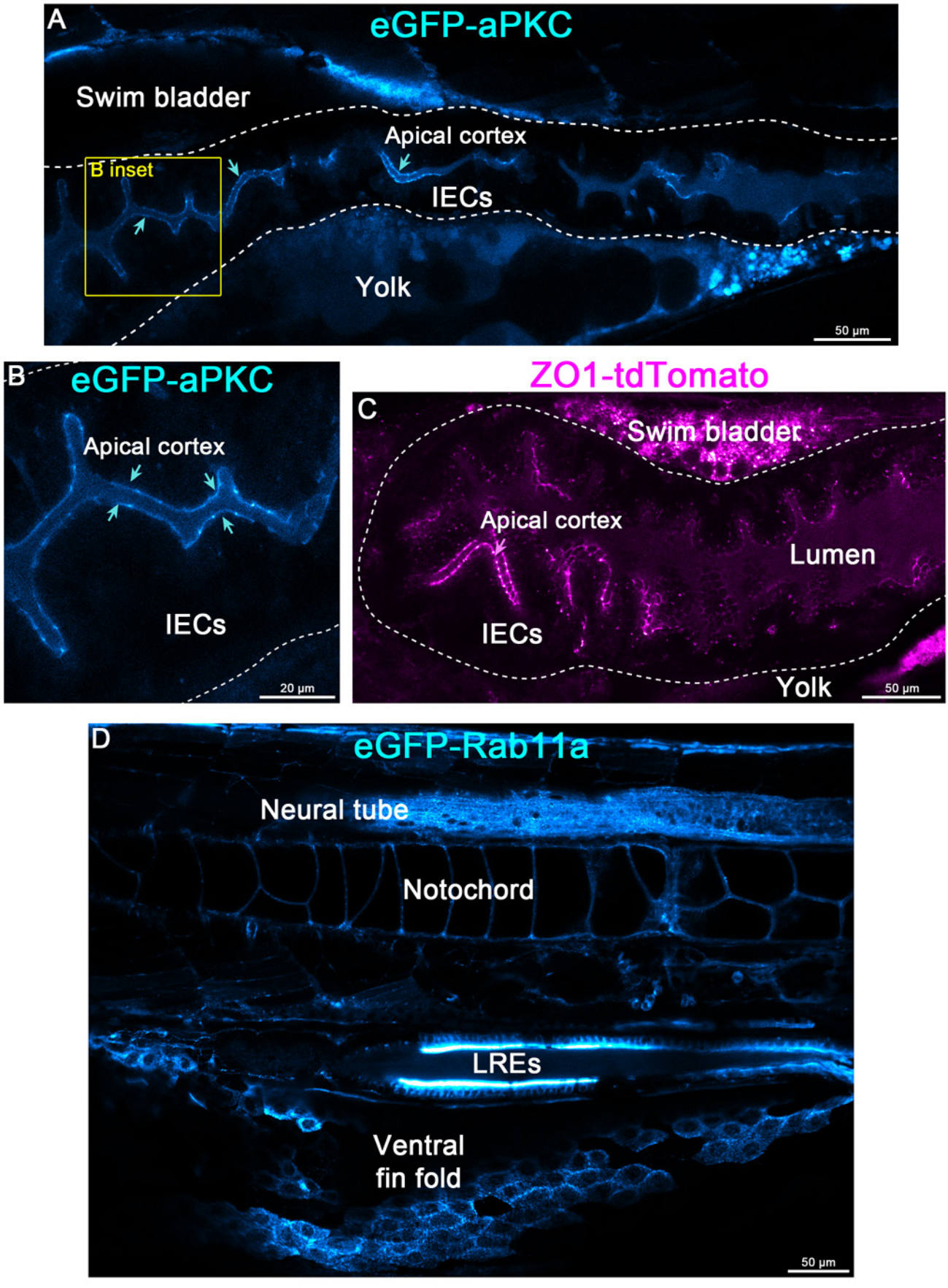


Fig. S1. Examples of mosaicism in injected embryos (F0s). (A-B) Live imaging of a 5 dpf eGFP-aPKC (encoded by *prkci/has*) F0 larva. Arrows point to the apical cortex. Scale bars are 50 µm **(A)** and 20 µm **(B)**. **(C)** Live imaging of a 5 dpf ZO1-tdTomato (encoded by *tjp1a*) F0 larva. Arrow points to the apical cortex. Scale bars is 50 µm. The dotted line marks the intestinal epithelium. **(D)** Live imaging of a 5 dpf eGFP-Rab11a F0 larva. The broad spatial expression patterns and high expression levels of eGFP-Rab11a allow for simple visual screening to test PCR donor variants to optimize endogenous tagging. Scale bars are 50 µm. Images were pseudo-colored with the ImageJ/FIJI Cyan Hot or Magenta Hot LUT to enhance visualization of lower expressing cell-types.

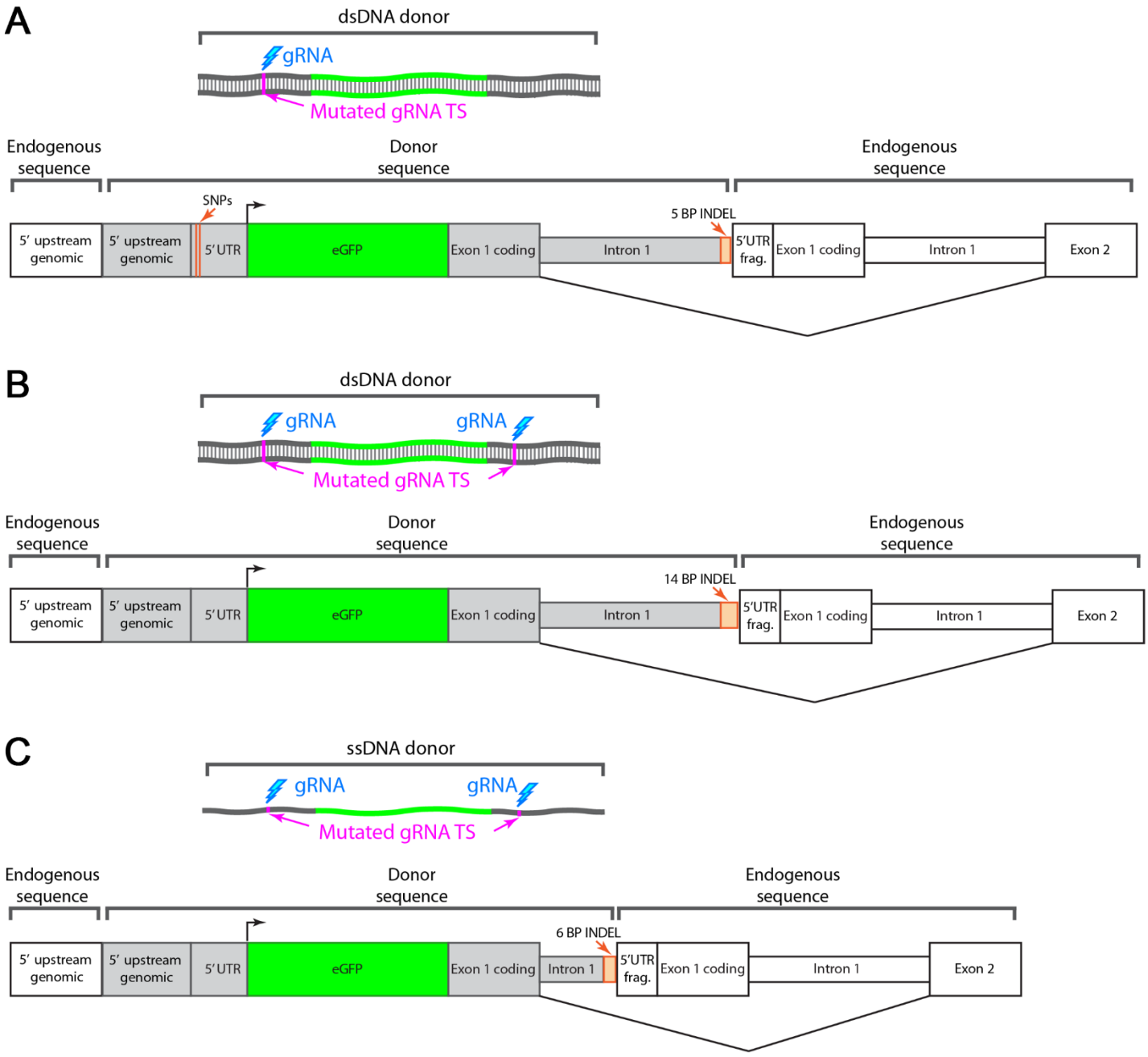
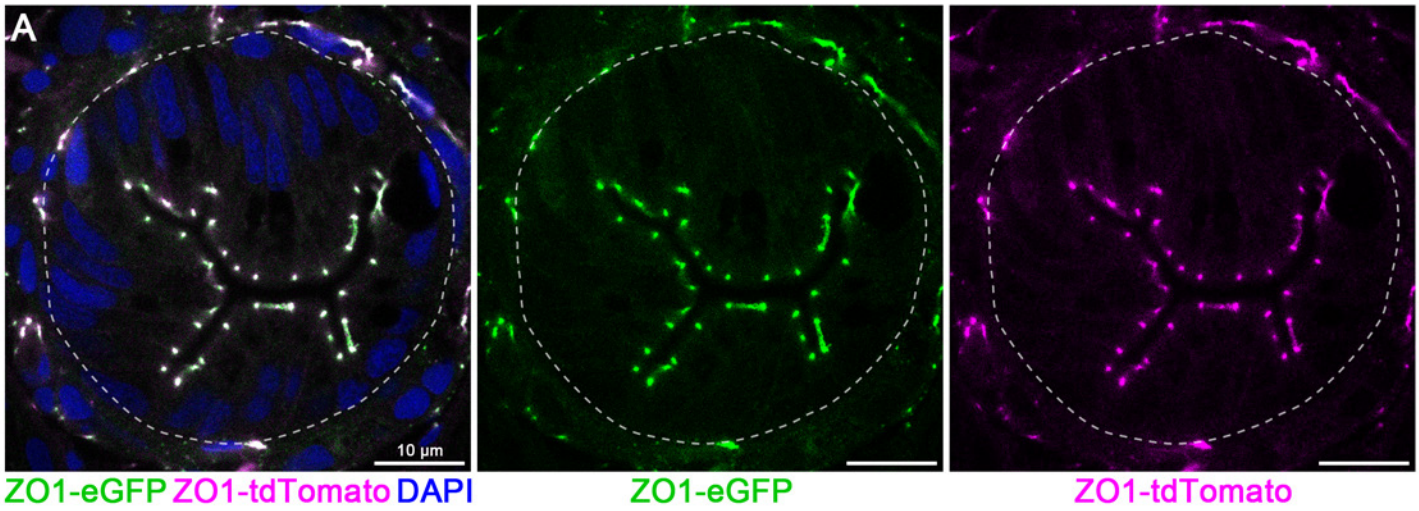
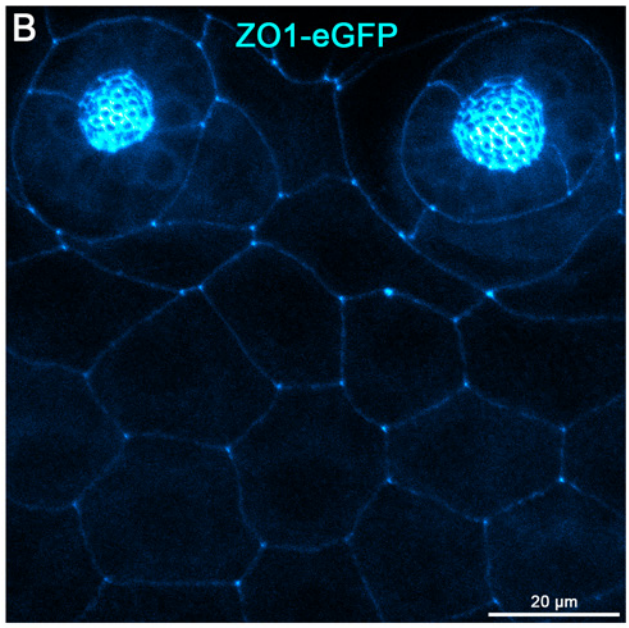


