Molecular dissection of Pax6 function: the specific roles of the paired domain and homeodomain in brain development

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Summary

The transcription factor Pax6 plays a key role during development of various organs, including the brain where it affects cell fate, cell proliferation and patterning. To understand how Pax6 coordinates these diverse effects at the molecular level, we examined the role of distinct DNA-binding domains of Pax6, the homeodomain (HD), the paired domain (PD) and its splice variant (5a), using loss- and gain-of-function approaches. Here we show that the PD is necessary for the regulation of neurogenesis, cell proliferation and patterning effects of Pax6, since these aspects are severely affected in the developing forebrain of the *Pax6^{Aey18}* mice with a deletion in the PD but intact homeo- and transactivation domains. In contrast, a mutation of the HD lacking DNA-binding (*Pax6^{4Neu}*) resulted in only subtle defects of forebrain development. We further demonstrate distinct roles of the two splice variants of the PD. Retrovirally mediated overexpression of Pax6 containing exon 5a inhibited cell proliferation without affecting cell fate, while Pax6 containing the canonical form of the PD lacking exon 5a affected simultaneously cell fate and proliferation. These results therefore demonstrate a key role of the PD in brain development and implicate splicing as a pivotal factor regulating the potent neurogenic role of Pax6.

Key words: Forebrain, Cortex, Neurogenesis, Proliferation, Regionalization, DNA-binding domains, Paired domain, Homeodomain, Mouse mutant

Introduction

The transcription factor Pax6 is conserved from invertebrates to vertebrates and regulates key developmental processes in several organs throughout phylogeny (Chi and Epstein, 2002). In all of these organs, Pax6 acts as a potent cell fate determinant, and often affects cell proliferation, yet it is not well understood how Pax6 coordinates these diverse effects at the molecular level in vivo. Pax6 contains two DNA-binding domains, the paired domain (PD) and a paired-like homeodomain (HD) linked via a glycine-rich domain, and acts as an activator of gene expression by a proline-, serine- and threonine-rich (PST) domain at the C-terminus (Bouchard et al., 2003). Both the PD and HD bind to specific DNA target sites (Chi and Epstein, 2002) and may also influence each other's binding to the DNA or cooperate on DNA binding (Duncan et al., 1998; Jun and Desplan, 1996; Mikkola et al., 2001; Mishra et al., 2002; Singh et al., 2000). Several splice variants have been discovered for the *Pax6* gene (Anderson et al., 2002; Duncan et al., 2000; Epstein et al., 1994; Kozmik et al., 1997; Mishra et al., 2002) resulting predominantly in a truncated form lacking the paired domain ('paired-less') and a form with a 14 amino acid (AA) insert into the PD [Pax6(5a), see Fig. 1]. The canonical PD (without the 14 AA insert) binds via its N-terminal PAI domain (Epstein et al., 1994) to DNA, while the insertion of the 14 AA from exon 5a (now designated as exon 5, but here still referred to as 5a) into the PAI domain [PD(5a)] abolishes its DNA-binding and favors DNA-binding of the C-terminal RED domain (Epstein et al., 1994) (Fig. 1A). Distinct consensus sites have been identified for these domains, with the N-terminal PAI domain binding to both consensus sites (P6CON; 5aCON), while the C-terminal RED domain can only activate targets with the 5aCON site (Epstein et al., 1994) (Fig. 1A). Notably, the Pax6(5a) form is required for aspects of postnatal differentiation in the eye (Singh et al., 2002), and an important role of the same modification of PD-mediated DNA binding was recently discovered in the developing compound eye of *Drosophila* (Dominguez et al., 2004).

Despite the crucial function of Pax6 in the developing brain, nothing is known about the specific roles of its DNA-binding domains, since analysis has so far been performed exclusively with the functional null alleles *Pax6^{Sey}*, *Pax6$^{Sey-Neu}$* in mice (Hill et al., 1991; Schmahl et al., 1993) and the *Pax6^{Sey}* rat (Matsuo et al., 1993), all of which contain a truncated Pax6 protein lacking the transactivator domain (TA) (Fig. 1E). The strongest effects of Pax6 mutation in the brain are manifested in the forebrain (Schmahl et al., 1993), where Pax6 expression starts at embryonic day (E) 9 in the mouse (Walther and Gruss,

1991). In the telencephalon, Pax6 is restricted to the dorsal region, and this region is misspecified in the *Pax6* mutants. Severe defects in patterning are indicated by the expansion of ventral transcription factors into the dorsal telencephalon or the loss of boundary structures restricting cell migration between these regions of $Pax6^{Sey}$–/– mice (Chapouton et al., 1999; Heins et al., 2002; Stoykova et al., 1996; Stoykova et al., 1997; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Notably these *Pax6* mutations also affect patterning in the hindbrain, spinal cord and the ventral diencephalon (Briscoe et al., 1999; Grindley et al., 1997; Osumi, 2001; Osumi et al., 1997; Stoykova et al., 1996) implying a widespread role of Pax6 in patterning.

In contrast, the recently discovered potent neurogenic role of Pax6 appears more restricted to the telencephalon, and seemingly contributes to the maintenance of neurogenesis into adulthood exclusively in this part of the brain (Alvarez-Buylla et al., 2001; Gage, 2002; Hack et al., 2004; Heins et al., 2002; Nakatomi et al., 2002). The absence of functional Pax6 not only severely impairs neurogenesis in the dorsal telencephalon, its overexpression is also sufficient to drive embryonic precursors, postnatal cortical astrocytes and adult neural stem cells towards neurogenesis (Hack et al., 2004; Heins et al., 2002). In addition, Pax6 is involved in the reduction of proliferation in the telencephalon, since its loss-of-function results in an increased number of precursors (Estivill-Torrus et al., 2002; Götz et al., 1998; Heins et al., 2002), while its gain-of-function reduces the number of progeny generated by a single progenitor cell (Heins et al., 2002). To gain a better understanding of potentially separate roles exerted by distinct DNA-binding domains of the Pax6 transcription factor, we examined here the function of its three DNA binding domains, the PD, PD(5a) and HD in the developing forebrain.

Materials and methods

Animals

$Pax6^{Sey}$ mice were maintained as heterozygotes on a mixed C57BL/6JxDBA/2J background, $Pax6^{Aey18}$ mice (J.G., unpublished) and $Pax6^{4Neu}$ mice were maintained on a C3HeB/FeJ background). $Pax6^{tm1Gfs}$ mice are referred here as *Pax6(5a)–/–* mice (Singh et al., 2002) and were maintained as homozygotes on a C57BL/6 background. Analysis was performed with embryos from embryonic day (E)10-16.

Immunohistochemistry and in situ hybridization

Embryonic brains were fixed in 4% paraformaldehyde in PBS (PFA) and sections were cut with a vibratome or with a cryostat after cryoprotection. Sections were processed for immunohistochemistry (Götz et al., 1998; Hartfuss et al., 2001), using the primary antibodies against the phosphorylated form of Histone H3 (PH3, rabbit, Biomol, 1:200), NeuN (mouse IgG1, 1:50, Chemicon), Mash1 (mouse IgG1, 1:2, kindly provided by F. Guillemot), Gsh2 (rabbit, 1:1000, kindly provided by K. Campbell), Ngn2 (mouse IgG2a, 1:10, kindly provided by D. Anderson), Olig2 (rabbit, 1:1000, kindly provided by D. Rowitch), Ki67 (rat Tec-3, 1:50, Dako), β-galactosidase (rabbit, 1:300, Cappel; mouse IgG2a, 1:500, Promega), O4 (mouse IgM, 1:1000, kindly provided by J. Price), GFAP (mouse IgG1, 1:200, Sigma), RC2 (mouse IgM, 1:500, kindly provided by P. Leprince), Pax6 (rabbit, 1:300, Babco), reticulon1 (mAb 9-4, 1:10, kindly provided by T. Hirata) and nestin (mouse IgG1, 1:4, Dev. Hybridoma Bank). The respective secondary antibodies were used from Jackson Immunoresearch, Inc. and Southern Biotechnology Associates, Inc. Specimens were mounted in Aqua Poly/Mount (Polysciences, Northampton, UK) and analyzed at a Confocal Microscope (Leica TCS 4NT; Leica Microsystems; Heidelberg). Digoxigenin-labeled RNA probe for SFRP2 was made and used as described (Chapouton et al., 2001).

