# Transducing Bioelectric Signals into Epigenetic Pathways During Tadpole Tail Regeneration

**AI-SUN TSENG AND MICHAEL LEVIN***
Center for Regenerative and Developmental Biology and Department of Biology, Tufts University, Medford, Massachusetts

ABSTRACT

One important component of the cell–cell communication that occurs during regenerative patterning is bioelectrical signaling. In particular, the regeneration of the tail in Xenopus laevis tadpoles both requires, and can be initiated at non-regenerative stages by, specific regulation of bioelectrical signaling (alteration in resting membrane potential and a subsequent change in sodium content of blastemal cells). Although standing gradients of transmembrane voltage and ion concentration can provide positional guidance and other morphogenetic cues, these biophysical parameters must be transduced into transcriptional responses within cells. A number of mechanisms have been described for linking slow voltage changes to gene expression, but recent data on the importance of epigenetic regulation for regeneration suggest a novel hypothesis: that sodium/butyrate transporters link ion flows to influx of small molecules needed to modify chromatin state. Here, we briefly review the data on bioelectricity in tadpole tail regeneration, present a technique for convenient alteration of transmembrane potential *in vivo* that does not require transgenes, show augmentation of regeneration *in vivo* by manipulation of voltage, and present new data in the Xenopus tail consistent with the hypothesis that the monocarboxlyate transporter SLC5A8 may link regeneration-relevant epigenetic modification with upstream changes in ion content. Anat Rec, 295:1541–1551, 2012. © 2012 Wiley Periodicals, Inc.

**Key words: regeneration; Xenopus; bioelectricity; voltage; epigenetic**

## THE *XENOPUS* LARVAL TAIL MODEL

The African clawed frog *Xenopus laevis* is a well-studied model for developmental biology (Beck and Slack, 2001; DeSimone et al., 2005; Koide et al., 2005; Beck et al., 2009; Vergara and Del Rio-Tsonis, 2009; Straka and Simmers, 2011). The *Xenopus* tadpole tail is a complex appendage containing multiple tissues including the spinal cord, muscle, notochord, and vasculature. During development, the tail can regenerate fully by 7–14 days post-amputation (dpa; Beck et al., 2003; Mochii et al., 2007; Tseng and Levin, 2008). A hallmark of this process is a swelling that forms at the amputation site by 1 dpa called the regeneration bud, which contains the lineage-restricted progenitor cells that are required for regeneration (Gargioli and Slack, 2004). Thus, tail regeneration is similar to tissue renewal in mammals. Notably, there is a "refractory period" during development (occurring during stages 45–47) in which tadpoles lose tail regenerative ability and are unable to regrow the appendage after amputation (Beck et al., 2003). The existence of this refractory period makes the *Xenopus* tadpole tail an excellent model for similar age-dependent

Grant sponsors: NIH, G. Harold and Leila Y. Mathers Charitable Foundation, U.S. Army Medical Research and Materiel Command (USAMRMC); Grant numbers: GM078484, W81XWH-10-2-0058, AR061988

*Correspondence to: Michael Levin, Center for Regenerative and Developmental Biology and Department of Biology, Tufts University, 200 Boston Avenue, Suite 4600, Medford, MA 02155. E-mail: michael.levin@tufts.edu

Received 23 November 2011; Accepted 12 April 2012.

DOI 10.1002/ar.22495
Published online 29 August 2012 in Wiley Online Library (wileyonlinelibrary.com).

changes in regenerative ability observed in human beings (Illingworth, 1974). The tadpole tail thus facilitates not only study of efficient endogenous regenerative mechanisms but also the opportunity to improve regeneration (which is difficult with some highly-regenerative model species such as planaria).

Recent studies in the last 10 years have identified multiple molecular mechanisms that regulate tail regeneration. TGF-β signaling is required for proper wound healing after tail amputation (Ho and Whitman, 2008). Well-known pathways including BMP, Notch, Wnt, and Fgf, are required to drive regenerative outgrowth, and inhibition of these activities will block regeneration (Beck et al., 2003; Beck et al., 2006; Lin et al., 2007). Interestingly, these functions appear to recapitulate their roles in tail development (Beck and Slack, 1999; Beck et al., 2001; Beck and Slack, 2002), thus suggesting that additional mechanisms may exist to initiate regeneration. Endogenous apoptosis is required during the first day after tail amputation for the initiation of regeneration (Tseng et al., 2007). The regulation of the levels of hyaluronan, an extracellular matrix component (Contreras et al., 2009) is also an early requirement for regeneration. More recent findings also indicate that systemic changes upon loss of the tail can regulate regeneration. For example, suppression of the immune response in *Xenopus* tadpoles promotes regeneration during the refractory period (Fukazawa et al., 2009). The extent of the inflammatory response also can influence regenerative ability (Franchini and Bertolotti, 2011). More broadly, while many efforts focus on the events local to the wound and regeneration bud, it is now clear that an important influence over regenerative events derives from tissues far anterior to the wound itself (Mondia et al., 2011), making the *Xenopus* tail an important future model for unraveling the mechanisms by which distant regions in the host organism contribute to local morphogenesis.

## BIOELECTRIC SIGNALS IN REGENERATION

Alongside the biochemical signals that underlie regenerative ability functions an important and powerful system of biophysical controls that orchestrates cell behavior into the patterning needs of the host organism. Bioelectricity refers to the slow changes in ion content and resting membrane voltage ($V_{mem}$) that are present in all cells. Distinct from the rapid action potentials of excitable nerve and muscle, and from the effects of external electromagnetic field exposure, it is now known that endogenous gradients of voltage serve as instructive signals regulating cell proliferation, differentiation, and migration (reviewed in Nuccitelli, 2003; McCaig et al., 2005; Blackiston et al., 2009; McCaig et al., 2009; Sundelacruz et al., 2009). Even more exciting than the control of position, type, and number at the level of individual cells, is the role of bioelectric signals in large-scale pattern formation during cancer suppression, embryogenesis, and regeneration (Lund, 1947; Nuccitelli et al., 1986; Levin, 2007, 2009). Standing gradients of voltage and ion fluxes among cells serve to regionalize tissues as pre-patterns for gene expression domains (Burr and Hovland, 1937; Burr and Sinnott, 1944; Vandenberg et al., 2011), provide coordinates to guide morphogenetic rearrangements (Shi and Borgens, 1995), dictate anatomical identity and polarity of actively patterning tissues (Marsh and Beams, 1949; Kurtz and Schrank, 1955; Marsh and Beams, 1957; Levin et al., 2002; Adams et al., 2006; Beane et al., 2011), and serve as master-regulators for initiating the development of specific organs and the regeneration of whole appendages (Borgens et al., 1979; Jenkins et al., 1996; Pai et al., 2012).

