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A novel role for Pax6 in the segmental organization of the hindbrain

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SUMMARY

Complex patterns and networks of genes coordinate rhombomeric identities, hindbrain segmentation and neuronal differentiation and are responsible for later brainstem functions. Pax6 is a highly conserved transcription factor crucial for neuronal development, yet little is known regarding its early roles during hindbrain segmentation. We show that *Pax6* expression is highly dynamic in rhombomeres, suggesting an early function in the hindbrain. Utilization of multiple gain- and loss-of-function approaches in chick and mice revealed that loss of Pax6 disrupts the sharp expression borders of *Krox20*, *Kreisler*, *Hoxa2*, *Hoxb1* and EphA and leads to their expansion into adjacent territories, whereas excess Pax6 reduces these expression domains. A mutual negative cross-talk between Pax6 and Krox20 allows these genes to be co-expressed in the hindbrain through regulation of the Krox20-repressor gene *Nab1* by Pax6. Rhombomere boundaries are also distorted upon Pax6 manipulations, suggesting a mechanism by which Pax6 acts to set hindbrain segmentation. Finally, FGF signaling acts upstream of the Pax6-Krox20 network to regulate *Pax6* segmental expression. This study unravels a novel role for Pax6 in the segmental organization of the early hindbrain and provides new evidence for its significance in regional organization along the central nervous system.

KEY WORDS: Pax6, Boundaries, Hindbrain, Rhombomere, Segmentation, Avian, Mouse

INTRODUCTION

During CNS development, the hindbrain is subdivided along its anterior-posterior (AP) axis into repetitive rhombomeres. Each rhombomere is a polyclonal cell lineage-restricted compartment of distinct genetic identity. The hindbrain develops into the brainstem and cerebellum and links the lower and upper parts of the CNS via extensive neural circuits. These are essential for processing sensory/motor information and controlling vital functions such as respiration. Furthermore, the hindbrain generates cranial nerves and neural crest, which form peripheral neuronal networks and craniofacial structures. These diverse fates are determined by a genetic program that generates, at much earlier stages, the fundamental patterns of rhombomeres (Lumsden, 2004; Lumsden and Krumlauf, 1996).

Multiple transcription factors (TFs) are localized to different segments and govern their identities through complex interactions. A key gene encodes the zinc-finger TF Krox20 (also known as Egr2), which is expressed in rhombomere (r) 3 and r5, and is crucial for their establishment. These segments disappear or expand upon loss or misexpression of Krox20, respectively (Giudicelli et al., 2001; Oxtoby and Jowett, 1993; Schneider-Maunoury et al., 1997). Krox20 is also a central factor for other rhombomeres owing to its regulatory relationship with Hox genes and Eph receptors (Krumlauf, 1994); Krox20 upregulates *Hoxa2*, *Hoxb2* and *Epha4* in r3/r5 and *Hoxb3* in r5, but inhibits other factors, such as *Hoxb1* (Giudicelli et al., 2001; Nonchev et al., 1996a; Nonchev et al., 1996b; Seitanidou et al., 1997). The basic leucine zipper TFs Vhnf1 and Kreisler (also known as Hnf1b and Mafb, respectively) are expressed in r5/r6. Their inactivation leads to r5/r6 specification defects, whereas their overexpression induces ectopic r5/r6 identities (Giudicelli et al., 2003; McKay et al., 1994; Moens et al., 1996; Prince et al., 1998). Vhnf1 upregulates *Kreisler*, which in turn induces *Krox20* and *Hoxb3* in r5 and *Hoxa3* in r5/r6 (Manzanares et al., 2002; Manzanares et al., 1999). Other TFs, such as Pbx/Meis, are also essential for hindbrain patterning via their synergistic activities with Hox factors (Aamar and Frank, 2004; Elkouby et al., 2012; Vlachakis et al., 2001; Wassef et al., 2008). Additional factors also modulate the expression of these TFs, such as Nab and Nlz (also known as Neurl1a), which colocalize with Krox20 but repress its transcription, leading to Krox20 restriction to the correct rhombomeres (García-Gutiérrez et al., 2011; Mechta-Grigoriou et al., 2000; Runko and Sagerström, 2003). Finally, Eph-ephrin signaling acts at the rhombomere interface and restricts cell intermixing by mediating repulsion. This system contributes to the formation of the sharp boundaries that are crucial for hindbrain segmental organization (Cooke et al., 2005; Mellitzer et al., 1999; Sela-Donenfeld et al., 2009; Xu et al., 1995).

Pax6 is a paired domain (PD) and homeodomain (HD) TF. It is central in neural development as it controls patterning and neurogenesis in multiple CNS regions (Osumi et al., 2008). In the forebrain, Pax6 regulates pretectum, thalamus and cortex patterning and is crucial for eye development (Ashery-Padan and Gruss, 2001; Hogan et al., 1986; Stoykova et al., 1996). In the spinal cord it sets the progenitor domains of ventral neurons (Bel-Vialar et al., 2007; Ericson et al., 1997). Along the hindbrain of E12 rat/E3 chick embryos, *Pax6* shows a uniform ventral^{high}-dorsal^{low} pattern and establishes progenitor domains of somatic motoneurons and V1 interneurons. In rat *Pax6* mutants, both neuronal populations are reduced together with loss of the abducens and hypoglossal nerves (Osumi et al., 1997; Takahashi and Osumi, 2002). Moreover, Pax6 regulates the migration and patterning of progenitor cells in the

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rhombic lip (Engelkamp et al., 1999; Landsberg et al., 2005). However, earlier roles of Pax6 during hindbrain segmentation are unknown.

We found that early chick and mouse embryos display segmental *Pax6* expression. Gain- and loss-of-function experiments demonstrated that Pax6 restricts the expression domains of various segmental genes and governs the AP organization of rhombomeres and boundaries. These findings contribute new understanding to how hindbrain segmentation is controlled, which is fundamental to the development of the various CNS regions where Pax6 is expressed.

MATERIALS AND METHODS

Embryos and genotyping

Loman chick eggs were incubated and treated as previously described (Weisinger et al., 2008). *Pax6*$^{+/-}$*/lacZ* mice were obtained from P. Gruss and A. Stoykova (Max-Planck Institute, Göttingen, Germany). *Pax6-LacZ* and *Pax6*$^{+/+}$ embryos were derived from intercrossing of heterozygous parents (St-Onge et al., 1997). Wild-type (WT) ICR mice were obtained from Harlan Laboratories (Jerusalem, Israel). Chick and mouse embryos were fixed in 4% paraformaldehyde, dehydrated in 100% methanol, and stored at −20°C. Genotyping of mice was performed on yolk sacs by PCR using the following primers: forward, 5′-GATTCTCCAGTTCAGGCACCAGGT-3′; reverse, 5′-TCCCAGTGGCTTGGACTCCTCAAG-3′; reverse, 5′-CCATTCAGGCTGCGCAACTGTTG-3′.

In ovo electroporation

pCIG-IRES-GFP, pCIG-Pax6-IRES-GFP, pCIG-Pax6-En-IRES-GFP, pCAGGS-RFP, pCAGGS-siRNAPax6-RFP, pBRE-lacZ, pAdRSVβgal-Krox20 and pAdRSVNab1-HA constructs (Bel-Vialar et al., 2007; Das et al., 2006; Giudicelli et al., 2001) (supplementary material Fig. S1A) were electroporated (2-4 μg/μl) into the hindbrain of 2- to 4-somite stage embryos and harvested 16-48 hours later, as described (Weisinger et al., 2008). FITC-conjugated morpholino (MO) oligonucleotides (GeneTools) were diluted in PBS to a working concentration of 2 mM and electroporated as described above. The sequences used were: Nab1-MO, 5′-CGCTGACGCCATCACGGATGACAGA-3′; control-MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

Bead implantation and cyclohexamide treatment

AG1-X2 beads (100-200 mesh, BioRad) were soaked in SU5402 (200 μM; Calbiochem) and implanted into the hindbrain lumen of chick embryos (Weisinger et al., 2010). Cyclohexamide (10 μg/ml; Sigma) was added to embryos ~6 hours after electroporation. Embryos treated with SU5402 or cyclohexamine were incubated for 16 or 12 hours, respectively, before fixation.

In situ hybridization (ISH), immunohistochemistry (IHC) and X-Gal staining

Whole-mount ISH was performed as described (Weisinger et al., 2008). Chick probes included *Kreisler*, *Pax6*, *Krox20*, *Hoxb1*, *Hoxa2*, *Cyp26b1*, *Cad7*, follistatin, *Fgf3* (Irving et al., 1996; Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld et al., 2009; Swindell et al., 1999; Weisinger et al., 2008) and *Nab1* (EST clone 651m17, MRC Geneservices, UK). Mouse probes included *Pax6*, *Krox20*, *Hoxb1*, *Hoxa2*, *Epha4*, *Fgf8* and *Kreisler* (Frohman et al., 1990; Hunt et al., 1991; Irving et al., 1996; Stoykova and Gruss, 1994; Theil et al., 2002; Tilleman et al., 2010). Double ISH was performed as described (Weisinger et al., 2008).

IHC on whole mounts or on frozen sections was performed as described (Kohl et al., 2012; Weisinger et al., 2008) using the following antibodies: rabbit anti-GFP/RFP (1:400; Molecular Probes), rabbit anti-EphA4 [1:200, D. Wilkinson, NIMR, London, UK (Irving et al., 1996)], mouse anti-chondroitin sulfate proteoglycan (1:50; Sigma), mouse anti-neurofilament associated protein 3A10 [1:4; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Pax6 (1:50; DSHB), sheep anti-fluorescein (1:2000; Roche) and rat anti-HA (1:400; Roche). Secondary antibodies included anti-mouse/rabbit Alexa Fluor 488/594 (1:400; Molecular Probes) or HRP (1:250; Sigma), and biotin-coupled goat anti-rat IgG (1:200; Jackson). Some embryos were stained with AEC (Lab Vision Corporation, Fremont, CA, USA) for HRP activity. Mouse X-Gal staining was performed as described (St-Onge et al., 1997).

Cell death and proliferation

Cell death was detected as described previously (Weisinger et al., 2008), using the In Situ Cell Death Detection Kit, POD (Roche) or rabbit anti-caspase 3 antibody (1:40; Cell Signaling). Mitosis index was detected using phosphorylated histone H3 (pH3) antibody (1:40; Santa Cruz Biotechnology) as described (Monsonego-Ornan et al., 2012).

Data analysis

Quantification of *Krox20*$^{+}$ domains was performed by measuring the mean area of *Krox20* expression of ten randomly chosen embryos/treatment using ImageJ software (NIH) and calculating the ratio of *Krox20*$^{+}$ areas in the electroporated side versus the control side. Quantification of mitotically active cells was performed by counting pH3^{+} cells from six randomly chosen embryos/treatment using ImageJ and calculating the ratio of pH3^{+} cells on control versus electroporated sides. Significance was determined using the unpaired Student's *t*-test.

RESULTS

Expression of *Pax6* in chick and mouse hindbrain

Initiation of *Pax6* expression in the chick hindbrain lags behind that in the spinal cord and forebrain (Fig. 1A). It begins as weak staining in r3 in 6-somite stage embryos (Fig. 1B) and by the 8- to 10-somite stage it strengthens and broadens in r3 from lateral to medial, excluding the ventral-most region (Fig. 1C,D). At 10 somites, *Pax6* appears faintly also in r5 (Fig. 1D) and becomes enhanced at 12 somites (Fig. 1E). High levels of *Pax6* remain in r3/r5 up to the 30-somite stage (Fig. 1F-J). From the 14-somite stage, *Pax6* also appears in r2/r4/r6 (Fig. 1F-I), although in a weaker and narrower dorsal-ventral (DV) pattern compared with that in r3/r5. *Pax6* is excluded from rhombomere borders at these stages. A marked change is seen in 35-somite embryos (Fig. 1J), in which *Pax6* becomes distributed in a longitudinal ventral^{high}-dorsal^{low} stripe along all rhombomeres, excluding the ventral-most and dorsal-most regions. Furthermore, enhanced *Pax6* expression is seen at rhombomere boundaries, where it covers a larger DV portion compared with the rhombomeres. Analysis of Pax6 protein revealed similar patterns in the segmented hindbrain (Fig. 1E′,I′; data not shown).