Fig. S2. Comparison of *eGFP-rab11a* donor integration sites in independently generated alleles. (A-C) 1-cell stage embryos were injected with *rab11a* KI cocktails using different donor-types and/or gRNAs targeting different genomic sites. Larvae showing mosaic eGFP-Rab11a expression were raised and outcrossed to WT fish, and the genomic integration site was analyzed by sequencing of 3 independently generated alleles. INDELs and SNPs present in the donor-derived sequence or at the integration boundaries are highlighted in orange. The intron 1 portion of the donor-derived sequence for panel C was 389 bp shorter than those of panels A–B (491 bp versus 102 bp). No differences in eGFP-Rab11a expression were observed between the 3 alleles. *TgKI(eGFP-rab11a)*^{pd1244}, which was used for all imaging experiments in this study, derives from the allele in panel C. Annotated sequence files for the 3 alleles are provided in Supplementary Files 5, 7, and 8. Noncoding variants present up- and downstream of the integration sites are not depicted and result from WT fish of the EK or AB/TL background being used to generate the different alleles.





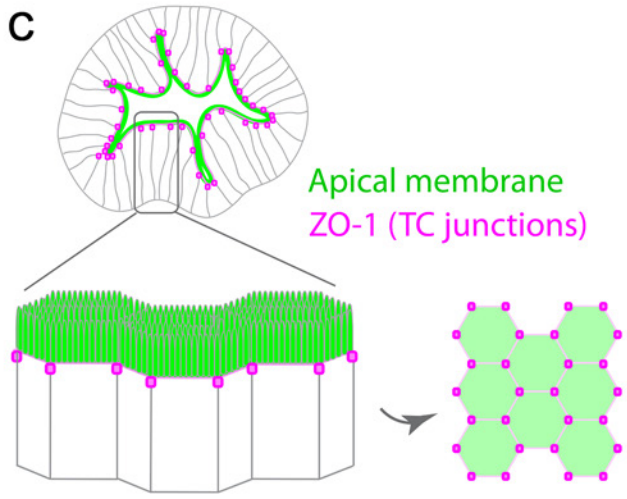


Fig. S3. Colocalization of compound heterozygous tagged ZO1 (*tjp1a*) alleles and localization to tricellular junctions. (A) Transverse sections of a *TgKI(tjp1a-eGFP)*^{pd1252};*TgKI(tjp1a-tdTomato)*^{pd1224} compound heterozygous larva at 5 dpf. Scale bars are 10 µm. **(B)** Live imaging of the epidermis of a 5 dpf *TgKI(tjp1a-eGFP)*^{pd1252} larva. Scale bars is 20 µm. **(C)** Schematic illustrating the enriched localization of endogenously tagged ZO1 to tricellular (TC) junctions.

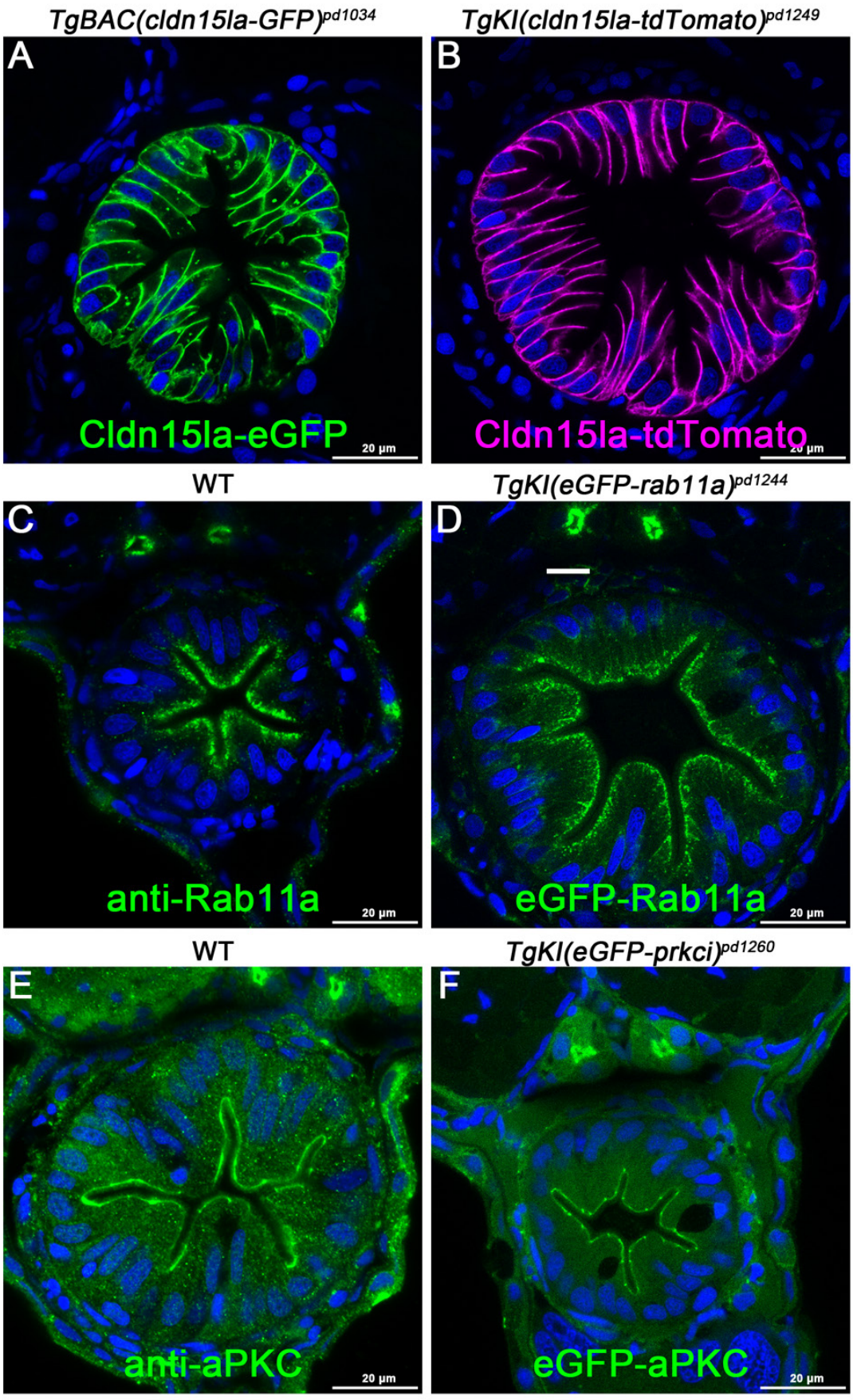
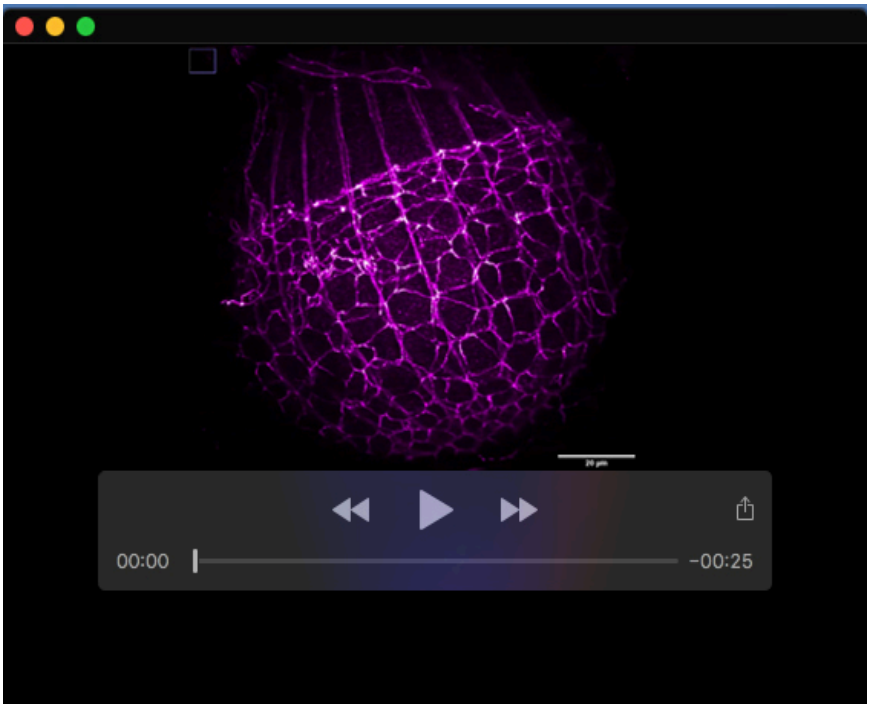
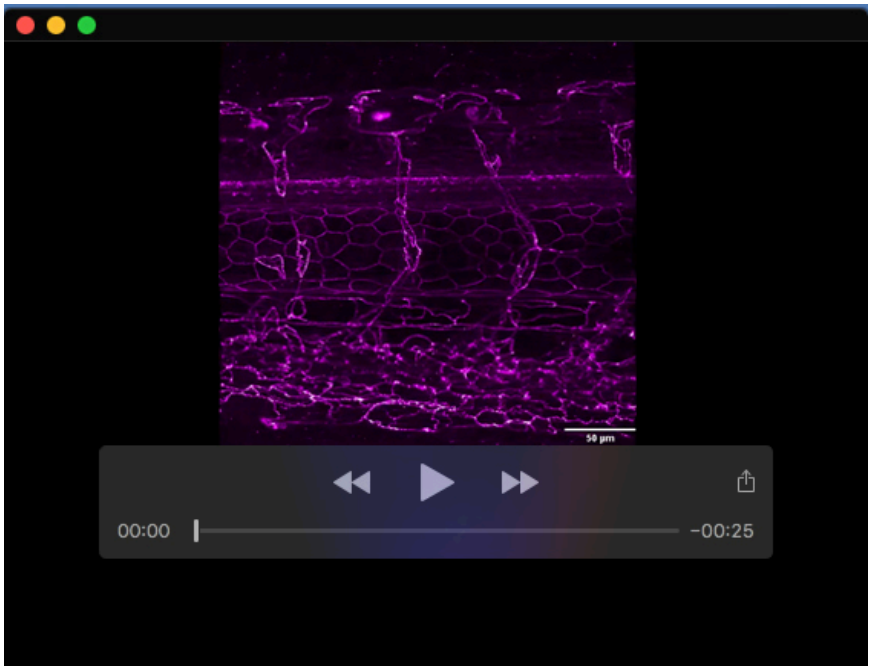


