

# Shh promotes direct interactions between epidermal cells and osteoblast progenitors to shape regenerated zebrafish bone

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## ABSTRACT

Zebrafish innately regenerate amputated fins by mechanisms that expand and precisely position injury-induced progenitor cells to re-form tissue of the original size and pattern. For example, cell signaling networks direct osteoblast progenitors (pObs) to rebuild thin cylindrical bony rays with a stereotypical branched morphology. Hedgehog/Smoothed (Hh/Smo) signaling has been variably proposed to stimulate overall fin regenerative outgrowth or promote ray branching. Using a photoconvertible *patched2* reporter, we resolve active Hh/Smo output to a narrow distal regenerate zone comprising pObs and adjacent motile basal epidermal cells. This Hh/Smo activity is driven by epidermal Sonic hedgehog a (Shha) rather than Ob-derived Indian hedgehog a (Ihha), which nevertheless functions atypically to support bone maturation. Using BMS-833923, a uniquely effective Smo inhibitor, and high-resolution imaging, we show that Shha/Smo is functionally dedicated to ray branching during fin regeneration. Hh/Smo activation enables transiently divided clusters of Shha-expressing epidermis to escort pObs into similarly split groups. This co-movement likely depends on epidermal cellular protrusions that directly contact pObs only where an otherwise occluding basement membrane remains incompletely assembled. Progressively separated pObs pools then continue regenerating independently to collectively re-form a now branched skeletal structure.

**KEY WORDS:** Zebrafish, Caudal fins, Regeneration, Ray branching, Bone patterning, Hedgehog signaling, Sonic hedgehog, Indian hedgehog, Osteoblasts, Calcification, Basal epidermis, Basement membrane, Smoothed inhibitor, Cyclopamine, BMS-833923

## INTRODUCTION

Unlike mammalian appendages, the adult zebrafish caudal fin perfectly restores tissue organization, size and shape in response to injury or resection (Tomini and Poss, 2014). Regeneration of the fin skeleton, which comprises bony rays (lepidotrichia) that extend along the proximal-distal axis, depends on the coordinated growth, differentiation and positioning of osteoblasts (Obs). Individual rays are formed by two semi-cylindrical bones, or hemi-rays, that are covered with Obs and segmented by regularly spaced joints. Furthermore, each lepidotrichia, excluding the most dorsal and ventral rays, bifurcates in a highly stereotypical manner. A near identical skeletal pattern is efficiently restored within 2 weeks of

fin amputation. Therefore, the zebrafish fin provides a tractable and simple model system with which to decipher mechanisms of regenerative skeletal patterning.

Bone regeneration is initiated after fin resection by Ob dedifferentiation that generates osteoblast progenitors (pObs) at the amputation site (Knopf et al., 2011; Singh et al., 2012; Sousa et al., 2011; Stewart and Stankunas, 2012; Stewart et al., 2014; Tu and Johnson, 2011). These pObs populate the lateral edges of the regenerative blastema that forms above each bony ray stump and surround a largely mesenchymal core cell population. The entire tissue is encased by a stratified epidermis. The Ob lineage remains highly organized for the duration of regeneration with distally located Runx2-expressing pObs and more proximal maturing Obs defined by *sp7* (*osterix*) expression (Stewart et al., 2014). This arrangement is maintained by spatially segregated Wnt and bone morphogenetic protein (BMP) signaling that promotes the opposing activities of Ob growth and differentiation, respectively (Stewart et al., 2014; Wehner et al., 2014). This balanced signaling network, however, does not explain how regenerated bones become bifurcated in the same pattern as the lost fin. Earlier studies show that ray branching requires a neighboring ray (Mari-Beffa et al., 1999) and transplantation of a non-branching dorsal (or ventral) ray to a medial position results in branching of the transplant (Murciano et al., 2002). These observations suggest that cell-cell interactions between adjacent tissues are essential for ray morphogenesis.

Hedgehog (Hh) signaling has been proposed to mediate ray branching during fin regeneration (Laforest et al., 1998; Quint et al., 2002; Zhang et al., 2012). The Hh ligand family, Sonic (Shh), Indian (Ihh) and Desert (Dhh) Hedgehogs, bind to their receptor, Patched (Ptch) on target cells (Fuse et al., 1999; Marigo et al., 1996; Stone et al., 1996). Binding of Hh to Ptch relieves Smoothed (Smo) inhibition, leading to transcriptional changes, including activation of *ptch* genes to form a negative-feedback loop (Briscoe and Thérond, 2013; Chen and Struhl, 1996; Ingham et al., 1991). During fin regeneration, *shha* transcripts are expressed in basal epidermal cells on each side of the distal regenerate, a pattern recapitulated by a *shha:GFP* reporter transgene (Laforest et al., 1998; Lee et al., 2009; Quint et al., 2002; Zhang et al., 2012). Preceding ray bifurcation, each *shha*-expressing cluster bisects into two discrete domains, presaging the splitting of underlying Obs and consequently ray branching (Laforest et al., 1998; Zhang et al., 2012). Laser ablation of *shha*-expressing basal epidermal cells delays branching, underscoring that epidermal-Ob interactions, possibly directing localized Ob proliferation, underlie regenerative bone patterning (Zhang et al., 2012). Shha is a candidate mediator of this signaling, as *ptch2* (previously called *ptc1*) is expressed in Obs adjacent to *shha*-expressing epidermal cells (Laforest et al., 1998; Murciano et al., 2007; Quint et al., 2002) and ectopic Shh promotes ray fusion and promiscuous bone formation (Quint et al., 2002). However, a role for Shha in ray branching has been questioned based on the suggestion that *shha*-expressing epidermal domains

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are constitutively split and that *shha* induction kinetics are inconsistent with Shha being the instructive ray bifurcation signal (Azevedo et al., 2012).

Additional and alternative roles for Hh/Smo signaling during fin regeneration are also possible. The Smo small-molecule inhibitor cyclopamine arrests proliferation of multiple cell types in the regenerate (Blum and Begemann, 2015; Lee et al., 2009; Quint et al., 2002; Wehner et al., 2014), suggesting Hh/Smo signaling contributes to general regenerative outgrowth. Furthermore, *ihha* is robustly expressed in blastemal Obs during regeneration (Avaron et al., 2006). Therefore, Ihha rather than Shha could account for *ptch2* induction in regenerating Obs and in the direct control of osteoblast growth and/or differentiation in a manner recapitulating the proposed developmental roles of Ihh (Abzhanov et al., 2007; Huycke et al., 2012; Lenton et al., 2011; Long, 2012).

We sought to resolve the role(s) of Hh/Smo signaling during fin bone regeneration. A dynamic *ptch2* transgenic reporter shows that Hh/Smo output is tightly restricted to a narrow band of distally extending basal epidermal cells and underlying pObs. These epidermal cells transiently split into two Shha-positive clusters on each side of the ray. We use viable *ihha*-null zebrafish to demonstrate that the Hh/Smo output in both cell types is Shha-driven and that Ob-expressed Ihha instead supports bone maturation, likely via an atypical signaling mechanism. We show that the small molecule Smo inhibitor, BMS-833923, avoids widespread off-target effects of the classic Smo inhibitor cyclopamine in zebrafish. Most strikingly, BMS-833923 unambiguously demonstrates that Shha/Smo signaling is required for ray branching morphogenesis and not regenerative outgrowth. Mechanistically, cellular protrusions from *shha*-expressing epidermal cells directly contact neighboring *ptch2*-expressing pObs at distal sites of incompletely assembled basal lamina. Rather than promoting local proliferation, the split clusters of motile Shha-positive basal epidermis progressively escort pObs into two separated pools that then independently continue regenerating to form a now bifurcated ray.

## RESULTS

### Hedgehog/Smoothed signaling output at the time of ray bifurcation is spatially and temporally restricted to osteoblast progenitors and basal epidermis

Zebrafish *patched2* (*ptch2*; previously named *ptc1*) is induced by Hedgehog/Smoothed (Hh/Smo) signaling (Concordet et al., 1996; Koudijs et al., 2008) and therefore its expression serves as a reporter of pathway activity. We used the *TgBAC(ptch2:Kaede)* *a4596* transgenic line (hereafter denoted as *ptch2:Kaede*), which recapitulates endogenous *ptch2* expression (Huang et al., 2012), to monitor Hh/Smo signaling during caudal fin regeneration. At 96 h post-amputation (hpa), when regenerating rays begin to branch, *ptch2:Kaede* was expressed in all reforming bony rays and was excluded from inter-ray regions. Kaede levels were highest towards the distal regenerate, where, at each ray, it split into two domains on both sides of the fin (Fig. 1A,B), reproducing the *ptch2* transcript pattern (Laforest et al., 1998; Murciano et al., 2007; Quint et al., 2002). We immunostained sections from *ptch2:Kaede* fins using Kaede and Runx2 antibodies to visualize sites of Hh/Smo activity relative to the position of Obs. At 72 hpa, *ptch2:Kaede* reporter activity was observed in distal and medial Runx2<sup>+</sup> Obs, and was absent in more proximal osteoblasts extending new bone (Fig. 1C–F; earlier time points preceding branching are shown in Fig. S1). Therefore, pObs activate Hh/Smo signaling as they are generated upon self-renewal and then lose this Hh/Smo response when they mature into quiescent and re-epithelialized Obs. Concurrently,

*ptch2:Kaede* was expressed in distal basal epidermal cells neighboring and extending beyond Runx2<sup>+</sup> pObs. At the onset of ray bifurcation, canonical Hh signaling therefore is spatially restricted to two cell types: pObs and basal epidermal cells.

