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The role of Pax6 transcription factor in mouse eye development

Role transkripčního faktoru Pax6 ve vývoji myšího oka

Ph.D. Thesis

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STATEMENT

This is to declare that I did not use this Ph.D. Thesis to acquire another academic degree. Further, I worked on the Ph.D. Thesis independently, under the supervision of Dr. Zbyněk Kozmík.

In Prague, 17. 3. 2016 …….

Mgr. Lucie Žílová

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ABSTRACT

The formation of the eye is a multistep process of complex morphogenetic events. It begins with the formation of the optic vesicle (OV) and its subsequent interaction with the overlying head surface ectoderm (SE). Reciprocal interaction between OV and SE evoke lens placode formation within the SE followed by coordinated invagination of both, the lens placode and OV. These events result in formation of lens, retina and retinal pigmented epithelium (RPE) with lens originating from the SE and retina/RPE originating from the OV. Early after the retinal domain is established, retinal progenitor cells start to differentiate in seven retinal cell types that are further stratified in the structure of the retina. The transcription factor Pax6 plays a pivotal role in eye formation in various animal species. In mammals, it is expressed from very early stages of eye development in OV and SE. As *Pax6*$^{-/-}$ mice are anopthalamic, with eye development arrested at OV/SE stage, much attention has been paid to elucidate the Pax6 function in different eye structures. However, whether and/or how Pax6 regulates the early signaling events leading to eye formation as well as the mechanism by which Pax6 regulates the differentiation of all retinal cell types is still only poorly understood.

Using the mouse as a model, we have found that Pax6 is required very early in eye development, to repress Wnt/β-catenin signaling pathway in the lens-forming surface ectoderm to allow the lens placode to be specified and OV and SE to invaginate and form the lens and the retina. This is mediated by the ability of Pax6 to directly activate the expression of inhibitors of Wnt/β-catenin signaling pathway in the presumptive lens ectoderm. To analyze Pax6 role specifically in OV compartment and its derivates, we generated a novel OV-specific Cre-expressing mouse line, *mRx-Cre*. Inactivation of *Pax6* selectively in the OV abolished formation of lens and retina, indicating that at the OV/SE stage, Pax6 expression is required not only in SE but also in the OV to ensure the lens-inductive ability of the OV. This indicates that coordinated action of Pax6 in both, SE and OV is crucial for the initiation of eye formation. Our study also showed that once the OV invaginates to form the retinal domain and retinal progenitor cells are specified, Pax6 is required for retinal progenitor cell proliferation, controlling the balance of cell cycle re-entry and preventing premature cell cycle exit. Beside its role in proliferation, our results also showed that Pax6 plays the crucial role in the initiation of a general retinal differentiation process as no retinal cell types are differentiated in *Pax6*-deficient retinae. Finally, we have focused on Pax6-regulated genes in retinal cell type differentiation. We identified two transcription factors of the Onecut transcription factor family, Onecut1 and Onecut2, to operate downstream of Pax6 to ensure the development of one particular retinal cell type, horizontal cells.

Alltogether, our data show that Pax6 regulates a various processes during eye development, starting from eye induction to proper differentiation of particular retinal cell types.

ABSTRAKT

Vývoj oka je komplexní proces zahrnující řadu dílčích morfologických změn. Na jeho počátku je vznik optického váčku a jeho následná interakce s hlavovým povrchovým ektodermem, která vede k přeměně části ektodermu na čočkovou ploténku. Čočková ploténka a optický váček následně koordinovaně invaginují za vzniku čočky, sítnice a sítnicového pigmentového epitelu. Zatímco povrchový ektoderm dává vznik čočce, optický váček dává vznik sítnici a pigmentovému epitelu. Sítnicové progenitorové buňky se krátce poté začnou postupně diferencovat do sedmi buněčných typů, které následně vytvářejí strukturu sítnice dospělého oka. U mnoha živočišných druhů hraje během vývoje oka klíčovou roli transkripční faktor Pax6. U savců je protein Pax6 produkován již v časných strukturách vyvíjejícího se oka, v optickém váčku a povrchovém ektodermu. U myší s mutací v genu *Pax6* (*Pax6*$^{-/-}$) dochází k zastavení vývoje oka ve stádiu kontaktu optického váčku a povrchového ektodermu a čočka ani sítnice se dále nevyvíjí. Z tohoto důvodu byla role transkripčního faktoru Pax6 velmi intenzivně studována. Nicméně způsob, jakým Pax6 reguluje signalizaci vedoucí ke spuštění vývoje oka, je jen málo prostudovaný. Stejně tak málo známý je i mechanismus, kterým Pax6 řídí diferenciaci jednotlivých sítnicových buněčných typů.

Za použití myši jako experimentálního modelu jsme zjistili, že ve velmi časných stádiích vývoje oka, protein Pax6 zprostředkovává inhibici signalizační dráhy Wnt/β-catenin v části hlavového povrchového ektodermu, ze které vzniká čočka. Tato inhibice je nezbytná pro vytvoření čočkové ploténky a následný vznik čočky a sítnice, a je dána schopností faktoru Pax6 aktivovat produkci několika známých inhibitorů Wnt/β-catenin signální dráhy v povrchovém ektodermu. Abychom mohli dále analyzovat úlohu faktoru Pax6 ve vývoji optického váčku, vytvořili jsme novou transgenní myší linii *mRx-Cre,* produkující rekombinázu Cre a umožňující inaktivaci genu specificky v optickém váčku. Za použití této linie jsme zjistili, že produkce proteinu Pax6 je nezbytná nejen v povrchovém ektodermu, ale také v optickém váčku, k zajištění správného vývoje čočky a sítnice. Když byla totiž produkce faktoru Pax6 v optickém váčku narušena, čočka ani sítnice se nevyvinula. Toto zjištění naznačuje, že pro správný vývoj oka je nezbytná koordinovaná aktivita faktoru Pax6 jak v povrchovém ektodermu, tak v optickém váčku. Naše studie také ukázala, že jakmile se optický váček jednou invaginuje a dá vzniknout sítnici, Pax6 dále řídí schopnost sítnicových progenitorových buněk opakovně se dělit a zabraňuje předčasnému ukončení tohoto procesu. Kromě role v dělení, Pax6 hraje také klíčovou úlohu v diferenciaci všech sítnicových buněčných typů. Pokud je Pax6 inaktivován, sítnicové progenitorové buňky zcela ztrácí schopnost diferencovat v jakékoliv známé sítnicové buněčné typy. V rámci studia role faktoru Pax6 při diferenciaci jsme se dále zaměřili na hledání kandidátních genů regulovaných faktorem Pax6, které by mohly hrát úlohu při řízení tohoto procesu. Naše studie identifikovala dva transkripční faktory, Onecut1 a Onecut2, jejichž inaktivace ve vyvíjející se sítnici odhalila, že tyto faktory jsou zodpovědné za vznik jednoho konkrétního buněčného typu, horizontálních buněk.

Závěrem lze říci, že naše studie ukázaly komplexní úlohu faktoru Pax6 v několika klíčových procesech vývoje oka zahrnujících proces vzniku čočky, dělení progenitorovýh buněk sítnice a jejich následnou diferenciaci.

CONTENT

1. PREFACE

The survival of humans likewise other living organisms, is strictly dependent on their ability to interact with the environment. This includes signal perception and its precise processing using sensory organs and central nervous system. As humans, we have five senses available from which the vision is of the most importance. This fact makes the eye one of the most precious sensory organ of our body. The eye formation is a complex process initiated very early in prenatal development and its disturbance can lead to severe eye abnormalities and loss of vision. Multiple congenital eye disorders including anophthalamia, micropthalmia, aniridia, colaboma, and retinal dysplasia stem from disruption of embryonic eye development. These are caused mainly by the malfunction of factors involved in regulation of eye development. One such factor is the transcription factor Pax6. Whereas heterozygous mutation in the *Pax6* causes aniridia and Peter`s anomaly in humans and "Small eye" phenotype in mice and rats, homozygous mutation causes anophthalamia. Uncovering the molecular processes during eye development, Pax6 is involved in, can help us to understand the origin of abnormalities connected to Pax6 malfunction.

This Ph.D. Thesis is focused on Pax6 role in mammalian eye development studied in mouse as a model. Thesis includes four publications, each dealing with different aspect of eye development:

1) **Lens morphogenesis is dependent on Pax6-mediated inhibition of the canonical Wnt/beta-catenin signaling in the lens surface ectoderm.**
   Machon O, Kreslova J, Ruzickova J, Vacik T, Klimova L, Fujimura N, Lachova J, Kozmik Z.
   Genesis. 2010 Feb;48(2):86-95.

2) **Generation of mRx-Cre transgenic mouse line for efficient conditional gene deletion in early retinal progenitors.**
   Klimova L, Lachova J, Machon O, Sedlacek R, Kozmik Z.
   PLoS One. 2013 May 7;8(5):e63029.

3) **Stage-dependent requirement of neuroretinal Pax6 for lens and retina development.**
   Klimova L, Kozmik Z.
   Development. 2014 Mar;141(6):1292-302.

4) **Onecut1 and Onecut2 transcription factors operate downstream of Pax6 to regulate horizontal cell development.**
   Klimova L, Antosova B, Kuzelova A, Strnad H, Kozmik Z.
   Dev Biol. 2015 Jun 1;402(1):48-60.

2. LIST OF ABBREVIATIONS

aa	amino acid
BMP	Bone Morphogenic Protein
bHLH	basic Helix-Loop-Helix
Cdk	Cyclin-dependent kinase
CNS	Central Nervous System
DNA	DeoxyriboNucleic Acid
EFTF	Eye Field Transcription Factor
E	Embryonic day
FGF	Fibroblast Growth Factor
G	Gap
GCL	Ganglion Cell Layer
HD	HomeoDomain
Hipk	Homeodomain Interacting Protein Kinase
HTH	Helix-Turn-Helix
ICN	IntraCellular domain of Notch
INL	Inner Nuclear Layer
LP	Lens Placode
MAPK	Mitogen-Activated Protein Kinase
M	Mitosis
OC	Optic Cup
ONL	Outer Nuclear Layer
OV	Optic Vesicle
P	Postnatal day
PD	Paired Domain
pNR	presumptive NeuroRetina
pOS	presumptive Optic Stalk
pRPE	presumptive Retinal Pigmented Epithelium
pRb	Retinoblastoma protein
PST	Prolin-Serin-Threonin
P6CON	Pax6 CONsensus binding site
RA	Retinoic Acid
Raldh	Retinaldehyd dehydrogenase
RBP-J	Recombining Binding Protein suppressor of hairless
RGC	Retinal Ganglion Cell
RPC	Retinal Progenitor Cell
RPE	Retinal Pigmented Epithelium
S	Synthesis
SE	Surface Ectoderm
Shh	Sonic hedgehog
TGF	Transforming Growth Factor

3. LITERATURE OVERVIEW

3.1 Overview of mammalian eye development

Proper eye development is strictly dependent on the coordinated formation of two main tissues of the eye: the retina and the lens. In vertebrates, eye development is initiated during early neurulation, when the eye field is specified within the patch of the head neural ectoderm to become the neural part of the eye. Soon after that, optic vesicles (OVs) evaginate from the wall of the rostral diencephalic wall toward the lens-competent head surface ectoderm (SE) (Figure 1a) (at embryonic day [E] 9). As the OV and SE come into the contact, series of reciprocal signals induce formation of the lens placode (LP) (at E9.5), a thickening of the SE that contacts the OV (Figure 1a). Once LP is formed, both LP and OV invaginate to form the lens vesicle and two-layered optic cup (OC) respectively (Figure 1b) (at E10). Outer layer of the OC gives rise to the retinal pigmented epithelium (RPE) while inner layer become specified to retina populated with mitotically active retinal progenitor cells (RPCs) (reviewed by Fuhrmann, 2010; Chow and Lang, 2001; Ogino et al., 2012) (Figure 1c). Margin between two layers of the OC differentiates into peripheral structures of the eye, the iris epithelium and ciliary body. The lens vesicle eventually separates from the SE and differentiates into the mature lens (Figure 1c) (at E11). Within the retina, RPCs differentiate in seven retinal cell types that together form the structure of the adult retina with three nuclear layers: i) retinal ganglion cells in the ganglion cell layer (GCL); ii) amacrine cells, bipolar cells, Müller glia cells and horizontal cells in the inner nuclear layer (INL); iii) rod and cone photoreceptors in the outer nuclear layer (ONL) (Young, 1985) (Figure 1c).

Since this particular work is focused mainly on the early stages of eye development concerning the process of lens induction, RPC proliferation and differentiation, the following sections are focused mainly on these aspects of eye development.

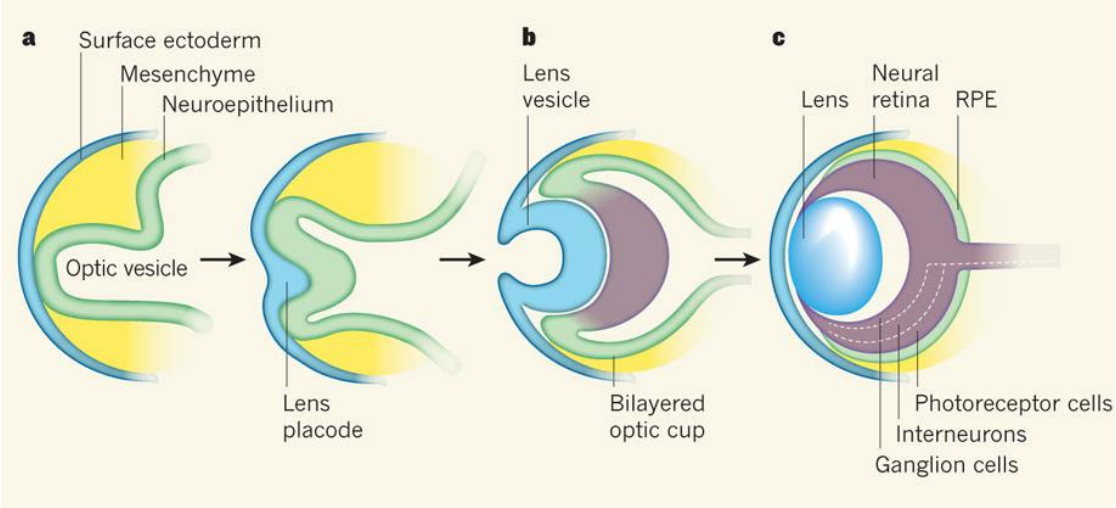

Figure 1. Schematic representation of mammalian eye development. (a) The optic vesicle forms as lateral evagination of the rostral diencephalic wall, contacts the head surface ectoderm and lens placode is formed. **(b)** Lens placode and optic vesicle invaginate to form the lens vesicle and bilayered optic cup. **(c)** Lens vesicle separates from the surface ectoderm and differentiates into the lens. Inner layer of the optic cup becomes the retina with tree retinal layers (ganglion cell layer occupied by ganglion cells, inner nuclear layer occupied by interneurons and outer nuclear layer occupied by photoreceptor cells); outer layer of the optic cup gives rise to the retinal pigmented epithelium. RPE, retinal pigmented epithelium. (Ali and Sowden, 2011).

3.2 Early development of the optic vesicle

As mentioned above, OV gives rise to the retina and RPE. The correct differentiation and alignment of individual eye tissues is dependent on complex interactions between three ectodermal derivates: the neuroectoderm represented by the OV, the neural crest represented by the extraocular mesenchyme and the head SE. Tissue-tissue interactions are mediated by coordinated action of extracellular factors and intrinsic factors. Several families of secreted signaling molecules, including Shh (Sonic hedgehog), Wnt, TGF-β (Transforming growth factor β) superfamily and FGFs (Fibroblast growth factor) are implicated in the control of inductive processes during eye formation as well as eye-field transcription factors (EFTFs), including Six3, Lhx2, Rx, Six6, Otx2 and Pax6 (reviewed by Fuhrmann, 2010; Chow and Lang, 2001; Ogino et al., 2012).

3.2.1 Evagination of the optic vesicle

OV neuroectoderm specification is initiated already before the OV evagination by the coordinated expression of EFTFs Six3, Lhx2, Rx, Six6, Otx2 and Pax6 within the region of the anterior neural plate (Li et al., 1997; Zuber et al., 2003). The first

morphological sign of eye formation is the evagination of the OVs. Although the molecular mechanism of the OV evagination is not completely understood, several studies performed in different vertebrate models suggest that retinal homeodomain transcription factor Rx (Rax) is involved in this process (Loosli et al., 2003; Mathers et al., 1997; Medina-Martinez et al., 2009; Rembold et al., 2006; Stigloher et al., 2006). *Rx* gene is one of the earliest genes expressed in the retinal lineage. In mouse, its expression is activated between embryonic day E7.5 and E8.0 in the anterior neural plate and later is strongly expressed in the OVs (Mathers et al., 1997). Rx is essential for the expression of other key regulators of early eye formation, such as Lhx2, Pax6, or Six3 that control the specification of retinal progenitor cells in the OV (Liu et al., 2010; Yun et al., 2009; Zhang et al., 2000; Zuber et al., 2003). In fish *Rx3*-null mutants, OV evagination is disrupted (Loosli et al., 2003) and *Rx*-deficient cells are excluded from OV domains in embryonic mouse chimeras consisting of wild-type and *Rx*-mutant cells (Medina-Martinez et al., 2009). In addition, studies performed in fish suggest that Rx regulates the cell movements that mediate OV evagination (Loosli et al., 2003; Rembold et al., 2006; Stigloher et al., 2006; Winkler et al., 2000). It has been shown that Rx is responsible for down-regulation of adhesive molecule Nlcam, that enables the cell movements during evagination (Brown et al., 2010).

3.2.2 Specification of the prospective retinal and RPE domains

During evagination, OV expands through the mesenchyme toward the SE (Figure 2A). At this stage, domains that will give rise to retina and RPE become specified within the OV. Dorsal region of the OV differentiates to the RPE and the distal/ventral to the retina (Hirashima et al., 2008; Kagiyama et al., 2005) (Figure 2). However, OV neuroepithelium stays bipotential with presumptive retina competent to generate RPE and RPE competent to generate retina (Araki and Okada, 1977; Horsford et al., 2005; Reh and Pittack, 1995; Rowan et al., 2004; Westenskow et al., 2010). The earliest known patterning genes of the OV are the LIM homeobox transcription factor Lhx2, homeobox transcription factor Chx10 and basic helix-loop-helix (bHLH) transcription factor Mitf (Burmeister et al., 1996; Green et al., 2003; Hodgkinson et al., 1993; Nguyen and Arnheiter, 2000; Yun et al., 2009; Zuber et al., 2003). Mitf specifies RPE domain, Chx10 the prospective retinal domain and Lhx2 is required for both, prospective retina and RPE specification. Mitf is first expressed in entire OV, but later, Mitf expression is down-regulated in the distal domain where

Chx10 expression is activated (Nguyen and Arnheiter, 2000) (Figure 2). Lhx2 operates upstream of Chx10 and Mitf as their expression is not initiated and eye development is arrested at OV/SE stage in Lhx2$^{-/-}$ mouse embryos (Yun et al., 2009; Zuber et al., 2003; Porter et al., 1997; Tetreault et al., 2009). Transcription factors of Pax family, Pax6 and Pax2 were shown to transcriptionally control the expression of Mitf and specification of RPE domain as Mitf is not expressed in the OV of *Pax6*$^{-/-}$ */Pax2*$^{-/-}$ compound mutant mice (Baumer et al., 2003). Beside intrinsic factors expressed by the OV, extraocular mesenchyme that surrounds evaginating OV is required for the maintenance and elevation of Mitf expression (Figure 2). Its removal interferes with Mitf expression and specification of RPE domain in chick and mouse OV (Fuhrmann, 2010; Fuhrmann et al., 2000; Kagiyama et al., 2005). It has been demonstrated that TGFβ family members (BMPs, activin) and Wnt ligands secreted by the mesenchyme are involved in this process (Fuhrmann et al., 2000; Fujimura et al., 2009; Grocott et al., 2011; Kagiyama et al., 2005).

Retina specification within the Mitf-expressing OV is mediated by FGF signaling (Cai et al., 2010; Gotoh et al., 2004; Hyer et al., 1998) (Figure 2 B). The FGF receptor activation leads to the expression of Chx10 in the distal OV (Gotoh et al., 2004; Cai et al., 2010). Chx10, in turn, is supposed to suppress Mitf expression to promote retinal development (Figure 2B) as its loss leads to persistent Mitf expression and retina to RPE transdifferentiation (Cai et al., 2010; Horsford et al., 2005; Rowan et al., 2004; Zhao et al., 2001). FGF ligands and receptors are abundantly expressed during eye development and their redundancy complicates the identification of the source of FGFs. However, as FGF1 and FGF2 are strongly expressed by the presumptive lens ectoderm (de Iongh and McAvoy, 1993; Pittack et al., 1997), it has been proposed that FGF signals from the SE are responsible for activation of FGF signaling in the distal OV and retinal specification (Figure 2B). Consistently, the removal of SE interferes with the retinal marker expression that can be restored by the source of FGF (Hyer et al., 1998; Pittack et al., 1997).

Beside FGF signaling, BMP signaling seems to be also involved in retina specification and/or maintenance of retinal domain (Figure 2B). BMP ligands and receptors are widely expressed in developing eye (Dudley and Robertson, 1997; Furuta and Hogan, 1998). While Bmp4 is expressed in distal surface of OV and later in dorsal retina, Bmp7 is expressed in the SE and periocular mesenchyme. *Bmp4*$^{-/-}$ and *Bmp7*$^{-/-}$ mouse embryos are anopthalamic and display the arrest in eye development at

OV/SE stage (Furuta and Hogan, 1998; Wawersik et al., 1999). Both, *Bmp4*$^{-/-}$ and *Bmp7*$^{-/-}$ OV display down-regulation of retina-specific genes and ectopic expression of pigmented genes such as Mitf (Huang et al., 2015; Morcillo et al., 2006) indicating that BMP signals (derived from SE and/or retina itself) are involved in retina specification. In accordance, inactivation of two BMP receptors (Bmpr1a and Bmpr1b) specifically in the presumptive retina results in down-regulation of Chx10 and abolished retinal differentiation (Murali et al., 2005). Moreover, recent studies performed in chick showed that BMP signals form SE (and developing lens) are crucial for specification of neural retina cells (Pandit et al., 2015).

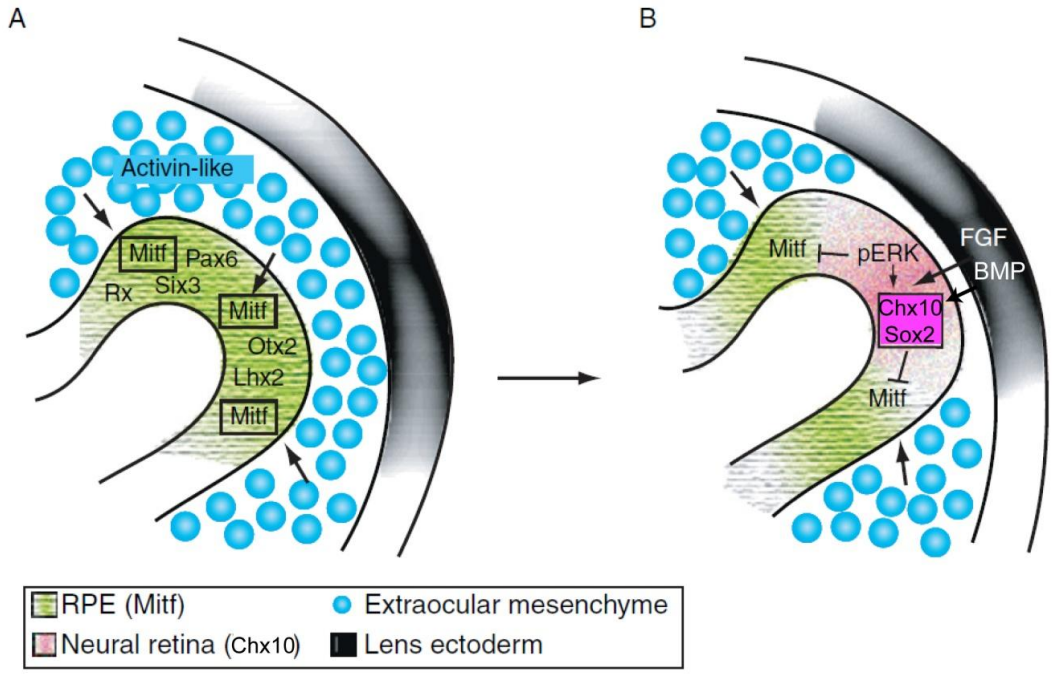

Figure 2. OV evagination and specification of the prospective retinal and RPE domains. (A) Early OV surrounded be extraocular mesenchyme producing activin-like molecules that induce Mitf expression in the entire OV. Proper response of the eye field to inducing signals is achieved by transcription factors Pax6, Lhx2, Six3, Otx2 and Rx **(B)** Later, OV contact SE. FGF and BMP signals from SE promote retinal specification within Mitf-expressing OV. FGFs from SE activate MAPK FGF signaling (pERK) in distal part of the OV leading to activation of Chx10 and Sox2 expression. Chx10 in turn is involved in Mitf downregulation in distal OV allowing the specification of the retina. Modified from Fuhrmann et al., 2010.

3.3 Lens induction and optic vesicle-to-optic cup transition

Early lens ablation experiments showed that preplacodal lens specification is crucial for the invagination of the OV and formation of the OC indicating that OC formation depends on proper lens formation (Hyer et al., 2003). As OV contacts the SE, reciprocal inductive signals elicit formation of LP within the SE and subsequent coordinated invagination of both, LP and OV to form the OC (Figure 3).

The first morphological sign of the lens development is the thickening of the SE and formation of the LP (at E9.0-E9.5) (Figure 3). The lens induction is a multistep process that requires several lens-inductive and lens-restrictive signals from the surrounding tissues such as evaginating OV and migrating neural crest cells (reviewed by Fuhrmann, 2010; Gunhaga, 2011). The genetic studies have identified multiple transcription factors and signaling pathways interacting in complex network orchestrating OV/SE-to-lens/OC transition (reviewed by Fuhrmann, 2010; Chow and Lang, 2001; Ogino et al., 2012). Among intrinsic factors, transcription factors Six3, Pax6 and Sox2 are strongly expressed in SE at the onset of the LP formation (Ashery-Padan et al., 2000; Grindley et al., 1995; Kamachi et al., 1998; Smith et al., 2009) (Figure 3). While Sox2 seems to be dispensable (Smith et al., 2009), both, Six3 and Pax6 are cell autonomously required for LP formation (Ashery-Padan et al., 2000; Liu et al., 2006). Most importantly, the timing and the proper level of Pax6 expression is crucial (Davis-Silberman et al., 2005; Davis et al., 2009; Duncan et al., 2004; Schedl et al., 1996). As Six3 was found to regulate Pax6 and Sox2 expression and Pax6 to regulate Six3 and Sox2 expression in the SE, it is supposed that Six3, Pax6 and Sox2 act in complex regulatory network to regulate each other to ensure proper lens development (Lang, 2004; Liu et al., 2006).

Concomitantly with the LP placode formation, OV becomes patterned into three domains (Figure 3): i) the prospective neuroretinal domain (central/distal OV) where Chx10 and Pax6 are expressed; ii) the prospective RPE domain (dorsal OV) where Mitf is expressed; iii) prospective optic stalk domain (ventral OV) where Pax2 is expressed. LP together with OV invaginate to form the lens and OC, respectively.

Among the signaling pathways, BMP and FGF were found to be essential for lens induction and coordinated OV-to-OC transition (Figure 3) as severe eye defects are associated with their inactivation (Faber et al., 2001; Furuta and Hogan, 1998; Garcia et al., 2011; Gotoh et al., 2004; Pan et al., 2006; Rajagopal et al., 2009; Sjodal

et al., 2007; Wawersik et al., 1999). As mentioned above, *Bmp4*$^{-/-}$ and *Bmp7*$^{-/-}$ mice exhibit arrest in eye development with no lenses being formed (Wawersik et al., 1999; Furuta and Hogan, 1998). This observation led to the assumption that beside the role in retinal specification, BMP signaling plays an essential role in the lens induction as well. Since Bmp4 is expressed in the distal surface of the OV that contacts the SE, it is the prime candidate to fulfill this role (Furuta and Hogan, 1998). Bmp4 expression in the OV was found to be positively regulated by transcription factor Lhx2 (Yun et al., 2009). In *Lhx2*-deficient OV, Bmp4 and Bmp7 are not expressed, effector molecules in SE are not activated and LP is not formed (Hagglund et al., 2011; Yun et al., 2009). Furthermore, OV-specific Bmp4 inactivation leads to arrested lens development as well (Huang et al., 2015), indicating that Bmp4 coming from the OV is essential for lens induction. Accordingly, inactivation of BMP receptors Bmpr1a and Acvr1 also abolished lens formation (Rajagopal et al., 2009). The mechanism by which BMP molecules regulate lens induction may include the activation (or elevation) of LP-specific gene expression as Sox2 and Pax6 expression is down-regulated when BMP signaling is disrupted (Furuta and Hogan, 1998; Wawersik et al., 1999).

The role of FGF signaling in the lens-inductive ability of the OV is less understood. Although mice with defective (Garcia et al., 2011; Gotoh et al., 2004; Pan et al., 2006) or suppressed (Faber et al., 2001) FGF signaling display severe lens defects, the specific ligand and its source have not been identified, probably due to a high degree of redundancy. It has been suggested that retina-derived N-cadherin could act as an alternative ligand for FGF receptor signaling in the lens (Smith et al., 2010). Similarly to BMP signaling, the mechanism of how FGF promotes the lens induction includes the stimulation of LP-specific gene expression, most importantly Pax6 expression that is essential for proper lens development (Faber et al., 2001; Gotoh et al., 2004). In addition, the expression of Bmp4 seems to depend on FGF signaling (Gotoh et al., 2004), indicating that interaction between BMP and FGF signaling is involved in lens induction.

The Wnt/β-catenin pathway, although not found to be directly involved in the lens-inductive ability of the OV, plays an important lens-restrictive role during eye development. In developing mouse eye, signaling is active in dorsal OV and later is restricted to extraocular mesenchyme, optic stalk and dorsal RPE with no activity in lens-forming SE or retina (Kreslova et al., 2007; Liu et al., 2006; Maretto et al., 2003). Wnt/β-catenin pathway activation results in the stabilization of the cytoplasmic β-

catenin and its subsequent accumulation in the nucleus. Nuclear β-catenin than associates with the TCF/Lef family of transcription factors to activate downstream target genes. Although the elimination of β-catenin in head SE results in formation of ectopic lentoid bodies in the periocular ectoderm, stabilization of β-catenin in lens-forming SE abrogates lens induction (Kreslova et al., 2007; Smith et al., 2005). In addition, Wnt pathway coreceptor Lrp6 becomes downregulated in the SE at the onset of eye development (Smith et al., 2005). These observations indicate that suppression of Wnt/β-catenin signaling in the SE is the prerequisite for the lens formation (Kreslova et al., 2007; Smith et al., 2005). However, how eye field transcription factors control the activity of Wnt/β-catenin pathway is poorly understood.

It is worth mentioning that the retinoic acid (RA) signaling plays also an important role in lens induction and OC morphogenesis (Duester, 2009). At early OV stage, strong RA signaling activity can be detected in the eye field (Mic et al., 2002) and its disruption leads to arrested eye development with no lens or retina formed (Bavik et al., 1996; Mic et al., 2004). It has been shown that RA signaling in the eye field is mediated by the specific expression of retinaldehyd dehydrogenases Raldh1, Raldh2, and Raldh3 (Duester, 2009), however, the exact mechanism of how RA signaling may contribute to OC morphogenesis has not been established.

It should be also noted that although there is an evidence that developing lens and its secreted signals are involved in retinal specification, recent studies reported the formation of fully differentiated retina in the absence of the lens or extraocular mesenchyme (Eiraku and Sasai, 2012; Eiraku et al., 2011; Nakano et al., 2012; Sasai, 2012). When cultured under specific conditions, mouse and human embryonic stem cells are able to form three dimensional OVs that further invaginate to form the OC with fully differentiated neurons. This indicates that patterning, morphogenesis and differentiation of the OV are at least partly ensured by the intrinsic mechanisms and do not require external cues. Nevertheless, more detailed analysis of factors present in three dimensional OV culture might help to resolve this issue.

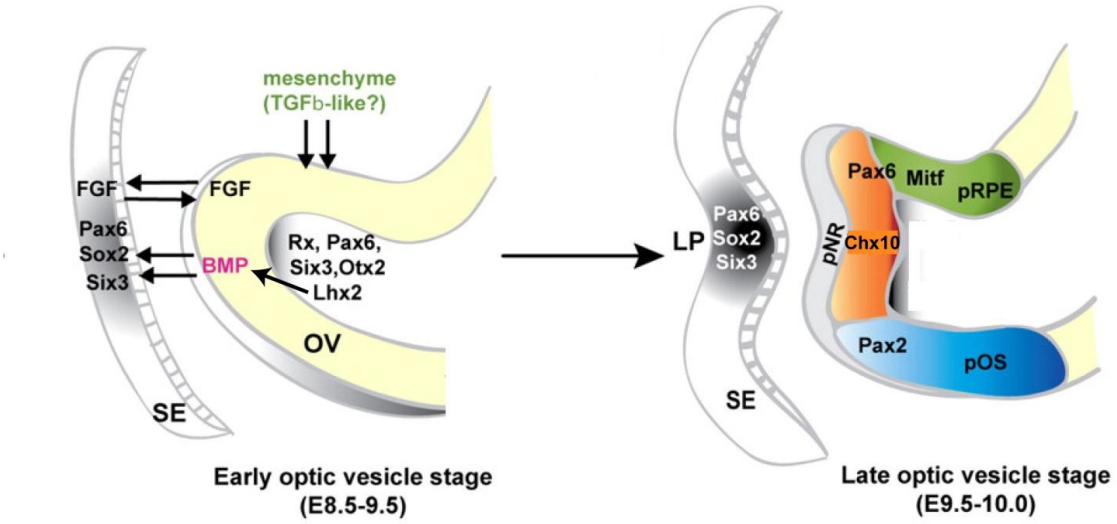

Figure 3. Lens induction and OV-to-OC transition. Reciprocal interaction between the OV and SE, elicits the formation of the LP within the SE. Subsequently, LP and OV invaginate to form the lens and OC. Lhx2 expressed by the OV induces the expression of BMP ligands that induce BMP activation in overlying SE. BMP together with FGF signaling elevates the expression of SE-specific transcription factors Six3, Pax6 and Sox2 and the LP is formed. At the same time, the OV diversifies into three domains: prospective neuroretinal domain expressing Chx10 and Pax6; prospective RPE domain expressing Mitf and Pax6 and prospective optic stalk domain expressing Pax2 and OV invaginates to form the OC. OV, optic vesicle; SE, surface ectoderm; PL, lens placode; OC, optic cup; pNR, prospective neuroretinal domain; pRPE, prospective RPE domain; pOS, prospective optic stalk domain. Modified from Yun et al., 2009.

3.4 Retinal progenitor cells

3.4.1 Retinal progenitor cell maintenance

As mentioned above, retinal domain is specified within the distal part of the OV closely contacting the SE. As OV invaginates, the cells located in the inner layer of the OC become specified in retinal progenitor cells (RPCs). RPCs are highly proliferative multipotent cells competent to give rise to all retinal neuron and glia cell types (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990). A defining feature of RPCs is co-expression of the transcription factors Rx, Pax6, Lhx2, Six3, Chx10, Sox2 and Hes1. These factors are expressed prior to the activation of neurogenic program and contribute to proliferative and retinogenic potential of RPCs (Burmeister et al., 1996; Grindley et al., 1995; 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). At the time of differentiation, the subpopulation of RPCs exits the cell cycle and transits from a proliferative state

towards a lineage-restricted neurogenic state. This is a gradual process with individual cell types generated through the course of retinogenesis in defined birth order (Young, 1985). Since retinogenesis is finished after birth in mouse, new progenitor cells are continuously generated in the neuroblastic (proliferative) layer in order to ensure stepwise differentiation of all retinal cell types (Mumm et al., 2005; Reese et al., 2011). Early in development, RPCs tend to divide symmetrically to increase the pool of progenitors in newly formed OC. When differentiation is initiated, cells begin to divide asymmetrically producing one cell that differentiates and a second that continue to divide as a progenitor cell. During later steps of retinogenesis, progenitor cells tend to undergo cell cycle exit that eventually leads to the depletion of the entire RPC pool (Dyer and Cepko, 2001a). The expansion of RPCs and precise timing of cell cycle exit thus must be coordinated to ensure temporal production of each of the seven classes of retinal cell types (reviewed by Agathocleous and Harris, 2009).

Growth-promoting and growth-inhibiting signals determine whether a progenitor cell will re-enter the cell cycle or exit the cell cycle in order to differentiate. In vertebrate retina, cyclin D1, pRb protein, KIP proteins $p57^{Kip2}$ and $p27^{Kip1}$ and INK4 protein Ink4d have been implicated in direct regulation of RPC proliferative potential (Das et al., 2009; Dyer and Cepko, 2000, 2001a; Geng et al., 2001; Levine et al., 2000; Levine and Green, 2004). Cyclin D1 is predominant D-type cyclin expressed in RPCs promoting cell cycle progression by activation of cyclin-dependent kinase Cdk4 (Das et al., 2009). However, several studies where compound cyclin D-deficient mice (cyclin D1/cyclin D3) were used indicate that some cyclin D-independent mechanism operates in RPCs to promote basal cell cycle progression as cyclin D-deficient RPCs still proliferate, although less efficiently than normal RPCs (reviewed by Levine and Green, 2004). Other proteins mentioned above have a growth inhibitory role during the RPC proliferative period. Cyclin-dependent kinase inhibitor proteins $p57^{Kip2}$, $p27^{Kip1}$ and Ink4d are expressed by the subsets of RPCs as they exit the cell cycle and $p57^{Kip2}$ and $p27^{Kip1}$ alone are sufficient to promote cell cycle exit when expressed in RPCs (Cunningham et al., 2002; Dyer and Cepko, 2000, 2001a, b). In addition, several pro-neurogenic bHLH transcription factors, including Mash1, NeudoD or Ngn1 were found to promote both, the cell cycle exit and neuronal fate determination, implying that the processes of cell type specification and cell cycle exit are tightly coupled (Farah et al., 2000; Ochocinska and Hitchcock, 2009).

