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Amputation-induced reactive oxygen species (ROS) are required for successful *Xenopus* tadpole tail regeneration

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Abstract

Understanding the molecular mechanisms that promote successful tissue regeneration is critical for continued advancements in regenerative medicine. Vertebrate amphibian tadpoles of the species *Xenopus laevis* and *Xenopus tropicalis* have remarkable abilities to regenerate their tails following amputation [1, 2], via the coordinated activity of numerous growth factor signaling pathways, including the Wnt, Fgf, BMP, notch, and TGFβ pathways [3-6]. Little is known, however, about the events that act upstream of these signalling pathways following injury. Here, we show that *Xenopus* tadpole tail amputation induces a sustained production of reactive oxygen species (ROS) during tail regeneration. Lowering ROS levels, via pharmacological or genetic approaches, reduces cell proliferation and impairs tail regeneration. Genetic rescue experiments restored both ROS production and the initiation of the regenerative response. Sustained increased ROS levels are required for Wnt/β-catenin signaling and the activation of one of its major downstream targets, *fgf20* [7], which, in turn, is essential for proper tail regeneration. These findings demonstrate that injury-induced ROS production is an important regulator of tissue regeneration.

To better understand the genetic and molecular mechanisms underlying *Xenopus tropicalis* tadpole tail regeneration, we recently performed a microarray screen examining gene expression during regeneration, which uncovered a number of coordinately upregulated genes involved in the production of ROS and H_2O_2[2]. Indeed, H_2O_2 and other ROS, traditionally viewed as harmful to cells, are now appreciated to have pleiotropic biological effects on various cellular processes, many of which could play roles during tissue regeneration [8, 9]. This prompted us to examine the production and role of ROS during vertebrate tail regeneration in *Xenopus* tadpoles.

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AUTHOR CONTRIBUTIONS NL designed and carried out most of the experiments in this study and co-wrote the manuscript. YC established the HyPerYFP assay in *Xenopus*, generated *spib* morpants, and assisted in many of the other experiments in the study. SI generated the *cyba* constructs, and PK generated the pHlourin constructs. KD performed western blot analyses on the tagged *cyba* constructs and helped prepare the manuscript. YK performed the C1-blastomere injections and cell-tracking analysis of the inflammatory cells. RL performed the whole-mount *in situ* hybridizations and whole-mount immunohistochemistry experiments. JG generated the initial finding that the antioxidant MC-186 could be used to lower ROS in *Xenopus*. EA supervised the project, aided with embryo experiments, and co-wrote the manuscript.

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We first sought to determine whether there was a change in ROS levels following *Xenopus* tadpole tail amputation and during the subsequent tail regeneration process. To image ROS *in vivo*, we used the ratiometric reporter fluorophore HyPerYFP [10]. This YFP variant possesses an oxidative sensitive OxyR domain that, following oxidation, causes a reversible conformational change in HyPerYFP and marked change in fluorescence excitation, a reaction that is particularly sensitive to H_2O_2 over other ROS [10]. Hence, a simple calculation of the HyPerYFP oxidized 490nm/reduced 402nm excitation ratio provides an *in vivo* assay of intracellular H_2O_2 or closely related ROS [11,12]. We generated several *Xenopus laevis* transgenic lines that express HyPerYFP ubiquitously from the CMV promoter, and the F0 founders successfully passed their transgenes to the F1 generation (Figure 1a, Supplementary Figure S1a) [13]. To assess any changes in H_2O_2 during regeneration, we amputated the tails of F1 or F2 HyPerYFP transgenic tadpoles, and found a marked increase in intracellular H_2O_2 following tail amputation (Figure 1b). Interestingly, the H_2O_2 levels remained high during the entire tail regeneration process, which lasts several days (Figure 1b). Titrations with exogenous H_2O_2 during tail regeneration suggested that regenerating tissues maintain a sustained level of intracellular H_2O_2 concentrations between 50μM and 200μM (Supplementary Fig. S1b).

To confirm these findings, we sought other means to detect ROS in regenerative tissue *in vivo*. Using the H_2O_2 sensitive fluorogenic dyes 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) and superoxide ($-O_2$) sensitive dihydroethidium (DHE) [14], we obtained similar results to those we obtained using the HyPerYFP probe (Supplementary Fig. S1c). Given that an increase in pH can lead to a change in the HyperYFP ratio [10], we next used a pH sensitive probe, pHluorin [15] and found that regenerating tails do not possess a pH level above 8.0 that would have generated a false-positive increased HyPerYFP ratio (Supplementary Fig. S1d). Together, these data and the HyPerYFP imaging results provide compelling evidence that tadpole tail regeneration is associated with a sustained presence of relatively high levels of H_2O_2 and/or other related ROS.

