Osmotic surveillance mediates rapid wound closure through nucleotide release

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Osmotic cues from the environment mediate rapid detection of epithelial breaches by leukocytes in larval zebrafish tail fins. Using intravital luminescence and fluorescence microscopy, we now show that osmolarity differences between the interstitial fluid and the external environment trigger ATP release at tail fin wounds to initiate rapid wound closure through long-range activation of basal epithelial cell motility. Extracellular nucleotide breakdown, at least in part mediated by ecto-nucleoside triphosphate diphosphohydrolase 3 (Entpd3), restricts the range and duration of osmotically induced cell migration after injury. Thus, in zebrafish larvae, wound repair is driven by an autoregulatory circuit that generates pro-migratory tissue signals as a function of environmental exposure of the inside of the tissue.

Introduction

Rapid epithelial wound closure is essential for metazoan life, as it restricts exposure of the inside of an organism to the noxious outside environment. Wound closure mechanisms operate efficiently in animals that occupy different habitats (e.g., land/river/sea), and whose epithelia are exposed to vastly different physicochemical environments (air, fresh/salt water, etc.). Wound closure shows striking similarities to morphogenetic processes, such as dorsal closure in Drosophila melanogaster (Redd et al., 2004). These developmental events are thought to be regulated by organism-intrinsic cues; i.e., the external environment does not instruct them. Given its robust functionality throughout phyla, and analogy to developmental mechanisms, it is intuitive (and in fact common) to regard wound repair as an intrinsically guided, postdevelopmental program. Yet, it remains unclear whether the conserved function of wound repair derives from an insulation against extrinsic influences, or an adaption to them. The question arises whether epithelial wound detection and repair are obligatory tissue-intrinsic processes, or if they also integrate information from the environment.

Zebrafish is a powerful system to study wound responses and their possible environmental adaption in the intact animal (Redd et al., 2004; Huttenlocher and Poznansky, 2008; Richardson et al., 2013). The tail fin fold of 2–4-d-old zebrafish larvae is a double-layered epithelium consisting of a basal epithelial layer that is attached to a basal lamina, and a suprabasal layer in which cells are connected by adherens and tight junctions (Fig. 1 a; Sonawane et al., 2009). This stratified skin fold protects the inside of the zebrafish (~270–300 mOsm, i.e., common vertebrate extracellular tonicity) from its natural hypotonic freshwater environment (~10 mOsm), analogous to the stratified linings of mouth and esophagus, which protect mammalian tissues from hypotonic salivary (~30 mOsm). The thinness and transparency of zebrafish tail fins facilitates interrogation of tissue damage detection mechanisms through pharmacologic/genetic perturbations and intravital microscopy. Using the zebrafish tail fin wounding assay, we previously demonstrated that a drop in interstitial osmotic pressure initiates eicosanoid-mediated leukocyte recruitment (Enyedi et al., 2013). In the present study, we asked whether osmotic signaling is an environmental master regulator of wound responses by examining its potential involvement in epithelial repair.

Results

Environmental hypotonicity triggers rapid wound closure in zebrafish larvae

To test for a role of external tonicity, we imaged wound closure in larval zebrafish tail fins after UV laser wounding of fish.
Figure 1. A transepithelial osmotic pressure gradient is required for rapid wound closure and barrier reconstitution of zebrafish tail fin wounds. (a) Simplified scheme of larval zebrafish tail fin epithelium ~3 dpf. Putative cell–cell contacts are indicated. (b, left) Representative time-lapse montage of zebrafish larvae immersed in hypotonic (Hypo) or isotonic (IsoNaCl, IsoSucrose) solutions at the indicated times after UV laser puncture injury. The actin cytoskeleton is labeled with GFP-Utr-CH. Bars, 50 µm. (b, right) Quantification of wound area as a function of time after injury. (c, left) Time-lapse montage of suprabasal AKT-PH-GFP (suprabasal). (c, right) Quantification of wound area as a function of time after injury. (d) Hypo + H2O2 or Iso + H2O2 solutions at the indicated times after UV laser puncture injury. The actin cytoskeleton is labeled with GFP-Utr-CH. Bars, 50 µm. (d) Quantification of oxidized area as a function of time after H2O2 addition.
immersed in either normal, hypotonic bathing medium or bathing medium that had been adjusted to the ionic composition and/or tonicity of vertebrate interstitial fluid (with the addition of NaCl or sucrose). The actin cytoskeleton and plasma membranes were labeled using GFP-utrophin-calponin homology domain (GFP-Utr-CH; Burkel et al., 2007), and AKT-pleckstrin homology domain (AKT-PH)–GFP (Kwon et al., 2007), respectively. Injection of mRNA into one-cell stage embryos led to ubiquitous labeling. In contrast, injection at the 4–8-cell stage gave rise to mosaic labeling of predominantly basal epithelial cells (Fig. S1 a). Basal epithelial cell labeling was also performed by injection of DNA constructs containing a fluorescent protein under the control of a basal cell–specific ΔNp63 promoter (Reischauer et al., 2009). Suprabasal labeling was achieved by expression via a keratin promoter (Gong et al., 2002). Several pulses of a micropoint laser (435 nm) were used to produce wounds on both sides of the epithelial fold. Importantly, these full-thickness wounds are unlikely to close by contraction of underlying structures, because those are ablated by the laser blast. In hypotonic fish bathing solution (standard E3 medium), closure of ~5,000-µm² puncture wounds was completed within ~20 min, i.e., ~5x faster than closure of similar sized lesions in Drosophila larvae (Geiger et al., 2011). Isotonicity (IsoNaCl or IsoSucrose) inhibited wound closure, with NaCl showing a more pronounced inhibition (Fig. 1 b and Video 1).

Isotonic inhibition of wound closure was reversible (Fig. 1 c and Video 2). We also tested whether isotonicity blocks restoration of barrier function. To this end, we amputated the tail fin tips of transgenic zebrafish larvae ubiquitously expressing a genetically encoded, reversible fluorescent H2O2 reporter (HyPer; Belousov et al., 2006) in isotonic medium. After the endogenous, injury-induced HyPer signal (Niethammer et al., 2009) had subsided, the Tg(actb2:HyPer) transgenic fish were mounted in isotonic agarose and overlaid with isotonic or hypotonic solution supplemented with H2O2. Intact tail fin skin is impermeable to both H2O and H2O2. H2O2 in the bathing solution probes wound permeability by eliciting a HyPer signal upon entering the fish. This signal was sustained upon isotonic, but not hypotonic, bath exposure, which is consistent with delayed barrier recovery in isotonic solution (Fig. 1 d and Video 3). Similar to the initial inflammatory response (Enyedi et al., 2013), rapid repair of zebrafish tail fin wounds depends on the osmotic difference between the freshwater environment and the interstitial fluid of the fish.

Environmental hypotonicity triggers migration of basal epithelial cells

Wound margin contraction through an actin cable (Martin and Lewis, 1992; Bement et al., 1993) or epithelial cell migration are the most plausible mechanisms for rapid wound closure in our model (see Discussion). To experimentally define the processes underlying isotonic inhibition of closure, we imaged the morphological responses of epithelial cells by expressing fluorescent markers for actin, myosin, and plasma membrane in different epithelial layers. Time-lapse videos revealed rapid formation of lamellipodia in the basal, but not the suprabasal, layer after injury in hypotonic solution (Fig. 2, a and b; Fig. 3 a; and Video 4). Notably, migrating cells that had arrived at the wound margin abruptly ceased migration, and underwent a drastic morphological change that involved cell rounding, apparent shrinkage, and extension of phosphatidylinositol (3,4,5)-trisphosphate (PIP3)–positive fingerlike protrusions (Fig. 2 a and Video 4). By terminating cell migration at the cell margin, and making room for subsequent rows of cells (through compaction of wound margin cells), this process may contribute to the orderly and sequential advancement of migrating basal cells toward the wound. Propagation of lamellipodia formation distal to the wound was strongly inhibited by isotonicity (IsoNaCl or IsoSucrose), with NaCl having a slightly more pronounced effect (Fig. 3 a and Fig. S1 b). Suppression of lamellipodia formation by isotonicity or cytochalasin D was paralleled by inhibition of basal cell translocation (Fig. 3 b) and wound closure (Fig. S2 a). Both the morphological transition of basal cells (see above) to a rounded shape at the wound margin and the propagated wave of basal cell crawling were absent in isotonic wounds, while fingerlike protrusions positive for PIP3 could be observed at the basal cell margin.

