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Permalink
https://escholarship.org/uc/item/10d348t4

Journal
PLoS biology, 9(5)

ISSN
1545-7885

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Publication Date
2011-05-24

DOI
10.1371/journal.pbio.1000621

Peer reviewed
Hydrogen Peroxide Promotes Injury-Induced Peripheral Sensory Axon Regeneration in the Zebrafish Skin

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Abstract

Functional recovery from cutaneous injury requires not only the healing and regeneration of skin cells but also reinnervation of the skin by somatosensory peripheral axon endings. To investigate how sensory axon regeneration and wound healing are coordinated, we amputated the caudal fins of zebrafish larvae and imaged somatosensory axon behavior. Fin amputation strongly promoted the regeneration of nearby sensory axons, an effect that could be mimicked by ablating a few keratinocytes anywhere in the body. Since injury produces the reactive oxygen species hydrogen peroxide (H$_2$O$_2$) near wounds, we tested whether H$_2$O$_2$ influences cutaneous axon regeneration. Exposure of zebrafish larvae to sublethal levels of exogenous H$_2$O$_2$ promoted growth of severed axons in the absence of keratinocyte injury, and inhibiting H$_2$O$_2$ production blocked the axon growth-promoting effects of fin amputation and keratinocyte ablation. Thus, H$_2$O$_2$ signaling helps coordinate wound healing with peripheral sensory axon reinnervation of the skin.

Introduction

Successful wound repair and regeneration requires coordination between the various cell types that make up the injured tissue. For example, following injuries that damage both epidermis and sensory endings, wounded epidermis promotes the regeneration of nerve fibers [1,2]. Conversely, complete epidermal wound healing requires the presence of sensory axons [1,3]. In amphibians, innervation of the wound epidermis by nerve fibers is also essential for limb regeneration and correlates with the establishment of signaling centers [4–6]. These observations imply that coordination between wound epidermis and sensory axons during healing and regeneration is regulated by molecular interactions between these cell types.

In mammals, peripheral axon regeneration is generally more robust than axon regeneration in the central nervous system. Nonetheless, reinnervation in the periphery can be slow or incomplete, depending on the extent of axonal injury and on interactions with surrounding cells [7,8]. Because nerve injury is often associated with damage of not only the nerve but also neighboring tissues, it has been difficult to separate autonomous and non-autonomous factors influencing axon regeneration in vivo. Recent studies in C. elegans and zebrafish have utilized laser ablation to precisely damage single axons in the peripheral nervous system, making it possible to assess the influence of non-neuronal tissues on axonal regeneration [9,10].

Tissue damage triggers a complex cascade of signals that activate inflammatory responses and promote tissue repair [11]. In fruit flies and zebrafish, the recruitment of immune cells to wounds is mediated by the small reactive oxygen species (ROS) hydrogen peroxide (H$_2$O$_2$), which emanates from the injury [12,13]. The role of H$_2$O$_2$ in oxidative stress has been well studied, as high levels can have deleterious effects on the maintenance of cell homeostasis [14]. In the nervous system, H$_2$O$_2$ can induce neurodegeneration through activation of pro-apoptotic pathways [15–17]. More recently it has come to be appreciated that H$_2$O$_2$ can act as a signaling molecule with specific developmental and physiological functions. H$_2$O$_2$ is thought to signal by oxidizing cysteine residues on target proteins, most notably phosphatases [18,19].

The larval zebrafish tail fin provides an accessible setting for investigating how peripheral axon regeneration is coordinated with the healing of injured tissue and for testing whether H$_2$O$_2$ plays a role in these interactions. During larval stages, zebrafish fins consist of a folded two-layered epithelium, surrounding muscle cells (Figure S1A). Zebrafish tail fins regenerate after amputation, both during larval development [20,21] and in adults [22], but sensory reinnervation of regenerated fins has not been explicitly assessed.

