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Supplemental Information

Local Dkk1 Crosstalk from Breeding Ornaments Impedes Regeneration of Injured Male Zebrafish Fins

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Inventory of Supplemental Information

SUPPLEMENTAL FIGURES AND TEXT

Supplemental Figures

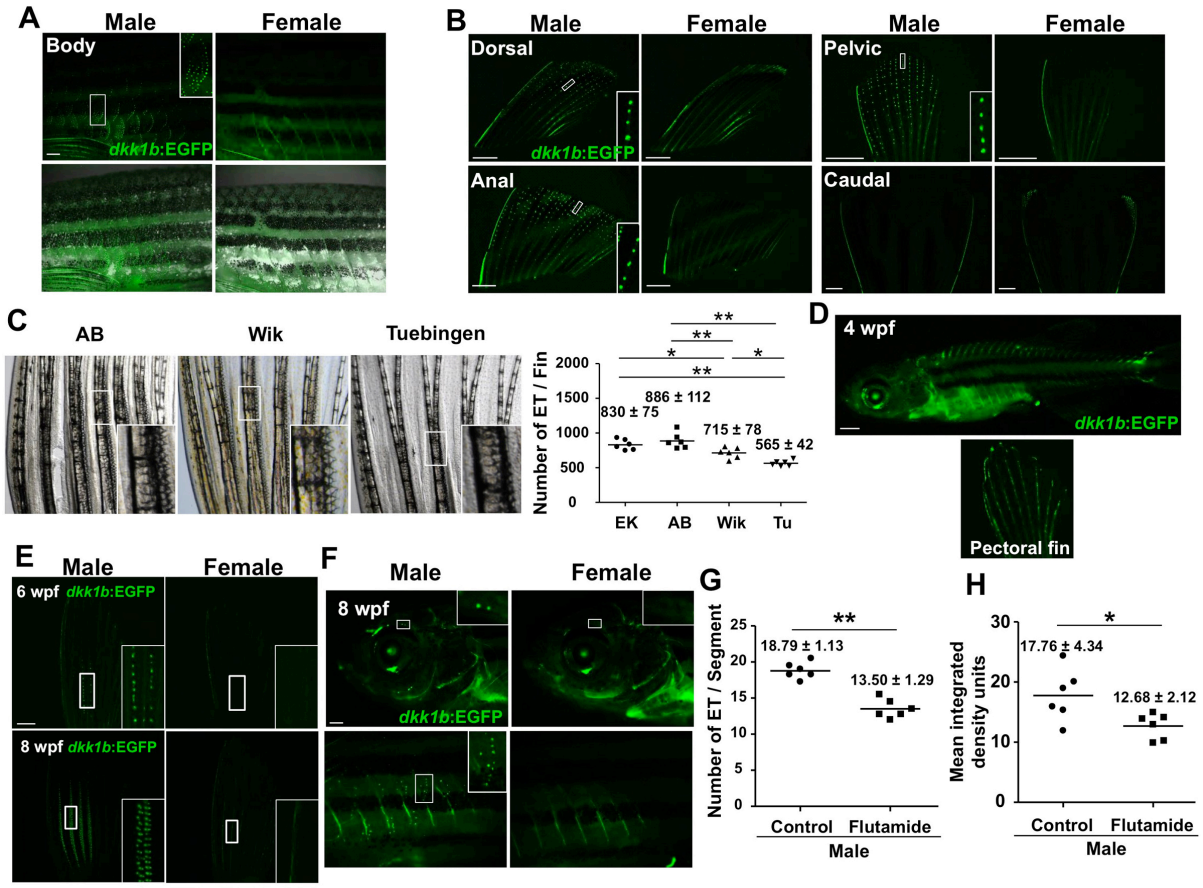

Figure S1, related to Figure 1. Sexually Dimorphic *dkk1b*:EGFP Expression Patterns

(A) Whole-mount images of male and female *dkk1b*:EGFP trunks. Males have many *dkk1b*:EGFP^{+} scale features, although posterior scales have much less. Both sexes show *dkk1b*:EGFP expression affiliated with bone.

