Supporting Information

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SI Methods

RNA Isolation and Microarrays. Fins were amputated from anesthetized adult zebrafish and reamputated 2 and 5 weeks later. Regeneration was allowed to occur at 27°C. Total RNA was isolated from pooled zebrafish fins ($n = 40 \times 3$ arrays) using TRI Reagent (Molecular Research Center). Small RNAs were isolated by passage over mirVana miRNA isolation kits (Ambion). RNA was labeled using Cy5 LabelIt kits (Mirus) and hybridized to arrays in triplicate, as described previously (1). Predicted mRNA targets were obtained by combining various available algorithms (miRanda, miRTar, and miRBase). A complete listing of all raw array data is provided in Table S1. All fold changes (FCs) and *P* values were calculated between active regeneration and adult fin time points. FCs, calculated by normalizing all values <0.1 to 0.1, are given in Table S2.

Northern Blot Analyses. Northern blot analyses were performed as described previously (2) with a U6 probe (TGAAGAGGCCATGCTAATCTTCTC) and an *miR-203a* probe (CAAGTGGTCCTAAACATTTCAC).

***In Situ* Hybridization Experiments.** Fins were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, embedded in agar, sectioned (16 μm) using a cryostat, and stored at −20°C until use. Serial sections were obtained so that one row of each type of experimental fin (at 0 dpa, 6 dpa, and 21 dpa) was present on each slide (VWR Superfrost plus charged slides). Slides were thawed at room temperature for 1 h, and the edges of the slides were sealed with a pap pen. Slides were washed in 1× PBS and incubated in proteinase K (final concentration, 10 μg/ml in 1× PBS) for 30 min at 37°C. Slides were washed quickly in 1× PBS and refixed with 4% PFA for at least 20 min, and then rewashed with 1× PBS. Sections were prehybridized for at least 1 h in prewarmed hybridization buffer (55% formamide, 5× SSC, 50 mg/ml of heparin, 5 mM EDTA, yeast tRNA, and 0.1% Tween-20) at the appropriate temperature (*miR-203*, 46°C; *lef-1*, 70°C). Hybridization was allowed to proceed overnight with probes generated as described previously (3, 4). Slides were then washed in a series of dilutions from 100% hybridization buffer without tRNA or heparin to 100% 2× SSC at the hybridization temperature, followed by two washes in 0.05× SSC for 30 min at the hybridization temperature. A final series of washes were performed at room temperature from 100% 0.05× SSC to 100% PBS with Tween 20 (PBST). Slides were then preblocked for 1 h at room temperature with AB block solution (1× PBST, 2% goat serum, 2 mg/ml of BSA), and then incubated overnight at 4°C in anti-digoxigenin antibody (1:5000 final dilution). Slides were washed three times for 5 min and six times for 15 min in 1× PBST at room temperature, followed by three 15-min rinses in NTMT (100 mM Tris-HCl [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, ddH_2O) at room temperature. The slides were then incubated in BM purple and kept in the dark, with staining allowed to proceed over time. When the desired staining level was obtained, the slides were rinsed in 1× PBST three times for 5 min and then visualized.

1. Thatcher EJ, Flynt AS, Li N, Patton JR, Patton JG (2007). MiRNA expression analysis during normal zebrafish development and following inhibition of the Hedgehog and Notch signaling pathways. *Dev Dyn* 236:2172–2180.
2. Sempere LF, Sokol NS, Dubrovsky EB, Berger EM, Ambros V (2003) Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and *Broad-Complex* gene activity. *Dev Biol* 259:9–18.
3. Wienholds E, *et al.* (2005) MicroRNA expression in zebrafish embryonic development. *Science* 309:310–311.
4. Thisse C, Thisse B, Schilling TF, Postlethwait JH (1993) Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail, and no tail mutant embryos. *Development* 119:1203–1215.