Previous reports in zebrafish have shown that epidermal injury results in the production of H_2O_2, which acts as a chemoattractant for inflammatory cells [11, 12]. Consistent with these reports, we found that epidermal wounding also caused an increase in ROS levels at the injury site, which remained elevated until wound healing was complete (Supplementary Fig. S2).

Given that tadpole tail amputation induces a massive recruitment of inflammatory cells to the site of injury [13] and inflammatory cells are known to produce high levels of ROS [9, 16], we asked whether the increase in ROS was due to the recruitment of inflammatory cells. Two pieces of experimental evidence argued against this possibility. First, we labeled the inflammatory cells of HyPerYFP transgenic tadpoles with RFP (see Methods) and found that the increase in ROS levels peaked within one hour post-amputation (hpa), while the major recruitment of inflammatory cells did not begin until 2 hpa (Figure 2a, Supplementary Video). Second, tadpoles with diminished inflammatory cells (morphants for *spib*, a transcription factor required for primitive myeloid cell development [17]; Figure 2c), showed no significant difference in the HyPerYFP ratios versus control morphant tadpoles following tail amputation (Figure 2d, e). These two sets of data strongly suggest that the sustained ROS levels during tail regeneration are largely produced by non-inflammatory wound resident cells, a finding consistent with previous reports examining zebrafish epidermal wounding [11].

To address the role of ROS during tail regeneration, we decreased ROS levels following amputation using several methods. We first used two chemicals that target the NADPH

Oxidase (NOX) enzyme complexes, a major source of cellular ROS [9] (Supplementary Fig. S3). We found that 2μM diphenyleneiodonium (DPI), a flavoprotein inhibitor, which targets the NOX subunit [18, 19] and 200μM apocynin (APO), which disrupts the assembly of the NOX complex [20], significantly reduced ROS levels by 12 hpa (Figure 3a; see Supplemental Fig. S3 for chemical structures and putative modes of action of the three inhibitors). Given that DPI and APO may have off target effects [19, 21], we used 5-50 times lower concentration of these inhibitors than others have used for similar experiments [1121]. In addition, we used a different method of lowering ROS, namely the therapeutic anti-oxidant and free radical scavenger MCI-186, (tradename Edaravone) [22, 23]. We found that 200μM MCI-186 also reduced ROS levels, although to a lesser extent than DPI or APO (Figure 3a). Notably, lowering amputation-induced ROS levels using these inhibitors resulted in an impairment of tail regeneration, as evidenced by shorter tail length at 72 hpa (Figure 3b). However, the failure of tail regeneration in ROS inhibitor treated tadpoles at 72 hpa could have simply been due to a delay in the regeneration program. To address this possibility, we cultured tadpoles following amputation for three days under ROS inhibition and then moved the tadpoles into fresh medium without the inhibitors until day 7 post-amputation, the time period needed for completion of tail regeneration (Figure 3c) [13]. This analysis showed that DPI or APO treatment over the first 3 days post-amputation (dpa) effectively precluded the regeneration program from reinitiating, even if the inhibitors were removed thereafter. In contrast, MCI-186, which had the least lowering effect on the HyPerYFP ratio, impaired or delayed regeneration while present, but in its absence, regeneration resumed such that after 7 days, the regenerated tails were largely similar to those in the DMSO treated controls (Figure 3c). These data suggested that NOX complex activity is required for the initiation of the regeneration program in the first 3 days post amputation, and regeneration is unable to recover thereafter, while the antioxidant scavenger merely delays the regeneration program while present.

We next sought to rescue the defects of ROS inhibitor treated tadpoles by the addition of exogenous H_2O_2 to the media. However, combining ROS inhibitors with prolonged, systemic exposure to H_2O_2, even as low as 50uM, for time periods longer than 24hours was toxic to tadpoles, thus precluding us from attempting regeneration phenotypic rescue experiments with exogenous H_2O_2. Given the difficulty we encountered attempting to rescue the chemical inhibitor derived phenotypes with exogenous H_2O_2, we turned to genetic perturbation approaches aimed at inhibiting NOX-mediated ROS production during tail regeneration. Our previous micro-array data suggested that the expression levels of cytochrome b-245 alpha polypeptide, *cyba* (also known as *p22phox,* a necessary subunit in NOX complexes 1, 2, and 4; Supplementary Figure S3b) [24], more than trebled following amputation and remained upregulated throughout regeneration (array target Str. 15394.1.S1_at, [13]). We confirmed the expression of *cyba* in newly amputated and regenerative tissue using RT-PCR and *in situ* hybridization (Supplementary Fig. S4). We then generated an antisense morpholino oligonucleotide (MO) designed to block the translation of *cyba* and, because antibodies recognizing the *Xenopus cyba* homologue are not available, we confirmed the efficacy of the MO using a C-terminal tagged *cyba-FLAG* epitope fusion construct (Figure 4a). Additionally, we generated an N-terminal myc-tagged version of *cyba* that was insensitive to the *cyba* MO knockdown effect, for use as a rescue construct (Figure 4b).

