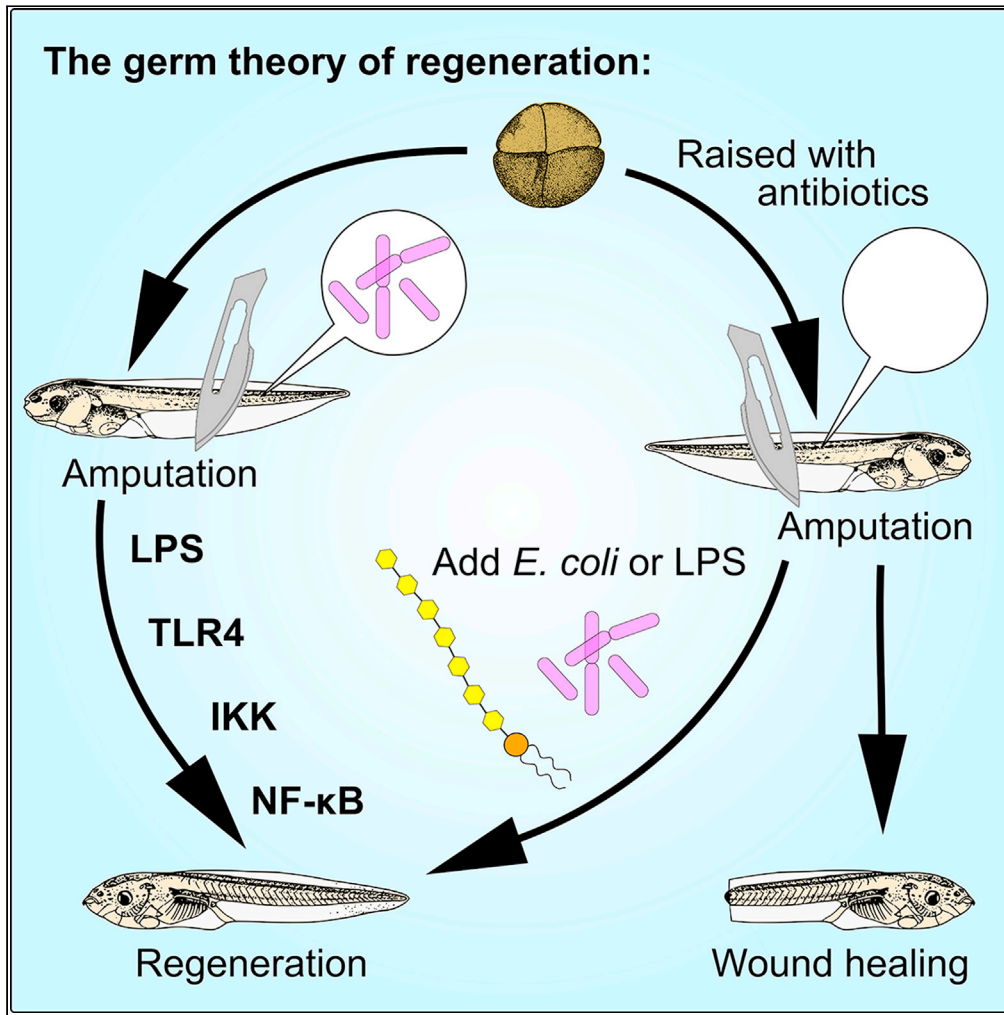


Article

Bacterial lipopolysaccharides can initiate regeneration of the *Xenopus* tadpole tail



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Highlights

NF-κB is rapidly and transiently activated in *Xenopus* tadpole tail regeneration

Activation of NF-κB rescues refractory tadpole tail regeneration

Bacterial LPS promote tadpole tail regeneration, whereas raising in antibiotics attenuates

NF-κB activation improves regeneration of tadpole hindlimbs but not older forelimbs



Article

Bacterial lipopolysaccharides can initiate regeneration of the *Xenopus* tadpole tailThomas F. Bishop^{1,2} and Caroline W. Beck^{1,3,*}

SUMMARY

Tadpoles of the frog *Xenopus laevis* can regenerate tails except for a short “refractory” period in which they heal rather than regenerate. Rapid and sustained production of ROS by NADPH oxidase (Nox) is critical for regeneration. Here, we show that tail amputation results in rapid, transient activation of the ROS-activated transcription factor NF- κ B and expression of its direct target *cox2* in the wound epithelium. Activation of NF- κ B is also sufficient to rescue refractory tail regeneration. We propose that bacteria on the tadpole’s skin could influence tail regenerative outcomes, possibly via LPS-TLR4-NF- κ B signaling. When raised in antibiotics, fewer tadpoles in the refractory stage attempted regeneration, whereas addition of LPS rescued regeneration. Short-term activation of NF- κ B using small molecules enhanced regeneration of tadpole hindlimbs, but not froglet forelimbs. We propose a model in which host microbiome contributes to creating optimal conditions for regeneration, via regulation of NF- κ B by the innate immune system.

INTRODUCTION

Tadpoles of the commonly used vertebrate model organism species *Xenopus laevis* exhibit variable regeneration at distinct life stages. When subjected to partial amputation at stages 40–44, the tadpole tail regenerates almost perfectly. However, between stages 45 and 47, there is much more variability, with many tadpoles not regenerating at all and others exhibiting reduced or pattern-deficient regeneration (Beck et al., 2003). In contrast, limb regeneration in this species undergoes a decline in re-patterning as the limb tissues differentiate (Dent, 1962). This phenomenon of regeneration-competent and regeneration-incompetent life stages in *Xenopus* has been widely leveraged, particularly for tail regeneration (for recent review, see Phipps et al. [2020]). This has resulted in the implication of several well-known developmental genetic pathways such as wnt, notch, shh, TGF β , BMP, FGF, and hippo in regeneration (Beck et al., 2003, 2006; Ho and Whitman, 2008; Lin and Slack, 2008; Lin et al., 2012; Taniguchi et al., 2014; Hayashi et al., 2014). There is also strong evidence for roles of epigenetic regulation (Tseng et al., 2011; Taylor and Beck, 2012) and biological processes such as membrane depolarization (Adams et al., 2007; Tseng et al., 2007), reactive oxygen species (ROS) production (Love et al., 2013), apoptosis (Tseng et al., 2007), metabolic reprogramming (Love et al., 2014), and extracellular matrix modulation (Contreras et al., 2009) in tail regeneration.

Successful tail regeneration is normally described in three stages and is best described for stages 40–44, within the first week of a tadpole’s life. First, following partial removal of the distal tail, the wound becomes covered with the wound epithelium, a thin layer of epithelial cells, recently identified by single-cell sequencing as a distinct population termed *regeneration organizing cells* (ROCs) (Aztekin et al., 2019). This process takes about 6–8 h, but the consensus is to include the first 24 h post-amputation (hpa) in this wound healing stage. The amputation event generates a rapid burst of ROS (Love et al., 2013) and an ROS-dependent depolarization event (Ferreira et al., 2016, 2018). Inhibitors of ROS or TGF β can disrupt this process (Ho and Whitman, 2008; Love et al., 2013). In the second stage of regeneration, from 1 to 2 days post-amputation (dpa), a regeneration bud forms beneath the wound epithelium. While often described as a blastema, this region is composed of precursor cells for the axial tissues of the tail: notochord and neural tube, with somites being regenerated from *pax7+* muscle satellite cells of the stump, as shown by grafting experiments (Gargioli and Slack, 2004). In the third stage, new tissue starts to differentiate, culminating in a fully functional tail by around 5–7 days.

Partially regenerating tails can be considered patterning or regrowth defects, whereas non-regenerating tails cover the wound with a full-thickness epidermis and the underlying dermis, essentially opting for

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scar-free wound healing (Beck et al., 2003). This indicates that early variation in response mechanisms in refractory stages can determine eventual regenerative outcomes of individuals. The role of the immune response in determining regenerative outcomes is well established, with an emerging picture of modulation between pro- and anti-inflammatory mechanisms determining regenerative outcomes in multiple model organisms. Inflammation is critical, whereas prolonged inflammation is detrimental (King et al., 2012; Mescher et al., 2013). Macrophages, phagocytic myeloid blood cells that can act as responders to innate immune threats, can produce both pro- and anti-inflammatory effectors. Removing macrophages by depletion in regeneration-competent species such as teleost fish, salamanders, and *Xenopus* can prevent regeneration (Godwin et al., 2013, 2017; Li et al., 2012; Petrie et al., 2014; Aztekin et al., 2020). The ROS-responsive, ROS-modulating transcription factor nuclear factor (NF)- κ B is a key player in innate immunity, modulating inflammatory responses (Dev et al., 2011). Activation of NF- κ B can dramatically alter the activity and function of a cell (Sun and Andersson, 2002) and is necessary for maintaining the undifferentiated state in human embryonic stem cells (Deng et al., 2016), human induced pluripotent stem cells (Takase et al., 2013), and mesenchymal stem cells (Chang et al., 2013). NF- κ B also facilitates proliferation of cancer cells (Sethi et al., 2008) and is required for zebrafish cardiac regeneration (Karra et al., 2015). Here, we show for the first time the broad, rapid, and transient nuclear localization of NF- κ B during the very early wound healing phase in regenerating *Xenopus* tadpole tails. The NF- κ B direct target *cox2* is expressed strongly in the ROCs of the wound epithelium of tails, and the magnitude of this response in developing limb buds correlated with the typical regenerative outcome for each stage. Two NADPH oxidase-encoding genes are also upregulated in regeneration-competent appendages, suggesting that the activation of NF- κ B may stimulate regeneration by prolonging the production of intracellular ROS by these enzymes. Intriguingly, we find that microorganisms can play a role in the initiation of tail regeneration. Commensal skin microorganisms offer a source of ligands for Toll-like receptor (TLR) pathway activation and consequent NF- κ B activity. Finally, we test the ability of a chemical treatment regimen designed to mimic brief NF- κ B activity to see if we can improve regeneration in limb buds and older limbs.

RESULTS

NF- κ B rapidly and transiently translocates to the nucleus on amputation of the *Xenopus* tadpole tail

It has been previously shown that the concentration of ROS greatly increases around the site of injury within minutes of tadpole tail amputation and that sustained ROS production is necessary for regeneration of the tail (Love et al., 2013). The pro-inflammatory transcription factor NF- κ B can be activated or inhibited by ROS, depending on context (reviewed in Lingappan [2018]) and can also modulate the level of ROS in cells, via upregulation of direct target genes such as the Nox genes that encode NADPH oxidases (reviewed in Morgan and Liu [2011]). As an ROS-enhancing, ROS-responsive transcription factor, NF- κ B has the potential to be involved in regulating the sustained ROS production that is required for successful regeneration. We therefore reasoned that canonical NF- κ B signaling may be involved in linking ROS production on injury to regenerative cellular responses in amphibians. Inactive NF- κ B is sequestered in the cytoplasm of all cells, bound to inhibitor of nuclear factor κ B (I κ B), which prevents transit into the nucleus (Karin and Lin, 2002). TNF α , IL-1 β , lipopolysaccharides (LPS), and other stress-related signals result in activation of I κ B kinase (IKK), which causes release of NF- κ B from I κ B (reviewed in Verstrepen et al. [2008]). I κ B is then degraded rapidly due to exposure of its PEST sequence (Karin and Lin, 2002). The resulting unmasking of the nuclear localization sequence on NF- κ B causes its rapid relocation to the nucleus, where it can upregulate direct target genes such as the pro-inflammatory gene *ptgs2* (*cox2*) and many others. The post-translational regulation of NF- κ B is therefore a rapid cellular response switch, since it does not depend on transcription or translation.