Retrovirus preparation and retroviral infection

The entire coding sequence of Pax6 (1873bp fragment) and Pax6(5a) (1915 bp fragment) was cloned in sense orientation into the *Bgl*II unique restriction site of the retroviral vector 1704 between the upstream LTR and the EMC IRES sequence (gift of J. E. Majors) (Ghattas et al., 1991). BOSC23 helper-free packaging cells (Pear et al., 1993) were used for viral production by means of transient transfection with the respective viral plasmid and resulted in a typical titre of 1×10^5/ml. Primary cells from cerebral cortex were isolated and cultured as described previously (Heins et al., 2002), and infected 2 hours after plating at a concentration giving no more than 50 clones per coverslip and analyzed after 1 week in vitro.

Data analysis

The quantification of neurons was performed in sections of the cerebral cortex at E14 from wild-type (WT) and homozygous mutants at corresponding rostral, intermediate and caudal levels stained for NeuN. The thickness of the NeuN-positive cortical plate was measured in confocal pictures taken in a defined (lateral) area of the cortex by first drawing a line at right angles to the ventricular surface (VS) from the VS to the pial surface. The length of this line was determined by the ImageJ program and served as measure of the total cortical thickness. A second line was drawn from the apical and basal side of the NeuN-positive band, and its length served as a measure for the thickness of the band of neurons, the cortical plate. The width of the cortical plate was calculated as the proportion of the overall thickness of the cerebral cortex (Table 1).

The quantification of PH3-labeled cells was performed by placing a 150-μm-wide square covering the entire cortical thickness parallel to the VS. All PH3-positive cells in this square were counted separately for cells lining the ventricle (VZ cells) and cells at abventricular positions (SVZ cells defined as PH3+ cells, five or more cell diameters away from the VS) (see Smart, 1976) (Table 1).

For the clonal analysis in vitro, all clones per coverslip were assessed for their cell type or size (Fig. 5) and the mean was calculated per coverslip (excluding coverslips with more than 50 clones; Fig. 5C,D). Group comparisons were made with the unpaired *t*-test and *P*-values smaller than 0.05 were considered significant (*), *P*-values smaller than 0.001 were considered highly significant (***).

Results

The paired domain, but not the homeodomain plays a crucial role in telencephalon development

To assess the role of the PD of Pax6 we analyzed the $Pax6^{Aey18}$ mutant mice, which are characterized by the loss of a splice acceptor site in front of exon 5a such that exon 5a and 6 are excluded and a large part of the PD is deleted, whereas the HD and TA remain unaffected (Fig. 1B) (J.G., unpublished). To examine specifically the role of the Pax6(5a) isoform we used the $Pax6^{tm1Gfs}$ mice [here referred to as *Pax6(5a)–/–*] carrying a targeted deletion of the exon 5a (Fig. 1C) (Singh et al., 2002). The $Pax6^{4Neu}$ mutation carries a point mutation in the third helix of the HD that abolishes its DNA-binding (Fig. 1D) (Favor et al., 2001). These mutants were compared to the $Pax6^{Sey}$–/– mice, characterized by a truncated form of Pax6 lacking the TA domain (Fig. 1E), which has been identified as a functional null by comparison to the *Pax6–/–* (e.g. Götz et al., 1998; Briscoe et al., 1999).

Table 1. Quantitative analysis of the phenotypes in neurogenesis and proliferation of mouse mutants with specific defects in Pax6 DNA-binding domains

	Thickness of cortical plate (neurons)/thickness of entire cortex	Proliferation/area (number of PH3+ cells per radial stripe of 150 μm) at ventricular surface	Proliferation/area (number of PH3+ cells per radial stripe of 150 μm) at subventricular zone	Proliferation/area (total number of PH3+ cells per radial stripe of 150 μm)	Percent abventricular mitosis per hemisphere
Wild type	0.25±0.039	13.9±4.2	4.9±2.6	18.8	24.2±5.2
$Pax6^{Aey18}$–/–	0.21±0.0311 $P<0.009$	12.8±2.5 $P<0.453$	13.3±2.9 $P<1.8\times10^{-7}$	26.1	42.0±8.7
Wild type	0.25±0.070	11.8±2.0	5.9±3.1	17.7	26.1±6.0
Pax6(5a)–/–	0.22±0.064 $P<0.1$	12.3±3.4 $P<0.661$	4.3±2.1 $P<0.143$	16.6	25.4±4.2
Wild type	0.23±0.055	15.1±5.3	6.3±4.5	21.4	29.7±7.4
$Pax6^{4Neu}$–/–	0.23±0.029 $P<0.8$	13.1±3.8 $P<0.311$	5.9±3.8 $P<0.847$	19	27.2±5.5
Wild type	0.23±0.035	16.3±6.3	7.8±6.7	24.1	16.0±6.9
$Pax6^{Sey}$–/–	0.17±0.041 $P<0.0007$	17.1±5.6 $P<0.831$	19.1±5.6 $P<9.8\times10^{-5}$	36.2	35.8±5.2

All values are given as mean±s.d., $n=12$.

Neurogenesis

Neurogenesis was examined in these mutants by immunohistochemistry for neuronal markers, such as NeuN (Fig. 2) or βIII-tubulin (data not shown). Neurons accumulate in the cortical plate (CP) forming a distinct band underneath the pial surface (Fig. 2A) at midneurogenesis, embryonic day (E) 14, in the WT cortex. The NeuN-positive band is distinctly smaller in the cortex of homozygous *Pax6*^{Aey18}–/– mouse mutants (Fig. 2A,B), reflecting a significant decrease in the number of neurons generated (Table 1). Since the neurogenesis phenotype of *Pax6*^{Aey18}–/– mice was reminiscent of the functional null allele *Pax6*^{Sey} (Fig. 2E), we quantified the thickness of the cortical plate relative to the total cortical thickness (see Materials and methods) in the lateral cortex at rostral, intermediate and caudal levels (Table 1). Notably the significant reduction of the thickness of the NeuN-positive band compared to WT in the *Pax6*^{Aey18}–/– cortex ($P<0.001$) and the comparable reduction observed in *Pax6*^{Sey}–/– cortex (Table 1), suggests that *Pax6*^{Aey18} is a functional null allele for cortical neurogenesis. In contrast, we found no reduction in neurogenesis in *Pax6(5a)–/–* mice (Table 1; Fig. 2C) or *Pax6*^{4Neu}–/– mice (Table 1; Fig. 2A,D). Thus, the PD, but not the HD of Pax6 is required for the regulation of neurogenesis in the cerebral cortex.

Cell proliferation

Reduction of neurogenesis in the developing telencephalon is accompanied by an increase in cell proliferation in mutants bearing functional null alleles of *Pax6* (Estivill-Torrus et al., 2002; Götz et al., 1998). The number of precursors was determined by immunostaining against the antigen Ki67 labeling actively dividing precursors in all phases of the cell cycle (Gerlach et al., 1997) and the phosphorylated form of histone H3 (PH3) (Hendzel et al., 1997). Since PH3 labels all

