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RESEARCH ARTICLE

Stage-dependent requirement of neuroretinal Pax6 for lens and retina development

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ABSTRACT

The physical contact of optic vesicle with head surface ectoderm is an initial event triggering eye morphogenesis. This interaction leads to lens specification followed by coordinated invagination of the lens placode and optic vesicle, resulting in formation of the lens, retina and retinal pigmented epithelium. Although the role of *Pax6* in early lens development has been well documented, its role in optic vesicle neuroepithelium and early retinal progenitors is poorly understood. Here we show that conditional inactivation of *Pax6* at distinct time points of mouse neuroretina development has a different impact on early eye morphogenesis. When *Pax6* is eliminated in the retina at E10.5 using an *mRx-Cre* transgene, after a sufficient contact between the optic vesicle and surface ectoderm has occurred, the lens develops normally but the pool of retinal progenitor cells gradually fails to expand. Furthermore, a normal differentiation program is not initiated, leading to almost complete disappearance of the retina after birth. By contrast, when *Pax6* was inactivated at the onset of contact between the optic vesicle and surface ectoderm in $Pax6^{Sey/flox}$ embryos, expression of lens-specific genes was not initiated and neither the lens nor the retina formed. Our data show that *Pax6* in the optic vesicle is important not only for proper retina development, but also for lens formation in a non-cell-autonomous manner.

KEY WORDS: Pax6, Retinal progenitor, mRx-Cre, Lens induction

INTRODUCTION

Proper eye development is dependent on the coordinated formation of two main tissues in the eye: the retina and the lens. Vertebrate eye development begins with invagination of the optic vesicle (OV) toward the lens-competent head surface ectoderm (SE). As OV contacts SE, a series of reciprocal inductive signals elicit formation of the lens placode (LP) and subsequent invagination of both LP and OV to form a two-layered optic cup (OC), with retinal pigmented epithelium (RPE) surrounding the retina (reviewed by Fuhrmann, 2010; Chow and Lang, 2001; Ogino et al., 2012). Genetic studies have identified multiple transcription factors and signaling pathways interacting in a complex network orchestrating early eye development (reviewed by Fuhrmann, 2010; Chow and Lang, 2001; Ogino et al., 2012; Xie and Cvekl, 2011). Among the signaling pathways, BMP (Furuta and Hogan, 1998; Rajagopal et al., 2009; Sjödal et al., 2007; Wawersik et al., 1999) and FGF (Faber et al., 2001; Garcia et al., 2011; Gotoh et al., 2004; Pan et al., 2006) were found to be essential for lens induction and coordinated OV-to-OC transition, as severe eye defects are associated with their inactivation.

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At the time the LP is formed, the dorsal region of the OV becomes specified to the retina and is populated with mitotically active retinal progenitor cells (RPCs) (Fuhrmann, 2010; Levine and Green, 2004). Lineage-tracing studies have shown that RPCs are multipotent, with a single progenitor cell competent to give rise to all retinal neuron and glia cell types (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990). The defining feature of RPCs is co-expression of the transcription factors Rx (Rax), Pax6, Lhx2, Six3/Six6, Chx10 (Vsx2) and Hes1, which are expressed prior to activation of the neurogenic program and contribute to the proliferative and retinogenic potential of RPCs (Burmeister et al., 1996; Grindley et al., 1995; Jean et al., 1999; Li et al., 2002; Liu et al., 2010; Marquardt et al., 2001; Mathers et al., 1997; Oliver et al., 1995; Porter et al., 1997; Tomita et al., 1996; Walther and Gruss, 1991). In a defined birth order, RPCs differentiate into seven retinal cell types: retinal ganglion cells, horizontal cells and cone photoreceptors differentiate first, followed by amacrine cells and rod photoreceptors, bipolar cells, and finally Muller glia cells (Young, 1985). As retinogenesis proceeds, RPCs are exposed to the changing environment of extrinsic cues (Cepko, 1999). These, in cooperation with intrinsic factors represented by transcription factors, most prominently of the basic helix-loop-helix (bHLH) and homeodomain class, regulate progenitor proliferation and operate to direct the bias towards particular cell types (Brown et al., 1998; Cepko, 1999; Hatakeyama and Kageyama, 2004; Inoue et al., 2002; Lillien, 1995; Morrow et al., 1999; Perron and Harris, 2000; Tomita et al., 1996).

At the time of neuronal differentiation, a subpopulation of progenitors undergoes a transition from the proliferative stage toward the lineage-restricted neurogenic stage, upon which they withdraw from the cell cycle and take up a neuronal or glial fate. Accordingly, a proper balance between cell cycle exit and re-entry is required to ensure the temporal generation of all retinal cell types (reviewed by Agathocleous and Harris, 2009). It is in cell cycle phase G1 that growth-promoting and growth-inhibiting signals determine whether a progenitor cell will exit or re-enter the cell cycle. In mammalian retina, the KIP proteins $p57^{Kip2}$ (Cdkn1c) and $p27^{Kip1}$ (Cdkn1b) and cyclin D1 have been implicated in direct regulation of progenitor proliferative potential (Das et al., 2009; Dyer and Cepko, 2000; Dyer and Cepko, 2001; Geng et al., 2001; Levine et al., 2000; Levine and Green, 2004), promoting either cell cycle exit or progression. The observation that some neurogenic factors promote both neuronal fate determination and cell cycle withdrawal implies that the processes of cell type specification and cell cycle exit are tightly coupled (Farah et al., 2000; Ochocinska and Hitchcock, 2009). However, the mechanism that orchestrates these complex events remains largely elusive.

The paired and homeodomain transcription factor Pax6 plays a pivotal role in both vertebrate and invertebrate eye development (Kozmik, 2005). Since *Pax6*-deficient ($Pax6^{-/-}$) mice are anophthalmic (Hill et al., 1991; Hogan et al., 1986), with eye development arrested at the OV stage, much attention has been paid

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to elucidation of *Pax6* function in the development of individual ocular structures (reviewed by Shaham et al., 2012). *Pax6* is expressed from very early stages of eye formation, in SE and OV, and later in developing lens, RPE and all mitotically active RPCs of the retina (Walther and Gruss, 1991). Conditional ablation of *Pax6* revealed its autonomous requirement for lens development (Ashery-Padan et al., 2000; Shaham et al., 2009) as well as for later retinal neurogenesis (Marquardt et al., 2001; Oron-Karni et al., 2008). Nevertheless, the autonomous role of *Pax6* in the progenitors of the OV and newly formed OC remains unresolved. Here we employed the Cre-loxP system to conditionally inactivate *Pax6* specifically in retina-committed eye progenitors.

RESULTS

Pax6 deletion in early RPCs results in strongly hypocellular retinae

Pax6 is expressed in both the OV and SE of the developing eye (Walther and Gruss, 1991). In $Pax6^{-/-}$ (*Sey/Sey*) mouse embryos, eye development is arrested at the OV stage and no eyes are formed (Hill et al., 1991; Hogan et al., 1986). Although *Pax6* expression in SE was found to be essential for lens induction (Ashery-Padan et al., 2000), the role of *Pax6* in the OV is less well understood. We used the *mRx-Cre* transgenic mouse line (Klimova et al., 2013) to conditionally inactivate *Pax6* in retina-committed progenitor cells selectively. Expression of *Cre* recombinase in the *mRx-Cre* line is controlled by regulatory sequences of the mouse *Rx* gene (supplementary material Fig. S1B). To monitor *mRx-Cre*, the *ROSA26R* reporter line was employed in which *Cre*-expressing cells can be traced by X-gal staining for *lacZ* expression (β-galactosidase) after Cre-mediated recombination (Soriano, 1999). Consistent with the expression pattern of the endogenous *Rx* gene (Mathers et al., 1997), strong Cre activity was observed in the OVs (Fig. 1A-C) of *mRx-Cre/ROSA26R* embryos at E9.0 and later in the neuroretina, RPE and optic stalk (Fig. 1D-F). Next, *mRx-Cre* mice were crossed to $Pax6^{fl/fl}$ mice to inactivate *Pax6* in the OV. $Pax6^{fl/fl}$/*mRx-Cre* mice were viable and fertile. The eyes of $Pax6^{fl/fl}$/*mRx-Cre* (i.e. *Pax6* loss-of-function) mutants were analyzed for the presence of Pax6 protein by immunohistochemistry (Fig. 1G-J). At E9.5, we observed decreased Pax6 levels in OV neuroepithelium of $Pax6^{fl/fl}$/*mRx-Cre* embryos, whereas Pax6 protein expression was very high in the SE and OV of $Pax6^{fl/fl}$ control embryos (compare Fig. 1G with 1H). One day later, very few $Pax6^{+}$ cells were detected in the neuroretina and optic stalk of $Pax6^{fl/fl}$/*mRx-Cre* (Fig. 1J). Importantly, the Pax6 protein levels remained unchanged in the SE and lens pit upon OV-specific Pax6 elimination (Fig. 1H,J).

