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Pax6 Is Required for the Multipotent State of Retinal Progenitor Cells

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Summary

The molecular mechanisms mediating the retinogenic potential of multipotent retinal progenitor cells (RPCs) are poorly defined. Prior to initiating retinogenesis, RPCs express a limited set of transcription factors implicated in the evolutionary ancient genetic network that initiates eye development. We elucidated the function of one of these factors, Pax6, in the RPCs of the intact developing eye by conditional gene targeting. Upon Pax6 inactivation, the potential of RPCs becomes entirely restricted to only one of the cell fates normally available to RPCs, resulting in the exclusive generation of amacrine interneurons. Our findings demonstrate furthermore that Pax6 directly controls the transcriptional activation of retinogenic bHLH factors that bias subsets of RPCs toward the different retinal cell fates, thereby mediating the full retinogenic potential of RPCs.

Introduction

During retinogenesis, the six principal cell types of the vertebrate retina derive from a common population of multipotent progenitor cells (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). Cell fate tracing experiments revealed that retinal progenitor cells (RPCs) retain the ability to generate different retinal cell types, even late in retinogenesis (Turner and Cepko, 1987; Jensen and Raff, 1997). The different retinal cell types are generated in a fixed chronological sequence. Retinal ganglion cells and horizontal cells are generated first, followed in overlapping phases by cone-photoreceptors, amacrine cells, rod-photoreceptors, bipolar cells, and finally Müller glia cells (Young, 1985). With progressing retinal development, RPCs appear to be exposed to changing extrinsic cues, often provided by previously generated cells, which seem to promote or inhibit the generation of particular cell types from the RPCs (Cepko, 1999). During retinogenesis, RPCs appear to be retained in a progenitor state by the action of Notch-Delta signaling and downstream effectors of Notch, like the bHLH transcription factor Hes1 (Tomita et al., 1996b; Henrique et al., 1997). The timing of retinal cell differentiation is thought to be controlled by the release from Notch signaling, presumably triggered by extrinsic signals (Dorsky et al., 1997). Additionally, intrinsic mechanisms are implicated in modulating the competence of RPCs to respond to inductive signals (Lillien, 1995) or to directly influence cell fate decisions (Ohnuma et al., 1999).

Similar to the ventricular zone of the developing central nervous system, the neuroblast layer of the developing mammalian retina is thought to contain both uncommitted (stem cell–like) progenitor cells and lineage-restricted progenitor cells, which are capable of undergoing a limited number of cell cycles, but display a bias in generating a more limited range of cell fates (Davis and Temple, 1994; Alexiades and Cepko, 1997; Lillien, 1998). Neuronal bHLH transcription factors are implicated in mediating changes in the intrinsic competence of neural progenitor cells and might participate in the transition from an uncommitted toward a biased progenitor cell (Lo et al., 1997; Edlund and Jessell, 1999; Guillemot, 1999). During retinogenesis, the bHLH factors Math5, Mash1, and Ngn2 are activated in subpopulations of RPCs and were shown to strongly bias RPCs toward particular cell fates (Tomita et al., 1996a; Kanekar et al., 1997; Brown et al., 1998; Morrow et al., 1999; Perron et al., 1999; Wang et al., 2001).

The most uncommitted RPCs (retinal stem cells) in the ciliary margin of the *Xenopus* retina were found to coexpress the transcription factors Pax6, Rx1, and Six3 prior to the activation of neurogenic genes (*Notch*, *Delta*, *bHLH*) and the subsequent steps toward retinal neurogenesis (Perron et al., 1998). Similarly, in the mouse retina, RPCs coexpress the transcription factors Pax6, Rx1(rax), Lhx2, Six3, and Six6(Optx2) prior to the onset of cell differentiation and during the ensuing stages of retinogenesis (Walther and Gruss, 1991; Oliver et al., 1995; Mathers et al., 1997; Jean et al., 1999). Pax6, Rx1, Six3, and Six6 have been implicated in a highly conserved genetic network that directs the initiation of eye development (Halder et al., 1995; Oliver and Gruss, 1997; Gehring and Ikeo, 1999). Forced expression of each of these factors in fish and frog results in mutual cross-activation and promotes the formation of ectopic retinal tissue (Mathers et al., 1997; Chow et al., 1999; Loosli et al., 1999; Zuber et al., 1999; Bernier et al., 2000). Null mutations in any of the genes encoding Pax6, Lhx2, and Rx1 display an early arrest of, or failure to initiate, optic vesicle formation, therefore obscuring the notion that the combined action of these factors might confer RPC identity. The requirement for Pax6 activity in both the lens and the retinal primordia (Glaser et al., 1994; Grindley et al., 1995; Ashery-Padan et al., 2000; Collinson et al., 2000) added a further level of complication to the study of a possible function in RPCs.

We elucidated the function of the Pax family transcription factor Pax6 (Gruss and Walther, 1992) in retinal

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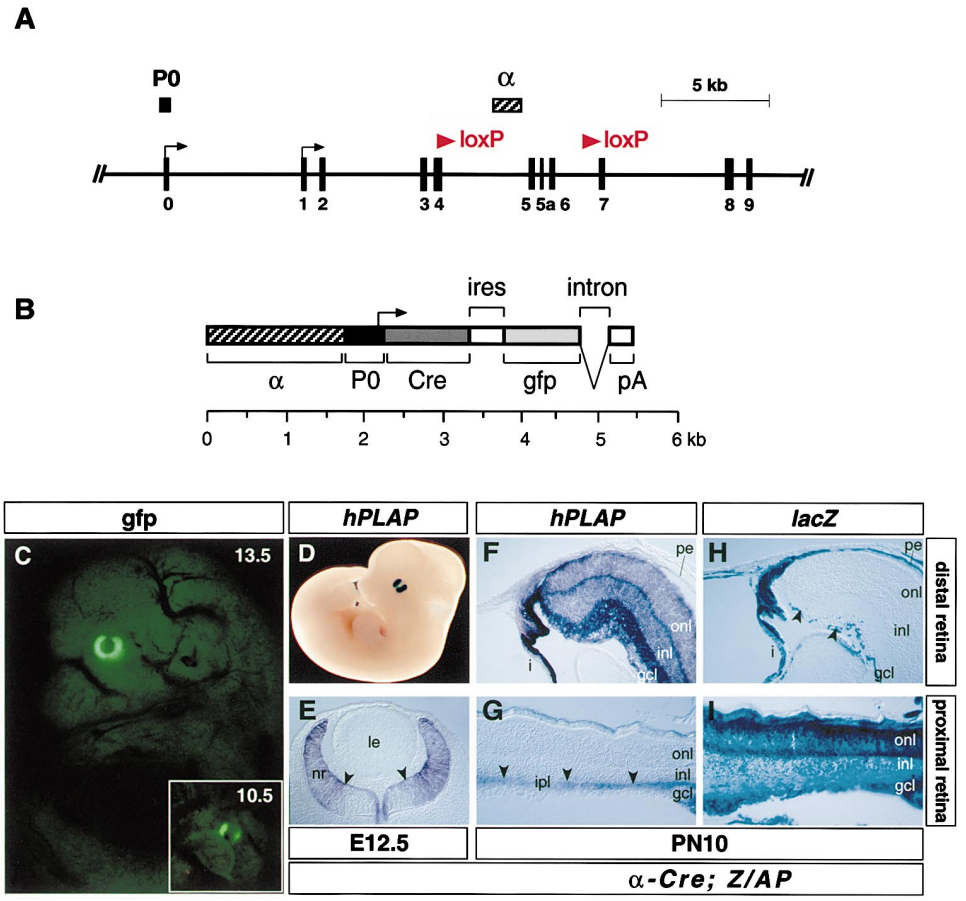

Figure 1. Conditional Inactivation of the *Pax6* Gene in Retinal Progenitor Cells

