Understanding the evolutionary transformation of fish fins into tetrapod limbs is a fundamental problem in biology. The search for antecedents of tetrapod digits in fish has remained controversial because the distal skeletons of limbs and fins differ structurally, developmentally, and histologically. Moreover, comparisons of fins with limbs have been limited by a relative paucity of data on the cellular and molecular processes underlying the development of the fin skeleton. Here, we provide a functional analysis, using CRISPR/Cas9 and fate mapping, of $5'$ hox genes and enhancers in zebrafish that are indispensable for the development of the wrists and digits of tetrapods. We show that cells marked by the activity of an autopodial hoxa13 enhancer exclusively form elements of the fin fold, including the osteoblasts of the dermal rays. In hoxa13 knockout fish, we find that a marked reduction and loss of fin rays is associated with an increased number of endochondral distal radials. These discoveries reveal a cellular and genetic connection between the fin rays of fish and the digits of tetrapods and suggest that digits originated via the transition of distal cellular fates.

The origin of tetrapod limbs involved profound changes to the distal skeleton of fins. Fin skeletons are composed mostly of fin rays, whereas digits are the major anatomical and functional components of the distal limb skeleton. One of the central shifts during the origin of limbs in the Devonian period involved the reduction of fin rays coincident with an expansion of the distal endochondral bones of the appendage. Because the distal skeletons of fins and limbs are composed of different types of bone tissue (dermal and endochondral, respectively) it remains unclear how the terminal ends of fish and tetrapod appendages are related and, consequently, how digits arose developmentally. Although the understanding of ectodermal signalling centres in fin buds and fin folds has advanced in recent years, that of the cells that form the skeletal patterns has remained elusive.

Hox genes, namely those of the HoxA and HoxD clusters, have figured prominently in discussions of limb development and origins. The ‘early’ and ‘late’ phases of HoxD and HoxA transcription are involved in specifying the proximal (arm and forearm) and distal (autopod) segments, respectively. Both fate map assays and knockout phenotypes in mouse limbs reveal an essential role for HoxA paralogues in the formation of the autopod. Mice engineered to lack Hoxa13 and Hoxd13 in limbs lack the wrists and digits exclusively. Moreover, the lineage of cells expressing Hoxa13 resides exclusively in the autopod of adult mice. Together, these lines of evidence reveal the extent to which $5'$ Hox genes are involved in, and serve as markers for, the developmental pattern of the wrist and digits. Unfortunately, as no such studies have yet been performed in fish, the means to find antecedents of autopodal development in fins has been lacking.

Analyses of $5'$ Hox expression in phylogenetically diverse wild-type fish as well as experimental misexpression in teleosts reveal that $5'$ Hox activity may be involved in patterning, and defining the extent of, the distal chondrogenic region of fish fins. Despite these advances, however, little is known about the contribution of different hox paralogues—individually and in combination—to the adult fin phenotype and the origin of cells that give rise to the distal fin skeleton. While previous studies have shown that osteoblasts of the fin rays in the caudal fin of zebrafish are derived from either neural crest or paraxial mesoderm, the source of osteoblasts in pectoral fin rays is currently unknown. Consequently, it remains unclear where the cellular and genetic markers of the autopod of the tetrapod limb reside in fish fins.

In order to bridge these gaps in knowledge, we followed the fates of cells marked by early and late phase hox enhancers to adult stages in pectoral fins. In addition, we engineered zebrafish that completely lacked each individual hoxa13 gene, and bred stable lines with multiple gene knockout combinations of hox paralogues. The power of these experiments is twofold: 1) to our knowledge, they represent the first functional analyses of hox activity in fins, and 2) they enable a direct developmental comparison to experiments performed in tetrapod limbs.

We performed in situ hybridization of hoxa13a, hoxa13b, and hoxd13a genes from 48–120 h post fertilization (hpf) in zebrafish to determine whether active hox expression has a role in the development of the pectoral fin fold. Hoxa13 genes in zebrafish are expressed in the distal fin mesenchyme at 48 hpf and weakly in the proximal portion of the pectoral fin fold from 72–96 hpf, indicating that hoxa13 genes are not actively expressed in the developing fold (Fig. 1a, b). Hox13a is expressed in the posterior half of the fin, but it becomes weak after 96 hpf (Fig. 1c). Hox expression is entirely absent in fins 10 days post fertilization (dpf) (Extended Data Fig. 1). As hox13 genes do not appear to have a main role in zebrafish fin fold development past 72–96 hpf, we sought to determine what structures hox-positive cells populate in the developing and adult folds.