The consequences of *Pax6* inactivation in retina-committed progenitor cells of $Pax6^{fl/fl}$/*mRx-Cre* embryos were first investigated at the histological level. The first manifestation of abnormal retina development was observed at E10.5. Already at this stage, the retina was thinner than in wild type (compare Fig. 2A with 2B), suggesting a decreased number of RPCs. We counted DAPI-stained ($DAPI^{+}$) cells per retinal section and found that the number of RPCs was decreased by 44±4.8% (± s.d.) in *Pax6*-deficient retinae (Fig. 2I). Hypocellularity became more obvious at E14.5, when the number of retinal cells reached only 19.7±3.1% of wild-type levels (Fig. 2C,D,I). At E16.5, *Pax6*-deficient retinae became progressively smaller (Fig. 2E-F′) and, whereas the retina in wild-type newborns was properly laminated (Fig. 2G), *Pax6*-deficient retinae reproducibly formed a thin layer around the lens with no sign of lamination (Fig. 2H,H′, red arrowheads). At postnatal stages, the eye was generally smaller with hardly distinguishable retinal cells (data not shown).

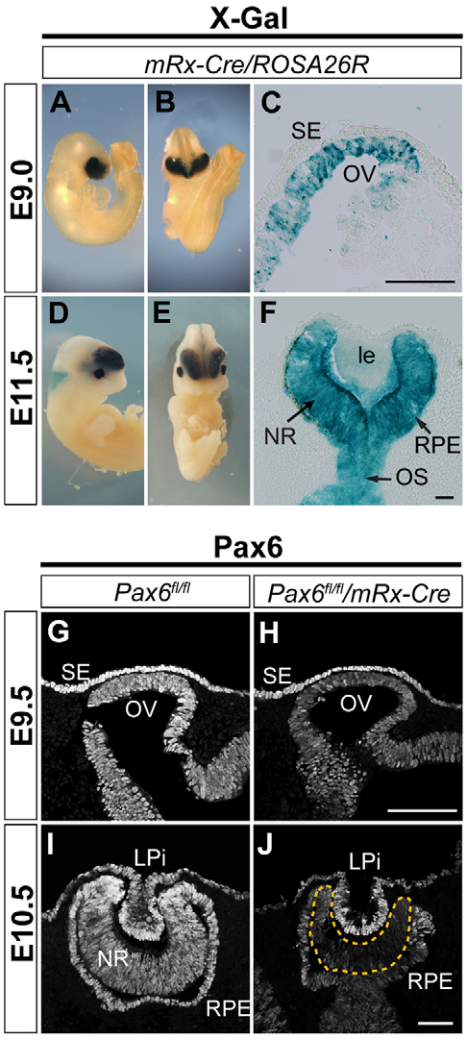

Fig. 1. *mRx-Cre*-mediated *Pax6* inactivation in early retinal progenitors. (A-F) *mRx-Cre* activity was assessed using the *ROSA26R* reporter mouse line. Whole-mounts or coronal sections were stained with X-gal at the indicated stages to show Cre activity in eye primordium. Side (A) and frontal (B) view of the embryo at E9.0. (C) Transverse section showing Cre activity in the optic vesicle. Side (D) and frontal (E) view of the embryo at E11.5. (F) Transverse section showing strong Cre activity in developing neuroretina, RPE and optic stalk. (G-J) Confocal images of transverse sections of wild-type ($Pax6^{fl/fl}$) (G,I) and *Pax6* loss-of-function mutant ($Pax6^{fl/fl}$/*mRx-Cre*) (H,J) eye primordium stained with Pax6 antibody at the indicated stages. The RPC population with inactivated Pax6 at E10.5 is indicated by the dashed line (J). NR, neuroretina; OV, optic vesicle; SE, surface ectoderm; LPi, lens pit; RPE, retinal pigmented epithelium; OS, optic stalk; le, lens. Scale bars: 50 µm.

Altered cell cycle length and disrupted balance between progenitor proliferation and cell cycle exit reduce the RPC population size

Tissue hypocellularity could be due to a decreased proliferation rate, premature cell cycle exit or cell death. To address this issue in *Pax6*-deficient retina, we first analyzed the proliferation potential of RPCs. The proportion of actively proliferating cells ($BrdU^{+}$/$DAPI^{+}$ per retinal section) was counted at several stages of embryonic development using incorporation of BrdU applied 1 hour prior to analysis. Despite the fact that *Pax6*-deficient retinae appeared strongly hypocellular, we did not observe a dramatic difference in the proportion of $BrdU^{+}$ cells relative to all retinal cells between E10.5 and E13.0 (Fig. 3A-D,I). However, at E14.5 a rapid decrease

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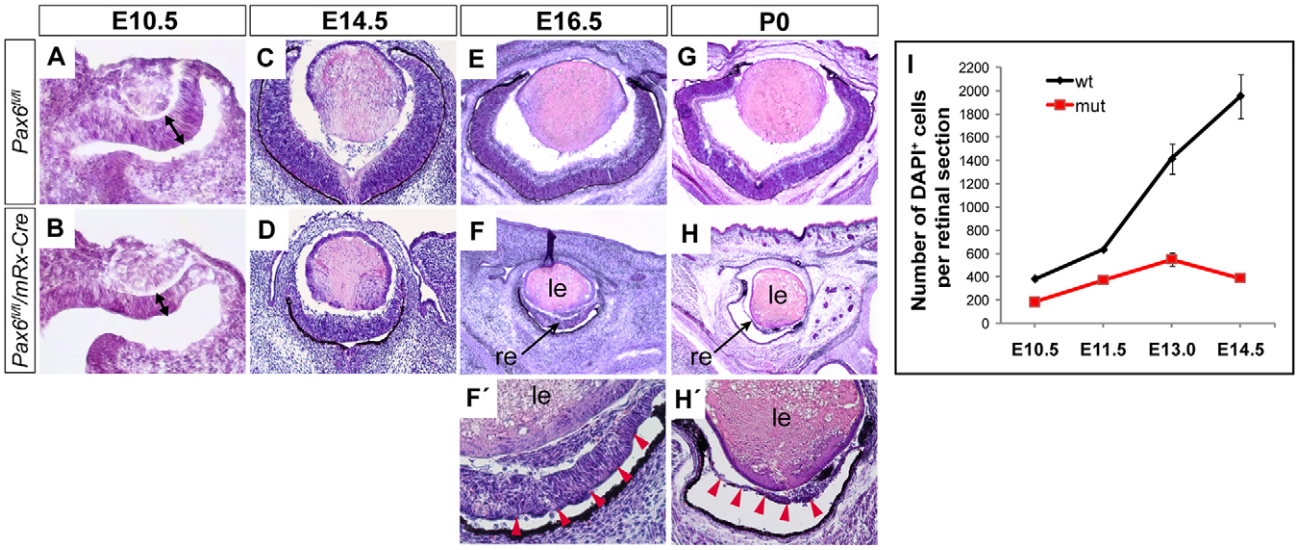

Fig. 2. Morphological consequences of Pax6 inactivation in early retinal progenitors. (A-H′) Coronal sections of wild-type (*Pax6*$^{fl/fl}$) (A,C,E,G) and *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) (B,D,F,H) eye were stained with Hematoxylin-Eosin at the indicated stages. Position of the retina is indicated with arrows for E16.5 and P0 (F,H) and with red arrowheads for magnifications (F′,H′). The double-headed arrows in A and B indicate thickness of the retina. (I) The growth of wild-type (wt) and *Pax6*-deficient (mut) retinae, assessed by the number of DAPI$^+$ cells per retinal section from three different retinae. Error bars indicate s.d. le, lens; re, retina.

of BrdU$^+$ retinal cells was observed (Fig. 3E,F,I). Only 9.1±2% of RPCs were BrdU$^+$, compared with 34±6.2% BrdU$^+$ cells in wild-type retinae (Fig. 3I). Although BrdU$^+$ cells were localized in the neuroblastic layer (NBL) throughout the whole retina of the wild-type OC (Fig. 3E), in *Pax6*-deficient retinae cycling cells were localized mostly in the central part of the retina with almost no BrdU$^+$ cells localized peripherally (Fig. 3F). From E16.5 onwards, almost no dividing BrdU$^+$ cells were found in *Pax6*-deficient retinae (Fig. 3H,I).

