Supplementary Data

Movie S1. Chondrocytes differentiate from *barx1*-lineage cells. Time-lapse video of *barx1*^{Gal4ff}*; UAS:*kikGR; *sox10:*DsRed embryo from 48 to ~90 hpf, showing DsRed expression (magenta) turning on in kikGR^{+} (green) *barx1*$^{+}$ lineage cells as the cells differentiate into chondrocytes.

Movie S2. Lateral but not medial chondrocytes in Meckel's cartilage are recently derived from *barx1*-lineage cells. Split-channel z-stack video of a *barx1*^{Gal4ff}*; UAS:*nlsGFP; *sox10:*DsRed larva at 6 dpf, showing colocalization of nlsGFP (green) with DsRed (magenta) in lateral but not medial chondrocytes in Meckel's cartilage. 40x ventral view with anterior to the left.

Movie S3. Lateral but not medial chondrocytes in the ceratohyal cartilage are recently derived from *barx1*-lineage cells. Split-channel z-stack video of a *barx1*^{Gal4ff}*; UAS:*nlsGFP; *sox10:*DsRed larva at 6 dpf, showing colocalization of nlsGFP (green) with DsRed (magenta) in lateral but not medial chondrocytes in the ceratohyal cartilage. 40x ventral view focused on one ceratohyal, with anterior to the left.

Movie S4. Chondrocytes in the hyomandibular but not the symplectic cartilage are recently derived from *barx1*-lineage cells. Split-channel z-stack video of a *barx1*^{Gal4ff}*; UAS:*nlsGFP; *sox10:*DsRed larva at 6 dpf, showing colocalization of nlsGFP (green) with DsRed (magenta) in chondrocytes of the hyomandibula and ceratohyal but not the symplectic, interhyal, or otic cartilages. 40x lateral view with anterior to the right.

Movie S5. PCCs emerge from *barx1*-lineage cells. Time-lapse video of a 36 hpf *barx1*^{Gal4ff}*; UAS:mCherryCAAX*; *sox9a:GFP* embryo from 36 to ~59 hpf, showing GFP expression (green) turning on in mCherryCAAX^{+} (magenta) *barx1*$^{+}$ lineage cells as they begin to mature into PCCs.

Table S1. Primers used to clone cDNAs for in situ probes

gene	forward primer (5'-3')	reverse primer (5'-3')	length (bp)	enzymes
cdh2	GCGTGAAAAGAGACTGGGTC	TAGGAATGGAGAAGCGTCCG	837	EcoRV, Sp6
lhx6	TCAGACGAGCCAGAGGAAAC	GATTCACAGGTCACCCATGC	800	EcoRV, Sp6
lhx8a	TTTGTCGCCAAGCTCTTCAC	AGGGGCAAAAGCAGTGTACT	775	EcoRV, Sp6
ncam1a	GACCCGCTACACTGAGCTTA	ATTGCTGTGCTGGAATACGG	894	BamHI, T7
pax9	CTGGACTCGGAACAGGTCAG	CCGTTATTGATCGAATGCCCA	697	BamHI, T7

