

# Unraveling Tissue Regeneration Pathways Using Chemical Genetics<sup>\*S</sup>

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Identifying the molecular pathways that are required for regeneration remains one of the great challenges of regenerative medicine. Although genetic mutations have been useful for identifying some molecular pathways, small molecule probes of regenerative pathways might offer some advantages, including the ability to disrupt pathway function with precise temporal control. However, a vertebrate regeneration model amenable to rapid throughput small molecule screening is not currently available. We report here the development of a zebrafish early life stage fin regeneration model and its use in screening for small molecules that modulate tissue regeneration. By screening 2000 biologically active small molecules, we identified 17 that specifically inhibited regeneration. These compounds include a cluster of glucocorticoids, and we demonstrate that transient activation of the glucocorticoid receptor is sufficient to block regeneration, but only if activation occurs during wound healing/blastema formation. In addition, knockdown of the glucocorticoid receptor restores regenerative capability to nonregenerative, glucocorticoid-exposed zebrafish. To test whether the classical anti-inflammatory action of glucocorticoids is responsible for blocking regeneration, we prevented acute inflammation following amputation by antisense repression of the Pu.1 gene. Although loss of Pu.1 prevents the inflammatory response, regeneration is not affected. Collectively, these results indicate that signaling from exogenous glucocorticoids impairs blastema formation and limits regenerative capacity through an acute inflammation-independent mechanism. These studies also demonstrate the feasibility of exploiting chemical genetics to define the pathways that govern vertebrate regeneration.

The promise of regenerative medicine is that therapies will be devised to promote the repair or replacement of damaged or diseased tissues and organs. This emerging field is approached from two distinct lines of attack. In recent years, stem cell-based models have been developed to generate a suite of differentiated cells for therapeutic applications. The use of high throughput chemical genetic screening to identify modulators of stem cell fate offers great promise (1). The alternative approach exploits the inherent regenerative capacity of non-mammalian models to define the molecular events that permit tissue regeneration (2). There are several regenerative animal models, including salamanders, newts, zebrafish, hydra, and flatworms, that are established to evaluate tissue regeneration (3–6). What is currently lacking is the availability of a vertebrate regeneration model that is amenable to rapid throughput assessments.

Zebrafish have the remarkable capability to regenerate their fins, optic nerve, scales, heart, and spinal cord (7). Adult caudal fin regeneration is the best studied model for dissecting the molecular signaling that controls regenerative growth and angiogenesis (7, 8). Comparative genomics indicate significant genetic conservation between mammals and lower vertebrates, which begs the question: what are the molecular differences that permit tissue regeneration in zebrafish and make mammalian tissues recalcitrant to regeneration? Answers to this question will provide a path for comparative studies in mammals. Zebrafish recover the lost caudal fin tissue after amputation through a process of epimorphic regeneration, and this occurs in a stepwise manner with the formation of an epithelial wound cap, followed by blastema formation and finally the regenerative outgrowth (Fig. 1) (5, 7). This complex regenerative process is orchestrated by sequential interactions between biomolecules and cells in a spatiotemporal manner. Global gene expression analysis on heart and fin regeneration in adult zebrafish illustrates the involvement of multiple signaling pathways mediated through the differential expression of hundreds of genes during this remarkable process (9–11). The identification of the signaling molecules that control these interactions will offer avenues to rapidly advance the field of regenerative medicine. The characterization of key regulators

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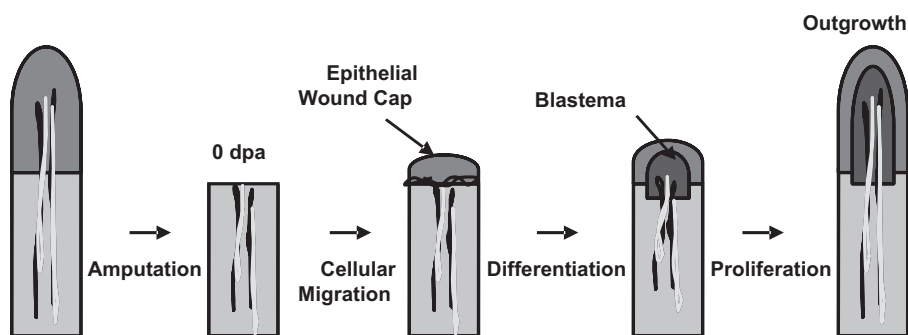


FIGURE 1. **Network of molecular signaling during regeneration.** The schematic diagram depicts the three steps of epimorphic regeneration: 1) wound healing, 2) blastema formation, and 3) regenerative outgrowth.

such as fibroblast growth factor and Wnt as critical factors during regeneration emphasizes the likely involvement of multiple signaling pathways in fin regeneration (12, 13). This again underscores the importance of a comprehensive approach to identify the full repertoire of molecular players required for tissue regeneration. Although adult zebrafish regeneration models have proven useful, many of the molecular and genetic tools that are useful for embryonic and larval studies are not easily applied to adult stage animals (7). Recent results indicate that these technical barriers may be overcome by using an early life stage regeneration model. Specifically, 2-day-old zebrafish larvae completely regenerate their fin primordia within 3 days following amputation (14–16). Because this life stage is inherently amenable to molecular and genetic manipulations such as transient and stable transgenics, genetic mutant screens, and chemical genetics, this model offers a powerful new way to identify novel regulators of tissue regeneration.

*In vivo* high throughput small molecule screening has the potential to target any biological process (17–23); however, this approach has not been applied in a vertebrate regenerative system. To probe tissue regeneration, an inhibitory screen was developed. The underlying premise is that if a chemical inhibits or modulates an essential molecular target, then regeneration will be impacted. The identification of the chemical target will thus help to reveal underlying molecular pathways that permit tissue regeneration. Previous larval fin regeneration studies demonstrated the feasibility of this general inhibitory approach; inhibition of fibroblast growth factor receptor-1 with SU5402 or activation of the aryl hydrocarbon receptor disrupted tissue regeneration (14–16). We report here for the first time an *in vivo* vertebrate regeneration assay that employs a rapid small molecule library screening to identify pathways essential for tissue regeneration. We also demonstrate that this regenerative platform is well suited to identify the molecular targets of small molecules and to define the molecular and cellular mechanism underlying the chemical response.