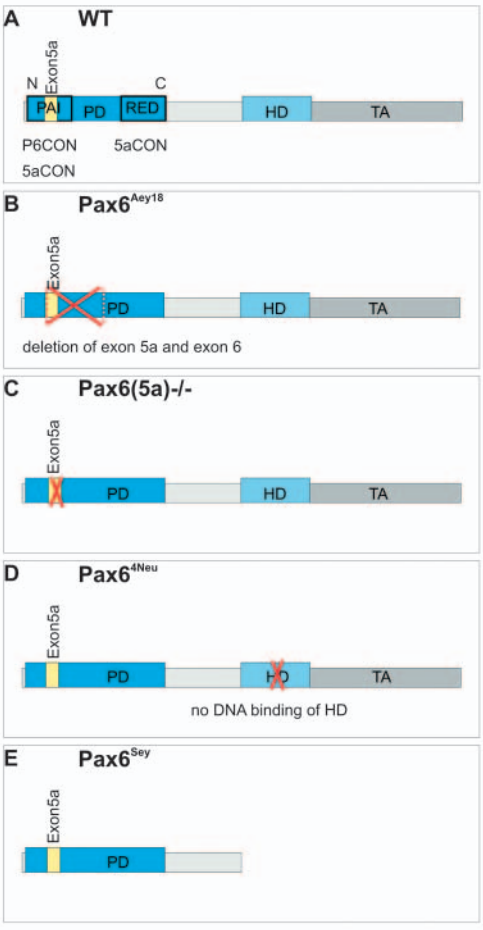

Fig. 1. Schematic drawings of the Pax6 mRNA in wild type (WT) and mice harboring mutations in the distinct DNA-binding domains of Pax6. (A) The mRNA of *Pax6* consists of two DNA binding-domains, the paired domain (PD) and the homeodomain (HD). The PD is subdivided in two independent DNA-binding domains, the N-terminal PAI and the C-terminal RED domain. The PAI domain is able to bind to P6CON (Pax6 consensus site) and 5aCON (Pax6(5a) consensus site), whereas the RED domain binds exclusively to 5aCON. By means of alternative splicing, 14 amino acids are inserted into the N-terminal PAI domain (see yellow box) and thereby abolish its DNA binding. The transactivation domain (TA), important for gene activation, is located at the C-terminal end of Pax6. (B) The *Pax6*^{Aey18} mutant is characterized by a large deletion in the PD (exon 5a and exon 6), whereas the HD and TA are still present. (C) The *Pax6(5a)–/–* lacks exon 5a in the PD due to gene targeting (Singh et al., 2002), while the rest of the PD, HD and TA are unaffected. (D) *Pax6*^{4Neu} mutant mice bear a point mutation in the HD that abolishes DNA binding of the HD, while PD and TA are intact (Favor et al., 2001). (E) The *Pax6*^{Sey} mutant mice are characterized by a truncated form of Pax6, lacking the HD and TA. These mutants have a comparable phenotype to the full gene deletion (*Pax6–/–*) and are therefore referred to as functional null alleles. PD, paired domain; HD, homeodomain; TA, PST-rich transactivator domain.

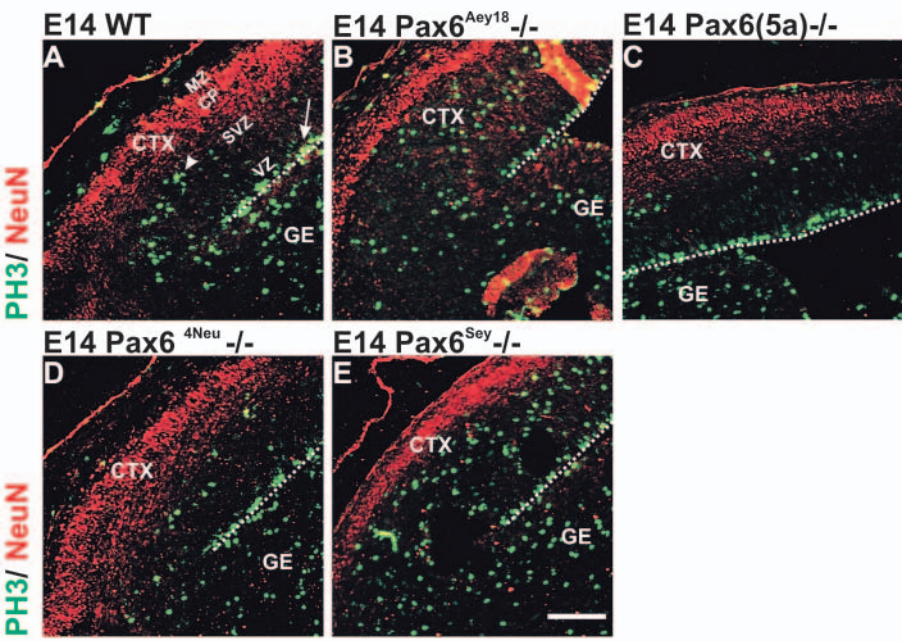

Fig. 2. Neurogenesis and cell proliferation in the cerebral cortex of mice harboring mutations in the distinct DNA-binding domains of Pax6. (A-E) Micrographs show coronal sections of the lateral cerebral cortex immunostained for NeuN (red) (neuronal marker) and PH3 (green) (marker for cells in M-phase) at embryonic day (E)14 in the respective mouse mutants as indicated in the panels. Note that the band of NeuN-positive cells is reduced in the PD mutant *Pax6*^{Aey18}*–/–* (B) compared to WT cortex (A), indicating a reduced neurogenesis that appears comparable in extent to the phenotype in the functional null *Pax6*^{Sey}*–/–* (E). In contrast, no such changes could be observed for the cortex of *Pax6(5a)–/–* (C) or the HD-mutant *Pax6*^{4Neu}*–/–* (D). In the mutants with an impaired neurogenesis (the PD mutant *Pax6*^{Aey18}*–/–* and *Pax6*^{Sey}*–/–*), we observed an increase in precursors (green PH3-positive cells in B,E), while no changes in comparison to WT (A) were seen in the cortex of *Pax6(5a)–/–* (C) and *Pax6*^{4Neu}*–/–* (D). Note that the PH3-positive cells were mostly increased in the subventricular zone (SVZ, arrowhead in A; increase in B and E), but not in the VZ (arrow in A). Thus, the PD of Pax6 is necessary and sufficient to mediate the effects of Pax6 on neurogenesis and cell proliferation in the cerebral cortex, while targets of the HD seem to play no role in these aspects. The dashed white line (A-E) indicates the ventricular surface of the cortex. MZ, marginal zone; CP, cortical plate ; SVZ, subventricular zone; VZ, ventricular zone; CTX, cortex; GE, ganglionic eminence. Scale bar: 100 μm.

precursors in late G2 and M-phase (Fig. 2), we could distinguish between precursors located in the VZ, that undergo interkinetic nuclear migration and perform M-phase at the ventricular surface (VS; arrow in Fig. 2A), from precursors located in the subventricular zone (SVZ) that undergo mitosis at abventricular positions (arrowhead in Fig. 2A) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Smart, 1976). In the *Pax6*^{Aey18}*–/–* mutant cortex the increase in the number of PH3-positive cells was obvious (Fig. 2A,B) and highly statistically significant (Table 1). Interestingly, the number of precursors in the VZ was not changed in the *Pax6*^{Aey18}*–/–* cortex, but the mutation results in a specific increase in SVZ precursors (Fig. 2A,B; Table 1). This phenotype is identical to the cortex of the *Pax6*^{Sey}*–/–* mice (Fig. 2E) that also exhibited a marked increase in SVZ precursor cells [2.7-fold increase in SVZ precursors in *Pax6*^{Aey18}*–/–* cortex compared to 2.4-fold increase in *Pax6*^{Sey}*–/–* cortex; Table 1 (Estivill-Torrus et al., 2002; Götz et al., 1998)], again supporting a functional null phenotype in the PD mutant *Pax6*^{Aey18}*–/–* mice. In contrast, no changes in proliferation, either in the VZ or SVZ, were detectable in mice solely lacking exon 5a (Fig. 2A,C; Table 1) or in the HD mutant *Pax6*^{4Neu}*–/–* (Fig. 2A,D; Table 1). Taken together, it appears that regulation of neurogenesis and proliferation does not involve the HD, but depends solely on PD function.

