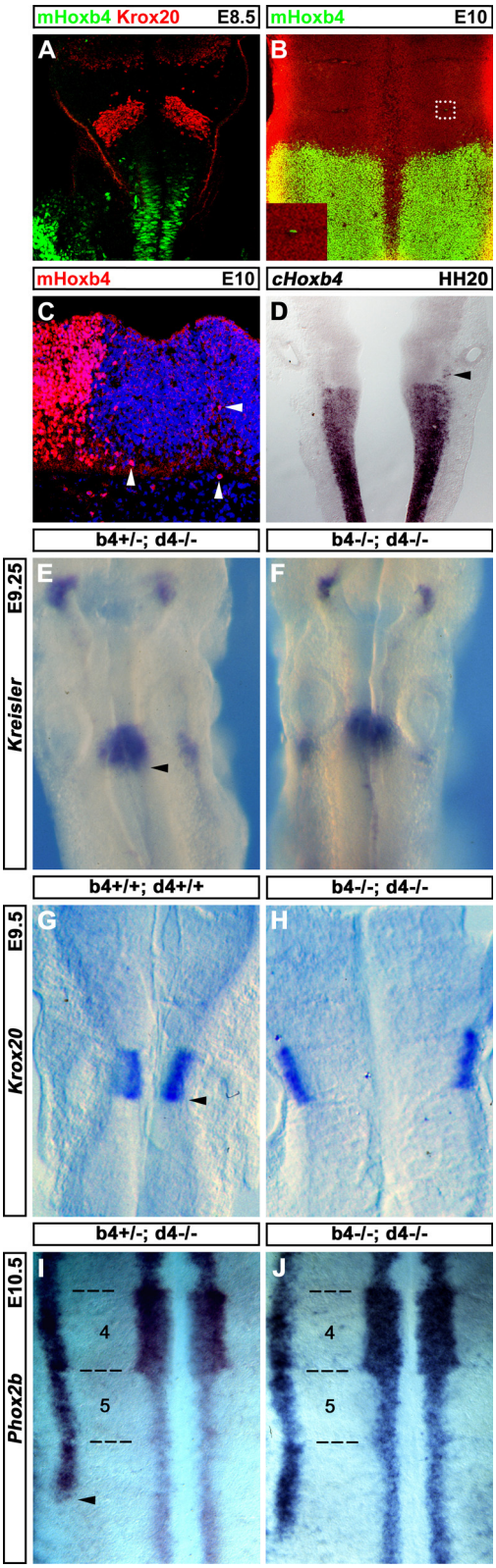
mHoxb4 Krox20 E8.5
A
mHoxb4 E10
B
mHoxb4 E10
C
cHoxb4 HH20
D
b4+/-; d4-/-
b4-/-; d4-/-
Kreisler E9.25
E
F
b4+/+; d4+/+
b4-/-; d4-/-
Krox20 E9.5
G
H
b4+/-; d4-/-
b4-/-; d4-/-
Phox2b E10.5
I
4
5
J
4
5

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).

