Pax6 regulates regional development and neuronal migration in the cerebral cortex

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Abstract

Mutations in the *Pax6* gene disrupt telencephalic development, resulting in a thin cortical plate, expansion of proliferative layers, and the absence of the olfactory bulb. The primary defect in the neuronal cell population of the developing cerebral cortex was analysed by using mouse chimeras containing a mixture of wild-type and *Pax6*-deficient cells. The chimeric analysis shows that *Pax6* influences cellular activity throughout corticogenesis. At early stages, *Pax6*-deficient and wildtype cells segregate into exclusive patches, indicating an inability of different cell genotypes to interact. At later stages, cells are sorted further based on telencephalic domains. *Pax6*-deficient cells are specifically reduced in the mediocaudal domain of the dorsal telencephalon, indicating a role in regionalization. In addition, *Pax6* regulates the process of radial migration of neuronal precursors. Loss of *Pax6* particularly affects movement of neuronal precursors at the subventricular zone/intermediate zone boundary at a transitional migratory phase essential for entry into the intermediate zone. We suggest that the primary role of *Pax6* is the continual regulation of cell surface properties responsible for both cellular identity and radial migration, defects of which cause regional cell sorting and abnormalities of migration in chimeras.
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Introduction

During development of the cerebral cortex, neurons are generated in the ventricular zone (VZ), a pseudostratified columnar epithelium that lies adjacent to the lateral ventricles. After the final cell division, newly postmitotic cells leave the VZ and migrate through the subventricular zone (SVZ) and the intermediate zone (IZ) to the cortical plate, the precursor of the cerebral cortex. Neurons generated later bypass the older ones and form the more superficial layers of the cortex in an inside-first outside-last sequence (Angevine and Sidman, 1961; Bayer and Altman, 1991; Rakic, 1974). Neuroblasts in their route to the cortical plate migrate along the surface of radial glia (Rakic, 1978).

Alleles of mouse small eye (*Pax6^{Sey}*) result from mutations in the *Pax6* gene (Hill et al., 1991). Studies of the developing telencephalon in homozygous *Pax6^{Sey/Sey}* mice show several abnormalities in the cerebral cortex, such as

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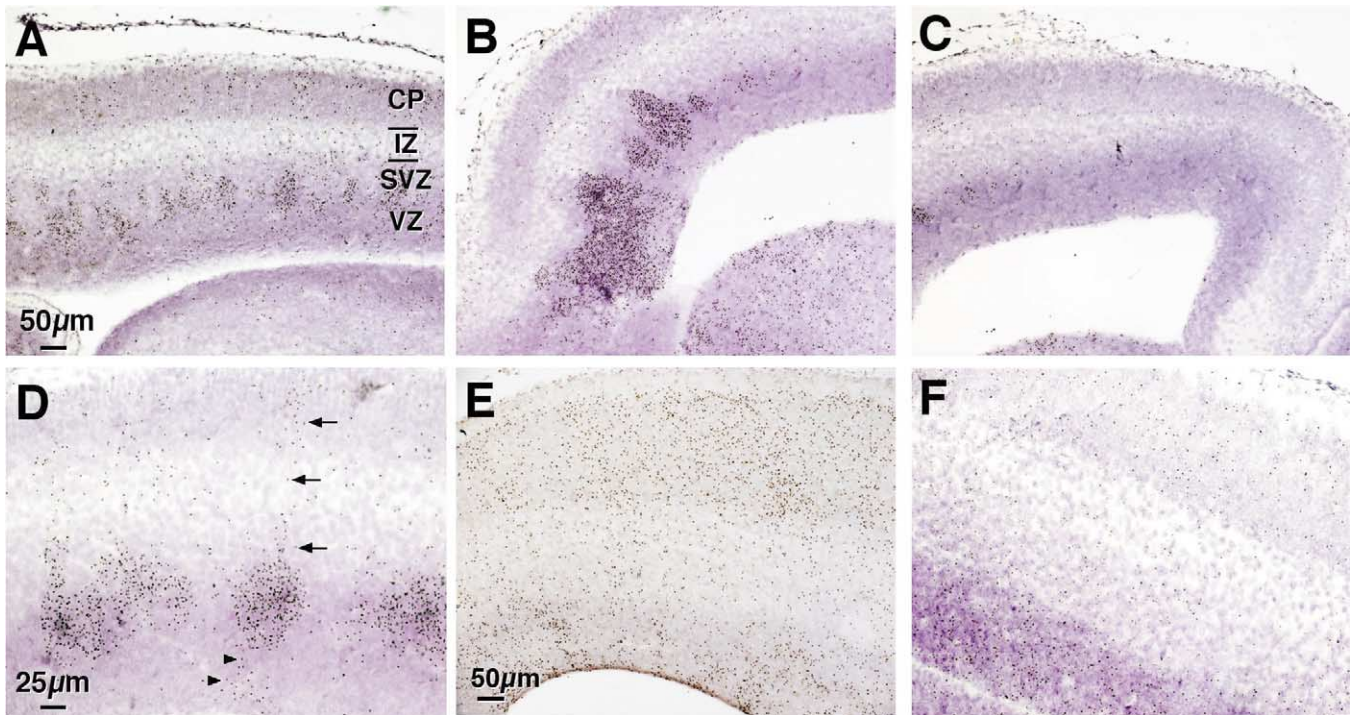
Fig. 1. Cell distribution in the chimeras at E16.5. (A–F) Sagittal sections of the developing telencephalon in chimeras at E16.5. Tg^+ cells are identified by a brown spot in the nucleus. (A–D) represent mutant chimeras of a low percentage and (E, F) are from wildtype chimeras. (A) Lateral sections showing accumulation of $Pax6^{-/-}$ cells in clusters throughout the proliferative region, mainly in the subventricular zone (SVZ). (B) Medial telencephalon showing higher accumulation of $Pax6^{-/-}$ cells in the rostral SVZ compared with (C) the caudal neocortex of the same chimera. (D) Higher magnification of the lateral telencephalon showing streams of $Pax6^{-/-}$ cells between the edge of the ventricular zone and the cluster and to the pial surface (indicated by arrows). Medial rostral (E) and caudal (F) neocortex of a control chimera shows distribution of Tg^+ cells throughout the entire telencephalic wall. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

expansion of the germinal epithelium, heterotopic germinal cells, and a thinner than normal cortical plate (Caric et al.,1997; Schmahl et al., 1993). Recent evidence suggests that cortical areas are established by homeodomain protein gradients in the VZ. One of these proteins is PAX6, which at early stages of cortical development, is expressed at high levels at the anterior and the lateral domains of the ventricular zone, reducing to lower levels at the mediocaudal region (Bishop et al., 2000; Walther and Gruss, 1991). Two studies have proposed that both *Pax6* and *Emx2,* expressed in a complementary gradient pattern in cortical progenitor cells, specify area identities in the neocortex (Bishop et al., 2000; Mallamaci et al., 2000). In addition, Pax6 has a role in dorsoventral patterning of the telencephalon (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001).

A difficulty in analysing telencephalon development in *Pax6*-deficient mice is that the telencephalon develops in an abnormal environment; thus, it is difficult to establish which are the primary telencephalic defects and which are secondary to the failure of other tissues or cell types. For example, *Pax6*$^{Sey/Sey}$ mice have no olfactory placodes, which are required for induction of the olfactory bulbs and, overall, for forebrain growth (Graziadei and Monti-Graziadei, 1992). Other telencephalic signaling centres are affected in the

Pax6$^{Sey/Sey}$ mutation. WNT signalling at both the cortical hem (caudal–medial edge of the cortical field) (Muzio et al., 2002a) and the lateral edge at the pallial and subpallial boundary is impaired (Kim et al., 2001). Thus, it is likely that forebrain defects are a result of both an intrinsic *Pax6* deficiency and a lack of induction by the olfactory placodes and cortical signaling centres. In addition, radial glia cells, that are required for migration of the neuronal precursors, are defective (Caric et al., 1997; Gotz et al., 1998). To overcome these problems, we have analysed fetal mouse aggregation chimeras containing a mixture of wildtype and *Pax6* mutant cells. In aggregation chimeras, the facial primordia, including the olfactory placodes, develop normally (Quinn et al., 1996), and mutant cells develop alongside wildtype cells in a more normal environment. Previously, chimeric analysis has been successfully used in defining the role of *Pax6* in the eye (Collinson et al., 2000, 2001; Quinn et al., 1996).

