Development 128, 3017-3027 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV3404

Synergy between *Hoxa1* and *Hoxb1*: the relationship between arch patterning and the generation of cranial neural crest

Anthony Gavalas, Paul Trainor*, Linda Ariza-McNaughton and Robb Krumlauf‡

Division of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

*Present address: Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, Missouri 64110, USA

‡Author for correspondence (e-mail: rek@stowers-institute.org)

Accepted 20 May 2001

SUMMARY

***Hoxa1* and *Hoxb1* have overlapping synergistic roles in patterning the hindbrain and cranial neural crest cells. The combination of an ectoderm-specific regulatory mutation in the *Hoxb1* locus and the *Hoxa1* mutant genetic background results in an ectoderm-specific double mutation, leaving the other germ layers impaired only in *Hoxa1* function. This has allowed us to examine neural crest and arch patterning defects that originate exclusively from the neuroepithelium as a result of the simultaneous loss of *Hoxa1* and *Hoxb1* in this tissue. Using molecular and lineage analysis in this double mutant background we demonstrate that presumptive rhombomere 4, the major site of origin of the second pharyngeal arch neural crest, is reduced in size and has lost the ability to generate neural crest cells. Grafting experiments using wild-type cells in cultured normal or double mutant mouse embryos demonstrate that this is a cell-autonomous defect, suggesting that the formation or generation of cranial neural crest has been uncoupled from segmental identity in these mutants. Furthermore, we show that loss of the second arch neural crest population does not have any adverse consequences on early patterning of the second arch. Signalling molecules are expressed correctly and pharyngeal pouch and epibranchial placode formation are unaffected. There are no signs of excessive cell death or loss of proliferation in the epithelium of the second arch, suggesting that the neural crest cells are not the source of any indispensable mitogenic or survival signals. These results illustrate that Hox genes are not only necessary for proper axial specification of the neural crest but that they also play a vital role in the generation of this population itself. Furthermore, they demonstrate that early patterning of the separate components of the pharyngeal arches can proceed independently of neural crest cell migration.**

Key words: Hox genes, Neural crest, Mouse mutants, Hindbrain segmentation, Pharyngeal arches, Cell transposition or grafting, Mouse embryo culture

INTRODUCTION

The neural crest is a transient cell population generated at the border between the neural plate and the presumptive epidermis. Their apposition is thought to induce the specification of neural crest cells (ncc) (Dickinson et al., 1995). Neural crest formation is a multistep process in which signals from both the non-neural ectoderm and non-axial mesoderm are involved (for a review see LaBonne and Bronner-Fraser, 1999). Neural crest cells delaminate from the neural tube and migrate along stereotyped routes to populate diverse regions in the embryo, where they give rise to most of the peripheral nervous system, melanocytes, smooth muscles and also, in the head, craniofacial cartilage and bone (Le Douarin, 1983). Neural crest induction takes place at the same time as anteroposterior (AP) pattering events that generate the nested expression domains of the Hox genes along the axis (Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000a).

The hindbrain is a good model system to study the relationship between both regional specification and neural crest induction. The hindbrain is a segmented structure transiently divided in seven lineage-restricted compartments, termed rhombomeres (r) (Fraser et al., 1990; Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996). The anterior limits of Hox gene expression coincide with rhombomere boundaries and display a two segment periodicity (Hunt et al., 1991a; Keynes and Krumlauf, 1994; Lumsden and Krumlauf, 1996; Wilkinson et al., 1989). The segmented structure of the hindbrain underlies the generation of distinct streams of neural crest cells that populate the pharyngeal arches (pa), which are the main metameric components of the developing head derived from all three germ layers (Noden, 1988). Neural crest cells that populate the first (pa1) and second (pa2) pharyngeal arches originate as distinct streams mainly from r1/r2 and r4, respectively. However, r3 and r5 also contribute a small population of ncc that exit the hindbrain at the junction with even-numbered rhombomeres and join the major streams emanating from the even segments (Golding et al., 2000; Köntges and Lumsden, 1996; Lumsden et al., 1991; Sechrist et al., 1993; Serbedzija et al., 1992). The generation of distinct

ncc streams is attributed to a combination of inter-rhombomeric signalling that leads to cell death in r3 and r5 (Graham et al., 1994; Graham et al., 1993; Graham et al., 1988), exclusion of crest cells from the adjacent mesenchyme (Farlie et al., 1999) and patterning information from the rhombomeres to the adjacent mesenchyme (Golding et al., 2000).

Tissue interactions in the pharyngeal arches are important for proper patterning. The migrating ncc ensheath the cranial paraxial mesoderm in each arch and provide signals that govern muscle identities (Köntges and Lumsden, 1996; Noden, 1983; Noden, 1986; Noden, 1988; Trainor and Tam, 1995; Trainor et al., 1994). Early transplantation experiments suggested that migrating ncc were pre-patterned according to their axial level of origin (Noden, 1983; Noden, 1988), but transplantation of rhombomeric tissue in mouse and chick have revealed the importance of cell community effects and an extensive degree of ncc plasticity in response to mesodermal signals in the arches (Grapin-Botton et al., 1995; Itasaki et al., 1996; Trainor and Krumlauf, 2000a; Trainor et al., 2000). However, ablations of the neural tube in the chick suggest that initial pharyngeal arch patterning may occur in the absence of substantial amounts of ncc (Veitch et al., 1999). Furthermore, studies in chick and mouse have shown that *Hoxa2* expression in the ncc is independently regulated from that in the neural epithelium (Maconochie et al., 1999; Mallo and Brandlin, 1997; Nonchev et al., 1996; Prince and Lumsden, 1994).

Analyses in mice with functional knockouts (KO) of Hox genes have suggested that patterning defects in the neuroepithelium or the pharyngeal arches are to a large extent independent (Rijli et al., 1998). The *Hoxa2* mutation resulted in re-specification of the second arch neural crest towards first-arch identity in the absence of neural epithelial defects in r4, the segment from which this population is derived (Gavalas et al., 1997; Gendron-Maguire et al., 1993; Rijli et al., 1993). Mutation of *Hoxb1* exclusively affected specification of the r4 neuroepithelium, even though *Hoxb1* is also expressed in the neurogenic crest of the second arch (Goddard et al., 1996; Hunt et al., 1991b; Murphy and Hill, 1991; Studer et al., 1996). Hypoplasias of ncc-derived elements in the *Hoxa1* mutants were attributed largely to segmentation defects in the hindbrain that resulted in a dramatically reduced r4 territory and a misplaced otocyst that occluded neural crest migration (Chisaka et al., 1992; Lufkin et al., 1991; Mark et al., 1993). The analysis of combined *Hoxa1* and *Hoxb1* null mutants revealed extensive synergy between the two genes. Prominent among the defects observed was the loss of all second arch-derived elements, owing to neural crest specification defects (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998).

The mechanisms responsible for generating these ncc defects remained unknown and they could be due to effects of the double mutation on any of the components of the pharyngeal arches, the neuroepithelium or a combination thereof. To address this problem, we have taken advantage of a mutation inactivating a 3′ retinoic acid response element (RARE) essential for early expression of *Hoxb1* in neural ectoderm (Marshall et al., 1994; Studer et al., 1998). Both *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ double mutants lose the ncc-derived second arch elements despite their retaining an r4-like territory. However, *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ mutants present additional defects in pharyngeal arch and cranial nerve patterning, suggesting a direct synergistic role of *Hoxa1* and *Hoxb1* in other aspects of pharyngeal arch development beyond ncc patterning itself (Gavalas et al., 1998). In order to exclude indirect effects of the mutant second arch environment on ncc development, we focused our analysis on the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ double mutants. The combination of the *Hoxa1* with the *Hoxb1 3′RARE* mutation resulted in an ectoderm-specific double mutation, leaving the other germ layers impaired only in *Hoxa1* function. As the migration route and gene expression patterns of the r4 ncc are not qualitatively affected in the *Hoxa1* mutants (Mark et al., 1993; Gavalas et al., 1998; this work), the mesodermal and pharyngeal arch environment remain permissive to ncc migration in this mutant background. Therefore, any additional ncc defects observed in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ double mutants should originate at the neuroepithelium itself.

In this study, using molecular and lineage analysis we have demonstrate that while the presumptive r4 territory is reduced but not absent, it is unable to generate ncc in this double mutant background. Grafting experiments in combination with mouse embryo culture also demonstrate that this is a cell-autonomous defect. These results suggest that Hox genes are not simply necessary for proper axial specification of the ncc, but that they also play a vital role in the generation of the ncc population itself.