To follow the fates of cells that experience early phase activity in the zebrafish fin, we modified our previously reported transgenesis vector to express Cre-recombinase driven by the zebrafish early-phase enhancer CNS65. This enhancer activates expression throughout the endochondral disk of pectoral fins from 31 to ~38 hpf (Fig. 2a and Extended Data Fig. 1). Stable lines expressing CNS65xCre were crossed to the lineage-tracing zebrafish line Tg(ubi:Switch) fish, in which cells that express Cre are permanently labelled with mCherry. At 6 dpf mesenchymal cells in which expression was driven by CNS65 at 38 hpf make up the entire endochondral disk of the pectoral fin (Fig. 2b). We also found mCherry-positive cells in the fin fold at 6 dpf and extensively at 20 dpf (Fig. 2b). These cells contained filamentous protrusions extending distally as well as nuclei positioned at the posterior side, both of which suggest that the cells were migrating distally out of the endochondral disk (Fig. 2b).

To determine the fate of late phase cellular activity, we employed the same fate-mapping strategy but used a late phase hoxa enhancer (e16) from the spotted gar (Lepisosteus oculatus) genome. We chose a hoxa enhancer because lineage-tracing data in mouse has shown that late phase Hoxa13 cells in the limb make up the osteoblasts of the wrist and digits exclusively, making it a bona fide marker of the autopod.
In addition, gar e16 (which has no sequence conservation in zebrafish) drives expression throughout the autopod in transgenic mice in a pattern that mimics the endogenous murine enhancer and Hoxa13 expression21,27. In transgenic zebrafish, gar e16 is active in the distal portion of the endochondral disk of the pectoral fin at 48 hpf, and ceases activity after approximately 55 hpf (Fig. 2a and Extended Data Fig. 1). When these transgenic zebrafish were crossed to Tg(ubi:Switch), at 6 dpf we detected the majority of mCherry-positive cells in the developing fin fold with a small number of cells lining the distal edge of the endochondral disk (Fig. 2c). At 20 dpf, the fin fold contained nearly all of the mCherry-positive cells, which had formed tube-like cells that appeared to be developing actinotrichia (Fig. 2c). In adult fish (90 dpf), late phase cells were restricted to the adult structures of the fin fold, where they composed osteoblasts that make up the fin rays, among other tissues (Fig. 2d). As the e16 enhancer is active only in the distal endochondral disk at 48 hpf, and the labelled cells end up hybridizing in the fin fold, a hypothesis supported by extensive filopodia in mCherry-positive cells projecting in the direction of the distal edge of the fin (Fig. 2c).

To explore the function of hox13 genes, we inactivated individual hox13 genes from the zebrafish genome by CRISPR/Cas9 and also made combinatorial deletions through genetic crosses of stable lines (Extended Data Fig. 2 and Extended Data Table 1–3). Homozygous null embryos for individual hox13 genes exhibited embryonic pectoral fins that were comparable in size with the wild type at 72 hpf (Extended Data Fig. 3). The shape and size of the fin fold and endochondral disk were also assayed by in situ hybridization for and1 and shha, which serve as markers for the developing fin fold and endochondral disk, respectively28 (Extended Data Fig. 3). In adult fins (~120 dpf), we observed no detectable difference in the length of fin rays of hox13a−/− mutants when compared to wild-type fish (Fig. 3d and Extended Data Fig. 4). However, both hox13a−/− and hox13b−/− single mutant fish retained fin rays that were shorter than the wild type, suggesting a role for hox13 genes in fin ray development (Fig. 3g, j and Extended Data Fig. 4). To determine the degree to which endochondral bones were affected, we used CT scanning technology for wild-type and mutant adult fish. Each single mutant, hox13a−/−, hoxa13b−/− or d13a−/−, had four proximal radials and 6–8 distal radials with similar morphology to those of wild-type zebrafish (Fig. 3c, f, i and Extended Data Fig. 4). We crossed heterozygous mutants to obtain fish that lacked all hox13 genes (hox13a−/−, hoxa13b−/−). The fin folds of hox13a−/−, hoxa13b−/− embryos were ~30% shorter than the wild type at 72 and 96 hpf, whereas the number of cells in the endochondral disk was ~10% greater (Extended Data Fig. 5). Adult fins of hox13−/− and hox13a−/−, hox13b−/− or hoxa13−/−, had four proximal radials and 6–8 distal radials with similar morphology to those of wild-type zebrafish (Fig. 3c, f, i and Extended Data Fig. 4). We crossed heterozygous mutants to obtain fish that lacked all hox13 genes (hox13a−/−, hoxa13b−/−). The fin folds of hox13a−/−, hoxa13b−/− embryos were ~30% shorter than the wild type at 72 and 96 hpf, whereas the number of cells in the endochondral disk was ~10% greater (Extended Data Fig. 5). Adult
hox13a<sup>−/−</sup> and hox13b<sup>−/−</sup> fish exhibited greatly reduced fin rays (Fig. 3m, Extended Data Fig. 4 and Supplementary Information). In contrast to dermal reduction, the endochondral distal radials of double mutants were significantly increased to 10–13 in number, often stacked along the proximodistal axis (Fig. 3o, Extended Data Fig. 4 and Supplementary Information, P = 0.0014, t-test comparing the means). A similar pattern was seen in triple knockout fish (mosaic for hox13b and hox13a) (Fig. 3p–r and Extended Data Fig. 4) along with altered proximal radials, implying that late phase hox genes are involved in patterning the proximal endochondral radials of fins, unlike their role in tetrapods (Fig. 3).