The amphibian larval tail has served as an excellent model for characterizing bioelectric signals that control regeneration. Ion fluxes in regenerating tails have been profiled using ion-selective vibrating probes (Reid et al., 2009) and fluorescent reporter dyes (Ozkucur et al., 2010). Moreover, the genetically-tractable *Xenopus laevis* system has allowed significant insight into the roles of ion flows in driving and controlling regeneration. Using a combination of ion imaging technology and molecular-genetic techniques, two sets of specific events have now been shown to be required for regeneration. The first is a repolarization of the regeneration bud driven natively by the V-ATPase proton pump (Adams et al., 2007).

The expression of the V-ATPase $H^+$ pump in the regeneration bud at the wound site is first observed by 6 hpa (hours post amputation) and remains through 2 dpa (Adams et al., 2007). Fluorescent imaging performed using the membrane voltage dye, bis-(1,3-dibutylbarbituric acid) pentamethine oxonol [DiBAC4(3)] (Epps et al., 1994) showed that at 6 hpa, the tail regeneration bud is highly depolarized. The activity of the V-ATPase pump results in a re-polarized regeneration bud by 24 hpa. Notably, refractory (non-regenerative) tail stumps remain depolarized at 24 hpa and do not re-grow the appendage. However, forced re-polarization of the refractory tail bud by ectopic expression of a yeast $H^+$ pump restores regeneration, demonstrating that it is the membrane potential of the regeneration bud that is the critical determinant of regenerative ability (Adams et al., 2007).

The second bioelectric event required for regeneration is a subsequent influx of sodium ions driven endogenously by the $Na_V1.2$ sodium channel that is expressed in the mesenchymal cells of the tail regeneration bud by 18 hpa (Tseng et al., 2010). Importantly, the expression of $Na_V1.2$ is dependent upon $V_{mem}$ re-polarization of the tail bud during regeneration. If the tail regeneration bud remains depolarized by 24 hpa (either due to absence of V-ATPase or a chemical modulation), $Na_V1.2$ fails to be expressed.

Together, V-ATPase and $Na_V1.2$ form the first molecularly-characterized bioelectric pathway for regulating regenerative growth. If the function of either of V-ATPase or $Na_V1.2$ is abrogated genetically or pharmacologically, regeneration does not occur. However, wound healing and proper development of the tadpole continue unperturbed—thus these are regeneration-specific events required for the rebuilding of this complex structure. Additionally, our studies demonstrate that the lack of regeneration resulting from the down-regulation of these bioelectric processes results from at least three factors: a failure to up-regulate mitotic proliferation of cells at the bud, loss of expression of important regeneration-associated genes (e.g., MSX-1 and Notch), and mispatterning of the innervation of the new regenerate tissue (Tseng et al., 2010).

Importantly, repolarization of $V_{mem}$ and sodium influx are not only necessary for regeneration but also

sufficient for inducing it under a range of non-regenerative conditions. Misexpression of a yeast-derived proton pump can initiate regeneration during the non-regenerative refractory period or the inactivation of the endogenous *Xenopus* V-ATPase (Adams et al., 2007). Similarly, an 1-hr treatment with a cocktail designed to drive a sodium influx can restore the regeneration of complete tails even after the formation of a thickened, non-regenerative wound epidermis (Tseng et al., 2010). This technology is being combined with *in vivo* bioreactors in attempts to impact limb regeneration in mammals (Hechavarria et al., 2010). However, to optimize the treatments to deliver the most regeneration-promoting bioelectrical signals, it is crucial to develop techniques for the tight temporal control of $V_{mem}$; indeed, the ability to experimentally set $V_{mem}$ to different levels in a patterning context *in vivo* is an important aspect of mapping out the bioelectric code that links biophysical properties to tissue outcomes. Here, we present a broadly usable technique that facilitates experimental control of $V_{mem}$ *in vivo*, show that modulation using this method can control the regenerative response in the tail, and propose a new hypothesis for how ion content may be converted into stable changes in transcription and cell behavior. Although future studies will use a definitive molecular-genetic analysis to test this model in various contexts, we show data consistent with the hypothesis that the SLC5A8 sodium/butyratre transporter is a mechanism by which some systems may link bioelectric events to epigenetic chromatin modifications.

## MODULATION OF GLYCL USING IVERMECTIN TO CONTROL $V_{MEM}$

The first demonstration of the role of $V_{mem}$ in initiating tadpole tail regeneration was performed by misexpression of the mRNA of a yeast hyperpolarizing pump (Adams et al., 2007). Although this method offers the benefit of molecular specificity, it has two limitations. First, since mRNA is introduced into the embryo long before amputation, it is difficult to confine its effects to only the events occurring at a specific timepoint after the injury. Second, any method involving this kind of "gene therapy" faces well-known safety and delivery efficiency barriers in application to biomedical contexts. To overcome these issues, and to facilitate a cell-specific method of voltage control, we designed a pharmacological strategy that takes advantage of endogenously expressed channels as a convenient "control knob" by means of which $V_{mem}$ can be regulated.

