AP2-dependent signals from the ectoderm regulate craniofacial development in the zebrafish embryo

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Summary

AP2 transcription factors regulate many aspects of embryonic development. Studies of AP2a (Tfap2a) function in mice and zebrafish have demonstrated a role in patterning mesenchymal cells of neural crest origin that form the craniofacial skeleton, while the mammalian Tfap2b is required in both the facial skeleton and kidney. Here, we show essential functions for zebrafish tfap2a and tfap2b in development of the facial ectoderm, and for signals from this epithelium that induce skeletogenesis in neural crest cells (NCCs). Zebrafish embryos deficient for both tfap2a and tfap2b show defects in epidermal cell survival and lack NCC-derived cartilages. We show that cartilage defects arise after NCC migration during skeletal differentiation, and that they can be rescued by transplantation of wild-type ectoderm. We propose a model in which AP2 proteins play two distinct roles in cranial NCCs: an early cell-autonomous function in cell specification and survival, and a later non-autonomous function regulating ectodermal signals that induce skeletogenesis.

Key words: Tfap2, Neural crest, Craniofacial, Danio rerio

Introduction

Vertebrate neural crest cells (NCCs) form all body pigmentation, peripheral neurons and glia, and most skull cartilage and bone. Some skeletogenic NCCs surround the brain to form the skull vault, while others migrate into the pharyngeal arches to form the jaw and larynx, and defects in these cells underlie many craniofacial birth defects. Cranial NCCs carry positional information from their origins alongside the midbrain and hindbrain, but also receive inductive signals from surrounding epithelia that determine skeletal fates (Graham, 2003; Santagati and Rijli, 2003; Trainor et al., 2003). Molecular mechanisms underlying these epithelial-mesenchymal interactions between NCCs and facial endoderm or ectoderm are largely unknown.

AP2 transcription factors are highly conserved DNA-binding proteins implicated in cranial NCC development. Three of the five members of this family in mammals (Tcfap2a, Tfap2b and Tcfap2g) are expressed in migrating NCCs (Moser et al., 1995; Hilger-Eversheim et al., 2000). Tfap2a–/– mutant mice have defects in the neural tube, body wall and limbs, and striking malformations of NCC-derived craniofacial structures (palate and ear ossicles) that correlate with defects in NCC survival (Schorle et al., 1996; Zhang et al., 1996; Morris-Kay et al., 1996). Craniofacial defects, however, could be indirectly due to disruptions in neural tube and epithelial morphogenesis in Tfap2a–/– mutants, because early NCC migration appears grossly unaffected (Schorle et al., 1996). Tfap2b–/– mutant mice and TFPAP2B–/– humans have renal epithelial apoptosis and patent ductus arteriosus (Char syndrome), and mild defects in the NCC-derived craniofacial skeleton (Chazaud et al., 1996; Moser et al., 1997b; Satoda et al., 2000). The only defects reported in Tcfap2g–/– mutant mice are placental (Auman et al., 2002). Thus, co-expression and conserved protein structures of Tfap2a and Tfap2b suggest that their functions are redundant, but specific requirements in NCCs remain unclear.

Insights into tfap2a function in NCCs have come from analysis of the zebrafish lockjaw (low) mutation, which eliminates tfap2a function but lacks the severe neural tube defects seen in Tcfap2a–/– mice. low–/– mutants have early defects in NCC specification, prior to migration, and later lack subsets of cartilage and pigment cells (Schilling et al., 1996; Knight et al., 2003). low/tfap2a is required for kit expression in pigment cell precursors and differentiation of early melanocytes and iridophores (Knight et al., 2004), and previous studies of the mammalian KIT promoter suggests that this regulation is direct (Huang et al., 1998). Similar to studies of the Hoxa2 promoter in mice (Maconochie et al., 1999), zebrafish low/tfap2a is required to activate transcription of Hox group 2 genes in NCCs of the second pharyngeal arch (hyoid), suggesting that tfap2a regulates the segmental identities of NCCs (Knight et al., 2004). Hyoid defects and fusions with the mandibular arch in low mutants also resemble embryos lacking hoxa2/hoxb2 gene functions (Hunter and Prince, 2002). These studies demonstrate a cell-autonomous role for tfap2a in subsets of NCC, but cannot account for more widespread defects in NCC survival and chondrogenesis in AP2-deficient embryos (Schorle et al., 1996; Knight et al., 2003; Barrallo-Gimeno et al., 2004). Thus, Tcfap2a may have additional, non-autonomous roles in the tissues with which NCCs interact.

