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Two *endothelin 1* effectors, *hand2* and *bapx1*, pattern ventral pharyngeal cartilage and the jaw joint

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SUMMARY

A conserved endothelin 1 signaling pathway patterns the jaw and other pharyngeal skeletal elements in mice, chicks and zebrafish. In zebrafish, *endothelin 1* (*edn1* or *sucker*) is required for formation of ventral cartilages and joints in the anterior pharyngeal arches of young larvae. Here we present genetic analyses in the zebrafish of two *edn1* downstream targets, the bHLH transcription factor Hand2 and the homeobox transcription factor Bapx1, that mediate dorsoventral (DV) patterning in the anterior pharyngeal arches.

First we show that *edn1*-expressing cells in the first (mandibular) and second (hyoid) pharyngeal arch primordia are located most ventrally and surrounded by *hand2*-expressing cells. Next we show that along the DV axis of the early first arch primordia, *bapx1* is expressed in an intermediate domain, which later marks the jaw joint, and this expression requires *edn1* function. *bapx1* function is required for formation of the jaw joint, the joint-associated retroarticular process of Meckel's cartilage, and the retroarticular bone. Jaw joint expression of *chd* and *gdf5* also requires *bapx1* function.

Similar to *edn1*, *hand2* is required for ventral pharyngeal cartilage formation. However, the early ventral arch *edn1*-dependent expression of five genes (*dlx3, EphA3, gsc, msxe* and *msxb*) are all present in *hand2* mutants. Further, *msxe* and *msxb* are upregulated in *hand2* mutant ventral arches. Slightly later, an *edn1*-dependent ventral first arch expression domain of *gsc* is absent in *hand2* mutants, providing a common downstream target of *edn1* and *hand2*. In *hand2* mutants, *bapx1* expression is present at the joint region, and expanded ventrally. In addition, expression of *eng2*, normally restricted to first arch dorsal mesoderm, expands ventrally in *hand2* and *edn1* mutants. Thus, ventral pharyngeal specification involves repression of dorsal and intermediate (joint region) fates. Together our results reveal two critical *edn1* effectors that pattern the vertebrate jaw: *hand2* specifies ventral pharyngeal cartilage of the lower jaw and *bapx1* specifies the jaw joint.

Key words: Zebrafish, *endothelin 1*, *hand2*, *bapx1*, Joints, Jaw, Pharynx, Pharyngeal arch, Branchial arch

INTRODUCTION

The skeleton of derived vertebrates consists of variously shaped bones and cartilages separated by joints. Experiments with the chick elbow joint showed that normal joints formed following excision of the embryonic forelimb distal to the joint; thus, joint specification appeared to have some local autonomy (Holder, 1977). Consistent with this idea, genetic analysis in the mouse has revealed that skeletal elements and joints are specified by partially separable mechanisms; whereas some genes are essential for joint formation, other genes are essential for particular bones (Brunet et al., 1998; Davis et al., 1995). However, these processes are intricately linked and share certain regulators. For instance, defects in joints and cartilage formation are both seen in *Gdf5* mutant mice (Gruneberg and Lee, 1973; Storm et al., 1994; Storm and Kingsley, 1996; Storm and Kingsley, 1999). Noggin (*Nog*) mutant mice lack joints, but also exhibit hyperplastic cartilage and defective cartilage maturation (Brunet et al., 1998). However, humans heterozygous for certain *NOG* mutations can have more specific joint defects, arguing these processes are at some level genetically separable (Gong et al., 1999).

GDF5 and another signalling molecule of the TGFβ superfamily, BMP5, are required for partially overlapping subsets of joints in the mouse axial skeleton, suggesting the skeleton is assembled piecemeal by partially redundant TGFβ signalling molecules (Storm et al., 1994; Storm and Kingsley, 1996; Storm and Kingsley, 1999). *Gdf5* negatively regulates its own expression, and thus refines where the joint is positioned (Storm and Kingsley, 1999). Despite being required for certain joints, GDF5 is not sufficient to induce ectopic joints (Storm and Kingsley, 1999; Merino et al., 1999; Francis-West et al.,

1999a). In chick embryos, another secreted molecule, Wnt14, is sufficient to induce ectopic joints, and in addition inhibits nearby joints (Hartmann and Tabin, 2001). Little is known about upstream factors that control the expression of these joint-promoting signaling molecules.

The jaw joint forms in the first, or mandibular, pharyngeal arch, articulating the upper and lower jaw. Work in mice, chicks and zebrafish has begun to unravel molecular mechanisms responsible for jaw development. In both mice and zebrafish, the secreted peptide endothelin 1 (encoded by the *edn1* or *sucker* gene in zebrafish) is required for development of the jaw, as well as skeletal elements of the second, or hyoid, pharyngeal arch (Kurihara et al., 1994; Miller et al., 2000; Miller and Kimmel, 2001). In mice, targeted inactivation of the Edn1 receptor, EdnrA, produces a similar phenotype as Edn1 inactivation, namely loss of the mandible and severe malformations of other pharyngeal skeletal elements (Clouthier et al., 1998). Pharmacological inactivation of EdnrA in chick embryos results in a similar disruption of lower jaw formation (Kempf et al., 1998). Thus, Edn1-EdnrA signaling is required for lower jaw formation in chicks as well as mice and fish. Within the early pharyngeal arch primordia, secretion of Edn1 from paraxial mesodermal cores and surrounding epithelia, both surface ectoderm and pharyngeal endoderm, is received by the EdnrA receptor, which is broadly expressed in the postmigratory cranial neural crest (CNC) cylinder. Edn1 signaling sets up a dorsoventral prepattern and promotes the specification of ventral fates within this cylinder of postmigratory CNC (reviewed by Kimmel et al., 2001a). In zebrafish, graded reduction of Edn1 function with *edn1* antisense morpholino oligonucleotides (Edn1-MOs) results in graded reduction in ventral pharyngeal cartilage formation. The pharyngeal joints also require Edn1 and are more sensitive to Edn1 reduction, because ventral cartilage, but not joints, form in animals injected with lower doses of Edn1-MOs (Miller and Kimmel, 2001). Thus, in zebrafish Edn1-signaling is required for both joint and ventral pharyngeal fates, with joint fates being more sensitive to Edn1 reduction.

The requirement for Edn1 in activating expression of *hand2* (also known as *dHAND*) in the ventral arch primordia is conserved between mice and zebrafish (Thomas et al., 1998; Miller et al., 2000). *hand2* mutant mice die before skeletal differentiation occurs with severe heart and circulatory defects (Srivastava et al., 1997; Thomas et al., 1998; Yamagishi et al., 2000). In the *hand2* mutant mouse pharyngeal arch primordia, CNC fails to adopt ventral fates and undergoes apoptosis (Thomas et al., 1998). Thus, *hand2* is an excellent candidate effector of Edn1-mediated pharyngeal arch patterning.

Here we present functional analysis of two *edn1*-dependent genes, *bapx1* and *hand2*, during zebrafish pharyngeal arch development. *edn1* expression is complementary to and surrounded by *hand2*-expressing ventral arch CNC, whereas *bapx1* expression defines an intermediate presumptive joint domain, ventral to some *dlx2* expression and dorsal to *hand2* expression. The loss of the jaw joint in *edn1* mutants can in part be explained by a failure to upregulate expression of *bapx1*, whose function is required for multiple aspects of skeletal development of the jaw joint region including the joint itself, the retroarticular process of Meckel's cartilage, and the retroarticular bone. In the developing jaw joint, *bapx1* is required for expression of *chordin* and *gdf5*. The loss of ventral pharyngeal cartilage in *edn1* mutants can in part be explained by a second *edn1* target gene, *hand2*. Similar to *edn1*, *hand2* is required for formation of almost all ventral pharyngeal cartilage. Despite this phenotypic similarity to *edn1* mutants, the early ventrally restricted *edn1*-dependent expression of *dlx3*, *EphA3*, *gsc*, *msxe* and *msxb* in cartilage precursors are all present in *hand2* mutants. Further, *msxe* and *msxb* are upregulated in the ventral arches of *hand2* mutants. However, similar to *edn1* mutants, *hand2* mutants lack late first arch expression of *gsc*. *bapx1* expression in *hand2* mutants is ectopically expanded ventrally, suggesting that *hand2* helps position the jaw joint by repressing expression of *bapx1*. Finally, we show that both *hand2* and *edn1* restrict *eng2* expression to dorsal mesoderm. Thus the specification of ventral pharyngeal arch fates involves the repression of other ventral, joint and dorsal arch fates. Collectively, our results identify *bapx1* and *hand2* as critical effectors of Edn1 in patterning the jaw joint and ventral pharyngeal cartilage.

MATERIALS AND METHODS

Fish maintenance

Fish were raised and staged as described (Westerfield, 1995; Kimmel et al., 1995). *edn1(sucker)*^{tf216b} (Piotrowski et al., 1996; Miller et al., 2000) were maintained on *AB background, whereas *hand2*^{s6} (*han*^{s6}) was inbred on the original background described by Yelon et al. (Yelon et al., 2000). *hand2*^{s6} heterozygotes were incrossed, and mutants sorted from clutches by lack of beating heart tissue at 28 hours postfertilization (hpf) (Yelon et al., 2000).

