

CORRECTION

Correction: Early bioelectric activities mediate redox-modulated regeneration (doi: 10.1242/dev.142034)

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The authors apologise to readers for this mistake.

Early bioelectric activities mediate redox-modulated regeneration

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ABSTRACT

Reactive oxygen species (ROS) and electric currents modulate regeneration; however, the interplay between biochemical and biophysical signals during regeneration remains poorly understood. We investigate the interactions between redox and bioelectric activities during tail regeneration in *Xenopus laevis* tadpoles. We show that inhibition of NADPH oxidase-mediated production of ROS, or scavenging or blocking their diffusion into cells, impairs regeneration and consistently regulates the dynamics of membrane potential, transepithelial potential (TEP) and electric current densities (J_1) during regeneration. Depletion of ROS mimics the altered TEP and J_1 observed in the non-regenerative refractory period. Short-term application of hydrogen peroxide (H_2O_2) rescues (from depleted ROS) and induces (from the refractory period) regeneration, TEP increase and J_1 reversal. H_2O_2 is therefore necessary for and sufficient to induce regeneration and to regulate TEP and J_1 . Epistasis assays show that voltage-gated Na^+ channels act downstream of H_2O_2 to modulate regeneration. Altogether, these results suggest a novel mechanism for regeneration via redox-bioelectric orchestration.

KEY WORDS: NADPH oxidases, Reactive oxygen species, Membrane potential, Transepithelial potential, Electric current density, Voltage-gated Na^+ channels, Regeneration, *Xenopus laevis*

INTRODUCTION

Understanding large-scale repair via regeneration is a fundamental issue in basic biology and regenerative medicine. Biochemical signals, such as Wnt and BMP pathways, have been extensively studied and have been shown to control regeneration (Alvarado and Tsonis, 2006; Stoick-Cooper et al., 2007). Reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), are suggested to act as signaling cues in the wound microenvironment (Sen and Roy, 2008; Veal et al., 2007). Upon wounding, the *Drosophila* embryo (Moreira et al., 2010), zebrafish larva (Niethammer et al., 2009) and *Xenopus* tadpole (Love et al., 2013) produce a H_2O_2 gradient that precedes the oxidative burst. This gradient, in fact, attracts immune cells toward the wound edges. In addition, a redox sensor has been identified in the leading edge of neutrophils (Wittmann et al., 2012; Yoo et al., 2011, 2012) and topical application of low doses of H_2O_2 enhances wound closure in mice (Loo et al., 2012). Importantly, ROS are required for the regeneration of planarian head

and tail (Pirotte et al., 2015), zebrafish caudal fin (Gauron et al., 2013) and heart (Han et al., 2014), *Xenopus* tadpole tail (Love et al., 2013), and gecko tail (Zhang et al., 2016).

In addition to ROS, biophysical signals such as the dynamic bioelectric activities measured during regeneration have been proposed to act as signaling cues (Levin, 2007; McCaig et al., 2005). It has been shown that transepithelial potential (TEP)-driven electric current densities (J_1) correlate with and are required for regeneration in amphibians (Borgens et al., 1977b; Jenkins et al., 1996; Nawata, 2001; Reid et al., 2009). Electrical stimulation induces some regeneration in non-permissive adult frog and rat limbs (Becker, 1972; Borgens et al., 1977a; Leppik et al., 2015; Smith, 1967). Moreover, engineered devices (Golding et al., 2016) partially induce mouse digit tip regeneration (Hechavarria et al., 2010) and are in clinical trials to improve paraplegia (Shapiro et al., 2005). Re-emerging bioelectric studies are revealing transduction mechanisms. V-ATPase-mediated repolarization of membrane potential (V_m) and voltage-gated Na^+ channel (VGSC or Na_v) 1.2-mediated sodium influx are necessary for and sufficient to induce *X. laevis* tadpole tail regeneration (Adams et al., 2007; Tseng et al., 2010). V-ATPase proton efflux activity correlates with regeneration rate and ability in zebrafish caudal fin regeneration (Monteiro et al., 2014). H^+/K^+ -ATPase-mediated V_m depolarization modulates planarian head regeneration (Beane et al., 2011). In addition, ion and V_m dynamics correlate with axolotl larvae tail regeneration (Özkucur et al., 2010).

Interestingly, redox and bioelectric activities control the expression and activity of signaling pathways, such as Wnt, FGF, BMP and Notch, in addition to cell behaviors, such as proliferation, apoptosis and innervation, which are required for regeneration (Adams et al., 2007; Gauron et al., 2013; Han et al., 2014; Love et al., 2013; Monteiro et al., 2014; Tseng et al., 2010). Redox (ROS) and bioelectric [V_m , TEP, electric fields (EF) and J_1] states therefore affect regeneration. But how do these signals integrate? Do they act in parallel or in series during regeneration? *Xenopus laevis* tadpole tail regeneration provides an ideal model with which to address these questions (Love et al., 2013; Reid et al., 2009). The tail comprises epidermis, muscle, blood vessels and spinal cord, making it a prime model for biomedical research (Beck et al., 2009; Deuchar, 1975). In addition, the fluctuating regenerative abilities of tails through developmental stages offer unique opportunities to study regeneration-deficient tails, in the refractory period (Beck et al., 2003), using the same model organism.

RESULTS

Do ROS modulate regeneration via the regulation of V_m , TEP, EF and/or J_1 , all of which are known to be required for regeneration? At the cellular level, NADPH oxidases in the cell membrane catalyze electron transfer from cytoplasmic NADPH to extracellular molecular O_2 , generating ROS and depolarizing V_m (Demaurex and Petheö, 2005; Lambeth and Neish, 2014). This dual property – electrogenic and catalytic – of NADPH oxidases led us to hypothesize a two-way regulation: (1) the activity of NADPH

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oxidases per se depolarizes V_m ; and (2) the ROS produced, especially H_2O_2 , regulate TEP, EF and J_i . To test this hypothesis, we performed descriptive spatiotemporal profiling and functional assays.

Injury-induced extracellular bioelectricity dynamically correlates with regeneration

We first mapped the extracellular bioelectricity, TEP, EF and J_i , during regeneration to select key spatiotemporal points at which to test the two-way regulation hypothesis. Regeneration can be divided into three phases, structurally completed in 7 days (Beck et al., 2009; Tseng et al., 2010): (1) wound healing [until ~6 h post-amputation (hpa)] – wound epithelium covers amputation plane; (2) regeneration bud formation (~24 hpa) – bud produces progenitor cells; and (3) regenerative outgrowth (~48 hpa and after) – outgrowth and patterning reestablishes tail structure and function (Fig. 1A, Fig. S5).

Before amputation, baseline trunk TEP was 28.64 ± 2.27 mV (inside positive; $n=9$) and the tail tip had small inward currents of -0.04 ± 0.005 $\mu A\ cm^{-2}$ ($n=7$) (Fig. 1B, Fig. S2C). EF were assumed to be 0 mV mm^{-1} , because over the measured distances (<1 mm), no consistent TEP gradients were detected. Upon amputation, a TEP short-circuit (to 0 mV) at the stump generated a strong lateral $EF_{Trunk \rightarrow Bud}$ of -85.50 mV mm^{-1} (calculated using uncut TEP) and a low-resistance pathway leaking outward currents (the injury current) of 13.27 ± 2.807 $\mu A\ cm^{-2}$ (at 0.08 hpa; $n=6$, $P=0.005$) in the bud (Fig. 1B–D). Spatial profiles showed a consistent anteroposterior (A/P) gradient either directly (for TEP) or inversely (for EF) proportional to the distance from the amputation plane and evolving circuits (for J_i and EF) during regeneration (Figs S2–S5). The spatial profile of J_i was intended to unveil circuit loops (primarily dorsoventral) more than gradients (cases of TEP and V_m).

Temporal profiles presented exponential-like curves, positive for TEP recovery and for EF (Fig. 1B,C), and negative for J_i (Fig. 1D), that correlated with regeneration. The dramatic TEP recovery (to $67.42 \pm 5.35\%$, $n=26$, $P<0.0001$ versus 1 hpa), decrease in EF (to -22.94 ± 4.89 mV mm^{-1} , $n=23$, $P=0.0002$ versus 1 hpa; 73% drop versus 0.08 hpa) and J_i reversal (to -0.10 ± 0.017 $\mu A\ cm^{-2}$, $n=27$, $P=0.005$ versus 0.08 hpa; 99% shift) by 6 hpa (Fig. 1B–D) match the wound epithelium formation phase and possible bud initiation (Fig. 1A,E, Fig. S5). Spatially, J_i reversals progressed in a centripetal way until 6 hpa, lastly in the bud (Fig. S4C), which is possibly due to centripetal healing. We descriptively termed this part of the curve as ‘slope’ (‘S’; Fig. 1B–D). Analogous TEP recovery is seen in wound healing assays *in vitro* (human skin) and *in vivo* (porcine skin) (Dubé et al., 2010). Similar drops in EF (75%) and J_i reversal (in the spinal cord) by 6 hpa occur in newt limbs and *X. laevis* tadpole tail regeneration, respectively (McGinnis and Vanable, 1986; Reid et al., 2009). From 6 to 48 (especially to 24) hpa, TEP, EF and J_i stabilization ($P \gg 0.05$ to all comparisons from 6 to 24 hpa; Fig. 1B–D) correlated with the regeneration bud formation phase (Fig. 1A,E, Fig. S5). We termed this part of the curve as ‘plateau’ (‘P’; Fig. 1B–D). From 48 hpa onwards, a final shift completed TEP recovery (Fig. 1B; bud versus trunk non-significant at 72 hpa, $n=11$, $P=0.106$; Fig. S2C), resolved EF (-10.74 ± 7.12 mV mm^{-1} at 72 hpa, $n=11$; Fig. 1C) and returned J_i to even lower than uncut baseline (-0.01 ± 0.002 $\mu A\ cm^{-2}$ at 48 hpa, $n=11$, $P<0.0001$; Fig. 1D). The very small J_i from 48 hpa might be due to the increased surface area to volume ratio, which is due to the animal growth. We therefore termed this part of the curve as ‘baseline’ (‘B’; Fig. 1B–D) and it correlated with regenerative

outgrowth phase (Fig. 1A,E, Fig. S5). Altogether, extracellular bioelectricity shows a dynamic correlation with regeneration and suggests that the bud at 6 hpa is a key spatiotemporal point to test the proposed hypothesis.