Despite being composed of different kinds of skeletal tissue, fin rays and digits share a common population of distal mesenchymal cells that experience late phase Hox expression driven by shared regulatory architectures and enhancer activities. In addition, loss of 5′ Hox activity results in the deletion or reduction of both of these structures. Whereas phylogenetic evidence suggests that rays and digits are not homologous in terms of morphology, the cells and regulatory processes in both the finfold and the autopod share a deep homology that may be common to both bony fish and jawed vertebrates.

Figure 3 | Adult fin phenotypes of hox13 deletion series. a–c, wild type. d–f, hox13a<sup>−/−</sup>. g–i, hox13b<sup>−/−</sup>. j–l, hox13a<sup>−/−</sup>, hox13b<sup>−/−</sup>. m–o, hox13a<sup>−/−</sup>, hox13b<sup>−/−</sup>. p–r, hox13a<sup>−/−</sup>, hox13b<sup>−/−</sup> and d13a<sup>0%</sup> (mosaic triple knockout; Methods and Extended Data Tables 3, 4). Each mutant hox sequence is found in Extended Data Tables 3, 4. a, d, g, j, m, p, Alizarin Red and Alcian Blue staining of pectoral fin. b, e, h, k, n, q, CT scanning of pectoral fins. Black: radials (endochondral bones); grey: fin rays (dermal bones). Note that hoxa13 single (g, h, j, k), double (m, n), and mosaic triple (p, q) mutant fins show shorter fin rays than wild type (a, b). Fins were scaled according to the bone staining pictures. c, f, i, l, o, r, Enlarged images of CT scanning without fin rays to reveal endochondral patterns. Dark grey: proximal radials, red: distal radials. Upper left side is the anterior and bottom right is the posterior side in each picture. And double knockout mutants have 10–13 distal radials (o and r, Extended Data Fig. 4, Supplementary Information). Third and fourth proximal radials started to fuse into one bone in hoxa13a<sup>−/−</sup>, hox13b<sup>−/−</sup> (o). Note that posterior distal radials are stacked along proximodistal axis (o). Posterior proximal radials are broken down into small parts in mosaic triple knockout (r). Scale bars are 2 mm. The size of specimens are not scaled in c, f, i, l, o, r and r to display the detail of distal radials. n = 3 fish for single and double mutants and n = 5 fish for mosaic triple mutant.

Two major trends underlie the fin-to-limb transition—the elaboration of endochondral bones and the progressive loss of the extensive dermal fin skeleton. In the combinatorial knockouts of hox13 genes, which in tetrapods result in a loss of the autopod, distal endochondral radials were increased in number while fin rays were greatly reduced. As a common population of cells in the distal appendage is involved in the formation of rays and digits, the endochondral expansion in tetrapod origins may have occurred through the transition of distal cellular fates and differential allocation of cells from the fin fold to the fin bud. The two major trends of skeletal evolution in the fin-to-limb transition may be linked at cellular and genetic levels.

Online Content | Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.
**METHODS**

All zebrafish work was performed according to standard protocols approved by The University of Chicago (ACUP #72074). No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

**Whole-mount in situ hybridization.** *In situ* hybridization for the *hox13, Cre, and* *shha* genes were performed according to standard protocols22 after fixation in 4% paraformaldehyde overnight at 4 °C. Probes for *hox13* and *shha* were as previously described.23 Primers to clone Cre and *and1* into vector can be found in Extended Data Tables 1 and 2. Specimens were visualized on a Leica M205FA microscope.