However, how these complex events are orchestrated during retinogenesis remains largely elusive.

3.4.2 Differentiation of retinal progenitor cells

Adult retina is composed of seven cell types that form sophisticated functional network. Light-sensitive photoreceptors transmit information through the bipolar cells to retinal ganglion cells (RGCs). RGCs than transmit visual information from retina to the visual centers of the brain. In addition, horizontal cells and amacrine interneurons specifically modify synaptic signal before it reaches RGCs. Defect in differentiation of any cell type can lead to severe retinal abnormalities and loss of vision.

RPCs start their differentiation soon after OC is formed. Retinal ganglion cells, horizontal cells and cone photoreceptors differentiate first, followed by amacrine cells, rod photoreceptors, bipolar cells and finally Müller glial cells (Young, 1985) (Figure 4A). Individual cell types are generated gradually in overlapping pattern with at least two retinal cell types generated at the same time. Neurogenesis is initiated at E11, in the center of OC and gradually progresses towards the retinal periphery (Hu and Easter, 1999) (Figure 4A).

Although RPCs retain their multipotency throughout retinal development (Turner and Cepko, 1987), they undergo a series of changes in intrinsic properties that control their competence to generate different retinal cell types. It is largely accepted that as the retinogenesis proceeds, RPCs are exposed to the changing environment of extrinsic cues that in cooperation with intrinsic factors regulate progenitor proliferation and direct the bias towards particular retinal cell fates (Cepko, 1999). The intrinsic factors are represented mainly by the bHLH class transcription factors (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). The bHLH factors comprise transcriptional activators as well as repressors that operate in an antagonistic manner during retinal development. Repressors represented most prominently by Hes family proteins Hes1 and Hes5, a homologs of *Drosophila hairy* and *Enhancer of split* genes, are key regulators of RPC proliferation and maintenance, keeping progenitor cells in an undifferentiated state. The mechanism of their action includes inhibition of neurogenesis by suppression of bHLH activators (Davis and Turner, 2001; Tomita et al., 1996). In *Hes1*-deficient retinae, cell proliferation is impaired and premature differentiation can be observed (Tomita et al.,

1996). Accordingly, Hes1 misexpression inhibits neuronal differentiation in the retina (Tomita et al., 1996). Hes1 and Hes5 are well known downstream effectors of Notch signaling (Ohtsuka et al., 1999), maintaining cells in an undifferentiated state (Gaiano et al., 2000). Upon stimulation by Notch ligand, the intracellular domain of Notch (ICN) is cleaved off, translocates into the nucleus where it forms the complex with RBP-J and Hes1/Hes5 repressors are expressed (Artavanis-Tsakonas et al., 1999; Honjo, 1996). Hes proteins than recruit corepressor Groucho to the promoters of differentiation-promoting genes and inhibit their expression (Davis and Turner, 2001) (Figure 5A). On the other hand, when neurogenesis is about to start, bHLH activators such as Math5, NeuroD, Math3 or Mash1 override the Hes repressors and promote neuronal differentiation (reviewed by Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008) (Figure 5B). Although bHLH transcription activators have been shown to cause strong cell autonomous bias toward particular retinal cell fates, their activity is dependent on co-expression with other factors, most prominently homeodomain class transcription factors, including Pax6, Six3, Prox1, Chx10 or Rx (reviewed by Ohsawa and Kageyama, 2008). These factors, expressed in early OV, are required for RPC proliferation and later on for differentiation of individual retinal cell types (Agathocleous and Harris, 2009; Burmeister et al., 1996; Furukawa et al., 2000; Marquardt et al., 2001). The homeodomain and bHLH transcription factors affect each other expression, creating a complicated transcriptional network that regulates retinal development. Transcription factors involved in cell fate determination are being identified, but how RPCs change their competence over time is not known. The following part summarizes some of the best characterized transcription factors involved in the differentiation of particular retinal cell types (Figure 6). It should be noted that almost all retinal cell types comprise of several subtypes. However, these will not be discussed here.

Retinal ganglion cells (RGCs): RGCs are the first retinal cell type to differentiate from RPCs. Later on, in adult retinae, they are localized in the GCL (Figure 4B) (Cepko et al., 1996; Young, 1985). RGC differentiation is governed by a well defined gene regulatory network of three transcription factors; Math5, Brn3b and Isl1 (Mu et al., 2008; Pan et al., 2008; Wu et al., 2015) (Figure 6). Math5 is essential to keep RPCs competent to generate RGCs as its targeted disruption leads to dramatic reduction of RGCs (Brown et al., 2001; Wang et al., 2001). However, Math5 alone is not sufficient to determine RGC fate. The POU domain transcription factor Brn3b and

LIM-homeodomain transcription factor Isl1, both operating downstream of Math5, are expressed by differentiating RGCs and activate genes essential for RGC differentiation (Liu et al., 2001; Mu et al., 2008; Pan et al., 2008; Yang et al., 2003). A recent study has shown that Brn3b and Isl1 are sufficient for RGC differentiation in the absence Math5 (Wu et al., 2015).

Amacrine cells: Amacrine cells are generated in the first wave of retinogenesis overlapping with retinal ganglion cells, horizontal cells and cone photoreceptors (Figure 4A). In adult retina, they are localized in the INL and GCL (Figure 4B). The bHLH transcription factors Math3 and NeuroD have been implicated in differentiation of amacrine cells (Figure 6) (Inoue et al., 2002). The expression pattern of Math3 and NeuroD overlaps with amacrine cell genesis and loss of amacrine cells was observed in *Math3/NeuroD* compound mutants (Inoue et al., 2002). Majority of cells that would normally become amacrine cells adopt the ganglion cell fate instead. This process is accompanied by upregulation of Math5, indicating antagonistic regulation between Math3/NeuroD and Math5 (Inoue et al., 2002). Although Math3/NeuroD are essential, they are not sufficient for amacrine cell differentiation (Hatakeyama et al., 2001; Inoue et al., 2002). The homeodomain transcription factors Pax6 and Six3 are expressed by differentiating amacrine cells as well. The combined expression of Math3/NeuroD with either Pax6 or Six3 is sufficient for amacrine cell genesis (Inoue et al., 2002). In addition, winged helix/forkhead transcription factor Foxn4 and homeodomain transcription factor Ptf1a, operating downstream of Foxn4, were also found to be required for proper amacrine cell differentiation (Figure 6). Experiments in mouse revealed, that while loss of Foxn4 results in a reduced number of amacrine cells, Foxn4 misexpression leads to effective generation of amacrine cells (Fujitani et al., 2006; Li et al., 2004).

Horizontal cells: Horizontal cells represent a population of retinal interneurons, localized in the INL (Figure 4B). Their differentiation is tightly connected with differentiation of amacrine cells as these cell types share a common Foxn4 and Ptf1a-expressing progenitor. Accordingly, the loss of Foxn4 or Ptf1a leads to complete loss of horizontal cells (Li et al., 2004; Fujitani et al., 2006). Although Foxn4 and Ptf1a are required for differentiation of both, amacrine and horizontal cells, homeodomain transcription factor Prox1 determine specifically the horizontal cell fate over the amacrine cell fate (Dyer et al., 2003) (Figure 6).

Bipolar cells: Bipolar cells are localized in the INL of adult retina (Figure 4B) and their differentiation is regulated by bHLH transcription factors Mash1 and Math3 and the homeobox transcription factor Chx10 (Hatakeyama et al., 2001) (Figure 6). In *Mash1/Math3* mutants, bipolar cells are completely lost (Tomita et al., 2000) as well as in *Chx10*-deficient retinae (Burmeister et al., 1996). However, misexpression of Chx10, neither Mash1 nor Math3 alone is sufficient for bipolar cell differentiation and only introduction of all three transcription factors at the same time can promote bipolar cell genesis (Hatakeyama et al., 2001).

Photoreceptor cells: Photoreceptor cells are localized in the ONL of the adult retina (Figure 4B) and are represented by two classes of cells; rods and cones. The cone-rod homeobox protein Crx is the earliest expressed photoreceptor determinant (Furukawa et al., 1997; Chen et al., 1997). 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). Besides Crx, Otx2 is a key regulator of the photoreceptor lineage (Nishida et al., 2003) as photoreceptor cells are selectively lost in Otx2-deficient retinae (Nishida et al., 2003). From bHLH factors, NeuroD missexpression was found to induce rod genesis (Inoue et al., 2002). In addition, homeodomain transcription factor Rx was identified to operate upstream of Otx2 and regulate photoreceptor development (Muranishi et al., 2011).

Müller glial cells: Müller glial cells are one of the last retinal cell types to differentiate. Since they are involved in the maintenance of retinal homeostasis and integrity, their bodies are spread through all retinal layers. Transcription factors Hes1, Hes5 and Hers2 were implicated in Müller glia development (Figure 6) as the reduction of their numbers can be observed in Hes-deficient retinae (Furukawa et al., 2000; Hojo et al., 2000). In addition, homeobox gene *Rx* is implicated in Müller glia development as well (Furukawa et al., 2000). Since misexpression of Rx, Hes1 or Hes5 leads to generation of Müller glia cells (Furukawa et al., 2000; Hojo et al., 2000; Mathers et al., 1997), and Rx, Hes1 and Hes5 proteins were shown to affect proliferation and maintenance of the RPC, it is largely accepted that RPCs that do not lose the expression of Hes1 and Hes5 eventually adopt the Müller glial cell fate. This

emphasizes the need of constantly present pool of undifferentiated progenitors throughout the retinogenesis (Deneen et al., 2006; Hojo et al., 2000).

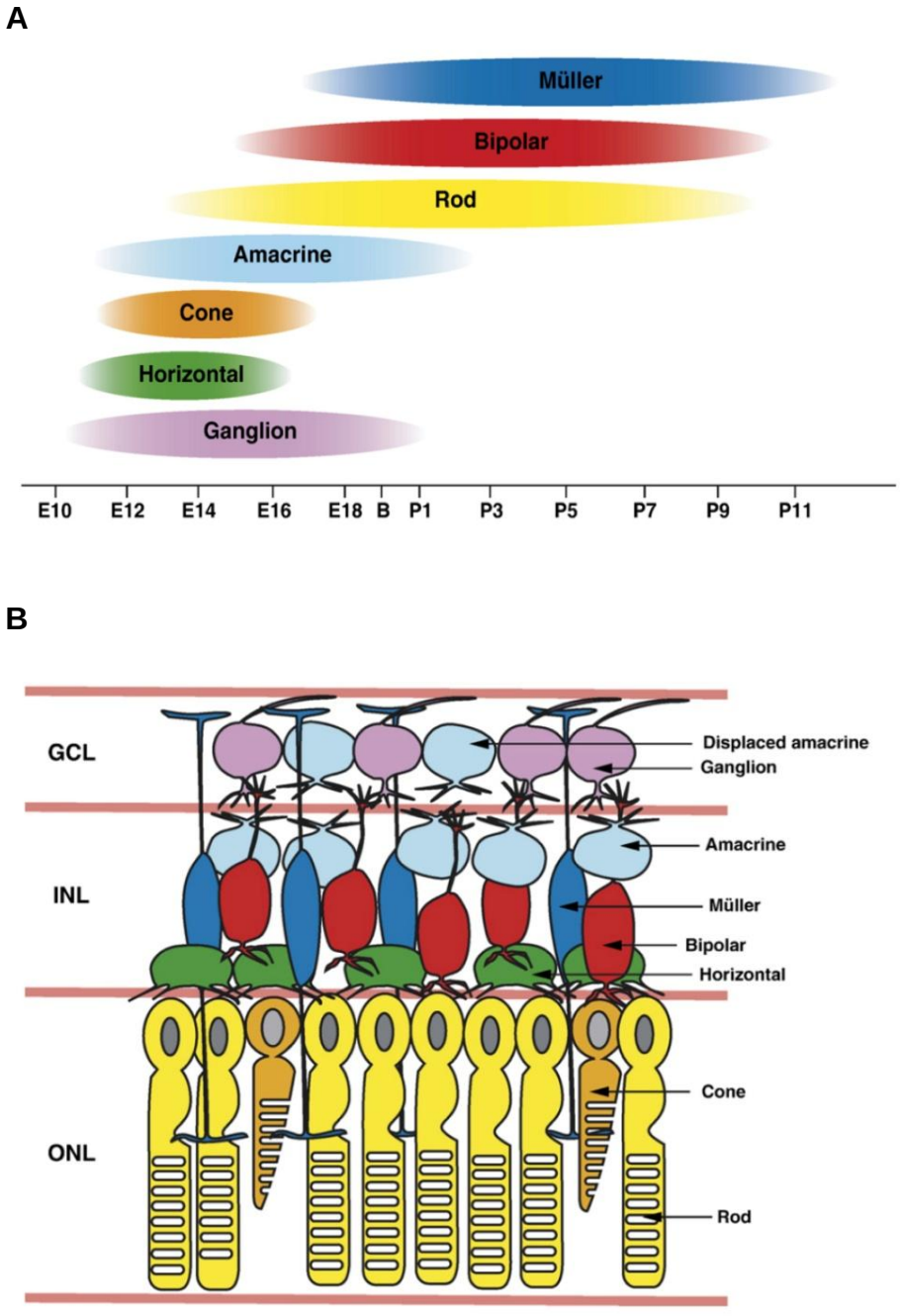

Figure 4. Schematic representation of retinal development and structure of the adult retina. (A) Retinal differentiation is initiated after embryonic day E10.5 and finished after birth, by postnatal day P11. Retinal cells are generated in a defined order with retinal ganglion cells generated first and Müller glial cells last. **(B)** Structural organization of the adult retina. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. E, embryonic day; P, postnatal day. Reproduced from Ohsawa and Kageyama, 2008.

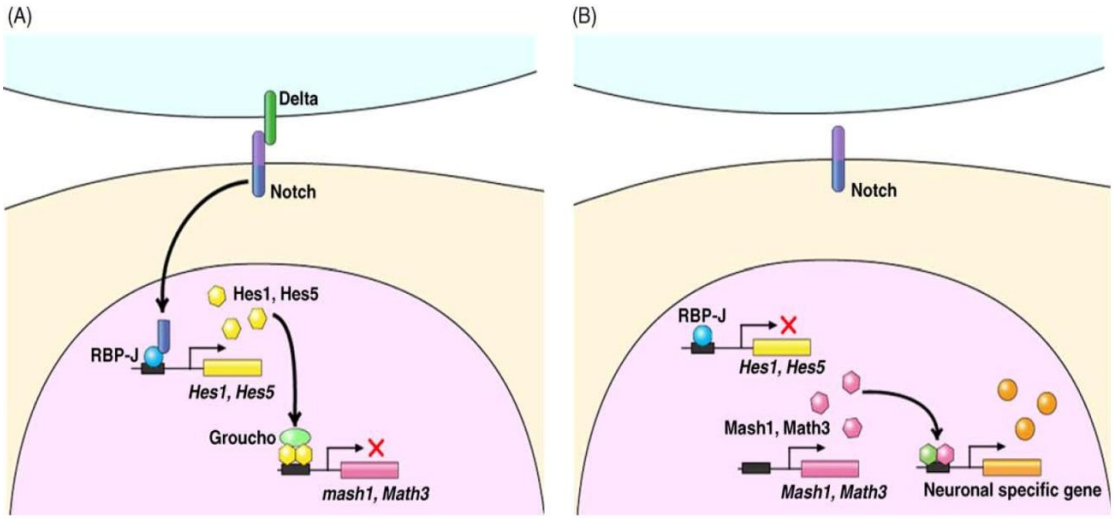

Figure 5. Notch signaling in neuronal differentiation. (A) After Notch receptor stimulation, intracellular domain of Notch receptor (ICN) is cleaved off and translocates into the nucleus. In the nucleus, ICN interacts with the DNA-binding protein RBP-J and stimulates the expression of Hes proteins (Hes1 and Hes5). Hes1 and Hes5 than recruit Groucho corepressor to the promoters of bHLH transcriptional activators, that are involved in neuronal differentiation. As a result, neuronal differentiation is inhibited. **(B)** In the absence of Notch receptor stimulation, Hes proteins are not expressed and bHLH activators, such as Mash1 or Math3, can be expressed. As a result, the expression of neuronal specific genes and differentiation is initiated. Reproduced from Hatakeyama and Kageyama, 2004.

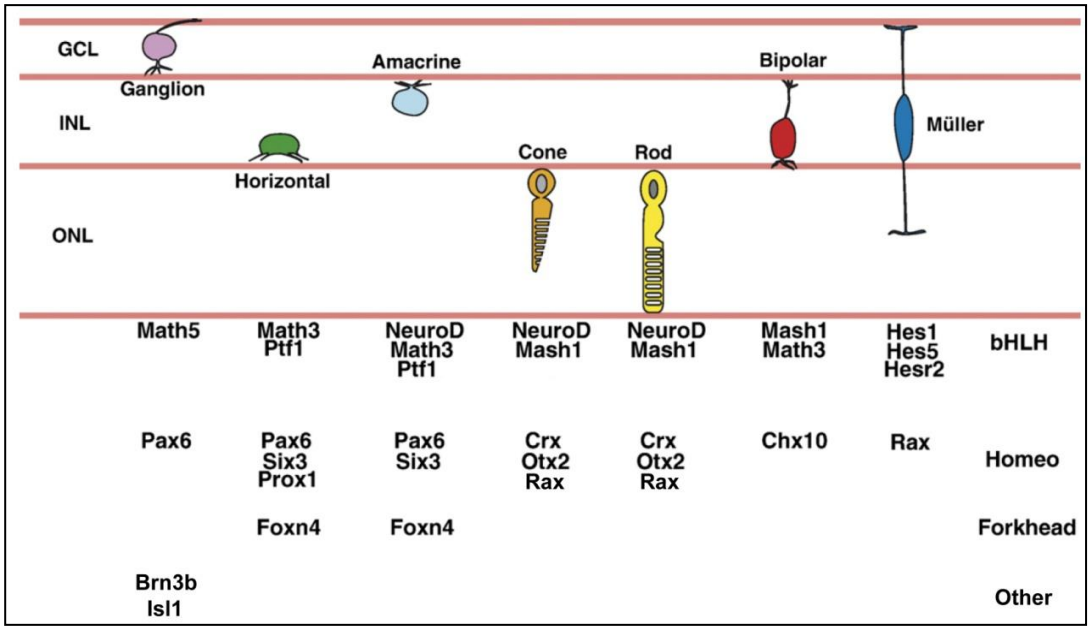

Figure 6. Transcription factors involved in the differentiation of particular retinal cell types. Mostly, cooperation of bHLH and homeodomain transcription factors is required for cell type specification in the retina. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Modified from Ohsawa and Kageyama, 2008.

3.5 Transcription factor Pax6 in eye development

3.5.1 Pax6 protein

Pax6 is the member of the PAX gene family, which encodes highly conserved transcription factors (reviewed by Kozmik, 2005). *Pax6* gene spans 28 kb and contains 16 exons that can produce several gene products (Glaser et al., 1992; Kammandel et al., 1999; Kim and Lauderdale, 2006) (Figure 7A). Although an alternative promoter usage and alternative splicing can produce several proteins with different functions, the most abundant and most intensively studied Pax6 variant is "canonical Pax6" (functional homolog of the *Drosophila* genes *Eyeless* and *Twin of Eyeless*) (Czerny et al., 1999; Quiring et al., 1994). In mammals, it is 422 amino acid (aa) protein with two highly conserved DNA binding domains: the bipartite paired domain (PD) and the paired-like homeodomain (HD) (Treisman et al., 1991; Wilson et al., 1995) (Figure 7B). The 128 aa PD, which is shared by all PAX genes, is encoded by exons 4-7 and consists of two subdomains, PAI and RED (Czerny et al., 1993; Xu et al., 1995). Both are represented by three α helices with two forming helix-turn-helix (HTH) DNA binding motif (Czerny et al., 1993; Epstein et al., 1994; Xu et al., 1999a; Xu et al., 1995). PD recognizes a bipartite binding site of about 17 nucleotides termed P6CON and binds to DNA as monomer (Czerny et al., 1993; Epstein et al., 1994) (Figure 7C). The specificity of PD is dependent on 3 aa (isoleucin-59, glutamine-61 and asparagine-64) located within the PAI subdomain (Czerny and Busslinger, 1995). The 61 aa paired-type HD, which is found in a majority of the homeodomain proteins, is encoded by exons 8-10 and binds as homo- or heterodimers to a palindromatic ATTA sequence (Czerny and Busslinger, 1995; Wilson et al., 1993; Wilson et al., 1995). Likewise PD, HD is represented by three α-helices with HTH motif (Xu et al., 1999a; Xu et al., 1995).

C-terminal region of the Pax6 is prolin-serin-threonin rich (PST) and is involved in transcription transactivation properties of Pax6 (Czerny and Busslinger, 1995; Glaser et al., 1994; Mikkola et al., 1999; Tang et al., 1998). PST carries several phosphorylation sites and it has been shown to be prone to phosphorylation by the MAPKs P38 and Erk2 and homeodomain-interacting protein kinase 2 (Hipk2) (Kim et al., 2006; Mikkola et al., 1999) (Figure 7B). Phosphorylation of specific residues was found to be required for Pax6-mediated transcription transactivation which is achieved by the recruitment of different co-activators to the enhancer via protein-protein

interaction (Kim et al., 2006; Mikkola et al., 1999; Tang et al., 1998). Apart from PST, the PD and HD domains have been also found to be involved in protein-protein interactions (Cvekl et al., 1999; Mikkola et al., 2001). Pax6 is known to act as transcription activator, however, situations where Pax6 was found to inhibit the expression of certain genes have been also reported. Pax6 inhibits expression of some crystallin genes in the lens and Crx expression in the retina (Duncan et al., 1998; Kralova et al., 2002; Oron-Karni et al., 2008). Although the exact mechanism is not known, it was suggested that inhibition is mediated via competition for promotor occupancy (Duncan et al., 1998).

Pax6 is characterized by highly complex spatiotemporal expression pattern. It is expressed in central nervous system (CNS), eye, olfactory system and pancreas, where it plays the pivotal role (reviewed by Hanson and Van Heyningen, 1995; Osumi et al., 2008; Simpson and Price, 2002). Expression of Pax6 in different times and tissues is achieved by the combined activity of several cis-regulatory elements. These are located upstream of the *Pax6* gene and in its introns (Kammandel et al., 1999; Kleinjan et al., 2004; Plaza et al., 1995; Williams et al., 1998; Xu et al., 1999b) (Figure 7A). In addition, more distant cis-regulatory elements downstream of Pax6 gene that contain several tissue-specific enhancers, were also identified (Griffin et al., 2002; Kleinjan et al., 2006) (Figure 7A). Some regulatory elements were found to be active in eye (Figure 7Ab, c, e, f, g, j, k, l, m, n), while others are active in CNS (Figure 7Ad, h, i, m, n, o), pancreas (Figure 7Aa, c) or in the olfactory region (Figure 7Aj, k, l). The combination of several DNA regulatory sequences enables intricate regulation and provides redundancy and robustness of the *Pax6* expression. Moreover, identification of tissue-specific enhancers provided us with the opportunity to perform tissue-specific gene inactivation using the Cre/LoxP system (Gu et al., 1994) which is nowadays widely used for gene function studies in eye development. It is based on the tissue-specific expression of the Cre recombinase that recognizes target sites (loxP sites) in targeted gene. Cre-mediated recombination than leads to gene inactivation specifically in the tissue, where Cre recombinase is expressed, leaving other tissues unaffected. For example, enhancer located in intron 4 of the *Pax6* gene (Figure 7Af) was used to generate the *α-Cre* line, in which the expression of Cre recombinase and gene inactivation occurs specifically in developing retina (Marquardt et al., 2001). On the other hand, DNA segment located upstream of the *Pax6* promotor (Figure 7Ab)

was used to generate *Le-Cre* line, in which Cre expression is localized in an early developing lens (Ashery-Padan et al., 2000).

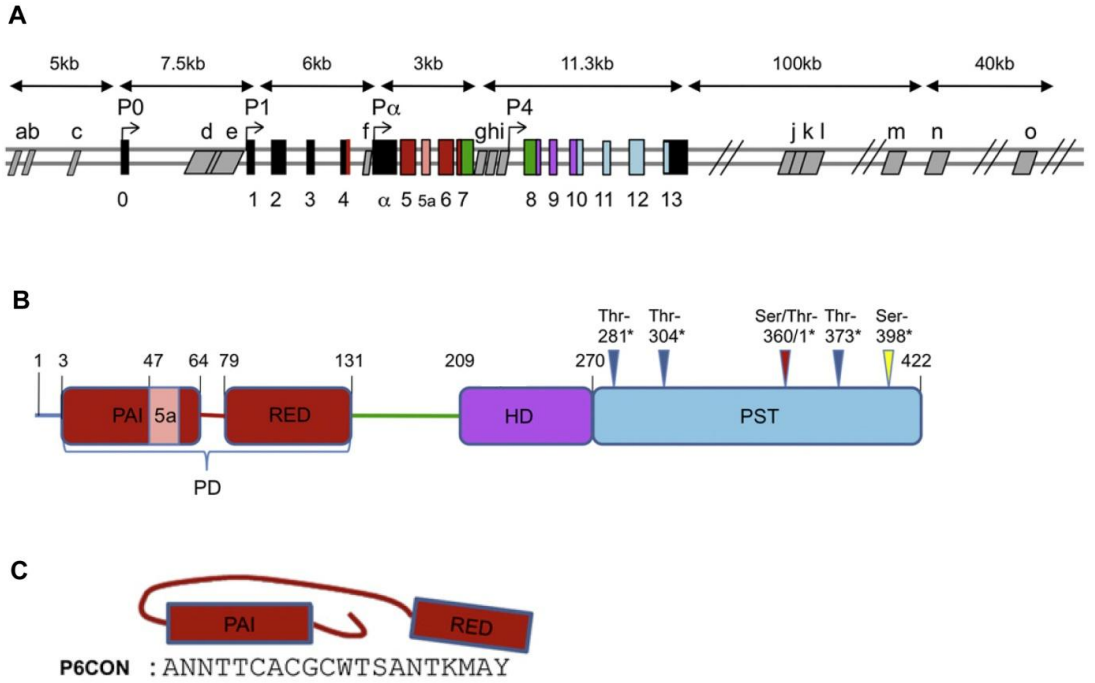

Figure 7. Structure of *Pax6* gene and Pax6 protein. (A) Structure of mouse *Pax6* gene. Exons are indicated by colored boxes with numbers; transcription start sites are indicated with arrows; regulatory sequences are indicated by gray boxes stained with letters (a-o). **(B)** Schematic structure of the transcription factor Pax6. Two DNA binding domains; the bipartite paired domain (PD) and the paired-type homeodomain (HD) are indicated with red and violet color, respectively. C-terminal prolin-serin-threonin (PST) domain is indicated with blue color. Phosphorylation sites within PST domain involved in transactivation properties of Pax6 are indicated with arrowheads. The numbers represent amino acid positions within the canonical Pax6 protein. **(C)** DNA specificity of the PD. The bipartite PD recognizes a bipartite consensus (P6CON) with PAI and RED recognition sequences. Adapted from Shaham et al., 2012.

3.5.2 Pax6 role in eye development

Pax6 plays key role in eye morphogenesis in both, insects and mammals since a mutation in the *Pax6* gene disrupts eye development both in flies (Quiring et al., 1994) and mice (Hill et al., 1991). In humans are mutations of *PAX6* cause of aniridia (lack of iris). Aniridia involves several abnormalities such as iris hypoplasia, ciliary body hypoplasia, cataract, dislocation of the lens, corneal opacity, or glaucoma (Lee et al., 2008). In the last decades, several mutations in *PAX6* locus connected with

aniridia were identified (The Human *PAX6* mutation Database; http://lsdb.hgu.mrc.ac.uk).

The function of Pax6 in eye development is highly conserved across the species (reviewed in Kozmik, 2005). Ectopic expression of human *Pax6* in *Drosophila* and *Xenopus* leads to the formation of ectopic eye structures (Halder et al., 1995; Chow et al., 1999; Zuber et al., 2003), indicating that Pax6 control highly conserved genetic pathway that initiates eye development. Based on those observations, *Pax6* was established as "master control gene" of eye development that is located at the top of a gene cascade triggering eye formation (Gehring and Ikeo, 1999). Although Pax6 alone can induce formation of ectopic eyes in flies or amphibians, the situation in mammals, however, seems to be more complex as other transcription factors and reciprocal interactions of several tissues are required for eye formation.

In mice, *Pax6* is one of the first EFTF expressed in the eye field. It is expressed in both, SE and OV; later in developing lens, RPE and all mitotically active RPCs of the OC (Walther and Gruss, 1991). As retina differentiates, Pax6 expression is restricted to retinal ganglion cells, amacrine cells, horizontal cells and Müller glial cells (de Melo et al., 2003; Roesch et al., 2008). In mice, naturally occurring homozygous lethal mutation named "Small Eye" (Sey) was found to be caused by mutation in the *Pax6* gene (Hogan et al., 1986). $Pax6^{Sey/Sey}$ ($Pax6^{-/-}$) homozygous embryos are anopthalamic, with eye development arrested at the OV/SE stage (Hill et al., 1991; Hogan et al., 1986). OV evagination occurs, expression of several EFTFs is maintained and retinal and RPE domains are initially established within the OV of *Pax6*-deficient embryos (Grindley et al., 1995; Marquardt et al., 2001). However, the OV does not further invaginate to form the OC and SE fails to differentiate into the lens (Hogan et al., 1986). In heterozygous mice ($Pax6^{Sey/+}$), lens and retina develop, although the lens is usually smaller and aberrantly connected to the cornea (Hogan et al., 1986).

Since Pax6 inactivation leads to arrest of early eye development (abrogating lens and retina formation), autonomous Pax6 roles in individual ocular tissues were left unresolved. However, the tissue-specific inactivation with the use of the Cre/LoxP system enabled to uncover the Pax6 functions in much higher details (reviewed by Shaham et al., 2012). Conditional ablation of Pax6 specifically in the lens-forming SE using *Le-Cre* mice revealed that Pax6 is required for the lens placode/lens formation

(Ashery-Padan et al., 2000). Pax6 role in developing retina was analyzed using *α-Cre* mice, which is active in distal parts of the retina at the time, when RPCs starts their differentiation program (Marquardt et al., 2001). When *Pax6* was inactivated, only one retinal cell type (amacrine cells) instead of all seven was generated. Pax6 was found to control the expression of several bHLH transcription factors, including Math5, Math3 or Ngn2. Those observations indicated that Pax6 plays the central role in retinal differentiation (Marquardt et al., 2001).

It should be noted here, that Pax6 was also found to play a role in later stages of eye development in derivatives of Pax6-expressing SE - lens and cornea as well as in iris and ciliary body, originating from OC (reviewed by Shaham et al., 2012). However, these will not be discussed here.

4. AIMS OF THE STUDY

Although much has been already revealed about the role of Pax6 transcription factor in eye development, there are still many questions to be answered. For example, whether and how Pax6 regulates the process of lens induction. Pax6 is expressed in both, OV and SE of the developing eye. Since the eye development is arrested at early OV/SE stage in *Pax6*-deficient embryos, it was not clear whether the cause of the defect is OV or SE. There is a strong evidence that Pax6 expression in lens-forming SE is essential for lens formation. However, the exact mechanism of how Pax6 regulates this process is not completely understood. The role of Pax6 in the OV has not been also addressed as the tool for gene manipulation in OV was not available. Therefore, it is still unknown whether Pax6 expression in OV is required for lens induction and retinal specification. In addition, Pax6 was found to activate several bHLH transcription factors involved in differentiation of retinal cell types during the later steps of retinal development. However, the complete Pax6-controlled gene regulatory network leading to differentiation of particular retinal cell types is far from being completely undersood.

The specific aims of this study were:

- To examine role of Pax6 in signaling events that regulate early steps of lens formation. Namely, to analyze a potential link between Pax6 and Wnt/β-catenin signaling pathway in the lens-forming surface ectoderm.

- To generate a Cre recombinase-expressing mouse line that enables inactivation of Pax6 in the developing optic vesicle and newly formed optic cup.

- To elucidate role of Pax6 in the optic vesicle and early optic cup.

- To identify new Pax6-regulated genes in retinal development and analyze their potential function in differentiation of the individual retinal cell types.

5. LIST OF METHODS

Nucleic acid manipulation

RNA isolation

DNA isolation

DNA purification for mouse pronuclear injection

polymerase chain reaction (PCR), quantitative PCR (qPCR)

PCR-based mouse genotyping

BAC recombineering

Tissue collection

Dissection and colection of embryonal and postnatal mouse tissues

Sample preparation for FACS sorting

Histology

Immunohistochemistry

β-galactosidase assay

RNA *in situ* hybridization on tissue sections

hematoxylin and eosin staining

fluorescence confocal microscopy

Chromatin immunoprecipitation assay

BrdU/EdU incorporation assay and measurement of cell cycle length

6. RESULTS

6.1 Lens morphogenesis is dependent on Pax6-mediated inhibition of the canonical Wnt/β-catenin signaling in the lens surface ectoderm.

During the process of lens induction, several lens-inductive and lens-restrictive signals cooperatively determine the proper position of the single lens within the eye. Although the OV is supposed to provide lens-inductive signals to the adjacent SE, Wnt/β-catenin signaling prevents lens formation in other parts of the head SE (Kreslova et al., 2007; Smith et al., 2005; Swindell et al., 2008). Accordingly, the Wnt/β-catenin signaling activity can be found in the broad region of the head SE excluding lens-forming SE (Kreslova et al., 2007; Swindel et al., 2008; Smith et al., 2005). Although it is well established that Wnt/β-catenin inactivation is the prerequisite for lens formation, the source of its inhibition and the role of EFTFs in its regulation is poorly understood.

In this study, we confirmed, that inhibition of Wnt/β-catenin signaling is essential for the lens formation and further provided the evidence that this inhibition is mediated by the transcription factor Pax6. It can be documented by the aberrant Wnt/β-catenin signaling activity accompanied by the loss of expression of Wnt/β-catenin inhibitors Sfrp1, Sfrp2 and Dkk1 in the presumptive lens ectoderm of $Pax6^{-/-}$ optic rudiment. In addition, chromatin immunoprecipitation and gene reporter assays revealed that expression of Sfrp1, Sfrp2 and Dkk1 is directly controlled by Pax6. This indicates that Pax6 plays the central role in the control of Wnt/β-catenin activity in the lens-forming SE.

My contribution to this work: I have performed chromatin immunoprecipitation experiment which results are depicted in Figure 5 of published paper Machon et al., 2010 (presented on pages 33 – 42 of this Thesis).

genesis 48:86–95 (2010)

ARTICLE

Lens Morphogenesis is Dependent on Pax6-Mediated Inhibition of the Canonical Wnt/Beta-Catenin Signaling in the Lens Surface Ectoderm

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Summary: Lens formation in mouse is critically dependent on proper development of the retinal neuroectoderm that is located close beneath the head surface ectoderm. Signaling from the prospective retina triggers lens-specific gene expression in the surface-ectoderm. Supression of canonical Wnt/β-catenin signaling in the surface ectoderm is one of the prerequisites for lens development because, as we show here, ectopic Wnt activation in the retina and lens abrogates lens formation. Wnt inhibiton is mediated by signals coming from the retina but its exact mechanism is unknown. We show that Pax6 directly controls expression of several Wnt inhibitors such as Sfrp1, Sfrp2, and Dkk1 in the presumptive lens. In accordance, absence of Pax6 function leads to aberrant canonical Wnt activity in the presumptive lens that subsequently impairs lens development. Thus Pax6 is required for down-regulation of canonical Wnt signaling in the presumptive lens ectoderm. genesis 48:86–95, 2010. **© 2009 Wiley-Liss, Inc.**

Key words: Wnt/β-catenin signaling; Pax6; lens

INTRODUCTION

Proper eye development depends on exact coordination of the formation of two main tissues in the eye ball: the retina and the lens (Bailey *et al*., 2006; Chow and Lang, 2001). The first indication of the vertebrate eye development is an evagination of the diencephalon toward the surface ectoderm to form the optic vesicle. Lens competent head surface ectoderm responds to signals from the optic vesicle which induces columnar thickening of the surface epithelium to form the lens placode. As the optic vesicle comes into contact with the surface ectoderm the optic vesicle becomes partitioned into the neural retina, the optic stalk, and the retinal pigment epithelium (RPE). Genetic analysis has identified a series of transcription factors and signaling pathways governing early stages of the eye development (Chow and Lang, 2001). This includes, among others, an evolutionarily highly conserved transcription factor Pax6 that has been considered as a key regulator of the eye development already in metazoans (Kozmik, 2005). *Pax6* mutations are associated with aniridia in humans and the small eye (Sey) phenotype in mice (Hanson and Van Heyningen, 1995). *Pax6* is expressed both in the retina and in the lens placode and *Pax6*-deficient embryos fail to develop the lens placode while the retina does not invaginate to form the optic cup (Grindley *et al*., 1995; Hogan *et al*., 1986). Conditional ablation of the *Pax6* in the lens placode revealed intrinsic function of *Pax6* the lens (Ashery-Padan *et al*., 2000). The expression of *Pax6* in lens is driven by an ectoderm enhancer (EE) (Kammandel *et al*., 1999; Williams *et al*., 1998). Targeted deletion of EE is accompanied with distinctive defects at every stage of lens development (Dimanlig *et al*., 2001). In addition, exact dosage of the Pax6 protein is required for lens placode formation (Collinson *et al*., 2000; Davis-Silberman *et al*., 2005; van Raamsdonk and Tilghman, 2000). The *Pax6* expression is maintained throughout retina development, from early formation of the optic vesicle to specification of the neuroretina and differentiation and timing of distinct retinal cell types. It has been shown that Bmp and Fgf signaling have key roles in lens development among others by controlling the expression of *Pax6* (Faber *et al*., 2001; Furuta and Hogan, 1998; Gotoh *et al*., 2004; Lovicu and McAvoy, 2005; Wawersik *et al*., 1999). Signaling through the canonical Wnt/β-catenin pathway leads to stabilization of

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the cytoplasmic β-catenin which then associates with the transcription factors belonging to the TCF/Lef family, and it results in the upregulation of TCF/Lef-dependent gene transcription. Wnt/β-catenin pathway entails a large set of molecules: multiple Wnt ligands and Frizzled receptors, LRP coreceptors, various soluble inhibitors as well as positively and negatively acting intracellular components (http://www.stanford.edu/~rnusse/wntwindow.html). To integrate such a complex regulation and to follow Wnt/β-catenin signaling in vivo, constructs with multimerized TCF/Lef binding sites have been used to generate transgenic reporters (DasGupta and Fuchs, 1999; Liu *et al*., 2003; Maretto *et al*., 2003). In the developing mouse eye, β-catenin mediated Wnt/β-catenin signaling is first active in the dorsal optic vesicle and later in restricted areas of the periocular mesenchyme, optic stalk, and in the dorsal retinal pigmented epithelium (RPE). In contrast, the neural retina and the lens are mostly negative for Wnt/β-catenin signaling activity with the exception of the ciliary margin (Fuhrmann *et al*., 2009; Kreslova *et al*., 2007; Liu *et al*., 2006; Maretto *et al*., 2003; Smith *et al*., 2005). Ectopic lentoid bodies were observed upon lens-specific deletion of β-catenin in the periocular region, which is responsive to Wnt/β-catenin signaling (Kreslova *et al*., 2007; Smith *et al*., 2005). These observations indicated that Wnt/β-catenin pathway acts within the periocular ectoderm to inhibit lens formation. Consistent with this, stabilization of β-catenin in central ocular ectoderm abbrogates lens induction (Smith *et al*., 2005). In $Rx^{-/-}$ mutants, the retina is not formed and this subsequently leads to abrogation of lens development (Mathers and Jamrich, 2000; Mathers *et al*., 1997). Since *Rx* is expressed only in the retina but not in the lens, it shows that retinal-specific gene expression is necessary for lens formation. Interestingly, it was shown recently that lens formation can be restored in *Rx*-deficient embryos experimentally, by the elimination of β-catenin expression in the head surface ectoderm (Swindell *et al*., 2008). These data further support the idea of a negative role of β-catenin in lens specification either through its function as a component of Wnt/β-catenin signaling or through its function in cell adhesion. Recently, conditional mutations of β-catenin and APC revealed roles for canonical Wnt/β-catenin signaling at later stages of lens development, namely during fiber cell differentiation (Martinez *et al*., 2009).