MATERIALS AND METHODS

Animals and embryo isolation for cultures

Embryos were obtained from 2-hour morning matings (8:00 am-10:00 am) of heterozygous *Hoxa1* mutants and *Hoxa1*$^{+/-}$*/Hoxb1 3′RARE*$^{-/-}$ mutants. The genotype of the embryos was determined using the polymerase chain reaction (PCR). The PCR conditions and primers for the *Hoxb1* 3′RARE mutation were as described (Studer et al., 1998). For the *Hoxa1* mutation, the conditions were the same and the primers used were 5′GCCATTGGCTGGTAGAGTCACGTGT3′ (common to both alleles), 5′GATGGAAGCCGGTCTTGTCGATCA-G3′ (specific for the mutant allele) and 5′CATGGGAGTCGAGAGG-TTTCCAGAG3′ (specific for the wild-type allele), which give rise to a 570 bp product for the wild-type allele and a 700 bp product for the mutated allele. Embryo culture conditions were as described previously (Trainor and Krumlauf, 2000b).

Whole-mount in situ hybridisation and histology

The following mouse cDNA templates were used: Hoxa2, Hoxb2, twist, Sox10, Fgf3, Fgf8, Shh, Bmp7, Ngn2 (Atoh4 – Mouse Genome Informatics), Pax1 and Crabp1. Antisense digoxigenin-labelled riboprobes were synthesised from linearised templates by the incorporation of digoxigenin-labelled UTP (Boehringer) using T3 or T7 or SP6 polymerase. Processing of the embryos and hybridisation with 500 ng ml^{-1} of the probe were as described previously (Gavalas et al., 1998). The preparation of Haematoxylin and Eosin stained sections was carried out using standard histological techniques.

Whole-mount detection of mitosis and apoptosis

Embryos were fixed overnight (o/n) in 4% paraformaldehyde in phosphate-buffered saline (PBS). For whole-mount detection of mitosis, embryos were bleached in 3% H_2O_2 in PBS for 10 minutes and subsequently washed 4×30 minutes in PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (PBST) followed by a 30 minute wash in PBST containing 5% foetal calf serum (PBSTN). Then, embryos were incubated o/n at 4°C with a rabbit polyclonal

antibody raised against the mitosis-specific phosphorylated histone 3 (Upstate Biotechnology Cat no 06-570) diluted 1:500 in PBST. The next day, embryos were washed seven times for 15 minutes each time with PBST and then 30 minutes with PBSTN. Then, the incubation with a goat anti-rabbit horseradish peroxidase-coupled secondary antibody (DAKO) diluted 1:400 in PBSTN was for o/n at 4°C. The following day embryos were washed seven time for 15 minutes each time in PBST and then incubated in 0.3mg/ml diaminobenzidine (DAB) in PBST for 10 minutes. The embryos were transferred in 35 mm diameter petri dishes and the reaction was carried out by incubating them in 0.3 mg/ml DAB/0.03% H_2O_2 in PBS. The reaction was followed in a dissection microscope and once complete (5-10 minutes) embryos were washed thoroughly in PBS, fixed in 4% paraformaldehyde in PBS for 2 hours and cleared o/n in 50% glycerol in PBT. Whole-mount detection of apoptosis was carried out using with the TUNEL method as described previously (Maden et al., 1997) and the final peroxidase reaction was as described above.

Ncc lineage tracing and transplantation experiments

Both techniques were carried out as described earlier (Trainor and Krumlauf, 2000b).

RESULTS

A territory with new characteristics, rx, forms in place of r4 in the *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ mutants

In previous analysis of *Hoxa1* and *Hoxb1* double mutants we found that the territory, which would normally form r4, has been altered in a manner not observed in either single mutant, and fails to express the appropriate r4 molecular markers (Gavalas et al., 1998; Studer et al., 1998). Our analysis of the double mutants also showed that defects in the r4-derived neural crest cells led to involution of the second arch and loss of its mesenchymal derivatives (Gavalas et al., 1998). However, the nature of these neural crest defects was unknown and could be related to processes controlling either the generation or the patterning potential of the second arch ncc. To address this issue, we have used a *Hoxb1 3′RARE*$^{-/-}$ (Studer et al., 1998) mutant to specifically eliminate *Hoxb1* function in ectoderm in a *Hoxa1*$^{-/-}$ genetic background (Lufkin et al., 1991).

The observed loss of second arch neural crest elements in the *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ mutants could be due to a loss of r4, the main territory of origin of second arch ncc (Lumsden et al., 1991; Sechrist et al., 1993). In the *Hoxa1* mutants, r4 is dramatically reduced when compared with wild type (Mark et al., 1993), but it does not lose the capacity to generate ncc (Mark et al., 1993). We examined whether in the double mutants a distinct territory persisted by performing double in situ hybridisation at 8.5 days post coitus (dpc) with riboprobes for *Krox20*, which marks r3 and r5 (Schneider-Maunoury et al., 1993), and *kreisler* (*kr*; Mafb – Mouse Genome Informatics), which marks r5 and r6 (Cordes and Barsh, 1994). A total of seven double mutant embryos were compared with four similarly stained *Hoxa1*$^{-/-}$ embryos and no differences were found in the extent of the territory not expressing *Krox20* and *kr* (Gavalas et al., 1998); compare Fig. 1b with 1c). The boundaries between expressing and non-expressing territories were ill-defined, owing to cell mixing but the presence of non-expressing cells was clear, particularly on the dorsal aspect of the hindbrain where ncc are generated.

We then examined whether this territory persisted at later stages by staining the hindbrains of double mutant and *Hoxa1*$^{-/-}$ embryos with *Hoxa2* and *Hoxb2* riboprobes at 9.5 dpc. We found that, whereas expression of *Hoxb2* is continuous throughout the r3-r6 territories in the wild-type and *Hoxa1*$^{-/-}$ embryos (Fig. 1d,e), there is a clear field of non-expressing cells that corresponds to the presumptive r4 territory in the double mutants. However, this same area expresses *Hoxa2* (Fig. 1j), thus defining a new territory of *Hoxa2*$^{+}$/*Hoxb2*$^{-}$ cells, which we designate as rx. Similar to earlier stages, the boundaries of this territory were ill defined and patches of non-expressing cells could be seen in r3. *Hoxb2*$^{-}$ cells could also

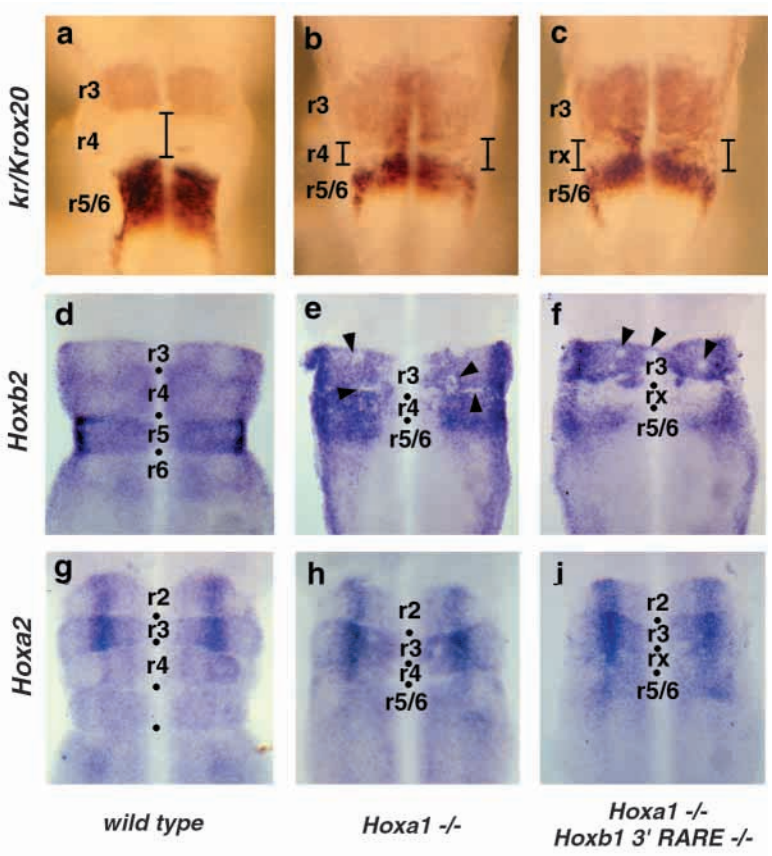

Fig. 1. A territory with new characteristics, rx, is formed in the place of presumptive r4 in the *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ embryos. Wild-type (a,d,g), *Hoxa1*$^{-/-}$ (b,e,h) and *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ (c,f,i) embryos were processed for whole-mount in situ hybridisation with kr/Krox20 (a-c), Hoxb2 (d-f) and Hoxa2 (g-i) antisense riboprobes. Double staining with *kr/Krox20* shows that the r4 territory of wild-type embryos defined by non-expressing cells (vertical bar in a) was significantly reduced but not eliminated, to similar extents in both *Hoxa1*$^{-/-}$ and *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ (vertical bars in b,c, respectively). *Hoxb2* expression is continuous throughout the r3-r6 region in both wild-type and *Hoxa1*$^{-/-}$ embryos (d,e) but not in *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ embryos (f). There are *Hoxb2* non-expressing cells intermingled with r3 cells in both *Hoxa1*$^{-/-}$ and *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ embryos (arrowheads in e,f). Expression of *Hoxa2* is continuous throughout the r2-r6 region in wild-type (g) as well as *Hoxa1*$^{-/-}$ (h) and *Hoxa1*$^{-/-}$/*Hoxb1 3′RARE*$^{-/-}$ (i) embryos.