The NF- κ B transcription factor is a dimer of two proteins, canonically p65 (RelA) and p50. To see if NF- κ B was activated during regeneration, we used a western blotting approach to detect RelA, the p65 component of NF- κ B, in nuclear and cytoplasmic fractions of 3-mm tail slices harvested at various times after partial tail amputation in stage 55 tadpoles (Figure 1A). We used older regeneration-competent tadpoles for this because of the need for a larger target from which to isolate protein. In uninjured control tail slices, RelA was detected only in the cytoplasmic fraction, whereas in tissue taken 1 h after amputation it was almost entirely nuclear. In later time points, RelA was detected only in the cytoplasm again. This suggests a rapid and transient relocation of NF- κ B to the nuclei in the majority of cells within 3 mm of the amputation plane.

Previous work, using prerefractory-stage *Xenopus* tadpole tails, has shown that ROS rapidly increase around the injury site, peak at about 20 min, and are sustained at a high level throughout the process of

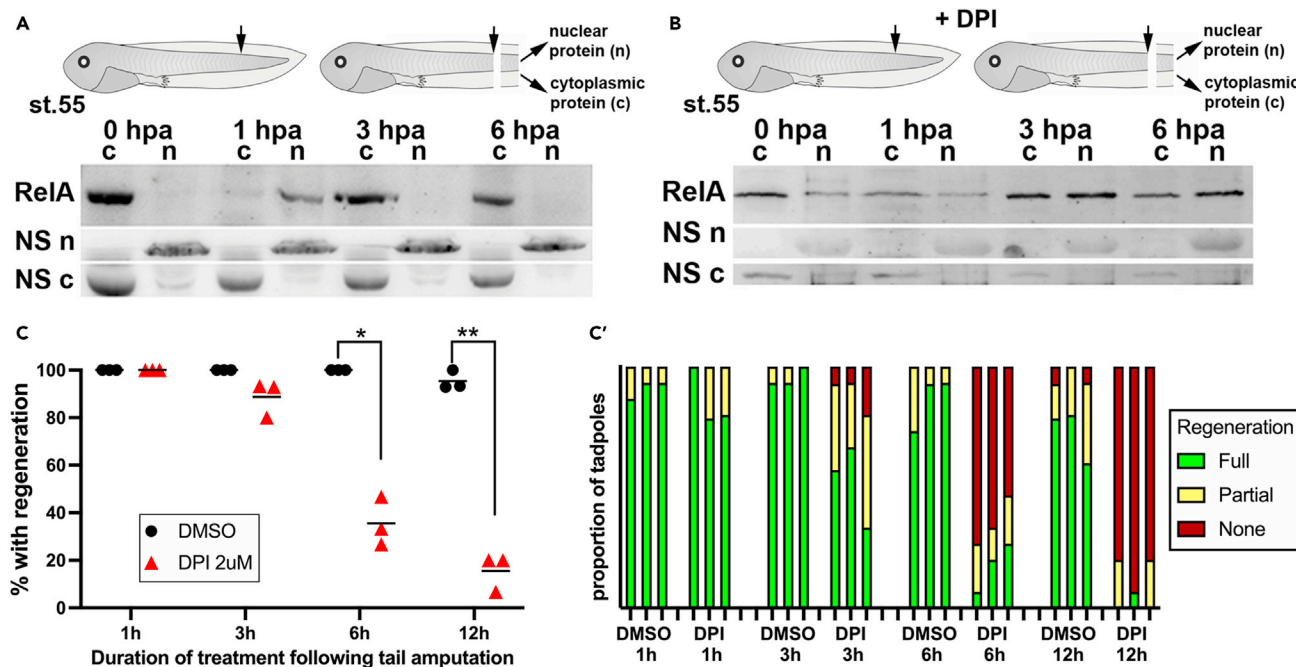


Figure 1. NF-κB rapidly and transiently translocates to the nucleus following tail amputation, and this requires active NADPH oxidase

(A–C) Western blots showing RelA in cytoplasmic (c) and nuclear (n) protein fractions from stage 55 tail sections as drawn, at indicated hours post amputation (hpa). Arrows indicate approximate site of cuts. Non-specific (NS) bands for each fraction are included as loading controls. (A) Untreated tadpoles (B) Tadpoles treated with 2 μM DPI from 1 h before amputation, to disrupt ROS production. (C) Graph showing percentage of tadpoles regenerating tails after 7 days, following chemical inhibition of NADPH oxidase (Nox) with 2 μM DPI treatment starting 1 h before amputation of the distal third of stage 43 tadpole tail, or 0.1% DMSO vehicle. Single cohort of tadpoles with N = 14–15 tadpoles per replicate Petri dish; points represent three replicates for each time point. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons of all means; adjusted p values *p < 0.05, **p < 0.01 are shown for treatments that differ significantly from the equivalent vehicle control. (C') Bar graphs indicate the distribution of phenotypes observed in (C). Raw data can be found in [Data S1](#).

tail regeneration, which takes 5–7 days (Love et al., 2013). To see how NF-κB cellular localization was affected by treatment with the Nox inhibitor, diphenyleneiodonium chloride (DPI), we repeated the tail regeneration western blotting experiment using tail tissue slices from tadpoles cultured in the presence of 2 μM DPI, starting 1 h pre-amputation (Figure 1B). RelA was mostly cytoplasmic but detectable in the nuclear fraction at low levels in unamputated control samples. However, this is likely due to a small amount of contamination from the cytoplasmic fraction, as evidenced by a similar profile in the non-specific cytoplasmic fraction. The rapid and transient relocation of RelA to the nucleus at 1 hpa seen in untreated tadpole tails was not detected, and instead we saw a slower shift, with RelA split between the nuclear and cytoplasmic fractions at 3 and 6 hpa (Figure 1B). These data suggest that NF-κB is regulated differently in the presence of DPI, correlating with the loss of regeneration potential observed by others (Love et al., 2013) and linking NADPH oxidase activity to NF-κB activation in our model system.

The previous study, in which tadpoles were raised in the presence of DPI for 3 days following amputation (Love et al., 2013), does not discriminate between the early burst and sustained ROS production. To see if ROS were required during the initial hours after amputation, shorter DPI treatments were tested (Figure 1C). DPI was added to stage 43 tadpoles 1 h before amputation, and washed off after 1, 3, 6, or 12 h after amputation. The shortest treatment, for 1 h before and 1 h after amputation, and covering the initial ROS burst reported by Love and colleagues, was not seen to reduce regeneration, with 100% of tadpoles able to regrow a tail within a week. Longer treatments with DPI resulted in greater numbers of tadpoles failing to mount a regenerative response; 11% failed to regenerate a tail after the 3 h, 64% after 6 h, and 84% after 12 h. Some reduction in regeneration was also seen in the DMSO vehicle controls after 12 h, with 4% of tadpoles failing to regenerate a tail in this group. This confirms the previous finding that Nox activity is required for tail regeneration in *Xenopus* (Love et al., 2013) as early as 3 h after tail amputation and further identifies the role of Nox in sustaining ROS levels after the initial burst.

Finally, we looked at the expression of three *Xenopus* NADPH oxidase catalytic subunit genes, *nox1*, *nox2* (*cybb*), and *nox4*, in regeneration-competent limb stages at various times after amputation at future ankle level (Figure S1). All three genes are direct targets of NF- κ B (Manea et al., 2010; Morgan and Liu, 2011) and could be responsible for sustained ROS production during regeneration, but their expression in regenerating appendages has not been described. *Nox1* expression could not be detected in control or amputated limbs. *Nox2* and *nox4* were expressed distally in stage 51 limbs at all time points from 6 hpa to 3 dpa (Figure S1A), which spans the processes of wound healing and blastema formation (Pearl et al., 2008). Expression of *nox2* was punctate and comparable to previously described distribution of neutrophils and macrophages in regenerating hindlimbs (Mescher et al., 2013), consistent with expression being confined to these migrating cells. Furthermore, *nox2* expression was punctate in intact stage 41 tails, appearing to concentrate at the cut surface of the wound from 6 h after tail amputation (Figure S1B). Expression of *nox4* was observed around the injury site, and in the regeneration bud, consistent with upregulated expression in regenerating tissue. None of the genes showed clear expression in the control uncut limbs indicating either or both *nox2* and *nox4* could be acting to drive sustained ROS production during tadpole appendage regeneration.

Activation of NF- κ B can improve tail regeneration in the refractory period, and expression of its direct target *cox2/ptgs2* in the wound epithelium correlates with regenerative success in limbs and tails

To see if activation of NF- κ B is sufficient to rescue regeneration of tadpole tails in the refractory period, we incubated tadpoles in 10 μ M prostratin for 30 min immediately after amputation. Prostratin activates NF- κ B via rapid activation of IKK (Williams et al., 2004). A greater percentage of the tadpoles did indeed regenerate in prostratin-exposed dishes versus vehicle controls (Figure 2A). When the tails were analyzed by phenotype, prostratin treatment resulted in significantly higher scores (Figure 2A': sibship#1 $p = 0.043$ and sibship#2 $p = 0.015$, unpaired t test). We then looked to see if *cox2* (*ptgs2*), a direct target of NF- κ B, was induced at the wound, using *in situ* hybridization of tails after partial amputation. In stage 52 tadpoles, which normally regenerate their tails following amputation of the distal third, *cox2* expression was seen at the leading edge of the wound from 6 hpa (Figure 2B). Expression of *cox2* in these distal epithelial cells was maintained until at least 3 dpa, after which it was found in the regenerating tip cells only. We also looked at the expression of *cox2* in limb buds at regeneration-competent (stage 51), partially competent (stage 53), and hypomorphic stages (stage 55). *Cox2* was strongly expressed in the wound edges at 6 hpa and in distal cells at 1 dpa (Figure 2C). This expression was gone by 2 dpa and was the strongest at 1 dpa in regeneration-competent stage 51 limbs, with a weaker and less broad expression in stage 53 and almost undetectable expression at stage 55. Together, these data suggest that NF- κ B transient activation occurs after partial limb or tail amputation, resulting in distal *cox2* expression, and that the magnitude of this response correlates positively with the completeness of regenerative success.