Somatosensation at larval stages in zebrafish is accomplished by two populations of neurons: trigeminal neurons, which are located in ganglia outside the hindbrain and innervate the skin of the head, and Rohon-Beard (RB) neurons, which are located in the dorsal spinal cord and innervate the skin of the trunk and tail (Figure 1A). The peripheral axons of somatosensory neurons arborize between the two epithelial layers that make up the larval skin, the outer periderm and inner basal cell layers [23]. Precisely severing a trigeminal peripheral axon after arborization is...
The observation that RB axons robustly reinnervate larval fins within a few days after amputation, despite the fact that trigeminal axon regeneration is limited after precise axotomy [24], could be explained in either of two ways: (1) fin injury and healing promote peripheral axon growth or (2) RB neurons innervating the tail possess greater structural plasticity than trigeminal neurons. To assess the intrinsic plasticity of RB axon arbors, we monitored axon behavior after precise laser axotomy with time-lapse imaging for 12 h (see Figure S1B for experimental procedures) [10] and traced the position of individual axon tips every 30 min. Axotomy of RB neurons induced a 2-fold increase in axon activity (axon tip displacement, including both growth and retraction) compared to uninjured axons (34.92±2.72 μm, n=24 versus 32.47±2.53 μm, n=13 axon tip displacement, *p<0.05; compare Figures 1C and 3A; quantification in Figure 3D, Videos S2 and S1, respectively), but, like trigeminal axons, regenerating RB axons avoided denervated territory (Figure S2) [24]. Notably, axon growth was balanced by retraction, so that total axon size did not substantially increase (Figure 3F; see Video S2). Like trigeminal axons [24], the ability of RB axons to reinnervate former territory in the fin was improved by inhibiting Rho kinase (unpublished data). Thus, the ability of RB axons to regenerate after fin amputation is likely not due to intrinsic regenerative capacity but is probably a specific response to tissue damage.

To further investigate the influence of tissue injury on RB axon regeneration, we compared the behavior of uninjured axon arbors (Figure 1C), precisely axotomized arbors (Figure 3A), and injured axons in amputated fins (Figure 1D). Fin amputation (Video S3) increased total axon activity (growth and retraction) more than axotomy alone (77.40±4.05 μm, n=26, ***p<0.001). Measuring the linear distance between an axon tip’s position just after amputation and its position 12 h later revealed that fin amputation promoted productive axon growth, since axon tips traveled farther after amputation than after precise axotomy (29.62±2.50 μm, n=8, versus 8.42±3.09 μm, n=8, **p<0.01; Figure 3E). Combining fin amputation with subsequent laser axotomy of a nearby RB axon branch increased the axon activity (83.74±3.09 μm, n=26, **p<0.01) and total growth (46.54±4.92 μm, n=13, ***p<0.001) even further (Figure 3B,D,E, Video S4), but the amount of retraction was not dramatically altered (Figure 3F). Amputating fins significantly improved the ability of regenerating axons to innervate denervated areas (14.11±7.02 μm, n=8 versus 60.24±13.06 μm, n=10, *p<0.05; Figure S2), which is likely important for allowing regenerating arbors to traverse the denervated zone that forms just proximal to the wound after amputation (Figure 1B, brackets). Thus, fin injury increases sensory axon activity, promotes growth (but not retraction), and allows axons to overcome their avoidance of denervated territories.

To determine the effective range of axon growth-promoting signals from injured tissue, we axotomized axons distant (>50 μm) from the amputation site (Figure 3C, Video S5). These axons did not grow significantly better than precisely severed axons in uninjured tissue, since neither axon activity nor linear growth distances were increased by distant amputation (axon activity: 38.67±2.85 μm, n=10, p=n.s.>0.05; linear distance = 15.63±4.42 μm, n=9; p=n.s.>0.05; Figure 3D,E). Thus, growth-promoting signals emanating from injured tissue likely function at short range. To define the time window during which axons can respond to regeneration-promoting signals, we axotomized RB arbors at different time points after amputation. Axon activity was most enhanced when axons were axotomized at 3 h post-amputation (114.6±7.04 μm, n=10, **p<0.01), but axotomy at 6 h post-amputation did not increase axon activity (61.20±6.45 μm, n=10, p=n.s.>0.03; Figure S3A), as compared to axotomy alone. This observation suggests that axon growth-promoting signals emanating from injured tissue likely function at short range. To define the time window during which axons can respond to regeneration-promoting signals, we axotomized RB arbors at different time points after amputation. Axon activity was most enhanced when axons were axotomized at 3 h post-amputation (114.6±7.04 μm, n=10, **p<0.01), but axotomy at 6 h post-amputation did not increase axon activity (61.20±6.45 μm, n=10, p=n.s.>0.03; Figure S3A), as compared to axotomy alone. This observation suggests that axon growth-promoting
signals are transiently emitted from the wound, rather than continuously from regenerating fin tissue. To assess whether the size of the severed arbor fragment influenced the amount of axon activity induced by amputation, we traced degenerated fragments in three dimensions to measure their total length and plotted length as a function of axon activity. Size of the axotomized arbor did not correlate with axon activity (Figure S3B).

