A pair of Sox: distinct and overlapping functions of zebrafish *sox9* co-orthologs in craniofacial and pectoral fin development

Yi-Lin Yan[1,*], John Willoughby[1,2,*], Dong Liu[1], Justin Gage Crump[1], Catherine Wilson[1], Craig T. Miller[1,3], Amy Singer[1], Charles Kimmel[1], Monte Westerfield[1] and John H. Postlethwait[1,†]

[1]Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA
[2]Biology Department, UMASS Amherst, MA 01002, USA
[3]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
*These authors contributed equally to this work
†Author for correspondence (e-mail: jpostle@uoneuro.uoregon.edu)

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Summary

Understanding how developmental systems evolve after genome amplification is important for discerning the origins of vertebrate novelties, including neural crest, placodes, cartilage and bone. *Sox9* is important for the development of these features, and zebrafish has two co-orthologs of tetrapod *SOX9* stemming from an ancient genome duplication event in the lineage of ray-fin fish. We have used a genotype-driven screen to isolate a mutation deleting *sox9b* function, and investigated its phenotype and genetic interactions with a *sox9a* null mutation. Analysis of mutant phenotypes strongly supports the interpretation that ancestral gene functions partitioned spatially and temporally between *Sox9* co-orthologs. Distinct subsets of the craniofacial skeleton, otic placode and pectoral appendage express each gene, and are defective in each single mutant. The double mutant phenotype is additive or synergistic. Ears are somewhat reduced in each single mutant but are mostly absent in the double mutant. Loss-of-function animals from mutations and morpholino injections, and gain-of-function animals injected with *sox9a* and *sox9b* mRNAs showed that *sox9* helps regulate other early crest genes, including *foxd3*, *sox10*, *snai1b* and *crestin,* as well as the cartilage gene *col2a1* and the bone gene *runx2a*; however, *tfap2a* was nearly unchanged in mutants. Chondrocytes failed to stack in *sox9a* mutants, failed to attain proper numbers in *sox9b* mutants and failed in both morphogenetic processes in double mutants. Pleiotropy can cause mutations in single copy tetrapod genes, such as *Sox9*, to block development early and obscure later gene functions. By contrast, subfunction partitioning between zebrafish co-orthologs of tetrapod genes, such as *sox9a* and *sox9b*, can relax pleiotropy and reveal both early and late developmental gene functions.

Key words: Chondrogenesis, Craniofacial, Gene duplication, Genome duplication, Limb morphogenesis, Skeletogenesis, Subfunction partitioning

Introduction

Conserved genetic pathways control development of chordate features (e.g. Bassham and Postlethwait, 2000; Satoh, 2003; Seo et al., 2004; Yasuo and Satoh, 1998). To shared chordate characters, vertebrates appended evolutionary novelties, including paired sense organs, cartilage, and bone (Gans and Northcutt, 1983; Northcutt and Gans, 1983; Shimeld and Holland, 2000). Several of these features derive from two tissues thought to be vertebrate innovations: neural crest, which gives rise to pigment cells, peripheral neurons, glia, craniofacial cartilage, and bone (e.g. Eisen and Weston, 1993), and epidermal placodes, which form ears, olfactory organs, lens, lateral line, and some cranial ganglia (Begbie and Graham, 2001). The evolutionary origin of developmental mechanisms leading to neural crest and placodes is not yet fully understood (Begbie and Graham, 2001; Graham and Begbie, 2000; Graham et al., 2004; Jeffery et al., 2004; Meulemans et al., 2003; Shimeld and Holland, 2000).

The transcription factor *Sox9* functions in the development of crest, placodes, cartilage, and bone. *Sox9* promotes crest-like behaviors in neural plate cells and biases cells towards glial and melanocyte fates (Cheung and Briscoe, 2003). *Sox9* also helps determine crest-derived chondrogenic lineages (Mori-Akiyama et al., 2003; Spokony et al., 2002; Yan et al., 2002), and it functions in morphogenesis and differentiation of cartilage and bone (Bi et al., 1999; Bi et al., 2001; Yan et al., 2002; Zelzer and Olsen, 2003). Despite these advances, the mechanisms by which *Sox9* acts in crest development are incompletely understood. *Sox9* also functions in placode development, including the otic placode (Liu et al., 2003; Saint-Germain et al., 2004), and human patients with mutations in *Sox9* sometimes lack olfactory bulbs (Houston et al., 1983). Given its role in crest and placode development, elucidating the mechanisms of *Sox9* action could help us understand the origin of vertebrate developmental innovations.

Our investigation of *Sox9* function exploits a special situation in teleost fish, the possession of two co-orthologs of tetrapod *Sox9* (Chiang et al., 2001; Cresko et al., 2003; Koopman et al., 2004; Li et al., 2002). These duplicated genes arose in an ancient genome duplication event that preceded the

teleost radiation (Amores et al., 1998; Koopman et al., 2004; Meyer and Schartl, 1999; Postlethwait et al., 1998; Postlethwait et al., 2000; Postlethwait et al., 2002; Taylor et al., 2003; Vogel, 1998; Wittbrodt et al., 1998). Although both genes still bind *Sox9*-binding enhancer sequences in DNA (Bell et al., 1997; Chiang et al., 2001; Ng et al., 1997), gene expression studies suggest that ancestral *Sox9* gene subfunctions partitioned between the two duplicates, leaving each with a subset of the original gene's functions (Chiang et al., 2001; Cresko et al., 2003; Li et al., 2002; Liu et al., 2003; Yan et al., 2002). This behavior is predicted to be frequent in the evolution of duplicated genes (Force et al., 1999; Hughes, 1994; Stoltzfus, 1999). Subfunction partitioning in teleosts may provide advantages for analysis (Postlethwait et al., 2004), and haploinsufficiency of *Sox9* mutations in mammals makes it difficult to obtain homozygous embryos for investigation (Bi et al., 2001; Mori-Akiyama et al., 2003; Sock et al., 2003); but we found that heterozygotes for mutations in zebrafish *sox9* co-orthologs are viable and produce homozygous mutant embryos.

To investigate the roles of *Sox9* in the development of neural crest and placodes, we conducted a genotype-driven screen for a mutation that deletes *sox9b* activity. We studied the phenotype of *sox9b* mutants, and compared them in single- and double-mutant combinations with *jellyfish* (*jef*^{hi1134}), a null mutation in *sox9a* (Yan et al., 2002). Results reveal distinct roles for *sox9a* and *sox9b* in development of crest, otic placode, cartilage, and bone, and illustrate how subfunction partitioning between teleost co-orthologs of human genes can facilitate the analysis of conserved gene function. Finally, we suggest that subfunction partitioning after duplications producing *Sox8*, *Sox9* and *Sox10* may have facilitated the evolutionary origin of neural crest and placodes.

Materials and methods

Genomic clones of *sox9b* (submitted as AY627769) were isolated by PCR from genomic DNA of 3 dpf (days post fertilization) zebrafish embryos using primers sox9b+296 CACCGGGACGAGCAGGAGAAGTT and sox9b-644 GTCTGGGCTGGTATTTGTAGTCTGGATGA obtained from the cDNA sequence AF277097, and they amplify most of the 5′ end of the gene, and primers sox9b+555 AGGGCGAGAAGCGTCCGTTTGT and sox9b-1695 AGCGCCACTGCAGATTAGATTGAA, which amplify most of the 3′ end.

Morpholino antisense oligonucleotides (MO) for *sox9b* (Gene Tools, Philomath, OR) were: intron 2 splice donor junction (e2i2) TGCAGTAATTTACCGGAGTGTTCTC, and intron 2 splice receptor junction (i2e3) GCCCTGAGACTGACCTGCACACACA. A mixture of both MOs (1 ng each) was injected into one-cell stage embryos. MOs for *sox9a* were as described (Yan et al., 2002).

In situ hybridization was performed as described (Jowett and Yan, 1996). Alcian blue and Alizarin red stained cartilage and bone in fixed larvae as described (Kimmel et al., 1998; Kimmel et al., 2003). Confocal imaging of live embryos and TUNEL assay was performed as described (Crump et al., 2004; Gavrieli et al., 1992).

The *sox9b*^{b971} mutation was made according to Fritz et al. (Fritz et al., 1996). Developing embryos were subjected to 150 to 300 rads of gamma radiation from a cesium-137 source at 2 hpf (hours post fertilization). 126 G0 females were squeezed to make G1 haploid embryos (Streisinger et al., 1981), of which 12 from each female were screened by PCR for loss of the *sox9b* 3′ UTR using primers sox9b+1461 CTCTGCCCGCTCACATCCAATACTC and sox9b-1695 AGCGCCAACTGCAGATTAGATTGAA. To control for sample loading, we simultaneously amplified the 3′UTR of *dlc* (NM_130944) using primers dlcF-GAGACTTGAAGACCCGAGGAAC and dlcR-AATAAAAGGCAAATACTCCACAG). From one G0 female, six of twelve haploids lacked the *sox9b* but not the *dlc* amplicon, and the mutation was inherited in Mendelian fashion. Deficiency mapping was done by PCR on DNA of haploid embryos from carrier females using Z-markers, gene-specific, and contig end-specific primers. To identify *b971* homozygotes, we amplified *sox9b* with *dlc* as internal control, and *jef*^{hi1134} (*sox9*^{hi1134}) heterozygotes were identified by PCR as described (Yan et al., 2002).

To make mRNA for rescue and ectopic expression experiments, we inserted *sox9a* and *sox9b* cDNA into pSP64T (Chiang et al., 2001; Krieg and Melton, 1984). The plasmid DNAs were linearized with *Bam*H1, and were transcribed in vitro with SP6 RNA polymerase using the SP6 mMESSAGE mMACHINE kit (Ambion). For mRNA injections, 50 pg mRNA was injected into 1-4-cell stage zebrafish embryos.

