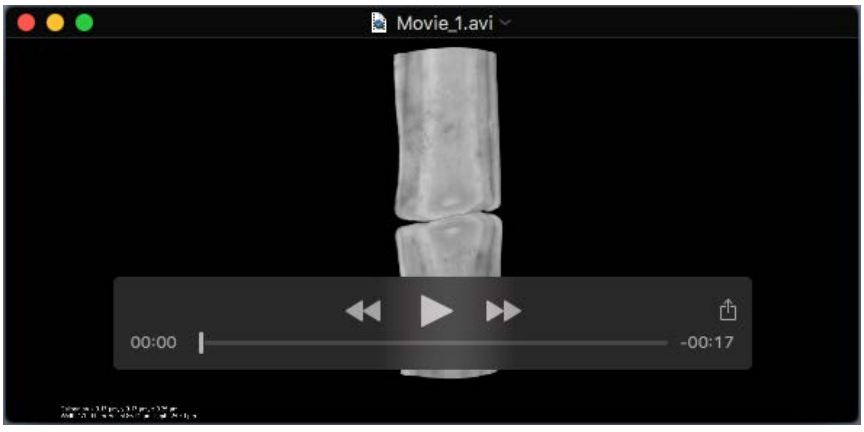
A Regulatory Pathway Involving Retinoic Acid and Calcineurin Demarcates and Maintains Joint Cells and Osteoblasts in the Fin Regenerate

Supplementary information

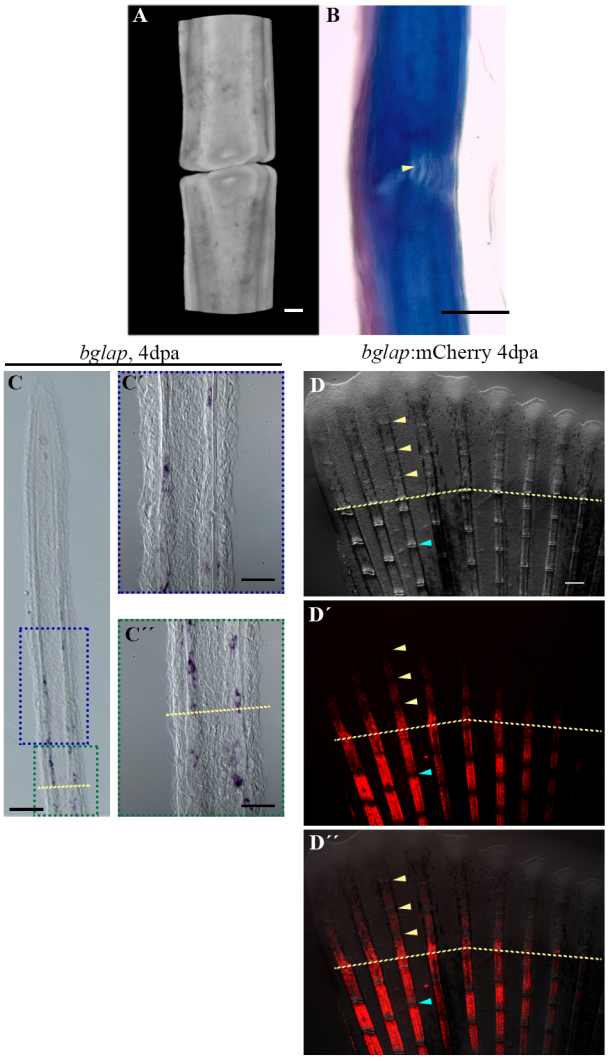
Table S1: Primers used for RT-PCR Analysis.