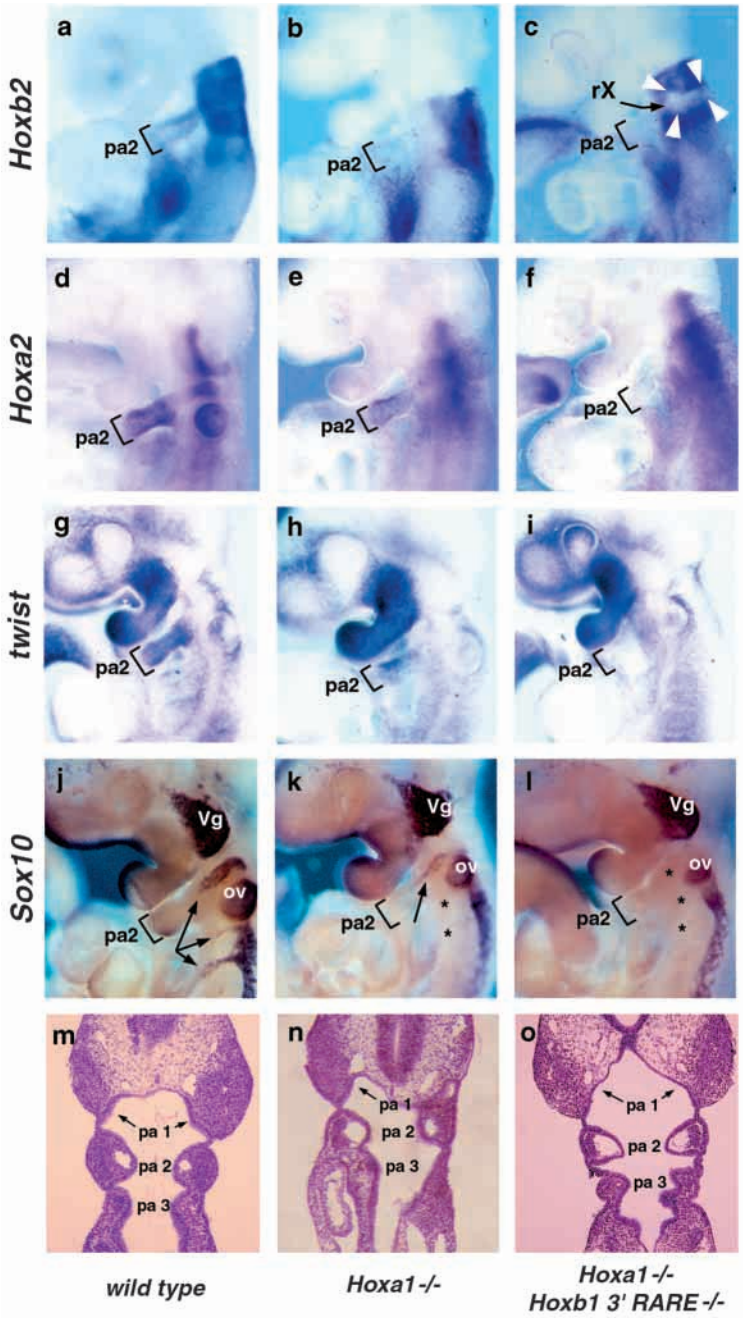

Fig. 2. Gradual loss of neural crest markers and reduction of the second arch mesenchymal population. Wild-type (a,d,g,j,m), *Hoxa1*$^{-/-}$ (b,e,h,k,n) and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (c,f,i,l,o) embryos were processed for whole-mount in situ hybridisation with Hoxb2 (a-c), Hoxa2 (d-f), twist (g-i), Sox10 (j-l) antisense riboprobes and histological staining of paraffin sections (m-o). (a-f) Expression of *Hoxb2* and *Hoxa2* was significantly reduced in the second pharyngeal arch (pa2) of *Hoxa1*$^{-/-}$ embryos (b,e) and completely lost in that of *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos (c,f). Expression of *Hoxb2* in the hindbrain of wild-type and *Hoxa1*$^{-/-}$ embryos was continuous but discontinuous in the hindbrain of *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos, suggesting the presence of a territory distinct from r3 and r5/r6 (noted with white arrowheads in c). (g-i) Expression of the mesenchymal ncc marker *twist* was reduced in the second pharyngeal arch of *Hoxa1*$^{-/-}$ embryos and was lost in that of *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos. (j-l) The neurogenic ncc marker *Sox10* was expressed in the proximal ganglia of wild-type embryos (arrows in j). Its expression was reduced in the facioacoustic ganglion of *Hoxa1*$^{-/-}$ embryos (arrow in k) and was lost in the more posterior ncc-derived ganglia of *Hoxa1*$^{-/-}$ embryos (asterisks in k) and in all the ncc-derived ganglia of *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos (asterisks in l). (m-o) The second pharyngeal arch mesenchymal population was reduced in the *Hoxa1*$^{-/-}$ embryos and further diminished but not extinguished in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos. ov, otic vesicle; Vg, trigeminal ganglion.

be observed interspersed in the r3 of the *Hoxa1*$^{-/-}$ embryos (arrowheads in Fig. 1e,f). *Hoxb2* remained upregulated in the dorsal edge of the rx territory. This does not necessarily represent r3 or r5 cells encroaching upon rx territory, as such upregulation is observed along the length of the caudal hindbrain in both *Hoxa1*$^{-/-}$ and double mutant embryos (Fig. 2e,f). Interestingly, this upregulation does not occur in the wild-type embryos (Fig. 1d) and it may represent an effect of the *Hoxa1* mutation itself.

Neural crest migratory defects and pharyngeal arch specification defects

To examine whether this novel rx territory is able to generate ncc, we have performed molecular analysis using general neural crest markers, as well as markers specifically expressed in the neurogenic and mesenchymal ncc.

Hoxa2 and *Hoxb2* are expressed in the migrating neural crest of the second arch and are the only Hox genes to be expressed in its mesenchyme (Hunt et al., 1991a; Hunt et al., 1991b). At 9.5 dpc, both genes are expressed in a continuous stream from r4 to the distal tip of the second arch (Fig. 2a,d). In the *Hoxa1*$^{-/-}$ mutants, expression of *Hoxa2*, as well as *Hoxb2*, is reduced in both the migrating neural crest and the mesenchyme of the arch (Fig. 2b,e). However, expression of both genes is completely abolished in the second arch of the double mutants (Fig. 2c,f). These results closely parallel the observed changes in the expression of *AP-2* and *Crabp1* (Gavalas et al., 1998). Interestingly, in the hindbrain of the double mutants expression of *Hoxb2* but not *Hoxa2* was lost in presumptive r4 (Fig. 2c,f). This further illustrates the existence of a territory (rx) positioned between the *Hoxb2*-expressing r3 and r5/r6 territories (Fig. 2c).

Twist encodes a basic helix-loop-helix transcription factor expressed in high levels in the mesenchyme of the pharyngeal arches and regulates the cellular phenotype and behaviour of head mesenchyme cells (Chen and Behringer, 1995). Twist expression is confined mainly at the periphery of the arch core (data not shown), suggesting that it marks mesenchymal ncc. The robust second arch expression seen in wild-type embryos (Fig. 2g) was reduced in *Hoxa1*$^{-/-}$ mutants (Fig. 2h) and it was completely abolished in the second arch of the double mutants (Fig. 2i).

Sox10 is an HMG-box transcription factor expressed in the neural crest cells that contribute to the peripheral nervous system (Kuhlbrodt et al., 1998). At 9.5 dpc, *Sox10* is expressed in all neural crest-derived ganglia of the cranial nerves (Fig. 2j). Consistent with a general reduction of the second arch