Injury-induced ROS production, availability and diffusion are necessary for regeneration

Next, we sought to clarify whether H_2O_2 is the main ROS involved in regeneration. We used drugs that target specific steps of the ROS ‘life cycle’ (see Fig. 6A): (1) production – by blocking NADPH oxidases with diphenyleioidonium (DPI) (O’Donnell et al., 1993); (2) availability – by scavenging ROS with MCI-186 (MCI) (Otomo, 2003); and (3) diffusion – by preventing entry of H_2O_2 into cells through aquaporins with $AgNO_3$ (Niemietz and Tyerman, 2002). As previously shown (Han et al., 2014; Love et al., 2013; Niethammer et al., 2009), we confirmed that these drugs decreased levels of intracellular ROS in the bud (Fig. S6).

Similarly to another study (Love et al., 2013), DPI-treated tadpoles showed significantly inhibited regeneration, with virtually all tails presenting either a weak (35%, sixfold increase relative to vehicle control 6%) or none (64%, >50-fold increase) phenotype [regeneration index (RI) reduced from 255, $n=233$, to 39, $n=74$, $P<0.0001$; Fig. 2A,A’]. To establish NADPH oxidases as the main ROS source for regeneration, we used the alternative drugs apocynin (Simons et al., 1990) and VAS2870 (VAS) (ten Freyhaus et al., 2006). Besides being less penetrant than DPI, both drugs significantly impaired regeneration (Fig. S7A). Scavenging ROS with the antioxidant MCI significantly impaired regeneration with a 83% decrease in full (11%) and a fourfold increase in weak (24%) phenotypes (RI reduced from 255 to 187, $n=43$, $P=0.005$; Fig. 2A,A’). More penetrant effects of MCI have previously been shown if quantified at 3 days post-amputation (dpa), but not at 7 dpa (Love et al., 2013). Most H_2O_2 diffuses through aquaporins, such as AQP3 and AQP8 (Bienert and Chaumont, 2014; Miller et al., 2010). Highly specific small-molecule drugs for aquaporins are not yet available (Verkman et al., 2014) so we used $AgNO_3$. $AgNO_3$ -treated tadpoles showed significantly impaired regeneration with a 69% reduction in the full phenotype (20%), and four- and fivefold increases in, respectively, the weak (25%) and the none (7%) phenotypes (RI reduced from 255 to 182, $n=65$, $P<0.0001$; Fig. 2A,A’). Penetrance on regeneration inhibition was therefore $DPI \gg AgNO_3 > MCI$ (Fig. 2A’). Altogether, H_2O_2 is probably the main ROS responsible for the impaired regeneration and it must diffuse into cells, likely via aquaporins, to be effective.

Love et al. have shown that ROS production for the first 3 days is enough to impair regeneration (Love et al., 2013). We tested the role for ROS earlier, in wound epithelium and regeneration bud form, because both are limiting factors of regeneration (Fig. S7B–C’). We started by testing DPI until 6 and 24 hpa. These experiments still robustly inhibited regeneration (until 6 hpa, RI reduced from 246, $n=94$, to 160, $n=52$, $P<0.0001$; until 24 hpa, RI reduced to 89, $n=50$, $P<0.0001$; Fig. S7B). VAS treatment confirmed this early requirement (Fig. S7A). Next, we extended the temporal screening and found that ROS are also necessary for the last phase of regeneration (48 hpa onwards), although not for morphogenesis (RI) but for growth (area) (Fig. S7B–B’). This is congruent with impaired proliferation produced by inhibition of NADPH oxidases (Gauron et al., 2013; Han et al., 2014; Love et al., 2013). Finally, by increasing DPI dose, we found that ROS are immediately necessary for regeneration (Fig. S7C,C’). Altogether, ROS presented multi-

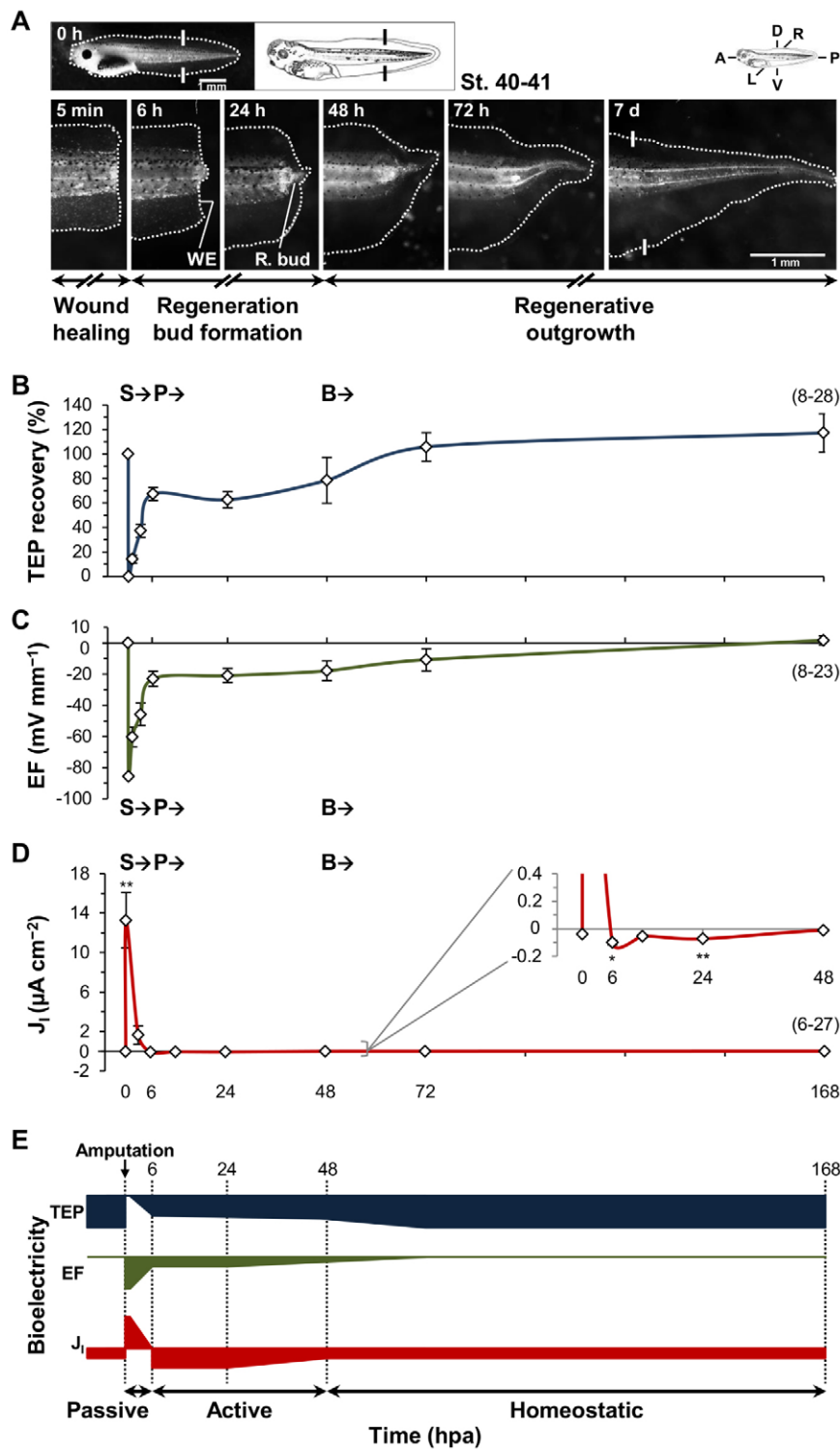


Fig. 1. Extracellular bioelectricity dynamically correlates with regeneration. (A) Regeneration time-lapse and phases of a representative tadpole tail amputated at stage 40-41. Major phenotypic structures annotated: wound epithelium (WE) and regeneration bud (R. bud). Photomicrographs of regenerating or regenerated tails are displayed in the same orientation as the whole organism showing anteroposterior (A/P), dorsoventral (D/V) and left-right (L/R) axes (top left scheme; applies to subsequent figures). White solid lines: amputation plane (black solid lines in schematic tadpole). Scale bars: 1 mm. (B) Temporal profile of TEP recovery in the regeneration bud in vehicle control (DMSO 0.1%). Calculated from data in Fig. S2C. (C) Temporal profile of lateral, trunk to bud, EF in vehicle control. Calculated from data in Fig. S2C. (D) Temporal profile of J_i in the bud in vehicle control. Conventional current flow is used, thus positive values are net outward and negative are net inward currents (applies to subsequent figures). Profiles B-D are descriptively divided into three parts: S, slope; P, plateau; and B, baseline. (E) Diagrammatic representation integrating the temporal dynamics of TEP recovery, $EF_{\text{Trunk} \rightarrow \text{Bud}}$ and J_i during regeneration. Profiles are divided into three phases according to the bioelectric activity: passive, active and homeostatic. Magnitudes are not absolute but present the relative dynamics in temporal directions and magnitudes. Same x-axis labels for B-D; same x-axis titles for B-E. For B-D, n specimens indicated in brackets.

phase pleiotropic effects during regeneration and NADPH oxidase-mediated ROS production is, to our knowledge, potentially the earliest biochemical limiting factor of regeneration.