To identify the origin of axon growth-promoting signals, we ablated individual muscle cells or keratinocytes in the fin of larvae expressing cell type-specific reporter transgenes that highlight each tissue [26,27]. Ablating muscle cells did not promote axon growth (25.72 ± 3.65 μm, n = 10; Figure 4A,E), but ablating ≥3 keratinocytes prior to axotomy provoked robust axon regeneration in both the fin (70.75 ± 6.14 μm, n = 11, Figure 4B,E) and head (73.81 ± 20.95 μm, n = 4; Figure 4C,D,E). However, ablating a single keratinocyte in either the fin (44.34 ± 2.35 μm, n = 10, *** p < 0.001) or the head (27.62 ± 1.94 μm, n = 14, ** p < 0.01) did not promote axon regeneration. This result suggests that a threshold of injury-induced signals is required to promote growth and reinnervation by RB and trigeminal axons.

The recently reported observation that zebrafish larval fin amputation produces high levels of hydrogen peroxide (H₂O₂) at the wound margin [13] prompted us to investigate whether H₂O₂ contributes to the promotion of axon regeneration by keratinocyte injury. By monitoring H₂O₂ with a chemical sensor (pentafluorobenzenesulfonyl fluorescein), we first verified that, like fin...
amputation (Figure 5A), laser ablating several keratinocytes produced detectable levels of H₂O₂ around the wound (Figure 5B). Ablation of 1–2 keratinocytes did not produce detectable levels of H₂O₂ at the wound margin (Figure 5C), indicating that the severity of the injury correlates with the amount of H₂O₂ produced.

To test whether H₂O₂ can promote axon regeneration, we added 3 mM H₂O₂ (0.01%) to the larval media (the highest concentration of H₂O₂ at which most embryos survived and developed normally, see Figure S4 for survival rates) (Figure 6A, Video S6). The addition of H₂O₂ to uninjured larvae significantly promoted some axon activity (untreated, uninjured: 32.47 ± 2.53 μm versus H₂O₂ uninjured: 72.30 ± 1.94 μm, *** p<0.001; Figure 6D). Adding H₂O₂ for 3 or 12 h to larvae in which RB axon arbors had been axotomized increased axon activity variably but significantly, compared to axotomy in untreated animals (3 h H₂O₂: 122.1 ± 0.81 μm, n = 6; 12 h H₂O₂: 101.4 ± 3.09 μm, n = 10, versus untreated 54.92 ± 2.72 μm, n = 24, ** p<0.01 each; Figure 6D). The linear growth distances of axotomized arbors were also increased by H₂O₂ (3 h: 43.50 ± 6.06 μm, n = 5; 12 h: 30.04 ± 2.25 μm, n = 8, versus untreated: 5.46 ± 3.78 μm, n = 5, ** p<0.01 each; Figure 6E). Thus, H₂O₂ is sufficient to promote axon regeneration and does not need to be present in a gradient for this effect, as has been proposed for its role in leukocyte recruitment [13].