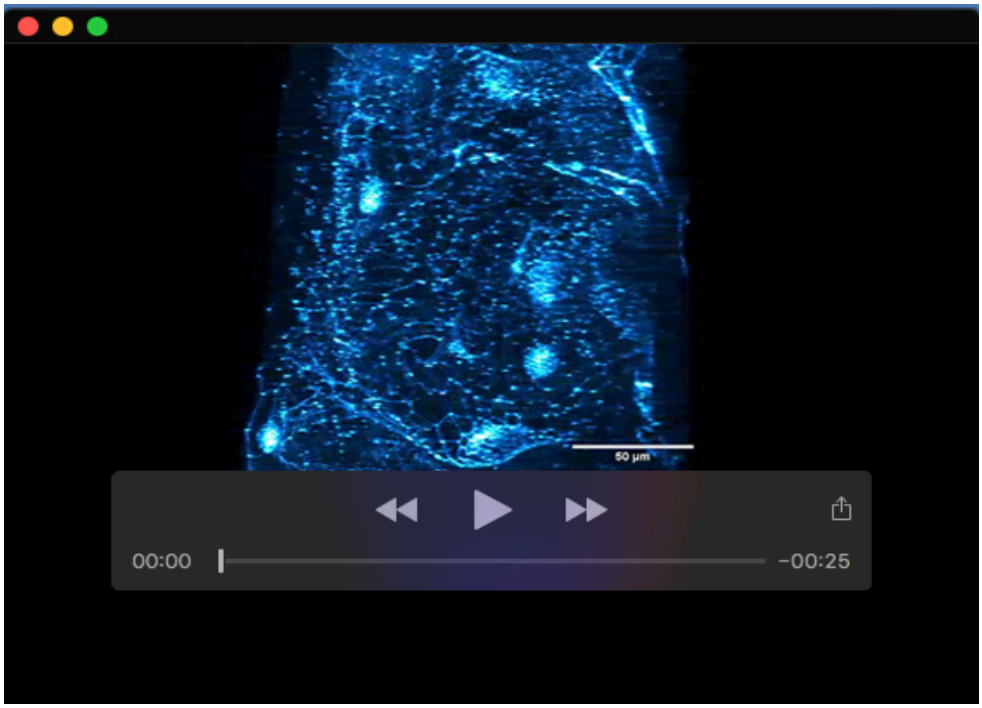
Fig. S4. Comparison of endogenously tagged proteins with transgene or antibody labeling. (A-B) Transverse section of the midgut of BAC transgenic line *TgBAC(cldn15la-GFP)*^{pd1034} (Alvers et al., 2014) and KI line *TgKI(cldn15la-tdTomato)*^{pd1249}. Both proteins are localized to the basolateral membrane, while the BAC transgenic line also shows sparse cytoplasmic labeling. **(C-D)** WT larvae stained with anti-Rab11a antibody (Cell Signaling 5589S) (Levic et al., 2020) resemble the KI line *TgKI(eGFP-rab11a)*^{pd1244}, with prominent labeling in the apical cytoplasm of IECs and the PN. **(E-F)** WT larvae stained with anti-aPKC antibody (Santa Cruz sc-216) (Bagnat et al., 2007) resemble the KI line *TgKI(eGFP-prkci)*^{pd1260}, with enriched labeling of the apical cortex of IECs and PN. DAPI is shown in blue for all panels.



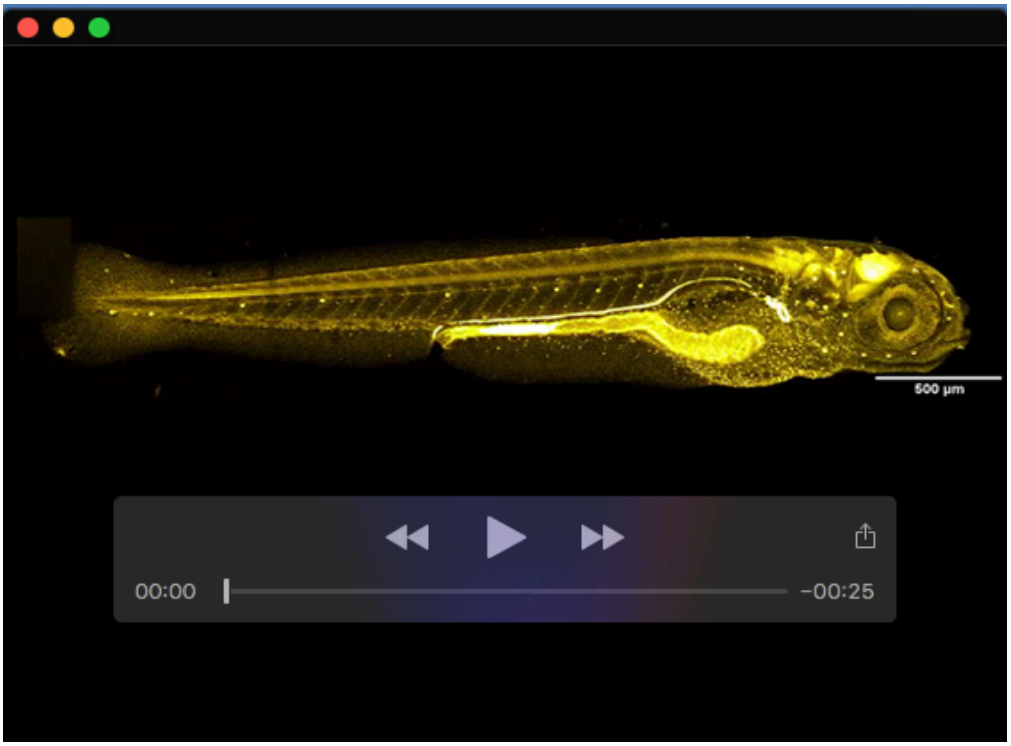
Movie 1. Rotating 3D reconstruction of ZO1-tdTomato expression in the lens. Data are from Fig. 2B.



