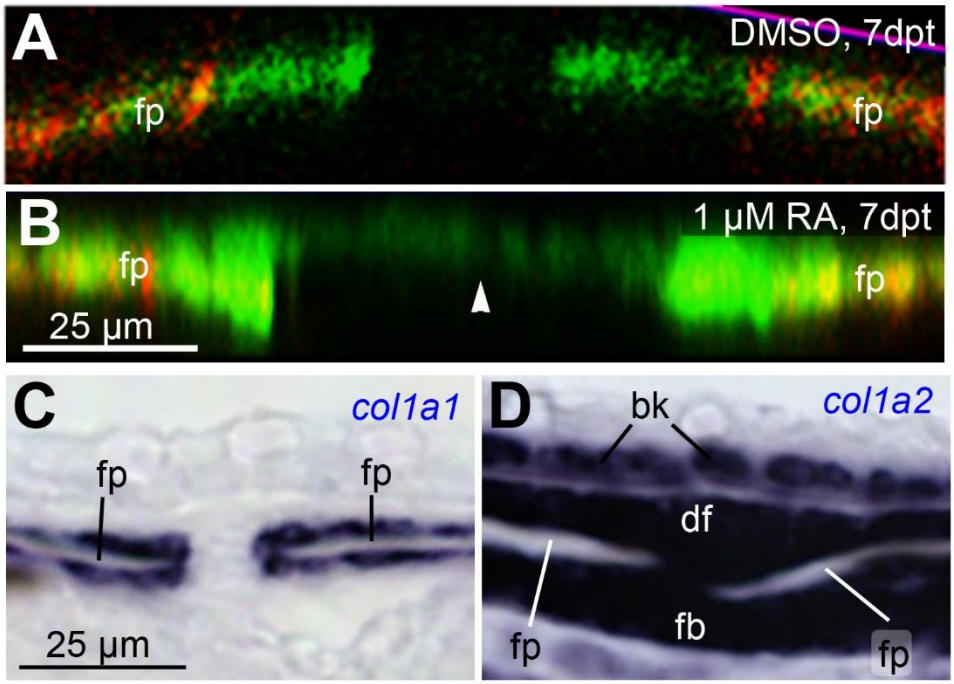

Fig. S1: RA-induced ectopic intercalvarial ossification predominantly occurs in the overlying dermis, consistent with the cutaneous *col1a2* expression. (A,B) SL8-9 fish treated with DMSO (A) or RA (B) for 7 days, after consecutive in vivo AR staining (red) before and calcein staining (green) after the treatment, as shown in Fig. 1D,E; virtual transverse sections obtained from 3D projections along confocal Z-stacks (ZEN2008 software; Zeiss) at positions indicated by arrowheads in Fig. 1D,E. The weak calcein staining between the frontal calvaria of the RA-treated fish (Fig. 1E; asterisk) predominantly occurs in regions slightly dorsal of the calvarial plates (indicated in (B) with arrowhead). According to H&E and Azan novum stainings (see for instance Fig. S5A), this is the dermis. (C,D) in situ hybridizations of untreated SL10 fish for *col1a1* (C) and *col1a2* (D). In contrast to *col1a1*, whose expression is restricted to bone-lining osteoblasts (C; compare with Figs. 2F and 5C), *col1a2* is expressed by basal keratinocytes (bf), dermal fibroblasts (df), consistent with former reports (Le Guellec and Sire, 2004), and in connective tissue fibroblasts (fb) between and below the calvarial plates. Upon RA-treatment, this *col1a2* expression became slightly reduced (data not shown). However, the reduction was much more moderate than that of *col1a1* in osteoblasts (see Figs. 2F,G and 5C,D). Abbreviations: bk, basal keratinocytes; df, dermal fibroblasts; fb, fibroblasts; fp, frontal calvarial plates