Here, the mouse chimera approach enabled analysis of neuronal precursors in the developing forebrain and showed that *Pax6* regulates forebrain development from the earliest stages affecting fundamental cell surface properties during corticogenesis. Analyses of later stages suggested that *Pax6* regulates a specific step in the radial migratory process. In

addition, neuronal precursors in the process of lateral migration are affected by the loss of Pax6. Both cell–cell recognition and neuronal migration are dependent on the composition of the cell surface; thus, we suggest that the primary role of *Pax6* in forebrain development is the regulation of fundamental cell surface properties.

Materials and methods

Mice and production of chimeras

Mice stocks, maintenance, and production of chimeras have been described previously (Quinn et al., 1996). Chimeras were made by aggregating eight-cell-stage embryos obtained from two different crosses. Two different small eye mutant alleles, $Pax6^{Sey}$ and $Pax6^{Sey-Neu}$, were used. Both small eye strains were pigmented and homozygous for $Gpi1^b$. $Pax6^{Sey/+}$ males were also homozygous for the re-iterated silent β-globin transgene marker TgN(Hbb-b1)83Clo (Quinn et al., 1996). Heterozygous $Pax6^{Sey-Neu/+}$ females were crossed to $Pax6^{Sey/+}$ males to produce embryos that were pigmented, homozygous for the glucose phosphate isomerase $Gpi1^b$ allele, hemizygous for the β-globin transgene ($Tg^{+/-}$, referred to as Tg^+), and differing at the small eye locus. These embryos were aggregated to (BALB/c $\times$ A/J) F_2 embryos (nonpigmented, homozygous for $Gpi1^a$ allele, noncarriers of the β-globin transgene, and wild type at the $Pax6$ locus) and cultured overnight before transfer into recipient pseudopregnant albino (homozygous for $Gpi1^c$ allele) females. Pregnancies were timed according to the pseudopregnant female, with the day of vaginal plug designated 0.5 day postcoitum (E0.5). Embryos were allowed to develop in utero to E10.5, E12.5, E14.5, E16.5, and E18.5.

Analysis of chimeras

Fetuses were dissected into cold phosphate-buffered saline (PBS). To determine proportion of cell contribution within chimeric fetuses, tissue samples from the tail and forelimbs were dissected into H_2O/glycerol 50:50 for GPI1 electrophoresis and scanning densitometry (West and Flockhart, 1994). The average percentage of GPI1B in the fetal tail and forelimb was used as an estimate of the overall contribution of cells derived from the $Pax6^{Sey-Neu/+} \times Pax6^{Sey/+}$ embryo. The head of each fetus was dissected, and the brain was fixed in ethanol: acetic acid (3:1 vol/vol) and processed to wax for analysis by DNA–DNA in situ hybridisation. The four groups of chimeras ($Pax6^{Sey-Neu/Sey} \leftrightarrow Pax6^{+/+}$, $Pax6^{Sey-Neu/+} \leftrightarrow Pax6^{+/+}$, $Pax6^{Sey/+} \leftrightarrow Pax6^{+/+}$, and $Pax6^{+/+} \leftrightarrow Pax6^{+/+}$) were distinguished by polymerase chain reaction and restriction enzyme analysis of fetal trunk DNA, as described in Quinn et al. (1996).
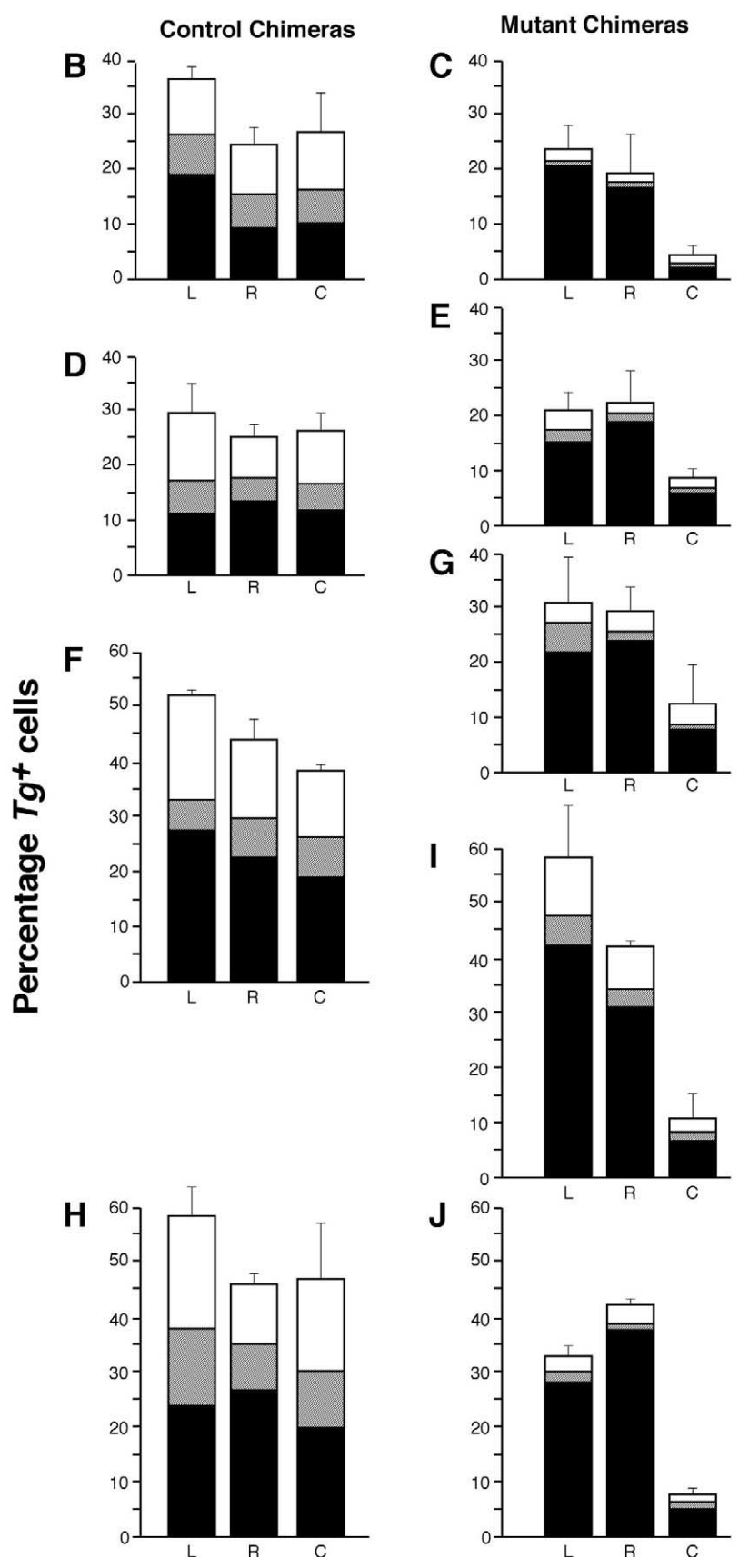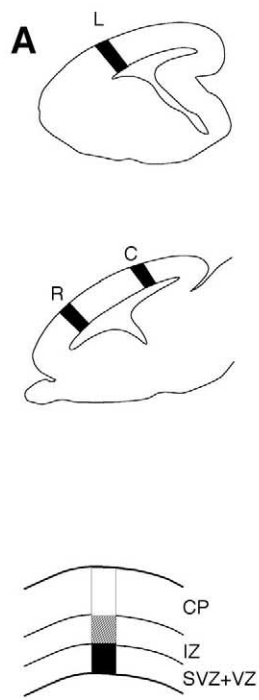
DNA–DNA in situ hybridisation