**Lineage tracing vector construction.** In order to create a destination vector for lineage tracing, we first designed a random sequence of 298 bp that contained a Smal site to be used in downstream cloning. This sequence was ordered as a gBlocks fragment (IDT) and ligated into the pCR8/GW/GTOPO TA cloning vector (Invitrogen). We then performed a Gateway LR reaction according to the manufacturers specifications between this entry vector and pXIG–Fos–GFP, which abolished an NcoI site present in the destination vector, and ligated in Cre with (primers in Extended Data Table 1), using the pCR8GW–Cre–pA–FRT–kan–FRT‘ (kind gift of M. L. Suster, Sars International Center for Marine Molecular Biology, University of Bergen, Bergen, Norway) as a template for Cre PCR and Platinum Taq DNA polymerase High Fidelity (Invitrogen). In order to add a late phase enhancer to this vector, we first ordered four identical oligos (IDT gBlocks) of the core e16 sequence from gar, each flanked by different restriction sites. Each oligo was then ligated into pCR8/GW/GTOPO, and sequentially cloned via restriction sites into a single pCR8/GW/GTOPO vector. This entry vector was used to template to PCR the final Lo-e16x4 sequence and ligate it into the Cre destination vector for *and1 and Smal*, creating Lo-e16x4–Cre. The early phase enhancer Dr-CNS65x3 was cloned into the destination vector using the same strategy. Final vectors were confirmed by sequencing. A full list of sequences and primers used can be found in Extended Data Table 1.

**Establishment of lineage tracing lines.** *AB* zebrafish embryos were collected from natural spawning and injected according to the Tol2 system as described previously23. Transposase RNA was synthesized from the PC2-zT2TP vector using the mMessage mMachine SP6 kit (Ambion). All injected embryos were raised to sexual maturity according to standard protocols. Adult F0 fish were outcrossed to wild-type *AB*, and the total F1 clutch was lysed and DNA isolated at 24 hpf for genotyping (see Extended Data Table 1 for primers) to confirm germline transmission of Cre plasmids in the F0 founders. Multiple founders were identified and tested for the strongest and most consistent expression via antibody staining and *in situ* hybridization. One founder fish was identified as best, and all subsequent experiments were performed using offspring of this individual fish.

**Lineage tracing crossing and detection.** Founder Lo-e16x4–Cre and Dr-CNS65x3–Cre fish were crossed to the Tgl(ubc:Switch) line (kind gift from L. L. Zon). Briefly, this line contains a construct in which a constitutively active promoter (*ubquitin*) drives expression of a loxP flanked GFP protein in all cells of the fish assayed. When Cre is introduced, the GFP gene is removed and the *shha* gene is inserted. Founder experiments were performed using offspring of this individual fish. When Cre is introduced, the GFP gene is removed and the *shha* gene is inserted.

**Results**

**Embryo microinjection.** Embryos were obtained by crossing *shha*+/−*hox13+/−*/+* fish and found in Extended Data Table 2. Injected eggs were raised to adult fish and genotyped by extracting DNA from tail fins. PCR products of each *hox13* gene were PCR amplified from adult fish, wild type: 98 bp, mutant; 53 bp + 57 bp. The final product size was confirmed by 3% agarose gel electrophoresis. The details of the mutant sequence are summarized in Extended Data Table 3a–c.

**Combination of stable and transient deletion of all *hox13* genes by CRISPR / Cas9.** Two gRNAs targeting exon 1 of *hoxa13b* and two gRNAs targeting exon 1 of *hoxa13a* were injected with *Cas9* mRNA into zebrafish one-cell eggs that were obtained from mating *hoxa13a−/−* and *hoxa13a−/−* to each other and raised to 96 hpf. After fixation by 4% PFA for 15 h, caudal halves were used for PCR genotyping. Pectoral fins of wild type and *hoxa13a−/−*, *hoxa13b−/−* were detached from the embryonic body and placed horizontally on glass slides. The fins were photographed with a Zeiss Axioscope microscope, and the fin fold length along the proximal/distal axis at the centre of the fin was measured using ImageJ. The resulting data were analysed by t-test comparing the means.

**Counting the cell number in endochondral disk.** Embryos were obtained by crossing *hoxa13a−/−*, *hoxa13b−/−* to each other and raised to 96 hpf. After fixation by 4% PFA for 15 h, caudal halves were used for PCR genotyping. Wild type
and hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> embryos were stained by DAPI (1:4,000 in PBS-0.1% Triton) for 3 h and washed for 3 h by PBS–0.1% Triton. Pectoral fins were detached from the embryonic body, placed on glass slides and covered by a coverslip. The DAPI signal was detected by Zeiss LSM 710 (Organismal Biology and Anatomy, the University of Chicago). Individual nuclei were manually marked using Adobe Illustrator and the number of nuclei was counted. The data were analysed by t-test comparing the means.