Here we describe a mutually antagonistic genetic interaction between the transcription factor Pax6 and Wnt/β-catenin signaling pathway occurring during the early stages of lens induction.

RESULTS

Ectopic Canonical Wn/β-Catenin Signaling in the Lens Ectoderm Suppresses Induction of Lens Fate

Previous studies suggested that formation of the lens placode within the surface ectoderm is strictly dependent on the absence of Wnt activity in the surface epithelium (Kreslova *et al*., 2007; Smith *et al*., 2005). To explore this idea further, we performed conditional activation of the canonical Wnt/β-catenin signaling in the embryonic mouse lens as well as in the retina by crossing LR-Cre mice (Kreslova *et al*., 2007) with $Catnb^{lox(ex3)}$ mice (Harada *et al*., 1999). The animals with the combined alleles are referred to as LR-Cre;$Catnb^{lox(ex3)}$. In the LR-Cre transgene construct, three copies of *Pax6* lens enhancer (EE) were cloned upstream of the Cre gene (Fig. 1c) (Kreslova *et al*., 2007). To visualize the activity of the LR-Cre transgene, LR-Cre mice were crossed with the R26R reporter mice (Soriano, 1999) which resulted in activation of β-galactosidase by Cre. As seen in Figure 1a, LR-Cre directs expression in the lens ectoderm and also in the surrounding head surface ectoderm from embryonic day (E) 9.5. At E10.5, LR-Cre activity expands to the involuted lens vesicle, the retina, and the presumptive cornea (Fig. 1b).

The activation of the canonical Wnt/β-catenin signaling in LR-Cre;$Catnb^{lox(ex3)}$ embryos was visualized in the BAT-gal reporter mouse with a triple combination of LR-Cre;$Catnb^{lox(ex3)}$; BAT-gal transgenes. BAT-gal reporter gene contains multiple TCF-binding sites coupled to a minimal promoter with the β-galactosidase gene and thus it serves as a specific and sensitive reporter of the canonical Wnt activity (Maretto *et al*., 2003). As expected, Wnt/β-catenin signaling was strongly induced in the area of the LR-Cre activity, i.e., in the head surface ectoderm including the lens placode after E9.5 (Fig. 2e–f). In contrast, wild type embryos exhibited Wnt reporter activity only in the dorsal optic vesicle (red arrow in Fig. 2e left) while the central optic vesicle and the lens placode showed no signs of the signal. Already at E9.5 stage, the lens placode was thinner in the LR-Cre;-$Catnb^{lox(ex3)}$ mutants (Fig. 2a,f; right panels) and development of the whole eye was severely disrupted at E11.5 and later (Fig. 2b–d,g–h). The lens was completely missing while the retina did not fold in around the missing lens and thus it was misshapen. Further, we used immunofluorescence staining with various lens markers to determine whether the lens fate was truly suppressed upon aberrant activation of the Wnt/β-catenin pathway. *Pax6* is normally expressed in the lens placode at E9.5 but its expression was greatly reduced in LR-Cre;$Catnb^{lox(ex3)}$ mutants (Fig. 3a). At E10.5 and E13.5, *Pax6* expression was detected in the misshapen retina but reduced in presumptive lens tissue (Fig. 3b,c). Similarly, Meis1 and Meis2, transcription factors acting upstream of Pax6 (Zhang *et al*., 2002), are specifically expressed in the surface ectoderm including the lens placode at E9.5. While the level of *Meis1* expression remains similar in the LR-Cre;$Catnb^{lox(ex3)}$ mutants, the level of *Meis2* expression appears to be reduced (Fig. 3d,e). Prox1 staining (Duncan *et al*., 2002; Wigle *et al*., 1999) at E10.5 revealed few cells in the severely affected placode that showed lens cell fate specificity (Fig. 3f, upper panel). The phenotype in the LR-Cre;$Catnb^{lox(ex3)}$ mutants varied among tested animals from almost com-

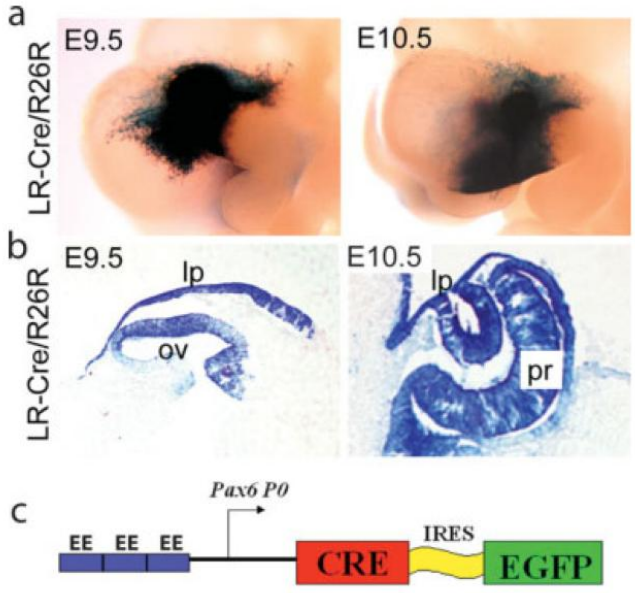

FIG. 1. Cre activity in the LR-Cre transgenic mouse. (**a**) The LR-Cre activity was monitored in the R26R reporter mouse crossed to LR-Cre with a strong staining in the lens ectoderm and the surrounding face ectoderm. (**b**) Sections of LR-Cre; R26R eyes showing the Cre activity in developing lens and retina. (**c**) Schematic depiction of the LR-Cre transgenic construct. EE, ectoderm enhancer; lp, lens placode; ov, optic vesicle; pr, presumptive retina.

plete absence to a partial reduction of the lens fate shown by the Prox1 immunofluorescence, depending on the level of Cre recombinase activity in a given embryo. However, immunostaining for β-crystallin lens marker revealed no signal (Fig. 3f, lower panel). Finally, α-crystallin is strongly expressed in the lens from E10.5, whereas no α-crystallin positive cells were detected in the LR-Cre;Catnb$^{lox(ex3)}$ mutants at E13.5 (Fig. 3g). These data altogether support the idea that Wnt/β-catenin signaling impairs formation of the lens as proposed by Smith *et al.*, (2005) and us (Kreslova *et al.*, 2007). Moreover, our results suggest that ectopic Wnt/β-catenin signaling disrupts lens induction upstream of Pax6 and Meis2.

Pax6 Regulates Expression of Wnt Inhibitors *Sfrp1* and *Sfrp2*

Our data shown above and the experiments by Smith *et al.* (2005) clearly document that induction of lens development is not compatible with active Wnt/β-catenin pathway. On the other hand, several *Wnt* genes and components of this pathway are expressed in the developing eye during the critical period of lens induction (Ang *et al.*, 2004; Chen *et al.*, 2004; Liu *et al.*, 2003; Stump *et al.*, 2003). Two independent mouse Wnt reporter lines TOP-GAL and BAT-gal show, however, absence of the canonical Wnt activity in the lens placode

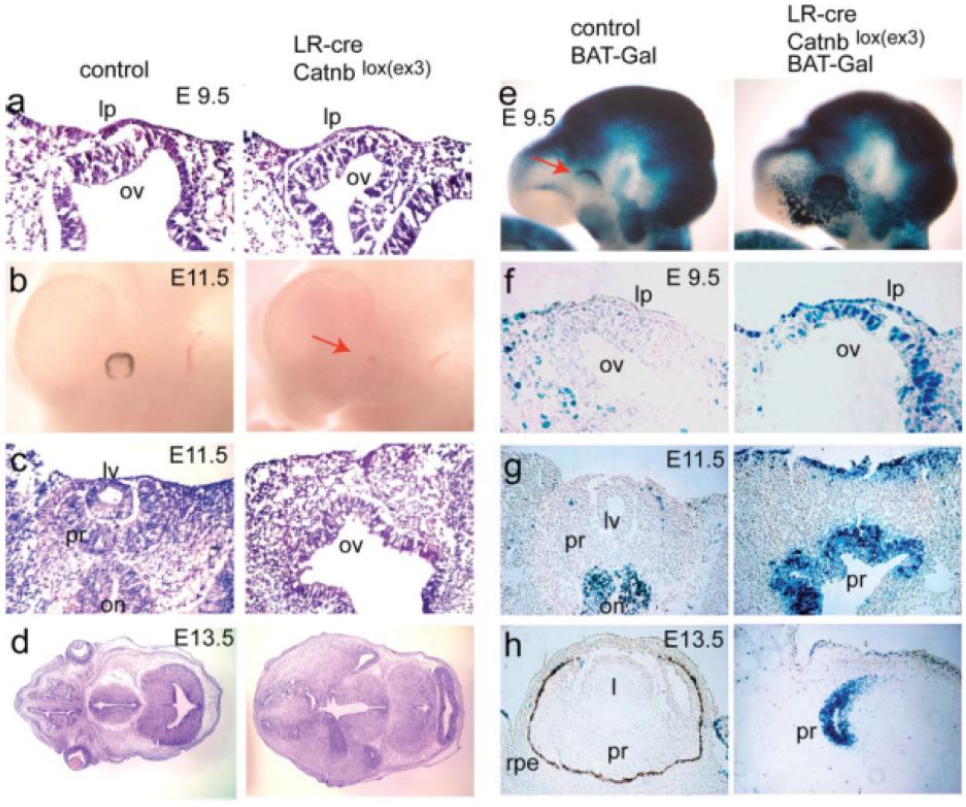

FIG. 2. Aberrant canonical Wnt/β-catenin signaling inhibits proper lens development. (**a**) Hematoxylin-eosin staining of the lens placode in controls and LR-Cre;Catnb$^{lox(ex3)}$ mutants showing thinner placodal layer at E9.5. (**b**) Missing lens in LR-Cre;Catnb$^{lox(ex3)}$ mutants at E11.5 with hematoxylin-eosin-stained section through the eye field (**c**). (**d**) Hematoxylin-eosin staining of E13.5 stage of wild type and LR-Cre; Catnb$^{lox(ex3)}$ mutant embryos. (**e**) High expression of BAT-gal Wnt reporter gene (blue) in LR-Cre; Catnb$^{lox(ex3)}$; BAT-gal compound mice in the surface ectoderm at E9.5. (**f**) Horizontal sections of the eye field of embryos shown in (e). (**g,h**) Ectopic activation BAT-gal (blue) in the retina and the surface ectoderm at E11.5 and E13.5 (h). l, lens; lp, lens placode; lv, lens vesicle; ov, optic vesicle; pr, presumptive retina; rpe, retinal pigment epithelium.

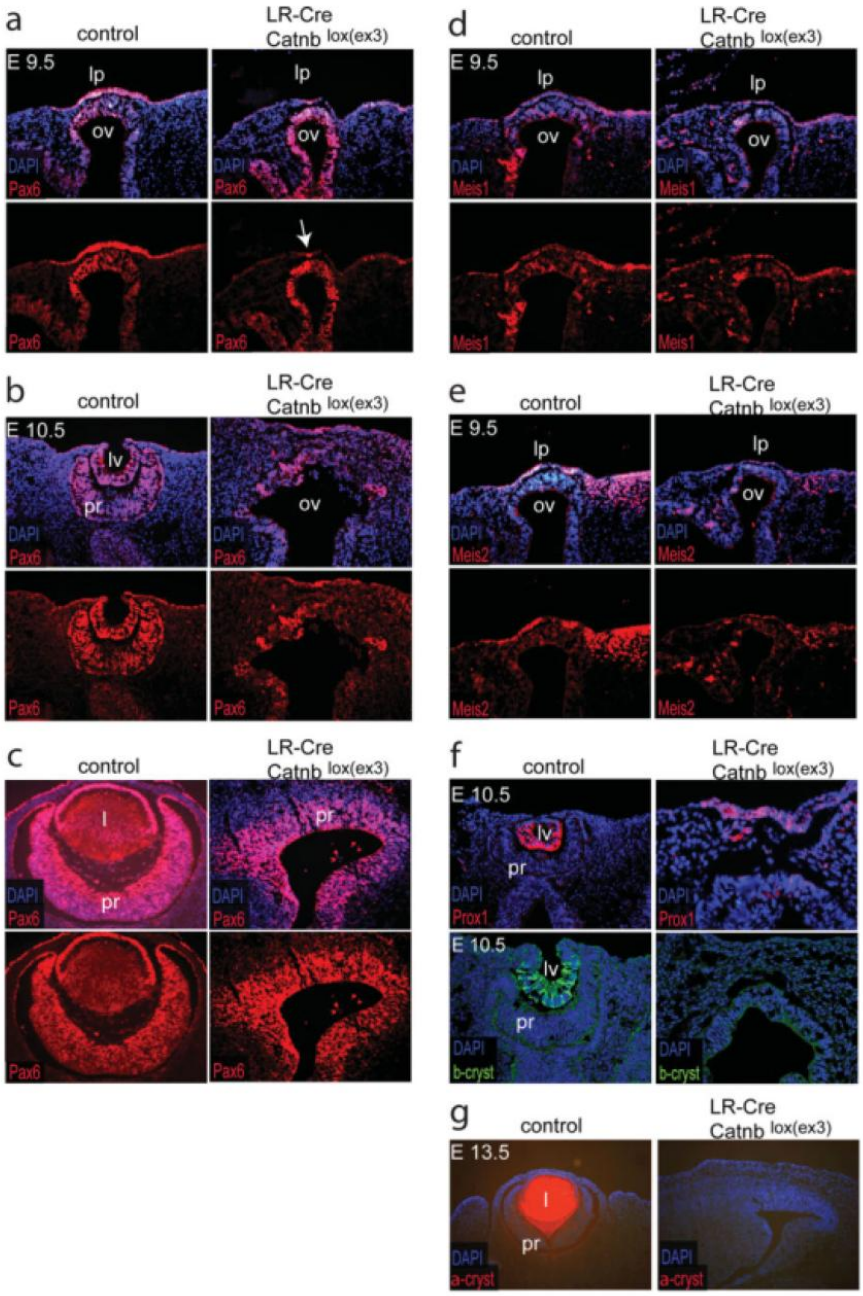

FIG. 3. Aberrant canonical Wnt/β-catenin signaling inhibits lens cell fate. Fluorescent immunostaining of various lens markers (in red or green) in LR-Cre;Catnb$^{lox(ex3)}$ mutants and controls with parallel DAPI counterstaining (in blue). (**a**) Pax6 expression at E9.5, at E10.5 (**b**) and at E13.5 (**c**). (**d**) Meis1 expression at E9.5. (**e**) Meis2 expression at E9.5. (**f**) Prox1 and β-crystallin expression at E10.5 (**g**) α-crystalin expression at E13.5. l, lens; lp, lens placode; lv, lens vesicle; ov, optic vesicle; pr, presumptive retina.

(Kreslova *et al.*, 2007; Smith *et al.*, 2005). This may be explained by a lack of appropriate Wnt receptors or other mediators in the lens, or by an active suppression of Wnt signals at various levels of the pathway. It is well known that Pax6 is a master gene during eye development (Kozmik, 2005). We therefore tested the possibility whether Pax6 may influence Wnt signaling by directing expression of some negative regulators of this pathway. In the developing telencephalon, *Pax6* is required for the expression of *Sfrp2* (Kim *et al.*, 2001) and recently, a similar dependence of *Sfrp2* on *Pax6* was reported in the embryonic eye (Duparc *et al.*, 2006). We performed whole mount in situ hybridization of Sfrp1 and Sfrp2 mRNA on wild type and Small eye (Sey/Sey; *Pax6*-null mutant) embryos. Both *Sfrp1* and *Sfrp2* are not detected in the optic cup at E9.5 and in the eye at E10.5 of Sey/Sey embryos (see Fig. 4). The absence of *Sfrp1* and *Sfrp2* signal cannot be explained by morphological changes in the eye of Sey/Sey mouse mutants between E9.5 and E10.5 because, for instance, *Sfrp1* mRNA is not detected also in the telencephalon of Sey/Sey and *Sfrp2* is absent in the spinal cord (arrows in Fig. 4). These tissues have only minor morphological defects in Sey/Sey at this stage that cannot account for the lack of expression. Normal

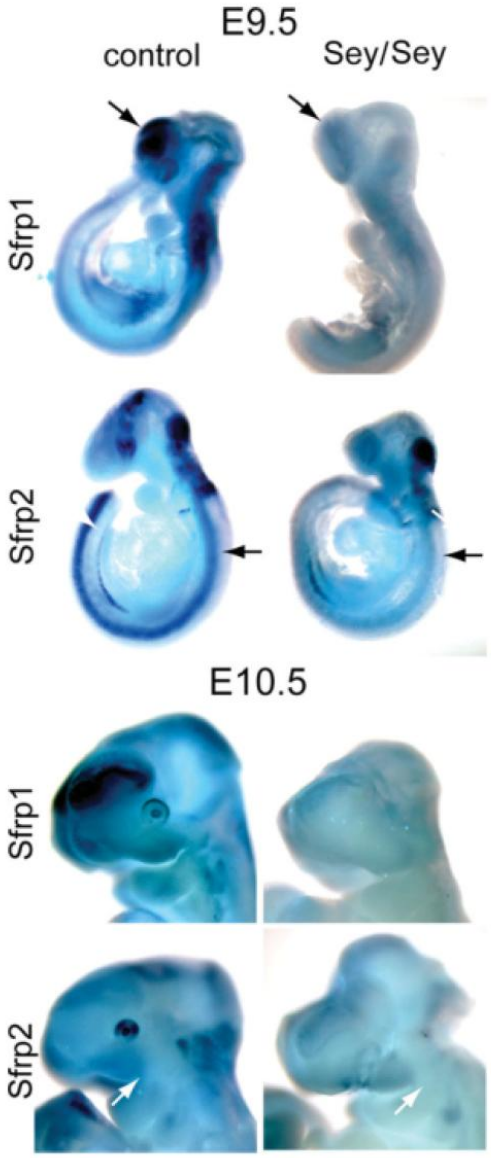

FIG. 4. Expression of *Sfrp1* and *Sfrp2* in wild type and Sey/Sey (Pax6-null) embryos by whole mount in situ hybridization. *Sfrp1* mRNA at E9.5 and E10.5 is normally seen in the optic vesicle and in the telencephalon of wild type embryos while in Sey/Sey embryos the signal disappears in both tissues. *Sfrp2* transcripts are detected in the optic vesicle, the midbrain and the spinal cord of wild type embryos but are lost in Sey/Sey mutants.

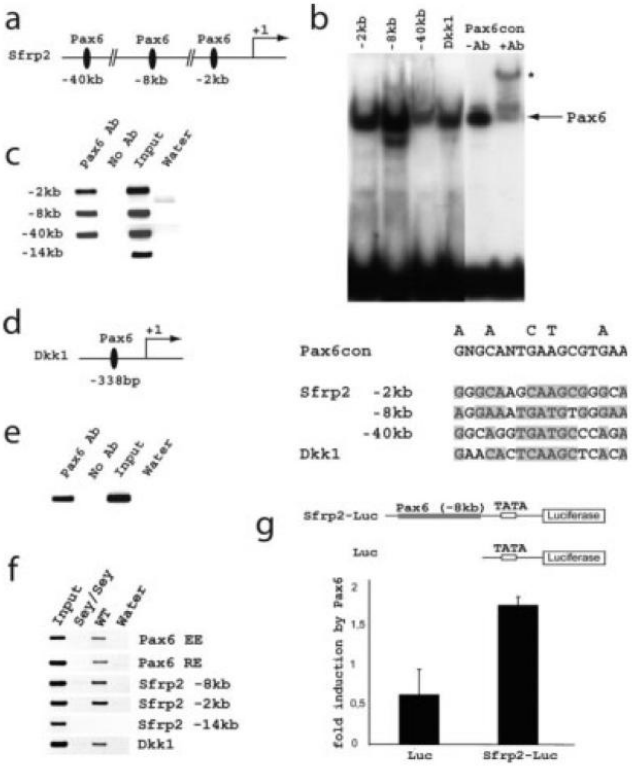

FIG. 5. Pax6 regulates transcription of *Sfrp2* and *Dkk1*. (**a**) Three putative binding sites for Pax6 reside in the *Sfrp2* gene. (**b**) Electrophoretic mobility shift assay showing that Flag-Pax6 binds to all three binding sites in the *Sfrp2* gene and to a binding site in *Dkk1* gene. Supershift using anti-Flag antibody is shown in the right lane. DNA sequence alignment of Pax6 binding sites is shown bellow. (**c**) Chromatin immunoprecipitation with anti-Pax6 antibody showing direct binding of Pax6 to three sites in the *Sfrp2* gene. (**d**) Pax6 binding site is present in the Dkk1 promoter. (**e**) Chromatin immunoprecipitation with anti-Pax6 antibody showing direct binding of Pax6 to a site in *Dkk1* promoter. (**f**) Chromatin immunoprecipitation with anti-Pax6 antibody showing no detectable signal in *Sey/Sey* ((Pax6-null) embryos. Previously characterized Pax6 binding sites (Mui *et al*., 2005) are used as controls (*Pax6* ectoderm enhancer, *Pax6* EE; *Pax6* retinal α-enhancer, *Pax6* RE). (**g**) Luciferase assay showing that Pax6 positively regulates expression upon binding to DNA fragment from *Sfpr2* gene.

levels of *Sfrp1* and *Sfrp2* are detected in Sey/+ heterozygote embryos (data not shown).

Does Pax6 control Wnt inhibitors directly? To answer this question, we tested for the presence of Pax6 binding sites in regulatory sequences of several known Wnt inhibitor genes. Computer analysis revealed three putative Pax6 binding sites at positions -40, -8, and -2 kb upstream of the transcription start site of the *Sfrp2* (Fig. 5a). Further, the electrophoretic mobility shift assay (EMSA) documented that Pax6 binds to respective binding sites in the *Sfrp2* locus in vitro (Fig. 5b) while chromatin immunoprecipitation assays using anti-Pax6 antibody confirmed Pax6 binding to the binding sites *in vivo* (Fig. 5c). We next tested a transcriptional effect of Pax6 binding to DNA in the *Sfrp2* locus by luciferase reporter assays. A 250-bp fragment including Pax6 binding site at position -8 kb upstream from the *Sfrp2* start site was cloned into a luciferase construct with a minimal TATA box containing promoter (Sfrp2-Luc). Coexpression of the *Pax6* cDNA with the *Sfrp2*-Luc plasmid increased the reporter activity approximately three-fold (Fig. 5g). Analysis of other Wnt inhibitor genes showed that the *Dkk1* locus contains a Pax6 binding site 338 bp upstream of the transcription start (Fig. 5d). Pax6 binds to this site both in vitro, as shown by the EMSA (Fig. 5b), and also in vivo as shown by chromatin immunoprecipitation assay (Fig. 5e). No positive signal in chromatin

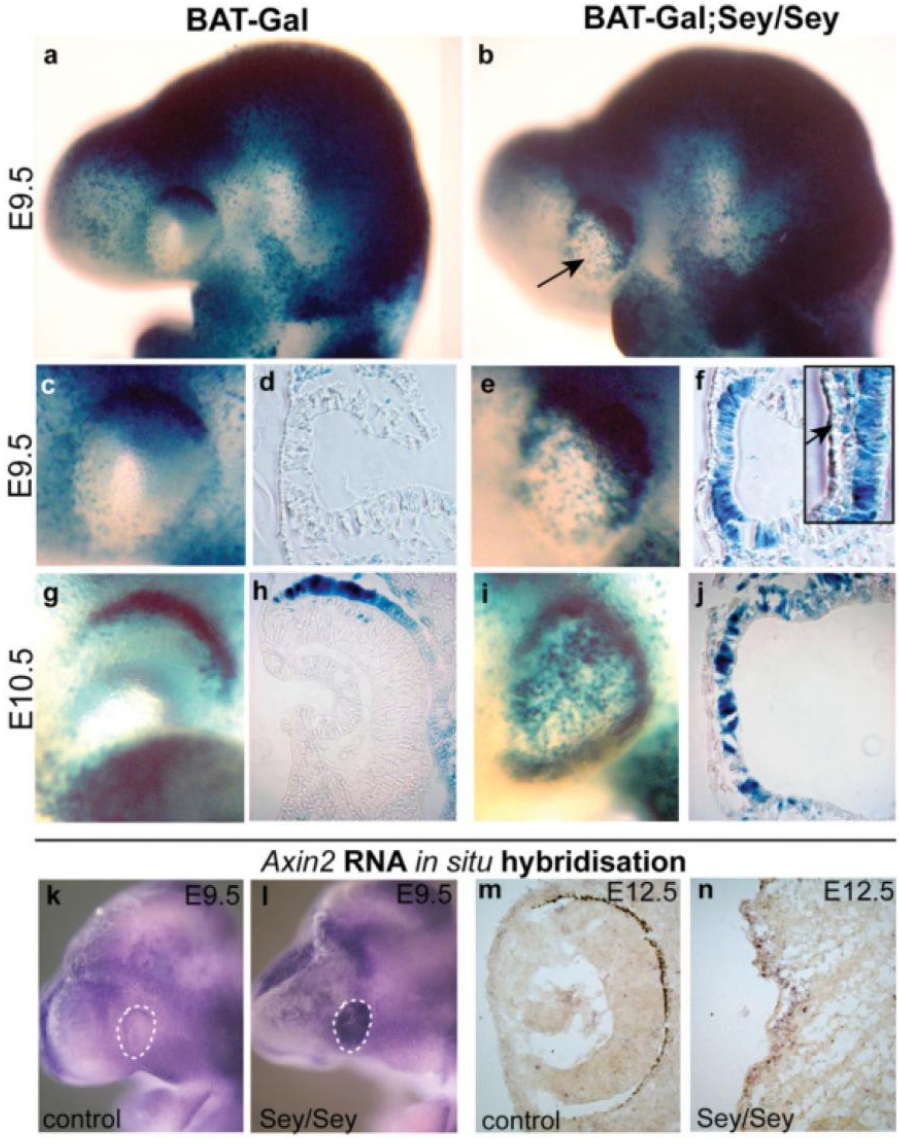

FIG. 6. Pax6 is involved in down-regulation of canonical Wnt//β-catenin signaling in the eye field. (**a–j**) Canonical Wnt activity was monitored using BAT-gal transgenic mice. (a,b) Whole-mount β-galactosidase staining of BAT-gal embryos at E9.5 in controls (a) and BAT-gal;Sey/Sey (b). (c,e) Detail of the eye field as in (a,b), note stronger staining of BAT-gal in Sey/Sey mutant background. (d,f) Sections of the lens placode with the underlying retina in BAT-gal controls (d) and BAT-gal;Sey/Sey (f). Inset in (f) shows a higher magnification of the lens placode and retina with the ectopic Wnt/β-catenin signaling. The arrow in the inset marks Wnt activation in the lens placode. (g–j) Whole-mount staining and sections of the eye at E10.5 in BAT-gal controls (g,h) and in BAT-gal;Sey/Sey (i, j). (**k–n**) Detection of *Axin2* mRNA in developing eyes of E9.5 and E12.5 wild type control embryos and Sey/Sey mutants showing ectopic activation of expression (oval dashed circle delineates the eye region in E9.5 embryos).

immunoprecipitation assays was obtained using Sey/Sey (Pax6-null) embryos (Fig. 5f). Taken together, these findings suggest that Pax6 may directly control transcription of several Wnt inhibitor genes in the developing eye.

Canonical Wnt/β-Catenin Signaling is Activated in the Embryonic Eye in the Absence of Pax6

Provided that active downregulation of the Wnt/β-catenin signaling is mediated by Pax6 during eye development, inactivation of Pax6 would be predicted to lead to ectopic Wnt activity in this tissue. We examined this possibility by crossing BAT-gal Wnt reporter mouse into Sey/Sey mutants. Embryos at E9.5 and E10.5 were stained for β-galactosidase enzymatic activity reflecting the canonical Wnt/β-catenin signaling in the BAT-gal mouse. As shown in Figure 6a,c,d, the optic cup is negative for the Wnt activity in control animals at E9.5 except for the epidermis forming a future dorsal eyelid. In BAT-gal; Sey/Sey littermates, however, many lens placodal cells (an arrow in the inset in Fig. 6f) as well as retinal progenitors within the eye field express β-galactosidase (Fig. 6b,e,f). At E9.5, the lens placode appears still intact in Sey/Sey embryos but it gradually disappears at E10.5 and later. In BAT-gal transgenic mice at E10.5, both the retina and the lens are again negative for β-galactosidase activity while in the Sey/Sey mutants, strong activation of Wnt reporter gene was seen in the eye field. The formation of the lens placode was compromised at this stage and Wnt-positive retina did not fold inward in Sey/Sey embryos. The expression of BAT-gal reporter gene in Sey/+ heterozygote embryos appeared unchanged as compared to wild type embryos (data not shown). BAT-gal reporter gene represents a sensitive yet artificial sensor of Wnt/β-catenin signaling. To further confirm that Wnt/β-catenin signaling is ectopically activated in Sey/

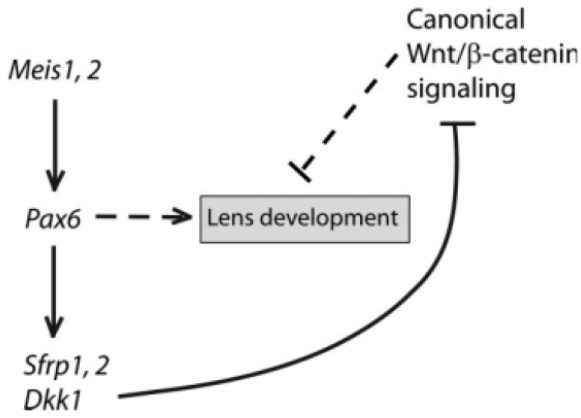

FIG. 7. Schematic diagram of the relationship between Pax6 transcriptional network and Wnt/β-catenin signaling depicting their positive and negative roles in lens development.

Sey mutant embryos we examined expression of *Axin2*, which is a natural target gene and universal readout of Wnt/β-catenin signaling (Jho *et al.*, 2002; Lustig *et al.*, 2002). A significant increase of *Axin2* expression is detected in Sey/Sey mutants at the placode stage of eye development (E9.5; Fig. 6k–l). The presence of *Axin2* mRNA in the aberrant lens surface epithelium of Sey/Sey embryos but not in normal lens at E12.5 (Fig. 6m,n) further confirms ectopic activation of Wnt/β-catenin signaling in the absence of Pax6 function.

In summary, our results suggest that one of the key roles of Pax6 during early eye development is to suppress canonical Wnt/β-catenin signaling by positively regulating genes encoding Wnt inhibitors.

DISCUSSION

In this study we describe a mutually antagonistic genetic interaction between the transcriptional network involving Pax6 and Wnt/β-catenin signaling pathway occurring during lens induction (see Fig. 7). We and others have previously shown that canonical Wnt signaling is active in the head surface ectoderm including the periocular mesenchyme surrounding the eye when it was monitored in TOP-gal or BAT-gal transgenic mice (Fuhrmann *et al.*, 2009; Kreslova *et al.*, 2007; Liu *et al.*, 2006; Smith *et al.*, 2005). The lens and the retina (except the dorsal RPE) were largely devoid of Wnt activity. Interestingly, conditional removal of β-catenin in the head surface ectoderm surrounding the eye with Wnt activity resulted in appearance of ectopic lentoid bodies (Kreslova *et al.*, 2007; Smith *et al.*, 2005). This led to the hypothesis that induction of the lens from the surface ectoderm requires down-regulation of canonical Wnt/β-catenin signaling in the lens placode. We show here that ectopic activation of canonical Wnt/β-catenin signaling in LR-Cre;Catnb$^{lox(ex3)}$ embryos abrogates lens formation which supports the idea that active canonical Wnt/β-catenin signaling is not compatible with proper lens development. Downregulation of Pax6 and Meis2 in LR-Cre;Catnb$^{lox(ex3)}$ embryos suggests that Wnt/β-catenin pathway acts at or upstream of these transcription factors (see Fig. 7). It is intriguing that we have observed a similar downregulation of Pax6 and Meis2 expression in developing telencephalon of D6-CLEF transgenic mice in which an ectopic Wnt signaling is mimicked (Machon *et al.*, 2007). Several Wnt molecules and their Frz receptors are expressed in the optic cup (Ang *et al.*, 2004) suggesting that an active Wnt suppressive mechanism takes place in the developing eye. Indeed, several inhibitors of Wnt pathway are strongly expressed in the optic cup, such as *Sfrp1*, *Sfrp2*, *Dkk1*, *Dkk2*, or *Dkk3* (Ang *et al.*, 2004; Diep *et al.*, 2004; Duparc *et al.*, 2006; Gage *et al.*, 2008). We therefore asked whether any of the major genetic regulators of eye development were involved in regulating canonical Wnt activity. We show that in the absence of Pax6 aberrant Wnt activity is detected in the retina and in the affected lens placode as measured by BAT-gal reporter and *Axin2 mRNA*. Failure of lens specification is well known phenomenon in Sey/Sey mice as well as in mice with a conditional deletion of the *Pax6* gene in the lens (Ashery-Padan *et al.*, 2000). Similar failure of lens fate determination was observed in mutant embryos LR-Cre;Catnb$^{lox(ex3)}$ with ectopic Wnt activity in the lens and retina. Expression of cellular markers such as Pax6, β-crystallin, and α-crystallin was not detected and involution of the optic cup did not occur. In that sense, phenotypic changes in the Pax6−/− lens and LR-Cre;Catnb$^{lox(ex3)}$ lens are similar. Does Pax6 have a direct role in suppressing Wnt activity in the lens primordium? In Sey/Sey mice, the expression of *Sfrp1* and *Sfrp2* disappeared both in the central nervous system and in the eye suggesting that Pax6 regulates these genes. Indeed, *Sfrp2* was absent in the developing eye (Duparc *et al.*, 2006) and in the embryonic forebrain (Kim *et al.*, 2001) in Sey/Sey mutant mice. We further show that Pax6 transcription factor binds to multiple regions in the *Sfrp2* gene and that it positively regulates expression *Sfrp2* reporter gene in transfection assays. *Sfrp1* expression remarkably overlaps with Pax6 in the eye and in the developing cortex (data not shown) indicating again a direct control of transcription of *Sfrp1*. Moreover, Pax6 binds to promoter of the *Dkk1* gene both in vitro and in vivo. Taken together these data suggest that Pax6 regulates expression of several Wnt inhibitors in the embryonic eye to ensure efficient suppression of canonical Wnt activity (see Fig. 7). It is likely that soluble Wnt inhibitors such as Sfrp1/2 or Dkk1 that are expressed in the retina suppress canonical Wnt/β-catenin signaling in the surrounding tissue, in particular in the lens where absence of Wnts is critical for lens cell specification. Furthermore, it has been shown previously that Wnt pathway coreceptor Lrp6 becomes sharply downregulated in the lens placode (and optic vesicle) between E9.5 and E10.5 indicating a negative role of Wnt signaling at the onset of eye development (Smith *et al.*, 2005). From this point of view it is especially interesting to note that Pax6 appears to regulate Wnt inhibitors with distinct modes of action.

Altogether, there appears to be a "multiple assurance" program to achieve an efficient inhibition of canonical Wnt/β-catenin signaling during the early stages of lens development.

Our data thus provide a novel concept of the role of Pax6 in lens development. Pax6 is known to play a positive role in lens fate determination by directly or indirectly regulating key transcription factors Six3 (Ashery-Padan *et al.*, 2000), Mab2l1 (Yamada *et al.*, 2003), Prox1 (Ashery-Padan *et al.*, 2000), and FoxE3 (Blixt *et al.*, 2007; Brownell *et al.*, 2000; Yoshimoto *et al.*, 2005). All available data point at a negative role of Wnt/β-catenin signaling in lens induction (see Fig. 7). We propose that another important role of Pax6 during the early stages of lens formation is to inhibit Wnt/β-catenin signaling by positively regulating expression of various classes of Wnt inhibitors.

