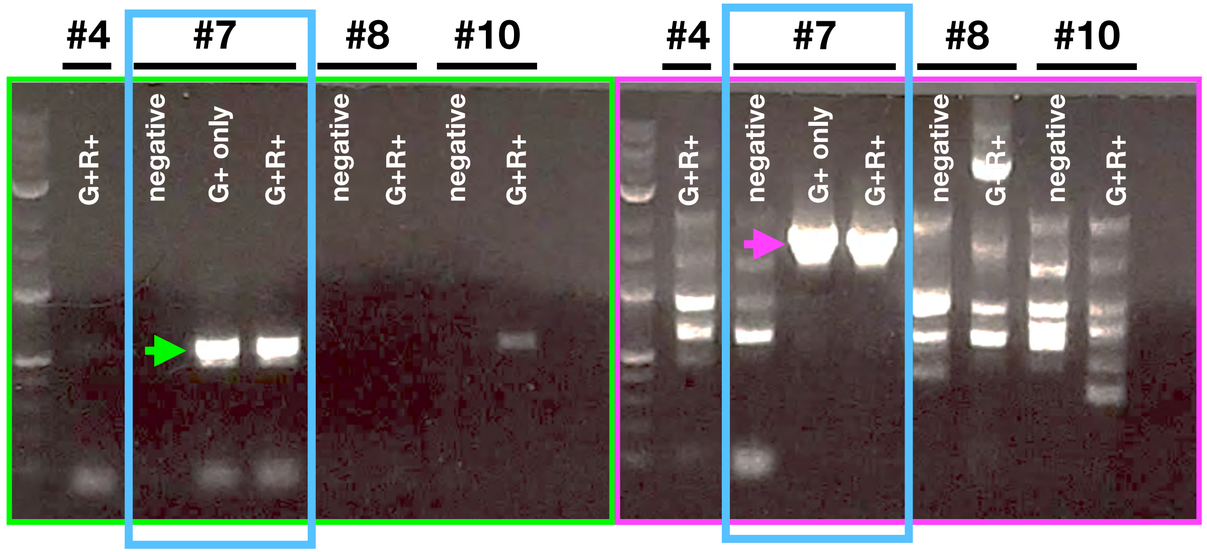
individual injected embryos



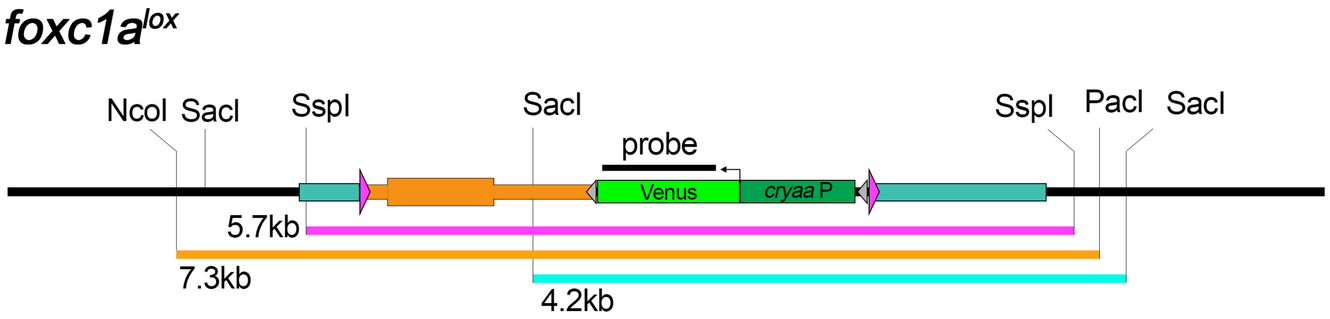
junctions validated by sequencing of shotgun-cloned fragments

- only see evidence for homologous knock-in in cryG+/cmlc2R+ embryos

embryos from putative founders



Identification and molecular characterization of germline founders



- total fish screened by outx: **45**
- mixed **Venus+**/**Cherry+** and **Venus only+**: **2***