Fixed brains embedded in wax were sectioned sagittally at 10 μm and mounted on slides coated with 3-aminopro-pyltriethoxysilane (Sigma). In situ hybridisation using a digoxygenin-labeled DNA probe was performed for visualisation of the β-globin transgenic cell marker (Keighren and West, 1993). The DNA probe was detected by diaminoben-zidine (DAB) staining for peroxidase-labeled antibody (nonradioactive DNA labeling and detection kit; Boehringer Mannheim). Slides were counterstained with hematoxylin and eosin and examined by bright-field and phase-contrast light microscopy. From each E16.5 chimeric brain, two nonconsecutive sagittal sections corresponding to the lateral brain and two nonconsecutive sagittal sections corresponding to the medial brain were analysed. In the medial neocortex, two fields were analysed: one in the rostral level (not including the RMS) and one in the caudal neocortex. In the lateral brain, the DNA in situ analysis showed no differences between rostral and caudal domains; thus, only one field was quantified in the rostral portion. The percentage of transgene-positive cells (Tg^+ cells) throughout the depth of the telencephalic wall was estimated by counting numbers of nuclei vs numbers of hybridisation signals in 160 $\times$ 160 μm squares using an eyepiece grid (Graticules Ltd). The percentage of Tg^+ cells within each of the three embryonic layers (cortical plate, intermediate zone, and proliferative layers) was calculated.

Immunohistochemistry

Sections were blocked in Tris-buffered saline (TBS) containing 1% normal serum, 1% bovine serum albumin and incubated overnight at 4°C with the following primary antibodies: anti-β tubulin isotype III (Sigma, 1:400), MAP2 (ICN, 1:300), NeuN (Chemicon, 1:100), and anti-polysia-lylated N-CAM (Pharmingen, 1:200). After rinsing in TBS, sections were incubated with the appropriate biotinylated secondary antibodies (Dako, 1:400) followed by avidin–biotin–HRP complex (Vector) and visualised with diamino-benzidine (Vector).

Results

The mutant embryos used for chimera production arose from matings in a $Pax6^{Sey-Neu/+} \times Pax6^{Sey/+}$ cross. Four possible chimeric genotypes were produced: the fetuses of interest containing a mixture of $Pax6^{-/-}$ cells and wild-type cells designated mutant chimeras, $Pax6^{Sey-Neu/Sey} \leftrightarrow Pax6^{+/+}$; and three classes of control chimeras: $Pax6^{Sey/+} \leftrightarrow Pax6^{+/+}$, $Pax6^{Sey-Neu/+} \leftrightarrow Pax6^{+/+}$, and $Pax6^{+/+} \leftrightarrow Pax6^{+/+}$. The global percentage of cells derived from the different aggregated embryos in the chimera was estimated by quantitative analysis of GPI1 isozyme composition of the limbs, with the percentage of the GPI1B isozyme representing the contribution

of cells in the chimera derived from the $Pax6^{Sey-Neu/+}$ × $Pax6^{Sey/+}$ cross. To identify the distribution of cells derived from this cross, DNA *in situ* hybridisation to the β-globin transgene, carried homozygously by the $Pax6^{Sey/+}$ male mice, was performed (labelled cells are referred to as Tg^+ cells). Thus, in the mutant chimeras, the $Pax6^{Sey-Neu/Sey}$ mutant cells (referred to as $Pax6^{-/-}$) were distinguished from the wild-type by the presence of a brown spot in the nucleus.

Accumulation of $Pax6^{-/-}$ cells in subdomains of the SVZ in E16.5 chimeras

We examined the forebrain of chimeras at embryonic day 16.5 (E16.5) to establish the distribution of cortical cells in the chimera. We produced 21 E16.5 chimeras of different genotypes that varied in composition from 15 to 75% GPI1B. In this analysis, we initially focused on $Pax6^{-/-}$ ↔ $Pax6^{+/+}$ chimeras in which the percent contribution of $Pax6^{-/-}$ cells was low or moderate (<50%). In these chimeras, the mutant cells accumulate in the SVZ, giving rise to an abnormal proliferative region (Fig. 1A; 15% GPI1B). Analysis of a number of cell clusters showed stripes of mutant cells that extended radially both from the clusters to the cortical plate and deeper from the germinal layer (VZ) to the clusters (Fig. 1D). Thus, the clusters appear to arise by aggregation of cells destined to populate the cortical plate. In chimeras with a high proportion of mutant cells (75% GPI1B; data not shown), the phenotype was very severe. The proliferative region was significantly thicker than normal and was composed of large aggregates of mutant cells, while the cortical plate and intermediate zone were dramatically reduced in thickness (data not shown).

The medial telencephalon at E16.5 showed a rostral to caudal difference in the extent of the accumulation of mutant cells in the SVZ. In the rostral SVZ, $Pax6^{-/-}$ cells formed large clusters (Fig. 1B). Within the same medial sections, the caudal neocortex contained $Pax6^{-/-}$ cells that were not distributed in apparent clusters in the SVZ or showed smaller clusters (Fig. 1C). In contrast, in the lateral chimeric forebrain, $Pax6^{-/-}$ cells were distributed throughout the SVZ, showing very little difference between rostral and caudal domains (Fig. 1A). This phenotype was found in all the mutant chimeras independent of the percentage of $Pax6^{-/-}$ cells in the chimera. In control chimeras, the Tg^+ cells were distributed throughout the VZ to the pial surface

in lateral neocortex and rostral (Fig. 1E) and caudal (Fig. 1F) medial neocortex with no detectable clustering as seen in the mutant chimeras.

The differential accumulation of $Pax6^{-/-}$ cells at E16.5 was quantified based on the percentage of Tg^+ cells throughout the width of the telencephalic wall in sagittal sections through the medial and lateral (L) neocortex. The medial sections were further analysed in both rostral (R) and caudal (C) regions of the neocortex (Fig. 2A). The telencephalic wall was subdivided into three embryonic zones (proliferative layers: VZ and SVZ, the IZ, and the cortical plate), and the contribution of Tg^+ cells to each layer was determined (Fig. 2B–J). We compared control and mutant chimeras over a range of GPI1B percentages [Fig. 2B–E, and G represent the lower-percentage (<50%) embryos; Fig. 2F, and H–J representing the higher-percentage embryos]. In the control chimeras (Fig. 2B, D, F, and H), there were no significant differences in the percentages of overall Tg^+ cells between the rostral (R) and caudal (C) fields analysed in the medial forebrain. However, in the same analysis, the mutant chimeras (Fig. 2C, E, G, I, and J) showed that, in the medial neocortex, there were differences between the overall percentage of Tg^+ cells in the rostral (R) and caudal (C) areas. The percentage in the caudal–medial forebrain was lower than the other two regions analysed and the GPI1-B percentage. In this area, the $Pax6^{-/-}$ cells were clearly underrepresented from the proliferative zone to the pial surface as compared with control chimeras. Furthermore, in the control chimeras (Fig. 2B, D, F, and H), in the three areas analysed (L, R, and C), the Tg^+ cells contributed similarly to the cortical plate and proliferative regions. In all of the mutant chimeras (Fig. 2C, E, G, I, and J), the majority of the Tg^+ cells in the rostral–medial forebrain (R) and lateral forebrain (L) were localised in the proliferative region, while in the cortical plate and intermediate zone, the Tg^+ cells were underrepresented. The mutant cells that accumulated in the proliferative region represented between 70 and 90% of the total Tg^+ in the telencephalic wall. Although in the quantitative analysis, the VZ and SVZ were both included as components of the proliferative region, most Tg^+ cells in the mutant chimeras resided in the SVZ near the IZ boundary at a distance from the ventricular cavity (Fig. 1A and B). Thus, this analysis in the mutant chimeras confirmed that there is underrepresentation of $Pax6^{-/-}$ cells in the caudal neocortex and an area-