We selected the glycine-gated receptor chloride channel (GlyCl) based on several considerations (Lynch, 2009). First, there exists a highly specific opener of the GlyCl channel—a compound called ivermectin (Shan et al., 2001). Second, chloride potential is close to resting $V_{mem}$ for many cell types, making it possible to easily depolarize or hyperpolarize cells by varying the external concentration of the chloride ion. Thus, by exposing a tissue, organ, or whole organism to ivermectin, the resting potential of any cell expressing GlyCl (either natively or by transfection) can be controlled by simply varying the extracellular concentration of chloride. The quantitative relationship is governed by the Goldman-Hodgkin-Katz equation:

$$V_m = \frac{RT}{F}\ln\left(\frac{p_K[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_K[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o}\right)$$

and can be summarized as follows: when extracellular chloride is low, $Cl^-$ ions will tend to leave cells through the open GlyCl (moving down its concentration gradient) and the cell will depolarize to a more positive $V_{mem}$ since negative charges are exiting. In contrast, a high extracellular chloride level will hyperpolarize the cell, as $Cl^-$ ions enter. This method was recently used to demonstrate the control of melanocyte transformation (Blackiston et al., 2011) and the induction of eye formation (Pai et al., 2012) by membrane voltage change in specific cells. The same technique can be used with any channel for which a specific activator is available (or which is known to be constitutively open). We thus applied this strategy to the control of voltage and regeneration outcome in the *Xenopus* tadpole tail.

## GLYCL-BASED MODULATION OF $V_{MEM}$ AFFECTS TAIL REGENERATION

First, we examined whether the GlyCl transporter, GlyCl-α1 [homologous to the mammalian glycine receptor subunit α-3 gene (also known as GLRA3)] (Blackiston et al., 2010) is expressed during regeneration. After tail amputation, expression of the GlyCl RNA is strongly detected in the regeneration bud at 24 hpa using *in situ* hybridization (Fig. 1A). Likewise, it is also highly expressed in the 24 hpa tail regeneration bud during the refractory period (Fig. 1A). Importantly, GlyCl is expressed in the deep bud mesenchyme (Fig. 1A, white arrowhead), and does not overlap with the wound epithelium in which the hyperpolarizing V-ATPase is present (Adams et al., 2007). These data show that GlyCl transporter is a suitable target for manipulation for $V_{mem}$ during both regenerative and non-regenerative states. We next asked whether modulating GlyCl function using the specific pharmacological channel opener, ivermectin, can alter regenerative ability.

After tail amputation at st. 40, most tails regenerate fully by 7 days (Table 1). Measurements of chloride levels are only available for early developmental stages (pre-tail formation), where they are in the range of 40–60 mM (Blackiston et al., 2011). Given the low level of $[Cl^-]_{ext}$ (10 mM) in *Xenopus* medium, treatment with ivermectin alone was shown to depolarize GlyCl-expressing cells during neurulation (Blackiston et al., 2011). Surprisingly, the same treatment did not alter tail regenerative ability. In contrast, treatment with 100 nM ivermectin and 75 mM $[Cl^-]_{ext}$ greatly reduced tail regeneration ability as measured by the Regeneration Index (RI = 76 as compared to RI = 291 for control siblings, $P < 0.001$; Fig. 1B). This treatment did not affect overall development but did induce hyper-pigmentation in the tadpoles as observed previously and is consistent with cell depolarization (Blackiston et al., 2010).

As the depolarization of the tail regeneration bud inhibits regeneration, we sought to confirm that the treatment with 100 nM ivermectin and 75 mM $[Cl^-]_{ext}$ changed the membrane voltage in those cells. Indeed, an examination of the physiology of the ivermectin-treated tadpoles at 24 hpa using DiBAC4(3) showed that these regeneration buds were relatively depolarized as

