Basal epidermis collective migration and local Sonic hedgehog signaling promote fin skeletal branching morphogenesis

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Basal epidermal Sonic hedgehog (Shh) signals short-range to progenitor osteoblasts to branch fin rays

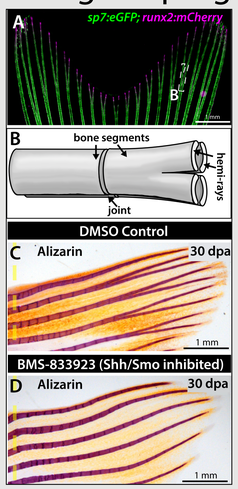

Fig. 1

Zebrafish caudal fins robustly develop and regenerate elaborately branched bony ray skeletons (**Fig. 1A**). Each bony ray is comprised of two hemi-rays and segmented by joints (**Fig. 1B**). Rays are enveloped in a multilayered epidermis. We recently showed Sonic hedgehog (Shh) signaling specifically promotes ray branching during adult caudal fin regeneration (**Fig. 1C, D**) by basal epidermal-initiated signaling that directs adjacent progenitor osteoblasts (pObs) into split pools (Armstrong et al., 2017). We investigated if and how Shh signaling similarly functions during developmental ray branching. We characterized Shha and receptor Ptch2 expression and activity in distal outgrowing regions of fin rays. We used the small molecule BMS-833923 (hereafter termed BMS), which chemically inhibits the downstream Hh target Smo, to demonstrate sustained Shh/Smo signaling is required for branching in all fins. Live time-lapse imaging and cell migration tracking revealed Hh/Smo signaling slows the migration of basal epidermal cells by apparent tethering to pObs. We conclude short-range Shh signaling positions pObs to form branch points by restraining collective migration of the basal epidermis.

Shh+ basal epidermal domains split followed by heterotypic Ptch2+ cells in branch point development

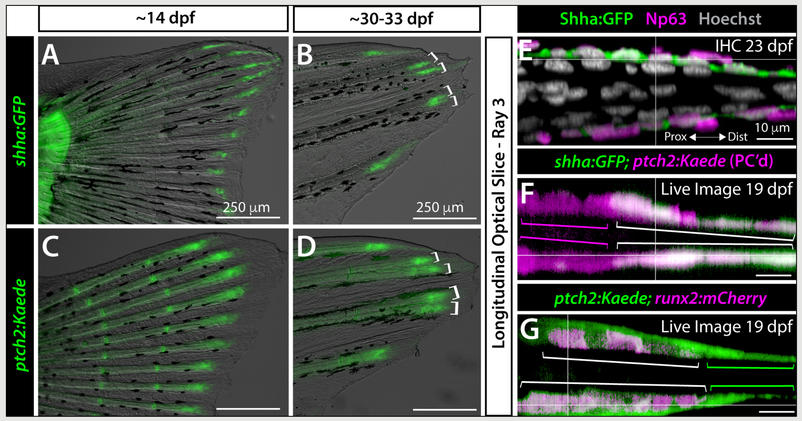

Fig. 2 Shha expression in the developing caudal fin is restricted to distal fin basal epidermis (bEp) in 14 dpf mid-larvae (A) until splitting immediately preceding ray branching (B) in 30-33 dpf juveniles. *ptch2:Kaede*, which reports on Hh-responsive cells and pathway activity, is expressed along ray lengths at 14 dpf (C) and follow splitting Shh+ bEps at 30-33 dpf (D). (E-G) Single optical slices of ray 3 distal regions. *shha:GFP* co-localizes with bEp marker Np63. *ptch2:Kaede* co-localizes with Shh+ bEps and Runx2+ progenitor osteoblasts (pObs).

Active Shh/Smo signaling is restricted to outgrowing distal ray regions

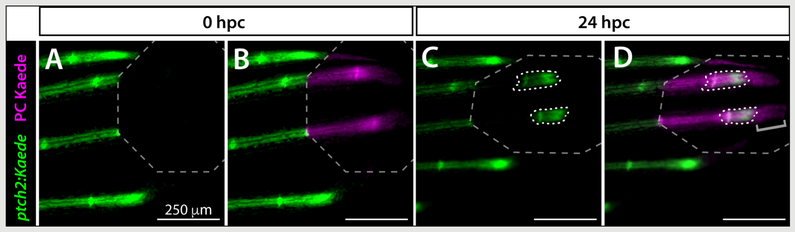

Fig. 3 The caudal fin of a 28 dpf *ptch2:Kaede* fish immediately after permanent photoconversion (0 hours post photoconversion, 0 hpc) in a distal region of interest (grey octagon, A, B). All green-emitting Kaede protein (A) is fully converted to red (B). 24 hours later (24 hpc), new green Kaede protein within the photoconverted ROI is expressed in bEps and pObs of distal outgrowing ray regions (C, D). Photoconverted bEps are distally displaced (grey brackets, D) to accommodate newly produced Kaede domains.