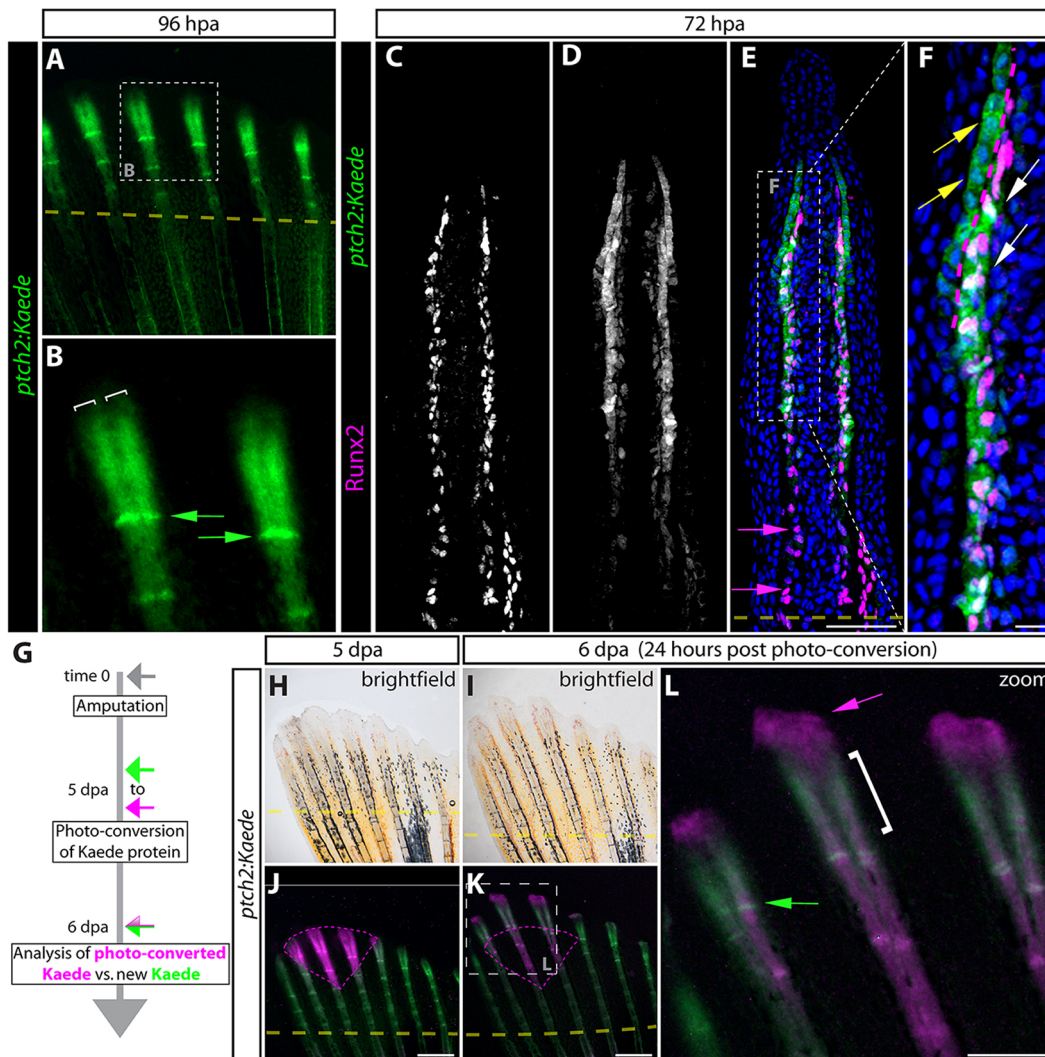
### Osteoblast progenitors and basal epidermis transiently encounter a distal field of active Hedgehog/Smoothed signaling

The ability to stably photoconvert the Kaede protein from green to red (Ando et al., 2002) allowed us to determine whether Hh/Smo signaling in pObs and basal epidermis was transient or continuous, and to follow the fate of Hh/Smo-responsive cells during regeneration. We performed Kaede photoconversion (the ‘pulse’) by illuminating a field containing distal regions of several rays of a 96 hpa *ptch2:Kaede* fish with 405 nm light (Fig. 1H,I). We then monitored both the perdurance of converted red Kaede and the appearance of new green Kaede during a 24 h ‘chase’ period. Active Kaede production was found in a narrow region distal to the photoconverted field corresponding to new bone segments, marked by intervening nascent joints, that re-formed during the chase period (Fig. 1I,K,L). By contrast, minimal new Kaede was produced in more proximal Obs that retained photoconverted red Kaede. We also detected a cell population at the extreme distal end of the regenerate that expressed only photoconverted Kaede (Fig. 1L). By observing confocal *z*-stacks, we determined these cells were previously Hh/Smo-responsive epidermal cells (Fig. S2A–H) that had migrated distally and possibly are then shed (Fig. S2I–K). We conclude that during the outgrowth phase of fin regeneration, Hh/Smo signaling is actively transmitted only in pObs and immediately adjacent basal epidermis. However, the fate of the Hh/Smo-responding cells in each lineage are distinct. After terminating Hh/Smo signaling, differentiating pObs remain relatively stationary where they progressively extend replacement bone. By contrast, the continuous distal displacement of basal epidermal cells directs their brief passage through the same ‘signaling zone’ before moving further distally as they downregulate Hh/Smo signaling.

### *shha* and *ihha* are transiently expressed in parallel bands of adjacent epidermis and osteoblast progenitors during fin regeneration

The dynamic *ptch2:Kaede*-marked Hh/Smo activity seen in the basal epidermis and Obs of regenerating fins could be driven by one or more of the Hh family members. By qRT-PCR on 96 hpa fins, we observed robust and comparable *shha* and *ihha* transcript levels. By contrast, *ihhb* was expressed at low levels (one-ninth that of *ihha*) and *shhb* mRNA was undetectable (Fig. 2A). *dhh* also is not expressed in regenerating fins (Avaron et al., 2006). We conclude that Shha and/or Ihha, which are transcribed in basal epidermis and Obs, respectively (Avaron et al., 2006; Laforest et al., 1998; Quint et al., 2002), are the only clear candidates to stimulate the *ptch2*-marked Hh/Smo activity seen in both cell types.

We used the *Tg(-2.4shha:GFP:ABC)sb15* (abbreviated *shha:GFP*) transgenic line (Ertzer et al., 2007), which recapitulates endogenous *shha* expression during fin regeneration (Lee et al., 2009; Zhang et al., 2012), to further resolve the spatial-temporal dynamics of *shha* transcription during fin regeneration. Using whole-mount analysis, we first observed faint GFP fluorescence in the distal regenerate at 48 hpa. This patch of *shha:GFP* expression split into two clusters on each side of the regenerating ray around 72 hpa (Fig. S3), as previously reported (Zhang et al., 2012). We used antibody staining of sectioned fins to observe *shha*-expressing epidermal cells relative to sp7- and Runx2-expressing



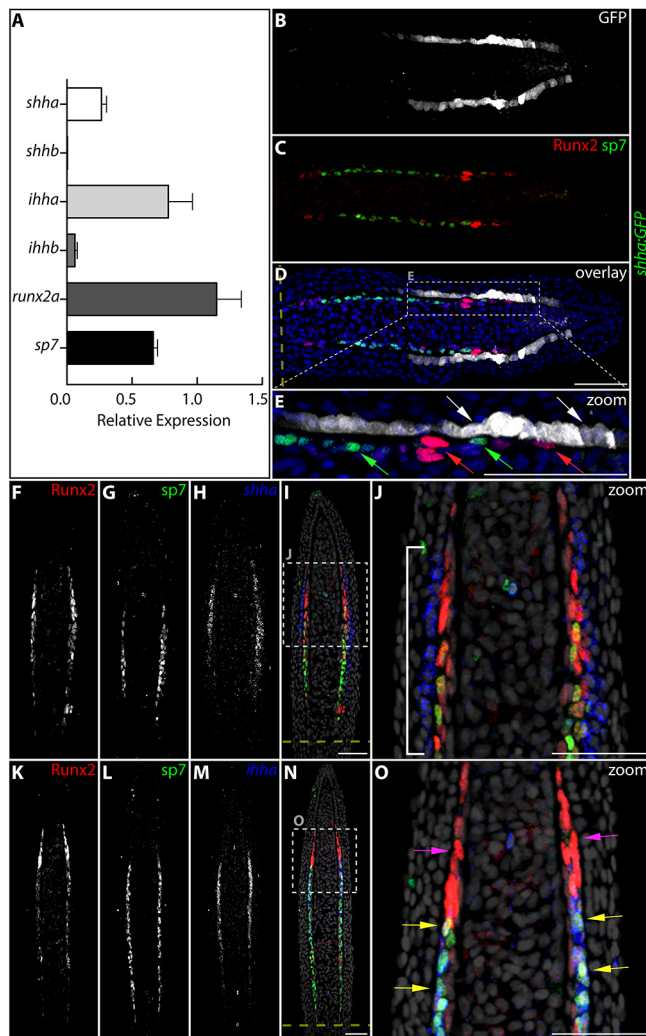
**Fig. 1. *ptch2:Kaede* expression and photoconversion reveals transient Hedgehog/Smoothed signaling restricted to distal osteoblast progenitors and basal epidermis during fin regeneration.** (A,B) Whole-mount images showing Kaede expression (green) in a 96 hpa fin from a *ptch2:Kaede* fish. (B) A high-magnification image of the boxed region in A. White brackets mark the split domains of Kaede at the distal end of the regenerate. Green arrows indicate Kaede<sup>+</sup> cells at newly re-forming joints. (C-F) Antibody-stained longitudinal fin sections from a 72 hpa *ptch2:Kaede* fish. Individual Runx2 (C) and Kaede (D) channels are shown in gray scale. Overlay images with Runx2 in magenta and Kaede in green are shown in E and F (a magnification of the boxed region in E). Nuclei are in blue. Yellow arrows indicate Kaede<sup>+</sup> basal epidermis; white arrows mark Kaede<sup>+</sup>/Runx2<sup>+</sup> Obs; magenta arrows indicate proximal Runx2<sup>+</sup> Obs lacking Kaede. The dotted magenta line indicates the boundary between Kaede<sup>+</sup> basal epidermis and pObs. (G) *ptch2:Kaede* photo-conversion experiment overview. (H-L) Whole-mount bright-field (H,I) and fluorescent (J-L) images of the regenerating caudal fin from a *ptch2:Kaede* photo-conversion experiment fish. (J) The fin is shown at 5 dpa, immediately after photoconverting Kaede protein in a distal field (tissue marked by the magenta dashed line). (K,L) The same fin 24 h post-conversion (6 dpa) imaged for Kaede expression. The dashed box in K marks the enlarged region in L. Unconverted and new Kaede is green; photo-converted Kaede is magenta. The green arrow marks cells in a forming joint that expressed Kaede within the previous 24 h. The white bracket indicates the narrow distal domain of new Kaede production since photoconversion. The magenta arrow indicates epidermis near the tip of the fin that had displaced distally while retaining photo-converted Kaede. Dashed yellow lines in A,E,J,K show amputation planes. Scale bars: 50  $\mu$ m in E; 10  $\mu$ m in F; 500  $\mu$ m in J,K; 250  $\mu$ m in L.

