Hox proteins drive cell segregation and non-autonomous apical remodelling during hindbrain segmentation

Fabrice Prin1, Patricia Serpente1, Nobue Itasaki2,* and Alex P. Gould1,‡

ABSTRACT
Hox genes encode a conserved family of homeodomain transcription factors regulating development along the major body axis. During embryogenesis, Hox proteins are expressed in segment-specific patterns and control numerous different segment-specific cell fates. It has been unclear, however, whether Hox proteins drive the epithelial cell segregation mechanism that is thought to initiate the segmentation process. Here, we investigate the role of vertebrate Hox proteins during the partitioning of the developing hindbrain into lineage-restricted units called rhombomeres. Loss-of-function mutants and ectopic expression assays reveal that Hoxb4 and its parologue Hoxd4 are necessary and sufficient for cell segregation, and for the most caudal rhombomere boundary (r6/r7). Hox4 proteins regulate Eph/ephrins and other cell-surface proteins, and can function in a non-cell-autonomous manner to induce apical cell enlargement on both sides of their expression border. Similarly, other Hox proteins expressed at more rostral rhombomere interfaces can also regulate Eph/ephrins, induce apical remodelling and drive cell segregation in ectopic expression assays. However, Krrox20, a key segmentation factor expressed in odd rhombomeres (r3 and r5), can largely override Hox proteins at the level of regulation of a cell surface target, EphA4. This study suggests that most, if not all, Hox proteins share a common potential to induce cell segregation but in some contexts this is masked or modulated by other transcription factors.

KEY WORDS: Hox, Segmentation, Cell segregation, Cell sorting, Cell affinities, Cell tension, Hindbrain, Rhombomeres, Apical polarity, Mouse, Chick

INTRODUCTION
The segregation of different cell populations into distinct territories is a central feature of embryonic development. Pioneering experiments showed that dissociated embryonic cells are able to reaggregate according to their tissue of origin (Townes and Holtfreter, 1955; Steinberg, 1963; Steinberg, 1970). This effect can be mimicked in vitro by qualitative and quantitative differences in cell-adhesion molecules such as cadherins, which can regulate tissue surface tension (Foty and Steinberg, 2005; Steinberg, 2007; Ninomiya et al., 2012). During the segmentation of embryonic epithelia, interfaces between different cell populations form a stable straight barrier to cell intermingling. Classic clonal analysis of the anteroposterior (AP) compartment boundary in the developing Drosophila wing epithelium first showed that segmental interfaces are lineage restrictions (Garcia-Bellido et al., 1973; Morata and Lawrence, 1975). Related lineage restrictions are also found in the neuroepithelium of the developing vertebrate hindbrain. These boundaries appear during hindbrain development between seven segmental units called rhombomeres (r1 to r7), which prefigure the metameric organization of adult cranial nerves (Vaage, 1969; Lumsden and Keynes, 1989; Fraser et al., 1990; Birgbauer and Fraser, 1994; Jimenez-Guri et al., 2010). Studies of insect and vertebrate epidermis have provided evidence that three interlinked mechanisms contribute to robust cell segregation: differential adhesion, actomyosin-dependent cortical tension and cell repulsion (reviewed by Dahmann et al., 2011; Battle and Wilkinson, 2012).

Grafting experiments in the chick indicate that hindbrain boundaries are generated when odd-numbered rhombomeres are placed next to even-numbered ones and there is less cell intermingling between odd-even than between odd-odd or even-even (Guthrie and Lumsden, 1991; Guthrie et al., 1993). One important family of cell-surface molecules that are differentially expressed between odd and even rhombomeres are the Eph receptor tyrosine kinases and their ligands, the ephrins (Nieto et al., 1992; Becker et al., 1994; Bergemann et al., 1995; Flenniken et al., 1996; Gale et al., 1996). At rhombomere interfaces, bi-directional Eph/ephrin signalling restricts cell intermingling by triggering mutual cell-cell repulsion and, within a rhombomere, Eph signalling can regulate cell affinity (Xu et al., 1995; Mellitzer et al., 1999; Xu et al., 1999; Cooke et al., 2001; Cooke et al., 2005). Two key transcription factors that regulate the odd and even properties of rhombomeres are Krrox20 (Egr2 – Mouse Genome Informatics) and kreisler/MafB. Krrox20 encodes a zinc-finger transcription factor that is specifically expressed in r3 and r5, and regulates the intermingling properties of cells from odd-numbered rhombomeres (Wilkinson et al., 1989; Voiculescu et al., 2001). The downstream targets of Krrox20 are thought to include the molecules responsible for mediating odd versus even rhombomere cell-surface properties. For example, EphA4 is highly expressed in r3 and r5, and has been shown to be a direct transcriptional target of Krrox20 (Theil et al., 1998). Kreisler encodes a transcription factor (MafB) that is expressed in r5 and r6 (Cordes and Barsh, 1994). In zebrafish genetic mosaics, cells lacking MafB activity are repulsed from wild-type r5 and r6 territory in an Eph-dependent manner (Moens et al., 1996; Cooke et al., 2001). Within r5, at least some of the cell segregation associated with kreisler activity is likely to involve Krrox20 (Frohman et al., 1993; McKay et al., 1994; Moens et al., 1996; Giudicelli et al., 2003; Sadl et al., 2003).