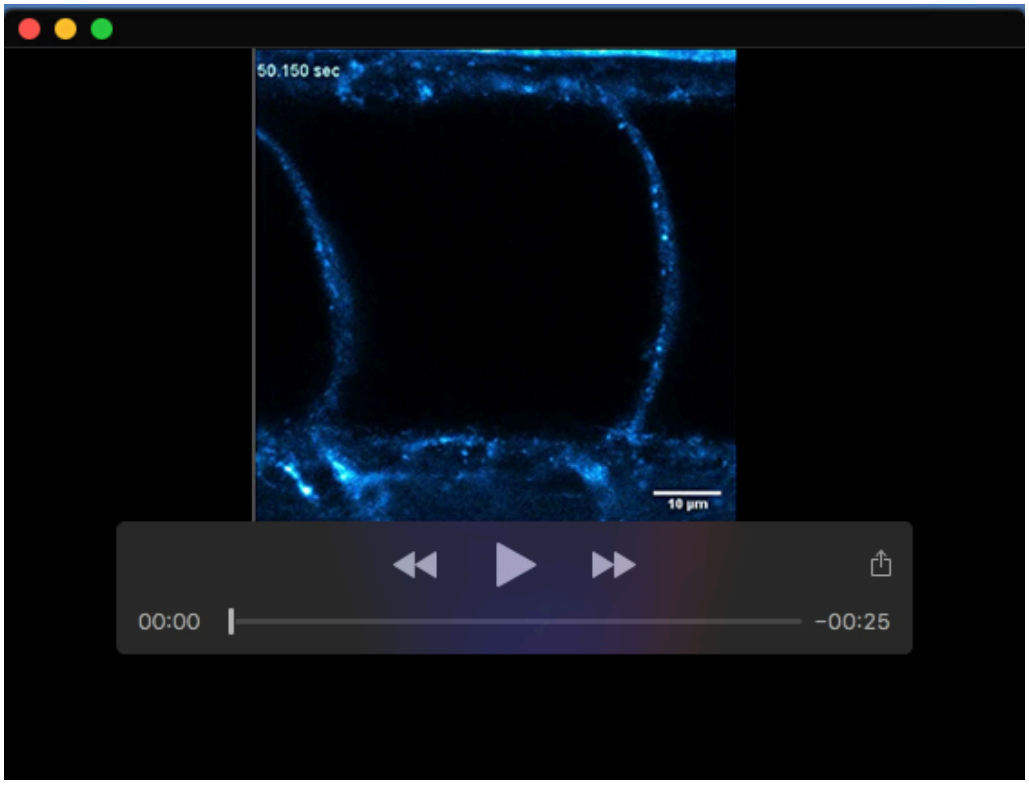
Movie 2. Rotating 3D reconstruction of ZO1-tdTomato expression in the embryonic trunk. Data are from Fig. 2C.



Movie 3. Rotating 3D reconstruction of ZO1-tdTomato expression in the otic capsule. Data are from Fig. 2D.



Movie 4. **Rotating 3D reconstruction eGFP-Rab11a expression in 5 dpf whole larvae.** Data are from Fig. 3A.



Movie 5. Live imaging of eGFP-Rab11a vesicle dynamics in notochord vacuole cells. Data are related to Fig. 3C. Data were acquired at 1 frame every 3 seconds.

Supplementary file 1. Sequence file for the genomic integration site of *TgKI(tjp1a-tdTomato)*^{pd1224}.

Click here to download Supplementary file 1

Supplementary file 2. Sequence file for the genomic integration site of *TgKI(tjp1a-eGFP)*^{pd1252}.

Click here to download Supplementary file 2

Supplementary file 3. Sequence file for the genomic integration site of *TgKI(cldn15la-tdTomato)*^{pd1249}.

Click here to download Supplementary file 3

Supplementary file 4. Sequence file for the genomic integration site of *TgKI(itgb1b-tdTomato)*^{sk108}.

Click here to download Supplementary file 4

Supplementary file 5. Sequence file for the genomic integration site of *TgKI(eGFP-rab11a)*^{pd1244}.

Click here to download Supplementary file 5

Supplementary file 6. Sequence file for the genomic integration site of *TgKI(eGFP-prkci)*^{pd1260}.

Click here to download Supplementary file 6

Supplementary file 7. Sequence file for the genomic integration site of the *eGFP-rab11a* allele presented in Fig. S2A.

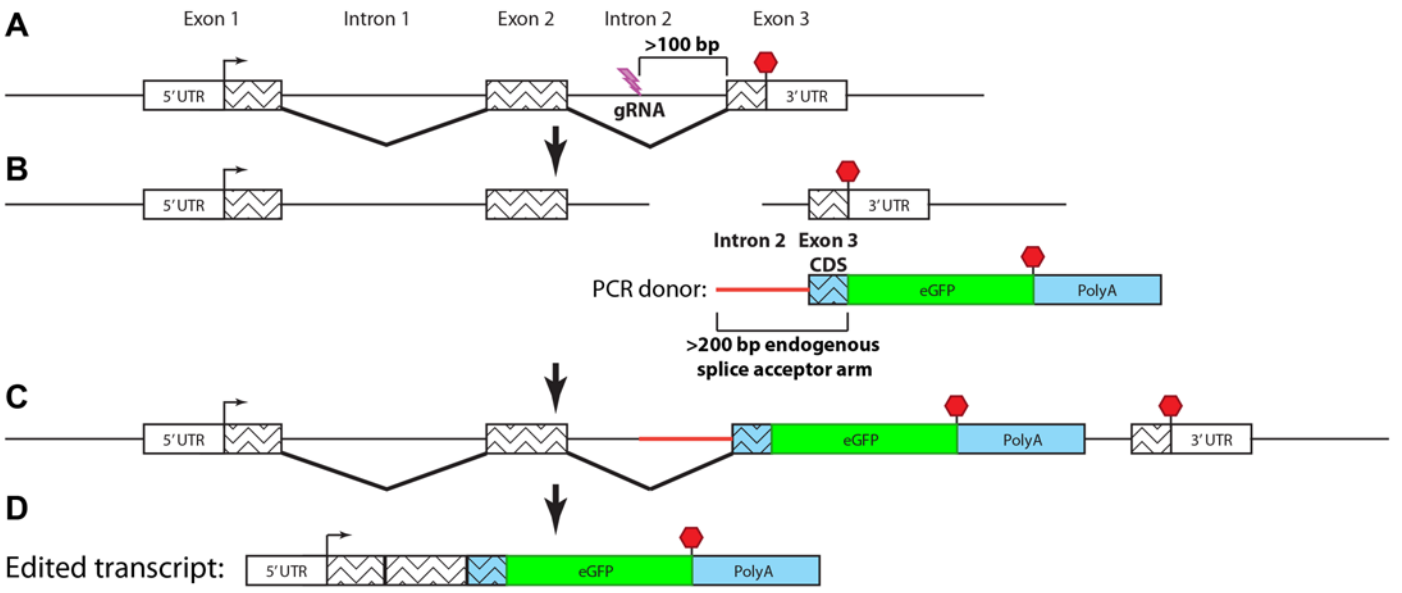
Click here to download Supplementary file 7

Supplementary file 8. Sequence file for the genomic integration site of the *eGFP-rab11a* allele presented in Fig. S2B.

Click here to download Supplementary file 8

Supplementary Materials and Methods

Part 1: C-terminal tagging using 5’ splice acceptor elements



1. Choose a target site
    - Examine the intron that immediately precedes the exon containing the stop codon for your gene of interest (GOI). An ideal candidate intron will be ≥300 bp.
    - Using CRISPRscan or a similar tool, try to identify a CRISPR target site in the middle of the intron, ideally ≥50 bp from the 5’ end and ≥100 bp from the 3’ end of the intron. Before proceeding, sequence the target site you plan to use to make sure your population of fish matches the reference sequence. Sequence variants are prevalent in non-coding regions.
    - If the last intron of your GOI doesn’t meet these criteria, then examine the next preceding intron and so on until you find a suitable target site. We have successfully targeted introns in this way for C-terminal tagging (targeting several introns away from the last exon and providing all of the intervening intron-exon sequence in the donor).
    - Design oligos for your target site and synthesize gRNAs, or simply order synthetic crRNA/tracrRNA. Appendix II has a protocol for synthesizing gRNAs. Consider making at least 2 different gRNAs for each target GOI. We often co-inject 2 gRNAs that target the intron within about 50-100 bp to increase the probability of generating a dsDNA break while also not deleting a large intron fragment.

2. Design and construct a donor plasmid.
    - This approach uses a 5’ endogenous splice acceptor element to mediate splicing of the fluorescent protein coding sequence into the target mRNA. We have generated a series of donor vectors that can be used for cloning (see Appendix I). They contain a gBait target site (an efficient gRNA target site against GFP that is not present in the WT genome), a multiple cloning site (MCS) followed by a fluorescent protein or other cDNA such as p2a-QF2, a stop codon,

another MCS, and the zebrafish *ubb* polyadenylation sequence. Note that the gBait target site is only needed if you plan to inject a plasmid donor instead of a PCR donor. Although we incorporated this into the donor vectors, we rarely use the gBait because we inject PCR donors. Although our donor vectors contain the *ubb* poly-adenylation site, it is possible to clone the 3'UTR of your GOI into the donor vector using the second MCS. Use one of our p2A-FP vectors if you want to make reporter lines.

