Damage-induced reactive oxygen species enable regenerative signalling by the rapid repositioning of Hedgehog expressing cells.

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Abstract

Aquatic vertebrates have been studied for their remarkable ability to regenerate limbs and tails after amputation. Previous studies indicate that reactive oxygen species (ROS) signalling is required to initiate regeneration, but the cellular mechanism by which this takes place is poorly understood. The developmental signalling molecules retinoic acid, Hedgehog, Wnt and FGF, have been shown to have pro-regenerative roles in many systems. However, whether these are playing roles that are specific to regeneration, or are simply recapitulating their developmental functions has remained unclear. To address these questions, we have analysed zebrafish larval tail regeneration. We find evidence that ROS released upon wounding mobilise notochord cells resulting in repositioning of these cells to the damage site. These notochord cells secrete Hedgehog ligands which are required for regeneration. Intriguingly, we find that Hedgehog signalling is not required for normal tail development suggesting that it plays a regeneration specific role. Our results provide a model for how ROS signalling initiates tail regeneration, and indicate that developmental signalling pathways can play regenerative functions that are not directly related to their known developmental roles. This understanding may facilitate development of regenerative therapies that involve the application of signalling molecules to damaged tissues or organs.

Introduction

The study of regenerative biology aims to understand the mechanisms and limitations of endogenous regenerative capacity. During vertebrate appendage regeneration in salamander limb, mouse digit, and *Xenopus* tadpole tail and zebrafish fin, a conserved sequence of events takes place after tissue is removed[1,2]. Immediately following tissue damage the wound is closed by active movement of the surrounding epithelia. Next, the epithelial covering of the wound thickens to form what is called the wound epithelia and cells migrate under the wound epithelia to form a tightly packed mass proliferative cells called the blastema. During the final phase of regeneration the blastemal cells and the wound epithelium proliferate and differentiate to give rise to the missing tissue.

How wounding initiates regeneration is a central question in regenerative biology. Tissue damage results in the immediate release of signals such as calcium, ATP, and reactive oxygen species (ROS) which act to stimulate wound closure and the immune response thus limiting detrimental effects of injury[3]. Of these initial signals, ROS stand out as a good candidate for the activation of regeneration. Upon wounding, calcium release results in a rapid burst of ROS that is likely to be produced by Duox, a NADPH Oxidase[3]. Then ROS, primarily in the relatively stable form of hydrogen peroxide, are thought to diffuse into the neighbouring tissue to act as a paracrine signal (referred to as ROS signalling). ROS exert their effects through the reversible oxidation of cysteine residues in key regulatory proteins[4]. Although precisely how ROS signalling is able to confer specific cellular responses is still poorly understood, studies focused upon the MAPK and Wnt pathways suggest that ROS levels may serve to modulate the activity of diverse signalling pathways[5,6]. These studies indicate that members of the thioredoxin family of redox sensors bind signalling pathway components in a ROS dependent manner.

The importance of ROS signalling to vertebrate regenerative biology has only recently become apparent. Research on zebrafish larvae has shown that ROS are required for axonal regeneration[7] and that ROS acts via a Src Family Kinase (SFK) to promote regeneration of the fin fold[8]. Other zebrafish studies have shown that adult heart and fin require ROS to regrow after wounding[9,10]. ROS have also been shown to act during

Xenopus tadpole regeneration[11]. Thus ROS signalling may act more generally as signal that serves to both coordinate the damage response and to initiate the regeneration program.

Another crucial question is how the regeneration of an organ differs from the initial development of that organ[12, 13]. For example, the mesenchyme of the developing limb bud resembles the blastema of the amputated limb in both morphology and its expression of *msx* genes. Similarly, the apical ectodermal ridge of the limb bud resembles the wound epithelium and both structures express *dlx* genes. This suggests that once these structures have appeared, regeneration follows the previously established developmental program. Developmental signalling pathways such as Wnt/β-Catenin, FGF, Hedgehog, Retinoic Acid (RA), Notch and BMP have been shown to play important roles during regeneration. We must therefore ask whether the regenerative roles of these pathways are unique to regeneration or are simply a recapitulation of earlier developmental roles.

To shed light on these questions, we have analysed how developmental pathways direct zebrafish larval tail regeneration. Here we provide evidence that Hedgehog signalling is a key regulator of tail regeneration, acting upstream of the Wnt/β-Catenin, FGF and RA signalling pathways. This finding is surprising given that Hedgehog signalling does not play a role in tail development. In addition, we propose that the source of the regenerative Hedgehog signal is notochord cells that are rapidly repositioned to the stump immediately following wounding. Our data suggest that this movement is dependent upon release of ROS from the wound site and requires SFK activity and microtubule polymerisation. Together these data suggest a model that ROS signalling initiates tail regeneration by relocating Hedgehog expressing notochord cells to the wound site.

Results:

Regeneration in zebrafish larvae has been studied in two contexts: fin fold excision and tail excision[14, 15, 16]. During fin fold excision, tissue removal is limited to epithelium and fin mesenchymal cells in the caudal region of the tail (Fig. S1a). On the other hand, tail excision involves partial removal of neural tube, notochord, muscle, pigment cells, blood vessels as well as the caudal fin fold (Fig. S1a). Within minutes after tail excision notochord cells move out of the notochord sheath to give rise to a cluster of cells (the "notochord bead)") that sit on the stump of the tail (Supplementary Movie 1). Formation of the notochord bead appears to be caused by contraction of the anterior/posterior body axis resulting in pressure build up in the notochord (Fig. S2). To determine the timing of regeneration after tail excision we examined the expression markers of the different stages during regeneration (Fig. S1b). By 24 hours post excision (hpe), *dlx5a* expression marks the forming wound epithelium, and by 48hpe the blastema is marked by strong expression of *msxc*. A previous study of fin fold regeneration found that the early blastema is marked by the RA synthesis gene *raldh2* and that RA signalling is required for regeneration[17]. Consistent with this, we find that *raldh2* is upregulated in the forming blastema at 24hpe. Increased expression of the muscle differentiation gene *myod* is seen between 48 and 81hpe suggesting that regrowth takes place during this interval. Although these genes are expressed during tail development, they are not detected immediately prior to excision (Fig. S1b). This indicates that tail excision reactivates expression of these genes. If operated fish are raised past larval development, they appear morphologically normal. However, skeletal visualisation reveals that the internal structure of the tail is modified perhaps due to a defect in notochord extension (Fig. S1c).

To begin to understand how developmental signalling coordinates regeneration, we first focused on the FGF pathway to identify ligands and downstream targets that are induced by tissue removal. Similar to previous studies that identified *fgf20a* as a damage-induced

ligand in the fin fold[18], we found that *fgf10a* transcripts are upregulated at 24hpe in cells adjacent to the extruded notochord bead (Fig. 1a). We also found that the target gene *pea3* is induced in the surrounding cells, indicating that these cells are responding to FGF signalling at this time (Fig. 1a). Neither gene is expressed strongly in unoperated tails at this time (Fig. S3a,b). The allele *tbvbo* encodes a null mutation in *fgf10a* that does not have a discernable effect on normal tail development (Fig. S4)[19]. Consistent with a role for FGF signalling in larval tail regeneration we found that *fgf10a*$^{-/-}$ larvae have reduced regenerative capacity (Fig. 1b). To determine whether the wound epithelium and blastema form properly, we tested levels of expression of *dlx5a* and *raldh2* in *fgf10a*$^{-/-}$ larvae and in fish treated with the FGF receptor inhibitor SU5402[20]. Both conditions did not show an observable difference in expression suggesting that FGF signalling does not play a role in the initial patterning of the regenerating tail (Fig. 1c). A previous study found that FGF signalling is required for regeneration specific proliferation in the fin fold[15], and similarly we found that the number of proliferating cells in *fgf10a*$^{-/-}$ larvae is reduced compared to wild-type fish (Fig. 1d). These results suggest that FGF signalling is required for damage-induced proliferation after tail excision.

We next investigated the role of Wnt/β-Catenin signalling during tail regeneration by screening for Wnt genes that are activated after excision. We found that *wnt10a* is upregulated starting at 18hpe in cells neighbouring the notochord bead and the Wnt/β-Catenin downstream target gene *tcf7* is upregulated also in this region (Fig. 2a). Neither gene is expressed strongly in unoperated tails at this time (Fig. S3c,d). To further investigate we used the Wnt/β-Catenin pathway inhibitor IWR-1[21] to turn off signalling following excision. Treatment with IWR-1 resulted in the absence of tail regrowth suggesting that Wnt/β-Catenin signalling is required for regeneration (Fig. 2b). To determine whether *dlx5a* or *raldh2* expression depend upon the Wnt/β-Catenin pathway, we used a heatshock inducible *dickkopf-1b* transgene (*hsp70l:dkk1b-GFP*)[22] to inhibit signalling and a glycogen synthase kinase antagonist (GskXV) to activate signalling[23]. We found that whereas inhibition abolished expression of both genes, pathway activation led to expansion of the expression domains of *dlx5a* and *raldh2* (Fig. 2c). Since both FGF and Wnt/β-Catenin pathways appear to act at the same time (24-48hpe), we wondered whether they might regulate each other's activity. By manipulation of both pathways we found that each pathway has a positive effect on the other pathway's activity (Fig. 2d,e). These data indicate that Wnt/β-Catenin signalling patterns the early regenerating tail and interacts with FGF signalling.