Obs at various times post-fin amputation (24-48 hpa are shown in Fig. S4A-I). At 72 hpa, 1 day ahead of the initiation of ray branching, *shha:GFP* expression was isolated to the basal epidermis adjacent and at least five cells distal to Runx2<sup>+</sup> pObs (Fig. 2B-E). However, using fluorescent *in situ* hybridization (FISH) coupled with Runx2 and *sp7* immunostaining, *shha* transcripts were actively produced only by basal epidermal cells directly neighboring distal Runx2<sup>+</sup> pObs (Fig. 2F-J). We conclude that the extreme distal *shha:GFP* expression likely represents migrating epidermal cells with residual GFP protein, rather than ongoing *shha* transcription. Overlapping GFP and Kaede protein expression in adherens junction-marked distal basal epidermal

cells in fins of 72 hpa *shha:GFP;ptch2:Kaede* fish further shows the basal epidermis transduces Hh signals only while neighboring pObs and ceases responding upon its continued distal displacement (Fig. S4J-M). A summary schematic showing the migratory and Hh/Smo pathway dynamics of both epidermal and Ob cells is shown in Fig. S5.

We also used FISH to resolve *ihha* transcription within hierarchically arranged regenerating Obs defined by Runx2 and/or *sp7* expression. The distal and proximal extents of *ihha* expression precisely corresponded with the distal-most *sp7* expressing and proximal-most Runx2-expressing Obs, respectively. Therefore, active *ihha* transcription is restricted to re-differentiating Runx2<sup>+</sup>



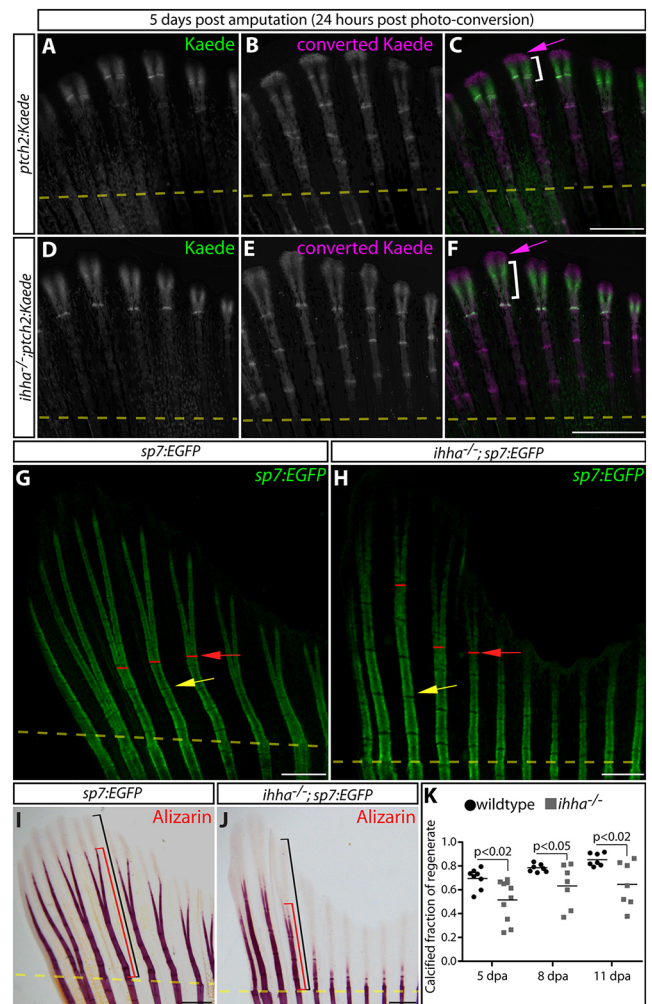


**Fig. 2. *shha* is briefly transcribed by distal migrating epidermal cells, whereas *ihha* is restricted to re-differentiating progenitor osteoblasts.** (A) qRT-PCR analysis of the relative expression levels of *shha*, *shhb*, *ihha*, *ihhb*, *runx2a* and *sp7* in 96 hpa fin tissue. The relative levels of the indicated transcripts are means of four fins normalized to *rpl8* expression. Error bars represent 1 s.d. (B-E) An immunostained fin section from a 72 hpa *shha:GFP* fish showing GFP (white), Runx2 (red) and sp7 (green) expression. Nuclei are in blue. E is a high-magnification view of the dashed box in D. (F-O) Fin sections from a 72 hpa fish stained by RNA *in situ* hybridization for *shha* (F-J) or *ihha* (K-O) transcripts (blue) and with Runx2 (red) and sp7 (green) antibodies. Single channels are shown in gray scale (F-I, K-N). Nuclei are gray in the overlay images (I, J, N, O). (J, O) Enlarged regions marked in I, N, respectively. The white bracket indicates the extent of epidermal cells expressing *shha* relative to pObs. Yellow arrows show Runx2<sup>+</sup>/sp7<sup>+</sup> Obs that express *ihha*; magenta arrows mark distal Runx2<sup>+</sup> pObs that lack *ihha* mRNA. The dashed yellow lines indicate amputation sites. Scale bars: 25 μm in J, O; 50 μm in E; 50 μm in B-D, F-N.

sp7<sup>+</sup> Obs (Fig. 2K-O) and not to Runx2<sup>+</sup> pObs nor the sp7<sup>+</sup> Obs that actively produce replacement bone.

### ***Ihha* is required to promote mineralization of regenerated bone**

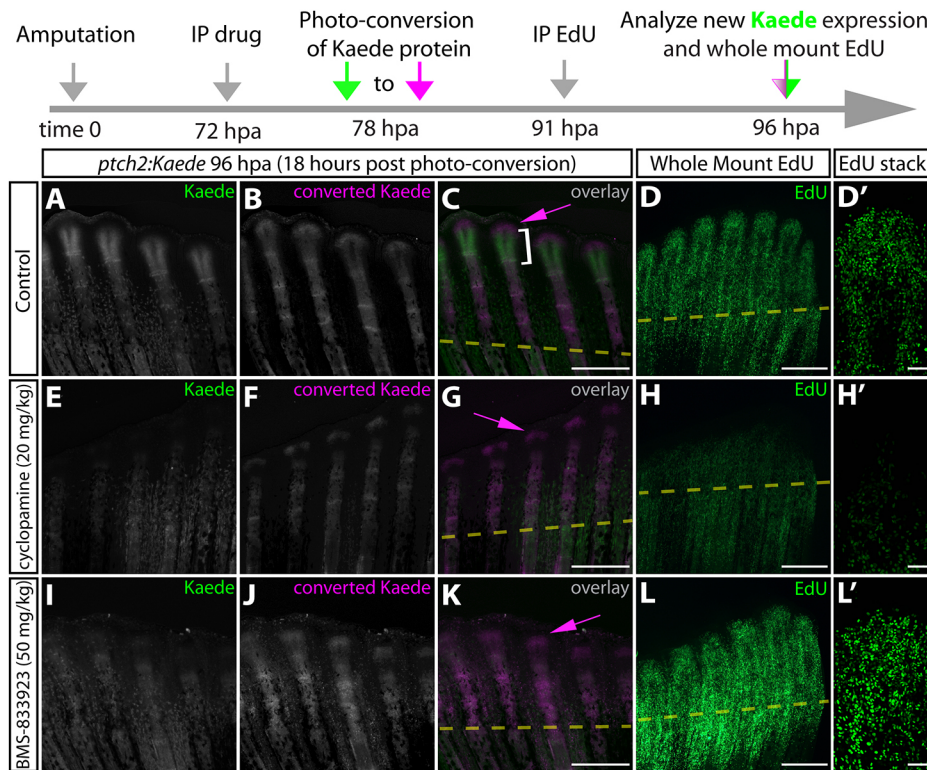
We used the *ihha*<sup>hu2131</sup> null allele (Hammond and Schulte-Merker, 2009) to determine whether Ihha is responsible for the *ptch2*-marked Hh/Smo activity in the epidermis and/or Obs of regenerating fins. A subset of *ihha*<sup>hu2131/hu2131</sup> (*ihha*<sup>-/-</sup>) homozygotes survived craniofacial, swim bladder and cloaca development defects