Patterning

Next we examined the role of the PD and HD in mediating dorso-ventral patterning in the forebrain. Previous analysis of the functional null *Pax6*^{Sey}*–/–* described an almost complete lack of neurogenin (Ngn) 2 expression in the dorsal telencephalon, while expression of the transcription factors Mash1, Gsh2 and Olig2, that is normally restricted to the ventral telencephalon, expands into the dorsal telencephalon in the absence of Pax6 function (Fig. 3D,D′, and data not shown) (see Stoykova et al., 2000; Torresson et al., 2000; Yun et al., 2001). Precisely this phenotype was observed in the cortex of *Pax6*^{Aey18}*–/–* mice (Fig. 3A,A′; see Fig. S1 in supplementary material) while no changes in dorso-ventral expression patterns of these transcription factors were observed in the cortex of the *Pax6(5a)–/–* (Fig. 3B,B′) and the HD mutant *Pax6*^{4Neu}*–/–* (Fig. 3C,C′; Fig. S1). Since *Ngn2* is a direct target of the canonical form of the PD (Scardigli et al., 2003), these findings further support the crucial role of the canonical PD of Pax6 in its regulation. Consistent with the gradient of Pax6 in the cortex, Ngn2 expression was still visible in the caudal-most and medial regions of the cortex in the *Pax6*^{Sey}*–/–* and *Pax6*^{Aey18}*–/–*, where Pax6 expression is lowest (see Fig. S2A,B,E in supplementary material).

The telencephalic region where these two domains of transcription factors normally abut differentiates various features of a boundary (Lumsden and Krumlauf, 1996) with formation of a radial glia fascicle and expression of specific diffusible molecules thereby inhibiting most, but not all, cells from crossing between the dorsal and the ventral telencephalon (Chapouton et al., 1999; Chapouton et al., 2001; Fishell et al., 1993). One characteristic marker is SFRP2, the soluble frizzled related protein 2, expressed specifically at the ventral-most region of the Pax6-positive territory (Chapouton et al., 2001; Kim et al., 2001). SFRP2 expression failed to appear in the *Pax6*^{Aey18}*–/–* (Fig. 4A,A′), the *Pax6*^{4Neu}*–/–* (Fig. 4C,C′) and *Pax6*^{Sey}*–/–* telencephalon (Kim et al., 2001) (Fig. 4D,D′), while it was normally expressed in the *Pax6(5a)–/–* telencephalon (Fig. 4B,B′). In contrast the boundary marker protein reticulon-1 (Hirata et al., 2002) was absent in the *Pax6*^{Aey18}*–/–* as in the *Pax6*^{Sey}*–/–*, but was still detectable in the *Pax6(5a)–/–* and in

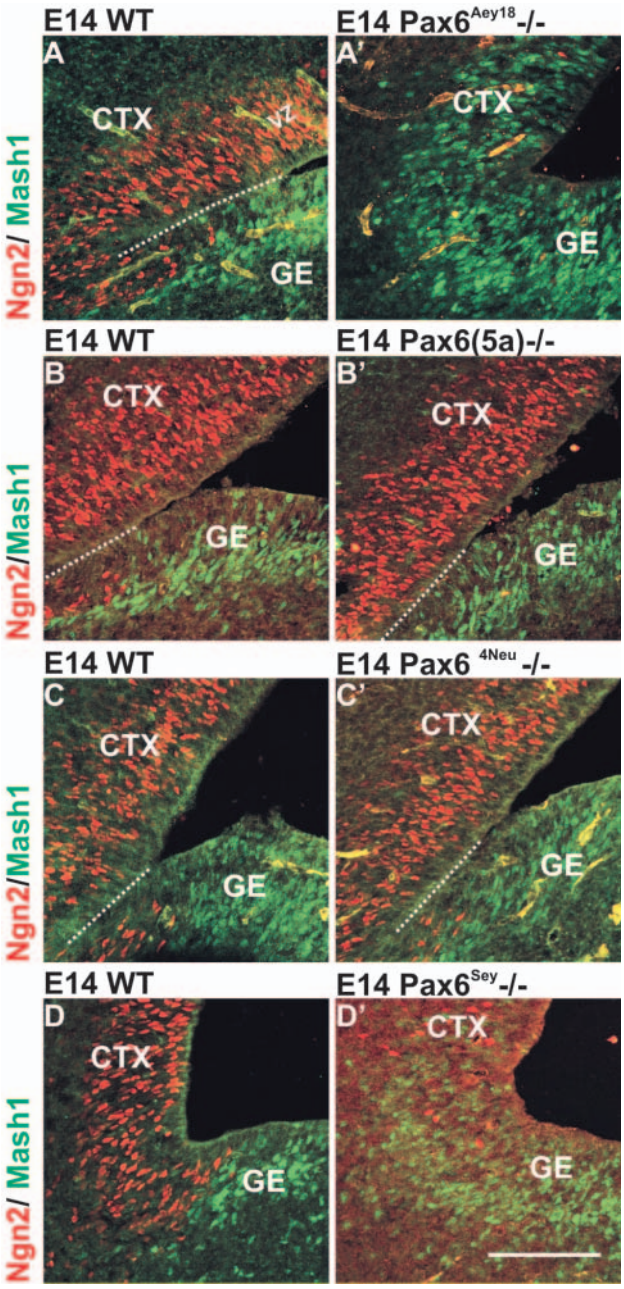

Fig. 3. Dorso-ventral patterning in the telencephalon of mice harboring mutations in the distinct DNA-binding domains of Pax6. Micrographs of coronal sections of the lateral telencephalon at embryonic day (E) 14. (A-D) In WT CTX, but not GE, precursors are Ngn2-immunopositive (red), while precursors in the GE, but not the CTX, are Mash1-immunoreactive (green in A-D). Panels from littermate mutant mice are depicted in the right column (A′-D′). Note that Ngn2-immunoreactivity is not detectable in the CTX of mutant mice with a large deletion in the PD (see Fig. 1B), the *Pax6*^{Aey18}*–/–* mice (A′) and the functional null allele *Pax6*^{Sey}*–/–* (E′), while it is unaffected in *Pax6(5a)–/–* (B′) and *Pax6*^{4Neu}*–/–* (C′). Conversely, Mash1-immunoreactivity spreads ectopically into the cortex of *Pax6*^{Aey18}*–/–* (A′) and *Pax6*^{Sey}*–/–* (D′) mice, but is not changed in *Pax6(5a)–/–* (B′) or *Pax6*^{4Neu}*–/–* (C′) telencephalon. Thus, the PD of Pax6 seems to be necessary and sufficient to exert patterning of the telencephalon. The dashed white line (A,B,B′,C,C′) indicates the ventricular surface. CTX, cortex; GE, ganglionic eminence. Scale bar: 100 μm.