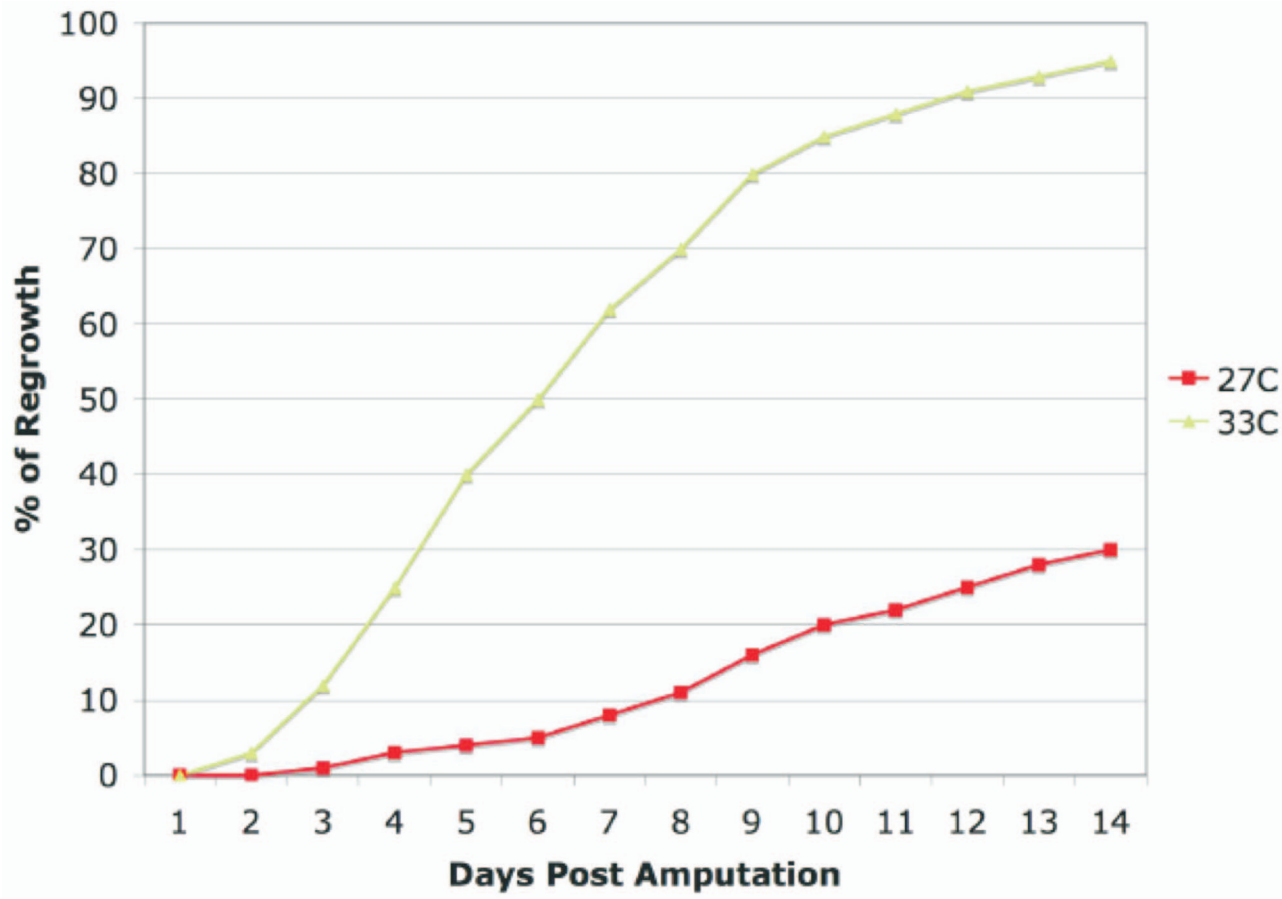

Fig. S1. Effect of temperature on regeneration: plot of regeneration growth over time at 27°C and 33°C. By 5 weeks, regeneration was nearly complete at both temperatures. The microarrays shown in Figs. S2 and S3 were conducted on fins undergoing regeneration at 27°C, whereas functional studies using gain of function and loss of function were conducted at 33°C. *miR-203* levels were dramatically down-regulated after amputation and remained low during regeneration, regardless of the temperature (see also Fig. S5).