To determine where Duox1 is required to promote axon regeneration, we created genetic chimeras by transplanting cells at the blastula stage from donor embryos injected with duox1-MO into uninjected host embryos (Figure 7A). Donor embryos were transgenic for a somatosensory GFP reporter (sensory:GFP) and host embryos were transgenic for a keratinocyte RFP reporter (Krt4:RFP; previously termed Krt8) [27,29]. At larval stages, we injected donor embryos with a morpholino targeting p53 (p53-MO) (Figures 5A, S5A) [13]. Injecting this morpholino into embryos prevented the promotion of axon activity by fin amputation (23.89 ± 3.29 μm, n = 11, ** p<0.01) (Figure 6B,F, Video S7). Interestingly, fin regeneration was also compromised in duox1 morphants, potentially reflecting a role for axon innervation in fin regeneration, similar to limb regeneration in amphibians [4]. Treating amputated morphant larvae with 1.5 mM H₂O₂ for 12 h rescued the deficit in axon reinnervation observed in the morphants (102.3 ± 5.6 μm, n = 5, ** p<0.01) (Figure 6C,F, Video S8). Due to the toxicity of prolonged H₂O₂ treatment, we unfortunately could not assess whether such rescued morphants also regenerated their fins. Blocking H₂O₂ production with the duox1-MO did not affect growth and retraction induced by axotomy alone (Figure 6D), suggesting that cell-intrinsic mechanisms through which axotomy induces axon activity may be regulated by different pathways. To minimize the possibility that duox1-MO toxicity inhibited axon growth following axotomy, we repeated this experiment with coinjection of a morpholino targeting p53, which inhibits apoptosis [28], as was done in a previous study with the duox1-MO [13]. Like in larvae injected with duox1-MO alone, axon growth promotion by amputation was blocked in larvae injected with both p53-MO and duox1-MO, compared to larvae injected with p53-MO alone (Figures 6F, S3B–D), suggesting that the duox1-MO’s effect on regeneration is not due to cellular toxicity. Together these results indicate that Duox1-mediated H₂O₂ production is necessary for the promotion of injury-induced axon growth.

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further increase activity when compared to untreated larvae. Conversely, knockdown of *duox1* did not significantly change axon activity after fin amputation in 30 hpf larvae (WT versus *duox1* MO, *p* = ns). These results support the notion that H2O2 acts by blocking axon growth inhibition, since it only influences regeneration at stages when inhibitors are present. Interestingly, a study in chick showed that axon growth promotion by skin wounds was also only effective at late developmental stages [1].

H2O2 promotes immune cell recruitment to wounds in developing fruit fly embryos [12] and during early inflammatory responses to fin amputation in larval zebrafish [13]. To test whether inflammation and axon growth are linked or independent effects of H2O2 signaling, we assessed axon growth in homozygous cloche mutants, which lack blood cells [30], and macrophage recruitment in larvae injected with *ngn1*-MO, which lack somatosensory neurons [31]. Amputation promoted axon growth in the absence of blood (91.16 ± 10.44 μm, *n* = 9; Figure S10) and macrophages homed to the wound in the absence of sensory neurons (1.5 ± 0.42 macrophages expressing hsC:GFP at the wound margin within 1 h of amputation, *n* = 6), similar to wildtype (1.0 ± 0.43 macrophages at the wound margin within 1 h of amputation, *n* = 7, *p* = ns). These two processes are independent of each other.

Our results demonstrate that skin injury promotes the growth of axons near the wound, an effect that is mediated by H2O2 (Figure 8). Keratinocyte ablation and genetic chimera experiments suggested that the axon growth-promoting effects of H2O2 require its production in keratinocytes. Similarly, in axolotl and chick, wound epidermis attracts axons [1,2] and damage to human skin co-cultured with rat dorsal root ganglia promotes regeneration of axons at the dermal/epidermal interface [32]. It will be interesting to determine whether H2O2 also plays a role in these phenomena. Intriguingly, H2O2 improves hippocampal neurite outgrowth in culture [33]. In *C. elegans*, a mutation in *pxn-2*, which encodes an extracellular peroxidase, improves regeneration of mechanosensory axons [34]. In zebrafish, H2O2 may be signaling directly to...
Figure 4. Keratinocyte damage promotes axon regeneration. Time-lapse sequences from 78–90 hpf. The rightmost panel shows axon tip trajectories (red) over the course of the time-lapse. (A) Ablating muscle cells (circles) in the fin of a transgenic reporter larva was accompanied by only limited regeneration of an axotomized arbor (arrow). (B) Ablating ≥3 keratinocytes (red) (circles) in the fin promoted axon regeneration after axotomy (arrow) and improved reinnervation of denervated territory (shaded area). (C) Axotomy of a trigeminal axon branch in the head (arrow) induced limited growth of the severed axon, but the denervated territory was avoided (shaded area). (D) Ablation of ≥3 keratinocytes and axotomy of a trigeminal axon (arrow) promoted robust growth of the severed axon and reinnervation of the denervated territory (shaded area). (E) Quantification of axon activity after keratinocyte and muscle cell ablations. Sample size for each group is indicated by the number in the bar. Error bars represent the standard error of the mean. For statistical analyses, we performed one-way ANOVA and Dunnett’s post-test to compare individual groups to control groups (ablation of 1 keratinocyte in the fin or the head) (asterisks above bars indicate significance compared to control) \( p = \text{ns} > 0.05, \ * p < 0.01, \ ** p < 0.001 \), krtc, keratinocyte.