Tcfap2a was previously known as keratin transcription factor, KTF-1, a regulator of epidermal differentiation in vitro.
Materials and methods

Animals and histology

Zebrafish embryos were obtained in natural crosses and staged according to Kimmel et al. (Kimmel et al., 1995). The low allele of tfap2a was isolated in the 1996 ENU mutagenesis screen in Tubingen (Schilling et al., 1996) and for this study was crossed into AB and fil1-GFP transgenic backgrounds (Lawson and Weinstein, 2002). low mutants were identified by their lack of early melanocytes at 25 hpf (Schilling et al., 1996; Knight et al., 2003). Cartilages were visualized by Alcian Blue staining and flat mounted (Kimmel et al., 1998; Javidan and Schilling, 2004).

Cloning and PCR amplification of tfap2b transcripts

Forward and reverse primers ap2b-7f (CGAGGGCAACGGACGGAGCC) and ap2b-1r (CCTTCCACCATAAACACC) were designed to EST sequences fp02d11.y1 (GenBank Accession Number BG737469) and fp60d9.x1 (GenBank Accession Number Bf671067), respectively. A 3.1 kb band corresponding to the full-length tfap2b (GenBank Accession Number DQ060246) was amplified by RT-PCR from 26 hpf embryos. This was cloned into pGEM T’Easy (Promega), completely sequenced in both directions using the Big Dye Terminator Sequencing reagent (ABI), and run on an ABI PRISM 310 sequencer (PE Applied Biosystems). RT-PCR amplification of tfap2b over exon 5 in morpholino-injected animals and for staging of tfap2b expression used 10 pmol each of primers ap2b-6f (AGAGCGGAGGTATCTGTG) and ap2b-6r (ATGTGACATTGGCTGCC) with a slightly lower annealing temperature of 50°C for 30 seconds.

Whole-mount in situ hybridization and immunohistochemistry

In situ hybridization was carried out as described previously (Thisse et al., 1993). Antisense probe for tfap2b was synthesized with SP6 RNA polymerase from the amplified tfap2b clone following linearization by NcoI. Probes and antibodies used were dlx2 (Akimenko et al., 1994), foxa2 (Prince et al., 1998), tfap2a and fgf8 (Furthauer et al., 1997), fli1 (Brown et al., 2000), gsc (Stachel et al., 1993), nkl2.3 (Lee et al., 1996), srf (Furthauer et al., 2002; Tsang et al., 2002), and son9b (Li et al., 2002). Sectioning was performed after in situ hybridization. Embryos were immered in 30% sucrose, embedded in 1% agar blocks, frozen in liquid nitrogen and cryostat sections cut at a thickness of 12 µm on a Leica cryostat.

Cell death detection assays

Apoptotic cell death was detected using a terminal transferase, dUTP nick-end labeling method (TUNEL) using several modifications suggested by the manufacturer (POD In Situ Cell Death Detection Kit, Roche). To avoid overfixing, embryos were dechorionated, and fixed in 4% PFA at 4°C overnight. They were then permeabilized with acetone at -20°C for 7 minutes and incubated in 2% goat serum at room temperature for 3 hours as a protein block prior to the TUNEL reaction. Apoptotic cells were counted in the pharyngeal region, between the posterior margin of the eye and anterior otic vesicle.

Morpholino injections

A morpholino ap2b-x.51 (GCCATTTTTCGACACCGCTCTGATC) was designed against the splice acceptor site of exon 5 in the tfap2b sequence at 1031 bp (Fig. 1B) by comparison with the corresponding genomic contig BX005121.8 from the zebrafish genome assembly (wwwensembl.org/D_rerio). The ap2b-x.51 morpholino was reconstituted in 0.2M KCl and 3-5 ng was injected into one- or two-cell stage embryos together with 3% TRITC-dextran to act as a lineage tracer.

Ectoderm cell transplantation

Wild-type donor animals were injected with a combination of 3% TRITC-dextran (neutral, 10,000 M) and 3% biotin-dextran (lysine-fixable, 10,000 M) at the one- to two-cell stage. Cells within a few cell diameters of the animal pole were transplanted at the late blastula stage, as cell-tracing studies have shown that this region forms cranial ectoderm (Kimmel et al., 1990). Transplants in this position never formed endoderm or mesoderm, and were generally segregated in either non-neural ectoderm or in the neural tube and NCCs. Cells were transplanted into this location in low mutants and in wild-type siblings, and allowed to develop to 25 hpf, when transplanted cells were visualized by rhodamine fluorescence. Each embryo was then either: (1) fixed in 4% PFA and processed individually by in situ hybridization for srf expression, and detection of the biotin-labeled donor cells with a peroxidase coupled avidin-streptavidin complex (Vectastain) using DAB as the colored substrate; or (2) raised to 4 days postfertilization and processed for detection and Alcian Blue staining for cartilage, as above.

