Published in final edited form as:
Development. 2008 December ; 135(24): 4037–4047. doi:10.1242/dev.028308.

Dual requirement for Pax6 in retinal progenitor cells

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Abstract

Throughout the developing central nervous system, pre-patterning of the ventricular zone into discrete neural progenitor domains is one of the predominant strategies used to produce neuronal diversity in a spatially coordinated manner. In the retina, neurogenesis proceeds in an intricate chronological and spatial sequence, yet it remains unclear whether retinal progenitor cells (RPCs) display intrinsic heterogeneity at any given time point. Here, we performed a detailed study of RPC fate upon temporally and spatially confined inactivation of Pax6. Timed genetic removal of *Pax6* appeared to unmask a cryptic divergence of RPCs into qualitatively divergent progenitor pools. In the more peripheral RPCs under normal circumstances, Pax6 seemed to prevent premature activation of a photoreceptor-differentiation pathway by suppressing expression of the transcription factor Crx. More centrally, Pax6 contributed to the execution of the comprehensive potential of RPCs: *Pax6* ablation resulted in the exclusive generation of amacrine interneurons. Together, these data suggest an intricate dual role for Pax6 in retinal neurogenesis, while pointing to the cryptic divergence of RPCs into distinct progenitor pools.

Keywords

Pax6; Retinal progenitor cells; Retinogenesis; Crx; Cre/*loxP*

INTRODUCTION

As in other regions of the CNS, neuronal diversity in the retina originates from a common pseudostratified layer of mitotically active neuroepithelium. The retinal neuroepithelium emerges from the distal tip of the optic vesicles (OVs), established as lateral protrusions

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/4037/DC1

from the ventral forebrain. Following contact with the presumptive lens ectoderm, the OVs invaginate to form the optic cups (OCs). The inner layers of the OCs, contain the retinal neuroepithelium. Retinogenesis is initiated with the first post-mitotic cells emerging from retinal progenitor cells (RPCs) in the central OC, and progresses towards the periphery. Most of the newly generated retinal cells appear to migrate vertically to their prospective layer. With the increasing proportion of postmitotic cells, new cells are generated in the proliferative zone, which is maintained until differentiation is completed (Marquardt, 2003). Cell birth-dating studies have revealed an intriguing sequential program of retinal cell-type specification, which is highly conserved among vertebrate species. Invariably, the first cell type to appear in all vertebrates is the ganglion cell, followed by partial overlap with the appearance of cone photoreceptors, amacrine and horizontal cells, while the bipolar interneurons and Müller glia cells appear last. Rod photoreceptor genesis occurs in parallel with that of the other cell types and peaks around birth (Carter-Dawson and LaVail, 1979; Rapaport et al., 2004; Sidman, 1961; Young, 1985).

Based on pivotal cell lineage studies, RPCs were concluded to be inherently multipotent (Fekete et al., 1994; Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). The importance of intrinsic determinants for cell-fate choices in the retina has been established by cell-dissociation and heterochronic aggregation experiments. (Belecky-Adams et al., 1996; Morrow et al., 1998; Rapaport et al., 2001; Reh and Kljavin, 1989; Watanabe and Raff, 1990). Based on these findings, the idea has emerged that overall retinogenesis progresses through gradual shifts in the competence of RPCs to respond to extrinsic cues (Cepko et al., 1996). However, the molecular mechanisms that underlie the postulated competence states of the RPCs remain largely elusive (Pearson and Doe, 2004).

An important class of cell fate determinants is the family of basic helix-loop-helix (bHLH) transcription factors, related to the *Drosophila* proneural genes *atonal* and *achete-scute* (Brown et al., 2001; Vetter and Brown, 2001). These factors were shown to bias progenitor cells toward distinct cell fates (Inoue et al., 2002; Wang et al., 2001). A number of homeodomain transcription factors act in direct conjunction with bHLH proteins to differentially affect cell-fate choices in RPCs (Inoue et al., 2002). These factors are expressed in the proliferating RPCs in conjunction with, and often preceding expression of the proneural bHLH factors (Hatakeyama and Kageyama, 2004; Hatakeyama et al., 2001).

The paired and homeodomain transcription factor Pax6 is a key player in early eye development across animal phyla (Halder et al., 1995). This protein has been shown to control retinal development and cell-fate choices, which are attributed in part to its regulation of bHLH genes (Marquardt et al., 2001; Philips et al., 2005). The function of Pax6 in mammalian retinogenesis is context dependent. In Pax6-null embryos, OVs are formed but the subsequent OC morphogenesis is prevented. Nevertheless, the Pax6-deficient OVs maintain expression of some retinogenic genes (*Rx, Chx10*) and appear to undergo premature neurogenesis based on the expression of pan-neuronal markers (Baumer et al., 2003; Grindley et al., 1995; Marquardt et al., 2001; Philips et al., 2005). This premature differentiation, however, is aborted, as fully differentiated neurons are not identified in the Pax6-deficient optic rudiment (Philips et al., 2005). In contrast to the arrested differentiation observed in the Pax6-null mutants, inactivation of Pax6 at the OC stage results in the

exclusive generation of amacrine interneurons at the expense of all other retinal cell types. Thus, at this later stage, Pax6 seems to be dispensable for the completion of neurogenesis but essential for RPC multipotency. The dynamics of amacrine cell genesis following Pax6 loss from RPCs has never been investigated and thus additional roles for Pax6 during earlier aspects of retinal cell-fate specification remain possible.

In this study, we performed a detailed investigation of RPC fate in different genetic Pax6-deficient models in mouse. Our results suggest an early co-existence of two distinct RPC populations that differ in their responsiveness towards Pax6 depletion. These findings therefore suggest a dual requirement for Pax6 in retinal neurogenesis, while uncovering early diversification of RPCs into intrinsically distinct progenitor pools.

MATERIALS AND METHODS

Mouse lines

In the *Pax6*^{lacZ} allele, the β-galactosidase-neomycin cassette was inserted instead of the genomic region containing the initiator ATG and exons 4-6 that encode the paired domain (St-Onge et al., 1997). The *Pax6*^{flox} allele contains *loxP*s flanking the regions deleted in the *Pax6*^{lacZ} allele (Ashery-Padan et al., 2000). The deletion of the *Pax6*^{flox} allele by Cre results in the *Pax6*^{del} allele (see Fig. S1 in the supplementary material). The α-*Cre*transgenic line contains the Pax6 P0 promoter and the peripheral retina enhancer (termed α) followed by *Cre* which was cloned 5′ of *IRES-introngfp-pA* (Marquardt et al., 2001). The *Chx10-Cre* mouse line contains a random integration of the *BAC-Chx10-Cre* transgene. This transgene includes a fusion gene of *Cre* and *GFP*, and an internal ribosome entry site (IRES) followed by human placental alkaline phosphatase (*AP*). This *GFPCre-IRES-AP* cassette was inserted into the first exon of *Chx-10 BAC* (Rowan and Cepko, 2004). The *Z/AP*-transgenic mice express the human AP gene following Cre-mediated excision (Lobe et al., 1999).

Immunofluorescence and BrdU-incorporation analysis

Immunofluorescence analysis was performed as previously described (Ashery-Padan et al., 2000). The primary antibodies were: mouse anti-BrdU (1:100, Chemicon), rabbit anti-cleaved caspase 3 (1:300, Cell Signaling), goat anti hAP (1:100, Santa-Cruz), mouse anti Isl1 (1:100, hybridoma-bank), rabbit anti-Pax6 (1:1000, Chemicon), mouse anti-syntaxin (1:500, Sigma) and rabbit anti-VC1.1 (1:500, Sigma). Secondary antibodies conjugated to rhodamine red-X or Cy2 (Jackson Laboratories). BrdU was injected 1.5 hours prior to sacrifice and conducted as described (Yaron et al., 2006). Slides were viewed with an Olympus BX61 fluorescent microscope or laser-scanning confocal microscope CLSM 410 (Zeiss) The image analysis was conducted with ‘AnalySIS’.