We injected 20ng of the *cyba atg* MO with 250pg of either *rfp* or *myc-cyba* mRNA into fertilized embryos and assessed the post-amputation ROS production and the regenerative response (Figure 4c,d). HyPerYFP imaging revealed that *cyba* morphants had ~33% reduction in amputation-induced ROS,, a loss that was rescued by co-injecting the morpholino insensitive N-terminal myc-tagged *cyba* variant. Notably, the decrease in ROS in *cyba* morphants correlated with a marked decrease in regenerative bud tissue formation,

an effect that was partially rescued using *myc-cyba* coexpression (Figure 4d). These data show that a portion of the amputation-induced ROS increase is mediated by the NOX-*cyba* complex, and that the regenerative response requires this enzymatic source of ROS.

We next wished to examine potential regenerative mechanisms that might be affected by the ROS produced following tail amputation and during regeneration. Intriguingly, previous reports had linked NOX-mediated H_2O_2 production with cell proliferation and growth factor signaling [25-28]. During *Xenopus* tail regeneration, a localized increase in cell proliferation in the injured and regenerating tail occurs at 24-36hpa, and these proliferating cells can be assayed by the presence of phospho-histone H3 (pH3), a marker for mitotic cells [2, 29]. To ask whether ROS production was necessary for cell proliferation during tail regeneration, we returned to the use of chemical inhibitors for these experiments due to the transient nature of *Xenopus* morpholino injections [30]. Treatment of regenerating tails with DPI and APO, and, to a lesser extent MCI-186, significantly decreased cell proliferation as assayed by pH3 staining at 36hours post-amputation (Figure 5a) and thus suggested a potential defect in growth factor signaling within these inhibitor treated tadpole tails.

Wnt and FGF signalling have been associated with increased cell proliferation during tissue regeneration [31-33]. To assess the dynamics of ROS and Wnt/β-catenin signaling during tail regeneration, we utilized a *X. tropicalis* Wnt/β-catenin signaling reporter line, which uses multimerized TCF optimal promoter (TOP) sites to drive the expression of destabilized GFP (dsGFP) (fluorescent protein half-life of 2hrs) [34]. We observed an absence of Wnt/β-catenin signaling at the wound site immediately after amputation, which was followed by a sustained activation of Wnt/β-catenin signaling from 24hpa, as assayed by the expression of the destabilized GFP reporter (Supplementary Figure S5a). Indeed, we found that inhibiting ROS production via DPI, APO, or MCI-186 treatment starting from amputation until 36hpa resulted in a marked decreased in $\mathrm{Wnt}/\tilde{\beta}$ catenin signaling, as evidenced by decreased Wnt/β-catenin directed dsGFP fluorescence (Figure 5b).

A previous report had shown that H_2O_2 modulates Wnt/β-catenin signaling *in vitro* via *nucleoredoxin* (*n×n*), a small redox sensitive protein from the thiorodoxin family [35]. Using *in situ* hybridization, we found that *n×n* was expressed during tail regeneration (Supplementary Fig. S5b). Though we have not specifically addressed the role of *n×n* during tail regeneration in this study, its expression provides a putative molecule linking changes in ROS levels with alterations in Wnt/β-catenin signaling activity.

fgf20 is a direct transcriptional target Wnt/β-catenin signaling [7] and it is markedly upregulated during *X. laevis* tail regeneration [3]. Furthermore, our previous microarray analysis showed that *fgf20* was the most highly upregulated *fgf* gene during *X. tropicalis* tail regeneration [2]. We confirmed this upregulation by whole-mount *in situ* hybridization, where we detected high expression levels of *fgf20* in the regenerative bud tissue, starting from 12hpa (Supplementary Figure S5c). Notably, we found that the expression of *fgf20* was decreased in tadpoles treated with the ROS inhibitors, DPI, APO, or MCI (Figure 5c, d). Given that *fgf20* is a major transcriptional target of Wnt/β-catenin signaling in *Xenopus*, these results further suggest that amputation-induced ROS are required for Wnt/β-catenin signaling in the early and intermediate stages of tail regeneration.