Three lines of evidence indicate that Krrox20-dependent segregation of odd versus even rhombomere cells cannot account for
all aspects of hindbrain segmentation. First, in both the grafting and the *in vitro* cell segregation experiments, cell miscibility is less efficient between cells from two different odd or even rhombomeres than between cells from the same rhombomere (Guthrie et al., 1993; Wizenmann and Lumsden, 1997). Second, *Krox20* mutants lack r3 and r5 territories but still form morphological grooves that express boundary markers at the positions of r1/r2, r2/r4, r4/r6 and r6/r7 (Schneider-Maunoury et al., 1997). And third, although there is an expression border of *Krox20* associated with all rhombomere interfaces from r2/r3 to r5/r6, this is not the case for the most rostral (r1/r2) and caudal (r6/r7) borders. In this regard, it is interesting that r7 has neither even nor odd character, as it can form a boundary with either r5 or r6 (Guthrie and Lumsden, 1991). Thus, aside from *Krox20*, there must be other AP-patterning genes that are important for regulating rhombomere-specific cell affinities.

Hox genes encode a conserved family of homeodomain transcription factors that are segmentally expressed and have well described functions in specifying AP positional identity in many different contexts (McGinnis and Krumlauf, 1992; Lumsden and Krumlauf, 1996; Alexander et al., 2009; Dasen and Jessell, 2009; Tschopp and Duboule, 2011). In the *Drosophila* embryo, as in the vertebrate hindbrain, the anterior expression borders of many Hox genes coincide with the position of lineage-restricted boundaries (Martinez-Arias and Lawrence, 1985; Vincent and O'Farrell, 1992).

Loss of Hox activity in *Drosophila* embryos can result in multiple adjacent segments/parasegments of identical AP character but, importantly, the boundaries between them remain intact (Lewis, 1978; Struhl, 1981). This indicates that *Drosophila* Hox genes are essential for segmental AP character but not for the subdivision of the embryo into lineage-restricted units. By contrast, loss of activity of vertebrate Hox genes not only alters AP identity but, in several single and multiple combinations, can also disrupt rhombomere boundaries (Carpenter et al., 1993; Gavalas et al., 1997; Helmbacher et al., 1998; Studer et al., 1998; Davenne et al., 1999; Rossel and Capecki, 1999; Barrow et al., 2000). Importantly, in some *Hox1* and *Hox2* mutant genotypes, the expression patterns of *Krox20* and kreisler are also strongly disrupted. Thus, the complex bidirectional regulatory interdependence between *Krox20*, kreisler and the anterior Hox genes makes it difficult to disentangle their respective outputs relevant to the segmentation process (reviewed by Tümpe1 et al., 2009). In particular, it is unclear whether Hox genes have any direct input, independent of *Krox20*, into early rhombomere interface formation.

In the present study, we use mouse genetics, retinoic acid (RA) treatments and chick electroporation to investigate the mechanism of segmentation at r6/r7, a caudal hindbrain boundary that does not require *Krox20*. We show that Hox genes of paralogue group 4 are necessary for the r6/r7 boundary and that they also mediate boundary suppression by exogenous RA. Single cell resolution studies of endogenous and artificial *Hox4*-*Hox4* interfaces using molecular and morphological markers, as well as measurements of cell-surface areas, demonstrate a non-cell autonomous mechanism for Hox4-induced neuroepithelial cell segregation, apical remodelling and boundary formation. Members of the Hox1, Hox2 and Hox3 groups all share with Hox4 the intrinsic ability to initiate cell segregation when ectopically expressed in the chick neuroepithelium. Co-electroporation experiments are then used to show that, during neuroepithelial cell segregation, *Krox20* can override an r5-resident Hox protein (Hox3) at the level of a common downstream target. The wider implications of this study for segmental partitioning at rhombomere interfaces are discussed.

**RESULTS**

**Hox4 genes are required for the r6/r7 boundary**

The murine r6/r7 boundary first becomes visible as a ridge on the apical/ventricular side (or a groove on the basal/pial side) of the neuroepithelium at around embryonic day 9.5 (E9.5). This is concomitant with a sharpening of the *Hoxb4* rostral limit of expression, a process involving feedback between *Hoxb4*, *Hoxd4* and *Rarb* (supplementary material Fig. S1A,B) (Serpente et al., 2005). In both mouse and chick, the position of the *Hoxb4* anterior expression border corresponds to the r6/r7 morphological boundary. *Hoxb4* cells are occasionally observed more anteriorly but these rare ‘escapers’ are largely excluded from r6 by localization to the pial surface or to the r5/r6 boundary (supplementary material Fig. S1B-D). To investigate the function of *Hox4* genes, we generated double mutant embryos (*Hoxb4*Δ/Δ; *Hoxd4*Δ/Δ) lacking the activities of the only two Hox4 paralogues expressed in r7 before E11 (Behringer et al., 1993; Gould et al., 1997; Morrison et al., 1997).

Neither the residual expression of kreisler in ventral r6 at E9.25 (Cordes and Barsh, 1994; Theil et al., 2002) nor *Krox20* in dorsal r5 at E9.5 (Wilkinson et al., 1989) were altered in double mutant embryos (supplementary material Fig. S1E-H). At E10.5, however, the r6/r7 posterior limit of the lateral *Phox2b* column (Pattyn et al., 1997) is caudally extended (supplementary material Fig. S11-J). Thus, *Hoxb4* and *Hoxd4* are not required to regulate the anterior segmentation genes *Krox20* or kreisler/Phox2b but they are essential for suppressing a later aspect of anterior neural character in r7.

We then tested the role of Hox4 genes in the r6/r7 segmentation process. In Hox4 double mutant embryos, the r1/2 to r5/6 boundaries remain visible in E9.5 sections as basal grooves with increased intercellular spacing but the r6/r7 boundary is absent (Fig. 1A,B). *Crabp1* mRNA is a neural crest marker and, at E9.5, it is strongly expressed in r2 and r4-6 (Maden et al., 1992; McKay et al., 1994). We find that, at E10.5, *Crabp1* marks all rhombomere boundaries but is more strongly expressed in those of r3/4 through to r6/r7 (Fig. 1C). In Hox4 double mutant embryos, there is a specific loss of r6/r7 but not the other *Crabp1*-expressing boundaries (Fig. 1D). The results thus far demonstrate that the r6/r7 segmentation mechanism does not involve *Krox20* or kreisler/Refa but it is strictly dependent upon an input from either *Hoxb4* or *Hoxd4*.