Primer Name	**Primer Sequence 5'-3'**
β-actin forward	ATGGATGAGGAAATCGCTGCCCTGGTC
β-actin reverse	CTCCCTGATGTCTGGGTCGTCCAAC
pthlha forward	CGTAATGCTGAGCCGGACA
pthlha reverse	TCACTGAACGCTTCATTCGGCT
pthlhb forward	AGCAGACAACGGCGTTCAGT
pthlhb reverse	GCATTTGGAAGGCACACGCT
pth1ra forward	TGTGCCAAATTCTTCCCCCA
pth1ra reverse	GAGCCGTCGAAAGTATCCGA

pth2R forward	CTTCTGTTCTCCGCGTCAGT
pth2R reverse	ATGCATGTGCTGCATGGTTG
pth1rb forward	AAGCATGGTGTCAGTGGAGG
pth1rb reverse	ACGCGTATCCTCTGTGGTTG

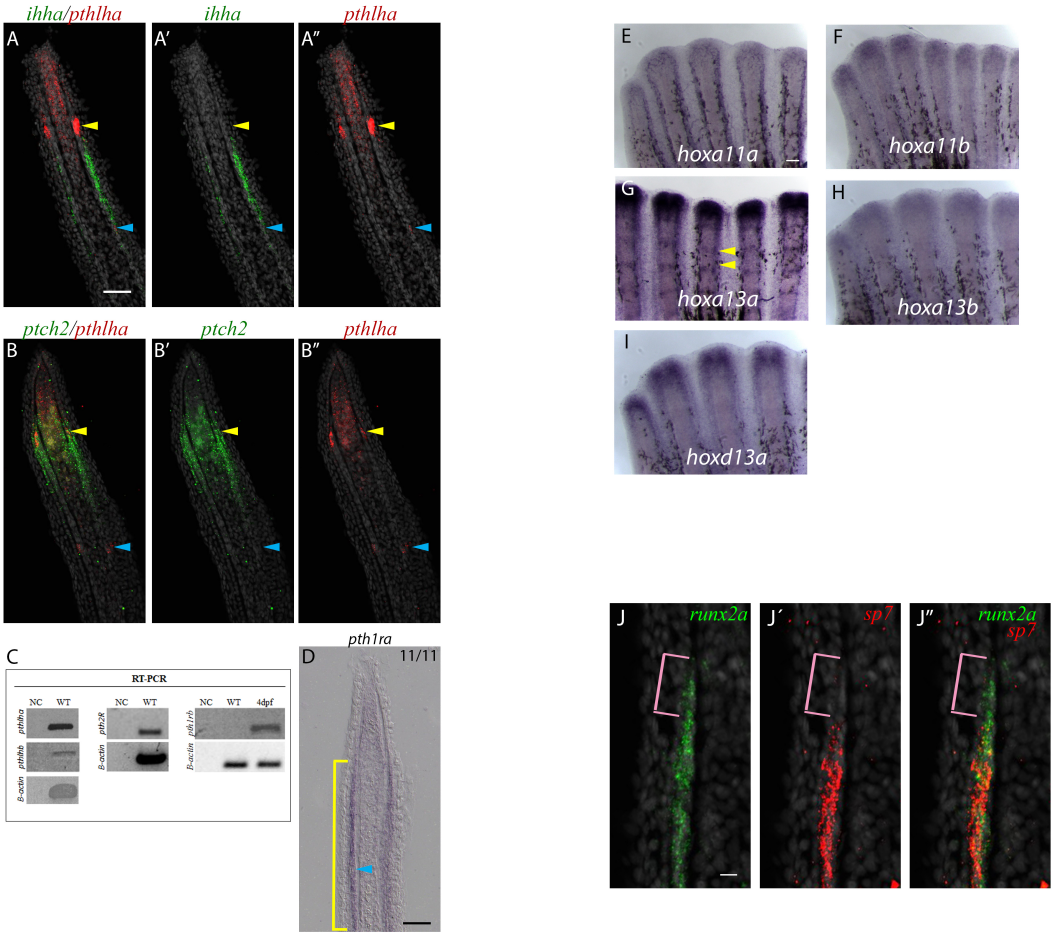


Movie 1: **Confocal 3D rendering of two fin ray segments.** 3D movie of Alizarin Red stained fin rays illustrates two consecutive segments in one hemiray that possess a concave shape.