Results

sox9 co-orthologs are expressed in shared and gene-specific domains

To interpret mutant phenotypes, we must first understand gene expression. The ovary provides *sox9b* as a maternal message that disappears during cleavage in zebrafish and *Xenopus* (Chiang et al., 2001; Li et al., 2002; Spokony et al., 2002). In 14 hpf embryos, the expression level of *sox9a* was high in the otic placode and somites (Fig. 1A). In contrast, the expression level for *sox9b* was high in the developing ear, eye, pre-chordal plate, and cranial and trunk premigratory neural crest (Fig. 1B). Each gene has specific expression domains, for example, *sox9b* is expressed at high levels in the cranial and trunk crest, and reciprocally, *sox9a* is expressed at high levels in the somites. Cross-sections anterior to the otic vesicle show that *sox9b* is expressed at higher levels than *sox9a* in cranial crest (Fig. 1C,F) (Li et al., 2002). Expression of *sox9b* downregulates in the crest shortly after cells begin to migrate.

By 26 hpf, *sox9a* expresses at higher levels than *sox9b* in the pharyngeal arches (Fig. 1D). In contrast, *sox9b* expresses in the developing eye and in a dorsal-ventral band in each hindbrain rhombomere (Fig. 1E). At 48 hpf (Fig. 1G,H), both genes are expressed in similar domains in the hindbrain, whereas the tectum and eyes express *sox9b*, but not *sox9a* (Chiang et al., 2001).

Pectoral fins and pharyngeal arches express both *sox9* genes (Chiang et al., 2001), but in different domains. In the pectoral fin bud at 48 and 68 hpf (Fig. 1G-J), precursors of the scapulocoracoid express *sox9a* but not *sox9b*. In contrast, the endochondral disc expresses *sox9b* at high levels, especially distally, but expresses *sox9a* at lower levels. In the arches, *sox9a* expresses at high levels in cartilage precursors deep in the arch and at lower levels in a surrounding single cell layer, the perichondrium (Fig. 1K,L), which later makes osteoblasts and forms a bone collar around the cartilage (Caplan and Pechak, 1987). In contrast, *sox9b* expresses in the ectoderm and endoderm of the epithelial sheath that surrounds the *sox9a*-expressing perichondrium and cartilage (Fig. 1M,N).

This analysis and earlier results (Chiang et al., 2001; Li et al., 2002; Liu et al., 2003; Yan et al., 2002), show that *sox9a* and *sox9b* transcripts accumulate in overlapping and unique domains. Gene expression, however, does not equate to gene function. The *jef* mutation destroys *sox9a* activity (Yan et al.,

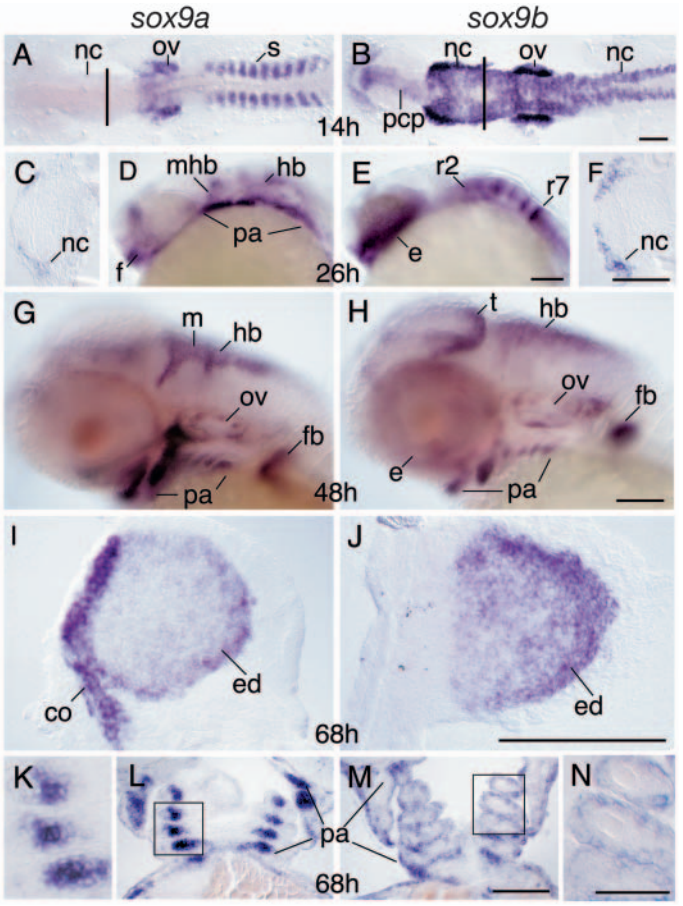

Fig. 1. Embryonic expression patterns. A,C,D,G,I,K,L, *sox9a*; B,E,F,H,J,M,N, *sox9b.* (A,B) 14 hpf, dorsal view. (C,F) Cross-sections of 14 hpf embryos at anterior-posterior level indicated by black bars in A and B. (D,E) Lateral views of 26 hpf embryos. (G,H) Lateral view, 48 hpf. (I,J) Pectoral fin bud at 68 hpf. (K-N) High (K,N) and low (L,M) magnification of sections of 68 hpf embryos through the arches. Abbreviations: co, scapulocoracoid; f, forebrain; fb, fin bud; e, eye; ed, endochondral disc; hb, hindbrain; m, midbrain; mhb, midbrain-hindbrain boundary; nc, neural crest; ov, otic vesicle; pa, pharyngeal arches; pcp, prechordal plate; r, rhombomeres; s, somites; t, tectum. Scale bars: in N, 50 μm for K,N; in B, 100 μm for A,B; in F, 100 μm for C-F; in H, 100 μm for G,H; in J, 100 μm for I,J; in M, 100 μm for L,M; in N, 100 μm for K,N.

2002), but to learn the role of *sox9b*, we mounted a genotype-driven screen.

b971 deletes *sox9b*

Using our previous strategy (Fritz et al., 1996), we conducted a genotype-driven screen for mutation of *sox9b*. Among 252 gamma ray-treated chromosomes, one lacked the *sox9b* PCR amplification product. This mutation, called *b971*, is inherited as a simple Mendelian recessive, and mapped to the lower end of LG3 (data not shown), the site of *sox9b* (Chiang et al., 2001). Haploid *b971* embryos were screened by PCR for loci previously mapped near *sox9b* (Barbazuk et al., 2000; Geisler et al., 1999; Hukriede et al., 1999; Knapik et al., 1998; Postlethwait et al., 2000; Postlethwait et al., 1998; Woods et al., 2000). Results showed that *b971* is a terminal deletion removing about 10 cM of the lower tip of LG3 (Fig. 2A). Embryos homozygous for *sox9b*^{b971} do not contain *sox9b* transcript (Fig. 2B,C), as expected for a deletion. The mutation also deletes an EST for *sox8* (fc23c10) (Cresko et al., 2003), but because it is not expressed at least up to hatching (data not shown), it is unlikely to affect the phenotype of *b971* embryos. To control for other ESTs deleted in *b971,* we treated wild types with *sox9b* morpholinos as explained below. Note that the splice-directed MOs caused *sox9b* transcript to accumulate in the nucleus rather than in the cytoplasm (Fig. 2D,E), suggesting that it remained in an unspliced form, as observed previously for *sox9a* (Yan et al., 2002), and confirming MO efficacy.

Cartilage phenotypes in Sox9-depleted animals

To understand *sox9b* function, we examined mutant phenotypes. Homozygous *sox9b*^{b971} embryos and double *sox9*

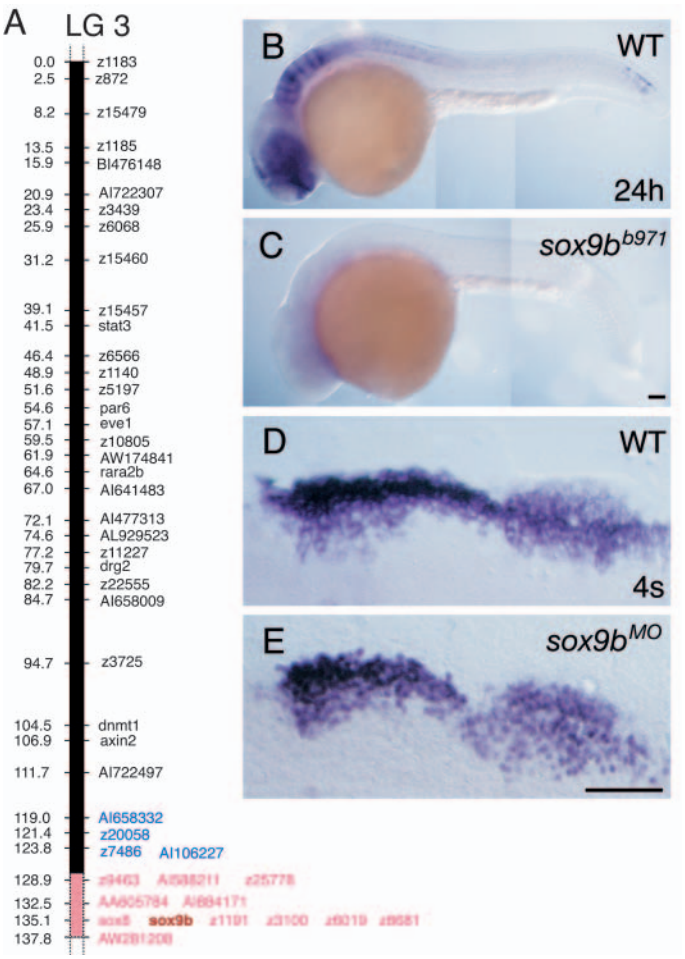

Fig. 2. *sox9b*^{b971} linkage map and *sox9b* expression, and *sox9b* morpholino-treated embryos. (A) LG3 (distance in cM), with red markers shown to be deleted, blue markers shown not to be deleted, and black markers untested. Region deleted, pale red. (B,C) Lateral views of 24 hpf embryos probed for *sox9b* expression. (B) wild type; (C) *sox9b*^{b971}. Mutant embryos lack *sox9b* expression. (D,E) Dorsal, right side view of cranial crest in four-somite stage embryos probed for *sox9b* expression. (D) Wild type; (E) embryo injected with *sox9b* splice-directed MO showing retention of *sox9b* transcript in the nuclei. Scale bars: in C, 100 μm for B,C; in E, 100 μm for D,E.

mutants do not straighten their body axis as wild types and *sox9a*^{hi1134} mutants do, giving a curly-down body axis (Fig. 3A,C,E). This phenotype could result from the brief expression of *sox9b* in the notochord at about 20 hpf. Animals injected with *sox9b* MOs also show the curly-down phenotype, supporting this hypothesis (Fig. 3D).