doi:10.1371/journal.pbio.1000621.g004
axons, altering the extracellular matrix, or eliciting a second signal from keratinocytes to promote axon growth, but does not require leukocytes (Figure 8). Assessing whether application of H$_2$O$_2$ to somatosensory neurons in culture can improve axon growth, as has been reported for hippocampal neurons in culture [33], could help resolve whether H$_2$O$_2$ acts directly or indirectly on axons to influence their regeneration.

In summary, we have found that wounded epidermis promotes somatosensory axon regeneration in zebrafish larvae and that H$_2$O$_2$ is a critical mediator of this effect. Since this effect does not require the presence of keratinocytes, we propose that H$_2$O$_2$ plays two independent roles during wound healing: promoting axon growth and mediating leukocyte recruitment. Thus, one signaling molecule emitted from injured tissue helps coordinate wound healing with functional recovery of skin.

Materials and Methods

Fish Lines and Maintenance
Zebrafish embryos were obtained from Nacre [35], AB (wildtype), Line Mu435;64 [26], cloche (clo$^{m109}$) [30], lysC:GFP [36], sensory:GFP [29], and islet2b:GFP [25] fish. Embryos and larvae were treated with 0.15 mM Phenylthiourea (PTU) to prevent pigment formation.

Transmission Electron Microscopy (TEM)
TEM was performed according to Rieger & Koster, CSH Protocols Vol. 2 (doi:10.1101/pdb.prot4772, 2007).

Zebrafish Larval Fin Amputation and Laser Axotomy
Fin amputation. Fin amputations were performed according to methods in Kawakami et al. [20]. Briefly, before amputation larvae were anesthetized in 0.01% Tricaine (Sigma, St. Louis, MO) and placed in a petri dish coated with 1.5% agarose. The distal one-third of the fin (posterior to the notochord) was amputated using a sterile syringe needle.

Axotomy. Two-photon laser axotomy was performed using a Zeiss LSM 510 META microscope system with a Chameleon laser. Details are described elsewhere [10].

Time-Lapse Microscopy
Zebrafish larvae were anesthetized in 0.01% Tricaine and mounted in a sealed chamber in 1.2% low-melting agarose (Sigma,
Figure 6. H$_2$O$_2$ promotes peripheral sensory axon growth in the skin. Time-lapse sequences from 78–90 hpf. The rightmost panel shows axon tip trajectories (red) over the course of the time-lapse; denervated territories are indicated by shaded areas. (A) Adding 3 mM H$_2$O$_2$ to the larval media for 12 h enhanced axon growth and promoted reinnervation of denervated territory after axotomy (arrow) in a non-amputated fin (see also Video S6). (B) Fin amputation did not promote axon growth in a duox1-morphant larva. The axon trajectories reflect mostly tissue movement during the time-lapse (see Video S7). (C) Adding 1.5 mM H$_2$O$_2$ rescued axon growth and improved reinnervation of denervated territory in amputated duox1-morphants (see also Video S8). (D, E) Quantification of axon activity (D) and linear axon growth (E) after axotomy in larvae treated with H$_2$O$_2$. (F) Quantification of axon activity after fin amputation. Sample size for each group is indicated by the number in the bar. Error bars represent the standard error of the mean. For statistical analyses, we performed one-way ANOVA and either Dunnett’s post-test to compare individual groups to controls (asterisks above bar indicate significance compared to control, the first column in each graph) or Bonferroni’s post-test to compare individual groups with each other (as indicated by brackets, $p=ns>0.05$, ** $p<0.01$). axo, axotomy; amp, amputation.