In situ hybridization

In situ hybridization was performed as previously described (Yaron et al., 2006). For the fluorescent in situ hybridization, we employed the HRP-conjugated sheep anti-digoxigenin Fab fragments (Roche) and the TSA kit (Perkins Elmer).

Measurements of the areas of Crx expression and quantification of BrdU incorporation

To define the borders of the *Pax6*$^{-}$*Crx*$^{+}$ (region 1) and *Pax6*$^{-}$*Crx*$^{-}$ (region 2), and to determine BrdU incorporation in each region, three serial sections (10 μm each) from each eye were analyzed and compared (an example in Fig. 2). On the first section, the Pax6 and VC1.1 expression domain was determined using specific antibodies and on the adjacent section, Crx transcripts were identified using in situ hybridization. In the *Pax6*$^{flox/flox}$;α-*Cre* mutants, the region which was *Pax6*$^{-}$*Crx*$^{+}$ was termed region 1, while the region that was *Pax6*$^{-}$*Crx*$^{-}$ was termed region 2. On the third sequential section, the proportion of BrdU^{+} cells in each region was determined. This analysis was conducted on three to four eyes for all genotypes and developmental stages, and for each eye the average value was calculated from two to four sections (number of eyes indicated in figure legends). To obtain total cell number in each domain, the measured 4′,6-diamidino-2-phenylindole (DAPI; 100ng/ml) area was divided by the average nucleus size to obtain an estimation of cell number (which was averaged to be 35 μm^2 by measuring the nuclear area for 40 clearly visible cells). The ratio of BrdU^{+} or caspase 3^{+} cells from total cell number was calculated for each section. To obtain control values, we calculated the parameters in the peripheral area of the OC corresponding to 30% of the length of the outer margin of the OC from the most distal tip to the optic nerve.

Quantification of the spatial distribution of Crx^{+}Pax6^{-} cells in the Pax6-deficient RPCs of the *Pax6*$^{flox/flox}$;*Chx10-Cre* embryos

Frozen sections were double labeled to detect the expression of Crx and Pax6 by fluorescent in situ hybridization and immunofluorescence analysis, respectively. For image analysis, the OC was arbitrarily divided into thirds based on the length of the outer margin of the OC. The area of Crx^{+}Pax6^{-} out of the total Pax6^{-} area was determined in each third (Fig. 5J). This analysis was conducted on central sections from six *Pax6*$^{flox/flox}$*;Chx10-Cre* eyes (11 sections in total).

Chromatin immunoprecipitation (ChIP)

Isolated mouse embryo eyes or limbs (E13) were used as a tissue source for ChIP. The dissociated cells were crosslinked in 1% formaldehyde for 15 minutes at room temperature. The ChIP-PCR was performed on ~100 eyes or 40 limbs according to the manufacturer's protocol (Upstate Biotechnology). The immunoprecipitations were performed overnight at 4°C using 5 μg of rabbit anti-Pax6 polyclonal IgG (Covance) or 5 μg of normal rabbit IgG (Santa Cruz Biotechnology). The PCR primer pairs used for the ChIP assay were: for the detection of Crx promoter, 5′TAAGCAGACGGTGCCCTTCC-3′ (forward), 5′-AGGAAATAG -GTCCCCTCACAC-3′ (reverse); and for the detection of the Crx 3′ UTR untranslated region, 5′-CACACCAGGAAAGGGCATGG-3′ (forward), 5′-TCTGCCTCTACCTCCCTCGTG-3′ (reverse).

RESULTS

Differential requirement for Pax6 indicates early divergence of OV progenitors

In the Pax6-deficient OV rudiment, no specific retinal cell type has been so far identified, prompting the conclusion that the loss of *Pax6* triggered a generic neurogenic program (Philips et al., 2005). Here, we performed a detailed investigation of *Pax6*-deficient OV retinal precursors, focusing on a potential regulatory relationship between Pax6 activity and the pathways for photoreceptor and amacrine specification (Garelli et al., 2006; Marquardt et al., 2001; Schedl et al., 1996; Toy et al., 2002). We analyzed, in both control ($Pax6^{+/+}$) and Pax6 null mutants ($Pax6^{lacZ/lacZ}$) (St-Onge et al., 1997), the expression of the homeoprotein Crx, an essential photoreceptor determinant and one of the earliest known exclusive markers for photoreceptor precursors (PRPs) (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). For the detection of amacrine precursors, we analyzed the expression of the carbohydrate epitope VC1.1, which, during early embryogenesis, labels the precursors of amacrine and horizontal cells (Alexiades and Cepko, 1997; Arimatsu et al., 1987; Naegele and Barnstable, 1991).

In E12.5 control retina, Crx (Fig. 1A,B) was detected in a few PRPs in the outer layers of the central OC, whereas VC1.1 (Fig. 1E,F) was observed in the inner layer of the central OC matching the location of ganglion and inner nuclear layer precursors. At a later stage (E15.5), in agreement with the central-to-peripheral progression of retinogenesis, their expression extended to the peripheral OC (Fig. 1C,D,G,H). At E12.5, the Pax6 protein was detected in most OC cells, including the $VC1.1^+$ cells (Fig. 1E,F). However, its expression was barely detected in the Crx-expressing cells (Fig. 1B). On E15.5, Pax6 expression was absent from the Crx^+ PRPs, but was low in the proliferative zone and high in the $VC1.1^+$ cells of the inner nuclear layer (Fig. 1C,D,G,H). Taken together, these results indicated that, during normal retinal development, Pax6 is co-expressed with VC1.1 but is excluded from Crx-expressing cells.

We next characterized the expression of Crx and VC1.1 in the $Pax6^{lacZ/lacZ}$ optic rudiment. *Crx* transcripts, which are normally detected in only a few cells on E12.5 were detected in most of the $Pax6^{lacZ/lacZ}$ OV neuroepithelium, including all cellular layers (Fig. 1I,J). This expanded expression domain of Crx was evident at later stages of development (E15.5) (Fig. 1K,L), and it was not accompanied by misexpression of photoreceptor-specific factors such as recoverin (data not shown) (Haverkamp and Wassle, 2000; Sharma and Ehinger, 1999). We concluded that the Crx^+ cells in the $Pax6^{lacZ/lacZ}$ optic rudiment do not differentiate to mature photoreceptors. These observations further indicate that neurogenesis is abrogated in the *Pax6*-null OV (Philips et al., 2005). Notably, *Crx* was expressed in a highly heterogeneous fashion, displaying high levels of expression in only a subset of cells of the $Pax6^{lacZ/lacZ}$ OV neuroepithelium (Fig. 1J,L).

Previous results have demonstrated that somatic loss of *Pax6* results in the exclusive differentiation of amacrine interneurons from *Pax6*-deficient RPCs (Marquardt et al., 2001). Thus, we tested the possibility of amacrine cell genesis in the $Pax6^{lacZ/lacZ}$ OV by analyzing the expression of VC1.1 (Alexiades and Cepko, 1997; Arimatsu et al., 1987; Naegele and Barnstable, 1991) (Fig. 1K-L). Interestingly, VC1.1 was detected in the $Pax6^{lacZ/lacZ}$ OV

neuroepithelium at E13 but not at E12.5, when it is normally expressed in the control retina (data not shown) (Fig. 1E). Thus, its expression in the *Pax6*$^{lacZ/lacZ}$ OV is delayed by ~1 day relative to normal onset. The expression of VC1.1 persisted at later developmental stages in a subset of *Pax6*$^{lacZ/lacZ}$ OV cells, indicating an initial commitment of these cells to the amacrine cell lineage (E15.5) (Fig. 1K,L). Moreover, the VC1.1 epitope was not co-expressed in most of the Crx-expressing cells on E15.5 (Fig. 1K,L), raising the possibility of two distinct responses of the OV to Pax6 loss.