  ***1** out of 2 with 5' and 3' KI PCR positive

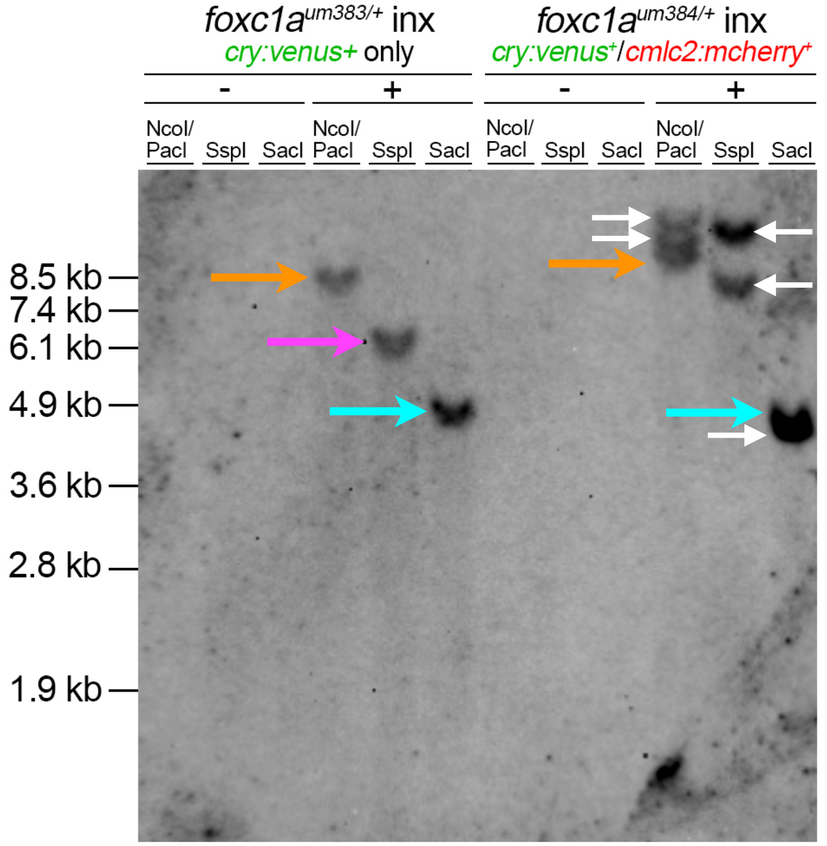
  Venus+: *foxc1a^um383*

  Venus+/Cherry+: *foxc1a^um384*

- **Venus+/Cherry+**: **7****

  ****1**/7 with 5' and 3' KI PCR positive, but not separable from cherry

- **Venus+** or **Cherry+**: **0**



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  Venus+: *foxc1a*^{um383}
  Venus+/Cherry+: *foxc1a*^{um384}

- **Venus+/Cherry+**: **7****

  ****1**/7 with 5’ and 3’ KI PCR positive, but not separable from cherry

- **Venus+** or **Cherry+**: **0**

knock-in at the ***rasa1a*** locus

- total fish screened by outx: **37**
- mixed **Venus+**/**Cherry+** and **Venus only+**: 2

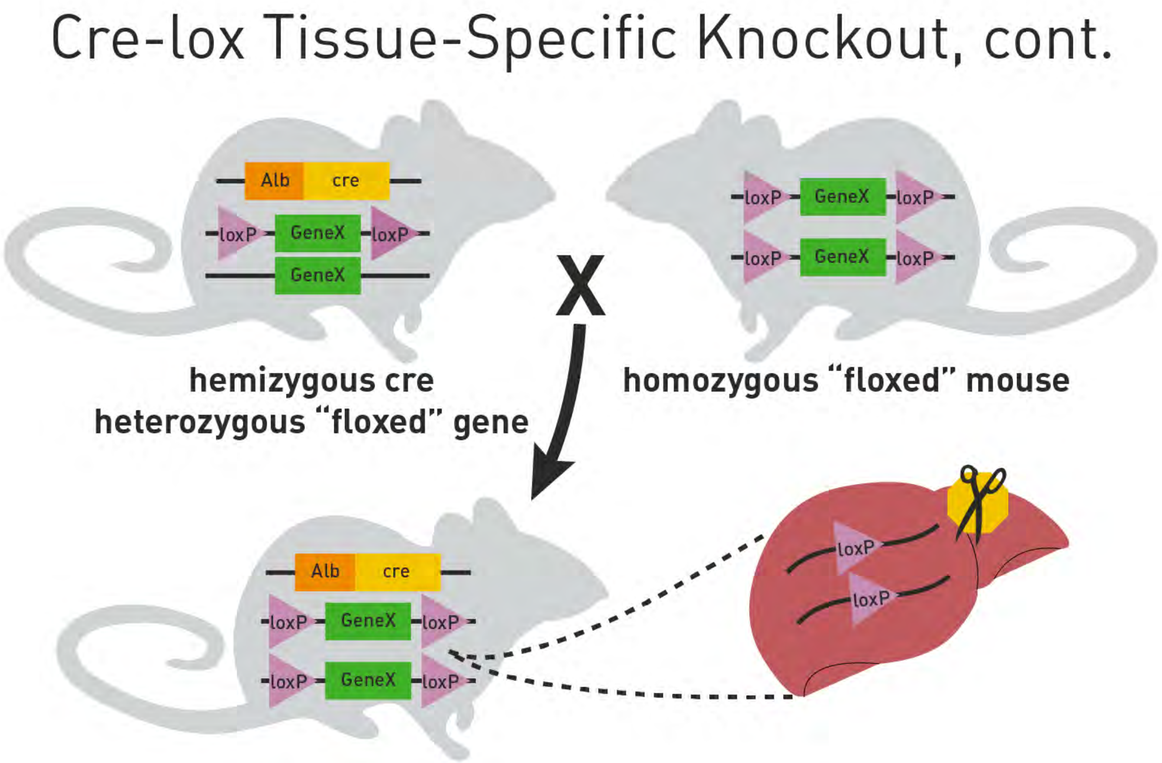
  Venus+: PCR-neg
  Venus+/Cherry+: only 5’ PCR-pos