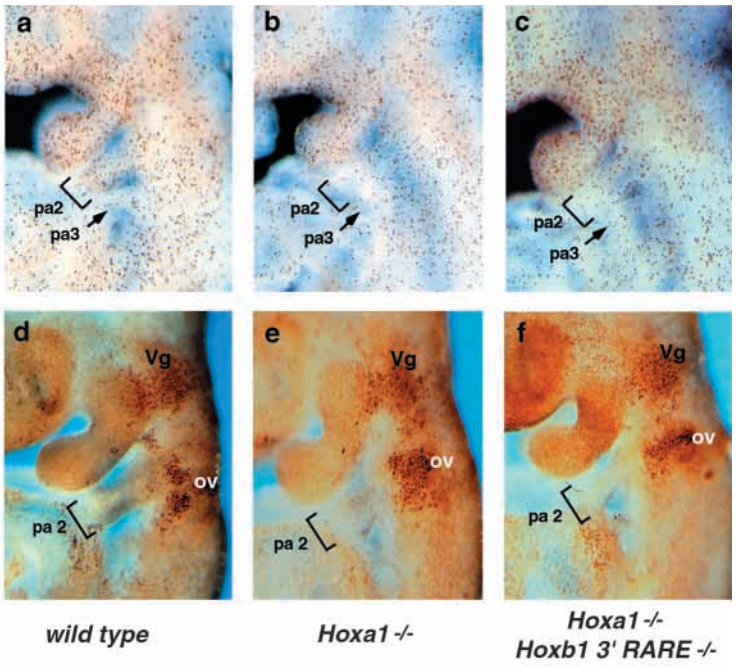

Fig. 3. Mitotic and cell death patterns in the second pharyngeal arch are not altered despite the loss of ncc. Wild-type (a,d), *Hoxa1*$^{-/-}$ (b,e) and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (c,f) embryos were processed for whole-mount immunohistochemical detection of mitosis (a-c) and cell death (d-f). (a-c) Reduced but considerable mitotic activity was still detected in the second pharyngeal arch (pa2) of both *Hoxa1*$^{-/-}$ and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos. (d-f**)** There was no increase of cell death in the second pharyngeal arch of either *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/ Hoxa1*$^{-/-}$ *Hoxb1 3′RARE*$^{-/-}$ embryos.

neural crest cell population in the *Hoxa1* mutants (Mark et al., 1993), expression of *Sox10* was reduced in the facioacoustic ganglion of the second arch (Fig. 2k, arrow). Furthermore, *Sox10* expression was totally abolished in the territories of the neural crest derived superior ganglia of nerves IX and X. This suggests that the loss of the proximal part of these nerves in the *Hoxa1* mutants arises owing to a defect in this population. In the double mutants, *Sox10* expression in the facioacoustic ganglion of the second arch was completely abolished (Fig. 2l).

The specific loss of expression of all neural crest markers examined in pa2 suggested that neural crest cells emanating from the normal presumptive r4 territory (rx) of the double mutants either failed to emigrate and populate the second arch, or were rapidly eliminated by cell death. Consistent with this idea, histological sections through the arch region of wild-type, *Hoxa1*$^{-/-}$ and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos show that despite the reduction (*Hoxa1*$^{-/-}$) or loss of the second arch ncc (double mutants), mesodermal cells could still migrate and populate pa2 (Fig. 2m-o).

Neural crest cells are not required for early survival and proliferation of pa2

In view of the apparent absence or elimination of migratory ncc moving into the second arch, we examined whether this could be manifested by ectopic cell death and/or proliferative defects during early arch development. We investigated cell proliferation by whole-mount immunohistochemistry using a polyclonal antibody against the phosphorylated form of histone 3 (H3), which is present only in mitotic cells (Hendzel et al.,

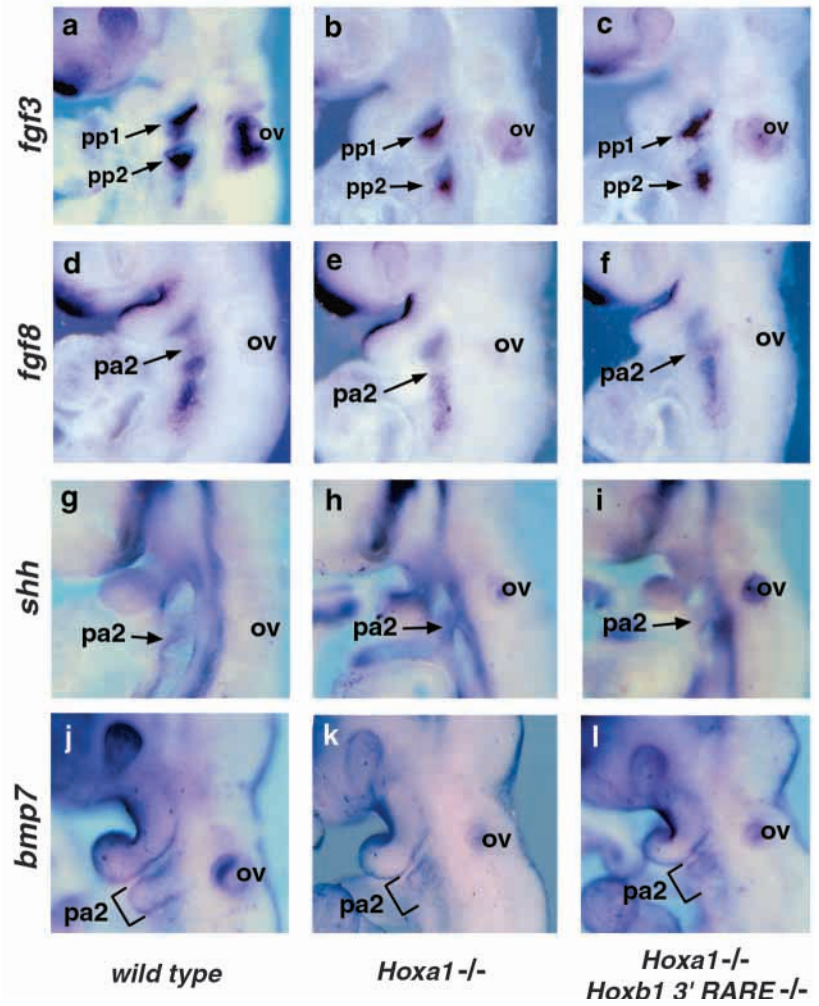

Fig. 4. Signalling molecules are correctly expressed in the second pharyngeal arch despite the loss of ncc. Wild-type (a,d,g,j), *Hoxa1*$^{-/-}$ (b,e,h,k) and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (c,f,i,l) embryos were processed for whole-mount in situ hybridisation with Fgf3 (a-c), Fgf8 (d-f), Shh (g-i) and Bmp7 (j-l) antisense riboprobes. (a-c), Expression of *Fgf3* in the first and second pharyngeal pouches (pp1 and pp2, respectively) was not changed in either *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos (arrows). (d-f) The weak expression of *Fgf8* on the second pharyngeal arch (pa2) was not abolished in either *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos (arrows). (g-i) Expression of *Shh* in the second pharyngeal arch (pa2) was not abolished in either *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos (arrows in). (j-l) Similarly, expression of *Bmp7* in the second pharyngeal arch (pa2) was not abolished in either *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos. ov, otic vesicle.

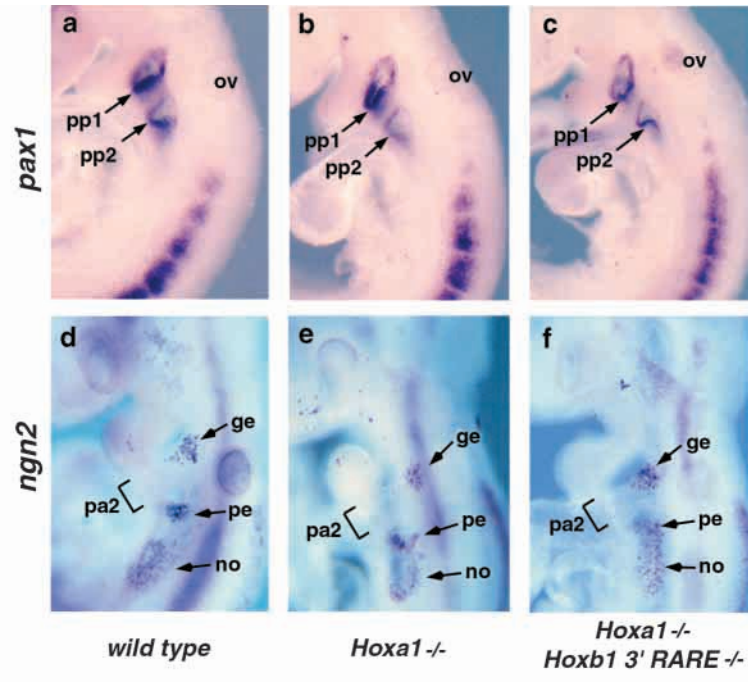

Fig. 5. Pharyngeal pouches and epibranchial placodes develop normally despite the loss of the second arch ncc. Wild-type (a,d), *Hoxa1*$^{-/-}$ (b,e) and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (c,f) embryos were processed for whole-mount in situ hybridisation with Pax1 (a-c) and Ngn2 (d-f) antisense riboprob*es*. (a-c) Expression of *Pax1* in the first and second pharyngeal pouches (pp1 and pp2 respectively) was not changed in either *Hoxa1*$^{-/-}$ *or Hoxb1 3′RARE*$^{-/-}$ embryos (arrows). (d-f) Expression of *Ngn2* in the second arch (pa2) geniculate (ge) and more posterior petrosal (pe) and nodose (no) ganglia revealed that their initial stages of development were not affected in either *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos (arrows).

1997). Interestingly, we found that proliferation in the second arch of both *Hoxa1* and *Hoxa1/Hoxb1 3′RARE* mutant embryos was reduced but not eliminated (Fig. 3a-c). This reduction in number of mitotic cells in the mutants was consistent with the smaller mesenchymal population in pa2. This demonstrates that even in the absence of ncc there are still mitotically active cells in pa2.