(A) The *Pax6*^{flox} allele: loxP sites (red arrowheads) and position of α enhancer, P0 promoter, and transcription start sites (arrows) are indicated. (B) Map of the α-*Cre* transgene. (C) gfp expression directed by α-*Cre*: E13.5 mouse embryo (α-*Cre* leaves a dorsal gap in expression). Inset: onset of gfp/Cre expression at E10.5. (D–I) Staining for hPLAP activity in whole-mount (D) or 10 μm cryosections (E–I) of α-*Cre; Z/AP* double transgenics. Staining for hPLAP (F and G) and β-gal (H and I) activity on adjacent sections. (H) arrowheads: β-gal^{+} blood vessels and astrocytes in the nerve fiber layer, both of which derive outside the nr. (G) The signal in the inner plexiform layer (ipl) corresponds to processes of hPLAP^{+} ganglion and amacrine cells in the distal retina. Abbreviations: β-gal: β-galactosidase; hPLAP: human placental alkaline phosphatase; i: iris; le: lens; onl, inl, gcl: outer nuclear, inner nuclear, ganglion cell layer; nr: neuroretina; pe: pigment epithelium

progenitor cells (RPCs) in the context of the intact developing eye by employing the Cre-loxP system (Tsien et al., 1996). After Cre-mediated loss of Pax6 activity in the developing neuroretina, principal RPC characteristics are maintained. However, the retinogenic potenital of Pax6-deficient RPCs becomes entirely restricted to only one of the six cell fates normally available to RPCs, resulting in the exclusive generation of amacrine interneurons. These cells clonally derive from the Pax6-deficient RPCs and differentiate in a wild-type-like fashion, while a selective elimination of the other retinal cell lineages by cell death or premature depletion of the RPC pool was not observed. Our results demonstrate that Pax6 activity in the RPCs is directly required for the transcriptional activation of retinogenic bHLH transcription factors in subsets of RPCs that are instrumental in imposing a bias on these progenitor cells. By directly controlling the transition from uncommitted RPC toward a lineage-restricted RPC intermediate, Pax6 mediates the full retinogenic potenital of RPCs and hence their multi-potency.

Results

Conditional Inactivation of Pax6 in the Neuroretina

To address the function of Pax6 in retinal progenitor cells (RPCs), we utilized the Cre-loxP system (Tsien et al., 1996) in order to accomplish the tissue-specific inactivation of the *Pax6* gene (Walther and Gruss, 1991). We have generated mice in which the endogenous *Pax6* gene has been replaced by gene targeting with a modified *Pax6* allele, *Pax6*^{flox}, in which exons 4–6, containing the translation start and the paired box, were flanked by two loxP sites (Figure 1A; see Ashery-Padan et al., 2000). To direct restricted inactivation of *Pax6* in the neuroretina, we placed the gene encoding the Cre recombinase under control of a retina-specific regulatory element (Figure 1A, "α"; Kammandel et al., 1999) of murine *Pax6*. We included in this construct an *IRES* (internal ribosomal entry site)-*gfp* reporter gene cassette (Zernicka-Goetz et al., 1997; Ashery-Padan et al., 2000) to encode Cre and gfp expression from a single bicistronic mRNA (Figures 1B and 1C). To define precisely the kinet-

ics and pattern of Cre recombinase activity driven by the α-*Cre-gfp* transgene (Figure 1B, referred to as α-*Cre*), we have generated α-*Cre; Z/AP* double transgenic animals (Figures 1D–1I). The *Z/AP* reporter line allows histochemical detection of Cre activity by excision of *lacZ* and concomitant activation of a ubiquitously expressed alkaline phosphatase (*hPLAP*) reporter gene (Lobe et al., 1999). Cre activity could be detected in the distal neuroretina (and iris), but at no stage in the lens or the retinal pigment epithelium or any other tissue (Figures 1D–1F). In α-*Cre; Z/AP* double transgenic animals, the hPLAP$^+$ cell progeny of the Cre-expressing RPCs contributed to all cells in the distal neuroretina of the mature eye (PN10, Figures 1F and 6A) while no β-galactosidase$^+$ cells, corresponding to cells that escaped Cre excision, could be detected in this area (compare Figures 1F–1H). Therefore, α-*Cre* directs Cre activity in the RPCs that give rise to all cells in the mature distal neuroretina. The mapping of the activity driven by the *Pax6* α enhancer in these precursor cells, moreover, reflects the endogenous expression of Pax6 in all uncommitted RPCs. Pax6 activity furthermore colocalized to all PCNA$^+$ (proliferating cell nuclear antigen) and cyclinD1$^+$ cells in the embryonic (E12.5 and E15.5) and postnatal neuroretina at PN8, which is shortly before completion of retinogenesis (data not shown), confirming the expression of endogenous Pax6 by all mitotic RPCs throughout retinogenesis.

Pax6$^{flox/flox}$; α-*Cre* (referred to as *flox/flox;* α-*Cre*) embryos and mice were generated by interbreeding, and retinal development was compared with their (wild-type) *Pax6$^{+/+}$*; α-*Cre* (referred to as +/+; α-*Cre*) littermates as a control. In *flox/flox;* α-*Cre* embryos, Pax6 was eliminated from the distal neuroretina around E10.5 (Figure 1C inset, data not shown), which is before onset of cell differentiation. The absence of Pax6 protein was confirmed by immunohistochemistry using antibodies against both the N-terminal and the C-terminal portions of the Pax6 protein (see Figures 2E and 2F, data not shown). Few if any Pax6$^+$ cells could be detected in the distal *flox/flox;* α-*Cre* neuroretina (referred to as *Pax6$^{flox\Delta}$* retina) after E11. However, the expression of Pax6 in the lens, corneal, and retinal pigment epithelium, as well as in the central portion of the retina, remained unaffected (Figures 2E and 2F). The elusion of these ocular tissues by Cre activity provided us with a unique tool to study Pax6 function in RPCs in the context and ambient environment of the intact eye.

Retinal Progenitor Cell Characteristics Are Maintained after Cre-Mediated Loss of Pax6 Activity

The coexpression of the transcription factors Rx1 (rax), Six3, Six6 (Optx2), Hes1, Lhx2, and Pax6 is a unique feature of RPCs (Walther and Gruss, 1991; Oliver et al., 1995; Mathers et al., 1997; Perron et al., 1998; Jean et al., 1999). High levels of expression of the corresponding mRNAs of all of these factors were observed in the *Pax6$^{flox\Delta}$* retina at stages spanning E11.5–E15.5 (Figures 2A–2D, data not shown). *Pax6$^{flox\Delta}$* RPCs therefore maintain the expression of Rx1, Six3, Six6, Hes1, and Lhx2, even 5 days after Pax6 inactivation was initiated. Furthermore, the gfp/Cre expression directed by the α enhancer of *Pax6* in the α-*Cre* transgene was maintained in the *Pax6$^{flox\Delta}$* retina, and the gfp$^+$Pax6$^-$ cells in this area exhibited the typical radial morphology of RPCs (data not shown). The *Pax6$^{flox\Delta}$* retina was observed to be hypocellular, which was already detectable at E12.5 (see Figure 2F) and was apparently due to reduced RPC proliferation in the *Pax6$^{flox\Delta}$* retina, as assayed by BrdU incorporation (1.5 hr pulse at E12.5). The ratio of BrdU-incorporating cells was observed to be at 0.12 (±0.05, see Experimental Procedures) in the *Pax6$^{flox\Delta}$* retina, as compared to 0.40 (±0.06) in the distal retina of control embryos. Similar values were obtained at E15.5 and E18.5 (data not shown). At the same time, significantly increased cell death, as assayed by the TUNEL method, could not be observed, neither in the embryonic (E12.5, E15.5) nor in the perinatal (PN0) *Pax6$^{flox\Delta}$* retina; ratios of apoptotic cells of 0.049 (±0.021) in the *Pax6$^{flox\Delta}$* retina compared to 0.044 (±0.026) in the distal retina of control embryos were determined at E12.5. Similar values were observed for the E15.5 retina (0.028 ± 0.016 and 0.029 ± 0.012 in the *Pax6$^{flox\Delta}$* and the control retina, respectively). Therefore, the inactivation of Pax6 did not lead to the disintegration or the selective loss of *Pax6$^{flox\Delta}$* RPCs or, eventually, their progeny. Taken together, these findings indicate that, by morphological criteria and the expression of early retinal determinants, in the absence of Pax6 activity in the neuroretina, principal RPC characteristics are maintained.