Results

Cloning and characterization of zebrafish tfap2b

We previously showed a requirement for tfap2a in NCC development (Knight et al., 2003; Knight et al., 2004), and here we have analyzed the expression and function of a zebrafish tfap2b. Two ESTs were identified by sequence comparison with mammalian Tcfap2b. A 3.1 kb fragment amplified from cDNA of 26 hpf embryos showed high similarity at the amino acid level to tfap2b genes from chick, mouse and human (E<0) compared with Tcfap2a genes from...
zebrafish (E<–141), mouse (E<–143) and human (E<–146), and was designated tfap2b. Alignment with other family members revealed that tfap2b shares the highly conserved DNA binding and heterodimerization domains characteristic of all AP2 proteins (data not shown). Zebrafish tfap2b genes are more similar in sequence to tfap2a (65%) than to tfap2g (60%), tfap2d (52%) or tfap2e (65%), and vertebrate Tfap2a and Tfap2b group together while Tfap2g is more distantly related (Fig. 1A).

Using the whole zebrafish genome assembly (www.ensembl.org/Danio_rerio), we found that tfap2b has the same exon-intron arrangement as tfap2a, including exon-intron boundaries within the essential DNA-binding domain (Fig. 1B). To test tfap2b function during development, we designed an antisense morpholino (ap2bMO) to the splice acceptor site of tfap2b exon5 at 1030 bp in the mRNA sequence, an approach that we previously used successfully to disrupt tfap2a function (Knight et al., 2003). We used RT-PCR to show that injection of 5 ng ap2bMO per embryo disrupts splicing of tfap2b exon5 (Fig. 1C). We then sequenced the larger PCR product from ap2bMO-injected animals at 24 hpf and found that splicing failed at the intron-exon5 boundary, causing the intron to remain in the mRNA transcript. Similar splicing defects caused by a morpholino to tfap2a (ap2aMO) phenocopy the lockjaw mutant phenotype (Knight et al., 2003). The highly conserved exon-intron structures of AP2 genes, coupled with their nearly identical DNA binding and heterodimerization domains, suggests that the disruption of tfap2b splicing in ap2bMO-injected animals reflects a similar abrogation of tfap2b gene function as for tfap2a.

**tfap2a and tfap2b are co-expressed in neural tube and ectoderm, but not neural crest**

Unlike tfap2a, which is first expressed in the non-neural ectoderm during gastrulation and later in NCCs (Fig. 2A), tfap2b expression is first detected at six- to seven-somite stages in the hindbrain (Fig. 2B). Later, tfap2b is expressed throughout the surface ectoderm and in migrating NCCs, while tfap2b expression remains restricted to the hindbrain (Fig. 2C,D). Both genes are co-expressed in the developing intermediate mesoderm and by 24 hours postfertilization (hpf) in the pronephric ducts. tfap2b expression was not detected during gastrulation or early somitogenesis by in situ hybridization, or by RT-PCR on cDNA prepared from embryos harvested hourly between 6 and 10 hpf (Fig. 1D).

Surprisingly, tfap2b is not expressed in NCCs at any stage. Migrating NCCs in the pharyngeal arches express tfap2a at 24 hpf (Fig. 2E,G). At this stage, tfap2b is weakly expressed in the arches, but not in NCCs (Fig. 2F,H), and expression is restricted to surface pharyngeal ectoderm (see Fig. 6). By contrast, tfap2a is expressed in both NCCs and more broadly throughout the surface ectoderm. Its expression overlaps that of tfap2b in the diencephalon and rhombencephalon (Fig. 2E-H). These patterns of expression are maintained, with tfap2b expression restricted to a subset of tfap2a-expressing cells in the pharyngeal ectoderm (Fig. 2LJ). tfap2a and tfap2b are also
co-expressed in the lens and retina (ganglion cell layer), midbrain (tectum and tegmentum), cerebellum and spinal cord. These expression domains persist until 52 hpf, whereas, by contrast, tfap2b mRNA is no longer detected in the pharyngeal ectoderm after 48 hpf (Fig. 2K,L).