Next, we investigated whether excessive cell death occurred in association with the absence of detectable neural crest cells. TUNEL whole-mount staining revealed that patterns of cell death in both single and double mutant backgrounds were remarkably similar to those of pa2 in wild-types embryo (Fig. 3d-f). The only detectable difference in the patterns of cell death in the mutants was seen around the otic vesicle, consistent with the underdevelopment of the otic vesicle in these mutants (Gavalas et al., 1998; Mark et al., 1993). Therefore, absence of gene expression in markers of migratory second arch ncc is not associated with a major change in mitogenic or survival signals within pa2.

Patterning in the arch epithelium does not depend on the presence of ncc

The above results suggest that in the absence of neural crest components other aspects of early arch patterning may not be affected. We examined a number of signalling molecules that

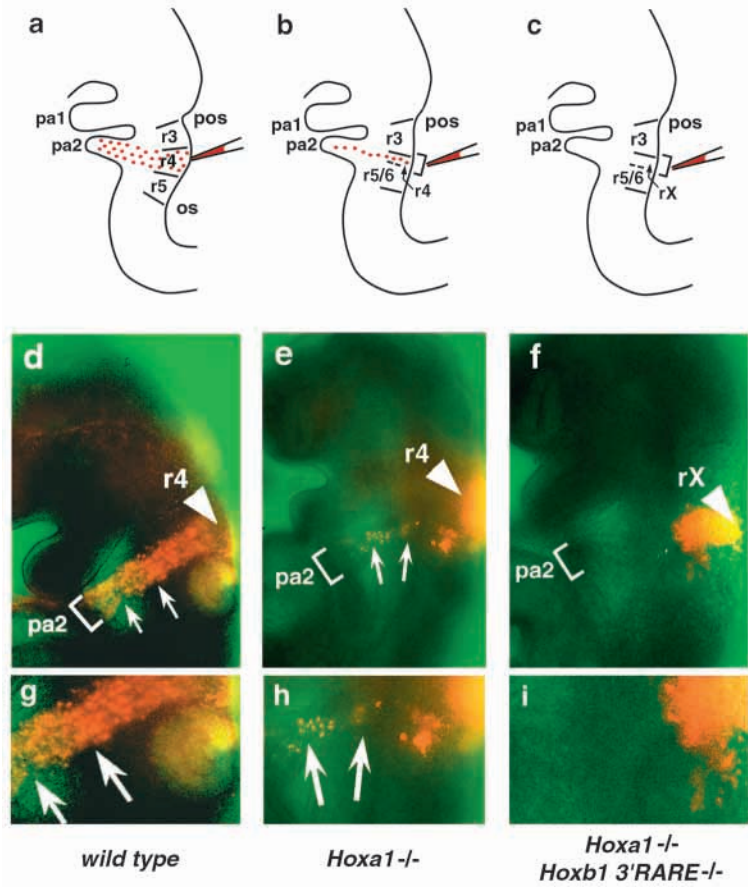

Fig. 6. Lineage analysis of the r4 cells confirms the lack of migrating ncc in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ mutants. Diagrammatic representation of the experimental procedure (a-c) and the results (d-i). Wild-type (a,d,g), *Hoxa1*$^{-/-}$ (b,e,h) and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (c,f,i) embryos are labelled with DiI at the presumptive r4 or rx territory (arrowheads in d-f) at the five-somite stage, left in culture for 30-32 hours and examined under fluorescence for ncc migratory patterns (arrows in d,e,g,h). The area of pa2 is shown in g-i under high-power magnification. Ncc migration is reduced in the *Hoxa1*$^{-/-}$ embryos and completely abolished in *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ embryos. The segmentation defects of *Hoxa1*$^{-/-}$ or *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ result in a less conspicuous preotic sulcus (pos in a-c) and an imperceptible otic sulcus (os in a-c). Brackets in b,e denote the area of the neuroepithelium that was labelled with DiI; brackets in d-f denote the pa2 neural crest migration domain. pa1 and pa2, pharyngeal arches 1 and 2, respectively.

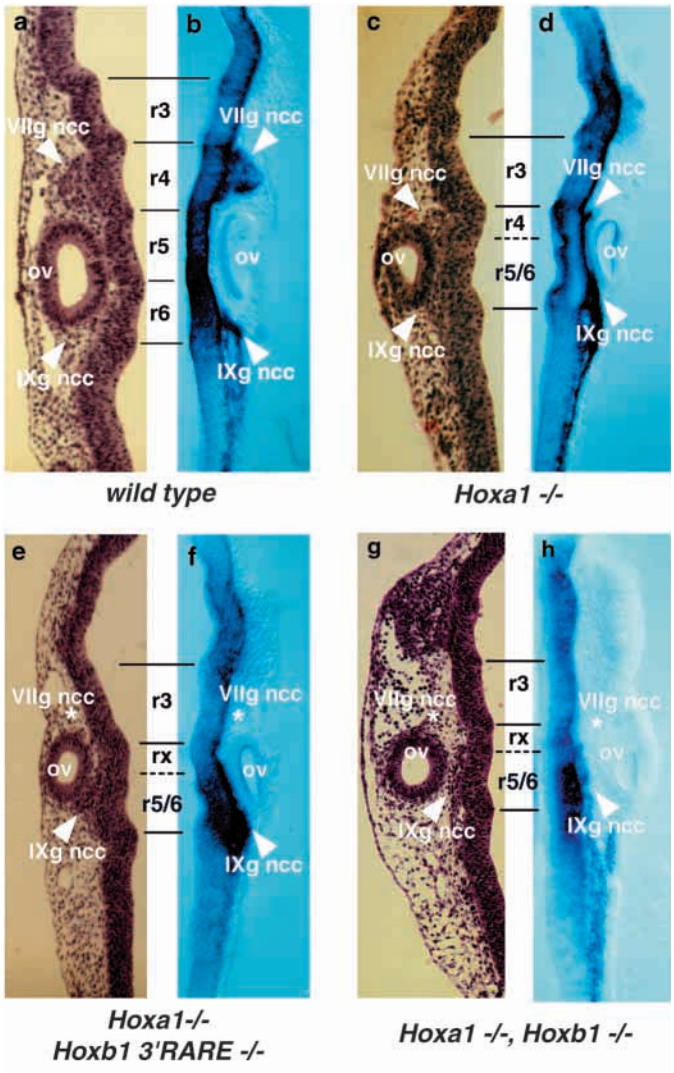

Fig. 7. Reduction of second pharyngeal arch delaminating ncc in the *Hoxa1*$^{-/-}$ and its loss in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ and *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ mutants. Wild-type (a,b), *Hoxa1*$^{-/-}$ (c,d), *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (e,f) and *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ (g,h) Haematoxylin/Eosin stained paraffin sections (a,c,e,g) or CRABPI antisense riboprobe stained vibratome sections (b,d,f,h) at the dorsal hindbrain level. Delaminating ncc of the VII and IX ganglia can be seen in the wild-type hindbrains (arrowheads in a,b) anteriorly and posteriorly, respectively, of the otic vesicle (ov). The delaminating population anterior of the otic vesicle is greatly reduced in the *Hoxa1*$^{-/-}$ mutants (arrowheads in c,d) and completely lost in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (e,f) and *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ mutants (g,h) (asterisks). The IXg ncc population still delaminates in all the mutants examined (arrowheads in c-h).

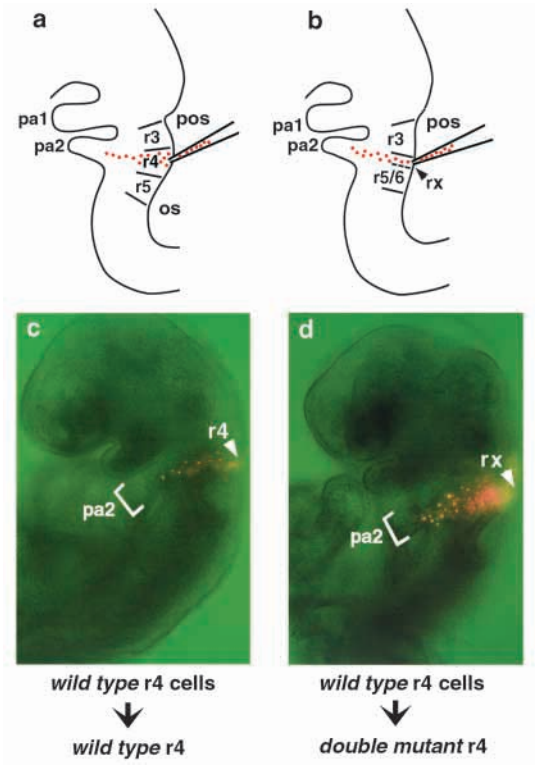

Fig. 8. Transplantation experiments demonstrate that the neural crest defect in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ mutants is cell-autonomous. Diagrammatic representation (a,b) of the experimental procedure and the results (c,d). DiI-labelled wild-type r4 cells were transplanted in the r4 of wild-type (a,c) or rx of*Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (b,d) recipient embryos at the five-somite stage. The embryos were cultured for 24 hours and then observed under fluorescence for migration patterns. The wild-type cells are able to migrate correctly into the second pharyngeal arch (pa2) of both wild-type (c) and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ (d) embryos, demonstrating that the ncc defect in the latter is cell-autonomous. os, otic sulcus; pos, preotic sulcus.