doi:10.1371/journal.pbio.1000621.g006
St. Louis, MO). Details of the mounting and imaging techniques are described elsewhere [10]. Larvae were imaged for 12 h using a 20x6 air objective. Stacks were scanned every 30 min in 3 μm intervals. Imaging was performed with 6–10 larvae per session on an LSM 510 confocal microscope (Zeiss) with an automated stage and Multitime software. Larvae were maintained at 28.5°C using a stage heater. Maximum intensity projections of confocal stacks were compiled using Zeiss software and further processed using Adobe Photoshop, NIH open source software Image J 1.34S (Abramoff, NIH Open Source software ImageJ, 2004), and Quick Time Player 7 Pro. For time-lapse imaging of peripheral sensory axon regeneration in H2O2 solution, 0.005%–0.01% H2O2 (1.5–3 mM) was added to the larval media 1 h prior to axotomy. Larvae were maintained in H2O2 solution for 3 or 12 h of time-lapse imaging. See also Figure S1B for timeline of experiments.

Plasmid Construction

All transgenes were constructed using the Gateway [Invitrogen] tol2kit created by the lab of Chi-Bin Chien [37].

**Tol2CREST3-Gal4VP16-14xUAS-EGFP**: The somatosensory neuron-specific CREST3 enhancer [38] was cloned into the 5’ Gateway vector (p5E), Gal4VP16-14xUAS [39] into the middle vector (pME), and EGFP-SV40pA into the 3’ vector (p3E). Elements were recombined together with the Tol2 destination vector (pDEST10). Tol2CREST3-LexA-LexAop-EGFP: LexAVP16-4xUAS and four copies of the LexAop [40] were cloned into the middle Gateway vector (pME) and recombined with p5E-CREST3 and p3E-EGFP-SV40pA to generate Tol2CREST3-LexA-4xLexAop-EGFP.

Plasmid and Morpholino Injections

Approximately 15 pg of CREST3-Gal4VP16-14xUAS-EGFP or CREST3-LexA-LexAop-EGFP plasmids were co-injected with 240 pg of Tol2 [41] transposase mRNA into 1-cell stage embryos of wildtype AB or Nacre strains or into the Gal4-UAS muscle reporter line Tg(Mü4435_64) [26], respectively. A similar amount of CREST3-Gal4VP16-14xUAS-EGFP was co-injected with 10 pg of Krt4:RFP and Tol2 transposase mRNA for keratinocyte ablations. To knock down expression of p53 [28], duox1 [13], and ngn1 [31], 50 nM of each modified antisense oligonucleotide was injected into 1-cell stage embryos.

Genotyping duox1 Morpholino-Injected Embryos by RT-PCR

Knockdown of duox1 by morpholino injection was verified with RT-PCR, using published primers [13]. Ten larvae at 3 dpf were pooled for RNA isolation and subsequent RT-PCR (see also Figure S3A).

Determination of Optimal H2O2 Concentration for Larval Experiments

To determine the sublethal concentration of H2O2 (Fisher Biotech, 30% in water) to use in larval experiments, we identified the maximum concentration at which 100% of larvae were viable.
Skin Injury Promotes Axon Regeneration

Axon activity was measured by tracing the movements of the 10 axon tips that grew most over a 12 h time window using Image J 1.34S and the Image J Manual Tracking software plugin (F. Cordelieres, Institut Curie, Orsay, France). Projected images were adjusted for movement of the specimen, using the Image J StackReg plugin (P. Thévenaz, Swiss Federal Institute of Technology, Lausanne, Switzerland). Statistical analyses were performed using Prism 4 (GraphPad Software Inc.). Unpaired, two-tailed Student’s t-tests were used for comparisons of two groups (Figures 1E and S2). One-way ANOVA and Dunnett’s (comparing groups to a control group) or Bonferroni’s (comparing groups to one another) post-tests were performed as indicated in each figure. Significance was set to p<0.05. All graphs show the standard error of the mean.

Axon Density Calculations
Confocal images were loaded into ImageJ software and converted to 8-bit images. A binary image was created and the mean pixel values in a 50x50 μm field in the distal fin portion were averaged to determine the axon density.

Quantification of Growth and Retraction
Images were exported as tiff files from the LSM software (Zeiss) and loaded into the ImageJ software. Axon tips were traced as described above and individual movements were designated as growth or retraction within each 30 min interval. The total length of growth and retraction for each axon was calculated for a 12-h period and a mean value of all traced axon tips derived (n = 4 axon tips/4 axons = 16 tracings total).