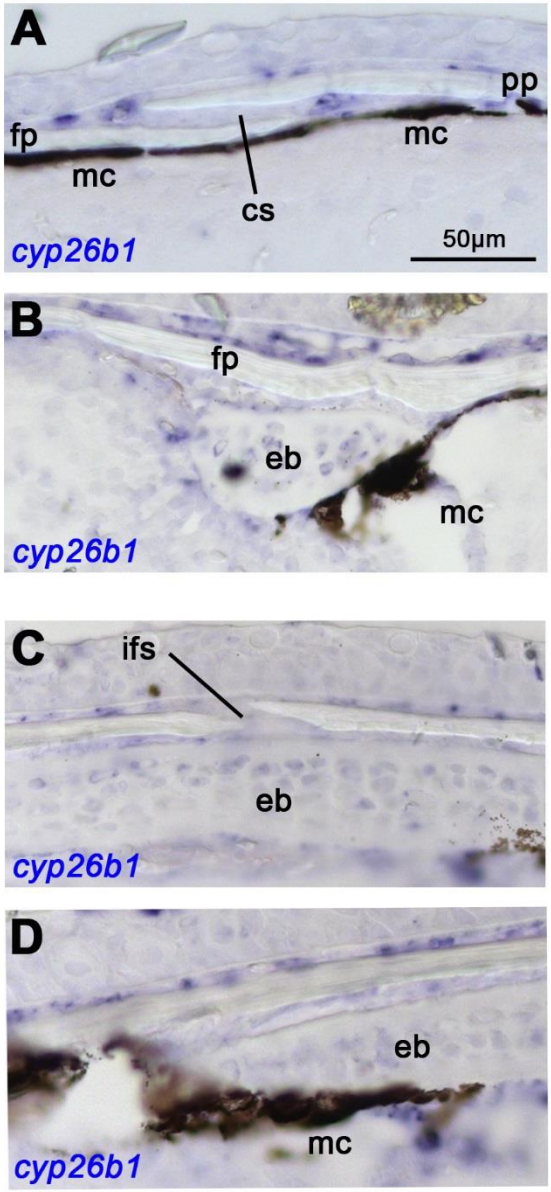

Fig. S2: *cyp26b1* displays differential expression in bone-lining cells of the calvarial plates. Panels (A,B) show sagittal sections, panels (C,D) transverse sections of untreated wild-type fish at SL10; *cyp26b1* in situ hybridization, revealing weak and differential *cyp26b1* expression in bone-lining cells. At the level of the sutures, where plates continue to grow (A,C), *cyp26b1* is more weakly expressed than in central regions of the plate (B,D), where no horizontal growth occurs. Within central regions, *cyp261* is expressed more strongly on the outer surface of the calvaria than on their inner surface (B,D), complementary to the asymmetric vertical growth of the calvaria, which preferentially occurs on their inner surface (see Fig. 1G). In conclusion, *cyp26b1* is preferentially expressed in bone-lining cells of regions that display no or reduced bony growth. Abbreviations: cs, coronal suture; eb, epiphyseal bar; fp, frontal plate; ifs, interfrontal suture; mc, melanocyte; pp, parietal plate.