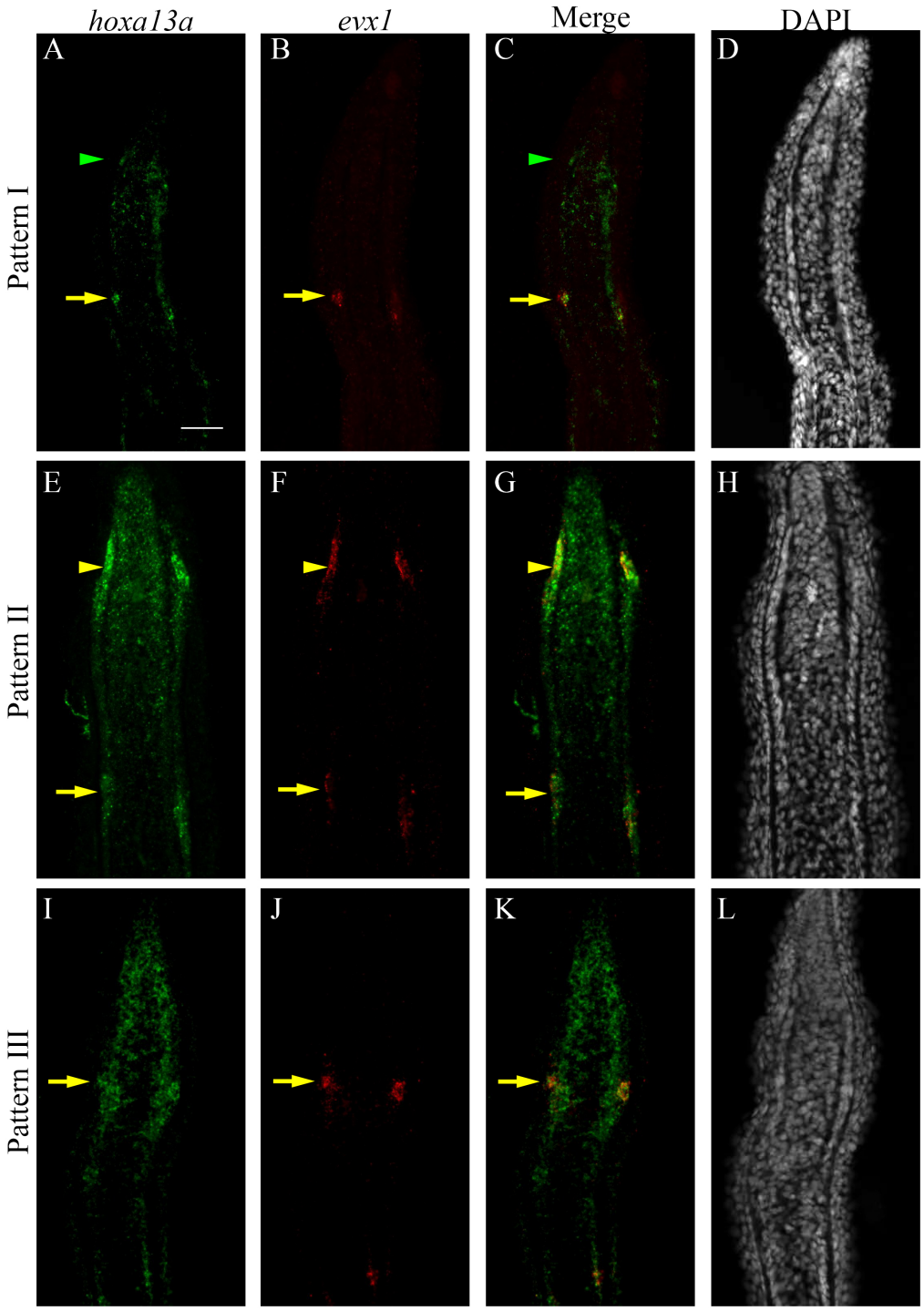
Supplementary figure 1

Fig. S1: Joint Structure. (A) Confocal 3D rendering of two fin ray segments separated by a joint. (B) Mallory staining illustrating two bone segments are connected by ligaments (yellow arrowhead). (C-C") ISH on longitudinal cryosections of 4dpa fin regenerates illustrate *bglap* expression in the proximal fin regenerate and stump osteoblasts. (C') Magnification of the proximal fin regenerate (blue box in C). (C") Magnification of the proximal fin regenerate and stump (green box in C). (D-D") *Tg(bglap:mCherry)* 4dpa regenerates illustrate mCherry expression in mature osteoblasts but not in joint regions in the fin regenerate (yellow arrowheads) and stump (blue arrowheads). (D) Brightfield only. (D') mCherry only. (D") merged. Amputation planes = dashed yellow line. Scale bars A=10µm, B=10µm, C=100µm, C'=50µm, C"=50µm, D-D''=200µm (shown in D).