Resident microbes may activate regeneration of tadpole tails

Xenopus laevis tadpoles can regenerate tails up to metamorphic climax, when the tail is resorbed, with the exception of stage 45–47, the refractory period (Beck et al., 2003). We and others have used this refractory period to search for mechanisms that improve regeneration. ROS, mainly in the form of H_2O_2 , are required for tail regeneration, with elevated endogenous H_2O_2 levels between 50 and 200 μ M estimated throughout regeneration, and detectable using transgenic reporter tadpoles (Love et al., 2013; Ferreira et al., 2016). Previously, it has been shown that incubation in exogenous H_2O_2 at up to 500 μ M for 48 h had no effect on tail regeneration at stage 40 (Love et al., 2013). However, incubating tadpoles in 50 μ M H_2O_2 for 24 h was shown by Ferreira et al. to rescue regeneration in refractory stage tadpoles (Ferreira et al., 2016). To see if local application of exogenous H_2O_2 could rescue regeneration in refractory-stage tadpoles tails, we “dipped” tadpole tails into serial dilutions of H_2O_2 for 3 s directly after amputation. While dipping in concentrations of at or below 0.03% H_2O_2 (approximately 10 mM) had no noticeable effect (data not shown), the brief exposure of the amputated tadpole tail tip to very high concentrations (0.1 and 1 M) prevented regeneration in 100% of tadpoles (Figure 3A). Interestingly, the tadpoles in this cohort, as well as those in sibship#1 in the prostratin experimental controls (77%, Figure 2A) also showed much higher rates of regeneration (79%, Figure 3A) than would be expected during the refractory period. Our previous discovery of this refractory period (Beck et al., 2003) used tadpoles grown from 4-cell stage in 50 μ M gentamicin sulfate, which we no longer routinely use for embryos that have not been injected. These observations together led us to propose a new hypothesis, that Gram-negative bacteria growing on the skin of tadpoles could influence regenerative capability. The NF- κ B activator, IKK, can itself be activated by the

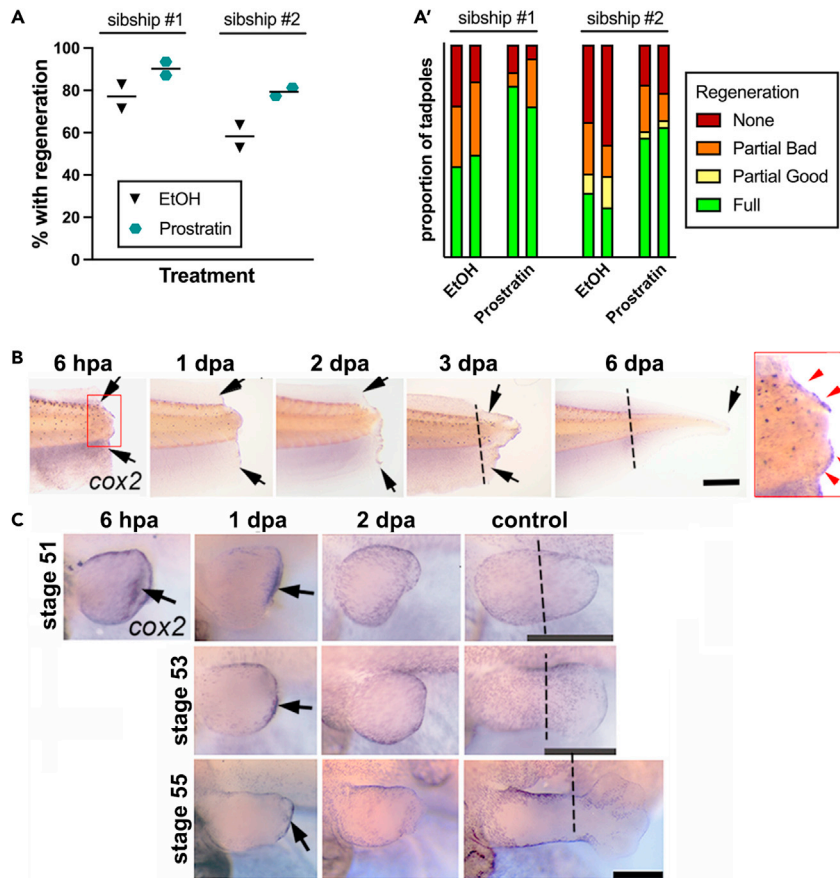


Figure 2. Activation of NF- κ B is sufficient for tadpole tail regeneration, and the direct target *cox2* is upregulated in the wound epithelium of regeneration-competent appendages

(A) Tadpoles in refractory stage 46 are more likely to regenerate a tail if exposed to 10 μ M prostratin, an indirect activator of NF- κ B, for 30 min after amputation, compared with vehicle-treated controls (0.1% EtOH). Points on scatterplots represent the percentage of regenerating tails with 28–34 individuals in a replicate Petri dish. Analysis by unpaired t test showed the effect of prostratin did not reach significance, although the trend is clear.

(A') Bar graphs indicate the distribution of phenotypes observed in (A). Sibships are separate cohorts of tadpoles (different parents). Raw data can be found in [Data S1](#).

(B) Representative examples of *cox2* expression (dark purple) in tails of stage 52 tadpoles at the indicated time post-amputation. Dotted lines indicate planes of amputation, and arrows indicate the limits of *cox2* expression in the distal epithelial cells, or tail tip in 6 dpa. Scale bar, 500 μ M. Red box shows a zoom of 6 hpa to show specific *cox2* expression localized to the wound epithelium (red arrowheads) as distinct from melanophores (scattered black dots).

(C) Representative examples of *cox2* expression in limb buds amputated at future ankle level at stages 51 (good regenerators, all 5 digits normally regenerate), 53 (3–5 digits regenerate), and 55 (hypomorphic regenerators, 0–3 digits regenerate). Examples are shown at various times after partial amputation, and dotted lines on controls indicate the plane of amputation. Black arrows indicate expression of *cox2* in the distal cells at 6 hpa and 1 dpa. Stronger and broader expression is seen at the most regeneration-competent stage, 51. Scale bars, 500 μ M.

binding of ligands from microorganisms to TLRs (Chow et al., 1999). This mechanism forms part of the innate immune system shared by most multicellular organisms. LPS are present on the surface of Gram-negative bacteria, and can bind to TLR4, eliciting activation of canonical NF- κ B signaling, via IKK. These same bacteria would be prevented from colonizing tadpoles grown in the presence of the aminoglycoside gentamicin. LPS that have been treated with H₂O₂ no longer function as TLR4 ligands (Cherkin, 1975). We therefore hypothesized that the very brief dipping of tails into very high concentrations of H₂O₂ was inhibiting regeneration by disabling TLR ligands of endogenous skin bacteria around the wound area, preventing TLR4 activation. In the absence of TLR4 activation, perhaps IKK activation of NF- κ B is insufficient to sustain the Nox-dependent ROS signaling required for successful regeneration.