Cloning *bapx1*

Degenerate PCR primers designed against the Nk3/Bap homeobox region 5′-TGGARMGNMGYTTYAAYCAYCA-3′ and 5′-TTRTAN-CKNCKRTTYTGRAACCA-3′ were used with zebrafish genomic DNA as a template and 48 cycles of: 94°C for 30 seconds, 48°C for 30 seconds and 72°C for 30 seconds, which generated a 117-bp band. This PCR product was cloned using a TOPO TA kit (Invitrogen) and sequencing revealed a Bapx1-like homeodomain. These primers and conditions were then used to screen DNA pools from an arrayed genomic DNA PAC library (Amemiya and Zon, 1999), which identified a single positive PAC, 91M18. This PAC was isolated and subcloned, and sequencing subclones yielded the sequence of the second exon and the 3′ end of the intron. Using gene-specific primers 5′-GATCTTGACCTGCGTCTCG-3′ and 5′-GCGTTATCTCTCCGG-ACCG-3′ from this PAC sequence to amplify a 72 bp band, phage dilution pools of a 15-19 hpf zebrafish cDNA library (Appel and Eisen, 1998) were screened by PCR. A single phage was isolated, which contained a 1357 bp insert, containing the first exon and predicted full-length ORF of a *bapx1* gene (Accession Number, AY225416).

Tissue labeling procedures

Alcian staining and in situ hybridizations were performed as described (Miller et al., 2000). After Alcian staining, some wholemounts were treated with a solution of 3% hydrogen peroxide and 1% potassium hydroxide for 10 minutes to remove pigmentation. Bone labeling using calcein was performed as described (Yan et al., 2002; Kimmel et al., 2003). For sectioning, embryos were embedded in Epon and sectioned at 5 microns.

A 1.2 kB PCR fragment of *bapx1* genomic DNA, containing the entire second exon and part of the 3′ UTR, was used for all in situs. *chd* and *gdf5* probes are described in Schulte-Merker et al. (Schulte-Merker et al., 1997) and Bruneau et al. (Bruneau et al., 1997),

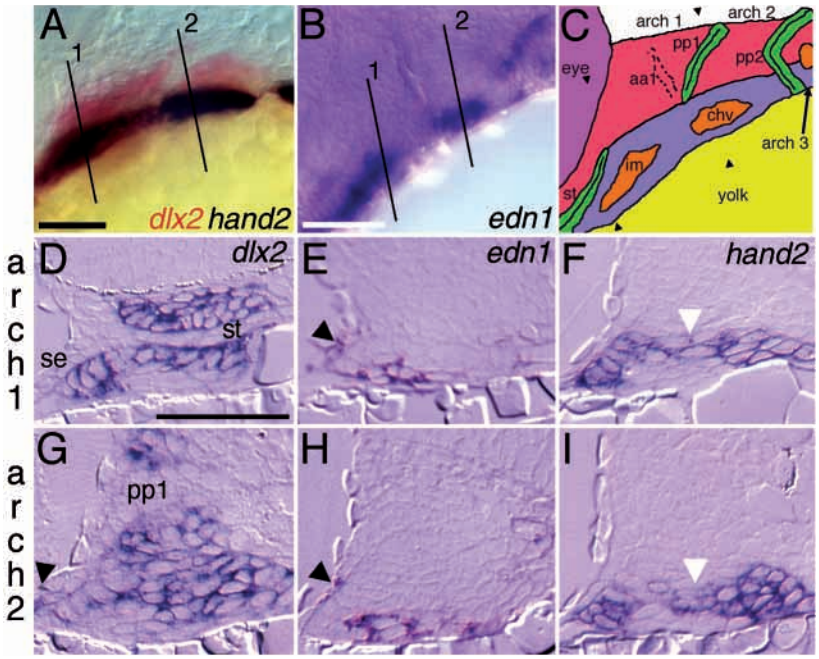

Fig. 1. *edn1* pharyngeal arch expression is ventrally confined. Lateral views of (A) *dlx2* and *hand2* expression in red and blue, respectively, at 28 hpf and (B) *edn1* expression at 36 hpf. (C) Schematic of zebrafish pharyngeal arch primordia from 28-36 hpf at a slightly dorsal-oblique lateral view. The first and second arch ventral myogenic arch cores (intermandibularis and constrictor hyoideus ventralis; shown in orange) (see Kimmel et al., 2001b) express *edn1* (Miller et al., 2000). The third arch myogenic core also expresses *edn1*. Pharyngeal epithelia, the stomodeum and pharyngeal pouches, are colored green. The first and second arches are labeled over the arches, with the postmigratory cranial neural crest (CNC) colored in red (*dlx2+dHAND–*) or blue (*dlx2+dHAND+*). Approximate section planes for the first two arches are indicated and numbered (A,B) or marked with arrowheads (C). (D-I) Transverse sections through the first two arches of 32 hpf embryos stained for *dlx2* (D,G), *edn1* (E,H) or *hand2* (F,I). *hand2*-expressing cells are a ventral subset of *dlx2*-expressing CNC cells, including cells just dorsal (white arrowheads) to the *edn1*-expressing ventral arch cores. Lateral surface ectoderm expresses both *dlx2* and *edn1* ventrally (arrowheads). chv, constrictor hyoideus ventralis; im, intermandibularis; pp1, pharyngeal pouch 1; se, surface ectoderm; st, stomodeum. Scale bars: 50 μm.

respectively. All other riboprobes are described or referenced in Miller et al. (Miller et al., 2000).

Morpholino oligo injections

edn1 morpholino oligo (edn1-MO) 5′-GTAGTATGCAAGTCCC-GTATTCCAG-3′ (31 to 7 nucleotides 5′ to predicted translation start site), (see Miller and Kimmel, 2001), bapx1 morpholino oligo (bapx1-MO1) 5′-GCGCACAGCCATGTCGAGCAGCACT-3′ (ATG start complementary sequence underlined) and bapx1-MO2 (5′-GCGG-AGCATTAGGGTTAAGATTACG-3′, complementary to 52 to 28 nucleotides 5′ of the predicted ATG start codon) were purchased from Gene Tools, Inc., and diluted to 25 mg/ml in 1 × Danieau buffer. Subsequent dilutions were made in 0.2 M KCl and 0.2% Phenol Red. These dilutions were injected into the yolk of 1-8 cell zebrafish embryos, approximately 5 nL per embryo. bapx1-MO1, which seemed less toxic and gave cleaner phenotypes (see below), was used for all phenotypic analyses. The inbred *AB line was used for all MO-injections into wild types.

RESULTS

edn1 expression in the first two arches is restricted to ventral pharyngeal tissues locally apposed to *hand2*-expressing cells

Along the dorsoventral (DV) axis of the zebrafish early larval pharyngeal skeleton, separate cartilages form dorsally and ventrally (such as the upper and lower jaw in the first arch), and are separated by joints at an intermediate position (such as the jaw joint in the first arch). In zebrafish, *edn1* patterns the DV pharyngeal arch axis: graded reduction of *edn1* in zebrafish results in joint loss with mild reduction and joint and ventral cartilage loss with more severe reduction. At high levels of *edn1* reduction, dorsal fates are also affected, although to a relatively lesser degree (Miller et al., 2000; Miller and Kimmel, 2001). Here we investigate the genetic circuitry downstream of *edn1* controlling DV pharyngeal arch patterning. We previously described a DV prepattern set up in the postmigratory CNC. Expression of *hand2*, which encodes a bHLH transcription factor, is confined to a ventral subset of *dlx2*-expressing CNC, defining a ventral domain of the prepattern. We also described *edn1* expression in ventral arch mesenchyme and epithelia (Miller et al., 2000). Here we extend those analyses and ask how, on a detailed cellular level, *edn1* expression in the ventral arches relates to this DV prepattern. For instance, does *edn1* expression extend past the DV interface, or is *edn1* expression contained within the ventral domain? We chose to focus on a postmigratory stage within the probable time of function of the Edn1 signal (Miller et al., 2000).

We analyzed the pharyngeal arch expression domains of *hand2*, *edn1*, and the more broadly expressed postmigratory CNC marker, *dlx2*, in serial sections of 32 hpf embryos stained for the expression of each of these genes (Fig. 1). These serial section studies strongly support our previous findings in whole-mounts (Miller et al., 2000), and add cellular resolution to the expression patterns. *hand2*-expressing cells are a ventral subset of *dlx2*-expressing CNC cells, and are closely apposed to cells of three different tissues expressing *edn1*: ventral surface ectoderm, ventral mesodermal cores and pharyngeal endodermal epithelia. In the first two arches, *hand2*-expressing cells cover dorsally the *edn1*-expressing arch cores, which are in extreme proximity to the yolk. *edn1* expression is not appreciably detected in the first pharyngeal pouch at this stage. The ventral surface ectodermal domain of *edn1* expression seems to extend to, but not beyond, the dorsal extent of the adjacent *hand2* expression domain (Fig. 1E,F,H,I). Thus, in the first two arches at this stage, *edn1* expression is restricted to the ventralmost tissues of the arches, appearing slightly more ventrally localized than *hand2* expression.