- **Venus+/Cherry+**: 18, PCR-neg
- **Venus+ only:** 2, **1 with 5’ and 3’ PCR-pos**
- **Cherry+ only**: 1

Summary I: Generation of conditional loxP-bearing alleles

- Applied original Hoshijima/Grunwald protocol to generate *gata2a*^{loxp}
  - identified points for **emphasis** and points for **improvement**
    - **sequence the locus from fish to be injected**
    - **use *cmlc2:cherry* marker to identify non-specific integration**
    - **use two CRISPRs to force homology outside of loxP sites**
    - **we like to use Cas12a (evidence for improved HDR over Cas9)**
    - **optimize each individual RNP dose to avoid "over-cutting"**
    - **confirm 5' and 3' knock-in in individual injected embryos**
    - **confirm knock-in/rule out non-specific integrants by Southern**
- Applied these improved knock-in conditions to generate loxP-flanked alleles in a single step
  - successful at two different loci: *foxc1a* and *rasa1a*

Now what do I do?

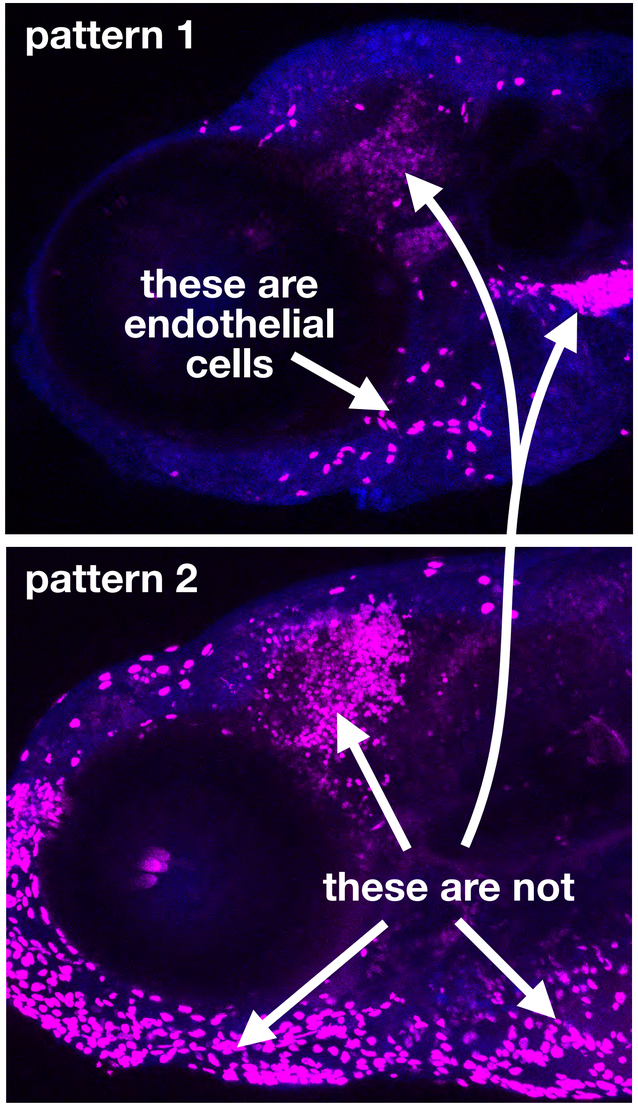


- cross *g.o.i.*^{loxp} onto a CreERT line
- use a "switch" transgene in *g.o.i.*^{loxp}*;CreERT* background to visualize knock-out cells

- Cre lines: 290 alleles using 189 different regulatory elements, 95 are CreERT

Caveats:

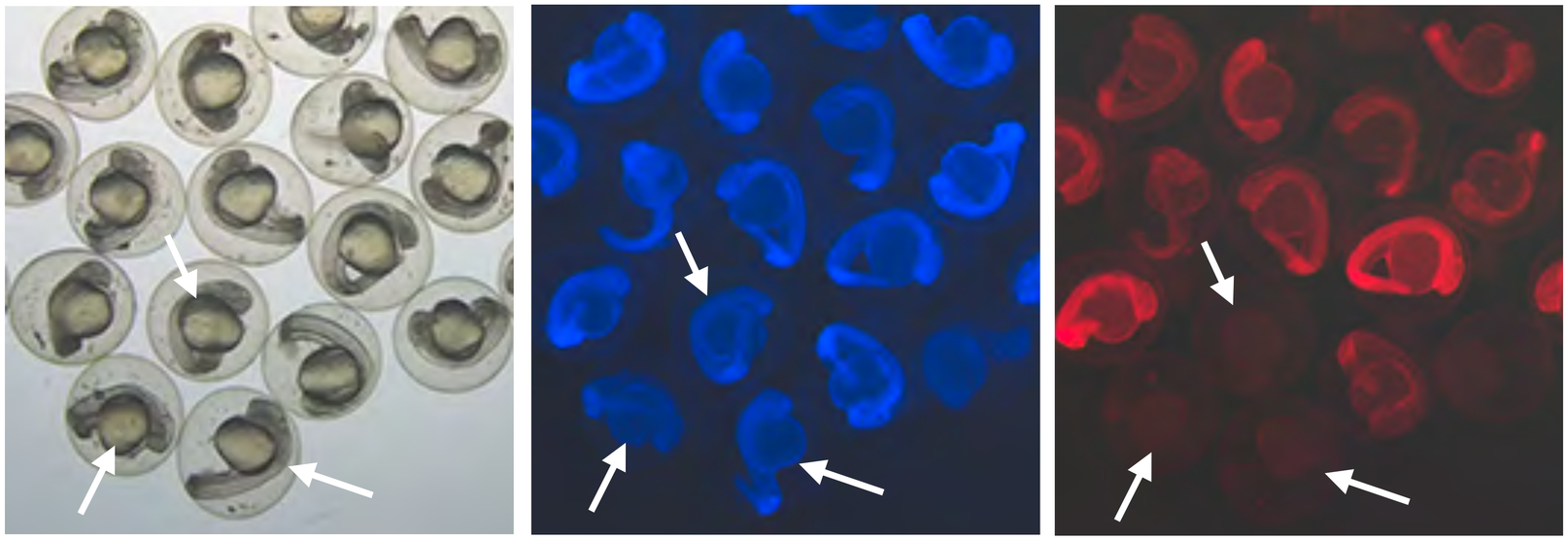
- most lines generated using Tol2 with heterologous marker gene (e.g. *cryaa:egfp*, *cmlc2:mcherry*)
- most still carrying multiple copies:
  - marker gene expression but no Cre expression
  - marker gene expression and unexpected patterns and/or mixed Cre expression patterns



A published "endothelial" CreERT line X
***Tg(ubb:loxP-cerulean-loxP;h2b-mcherry)**^{jhu66}*

Similar issues with Switch lines: Variable loxP recombination at different insertions

***Tg(actb2:loxp-CFP-loxp;mcherry)* + *creert* mRNA + 4OHT**



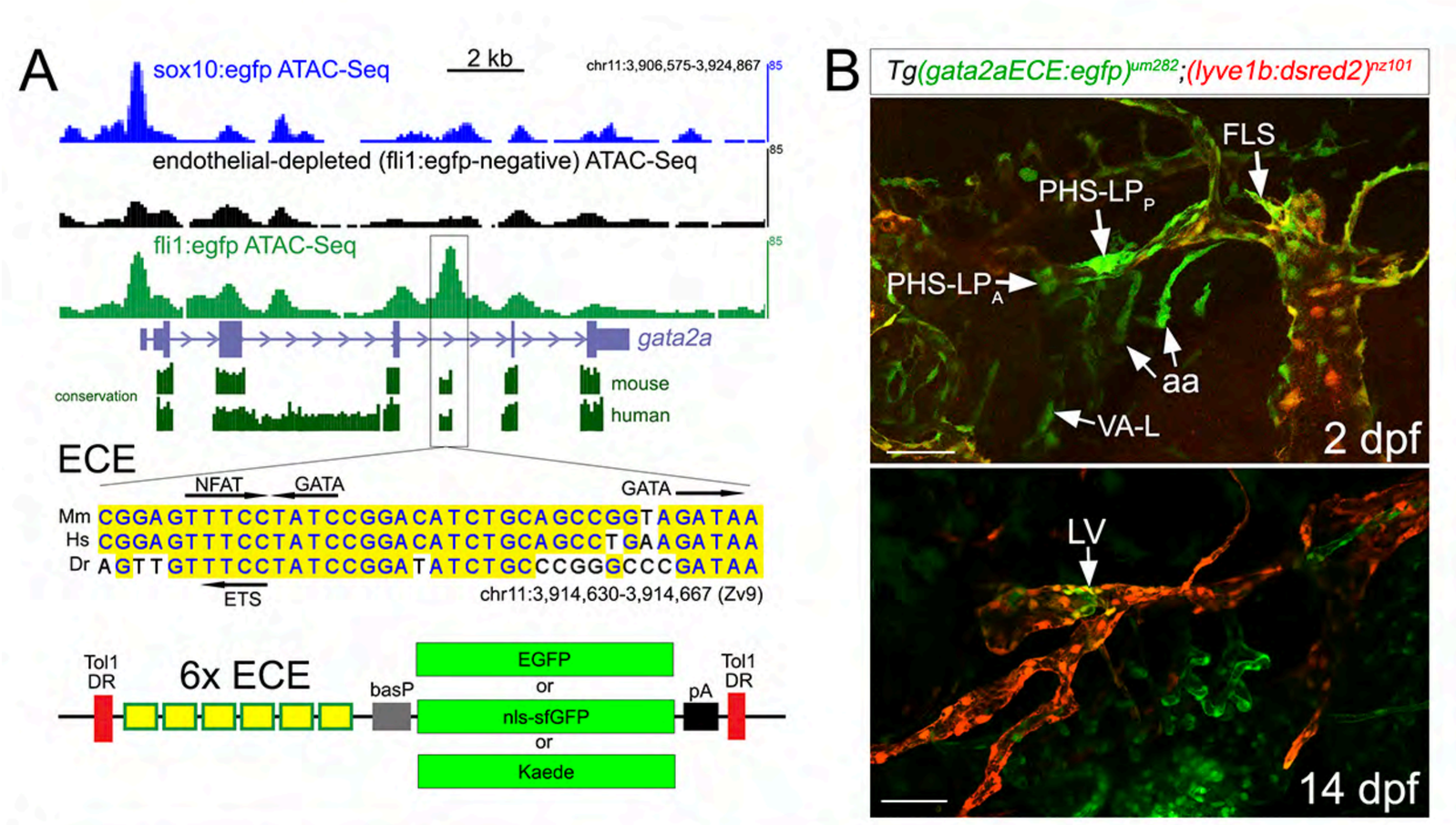
- have also seen variable expression across same cell type in different switch lines using “ubiquitous” *ubb* promoter