Fig. 2. Quantitative analysis of the distribution of Tg^+ cells in E16.5 chimeras. (A) Representation of sagittal views of the telencephalon showing the lateral (L), medial–rostral (R), and medial–caudal (C) areas analysed and a representation of the neocortex with the embryonic regions analysed in the quantification: cortical plate (CP), intermediate zone (IZ), and proliferative zone (SVZ + VZ). The histograms show the average percentages (±S.E.M.) of Tg^+ cells through the width of the telencephalic wall in control chimeras (B, D, F, and H) and mutant chimeras (C, E, G, I, J) with different percentages of chimerism (B–E, G contain less than 50% mutant cells by GPI1 analysis) (F, H–J contain greater that 50% mutant cells by GPI1 analysis). In all histograms, the y-axis depicts the percentage of Tg^+ cells. Each bar (total percentage of Tg^+ cells in the telencephalic wall) is divided, showing the percentage of Tg^+ cells in the cortical plate (white), intermediate zone (pattern), and proliferative zone (black) that contribute to the total. In control chimeras, the Tg^+ cells contributed to L, R, and C domains and were distributed between the three different embryonic layers. In mutant chimeras, Tg^+ cells contributed to the L and R domains but were localised mainly in the proliferative region; this accumulation correlated with a diminution of Tg^+ cells in CP and IZ. In all the mutant chimeras, the total percentage of Tg^+ cells in the medial–caudal neocortex was reduced.
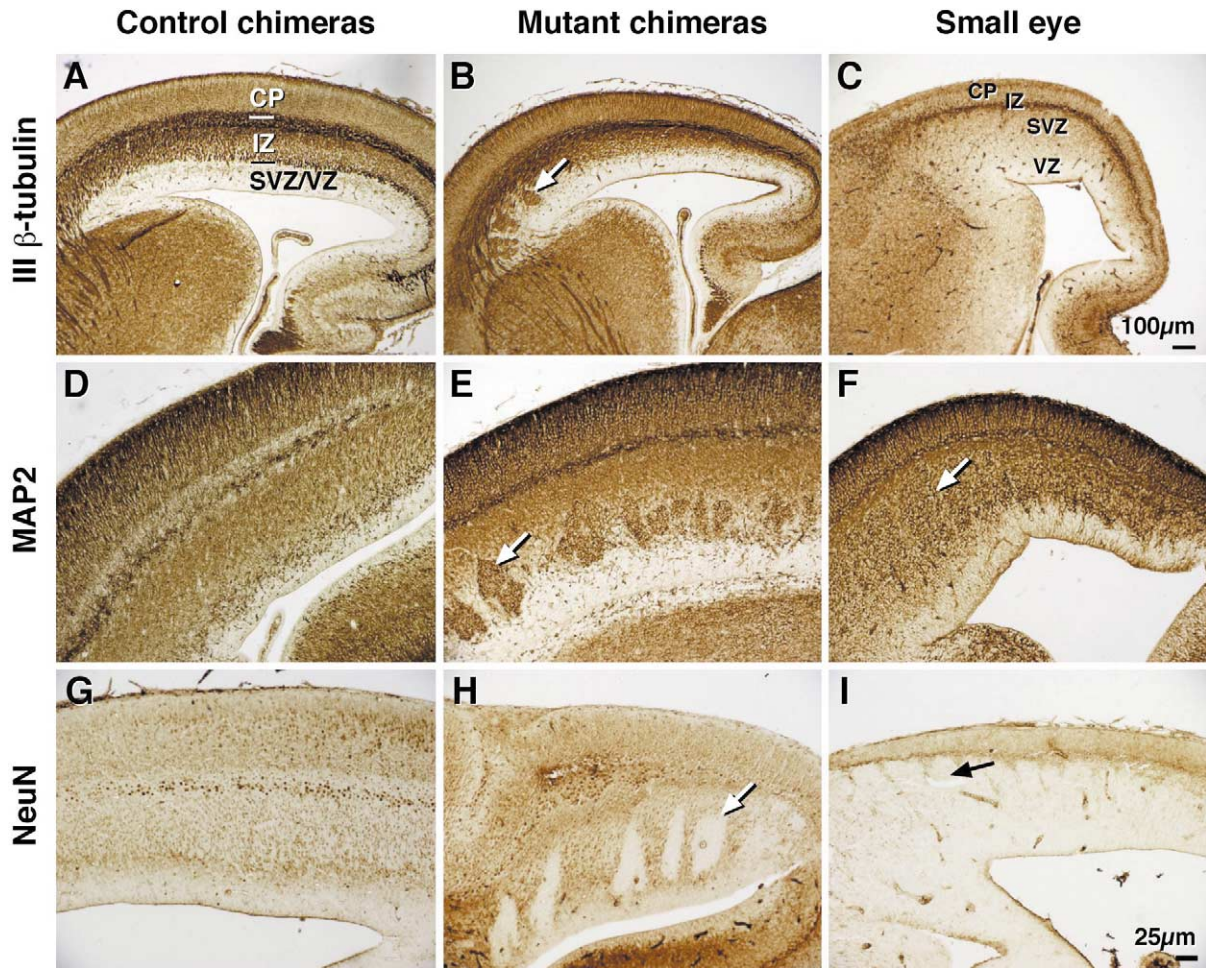
Fig. 3. Neuronal precursors are affected in the mutant chimeras. Expression pattern of class III β-tubulin (A–C), MAP2 (D–F), and NeuN (G–I) in E16.5 control chimeras (A, D, G), mutant chimeras (B, E, H), and small eye mutants (C, F, I). All examples represent sagittal section of the forebrain; rostral is on the left and caudal on the right. (A) Pattern of expression of class III β-tubulin in sagittal section of a control chimera. (B) In the mutant chimeras, the abnormal clusters of $Pax6^{-/-}$ cells (arrow) that accumulate through the SVZ are class III β-tubulin immunoreactive. (C) In the small eye mutant, the CP, the reduced IZ, and the engrossed SVZ are class III β-tubulin immunoreactive. MAP2 expression pattern in a (D) control chimera, (E) mutant chimera showing positive immunoreaction in $Pax6^{-/-}$ cells of the SVZ (arrow) and (F) in the small eye mutant (arrow indicates the abnormal SVZ). NeuN expression pattern in (G), the control chimera showing immunopositive cells through the cortical plate, subplate, and IZ and no expression in the proliferative regions. (H) In the mutant chimera, $Pax6^{-/-}$ cells are negative for NeuN, as is in all the proliferative region in (I), the small eye mutant. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

specific accumulation of $Pax6^{-/-}$ cells in the proliferative zone in rostral and lateral cortex.