To understand the role of *sox9b* in cartilage development, we stained mutants with Alcian blue. Results showed that homozygous *sox9b*^{b971} larvae have reduced first and second pharyngeal arches and neurocranial cartilages (Fig. 3F,H). Ventral views show that *sox9a*^{hi1134} animals retain only a few cells of the ceratohyal (Fig. 3K,L), but *sox9b*^{b971} larvae preserve portions of several cartilages (Fig. 3M). This shows that *sox9a* plays a more important role than *sox9b* in the development of many pharyngeal cartilages. Although both single mutants retain portions of some pharyngeal cartilages, double mutants lack all pharyngeal cartilages (Fig. 3J,O). We conclude that *sox9a* and *sox9b* have both redundant and gene-specific functions in pharyngeal cartilage development.

The neurocranium responds like the arches. Mutations in *sox9a* delete all neurocranial cartilages except a few unidentified nodules (Fig. 3P,Q). In contrast, in *sox9b*^{b971} mutants, the neurocranium, including rostral ethmoid plate, anterior basicranial commissure cartilages, posterior basicapsular commissures, and occipital arches, is merely reduced (Fig. 3R). Animals injected with *sox9b* MO show a similar phenotype (Fig. 3I,S). Double mutants lack all traces of the neurocranium (Fig. 3T).

The *sox9* genes also have overlapping and distinct roles in the pectoral fin. The wild-type pectoral appendage includes cleithrum (dermal bone), scapulocoracoid (chondral bone), endochondral disc (or endoskeletal disc), and dermally derived actinotrichia (Grandel and Schulte-Merker, 1998) (Fig. 3U). Lack of *sox9a* function deletes the scapulocoracoid, but not the cleithrum, endochondral disc, or actinotrichia (Fig. 3V). As with *sox9a*, lack of *sox9b* leaves the cleithrum unchanged, but also leaves the scapulocoracoid intact, while decreasing the size of the endochondral disc and the number of actinotrichia, although those remaining are of normal length (Fig. 3W). Animals treated with *sox9b* MO have the same defect (Fig. 3X), showing that this mutant phenotype is due to lack of *sox9b* function. In the double mutant, the scapulocoracoid, endochondral disc, and nearly all of the actinotrichia disappear, leaving only the cleithrum (Fig. 3Y). The phenotype of the double mutant is more extreme than the sum of the phenotypes of the single mutants: the endochondral disc and most actinotrichia are missing from the double mutant.

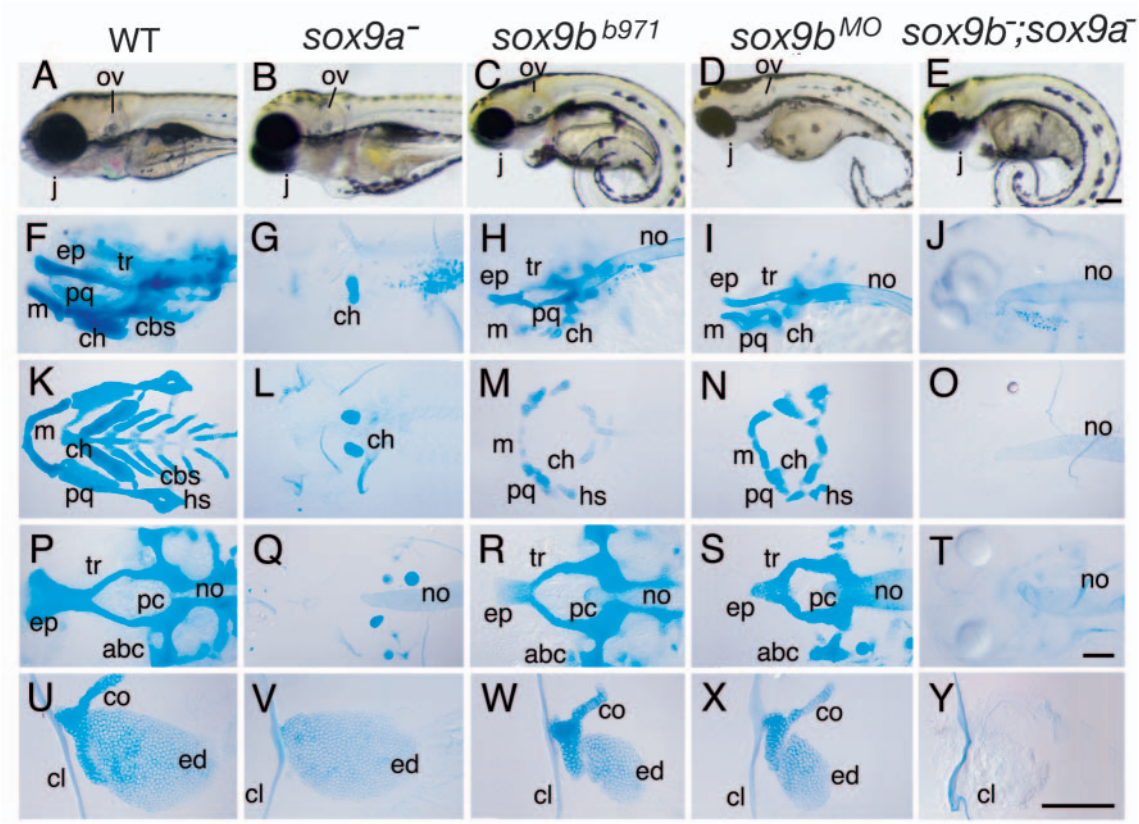

Fig. 3. Wild-type, *sox9* mutants, and *sox9b*-morpholino-treated animals. (A-E) Live larvae; (F-J) lateral view of head of Alcian-stained larvae. (K-O) Flat mount of Alcian-stained pharyngeal arches. (P-T) Flat mount of Alcian-stained neurocranium. (U-Y) Alcian-stained pectoral fin bud. All animals at 4 dpf. (A,F,K,P,U) Wild type; (B,G,L,Q,V) *sox9a* mutant, showing deletion of most cranial cartilage and scapulocoracoid; (C,H,M,R,W) *sox9b*^{b971}; neurocranium, arches, and endochondral disc reduced; (D,I,N,S,X) *sox9b* MO-injected animals phenotypically similar to *sox9b*^{b971}; (E,J,O,T,Y) double mutant, arches, neurocranium, and fin deleted. Abbreviations: abc, anterior basicranial commissure; cbs, ceratobranchials; ch, ceratohyal, m, Meckel's cartilage; cl, cleithrum; co, scapulocoracoid; ed, endochondral disc; ep, ethmoid plate; hs, hyosymplectic; j, jaw; ov, otic vesicles; no, notochord; pc, parachordal; pq, palatoquadrate; tr, trabecula. Scale bars: in E, 100 μm for A-E; in T, 100 μm for F-T; in Y, 100 μm for U-Y.

Because fin bud development was substantially altered in *sox9* mutants, we wondered whether the conserved genetic pathway that controls appendage patterning (Capdevila and Izpisua Belmonte, 2001; Richardson et al., 2004; Tanaka et al., 2000) was intact in double mutants. We therefore examined the expression patterns of two components, *shh*, which is expressed in the zone of polarizing activity (Neumann et al., 1999), and *dlx2a*, which is expressed in the apical ectodermal ridge (Akimenko et al., 1994). In the pectoral fin buds of 34 and 52 hpf embryos, expression of both genes was highly similar in normal animals, single mutants, and double *sox9* mutants (Fig. 4). We conclude that *sox9* activity, although essential for the development of the cartilaginous components of the proximal fin bud, is not required for the establishment of the main signaling centers in appendage development.

Can wild-type *sox9b* mRNA rescue *sox9b*^{b971} animals?

Morpholino experiments supported the hypothesis that phenotypes observed in *sox9b*^{b971} animals are due to the lack of *sox9b* function. Because the *sox9b*^{b971} mutation deletes several ESTs (Fig. 2A), however, we wanted to confirm that phenotypes we observed are due to deletion of *sox9b*. To test whether wild-type *sox9b* activity could rescue *sox9b*^{b971} phenotypes, we injected in vitro-synthesized wild-type *sox9b* mRNA into one- to four-cell stage homozygous *sox9b*^{b971} embryos and their phenotypically wild-type siblings. Despite the mosaic distribution of injected transcripts (Ekker, 1999), and the late phenotype we are examining, which results in mRNA dilution and gives opportunities for mRNA degradation, *sox9b* mRNA caused substantial rescue of Alcian stained pharyngeal cartilages (Fig. 5A,C,E,G,I) and rostral extension of the ethmoid plate in the neurocranium (Fig. 5B,D,F,H,J). We conclude that the cartilage phenotypes observed in the craniofacial skeleton of homozygous *sox9b*^{b971} animals result from loss of *sox9b* function.

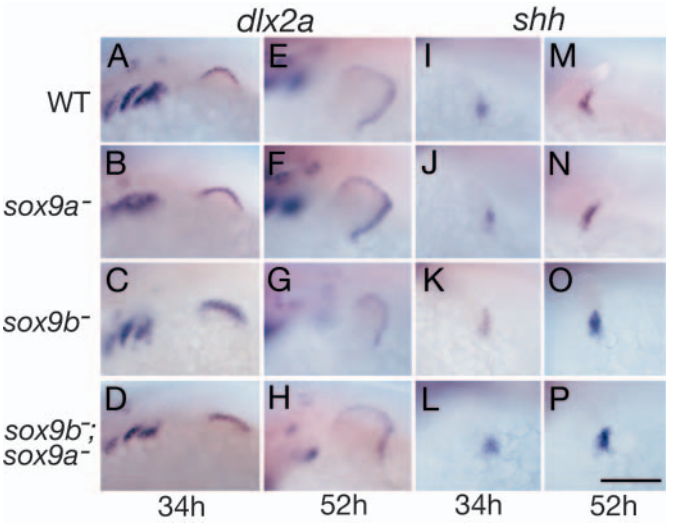

Fig. 4. Appendage developmental control genes in *sox9* mutants. (A-H) The apical ectodermal ridge gene *dlx2a*. (I-P) The zone of polarizing activity gene *shh*. (A-D,I-L) 34 hpf; (E-H,M-P) 52 hpf. (A,E,I,M) Wild type; (B,F,J,N) *sox9a*$^{-}$; (C,G,K,O) *sox9b*^{b971}; (D,H,L,P) *sox9a*$^{-}$; *sox9b*^{b971} double mutant. Scale bar: in P, 100 μm.