Pax6 Is Required for the Activation of Retinogenic bHLH Transcription Factors in Subsets of Retinal Progenitor Cells

The bHLH transcription factor Math5 is expressed in a subpopulation of RPCs and is required for the subsequent activation of Brn3b in differentiating retinal ganglion cells (Figure 2K; Brown et al., 1998; Wang et al., 2001). The POU domain transcription factor Brn3b itself is required for the differentiation of retinal ganglion cells (Gan et al., 1996; McEvilly et al., 1996). In the *Pax6$^{flox\Delta}$* retina, Math5 expression failed to be initiated (Figure 2L), consistently leading to the absence of Brn3b$^+$ cells (Figures 4J–4L, data not shown). The bHLH transcription factor Mash1 is expressed in RPCs in the neuroblast layer (Figure 2I) and has been shown to be required for normal bipolar cell differentiation (Tomita et al., 1996a). In the *Pax6$^{flox\Delta}$* retina, the expression of Mash1 was not observed (Figure 2J). Furthermore, the expression of Ngn2, a bHLH factor implicated in retinal neurogenesis (Gradwohl et al., 1996; Perron et al., 1999), could not be detected in the *Pax6$^{flox\Delta}$* RPCs (Figures 2M and 2N). Interestingly, at the same time, the *Pax6$^{flox\Delta}$* retina displayed high levels of NeuroD expression (Figures 2O and 2P). NeuroD has been shown to strongly promote amacrine cell genesis and to be required for the normal differentiation of this cell type (Morrow et al., 1999).

We next addressed whether, in the wild-type situation, the RPC subpopulations defined by the expression of retinogenic bHLH factors coexpress Pax6 by taking advantage of a previously generated *Ngn2^{gfp}* knockin mouse line, in which gfp expression faithfully recapitulates the endogenous expression of Ngn2 (G. Gradwohl and F. G., unpublished data). The majority of the gfp$^+$ cells in the *Ngn2^{gfp}* retina displayed the characteristic

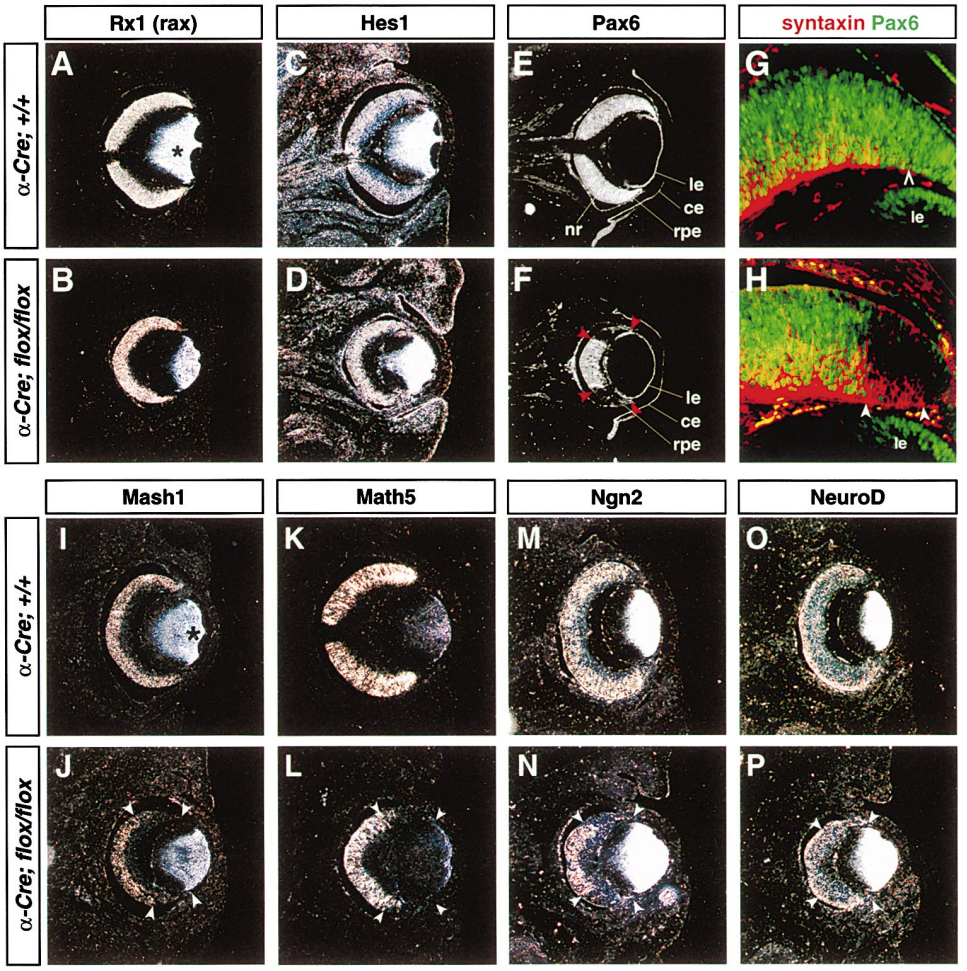

Figure 2. Retinal Progenitor Cell Characteristics Are Maintained after Loss of Pax6 Activity, but Pax6 Is Required for Activation of Retinogenic Gene Expression in Subsets of RPCs

Radioactive in situ hybridization (A–D, I–P) and immunohistochemistry (E–H) on 6 μm paraffin sections at E14.5. Expression of Rx1, Hes1, and Pax6 in the wild-type neuroretina (nr) (A, C, and E). Rx1 and Hes1 expression is maintained in the *Pax6*$^{flox\Delta}$ nr (B and D). (F) Pax6 is eliminated from the (distal) *Pax6*$^{flox\Delta}$ nr, but not from cornea (ce), lens (le), and retinal pigment epithelium (rpe). syntaxin^{+} cells (red) start to appear in the distal control (G, arrow) and the *Pax6*$^{flox\Delta}$ (H) nr at E14. (H) Closed arrows: Pax6^{-}syntaxin^{+} cells. Mash1 (I) and Math5 (K) are normally localized to subsets of RPCs, but both fail to be expressed in the *Pax6*$^{flox\Delta}$ nr (J and L). (M–N) Ngn2 expression is absent from the *Pax6*$^{flox\Delta}$ nr (arrows). However, NeuroD is present in the adjacent section (N) while Pax6 is not (data not shown). Note that the white reflection in the lens (asterisk) is due to reflection by lens fibers in dark field microscopy.

radial morphology of RPCs and most of the gfp^{+} cells were Pax6^{+} (Figure 3A). Double labeling experiments, moreover, showed that most Mash1^{+} cells colocalized with Pax6 (data not shown). However, the Ngn2^{+} RPC subpopulation did not coexpress Mash1 (Figure 3B). Therefore, Ngn2 and Mash1 are expressed in two nonoverlapping RPC populations that coexpress Pax6.

The coexpression of Pax6 with Ngn2 and Mash1 in RPC subpopulations and the strict requirement for Pax6 activity to initiate Ngn2, Mash1, and Math5 expression in RPCs raised the possibility that Pax6 directly controls the transcriptional activation of retinogenic genes. To address this possibility, we performed electrophoretic mobility shift assays (EMSA) on an array of candidate binding sites for Pax6 (Epstein et al., 1994) within a retina-specific enhancer (*E2*$_{Ngn2}$) of murine *Ngn2* (Figure 3D, R. Scardigli et al., submitted). In transgenic mouse embryos, *E2*$_{Ngn2}$ mediated reporter gene activity recapitulates the endogenous expression of Ngn2 in the retina (Figure 3C). By utilizing this approach, we have identified a sequence (E2.3) in the *Ngn2* enhancer to which Pax6 protein bound with a high affinity and another site (E2.1) to which Pax6 bound with lower, but substantial affinity (Figure 3E). Similarly, robust Pax6 binding was identified for a 37 bp sequence in an enhancer of the *Mash1* gene that was previously shown to direct expression in diencephalon and retina in vivo (*E*$_{Mash1}$, Verma-Kurvari et al., 1998), as well as to two clustered sites in the promoter region of the *Math5* gene (T. M., N. A., J. Johnson, L. Gan, and P. G., unpublished results). In contrast, by utilizing the same approach, we could not detect substantial binding of Pax6 protein to the promoter region of the murine *NeuroD* gene (data not shown).

