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Supplemental Information

Retinoic Acid Production by Endocardium and Epicardium Is an Injury Response Essential for Zebrafish Heart Regeneration

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3 Supplemental Figures

Figure S1. Expression data to support dynamism of endocardial activation after cardiac injury in zebrafish, including a second marker, *heg*, and an early, 1 hour post-injury *raldh2* expression timepoint. These data supplement expression data in Figure 1.

Figure S2. Expression data for retinoic acid signaling pathway, as assessed by flow cytometric sorting of cardiac cells and RT-PCR. These data indicate RA receptor expression in cardiac cells, substantiating functional approaches in Figure 3.

Figure S3. Additional characterization of transgenic reagents to block retinoic acid signaling, including embryonic phenotypes, transgene product localization, proliferation tests in an additional line, and TUNEL data. These data provide useful additional information to supplement Figure 3.

Supplemental Experimental Procedures

Descriptions of transgenic animal construction, cloning of *polypterus raldh2*, antibodies used, and cell sorting and PCR experiments.

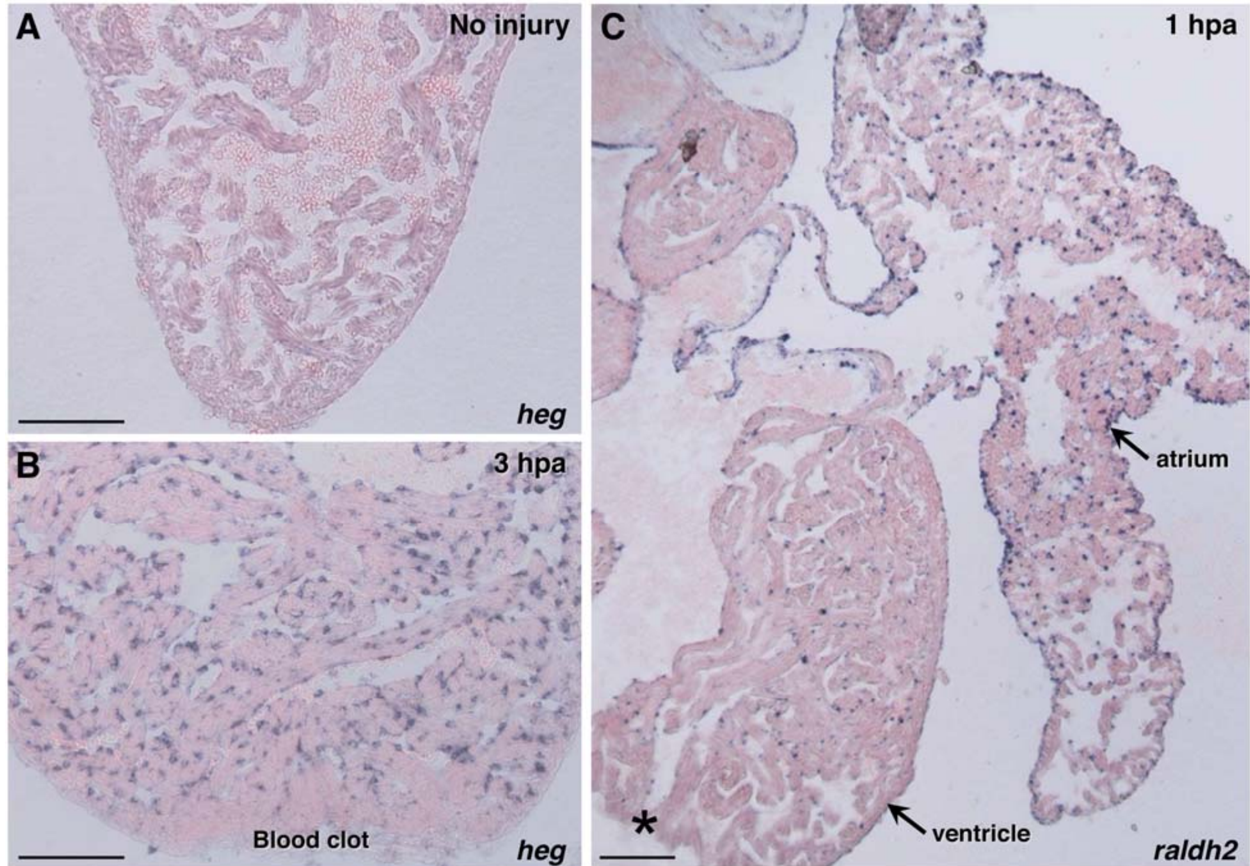


Figure S1. Organ-wide Endocardial Response to Removal of the Ventricular Apex

(A) *heg in situ* hybridization shows weak or no detectable expression in the uninjured ventricle. Scale bar = 100 μm (A-C).