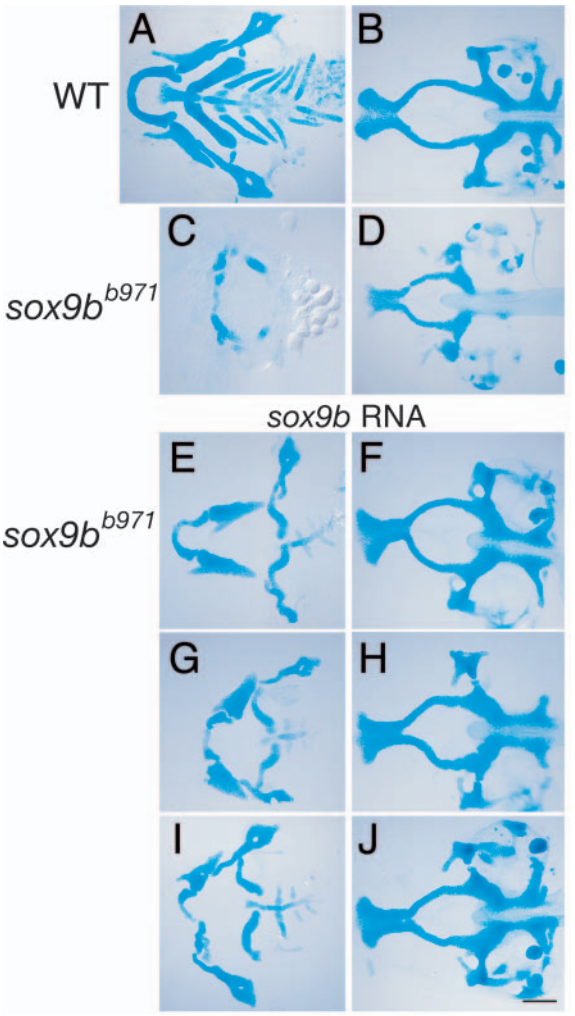

Fig. 5. Injection of *sox9b* mRNA partially rescues the mutant phenotype. (A,B) Wild type; (C,D) Control *sox9b*^{b971}; (E-J) *sox9b* mRNA-injected *sox9b*^{b971} larvae, showing partial rescue of the craniofacial skeletal phenotype of *sox9b*^{b971}. Partial rescue occurred in 14 of 85 injected four-day-old homozygous mutant larvae. (A,C,E,G,I) Pharyngeal arches; (B,D,F,H,J) Neurocranium. Scale bar: in J, 100 μm.

sox9 mutations and bone development

To understand the role of *sox9* in craniofacial bone development, we studied *runx2b* in *sox9* mutants. In mammals, *RUNX2* acts as the master regulatory gene for osteoblastogenesis (Karsenty and Wagner, 2002). Wild-type embryos at 2 dpf (Fig. 6A) express *runx2b* in precursors of the bony skeleton (Flores et al., 2004). This pattern is nearly normal in *sox9a*^{hi1134} mutants (Fig. 6B), but in *sox9b*^{b971} mutants, in wild-type animals injected with *sox9b* MOs, and in double mutants, *runx2b* expression is substantially reduced or missing from pharyngeal arches (Fig. 6C-E).

To directly study bone development, we stained living larvae with Alizarin at 5-7 dpf, a stage at which many bones have begun to differentiate in wild types (Fig. 6F). In *sox9a*^{hi1134} mutants (Fig. 6G), some dermal bones were somewhat smaller than normal (dentary, opercular, maxillary), whereas others

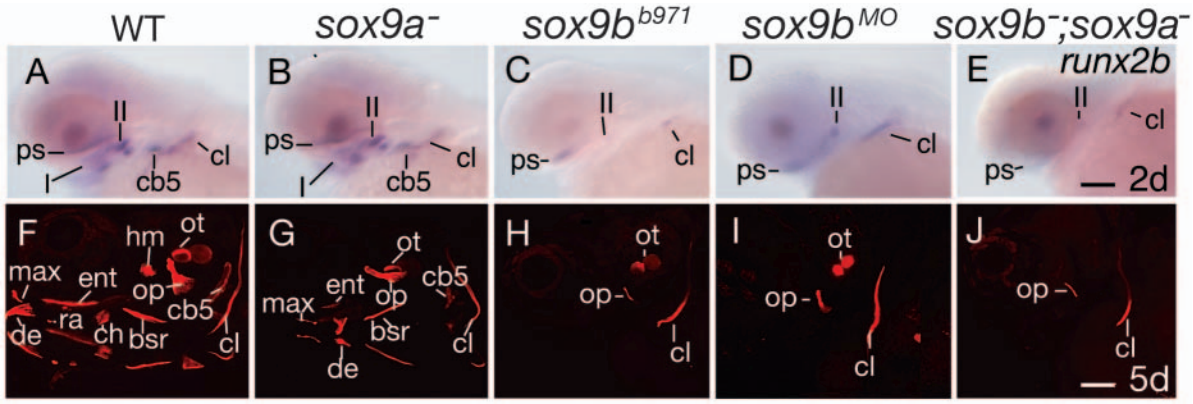

Fig. 6. Bone development. (A-E) Lateral view of *runx2b* expression at 2 dpf. (F-J) Alizarin red staining on live larvae at 5 dpf. (A,F) Wild type; (B,G) *sox9a*$^{-}$; (C,H) *sox9b*^{b971}; (D,I) *sox9b* MO injected; (E,J) double mutant. Abbreviations: I, first pharyngeal arch; II, second pharyngeal arch; bsr, brachiostegal rays; cb5, ceratobranchial arch 5; ch, ceratohyal; cl, cleithrum; de, dentary; ent, entopterygoid; hm, hyomandibular; max, maxilla; op, opercle; ot, otolith; ps, parasphenoid; ra, retroarticular. Scale bar: in J, 100 μm.

were relatively unaffected (cleithrum). In contrast, many cartilage replacement bones were completely missing (ceratohyal, and hyomandibular), and others were much smaller than normal (ceratobranchial-5) in *sox9a*^{hi1134} mutants. In *sox9b*^{b971} and animals treated with *sox9b* MOs (Fig. 6H,I), however, all cartilage-replacement bones and most dermal bones were missing, with the exception of the cleithrum and the opercle, which were reduced. Double mutants had bone defects similar to *sox9b*^{b971} single mutants (Fig. 6J), suggesting that the residual dermal bones remaining in *sox9b*^{b971} mutants are not due to redundancy with *sox9a*. We conclude that *sox9a* is more important for the formation of cartilage-replacement bones, and *sox9b* is important in the formation of both cartilage-replacement and dermal bones in the cephalic and pectoral fin skeletons.

sox9 mutations and pigment cell development

In addition to craniofacial cartilages, neural crest forms melanocytes, xanthophores, and iridophores (Rawls et al., 2001). A striking feature of *sox9b*^{b971} homozygotes and double mutants is the presence of melanocytes with dispersed melanosomes even in the light (Fig. 3C; Fig. 7A,C,D). To learn whether *sox9b*^{b971} affects melanocyte development, we examined expression of dopachrome tautomerase (*dct*), an enzyme of melanin biosynthesis (Kelsh et al., 2000), and found that the number, size, and distribution of *dct*-expressing cells was normal in *sox9b*^{b971} animals (Fig. 7E-H). This suggests that the melanocyte phenotype may be a function of cell physiology rather than development. Xanthophores appeared normal in *sox9b*^{b971} animals, as did the distribution of cells expressing xanthine dehydrogenase mRNA (data not shown). Dark field microscopy showed that *sox9a*^{hi1134} mutants have the normal number and distribution of iridophores (Fig. 7I,J), but *sox9b*^{b971} and double mutants had reduced populations of iridophores (Fig. 7K,L). Melanocyte and iridophore phenotypes were like *sox9b*^{b971} mutants in animals injected with *sox9b*-MO (Fig. 7C,K; data not shown). We conclude that *sox9b* is essential for normal development of iridophores and for the distribution of melanosomes within melanocytes.

The roles of *sox9* in otic vesicle development

Mutant phenotypes showed that *sox9* co-orthologs are important for development of the ear, a derivative of the otic placode. In *sox9a*^{hi1134} mutants, the otic vesicle and otoliths are nearly normal (Fig. 7M,N) (Liu et al., 2003; Yan et al., 2002). In *sox9b*^{b971} mutants, the ear is smaller than normal and forms small, but otherwise normal otoliths (Fig. 7O), confirming our previous analysis using *sox9b* MOs (Liu et al., 2003). The otic vesicle in double mutants, however, was usually missing, or if present, consisted of vestiges lacking normal otoliths (Fig. 7P). Antibody to Pax2, an otic placode marker (Puschel et al., 1992), failed to label the otic region in double mutants (data not shown), suggesting that the otic placode does not form in *sox9*-deficient zebrafish. Alizarin staining confirmed the absence of otoliths in double mutants (Fig. 6J). The enhanced ear phenotype of the double mutant compared to either single mutant suggests partially overlapping functions of *sox9* co-orthologs in otic placode development.

What is the position of *sox9* in the hierarchy of crest gene expression?