**Adult fish skeletal staining.** Skeletal staining was performed as previously described<sup>33</sup>. Briefly, fish were fixed in 10% neutral-buffered formalin overnight. After washing with milli-Q water, solutions were substituted by 70% EtOH in a stepwise fashion and then by 30% acetic acid/70% EtOH. Cartilage was stained with 0.02% alcian blue in 30% acetic acid/70% EtOH overnight. After washing with milli-Q water, the solution was changed to a 30% saturated sodium borate solution and incubated overnight. Subsequently, specimens were immersed in 1% trypsin/30% saturated sodium borate and incubated at room temperature overnight. Following a milli-Q water wash, specimens were transferred into a 1% KOH solution containing 0.005% Alizarin Red S. The next day, specimens were washed with milli-Q water and subjected to glycerol substitution. Three replicates for each genotype were investigated.

**PMA staining and CT scanning.** After skeletal staining, girdles and pectoral fins were manually separated from the body. Girdles and fins were stained with 0.5% PMA (phosphomolybdic acid) in milli-Q water for 16 h and followed by washes with milli-Q water. Specimens were placed into 1.5 ml Eppendorf tubes with water and kept overnight to settle in the tubes. The next day, tubes containing specimens were set and scanned with the UChicago PaleoCT scanner (GE Phoenix v/tome/x 240kv/180kv scanner) (http://luo-lab.uchicago.edu/paleoCT.html), at 50 kVp, 160μA, no filtration, 5×-averaging, exposure timing of 500 ms per image, and a resolution of 8μm per slice (512 μm<sup>3</sup> per voxel). Scanned images were analysed and segmented using Amira 3D Software 6.0 (FEI). Three replicates for single and double homozygotes and five for mosaic triple knockout were investigated.