(B) *heg* is induced by 3 hours post-amputation (hpa) in endocardial cells throughout the ventricle (and atrium, not shown).

(C) *raldh2 in situ* hybridization at 1 hpa, indicating induction in many atrial endocardial cells, but fewer ventricular endocardial cells. The injured ventricular apex is marked by an asterisk.

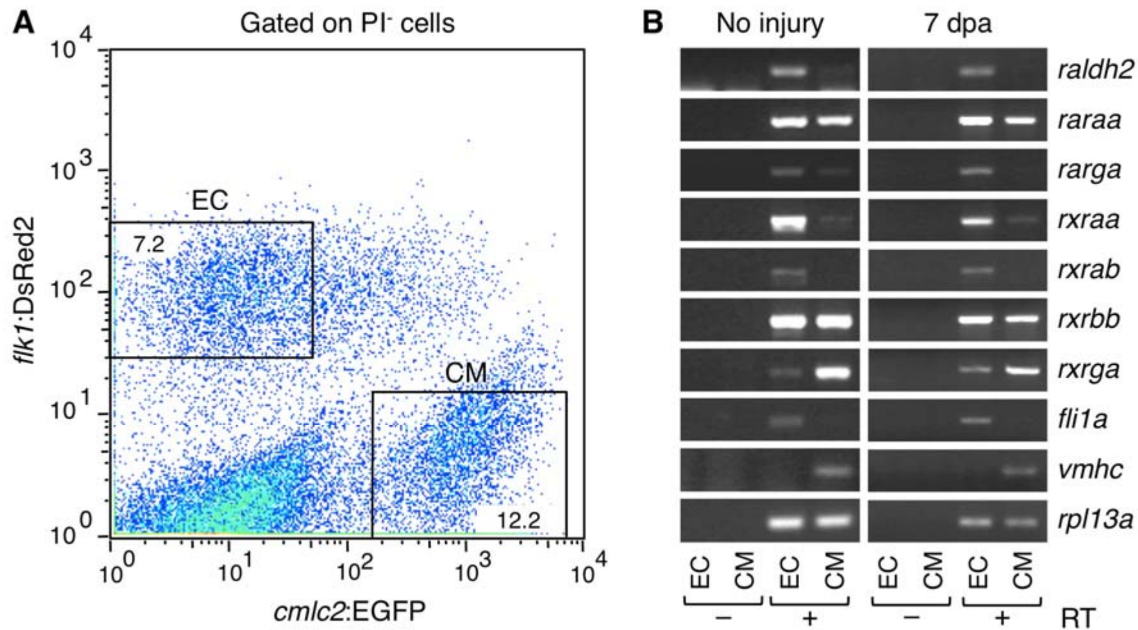
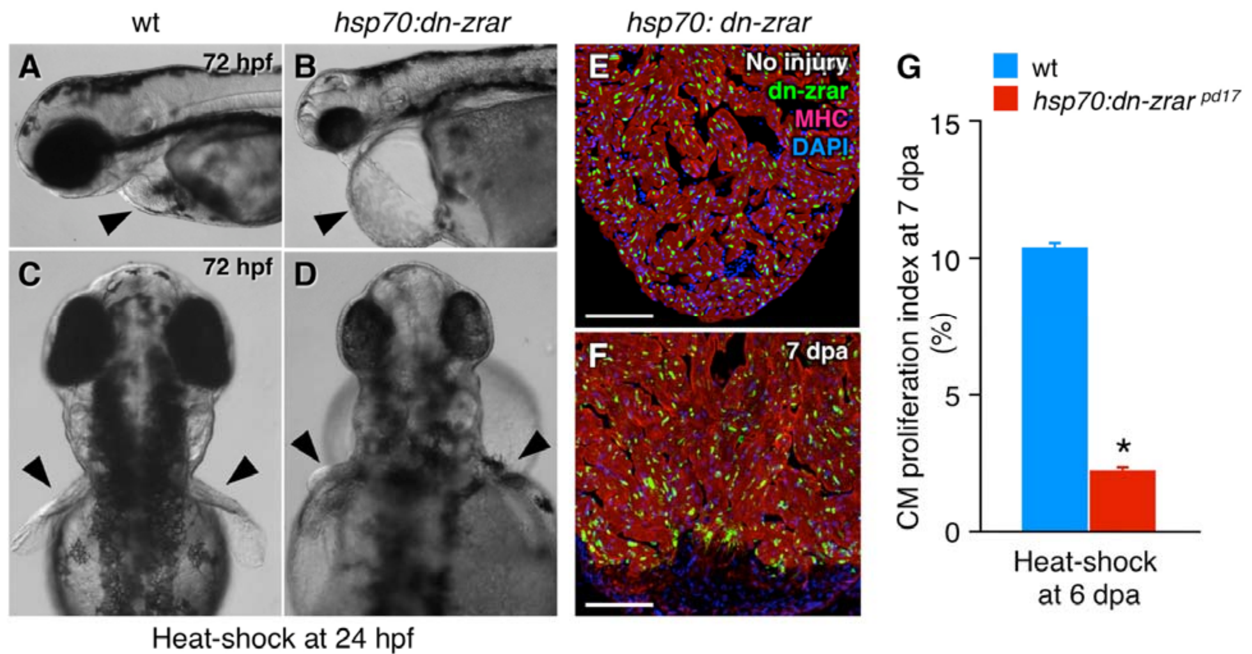


