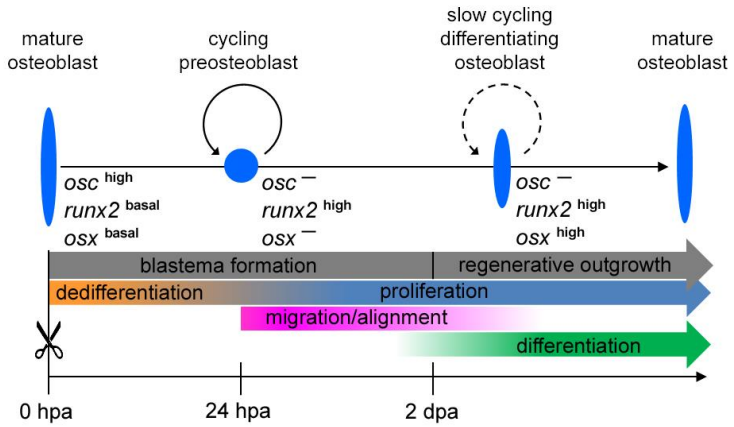

Fig. S1. Overview of osteoblast differentiation states during fin regeneration.

Wound epidermis formation, which overlaps with blastema formation, is not shown.

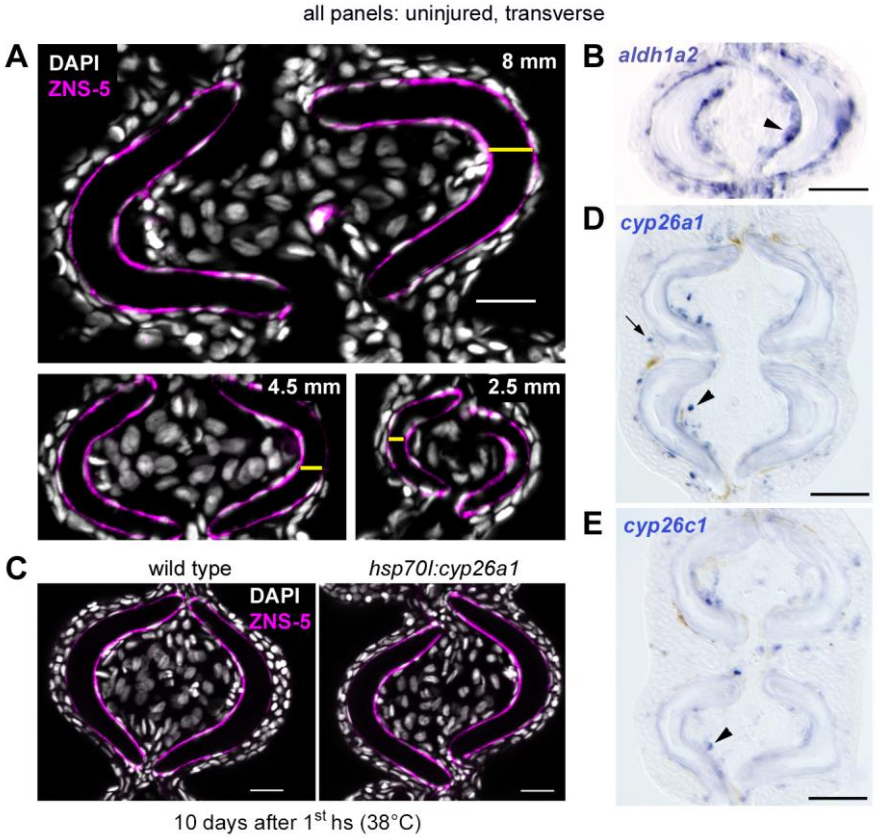

Fig. S2. Hemiray thickness increases during fin growth; RA signaling is not required for osteoblast survival.