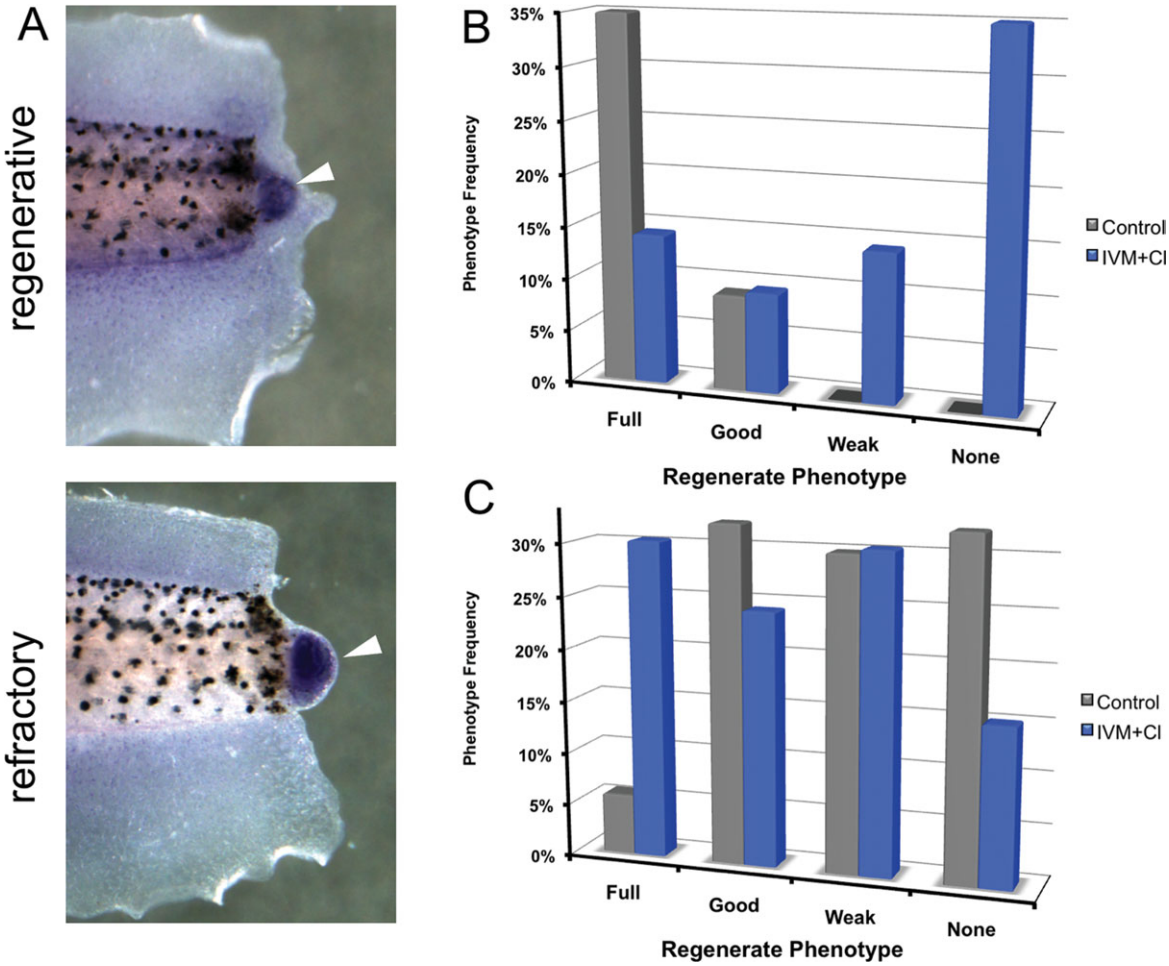


Fig. 1. Manipulation of $Cl^-$ flux can modulate regenerative ability. **A**: RNA *in situ* of GLRA3 (GlyCl) after tail amputation. GLRA3 mRNA is expressed at 24 hpa in the regeneration bud during both regenerative (st. 40) and non-regenerative states. White arrowheads indicate expression domain. For all images, anterior is to the left and dorsal is up. **B**: Treatment with 100 nM ivermectin in the presence of 75 mM $[Cl^-]_{ext}$ significantly reduced regenerative ability (Table 1, $P < 0.01$ using the Mann-Whitney *U* test). The Regeneration Index (RI – see Methods) is a measure of the quality of regeneration and ranges from 0 (no regeneration) to 300 (complete regeneration). **C**: In contrast, the same treatment promoted regeneration during the non-regenerative refractory period (Table 1, $P < 0.01$ using the Mann-Whitney *U* test).

**TABLE 1. The effect on tail regeneration from modulation of Cl flux**

| | | Phenotype frequency (%) | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Stage | Full | Good | Weak | None | N | RI |
| Control | 41 | 40 | 4 | 0 | 0 | 44 | 291 |
| 75 mM $Cl^-$ and 100 nM Ivm | 41 | 6 | 4 | 6 | 26 | 42 | 76 |
| Control | 46 | 5 | 28 | 26 | 28 | 87 | 111 |
| 75 mM $Cl^-$ and 100 nM Ivm | 46 | 10 | 8 | 10 | 15 | 33 | 170 |

Summary of the data obtained when tadpoles with amputated tails were treated with or without Ivermectin and $[Cl^-]$. Shown are the phenotypic frequencies obtained for each class of tail regenerates observed.

compared to their control, untreated siblings ($P << 0.001$; Fig. 2A,B). We conclude that treatment with ivermectin and variable levels of chloride are indeed an effective way to probe the relationship between regenerative ability and $V_{mem}$ levels. However, the depolarization (and its related phenotypes) observed from treatment using relatively high chloride levels during regenerative stages predicts that at st. 40–41 the intracellular $Cl^-$ concentration in the bud may be quite high. Investigation of time-dependent changes of chloride levels in a small tissue such as the bud will require development and *in vivo* calibration of reliable fluorescent indicators of chloride content (Arosio et al., 2010; Waseem et al., 2010).

We next tested the ability of induced hyperpolarization to promote regrowth under non-regenerative conditions. When tails were amputated during the refractory period and the tadpoles were treated with 100 nM ivermectin and 75 mM $[Cl^-]_{ext}$, we observed an