Pax6 expression was also studied in wild-type (WT) mice by ISH, and in *Pax6-LacZ* mutants, in which the tracing of endogenous *Pax6* is evident by *lacZ* staining (St-Onge et al., 1997). In both mice at E8.0, *Pax6* is evident in the spinal cord and forebrain but excluded from the hindbrain (Fig. 1N; data not shown), whereas at E8.5 *Pax6* becomes upregulated in r3/r5 (Fig. 1K; data not shown). In E9-9.5 mouse embryos, *Pax6* is found in all rhombomeres, yet it remains enhanced and broader in r3/r5 compared with other segments (Fig. 1L,M,O,P). The segmental and dynamic pattern of *Pax6* expression in the mouse hindbrain, which has also been reported in early fish, rat and frog hindbrain (Derobert et al., 2002; Qiu et al., 2009; Takahashi and Osumi, 2011), is compatible with that observed in chick (Fig. 1A-I).

A negative cross-talk between Pax6 and Krox20 mediated via Nab1

The rhombomeric *Pax6* expression suggests a role in hindbrain segmentation. To test this, gain- and loss-of-function experiments were conducted in chick and mice. Various pCAGG plasmids, previously used to misexpress or inhibit Pax6 in the spinal cord (Bel-Vialar et al., 2007; Das et al., 2006; Matsunaga et al., 2000), were utilized. These included green/red fluorescent protein cDNAs as controls (GFP/RFP), *Pax6* full-length cDNA for overexpression

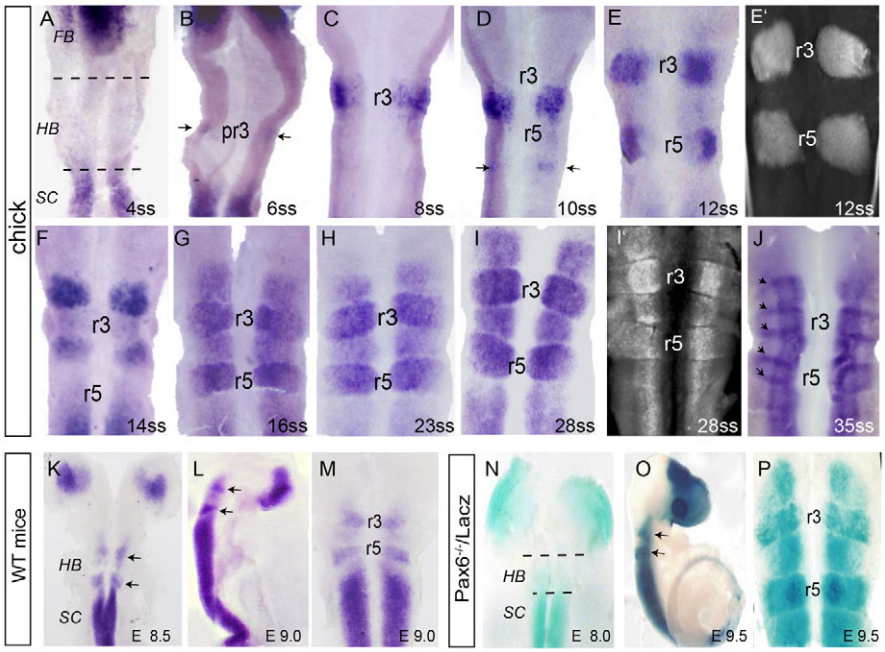

Fig. 1. Expression of *Pax6* in chick and mouse hindbrain. (**A-P**) *In situ* hybridization (ISH) (A-M), immunohistochemistry (IHC) (E′,I′) or *lacZ* staining (N-P) were performed on chick (A-J), wild-type (WT) mice (K-M) and *Pax6-LacZ* mice (N-P) to detect *Pax6* mRNA (A-M) and Pax6 protein expression (E′,I′) at different stages in whole embryos (A,B,K,L,N,O) or flat-mounted hindbrains (C-J,M,P). Arrows indicate *Pax6* expression sites. HB, hindbrain; SC, spinal cord; FB, forebrain; pr, presumptive rhombomere; r, rhombomere; E, embryonic day; SS, somite stage.

(Pax6-GFP), *Pax6* HD and PD cDNA fused to the Engrailed domain to act as a dominant repressor (Pax6-En-GFP), and siRNA to knock down Pax6 (Pax6-siRNA-RFP) (supplementary material Fig. S1A). Plasmids were unilaterally electroporated into the hindbrain of 2- to 4-somite embryos in order to target stages at which endogenous Pax6 has been initiated by the time of plasmid expression. Unless indicated otherwise, embryos were harvested 16-20 hours later, at the 15- to 18-somite stage. The ability of these constructs to express exogenous Pax6 or to downregulate endogenous Pax6 was confirmed (supplementary material Fig. S1B-E).

Krox20 is a central TF expressed in r3/r5 (Oxtoby and Jowett, 1993; Swiatek and Gridley, 1993). Detailed examination of *Krox20* dynamics demonstrates its initiation prior to *Pax6* (supplementary material Fig. S2A,C; Fig. 1A,D), although subsequently *Krox20* and *Pax6* overlap in r3/r5 (supplementary material Fig. S2B-D; Fig. 1C-F). Also at later stages, when Pax6 appears in other segments, Pax6 remains higher in r3/r5 (Fig. 1G-I). The comparable expression of these genes suggested their possible interaction; hence, we determined whether manipulation of Pax6 affects *Krox20*. Whereas *Krox20* was restricted to r3/r5 in controls (Fig. 2A-B′; n=21/21) and in the control side of Pax6-manipulated chick embryos (Fig. 2C-H, left side), hindbrains expressing ectopic Pax6 showed dramatic reduction in *Krox20*-expressing (*Krox20*$^+$) domains (Fig. 2C-D′; n=23/26). Conversely, embryos expressing Pax6-En-GFP or Pax6-siRNA-RFP showed expansion in *Krox20*$^+$ territories into adjacent segments (Fig. 2E-H′; n=36/43 for Pax6-En-GFP, n=12/15 for Pax6-siRNA-RFP) and fuzzy boarders of r3/r5 with an overall increase in the segment size. In many cases, *Krox20* expression levels appeared elevated compared with contralateral rhombomeres (Fig. 2F,H,J). The severity of the effects was variable between embryos, probably owing to differences in electroporation efficiency or intrinsic variability in cell mixing. Alternatively, this might have resulted from some ectopic *Krox20*$^+$ cells that switched their identities so that it was similar to that of neighboring cells. Pax6 effects appeared both cell-autonomous and non-cell-autonomous, as the loss or expansion of *Krox20* expression did not always coincide with Pax6-GFP, Pax6-En-GFP or Pax6-siRNA-RFP expression (Fig. 2E-H). Quantification of these results revealed a ~50% decrease and ~30% increase in *Krox20*$^+$ regions upon Pax6 overexpression and knockdown, respectively (Fig. 2N). Together, these data show a negative effect of Pax6 on *Krox20* to restrict its expression domains in the hindbrain.

To confirm these Pax6 effects, a rescue experiment was performed. Chick embryos were electroporated with Pax6-GFP, Pax6-En-GFP or with both constructs to antagonize each action. As before, *Krox20*$^+$ domains dramatically decreased or increased in embryos expressing Pax6-GFP (Fig. 2I; n=5/5) or Pax6-En-GFP (Fig. 2J; n=10/10), respectively. However, relatively normal *Krox20* patterns were re-established in the rescued embryos (Fig. 2K; n=5/5).

Since Pax6 manipulations impaired normal *Krox20* domains, Pax6 effects on cell death and proliferation were examined. Analysis of cell death was performed in chick embryos expressing each plasmid and revealed similar low-level cell death in each type of treated hindbrain (supplementary material Fig. S3A-C; n=12 for each). Analysis of mitosis revealed no marked differences in controls compared with Pax6-manipulated embryos (supplementary material Fig. S3D-F; n=9 for each). Measuring the area (width × length) of electroporated versus contralateral hindbrain sides indicated no marked differences in size (data not shown). Hence, the perturbed expression of *Krox20* upon Pax6 manipulations cannot be attributed to major changes in cell death or proliferation.

We next determined whether Pax6 is involved in setting *Krox20*$^+$ domains in mice. Hindbrains obtained from E8.5 *Pax6*$^{+/+}$ mice showed normal *Krox20* expression in r3/r5, with somewhat higher levels in r5. At E9.0, *Krox20* was downregulated in r3 but was still sharply defined in expression in r5 (Fig. 2O,Q; n=4 for each stage) (see also Voiculescu et al., 2001). *Pax6-LacZ* mutants showed enlarged *Krox20*$^+$ domains and enhanced staining in r3/r5, together with an irregular shape to the r3/r5 borders (Fig. 2P,R; n=4 for E8.5, n=5 for E9.0). This result agrees with recent data from *Pax6* mutant rats, in which *Krox20* expression in r5 was enlarged (Numayama-Tsuruta et al., 2010). Notably, the expression of the mid-hindbrain boundary gene *Fgf8* remained similar in *Pax6*$^{+/+}$ and *Pax6-LacZ* embryos, as did the size and general morphology of these hindbrains (Fig. 1S,T; n=4 for each; data not shown). These observations