(A) Sections of uninjured fins of different fin lengths reveal correlation between fin length and hemiray thickness. Fin lengths: 8, 4.5 and 2.5 mm. Yellow bars: Hemiray thickness. (B) ISH demonstrates expression of *aldh1a2* in proximity to hemirays (arrowhead). (C) Inhibition of RA signaling does not interfere with survival of mature osteoblasts. IHC for ZNS-5 demonstrates a similar number of osteoblasts in wild type and *hsp70l:cyp26a1* fish upon 10 days of heat-shock treatment. (D and E) ISH demonstrates expression of *cyp26a1* (D) and *cyp26c1* (E) in single epidermal (arrow) and mesenchymal (arrowhead) cells in uninjured fins. Scale bars: 20 µm in A, C; 50 µm in B, D and E. hs, heat-shock.

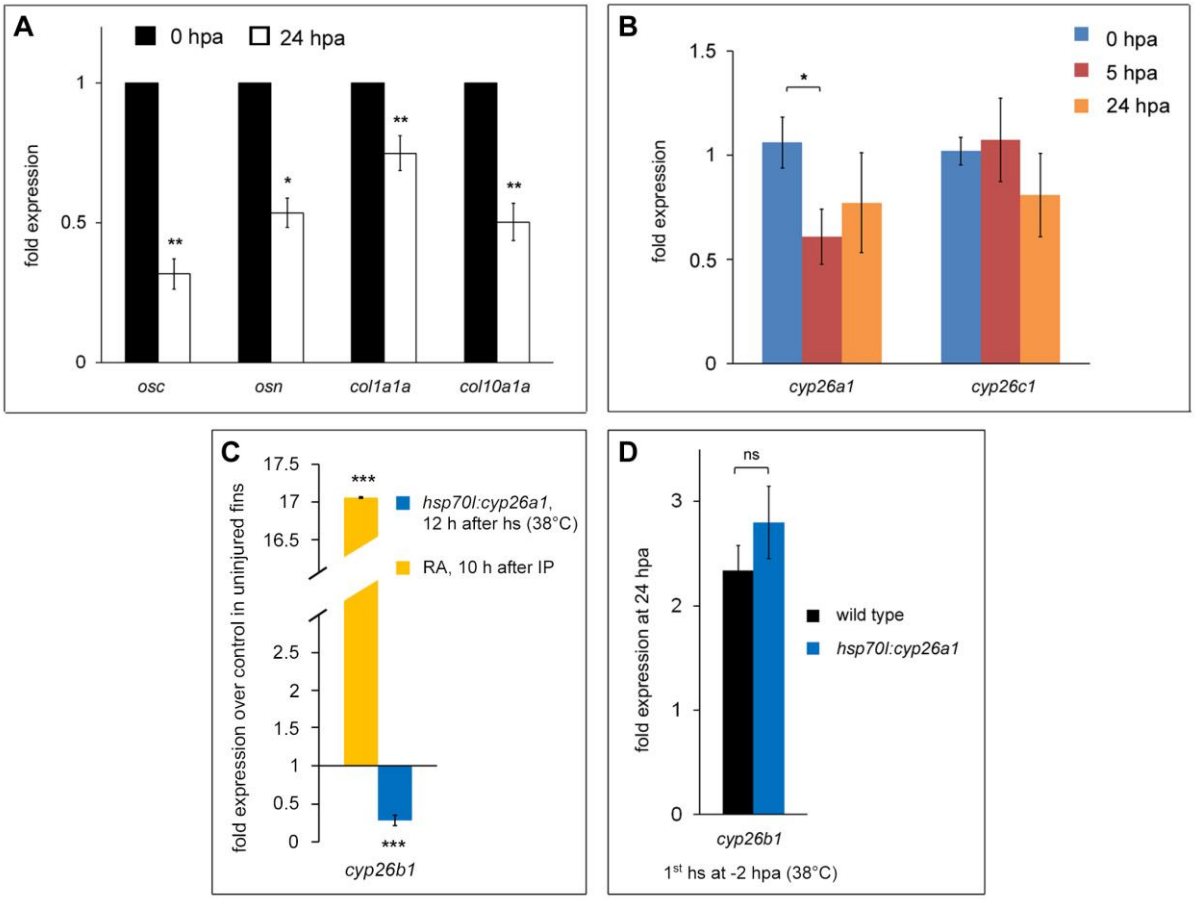

Fig. S3. Stump osteoblasts downregulate bone matrix genes; upregulation of *cyp26b1* does not require RA signaling.