To examine the ability of Pax6 to activate transcription of genes encoding retinogenic bHLH factors, we transfected *COS-7* cells with an *E2*$_{Ngn2}$*-lacZ* reporter con-

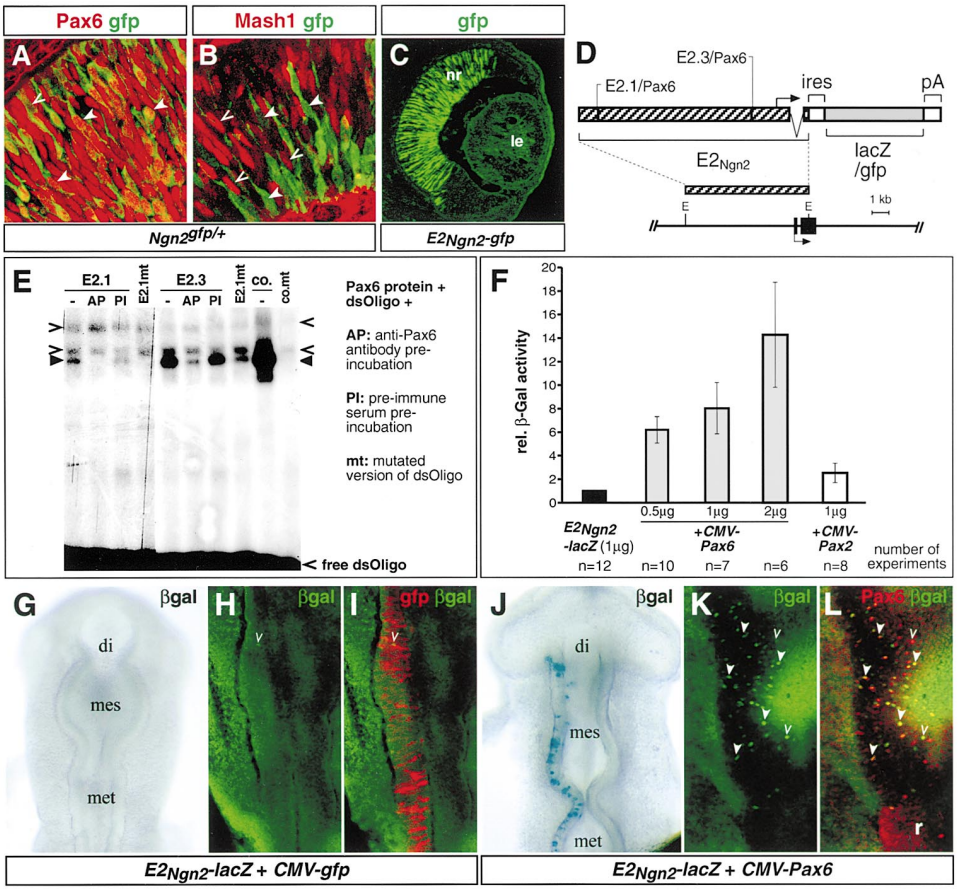

Figure 3. Pax6 Directly Activates Retinogenic Gene Expression In Vitro and In Vivo

(A) Ngn2 (gfp$^+$) and Pax6 expression (red nuclei) in E14.5 *Ngn2$^{gfp/+}$* retina. Closed arrow: strongly Pax6$^+$gfp$^+$; open arrow: few gfp$^+$ RPCs with low Pax6. (B) Mash1 (open arrow) and gfp/Ngn2 (closed arrow) are mutually exclusive. (C) gfp expression in E12.5 *E2$_{Ngn2}$-gfp* retina. (D) Transgene directing *gfp* or *lacZ* under control of *E2$_{Ngn2}$*. Positions of E2.1 and E2.3 Pax6 binding sites and of *E2$_{Ngn2}$* in the *Ngn2* locus are indicated. (E) EMSA with ^{32}P-labeled double-stranded (ds) oligonucleotides. Preincubation with rabbit anti-Pax6 (AP) blocks formation of specific Pax6/dsOligo complexes (closed arrows), while rabbit preimmune serum (PI) or rabbit anti-Pax2 (not shown) has no such effect (some interference of general protein/dsOligo complex formation was always observed with crude PI). Pax6 protein does not bind to mutated dsOligos E2.1mt, E2.3mt. Open arrows: unspecific binding by putative reticulocyte extract components. Co.: control dsOligo, co.mt: RE3 mvt (RE3, Schwarz et al., 2000). (F) Pax6 activates transcription via *E2$_{Ngn2}$* in cotransfected *COS-7* cells. (G–L) In ovo electroporation of Pax6 activates *Ngn2* expression in chick embryos. (G–I) After *E2$_{Ngn2}$-lacZ*+*CMV-gfp* coelectroporation, nearly no β-gal$^+$ cells are detected (H and I, arrow). gfp immunoreactivity reveals efficient targeting to the mesencephalon (I). Coelectroporation with *CMV-Pax6*: Pax6$^+$ cells upregulate β-gal (J–L, closed arrows). Open arrows: β-gal^{high}Pax6^{low} or Pax6^{low}β-gal^{high} cells (r: endogenous Pax6 in rhombomere). di: diencephalon; mes: mesencephalon; met: metencephalon.

struct, alone or plus *CMV-Pax6* (Figure 3F). Control transfected cells (*E2$_{Ngn2}$-lacZ* alone or plus control plasmid DNA) displayed a very low basal level of β-galactosidase activity (Figure 3F). The cotransfection of *E2$_{Ngn2}$-lacZ* with increasing amounts of *CMV-Pax6* led to a strong elevation of β-galactosidase activity in a concentration-dependent manner (Figure 3F). Similar results were obtained with *3T3* cells (data not shown). At the same time, the transfection of cells with Pax2 (*CMV-Pax2*), which binds to a consensus sequence similar to that of Pax6 or with *CMV-Pax3* or *CMV-Pax7* (data not shown), led to only a moderate increase in β-galactosidase activity (Figure 3F), underlining that the activation of *E2$_{Ngn2}$* was specific to Pax6. In both *COS-7* and *3T3* cells, *E$_{Mash1}$-lacZ* displayed very high levels of basal activity, thereby obscuring analysis of a possible activation by Pax6 (data not shown). To examine whether Pax6 is able to activate expression from the *Ngn2* promoter in the context of the embryo, we performed in ovo electroporation of chick embryos. The coelectroporation of *E2$_{Ngn2}$-lacZ* plus *CMV-Pax6* to the mesencephalon (which in the normal situation is Pax6 and Ngn2 negative, see Figure 3L) led to a strong activation of *lacZ* expression (Figures 3J–3L) in contrast to the control coelectroporation of *E2$_{Ngn2}$-lacZ* plus *CMV-gfp* (Figures 3G–3I). The forced expression of Pax6 in the mesencephalon, moreover, led to ectopic upregulation of endogenous *Ngn2* expression, as assayed by in situ hybridization using a probe for chick *Ngn2* (data not shown). Taken together, Pax6 is required for the initiation of the differentiation programs that normally bias subsets of RPCs toward the distinct retinal cell fates, with the apparent exception of the program directing amacrine cell genesis. The interaction of Pax6 with the promoters of genes

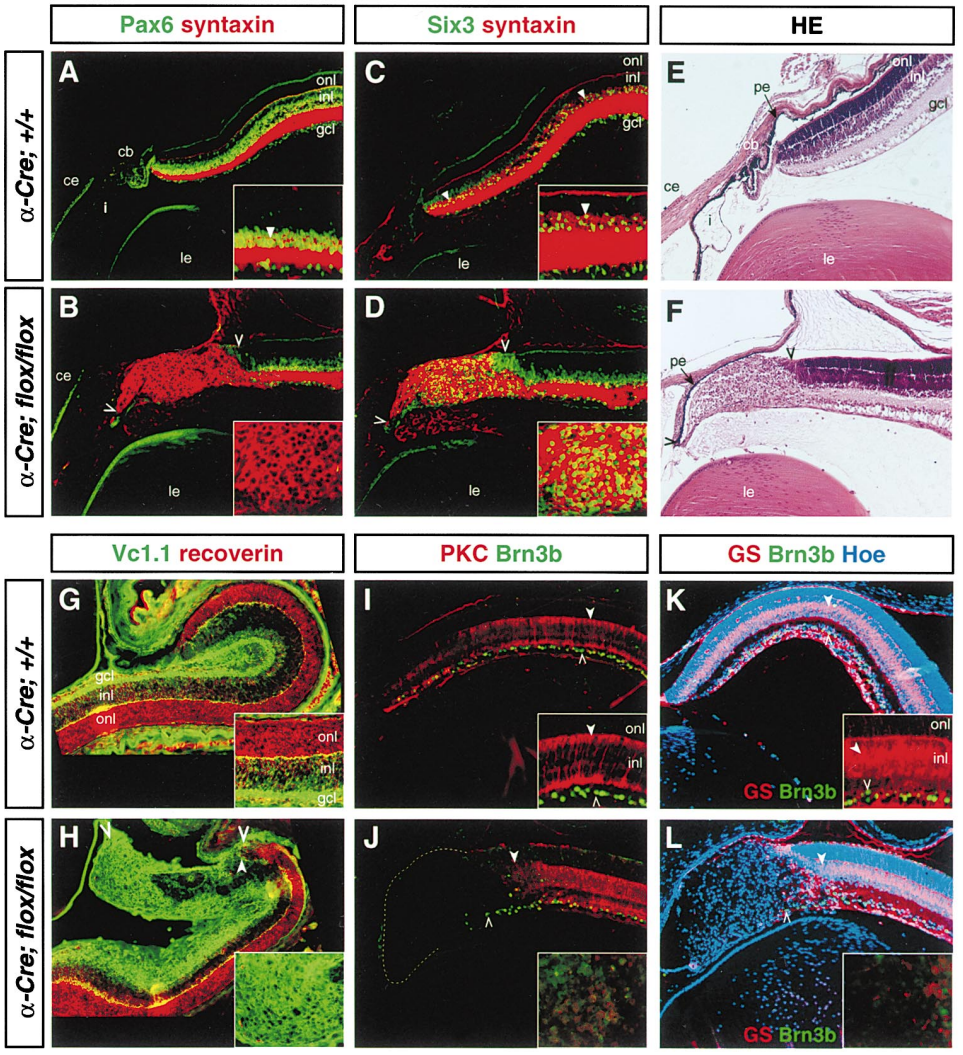

Figure 4. Loss of Pax6 Activity in Retinal Progenitor Cells Leads to the Exclusive Generation of Amacrine Cells