- Using genomic DNA, clone a fragment of your GOI from the final ~300 bp (or greater) of the intron you are targeting to the end of the coding sequence in the final exon but without the stop codon. As mentioned above, this fragment can encompass multiple introns and exons if necessary. Clone this fragment into the donor vector above so that the coding sequence of the gene is in frame with the fluorescent protein sequence. Ensure that the beginning of the fragment you clone is intron sequence and not a previous exon (the donor can encompass multiple introns-exons, but it should begin with intron sequence)
- This is optional, but if your gRNA target site is in your donor plasmid, mutating it is recommended. This seems to enhance efficiency of KI; however we have generated several stabile KI lines even when not mutating the gRNA target site in the donor. If wanted, use Q5 site-directed mutagenesis kit to introduce several mismatches, at least 3 in the core sequence (the 12 bp before the PAM site) and 1 in the PAM site. More than 2 mismatches in the core sequence was shown to abolish targeting efficiency (PMID: 23873081). NEBasechanger can be used to design the oligos.

3. Generate a PCR donor from your donor plasmid
    - Set up the following PCR using the primers F1-pUC19-intron-KI, 5'-GCGATTAAGTTGGGTAACGC-3' and R1-pUC19-intron-KI, 5'- TCCGGCTCGTATGTTGTGTG-3'. These primers will work for all of our C-terminal donor vectors.

– Plasmid donor (10 ng/uL)		1 uL
– Q5 polymerase buffer (5x)		5 uL
– dNTP mix (10 mM)		0.5 uL
– Forward primer (10 uM)		1.25 uL
– Reverse primer (10 uM)		1.25 uL
– Water		15.75 uL
– Q5 Polymerase		0.25 uL
– Total volume		25 uL (if needed, scale up and divide into multiple tubes)
– Anneal:	64°C	0:20
– Extension:	72°C	2:00
– Cycles	35	

    - After PCR, run ~5 uL on a 1% TBE agarose gel to check the product. For unknown reasons, we sometimes observe a non-specific faint band at 1700 bp even after testing 4 different primer

combinations for the tdTomato donor plasmid. This second non-specific band has not interfered with efficiency of knock-in.

- Use the remaining 20 uL to purify the PCR donor using the Nucleospin Gel and PCR Clean-up kit (Machery-Nagel). It is not necessary to gel purify the band (just do PCR clean-up).
  - Add 2x volume of Buffer NT1, mix, and then transfer to a column
  - Centrifuge at 11K x g for 30 seconds and then discard flow through
  - Add 600 uL of Buffer NT3 to the column, centrifuge as before, and discard flow through
  - Repeat the NT3 wash step a second time
  - Repeat the NT3 wash step a third time
  - Pipette out and discard the flow through
  - Centrifuge the dry column at 11K x g for 30 seconds
  - Transfer the column to a clean microcentrifuge tube
  - Place the column/tube to a heat block at 60°C and let sit for 10 minutes
  - Add 20 uL of RNase-free water to the column and let sit for 1 minute
  - Centrifuge the column at 11K x g for 1 minute
  - The expected concentration is approximately 50-100 ng/uL x 20 uL

4. Injections and screening

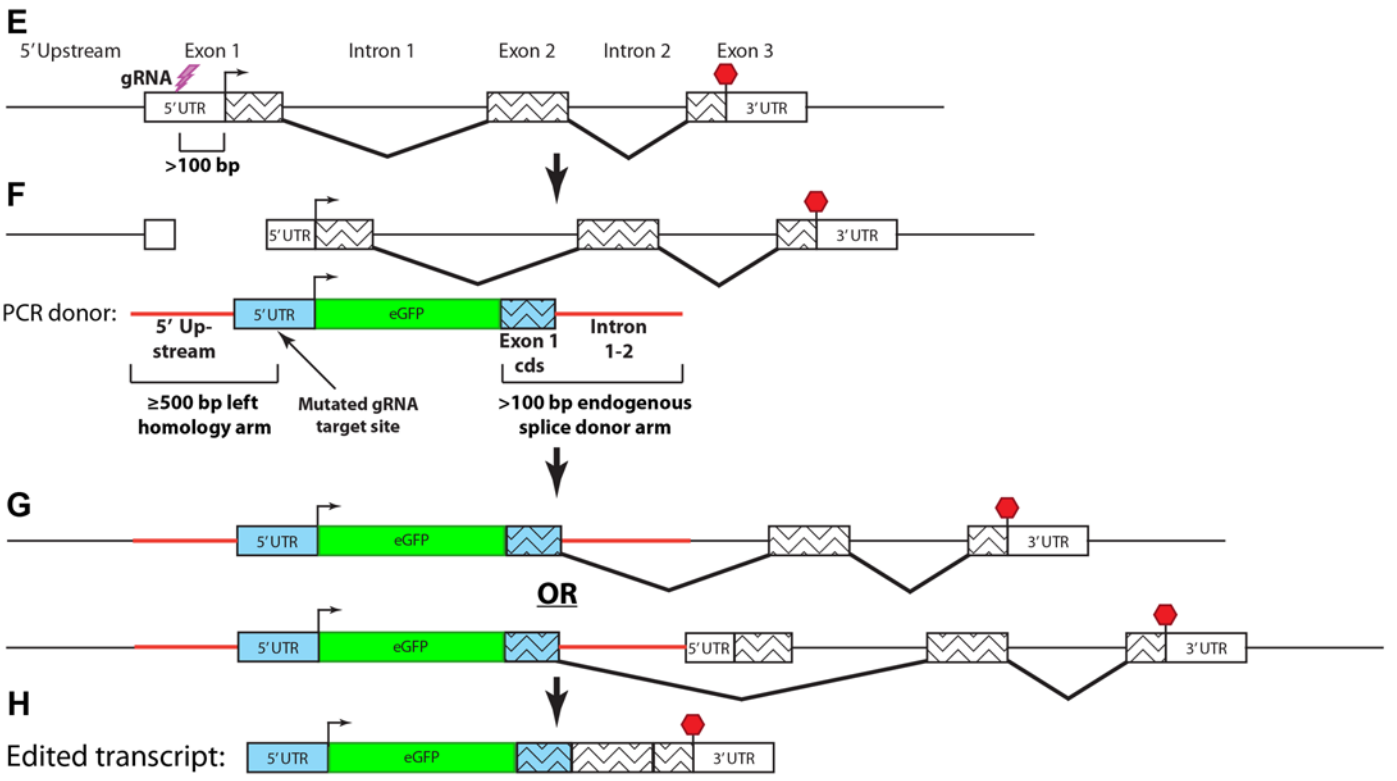
- Prepare the following injection working mix (if you are using a crRNA, see Appendix III):
  - PCR donor 5-10 ng/uL
  - Target site gRNA 1 35-50 ng/uL
  - Target site gRNA 2 35-50 ng/uL (optional; only 1 gRNA is required)
  - Phenol Red 0.05%
  - Cas9-NLS protein 300-500 ng/uL (PNABio catalog no. CP01)
- Inject as many embryos as you can manage (n=500 that are healthy at the end of day 0 is a good starting point). Inject only early 1-cell stage embryos as soon as possible after fertilization.
- Screen the embryos for fluorescence starting at 1 dpf or at a stage when you know your GOI will be most highly expressed. This is the most time-consuming and tedious part of this protocol as many proteins are present at much lower levels than we are accustomed to viewing by overexpression in transgenic lines. If you suspect expression with a dissecting microscope, then confirm this using live confocal imaging.
- We have observed F0 efficiency rates ranging from 0.5-2.5% depending on the gene.
- If you can't see any expression, try these steps:
  - first check if your gRNA gives good activity using a heteroduplex mobility shift assay as described in Appendix II.
  - If your gRNA seems to be effective, then consider quickly imaging about 50-100 healthy larvae at 5 dpf by confocal (or when you expect to see expression). We typically observe about 1-2% of injected fish showing expression that is non-mosaic enough to sort them on a dissecting scope. Genotyping indicates that the efficiency is typically closer to about

4-5%, so there are a lot of fish that are so mosaic that they are missed but could probably be found by confocal imaging. Assuming an efficiency of rate of 5%, it should be possible to randomly find 2-3 expressing fish if you image 50 larvae.

  - The last resort is to raise unscreened fish and then genotype when they are adults.

- If you are able to raise and outcross only the fish that showed expression, it's safe to assume you'll get a stable line. Of those F0's that showed broad expression as embryos or larvae, we have observed that 60-100% provide F1's with expression. The stable F1's are also much easier to screen compared to the F0's that show mosaic expression.
- Outcross F1 founder fish and sequence the gDNA integration and spliced transcript in the F2 generation. You should observe Mendelian segregation at the F2 generation.

Part 2: N-terminal tagging using 3’ splice donor elements



1. Choose target sites
    - With this approach, you will select target sites in non-coding areas that are upstream of the insertion site (e.g. in the 5’UTR or further upstream). It is also possible to target downstream of the insertion site in intron 1, but upstream targeting seems to be more effective.
    - Try to select target sites that are less likely to be disruptive. The targeted region will be repaired by the donor, but there may be small indels at either the 5’ or 3’ end of each target site. In general, try to stay about 50-100 bp upstream of the start codon. It is possible that target sites that are very far from the insertion site will decrease KI efficiency (e.g. cutting 600 bp away from the insertion site in a large intron may not work as well as cutting only 200 bp away). We have not tested this in zebrafish, but it is generally accepted that cutting closer to the insertion site increases efficiency of KI for large insertions such as fluorescent protein sequences.
    - Design gRNA target sites, following guidelines described in Part 1 (e.g. make sure you sequence the gRNA target sites you plan to use in your population of fish).