We could detect recruitment of actin and myosin II at the wound margin by imaging ubiquitously expressed GFP-Utr-CH (Fig. 1 b) and mKate2-labeled myosin regulatory light chain (MRLC-mKate2; Fig. 3 c), or suprabasally expressed GFP-Utr-CH (Fig. S1 c). Together, these data indicate the formation of a “purse string” (Martin and Lewis, 1992; Bement et al., 1993) in the suprabasal layer. We cannot formally exclude purse string formation in the basal epithelial sheet, but our experiments do not bear evidence for it. Unlike at the rounded suprabasal margin (Fig. 2 a, white arrow; and Video 4), fingerlike protrusions appear to predominate at the basal wound margin as mentioned above (Fig. 2 a, yellow arrow; and Video 4). Actin and myosin recruitment to the wound margin seemed little affected by isotonicity (Figs. 1 b and 3 c). Likewise, wound margin rounding, which is indicative of a functional purse string, was visible in isotonicity (Fig. 1, b and c; and Videos 1 and 2). Thus, spatially separated wound closure mechanisms (i.e., suprabasal purse string contraction and basal cell migration) mediate rapid wound closure in zebrafish larvae.

Morphodynamic profiling of epithelial sheet motion by particle image velocimetry (PIV)

Wound area assays (e.g., Fig. 1 b) integrate the functional contributions of all force-generative processes operant in an injured...
motility in response to hypotonic exposure, we decided to directly measure wound-induced epithelial sheet motility by PIV analysis of the suprabasal layer (Fig. 4a).

tissue, and their spatial coordination, as well as their mutual interactions into a single endpoint measurement. To obtain more specific information about the mechanisms that initiate cell

Figure 2. Epithelial cell layers within the larval zebrafish epidermis exhibit distinct morphological wound closure mechanisms. (a) Time-lapse images of a representative 2.5–3-dpf zebrafish larva at the indicated times after UV laser-induced injury. (a, top) Transgenic Tg(krt4:AKT-PH-GFP) expression of AKT-PH-GFP driven in the suprabasal cell layer (green) is observed simultaneously with mosaic AKT-PH-mKate2 in underlying basal epidermal cells (4–8-cell-stage mRNA injection; red) after puncture wounding in hypotonic E3 medium. Basal cells form lamellipodia and translocate collectively toward the wound, whereas the suprabasal cells translocate and elongate without visible lamellipodia. Note that basal cells at the margin can protrude across the wound opening (yellow arrow), whereas suprabasal cells at the margin align to form a smooth wound edge indicative of contractile “purse string” closure (white arrow). Basal and suprabasal cells maintain a largely consistent proximity; representative center of mass tracks for basal (yellow) and suprabasal (white) cells are shown (10’, top right panel). Bars, 50 µm. (a, bottom) Enlargement of a region in the top panel; basal cell (yellow x) and neighboring suprabasal cell (white x) correspond to upper tracks in top right panel. Bars, 25 µm. All images are from a partial z projection to capture an individual epidermal bilayer. See Video 4. (b) Representative images of a 2.5–3-dpf zebrafish larva mosaically expressing GFP under the control of a basal cell–specific ΔNp63 promoter, immersed in hypotonic bathing solutions shown at indicated times after UV laser cut injury. Broken white line, position of wound. Broken yellow lines, outlines of representative lamellipodial protrusions. Bars, 25 µm.
Some residual motion remained after isotonic injury (Fig. 4b, red curve), which was further suppressed by preincubation with the Rho kinase inhibitor Y27632 (Fig. 4b, green curve), a compound known to abrogate purse string formation (Abreu-Blanco et al., 2011). Interestingly, Y27632 prolonged sheet motion after hypotonic but not isotonic injury (Fig. 4b, compare blue and gray curves), and appeared to interfere with coordination of tissue movements, at least to some degree. Specifically, closure movements appeared less regular, and not as concentrically balanced around the wound as in the control (Video 5). However, it remains unclear whether this effect is caused by the abrogation of the

Tissue velocity was measured as a function of time after injury (global PIV; Fig. 4b) and/or distance from the wound (spatial PIV; Fig. 4c). Averaging the motion profiles of multiple animals permitted quantitative comparison of dynamic “tissue-motion phenotypes” after experimental perturbations. Spatial PIV revealed that tissue motion spreads \(~150–200\) µm away from the wound after hypotonic injury (Fig. 4c), which is consistent with the length scale of basal cell translocation (Fig. 3b). Highlighting statistical significant differences by subtracting hypotonic and isotonic velocity maps (Fig. 4c) showed that isotonicity reduced amplitude and spatiotemporal spreading of motion through the tissue, in line with our morphological observations. Some residual motion remained after isotonic injury (Fig. 4b, red curve), which was further suppressed by preincubation with the Rho kinase inhibitor Y27632 (Fig. 4b, green curve), a compound known to abrogate purse string formation (Abreu-Blanco et al., 2011). Interestingly, Y27632 prolonged sheet motion after hypotonic but not isotonic injury (Fig. 4b, compare blue and gray curves), and appeared to interfere with coordination of tissue movements, at least to some degree. Specifically, closure movements appeared less regular, and not as concentrically balanced around the wound as in the control (Video 5). However, it remains unclear whether this effect is caused by the abrogation of the
Figure 4. **Quantitative analysis of wound-induced epithelial sheet movement by PIV.** (a) Representative example of PIV analysis (PIVlab, MATLAB) of epithelial sheet movement after UV puncture injury of a $Tg(krt4:AKT-PH-GFP)$ larva exhibiting plasma membrane labeling in the suprabasal layer. Green arrows, velocity vectors derived by comparing particle movements between subsequent frames. Red area, extra-tissue area excluded from analysis. Bar, 50 µm. (b) Global PIV analysis of UV-puncture wounded $Tg(krt4:AKT-PH-GFP)$ larvae immersed in solutions of indicated composition ($Y = Y27632$, Rho-kinase inhibitor, 100 µM). The graph displays the mean of all velocity vector magnitudes within the unmasked field of view as a function of time after injury. (c) Spatial PIV analysis representing the Iso and Hypo datasets from b as spatially resolved 3D plots. (i) and (ii) Averaged spatial PIV plots of the indicated number of $Tg(krt4:AKT-PH-GFP)$ larvae after UV-laser puncture injury in hypotonic or isotonic medium. Tissue velocities are color-coded (blue to green to yellow to red), and represented as a function of time after injury (x axis) and distance from injury site (y axis). (iii) Differential plot derived by subtraction of the indicated velocimetry plots, and t test filtering of statistically significant differences between groups (unpaired t test, $P < 0.05 =$ significant difference). Statistically significant velocity differences are color-coded (turquoise to pink). Pink, positive values. Turquoise, negative values.
purse string, or decreased tissue tension in general. The fact that lamellipodial cell migration occurs only in the basal layer, but that motion is detected in the suprabasal layer even when the purse string is inhibited, suggests that the layers are mechanically coupled (e.g., desmosomes; Fig. 1 a; Sonawane et al., 2009) and that movement is largely promoted by the basal cells (Fig. S2 b). Supportive of this idea, basal and superficial cells maintain relative positions during movement (Fig. 2 a and Video 4). We therefore conclude that the superficial layer is dragged toward the wound primarily by osmotically induced basal cell migration, and potentially assisted (e.g., through spatial coordination) by an intrinsically triggered purse string contraction of the suprabasal wound margin.

Environmental hypotonicity stimulates wound-induced ATP release

Next, we investigated the mediators of basal cell migration after hypotonic injury. We previously showed that osmotically induced arachidonic acid release stimulates leukocyte migration to tail fin wounds (Enyedi et al., 2013). Arachidonic acid produced sporadic increases in plasma membrane activity (“wobbling”) of the basal cells, but unlike hypotonic solution, it did not induce wound-polarized lamellipodia or directional cell migration after isotonic injury (Video 6). This suggests that the mechanisms of rapid epithelial defense and repair diverge downstream of the shared osmotic trigger.