(A) Fin amputation causes downregulation of bone matrix genes. qPCR analysis at 0 and 24 hpa. (B) Expression of *cyp26a1* and *cyp26c1* is unchanged or temporarily downregulated upon fin amputation. qPCR analysis at different time points after amputation. All not significant unless noted otherwise. (C) RA injection upregulates expression of *cyp26b1* in uninjured fins. Inhibition of RA signaling in *hsp70l:cyp26a1* fish downregulates expression. qPCR analysis. (D) Inhibition of RA signaling in *hsp70l:cyp26a1* fish does not prevent *cyp26b1* upregulation upon fin amputation. qPCR analysis at 24 hpa. Data are represented as mean±s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant. h, hours. hs, heat-shock.

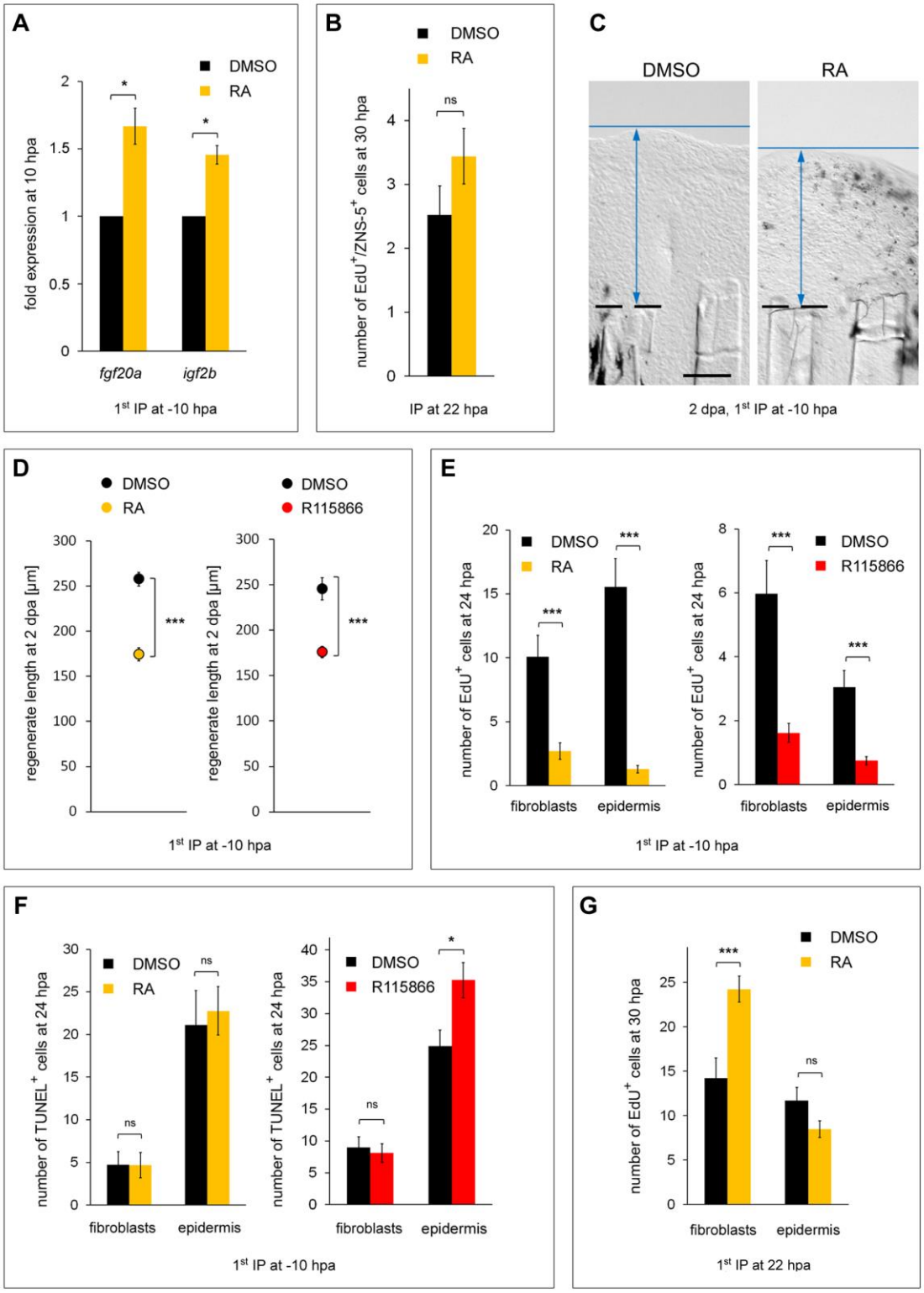

Fig. S4. Stump osteoblasts require Cyp26b1 activity for dedifferentiation but not for subsequent proliferation