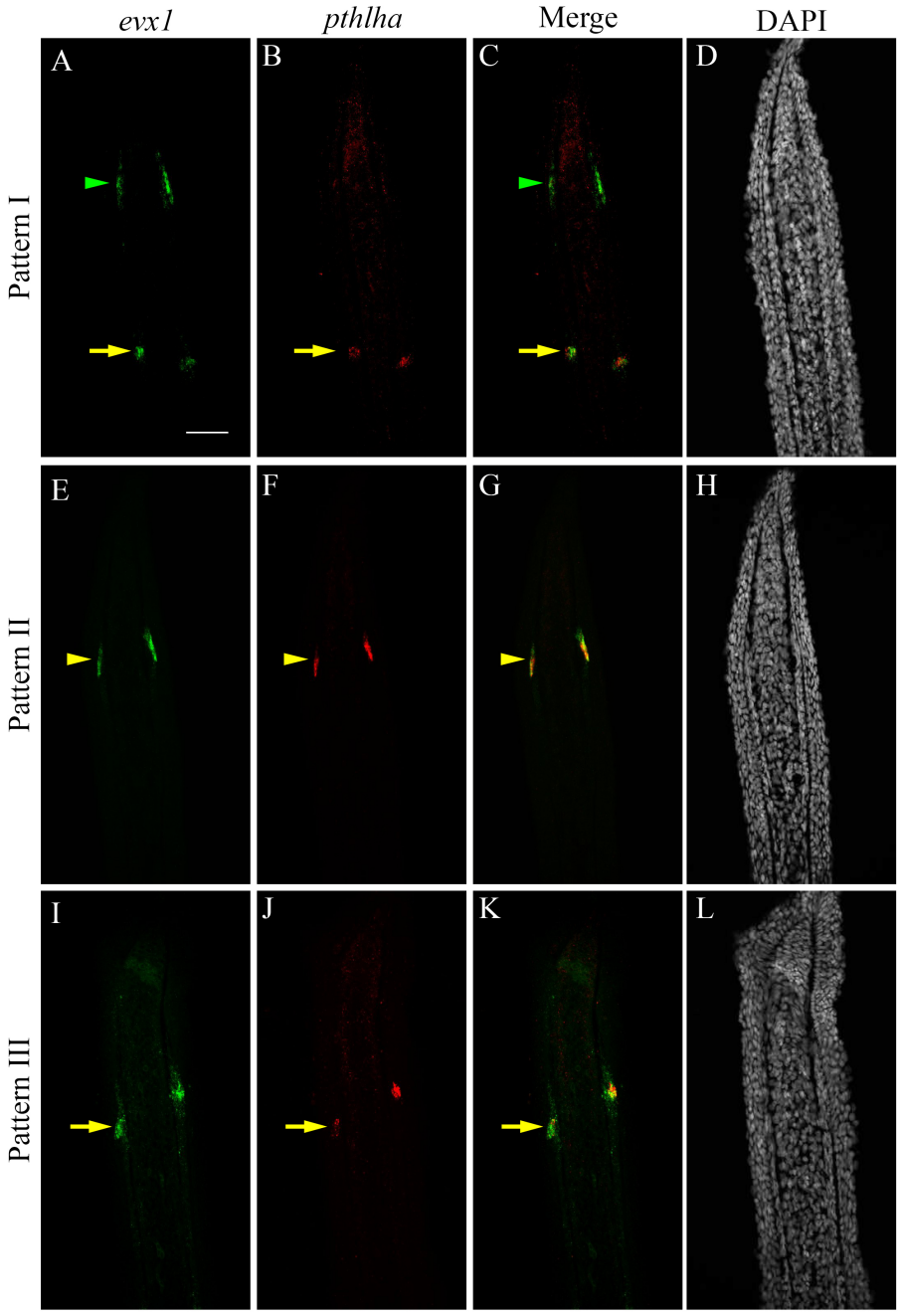
Supplementary figure 2

Fig. S2: Gene expression analysis of *hox* genes, *pthlha, ihha, pth1rb* and *ptch-2* at 4dpa. (A-A”) *pthlha* is expressed at the level of joints (blue and yellow arrowheads in A-A” and B-B”) while *ihha* is only found in osteoblasts (A-A”). (B-B”) *ptch2* is expressed in joint cells (yellow arrowheads) and differentiating osteoblasts, but not mature joints (blue arrowheads). (C) RT-PCR indicates *pthlha, pthlhb,* and *pth2r* are expressed in the 4dpa fin regenerates. *pth1rb* is not expressed in 4dpa wildtype fin regenerates, but is expressed in 4 days post fertilization (dpf) larvae. *β-actin* was used as the housekeeping control. NC = Negative Control. WT = wildtype. (D) In 11/11 sections, *pth1ra* is faintly expressed in differentiating osteoblasts (yellow bracket) and joint-forming cells (blue arrowhead). (E-I) ISH on whole mount fin regenerates indicate only *hoxa13a* is strongly expressed in joint regions (yellow arrowheads). Although faint staining appears in joints for *hoxa13b* (H) and *hoxd13a* (I), it is likely background as 4dpa fin regenerate sections do not show expression. (J-J”) Magnified images from Fig. 3D-D” to further illustrate the absence of *sp7* expression in the most distal *runx2a* expression domain (pink brackets). Scale bar A-B” = 50µm (shown in A), J-J’’=10µm (shown in J); D =50µm, E-I =100µm (shown in E).