Many cells respond to osmotic swelling with nucleotide secretion (Hoffmann et al., 2009). Nucleotide triphosphates (NTPs), such as ATP, are present in the cytoplasm of all cells at high concentrations ([ATP] ~5 mM; Beis and Newsholme, 1975). Vesicular NTP concentrations can be an order of magnitude higher ([ATP] ~90 mM; Johnson, 1988). NTPs may enter the interstitial space via cell lysis, exocytosis, or nucleotide conducting pores. In mammalian cell culture wound healing models, ATP has been found to mediate cell migration via P2Y2 receptor signaling (Yin et al., 2007; Block and Klarlund, 2009) and that movement is largely promoted by the basal membrane-impermeable DNA-selective antagonist polyoxometalate (POM)/compound 7 (Müller et al., 2011). For genetic interference, we designed a translation-blocking morpholino (entpd3 MO1) and a splice-blocking morpholino (entpd3 MO2) against zebrafish entpd3. entpd3 MO1 produced no major morphological defects besides cardiac edema (Fig. S4 c). Entpd3 MO2 produced a truncated entpd3 mRNA, which exhibited no gross morphological defects (Fig. S4 c). Both morpholinos had no obvious morphological effects on the tail fin epithelium (Fig. S4 c). To chemically interfere with extracellular nucleotide hydrolysis, we used adenosine 5’-γ-thio) triphosphate (ATPγS; a slow-hydrolyzing ATP analogue that, owing to its structural analogy to ATP, is expected to competitively inhibit all ATP hydrolyzing enzymes in the extracellular space) and the ENTPD subgroup-selective antagonist polyoxometalate (POM)/compound 7 (Müller et al., 2006).

Entpd3 knockdown by both morpholinos consistently increased the range and duration of wound-induced epithelial sheet motion (Fig. 6, a and b; and Fig. S4 d). This motion phenotype could be partially rescued by co-injecting entpd3 MO1 with morpholino-resistant entpd3 mRNA (Fig. 6 b, blue vs. red curve), arguing against an off-target effect. Enhanced tissue motion was not observed upon injection of an entpd3 MO1 five-nucleotide mismatch control morpholino (MO1 5 mm), excluding nonspecific morpholino effects (Fig. 6 c). Wound-induced epithelial sheet motion was blunted by the addition of apyrase to the fish bathing medium (Fig. 6 d). The genetic
results were corroborated by the pharmacologic inhibition data. As expected, ATPγS produced a dramatic increase in wound-induced tissue motion, which is consistent with its ability to compete with extracellular ATP hydrolysis in general (i.e., not only with ENTPD-mediated hydrolysis; Fig. 6, e and f). POM produced a similar, though less pronounced, motion phenotype (i.e., prolonged epithelial migration), which is consistent with its more restricted target selectivity (as compared with ATPγS; Fig. 6 g). As expected, POM and ATPγS (Video 8) promoted lamellipodia formation in cells far from the wound margin in hypotonicity. The migratory response that ATPγS elicited in hypotonic solution was often so massive that it globally distorted the tissue structure, producing sample drifts (which were corrected by computational image registration when possible; Video 8). Importantly, these dramatic epithelial migration phenotypes were not induced by pharmacological inhibitor treatment per se, but only in conjunction with hypotonicity (Fig. 6, f and g). This is consistent with the idea that hypotonic, but not isotonic, injury leads to the release of a pro-migratory signal that is NTPase sensitive. Interestingly, although inhibition of interstitial NTP breakdown generally increased sheet motion, aberrant motion typically did not lead to faster wound closure. Rather, it tended to antagonize the normal spatially and highly coordinated, concentric closure movements. Not unexpectedly, efficient wound closure likely requires both epithelial sheet motility (in this case driven by lamellipodia formation in the basal epithelial layer) and proper spatial coordination of collective cell migration.
Figure 6. Epithelial movements in response to hypotonic injury are regulated by extracellular NTP hydrolysis. (a, i and ii) Spatial PIV analysis of the indicated number of Tg(krt4:AKT-PH-GFP) larvae subjected to UV laser cut injury in hypotonic medium, with or without translation morpholino-mediated
ATP reconstitutes cell migration in the absence of environmental hypotonicity

To mimic transient ATP concentration peaks that cells in a tissue may experience immediately after nonlytic ATP release by exocytosis or channel conductance, we supplemented isotonic bathing solution with cytoplasmic levels of ATP. This strongly stimulated basal cell migration in isotonicity as indicated by induction of lamellipodial protrusions toward the wound, and velocimetry analysis (Fig. 7, a and b; and Video 9). As expected, the response was abrogated by pre-incubating the ATP solution with apyrase before adding it to the larvae (Video 9). This excludes the possibility that contamination of the ATP solution stimulated motility. UTP, like ATP, stimulated basal cell motility (Fig. S5 a). The finding that physiologically relevant extracellular concentrations of ATP or UTP can promote epithelial cell motility after isotonic wounding is consistent with secreted NTPs providing a wound-relevant chemokinetic signal. This also shows that isotonicity blocks a migratory stimulus for epithelial cells, but not their general ability to migrate. Although UTP potently induced basal cell migration, our data do not determine whether it is actually released, as luciferase is highly specific for ATP, but not UTP (Moyer and Henderson, 1983). UTP and ATP can be rapidly interconverted by ecto-nucleoside diphosphokinases in the extracellular space of tissues (Lazarowski et al., 1997).

The NTP sensing mechanism underlying basal epithelial responses in our system remains unclear. Our current data indicate that it differs from previously reported purinergic mechanisms involved in wound repair in vitro. Suramin, a nonspecific P2 receptor inhibitor commonly used to block P2Y2, had little effect on rapid wound closure, even at high concentrations (Fig. S5 b). The agonist profile of basal epithelial migration in our system does not match known P2 receptor profiles (Table S1 and Videos 9 and 10). Furthermore, tail fin wound closure occurs much faster than P2Y2-dependent monolayer healing in cell culture. Phylogenetic variations of P2Y specificity or formation of receptor heterodimers may account for this noncanonical behavior. Alternatively, unknown nucleotide receptors may exist. The unexpected discovery of novel ATP receptors in plants, which do not bear sequence similarity to traditional P2 receptors (Choi et al., 2014), underlines this possibility. Our ongoing efforts are directed toward identifying the molecular specifics of NTP release and sensing in our model.

### Discussion

Purse string contraction of the wound margin and basal sheet phenomena are the two most plausible wound closure mechanisms that are relevant to our model. Alternative wound closure phenomena, such as cell or matrix contraction, are unlikely to contribute because of the rapid nature of the wound closure and thick thickness loss of cells and matrix in the wound region. Although we observe fingerlike protrusions at the wound margin, the almost perfectly circular shape of the wound during closure and its remarkably rapid kinetics argue against a slower zippering mechanism.

Pharmacological Rho kinase inhibition reveals that our laser puncture wounds largely close in the absence of a purine string, albeit not as well coordinated as in the control samples and with a higher incidence of “jagged” and deformed wound margins. Hence, the contractile actin cable cannot be the major mediator of osmotically stimulated wound closure. Likewise, our data do not bear evidence that purine string formation or contraction depends on osmotic cues. The actin cable still forms and rounds up at the wound margin after isotonic injury (Fig. 1 b), which indicates intrinsically stimulated local force generation at the margin even in the absence of the hypotonic trigger. In contrast, we find that lamellipodia formation, migration of basal epithelial cells, and epithelial sheet movement are strongly inhibited by environmental isotonicity. Collectively, this argues that rapid wound closure is mainly mediated by hypotonically induced basal cell migration in our model, although basal cell-specific inhibition of lamellipodial migration would be required to test the contribution of the basal cells to wound closure more directly. To our knowledge, this is the first study delineating different wound closure mechanisms in a complex, bilayered epidermis. Intriguingly, contractile- and actin polymerization-mediated modes of wound closure appear to be spatially separated between different tissue layers, and show different environmental sensitivity. In motile cells, Rho-driven myosin contractility and Rac-driven actin polymerization are believed to antagonize each other, requiring spatial front-back separation to allow coordinated movements. Spatial separation of these mechanisms within multicellular structures may facilitate coordinated tissue movements during wound closure.

Consistent with preceding cell culture studies (Praetorius and Leipziger, 2009), we find that environmental hypotonicity induces nucleotide release at the wound site, most likely through

knockdown of entp3 mRNA (entp3 MO1; ~19 ng). [b] Global PIV analysis of the datasets in panel a additionally including morpholino-rescue data (blue curve). [c] Global PIV analysis of MO1 five-nucleotide mismatched control morpholino (entp3 MO1 5 mm; ~19 ng). [d] Global PIV analysis of Tg(krt4: AKT-PH-GFP) larvae subjected to mechanical tail fin tip amputation in isotonic solution ± potato apyrase (50 U/ml). After a 10-min preincubation in isotonic mounting agarose with or without apyrase, fish were overlaid with a bolus of hypotonic solution ± POM (ENTPD inhibitor, 100 µM). After a 10-min preincubation in isotonic mounting agarose ± POM, fish were overlaid with a bolus of hypotonic (to initiate the wound response) or isotonic solution ± POM. Note that velocimetry analysis does not include the isotonic preincubation period [i.e., t = 0’ in the plot is 10’ after injury]. See also Video 8.
nonlytic cell swelling. Notably, our luciferase/luciferin bathing technique is limited by diffusion of luciferase (dimer mol wt \( \approx 120 \text{ kDa} \)) and luciferin from the bathing medium through the open wound into the tissue. While highlighting the fraction of ATP directly released into the bathing solution, our current measurements may underestimate the actual ATP release pattern due to the limited availability of luminescence-generating enzyme/substrate inside the tissue. Future studies, e.g., with transgenically expressed, membrane-bound luciferase, may allow measurement of ATP release deeper within the tissue.