We then asked whether *fgf20*, one of many *fgfs* expressed during tail regeneration [3, 13], was itself necessary for tadpole tail regeneration. In zebrafish, *fgf20* has been shown to be essential for the formation of regenerative blastema tissue following tail fin amputation [31], however, its role during Xenopus tadpole tail regeneration had not been addressed. To address the role of *fgf20* in the regenerative response, we designed two antisense morpholinos targeting two separate splice junctions in *X. tropicalis fgf20* (Supplementary

Fig. S5c), and we found that both morpholinos were similarly efficient in reducing *fgf20* transcript levels as assayed by RT-PCR (Figure 5d). Following tail amputation, *fgf20* morphant tadpoles were able to heal the wound at the amputation site, but they failed to mount a full regenerative response. More specifically, we noted a significant defect in the regeneration of the axial tissues of the tail, corresponding to the tissue that expresses *fgf20*, and overall tail regrowth was significantly reduced in *fgf20* versus control morphants tadpoles (Figure 5e,f). These data show that *fgf20* function is required for the regeneration of the axial tissues of the tail, but not for the healing and regeneration of the epidermal tissues.

Thus, our data show that *Xenopus* tadpole tail regeneration requires the sustained production of H_2O_2 or closely related ROS, especially during the first 72 hours following amputation. ROS are likely to have pleiotropic effects on cellular physiology, including metabolism, motility, proliferation and signaling, due to the potential global effects that oxidation might incur on protein function [8, 9]. In our study, we focused on Wnt signaling and cell proliferation due to the known role that oxidation has on these aspects of cell biology and their previously established roles during tail regeneration. Our finding that a change in ROS levels is required for proper Wnt signaling during tadpole tail regeneration is particularly interesting. It is generally recognized that Wnt signaling plays a critical role in almost every studied regeneration system, from Hydra to mammals, yet very little is known about what controls the activation of Wnt signalling following injury [36, 37]. Thus, our work suggests that increased production of ROS plays a critical role in facilitating Wnt signalling following injury, and thus allows the regeneration program to commence. Given the ubiquitous role of Wnt signalling in regenerative events [37], this finding is intriguing as it might provide a general mechanism for injury induced Wnt signalling activation across all regeneration systems, and furthermore, manipulating ROS may provide a means to induce the activation of a regenerative program in those cases where regeneration is normally limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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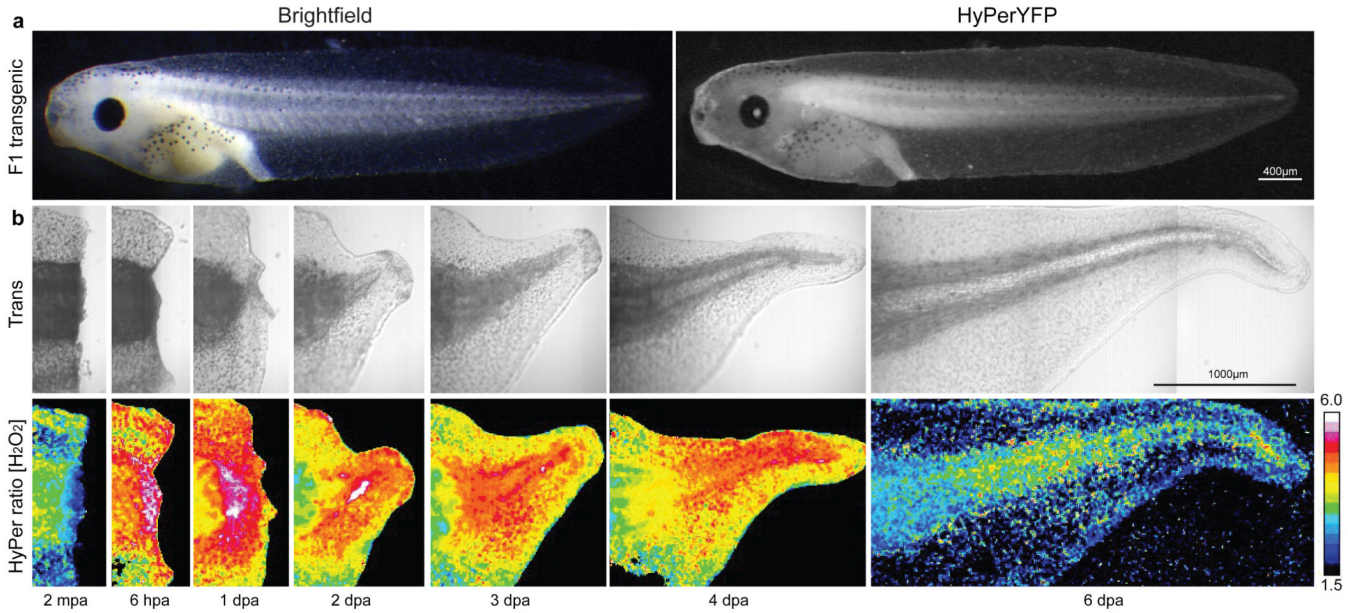

Figure 1.