(A) RA injections starting at -10 hpa do not prevent upregulation of *fgf20a* and *igf2b* expression in the fin stump. qPCR analysis at 10 hpa. (B) Proliferation of stump osteoblasts

is unaffected upon injection of RA at 22 hpa. $EdU^+/ZNS\text{-}5^+$ cells per section at 30 hpa. (C-F) Both RA and R115866 injections starting at -10 hpa slow down regeneration (C and D) and negatively impact proliferation of fibroblasts and epidermal cells (E), but do not increase cell death (F). (C) Fixed regenerates of RA-injected and control fish at 2 dpa. (D) Regenerate length of RA- and R115866-injected fish. (E and F) EdU^+ (E) or $TUNEL^+$ (F) cells per section at 24 hpa. (G) RA injection at 22 hpa promotes proliferation of fibroblasts. EdU^+ cells per section at 30 hpa. Data are represented as mean±s.e.m. *$p < 0.05$, ***$p < 0.001$. ns, not significant. Dashed lines indicate amputation plane.

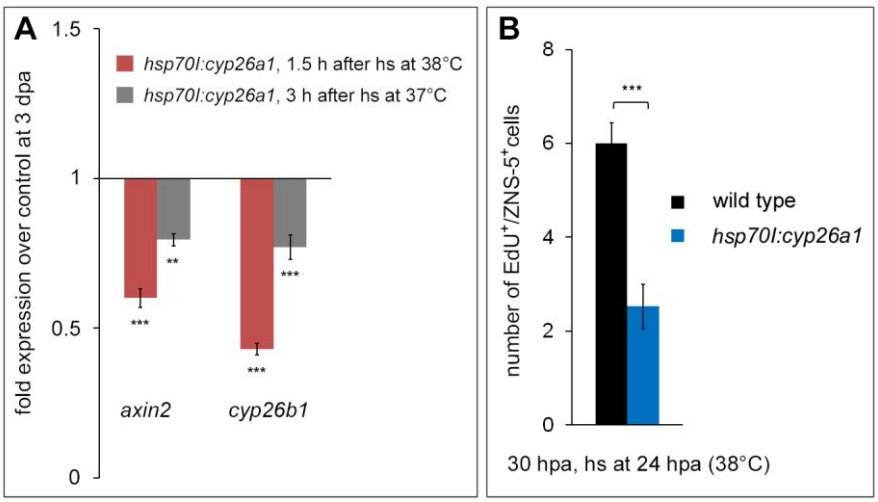

Fig. S5. Osteoblast proliferation in the stump requires RA signaling.

(A) Comparison of *axin2* and *cyp26b1* downregulation during regenerative outgrowth between *hsp70l:cyp26a1* fish that received a heat-shock at 37°C and *hsp70l:cyp26a1* fish that received a 38°C heat-shock. qPCR analysis at 3 dpa. (B) Inhibition of RA signaling in *hsp70l:cyp26a1* fish causes downregulation of osteoblast proliferation in the stump. $EdU^+/ZNS\text{-}5^+$ cells per section at 30 hpa. Data are represented as mean±s.e.m. **p < 0.01, ***p < 0.001. hs, heat-shock.