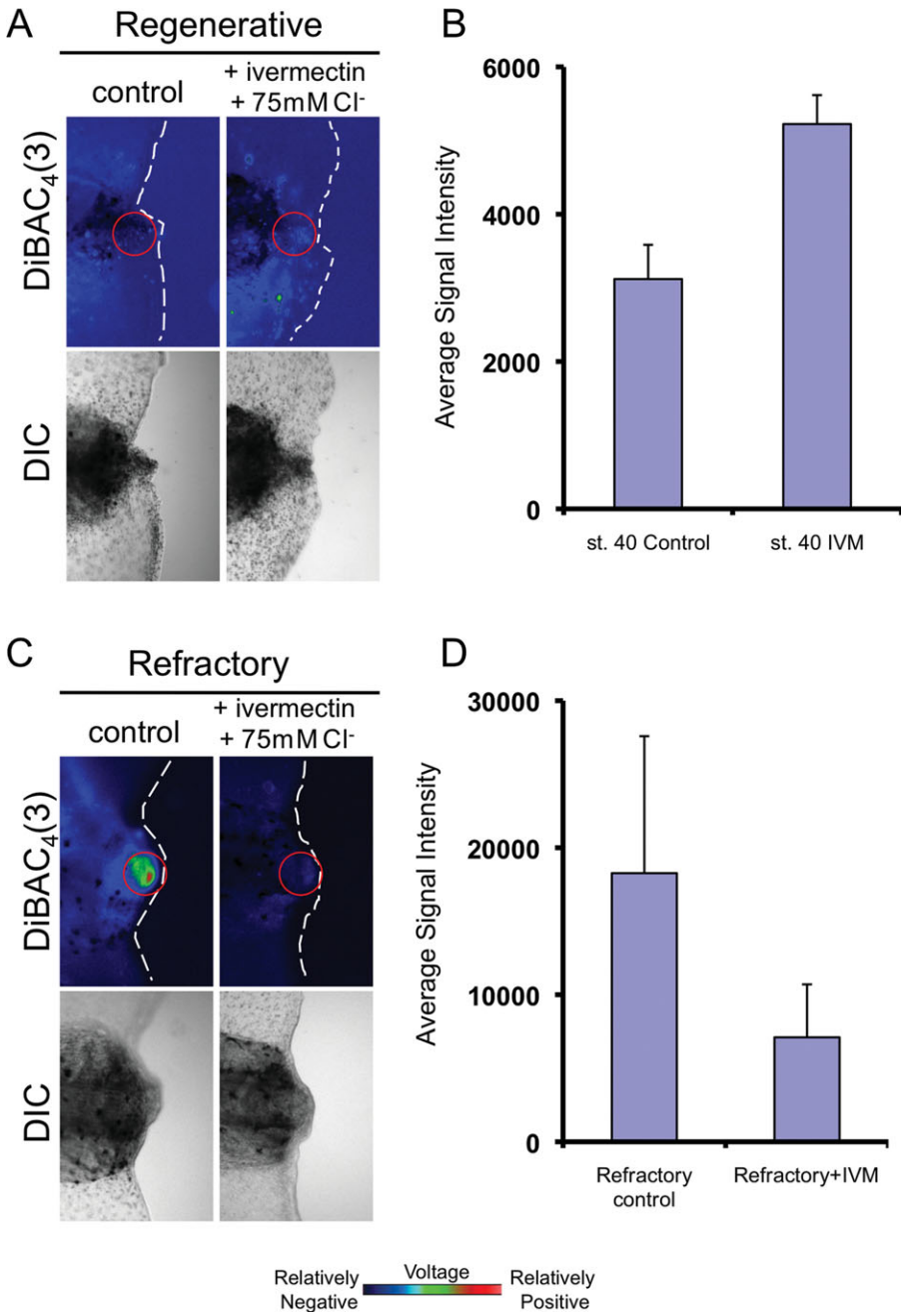


Fig. 2. Modulation of $Cl^-$ flux alters membrane voltage. St. 40 (**A** and **B**) or refractory (**C** and **D**) tadpole tails were amputated and exposed to 100 nM ivermectin and 75 mM $[Cl^-]_{ext}$. Membrane voltage assays using $DiBAC_4(3)$ was performed at 24 hpa. A and C: White dashed lines demarcate amputation plane and red circles outline the regeneration bud. Images are pseudo-colored in blue-green-red. Darker pixels (blue) indicate relative hyper-polarization whereas brighter pixels (green to red) show relative depolarization. B and D: Relative quantification of signal intensity in the regeneration bud. Sample sizes are as follows: st. 40 control (N = 10), st. 40 treated (N = 9), refractory control (N = 15), and refractory treated (N = 15). Error bars indicate standard deviation.

increase of regeneration of the tails as compared to the untreated control siblings (Fig. 1C, $P < 0.005$, and Table 1). Since the hyper-polarization of the tail regeneration bud promotes regeneration during the refractory period, we examined the $V_{mem}$ state resulting from the treatment of refractory tadpoles with 100 nM ivermectin and 75 mM $[Cl^-]_{ext}$. Non-regenerative refractory tail stumps are highly depolarized at 24 hpa (Fig. 2C) In contrast, the ivermectin-treated refractory tail regeneration buds are relatively hyper-polarized ($P << 0.001$; Fig. 2C,D), confirming directly the ability of ivermectin treatment to modify intracellular state as a function of chloride levels.

Together, our results suggest that induction of targeted $V_{mem}$ changes by pharmacological modulation of specific ion translocators is an effective way to regulate

regenerative ability (although, significant work remains to optimize these techniques and reduce outcome variability, which presumably arises from the inherent physiological heterogeneity among all organisms). "Physiomic" profiling of key tissues for intracellular ion content is an important area for future work, both for understanding endogenous physiological states and the design of strategies for experimental manipulations of growth. Moreover, GlyCl-expressing cells are important for induction of tail repair in this system; future work will characterize their identity and specific role. Likewise, application of ivermectin and/or control of medium ionic content at different stages post-amputation offers the potential to dissect the temporal aspects of voltage control.

## HOW DOES BIOELECTRICITY INTERFACE WITH TISSUE OUTCOMES?

For voltage changes to control complex tissue outcomes, this bioelectrical signal must translate into transcriptional responses. Mechanisms for transduction of voltage gradients and ion flows into second-messenger cascades and changes of gene expression include: conformational changes in integrin signaling (Olivotto et al., 1996; Arcangeli, 2005), activation of calcium influx through voltage gated calcium channels (Sasaki et al., 2000; Deisseroth et al., 2004; Siu, 2004), regulation of small morphogen movement in and out of cells by voltage-powered transporters (Fukumoto et al., 2005; Blackiston et al., 2011), and voltage regulation of phosphatase activity (Murata et al., 2005; Okamura and Dixon, 2011). What other mechanisms might be involved?

It was recently shown that a transient perturbation of physiological (gap junction-based) connectivity among cells in regenerating planarian flatworms results in a stable alteration of their target morphology. Worm fragments treated with a non-genotoxic gap junction inhibitor regenerate as double-headed, possessing an ectopic second head at the posterior blastema instead of a tail (Nogi and Levin, 2005). Remarkably, upon further bisection, but with no further exposure to any reagents, such two-headed worms continue to regenerate as bipolar worms with a head at each end (Oviedo et al., 2010). Another example of physiological events resulting in specific, long-term patterning changes of regenerative repair is trophic memory in deer (Bubenik and Pavlansky, 1965; Goss, 1990). Injury made to a specific point in an antler rack results in ectopic tines in that precise location over several subsequent years as the antlers are shed and the entire structure regenerated (with modifications according to injuries sustained in previous years!). Such permanent re-setting of anatomical structure from a transient, physiological (not genomic) perturbation suggests the possibility that epigenetic modification may be involved in control of regenerative pattern formation (Barrero and Izpisua Belmonte, 2011).