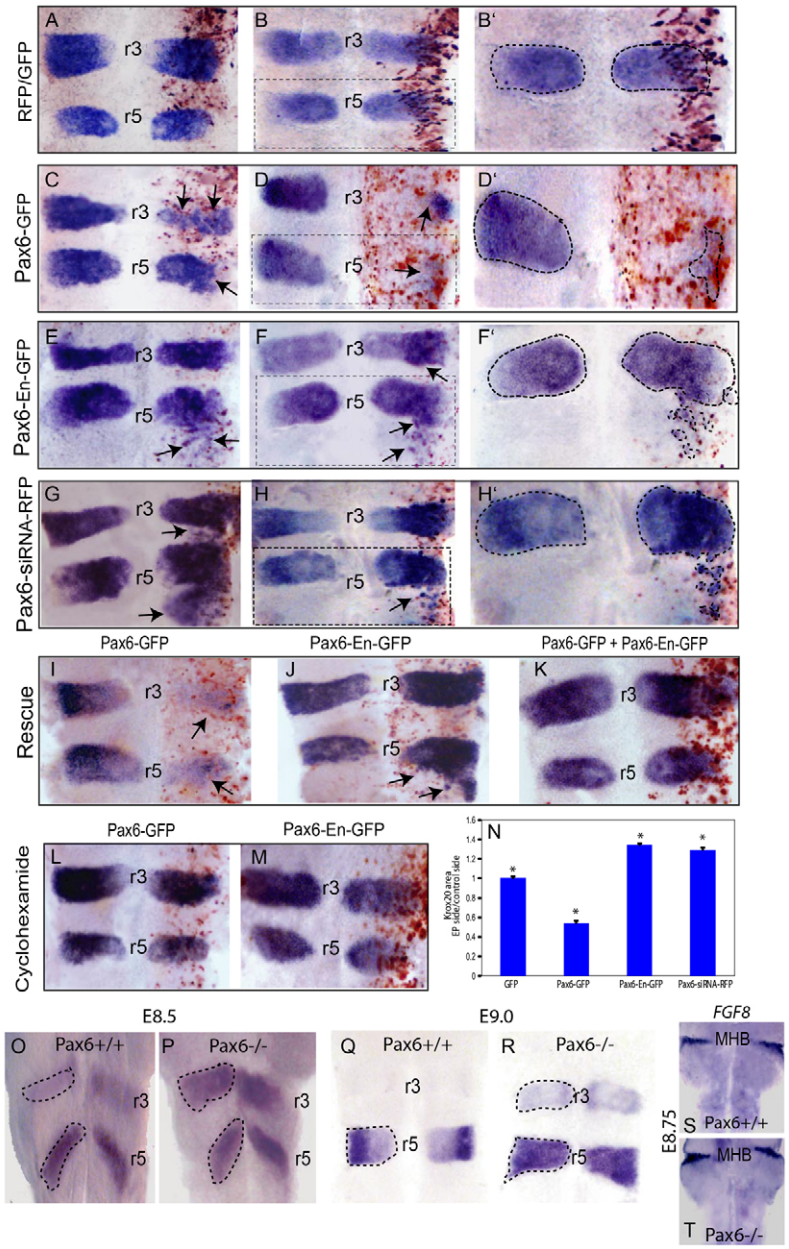

Fig. 2. Alterations in *Krox20* expression domains upon Pax6 loss- and gain-of-function. (**A-M**) Flat-mounted hindbrains of chick embryos that were electroporated in the right side with control RFP (A), control GFP (B,B′), Pax6-GFP (C-D′,I,L), Pax6-En-GFP (E-F′,J,M), Pax6-siRNA-RFP (G-H′), and both Pax6-GFP and Pax6-En-GFP (K) constructs and subject to ISH to detect *Krox20*. (L,M) Embryos were treated with cyclohexamide. (B′,D′,F′,H′) Enlargements of boxed regions in B,D,F,H. (**N**) Quantification of *Krox20*$^{+}$ areas [ratio of electroporated (EP) to control side] with the different treatments. Error bars indicate s.d. **P*<0.01. (**O-T**) *Krox20* (O-R) and *Fgf8* (S,T) expression in flat-mounted hindbrains of *Pax6*$^{+/+}$ (O,Q,S) or *Pax6*$^{-/-}$ (P,R,T) mouse embryos. In all images, brown staining shows RFP/GFP-expressing cells, dashed ellipses indicate the boundaries of *Krox20*$^{+}$ domains and arrows mark distorted *Krox20* expression. MHB, midbrain-hindbrain boundary.

exclude the possibility of *Krox20* effects resulting from a general developmental defect or delay in the *Pax6* mutants, and suggest conserved roles of Pax6 in restricting *Krox20* domains to r3/r5 in different species.

Pax6 serves mainly as a transcriptional activator (Ericson et al., 1997; Osumi et al., 2008). Hence, the negative (and non-cell-autonomous) effect of Pax6 argues against the possibility of Pax6 as a direct repressor of Krox20. Consistent with this, if Pax6 acts as a repressor we would expect both the Pax6-GFP and Pax6-En-GFP constructs to repress *Krox20* and for Pax6-siRNA to enhance *Krox20*, contrary to our findings. Further support for the indirect activity of Pax6 on *Krox20* was provided by treating embryos several hours after electroporation with cyclohexamide to prevent *de novo* protein synthesis. *Krox20* patterns remained unaffected (Fig. 2L,M; *n*=16/16 for each treatment), indicating that the negative effect of Pax6 on *Krox20* is indirect and requires other mediators.

Nab1/2 are zinc-finger proteins that directly antagonize *Krox20* transcriptional activity (LeBlanc et al., 2006; Russo et al., 1995; Svaren et al., 1996). A negative-feedback loop has been found between these proteins in the hindbrain; Nab1/2 are expressed in r3/r5 and repress *Krox20* transcription in these segments, whereas Krox20 positively regulates Nab1/2 expression (Desmazières et al., 2009; Mechta-Grigoriou et al., 2000). This cross-talk was suggested to ensure an equilibrated *Krox20* expression, which is required to control its different activities (such as proliferation versus regulation of gene expression). As Pax6 recapitulates *Krox20* patterns in r3/r5 during early stages, and yet it represses *Krox20* via an indirect mechanism, we examined whether Pax6 functions through the induction of Nab1. *Nab1* was expressed normally in r3/r5 in control chick embryos (Fig. 3A,A′; *n*=8/8) and in the control side of Pax6-manipulated hindbrain (Fig. 3B,C, left). Strikingly, overexpression of Pax6 resulted in upregulation and expansion of *Nab1* into other segments (Fig. 3B; *n*=7/8), whereas Pax6-En substantially reduced *Nab1* within its normal domains (Fig. 3C; *n*=6/6). These results indicate that Pax6 induces the expression of the Krox20-repressor Nab1, which in turn may act to limit *Krox20*$^{+}$ domains.

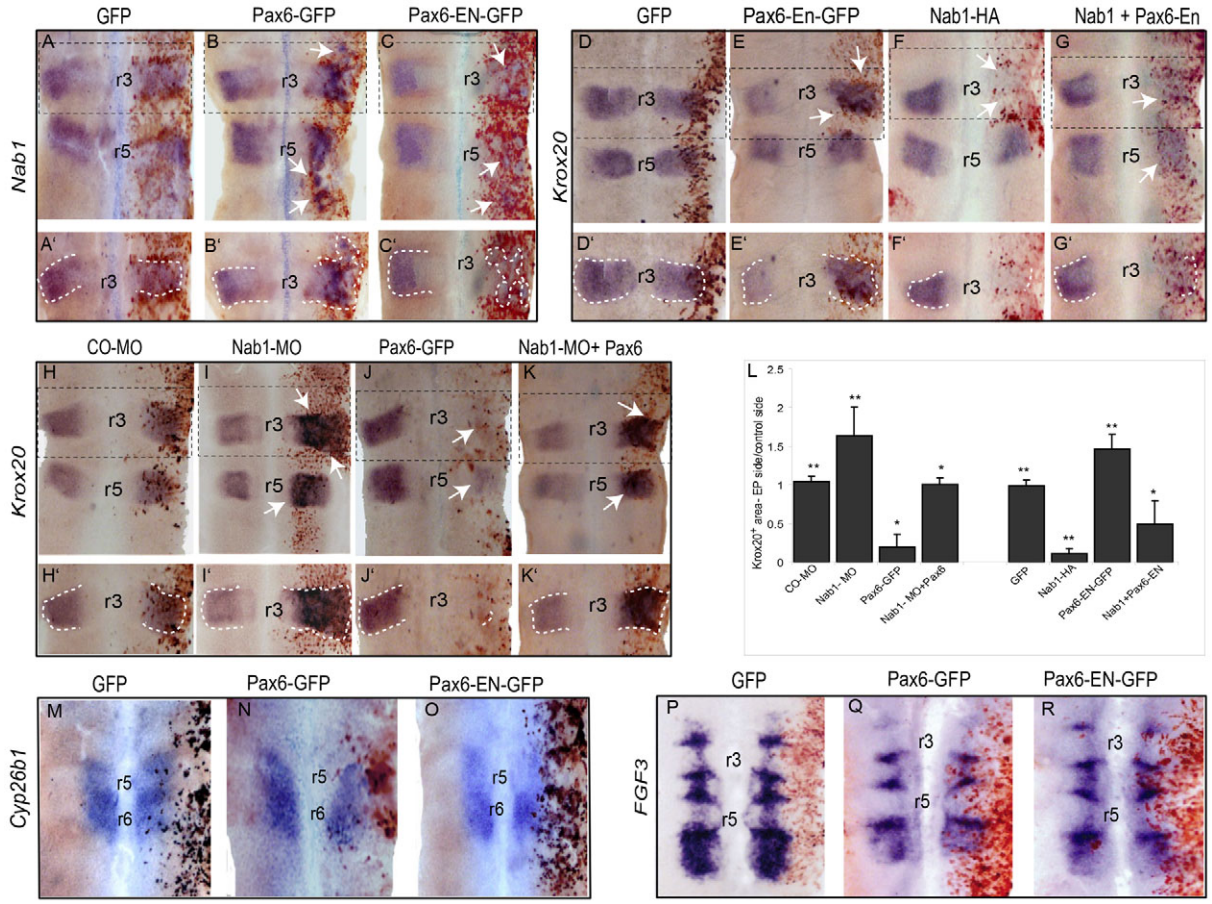

Fig. 3. Pax6 upregulates *Nab1* to restrict *Krox20* expression domains. (**A-K,M-R**) Flat-mounted hindbrains of chick embryos that were electroporated in the right side with control GFP (A,A′,D,D′,M,P), Pax6-GFP (B,B′,J,J′,N,Q), Pax6-En-GFP (C,C′,E,E′,O,R), Nab1-HA (F,F′), control-MO (H,H′), Nab1-MO (I,I′) both Pax6-En-GFP and Nab1-HA (G,G′), and both Pax6-GFP and Nab1-MO (K,K′) constructs and subject to ISH to detect *Nab1* (A-C′), *Krox20* (D-K), *Cyp26b1* (M-O) and *Fgf3* (P-R). Brown staining indicates cells expressing GFP, HA or MO-FITC. (A′-K′) Views of the boxed regions in A-K. White dashed lines indicate *Nab1* (A-C) or *Krox20* (D-K) boundaries. White arrows indicate altered *Nab1* (A-C) or *Krox20* (D-K) expression. (**L**) Quantification of *Krox20*$^{+}$ areas with the different Nab1 treatments. Error bars indicate s.d. **P*<0.05, ***P*<0.01.

To confirm such a triple cross-talk, we tested whether Pax6 is capable of affecting *Krox20* in embryos depleted of Nab1. FITC-conjugated morpholino antisense oligonucleotides (MOs) directed against the 5′UTR of chick *Nab1* (Nab1-MO), or control FITC-conjugated MO (control-MO), were electroporated into chick hindbrain alone or together with Pax6-GFP plasmid and examined for *Krox20* expression. Control-MO did not alter *Krox20* expression (Fig. 3H,H′; *n*=8/8), whereas Nab1-MO resulted in a dramatic increase in the size and intensity of the *Krox20* domains (Fig. 3I,I′; *n*=9/10). Conversely, similar to our previous data (Fig. 2), overexpression of Pax6 led to reduced *Krox20*$^{+}$ domains (Fig. 3J,J′; *n*=6/6). However, the Pax6 effect was completely reversed in the background of the Nab1 morphants (Fig. 3K,K′; *n*=12/12), which demonstrated expanded *Krox20*$^{+}$ domains, although less so compared with single Nab1-MO embryos. Quantification of this rescue experiment is shown in Fig. 3L and the exclusion of any MO side-effect on cell death or proliferation is provided in supplementary material Fig. S3. These results suggest that Nab1 is downstream of Pax6 in mediating its inhibitory effect on *Krox20* expression, which is lost upon Nab1 knockdown.

Next, we performed the opposite experiment to examine whether overexpression of Nab1 is sufficient to rescue the effect of dominant-negative Pax6 on *Krox20*. Chick embryos of similar stages as above were electroporated with plasmids encoding Pax6-En-GFP, Nab1-HA or both. *Krox20* expression domains and levels were unaffected in controls (Fig. 3D,D′; *n*=5/5) and increased with Pax6-En (Fig. 3E,E′; *n*=4/4, similar to the experiment in Fig. 2), whereas excess Nab1 induced substantial reduction in *Krox20* expression (Fig. 3F,F′; *n*=9/10) [as also shown previously (Desmazières et al., 2009; Mechta-Grigoriou et al., 2000)]. Strikingly, the expansion of *Krox20* by Pax6 was completely reversed in embryos co-expressing Nab1 and Pax6-En (Fig. 3G,G′; *n*=7/8), which demonstrate loss in *Krox20* expression, albeit somewhat less so compared with the single Nab1-expressing embryos, as expected when using two plasmids oppositely affecting *Krox20*. Quantification of these results is provided in Fig. 3L. Notably, the effect of Nab1 gain- and loss-of-function on *Krox20* expression seems both cell-autonomous and non-cell-autonomous, raising the possibility that the initial effects of Nab1 on *Krox20* lead to secondary cell-autonomous and non-cell-autonomous effects of Krox20 on its own regulation or that cells may lose their identities (Giudicelli et al., 2001). Altogether, these data provide the first evidence for a Pax6-Nab1-Krox20 network by showing that the Krox20-repressor Nab1 is induced by Pax6 and acts downstream of it to restrict *Krox20* expression to its proper domains and levels.