The third developmental pathway that we have investigated is the Hedgehog signalling pathway. We first tested whether the Hedgehog pathway influences regeneration using the inhibitor cyclopamine[24] (Fig. 3a,b). Cyclopamine treatment following tail excision resulted in loss of regeneration and loss of *dlx5a* and *msxc* expression. Consistent with an early role for Hedgehog signalling we found that treatment from -12hpe to 12hpe is sufficient to block regeneration. To control for off-target effects of the cyclopamine treatment, we used another Hedgehog pathway inhibitor, LDE225[25], and obtained similar results (Fig. 3a,b). To determine how Hedgehog signalling controls regeneration, we tested for expression of Wnt, FGF and RA genes after cyclopamine treatment and found that their expression is abolished (Fig. 3c). Consistent with Hedgehog signalling acting upstream of FGF signalling we found that cyclopamine treatment strongly reduced proliferation after tail excision (Fig. 3d). Given the early timing of the Hedgehog signalling requirement, it is possible that this pathway acts upstream of the other developmental pathways. To test this, we performed chemical epistasis by treating regenerating fish with cyclopamine to block the Hedgehog pathway while activating the Wnt/β-Catenin pathway with GskXV. We found that GskXV treatment is sufficient to restore expression of the regeneration markers *dlx5a*, *msxc* and *raldh2* indicating that the Hedgehog and Wnt/β-Catenin pathways form a linear pathway (Fig. 3e). Together these

data present a model that the Hedgehog pathway plays a key role during regeneration by activating the Wnt/β-Catenin, FGF and RA pathways.

To determine the source and timing of Hedgehog signalling, we looked at expression of Hedgehog ligands as well as the downstream target *patched1* (*ptch1*). We found that two Hedgehog ligands (*sonic hedgehog a*, *shha* and *indian hedgehog b*, *ihhb*) are strongly expressed in the notochord bead (Fig. 4a and Fig. S5a). Although this expression appears to be limited to the cells in the bead, this restricted detection is an artefact of the wholemount in situ hybridisation method: When fixed larvae are cut along the coronal axis or obliquely to reveal the notochord prior to hybridisation, then expression of *ihhb* is detected in the notochord before and after excision (Fig. 4b). This artefact is likely to be because notochord sheath cells deposit a dense extracellular matrix that may restrict penetration of components during in situ hybridisation. Consistent with this model, we find that there is a low level of expression of *ptch1* around the caudal tip of the notochord prior to excision (Fig. S3f), and high level expression after the notochord bead has formed (Fig. S5a). To further test whether Hedgehog signalling acts upstream of other developmental pathways, we looked to see whether manipulation of any of these pathways affects the wound-induced expression of *ptch1* and found that none of the treatments altered its expression (Fig. S5b). Together these data suggest the model that formation of the notochord bead provides for a novel source of Hedgehog signalling that acts upstream of other developmental pathways.

In the fin fold excision model of regeneration, the notochord is not injured suggesting that the initiation of regeneration may take place by a mechanism that does not rely upon the notochord bead and Hedgehog signalling. To test this we treated fish with cyclopamine after fin fold excision. We found a mild effect in that the fin fold regenerates at a slower rate when Hedgehog signalling is blocked (Fig. S6a). Consistent with this we did not detect upregulation of *ihhb* or *ptch1* after fin fold excision (Fig. S6b). Although these results suggest that the Hedgehog pathway may act during fin fold regeneration, they indicate that it is unlikely to play the same crucial role that it does during tail regeneration.

Given that ROS signalling has been proposed to activate regeneration in different models, it is possible that ROS initiate larval tail regeneration upstream of Hedgehog signalling. High levels of ROS are synthesized in cells along the edge of the stump starting immediately after excision (Fig. S7a). To begin to test the role of ROS we blocked their generation with the flavoenzyme inhibitor diphenylene iodonium (DPI) which inhibits NADPH oxidases[3]. Treatment with 150μM DPI from 1 hour prior to excision until 1hpe is sufficient to reduce regeneration by 50% (Fig. S7b,c) and strongly reduces wound-induced ROS levels (Fig. S7a,d). DPI may affect other signalling pathways besides ROS that involve flavoenzymes or indeed may have unrelated off-target effects[26]. To control for these effects of the DPI treatment, we utilised the ROS scavenger MCI186[11] which also results in reduced ROS levels and regenerated tail length (Fig. S7d,e). The effect of MCI 186 is milder that that of DPI but increasing levels of MCI 186 results in toxicity, so we kept the levels of MCI 186 below this threshold. Consistent with ROS signalling activating regeneration upstream of the Hedgehog pathway, we found that wound-induced expression of *ptch1*, *raldh2*, *tcf7* and *pea3* is reduced following DPI treatment (Fig. S7f).

We next sought to determine whether ROS signalling potentially regulates regeneration by promoting the formation of the notochord bead. When we measured the size of the notochord bead at 4hpe, we found that DPI reduced bead formation, but that MCI treatment did not (Fig. 5a). As SFKs have been proposed to act downstream of ROS signalling during fin fold regeneration, we tested whether the SFK inhibitor PP2[27] influences bead size. We also used nocodazole which interferes with microtubule

214 polymerisation in an attempt to block bead formation by an independent mechanism. We
215 found that both compounds had a strong effect on bead formation (Fig. 5a). To assess
216 how these inhibitors affect wound-induced activation of Hedgehog pathway, we analysed
217 levels of *ptch1* and *ihhb* expression. We found that these compounds reduce the amount
218 of *ihhb* transcripts in the notochord bead (Fig. 5b,c) and reduce *ptch1* to levels similar to
219 those seen with DPI treatment (Fig. S8). Although nocodozole treatment would also be
220 predicted to affect ciliogenesis and thus Hedgehog signal transduction, the observation
221 that treatment blocks bead formation and Hedgehog ligand expression suggest that
222 nocodozole exerts its effect upstream of ciliogenesis. Intriguingly, DPI treatment can
223 result in the loss of *ihhb* expression even when the bead is fully extruded suggesting that
224 a burst of ROS production may be required for both notochord extrusion and expression
225 of *ihhb* in the notochord bead (Fig. S9). Together these data suggest that ROS/SFK
226 dependent bead formation is a necessary step in the regeneration of the tail.
227
228 To further control for potential unintended effects of chemical treatments we decided to
229 test whether we could rescue DPI and PP2 treatment by activating Wnt/β-Catenin
230 signalling as we have done for cyclopamine treatments (Fig. 3e). We treated fish at the
231 time of excision with either DPI or PP2 to inhibit bead formation, allowed the fish to
232 recover and then the next day activated WNT/β-Catenin signalling with GskXV. Rescue
233 was quantified by measuring the area of *raldh2* expression after RNA in situ analysis.
234 Both DPI and PP2 treated fish show a significant level of recovery of *raldh2* expression
235 after treatment with GskXV (Fig. S10). This experiment suggests that the effects of DPI
236 and PP2 are not simply due to toxic off-target effects and support the hypothesis that
237 ROS and SFKs act upstream of Wnt/β-Catenin signalling. In addition we attempted to
238 rescue formation of the notochord bead by supplementing the fish water with H_2O_2 during
239 DPI treatment (data not shown). These tests were unsuccessful perhaps because the
240 timing, concentration or distribution of endogenous ROS is difficult to mimic with
241 exogenously provided H_2O_2. Given these limitations, the DPI treatments should be
242 interpreted with caution and further analysis of ROS signalling is needed to confirm its
243 role in regeneration.
244
245 Having found that DPI and PP2 both strongly reduce notochord bead extrusion, we
246 decided test whether these compounds affect early cell shape changes seen directly after
247 tail excision (Supplementary Movie 1). Within the first few minutes after excision there
248 is a change in the curvature of the cell membranes which initially bow towards the
249 anterior, then change to bow towards the posterior (Fig. S2). This change suggests that
250 these cells are passively being forced towards the open end of the notochord perhaps due
251 to increased pressure within the notochord. To quantify this change we chose to measure
252 the Menger curvature which is the inverse of the radius of a circle that approximates the
253 curved arc of the cell membrane. A cell membrane that runs perpendicular to the
254 notochord sheath will result in a Menger curvature of 0, one bowed to the posterior
255 results in a positive value and one bowed to the anterior a negative value. We measured
256 changes in notochord cell curvature during the first 20 minutes after excision and found
257 that while control fish show a dramatic change in curvature, those treated with DPI or
258 PP2 resemble uncut fish (Fig. S11).
259
260 A second early morphological change after tail excision is contraction of the trunk along
261 the anterior/posterior axis (Supplementary Movie 1). Contraction along this axis could
262 result in increased pressure within the notochord that may result in expulsion of cells
263 from the open end of notochord. We measured trunk length of individual animals before
264 excision and then measured the same animal again at 2hpe (Fig. S12). We found that
265 whereas untreated fish contract by an average of 4.4%, DPI and PP2 only contract by
266 1.7% and 1.2% respectively. Taken together these data suggest that ROS/SFK signalling
267 is required for early cell movements immediately following tail excision.