Neuronal precursors are affected in the mutant chimeras

To determine which cell type was affected in the chimeras, we used markers that distinguish neuronal precursors. We used the neuron-specific class III β-tubulin isotype. At E16.5 in the control chimeras, there is immunoreactivity throughout the CP, subplate, and IZ with an intensely stained band of cells at the border between the SVZ and the IZ (Fig. 3A). In both stages of mutant chimeras analysed (E16.5 and E18.5; see below), the clustered $Pax6^{-/-}$ cells expressed this early neuronal marker (Fig. 3B). In order to determine the stage of differentiation of $Pax6^{-/-}$ cells, we

used MAP2, a cytoskeletal component important in maintaining a differentiated neuronal cellular structure. In all the E16.5 chimeras, strong immunoreaction for MAP2 was shown in cell bodies and dendrites through the neocortex, subplate neurons, and cells in the IZ and SVZ (Fig. 3D and E). In the mutant chimeras, the clusters of $Pax6^{-/-}$ cells were additionally immunoreactive for this neuronal marker (Fig. 3E). In contrast, NeuN (Mullen et al., 1992) is expressed in older neurons and is found throughout the cortical plate, in particular the early formed superficial layer and subplate, and intermediate zone, with low expression in the proliferative regions (Fig. 3G). In the mutant chimeras, the expression in the cortical plate was similar to the controls. Interestingly, $Pax6^{-/-}$ cells that accumulated in the SVZ were negative for NeuN immunostaining (Fig. 3H). The
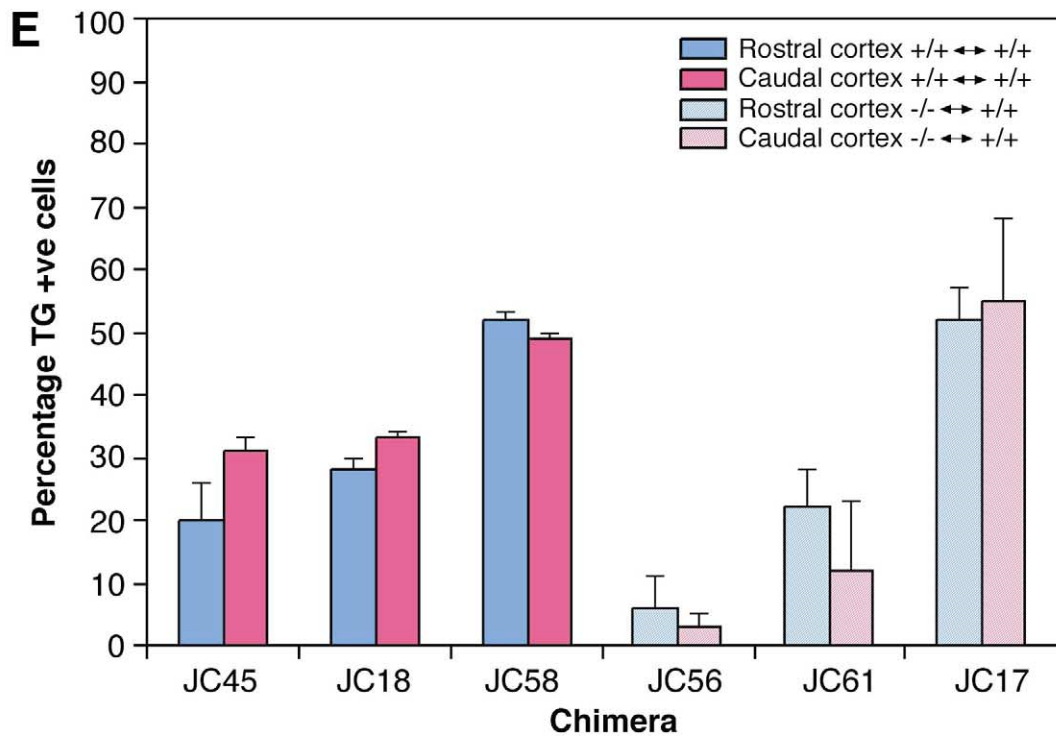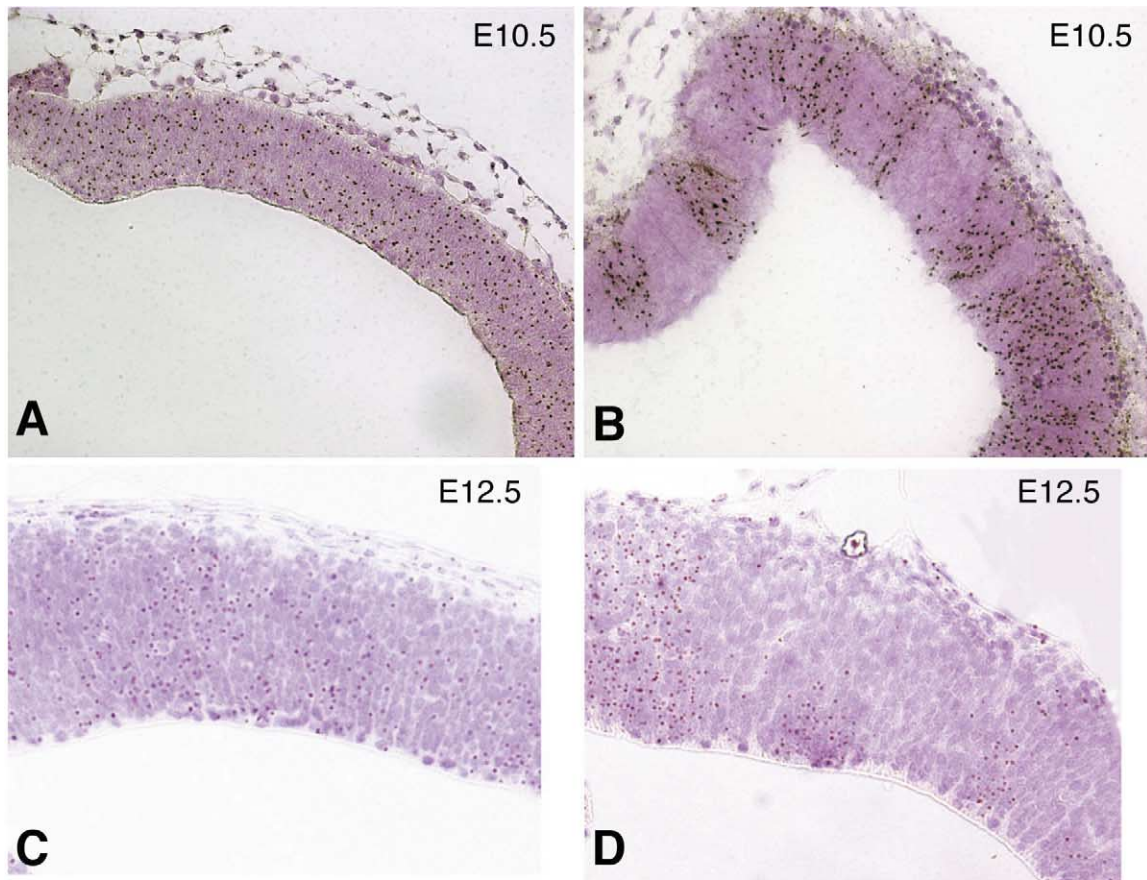
Fig. 4. Analysis of cell distribution at early stages of corticogenesis. Sections of E10.5 (A, B) and E12.5 (C, D) dorsal telencephalon comparing distribution of Tg^+ cells in $+/+\leftrightarrow+/+$ wildtype (A, C) and $Pax6^{-/-} \leftrightarrow +/+$ mutant (B, D) chimeras. In (B) and (D), Tg^+ cells are segregated into exclusive cell patches. Histogram (E) of the analysis of the regional distribution of Tg^+ cells at E12.5 in $+/+\leftrightarrow+/+$ wildtype and $Pax6^{-/-}\leftrightarrow+/+$ mutant chimeras. Medial sections were taken, and the Tg^+ cell distribution was analysed in rostral and caudal regions.

identification of the $Pax6^{-/-}$ cell clusters was clear; however, adjacent sections were used for the DNA in situ hybridisation to confirm their identity (data not shown). These results suggest that the clustered mutant cells are neuronal, although they do not express the late neuronal marker (NeuN) apparent in cells that leave the proliferative zone.