**Misexpression of Hox4 proteins suppresses r6/r7 and more anterior boundaries**

We next used exogenous retinoic acid (RA) as an indirect way of misexpressing Hoxb4 and Hoxd4 across the r6/r7 interface. RA suppresses varying numbers of hindbrain boundaries, depending upon the treatment stage (Wood et al., 1994; Nittenberg et al., 1997; Dupé and Lumsden, 2001). At E9.25, it expands murine Hox4 expression ectopically into the anterior hindbrain (Conlon and Rossant, 1992; Morrison et al., 1997). To map accurately the anterior limit of Hox4 expression induced by RA at E9.25, we used a transgenic reporter for Hox4-6 proteins: LNE-LacZ (Gould et al., 1997; Gould et al., 1998). *LNE-LacZ* reveals that RA treatment at E9.25 induces ectopic expression of Hoxb4 and Hoxd4 up to r3/4 in controls but, in Hox4 double mutants, the remaining Hox4-6 genes can only be RA induced up to r5/6 (Fig. 1E,F). Hence, the effects of misexpressing Hoxb4/Hox4 either side of the r6/r7, r5/6 and r4/5 boundaries can be deduced by comparing RA-treated controls with Hox4 double mutants. RA-treated controls show widespread upregulation of *Crabp1* (Leonard et al., 1995) and we find that the distinct stripes of expression at the r4/5, r5/6 and r6/r7 boundaries are missing (Fig. 1G). RA-treated Hox4 double mutants show a similar widespread *Crabp1* upregulation but the r4/5 and r5/6 stripes
of expression are now restored (Fig. 1H). Thus, RA-mediated suppression of the r4/5 and r5/6 boundaries is dependent upon ectopic expression of Hoxb4 and Hoxd4. The RA results also imply that inducing Hox4 expression either side of rhombomere boundaries is sufficient to suppress them.

To test more directly the effects of Hox4 misexpression on segmentation, we used in ovo electroporation of chick embryos at the 7- to 12-somite stage (HH stages 9-11), when rhombomere boundaries are forming (Vaage, 1969; Lumsden, 1990). By HH stages 19-21 (~48 hours later), hindbrains expressing widespread ectopic murine Hoxb4 (mHoxb4) showed a dramatic disruption of morphological segmentation (Fig. 2A,B′). Expression of Fgf3 mRNA 1 day after electroporation in rhombomere boundaries is reduced or absent on the electroporated side. Residual Fgf3+ cells are displaced by the mouse Hoxb4+ electroporated cells (insets). (C,C′) Flat-mouted chick hindbrains co-electroporated with separate mouse Hoxb4 and GFP plasmids. GFP+ electroporated cells (green) and the distribution of Cspg1 are shown. Cspg1 highlights rhombomere boundaries and is also detected around groups of mouse Hoxb4+ electroporated cells.
Hox4 proteins regulate multiple cell adhesion/repulsion genes

To identify hindbrain targets of Hox4 proteins, we surveyed a panel of genes implicated directly or indirectly in cell adhesion and/or repulsion using the chick electroporation assay. Ectopic expression of mouse Hoxb4 rapidly and strongly repressed the key segmentation gene Krox20 as well as its direct target Epha4 (Theil et al., 1998) within r3 and r5 (supplementary material Fig. S2A-H). Ephrin B2 (Efnb2 – Mouse Genome Informatics), which encodes a known ligand for EphA4, is also moderately downregulated by ectopic mouse Hoxb4 (supplementary material Fig. S2I-L). Negative regulation by mouse Hoxb4 is specific for a subset of Eph/ephrin genes, as Epha3 expression is not detectably suppressed (data not shown). We also found that ectopic mouse Hoxb4 regulates ephrin A5 (Efna5 – Mouse Genome Informatics) expression in a context-dependent manner: downregulating it in dorsal r1 but upregulating it in more posterior hindbrain regions (supplementary material Fig. S2M-T). Hence, ectopic Hoxb4 can regulate Krox20 as well as three genes implicated in cell adhesion/repulsion: Epha4, ephrin A5 and ephrin B2. Although only two of these target genes (ephrin A5 and ephrin B2) are substantially co-expressed with endogenous Hoxb4 in r7, they are all candidates for mediating the boundary suppressing effects of ectopic Hoxb4 in the anterior hindbrain.

We next focused on two putative cell-adhesion/repulsion genes that are differentially expressed in r6 versus r7, and so may be relevant transcriptional targets of endogenous Hoxb4. In both chick and mouse hindbrain, the leucine-rich repeat transmembrane protein LRRTM3 is expressed in dorsal r7 but not in dorsal r6 (Haines and Rigby, 2007) (Fig. 3A). In the chick hindbrain, mouse Hoxb4 misexpression leads to ectopic Lrrtm3 upregulation in the dorsal anterior hindbrain (Fig. 3A,B). In both chick and mouse, Epha7 expression in the hindbrain has a posterior limit at the r6/r7 boundary (Araujo and Nieto, 1997). In the mouse, we found that Hoxb4 or Hoxd4 are required to repress inappropriate Epha7 expression in r7 (Fig. 3C,D). Consistent with this, ectopic mouse Hoxb4 in the chick represses Epha7 expression in r1-r6 (Fig. 3E,F). Hence Lrrtm3 and Epha7 are positive and negative Hox4 targets in r7, respectively, and Hox4 ensures their differential expression either side of the r6/r7 boundary.