Given the complex signalling interactions that take place during regeneration, we wondered whether these interactions are also required during normal tail development. Although interactions between the FGF, Wnt/β-Catenin and RA pathways are crucial for tail formation in chick, mouse and zebrafish[28, 29, 30, 31], roles for ROS and Hedgehog signalling have not been described. To test whether ROS act during tail development, we treated larvae with DPI during axis elongation and found that morphologically tails are unaffected by this treatment (Fig. 6a). Likewise, expression of Hedgehog ligands is not affected by DPI treatment during tail development (Fig. 6b). To functionally test whether Hedgehog signalling acts on the tail bud, we treated fish with cyclopamine and found that blocking Hedgehog signalling does not affect *raldh2* or *tcf7* levels (Fig. 6c). Consistent with this, we found that cyclopamine treated fish have relatively normal tail development except for the formation of U-shaped somites due to a known function of Hedgehog signalling in somite patterning (Fig. 6d)[24]. Furthermore, fish carrying null mutations in the Hedgehog receptor gene *smoothened* (*smo*^{b577})[32] form a tail, but lack Hedgehog signalling (Fig 6d). *smoothened* is absolutely required for Hedgehog signalling as it has no paralogs and there is no evidence for its redundancy with other genes. The possibility that low levels of maternal Smoothened activity may play a role in tail development is very unlikely as maternal-zygotic *smoothened* mutants form a tail[33]. These data indicate that proposed regulatory interactions mediated by ROS and Hedgehog do not act during normal tail development and have specific functions during tail regeneration.

Discussion

In the course of this study, we have uncovered a mechanism by which damage-induced ROS signalling may initiate regeneration (Fig. 7). Our model does not exclude the involvement of other signals such as TGFβ, EGF (Epidermal Growth Factor), hypoxia and non-canonical WNT signalling which are also likely to play roles in larval tail regeneration[14, 22, 34, 35]. Rather this model is intended to provide a framework for future analysis of larval tail regeneration in fish. Surprisingly our data suggest that ROS influence regeneration by causing the rapid repositioning of Hedgehog-expressing notochord cells to the site of the wound. Prior to injury, notochord cells express Hedgehog ligands but this expression has little or no direct effect on cells neighbouring the notochord as judged by expression of *ptch1*. Once the sheath is breached and notochord cells are extruded to form a bead, high levels of Hedgehog ligands signal to the surrounding tissue. Another surprising result is that although Hedgehog signalling is required for tail regeneration, it is dispensable for tail development. Thus the Hedgehog pathway plays a regeneration-specific role in this context and acts as a relay between the immediate damage response and the expression of signalling pathways known to coordinate both development and redevelopment of the tail.

The important role played by the notochord extrusion and Hedgehog pathway activation in zebrafish larvae may be conserved during tail regeneration in other aquatic vertebrates such as frogs and salamanders. One study has shown that in *Xenopus* tadpoles a notochord bead forms at the stump within the first few hours of tail excision, and that these extruded notochord cells express Hedgehog ligands from 24-48hpe[36]. This study went on to show that tadpoles treated with cyclopamine immediately following tail excision have reduced regeneration. Another study has shown that ROS signalling is required for induction of FGF and Wnt/β-Catenin signalling at 36hpe[11]. Based upon this timing, it is possible that Hedgehog signalling from the notochord bead also acts to link ROS signalling to redevelopment of the tadpole tail. The picture from axolotl is intriguing, as although Hedgehog signalling is important for axolotl tail regeneration, the expression of *Sonic hedgehog* is restricted to the floor plate of the neural tube[37]. It would be fascinating if the Hedgehog pathway has maintained its role as a key regulator of tail

regeneration in axolotl despite its expression being limited to the floor plate. In addition to its role in tail regeneration, Hedgehog ligands *shha*, *ihhb* and *desert hedgehog* have been shown to be upregulated during heart regeneration where they direct epicardial regeneration[38] and Hedgehog signalling activates Wnt/β-Catenin genes during newt limb regeneration[39]. As these tissues also require ROS production to regenerate, it will be interesting to test whether the Hedgehog pathway acts to link ROS signalling to redevelopment in these contexts.

In our model of tail regeneration, we have placed the FGF, Wnt/β-Catenin and RA pathways in a in a "redevelopment" module because broadly speaking these pathways interact in a similar ways during development and regeneration (Figure 7). This makes sense because it is unlikely that organisms would evolve entirely novel mechanisms to regrow tissue, when a pre-existing developmental module would suffice. However, this may be an oversimplification as there are some fundamental differences between the origins and movement of precursor cells during development and redevelopment. Our preliminary comparison of cross talk between these pathways also suggests that developmental interactions are present but in an altered form during redevelopment (data not shown).

In contrast, Hedgehog signalling plays a novel role during tail regeneration, raising the question of how Hedgehog signalling has evolved to act upstream of the redevelopment module. If in nature larvae are often losing the end of their tails, then it is reasonable to suggest that Hedgehog regulation of the redevelopment module has evolved due to selective pressure. Alternatively, Hedgehog signalling may interact with FGF, Wnt/β-Catenin and RA pathways during another developmental process that is unrelated to tail regeneration. In this case its regulatory role could have evolved and be maintained by selection for this unrelated developmental process. This second model does not require selective pressure to evolve Hedgehog's regenerative role, and rather suggests that an existing developmental signalling network has been co-opted to serve during regeneration.

A related question is how ROS/SFK signalling has evolved to regulate notochord extrusion. Here we propose that ROS cause contraction along the anterior/posterior axis which results a build-up of pressure within the trunk of the fish. Given that the notochord sheath forms a tube-like structure, compaction along the anterior/posterior axis could build up pressure and force notochord cells rapidly out of the open end. The driving force for contraction may be the mass movement of epithelial cells towards the stump that has been described after fin fold excision[40]. The molecular mechanism for notochord bead extrusion is likely to involve ROS signalling through SFKs as well as microtubule polymerisation. Further evidence for this mechanism comes from a range of models. Studies in *Xenopus* have shown that microtubule polymerisation is required for wound closure[41] and ROS have been shown to play a role in wound closure in *C.elegans*[42] and more recently in zebrafish[43]. *Yoo et al* have shown that the SFK Lyn acts as a receptor for ROS signalling[27] and several studies have suggested the model that SFKs directly phosphorylate microtubules to promote their polymerisation and/or stabilisation[44, 45, 46]. Thus, it is possible that the notochord bead results from ROS-dependent morphological changes that pressurise the notochord forcing cells out of the open end. Once formed, the notochord bead then acts as a novel source of Hedgehog signalling to promote tail redevelopment.

Figures

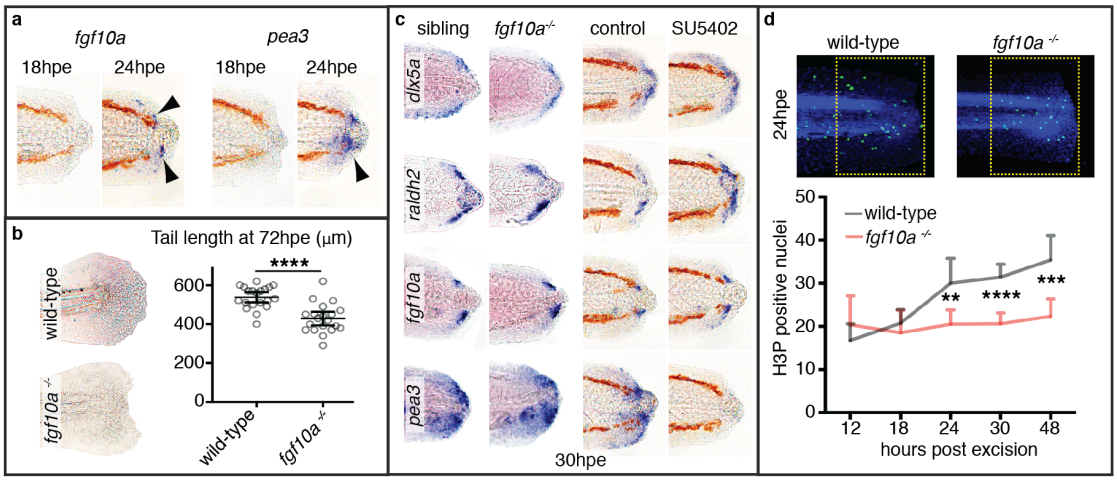

Figure 1: FGF signalling is required for cell proliferation during larval tail regeneration. a, Arrowheads point to expression of the FGF ligand, *fgf10a*, and the downstream target, *pea3*, which become evident by 24hpe. **b,** *fgf10a*$^{-/-}$ fish have reduced tail regrowth compared to wild-type fish. **c,** Expression of *dlx5a* and *raldh2* are not strongly affected when FGF signalling is reduced. SU5402 treatment was done from 26 hpe to 30hpe at 10µM concentration. This treatment is sufficient to abolish detection of the FGF downstream target *pea3*. **d,** Whereas in wild-type fish DNA mitosis increases after tail excision, in *fgf10a*$^{-/-}$ fish mitosis remains constant. Fish were stained with Phospho-Histone H3 (ser10) antibody in green.