Retinoic acid (RA) is central hindbrain AP regulator (Dupé and Lumsden, 2001; Glover et al., 2006; Niederreither et al., 2000). Reduced or excess RA signal switches rhombomeres into more anterior or posterior identities, respectively. As with Krox20, RA inhibition results in expansion of r3 and loss of r5, whereas excess RA causes enlargement of r5 at the expense of r3 (Abu-Abed et al., 2001; Dupé and Lumsden, 2001; Hernandez et al., 2007; Morriss-Kay et al., 1991; Niederreither et al., 2000). Recent microarray data obtained from E11.5 *Pax6* mutant rats revealed reduction in the mRNA of the RA-degrading enzyme Cyp26b1 compared with WT, suggesting that Cyp26b1 is downstream of Pax6 at that stage. Moreover, r5, but not r3, was expanded in the rat *Pax6* mutant, indicating that RA signaling is enhanced leading to general hindbrain posteriorization (Numayama-Tsuruta et al., 2010). Based on this study, we analyzed whether Pax6 affects *Cyp26b1* in the early chick hindbrain. Embryos were electroporated with GFP, Pax6-GFP or Pax6-En-GFP and examined for *Cyp26b1*, which is expected in r5/r6 at the stage examined (16-18 somites) (Reijntjes et al., 2003). No change was found in *Cyp26b1* patterns in either treatment (Fig. 3N-O; n=6/6 for each). Fibroblast growth factor 3 (Fgf3), which displays a segmental pattern in the hindbrain (Mahmood et al., 1995; Weisinger et al., 2008), was previously shown to be directly affected by RA (Niederreither et al., 2000). Yet, electroporation of either of the constructs did not affect *Fgf3*, which remained normal in expression to the expected stage (Fig. 3P-R; n=17/18 for each). These results argue against the possibility that RA signaling mediates Pax6 effects on *Krox20* in the early chick hindbrain, and are at variance with its suggested effect at much more advanced stages in the rat. They also fit with our data showing that *Krox20*$^{+}$ domains are affected in both r3 and r5 upon Pax6 manipulation, rather than only in r5, as would be predicted upon excess RA signaling. Additionally, the lack of effect on *Cyp26b1* and *Fgf3* confirms the specific effect of Pax6 on *Krox20* rather than on any gene examined. As the patterns of *Fgf3* and *Cyp26b1* change dynamically at subsequent developmental stages, these results also suggest that Pax6 manipulations do not lead to a general developmental delay in the hindbrain.

Since Pax6 overlaps with *Krox20* in r3/r5, yet it negatively regulates *Krox20*, we asked how these factors can co-exist in r3/r5. One possible scenario is a double negative-feedback loop that would result in mutual Krox20 and Pax6 repression leading to their balanced expression. We examined how excess Krox20 affects *Pax6* expression by electroporating chick embryos at 2-4 somites with control-βgal or with pAdRSVβgal-Krox20 plasmids (Giudicelli et al., 2001). Embryos were analyzed for *Pax6* 18 hours later. Control embryos showed intense *Pax6* in r3/r5 and lower expression in other segments (Fig. 4A,A′; n=10/10), as expected at this stage (Fig. 1G). Krox20 misexpression resulted in downregulation of *Pax6* in the electroporated side, as compared with the contralateral side or control embryos (Fig. 4B,B′; n=12/15). Since *Pax6* expression was slightly masked by the *lacZ* staining (Fig. 4A,B), we also co-electroporated the pAdRSVβgal-Krox20 plasmid with the pCAGG-GFP construct (in a 10:1 ratio) and stained for *Pax6* and the less obtrusive GFP. Similar loss of *Pax6* was observed (Fig. 4D,D′; n=5/7), in comparison to controls (Fig. 4C,C′; n=10/10). These results demonstrate a negative effect of Krox20 on *Pax6* in r3/r5, indicating a bi-directional negative regulatory cross-talk between these genes.

Disrupted EphA4 expression and impairment of boundaries upon Pax6 manipulation

EphA4 is a direct target of Krox20 (Theil et al., 1998). The interaction between EphA4 and ephrins at rhombomere interfaces

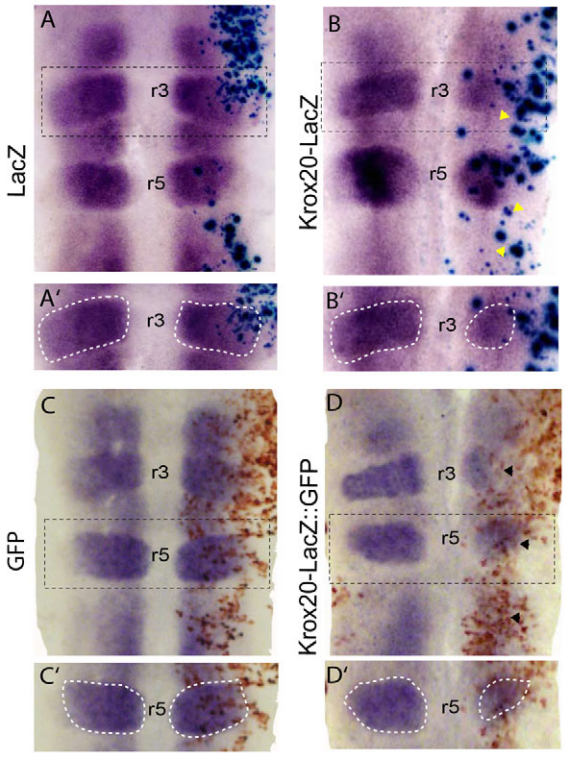

Fig. 4. Ectopic Krox20 expression inhibits *Pax6* expression. (**A-D′**) Flat-mounted chick hindbrains that were electroporated in the right side with control *lacZ* (A,A′), Krox20-lacZ (B,B′), control-GFP (C,C′) and both Krox20-lacZ and control-GFP (D,D′) constructs were analyzed by ISH for *Pax6* expression. (A′-D′) Enlargements of the boxed regions in A-D. Blue (A-B′) and brown (C-D′) dots indicate *lacZ* and GFP-expressing cells, respectively. White dashed lines indicate boundaries of *Pax6* expression. Arrowheads indicate abnormal *Pax6* expression.

prevents intersegmental cell mixing and results in the formation of sharp borders (Cooke et al., 2005; Sela-Donenfeld et al., 2009; Xu et al., 1995). As the sharply defined *Krox20* expression in r3/r5 borders is distorted upon Pax6 gain- and loss-of-function (Fig. 2), we analyzed whether EphA4 is affected. Normal EphA4 expression was shown in r3/r5 in controls (Fig. 5A,A′; n=17/17) and in the control side of Pax6-manipulated chick embryos (Fig. 5B,C). Pax6 overexpression led to decreased EphA4 expression and distortion of the r3/r5 sharp margins (Fig. 5B,B′; n=26/34). Conversely, EphA4^{+} cells extended into adjacent territories and the sharp borders of r3/r5 were lost upon expression of Pax6-En (Fig. 5C,C′; n=24/29), as also found with Pax6-siRNA (data not shown). EphA4 was also examined in E9.5 mice. *Pax6*$^{+/+}$ mice showed clear *Epha4* expression in r3/r5 (Fig. 5D,D′; n=8) and lower expression in other segments. *Pax6* mutants showed enhanced and expanded expression of *Epha4*, accompanied by larger r3/r5 territories and non-sharp boundaries (Fig. 5E,E′; n=9). Noticeably, *Epha4* expression seemed broader and less constricted also in other hindbrain areas (i.e. r6/r7 border) in the *Pax6* mutants. These data indicate that Pax6 limits EphA4 expression domains in chick and mouse, consistent with the mode of action of Pax6 on *Krox20* (Fig. 2).

Rhombomere boundaries display specialized cellular properties (Heyman et al., 1995) and require Eph-ephrin signaling in order to form. Perturbed Eph-ephrin interaction leads to distorted segmental borders and an absence of boundary cells (Sela-Donenfeld et al.,

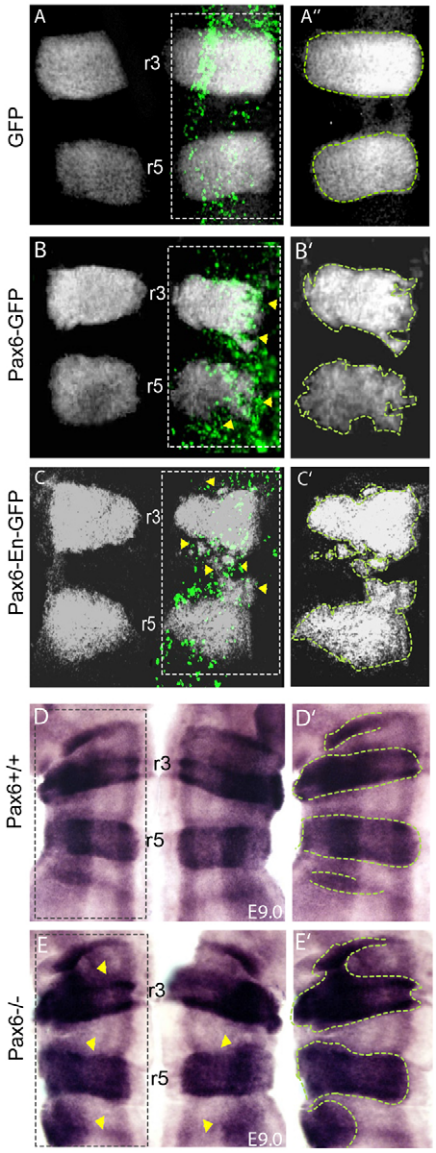

Fig. 5. EphA4 expression domains are altered upon Pax6 gain- and loss-of-function. (**A-C′**) Flat-mounted chick hindbrains that were electroporated in the right side with control-GFP (A,A′), Pax6-GFP (B,B′) and Pax6-En-GFP (C,C′) constructs and stained with anti-EphA4 antibody. Gray or green staining indicates cells expressing EphA4 or GFP, respectively. (**D-E′**) Flat-mount hindbrains of *Pax6*$^{+/+}$ and *Pax6*$^{-/-}$ mice analyzed by ISH to detect *Epha4* mRNA. (A′-E′) Views of the boxed regions in A-E. Dashed areas (A′-E′) indicate boundaries of *Epha4*$^{+}$ domains. Arrowheads mark abnormal EphA4 patterns.

2009; Sela-Donenfeld and Wilkinson, 2005; Xu et al., 2000). Based on Pax6 effects on EphA4 (Fig. 5), the appearance of hindbrain boundaries was examined. The matrix protein chondroitin sulfate proteoglycan (CSPG) (Heyman et al., 1995) was typically demonstrated at boundary cells in control chick embryos (Fig. 6A,A′; *n*=15/15) but was largely disrupted in the hindbrain side expressing Pax6-GFP or Pax6-En-GFP (Fig. 6B-C′; *n*=13/15 or 9/12, respectively), as compared with the contralateral side. Equally, the neurofilament protein 3A10 (Guthrie et al., 1991) was distributed normally in axons at rhombomere boundaries of control embryos (Fig. 6D,D′; *n*=12/12) and in the control side of Pax6-manipulated embryos (Fig. 6E,F, left side). However, significantly less 3A10 accumulation was evident at boundaries when electroporated with Pax6 plasmids (Fig. 6E-F′; *n*=11/14 and 13/16 for Pax6-GFP and Pax6-En-GFP, respectively). In addition, axons and cell bodies within rhombomeres, which are also marked by 3A10, seemed disorganized. These data, together with our previous results, confirm that impairment of Pax6 activity leads to disruption of the sharp domains of r3 and r5 and to loss of repetitive boundaries. This is compatible with previous studies in which distorted hindbrain boundaries were associated with impaired segmentation and neuronal organization (Sela-Donenfeld et al., 2009; Xu et al., 1995).