Shh-mediated ray branching is conserved in all fins

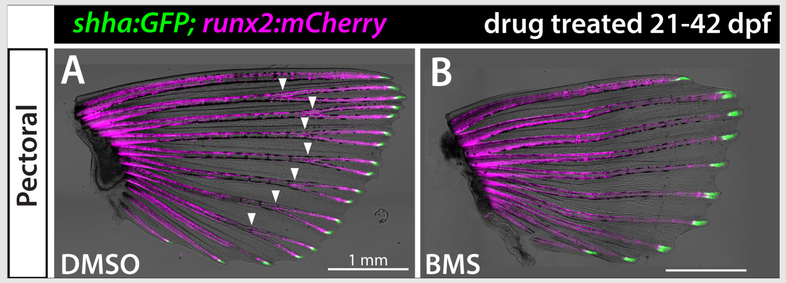

Fig. 4 Developing *shha:GFP;runx2:mCherry* pectoral fins treated with BMS (B) do not form branched rays as compared to DMSO-treated controls (A, *n*=6 per group). *runx2:mCherry* is expressed in progenitor osteoblasts (pObs) and perdures along ray lengths. Shh+ bEps and Runx2+ pObs are expressed in distal ray regions of all fins (caudal, dorsal, anal, pelvic, and pectoral). Ray branching of all fins is inhibited by BMS (data not shown).

Sustained Shh/Smo signaling promotes ray branching during fin development

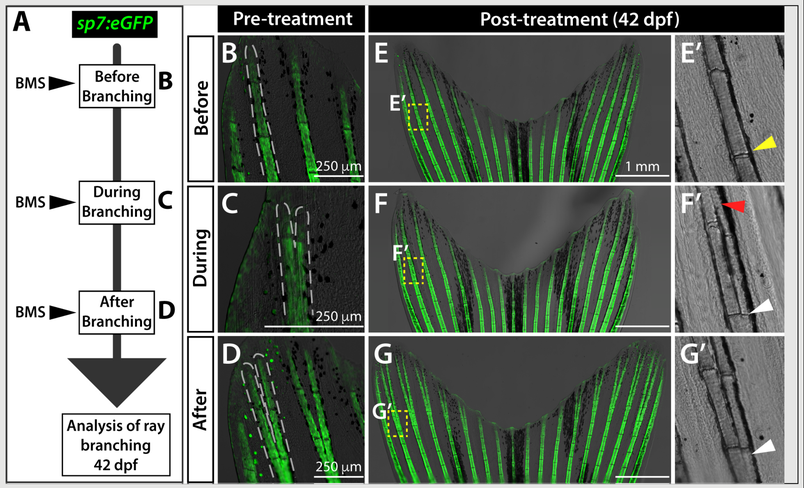

Fig. 5 Staggering treatment of *sp7:EGFP* caudal fins with BMS (schematic in A) to before (B), during (C), and after branching (D) reveals semi-branched rays treated with BMS re-fuse back into one ray (F, red arrowhead in F'), whereas 'before' fins remain unbranched (E, E', yellow arrowhead indicates lack of typical branching) and 'after' fins remain branched (G, G'). White arrowheads in I' and K' designate the start of branching. *n*= 4-5 per group.

Shh+ basal epidermis and progenitor osteoblasts are closely associated in live, developing rays

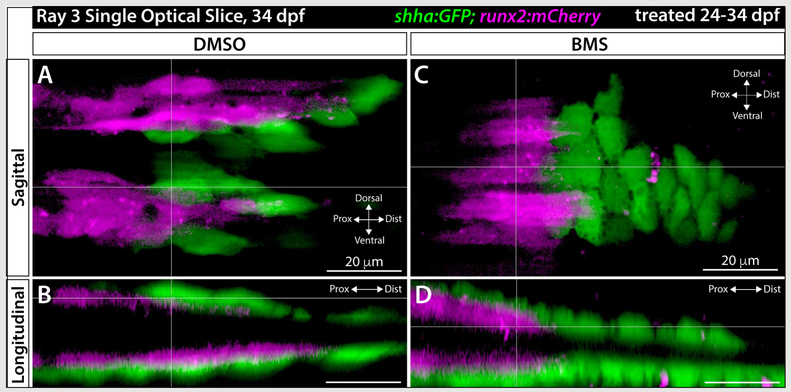

Fig. 6 Confocal single optical slices of dorsal ray 3 in live 34 dpf *shha:GFP;runx2:mCherry* fish. Shh+ bEps overlying each hemi-ray of Runx2+ pObs share extensive surface contacts (A, B) that are not disrupted by BMS treatment (C, D).