NADPH oxidase-driven electron flow depolarizes V_m in the regeneration bud at 6 hpa

Cellular bioelectricity has previously been characterized in the same *X. laevis* model (Adams et al., 2007) and in axolotl larvae (Özkucur

et al., 2010). These studies showed a robust V_m depolarization in the bud at 6 hpa that should repolarize by 24 hpa for complete regeneration (Adams et al., 2007). However, the origin of depolarization is unknown. V_m depolarization (Adams et al., 2007) and H_2O_2 concentration (Love et al., 2013) appear to overlap spatiotemporally. Activity of NADPH oxidases generates an electron flow that depolarizes V_m in immune cells that can be used as a direct quantification of holoenzyme activity (Demaurex and Petheö, 2005).

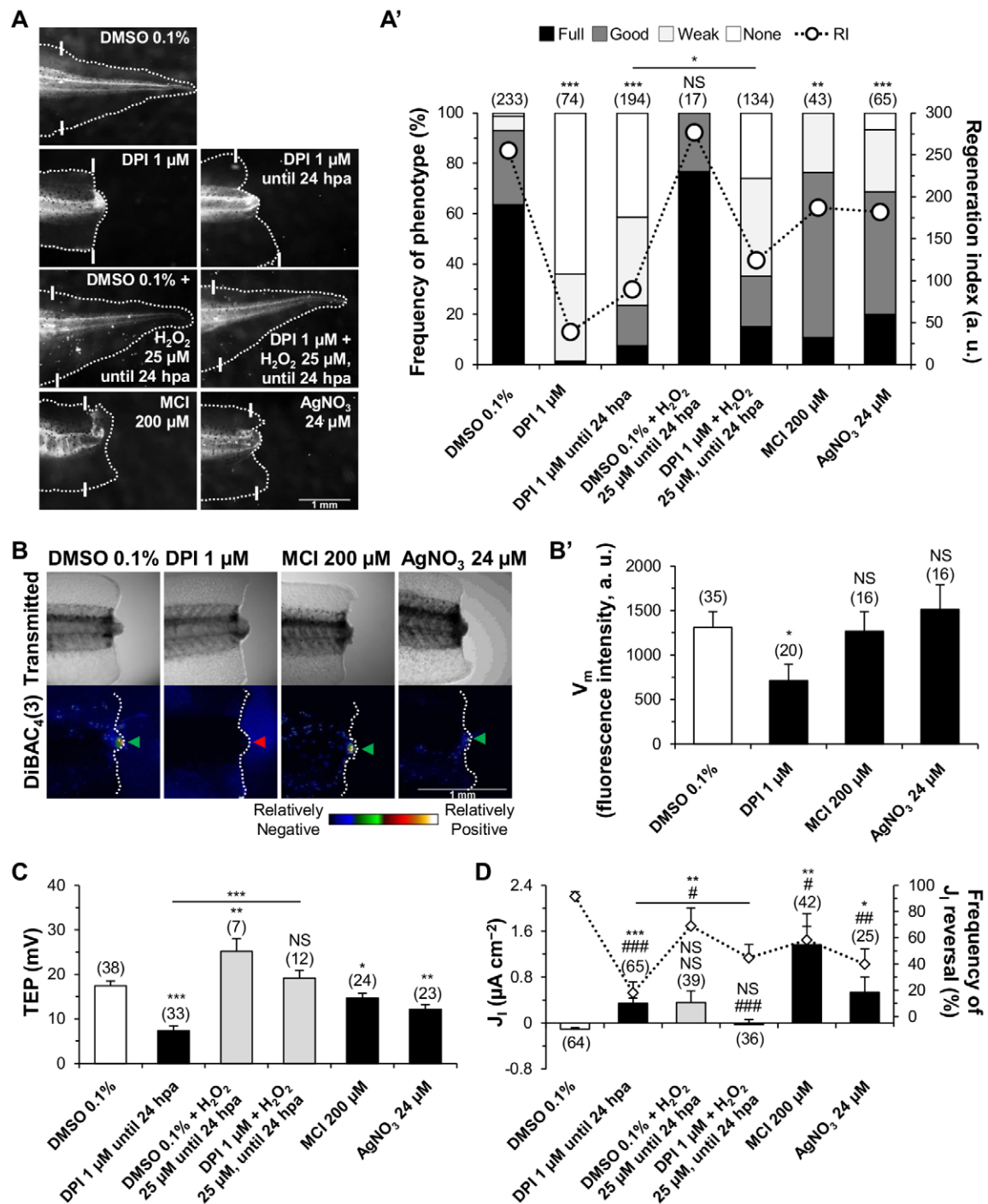


Fig. 2. Electrogenic property of NADPH oxidases is necessary for V_m depolarization and catalytic property for regeneration, TEP increase and J_1 reversal. (A,A') Loss and rescue of regeneration by ROS and H₂O₂ modulation. (A) Representative 7 dpa tails from vehicle-control (DMSO 0.1%) and pharmacologically treated tadpoles. White lines: amputation plane. (A') Qualitative and quantitative analyses of regeneration efficiency for the different conditions tested. RI, regeneration index. (B,B') Activity of NADPH oxidases per se depolarizes V_m . (B) Representative tails under transmitted light (top panels) and fluorescence imaging (bottom panels) of membrane potential-sensitive dye DiBAC₄(3) in vehicle control and after pharmacological treatment at 6 hpa. Green arrowhead, V_m depolarization (relatively positive) in the bud; red arrowhead, no V_m depolarization in the bud. (B') Semi-quantitative analysis of V_m for the different conditions tested. (C,D) Pharmacological loss and rescue of regeneration are correlated with variations of TEP (C) and J_1 (D). In D, frequencies of current reversals (mean percentage of inward J_1 , diamonds) also correlated with pharmacological treatment. * P <0.05 versus J_1 magnitude; # P <0.05 versus J_1 reversals; **,### P <0.01; ***,### P <0.001. a.u., arbitrary units. Scale bars: 1 mm; n values are indicated in brackets.

To test whether the activity of NADPH oxidases induced the V_m depolarization, we blocked them with DPI and imaged V_m with the membrane potential-sensitive dye DiBAC₄(3) (Fig. S8A). DPI-treated tails at 6 hpa were, as in vehicle controls, still depolarized in

the bud in relation to the rest of the tail (bud>>shoulder>trunk; Fig. S8A'), but had a significantly decreased depolarization (46% reduction, $n=20$, $P=0.033$; Fig. 2B,B'). Consequently, the spatial A/P gradient was also reduced (Fig. S8A').

To exclude a possible effect of ROS/H₂O₂ in V_m, we next scavenged and prevented them from diffusing into cells as before. MCI- and AgNO₃-treated tails showed no V_m variation in the bud at 6 hpa ($n=16$, $P>0.05$ to both; Fig. 2B,B'). Therefore, V_m depolarization in the bud at 6 hpa is originated by the activity of NADPH oxidases per se.

H₂O₂ regulates TEP increase and J₁ reversal in the regeneration bud

Next, we tested whether H₂O₂ regulates TEP, EF and J₁. To achieve this, we blocked ROS/H₂O₂ production, availability and diffusion as before and measured TEP and J₁ in the bud at 6 hpa. DPI-treated tails showed a significantly decreased TEP (58% reduction, from 17.42±1.11, $n=38$, to 7.33±1.08 mV, $n=33$, $P<0.0001$; Fig. 2C). DPI reversed the direction of some TEPs to negative inside (e.g. 12% of total at 6 hpa). MCI- and AgNO₃-treated tadpoles also had significantly decreased TEPs (MCI, 16% reduction, $n=24$, $P=0.046$; AgNO₃, 30% reduction, $n=23$, $P=0.002$; Fig. 2C).

Drug-treated shoulder and trunk (amputated or uncut) TEP shifted in the same way as bud TEP (Figs S8B, S12C,D), suggesting a systemic baseline shift and effect on housekeeping translocators. As a consequence, spatial A/P gradients remained relatively unaffected (Fig. S8B), leading to constant TEP recoveries and long-range EF_{Trunk→Bud} (Fig. S12E). However, short-range EF_{Shoulder→Bud} presented increased magnitudes (Fig. S16A), potentially powered by J₁ direction (below). For these reasons, we mainly compared absolute TEP in bud.

The TEP decrease, caused by depleted H₂O₂, anticipated an effect of H₂O₂ on J₁. Almost all (91.60±3.38%) vehicle-control bud currents reversed at 6 hpa, giving a net inward J₁ (−0.10±0.025 μA cm^{−2}, $n=64$). Only 18.05±8.32% reversed in DPI-treated tadpoles, maintaining a significant outward J₁ (0.34±0.096 μA cm^{−2}, $n=65$, $P<0.0001$; Fig. 2D). Indeed, steady outward J₁ were measured for days (Fig. S9A). Further analysis showed the reversibility of DPI-prevented reversal (Fig. S9B). MCI and AgNO₃ treatments also prevented reversal at 6 hpa with significant outward J₁ (MCI, $n=42$, $P<0.01$, 58.33±10.41% reversal; AgNO₃, $n=25$, $P=0.026$, 40.00±11.55% reversal; Fig. 2D). Unlike DPI, J₁ in MCI- and AgNO₃-treated tails reversed by 24 hpa (Fig. S8C), mimicking the refractory period (Fig. S11C).

