

## Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6

Nicole Bäumer<sup>1</sup>, Till Marquardt<sup>1,\*</sup>, Anastassia Stoykova<sup>1</sup>, Derek Spieler<sup>2</sup>, Dieter Treichel<sup>1</sup>, Ruth Ashery-Padan<sup>1,†</sup> and Peter Gruss<sup>1,‡</sup>

<sup>1</sup>Department of Molecular Cell Biology, Max-Planck-Institute of Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

<sup>2</sup>Department of Developmental Biology, Max-Planck-Institute of Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

\*Present address: The Salk Institute for Biological Studies, Gene Expression Laboratory, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

†Present address: Sackler Faculty of Medicine, Department of Human Genetics and Molecular Medicine, Tel Aviv University, Ramat Aviv 69978, Tel Aviv, Israel

‡Author for correspondence (e-mail: peter.gruss@mpg-gv.mpg.de)

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### SUMMARY

The transcription factors Pax2 and Pax6 are co-expressed in the entire optic vesicle (OV) prior and concomitant with the establishment of distinct neuroretinal, retinal, pigmented-epithelial and optic-stalk progenitor domains, suggesting redundant functions during retinal determination. Pax2; Pax6 compound mutants display a dose-dependent reduction in the expression of the melanocyte determinant *Mitf*, accompanied by transdifferentiation of retinal pigmented epithelium (RPE) into neuroretina (NR) in Pax2<sup>-/-</sup>; Pax6<sup>+/-</sup> embryos, which strongly resembles the phenotype of *Mitf*-null mutants. In Pax2<sup>-/-</sup>; Pax6<sup>-/-</sup> OVs *Mitf* fails to be expressed and NR

markers occupy the area that usually represents the *Mitf*<sup>+</sup> RPE domain. Furthermore, both, Pax2 and Pax6 bind to and activate a *MITF* RPE-promoter element in vitro, whereas prolonged expression of Pax6 in the Pax2-positive optic stalk leads to ectopic *Mitf* expression and RPE differentiation in vivo. Together, these results demonstrate that the redundant activities of Pax2 and Pax6 direct the determination of RPE, potentially by directly controlling the expression of RPE determinants.

Key words: Eye development, Pigmented retina, Regionalization, Pax2, Pax6, *Mitf*

### INTRODUCTION

The vertebrate eye is a highly specialized neurosensory organ. In the mouse, eye development starts on embryonic day 8.5 (E8.5) when the OV grows out from the anterior forebrain. After contacting the surface ectoderm by E9.5, both the OV and the surface ectoderm invaginate. This forms the two-layered optic cup from the OV and the lens pit and subsequently the lens vesicle from the surface ectoderm (reviewed by Chow and Lang, 2001). Although the very proximal part of the OV will narrow to surround the optic nerve, the outer layer of the optic cup will become the RPE and the inner layer will differentiate into the NR with its different cell types (Young, 1985).

Initially, the cells of the early OV are bipotent because they can differentiate into either NR or RPE cells. The first indication for the determination of different areas within the developing eye is apparent at the OV stage. Here, the microphthalmia-associated transcription factor *Mitf*, which belongs to the family of basic helix-loop-helix (bHLH)/leucine zipper transcription factors, is first expressed throughout the entire OV (Bora et al., 1998; Nguyen and Arnheiter, 2000). Subsequently, the *Mitf* expression is

downregulated in the distal portion of the OV to mark the region of the presumptive RPE in the proximal-dorsal portion of the OV (Nguyen and Arnheiter, 2000). In the adjacent, distal OV, the presumptive NR region (Burmeister et al., 1996; Liu et al., 1994), the paired-like homeodomain transcription factor *Chx10* starts to be expressed at the same time. Later on *Mitf* and *Chx10* continue to be expressed in adjacent regions of the eye, suggesting a reciprocal regulation between these two factors (Nguyen and Arnheiter, 2000). Interestingly, in *Mitf*-mutant eyes, portions of the outer RPE layer transdifferentiate into NR instead of RPE, reflecting the important roles of *Mitf* in promoting the RPE differentiation and suppressing NR characteristics (Mochii et al., 1998; Bumsted and Burnstable, 2000; Nguyen and Arnheiter, 2000). *Chx10*-mutant NRs are generally hypocellular and lack bipolar cells (Ferda Perkin et al., 2000; Burmeister et al., 1996).