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Extended Data Figure 1 | Cre in situ hybridization of lineage tracing fish. a, Cre is expressed only from 31 hpf to 38 hpf in Dr-CNS65x3-Cre, whereas it is expressed from 38 hpf to 55 hpf in Lo-e16x4-Cre. These temporal expression patterns of Cre indicate that our transgenic lineage tracing labelled the cells which experienced only early or late phase hox. Scale bars are 100 μm. b, Cre expression pattern from 48–120 hpf in independent Lo-e16x4-Cre lines (different founders from a). The fin is outlined by a dashed white line. The expression patterns from different founders were investigated and all expression ceases before 72 hpf. Our in situ results indicate that Lo-e16x4-Cre marks only the cells that experienced late phase hox expression from 38–55 hpf. n = 5 embryos for all stages. Scale bars are 100 μm. c, The expression pattern of and1 and hox13 genes in wild type (10 dpf) and also Cre in Lo-e16x4-Cre line (10 dpf and 3 months, n = 10). Whereas and1 expression can be observed in fin fold (positive control, black arrow), hox13 genes are not expressed at 10 dpf in the wild type. Cre is not expressed at 10 dpf and at 3 months in the fin, indicating that Lo-e16x4-Cre activity is limited to only early embryonic development (38–55 hpf). Three month fins were dissected from the body of Lo-e16x4-Cre lines and subjected to in situ hybridization (n = 3). Scale bars are 500 μm at 10 dpf and 3 months.
PCR products of *hoxa13a*, *hoxa13b* or *hoxd13a* were subjected to a T7E1 assay (Methods) and confirmed by gel electrophoresis. a, The result of the *hoxa13a*, *hoxa13b* or *hoxd13a* T7E1 assay for ten adult fish. 'M.' is a 100 bp DNA ladder marker (NEB). In the *hoxa13a* gel picture, 810 bp (black arrowhead) is the wild-type band as observed in cont. lane (wild type without gRNA injection). All ten fish showed smaller and bottom shifted products (red arrowheads) compared to negative control fish, indicating that all fish have mutations in the target region of *hoxa13a*. In the *hoxa13b* gel picture, 1,089 bp is the wild-type band. All ten fish into which *hoxa13b* gRNAs were injected showed smaller and bottom shifted products compared to negative control fish, indicating that all fish have mutations in the target region of *hoxa13b*. In the *hoxd13a* gel picture, 823 bp is the wild-type band. Eight of ten fish showed smaller and bottom shifted products, indicating that 80% of fish have mutations in the target region of *hoxd13a*. b, The efficiency of CRISPR/Cas9 deletion for *hox13* in zebrafish. Almost all adult fish into which gRNAs and Cas9 mRNA were injected have mutations at the target positions. c, The efficiency of germline transmission of CRISPR/Cas9 mutant fish. Identified mutant fish were outcrossed to wild-type fish to obtain embryos and confirmed germline transmission. Obtained embryos were lysed individually at 48 hpf, genotyped by T7E1 assay and sequenced. Because of CRISPR/Cas9 mosaicism, some different mutation patterns, which result in a non-frameshift or frameshift mutation, were observed.
Extended Data Figure 3 | Embryonic phenotypes of hox13 deletion mutants. a, e, i, m, q. Whole body pictures at 72 hpf. a, Wild type, e, hoxa13a−/− (4 bp del./4 bp del.), i, hoxa13b−/− (4 bp del./14 bp ins.), m, hoxd13a−/− (5 bp ins./17 bp del.), and q, hoxa13a−/−, hoxa13b−/− double homozygous embryo (8 bp del./29 bp del., 14 bp ins./14 bp ins.). The details of mutant sequences are summarized in Extended Data Table 3. Wild-type and single homozygous fish for hoxa13a or hoxa13b were treated by PTU to inhibit pigmentation. The body size and length of mutant embryos are relatively normal at 72 hpf. n = 5 embryos for all genotypes. b, f, j, n, r, Bright field images of pectoral fins. Pectoral fins were detached from the body and photographed (Methods). Hoxa13a−/−, a13b−/− double homozygous embryo shows 30% shorter pectoral fin fold compared to wild type (r, see also Extended Data Fig. 5). n = 5 embryos for all genotypes. c, g, k, o, s, and l in situ hybridization at 72 hpf. Hox13 mutants show normal expression patterns, which indicates that fin fold development is similar to wild type in these mutants. n = 3 embryos for all genotypes. d, h, l, p, t, shha in situ hybridization at 48 hpf. Hox13 mutants show a normal expression pattern that is related to relatively normal anteroposterior asymmetry of adult fin (Fig. 3, Extended Data Fig. 4 and Supplementary Information). n = 3 embryos for all genotypes. Scale bars are 1 mm (a), 200 μm (b, c) and 100 μm (d).
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | Phenotype of adult hox13 mutant fish. a, c, e, g, i, k, m. Whole body morphology of hox13 deletion mutants were photographed at 4 months old; hox13a<sup>−/−</sup> (8 bp del./29 bp del.), hox13b<sup>−/−</sup> (4 bp del./14 bp ins.), hoxd13<sup>a−/−</sup> (5 bp ins./10 bp ins.), hox13a<sup>−/−</sup>, hox13b<sup>−/−</sup> double homozygous fish (8 bp del./29 bp del., 14 bp ins./14 bp ins.) and triple knockout (k, m, mosaic for hoxa13a hoxa13b and hoxd13a) fish (Methods). n = 3 fish for wild type, single and double mutants and n = 5 fish for triple mosaic mutants (same specimens were used as in Fig. 3). The details of mutant sequences are summarized in Extended Data Table 3. Each homozygous mutant fish shows normal morphology at 4 months old except for slightly short pectoral fin rays of hox13a<sup>−/−</sup> or a13b<sup>−/−</sup> single mutants. Hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> double homozygous fish shows a severe reduction of fin rays in pectoral, pelvic, dorsal and anal fins compared with wild type. The triple knockout (mosaic for hoxa13a, hoxa13b and hoxd13a) fish also showed a reduction in fin rays. Scale bar is 5 mm. Owing to the size of the adult fish, three different pictures for anterior, centre and posterior of the body were merged to make whole-body pictures. b, d, f, h, j, l, n. Bone staining pictures of mutant fish. The endochondral bones of pectoral fins are shown. Whereas single homozygous fish show relatively normal proximal radials (b, d, f, h and Fig. 3), double homozygous mutants show fused third and fourth proximal radials (j). One triple knockout (mosaic for hoxa13a, hoxa13b and hoxd13a, 0, 25, 50%) fish had fused third and fourth proximal radials (i), but another triple knockout (0, 0, 0%) had more broken down proximal radials (n). n = 3 fish for wild type, single and double mutants and n = 5 fish for triple mosaic mutants (same specimens were used as in Fig. 3). The scale bar is 500 μm. o, p. Examples of counting distal radials in wild-type and hox13a<sup>−/−</sup> double homozygous fish. First distal radials are not shown in CT segmentation because of a fusion with first fin ray. q. The number variation of distal radials in mutant fish. Multiple fins were investigated in wild type (25 fish/50 fins), hoxa13a<sup>−/−</sup> (4 bp del./4 bp del., 3 fish/6 fins), hoxa13b<sup>−/−</sup> (4 bp del./14 bp ins., 3 fish/6 fins), hoxd13a<sup>−/−</sup> (5 bp ins./17 bp del., 3 fish/6 fins), hox13a<sup>−/−</sup>, hox13b<sup>−/−</sup> double homozygous (8 bp del./29 bp del., 14 bp ins./14 bp ins., 3 fish/6 fins) and triple knockout (mosaic for hoxa13a, hoxa13b and hoxd13a) fish (five fish/10 fins). The number of distal radials increased to 10 and 13 in double and triple mutants, respectively. The difference in distal radial number between wild-type and double homozygous or wild-type and triple knockout fish (mosaic for hoxa13a, hoxa13b and hoxd13a) is statistically significant (P = 0.0014 or P = 0.00001, respectively, t-test comparing the means, two-tailed distribution).
**Extended Data Figure 5 | Analysis of embryonic fin fold and endochondral disk in hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> embryos.**

**a.** A bright field image of wild-type and hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> pectoral fins at 72 hpf. Pectoral fins were detached from the body and photographed (Methods). Scale bar is 150 μm.