Our in situ hybridization experiments (Fig. 1B) showed that *sox9b* is expressed at high levels in premigratory neural crest about as early as other early crest markers such as *foxd3* (formerly *fkd6*) (Odenthal and Nusslein-Volhard, 1998), *snai1b* (formerly *snail2*) (Thisse et al., 1995), and *tfap2a* (formerly *AP2alpha*) (Barrallo-Gimeno et al., 2004; Knight et al., 2003; Knight et al., 2004; O'Brien et al., 2004). Double in situ hybridization experiments with *sox9b* and either *foxd3* or *snai1b* showed that these three genes are expressed in largely overlapping cell populations in this domain (data not shown).

Results showed that at the three-somite stage, *sox9a*^{hi1134} embryos express *snai1b* nearly normally (Fig. 8A,B), but *sox9b*^{b971} embryos have a substantially smaller *snai1b* expression domain (Fig. 8C). The same results were obtained with *foxd3* and *sox10* (data not shown). We used three different combinations of mutations and MOs to generate animals lacking activity of both *sox9a* and *sox9b* (*sox9b*^{b971};*sox9a-MO* and *sox9b-MO*;*sox9a*^{hi1134}, and *sox9b*^{b971};*sox9a*^{hi1134} double mutants). All combinations showed greatly reduced expression of *snai1b* (Fig. 8D shows only the double mutant, and results

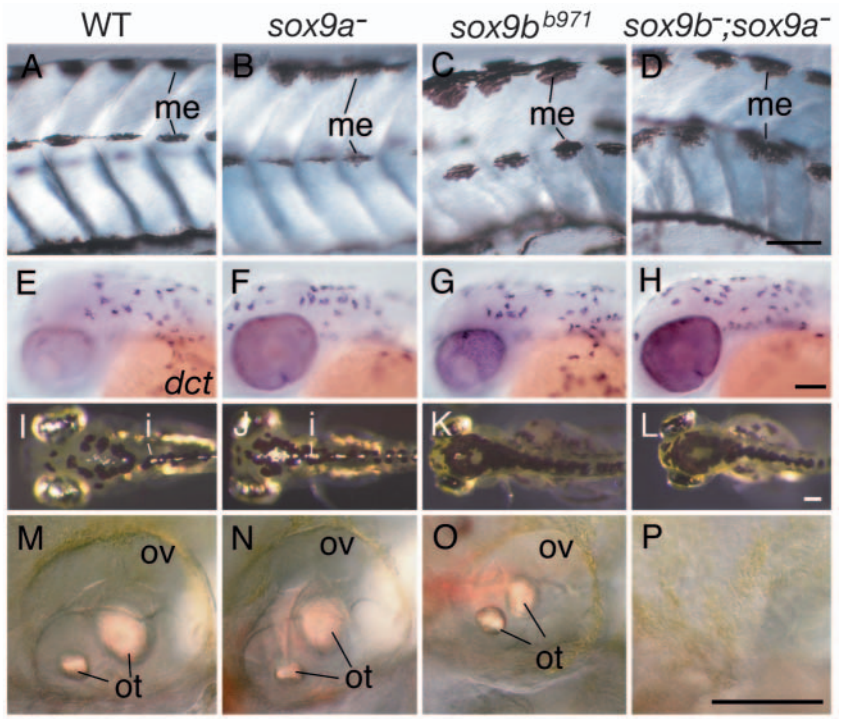

Fig. 7. Pigment cells and ears in mutants and wild types. (A-D) Trunk melanocytes, lateral view at 4 dpf, showing large melanocytes in *sox9b*^{b971} and double mutant. (E-H) *dct* expression at 26 hpf, showing little difference among genotypes; (I-L) iridophores at 4 dpf, showing fewer iridophores in *sox9b*^{b971} and double mutant. (M-P) ear at 4 dpf, showing small ear in *sox9b*^{b971} and no ear in double mutant. (A,E,I,M) Wild type; (B,F,J,N) *sox9a*$^{-}$ mutant; (C,G,K,O) *sox9b*^{b971}; (D,H,L,P) double mutant. Abbreviations: i, iridophore; me, melanocyte; ot, otolith; ov, otic vesicle. Scale bars: in D 100 μm for A-D; in H, 100 μm for E-H; in L, 100 μm for I-L; in P, 100 μm for M-P.

with *foxd3* and *sox10* expression were similar to *snail1b* at the three-somite stage, data not shown). We conclude that *sox9b* function is required for full development of the *snai1b*-, *foxd3*- and *sox10*-expressing neural crest subpopulation in three-somite stage embryos.

The transcription factor *tfap2a* is expressed early in crest development in zebrafish and other vertebrates (Barrallo-Gimeno et al., 2004; Knight et al., 2004; Knight et al., 2003; Luo et al., 2003; O'Brien et al., 2004) (http://zfin.org). We examined *tfap2a* expression in zebrafish embryos homozygous for *sox9a* or *sox9b* mutations either singly or in double mutant combinations, and found no difference from wild type (data not shown), suggesting that *sox9* genes are not upstream of *tfap2a* in zebrafish.

To learn whether *sox9* genes play a role in migrating neural crest, we examined *dlx2a*, which is expressed in a subset of premigratory, and postmigratory crest that forms pharyngeal arches (Akimenko et al., 1994; Kelsh and Raible, 2002; Miller et al., 2000). Results showed that *dlx2a*-expressing cells were unaffected in *sox9a*^{hi1134} embryos (Fig. 8E,F), even though *sox9a* and *dlx2a* are expressed in very similar domains. In contrast, the *dlx2a*-expression domain was somewhat smaller in *sox9b*^{b971} embryos and double mutants (Fig. 8G,H). This shows that *sox9* function is not essential for cranial crest migration in zebrafish, but that *sox9b* may help determine the size of the *dlx2*-expressing cell population.

To discover the effect of *sox9* on chondrocyte differentiation, we investigated *col2a1* (Yan et al., 1995), which encodes a major collagen of cartilage. Embryos homozygous for *sox9a*^{hi1134} or wild-type embryos injected with *sox9a* MO (data not shown) had, by 68 hpf, little expression in the pharyngeal arches, otic vesicle, and eye capsule (Fig. 8I,J). In contrast, *sox9b*^{b971} homozygotes (Fig. 8K) or wild-type

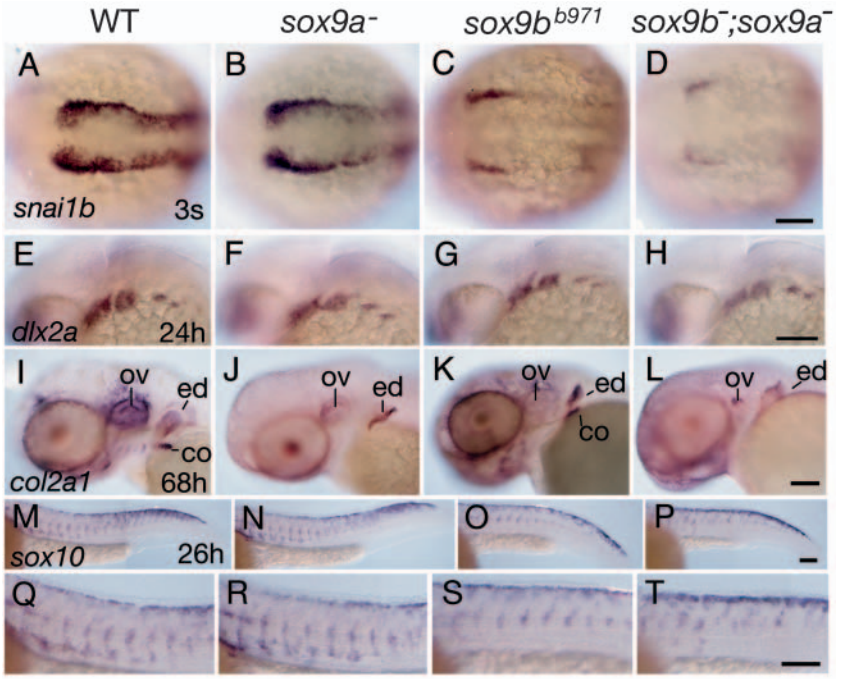

Fig. 8. Expression of markers for neural crest and collagen in wild-type and mutant embryos. (A-D) Dorsal view of *snail1b* expression at three-somite stage showing decreased expression in *sox9b*^{b971} and double mutant; (E-T) lateral views; (E-H) *dlx2a* expression at 24 hpf slightly reduced in *sox9b*^{b971} and double mutant; (I-L) *col2a1* expression at 68 hpf showing lack of scapulocoracoid in *sox9a* mutant and double mutant and smaller endochondral disc in *sox9b*^{b971} and double mutant; (M-T) *sox10* expression in trunk and tail of 26 hpf embryos showing decreased expression. (A,E,I,M) Wild type; (B,F,J,N,R) *sox9a*$^{-}$ mutant; (C,G,K,O,S) *sox9b*^{b971}; (D,H,L,P,T) double mutant. (Q-T) Higher magnification of M-P. Abbreviations: co, scapulocoracoid; ed, endochondral disc; ov, otic vesicle. Scale bars: in D 100 μm for A-D; in H, 100 μm for E-H; in L, 100 μm for I-L; in P, 100 μm for M-P; in T, 100 μm for Q-T.

embryos injected with *sox9b* MO (data not shown) had much smaller *col2a1* expression domains in the arches. In the pectoral fin, sox9a mutants lacked *col2a1* expression in the scapulocoracoid and had reduced expression in the endochondral disc, whereas sox9b mutants had a smaller *col2a1* domain in the endochondral disc but a normal scapulocoracoid domain. In the double mutant (Fig. 8L) and in *sox9a*^{hi1134} embryos injected with *sox9b* MO (data not shown), *col2a1* expression was greatly reduced in the arches and ear, was absent from the scapulocoracoid region, and was reduced in the endochondral disc. We conclude that *sox9a* and *sox9b* are required either for the expression of *col2a1* in overlapping sets of cartilaginous cells, or for the survival and/or proliferation of cells that normally express *col2a1*.