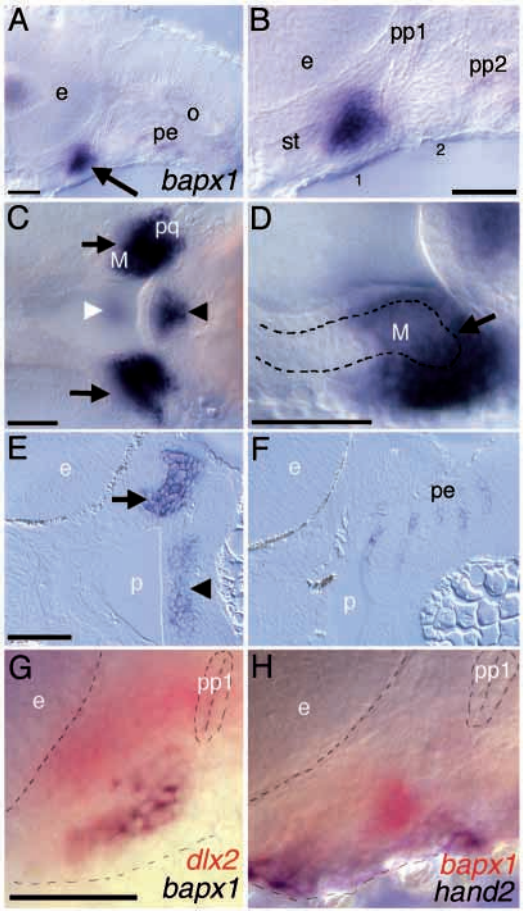

Fig. 2. *bapx1* is expressed at the developing jaw joint. (A-H) *bapx1* expression in wholemount (A-D,G,H) and sectioned (E,F) wild-type embryos. (A) At 32 hpf, expression is seen in a cluster of first arch mesenchyme (arrow) as well as pharyngeal endoderm. (B) A higher magnification of A, showing that this domain appears to be at an intermediate dorsoventral position within the first arch. (C) Ventral view of 54 hpf embryo, showing bilateral first arch joint domains (arrows), first arch midline domain (white arrowhead), second arch midline domain (black arrowhead). (D) High magnification ventral-lateral oblique view of 54 hpf embryo showing the posterior end of the lower jaw (M) is surrounded by *bapx-1*-expressing cells, with apparent expression in cells in the posterior end of Meckel's cartilage (outlined with broken line). The jaw joint appears to be filled with and surrounded by *bapx1*-expressing cells. The second arch midline domain (arrowhead) and pharyngeal endoderm domains are also present. (E,F) Horizontal sections of *bapx1* expression at 48 hpf showing (E) first arch jaw joint (arrow) and second arch midline domain (arrowhead), and (F) pharyngeal endoderm expression in pouches 2 through 6. (G,H) Two-colored in situ hybridization of expression of (G) *dlx2* in red and *bapx1* in blue at 32 hpf and (H) *bapx1* in red and *hand2* in blue at 30 hpf. *bapx1* expression is ventral to a portion of *dlx2* expression and dorsal to *hand2* expression. The outline of the eye in the upper left, the first pharyngeal pouch in the upper-right corner, and the edge of the embryo towards the bottom are delineated. e, eye; M, Meckel's cartilage; p, pharynx; pe, pharyngeal endoderm; pp1, pharyngeal pouch 1; pp2, pharyngeal pouch 2; st, stomodeum. Scale bars: 50 μm.

A zebrafish *bapx1* gene is expressed at the first arch joint, and this expression domain requires *edn1* function

In this DV postmigratory CNC prepattern, approximately the ventral third of the *dlx2*-expressing CNC cylinder expresses *hand2*, and probably includes precursors of the ventral cartilages. We hypothesized that joint-forming cells, although slightly farther away from the Edn1 source, respond to Edn1 signaling, given the requirement of *edn1* for pharyngeal joint primordia (Miller and Kimmel, 2001). We thus began a search for markers of pharyngeal joint primordia. In *Xenopus* embryos, the *bapx1/bagpipe*-related NK3 superfamily homeobox gene *Xbap* is expressed in a large region in the intermediate first arch, encompassing the jaw joint region (Newman et al., 1997). Using degenerate PCR with a zebrafish genomic DNA library, followed by gene-specific PCR with a 15-19 hpf zebrafish cDNA library, we cloned a zebrafish *bapx1* gene. Phylogenetic analyses of this zebrafish and other *bagpipe*-related genes reveals that this zebrafish gene is orthologous to *Xbap* and other vertebrate *bapx1* (*nkx3.2*) genes (data not shown).

Similar to expression of the *Xenopus Xbap* gene (Newman et al., 1997), expression of zebrafish *bapx1* is present in mesenchyme of the mandibular arch primordia. Mandibular expression begins at approximately 30 hpf (see Fig. 2H), and at 32 hpf a large patch of expression is present in the posterior intermediate first arch postmigratory CNC cylinder (Fig. 2A,B). This domain persists through 54 hpf. By this stage, these intermediate *bapx1* domains clearly mark the jaw joints because the upper and lower jaw cartilages have begun to chondrify, and *bapx1* expression is present in cells within and surrounding the jaw joint (Fig. 2C,D). By 54 hpf, additional *bapx1* expression domains are present in the midline of the first two arches (Fig. 2C). Expression is also detected in pharyngeal endodermal epithelia at 32 through 54 hpf (Fig. 2A,B,F). Other *bapx1* expression domains include putative sclerotomal derivatives at 48 hpf, the pectoral fin at 54 hpf, and cells closely apposed to the eye at 36 and 42 hpf (data not shown).

We next asked how *bapx1* expression in the putative joint region primordium relates to the *dlx2/hand2* DV prepattern in the zebrafish pharyngeal arch primordia. Double-labeling experiments show that *bapx1* expression is ventral to a large dorsal domain of *dlx2*-expressing CNC cells and dorsal to *hand2*-expressing CNC cells (Fig. 2G,H). Thus at this early stage, from 30-32 hpf, in the first arch primordia, *bapx1* marks an intermediate or presumptive joint region.

bapx1 expression in the developing jaw joint primordium requires *edn1* function

Because the jaw joint region is particularly affected in 4-day old animals with reduced *edn1* function (Miller and Kimmel, 2001), we next asked whether *bapx1* downregulation prefigures the *edn1* mutant phenotype. At 36 hpf, first arch mesenchymal *bapx1* expression is undetectable in *edn1* mutants (Fig. 3A,B). These defects are not simply because of developmental delay, because by 54 hpf, the midline domains of *bapx1* expression in both arch one and two are present in *edn1* mutants, whereas the jaw joint domains of *bapx1* expression remain undetectable (Fig. 3C,D).

bapx1 is required for the jaw joint

Injecting morpholino antisense oligos (MOs) into embryos has

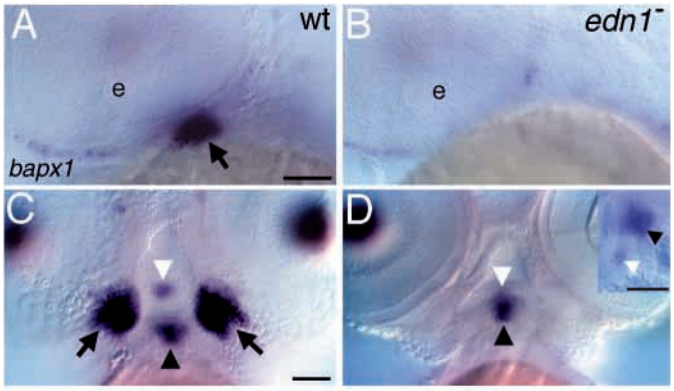

Fig. 3. *edn1* function is required for jaw joint *bapx1* expression. (A-D) *bapx1* expression in wild-type (A,C) and *edn1* mutant larvae (B,D). (A,B) Lateral views of 36 hpf embryos, focused on the first arch joint domain (arrow in A). First arch mesenchymal expression of *bapx1* is absent in *edn1* mutants (B). Cells apposed to the eye and the first pharyngeal pouch express *bapx1* in both wild types and mutants. (C,D) Ventral views of 54 hpf embryos. Joint domains (arrows in C,D) are abolished in *edn1* mutants, whereas the first and second arch midline domains (white and black arrowheads, respectively, in C and D) are present. Inset (D) shows lateral view of same embryo, showing both midline domains are present. Scale bars: 50 μm.