The paired and homeodomain transcription factor Pax6 is assumed to be a 'master regulator' of eye development (Gehring and Ikeyo, 1999; Ashery-Padan and Gruss, 2001) because forced expression of Pax6 alone is sufficient to induce ectopic eyes in fly and frog embryos (Halder et al., 1995; Chow et al., 1999). When Pax6 is lacking no functional eye structures

form in organisms as different as mouse, human, rat, frog and fly (reviewed by Gehring and Ikeo, 1999). Mice heterozygous for *Pax6*-null mutations exhibit a *small eye* phenotype, which is characterized by multiple ocular abnormalities, such as microphthalmia, lens cataracts and iris defects, and human *PAX6* mutations lead to *Aniridia*, as well as multiple lens and corneal defects (Hill et al., 1991; Glaser et al., 1992; Glaser et al., 1994). *Pax6* is expressed highly throughout the early OV and the surface ectoderm, and remains expressed in all eye components at the optic-cup stage, including lens vesicle, outer and inner optic cup layers, and optic stalk (Walther and Gruss, 1991). Later, *Pax6* expression becomes restricted to the lens, corneal and conjunctive epithelia, iris and inner portion of the NR (Walther and Gruss, 1991). Conditional inactivation of *Pax6* in the surface ectoderm leads to a specific ablation of the lens (Ashery-Padan et al., 2000). Furthermore, the conditional elimination of *Pax6* in the distal NR causes a complete failure of differentiation of all NR cell types except amacrine cells (Marquardt et al., 2001). However, the function of *Pax6* during the early phase of OV genesis has not been studied extensively (Grindley et al., 1995).

At early stages of eye development, the Pax-family member *Pax2* is co-expressed with *Pax6* in the OV, but is absent in the surface ectoderm (Nornes et al., 1990) (this study). During optic nerve formation at ~E12.5, *Pax2* expression becomes restricted to the ventral NR that surrounds the closing optic fissure and the presumptive glia cells of the optic nerve (Nornes et al., 1990; Torres et al., 1996). After E12.5, *Pax2*-deficient mice exhibit a severe retinal coloboma – a failure to close the choroid fissure. Furthermore, eyes are achiasmatic and the retinal ganglion cell axons project only ipsilaterally (Torres et al., 1996).

Previously, it has been proposed that *Pax2* and *Pax6* might be engaged in reciprocal negative regulation, thereby resulting in the delineation of the optic-cup versus the optic-stalk domains (Schwarz et al., 2000). The co-expression of *Pax2* and *Pax6* at high levels throughout the entire OV prior to and concomitant with the establishment of the distinct progenitor domains of the NR, RPE and optic stalk (i.e. Martinez-Molares et al., 2001) (this study) prompted us to examine whether *Pax2* and *Pax6* do synergize during early retinal development. Despite the early arrest of OV development in *Pax6*-null mutants, we show that the establishment of distinct NR, RPE and optic-stalk-progenitor domains is independent of *Pax6* activity. Similarly, *Pax2* is dispensable for the formation of the distinct progenitor domains in the OV. However, *Pax2*; *Pax6* compound mutants displayed a dose-dependent reduction in the expression of the RPE determinant *Mitf*, accompanied by transdifferentiation of RPE into NR in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> embryos. This resembles the phenotype of *Mitf*-null mutants. In *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs, *Mitf* fails to be expressed, with NR markers occupying the area usually representing the *Mitf*<sup>+</sup> RPE domain. Furthermore, we show that *Pax2* and *Pax6* both bind to and activate a *MITF*-RPE promoter element in vitro. Moreover, the prolonged expression of *Pax6* in the *Pax2*-positive optic stalk in transgenic mice leads to the ectopic expression of *Mitf* and RPE differentiation. Together, these results demonstrate that the redundant activities of *Pax2* and *Pax6* are required and sufficient to direct the determination of RPE, and that this might be achieved by directly controlling the expression of RPE determinants such as *Mitf*.