EXPERIMENTAL PROCEDURES

Mouse Lines

LR-Cre activity was assayed by crossing LR-Cre mice (Kreslova *et al.*, 2007) with the R26R reporter mouse line (Soriano, 1999). The R26R mice were purchased from Jackson laboratory (stock no. 003309). Sey^{1Neu} loss-of-function allele of Pax6 (Hill *et al.*, 1991) was provided by Dr. J. Favor. To determine the canonical Wnt pathway during eye development, we used BAT-gal reporter line (Maretto *et al.*, 2003; kindly provided by H. Edlund). For ectopic activation of β-catenin-mediated Wnt pathway, $Catnb^{lox(ex3)}$ mice were used (Harada *et al.*, 1999; kindly provided by Dr. M. M. Taketo).

Tissue Collections and Histology

All mouse embryos were obtained from timed pregnant females where the vaginal plug was defined as the embryonic day 0.5 (E0.5).

Embryos were harvested in cold PBS, fixed in either 4% paraformaldehyde for minimum 1 h up to overnight depending on the nature of primary. Tissues were cryoprotected in 30% sucrose in PBS overnight at 4°C, embedded and frozen in OCT (Tissue Tek, Sakura Finetek). A 6–8-μm horizontal crysections were prepared before immunohistochemistry.

X-Gal Staining

The β-galactosidase assay was carried out as described by. After the fixation in 0.4% paraformaldehyde in PBS, cryosections were directly stained with the staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg ml^{-1} X-gal).

For whole-mount staining, fixed embryos were washed three times in the rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris pH 7.3, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40), and incubated for several hours up to overnight at 37°C in staining solution.

Immunohistochemistry

The cryosections were refixed for 10 min in 4% paraformaldehyde, washed with PBS, permeablized with PBS/0.1%Triton X-100 (PBT) for 15 min prior to blocking. Sections were blocked for 3 0min in 10%BSA/PBT, incubated overnight with primary antibodies, washed three times with PBS, incubated 1 h at room temperature with secondary antibodies, washed three times with PBS, and mounted in Vectashield with DAPI (Vector Laboratories). Primary antibodies were used: anti-α-crystallin (kind gift from Dr. Sam Ziegler), anti-β-crystallin (kind gift from Dr. Sam Ziegler), anti-Pax6 (Covance), anti-Prox1 (Chemicon), anti-Meis1, and anti-Meis2 (kind gift from Dr. A. Buchberg). Secondary antibodies were used: anti-mouse or anti-rabbit Alexa488 or 594 (Molecular Probes).

In Situ Hybridization

In situ hybridization on 8-μm thick cryosections was carried out according to standard protocols and hybridization was incubated overnight at 68°C. Plasmids for antisense probes Sfrp1/2 were kindly provided by S. Pleasure. Axin2 antisense probe was prepared using mouse Axin2 cDNA cloned into pBluescript.

Luciferase Assay

A 250-bp DNA fragment from Sfrp2 gene containing Pax6 consensus binding site was cloned into pGL3-TATA. This reporter plasmid was cotransfected into HEK293 cells with a mouse Pax6 expression vector. Luciferase activity was measured 48 hours after transfection according to manufacture's protocol (Promega).

Electroforetic Mobility Shift Assay (EMSA)

EMSA with the full-length FLAG-tagged Pax6 was performed using double-stranded oligonucleotides comprising Pax6 binding sites from mouse *Sfrp2*, *Dkk1* genes in comparison with Pax6 consensus binding site. Supershift experiment was performed by incubating protein-DNA complexes in the presence of anti-Flag M2 antibody (Sigma) prior to loading onto 6% gel.

Chromatin immunoprecipitation

A chromatin immunoprecipitation assay was performed according to the manufacturer's protocol (Upstate Biotech) with modifications. The head region of E12.5 C57Bl6 or Sey^{1Neu}/Sey^{1Neu} embryos was homogenized in 1% formaldehyde in phosphate-buffered saline and cross-linked at 37°C for 15 min. Cross-linking was stopped by adding glycine (0.125 M) and incubating at room temperature for 5 min. Cross-linked cells were washed twice with cold phosphate-buffered saline containing fresh protease inhibitors, pelleted, and resuspended in 2 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) with protease inhibitors. Samples were incubated on ice for 10 min, and lysates were sonicated on an ice water bath to produce 150–500 bp of DNA fragments. Cell debris was removed by centrifugation for 10 min at

14,000 rpm at 4°C, and the supernatant was diluted ten times with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) containing protease inhibitors. Thirty micrograms of sonicated chromatin was precleared with 50 μl of protein A(G)/agarose slurry (Upstate Biotech) for 1 h at 4°C. Beads were pelleted by centrifugation for 5 min at 3,000 rpm at 4°C. The supernatant was incubated either with 5 μg of anti-Pax6 antibody (Covance) or with no antibody (no antibody control) overnight at 4°C. Thirty microliter of protein A(G)/agarose slurry (Upstate Biotech) was added, and samples were rocked at 4°C for 1 h. After washing for 5 min at 4°C twice in low salt buffer (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), twice in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), four times in LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate,1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), immunocomplexes were eluted twice with 100 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS) for 15 min at room temperature. Immunoprecipitated DNA was decrosslinked overnight at 65°C in the presence of proteinase K (0.06 U μl^{-1}, Roche Applied Science) and 250 mM NaCl. Samples were purified using a MinElute reaction cleanup kit (Qiagen), and 1/20th of eluate was used for PCR. PCR was performed as follows: 95°C 2 min for 1 cycle; then 95°C 30 s, 60°C 30 s, and 72°C 30 s for 40 cycles; and finally 72°C 5 min. The primers used for PCR analyses were as follows:

Sfrp2/-2kb: 5′TGTCACACAAGCATGCAATTGGC,
5′CGCGGGCCTAGGCATTGTTTG,
Sfrp2/-8kb: 5′CTTGCTTGCGTTTTCTCACAT,
5′TACACAAGGAATTCACATGGC,
Sfrp2/-14kb: 5′CCTAATGGGGTCAGTTCCTCC,
5′GGACACATGACAGGACACCAG,
Sfrp2/-40kb: 5′GGAAGCAGCCTTCTGAGCAGAG,
5′CCAGCTGTCTAGAAGGGATTAG,
Dkk1: Dkk1-52 5′AAGTCCTCCCTGCTTCCGAC,
Dkk1-31 5′GGTGGAGTCTCTGGCTGCCA,
Pax6 EE: 5′CTAAAGTAGACACAGCCTT,
5′GGAGACATTAGCTGAATTC,
Pax6 RE: 5′AGTGACAAGGCTGCCACAAG,
5′ CTCCTGATAAATTGACTCCAG.

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6.2 Generation of mRx-Cre transgenic mouse line for efficient conditional gene deletion in early retinal progenitors.

Early eye morphogenesis is driven by coordinated formation of the OC and the lens. In the last decades, studies where classical mouse knockouts were used brought valuable information about the impact of different transcription factors on overall eye development. However, inactivation of these factors often leads to embryonic lethality leaving the role of the gene in desired periods of development unresolved. Moreover, OV and SE reciprocal interaction is required for OC formation and some developmentaly essential genes are expressed in both, OV and SE. This fact complicates the interpretation of the phenotypic data obtained using classical knockout strategy. This is particularly the case of Pax6 (Hogan et al., 1986; Hill et al., 1991; Grindley et al., 1995; Walther and Gruss, 1991). To better understand the molecular mechanisms that regulate OV and SE autonomous development, the role of particular factors needs to be evaluated separately in OV and SE. In such situations, Cre/LoxP system is employed.

In this study, we used the regulatory sequences of mouse *Rx* gene to generate *mRx-Cre* line to direct the *Cre* expression to early OV and its derivates. Our analysis showed that *mRx-Cre*-mediated deletion can be observed in developing eye already from E8.5 onwards and later in all RPCs of the newly formed OC. Specificity, early onset and strength of the Cre-recombinase expression indicates that *mRx-Cre* line is an optimal tool for gene inactivation exclusively in the developing OV/retina.

My contribution to this work: I have generated the majority of the experimental data and wrote the manuscript of published paper Klimova et al., 2013 (presented on pages 44 – 49 of this Thesis).

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Generation of *mRx-Cre* Transgenic Mouse Line for Efficient Conditional Gene Deletion in Early Retinal Progenitors

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Abstract

During mouse eye development, all retinal cell types are generated from the population of retina-committed progenitors originating from the neuroepithelium of the optic vesicle. Conditional gene inactivation provides an efficient tool for studying the genetic basis of the developing retina; however, the number of retina-specific Cre lines is limited. Here we report generation of the *mRx-Cre* BAC transgenic mouse line in which the expression of *Cre* recombinase is controlled by regulatory sequences of the mouse *Rx* gene, one of the earliest determinants of retinal development. When *mRx-Cre* transgenic mice were crossbred with the *ROSA26R* or *ROSA26R-EYFP* reporter lines, the Cre activity was observed in the optic sulcus from embryonic day 8.5 onwards and later in all progenitors residing in the neuroepithelium of the optic cup. Our results suggest that *mRx-Cre* provides a unique tool for functional genetic studies in very early stages of retinal development. Moreover, since eye organogenesis is dependent on the inductive signals between the optic vesicle and head surface ectoderm, the inductive ability of the optic vesicle can be analyzed using *mRx-Cre* transgenic mice.

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Introduction

Mammalian eye organogenesis is a multistep process of complex morphological events that involves interaction of the forebrain-derived optic vesicle (OV) with lens-competent head surface ectoderm (SE). If sufficient, this interaction leads to the coordinated invagination of both OV and SE resulting in formation of the optic cup (OC) [1]. The inner layer of OC is populated by retinal progenitor cells that further differentiate into seven retinal cell types: ganglion cells, amacrine cells, bipolar cells, horizontal cells, cone and rod photoreceptors and Muller glia cells. In previous three decades, several genes have been identified to play a crucial role in this process using the classical knockout strategies [2,3,4]. However, in some cases inactivation of such genes can lead to very early arrest of the eye development or even to the embryonic lethality, as can be demonstrated for the *Pax6* gene [5,6]. This fact makes the dissection of cell autonomous function of a gene in early retinal progenitors of the OV and OC complicated. Nevertheless, the introduction of Cre-loxP-mediated tissue-specific gene inactivation can bypass embryonic lethality and allow functional study of the developmentally essential genes [7]. To perform conditional gene inactivation in retinal progenitors, utilization of a few *Cre* recombinase-expressing mouse lines has been reported. However, these lines display certain limitations and do not always offer sufficient strength at early stages [8,9,10,11] or the required specificity [12,13].

The homeodomain transcription factor *Rx* gene is one of the earliest genes expressed in the retinal lineage. It has been shown to be activated between embryonic day (E) 7.5 and E8.0 in the anterior neural plate and later strongly expressed in the optic vesicles and ventral forebrain [14,15]. Rx function is essential for vertebrate eye development. Loss of the *Rx* function results in loss of eyes in various vertebrate species [14,15,16,17,18,19,20], suggesting its conserved role in the eye development. The early onset of *Rx* expression in the retinal primordium suggests that the *Rx* locus could be utilized for driving the *Cre* expression at very early stages of retinal development.

One such *Cre*-expressing line, taking advantage of *Rx* expression, has been generated previously [12]. Based on the similarity in the *Rx* expression pattern in Japanese killifish medaka and mouse [20], a 4-kb DNA fragment upstream of the medaka *Rx3* gene has been identified to contain an evolutionarily conserved region that can direct the *Cre* expression in the mouse retina [12]. However, when we reinvestigated this line we found that beside its activity in optic vesicles, it has a much broader scope of activity and this certain nonspecificity may complicate its use. Here, we have taken advantage of the *Rx* expression and have generated two transgenic mouse lines, *MB31-Cre* and *mRx-Cre*, and compared their recombination potential with that of *Rx-Cre*. Our data demonstrate that among the three analyzed lines, *mRx-Cre* represents an ideal tool for gene manipulation in early retinal progenitors as judged by its specificity, strength and early onset.

Results and Discussion

In order to perform gene inactivation specifically in retinal progenitors of the optic vesicle we first reinvestigated the previously generated *Rx-Cre* [12]. The *Rx-Cre* transgene is schematically depicted in Figure 1A. To define the kinetics and pattern of the Cre recombinase activity driven by *Rx-Cre*, transgenic mice were bred with the *ROSA26R* reporter mouse strain to generate *Rx-Cre; ROSA26R* double transgenic animals. These mice enabled detection of the Cre activity and lineage tracing of *Cre*-expressing cells using X-gal staining. Upon Cre recombination, the expression of *LacZ* under the ROSA promoter is activated by the removal of a stop cassette [21]. The *Rx-Cre; ROSA26R* embryos reproducibly showed a broad area of recombination (Figure 2A,D). Already at E9.0 X-gal staining was observed both in OV and in the overall head region, also targeting head surface ectoderm (Figure 2A,A'). By E10.5 expression was detected in the neuroepithelium of the optic cup and also in the invaginating structure of the lens pit (Figure 2D'). At the early stages of vertebrate eye development, some genes are expressed in both SE and OV that interact and induce their mutual development. To study such genes it is therefore essential to perform gene inactivation in both tissues separately in order to dissect their cell autonomous functions. For this reason, Cre activity in the ectodermal compartment can be undesirable. We thus analyzed recombination in ectoderm-derived structures in more detail using the *ROSA26R-EYFP* reporter line [22]. The Cre-mediated recombination in *ROSA26R-EYFP* results in the expression of fluorescent protein *EYFP* which enables a better signal resolution at the single cell level than *ROSA26R*. The analysis of *Rx-Cre; ROSA26R-EYFP* embryos confirmed Cre activity in SE and invaginating lens vesicle (Figure 3A,A'). Expression of *EYFP* also offered the opportunity to determine the degree of mosaic recombination based on the proportion of $EYFP^+$ and $DAPI^+$ retinal progenitor cells. The pattern of *EYFP* expression revealed that at E10.5 a considerable amount of cells residing in the retina escaped to *Rx-Cre*-mediated deletion (Figure 3A').

To establish a genetic tool for directing gene inactivation selectively to the OV compartment, we decided to generate a new *Cre*-expressing line. We employed the same strategy as Swindell *et al* [12], having in mind that a nonspecific expression pattern of the short transgene *Rx-Cre* may depend on its integration site into the genome. The 4-kb DNA fragment upstream of the medaka *Rx3* gene was used to drive the expression of *Cre* and generate *MB31-Cre* (Figure 1B). The coding sequence for fluorescent protein EGFP was linked to *Cre* via the *IRES* sequence for monitoring the recombinase expression. Pronuclear injection yielded nine transgenic founders with three animals exhibiting Cre activity specifically localized in the retina (not shown). One founder was chosen for further analysis using *ROSA26R* and *ROSA26R-EYFP* reporter lines. We were able to detect Cre activity in *MB31-Cre; ROSA26R* embryos as early as at E9.0, specifically in the OV compartment (Figure 2B,B'). At E10.5, as the optic vesicle had invaginated to form the optic cup, the $X\text{-gal}^+$ progeny of *Cre*-expressing cells contributed to cells in the retina and retinal pigmented epithelium (Figure 2E,E'). In all analyzed embryos, no Cre activity was observed in ectodermal derivatives such as SE or lens pit or in other parts of the embryo (Figure 2B,E). This remarkable specificity was also demonstrated using *MB31-Cre; ROSA26R-EYFP* transgenic embryos, where EYFP was specifically localized in the retinal tissue (Figure 3B) and no $EYFP^+$ cells were observed in the invaginating lens pit at E10.5 (Figure 3B'). These results indicate that *MB31-Cre* directed the recombinase activity specifically to the developing retina. However, it should be noted

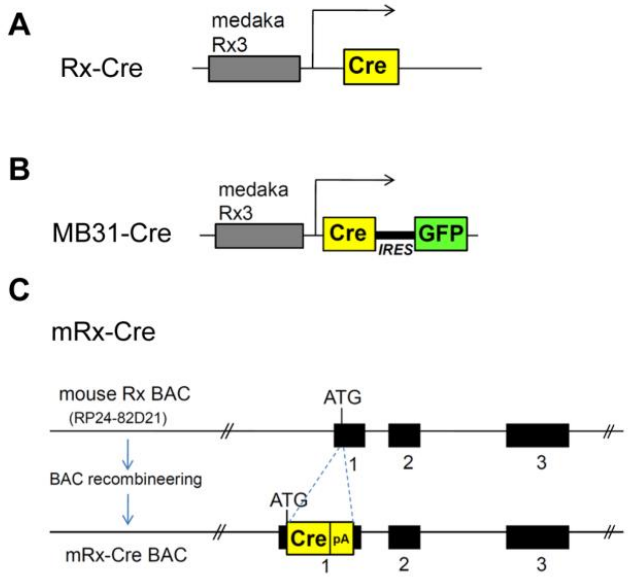

Figure 1. Schematic representation of *Cre* recombinase-expressing transgenic mouse lines used in this study. (A) The *Rx-Cre;* with a 4-kb DNA fragment upstream of the medaka *Rx3* gene driving *Cre* expression [12]. (B) To generate *MB31-Cre,* the 4-kb DNA fragment upstream of the medaka *Rx3* gene was linked to the coding region of *Cre* recombinase. The *IRES* sequence was used to connect *Cre* expression with EGFP to monitor the transgene expression. (C) To generate *mRx-Cre BAC,* a BAC containing 200 kb covering the *Rx* locus was modified by BAC recombineering. The *Cre* coding region (Cre-pA) was inserted into the *Rx* translational initiation start site (ATG). The exons are indicated with black boxes.
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that at E10.5, in both *MB31-Cre; ROSA26R* and *MB31-Cre; ROSA26R-EYFP* embryos, Cre-mediated deletion did not target the whole progenitor cell population since not all retinal cells were $X\text{-gal}^+$ or $EYFP^+$, respectively (Figure 2E' and Figure 3B'). Although the 4-kb DNA fragment upstream of the medaka *Rx3* gene directed the *MB31-Cre* transgene expression specifically to the retina, it did not reconstitute the expression with regard to the onset and strength observed in the mouse *Rx* gene expression [14]. This may be attributed to the differences in the regulation of *Rx* expression in medaka and mice or to the insufficiency of the 4-kb fragment to cover all the regulation of *Rx*. It is possible that additional *cis*-regulatory elements are required for proper spatio-temporal expression of the *Rx* gene in mice. To circumvent these problems we decided to generate another *Cre*-expressing line, referred to as *mRx-Cre,* in which the *Cre* expression is driven by mouse *Rx* gene regulatory sequences.

To generate *mRx-Cre* transgenic mouse we selected a BAC clone (RP24-82D21) containing the entire mouse *Rx* gene as well as 95 kb upstream of the *Rx* translational start site and 100 kb downstream of the locus. We employed the method of BAC recombineering [23] to insert the *Cre* recombinase coding sequence into the first ATG of *Rx* gene (Figure 1C). As the 200-kb BAC clone is supposed to carry all *cis*-regulatory sequences ensuring proper spatiotemporal expression, the expression pattern of *Cre* recombinase should imitate that of the endogenous *Rx* gene. The *Cre*-inserted BAC was used for pronuclear injection to generate *mRx-Cre* transgenic mice. We obtained three founders showing expression in the developing retina (not shown). One of them was chosen for further analysis using the *ROSA26R* reporter line. As already mentioned, endogenous *Rx* expression starts

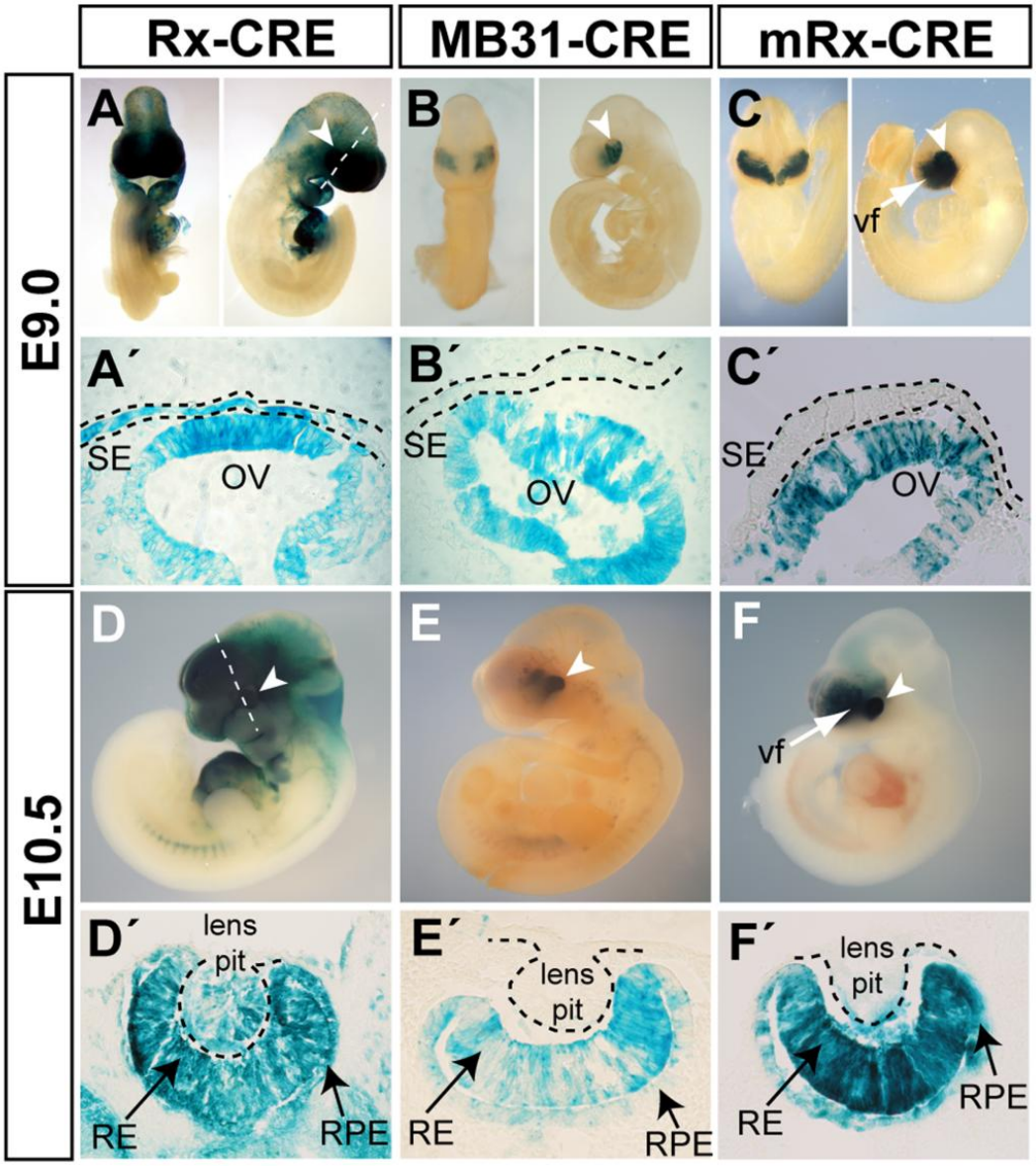

Figure 2. ***In vivo*** **activity of** ***Rx-Cre, MB31-Cre*** **and** ***mRx-Cre*** **transgene products assessed using the** ***ROSA26R*** **line.** Whole-mounts (A–F) or coronal sections (A′–F′) were stained with X-gal at indicated stages to show the Cre activity in the eye primordium (white arrowheads). Surface ectoderm and developing lens are indicated with dashed lines. SE – surface ectoderm; OV – optic vesicle; RPE – retinal pigmented epithelium; RE – retina; vf – ventral forebrain.
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between E7.5 and E8.0 [14]. In agreement, we observed the mRx-Cre activity already from E8.5 in the optic sulcus, albeit with a frequent appearance of mosaic recombination (Figure 4A) of *mRx-Cre; ROSA26R* embryos. This slight delay can be explained by a short intermission between the *Cre* expression onset and recombination seen in *ROSA26R*. Consistent with the expression pattern of the endogenous *Rx* gene [14], strong X-gal staining was observed in the optic vesicles of E9.0 *mRx-Cre; ROSA26R* embryos (Figure 2C). Strikingly, at E10.5, the X-gal$^+$ progeny of *Cre*-expressing cells contributed to all cells of the retina and the majority of retinal pigmented epithelium cells (Figure 2F′). Therefore, *mRx-Cre* directed Cre activity in all optic vesicle derivates, mainly to the forming retina. This almost absolute recombination rate was further confirmed in *mRx-Cre; ROSA26R-EYFP* eyes at E10.5 showing that virtually all retinal progenitor cells were EYFP$^+$ (Figure 3C′). The observation that *mRx-Cre* targets all retinal progenitors in the early stages of eye development was further documented by uniform X-gal staining of all cellular layers of the *mRx-Cre; ROSA26R* adult retina (Figure 4E,E′). Importantly, no EYFP$^+$ cells were observed in the invaginating lens pit of *mRx-Cre; ROSA26R-EYFP* embryos (Figure 3C′). In addition, no cells showing recombination were observed in other parts of the embryo than eye, ventral forebrain and hypothalamus. Beside strong β-galactosidase activity in OV-derived structures, a strong although mosaic activity was found in the ventral part of forebrain and in prospective hypothalamus (Figure 4B–D′). Although strong X-gal staining was observed in E15.5 hypothalamus and forebrain after whole-mount staining

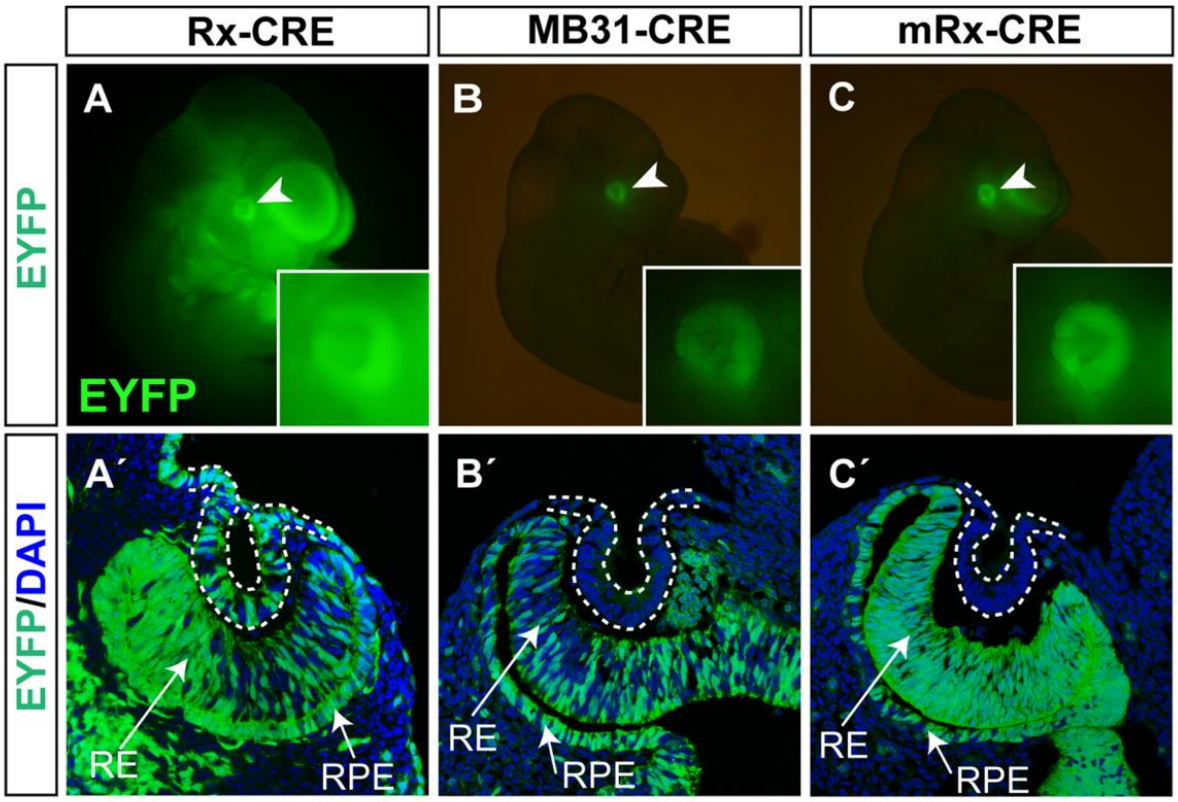

Figure 3. Activity of *Rx-Cre*, *MB31-Cre* and *mRx-Cre* in the eye primordium analyzed using the *ROSA26R-EYFP* reporter line. (A–C) Whole-mounts showing *EYFP* expression (green) in the overall embryo at E10.5. (A′–C′) Coronal sections through the eye region co-stained with DAPI (blue) showing Cre activity in the retina, retinal pigmented epithelium and invaginating lens pit (dashed line) at E10.5.
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(Figure 4D–D'), sectioning of E15.5 and adult brains revealed that the *mRx-Cre* activity in the forebrain/cortex is strongly mosaic (Figure 4D',F). In contrast, the rate of recombination in the hypothalamus appeared very high and we propose that this driver line may be used for genetic studies in the hypothalamus.

In conclusion, we have generated two *Cre*-expressing transgenic mice based on *Rx* regulatory sequences and provided their comparison with previously reported and widely used *Rx-Cre* [12]. In our hands, *mRx-Cre* appears the optimal Cre-driver for retinal progenitors, exhibiting very early onset, strength, specificity, and very low degree of mosaicism. Furthermore, *mRx-Cre* provides a useful tool for the studies of molecular mechanisms facilitating the interaction between OV and SE since it shows selective activity in OV only.

Materials and Methods

Ethics Statement

Housing of animals and *in vivo* experiments were performed after approval by the Animal Care Committee of the Institute of Molecular Genetics (study ID#174/2010) and in compliance with national and institutional guidelines (ID#12135/2010-17210).

Mouse Lines

mRx-Cre. A 200-kb Bacterial Artificial Chromosome (BAC) (RP24-82D21) harboring all coding exons, 5′ and 3′ region of the mouse *Rx* gene was purchased from Children's Hospital Oakland Research Institute. To generate *mRx-Cre* BAC, the open reading frame of Cre recombinase was inserted into the exon 1 containing the translation initiation codon of *Rx* using a method of BAC recombineering [23]; http://web.ncifcrf.gov/research/brb/protocol.aspx). The recombineering construct pCS-Cre-FRT-neo-FRT was generated by replacing the *DsRed* coding sequence in pCS2+DsRed+FRTKanFRT (provided by James D. Lauderdale [24]) by the *Cre* coding sequence. The Cre-FRT-kan-FRT targeting cassette was PCR-amplified from pCS-Cre-FRT-neo-FRT using *Rx* forward and reverse targeting primers: mRxCreF: 5′-AGGGAACCGGGCATCGAGCTCCAGTTTG-CAAAGTGCACTCCCTCCTCACCATGTCCAATTTACT-GACCGTACA-3′; mRxCreR: 5′CTTGGTAAAGCCCAG-GATGGCTTCGA TGCTGTGCAAACGCGACGTCTC-TATTCCAGAAGTAGTGAGGAG-3′. The PCR product was purified using Qiaex Gel extraction Kit (Qiagen) and treated with *Dpn*I to dispose of the template plasmid backbone. The PCR product was then electroporated into RP24-82D21 BAC-carrying bacterial strain EL250, and double resistant colonies (Cm^r, Kan^r) were tested for homologous recombination by PCR (primers: F: 5′-AGCACCAAAGCTCCAGTTACC-3′; R: 5′-CGTTGCATCGACCGGTAATGCA-3′). The kanamycin resistance cassette was further removed by induction of *flipase* activity in EL250 cells and the colonies were tested for kanamycin sensitivity. Modified *mRx-Cre* BAC was isolated, treated with *Not*I and applied to a Sepharose 4B-CL column to remove the BAC backbone according to the protocol (http://www.med.umich.edu/tamc/BACcol.html). Fractions were collected and insert integrity was analyzed using pulsed field gel electrophoresis. DNA was used for pronuclear injection. Injection gave us three founders one of which was chosen for further analysis. Mice were genotyped using

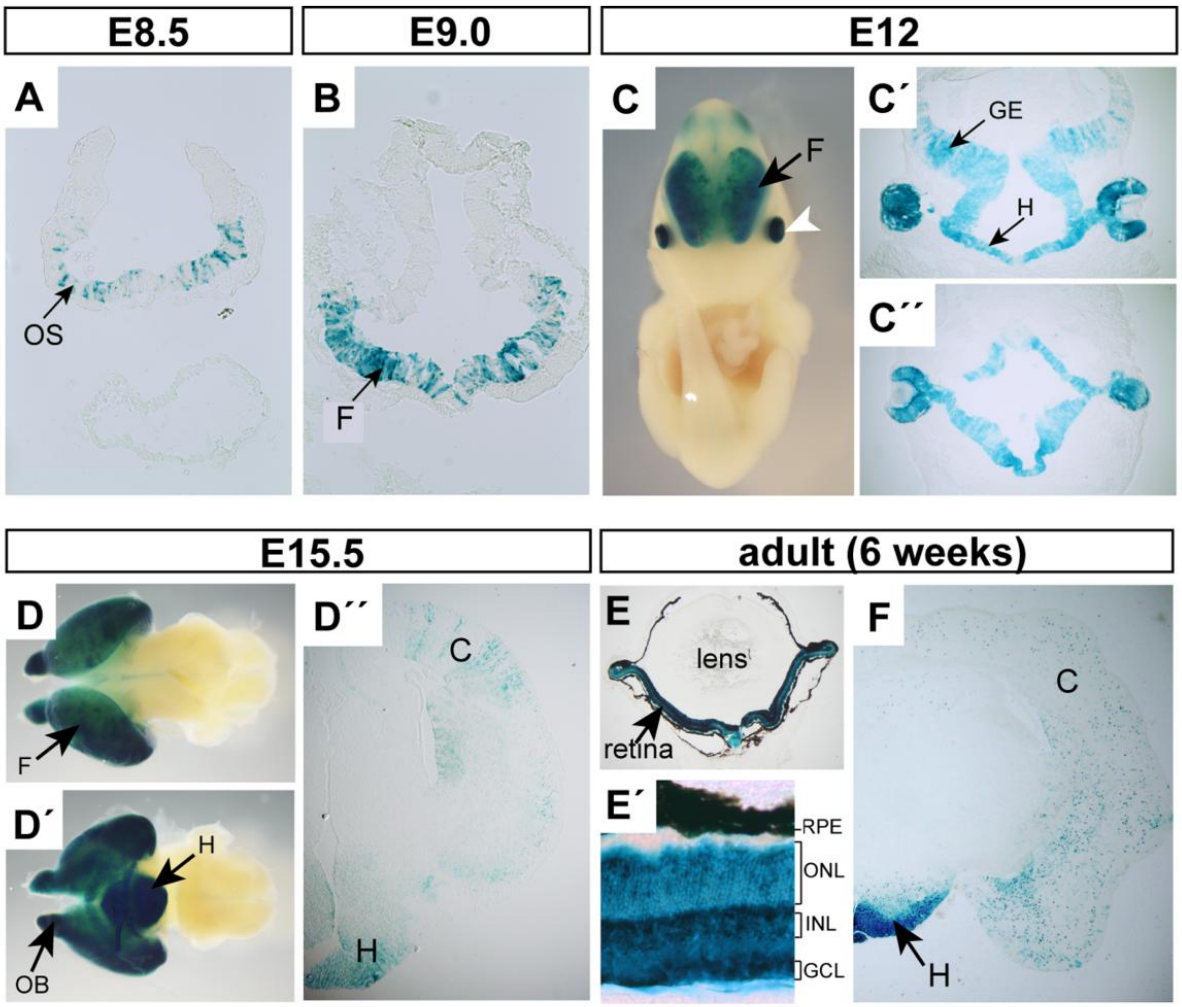

Figure 4. The *mRx-Cre* activity in the eye, forebrain and hypothalamus analyzed using the *ROSA26R* reporter line. Whole-mounts or sections were stained with X-gal at indicated stages to show the *mRx-Cre*-mediated Cre activity. (A) The X-gal^{+} cells were first observed in the optic sulcus of E8.5 embryo. (B–D′) The Cre activity in developing brain. Whole-mounts (C, D, D′), coronal sections (C′, D′) and transversal section (C′) showing Cre activity in embryonic brain. (F) Coronal section of adult brain showing Cre activity in the hypothalamus and cortex. (E–E′) Sections through the adult eye showing strong uniform Cre activity in all layers of the retina. OS-optic sulcus; F-forebrain; GE-ganglionic eminences; H-hypothalamus; OB-olfactory bulbs, C-cortex; RPE-retinal pigmented epithelium; ONL-outer nuclear layer; INL-inner nuclear layer; GCL-ganglion cell layer.
doi:10.1371/journal.pone.0063029.g004

primers that recognize the recombination junction, with forward primer located upstream of the *Rx* translation start site (F: 5′-AGCACCAAAGCTCCAGTTACC-3′) and reverse primer located in *Cre* recombinase (R: 5′-CGTTGCATCGACCGGTAATGCA-3′). For analysis of Cre activity, the eighth generation from the original founder was used for the generation of presented embryos. For stages E8.5–E10.5, twenty embryos from three to five independent litters were used. For stages E12–E15.5, eight embryos from three litters were used. For adult stages (6 weeks), tissues from three independent animals were used. All embryos (tissues) reproducibly exhibited the same expression pattern with highly comparable strength. Presented expression pattern was stable from F1 generation.

MB31-Cre. The coding region of Cre recombinase was cloned into pIRES2-EGFP (Clontech) and the Cre-IRES-EGFP cassette was fused to the medaka *Rx* gene promoter (provided by Jochen Wittbrodt). DNA was used for pronuclear injection and nine founders were analyzed for activity using *ROSA26R* mice. The least mosaic line, designated *MB31*, was characterized further. For analysis of Cre activity, the fifteenth generation from the original founder was used for the generation of presented embryos. For stages E9.5 and E10.5, twenty embryos from three to five independent litters were used. All embryos reproducibly exhibited the same expression pattern with highly comparable strength.

The *Rx-Cre* mice were described previously and were provided by Milan Jamrich [12]. For analysis of Cre activity, fifteen embryos from three litters were used. All embryos exhibited comparable expression pattern. Both *Rx-Cre* and *MB31-Cre* were genotyped using primers: F: 5′-CATTTGTGAAGTGCTTGAAGGAAT-3′; R: 5′-AGAGGAAGGCAGCACTGATGAAA-3′.

Generation of *ROSA26R* (stock no. 003309) and *ROSA26R-EYFP*, both purchased from Jackson laboratory, was described previously [21,22]. The genotype was determined by PCR analysis of genomic DNA obtained from tail biopsies.