Immunohistochemistry on serial 8 μm paraffin sections of PN9–10 eyes. (A, inset) In the wild-type, all syntaxin$^+$ amacrine cells coexpress Pax6 (arrow). (C, inset) Six3$^+$ nuclei (arrows) colocalize with syntaxin$^+$ cells. (B, inset) In the *Pax6*$^{flox\Delta}$ nr (open arrows), all cells are syntaxin$^+$Pax6$^-$. (D, inset) However, most of the syntaxin$^+$ cells are Six3$^+$. (E) In the control, HE histological staining reveals lamination into outer (onl) and inner nuclear (inl) and ganglion cell layer (gcl). (F) Laminar organization is absent in the *Pax6*$^{flox\Delta}$ nr. (I) In the control, all photoreceptors are recoverin$^+$ (G, inset), while amacrine cells are Vc1.1$^+$. (H) In the *Pax6*$^{flox\Delta}$ nr, recoverin is absent, but Vc1.1 is present in all cells (H, inset). (I) Bipolar cell bodies and processes are PKC$^+$ (I, inset, filled arrow), while the nuclei of ganglion cells are Brn3b$^+$ (open arrows). (J) The *Pax6*$^{flox\Delta}$ nr is negative for PKC and Brn3b. (K) Glutamine synthetase (GS) labels Müller glia cell bodies (K, inset, filled arrows) and processes. The cells in the *Pax6*$^{flox\Delta}$ nr (nuclei are Hoechst$^+$) are devoid of GS and Brn3b (L). Abbreviations: cb, ciliary body; ce, corneal epithelium; i, iris; le, lens; nr, neuroretina; pe, pigmented epithelium.

encoding for retinogenic bHLH factors and strong transcriptional activation mediated by interacting with the *E2*$_{Ngn2}$ enhancer in vitro and in vivo finally demonstrates the ability of Pax6 to initiate retinogenic gene expression.

Loss of Pax6 Activity in Retinal Progenitor Cells Leads to the Exclusive Generation of Amacrine Interneurons

We next followed the fate of *Pax6*$^{flox\Delta}$ cells into postnatal stages (PN0–20), when in the wild-type situation all retinal cell types have been generated (the last retinal cells leave the cell cycle at PN10; Young, 1985). Histological analysis of the postnatal *Pax6*$^{flox\Delta}$ retina (PN9) revealed an accumulation of cells that lacked the normal laminar organization of the mature wild-type retina (Figures 4E and 4F). In the *Pax6*$^{flox\Delta}$ retina, we failed to detect retinal ganglion cells (Brn3b, Thy-1; Soucy et al., 1998; Figures 4I–4L and 7A), rod and cone photoreceptors (recoverin, protein kinase C, rhodopsin kinase; Haverkamp and Wässle, 2000; Sears et al., 2000; Figures 4G, 4H, and 7A), bipolar cells (protein kinase C, recoverin; Soucy et al., 1998; Figures 4I and 4J), horizontal cells (NF$_{165\ kDa}$; Burmeister et al., 1996; Figure 7A), as well as Müller glia cells (glutamine synthetase, Rx1; Haverkamp and Wässle, 2000; Furukawa et al., 2000; Figures 4K, 4L, and 7A). The absence of these retinal cell types was consistent at all stages analyzed (E11–12.5, E14–15.5,

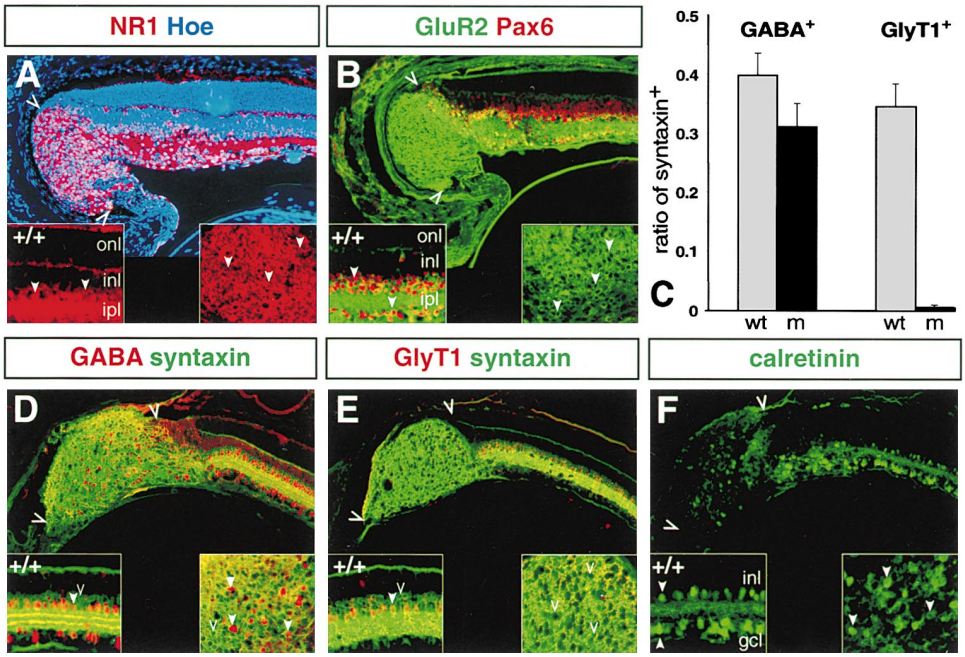

Figure 5. Subtype Identities and Neurotransmitter Phenotypes of Amacrine Cells in the *Pax6*$^{flox\Delta}$ Neuroretina

Immunhistochemistry on 10 μm cryosections (A and B) and 6 μm (D–F) paraffin sections of PN10 *flox/flox;* α*-Cre* eyes. (A and B) All cells of the *Pax6*$^{flox\Delta}$ neuroretina (nr) (open arrows) were positive for NR1 (A, arrows in inset; cell nuclei are labeled with Hoechst) and GluR2 (B, green, arrows), but negative for Pax6 (B, red nuclei). Left insets depict the expression in the wild-type (+/+) control retina. (C) Ratios of GABAergic (GABA) and glycinergic (GlyT1) amacrine cells in the control (wt) and the *Pax6*$^{flox\Delta}$ (m) nr were determined by coimmunostaining of syntaxin (open arrows, D and E insets) with GABA and GlyT1 (closed arrows, D and E insets) antibodies, respectively (see Experimental Procedures). (F) In the wild-type, calretinin is localized to cholinergic amacrine cells (inset, arrows). Calretinin$^+$ cells are abundant in the *Pax6*$^{flox\Delta}$ nr (arrows).

PN0–2, PN8–9, PN20). Remarkably, virtually all cells of the *Pax6*$^{flox\Delta}$ retina were strongly positive for the amacrine cell markers syntaxin and Vc1.1 (Figures 4A, 4B, 4G, and 4H). Since, at the same time, the absence of all other retinal cell types was observed, the inactivation of Pax6 in RPCs evidently led to the exclusive generation of amacrine interneurons. During normal retinal development, Rx1, which initially is coexpressed with Six3 and Pax6 in RPCs (Mathers et al., 1997; Perron et al., 1998; Figures 2A and 2E), becomes extinguished in the amacrine cell differentiation pathway (Furukawa et al., 2000; data not shown), while the expression of Six3 (and Pax6) is maintained at a high level in this cell type (Figures 4A–4C). We consistently observed that the nuclei of most of the syntaxin$^+$ cells in the *Pax6*$^{flox\Delta}$ retina were strongly positive for Six3 (Figure 4D), while having downregulated Rx1 by this stage (data not shown), indicating an expression profile characteristic for the normal amacrine cell differentiation pathway.