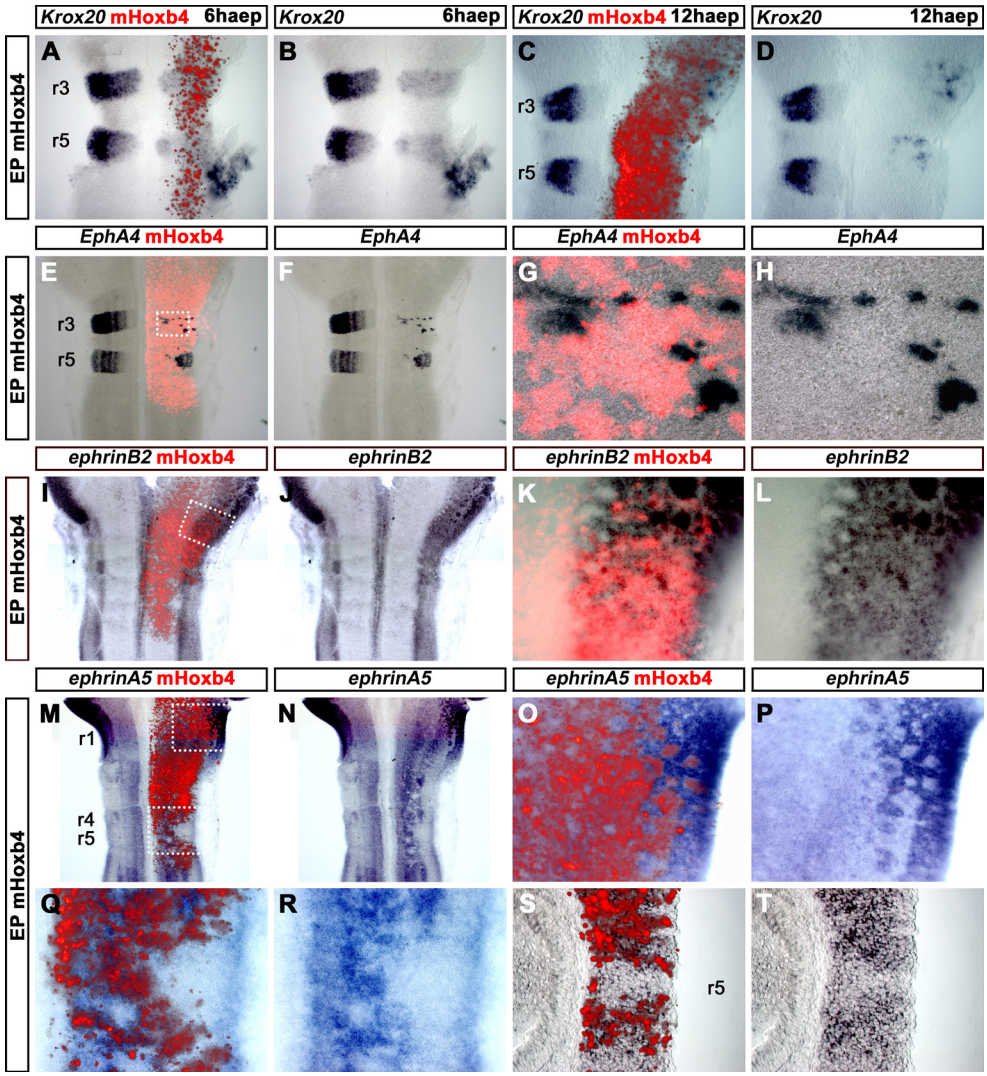
Krox20 mHoxb4 6haep
Krox20 6haep
Krox20 mHoxb4 12haep
Krox20 12haep
EP mHoxb4
A
B
C
D
r3
r5
EphA4 mHoxb4
EphA4
EphA4 mHoxb4
EphA4
E
F
G
H
ephrinB2 mHoxb4
ephrinB2
ephrinB2 mHoxb4
ephrinB2
I
J
K
L
ephrinA5 mHoxb4
ephrinA5
ephrinA5 mHoxb4
ephrinA5
M
N
O
P
r1
r4
r5
Q
R
S
T

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-specifc 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).

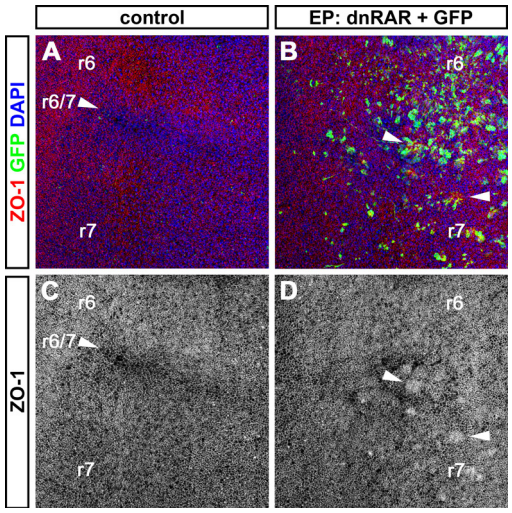

Figure S3. dnRAR induces apical remodelling and disrupts the r6/r7 boundary.