Pax6 affects the segmental expression of *Kreisler*, *Hoxa2* and *Hoxb1*

As Pax6 manipulations induce distorted expression of Krox20 and EphA4, the intercrossing of cells between segments and loss of boundaries, we examined whether other landmark genes, upstream or downstream of *Krox20*, are affected by Pax6 in chick and mice.

Kreisler is first evident in r5 at the 4-somite stage, strengthens at the 6-somite stage, and expands to r6 in 9-somite embryos, remaining in r5/r6 to later stages (supplementary material Fig. S2E-H) (Grapin-Botton et al., 1998; McKay et al., 1994). Noticeably, *Kreisler* precedes *Pax6* in expression (Fig. 1A), whereas later they overlap in r5. Testing Pax6 effects on *Kreisler* revealed its normal expression in r5/r6 in control chick embryos (Fig. 7A; *n*=17/17) and in the control side of Pax6-manipulated embryos (Fig. 7B,C, left side). However, Pax6 misexpression led to marked loss in *Kreisler*$^{+}$ domains (Fig. 7B; *n*=20/26). Conversely, Pax6-En disrupted the sharp r5/r6 borders of *Kreisler* and showed *Kreisler*$^{+}$ cells in neighboring segments (Fig. 7C; *n*=18/24). The inhibiting effect of Pax6 on *Kreisler* was recapitulated in mice. *Pax6*$^{+/+}$ mice showed normal *Kreisler* expression in r5/r6, whereas *Pax6* nulls demonstrated enlarged *Kreisler*$^{+}$ domains, fuzzy r4/r5 and r6/r7 borders and the appearance of *Kreisler*$^{+}$ cells in r4 (Fig. 7D,E; *n*=7 and *n*=8, respectively). We next tested whether the effects of Pax6 on *Kreisler* are mediated through its regulation of *Nab1* and *Krox20* (Fig. 3). In contrast to the expanded *Kreisler* domains found in the Pax6-En experiment, a clear reduction in *Kreisler* was evident upon Nab1 misexpression. Moreover, Nab1 reversed the effect of Pax6-En, such that *Kreisler* territories remained reduced, rather than enlarged, in embryos co-expressing both plasmids (supplementary material Fig. S4). This implicates the Pax6-Nab1 interaction in governing the spatial expression of several hindbrain genes.

Analysis of group 1 and 2 Hox genes was also performed. *Hoxb1* is expressed from r4 posteriorly in chick embryos of 2-6 somites (supplementary material Fig. S2M,N) (Gavalas et al., 2003), and subsequently remains in r4 and r7 (supplementary material Fig. S2O,P). *Hoxa2 is* expressed along the hindbrain of 4- to 6-somite embryos, with an anterior border at presumptive r2 (supplementary material Fig. S2I,J) (Barrow et al., 2000; Maconochie et al., 2001). Although this pattern is retained, *Hoxa2* is also later enhanced in other segments (supplementary material Fig. S2K,L). Comparing these genes with *Pax6* reveals that *Pax6* initiates slightly later than the Hox genes and that their distribution overlaps in some segments.

Examination of the effect of Pax6 on *Hoxb1* showed normal r4 localization of *Hoxb1* expression in controls (Fig. 7F; *n*=25/25) and control sides of Pax6-manipulated chick hindbrains. Embryos misexpressing Pax6 showed a reduced *Hoxb1* domain, whereas some expansion in *Hoxb1* and disruption of its sharp borders were evident with Pax6-En electroporation (Fig. 7G,H; *n*=14/20 and

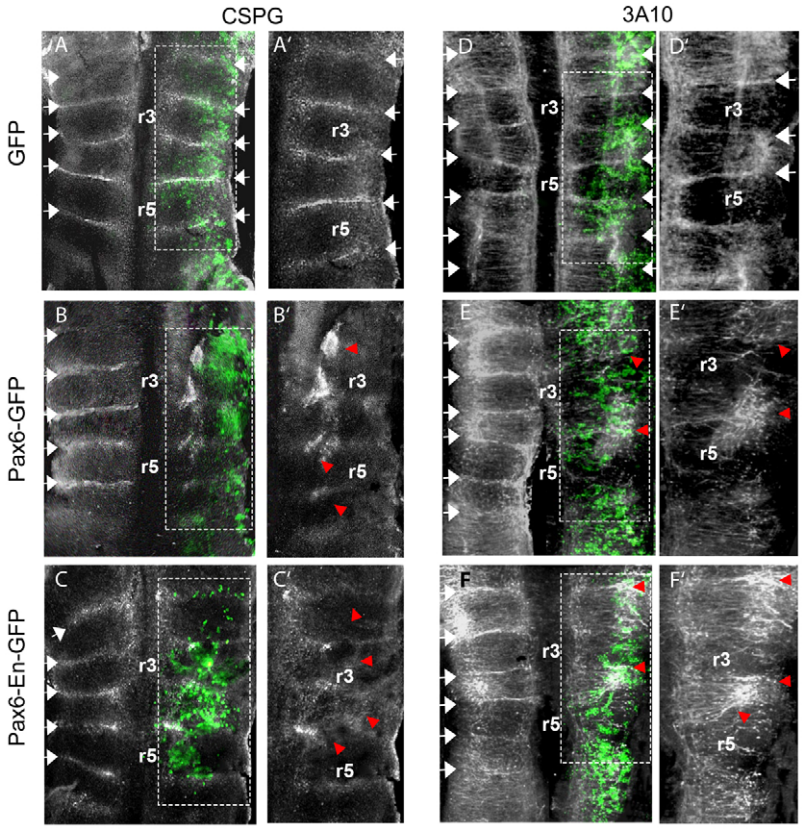

Fig. 6. Distorted boundaries upon Pax6 gain- and loss-of-function. (**A-F′**) Flat-mounted chick hindbrains that were electroporated in the right side with control-GFP (A,D), Pax6-GFP (B,E) and Pax6-En-GFP (C,F) constructs and stained with anti-CSPG (A-C) or 3A10 (D-F) antibodies (gray). GFP-expressing cells are in green. (A′-F′) Enlargements of the boxed regions in A-F. Arrows indicate normal boundaries and arrowheads mark distorted boundaries.

17/20, respectively). Similarly, *Pax6* mutant mice exhibited an expansion of *Hoxb1*$^{+}$ domains in the hindbrain compared with *Pax6*$^{+/+}$ embryos (Fig. 7J,I; *n*=5 and 4, respectively).

Similar effects were found on *Hoxa2* patterns. Control chick embryos showed normal *Hoxa2* expression from r2 and caudally, with enhanced r3-r5 staining (Fig. 7K,K′; *n*=12/12), as also shown in the control side of Pax6-manipulated embryos. Pax6 misexpression resulted in a clear reduction of *Hoxa2* in these segments, whereas embryos expressing Pax6-En showed some expansion and irregular borders of the *Hoxa2*$^{+}$ domains (Fig. 7L-M′; *n*=17/17 and 9/12, respectively). This effect seemed more subtle compared with other segmental genes, probably owing to masking by the basal *Hoxa2* expression level present along the hindbrain. Examination of *Hoxa2* in *Pax6*$^{+/+}$ mice showed sharply defined expression in r3 and fainter expression also in r5. In *Pax6* nulls, *Hoxa2* domains became less confined to r3 and r5 with fuzzier borders of expression along the hindbrain (Fig. 7N-O′; *n*=4 and 5 for *Pax6*$^{+/+}$ and *Pax6-LacZ*, respectively).

Together, these results demonstrate that misexpression or knockdown of Pax6 disrupts the sharp segmental patterns of *Kreisler*, *Hoxb1* and *Hoxb2* by decreasing their domains or distorting their expression borders and expanding their territories, respectively, suggesting a broad Pax6 activity that limits the expression domains of multiple hindbrain genes in chick and mice.

Pax6 expression is regulated by FGF signaling

The FGF pathway, mediated by Fgf3, upregulates *Krox20* expression in chick (Aragon and Pujades, 2009; Labalette et al., 2011; Marín and Charnay, 2000; Weisinger et al., 2010). Based on the *Pax6-Krox20* cross-talk (Figs 2-4), we examined whether FGF signaling also regulates *Pax6* expression. Comparison between *Fgf3* and *Pax6* expression patterns revealed their similarities to *Fgf3/Krox20* patterns (Marín and Charnay, 2000; Weisinger et al., 2010; Weisinger et al., 2008); *Fgf3* precedes *Pax6*, whereas slightly later *Fgf3* is found in r4-r6 and *Pax6* in r3 (Fig. 8A-C). We next blocked FGF signaling and analyzed *Pax6* expression. Control beads, or beads soaked with SU5402 (a chemical inhibitor of FGF receptors), were implanted into the hindbrain of 2- to 4-somite chick embryos (Weisinger et al., 2012), which were analyzed 16 hours later. Whereas controls demonstrated normal *Pax6* expression, SU5402 led to *Pax6* downregulation (Fig. 8D-G; *n*=11/11 and 12/17, respectively). The SU5402 effect was local, as it did not alter *Pax6* at a distance from the hindbrain [i.e. in the forebrain/spinal cord; Fig. 8E,F), and SU5402 did not affect the expression of two other hindbrain genes, follistatin (*Fst*) and cadherin 7 (*Cad7*) (Fig. 8H-K; *n*=10/12 and 5/5, respectively) (see also Weisinger et al., 2010; Weisinger et al., 2012). These results confirm the specificity of SU5402 treatment on *Pax6* and indicate that FGF signaling is involved in the upregulation of *Pax6* in the hindbrain.