Cell death, as a possible cause of hypocellularity, was analyzed on retinal sections between E10.5 and E15.5 using an antibody against cleaved caspase 3 (supplementary material Fig. S2A-J). Although we revealed no significant difference between *Pax6*-deficient and wild-type retinae at E10.5-E12.5, increased cell death was observed at E13.5 and E14.5 (supplementary material Fig. S2K).

Because the BrdU incorporation assay between E10.5 and E13.0 did not indicate perturbed S-phase re-entry and since no increase in cell death was observed in *Pax6*-deficient RPCs of these stages, we next analyzed a potential M-phase arrest and the length of RPC cell cycle as potential contributors to the phenotype. For M-phase arrest, staining for phosphorylated histone H3 (PH3) was performed at E10.5, E11.5 and E14.5 (supplementary material Fig. S3A). No difference in the proportion of PH3$^+$ cells was observed at E10.5 and E11.5. However, a decreased proportion of PH3$^+$ cells was observed in *Pax6*-deficient retinae at E14.5 (supplementary material Fig. S3B). This decrease corresponds to the decreased proliferation rate at E14.5 observed in the BrdU incorporation assay (Fig. 3I). To measure the length of the cell cycle, we used window labeling based on two thymidine analogs that can be differentially detected (Burns and Kuan, 2005; Das et al., 2009). Retinal sections of E11.5 and E13.0 embryos were co-stained for BrdU, EdU and Pcna (Fig. 3J; data not shown) and the lengths of the whole cell cycle (T_c), S phase (T_s) and G1+G2+M phase (T_c–T_s) were determined as previously described (Das et al., 2009). At both stages analyzed, T_c of *Pax6*-deficient RPCs was significantly prolonged compared with wild-type littermates (Fig. 3K). The prolonged T_c was not caused by a lengthened S phase, as T_s was unchanged, but instead T_c–T_s was increased (Fig. 3K). Furthermore, quantification of EdU$^+$ cells relative to Pcna$^+$ proliferating progenitors showed that overall progenitor proliferation was also affected at E11.5 and E13.0 (Fig. 3K′), at stages when no significant difference in the proportion of BrdU$^+$ cells relative to all retinal cells was detected in *Pax6*-deficient retinae (Fig. 3I). This difference can be attributed to neurogenesis in wild-type retinae, which decreases the fraction of BrdU$^+$ cells relative to all (DAPI$^+$) retinal cells. Taken together, these data indicate that Pax6 positively regulates progression through the RPC cell cycle.

During development, decreased proliferation usually coincides with cell cycle exit and subsequent differentiation. To address whether premature cell cycle exit might contribute to the phenotype observed in *Pax6*-deficient retina, we analyzed the expression of cyclin D1 and of the cyclin-dependent kinase inhibitors p27^{Kip1} and p57^{Kip2}, which are known regulators of RPC proliferation. At E14.5, the stage when the decrease in BrdU$^+$ cells was noted (Fig. 3F), we observed a decreased level of cyclin D1 and p27^{Kip1} and elevated expression of p57^{Kip2} (Fig. 3L-S). For cyclin D1 and p57^{Kip2}, expression changes were obvious mostly in the distal parts of retinae (Fig. 3M,Q,S), in a pattern complementary to that of BrdU staining (Fig. 3F). This suggested that peripherally located cells have just left the cell cycle, as they were p57^{Kip2+}, cyclin D1$^-$ and BrdU$^-$. To address the possible gradual peripheral-to-central progression of this phenomenon, we analyzed the retinae of E16.5 embryos. Whereas no p57^{Kip2+} cells were detected in any wild-type retina, p57^{Kip2} was detected in the majority of *Pax6*-deficient retinal cells (Fig. 3V,W). At the same time, cyclin D1 was downregulated in *Pax6*-deficient retinae (compare Fig. 3T with 3U). These results indicate that Pax6 depletion from early retinal progenitors dramatically restricted their proliferation potential and shifted RPCs to forced cell cycle exit.

Pax6-deficient RPCs maintain RPC characteristics but are unable to proceed through the general differentiation program

As *Pax6*-deficient retinae exhibit severe proliferation defects, we next analyzed whether retinal progenitor characteristics were

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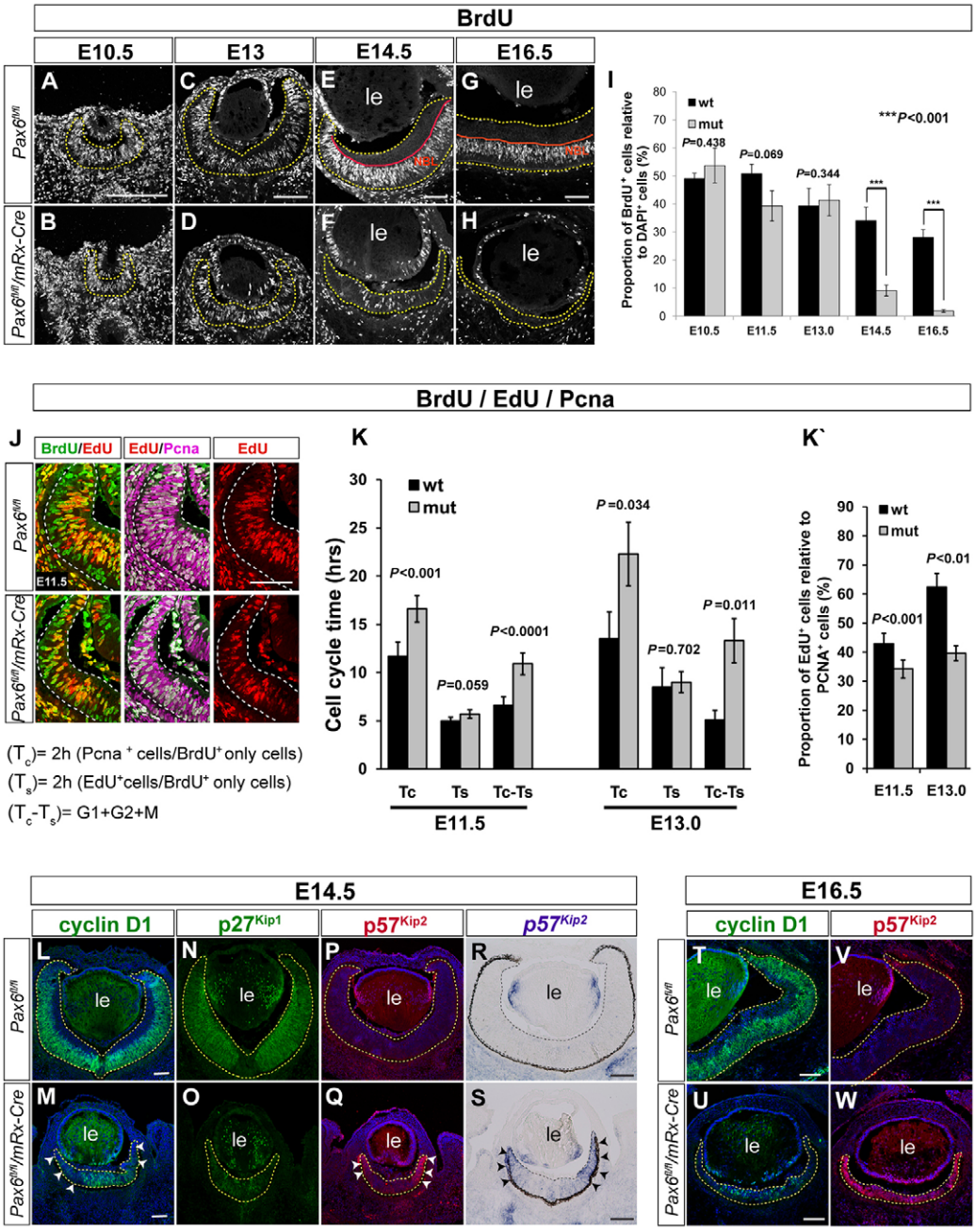