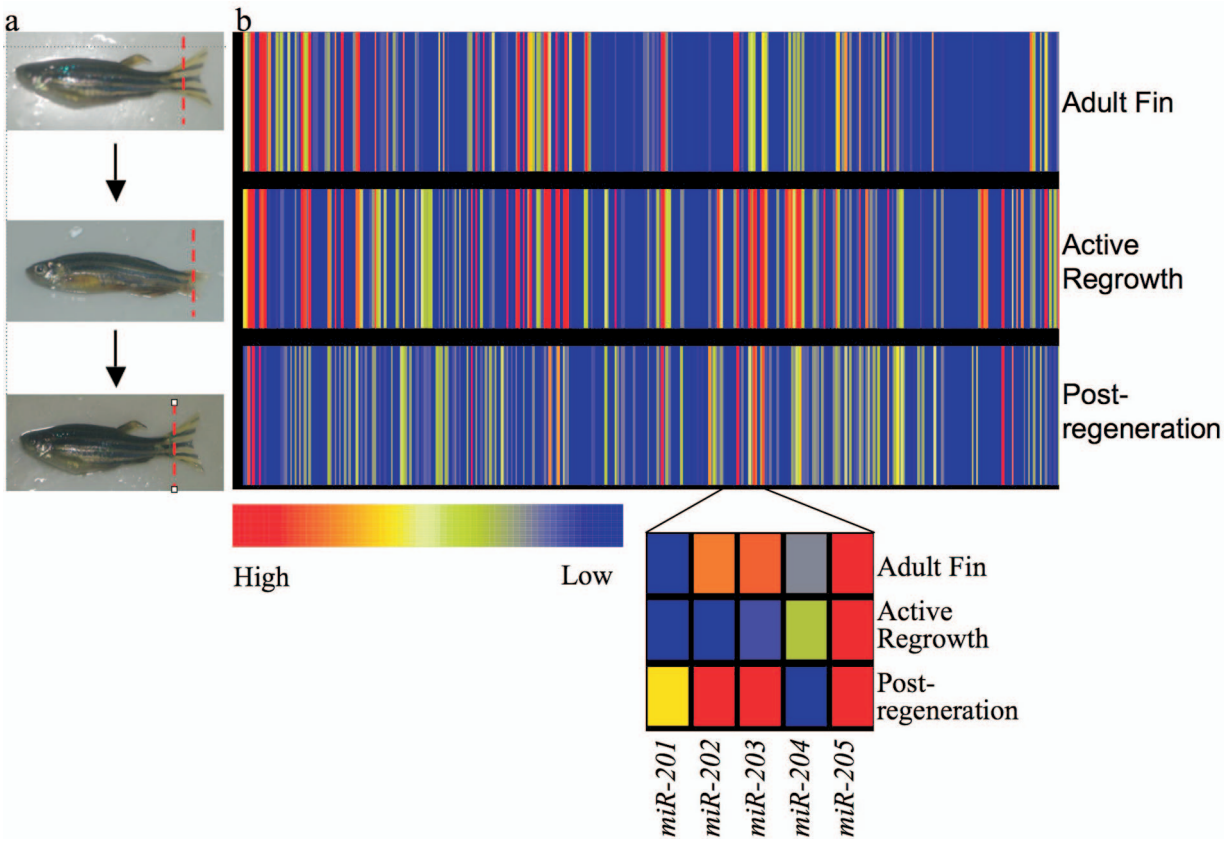

Fig. S2. miRNA Microarray expression analysis. (*a*) Caudal fins were amputated from 120 adult zebrafish and allowed to regenerate for 2 or 5 weeks before reamputation and isolation of RNA. (*b*) Microarrays were performed to examine the expression of 346 miRNAs, and heat maps were generated to illustrate the expression of individual miRNAs. Red indicates a high level of expression, and different shades of blue indicate decreasing expression levels. Changes in expression for a subset of miRNAs across the three time points are depicted (*Bottom*) to illustrate that each of the lines in the global heat maps (*Top*) represent a single miRNA.