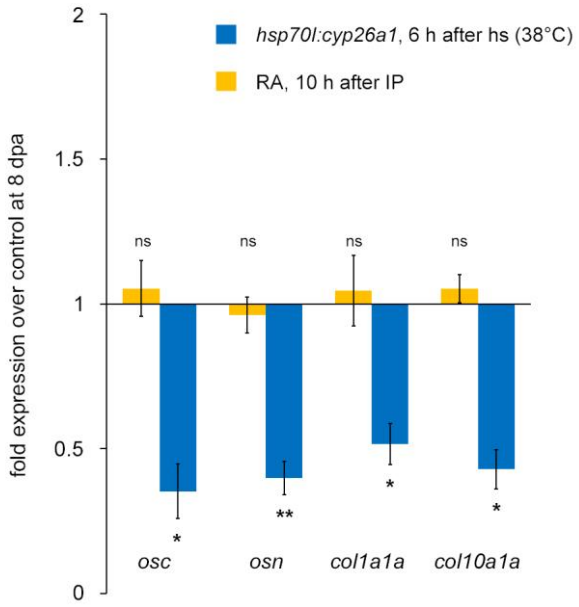

Fig. S6. Expression of bone matrix genes at 8 dpa requires RA signaling.

Inhibition of RA signaling in *hsp70l:cyp26a1* fish at 8 dpa causes downregulation of *osc*, *osn*, *col1a1a* and *col10a1a* expression. Conversely expression levels are unchanged in RA injected fish. qPCR analysis. Data are represented as mean±s.e.m. *p < 0.05, **p < 0.01. ns, not significant.

Supplementary Materials and Methods

qPCR analysis

For RNA extraction from uninjured fins, a 1 mm wide tissue stripe from the middle of the fin was harvested. For RNA extraction from 0-24 hpa, tissue within 1 mm proximal to the amputation plane was harvested. At 3, 4 and 8 dpa, tissue distal to the amputation plane was used. To analyze expression levels of bone matrix genes at 4 or 8 dpa, *osx:gfp* and *osc:gfp* (or *hsp70l:cyp26a1*, *osx:gfp* (*osc:gfp*) double transgenic) fish were used and the distal GFP-free tissue was carefully removed prior to RNA extraction by using an injection needle. Tissues from 4-10 fins were pooled for each RNA sample. Total RNA was extracted with Trizol reagent (Invitrogen) or TriPure (Roche) and treated with DNase I. Equal amounts of total RNA from each sample were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) or Maxima Reverse Transcriptase (Thermo Scientific) using anchored oligo(dT) primers. 3-4 RNA samples were reverse transcribed per Experiment (Table S2 and S3). Quantitative real-time PCR (qPCR) was performed using a C1000 thermal cycler combined with a CFX96 real-time PCR detection system (Bio-Rad) and Maxima SYBR Green qPCR Master Mix (Thermo Scientific). Primers are listed in Table S1. qPCR reactions for each cDNA pool and each target gene were performed in triplicate. qPCR data were analyzed using the CFX Manager software (Bio-Rad). *ef1a*, *tbp* and *actb1* were used as reference genes. Expression levels were normalized to expression levels of two reference genes (expression stability: Mean M <0.5) and expression ratios were calculated relative to control samples. Uninjured fins (= 0 hpa) were used as control for comparisons of expression levels between different time points. Regenerates of heat-shocked non-transgenic siblings were used as control if expression levels were examined in *hsp70l:cyp26a1* fish and regenerates of vehicle treated fish were used as control if expression levels were examined in RA- or R115866 treated fish. Reference genes were used in different combinations, depending on the treatment condition and regeneration stage. If normalization to different reference genes gave conflicting results (expression stability:

Mean M ≥0.5), results were verified by normalization to the input RNA amount by performing RiboGreen or Qubit assays (Invitrogen).

Imaging and measurements

Images were captured with the Zeiss AxioVision or Zeiss ZEN software on a Zeiss Stemi 2000-C stereomicroscope equipped with an AxioCam ERc5s, a Leica MZ10F stereomicroscope equipped with an AxioCam MRc or a Zeiss Axio Imager.M2 equipped with a AxioCam MRc or a AxioCam MRm. For fluorescent microscopy of IHC or EdU stained sections and whole mounts, structured illumination microscopy were used (Zeiss ApoTome.2, Zeiss Axio Imager.M2). Zeiss ZEN and Adobe Photoshop were used for image processing and length measurements. All length measurements were performed on the third dorsal and the third ventral fin ray. Measurements of hemiray thickness were performed on the third and fourth dorsal and the third and fourth ventral fin rays. Fin length was determined by measuring the length of the third ventral ray (peduncle to distal tip).