Colorless (*sox10*) is expressed early in most premigratory neural crest, a subpopulation of trunk crest cells migrating in the medial pathway (Dutton et al., 2001), and in craniofacial ectomesenchyme (Kelsh and Raible, 2002). Expression of *sox10* was nearly normal in trunk crest of homozygous *sox9a*^{hi1134} embryos (Fig. 8M,N,Q,R). In homozygous *sox9b*^{b971} (Fig. 8O,S), however, and in animals abrogated for function of both *sox9* co-orthologs (double mutants (Fig. 8P,T); *sox9b*^{b971};*sox9a*-MO, and *sox9b*-MO;*sox9a*^{hi1134} (data not shown)), the extent of the *sox10* expression domain in the trunk was reduced, and *sox10*-expressing cells did not migrate as far ventrally as normal. Results for *crestin* (Luo et al., 2001; Rubinstein et al., 2000) were similar (data not shown). We conclude that *sox9b* and to a lesser extent, *sox9a*, is required for full development of *sox10*- and crestin-expressing neural crest.

To complement these loss-of-function experiments, we conducted gain-of-function experiments to investigate regulatory interactions among early crest genes. In wild-type embryos injected with *sox9a* or *sox9b* mRNAs, the injected mRNA was mosaically distributed in a portion of each embryo (compare Fig. 9A and B, and Fig. 9D and F). Injection of *sox9a* mRNA caused ectopic expression of *sox9b* (Fig. 9E), and vice versa (Fig. 9C). This suggests that *sox9a* and *sox9b* have a mutually positive regulatory influence on each other, confirming our earlier results for ear development (Liu et al., 2003; Hans et al., 2004). Injection of *sox9* mRNAs into zebrafish early cleavage stage embryos showed that both *sox9a* and *sox9b* caused ectopic expression or rostral extension of the expression domains for *foxd3*, *sox10* and *snai1b* (Fig. 9 G-O). We conclude that *foxd3*, *sox10* and *snai1b* can be upregulated by *sox9* genes in crest development, consistent with the results from the loss-of-function experiments.

The roles of *sox9* genes in cartilage morphogenesis

In living 48 hpf Bodipy ceramide-stained embryos imaged by confocal microscopy, we could not unambiguously distinguish wild-type from mutant embryos (Fig. 10A-D′). By 72 hpf, chondrocytes were stacking in orderly rows in wild-type embryos, but in *sox9a*^{hi1134} mutants, chondrocytes failed to stack (Fig. 10E-F′). Mutants homozygous for *sox9b*^{b971} had fewer chondrocytes, which, however, did stack (Fig. 10G,G′). Double mutants showed fewer chondrocytes and no stacking (Fig. 10H,H′). We conclude that *sox9a* and *sox9b* play different roles in cartilage morphogenesis, with *sox9a* necessary for stacking and *sox9b* essential for attaining the proper number of chondrocytes.

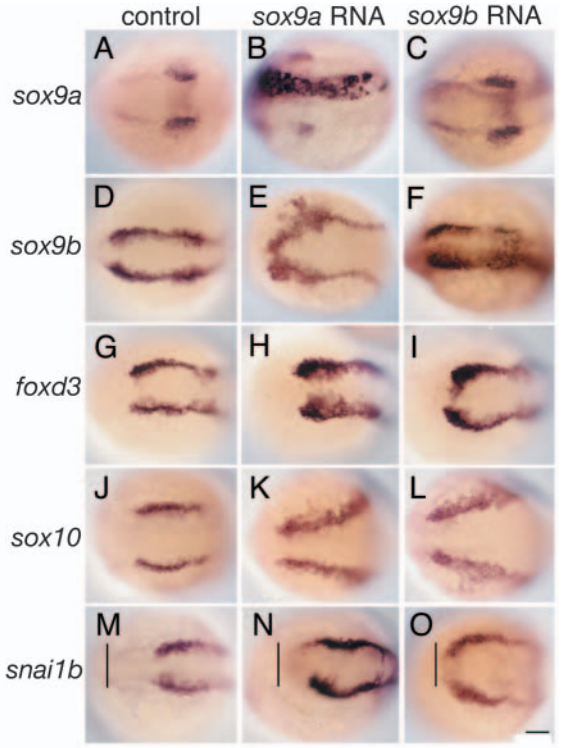

Fig. 9. Ectopic expression of *sox9a* and *sox9b* mRNAs causes ectopic expression of other neural crest genes. (A,D,G,J,M) Uninjected controls. (B,E,H,K,N) Embryos injected with *sox9a* mRNA. (C,F,I,L,O) Embryos injected with *sox9b* mRNA. (A-C) Embryos probed for *sox9a*. (D-F) *sox9b* probe; (G-I) *foxd3* probe; (J-L) *sox10* probe; (M-O) *snai1b* probe; vertical line indicates anterior border of the neural plate. Scale bar: in O, 100 μm.

Small cartilages in *sox9b*^{b971} mutants could be due to slow cell proliferation rates or elevated death of cartilage progenitor cells, among other possibilities. To test the cell death hypothesis, we performed TUNEL assays on various genotypes at 24 hpf. The results showed (Fig. 11) that homozygotes for *sox9b*^{b971} or double mutants or animals injected with *sox9b*-MO had increased TUNEL staining in the central nervous system and pharyngeal cartilage precursors (Fig. 11C- E). This result shows that *sox9b* helps prevent cell death but *sox9a* does not. In mouse limb buds, *Sox9* also suppresses apoptosis (Akiyama et al., 2002). This result does not rule out the possibility that cell proliferation may also be diminished in *sox9b* mutants.

Discussion

How do developmental mechanisms evolve after genome duplication? This question is important for understanding the origin of vertebrates, because genome duplication may have preceded the evolution of vertebrate features such as neural crest and placodes (Force et al., 2002; Hokamp et al., 2003; Holland et al., 1994; Hughes, 1999; Larhammar et al., 2002; Ohno, 1970; Sidow, 1996; Skrabanek and Wolfe, 1998). Furthermore, related human diseases are sometimes caused by mutations in paralogous genes that arose in ancient genome amplifications, such as campomelic dysplasia (*SOX9*) and

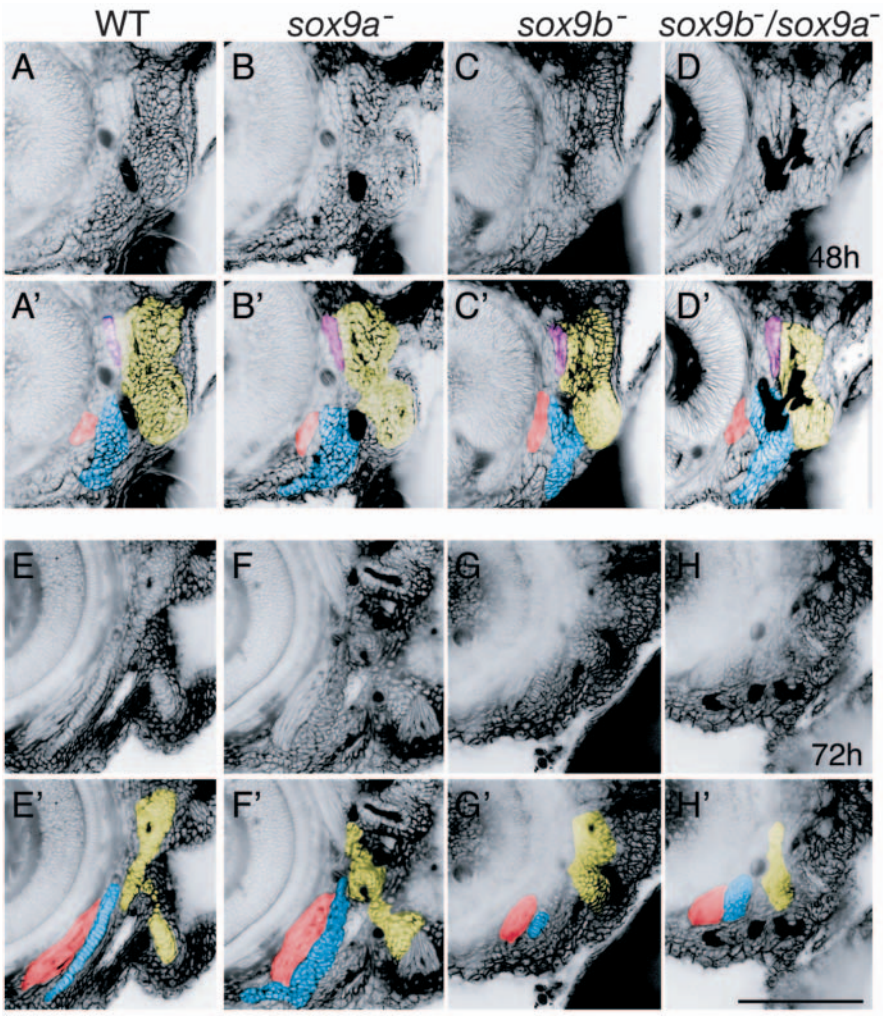

Fig. 10. Differential functions of *sox9* duplicate genes in the morphogenesis and growth of chondrogenic crest. Confocal micrographs of the pharyngeal arches of wild type (A,A′,E,E′), *sox9a*$^{-}$ (B,B′,F,F′), *sox9b*^{b971} (C,C′,G,G′), and *sox9b*^{b971}; *sox9a*$^{-}$. (D,D′,H,H′) animals stained with the vital dye, BODIPY-ceramide as described (Yan et al., 2002) to label interstitial spaces. Images are color reversed so cells appear white and interstitial spaces black: original images (A-H) have been psuedocolored (A′-H′) to highlight arch features. (A-D′) At 48 hours, prechondrogenic condensations of crest cells are seen in the first (blue) and second (yellow) arches. The adductor mandibulae muscle core (red) and first endodermal pouch (purple) are shown for reference. Prechondrogenic condensations are normal in both single mutants (B-C′) and reduced in size in the double mutant (D,D′). (E-H′) The same individual animals in A-D′ were imaged at 72 hours. In wild-type animals (E,E′), chondrocytes have arranged themselves into characteristic stacks and are easily visualized by their thicker cell matrix. First arch cartilages include Meckel's (m) and palatoquadrate (pq), and second arch cartilages include the hyosymplectic (hs) and ceratohyal (ch). As described in Yan et al. (Yan et al., 2002), in *sox9a*$^{-}$ animals, crest condensations are present but fail to differentiate and undergo morphogenesis to form stacks of chondroctyes (F,F′). In contrast, in *sox9b*^{b971} animals (G,G′), chondrocytes are greatly reduced in number, yet can still form small stacks (e.g. pq). The *sox9b*^{b971}; *sox9a*$^{-}$ double mutants show an additive effect including both morphogenetic and growth defects: condensations are smaller in size and do not form stacks of chondrocytes. Total animals examined: n_{wt}=3, n_{sox9a}=1, n_{sox9b}=4, $n_{sox9a;sox9b}$=5. Scale bar: in I′, 100 μm.