Mosaic expression of Hox4 proteins initiates neuroepithelial cell segregation

In electroporated chick hindbrains with a high degree of mosaicism, we were intrigued by a marked difference in the distribution of mouse Hoxb4+ compared with GFP+ control cells. Whereas control GFP+ cells are interspersed with many non-labelled neighbours in a salt-and-pepper pattern, mouse Hoxb4+ cells in the midbrain and hindbrain tend to form coherent groups (Fig. 4A-D; data not shown). Despite high electroporation-to-electroporation variability, an automated cell-counting algorithm (Mirenda et al., 2007) (see Materials and methods) revealed that mouse Hoxb4+ electroporated cells tend to form small groups (four cells or fewer) less frequently and large groups (at least 15 cells) significantly more frequently than control electroporated cells (Fig. 4E). We then generated Hox4 mosaics in a second way, by electroporating a dominant-negative (dn) RAR construct that interferes with the expression of RAR target genes (Blumberg et al., 1997). One direct target is Hoxb4, which is strongly downregulated within its endogenous expression domain by dnRAR (Gould et al., 1998). We find that the dnRAR method also produces large coherent groups of electroporated cells, but they are chick Hoxb4+ rather than mouse Hoxb4+ (Fig. 4F,G). Moreover, dnRAR electroporated cells can also disrupt the endogenous chick r6/r7 boundary (supplementary material Fig. S3A,D). The finding that large cell groups are generated with either mosaic approach indicates that they are unlikely to be an artefact resulting from overly high mouse Hoxb4 expression. Importantly, the observation that either Hox4+ or Hox4− cells can form cell groups suggests that their formation is triggered by a Hox expression interface, rather than by an intrinsic property of Hox4+ cells per se. The underlying mechanism is therefore likely to correspond to cell segregation. Consistent with this, we find no evidence for alternative mechanisms involving increased cell proliferation and/or cell competition-like processes associated with apoptosis (supplementary material Fig. S4A-J; data not shown).

Hoxb4 regulates apical cell surface area in a non-autonomous manner

The results thus far provide evidence that a Hox4+/Hox4− expression interface is necessary and sufficient to promote cell segregation and features of a rhombomere boundary. Previous studies have shown
of mouse Hoxb4+ cell clusters, correlating with increased apical constriction and neuroepithelial curvature (Fig. 5D-F). In striking contrast, where mouse Hoxb4+ cells locally outnumber and surround a cluster of mouse Hoxb4− cells, then strong apical N-cadherin staining, pronounced apical constriction and neuroepithelial curvature tend to be observed in the mouse Hoxb4− cell population (Fig. 5G-I). Pan-cadherin stainings also revealed that, even in rare cases, when the curvature in the minority mouse Hoxb4+ or mouse Hoxb4− cell populations is very extreme, the neuroepithelium and the basement membrane remain intact (supplementary material Fig. S5A-E; data not shown). We conclude that when cells segregate within the neuroepithelium in the ectopic Hoxb4 assay, apical constriction at the centres of these clusters can involve either the Hoxb4+ or the Hoxb4− cell population.

Rhombomere boundaries are associated with apical enlargement (Guthrie et al., 1991; Takahashi and Osumi, 2011) but we found that a Hoxb4+/Hoxb4− interface can drive nearby apical constriction. It is therefore important to define, with high resolution, how cells with small and large apices map onto the endogenous Hoxb4 expression border. Using the tight junction associated protein ZO-1 as a marker, we observed a zone of cells with large apical surfaces at the mouse and chick r6/r7 boundary, spanning the wiggly mouse Hoxb4+/Hoxb4− interface (supplementary material Fig. S6A-E). Cells with large apical areas, more than double the typical inter-rhombomeric value, extend approximately three or four cells on either side of the mouse Hoxb4+/Hoxb4− interface in ventrolateral regions (Fig. 6A-C) but this varies as a function of DV position. Importantly, r6/r7 apical enlargement is reduced in Hoxb4+/−; Hoxd4−/+ (but not in Hoxb4+/−; Hoxd4−/−) embryos, and is no longer detectable in complete Hox4 double mutants (Fig. 6D-J). Hence, Hox4 genes are required in a dose-dependent manner to drive apical enlargement on both sides of the murine r6/r7 boundary. Similarly, in the chick hindbrain, r6/r7 apical enlargement was abrogated by indirectly blocking Hox4 expression via electroporation with dnRAR (supplementary material Fig. S3). Moreover, ZO-1 analysis of artificial chick Hoxb4+/Hoxb4− expression borders, created by Hoxb4 electroporation in the chick, confirms the earlier cadherin results and shows clearly that segregated Hoxb4+ or Hoxb4− cell clusters can undergo strong apical constriction at their centres (supplementary material Fig. S6F-K). This chick analysis also reveals strong apical enlargement in cells on both sides of an artificial Hoxb4 border (Fig. 6K,L; supplementary material Fig. S6F-K). Together, the mouse and chick analyses demonstrate that a Hox4 boundary is necessary and sufficient to induce apical enlargement. They also show that Hox4 proteins can regulate apical remodelling (constriction and enlargement) in both cell and non-cell autonomous manners.