Spatially, drug-sustained outward current at 6 hpa precluded an evolving electrical circuit (Figs S8C, S9C) and probably contributed to maintaining EF direction (cathode in the bud) and to increasing EF magnitude (Fig. S16A). Importantly, the penetrance of the drugs on TEP decrease and J₁ reversal was equal to impaired regeneration (DPI≫AgNO₃>MCI; Fig. 2A',C,D). Altogether, the TEP increase and J₁ reversal are regulated by H₂O₂ that diffuses into cells, likely via aquaporins.

Exogenous H₂O₂ rescues regeneration, TEP increase and J₁ reversal

After demonstrating that H₂O₂ is required for regeneration and regulates TEP and J₁, we performed stimulation assays. We first attempted to rescue DPI-impaired regeneration, TEP and J₁ using exogenous H₂O₂. To impose a suitable spatiotemporal redox state was challenging due to diverse cell responses (physiological and biochemical) to disparate ROS levels. Briefly, high levels might produce oxidative stress, whereas low levels produce more signaling effects (Lambeth and Neish, 2014). Long-term exposure to H₂O₂ could turn counterproductive by setting too much of a steady state in the very dynamic process of regeneration. In addition, regeneration-specific events are more likely to be present within 24 hpa.

Therefore, we focused on application of H₂O₂ in low doses (μM range) and for a short exposure (first 24 h).

H₂O₂ until 24 hpa significantly rescued DPI-impaired regeneration, TEP increase and J₁ reversal (Fig. 2A,A',C,D). H₂O₂ increased twofold the frequency of full phenotypes compared with DPI-treated tails (from 7 to 15%) and decreased 37% the frequency of none phenotypes (from 41 to 26%). Overall, regeneration was significantly rescued (RI from 90, $n=194$, to 124, $n=134$, $P=0.016$; Fig. 2A,A'). Long-term (7 days) exposure to H₂O₂ did not rescue regeneration (Fig. S13), pointing to the importance of redox state dynamics (Loo et al., 2012).

TEP magnitude in the bud at 6 hpa was entirely rescued to values slightly higher than vehicle control [dimethyl sulfoxide (DMSO): 17.42±1.11 mV, $n=38$; DPI+H₂O₂: 19.15±1.72 mV, $n=12$; $P=0.435$; Fig. 2C]. H₂O₂ significantly rescued 161% of TEP (from 7.33±1.08, $n=33$, to 19.15±1.72 mV, $n=12$, $P<0.0001$; Fig. 2C) and enhanced 45% of TEP (from 17.42±1.11 to 25.20±2.84 mV, $n=7$, $P=0.009$; Fig. 2C). As for DPI, MCI and AgNO₃, shoulder and trunk (amputated or uncut) TEP shifted in the same way as bud TEP (Figs S8B, S12C-E).

J₁ magnitude and J₁ reversal in the bud at 6 hpa were dramatically rescued to values closer to vehicle control (DMSO: −0.10±0.025 μA cm^{−2}, 91.60±3.38% reversal, $n=64$; DPI+H₂O₂: −0.02±0.086 μA cm^{−2}, 44.76±10.05% reversal, $n=36$; $P=0.395$; Fig. 2D). H₂O₂ significantly rescued 105% of J₁ magnitude to inward current (from 0.34±0.096, $n=65$, to −0.02±0.086 μA cm^{−2}, $n=36$, $P=0.005$; Fig. 2D). Importantly, current reversals more than doubled (from 18.05±8.32 to 44.76±10.05%, $P=0.029$; Fig. 2D). Unlike the changes induced in TEP, H₂O₂ neither enhanced J₁ magnitude nor J₁ reversal frequency in vehicle control. Nonetheless, most currents still reversed by 6 hpa (69.05±13.52%), resulting in a large variance (0.36±0.204 μA cm^{−2}, $n=39$, $P=0.114$; Fig. 2D). Altogether, short-term exposure to low-dose H₂O₂ rescued DPI-impaired regeneration, TEP increase and J₁ reversal. In addition, these results suggest that the electrogenicity of NADPH oxidases may not affect TEP and J₁.

Exogenous H₂O₂ induces regeneration, TEP increase and J₁ reversal in the refractory period

The *X. laevis* non-regenerative refractory period provides a unique advantage among regeneration models. We explored it to test whether we could induce regeneration, TEP and J₁ using exogenous H₂O₂. First, we verified whether the refractory period has impaired bioelectricity. Remarkably, TEP decrease and postponed J₁ reversal in the bud mimicked the effects of depleted ROS (Fig. S11). Blocked J₁ reversal has previously been shown in the spinal cord (Reid et al., 2009). Application of H₂O₂ until 24 hpa increased the frequency of full phenotypes by 2.4-fold (from 13 to 31%) and decreased the frequency of none phenotypes by 62% (from 39 to 15%). Overall, regeneration was significantly induced (RI from 110, $n=55$, to 174, $n=51$, $P=0.004$; Fig. 3A,A'). As for rescue, long-term (7 days) exposure to H₂O₂ did not induce regeneration (Fig. S13).

The magnitude of the TEP in the bud at 6 hpa increased to values slightly higher than those found during the regenerative period (DMSO stage 40-41: 23.10±1.74 mV, $n=14$; DMSO stage 45-46+H₂O₂: 25.92±1.54 mV, $n=18$; $P=0.235$; Fig. 3B). H₂O₂ raised the TEP by 115% (from 12.04±1.45, $n=14$, to 25.92±1.54 mV, $n=18$, $P<0.0001$; Fig. 3B). In addition, H₂O₂ shoulder and trunk (amputated or uncut) TEP shifted in the same way as bud TEP (Figs S10A and S12C-E).

J₁ magnitude and J₁ reversal in the bud at 6 hpa were dramatically induced to values closer to those found during the regenerative

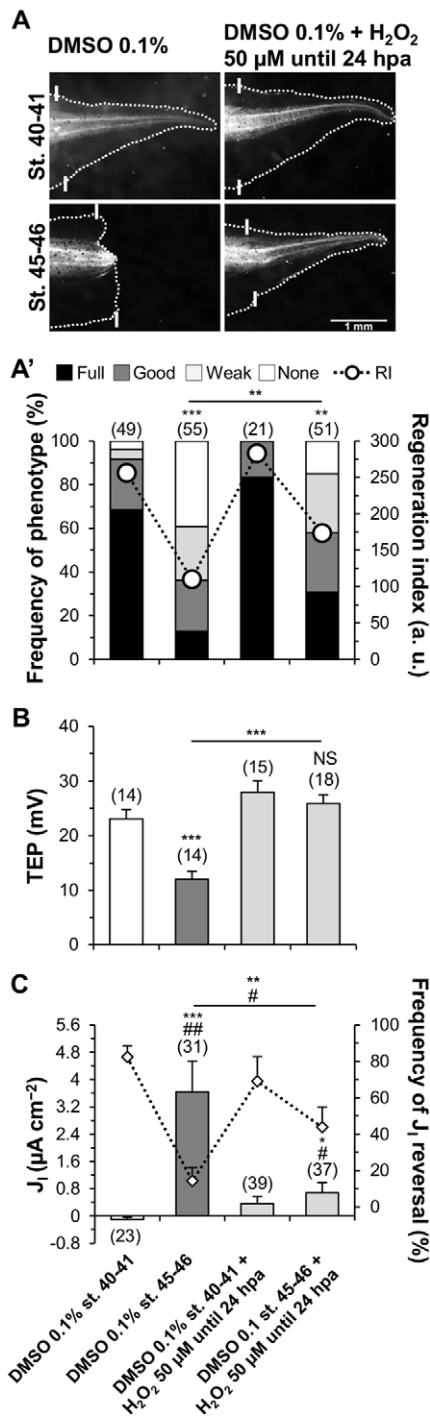


Fig. 3. Exogenous H₂O₂ is sufficient to induce regeneration, TEP increase and J₁ reversal during the refractory period. (A,A') Induction of regeneration by exogenous H₂O₂. (A) Representative 7 dpa tails from tadpoles amputated during the regenerative (stage 40-41) or refractory period (stage 45-46). Vehicle-control (DMSO 0.1%) and H₂O₂-treated (50 µM until 24 hpa) results are shown. White solid lines, amputation plane. Scale bar: 1 mm. (A') Qualitative and quantitative analyses of regeneration efficiency for the different conditions tested. RI, regeneration index; a.u., arbitrary units. (B,C) Exogenous H₂O₂-mediated induction of regeneration is correlated with variations of TEP (B) and J₁ (C). In C, frequencies of current reversals (mean percentage of inward J₁, diamonds) also correlated with pharmacological treatment. **P*<0.05 versus J₁ magnitude; #*P*<0.05 versus J₁ reversals; ***P*<0.01; ****P*<0.001. Horizontal axis labels in C also apply to A',B. *n* values are indicated in brackets.

period (DMSO stage 40-41: $-0.10 \pm 0.065 \mu\text{A cm}^{-2}$, $82.34 \pm 6.27\%$ reversal, $n=23$; DMSO stage 45-46+H₂O₂: $0.68 \pm 0.296 \mu\text{A cm}^{-2}$, $43.81 \pm 11.18\%$ reversal, $n=37$; *P*=0.013; Fig. 3C, Fig. S10B). H₂O₂ significantly decrease J₁ magnitude in 81% to a still outward current (from 3.64 ± 0.897 , $n=31$, to $0.68 \pm 0.296 \mu\text{A cm}^{-2}$, $n=37$, *P*=0.003; Fig. 3C). Importantly, current reversals increased threefold (from 14.29 ± 7.19 to $43.81 \pm 11.18\%$, *P*=0.044; Fig. 3C).