Fig. 3. Cell cycle dynamics is affected in *Pax6*-deficient retinae. (A-I) Following a 1 hour BrdU pulse, eye sections of wild-type (*Pax6*$^{fl/fl}$) and *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) retinae were stained for BrdU incorporation at the indicated stages. Retina is indicated by the dashed line. The solid red line indicates the interface between the differentiating and neuroblastic layers in wild-type retina (E,G). (I) The proportion of BrdU^{+} cells relative to all retinal (DAPI^{+}) cells in wild-type (wt) and *Pax6*-deficient (mut) retinae at the indicated stages. *n*=8 sections from four retinae (E10.5); *n*=4 sections from three retinae (E13, E14.5, E16.5). (J-K′) Cell cycle length determined by BrdU, EdU and Pcna triple staining. Pregnant females were injected 2.5 hours and 0.5 hours prior to dissection with BrdU and EdU, respectively. (J) Single fields of eye sections stained with BrdU, EdU and for Pcna. Beneath are the formulae for cell cycle (T_c), S phase (T_s) and G1+G2+M phase (T_c–T_s) length determination. (K) Quantification of average T_c, T_s and G1+G2+M phase time in wild-type (wt) and *Pax6*-deficient (mut) retinae at E11.5 and E13.0. (K′) The proportions of EdU^{+} cells relative to proliferating progenitor (Pcna^{+}) cells in wild-type (wt) and *Pax6*-deficient (mut) retinae at E11.5 and E13.0. *n*=9 sections from three retinae (E11.5) and *n*=3 sections from three retinae (E13.0) were used. (L-Q) Sections stained with antibodies against cyclin D1, p27^{Kip1} and p57^{Kip2} at E14.5. (R,S) *p57^{kip2}* mRNA expression at E14.5. (T-W) Sections stained with antibodies against cyclin D1 and p57^{Kip2} at E16.5. Areas with changed expression are indicated by arrowheads. Error bars indicate s.d. *P*-values are by Student's *t*-test. NBL, neuroblastic layer; le, lens. Scale bars: 100 µm.

maintained in mutants. We assessed the expression of known markers such as Rx, Lhx2, Chx10, Sox2, Six3, Hes1 and cyclin D1 at E10.5, when the cell number was already decreased. However, the expression of none of these factors was significantly changed (Fig. 4A-G′).

It has been reported that conditional inactivation of *Pax6* in the distal parts of the OC at later stages (E12) of retinogenesis using *α-Cre* leads to exclusive generation of amacrine cells (Marquardt et al., 2001; Oron-Karni et al., 2008). In accordance, generation of amacrine cells was observed in *Pax6*$^{fl/fl}$*/α-Cre* mice, as documented by staining

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for the amacrine cell marker syntaxin and Vc1.1 (HNK-1) immunoreactivity in the distal part of *Pax6*$^{fl/fl}$/*α-Cre* retinae (supplementary material Fig. S1C). We therefore tested the differentiation potential of RPCs in which *Pax6* is absent from E10.5 in the whole retina. We used RNA *in situ* hybridization to analyze the expression of the pro-neural bHLH transcription factors Atoh7, Ngn2 (Neurog2), Neurod1, Mash1 (Ascl1) and Math3 (Neurod4), which that have been shown to initiate the differentiation program and exert bias towards particular cell fates. The expression of these bHLH factors was not initiated at E14.5 (Fig. 4H′-L′), suggesting that not only proliferation but also the retina-specific differentiation program was severely affected in the absence of *Pax6*.

To test whether general neuronal differentiation took place in the mutants, retinae were stained with antibody against the pan-neuronal marker acetylated beta III tubulin (Tuj1, also known as Tubb3), which marks differentiating cells and reveals formation of the differentiated cell layer (DCL) (Sharma and Netland, 2007; Sigulinsky et al., 2008). Although strong Tuj1 staining was observed in the DCL of wild-type retinae at E14.5 and E16.5 (Fig. 4M,N), only a very few Tuj1^{+} cells were detected in *Pax6*-deficient retinae, as DCL was not established at all (Fig. 4M′,N′, arrowheads). In addition, it should be noted that staining for the early amacrine cell-specific factor bHLHb5 (Bhlhe22) and Vc1.1 immunoreactivity at E15.5 revealed no appearance of amacrine cells in the *Pax6*$^{fl/fl}$/*mRx-Cre* retina (supplementary material Fig. S4A-D). Taken together, these results indicate that the overall differentiation potential of *Pax6*-deficient early progenitors is severely compromised.

Pax6-deficient RPCs transiently upregulate *Crx* expression but do not accomplish photoreceptor differentiation

Previous studies have shown that *Pax6* inactivation in RPCs located in the most peripheral region of the OC leads to premature activation of the photoreceptor differentiation program (Oron-Karni et al., 2008). This process is accompanied by upregulation of cone-rod homeobox protein (Crx), which is the earliest expressed photoreceptor determinant (Furukawa et al., 1997; Chen et al., 1997). We therefore analyzed early RPCs in *Pax6*$^{fl/fl}$/*mRx-Cre* for the presence of *Crx* transcripts. Already at E10.5, following Pax6 protein elimination, *Crx* mRNA was detected throughout the invaginating *Pax6*-deficient retina (Fig. 5B), whereas in wild-type controls *Crx* expression was not detectable (Fig. 5A). In E13.5 control retina, Crx protein was immunohistochemically detected in a few photoreceptor-committed cells of the OC (Fig. 5C). Strikingly, in *Pax6*-deficient retina of the same stage, Crx protein was produced by virtually all RPCs (Fig. 5D). At the protein level, elevated *Crx* expression was reproducibly detected between E11.5 and E14.5 in *Pax6*-deficient RPCs (supplementary material Fig. S4F,H).

Crx protein is known to enhance the expression of photoreceptor-specific genes (Hennig et al., 2008; Chen et al., 1997; Mitton et al., 2000; Peng and Chen, 2005); however, Crx alone does not determine the specific photoreceptor cell fate and is supposed to activate transcription in cooperation with other transcription factors (Akagi et al., 2005; Furukawa et al., 1999; Hennig et al., 2008). To further test the ability of Crx to induce photoreceptor differentiation, we analyzed *Pax6*-deficient RPCs for the expression of Otx2, a key regulator of the photoreceptor lineage (Nishida et al., 2003). Otx2 expression failed to be activated, with the exception of a few cells in the most distal part of the OC (Fig. 5F). To rule out a possible delay of Otx2 expression in *Pax6*-deficient retinae, the expression of Otx2 and its photoreceptor-specific target Blimp1 (Prdm1) was analyzed at E15.5. Although the expression of both Otx2 and Blimp1 was apparent in the outer layer, where differentiating photoreceptors reside in wild-type retinae (Fig. 5I,K), their