To identify the mechanism by which Hox4 proteins act, we tested whether any of the Hox4 target genes identified in this study are themselves sufficient to induce chick neuroepithelial cell segregation. Ectopic expression of several targets had little or no discernible effect, indicating that they are not sufficient to induce cell segregation under the conditions of our ectopic electroporation assay (data not shown). However, we observed that ephrin B2 and the closely related protein ephrin B1, were each sufficient to induce cell segregation and to disrupt endogenous rhombomere boundaries (supplementary material Fig. S7A-C). As with Hoxb4, either the electroporated (ephrin B2+) or the non-electroporated (ephrin B2−) cells can form large cell groups, depending upon their relative frequency. We also observed that ephrin B2+ and ephrin B2− cell clusters displayed apical remodelling features similar to those seen with Hoxb4 clusters (supplementary material Fig. S7D-I). These findings demonstrate that ectopic expression of a Hox4
Many Hox proteins are sufficient to drive neuroepithelial cell segregation

To determine whether cell segregation and apical remodelling are unique to Hox4 or whether they are a general property of all Hox proteins, we examined several different rhombomere boundaries. At the chick r3/4 boundary, we observed a strong increase in ZO-1 apical cell areas in a strip of several cells wide (Fig. 7A,B). To map accurately the apical cell areas onto segmental gene expression, we used Epha4 and Hoxa3 to mark the chick and mouse r4/r5 interfaces respectively. In both species, this revealed that the zone of apical enlargement straddles the r4/r5 gene expression interface, extending several cells on either side (Fig. 7C-F). These results strongly suggest that apical enlargement spanning a gene expression border is a common feature of many rhombomere interfaces, including those that are Krox20 dependent. This raises the issue of whether Hox proteins contribute to apical remodelling and to cell segregation at Krox20 interfaces. To address this key issue, we surveyed a panel of Hox and other neural transcription factors for their ability to induce neuroepithelial cell segregation in chick electroporation assays. Neither GFP nor Sox2 expression led to detectable cell segregation but, consistent with previous results (Giudicelli et al., 2001), ectopic Krox20 generated medium-to-large patches of Epha4-expressing cells in even rhombomeres (Fig. 7G). Strikingly, when Hox proteins were surveyed, not only Hoxd4 but also those expressed in more anterior regions of the hindbrain (mouse Hoxb1, mouse Hoxa2, mouse Hoxa3 and human HOXB3) were sufficient to induce large neuroepithelial cell clusters (Fig. 7G). We also examined apical profiles in clusters of Hoxa2-, Hoxa3- or Krox20-expressing cells and, in each case, observed central apical constriction and peripheral apical enlargement similar to that seen with Hoxb4 (supplementary material Fig. S8). We note, however, that if two neuroepithelial cell populations ectopically expressing a different Hox protein are confronted, then they tend to intermingle within mixed clusters (data not shown). This suggests that either Hox proteins all induce similar neuroepithelial cell affinities or that the high gene expression in this ectopic assay masks any intrinsic differences between Hox proteins. Either way, this survey demonstrates that Hox proteins of all four paralogue groups expressed within the hindbrain and Krox20 share the potential to drive neuroepithelial cell segregation and apical remodelling.

Krox20 can largely override Hoxa3 at the level of a downstream target: Epha4

The above findings prompt the question of how does the cell segregation function of Krox20 relate to those of Hox proteins expressed endogenously in r3 and/or r5? We therefore ectopically co-expressed Krox20 with an r5 resident (Hoxa3) or a non-resident (Hoxb4) Hox protein. A previous study showed that ectopic expression of Krox20 alone, not only activates Epha4 in electroporated cells but also induces Krox20 and Epha4 several cell diameters away, via non-cell autonomous auto-activation (Giudicelli et al., 2001). Consistent with this, we observe that Krox20 electroporated and non-electroporated cells both contribute to large coherent patches of Epha4+ tissue in even rhombomeres (supplementary material Fig. S9A,B). By contrast, ectopic
expression of Hoxa3 or Hoxb4 alone leads to repression of Epha4 within odd rhombomeres (supplementary material Fig. S9C,D,G,H). Given that Krox20 and Hox proteins have different activities, we were able to use co-electroporation to conduct epistasis tests at the level of competition for a common downstream target: Epha4. In both the Krox20/Hoxa3 or the Krox20/Hoxb4 combinations, we observed that co-electroporated cells strongly expressed Epha4 in even rhombomeres but that the non-cell autonomous range of Epha4 induction in non-electroporated cells was greatly restricted (supplementary material Fig. S9E,F,I,J). Hence, ectopic Krox20 predominates over ectopic Hox protein with respect to cell-autonomous activation rather than repression of Epha4. Hox proteins can, however, restrict the non-cell autonomous range of Epha4 induction by Krox20. Together, the results of the ectopic co-expression assays indicate that Krox20 and Hox proteins can both induce apical remodelling but that they regulate Epha4 in opposite ways. They also suggest that Krox20 may promote the cell segregation properties of odd rhombomeres, at least in part by overriding the ability of resident r3/r5 Hox proteins to regulate cell-surface molecules such as Epha4.