Solutions:

- Try to generate new CreERT lines
  - breed out to single copy and confirm specificity

- Breed out available switch lines to single copy and confirm recombination

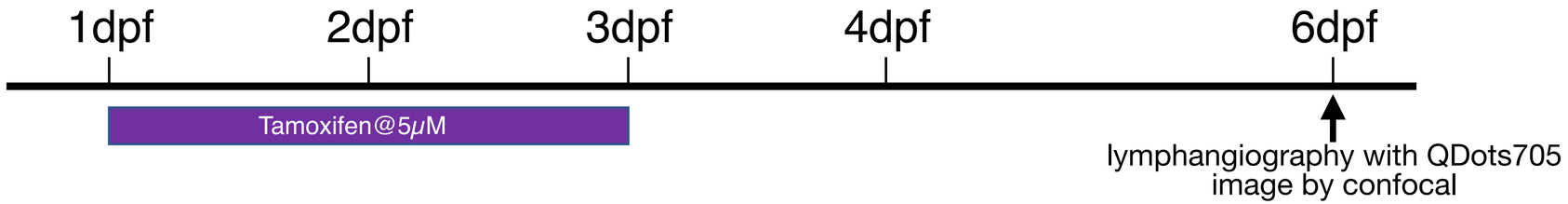
Developing a new endothelial/lymphatic CreERT line