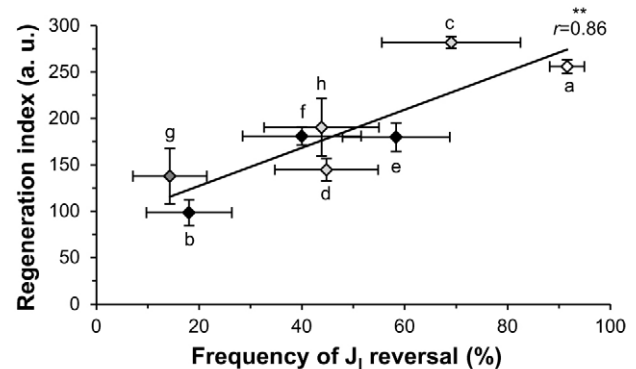
We then attempted to induce ectopic tail formation during the regenerative period. Upon dorsal incisions, H₂O₂ treatment for 24 h slightly induced significant ectopic (abnormal or complete) tail formation (up to a frequency of 19%) in a dose-independent way (Fig. S15). Altogether, short-term exposure to low-dose H₂O₂ is sufficient to induce regeneration, TEP increase and J₁ reversal during the refractory period, in addition to inducing ectopic tail formation in the regenerative period.

Regeneration efficiency correlates linearly with TEP magnitude and J₁ reversals

Following the parallel responses of regeneration, TEP and J₁ to the different conditions used, we tested whether bioelectricity predicts regeneration efficiency. Plotting all conditions together showed a significant linear correlation of regeneration efficiency with TEP magnitude ($r=0.74$, *P*=0.036; Fig. S14) and with the frequency of J₁ reversals ($r=0.86$, *P*=0.006; Fig. 4).

Voltage-gated Na⁺ channels are required for H₂O₂-modulated regeneration

To unveil the mechanisms by which H₂O₂ modulates regeneration, we searched for ion translocators that ideally affect regeneration and bioelectricity. Na_v1.2 is required for *X. laevis* tadpole tail regeneration via Na⁺ influx control (Tseng et al., 2010). Therefore, we tested whether Na_v channels act downstream of H₂O₂ using complementary epistasis assays. First, we verified that the inhibition of Na_v channels impairs regeneration. As expected (Tseng et al., 2010), the Na_v inhibitor tricaine (Frazier and



- ◇ (a) DMSO 0.1% st. 40-41
- ◆ (b) DPI 1 µM st. 40-41 until 24 hpa
- ◇ (c) DMSO 0.1% st. 40-41
- ◆ (d) DPI 1 µM st. 40-41 + H₂O₂ 25 µM, until 24 hpa
- ◆ (e) MCI 200 µM st. 40-41
- ◆ (f) AgNO₃ 24 µM st. 40-41
- ◇ (g) DMSO 0.1% st. 45-46
- ◇ (h) DMSO 0.1% st. 45-46 + H₂O₂ 25 µM until 24 hpa

Fig. 4. Regeneration efficiency is directly proportional to J₁ reversals. Compilation of the different conditions tested. Data of regeneration index are from Figs 2A', 3A' and data of frequency of J₁ reversal are from Figs 2D, 3C. Most crossed data are matched siblings. Solid line, linear regression ($r^2=0.75$); *r*, correlation coefficient.

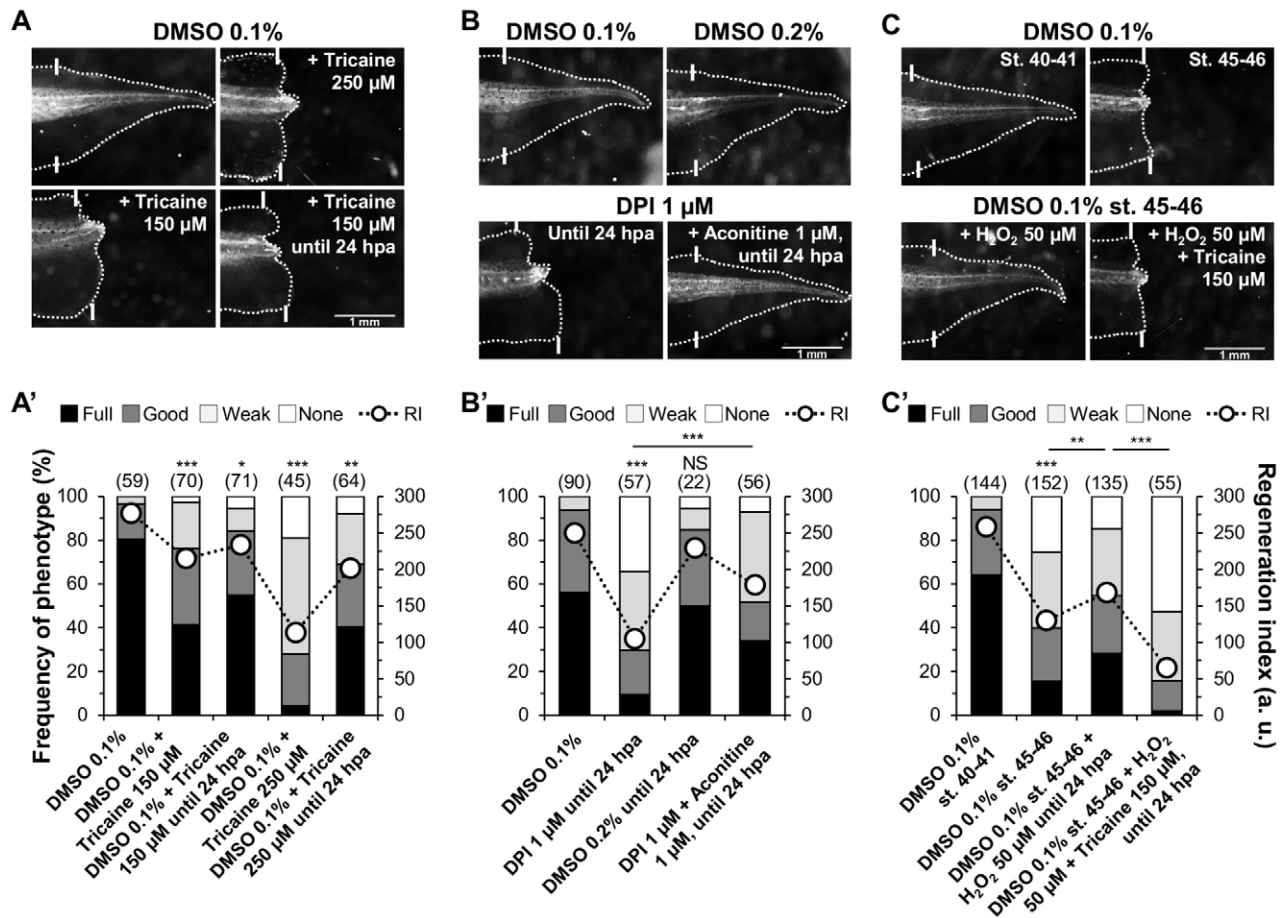


Fig. 5. H₂O₂ modulates regeneration via voltage-gated Na⁺ (Na_v) channels. (A,A') Na_v channel inhibition with tricaine impaired regeneration. (A) Representative 7 dpa tails treated with vehicle (DMSO 0.1%) or tricaine (250 or 150 μ M) from amputation or until 24 hpa. (A') Qualitative and quantitative analyses of regeneration efficiency for the different conditions tested. (B,B') Na_v channel activation with aconitine rescued DPI-impaired regeneration. (B) Representative 7 dpa tails treated with vehicle (DMSO 0.1% and DMSO 0.2%) or DPI (1 μ M), alone or with aconitine (1 μ M), until 24 hpa. (B') Qualitative and quantitative analyses of regeneration efficiency for the different conditions tested. (C,C') Na_v channel inhibition abolished H₂O₂-induced regeneration in the refractory period. (C) Representative 7 dpa tails amputated from tadpoles during the regenerative (stage 40-41) or refractory (stage 45-46) periods. Tails were treated until 24 hpa with vehicle, with H₂O₂ (50 μ M) alone, or with H₂O₂ (50 μ M) plus tricaine (150 μ M). (C') Qualitative and quantitative analyses of regeneration efficiency for the different conditions tested. Primary vertical axis title in A' also applies to B', C'; secondary vertical axis title in C' also applies to A', B'. RI, regeneration index; a.u., arbitrary units. White solid lines, amputation plane. Scale bars, 1 mm; *n* values are indicated in brackets.

Narahashi, 1975) significantly impaired regeneration (250 μ M: RI reduced from 277, *n*=59, to 113, *n*=45, *P*<0.0001; 250 μ M until 24 hpa: RI reduced to 202, *n*=64, *P*=0.008). A lower dose (150 μ M) had less penetrant effects (Fig. 5A,A'). Next, we designed the epistasis assays: (1) rescue DPI-impaired regeneration using the Na_v activator aconitine; and (2) loss H₂O₂-induced regeneration in the refractory period using the Na_v inhibitor tricaine.

Treatment with aconitine (Ameri, 1998) until 24 hpa significantly rescued DPI-impaired regeneration (Fig. 5B,B'). Aconitine increased 3.6-fold the frequency of tails with full phenotypes in relation to DPI-treated tails (from 9 to 34%) and decreased by ~80% the frequency of none phenotypes (from 34 to 7%). Overall, regeneration was significantly rescued (RI from 105, *n*=57, to 179, *n*=56, *P*<0.0001; Fig. 5B,B'). Interestingly, aconitine (in the μ M range) was lethal in the absence of DPI, pointing to antagonistic competition.