The mechanisms of swelling-induced ATP release in cells are incompletely understood even in simplified cell culture systems. Besides osmotic cell lysis, hemi-channel conduction (e.g., via connexins or pannexins) and vesicular NTP release appear to be important. Our current results do not argue for cell lysis being a mediator of wound-induced ATP release. Whether any of the other known nonlytic mechanisms accounts for wound-induced ATP release in zebrafish tail fins remains to be investigated.

If extracellular NTPs are endogenous mediators of basal cell migration in our model, epithelial sheet motion should be inhibited by increasing extracellular NTP breakdown. Likewise, decreasing extracellular NTP hydrolysis should enhance epithelial sheet motility. Collectively, our genetic and pharmacologic perturbations of extracellular nucleotide metabolism, including morpholino-mediated knockdown of the most abundant ENT-PDase of basal epithelial cells, (entpd3), confirm these predictions. ATP bathing triggers basal cell migration, but does not fully reconstitute hypotonic wound closure. A possible explanation for this could be that ATP bath application is unlikely to recapitulate the endogenous, spatiotemporal ATP release pattern, which may be crucial, e.g., for proper spatial coordination of collective movements. In addition, ubiquitous exposure of larvae to high ATP concentrations on the outside, apical surface of suprabasal cells may adversely affect the behavior of the suprabasal layer during closure. Finally, our data do not exclude that there are other unknown, hypotonically triggered processes, in addition to NTP release, that contribute to rapid wound closure.

In this study, we have identified an osmotic signaling circuit that initiates extracellular ATP release in response to a drop of interstitial osmotic pressure. This autoregulatory mechanism adjusts wound responses to wound size by coupling basal cell migration to environmental exposure (Fig. 7 c). Intriguingly, the same osmotic cue that mediates rapid wound detection by leukocytes via eicosanoids (Enyedi et al., 2013) also mediates rapid wound closure by nucleotides. Only very few reports have addressed potential environmental contributions to tissue healing and regeneration, including classic work by Goldfarb and Loeb (Loeb, 1891; Goldfarb, 1907, 1914; Radice, 1980; Fuchigami et al., 2011). To date, wound repair is still predominantly viewed as an organism-intrinsic process driven by cell damage, lack of contact inhibition, or altered mechanical signaling at tissue edges. This study advances this concept by illuminating how environmental surveillance regulates rapid epithelial repair through osmotic signaling in vivo. Transepithelial gradients of osmotic pressure, comparable to those that zebrafish experience in freshwater, exist in the human mouth and esophagus. Furthermore, steep transepithelial pH gradients occur in the stomach. It will be interesting to determine whether these chemical gradients mediate environmental surveillance of mammalian wet epithelia.

**Materials and methods**

**General zebrafish procedures**

Adult AB wild-type (wt), casper (White et al., 2008), and transgenic zebrafish strains were maintained according to institutional animal guidelines, and as described previously (Nusslein-Volhard and Dahm, 2002). Zebrafish larvae were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\(_2\), and 0.33 mM MgSO\(_4\)). For wounding assays, 2.5–3 days postfertilization (dpf) larvae were anesthetized using 0.2 mg/ml tricaine (Sigma-Aldrich) in E3 for at least 20 min before and during laser wounding and imaging. To suppress skin pigmentation, larvae were maintained in E3 medium supplemented with 0.2 mM N-phenylthiourea (PTU; Sigma-Aldrich) from 1 dpf until the start of the experiment.

**Reagents**

The following nucleotides, nucleotide analogues, or metabolites were used at a final concentration of 5 mM in isotonic E3 medium: adenosine 5'-triphosphate disodium salt hydrate (ATP; A26209; Sigma-Aldrich), adenosine 5'-[γ-thio]triphosphate tetralithium salt (ATP-S; 4080; Tocris Bioscience), 2'-[3']-O-[4-Benzoylbenzoyl]-adenosine 5'-triphosphate triethylammonium salt (Be-ATP; B6396; Sigma-Aldrich), adenosine 5'-diphosphate sodium salt [ADP; A2754; Sigma-Aldrich], adenosine 5'-[β-thio]diphosphate triethylammonium salt (ADP[S]; AR016; Sigma-Aldrich), adenosine 5'-monophosphate sodium salt [AMP; A1752; Sigma-Aldrich], adenosine (A9251; Sigma-Aldrich), uridine 5'-triphosphate trisodium salt hydrate (UTP; U6625; Sigma-Aldrich), uridine 5'-[γ-thio]triphosphate trisodium salt (UTP-S; 3279; Tocris Bioscience), uridine 5'-diphosphate disodium salt hydrate (UDP; 94330; Sigma-Aldrich), 3'-[2-Oxo-2-pheynethyl]uridine-5'-diphosphate disodium salt (3'-phenacyl UDP; PSB 0474; Santa Cruz Biotechnology, Inc.), uridine 5'-diphosphoglucone disodium salt hydrate (UDP-glucose; U4625; Sigma-Aldrich), uridine 5'-monophosphate (UMP; U1752; Sigma-Aldrich), and uridine (U3750; Sigma-Aldrich). Arachidonic acid (A9673; Sigma-Aldrich) was used at a final concentration of 10 µM in isotonic E3 medium. The following inhibitor compounds were used: cell-permeable Cytochalasin D (100 µM; 1233; Tocris Bioscience) and Y-27632 (100 µM, 10005583; Cayman) were dissolved DMSO (DB418; Sigma-Aldrich) and diluted in the indicated E3 medium. Cell-impermeable POM ([K\(_5\)H\(_3\)Th\(_{12}\)Co\(_{4}\)]; 100 µM; Müller et al., 2006), apyrase (50 U/ml; A6410; Sigma-Aldrich), ATP-S (5 mM; 4080; Tocris Bioscience), or Suramin (1 mM; 52671; Sigma-Aldrich) were dissolved in the indicated E3 medium. The following reagents were used for luminescence imaging: firefly luciferase (19506; Sigma-Aldrich) and sodium alamethicin (6882; Sigma-Aldrich), and SYTOX Orange (Life Technologies). Hydrogen peroxide solution (H\(_2\)O\(_2\); 216763; Sigma-Aldrich) was diluted to 1 mM in E3 medium for the barrier reconstruction assay.