Tissue Collections and Histology

Mouse embryos were harvested at several developmental stages from timed pregnant females. The morning of vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos were fixed in 4% paraformaldehyde (w/v) on ice for time depending on embryonic stage (from 20 minutes up to 2 hours). Embryos were washed several times with cold PBS, cryopreserved by overnight incubation in 30% sucrose (w/w), frozen in OCT (Tissue Tek, Sakura Finetek) and sectioned.

X-Gal Staining

For β-galactosidase assay, embryos were fixed on ice in 0.4% formaldehyde (w/v) in PBS, washed 3×20 minutes with the rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris pH 7.3, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) and incubated in X-Gal staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg/ml X-gal) overnight at room temperature. For sectioning, embryos were re-fixed in 4% parformaldehyde (w/v), washed with PBS, cryopreserved in 30% sucrose (w/w) and embedded in OCT (Tissue Tek, Sakura Finetek).

Immunohistochemistry

For observation of *EYFP* expression, the cryosections were permeabilized with PBT (PBS with 0.1% Tween-20) for 15 minutes, washed 3×10 min with PBT, stained 10 min with DAPI (1 µg/ml) in PBT and mounted into Mowiol (Sigma).

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ZK LK. Performed the experiments: OM JL LK. Analyzed the data: LK OM ZK. Contributed reagents/materials/analysis tools: ZK RS. Wrote the paper: LK OM ZK.

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

It has been reported that Pax6 plays an important role in retina cell type differentiation (Marquardt et al., 2001; Oron-Karni et al., 2008). Conditional Pax6 inactivation in differentiating retinal cells leads to disruption of differentiation process and exclusive generation of only one out of the seven retinal cell types, the amacrine interneurons. This led to the assumption, that Pax6 plays the role in RPC multipotency. However, the role of Pax6 in earlier retinal structures such as OV or RPCs in a newly formed OC has not been addressed as the tool for efficient gene inactivation in this tissue was not available.

In this study, we used the *mRx-Cre* line to inactivate *Pax6* specifically in the OV and just established RPCs of the OC. Interestingly, we have found that OV-expressed Pax6 is essential for development of both eye tissues: the lens and the retina. When Pax6 is inactivated during OV/SE stage, eye development is arrested, no lens is formed and OV does not invaginate to form the OC. This represents a striking fact since it has been believed, that OV-expressed Pax6 has no impact on the process of lens induction. On the other hand, when Pax6 was inactivated in a developing retina, after the OV-to-OC transition, lens and retina did form, but the RPC proliferation was severely disrupted. Proliferation analysis showed that *Pax6*-deficient RPCs up-regulate the expression of cell cycle inhibitor $p57^{Kip2}$ and prematurely leave the cell cycle that leads to the complete depletion of RPC pool in early development and almost complete loss of retinal cells by birth. In addition, our data clearly showed that Pax6 is required for the differentiation of all retinal cell types. Taken together we have shown that Pax6 is implicated in various processes of eye development, including lens induction and the differentiation of particular retinal cell types.

My contribution to this work: I have generated all the experimental data and wrote the manuscript of published paper Klimova and Kozmik 2014 (presented on pages 51 – 67 of this Thesis).

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RESEARCH ARTICLE

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

Lucie Klimova and Zbynek Kozmik*

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).

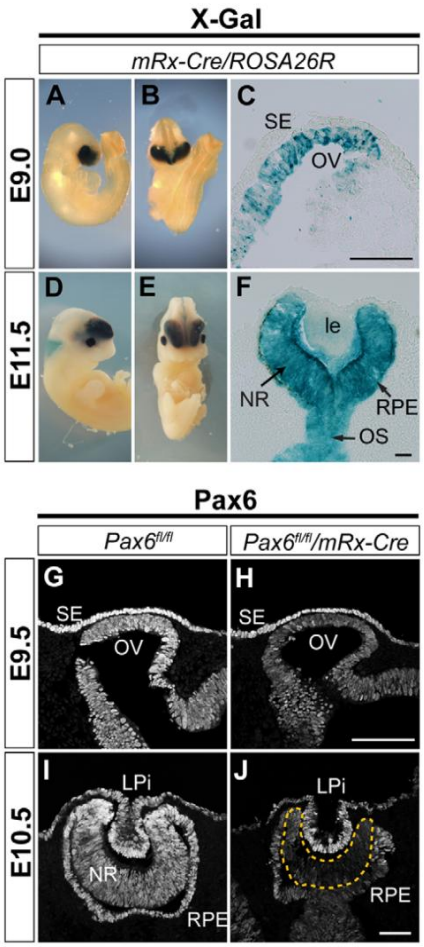

Fig. 1. *mRx-Cre*-mediated *Pax6* inactivation in early retinal progenitors. (A-F) *mRx-Cre* activity was assessed using the *ROSA26R* reporter mouse line. Whole-mounts or coronal sections were stained with X-gal at the indicated stages to show Cre activity in eye primordium. Side (A) and frontal (B) 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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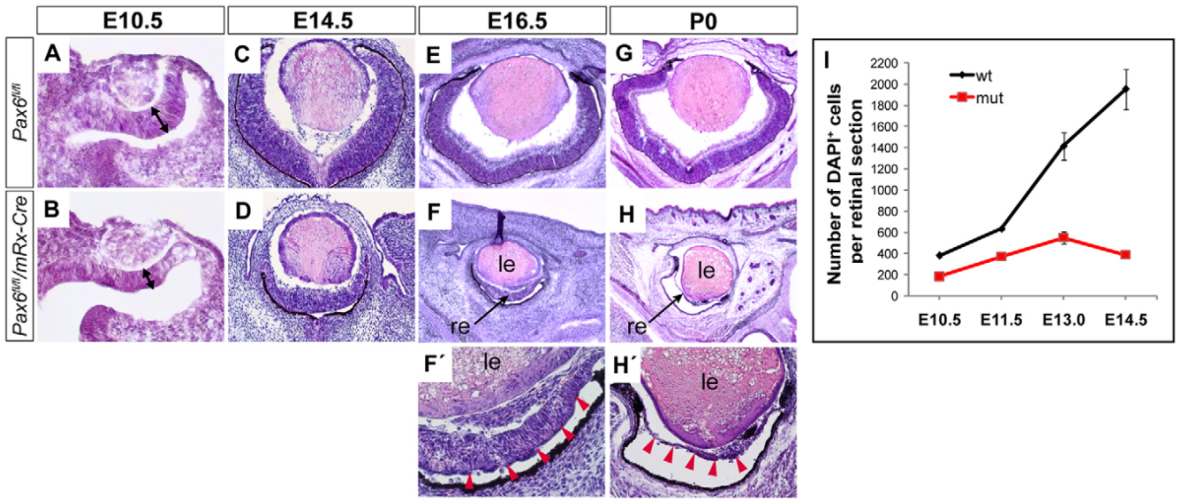

Fig. 2. Morphological consequences of Pax6 inactivation in early retinal progenitors. (A-H′) Coronal sections of wild-type (*Pax6*$^{fl/fl}$) (A,C,E,G) and *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) (B,D,F,H) eye were stained with Hematoxylin-Eosin at the indicated stages. Position of the retina is 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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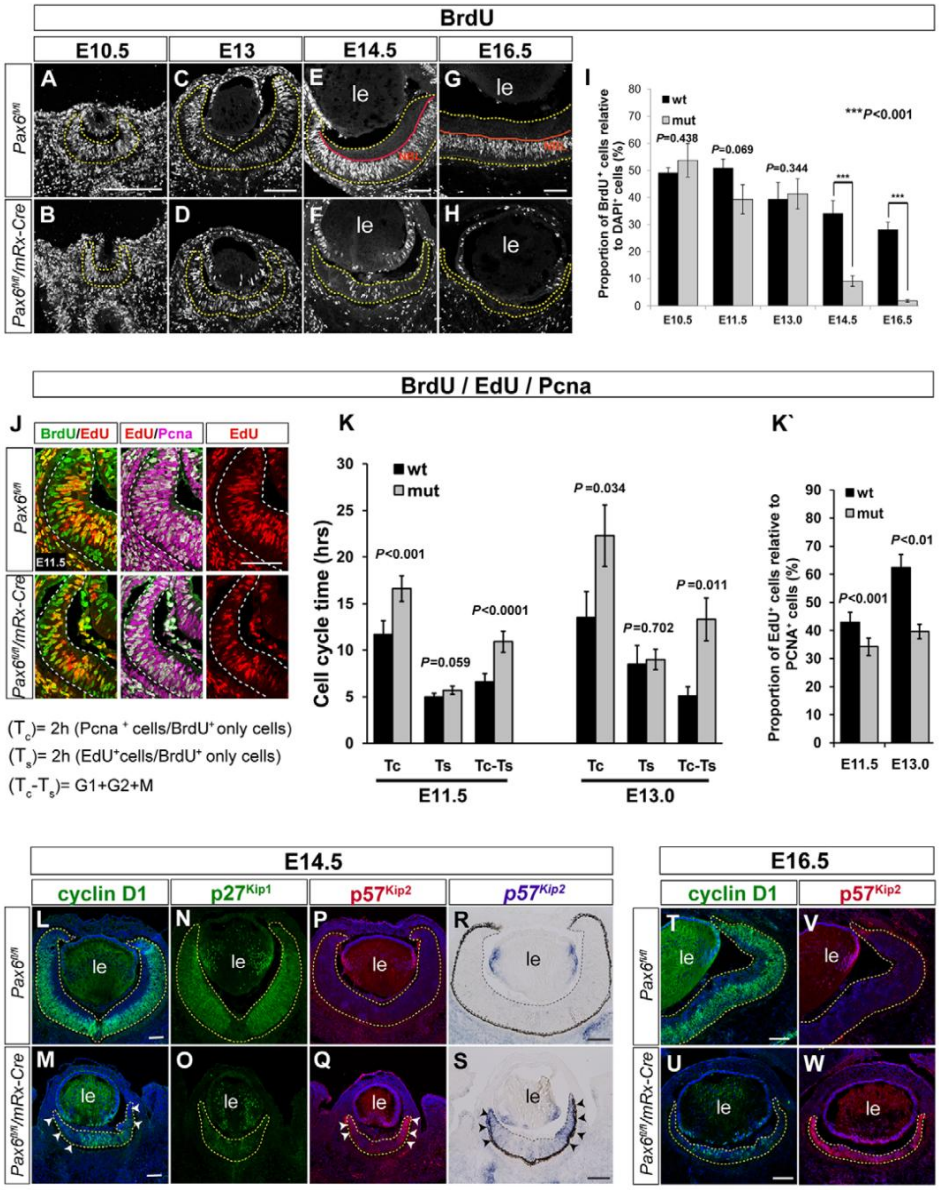

Fig. 3. Cell cycle dynamics is affected in *Pax6*-deficient retinae. (A-I) Following a 1 hour BrdU pulse, eye sections of wild-type (*Pax6*$^{fl/fl}$) and *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) retinae were stained for BrdU incorporation at the indicated stages. Retina is indicated by the dashed 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

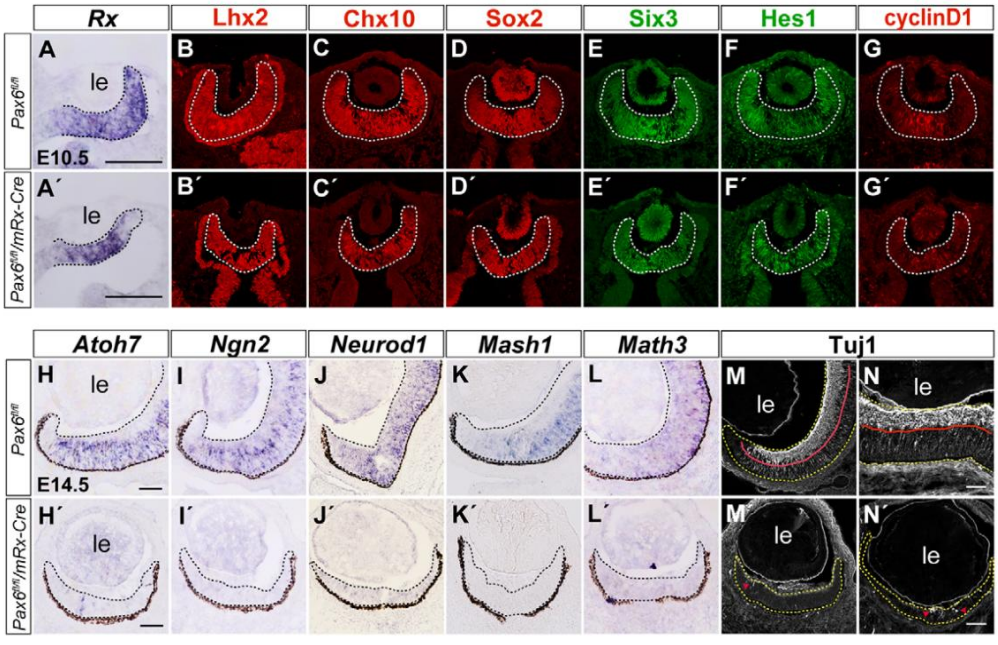

Fig. 4. Retinal progenitor characteristics are maintained but the differentiation program is not initiated in *Pax6*-deficient retina. (A-G′) Expression of the indicated genes (mRNA, A,A′) and proteins (B-G′) involved in RPC pool maintenance analyzed at E10.5. (H-L′) Expression of the indicated genes (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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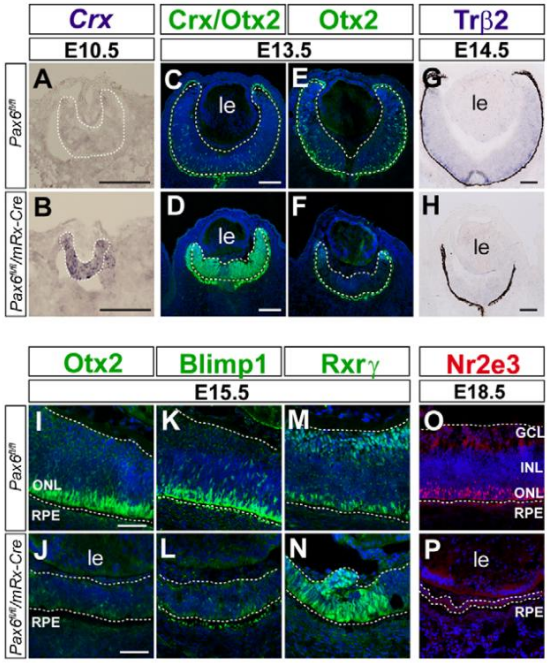

Fig. 5. Expression of photoreceptor-specific factors in *Pax6*-deficient retina. (A,B) *Crx* mRNA expression at E10.5 showing elevated levels in *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) retina (B). (C-F) Confocal images showing Crx (C,D) and Otx2 (E,F) protein expression using Crx/Otx2 and Otx2 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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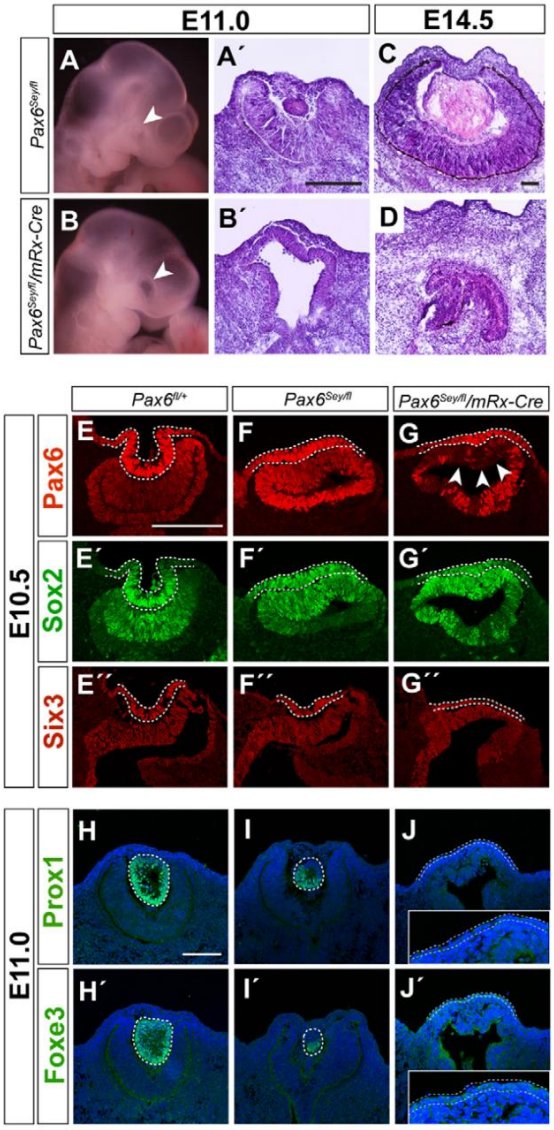

Fig. 6. When Pax6 is eliminated from OV before its transition to the OC **then the lens is not formed.** (A-D) Morphological consequences of OV-specific *Pax6* inactivation. Heads with the eye region indicated (arrowheads) are shown for control (*Pax6*$^{Sey/fl}$) (A) and mutant (*Pax6*$^{Sey/fl}$*/mRx-Cre*) (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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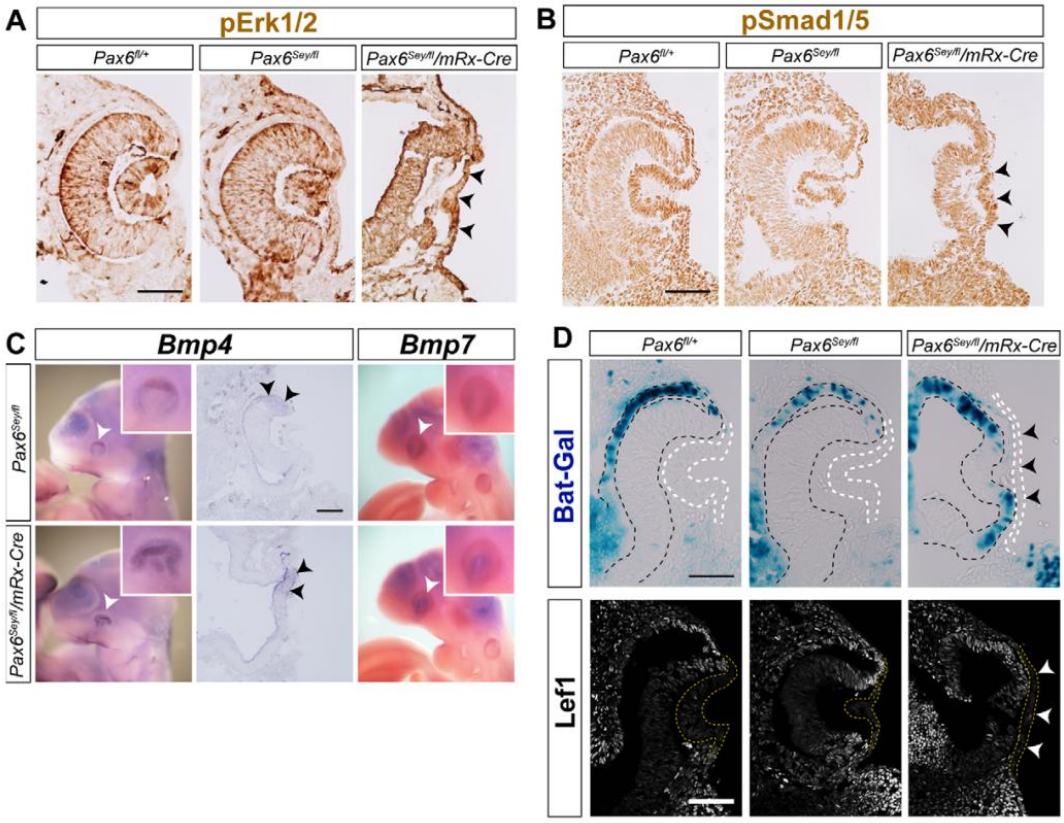

Fig. 7. The *Pax6*-dependent lens-inductive ability of OV neuroepithelium is not dependent on FGF, BMP or Wnt/β-catenin signaling. (A) pErk1/2 detection in control (*Pax6*$^{fl/+}$ and *Pax6*$^{Sey/fl}$) and mutant (*Pax6*$^{Sey/fl}$*/mRx-Cre*) E11.0 eyes of littermate embryos. (B) pSmad1/5 detection 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).

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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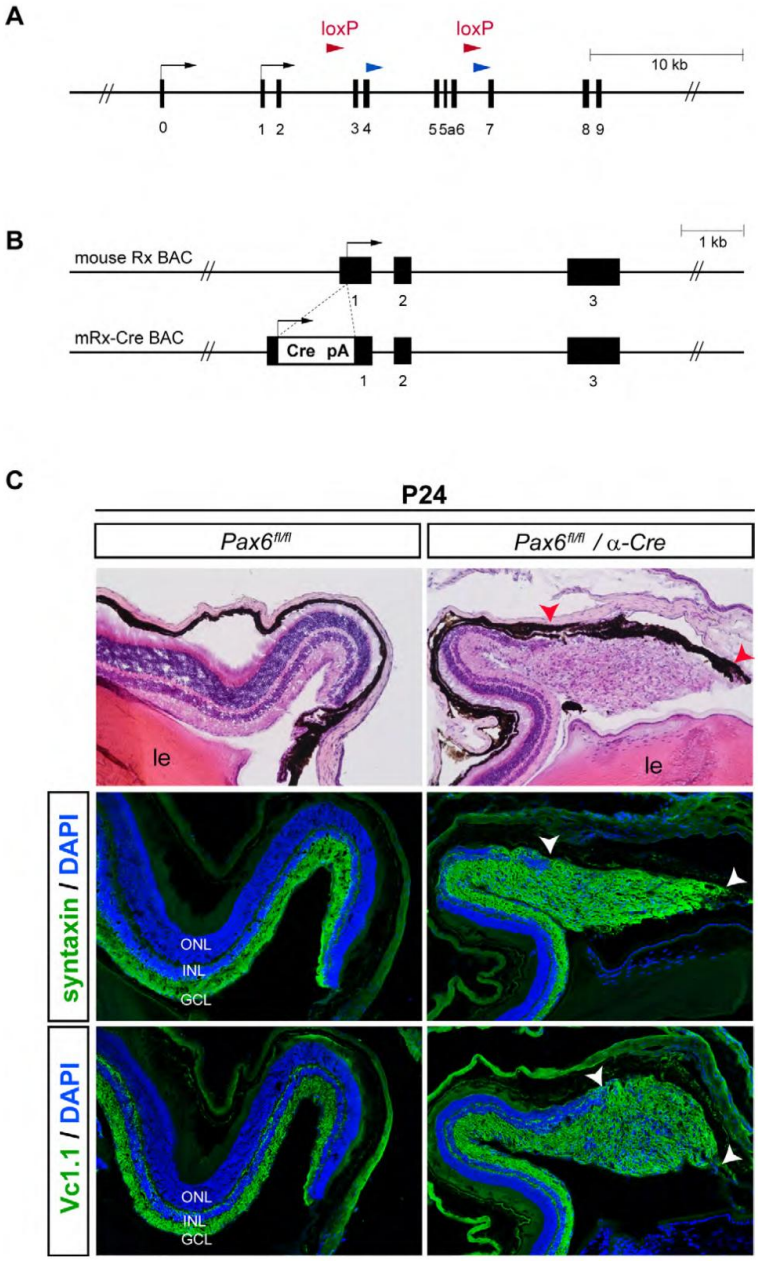
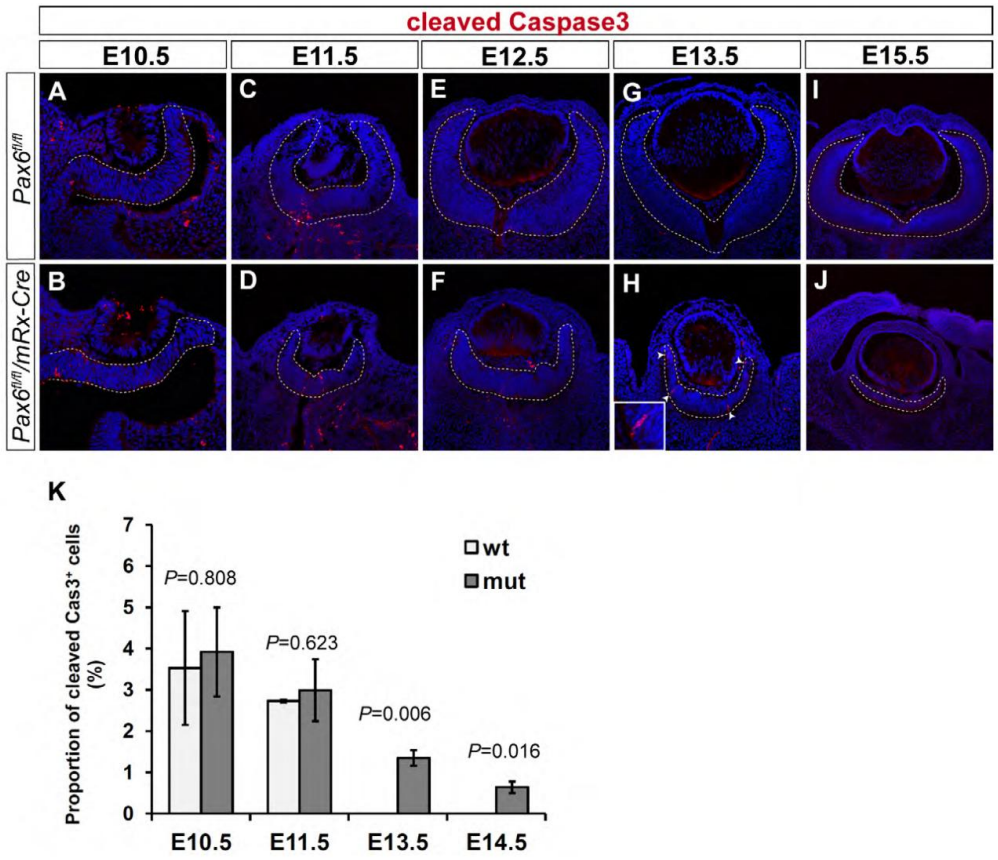
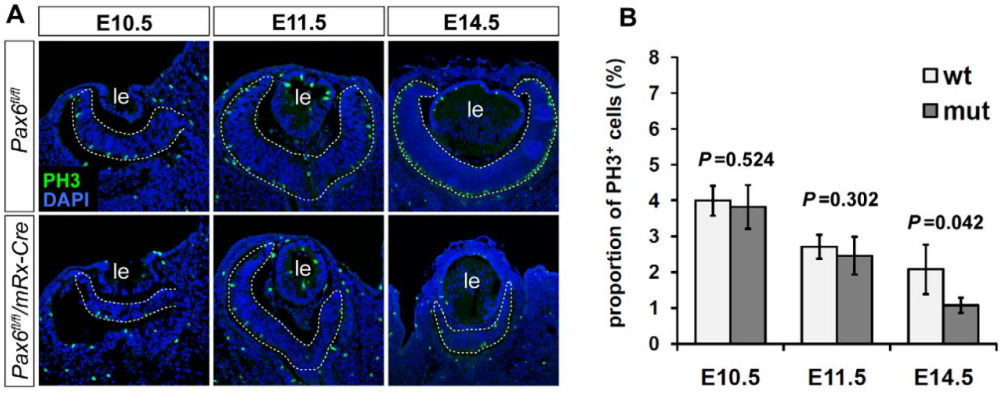


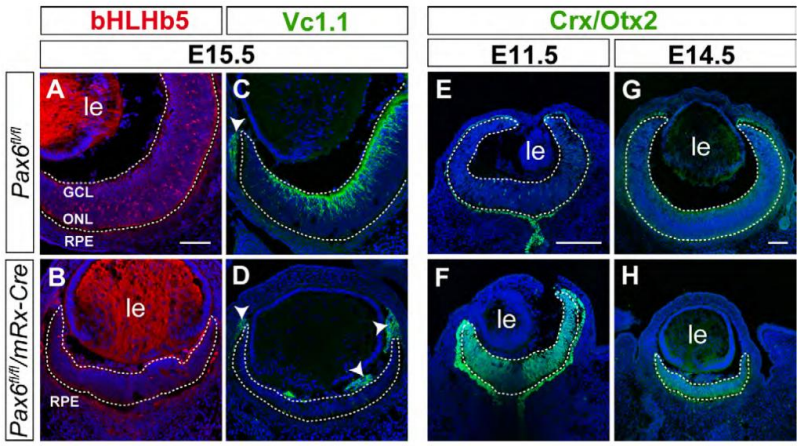
Supplementary Fig. S1. Schematic representation of mouse lines *Pax6$^{fl/fl}$* and *mRx-Cre* used in this study. (A) To generate *Pax6$^{fl/fl}$*, loxP sites flanking exons 3-6 (red arrowheads) were introduced into *Pax6* locus by homologous recombination in embryonic stem cells. The paired domain of Pax6 is encoded by exons 5, 5a, 6 and 7. Blue arrowheads represent positions of loxP sites in *Pax6$^{fl/fl}$* mice generated previously (Ashery-Padan et al., 2000). Details of gene targeting are available upon request. (B) To generate *mRx-Cre*, BAC containing 200kb covering the *Rx* locus was modified by BAC recombineering. The *Cre* coding region (Cre-pA) was inserted into the *Rx* translational initiation start site (ATG). Exons are indicated by black boxes. (C) *Pax6$^{fl/fl}$* mice generated in this study were crossed with *α-Cre* to show that amacrine cells are generated in *Pax6$^{fl/fl}$*/*α-Cre* mutants as previously reported (Marquardt et al., 2001). Adult retinal sections were stained with antibodies against amacrine cell markers syntaxin and Vc1.1 (HNK-1 epitope); retinal areas with amacrine cells are indicated with arrowheads. le, lens; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.



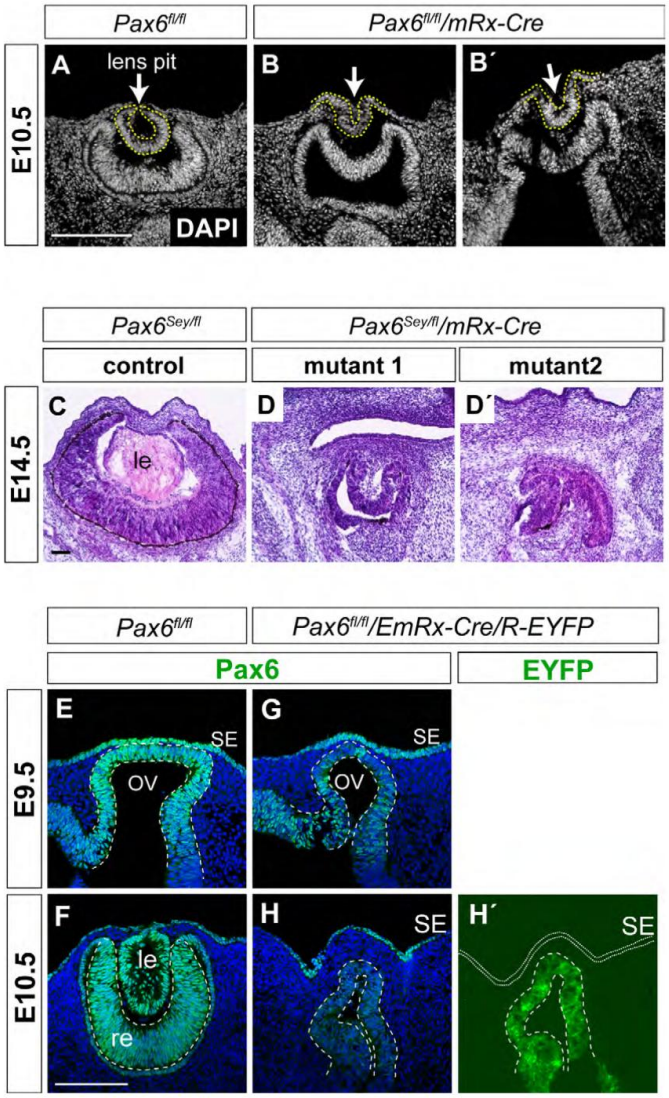
Supplementary Fig. S2. Apoptosis in *Pax6*-deficient retina. (A-J) Retinal sections of wild-type (*Pax6*$^{fl/fl}$) and mutant (*Pax6*$^{fl/fl}$*/mRx-Cre*) embryos were stained with antibody against cleaved Caspase3 (Cas3) at indicated stages. Retina is indicated with dashed line. (K) Quantification of apoptotic cells determined as proportion of Cas3^{+} cells versus DAPI^{+} cells in wild-type (wt) and *Pax6*-deficient (mut) retinae. Error bars indicate s.d. *P*-values are by Student's *t*-test.



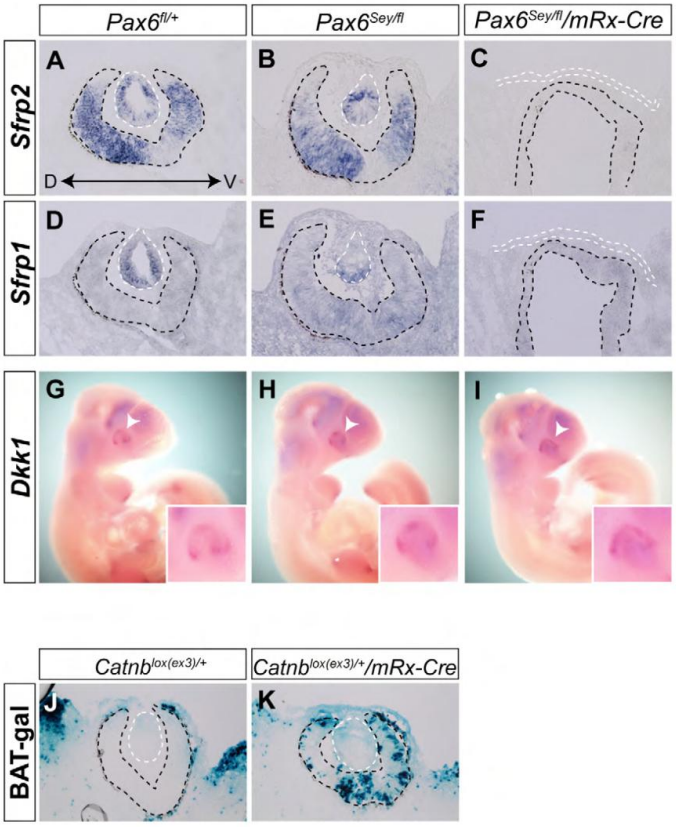
Supplementary Fig. S3. M-phase cell cycle arrest does not contribute to early proliferation phenotype in *Pax6*-deficient retina. (A) Sections stained with antibody against phosphorylated histone H3 (PH3) at E10.5, E11.5 and E14.5. (B) Quantification of M-phase cells determined as proportion of $PH3^+$ cells versus $DAPI^+$ cells in wild-type (wt) and *Pax6*-deficient (mut) retinae (indicated with dashed lines). Error bars indicate s.d. *P*-values are by Student's *t*-test. le, lens



Supplementary Fig. S4. Expression of bHLHb5, Vc1.1 and Crx in *Pax6*-deficient retina. (A-D) Confocal images showing bHLHb5 (A,B) and Vc1.1 (HNK-1 epitope) (C,D) immunoreactivity in wild-type (*Pax6*$^{fl/fl}$) and *Pax6*-deficient (*Pax6*$^{fl/fl}$*/mRx-Cre*) retina at E15.5. Arrowheads indicate Vc1.1 immunoreactivity in non-retinal tissue (B,C). (E-H) Crx protein expression assessed using Crx/Otx2 antibody at E11.5 (E, F) and E14.5 (G,H). Dashed lines indicate the position of retina. le, lens; GCL, ganglion cell layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium. Scale bar: 100 µm.



Supplementary Fig. S5. Pax6 elimination from early RPCs or OV neuroepithelium interferes with optic cup/lens pit morphogenesis. (A-B′) Transversal sections of E10.5 wild-type (*Pax6$^{fl/fl}$*) and mutant (*Pax6$^{fl/fl}$/mRx-Cre*) eyes of littermate embryos stained with DAPI (grey). Forming lens pit is indicated with dashed line. (C-D′) Section of control (*Pax6$^{Sey/fl}$*) and mutant (*Pax6$^{Sey/fl}$/mRx-Cre*) eyes of littermate embryos at E14.5 stained with hematoxylin-eosin. (E-H′) Sections of control (*Pax6$^{fl/fl}$*) and mutant (*Pax6$^{fl/fl}$/EmRx-Cre*) eyes stained with antibody against Pax6 at indicated stages. (H′) Expression of EYFP showing area of Cre-mediated deletion visualized using *R-EYFP* reporter mouse line. OV, optic vesicle; SE, surface ectoderm; le, lens; re, retina. Scale bar: 100 µm.



Supplementary Fig. S6. Wnt/β-catenin inhibitors *Sfrp1* and *Sfrp2* are downregulated upon OV-specific *Pax6* inactivation but Wnt signaling is not responsible for the arrested lens development. (A-F) *Sfrp2* (A-C) and *Sfrp1* (D-F) mRNA expression in control (*Pax6^{fl} and Pax6$^{Sey/fl}$*) and mutant (*Pax6$^{Sey/fl}$/mRx-Cre*) E11.0 eyes. (G-I) *Dkk1* mRNA expression at E10.5; eye region indicated with arrowheads. (J,K) Activity of Wnt/β-catenin signaling assessed using a BAT-gal reporter mouse in control (*Catnb$^{lox(ex3)/+}$*) (J) and retinal mutant (*Catnb$^{lox(ex3)/+}$/mRx-Cre*) (K) with activated Wnt/β-catenin pathway in developing neuroretina. Retina is indicated with black dashed line; lens or the corresponding tissue with white dashed line.