To further define the fate acquired by the *Pax6*$^{lacZ/lacZ}$ OV cells, we characterized the expression of the transcription factor Isl1, which is expressed by a subset of amacrine cells (Galli-Resta et al., 1997). In *Pax6*$^{lacZ/lacZ}$, Isl1^{+} cells were detected in the optic rudiment, supporting initiation of the amacrine differentiation pathway in the Pax6-mutant cells. Moreover, as previously indicated (Philips et al., 2005), at all of the tested stages, the *Pax6*$^{lacZ/lacZ}$ OV neuroepithelium was deficient for markers of other retinal cell types, such as the ganglion cell marker Pou4f2 and the horizontal cell marker neurofilament Nf165 (Aramant et al., 1990; Gan et al., 1996; Xiang et al., 1993) (data not shown). To further determine whether the VC1.1 and Isl1 cells identified in the *Pax6*$^{lacZ/lacZ}$ OV give rise to mature amacrine interneurons, we analyzed the expression of the selective amacrine cell marker syntaxin and the pan-neural marker β-III tubulin (Brandstatter et al., 1996). Syntaxin and β-III tubulin were not detected in the *Pax6*$^{lacZ/lacZ}$ OV, although in the normal retina their expression was evident (E14, data not shown). These findings suggest that the VC1.1 cells in the Pax6-null retina are amacrine precursors, which are unable to differentiate to mature neurons.

Taken together, at the earliest stages preceding retinogenesis, Pax6 loss seems to expose two cryptic populations of RPCs: one seems to prematurely misexpress Crx, whereas in the other VC1.1 expression is delayed. Moreover, during early retinogenesis, Pax6 seems to be required for completion of neurogenesis: despite the apparent upregulation of the two early cell fate-specification programs to photoreceptors and amacrine cells, the *Pax6*-deficient OV progenitor populations were eventually abrogated in their capacity to terminally differentiate into mature neurons.

Divergent function of Pax6 within two distinct subsets of RPCs

The spatial and temporal roles of Pax6 were investigated by establishing the *Pax6*^{flox} allele (Materials and methods) (Ashery-Padan et al., 2000). Cre-mediated deletion of *Pax6*^{flox} results in the *Pax6*^{del} allele (see Fig. S1 in the supplementary material). Corresponding to the loss of Pax6 activity, the *Pax6*$^{del/del}$ embryos exhibit the same phenotype as the *Pax6*$^{lacZ/lacZ}$ mutants; developmental arrest at the OV stage, premature misexpression of Crx, and delayed expression of the VC1.1 epitope (data not shown) (see Fig. S1 in the supplementary material).

In contrast to the differentiation arrest of the *Pax6*$^{lacZ/lacZ}$ and *Pax6*$^{del/del}$ optic rudiments, the selective removal of Pax6 from the OC after E10.5 resulted in the generation of mature amacrine cells (see Fig. S3 in the supplementary material) (Marquardt et al., 2001). We therefore investigated the potential differences in the requirement for *Pax6* in early (OV) and later phase (OC) RPCs. To this end, we analyzed the expression of VC1.1 and Crx, in

control (*Pax6*$^{flox/flox}$) and *Pax6*$^{flox/flox}$;α-*Cre* littermates (Fig. 2). The region of Pax6 depletion in the *Pax6*$^{flox/flox}$;α-*Cre* OC was determined by antibody labeling (Fig. 2A-H).

Interestingly, the expression of VC1.1 in the *Pax6*$^{flox/flox}$;α-*Cre* OC was found to be similar to its distribution in the control (Fig. 2A): VC1.1 expression was detected in the central OC and initially displayed little overlap with the region of Pax6 inactivation (Fig. 2B). Thus, reminiscent of the situation in the *Pax6*$^{lacZ/lacZ}$ OV, VC1.1 is not prematurely upregulated in *Pax6*-deficient RPCs of the *Pax6*$^{flox/flox}$;α-*Cre* retina. During subsequent developmental stages, VC1.1 expression displayed a gradual central-to-peripheral expansion in the *Pax6*$^{flox/flox}$;α-*Cre* OC (Fig. 2B,D,F), and was detected in Pax6^{-} cells (Fig. 2F, inset), although its expression was delayed in comparison with that observed in the control retina (Fig. 2C,E,G).

Intriguingly, the peripheral OC of *Pax6*$^{flox/flox}$;α-*Cre* mutants displayed dramatic precocious upregulation of Crx expression (Fig. 2J). This expression was already detected on E12, i.e. about 48 hours prior to normal onset of Crx in this region (Fig. 2K). Moreover, in the *Pax6*$^{flox/flox}$;α-*Cre* peripheral OC, the precocious Crx^{+} cells were localized throughout the basal-apical extent of the retina (Fig. 2J,L,N), as opposed to the normal restriction of Crx expression to the PRPs located in the outer layer of the OC (Fig. 2K,M,O).

In the peripheral *Pax6*$^{flox/flox}$;α-*Cre* retina, most Pax6^{-} cells expressed Crx by E12. By contrast, a distinct population of Pax6^{-} RPCs located toward the center of the OC showed no detectable upregulation of Crx (compare Fig. 2B,J, indicated zones '1' and '2' and see diagram in Fig. 2). Quantitative analysis revealed that the proportion of *Crx*$^{+}$*Pax6*$^{-}$ cells (region 1) constituted 65% (s.d.=9%) of the total number of Pax6-deficient cells on E12 (regions 1+2) (Fig. 2Y). Moreover, we did not detect any overlap between Crx expression and that of VC1.1 in Pax6-deficient retinal cells (compare Fig. 2B,D,F,H with Fig. 2J,L,N,P). Thus, in striking similarity to the situation found in the Pax6-deficient OV neuroepithelium of *Pax6*$^{lacZ/lacZ}$ embryos, these data indicate the existence of two distinct progenitor populations within the OC that differ in their requirement for Pax6 activity. The first, more peripherally located population (Fig. 2B,J, region 1) precociously upregulates Crx following Pax6 inactivation, whereas the second (Fig. 2B,J, region 2), centrally located population does not display Crx expression following loss of Pax6.

Differential impacts of Pax6 removal on the proliferation of region-1 and region-2 retinal progenitor pools

At subsequent developmental stages, we observed a gradual shift in the relative proportions of the two distinct OC subpopulations of regions 1 and 2 of the *Pax6*$^{flox/flox}$;α-*Cre* OC. At E14, the proportion of *Crx*$^{+}$*Pax6*$^{-}$ cells in regions 1 and 2 was reduced to 60% (s.d.=4%) (Fig. 2Y). By E16, this proportion dropped further to 40% (s.d.=10%) (Fig. 2Y). Eventually, on E18, the *Crx*$^{+}$*Pax6*$^{-}$ cells constituted only a very small portion of the total number of Pax6^{-} cells in the peripheral *Pax6*$^{flox/flox}$;α-*Cre* OC (Fig. 2P), whereas VC1.1 and syntaxin (Fig. 2H,X) were detected in many cells at the retinal periphery and their expression extended across the apical-basal axis of the OC, although normally these markers are detected in the inner layers, corresponding to the location of amacrine cells (Fig. 2G,W). Some cells in the peripheral *Pax6*$^{flox/flox}$;α-*Cre* OC were negative for both VC1.1 and

syntaxin on E18 (Fig. 2H,X). At postnatal stages, Crx was not detected in the *Pax6*$^{-}$ deficient OC periphery, whereas the *Pax6*$^{-}$ cells expressed syntaxin within the peripheral retina, in accordance with their eventual differentiation into amacrine interneurons (see Fig. S3 in the supplementary material) (Marquardt et al., 2001).