Neurotransmitter Phenotypes and Subtype Identities of Amacrine Interneurons Generated from Pax6-Deficient Retinal Progenitor Cells

Since syntaxin and Vc1.1 are expressed by all mature amacrine cells as well as amacrine cell progenitors, we tested the *Pax6*$^{flox\Delta}$ retina for the expression of βIII-tubulin, which is upregulated in neurons after exiting the cell cycle and NeuN, a marker for mature neurons (Lee et al., 1995; Magavi et al., 2000). The syntaxin$^+$Pax6$^-$ cells in the *Pax6*$^{flox\Delta}$ retina expressed high levels of βIII-tubulin and lower levels of NeuN, which is characteristic for mature amacrine cells in the wild-type retina (Figure 7A, data not shown). The *Pax6*$^{flox\Delta}$ cells displayed neurotransmitter phenotypes of terminally differentiated amacrine interneurons, including the expression of the NMDA and AMPA glutamate receptors NR1 and GluR2 (Figures 5A and 5B), as well as decarboxylase (GAD_{65}, Figure 7A). Likewise, approximately normal numbers of GABAergic, cholinergic (see below), and dopaminergic amacrine cells were generated in the *Pax6*$^{flox\Delta}$ retina, as well as cells positive for the neuropeptide SubstanceP (Figure 7A, data not shown). Therefore, the Pax6$^-$ cells in the *Pax6*$^{flox\Delta}$ retina constitute terminally differentiated amacrine cells rather than amacrine precursor cells that are retained in an undifferentiated state.

GABAergic and glycinergic constitute the two major nonoverlapping populations of amacrine cells (comprising about three fourths of the total population), while most of the other amacrine cells are of yet unidentified classes (Strettoi and Masland, 1996; Haverkamp and Wässle, 2000). In the wild-type retina, about 39.7% (±3.8, see Experimental Procedures) of syntaxin$^+$ amacrine cells were GABA$^+$ (γ-ammino butric acid), while 34.5% (±3.8) were positive for glycine transporter 1 (GlyT1), which is similar to the numbers found in other mammalian species (Figures 5C–5E, Strettoi and Masland, 1996; Menger et al., 1998). The relatively lower proportion of GlyT1$^+$ cells detected by our counting might be due to species differences or to the different stages assayed. In the *Pax6*$^{flox\Delta}$ retina, 31.1% (±3.9) of the syntaxin$^+$ amacrine cells are GABA$^+$ (Figures 5C and

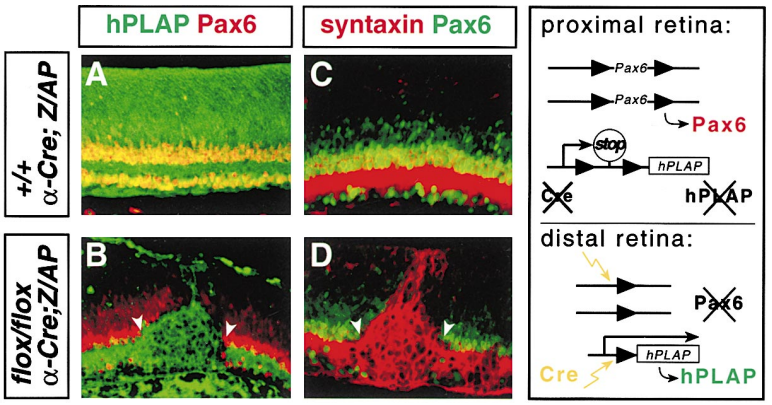

Figure 6. Cell Fate Tracing of Pax6-Deficient Retinal Progenitor Cells

(A–D) Immunohistochemistry on coronal 8 μm paraffin sections of PN1 *Z/AP; α-Cre; +/+* control (A and C) and *Z/AP; α-Cre; flox/flox* (B and D) eyes. (A) In the wild-type background, the hPLAP$^+$ cell progeny contributed to all cells of the distal neuroretina (nr), including Pax6$^+$ cells (yellow Pax6$^+$hPLAP$^+$ nuclei). (B) In the *Pax6$^{flox/flox}$* background, the hPLAP$^+$ cell progeny are localized to Pax6$^-$ cells in the distal nr, but not to the normally organized (more proximal, Pax6$^+$) nr. Immunohistochemistry on adjacent section reveals presence of syntaxin$^+$ amacrine cells (D) in the area corresponding to the hPLAP$^+$Pax6$^-$ cells (B). Scheme depicting molecular mechanisms leading to hPLAP versus Pax6 activity in the proximal and distal *Z/AP; α-Cre; Pax6$^{flox/flox}$* retina.

5D). Interestingly, GlyT1$^+$ cells were dramatically underrepresented in the *Pax6$^{flox\Delta}$* retina, 0.4% (±0.3, Figures 5C and 5E). However, the almost complete absence of glycinergic amacrine cells was apparently not accompanied by a proportional increase in the number of GABAergic amacrine cells. At the same time, all cells of the *Pax6$^{flox\Delta}$* retina displayed definitive characteristics of terminally differentiated amacrine cells (see above). These observations might indicate a concommitant increase in the proportion of "unidentified" amacrine cell classes. The observed larger number of Six3$^+$syntaxin$^+$ versus Six3$^-$syntaxin$^+$ amacrine cells in the *Pax6$^{flox\Delta}$* retina (compare insets Figures 4C and 4D) possibly reflects such a shift. Starburst amacrine cells are cholinergic and constitute an important subpopulation of GABAergic amacrine cells and express the calcium binding protein calretinin (Figure 5F), as well as the LIM homeodomain factor Isl1 (Galli-Resta et al., 1997). Isl1$^+$Pax6$^-$ and calretinin$^+$Pax6$^-$ cells are detected in the distal mutant retina, as well as choline acetyl transferase (ChAT) positive cells (Figure 7A, data not shown). Together, these results demonstrate that the *Pax6$^{flox\Delta}$* RPCs generate terminally differentiated amacrine interneurons, which, with the exception of the glycinergic class, are specified into their various amacrine cell subclasses.

Amacrine Cells Clonally Derive from *Pax6$^{flox\Delta}$* Retinal Progenitor Cells

In *flox/flox; α-Cre* embryos and mice, the *Pax6$^{flox\Delta}$* cells in the distal neuroretina are found in immediate proximity to Pax6$^+$ cells in the proximal retina, the latter of which have not been submitted to Cre recombination. Although no overt migration of (Pax6$^+$) cells into the *Pax6$^{flox\Delta}$* retina could be observed during embryogenesis, the intermingling between these two artificially generated neighboring cell populations could nevertheless not be ruled out. By generating *flox/flox; α-Cre; Z/AP* mice, we were able to follow the fate of the progeny of RPCs in which Cre activity resulted in the loss of Pax6 (Figures 6A–6D). In a given cell of this genotype, Cre activity led to constitutive hPLAP expression by the *Z/AP* transgene, which eventually will be inherited by the progeny of this cell. As has been observed above (Figures 1F and 1H), in the *+/+; α-Cre; Z/AP* genotype, the hPLAP$^+$ cell progeny contributed to all cells of the distal neuroretina (Figure 6A). However, in *flox/flox; α-Cre; Z/AP* littermates, the hPLAP$^+$ cell progeny contributed nearly exclusively to syntaxin$^+$Pax6$^-$ (Figure 6D) cells in the *Pax6$^{flox\Delta}$* retina (Figure 6B). As expected, only few cells in the neighboring (Pax6$^+$) proximal retina displayed hPLAP activity (Figure 6B). Therefore, the syntaxin$^+$Pax6$^-$ cells in the *Pax6$^{flox\Delta}$* retina clonally derive from Pax6-deficient (*Pax6$^{flox\Delta}$*) RPCs.