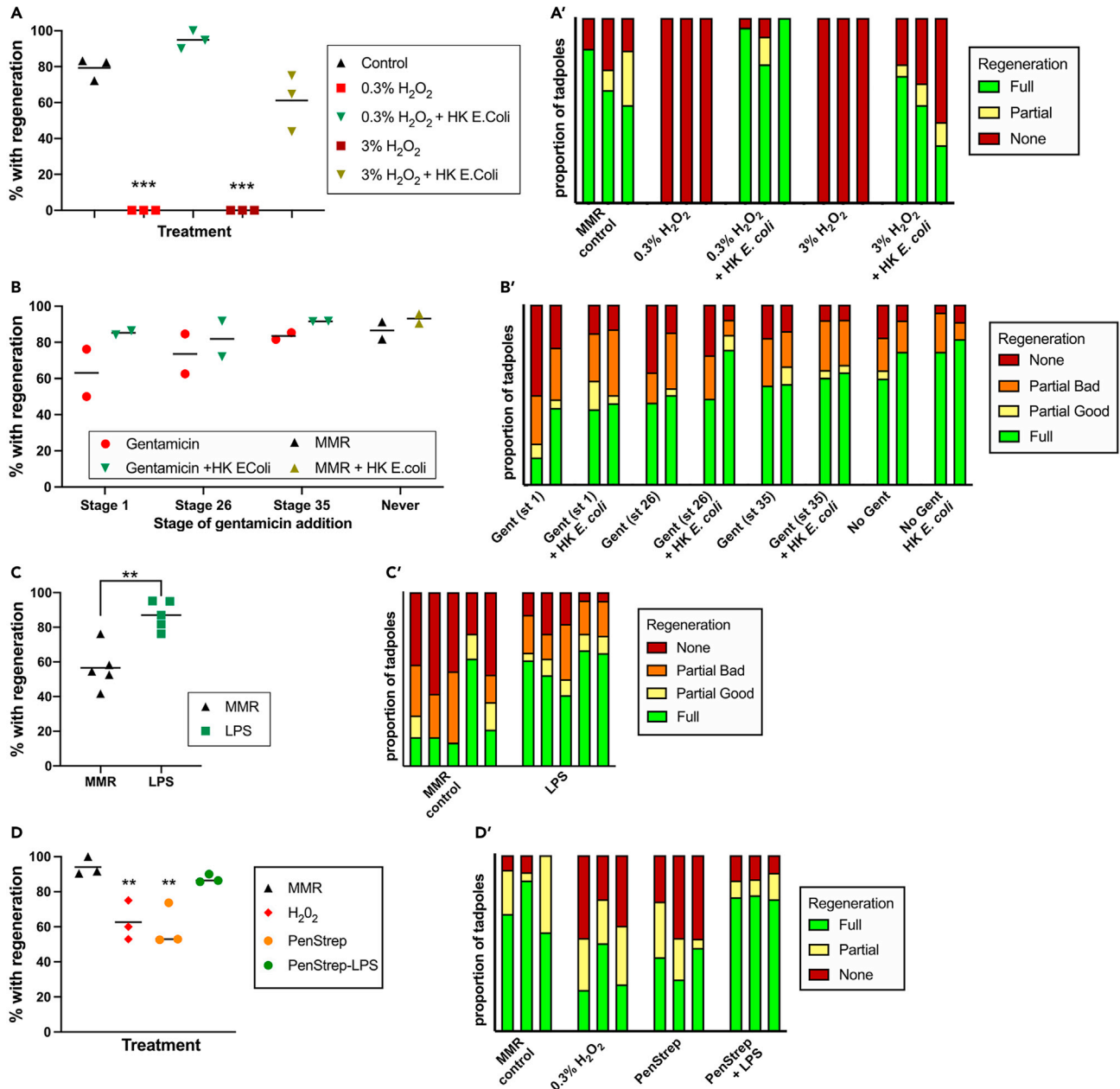


Figure 3. Resident microbes may activate regeneration of tadpole tails

(A–D) Scatterplots showing percentage of tadpoles regenerating after amputation of the distal third of the tail at stage 46, with points representing percentage of tadpoles regenerating in each replicate dish, and stacked bar graphs of the same data by regeneration phenotype (A'–D'). Raw data can be found in [Data S1](#). (A) Local brief application of either 0.3% or 3% H₂O₂ to tadpole tail stumps immediately after amputation prevents regeneration completely. This effect can be reversed by addition of heat-killed (HK) *E. coli*. Data were analyzed using one-way ANOVA and Tukey's multiple comparisons test, ***p < 0.001. Each point represents an experimental dish with N = 14–20 tadpoles. (B) Raising tadpoles in the broad-spectrum antibiotic gentamicin (100 µg/mL) to prevent skin bacterial colonization and growth prevents some tadpoles from regenerating. The later the treatment is started, the more tadpoles regenerate. In each case, more tadpoles regenerate if LPS-containing HK *E. coli* is added just after amputation. Analysis by one-way ANOVA and Tukey multiple comparisons test did not reveal any significant p values, but the data trends are clear. Each point represents an experimental dish with N = 21–48 tadpoles. (C) Significantly more tadpoles regenerate when commercial purified LPS (50 µg/mL, from *E. coli* 0111:B4) are added to the medium just after tail amputation, for 1 h (unpaired t test, p = 0.002). Each point represents an experimental dish with N = 19–24 tadpoles. (D) Regeneration decreased when tadpoles were either soaked in 0.3% H₂O₂ for 2 min before amputating the tail or raised with antibiotics (N = 12 to 22, **p < 0.01, one-way ANOVA, Dunnett's multiple comparisons with controls/MMR). Adding exogenous LPS immediately after amputation rescues the effect of raising in antibiotics.

To test this, heat-killed K12 *Escherichia coli* (Gram-negative bacteria with LPS, method from Pradhan et al. [2012]) was added to see if it could rescue regeneration in H₂O₂ tail-dipped tadpoles. Heat-killed *E. coli* rescued regeneration in 95% of tadpoles treated with 0.3% H₂O₂ and 61% of tadpoles treated with 3% H₂O₂ (Figure 3A). We then raised embryos in gentamicin from day 0 (stage 1, fertilized egg), stage 26, or stage 35 and compared their regeneration rate with or without heat-killed *E. coli* (Figure 3B). Since LPS function well as a ligand even when bacteria are killed, we expected there to be more bacteria, and therefore more LPS available to activate NF- κ B, in the later treated tadpoles. More tadpoles regenerated the later gentamicin was added to media, supporting this model. In each case, addition of heat-killed *E. coli* immediately after amputation increased the percentage of tadpoles regenerating, although this did not reach significance. We then purchased commercially purified LPS from *E. coli*, (strain 0111:B4) and showed that 50 μ g/mL added after tail amputation significantly increased regeneration from 57% of tadpoles to 87% of tadpoles, $p = 0.002$, unpaired t test (Figure 3C). A significant increase in tail regeneration was also elicited by commercially purified LPS from *Pseudomonas aeruginosa* (Figure S2).

To see if exogenous H₂O₂ could inhibit regeneration by altering some aspect of the tadpole's skin (such as commensal bacteria), as well as by exposing the amputation wound site, we immersed refractory stage tadpoles in 0.3% H₂O₂ for 2 min, rinsed three times in Marc's modified ringers (MMR), and then amputated tails. Regeneration of these "sterilized" tadpoles were compared with regeneration of tadpoles from the same batch raised in antibiotics (Figure 3D). Both treatments resulted in a significant and comparable reduction in the percentage of tadpoles that regenerated tails, and *E. coli* LPS addition immediately after amputation rescued this effect in antibiotic-treated tadpoles. Importantly, we showed that heat-killed *E. coli* extracts work as well as commercial LPS to rescue the effect of raising tadpoles in antibiotics immediately after fertilization and jelly coat removal. Comparing the percentage regeneration means \pm SE from the relevant first two data columns in Figure 3B (MMR control = 86.6 ± 4.8 , antibiotic raised = 63.1 ± 13.1 , antibiotic raised rescue with HK *E. coli* = 85.3 ± 1.1) with those in Figure 3D (MMR control = 93.9 ± 3.1 , antibiotic raised = 59.7 ± 7.0 , antibiotic raised rescue with LPS = 87.4 ± 1.3) shows a robust, reproducible, and equivalent rescue.

To determine if the effect raising tadpoles in gentamicin has on tail regeneration was due to suppressing bacterial colonization of the skin, rather than any off-target effects, we investigated the effect of other antibiotics. Penicillin/streptomycin, commonly used in cell and tissue culture, was added to tadpoles from stages 2–4, and tadpoles raised in the presence of these antibiotics were compared with siblings raised either with no antibiotics or in 50 μ g/mL gentamicin, in cohorts of tadpoles from two different mothers. The distal third of the tail was amputated from all tadpoles at stage 46, and the percentage of tadpoles regenerating a tail after 5 days was calculated for each replicate Petri dish (Figure 4A). Mean regeneration percentage was high in both of these cohorts, at 94 and 93. As expected from previous results, significantly fewer tadpoles regenerated when they were raised in gentamicin (means 51% and 64%). Penicillin- and streptomycin-raised tadpoles were also significantly worse at regenerating (means 40% and 59%). Both gentamicin and streptomycin are aminoglycosides, which are a class of antimicrobials that inhibit bacterial protein synthesis (reviewed in Krause et al. [2016]). Gentamicin has been shown to inhibit migration of canine corneal cells (Hendrix et al., 2001), alter the function of neutrophils (Umeki, 1995), and cause kidney nephrotoxicity (Swan, 1997). To ensure that the effect on regeneration was the result of antimicrobial action and not off-target effects on the tadpoles themselves, such as affecting migration of ROCs (Aztekin et al., 2019) we compared the regeneration ability of tadpoles raised with no antibiotics, but which then had antibiotics added on amputation, with tadpoles raised in antibiotics (Figure 4B, C). Significantly fewer tadpoles raised in either penicillin and streptomycin or gentamicin regenerated compared with controls, whereas tadpoles that only had these antibiotics added from the time of amputation regenerated as well as controls. This shows that, at the levels used here, the antibiotics are not directly affecting the ability of tadpoles to regenerate tails. Furthermore, the effect on regeneration is not seen when tadpoles are raised in antibiotics that predominantly target gram-positive bacteria, such as penicillin G (Figure S3).

Brief activation of NF- κ B activator, IKK, using chemical genetics, can enhance hindlimb regeneration at stage 56 but not forelimb regeneration in older tadpoles

Having established that brief NF- κ B activation could be a driver of regeneration in tadpole tails, we then turned to the tadpole limb, which exhibits an ontogenic decline in regenerative potential (Dent, 1962; Beck et al., 2009). Modulation of inflammation has been implicated in successful regeneration (Mescher et al 2013, King et al., 2012). A week-long exposure to the Cox2 inhibitor celecoxib improved regeneration in stage 54–55 tadpole hindlimbs following knee-level amputation (King et al., 2012). We wanted to see if

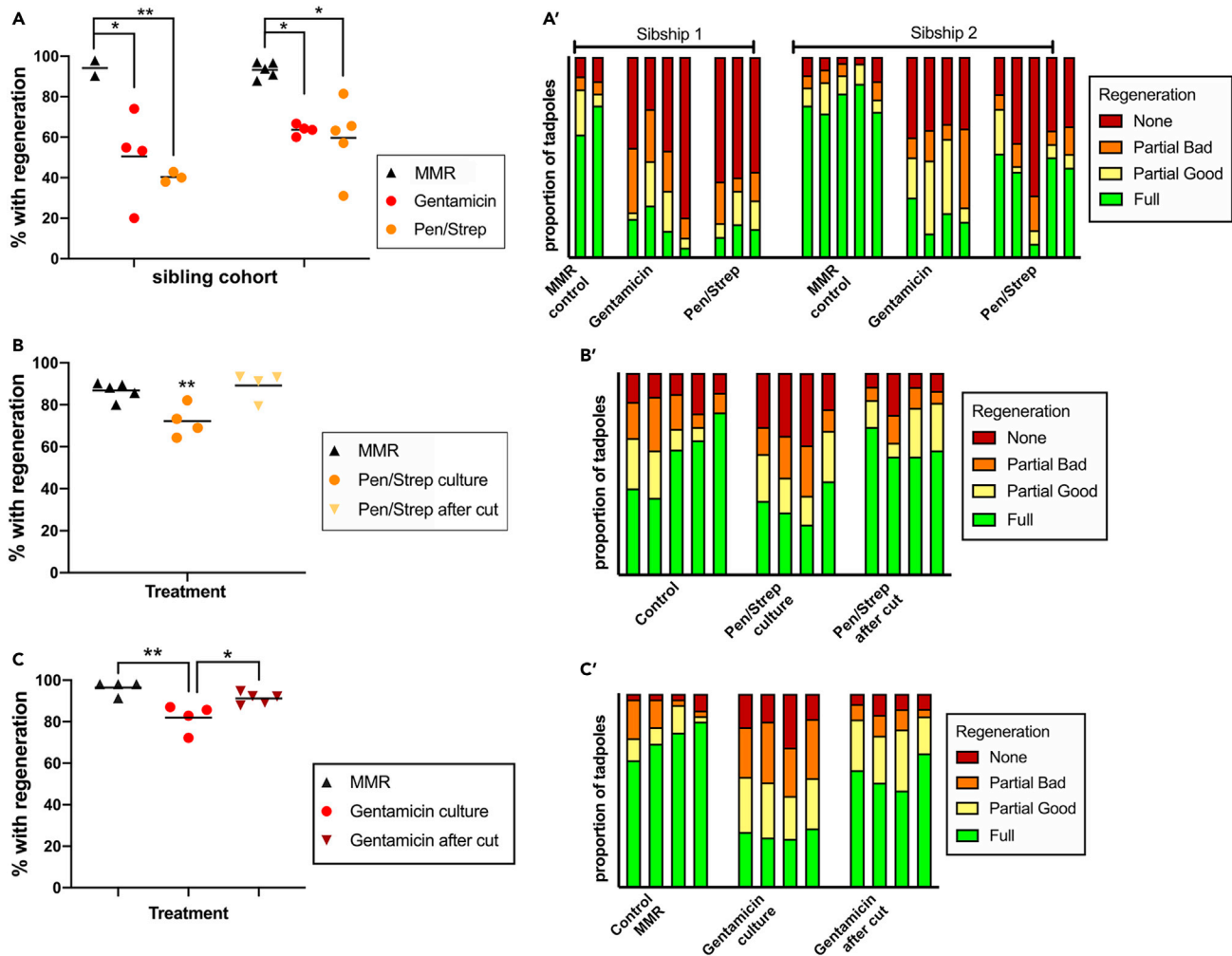


Figure 4. Raising tadpoles in antibiotics influences tail regeneration

(A–C) Scatterplots showing percentage of tadpoles regenerating after amputation of the distal third of the tail at stage 46, percentage of tadpoles regenerating in each replicate dish, and stacked bar graphs of the same data by regeneration phenotype (A'–C'). Raw data can be found in [Data S1](#). (A) Raising tadpoles in either gentamicin 50 $\mu\text{g}/\text{mL}$ or penicillin/streptomycin from 2- to 4-cell stage significantly reduces the number of tadpoles that regenerate tails in the refractory period in two sibling cohort groups (two-way ANOVA, Tukey's multiple comparisons test all means). Each point represents an experimental dish with $N = 20\text{--}33$ tadpoles. (B and C) Antibiotics do not directly alter the regeneration process of *Xenopus* tadpole tails. Culturing embryos from 2 to 4 cells in penicillin/streptomycin (B) or 100 $\mu\text{g}/\text{mL}$ gentamicin (C) significantly reduces the number of tadpoles regenerating, but adding the same antibiotics after cutting does not hinder regeneration (one-way ANOVA, Tukey's multiple comparisons test of all means). Each point represents an experimental dish with $N = 28\text{--}34$ tadpoles. * $p < 0.05$, ** $p < 0.01$.