(B) Whole-mount images of *dkk1b*:EGFP dorsal, anal, and pelvic fins. *dkk1b*:EGFP^{+} ET are present on distal fin regions in males, but not females. Male caudal fins do not have detectable *dkk1b*:EGFP^{+} ET. Insets show higher

magnification views of ET in male fins. Note that the exterior of the anterior fin ray in dorsal, anal, and pelvic fins expresses *dkk1b*:EGFP, as well as the most dorsal and ventral rays of the caudal fin.

(C) (Left) Whole-mount images of pectoral fins from 4-6-month-old AB, Wik and Tuebingen (Tu) strain males. All tested male strains had pectoral fin ET. (Right) Quantification of average numbers of ET per fin. n = 6 fins for each strain. Data are mean ± s.d.*P < 0.05, **P < 0.001 by two-tailed Student's t-test.

(D) *dkk1b*:EGFP expression patterns at 4 weeks post-fertilization (wpf). At 4 wpf, *dkk1b*:EGFP^{+} cells are located near bone in the trunk (Top) and pectoral fins (Bottom).

(E) *dkk1b:*EGFP^{+} pectoral fins at 6 and 8 weeks post-fertilization (wpf), when male-specific ET begin to become prominent. Insets are high magnification views of boxes.

(F) *dkk1b*:EGFP^{+} ET are detectable in the head and scales of male zebrafish at 8 wpf. Insets are high magnification views of boxes.

(G) Quantification of the average number of ET per ray segment, from 4 quantified segments per fish. ET were counted from two segments located just proximal to the bifurcation point on the 3rd and 4th fin rays (from the anterior).

(H) Quantification of mean *dkk1b*:EGFP fluorescence in ET (see Materials and Methods). n = 6. Data are mean ± s.d. *P < 0.05, **P < 0.001 by two-tailed Student's t-test.

Scale bars = 1 mm (A, B, D, E); 500 μm (F)