**Fig. 3. *Ihha* promotes the efficient calcification of regenerated bone by non-canonical Hedgehog signaling.** (A-F) Whole-mount Kaede fluorescence images of 5 dpa caudal fins from *ptch2:Kaede* (A-C) and *ihha*<sup>-/-</sup>; *ptch2:Kaede* (D-F) fish 24 h after photoconversion. Photoconverted pre-existing Kaede is magenta; Kaede produced after photoconversion is green. Magenta arrows indicate extreme distal epidermal tissue exclusively expressing converted Kaede. The white brackets indicate the domain of newly expressed Kaede protein. (G, H) Whole-mount images showing GFP-expressing osteoblasts in fins from *sp7:EGFP* and *ihha*<sup>-/-</sup>; *sp7:EGFP* fish at 11 dpa. Yellow arrows mark newly formed joints. Red lines and arrows denote points of ray bifurcation. (I, J) Bright-field images of Alizarin Red-stained *sp7:EGFP* (I) and *ihha*<sup>-/-</sup>; *sp7:EGFP* (J) fins at 11 dpa. Black and red brackets show the total length of the regenerate and the extent of mineralization from the site of amputation, respectively. Yellow dashed lines in all panels show amputation positions. (K) Quantification of the relative extent of calcified regenerated bone in *sp7:EGFP* versus *ihha*<sup>-/-</sup>; *sp7:EGFP* fish at 5, 8 and 11 dpa. Means and data points representing individual fish are shown. Significant differences between control and *ihha*-null fish ( $P < 0.05$ ) were determined by two-tailed Student's *t*-tests. Scale bars: 500 μm.

(Hammond and Schulte-Merker, 2009; Huycke et al., 2012; Korzh et al., 2011; Parkin et al., 2009), allowing adult regeneration studies. The pattern of *ptch2:Kaede* fin expression in 5 dpa *ihha*-null fish was normal and, unexpectedly, levels were modestly increased rather than decreased (Fig. 3A-F). *shha*, *shhb* and *ihhb* transcript levels were not altered in regenerating fins of *ihha*-deficient fish, arguing against compensatory expression of related Hh genes (Fig. S6A). By contrast, *ihha* transcripts were reduced, likely due to nonsense-mediated mRNA decay (Fig. S6A). We deduce that the epidermal and Ob Hh/





**Fig. 4. BMS-833923, a newly identified potent zebrafish Smoothed inhibitor, shows that Hedgehog/Smo signaling does not impact fin regenerative outgrowth.** (A-L') Whole-mount fluorescence images of 96 hpa fins from *ptch2:Kaede* fish 18 h after Kaede photoconversion and 5 h after EdU injection. Regenerating fish were treated for 24 h with DMSO (A-D'), cyclopamine (E-H') or BMS-833923 (I-L'). Newly produced Kaede and converted Kaede are green and magenta, respectively, in overlay images (C, G, K). Magenta arrows indicate cells with converted Kaede. White brackets mark the domain of new Kaede expression in the 18 h since photoconversion. (D, H, L) Fins from the same fish shown for Kaede fluorescence processed to reveal EdU-incorporating nuclei in green. (D', H', L') Confocal stack images showing EdU incorporation at the distal aspect of single re-forming rays. The timeline at the top of the figure details the experimental design. Scale bars: 500  $\mu$ m.

Smo activity during fin regeneration is *Ihha* independent and therefore Shha driven. Furthermore, any essential *Ihha* signaling is not driven by Smo-mediated transcriptional changes canonically represented by *ptch2:Kaede* expression.

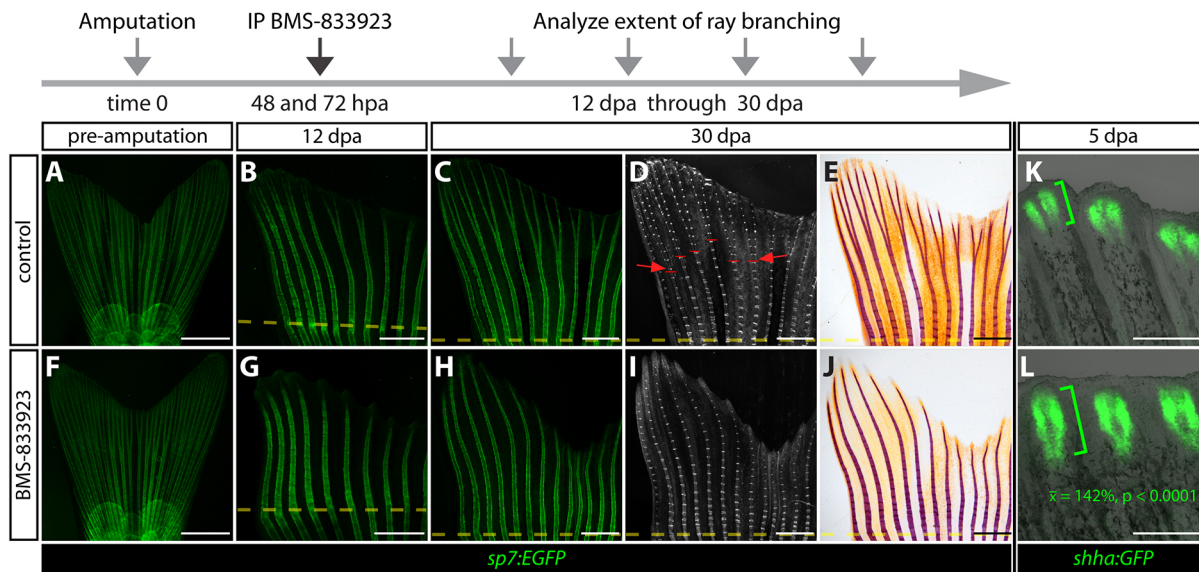
Fins from *ihha*<sup>-/-</sup> fish regenerated grossly normally following resection, including restoring organized bones with branched rays as shown by *Tg(sp7:EGFP)b1212* (hereafter referred to as *sp7:EGFP*) expression (Fig. 3G,H). However, using whole-mount Alizarin Red and von Kossa staining of tissue sections, the majority of *ihha*-deficient fish still displayed qualitative and quantitative calcification defects 6 weeks after amputation (Fig. 3I-K, Fig. S6B-J). Fin ray calcification was largely normal in unamputated *ihha*<sup>-/-</sup> adult fish, indicating that *Ihha* non-redundantly promotes regenerative but not developmental bone maturation (Fig. S6K-N). Consistent with a late role for *Ihha* in bone regeneration, *ihha*<sup>-/-</sup> zebrafish had no change in Ob numbers, proliferation rate or *Runx2/sp7* expression during the fin regenerative response (Fig. S6O-Q). Furthermore, BMP signaling, as monitored by pSmad1/5/8 staining, was intact in the absence of *ihha* (Fig. S7). We conclude that *Ihha* expressed in re-differentiating Obs acts in parallel with the core differentiation regulatory network to support maturation of subsequently formed fully differentiated Obs.

#### Smoothed inhibitor BMS-833923 blocks Hedgehog signaling in zebrafish and avoids non-specific anti-proliferative effects of cyclopamine

The Shha-driven Hh/Smo signaling we observed in a narrow distal band of neighboring basal epidermis and Obs does not adequately explain the widespread proliferation block observed when exposing regenerating zebrafish to the Smo-inhibitor cyclopamine (Blum and Begemann, 2015; Lee et al., 2009; Quint et al., 2002; Wehner et al., 2014). We surmise that this phenotype, which is the primary evidence supporting the hypothesis that Hh promotes regenerative outgrowth, is an off-target effect similar to that reported when using

cyclopamine to study zebrafish germ cell development (Mich et al., 2009). Therefore, we performed a screen of seven additional Smo small-molecule inhibitors to determine whether any would reduce *ptch2:Kaede* expression and recapitulate developmental defects seen in *smo*-null embryos without blocking global cell proliferation.

We amputated *ptch2:Kaede* fins and intraperitoneally injected cyclopamine (20 mg/kg), BMS-833923 (50 mg/kg), Vismodegib (75 mg/kg), Erismodegib (50 mg/kg), SANT-1 (50 mg/kg), Taladegib (8.25 mg/kg), glasdegib (50 mg/kg) or PF-05274857 (100 mg/kg) at 72 hpa. We photoconverted the Kaede protein 6 h later (78 hpa) and analyzed fins at 96 hpa (24 h after drug delivery) for new Kaede protein expressed during the drug exposure period ( $n \geq 4$  for each group). As expected, cyclopamine-treated fins produced no new Kaede expression (Fig. 4A-C,E-G). Among the other compounds tested, only BMS-833923 prevented new Kaede expression (Fig. 4I-K, Fig. S8). Whole-mount EdU analysis indicated that cyclopamine treatment rapidly arrested DNA synthesis in cells throughout the regenerating fin and correspondingly halted regenerative outgrowth (Fig. 4D,H). Importantly, this proliferation arrest was not observed in BMS-833923-treated fins (Fig. 4L). We could not identify a cyclopamine dose that separated its effects on *ptch2:Kaede* expression and cell proliferation. By contrast, even doses of BMS-833923 exceeding those necessary to block *ptch2:Kaede* had no appreciable effect on proliferation (Fig. S9). Furthermore, BMS-833923 treatment of early zebrafish embryos more closely recapitulated the gross developmental defects seen in *smo*-null zebrafish than did cyclopamine exposure (Fig. S10) (Aanstad et al., 2009; Chen et al., 2001; Lewis and Eisen, 2001; Loucks et al., 2007; Varga et al., 2001; Wolff et al., 2003). Notably, BMS-833923 phenocopied *smo* mutants in preventing the development of Engrailed-expressing muscle pioneer cells (Aanstad et al., 2009; Barresi et al., 2000; Wolff et al., 2003). Therefore, BMS-833923 exhibits preferred characteristics over cyclopamine as a small-molecule inhibitor for Hh/Smo signaling studies in zebrafish.