## MATERIALS AND METHODS

### Transgenic and targeted mice

*Pax2*<sup>+/-</sup>; *Pax6*<sup>lacZ/+</sup> (*Pax6*<sup>+/-</sup>), *lens-Cre* and *Pax6*<sup>lox</sup> mice were generated previously (see Torres et al., 1995; St-Onge et al., 1997; Ashery-Padan et al., 2000). In the *pPax2*<sup>*Pax6*</sup> mice (Schwarz et al., 2000), a 9.3 kb genomic fragment of the 5' region of the first exon of *Pax2* was fused *NotI* (blunt) to full-length *Pax6* cDNA in the vector pBSKSII<sup>+</sup>.

DNA from either the yolk sac or tail of *Pax6*<sup>lac</sup> embryos was genotyped by PCR as described (Bäumer et al. 2002).

DNA from either the yolk sac or tail of *Pax2*<sup>-/-</sup> embryos was genotyped using two PCRs. The following primers were used to identify the mutant allele: Neo-f4, 5'-CTTCTATCGCCTTCTT-GACG-3'; Pax2-r3, 5'-TCCAGCCATTACTTGAACG-3'. A band of 600 bp indicated the existence of a *Pax2*-mutant allele. Each PCR assay contained 1 µg of DNA, 1/10 vol of HotStarTag buffer (Qiagen), 200 µM dNTP mix, 10 µM of Neo-f4 primer, 23 µM of Pax2-r3 primer and 1 U HotStarTaq polymerase (Qiagen). Cycling conditions were 95°C for 15 minutes, followed by 34 cycles of 94°C for 45 seconds, 58°C for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 5 minutes.

For the identification of the wild-type allele, the following primers were used: Pax2-f, 5'-CGGGGCTGCGTTGCTGACTG-3'; Pax2-r, 5'-GCTTTGCAGTGCATATCCATCG-3'. A band of 300 bp indicated the existence of a *Pax2* wild-type allele. Each PCR assay contained 1 µg of DNA, 1/10 vol of PCR buffer (Biotherm), 200 µM dNTP mix, 0.33 pmol µl<sup>-1</sup> of each primer and 1.5 U Taq polymerase (Biotherm). Cycling conditions were 94°C for 2 minutes, 80°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, then 72°C for 5 minutes.

### Immunohistochemistry

The embryos were fixed for 30 minutes in 4% PFA/PBS (pH 7.8), washed with PBS, incubated in cold 30% Sucrose/PBS over night and frozen in Tissue Freezing Medium (Jung). Sections of 6-10 µm were air-dried and stored at -80°C.

For antibody staining, the sections were washed in PBS (3 X 5 min), blocked with 1% BSA (IgG-free, Sigma), 0.05% Tween-20 in PBS for 30 minutes at room temperature. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Primary antibodies: 1:300 rabbit anti-β-Gal (Cappel); 1:20 monoclonal mouse anti-*Pax6* (DSHB); 1:200 rabbit anti-*Pax2* (Babco); 1:150 rabbit anti-*Mitf* (gift of H. Arnheiter); 1:500 rabbit anti-*Chx10* (gift of R. McInnes); 1:2000 rabbit anti-*Otx2* (gift of F. Vaccarino); 1:75 goat anti-*Brn3b* (Santa Cruz). After three, 5-10 minute washes in PBS, the secondary antibody was applied in blocking solution for 1 hour [1:500 Alexa 568 goat anti rabbit (Molecular Probes); 1:60 FITC goat anti mouse (Southern Biotechnology)]. After three washes with PBS, counterstaining was performed with Dapi and the sections were embedded with Mowiol.