**b.** The difference in fin fold length between wild-type and hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> embryos. The length of the fin fold was measured in wild-type (n = 8) and hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> double homozygous (n = 5) embryos at 72 hpf and 96 hpf (Methods). The length of the fin folds was decreased to about 70% of wild type in double homozygous embryos (72 hpf; P = 0.006, 96 hpf; P = 0.004, t-test comparing the means, one-tailed distribution, see Source Data). The error bars indicate s.e.m.

**c, d.** Images of DAPI staining of wild-type (c) and hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> mutant (d) pectoral fins captured by confocal microscopy. White circles indicate nuclei in the endochondral disks. Scale bar is 200 μm.

**e.** The average number of cells in the endochondral disk of wild-type and hoxa13a<sup>−/−</sup>, hoxa13b<sup>−/−</sup> mutant fins (see Methods and Source Data). The difference is statistically significant (P = 0.041 by Student’s t-test, one-tailed distribution). The error bars indicate s.e.m.
Extended Data Table 1  | Primers and oligos sequence for lineage tracing

Lineage tracing oligos

|CRE_PCR_F_Nco| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_PCR_R_BglII| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_Genotype_F| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_Genotype_R| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_Prome_F| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_Prome_R| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_Prome_rob_F| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|CRE_Prome_rob_R| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |

Primers and oligos sequence for lineage tracing

|Random_Oligo_Smal| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |
|Random_Oligo_Smal| 5'-GGGTACCTAGACTGACACGCGTGTTTGACCTCGGTTGCTTCGGGATCC-3' |

PCR primers and oligos for construction of lineage tracing vectors are listed (See Methods). Restriction enzyme sites that were used for ligating oligos are highlighted in italics and bold in oligo sequence.
Extended Data Table 2 | PCR primers for CRISPR/Cas9 deletion, T7E1 assay, genotypes and gene cloning

**CRISPR gRNA oligos**

zebra *hoxa13a* _gRNA1_F

5'- AATTAACTACGACTCATAAGGGAATACAACTCAGTTTAGGAGCTAGAAATAGC -3'

zebra *hoxa13a* _gRNA2_F

5'- AATTAACTACGACTCATAAGGGAATACAACTCAGTTTAGGAGCTAGAAATAGC -3'

zebra *hoxa13b* _gRNA1_F

5'- AATTAACTACGACTCATAAGGGAATACAACTCAGTTTAGGAGCTAGAAATAGC -3'

zebra *hoxa13b* _gRNA2_F

5'- AATTAACTACGACTCATAAGGGAATACAACTCAGTTTAGGAGCTAGAAATAGC -3'

zebra *hoxa13b* _gRNA_R

5'- AATTTTCTCAGGGCGCTTTTCAAGTGATGAAAGGACAGACATTAGCTATTATTCTAGCTAAAC -3'

**T7 assay primers**

zebra *hoxa13a* _Cont_F

5'- CTGCAGCGGGTGATTCTG -3'

zebra *hoxa13a* _Cont_R

5'- CTCCTTTACCCGTCGGTTTT -3'

PCR product: 810 bp

zebra *hoxa13b* _Cont_F

5'- GAAGCTTATCACTAGAATCTTTACAGC -3'

zebra *hoxa13b* _Cont_R

5'- TTTT TCTCAGGGCCTAAAGGT -3'

PCR product: 1089 bp

zebra *hoxa13b* _Cont_F

5'- TTCTTCTAGGGCGCTTTTAAAGGT -3'

PCR product: 823 bp

**Genotype primers for single (hoxa13a or a13b) and double (hoxa13a, a13b) mutants**

zebra *hoxa13a* _8 bp del_F

5'- GCCAAGGAGTTTGCCTTGTA -3'

zebra *hoxa13a* _8 bp del_R

5'- TGACGACTTCCAACGTTC -3'

PCR product: wild-type 231 bp, mutant (cut by Ava1) 111 +119 bp

zebra *hoxa13a* _29 bp del_F

5'- CAGGCAATAAGCGGGCCTT -3'

zebra *hoxa13a* _29 bp del_R

5'- GTGCAGTAGACCTGTCCGTT -3'