Overall, the peripheral *Pax6*$^{flox/flox}$*;*α*-Cre* retina displayed a markedly reduced size relative to the control retina, in agreement with the reduced mitotic rate of the *Pax6*$^{-}$ deficient RPCs (Marquardt et al., 2001). This raised the possibility that the observed shift in the relative proportion of region 1 and 2 progenitors in the *Pax6*$^{flox/flox}$*;*α*-Cre* was due to differential impacts of Pax6 deficiency on the mitotic rates of the distinct progenitor populations. Alternatively, loss of Pax6 may also differentially affect the subsequent survival of region 1 and 2 cells. To address these possibilities, we performed quantitative analysis of cell proliferation in *Pax6*$^{flox/flox}$*;*α*-Cre* and control retinas through BrdU pulse chase assays on E12-16 (Fig. 2Q-V,Z). Following a 1.5 hours BrdU pulse, at E12, both region 1 and 2 progenitors in the *Pax6*$^{flox/flox}$*;*α*-Cre* retina displayed similarly reduced BrdU incorporation relative to control peripheral retinas (14.7%, s.d.=2.1% and 16.9%, s.d.=2% BrdU^{+} cells, respectively, versus 25.5%, s.d.=3.6% in the control) (Fig. 2Z). However, a marked difference in the relative incorporation of BrdU was detected in the E14 *Pax6*$^{flox/flox}$*;*α*-Cre* retina, with only 10.3% BrdU^{+} (s.d.=2.6%) cells in region 1, compared with 19.3% (s.d.=1%) BrdU^{+} cells in region 2, and 23.8% (SD=1.8%) in the control peripheral retina (Fig. 2Z). These results indicated that in the *Pax6*$^{flox/flox}$*;*α*-Cre* retina, the marked expansion of region 2 progenitors relative to region 1 is due to the different impacts of loss of Pax6 activity on their proliferation.

To address whether differential rates of apoptosis may additionally account for the observed relative shifts in OC population sizes, we performed immunodetection of cleaved caspase 3 (Ccaspase 3) in *Pax6*$^{flox/flox}$*;*α*-Cre* and control retinas (Di Cunto et al., 2000). We did not detect any significant increase in the number of Ccaspase 3^{+} cells in the *Pax6*$^{flox/flox}$*;*α*-Cre* compared with the control OCs at E14 and E16 (data not shown). This indicates that the observed relative shifts in OC population sizes are due to differences in mitotic rate, rather than to selective elimination through apoptosis. The proliferation arrest during early embryogenesis of region 1 cells and some cell loss due to apoptosis, are consistent with the eventual elimination of *Pax6*$^{-}$*;Crx*$^{+}$ cells (Fig. 2P; see Fig. S3 in the supplementary material)

Pax6 controls different neurogenic programs in region 1 and region 2 RPCs

Previous data have indicated that the retinal expression of a number of proneural bHLH factors depends on Pax6 function (Marquardt et al., 2001; Scardigli et al., 2003). We therefore further investigated the impact of *Pax6* inactivation on the expression of selected bHLH factors in both region 1 (*Pax6*$^{-}$*Crx*$^{+}$) and region 2 (*Pax6*$^{-}$*Crx*$^{-}$) pools. This analysis was performed on E15, when the expression of most bHLH factors and Crx has progressed into the OC periphery corresponding to regions 1 and 2 (Fig. 3A-E; see Fig. S2 in the supplementary material). The region of Pax6 loss was determined by detection of Pax6 with antibodies or by monitoring the expression of hAP activity in the *Pax6*$^{flox/flox}$*;*α*-Cre;Z/AP* OCs (Fig. 3; see Fig. S2 in the supplementary material). In the *Pax6*$^{flox/flox}$*;*α*-Cre* embryos,

Crx misexpression was detected only in the peripheral compartment of the Pax6-depleted OC (Fig. 3F; see Fig. S2 in the supplementary material), whereas Atoh4 expression was abolished in all of the Pax6-depleted compartments, in agreement with previous reports on the direct regulation of Atoh4 by Pax6 (Fig. 3G; see Fig. S2 in the supplementary material) (Marquardt et al., 2001; Scardigli et al., 2003). We next compared the expression pattern of additional proneural bHLH factors in the two Pax6 mutant regions: region 1, which is defined as the *Pax6*$^{-}$*Atoh4*$^{-}$*Crx*$^{+}$ domain (Fig. 3F,G); and the *Pax6*$^{-}$*Atoh4*$^{-}$*Crx*$^{-}$demarcated region 2 (Fig. 3F,G). On adjacent sections, analysis of the expression of *Atoh3, Neurod1* and *Atoh7* revealed their downregulation in the *Pax6*$^{flox/flox}$;α-*Cre* peripheral OC (Fig. 3H-J). The normal expression of *Atoh3*, in the photoreceptor layer (Fig. 3C), was virtually extinguished from both region 1 and region 2 progenitors (Fig. 3H), thus resembling the loss of *Atoh4* from the Pax6-deficient cells (Fig. 3G). Interestingly, the expression of *Neurod1* appeared to be differentially affected in regions 1 and 2 of *Pax6*$^{flox/flox}$;α-*Cre* mutants, being severely diminished in region 1 progenitors. At the same time, low levels of *Neurod1* expression were maintained in region 2, similar to its expression in the neuroblast layer of the control retina (compare Fig. 3D with Fig. 3I). However, the characteristically high levels of *Neurod1* expression in the presumptive photoreceptor-containing outer nuclear layer was severely diminished in both regions 1 and 2 of the *Pax6*$^{flox/flox}$;α-*Cre* (Fig. 3I). Similar to the neuroblast layer expression of *Neurod1*, *Atoh7* mRNA levels displayed marked differences between regions 1 and 2 of the *Pax6*$^{flox/flox}$;α-*Cre*. Whereas *Atoh7* expression was almost completely lost from region 1 RPCs, region 2 RPCs displayed persistent, albeit reduced levels of *Atoh7* mRNA compared with the peripheral control retina (compare Fig. 3E with Fig. 3J). Retinal cell fate depends on the combination of bHLH factors expressed within the cells; for example, *Neurod1* has previously been implicated in controlling amacrine cell genesis (Inoue et al., 2002; Ohsawa and Kageyama, 2008). The persistent *Atoh7* and *Neurod1* expression in *Pax6*$^{-}$*Crx*$^{-}$ region 2 RPCs thus suggests the funneling of these progenitors towards an amacrine fate through loss of most of the other essential neurogenic programs (Marquardt et al., 2001). Taken together, these data indicate that Pax6 controls different sets of neurogenic programs in two inherently distinct subsets of OC progenitors.

Pax6^{-} *Crx*$^{+}$ region 1 progenitor cells initiate, but do not complete, a photoreceptor-specification program

The massive upregulation of Crx in region 1 RPCs of the *Pax6*$^{flox/flox}$;α-*Cre* retina suggested premature acquisition of the photoreceptor cell fate by these progenitor cells. To further test this idea, we investigated the expression of a number of factors associated with the photoreceptor-differentiation pathway in the *Pax6*$^{lacZ/lacZ}$ and *Pax6*$^{flox/flox}$;α-*Cre;Z/AP*. In the latter model, the region of Pax6 inactivation was monitored by detection of hAP expression from the Z/AP-transgene (Fig. 4J). The paired-like homeodomain protein Rx is one of the earliest known RPC markers, is essential for initiating retinal development and has been implicated in eventual regulation of photoreceptor-specific gene expression (Bailey et al., 2004; Kimura et al., 2000; Wang et al., 2004). In both the *Pax6*$^{lacZ/lacZ}$ OV and the *Pax6*$^{flox/flox}$;α-*Cre;Z/AP* mutants, the expression of *Rx* was maintained at levels similar to the stage-matched control retina (not shown) (Baumer et al., 2003; Marquardt et al., 2001). In contrast to *Rx*, the expression of *Otx2*, a member of the *otd/Otx* gene family essential for

photoreceptor differentiation (Fig. 4B) (Nishida et al., 2003), was virtually undetectable in the *Pax6*-deficient RPCs of *Pax6*$^{flox/flox}$;α-*Cre;Z/AP* mutants (Fig. 4H), as well as in the distal neuroretinal portion of the *Pax6*$^{lacZ/lacZ}$ OV (Fig. 4E). Similarly, the expression of *Trb2* (thyroid hormone receptor β 2), which regulates M and S opsin expression in cones and is expressed in the PRPs layer on E15 (Fig. 4C) (Applebury et al., 2007; Ng et al., 2001; Shibusawa et al., 2003), was absent from *Pax6*$^{lacZ/lacZ}$ OVs (Fig. 4F) and from both region 1 and 2 progenitors in *Pax6*$^{flox/flox}$;α-*Cre;Z/AP* mutants (Fig. 4I), despite the detection of high levels of *Crx* RNA in region 1 cells on adjacent sections of the same specimen (Fig. 4G). Together, these data indicate that the *Pax6*$^{-}$*Crx*$^{+}$ region 1 progenitors in the *Pax6*$^{flox/flox}$;α-*Cre* retina initiate an early photoreceptor specification program, but eventually fail to enter terminal differentiation towards mature photoreceptor neurons.