tfap2a and tfap2b are both required for craniofacial development

To test tfap2b function, 3 ng/embryo of ap2bMO was injected into wild-type embryos at the one- to two-cell stage. This effectively blocked splicing of the tfap2b transcript (Fig. 1C) and injected embryos appeared healthy at 72 hpf (Fig. 3A,C). This was also true for injections of 5-10 ng/embryo. By contrast, injection of 3 ng/embryo of ap2bMO into low mutants (hereafter referred to as ‘AP2a/b-deficient’) caused more severe craniofacial defects than low alone. In low mutants, the jaw hangs ventrally and the hyoid and branchial arches are reduced (Fig. 3B). By contrast, AP2a/b-deficient embryos lack virtually all pharyngeal cartilages (Fig. 3D). low mutant embryos were distinguished from ap2bMO-injected wild-type siblings by a lack of melanocytes at 26 hpf. This was further confirmed by PCR identification from tail DNA of ap2bMO-injected mutants using a restriction site created by the low lesion in exon 5 of tfap2a (data not shown) (Knight et al., 2003).

Closer examination of cartilage confirmed that ap2bMO has no effect in the presence of a functional tfap2a gene. In low mutants alone, both dorsal (hyosymplectic, hs) and ventral (ceratohyal, ch) hyoid cartilages are reduced, and remnants of the hyoid fuse with the mandibular arch (Fig. 3F). By contrast, AP2a/b-deficient larvae also lack an anterior neurocranium and much of the mandibular arch (Fig. 3H). The posterior neurocranium and pectoral fin skeletons, which are derived from mesoderm, are not affected. Rather, defects are restricted to NCC-derived cartilage, suggesting that tfap2a and tfap2b play redundant roles in the development of skeletogenic NCCs.

tfap2b is not required for early neural crest cell specification

To determine if tfap2b, like tfap2a, is required during early NCC development, we examined dlx2 and hoxa2 expression in the pharyngeal arches. At 26 hpf, dlx2 expression marks migrating NCCs in mandibular, hyoid and branchial NCCs (m, h and b in Fig. 4A). hoxa2 is co-expressed in hyoid and branchial NCCs (Fig. 4D). Both are reduced in the hyoid arch to a few ventral cells in low mutants. By contrast, mandibular expression of dlx2 is unaffected (Fig. 4A,B) (Knight et al., 2003). ap2bMO injections into low mutant embryos did not enhance this phenotype (Fig. 4C,F) or disrupt dlx2 or hoxa2 expression (data not shown). Thus, unlike tfap2a, tfap2b is not required for early homeobox gene activation in NCCs.

To test later requirements for tfap2b in the arches, we examined expression of the ETS-domain transcription factor fli1 in AP2a/b-deficient embryos (Brown et al., 2000). fli1 is first expressed in NCCs at 22 hpf, after migration, and also in vascular endothelia (Fig. 4G-I). In low mutants, fli1 expression is slightly reduced in the hyoid and branchial arches (Fig. 4H), but not in the mandibular arch, similar to dlx2. Injection of up to 5 ng of ap2bMO had no effect on fli1 expression in wild-type controls (data not shown). However, ap2bMO nearly eliminated fli1 expression in the arches when injected into low mutants (Fig. 4I). These results are consistent with a requirement for tfap2b in NCCs after migration into the arch primordia.
AP2 and craniofacial development

To address requirements for tfap2b in skeletogenic NCCs, we examined goosecoid (gsc) and sox9a expression (Fig. 4J-O). Both genes play important roles in skeletal differentiation (Rivera-Perez et al., 1999). gsc expression is reduced in the dorsal hyoid arch in low mutants, and this correlates with loss of hoxa2 expression and hyosymplectic cartilage. Expression in the ventral arch is unaffected (Fig. 4K) (Knight et al., 2003). Injection of up to 5 ng of ap2bMO has no effect on gsc expression (data not shown). However, in AP2a/b-deficient embryos, gsc expression is eliminated throughout the hyoid (Fig. 4L). This suggests that tfap2b is required for the specification of NCC skeletal progenitors.

Unlike low/tfap2a–/– mutants alone, AP2-deficient embryos also show defects in the neurocranium. Two clusters of cartilage precursors in the pharyngeal arches, and a third pair dorsal to the mouth that form the neurocranium, express sox9a at 52 hpf (Fig. 4M). low mutants lack sox9a expression in the hyoid, and mandibular domains remain split at the midline, but do not affect the neurocranium (Fig. 4N). ap2bMO injections alone have no effect on the neurocranium (data not shown), but cause a complete loss of neurocranial sox9a expression when injected into low (Fig. 4O). These results demonstrate requirements for AP2 function throughout the NCCs that form the neurocranium and pharyngeal arches.