**Fig. 5. BMS-833923 demonstrates that Hedgehog/Smoothed signaling is dedicated to bony ray branching during the outgrowth phase of fin regeneration.** (A–J) Whole-mount images of fins from the same two *sp7:EGFP* fish acquired prior to amputation and at 12 and 30 days post-amputation (dpa). The fish are treated with either control DMSO or BMS-833923 at 48 and 72 hpa. (A–C, F–H) Fluorescence images showing osteoblast GFP expression in green. (D, I) Rotterman contrast images. Periodic high-contrast patches along the rays are joints. Red lines and arrows mark ray bifurcation points. (E, J) Bright-field images of Alizarin Red-stained fins collected from the same fish to visualize mineralized bone. Amputation planes are indicated with dashed yellow lines. (K, L) Wide-field whole-mount GFP fluorescence and bright-field overlay images of 5 dpa fins from *shha:GFP* fish following injections with DMSO or BMS-833923 at 48 and 72 hpa. Green brackets indicate the extent of epidermal *shha:GFP* expression along the proximal-distal axis. Student's *t*-test shows the 142% mean increase in the length of the *shha:GFP* domain is significant [ $n=5$  control and 10 BMS-833923-treated fish (60 scored rays),  $P<0.0001$ ]. Scale bars: 2 mm in A, F; 1 mm in B–E, G–J; 500  $\mu$ m in K, L.

### Hedgehog/Smoothed signaling is dedicated to ray bifurcation during the outgrowth phase of fin regeneration

To test potential Shha/Smo signaling contributions to ray branching, we treated *sp7:EGFP* zebrafish with BMS-833923 at 48 and 72 hpa, and monitored the course of regeneration. At 12 dpa, when all regenerated fins from control treated fish had bifurcated lepidotrichia, BMS-833923-treated fish dramatically lacked branched rays (Fig. 5A–B, F–G). By contrast, BMS-833923 exposure did not disrupt regenerate outgrowth (control mean=3.30 mm; BMS-833923 mean=3.07 mm;  $n=5$ ,  $P>0.212$ ). This specific ray branching defect persisted through 30 dpa when fin regeneration, including bone growth, overt differentiation and joint formation, was otherwise completely normal ( $n=12$ ; Fig. 5C–D, H–I). The permanent blockage of ray branching may reflect prolonged Hh/Smo inhibition by BMS-833923; *ptch2:Kaede* zebrafish treated at 48 and 72 hpa failed to express new Kaede protein through 9 dpa (Fig. S11). Bone mineralization, which was disrupted in *ihha*<sup>-/-</sup> zebrafish, was unaffected by Hh/Smo inhibition initiated after 48 hpa (Fig. 5E, J), reinforcing the possibility that Ihha does not function through Smo-dependent signaling to promote regenerative bone maturation. Furthermore, we conclude that Hh/Smo signaling driven by basal epidermal Shha is primarily dedicated to directing ray branching after the initiating steps of fin regeneration are complete.

BMS-833923-exposed *shha:GFP* zebrafish (treated at 48 and 72 hpa) retained two distinct GFP-positive epidermal patches adjacent to each hemiray at 4 and 5 dpa. However, the *shha:GFP*-expressing domain was expanded along the proximal-distal axis (mean length=142%,  $n=5$  control and 10 BMS-833923-treated fish,  $P<0.0001$ ; Fig. 5K, L), reminiscent of a previous report using cyclopamine (Quint et al., 2002). Collectively, we conclude that: (1) Shha/Smo signaling is not required to generate split clusters of *shha*-expressing epidermis; (2) in the absence of Smo activity, *shha*-expressing epidermal splitting is not sufficient to promote ray

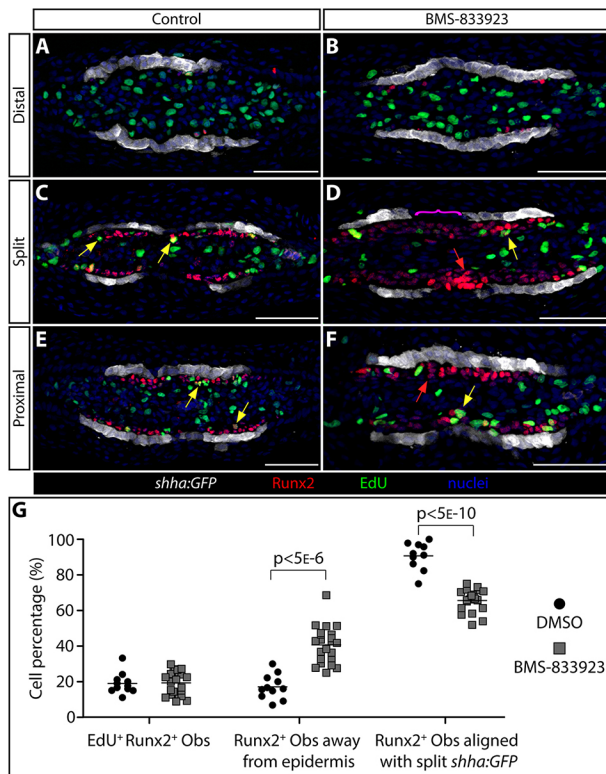
branching; and (3) negative feedback restricts *shha* expression to a short stretch of distal basal epidermis.

The onset of *shha* expression splitting precedes observable ray branching and the split pattern persists for several days while branching unfolds (Laforest et al., 1998; Zhang et al., 2012). To assess whether Shh/Smo signaling is continuously required for ray bifurcation, we inhibited Hh/Smo signaling at 5 dpa, 2 days post-splitting of *shha:GFP*-expressing epidermis (Fig. S3; Zhang et al., 2012). Fins from these BMS-833923-exposed fish had normal regenerative outgrowth with limited and delayed ray bifurcation in the longest rays (rays 3–6, both dorsal and ventral sides) at 40 dpa ( $n=10$ ; Fig. S12). Therefore, the process of Shh/Smo-induced ray branching transpires over several days, suggesting a progressive rather than switch-like mechanism.

### Shh promotes ray bifurcation by directing pObs to migrate into split pools

Shh/Smo signaling has been speculated to direct branching morphogenesis by inducing local proliferation of pObs underlying the split *shha*-expressing basal epidermis (Zhang et al., 2012). Alternatively, Shh/Smo could direct pObs to migrate into split pools that then continue regenerating independently to form branched rays. To distinguish between these possibilities, we treated *shha:GFP* zebrafish with BMS-833923 at 48 and 72 hpa, and then analyzed the proliferation (by EdU incorporation) and arrangement of Runx2<sup>+</sup> pObs at 96 hpa in multiple transverse sections along the proximal-distal axis. We tracked individual rays in control and BMS-833923-treated animals from a relatively proximal position, where *shha:GFP* initiated in a single basal epidermal field, through the region of *shha:GFP* domain splitting, to far distal sections that lacked Runx2<sup>+</sup> pObs (Fig. 6A–F). Given our *ptch2:Kaede* cell tracing showed basal epidermal cells migrate distally, we conclude that *shha* transcriptional initiation precedes





**Fig. 6. Shha-driven Smoothened signaling directs progenitor osteoblasts to migrate in step with transiently split basal epidermal clusters at the onset of ray branching.** (A-F) Runx2 and EGFP immunostaining (red and white, respectively) and EdU incorporation (2 h treatment, green) on transverse 96 hpa fin sections from individual *shha:GFP* fish treated at 48 and 72 hpa with DMSO (A,C,E) or BMS-833923 (B,D,F). Nuclei are blue. (A,B) Far distal sections beyond the distal-most pObs. (C,D) Sections from positions where *shha*-expressing epidermal cells have split into two clusters on each side of the fin. (E,F) Further proximal sections where *shha* is first induced by distal migrating epidermal cells. Yellow arrows indicate Runx2<sup>+</sup>/EdU<sup>+</sup> cells located more than one cell layer from *shha:GFP*-expressing basal epidermis. Red arrows indicate Runx2<sup>+</sup> pObs two or more cell layers distant from *shha:GFP*-positive epidermal cells. The magenta bracket in D highlights Runx2<sup>+</sup> pObs that span the junction between split *shha:GFP* domains in BMS-833923-treated fish. (G) Quantification of pOb proliferation and the relative position of pObs to epidermal cells in regenerating fins from the above and similar DMSO versus BMS-833923 treated *shha:GFP* fish. Only images at 'split' positions are scored. Left plots: the fraction of EdU incorporating Runx2<sup>+</sup> pObs. Middle plots: the fraction of pObs not directly adjacent to GFP<sup>+</sup> epidermal cells. Right plots: the fraction of pObs aligned with split clusters of *shha:GFP*-expressing epidermis. Each data point represents a scored independent section (11 individual rays from five DMSO-treated fish and 23 rays from seven BMS-833923-treated fish). Two-tailed Student's *t*-tests were used to determine statistically significant differences ( $P < 0.05$ ) between the means of control versus small-molecule-treated samples. Scale bars: 50  $\mu$ m.

the physical assortment of *shha*-expressing epidermal cells into distinct clusters. Furthermore, as anticipated from our whole-mount studies, both *shha* induction and epidermal movements are Smo independent. Finally, *shha*-expressing epidermal cells only transiently split as the distal-most basal epidermal cells that retained GFP protein but no longer actively produce *shha* re-merged into a single population.