are expressed in the surface ectoderm and pharyngeal endoderm encapsulating the arch mesenchyme (Francis-West et al., 1998; Rijli et al., 1998), as reduction or changes in their expression could contribute to the developmental defects found in these mutants. At 9.25 dpc, fibroblast growth factor 3 (*Fgf3*) showed restricted strong expression in part of the second pharyngeal pouch and weak expression in the surface ectoderm of the second arch (Fig. 4a; Mahmood et al., 1995; Wilkinson et al., 1988). This expression pattern did not change appreciably in either *Hoxa1* or *Hoxa1/Hoxb1 3′RARE* mutants (Fig. 4b,c). At the same stage *Fgf8* was also expressed in the surface ectoderm of the second arch (Fig. 4d; Heikinheimo et al., 1994) and this expression was not affected in either of the two mutants examined (Fig. 4e,f). Targeted inactivation of *Shh* in the mouse resulted in cyclopia and loss of pharyngeal arch structures (Chiang et al., 1996), whereas sonic hedgehog (Shh) inhibition in the head mesenchyme lead to loss of pharyngeal arch structures (Ahlgren and Bronner-Fraser, 1999). Therefore, we examined *Shh* expression in the mutants but no changes were detected in either *Hoxa1* or *Hoxa1/Hoxb1 3′RARE* mutants (Fig. 4g-i). At 9.5 dpc, expression of bone morphogenetic protein 7 (*Bmp7*) extended over the whole arch and this generalised expression persisted in both types of mutants examined (Fig. 4j-l).

Next, we examined the expression of pharyngeal pouch and placode specific markers. *Pax1* belongs to the *Pax1/Pax9* subfamily of paired homeodomain transcription factors (Muller et al., 1996; Peters et al., 1998) and at 9.25 dpc specifically labelled the endoderm of first and second pharyngeal pouches (Fig. 5a). *Pax1* expression in the mutants was indistinguishable from that of the wild-type embryos (Fig. 5a-c). The epibranchial placodes are formed by local

thickenings of the surface ectoderm and they give rise to all the cells of the inferior ganglia of the cranial nerves (Le Douarin et al., 1986). *Ngn2* is a bHLH transcription factor necessary for determination of the epibranchial placodes (Fode et al., 1998). We found that all ganglia derived from epibranchial placodes, including the second arch geniculate ganglion, expressed *Ngn2* and were formed correctly in both *Hoxa1* and *Hoxa1/Hob13′RARE* mutants (Fig. 5d-f). Taken together, these results suggest that important aspects of the signalling and patterning in the second arch have not been altered in the *Hoxa1/Hoxb1 3′RARE* mutants.

Lineage analysis of neural crest cell migration in *Hox* mutant embryos

As early arch patterning is not affected in the double mutants and we found no evidence for increased cell death, we re-examined whether any neural crest cells that were not detected by molecular markers were migrating from the r4-like territory. To address this problem, we took advantage of our previous fate map of cranial regions (Trainor and Tam, 1995) and performed lineage analysis of neural crest cells migrating from the future r4 or rx territory of cultured wild-type and mutant mouse embryos. At 8.25 dpc (five somite embryos) the pre-rhombomeric territories in wild-type embryos can be distinguished based on the position of the preotic (pos) and otic sulci (os) (Fig. 6a). DiI injections into the dorsal region of future r4 in wild-type embryos, followed by embryo culture for 30-32 hours, revealed extensive labelling of cells emigrating from r4 into pa2 (Fig. 6d).

In the *Hoxa1* and *Hoxa1/Hoxb1RARE* mutants, the presumptive r4 (rx) territory is reduced in size, the pos is significantly smoother and the os has all but disappeared. Therefore, to investigate cell migration in the Hox mutants, DiI injections were made in a broad enough region to cover the presumptive r4 (rx) territory and the edges of neighbouring territories r3 and r5/6 (area labelled is denoted by brackets in Fig. 6b,c). This approach ensures that despite the loss of morphological landmarks, the r4 (rx) territories and the neighbouring r3 and r5 populations, which (in the wild-type) contribute to the ncc populating pa2 (Sechrist et al., 1993), will be labelled. In the *Hoxa1* mutants, neural crest cells migrated into pa2, but they were greatly reduced in number compared with wild type (compare Fig. 6d,g with Fig. 6e,h). In contrast, lineage analysis in the double mutants demonstrated the complete absence of migratory cells derived from rx (Fig. 6f,i). The reduction in migratory cells in the *Hoxa1* mutants and their absence in the double mutants directly correlated with the changes in the expression patterns of neural crest molecular markers. Together, these results strongly suggest that the rx territory in the *Hoxa1/Hoxb1 3′RARE* mutant background has lost the ability to generate migratory neural crest cells.

Neural crest cells fail to delaminate from the presumptive r4 territory

The loss of migrating ncc into the second arch could result either from a failure to delaminate or from trapping of the cells in the adjacent mesenchyme. To distinguish between the two possibilities, we examined histological sections of hindbrain preparations. In wild-type embryos at 9.25 dpc, delaminating ncc can be seen both histologically (Fig. 7a) and as *CrabpI*-expressing cells in two distinct streams adjacent to r4 and r6 (Fig. 7b). In the *Hoxa1* mutants, we detected r4- and r6-derived streams of emigrating ncc (Fig. 7c,d). In the caudal hindbrain the streams are continuous, owing to the drastic reduction of the r5 territory, and delaminating cells can be seen both anterior and posterior to the otic vesicle (Fig. 7c,d; Mark et al., 1993). In contrast, in the *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ double mutants, there are no delaminating cells from the presumptive r4 (rx) territory (Fig. 7e,f). This situation is very similar to that observed in the *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ full double mutants (Fig. 7g,h), suggesting that this is a defect arising in the neuroepithelium itself.

The neural crest migratory defects are cell autonomous

The observation that no ncc are delaminating from the hindbrain at the level of the presumptive r4 could arise through either an intrinsic defect in the ability of the neuroepithelium to generate migrating ncc or a non-permissive mesenchymal environment. The latter possibility appears less likely as the *Hoxb1 3′RARE* mutation specifically eliminates function in the neural ectoderm and these defects are not seen in the *Hoxa1* mutation alone. To directly investigate whether these defects are intrinsic to the neuroepithelial cells, we performed cell transposition experiments in wild-type and double mutant embryos. Although it would have been desirable to transpose rx cells into wild-type embryos to test their potential, the lack of segmentation and morphological landmarks, and the small size of the territory rx preclude the reliable isolation of rx cells (and, thus, this experiment). Therefore, DiI labelled r4 cells from wild-type embryos were orthotopically grafted into either dorsal r4 of wild type-embryos or the rx region of double mutant embryos at 8.25 dpc (Fig. 8a,b). The migration patterns of these labelled wild-type r4 cells following transplantation into rx or r4 were examined after 24 hours in culture. In both wild-type and *Hoxa1/Hoxb1 3′RARE* embryos we observed that the grafted cells generated ncc that was able to migrate in the second arch (Fig. 8c,d). This shows that the arch environment in the double mutant embryos is permissive for neural crest cell migration, strongly suggesting that the failure of the neuroepithelium to generate migrating neural crest is caused by cell autonomous defects.

DISCUSSION

The combined loss of function of *Hoxa1* and *Hoxb1* results in loss of second pharyngeal arch derivatives and neural crest specification defects (Gavalas et al., 1998). The loss of these structures could have arisen not only from failure of the ncc to migrate or integrate properly in the arch environment, but also from patterning defects of the arch mesoderm and epithelium. In this study, we addressed this issue genetically by introducing the *Hoxb1 3′RARE* mutation in the *Hoxa1* mutant background, which results in loss of function of both *Hoxa1* and *Hoxb1* only in the neural ectoderm. As *Hoxa1*$^{-/-}$*/Hoxb1*$^{-/-}$ mutants have additional arch and cranial nerve defects, compared with *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ mutants (Gavalas et al., 1998), this genetic approach allows us to eliminate defects in the arch environment that indirectly affecting ncc patterning. Using a combination of molecular markers, lineage analysis and cell grafting, we demonstrated that the neuroepithelium of these

mutants had cell-autonomous defects that prevent the presumptive r4 (rx) territory from generating neural crest cells. This does not prevent correct early arch patterning from taking place, and suggests that these processes can be uncoupled in vertebrates. Furthermore, our observations imply that Hox genes are not only involved in regulating the specification of axial (AP) identities in the hindbrain and neural crest tissues, but also in controlling the generation of cranial neural crest cells. These results raise seceral interesting questions with respect to the way in which these processes are related.