## EXPERIMENTAL PROCEDURES

**Screening for Inhibitors of Larval Fin Regeneration**—Fertilized eggs were obtained from AB strain zebrafish (University of Oregon, Eugene, OR) for all of the experiments. All embryos were raised in our laboratory according to standard procedures.

Two-day-old embryos were dechorionated and anesthetized with 3-aminobenzoic acid ethyl ester (tricaine). The larvae were laid on an agar plate, and the caudal fin primordia were amputated with a surgical blade just posterior to the notochord. Two amputated larvae were arrayed per well in 96-well plates containing 50  $\mu$ l of E3 embryo buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>). The small molecules (2,000 bioactives from MicroSource Discovery Systems, Gaylordsville, CT) were added individually to the test wells at a final concentration of 25  $\mu$ M. The amputated larvae were incubated for 3 days at 28 °C. Three days post-amputation (dpa)<sup>2</sup> the larvae were anesthetized and assessed visually to score regenerative progression. After the primary screen, beclomethasone dipropionate (beclomethasone, Sigma) was used as a prototype glucocorticoid receptor (GR) agonist. For all functional studies, beclomethasone was used at 1  $\mu$ M final concentration.

**Adult Zebrafish Study**—Adult male zebrafish (AB strain) were pre-exposed for 1 day to vehicle or beclomethasone with waterborne concentrations ranging from 0.05 to 0.0005 mg/liter. The concentration used for the study reported here is 0.005 mg/liter ( $n = 6$ ). After pre-exposure, the fish were anesthetized, and their caudal fins were surgically amputated. The fish were transferred back to the tanks and were continuously exposed to vehicle or beclomethasone until the end of the study. The exposure solutions were changed daily.

**Quantitative Real-time PCR**—The larvae were amputated at 2 days postfertilization (dpf) and exposed to vehicle or beclomethasone, and the regenerating fin tissue was specifically isolated at 1 dpa. Total RNA was extracted from the regenerating fin tissue using the RNAqueous Micro kit (Ambion, Austin, TX). Three groups per treatment, each composed of 150 larval fins, were pooled to make an individual replicate. For RNA from whole embryo, the amputated larvae exposed to vehicle or beclomethasone at 2 dpf were sampled at 3 dpf. Total RNA was isolated from triplicate groups of whole embryos using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Data were quantitatively expressed as the -fold change in the mRNA levels between beclomethasone-exposed embryos and vehicle-exposed embryos after normalizing to  $\beta$ -actin abundance. The  $p$  values represent the significant difference of transcript level between groups as calculated using one-way analysis of variance (ANOVA) and Tukey's method (SigmaStat, Chicago, IL) (see Fig. 4). To quantify GR transcript morpholino knockdown, RNA was isolated from the control or GR morphants at 2 dpf (see Fig. 5C). The transcript levels were

<sup>2</sup> The abbreviations used are: dpa, days postamputation; GR, glucocorticoid receptor; dpf, days postfertilization; ANOVA, analysis of variance; MO, morpholino; BrdUrd, bromodeoxyuridine; hpa, hours postamputation; GFP, green fluorescent protein; qRT, quantitative real-time; GILZ, glucocorticoid-induced leucine zipper.

## Chemical Genetics and Tissue Regeneration

normalized to  $\beta$ -actin abundance. The  $p$  value represents significance as determined by one-way ANOVA. For all of the experiments, cDNA was synthesized from 3  $\mu$ g of total RNA per group using Superscript II (Invitrogen) and oligo(dT) primers in a 20- $\mu$ l volume. Quantitative real-time (qRT)-PCR was conducted using gene-specific primers with the Opticon-2 real-time PCR detection system (MJ Research, Waltham, MA). Briefly, 1  $\mu$ l of cDNA was used for each PCR in the presence of SYBR Green using a DyNAmo SYBR Green qPCR kit according to the manufacturer's instructions (Finnzymes, Espoo, Finland). Agarose gel electrophoresis (see Fig. 5C) and thermal denaturation (melt curve analysis) were conducted to ensure formation of specific products. Primer sequences used were as follows: FK506 binding protein 5 (FKBP5), 5'-CACGTTACAAACACACTGC-3', 5'-ATCAAACGAACAAGCGGGTC-3'; glucocorticoid-induced leucine zipper (GILZ), 5'-CGACTGTTTTATATGGGCTG-3', 5'-TCTTCAGACACCAACATGCC-3'; SRY-box-containing gene 9b (SOX9b), 5'-TGACGAGTTGTTCTCCAGAG-3', 5'-AGGCCACACGTCTATAACCC-3'; and GR, 5'-CAAATGGGCTAAAGCTCTGC-3', 5'-TCTTCAACCCATCCTTCGGC-3'.

**Morpholinos**—Antisense repression of GR was performed using splice variant morpholino (MO) oligonucleotides (Gene Tools, Philomath, OR). A putative zebrafish GR ortholog was identified (GenBank<sup>TM</sup> accession number AB218424). Because there were three predicted transcripts based on alternative splicing, a MO was designed at an intron-exon boundary that was conserved between predicted transcripts. The sequence of GR splice variant MO was 5'-CTGCTTCATGTATTTTAGGTTCCG-3'. The sequence of Pu.1 MO is 5'-GATATACTGATACTCCATTGGTGGT-3' (Gene Tools). Morpholinos were diluted to 3 mM in 1 $\times$  Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5 mM HEPES, pH 7.6) as described (44). A standard control morpholino (5'-CTCTTACCTCAGTTACAATTTATA-3', Gene Tools) was used as the control morpholino (Control-MO). Approximately 2 nl of the appropriate MO solution was microinjected into the embryos at the 1–2-cell stage. The 3' end of the MOs was fluorescein-tagged to screen microinjection success at 24 hpf. The control and GR morphants at 2 dpf were amputated, exposed to vehicle or beclomethasone, and raised for 3 days at 28 °C. The control and Pu.1 morphants were amputated and allowed to grow for 3 days at 28 °C.