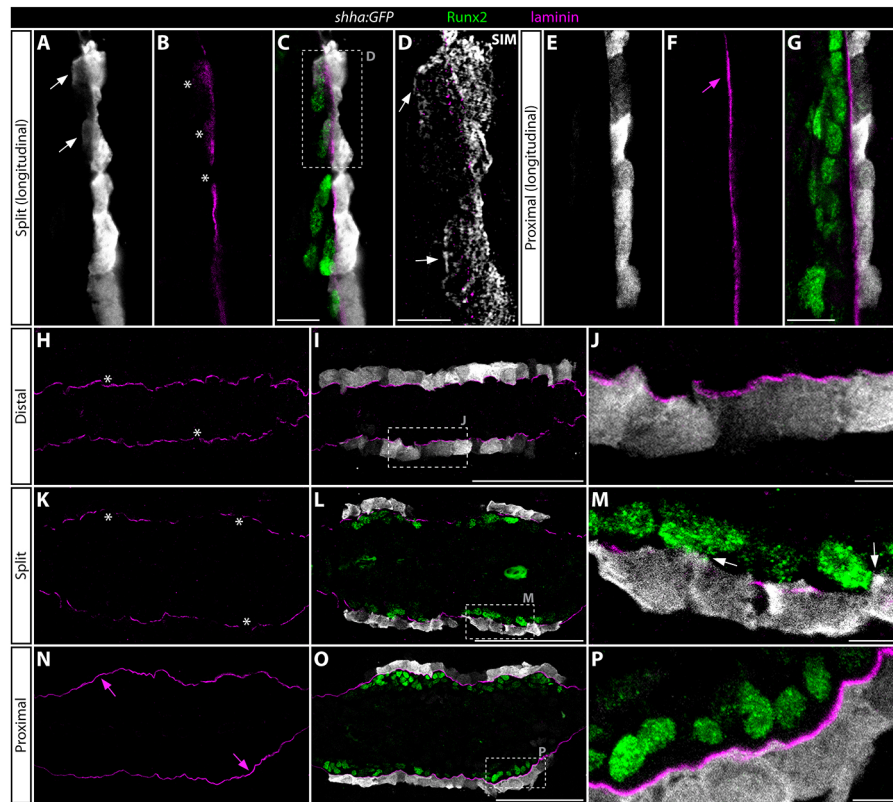
At sites of transiently split *shha:GFP*-expressing epidermis, most Runx2<sup>+</sup> pObs in control fins were arranged in a single layer directly adjacent to GFP-positive epidermis (Fig. 6C, additional repeats in Fig. S13). By contrast, Runx2<sup>+</sup> pObs in BMS-833923-treated fins were up to several cell layers thick and spanned the entire junction

between split *shha:GFP* domains (Fig. 6D). At a more proximal position prior to the splitting of *shha:GFP*-expressing epidermis, Runx2<sup>+</sup> pObs in BMS-833923 treated fins were four or more cell layers distant from the epidermis (Fig. 6E). In some cases, this transverse section analysis appeared to indicate BMS-833923 exposed fins had a larger population of Runx2<sup>+</sup> pObs. However, a quantitative analyses of longitudinal sections from multiple animals revealed that overall numbers of distal Runx2<sup>+</sup> pObs were unaffected by BMS-833923 treatment (Fig. S14A-C). Rather, the pOb pool was incompletely extended along the proximal-distal axis in the absence of Hh/Smo signaling. Furthermore, while Hh/Smo inhibition led to disorganized Runx2<sup>+</sup> pObs, it did not alter the fraction of EdU-incorporating proliferating Runx2<sup>+</sup> Obs (Fig. 6G), including following acute BMS-833923 treatment (Fig. S14D-F). In addition, the proliferative rate of Runx2<sup>+</sup> pObs was not correlated with their proximity to *shha:GFP*-expressing epidermis ( $P > 0.868$ , two-tailed Fisher's exact test,  $n = 336$  for layer 1 Runx2<sup>+</sup> cells and  $n = 73$  for Runx2<sup>+</sup> cells in layers 2 and over). We conclude that Shh/Smo signaling aligns underlying pObs with *shha*-expressing basal epidermis and then guides pObs into split pools to initiate ray branching during fin regeneration.

### **Shha-expressing basal epidermal cells directly contact and recruit underlying osteoblast progenitors**

Emerging studies show that, in some contexts, Shh/Smo signaling is highly localized and even mediated by direct cell-to-cell contacts through cell surface-retained Shh protein (Sanders et al., 2013). Therefore, we used antibody staining and confocal microscopy, including structured illumination microscopy (SIM, Gustafsson, 2000) of sectioned regenerating *shha:GFP* fins to explore the relative positioning of basal epidermis and pObs. Strikingly, at the point of epidermal splitting, GFP<sup>+</sup> basal epidermal cells directly appose neighboring Runx2<sup>+</sup> pObs, including through extended cellular protrusions that contact and occasionally envelop pObs (Fig. 7A-D,K-M). Antibody staining of regenerating fin sections prepared from permanently labeled epidermal mosaic fish (Stewart and Stankunas, 2012) confirmed that basal epidermal-originating processes directly contact Runx2<sup>+</sup> pObs (Fig. S15).

The basal epidermis and underlying mesenchyme of regenerating fins seemingly are separated by a continuous laminin  $\beta$ 1a-containing (Lamb1a) basement membrane (Chen et al., 2015) that should impede epithelial-stromal interactions (Kelley et al., 2014). However, our confocal analysis of antibody-stained longitudinal sections revealed diffuse laminin staining in distal regions featuring basal epidermal-pOb contacts (Fig. 7B). At more proximal positions, laminin expression defined an unbroken barrier between basal epidermis and neighboring pObs (Fig. 7E-G). Immunostaining of serial transverse sections from 96 hpa *shha:GFP* fins confirmed extensive laminin gaps exclusively where Runx2<sup>+</sup> pObs and *shha:GFP*-expressing epidermal cells interacted (Fig. 7K-P). Given the continuous distal displacement of epidermal cells and especially high *lamb1a* transcript levels in the distal regenerate (Chen et al., 2015), we suggest that this region is the active site of ongoing basal lamina assembly associated with fin regenerative outgrowth. Intriguingly, far distal regions had a more robust basement membrane than at the point of *shha:GFP* domain splitting (Fig. 7H-J). We propose that basal lamina gaps at the proximal/distal position defined by active Shha/Smo signaling enable direct interactions between Shha-positive epidermal cells and Ptch2-expressing pObs. These Hh/Smo-reinforced interactions coupled with epidermal movements then escort pObs into separated pools to initiate ray branching (Fig. 8).



**Fig. 7. *shha:GFP*-expressing basal epidermal cells extend cellular protrusions through incompletely assembled basement membrane to contact *Runx2*<sup>+</sup> progenitor osteoblasts.** (A–P) GFP (white), laminin (magenta) and *Runx2* (green) antibody stained fin sections from 96 hpa *shha:GFP* fish. All images are 1 airy unit (~1 μm) single optical confocal sections, except D, which is a structured illumination microscopy (SIM) image representing an ~100 nm section. (A–D) Longitudinal section showing the distal regenerate where the *shha*-expressing basal epidermis is split into two clusters and Hh/Smo signaling is activate in both pObs and basal epidermis. (C) An overlay showing *Runx2* staining together with A and B. (D) A high-magnification SIM image of the boxed area in C. (E–G) A proximal field from the same longitudinal section shown in A–D. (H–P) Transverse sections representing three positions along the proximal-distal axis from a single regenerating ray. (H–J) An extreme distal section beyond the distal extent of *Runx2*<sup>+</sup> pOb pools. (K–M) Section from a position where *shha:GFP*-expressing basal epidermis is split into two clusters on each side of the regenerating fin. (N–P) A further proximal section where *shha* expression has initiated in epidermal cells but prior to their division into split clusters. The dashed boxes in I, L and O mark regions shown at higher magnification in J, M and P, respectively. White arrows indicate cellular protrusions from *shha:GFP*<sup>+</sup> cells that contact or enshroud *Runx2*<sup>+</sup> osteoblasts. White asterisks mark gaps in the basal lamina. Magenta arrows show a continuous laminin-containing basement membrane that physically separates the basal epidermis from *Runx2*<sup>+</sup> pObs. Scale bars: 5 μm in D, J, M, P; 50 μm in C, G, I, L, O.