**Plasmid construction and in vitro transcription**

To observe cell/tissue dynamics and morphology, the PH domain of human AKT1 (amino acids 1–147) fused with GFP was used (18836; Addgene; Kwon et al., 2007), or the AKT1 PH-domain was fused in-frame with the far-red fluorescent protein mKate2 (Evrogen). The AKT-PH-GFP was subcloned from pcDNA-AKT-PH-GFP (18836; Addgene) into pcCS2 [Sp6 promoter] with EcoRI–XbaI. The AKT-PH cassette was PCR cloned from pcDNA-PHTK-GFP into pcCS2+ with Clal–EcoRI upstream of mKate2. To observe myosin II localization during wound closure, human myosin regulatory light chain 1 [mrc1c] was PCR amplified from pEGFP-nrlc1c (35680; Addgene; Beach et al., 2011) and cloned into the promoterless middle entry vector pME (tol2kit) with Xhol–HindIII, in frame with mKate2. mrc1c–mKate2 was PCR amplified from pME and cloned into pcCS2+ with Xhol–NcoI. pcCS2+GFP-Uroplakin colominin homology domain (GFP-UrhCH) was created as described previously (26737; Addgene; Burk et al., 2007). In brief, UrCH (amino acids 1–296) was cloned into the BspE1–Xhol sites of the pcCS2-EGFP plasmid [sp6 promoter]. Entpd3 M01 insensitive was cloned using the following primers (mutations in lower case): Entpd3 mut_fwd, 5'–AGCCTTAGATGAcAaAgaaGaTcCataCTCAGACACCTCTAC3'; and Entpd3_rev, 5'–GCTTCTAGAACTCCCAATACGAGCACC3' into the HindIII and XbaI sites of pME, then subcloned into Clal–XbaI sites of pcCS2+. In vitro transcription of mRNA from pcCS2+ plasmids was performed using the mMessage mMACHINE Sp6 kit (Life Technologies), following plasmid
Figure 7. ATP reconstitutes basal cell migration and epithelial sheet movement in the absence of a transepithelial osmotic gradient. (a) Representative time-lapse images of zebrafish larvae exhibiting mosaic plasma membrane AKT-PH-mKate2 labeling of predominately basal cells (4–8-cell-stage mRNA injection). Larvae were subjected to UV-laser-cut wounding in isotonic mounting agarose. After 10 min of isotonic preincubation (red time indices), a bolus of isotonic solution ± 5 mM ATP was added to the imaging dish. Yellow x, representative morphological response after addition of isotonic solution ± 5 mM ATP. Note that formation of AKT-PH-mKate2–rich membrane protrusions [yellow broken line] after iso-iso/ATP, but not iso-iso shifting. The same representative iso-iso control and data set were used in Fig. S5 a. See also Video 9. Bars: (main panels) 50 µm; (inset) 10 µm. (b) Global PIV analysis of the indicated number of larvae exhibiting ubiquitous plasma membrane labeling (one-cell stage AKT-PH-mKate2 mRNA yolk injection). Larvae were subjected to UV-laser-cut wounding in isotonic mounting agarose. After 10 min of isotonic incubation, a bolus of isotonic medium ± 5 mM ATP was added to the sample. (c) Proposed circuitry scheme of tissue intrinsic and environmentally triggered branches of the wound response in zebrafish tail fins. Tissue-intrinsic mechanisms include purse-string contraction (not depicted). Environmentally dependent osmotic surveillance through secretion of nucleotides (epithelial cells) and eicosanoids (leukocytes) is depicted.
linearization with either NolI or Asp718. Akt-PH-GFP was subcloned from pcDNA-AKT-PH-GFP [18836; Addgene®] into EcorI-XbaI of pME, and Akt-PH-KmTe2 was subcloned from pCS2+Akt-PH-KmTe2 into Clal-XbaI of pME. GFP-UtrCH was PCR amplified from pCS2+GFP-UtrCH and cloned into pME. To construct plasmids for transient mosaic expression or the generation of stable transgenic zebrafish, the tol2kit system was used (Kwan et al., 2007); pME-AKT-PH-GFP, pME-AKT-PH-mKate2, or pME-GFP-UtrCH were combined with plasmids containing either the actb2 (Higashijima et al., 1997) or keratin4 (kt4; previously kt8; Gong et al., 2002) promoters and an SV40 poly-A sequence into the pDestTol2CG2 backbone (Kwan et al., 2007) using gateway cloning (Invitrogen).

To create Tg(krt4:AKT-PH-GFP), Tg(krt4:AKT-PH-mKate2), or Tg(kRtP63:Gol4:UAS-GFP) transgenic zebrafish, 25 pg of each construct was co-injected with 25 pg transposable mRNA into the cytosol of one-cell stage casper embryos (White et al., 2008). Potential transgenic larvae were selected based on heart-specific expression of EGFP driven by the cardiac myosin light chain (mlc) promoter, or by mosaic GFP expression from the Tg(krt4:mlc-GFP) transgenic background. To observe cells in the suprabasal epidermal layer exclusively, cDNA-encoding AKT-PH-GFP, AKT-PH-mKate2, or GFP-UtrCH was expressed under the control of the krt4 promoter (see "Plasmid construction and in vitro transcription"). Labeling of plasma membranes in the basal cell layer was achieved by mosaic expression of AKT-PH-GFP or AKT-PH-KmTe2. To this end, mRNA (0.5–1 ng) was injected into the yolk of 4–8-cell-stage embryos. 2.5–3-dpf larvae with strong, mosaic expression in the tail fin were crossed to obtain the 2.5–3-dpf transgenic larvae used in the wounded experiments.

**Epidermal layer-specific cell labeling**

One-cell stage yolk injections of mRNA (0.5–1 ng) encoding GFP-UtrCH, MRIC-mKate2, or AKT-PH-KmTe2 were used to visualize F-actin, Myosin II, or plasma membrane (PIP3), respectively, in both epidermal layers (basal and suprabasal). To observe cells in the suprabasal epidermal layer exclusively, cDNA-encoding AKT-PH-GFP, AKT-PH-KmTe2, or GFP-UtrCH was expressed under the control of the krt4 promoter (see "Plasmid construction and in vitro transcription"). Labeling of plasma membranes in the basal cell layer was achieved by mosaic expression of AKT-PH-GFP or AKT-PH-KmTe2. To this end, mRNA (0.5–1 ng) was injected into the yolk of 4–8-cell-stage embryos. 2.5–3-dpf larvae with strong, mosaic expression in the tail fin were selected for experiments. Basal cells are distinguishable from superficial cells by morphological characteristics, including cell shape and the degree of enrichment of membrane markers such as PH-KmTe2 or PH-GFP (Fig. 2). This basal cell labeling strategy was validated by injecting AKT-PH-KmTe2 mRNA into the yolk of 4–8-cell stage Tg(krt4:AKT-PH-GFP) embryos, which were co-injected with 25 pg transposable mRNA into the cytosol of one-cell stage larvae expressing PH-AKT-GFP in the basal cells were z-projected (maximum-intensity projection), convoluted (3 × 3 × 1 matrix, Fiji "Convolve" command), background subtracted, and contrast enhanced in Fiji. Tissue velocities were measured by calculating particle motion between subsequent images of the time lapse using MATLAB (R2010b; MathWorks) with the open source PIV analysis software PIVlab v1.32 (developed by W. Thielicke and S. Stalham). Briefly, in brief, the program plots each frame onto three images captured from different displacement distances of cells was plotted as a function of binned (12 µm), initial wound distance of these cells (Dw/2). Negative values in the binned displacement data are attributed to sample drift. The wound area was assessed in 2.5–3-dpf Tg(krt4:AKT-PH-GFP), or in GFP-UtrCH mRNA-injected larvae, ~5,000 µm² wound punctures were generated by 3–4 rapid successive UV laser pulses, which produced wounds on both sides of the epithelial fold (i.e., two wounds). The area of the wound proximal to the objective lens was measured by using the polygon selection tool in Fiji to outline the wound margin. The Tg(krt4:AKT-PH-GFP) dataset, which was part of the wound area quantification (Fig. 1 b), was used for velocimetry analysis in Fig. 4.

**Velocimetry analysis**

Spinning disc confocal time-lapse stacks of wounded tail fins expressing PH-AKT-GFP or PH-AKT-KmTe2 in the suprabasal epithelial layer (Tg(krt4:AKT-PH-GFP) or Tg(krt4:AKT-PH-KmTe2)) were z-projected (maximum-intensity projection), convoluted (3 × 3 × 1 matrix, Fiji "Convolve" command), background subtracted, and contrast enhanced in Fiji. Tissue velocities were measured by calculating particle motion between subsequent images of the time lapse using MATLAB (R2010b; MathWorks) with the open source PIV analysis software PIVlab v1.32 (developed by W. Thielicke and S. Stalham). Briefly, the program plots each frame onto three images captured from different displacement distances of cells was plotted as a function of binned (12 µm), initial wound distance of these cells (Dw/2). Negative values in the binned displacement data are attributed to sample drift. The wound area was assessed in 2.5–3-dpf Tg(krt4:AKT-PH-GFP), or in GFP-UtrCH mRNA-injected larvae, ~5,000 µm² wound punctures were generated by 3–4 rapid successive UV laser pulses, which produced wounds on both sides of the epithelial fold (i.e., two wounds). The area of the wound proximal to the objective lens was measured by using the polygon selection tool in Fiji to outline the wound margin. The Tg(krt4:AKT-PH-GFP) dataset, which was part of the wound area quantification (Fig. 1 b), was used for velocimetry analysis in Fig. 4.
movements, these time-lapse stacks were registered (“StackReg-> Rigid Body” command; Fiji) before PIVlab analysis.

This normalization enabled averaging of spatiotemporal tissue motion profiles over multiple animals, and comparison between different experimental groups. For statistical comparison, averaged velocity maps of different experimental groups were subtracted as indicated, and the corresponding (i.e., with regards to distance from wound margin, and time after injury) velocity values of different experimental groups were tested for significant differences using an unpaired Student’s t-test (i.e., P < 0.05). All statistical insignificant velocity differences (P > 0.05) in the subtraction map were set to zero to highlight only statistically significant differences. Means of global velocity vector magnitudes for time-lapse frames were extracted from PIVlab, averaged in Excel, and plotted using MATLAB. The differences in velocity ranges depend on the acquired laser sheet and the type of wound (amputation, cut, or puncture) required to deliver selected compound into the tissue. For example, wounding regimes requiring isotonic to hypotonic shifting to deliver cell-impermeable compounds exhibit velocity ranges that differ from wounding regimes that deliver cell-permeable compounds where shifting was not used. Amputation wounds to deliver larger molecules (e.g., apyrase) also have different velocity profiles than cut or puncture wounds.