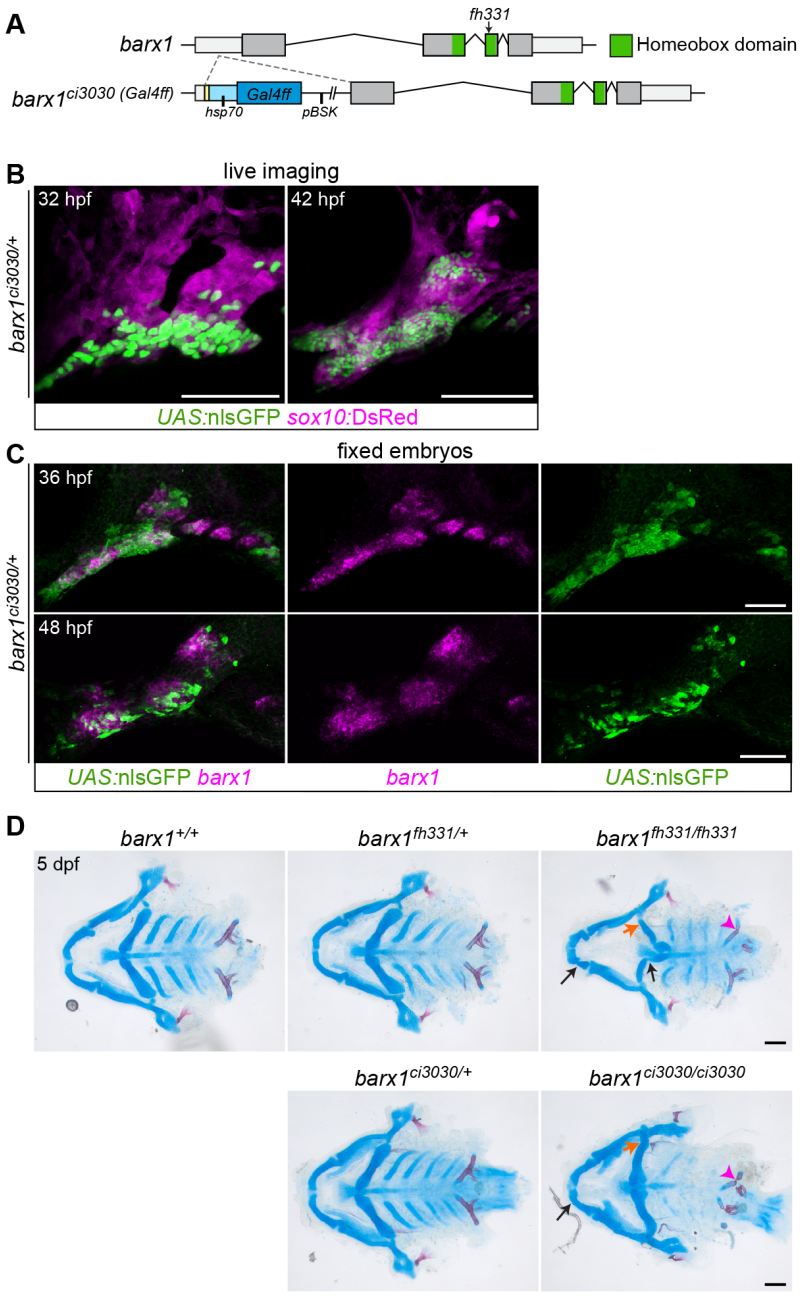

Fig. S1. The *barx1* Gal4ff knockin allele drives expression reminiscent of endogenous *barx1* and is a hypomorphic allele. A, Structure of the zebrafish *barx1* gene, with the locations of the homeobox and the *fh331* nonsense allele indicated. The *ci3030* allele is a knockin of a pBSK plasmid containing a minimal *hsp70l* promoter upstream of the Gal4ff coding sequence into the *barx1* 5'UTR. **B**, Live imaging of *barx1*^{ci3030}; *UAS:nlsGFP; sox10:DsRed* larvae at 32 and 42 hpf, showing discrete GFP expression within the DsRed^{+} pharyngeal arches. **C**, In situ hybridization for *barx1* in *barx1*^{ci3030}; *UAS:nlsGFP*

embryos at 36 and 48 hpf confirms strong colocalization between GFP and the endogenous mRNA. **D**, Alcian blue and Alizarin red staining of 5 dpf larvae carrying either wild-type or mutant *barx1* alleles. The ectopic joints in Meckel's cartilage and the ceratohyal (black arrows) apparent in the *fh331* mutant are not clearly defined in the *ci3030* mutant, though the latter does present the characteristic diminution of the dorsal half of the ceratohyal (orange arrows) and an ectopic joint in the fifth ceratobranchial with tooth detachment (pink arrowhead). Scale bars in B-C = 50 μm; in D = 100 μm.