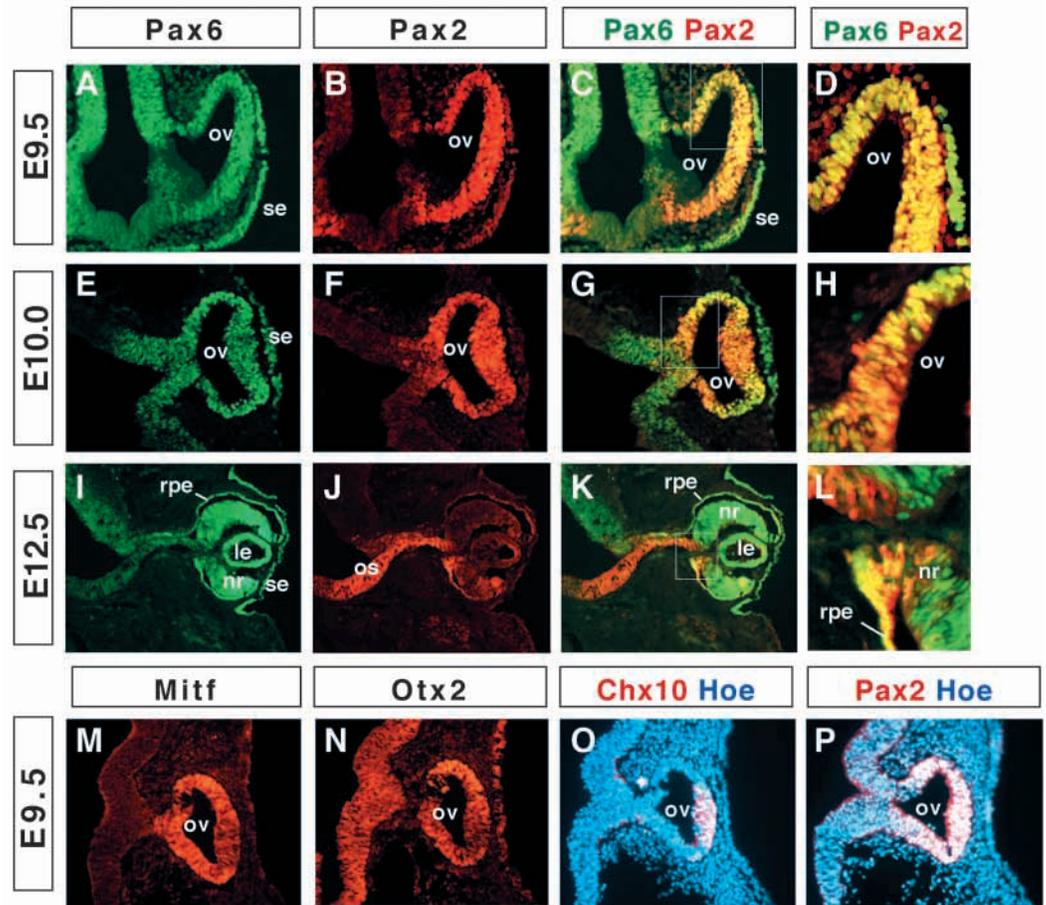
Hematoxylin-Eosin (HE) stainings were performed using standard protocols.

### Bandshift assays

*Pax6* and *Pax2* proteins were overexpressed using SP6 promoter-coupled *Pax2* and *Pax6* cDNA in the TNT in vitro transcription and translation system (Promega) according to the manufacturer's protocol.

Double-stranded oligonucleotides (see Fig. 5B for sense sequences) were end-labeled using polynucleotide kinase and γ-[<sup>32</sup>P]-ATP. The binding reaction was performed for 1 hour on ice in retard buffer (40 mM HEPES-NaOH, pH 7.6, 8% Ficoll, 10 mM MgCl<sub>2</sub>, 80 mM NaCl, 0.2 mM EDTA, 1 mM DTT) with 5 µg poly-dI-dC, 25,000 cpm of the double-stranded oligonucleotide and 2-10 µl of either *Pax2* or *Pax6* TNT protein. To test the binding specificity, either *Pax6* or *Pax2* polyclonal rabbit antibodies (Babco) were preincubated 1:10 in retard