Treatment with tricaine until 24 hpa significantly impaired H₂O₂-induced regeneration (Fig. 5C,C'). Tricaine virtually eliminated full phenotypes in relation to H₂O₂-treated tails (from 30 to 2%) and increased 3.4-fold the frequency of none phenotypes (from 17 to

57%). Overall, regeneration was significantly lost (RI from 168, *n*=135, to 65, *n*=55, *P*<0.0001; Fig. 5C,C'). Therefore, Na_v channels act downstream of H₂O₂ and mediate H₂O₂-modulated regeneration – a new mechanism.

DISCUSSION

Bioelectric and, more recently, redox activities have been shown, independently, to modulate regeneration in widespread models: planaria (Beane et al., 2011; Pirotte et al., 2015), zebrafish (Gauron et al., 2013; Monteiro et al., 2014) and amphibians (Love et al., 2013; Reid et al., 2009). Seeking a biochemical and biophysical integration, we aimed to determine whether and how these pervasive activities interact during regeneration.

Redox-modulated regeneration is mediated by early bioelectric activities

Do ROS modulate regeneration via regulation of bioelectric activities that are known to be required for regeneration? Our results support the two-way regulation hypothesis proposed to address this question. First we mapped the spatiotemporal dynamics of extracellular

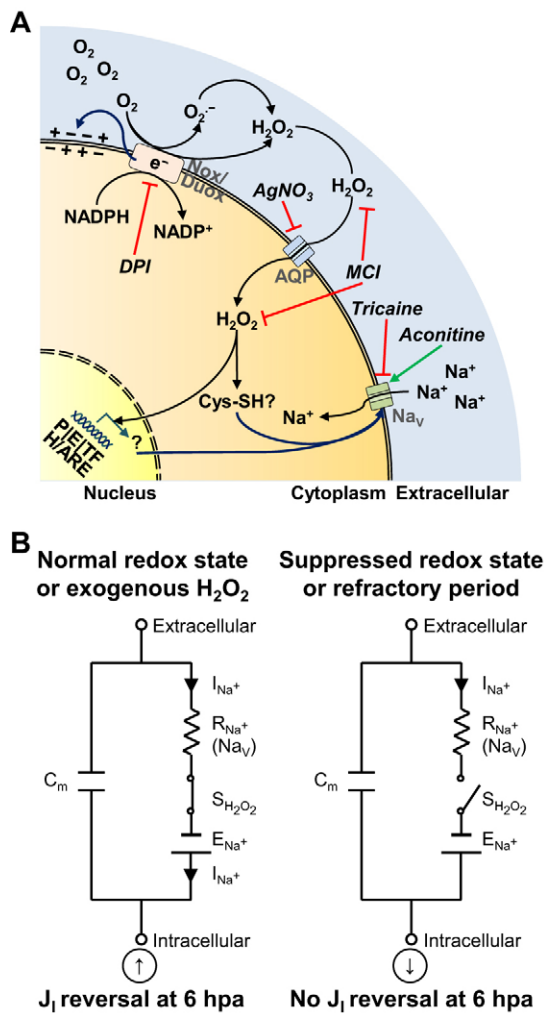


Fig. 6. Biochemical and bioelectrical integration during early regeneration via H_2O_2 . (A) Drug targets and H_2O_2 effects on bioelectricity at distinct steps during a putative H_2O_2 'life cycle'. From top to bottom, steps are production of H_2O_2 from NADPH oxidases, availability and diffusion of produced H_2O_2 , and H_2O_2 -mediated influences on bioelectric activities or parameters (V_m , TEP, EF, J_1 and Na_v). A representative cell potentially from wound epithelium (overlying bud) and/or mesenchymal bud of a tadpole amputated at stage 40-41. It is also possible that one cell produces H_2O_2 and another takes it up to regulate its bioelectric state. For simplicity, we present everything in the same hypothetical cell. e^- , electrons; Nox/Duox, NADPH oxidase families (Nox1, Nox2, Nox4 and Nox5 produce superoxide anion (O_2^-), whereas Duox1 and Duox2 produce H_2O_2 directly); AQP, aquaporin; Na_v , voltage-gated Na^+ channel; Cys-SH, cysteine (Cys) residues of proteins containing thiol (-SH) groups suitable for oxidation; P, promoters; E, enhancers; TF, transcription factors; H/A/E, hypoxia and antioxidant responsive elements; black arrows, 'life cycle' of H_2O_2 , from upstream O_2 to downstream effect; blue arrows, redox-mediated bioelectric outcome; green arrow, pharmacological activation; red lines, pharmacological inhibition. Drugs are in italic. (B) H_2O_2 is a switcher in the electrical equivalent circuit. Simplified circuit in a membrane patch of, potentially, a wound epithelium (overlying bud) and/or mesenchymal bud cell of a tadpole amputated during the regenerative (stage 40-41) or refractory (stage 45-46) period. In the absence of H_2O_2 (switcher of Na_v channels), putative Na^+ current (charge carrier) does not cross the membrane through Na_v channels and current reversal does not occur at 6 hpa. Suppressed redox state mimics the refractory period circuit. Exogenous H_2O_2 rescues (from depleted ROS) and induces (from the refractory period) J_1 reversal. Na^+ , charge carrier; C_m , capacitance of membrane; E_{Na^+} , electromotive force driving Na^+ ; I_{Na^+} , current of Na^+ ; R_{Na^+} , resistance to Na^+ flux (synonymous of Na_v channels); $S_{H_2O_2}$, switcher H_2O_2 . Arrows inside circles indicate net inward (down arrow) or outward (up arrow) current measured extracellularly.

bioelectricity (Fig. 1, Figs S2-S5) and the temporal requirement for NADPH oxidase-mediated ROS production (Fig. 2A,A', Fig. S7). These profiles identified the bud at 6 hpa as key to testing the proposed hypothesis, owing to its bioelectric state. Next, we blocked NADPH oxidases and imaged V_m . We found that V_m depolarization resulted from NADPH oxidase activity per se (Fig. 2B,B'). This supported the first part of the two-way regulation hypothesis, arguing that the electrogenic property of NADPH oxidases is the mechanism by which V_m depolarization occurs. As depolarization is not required for regeneration (Adams et al., 2007), we then focused on the extracellular bioelectricity. Drug-depleted ROS levels decreased TEP and prevented or postponed J_1 reversal, maintaining a steady outward current at 6 hpa (Fig. 2C,D). This mimicked the altered TEP and J_1 measured during the refractory period (Fig. S11). Exogenous H_2O_2 rescued and induced regeneration, TEP and J_1 (Figs 2, 3). It also induced ectopic tail formation (Fig. S15). Regeneration efficiency was directly proportional to TEP increase and J_1 reversals (Fig. 4, Fig. S14). These results supported the second part of the two-way regulation hypothesis, arguing that H_2O_2 regulates TEP, EF and J_1 . Ion translocators are known to mediate bioelectricity (McCaig et al., 2005), thus H_2O_2 is likely to be epistatic to translocators. Indeed, epistasis assays revealed that Na_v channels act downstream of H_2O_2 (Fig. 5). Altogether, the convergent results from disparate methods provide considerable consilience to conclude that redox-modulated regeneration is mediated by early bioelectric activities (Figs 6, 7).

Integrating bioelectric activities in the context of regeneration

Endogenous electric activities arise due to energetically expensive resting electrochemical potentials, primarily V_m and TEP. Can these biological batteries be, in part, dormant sensors and effectors ready to detect and to react to ubiquitous injuries? In fact, the idea that endogenous batteries play a role in wound healing (primarily guiding closure) is not entirely new (Barker et al., 1982; Borgens, 1984; Lykken, 1971). For an integrative view, we propose three phases of extracellular bioelectric contributions in regeneration: passive, active and homeostatic (Fig. 1E, Fig. S5).

Passive (until 6 hpa)

Barrier breaking disrupts the in/out discontinuum so ions leak down their electrochemical gradients, resulting in injury current and lateral EF (primary) with the cathode at the amputation plane. The trunk TEP remains unchanged with amputation, thus the A/P gradient is resolved at this position. Thereby, the 'bioelectric microenvironment' – spatial range of physiologically meaningful bioelectricity – capable of influencing regeneration spans $\sim 335 \mu m$ anterior to the amputation plane. Importantly, this microenvironment matches maximum mobilization distances, $\sim 400 \mu m$, of cells for wound closure in urodeles (Lash, 1955).