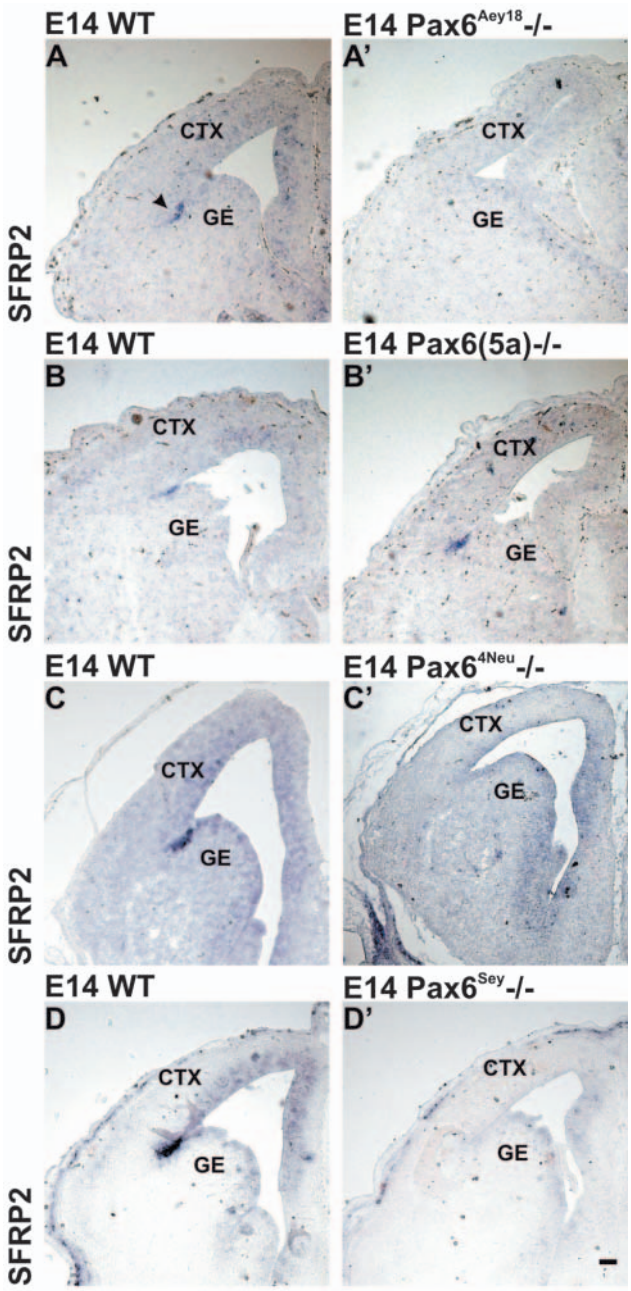

Fig. 4. *SFRP2* expression in the pallio-subpallial boundary between the dorsal (CTX) and ventral (GE) telencephalon of mice harboring mutations in the distinct DNA-binding domains of Pax6. Micrographs depict in situ-hybridization for *SFRP2* mRNA in coronal sections of the telencephalon of WT and mutant littermates as indicated in the panel at embryonic day (E) 14. Note that *SFRP2* expression is highest at the boundary between CTX and GE in WT mice (A,B,C,D; arrowhead in A), and *Pax6(5a)–/–* (B′), while its expression is lost at this position in the Pax6 mutation with a large deletion in the PD *Pax6*^{Aey18}*–/–* (A′), the mutation with the defect HD *Pax6*^{4Neu}*–/–* (C′) and the functional null allele *Pax6*^{Sey}*–/–* (D′). Thus, both, DNA binding of the PD and the HD of Pax6 are required for *SFRP2* expression at the pallial-subpallial boundary. CTX, cortex; GE, ganglionic eminence. Scale bar: 100 μm.

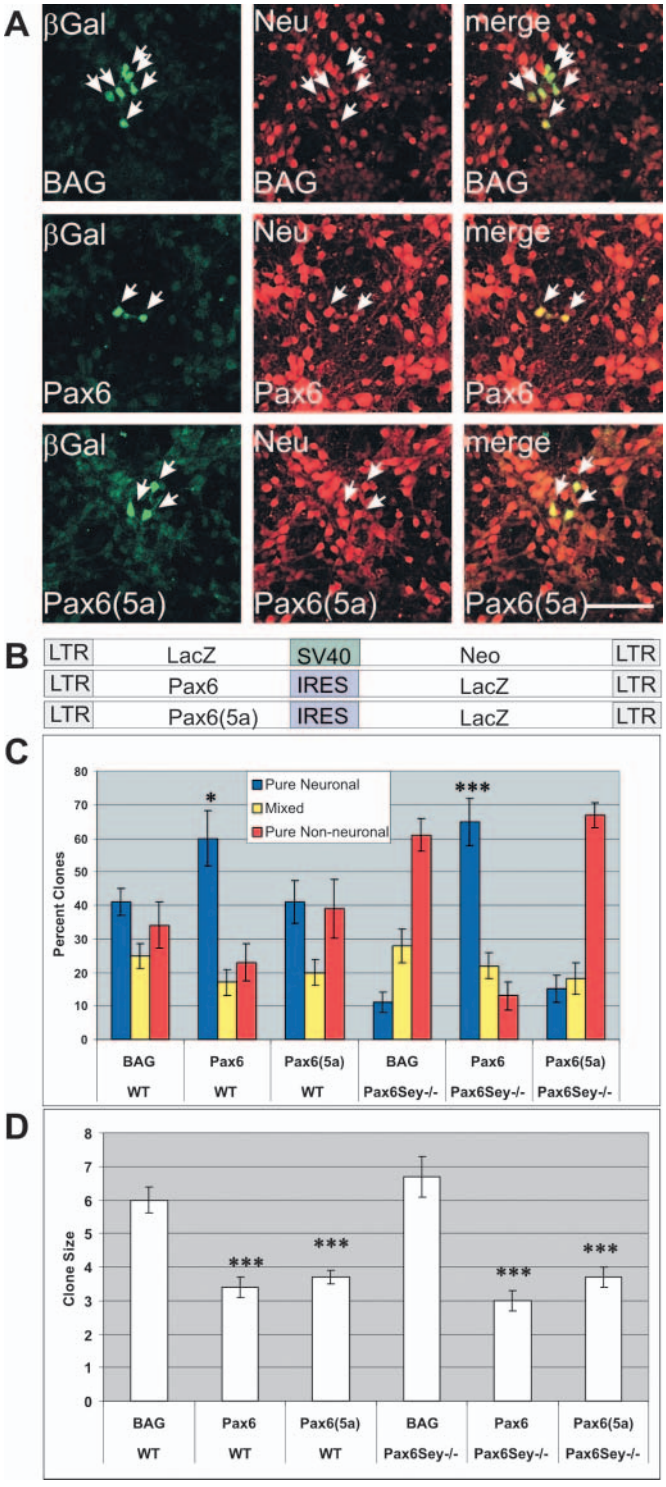

Fig. 5. Retroviral overexpression of Pax6(5a) reveals a distinct influence on cell proliferation without effects on cell fate. (A) Corresponding micrographs of a clone of cells (indicated by arrow), isolated from the embryonic day (E) 14 cortex, infected with the BAG control retrovirus (upper row), Pax6-containing retrovirus (middle row) and Pax6(5a)-containing virus (lower row), cultured for 7 days in vitro and immunostained against β-galactosidase (β-gal, green), NeuN (red). Scale bar: 50 μm. (B) Schematic drawings of the retroviral constructs used for control, Pax6 (canonical form of PD) and Pax6(5a) overexpression together with the marker gene *lacZ*. (C) Histogram depicting the clone type of either control (BAG), Pax6- or Pax6(5a)-infected cells isolated from E14 WT (bars to the left) or *Pax6^{Sey}–/–* mutant cortex (bars to the right) and cultured for 7 days. Note that the percentage of pure neuronal clones (blue bars) increases significantly (compared to WT control, *t*-test, see Materials and methods; error bars=s.e.m.) in the cells transduced with virus containing Pax6 with the canonical form of the PD, at the expense of the mixed (yellow bars) and pure non-neuronal clones (red bars). In contrast, Pax6(5a) exerted no effect on the clone type, even in the absence of functional Pax6 in *Pax6^{Sey}–/–* cortical cells. (D) Histogram depicting the mean size of clones (=the number of β-galactosidase-positive cells per clone, i.e. the number of cells generated by a single infected precursor) in the cultures described in C. Note that the clone size was reduced after transduction of cortical cells with Pax6 and Pax6(5a). Pax6(5a) was sufficient to reduce cell proliferation, suggesting that this effect is mediated by the 5aCON site. Numbers of clones analyzed: WT Ctrl: 395, WT Pax6: 243, WT Pax6(5a): 268, *Pax6^{Sey}–/–* Ctrl: 353, *Pax6^{Sey}–/–* Pax6: 229, *Pax6^{Sey}–/–* Pax6(5a): 219.

the HD mutant *Pax6^{4Neu}–/–* (data not shown). Taken together, these results suggest that the HD and the PD cooperate on some, but not all aspects of differentiation of the dorso-ventral telencephalic boundary, while the other effects of Pax6 on dorso-ventral patterning mediated by region-specific expression of transcription factors are predominantly regulated by the PD.