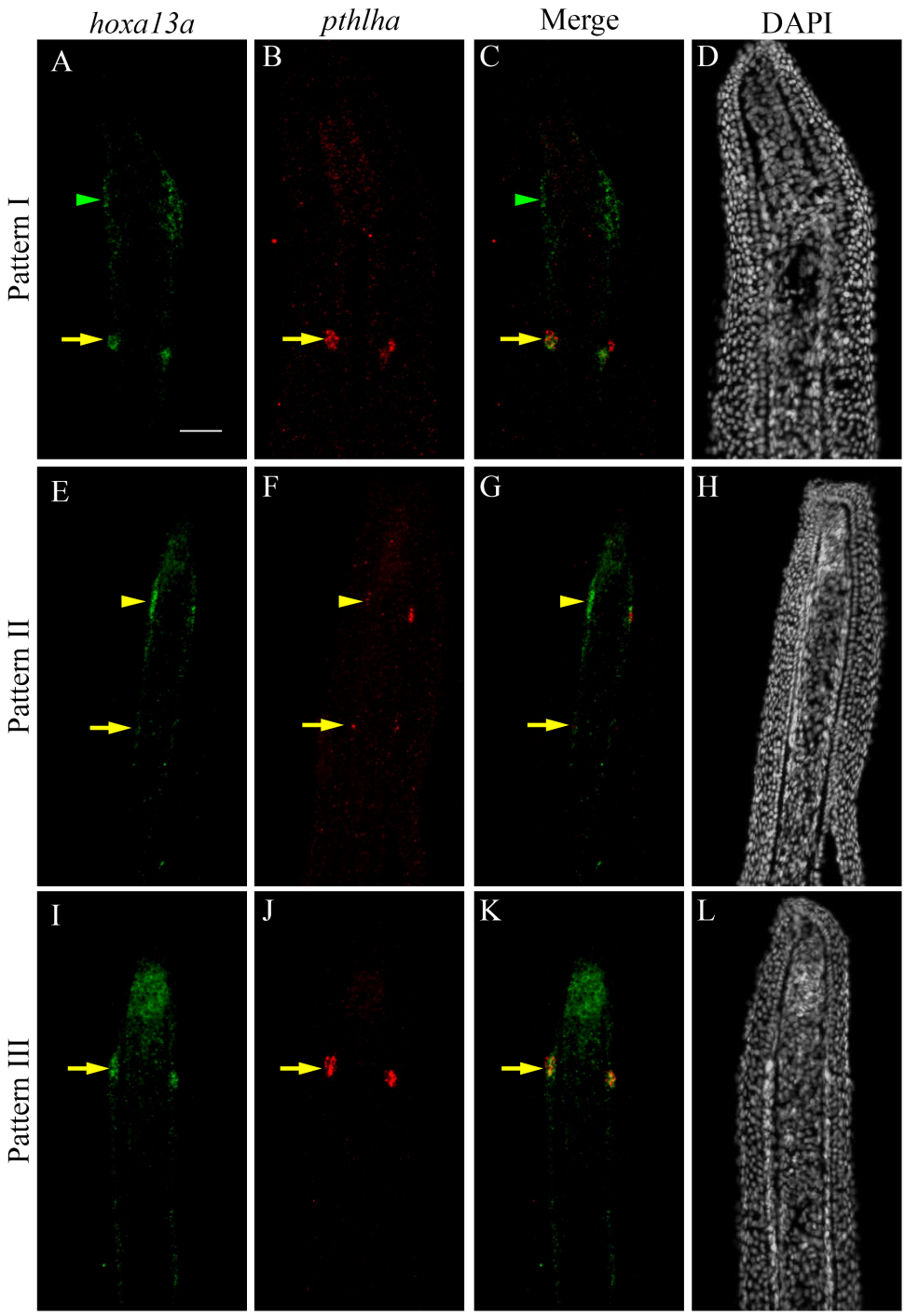
Supplementary figure 3

Fig. S3: Relative expression patterns of *hoxa13a* and *evx1*. (A-L) Double FISH (A-C, E-G, I-K) and DAPI counterstains (D, H, L) on longitudinal cryosections of 4dpa fin regenerates. (A-C, E-G, I-K) In joint-forming cells, *evx1* and *hoxa13a* are always co-expressed (yellow arrows). (A-H) In presumptive joint cells, *hoxa13a* is expressed either alone (Pattern I: A-D, green arrowheads) or is co-expressed with *evx1* (Pattern II: E-H, yellow arrowheads). (I-K) In Pattern III: *hoxa13a* and *evx1* are co-expressed in joint-forming cells when presumptive joint cells are not present (yellow arrows). (A, E, I) *hoxa13a* alone. (B, F, J) *evx1* alone. (C, G, K) *hoxa13a* and *evx1* expression merged. Scale Bars = 50μm (shown in A). These images are single image views for the merged images in Fig.4A-A".