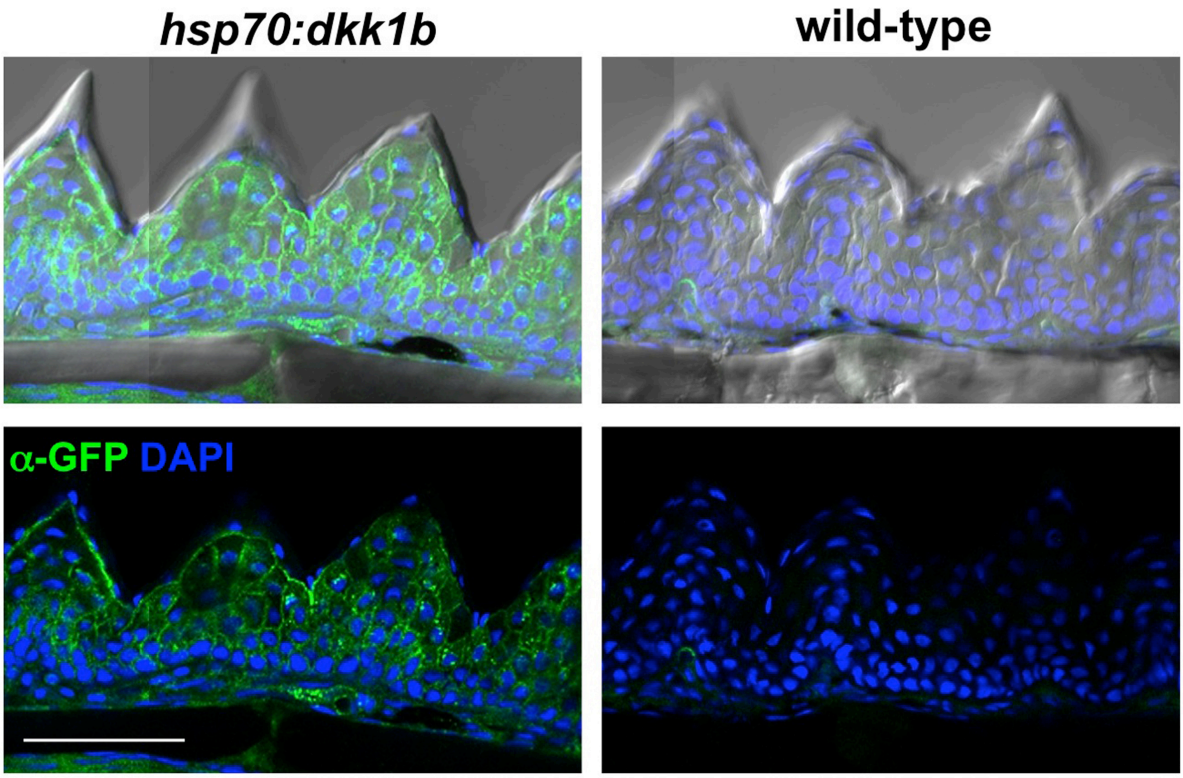

Figure S2, related to Figure 3. Ubiquitous Dkk1b-GFP Expression in *hsp70:dkk1b* ET After a Single Heat-Shock

Wild-types did not show GFP signal after heat-shock. An antibody was used to detect GFP in this experiment. Scale bar = 50 μm

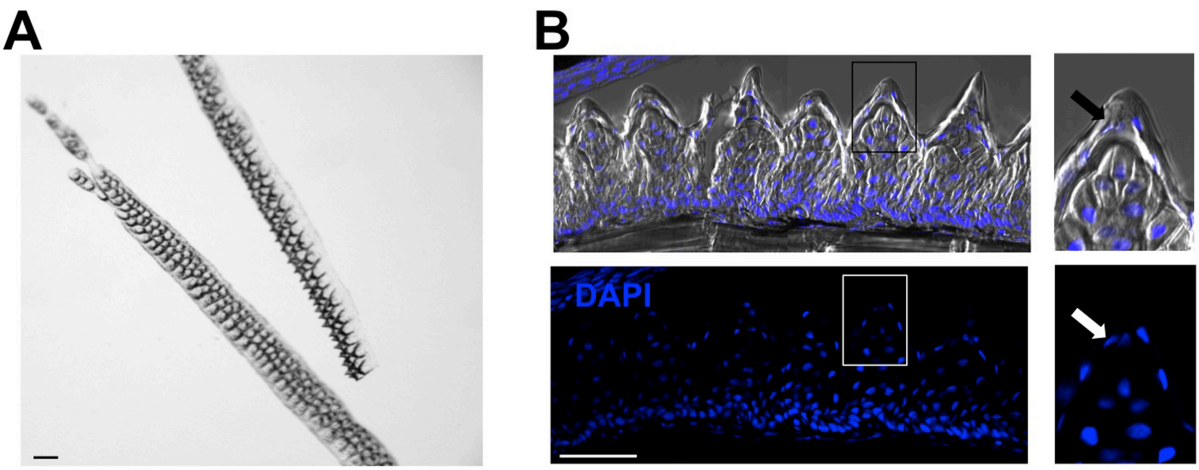

Figure S3, related to Figure 4. Rigid Keratinized Cuticles of Male Pectoral Fins

(A) Image of cuticles discarded from male pectoral fins after breeding.

(B) Section images of male pectoral fin. Arrow in inset points to cuticle cell nucleus.