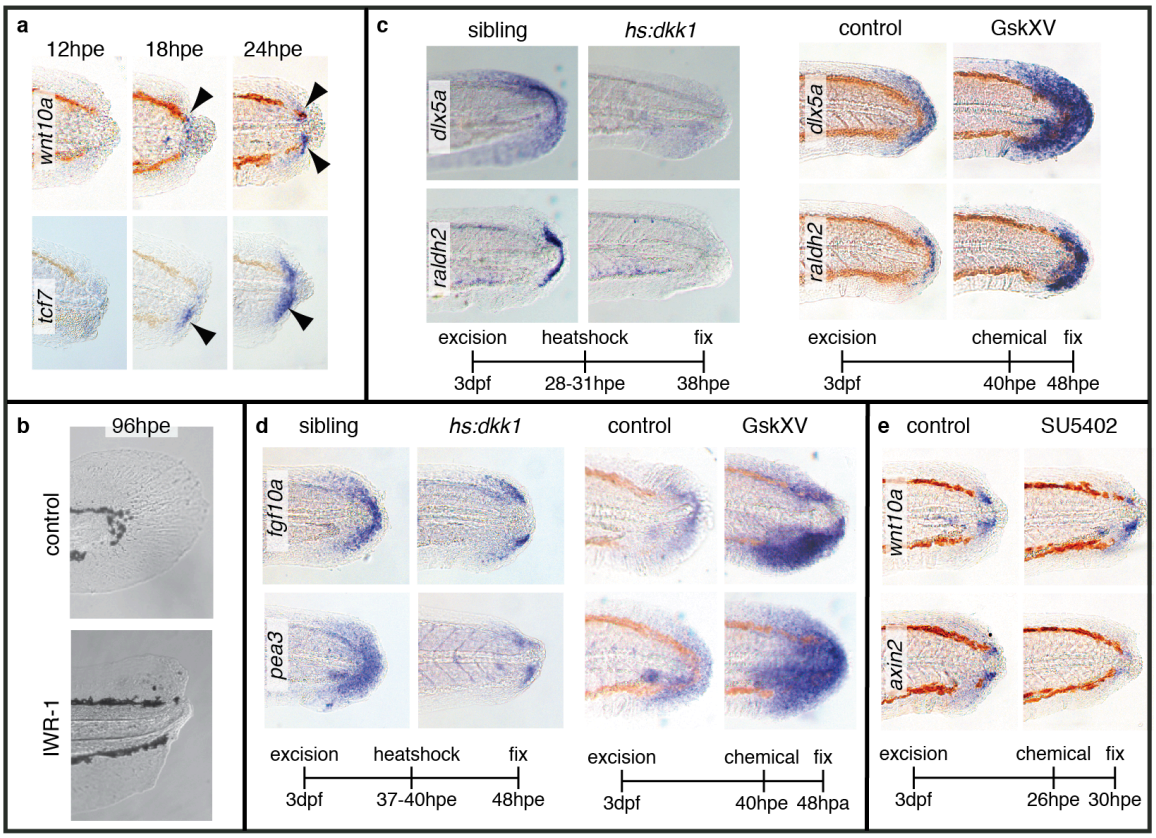

Figure 2: Wnt/β-Catenin signalling is required for patterning during larval regeneration.
a, Expression of the Wnt ligand, *wnt10a*, and the downstream target, *tcf7*, are upregulated by 18hpe (arrowheads). **b,** Larvae treated with 10μM IWR-1 continuously after excision show no signs of regeneration at 96hpe. **c,** Expression domains of *dlx5a* and *raldh2* are abolished when Wnt/β-Catenin signalling is inhibited and increased when Wnt/β-Catenin signalling is up-regulated. **d,** Expression of *fgf10a* and *pea3* are down-regulated in *hs:dkk1* larvae and up-regulated in fish treated with GskXV. **d,** Expression of the Wnt/β-Catenin target gene *axin2* is reduced in fish treated with SU5402.

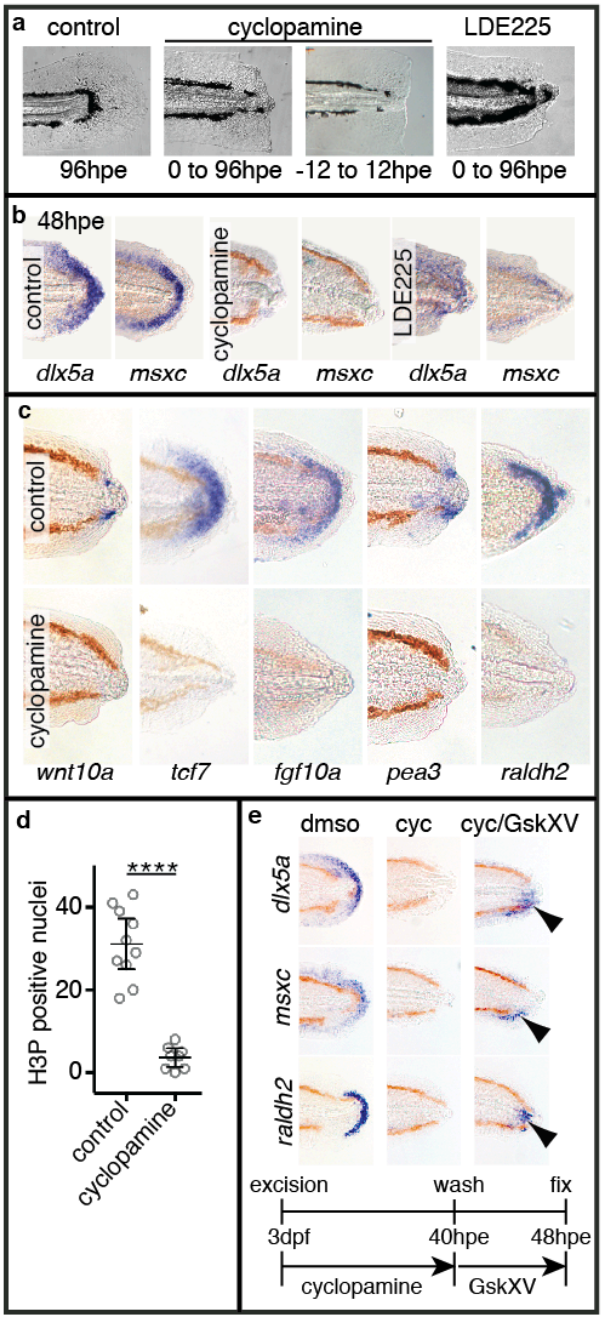

Figure 3: Hedgehog signalling plays a central role during larval tail regeneration.
a, Continuous inhibition with the Hedgehog pathway inhibitors cyclopamine and LDE225 blocks regeneration. Also, pulse treatment around the time of tail excision blocks regeneration at 96hpe. **b,** Larvae treated with cyclopamine and LDE225 from 0-48hpe have reduced expression of *dlx5a* and *msxc*. **c,** Wound-induced expression of markers for the Wnt/β-Catenin, FGF and RA pathways is lost after treatment with cyclopamine. Larvae in the *wnt10a* and *pea3* panels were treated with cyclopamine from 0-30hpe. *tcf7, fgf10a* and *raldh2* panels were treated with cyclopamine from 0-48hpe. **d,** Cell proliferation is inhibited by treatment with cyclopamine. This analysis was done as in Figure 1d. **e,** Upregulation of Wnt/β-Catenin signalling after cyclopamine treatment restores expression of *dlx5a*, *msxc* and *raldh2*. Arrowheads indicate partial rescue of marker expression.

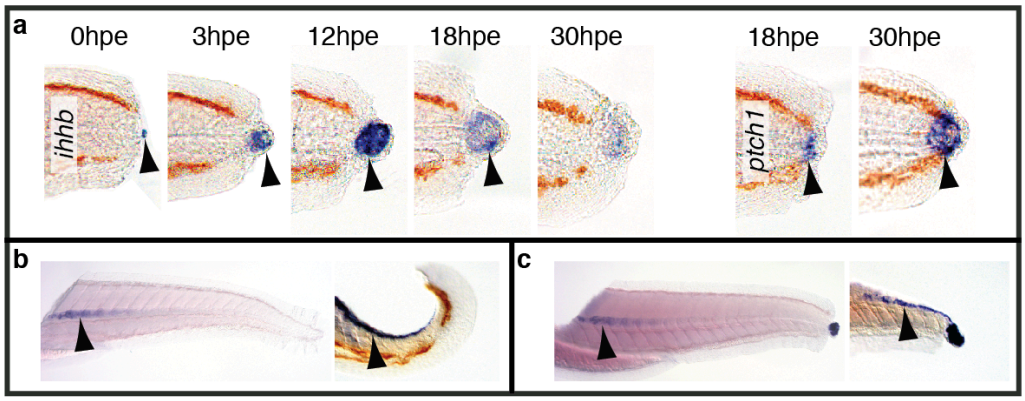

Figure 4: The Hedgehog pathway is activated immediately after tail excision. **a,** *ihhb* expression is detected within the notochord bead and continues until 30hpe. Expression of *ptch1* is upregulated by 18hpe and continues after 30hpe. Arrowheads point to expression domains. Oblique and coronal sections reveal that *ihhb* is expressed in the notochord before (**b**) and after (**c**) tail excision. Arrowheads point to expression in the notochord.