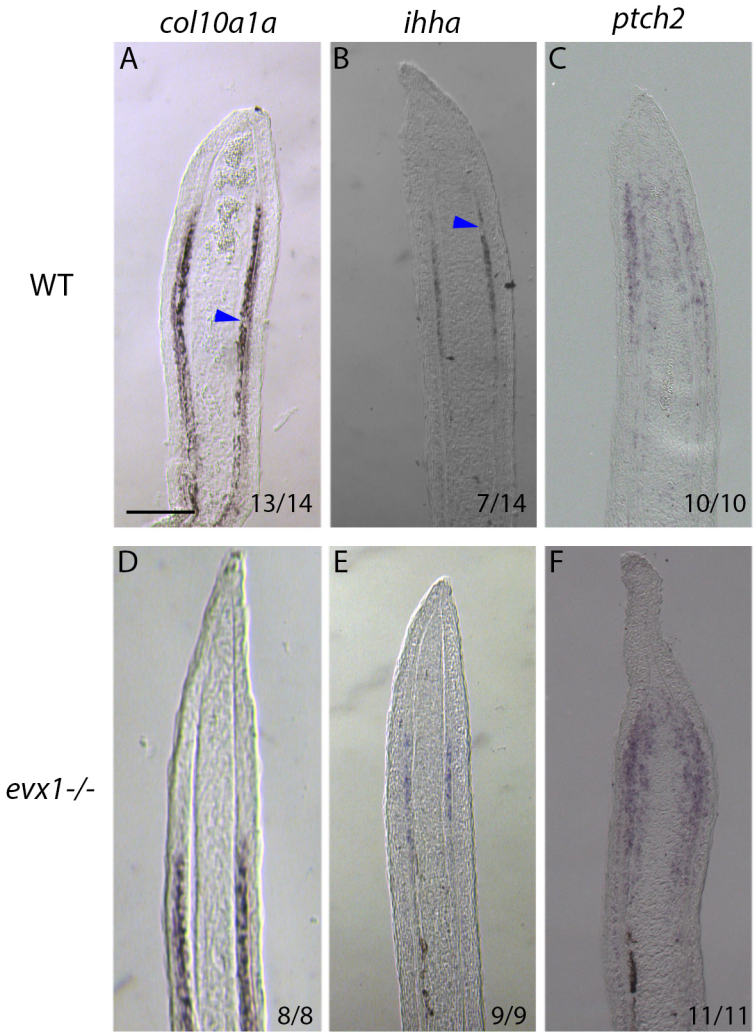
Supplementary figure 4

Fig. S4: Relative expression patterns of *evx1* and *pthlha*. (A-L) Double FISH (A-C, E-G, I-K) and DAPI counterstains (D, H, L) on longitudinal cryosections of 4dpa fin regenerates. (A-C, I-K) In joint-forming cells, *evx1* and *pthlha* are always co-expressed (yellow arrows). In presumptive joint cells, *evx1* is expressed either alone (Pattern I: A-D, green arrowheads) or is co-expressed with *pthlha* (Pattern II: E-H, yellow arrowheads). (I-L) In Pattern III: *evx1* and *pthlha* are expressed in joint-forming cells when presumptive cells are not yet present (yellow arrows). (A, E, I) *evx1* expression alone. (B, F, J) *pthlha* expression alone. (C, G, K) *evx1* and *pthlha* expression merged images. Scale Bars = 50μm (shown in A). These images are single image views for the merged images in Fig.4B-B".