Table 1. *bapx1* is required for the jaw joint

	Concentration of *bapx1*-MO (mg/ml)	Joint region phenotype		Percentages	
		Joint loss	Joint+RAP loss	No BH	Small BH
Uninjected		0 (0/100)	0 (0/100)	0	0
MO1	1	51 (108/213)	1 (2.5/213)	0	16
	3	80 (147/183)	23 (42/183)	0	70
MO2	1	2 (1/48)	0 (0/48)	0	2
	2	88 (66/75)	23 (17/75)	48	52
MO1+2	0.5 each	77 (51/66)	5 (3/66)	3	97
	1.5 each	98 (59/60)	55 (33/60)	67	33

Animals were fixed at 4 days and Alcian stained, then scored in wholemounts. Both left and right sides of each animal were scored, and counted as half an animal. Joint loss animals lacked the joint, but had the cartilaginous retroarticular process (RAP) of Meckel's cartilage, whereas joint + RAP loss animals lacked both the joint and RAP. No animals were observed to lack the process yet have the joint. BH, basihyal.

been shown to be highly successful at reducing gene function *in vivo* (reviewed by Heasman, 2002). This technique efficiently works to downregulate genes involved in zebrafish head skeletal development, as shown by highly penetrant phenocopy of the *edn1* mutant phenotype upon injection of edn1-morpholinos (Miller and Kimmel, 2001). To assess *bapx1* function in pharyngeal skeletal patterning, morpholino oligos complementary to the region around the predicted translation start site of *bapx1* were injected. Animals injected with bapx1-MOs display dose-dependent loss of the jaw joint (Fig. 4, Table 1). Injection of 1 mg/ml (total of 5 ng) of bapx1-MO1 resulted in 51% of injected animals lacking jaw joints, whereas injection of 3 mg/ml (total of 15 ng) raised this frequency to 80% (Table 1). The loss of features of the jaw joint region in bapx1-MO1-injected animals was graded in severity. Frequently the cartilaginous retroarticular process (RAP), which projects ventrally from the posterior end of Meckel's cartilage, was present, but just dorsally, the jaw joint itself was lost with the dorsal and ventral cartilages locally fused (Table 1, Fig. 4A-H). In more severely affected animals RAP was missing, and the joint was completely missing and filled in with ectopic cartilage (Fig. 4C,D). Unlike animals with mild *edn1* reduction, and consistent with the absence of second arch joint *bapx1* expression, the second arch joint is unaffected. However, the second arch midline skeletal element, the basihyal, which is prefigured by *bapx1*-expressing cells (see Fig. 3C), was characteristically reduced in bapx1-MO1-injected animals (Table 1).

To confirm the specificity of this morpholino, we injected a second non-overlapping bapx1-MO (bapx1-MO2). Although injection of MO2 caused other possibly non-specific phenotypes including loss of the branchial cartilages (data not shown, see Discussion), this MO also caused highly penetrant, dose-dependent loss of the jaw joint (Table 1). To further confirm the specificity of these jaw joint region MO phenotypes, we injected these two bapx1-MOs together, at relatively lower concentrations. These combinatorial injections caused more frequent jaw joint loss at concentrations lower than that of the singles alone. Although injection of 5 ng of MO1 and MO2 alone resulted in 51% and 2% loss of the jaw joint, respectively, injecting half as much of each MO combinatorially enhanced the penetrance of this phenotype to 77%. Coinjection of MO1 and MO2 also enhanced the basihyal phenotype, resulting in frequent deletion of this element (Table 1). These phenotypic enhancements further suggest that these non-overlapping MOs are specifically reducing function of *bapx1*.

Slightly later in development, at around 6 dpf, the retroarticular bone (RAB) begins ossifying perichondrally on RAP of Meckel's cartilage (Fig. 4I) (Cubbage and Mabee, 1996) (C. B. K., unpublished). Because RAP is often missing in bapx1-MO-injected fish (see above), we asked if skeletal defects in bapx1-MO-injected animals also included defects in RAB. To examine potential defects in pharyngeal bones of bapx1-MO-injected animals, we stained bones in uninjected and injected larvae with the fluorescent dye Calcein (Kimmel et al., 2003; Yan et al., 2002). In bapx1-MO-injected animals, we observed a highly penetrant RAB loss (Fig. 4J; 111/117, or 95% of injected animals lacking RAB vs. 14/51 or 28% of uninjected siblings lacking RAB). Thus, *bapx1* is required for at least three aspects of skeletal development in and around the jaw joint: (1) local inhibition of chondrogenesis at the jaw joint, (2) specification of the RAP of Meckel's cartilage, and (3) ossification of the RAB.

To provide a potential genetic mechanism for *bapx1*'s role in jaw joint development, we asked whether markers of tetrapod limb joints also mark the zebrafish jaw joint, and whether such domains require *bapx1* function. In tetrapods, *Gdf5*, which encodes a TGFβ-related signaling molecule, is expressed in early cartilage condensations and later in developing joints (Chang et al., 1994; Storm et al., 1994; Storm and Kingsey, 1996; Merino et al., 1999; Francis-West et al., 1999a). A subset of mouse appendicular and axial joints requires *Gdf5* function (Storm et al., 1994; Storm and Kingsley, 1996; Merino et al., 1999). Another marker of developing tetrapod limb joints is the BMP-antagonist *chordin* (*chd*) (Francis-West et al., 1999b; Scott et al., 2000). In 56 hpf zebrafish, *chd* expression, which is localized to the jaw joint in

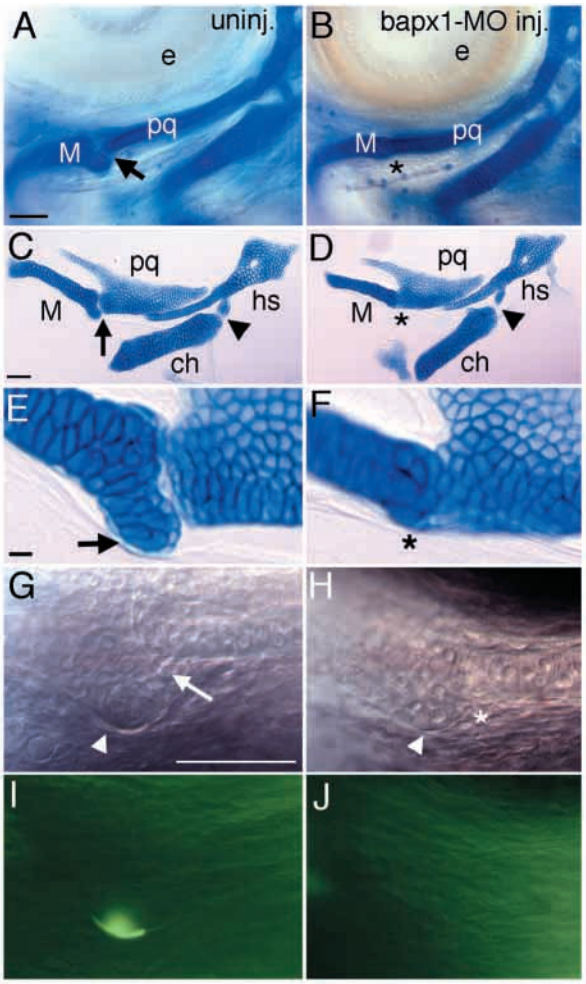

Fig. 4. *bapx1* is required for patterning the first arch joint region. Lateral views of pharyngeal cartilages (A-H) and bones (I,J) in uninjected (A,C,E,G,I) and bapx1-MO-injected (B,D,F,H,J) larvae. Wholemounted (A,B) and flat-mounted (C-F) Alcian Green-stained pharyngeal cartilages in animals at 4 days. The first arch joint (arrow in A,C) is eliminated upon *bapx1* downregulation (asterisk in B,D), whereas the second arch joint is unaffected (arrowheads in C,D). Panel D is a montage of two Nomarski focal planes of the same flat-mount preparation. (E,F) Higher magnification of jaw joint region showing retroarticular process (RAP) (arrow in E) is severely reduced (asterisk in F) in bapx1-MO-injected animals. (G,H) Nomarski images of live animals revealing that the jaw joint (arrow) is missing (asterisk) and the RAP of Meckel's cartilage (arrowhead) is reduced in bapx1-MO-injected animals. (I,J) Fluorescent images of the same fish as in G,H stained with calcein, which fluorescently labels calcified bone. In uninjected animals, the retroarticular bone (RAB) has formed on the RAP of Meckel's cartilage, and this bone is missing in bapx1-MO-injected animals. The second branchiostegal ray (BSR2) (C. B. K., unpublished), a bone that normally forms slightly later than RAB, was actually more frequently observed in bapx1-MO-injected animals (95/121 or 79% of injected animals had BSR2, whereas 29/52 or 56% of uninjected siblings had BSR2). This indicates that the loss of RAB is specific and not a result of developmental delay. ch, ceratohyal; e, eye; hs, hyosymplectic; m, Meckel's; pq, palatoquadrate. Scale bars: 50 μm for all except (E,F): 10 μm.

wild types (Fig. 5A), is absent in bapx1-MO-injected animals (Fig. 5B). At 78 hpf, *gdf5* expression is detected in cells within the jaw joint, and these expression domains are severely reduced in bapx1-MO-injected animals. *gdf5* expression was also detected in a triangular cluster of cells at the second arch ventral midline, seemingly prefiguring the unpaired ventral midline basihyal cartilage, and similar to the second arch midline domain of *bapx1* expression at 54 hours (Fig. 5C, Fig. 2C). This second arch midline domain of *gdf5* expression was also downregulated in bapx1-MO-injected animals (Fig. 5D), correlating with the reduced basihyal cartilage phenotype (Table 1). These expression defects in bapx1-MO-injected animals were specific to the joint region, because other expression domains of both genes, including cells around the second arch joint for both genes, the heart and ears for *chd*, and ceratohyal and palatoquadrate perichondrial cells for *gdf5*, were not affected by bapx1-MO injection. Thus, *bapx1* is required for development of the jaw joint region: RAP, RAB and the jaw joint itself all require *bapx1* function, as does the early expression of two genes, *chd* and *gdf5*, within the developing jaw joint.