H2O2 barrier reconstitution assay

To measure epithelial barrier integrity, Tg(actb2:HyPer) larvae were used to visualize the penetration of exogenous H2O2, a molecule with similar physicochemical properties as H2O, through the wound into the cytosol of the cells in the tail fin. The 2.5–3-dpf larvae were subjected to tail fin amputation in standard E3 saline (using a needle knife [Fine Science Tools]), and were embedded in a small volume of 1% isotonic low-melting agarose (~300 µl) in a glass-bottom dish (MatTek Corporation) 1 h later. This waiting period was required for the endogenous, wound-induced H2O2 signal to diminish, allowing the consecutive measurement to assess the effect of exogenously added H2O2. At least a 10x agarose volume equivalent of standard-E3 (hypotonic) or IsoNaCl-E3 (3–4 ml) containing 1 mM H2O2 was added on top of the agarose pad when the acquisition started. Every minute, H2O2 fluorescence was excited using LED light and 438/57 and 475/28 excitation filters (Lumencor). Emission was acquired using a 535/30 emission filter (Chroma Technology Corp.). Images were acquired at room temperature (~26°C) using the NIS-Elements (Nikon) software. To quantify cell lysis in response to hypotonicity, a circular region of interest (100 µm) was drawn around each individual ATP flash through the time-lapse (Fiji), to measure the intensity of luciferase luminescence and the local SYTOX orange fluorescence. The intensity measurements start one frame before, and end 1–2 frames after each luminescence peak (40 s per frame). Color-coded traces correspond to matching measurements of luminescence and fluorescence, respectively.

To test the effect of tonicity on light generation by luciferase, luminescence was measured in 5-µl drops of isotonic (145 mM NaCl) or hypotonic E3 (5 mM NaCl) supplemented with firefly luciferase (0.5 mg/ml), sodium luciferin (150 µM), and MgSO4 (10 mM), and indicated concentrations of ATP (10–100 µM). Luminescence was acquired using the same settings as above, except that a 4x Plan-Apochromat NA 0.2 air objective lens was used. Background of drop images was acquired in a rectangular area outside the drop, and subtracted from the drop image. A rectangular region covering the center of the drop was measured to obtain the mean luminescence using Fiji. Three drops per ATP concentration were measured. Mean luminescence and standard deviation of triplets was plotted as a function ATP concentration, and toxicity (Fig. S3).

Cell sorting and semi-quantitative RT-PCR

Basal cell sorting was performed by disaggregating ~200 Tg(AN-p63: Gal4, UAS-GFP) transgenic 2.5–3-dpf larvae into a single cell suspension as described previously (Bertrand et al., 2007). In brief, larvae were anesthetized using 0.2 mg/ml tricaine (Sigma-Aldrich) in E3, dissociated using Liberase TM (Roche; 13 U/ml, 15 min at 32°C), and disrupted mechanically with a pestle. Cell suspensions were passaged through a 40-µm nylon mesh and washed twice in FACS buffer (centrifugation at 250 g for 5 min).