Quantification of cell proliferation and cell death

For quantification of cell proliferation and cell death at 24 and 30 hpa, EdU- or TUNEL-labeled epidermal cells and osteoblasts were counted within one segment length proximal to the amputation plane. Labeled stump fibroblasts were counted inside a defined area of 50 x 200 µm adjacent to the amputation plane. For quantifications at 3 dpa, labelled cells were counted in the tissue distal to the amputation plane and normalized to the regenerate length.

Cryosectioning

For cryosectioning, fixed fins were decalcified in 10 mM EDTA in PBT (phosphate buffered saline (PBS) containing 0.1 % Tween20) and embedded in 1.5% agar, 5% sucrose in PBS. Embedded fins were saturated in 30% sucrose in PBS and subsequently snap-frozen in Tissue-Tek O.C.T. Compound (Sakura) in liquid nitrogen. Sections were cut at 16 µm.

Immunohistochemistry

For IHC, fins were fixed in 4% Paraformaldehyde (PFA) in PBS for 3 hours at room temperature or over night at 4°C, transferred to MeOH and stored at –20°C. Fins were rehydrated and cryosectioned or directly subjected to whole mount IHC. Sections or fins were washed in PBT, permeabilized in PBTx (PBS containing 0.3% Triton X-100) (30 min for sections and 1 hour for whole mounts), blocked in 2% blocking reagent (Roche) in PBT and subsequently incubated with primary antibodies diluted in 2% blocking reagent over night at 4°C. After several washes in PBT, tissue was incubated with secondary antibodies diluted in PBT for 3-4 hours at room temperature or over night at 4°C. Whole mounts were transferred to 70% glycerol in PBS for imaging. Sections were counterstained with DAPI and mounted using Mowiol containing DABCO. The following antibodies and dilutions were used: mouse ZNS-5 (Zebrafish International Resource Center) 1:1500, rabbit anti-GFP (Invitrogen, A6455) 1:500. Alexa- (Invitrogen) or Atto- (Sigma) labeled secondary antibodies were used. INT/BCIP (Roche) was used to detect AP-coupled antibodies.

TUNEL labeling

TUNEL labeling was performed in combination with IHC for ZNS-5. Fins were fixed in 4% PFA in PBS for 3 hours at room temperature or over night at 4°C, transferred to MeOH and stored at –20°C. Fins were rehydrated and cryosectioned. Sections were washed in PBS, permeabilized for 30 min in PBTx and equilibrated in terminal deoxynucleotidyl transferase (TdT) buffer (200 mM potassium cacodylate, 25 mM Tris, 0.05% Triton X-100, 1 mM CoCl2, pH 7.2). The buffer was subsequently replaced with TdT buffer containing 0.5 µM fluorescein-12-dUTP, 40 µM dTTP and 0.02 units/µl TdT (all Thermo Scientific). Slides were incubated at 37°C for 1-2 hours and washed in PBS. Sections were blocked in 2% Blocking Reagent in PBS and incubated with anti-fluorescein-AP-coupled antibody (1:2000, Roche) and ZNS-5 antibody (1:1500) in 2% blocking reagent at 4°C over night. After several washes in PBT, TUNEL labeled cells were detected with NBT/BCIP. AP activity was subsequently quenched with 100 mM glycin (pH 2.2). Sections were washed in PBS, blocked in 2%

blocking reagent and incubated with anti-mouse-AP-coupled antibody (1:500, Sigma). ZNS-5 labeled cells were detected with INT/BCIP (Roche). Sections were mounted using Mowiol.

EdU labeling

For EdU labelling, fish were IP injected with approximately 20 µl of 2.5 mg/ml EdU (Jena Bioscience) in PBS 1 hour (for analyses at 24 or 30 hpa) or 30 min (for analyses at 3 dpa) prior to fixation. Fins were fixed in 4% PFA in PBS for 3 hours at room temperature or over night at 4°C, transferred to MeOH and stored at –20°C. Fins were rehydrated, washed in PBT and permeabilized in PBTx for 30 min. Subsequently, fins were equilibrated in 100 mM Tris/HCl pH 8 and EdU was detected using a copper-catalyzed azide-alkyne click chemistry reaction (0.6 µM Cy3- or Fluor488- labeled azides (Jena Bioscience), 100 mM Tris, 1 mM CuSO4, 100 mM ascorbic acid, pH 8) with 20 min incubation time. Labeled fins were cryosectioned. For EdU/IHC double staining, EdU labeling was performed on whole mounts and IHC was subsequently performed on sections.

