Conditional Mutagenesis of Zebrafish *tcf21*

Joshua Schaaf[1], Leonard Burg[1], Shannan Lowe[1], Noah Goff[1], and Darius Balciunas[1]

1. Department of Biology, Temple University

SSP SCIENCE SCHOLARS PROGRAM

College of Science and Technology TEMPLE UNIVERSITY

Undergraduate RESEARCH PROGRAM

Introduction

Many eukaryotic genes serve functions in multiple biological processes, at different times and in different tissues. These pleiotropic genes prove difficult to analyze with traditional knock-outs due to their widespread functions. Conditional mutagenesis is needed to test for the necessity of these genes; however, conditional gene inactivation has until recently only been feasible in the mouse. This led other model systems to use substitute approaches such as overexpression of dominant negatives and antisense-based tools[3]. Mutant *tcf21* is known to be embryonic lethal in zebrafish[2]. It is also expressed specifically in epicardial cells post-amputation of a section of the zebrafish heart[2], causing some speculation around its necessity for heart regeneration. Complete conditional gene inactivation has previously been successfully described in zebrafish *fleer* and *tbx20*[1], but has yet to be implemented in zebrafish *tcf21*. Described here is a straightforward approach for integrating loxP sequences at *tcf21* using CRISPR/Cas9 technology paired with oligonucleotide templates for homology directed repair.

Conditional Gene Inactivation

The loxP sequence:

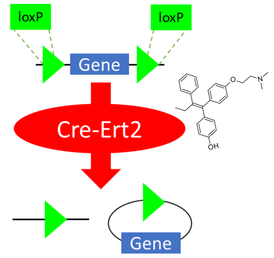
- 34 bp
- Directional
- Bacteriophage P1

5′ ataacttcgtataatgtatgctatacgaagttat 3′

Palindromic | Asymmetric (Gives Direction) | Palindromic

Cre-loxP Interaction:

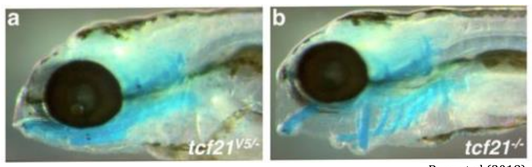
- Cre-Ert2 fusion + 4-hydroxytamoxifen



Why *tcf21*?

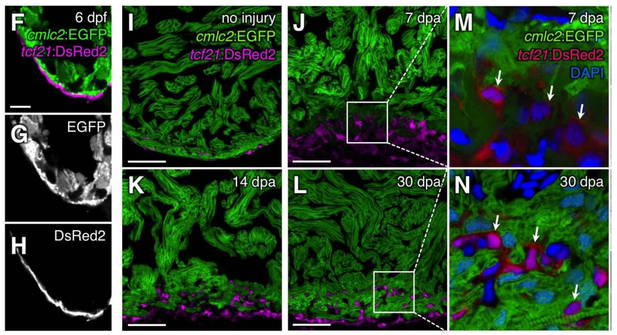
Mutant *tcf21 is* embryonic lethal & epicardial expression:

- Pharyngeal arches (Alcian Blue)



Burg et.al (2018)

- Expression in zebrafish heart pre/post amputation

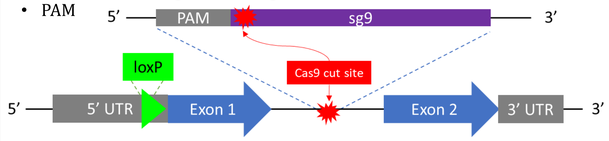


Kikuchi et.al (2011)

CRISPR/Oligo HDR

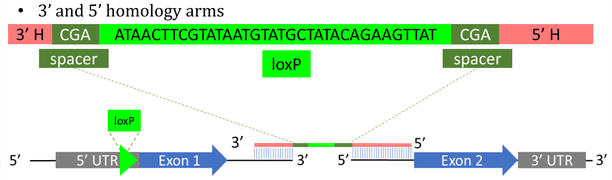
Using CRISPR/Cas9 to induce a DSB:

- Guide RNA has region homologous to desired DSB location
- PAM



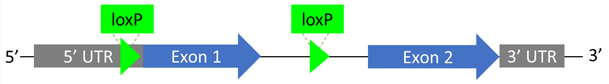
Oligonucleotide mediated homology directed repair:

- HDR vs NHEJ (majority)
- 3′ and 5′ homology arms



Expected final integration of loxP sequences on *tcf21*:

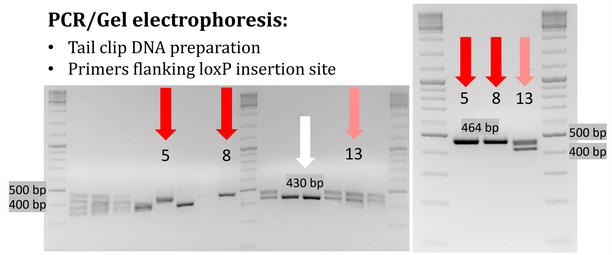
- loxP in non-coding region
- Flanking essential part of gene



Genotyping for 5' UTR loxP

PCR/Gel electrophoresis:

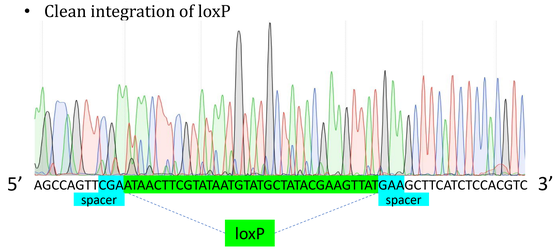
- Tail clip DNA preparation
- Primers flanking loxP insertion site



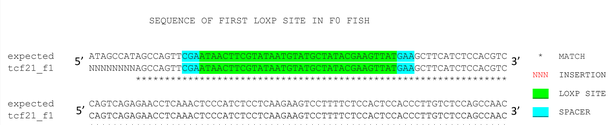
Checking for 5′ UTR loxP

Sanger sequencing of fish 5 amplicon:

- Forward *tcf21* primer
- Clean integration of loxP



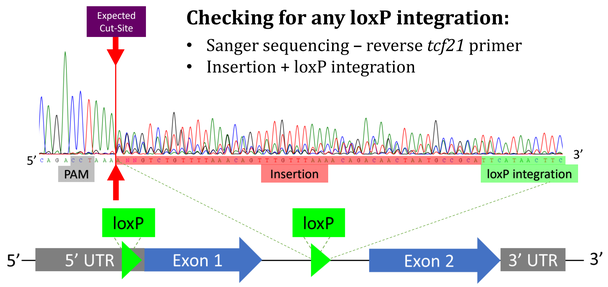
- Sequence alignment



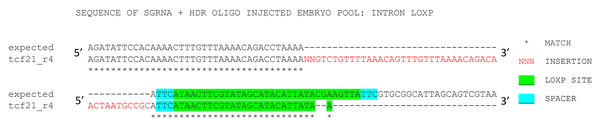
Intron loxP HDR Activity

Checking for any loxP integration:

- Sanger sequencing – reverse *tcf21* primer
- Insertion + loxP integration

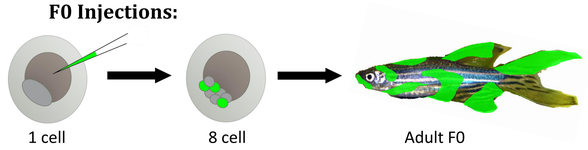


- Possible sequence

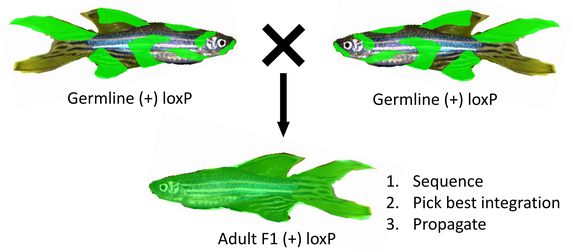


Injected Fish Mosaicism

F0 Injections:



- Cross mosaic fish positive for germline edit

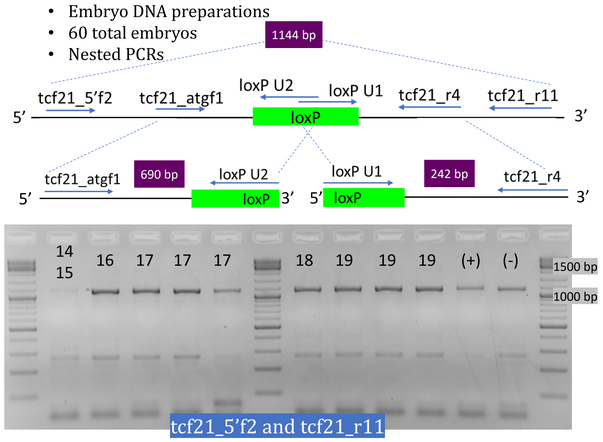


1. Sequence
2. Pick best integration
3. Propagate

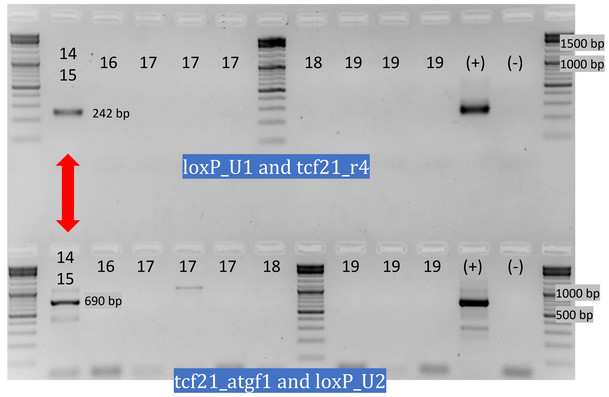
Germline Transmission 1

Injected F0 fish:

- Embryo DNA preparations
- 60 total embryos
- Nested PCRs



Germline Transmission 2

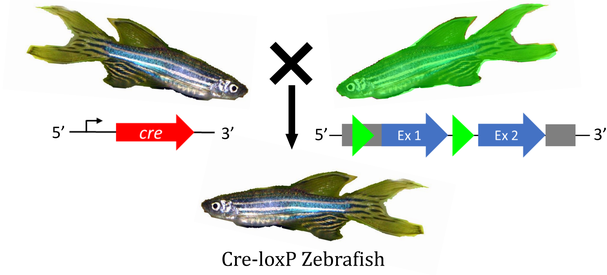


14 & 15 are being in-crossed, and the DNA is being sequenced.

Creating Conditional Mutant

Cross 'floxed' *tcf21* fish with specific *Cre* driver:

- Cre-Ert2: inactive until 4-hydroxytamoxifen is present



Cells with active Cre-Recombinase: 5′ Ex 2 3′

1. Transcribed
2. Translated
3. Protein Degraded

Conclusions

- Confirmed successful integration of 5′ UTR loxP site in *tcf21*
  - Sanger sequencing/alignment
  - Created a line of zebrafish homozygous for 5′ UTR loxP
- Confirmed oligonucleotide mediated HDR with sanger sequencing for intron loxP
- Genotyped injected zebrafish homozygous for 5′ UTR loxP
  - ~19 f0 injected fish genotyped by germline transmission
  - 1 pair f0 fish positive for germline transmission of the intron loxP

Future Work

- More f0 fish are ready to be screened
  - Continuation of germline transmission genotyping
- In-cross and sequence DNA from 14 & 15 progeny
- Cross fully 'floxed' *tcf21* zebrafish with a zebrafish with *Cre*-Ert2 driver
  - Observe phenotype(s) of conditionally knocked-out *tcf21* post cardiac injury
  - Genotype conditional mutants post tamoxifen treatment

Literature Cited

1. Burg L, Palmer N, Kikhi K, Miroshnik ES, Rueckert H, Gaddy E, et al. (2018) Conditional mutagenesis by oligonucleotide-mediated integration of loxP sites in zebrafish. PLoSGenet 14(11): e1007754.https://doi.org/10.1371/journal.pgen.1007754
2. Kikuchi, K., et al. "tcf21+ Epicardial Cells Adopt Non-Myocardial Fates during Zebrafish Heart Development and Regeneration." *Development*, vol. 138, no. 14, 2011, pp. 2895–2902., doi:10.1242/dev.067041.
3. Aidas N and Stephen C. Ekker. "Effective Targeted Gene 'Knockdown' in Zebrafish." *Nature Genetics*, vol. 26, no. 2, 2000, pp. 216–220., doi:10.1038/79951.