Gain-of-function experiments reveal a specific role of the PD5a in proliferation but not in cell fate

Since the canonical form of the PD can bind to both P6CON and 5aCON (Epstein et al., 1994), we were concerned whether upregulation of canonical Pax6 in the *Pax6(5a)–/–* may compensate for the lack of the Pax6(5a) isoform. Indeed, real-time RT-PCR analysis revealed a relative increase in *Pax6* mRNA in the *Pax6(5a)–/–* cortex that may be sufficient to compensate for the small amount (10-20%) of the Pax6(5a) isoform during most of neurogenesis in the developing cortex (see Fig. S3 in supplementary material). We therefore chose gain-of-function experiments to directly assess the specific effect of the Pax6(5a) isoform. Replication-incompetent retroviral vectors were used to overexpress Pax6(5a) in individual precursor cells to examine the influence on the progeny of these individual precursors. As indicated in Fig. 5A, single precursor cells from the cerebral cortex isolated at E14 were infected with one of the retroviral vectors carrying the LacZ marker gene encoding for β-galactosidase (βGal) (Fig. 5B), and their progeny were detected as distinct clusters of βGal immunoreactive cells, each representing a clone, after one week in vitro (Heins et al., 2001; Heins et al., 2002; Williams et al., 1991). Besides the control construct, viral vectors containing the canonical form of Pax6 (Hack et al., 2004; Heins et al., 2002) or the Pax6(5a) isoform (see Materials and methods), an IRES sequence and the marker gene (Fig. 5B) were used. This experiment assessed the cell-autonomous effects of the two isoforms of the PD on cell fate and cell proliferation by analysing the type and number of descendants generated by a single infected precursor cell.

The cell types generated by infected precursors were identified by immunocytochemistry using anti-NeuN for neurons, O4 for oligodendrocytes, anti-GFAP for astrocytes

and RC2 or anti-nestin for undifferentiated precursors. Clones were classified as pure neuronal clones when all cells of a cluster were NeuN-positive (examples in Fig. 5A), as mixed when only some cells of a cluster were NeuN-positive, and as non-neuronal when no cell of a cluster was NeuN-positive (Heins et al., 2001; Heins et al., 2002). Note that Pax6 containing the canonical PD potently increased neurogenesis and the number of neuronal clones as described previously (Heins et al., 2002), while Pax6(5a) overexpression had no effect on neuronal or glial cell fate (Fig. 5C). While the canonical form of Pax6 almost completely suppressed the generation of oligodendrocytes (0% of clones, n=81) or astrocytes (3% of clones, n=249), precursors infected with the Pax6(5a) form still generated control levels of oligodendrocyte clones (7% of clones, n=103) or astrocytes (11% of clones, n=137). One transcription factor involved in cell fate and the regulation of patterning is Mash1 (Casarosa et al., 1999) that normally is reduced after Pax6 overexpression (Heins et al., 2002). Only cells infected with the virus containing Pax6 with the canonical PD downregulated Mash1 (2% of clones, control 14%) while no change in Mash1-immunoreactive cells could be observed in the Pax6(5a)-infected cells (18% of clones). The absence of any effect of Pax6(5a) on cell fate was further confirmed in rescue experiments performed in cortical cells from *Pax6^{Sey}*–/– mice (Fig. 5C), where the low degree of neurogenesis could only be rescued by introduction of the canonical form of Pax6, while the Pax6(5a) form showed no effect at all (Fig. 5C). Taken together, these data demonstrate that even after overexpression in a Pax6 functional null background, Pax6(5a) is not able to influence cell fate of cortical progenitors. This suggests that the targets important for the mediation of the potent neurogenic role of Pax6 are regulated by the consensus site that is exclusively bound by the canonical PD of Pax6, i.e. the P6CON site.

In contrast to the lack of effect on cell fate, Pax6(5a) retroviral transduction exerted a potent effect on cell proliferation. The clone size reflects the number of the progeny generated by a single infected precursor and is significantly reduced by overexpression of both forms of Pax6, the canonical PD or the PD with the 5a insert (Fig. 5D). Pax6(5a) overexpression leads to a reduction in clone size independent of their cell type, i.e. in both neuronal and glial clones. While neuronal clones were typically small (see examples in Fig. 5A) (Heins et al., 2001; Heins et al., 2002), oligodendrocyte-containing clones are much larger and had a mean clone size of 16 cells in cultures infected with the control virus but only 11 cells in Pax6(5a)-infected cultures. Thus, Pax6(5a) reduces cell proliferation independent of the cell fate, while the canonical form of Pax6 couples both effects.

Discussion

Our functional analysis of the distinct DNA-binding domains of Pax6 showed that the canonical form of the PD regulates coordinately, neurogenesis, cell proliferation and patterning in the developing telencephalon, while the spliced form of the PD [Pax6(5a)] affects specifically cell proliferation without any effects on cell fate and regionalization (Fig. 6). In contrast, mutation of the HD affected only subtle aspects of the boundary delineating the dorsal and ventral telencephalon. Since the canonical form of the PD binds to both Pax6 consensus sites (P6CON, 5aCON) and the Pax6(5a) isoform binds exclusively to the 5aCON site (Epstein et al., 1994), our results suggest that the regulation of neurogenesis and the forebrain patterning is mediated via target genes containing P6CON sites, whereas the regulation of proliferation is mediated by target genes containing 5aCON sites (Fig. 6).

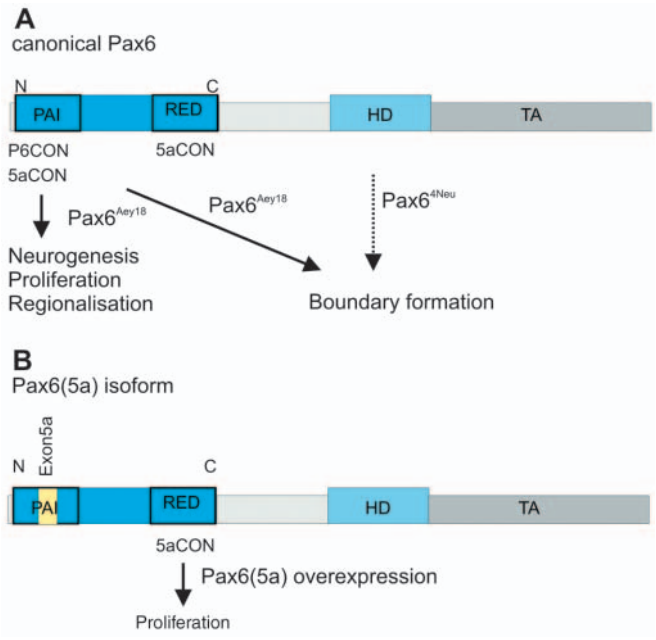

Fig. 6. Summary of the specific roles of the Pax6 DNA-binding domains in telencephalic development. Analysis of the *Pax6^{Aey18}*–/– mutant showed that the PD of Pax6 (A,B) is important for the regulation of neurogenesis, proliferation and regionalization in the developing telencephalon and that it contributes to the formation of the pallio-subpallial boundary. The lack of a phenotype in neurogenesis and proliferation in the forebrain of *Pax6^{4Neu}*–/– mice showed that the HD has no role in these processes (A), but is involved in some aspects of the pallio-subpallial boundary formation (SFRP2 expression). The Pax6(5a) isoform (B) is generated by alternative splicing leading to the insertion of a 14 AA insert in the N-terminal PAI subdomain of the PD, abolishing DNA binding and thereby favoring DNA binding via the RED domain. Retroviral overexpression revealed the anti-proliferative role of the Pax6(5a) isoform in cortical cells.

Homeodomain targets play minor roles in the developing telencephalon

Our analysis of the *Pax6^{4Neu}*–/– mutant, which carries a point mutation in the third helix of the HD that abolishes its DNA binding (Favor et al., 2001), revealed that DNA binding of the HD plays no role in the regulation of neurogenesis and cell proliferation in the developing forebrain, but is required together with the PD for the differentiation of one aspect of boundary formation in the forebrain, namely the expression of *SFRP2*. SFRP2 is a wnt-inhibitor expressed at the border between the dorsal and the ventral telencephalon. In both, the PD (*Pax6^{Aey18}*–/–) and HD (*Pax6^{4Neu}*–/–) mutant mice SFRP2 failed to be expressed at this position, while it was apparent in other parts of the brain. Other aspects of boundary differentiation, such as reticulon-1 expression or high BLBP-content in fasciculating radial glia fibers (data not shown), were

normal in the HD mutant *Pax6^{4Neu}–/–*, suggesting that DNA binding of the HD is required only for some aspects of boundary specification. Since both HD and PD mutations fail to regulate SFRP2, either both DNA-binding domains may need to bind cooperatively to mediate transcription of SFRP2, or an intact PD is required for the appropriate modulation of HD DNA binding as previously demonstrated in vitro (Jun and Desplan, 1996; Singh et al., 2000; Mishra et al., 2002). These results further support the multitude of regulatory mechanisms contributing to the boundary formation at this position that may also act as an organizing centre in the developing telencephalon (Assimacopoulos et al., 2003).