we could increase this effect by first activating NF- κB . We controlled our experiments by amputating both hindlimbs at the same level and varying the left- and right-side treatment. We used a double-stage treatment, with immediate exposure of the stump to the IKK (and therefore NF- κB) activator prostratin, followed by treatment with the Cox2 inhibitor celecoxib, which turns off the inflammatory pathway by inhibiting IKK (Sareddy et al., 2012). In our experiments with stage 56 limbs, treatment with prostratin or vehicle control resulted in 0–1 toes regenerating, and celecoxib treatment, beginning 17 min after amputation, only increased the number of regenerating toes when it was preceded by prostratin pretreatment (mean 2 toes, range 0–3; Figure 5). Brief activation of NF- κB after limb amputation followed by a high dose of celecoxib therefore improved regeneration outcomes, but was not able to produce perfect regenerates.

The same chemical genetic approach for brief NF- κB activation was then used in older animals. Once *Xenopus* tadpoles have lost their tails at metamorphosis, they rely on hindlimbs for swimming, so it is better to amputate forelimbs. Although forelimbs are used for feeding, they can easily manage without if food is plentiful. Forelimbs

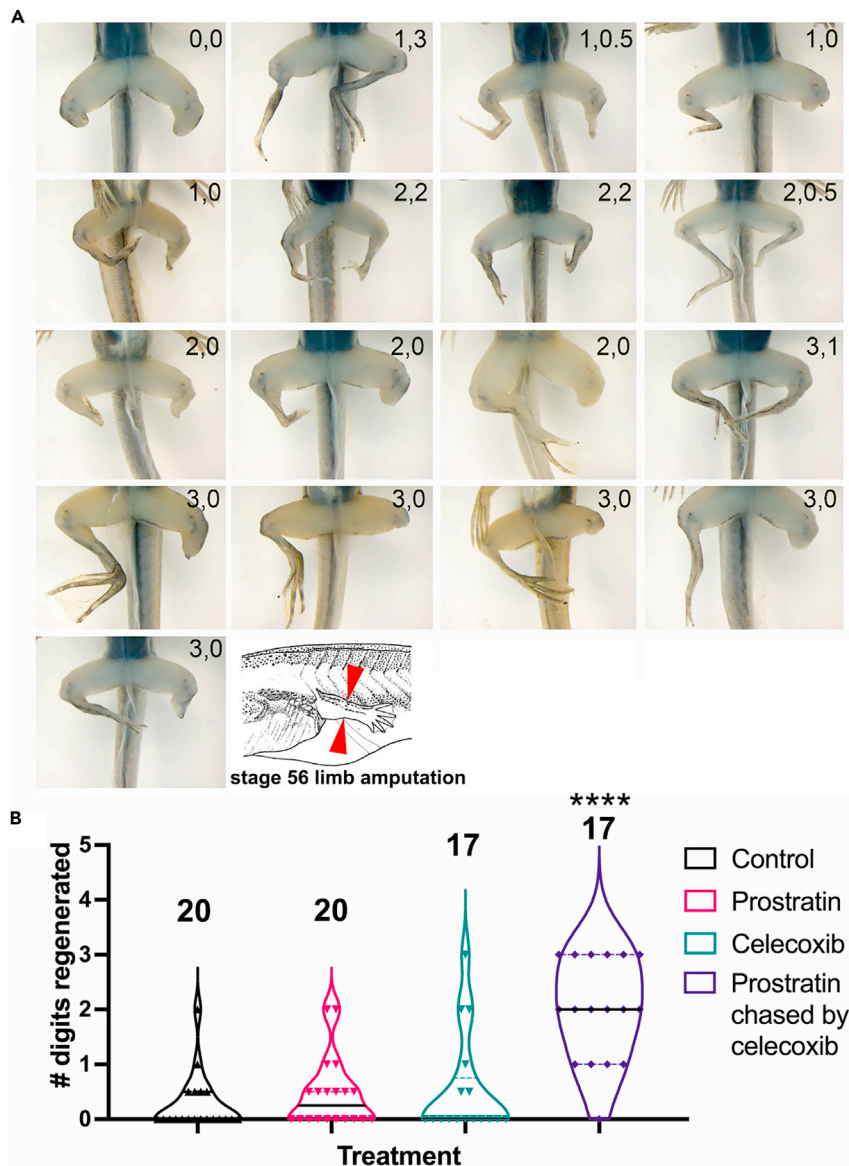


Figure 5. Short-term chemical genetic activation of NF- κ B enhances hindlimb regeneration at stage 56, but regeneration is limited to 3 digits

(A) Stage 56 hindlimbs were amputated at knee level (red arrowheads). The right limb was treated with 1 μ L topical 100 μ M prostratin in 1% ethanol and the left limb treated with vehicle (1% ethanol), for 17 min. Both limbs were then treated with 40 μ M celecoxib for 90 min. Tadpoles were allowed to regenerate until stage 58 and are viewed from the ventral side (so the left control hindlimb appears on the right of the panels). Numbers in top right of each image indicate the score for each limb (right, prostratin and celecoxib; left, vehicle and celecoxib).

(B) Violin plot to show number of digits regenerated after immediate treatment of stage 56 limb stumps with 1% ethanol for 17 min (control) or 100 μ M prostratin for 17 min ($n = 20$), or the same two treatments followed by 40 μ M celecoxib for 90 min ($n = 17$, tadpoles shown in A). Prostratin followed by celecoxib after 17 min resulted in significantly more digits regenerating than any other treatments, one-way ANOVA and Tukey's multiple comparisons to all means, **** $p < 0.0001$, median shown by solid line and quartiles by dotted lines. Number above violin indicates size of sample (N).

regenerate only a hypomorphic spike (Suzuki et al., 2006); although they form a wound epidermis and blastema, the spike only consists of a single cartilage extension encased in skin, with nerves and blood vessels: no bone, muscle, joints, or dermis is seen. Even this hypomorphic response can be inhibited, overexpression of the BMP inhibitor *Noggin* following amputation midway through the forearm results in formation of a stump (Beck et al.,

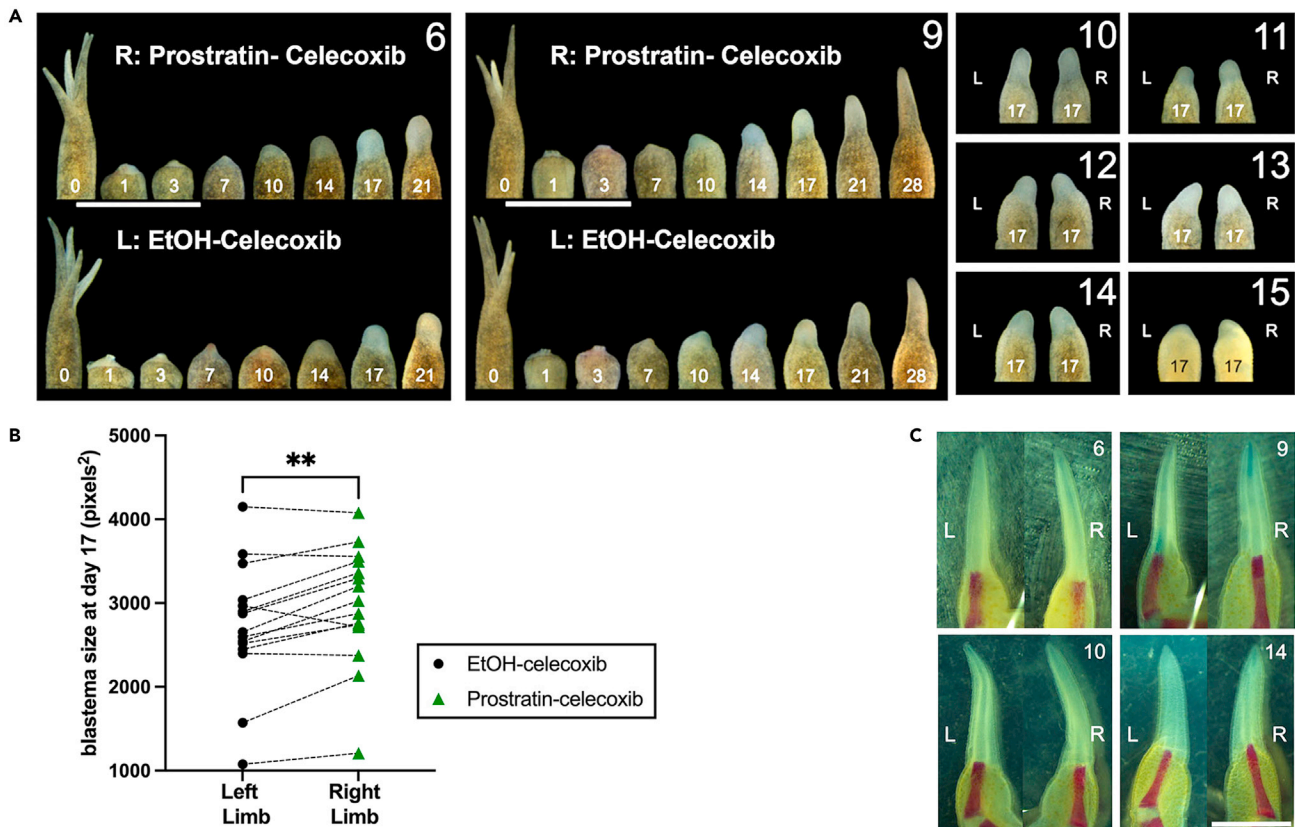


Figure 6. Brief activation of NF- κ B using chemical genetics (prostratin for 20 min followed by celecoxib) does not enhance forelimb regeneration, but does increase blastema size

(A) Numbers on limbs indicate days after amputation. A full stage series is shown for froglets 6 and 9, with just the 17 dpa limbs, which have clear blastemas, shown for froglets 10, 11, 12, 13, 14, and 15. In each case the left forelimb has been treated with vehicle (1% EtOH) and the right one with 100 μ M prostratin in 1% EtOH for 20 min following amputation at the mid forearm level, and then with 40 μ M celecoxib for 90 min. Scale bar, 5 mm.