## ROLE OF EPIGENETICS IN REGENERATION

Multiple studies suggest that during appendage regeneration of the *Xenopus* tadpole tail (Gargioli and Slack, 2004), axolotl limb (Kragl et al., 2009), and the mouse digit tip (Rinkevich et al., 2011), the injured organs use lineage-specific progenitor cells to regrow the lost tissues. How differentiated cells return to a highly proliferative state is still largely a mystery. It is well known, however, that epigenetics (or chromatin remodeling) is critical for the establishment of many biological events including X chromosome inactivation, imprinting, cell reprogramming, and carcinogenesis (Hajkova et al., 2008; Bao et al., 2009; Dvash and Fan, 2009; Brower, 2011). Chromatin remodeling includes modifications of histones such as methylation and acetylation, and DNA methylation, and results in altering the accessibility of DNA for transcription. Epigenetic regulation of chromatin has been shown to be a critical element in the regulation of regenerative states and the mechanisms underlying this process are starting to be elucidated.

An early and important link showing the potential of epigenetic control to modify cellular states was the demonstration of the conversion of 10T1/2 embryonic fibroblasts to muscle by treatment with 5-azacytidine, a methyltransferase inhibitor (Taylor and Jones, 1982). Supporting the hypothesis that regulation of cellular differentiation is dependent upon DNA methylation, expression of an antisense RNA against the DNA methyltransferase, Dnmt-1, also promoted conversion of 10T1/2 cells to the myogenic phenotype (Taylor and Jones, 1982).

More recently, a demonstration of the relationship between chromatin modification and organ regeneration utilized the age-dependent tissue repair capacity of *Xenopus*. In contrast to the young tadpole limb, the froglet limb shows limited regeneration and regrows only a cartilaginous spike after amputation. One explanation for the defect in limb patterning could be due to the absence of anterior-posterior patterning gene Sonic hedgehog (Shh) during froglet regeneration. Yukushiji and colleagues showed that while Shh is expressed during proper tadpole limb regeneration, it is not expressed in the regenerating froglet limb (Yakushiji et al., 2007). An examination of the limb enhancer region of regulating Shh expression showed that this sequence is hypomethylated in the tadpole but hypermethylated in the froglet (Yakushiji et al., 2007). In addition, the urodele axolotl, which can regrow a complete limb, shows low levels of methylation at the same conserved Shh limb enhancer. The strong correlation of DNA methylation state to Shh expression demonstrates that control of the regenerative state is in part, regulated by epigenetic mechanisms. Consistent with this hypothesis, a causal role for DNA methylation regulation in altering regenerative capacity has been demonstrated genetically in the zebrafish. It was discovered that zebrafish carrying loss-of-function alleles of Dnmt-1 showed increased ability to regenerate pancreatic beta cells in response to targeted ablation (Anderson et al., 2009). Together, these studies suggest that the directed manipulation of epigenetic states may be a successful approach towards improving regenerative ability.

## A ROLE FOR HDAC ACTIVITY IN REGENERATION

If epigenetic mechanisms are important for guiding regeneration, how might these mechanisms link to upstream bioelectric controls? One system in which such a pathway has been elucidated is the orientation of the early embryonic left-right axis by an endogenous Left to Right voltage gradient. Recent work demonstrated that

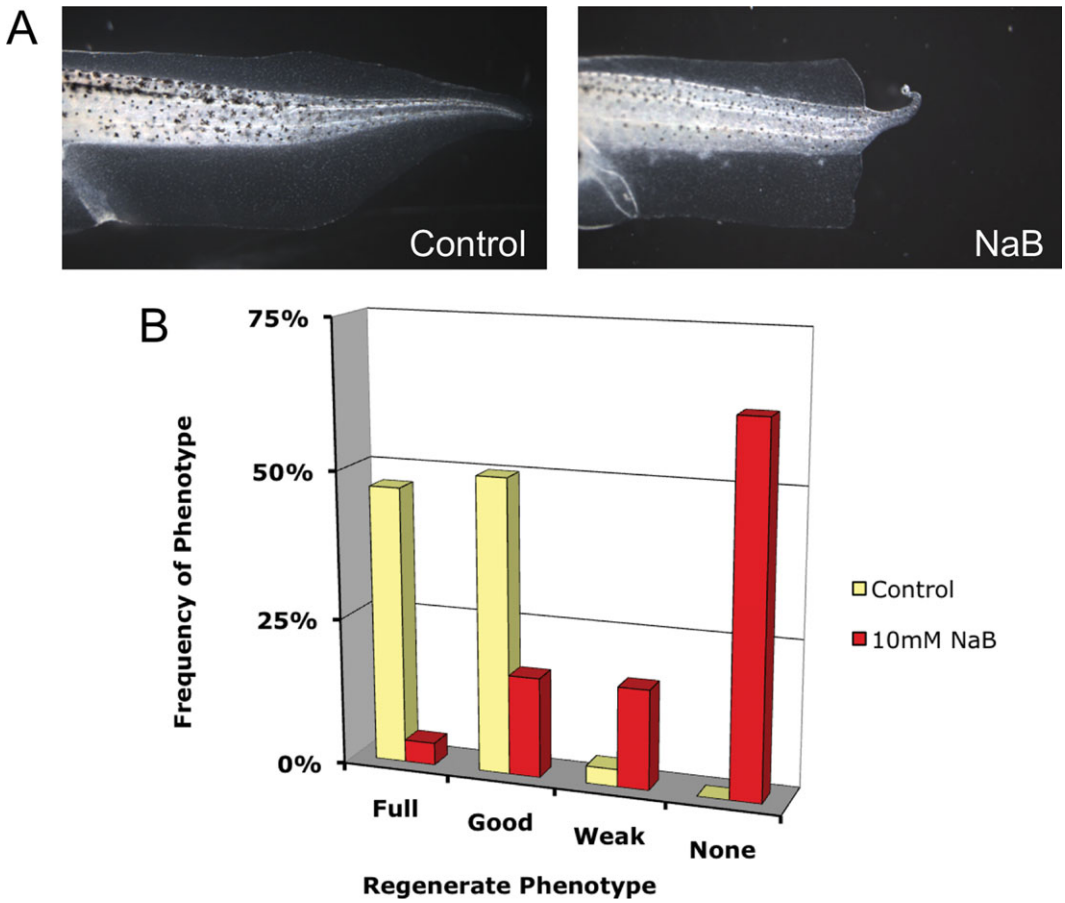


Fig. 3. The HDAC inhibitor, sodium butyrate, blocks tail regeneration. **A**: Tadpoles were treated with or without sodium butyrate (NaB) after tails were amputated at st. 40. Left panel shows a control that regenerated fully after 7 dpa. In contrast, a regenerate treated with NaB grew poorly (Right panel). **B**: Graph showing quantification of the regenerate phenotypes. Treatment with 10 mM NaB after tail amputation significantly reduces regenerative ability (RI = 62, N = 53) as compared to control siblings (RI = 244, N = 34, $P < 0.05$).