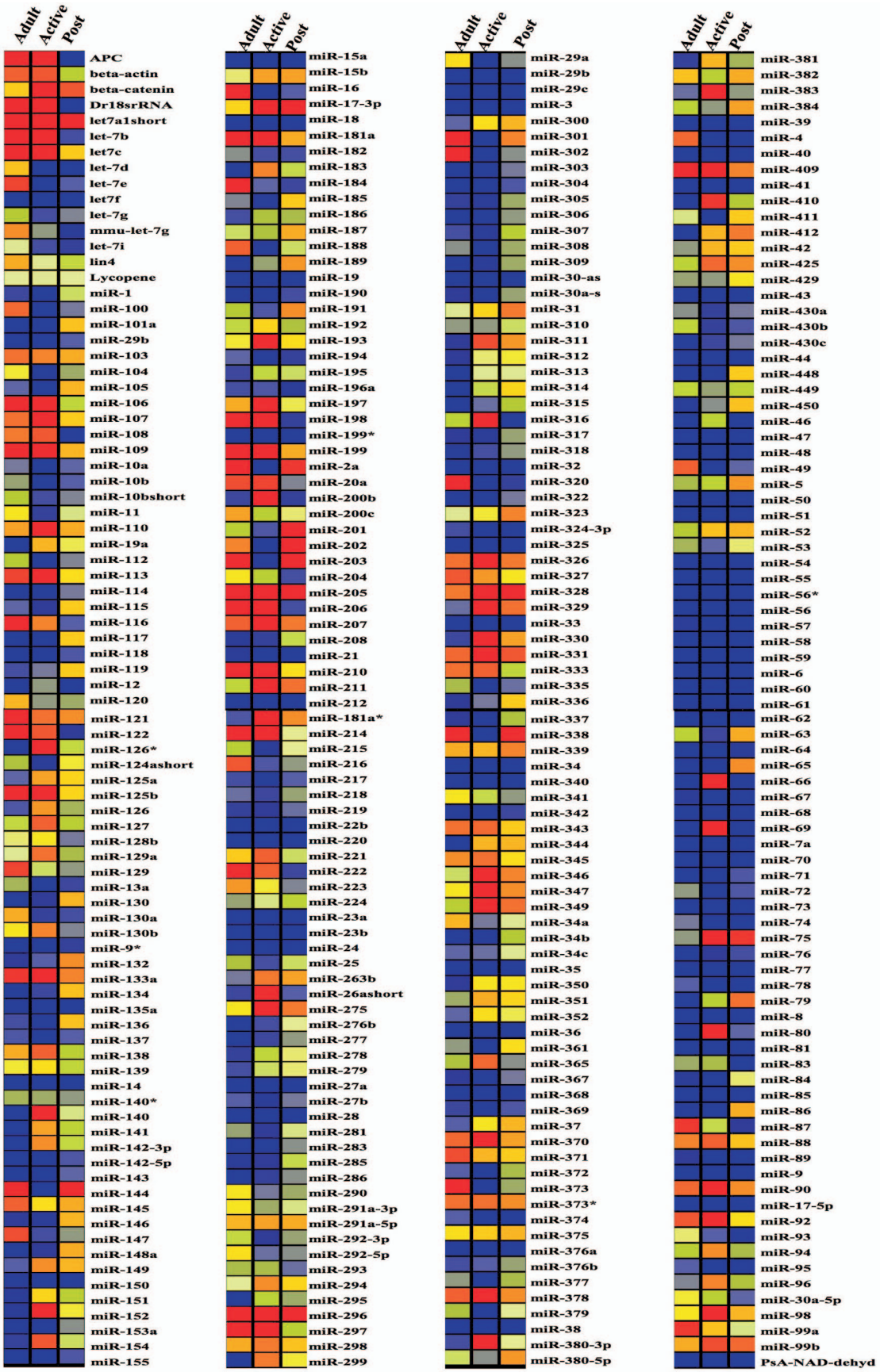

Fig. S3. Microarray expression profiles. Detailed heat maps from individual miRNAs are as shown. Red indicates high expression; blue indicates little to no expression.