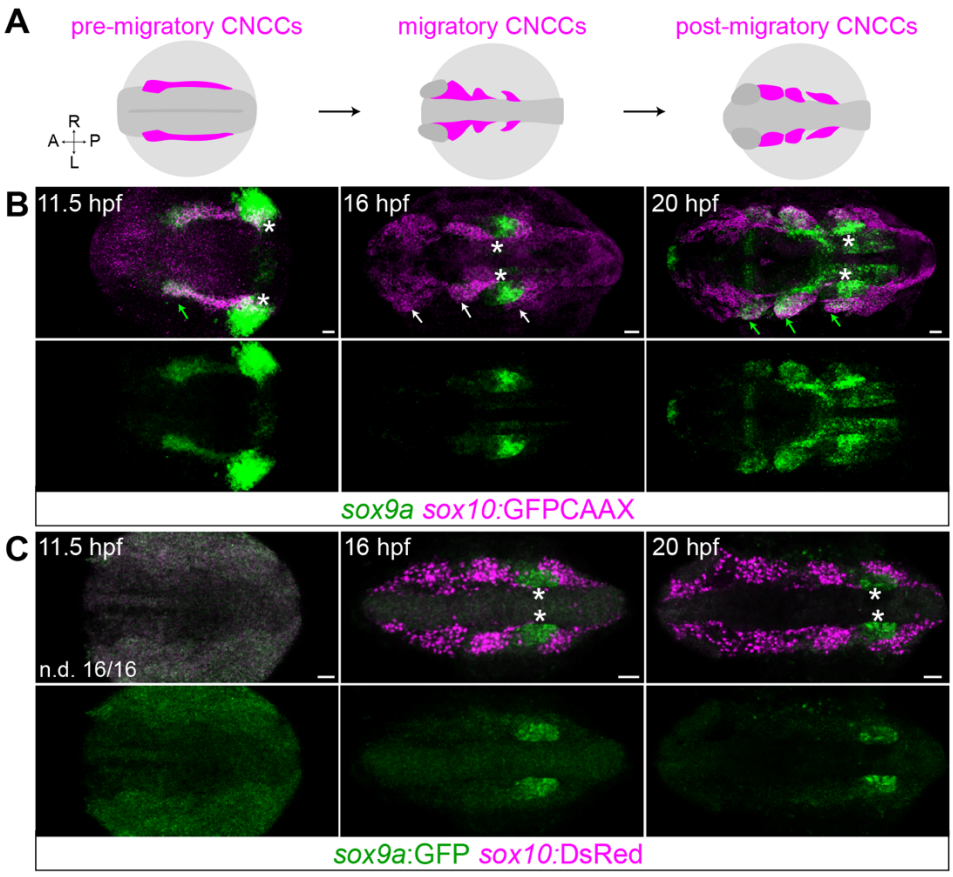

Fig. S2. Waves of *sox9a* expression in pre-migratory and post-migratory cranial neural crest. A, Schematics indicating the position of CNC cells (magenta) at pre-migratory (~11.5 hpf), migratory (14-16 hpf), and post-migratory (20 hpf) stages. Dorsal view. **B**, In situ hybridizations for *sox9a* (green) in embryos carrying a *sox10:GFPCAAX* transgene that labels all CNC cells (magenta). *sox9a* mRNA is expressed in the otic placode (*) and pre- and post-migratory CNC cells (green arrows) but not in migrating CNC cells (white arrows). **C**, Fixed transgenic embryos from the same stages show that the *sox9a:*GFP reporter does not recapitulate early *sox9a* expression in pre- and post-migratory CNC but is expressed in the otic placode. Neither *sox9a:*GFP nor *sox10:*DsRed was detected (n.d.) in any 11.5 hpf embryo (n = 16 screened by confocal; from the same clutch as the 16 and 20 hpf embryos shown). Images are maximum intensity projections. Scale bars = 50 μm.