Figure S2. Expression Analyses of RA Signaling Components

(A) Endocardial cells (EC) and cardiomyocytes (CM) were purified by FACS from dissociated uninjured or 7 dpa *cmlc2:EGFP; flk1:DsRed2* ventricles. A representative plot from 7 dpa ventricles is shown. Cells stained by propidium iodide (PI) are excluded as dead cells. Percentages of the total PI-negative cell population that were gated are shown.

(B) Expression of RA signaling components was examined by RT-PCR in purified endocardial cells (EC) and cardiomyocytes (CM). Endocardial (*fli1a*) and cardiomyocyte markers (*vmhc*) were used to confirm the specificity of cell sorting. *ribosomal protein L13a* (*rpl13a*), was used as a control. The amplified products were not detectable without reverse transcriptase (RT), confirming that bands are amplified from cDNAs of the target genes but not from genomic DNA contaminants. *raldh2* transcripts detected in samples of uninjured ventricles are likely accentuated by the severe trauma of ventricular cell extraction and dissociation.



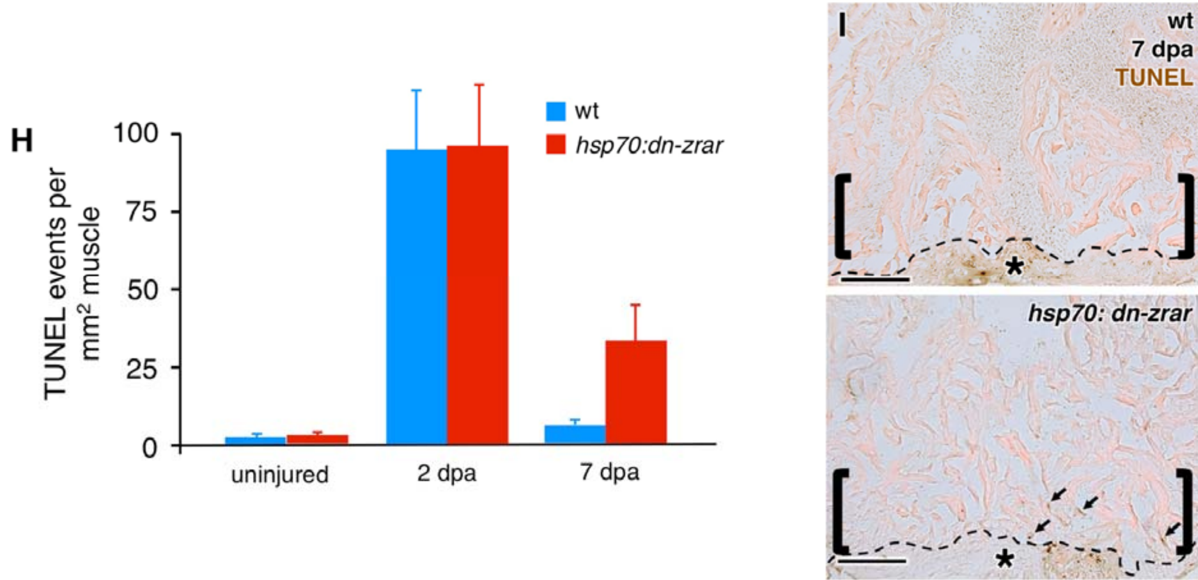


Figure S3. Effects of *dn-zrar* Induction during Embryogenesis and Regeneration

(A-D) A single heat-shock at 37°C for 30 minutes was delivered to *hsp70:dn-zrar* or wild-type (wt) embryos at 24 hours post-fertilization (hpf) as described previously (Lee et al., 2005). At 72 hpf, cardiac edema (B, arrowheads) and pectoral fin reductions (D,

arrowheads) were observed in *hsp70:dn-zrar* embryos, but not in wt (A, C, arrowheads). Cardiac edema was observed in all embryos with pectoral fin defects.

(E, F) Cardiomyocyte expression and nuclear localization of the dn-zrar inhibitory product (green) in sections of uninjured (E) and regenerating (F) ventricles. Most or all cardiomyocytes show strong nuclear dn-zrar expression one day after a heat-shock. Scale bars = 100 μm in (E, F, H, and I).