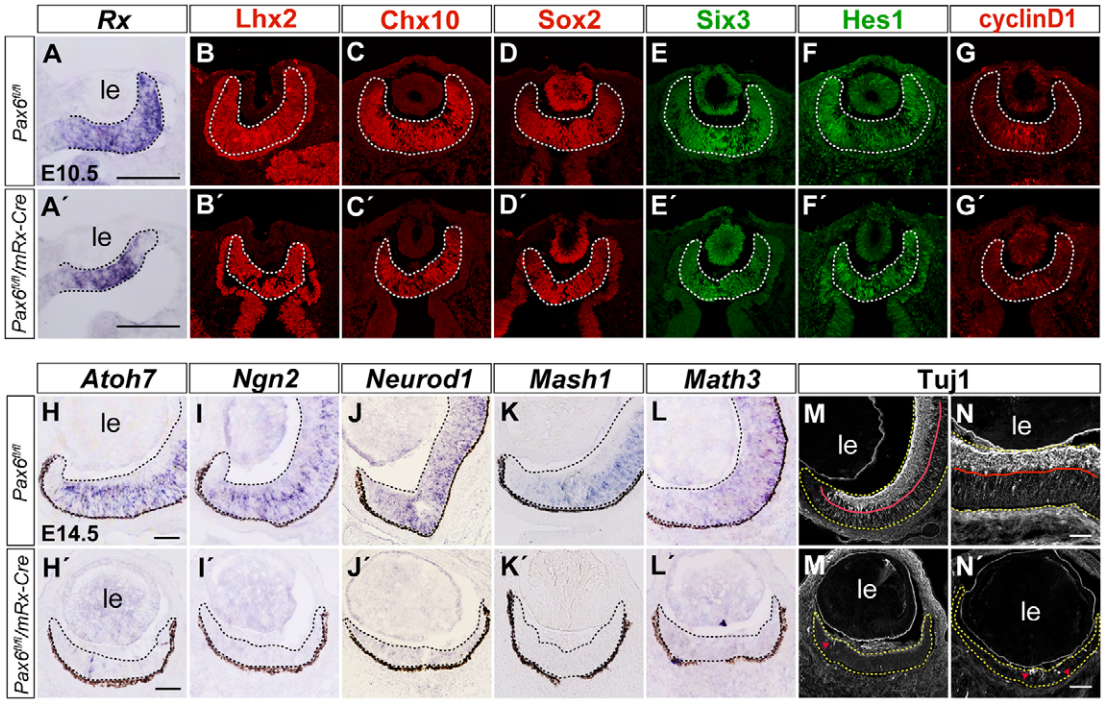

Fig. 4. Retinal progenitor characteristics are maintained but the differentiation program is not initiated in *Pax6*-deficient retina. (A-G′) Expression of the indicated genes (mRNA, A,A′) and proteins (B-G′) involved in RPC pool maintenance analyzed at E10.5. (H-L′) Expression of the indicated genes (mRNA) at E14.5. (M-N′) Sections stained with antibody against the pan-neuronal marker Tuj1 at E14.5 (M,M′) and E16.5 (N,N′). Tuj1^{+} cells in *Pax6*-deficient retina are indicated by red arrowheads. The solid red line indicates the interface between differentiating and neuroblastic cell layers. Retina is indicated with a dashed line. le, lens. Scale bars: 100 µm.

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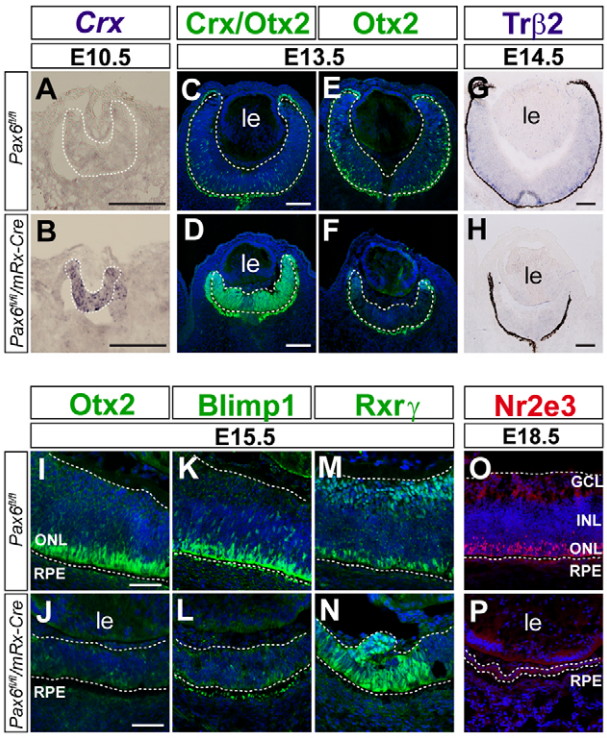

Fig. 5. Expression of photoreceptor-specific factors in *Pax6*-deficient retina. (A,B) *Crx* mRNA expression at E10.5 showing elevated levels in *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) retina (B). (C-F) Confocal images showing Crx (C,D) and Otx2 (E,F) protein expression using Crx/Otx2 and Otx2 only antibody at E13.5. (G,H) *Trβ2* mRNA expression at E14.5. (I-N) Protein expression of Otx2 (I,J), Blimp1 (K,L) and Rxrγ (M,N) at E15.5. (O,P) Nr2e3 protein expression at E18.5. Retina is indicated with dashed line. le, lens; RPE, retinal pigmented epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 100 µm.

expression was not initiated in *Pax6*$^{fl/fl}$*/mRx-Cre* (Fig. 5J,L). We observed elevated levels of the cone-specifying nuclear receptor retinoid X receptor-γ (Rxrγ) (Fig. 5N); however, other factors essential for cone lineage specification, such as thyroid hormone nuclear receptor TRβ2 (Thrb), or for rod specification, such as Nr2e3, were not expressed upon *Pax6* inactivation (Fig. 5H,P). In summary, although Crx expression was efficiently induced by all *Pax6*-deficient RPCs, these cells were not able to fully accomplish the photoreceptor differentiation program, as they failed to express other factors indispensable for this process.

The absence of *Pax6* in the OV during OV-SE tissue interaction leads to lens development arrest

Despite the fact that *Pax6*$^{fl/fl}$*/mRx-Cre* animals did not exhibit severe lens defects in general (Fig. 2), we occasionally observed morphological abnormalities interfering with lens pit/OC formation in these mutants (supplementary material Fig. S5A,B′). Since SE and OV continuous interaction is required for lens and OC morphogenesis, our observation led us to hypothesize that, at the OV stage, OV-expressed *Pax6* might also play a role in lens formation. To test whether earlier removal of Pax6 protein in the OV enhances the lens phenotype, we introduced a single *Sey* allele (*Pax6*$^{Sey/+}$) into the *mRx-Cre/Pax6*^{fl} background. Under these conditions, only one allele of *Pax6* has to be recombined since the second allele is genetically inactive in *Sey*. Although there are several lens phenotypes associated with the inactivation of one *Pax6* allele, including small lens size or its incomplete separation from the overlying ectoderm, the lens is always formed (Hogan et al., 1986). This genetic combination resulted in downregulation of Pax6 protein levels in the OV neuroepithelium of *Pax6*$^{Sey/fl}$*/mRx-Cre* embryos before its transition to the OC (Fig. 6G). Note that this effect can be partially attributed to slightly delayed OC/lens pit morphogenesis in *Pax6*$^{Sey/fl}$ embryos (Fig. 6F). At E11.0, *Pax6*$^{Sey/fl}$*/mRx-Cre* embryos reproducibly exhibited defective lens/OC formation (Fig. 6B,B′). In all embryos analyzed, the lens was completely missing, and the OV either remained arrested or occasionally showed invagination into a small OC-like structure (Fig. 6D; supplementary material Fig. S5D,D′). The arrest of eye development can also be observed when Pax6 protein is eliminated specifically in the OV neuroepithelium already at E9.5 using an earlier deleting founder of *mRx-Cre* (*EmRx-Cre*) (supplementary material Fig. S5E-H′). To further test whether the lens fate was established in *Pax6*$^{Sey/fl}$*/mRx-Cre* embryos, we analyzed expression of the LP-specific transcription factors Pax6, Six3 and Sox2. Although their expression in the SE was maintained (Fig. 6G-G″), indicating that the LP was initially formed, expression of the lens differentiation genes *Foxe3* and *Prox1* was not initiated and the LP did not invaginate to form the lens vesicle (Fig. 6J,J′).