2. Design and construct a donor plasmid
    - This approach requires more effort to make a donor plasmid compared to C-terminal tagging, but the method works well. The donor plasmid needs to have a 5’ homology arm ≥500 bp in length and a 3’ splice donor element. There are several ways of making this type of construct.

- Option 1 uses traditional cut-and-paste cloning.
  - Clone a fragment of gDNA that spans from ≥500 bp upstream of the insertion site and ≥500 bp downstream from the insertion site. Make sure that the gene fragment you clone begins and ends in non-coding regions.
    - Clone this fragment into a vector using restriction digest/ligation or clone into a gateway vector like pDONR221 using a BP reaction.
    - Identify the desired insertion site where the gene needs to be tagged, and use Q5 site directed mutagenesis (Q5 SDM) to introduce a small multiple cloning site (MCS) in frame with the coding sequence. The restriction sites should not be in the vector or gDNA fragment being cloned.
      - Here is an example using AgeI/SpeI sites:

Original sequence

5'UTR coding

tttgttttgatcgagATGGGGACACGAGAC

Sequence after SDM

5'UTR MCS coding

tttgttttgatcgagcctACCGGTcttACTAGTtccATGGGGACACGAGAC

      - Here are some other small MCS's commonly used:
        - AgeI/SpeI cctACCGGTcttACTAGTtcc
        - NheI/SacI cctGCTAGCcttGAGCTCtcc
        - SacII/XhoI cctCCGCGGcttCTCGAGtcc

  - Next, clone in eGFP-nostop or another FP. For most FPs, you can use these primers with a Tm of 67°C with Q5 polymerase, but be sure to add restriction sites to the beginning of the primer sequence
    - For N-terminal tagging
      - f_GFP_Kozak gccgccaccATGGTGAGCAAGGGCGAGGA
      - r_GFP_noX CTTGTACAGCTCGTCCATGCC
  - Next, mutate the gRNA target site in your plasmid so that you do not cut off the homology arm of your DNA donor when you inject. This is not absolutely required, but we have observed a 2-fold higher efficiency when the target site is mutated in the donor construct.
    - Use Q5 SDM to introduce several mismatches, at least 3 in the core sequence (the 12 bp before the PAM site) and 1 in the PAM site. More than 2 mismatches in the core sequence was shown to abolish targeting efficiency (PMID: 23873081).

- Option 2 uses seamless cloning, which we do with InFusion cloning (Takara Bio).
  - Use the web app from Takara to design your construct (https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools)
  - Identify a convenient backbone vector with a multiple cloning site (e.g. pCS2+).
  - Paste the plasmid sequence into the web app and choose a restriction site for linearization. Alternatively, you can linearize the plasmid by PCR.
  - Paste the insertion sequences you want to fuse into the app. Here is an example where the donor insert includes a 5' homology arm with a mutated gRNA target site, eGFP, exon 1 coding sequence, and a 3' splice donor element all in one reaction.
    - For insert 1, paste ≥500 bp of sequence spanning from a 5' upstream region to the gRNA target, but mutate the PAM sequence of the gRNA target site (this will make the reverse primer mutagenic).
    - For insert 2, paste the sequence immediately following the gRNA target site through the end of the 5'UTR.
    - For insert 3, paste in the fluorescent protein coding sequence.
    - For insert 4, paste ≥500 bp of sequence spanning from the beginning of the exon coding sequence at the ATG start site through the middle of intron 1.
  - Using the primers obtained from the app, PCR amplify inserts 1, 2, and 4 from gDNA. PCR amplify insert 3 from a plasmid containing the fluorescent protein coding sequence.
  - Column-purify the PCR products and check their sizes on an agarose gel. Plan for a final concentration of ~15-50 ng/µL depending on number of base pairs (larger ones need to be more concentrated). Adjust PCR conditions accordingly.
  - Linearize the destination vector, either by restriction digest or PCR according to how you designed the cloning.
  - For any PCR products that were amplified from plasmid DNA as template, treat the purified product with DpnI (1 uL in 1X cutsmart buffer for 30 minutes at 37C) and then purify them once more.
  - Set up the InFusion reaction
    - Use a 2:1 molar ratio of insert:vector, and use 25-50 ng of vector.
    - Reaction

Vector	X µL
Insert	X µL
Water	X µL
5X InFusion master mix (Takara 638948)	2 µL
Total volume	10 µL

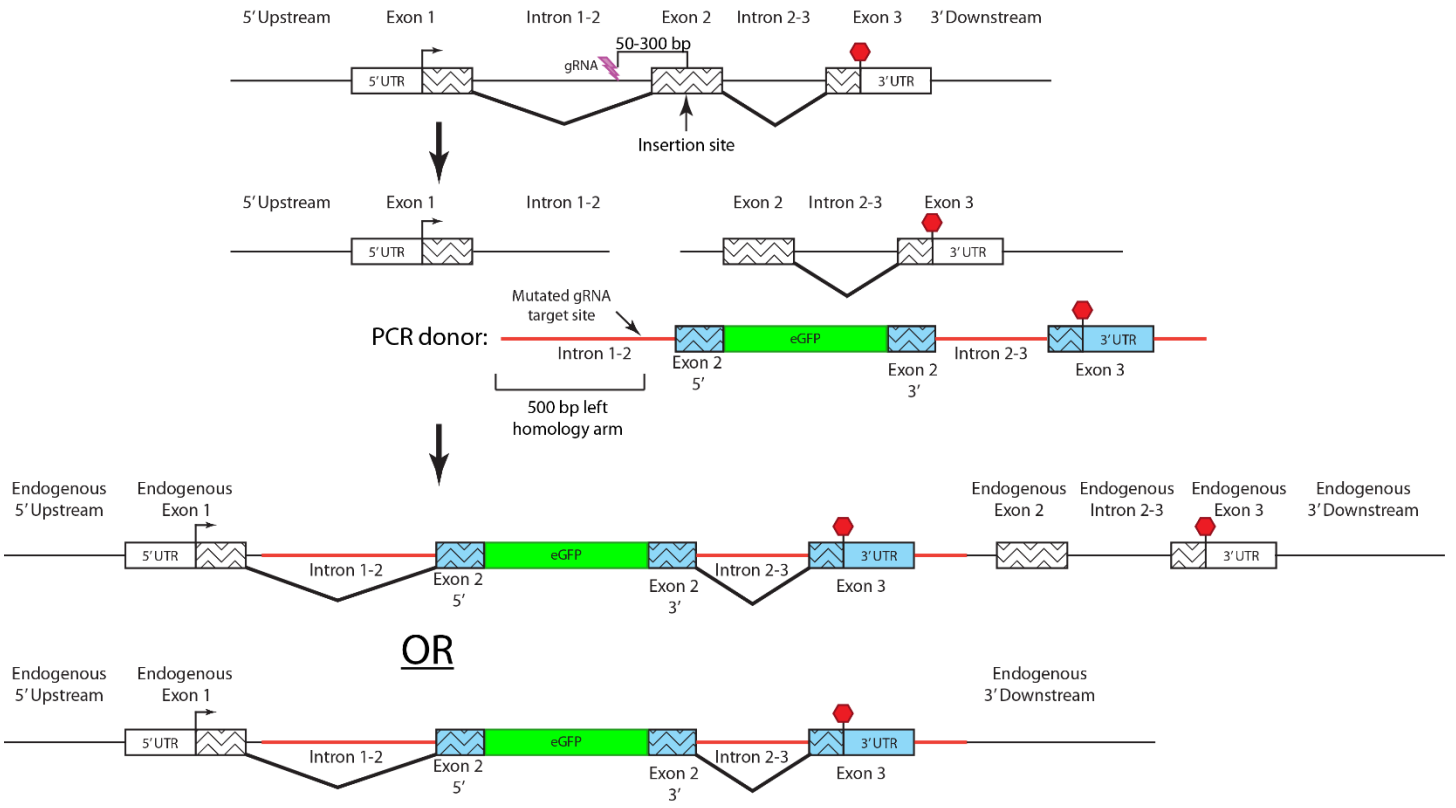
  - Incubate at 50°C for 15 minutes, and then place on ice. Do not exceed 15 minutes. You can freeze the reaction or transform the product immediately.
  - Transform 2.5 uL of the InFusion reaction, obtain colonies, and screen/sequence them.

- Typically, 20-100% of the colonies have the correct insertions if the DNA fragments used are of high purity, but efficiency depends on the number of insertions being used. If you did not perform DpnI treatment on PCRs amplified from plasmid, then many of your transformants may have the original circular plasmid that you used for PCR.