(G) A second line of *hsp70:dn-zrar* was analyzed at 7 dpa for cardiomyocyte (CM) proliferation, after a single heat-shock at 6 dpa. wt, clutchmate control. Data are mean ± SEM of 4 animals analyzed (2364 wt and 3038 transgenic cardiomyocytes analyzed). *$p < 3 \times 10^{-8}$, Student's t-test.

(H, I) Apoptosis was examined by TUNEL assay and quantified in wt and *hsp70:dn-zrar* ventricles one day after a single heat-shock. TUNEL signals were quantified at the apex for uninjured ventricles, and at the apical injury site at 2 dpa and 7 dpa. TUNEL-positive CMs were rare in uninjured animals, and appeared at similar frequencies at 2 dpa in wt and *hsp70:dn-zrar* injuries. Unlike 7 dpa wt ventricles, some 7 dpa transgenic ventricles displayed several TUNEL-positive cells within the myocardium (arrows in bracketed area of (I)), but overall there were no significant differences at the tested timepoints. Apoptotic cells were detected in the clot (asterisk) in both wt and transgenic ventricles. The dotted lines indicate approximate borders of the clot. Data are mean ± SEM of 3-9 animals analyzed.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Construction of Transgenic Animals

flk1:DsRed2

DsRed2 cDNA was cloned behind a 7 kb *flk1* promoter fragment (Jin et al., 2005), and the entire cassette was flanked with I-SceI sites for transgenesis using the meganuclease method (Thermes et al., 2002). The full name of this transgenic line is *Tg(flk1:DsRed2)*^{pd27}.

hand2:EGFP and gata5:EGFP

The first exon of *hand2* in the BAC clone CH211-15E22, and second exon of *gata5* in the BAC clone DKEYP-73A2 were replaced with EGFP at the translational initiation site by Red/ET recombineering (GeneBridges). The EGFP cassettes were amplified by PCR from *pcrii-egfp-frt-kan-frt* (provided by A. Nechiporuk). Constructs were linearized, and purified DNA was injected into embryos at the single-cell stage as described (Higashijima et al., 1997). The full names of these transgenic lines are *Tg(hand2:EGFP)*^{pd24} and *Tg(gata5:EGFP)*^{pd25}.