Since *mRx-Cre*-mediated gene manipulation was performed in the OV and not in the SE, a non-cell-autonomous process is likely to regulate lens development in a fashion dependent on OV-expressed *Pax6*. The BMP and FGF signaling pathways are known to play an important role in the lens-inductive ability of OV. Using antibody staining we examined the intracellular mediators of these pathways: phosphorylated Erk proteins (pErk1/2; also known as pMapk3/1) for FGF signaling and phosphorylated Smad proteins (pSmad1/5) for BMP signaling in *Pax6*$^{Sey/fl}$*/mRx-Cre* LP. In both wild type and the *Pax6*$^{Sey/fl}$*/mRx-Cre* mutant, strong pErk1/2 staining was observed in the LP/lens (Fig. 7A), indicating that FGF signaling was unaffected even when lens formation was disrupted. Similarly, we did not observe any significant difference in pSmad1/5 levels in the LP/lens between wild-type and *Pax6*$^{Sey/fl}$*/mRx-Cre* mutant eyes (Fig. 7B). Furthermore, the expression of BMP ligands essential for eye development, *Bmp4* and *Bmp7*, was not abolished, indicating that BMP signaling was not grossly affected upon OV-specific *Pax6* inactivation (Fig. 7C).

Unlike BMP and FGF, activation of the Wnt/β-catenin pathway has been shown to inhibit lens fate since stabilization of β-catenin in lens primordium prevents lens formation (Kreslova et al., 2007; Machon et al., 2010; Smith et al., 2005). Activation of the Wnt/β-catenin pathway results in the accumulation of β-catenin in the nucleus, which allows the TCF/Lef family of transcription factors to activate downstream target genes. We therefore assayed the activity of the canonical Wnt/β-catenin pathway using the *BAT-gal* reporter mouse line carrying *lacZ* driven by multimerized TCF/Lef binding sites (Maretto et al., 2003). Although we observed decreased mRNA expression of known Wnt/β-catenin inhibitors *Sfrp1* and *Sfrp2* in *Pax6*$^{Sey/fl}$*/mRx-Cre* E10.5 eyes (supplementary material Fig. S6A-F), expression of another Wnt inhibitor, *Dkk1*, remained unchanged (supplementary material Fig. S6G-I), and *BAT-gal* reporter mice did not exhibit overall aberrant Wnt activation in the LP (Fig. 7D, arrowheads). Since the *BAT-gal* reporter does not always display sufficient sensitivity (Barolo, 2006), we used staining for Lef1 as a target of Wnt signaling (Planutiene et al., 2011; Wu et al., 2012). As with *BAT-gal* reporter activity, Lef1 was not aberrantly expressed in the LP of *Pax6*$^{Sey/fl}$*/mRx-Cre* mutants (Fig. 7D), indicating no activation of Wnt signaling. By contrast, aberrant Wnt signaling activity was observed in the OV. However, this is unlikely to cause

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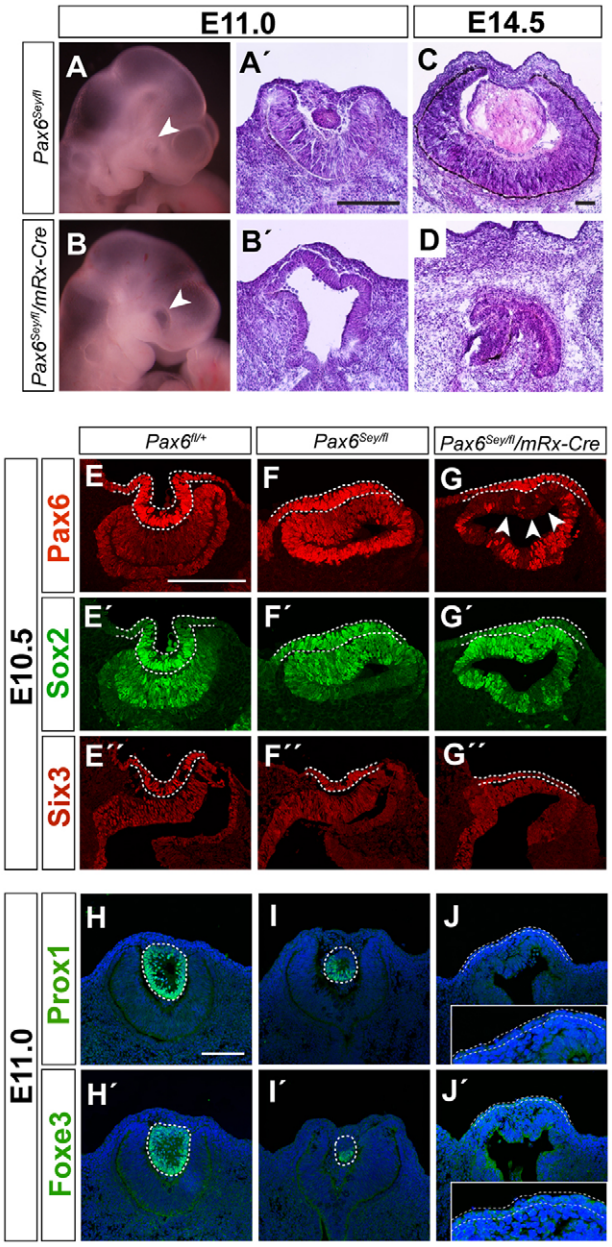

Fig. 6. When Pax6 is eliminated from OV before its transition to the OC then the lens is not formed. (A-D) Morphological consequences of OV-specific *Pax6* inactivation. Heads with the eye region indicated (arrowheads) are shown for control (*Pax6*$^{Sey/fl}$) (A) and mutant (*Pax6*$^{Sey/fl}$*/mRx-Cre*) (B) embryos at E11. Hematoxylin-Eosin-stained sections of E11.5 (A′,B′) and E14.5 (C,D) eyes showing the absence of lens development upon OV-specific *Pax6* elimination. (E-G″) Confocal images showing expression of Pax6, Sox2 and Six3 protein in control (*Pax6*$^{fl/+}$ and *Pax6*$^{Sey/fl}$) and mutant (*Pax6*$^{Sey/fl}$*/mRx-Cre*) E10.5 eyes. Lens pit/lens placode are indicated with a dashed line. Decrease of the Pax6 protein level in OV neuroepithelium of *Pax6*$^{Sey/fl}$*/mRx-Cre* eyes is indicated by arrowheads (G). (H-J′) Expression of Prox1 and Foxe3 protein at E11.0. The lens or the corresponding region is indicated with a dashed line. Insets (J,J′) are magnifications of the SE area. Scale bars: 100 µm.

the lens development arrest, since intentional activation of the Wnt/β-catenin pathway in OV neuroepithelium in *Catnb*$^{lox(ex3)}$ mice (Harada et al., 1999) did not interfere with lens formation (supplementary material Fig. S6K). In summary, our findings suggest that, although OV-expressed *Pax6* is essential for lens formation, this process is independent of FGF, BMP and Wnt/β-catenin signaling.

DISCUSSION

In our study, we focused on *Pax6* function in retina-committed eye progenitors of the OV and early OC. The current model assumes that prospective retina-expressed *Pax6* is dispensable for lens and OC development (Fujiwara et al., 1994). In addition it is known that, at the stage of neurogenesis, *Pax6* loss leads to the exclusive generation of amacrine interneurons, indicating *Pax6* requirement for progenitor cell multipotency (Marquardt et al., 2001). Here we show that, in prospective retina, *Pax6* is required for three crucial processes: lens induction, propagation of early retinal progenitors, and initiation of the retinal differentiation program.