We used a transgenic line in which the flil promoter drives eGFP (flil-GFP), to correlate NCC and skeletal defects (Lawson and Weinstein, 2002). This flil-GFP transgene was crossed into lowts213 and used to visualize NCCs in the pharyngeal arches of 36 hpf individuals, that were then raised to 4 dpf and stained for cartilage (Fig. 5). In this line, the flil-GFP co-localizes with dlx2 expression in NCCs (data not shown). flil-GFP expression was reduced in low mutants (Fig. 4).
Development (Fig. 7A-I). In the lowts214 examined apoptosis in the ectoderm using TUNEL labeling and ectoderm? AP2 proteins play important roles in cell survival, pharyngeal ectoderm, and redundant with that of results suggest that function is required in the tfap2b expression is restricted to ectoderm (Fig. 6C). These overlaps in NCCs with dlx2 expression (Fig. 6A). By contrast, tfap2b expression is restricted to ectoderm (Fig. 6C). These results suggest that tfap2b function is required in the pharyngeal ectoderm, and redundant with that of tfap2a.

Do AP2a/b-deficient embryos have defects in pharyngeal ectoderm? AP2 proteins play important roles in cell survival, and Tcfap2a mutant mice show elevated apoptosis. We examined apoptosis in the ectoderm using TUNEL labeling (Fig. 7A-I). In the low52T4 allele of tfap2a, a brief period of apoptosis occurs in NCCs between 10 and 14 somites (Knight et al, 2003), and apoptosis is more widespread in the mob510 allele (Barallo-Gimeno et al., 2004). We performed TUNEL labeling in either low mutants alone (Fig. 7B,E,H) or AP2a/b-deficient embryos (Fig. 7C,F,I) carrying the fli1-GFP transgene. Colocalization of GFP and TUNEL showed that apoptosis was excluded from NCCs, and confined to the ectoderm. The number of TUNEL labeled cells in pharyngeal ectoderm at 28 hpf was similar in low (4.5±2.62; n=4) and wild-type controls (4.3±2.32; n=6), and only slightly increased in embryos injected with the ap2b MO alone (7.3±4.49; n=5). By contrast, TUNEL labeling was dramatically elevated in AP2a/b-deficient embryos (75.8±16.22; n=6). Large numbers of apoptotic NCCs were observed along the dorsal edges of the spinal cord in the tail, and this correlates with pigment cell defects in the tail in low mutants (Fig. 7G-I) (Knight et al, 2004). Elevated ectodermal apoptosis was observed not only in the pharyngeal arches in AP2-deficient animals, but also in olfactory epithelia and in the skin overlying the hindbrain (data not shown).

By contrast, other epithelia such as the pharyngeal endoderm are unaffected by the loss of AP-2. The NK homeobox gene, nkx2.3, marks the pharyngeal
pouch endoderm at 34 hpf (Fig. 7J), and this endoderm forms a normal series of pouches in low mutants and in AP2a/b-deficient embryos (Fig. 7K,L).

Ectodermal grafts rescue AP2-dependent cartilages

To determine if tfap2a and tfap2b are required in ectoderm, we performed transplants. Ectodermal precursors were transplanted at blastula stages, from fluorescently labeled wild-type donor embryos [injected with tetramethylrhodamine (TRITC) and biotin-conjugated dextran], into unlabeled low mutants injected with ap2bMO (Fig. 8A). Cells placed near the animal pole gave rise to scattered clones in the skin of the head at later stages. Transplanted cells contributed to the olfactory epithelia (n=34; Fig. 8E), retina (n=36; Fig. 8F,G) and neural tube (n=6), as well as the pharyngeal ectoderm (n=14; Fig. 8E-G), but not to the mesoderm or endoderm. Of 14 low mutant hosts with transplanted wild-type pharyngeal ectoderm, four showed unilateral rescue of hyoid arch development coinciding with the transplanted cells (Fig. 8, left and center columns). Similar rescues were achieved in 5/9 transplants of pharyngeal ectoderm into low mutants injected with ap2bMO (Fig. 8, right columns).
column), and in these cases we also observed rescue of *fli1-GFP* expression on the transplanted side (Fig. 8J). Transplanted cells were clearly situated in the ectoderm when TRITC was compared with *fli1-GFP* (compare Fig. 8G and 8J) and later biotin-labeling compared with cartilage at 4 dpf (Fig. 8H,I). In each case, either the ceratohyal, hyosymplectic or ceratobranchial cartilages (or in some cases all three) were rescued on one side adjacent to transplanted ectodermal cells (Fig. 8K-P). In some cases, transplants also rescued more dorsal defects in the neurocranium, indicating that the ectoderm also influences these cartilages. This demonstrates that *tfap2a* and *tfap2b* are required non-autonomously in the ectoderm to regulate NCC-derived cartilage differentiation, in addition to the well-documented, cell-autonomous roles of *tfap2a* in NCCs.