**Fig. 1.** The expression domains of Pax2 and Pax6 entirely overlap with markers of the early OV progenitor domains. Immunohistochemical analysis of the expression of Pax6 (green, A,E,I) and of Pax2 (red, B,F,J) in serial, 6  $\mu$ m sections. C, G and K show overlays of the respective Pax2 and Pax6 expression. D, H and L are at higher magnifications. Pax2 and Pax6 expression overlaps in the early OV at E9.5 (A-D) and at E10.0 (E-H). (I-L) At E12.5, Pax2 is restricted mainly to the optic stalk but Pax6 is still expressed in all distal eye components. (M-P) At E9.5, the segregation of RPE markers, such as Mitf (M) and Otx2 (N) is detectable, in contrast to the NR marker Chx10 (O). The adjacent section (P), stained for Pax2, reveals the overlap of Pax2 and Pax6 (see also A and B) with both RPE and NR markers. le, lens; nr, neural retina; os, optic stalk; ov, optic vesicle; rpe, retinal pigmented epithelium; se, surface ectoderm.



buffer with poly-dI-dC and protein for one hour on ice. The probes were run over an 8% polyacrylamide gel. The gel was exposed to a Kodak Biomax film over night.

#### Cell culture

Co-transfection experiments of Cos-7 cells were performed as described (Marquardt et al., 2001). The cDNAs of *Pax2* (Chalepakis et al., 1990), *Pax6* (Walther and Gruss, 1991) and *Pax1* (Chalepakis et al., 1990) were cloned to a hCMV promoter. The promoter of the *MITF* exon A (Udono et al., 2000) was a generous gift of S. Shibahara.

## RESULTS

### Pax2 and Pax6 are co-expressed in the early OV with both RPE and NR markers

Although several reports showed that *Pax2* and *Pax6* are expressed in the early OV (Nornes et al., 1990; Walther and Gruss, 1991), whether their expression domains overlap tightly has not been addressed in detail. We found that Pax2 and Pax6 are co-expressed at high levels in virtually all cells of the early OV at E9.5 (Fig. 1A-D; Fig. 4A) and E10.0 (Fig. 1E-H), whereas Pax2 immunoreactivity was never detected in the Pax6-positive surface ectoderm (Fig. 1B,F,L; Fig. 4A). By E12.5, Pax6 expression started to be downregulated in the optic stalk region, but the signal was still abundant in the NR, the RPE, the lens vesicle and the surface ectoderm (Fig. 1I). At the same stage, Pax2 expression decreased in the distal eye compartments, but was maintained in the ventral NR, some

proximal cells of the RPE and in the optic stalk (Fig. 1J; Fig. 2D). The distribution of Pax2 protein reported here refines the previously reported expression dynamics of *Pax2* mRNA (see Nornes et al., 1990; Martinez-Morales et al., 2001). Therefore, the expression domains of Pax2 and Pax6 mostly overlap during the OV and early optic-cup stages, but become largely exclusive at later stages.