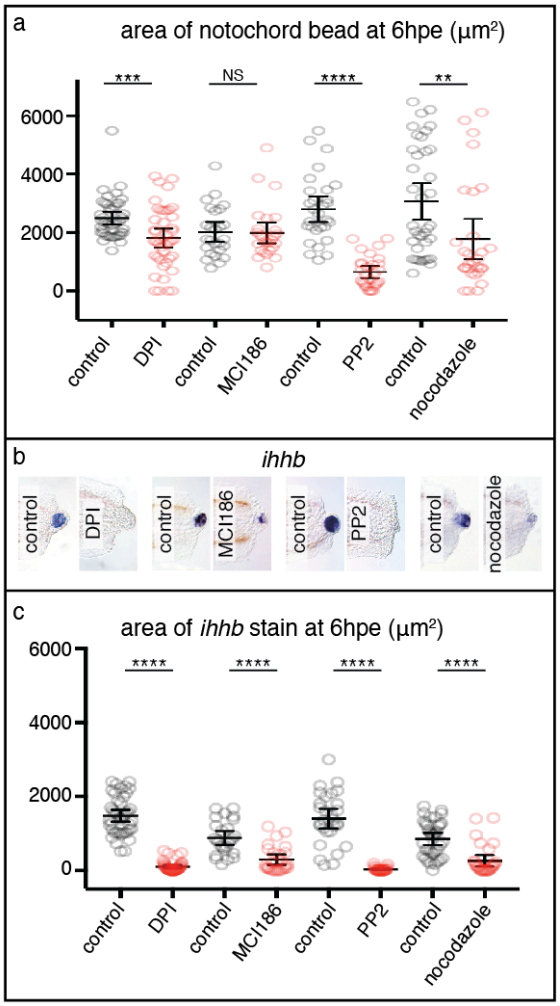

Figure 5: Comparison of the effects of DPI, MCI 186, PP2 and nocodazole on notochord bead extrusion and *ihhb* expression.
a, Bead extrusion is affected strongly by PP2 treatment and not by MCI 186 treatment. DPI and nocodozole have intermediate effects on extrusion. **b,** *ihhb* expression after different treatments. **c,** Quantification of *ihhb* stain from panel b shows that all treatments reduced the level of expression in the notochord bead. DPI treatment started at 1 hour prior to wounding and ended at 1hpe, after which larvae were washed in E3 buffer and incubated until 6hpe. MCI186 treatment started at 0hpe and ended at 3hpe. PP2 treatment started at 1 hour prior to wounding and ended at 6hpe. Nocodazole treatment started at 0hpe until 6hpe.

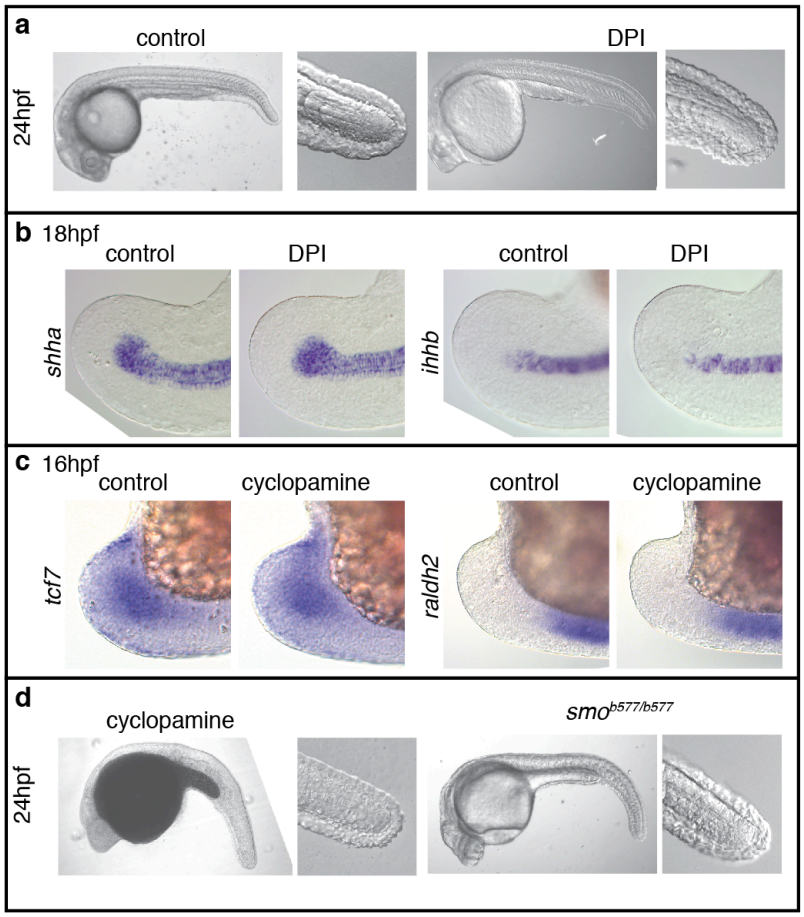

Figure 6: ROS and Hedgehog signalling do not have similar roles during tail development.
a, DPI treatment from 14-24hpf does not affect larval tail morphology. **b,** *shha* and *ihhb* expression in the tail is not affected by DPI treatment at from 14-18hpf. **c,** *tcf7* and *raldh2* expression in the tail bud is not affected by treatment with cyclopamine from 8-16hpf. **d,** Loss of Hedgehog signalling does not affect tail formation. Larvae were treated with cyclopamine from 8-10hpf. Homozygous *smo*^{b577} mutants lack zygotic Hedgehog signalling activity.

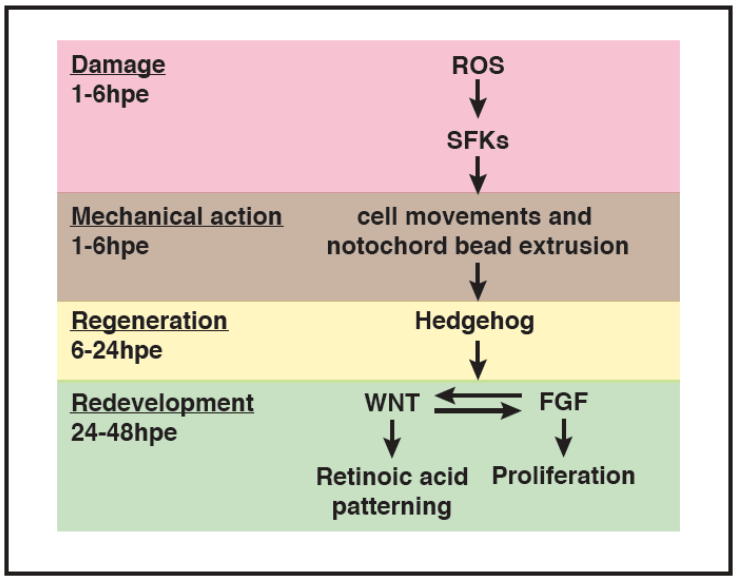

Figure 7: A model for tail regeneration.

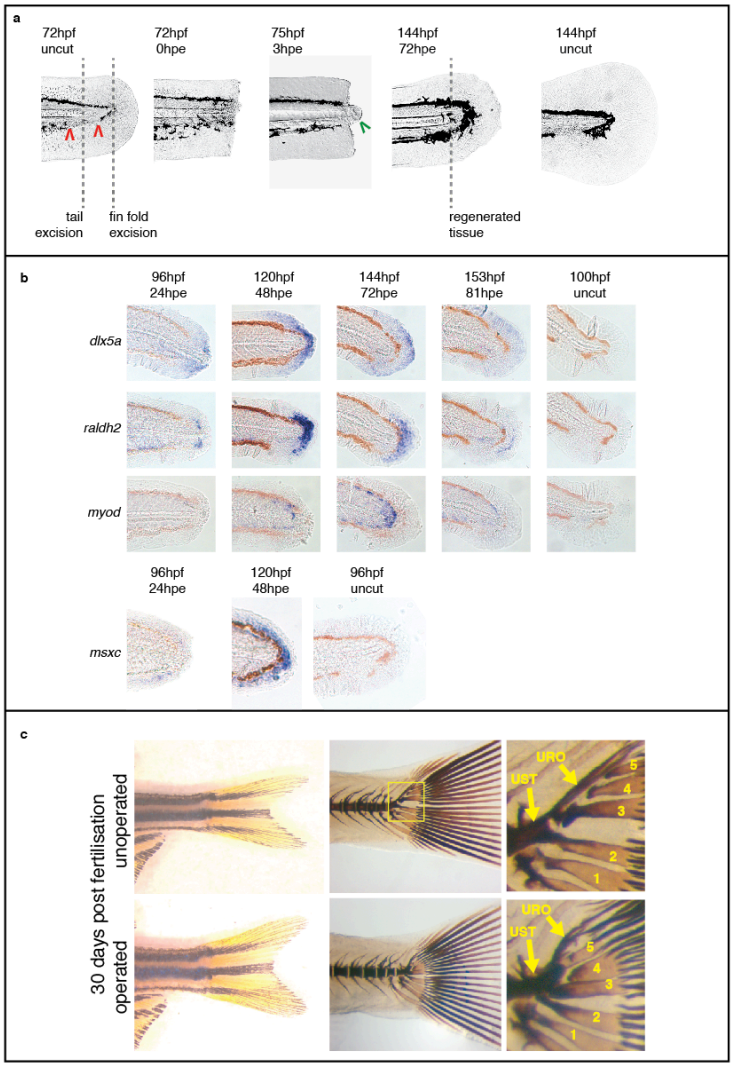

Figure S1: Larval tail regeneration. a, When cut in the pigment gap (zone between the two red arrows), the notochord bead (the green arrow) is extruded within 3hpe and the tail regenerates within 4-5 days. The position of fin fold excision is shown for comparison. **b,** Different stages of regeneration are marked by the expression of *dlx5a*, *raldh2*, *myod* and *msxc*. Uncut fish are shown to the right for comparison. **c,** Live images of the tail after metamorphosis is complete (left panels) and Von Kossa stain showing the failure of the notochord to extend dorsally (right panels). In unoperated fish the uroneural and hypurals 3-5 form around and are attached to the notochord which grows dorsally as the adult tail forms. After excision these bones all form on the urostyle suggesting that the dorsal flexure of the notochord has not occurred. Abbreviations: urostyle (ust), uroneural (uro) and hypural 1-5 (1-5).