DISCUSSION
Non-cell-autonomous roles for Hox4 proteins during boundary formation

The segregation of cells into two non-intermingling populations is thought to drive subsequent specializations, including increased apical cell area, actomyosin enrichment and the expression of local organizer signals (reviewed by Dahmann et al., 2011; Battle and Wilkinson, 2012). Three lines of evidence were provided that a Hox4+/Hox4– expression border is both necessary and sufficient to stimulate cell segregation and thus to define the site of a future rhombomere boundary. First, Hoxb4 and Hoxd4 are expressed on the posterior side of the r6/r7 interface and are strictly required for apical remodelling and other features of the r6/r7 boundary. Second, either direct or RA-induced misexpression of Hox4 proteins on both sides of r6/r7 or other presumptive rhombomere boundaries is sufficient to suppress their formation. And third, artificial Hox4 borders stimulate cell segregation, apical remodelling and molecular characteristics of boundaries. Consistent with a key role in cell segregation, we found that Hox4 proteins regulate multiple genes encoding cell surface molecules implicated in adhesion/repulsion such as Lrrtm3 and several Eph/ephrin proteins. Although many of these may be needed to mediate the neuroepithelial functions of Hoxb4, at least one of them, ephrin B2, is sufficient to induce cell segregation and apical remodelling in our ectopic expression assay.
The mechanistic links between apical remodelling and cell segregation are likely to be complex and challenging to disentangle. Nevertheless, when cell segregation is first detected after Hoxb4 electroporation, apical constriction at the cluster centre is already very pronounced whereas apical enlargement at the cluster periphery is only minimal (data not shown). Hence, strong apical enlargement is not a prerequisite for cell segregation and it may even be a consequence of nearby apical constriction. At both endogenous and artificial Hoxb4 expression interfaces, Hoxb4+ cells drive apical enlargement, not only in themselves but also in their Hoxb4- neighbours several cell diameters away. Possible non-cell autonomous mechanisms accounting for this include the spread of a secreted interface-derived signal or mechanical propagation of increased tension over several cell diameters. In many developing epithelia, enlarged apical cell profiles are known to be associated with increased intercellular surface tension (reviewed by Lecuit and Lenne, 2007). Enlarged apical cell profiles have also been observed in the row of cells on either side of the insect AP compartment boundary, where they are associated with increased actomyosin-dependent intercellular surface tension along the boundary (Landsberg et al., 2009). Intercellular surface tension also appears to play a role in chick hindbrain segmentation, where myosin II inhibitor experiments and finite element modelling implicate isotropic contraction within rhombomeres and weak circumferential contraction at rhombomere interfaces (Filas et al., 2012). Future studies will be needed to measure how cell bond tensions vary as a function of distance from the gene expression borders at rhombomere interfaces.

An ancestral function for Hox proteins in epithelial cell segregation?

This study compared factors driving boundary formation at r6/r7 with those at the four odd-even rhombomere interfaces associated with a Krox20 expression border (r2/r3 to r5/r6). Although the r6/r7 interface is a bona fide lineage restriction (Fraser et al., 1990; Birgbauer and Fraser, 1994), we showed that it is less straight and displays less pronounced apical remodelling than other interfaces. This correlates, in the chick, with weaker Fgf3 and Pax6 expression at r6/r7 than at the Krox20 boundaries and manipulations of either gene at early somite stages can alter segmentation and boundary gene expression (Marín and Charnay, 2000; Weisinger et al., 2012; Kayam et al., 2013). However, when we ectopically expressed Pax6 or Fgf3 (or inhibited Fgf signalling) at the later developmental stage used in our Hox electroporations, apical remodelling at rhombomere boundaries was not detectably altered (data not shown).

Evidence from numerous gain-of-function electroporations showed that ectopic expression borders of Krox20 or of hindbrain Hox proteins from paralogue groups 1 to 4 are sufficient to initiate cell segregation and apical remodelling. This raises the issue of whether, during normal r3 and r5 development, it is Krox20 or resident Hox proteins such as Hoxa3 that primarily regulate cell segregation. To address this, co-electroporation was used to bypass any cross regulation between the Krox20 and Hoxa3 loci and to reveal interactions at the level of Epha4, a common target for Krox20 and Hoxa3 proteins. The finding that Krox20 activation tends to ‘win out’ over Hoxa3 repression of Epha4 suggests that phenotypic suppression of Hox proteins (González-Reyes et al., 1990) is an important mechanism of action for Krox20 during cell segregation. Intriguingly, however, we found that Hox proteins were able to restrict the auto-activation of Epha4, and thus probably of Krox20 (Giudicelli et al., 2001), in non-electroporated cells. This suggests that one function of Hox expression in even rhombomeres could be to block the spread of...
non-cell autonomous auto-activation of Krox20/Epha4 across the odd/even rhombomere interface. Testing this hypothesis will likely require approaches in which Hox and Krox20 protein expression can be controlled in more physiological and precise ways than are possible with our current electroporation assay.

Future studies may reveal whether the role for vertebrate Hox proteins in neuroepithelial cell segregation is related to their earlier function in the ordered migration of ingressing epiblast cells during gastrulation (Imura and Pourquié, 2006). Either way, the possibility of a conserved role for the Hox protein family in epithelial cell segregation is strongly suggested when our vertebrate Hox study is taken together with previous Drosophila Hox studies (Garcia-Bellido, 1968; Morata and Garcia-Bellido, 1976; Estrada and Sánchez-Herrero, 2001; Gandille et al., 2010; Curt et al., 2013).

Thus, in addition to their numerous evolutionarily diverse functions, Hox proteins (and perhaps other homeodomain proteins) may share an ancestral role in cell segregation. In some developmental contexts, this Hox function may suffice to prevent enough cell intermingling but, in others, it could have been largely overridden during evolution by derived segmentation genes such as Krox20, engrailed and hedgehog.

MATERIALS AND METHODS

Mouse breeding, genotyping and retinoic acid treatment
Animal work was approved by the NIMR local Ethical Review Process and was licensed and conducted under appropriate authority granted by the UK Home Office under the Animals (Scientific Procedures) Act 1986. Hoxb4 and Hoxd4 mutant embryos and the Late Neural Enhancer LacZ (LNE-LacZ) line were generated and genotyped as described (Gould et al., 1997; Gould et al., 1998; Serpente et al., 2005). All-trans retinoic acid (25 μg/ml stock in DMSO) was diluted 1:10 in sesame oil and 200 μl per pregnant dam (~25 mg/kg bodyweight) administered by gavage at E9.25 as described (Gould et al., 1998).