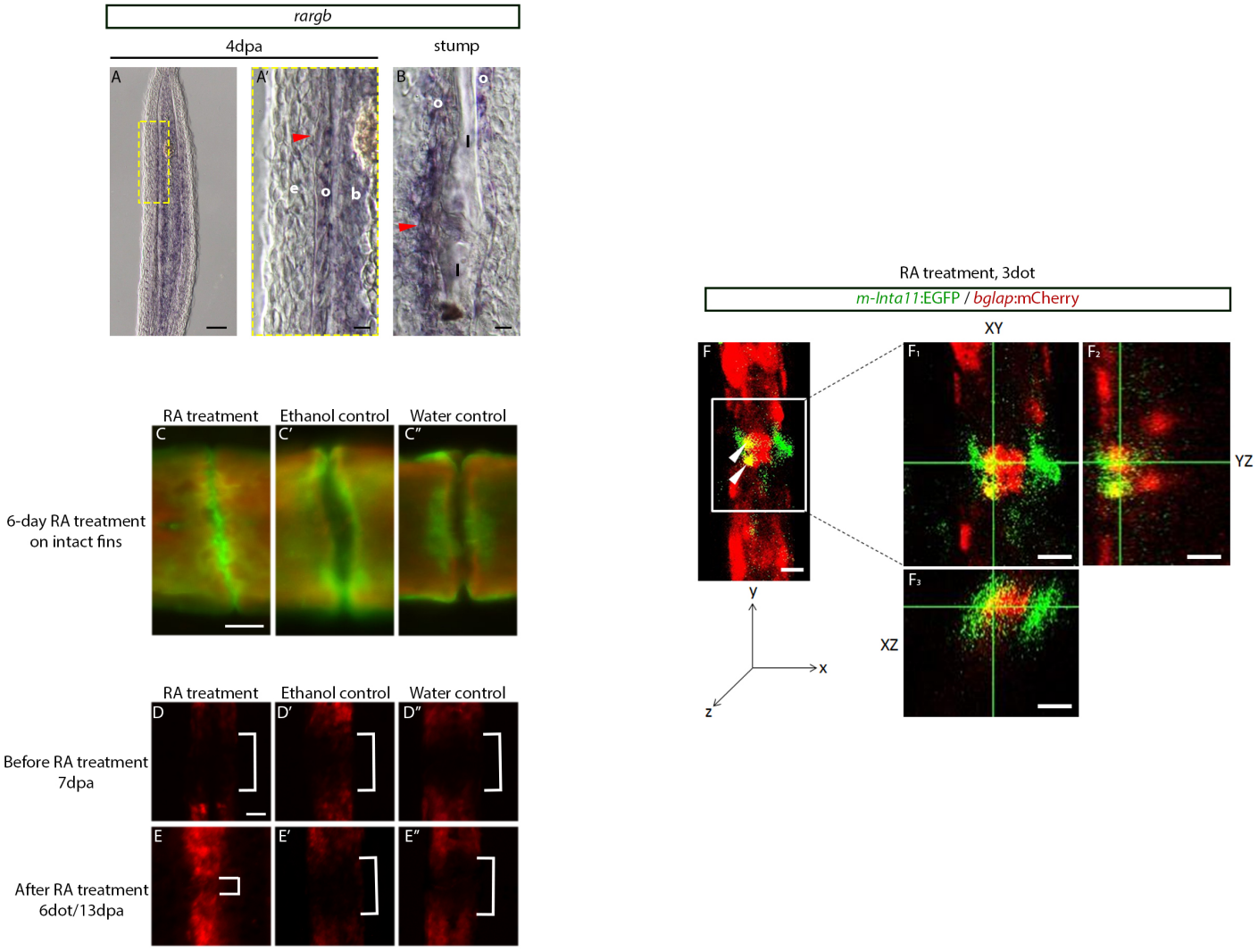
Supplementary figure 5

Fig. S5: Relative expression patterns of *hoxa13a* and *pthlha*. (A-L) Double FISH (A-C, E-G, I-K) and DAPI counterstains (D, H, L) on longitudinal cryosections of 4 dpa fin regenerates. (A-C, E-G, I-K) In joint-forming cells, *hoxa13a* and *pthlha* are always co-expressed (yellow arrows). In presumptive joint cells, *hoxa13a* is expressed either alone (Pattern I: A-D, green arrowheads) or is co-expressed with *pthlha* (Pattern II: E-H, yellow arrowheads). (I-L) In Pattern III: *hoxa13a* and *pthlha* are expressed in joint-forming cells when presumptive cells are not yet present (yellow arrows). (A, E, I) *hoxa13a* expression alone. (B, F, J) *pthlha* expression alone. (C, G, K) *hoxa13a* and *pthlha* expression merged. Scale Bars = 50μm (shown in A). These images are single image views for the merged images in Fig.4C-C".



Supplementary figure 6

Fig. S6: Gene expression analyses in wildtype and *evx1*$^{-/-}$ loss of function mutants. In wildtype longitudinal cryosections *col10a1a* (A) and *ihha* (B) are expressed in osteoblasts with gaps corresponding to joint cells (blue arrowheads). There is no change in *ptch2* expression between wildtype (C) and *evx1*$^{-/-}$ mutants (F). However, in *evx1*$^{-/-}$ mutants, *col10a1a* (D) and *ihha* (E) are expressed in a continuous pattern without the gaps corresponding to the position of joints. Scale bars for all panels = 100µm (shown in A). Numbers in each panel represent the number of sections with the expression pattern over the total number of sections analyzed.