hand2 is required for ventral pharyngeal cartilage development

Although *bapx1* is a required *edn1* effector of joint patterning, ventral pharyngeal cartilage formation outside of the joint region is largely unaffected in bapx1-MO-injected animals. Thus, we next sought an *edn1* effector of ventral cartilage patterning. We focused on *hand2*, an excellent candidate for three reasons. First, in the mouse embryo, *hand2* expression requires *Edn1* function and *hand2* is required for ventral pharyngeal arch patterning (Thomas et al., 1998). Second, *hand2* expression requires *edn1* function in zebrafish, correlating with the *edn1* mutant ventral cartilage loss (Miller et al., 2000). Third, as shown above, *hand2* expression exquisitely complements *edn1* expression in the ventral pharyngeal arch primordia.

hand2 mutant zebrafish were found in a screen for mutations affecting heart development, including a null allele completely deleting the *hand2* locus (han^{s6}) (Yelon et al., 2000). Despite lacking a heart and circulating blood, han^{s6} mutants (for clarity hereafter referred to as *hand2* mutants) live for at least four days and make differentiated pharyngeal cartilage in the mandibular and hyoid arches (Fig. 6A,B). Unlike mutants of the anterior arch class (e.g. *edn1* mutants) cartilages of the more posterior pharyngeal arches never develop. Dorsal anterior arch cartilages of reduced size, but relatively normal shape, form in *hand2* mutants, whereas only a tiny amount of ventral cartilage forms (Fig. 6C-I). Mutant dorsal cartilages have readily recognizable components, including the pterygoid process and parallel stacks of chondrocytes forming the lateral plate of the palatoquadrate cartilage in arch one, and the symplectic (SY) and hyomandibular regions of the hyosymplectic cartilage in arch two. Mutant ventral cartilages, in contrast, are almost absent in the anterior arches (Fig. 6C-I). Although similar to *edn1* mutants, *hand2* mutants lack jaw joints, less cartilage is present ventrally in *hand2* mutants, and the two upper jaws typically are connected by a small disorganized cartilage bridge spanning the midline (Fig. 6C-E; Table 2). In contrast and also unlike *edn1* mutants, the *hand2*

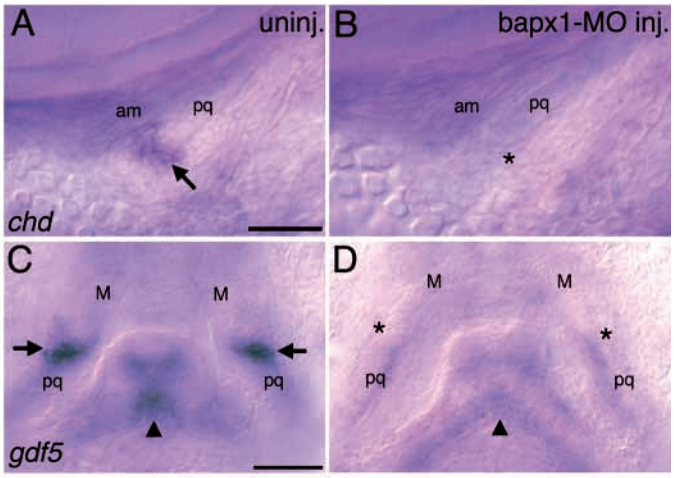

Fig. 5. *bapx1* is required for jaw joint expression of *chd* and *gdf5*. (A-B) Lateral views of wild-type wholemounted embryos at 56 hpf. (A) In uninjected animals, *chd* is expressed at the jaw joint (arrow) and (B) this domain appears to be absent in bapx1-MO-injected animals (asterisk). The jaw-closer muscle, the adductor mandibulae, which connects the upper and lower jaw, is present and grossly unaffected in bapx1-MO-injected animals. (C,D) Ventral views of wild-type wholemounted embryos at 78 hpf. (C) In uninjected animals, *gdf5* is expressed at the first arch joint (arrows) and (D) these domains are severely downregulated in bapx1-MO-injected animals. Second arch midline expression of *gdf5* (arrowheads) is also downregulated in bapx1-MO-injected animals. am, adductor mandibulae; M, Meckel's; pq, palatoquadrate. Scale bars: 50 μm.

Table 2. *hand2* is required for ventral pharyngeal cartilage and the jaw joint

Phenotype	Percent *hand2* mutants
Severely reduced dorsal cartilage	
Arch 1: PQ + PTP	0.0 (0/135)
Arch 2: HM + SY	0.5 (0.5/135)
Severely reduced ventral cartilage	
Arch 1: M	100.0 (135/135)
Arch 2: CH	100.0 (135/135)
Arches 3-7	100.0 (135/135)
Morphological jaw joint loss	97.8 (132/135)
Arch 2 joint loss	0.0 (135/135)

Animals were fixed at 4 days and Alcian stained, then scored in wholemounts. Both left and right sides of each animal were scored, and counted as half an animal. bh, basihyal.

mutant second arch has well-formed morphological joints between the dorsal and severely reduced ventral second arch cartilages (Fig. 6C-I). Hence the differential requirement of *hand2* for joint formation reveals a key patterning difference between arch one and two.

A complex role of *hand2* in ventral pharyngeal arch patterning

We previously showed that *edn1* function is required for the ventral arch expression of *hand2* and four other genes: *dlx3*, *EphA3*, *gsc* and *msxe* (Miller et al., 2000; Miller and Kimmel; 2001). Homologs of *dlx3*, *gsc* and *msxe* are all required for proper mammalian craniofacial development (Kula et al., 1996; Price et al., 1998; Rivera-Perez et al., 1995; Yamada et al., 1995; Jabs et al., 1993; Satokata and Maas, 1994; Satokata et al., 2000). *EphA3*, the Ephrin transmembrane receptor tyrosine kinase, is expressed in Meckel's cartilage of rats (Kilpatrick et al., 1996). Thus, all four of these genes have potentially conserved pharyngeal arch domains transducing the Edn1 signal.

Because absence of ventral cartilage is shared between *hand2* and *edn1* mutants (Piotrowski et al., 1996; Kimmel et al., 1996; Miller et al., 2000) and because in the mouse pharyngeal arch *msx1* expression requires *hand2*, we expected that ventral arch expression of some or all of these *edn1* target genes would also require *hand2*. Instead, early ventral arch expression of *dlx3*, *EphA3*, *gsc* and *msxe* are robustly present in *hand2* mutants (Fig. 7). We see three classes of effects on these genes in *hand2* mutants: no effect or mild upregulation, arch-specific requirement and clear upregulation. In the first class, the ventrally restricted pharyngeal arch expression domains of *dlx3* and *EphA3* expression are present in *hand2* mutants, and appear slightly upregulated (Fig. 7A-D).

In the second class, *gsc* expression requires *hand2* function in ventral arch one, but not ventral arch two. At 32 hpf, dorsal and ventral domains of *gsc* expression are present in the second arch, and both domains are present in *hand2* mutants at 32 hpf (Fig. 7E,F). Later at 38 hpf, a ventral arch one domain of *gsc* expression is present, and this domain of *gsc* expression is absent in *hand2* mutants (Fig. 7G,H). Both the early second arch ventral domain and the late first arch ventral domain of *gsc* expression are missing in *edn1* mutants (Miller et al., 2000) (data not shown).

In the third class, two *msx* genes are upregulated in *hand2* mutants. Expression of *msxe* in ventral first and second arch CNC at 30 hpf is present in *hand2* mutants and appears upregulated, possibly in other cells, but seemingly in the same cells that express *msxe* at lower levels in wild types (Fig. 7I,J). A second zebrafish *msx* gene, *msxb*, is more sparsely and weakly expressed in wild-type ventral arch CNC at 30 hpf (Fig. 7K). This expression, similar to that of *msxe*, requires *edn1* but not *hand2* function (Fig. 7L,M). Similar to *msxe*, but more dramatically so, *msxb* expression appears upregulated in *hand2* mutants. This *msxb* upregulation in *hand2* mutants appears, similar to the *msxe* upregulation, to involve upregulation of transcription in cells that normally express *msxb* at lower levels, and in addition seems to involve the ectopic expression of *msxb* in ventral CNC cells in which *msxb* expression is normally not detectable by *in situ* hybridization (i.e. in cells in the anterior ventral first and second arch, compare Fig. 7K,L). Thus, in stark contrast to *edn1* mutants, and despite ventral cartilage being almost absent later, early expression of *dlx3*, *EphA3*, *gsc*, *msxe* and *msxb* are all present in the ventral arches of *hand2* mutants. However, similar to *edn1* mutants, *hand2* mutants lack later first arch ventral *gsc* expression. These findings reveal complexity in the genetic network controlling ventral pharyngeal cartilage development. Based on the genes we have examined, we suggest that most *edn1*-dependent signaling in the early pharyngeal arch primordia occurs independently of *hand2* function.