hsp70:dn-zrar

The zebrafish dominant-negative RAR (*dn-zrar*) was designed based precisely on the dominant-negative form of human RARα (α403*) (Damm et al., 1993). The zebrafish *raraa* isoform was chosen because Rar a is the most similar isoform to human RARα (81% amino acid similarity), and is the major isoform expressed in adult zebrafish hearts (Figure S2B). Despite the low similarity (10%) between the first 50 N-terminal amino acids, the remainder of Raraa sequence is almost identical. Thus, we truncated a nearly identical C-terminal region that is deleted from human RARα in α403*. We

amplified the truncated version of *rar* a by PCR with the primers 5'-CCGCTCGAGCCGCCACCATGTATGAGAGTGT-3' and 5'-CCGGAATTCACGGAATCTCCATCTTCAGGG-3' from a mixed cDNA pool of 24 and 56 hpf EK/AB embryos, and subcloned it into pEGFP-N3 (Clontech), fusing EGFP to the truncated C-terminus. The chimeric *dn-zrar* was excised and subcloned behind a heat-inducible *hsp70* promoter (Halloran et al., 2000). The entire *hsp70:dn-zrar* cassette was flanked with 2x core insulator elements and I-SceI sites. The full name of this transgenic line is *Tg(hsp70l:dn-zrar-egfp)*^{pd18}; a second line used in the study from a different founder is *Tg(hsp70l:dn-zrar-egfp)*^{pd17}.

hsp70:cyp26a1

EGFP from *phsp70/4:egfp* (Halloran et al., 2000) was replaced by full-length zebrafish *cyp26a1* cDNA, and the entire cassette was inserted into the I-SceI backbone vector (Thermes et al., 2002). Supercoiled plasmid DNA containing the transgene was injected together with I-SceI meganuclease into one-cell-stage embryos to create mosaic founder fish. A detailed characterization of *hsp70/4:cyp26a1* animals will be reported elsewhere (Blum and Begemann, submitted for publication). The full name of this transgenic line is *Tg(hsp70l:cyp26a1)*^{kn1}.

Cloning of *Polypterus senegalus raldh2*

Internal organs were collected from juvenile fish and homogenized in TRIzol reagent (Invitrogen). Based on the manufacture's protocol, total RNA was purified, and 1 μg of this was used to synthesize first-strand cDNA by Superscript III reverse transcriptase with oligo-dT primers (Invitrogen). Primers used for the PCR reaction to amplify the

polypterus *raldh2* were 5'-GCACAATGACTTCCAGTAAAATTGA-3' and 5'-TATAATAGTATTTACAGGGTTTCAAGTGGA-3', which were designed based on polypterus *raldh2* sequence (R.D.D., unpublished results). PCR reactions with the primers amplified the approximately 2.1 kb fragment that includes the entire coding sequence and 3' UTR fragment. The DNA fragment was gel purified, cloned into pBluescript II KS (+/-) vector (Stratagene), and verified by DNA sequencing. The Genbank accession number is HQ876171.

Antibodies

For immunofluorescence, antibodies were used against zebrafish (zf) Raldh2 (rabbit) at a 1:500 dilution (Abmart), GFP (chicken; AVES Labs) at 1:500, DsRed (rabbit; Clontech) at 1:500, Mef2 (rabbit; Santa Cruz Biotechnology) at 1:75, and PCNA (mouse; Sigma) at 1:200. The antibody against zebrafish Raldh2 was affinity-purified from sera of immunized rabbits with zebrafish Raldh2 peptides (Abmart). Secondary antibodies used in this study were: Alexa Fluor 594 goat anti-mouse IgG (H+L) for anti-PCNA and anti-Mef2, Alexa Fluor 594 goat anti-rabbit IgG (H+L) for anti-DsRed and anti-zf Raldh2, Alexa Fluor 488 goat anti-rabbit IgG (H+L) for anti-Mef2, anti-PCNA and anti-zf Raldh2, Alexa Fluor 488 goat anti-chicken IgG (H+L) for anti-GFP. All secondary antibodies were from Invitrogen and used at a 1:200 dilution.