DISCUSSION

Pax6 expression was previously described in a longitudinal ventral^{high}-dorsal^{low} pattern in the hindbrain, similar to that in the spinal cord. Regulatory roles of Pax6 were attributed in establishing ventral neuronal domains in these two CNS regions (Bel-Vialar et al., 2007; Bertrand et al., 2000; Ericson et al., 1997; Numayama-Tsuruta et al., 2010; Osumi et al., 1997; Takahashi and Osumi, 2002). Here, we investigated whether Pax6 functions at much earlier hindbrain stages, when it displays segmental expression. Pax6 was found to be required to set the precise domains of key hindbrain genes (*Krox20*, *Kreisler*, *Hoxa2*, *Hoxb1*, EphA4) in specific segments in chick and mice; whereas excess Pax6 decreased their segmental distribution, Pax6 knockdown enhanced and expanded their expression into adjacent domains. Investigation of the

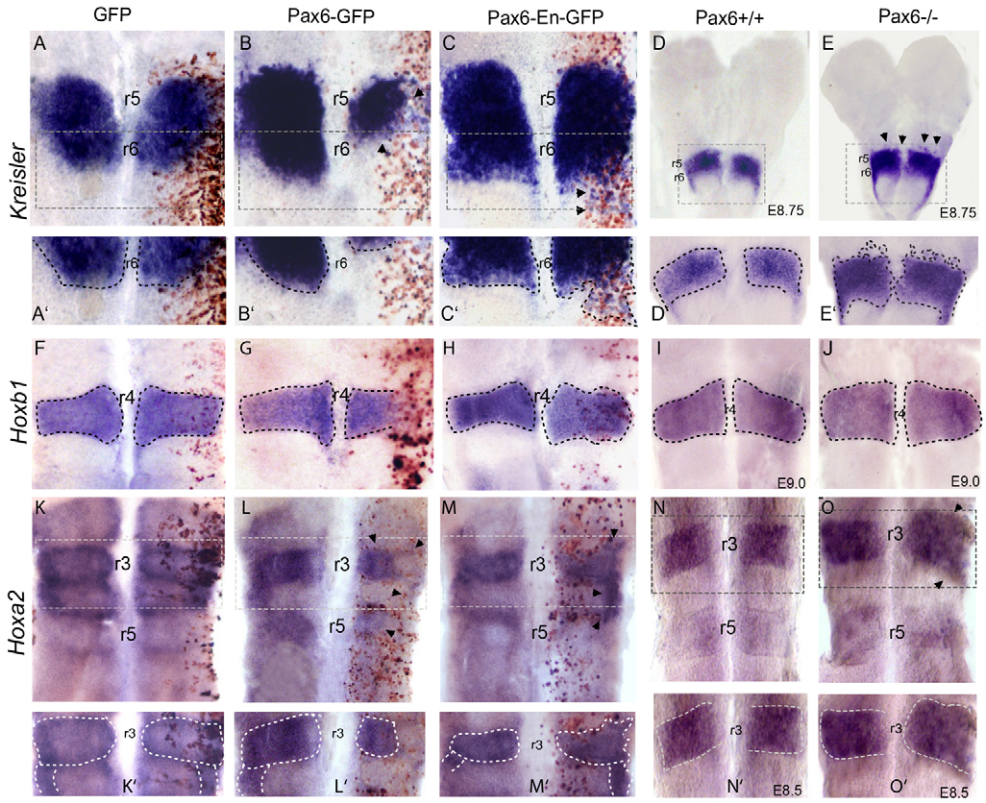

Fig. 7. Pax6 affects the segmental expression of *Kreisler*, *Hoxb1* and *Hoxa2*. (**A-O′**) Flat-mounted views of chick hindbrains (A-C,F-H,K-M) electroporated in the right side with control-GFP (A,F,K), Pax6-GFP (B,G,L) or Pax6-En-GFP (C,H,M) constructs, or of $Pax6^{+/+}$ (D,I,N) and $Pax6^{-/-}$ (E,J,O) mouse hindbrains. ISH was performed to detect *Kreisler* (A-E), *Hoxb1* (F-J) and *Hoxa2* (K-O). Brown dots (A-C,F-H,K-M) mark GFP-expressing cells. (A′-E′,K′-O′) Enlargements of the boxed regions in A-E,K-O. Dashed lines (A′-E′,F-J,K′-O′) mark expression borders. Arrowheads indicate irregular gene expression.

mechanism through which Pax6 limits *Krox20* expression revealed the upregulation of the *Krox20*-repressor *Nab1* by Pax6. A double negative-feedback regulatory loop was found between Pax6 and Krox20 that enabled their co-expression in hindbrain segments. Furthermore, a role for FGF signaling in inducing their expression was found. Consistent with the activity of Pax6 in setting sharp borders of expression of segmental genes, rhombomere boundaries became distorted upon Pax6 manipulation. This study unraveled a new AP role for Pax6 in the segmental organization of the early hindbrain. A summary of the main phenotypes and a schematic illustration of our results are presented in Table 1 and Fig. 9.

Pax6 as a guardian of sharply defined hindbrain segments

A small number of previous studies have suggested Pax6 involvement in hindbrain AP patterning; Pax6 was found to regulate *Hoxd4* in mouse/zebrafish spinal cord (Nolte et al., 2006) and its depletion reduced *Hoxd4* expression. Yet, the anterior border of *Hoxd4* expanded into r6 in *Pax6* nulls/morphants. This could not be explained by positive regulation of *Hoxd4* by Pax6 and suggested its additional, previously underinvestigated role in hindbrain segmentation. Furthermore, microarray analysis performed on E11.5 WT and *Pax6* null rats showed some increase in the expression domains of *Krox20* and *Epha4* in the mutants (Numayama-Tsuruta et al., 2010). Our work substantiated these findings by examining chick and mouse embryos at much earlier stages than in the above studies, during which hindbrain segmentation is established, and showed a clear expansion and loss of sharp segmentation of multiple hindbrain genes upon Pax6 loss. We illuminate these previous results by directly demonstrating a novel role for Pax6 in setting the precise domains of hindbrain segments, which is mediated, at least in part, by positively regulating the repressor gene *Nab1*.

Possible mechanisms of Pax6 activity

One mechanism by which Pax6 might act is by establishing inter-rhombomeric boundaries. Pax6 manipulations disrupt the segmental restriction of genes and allow cell intermixing. Concomitantly, rhombomere boundaries are impaired. The effect of Pax6 on EphA4 might suggest how boundaries are lost because interfering with Eph-ephrin signaling eliminates boundary cell formation, which is associated with cell crossing and loss of sharp rhombomere borders (Cooke et al., 2005; Sela-Donenfeld et al., 2009; Xu et al., 1995). The accumulation of Pax6 at hindbrain boundaries at later stages (Heyman et al., 1995; Sela-Donenfeld et al., 2009; Xu et al., 1995) further supports a role for Pax6 in stabilizing hindbrain boundaries. Yet, whether the effect of Pax6 on EphA4 is direct or is mediated by the effect on its upstream regulator Krox20, or both, is not clear. Notably, Pax6 was recently suggested to regulate boundary cell specification in the rat hindbrain (Takahashi and Osumi, 2011). They showed Pax6 expression in rhombomeres and exclusion from boundaries (in contrast to in other vertebrates), and the loss of some boundary markers [PLZF (Zbtb16), Wnt5a] and expansion of others (Cad7) in *Pax6* nulls, together with hindbrain morphological disorganization. That work suggested that Pax6 represses the expansion of boundaries into rhombomeres and neural differentiation in the rat hindbrain, by an unknown mechanism. Consistent with these findings, we found distorted expression of boundary markers and segmental disorganization upon Pax6 manipulation in early staged chick and mice. Moreover, we suggest that Pax6 might control boundary formation through its early activity in stabilizing the segmental borders of hindbrain genes. Consistent with our findings, Pax6 was reported to regulate boundary formation in between the dorsal and ventral telencephalon (Haubst et al., 2004) through upregulating Sfrp2 (a Wnt signaling inhibitor), which in turn prevents cell crossing. Intriguingly, Pax6 induces Sfrp2 also in the spinal cord to restrict Wnt signaling and

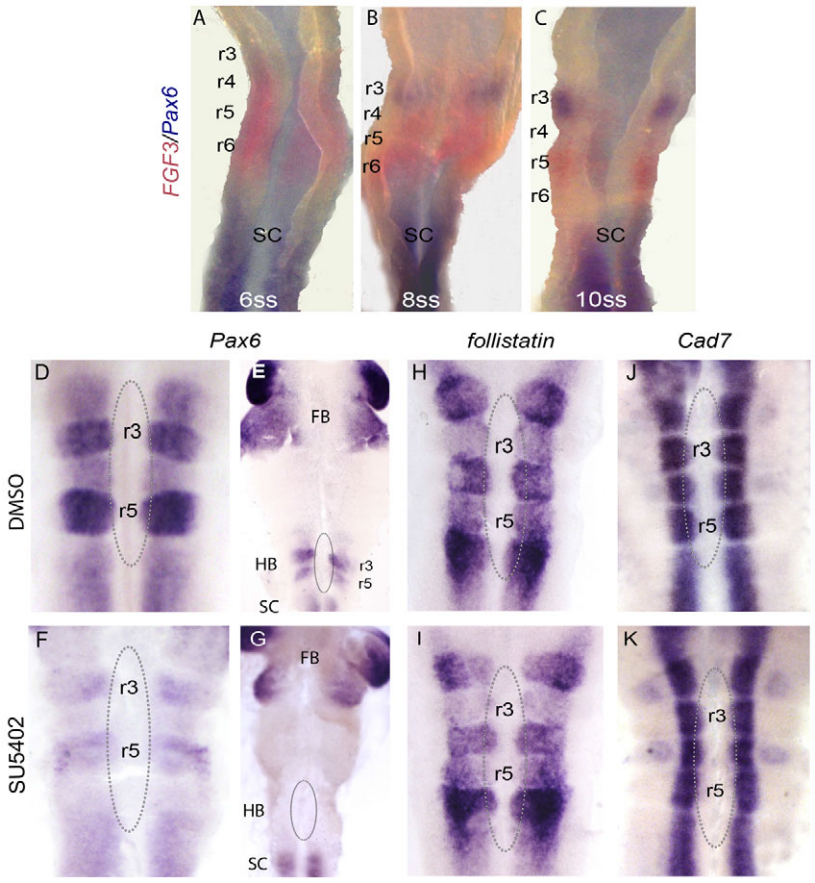

Fig. 8. *Pax6* expression is regulated by FGF signaling. (**A-C**) Double ISH in chick hindbrain at sequential early stages shows *Pax6* (blue) and *Fgf3* (red) expression. (**D-K**) Flat-mounted hindbrain of chick embryos grafted with DMSO-soaked (D,F,H,J) or SU5402-soaked (F,G,I,K) beads and analyzed by ISH to detect *Pax6* (D-G), follistatin (H,I) or *Cad7* (J,K). Ellipses mark bead localization.

sets sharp boundaries of expression of DV-specific genes (Ericson et al., 1997).

Differential cell adhesion is an effective mechanism for compartmentalization, which might also mediate Pax6 activity. The early expression of Pax6 in r3/r5 might regulate distinct adhesion properties in these cells. In such a scenario, the expected phenotypes of excess or reduced Pax6 levels will include enhanced or reduced adhesion of r3/r5 cells, their segregation or spreading, respectively, and prevention of sharp segmental borders and boundary cell formation, as we indeed demonstrated. Pax6 regulates adhesion molecules in the CNS, such as L1, tenascin and cadherins (Duparc et al., 2006; Osumi, 2001; Osumi et al., 2008; Stoykova et al., 1997; Takahashi and Osumi, 2011; Tyas et al., 2003). As some of these adhesion molecules are expressed in the hindbrain (Liu et al., 2001; Numayama-Tsuruta et al., 2010; Takahashi and Osumi, 2008), it would be of interest to test whether Pax6 controls their expression at early stages. Intriguingly, the effect of Pax6 on EphA4 might suggest one such mechanism, as EphA4 was previously reported to affect adhesion within rhombomeres (in addition to its boundary function) (Cooke et al., 2005).

As Pax6 was found to restrict the expression of multiple genes, another possibility is that it acts as a general repressor. Yet, Pax6 mostly acts as an activator during development (reviewed by Osumi et al., 2008) [but see Weasner et al. (Weasner et al., 2009)]. Moreover, as Pax6 was found to require *de novo* protein synthesis and to positively induce *Nab1* expression, we disfavor such a possibility. Additionally, the observation that Pax6 is not evenly distributed in all segments and yet it affects multiple segmental genes in both cell-autonomous and non-cell-autonomous fashions, does not fit with a general repressor activity. Furthermore, despite Pax6 effects on gene restriction, we do not observe such as global misspecification, switching in segmental identities, duplication or loss of segments. The lack of such phenotypes argues against Pax6 as global repressor of multiple genes that acts to specify segmental

Table 1. Summary of phenotypes of segmental genes and boundary markers upon Pax6 gain- and loss-of-function in chick embryos

	Boundary markers		Segmental genes		
Treatment	No effect	Distortion or loss of boundary	No effect	Expansion in expression or loss of sharp borders	Reduced expression
GFP	27/27 (100%)	0	124/129 (96%)	0	0
Pax6 gain-of-function	5/29 (17%)	24/29 (83%)	29/129 (22.5%)	0	100/129 (77.5%)
Pax6 loss-of-function	6/28 (21%)	22/28 (79%)	29/149 (19.5%)	123/149 (82.5%)	0

Data show the number of chick embryos that exhibit normal or distorted boundaries, as evaluated by CSPG and 3A10 staining, as well as normal, reduced or expanded expression of the segmental genes *Krox20*, EphA4, *Kreisler*, *Hoxa2* and *Hoxb1*. The percentage showing the phenotype is indicated. Gain-of-function refers to electroporation of Pax6-GFP and loss-of-function refers to electroporation of Pax6-En-GFP and Pax6-siRNA plasmids.