Discussion

Early and Late Functions of Pax6 in Eye Development

By employing the Cre-loxP strategy to conditionally inactivate Pax6 in the lens primordium, we recently demonstrated that Pax6 activity in the head surface ectoderm is autonomously required for lens formation (Ashery-Padan et al., 2000). A remarkable outcome of this study was that the complete absence of lens structures nevertheless permitted the formation of a properly laminated retina containing all retinal cell types, indicating a striking independence of retinogenesis from the interaction with the developing lens. The developmental arrest in optic vesicle formation in the *Pax6$^{-/-}$* mutant can therefore be attributed to an autonomous function of Pax6 during early optic vesicle genesis. Despite the continued expression of a number of factors indicative for early retinal development (Figure 7B, data not shown), the absence of Pax6 activity in the *Pax6$^{-/-}$* optic vesicle led to a failure in the transition from the neuroectoderm of the optic vesicle toward the establishment of RPC identity. Consequently, neurogenesis, including the upregulation of neurogenic bHLH factors (NeuroD, Ngn2) and the appearance of βIII-tubulin$^+$ or syntaxin$^+$ differentiating neurons, was not observed in the *Pax6$^{-/-}$* optic vesicle rudiments (data not shown). Additionally, the *Pax6* α enhancer fails to be activated in *Pax6$^{-/-}$ α-Cre* optic vesicles (data not shown). Our results finally demonstrate that once RPC identity is established, Pax6 becomes dispensable for the maintenance of retinal identity. During the ensuing stages of retinogenesis, the continued presence of Pax6 activity eventually ensures

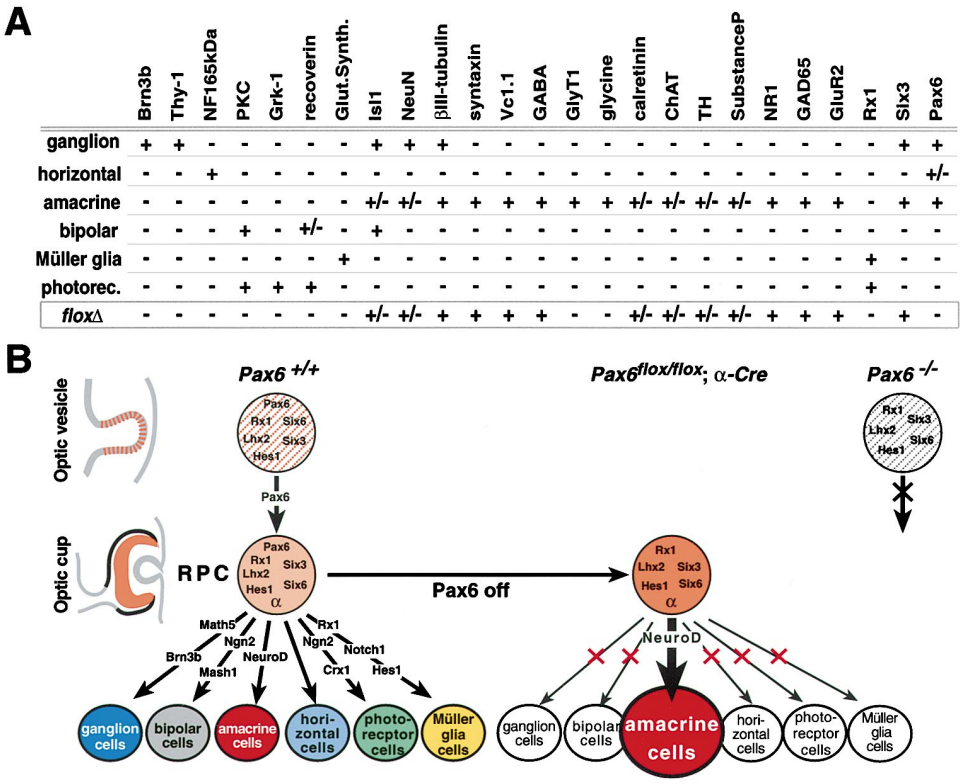

Figure 7. Multipotent Retinal Progenitor Cells Require Pax6 to Exert their Full Retinogenic Potential

(A) Table summarizing the expression profile of the cell types of the mature wild-type and the cells of the *Pax6*$^{flox\Delta}$ neuroretina (depicted as "*flox*Δ"). The expression of cell type specific markers analyzed in this study are noted as positive (+), negative (−), weak/few (+/−).

(B) Scheme depicting the two levels of Pax6 requirement during retinal determination. See Discussion for a description.

the maintenance of the potential of RPCs to generate all retinal cell types (Figure 7B).

Retinogenesis from Pax6-Deficient Retinal Progenitor Cells

During normal retinogenesis, Pax6 becomes downregulated in most cell lineages, but is maintained at a high level in all amacrine cells (see Figure 4A). The observation of wild-type-like amacrine cell genesis in the *Pax6*$^{flox\Delta}$ retina (see Figures 2G and 2H, data not shown) and the generation of terminally differentiated amacrine cells demonstrates the dispensability of Pax6 activity for the differentiation and maintenance of this cell type. A similar observation was made for the endocrine lineage of the developing pancreas, where Pax6 activity is present at a high level in both α and β cells, but only the first fail to be generated in Pax6 null mutant mice (St. Onge et al., 1997). However, the observed near absence of glycinergic amacrine cells in the *Pax6*$^{flox\Delta}$ retina indicates that Pax6 activity is indeed not dispensable for all amacrine cell classes.

In the wild-type situation, the onset of amacrine cell genesis is preceded by or coincides with the differentiation of retinal ganglion cells, cone photoreceptors, and horizontal cells (Young, 1985), all of which fail to be generated in the *Pax6*$^{flox\Delta}$ retina. Hence, Pax6 inactivation does not lead to the preferential generation of the only cell fate available by the time inactivation occurs. Furthermore, bipolar and Müller glia cells start to differentiate at E14 and E16 (Young, 1985), while in the *Pax6*$^{flox\Delta}$ retina, the first differentiating (syntaxin^{+}βIII-tubulin^{+}) cells appear around E14 (Figures 2G and 2H, data not shown). Only 1 day following birth (PN1), the majority of the cells in the *Pax6*$^{flox\Delta}$ retina were observed to constitute differentiated amacrine cells (Figure 6D, data not shown), that is 2 days before the peak, but 4–6 days after onset of Müller glia and bipolar cell differentiation (Young, 1985). The gradual appearance of syntaxin^{+} cells, moreover, indicates that the inactivation of Pax6 does not lead to an immediate switch to a lineage-restricted (amacrine and horizontal cell generating, Alexiades and Cepko, 1997) RPC population. The absence of late-born cell types in the *Pax6*$^{flox\Delta}$ retina, therefore, cannot be attributed to a premature depletion of the *Pax6*$^{flox\Delta}$ RPC pool. The normal timing of cell differentiation possibly reflects the largely unaffected Notch signaling in the *Pax6*$^{flox\Delta}$ retina, as indicated by the initiation of Hes1, Notch1, and Delta-like1 expression (albeit the latter two at lower levels, data not shown). Finally, the severely reduced RPC proliferation or premature cell cycle exit in *Chx10* and *Hes1* mutant mice (Burmeister et al., 1996; Tomita et al., 1996b), or the block of DNA synthesis in *Xenopus* embryos (Harris and Hartenstein, 1991), all had little effect on the determination of retinal cell fate. Therefore, the observed restriction to the amacrine cell fate in the *Pax6*$^{flox\Delta}$ retina cannot be indirectly attributed to a decreased level of RPC proliferation. The

Pax6$^{flox\Delta}$ RPCs rather appear to be intrinsically restricted in their retinogenic potential to only one of the different retinal cell fates normally available to RPCs.

Pax6 Controls the Retinogenic Potential of Retinal Progenitor Cells

Our results indicate that the direct control of retinogenic bHLH factor expression constitutes a general mechanism of how Pax6 executes its function in retinal neurogenesis. bHLH transcription factors have been shown to play an essential role in the differentiation of retinal cell types from RPCs (Tomita et al., 1996a; Kanekar et al., 1997; Perron et al., 1999; Wang et al., 2001). However, no overt defect could so far be detected in the retina of Ngn2-deficient mice (Fode et al., 2000; our unpublished observations), which possibly is due to compensation by upregulation of Mash1 (C. Schuurmans and F. G., unpublished data), as has been previously demonstrated for the cerebral cortex (Fode et al., 2000). Our preliminary results indicate that *Ngn2; Mash1* double mutants display a complete absence of bipolar cells (T. M., R. S., F. G., and P. G., unpublished results). Since Mash1 null mutants alone exhibit reduced (but not absent) bipolar cell differentiation (Tomita et al., 1996a) , this observation suggests that during normal retinogenesis, bipolar cells evolve from two nonoverlapping RPC populations that are defined by the activity of Ngn2 and Mash1, which in turn require Pax6 for their expression.