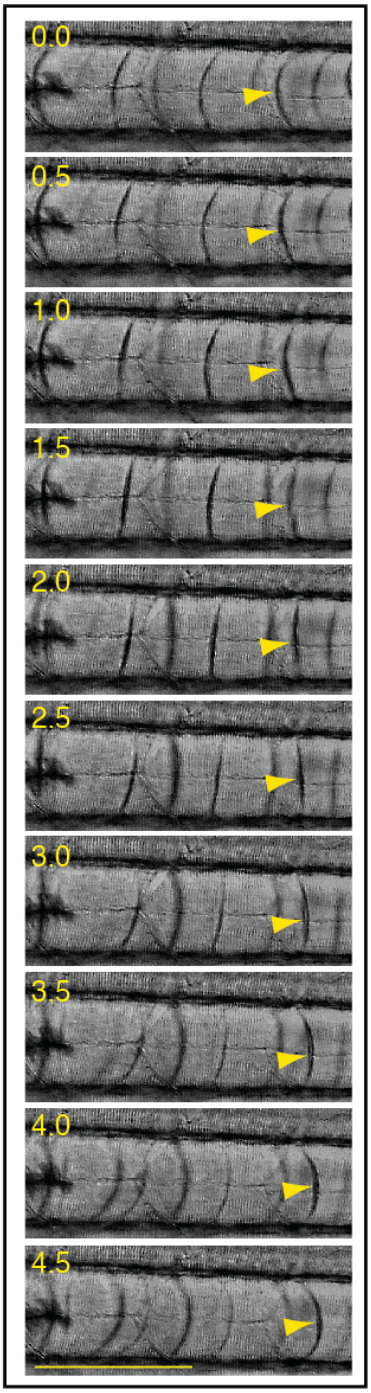

Figure S2: There appears to be a change in pressure within the notochord at the start of extrusion. Close-up of notochord after tail excision to show cell membranes are bowed towards anterior at the start and then become bowed towards the posterior. Arrowhead marks a cell membrane. Images captured every 30 seconds as in Supplemental Movie 1. Scale bar = 100μm.

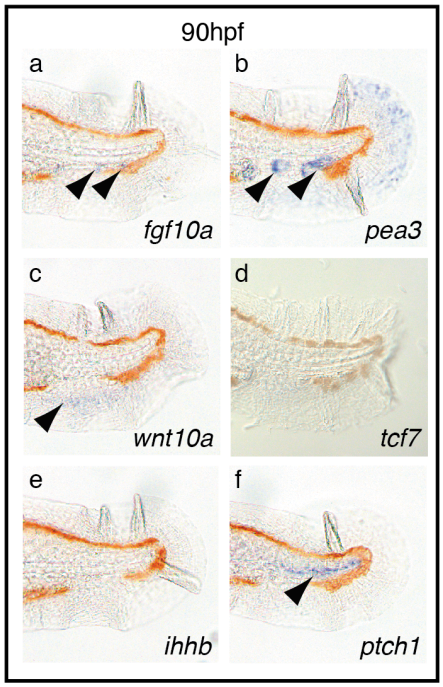

Figure S3: Expression of signalling genes in the larval tail.
a, *fgf10a* is expressed weakly in neuromasts (arrowheads). **b,** *pea3* is expressed in neuromasts (arrowheads) and has weak expression in the fin fold. **c,** *wnt10a* has weak expression in the ventral fin fold (arrowhead). **d,** *tcf7* has no detectable expression. **e,** *ihhb* has no detectable expression. **f,** *ptch1* has weak expression in tissue neighbouring the notochord (arrowhead).

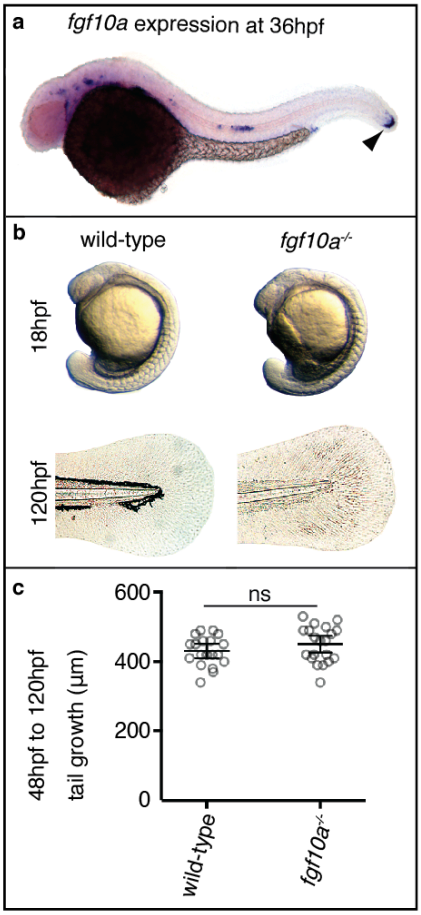

Figure S4: *fgf10a* does not act during tail development. a, Arrowhead indicates *fgf10a* expression in the tail. **b,** Development of the *fgf10a*$^{-/-}$ larval tail is indistinguishable from wild-type. **c,** Measurement of tail growth does not show a significant difference between *fgf10a*$^{-/-}$ and wild-type fish.

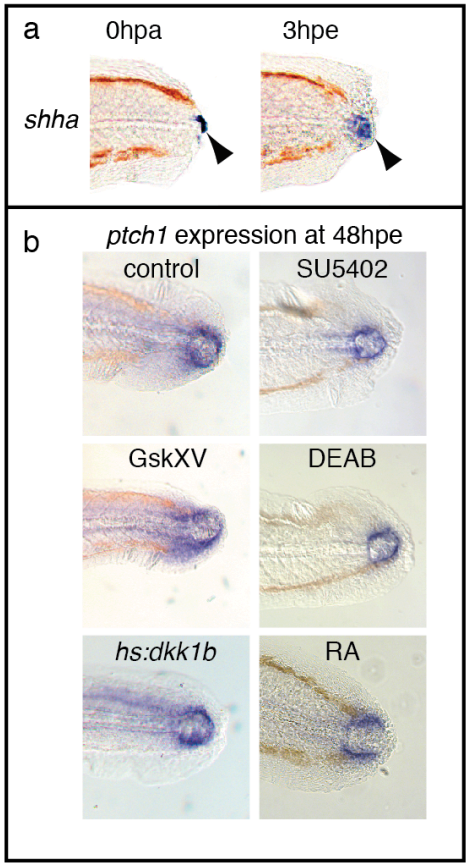

Figure S5: *shha* is expressed in the notochord bead and Hedgehog signalling is unaffected by manipulation of other developmental pathways.
a, *shha* is expressed in the notochord bead (arrowheads). **b,** The activity of the Hedgehog pathway was monitored by in situ hybridisation for *ptch1*. Larvae were treated with GskXV at 12.5µM from 40-48hpe to activate the Wnt/β-Catenin pathway. *hs:dkk1b-GFP* fish were heatshocked for three hours at 39°C at 28hpe and allowed to recover for 17 hours at 28.5°C to inhibit the Wnt/β-Catenin pathway. Larvae were treated with SU5402 from 44-48hpf to inhibit the FGF pathway. Larvae were treated with DEAB from 1-48hpe to block the RA pathway. Larvae were treated with RA from 43.5-48hpf to activate the RA pathway.

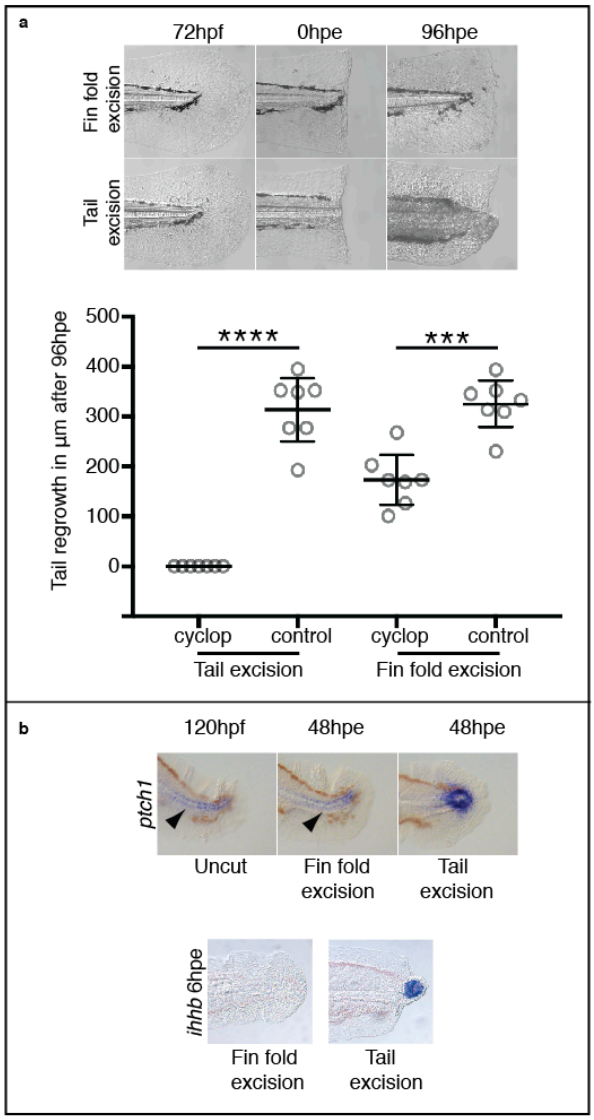

Figure S6: Hedgehog signalling does not play a crucial role in fin fold regeneration. a, Treatment with 20µM cyclopamine is sufficient to block regeneration and has a milder affect on fin fold excision. Regrowth was measured from images in Fiji/ImageJ software by placing a rectangle parallel to the body that started at the end of the notochord and finished at the caudal end of the fin fold. **b,** Neither *ptch1* nor *ihhb* are noticeably upregulated following fin fold excision. Arrowheads indicate low levels of *ptch1* expression in unoperated and fin fold excision larvae.