**In Situ Hybridization**—Whole mount *in situ* hybridization was conducted on the regenerating fin at different time points as described previously (45, 46). The *msxe* and *dlx5a* probes were obtained from Atsushi Kawakami (14). The *junbl* probe was a gift from Atsushi Kawakami.<sup>3</sup>

**Bromodeoxyuridine (BrdUrd) Incorporation Analysis by Immunohistochemistry**—The amputated larvae that were exposed to vehicle or beclomethasone were incubated with BrdUrd (Roche Applied Science) for 6 h starting from 6 h post-amputation (hpa) or 24 hpa. The larvae were labeled with BrdUrd (10 mM) in a 96-well plate with one larva in each well at 28 °C. After 6 h of incubation with BrdUrd, the animals were

euthanized with tricaine and fixed in 4% paraformaldehyde overnight. The fixed larvae were dehydrated with methanol and then stored in methanol at –20 °C. For performing immunohistochemistry, the larvae were rehydrated using a graded methanol/PBST (phosphate-buffered saline and 0.1% Tween-20) series. The larvae were then treated with Proteinase K (10  $\mu$ g/ml) in PBST for 20 min at room temperature and then rinsed several times with PBST. The larvae were refixed in 4% paraformaldehyde for 30 min and then rinsed several times in water. This was followed by quick rinses in 2 N HCl, then incubation in 2 N HCl at room temperature for 1 h, and washing with PBST several times. The larvae were then blocked with 1% normal goat serum in PBST for 1 h at room temperature and then incubated with anti-BrdUrd antibody (1:100, G3G4, Developmental Studies Hybridoma Bank, Iowa City, IA) overnight at 4 °C. After four 30-min washes with PBST, the larvae were incubated with a secondary antibody (1:1000, Alexa 546-conjugated goat anti-mouse, Molecular Probes, Eugene, OR) for 4 h at room temperature. The larvae were then washed four times for 30 min in PBST and visualized by epifluorescence microscopy. The BrdUrd-labeled fluorescent cells were quantified with the images using the Image-Pro Plus software program (Media Cybernetics, Inc., Silver Spring, MD).

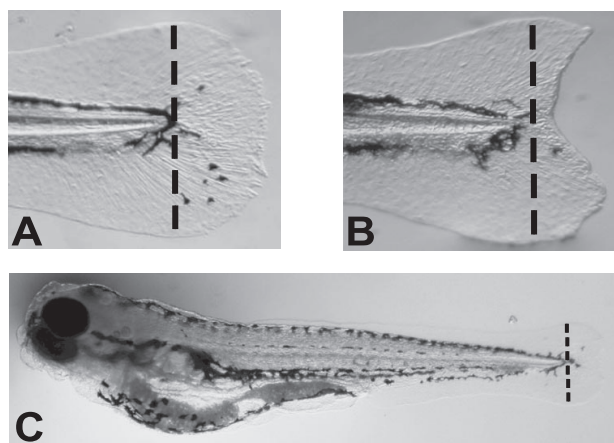
**Morphologic Characterization by Histological Analysis**—The amputated larvae exposed to vehicle or beclomethasone were euthanized and fixed in 4% paraformaldehyde at 1 dpa. The fixed larvae were postfixed in 1% osmium tetroxide and embedded in Embed 812-Araldite 502 resin. Semithin serial sections were cut at 1  $\mu$ m, stained with toluidine blue, and analyzed by light microscopy.

**Neutrophil and Macrophage Migration Assay**—The Tg(BACmpo:gfp)<sup>i114</sup> transgenic line larvae were anesthetized and amputated at 3 dpf as described above. At the time points indicated, amputated larvae were taken from predetermined wells, the numbers of green fluorescent protein (GFP)-positive neutrophils were counted (39), and in the same fish the number of neutral red positive macrophages were assessed. For macrophage count, the amputated larvae at 3 dpf were exposed to neutral red at a final concentration of 2.5  $\mu$ g/ml for 4 h prior to assessment. The macrophages were stained red and were counted at the amputation site as illustrated in Fig. 9, C, D, G, and H.

## RESULTS AND DISCUSSION

**Development of Rapid Throughput Zebrafish Regeneration Assay**—Because regeneration is accomplished by an orchestrated coordination of multiple pathways and signaling events, a vertebrate regeneration assay was developed to identify small molecules that specifically modulated tissue regeneration. To demonstrate the power of this approach, a 2000-member structurally diverse bioactive small molecule library was screened to identify inhibitors of regeneration. Two-dpf larvae were amputated, transferred to 96-well plates, and continuously exposed to individual chemicals. At 3 dpa, the larvae were microscopically imaged to assess regenerative progression. A total of 17 small molecules (~0.8% of the library) inhibited tissue regeneration. These inhibitory chemicals comprised several different functional classes such as anti-inflammatory, keratolytic, cyto-

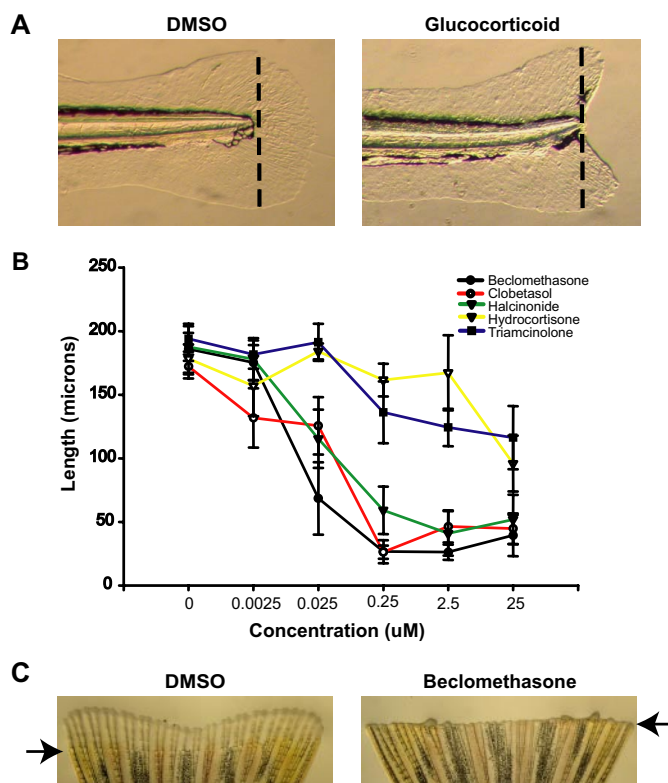
<sup>3</sup> T. Ishida and A. Kawakami, manuscript in preparation.