We have used the same neuronal markers in the *Sey* mutants ($Pax6^{Sey}/Pax6^{Sey}$) (Fig. 3C, F, and I). Cells of the abnormal SVZ were immunopositive for class III β-tubulin (Fig. 3C) and MAP2 (Fig. 3F), but negative for NeuN (Fig. 3I), suggesting that the clusters seen in the chimeras are equivalent to those reported in the mutant. Furthermore, cells that did leave the proliferative region expressed NeuN, suggesting that the *Pax6* mutation does not completely block cell differentiation, but that Pax6 is required for efficient migration.

Distribution of wild-type and $Pax6^{-/-}$ mutant cells at other stages in development

Neurogenesis begins at around E12.5 in the mouse telencephalon, and this stage was examined for an early *Pax6* role in the corticogenic precursors. At this stage, we detected no difference in the rostral to caudal distribution of $Pax6^{-/-}$ cells in the $Pax6^{Sey-Neu/Sey} \leftrightarrow Pax6^{+/+}$ chimeras (Fig. 4C–E). Analysis of cell number suggested that there is no difference in the percentage of $Pax6^{-/-}$ cells comparing rostral and caudal regions (Fig. 4E). However, the $Pax6^{-/-}$ cells are segregated into patches both rostrally and caudally with very little intermixing of the $Pax6^{-/-}$ and wildtype cells (Fig. 4C and D).

The stage that segregation of $Pax6^{-/-}$ and wildtype cells occurs was traced further back to E10.5 well before neurogenesis. At these early stages of corticogenesis, the mutant and wildtype cells reside in well-segregated patches (Fig. 4A and B). Thus, *Pax6* has an influence on cortical cells during development of the telencephalon at these initial stages. Such segregation of cells suggests that cell–cell recognition is affected, indicating a fundamental cell surface difference in mutant and wildtype cells at these stages.

A later stage of development (E18.5) was examined to define the culmination of the phenotype at E16.5. Cell distribution analysis in sagittal sections throughout the brain showed that the irregular clusters of cells (Fig. 5B) were, as at E16.5, entirely composed of $Pax6^{-/-}$ mutant cells (Fig. 5D). Serial sections showed that these aggregates, however, were found throughout the SVZ of the telencephalon. Accumulation of mutant cells in the SVZ (Fig. 5D) was associated with a reduction of mutant cells in the cortical plate (Fig. 5B). In the control chimeras, the telencephalon had a normal appearance (Fig. 5A and C). In these chimeras, Tg^+ cells were distributed throughout the width of the telencephalon from the VZ to the cortical plate. Thus, the analysis of cell distribution in the E18.5 mutant chimeras showed both

a reduction of mutant cells in the cortex and accumulation in clusters throughout the SVZ.

$Pax6^{-/-}$ cells in lateral migratory pathways

During development, neuroblast migration also occurs tangentially to the brain surface (Fishell et al., 1993; O'Rourke et al., 1992, 1995; Walsh and Cepko, 1992;). *Pax6* is expressed in the lateral portion of the proliferative region that gives rise to tangentially oriented migratory pathways in the chimeras. The distribution of the $Pax6^{-/-}$ cells in these pathways was examined. $Pax6^{-/-}$ cells were found through the entire rostral migratory stream (RMS) (Fig. 6B). However, the $Pax6^{-/-}$ cells formed abnormal clusters through all the RMS continuous with the neocortical SVZ (Fig. 6B). To confirm that $Pax6^{-/-}$ cells were found in other tangential streams, the lateral cortical stream (LCS) (Bayer et al., 1991) was also examined. This stream, found at the junction of the neocortex and the basal ganglia, contained $Pax6^{-/-}$ cells in the mutant chimeras (Fig. 6D). Together, these results show that $Pax6^{-/-}$ cells are localised throughout the lateral pathways, presumably migrating from the anterior lateral ventricle; however, the $Pax6^{-/-}$ cells remain segregated within these streams of migrating cells.

Discussion

Pax6 required for neocortical regionalisation

During development of the cerebral cortex, migration along radial glia cells is considered the primary guidance system (Rakic, 1972). In homozygous $Pax6^{Sey/Sey}$ mutant mice, previous studies showed that radial glial cells are present in the neocortex (Caric et al., 1997; Gotz et al., 1998), but exhibit altered morphology, tenascin expression, and cell cycle characteristics (Gotz et al., 1998; Heins et al., 2002). The morphological defects are cell-autonomous and may contribute to the failure of migration in the homozygous mutant cerebral cortex (Gotz et al., 1998; Heins et al., 2002). Previously, we concluded in analysis of homozygous $Pax6^{Sey/Sey}$ mutants that *Pax6* had nonautonomous effects on neuronal migration (Caric et al., 1997). Here, the nature of the *Pax6* defect on neuronal migration in mouse chimeras is addressed. Based on the hypothesis that mutant cortical cells in the presence of wildtype cells would display their full developmental potential, we have been able to focus on *Pax6* functions in corticogenesis predicted to be cell-autonomous.

These chimera studies showed that, initially, *Pax6* activity could be traced to a stage prior to neurogenesis. At E10.5, at least a day before neurogenesis begins, $Pax6^{-/-}$ and wildtype cells have fully segregated into distinct patches in the telencephalon. This cell-segregation phenomenon is typically associated with cell surface differences

(Godt and Tepass, 1998). Two days later, at early stages of neurogenesis, the cell types remain segregated. At these stages, the mutant and wildtype cell patches are spread throughout the dorsal telencephalon. Thus, at pre- and early neurogenic stages, *Pax6* imparts genetic information, which in the chimeras, is detected as affecting cell–cell recognition.

At later, neurogenic stages, *Pax6* has a cell-autonomous role on migrating neuroblasts. Many $Pax6^{-/-}$ cells at E16.5 were found in clusters residing at the SVZ/IZ boundary. These cell clusters were identified by immunohistochemistry (discussed below) as containing postmitotic neuronal precursors. In addition, accumulation of these clusters showed a significant regional bias within the neocortex. The density of cells in the SVZ was highest in the rostral and lateral regions and lowest in the mediocaudal region. These regional differences were not detected at E12.5, but do occur by E14.5 (data not shown) at the peak of neurogenesis.

Neocortical regionalisation was proposed to be controlled by both intrinsic (Rakic, 1988; Miyashita-Lin et al., 1999) and extrinsic mechanisms (O'Leary, 1989), with recent evidence suggesting an important role for molecular determinants intrinsic to the proliferative zone of the neocortex. Several molecular gradients have been described (Donoghue and Rakic, 1999), and it has been proposed that the *Pax6* gradient in combination with the complementary *Emx2* expression gradient (high caudomedially, low rostro-laterally) are responsible for neocortical arealization (Bishop et al., 2000; Mallamaci et al., 2000). The graded expression may control local identity, proliferation, and/or radial migration from the proliferative region to the neocortex, and in that way may control the cortical regionalization.

The proposed role for *Pax6* in telencephalic regionalization is based on the *Pax6* expression gradient and on studies of gross morphological defects in the homozygous $Pax^{Sey/Sey}$ mutant embryo (Bishop et al., 2000). Using chimeras and focusing on those that have a low contribution of mutant cells (i.e., less than 40%) has enabled analysis of the role of *Pax6* in regionalization in an environment that more nearly reflects wildtype. We found that the differential accumulation of $Pax6^{-/-}$ cells inversely correlates with the *Pax6* gradient of expression (Bishop et al., 2000; Walther and Gruss, 1991). In the mediocaudal region, which shows the lowest expression of *Pax6*, $Pax6^{-/-}$ cells are preferentially lost or sorted out. Conversely, in the mediorostral and the lateral regions, areas of highest *Pax6* expression, $Pax6^{-/-}$ cell numbers are appreciably higher.