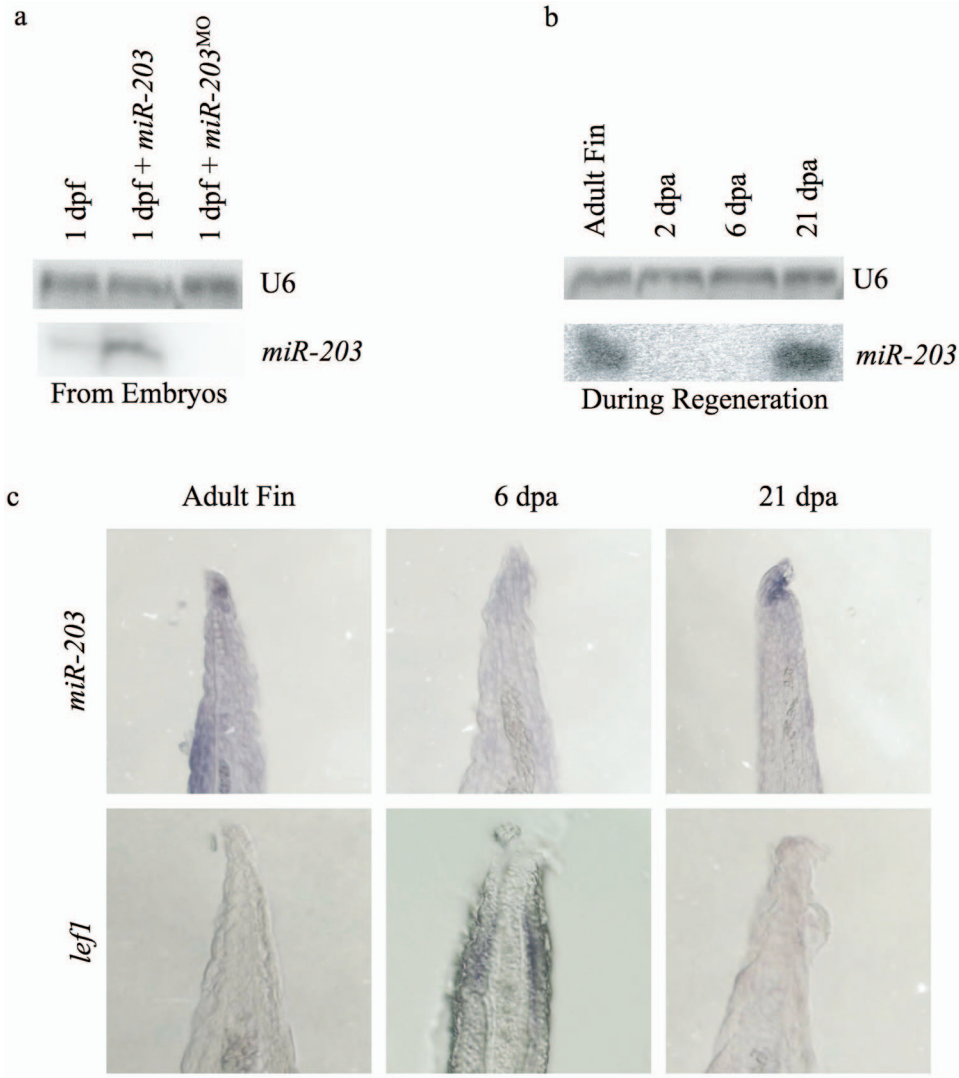

Fig. S4. Analysis of *miR-203* expression during embryogenesis and regeneration. (*a*) Northern blot analyses using a probe against *miR-203* were performed on RNA from embryos extracted at 1 day postfertilization (dpf), 1 dpf after injection of *miR-203* at the single-cell stage, and 1 dpf after injection of *miR-203*^{MO} at the single-cell stage. (*b*) Northern blot analyses were performed on RNA isolated from adult fins (0 dpa) and at 2 dpa, 6 dpa, and 21 dpa using probes against *miR-203*. (*c*) *In situ* hybridizations using locked nucleic acid probes against *miR-203* on adult fin sections at the indicated times after amputation. Each section shows a single lepidotrichia with adjacent mesenchymal tissue. *miR-203* levels were evident in adult fin (0 dpa) both at the distal tip and in the mesenchymal tissue, whereas the levels of *lef1* were barely above background. In contrast, at 6 dpa, *miR-203* levels were mostly undetectable, whereas the signal for *lef1* was readily apparent. As a control, fins also were stained at 21 dpa, at which time *miR-203* levels were readily detectable, especially in the distal tip, and *lef1* levels were at background levels or lower.