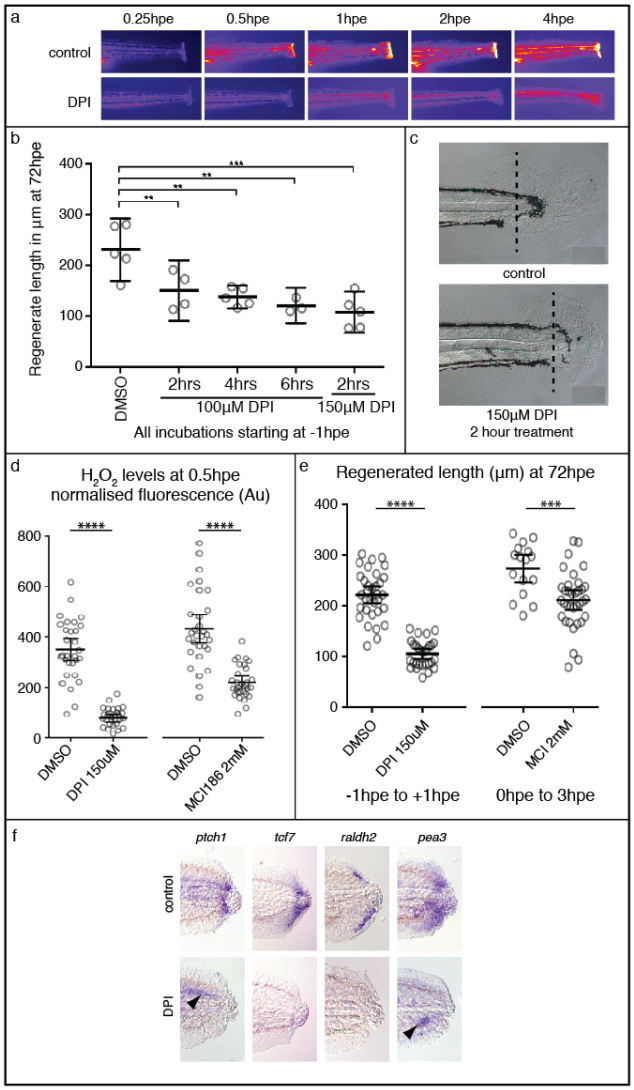

Figure S7: ROS activity immediately after tail excision. a, Time course showing production of ROS after tail excision is reduced in larvae treated with 150µM DPI from 1 hour prior to excision. Larvae were bathed in 10µM PFBS-F (205429, Santa Cruz) to detect ROS. **b,** Optimisation to show that DPI treatment for as little as 2hrs (1hr pre-treatment and 1hr post-treatment) is sufficient to reduce tail regrowth by >50%. **c,** Representative larval tails showing the extent of tail regrowth after DPI treatment. **d,** Quantification of PFBS-F fluorescence shows that DPI treatment has a stronger effect on ROS levels than MCI186. **e,** Comparison of the efficacy of DPI to MCI186 in regards to regenerated tail length. **f,** DPI treatment inhibits wound-induced activation of the Hedgehog, Wntβ-Catenin, RA and FGF pathways. Larvae were pre-treated with 150 µM DPI for one hour and for 6hpe in 100µM DPI. Larvae were then washed in E3 buffer and incubated until 24hpe or 48hpf in the case of *tcf7*. Arrowheads point to expression of *pea3* and *ptch1* that is found in unoperated animals (Supplementary Fig. S2).

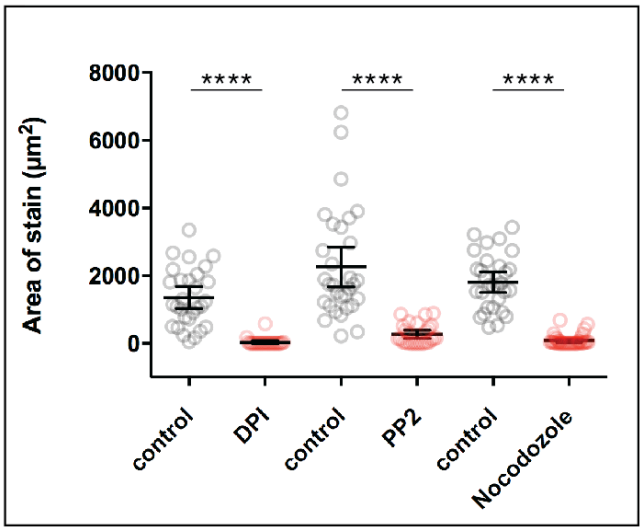

Figure S8: Wound-induced *ptch1* expression is reduced at 24hpe after treatment with inhibitors. Samples were treated as in Figure 5c.

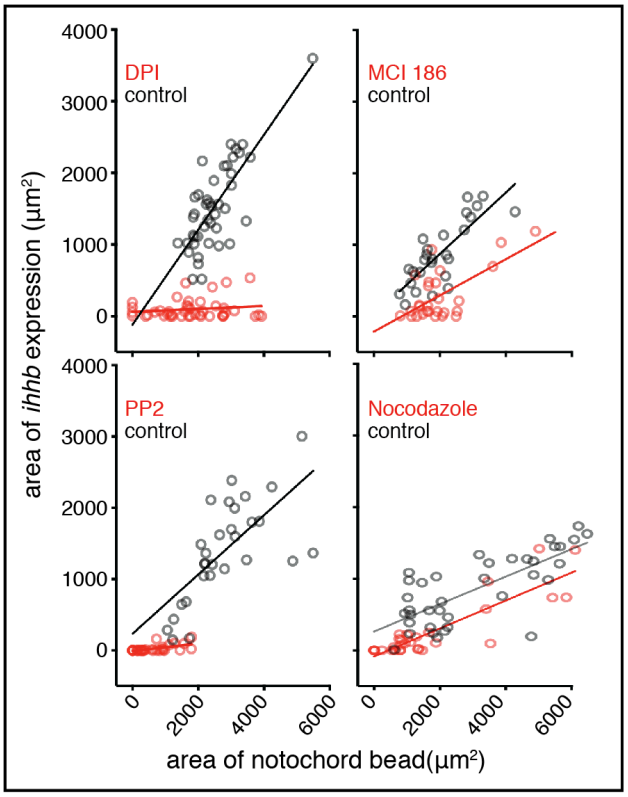

Figure S9: Treatment with DPI, MCI 186, PP2 or nocodazole and comparison of bead size to expression of *ihhb*. Data from panels 5a and 5c plotted together for direct comparison. Regression lines suggest that nocodazole has the strongest correlation and DPI has the weakest.