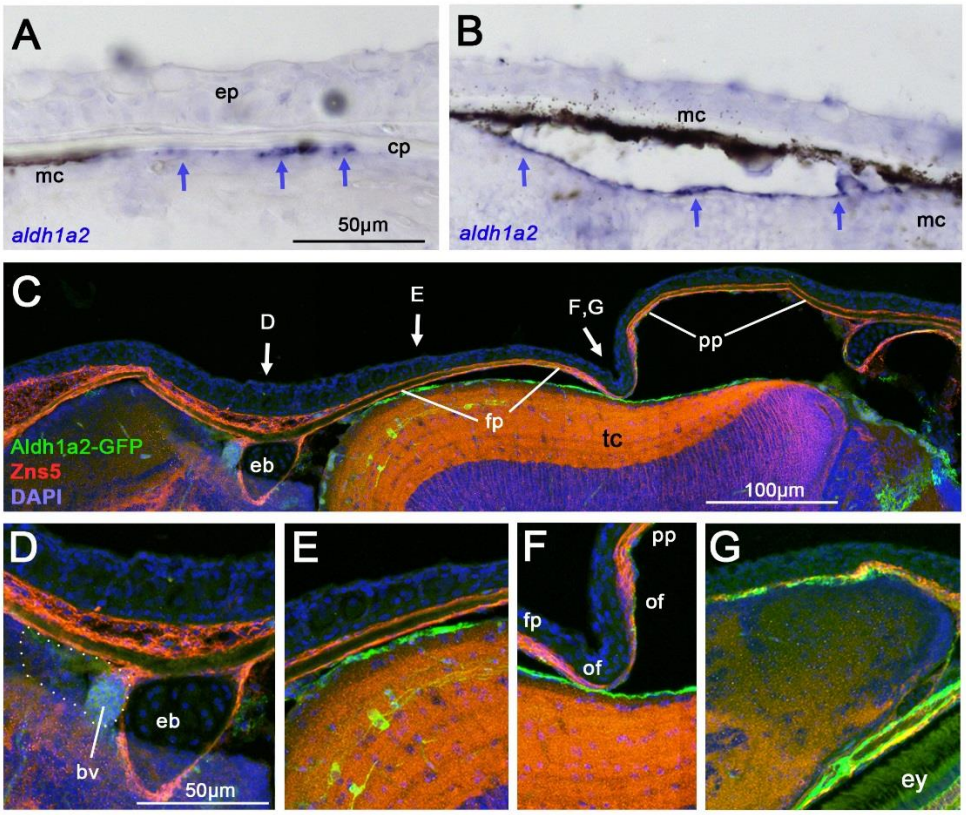

Fig. S3: *aldh1a2* displays differential expression in the meninges underlying the calvarial plates. Panels (A,B,G) show transverse sections, panels (C-F) sagittal sections of untreated fish at SL9 (A,B) or SL10-11 (C-G). (A,B) *aldh1a2* in situ hybridization; *aldh1a2* signals are marked with arrows; melanocytes of the meninges (mc) (Goldgeier et al., 1984) and the calvarial plate (cp) are indicated. Note that the aldh1a2+ cells are positioned between or even underneath the meningeal melanocytes. (C-G) Double immunofluorescence for *tg(aldh1a2:aldh1a2-GFP)*-driven GFP (Pittlik and Begemann, 2012) and the osteogenic cell marker ZNS5, counterstained with DAPI. Panel (C) shows an overview, and panels (D-F) magnified views of regions indicated in (C); panel (G) shows transverse sections at the level within the region shown in (F). (F,G) are directly at the osteogenic fronts (of) of the frontal (fp) and parietal plates (pp), which have not met and formed a suture as yet; (D) is at the level of the epiphyseal bar (eb), thus, within central regions of the frontal plate and very remote from the osteogenic front, and (E) is in an intermediate position closer to the frontal pate osteogenic front. In (D), the distinct and highly vascularized tissue directly anterior of the epiphyseal bar, which displays strong RA-induced *cyp26b1* expression (Fig. 7I-L), is outlined. The Aldh1a2-GFP signal is much stronger in regions close to the osteogenic front than in very remote regions. In addition, it is not localized within the ZNS5-positive osteogenic cells themselves, but in cells below them, at about the same level like the meningeal melanocytes. This expression pattern correlates with the pattern of calvarial growth, with horizontal growth occurring at the osteogenic fronts (see Fig. 1D), and vertical growth preferentially occurring on the inner surface of the calvarial plates (see Fig. 1G). Abbreviations: bv, blood vessel; cp, calvarial plate; eb, epiphyseal bar; ep, epidermis; ey, eye: fp, frontal plate; mc, melanocytes; of, osteogenic front; pp, parietal plate, tc, optic tectum.