(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.

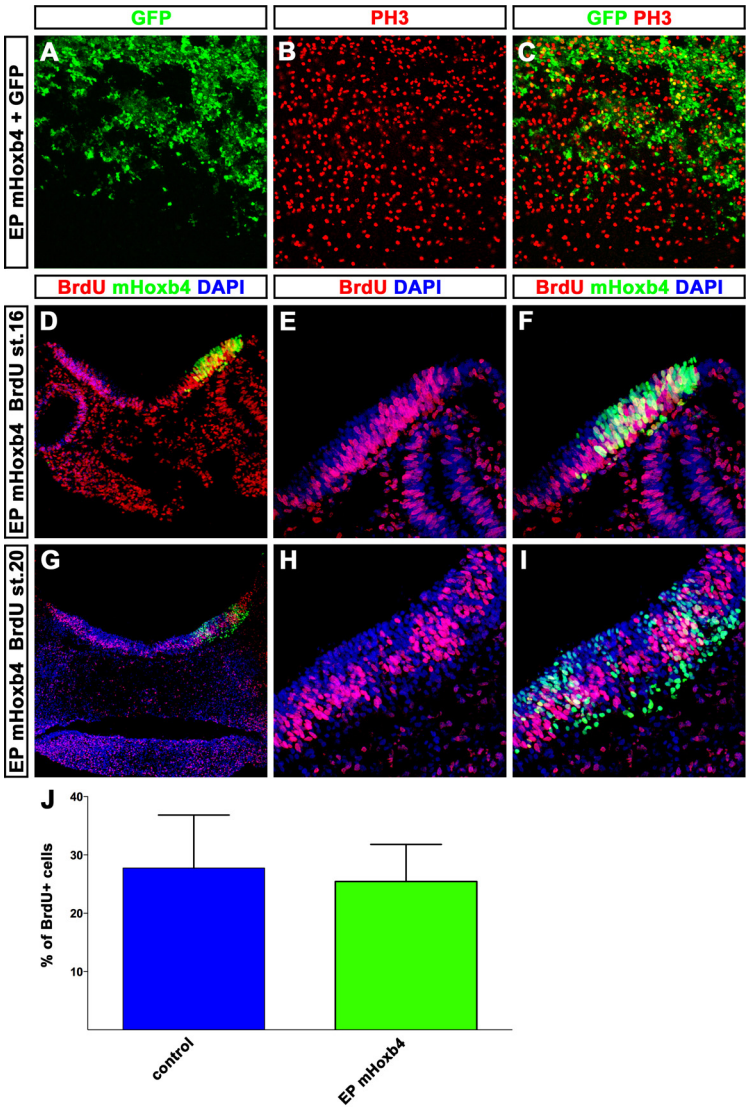

Figure S4. mHoxb4 misexpression does not alter neuroepithelial proliferation.
(A-C) Distribution of mitotic cells in hindbrains 2 days after *mHoxb4* and *GFP* coelectroporation. Single confocal section showing GFP⁺ and PH3⁺ cells.
(D-I) BrdU incorporation following one hour pulse at one day (D-F) or 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).