Active (6-48 hpa, stronger until 24 hpa)

Presumptive barrier restoration re-establishes the discontinuum so ions diffuse with or without energy costs through the wound epithelium. Leaky epithelia are not highly efficient ion-flux barriers due to paracellular movement. Thus, the timing from passive to active activity is difficult to determine precisely. Rather, according to the TEP increase and J_1 reversal frequency, a transition should occur from 1 to 6 hpa. Epithelial resistance plus J_1 reversal leads to an exponential build-up of TEP, provoking an equivalent drop in EF. The current reversal possibly reversed short-range EF (secondary) locally in the bud (to anode). The plateau

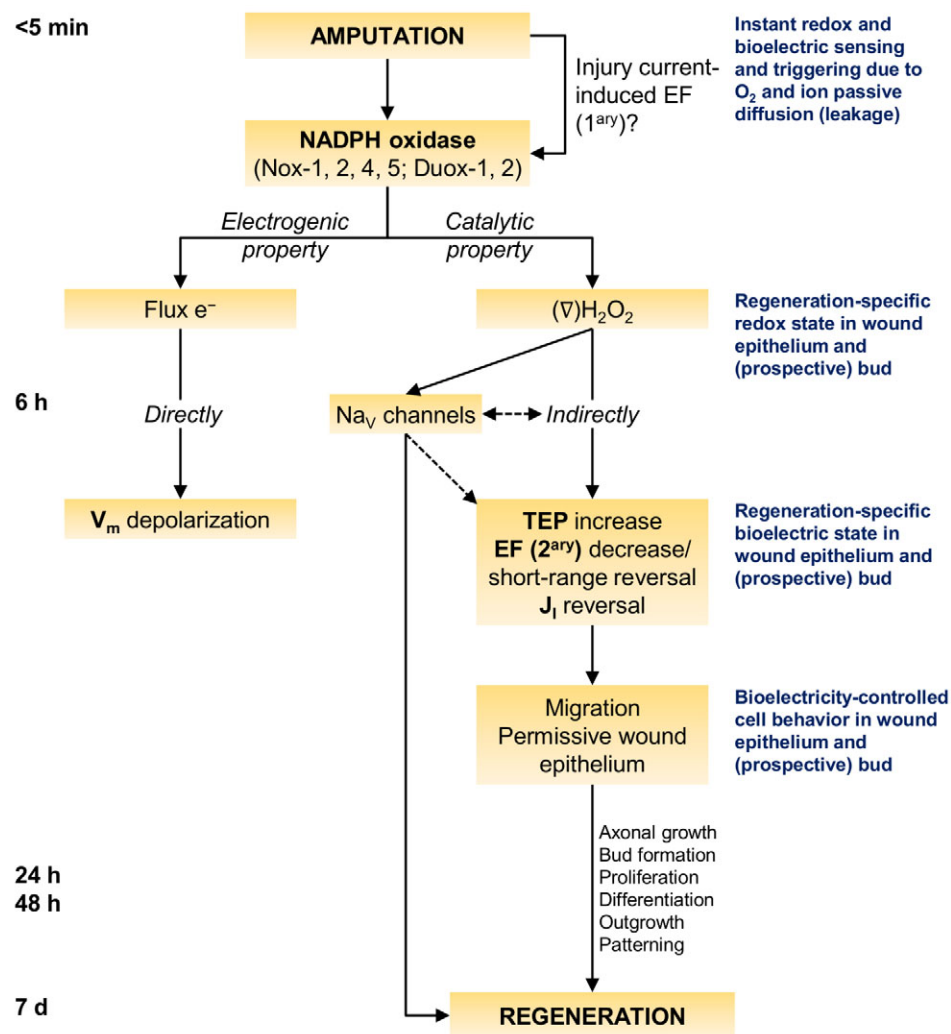


Fig. 7. Stepwise model of redox and bioelectric integration during regeneration. NADPH oxidase-mediated electron flow and H_2O_2 production independently regulate different bioelectric activities. This dual property – electrogenic and catalytic – of NADPH oxidases explicitly represents the proposed two-way regulation hypothesis. The focus is on the early timing of 6 hpa, when the hallmark H_2O_2 -switched current reversal occurs in regenerative tadpoles. A redox-bioelectric feedback module may be present, where bioelectric signals (instant primary EF) might activate redox signals (NADPH oxidases and generated H_2O_2), which in turn regulate bioelectricity (V_m , TEP, secondary EF and J_1) to modulate regeneration. Bioelectrically controlled cell migration and subsequent permissive wound epithelium is suggested as a mechanism by which, ultimately, H_2O_2 may control regeneration. e^- , electrons; ∇ , gradient.

part in both TEP and J_1 suggests that inward current magnitude should go beyond TEP recovery.

Homeostatic (from 48 hpa)

Ion fluxes should now be mainly for housekeeping functions, as TEP and J_1 return to uncut baseline, resolving EF. Uncut and complete regeneration tails have an inside-positive TEP and a small inward J_1 , in agreement with the salt uptake (osmoregulatory) function of amphibian skin (Koefoed-Johnsen and Ussing, 1958). Remarkably, TEP recovers to the new stage-specific level, not to the level before amputation (Fig. S2C), implying systemic control synchronizing development and regeneration. Bioelectric and regeneration phases dynamically correlate (Figs 1A,E, 4, Fig. S5), suggesting a control of regeneration by bioelectric events.

Is NADPH oxidase-driven V_m depolarization an injury-specific by-product of ROS production?

We showed that the characteristic V_m depolarization in the bud at 6 hpa mechanistically mimics the well-known NADPH oxidase-driven depolarization in immune cells during an oxidative burst (Bankers-Fulbright et al., 2003). This makes it attractive to speculate that V_m depolarization is a by-product of ROS production, which is reinforced by the non-requirement of V_m depolarization for

regeneration. V_m should be repolarized by H^+ efflux at 24 hpa to allow regeneration (Adams et al., 2007). We noticed that, irrespective of the drugs used, bud repolarization occurred by 24 hpa (Fig. S8A'), showing that redox state modulates regeneration in a V_m -independent way (Fig. 7). Similarly, robust V_m depolarization around the wound was measured in the caudal fin inter-ray wound model in zebrafish (F.F., unpublished). Importantly, Gauron et al. showed increased ROS levels produced by NADPH oxidases around wounds of the same model (Gauron et al., 2013). Thus, NADPH oxidase-driven V_m depolarization might be a universal injury-specific by-product of ROS production. However, depolarization is not excluded from having functions (Tseng et al., 2010) not addressed in the present study. In the past two decades, non-injury related V_m polarizations are being unveiled as being key in diverse developmental and patterning processes (Levin, 2014). Future work may unveil whether the activities of NADPH oxidases also have a role in these contexts, when ROS production is involved.

Electric current reversal: a potential hallmark of regeneration switched on by H_2O_2

Reid et al. showed a correlation between electric current reversals (from outward to inward) and *X. laevis* tadpole tail regeneration. Ion substitution assays revealed that Na^+ contributed to approximately

two-thirds of the reversed current. However, neither ion nor translocator modulations were able to actually maintain the outward current (Reid et al., 2009). Remarkably, using non-ionic modulation, we either prevented or postponed current reversal in the bud at 6 hpa, mimicking the refractory period. This led to a dramatic inhibition of regeneration. We showed that H_2O_2 diffuses into the cell and stimulates the reversal. Thus, in an electrical equivalent circuit, we propose that H_2O_2 is a switcher that permits Na^+ -carried current to diffuse into the stump (Fig. 6B). In the same model, Tseng et al. unveiled part of the molecular basis for Na^+ influx, the channel $Na_v1.2$ (Tseng et al., 2010). We found that H_2O_2 acts upstream of the Na_v channel family. Future work is necessary to reveal the mechanism of this activation, which can be indirect (e.g. redox-sensitive pathways or gene transcription of channels proteins/regulators) or direct (e.g. oxidation of channels cysteine residues, if available) (Fig. 6A). $Na_v1.2$ is present by 18 hpa in the bud, accumulating cations there (Tseng et al., 2010). The mismatch with our measured reversal at 6 hpa might imply other Na_v channel(s) in action, possibly in the wound epithelium. This would complete the path of Na^+ from outside to bud, as barrier restoration prevents most ion leakage and reduces paracellular flux. In principle, however, some Na^+ transporter(s) should be present in the wound epithelium to move the ions against the electrochemical gradient. The sharp TEP increase until 6 hpa supports this and might suggest the ubiquitous Na^+/K^+ -ATPase as a candidate (Dubé et al., 2010). Thus, it is possible that H_2O_2 (or other ROS) modulates additional ion translocators (Ma, 2011; Matalon et al., 2003). Exogenous H_2O_2 rescued and induced regeneration and induced ectopic tails, suggesting that H_2O_2 provides indispensable morphogenetic information. H_2O_2 robustly rescued and induced TEP increase and J_1 reversal at 6 hpa. Therefore, we propose that H_2O_2 -switched current reversal is a hallmark of regeneration. As a limiting and stimulating factor of regeneration, this hallmark can be used as a diagnostic characteristic of regeneration and as a marker and prognosis of its efficiency (Fig. 4). Furthermore, it can be used to promote regeneration in regenerative-deficient animals.

J_1 reversal prior to blastema formation was also seen in newt limbs (unquantified frequency, apparently occasional due to net outward current) (Borgens et al., 1977b) and tails (Nawata, 2001), and in *X. laevis* tadpole limb regeneration (G.L., unpublished). Reversal in newt tails was assumed to be a return to baseline (Nawata, 2001), despite the higher magnitude than before amputation. Surface potential measurements revealed stump potential reversal (from positive to negative) prior to blastema formation in urodels, but not in non-permissive anuran limb regeneration (Becker, 1961; Rose and Rose, 1974), showing a correlation with regeneration. However, another study found potential reversal in both taxa (Lassalle, 1979). We measured instead transepithelial potentials that, apart from sporadic reversals, had no overall mean reversal. Short-range $EF_{Shoulder \rightarrow Bud}$ presented more reversal frequency (21%) than did long-range $EF_{Trunk \rightarrow Bud}$ (9%) at 6 hpa (increasing thereafter; Fig. S3C), indicating the predominance of local reversal (in the bud). Indeed, in newt limbs, the surface potential reversal occurred only a short distance from the amputation plane (Rose and Rose, 1974). The small and soft bud hindered the spatial resolution required to fully characterize local reversals. Unambiguous current reversal supports the short-range potential and EF reversals in the bud. Altogether, our results showed a close correlation between reversals, bud formation and regeneration (Figs 4, 6, 7).