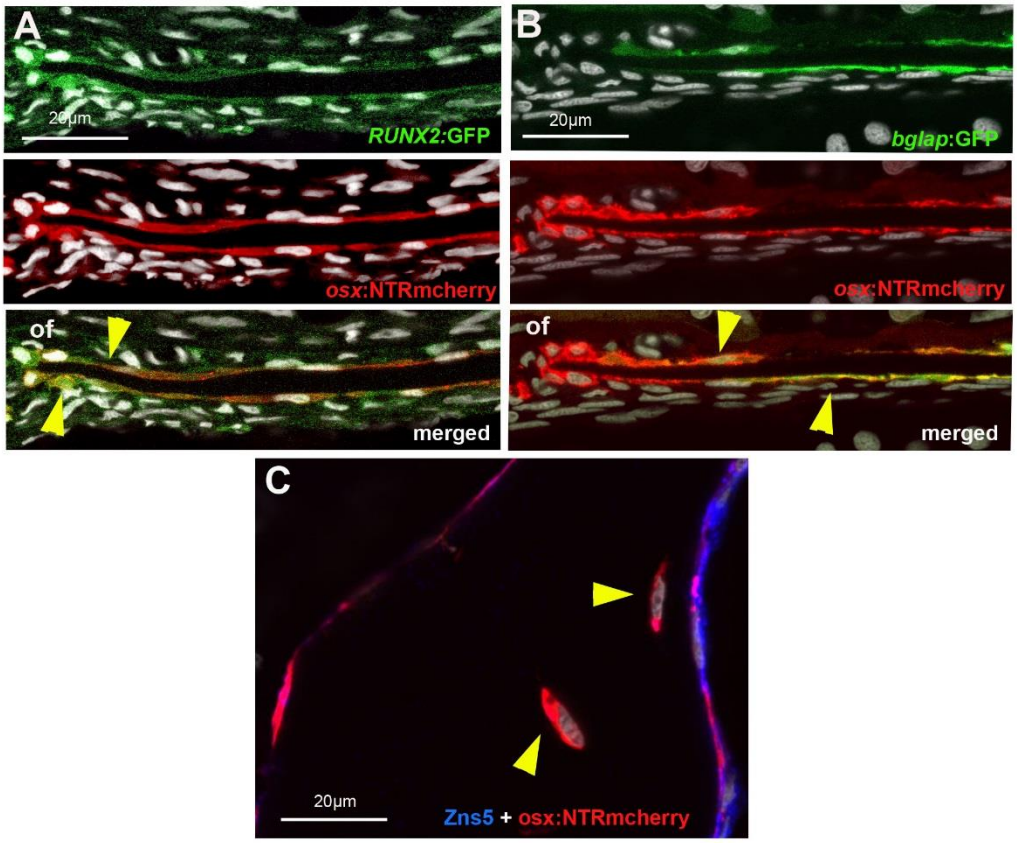

Fig. S4: *osx:NTRmCherry*-encoded protein is expressed in osteogenic cells of all differentiation stages. (A,B) Double fluorescence of *tg(osx:NTRmCherry)* (red; upper row) together with *tg(RUNX2:EGFP)*, marking osteoblast progenitors (Kague et al., 2012) (A) or with *tg(bglap:GFP)* (Vanoevelen et al., 2011) (B), marking mature osteoblasts (green, middle row); counterstained with DAPI (white); transverse sections through frontal plate. Osteogenic front (of) is indicated. Yellow arrowheads in the merged images (lower row) point to *osx+runx2* double positive close to the osteogenic front (A), and to *osx+bglap* double positive bone-lining cells more remote from the osteogenic front (B). (C) Double immunofluorescence for *tg(osx:NTRmcherry)*-driven RFP and the osteogenic cell marker ZNS5; transverse section through the orbital bone of SL22 wild-type fish: in addition to its co-expression with ZNS5 in bone-lining cells, the *osx* transgene is expressed in isolated cells within the mineralized bone, which most likely are terminally differentiated osteocytes (Franz-Odendaal et al., 2006).