Neural crest formation versus AP identity

Simultaneous loss of *Hoxa1* and *Hoxb1* function does not eliminate the presumptive r4 (rx) territory; however, its precise AP identity is unknown. This cell population does express *Hoxa2,* but not *Krox20*, *kr* or *Hoxb2* (Gavalas et al., 1998; Studer et al., 1998). This suggests that rx may have a r2-like character, which is in line with the previous findings that r4 adopts an r2-like identity in single *Hoxb1* mutants (Goddard et al., 1996; Studer et al., 1996). However, as demonstrated in this paper, unlike r2, rx in the double mutants lacks the ability to generate migratory neural crest cells. One explanation would be that cells in this territory have been locked in an immature neuroepithelial state; however, we have previously found that motoneurones can be generated from these cells and send projections into the periphery in a even-like segmental pattern (Gavalas et al., 1998). Therefore, in this double mutant context where *Hoxa1* and *Hoxb1* have been jointly eliminated from only the neuroepithelium, it appears that the formation or generation of cranial neural crest has been uncoupled from segmental identity. Neural crest is still generated at more posterior levels, most probably due to functional compensation from other Hox genes.

The functional loss of *Hoxa1* and *Hoxb1* may have interfered with neural crest induction by affecting the competence of ectodermal cells to respond to neural crest-inducing signals such as Bmps and Wnts. Bmp signalling is indispensable for ncc generation, as the combination of *Bmp5* and *Bmp7* mutations (Solloway and Robertson, 1999), and interference with Bmp signalling resulted in severe depletion of ncc (Kanzler et al., 2000; Sela-Donenfeld and Kalcheim, 2000). Wnt signalling is required for maintenance and/or proliferation of neural crest, as shown by the effects of the combined *Wnt1* and *Wnt3a* mutations (Ikeya et al., 1997). Expression of genes specifically involved in neural crest generation such as noelin 1 may also have been affected (Barembaum et al., 2000). Alternatively neural crest cells may have been induced, but be unable to go through the epithelial-to-mesenchymal transition. This may implicate Hox genes in the switch to the expression of migration-specific cadherins and Rho-family GTPases necessary for this process (Liu and Jessell, 1998; Takeichi, 1995).

Neural crest cells and the arch environment

Surprisingly, both the molecular markers and DiI lineage analysis indicates that in the mutants there is no ncc in-filling from adjacent rhombomeres. This is in contrast to the findings in the chick that physical ablation of this population can be compensated for by in-filling of surrounding neural crest populations (Couly et al., 1996; Hunt et al., 1995; Saldivar et al., 1997). This difference may be due to intrinsic species-specific differences or the in-filling may be a consequence of the experimental procedure itself. Alternatively, head mesenchyme adjacent to the hindbrain can form exclusion zones that help guide ncc in forming distinct migratory streams (Farlie et al., 1999; Golding et al., 2000). As a result of the combined mutations, it could be argued that this exclusion zone has expanded, precluding rx ncc migration. However, the finding that wild-type cells when transplanted into the presumptive r4 territory of the double mutant embryos can generate neural crest cells that migrate into pa2, excludes this possibility.

Fate-mapping studies in the chick have suggested that the odd-numbered rhombomeres contribute ncc to the main streams emanating from the even-numbered rhombomeres (Sechrist et al., 1993). In the double mutants examined here, based on both the molecular and cell-tracing analysis, r3 is unable to contribute ncc that will migrate in the second arch. This suggests that r3 in the mouse does not generally contribute appreciable amounts of ncc to the second arch. However, there are patterning defects in r3 of both *Hoxa1*$^{-/-}$ and *Hoxa1*$^{-/-}$*/Hoxb1 3′RARE*$^{-/-}$ mutant embryos (Gavalas et al., 1998; Helmbacher et al., 1998), which most probably result from intermingling of misspecified r4 cells with the r3 territory. This may alter the normal ability of r3 to contribute ncc to the second arch.

In the chick, after extensive deletion of the neural tube, arch patterning appears to be unaffected (Veitch et al., 1999). However, the lack of DiI labelling in these experiments left open the possibility of limited ncc in-filling from anterior and posterior regions of the neural tube. Our demonstration of a complete loss of the second arch neural crest cell population has allowed us to assess the direct role of these cells on arch patterning in the mouse. We found that, in its absence, there is no excessive cell death or arrest of proliferation in the arch, implying that this population is not the source of indispensable survival or mitogenic signals. Expression of signalling molecules in the arch epithelium was not affected. The presence of mesoderm/mesenchyme in pa2 in the absence of neural crest cell migration from rx also implies that ncc are not essential for their migration into the arches. Furthermore pouch formation and ectodermal placode development were normal, highlighting the fact that ncc are not essential for these events. As these processes depend upon proper endoderm patterning (Begbie et al., 1999), it appears that endoderm is relatively normal in the double mutants examined here.

This does not imply that *Hoxa1* and *Hoxb1* do not have normal roles in endoderm patterning, as they are expressed in this tissue (Hunt et al., 1991b; Murphy and Hill, 1991). However, the *Hoxb1 3′RARE* mutation in the neural enhancer flanking this gene that we used in this study, specifically eliminated expression from only the ectoderm and not the endoderm (Marshall et al., 1994; Studer et al., 1998), which is regulated by a separate control element (Huang et al., 1998). Importantly, endoderm patterning and *Hoxa1* and *Hoxb1* expression are altered when mouse embryos are treated with a retinoid antagonist, suggesting that these genes do have a role in early endoderm patterning (Wendling et al., 2000).

In conclusion, it is becoming increasingly evident that arch development involves the formation of main components, which are initially patterned in a relatively independent manner, and that subsequent growth and differentiation is

tightly linked to the integration of tissue interactions between these components. This is consistent with the well-established instructive roles of neural crest cells in patterning muscle tissue (Köntges and Lumsden, 1996; Noden, 1988). How the formation and early patterning of the pharyngeal arches are coupled to the regulation of ncc formation and segmental identity will be an important issue to be addressed.

For gifts of plasmids we thank P. Charnay (*Krox20*), G.S. Barsh (*kr*), M. Wegner (*Sox10*), F. Perrin-Schmitt (*twist*), S. Mansour (*Fgf3)*, G.R. Martin (*Fgf8)*, A. McMahon (*Shh*, *Bmp7*), F. Guillemot (*Ngn2)*, P. Gruss (*Pax1*) and P. Chambon (*Crabp1*). We are grateful to P. Chambon for providing the *Hoxa1* mouse line. We also thank Helena Grigorieva for the histology and the staff of the NIMR animal house for mouse husbandry. A. G. was supported by an EMBO postdoctoral fellowship, and P. T. by EMBO and HFSP postdoctoral fellowships. This work was funded by Core MRC Programme support to R. K. Correspondence and requests for materials should be addressed to R. K.

REFERENCES

Ahlgren, S. C. and Bronner-Fraser, M. (1999). Inhibition of sonic hedgehog signaling in vivo results in craniofacial neural crest cell death. *Curr. Biol.* **9**, 1304-1314.

Barembaum, M., Moreno, T. A., LaBonne, C., Sechrist, J. and Bronner-Fraser, M. (2000). Noelin-1 is a secreted glycoprotein involved in generation of the neural crest. *Nat. Cell Biol.* **2**, 219-225.

Begbie, J., Brunet, J. F., Rubenstein, J. L. and Graham, A. (1999). Induction of the epibranchial placodes. *Development* **126**, 895-902.

Chen, Z. F. and Behringer, R. R. (1995). twist is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9**, 686-699.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Chisaka, O., Musci, T. and Capecchi, M. (1992). Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6*. *Nature* **355**, 516-520.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-1034.

Couly, G. F., Grapin-Bottom, A., Coltey, P. and Le Douarin, N. M. (1996). The regeneration of the cephalic neural crest, a problem revisited: the regenerating cells originate from the contralateral or from the anterior and posterior neural folds. *Development* **122**, 3393-3407.

Dickinson, M. E., Selleck, M. A., McMahon, A. P. and Bronner-Fraser, M. (1995). Dorsalization of the neural tube by the non-neural ectoderm. *Development* **121**, 2099-2106.

Farlie, P. G., Kerr, R., Thomas, P., Symes, T., Minichiello, J., Hearn, C. J. and Newgreen, D. (1999). A paraxial exclusion zone creates patterned cranial neural crest cell outgrowth adjacent to rhombomeres 3 and 5. *Dev. Biol.* **213**, 70-84.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. and Guillemot, F. (1998). The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20**, 483-494.

Francis-West, P., Ladher, R., Barlow, A. and Graveson, A. (1998). Signalling interactions during facial development. *Mech. Dev.* **75**, 3-28.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.

Gavalas, A., Davenne, M., Lumsden, A., Chambon, P. and Rijli, F. (1997). Role of *Hoxa-2* in axon pathfinding and rostral hindbrain patterning. *Development* **124**, 3693-3702.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F., Krumlauf, R. and Chambon, P. (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). Hoxa-2 mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Goddard, J., Rossel, M., Manley, N. and Capecchi, M. (1996). Mice with targeted disruption of *Hoxb1* fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217-3228.