Since in the rostral and lateral domains (regions of highest Pax6 expression) there is no wholesale exclusion of cells, it appears that *Pax6* is not required for regionalisation in these neocortical domains. In contrast, loss of $Pax6^{-/-}$ cells in the mediocaudal region suggests a regional dependence on *Pax6* expression. We suggest therefore that this dependence on Pax6 requires coexpression of other areal

determinants. For example, *Pax6* may be required in the caudal region but in combination with high concentrations of Emx2. Clearly, during corticogenesis, *Pax6* and *Emx2* operate in combination, as evidenced by the *Pax6/Emx2* double mutant (Muzio et al., 2002b). Thus, *Pax6* is necessary but only in combination with other factors for normal caudal development at these stages. The apparent reduction in the size of the rostral cortex previously reported in the *Sey* mutants (Bishop et al., 2000) may result from the loss of olfactory bulbs and placodes which are rescued in the chimeras.

Cell-autonomous requirement for Pax6 in neuronal migration

Cells deficient for *Pax6* accumulate at the SVZ/IZ boundary. These clusters of mutant cells are committed to a neuronal lineage as shown by expression of class III β-tubulin and MAP-2; however, these cells were not immuno-reactive for NeuN. These three neuronal markers provide graded indices of neuronal differentiation, NeuN antibody marking cells that have moved into the IZ. Although committed to a neuronal lineage, the $Pax6^{-/-}$ cells found in the clusters lack later neuronal markers characteristic of cells that have moved from the proliferative regions.

The lack of NeuN expression is likely to be due to the position of the cells clustered at the SVZ/IZ boundary. Based on observations in the (nonchimeric) $Pax6^{Sey/Sey}$ mutant forebrain, cells in the IZ and cortical plate express NeuN, suggesting that mutant cells have the capacity for expression and arguing that expression is dependent on position of the cell along the migratory route. Transplantation experiments (Caric et al., 1997) further suggest that *Pax6* mutant cells possess the capacity to terminally differentiate (to at least some cell types), having occupied a position in the cortical plate. Thus, the mutant cells appear to express markers relevant to their position, and upon reaching the cortical plate, express the repertoire of genes required for differentiation. We suggest that the neuronal property disrupted by the *Pax6* mutation is the efficient migration of neuroblasts, and in the absence of *Pax6*, neuroblast migration is impeded in the SVZ at the point of entry to the IZ.

Two modes of radial migration were proposed for development of the cerebral cortex (Nadarajah et al., 1999): radial glia-mediated locomotion and soma translocation. Clearly, in the chimera, $Pax6^{-/-}$ neuroblasts migrate away from the ventricular edge to the SVZ/IZ boundary. It is not clear which mode of cellular translocation, if defective, would abandon cells at the SVZ/IZ boundary. Interestingly, Altman and Bayer (1990) suggested that some (or perhaps all) neurons interrupt their radial migration for a significant time period within the SVZ, indicating a transitional phase at this boundary. We suggest that radial migration requires additional genetic input at the point of the SVZ/IZ transition
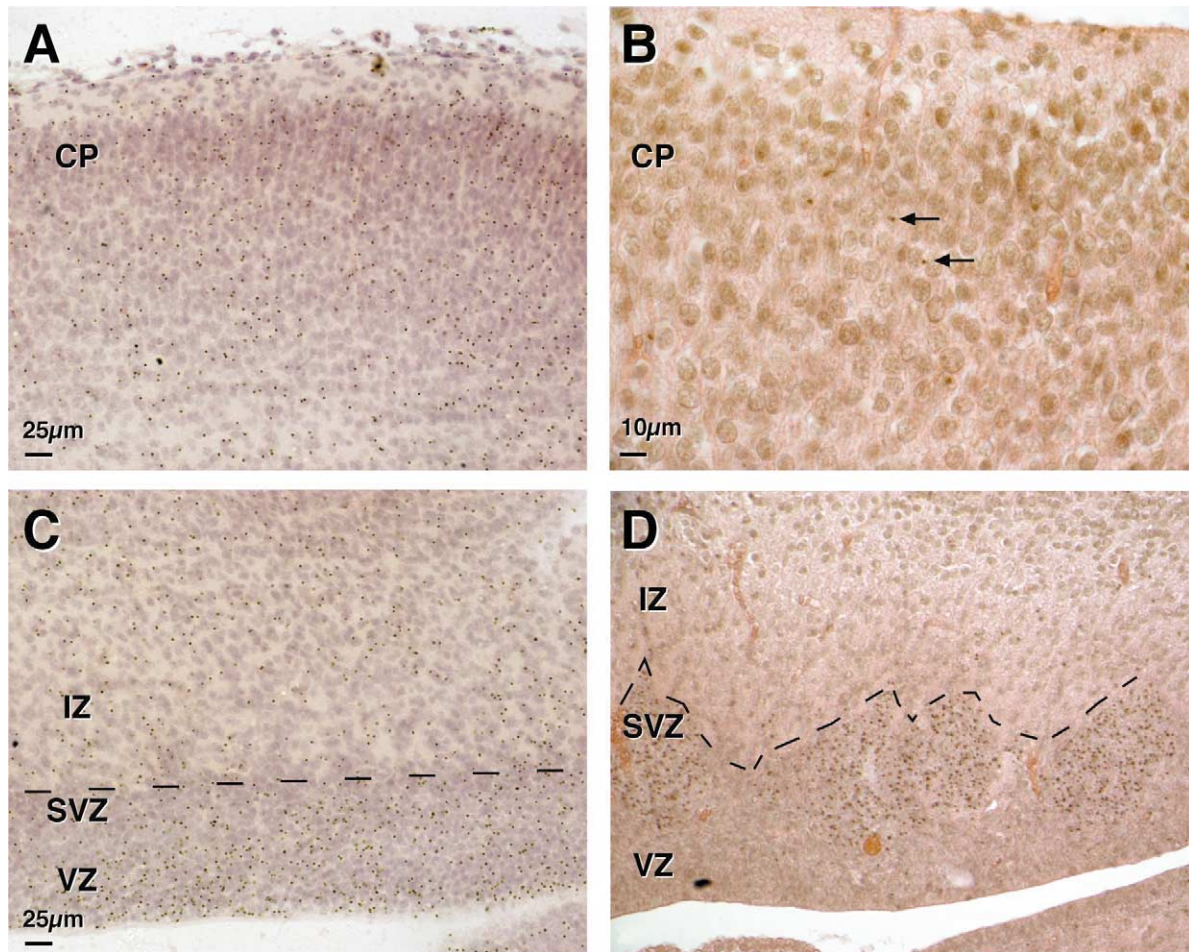
Fig. 5. Cell distribution in the chimera at E18.5. Comparative analysis of cell distribution between control (A, C) and mutant (B, D) chimeras with similar GPI1B percentage. (A) DNA in situ analysis in a 36% GPI1B control chimera shows distribution of Tg^+ cells throughout the cortical plate, (C) IZ, and proliferative layers. (D) DNA in situ analysis in a 32% GPI1B mutant chimera, showing that the abnormal clusters of cells in the SVZ are constituted of $Pax6^{-/-}$ cells. Accumulation of $Pax6^{-/-}$ cells in the proliferative region is associated with a diminution of $Pax6^{-/-}$ cells in the cortical plate (B). Broken lines in (C) and (D) mark the upper edge of the subventricular zone. Scale bars, 25 μm (A, C, D); 10 μm in (B). CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

and that *Pax6* plays a role in this process. In the absence of *Pax6,* the period and number of migrating cells that are impeded at the boundary increase significantly, leading to accumulation into large clusters.