**FIGURE 2. Rapid screening for inhibitors of larval fin regeneration.** The 2-dpf larvae were surgically amputated and exposed to the chemicals in a 96-well plate continuously for 3 days at 28 °C. Larvae were screened for inhibitors of larval fin regeneration at 3 dpa. *A*, a typical example of complete regeneration; *B*, impaired regeneration; *C*, a larvae that is able to regenerate its fin but displays overt embryo toxicity.

chrome P-450 inhibitor, etc. Representative images of complete and impaired regeneration are depicted (Fig. 2, *A* and *B*). Although a number of small molecules produced overt toxicity at the test concentration (25  $\mu$ M) leading to systemic edema by the end of the assay, these animals completely regenerated their fin tissue (Fig. 2*C*). This specificity indicates that a toxic response can be uncoupled from the regeneration response.

**Identification of Glucocorticoids as Modulators of Regeneration**—The positive “hits” were of different chemical classes, and using structure–function analysis, a major cluster of compounds was identified as glucocorticoids (five of the hit compounds). Although the main focus of this study was to demonstrate the feasibility of using small molecules to probe tissue regeneration, we also wanted to demonstrate the ability to identify small molecule targets rapidly during early life stages. We selected glucocorticoids for further studies because this was the largest cluster of hits. Glucocorticoids consistently and specifically inhibited regeneration without inhibiting normal growth, creating a V-shaped fin (Fig. 3*A*). This characteristic morphology occurs because the tissue lateral to the amputation plane continues to grow and partially collapses around the amputation plane. Glucocorticoids are steroid hormones that exert most of their actions by binding the GR (24–26). From mammalian studies it is clear that there are several isoforms of GR because of differential splicing and alternative translation initiation sites, of which GR $\alpha$  and GR $\beta$  are the best-studied (26–28). Binding of glucocorticoid activates GR $\alpha$  transcriptional activity, leading to the initiation or repression of transcription, whereas GR $\beta$  is not able to bind ligands and has a dominant negative activity through inhibition of GR $\alpha$  transcriptional activity (29). In addition to the classical genomic model of GR activation, nongenomic activities have also been reported at high concentrations of glucocorticoids (30, 31). To broadly analyze the mechanism of action from the positive hits, dose response studies were completed with selected glucocorticoids by measuring the length of maximum outgrowth. The length of maximum outgrowth is the distance from the center of the amputation plane to the tip of the regenerating fin (15),



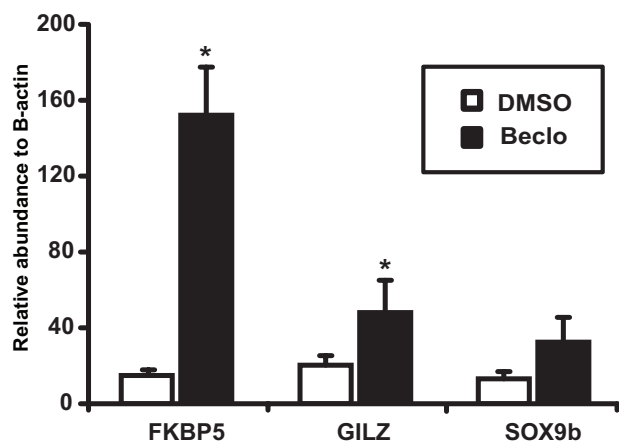
**FIGURE 3. Glucocorticoids inhibit regeneration.** *A*, the caudal fin primordia of larvae at 2 dpf were amputated and exposed to vehicle or glucocorticoid continuously for 3 days at 28 °C. The regeneration images were captured at 3 dpa, and the broken line indicates the plane of amputation. These images are representative of 12 larvae. *B*, the tissue regenerate length from the original plane of amputation was measured at 3 dpa in the presence of the indicated concentrations of selected glucocorticoids. The regenerate length was calculated from the center of the plane of amputation to the tip of the regenerating fin. *C*, the inhibitory effect of glucocorticoids on regeneration was also assessed in adults to determine whether chemical hits from the larval screen would be predictive for the better established adult regeneration model. Adult zebrafish were amputated and continuously exposed to beclomethasone (0.005 mg/liter) ( $n = 6$ ). The images were captured at 5 dpa, and the arrow indicates the plane of amputation. *DMSO*, dimethyl sulfoxide.

and the IC<sub>50</sub> for regeneration ranged from 200 to 400 nM (Fig. 3*B*). Beclomethasone dipropionate has a potent inhibitory effect on regeneration at the lower nanomolar range; hence, this small molecule was selected for further experiments.

Because the adult fin regeneration model is more widely studied, it was important to determine whether chemical hits identified in the larval screen would be predictive for the adult fin regeneration responses. Therefore, the regeneration response to glucocorticoid exposure on fin regeneration was also assessed in adult zebrafish. Similar to the larvae, beclomethasone exposure inhibited adult caudal fin regeneration, and small projection-like structures were observed at the plane of amputation (Fig. 3*C*). The similar inhibitory response suggested that a common underlying molecular target was targeted by this chemical.

**Beclomethasone Induces Expression of GR Target Genes**—Because the IC<sub>50</sub> of glucocorticoids for regeneration was in the nanomolar range, it is highly likely that the glucocorticoids are acting via activation of the GR. To test whether beclomethasone activates the GR pathway, three primary GR target genes were identified, including *gilz*, *fkbp5*, and *sox9b* (32–35). To

## Chemical Genetics and Tissue Regeneration



**FIGURE 4. Expression of GR primary target genes is induced by beclomethasone.** Two-day-old larvae were amputated and exposed to vehicle or beclomethasone (*Becl*). qRT-PCR was conducted on the regenerating fin tissue using gene-specific primers for the primary GR target genes *fkbp5*, *gilz*, and *sox9b*. The abundance of the message levels was normalized to  $\beta$ -actin expression. The expression of *fkbp5* ( $p < 0.004$ ) and *gilz* ( $p < 0.024$ ) was induced significantly in beclomethasone-exposed larval fin tissue. The respective values represent the mean  $\pm$  S.E., and the asterisks indicate statistically significant differences (one-way ANOVA,  $n = 3$ ). DMSO, dimethyl sulfoxide.

analyze the expression of these genes, qRT-PCR was conducted using RNA from the larval regenerating fin tissue at 1 dpa in the presence and absence of beclomethasone. All three genes were significantly induced in response to beclomethasone exposure, indicating that the GR is activated by glucocorticoids in this regeneration model (Fig. 4). Similarly, qRT-PCR analysis was performed in the whole embryo at 3 dpf after exposure to beclomethasone at 2 dpf, and the global expression of those genes was similar in pattern to the regenerating fin tissue (supplemental Fig. 1). These results suggest that beclomethasone is activating the GR and that the inhibitory effect on regeneration could be mediated through inappropriate GR activation.