Table S1. Primary antibodies and RNA probes

Primary antibodies

Antibody	Host	Dilution	Source
Pax6	Rabbit	1:500	Covance (PRP-278P)
Sox2	Goat	1:400	Santa-Cruz (sc-17320)
cyclin D1	Mouse	1:500	Santa-Cruz (sc-450)
$p27^{Kip1}$	Mouse	1:1000	BD Biosciences (610241)
$p57^{Kip2}$	Goat	1:70	Santa-Cruz (sc-1039)
Tuj1	Mouse	1:1500	R&D Systems (MAB1199)
cleaved caspase 3	Rabbit	1:300	Cell Signaling (D175)
PH3	Rabbit	1:1000	Upstate (06-570)
Lef1	Rabbit	1:500	Cell Signaling (C12A5)
Pcna	Mouse	1:3000	Sigma, P8825
Crx/Otx2	Rabbit	1:500	Kind gift from Dr Craft (Zhu and Craft, 2000)
Six3	Rabbit	1:2000	Kind gift from Dr P. Bovolenta (unpublished)
Otx2	Rabbit	1:300	R&D Systems (BAF1979)
Blimp1	Rat	1:300	Santa-Cruz (sc-47732)
Rxrγ	Rabbit	1:1500	Santa-Cruz (sc-555)
Nr2e3	Rabbit	1:100	Kind gift from Dr S. Chen (Chen et al., 2005)
bHLHb5	Goat	1:400	Santa-Cruz (sc-6045)
Vc1.1 (HNK-1)	Mouse	1:100	Sigma (C6680)
Lhx2	Goat	1:1000	Santa-Cruz (sc-19344)
Hes1	Rabbit	1:1000	Kind gift from Dr N. Brown (Lee et al., 2005)
Chx10	Sheep	1:800	Exalpha (X1180P)
Foxe3	Rabbit	1:1000	Kind gift from Dr Carlsson (Blixt et al., 2007)
Prox1	Rabbit	1:2000	Chemicon (AB5475)
BrdU	Rat	1:100	Abcam (AB6326)
pErk1/2	Rabbit	1:3000	Cell Signaling (9101S)
pSmad1/5	Rabbit	1:2000	Invitrogen (700047)

RNA probes

Gene	Source
Neurod1	Open Biosystems
$p57^{Kip2}$	Open Biosystems
Rx	Open Biosystems
Trβ2	Open Biosystems
Crx	Open Biosystems
Atoh7	Kindly provided by Dr Brown (Brown et al., 1998)
Math3	Kindly provided by Dr Brown (Farah et al., 2000)
Mash1	Kindly provided by Dr Brown (Brown et al., 1998)
Bmp4	Kindly provided by Dr Hogan (Jones et al., 1991)
Bmp7	Kindly provided by Dr Hogan (Lyons et al., 1995)
Ngn2	Kindly provided by Dr J. Rubenstein (Sommer et al., 1996)
Sfrp1	Kindly provided by Dr S. Pleasure (Rattner et al., 1997)
Sfrp2	Kindly provided by Dr S. Pleasure (Rattner et al., 1997)
Dkk1	Kindly provided by Dr S. Krauss (Diep et al., 2004)

6.4 Onecut1 and Onecut2 transcription factors operate downstream of Pax6 to regulate horizontal cell development.

Mouse retina is composed of seven retinal cell types: retinal ganglion cells, amacrine cells, bipolar cells, horizontal cells, rod and cone photoreceptor cells and Müller glial cells. Several studies clearly showed that Pax6 plays a crucial role in RPC differentiation (Klimova and Kozmik, 2014; Marquardt et al., 2001). However, less is known about the identity of Pax6-regulated genes that participate in this process.

In this study we focused on searching for Pax6-regulated genes during the course of retinogenesis. We used *Pax6*-deficient and *Pax6*-wild-type embryonal retinal cells and analyzed them for differentially expressed genes that might play the role in neuronal development. Among the identified genes we found two transcription factors of Onecut transcription factor family Onecut1 and Onecut2 which role in retinal development was largely unknown. Inactivation of Onecut1 and Onecut2 in developing mouse retina revealed their essential role in generation of one particular retinal cell type, horizontal cell interneurons. In addition, more detailed analysis showed that Onecut factors are involved in generation of other retinal cell types including cone photoreceptors, amacrine cells, bipolar cells and Müller glia cells as their numbers were reduced in *Onecut*-deficient retinae. Taken together this data indicates that Onecut transcription factors, operating downstream of Pax6, regulate generation of several retinal cell types.

My contribution to this work: I have generated the experimental data depicted in Figures 1, 2, 4, and 5 and I wrote the manuscript of published paper Klimova et al., 2015 (presented on pages 69 – 86 of this Thesis).

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Onecut1 and Onecut2 transcription factors operate downstream of Pax6 to regulate horizontal cell development

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ABSTRACT

Genetic studies of the last decades strongly indicated that generation of particular retinal cell types is governed by gene regulatory networks of transcription factors and their target genes. The paired and homeodomain transcription factor Pax6 plays a pivotal role in retinal development as its inactivation in the retinal progenitor cell population leads to abolished differentiation of all retinal cell types. However, until now, only a few transcription factors operating downstream of Pax6 responsible for generation of individual retinal cell types have been identified. In this study, we identified two transcription factors of the Onecut family, Onecut1 and Onecut2, as Pax6 downstream-acting factors. Onecut1 and Onecut2 were previously shown to be expressed in developing horizontal cells, retinal ganglion cells and cone photoreceptors; however, their role in differentiation of these cell types is poorly understood. In this study, we show that the horizontal cell genesis is severely disturbed in *Onecut*-deficient retinae. In single *Onecut1* and *Onecut2* mutants, the number of horizontal cells is dramatically reduced while horizontal cells are completely missing in the *Onecut1/Onecut2* compound mutant. Analysis of genes involved in the horizontal cell genesis such as Foxn4, Ptf1a, Prox1 and Lim1 showed that although horizontal cells are initially formed, they are not maintained in *Onecut*-deficient retinae. Taken together, this study suggests the model in which Pax6 regulates the maintenance of horizontal cells through the activation of Onecut1 and Onecut2 transcription factors.

Introduction

Proper visual perception is strictly dependent on coordinated differentiation and correct assembly of multiple cell types in the structure of the neuroretina. During the course of retinogenesis seven retinal cell types are generated from the common retinal progenitor cell (RPC) population (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990) and further organized in three retinal layers: retinal ganglion cells (RGCs) in the ganglion cell layer (GCL); amacrine cells (ACs), bipolar cells, Müller glia cells and horizontal cells (HCs) in the inner nuclear layer (INL); rod and cone photoreceptors in the outer nuclear layer (ONL). The birth order is conserved among vertebrate species, suggesting the existence of a common molecular mechanism governing this process. It is largely accepted that as the retinogenesis proceeds, RPCs are exposed to the changing environment of extrinsic cues (Cepko, 1999) that in cooperation with intrinsic factors direct retinal neurogenesis (Brown et al., 1998; Cepko, 1999; Hatakeyama and Kageyama, 2004; Inoue et al., 2002; Lillien, 1995; Perron and Harris, 2000; Tomita et al., 1996). Among intrinsic factors, mainly transcription factors of the basic helix-loop-helix (bHLH) and homeodomain class have been found to promote a strong cell-autonomous bias toward particular cell fates whereas inhibiting others (Hatakeyama and Kageyama, 2004). Although genetic studies have identified many transcription factors involved in the process of retinogenesis, the complete map of gene regulatory networks (GRNs) that mediate differentiation of all individual cell types is not completely understood.

The paired and homeodomain transcription factor Pax6 plays a pivotal role in retinal development. It is expressed from very early stages, in all mitotically active RPCs (Walther and Gruss, 1991). Later, its expression is restricted to differentiated retinal ganglion cells, amacrine cells, horizontal cells and Müller glial cells (de Melo et al., 2003; Roesch et al., 2008). Pax6 is assumed to be required for RPC multipotency as its early inactivation results in failure of acquisition of any specific retinal cell fate (Klimova and Kozmik, 2014). Since Pax6 was found to control expression of several bHLH transcription factors, it has been suggested that Pax6 regulates the complex GRN in the retina (Hatakeyama and Kageyama, 2004; Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008; Riesenberg et al., 2009). However, until now only a few Pax6 downstream-acting factors potentially accounting for the severe differentiation defect observed in *Pax6*-deficient retinae have been identified.

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Onecut proteins (Oc) belong to an ancient superclass of homeobox transcription factors. They are represented by three members in mammals, Onecut1 (Oc1), Onecut2 (Oc2) and Onecut (Oc3) (Jacquemin et al., 1999; Lannoy et al., 1998; Vanhorenbeeck et al., 2002). Originally, they were identified as factors controlling cell differentiation in endoderm-derived tissues such as liver and pancreas (Clotman et al., 2005, 2002; Jacquemin et al., 2000, 2003; Margagliotti et al., 2007; Pierreux et al., 2006). However, in the last decade, there has been growing evidence that Oc play a role in the nervous system development as well. Oc have been found to regulate neuronal identity, migration, organization and maintenance in different regions of the central nervous system (Audouard et al., 2013; Espana and Clotman, 2012a, b; Hodge et al., 2007; Chakrabarty et al., 2012; Roy et al., 2012; Stam et al., 2012). Among the neuronal tissues, their expression was also observed in the retina (Haworth and Latinkic, 2009; Wu et al., 2012). Although detailed analysis of Oc expression in the mouse revealed expression of Oc1 and Oc2 in developing retinal ganglion cells, horizontal cells and cone photoreceptors (Emerson et al., 2013; Wu et al., 2013, 2012) their role in differentiation of these retinal cell types has not been completely understood.

Horizontal cells represent a population of retinal interneurons that modulate signaling between photoreceptors and bipolar cells. Like other retinal cell types, HCs are derived from the RPC population (Turner et al., 1990). Transcription factors Foxn4, RORβ1, Ptf1a, Prox1, Ap2, Sall3 and Lim1 were found to be required for differentiation and proper localization of HCs in the retina (Bassett et al., 2012; de Melo et al., 2011; Dyer et al., 2003; Fujitani et al., 2006; Li et al., 2004; Liu et al., 2013; Nakhai et al., 2007; Poche et al., 2007). Foxn4, one of the earliest HC determinants, controls expression of Ptf1a and Prox1 to promote the HC fate (Fujitani et al., 2006; Li et al., 2004). However, it has been suggested that co-expression of Foxn4 and Pax6 in mitotic RPCs is crucial for acquisition of competence for the genesis of HCs (Li et al., 2004). Although the Foxn4 role in HC development has been well established (Fujitani et al., 2006; Li et al., 2004), a potential role of Pax6 remains largely elusive. Here we show that Pax6 regulates the HC development through activation of two transcription factors of the Oc transcription factor family, Oc1 and Oc2.

Materials and methods

Mouse lines

For retina-specific inactivation of *Pax6*, *α-Cre* (Marquardt et al., 2001), *mRx-Cre* (Klimova et al., 2013) and $Pax6^{fl/fl}$ (Klimova and Kozmik, 2014) mice were used. To inactivate *Oc1* and *Oc2*, $Oc1^{fl/fl}$ (Zhang et al., 2009), $Oc2^{+/-}$ (Clotman et al., 2005) mice were used.

Tissue collections and histology

Mouse embryos were harvested from timed pregnant females. The morning of vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos were fixed in 2% or 4% paraformaldehyde (w/v) in PBS on ice for the time depending on embryonic stage (from 20 min to 4 h). Embryos were washed with cold PBS, cryopreserved by overnight incubation in 30% sucrose (w/w), frozen in OCT (Tissue Tek, Sekura Finetek) and sectioned.

Immunohistochemistry and β-galactosidase staining

The cryosections were permeabilized with PBT (PBS with 0.1% Tween-20) for 15 min, blocked with 10% BSA in PBT for 30 min and incubated with primary antibody overnight at 4 °C. Primary antibodies used were: rabbit anti-Pax6 (Covance, PRB-278P, 1:500), mouse anti-Pax6 (DSHB, clone P3U1, 1:2000), mouse anti-Pax6 (Santa-Cruz, sc-53108, 1:50), mouse anti-Prox1 (Millipore, MAB5652, 1:1000), mouse anti-Lim1/2 (DSHB, clone 4F2-c, 1:250), rabbit anti-Oc1 (HNF6) (Santa-Cruz, H-100, sc-13050, 1:500), sheep anti-Oc2 (R&D Systems, AF6294, 1:500), rabbit anti-Ptf1a (AB2153, provided by Dr. Ole Madsen, Beta Cell Biology Consortium, 1:250), goat anti-Brn3b (Santa-Cruz, C-13, sc-6026X, 1:2000), mouse anti-Isl1/2 (DSHB, clone 40.2D6-c, 1:200), rabbit anti-Brn3a (provided by Dr. E. Turner, 1:4000), mouse anti-Calbindin-D-28K (Sigma-Aldrich, clone CB-955, C9848, 1:2500), rabbit anti-Calretinin (Sigma-Aldrich, C7479, 1:750), rabbit anti-Ap2α (Santa-Cruz, C-18, sc-184, 1:1000), mouse anti-Ap2α (DSHB, clone 3B5, 1:1000), RXRγ (Santa-Cruz, Y-20, sc-555X, 1:3000), rabbit anti Tbr2 (Abcam, ab23345, 1:500). Sections were washed 3 × 10 min with PBT, incubated with secondary antibody (Molecular Probes) for 1 h, washed 3 × 20 min in PBT, washed 10 min with DAPI (1 μg/ml) in PBT and mounted into Mowiol (Sigma). The paraffin sections were deparaffinized and rehydrated. For immunohistochemical analysis, dewaxed sections were incubated for 20 min in citrate buffer (10 mM, pH 6.0) at 98 °C in a steam bath. Sections were washed 3 × 10 min with PBT, treated with 1.5% H_2O_2 in 10% methanol in PBS for 25 min, again washed 3 × 10 min with PBT, blocked with 10% BSA in PBT for one hour and incubated with primary antibody overnight at 4 °C. Primary antibody was detected with biotinylated anti-mouse, anti-rabbit secondary antibody (Vector Laboratories) and subsequently visualized with Vectastain ABC Elite kit and ImmPACT DAB substrate (all Vector Laboratories).

For β-galactosidase staining, embryos were fixed in 2% PFA, sections were washed with the rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris pH 7.3, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) and incubated in X-Gal staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg/ml X-gal) at 37 °C.

In situ hybridization

For RNA antisense probe synthesis, plasmid carrying Foxn4 cDNA (nucleotides 492–1562 of NM_148935) was used. The antisense mRNA probe was synthesized using RNA polymerase and digoxigenin-labeled nucleotides (Roche) according to the manufacturer's instructions. RNA *in situ* hybridization was carried out as previously described (Fujimura et al., 2009).

Quantitative RT-PCR (qRT-PCR)

For analysis of differentially expressed genes in wild-type and Pax6-deficient retinal cells, E12.5 embryonic eyes were dissected and washed in cold PBS and treated with 0.5% trypsin in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies) for 4 min at 37 °C to prepare single-cell suspension. Cells were subsequently washed with DMEM supplemented with 20% fetal bovine serum (FBS) (Gibco, Life Technologies), transferred to FBS-free DMEM, and $EGFP^+$ retinal cells were FACS sorted. FACS-sorted cells were subjected to RNA isolation using the RNeasy Micro isolation system (Qiagen) according to the standard manufacturer's protocol. Isolated RNA was subjected to reverse transcription and amplification using Ovation® Pico WTA Systems V2 (Nugene) and the resulting cDNA was used for qRT-PCR. Two independent samples for both wild-type and Pax6-deficient retinae were used. Every sample was represented by 20 embryonic eyes originating from 2–3 litters collected in one experiment.

To analyze mRNA expression in Onecut mutant retinae, retinae from E14.5 embryonic eyes were dissected and total RNA was isolated with Trizol® Reagent (Life Technologies). Random-primed cDNA was generated from 200 ng of total RNA using SuperScript VILO cDNA Synthesis kit (Life Technologies). At least four different

embryos originating from two litters were used for retina dissection and subsequent RNA isolation per each genotype.

qRT-PCR was run in the LightCycler® 480 Instrument (Roche) using a LightCycler® 480 DNA SYBR Green I Master (Roche) according to the standard manufacturer's protocol; typically, 5 μl reaction mixture was used. PCR reactions were performed in technical duplicate for each primer set of primers, with four different cDNAs. Crossing point (Cp) values were calculated by LightCycler® 480 Software (Roche) using the second-derivate maximum algorithm. The average Cp values of all technical replicates were normalized by Cp values of a housekeeping gene. Statistical significance of the change in mRNA expression was calculated by a two-tailed Student's *t*-test. Finally, the change in mRNA expression was presented as the ratio $Oc1^{-/-}$/wild-type ($Oc2^{-/-}$/wild-type, $Oc1^{-/-}$/$Oc2^{-/-}$/wild-type) retinae in log with base 2 scale. Sequences of used primers are listed in Supplementary Table 1.

Quantification of marker-positive cells

To analyze the number of HCs, eyes were sectioned, immunostained, and the number of marker-positive HCs per whole central retinal section was counted and normalized to wild-type control. For a single eye, a minimum of five sections were used; for each genotype, a minimum of eight individual retinae was analyzed. Statistical significance was assessed by Student's *t*-test.

To analyze the number of other retinal cell types, the number of marker-positive cells from a single eye field of the central retinal section was counted. Eye fields localized in the same distance from the optic nerve were selected. The number of marker-positive cells per defined retinal area was counted and normalized to wild-type control. For a single eye, a minimum of five eye fields; for each genotype, a minimum of four retinae were used. Statistical significance was analyzed by Student's *t*-test.

Electrophoretic mobility shift assays (EMSAs)

In silico analysis to identify putative Pax6 binding sites in *Oc1* locus was performed using high-quality transcription factor binding profile database JASPAR (http://jaspar.genereg.net; JASPAR: an open-access database for eukaryotic transcription factor binding profiles) (Sandelin et al., 2004). Electrophoretic mobility shift assays (EMSAs) with the full-length Pax6 was performed as previously described (Kozmik et al., 1997) using double-stranded oligonucleotides comprising binding sites shown in Supplementary Table 1. For competition experiment, wild-type and mutated Pax6 consensus binding sites were used as specific or non-specific competitor, respectively.

Cell culture, transient transfection and luciferase reporter assay

293T cells were cultured in Dulbecco's modified Eagle's medium (SIGMA) supplemented with 10% fetal bovine serum (PAA laboratories), 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (SIGMA). Transient transfection was performed using Fugene 6 (Roche) according to the manufacturer's protocol. Cells were plated in 24-well plates 24 h prior to transfection. Typically, 100 ng of the firefly luciferase reporter gene containing sites BC and DE introduced upstream of minimal TATA containing promoter in pGL3 vector (Kozmik et al., 1997) was co-transfected with 50 ng of Pax6 expression plasmid. The total amount of DNA transfected per well was 300 ng and was adjusted with pUC18. A β-galactosidase expression plasmid was co-transfected to normalize the transfection efficiency. Triplicate assays were performed to obtain standard deviations. Two days after transfection, the cells were lysed in 100 μl of 1 × passive lysis buffer (Promega). Luciferase reporter assays were performed using a Luciferase Reporter assay kit (Promega). β-galactosidase was detected with the Galacto-Star system (Applied Biosystems).

Results

Oc1 and Oc2 transcription factors operate downstream of Pax6 in retinal development

Pax6 plays a pivotal role in retinal development as *Pax6*-deficient retinae display severe differentiation defects (Klimova and Kozmik, 2014; Marquardt et al., 2001). This is by large attributed to the Pax6 ability to regulate expression of the transcription factors that mediate differentiation of particular retinal cell types (Marquardt et al., 2001; Oron-Karni et al., 2008; Riesenberg et al., 2009). Although some Pax6 downstream-acting genes have been previously identified, the complete transcriptional network leading from Pax6-expressing RPCs to all differentiated retinal cell types remains unresolved. In the search for Pax6-regulated genes in mouse retinal development, we screened Pax6 wild-type and Pax6-deficient retinal cells for differentially expressed genes (Fig. 1A). To inactivate *Pax6* in retinal cells, we generated conditional *Pax6* retinal mutants. We used mice with floxed *Pax6* allele ($Pax6^{flox/flox}$) (Klimova and Kozmik, 2014) and distal retina-specific *α-Cre* mice as a deleter (Marquardt et al., 2001) to generate $Pax6^{flox/flox}$/*α-Cre* ($Pax6^{-/-}$) retinal mutants. The *α-Cre*-mediated recombination leads to Pax6 elimination in distal parts of the retina at E12.5. At this stage the subpopulation of RPCs proceeds through the differentiation process, and thus analysis of this stage enables identification of both progenitor-cell-specific and differentiation-specific genes. As EGFP is expressed along with the Cre recombinase from *α-Cre* transgene (Marquardt et al., 2001), $EGFP^{+}$/$Pax6^{-/-}$ retinal cells were FACS sorted from E12.5 $Pax6^{flox/flox}$/*α-Cre* embryonic eyes and subjected to qRT-PCR for candidate Pax6-regulated genes (Fig. 1A). As a control, Pax6 wild-type retinal cells from $Pax6^{wt/wt}$/*α-Cre* ($EGFP^{+}$/$Pax6^{+/+}$) eyes were used. We established several criteria for Pax6-regulated candidate genes. We searched for: i) evolutionarily conserved ii) neuronal tissue-specific iii) transcriptional factors and iv) with a role in cell type specification. Based on these criteria, Oc1 and Oc2 transcription factors were identified as potential Pax6-regulated genes. The qRT-PCR performed with wild-type and Pax6-deficient retinal cells at E12.5 clearly showed a decrease of *Oc1* and *Oc2* mRNA expression corresponding with decreased expression of *Pax6* (Fig. 1B). Accordingly, immunohistochemical analysis showed a dramatic decrease of Oc1 and Oc2 protein levels (Fig. 1C). Although the expression of both Oc1 and Oc2 proteins was apparent in Pax6 wild-type ($Pax6^{flox/flox}$) retinae at E13.5, their expression was lost in the distal parts of Pax6 mutant ($Pax6^{flox/flox}$/α-*Cre*) retinae (Fig. 1C).

Oc1 and Oc2 proteins were previously found to be co-expressed in developing retinal ganglion cells and horizontal cells (Wu et al., 2012). The main prerequisite for target gene regulation is co-expression of selected proteins within the identical cell population. To find out whether Pax6 and Oc1 and Oc2 proteins are co-expressed during retinal development, retinal sections from E14.5 and postnatal (P18) retinae were co-stained with Pax6 and Oc1 or Pax6 and Oc2 antibody (Fig. 2). At E14.5 Pax6 is expressed virtually by all retinal cells and its co-expression with Oc1 and Oc2 was apparent in all Oc1 and Oc2-expressing cells (Fig. 2A and B). Based on previous observations, these cells represent cycling RPCs or differentiating ganglion and horizontal cells (Wu et al., 2012). In postnatal retinae (P18), Pax6 was expressed in RGCs localized in GCL and ACs and HCs localized in INL (Fig. 2C and D). At the same stage, Oc2 along with Oc1 was expressed in the HC population, where they were co-expressed with Pax6 (Fig. 2C–E). These data show that transcription factors Oc1 and Oc2 are co-expressed with Pax6 within the same populations during retinal development. Moreover, the absence of *Oc1* and *Oc2* gene products in Pax6-deficient retinal cells strongly indicates that *Oc1* and *Oc2* operate downstream of Pax6.

To provide a more direct link between Pax6 and Onecut expression, we performed *in silico* analysis of the upstream

regulatory region of *Oc1* and revealed several putative binding sites for Pax6 (Fig. 3A and B). Binding of the full-length Pax6 to individual sites was experimentally verified by electrophoretic mobility shift assays (EMSAs) in the presence of specific and nonspecific competitor DNA (Fig. 3C). Putative binding sites located in the −8 kb (site A), −4 kb (sites B and C) and −2.5 kb (sites D and E) region upstream of the *Oc1* gene transcription start site were recognized by full-length Pax6 specifically, since complex formation was efficiently inhibited by the presence of unlabeled Pax6 consensus binding site but not in the presence of mutant Pax6 binding site (Fig. 3C). To see whether the sites identified in the upstream regulatory region of *Oc1* gene by EMSA can function as Pax6-dependent regulatory modules, their sequences (listed in Supplementary Table 1) were introduced into the minimal reporter gene containing TATA element and such reporters were tested by co-transfection with an expression vector encoding Pax6. As shown in Fig. 3D, Pax6 activates transcription of reporter genes carrying sites BC and DE but the reporter gene is devoid of any binding site. Combined, our data show that the upstream regulatory region of *Oc1* gene contains several binding sites that are recognized by Pax6 *in vitro* and that can mediate Pax6-dependent regulation in transient reporter assays *in vivo*.

Oc1 and Oc2 are essential for horizontal cell development

Oc1 and Oc2 have been previously found to be expressed during the development of RGCs, HCs and cone photoreceptors in mammalian retina (Emerson et al., 2013; Wu et al., 2012). To investigate their potential role in differentiation of these retinal cell types, *Oc1* and *Oc2* were inactivated in the mouse retina (Fig. 4). To inactivate *Oc1*, mice with the floxed allele of *Oc1* ($Oc1^{flox/flox}$) (Zhang et al., 2009) were crossed with retina-specific *mRx-Cre* (Klimova et al., 2013) to generate $Oc1^{flox/flox}$/*mRx-Cre* ($Oc1^{-/-}$) retinal mutants. In *mRx-Cre* the Cre-mediated recombination is performed in the presumptive retinal tissue at E9.5. This enables inactivation of *Oc1* early before the differentiation program is initiated (Klimova et al., 2013). To inactivate *Oc2*, $Oc2^{+/-}$ mice (Clotman et al., 2005) were used. Since $Oc2^{-/-}$ mice fail to thrive during early postnatal period and display high levels of mortality before weaning (Dusing et al., 2010), heterozygote knockout mice were crossbred to obtain $Oc2^{-/-}$ embryos and postnatal mice. To inactivate *Oc1* along with *Oc2* to generate $Oc1^{-/-}/Oc2^{-/-}$ retinae, $Oc2^{+/-}$ mice were crossed to $Oc1^{flox/flox}$/*mRx-Cre* background. The efficiency of Oc1 and Oc2 inactivation was analyzed by immunohistochemistry of E14.5 retinal sections using Oc1 and Oc2-specific antibody (Fig. 4A–H). Although Oc1 and Oc2 expression was apparent in wild-type retinae (Fig. 4A and E), their expression was lost in corresponding mutants (Fig. 4B–D, F–H). The consequence of Oc1 and Oc2 inactivation was first investigated at the histological level in retinae from $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ mice at P18, at the stage when retinogenesis is complete. Although the wild-type retina displayed proper lamination with distinctly formed layers of retinal cells (Fig. 4I), in $Oc1^{-/-}$ and $Oc2^{-/-}$ retinae, the outer plexiform layer (opl), localized between INL and ONL, was thinned (Fig. 4J and K). In $Oc1^{-/-}/Oc2^{-/-}$ double mutant the opl was not even distinguishable and INL and ONL fused to form a single layer (Fig. 4L). In additionally, INL and inner plexiform layer (ipl) of the $Oc1^{-/-}/Oc2^{-/-}$ double

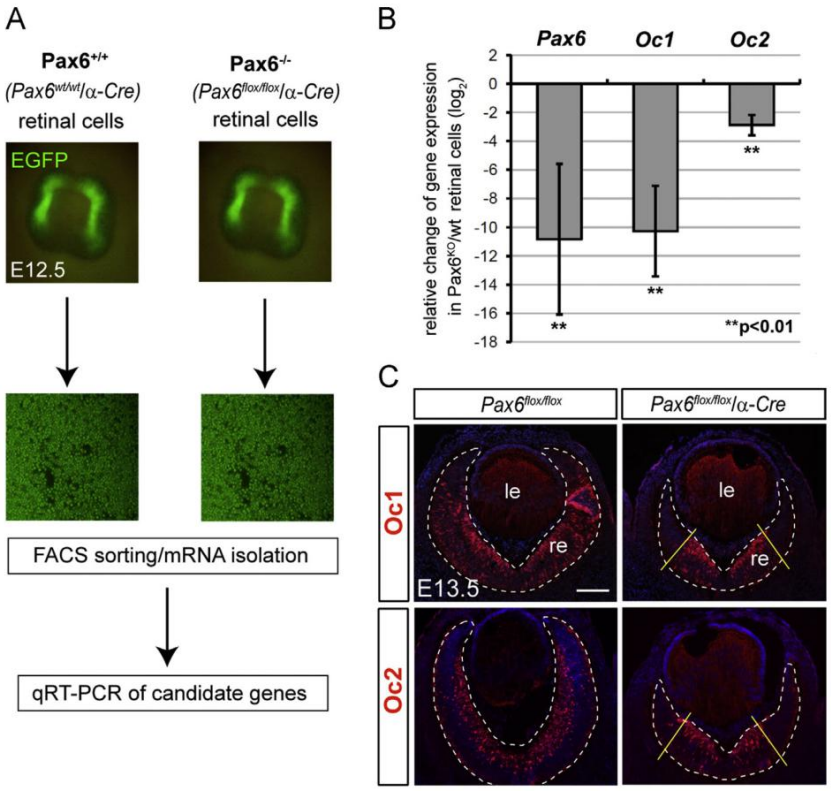


Fig. 1. Transcription factors Oc1 and Oc2 operate downstream of Pax6 in retinal development. (A) Experimental strategy used to search for Pax6-regulated genes in retinal development. E12.5 embryonic eyes of $Pax6^{wt/wt}$/*α-Cre* Pax6 wild-type ($EGFP^{+}/Pax6^{+/+}$) and $Pax6^{flox/flox}$/*α-Cre* Pax6 retinal mutants ($EGFP^{+}/Pax6^{-/-}$) were dissected, transformed to single-cell suspension and subjected to FACS to obtain $Pax6^{+/+}$ and $Pax6^{-/-}$ retinal cells. mRNA from $Pax6^{+/+}$ and $Pax6^{-/-}$ retinal cells were isolated and processed by qRT-PCR using primers specific for candidate Pax6-regulated genes. (B) qRT-PCR analysis showing the changes of relative mRNA expression of *Pax6* and candidate transcription factors *Oc1* and *Oc2* in $Pax6^{+/+}$ and $Pax6^{-/-}$ (Pax6KO) retinal cells. Error bars indicate standard deviation, *p*-Values are calculated by Student's *t*-test. (C) Immunohistochemical detection of Oc1 and Oc2 proteins in Pax6 wild-type ($Pax6^{flox/flox}$) and Pax6-deficient ($Pax6^{flox/flox}$/*α-Cre*) distal retina. The area of α-Cre-mediated inactivation of *Pax6* in $Pax6^{flox/flox}$/*α-Cre* retinae is demarcated with a solid yellow line. Retina is indicated with a dashed line. le, lens; re, retina.

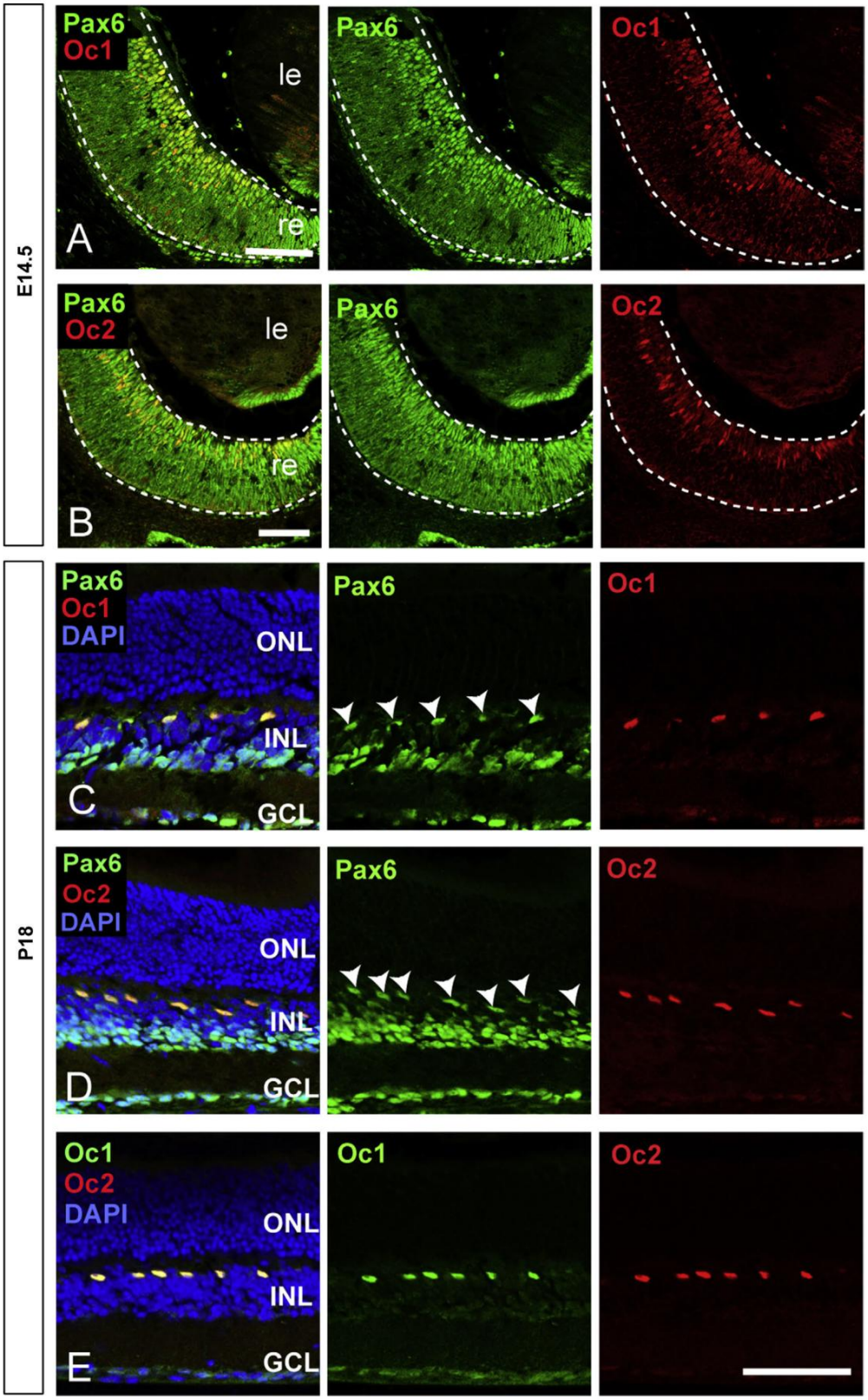


Fig. 2. Oc1 and Oc2 are co-expressed with Pax6 during retinal development. (A, B) Retinal sections of E14.5 eyes stained with Pax6-, Oc1- and Oc2-specific antibody showing the co-expression of Pax6 and Oc1 (A) and Pax6 and Oc2 (B) in retinal cells. (C–E) Retinal sections of P18 eyes stained with Pax6-, Oc1- and Oc2-specific antibody showing co-expression of Pax6 and Oc1 (C), Pax6 and Oc2 (D) and Oc1 and Oc2 (E) in horizontal cells localized between the inner and outer nuclear layers. Retinae at E14.5 are indicated with a dashed line. Arrowheads in (C) and (D) indicate cells that co-express Pax6 and Oc1 or Oc2, respectively. le, lens; re, retina; RPE, retinal pigmented epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

mutant was thinned as well (Fig. 4L). As the opl is the layer where the projections of horizontal cells are normally present and HCs are the only Oc-expressing cell type in the adult retina (Wu et al., 2012), histological analysis indicated a defect in the HC population. To analyze whether HCs are present in mutant retinae, sections of *Oc1*$^{-/-}$, *Oc2*$^{-/-}$, *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ and wild-type P18 eyes were analyzed for expression of HC-specific markers (Fig. 5). A calcium binding protein, Calbindin, is expressed in all HCs and a subset of amacrine cells (Dyer et al., 2003; Poche et al., 2007), along with activating protein-2 Ap2α (Bassett et al., 2012, 2007). On the other hand, expression of lim-homeodomain transcription factor Lim1, Oc1 and Oc2 is found exclusively in HCs at this stage (Liu et al., 2000; Poche et al., 2007; Wu et al., 2012). Staining for all indicated proteins was dramatically reduced in all mutants when compared with the wild-type control (Fig. 5A) indicating a defect in HC development. To quantify the decrease of horizontal cells in particular mutants, retinal sections were stained with Ap2α antibody and the number of Ap2α^{+} cells localized at the outer face of the INL was counted. Whereas the quantification showed a dramatic decrease of HCs in single *Oc1*$^{-/-}$ and *Oc2*$^{-/-}$ mutants (80.2 ± 2.8% for *Oc1*$^{-/-}$; 84.7 ± 4.3% for *Oc2*$^{-/-}$), in retinae of double mutants, only a few if any Ap2α^{+} HCs were found (Fig. 5B). The same result was obtained when Calbindin was used for quantification of HCs (data not shown). These data show that Oc1 and Oc2 inactivation leads to HC loss.