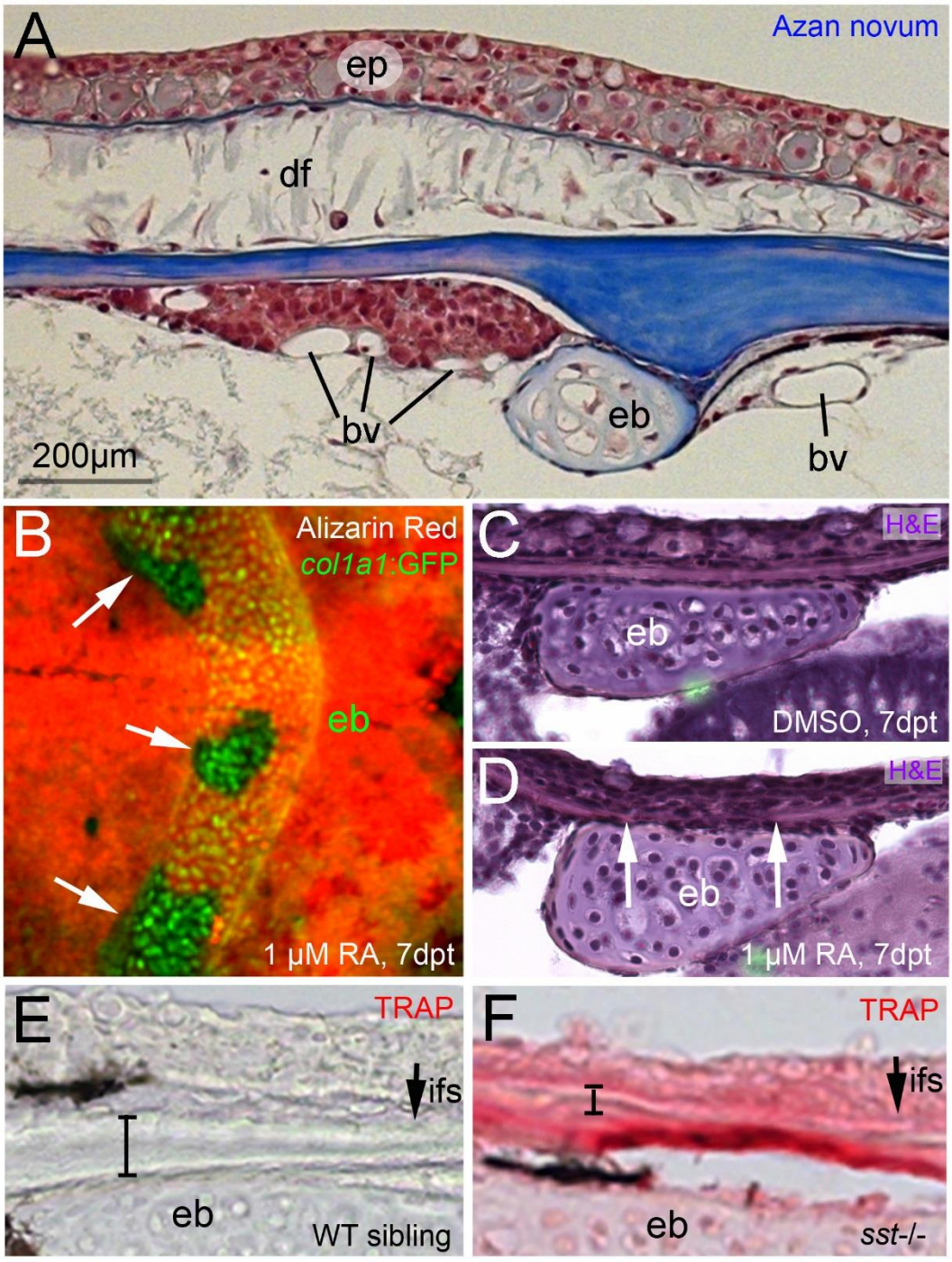

Fig. S5: RA-induced calvarial fragmentation occurs primarily at the level of the epiphyseal bar. (A) Azan novum staining of sagittal section through head of 1 year old untreated fish; anterior to the left; region of epiphyseal bar. Directly anterior of the epiphyseal bar is a distinct tissue that is tightly associated with the calvarial plate, suggesting that it is extracephalic. It is highly vascularized, and was not found at any other position along the anterior-posterior length of the brain case. Compare also with Fig. S3D and with Fig. 7I-L, showing that this tissue displays strong *cyp26b1* expression upon RA treatment. (B) *tg(col1a1:GFP)* fish (Kague et al., 2012) after RA-treatment and in vivo alizarin red staining; dorsal view on head region. The holes

directly above the GFP-positive epiphyseal bar are indicated with arrows. (C,D) Sagittal sections through epiphyseal bar region of fish after treatment with 1µM DMSO (B) or 1µM RA (C); H&E staining; in (D) the borders of the calvarial hole are indicated with arrows. (E,F) Magnified views of images shown in Figure 3P,Q; transverse sections through epiphyseal bar region of wild-type sibling (D) and *sst* mutant (E); TRAP staining; the interfrontal suture (ifs) is indicated with an arrow, the thickness of the calvarial plate with a bar. Abbreviations: bv, blood vessel; df, dermal fibroblast; eb, epiphyseal bar; ep, epidermis; fp, frontal plate; ifs, interfrontal suture.