Pax6 binds the Crx promoter in the embryonic mouse retina

The dramatic change in *Crx* expression in both *Pax6*$^{lacZ/lacZ}$ and *Pax6*$^{flox/flox}$;α-*Cre* mutants, together with the early appearance of Crx close to the onset of Pax6 inactivation, suggested direct inhibition of Crx expression by Pax6 in a subpopulation of RPCs. A 2 kb region has been shown to contain crucial regulatory sequences required for full expression of Crx in the developing retina (Furukawa et al., 2002). The 300 bp sequence adjacent to the transcription start site is conserved among mammals (81% conservation between mice and humans). In addition, this region includes putative binding sites for paired-type homeodomain-containing proteins (Nishida et al., 2003; Tatusova and Madden, 1999). To establish whether Pax6 binds directly to the proximal *Crx* promoter in vivo, we performed a ChIP analysis using a specific antibody against Pax6 to immunoprecipitate chromatin from embryonic (E13) mouse eyes (Fig. 4K). The sequences of the *Crx* promoter were amplified from the immunoprecipitated chromatin by PCR. We also used the *Crx* 3′ UTR sequences and *Optimidin* intron 6 sequences as reference regions (Grinchuk et al., 2005). In the chromatin prepared from E13 retina, Pax6 was found to occupy the *Crx* promoter region but not its 3′ UTR or the *Optimidin* intron 6 sequences (Fig. 4K, not shown). Binding of Pax6 to Crx promoter was not identified in the limb chromatin where Pax6 is not expressed, and the binding was not detected with non-specific IgG (Fig. 4K). In addition, we tested one putative binding site for Pax6 [chr7:16465201-16465450; predicted by MatInspector (Cartharius et al., 2005)] but this site did not bind in vitro to Pax6 by electromobility shift assay (EMSA; data not shown). We therefore conclude that Pax6 interacts directly with the Crx promoter region in the embryonic retina, and that Pax6 activity on the Crx promoter possibly requires additional co-factors or occurs at different Pax6-binding site than the one indicated by the in silico prediction. The ChIP data combined with gene ablation studies suggest direct, albeit context-dependent, regulation of Crx by Pax6 in retinal progenitor cells.

The aberrant expression of Crx in the Pax6 mutants reflects an intrinsic requirement for Pax6 in RPCs of the OC periphery

The α-*Cre* transgene mediates recombination within the OC periphery, including retinal progenitors and non-neuronal progenitors that are destined to iris and cilliary body fates (Davis-Silberman and Ashery-Padan, 2008; Marquardt et al., 2001). The misexpression of Crx, which is observed in the most peripheral OC, may therefore represent a unique role for

Pax6 in the population of non-neuronal progenitors rather than a novel function in retinal neurogenesis. To explore this possibility, we employed the *Chx10-Cre*-transgenic mouse line (Rowan and Cepko, 2004). The recombination pattern mediated by this transgene is a mosaic, yet it overlaps with *Chx10* expression domains; at mid-gestation (E14), the *Chx10-Cre* recombination has been reported to occur in the OC in patches of neuronal precursors but to be excluded from the most distal tips where the non-neuronal progenitors reside (Rowan and Cepko, 2004). We characterized the phenotype of the *Pax6$^{flox/flox}$;Chx10-Cre* OC on E14. At this stage, Pax6 is normally detected in most cells of the OC; however, it is reduced to almost undetectable levels in the Crx-expressing PRPs (Fig. 5A,B). In the *Pax6$^{flox/flox}$;Chx10-Cre* mutants, the recombination pattern was monitored by detection of Pax6 protein loss (Fig. 5C-E). Corresponding with the reported pattern of *Chx10-Cre* activity, Pax6 was depleted from patches of RPCs in both the distal and proximal OC, but its expression was maintained in the most distal non-neuronal progenitors (Fig. 5C). In some of the Pax6-depleted regions, Crx misexpression was detected across the apical-basal OC; however, there were patches of Pax6-deficient cells that did not upregulate Crx (Fig. 5C,D, white arrowheads). At E14, however, most of the Pax6^{-}Crx^{-} cells had not yet upregulated VC1.1 expression (data not shown), corresponding with the delayed expression of VC1.1 observed in the *Pax6$^{flox/flox}$;α-Cre* mutants (Fig. 2D). However, the expression of VC1.1 was evident in *Pax6*$^{-}$ cells of the *Pax6$^{flox/flox}$;Chx10-Cre* mutants on E16, whereas in the normal retina at this stage, all of the VC1.1 cells co-expressed Pax6 (Fig. 5F,H). Moreover, there was no overlap in the expressions of VC1.1 and Crx in the *Pax6$^{flox/flox}$;Chx10-Cre* mutants, similar to their separate distribution in the control and *Pax6$^{flox/flox}$;a-Cre* mutants (Fig. 5G,I; Fig. 2F,N).

Taken together, the two distinct responses identified in the *Pax6$^{flox/flox}$;α-Cre* OC following loss of Pax6 were identified in the *Pax6$^{flox/flox}$;Chx10-Cre* mutants (Pax6^{-}Crx^{+} and Pax6^{-}Crx^{-} VC1.1^{+}), although the recombination mediated by *Chx10-Cre* did not include the non-neuronal progenitors of the OC. We therefore conclude that the two phenotypes observed in the OC following Pax6 loss reflect the distinct roles of Pax6 in the RPCs. Notably, the Pax6^{-}Crx^{+} cells were detected both adjacent to, and at a distance from, non-recombined Pax6-expressing cells (Fig. 5C-E). This demonstrates that normal cells do not inhibit Crx misexpression in adjacent mutant cells and provides further support for the notion that the two distinct phenotypes of the Pax6-deficient OC represent different intrinsic requirements for Pax6 in the developing retina.

To determine the eventual phenotype of Pax6-deficient RPCs in the *Pax6$^{flox/flox}$;Chx10-Cre* mutants, we traced the mutant cells with *Z/AP* and determined their neuronal phenotype by co-labeling with antibodies to the amacrine-specific marker syntaxin or the photoreceptor determinant, recoverin. The phenotype of the hAP^{+} cells in the *Pax6$^{flox/flox}$;Chx10-Cre;Z/AP* retina was similar to that observed in the *Pax6$^{flox/flox}$;α-Cre* mice (see Fig. S3 in the supplementary material) (Marquardt et al., 2001). In regions where hAP was detected across the retina, thus originating from Pax6-deficient RPCs, the laminar organization was lost and most cells co-expressed hAP and syntaxin, but not recoverin (see Fig. S3 in the supplementary material). This further demonstrates that, regardless of the location of Pax6-deficient RPCs in the central or peripheral OC, the Pax6-deficient RPCs that maintain the

differentiation potential are eventually restricted in their differentiation capacity and differentiate exclusively into amacrine interneurons.

In the *Pax6$^{flox/flox}$;α-Cre*, the Pax6^{-}Crx^{-} cells were consistently localized toward the center of the OC, whereas the Pax6^{-}Crx^{+} cells were identified more distally (Fig. 2). We therefore asked whether the central or peripheral position of the mutated cells in *Pax6$^{flox/flox}$;Chx10-Cre* is predictive of their eventual phenotype (*Pax6^{-}Crx^{-}* or *Pax6^{-}Crx^{+}*). The proportion of the *Pax6^{-}Crx^{+}* area relative to the total Pax6-deficient area was measured in the peripheral and central thirds of the OCs and the average values were calculated. In the peripheral third of the OC, 79% (s.d.=18%) (Fig. 5J) of the Pax6-deficient regions were *Crx^{+}*, while in the central third, only 34% (s.d.=10%) (Fig. 5J) of the Pax6-deficient domain misexpressed Crx; this difference was highly significant (Fig. 5J). Thus, the phenotypic outcome of Pax6-deficient RPCs correlated with the location of the cells within the OC; mutation in Pax6 in the peripheral OC is most likely to result in misexpression of Crx, whereas Pax6 deletion more centrally is likely to result in differentiation of the Pax6-deficient cells to amacrine interneurons.