Previous study has shown that apart from HCs, Oc1 and Oc2 are strongly expressed by differentiating RGCs (Wu et al., 2012). It has been suggested that Oc proteins might play an important role during RGC differentiation as well (Wu et al., 2012). Based on that, we selected several markers of RGCs and assessed *Oc1*$^{-/-}$, *Oc2*$^{-/-}$, *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ and wild-type E14.5 and P18 retinae for their expression (Supplementary Fig. S1). At E14.5, immunohistochemistry was used to analyze the protein expression of Brn3a, Brn3b, Isl1 and Tbr2 (Supplementary Fig. S1A) and qRT-PCR was used to assess the expression of *Pax6*, *Math5*, *Brn3b*, *Isl1*, *Tbr2*, *Oc1*, *Oc2* (Supplementary Fig. S1B). At P18, immunohistochemistry was used to analyze the expression of Brn3a, Brn3b, Isl1, Tbr2 and Pax6 (Supplementary Fig. S1C) and the number of Brn3b^{+}, Pax6^{+} and Tbr2^{+} cells localized in GCL was counted to quantify the number of RGCs (Supplementary Fig. S1D). This analysis showed that mRNA expression of *Oc1* and *Oc2* was dramatically reduced in the corresponding mutants (Supplementary Fig. S1B), as expected, and their expression levels in particular mutants did not indicate any interaction between *Oc1* and *Oc2*. Along with *Oc1* and *Oc2*, the expression of the other Onecut factor, *Oc3* was dramatically reduced in the *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ double mutant (Supplementary Fig. S1B), indicating *Oc3* dependence on *Oc1* and *Oc2* expression. Although immunohistochemical analysis at E14.5 and P18 and qRT-PCR at E14.5 did not reveal any conspicuous difference in the expression of *Math5*, *Brn3a*, *Brn3b* and *Isl1* (Supplementary Fig. S1A–C), the expression of *Tbr2* was dramatically reduced in Oc-deficient retinae (Supplementary Fig. S1A–D). At E14.5, Tbr2 was not detectable and the number of Tbr2^{+} cells at P18 was reduced by 63% (± 10.9%) in the *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ double mutant retinae

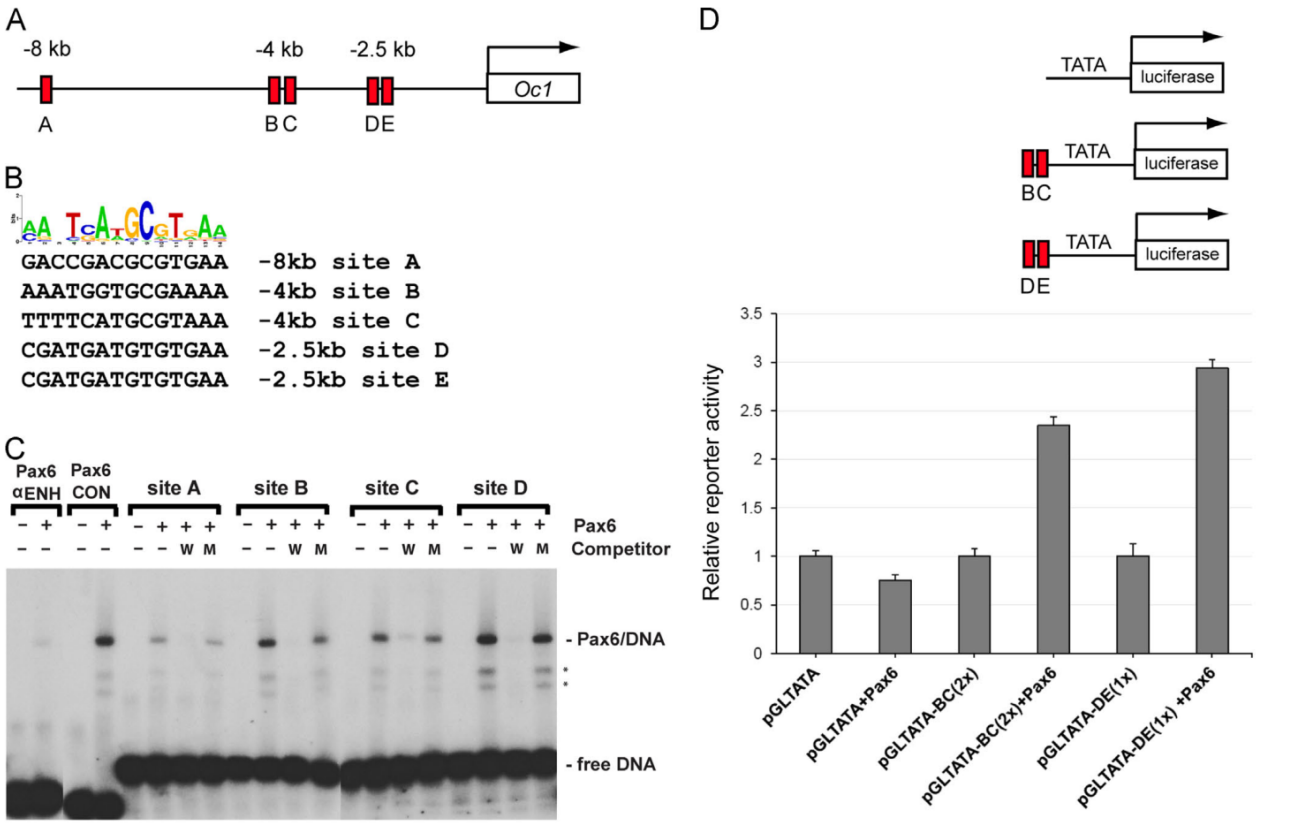


Fig. 3. Multiple Pax6 binding sites are present in the upstream regulatory region of *Oc1* gene. (A) Schematic diagram of *Oc1* upstream regulatory region indicating position of Pax6 binding sites A–E. (B) Alignment of sites A–E with the recognition sequence logo of Pax6. (C) Electrophoretic mobility shift assay (EMSA) with binding sites A–D (nucleotide sequence of Pax6 binding site E is identical to site D). The specific complex of Pax6 with its binding sites is competed by wild-type Pax6 consensus site (W) but not by the mutant binding site (M). Asterisks denote DNA-binding competent degradation products of Pax6. Binding to a functional Pax6 binding site derived from retina-specific α enhancer (Schwarz et al., 2000) and high-affinity consensus Pax6 binding site is shown for comparison. (D) Indicated luciferase reporter genes were co-transfected with Pax6-encoding expression vector or with empty expression vector. Triplicate assays were performed to obtain standard deviations and transfection efficiency was normalized by co-transfection of β-galactosidase expression plasmid.

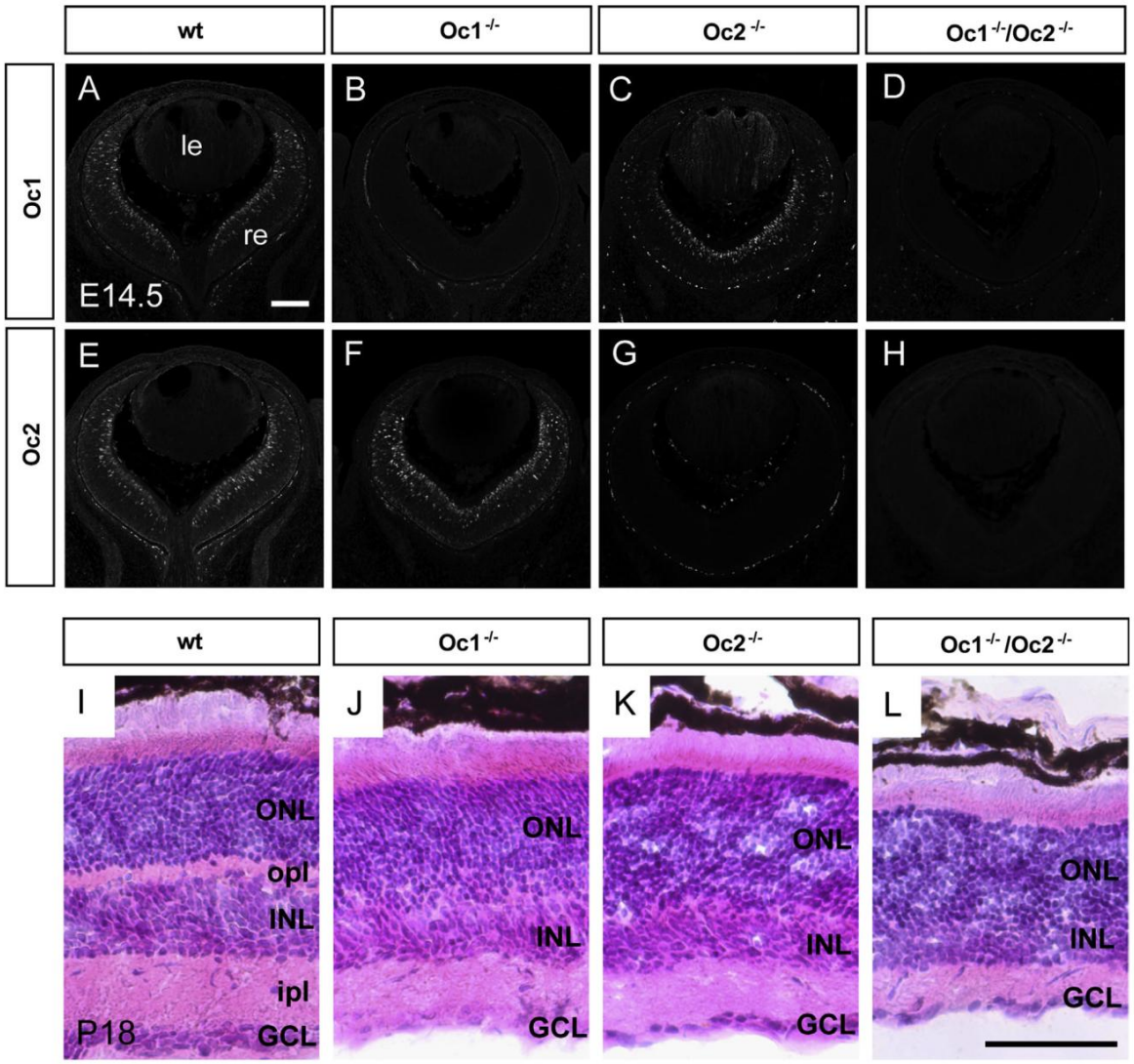


Fig. 4. Oc1 and Oc2 inactivation in the mouse retina. (A–H) Retinal sections of E14.5 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eye stained with Oc1- and Oc2-specific antibody showing the efficiency of Oc protein elimination in the corresponding mutants. (I–L) Retinal sections of P18 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eyes stained with hematoxylin-eosin showing a reduced outer plexiform layer in Oc mutants. le, lens; re, retina; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; opl; outer plexiform layer; ipl, inner plexiform layer.

(Supplementary Fig. S1A, C and D). However, quantification of RGCs at P18 showed no difference in the number of $Brn3a^+$ RGCs and only a slight reduction of $Pax6^+$ RGCs (by $9.8\% \pm 1.1\%$) (Supplementary Fig. S1D) in $Oc1^{-/-}/Oc2^{-/-}$ double mutant retina. Although Pax6 is expressed by most RGCs and Brn3a by ~80% of RGCs (Xiang et al., 1995), Tbr2 is expressed by a small subset of RGCs (~10%) (Mao et al., 2008; Sweeney et al., 2014). Our data indicate that Oc proteins are involved in generation of $Tbr2^+$ RGCs.

Recently, Oc factors have been found to be involved in cone differentiation in mouse retina (Emerson et al., 2013; Sapkota et al., 2014). Thus we examined the expression of an early cone-specific marker Rxrγ (Roberts et al., 2005) in E14.5 and P18 wild-type and Oc-deficient retinae. In accordance with previous works, we found Rxrγ protein expression absent in $Oc1^{-/-}/Oc2^{-/-}$ E14.5 (Supplemetary Fig. S2A) and quantification at P18 showed decreased number of $Rxr\gamma^+$ cells (by 23% $\pm$1.9%) in $Oc1^{-/-}/Oc2^{-/-}$ retinae indicating that number of cone photoreceptors is reduced upon Oc inactivation. Among other retinal cell types, we observed decreased numbers of ACs immunoreactive for Calretinin (decreased by $61.5 \pm 0.5\%$), Pax6 (decreased by $17.6 \pm 4.8\%$) and Isl1 (decreased by $25 \pm 1.6\%$). As recent study revealed defects in generation of other retinal cell types as well (Sapkota et al., 2014) we additionally analyzed the number of bipolar (BC) and Müller glia cells in Oc-deficient retinae. BCs immunoreactive for Chx10 were decreased by $27 \pm 3.7\%$ and Müller glia cells immunoreactive for Lhx2 were decreased by $27 \pm 2.7\%$ (Supplementary Fig. S2B) in $Oc1^{-/-}/Oc2^{-/-}$ retinae. Although for Calretinin, Pax6, Isl1 and Chx10, the decrease was observed in the $Oc1^{-/-}/Oc2^{-/-}$ double mutant only, for Lhx2, the decrease was observed in single Oc1 and Oc2-deficient retinae as well. As ACs, BCs and Müller glial cells are present in the INL, their reduction might contribute to the reduced thickness of INL and ipl observed in $Oc1^{-/-}/Oc2^{-/-}$ double mutant retinae (Fig. 4L). Taken together, our data indicate that Oc loss has an impact on majority of retinal cell types.

Although HCs are absent postnatally, their differentiation program is initiated in Oc-deficient embryonic retinae

Like other retinal cell types, HCs are generated from a common RPC population. Several transcription factors have been shown to

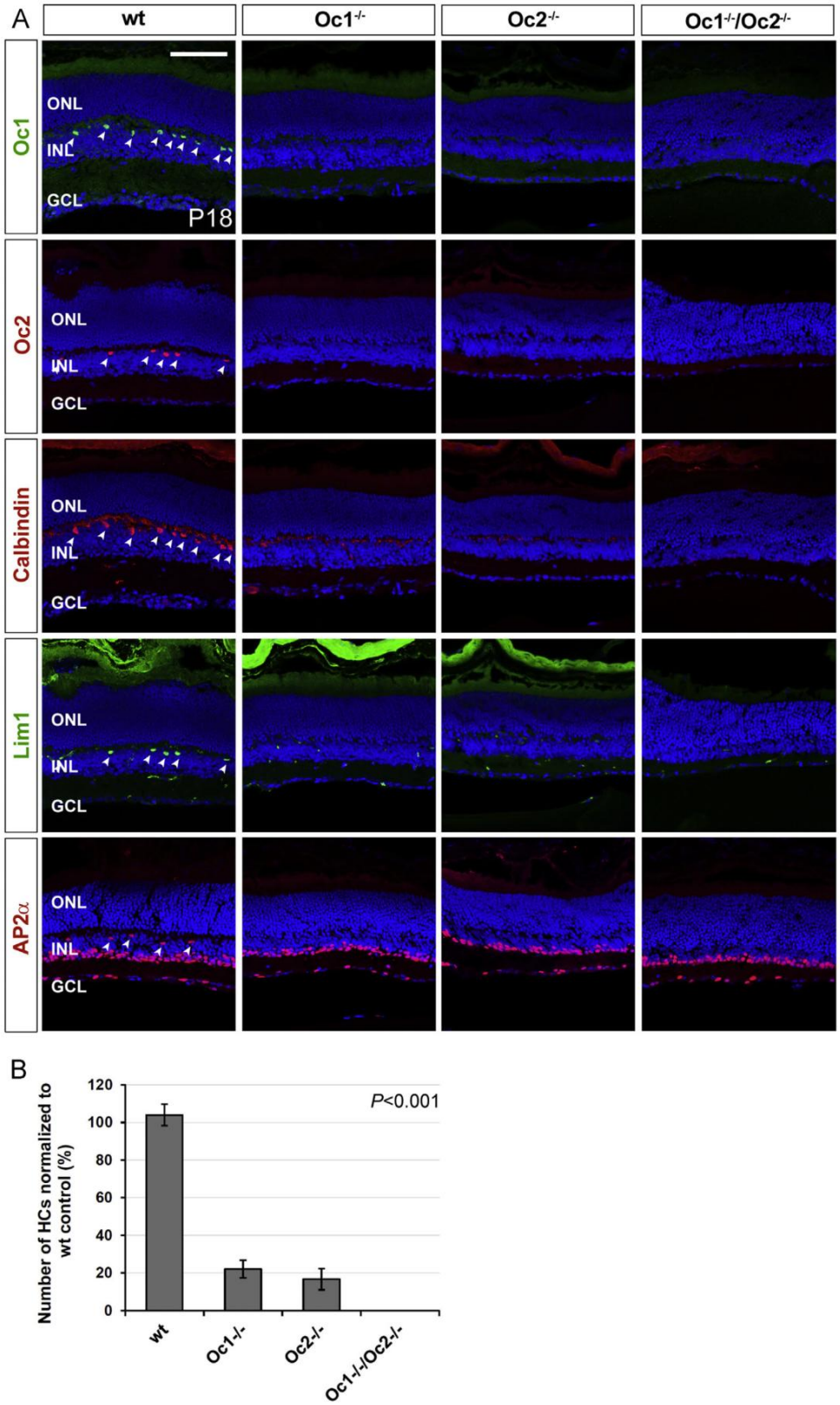


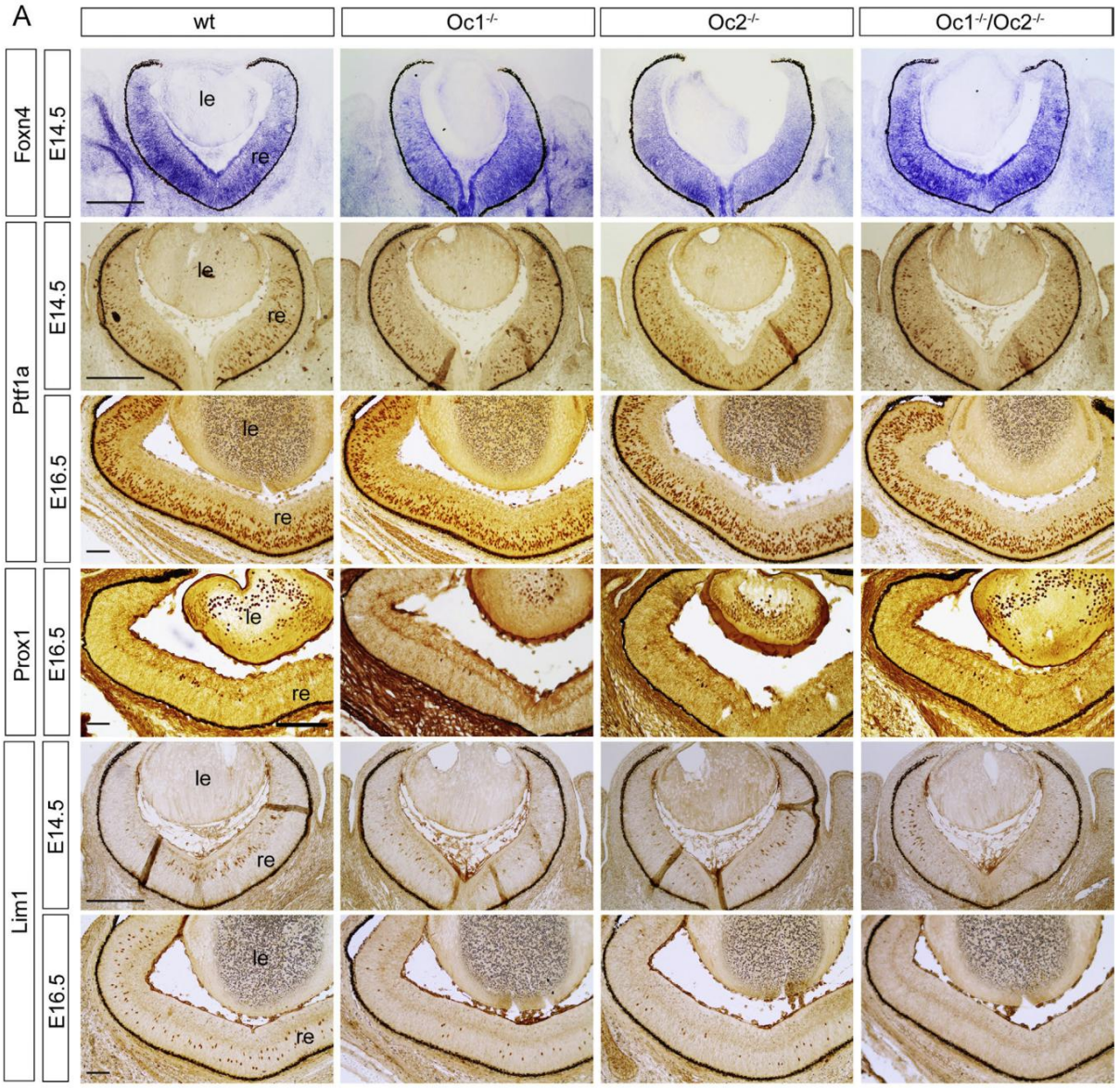
Fig. 5. Oc1 and Oc2 are essential for horizontal cell development. (A) Retinal section of P18 wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ eyes stained with antibodies against HC markers Oc1, Oc2, Calbindin, Lim1 and Ap2α. (B) Quantification of the number of HCs in wild-type (*wt*), $Oc1^{-/-}$, $Oc2^{-/-}$, $Oc1^{-/-}/Oc2^{-/-}$ and $Oc1^{+/-}/Oc2^{+/-}$ retinae assessed as the number of $Ap2\alpha^{+}$ cells localized at the outer face of the inner nuclear layer. Error bars indicate standard deviation, *p*-values are calculated by Student's *t*-test. Arrowheads indicate HCs stained for HC-specific marker. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

be required for HC development, including Foxn4, Ptf1a, Prox1 and Lim1 (Dyer et al., 2003; Fujitani et al., 2006; Li et al., 2004; Nakhai et al., 2007). HCs share initial steps of differentiation with ACs. In HC/AC common precursors, Foxn4 operates upstream of Ptf1a to control both HC and AC fates (Fujitani et al., 2006; Li et al., 2004). On the other hand, transcription factors Prox1 and Lim1 are required specifically for development and proper laminar positioning of HCs (Dyer et al., 2003; Poche et al., 2007). As simultaneous Oc1 and Oc2 inactivation is associated with complete loss of HCs, we analyzed the relationship of Oc1 and Oc2 with other transcription factors implicated in HC development. We assessed retinae from E14.5 and E16.5 wild-type, $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ mutant retinae for the protein expression of Ptf1a, Lim1 and Prox1 (Fig. 6A, Supplementary Fig. S3). *Foxn4* expression was analyzed by *in situ* hybridization at E14.5 (Fig. 6A). Finally, we processed mRNA from E14.5 wild-type, $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ mutant retinae by qRT-PCR to quantify the mRNA expression of *Foxn4*, *Ptf1a*, *Prox1* and *Lim1* (Fig. 6B). Embryonal stages for analysis of particular gene products were selected with respect to the onset of their expression and the onset of HC genesis. Whereas qRT-PCR and immunohistochemical analysis did not reveal any significant changes of *Foxn4* and *Ptf1a* expression, the expression of *Lim1* was reduced in $Oc1^{-/-}$, $Oc2^{-/-}$ and $Oc1^{-/-}/Oc2^{-/-}$ retinae (Fig. 6A and B). At the mRNA level, slight reduction of *Lim1* was apparent at E14.5 (Fig. 6B), whereas Lim1 protein expression remained unchanged at this stage when compared with wild-type control (Fig. 6A). At E16.5, the Lim1 protein expression was reduced in $Oc1^{-/-}$ and $Oc2^{-/-}$ with no expression observed in $Oc1^{-/-}/Oc2^{-/-}$ mutant retinae (Fig. 6A). These data indicate that although the Lim1 expression was properly initiated, it was not maintained in Oc-deficient retinae. For *Prox1*, qRT-PCR did not indicate any change in the mRNA expression (Fig. 6B). Immunohistochemical analysis performed at E14.5 and E16.5 showed that at both stages Prox1 protein expression was maintained, although slightly weaker expression was observed at E14.5 (Fig. 6A; Supplementary Fig. S3). As $Prox1^{+}$/$Lim1^{+}$ HCs are initially generated in Oc-deficient retinae, the data presented above indicate that although Oc1 and Oc2 factors are essential for HC development, they do not regulate the HC development through activation of Foxn4, Ptf1a, Prox1 or Lim1. To introduce Oc1 and Oc2 factors to the existing GRN of HC development, we finally analyzed the relationship between Pax6 and Foxn4 as Oc1 has been previously found to be dependent on Foxn4 expression as well (Wu et al., 2013). The qRT-PCR performed with E12.5 FACS-sorted $Pax6^{-/-}$ ($Pax6^{flox/flox}$/*α-Cre*) and $Pax6^{+/+}$ ($Pax6^{wt/wt}$/*α-Cre*) retinal cells showed that the expression of *Foxn4* is not dependent on Pax6 (Supplementary Fig. S4), indicating a Foxn4-independent mechanism of Pax6-mediated Oc1 and Oc2 activations.

As Lim1 expression was lost soon after HCs have been generated in Oc-deficient retinae, Oc factors might regulate HC development through the maintenance of HC-specific expression at later stages. To examine when HCs are lost, we analyzed E18.5 wild-type and Oc-deficient retinae for the expression of HC-specific genes *Prox1* and *Oc2* (Fig. 7). As *Oc2* knockout mice were generated by the insertion of *LacZ* into the *Oc2* coding sequence (Clotman et al., 2005), we used β-galactosidase expression to trace the expression of *Oc2*. In E18.5 wild-type ($Oc2^{+/-}$) retinae, the expression of Prox1 and β-galactosidase was apparent in the outer neuroblastic layer where HCs are localized (Fig. 7). However, in $Oc1^{-/-}$ and $Oc2^{-/-}$ retinae, number of $Prox1^{+}$ cells was reduced and in $Oc1^{-/-}/Oc2^{-/-}$ mutant retinae Prox1 expression was not detectable and HCs were lost as no β-galactosidase staining was observed in corresponding retinal area (Fig. 7). These data indicated that HCs were lost soon after they have been generated and that Oc1/Oc2 play the role in HC maintenance.

Discussion

The transcription factor Pax6 is essential for eye development in various animal species (Kozmik, 2005). Studies in which the conditional gene inactivation approach was used showed that in mammalian retina Pax6 plays a central role in RPC multipotency (Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008). Although generation of all retinal cell types requires Pax6, little is known about the identity of Pax6 downstream-acting factors that mediate differentiation of the particular retinal cell types. Previous studies showed that several pro-neural bHLH transcription factors including Math5, Ngn2, Mash1, Math3 and Neurod1 are dependent on Pax6 expression in the mouse retina (Klimova and Kozmik, 2014; Marquardt et al., 2001; Riesenberg et al., 2009). Accordingly, the enhancer element of *Math5* was found to be bound by Pax6, suggesting direct regulation by Pax6 (Marquardt et al., 2001; Riesenberg et al., 2009). As Math5 has been implicated in RGC genesis (Brown et al., 2001; Wang et al., 2001), Neurod1 in photoreceptor (Morrow et al., 1999), Neurod1 with Math3 in AC (Inoue et al., 2002) and Mash1 with Math3 in BC genesis (Tomita et al., 2000), the dependence of these bHLH factors on Pax6 may contribute to the loss of several cell types in Pax6-deficient retinae. However, how Pax6 contributes to HC development has not been studied. Horizontal cells are generated in the first wave of retinogenesis starting around E11 (Hatakeyama and Kageyama, 2004). At this stage, Foxn4 is expressed in mitotic progenitors that give rise to HC/AC common precursors (Li et al., 2004). Although Foxn4 inactivation clearly showed that Foxn4 is essential for HC differentiation, it has been suggested that an additional progenitor-expressed factor, most probably Pax6, may contribute to HC genesis as Foxn4 alone promotes the AC fate only when over-expressed in retinal progenitors (Li et al., 2004). Accordingly, it was previously shown that co-expression of Math3 and Pax6 promotes HC formation (Inoue et al., 2002). However, because HCs develop normally in Math3-null mice (Inoue et al., 2002), other genes may be involved in HC development. In this study we searched for Pax6 downstream-acting transcription factors that might execute this task and identify two transcription factors of the Oc family, Oc1 and Oc2. In *Oc1* and *Oc2* mutants, the number of HCs was dramatically reduced and no HCs developed when both *Oc1* and *Oc2* were inactivated, indicating their essential role in HC genesis. It goes along with the recent studies that reported a decrease of HCs in Oc-deficient retinae (Sapkota et al., 2014; Wu et al., 2013). As Oc1 expression was found to be dependent on Foxn4 expression as well (Wu et al., 2013), and Foxn4 and Pax6 are expressed independently of each other (Li et al., 2004; this study), Pax6 and Foxn4 might cooperate to activate *Oc1* and *Oc2* expression in the retina (Fig. 8). Accordingly, Pax6 or Foxn4 alone do not promote HC genesis when over-expressed in P0 or E17.5 retinal progenitors (Inoue et al., 2002), indicating that none of them is sufficient although both are necessary (Klimova and Kozmik, 2014; Li et al., 2004; Marquardt et al., 2001). Apart from Foxn4 and Pax6, bHLH pancreas-specific transcription factor 1a (Ptf1a) and homeodomain protein Prox1 are required for HC differentiation (Dyer et al., 2003; Fujitani et al., 2006). Ptf1a operates downstream of Foxn4 and is transiently expressed in post-mitotic AC/HC precursors (Fujitani et al., 2006) (Fig. 8). While Ptf1a and Foxn4 specify the HC/AC common precursor and are required for differentiation of both cell types (Fujitani et al., 2006; Li et al., 2004; Nakhai et al., 2007), Prox1, acting downstream of Ptf1a and Foxn4 (Fujitani et al., 2006; Li et al., 2004), is required specifically for acquisition of HC fate (Dyer et al., 2003). Prox1 is the only factor identified to date that is both necessary and sufficient for the HC fate determination (Dyer et al., 2003). Oc1 and Oc2 appear to be dispensable for the initial steps of HC differentiation as the expression of Foxn4, Ptf1a, Prox1 and Lim1 is



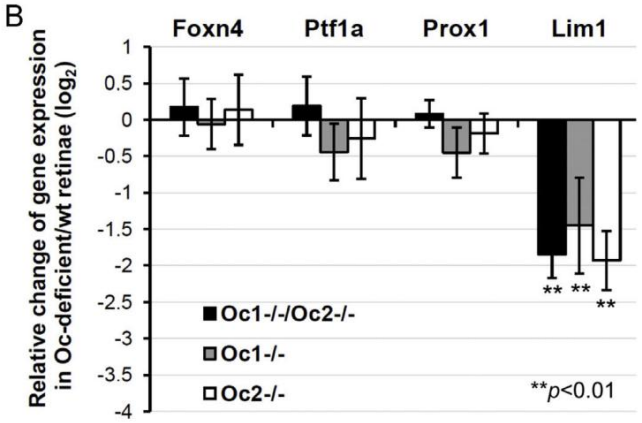


Fig. 6. Horizontal cell differentiation is initiated in Oc1 and Oc2-deficient retinae. (A) Retinal sections of E14.5 (and E16.5) wild-type (*wt*), *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ eyes assessed for the expression of HC-specific factors Foxn4, Ptf1a, Prox1 and Lim1. *In situ* hybridization was used to analyze the mRNA expression of *Foxn4* and immunohistochemistry was used to analyze the protein expression of Ptf1a, Prox1 and Lim1. (B) Quantification of *Foxn4*, *Ptf1a*, *Prox1* and *Lim1* mRNA expression at E14.5 assessed by qRT-PCR. Whole retinae from E14.5 wild-type (*wt*), *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ eyes were dissected, subjected to RNA isolation and processed by qRT-PCR. Error bars indicate standard deviation, *p*-Values are calculated by Student's *t*-test. le, lens; re, retina. Scale bar 200 μm.

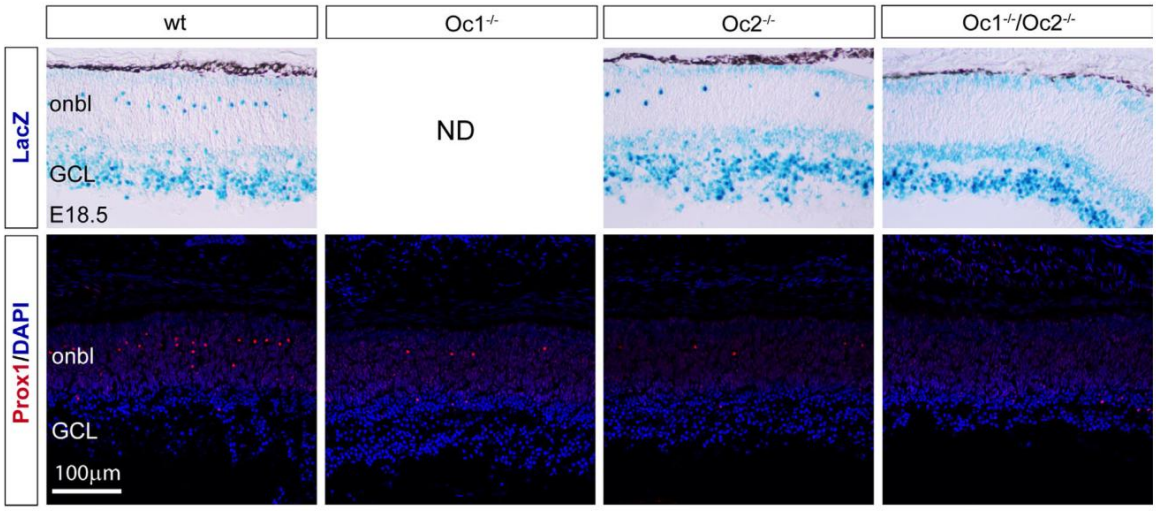


Fig. 7. Oc factors are involved in the maintenance of HCs. Retinal sections of E18.5 *Oc2*$^{+/-}$ (*wt*), *Oc1*$^{-/-}$, *Oc2*$^{-/-}$ and *Oc1*$^{-/-}$/*Oc2*$^{-/-}$ eyes assessed for the expression of HC-specific factors Oc2 and Prox1. As *Oc2* knockout mice were generated by the insertion of *LacZ* into the *Oc2* coding sequence (Clotman et al., 2005), β-galactosidase expression was used to trace the expression of *Oc2*. β-galactosidase and Prox1 staining shows that HCs are lost already at E18.5.

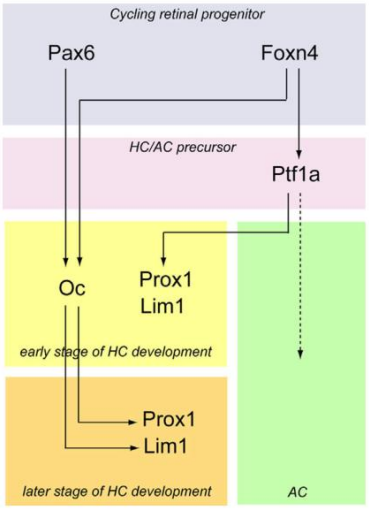


Fig. 8. Schematic representation of the Pax6, Oc1 and Oc2 role in HC development. Pax6 and Foxn4 are co-expressed in cycling retinal progenitor cells that give rise to both HCs and ACs (Li et al., 2004). In the common progenitor of HCs and ACs, Foxn4 stimulates expression of Ptf1a (Fujitani et al., 2006), which is required for differentiation of both cell types (Fujitani et al., 2006; Nakhai et al., 2007). To specify the HC fate, Pax6 and Foxn4 stimulate expression of Oc1/Oc2 in HC precursors (this study; Wu et al., 2013). At early stages of HC development, the expression of Prox1 and Lim1 is not dependent on Oc expression. At later stages of HC development, Oc factors are required to maintain the HC identity most likely through the regulation of Lim1 and Prox1 expression. The sequence of events leading to differentiation of HCs is indicated by solid arrows, events leading to differentiation of ACs with dashed arrow.

initiated in Oc-deficient retinae. Recent studies have indicated that Prox1 and Lim1 expression is not maintained upon Oc inactivation, suggesting the model in which Oc factors operate upstream of Prox1 to direct HC differentiation (Sapkota et al., 2014; Wu et al., 2013). However, we used three independent approaches to analyze the expression of Prox1 in *Oc*-deficient retinae and found that Prox1 (as well as Lim1) expression is initiated and Prox1^{+}/Lim1^{+} HCs are initially generated. Consistently, RNA-seq data presented by Sapkota et al. (2014) indicated only very slight decrease of Prox1 expression in Oc-deficient retinae.

Studies of the Oc role in different parts of the nervous system indicate that Oc factors are responsible for the maintenance of specific cell fate and survival rather than for the initial steps of differentiation (Espana and Clotman, 2012a, 2012b; Stam et al., 2012). A similar phenomenon can be observed in the retina as well. The expression of the main determinants of HC fate is initiated, indicating that the differentiation program of HCs is initiated properly but not maintained in the absence of *Oc1* and *Oc2*. As Lim1 and Prox1 expression is lost soon after HCs have been generated, the maintenance of HCs may require the continuous Lim1 and Prox1 expression at later stages of HC development (Fig. 8). Accordingly, Oc1 misexpression in postnatal mouse retina is sufficient to induce Lim1 expression (Emerson et al., 2013). The Pax6-regulated Oc pathway might then represent the branch in HC GRN, ensuring rather the maintenance of HC fate than the initial cell fate determination (Fig. 8). However, considering that HCs represent only approximately 0.2% of all retinal cells in the mouse (Ajioka et al., 2007), it is complicated to determine whether these cells died or adopted an alternative retinal cell fate. Further studies need to be executed to answer this issue.

Apart from HCs, strong expression of Oc1 and Oc2 was observed in developing RGCs (Wu et al., 2012). RGCs are the first retinal cell type to differentiate in the mammalian retina (Cepko et al., 1996; Young, 1985) and their differentiation is governed by a well-defined GRN of three transcription factors; Math5, Brn3b and Isl1. Whereas Math5 keeps RPCs competent to generate RGCs, Brn3b and Isl1 function downstream of Math5 and activate genes essential for RGC differentiation (Mu et al., 2008; Pan et al., 2008). As Oc1 and Oc2 expression was found to overlap with Math5 as well as with Brn3b and Isl1, the Oc role in establishing RGC fate was suggested (Wu et al., 2012). Since Oc1 and Oc2 expression appeared to be independent of Isl1, Brn3b and Math5, it was assumed that Oc regulate RGC genesis independently of these factors and represent a novel pathway in the RGC GRN (Wu et al., 2012). Accordingly, our analysis showed that *Oc1* and *Oc2* expression is dependent on Pax6 expression, integrating Oc1 and Oc2 downstream of Pax6, to the parallel with that of Math5 in RGC GRN. Interestingly, Oc inactivation seems to have only a negligible impact on RGC differentiation in general. On the other hand, a small subset of RGCs (~10%), Tbr2^{+} RGCs, is severally affected (Sapkota et al., 2014; this study). It is represented by RGC types that send axonal projections to non-image forming areas of brain,

such as Opn4/melanopsin-expressing photosensitive RGCs (ipRGCs) (Mao et al., 2008, 2014; Sweeney et al., 2014). Oc factors are then likely to be involved specifically in generation of this RGC subpopulation.

Until now, Oc expression has been observed in differentiating HCs, RGCs and cone photoreceptors (Emerson et al., 2013; Wu et al., 2012). However, analysis of other retinal cell types in Oc-deficient retinae indicates Oc's role in differentiation of bipolar cells, amacrine cells and Müller glia cells as well (Sapkota et al., 2014; this study). Since we do not have detailed information about Oc expression in such retinal cell types, we cannot distinguish whether the effect of Oc loss is caused by cell-autonomous or non-cell-autonomous mechanism. As Oc1 and Oc2 expression in RPCs has been previously reported (Emerson et al., 2013; Wu et al., 2012), it raises the question whether Oc factors could be transiently expressed in progenitors of retinal cell types other than HCs, RGCs and cone photoreceptors. More comprehensive analysis combined with the tracing of Oc-expressing cells would help to resolve this issue.