(B) Blastema size was estimated from photographs at 17 dpa in $n = 15$ froglets using ImageJ. Left control limbs formed significantly smaller blastemas, $p = 0.0017$, paired t test. Left and right limbs from the same froglet are connected by a dotted line.

(C) Bone and cartilage were stained in 5 froglets, 2 months after amputation; 4 sets of forelimbs are shown here. The alizarin red bone (red) ceases at the point the forelimbs were amputated, and a cartilage spike formed, with no joints or branches, in each case (pale blue). Numbers on the top right indicate froglet number; L and R indicate left and right forelimb, respectively; scale bar, 2 mm, $**p < 0.01$. Raw data can be found in [Data S1](#).

2009). Froglets were subjected to amputation midway through both forearms, followed by topical treatment of stumps for 20 min with either 100 μ M prostratin or 1% ethanol (vehicle control). They were then treated by immersion in 40 μ M celecoxib for 90 min. Both treatments resulted in the eventual formation of hypomorphic spikes (Figure 6A), although the average size of the blastema in the prostratin and celecoxib-treated right forelimbs was larger than controls in 11/15 froglets at 17 dpa (paired t test $p = 0.017$, Figure 6B). Five froglets were stained for bone and cartilage, but no bone, joints, or multiple cartilage spikes was observed (Figure 6C). Therefore, using prostratin chased by celecoxib to briefly activate NF- κ B does not rescue forelimb regeneration in *Xenopus*, but does increase the initial blastema size. The prostratin analog DPP (13-deoxyphorbol 12-phenylacetate) has been reported to bind 10 times more strongly than prostratin to protein kinase C, and is also an IKK activator. When DPP was used instead of prostratin, the resulting blastemas were consistently larger than vehicle-treated controls, and one froglet produced two small bone fragments distinct from the uncut bone, but patterned limb regeneration was not achieved (Figure S4).

DISCUSSION

NF- κ B as an early responder to tail amputation

Regeneration of the *Xenopus* tadpole tail can be divided into three distinct phases: an early wound healing stage, which takes place between amputation and 6 hpa, an intermediate phase in which the regeneration

bud is established (around 24 hpa), and a late phase from 2–7 dpa, in which replacement of lost tissues is completed (Beck et al., 2009; Ferreira et al., 2018). The rapid translocation of NF- κ B to the nucleus of distal tail cells within 1 h of tail amputation suggests that this transcription factor is an early response to injury. This early, transient translocation appears to be dependent on Nox activity, since tadpoles exposed to the Nox inhibitor, DPI, had a more gradual RelA translocation, with nuclear levels peaking at 3–6 hpa. This suggests that ROS can modulate NF- κ B during early wound healing in regenerating tadpole tails. A downstream direct target of NF- κ B, *ptgs2* (*cox2*) was upregulated in the wound epithelial cells of regeneration-competent tadpole tails and early limb buds 6 h after amputation, potentially in the ROCs identified recently by Aztekin et al. (2019). ROCs express many signaling factors, such as wnt, FGF, BMP, Notch, and TGF β and have been shown to play a critical role in regenerative competence and the production of a regeneration bud/blastema, at least in tails. *Cox2* encodes a cyclo-oxygenase enzyme, which regulates formation of the inflammatory molecule prostaglandin E2. *Cox2* is known to be upregulated in lizard (*Gekko japonicus*) autotomized tail stumps (Xu et al., 2019). The *Cox2* inhibitor indomethacin was shown to retard regeneration in *G. japonicus* tails, in a wnt signaling-dependent manner (Xu et al., 2019). Prostaglandin E2 also inhibits NF- κ B activity, via IKK in fibroblasts (Gomez et al., 2005), suggesting a mechanism whereby the pro-inflammatory response can be self-regulating (Figure 7). In support of this Pearl et al. (2008) found that regeneration-competent 3 dpa limb blastemas have significantly reduced *ptgs2* (*cox2*) expression, compared with noggin-blocked non-regenerating controls. Together this suggests that early *cox2* in ROCs is a marker of successful regeneration, but that prolonged expression prevents the formation of a regeneration blastema.

Interestingly, recent work has identified an early role in regeneration for the hypoxia-induced transcription factor, HIF1 α (Ferreira et al., 2018). In Ferreira et al.'s model, tail amputation leads to an O₂ influx within 5 min, which leads to ROS increase, and HIF1 α activity, causing membrane polarity reversal. The regeneration-specific redox state in the wound epithelium and regeneration bud is established in the first hour by NADPH oxidases. Notably, this early time frame correlates with our observations of NF- κ B rapid and transient relocation to the nucleus. Like NF- κ B, HIF1 α is regulated post-translationally, so is capable of rapid responses, and the two pathways are intertwined as IKK β can regulate hypoxia-induced HIF1 α and downstream targets such as *Cox2* in mouse macrophages (Rius et al., 2008) and NF- κ B can also be activated by hypoxia (D'ignazio and Rocha, 2016).

NF- κ B as a potential potentiator of the wound-responsive ROS production, via induction of Nox genes

We found that two direct targets of NF- κ B, which code for the NADPH oxidases thought to be the primary drivers of the sustained ROS signaling required for regeneration, become expressed by 6 hpa in limb buds. *nox4* is expressed in distal wound cells, so expression seems to be induced specifically by amputation. *Nox2* is expressed in punctate cells, which resemble the phagocytic cells recruited to the wound. Either Nox could be acting to drive sustained ROS production during tadpole appendage regeneration. However, preventing inflammatory cell recruitment to the site of injury does not significantly alter ROS production in regenerating tadpole tails (Love et al., 2013), suggesting that *nox2* does not substantially contribute to overall ROS production during tail regeneration. *Nox4* is constitutively active, and its main regulation is via transcriptional upregulation. Interestingly, *Nox4* can be directly upregulated by TLR4 signaling (Park et al., 2004; Suzuki et al., 2012), which supports our model.

Bacteria on tadpole skin could influence tail regeneration via LPS-TLR4-NF- κ B signaling

The innate immune system is well established in stage 46 tadpoles, but the adaptive immune system is not yet online (Robert and Ohta, 2009). Here, we have shown that raising tadpoles in antibiotics that select against gram-negative bacteria or brief local exposure of the cut tail to H₂O₂ biases tail-amputated tadpoles toward wound healing and that raising them without antibiotics, or post-amputation exposure to LPS, prostratin, or heat-killed *E. coli* is more likely to result in a regenerative response. We propose that LPS from gram-negative commensal skin microbiota activate NF- κ B via TLR4, which is expressed in macrophages and neutrophils. The critical role of macrophages is well established not only for epimorphic appendage regeneration in fish and amphibians but also for the rare cases of mammalian epimorphic regeneration. Macrophage depletion prevented both ear punch hole closure in the African spiny mouse *Acomys cahirinus* (Simkin et al., 2017a) and digit tip regeneration in *Mus musculus* (Simkin et al., 2017b). Prolonged myeloperoxidase activity (a marker of inflammation and neutrophils) was associated with fibrotic scarring, whereas prolonged ROS was associated with successful regeneration. *Xenopus* tadpole tail regeneration also requires prolonged ROS, which we suggest could be regulated via NF- κ B, as mentioned previously (Figure 7).

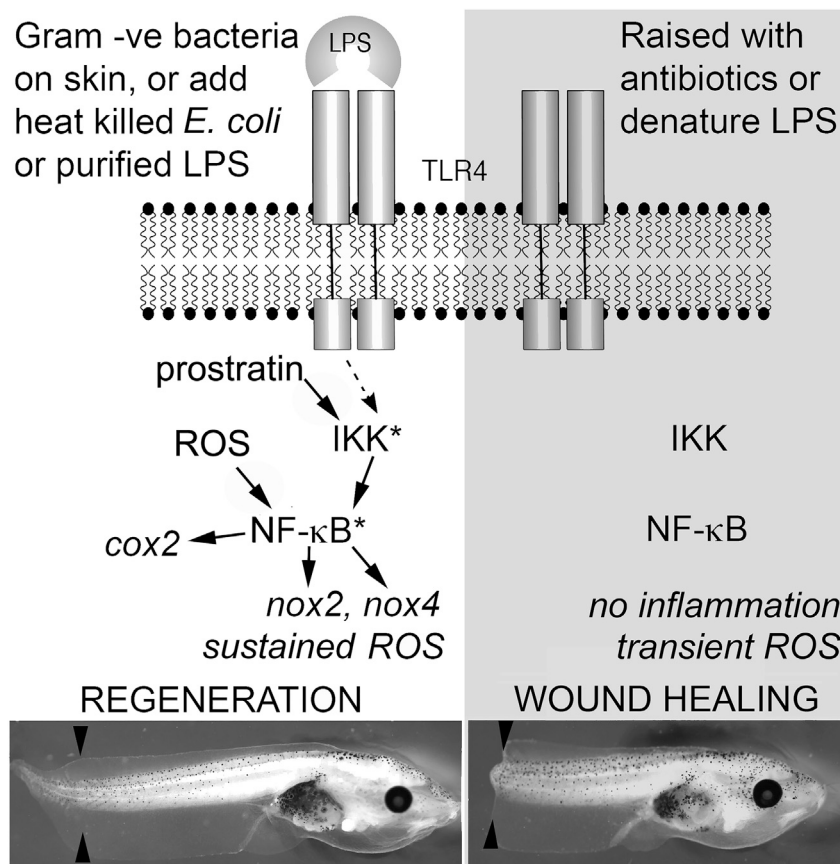


Figure 7. Model for how skin bacteria could result in tail regeneration in refractory-stage tadpoles

ROS are produced rapidly when the tail is cut. We propose a model where gram-negative bacteria on tadpole skin can activate TLR4 receptors on tissue-resident macrophages when the tail is cut. This leads to rapid activation of the NF-κB transcription factor, which in turn upregulates expression of NADPH oxidases (Nox2, Nox4) allowing sustained production of ROS, as well as upregulation of the pro-inflammatory enzyme Cox2. In this model, raising tadpoles with antibiotics to prevent Gram-negative bacteria colonization of skin or denaturing LPS on skin would result in reduced activation of TLR4, no activation of NF-κB, and loss of sustained ROS.