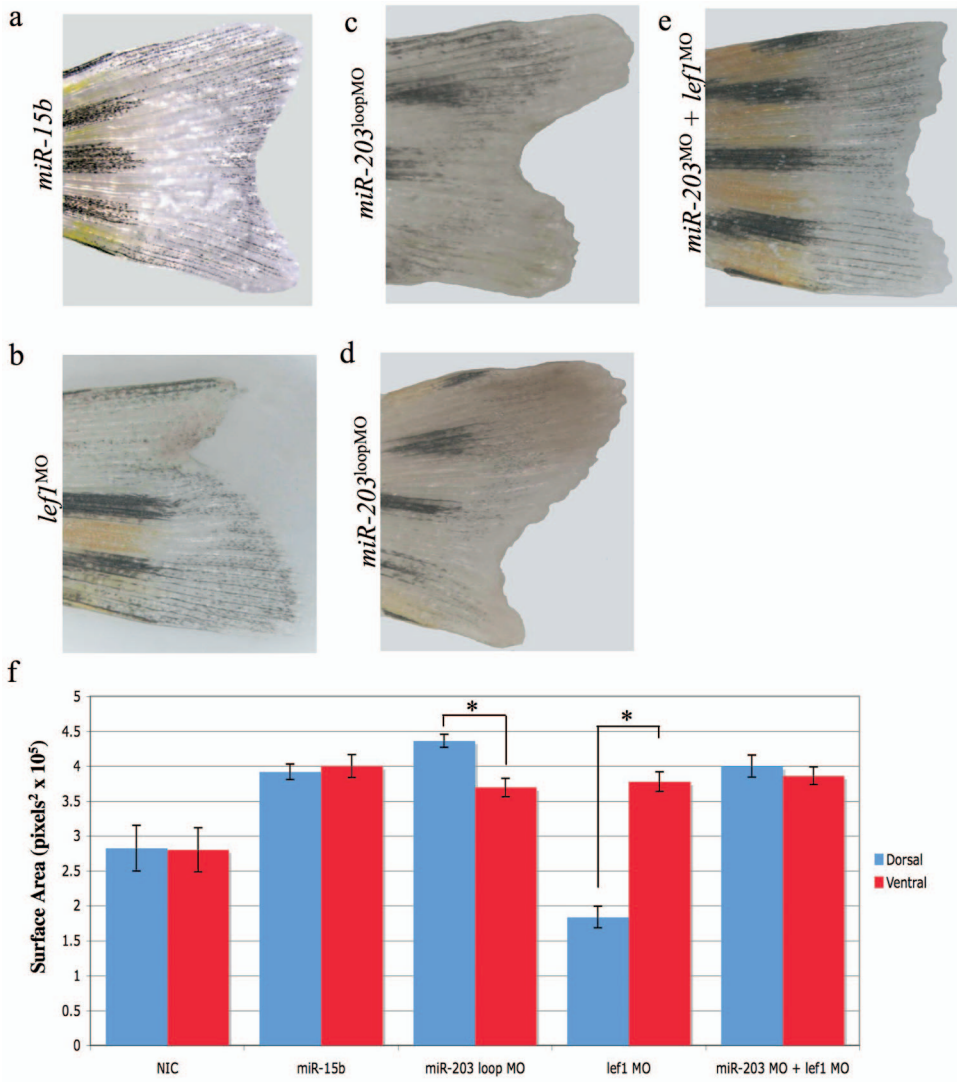

Fig. S5. Regeneration control experiments. (*a*) *miR-15b*, expressed ubiquitously during regeneration, was injected into the dorsal halves of fins at 2 dpa, and regeneration was allowed to proceed for 6 days ($n = 6$). (*b*) A morpholino against the translational start site of *lef1* was injected into the dorsal half of fins at 2 dpa ($n = 12$; $P \ll .001$), and regeneration was allowed to proceed until 6 dpa. (*c* and *d*) A morpholino against the pre-*miR-203* loop region was injected into the dorsal halves of fins at 2 dpa ($n = 12$), and regeneration was allowed to proceed until 6 dpa. (*e*) The overgrowth phenotype observed in the *miR-203*^{MO} injections was rescued by co-injection of *lef1*MO ($n = 12$). (*f*) Quantitative analysis of regeneration. Surface area was calculated using Image J. Asterisks indicate significance of $P \geq .001$.

