PNAS
www.pnas.org

Supplementary Information for

An Fgf-Shh positive feedback loop drives growth in developing unpaired fins.

M. Brent Hawkins[1], David Jandzik[1], Frank J. Tulenko, Amanda N. Cass, Tetsuya Nakamura, Neil H. Shubin, Marcus C. Davis, David W. Stock

[1]Co-corresponding authors: M. Brent Hawkins, David Jandzik

Email: MichaelBrent.Hawkins@childrens.harvard.edu; david.jandzik@uniba.sk

This PDF file includes:

Supplementary Information Text

Extended Methods

Animal sources and care

Channel catfish: Newly fertilized channel catfish were purchased from Osage Catfisheries (Osage Beach, MO). Embryos were raised in a recirculating incubator system at a temperature of 26.6°C with constant water flow agitation to mimic aeration by the male parent. Staging of embryos and larvae was performed following Armstrong and Child (1). Experiments were assessed and approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee (IACUC).

American paddlefish: Fertilized eggs were purchased from Osage Catfisheries (Osage Beach, MO) and raised in freshwater tanks at 18°C following Tulenko *et al*. (2). Experiments were assessed and approved by the Kennesaw State University and James Madison University IACUCs.

Little skate: Fertilized eggs were purchased from Marine Biological Laboratory (Woods Hole, MA) and fixed by 4% PFA at 4°C for whole mount *in situ* hybridization. Experiments were approved by the University of Chicago IACUC.

Pharmacological treatments

SU5402 is an inhibitor of Fgf signaling (3). Solid SU5402 (Tocris, Catalog No. 3300) was resuspended in dimethylsulfoxide (DMSO) to a concentration of 10 mM and stored in aliquots at -20°C. Cyclopamine is an inhibitor of Hh signaling (4). Solid cyclopamine (LC Labs, Product No. C-8700) was resuspended in 100% ethanol to a concentration of 10 mM and stored in aliquots at -20°C. Pharmacological treatments were carried out in Nunc 24-well tissue culture plates (ThermoFisher, Catalog No. 144530) in an incubator set to 26.6°C. During treatment, catfish larvae were maintained at a density of one individual per well in 1 mL of 30% Danieau's medium. For the 50 μM drug treatments, media for treated animals contained inhibitor plus 1% vehicle (DMSO for SU5402 and 100% ethanol for cyclopamine) while control animal media contained 1% vehicle only. Animals were treated for 8 hours beginning at Stage 39, then euthanized by MS222 overdose and processed for *in situ* hybridization. For the lower dosage experiments (10 μM cyclopamine and 25 μM SU5402) to assess morphology, final vehicle concentration for control and treatment animals was 0.5%. Following treatment with the lower dosages for 8 hours beginning at Stage 39, animals were raised to older ages to examine skeletal development.

Fixation and *in situ* hybridization

Channel catfish: After euthanasia, animals were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Following fixation, animals were washed twice for 5 minutes each in PBS plus 0.1% Tween, then washed twice for 5 minutes each in 100% methanol, and then stored at -20°C in fresh 100% methanol. *In situ* hybridization was performed following Jackman *et al*. (5) with minor modifications. For riboprobe synthesis, RNA was extracted from two 14-day old juveniles using the Promega RNAwiz kit and cDNA was synthesized using the Invitrogen SuperScript II kit with random hexamer primers. The degenerate primer pair 5'-GCNTAYAARCARTTYATHCC-3' and 5'-SWRTACCARTGNACNCCRTC-3' was used to amplify a 1013 bp region of *shha* and the degenerate primer pair 5'-TTYGCNTTYTGYTAYTAYGC-3' and 5'-GGRTARTTDATRAARTCRAA-3' was used to amplify a 540 bp region of *fgf8a* for cloning and probe synthesis.

American paddlefish: Embryos and larvae were fixed overnight in Carnoy's solution and then dehydrated to 100% ethanol and stored at -20°C. *In situ* hybridization was performed following

Tulenko *et al*. (2). Cloning and synthesis of riboprobes for *shh* and *fgf8* are described in Davis *et al*. (6) and Tulenko *et al*. (7).

Little skate: To generate *in situ* hybridization probe complementary to *fgf8*, a partial coding sequence (nucleotides 53-561 of GenBank EU574737.1) was amplified from stage 29 cDNA by PCR and cloned into pCR™II-TOPO® vector (Invitrogen). *In situ* hybridization was performed as previously described (8).

Skeletal staining

After fixation as described above, channel catfish juveniles were cleared and stained for cartilage with Alcian Blue and mineralized tissue with Alizarin Red following the protocol of Miyake and Hall (9).

Cryosectioning

After whole mount *in situ* hybridization skate embryos were refixed using 4% PFA and preserved until sectioning. The embryos were briefly rinsed two times with PBS plus 0.1% Tween and then stepped through PBS washes with increasing sucrose concentrations of 10, 15, and 20% of sucrose for two hours each. After the 20% sucrose wash, the solution was replaced with O.C.T. compound (Sakura) and the embryos were frozen and sectioned at 8 µm.

SI References

1. P. B. Armstrong, J. S. Child, *Stages in the development of Ictalurus nebulosus* (Syracuse University Press, 1962).
2. F. J. Tulenko, *et al.*, HoxD expression in the fin-fold compartment of basal gnathostomes and implications for paired appendage evolution. *Sci. Rep.* **6**, 22720 (2016).
3. M. Mohammadi, *et al.*, Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955–960 (1997).
4. M. K. Cooper, J. A. Porter, K. E. Young, P. A. Beachy, Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603–1607 (1998).
5. W. R. Jackman, B. W. Draper, D. W. Stock, Fgf signaling is required for zebrafish tooth development. *Dev. Biol.* **274**, 139–157 (2004).
6. M. C. Davis, R. D. Dahn, N. H. Shubin, An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish. *Nature* **447**, 473–476 (2007).
7. F. J. Tulenko, *et al.*, Fin-fold development in paddlefish and catshark and implications for the evolution of the autopod. *Proceedings of the Royal Society B: Biological Sciences* **284**, 20162780 (2017).
8. J. A. Gillis, *et al.*, Electrosensory ampullary organs are derived from lateral line placodes in cartilaginous fishes. *Development* **139**, 3142–3146 (2012).
9. T. Miyake, B. K. Hall, Development of in vitro organ culture techniques for differentiation and growth of cartilages and bones from teleost fish and comparisons with in vivo skeletal development. *J. Exp. Zool.* **268**, 22–43 (1994).