Cell sorting of GFP-positive cells was performed on a FACS (Aria III; BD) using 488-nm excitation and 530/30-nm emission wavelengths. mRNA was extracted with aligol (di)25 Dyna Beads (Invitrogen), followed by cDNA synthesis with RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and PCR Pfir Hi Hot Start II DNA polymerase (Thermo Fisher Scientific) using the following gene primers (PCR cycle numbers referred to in Fig. S4 are indicated below).

```plaintext
entpd1_Fwd: 5'-ATAGTCCCTGGA-TGGCAGGCTC-3',
entpd1_Rev: 5'-GGGGTTCGCTGTGTCTG-3' (33 PCR cycles).
entpd2a.1_Fwd: 5'-ACATCAA-GGGTCACCCAGGCT-3',
entpd2a.1_Rev: 5'-GATGACCTGACCGGCG-3' (35 PCR cycles).
entpd2a.2_Fwd: 5'-GCACTACGC-3',
entpd2a.2_Rev: 5'-AAGAGCACCCGAGG-3' (33 PCR cycles).
entpd2b_Fwd: 5'-GGACGACAG-TATCCCTCACAAGC-3',
entpd2b_Rev: 5'-GCTGTGTAACCTCCTCTC-TAGGT-3' (41 PCR cycles).
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Luminescence imaging

Zebrafish tail fin cats were performed on anesthetized 2.5–3-dpf larvae in a glass-bottom dish with a drop (~150 µl) of isotonic fish water (IsoNaCl-E3) supplemented with firefly luciferase (~1.7 mg/ml) and co-luciferin (100 µM), MgSO4 (10 mM), and SYTOX Orange (~7 µM) using a needle knife. For larval mounting, 100 µl of warm 1.5% low-melting agarose in IsoNaCl-E3 was mixed into the drop containing the luminescence mix and the wounded larva. After agar solidification, the dish was transferred to an inverted microscope (Eclipse Ti; Nikon) equipped with a 20x Plan-Apochromat NA 0.75 air objective lens, an Andor Clara charge-coupled device (CCD) camera, and a motorized stage. Hyper ratio images (E500/E420) were created by dividing median filtered and background subtracted YFP500 and YFP420 images using Fiji, as described previously (Niethammer et al., 2009; Eyedvi and Niethammer, 2013). Oxidized tissue surface area was measured using Fiji by thresholding for HyPer ratio values over 0.64.

Cellular inhibitors treatment and morpholino injections

Cell-permeable pharmacological inhibitors were applied to 2.5–3-dpf zebrafish by preincubation for 60 min in standard E3 or isotonic E3 naive medium supplemented with the following compounds: Cytoschalin D (100 µM) or 1’-27632 (100 µM). For UV laser wound- ing, larvae were mounted in 1% low-melting-point agarose (Hyo-E3 or IsoNaCl-E3) and overlaid with the respective medium containing the indicated concentration of amylases and inhibitors. For inhibition experiments with POM (100 µM), ATP5-S (5 µM), or Suramin (1 µM), larvae were mounted, laser-wounded in 1% IsoNaCl-E3 agarose supplemented with each compound, and incubated (10 min) with a drop of IsoNaCl-E3 placed at the interface between the dipping objective lens and mounted sample (containing the respective compound). The preincubation period was required for sufficient infusion of the water-soluble compound through the open wound (i.e., kept open with isotonic E3).
A 10× bolus of standard E3 or IsoNaCl E3 medium supplemented with each respective compound was then added to the imaging dish. Apyrase (50 U/ml) inhibition was performed using the identical shifting regimen described for POM, Suramin, or ATP-S, with the exception that tail fin amputation (needle knife; Fine Science Tools) was used to provide a larger opening for apyrase to infuse the tissue (osmolarity readings of the apyrase solutions were identical to the control solutions, ruling out salt contamination in the apyrase stocks). For the PIVlab analysis of ATP-S treatment, ATP-S was not administered in the agarose, but added to the interface drop and the shifting medium to prevent excessive drifting of the sample. Imaging was performed for the indicated time using a laser spinning disk confocal microscope (see "Confocal imaging and laser wounding"). Basal cell migration or suprabasal tissue movement were assessed for 20–30 min after shifting. Hydrophilic compounds were dissolved in DMSO, which was applied to samples at a maximal final concentration of 1%. 2.3 nl of 1 mM (~19 ng) translation-blocking morpholino (5′-GACTGAGCTCTCTATGATCGAC-3′; MO1; Gene Tools, LLC), translation-blocking five-nucleotide mismatch control morpholino (5′-GAAGTOGACACTATATACATGAC-3′; MO2; Gene Tools), targeting the exon3-intron3 of zebrafish entpd3, were injected into the yolk of one-cell-stage embryos. For UV laser wounding, larvae were mounting in 1% low-melting-point agarose (HypoE3 or IsoNaCl-E3) and overlaid with the indicated medium, UV-cut-wounded, and imaged for the indicated time using a laser spinning disk confocal microscope (see "Confocal imaging and laser wounding"). To confirm knockdown efficiency of MO2, mRNA from 2.5–3 df-old morphant larvae (n = ~20) were extracted using oligo (dT)25 Dyna Beads (Invitrogen). cDNA synthesis was performed with the ReverAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and PCR Phire Hot Start II DNA polymerase (Thermo Fisher Scientific), using the following primers: 5′-ATGACATAAAGGAGTCCGATG-3′ and 5′-CTTTTGCGTCTTACCCAATC-3′ targeting the exon3-intron3 of zebrafish entpd3 mRNA. Video 8 demonstrates enhanced basal cell migration in the presence of an ENTPDases inhibitor (POM 7) and the pan eNTPase inhibitor ATP-S. Video 9 shows that ATP, but not stabilized ATP or ATP metabolites, can reconstitute basal cell migration in isotonic medium. Video 10 shows that UTP, but not stabilized UTP or UTP metabolites, can reconstitute basal cell migration in isotonic medium. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201408049/DC1.

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References


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Figure S1. Labeling distinct epidermal cell populations reveals that basal epidermal cells migrate beneath the suprabasal cell layer in response to decreased osmotic pressure. (a) Schematic representation of the larval zebrafish tail fin epidermis at 3 dpf, highlighting the epithelial labeling methodology performed. (A, A) One-cell-stage embryonic mRNA injection (yolk sac) permits labeling of both epidermal layers. (A, B) Stable transgenic or transient expression driven by the krt4 promoter results in fluorescent marker expression in the suprabasal (surface) layer exclusively. (A, C) mRNA injection into yolk of 4–8-cell embryos, or DNA plasmid injection into one-cell-stage embryos, followed by epifluorescence selection, labels predominantly basal epidermal cells in a mosaic pattern. (b and c) Time-lapse images of representative 2.5–3-dpf zebrafish larvae at the indicated times after UV laser–induced injury. (b) Mosaically labeled AKT-PH-mKate2 larvae puncture wounded in either hypotonic (hypo) or isotonic (IsoNaCl or IsoSucrose) E3 medium (white broken line, wound margin). (b, left) Wounding in hypotonic E3 medium revealed propagated lamellipodia formation (yellow broken lines) and translocation toward the wound. (b, center) Wounding in IsoNaCl failed to induce noticeable protrusions. (b, right) Wounding in IsoSucrose exhibited small or sporadic lamellipodia in basal cells usually closer to the wound (yellow dotted lines), whereas lamellipodia formation in more distal regions was inhibited. Yellow x, representative morphological features of cells at comparable distances from the wound margin in the hypotonic and isotonic treatments. (c) Cut-wounded larva injected with krt4:GFP-UtrCH DNA for transient expression in the suprabasal epidermal layer. Actin becomes enriched on cell sides facing the wound (white broken line). At the same time, cells form a smooth wound margin (compare yellow x in 0’ and 2’ and magnifications [right]). Note that some margin cells present at time 0’ (red x’s) are extruded from the tissue by 2’ Bars: (b, main panels) 50 µm; (b, insets) 10 µm; (c, two left panels) 50 µm; (c, enlarged panels on the right) 25 µm.
Figure S2. Basal epithelial cell migration is the primary driving force behind rapid wound closure. (a and b) Representative time-lapse images of 2.5–3 dpf Tg(krt4:AKT-PH-GFP) transgenic zebrafish larvae labeled exclusively in the suprabasal layer. Larvae were mounted in 1% low-melting-point hypotonic or isotonic agarose and UV laser wounded to produce round puncture wounds. (a) Larvae wounded in ±100 µM cytochalasin D, an inhibitor of actin dynamics. Note the complete inhibition of wound closure after 10 min compared with the control. Hypo controls in a and b were from the same dataset. (b) Larvae were wounded in either Hypo or Iso E3 medium with or without 100 µM Rho kinase (Y27632) inhibitor. (b, bottom) At 10 min after injury, both isotonic wounds fail to close in the absence of basal cell migration (insets), but wound margin contraction is additionally absent in isotonic samples treated with Y27632 (jagged margin; yellow arrow), in comparison to iso control (rounded wound margin). (b, top) In the presence of hypotonically induced basal cell lamellipodial migration (insets), both hypo Y27632 and hypo control samples exhibit substantial wound closure by 10 min. See also Video S5. All insets capture representative AKT-PH-mKate2 mosaicically labeled basal cells (4–8-cell mRNA injections) from independent samples under identical treatments as the larger images. Inset images were captured at 2.5 min after wounding, and at similar distances from the wound (~100 µm); lamellipodia are delimited with yellow broken lines, and cell bodies are marked with a yellow x. Representative images of at least n = 3 larvae are shown. Some representative panels are from the same dataset used to quantify wound closure in Fig. 1. Bars: (main panels) 50 µm; (insets), 10 µm.
Figure S3. The effect of tonicity on light generation by firefly luciferase. (a) Mean luminescence of hypotonic (5 mM NaCl) or isotonic (145 mM NaCl) E3 solutions at the indicated concentrations of ATP (for details see Materials and methods). On average, luminescence is quenched approximately twofold in isotonic solutions. Error bars indicate standard deviation (n = 3 technical replicates). (b) Isotonic quenching of luminescence cannot account for lack of ATP signal detection during isotonic incubation of wounded fish. Profile plots of all detectable luminescence signals in a typical in vivo luminescence experiment are shown. The intensity measurements were performed on one representative sample from the dataset depicted in Fig. 5. Black lines, profile plots centered on signal maximum (profile thickness = 10 pixels). Blue line, detection limit as defined as five times the standard deviation of baseline noise. Red line, quenching threshold as defined as detection limit multiplied with the quenching factor determined in a. Signals above the quenching threshold should be detectable in our assay regardless of the tonicity of the medium.