### AP2 regulates Fgf signaling from the ectoderm

What ectodermal signal is disrupted in AP2a/b-deficient embryos? Shh, Bmp4 and Fgf8 are expressed in the facial ectoderm, and all three have been implicated in NCC patterning, proliferation and survival (Bachler and Neubeuser, 2001). *tfap2a* mutants lack Shh expression in the posterior ectoderm of the hyoid arch (Knight et al., 2003), but injection of *ap2b-MO* into *low* mutants does not cause any defects in *shh* expression. Likewise, *bmp4* expression is only slightly reduced in the ventral ectoderm in AP2a/b-deficient embryos (data not shown). To address requirements for AP-2 in regulating Fgfs, we examined *fgf3* and *fgf8* expression, both of which have been implicated in craniofacial development in zebrafish (Roehl and Nusslein-Volhard, 2001; David et al., 2002). *fgf8* is expressed in the mandibular ectoderm and more weakly in more posterior arch ectoderm at 28 hpf. Expression of both *fgf3* and *fgf8* persists in AP2a/b-deficient embryos (Fig. 9A,B).

Other Fgf proteins (*Fgf9*, *Fgf17* and *Fgf18*) expressed in cranial ectoderm in mammals have not been well characterized in zebrafish. Therefore, we examined expression of the interleukin receptor-related Fgf target *sef* as a general indicator of responses to Fgf in the arches. *sef* is expressed in cranial NCCs in the arches beginning at 18 hpf, and near other sources of Fgfs at the mid-hindbrain boundary and in the pectoral fin buds (Fig. 9C). Expression of *sef* is lost in the first arch (mandibular) and strongly reduced in the second arch (hyoid) in AP2a/b-deficient embryos, while other domains of expression are unaffected (Fig. 9D). This appears to reflect a loss of Fgf produced by the ectoderm, as transplantation of wild-type pharyngeal ectoderm partially rescues *sef* expression on the grafted side (8/18; Fig. 9E). Our results implicate Fgfs as one component of the ectodermal signals that require the combined functions of Tfap2a and Tfap2b.

### Discussion

We have identified a zebrafish *tfap2b* required for craniofacial development that acts together with *tfap2a* in the pharyngeal ectoderm to promote skeletal development of NCCs. Two lines of evidence lead to these conclusions: (1) *tfap2b* is expressed in the ectoderm, but not in NCCs, yet it functions redundantly with *tfap2a* in cartilage development; (2) transplantation of wild type ectoderm into an AP2a/b-deficient embryo partially rescues NCC-derived cartilage. Based on our morpholino studies, *tfap2b* is not required for early NCC specification, but only later after migration. We propose that AP2 genes act in two separate aspects of NCC development (Fig. 10). First, *tfap2a* regulates specification of skeletal precursor cells and their segmental identities, through regulation of Hox group 2 genes (Knight et al., 2003). Second, *tfap2a* and *tfap2b* together control Fgfs and perhaps other signals from the ectoderm that regulate skeletogenesis. The coordinated action of these pathways mediates the spatiotemporal formation of craniofacial cartilage and bone.

### Redundancies between *tfap2a* and *tfap2b*

In mice, Tcfap2a and Tcfap2b are co-expressed in many embryonic tissues such as NCCs, yet have unique requirements in development. Loss-of-function mutations in *Tcfap2a* disrupt neural tube closure and craniofacial NCCs (Schorle et al., 1996; Zhang et al., 1996). By contrast, mutations in *Tcfap2b* or human TFAP2B cause renal failure and craniofacial defects associated with Char syndrome (Moser et al., 1997b; Satoda et al., 2000). AP2 proteins may function redundantly in tissues where they are co-expressed, and our results support this hypothesis for the ectoderm (Moser et al., 1997a). 