Pax6 role in radial vs tangential migration

Our experiments further suggest that *Pax6* affects both radial and tangential migration of neuronal precursors. Segregation of wild type and $Pax6^{-/-}$ cells was found in both long tangential migratory streams that were analysed, the LCS and the RMS. Previous time-course studies suggest abnormalities in the LCS in homozygous $Pax6^{Sey/Sey}$ mutants (Brunjes et al., 1998). Although there is no conclusive information concerning the origin of the migrating cells through this pathway, i.e., the VZ of the LGE, the neocortical VZ, or both (Bayer and Altman, 1991; De Carlos et al., 1996), *Pax6* is expressed in this cortiostriatal boundary

domain extending to the VZ of the LGE (Stoykova et al., 1997; own data). The substrate used by these migrating neurons from the corticostriatal sulcus is not clear, even though curved radial glial fibers have been shown throughout this pathway in Golgi preparations (De Carlos et al., 1996; Smart and Sturrock, 1979) and by RC2 colocalisation (Edwards et al., 1990). Alternately, tangential migration might occur along axonal pathways as in other brain regions. The RMS originates from the rostrolateral part of the lateral ventricle. Neurons migrating through this stream reach the olfactory bulb, where they differentiate into interneurons (Luskin, 1993). Neurons migrate in a neurophilic mode, moving along one another in chain formations (Lois et al., 1996; Wichterle et al., 1997). There were phenotypic differences between the mutant and wildtype cells found in the LCS and RMS. In the RMS, the aggregates of mutant cells were continuous with and had the same appearance as the clusters found through the SVZ of the neocortex. These
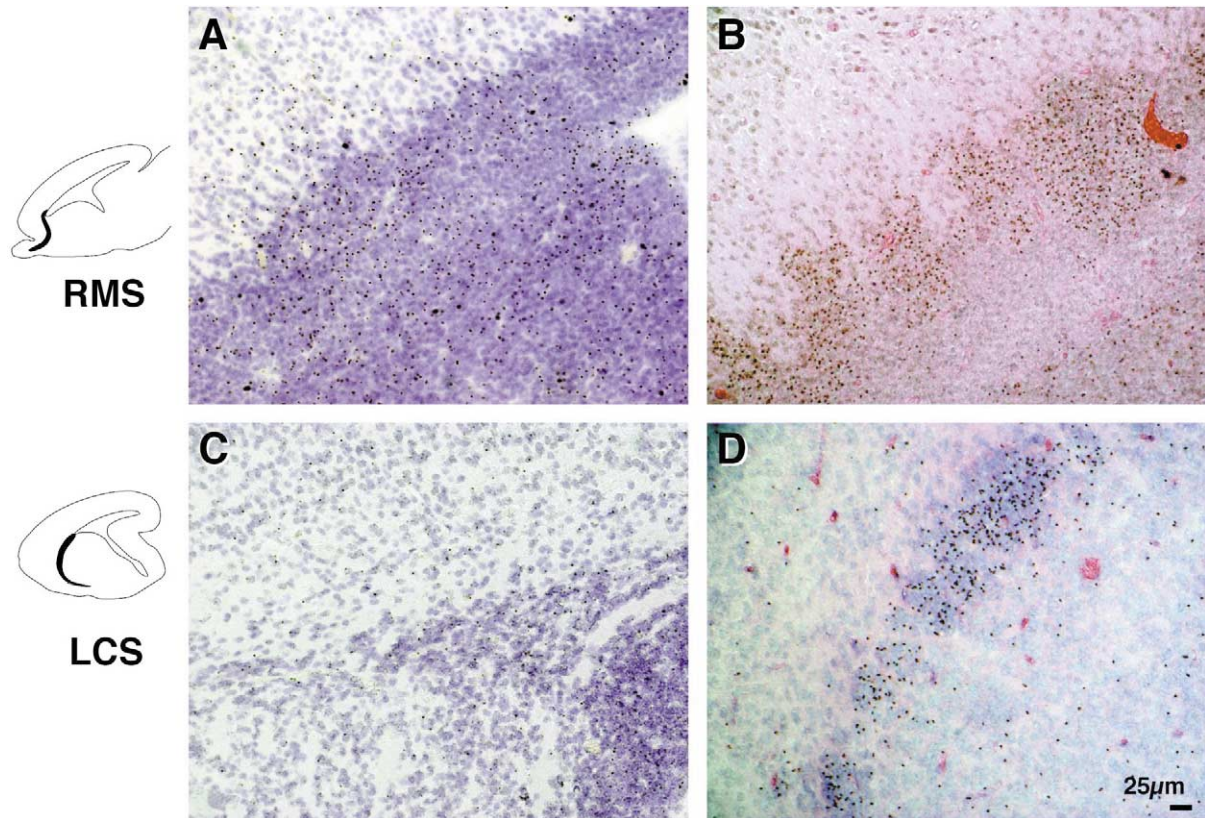
Fig. 6. Segregation between wild type and $Pax6^{-/-}$ cells in lateral migratory pathways. (A, C) Distribution of Tg^+ cells in a control chimera through (A) the rostral migratory stream (RMS) and (C) through the lateral cortical stream (LCS). (B, D) Distribution of $Pax6^{-/-}$ cells in mutant chimeras in both the (B) RMS and (D) LCS. Note that the distribution of $Pax6^{-/-}$ cells through the RMS is similar to the aggregates found in the neocortical SVZ. Segregation between wild type and $Pax6^{-/-}$ cells was found in both pathways. Scale bar, 25 μm.

observations indicate that *Pax6* affects cellular interactions required for neurons to migrate both tangentially and radially.

Cell surface changes in the neuronal precursors

We suggest that the primary role for *Pax6* during brain development is in the control of cell surface properties, and this is the basis for cell segregation found in the early stage chimeras. Furthermore, *Pax6* regulates components of the surface which are required for normal migration and are critical for movements of cells into the intermediate zone. Previous in vitro studies have suggested changes in the adhesive properties of the $Pax6^{-/-}$ cortical cells as they segregated from the wildtype in short-term segregation assays (Stoykova et al., 1997). Chimeric analysis of the function of *Pax6* in the developing eye has shown segregation of wild-type and $Pax6^{-/-}$ cells, supporting the role of *Pax6* in controlling cell surface properties (Collinson et al., 2000; Quinn et al., 1996). These cell surface properties are important for cell–cell interactions, which we suggest play a part in the migration process.

Appropriate radial guided neuronal migration to the cortical plate involves several processes, such as neuron–glial

recognition, neuron–glial adhesion, active migration, as well as arrest of migration once the neuron arrives to the correct location. Cell adhesion molecules (CAMs) such as the immunoglobulin (Ig) and cadherin families, as well as integrins, mediate recognition and adhesion. Previous studies have demonstrated that several CAMs are regulated by Pax proteins (Edelman and Jones, 1998); in particular, in vitro experiments have shown that *Pax6* activates L1 expression in neural cells (Meech et al., 1999). Additional evidence for a link between *Pax6* and CAMs comes from the altered expression of R-cadherin in the developing cortex of the small eye mutant (Stoykova et al., 1997).

The chimeras provide the opportunity to examine mutant cell behaviour in the presence of wildtype cells and determine the earliest stages at which there is a clear requirement for *Pax6*. In the presence of wildtype cells undergoing the presumed normal migratory process, mutant neuronal precursors were identified and found to be deficient at distinct stages of this process. The effects attributed to the lack of *Pax6* in the chimeras appear to have a basis in abnormal cell–cell interactions. We suggest a fundamental role for *Pax6* during neurogenesis is the continued regulation of components at the cell surface.

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