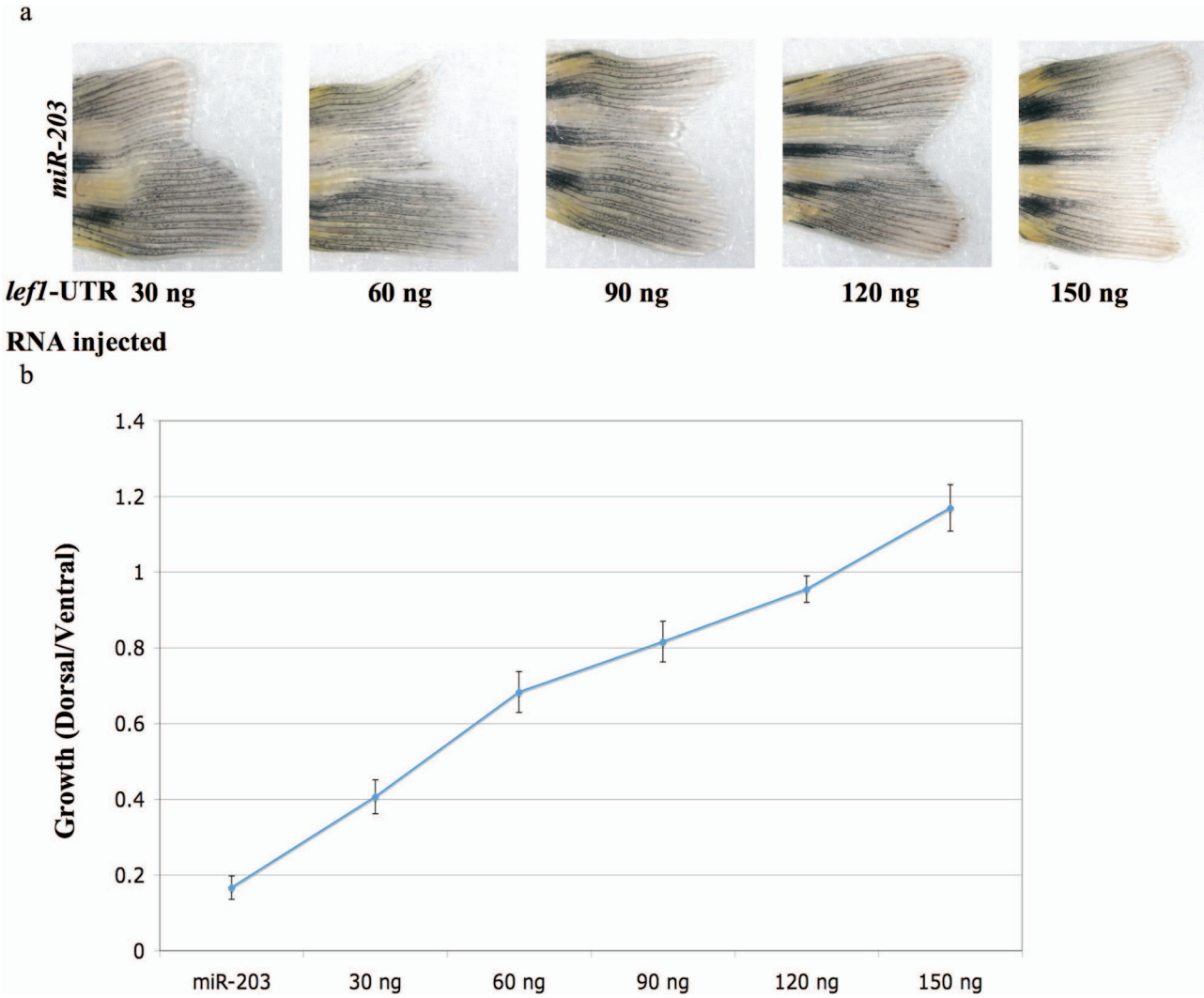

Fig. S6. Dose-dependent rescue of *miR-203* overexpression. Fins were injected with *miR-203* at 2 dpa, followed by co-injection of *lef1* mRNAs lacking recognition elements for *miR-203*. A total of 100 ng of *miR-203* was injected along with the indicated amount of *lef1* mRNA. At 6 dpa, fins were photographed and measured ($n = 6$).

Other Supporting Information Files

Table S1 (PDF)
Table S2 (PDF)