DISCUSSION

This study provides several new insights into the process of neurogenesis in the developing mammalian retina and the involvement of Pax6 in these events. First, our findings reveal an early, Pax6-independent subdivision of RPCs into inherently distinct progenitor pools. Second, our data indicate that Pax6 is required up until the optic-cup stage for the spatial distribution and neurogenic potential of the two RPC populations. Finally, this study uncovers a dual requirement for Pax6 during retinal neurogenesis at the optic-cup stage: for the RPCs in the OC periphery, Pax6 is required for the completion of neurogenesis and for the inhibition of Crx expression. By contrast, in the more centrally located RPCs, Pax6 is dispensable for neurogenesis but is essential for their multipotency.

An early Pax6-independent subdivision of RPCs into distinct progenitor pools

In *Pax6*-null mutants, the retinal neuroepithelium of the OV rudiment displayed two distinct subsets of progenitors that differed in their phenotype: in one population, premature upregulation of Crx was observed, whereas in the other, Crx was not expressed and the appearance of VC1.1 was delayed. The detection of two distinct phenotypes in the *Pax6* null optic-rudiment indicated a prior distinction of discrete RPC subsets, well before the normal onset of retinal cell differentiation and that these progenitor populations emerge independently of Pax6 activity during early stages of retinogenesis.

Previous data have indicated that the early subdivision of the OV neuroepithelium into spatially separate optic stalk, neuroretinal and pigment epithelial progenitor fields requires the activity of signaling pathways such as Shh from the midline, TGFβ signaling from the extra-ocular mesenchyme and Fgfs from the surface ectoderm (Fuhrmann et al., 2000; Macdonald and Wilson, 1997; Nguyen and Arnheiter, 2000). The spatial distinction into these principal progenitor domains was found to be maintained in the OV of *Pax6*-null mutants, but was lost upon elimination of both *Pax6* and *Pax2* (Baumer et al., 2003; Grindley et al., 1995). Here, we found that the early elimination of Pax6 in the *Pax6$^{lacZ/lacZ}$*

mutant leads to a loss of spatial separation between regions 1 and 2 RPCs within the presumptive neuroretinal domain. Both the *Crx*$^{+}$*VC1.1*$^{-}$ and the *Crx*$^{-}$*VC1.1*$^{+}$ RPC pools were found to be intermixed within the *Pax6*$^{lacZ/lacZ}$ OV, in contrast to their spatial separation in the *Pax6*$^{flox/flox}$*;α-Cre* and *Pax6*$^{flox/flox}$*;Chx10-Cre* retinas. The present study thus suggests that early patterning of the OV and formation of the OC are accompanied by the establishment of distinct subpopulations of RPCs within the neuroretina. The dependency on Pax6 for this regionalization of the RPCs during early stages of eye development may directly relate to the function of Pax6 in the OV, or it may reflect a secondary outcome of the arrest in OC formation or absence of the lens, which has been shown in previous studies to be required for the morphology of the OC (Ashery-Padan et al., 2000). Together, the establishment of regional distinctions between RPCs along the proximodistal axis of the neuroretina appears to depend, directly or indirectly, on an early phase of Pax6 activity – a dependency that ceases once the optic-cup stages are reached.

Dual requirements for Pax6 within the two subpopulations of RPCs

Recent studies have shown that in the developing neocortex there are several distinct neurogenic progenitor cells that are multipotent, including the radial glia and intermediate progenitor cells (Hevner, 2006; Pontious et al., 2008). Within these populations, Pax6 is expressed and plays different roles, depending on the temporal and spatial context (Guillemot, 2005; Pinto and Gotz, 2007; Warren et al., 1999). Moreover, a dual role for Pax6 has been reported in the generation of neurons of the adult olfactory bulb, where Pax6 was found to initially regulate the establishment of the neuronal lineages and, subsequently, their specification toward a periglumerular cell fate (Hack et al., 2005). In contrast to the developing neocortex, differences among RPCs have not yet been recognized in the developing retina. However, there are several lines of evidence supporting distinct transient states of these cells: first, considering the central-peripheral pattern of differentiation, it is likely that the RPCs located adjacent to differentiating neurons at the central OC are exposed to different cues from the RPCs located far from the differentiation front, at the OC periphery. Second, recent findings have shown the differential expression of genes in the central versus peripheral regions (Adler and Canto-Soler, 2007; Koso et al., 2006; Koso et al., 2007). Finally, in this study, two distinct phenotypes of RPCs were identified after Pax6 inactivation in the OC, including differences in the expression Crx, the expression profile of proneural bHLH genes, proliferation index and neurogenic potential. Moreover, these different phenotypes were correlated to the location of the cells along the central-peripheral axis of the OC. Together, these findings indicate an important distinction between Pax6 activities within adjacent RPC pools, suggesting an inherent difference between RPC populations. Considering that all retinal cell types eventually populate both central and peripheral retina in the adult, it seems likely that the differences documented here between central and peripheral OC RPCs primarily reflect distinct differentiation stages of the multipotent progenitor pools, similar to the transient states observed in cortical neurogenesis (Hevner, 2006; Pontious et al., 2008) rather than differences in cell specification. In this case, the role of Pax6 is to promote the maturation of progenitor cells and their eventual differentiation to all of the retinal cell types.

Analogous to the early intrinsic differences identified here between distal and proximal RPCs, recent studies have found a regional distribution of components of the Wnt, Hedgehog, BMP and Notch signaling pathways along the proximal-distal axis of the OC (Adler and Canto-Soler, 2007; Yaron et al., 2006). Similarly, the stem-cell epitope CD15 was found to be transiently expressed in a Wnt-dependent manner within a subset of RPCs located at the retinal periphery (Koso et al., 2007). These factors may therefore create focal differences among RPCs and underlie the intrinsic differences that were exposed here following loss of *Pax6*, although their precise role and their regulatory relationship with Pax6 remain to be addressed.

Involvement of Pax6 in the transcriptional network regulating photoreceptor differentiation in mammals

The iterative deployment of Pax6 in the process of eye formation in evolutionarily distant organisms, suggests that there are common transcriptional targets for Pax6 in the different species, such as the regulation of opsin gene expression (Arendt et al., 2004; Zuker, 1994; Gehring, 2005). In support of this idea, *eyeless*, the fly homolog of *Pax6*, was found to be expressed in photoreceptors and was subsequently shown to regulate the expression of the *Drosophila* rhodopsin genes in these cells (Papatsenko et al., 2001; Quiring et al., 1994; Sheng et al., 1997). In vertebrates, however, this role for Pax6 does not appear to be conserved, in line with the rapid downregulation of Pax6 expression in differentiating photoreceptors during vertebrate retinogenesis. Moreover, our ChIP data indicate selective binding of Pax6 protein to the *Crx* promoter region, supporting its role as a direct transcriptional repressor of photoreceptor fate. The current study reveals the complex involvement of Pax6 in the transcriptional network leading to photoreceptor differentiation in mammals (Fig. 6). Surprisingly, although in both regions Pax6 is essential for completion of the photoreceptor-differentiation program, its regulation of the genes involved in the photoreceptor lineage is different in the two regions of the OC: in region 1 it plays a role in inhibiting the onset of Crx expression, whereas in region 2 it is required for the expression of Crx. Thus, based on these findings, the ancestral role of Pax6 in regulating opsin expression appears to have switched to a different, more complex, level of control over key retinogenic programs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Valerie A. Wallace, Takahisa Furukawa, Ryoichiro Kageyama, Tom Reh and Meredithe L. Applebury for providing us with the constructs for preparing in situ probes and T. M. Jessell and the Developmental Studies Hybridoma Bank for Isl1 antibody. We are grateful to Peter Gruss for the mouse lines that were established in his laboratory and to Luc St-Onge for the *Pax6*$^{lacZ/lacZ}$ mice, to Connie Cepko for the Chx10-Cre mice and to Leonid Mittelman for confocal images. Research in R.A.-P.'s laboratory is supported by the Israel Science Foundation, German Israeli Foundation, AMN foundation, the Glaucoma Research Foundation, Israeli Ministry of Health, and E. Matilda Ziegler Foundation. T.M. was supported by the DFG Emmy Noether Young Investigator Program. A.C. was supported by NIH grant EY012200 and an Irma T. Hirschl Career Scientist Award.