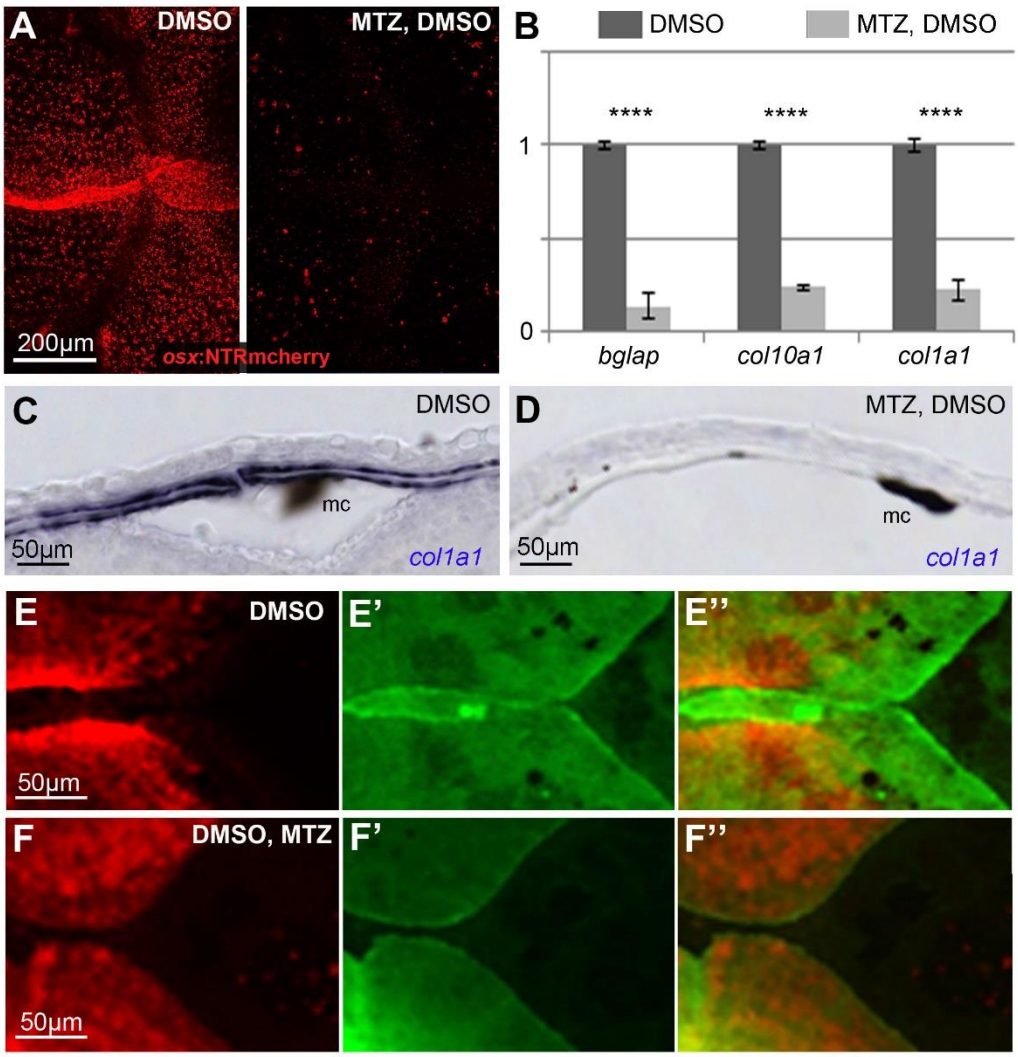

Fig. S6: Metronidazole treatment of *tg*(*osx*:*NTRmcherry*) transgenics leads to efficient osteogenic cell ablation and impaired bone formation. (A) Red channel of images shown in Fig. 4G,J, with dorsal views an calvarial plates of *osx*:*NTRmcherry* transgenic fish after treatment with DMSO (left panel) or MTZ (right panel), revealing ablation of approximately 95% of all *osx* transgenic / osteogenic cells. (B) qRT-PCR analysis of isolated calvarial plates from SL11-12 *tg*(*osx*:*NTRmcherry)* fish. Expression of osteoblast marker genes *bglap, col10a1* and *col1a1* is strongly reduced after osteogenic cell ablation / MTZ-treatment, compared to the DMSO-treated controls. (C,D) in situ hybridization, revealing loss of *col1a1* expression along calvarial plates after treatment with DMSO and MTZ (D), compared to DMSO treatment alone (D). (E,F) Magnified dorsal view of central head region of SL9-10 non-transgenic sibling control fish treated with DMSO (E-E''; n=5/5), and *tg(osx:NTRmcherry)* transgenic treated with DMSO + MTZ (F-F''; n=6/6) for 7 days, after consecutive in vivo alizarin red staining (red; E,F) before and calcein staining (green; E',F') after the treatment. Panels (E'',F'') show merged images. After osteogenic cell ablation, growth at the osteogenic fronts of the frontal calvarial plates ceases completely (compare with Fig. 1D-F for the strongly reduced, but not completely ceased calvarial growth upon RA-treatment of wild-type fish).

Supplemental Materials and Methods

In vivo bone staining

The vital stain protocol has been modified from (Kimmel et al., 2010). Alizarin red (Sigma-Aldrich, St. Louis, MO) or calcein (Sigma-Aldrich, St. Louis, MO) solutions were prepared by diluting either dye to a final concentration of 100 mg/l in fish water. The Alizarin red solution was further supplemented with 1mM Hepes (Carl Roth, Karlsruhe, D), and the final pH was adjusted to 6.5. The in vivo staining was conducted in the dark for 1-3 hours prior to the treatment/imaging. Subsequent to the staining, fish were rinsed 3x 5 minutes in fish water.