hand2 represses joint and dorsal arch fates

Next we asked whether *hand2* mutants, similar to *edn1* mutants that also lack jaw joints, have early *bapx1* expression defects. Despite lacking a jaw joint, *hand2* mutants have expanded

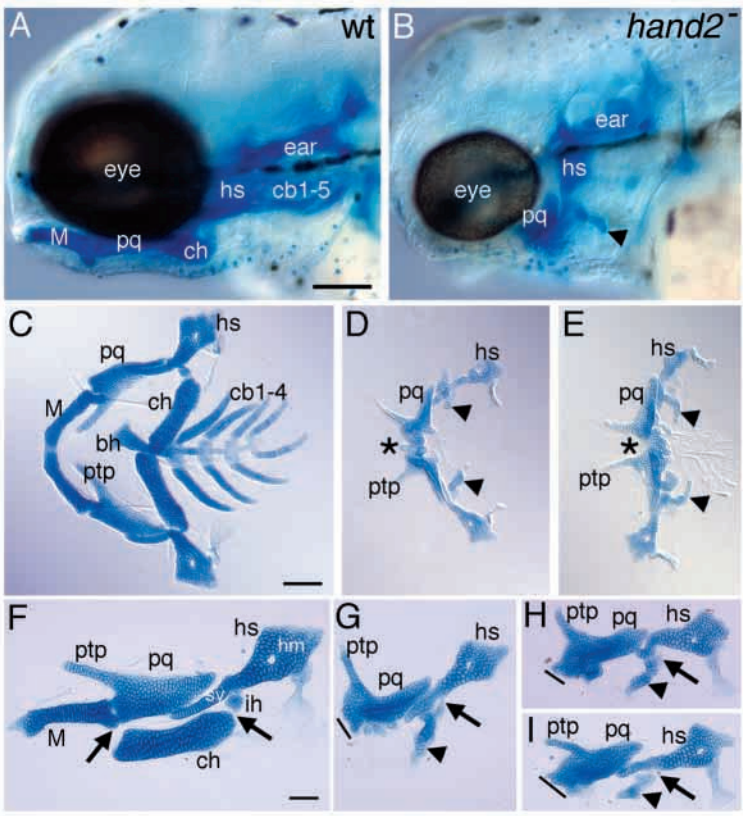

Fig. 6. *hand2* is required for ventral pharyngeal cartilage and the jaw joint but not for the second arch joint. (A,B) Lateral views of wholemount 4-day old wild type (A) and *hand2* mutant (B) larvae stained with Alcian Green, revealing severe loss of ventral pharyngeal cartilage in the mutant. (C-I) Flat-mounted pharyngeal cartilages from wild type (C,F) and *hand2* mutants (D,E,G-I). (C-E) Flat-mounted pharyngeal cartilages from the first six arches in wild type (C), and the entire pharynx in two different *hand2* mutants (D,E). In *hand2* mutants, ventral cartilages (arrowheads) are severely reduced. The two upper jaws are connected by a continuous cartilaginous bridge (asterisk), although some hints of partial jaw joint formation are evident (see Table 2). Branchial cartilages are also absent in *hand2* mutants. The upper hyomandibular region of the hyosymplectic in E was slightly nicked during dissection and resembled the contralateral counterpart. (F-I) Flat-mounted pharyngeal cartilages from one side of the first and second arches. The prominent ventral cartilages (M, ch) present in wild type (F) are absent in *hand2* mutants, although a small jumbled mass of ventral second arch cartilage is invariably present (arrowheads, see Table 2). (G-I). The dorsal cartilages are relatively well patterned with distinctive PTP and SY elements visible. The second arch joint (arrows) is present in *hand2* mutants. Because of the highly penetrant jaw joint loss (see Table 2), the *hand2* mutants in G-I were cut during dissection in order to flat-mount; the line indicates the plane of cutting and where the upper jaw was fused to its contralateral counterpart (asterisk in D,E). bh, basihyal; cb1-4, ceratobranchial 1-4; ch, ceratohyal; hm, hyomandibula; hs, hyosymplectic; ih, interhyal; M, Meckel's; pq, palatoquadrate; ptp, pterygoid process of palatoquadrate; sy, symplectic. Scale bars: 100 μm in A-E, 50 μm in F-I.

intermediate first arch *bapx1* expression (Fig. 8B). However and remarkably, *bapx1* expression ectopically expands into the ventral first arch of *hand2* mutants, where *hand2* is normally expressed (Fig. 8B). To determine whether this ectopic *bapx1* expression in *hand2* mutants requires Edn1 function, we injected Edn1 morpholinos (Edn1-MOs) (Miller and Kimmel, 2001) into clutches of *hand2* mutants to obtain animals lacking both *hand2* and *edn1* function. The expansion of *bapx1* expression in *hand2* mutants is *edn1*-dependent, because *hand2* mutants injected with Edn1-MOs, similar to *edn1* mutants, completely lack first arch mesenchymal expression of *bapx1* (Fig. 8C).

The expanded domain of *bapx1* expression in *hand2* mutants suggests *hand2* functions to repress the specification of joint fates. Because the joint forms at an intermediate DV location (see above), we finally asked whether even more dorsal fates are also repressed by *hand2*. Although we currently know of no marker restricted to dorsal arch postmigratory CNC in zebrafish, *engrailed2* (*eng2*) expression is restricted to dorsal first arch myoblasts (see Hatta et al., 1990; Ekker et al., 1992; Kimmel et al., 2001b). In both *hand2* and *edn1* mutants, *eng2* expression expands ventrally, apparently revealing an unsubdivided arch mesodermal core (Fig. 8D-F). Thus, *edn1* and *hand2* specify ventral fates at least in part by repressing dorsal arch fates.

DISCUSSION

We show that two *edn1* target genes, *hand2* and *bapx1*, pattern ventral pharyngeal cartilage and the jaw joint during zebrafish development. Although shown to be required for patterning throughout the DV extent of the pharyngeal arch, *edn1* expression is restricted to ventral tissues complementary to *hand2* expression. First arch *bapx1* expression defines an intermediate or presumptive joint domain that requires *edn1* function. We uncover multiple roles of *bapx1* in patterning the jaw joint region, including a requirement for morphological jaw joint formation and early expression of *chd* and *gdf5* in the developing jaw joint. We show that a second *edn1* target gene, *hand2*, is required for ventral cartilage formation. The early, ventrally restricted, *edn1*-dependent pharyngeal arch expression of *dlx3*, *EphA3*, *gsc*, *msxe* and *msxb* does not require *hand2*. Instead, both *msx* genes appear upregulated in *hand2* mutants. Moreover, *bapx1* and *eng2* expression expand ventrally in *hand2* mutants. Thus, the specification of ventral pharyngeal fates is achieved at least in part through the repression of joint and dorsal fates.

Gene expression defines three domains within postmigratory pharyngeal arch CNC

The serial section analyses we present here place *edn1*-expressing cells as ventrally confined at a postmigratory stage. The ventral surface ectoderm, paraxial mesodermal arch cores, and pharyngeal endodermal epithelia expression domains of *edn1* are all complementarily contained within the *hand2*-expressing ventral region of the arch primordia. Because the Edn1 signal is thought to be secreted and Edn1 function is required for patterning throughout the DV extent of the pharyngeal arch, perhaps Edn1 acts as a morphogen in patterning DV fates in the pharynx.

Our finding that a day before chondrogenesis, *bapx1* is expressed in an intermediate region of the first arch, ventral to some *dlx2* expression and dorsal to *hand2* expression, raises the possibility that at least three domains of the resultant