Scale bars = 100 μm (A); 50 μm (B)

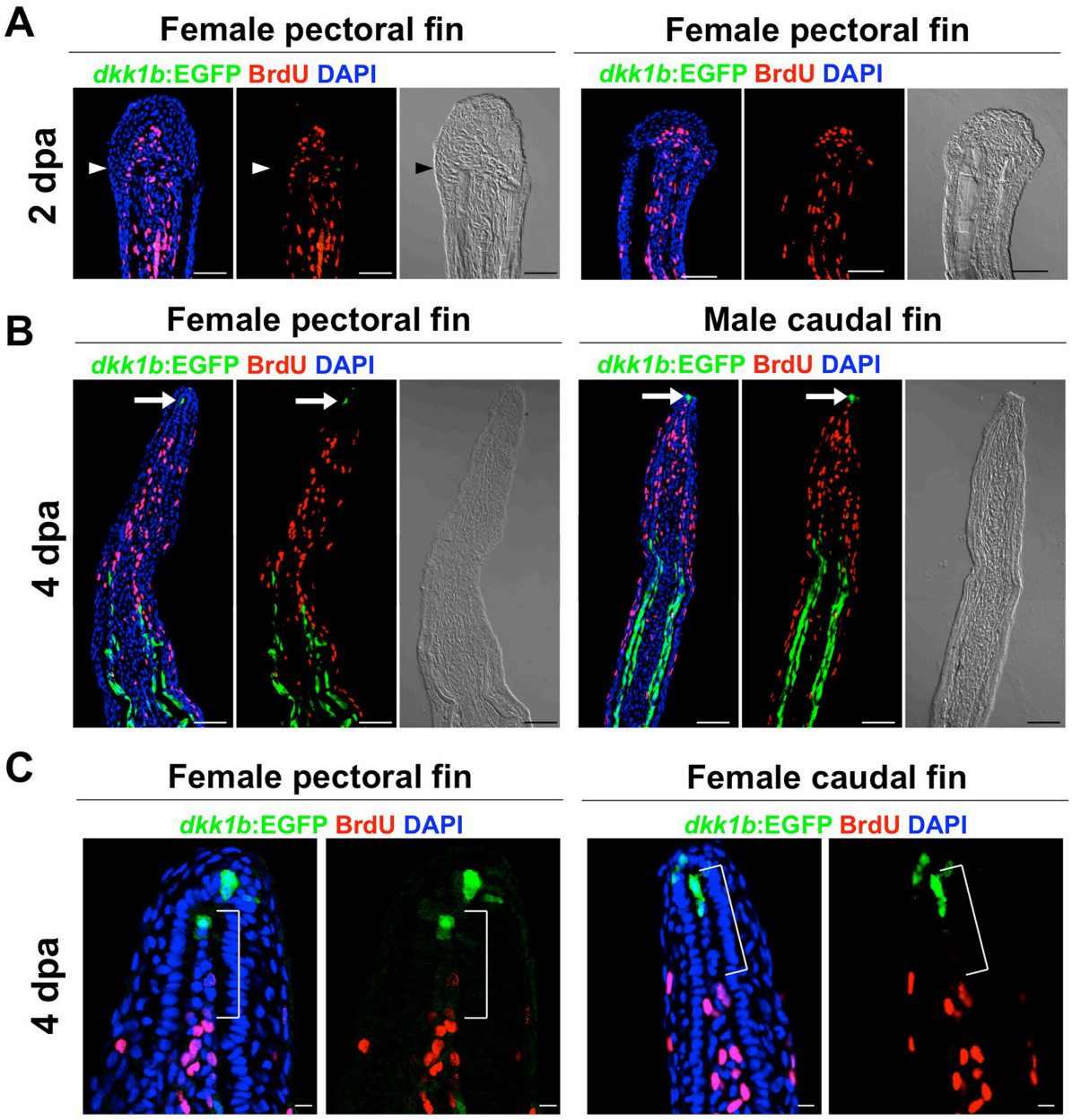

Figure S4, related to Figure 5. Additional Confocal Images of *dkk1b*:EGFP Expression During Fin Regeneration

(A) *dkk1b*:EGFP is low or undetectable at 2 dpa (blastema formation).