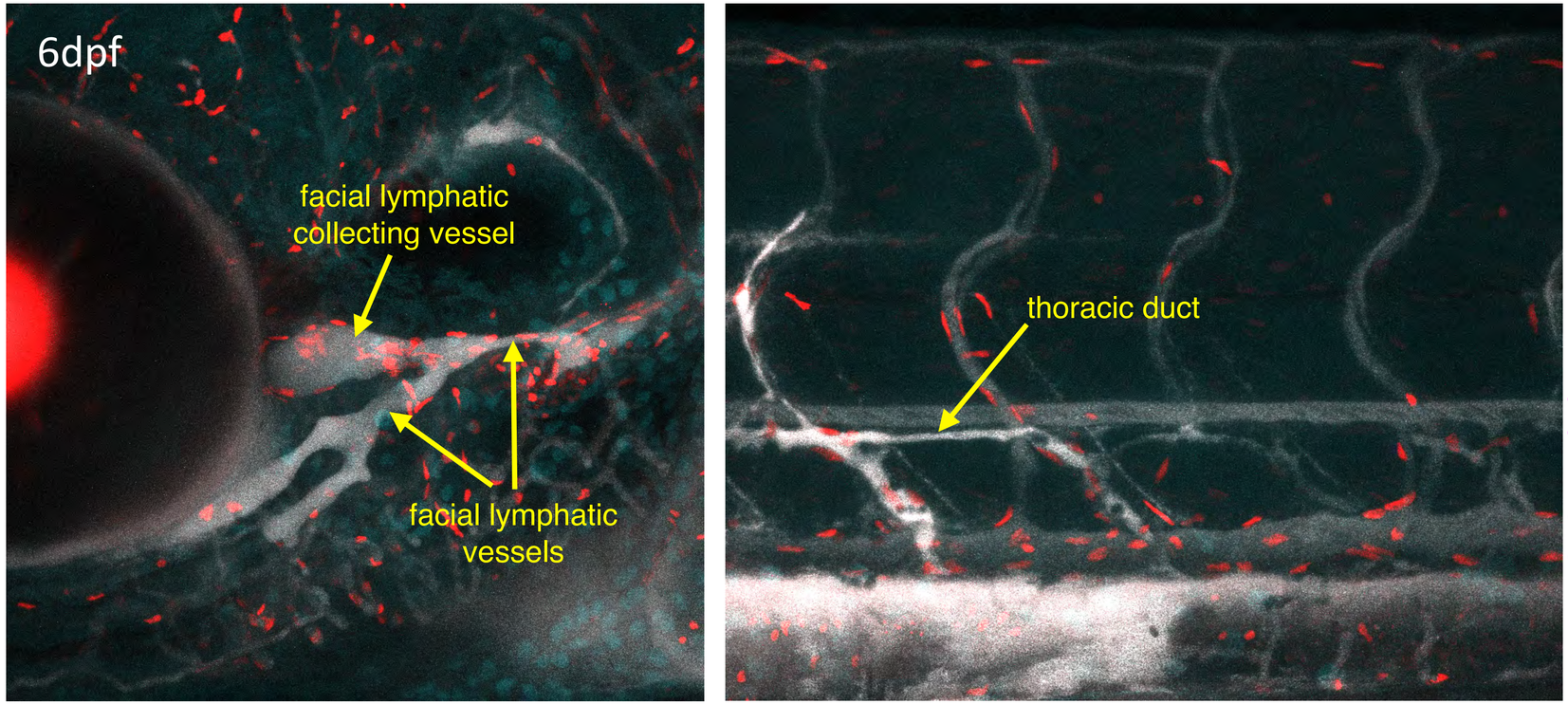


Shin et al. (2019) Developmental Cell, 51(3):374-386

Tg(gata2aECE:creERT) validation

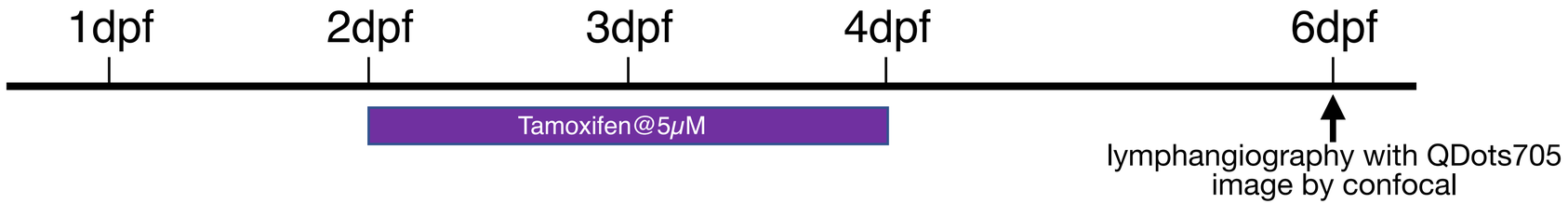
Tg(gata2aECebasp:creERT2;cryR) ♂ x *Tg(ubi:loxP:blue:nucRed)* ♀ (bred to single copy)