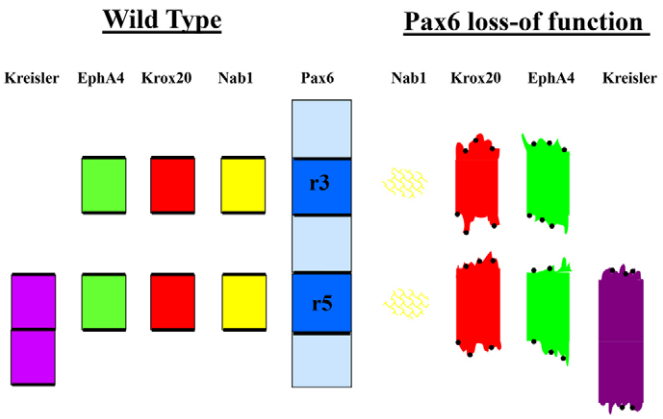

Fig. 9. The role of Pax6 in hindbrain segmental organization. Hindbrain of an 18-somite embryo, reflecting the stage at which most analyses were performed. In WT (left) Pax6 is predominantly expressed in r3/r5, although weaker expression is evident in the other segments. Sharp segmental expression of Nab1, Krox20, EphA4 and Kreisler is evident, with clear inter-rhombomeric boundaries (in black). In Pax6 loss-of-function (right), expression of Nab1 is lost whereas domains of Krox20, EphA4 and Kreisler expand into adjacent territories, concomitant with perturbed rhombomere boundaries (black dots). This model suggests that Pax6 is required for hindbrain segmental organization by restricting the expression domains of multiple hindbrain genes to their correct regions, together with its effect on the formation of inter-rhombomeric boundaries.

identities, and supports its role in guarding the segmental domains of hindbrain genes.

As multiple segmental genes are similarly affected by Pax6, an additional option, which is not mutually exclusive with the others, is that Pax6 modulates one gene (e.g. *Krox20* through *Nab1*), which in turn affects, directly or indirectly, all the others. The regulatory interactions between these genes are highly complex. For instance, Hoxa2 is a direct target of Krox20 in r3/r5, Krox20 in r5 (but not r3) is maintained by Kreisler (Manzanares et al., 1999; Nonchev et al., 1996a), and Krox20 and Hoxb1 inhibit the expression of each other in r4 and r3/r5, respectively, but Hoxb1 is also required for the early initiation of Krox20 in r3 (Garcia-Dominguez et al., 2006; Giudicelli et al., 2001; Wassef et al., 2008). Our finding that *Kreisler* is reduced upon Nab1 misexpression supports this possibility by suggesting that Nab1 inhibition of *Krox20* leads to a change in the segmental identities of r3/r5, which eventually results in the downregulation of *Kreisler*. Further elucidation of how Nab1 is induced by Pax6 and affects the expression of multiple hindbrain genes, as well as the identification of additional downstream targets of Pax6, are required in order to test such a hypothesis.

Finally, we show that exogenous Pax6 plasmids can enforce modifications in gene expression both cell-autonomously and non-cell-autonomously. Interestingly, in addition to its established cell-autonomous roles, Pax6 has demonstrated an unexpected paracrine effect in different CNS tissues (Di Lullo et al., 2011; Lesaffre et al., 2007). Whether Pax6 acts similarly in the early hindbrain is not known and requires further understanding of how Pax6 acts as a signaling molecule. An additional explanation for such dual effects of Pax6 on hindbrain genes is suggested by considering the fact that Krox20 patterns the hindbrain through cell-autonomous and non-cell-autonomous mechanisms (Giudicelli et al., 2001). Its non-cell-autonomous activity was identified from the ability of cells electroporated with Krox20 to induce the expression of endogenous Krox20 in surrounding, non-electroporated cells, by an unknown manner. Future studies will evaluate whether Pax6 acts similarly. The finding that ectopic Pax6 sequences do not necessarily overlap with its effect on the expression of segmental genes might also suggest an option for change in cell fate upon electroporation, accompanied with loss or overexpression of the examined gene, as well as with intermingling with neighboring cells. For example, misexpression of Pax6, which upregulates *Nab1* and antagonizes Krox20, might lead to a change in cell identity and in the adhesion properties of the electroporated cell. Such an activity of exogenous Pax6 vectors awaits future evaluation.

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The authors declare no competing financial interests.

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References

Aamar, E. and Frank, D. (2004). Xenopus Meis3 protein forms a hindbrain-inducing center by activating FGF/MAP kinase and PCP pathways. *Development* **131**, 153-163.
Abu-Abed, S., Dollé, P., Metzger, D., Beckett, B., Chambon, P. and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.* **15**, 226-240.
Aragon, F. and Pujades, C. (2009). FGF signaling controls caudal hindbrain specification through Ras-ERK1/2 pathway. *BMC Dev. Biol.* **9**, 61.
Ashery-Padan, R. and Gruss, P. (2001). Pax6 lights-up the way for eye development. *Curr. Opin. Cell Biol.* **13**, 706-714.
Barrow, J. R., Stadler, H. S. and Capecchi, M. R. (2000). Roles of Hoxa1 and Hoxa2 in patterning the early hindbrain of the mouse. *Development* **127**, 933-944.
Bel-Vialar, S., Medevielle, F. and Pituello, F. (2007). The on/off of Pax6 controls the tempo of neuronal differentiation in the developing spinal cord. *Dev. Biol.* **305**, 659-673.
Bertrand, N., Médevielle, F. and Pituello, F. (2000). FGF signalling controls the timing of Pax6 activation in the neural tube. *Development* **127**, 4837-4843.
Cooke, J. E., Kemp, H. A. and Moens, C. B. (2005). EphA4 is required for cell adhesion and rhombomere-boundary formation in the zebrafish. *Curr. Biol.* **15**, 536-542.
Das, R. M., Van Hateren, N. J., Howell, G. R., Farrell, E. R., Bangs, F. K., Porteous, V. C., Manning, E. M., McGrew, M. J., Ohyama, K., Sacco, M. A. et al. (2006). A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev. Biol.* **294**, 554-563.
Derobert, Y., Baratte, B., Lepage, M. and Mazan, S. (2002). Pax6 expression patterns in Lampetra fluviatilis and Scyliorhinus canicula embryos suggest highly conserved roles in the early regionalization of the vertebrate brain. *Brain Res. Bull.* **57**, 277-280.
Desmazières, A., Charnay, P. and Gilardi-Hebenstreit, P. (2009). Krox20 controls the transcription of its various targets in the developing hindbrain according to multiple modes. *J. Biol. Chem.* **284**, 10831-10840.
Di Lullo, E., Haton, C., Le Poupon, C., Volovitch, M., Joliot, A., Thomas, J. L. and Prochiantz, A. (2011). Paracrine Pax6 activity regulates oligodendrocyte precursor cell migration in the chick embryonic neural tube. *Development* **138**, 4991-5001.
Duparc, R. H., Boutemmine, D., Champagne, M. P., Tétreault, N. and Bernier, G. (2006). Pax6 is required for delta-catenin/neurojugin expression during retinal, cerebellar and cortical development in mice. *Dev. Biol.* **300**, 647-655.