(B) *dkk1b*:EGFP expression during female pectoral fin (left) and male (right) caudal fin regeneration at 4 dpa (regenerative outgrowth). *dkk1b*:EGFP is expressed in a small cluster of cells located in and near the distal-most regions of the blastema

(arrows) at 4 dpa, and in areas of osteoblast patterning. Note that *dkk1b*:EGFP expression is associated with non-proliferative ($BrdU^+$) regions. Amputation sites are proximal to imaged areas in (B).

(C) High-magnification view of the distal tips of regenerating fins at 4 dpa. Brackets indicate a region of BrdU-low mesenchyme underlying the *dkk1b*:EGFP domain. Green: EGFP immunofluorescence; red: BrdU immunofluorescence; blue: DAPI. Scale bars = 50 μm (A, B); 10 μm (C)

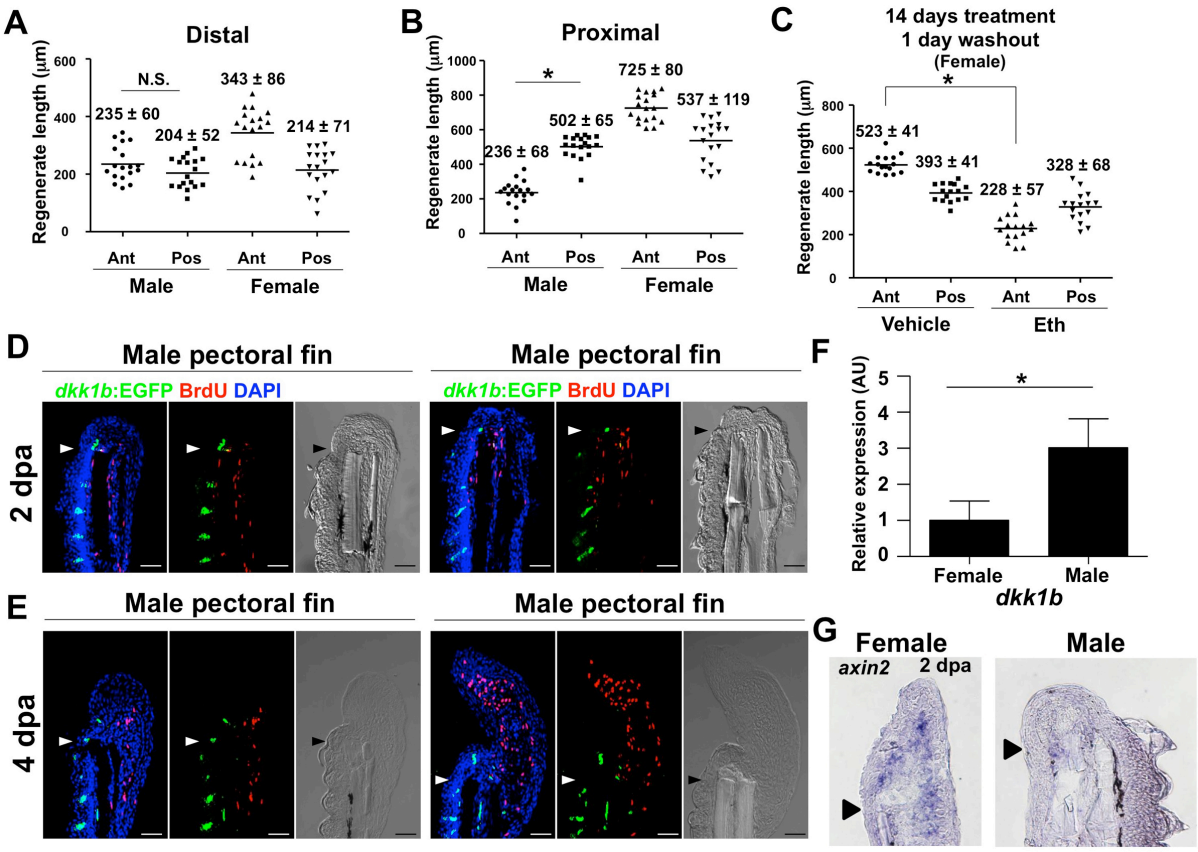

Figure S5, related to Figure 6. Additional Evidence that Dkk1b-Producing ET Inhibit Regeneration

(A) Quantification of regenerate lengths after distal amputations of 10-20% of pectoral fins. n = 18. Data are mean ± s.d. 'N.S.': not significant.