In situ hybridization

Digoxigenin (DIG)- or fluorescein labeled RNA antisense probes were synthesized from cDNA templates: *aldh1a2* (Grandel et al., 2002), *cyp26a1* (Kudoh et al., 2002), *cyp26b1* (Hernandez et al., 2007), *cyp26c1* (Gu et al., 2005). WISH or ISH on sections was performed as previously described (Blum and Begemann, 2012). For double WISH DIG- and fluorescein-labeled probes were hybridized simultaneously. Fins were first incubated with anti-DIG-AP coupled antibody and color reaction was performed with BCIP/NBT. AP activity was quenched with 100 mM glycin (pH 2.2) and fluorescein was detected by using anti-fluorescein-AP coupled antibody and INT/BCIP as substrate.

TRAP staining

For TRAP staining, fins were fixed in 4% PFA in PBS for 3 hours at room temperature or over night at 4°C, washed in PBT and permeabilized in PBTx for 30 min. Subsequently, fins were equilibrated in TRAP Puffer (0.1M NaAcetate, 0.1M acetic acid, 50mM NaTartrate) and

color reaction was performed in TRAP buffer containing 0.1 mg/ml Naphtol AS-MX phosphate (Sigma) and 0.3 mg/ml Fast Red Violet LB (Sigma). Labeled fins were transferred to 70% glycerol in PBS for imaging or were cryosectioned.

Hematoxylin staining

Fins were fixed in 4% PFA in PBS, transferred to methanol and stored at –20°C. Fins were rehydrated prior to cryosectioning. Sections were stained in Mayer's Hematoxylin Solution (Sigma) for 3-5 minutes, washed in water and cleared in 0.37% HCl in 70% ethanol for 5-10 seconds.

Alizarin Red staining

For Alizarin Red staining, fins were fixed in 4% PFA in PBS, transferred to MeOH and stored at -20°C. Fins were rehydrated, washed in PBT and stained in 0.1% Alizarin Red in 0.5% KOH overnight. Excess dye was removed by several washes in 0.5% KOH. Stained fins were transferred to 70% glycerol in 0.5% KOH for imaging.

Supplementary References

Grandel, H., Lun, K., Rauch, G.-J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Küchler, A. M., Schulte-Merker, S., Geisler, R., et al. (2002). Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development* **129**, 2851–65.

Gu X, Xu F, Wang X, Gao X, Z. Q. (2005). Molecular cloning and expression of a novel CYP26 gene (cyp26d1) during zebrafish early development. *Gene Expr. Patterns* **5**, 733–739.

Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335–46.

Table S1. Primer sequences for qPCR experiments.

Gene	Forward primer	Reverse primer
aldh1a2	GAGAGAGACAGTGCTTACCTTGC	CACAAAGAAGCAGGGGAGG
axin2	GCAGCACAGTTGATAGCCAG	GTCTTGGCTGGCACATATCC
bactin1	TTGCTCCTTCCACCATGAAG	CTTGCTTGCTGATCCACATC
bmp2b	CTGCTGACCACAAGTTTTCG	CAAAGACAGCAGCAATCCC
col10a1a	GCATTCTTCTTCTCCTGGTG	CCTGAACCCCAACCCCC
col1a1a	CAAAACAACGAAAACATCCC	GCATTTGGTTTCGCTCTTTC
cyp26a1	GATGGGAGCTGATAATGTG	CCTGAACCTCCTCTCTGACC
cyp26b1	GCTGGCTGCGTGTTTAGTG	GCCGTCCCAGTAGATGAGTC
cyp26c1	GCAGGAGACAAGGAGGAGG	GCTTCTGCCGTCTCGTGTG
dkk1b	ATGCCAGAGACACTAAATGAACA	TATGAAGGAAACCAGTTGAAAAA
ef1a	TACGCCTGGGTGTTGGACAAA	TCTTCTTGATGTATCCGCTGAC
fgf20a	AAAAGCTGTCAGCCGAGTGT	TGGACGTCCCATCTTTGTTG
igf2b	GCAGGTCATTCCAGTGATGC	TCTGAGCAGCCTTTCTTTGC
osc	CCTGATGACTGTGTGTCTGAG	CGCTTCACAAACACACCTTC
osn	GTGGAGGATGTTATTGCTGAG	GGGGCAGGTCAAAGGGTC
runx2a	GATTTGTGCTCCCGCTTTAG	CTGCTGGACGGCGGACTG
runx2b	GGAGTGGAGGGAGATGGAAG	TAGCGAGTGGAAGAGTACAGATTG
tbp	CGGTGGATCCTGCGAATTA	TGACAGGTTATGAAGCAAAACAACA