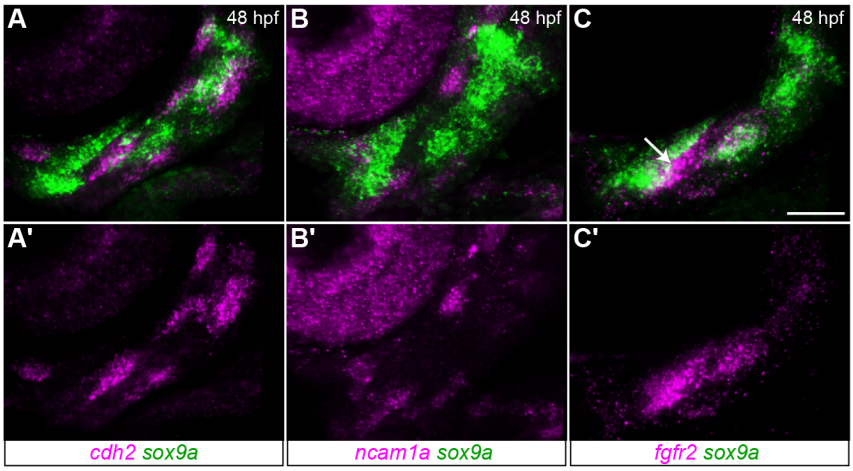

Fig. S3. Amniote precartilaginous condensation markers are not enriched in early zebrafish precartilaginous condensations. A-B, Double in situ hybridizations for *cdh2* and *ncam1a* with *sox9a* reveal no co-expression in nascent PCCs at 48 hpf. **C**, *fgfr2* overlaps with *sox9a* in the parts of the PCCs that initiate chondrogenesis early, particularly the posterior edge of palatoquadrate (arrow). Single channels are shown in A'-C'. Scale bar = 50 μm.

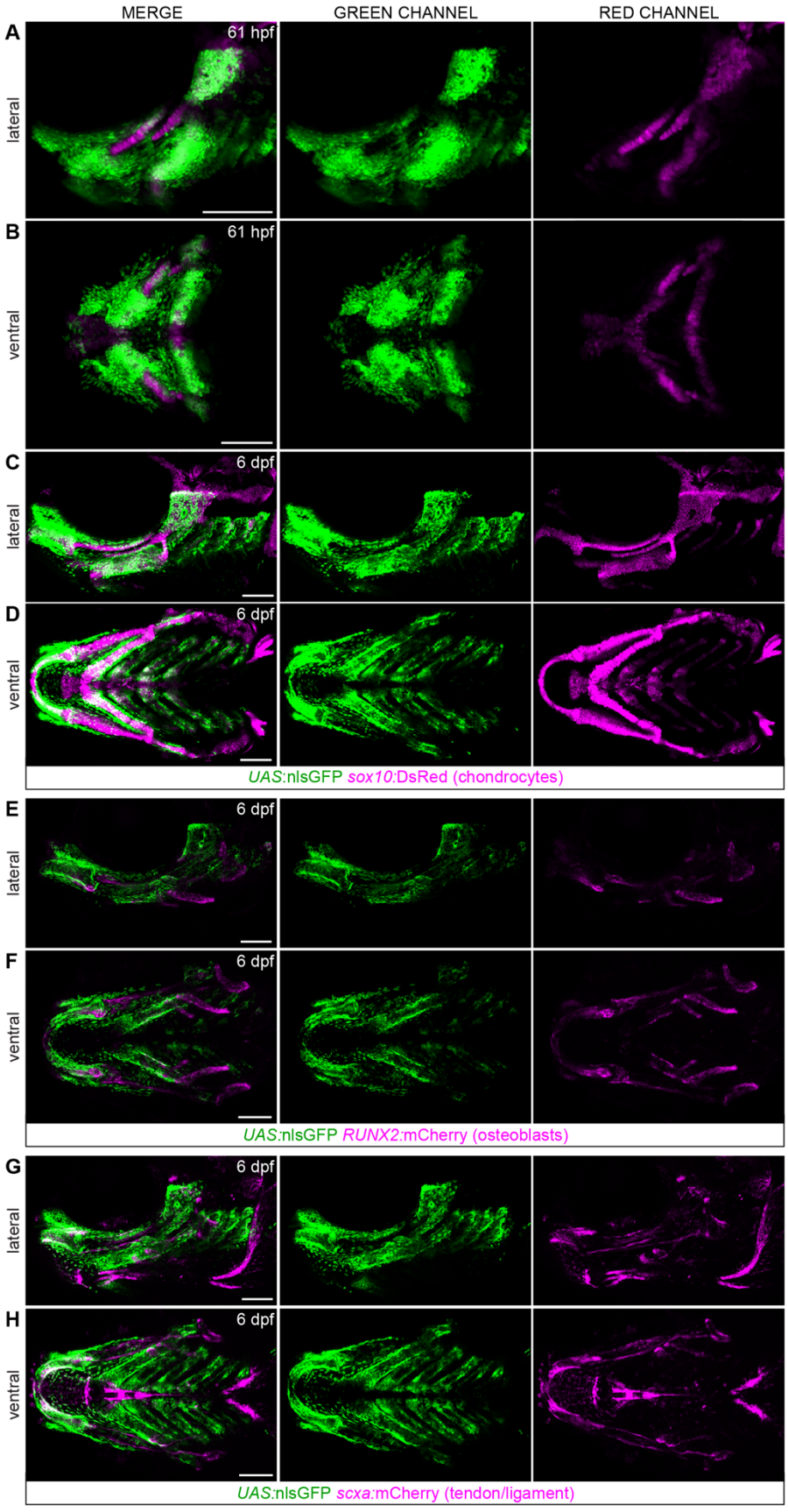
MERGE
GREEN CHANNEL
RED CHANNEL
A
61 hpf
lateral
B
61 hpf
ventral
C
6 dpf
lateral
D
6 dpf
ventral
UAS:nlsGFP sox10:DsRed (chondrocytes)
E
6 dpf
lateral
F
6 dpf
ventral
UAS:nlsGFP RUNX2:mCherry (osteoblasts)
G
6 dpf
lateral
H
6 dpf
ventral
UAS:nlsGFP scxa:mCherry (tendon/ligament)

