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 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 Vhnfl and Kreisler (also known as Hnf1b and Maft, 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). Vhnfl 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 Nub and Nilz (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; Mecha-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-Viliar et al., 2007; Ericson et al., 1997). Along the hindbrain of E12 rat/E3 chick embryos, Pax6 shows a uniform ventralhigh-dorsallow 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
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′-GATTCTCCAGTCCAGGCACCAGGT-3′; reverse, 5′-TCCCCAGTGCTGAGCTTCCTCAG-3′; reverse, 5′-CCATCGCTGGCTGCAAACCTTGG-3′.

In ovo electroporation

pCIG-IRES-GFP, pCIG-Pax6-IRES-GFP, pCIG-Pax6-En-IRES-GFP, pCAGGS-RFP, pCAGGS-snRNA-Pax6-RFP, pBRE-lacZ, pDARS-Vgfla-Krox20 and pAdRSV/Nab1-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 and harvested 16–48 hours later, as described (Weisinger et al., 2008). Whole-mount ISH was performed as described (Weisinger et al., 2008).

In situ fixation.

Embryos ~6 hours after electroporation. Embryos treated with SU5402 or (Weisinger et al., 2010). Cyclohexamide (10 µg/ml; Sigma) was added to AG1-X2 beads (100–200 mesh, BioRad) were soaked in SU5402 (200 µM; Bevlow technologies) as described (Monsonego-Ornan et al., 2012).

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). High levels of Pax6 remain in r3/r5 up to the 30-somite stage (Fig. 1F). From the 14-somite stage, Pax6 also appears in r2/r4/r6 (Fig. 1F), 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. 1I), 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-J, 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 (Derober et al., 2002; Qu 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

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).
(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 x 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 r5 but was still sharply defined in expression in r5 (Fig. 2O,Q; n=4 for each stage) (see also Viouculescu 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. 1. Expression of Pax6 in chick and mouse hindbrain. (A-P) in situ hybridization (ISH) (A-M), immunohistochemistry (IHC) (E’-J’) 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’-J’) 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.
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. 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, n = 8/8) and in the control side of Pax6-manipulated hindbrain (Fig. 3B, 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.
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-expressing 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.

Fig. 3. Pax6 upregulates Nab1 to restrict Krox20 expression domains. (A–K) 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′) 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.
Pax6 role in hindbrain segments

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; Morriess-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 expression 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 expression 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 pAdRSV/βgal-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 pAdRSV/βgal-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 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., 2009). 

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.
side expressing Pax6-GFP or Pax6-En-GFP (Fig. 6B,C) embryos (Fig. 6D,D/H11032) the neurofilament protein 3A10 (Guthrie et al., 1991) was 9/12, respectively), as compared with the contralateral side. Equally, (Fig. 6A,A/H11032)/H11032) indicated boundaries of EphA4+ regions in A-E. Dashed areas (A, Epha4+E/H11032) Views of the boxed regions in A-E. Dashed areas (A’-E’) indicate boundaries of EphA4+ domains. Arrowheads mark abnormal EphA4 patterns.

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.

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. S2L,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...
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; Marin and Charnay, 2000; Weisinger et al., 2010). Fgf3/Krox20 patterns (Marín and Charnay, 2000; Weisinger et al., 2010; Weisinger et al., 2008; Weisinger et al., 2012) 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-Viallar 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
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, rhombomeric 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
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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Boundary markers</th>
<th>Segmental genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No effect</td>
<td>Distortion or loss of boundary</td>
</tr>
<tr>
<td>GFP</td>
<td>27/27 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Pax6 gain-of-function</td>
<td>5/29 (17%)</td>
<td>24/29 (83%)</td>
</tr>
<tr>
<td>Pax6 loss-of-function</td>
<td>6/28 (21%)</td>
<td>22/28 (79%)</td>
</tr>
</tbody>
</table>

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.
<table>
<thead>
<tr>
<th>Wild Type</th>
<th>Pax6 loss-of-function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kreisler</td>
<td>EphA4</td>
</tr>
<tr>
<td>r3</td>
<td>r5</td>
</tr>
</tbody>
</table>

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.

**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.

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 (Manzanoares 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; Wassel 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 gene expression 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 identities, and supports its role in guarding the segmental domains of hindbrain genes.

**Acknowledgements**

We thank Sophie Bel-Vialar for the pCIG-Pax6 plasmids; Patrick Charney for the pAdRSVgal-Krox20 and pAdRSVNab1-HA vectors; Chaya Kalcheim for the pBRE-lacZ plasmid; ARK-Genomics for the Pax6-RNAi vector; DSHB for antibodies; Peter Gruss and Anastassia Stoykova for the heterozygote Pax6-LacZ mice; James Briscoe, Peter Charney, Robb Krumlauf, Eldad Tzahor, David Wilkison, Paul Trainor, Tom Schultheiss, Anthony Gavalas, Suzanne Mansour and Cliff Tabin for probes and antibodies; and Ruth Ashery-Padan for helping with the mice.

**Funding**

This study was supported by the Israel Science Foundation [161/07 and 133/11 to D.S.D.] and the Israel Science Foundation [1391/11 to C.B.]. G.K. was supported by a fellowship from the Robert H. Smith Fund.

**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl; doi:10.1242/dev.089136/-/DC1

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