3. Generate a PCR donor from your donor plasmid
    - Set up the following PCR using the primers that flank the donor insert in your plasmid.
        - Plasmid donor (10 ng/uL) 1 uL
        - Q5 polymerase buffer (5x) 5 uL
        - dNTP mix (10 mM) 0.5 uL
        - Forward primer (10 uM) 1.25 uL
        - Reverse primer (10 uM) 1.25 uL
        - Water 15.75 uL
        - Q5 Polymerase 0.25 uL
        - Total volume 25 uL (if needed, scale up and divide into multiple tubes)

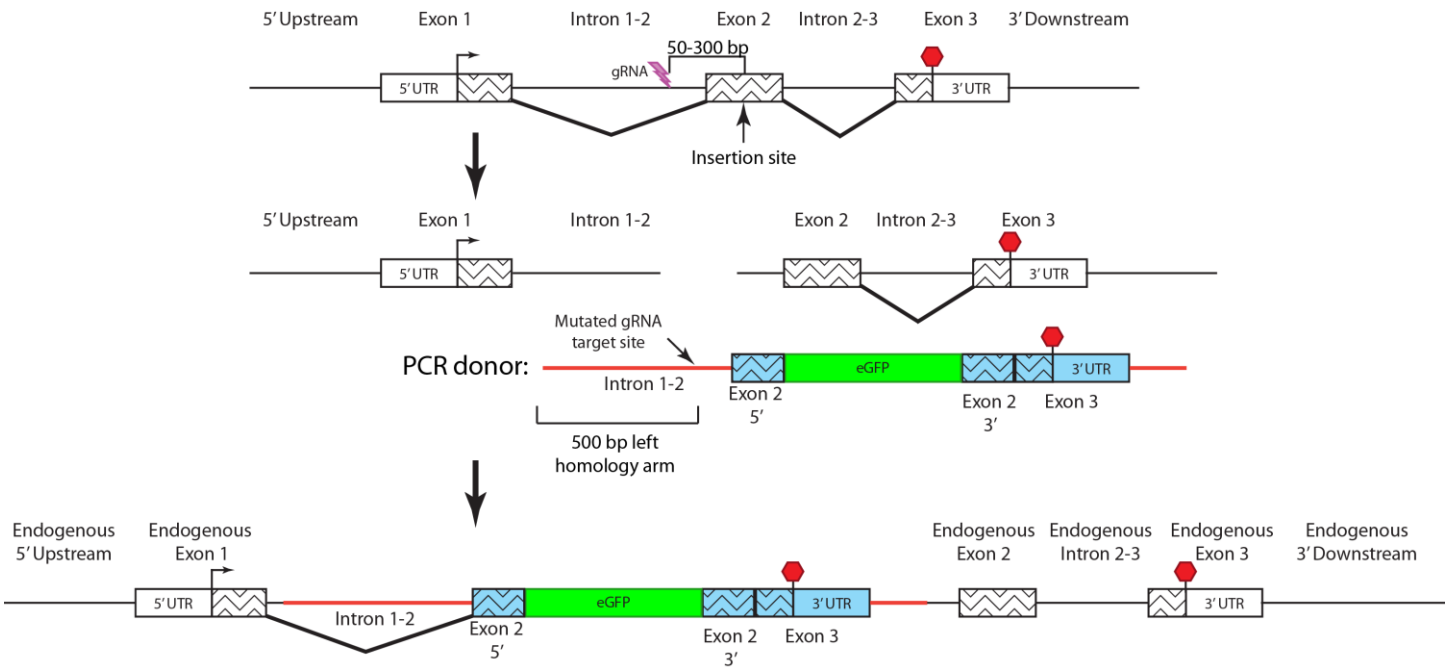
        - Anneal: X°C 0:20
        - Extension: 72°C X:XX
        - Cycles 34
    - After PCR, run ~5 uL on a 1% TBE agarose gel to check the product.
    - Use the remaining 20 uL to purify the PCR donor using the Nucleospin Gel and PCR Clean-up kit (Machery-Nagel) as described in Part 1.
4. Injections and screening
    - Prepare the following injection working mix (if you are using a crRNA, see Appendix III):
        - dsDNA PCR donor 5-10 ng/uL
        - Target site gRNA 35-50 ng/uL
        - Phenol Red 0.05%
        - Cas9-NLS protein 300-500 ng/uL (PNABio catalog no. CP01)
    - Follow the injection and screening procedure described in Part 1.

Part 3: Internal tagging using partial gene replacement or cDNA KI



1. Choose target sites
    - With this approach, you will select target sites in non-coding areas that are upstream of the insertion site.
    - Follow the same design principles as in Part 2 (N-terminal tagging), choosing gRNA target sites that are 100-200 bp away from intron-exon boundaries so as not to affect splicing.
    - Design gRNA target sites as described in Parts 1-2.
2. Design and construct a donor plasmid
    - Clone a fragment of gDNA that spans from about 500 bp upstream of the gRNA target site to about 100-200 bp downstream of the predicted poly-adenylation site. You can use this web app (http://dnafsminer.bic.nus.edu.sg/PolyA.html) to predict where the poly-A site is in your gene. Make sure that the gene fragment you clone begins and ends in non-coding regions.
    - This will likely be a large fragment to clone (2-3 kb or larger) and, depending on how large your gene is and where you need to tag, this approach may be challenging or not feasible. InFusion cloning can simplify this by allowing you to seamlessly clone several gDNA fragments together.

- If it is not possible to clone a genomic DNA fragment because of its size, another approach would be clone and KI a cDNA fragment of your GOI:



- Follow the guidelines in Part 2 to design your donor plasmid.

3. Generate a PCR donor from your donor plasmid
    - Follow the description in Part 2 to generate a dsDNA PCR donor to use for injections.
4. Injections and screening
    - Prepare the following injection working mix (if you are using a crRNA, see Appendix III):
        - dsDNA PCR donor 5-10 ng/uL
        - Target site gRNA 35-50 ng/uL
        - Phenol Red 0.05%
        - Cas9-NLS protein 300-500 ng/uL (PNABio catalog no. CP01)
    - Follow the injection and screening procedure described in Parts 1-2.

Appendix I. Donor vectors for C-terminal last exon replacement or internal cDNA replacement

- We have made the following donor vectors for C-terminal tagging:
  - pUC19-TgKI-MCS-eGFP-MCS-polyA
  - pUC19-TgKI-MCS-eGFP_Y40N-MCS-polyA (the gBait site is mutated with a favorable substitution found in many GFP derivatives)
  - pUC19-TgKI-MCS-mScarlet-MCS-polyA
  - pUC19-TgKI-MCS-mLanYFP-polyA
  - pUC19-TgKI-MCS-tdTomato-polyA
  - pUC19-TgKI-MCS-p2A-eGFP-MCS-polyA
  - pUC19-TgKI-MCS-p2A-mScarlet-MCS-polyA
  - pUC19-TgKI-MCS-p2A-tdTomato-polyA
  - pUC19-TgKI-MCS-p2A-Venus-PEST-MCS-polyA
  - pUC19-TgKI-MCS-p2A-QF2-MCS-polyA
- Last intron-exon fragments and 3'UTR fragments (if desired) can be cloned into the 5' and 3' MCS, respectively, using cut-and-paste or InFusion seamless cloning.
- Note that the tdTomato and mLanYFP vectors do not contain a second MCS after the fluorescent protein coding sequence.
- These plasmids have been deposited to Addgene and should be available by Fall of 2021.
- The plasmids generally have the following layouts:
  - pUC19-TgKI-MCS-mScarlet-MCS-polyA

gBait — Clone targeted intron (~300 bp) through last exon no stop sequence here — no start mScarlet

```
GGCGAGGGCGATGCCACCTACGG GA TGC GGC CGC ACC GGT GGA TCC GAA TTC GCT AGC CCG CGG TCT AGA CTC GAG GTG AGC AAG ...
                        NotI  AgeI  BamHI EcoRI NheI SacII XbaI  XhoI
```

mScarlet stop — If needed, clone the 3'UTR for your GOI here — *ubb* PolyA

```
... CTG TAC AAG TAA ACT AGT ATC GAT AAG CTT GAG CTC GTC GAC ATCATTCTCAGTATCCCCTGCTTATGCACTCACCACTTTTT...
                    SpeI  ClaI  HindIII SacI  SalI
```

  - pUC19-TgKI-MCS-p2A-QF2-MCS-polyA

gBait — Clone targeted intron (~300 bp) through last exon no stop sequence here — p2A peptide

```
GGCGAGGGCGATGCCACCTACGG GA TGC GGC CGC ACC GGT GGA TCC GAA TTC GCT AGC CCG CGG TCT AGA CTC GAG GGA TCC GGA ...
                        NotI  AgeI  BamHI EcoRI NheI SacII XbaI  XhoI
```

P2A peptide — QF2 — If needed, clone the 3'UTR for your GOI here — *ubb* PolyA

```
... AAC CCC GGT CCT ATG CCA CCC AAG ... TAC GAA CAG TGA ACT AGT ATC GAT AAG CTT GAG CTC GTC GAC ATCATTCTCAGTATCCCCT...
                                        SpeI  ClaI  HindIII SacI  SalI
```

Appendix II. gRNA synthesis and injection protocol

Part 1: Designing, synthesizing, and testing gRNAs

1. Design oligos for target site
   - Order a PAGE purified oligo for the universal sgRNA scaffold
   - 5'-ttttgcaccgactcggtgccacttttcaagtTgataaCggactagccttattttaacttgctatttctagctctaaaac-3'
   - Use http://www.crisprscan.org (Moreno-Mateos, et al., 2015, Nature Methods) to identify target sites for your gene of interest.
     - This app uses an algorithm for scoring target sites based on experimental data from zebrafish. Ideally you should try to identify canonical GGN18NGG sites, but the tool will also find alternative sites such as Gg18NGG (mismatch in the second base).
   - Gene-specific oligo: AATTAATACGACTCACTATAGGN18gttttagagctagaaatagc, where N18 is the following sequence from the target sites you identified GGXXXXXXXXXXXXXXXXXXXGG (*although the universal scaffold oligo is PAGE purified, this gene specific oligo does not have to be PAGE purified).
   - Example

```
template:          AATTAATACGACTCACTATAGGN18                gttttagagctagaaatagc
oligo:             AATTAATACGACTCACTATAGGCTAGTCTGGTCTGCGCTAgttttagagctagaaatagc
target site:                          GGCTAGTCTGGTCTGCGCTAAGG
pasted sequence:                        CTAGTCTGGTCTGCGCTA
```

2. Design and order primers for analyzing indels using the heteroduplex mobility shift assay (HDA, protocol below).
   - Obtain genomic DNA sequence from ensembl or NCBI and design PCR primers to amplify a 200-400 bp product containing the target site in the middle. Smaller fragments are preferred.
   - Order 2 sets of primers per target site. You will first test the primers to ensure that you get only 1 clean band with the PCR so that you can directly use the PCR product for the HDA without any prior cleanup or purification. 2 sets of primers will allow you to try 4 primer combinations.
3. gRNA synthesis
   - If you are making only a single gRNA, then it is recommended to make 4x volumes for steps 1-2. This eliminates the need to pipette and mix very small volumes.
   - If you are making more than 2 gRNAs, then you can make master mixes for each step. However, it is very important that after each step you mix thoroughly by pipetting.