The canonical form of the paired domain is necessary and sufficient for the regulation of neurogenesis and cell proliferation

The novel Pax6 mutation *Pax6^{Aey18}* (J.G., unpublished) with a PD lacking most of its DNA-binding domain but intact HD and TA resulted in the same telencephalic phenotype as in the Pax6 functional null allele *Pax6^{Sey}–/–* (Estivill-Torrus et al., 2002; Götz et al., 1998; Stoykova et al., 1996; Stoykova et al., 2000). Even at the quantitative level, no differences in neurogenesis and cell proliferation were detectable between *Pax6^{Aey18}–/–* and *Pax6^{Sey}–/–* cortices, suggesting that the PD alone is necessary and sufficient for all of these aspects of cortical development. In addition, both genotypes expressed similar severity in the disturbance of patterning in the telencephalon. Our results therefore demonstrate that targets of the PD are necessary and sufficient to regulate cell proliferation and cell fate, comprising neurogenesis and patterning, in the developing forebrain.

Targets of the 5aCON site are involved in the regulation of cell proliferation in the developing telencephalon

Given the predominant role of the PD in exerting the Pax6 functions in forebrain development, we further determined the specific role of the 5a splice insert into the PD that shifts DNA binding from the N-terminal to the C-terminal domain of the PD (Epstein et al., 1994), by analysis of the *Pax6(5a)–/–* mice (Singh et al., 2002). We did not detect any changes in neurogenesis, cell proliferation and regionalization in the *Pax6(5a)–/–*. This finding has to be interpreted with caution, since the mRNA of the canonical form of *Pax6* was upregulated (1,4-fold) in amounts sufficient to compensate for the lack of Pax6(5a) in *Pax6(5a)–/–* mice. Messenger RNA of *Pax6(5a)* comprises about 10-20% of the total *Pax6* mRNA (E10-E12, see Fig. S3 in supplementary material), a ratio previously observed to be rather effective for transcriptional regulation (Chauhan et al., 2004). From our in vivo analysis we can therefore only conclude that there are no specific roles of the Pax6(5a) isoform that could not also be exerted by the canonical PD in the developing forebrain. Indeed, Pax6(5a) affects solely a subset of the functions of the canonical form of Pax6. Overexpression of Pax6(5a) in individual cortical precursor cells showed a specific and cell-autonomous effect on the number of progeny of a single precursor, without affecting cell fate even in the absence of functional Pax6 in the *Pax6^{Sey}–/–* background. This phenotype, the reduction of clone size, can be due to three mechanisms – an increase in cell death, an increase in postmitotic cells or an increase in asymmetric rather than symmetric cell divisions. We observed no difference in cell death as analyzed by DAPI staining, but discovered a significant reduction of proliferating cells in the Pax6-transduced clones already 2 days after infection (71% proliferating cells amongst all control virus infected cells compared to 53% after Pax6 overexpression). Thus, Pax6 and Pax6(5a) overexpression increases the number of cells leaving the cell cycle either due to an increase in asymmetric cell division or a lengthening of cell cycle (Calegari and Huttner, 2003). Both effects could also explain the increase in the number of precursors seen in the Pax6 loss-of-function mutations (Götz et al., 1998; Estivill-Torrus et al., 2002). Further experiments are needed to clarify the exact role of Pax6 on cell cycle length or the mode of cell division in proliferating precursors. Since the Pax6(5a) form is sufficient to induce these changes in proliferation and binds exclusively to the 5aCON site, these data demonstrate that targets of the 5aCON site are specifically involved in the regulation of cell proliferation, while targets of the P6CON site seemingly regulate neurogenesis. The latter is consistent with the role of Ngn2, a target containing the P6CON site, in neurogenesis (Bertrand et al., 2002).

PD, PD5a and HD act in a context-specific manner during forebrain and eye development

The effect on cell proliferation depends on the CNS region – while Pax6 negatively regulates proliferation in the telencephalon, it promotes proliferation in the vertebrate (Marquardt et al., 2001) and invertebrate (Dominguez et al., 2004) eye. This is also the case for the Pax6(5a) isoform. The deletion of exon 5a results in reduced number of iris and lens fiber cells in the eye (Singh et al., 2002), consistent with its proliferation promoting effect. Similarly, in the *Drosophila* eye *eyegone* and the murine *Pax6(5a)* positively affect cell proliferation (Dominguez et al., 2004). Thus, the positive effect of the 5aCON targets on cell proliferation in the eye is widespread across vertebrates and invertebrates, while the Pax6(5a)-mediated reduction of cell proliferation observed in precursors of the developing mouse telencephalon seems to be more specific and may have evolved more recently. The HD of Pax6 plays an important role in the eye (Favor et al., 2001), but not in the developing forebrain. Favor and colleagues (Favor et al., 2001) showed that the *Pax6^{4Neu}* mutation leads to severe defects in eye formation with homozygous mice developing no eye, except a remnant of the retinal neuroepithelium (pseudo-optic cup), suggesting that targets of the HD are important for both the early role of Pax6 in the surface ectoderm for lens formation, and later processes during retina specification (Ashery-Padan and Gruss, 2001; Ashery-Padan et al., 2000; van Heyningen and Williamson, 2002). Our analysis has further shown reduced proliferation and Ngn2 immunoreactivity in the remnant of the retinal neuroepithelium in the *Pax6^{4Neu}–/–* (data not shown), similar to the phenotype in the PD mutant *Pax6^{Aey18}–/–*. Both the PD and HD are thus important for the regulation of proliferation and cell fate in the retina, while the HD plays no role in these aspects in the telencephalon. In conclusion our results thus imply the selective use of PD, PD5a and HD targets as one mechanism that may contribute to the region-specific differences in Pax6 function within the CNS as well as in different organs.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/24/6131/DC1

References

Alvarez-Buylla, A., Garcia-Verdugo, J. M. and Tramontin, A. D. (2001). A unified hypothesis on the lineage of neural stem cells. *Nat. Rev. Neurosci.* **2**, 287-293.

Anderson, T. R., Hedlund, E. and Carpenter, E. M. (2002). Differential Pax6 promoter activity and transcript expression during forebrain development. *Mech. Dev.* **114**, 171-175.

Ashery-Padan, R. and Gruss, P. (2001). Pax6 lights-up the way for eye development. *Curr. Opin. Cell Biol.* **13**, 706-714.

Ashery-Padan, R., Marquardt, T., Zhou, X. and Gruss, P. (2000). Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* **14**, 2701-2711.

Assimacopoulos, S., Grove, E. A. and Ragsdale, C. W. (2003). Identification of a Pax6-dependent epidermal growth factor family signaling source at the lateral edge of the embryonic cerebral cortex. *J. Neurosci.* **23**, 6399-6403.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Bouchard, M., Schleiffer, A., Eisenhaber, F. and Busslinger, M. (2003). Evolution and Function of *Pax* Genes. In *Encyclopedia of the Human Genome* (ed. D. N. Cooper), pp. 1-7. London: Macmillan.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.

Calegari, F. and Huttner, W. B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. *J. Cell Sci.* **116**, 4947-4955.

Casarosa, S., Fode, C. and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525-534.

Chapouton, P., Gartner, A. and Götz, M. (1999). The role of Pax6 in restricting cell migration between developing cortex and basal ganglia. *Development* **126**, 5569-5579.

Chapouton, P., Schuurmans, C., Guillemot, F. and Götz, M. (2001). The transcription factor neurogenin 2 restricts cell migration from the cortex to the striatum. *Development* **128**, 5149-5159.