Golding, J., Trainor, P., Krumlauf, R. and Gassman, M. (2000). Defects in pathfinding by cranial neural crest cells in mice lacking the Neuregulin receptor ErbB4. *Nat. Cell Biol.* **2**, 103-109.

Graham, A., Heyman, I. and Lumsden, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119**, 233-245.

Graham, A., Papalopulu, N., Lorimer, J., McVey, J., Tuddenham, E. and Krumlauf, R. (1988). Characterization of a murine homeo box gene, *Hox 2.6*, related to the *Drosophila deformed* gene. *Genes Dev.* **2**, 1424-1438.

Graham, A., Francis-West, P., Brickell, P. and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684-686.

Grapin-Botton, A., Bonnin, M.-A., Ariza-McNaughton, L., Krumlauf, R. and LeDouarin, N. M. (1995). Plasticity of transposed rhombomeres: Hox gene induction is correlated with phenotypic modifications. *Development* **121**, 2707-2721.

Heikinheimo, M., Lawshe, A., Shackleford, G., Wilson, D. B. and MacArthur, C. A. (1994). *Fgf-8* expression in the post-gastrulation mouse suggests roles in the development of face, limbs and central nervous system. *Mech. Dev.* **48**, 129-138.

Helmbacher, F., Pujades, C., Desmarquet, C., Frain, M., Rijli, F., Chambon, P. and Charnay, P. (1998). *Hoxa1* and *Krox20* synergize to control the development of rhombomere 3. *Development* **125**, 4739-4748.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.

Huang, D., Chen, S., langston, A. and Gudas, L. (1998). A conserved retinoic acid responsive element in the murine *Hoxb-1* gene is required for expression in the developing gut. *Development* **125**, 3235-3246.

Hunt, P., Gulisano, M., Cook, M., Sham, M., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991a). A distinct *Hox* code for the branchial region of the head. *Nature* **353**, 861-864.

Hunt, P., Wilkinson, D. and Krumlauf, R. (1991b). Patterning the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating neural crest. *Development* **112**, 43-51.

Hunt, P., Ferretti, P., Krumlauf, R. and Thorogood, P. (1995). Restoration of normal Hox code and branchial arch morphogenesis after extensive deletion of hindbrain neural crest. *Dev. Biol.* **168**, 584-597.

Ikeya, M., Lee, S. M., Johnson, J. E., McMahon, A. P. and Takada, S. (1997). Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* **389**, 966-970.

Itasaki, N., Sharpe, J., Morrison, A. and Krumlauf, R. (1996). Reprogramming *Hox* expression in the vertebrate hindbrain: Influence of paraxial mesoderm and rhombomere transposition. *Neuron* **16**, 487-500.

Kanzler, B., Foreman, R. K., Labosky, P. A. and Mallo, M. (2000). BMP signaling is essential for development of skeletogenic and neurogenic cranial neural crest. *Development* **127**, 1095-1104.

Keynes, R. and Krumlauf, R. (1994). Hox genes and regionalization of the nervous system. *Annu. Rev. Neurosci.* **17**, 109-132.

Köntges, G. and Lumsden, A. (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.

Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M. (1998). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18**, 237-50.

LaBonne, C. and Bronner-Fraser, M. (1999). Molecular mechanisms of neural crest formation. *Annu Rev Cell Dev Biol* **15**, 81-112.

Le Douarin, N. (1983). *The Neural Crest.* Cambridge: Cambridge University Press.

Le Douarin, N., Fontaine-Perus, J. and Couly, G. (1986). Cephalic ectodermal placodes and neurogenesis. *Trend Neurosci.* **9**, 175-180.

Liu, J. P. and Jessell, T. M. (1998). A role for rhoB in the delamination of neural crest cells from the dorsal neural tube. *Development* **125**, 5055-5067.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M. and Chambon, P. (1991).

Disruption of the *Hox-1.6* homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66**, 1105-1119.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

Maconochie, M., Krishnamurthy, R., Nonchev, S., Meier, P., Manzanares, M., Mitchell, P. and Krumlauf, R. (1999). Regulation of *Hoxa2* in cranial neural crest cells involves members of the *AP-2* family. *Development* **126**, 1483-1494.

Maden, M., Graham, A., Gale, E., Rollinson, C. and Zile, M. (1997). Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids. *Development* **124**, 2799-2805.

Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C. and Mason, I. (1995). Multiple roles for FGF-3 during cranial neural development in the chicken. *Development* **121**, 1399-1410.

Mallo, M. and Brandlin, I. (1997). Segmental identity can change independently in the hindbrain and rhombencephalic neural crest. *Dev. Dyn.* **210**, 146-156.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119**, 319-338.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature* **370**, 567-571.

Muller, T. S., Ebensperger, C., Neubuser, A., Koseki, H., Balling, R., Christ, B. and Wilting, J. (1996). Expression of avian Pax1 and Pax9 is intrinsically regulated in the pharyngeal endoderm, but depends on environmental influences in the paraxial mesoderm. *Dev. Biol.* **178**, 403-17.

Murphy, P. and Hill, R. E. (1991). Expression of the mouse labial-like homeobox-containing genes, Hox 2.9 and Hox 1.6, during segmentation of the hindbrain. *Development* **111**, 61-74.

Noden, D. (1983). The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev. Biol.* **96**, 144-165.

Noden, D. (1986). Patterning of avian craniofacial muscles. *Dev. Biol.* **116**, 347-356.

Noden, D. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103**, 121-140.

Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P. (1996). Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**, 543-554.

Peters, H., Neubuser, A., Kratochwil, K. and Balling, R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev.* **12**, 2735-2747.

Prince, V. and Lumsden, A. (1994). Hoxa-2 expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-923.

Rijli, F., Gavalas, A. and Chambon, P. (1998). Segmentation and specification in the branchial region of the head: The role of *Hox* selector genes. *Int. J. Dev. Biol.* **42**, 393-401.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell* **75**, 1333-1349.

Rossel, M. and Capecchi, M. (1999). Mice mutant for both *Hoxa1* and *Hoxb1* show extensive remodeling of the hindbrain and defects in craniofacial development. *Development* **126**, 5027-5040.

Saldivar, J. R., Sechrist, J. W., Krull, C. E., Ruffin, S. and Bronner-Fraser, M. (1997). Dorsal hindbrain ablation results in the rerouting of neural crest migration and the changes in gene expression, but normal hyoid development. *Development* **124**, 2729-2739.

Schneider-Maunoury, S., Topilko, P., Seitanidou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993). Disruption of *Krox-20* results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-1214.

Sechrist, J., Serbedzija, G. N., Scherson, T., Fraser, S. E. and Bronner-Fraser, M. (1993). Segmental migration of the hindbrain neural crest does not arise from its segmental generation. *Development* **118**, 691-703.

Sela-Donenfeld, D. and Kalcheim, C. (2000). Inhibition of noggin expression in the dorsal neural tube by somitogenesis: a mechanism for coordinating the timing of neural crest emigration. *Development* **127**, 4845-4854.

Serbedzija, G., Fraser, S. and Bronner-Fraser, M. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116**, 297-307.

Solloway, M. J. and Robertson, E. J. (1999). Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* **126**, 1753-1768.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F., Chambon, P. and Krumlauf, R. (1998). Genetic interaction between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-1036.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384**, 630-635.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619-627.

Trainor, P. A. and Tam, P. P. L. (1995). Cranial paraxial mesoderm and neural crest of the mouse embryo-codistribution in the craniofacial mesenchyme but distinct segregation in the branchial arches. *Development* **121**, 2569-2582.

Trainor, P. and Krumlauf, R. (2000a). Patterning the cranial neural crest: Hindbrain segmentation and *Hox* gene pasticity. *Nat. Rev. Neurosci.* **1**, 116-124.

Trainor, P. and Krumlauf, R. (2000b). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nat. Cell Biol.* **2**, 96-102.

Trainor, P., Manzanares, M. and Krumlauf, R. (2000). Genetic interactions during hindbrain segmentation in the mouse embryo. In *Results and Problems in Cell Differentiation*. Vol. 30 (ed. A. Goffinet and P. Rackic), pp. 51-89. Berlin: Springer-Verlag.

Trainor, P. A., Tan, S. S. and Tam, P. P. L. (1994). Cranial paraxial mesoderm-regionalization of cell fate and impact on craniofacila development in mouse embryos. *Development* **120**, 2925-2932.

Veitch, E., Begbie, J., Schilling, T. F., Smith, M. M. and Graham, A. (1999). Pharyngeal arch patterning in the absence of neural crest. *Curr. Biol.* **9**, 1481-1484.

Wendling, O., Dennefeld, C., Chambon, P. and Mark, M. (2000). Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. *Development* **127**, 1553-1562.

Wilkinson, D., Peters, G., Dickson, C. and McMahon., A. (1988). Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse. *EMBO J.* **7**, 691-695.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.