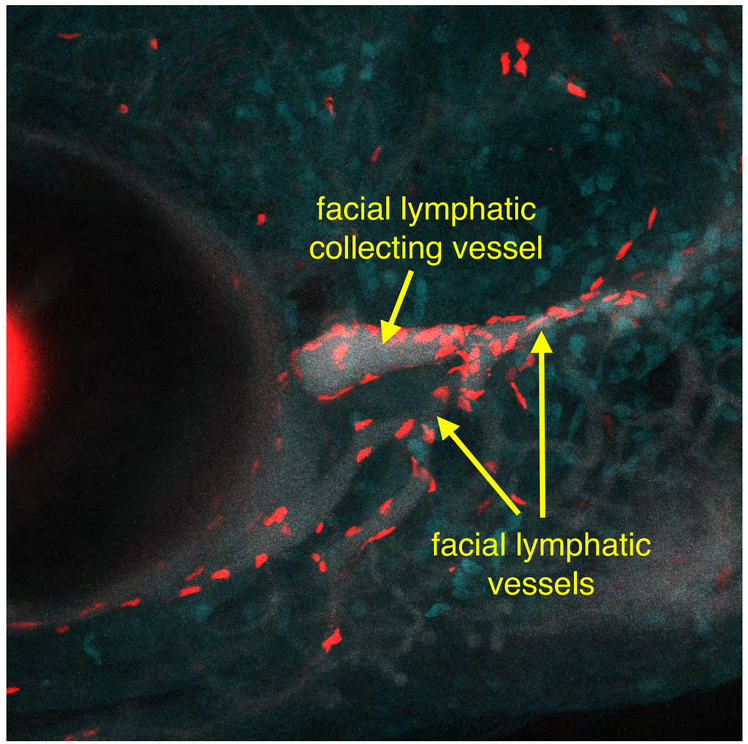


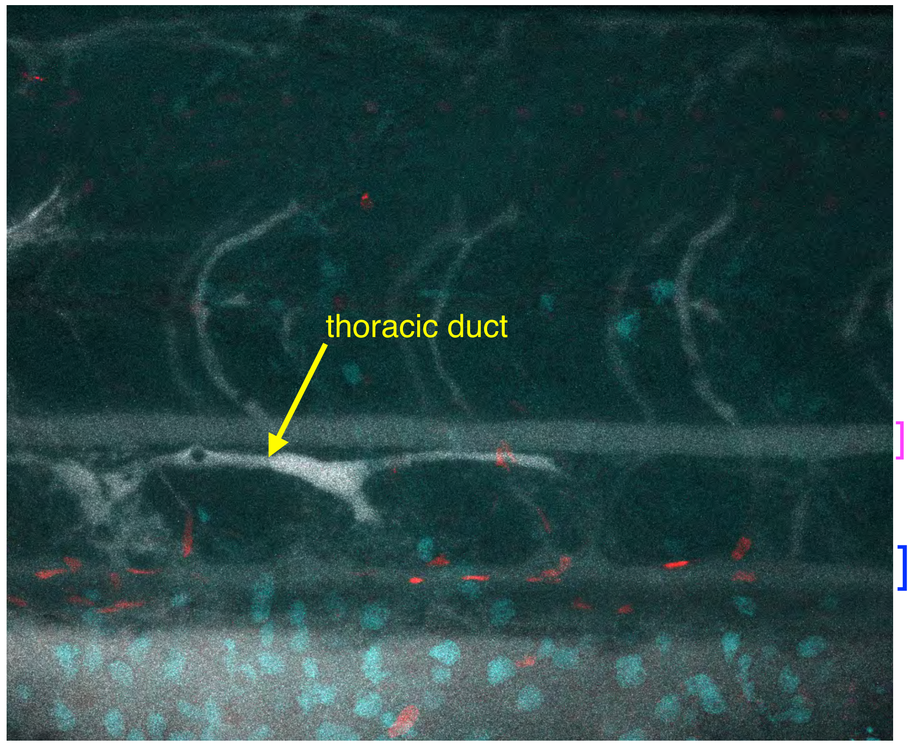


Tg(gata2aECE:creERT) validation

Tg(gata2aECebasp:creERT2;cryR) ♂ x *Tg(ubi:loxP:blue:nucRed)* ♀ (bred to single copy)





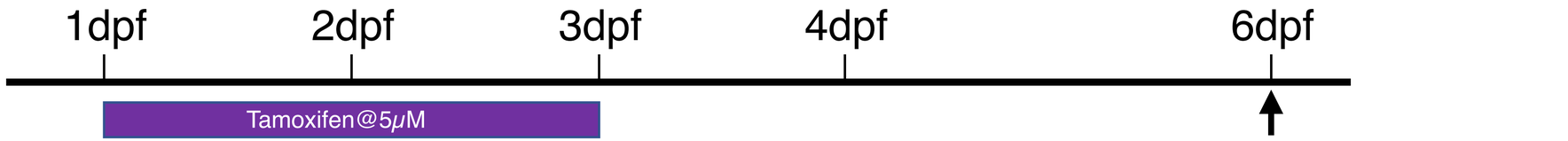


] dorsal aorta

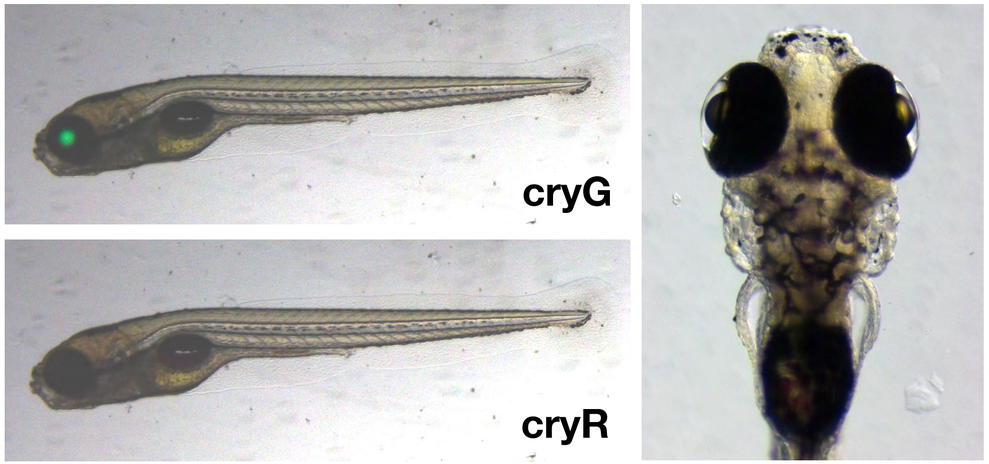
] posterior cardinal vein

Defining the developmental window for *gata2a* necessity in lymphatic function

gata2a^{loxpCryG};*Tg(gata2aECebasp:creERT2;cryR)* in-cross



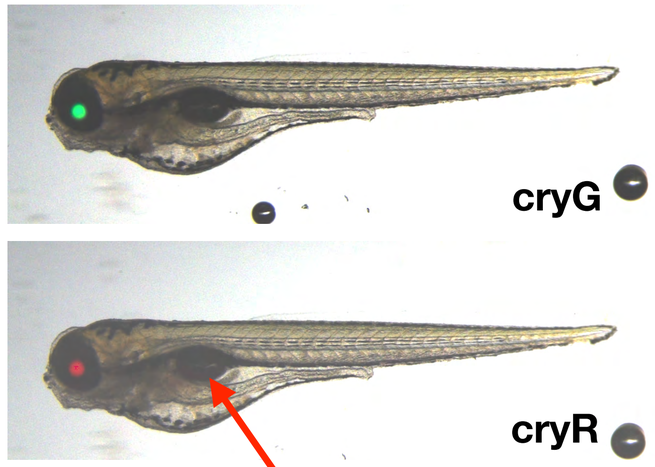
***gata2a*$^{fl/\ fl}$**
= unrecombined floxed allele