Tissue sectioning and *in situ* hybridization

Samples fixed in 4% PFA (Sigma-Aldrich, St. Louis, MO) and stored in 100% MeOH at -20°C were progressively rehydrated to PBST (PBS/1% Tween-20), embedded in a solution of PBS containing 1.5% agarose and 15% sucrose, and incubated overnight at 4°C in 30% sucrose in PBS. Tissue blocks covered in cryomatrix (VWR, Radnor, PA) were snap frozen in 2-methyl butane (Isopentane; Carl Roth, Karlsruhe, D), at -80°C. Sections of 14μm thickness were collected on coated glass slides (Ultra Plus; VWR, Radnor, PA) and stored at –20°C until used. At least an hour prior to usage, frozen cryostat sections were thawed at room temperature.

For the *in situ* hybridization, the slides were heated at 58°C for 10 min, fixed in 4%PFA for 5 min, and washed twice in PBST (PBS with 1% Tween20). The slides were then incubated for 15 min in 0.3% Triton (Sigma-Aldrich, St. Louis, MO) in PBS, washed twice in PBST, and permeabilized with a 10 μg/ml Proteinase K solution at 37°C for 30 min. Permeabilization step was followed by 2 washes of 5 min each in PBST, and the slides were subsequently incubated in acetylation solution (750μl triethanolamine (Sigma-Aldrich, St. Louis, MO) and 162μl acetic anhydride (Supelco, Bellefonte, PA) in 60ml of water) for 5 minutes, followed by 2x 5 min washes in PBST. Next, the slides were taken through a serie of ethanol dilutions in water (50% EtOH, 70% EtOH, 95% EtOH and 100% EtOH, 3 minutes each), allowed to dry for 3 minutes at RT, and pre-hybridized at 58°C for 3-4 hours in a solution containing 50%

deionized formamide, 5x SSC (20x SSC stock solution; 3.0M NaCl, 0.3M citric acid), 0.1% Tween-20, 50 μg/ml heparin, 9.2mM citric acid, 200 μg/ml yeast tRNA and 2.5% dextran Sulfate. Probes diluted in pre-hybridization solution, to a final concentration of 1 ng/μl, were subsequently added for overnight at 58°C.

The next day, the hybridized slides were washed at 58°C for 15 minutes in a series of washing solutions (2 washes of 15 minutes each in 2x SSC, 2 washes of 15 minutes each in a solution of 50% formamide / 50% 2x SSC, and 2 washes of 15 minutes each in 0.2% SSC). This was followed by two 10-minute washes in PBST (or TNT solution for the fluorescent *in situ* hybridization). The samples were then incubated for at least 2 hours at RT in blocking solution, consisting of PBST supplemented with 10% calf serum (or TNTB consisting of a 1/10 dilution of Roche blocking agent in TNT). The slides were subsequently incubated at 4°C overnight in the corresponding blocking solution to which anti-digoxigenin antibody conjugated to Alkaline Phosphatase (AP) (Roche, Basel, CH, purchased from Sigma-Aldrich, 1109274910), or anti-digoxigenin antibody conjugated to peroxide (POD) (Roche, Basel, CH, purchased from Sigma-Aldrich, 11633716001) has been added to a dilution of 1/3000 or 1/200, respectively.

To wash out unbound antibody, slides were put through 6 washes of 15 minutes each in PBST (or TNT). Prior to the chromogenic reaction (sections incubated with anti-DIG conjugated to AP), the sections were equilibrated in two 5-minute washes in equilibration buffer (100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl) plus 0.1% Tween-20. Sections were stained in a solution containing 5-Bromo-4-Chloro-3-Indolyl phosphate (BCIP) and Nitro Blue Tetrazodium (NBT) mix (Roche, Basel, CH), diluted 1/100 in the equilibration buffer. The staining was performed in the dark, at RT, until a suitable color was obtained. For fluorescent *in situ* hybridization, the TSA reaction was carried for an hour, in the dark, in TSA buffer solution (Invitrogen, Carlsbad, CA) containing 0.001% H_2O_2, and the first fluorescent TSA substrate (Invitrogen, Carlsbad, CA). The reaction was stopped using 1% H_2O_2 in TNT buffer, followed by a 2 hours blocking step at RT, in TNTB solution, and incubated for ON at 4°C, in a solution containing anti-Fluorescein antibody conjugated to Peroxidase (POD) (Roche, Basel, CH; purchased from Sigma-Aldrich, 11426346910) diluted 1/200 in TNTB. The same washes and TSA reaction steps were followed for the second antibody, with staining carried out for one hour in the dark. After staining, the samples