Production of ROS during *Xenopus* tadpole tail regeneration. **(a)** Panels show brightfield and fluorescence images of a tadpole derived from the F1 generation of a transgenic *Xenopus laevis* line that expresses the H_2O_2 sensor HyPerYFP ubiquitously [10]. **(b)** Panels show HyPerYFP imaging of a representative regenerating tadpole tail. [H_2O_2] is derived from the excitation ratio of HyPerYFP490nm/HyPerYFP402nm. mpa, minutes post-amputation; hpa, hours post-amputation; dpa, days post-amputation. Due to the size of the regenerated tail, the 6-day after amputation time point panels are derived from the merging of three images.

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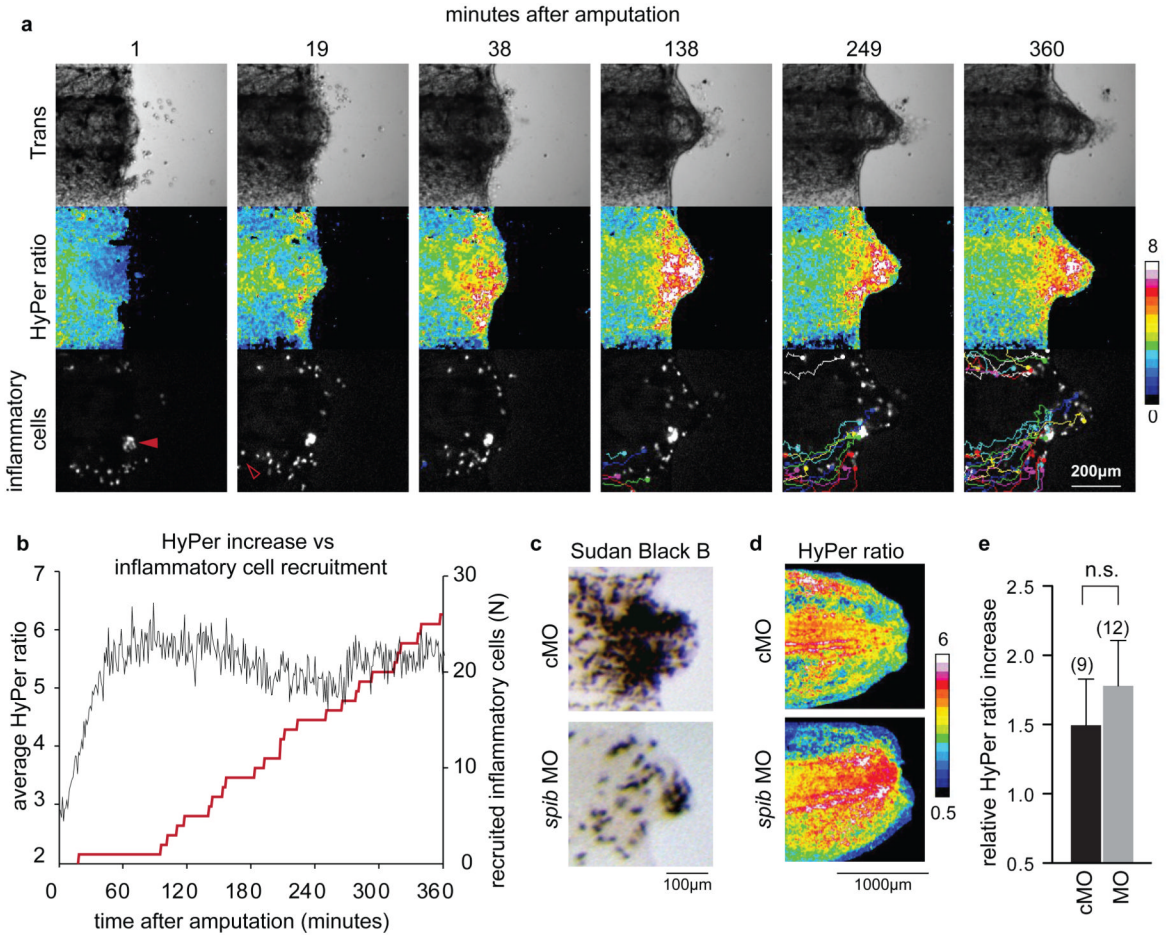

Figure 2.