In different systems studied, Oc1 and Oc2 show functional redundancy as they are expressed in a partially overlapping pattern and share biochemical activities (Clotman et al., 2005; Espana and Clotman, 2012a, b; Jacquemin et al., 1999; Margagliotti et al., 2007; Vanhorenbeeck et al., 2002, 2007). In neuronal populations, including A13 neurons, Locus Coereleus neurons and Renshaw cells, inactivation of *Oc1* or *Oc2* alone results in no or mild defective phenotype only, when compared with compound *Oc1*/*Oc2* mutants (Espana and Clotman, 2012a, 2012b; Stam et al., 2012). Interestingly, in the retina, the degree of redundancy appears lower as inactivation of a single *Oc* gene leads to dramatic reduction of HCs. Although the phenotypes of *Oc1* and *Oc2* share general characteristics and Oc1 and Oc2 probably exert overlapping functions, we cannot formally exclude the possibility that Oc1 and Oc2 may display distinct properties during the HC development. Further, more detailed analysis and comparison of Oc1 and Oc2 target genes that mediate HC genesis might resolve this issue.

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While this article was under review the manuscript Sapkota et al. was published describing the analysis of Oc1/Oc2 double K.O. mice. To address some discrepancies in our initial submission immunohistochemical stainings at P18 were performed for the following markers: Tbr2, Rxrγ, Chx10 and Lhx2.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.02.023.

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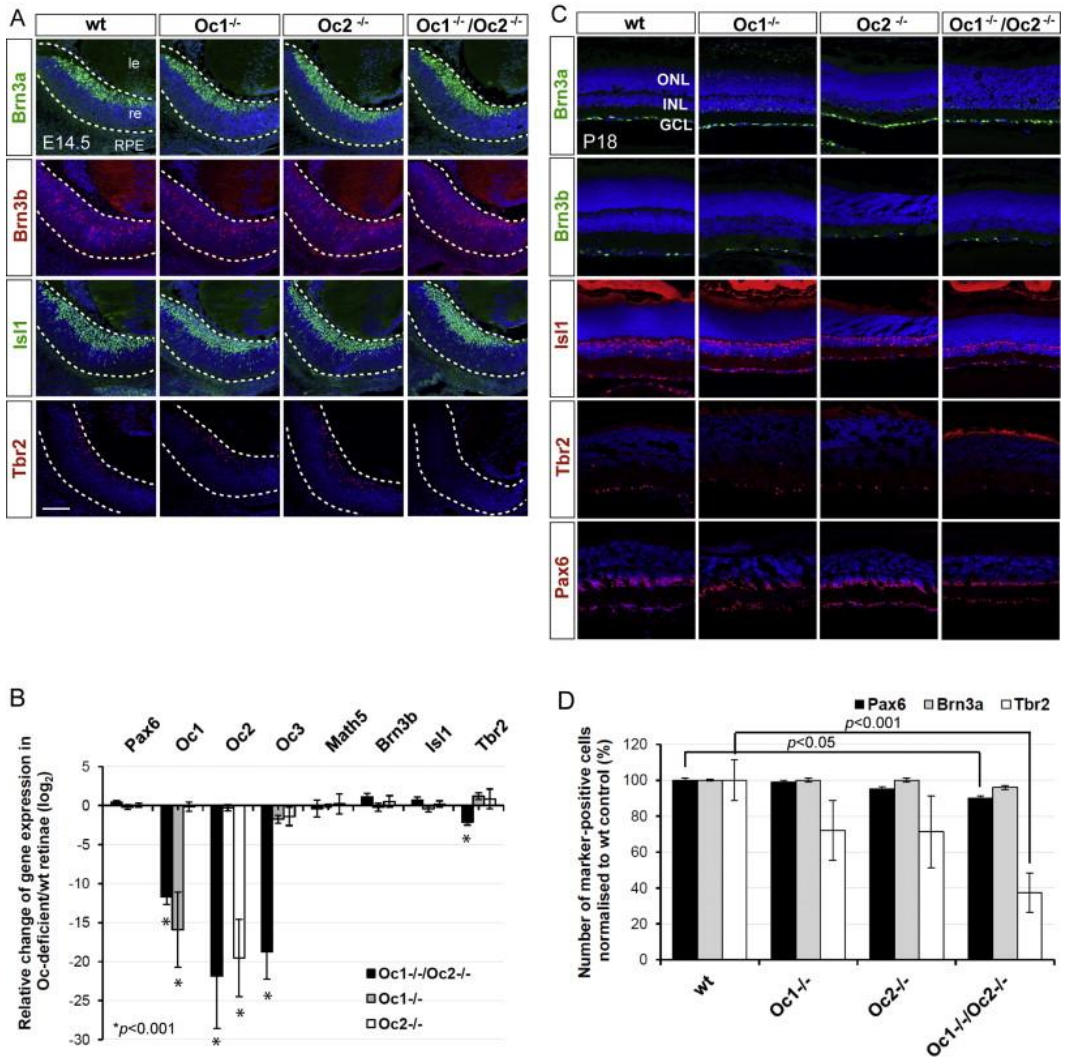
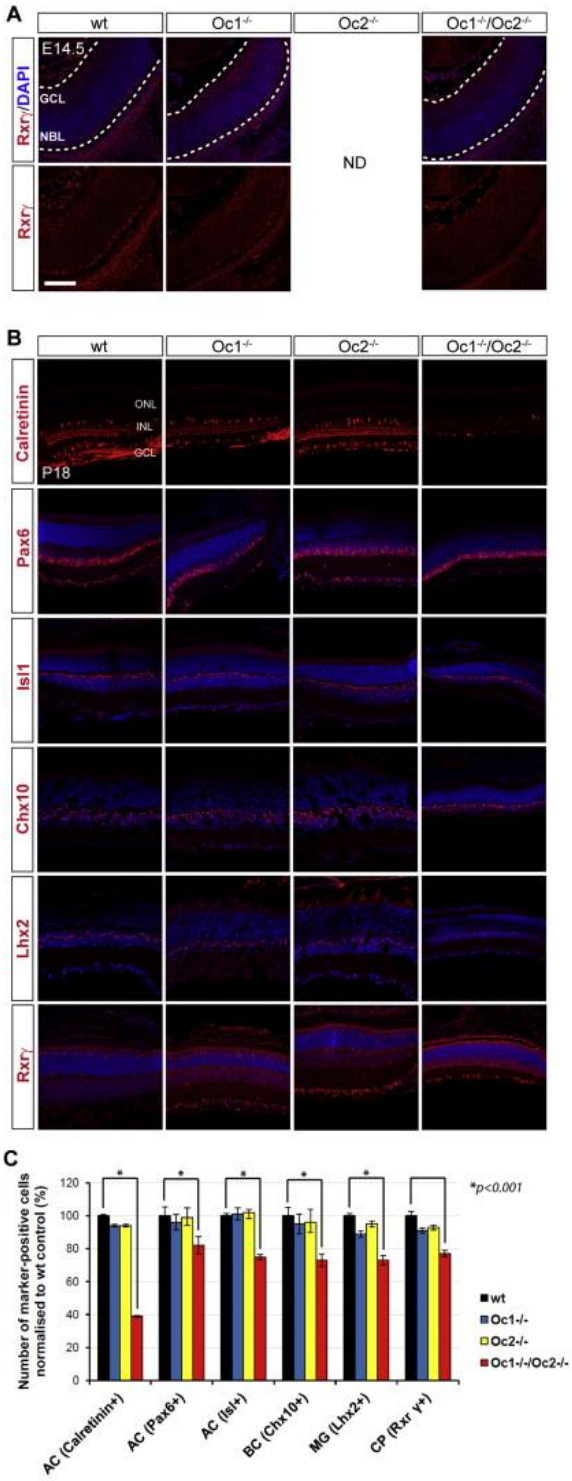
Supplementary Table 1.

Sequences of oligonucleotides used for qRT-PCR, EMSA and Luciferase reporter assay.

Primers for qRT-PCR		
Gene	**Forward primer**	**Reverse primer**
Pax6	ACAGCGGAGTGAATCAGC	GCAGAATTCGGGAAATGTCG
Oc1	CCCTGGAGCAAACTCAAGTC	TTGGACGGACGCTTATTTTC
Oc2	AGAGGGTTCTATGCCGGTCT	GGGATTTCTTCTGCGAGTTG
Oc3	CCCTGGAGCAAGCTCAAAT	ACACGAGGCGTTGCTTCTT
Math5	CAGGACAAGAAGCTGTCCAA	GGGTCTACCTGGAGCCTAGC
Brn3b	CGGAGAGCTTGTCTTCCAAC	ATGGTGGTGGTGGCTCTTAC
Isl1	ACGTGCTTTGTTAGGGATGG	TCACGAAGTCGTTCTTGCTG
Foxn4	AAGTGCTTCGAGAAGGTGGA	GGCTTTAGGCTCTCCTCGTT
Tbr2	GGCAAAGCGGACAATAACAT	AGCCTCGGTTGGTATTTGTG
Ptf1a	AACCAGGCCCAGAAGGTTAT	CCACACTTTAGCTGTACGGATG
Lim1	AAGGAGCGAAGGATGAAACA	CTTGCGGGAAGAAGTCGTAG
Actin	GTCCACACCCGCCACCAGTT	TTCTCCATGTCGTCCCAGTTG
Oligonucleotides for EMSA (top strand shown)		
1433A, -8kb	actggggcccagttcacgcgtcggtctccttcgcccacttc	
1479A, -4kb	ggtttaaacccattttcatgcgtaaaacaatttttaggaag	
1480A, -4kb	aaaagacaaaagaaatggtgcgaaaaggctgggtttaaacc	
1481A, -2.5kb	tagcaccatgaacgatgatgtgtgaactaatacccgatgat	
Pax6 wild-type binding site	aagaggtgcgcaatcaagcgtgaagtcatga	
Pax6 mutant binding site	aagaggtgcgccagccacacggaagtcatga	
Pax6 α-enhancer	aagaggttccggatcatgcatggagtcatga	
Luciferase reporter assay		
1488A, -4kb	gataaaagacaaaagaaatggtgcgaaaaggctgggtttaaacccattttcatgcgtaaaacaatttttaggaag	
1489A, -2.5kb	tggtagcaccatgaacgatgatgtgtgaactaatacccgatgatgtgtgaactaattctttctttc	

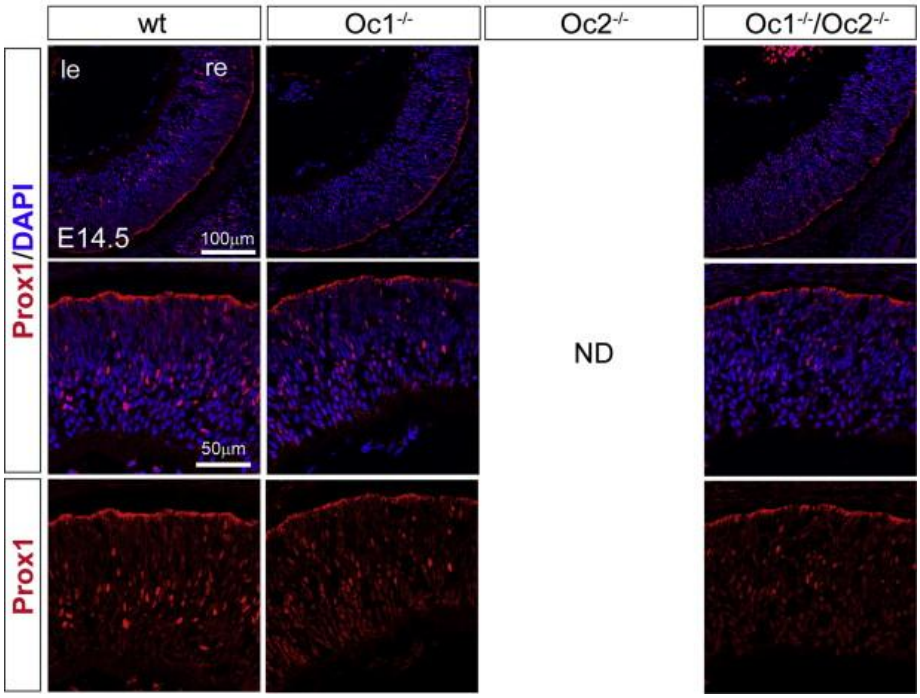


Supplementary Fig. S1.
Expression of RGC markers in Oc-deficient retinae. (A) Retinal sections of E14.5 wild-type (*wt*), *Oc1−/−*,*Oc2−/−* and *Oc1−/−/Oc2−/−* eyes stained with antibodies against RGC markers Brn3a, Brn3b, Isl1 and Tbr2. (B) qRT-PCR analysis showing the relative mRNA expression of *Oc1*, *Oc2* and *Oc3* and ganglion cell-expressed genes *Pax6*, *Math5*, *Brn3b*, *Isl1* and *Tbr2* in wild-type (*wt*), *Oc1−/−*, *Oc2−/−* and *Oc1−/−/Oc2−/−*E14.5 retinae. (C) Retinal sections of P18 wild-type (*wt*), *Oc1−/−*, *Oc2−/−* and *Oc1−/−/Oc2−/−* eyes stained with antibodies against Brn3a, Brn3b, Isl1, Tbr2 and Pax6. (D) Quantification RGCs in P18 wild-type (*wt*), *Oc1−/−*, *Oc2−/−* and *Oc1−/−/Oc2−/−* retinae assessed as the number of $Pax6^+$ (black bars), $Brn3b^+$ (gray bars) and $Tbr2^+$ (white bars) cells localized in the ganglion cell layer. Error bars indicate standard deviation,*p*-Values are calculated by Student's *t*-test. Retinal area at E14.5 is indicated by the dashed line. le, lens; re; retina; RPE, retinal pigmented epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 100 µm.

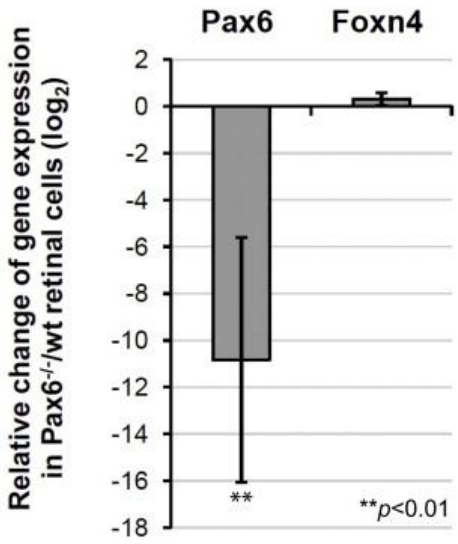


Supplementary Fig. S2.

Expression of photoreceptor cell, amacrine cell, bipolar cell and Müller glia cell markers in Oc-deficient retinae. (A) Retinal sections of E14.5 wild-type (*wt*), *Oc1−/−*, *Oc2−/−* and *Oc1−/−/Oc2−/−* eyes immunostained for early cone photoreceptor marker Rxrγ. (B) Retinal sections of P18 wild-type (*wt*), *Oc1−/−*, *Oc2−/−* and *Oc1−/−/Oc2−/−* eyes stained with antibodies against Calretinin, Pax6, Isl1, Chx10, Lhx2 and Rxrγ. (C) Quantification of Calretinin$^+$, Pax6$^+$ and Isl1$^+$ amacrine cells (AC); Chx10$^+$ bipolar cells (BC); Lhx2$^+$ Müller glia cells (MG) and Rxrγ$^+$ cone photoreceptors (CP) in P18 wild-type (*wt*) (black bars), *Oc1−/−*(blue bars), *Oc2−/−* (yellow bars) and *Oc1−/−/Oc2−/−* (red bars) retinae. Error bars indicate standard deviation, *p*-Values are calculated by Student's *t*-test. Retinal area at E14.5 is indicated by the dashed line. GCL, ganglion cell layer; NBL, neuroblastic layer.



Supplementary Fig. S3.
Prox1 expression in E14.5 Onecut-deficient retinae. Retinal sections of E14.5 wild-type (*wt*), *Oc1−/−* and*Oc1−/−/Oc2−/−* eyes immunostained with Prox1-specific antibody.le, lens; re, retina.



Supplementary Fig. S4.
The expression of Foxn4 is independent of Pax6 expression in the developing retina. Quantification of mRNA expression of *Pax6* and *Foxn4* assessed by qRT-PCR. E12.5 embryonic eyes of *Pax6wt/wt/α-Cre*Pax6 wild-type ($EGFP^{+}/Pax6^{+/+}$) and *Pax6flox/flox/α-Cre* Pax6 retinal mutants ($EGFP^{+}/Pax6^{-/-}$) were dissected, transformed to single-cell suspension and subjected to FACS to obtain $Pax6^{+/+}$ and $Pax6^{-/-}$retinal cells. mRNA from $Pax6^{+/+}$ and $Pax6^{-/-}$ retinal cells was isolated and processed by qRT-PCR using primers specific for Pax6 and Foxn4. Error bars indicate standard deviation, *p*-Values are calculated by Student's *t*-test.

7. DISCUSSION

Studies presented in chapter 6 of this Thesis were focused on the role of Pax6 transcription factor in different aspects of eye development. The results of this investigation extend the current knowledge about the involvement of Pax6 in three crucial processes: i) lens induction, ii) proliferation of retinal progenitor cells and iii) retinal differentiation.

The role of Pax6 in lens induction

The process of eye morphogenesis has fascinated developmental biologists from the beginning of the last century. The correct alignment of individual eye tissues is dependent on complex interactions between the head SE, OV and extraocular mesenchyme. From these, the interaction between OV and SE has been subjected to extensive studies questioning whether the formation of the lens is dependent on the by-standing retina. Ablation experiments in amphibians and later genetic studies in mice brought the evidence that retina-specific expression is required for the lens formation (Brownell et al., 2000; Mathers et al., 1997; Porter et al., 1997; Spemann, 1901; Yun et al., 2009). However, the molecular mechanism underlying this process is still far from being completely understood.

In mammals, the transcription factor Pax6 has been one of the prime candidates to be involved in the process of lens induction. *Pax6*$^{-/-}$ embryos are anopthalamic with arrest in eye development at the OV/SE stage and neither lens nor OC are formed (Hill et al., 1991; Hogan et al., 1986). There is a clear evidence, that the SE-expressed Pax6 is required for lens development (Collinson et al., 2000; Fujiwara et al., 1994; Grindley et al., 1995; Quinn et al., 1996). It is supposed that Pax6 ensures the competence of SE to respond to the signals from the OV and controls the expression of some key lens-specific transcription factors, including Six3, Prox1 and FoxE3 (Ashery-Padan et al., 2000; Yamada et al., 2003; Blixt et al., 2007; Brownell et al., 2000; Yoshimoto et al., 2005). Since Pax6 expression in SE is crucial for lens development, it has been suggested that loss of Pax6 in SE is responsible for the severity of the phenotype observed in *Pax6*$^{-/-}$ embryos (Collinson et al., 2000; Fujiwara et al., 1994; Grindley et al., 1995; Quinn et al., 1996). However, while conditional inactivation of *Pax6* specifically in SE prevents lens formation, it does not

prevent the formation of the OC and retina with fully differentiated neurons (Ashery-Padan et al., 2000). This implicates that Pax6 expressed in the OV should be involved in process of OC/retina formation as well. To assess the role of Pax6 in the OV, Fujiwara and colleagues (Fujiwara et al., 1994) used tissue transplantation experiments in rats. They combined *Pax6*-deficient OV with *Pax6*-wild type SE at selected developmental stage and analyzed the result of their interaction. In their experiments, the lens and retina were formed leading to the assumption that OV-expressed Pax6 is not involved in lens induction as well as in early stages of OC/retina formation (Fujiwara et al., 1994). Although this conclusion has not been further tested genetically, it has been accepted for twenty years (reviewed by Ashery-Padan and Gruss, 2001; Lang, 2004; Mathers and Jamrich, 2000; Ogino and Yasuda, 2000). In mice, genetic studies depend on tools used for manipulation of a gene function. Although, several studies reported generation of OV/retina-specific *Cre*-expressing mouse lines (Furuta et al., 2000; Marquardt et al., 2001; Rowan and Cepko, 2004; Sato et al., 2007; Swindel et al., 2006; Kreslova et al., 2007), majority of them have certain limitations that hindered their use for OV-specific gene inactivation. In our study, we generated *mRx-Cre* transgenic mouse line to inactivate selected gene specifically in the OV. This tool enabled us to dissect the function of Pax6 specifically in OV. We brought the evidence that OV-expressed Pax6 is required for lens induction as well as for early OC/retina formation (Klimova and Kozmik, 2014; Klimova et al., 2013). In *Pax6*-OV mutants, the eye development was arrested at the OV/SE stage similarly to the *Pax6*$^{-/-}$ (*Sey*) phenotype. This indicates that in *Pax6*$^{-/-}$ embryos, the defect in eye formation is attributed to the *Pax6* function in both, OV and SE (Klimova and Kozmik, 2014). Our observation strongly supports previous experiments performed in chick, in which electroporation of anti-Pax6 morpholinos specifically to OV resulted in defected lens and retina formation (Canto-Soler and Adler, 2006).

How does OV-expressed *Pax6* regulate the lens formation? There is strong evidence that signaling from the OV is essential for the activation of lens-specific gene expression in 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). It has been reported, that the genetic inactivation of BMP ligands *Bmp4* and *Bmp7* results in the abrogation of lens development (Furuta and Hogan, 1998; Wawersik et al., 1999). Expression of the *Bmp7* and *Bmp4* in the OV is regulated by transcriptional factor

Lhx2 (Yun et al., 2009). Lhx2 deletion disrupts the signaling from OV to SE and the lens is not formed (Hagglund et al., 2011; Yun et al., 2009). BMP activity, on the other hand, is apparently independent of *Pax6*, as the expression of *Bmp4*, *Bmp7* and activation of downstream effectors in the SE is not affected upon OV-specific *Pax6* inactivation (Klimova and Kozmik, 2014). In accordance, independent function of *Pax6* and *Bmp4* was previously documented (Furuta and Hogan, 1998). FGF signaling is also involved in lens induction and its abrogation results in severe lens defects (Faber et al., 2001; Gotoh et al., 2004; Pan et al., 2006). However, our study showed that *Pax6* is not required for FGF activation in the LP and that FGF pathway is not involved in *Pax6*-mediated OV lens-inductive ability (Klimova and Kozmik 2014).

The Wnt/β-catenin signaling pathway plays an important lens-restrictive role during the eye development. Ectopic activation of Wnt/β-catenin signaling in lens-forming SE abrogates lens formation, indicating that Wnt activity needs to be tightly restricted to allow proper lens development (Machon et al., 2010; Smith et al., 2005). Lens and retina does not form when Wnt/β-catenin pathway is aberrantly activated in the SE during the onset of eye development (Machon et al., 2010; Smith et al., 2005). This eye phenotype is strikingly similar to that observed in *Pax6*$^{-/-}$ embryos (Hill et al., 1991; Hogan et al., 1986). Interestingly, our study showed that the Wnt/β-catenin pathway is aberrantly activated in the eye primordium of *Pax6*$^{-/-}$ embryos (Machon et al., 2010). Several Wnt molecules and their receptors are expressed in developing eye (Ang et al., 2004). Some of them are involved in the inhibition of Wnt/β-catenin signaling such as Sfrp1, Sfrp2, Dkk1, Dkk2 and Dkk3 (Ang et al., 2004; Diep et al., 2004; Duparc et al., 2006; Gage et al., 2008). Since *Pax6* was found to directly control the expression of Wnt antagonists *Sfrp1*, *Sfrp2* and *Dkk1*, the model in which *Pax6* controls the Wnt/β-catenin activity in SE was proposed (Machon et al., 2010). However, it is not clear whether the source of Wnt/β-catenin inhibition is the SE or OV since Pax6 is expressed in both tissues. It is likely that soluble Wnt inhibitors that are expressed in the OV/retina suppress Wnt activity in the surrounding tissues including the SE. OV-expressed *Pax6* controls the expression of *Sfrp1* and *Sfrp2* in both, SE/lens placode and OV/retina (Klimova and Kozmik, 2014). Nevertheless, the loss of *Sfrp1* and *Sfrp2* is not accompanied by the release of Wnt/β-catenin repression and aberrant activation in the SE (Klimova and Kozmik 2014). In addition, recent study showed that Sfrp1 and Sfrp2 are not involved in Wnt/β-catenin pathway silencing during the process of lens induction (Sugiyama et al., 2013). Together, this

indicates that Sfrp1 and Sfrp2 are not involved in Pax6-mediated Wnt/β-catenin inhibition and that SE-expressed *Pax6* plays an additional cell-autonomous role in controlling Wnt/β-catenin activity in lens-forming SE.

Although Pax6 inactivation in OV disrupts lens formation, the effect of Pax6 is apparently not mediated by BMP, FGF or Wnt/β-catenin signaling pathways. This indicates that additional yet not well defined signals might be involved in the process of lens induction. RA signaling is supposed to play an important role in eye induction as well (Duester, 2009). Since we did not analyze the RA signaling in *Pax6*-OV mutants, we cannot formally exclude the possibility that RA signaling is involved in Pax6-mediated lens induction. Further identification of molecules acting downstream of Pax6 in the OV might help us to understand the molecular mechanism of how Pax6 regulates this complex process.

The role of *Pax6* in the proliferation of retinal progenitor cells

The development of any neuronal tissue is initiated with an extensive propagation of progenitor cell pool. At the onset of neurogenesis, a subset of progenitors exits the cell cycle and differentiates into neurons while the rest keeps proliferating to ensure differentiation of late-born neuronal cell types. Pax6 has been found to be involved in the proliferation of neuronal progenitors in various neuronal tissues (Estivill-Torrus et al., 2002; Gotz et al., 1998; Marquardt et al., 2001; Philips et al., 2005; Warren et al., 1999; Warren and Price, 1997). However, its role in these tissues and different developmental stages does not appear to be uniform but rather context dependent. For instance, Pax6 has been shown to negatively regulate proliferation of early cortical progenitors (Estivill-Torrus et al., 2002; Gotz et al., 1998; Warren et al., 1999), but it seems that it does have the opposite effect on proliferation of the progenitor population in developing diencephalon and eye primordium (Philips et al., 2005; Warren and Price, 1997). This should be also true for mammalian retina, as conditional inactivation of *Pax6* in RPCs of the OC results in hypocellularity accompanied by a decreased proportion of cells in S-phase (Farhy et al., 2013; Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008). In accordance, our study showed a dramatic reduction of RPCs upon Pax6 inactivation (Klimova and Kozmik, 2014). Analysis of apoptosis revealed that *Pax6* does not regulate death or survival of RPCs, supporting its presumable role in the control of progenitor proliferation. How does Pax6 regulate RPC proliferation? Our

work and that of others have demonstrated that the expression of several components of cell cycle machinery is affected in *Pax6*-deficient retinae (Farhy et al., 2013; Klimova and Kozmik, 2014). It is thus possible that Pax6 affects progenitor proliferation through the control of the general components of the cell cycle machinery as has been proposed in other studies (Estivill-Torrus et al., 2002; Farah et al., 2000; Holm et al., 2007; Ochocinska and Hitchcock, 2009). Our study showed that following *Pax6* loss, the expression of cyclin D1 is down-regulated while the expression of cyclin kinase inhibitor $p57^{Kip2}$ is up-regulated (Klimova and Kozmik, 2014). Since $p57^{Kip2}$ was found to be both necessary and sufficient to induce the cell cycle exit when expressed in RPCs (Dyer and Cepko, 2000), the pro-proliferative effect of *Pax6* in retina might be mediated by the inhibition of premature cell cycle exit. Whether is $p57^{Kip2}$ a direct transcriptional target of Pax6 remains to be elucidated. Mechanism of $p57^{Kip2}$–mediated cell cycle exit may include blocking of phosphorylation of retinoblastoma protein (Sherr and Roberts, 1995). Alternatively, it has been suggested that $p57^{Kip2}$ effect might be mediated through the binding of $p57^{Kip2}$ to cyclin D1 and inactivation of its associated kinase, as their direct interaction was demonstrated in mitotic retinal progenitors (Dyer and Cepko, 2000). However, we do not favor the latter possibility because in our mutants, cyclin D1 protein levels were dramatically down-regulated in areas corresponding to those with elevated $p57^{Kip2}$ expression. It is thus more likely that cyclin D1 became down-regulated as a result of $p57^{Kip2}$-mediated cell cycle exit. Nevertheless, we cannot exclude the possibility that Pax6 promotes the RPCs proliferation via regulation of both, $p57^{Kip2}$ and *cyclinD1*.

In addition, we have also found that *Pax6* positively regulates the progression through the cell cycle by controlling its length. Our study showed that the total length of the cell cycle is significantly increased in *Pax6*-deficient retinae (Klimova and Kozmik, 2014). Interestingly, we did not see any difference in the length of the S phase between the controls and Pax6 conditional mutants, but the cumulative time spent in the G1, G2 and M phase was significantly prolonged after *Pax6* ablation indicating Pax6 role in those phases of the cell cycle. However, the molecular mechanism of how Pax6 executes this function remains still unknown.

It is worth mentioning that all *Pax6*-deficient RPCs up-regulate the expression of photoreceptor-specific transcription factor Crx. An interesting question remains, whether aberrant Crx activation itself cannot be involved in the cell cycle exit

induction. However, during normal photoreceptor genesis, transcription factor Otx2 is expressed immediately prior to the cell cycle exit and has been suggested to be involved in its induction; in contrast, Crx expression is initiated in post-mitotic cells only, making its natural role in the cycle exit unlikely (Garelli et al., 2006; Muranishi et al., 2011; Nishida et al., 2003). Furthermore, early photoreceptor precursors do not make use of $p57^{Kip2}$ to exit the cell cycle as the wave of photoreceptor differentiation (from E11) precedes the wave of $p57^{Kip2}$ expression (from E14.5) in the developing retina (Dyer and Cepko, 2001b; Furukawa et al., 1997; Chen et al., 1997; Nishida et al., 2003).

The role of Pax6 in regulation of retinal differentiation

The population of RPCs is established once the OV starts to invaginate to form the OC. Soon after that RPCs start to differentiate in seven retinal cell types. In *Pax6*$^{-/-}$ embryos, the eye development is arrested at the OV stage and OC with population of RPCs is not formed (Hill et al., 1991; Hogan et al., 1986). This situation substantially complicates the studies of *Pax6* role in acquisition of specific retinal cell fates. Analysis of the arrested OV rudiment of the *Pax6*$^{-/-}$ embryos revealed that Pax6 prevents premature differentiation, as neurogenesis is initiated prematurely in *Pax6*$^{-/-}$ embryos (Philips et al., 2005). Other studies have indicated that some retinal progenitor characteristics are maintained in *Pax6*$^{-/-}$ arrested OV (Baumer et al., 2003; Bernier et al., 2001). Nevertheless, since the *Pax6*$^{-/-}$ OV becomes defective even before it contacts the SE, this system is clearly far from being the optimal tool for studying the role of Pax6 in retinal neurogenesis (Grindley et al., 1995).

In more recent studies, the conditional gene inactivation was used to uncover the role of Pax6 in retinal differentiation. Our work and that of others have shown that Pax6 is essential for RPC multipotency and acquisition of individual retinal cell fates (Klimova et al., 2015; Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008). In *Pax6*-deficient OC, general differentiation program is abolished and no specific retinal cell type is generated (Klimova and Kozmik, 2014). Interestingly, all other retinal progenitor characteristics, assessed by the expression of Rx, Lhx2, Six3, Chx10, Sox2 and Hes1, are maintained in *Pax6*-deficient retina pointing solely to a Pax6 role in RPC differentiation (Klimova and Kozmik, 2014; Marquardt et al., 2001). How could a single transcription factor regulate differentiation of multiple retinal lineages? Genetic studies of the last decades strongly

indicated that generation of individual retinal cell types is governed by gene regulatory networks of transcription factors and their target genes. Out of these, a various combinations of the bHLH and homeodomain class transcription factors were found to play a pivotal role (reviewed by Ohsawa and Kageyama, 2008). Although Pax6 does not seem to regulate the expression of other homeodomain transcription factors such as Six3, Chx10 or Rx in the retina, it was found to operate upstream of several bHLH activators, including Math5, Mash1, Math3, Ngn2 and NeuroD (Hatakeyama and Kageyama, 2004; Klimova and Kozmik, 2014; Marquardt et al., 2001; Oron-Karni et al., 2008; Riesenberg et al., 2009). Math5 is a key regulator of RGC development as its absence results in almost complete loss of RGCs (Brown et al., 2001; Wang et al., 2001). Since Math5 operates upstream of other regulators of RGC genesis, including Brn3b and Isl1 (Liu et al., 2001; Yang et al., 2003), its dependence on Pax6 thus might contribute to the loss of RGCs in *Pax6*-deficient retina. Accordingly, in *NeuroD* and *Math3* compound mutants, amacrine cells are selectively lost (Inoue et al., 2002) and in *Mash1* and *Math3* compound mutants, bipolar cells are lost (Tomita et al., 2000). The absence of above mentioned bHLH transcription factors in the *Pax6*-deficient retina is the most probable cause of the observed loss of RGCs, amacrine and bipolar cells.

However, it appears that during differentiation of the photoreceptors Pax6 plays a dual role. On one hand, Pax6 negatively regulates the expression of photoreceptor-specific transcription factor Crx to prevent the premature photoreceptor differentiation (Klimova and Kozmik, 2014; Oron-Karni et al., 2008). On the other hand, although efficiently expressed by all progenitors, Crx does not promote photoreceptor differentiation in the absence of *Pax6* (Klimova and Kozmik, 2014; Oron-Karni et al., 2008). This indicates that *Pax6* is required for the expression of another factor(s) involved in photoreceptor genesis. Good candidate would be the Otx2, a key regulator of photoreceptor lineage acting upstream of Crx (Akagi et al., 2004; Nishida et al., 2003), that has been shown to cooperate on trans-activation of photoreceptor-specific genes (Peng and Chen, 2005). Recent study have identified the retinal enhancer of Otx2 and carefully analyzed the mechanism of how Otx2 is regulated. However, Pax6 was not found as a potent trans-activator of Otx2 retinal enhancer (Muranishi et al., 2011). It appears that the enhancer is negatively regulated by Hes1 (Muranishi et al., 2011), the effector of Notch signaling expressed by undifferentiated RPCs (Tomita et al., 1996). Since Hes1 expression is maintained

upon *Pax6* inactivation (Klimova and Kozmik, 2014; Marquardt et al., 2001) the persistent activity of Notch pathway in general may prevent activation of differentiation program and Otx2 expression in *Pax6*-deficient retina. However, the molecular mechanism of how the activity of Notch signaling is attenuated remains to be elucidated.

Although the loss of bHLH activators undoubtedly contributed to the differentiation defect in *Pax6*-deficient retinae, it does not fully explain why all retinal cell types are lost upon *Pax6* inactivation. This indicates that other Pax6-regulated factors may operate in the retina to ensure proper differentiation of all retinal cell types. For example, Pax6 has been found to be expressed in developing and mature HCs (de Melo et al., 2003; Roesch et al., 2008), its potential role in the horizontal cell genesis, however, has not been addressed. We have searched for novel Pax6-regulated transcription factors that might play the role in retinal development and identified two transcription factors of Onecut family, Onecut1 and Onecut2 (Klimova et al., 2015). These are expressed in mostly overlapping pattern; in RPCs and in developing horizontal cells, RGCs, and cone photoreceptors (Emerson et al., 2013; Wu et al., 2012). Our work and that of others have shown that in *Onecut1*- and *Onecut2*-deficient retinae, the number of horizontal cells is reduced while horizontal cells are completely missing in *Onecut1/Onecut2*-deficient retina (Goetz et al., 2014; Klimova et al., 2015; Sapkota et al., 2014; Wu et al., 2013). This suggests that Onecut factors play an important role in horizontal cell genesis. Interestingly, horizontal cells seem to initiate their differentiation properly as the expression of main regulators of horizontal cell differentiation, including Foxn4, Ptf1a, Prox1 and Lim1 is initiated in *Onecut*-deficient retinae (Klimova et al., 2015). Pax6-regulated Onecut pathway thus ensures rather the maintenance of horizontal cell fate than the initial fate determination. Interestingly, although the loss of horizontal cells in *Onecut*-deficient retinae was obvious, more detailed analysis revealed that the numbers of cone photoreceptors, amacrine cells, bipolar cells, and Müller glial cells are reduced indicating the role of Onecut factors in generation of these cell types (Klimova et al., 2015; Sapkota et al., 2014). Together, Onecut factors seem to represent potent regulators of retina differentiation and their dependence on Pax6 may contribute to the loss of several retinal cell types in *Pax6*-deficient retina.

8. CONCLUSIONS

We have used a Cre/LoxP system to analyze the role of Pax6 transcription factor in various processes of eye development. Our studies showed that Pax6 plays a pivotal role in several aspects of eye development, including lens induction, proliferation of RPCs and differentiation of individual retinal cell types. Results summarized in this Ph.D. Thesis have significant implications for understanding mammalian eye development and extend our knowledge about the role of Pax6 in this complex process.

Our results can be summed up as follows:

- We have provided the evidence that Pax6 is involved in the negative regulation of Wnt/β-catenin signaling pathway in the lens-forming surface ectoderm. Gene reporter assays and chromatin immunoprecipitation assays revealed that Pax6 directly controls the expression of Sfrp1, Sfrp2 and Dkk1 to promote lens formation.

- We generated *mRx-Cre* transgenic mouse line that with the use of Cre/LoxP system enables gene inactivation specifically in the optic vesicle and it derivates.

- Using *mRx-Cre* mouse line to inactivate Pax6 in optic vesicle and newly formed retinal progenitor cells of the optic cup, we have revealed that in prospective retina, Pax6 is required for three crucial processes: i) lens induction, ii) propagation of early retinal progenitor cells and iii) initiation of the retinal differentiation program.

- We identified Onecut1 and Onecut2 transcription factors as potential targets of Pax6 in retinal development. We have shown that Onecut factors operate downstream of Pax6. The analysis of *Onecut1/Onecut2*-deficient retinae revealed that Onecut factors are essential for horizontal cell maintenance.

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