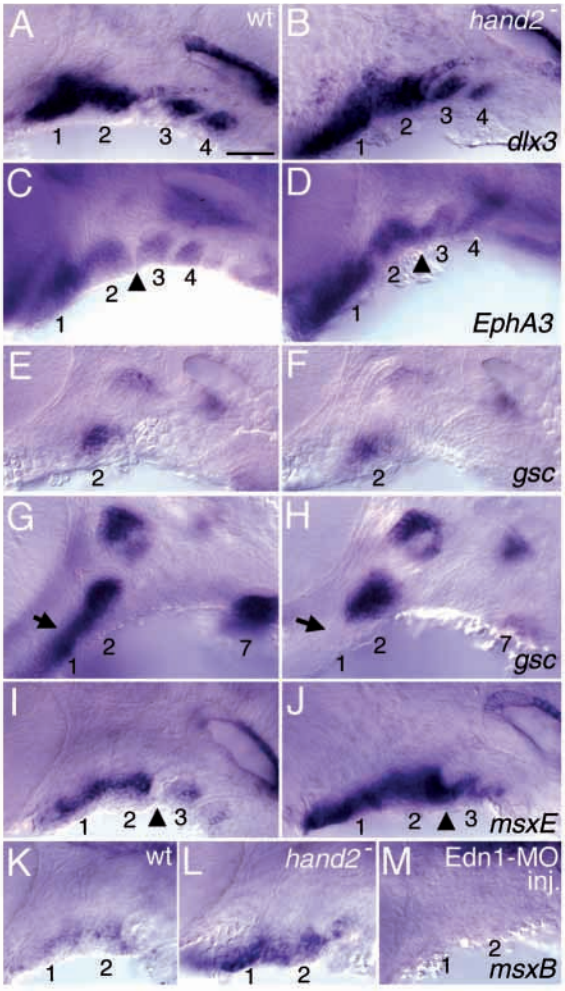

Fig. 7. *hand2* is not required for early ventral pharyngeal arch expression of *edn1* targets. Lateral views of deyolked wild type (A,C,E,G,I,K), *hand2* mutant (B,D,F,H,J,L) and edn1-MO-injected (M) animals. In each panel, the eye is in the upper left and the ear is in the upper right. (A,B) The ventrally restricted pharyngeal arch expression domain of *dlx3* at 30 hpf (A) does not require *hand2* function (B). (C,D) *EphA3* expression at 32 hpf (C) also does not require *hand2* function (D). (E-H) *gsc* expression. (E,F) Early dorsal and ventral hyoid expression domains of *gsc* at 32 hpf (E) are present in *hand2* mutants (F). (G,H) At 38 hpf, ventral arch one expression of *gsc* is absent in *hand2* mutants (arrows), whereas both dorsal and ventral arch two expression are still present (H). The seventh pharyngeal arch expression domain is downregulated in *hand2* mutants. (I,J) *msxe* expression at 30 hpf. *hand2* mutants have upregulated ventral arch *msxe* expression. (K-M) *msxb* expression at 30 hpf. Ventral pharyngeal arch expression of *msxb* (K) requires Edn1 signalling (M), but is upregulated in *hand2* mutants (L). Relevant pharyngeal arches are numbered in each panel. The second pharyngeal endodermal pouch fails to completely separate the second and third arches in *hand2* mutants (arrowheads in C,D and I,J). Scale bar: 50 μm.

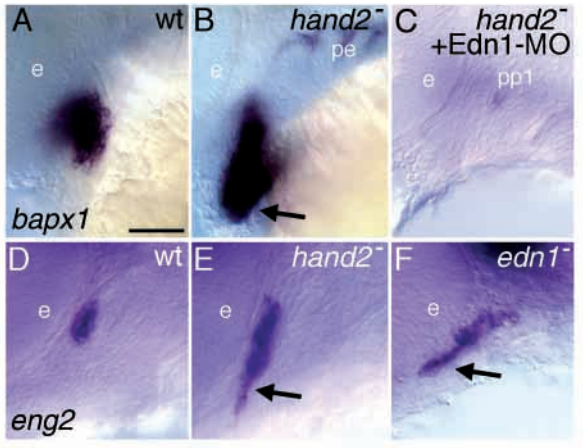

Fig. 8. *hand2* represses joint and dorsal pharyngeal fates. Lateral views of wholemount wild type (A,D), *hand2* mutant (B,E), *hand2* mutant injected with Edn1-MOs (C) and *edn1* mutant (F). (A-C) *bapx1* expression at 36 hpf is present at its normal location and expanded ventrally in *hand2* mutants (arrow). This expansion of *bapx1* in *hand2* mutants is *edn1*-dependent, as *hand2* mutants injected with Edn1-MOs lack all first arch mesenchymal expression of *bapx1*, whereas the first pharyngeal pouch expression domain is present (C). We found no evidence for the converse relationship, as *hand2* expression appeared unaffected in bapx1-MO-injected animals (data not shown). (D-F) *eng2* expression at 30 hpf, localized to constrictor dorsalis, the dorsal first arch mesodermal core (see Kimmel et al., 2001b) expands ventrally in both *hand2* and *edn1* mutants. e, eye; pe, pharyngeal endoderm; pp1, pharyngeal pouch 1. Scale bars: 50 μm.

skeleton (dorsal, joint and ventral) fate map to stereotypical domains of the pharyngeal arch primordia. Preliminary experiments with uncaged fluorescein support this idea, because uncaged dorsal and ventral spots in the arch primordia gave rise to labeled cells in dorsal and ventral cartilages, respectively (C. T. M., C. B. K., S. Cheesman and S. Hutchinson, unpublished). Powerful new tools including green fluorescent protein (GFP) lines and 4D confocal microscopy promise to allow high resolution fate mapping of the postmigratory CNC cylinders (J. G. Crump and C. B. K., unpublished), and will test the proposals that the DV prepattern prefigures dorsal and ventral cartilages, and that the early intermediate *bapx1* expression domain at 30 hpf prefigures the jaw joint.

An *edn1* target, *bapx1*, is required for patterning the intermediate jaw joint region of the first arch

Our bapx1-morpholino experiments reveal multiple requirements for *bapx1* in formation of the jaw joint region. Morphologically, the jaw joint itself, the nearby RAP of Meckel's cartilage, and the RAB all require *bapx1* function. These phenotypes, all associated with the jaw joint region, suggest *bapx1* functions to specify multiple fates within the intermediate first arch primordia. Because all three of these skeletal phenotypes are seen in *edn1* mutants (Miller et al., 2000; Kimmel et al., 2003), and *bapx1* first arch expression requires *edn1* signaling, *bapx1* is a critical effector of *edn1* in patterning the intermediate first arch.

bapx1 expression was detected at the ventral midline of the second arch, i.e. in the position of the later basihyal cartilage, consistent with the report of *Xenopus Xbap* expression (Newman et al., 1997). Midline expression of *gdf5* in the second arch was downregulated in bapx1-MO-injected animals, providing a potential earlier molecular correlate of the basihyal reduction phenotype. Thus, in zebrafish, *bapx1* is

required for jaw joints and a specific ventral midline cartilage, the basihyal.

Although mammalian *Bapx1* is expressed in mandibular arch mesenchyme and joints in the appendicular skeleton, no defects have been reported in these tissues in *Bapx1* mutant mice. *Bapx1* mutant mice have defective vertebrae and basal skulls, and are asplenic (Tribioli et al., 1999; Lettice et al., 1999; Akazawa et al., 2000). Calcein labeling revealed no detectable differences in early stages of vertebral formation in bapx1-MO injected-animals (data not shown, but see below) and we did not examine spleen development in bapx1-MO-injected zebrafish.

Although our morphological and molecular analysis of bapx1-MO-injected animals demonstrate that *bapx1* is required for the jaw joint, we cannot be certain that MO injections completely eliminate *bapx1* function, especially at later larval stages when the MO is probably significantly diluted. Thus, the lack of detectable differences in early vertebral development in bapx1-MO-injected animals could reflect the late stage this event occurs and the dilution of the injected MO. Once true genetic nulls of zebrafish *bapx1* are discovered, the role of *bapx1* in patterning the axial skeleton and spleen in zebrafish can be examined.

Morphologically, synovial joints are associated with a complex array of cell types, including articular cartilage, tendons and ligaments (see Kingston, 2000). Once markers are discovered for these tissue types in zebrafish, their formation can be assayed in anterior arch mutants and bapx1-MO-injected animals. Although GDF5 is insufficient to induce ectopic joints, GDF5 is sufficient to induce tendons and ligaments (Wolfman et al., 1997). Perhaps *gdf5* and/or *bapx1* in the developing jaw joint region also function to pattern connective tissues.

In chicks, RAP is derived from CNC emanating from the r4 level (Kontges and Lumsden, 1996). Thus, it will be particularly interesting to determine the axial level of origin of RAP in zebrafish. Genetic null alleles of *bapx1* will allow mosaic analyses to determine which phenotypes (e.g. RAP loss) are cell-autonomous.

A second *edn1* target, *hand2*, is required for ventral pharyngeal cartilage formation

In zebrafish, the *hand2* mutant cartilage phenotype resembles the *edn1* mutant phenotype (i.e. ventral cartilage in the first two arches is severely reduced, displaced ventrally and posteriorly, and the jaw joint is missing). However, one notable difference is that the dorsal cartilages in *hand2* mutants are less affected than in *edn1* mutants. Organized stacks of palatoquadrate and SY chondrocytes are seen in *hand2* mutants, but not in *edn1* mutants, which typically lack the SY cartilage altogether (Kimmel et al., 1998; Miller et al., 2000). Thus, *edn1* affects a broader pharyngeal arch domain than *hand2* (see below).