Amputation-induced ROS production does not depend on inflammatory cells. **(a)** Frames from the Supplementary Video showing transillumination (Trans), H_2O_2 production (HyPerYFP), and inflammatory cells (labeled with RFP) recruitment during the first 6 hrs following amputation. Closed red arrow points to blood clot that quickly forms in the distal site of the dorsal aorta following tail amputation [2]. Open red arrow points to first inflammatory cell recruited into the area under examination. The colored lines show the migratory paths of the recruited inflammatory cells. **(b)** Quantification showing the change in average HyPerYFP ratio in relation to the number or recruited inflammatory cells into the area examined in the Supplementary Movie. **(c)** Sudan Black B staining of inflammatory cells in regenerative bud tissue at 24hpa, showing the decreased number of inflammatory cells in *spib* versus control morphant tadpoles. **(d)** Representative HyPerYFP imaging of control and *spib* morphants at 24 hours after tail amputation. **(e)** Quantification of H_2O_2 production using the HyPerYFP probe in control and *spib* morphants. Error bars indicate standard deviation (s.d.) of the mean. n tadpole tails analyzed indicated by brackets. n.s.; $P > 0.05$.

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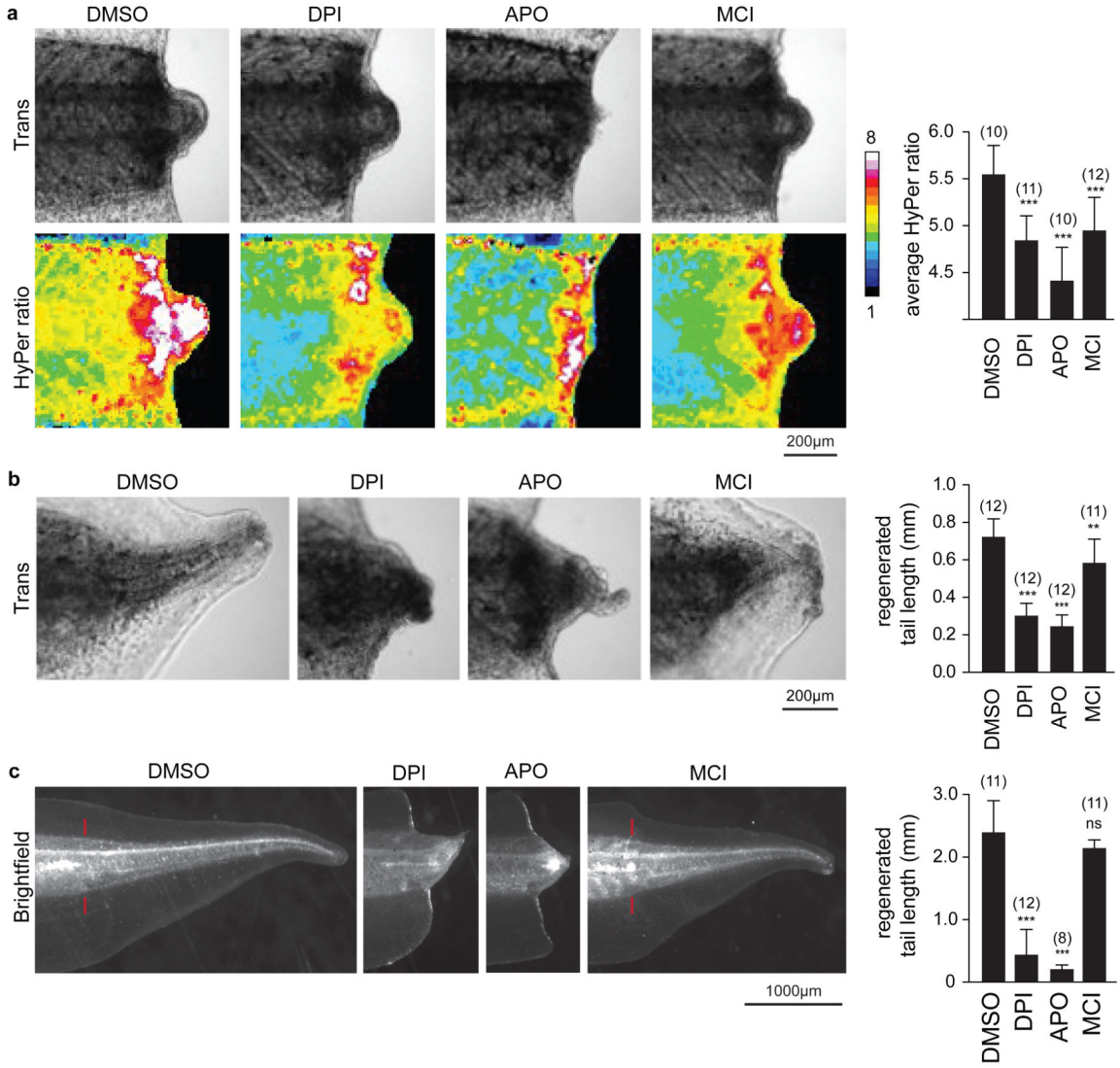

Figure 3.