Supplementary figure 7

Fig. S7: Retinoic Acid Treatment leads to bone deposition and osteoblast encroachment in joint regions. (A) ISH on 4dpa fin regenerates indicating *rargb* is expressed in osteoblasts and blastema of the 4dpa fin regenerate. (A') Magnified image from the yellow box in A indicating *rargb* is expressed in osteoblasts, blastema, and joint-forming cells (red arrowhead). (B) *rargb* is also expressed in mature joint cells (red arrowhead) surrounding the lepidotrichia. (C) Calcein (green) and alizarin red (red) stains illustrate that 6dot with RA results in new bone deposition (green) in joints of intact fins when compared to ethanol (C') and water (C") controls. (D-D") Prior to RA treatment, *Tg(bglap:mCherry)* fin regenerates do not possess mCherry expressing osteoblasts in joint regions (white brackets). (E) 13dpa/6dot with RA: mCherry-expressing osteoblasts are observed in the joints (white bracket). No mCherry-expressing osteoblasts are in joint regions in ethanol (E', D') and water (E", D") controls (white brackets). (F) Confocal image of *Tg(m-Inta11:EGFP; bglap:mCherry)* following 3 dot with RA. Image of the XY (F_1), YZ (F_2), and XZ (F_3) planes illustrating a joint cell co-expressing EGFP and mCherry (yellow). osteoblasts (o), blastema (b), lepidotrichia (l), basal epidermis (e). Scale Bars: A=50μm; A'-B=10μm; C-C''=10μm (shown in C); D-E''=50μm (shown in D); F-F_3=20μm.