Waardenburg syndrome (*SOX10*), or osteogenesis imperfecta (*COL1A1*) and spondyloepiphyseal dysplasia (*COL2A1*). The general principles underlying these mechanisms will become evident by investigating mutations in pairs of gene duplicates.

The discovery of a genome duplication in zebrafish ancestry (Amores et al., 1998; Postlethwait et al., 1998), and the revelation that it may have occurred before the teleost radiation (Amores et al., 1998; Koopman et al., 2004; Meyer and Schartl, 1999; Postlethwait et al., 1998; Postlethwait et al., 2000; Postlethwait et al., 2002; Taylor et al., 2003; Vogel, 1998; Wittbrodt et al., 1998) provides opportunities to learn how developmental pathways evolve after all genes of the pathway are duplicated. In the parallel model, duplicated genes sort into two parallel but non-interacting pathways, for example different duplicates act exclusively in different cell types. Under the network model, a reticulated pathway of regulation would interweave the functions of gene duplicates at several levels of the pathway.

In mammals, *Bmp2*, *Msx2*, *Sox9*, *Col2a1*, *Runx2* and *Col1a1* interact in the developmental pathway leading from neural crest to cartilage and bone (Healy et al., 1999; Mori-Akiyama et al., 2003; Takahashi et al., 2001). Zebrafish has duplicate copies of these six genes (Chiang et al., 2001; Ekker et al., 1997; Fisher et al., 2003; Flores et al., 2004; Martinez-Barbera et al., 1997; Postlethwait, 2004; Yan, et al., 1995; Yan et al., 2002), and we have begun to investigate their regulatory interactions to test the parallel and network models for the evolution of developmental pathways after genome duplication. In experiments reported here, we made null-activity animals for one of the two *sox9* co-orthologs, and compared them to mutants for the other *sox9* co-ortholog. The network model appears to receive initial support because *Sox9* co-orthologs perform redundant functions in development of the ear, endochondral disc, and pharyngeal arches.

Sox9 expression patterns and subfunction partitioning

Our side-by-side comparison of *sox9a* and *sox9b* expression patterns extends earlier reports (Chiang et al., 2001; Li et al., 2002; Liu et al., 2003; Yan, et al., 2002), and shows that even when *sox9a* and *sox9b* are expressed in the same organs, the precise cell type or timing can be different. For example, in the pectoral appendage, *sox9a* is expressed at high levels in the girdle and at low levels in the endochondral disc, while reciprocally, *sox9b* is not expressed in the girdle but is expressed at high levels in the endochondral disc. In mouse, *Sox9* is expressed in both the limb girdle and the endochondral elements of the forelimb (Wright et al., 1995), suggesting that

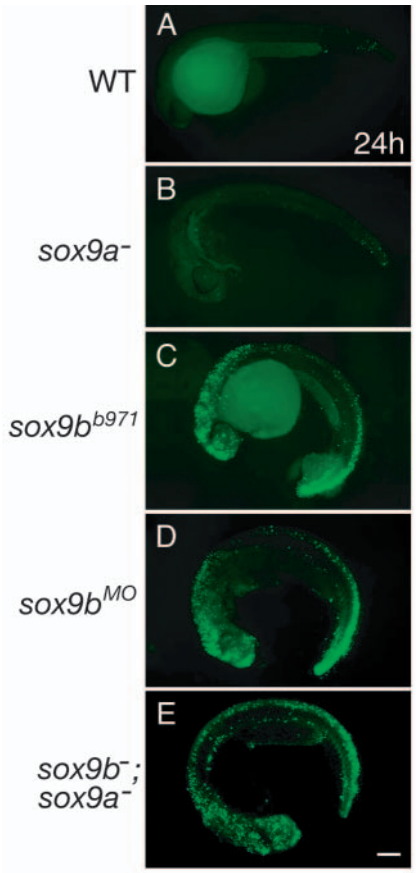

Fig. 11. Cell death in wild-type and mutant embryos. Lateral views of TUNEL-labeled embryos at 24 hours. (A) wild type; (B) homozygous *sox9a*$^{-}$ embryo; (C) homozygous *sox9b*^{b971} mutant embryo; (D) *sox9b*-MO-treated wild-type embryos; (E) double mutant. Cell death is increased in *sox9b*^{b971} mutants, sox9b MO-treated embryos, and in double mutants. Scale bar: in E, 100 μm.

evolution has divided up ancestral roles of *Sox9* between zebrafish co-orthologs. Likewise, *sox9a* and *sox9b* are both expressed in the arches, but we show here that these are in different cell types, either the central mesenchyme and perichondrium, or the surrounding epithelial sheath. Embryos express *sox9b* at high levels in the early neural crest in both the head and trunk as in tetrapod embryos, but they express *sox9a* at much lower levels than *sox9b* in that domain. In contrast, zebrafish embryos express *sox9a* at high levels in the somites as in tetrapods (Healy et al., 1999; Peters et al., 1999), but *sox9b* is not expressed in this domain. Together, these data show that the expression patterns of *sox9a* and *sox9b* tend to sum to the expression pattern in the mouse.

Parsimony suggests that expression domains shared by tetrapod and teleost *Sox9* genes probably existed 450 million years ago in their last common ancestor. This supports the conclusion that specific independent regulatory elements were driving those expression domains in the last common ancestor of fish and tetrapods, and that those elements became partitioned between the two zebrafish genes over time (Force et al., 1999; Hughes, 1994; Postlethwait et al., 2004; Stoltzfus, 1999). Comparative sequence analysis can identify candidates for such regulatory elements. For example, five regions of non-coding sequence conserved with human and mouse have been identified flanking *fugu Sox9a* (Bagheri-Fam et al., 2001; Koopman et al., 2004), and these are candidates for regulatory elements driving expression in the limb and vertebral column (Wunderle et al., 1998). The subfunction partitioning model would predict that *sox9a* and *sox9b* in zebrafish would have different conserved sequences, and that comparative analysis would reveal candidates for these elements. Partitioned conserved non-coding sequences have already been identified for some zebrafish duplicates of mammalian genes (Postlethwait et al., 2004). Morpholino knockdown experiments for zebrafish duplicates of human *HOXB1* have shown that the two fish duplicated genes have distinct and partially redundant functions, and study of conserved non-coding sequences has shown that the co-orthologs have suffered complementary degenerative mutations (McClintock et al., 2002).

The b971 mutation deletes *sox9b*

Our reverse genetics mutation screen identified a terminal chromosome deletion that removed *sox9b* and surrounding loci. We analyzed all known loci in this region (http://zfin.org/cgi-bin/mapper_select.cgi) for those that might play a role in the phenotype, and only *sox8* (Cresko et al., 2003) is an obvious candidate for crest development (Cheung and Briscoe, 2003). Because *sox8* is not expressed in neural crest derivatives in wild-type zebrafish embryos, and homozygous *Sox8* knockout mice survive with no abnormalities in neural crest derivatives (Sock et al., 2001), we conclude that *sox8* does not contribute to the phenotypes we observe.

To further test whether the *sox9b*^{b971} phenotype is due to lack of *sox9b*, we increased wild-type *sox9b* activity in mutants and decreased *sox9b* activity in wild types. Splice-blocking *sox9b* MOs phenocopied each aspect of the *sox9b*^{b971} phenotype, as expected if *sox9b* loss is responsible for the phenotypes we observed. Reciprocally, mRNA injections mosaically rescued Alcian blue-stained cartilage in *sox9b*^{b971} mutants, despite the late phenotype, which gives ample opportunity for mRNA dilution and degradation. These tests confirmed that the effects we observed are due to the lack of *sox9b* rather than adjacent loci.

The role of *sox9* genes in the genetic hierarchy of neural crest development

According to a recent model, neural folds originate at a threshold level of a BMP gradient and form neural crest due to Wnt, FGF, and retinoic acid signals (Aybar et al., 2002; Lewis et al., 2004; Tribulo et al., 2003). Early cranial crest expresses *sox9b*, *snai1b* (Thisse et al., 1995), *foxd3* (Odenthal and Nusslein-Volhard, 1998) and *tfap2a* (Barrallo-Gimeno et al., 2004; Knight et al., 2003; Knight et al., 2004; Luo et al., 2003; O'Brien et al., 2004) (http://zfin.org). *Tfap2a* may respond directly to crest-inducing signals in *Xenopus* (Luo et al., 2003). Expression of *sox9b* in premigratory neural crest is normal in zebrafish *tfap2a* mutants (Knight et al., 2003), showing that *tfap2a* does not regulate the induction of *sox9b* expression. Reciprocally, we found that *tfap2a* expression in premigratory crest is normal *sox9b*^{b971} mutants, as it is in mouse (Mori-Akiyama et al., 2003); thus, *sox9b* does not induce *tfap2a.* Because neither of these genes induces the other, each must respond independently to the diffusible signals that specify

neural crest, or each gene is controlled by different factors in parallel pathways. After cranial crest begins to migrate, expression domains of *sox9a* and *sox9b* decrease in *tfap2a* mutants (Barrallo-Gimeno et al., 2004; Knight et al., 2004). This shows that *tfap2a* is required to establish the full range of *sox9a*- and *sox9b*-expressing cells.