## DISCUSSION

### Active Hh/Smo signaling during fin regeneration is tightly restricted to distally migrating basal epidermis and adjacent osteoblast progenitor cells

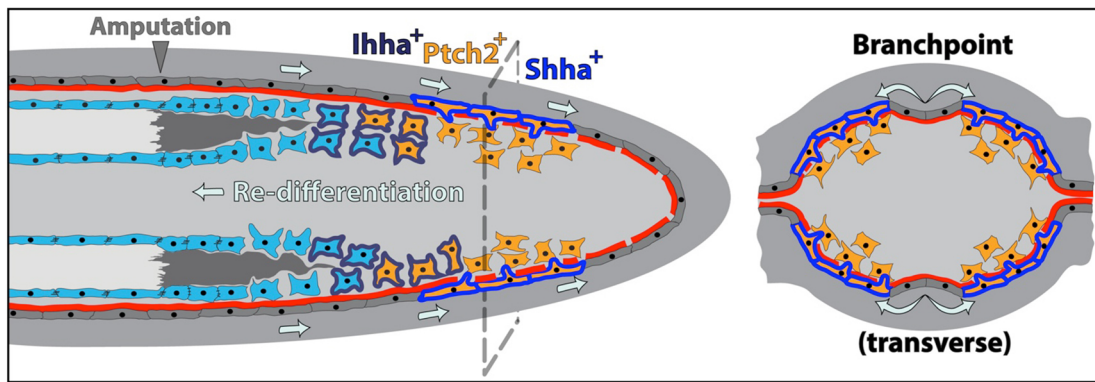
Our *ptch2:Kaede* photoconversion experiments monitoring Hh/Smo-responsive cells at the time of ray branching resolve active Hh/Smo signaling to a narrow stretch of distal basal epidermal cells and neighboring *Runx2*<sup>+</sup> pObs. This highly restricted nature of Hh/Smo-active cells in the regenerating fin suggests a short-range communication mode and tight pathway control that is inconsistent with the proposition that Hh/Smo signaling is a major regulator of proliferative outgrowth (Lee et al., 2009; Quint et al., 2002; Wehner et al., 2014). These experiments also highlight the distinct advantage afforded by photoconvertible reporter proteins when monitoring gene expression and cell signaling dynamics. Without photoconversion, *ptch2*-driven Kaede, a relatively stable protein, misleadingly appears to be broadly expressed in both the basal epidermis and in Obs. Furthermore, by effectively enabling lineage tracing, Kaede photoconversion shows that Hh/Smo activity is continuously maintained at the same relative position in both Ob and epidermal lineages, attributable to distal-migrating epidermis that provides a constant source of new *shha*-expressing cells.

The distal movement of basal epidermis, concurrent with regenerative outgrowth is consistent with the observation that the fin epidermis largely proliferates proximally to the site of amputation (Poleo et al., 2001). We propose this proximal expansion pushes continuously generated basal epidermal cells to displace distally. During this effective ‘migration’, basal epidermal cells transiently upregulate *shha* to generate a zone of active Hh/Smo signaling in both basal epidermis and adjacent Obs. Conversely, proliferative pObs in the active zone remain ‘in place’ as the regenerate extends distally. pObs that escape self-renewing Wnt signals similarly lose Hh/Smo activity while upregulating BMP and other differentiation pathways that promote them to progressively extend reforming bone (Stewart et al., 2014).

### Ihh promotes mineralization of regenerated bone through non-canonical signaling

Ihh promotes Ob proliferation and/or differentiation during bone development (Abzhanov et al., 2007; Huycke et al., 2012; Lenton et al., 2011; Long, 2012). Expanding on previous fin regeneration studies (Avaron et al., 2006), we found that *ihh* is expressed distinctly in pObs as they re-differentiate and acquire *sp7* expression but not in *Runx2*<sup>+</sup>/*sp7*<sup>-</sup> self-renewing pObs or fully differentiated





**Fig. 8. Model showing how cell movements, Hh/Smo pathway dynamics and direct cell-to-cell interactions between neighboring basal epidermal cells and progenitor osteoblasts induce ray bifurcation during fin regeneration.** Basal epidermal cells generated proximal to the caudal fin amputation site continuously advance distally along a mature basal lamina. During this effective migration, groups of basal epidermal cells overlying the regenerating blastema transiently upregulate *shha* expression (cells outlined in blue) and then split into two clusters on each side of the ray. *Shha* drives active Hh/Smo signaling marked by *ptch2* expression (orange cells) in a narrow distal zone of *shha*-expressing epidermal cells and adjacent progenitor osteoblasts (pObs). As fin outgrowth proceeds, some pObs escape self-renewal signals, initiate re-differentiation and progressively extend reforming bone. Fully differentiated Obs secrete new bone matrix, enabled by the earlier non-canonical activity of Ob-expressed *Ihha* (cells outlined in dark blue). The interface between Hh/Smo-responsive Obs and basal epidermis is the active site of basal lamina assembly associated with fin regenerative outgrowth. Coinciding with the epidermal 'branchpoint', this incompletely assembled basement membrane enables *shha*-expressing basal epidermal cells to extend cellular protrusions that contact neighboring pObs. This direct binding activates Smo-dependent signaling (including further *ptch2* expression) and progressively escorts pObs into physically separated pools. The newly divided pools of *Runx2*<sup>+</sup> pObs then continue regenerating independently to produce a now bifurcated ray.

*Runx2*<sup>-</sup>/*sp7*<sup>+</sup> Obs. This unique pattern implies a role for *Ihha* in early steps of bone re-differentiation. However, our analysis of fin regeneration in viable homozygous null *ihha* zebrafish demonstrates that *Ihha* does not control pOb proliferation, *sp7* expression, BMP signaling or organization of re-differentiated Obs. Instead, the deficient accumulation of calcified bone in *ihha*-null fish suggests *Ihha* functions in re-differentiating pObs to prime their later mineralization, likely acting in parallel with the BMP/*sp7* differentiation network.

*Ihha*<sup>-/-</sup>;*ptch2:Kaede* regenerating fins did not show decreased *Kaede* expression, indicating that Hh/Smo activity in the basal epidermis and Obs is driven solely by *Shha*, the only other appreciably expressed Hh ligand. Furthermore, given *ihha*-deficient zebrafish did show a bone maturation defect during fin regeneration that was not recapitulated by BMS-833923 exposure, *Ihha* may act by Smo-independent non-canonical Hh signaling (Jenkins, 2009). Finally, the modest expansion of *ptch2:Kaede* in *ihha*<sup>-/-</sup> regenerating fins suggests that upregulation of *Ihha* in differentiating Obs negatively regulates Hh/Smo output in surrounding cells to constrain pathway activity to the observed narrow distal zone. Expanded *shha:GFP* expression in BMS-833923-treated animals further indicates that negative-feedback networks restrict epidermal *shha* expression and therefore Hh/Smo output.

#### The Smo inhibitor BMS-833923 avoids the off-target anti-proliferative effects of cyclopamine in zebrafish

Cyclopamine and smoothened agonist exposure experiments suggest mitogenic Hh/Smo signaling supports regenerative fin outgrowth (Blum and Begemann, 2015; Lee et al., 2009; Quint et al., 2002; Wehner et al., 2014). However, our observation that only two small distal cell populations undergo active Hh/Smo signaling during fin regeneration is incongruous with this model. By screening recently developed Smo inhibitors, we discovered that BMS-833923 (Akare et al., 2014) is as effective as cyclopamine in blocking zebrafish Hh/Smo activity. However, BMS-833923 more faithfully recapitulates *smo* mutant phenotypes during development, including blocking development of muscle pioneer cells (Aanstad et al., 2009; Barresi

et al., 2000). Crucially, unlike cyclopamine, BMS-833923 did not inhibit cell proliferation during fin regeneration and, instead, dramatically and specifically abrogates ray branching. We conclude that the widespread anti-proliferative effects of cyclopamine on regenerating fins is not due to Hh/Smo inhibition but rather reflects unknown off-target effects of the compound.

A non-specific anti-proliferative effect of cyclopamine is consistent with the observation that ablation of *shha*-expressing basal epidermal cells does not affect overall regenerative outgrowth of the fin (Zhang et al., 2012). Consistent with cyclopamine having off-target effects in zebrafish, cyclopamine-induced aberrant migration of primordial germ cells occurs in a Smo-independent manner (Mich et al., 2009). Furthermore, high doses of cyclopamine ( $\geq 10 \mu\text{M}$ ) inhibit proliferation in cells that lack detectable Smo expression (Zhang et al., 2009). We advocate that the many studies using cyclopamine in zebrafish, including those that have led to fin regeneration models incorporating mitogenic roles of Hh/Smo signaling, be revisited and/or interpreted with caution.

#### *Shha*-expressing basal epidermal cells directly contact and recruit *Runx2*<sup>+</sup> osteoblast progenitors to promote ray branching

Inhibition of Hh/Smo signaling using BMS-833923 from 48–96 hpa strikingly blocks ray branching without disrupting regenerative bone growth or maturation. Given the distinct bone maturation defects in *ihha*<sup>-/-</sup> mutants and the unappreciable expression of other Hh ligands, we conclude that *Shha*-driven Hh/Smo signaling induces ray bifurcation. Consistent with this notion, ablation of *shha*-expressing basal epidermal cells significantly delays ray branching (Zhang et al., 2012). However, using PCNA staining to identify cycling cells, Zhang et al. concluded that the *shha:GFP*-expressing basal epidermis induces localized Ob proliferation to direct ray branching. In contrast, by quantitative EdU incorporation studies, we found that *Runx2*<sup>+</sup> pOb proliferation at the ray branching site is unaffected by Hh/Smo inhibition. Furthermore, pOb proliferative rates are independent of proximity to *Shha*-

expressing epidermal cells. However, in the absence of Shha/Smo signaling, pObs, but not *shha:GFP*<sup>+</sup> basal epidermal cells, fail to form two spatially distinct pools that precede branching events. Therefore, Shha/Smo signaling promotes ray branching by directing the cellular migration rather than localized proliferation of pObs.