Fig. S4. The *barx1*-lineage marker is co-expressed broadly with a chondrocyte reporter but minimally with osteoblast and connective tissue reporters. Merged-channel images from Fig. 3A-D are shown here with their respective single-channel components to better illustrate the overlap or lack thereof between *barx1*^{Gal4ff}*; UAS:*nlsGFP and sox10:*DsRed* (chondrocytes; shown at 61 hpf (**A-B**) and 6 dpf (**C-D**), *RUNX2:*mCherry (osteoblasts, **E-F**), and *scxa*:mCherry (tendon/ligament, **G-H**). Scale bars = 100 μm.

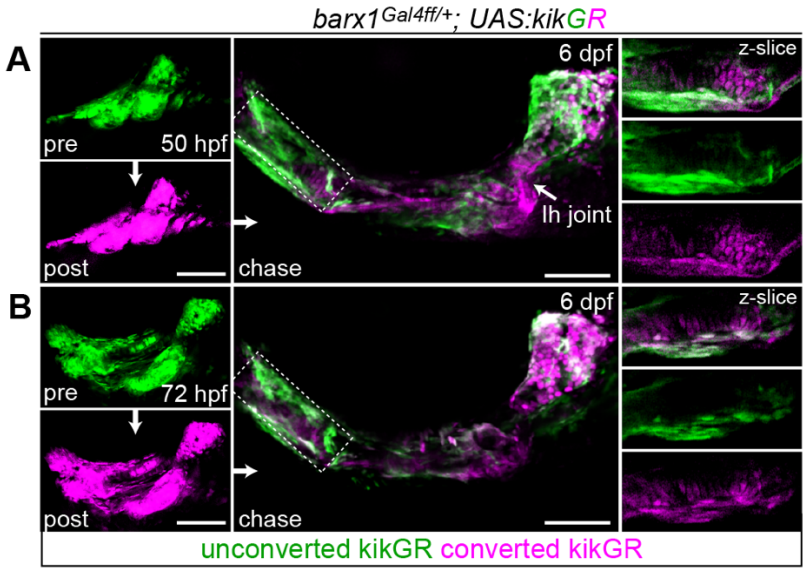

Fig. S5. Restricted domains of new protein production from the *barx1* driver after 50 hpf. *barx1*^{Gal4ff}; *UAS:kikGR* embryos were exposed to a UV laser at 50 (**A**) or 72 hpf (**B**) to irreversibly photoconvert green kikGR protein ("pre") to red ("post"). They were then chased to 6 dpf and reimaged. Newly synthesized kikGR protein (green) was predominantly limited to perichondral and mesenchymal cells (see single-channel z-slice images to the right of the main panels). However, some green chondrocytes were observed in the Hm cartilage, particularly in the larvae exposed to UV at 50 hpf, indicative of continued Gal4-induced kikGR expression after 50 hpf. Conversely, no green protein was observed in the Ih joint region. Images are maximum intensity projections (mips) unless otherwise noted. Scale bars A-H = 100 μm.