Previous work has shown that tissue-resident macrophages are present in *Xenopus* tails at the stages used here (Paredes et al., 2015). In studies of *Xenopus* limb bud regeneration, neutrophils are the first to arrive at the wound site, during the establishment of the blastema, with macrophages coming in later and persisting longer, well into the re-patterning stage (reviewed in Mescher [2017]). This also correlates well with the requirement for an initial inflammatory response to wounding that must be dampened down in order for regeneration to proceed. Promoting regeneration by modulation of inflammation is a well-established regenerative medicine strategy (Julier et al., 2017). Our attempts to promote better limb bud regeneration using chemicals to mimic this inflammation “on-off” environment was partially successful, but despite resulting in an initially larger blastema, the treatment did not elicit regeneration in older limbs.

A recent single-cell analysis of tadpole tail regeneration found two populations of “myeloid” cells, termed Myeloid1 and Myeloid2, which are required for regeneration (Aztekin et al., 2020). TLR4 is expressed in both populations, but to a higher degree in Myeloid1, associated with pro-inflammatory markers. We propose that, during the refractory period, LPS from skin bacteria activates TLR4 in tissue-resident macrophages, accounting for the decreased regeneration seen in cohorts of tadpoles raised in antibiotics. Critically, this method of activating innate immunity could elicit a strong, but short-lived, inflammatory response. Previously, it was shown that inhibiting the adaptive immune response rescued refractory-stage tadpoles (Fukazawa et al., 2009), suggesting that the role of NF-κB in regeneration is complex. In another model of regeneration, the zebrafish heart, NF-κB is both necessary and activated (Karra et al., 2015).

Our results suggest the exciting possibility that the microbiome of the skin could influence vertebrate regeneration. A similar role for the microbiome has been demonstrated in a key invertebrate regeneration model organism, planarians (flatworms). *Schmidtea mediterranea* with an over-representation of Proteobacteria failed to regenerate (Arnold et al., 2016). Our antibiotic-raised tadpoles had altered regenerative success, which could be due to an altered microbiome. The robustness of regeneration in pre-refractory tadpole tails suggests that these younger tadpoles rely on a different trigger to bacterial LPS. Older, post-refractory tadpoles are feeding and would not be free of LPS even if raised in antibiotics. Future work will be needed to confirm the role of TLR4, LPS, and microbiota in the regeneration in *Xenopus* and other models capable of appendage regeneration.

Limitations of the study

The ability to detect NF- κ B in cytoplasmic and nuclear extracts of *Xenopus* tails limited our ability to directly test during the refractory stage, when the tail is much smaller. Ideally in future, immunohistochemistry could be used to directly assess NF- κ B localization in individual cells. LPS activation of NF- κ B has not been demonstrated in frogs, but the canonical pathway LPS-TLR4-NF- κ B pathway is conserved in fish (Correa et al., 2004). While this manuscript was under review, work in the regeneration-competent axolotl has demonstrated conservation of the LPS-TLR4 response in amphibian macrophages (Debuque et al., 2021). In future, it may be possible to demonstrate this in *Xenopus* using similar approaches. In our model, the role of TLR4 is inferred only, and direct demonstration of the role of TLR4 is outside the scope of this study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.103281>.

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AUTHOR CONTRIBUTIONS

T.F.B., conceptualization and investigation; T.F.B. and C.W.B., methodology, formal analysis, writing, reviewing, and editing; C.W.B., data curation, resources, and supervision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Digoxygenin-AP, Fab fragments, from sheep	Roche	#11093274910
Rabbit anti-NF- κ B p65, polyclonal antibody	Invitrogen	#PA5-16545
Goat anti-rabbit IgG (H+L)-HRP conjugate	Bio-Rad	#1706515
Bacterial and virus strains		
DH10B <i>E. coli</i>	ThermoFisher Scientific	EC0113
Chemicals, peptides, and recombinant proteins		
Celecoxib	Sigma Aldrich	Cas 169590-42-5 #SML3031
Prostratin	Sigma Aldrich	#P0077
DPI (Diphenyleiiodonium chloride)	Sigma Aldrich	Cas 4673-26-1 #D2926
DPP (12-deoxyphorbol 13-phenylacetate)	Sigma Aldrich	Cas 54662-30-5 #D7821
LPS (lipopolysaccharides) from <i>P. Aeruginosa</i> 10	Sigma Aldrich	#L9143
LPS (lipopolysaccharides) <i>E. coli</i> 0111:B4	Sigma Aldrich	#L3024
Experimental models: Organisms/strains		
<i>Xenopus laevis</i> , wild type	Own colony	
Oligonucleotides		
TCCGTTTTCCAGGGCAGTG	Sigma Aldrich NZ	<i>Nox1.L 5'</i>
AGATTGGACGCCCATAGCTG	Sigma Aldrich NZ	<i>Nox1.L 3'</i>
GAATGATCGTACTACCCGC	Sigma Aldrich NZ	<i>Ptgs2.S 5' (Cox2)</i>
TTAAAGTTCGGATGTGTGC	Sigma Aldrich NZ	<i>Ptgs2.S 3' (Cox2)</i>
CTATGACGAGGGCGAAGAT	Sigma Aldrich NZ	<i>Cybb.L 5' (Nox2)</i>
TCATCCAGCCAGTGAGGTA	Sigma Aldrich NZ	<i>Cybb.L 3' (Nox2)</i>
TAGGCAGGAATCCAGTGATGG	Sigma Aldrich NZ	<i>Nox4-like.S 5'</i>
CACTCCGCAACAGAAGTGA	Sigma Aldrich NZ	<i>Nox4-like.S 3'</i>

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Caroline Beck (caroline.beck@otago.ac.nz).

Materials availability

Plasmids generated in this study are available upon request.

Data and code availability

- Raw data for all graphs and charts as well as statistical analyses are available in [Data S1](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal ethics

All animal experiments were approved by the University of Otago Animal Ethics Committee under AEC56/12, AUP86/14 and AUP01/19

Experimental model animals

All *X. laevis* tadpoles and froglets used in these experiments were unsexed as they are sexually monomorphic up until sexual maturation. The developmental stages (Nieuwkoop and Faber, 1956) are indicated in the specific methods for each experiment. Animals from the same sibship were randomly assigned to treatment and control groups and when multiple dishes or tanks were required, an equal number of animals from each was assigned to each treatment group. Adult *X. laevis* wild type were bred and housed at the University of Otago's Zoology Department, in re-circulating Marine Biotech XR3 aquaria in a dedicated controlled temperature room at 18°C. They are fed salmon pellets twice a week, and 10% of the total water is changed each day from a tap water supply filtered through carbon to remove chlorine. Tadpoles > stage 48 are housed in an XR1 aquarium and fed spirulina powder daily until metamorphosis. Froglets up to sexual maturity are fed size appropriate salmon pellets daily.

Experimental bacterial strains

Dh10b *Escherichia coli* were grown in 250mL flasks containing 10mL Luria broth overnight at 37°C with shaking at 225rpm.

METHOD DETAILS

Xenopus breeding

Adult female *Xenopus laevis* were induced to lay eggs by injection of 500u HCG (Chorulon) per 75g body-weight, into the dorsal lymph sac, and placed at 18°C 16 hours before eggs were required. Eggs were fertilised with homogenised testis, dejellied in 2% Cysteine HCl pH 7.9 immediately after embryo rotation, washed well and raised in petri dishes in 0.1x MMR (Marc's Modified Ringers: 10x stock is pH corrected to 7.8 and contains 1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 20 mM CaCl₂; 50 mM HEPES, 1 mM EDTA pH 8.0).

Western blots

Stage 55 tadpoles, n = 4 for each timepoint, were anaesthetised by placing in 1/4000 MS222 with subjected to removal of the distal 40% of the tail with a scalpel blade. For the zero hour timepoint, a 3mm slice of tail was harvested immediately, for the 1, 3 and 6 hour samples tadpoles were re-anaesthetised before cutting the distal 3mm of the tail stumps. For DPI treatments, the tadpoles were incubated with 2µM DPI in 0.1% DMSO for 1 hour prior to the first amputation, and continually exposed until the samples were collected. The four 3mm tail samples for each treatment and timepoint were pooled and homogenised in a bead beater in CER I (NE-PER) reagent, Halt protease inhibitor cocktail (Thermo Fisher) and EDTA. Cytoplasmic and nuclear fractions were extracted using NE-PER and cytoplasmic extraction reagents according to instructions (Thermo Fisher). Nuclear extracts were diluted 1:1 in PBS. Samples were prepared with sample loading buffer and heated to 100°C for 3 minutes. 10ul of each sample was run on 1.2% SDS-PAGE gels along with a Precision Plus Protein dual colour standard (Bio-Rad), and transferred to PVDF membranes. Membranes were blocked for 25 minutes with 2% BSA in PBS/Tween and incubated for 2 hours with 1:200 rabbit-anti NF-κB p65 polyclonal antibody (Pierce), then for 1 hour with 1:5000 Goat anti-rabbit IgG (H+L) HRP conjugate (Bio-Rad). Reactivity was detected with Clarity Western ECL substrate (Bio-Rad).

Tail regeneration

Tadpoles were raised in batches of 40–60 in 10mm petri dishes with 30 ml 0.1 x MMR, and staged according to (Nieuwkoop and Faber, 1956). Tadpoles > stage 45 were first briefly anaesthetised in 1:4000 w/v MS222 before removing one-third of the tail using a clean scalpel blade. Young tadpoles < stage 44 were amputated without anaesthesia. Tadpoles were then divided into treatment groups and allowed to regenerate at 24°C for 5–7 days, before being scored as NR (no regeneration, stump covered with full thickness epidermis), PB (Partial bad, regenerated but not functional, one or more tissues missing, poor outgrowth), PG (partial good, all tissues present, good outgrowth but may have dorsal or ventral fin defect and/or bend) or FR (full regeneration, no defects). Data are presented as replicate dish scatter plots as % regeneration which includes PB, PG and FR categories, and as stacked column graphs to show categorical data. Raw data can be found in [Data S1](#). Treatments were as follows. DPI was added at 2µM in 0.1% DMSO from 1 hour before amputation to the completion treatment time (1, 3, 6 or 12 hours). Prostratin (Sigma) was added to tadpoles in 0.1x MMR as a 10µM solution in 0.1% EtOH. Post-amputation treatments with localised H₂O₂ were performed on stage 46 tadpoles, making sure to first wash off any MS222 with 3 x 0.1MMR washes, as MS222 forms a toxic product in combination with H₂O₂. Tadpoles were individually sucked up into an unmodified 3ml plastic pasteur pipette, head first, with the tail projecting from the end. Tails

were then dipped in H₂O₂ solution for 3 seconds and then washed 3 x with 0.1x MMR before being placed in their appropriate treatment dishes. Pre-amputation H₂O₂ experiments involved placing tadpoles in 0.3% H₂O₂ for 2 minutes, with thorough washing in 0.1xMMR before anaesthesia.