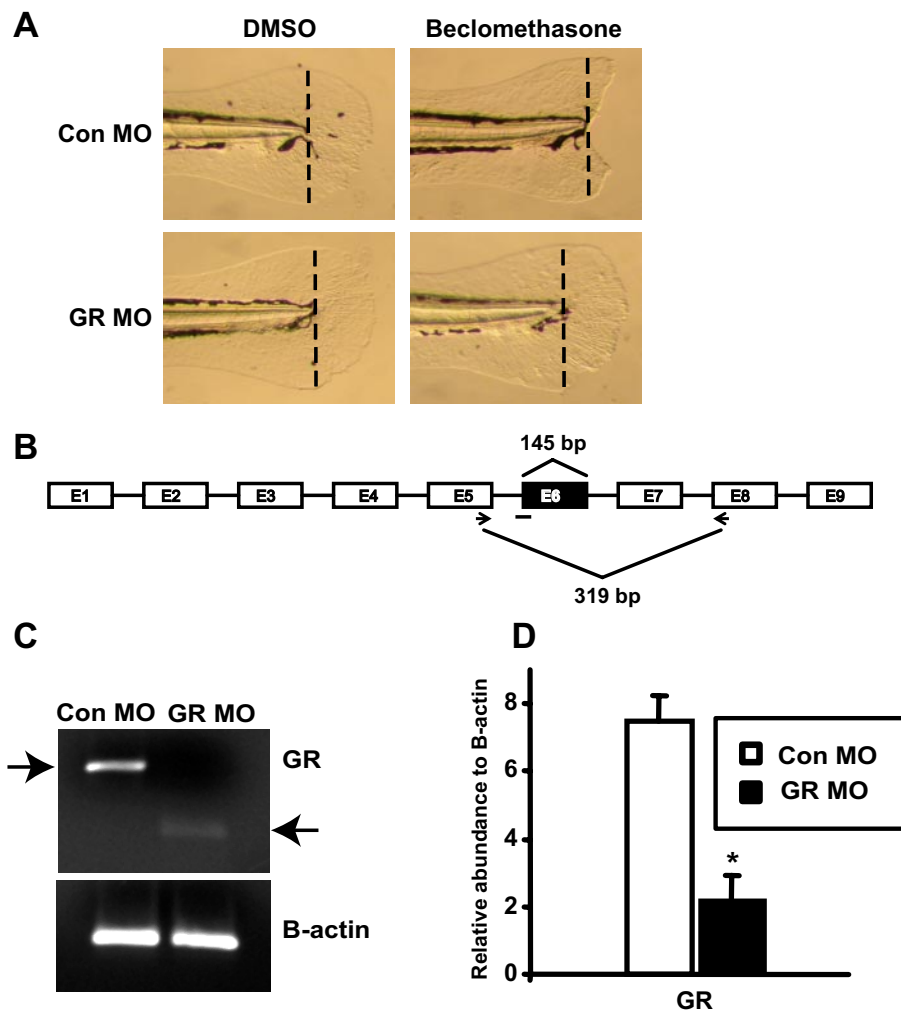
**GR Activation Is Required for Beclomethasone to Inhibit Regeneration**—To directly determine whether the GR was the molecular target of beclomethasone and to determine whether the GR was necessary for the glucocorticoid-mediated inhibition of regeneration, a morpholino was designed to block splicing of the exons encoding the well conserved GR ligand-binding domain (Fig. 5B). Transient knockdown of GR did not elicit obvious early developmental defects, suggesting that the GR is not essential for early embryonic development (data not shown). The standard control morpholino-microinjected larvae (morphants) and GR morphants were amputated at 2 dpf and continuously exposed for 3 days to vehicle or beclomethasone. It is noteworthy that the control and GR morphants exposed to vehicle completely regenerated their fin tissue, emphasizing that endogenous GR is not required for larval fin regeneration (Fig. 5A). Control morphants exposed to beclomethasone failed to regenerate, whereas knockdown of GR completely restored regenerative capacity in the presence of beclomethasone (Fig. 5A). These results indicate that impaired regeneration by beclomethasone is mediated through ligand-activated GR. To confirm GR morpholino efficacy, qRT-PCR was performed with GR-specific primers in control and GR morphants. The GR primary transcript was detected in the control morphants but was not detected in the GR morphants,

indicating efficient knockdown of GR transcripts (Fig. 5C). The predicted misspliced transcript was also detected as a lower band (Fig. 5C), and the size of this product is consistent with the loss of the targeted exon. Additionally, qRT-PCR analysis revealed significant reduction in the GR transcript between control and GR morphants (Fig. 5D). These results indicate that the molecular target for glucocorticoids identified in the small molecule screen is the GR. Collectively, inappropriate ligand-activated GR blocks tissue regeneration, and the next goal was to begin to identify the events downstream of GR that mediate this response.

**Glucocorticoids Target Early Stages of Regeneration**—A significant advantage of chemical genetics is that the initiation and termination of the chemical exposure can be tightly controlled; therefore, the screen can be designed to probe any stages of this complex process. We have reported previously that aryl hydrocarbon receptor ligands block regeneration at both early and late stages in adult and larval zebrafish (15, 36). To define the regenerative stage that is most responsive to GR activation, beclomethasone ( $1 \mu\text{M}$ ) was added beginning at a number of distinct time windows postamputation. Larvae that were exposed to beclomethasone immediately following amputation for just 4 h failed to regenerate (Fig. 6, A and B). However, larvae exposed to beclomethasone beginning at 4 hpa and then continuously until 3 dpa were nonresponsive, as they completely regenerated their fins. These data indicate that glucocorticoids exclusively target early stages of regeneration, which encompass wound healing/blastema stages in larvae (Fig. 6, C and D). In adult zebrafish, beclomethasone exposure for just 1 dpa was not sufficient enough to block regeneration when assessed at 5 dpa (data not shown), indicating slight differences in the regenerative window of sensitivity. The differential response in adult zebrafish could be due to variations in the pharmacokinetics, metabolism, and drug efficiency in the complex adult regeneration system.