Pharmacologically lowering ROS impairs tail regeneration. **(a)** Panels show representative HyPerYFP imaging and quantification of tadpole tails treated with DMSO, 2μM DPI, 200μM apocynin (APO), or 200μM MCI-186 (MCI) treatments at 12 hours after amputation. **(b)** Representative tails and quantification of regenerated tail length at 72 hours after amputation following the indicated inhibitor treatments. **(c)** Images and quantification of tadpoles that were exposed to DMSO, DPI, APO, or MCI (same doses as above) from 0-72hpa, and then cultured in normal media until 7dpa. Error bars indicate standard deviation of the mean (s.d.) of (n) specimens. Red lines indicate initial point of amputation. Significance was determined using one-way ANOVA versus DMSO control. **, $P < 0.01$; ***, $P < 0.001$; no significance (n.s.), $P > 0.05$.

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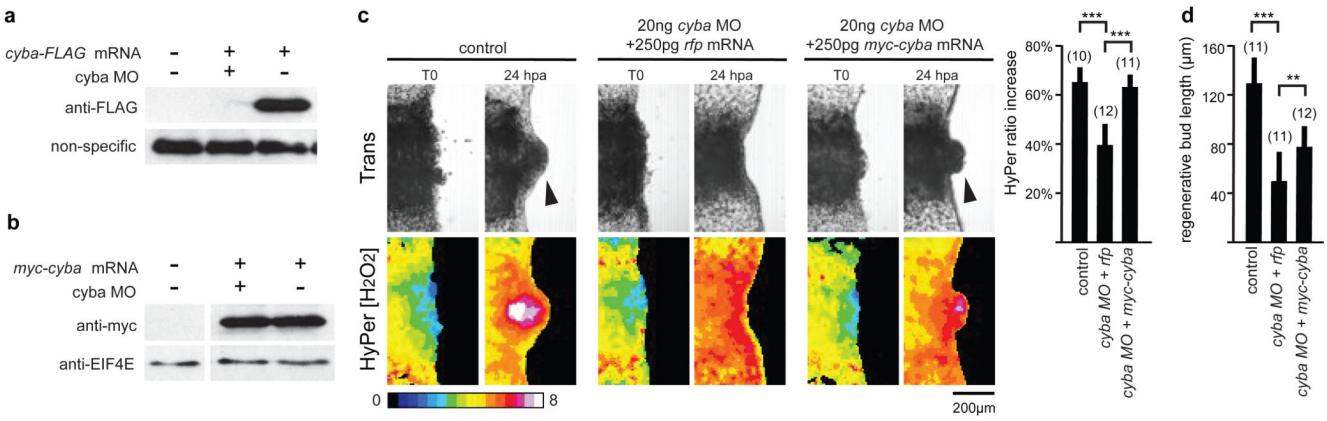

Figure 4.

Morpholino mediated knockdown of *cyba* results in lowered amputation-induced ROS production and decreased regenerative tissue formation. **(a)** Western-blot against the FLAG epitope in *cyba-flag* mRNA injected *cyba* morphant or control embryos. **(b)** Western-blot against the myc epitope in *myc-cyba* mRNA injected *cyba* morphant or control embryos. **(c)** Representative transillumination and HyPerYFP imaging at amputation (T0) and 24 hpa in control, *cyba* morphants, and *myc-cyba* rescue *cyba* morphants, showing inhibition of ROS production and regenerative tissue formation by the *cyba* MO and rescue by co-injection of the MO-resistant *myc-cyba* mRNA. Black closed arrow shows regenerative bud. The quantification of HyPerYFP ratio increases following amputation in control, *cyba* morphants, and *myc-cyba* rescue construct injected *cyba* morphants is shown to the right of the panels. **(d)** Quantification of regenerative bud formation in control, *cyba* morphants, and *myc-cyba* rescue construct injected *cyba* morphants. Error bars indicate standard deviation (s.d.) of (n) specimens. Significance was determined using one-way ANOVA versus control. **, $P < 0.01$; ***, $P < 0.001$.