Lyophilised LPS from *E.coli* 0111:B4 or *P.aeruginosa* 10 (both from Sigma) was suspended in water and used at 50ug/ml for 1 hour after amputation. Antibiotics were prepared with sterile water and stored as frozen aliquots as 500 or 1000x stocks. Gentamicin sulphate, streptomycin and penicillin G were purchased from Sigma, and Penicillin/streptomycin for cell culture from Roche, and used as recommended. If no stage is indicated, antibiotics were added from 2-4 cell stage and changed at least every two days, and for at least one day following amputation. Heat killed Dh10b *E.coli* were prepared from a 10ml stationary phase liquid culture, grown in Luria broth, centrifuged at 13kRPM and resuspended in PBS three times. The cells were then heated to 70°C for 1 hour, pelleted and resuspended in 2ml 0.1 x MMR. This suspension was added to tadpoles at 1:100 dilution.

Hindlimb regeneration

Stage 51–55 tadpoles were anaesthetised with 1:4000 MS222 and placed on moist paper towels. One hindlimb was amputated using Vannas iridectomy scissors at the approximate level of the ankle. Tadpoles were returned to clean dechlorinated water in 1 litre tanks with air bubbling to aid recovery, then returned to a recirculating tank in an aquarium until fixed for *in situ* hybridisation. Stage 56 hindlimbs were anaesthetised and amputated at knee level, as above, then either 100uM prostratin in 1% ethanol (right limb) or vehicle control (left limb) was added topically with a pipette (1µl) to each stump, for 17 minutes. Half of the tadpoles were then treated with 40uM celecoxib in 0.04% DMSO, in their recovery water, for 90 minutes. Digits were counted as in (Slack et al., 2004), scoring 1 for each individual digit, 0.5 for a spike and 0 for a stump.

Forelimb regeneration

Stage 66, recently metamorphosed froglets were anaesthetised in 1:4000 MS222, and placed on moist paper towels. Both forelimbs were amputated midway through the forearm using a scalpel blade, and the limb stumps raised up by placing 10µl tips under their armpits. Either 100µM prostratin in 1% ethanol (right limb) or vehicle control (left limb) was added topically with a pipette (1µl) to each stump, for 20 minutes. Froglets were regularly moistened with 0.1 x MMR containing MS222 to prevent desiccation. They were then moved to petri dishes containing 40µM celecoxib in 0.04% DMSO with heads propped above the water level to allow breathing, until they were able to move. Once recovered from anaesthetic, they were placed in recovery tanks containing 40µM celecoxib in 0.04% DMSO for a total of 90 minutes. For DPP experiments, 750µM 12-deoxyphorbol 13-phenylacetate in 10% DMSO replaced prostratin, and 10% DMSO was used as vehicle control. Regenerating limbs were photographed at days 0, 1, 3, 7, 10, 14, 17, 21 and 28. Blastema area was calculated from 17 dpa photograph using Image J to count pixels (Schneider et al., 2012).

Cartilage and bone staining

Staining of bone and cartilage in froglets has been previously described (Barker and Beck, 2009), and was undertaken 2 months after amputation. Briefly, froglets had been fixed and stored in 4% formaldehyde (v/v) in PBS. They were cut in half just below the forelimbs and the posterior ends discarded. The viscera were removed from the abdominal cavities and the froglets were rinsed in PBS. They were bleached using 5% H₂O₂ in PBS under a warm light bulb on a nutator during the day and kept stationary at night, for three days with solutions changed daily. Once the pigment had been completely bleached, froglets were rinsed several times with PBS and bubbles were removed from the eyes using fine forceps. Froglets were washed briefly in 70% EtOH in PBS and stained with Alcian Blue stain for cartilage (10 mg Alcian Blue [BDH laboratory supplies] in 60 mL of EtOH + 40 mL acetic acid) for 8 hours. Tissue was washed three times with 70% EtOH (v/v) in PBS over a period of 2 hours and then macerated using 1% KOH for 5 days with daily KOH changes. They were then stained for mineralized bone using Alizarin Red S (10 mg in 100 mL of 1% KOH) for 2 h before soaking overnight in 1% KOH. Bone and cartilage stained samples were cleared and stored in a 1:1 ratio of glycerol (BDH laboratory supplies) and 95% EtOH.

Whole mount *in situ* hybridisations

Coding regions of *ptgs2* (*cox2*), *nox1*, *cybb* (*nox2*) and *nox4* were amplified using primers (Table 1) from stage 12 *X. laevis* cDNA using Pwo DNA polymerase (Roche) and ligated into the cloning site of Pcr4-TOPO vector (Invitrogen Life Sciences). Insertions and their direction were verified by Sanger sequencing. Plasmids were linearised with NotI and digoxigenin labelled antisense RNA probes made

Table 1. Primers used for whole-mount *in situ* hybridization probes

Gene	5' primer	3' primer	Probe
Nox1.L	TCCGTTTTCCAGGGCAGTG	AGATTGGACGCCCATAGCTG	<i>nox1</i>
<i>Ptgs2.S</i>	GAATGATCGTACTACCCGC	TTAAAGTTCGGATGTGTGC	<i>cox2</i>
<i>Cybb.L</i>	CTATGACGAGGGCGAAGAT	TCATCCCAGCCAGTGAGGTA	<i>nox2</i>
Nox4-like.S ^a	TAGGCAGGAATCCAGTGATGG	CACTCCCGCAACAGAAGTGA	<i>nox4</i>

^aGene LOC108710019, not found in Xenbase, but is located on chromosome 2S according to NCBI.

by run off transcription with T3 RNA polymerase and DIG-NTP mix (Roche). Template DNA was removed using DNaseI and probes precipitated using 2.5M LiCl. Limb *in situ* were performed as described in (Pearl et al., 2008), and tails as in (Beck et al., 2003).

Briefly, tissue was fixed for 2–4 hours in 20ml glass scintillation vials using 4% paraformaldehyde/PBSA (Oxoid), at room temperature with gentle nutation, then dehydrated with 75% EtOH in PBSA for 10 minutes followed by 100% EtOH. For limb samples, tails, viscera and heads were removed, samples were placed in fresh 100% EtOH and stored at 30°C until required. Tadpoles were removed from -20°C and allowed to warm to room temperature before being rehydrated in 75% EtOH/PBSA for 10 m, 50% EtOH/PBSA for 10 minutes and PBSA 3 × 5 minutes. Tadpoles were permeabilised with proteinase K in PBS as follows: stage 51–10 µg/ml for 15 minutes, stage 53–20 µg/ml for 12 minutes, stage 54–20 µg/ml for 15 minutes, stages 55 & 56–40 µg/ml for 15 minutes, stages 57, 58 & 59–40 µg/ml for 30 minutes. Proteinase K was removed and replaced with 0.1 M triethanolamine pH 7.8 for 5 minutes. then 0.1 M triethanolamine pH 7.8 with 0.25% acetic anhydride (Sigma) (v/v) for 5 minutes with swirling. Another volume of acetic anhydride was added (to make it 0.5%) for 5 minutes with swirling. The samples were washed in PBSA with 0.1% tween20 (PBSAT) for 2 × 5 minutes before being re-fixed in 4% formaldehyde/PBS for 20 minutes and washed 5 × 5 minutes at room temperature with nutation. Samples were suspended in 20% hyb buffer (v/v) in PBS and left to settle and 20% hyb buffer was replaced with 100% hyb buffer (50% (v/v) formamide (Roche); 5 × SSC; 1 mg/ml yeast RNA (Roche); 100 µg/ml heparin; 1 × Denhardt's; 0.1% Tween 20; 0.1% CHAPS; 10 mM EDTA pH 8) and stored at -20°C. 20x SSC buffer is 3 M NaCl; 0.3 M Na Citrate.

For hybridisation to antisense DIG-UTP labelled probes, samples were placed in glass Packard vials with silicone sealed lids in new hyb buffer pre-warmed to 60°C and placed in the incubator at 60°C with rocking for 2 hours. The hyb buffer was replaced with 1 ml of DIG labelled RNA probe which had been denatured at 80°C for 3 m and made up to 1 µg/ml with 60°C hyb buffer. Samples were then incubated at 60°C overnight with very gentle shaking to hybridise the probes. The probe was washed off with 2 × 10 minute washes with hyb buffer, 3 × 20 minutes with 2 × SSC 0.1% tween 20 (v/v), and 2 × 30 minutes with 0.2 × SSC 0.1% tween 20 (v/v), (60°C with rocking). The samples were then returned to room temperature and washed with MABT (10 × MAB is 1M Maleic acid (Sigma) pH 7.8; 1.5 M NaCl. Adjusted to pH 7.5 with solid NaOH) for 2 × 15 minutes. MABT is 1 × MAB with 0.1% tween20. The samples were preincubated in 2% Boehringer Mannheim blocking reagent (w/v) in MABT for 30 minutes followed by 2% blocking reagent (w/v) and 20% lamb serum (Invitrogen) (v/v) in MABT for 2 hours. Finally, samples were incubated in 1/2000 anti-DIG fab fragments (Roche) coupled to alkaline phosphatase in 2% blocking reagent (w/v) and 20% lamb serum (v/v) in MABT, overnight at 4°C with nutation. Remaining steps were room temperature. Samples were washed in MABT, 3 × 15 minutes and then 6 × 30 minute. Samples were washed with alkaline phosphatase (AP) buffer (100 mM Tris Cl pH 9.5; 50 mM MgCl₂, 100 mM NaCl; 0.1 % (v/v) Tween 20) for 3 minutes and replaced with fresh AP buffer for 10 m (RT with nutation). AP buffer was replaced with 3.4% (w/v) NBT-BCIP tablet (Roche) and left in the dark until purple colour developed (1-3 h). Samples were washed 2 × 15 m in PBST (RT with nutation) and fixed in 4% formaldehyde (v/v) in PBS.

QUANTIFICATION AND STATISTICAL ANALYSIS

All regeneration data and statistical analysis with p values is available in [Data S1](#). Graphs were prepared and data analysed using GraphPad Prism v 9. Statistical tests are listed for each figure in the accompanying legend, significance in each case is taken as p < 0.05. Briefly: [Figure 1C](#), 2-way ANOVA with Sidak's multiple corrections all means; [Figures 2A and 3C](#) unpaired T test; [Figures 3A and 3B](#), [4A–C](#), [5B](#) and [S3](#) 1-way ANOVA with Tukey's comparisons to all means; [Figures 6B and S4B](#) paired T test: [Figure S3](#) 1-way ANOVA with Dunnet's multiple comparisons to the control mean.