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**Fig. 9.** *tfap2a* and *tfap2b* are required for Fgf signaling in NCCs. Lateral views, anterior towards the left. (A,B) At 32 hpf, *fgf8* mRNA is detected in wild type in the ectoderm covering the arches, in addition to the mid-hindbrain boundary (mhb); this expression remains in AP2a/b-deficient embryos. Arrows in A,B indicate ectodermal expression of *fgf8*. (C,D) At 28 hpf in wild type, *sef* mRNA is detected in NCCs; this expression is severely reduced in AP2a/b-deficient embryos. (E,F) Mosaic embryos in which biotin-labeled, wild-type ectodermal cells (brown, arrows) were transplanted on one side of the head and partially restore *sef* expression (blue). (E) Dorsal view, anterior towards the left showing transplanted side with substantially increased levels of *sef* expression, and lack of expression on the contralateral, control side (arrows). (F) A transverse section through the embryo shown in E at the level of the hyoid arch showing grafted wild-type ectoderm (brown) and rescued *sef* expression in underlying NC (blue). Arrowhead in F indicates transplanted ectoderm. m, mandibular arch; h, hyoid arch; b, branchial arches. Scale bars: 50 µm.
Development, which shares nearly identical DNA-activated by tfap2b from address this issue in NCCs, we morpholino-depleted albeit with different affinity (Maconochie et al., 1999). To Knight et al., 2004).

Arches in the tfap2a promotes Hoxa2 expression in NCCs (A) Initially, tfap2a acts autonomously within NCCs to regulate hoxa2, dlx2 and kit expression. (B) Later, tfap2a and tfap2b act together in the surface ectoderm (blue) to regulate Fgf8 and possibly other Fgfs that activate sef expression and control cartilage development, together with Fgf3 signaling from the pharyngeal endoderm (yellow).

tfap2b is only co-expressed with tfap2a in a restricted domain within the pharyngeal ectoderm. Few studies have addressed AP2 functions in ectoderm as the skin appears to develop normally in Tfap2a/– and Tfap2b/– mutant mice (Schorle et al., 1996). Disruption of tfap2b alone has no effect on zebrafish embryos, but enhances defects in ectodermal cell survival and craniofacial cartilage caused by loss of tfap2a alone, suggesting that the two AP2 proteins can compensate for one another. tfap2a and tfap2b are also co-expressed in the pronephric ducts, and in several regions of the CNS, where they may also be redundant.

Separate requirements for AP2 genes in early versus late NCC development

tfap2a promotes Hoxa2 expression in NCCs that populate the hyoid arch (Maconochie et al., 1999; Knight et al., 2003; Knight et al., 2004). hoxa2 expression may similarly be activated by tfap2b, which shares nearly identical DNA-binding domains and binds a NCC-specific Hoxa2 enhancer, albeit with different affinity (Maconochie et al., 1999). To address this issue in NCCs, we morpholino-depleted tfap2b from low (tfap2a) mutants, examined hoxa2 mRNA, and found that the loss of tfap2b had no effect. This suggests that tfap2b does not compensate for tfap2a in activating hoxa2, but only later in cartilage induction. Our data also argue against a role for AP2 genes in NCC migration. NCCs migrate into all of the arches in the low allele of tfap2a (Knight et al., 2003), though some cells fail to migrate into the hyoid arch in the mob610 allele (Barrallo-Gimeno et al., 2004) and we show that depletion of Tfap2b protein in low does not enhance defects in early markers of migrating NCCs such as hoxa2 and dlx2.

Rather, we argue that tfap2b regulates NCC specification after migration into the arches as cells condense to form cartilage. In contrast to hoxa2, expression of fli1 is reduced in AP2a/b-deficient embryos, compared with low alone, and we used a fli1-GFP transgene to show that this prefigures condensation defects. Later markers of differentiating cartilage, such as gsc and sox9a, are almost completely eliminated in these embryos. Surprisingly, AP2a/b-deficient embryos also have defects in the neurocranium. As both pharyngeal and neurocranial cartilages derive from NCCs (the posterior head skeleton is mesodermal) these results suggest that AP2 genes are required for the development of other NCC-derived cartilages. tfpa2a and tfap2b may be partially redundant in more anterior cartilages of the mandibular arch and neurocranium, which are not disrupted in tfap2a/– mutants alone.