Table S2. Number of specimens used in quantitative and nonquantitative experiments (Figs. 1-6). Numbers for corresponding experiments, which are not shown in the figure, are included. For nonquantitative experiments: the first number indicates the number of specimens showing the phenotype, the second number the total number.

Figure	n=
1B	33-46 rays per position along the proximodistal axis in adult fish; 27-39 rays per fin length class
1D	3 cDNA samples per condition
2A	3 cDNA samples per time point
3A	3 cDNA samples per condition
3B	RA: DMSO= 15 sections (4 fins), RA= 35 sections (5 fins); R115866: DMSO= 35 sections (6 fins), R115866=53 sections (6 fins)
3D	RA: DMSO= 36/175 rays, RA= 181/237 rays; R115866: DMSO= 35/211 rays, RA= 122/223 rays
4D	wild type= 24 sections (6 fins), *hsp70l:cyp26a1*= 32 sections (10 fins); DMSO= 31 sections (8 fins), RA=29 sections (6 fins)
5C	RA: DMSO= 12 rays, RA= 12 rays; R115866: DMSO= 12 rays, R115866= 12 rays
5F	proximal= 7 rays, distal=7 rays
5G	proximal= 7 rays, distal=8 rays
5H	proximal= 13 rays, distal=12 rays
5I	3 cDNA samples per condition
6A	3-4 cDNA samples per condition
6B	wild type= 16 rays (8 fins), *hsp70l:cyp26a1*= 16 rays (8 fins);
6C	RA: DMSO= 0/5 fins , RA= 6/6 fins; R115866: DMSO= 0/6 fins, R115866= 6/6 fins
6F	RA: DMSO= 0/6 fins , RA= 5/6 fins; R115866: DMSO= 0/5 fins, R115866= 4/5 fins
6G	DMSO= 0/5 fins , RA= 6/6 fins

Table S3. Number of specimens used in quantitative and nonquantitative experiments (Figs. S1-4). Numbers for corresponding experiments, which are not shown in the figure, are included. For nonquantitative experiments: the first number indicates the number of specimens showing the phenotype, the second number the total number.

Figure	n=
S2C	wild type= 5/5 fins, *hsp70l:cyp26a1*= 5/5 fins
S3A	3 cDNA samples per time point
S3B	3 cDNA samples per time point
S3C	3 cDNA samples per condition
S3D	3 cDNA samples per condition
S4A	3 cDNA samples per condition
S4B	DMSO= 25 sections (5 fins), RA= 25 sections (4 fins)
S4D	RA: DMSO= 30 rays, RA= 35 rays; R115866: DMSO= 24 rays, R115866= 26 rays
S4E	RA: DMSO= 15 sections (4 fins), RA= 35 sections (5 fins); R115866: DMSO= 35 sections (6 fins), R115866=53 sections (6 fins)
S4F	RA: DMSO= 16 sections (5 fins), RA= 25 sections (6 fins); R115866: DMSO= 27 sections (7 fins), R115866=30 sections (7 fins)
S4G	DMSO= 25 sections (5 fins), RA= 25 sections (4 fins)
S5A	3 cDNA samples per condition
S5B	wild type= 17 sections (6 fins), *hsp70l:cyp26a1*= 17 sections (6 fins)
S6	3 cDNA samples per condition