**Beclomethasone Impairs Wound Epithelium and Blastema Formation**—Because the sensitive window of exposure in larval zebrafish is within the first 4 h of regeneration, we performed *in situ* localization with a molecular marker that defines the wound epithelium, which is the first step of regeneration that occurs after amputation of the fin tissue. The expression of the basal wound epidermis marker *dlx5a* (10, 14) was used to assess whether beclomethasone exposure affected the formation of an intact wound epithelium. The larvae exposed to vehicle revealed a strong expression of *dlx5a* at 1 dpa in the wound epithelium of the regenerating fin, whereas the larvae exposed to beclomethasone failed to express *dlx5a* (Fig. 7, A and B). This indicates that the wound epithelium is not properly formed in the beclomethasone-exposed larvae and that the wound epithelium could be the primary target of GR activation, which indeed supports the sensitivity of the critical window of early exposure.

To further characterize whether blastema formation is impacted by beclomethasone, we assessed the expression of two blastema markers *msxe* (14) and *junbl*<sup>3</sup> by *in situ* hybridization. The normal expression of *msxe* in the blastema region just beneath the plane of amputation was present in the vehicle-exposed larvae at 1 dpa, but *msxe* expression was absent in the beclomethasone-exposed larvae (Fig. 7, C and D). Similarly, the



**FIGURE 5. Activation of GR is required for inhibition of regeneration by beclomethasone.** *A*, control (*Con MO*) and GR morphants (*GR MO*) were amputated and exposed to vehicle or beclomethasone at 2 dpf for 3 days. The images were acquired on 3 dpa. *B*, the bold line at the intron-exon (I5-E6) boundary designates the gene sequence targeted with the GR splice variant MO. The arrows at E5 and E8 indicate the forward and reverse primers designed for qRT-PCR to analyze the splice-blocking efficiency. The splice variant MO should result in the loss of E6 to get a smaller misspliced PCR product. *C*, the analysis of GR transcript in control and GR morphants after qRT-PCR followed by agarose gel electrophoresis is shown. The upper arrow denotes the PCR product for the primary GR transcript, and the lower arrow points to the misspliced GR variant after the loss of targeted E6.  $\beta$ -Actin expression was used as the loading control. *D*, the results of qRT-PCR analysis for the GR gene between control and GR morphants are shown. The relative abundance of GR mRNA levels is illustrated ( $p < 0.045$ ), and the asterisk indicates statistically significant differences (one-way ANOVA,  $n = 3$ ). DMSO, dimethyl sulfoxide.

*junbl* blastema marker, which was highly expressed in the blastema of the vehicle-exposed larvae at 1 dpa, was completely absent in beclomethasone-exposed larvae (Fig. 7, *E* and *F*), suggesting that GR activation by beclomethasone inhibits blastema formation, which is essential for fin regeneration. Additionally, we assessed the formation of blastema at 1 dpa by light microscopic analysis of semithin sections of vehicle- or beclomethasone-exposed larvae. In the vehicle-exposed larva, a solid dense blastema was formed immediately proximal to the amputation site (Fig. 7*G*). In the beclomethasone-exposed larvae, cells were very loosely arranged and separated by wide empty spaces (intercellular spongiosis/edema) immediately proximal to the amputation site, resulting in the formation of abnormal blastema (Fig. 7, *H1* and *H2*). In addition, many of the epithelial cells in beclomethasone-treated larvae displayed cytoplasmic vacu-

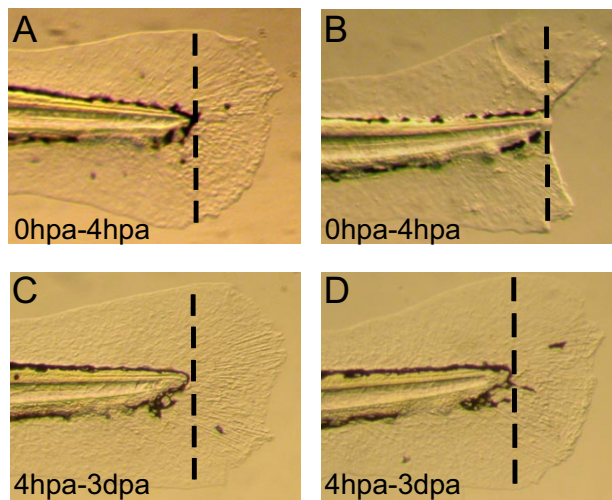
olation, and the epithelium was separated from deeper cell layers. Altogether, these results indicate that inappropriate GR activation impacts signaling molecules critical for wound healing and blastema formation.

**Cell Proliferation Is Affected by Beclomethasone**—To determine the impact of GR activation on early regeneration stage cellular proliferation, BrdUrd incorporation proliferation studies were completed at 6–12 and 24–30 hpa, reflective of pre- and post-blastema formation in larval fin regenerates. At 6–12 hpa, many BrdUrd-labeled cells were observed just beneath the plane of amputation in the vehicle-exposed larvae, whereas there was significant reduction in the number of proliferating cells in the beclomethasone-exposed larvae (Fig. 8*A*). There was also a significant reduction in cellular proliferation in the beclomethasone-exposed larvae compared with vehicle at 24–30 hpa (Fig. 8*A*) and at 12–24 hpa (data not shown). Similar to previous reports, we also observed a cluster of BrdUrd-labeled cells at the posterior and ventral side of the notochord that is likely to contribute to the normal development of the caudal fin region (14). Irrespective of the chemical treatment, there was no difference in the number of cells at the ventral side of the notochord, suggesting that the normal growth of the caudal fin region is not affected by beclomethasone; rather, the inhibitory effect is very specific to the regenerating fin tissue.

Altogether, these results indicate that GR activation results in the inhibition of cell proliferation at multiple regenerative stages.