In ovo electroporation of chick embryos

Embryos were electroporated at HH stage 9-11 (Hamburger and Hamilton, 1992) as described (Itasaki et al., 1999) but using five 50 ms square pulses of 18 V and analysed 2 days after electroporation, unless otherwise stated. To target the ventral hindbrain, the positive electrode was placed under the embryo and five 50 ms square pulses of 10 V were used. Electroporated constructs were injected at 1-2.5 μg/μl for each construct except that pCAGGS-GFP (Momose et al., 1999) was used at 0.6-1 μg/μl in co-electroporations. The plasmids used were a bicistronic IRES myristylated GFP (mGFP) version (McLarren et al., 2003) or a bicistronic IRES nuclear GFP (nlsGFP) version (pCIG) (Megason and McMahon, 2002) of pCAGGS with or without insertion of a mouse Hoxb4 cDNA (a gift from S. Guthrie) or a dominant-negative RARα1 construct (Gould et al., 1998). Other electroporated constructs used were: Sox2 (a gift from L. Lovell-Badge, NIMR, London, UK), pAdRSV-Krox20 (Giudicelli et al., 2001), pβ3Act-pA-mHoxb1 (Pöpperl et al., 1995), pCIG-mHoxa2 (Gouti et al., 2011), pCAGGS-mHoxa3 and pCAGGS-HOXB3 (Guidato et al., 2003), pCAGGS-mHoxd4 [a gift from J. Chilton (Peninsula Medical School, Plymouth, UK) and S. Guthrie (King’s College London, UK)], and pCIG-mEphrinB1 and pCIG-mEphrinB2 (gifts from A. Davy, Cribbs and E. Sanchez-Herrero for communicating results prior to publication). For BrdU labelling, pixel counts using the ROI tool were made at identical dorsoventral levels of electroporated and contralateral sides. Graphs and statistical analyses utilized Prism (GraphPad).

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Competing interests

The authors declare no competing financial interests.

Author contributions

F.P., P.S., N.I. and A.P.G. conceived and designed the experiments. F.P., P.S., N.I. and A.P.G. performed the experiments. F.P. and A.P.G. wrote the manuscript.

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Supplementary material

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References


Figure S1. Hox4 genes are expressed in r7 and required for its regional identity. (A) E8.5 mouse hindbrain showing Hoxb4 and Krox20 protein expression. Cells express different levels of Hoxb4 and are intermingled with non-expressing cells. (B, C) E10 flat-mounted mouse hindbrains (B) and para-sagittal cryosection (C) showing Hoxb4 expression. Hoxb4+ cells delineate a sharp r6/r7 boundary. The few Hoxb4+ cells detected anterior to this limit are located at the level of the next boundary (dotted box and inset in B, arrowhead in C) or at the pial surface of the hindbrain (arrowheads in C). (D) Coronal cryosection through st.20 chick head showing cHoxb4 mRNA distribution in the hindbrain with a sharp anterior border at the r6/r7 boundary. The few cHoxb4+ cells detected anterior to this border are located at the pial side of the hindbrain (arrowhead). (E,F) Dorsal view of mouse hindbrains at E9.25 showing normal Kreisler mRNA expression in r6 for both Hoxb4+/−; Hoxd4+/− and Hoxb4+/−; Hoxd4−/− genotypes. Arrowhead in (E) shows r6/r7 border. (G,H) Flat-mounts of E9.5 mouse hindbrains showing Krox20 mRNA expression in wild type (G) and Hoxb4+/−; Hoxd4−/− (H) embryos. Arrowhead in (G) shows r5/6 border. (I,J) Flat-mounts of E10.5 mouse hindbrains showing Phox2b mRNA expression in Hoxb4+/−; Hoxd4−/− (I) and Hoxb4+/−; Hoxd4−/− (J) embryos. In the absence of all Hoxb4 and Hoxd4 paralogues at this later stage, the lateral expression of Phox2b, normally stopping at the r6/r7 boundary (arrowhead in I), is extended posteriorly (J).
**Figure S2. Ectopic Hoxb4 modulates Krox20 and Eph/ephrin expression.**

(A-D) Flat-mounted chick hindbrains 6 hours (A,B) or 12 hours (C,D) after mHoxb4 electroporation (haep), showing the expression of Krox20 mRNA. For each pair of panels, the channel showing the mHoxb4 electroporated cells (red) is omitted from the right panel.

(E-T) Distribution of Eph and ephrin mRNAs in chick hindbrains two days after mHoxb4 electroporation. Flat-mounted chick hindbrains (E-R) or a coronal section (S,T) are shown and, for each pair of panels, the channel showing the mHoxb4 electroporated cells (red) has been omitted from the right panel. (E-H) EphA4 expression in r3 and r5 is disrupted by mHoxb4+ cells (E,F) and higher magnification of the r3 region (dotted box) shows that EphA4 mRNA is largely undetectable in mHoxb4 electroporated cells (G,H).

(I-L) ephrinB2 expression in the dorsal hindbrain is reduced by mHoxb4 electroporation (I,J) and higher magnification of the r1 region (dotted box) shows moderate downregulation of ephrinB2 expression in mHoxb4+ cells (K,L).

(M-P) ephrinA5 expression is altered by mHoxb4 electroporation in a region-specific manner (M,N). Higher magnification of the r1 region (upper dotted box) shows strong downregulation of ephrinA5 expression in mHoxb4+ cells (O,P). However, higher magnification of the r4/r5 region (lower dotted box) shows upregulated ephrinA5 expression in mHoxb4+ cells, both in flat mounts (Q,R) and in coronal sections (S,T).
Figure S3. dnRAR induces apical remodelling and disrupts the r6/r7 boundary.

(A,B) Confocal z-projections of the apical zone of the r6/r7 region of flat-mounted hindbrains after co-electroporation of dnRAR and GFP plasmids. Control (A,C) and electroporated (B,D) sides of the hindbrain show DAPI stained nuclei (blue), GFP+ electroporated cells (green) and ZO-1 immunostaining (red in A,B, white in C,D). The r6/7 boundary is visible as a line of enlarged cell apices on the control side but this is not apparent on the electroporated side, where GFP-expressing dnRAR cells form apically constricted clusters.
Figure S4. mHoxb4 misexpression does not alter neuroepithelial proliferation.