a histone de-acetylation mechanism converts the very early voltage gradient into asymmetric gene expression at much later stages (Carneiro et al., 2011) by rightward electrophoretic transport of serotonin—a cofactor for a histone deacetylase. Embryos expressing a maternal dominant-negative HDAC (mHDAC) isoform lost the normally consistent left-right placement of organs including the heart and viscera (a phenotype known as heterotaxia). Moreover, pharmacological reduction of HDAC activity using the inhibitor, sodium butyrate, also induced heterotaxia, characterized by increased histone acetylation and aberrant expression of the conserved left-right asymmetry marker, *Nodal related 1* (Xnr-1) gene. Notably, normal asymmetric Xnr-1 expression is regulated by an intronic regulatory element that becomes highly acetylated and shows an increase in the zH3K4me2 modification upon early HDAC inhibition, which is linked to a repressive chromatin state. Subsequent biochemical and mutagenesis experiments demonstrated that mHDAC acts in a complex with a known partner, the transcriptional repressor Mad3, to regulate left-right patterning. Interestingly, the activity of the mHDAC/Mad3 complex is dependent upon binding of serotonin during early embryogenesis. As previous studies (Yakushiji et al., 2007; Anderson et al., 2009; Stewart et al., 2009) had demonstrated that epigenetic modifications can influence regeneration, we then asked whether HDAC activity is also required for *Xenopus* tadpole tail regeneration.

*Xenopus laevis* have multiple HDACs, and RNA *in situ* hybridization experiments showed that the Class I HDAC, HDAC1, but not the Class II HDAC, HDAC6, was expressed in regenerating tails (Tseng et al., 2011). Mad3 was similarly expressed during tail regeneration. Pharmacological inhibition of HDACs using the well-characterized inhibitors Trichostain A (TSA) and Valproic Acid blocked regeneration and increased histone H4 acetylation levels in the non-regenerating tail stump. A dominant-negative form of Mad3 also recapitulated the loss of regenerative function. These results suggest that HDAC and Mad3 potentially act together in a complex since Mad3 itself has no functional activity by itself. To ask what occurs downstream of HDAC inhibition, we assayed RNA expression of genes known to drive regenerative outgrowth in the tail. Consistent with observations from other systems, both Msx1 and Notch1 showed aberrant expression in the 24 hpa regeneration bud when HDAC activity was blocked using TSA. Together, the data show that HDAC and Mad3 activity are required during appendage regeneration (Tseng et al., 2011). Furthermore,

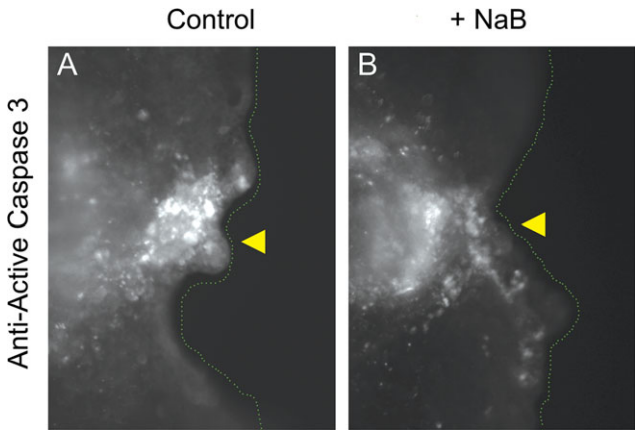


Fig. 4. Caspase 3 activity does not change appreciably after sodium butyrate treatment. **A**: Control tail regenerate at 24 hpa. **B**: Tail regenerate treated with 20 mM NaB at 24 hpa. Apoptosis was assayed using an anti-activated Caspase 3 antibody. Yellow arrowheads indicate regeneration buds.

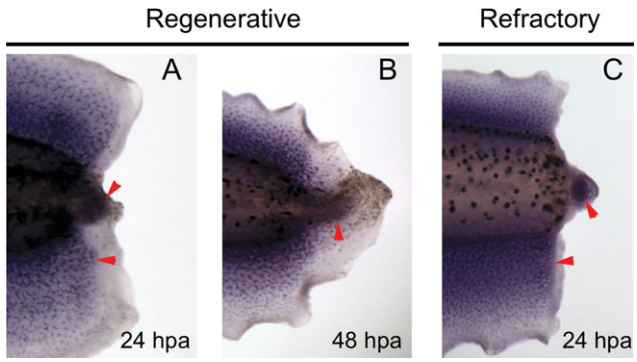


Fig. 5. SLC5A8 (SMCT1) expression during regeneration. **A** and **B**: RNA *in situ* showed that after st. 40 tail amputation, SLC5A8 RNA is detected in the regeneration bud at 24 hpa and 48 hpa. **C**: Similarly, SLC5A8 is also expressed at 24 hpa during the refractory period. Red arrowheads indicate expression domain.

HDAC and Mad3 may act in a complex together to regulate acetylation levels in regenerative gene networks.