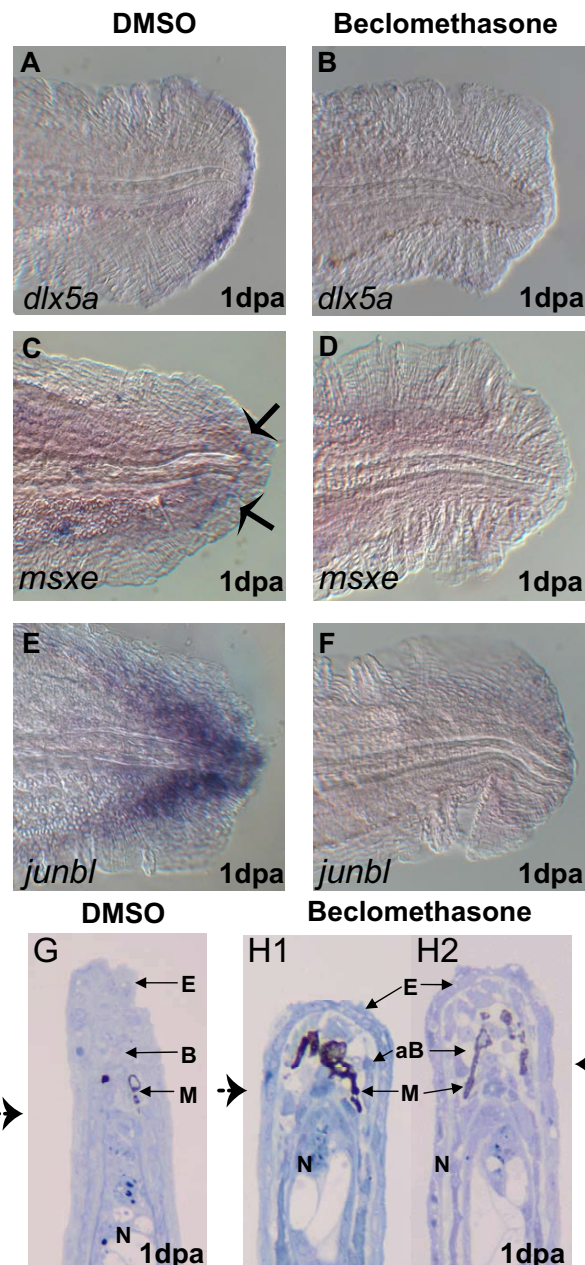
**Neutrophils and Macrophages Are Not Essential for Regeneration**—Of the multiple actions of glucocorticoids, perhaps the best understood is the immunosuppressive effects on the acute-phase inflammatory response (26, 37). It is known that amputation of the caudal fin in zebrafish induces neutrophilic inflammation (38, 39), and work from other model systems suggests that newly recruited neutrophils and macrophages secrete cell-signaling molecules, such as growth factors and cytokines, that are considered important for wound healing and tissue repair. We therefore hypothesized that the action of glucocorticoids on inhibiting regeneration might occur via inhibition of macrophage or neutrophil migration to the site of

## Chemical Genetics and Tissue Regeneration



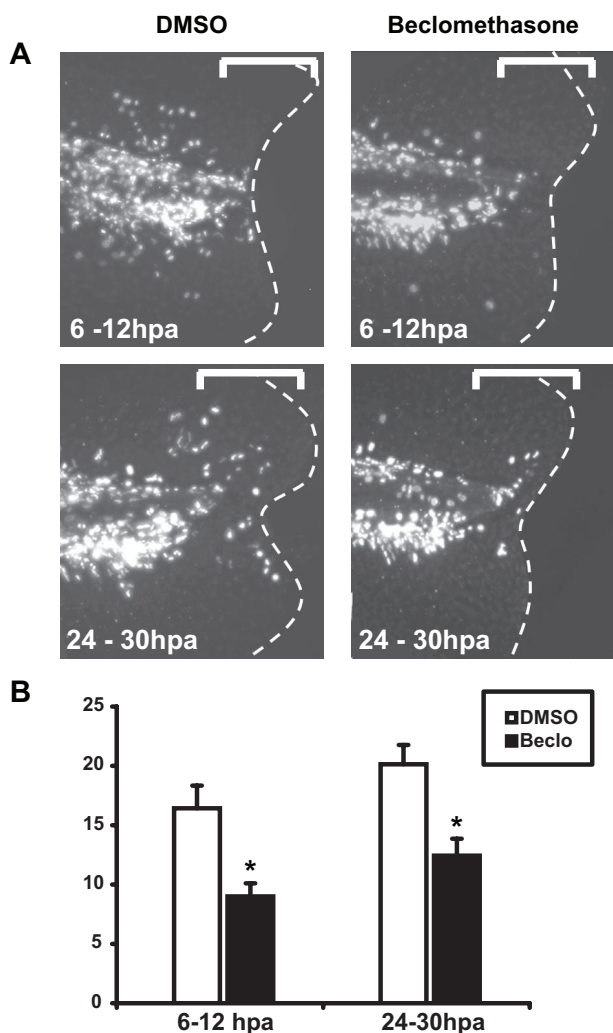
**FIGURE 6. Beclomethasone specifically impacts early stages of regeneration.** The larvae were amputated at 2 dpf and exposed to vehicle (A) or beclomethasone (1  $\mu\text{M}$ ) (B) until 4 hpa and then raised in fish water for 3 days. Images were acquired 3 days later (3 dpa). Larvae were amputated at 2 dpf and were allowed to regenerate for just 4 h before continuous exposure to vehicle (C) or beclomethasone (1  $\mu\text{M}$ ) (D) and the image acquisition at 3 dpa. Beclomethasone was used at varying concentrations from 0.25 to 25  $\mu\text{M}$  to confirm these results (data not shown). Twelve larvae were used per group.

injury. To assess whether beclomethasone affects the infiltration of neutrophils or macrophages to the amputation site, we utilized the Tg(BACmpo:gfp)<sup>i114</sup> line, which expresses GFP under the control of the neutrophil-specific myeloperoxidase (mpo/mpx) regulatory region (39). Tg(BACmpo:gfp)<sup>i114</sup> larvae were amputated at 72 hpf and exposed to vehicle or beclomethasone as described above. There was a modest reduction in the number of neutrophils (GFP-positive cells) at the amputation site of the beclomethasone-exposed larvae at 4 and 8 hpa compared with vehicle-exposed larvae, raising the possibility that the glucocorticoid response could be mediated by a small reduction in neutrophils (Fig. 9, A and B). Similarly, the number of macrophages that migrated to the amputation site was quantified by neutral red staining, and there was no significant difference in the number of macrophages between vehicle- and beclomethasone-exposed larvae (Fig. 9, C and D). It remained a possibility that the block in regeneration by glucocorticoids was mediated either by a reduction in neutrophils at the amputation site or by the suppression of important regenerative cytokine produced by invading inflammatory cells. To determine directly the importance of myeloid cells in larval fin regeneration, we used antisense morpholinos to target Pu.1, a transcription factor required to permit myeloid cell development (40–42). After amputation, Pu.1 morphants completely regenerated their caudal fin indistinguishably from control morphants, indicating that neutrophils and macrophages are not required for normal larval fin regeneration (Fig. 9, E and F). This significantly extends the findings of wound healing studies in the Pu.1 null mice where tissue repair proceeds in the complete absence of neutrophils and macrophages (43). The efficiency of Pu.1 MO was confirmed by assessing the GFP-positive cells for neutrophils and performing neutral red staining for macrophages. Both neutrophils and macrophages were detected in the control morphants but were completely absent at the amputation site in the Pu.1 morphants at 8 hpa