Separate requirements for AP2 genes in NCCs and in surface ectoderm

Tfap2a is required cell-autonomously for NCC specification, prior to migration into the pharyngeal arches (Knight et al., 2003; Knight et al., 2004), and directly regulates hoxa2 and kit transcription (Maconochie et al., 1999). Here, we show an additional non-autonomous requirement for AP2 proteins in the pharyngeal ectoderm. To achieve this, we grafted ectodermal precursors from wild-type donors into either low or AP2a/b-deficient embryos and analyzed the cartilage pattern. Grafts that included pharyngeal ectoderm rescued cartilage unilaterally on the transplanted side, including restoration of dorsal first (palatoquadrate) and second (hyosymplectic) arch cartilages, as well as their separation from the neurocranium. This is a dramatic example of a non-autonomous role for ectoderm in cartilage development and is consistent with previous studies in mice implicating the surface ectoderm in arch patterning (Trumpp et al., 1999; Trokovic et al., 2003; Macatee et al., 2003). A non-autonomous requirement for tfap2a has previously been suggested for NCC-derived melanocytes (O’Brien et al., 2004). These requirements in ectoderm appear to act separately and in parallel to chondrogenic signals from the pharyngeal endoderm (Hall, 1980; David et al., 2002), and AP2-deficient embryos show no defects in endoderm.

Ectodermal signals may also regulate Hox gene expression in the arches and arch identities. Cartilage defects in low (tfap2a) mutant zebrafish resemble homeotic defects caused by loss of Hox group 2 gene function, in which hyoid cartilages are transformed to a mandibular fate (Knight et al., 2004). This could be caused directly by loss of Hoxa2 transcriptional activation by AP2 proteins in NCCs, or indirectly by defects in surrounding tissues. Our results indicate both direct and indirect roles for tfap2a, as ectodermal grafts rescue cartilage formation as well as homeotic transformations of the hyoid skeleton in low mutant embryos (Knight et al., 2004). This suggests that Hox genes initially specify the segmental identities of migrating NCCs, but then require ectodermal signals to maintain this identity (Trainor and Krumlauf, 2000; Schilling et al., 2000). Defects in Hoxa2–/– mutant mice have
been interpreted as intrinsic changes in NCC, but Hoxa2 expression is lost in both ectoderm and NCCs, and the role of the ectoderm has been largely ignored. In Hoxa2 mutants, ectopic expression of mandibular arch genes in the hyoid arch results from defects in reciprocal signals between NCCs and ectoderm (Bobola et al., 2003). Our results suggest that the ectoderm maintains Hox expression in NCCs during migration, and this depends on AP2 genes.

Tcfap2a expression begins during gastrulation in the non-neural ectoderm and persists in the skin throughout embryonic development. We show that AP2-deficient zebrafish have neural ectoderm and persists in the skin throughout embryonic and this depends on AP2 genes.

Fgf functions in establishing pharyngeal arch identity. Hoxa2 represses Fgf activated genes such as Pitx1 and Lhx6 in NCCs of the hyoid arch (Bobola et al., 2003). In Hoxa2+/− mutants, these genes are ectopically expressed and the hyoid arch is transformed to a mandibular fate. We previously described similar transformations in low mutant zebrafish. Our data suggest that these are due to an early function for tcfap2a in regulating the expression of hoxa2 directly in NCCs, while a tfap2b-dependent signal from the ectoderm (possibly an Fgf) is involved only later in chondrogenesis.

**Conserved functions for AP2 transcription factors in vertebrate development**

NCCs arose at the origin of vertebrates and many of the genes involved in NCC patterning evolved from ancestral genes that had more ancient roles in patterning other tissues. In the invertebrate chordate, amphioxus, an AP2 gene is expressed in the non-neural ectoderm, indicating that this is a conserved feature of AP2 genes in all chordates (Meulemans and Bronner-Fraser, 2002). Pharyngeal arches also have an origin prior to that of the NCCs, as evidenced by the presence of branchial gills in amphioxus and appendicularian urochordates. The pharyngeal arches are initially patterned independently of NCCs as shown by NCC extirpations in the chick, in which ectodermal and endodermal patterning is not perturbed (Veitch et al., 1999). Given that AP2 genes have ancient roles in patterning of the ectoderm and that pharyngeal arches arose prior to the NCCs, it is tempting to speculate that AP2 genes were involved in patterning the pharyngeal ectoderm prior to the origin of NCCs. Thus, when NCCs first gained their migratory behavior, they retained some of their identity from their site of origin in the hindbrain through NCC-specific genes, such as tfap2a, but were influenced by signals already present in the arch environment into which they migrated, including those regulated by AP2.

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**References**