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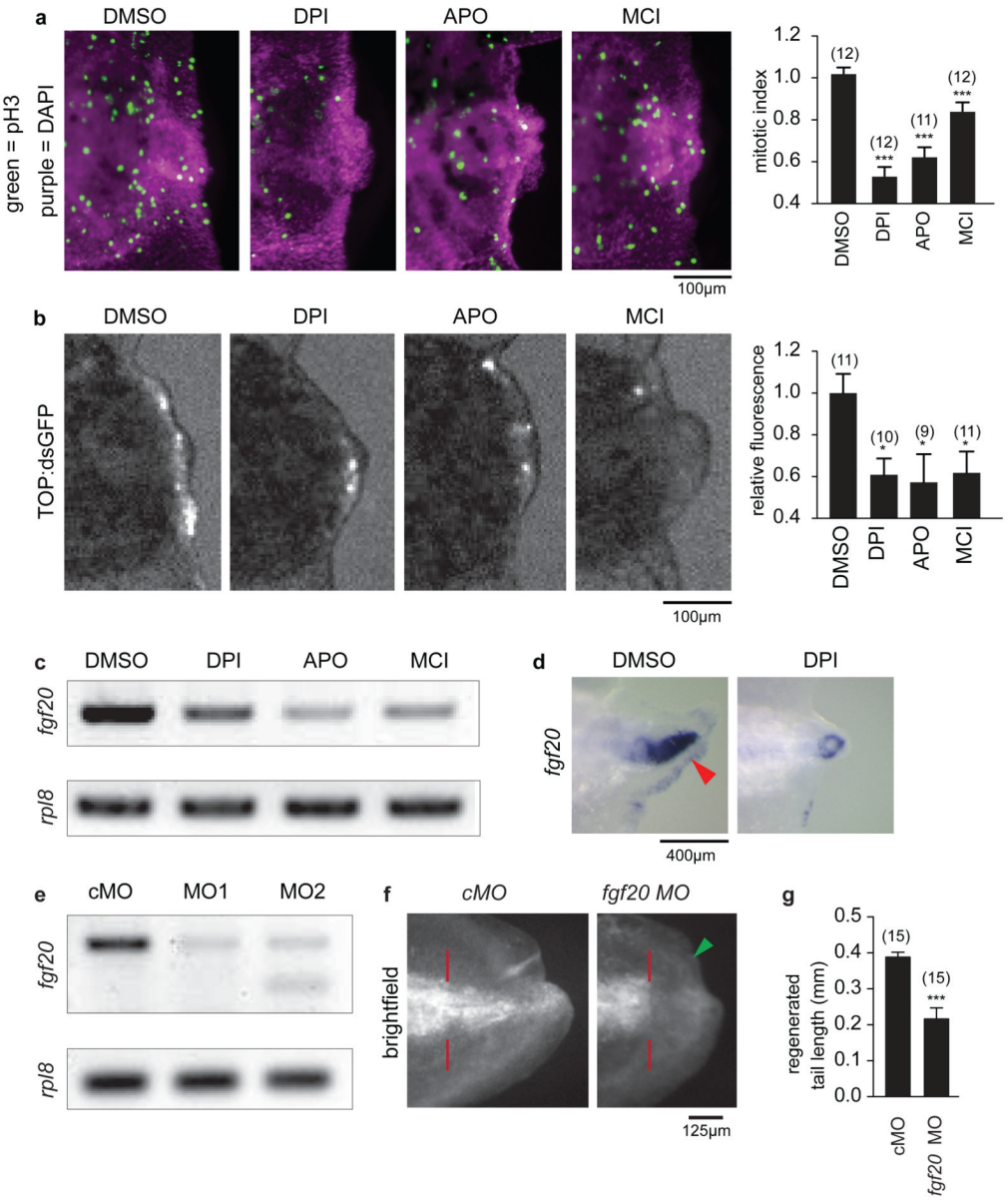

Figure 5.

Amputation induced ROS are important for proper growth factor signaling during tail regeneration. **(a)** Representative images and quantification of *X. laevis* tadpole tail mitotic cells at 36hpa when cultured in control (DMSO) or ROS inhibitors DPI (2μM), APO (200μM), and MCI (200μM). **(b)** Representative images and relative Wnt/β-catenin signaling reporter dsGFP fluorescence at 36hpa in control (DMSO) or ROS inhibitors DPI (2μM), APO (50μM), and MCI (200μM) treated *X. tropicalis* tadpoles. **(c)** RT-PCR reactions amplifying *fgf20* or reference gene *rpl8* in control (DMSO) or ROS inhibitors DPI (2μM), APO (50μM), and MCI (200μM) at 36hpa in *X. tropicalis* tadpoles. **(d)** *In situ* hybridization of *fgf20* in DMSO controls versus DPI treated *X. tropicalis* tadpole tails during the tail regrowth phase of tail regeneration. **(e)** RT-PCR reactions detecting *fgf20* in *X. tropicalis* control or *fgf20* morphants. **(f)** Representative regenerated control or *fgf20* morphant tadpole tails at 48hpa. **(g)** Quantification of regenerated tail length in control or *fgf20* morphants. Error bars indicate standard error of the mean (s.e.m.) of (n) specimens.

Significance was determined using one-way ANOVA or unpaired t-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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