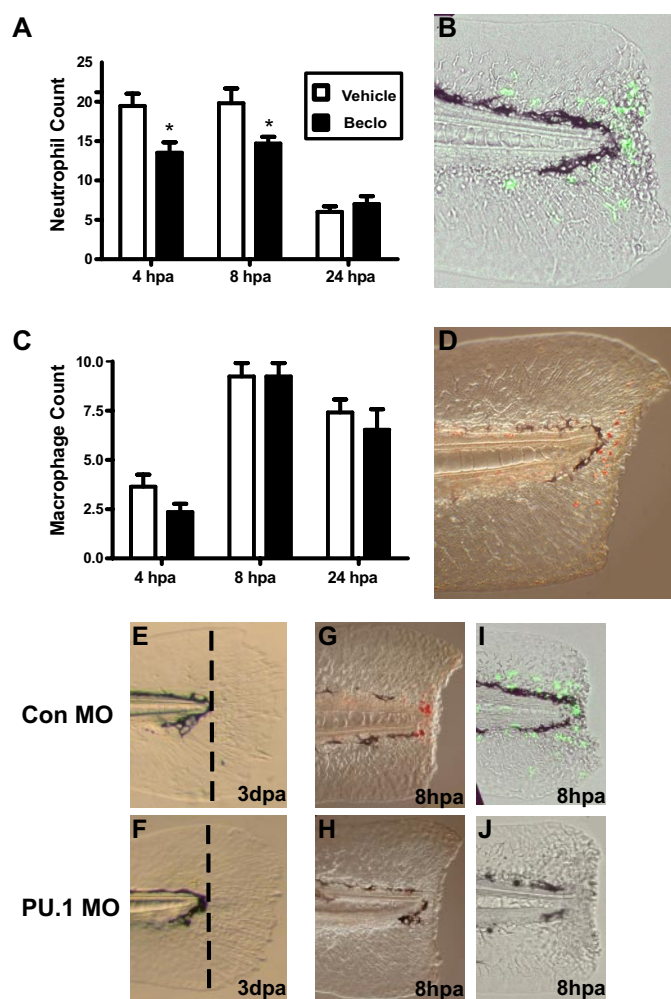
**FIGURE 7. Wound healing and blastema formation are affected by beclomethasone.** *In situ* hybridization was performed with amputated larvae exposed to vehicle or beclomethasone using different molecular markers at 1 dpa. The expression of wound epithelium and blastema markers in the regenerating fin at 1 dpa exposed to vehicle or beclomethasone with *dlx5a* (A and B), *msxe* (C and D), or *junbl* (E and F) are shown. G, images are of methylene blue-stained semithin sections of larvae taken at  $\times 400$  by light microscopy. Two images of the same beclomethasone-exposed larvae sectioned at different levels are depicted in H1 and H2. Orientation of beclomethasone-treated larvae within the blocks was slightly oblique. The level of the amputation site was identified by the presence of multifocal necrosis with the notochord. The arrowheads indicate the plane of amputation. B, blastema; aB, abnormal blastema; E, epithelium; M, melanocyte; N, notochord; DMSO, dimethyl sulfoxide.

(Fig. 9, G–J). These results illustrate for the first time that acute inflammation through neutrophils and macrophages is not absolutely required for the initiation of regeneration in zebrafish. Moreover, the similarity in the functional role of the Pu.1 gene during wound healing between mice and zebrafish underscores the conserved function of genes across different vertebrate animals.



**FIGURE 8. Proliferation of cells is impacted by beclomethasone at different regenerative stages.** *A*, amputated larvae exposed to vehicle or beclomethasone were incubated with BrdUrd at 6–12 and 24–30 hpa. BrdUrd-labeled cells between vehicle- or beclomethasone-exposed larvae at 6–12 and 24–30 hpa are shown. The images are representative of seven larvae and the experiment was repeated twice. The brackets represent the area analyzed for the count of proliferating cells. The dashed line represents the boundary of the regenerating fin. *B*, quantification of the cell proliferation between vehicle- and beclomethasone-exposed larvae is shown. The respective values represent the mean  $\pm$  S.E. (one-way ANOVA and Tukey's method,  $n = 7$ ). There was significant reduction (\*) in the number of proliferating cells in the beclomethasone-exposed larvae compared with vehicle at both time points ( $p < 0.005$ ). All parameters were measured using the Image Pro-Plus software. *DMSO*, dimethyl sulfoxide; *Beclomethasone*, beclomethasone.

**Conclusions**—Numerous human conditions could be improved significantly if therapies that encourage tissue regeneration were available. Our findings illustrate the power of *in vivo* chemical genetics to identify novel bioactive compounds and their molecular targets that together function to modulate tissue regeneration. The genetic and molecular utilities of this early life stage regeneration model such as transient and stable transgenics, genetic mutant screens, rapid antisense repression, and the controlled use of chemical genetics make this vertebrate regeneration model an outstanding discovery platform. The results generated can be rapidly evaluated in other regeneration models. By utilizing the power of comparative approaches, the most exciting outcome will be a molecular



**FIGURE 9. Neutrophils and macrophages are not critical for regeneration.** *A*, neutrophil migration assay was performed on vehicle- or beclomethasone-exposed larvae at various time points. *B*, an image depicting the neutrophil migration to the wound site. The GFP-positive cells were counted as part of the development of this assay. *C*, macrophage migration assay was performed as described for *A* using neutral red staining. *D*, a representative image showing the macrophages stained red at the amputation site. Control morphants (*E*) and Pu.1 morphants (*F*) completely regenerate the fin tissue. Images of control (*G*) and Pu.1 (*H*) morphants stained with neutral red at 8 hpa. Twelve larvae were used for each group. Epifluorescent overlay images illustrating amputation-induced neutrophil migration in control (*I*) and Pu.1 morphants (*J*). Statistical analysis was conducted by one-way ANOVA with Bonferroni's post-test correction for multiple comparisons (Prism, GraphPad).

explanation for the observed differences in regenerative capacity across taxa and will reveal pathways for therapeutic interventions.

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