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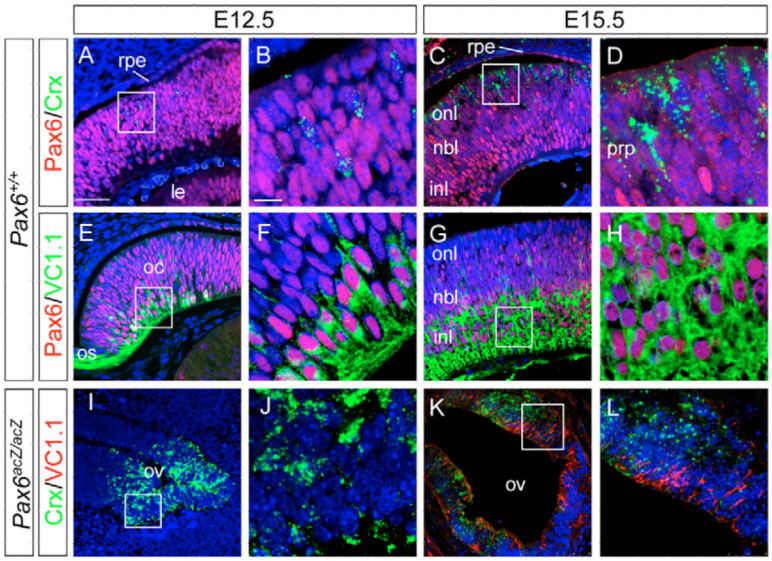

Fig. 1. Differential response to Pax6 loss in subpopulations of *Pax6*$^{lacZ/lacZ}$ OV progenitors

(**A-L**) Crx expression was characterized by fluorescent in situ hybridization (A-D,I-L, green), the distribution of VC1.1 epitope (E-H, green K,L; red) and Pax6 (A-H, red) was monitored by antibody labeling, in the control *Pax6*$^{+/+}$ (A-H) and *Pax6*$^{lacZ/lacZ}$ (I-L) embryos. The insets in A,E,I,C,G,K are enlarged in B,F,J,D,H,L. inl, inner nuclear layer; le, lens; nr, neuroretina; oc, optic cup; os, optic stalk; prp, prospective photoreceptor layer; rpe, retinal pigmented epithelium. Scale bars: in A, 50 μm for A,E,I,C,G,K; in B, 10 μm for B,F,J,D,H,L.

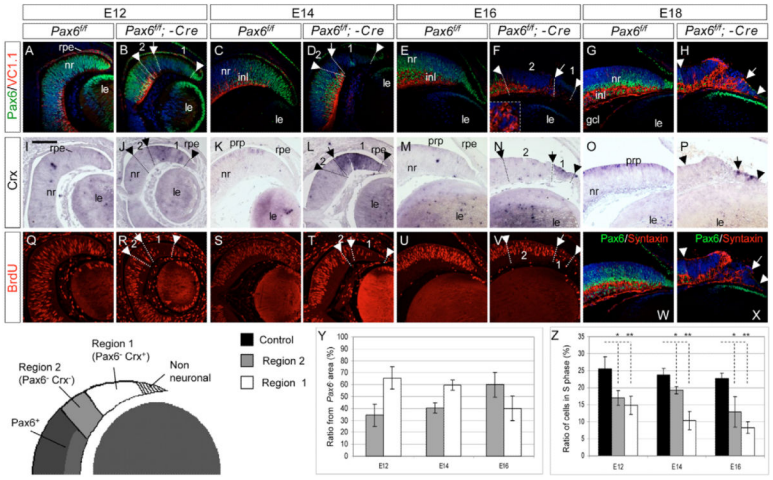

Fig. 2. Pax6 plays a unique role in each of two spatially distinct subsets of RPCs in the *Pax6*$^{flox/flox}$;*α-Cre* OC

The expression of Pax6, VC1.1 (**A-H**; green and red, respectively), Crx (**I-P**), BrdU (**Q-V**) and syntaxin (**W**,**X** red) were characterized on adjacent sections by antibody labeling (A-H,Q-X) or in situ hybridization (I-P) in control (*Pax6*$^{flox/flox}$) and mutant (*Pax6*$^{flox/flox}$;α-*Cre*) retinas in the course of eye development. In the *Pax6*$^{flox/flox}$;α-*Cre* OC, Pax6 was eliminated from the peripheral regions (B,D,F,H; the Pax6-deficient domain is flanked with arrowheads). Two spatially distinct populations of Pax6-deficient RPCs were identified (diagram): the *Pax6*$^{-}$ cells that are located in the OC periphery upregulate Crx, whereas the *Pax6*$^{-}$ cells located towards the central OC do not upregulate Crx. The border between the two Pax6-deficient cell types is indicated with an arrow and a broken line, and their margins are marked with arrowheads (labeled as region 1 or region 2, respectively). (**Y**) The percentage of the Crx-expressing domain (region 1, white bars) and the Crx^{-} domain (region 2, gray bars) relative to the total Pax6-deficient area was calculated for E12, E14, E16 OCs (*n*=4 eyes for all embryonic stages). (**Z**) Significant reduction in the percentage of BrdU^{+} cells was detected for both Pax6-deficient regions at all stages of development (**P<0.005 and *P<0.05 by Student's *t*-test; *n*=4 eyes for all *Pax6*$^{flox/flox}$;α-*Cre* retinas and three eyes for controls). The reduction in the proliferation index was significantly more extensive in region 1 than in region 2 at E14 (P<0.01 by Student's *t*-test). inl, inner nuclear layer; gcl, ganglion cell layer; le, lens; nr, neuroretina; prp, prospective photoreceptor layer; rpe, retinal pigmented epithelium. Scale bar: 100 μm.

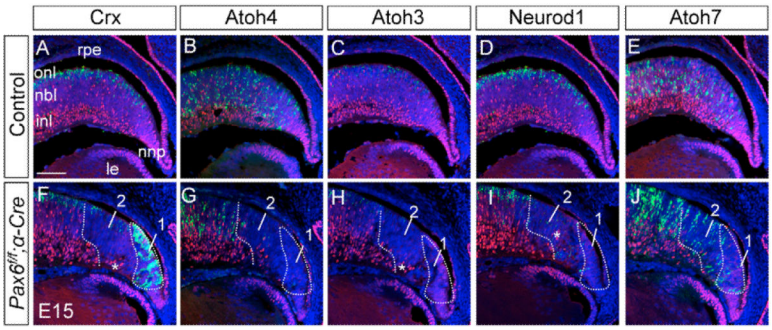

Fig. 3. Altered expression profile of bHLH transcription factors in the two regions of Pax6 mutant RPCs

(**A-J**) At E15, the expression in control (A-E) and *Pax6*$^{flox/flox}$;α-*Cre* (F-J) of Crx (A,F), Atoh4 (B,G), Atoh3 (C,H), Neurod1 (D,I) and Atoh7 (E,J) was characterized on adjacent sections by fluorescent in situ hybridization (green). On the same sections, Pax6 expression was determined by indirect immunofluorescence analysis (red). In the control, Crx was detected in the prospective photoreceptor layer (A), while in the *Pax6*$^{flox/flox}$;α-*Cre*, Crx was upregulated in the peripheral region of the Pax6-deficient OC (F, surrounded by broken lines labeled 1), but was downregulated in the mutated RPCs that are located more centrally (F, surrounded by broken lines labeled 2). inl, inner nuclear layer; le, lens; nbl, neuroblast layer; nnp, non-neuronal progenitors; rpe, retinal pigmented epithelium. Asterisk indicates cells that escaped the recombination. Scale bar: 75 μm.