In tetrapods, *Snail* is expressed in about the same temporal and spatial pattern as *Sox9* and is the earliest known gene expressed in *Xenopus* neural crest (Essex et al., 1993; Spokony et al., 2002). Zebrafish has two co-orthologs of *Snail*, called *snai1a* and *snai1b* (formerly *sna2*), as well as *snai2* (formerly *slug*) (Locascio et al., 2002), but only *snai1b* is expressed at high levels in premigratory neural crest (Locascio et al., 2002; Thisse et al., 1993; Thisse et al., 1995). Because the *snai1b* expression domain in premigratory crest was reduced in zebrafish embryos lacking *sox9b* function, we conclude that *sox9b*, but not *sox9a*, is required for full development of the *snai1b*-expressing neural crest population. In *Sox9* MO-treated *Xenopus* embryos (Spokony et al., 2002), *Snail* expression is also diminished, suggesting that the dependence of *Snail* on *Sox9* activity may be general among vertebrates.

To learn whether *sox9* plays a role in migrating neural crest, we examined the expression of *dlx2a*, which is expressed in a subset of premigratory, migratory, and postmigratory crest that forms the pharyngeal arches (Akimenko et al., 1994; Kelsh and Raible, 2002; Miller et al., 2000). The *dlx2a*-expression domain was smaller in *sox9b*, but not *sox9a* mutants, showing that *sox9* activity is not required for cranial crest migration, but that *sox9b* function is essential to establish the full size of the *dlx2*-expressing cell population.

sox10 is expressed in most premigratory neural crest in a subpopulation of trunk crest cells that migrates in the medial pathway (Dutton et al., 2001) and in postmigratory craniofacial precursors (Kelsh and Raible, 2002). Likewise, *crestin* is expressed in premigratory and migratory neural crest, overlapping the initial *sox10* and *sox9b* expression pattern (Li et al., 2002; Luo et al., 2001; Rubinstein et al., 2000). Activity of *sox9b*, but not *sox9a*, was necessary for full ventral migration of *sox10*- and *crestin*-expressing cells in the trunk and tail, but apparently not in the head.

These experiments, taken with results from tetrapods (Cheung and Briscoe, 2003; Mori-Akiyama et al., 2003; Spokony et al., 2002), show that *Sox9* activity is essential for the establishment of the chondrogenic crest in vertebrates generally. On the other hand, among pigment cells, *sox9* appears to act only in iridophore development. Although melanocytes appeared enlarged in *sox9b* mutants, melanocyte precursors were normal. The melanocyte phenotype may be due to defective eyes in *sox9* mutants because blind fish secrete more alpha-melanocyte stimulating hormone, which causes them to become more darkly pigmented than normal fish (Rodrigues and Sumpter, 1984).

The roles of zebrafish *sox9* genes in skeletogenesis

In mammals, Sox9 protein binds to a chondrocyte-specific enhancer in an intron of *Col2a1*, and stimulates its transcription (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhou et al., 1998). Zebrafish *col2a1* is co-expressed in several domains with *sox9a* and *sox9b*. Reduction of *col2a1* expression in mutants correlated well with the *sox9* co-ortholog predominantly expressed in each domain. In regions where the *sox9* duplicates were co-expressed, double mutants summed the effect of the two homozygous mutations on *col2a1* expression. This indicates that *sox9a* and *sox9b* act in the differentiation of chondrocytes in similar ways in overlapping sets of cartilaginous cells.

In the pectoral fin, *sox9* co-orthologs have additive and exclusive roles. The scapulocoracoid, a chondral bone, expresses *sox9a* at high levels, and the *sox9a* mutant lacks this skeletal element. Likewise, human campomelic dysplasia patients are *SOX9* heterozygotes and have hypoplastic scapulae (Houston et al., 1983; Mortier et al., 1997). In contrast, *sox9b* is not expressed in the scapulocoracoid, and this element is nearly normal in the *sox9b* mutant. The fin's endochondral disc is nearly normal in *sox9a* mutants, but the distal portion is greatly reduced in *sox9b* mutants, again reflecting gene expression patterns. In the proximal portion of the endochondral disc, the two *sox9* orthologs play additive roles because the double mutant lacks this cartilage. Double mutants retain Alcian staining in the anlage of the cleithrum, a dermal bone.

Zebrafish *sox9* co-orthologs play different roles in chondrocyte morphogenesis and survival. By imaging live developing embryos, we confirmed that *sox9a* does not regulate prechondrocyte cell number or condensation (a role of *Sox9* in mouse) (Bi et al., 2001)), but does control the stacking of chondrocytes in orderly cell rows (Yan et al., 2002). On the other hand, *sox9b* does not control stacking, but is necessary for pharyngeal cartilages to acquire their normal cell number, either by suppressing cell death or increasing cell proliferation. Although chondrocytes stack in *sox9b* mutants, individual pharyngeal cartilage organs are not well shaped, showing that *sox9b* activity is important for fine aspects of cartilage patterning.

Analysis of zebrafish mutants showed that *sox9a* and *sox9b* play different roles in development of the bony skeleton. The *sox9a* gene is expressed in the mesenchyme and perichondrium of branchial arches, while *sox9b* is expressed in the epithelium that surrounds each arch, and is required for the differentiation of these cartilages into bone. This suggests that a *sox9b*-dependent signal emanating from the pharyngeal pouch epithelium may trigger the *sox9a*-expressing perichondrium to differentiate into osteoblasts and form bone.

Runx2 encodes a transcription factor expressed in pre-hypertrophic and hypertrophic chondrocytes and osteoblasts (Takeda et al., 2001), and it promotes chondrocyte maturation and osteoblast differentiation (Inada et al., 1999; Komori et al., 1997; Otto et al., 1997). Expression of *runx2b* was normal in *sox9a* mutants, but was severely reduced in the arches of *sox9b* mutants, suggesting that *sox9b* exerts its effect on bone formation by supporting *runx2b* expression. This suggests that *sox9b* in the arch epithelium, but not *sox9a* in the central chondrocytes, is required for *runx2b* expression in the periochondrium, which in turn is necessary for the perichondrium to differentiate into osteocytes and finally bone. Only a portion of two dermal bones, the opercle in the craniofacial skeleton and the cleithrum in the pectoral girdle, are largely independent of *sox9* activity.

Chimeric mice bearing *Sox9*-deficient cells also show interactions of *Runx2* and *Sox9*. Depleting *Sox9* in the nasal region causes an expansion of the *Runx2* expression domain, suggesting that *Sox9* negatively regulates *Runx2* in these cells

(Mori-Akiyama et al., 2003). In contrast, *Sox9*-deficient mouse limb buds lack *Runx2* expression, suggesting that *Sox9* positively regulates *Runx2* in limb buds. *Sox9* may positively regulate *Runx2* in cases in which *Sox9*-deficient cells fail to complete chondrocyte differentiation and become osteoblasts (Mori-Akiyama et al., 2003). In other situations, lack of *Sox9* activity may block cells from later steps necessary for bone formation. Reciprocally, *Sox9* expression is expanded in mouse tissue culture cells lacking *Runx2* and in the limbs of *Runx2* mouse mutants, showing that *Runx2* is a negative regulator of *Sox9* (Stock et al., 2004; Yoshida et al., 2002). While *Runx2* is expressed in the perichondrium in mouse (Bronckers et al., 2003), *Sox9* is not (Smits et al., 2001), demonstrating that in this tissue, *Sox9* cannot be a direct transcription factor for *Runx2*. Subfunction partitioning between the two recently described *Runx2* co-orthologs in zebrafish (Flores et al., 2004) may facilitate analysis of these problems.

Null mutations in genes that are essential at several developmental stages (pleiotropy) will block development at the earliest essential subfunction, which can obscure later gene roles. In some cases, conserved temporal subfunctions have partitioned between teleost duplicates. For example mouse *Nodal* mutants are blocked in gastrulation (Conlon et al., 1994; Varlet et al., 1997), masking later gene functions. Zebrafish has two *Nodal* genes (Blader and Strähle, 1998; Dougan et al., 2003; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998), and mutant analysis reveals both early and late functions. Likewise, in *Xenopus Sox9* knockdown embryos, a role for *Sox9* in chondrocyte stacking was obscured because early *Sox9* function (which in zebrafish partitions to *sox9b*) is required for the formation of the cells that stack (Spokony et al., 2002). Thus, subfunction partitioning in zebrafish permitted ready identification of late gene functions obscured in tetrapods by early gene functions and by haploinsufficiency.

Subfunction partitioning, *sox9* and *sox10*

sox8, *sox9* and *sox10* belong to the SoxE gene family and are expressed in overlapping domains in neural crest, heart, limb buds, glia, and gonads (Bell et al., 2000; Bowles et al., 2000; Cheng et al., 2001; Chimal-Monroy et al., 2003; Schepers et al., 2000; Sock et al., 2001). Premigratory crest expresses both *Sox9* and *Sox10*, but *Sox9* is required for ectomesenchymal crest, and *Sox10* for non-ectomesenchymal crest (Akiyama et al., 2002; Dutton et al., 2001). We propose that an ancestral non-vertebrate chordate possessed a single *Sox9/10* gene that was expressed in cells at the neural plate border and was essential for specifying pigment cells, mesenchyme, glia, and some neurons (see Jeffery et al., 2004). After gene duplication prior to the divergence of zebrafish and human lineages (Bowles et al., 2000; Chiang et al., 2001; Dutton et al., 2001; Li et al., 2002), subfunction partitioning (Force et al., 1999) may have resulted in ectomesenchymal functions largely partitioning to *Sox9*, and non-ectomesenchymal functions mainly segregating to *Sox10*, but with some overlap due to the retention of a few ancestral subfunctions by both genes. This proposed process is analogous to subfunction partitioning between the zebrafish *sox9* duplicates documented here. Because the advent of neural crest is a key to the evolution of vertebrates, the duplication of *SoxE* and subfunction partitioning among its descendent genes may have been a crucial step in vertebrate origins. This hypothesis makes specific predictions about the roles of *SoxE* in the development of non-vertebrate chordates that have yet to be tested.

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