(B) Quantification of regenerate length after proximal amputation of 50% of pectoral fins. n = 18. Data are mean ± s.d.*P < 0.001 by one-way ANOVA with Bonferroni's posttest. Anterior regenerates are smaller than posterior regenerates after proximal amputations through the ET field, but not after amputation distal to the ET field. Note that growth rates during fin regeneration are position-dependent, with distal regenerates growing slower than proximal regenerates (Lee et al., 2005).

(C) Adult females treated with vehicle or Eth for 14 days prior to one day of washout had specific defects in anterior regeneration that mirrored male defects. n = 16. Data are mean ± s.d.; *P < 0.001 by one-way ANOVA with Bonferroni's posttest.

(D-E) Additional confocal images of *dkk1b*:EGFP in amputated anterior regions of male pectoral fins. (D) *dkk1b*:EGFP^{+}ET cells are located over and/or dorsally adjacent to the amputation place. Note that there are only few BrdU^{+} proliferating cells (red) near the amputation plane. (E) *dkk1b*:EGFP expression in dysmorphic 4 dpa regenerates. Proliferation appears to be directed away from *dkk1b*:EGFP^{+}ET. Green: EGFP immunofluorescence; red: BrdU immunofluorescence; blue: DAPI. Scale bars = 50 μm.

(F) Increased expression of *dkk1b* in the anterior regions of 2 dpa male pectoral fin tissue compared with females, as assessed by quantitative PCR (n = 3). Error bars indicate standard deviation. Data are normalized to *actb2* (*beta-actin2*). AU = arbitraty units. *P < 0.05 by two tailed Student's t-test.

(G) *axin2* expression at 2 dpa in female (Left) and male (Right) pectoral fins, as assessed by in situ hybridization. Note that *axin2* (violet) is clearly detectable in the female regenerate, but weak in the male sample. Arrowheads indicate amputation plane.