Cell Sorting and RT-PCR

Adult zebrafish ventricles were extracted from uninjured or injured animals at 7 dpa and placed in ice-cold Hanks' Balanced Salt Solution without calcium and magnesium

(HBSS) (GIBCO) before carefully removing the atria and outflow tracts. Ventricles were cut into several pieces with a razor, washed to remove blood cells, and placed into a 1.5-ml microcentrifuge tube with HBSS containing 1 mg/ml collagenase type 2 (Worthington). The tube was incubated at room temperature for 30 minutes with rotation, and pipetted gently every 10 minutes. After incubation, dissociated cells were washed twice and suspended with ice-cold HBSS containing propidium iodide (PI). After filtration, cells were phenotyped and sorted by the FACSVantage SE (BD Biosciences Flow Cytometry Systems). Dead cells (PI-positive) were excluded from sorting, and DsRed2^{+}EGFP^{-} or DsRed2^{-}EGFP^{+} cells are sorted directly into 1.5-ml microcentrifuge tubes with 1 ml TRIzol reagent (Invitrogen). DsRed2^{+}EGFP^{+} cells in the plot were also excluded from the sorting, since these are likely to be doublet cells from incomplete dissociation (note that DsRed2^{+}EGFP^{+} cells were not detected in tissue sections at 7 dpa (Figure 2A). Fifteen ventricles of *flk1:DsRed2; cmlc2:EGFP* fish (15,000-20,000 DsRed2^{+}EGFP^{-} or DsRed2^{-}EGFP^{+} cells) were collected and analyzed with FlowJo software (Treestar). Total RNA was purified based on a manufacturer's protocol, and 250 ng total RNA from DsRed2^{+}EGFP^{-} or DsRed2^{-}EGFP^{+} cells was used to synthesize first-strand cDNA using Superscript III reverse transcriptase with oligo-dT primers (Invitrogen). Genes of interest were amplified by PCR based on the manufacturer's protocol (Invitrogen).

The primers for PCR were: *raldh2*, 5'-TGAAGTTGAACTGCCAGGAGAGGT-3' and 5'- AGACCTTGCCGCTCACAGAATCAT-3'; *raraa*, 5'-CCCTGGATGTGGATTTATGG-3' and 5'-GCTGATTAGCGAAAGCGAAC-3'; *rarga*, 5'-GCTGGTCAACAAAGTGAGCA-3' and 5'-TGAGTCCGGTTGAGGGTTAG-3'; *rxraa*, 5'-GCATCTCCTGGAGTGGGTTA-3'

and 5'-TGGACGGCTTCTCTCTTCAT-3'; *rxrab*, 5'-CCATGGGGATGAAGAGAGAA-3' and 5'-TTCACAGCTATGGAGCGATG-3'; *rxrbb*, 5'-CAGCTGTTCACTCTGGTGGA-3' and 5'-GACCTTTGGCATCTGGGTTA-3'; *rxrga*, 5'-AGGTTGGAACGAGCTGCTTA-3' and 5'-GCTGATCCGGGTAGTTGTGT-3'; *fli1a*, 5'-ACTTCCTGAGACTCACCAGCGTTT-3' and 5'-TTTCCGCTGTGCATGTTGTTCGAC-3'; *vmhc*, 5'-AGCAACTTGGTGAGGGAGGAAAGA-3' and 5'-TTCTCGGCTAGTTTGCGCTCGATA-3'; *rpl13a*, 5'-TCTGGAGGACTGTAAGAGGTATGC-3' and 5'-AGACGCACAATCTTGAGAGCAG-3'. Reactions used 42 amplification cycles, except for *fli1a* and *rpl13a* (30 cycles).

REFERENCES

Damm, K., Heyman, R.A., Umesono, K., and Evans, R.M. (1993). Functional inhibition of retinoic acid response by dominant negative retinoic acid receptor mutants. Proceedings of the National Academy of Sciences of the United States of America *90*, 2989-2993.

Halloran, M.C., Sato-Maeda, M., Warren, J.T., Su, F., Lele, Z., Krone, P.H., Kuwada, J.Y., and Shoji, W. (2000). Laser-induced gene expression in specific cells of transgenic zebrafish. Development (Cambridge, England) *127*, 1953-1960.

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y., and Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Developmental biology *192*, 289-299.

Jin, S.W., Beis, D., Mitchell, T., Chen, J.N., and Stainier, D.Y. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development (Cambridge, England) *132*, 5199-5209.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M., and Poss, K.D. (2005). Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration. Development (Cambridge, England) *132*, 5173-5183.

Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J., and Joly, J.S. (2002). I-SceI meganuclease mediates highly efficient transgenesis in fish. Mechanisms of development *118*, 91-98.