were washed several times at RT in TNT, mounted with a cover slip in Mowiol/DAPI solution (Carl Roth, Karlsruhe, D), and allowed to dry for overnight in the dark, at room temperature, prior to imaging.

After staining, the samples were either washed in PBST, fixed in 4% PFA for 2 hours, mounted with a cover slip in Mowiol/DAPI solution, and allowed to dry for ON at RT, prior to imaging, or used for a regular immuno-histochemistry.

Antisense RNA probes were generated via in vitro transcription with Digoxigenin or Fluorescein RNA labeling mix (Roche, Basel, CH; purchased from Sigma-Aldrich, 11277073910, 11685619910) and the following templates, as described: *col1a1a* (Laue et al., 2011), *col10a1* (Laue et al., 2008), *phex* (Laue et al., 2011), *spp1* (Laue et al., 2008), *cyp26b1* (Laue et al., 2008), *aldh1a2* (Begemann et al., 2001), *aldh1a3* (Pittlik et al., 2008), *aldh8a1* (Liang et al., 2008). For *acp5a*, EST cb576 (Zebrafish International Resource Center ZIRC, Eugene, OR) was digested with *Not*I and transcribed with T7 RNA polymerase.

Immunohistochemistry

Immunohistochemistry was carried out by permeabilizing thawed cryostat sections in ice-cold acetone for 20 min, followed by 5x 5 minutes washes in PBST. Subsequently, samples were blocked in 10% calf serum in PBST for 2 hours, and then incubated overnight at 4°C with the primary antibodies. Primary antibodies used were: mouse Zns-5 (ZDB-ATB-081002-37; Zebrafish International Resource Center ZIRC, Eugene, OR), chicken anti-GFP (ThermoFisher Scientific, A10262), rabbit anti-RFP (MBL International Corporation, PM005), each diluted in blocking solution to 1:200. Afterwards, samples were washed 5 times in PBST, 10 minutes each, and incubated for 4 hours at room temperature with fluorescently labeled secondary bodies. Secondary antibodies used were: goat-anti-mouse Cy3 (ThermoFisher Scientific, A10521), goat anti-rabbit Cy3 (ThermoFisher Scientific, A10520), goat anti-rabbit AlexaFluor-488 (ThermoFisher Scientific, A-11034), goat-anti-chicken AlexaFluor-488 (ThermoFisher Scientific, A-11039), each diluted 1:200 in blocking solution. Samples were subsequently washed at room temperature, 5x in PBST, for 10 minutes each, then mounted with Mowiol/DAPI mixture and left to dry at room temperature over night.

qRT-PCR

Calvaria isolated from fish treated with RA or DMSO were immersed in Trizol for RNA extraction and cDNA synthesis according to standard protocols. The levels of gene expression were determined via qRT-PCR with a 7500 Fast Real Time PCR System and premade or customized TaqMan Gene Expression Assays (Applied Biosystems, Waltham, MA), normalized against the products of the ribosomal protein S23 gene (*rps23*).

Reference numbers of used premade TaqMan Gene Expression Assays were:

rps23: Dr03430371_m1

acp5a: Dr03140272_m1

ctsk: Dr03423909_m1

spp1: Dr03108261_m1

phex: Dr03092609_m1

ankha: Dr03135792_m1

ankhb: Dr03144647_m1

col1a1a: Dr03150834_m1

col10a1: Dr03143511_m1

Primer sequences of costumized TaqMan Gene Expression Assays were (forward, reverse, probe):

bglap: TCTCCTAATCATGAAGGTGTGTTTG, GGCGTCATACCAGAAGAATGG, ACGACAGATGCAGTGTGT

rank: GCAAGACTGAGAAGCAGTTAGGAA, GGCGTCATACCAGAAGAATGG, ACGACAGATGCAGTGTGT

rankl: TGCTGCAGGTCGCGTCTAG, AGATCGACCTCTCGGAGATAACC, TGGCGATTCTGTTGCAC