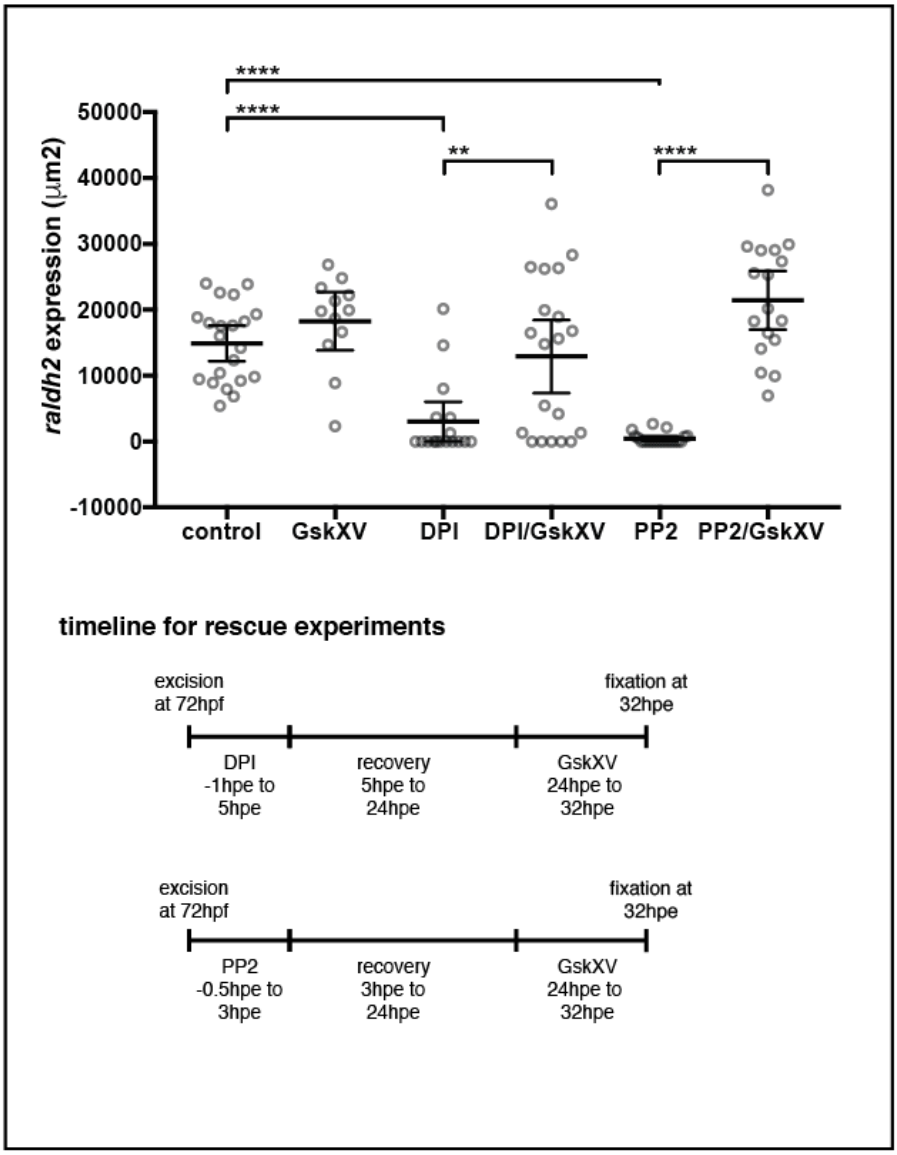

Figure S10: Treatment with DPI and PP2 is partially rescued by upregulation of the Wnt/β-Catenin pathway. Larvae were operated on at 72hpf and treated with DPI, PP2 alone or in combination with GskXV. For DPI treatments, larvae were pre-treated for one hour prior to excision at 150µM concentration, then in 100µM for 5hpe. For PP2 treatments, larvae were pre-treated for thirty minutes prior to excision at 20µM concentration, then in 20µM for 3hpe. Larvae were then treated with 10µM GskXV from 24hpe to 32hpe and stained for *raldh2* expression as a marker of the blastema.

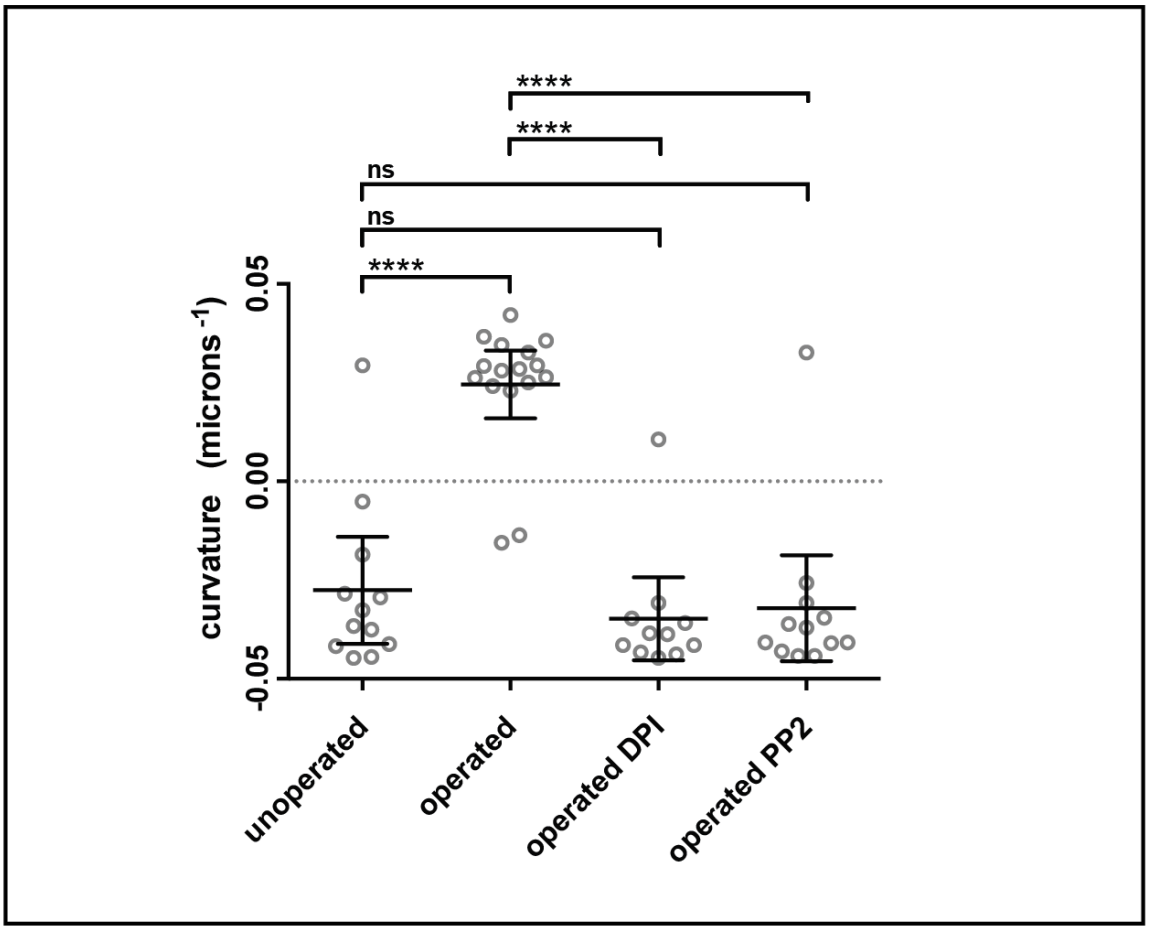

Figure S11: Menger curvature measurements show that notochord cell shape changes are altered by treatment with DPI or PP2. Fish were pretreated for two hours with DPI or PP2 at 28.5°C. Then fish were operated and incubated at 21°C with imaging taking place at twenty minutes post excision.

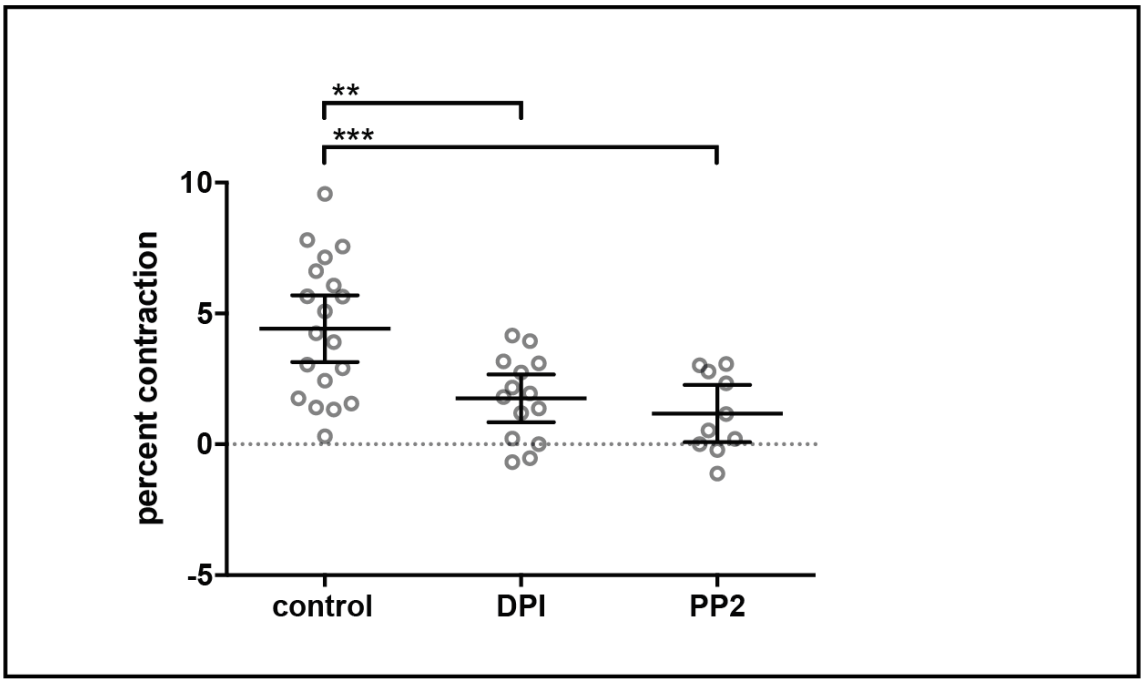

Figure S12: Wound induced contraction of the trunk is reduced by treatment with DPI or PP2. Fish were pretreated for two hours with DPI or PP2. Then fish were imaged immediately prior to tail excision, incubated individually for a further two hours in DPI or PP2 and imaged again.

Supplemental Movie 1. Tissue movement following tail excision.
This movie shows the rapid repositioning of notochord cells to form the notochord bead. The white arrow marks one cell's movement (1.5μm/minute). Note that the notochord cell membranes are bowed towards the anterior at the start of the movie and rapidly change to bow posteriorly within the first five minutes. This may indicate that pressure is building up within the notochord, and that subsequent notochord cell movement is passive rather than an active migration.

Green dots mark reference points within the fin fold epithelia and show the movement of the tissue towards the posterior. Over the duration of the movie the path between these dots shrinks by 11%. Red dots mark individual mesenchymal cells, these move together by 9% during the movie. Measurement of the length of the dorsal edge of the body using melanocyte position also shows a contraction of 9%. Together these indicate that the trunk shrinks by approximately 10% along the anterior/posterior axis during the first three hours after excision. Measurements along the dorsal/ventral axis of each somite show that the trunk becomes wider by approximately 7%. Consistent with this, the angle of the somites increases by 5% on average.

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