The role of *Pax6* in proliferation of early retinal progenitors

Although *Pax6* has been found to be involved in neural progenitor proliferation, the response to *Pax6* loss seems to be dependent on the developmental context. *Pax6*$^{-/-}$ mutants display an increased number of early cortical progenitors in S phase (Estivill-Torrus et al., 2002; Götz et al., 1998; Warren et al., 1999), but a reduction in proliferation was observed in the diencephalon and optic rudiment (Philips et al., 2005; Warren and Price, 1997). Conditional inactivation of *Pax6* in RPCs of the peripheral OC at E12 results in hypocellularity accompanied by a decreased proportion of cells in S phase (Marquardt et al., 2001; Oron-Karni et al., 2008), indicating a pro-proliferative effect of *Pax6* in RPCs. The molecular mechanism of how *Pax6* regulates cell proliferation remains elusive. One possibility includes the regulated expression or function of general components of the cell cycle machinery either directly by Pax6 or indirectly by some of its targets (Cvekl et al., 1999; Estivill-Torrus et al., 2002; Farah et al., 2000; Holm et al., 2007; Ochocinska and Hitchcock, 2009). Here, we show that after *Pax6* inactivation the cyclin-dependent kinase inhibitor p57^{Kip2} exhibits aberrant accumulation. This process was accompanied by RPC incompetence to re-enter S phase and by downregulation of cyclin D1. Although cyclin D1 is normally expressed by cycling RPCs, promoting progression through the cell cycle, its expression is rapidly downregulated in emerging postmitotic cells (Barton and Levine, 2008; Das et al., 2009; Dyer and Cepko, 2001). By contrast, the expression of *p57*^{Kip2} is upregulated in a small subset of RPCs between E14.5 and E17.5 as they exit the cell cycle (Dyer and Cepko, 2000). Loss-of-function and overexpression studies performed in the mouse retina demonstrated that p57^{Kip2} is both necessary and sufficient to induce cell cycle exit (Dyer and Cepko, 2000). Thus, the pro-proliferative effect of *Pax6* in the retina might be mediated, at least in part, by the inhibition of premature cell cycle exit through regulation of p57^{Kip2} protein levels. As we also observed upregulation of *p57*^{Kip2} mRNA, the negative control appears to occur at the transcriptional level. The mechanism by which p57^{Kip2} mediates cell cycle exit in *Pax6*-deficient RPCs might include blocking of phosphorylation of the retinoblastoma protein (reviewed by Sherr and Roberts, 1995).

It is worth noting that, before *Pax6*-deficient RPCs exit the cell cycle, a p57^{Kip2}/cyclin D1-independent mechanism regulates the proliferation rate. The cell cycle length of the *Pax6*-deficient RPC population is significantly increased relative to that of the wild-type RPC population at E11.5 and E13. In contrast to *Pax6*-deficient cortical progenitors manifesting prolonged S phase (Estivill-Torrus et al., 2002), the cumulative time spent in the G1, G2 and M phases was increased in *Pax6*-deficient RPCs, indicating Pax6 function in these phases of the cell cycle.

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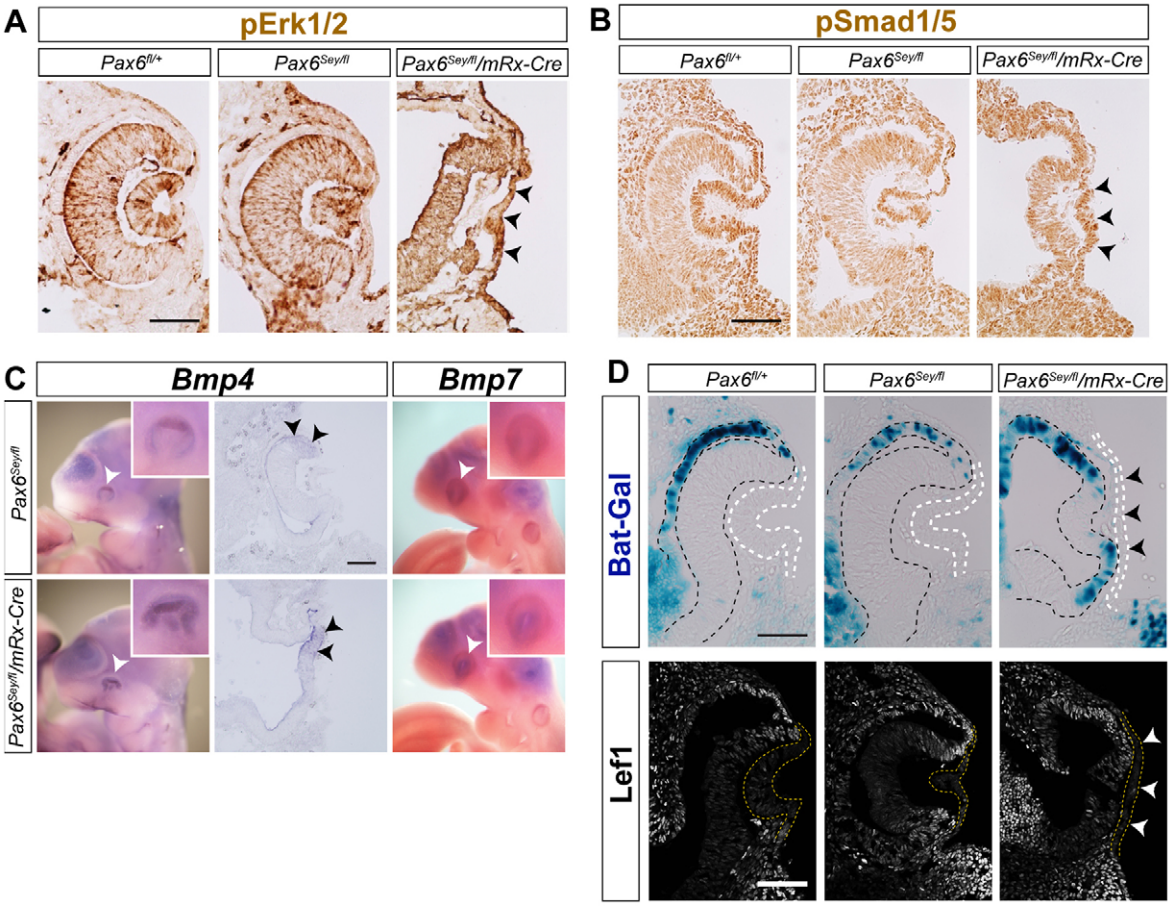

Fig. 7. The *Pax6*-dependent lens-inductive ability of OV neuroepithelium is not dependent on FGF, BMP or Wnt/β-catenin signaling. (A) pErk1/2 detection in control (*Pax6*$^{fl/+}$ and *Pax6*$^{Sey/fl}$) and mutant (*Pax6*$^{Sey/fl}$*/mRx-Cre*) E11.0 eyes of littermate embryos. (B) pSmad1/5 detection in E10.5 eyes of littermate embryos. (C) *Bmp4* (left) and *Bmp7* mRNA expression on whole-mount littermate embryos. Insets are magnifications of the eye region (white arrowheads). The right-hand *Bmp4* panel shows coronal sections of the same samples (black arrowheads indicate the area with *Bmp4* expression). (D) Activity of Wnt/β-catenin signaling at E10.5 as assessed using a BAT-gal reporter mouse (top) and expression of the Wnt/β-catenin target Lef1 (bottom). Arrowheads in *Pax6* mutants indicate the position of the region corresponding to lens placode (A,B,D). Dashed lines (D) indicate retina and RPE (black) and area corresponding to developing lens (white and yellow). Scale bars: 100 µm.

The role of *Pax6* in differentiation into multiple retinal cell types

Once the OV starts to invaginate to form the OC, the population of RPCs is established. Previous studies have indicated that some retinal progenitor characteristics are maintained in the arrested OV rudiment of germline *Pax6*$^{-/-}$ embryos (Bäumer et al., 2003; Bernier et al., 2001). The *mRx-Cre* line allows inactivation of *Pax6* precisely at the time when the RPC population is being established and before the differentiation program has been initiated (at E10). Our analysis shows that *Pax6* is absolutely essential for the generation of all retinal cell types, since no sign of general neuronal differentiation was observed upon *Pax6* inactivation, pointing out a specific *Pax6* role in the maintenance of RPC multipotency. This can be explained by the ability of Pax6 to activate the expression of proneurogenic bHLH factors, including Atoh7, Mash1, Math3, Ngn2 and Neurod1 (Hatakeyama and Kageyama, 2004; Marquardt et al., 2001; Oron-Karni et al., 2008; Riesenberg et al., 2009) (this study).