100% (n=5/5)
100% (n=8/8)

cryR-only also 100% normal

***gata2a*$^{i\Delta EC}$**
= endothelial specific knockout



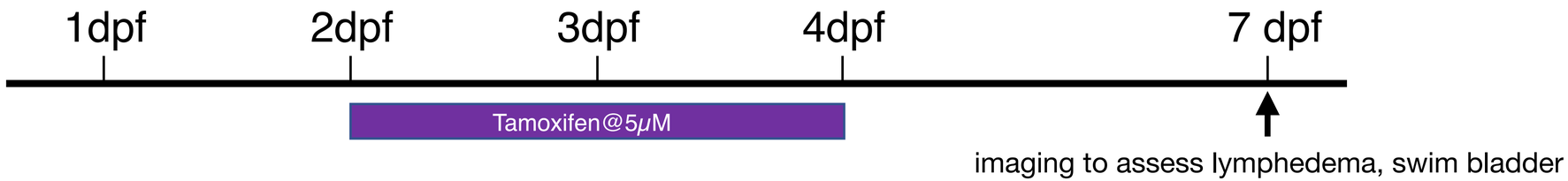
inflated swim bladder and normal circulation (unlike *gata2a*^{um27})

67% (n=14/21)
70% (n=7/10)

penetrance same as *gata2a*^{um27}

Defining the developmental window for *gata2a* necessity in lymphatic function

gata2a^{loxpCryG}*;Tg(gata2aECebasp:creERT2;cryR)* in-cross



gata2a$^{i\Delta FLEC}$

= endothelial specific knockout

10% (n=1/10)

Summary II

- developed a new endothelial-specific inducible Cre line
  - single copy, low/no background
  - used this line to demonstrate EC autonomous role for *gata2a* in lymphatic development
  - defined a potential early window for *gata2a* requirement

- identified a number of caveats with available Cre and switch lines
  - issues mostly stem from multiple Tol2 inserts
  - these issues can lead to significant problems when applying with conditional knockout alleles

Ongoing efforts

- Defining a “S.O.P.” for confirming knockout/assessing degree of knockout
  - FACS sorting switched cells, qPCR for floxed exon, qRT-PCR for target gene
  - preliminary evidence suggests not all cells have knockout
- possible difference in rates of recombination between conditional allele and switch marker line
  - developing iSure-Cre trangenic lines
    - Chacon et al, 2019 Nature Communication, 10:2262
- using our new conditional lines to do some cool experiments!!!

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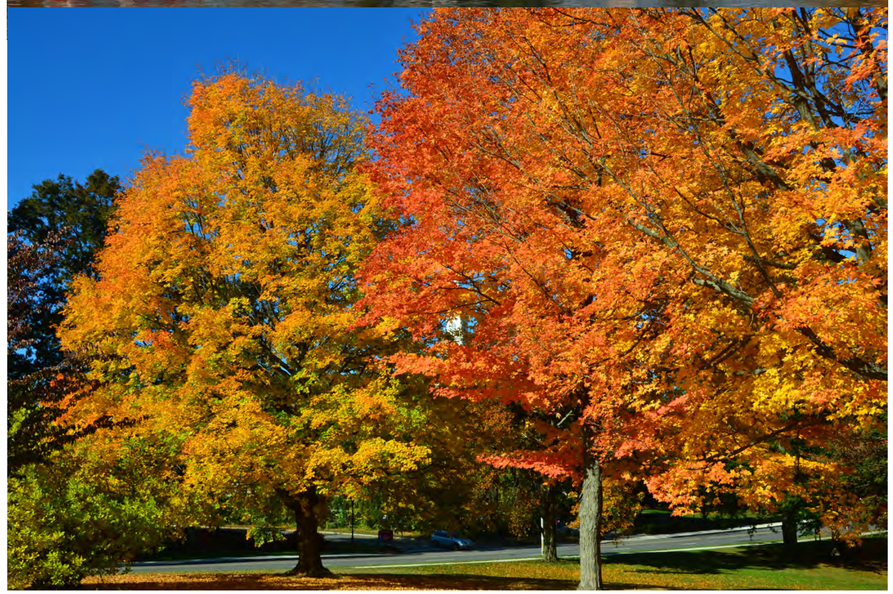
R21 OD030004



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