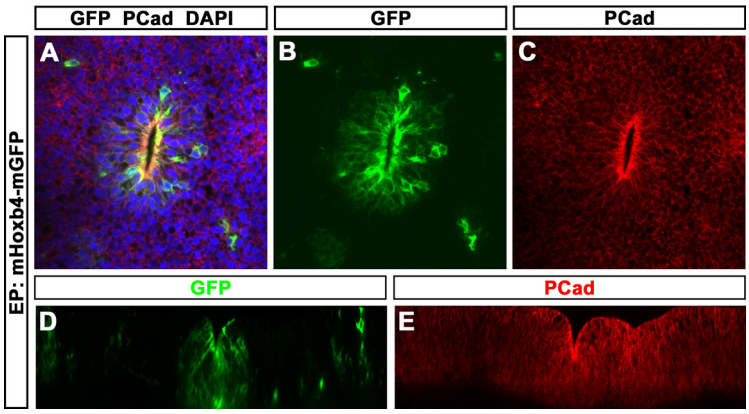

Figure S5. Hoxb4 cell clusters can form neuroepithelial invaginations with strong apical Cadherin staining.
(A-E) Flat-mounted chick hindbrains electroporated with *mHoxb4-ires-myrGFP*. Panels show a single mHoxb4+ cell cluster in r5 with strong pan-cadherin (PCad) staining at the centre (apical surface) of a pronounced invagination. An XY view (A-C) and a YZ section (D-E) of the same cluster are shown.

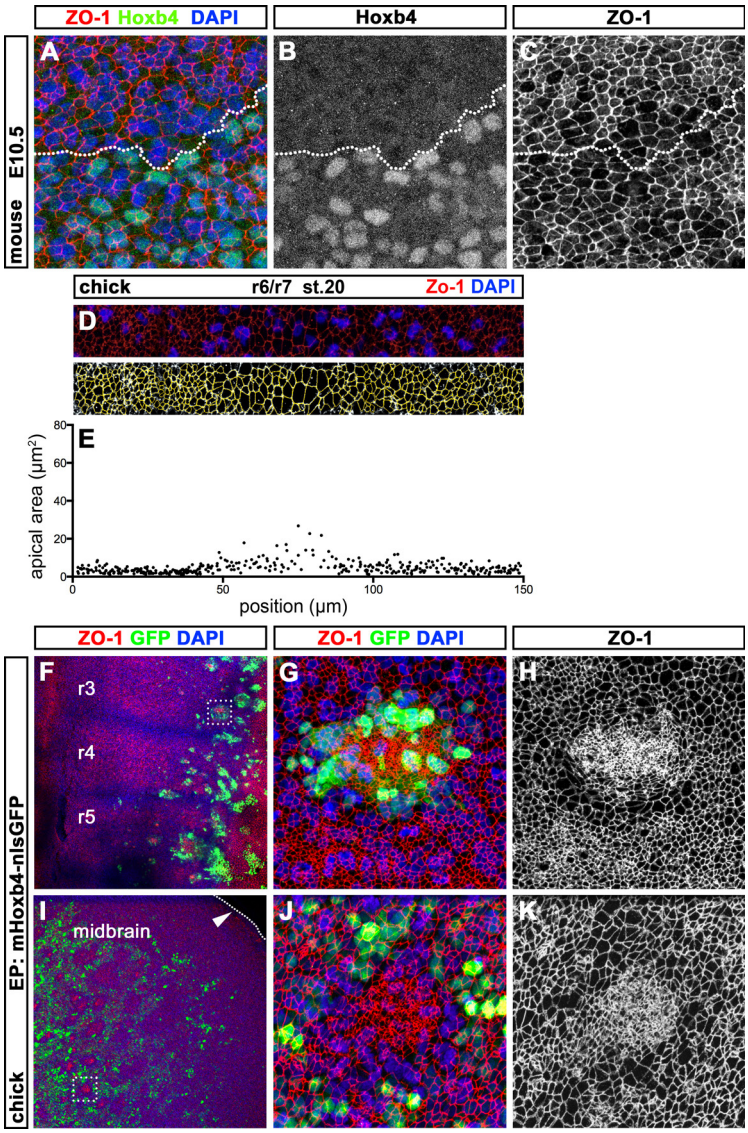
ZO-1 Hoxb4 DAPI
Hoxb4
ZO-1
mouse E10.5
A
B
C
chick
r6/r7 st.20
Zo-1 DAPI
D
E
apical area (µm²)
position (µm)
ZO-1 GFP DAPI
ZO-1 GFP DAPI
ZO-1
chick
EP: mHoxb4-nlsGFP
F
r3
r4
r5
G
H
I
midbrain
J
K

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 (μm^2) 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.