Our observation that Pax6 is indispensable for neuronal differentiation in the retina is seemingly inconsistent with previous studies. Marquardt and colleagues (Marquardt et al., 2001) showed that inactivation of *Pax6* at the OC stage using *α-Cre* leads to the exclusive generation of amacrine interneurons. Further detailed analysis revealed two populations of RPCs that differentially responded to *Pax6* loss: whereas progenitors located more centrally in the OC adopted amacrine cell fate, those located peripherally activated expression of *Crx* (Oron-Karni et al., 2008). Nevertheless, our data show that *Pax6* is also indispensable for amacrine cell genesis, as Neurod1, Math3, Atoh7 and other amacrine cell-specific factors are not expressed in the absence of *Pax6*. This difference can be attributed to the timing of Pax6 inactivation. When using *mRx-Cre*, Pax6 is completely eliminated before the differentiation program is initiated (E10) (this study); for *α-Cre* (Marquardt et al., 2001; Oron-Karni et al., 2008), Pax6 is eliminated 2 days later (E12) (Riesenberg et al., 2009) (our observation). At E12 the differentiation program has already been initiated, as some proneurogenic factors, including Neurod1 and Atoh7, are expressed (reviewed by Hatakeyama and Kageyama, 2004). The amacrine cell genesis is likely to be the result of biphasic inactivation of *Pax6* by *α-Cre* with respect to the onset of neurogenesis. Since progenitors located in the central OC differentiate earlier, the presence of two populations of RPCs in the OC of *α-Cre/Pax6*$^{fl/fl}$ conditional mutants, with the amacrine cell population located more centrally, then apparently reflects the different degree of neuronal differentiation along the central-to-peripheral axis (Oron-Karni et al., 2008).

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The role of *Pax6* in the lens-inductive ability of the OV

In $Pax6^{-/-}$ embryos eye development is arrested at the OV stage and neither lens nor OC is formed (Grindley et al., 1995; Hill et al., 1991; Hogan et al., 1986). Since *Pax6* is expressed in both SE and OV (Walther and Gruss, 1991), it was not clear which component is the source of the defect. Several studies indicated that SE-expressed *Pax6* might be responsible (Collinson et al., 2000; Fujiwara et al., 1994; Grindley et al., 1995; Quinn et al., 1996), leading to a general acceptance of the notion that *Pax6* activity in the OV is, by and large, not required for lens formation (reviewed by Ashery-Padan and Gruss, 2001; Lang, 2004; Mathers and Jamrich, 2000; Ogino and Yasuda, 2000). However, such a conclusion has not been tested genetically. Experiments in which anti-*Pax6* morpholinos were electroporated into chick embryo OV indicated that OV-expressed *Pax6* might play an essential role in retina and lens formation as well (Canto-Soler and Adler, 2006). Conditional inactivation of *Pax6* in the SE revealed that SE-expressed *Pax6* is autonomously required for LP/lens but not retina formation (Ashery-Padan et al., 2000). In this study we present evidence that early expression of *Pax6* in the OV is indispensable for the development of both tissue components: cell-autonomously for OC/retina development and non-cell-autonomously for lens formation. In OV *Pax6* mutants, eye development was arrested at the OV stage in a manner morphologically reminiscent of the $Pax6^{-/-}$ (*Sey*) phenotype. Thus, in $Pax6^{-/-}$ embryos, the defect in eye formation is apparently attributable to *Pax6* function in both OV and SE (this study) (Canto-Soler and Adler, 2006), in sharp contrast to the current, prevailing view (Ashery-Padan and Gruss, 2001; Ogino and Yasuda, 2000). Interestingly, *Pax6* is required for lens formation only before the OV-to-OC transition. Once the lens pit starts to emerge from the LP, lens development is no longer dependent on OC-expressed *Pax6*. This accords with the idea that lens development becomes independent of OV/OC when the lens has reached a certain developmental stage (Adler and Canto-Soler, 2007; Lang, 2004).

How *Pax6* regulates the ability of OV to induce lens formation remains elusive. It has been demonstrated that lens formation is dependent on the deposition of molecules of the extracellular matrix between the LP and OV, and that this process is dependent on *Pax6* expression (Huang et al., 2011). There is good evidence that signaling from the OV is essential for the activation of lens-specific expression in the SE and subsequent lens formation (Faber et al., 2001; Furuta and Hogan, 1998; Kamachi et al., 1998; Wawersik et al., 1999; Yun et al., 2009). We have not been able to detect conspicuous changes in components of the BMP, FGF and Wnt/β-catenin pathways. It remains possible that the causal changes in $Pax6^{Sey/fl}$/*mRx-Cre* are transient or too subtle to be detected in our analysis. Alternatively, additional, as yet ill-defined signals might be involved in lens induction. The identification of further molecules acting downstream of *Pax6* in the OV might help uncover the molecular details of this process.

MATERIALS AND METHODS

Mouse lines

A mouse with floxed *Pax6* alleles ($Pax6^{fl/fl}$) was generated by homologous recombination in embryonic stem cells, with loxP sites flanking exons 3 and 6 (supplementary material Fig. S1A). *ROSA26R* (Soriano, 1999), *ROSA26R-EYFP* [*R-EYFP* (Soriano, 1999; Srinivas et al., 2001)], Sey^{1Neu} (Hill et al., 1991), *BAT-gal* reporter line (Maretto et al., 2003), $Catnb^{lox(ex3)}$ (Harada et al., 1999) and *mRx-Cre* (Klimova et al., 2013) (supplementary material Fig. S1B) mice have been described previously.

Tissue collections and histology

Mouse embryos were harvested from timed pregnant females. The morning of vaginal plug was considered embryonic day (E) 0.5. Embryos were fixed in 4% (w/v) paraformaldehyde (PFA), washed with PBS, cryopreserved in 30% (w/w) sucrose, frozen in OCT (Tissue Tek, Sekura Finetek) and sectioned.

Immunohistochemistry, *in situ* hybridization and β-galactosidase assay

Cryosections for immunohistochemistry were permeabilized with PBT (PBS with 0.1% Tween 20), blocked with 10% BSA in PBT and incubated with primary antibody overnight at 4°C. Primary antibodies are listed in supplementary material Table S1. Sections were washed with PBT, incubated with secondary antibody (Molecular Probes) coupled to Alexa fluorophore, counterstained with DAPI and mounted in Mowiol (Sigma). To detect phosphorylated Erk (pErk), the TSA Indirect Tyramide Signal Amplification Kit (Perkin Elmer) was used. To detect phosphorylated Smad1/5 (pSmad1/5), the Vectastain ABC Kit (Vector Labs) was used. Antisense mRNA probes for *in situ* hybridization were synthesized using RNA polymerase and digoxigenin-labeled nucleotides (Roche) following the manufacturer's instructions (for a list of probes see supplementary material Table S1). RNA *in situ* hybridization and X-gal staining were carried out as previously described (Fujimura et al., 2009).

BrdU incorporation assay and measurement of cell cycle phase length

For analysis of all proliferation markers, a minimum of three animals from two individual litters were used. Wild-type littermates were used as control. To determine the proportion of actively proliferating RPCs, timed pregnant females were injected 1 hour prior to dissection with BrdU (0.1 mg/g body weight). Embryos were fixed with 4% PFA, cryopreserved in 30% sucrose, embedded in OCT and sectioned. Antigen retrieval was performed by microwave heating in 10 mM sodium citrate (pH 6.5) followed by incubation in 2 M HCl and neutralization (0.1 M borate buffer pH 8.3). Sections were blocked in 10% BSA and incubated overnight at 4°C with anti-BrdU antibody (Abcam, ab6326; 1:100). The cell proliferation rate was always calculated from at least two central sections per individual eye as the ratio of $BrdU^+$ cells versus $DAPI^+$ cells and statistical significance analyzed by Student's *t*-test.

To determine the cell cycle rate at E11.5 and E13, two thymidine analogs were used as previously described (Das et al., 2009). Pregnant females were injected 2.5 hours and 0.5 hours prior to dissection with BrdU and EdU (5-ethynyl-2′-deoxyuridine), respectively. BrdU was detected using anti-BrdU antibody, EdU using the Click-it Reaction (Molecular Probes), and Pcna staining was used to identify all cycling RPCs. The length of the cell cycle (T_c) and of S phase (T_s) in hours (h) was determined by: T_c=2h($Pcna^+$ cells/$BrdU^+$ only cells); T_s=2h(EdU^+ cells/$BrdU^+$ only cells). The combined length of G1, G2 and M phase was calculated as: T_c–T_s. Cell counts were performed from a single field of central sections of the eye (as depicted in Fig. 3J). Three fields per individual eye were counted. T_c and T_s for individual fields were determined and analyzed by Student's *t*-test.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.K. and Z.K. designed experiments, analyzed data and wrote the manuscript. L.K. performed experiments.

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Supplementary material

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