Dupé, V. and Lumsden, A. (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.
Elkouby, Y. M., Polevoy, H., Gutkovich, Y. E., Michaelov, A. and Frank, D. (2012). A hindbrain-repressive Wnt3a/Meis3/Tsh1 circuit promotes neuronal differentiation and coordinates tissue maturation. *Development* **139**, 1487-1497.
Engelkamp, D., Rashbass, P., Seawright, A. and van Heyningen, V. (1999). Role of Pax6 in development of the cerebellar system. *Development* **126**, 3585-3596.
Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.
Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse Hox-2.9 gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110**, 589-607.
Garcia-Dominguez, M., Gilardi-Hebenstreit, P. and Charnay, P. (2006). PIASxbeta acts as an activator of Hoxb1 and is antagonized by Krox20 during hindbrain segmentation. *EMBO J.* **25**, 2432-2442.
García-Gutiérrez, P., Juárez-Vicente, F., Gallardo-Chamizo, F., Charnay, P. and García-Domínguez, M. (2011). The transcription factor Krox20 is an E3 ligase that sumoylates its Nab coregulators. *EMBO Rep.* **12**, 1018-1023.
Gavalas, A., Ruhrberg, C., Livet, J., Henderson, C. E. and Krumlauf, R. (2003). Neuronal defects in the hindbrain of Hoxa1, Hoxb1 and Hoxb2 mutants reflect regulatory interactions among these Hox genes. *Development* **130**, 5663-5679.
Giudicelli, F., Taillebourg, E., Charnay, P. and Gilardi-Hebenstreit, P. (2001). Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms. *Genes Dev.* **15**, 567-580.
Giudicelli, F., Gilardi-Hebenstreit, P., Mechta-Grigoriou, F., Poquet, C. and Charnay, P. (2003). Novel activities of Mafb underlie its dual role in hindbrain segmentation and regional specification. *Dev. Biol.* **253**, 150-162.
Glover, J. C., Renaud, J. S. and Rijli, F. M. (2006). Retinoic acid and hindbrain patterning. *J. Neurobiol.* **66**, 705-725.
Grapin-Botton, A., Bonnin, M. A., Sieweke, M. and Le Douarin, N. M. (1998). Defined concentrations of a posteriorizing signal are critical for MafB/Kreisler segmental expression in the hindbrain. *Development* **125**, 1173-1181.
Guthrie, S., Butcher, M. and Lumsden, A. (1991). Patterns of cell division and interkinetic nuclear migration in the chick embryo hindbrain. *J. Neurobiol.* **22**, 742-754.
Haubst, N., Berger, J., Radjendirane, V., Graw, J., Favor, J., Saunders, G. F., Stoykova, A. and Götz, M. (2004). Molecular dissection of Pax6 function: the specific roles of the paired domain and homeodomain in brain development. *Development* **131**, 6131-6140.
Hernandez, R. E., Putzke, A. P., Myers, J. P., Margaretha, L. and Moens, C. B. (2007). Cyp26 enzymes generate the retinoic acid response pattern necessary for hindbrain development. *Development* **134**, 177-187.
Heyman, I., Faissner, A. and Lumsden, A. (1995). Cell and matrix specialisations of rhombomere boundaries. *Dev. Dyn.* **204**, 301-315.
Hogan, B. L., Horsburgh, G., Cohen, J., Hetherington, C. M., Fisher, G. and Lyon, M. F. (1986). Small eyes (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. *J. Embryol. Exp. Morphol.* **97**, 95-110.
Hunt, P., Gulisano, M., Cook, M., Sham, M. H., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991). A distinct Hox code for the branchial region of the vertebrate head. *Nature* **353**, 861-864.
Irving, C., Nieto, M. A., DasGupta, R., Charnay, P. and Wilkinson, D. G. (1996). Progressive spatial restriction of Sek-1 and Krox-20 gene expression during hindbrain segmentation. *Dev. Biol.* **173**, 26-38.
Kohl, A., Hadas, Y., Klar, A. and Sela-Donenfeld, D. (2012). Axonal patterns and targets of dA1 interneurons in the chick hindbrain. *J. Neurosci.* **32**, 5757-5771.
Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.
Labalette, C., Bouchoucha, Y. X., Wassef, M. A., Gongal, P. A., Le Men, J., Becker, T., Gilardi-Hebenstreit, P. and Charnay, P. (2011). Hindbrain patterning requires fine-tuning of early krox20 transcription by Sprouty 4. *Development* **138**, 317-326.
Landsberg, R. L., Awatramani, R. B., Hunter, N. L., Farago, A. F., DiPietrantonio, H. J., Rodriguez, C. I. and Dymecki, S. M. (2005). Hindbrain rhombic lip is comprised of discrete progenitor cell populations allocated by Pax6. *Neuron* **48**, 933-947.
LeBlanc, S. E., Jang, S. W., Ward, R. M., Wrabetz, L. and Svaren, J. (2006). Direct regulation of myelin protein zero expression by the Egr2 transactivator. *J. Biol. Chem.* **281**, 5453-5460.
Lesaffre, B., Joliot, A., Prochiantz, A. and Volovitch, M. (2007). Direct non-cell autonomous Pax6 activity regulates eye development in the zebrafish. *Neural Dev.* **2**, 2.
Liu, Q., Marrs, J. A., Chuang, J. C. and Raymond, P. A. (2001). Cadherin-4 expression in the zebrafish central nervous system and regulation by ventral midline signaling. *Brain Res. Dev. Brain Res.* **131**, 17-29.
Lumsden, A. (2004). Segmentation and compartition in the early avian hindbrain. *Mech. Dev.* **121**, 1081-1088.
Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.
Maconochie, M. K., Nonchev, S., Manzanares, M., Marshall, H. and Krumlauf, R. (2001). Differences in Krox20-dependent regulation of Hoxa2 and Hoxb2 during hindbrain development. *Dev. Biol.* **233**, 468-481.
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.
Manzanares, M., Trainor, P. A., Nonchev, S., Ariza-McNaughton, L., Brodie, J., Gould, A., Marshall, H., Morrison, A., Kwan, C. T., Sham, M. H. et al. (1999). The role of kreisler in segmentation during hindbrain development. *Dev. Biol.* **211**, 220-237.
Manzanares, M., Nardelli, J., Gilardi-Hebenstreit, P., Marshall, H., Giudicelli, F., Martínez-Pastor, M. T., Krumlauf, R. and Charnay, P. (2002). Krox20 and kreisler co-operate in the transcriptional control of segmental expression of Hoxb3 in the developing hindbrain. *EMBO J.* **21**, 365-376.
Marín, F. and Charnay, P. (2000). Hindbrain patterning: FGFs regulate Krox20 and mafB/kr expression in the otic/preotic region. *Development* **127**, 4925-4935.
Matsunaga, E., Araki, I. and Nakamura, H. (2000). Pax6 defines the di-mesencephalic boundary by repressing En1 and Pax2. *Development* **127**, 2357-2365.
McKay, I. J., Muchamore, I., Krumlauf, R., Maden, M., Lumsden, A. and Lewis, J. (1994). The kreisler mouse: a hindbrain segmentation mutant that lacks two rhombomeres. *Development* **120**, 2199-2211.
Mechta-Grigoriou, F., Garel, S. and Charnay, P. (2000). Nab proteins mediate a negative feedback loop controlling Krox-20 activity in the developing hindbrain. *Development* **127**, 119-128.
Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.
Moens, C. B., Yan, Y. L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). valentino: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.
Monsonego-Ornan, E., Kosonovsky, J., Bar, A., Roth, L., Fraggi-Rankis, V., Simsa, S., Kohl, A. and Sela-Donenfeld, D. (2012). Matrix metalloproteinase 9/gelatinase B is required for neural crest cell migration. *Dev. Biol.* **364**, 162-177.
Morriss-Kay, G. M., Murphy, P., Hill, R. E. and Davidson, D. R. (1991). Effects of retinoic acid excess on expression of Hox-2.9 and Krox-20 and on morphological segmentation in the hindbrain of mouse embryos. *EMBO J.* **10**, 2985-2995.
Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dollé, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.
Nolte, C., Rastegar, M., Amores, A., Bouchard, M., Grote, D., Maas, R., Kovacs, E. N., Postlethwait, J., Rambaldi, I., Rowan, S. et al. (2006). Stereospecificity and PAX6 function direct Hoxd4 neural enhancer activity along the antero-posterior axis. *Dev. Biol.* **299**, 582-593.
Nonchev, S., Maconochie, M., Vesque, C., Aparicio, S., Ariza-McNaughton, L., Manzanares, M., Maruthainar, K., Kuroiwa, A., Brenner, S., Charnay, P. et al. (1996a). The conserved role of Krox-20 in directing Hox gene expression during vertebrate hindbrain segmentation. *Proc. Natl. Acad. Sci. USA* **93**, 9339-9345.
Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P. (1996b). Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**, 543-554.
Numayama-Tsuruta, K., Arai, Y., Takahashi, M., Sasaki-Hoshino, M., Funatsu, N., Nakamura, S. and Osumi, N. (2010). Downstream genes of Pax6 revealed by comprehensive transcriptome profiling in the developing rat hindbrain. *BMC Dev. Biol.* **10**, 6.
Osumi, N. (2001). The role of Pax6 in brain patterning. *Tohoku J. Exp. Med.* **193**, 163-174.
Osumi, N., Hirota, A., Ohuchi, H., Nakafuku, M., Iimura, T., Kuratani, S., Fujiwara, M., Noji, S. and Eto, K. (1997). Pax-6 is involved in the specification of hindbrain motor neuron subtype. *Development* **124**, 2961-2972.
Osumi, N., Shinohara, H., Numayama-Tsuruta, K. and Maekawa, M. (2008). Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. *Stem Cells* **26**, 1663-1672.
Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.
Prince, V. E., Moens, C. B., Kimmel, C. B. and Ho, R. K. (1998). Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. *Development* **125**, 393-406.
Qiu, R., Liu, Y., Wu, J. Y., Liu, K., Mo, W. and He, R. (2009). Misexpression of miR-196a induces eye anomaly in Xenopus laevis. *Brain Res. Bull.* **79**, 26-31.

Reijntjes, S., Gale, E. and Maden, M. (2003). Expression of the retinoic acid catabolising enzyme CYP26B1 in the chick embryo and its regulation by retinoic acid. *Gene Expr. Patterns* **3**, 621-627.
Runko, A. P. and Sagerström, C. G. (2003). Nlz belongs to a family of zinc-finger-containing repressors and controls segmental gene expression in the zebrafish hindbrain. *Dev. Biol.* **262**, 254-267.
Russo, M. W., Sevetson, B. R. and Milbrandt, J. (1995). Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc. Natl. Acad. Sci. USA* **92**, 6873-6877.
Schneider-Maunoury, S., Seitanidou, T., Charnay, P. and Lumsden, A. (1997). Segmental and neuronal architecture of the hindbrain of Krox-20 mouse mutants. *Development* **124**, 1215-1226.
Seitanidou, T., Schneider-Maunoury, S., Desmarquet, C., Wilkinson, D. G. and Charnay, P. (1997). Krox-20 is a key regulator of rhombomere-specific gene expression in the developing hindbrain. *Mech. Dev.* **65**, 31-42.
Sela-Donenfeld, D. and Kalcheim, C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development* **126**, 4749-4762.
Sela-Donenfeld, D. and Wilkinson, D. G. (2005). Eph receptors: two ways to sharpen boundaries. *Curr. Biol.* **15**, R210-R212.
Sela-Donenfeld, D., Kayam, G. and Wilkinson, D. G. (2009). Boundary cells regulate a switch in the expression of FGF3 in hindbrain rhombomeres. *BMC Dev. Biol.* **9**, 16.
St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P. (1997). Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* **387**, 406-409.
Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395-1412.
Stoykova, A., Fritsch, R., Walther, C. and Gruss, P. (1996). Forebrain patterning defects in Small eye mutant mice. *Development* **122**, 3453-3465.
Stoykova, A., Götz, M., Gruss, P. and Price, J. (1997). Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. *Development* **124**, 3765-3777.
Svaren, J., Sevetson, B. R., Apel, E. D., Zimonjic, D. B., Popescu, N. C. and Milbrandt, J. (1996). NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli. *Mol. Cell. Biol.* **16**, 3545-3553.
Swiatek, P. J. and Gridley, T. (1993). Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. *Genes Dev.* **7**, 2071-2084.
Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M. and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216**, 282-296.
Takahashi, M. and Osumi, N. (2002). Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. *Development* **129**, 1327-1338.
Takahashi, M. and Osumi, N. (2008). Expression study of cadherin7 and cadherin20 in the embryonic and adult rat central nervous system. *BMC Dev. Biol.* **8**, 87.
Takahashi, M. and Osumi, N. (2011). Pax6 regulates boundary-cell specification in the rat hindbrain. *Mech. Dev.* **128**, 289-302.
Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P. and Wilkinson, D. G. (1998). Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. *Development* **125**, 443-452.
Theil, T., Ariza-McNaughton, L., Manzanares, M., Brodie, J., Krumlauf, R. and Wilkinson, D. G. (2002). Requirement for downregulation of kreisler during late patterning of the hindbrain. *Development* **129**, 1477-1485.
Tilleman, H., Hakim, V., Novikov, O., Liser, K., Nashelsky, L., Di Salvio, M., Krauthammer, M., Scheffner, O., Maor, I., Mayseless, O. et al. (2010). Bmp5/7 in concert with the mid-hindbrain organizer control development of noradrenergic locus coeruleus neurons. *Mol. Cell. Neurosci.* **45**, 1-11.
Tyas, D. A., Pearson, H., Rashbass, P. and Price, D. J. (2003). Pax6 regulates cell adhesion during cortical development. *Cereb. Cortex* **13**, 612-619.
Vlachakis, N., Choe, S. K. and Sagerström, C. G. (2001). Meis3 synergizes with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* **128**, 1299-1312.
Voiculescu, O., Taillebourg, E., Pujades, C., Kress, C., Buart, S., Charnay, P. and Schneider-Maunoury, S. (2001). Hindbrain patterning: Krox20 couples segmentation and specification of regional identity. *Development* **128**, 4967-4978.
Wassef, M. A., Chomette, D., Pouilhe, M., Stedman, A., Havis, E., Desmarquet-Trin Dinh, C., Schneider-Maunoury, S., Gilardi-Hebenstreit, P., Charnay, P. and Ghislain, J. (2008). Rostral hindbrain patterning involves the direct activation of a Krox20 transcriptional enhancer by Hox/Pbx and Meis factors. *Development* **135**, 3369-3378.
Weasner, B. M., Weasner, B., Deyoung, S. M., Michaels, S. D. and Kumar, J. P. (2009). Transcriptional activities of the Pax6 gene eyeless regulate tissue specificity of ectopic eye formation in Drosophila. *Dev. Biol.* **334**, 492-502.
Weisinger, K., Wilkinson, D. G. and Sela-Donenfeld, D. (2008). Inhibition of BMPs by follistatin is required for FGF3 expression and segmental patterning of the hindbrain. *Dev. Biol.* **324**, 213-225.
Weisinger, K., Kayam, G., Missulawin-Drillman, T. and Sela-Donenfeld, D. (2010). Analysis of expression and function of FGF-MAPK signaling components in the hindbrain reveals a central role for FGF3 in the regulation of Krox20, mediated by Pea3. *Dev. Biol.* **344**, 881-895.
Weisinger, K., Kohl, A., Kayam, G., Monsonego-Ornan, E. and Sela-Donenfeld, D. (2012). Expression of hindbrain boundary markers is regulated by FGF3. *Biol. Open* **1**, 67-74.
Xu, Q., Alldus, G., Holder, N. and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. *Development* **121**, 4005-4016.
Xu, Q., Mellitzer, G. and Wilkinson, D. G. (2000). Roles of Eph receptors and ephrins in segmental patterning. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 993-1002.