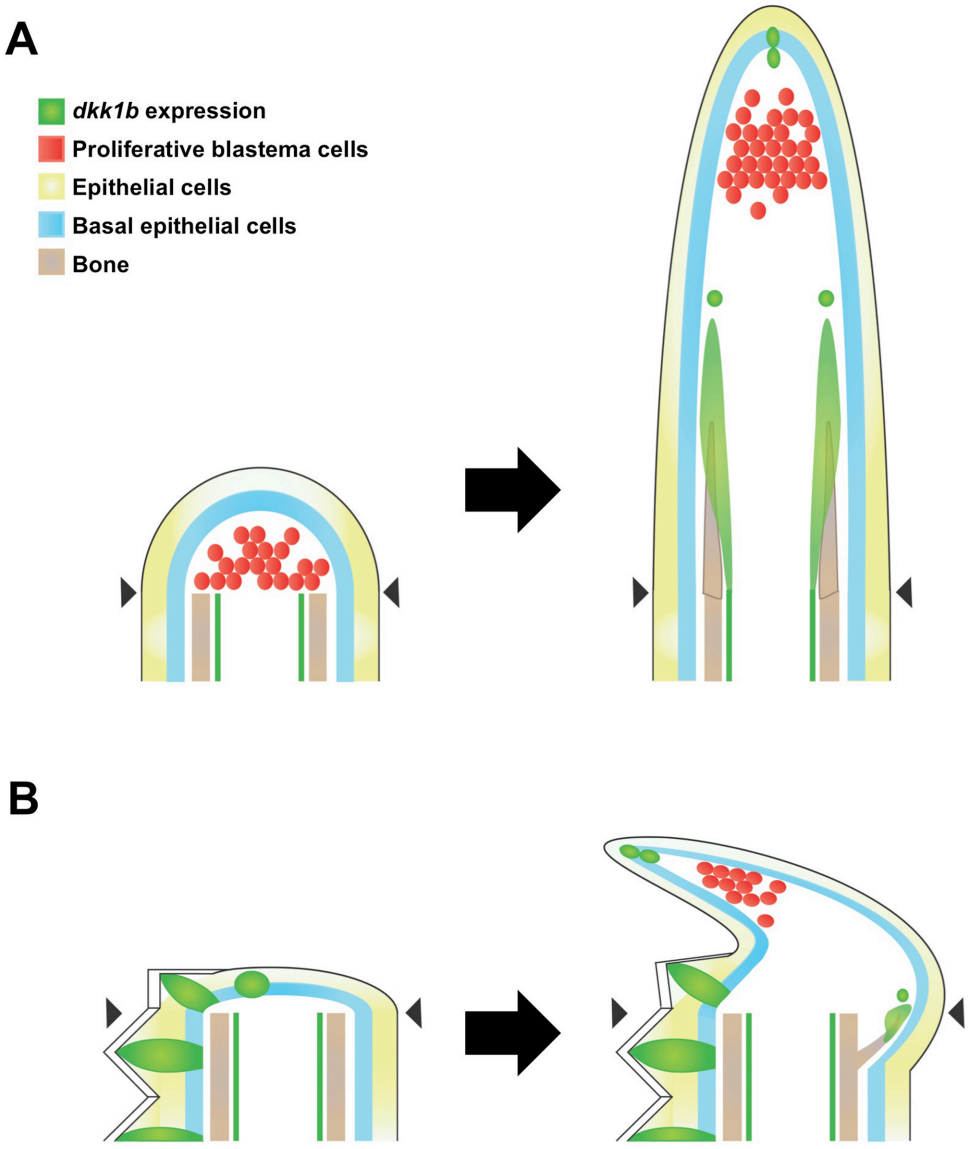

Figure S6. Cartoon Model of Dkk1b Production and Consequences During Zebrafish Fin Regeneration

(A) Regeneration in fins other than male pectoral fins. Dkk1b-producing cells are negligible in the regenerate at 2 dpa prior to regenerative outgrowth (left), but appear adjacent to less proliferative mesenchymal regions by 4 dpa (right). Because Dkk1b has inhibitory effects on blastemal proliferation and fin regeneration, its presence in one or both of these areas is likely to represent an inhibitory component of the regeneration niche.

(B) Regeneration in male pectoral fins. Male pectoral fin ET constitutively produce Dkk1b, positioning this inhibitor in the site of regeneration at 2 dpa (left). By 4 dpa (right), interference by ET-generated Dkk1b inhibits blastema formation, directing initial growth ventrally and potentially mispatterning the regenerate.

Supplemental Tables

Embryo Number	Control (No amputation)	Amputation					
		Female			Male		
		Caudal	Anal	Pectoral	Caudal	Anal	Pectoral
>50	14	7	9	7	12	11	0
11 - 50	3	1	1	0	2	6	0
1 - 10	1	0	0	2	2	0	9
None	2	4	3	4	3	2	13
Total Trials	20	12	13	13	19	19	22

Table S1, related to Figure 2B. Results of Mating Tests After Various Amputation Injuries in Male and Female Zebrafish

Numbers represent the number of mating pairs out of the total trials that yielded a certain number (first column) of embryos. Representative charts are shown in Figure 2B.