Our observation that *shha:GFP*-expressing basal epidermal cells directly contact underlying pObs, including through cellular protrusions, suggests Shh/Smo-promoted pOb migration is driven by simple and direct intercellular interactions. A model that very short-range signaling by Shha drives ray branching conflicts with the widely held paradigm that Shh works as a long-range morphogen [e.g. to pattern the spinal cord (Briscoe and Théron, 2013)]. However, several other recent studies demonstrate exceptions to this rule. In the embryonic chick limb, Shh remains tightly associated with its producing cells and likely acts through its retention on long cytoplasmic extensions (Sanders et al., 2013). In fly imaginal discs, localized Hh acts as a short-range signal (Ayers et al., 2010). At the simplest level, membrane-retained epidermal Shha may interact with Ptc2 on pObs to produce the observed cell-cell adhesion that promotes bone branching. As such, ‘positive’-feedback activation of *ptch2* to reinforce cell interactions could be the major or even only relevant target gene of Shha/Smo signal transduction. Alternatively, Hh/Smo activation in both epidermal and pObs could direct the transcriptional upregulation of more traditional cell-adhesion molecules. Regardless, our demonstration of direct cell-cell Shh-promoted interactions during osteoblast patterning suggests similar mechanisms underlie Hh/Smo signaling roles in other regeneration, developmental or disease contexts.

#### Incompletely assembled basement membrane enables localized Ob recruitment by epidermal cellular protrusions

The physical separation of epidermis and mesenchyme by basement membrane in the zebrafish fin is thought to provide spatially restricted and efficient epithelial-mesenchymal signaling during development and regeneration (Lee et al., 2009; Tornini and Poss, 2014). Recent work establishes that Lamb1a is a major component of the basal lamina in the regenerating caudal fin and its function is required to ensure polarity of the basal epithelium during regeneration (Chen et al., 2015). We demonstrate that direct basal epidermis-pOb interactions are enabled by the lack of a robust Lamb1a-containing basement membrane at the forming branch site. In further support, electron microscopy studies show an irregular epidermal-blastemal interface towards the distal tip of regenerating fins where basal epidermal cells extend cellular processes (or ‘digitations’) that contact underlying mesenchyme (Becerra et al., 1996; Géraudie and Singer, 1992). We suggest these distal basal lamina gaps reflect the active site of basement membrane extension that is associated with regenerative outgrowth. Newly synthesized Lamb1a progressively self-organizes to help establish a robust basement membrane that precludes prolonged contacts between the basal epidermis and re-differentiating pObs. Notably, the relatively more robust distal-most basal lamina could reflect persistent material established by the wound epidermis at the onset of regeneration.

#### Modular and orthogonal signaling networks cooperate to regenerate functional bone

Our study of Hh/Smo signaling during zebrafish fin regeneration illustrates how distinct signaling networks can produce independent modules that cooperate to regenerate a bone of the proper size and shape. A Wnt/BMP network establishes a system of balanced pOb growth and differentiation that allows the progressive reformation of mature bone (Stewart et al., 2014). Simultaneously, the Shh/Ptc2/

Smo network acts orthogonally to control the shape of the bone by periodically splitting regenerating pObs into physically separated pools. Of relevance for regenerative medicine, these signaling networks are likely conserved but tightly restrained in adult mammals, including humans. Notably, Shh signaling has long been appreciated to control bone patterning during chick and mouse limb development (Capdevila and Izpisua Belmonte, 2001; Riddle et al., 1993). Therefore, the localized delivery of Shh, perhaps immobilized on scaffolds, could guide therapeutic osteoblast stem cells expanded by Wnt signaling to reshape severely damaged or diseased bone.

## MATERIALS AND METHODS

### Zebrafish

*Danio rerio* wild-type AB, *ihhd*<sup>hu2131</sup> (Hammond and Schulte-Merker, 2009), *Tg(sp7:EGFP)b1212* (DeLaurier et al., 2010), *TgBAC(ptch2:Kaede)a4596* (Huang et al., 2012) and *Tg(-2.4shha:gfp:ABC)sb15* [previously known as *Tg(-2.2shha:gfp:ABC)* (Ertzer et al., 2007; Shkumatava et al., 2004) lines were maintained at 28–29°C (Westerfield, 2007). All experiments were approved by the University of Oregon Institutional Animal Care and Use Committee (IACUC). The *ihhd*<sup>hu2131</sup> allele was genotyped by PCR (primers: 5'-CTGT-GCCACCGTCACTC-3' and 5'-GCTACATTGGACTAAACTGCAT-3') with subsequent *NspI*-mediated digestion of the PCR products. Adult zebrafish were anesthetized by immersion in water containing 0.6 mM Tricaine-s (Western Chemical). Caudal fins were amputated two segments proximal to the first branch point of the fin rays using a razor blade.

### Kaede photoconversion and imaging

*ptch2:Kaede* fish were anesthetized and immediately viewed on a glass slide using a Nikon Eclipse Ti wide-field inverted microscope. A desired field containing Kaede-expressing tissue was photoconverted by a 2-min illumination using a DAPI filter set. For the experiments in Fig. 1A–E, Kaede was photoconverted using a 405 nm laser for 2 min. Fish were left in the dark for indicated periods and then re-imaged for both green and red Kaede fluorescence. Imaging used either a Nikon Eclipse Ti wide-field inverted microscope or a Leica M165 FC stereomicroscope, except for the experiments in Fig. S2A–H, where optical section *z*-stacks were collected using a Zeiss LSM 880 laser scanning confocal microscope. For further details, see the supplementary Materials and methods.

### Small-molecule treatments

BMS-833923 (Cayman Chemical), cyclopamine (LC Labs), Vismodegib (Chemie Tek), Erismodegib (Chemie Tek), SANT-1 (Cayman Chemical), Taladegib (Cayman Chemical), Glasdegib (Selleck Chemicals) and PF-05274857 (Cayman Chemical) were dissolved in DMSO. Immediately prior to intraperitoneal injections, stock solutions were diluted 1:10 in injection buffer (50% PEG-400, 5% Propylene Glycol, 0.5% Tween-80). Unless otherwise noted, doses were: BMS-833923, 40 mg/kg; cyclopamine, 20 mg/kg; Vismodegib, 75 mg/kg; Erismodegib, 50 mg/kg; SANT-1, 50 mg/kg; Taladegib, 8.25 mg/kg; Glasdegib, 50 mg/kg; and PF-05274857, 100 mg/kg. For each treatment, cohorts of at least five animals were used.

### Section immunostaining

Immunostaining was performed on frozen or paraffin wax-embedded sections using the indicated antibodies and then visualized by confocal microscopy. For further details, see the supplementary Materials and methods.

### Whole-mount fin fluorescent imaging

EGFP and Kaede expression in intact fins was detected by epifluorescence on a stereo or inverted wide-field microscope. For further details, see the supplementary Materials and methods.

### Quantitative RT-PCR

Quantitative changes in gene expression were determined by real-time PCR using cDNA prepared from total RNA extracted from regenerating fins. For further details, see the supplementary Materials and methods.



### Fluorescent *in situ* hybridization

RNA *in situ* hybridizations used digoxigenin-labeled antisense RNA probes and frozen fin sections. For further details, see the supplementary Materials and methods.

### Histological staining

Histological staining was performed on frozen or paraffin wax-embedded fin sections followed by imaging using bright-field microscopy. For further details, see the supplementary Materials and methods.

### Embryo small molecule treatments

*ptch2:Kaede* embryos (1.5 hpf) were exposed to compounds or to vehicle added directly to the fish water. For further details, see the supplementary Materials and methods.

### Embryo immunostaining

Fixed and processed embryos were incubated overnight with the indicated antibodies, developed and imaged by confocal microscopy. For further details, see the supplementary Materials and methods.

### *In vivo* 5-ethynyl-2'-deoxyuridine (EdU) labeling

Fish were injected intraperitoneally with EdU, which was subsequently detected on fin sections using the Click-iT proliferation assay kit (Thermo Fisher). For further details, see the supplementary Materials and methods.

### Statistical analyses and replicates

Student's *t*-tests and Fisher's exact tests were used to determine statistically significant differences between sample sets. For further details, see the supplementary Materials and methods.

### Structured illumination microscopy (SIM)

Super-resolution structured illumination (SR-SIM) on antibody stained fin sections was performed using a Zeiss ELYRA S.1. microscope. For further details, see the supplementary Materials and methods.

### Cre/lox-labeled epidermal mosaic fish

For further details, see the supplementary Materials and methods.

### Acknowledgements

We thank the University of Oregon Zebrafish Facility for animal care; C. Kimmel for *Tg(sp7:EGFP)b1212* and *ihha<sup>hu2131</sup>* fish; S. Megason for providing the *TgBAC(ptch2:Kaede)a4596* fish developed in A. Schier's lab and the *Tg(-2.4shha:gfp:ABC)sb15* fish developed in U. Strahle's lab; J. Postlethwait for *shha* and *ihha* cDNA plasmids; K. Poss for the Lamb1a antibody staining protocol; G. Yette for generating epidermal mosaic fish; and J. Nichols, C. Kimmel, C. Doe and the Stankunas lab for input.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

B.E.A., S.S. and K.S. designed experiments; B.E.A., A.H. and S.S. performed experiments; B.E.A., S.S. and K.S. prepared and wrote the manuscript.

### Funding

B.E.A. was supported by a National Institutes of Health training grant from the National Institute of General Medical Sciences (5T32GM007413). The National Institutes of Health provided research funding (1R01HL115294 from the National Heart, Lung, and Blood Institute to K.S. and 5R03AR067522 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases to S.S.). Deposited in PMC for release after 12 months.

### Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.143792.supplemental>

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