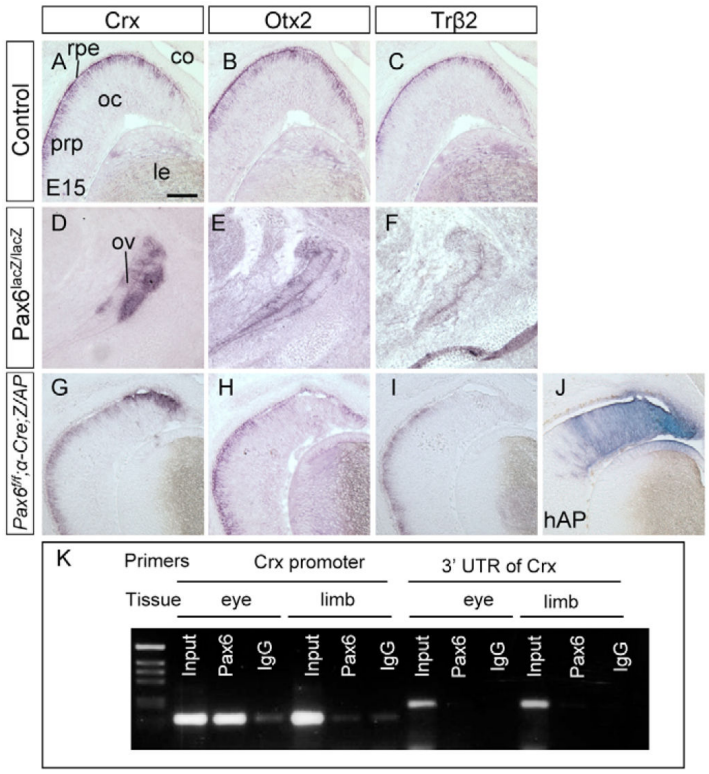

Fig. 4. *Pax6*$^{-}$ *Crx*$^{+}$ RPCs do not complete the photoreceptor-specification program

(**A-I**) The expression pattern of factors involved in photoreceptor differentiation; Crx (A,D,G), Otx2 (B,E,H) and Trβ2 (C,F,I) in control (A-C), *Pax6*$^{lacZ/lacZ}$ (D-F) and *Pax6*$^{flox/flox}$*;a-Cre;Z/AP* (G-I) E15 eyes. (**J**) The region of Pax6 inactivation was determined by detection of human alkaline phosphatase (hAP) expressed from the *Z/AP* reporter (J is adjacent to G-I). (**K**) Chromatin immunoprecipitation (ChIP) was conducted on chromatin from E13 eyes with Pax6 or rabbit IgG (IgG). PCR amplification was carried out with specific primers for detection of the Crx promoter. The Crx 3′ UTR sequence was amplified as a control that does not bind Pax6 in vivo. The same pairs of primers were used for amplification of the chromatin samples prior to immunoprecipitation (input lane). When ChIP was conducted on limb tissue, where Pax6 is not expressed, no amplification of Crx promoter sequences was detected. co, cornea; le, lens; oc, optic cup; ov, optic vesicle; prp, photoreceptor layer; rpe, retinal pigmented epithelium. Scale bar: 100 μm.

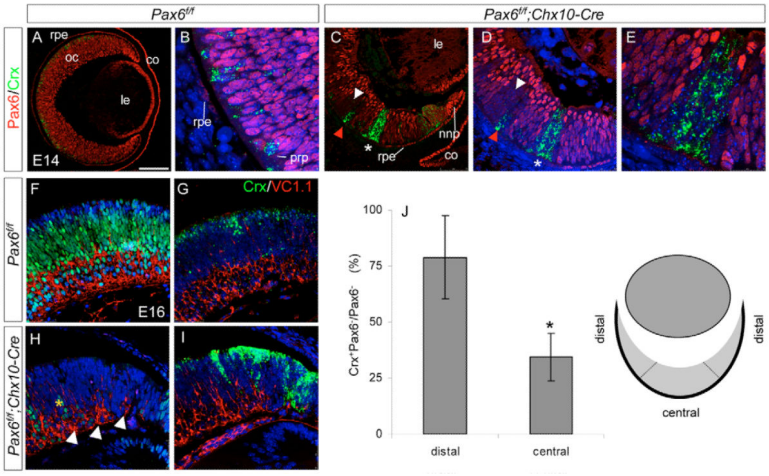

Fig. 5. The misexpression of Crx is a cell-autonomous response to Pax6 loss in RPCs

(**A-E**) Pax6 and Crx were detected on the same sections from control (A,B) and *Pax6*$^{flox/flox}$*;Chx10-Cre* (C-E) E14.5 eyes using indirect immunofluorescence analysis (Pax6, red) or fluorescent in situ hybridization (Crx, green). Only in some of the Pax6-deficient cells was misexpression of Crx detected, whereas other cells were negative for both Pax6 and Crx (white arrowhead in C,D). The *Pax6*$^{-}$*Crx*$^{+}$ cells (D,E) were detected both adjacent to Pax6-expressing cells (white asterisk) or in regions distant from Pax6 expression (red arrowhead). (**F-I**) At E16.5, the expression of VC1.1 (red) was compared with Pax6 protein (F,H green) or Crx transcripts (G,I green) in control (F,G) and *Pax6*$^{flox/flox}$*;Chx10-Cre* embryos (H,I). (**J**) The distribution of *Pax6*$^{-}$*Crx*$^{+}$ cells along the central-peripheral regions of the OC was quantified. A significant difference in the proportion of Crx-expressing cells in the Pax6-deficient areas between central and peripheral OC was identified (**P*<0.001 by Student's *t*-test, *n*=6 eyes). The scheme illustrates the arbitrary division of the OC into central/peripheral regions. co, cornea; le, lens; nnp, non-neuronal progenitors; oc, optic cup; prp, photoreceptor layer; rpe, retinal pigmented epithelium. Scale bar: in A, 200 μm; in B,E, 25 μm; in C, 75 μm; in D,F,G,H,I, 50 μm.

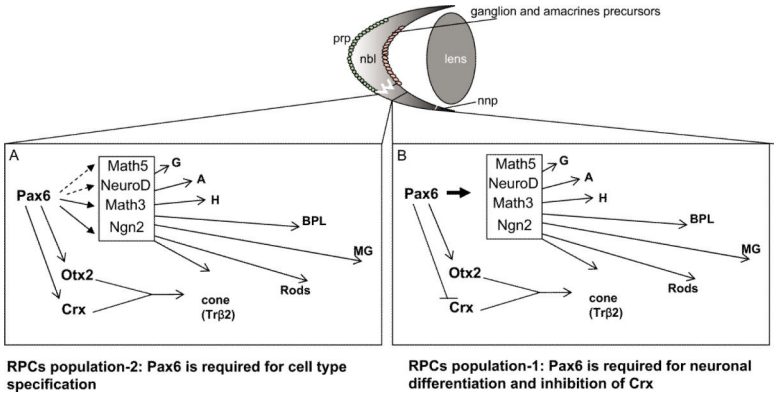

Fig. 6. A model summarizing Pax6 functions in retinal progenitor cells

(**A**) In the central retina, close to the differentiation front (arrows), Pax6 is required for the normal expression profile of transcription factors that play a role in the execution of specific retinal lineages but is dispensable for the completion of neurogenesis. (**B**) In the peripheral RPCs, Pax6 inhibits Crx expression and at the same time is essential for proneural gene expression and completion of the neurogenic program. The involvement of Pax6 in the regulation of Crx and Otx places Pax6 upstream in the transcriptional network that regulates PR specification and differentiation. The requirement for Otx and Crx encompasses the determination and specification of both rod and cone photoreceptors. Later, the terinoids and thyroid hormone (e.g. Trβ2) nuclear receptors function within the cone precursors for the distinction between the M and S cone types (Hennig et al., 2008). nnp, non-neuronal progenitors; nbl, neuroblast layer; prp, photoreceptor precursor layer.