PCR product: wild-type 110 bp, mutant 81 bp

zebra *hoxa13b* _14 bp ins_F

5'- TACACTGGTTCGCAGCAAAA -3'

zebra *hoxa13b* _14 bp ins_R

5'- GATTGACCCGGTGATGTTTC -3'

PCR product: wild-type 98 bp, mutant (cut by Bcc1) 53 + 57 bp

**Cloning primers**

Danio_and1_F

5'-ACCTGCTCTGCTCCAGTTA -3'

Danio_and1_R

5'- CACATCTCCTTGAGGGGAAA -3'

For synthesis of gRNAs, each forward primer and common reverse primer ('zebra gRNA_R') were hybridized and used as templates. For genotype of single and double mutants, PCR products were treated by the enzymes indicated.
Extended Data Table 3 | List of hox13 mutant sequences

a. hoxa13a

| Sequence | Description |
|----------|-------------|
| TCCAGCAATAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCACTGCA | wild-type |
| TCCAGCAATAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCA | -22bp del / 29bp del. |
| TCCAGCAATAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCA | 14bp insertion |
| TCCAGCAATAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCA | 8bp insertion |

b. hoxa13b

| Sequence | Description |
|----------|-------------|
| CTAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCA | wild-type |
| CTAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCA | 7bp insertion |
| CTAAGCAATGCTGGATCCCGACTCAGTAGGCTTACTGCGATGGATTACTACAACGACCACTGCA | 11bp deletion |

.. (continued)
Extended Data Table 4 | Genotyping of progeny from mutant crosses

| Genotype | 72 hpf | 3 months |
|----------|--------|----------|
| hoxa13a+/--; hoxa13a+/+ | 9 (25.0%) | 9 (21.4%) |
| hoxa13a+/-- | 17 (47.2%) | 20 (47.6%) |
| hoxa13a+/+; hoxa13a+/+ | 10 (27.8%) | 13 (31.5%) |
| Total | 36 | 42 |

| Genotype | 72 hpf | 3 months |
|----------|--------|----------|
| hoxa13b+/--; hoxa13b+/+ | 8 (25.0%) | 20 (32.3%) |
| hoxa13b+/-- | 20 (62.5%) | 32 (51.6%) |
| hoxa13b+/+; hoxa13b+/+ | 12 (37.5%) | 10 (16.1%) |
| Total | 32 | 62 |

| Genotype | 72 hpf | 3 months |
|----------|--------|----------|
| hoxd13a+/--; hoxd13a+/+ | 8 (25.9%) | 18 (57.1%) |
| hoxd13a+/-- | 18 (51.4%) | 11 (57.9%) |
| hoxd13a+/+; hoxd13a+/+ | 9 (28.7%) | 3 (15.8%) |
| Total | 36 | 19 |

**a** Breeding data in hox13 single mutants. Single heterozygous fish were crossed with each other to obtain embryos and next generations. Embryos (72 hpf) or adult fish (3 months) were genotyped by T7E1 assay and sequenced. The number of each genotype and percentages are shown. The ratio of each genotype approximately follows Mendelian ratio.

**b** Breeding data for double hoxa13 mutants. Double heterozygous fish (hoxa13a+/-; hoxa13b+/-) were crossed to obtain embryos and next generations. Embryos (72 hpf) or adult fish (three months) were genotyped by PCR followed by enzyme digestion (Methods) or sequencing. The number of each genotype and percentage are shown. The ratio of each genotype approximately follows Mendelian ratio.

**c** The efficiency of triple knockout (mosaic for hoxa13a, hoxa13b and hoxd13a) in zebrafish (See Methods). The number of normal adult fish and short-finned fish from negative control injection (Cas9 mRNA without gRNAs) or triple knockout injection (Cas9 mRNA with gRNAs) are shown. Genotypes for short-finned fish were calculated from deep sequencing of each allele and shown as a percentage of normal alleles in d.

| Genotype | total adult fish | short finned fish | % of normal alleles |
|----------|------------------|-------------------|---------------------|
| Negative control; Cas9 only | 96 | 0 | 0.00 |
| Cas9/hoxa13b and d13a gRNAs | 161 | 7 | 4.35 |

**d** Genotype of short fin fish (The percent of normal alleles are shown):

| Genotype | #1 | #2 | #3 | #4 | #5 | #6 | #7 |
|----------|----|----|----|----|----|----|----|
| hoxa13a | 20% | 50% | 0% | 0% | 25% | 25% | 0% |
| hoxa13b | 20% | 0% | 25% | 0% | 0% | 0% | 0% |
| hoxd13a | 100% | 67% | 50% | 25% | 30% | 100% | 0% |