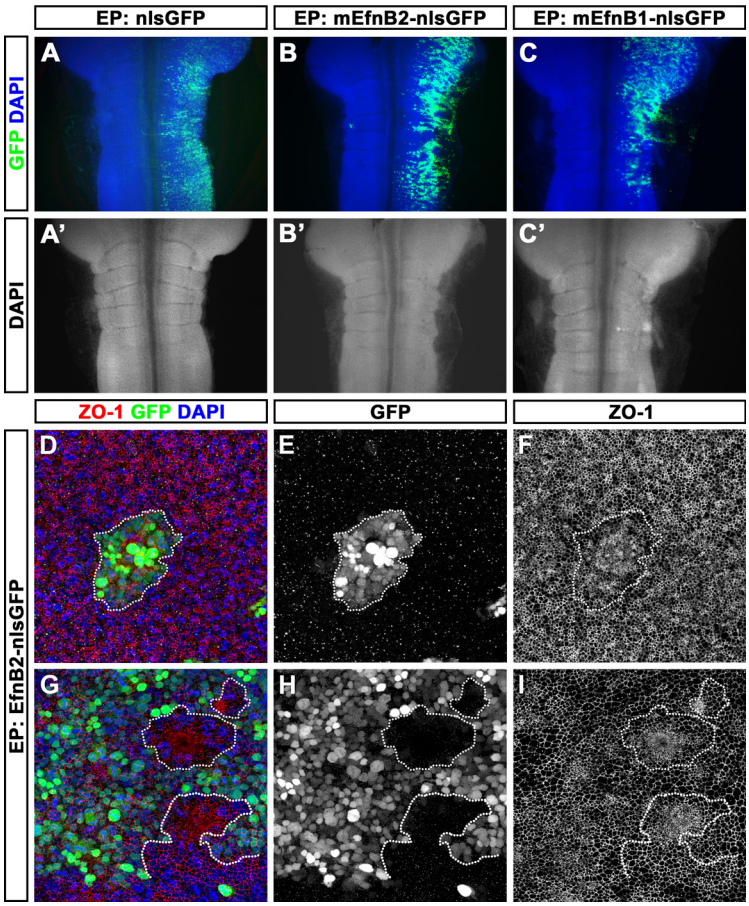

Figure S7. Ectopic ephrinB drives cell segregation, apical remodelling and boundary disruption.

(A-C') Flat-mounted hindbrains electroporated with plasmids epxressing *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).