The exclusive commitment of *Pax6*$^{flox\Delta}$ RPCs to the amacrine cell fate is apparently due to the Pax6-independent activation of the amacrine cell differentiation program, for which the expression of NeuroD is indicative (Morrow et al., 1999; Figure 7B). Interestingly, in most areas of the developing CNS, NeuroD expression was observed to be dependent on the activity of Neurogenins (Guillemot, 1999). Since Ngn2 fails to be expressed in *Pax6*$^{flox\Delta}$ RPCs, the continued expression of factors like Six3 appears to be sufficient to mediate transcriptional activation of NeuroD. Most interestingly, although only retinal ganglion cells fail to be generated in the retina of Math5-deficient mice, the abolishment of this Pax6-dependent cell differentiation pathway alone results in a similar, yet partial, shift toward the amacrine cell fate (Wang et al., 2001). Future studies will have to address whether this cell fate shift is similarly mediated by an upregulation of NeuroD in the RPCs that normally express Math5.

The selection of subsets of RPCs to activate retinogenic bHLH transcription factors presumably involves the interplay of long-range extrinsic cues (like Shh- or EGF-mediated signaling, Lillien, 1998) and short-range cellular interactions, as mediated by Notch-Delta signaling (Dorsky et al., 1997). However, for their subsequent transcriptional activation and thereby the progression from undetermined to lineage-restricted RPC, the presence of Pax6 activity in the RPCs appears to be strictly prerequisite. These factors then impose a bias on these subsets of RPCs, thereby leading to the transition from uncommitted RPC toward a lineage-restricted RPC intermediate. By directly controlling this transition process, Pax6 mediates the full retinogenic potential of RPCs and hence, their multi-potency (Figure 7B).

Acquisition of Retinal Progenitor Cell Identity

In the developing retina of amphibia and fish, retinal cells are generated in the ciliary marginal zone (CMZ) in the peripheral retina and continue to be added throughout adult life (Wetts et al., 1989). In higher vertebrates, the regenerative capacity of the adult retina has been lost. Recently, however, evidence has been provided that retinal stem cells can be retrieved from the pigmented ciliary body of the adult mammalian retina, possibly reflecting an evolutionary homology to the CMZ of lower vertebrates (Tropepe et al., 2000). The least determined RPCs (retinal stem cells) in the peripheral CMZ were found to coexpress the transcription factors Pax6, Rx1, and Six3, prior to the activation of proneural genes and the subsequent steps toward retinal neurogenesis (Perron et al., 1998). In the mammalian retina, RPCs similarly express a limited set of transcription factors, Pax6, Rx1, Six3, Six6, and Lhx2, prior and during the ensuing steps of retinogenesis (Walther and Gruss, 1991; Oliver et al., 1995; Mathers et al., 1997; Jean et al., 1999). Pax6, Rx1, Six3, and Six6 have been implicated to constitute key components of an evolutionary conserved genetic network that initiates eye development (Halder et al., 1995; Oliver and Gruss, 1997; Gehring and Ikeo, 1999; Loosli et al., 1999).

Given their role in initiating the events that ultimately lead to retinal development, the characteristic expression profile of Pax6, Rx1, Six3, Six6, and Lhx2 indicated an additional requirement for the combined action of these factors in conferring and/or maintaining RPC identity. In this study, we could assign an essential role for one member of this limited set of transcription factors in mediating the retinogenic potential of RPCs, thereby strongly supporting this assumption. Pax6-deficient RPCs maintain retinal characteristics, including the expression of Rx1, Six3, Six6, and Lhx2, and undergo neuronal differentiation, although their potential has become restricted to one single cell fate. These observations strongly indicate that the continued functions of these other factors are sufficient to maintain retinal identity. Factors like Six3 might be essential to permit the generation of amacrine cells in the absence of Pax6 function. The challenge lies now in addressing the function of each of the other members of this class of factors in RPCs, like Rx1, Six3, Six6, and Lhx2, in mediating and maintaining the retinogenic potential of RPCs.

Experimental Procedures

Transgenic and Targeted Mice

A 2.2 kb enhancer-minimal-promoter cassette (containing P0 promoter and 1.8 kb of *Pax6* intron 4) was cloned 5′ of *IRES-intron-gfp-pA* (Ashery-Padan et al., 2000) in pSL1180 (Pharmacia). Eventually, *Cre* (gift from K. Rajewsky) was inserted to obtain α-*P0-Cre-IRES-intron-gfp-pA* (α-*Cre-gfp*). To obtain *E2*$_{Ngn2}$*-lacZ*, a 7610 bp *EagI* fragment, including 5′ flanking sequences and 144 bp of *Ngn2* coding sequence (R. Scardigli et al., submitted) was cloned into *pINL* (Fode et al., 2000). Microinjection and retransfer were carried out according to Hogan et al., 1994. α-*Cre* mice and embryos were identified by gfp fluorescence. For generation of *Pax6*^{flox} mice, see Ashery-Padan et al., 2000.

Electrophoretic Mobility Shift Assay

EMSAs were carried out as described (Schwarz et al., 2000). Preincubation with 1:10 diluted rabbit polyclonal Pax6 antibody or rabbit preimmune serum was carried out for 1 hr on ice prior to binding.

Sequences of the (sense) oligonucleotides used in the EMSAs are given below. Nucleotide positions and mutations in the core motif of the consensus sequence (Epstein et al., 1994) are indicated:

E2.1 (408 bp): 5′-TAGTAGACACTATTTTCAAGCCTCTATAGTTTA AAC-3′;
E2.1mut: 5′-TAGTAGACACTATTTTCACTCGAGTATAGTTTAAAC-3′;
E2.3 (5867 bp): 5′-CCCCAAATTACGTCAAGGCGTGAAGCCTCACT GTGT-3′;
E2.3mut: 5′-CCCCAAATTACGTCACTCGAGGAAGCCTCACTGTGT-3′.

Transfections of Cells and In Ovo Electroporation of Chick Embryos

80% confluent *COS-7* or *3T3* cells were transfected with 1.0 μg *E2$_{Ngn2}$-lacZ*, 0.1 μg *CMV-luciferase*, and 0.5–2.0 μg *CMV-Pax6/2/7* and *pBSKS* (Stratagene) to a total of 3.0 μg DNA using Lipofectamine (Gibco). β-gal activity was determined in a classic enzyme assay and normalized to transfection efficiency by Luciferase activity (Promega). In ovo electroporation of 36–48 hr old chick embryos was carried out as described (Funahashi et al., 1999). *E2$_{Ngn2}$-lacZ* (1 μg/μl) was coinjected with *CMV-gfp* (0.1–4 μg/μl, Clontech) alone or with *CMV-Pax6* (1–4 μg/μl). Embryos were allowed to develop for 6 hr.

BrdU and TUNEL Labeling

Pregnant mice were injected with 0.14 mg/g body weight BrdU/PBS. BrdU incorporation was detected on 6 μm paraffin sections according to the manufacturer (Boehringer Mannheim). Apoptotic cells were detected with the ApoTag kit (Intergen). BrdU$^+$ and TUNEL$^+$ were counted against Hoechst$^+$ cell nuclei (=total cell number). At E12.5, 19 sections from 10 mutant and 9 sections from 6 control eyes were analyzed for BrdU and TUNEL. For E15.5, 18 sections from 6 mutant and 8 sections from 4 control eyes were counted.

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry was carried out as described (Ashery-Padan et al., 2000). Dilutions and sources of antibodies (see Figure 7A) are available on request. All secondary antibodies were from Molecular Probes (always 1:500). Staining for hPLAP and β-gal activity was carried out as described (Lobe et al., 1999). Cell counting was carried out using AnalySIS (collected with Olympus B-201 or Zeiss LSM-410-invert). GABA$^+$ and GlyT1$^+$ were counted against syntaxin$^+$ cells (=total number of amacrine cells). 19 sections of 6 control eyes, while 12 sections of 3 mutant eyes were counted for each marker. ISH on paraffin sections using ^{35}S-labeled antisense RNA probes was carried out according to Kessel and Gruss, 1991. The probes used in this study were as follows: Six3 (Oliver et al., 1995), Six6 (Jean et al., 1999), Lhx2 (Porter et al., 1997), Rx1 (G. Bernier and P. G., unpublished data), Hes1 (Tomita et al., 1996b), Mash1 (Lo et al., 1997), NeuroD (Lee et al., 1995), Ngn2 (Sommer et al., 1996), Math5 (Brown et al., 1998).

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