Figure S4. **entpd3** is the predominant ecto-NTP diphosphohydrolase (ENTPD/NTPDase) in the zebrafish epidermis, and regulates tissue velocity during wound closure. Epidermal mRNA was isolated by dissociating and FACS sorting 2.5–3-dpf transgenic zebrafish expressing GFP in basal cells via Tg(Np63:Gal4;UAS:GFP) (Materials and method section). (a) mRNA levels were compared between known extracellular NPTDases using semiquantitative RT-PCR, where the strongest detectable mRNA expression in the epidermis was exhibited by **entpd3**. Note the expression of the epidermal-enriched **tp63** transcript. (b) For each primer set, PCR cycle number was adjusted to reveal the relative abundance of each **entpd** in the tp63-positive (p63+) sorted population relative to mRNA levels present in whole larvae (w; Materials and methods section). Note, the separation of panels in b indicates independent PCR experiments. Panels in a and b are representative of n = 2 independent sorting experiments. (c) Representative whole images of 2.3–3-dpf Tg(krt4:AKT-PH-GFP) **entpd3** morphants. Top left, control; top right, splice-blocking morphant MO2 (~19 ng); bottom left, translation-blocking MO1 morphant (~19 ng); bottom right, translation-blocking MO1 five-nucleotide mismatch control morphant MO1 5 mm (~19 ng). Bar, 500 µm. (d) Global PIV analysis of the indicated number of Tg(krt4:AKT-PH-GFP) larvae subjected to UV-laser-cut injury in Hypo or Iso medium, with or without splice-blocking morpholino-mediated knockdown of **entpd3** (entpd3 MO2, ~19 ng). (Inset) RT-PCR of **entpd3** MO2 knockdown; note the downshift associated with aberrant splicing and nucleotide deletion in the **entpd3** coding sequence (see Materials and methods).
Figure S5. **UTP reconstitutes basal cell migration, and suramin fails to inhibit hypotonic wound closure.** (a) Representative time-lapse images of 2.5–3-dpf zebrafish larvae exhibiting mosaic membrane labeling in predominantly the basal layer by injection of mRNA encoding AKT-PH-mKate2 into the yolk of 4–8-cell stage embryos. Larvae were mounted in 1% low-melting-point isotonic agarose. After a 10-min incubation in isotonic E3 medium (red time indices), mounted larvae were covered with a bolus (~10x agarose volume equivalent) of isotonic E3 ± 5 mM UTP. Yellow x, representative morphological response after addition of isotonic solution ± 5 mM UTP. Note the lamellipodial protrusions that developed shortly after UTP addition (yellow broken line). See also **Video 1**. Representative iso-iso control sample and dataset were also used for ATP experiment shown in Fig. 7a. (b) Time-lapse images of 2.5–3-dpf larvae puncture-wounded with a UV laser in Iso medium containing 1% low-melting-point agarose in the presence or absence of 1 mM suramin (nonselective P2 receptor antagonist). After a 10-min incubation to permit suramin infusion into the wound (red time indices), an ~10x bolus of hypotonic E3 ± 1 mM Suramin was added to the sample. (Right) Wound area quantification as a function of time after injury. Error bars indicate SEM of the indicated (n) number of larvae. Bars: (a, main panels, and b) 50 µm; (a, insets) 10 µm.
Video 1. **Isotonicity inhibits rapid wound closure in the larval zebrafish tail fin.** 2.5–3-dpf zebrafish tail fins expressing GFP-Utr-CH were UV-laser-wounded in hypotonic, isotonic (NaCl), or isotonic (sucrose) E3 medium. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Imaging begins ~0.5–1 min after wounding. Frames were taken every 30 s. Video stills are in Fig. 1b. Bar, 50 µm.

Video 2. **Isotonic inhibition of rapid wound closure is reversible.** 2.5–3-dpf zebrafish tail fins expressing AKT-PH-GFP in the suprabasal epidermal layer (krt4 promoter) were UV-laser-wounded in isotonic (NaCl) medium and shifted after 40 min into hypotonic E3 medium. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Imaging begins ~0.5–1 min after wounding. Frames were taken every 30 s. Video stills are in Fig. 1c. Bar, 50 µm.

Video 3. **Hypotonicity triggers rapid barrier recovery of the wounded tail fin epidermis.** 2.5–3-dpf transgenic Tg(actb2:HyPer) larval tail fins amputated in isotonic (NaCl) medium and shifted into hypotonic or isotonic medium supplemented with 1 mM H$_2$O$_2$. Images were acquired by time-lapse epifluorescence microscopy (Eclipse Ti; Nikon). H$_2$O$_2$ shifting and imaging starts 1 h after wounding. Frames were taken every minute. Color-coded HyPer ratios (E$_{500}$/E$_{420}$) are depicted. Red, high [H$_2$O$_2$]. Blue, low [H$_2$O$_2$]. Video stills are in Fig. 1d. Bar, 100 µm.

Video 4. **Distinct wound responses in the bilayered tail fin epidermis.** 2.5–3-dpf UV-laser-wounded zebrafish tail fins displaying individually labeled suprabasal (green, Tg(krt4:AKT-PH-GFP)) and basal (red, AKT-PH-mKate2 mRNA-injected) cell layers wounded in normal hypotonic E3 medium. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Frames were taken every 30 s. Imaging begins ~0.5–1 min after wounding. The video is representative of n = 4. Video stills are in Fig. 2a. Bars, 50 µm.

Video 5. **The effect of Rho-kinase inhibition on wound closure.** 2.5–3-dpf Tg(krt4:AKT-PH-GFP) zebrafish tail fins expressing AKT-PH-GFP in the suprabasal epidermal layer were UV-laser-wounded in hypotonic medium in the presence/absence of Rho kinase inhibitor (Y = Y27632, 100 µM). Frames were taken every 30 s. Imaging begins ~0.5–1 min after wounding. The video is representative of n = 5. Video stills from the same dataset are in Fig. S2b. Bar, 50 µm.

Video 6. **Arachidonic acid does not reconstitute directional cell migration after isotonic injury.** 2.5–3-dpf zebrafish tail fins expressing AKTPH-mKate2 in basal cells (AKTPH-mKate2 mRNA injected), UV-wounded in isotonic (NaCl) medium, and then shifted to isotonic medium ± 10 µM arachidonic acid (AA). Shifting occurs at ~10 min. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Frames were taken every 30 s. Imaging begins ~0.5–1 min after wounding. The video is representative of n = 3 different larvae. Bar, 50 µm.
Video 7. Hypotonic medium triggers ATP release at wounded tail fins. Luciferase/luciferin luminescence imaging of 2.5–3-dpf wt larval tail fins subjected to tip amputation in isotonic (NaCl) medium, and shifted to hypotonic medium after 20 min. Green, ATP-dependent luminescence. Red, staining of damaged cells by SYTOX Orange. Images were acquired by time-lapse epifluorescence microscopy (Eclipse Ti; Nikon). Frames were taken every 40 s. Imaging begins ~3 min after wounding. The video is representative of n = 6 different larvae. Video stills are in Fig. 5 a. Bars, 100 µm.

Video 8. Pharmacological inhibition of ENTPDases and broad-spectrum inhibition of ecto-ATPase enhances basal cell migration. 2.5–3-dpf UV-laser-wounded zebrafish tail fins expressing AKT-PH-mKate2 in basal cells wounded in isotonic (NaCl) medium with 100 µM POM/compound 7 inhibitor, and then shifted to hypotonic medium with 100 µM POM (center), or wounded in isotonic (NaCl) medium with 5 mM ATPyS, and then shifted to hypotonic medium with 5 mM ATPyS (right). Isotonic to hypotonic control is shown (left). Shifting occurs at ~10 min. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Frames were taken every 30 s. Imaging starts ~0.5–1 min after wounding. Rigid body registration was performed (Fiji). Responses are representative for at least n = 3 different larvae. Bar, 50 µm.

Video 9. ATP, but not stabilized analogues or hydrolysis products of ATP, reconstitutes basal epithelial cell migration to the wound in isotonic medium. A composite movie of 2.5–3-dpf zebrafish tail fins expressing AKT-PH-mKate2 in basal cells and UV-laser-wounded in isotonic (NaCl) medium, then shifted to isotonic medium containing 5 mM of indicated compounds. Samples were shifted after ~10 min. (left) Looped isotonic control movie for comparison. (right) Basal cell responses to ATP, Apyrase-treated ATP, ATPyS, Bz-ATP, ADP, ADPβS, AMP, and adenosine. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Frames were taken every 30 s. Imaging begins at ~0.5–1 min after wounding. Video stills are in Fig. 7 a. The isotonic control is identical in Videos 9 and 10. Responses are representative for at least n = 3 different larvae. Bars, 50 µm.

Video 10. UTP, but not stabilized analogues or hydrolysis products of UTP, reconstitutes basal epithelial cell migration to the wound in isotonic medium. A composite movie of 2.5–3-dpf zebrafish tail fins expressing AKT-PH-mKate2 in basal cells and UV laser wounded in isotonic (NaCl) medium, then shifted to isotonic medium containing 5 mM of the indicated compounds. Samples were shifted after ~10 min. (left) Looped isotonic control movie for comparison. (right) Basal cell responses to UTP, UTPyS, UDP, 3-phenacyl UDP, UDP glucose, UMP, and Uridine. Images were acquired by time-lapse spinning disk confocal microscopy (Eclipse FN1; Nikon). Frames were taken every 30 s. Imaging begins at ~0.5–1 min after wounding. Video stills are in Fig. 5 a. The isotonic control is identical in Videos 9 and 10. Responses are representative for at least n = 3 different larvae, except for 3-phenacyl UDP (n = 2). Bars, 50 µm.

Table S1. Pharmacological agonist profile of known P2 receptors

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>P2Y1, P2Y2, P2Y4, P2Y11, P2Y12, P2Y13, P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7</td>
</tr>
<tr>
<td>ADPβ</td>
<td>P2Y1, P2Y6, P2Y12, P2Y13</td>
</tr>
<tr>
<td>AMPβ</td>
<td>A1, A2A, A2B, A3</td>
</tr>
<tr>
<td>UTPβ</td>
<td>P2Y2, P2Y4, P2Y6</td>
</tr>
<tr>
<td>UDPβ</td>
<td>P2Y6, P2Y14</td>
</tr>
<tr>
<td>ATPySβ</td>
<td>P2Y1, P2Y2, P2Y11, P2Y12, P2X1, P2X2, P2X5</td>
</tr>
<tr>
<td>BzATPβ</td>
<td>P2X1, P2X3, P2X7</td>
</tr>
<tr>
<td>ADPβSβ</td>
<td>P2Y1, P2Y11, P2Y12, P2Y13</td>
</tr>
<tr>
<td>UTPySβ</td>
<td>P2Y2, P2Y4</td>
</tr>
<tr>
<td>3-PA-UDPβ</td>
<td>P2Y6</td>
</tr>
<tr>
<td>UDP-GluCβ</td>
<td>P2Y14</td>
</tr>
</tbody>
</table>

Underlining indicates P2 receptors that respond to both ATP and UTP. Main source: IUPHAR/BPS. ATP, Adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; A, adenosine; UTP, uridine 5'-triphosphate; UDP, uridine 5'-diphosphate; ATPyS, adenosine 5'-[β-thio]triphosphate; Bz-ATP, 2'-[3'-O-4-Benzoylbenzoyl]adenosine 5'-triphosphate; ADPβS, adenosine 5'-[β-thio]diphosphate; UDP, uridine 5'-[β-thio]diphosphate; 3-PA-UDP, 3'-[2-0xo-2-phenylethyl]uridine-5'-diphosphate; UDP-glucose, uridine 5'-diphosphoglucose.

*Ligand elicits migration response.

*Ligand elicits no migration response.