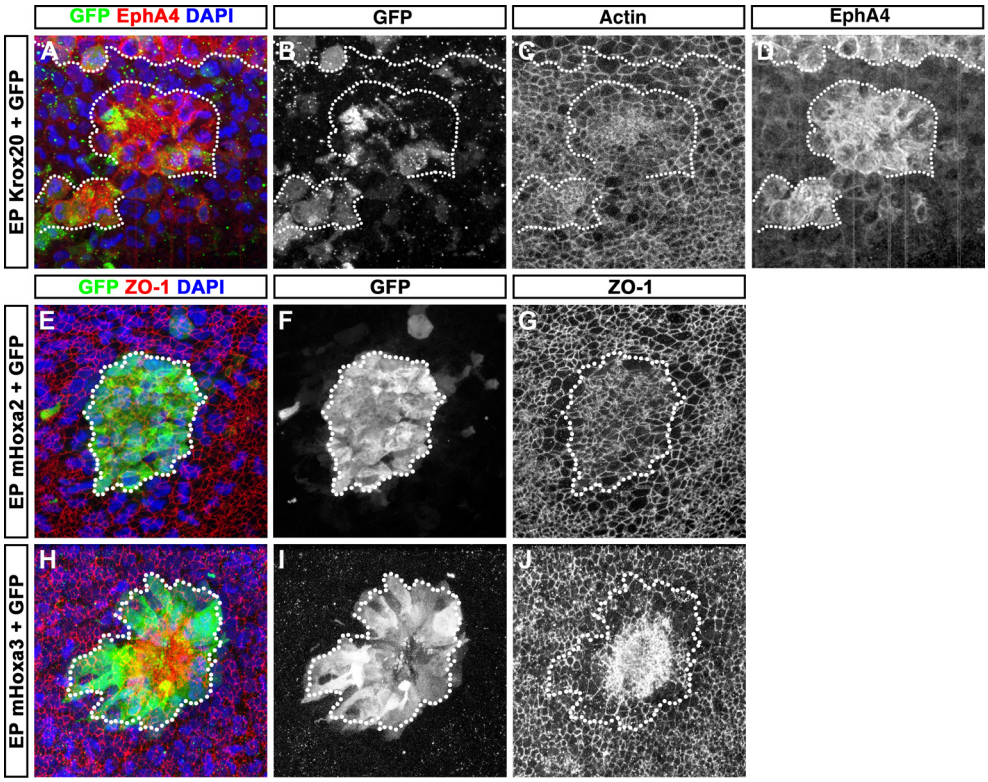

Figure S8. Ectopic Krox20, Hoxa2 or Hoxa3 induces apical remodelling.
(A-J) Confocal z-projections of the apical and subapical zones of flat-mounted hindbrains after co-electroporation with plasmids expressing Krox20 (A-D) or mHoxa2 (E-G) or mHoxa3 (H-J) and also a plasmid expressing GFP. Images show 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.

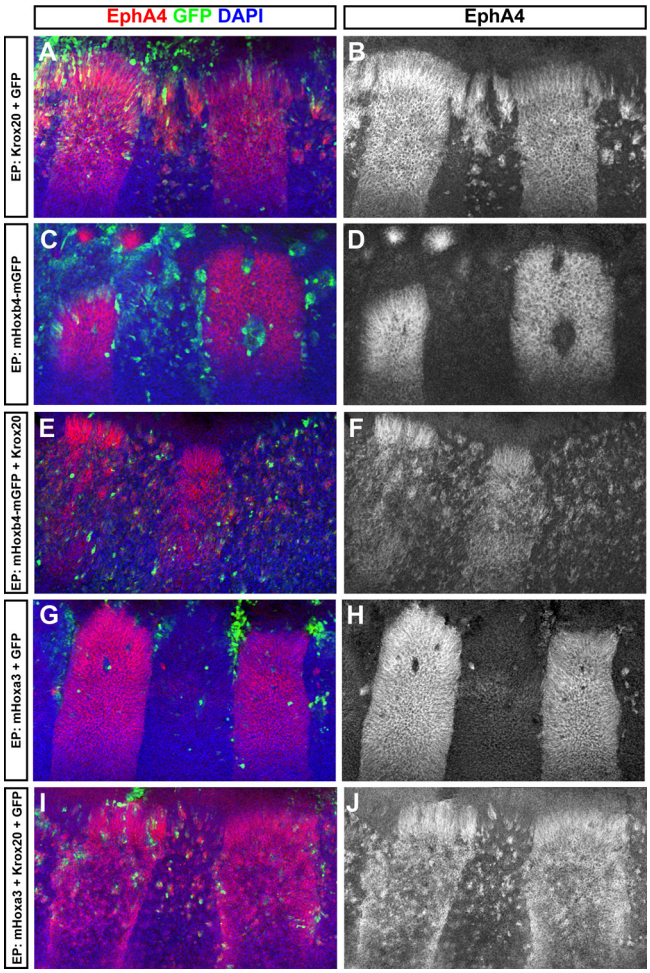

Figure S9. Krox20 activation of EphA4 overrides Hoxa3 repression of EphA4.

(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.