Chauhan, B. K., Yang, Y., Cveklova, K. and Cvekl, A. (2004). Functional interactions between alternatively spliced forms of Pax6 in crystallin gene regulation and in haploinsufficiency. *Nucleic Acids Res.* **32**, 1696-1709.

Chi, N. and Epstein, J. A. (2002). Getting your Pax straight: Pax proteins in development and disease. *Trends Genet.* **18**, 41-47.

Dominguez, M., Ferres-Marco, D., Gutierrez-Avino, F. J., Speicher, S. A. and Beneyto, M. (2004). Growth and specification of the eye are controlled independently by Eyegone and Eyeless in Drosophila melanogaster. *Nat. Genet.* **36**, 31-39.

Duncan, M. K., Haynes, J. I., 2nd, Cvekl, A. and Piatigorsky, J. (1998). Dual roles for Pax-6: a transcriptional repressor of lens fiber cell-specific beta-crystallin genes. *Mol. Cell Biol.* **18**, 5579-5586.

Duncan, M. K., Cvekl, A., Li, X. and Piatigorsky, J. (2000). Truncated forms of Pax-6 disrupt lens morphology in transgenic mice. *Invest. Ophthalmol. Vis. Sci.* **41**, 464-473.

Epstein, J. A., Glaser, T., Cai, J., Jepeal, L., Walton, D. S. and Maas, R. L. (1994). Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. *Genes Dev.* **8**, 2022-2034.

Estivill-Torrus, G., Pearson, H., van Heyningen, V., Price, D. J. and Rashbass, P. (2002). Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development* **129**, 455-466.

Favor, J., Peters, H., Hermann, T., Schmahl, W., Chatterjee, B., Neuhauser-Klaus, A. and Sandulache, R. (2001). Molecular characterization of Pax6(2Neu) through Pax6(10Neu): an extension of the Pax6 allelic series and the identification of two possible hypomorph alleles in the mouse Mus musculus. *Genetics* **159**, 1689-1700.

Fishell, G., Mason, C. A. and Hatten, M. E. (1993). Dispersion of neural progenitors within the germinal zones of the forebrain. *Nature* **362**, 636-638.

Gage, F. H. (2002). Neurogenesis in the adult brain. *J. Neurosci.* **22**, 612-613.

Gerlach, C., Golding, M., Larue, L., Alison, M. R. and Gerdes, J. (1997). Ki-67 immunoexpression is a robust marker of proliferative cells in the rat. *Lab. Invest.* **77**, 697-698.

Ghattas, I. R., Sanes, J. R. and Majors, J. E. (1991). The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell Biol.* **11**, 5848-5859.

Götz, M., Stoykova, A. and Gruss, P. (1998). Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* **21**, 1031-1044.

Grindley, J. C., Hargett, L. K., Hill, R. E., Ross, A. and Hogan, B. L. (1997). Disruption of PAX6 function in mice homozygous for the Pax6Sey-1Neu mutation produces abnormalities in the early development and regionalization of the diencephalon. *Mech. Dev.* **64**, 111-126.

Hack, M. A., Sugimori, M., Lundberg, C., Nakafuku, M. and Götz, M. (2004). Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol. Cell Neurosci.* **25**, 664-678.

Hartfuss, E., Galli, R., Heins, N. and Götz, M. (2001). Characterization of CNS precursor subtypes and radial glia. *Dev. Biol.* **229**, 15-30.

Haubensak, W., Attardo, A., Denk, W. and Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 3196-3201.

Heins, N., Cremisi, F., Malatesta, P., Gangemi, R. M., Corte, G., Price, J., Goudreau, G., Gruss, P. and Götz, M. (2001). Emx2 promotes symmetric cell divisions and a multipotential fate in precursors from the cerebral cortex. *Mol. Cell Neurosci.* **18**, 485-502.

Heins, N., Malatesta, P., Cecconi, F., Nakafuku, M., Tucker, K. L., Hack, M. A., Chapouton, P., Barde, Y. A. and Götz, M. (2002). Glial cells generate neurons: the role of the transcription factor Pax6. *Nat. Neurosci.* **5**, 308-315.

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

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522-525.

Hirata, T., Nomura, T., Takagi, Y., Sato, Y., Tomioka, N., Fujisawa, H. and Osumi, N. (2002). Mosaic development of the olfactory cortex with Pax6-dependent and -independent components. *Brain Res. Dev. Brain Res.* **136**, 17-26.

Jun, S. and Desplan, C. (1996). Cooperative interactions between paired domain and homeodomain. *Development* **122**, 2639-2650.

Kim, A. S., Anderson, S. A., Rubenstein, J. L., Lowenstein, D. H. and Pleasure, S. J. (2001). Pax-6 regulates expression of SFRP-2 and Wnt-7b in the developing CNS. *J. Neurosci.* **21**, RC132.

Kozmik, Z., Czerny, T. and Busslinger, M. (1997). Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. *EMBO J.* **16**, 6793-6803.

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

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P. (2001). Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* **105**, 43-55.

Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo, N., Taniguchi, S., Doi, H., Iseki, S. et al. (1993). A mutation in the Pax-6 gene in rat small eye is associated with impaired migration of midbrain crest cells. *Nat. Genet.* **3**, 299-304.

Mikkola, I., Bruun, J. A., Holm, T. and Johansen, T. (2001). Superactivation

of Pax6-mediated transactivation from paired domain-binding sites by dna-independent recruitment of different homeodomain proteins. *J. Biol. Chem.* **276**, 4109-4118.

Mishra, R., Gorlov, I. P., Chao, L. Y., Singh, S. and Saunders, G. F. (2002). PAX6, paired domain influences sequence recognition by the homeodomain. *J. Biol. Chem.* **277**, 49488-49494.

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T. and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* **131**, 3133-3145.

Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., Tamura, A., Kirino, T. and Nakafuku, M. (2002). Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* **110**, 429-441.

Noctor, S. C., Martinez-Cerdeno, V., Ivic, L. and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* **7**, 136-144.

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.

Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* **90**, 8392-8396.

Scardigli, R., Bäumer, N., Gruss, P., Guillemot, F. and Le Roux, I. (2003). Direct and concentration-dependent regulation of the proneural gene *Neurogenin2* by Pax6. *Development* **130**, 3269-3281.

Schmahl, W., Knoedlseder, M., Favor, J. and Davidson, D. (1993). Defects of neuronal migration and the pathogenesis of cortical malformations are associated with Small eye (Sey) in the mouse, a point mutation at the Pax-6-locus. *Acta Neuropathol. (Berl)* **86**, 126-135.

Singh, S., Stellrecht, C. M., Tang, H. K. and Saunders, G. F. (2000). Modulation of PAX6 homeodomain function by the paired domain. *J. Biol. Chem.* **275**, 17306-17313.

Singh, S., Mishra, R., Arango, N. A., Deng, J. M., Behringer, R. R. and Saunders, G. F. (2002). Iris hypoplasia in mice that lack the alternatively spliced Pax6(5a) isoform. *Proc. Natl. Acad. Sci. USA* **99**, 6812-6815.

Smart, I. H. (1976). A pilot study of cell production by the ganglionic eminences of the developing mouse brain. *J. Anat.* **121**, 71-84.

Stoykova, A., Fritsch, R., Walther, C. and Gruss, P. (1996). Forebrain patterning defects in Small eye mutant mice. *Development* **122**, 3453-3465.

Stoykova, A., Gotz, 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.

Stoykova, A., Treichel, D., Hallonet, M. and Gruss, P. (2000). Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. *J. Neurosci.* **20**, 8042-8050.

Toresson, H., Potter, S. S. and Campbell, K. (2000). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. *Development* **127**, 4361-4371.

van Heyningen, V. and Williamson, K. A. (2002). PAX6 in sensory development. *Hum. Mol. Genet.* **11**, 1161-1167.

Walther, C. and Gruss, P. (1991). Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Williams, B. P., Read, J. and Price, J. (1991). The generation of neurons and oligodendrocytes from a common precursor cell. *Neuron* **7**, 685-693.

Yun, K., Potter, S. and Rubenstein, J. L. (2001). Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. *Development* **128**, 193-205.