Quantifying Degenerating Axon Fragment Size
The detached distal portions of axonized axons were traced using NeuroLucida software (MBF Bioscience) to determine the total combined length of all the branches in the detached arbor. The length was plotted against axon activity of the parent axon during the regeneration phase (12 h).

Quantification of Escape Behavior
Larvae were placed in a petri dish and tapped with an insect pin at the distal tip of the caudal fin and escapes were recorded. Two groups were compared: wildtype uninjured larvae at 6 dpf and age-matched wildtype larvae whose fins were amputated 3 dpf.

Supporting Information
Figure S1  Ultrastructure of a larval fin and experimental design. (A) Transmission electron micrograph of a sagittal section through
Figure S2 Quantification of peripheral RB sensory axon reinnervation of denervated territories in the caudal fin. Example tracings are indicated above the bars (see Figure 3 and methods for details). Reinnervation was significantly increased when an axon branch was amotomized after fin amputation as compared to axotomy in non-amputated fins (60.24±13.06 µm versus 14.11±7.02 µm, * p<0.05; unpaired, two-tailed Student’s t-test). Found at: doi:10.1371/journal.pbio.1000621.s002 (0.03 MB TIF)

Figure S3 The relative timing of injury and axotomy, but not the size of the severed axon fragment, affects axon regeneration. (A) Quantification of axon regeneration at different time points after axotomy. Axon activity significantly increased when axotomy was performed at 1 hpamp (83.74±5.09 µm, ** p<0.01) and 3 hpamp (114.6±5.70 µm, ** p<0.01), but axotomy at 6 hpamp (61.20±6.45 µm, p = ns>0.05) did not significantly promote axon activity when compared to axotomy alone (54.92±2.72 µm). For statistical analyses, we performed one-way ANOVA and Dunnett’s post-test to compare individual groups to the control group (first column). (B) Correlation between axotomized arbor size and axon activity. The total length of axotomized arbors is plotted as a function of axon activity, showing that axon activity did not correlate with the size of axotomized arbors. hpamp, hours post amputation; Ax, axotomy. Found at: doi:10.1371/journal.pbio.1000621.s003 (0.34 MB TIF)

Figure S4 Survival rates of larvae after treatment with H2O2 for 12 h. Most of the larvae survived at 0.01% (3 mM) or less. Found at: doi:10.1371/journal.pbio.1000621.s004 (0.46 MB TIF)

Figure S5 Knockdown of duox1 blocks the growth-promoting effects of amputation in p53 morphant larvae. (A) RT-PCR showing knockdown of duox1 wildtype transcript after morpholino injection as in [13]. Arrows point to the relevant bands. (B–D) Time-lapse sequences from 78–90 hpf. The rightmost panel shows axon tip trajectories (red) over the course of the time-lapse; denervated territories are indicated by shaded areas. (B) Enhanced axon growth in a p53 control-MO-injected larval fin after amputation (dotted line) and reinnervation of denervated territory (shaded area). (C) Co-injection of p53-MO and duox1-MO prevented axon growth and reinnervation after amputation. (D) Rescue of axon growth inhibition and reinnervation in p53-MO/duox1-MO double morphants in the presence of 1.5 mM H2O2. See quantification in Figure 6F. Found at: doi:10.1371/journal.pbio.1000621.s005 (5.71 MB TIF)

Figure S6 Quantification of axon behavior at 30 hpf. None of the groups differed significantly from the control group (untreated uninjured: 99.77±4.96 µm versus untreated 3 mM H2O2: 111.1±2.03 µm, p = ns>0.05; untreated axotomy: 135.1±4.53 µm versus 3 mM H2O2 axotomy: 124.1±2.73 µm, p = ns>0.05; untreated amputated: 132.7±9.43 µm versus duox1-MO amputated: 119.6±7.19 µm, p = ns>0.05). One-way ANOVA and Bonferroni’s post-test were used to compare all groups (p = ns>0.05, ** p<0.01). Found at: doi:10.1371/journal.pbio.1000621.s006 (2.93 MB TIF)

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: SR AS. Performed the experiments: SR. Analyzed the data: SR. Wrote the paper: SR AS.
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