Early *edn1*-dependent expression of *dlx3*, *EphA3*, *gsc*, *msxe* and *msxb* occurs in ventral postmigratory CNC of *hand2* zebrafish mutants, showing that none of these genes are sufficient for ventral cartilage formation. Late ventral arch one *gsc* expression fails to be initiated in *hand2* mutants (Fig. 2H,I). This defect, shared with *edn1* mutants, may contribute to the shared loss of ventral cartilage and/or the jaw joint in *hand2* and *edn1* mutants.

hand2 represses joint, dorsal and ventral fates

Given the phenotypic similarity of ventral cartilage reduction in *edn1* and *hand2* mutants and that in the mouse pharyngeal arch expression of *msx1* requires *hand2* function (Thomas et al., 1998), we expected that a subset of the *edn1*-dependent ventral genes would also require *hand2*. Although late ventral arch one *gsc* expression failing to be initiated in *hand2* mutants meets this prediction, it is perhaps surprising that not only are the early *edn1*-dependent genes expressed, but that *msxe* and *msxb* are upregulated. Precedents exist for Hand2 functioning as a repressor, because in the mouse limb bud, Hand2 represses expression of *Gli3* and *Alx4* (te Welscher et al., 2002). We suggest that in the zebrafish pharyngeal arches, the repression of *msxe* by *hand2* is largely in the same ventral arch cells that in wild types express both genes. *msxb*, however, appears to be upregulated in *hand2* mutants both in cells that in wild types express *msxb*, and in cells that in wild types do not contain detectable *msxb* transcript by in situ hybridization (e.g. cells in the anterior ventral first two arches, see Fig. 7). Once antibodies specific to zebrafish Hand2, MsxB and MsxE are available, double-labeling experiments in sections could reveal the exact cellular relationship of these expression domains in pharyngeal arches of wild types and *hand2* mutants. The difficulty in establishing orthology of the zebrafish *msx* genes with mammalian *msx* genes, two of which are required for craniofacial development, and one of which is downstream to Edn1 signaling, complicates predicting the head skeletal consequences of altered *msxb* and *msxe* expression in zebrafish (Ekker et al., 1997; Satokata and Maas, 1994; Satokata et al., 2000; Thomas et al., 1998). Functional analyses of the zebrafish *msx* genes in head skeletal patterning could reveal specific roles, if any, for *msxb* and *msxe*. Because *msx1* and *msx2* in mice have redundant roles in chondrogenesis and osteogenesis (Satokata et al., 2000), perhaps the zebrafish *msx* genes do also.

Hu et al. (Hu et al., 2001) show that *msx* genes maintain cells in a proliferative state by blocking exit from the cell cycle, thus inhibiting differentiation. These findings could provide an explanation for why the same phenotype (ventral cartilage loss) is seen in *edn1* and *hand2* mutants, despite opposite effects on *msx* expression. Perhaps in *edn1* mutants, the early failure to specify the entire ventral arch domain, including expression of *msxe* and *msxb*, results in the almost complete absence of tissues derived from this domain because of lack of proliferation. Conversely, in *hand2* mutants, perhaps excess and ectopic *msx* expression prevents ventral arch CNC from differentiating into chondrocytes.

The ventrally expanded expression domain of *bapx1* in *hand2* mutants indicate that the ventral first arch in *hand2* mutants has partially adopted a joint fate. However, because the jaw joint later fails to form in *hand2* mutants, *bapx1* appears to be insufficient for formation of a differentiated jaw joint in this context. Because the expanded ventral domain of *bapx1* in *hand2* mutants also requires *edn1* function, the positioning of *bapx1* to the intermediate first arch is accomplished at least in part through the positive regulation of *edn1* and the repression ventrally by *hand2*.

In wild-type zebrafish embryos, *eng2* expression in the head periphery is restricted to a dorsal first arch paraxial mesodermal core, constrictor dorsalis (reviewed by Kimmel et al., 2001b). Expression of *eng2* expands ventrally in both *hand2* and *edn1*

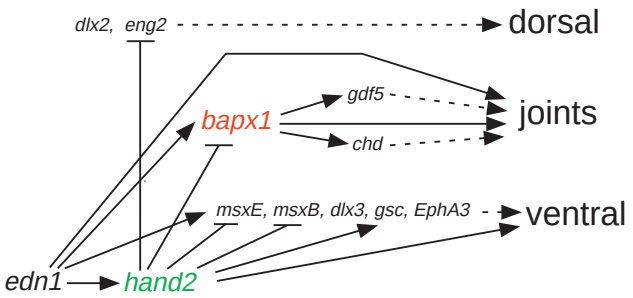

Fig. 9. A genetic model for dorsal/ventral pharyngeal arch patterning. Two *edn1* downstream target genes, *bapx1* (red) and *hand2* (green), specify joints and ventral pharyngeal fates, respectively. *edn1* is also required for the second arch joint, which is *bapx1*-independent, so an additional arrow is drawn from *edn1* to joint fates. The early ventral expression of *msxe*, *dlx3*, *gsc* and *EphA3* requires *edn1* but not *hand2*. The later (38 hpf, see Fig. 7H,I) arch one ventral, but not the early arch two ventral, *gsc* expression requires both *hand2* and *edn1*. In the first arch, *hand2* represses the expression of dorsally restricted *eng2* and joint-restricted *bapx1*. *bapx1* positively regulates *gdf5* and *chd*, potential effectors of jaw joint development. Although both *edn1* and *hand2* repress *eng2*, we parsimoniously propose this is through *hand2*. We propose the role of *dlx2* in patterning the dorsal pharyngeal arches is conserved between mice (Qiu et al., 1995; Qiu et al., 1997; Panganiban and Rubenstein, 2002) and zebrafish. In zebrafish, *dlx2* appears to be expressed throughout the entire postmigratory cranial neural crest (CNC) cylinder (Miller et al., 2000; Kimmel et al., 2001a; Kimmel et al., 2001b). The ventral subdomain of *dlx2* expression which requires *edn1* function (Miller et al., 2000) also appears to require *hand2* function (data not shown), not diagrammed here for simplicity. *dlx6* helps transduce the Edn1 signal to *hand2* in mice (Charité et al., 2001) (also not shown here). Proposed functions not yet experimentally tested are indicated by broken lines. The dorsoventral pharyngeal arch axis in tetrapods is sometimes referred to as the proximal-distal axis.

mutants, showing that mandibular mesoderm is dorsalized in *edn1* and *hand2* mutants, and indicating that in the early pharyngeal arch primordia, these ventral specifiers repress dorsal arch fates. Testing the initial proposal of Piotrowski et al. (Piotrowski et al., 1996) that dorsal skeletal identity is expanded ventrally in anterior arch mutants, awaits the identification of zebrafish dorsal-specific postmigratory CNC markers. Excitingly, a dorsal second arch dermal bone, the opercle, expands in animals with reduced *edn1* function, providing another line of evidence that Edn1 signaling represses dorsal pharyngeal arch fates (Kimmel et al., 2003).

Model for DV pharyngeal arch patterning

The downregulation of *bapx1* expression in the jaw joint primordium and of *dlx3*, *EphA3*, *gsc*, *msxe* and *msxb* expression ventrally (this work) (Miller et al., 2000) in *edn1* mutants reveals that Edn1 patterns both ventral and joint fates. *edn1* expression appears contained within the ventral arch and at least some *bapx1* expression does not overlap with *hand2* expression. This raises the hypothesis that Edn1 functions as a morphogen in patterning the arch primordia, with the ventrally localized secreted Edn1 signal specifying ventral and joint fates at high and intermediate thresholds, respectively. An Edn1 gradient model, combined with the repression of *bapx1* by *hand2*, provides an attractive mechanism for the positioning of the jaw joint. Analysis of dermal bone phenotypes in *edn1* mutants is consistent with a gradient model (Kimmel et al., 2003). Embryological studies involving focal misexpression of Edn1 would directly test the gradient model.

Based on our results, we propose the following genetic model for DV pharyngeal arch patterning (Fig. 9). Specification of ventral is in part performed by the *edn1*-dependent activation of *hand2*. Specification of the jaw joint is performed by the positive regulation of *bapx1* by *edn1*, acting at a distance from the ventral *edn1* source. In the first arch, *hand2* restricts the jaw joint by repressing *bapx1* in the ventral cartilage-forming domain, hence delimiting the position of the jaw joint. *bapx1* positively regulates jaw joint expression of *chd* and *gdf5*, which might play roles in pharyngeal joint development.

Potential evolutionary implications of *bapx1* expression

The localized expression of *bapx1*, *chd* and *gdf5* to the zebrafish jaw joint (this work) and tetrapod appendicular joints (Tribioli et al., 1997; Francis-West et al., 1999a; Francis-West et al., 1999b; Scott et al., 2000; Storm and Kingsley, 1996; Merino et al., 1999) combined with the requirements of *bapx1* (this work) and *gdf5* for formation of particular joints (Storm and Kingsley, 1996) suggests that a conserved genetic network controls joint formation in both the pharyngeal and appendicular skeleton. Whether pharyngeal or appendicular joints arose first during evolution is currently not clear, but some early vertebrates such as placoderms and acanthodians had a clearly functional jaw joint (Janvier, 1996).

Because *bapx1* in vertebrates and invertebrates is expressed in gut-associated mesenchyme (Tribioli et al., 1997; Tribioli and Lufkin, 1999; Azpiazu and Frasch, 1993), this is probably an ancient role for *bapx1*, one present before the jaw or skeletal joints evolved. The localized expression of *bapx1* in the jaw joint is particularly fascinating given the transformation this region underwent during vertebrate evolution. The expression and function of zebrafish *bapx1* suggests a specific genetic network exists for jaw joint formation and immediately raises the question of whether agnathan lampreys have a first arch mesenchymal *bapx1* expression domain. If so, perhaps modification of *bapx1* downstream targets, or modification of Bapx1 function, facilitated evolution of the jaw. If not, perhaps co-opting *bapx1* expression in the first arch of agnathans played a role in the appearance of jaws and joints during gnathostome evolution.

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