(A-C) Distribution of mitotic cells in hindbrains 2 days after mHoxb4 and GFP coelectroporation. Single confocal section showing GFP+ and PH3+ cells.

(D-I) BrdU incorporation following one hour pulse at one day (D-F) or 2 days (G-I) after mHoxb4 electroporation. Electroporated cells (mHoxb4) and tissue morphology (DAPI) are shown. E, F and H, I are higher magnifications of the electroporated side of the transverse hindbrain sections in D and G, respectively.

(J) The frequency of BrdU+ cells for electroporated (EP mHoxb4, n=5) and non-electroporated (control, n=5) sides. Error bars: SD, P=0.9365 (Mann-Whitney test).
Figure S5. Hoxb4 cell clusters can form neuroepithelial invaginations with strong apical Cadherin staining.

(A-E) Flat-mounted chick hindbrains electroporated with mHoxb4-ires-myrGFP. Panels show a single mHoxb4+ cell cluster in r5 with strong pan-cadherin (PCad) staining at the centre (apical surface) of a pronounced invagination. An XY view (A-C) and a YZ section (D-E) of the same cluster are shown.
**Figure S6. Apical enlargement at endogenous and artificial Hoxb4 interfaces.**

(A-C) Flat-mounted E10.5 mouse hindbrain showing a high magnification confocal z-projection of the r6/r7 region with DAPI-stained nuclei (blue) and the expression of Hoxb4 (green) and ZO-1 (red). Cells either side of the wiggly Hoxb4+/Hoxb4- interface (dotted line) show enlarged apical profiles.

(D) The chick r6/r7 region at stage 20 showing a confocal z-projection of the apical and subapical zones at a lateral level of the hindbrain with DAPI stained nuclei (blue) and ZO-1 expression (red). ZO-1 apical profiles used for the analysis in E are shown in yellow in the bottom panel. Anterior is to the left.

(E) Quantification of cell apical areas (µm²) along the anteroposterior axis.

(F-K) Flat-mounted chick hindbrains electroporated with mHoxb4-nlsGFP plasmid. Confocal z-projections of hindbrain (F) and midbrain (I) with higher magnifications of the indicated regions (dotted boxes) also shown (G,H,J,K). Images show electroporated cells expressing nuclear GFP (green), DAPI-stained nuclei (blue) and ZO-1 expression (red or white). Confocal z-projections allow deep nuclei to be imaged together with their superficial apical profiles (G,H,J,K). Arrowhead in I indicates the anterior-lateral edge (dotted line) of the flatmounted midbrain (tectum). Both Hoxb4+ (G,H) and Hoxb4- (J,K) cell clusters show strong central apical constriction and peripheral apical enlargement, the latter encompassing electroporated and non-electroporated cells.
Figure S7. Ectopic ephrinB drives cell segregation, apical remodelling and boundary disruption.

(A-C') Flat-mounted hindbrains electroporated with plasmids expressing *nlsGFP* (A, A'), *ephrinB2-ires-nlsGFP* (EfnB2) (B, B') or *ephrinB1-ires-nlsGFP* (EfnB1) (C, C'). Electroporated GFP+ cells (green) expressing ephrinB2 or ephrinB1 (B, C) but not GFP alone (A) segregate into large clusters associated with disrupted rhombomere boundaries (visible with DAPI).

(D-I) Confocal z-projections of the apical zone of flat mounted hindbrains electroporated with *ephrinB2-nlsGFP* at the level of the midbrain (D-F) or r3 (G-I). Electroporated cells are marked with nuclear GFP (green D, G, white E, H), nuclei with DAPI (D,G) and cell apical profiles with ZO-1 (red D, G, white F, I). Areas delimited by a dotted line mark interfaces between ectopic ephrinB2+ and ephrinB2- cells.

When ephrinB2+ electroporated cells are in the minority, they segregate into clusters with central apical constriction and peripheral apical enlargement that extends to non-electroporated neighbours (D-F). When ephrinB2- cells are in the minority, they segregate into clusters that are associated with apical remodelling (G-I).
Figure S8. Ectopic Krox20, Hoxa2 or Hoxa3 induces apical remodelling. (A-J) Confocal z-projections of the apical and subapical zones of flat-mounted hindbrains after co-electroporation with plasmids expressing Krox20 (A-D) or mHoxa2 (E-G) or mHoxa3 (H-J) and also a plasmid expressing GFP. Images show electroporated cells expressing GFP (green or white), DAPI-stained nuclei (blue), EphA4 or ZO-1 expression (red or white) and actin expression (white). High magnifications show the r4 region with the r3/r4 interface (dotted line) indicated (A-D) or they show the midbrain region (E-J). Cell clusters expressing EphA4 or GFP (dotted line) display marked apical constriction at their centres and apical enlargement at their peripheries, and also in neighbouring cells.
Figure S9. Krox20 activation of EphA4 overrides Hoxa3 repression of EphA4.

(A-J) Flat-mounted hindbrains after electroporation/co-electroporation with plasmids expressing GFP and Krox20 (A,B), mHoxb4 (C,D), mHoxb4 and Krox20 (E,F), mHoxa3 (G,H), or mHoxa3 and Krox20 (I,J). Confocal z-projections show GFP-expressing electroporated cells (green), DAPI-stained nuclei (blue) and EphA4 expression (red or white). Anterior is to the left and the positions of r3 and r5 are marked by stripes of EphA4 expression. Ectopic Krox20 induces large patches of EphA4 in even rhombomeres, containing both electroporated (GFP+) and non-electroporated (GFP-) cells. Ectopic Hoxb4 or Hoxa3 represses EphA4 in odd rhombomeres. Co-expression of Krox20 and Hoxb4, or Krox20 and Hoxa3, activates EphA4 in even rhombomeres but in smaller patches than with Krox20 alone.