1. Anneal oligos
   a. Prepare the following (If only making 1 gRNA, make this step 4x volume)

– Target site oligo (10uM)	1 uL	4 uL
– Sg Scaffold oligo (10uM)	1 uL	4 uL
– NEB buffer 2.1 (10x)	0.5 uL	2 uL
– Water	2 uL	8 uL

   b. Mix well and then heat at 98°C for 1 min and cool to 37°C at 0.1°C per second

2. Template DNA extension; if only making 1 gRNA, make this step 4x volume (red font)
    a. Prepare the following (the T4 polymerase quality is critical for this step)

– Annealed oligo mix from above	4.5 uL	18 uL
– dNTP (*500 uM)	0.5 uL	2 uL
– T4 DNA polymerase (NEB)	0.5 uL	2 uL

    b. Mix well and then incubate at 12°C for 20 minutes
    c. Incubate at 75°C for 20 minutes to inactivate enzyme

3. IVT RNA synthesis using the T7 Megashortscript kit
    a. Prepare the following, note that this is a full reaction (black font) but we typically do half reactions (blue font)

– Template DNA from steps 1-2 above	5 uL	2.5 uL
– T7 reaction buffer (10X)	2 uL	1 uL
– Water	3 uL	1.5 uL
– ATP (75 mM)	2 uL	1 uL
– CTP (75 mM)	2 uL	1 uL
– GTP (75 mM)	2 uL	1 uL
– UTP (75 mM)	2 uL	1 uL
– T7 enzyme mix	2 uL	1 uL

    b. Mix by pipetting and then incubate at 37°C for 1 hour

4. Precipitate gRNA
    a. Precipitation is sufficient to obtain high quality gRNAs. The gRNAs are much more concentrated than the template DNA oligos, so there is no need to isolate the gRNAs from the template.
    b. Prepare the following and then mix

– RNA synthesis reaction from above	10 or 20 uL
– Water	275 uL
– Ammonium acetate stop sol'n (kit)	15 uL
– Isopropanol	300 ul

    – Vortex for 5 seconds
    – Incubate at -20°C for ≥ 30 minutes or overnight
    – Centrifuge at 14k RPM at 4°C for 10 minutes
    – Pipette off supernatant
    – Add 500 uL of 75% ethanol and mix well
    – Centrifuge at 13k RPM at room temperature for 5 min.
    – Pipette off supernatant
    – Centrifuge for 30 seconds to collect residual ethanol to bottom of tube
    – Pipette off remaining ethanol with 10 uL tip, careful not to disturb the pellet

- Dry the pellet by evaporation for about 1-5 minutes or as soon as pellet no longer looks white and becomes translucent
- Add 20-40 uL of nuclease-free water to resuspend the pellet.
- Gently resuspend the RNA pellet and briefly centrifuge
- Prepare 2 aliquots and store at -80°C

4. Assess RNA quality
    a. Measure concentration on nano-drop
        i. Expected concentration is 200-400 ng/uL x 20-40 uL
        ii. Expected 260/280 is 2.0-2.2
        iii. Expected 260/230 is 2-4.
    b. Run 500 ng of gRNA on a 2% TBE agarose gel
        i. You should see a broad band at 100-200 bp and a sometimes a lighter band above 200 bp when run on a TBE gel
5. Inject gRNA
    a. Coinject 50 pg of gRNA and 500 pg of Cas9-NLS protein. Increase or decrease gRNA concentration as needed.
6. Genotype embryos
    a. Choose 8 healthy injected embryos and 6 noninjected control sibling embryos and prepare genomic DNA (any stage from 1-5 dpf is acceptable)
        i. Anesthetize embryo and add to 30 uL of lysis buffer (10 mM Tris, pH 8.0, 50 mM KCl, 0.3% tween) + 3 uL of Proteinase K stock solution (from 10 mg/mL stock)
        ii. Heat at 55°C for 20 minutes
        iii. Heat at 98°C for 10 minutes
        iv. Vortex, spin down, and store at -20°C
    b. Heteroduplex mobility shift assay
        i. PCR amplify target region using the primers you tested in step 2
        ii. Melt and rehybridize using the following thermal cycler settings

1.	95°C	5 minutes
2.	95°C to 85°C	-2°C per second
3.	85°C to 25°C	-0.1°C per second
4.	16°C	hold

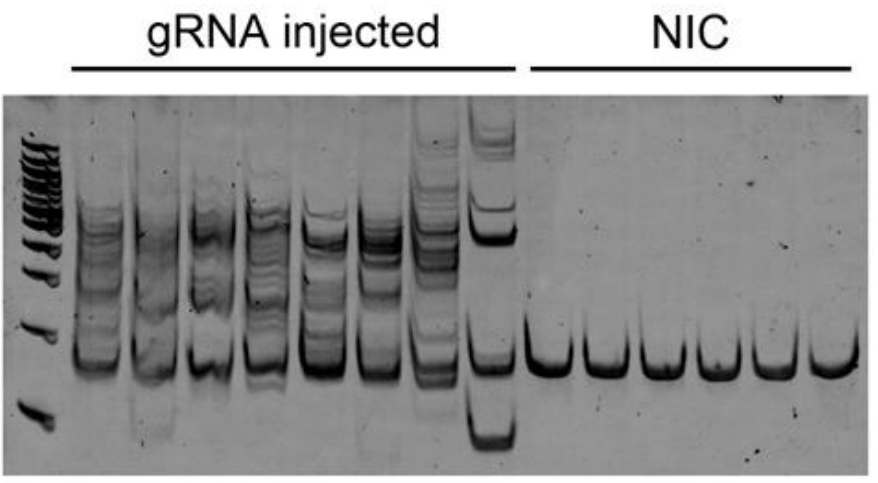
        iii. Prepare 10% acrylamide gel mix (make 20 mL for 2 small gels with the biorad casting plates with 1.5 mm spacers and a 15 well comb)

1.	30% 29:1 acrylamide:bis-acyrl.	6.9 mL
2.	Water	9 mL
3.	TBE (5x)	4 mL
4.	APS (10% w/v, store at -20c)	100 uL

5. TEMED 10 uL
6. Invert to mix

iv. Pour acrylamide gel (we do this with the Biorad gel casting rig)
    1. Press the glass plates together. Some choose to apply a thin layer of Vaseline to the raised sides of the bottom plate to prevent them from leaking.
    2. Put plates into clamp, close the clamp, and place unit into apparatus. Acrylamide leakage is usually caused by chipped glass plates.
    3. Pipette the acrylamide solution into the mold
    4. Insert comb; some choose to place a pencil into the top clamp to firmly seal the bottom of the glass
    5. Let sit at room temp for at least 40 minutes. Gels can be stored in a ziplock bag with 1x TBE buffer at 4°C for several weeks.
    6. Carefully remove the comb. The remaining liquid in the wells will be displaced when you add buffer
    7. Assemble the electrophoresis apparatus, fill the middle with 1x TBE, and add about 200 mL of 1x TBE to the outer chamber

v. Run, stain, and analyze gel
    1. Add DNA loading buffer to your PCR samples and then load samples into gel
    2. Run at 150V for 30-60 minutes
    3. Disassemble and gently pry apart the glass plates
    4. Stain the gel in ethidium bromide solution (1:2,000 1mg/mL stock in 1X TBE) for 5 minutes
    5. Remove staining solution and replace with 1x TBE, incubate for 1-5 minutes
    6. Analyze on gel doc. NIC samples should be a single band at the expected size, while gRNA-injected samples should show upper bands of various sizes do to the formation of heteroduplexes that migrate more slowly (see image below)



Appendix III: Assembling a synthetic crRNA/tracrRNA/Cas9 complex

1. Resuspend tracrRNA and crRNA to 100 µM in water. Store at -80°C.
2. Prepare stock mix:
    - tracrRNA 10 µL
    - crRNA 10 µL
    - Duplex buffer (IDT) 13.3 µL
    - Total volume 33.3 µL
3. Incubate at 95°C for 5 minutes and then cool to room temperature. Store at -80°C.
4. The stock concentration is approximately 515 ng/µL RNA duplex.
5. Assemble injection working mix:
    - tracrRNA/crRNA duplex 35-50 ng/µL
    - PCR donor 5-10 ng/µL
    - Phenol red 0.05%
    - Cas9-NLS protein 300-500 ng/µL (PNABio catalog no. CP01)
6. Incubate at 37°C for 5 minutes and then put on ice and use for injections immediately.