## A MECHANISM FOR BUTYRATE INHIBITION OF TAIL REGENERATION VIA SMCT1

In thinking about ways in which voltage gradients and ion concentration levels could control downstream epigenetic modifications, we considered how influx of endogenous HDAC-modifying molecules might be coupled to ion flows. An excellent candidate for this function is the $Na^+$-coupled monocarboxylate transporter, SLC5A8/SMCT1. Mammalian SMCT1 was originally identified as a tumor suppressor acting in the colon (Li et al., 2003). It is expressed in normal colon cells but silenced in cancer. Studies have suggested that SMCT1 is a tumor suppressor because it has the ability to import the well-known HDAC inhibitor, butyrate (Fagot et al., 1994; Mandal et al., 1997; Miyauchi et al., 2004). The known importance of voltage gradients in neoplastic conversion (Fraser et al., 2005; House et al., 2010; Roepke et al., 2010; Becchetti, 2011) is likewise consistent with a possible role for such transporters in cancer. Thus, we asked whether SMCT1 might be a transporter that imports butyrate into the amputated tail.

We first assayed the effect of Sodium Butyrate (NaB) on regenerating *Xenopus* tadpoles. Treatment of 10 mM NaB immediately after tail amputation strongly inhibited regeneration, reducing the regeneration index by over 70% (RI = 62 as compared to RI = 244 for control siblings; Fig. 3), demonstrating that NaB has the same effect as the other two HDAC inhibitors (TSA and valproic acid) in regeneration. One possible explanation for the inhibition of regeneration could be due to the known ability of butyrate to promote apoptosis (Hague et al., 1993). Furthermore, we have previously demonstrated that pharmacological modulation of apoptosis can alter tail regenerative ability (Tseng et al., 2007). Thus, we assessed the pattern of programmed cell death in amputated tails using an antibody to activated Caspase-3, a marker of apoptosis (Janicke et al., 1998). In 24 hpa tail regeneration buds, there was no observable difference in the activated Caspase-3 expression pattern between untreated controls and those tails treated with NaB (Fig. 4). Our data suggest that at the concentration of sodium butyrate which is sufficient to significantly inhibit tail regeneration, apoptosis levels remains unaffected and is unlikely to play a role in this mechanism.

The inhibition of regeneration by butyrate indicates that there is likely a transporter that is required to increase intracellular butyrate concentration. Using RNA *in situ* hybridization, we next determined that *Xenopus* SMCT1 is expressed in tail regeneration during both the regenerative (Fig. 5A,B) and refractory stages (Fig. 5C). Thus, its expression is consistent with a proposed role in tying voltage and sodium level changes to alterations in regeneration-relevant genes via an epigenetic mechanism involving HDAC.

## CONCLUSION

Our experiments have provided the first pilot data consistent with this new hypothesis, to motivate investigation of such transporters in various systems in which bioelectric events drive regenerative outcomes. Future study will have to genetically and physiologically test this idea, by gene-specific gain- and loss-of-function analyses of SMCT1 and the careful tracking of butyrate levels *in vivo* under controlled modulation of voltage and sodium levels in the wound tissues. Understanding mechanisms by which ion flows control long-term changes in gene expression will shed new light on the evolution and cell biology of morphogenetic pathways, accelerate the development of biomedical interventions that capitalize on bioelectricity, and suggest new modules for synthetic biology.

## METHODS

### Tail Regeneration Assay

*Xenopus laevis* larvae were cultured via approved protocols (Institutional Animal Care and Use Committee, #M2008-08). Tails at stages (st.) 40–41 (regenerative) or 45–47 (refractory) (Nieukoop and Faber, 1967) were amputated at the midpoint between the anus and the tip. Tadpoles were separated into control or treated groups, to which ivermectin (Sigma) and *N*-Methyl-D-glucamine Hydrochloride (to increase $[Cl^-]_{ext}$ concentration to 75

mM, TCI America) or Sodium Butyrate (Alfa Aesar) was added in 0.1X MMR at 22°C for 7 days and scored for tail regeneration. To quantify and compare regeneration in groups of tadpoles treated with or without pharmacological agents, a composite regeneration index (RI), ranging from 0 (no regeneration) to 300 (complete regeneration) was calculated as described previously (Adams et al., 2007). Tail regenerates are scored into four phenotype categories: (full, good, weak, and none). For example, a group of tails in which >80% were fully regenerated would have an RI ranging from 240 to 300; if full regeneration occurred in 10% of the animals within the group, the RI would range from 0 to 30.

### *In Situ* Hybridization and Immunostaining

Wholemount RNA *in situ* hybridization was performed according to standard protocols (Harland, 1991) with probes to *Xenopus* GlyCl-α1 [homologous to the mammalian glycine receptor subunit α-3 gene (GLRA3)] (Blackiston et al., 2010) and SMCT1 (Costa et al., 2003). An anti-active Caspase-3 antibody (Abcam #AB13847) was used to detect apoptosis. For both procedures, *Xenopus* embryos were fixed overnight in MEMFA buffer and processed accordingly (Sive, 2000).

### Fluorescent Imaging of Membrane Voltage

Tadpoles were incubated for 60 min in 0.1XMMR containing the membrane voltage dye, bis-(1,3-dibutylbarbituric acid) pentamethine oxonol [$DiBAC_4(3)$], as described previously (Adams et al., 2007; Oviedo et al., 2008; Blackiston et al., 2011). Images were collected at 1dpa with an Olympus BX-61 microscope using the Ex470/20 filter. IPLab software (BD Biosciences) was used to quantitate relative signal intensity at the regeneration bud. Student's t-tests were used for comparison.

### Statistical Analysis

To compare tail regeneration experiments, raw data from scoring was used. Comparison of two treatments was analyzed with Mann–Whitney *U* test for ordinal data with tied ranks, using normal approximation for large sample sizes. Multiple treatments were compared using a Kruskal–Wallis test, with Dunn's Q corrected for tied ranks.

## ACKNOWLEDGEMENT

The authors thank Punita Koustubhan, Amber Currier, and Claire Stevenson for animal husbandry assistance, Joan Lemire for technical assistance, Aaron Zorn for the *Xenopus* SLC5A8 transporter clone, and Michael Romero for many helpful discussions on this topic.

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