Shh signaling restrains collective migration of bone-associated basal epidermal cells

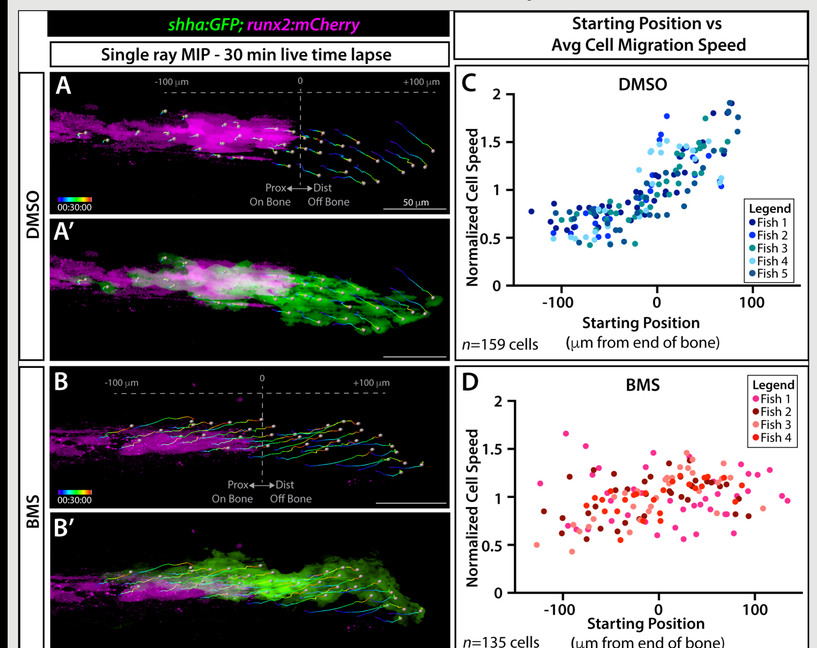

Fig. 7 Dorsal Ray 3 MIP of representative 23 dpf *shha:GFP;runx2:mCherry* fish at the end of 30 min time lapse imaging (A, A', B, B'). Colored tracks show displacement of individual *Shha:GFP+* bEps over 30 minutes. Grey dashed lines indicate the end of the ray as marked by the most distal Runx2+ pOb (Position "0"). *n*=159 *Shha:GFP+* bEps (grey spheres, A-A') from 5 fish were tracked from DMSO-treated fish and *n*=135 from 4 BMS-treated fish. Cell starting positions (X-displacement from most distal pOb in microns at time 0 min) are plotted against individual cell speeds (C, D). *Shha:GFP+* bEps located past the end of the bony ray in control fish undergo a rapid increase in migration speed (C) which is not apparent in BMS-treated fish (D). Colors represent cells from the same fish and different colors indicate different fish.

Conclusions & Future Directions

In this study we confirm Shh signaling is required for developmental fin ray branching as it is in regeneration. We demonstrated Shh is expressed in a subpopulation of basal epidermal cells (bEps) located immediately adjacent to progentior osteoblasts (pObs) in distal growing ray regions (Fig. 8). Shh+ bEps act locally on responsive Ptch2+ bEps and pObs. Chemical inhibition using BMS revealed a sustained requirement for Shh/Smo signaling in ray branching of all fins, suggesting Shh-directed ray branching precedes the evolution of paired fins. We used live imaging to show Shh+ bEps and pObs share direct and extensive surface contacts. Time lapse imaging and cell tracking revealed Shh+ bEps overlaying pObs have slowed rates of distal migration compared to Shh+ bEps located past the bony ray's distal end. We conclude localized heterotypic interactions between Shh+ bEps passing over distal pObs restrain their migration and slowly displace pObs into split pools to form branch points.

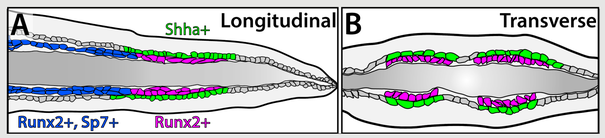

Fig. 8 Schematic sections of a branching ray. Shh+ bEps (green) are adjacent to Runx2+ pObs (magenta) of either hemi-ray. Proximally maturing bone is marked by Runx2/Sp7.

How does collective epidermal migration direct skeletal patterning? We propose a model in which heterotypic adhesion between Shh+ bEps and pObs enables skeletal positioning. We hypothesize the Shh ligand is membrane-retained instead of secreted and may itself act as an adhesive molecule with Ptch2+ pObs. We plan to test this hypothesis with primary cell culture adhesion assays. We are additionally investigating how *shha* is locally activated.

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