Embryo Number	Control	Amputation (Male)				Cuticle (-)
		Pec - One	All Exc Pec	Pec - 20%	Pec - 90%	
> 50	15	17	7	12	1	0
11 - 50	3	1	4	3	0	0
1 - 10	0	0	2	1	3	2
None	2	4	6	2	14	15
Total trials	20	22	19	18	18	17

Table S2, related to Figure 2C. Results of Mating Tests After Various Pectoral Fin Injuries

'Pec – One' indicates that one of the pectoral fins was completely removed. 'All Exc Pec' refers to amputation of all fins except pectoral fins. Pec - 20% and - 90% indicate that 20% or 90% of both fins were amputated, respectively. 'Cuticle (-)' refers to physical removal of cuticles located on ET. Numbers represent the number of mating pairs out of the total trials that yielded a certain number (first column) of embryos. Representative charts are shown in Figure 2C.

Embryo Number	Control (Caudal fin Amputation)	Pectoral fin amputation		
		Good+ Good Good + Mild Good + Severe	Mild+ Mild Mild + Severe	Severe
> 50	66	36	7	0
11 - 50	5	10	4	0
1 - 10	2	0	2	2
None	2	1	3	11
Total Trials	75	47	16	13

Table S3, related to Figure 7A. Results of Mating Tests With Males One or Two Months After Amputation of Caudal or Pectoral Fins

Pectoral fin regeneration phenotypes were scored as in Figure 7A. Males with well regenerated fins mated more effectively than those with defective regeneration. Numbers represent the number of mating pairs out of the total trials that yielded a certain number (first column) of embryos. Representative charts are shown in Figure 7C.

Supplemental Movie Legends

Movie S1. Zebrafish Mating Behaviors

Time 0:00-0:07: Zebrafish mating behaviors acquired by high-speed video. Video indicating behaviors of mating pair as described in Figure 2A.

Time 0:08-0:30: Mating behavior by a male with intact pectoral fins. Video indicating male can use both pectoral fins during mating behavior.

Time 0:31-0:44: Mating behavior by a male in which the one pectoral fin was amputated. When the female is positioned at the side of an intact male pectoral fin, the male can successfully spawn.

Time 0:45-0:53: Failed spawning by a male in which the one pectoral fin was amputated. When the female is positioned at the side of the amputated male pectoral fin, the male cannot grasp her and she easily escapes.

Time 0:54-1:16: Failed spawning by a male in which both pectoral fins were amputated. Although the male pursues the female, he is unable to grasp her and stimulate laying.

Supplementary Experimental Procedures

In situ Hybridization

In situ hybridization on cryosections of 4% paraformaldehyde-fixed fins was performed as described previously (Lee et al., 2009). To generate a digoxigenin-labeled probe for *axin2*, we generated a fragment of *axin2* cDNA using primer sequences: 'Forward: TGGCAGTTCAGCATTTCCAATGGA' and 'Reverse: TCCAGGACAAGGCTACTGGTT'.

RNA Isolation and Quantitative PCR

RNA was isolated from dissected anterior regions of pectoral fins at 2 dpa, using Tri-Reagent (Sigma). cDNA was synthesized from 1 μg of total RNA using the Roche First Strand Synthesis Kit. Quantitative PCR was performed using the Roche LightCycler 480 and the Roche LightCycler 480 Probes Master. All samples were analyzed in biological triplicates and technical duplicates. Primers sequences and probe numbers for *actb2* and *dkk1b* were *actb2* forward primer: AGAGCTACGAGCTGCCTGAC; *actb2* reverse primer: TACCGCAAGATTCCATACCC; *actb2* probe number: 104; *dkk1b*forward primer: CTGAACTTCGCCCTCGATAC; *dkk1b* reverse primer: CCGCATTCCTCATCACTCTC; *dkk1b* probe number: 66.

Supplemental References

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 *132*, 5173-5183.

Lee, Y., Hami, D., De Val, S., Kagermeier-Schenk, B., Wills, A.A., Black, B.L., Weidinger, G., and Poss, K.D. (2009). Maintenance of blastemal proliferation by functionally diverse epidermis in regenerating zebrafish fins. Dev.Biol. *331*, 270-280.