How can the H_2O_2 -switched current reversal mechanistically impact regeneration? Cell migration precedes proliferation (Adams et al., 2007) and is essential for wound closure in the first phase of

regeneration (Beck et al., 2009). Thus, the early and vectorial J_1 reversal suggests migration as a prime candidate. However, other cell behaviors are not excluded. Cumulative evidence shows that endogenous or exogenous EF act as guiding cues for migration during wound healing (McCaig et al., 2005; Zhao et al., 1996, 2006). Importantly, most cells, including keratinocytes and neurons, migrate or grow towards the cathode (Song, 2004; Sun et al., 2013). ROS-depleted and refractory period tails at 6 hpa had a sustained outward J_1 that led to lower bud TEP and higher short-range EF, establishing a long-term cathode at amputation plane (Figs 2C, 3B, Figs S11, S16A). Therefore, we postulate that a non-reversed J_1 by 6 hpa might lead to cell overmigration. In short, cells are deluded into behaving as if the wound was still open (Fig. S16B). Wound epithelium from the refractory period is thicker than regenerative *X. laevis* tadpole tails (Beck et al., 2003; Reid et al., 2009; Tseng et al., 2010). Mature skin flapped or grafted to amputated newt limbs or children's fingertips blocks regeneration (Illingworth, 1974; Mescher, 1976). Interestingly, these skin-like structures have altered currents (Altizer et al., 2002; Reid et al., 2009) (Fig. S11) and might disrupt epithelial-mesenchymal crosstalk (Lee et al., 2009), both of which are important for regeneration. Thereby, cell overmigration might produce a non-permissive refractory-like wound epithelium. This might explain why J_1 reversal by 24 hpa in MCI- and $AgNO_3$ -treated tails (Fig. S8C) was not sufficient for regeneration (Fig. 2A,A'). Although we cannot exclude direct ROS-induced chemotaxis (Hurd et al., 2012; Pan et al., 2011), ROS-modulated electrotaxis might be the major cue (Zhao, 2009). This rationale could apply to axonal sprouting and to axonal growth (electrotropism). Neuronal presence is a long-recognized limiting and stimulating factor in regeneration (Kumar and Brookes, 2012; Sidman and Singer, 1951). Importantly, applied currents enhance nerve growth and regeneration in adult frogs (Borgens et al., 1979). Observed J_1 reversal per se or the establishment of a steady inward current might be independent cues. Reversal can be a transitory electric pole discretely sensed by cells. Therefore, J_1 reversal may fine-tune (mitigate) migration of cells and axonal growth, setting the conditions for or triggering bud initiation around 6 hpa and subsequent proliferation and *de novo* tail formation. Indeed, a permissive epithelium neuronally supplied (Thornton, 1954) is the basis of the accessory limb formation in axolotls (Endo et al., 2004). H_2O_2 -induced ectopic tails might thus result from bioelectric effects on neuronal tissue.

Towards a comprehensive model of redox and bioelectric integration during regeneration

We aimed to conceptualize a comprehensive model that integrates redox and bioelectric states during regeneration (Fig. 7). We showed that NADPH oxidase-mediated ROS production is pleiotropic (early on it affects morphogenesis; later on it affects growth) and is required immediately upon amputation. Thus, an elusive ultra-fast signal needs to trigger the activity of NADPH oxidases. Probably the fastest injury-induced signals are bioelectric in nature. Exogenous EF activate NADPH oxidases in cells *in vitro* (Chatterjee et al., 2012; Li et al., 2013). Thus, we propose that endogenous EF activate NADPH oxidases (Fig. 7). Another candidate is Ca^{2+} signaling. Wounding induces fast Ca^{2+} influx and Ca^{2+} flashes in *X. laevis* oocytes and *Drosophila* embryo, respectively (Luxardi et al., 2014; Razzell et al., 2013). Importantly, those flashes activate a NADPH oxidase (Duox). Excitingly, this points to a feedback module, where bioelectric signals (primary EF and/or Ca^{2+}) might activate redox signals (NADPH oxidases and generated H_2O_2) that in turn regulate bioelectricity (V_m , TEP,

secondary EF and J_1) to modulate regeneration (Fig. 7). This might be a starting point from which to test whether bioelectricity-modulated redox activities also occur during regeneration. By 6 hpa, the electrogenic property of NADPH oxidases depolarizes V_m and the catalytic property stimulates TEP recovery and switches on J_1 reversal (Figs 6B, 7). H_2O_2 -modulated regeneration is then V_m independent. However, both paths might interact. For example, preventing V_m repolarization downregulates $Na_v1.2$ expression (Tseng et al., 2010). H_2O_2 diffusion into cells revealed for the first time, to our knowledge, that aquaporins are potential new targets to modulate epimorphic regeneration (Fig. 6A). H_2O_2 -switched current reversal, potentially via Na_v channels, may fine-tune cell migration and axonal growth that is essential for regeneration (Figs 6, 7).

Although the evidence supporting H_2O_2 is compelling, the role of other ROS in controlling bioelectric and regenerative outcomes should not be excluded (Li et al., 2013). Moreover, the possible importance of a H_2O_2 extracellular gradient is not excluded by this study. Indeed, it might be translated into a gradient of bioelectric activity that can be a blueprint for positional information and axes patterning (Jaffe, 1981; Tseng and Levin, 2013). Engineered solutions for controlled release of H_2O_2 (Garland et al., 2014) may prove useful for future work, especially in non-aquatic models.

In conclusion, this study unveils the interplay between biochemical and biophysical signals during regeneration, suggesting a novel mechanism that mediates regeneration: early H_2O_2 -switched J_1 reversal, potentially via Na_v channels. It also highlights the opportunities for interdisciplinary integration of apparently disparate states (Serena et al., 2009; Tandon et al., 2014) that may reserve promising advances for translational medicine.

MATERIALS AND METHODS

Animals and surgery

Animal procedures and pharmacological treatments were approved by local Institutional Animal Care and Use Committee (protocols 16822 and 18601). *Xenopus laevis* (Daudin, 1802) tadpoles acquired from Xenopus Express (www.xenopus.com) were staged (Nieuwkoop and Faber, 1967), sorted and transferred to fresh Marc's modified Ringer (MMR) $0.1\times$ culture medium, composed of (in mM): NaCl 10, $CaCl_2\cdot 2H_2O$ 0.2, KCl 0.2, $MgCl_2\cdot 6H_2O$ 0.1 and HEPES 0.5 (pH 7.1–7.2) (Sigma-Aldrich). Surgery was executed as previously described (Adams et al., 2007; Reid et al., 2009). For further details, see Supplementary Materials and Methods.

Tail regeneration assay

Tail regeneration assays were performed as previously described (Adams et al., 2007; Tseng et al., 2010). For further details, see Supplementary Materials and Methods.

Ectopic tail induction

Ectopic tails were induced by a dorsal incision severing down to the spinal cord (inclusive; Fig. S15A) (Sugiura et al., 2009). For further details, see Supplementary Materials and Methods.

Pharmacological modulations

Drugs and respective vehicle reconstituted in MMR $0.1\times$ were applied via immersion (bath) at the doses and exposures specified. Most drugs were stocked in DMSO (Sigma-Aldrich, cat. no. D2650) at $-20^\circ C$: DPI (Sigma-Aldrich, cat. no. D2926) 1 and 10 mM; MCI (Cayman chemical, cat. no. 13320) 200 mM; $AgNO_3$ (Sigma-Aldrich, cat. no. 209139) 24 mM; apocynin (Sigma-Aldrich, cat. no. W508454) 200 mM; VAS (EMD Millipore, cat. no. 492000) 1.4 mM; and aconitine (Alamone Labs, cat. no. A-150) 1 mM. Vehicle (DMSO) had non-significant effects on readouts (Fig. S1). H_2O_2 (EMD Millipore, cat. no. HX0635) was kept as a 1 M stock

in deionized water at $4^\circ C$. Occasionally, stocks of 25 and 50 mM were kept for short periods at $-20^\circ C$. Tricaine was kept as a 1 mM stock in MMR $0.1\times$ (pH readjusted to 7.2) at $-20^\circ C$. Working solutions were freshly prepared. For further details, see Supplementary Materials and Methods.

ROS and V_m fluorescence imaging

Intracellular relative ROS and V_m were measured non-invasively by fluorescence imaging of vital dyes as previously described (Adams et al., 2007; Owusu-Ansah et al., 2008). For further details, see Supplementary Materials and Methods.

Glass microelectrode measurement

Trans epithelial potential was measured invasively by glass microelectrode impalement through the epithelial layers as previously described (Luxardi et al., 2014; McCaig and Robinson, 1982). For further details, see Supplementary Materials and Methods.

Vibrating probe measurement

Extracellular net electric current density was measured non-invasively with a locked-in one dimensional vibrating probe, prepared and used as previously described (Reid et al., 2007, 2009). For further details, see Supplementary Materials and Methods.

Statistical analysis

Regeneration efficiencies were compared using the Fisher's exact test. Frequencies (in J_1 reversals and ectopic tails) were compared using the Mann-Whitney U -test. Correlations were tested with the Pearson's correlation coefficient r . Linear regressions (coefficient of determination r^2) were applied to fit data to a statistical model. All remaining comparisons were analyzed using the unpaired Student's t -test.

Data are presented as mean \pm s.e.m. Sample size (n , biological replicates) used in each experiment are stated in figures and text. At least two independent batches of tadpoles were used per readout. Differences were significant if $P<0.05$ and the levels of significance are: NS, non-significant; * $P<0.05$; ** $P<0.01$ and *** $P<0.001$. Means and standard errors were calculated and plotted using Excel, and statistics were performed using GraphPad Prism 5 (GraphPad Software). For further details, see Supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.F. and M.Z. conceived the study. F.F., G.L. and M.Z. designed the experiments. F.F. performed the experiments and analyzed the results. G.L. and B.R. provided technical training and guidance, and helped to interpret some results. F.F., G.L. and M.Z. outlined the manuscript. F.F. wrote the manuscript. All authors read, commented and agreed with the final version of the manuscript.

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Supplementary information

Supplementary information available online at
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