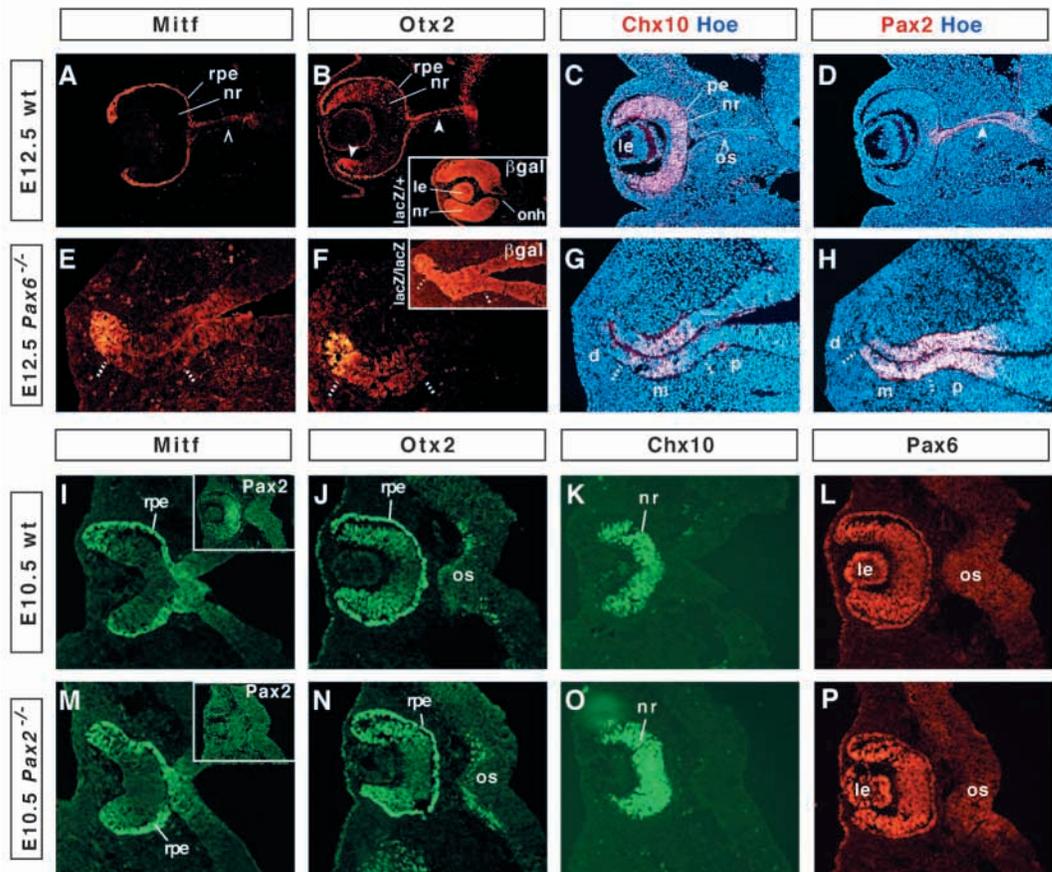
At E9.5, different regionalization markers in the early eye can be used to distinguish immunohistochemically between the presumptive RPE and the presumptive NR in the OV. The RPE domain in the medial region of the OV expressed Mitf and Otx2 transcription factors (Fig. 1M,N) (see also Nguyen and Arnheiter, 2000; Martinez-Morales et al., 2001) and the NR domain in the distal part of the OV is Chx10-positive (Fig. 1O; Burmeister et al., 1996). Because both progenitor domains co-express Pax2 and Pax6 (Fig. 1C,D,L; Fig. 4A), a function of these Pax-family transcription factors during the early stages of retinal determination seemed likely.

### The activity of Pax2 or Pax6 is dispensable for proximo-distal patterning of the early OV

To elucidate the early function of Pax2 and Pax6 during OV formation, we examined the patterning of *Pax6*<sup>-/-</sup> and *Pax2*<sup>-/-</sup> OVs. At E12.5 the RPE determinant Mitf is completely restricted to the RPE in the wild-type eye (Fig. 2A), whereas Chx10 remained confined to the NR (Fig. 2C). Remarkably, expression of both Mitf and Chx10 is initiated in the *Pax6*<sup>-/-</sup> OV (Fig. 2E,G), although neither retinal neurogenesis nor RPE

**Fig. 2.** Pax2 and Pax6 activity is not required for the regionalization of the OV. Immunohistochemistry of serial, 6  $\mu$ m sections at E12.5 (A-H) and E10.5 (I-P). In the wild-type eye at E12.5, Mitf expression is confined to the RPE and some cells in the dorsal optic stalk (A, arrowhead). By contrast, Otx2 is expressed in the RPE and the dorsal optic stalk, with a sharp, distal-proximal gradient in the distal NR arrowheads, (B). Chx10 is restricted to the NR (C) and Pax2 mainly to the optic stalk (D, arrowhead). (E) At E12.5, Mitf expression in the *Pax6*<sup>-/-</sup> mutant eye is confined to the distal tip of the OV. Otx2 is expressed highly in the same region (F) but less in the medial portion of the OV where Chx10 is expressed at a high level (G). (H) The proximal region of the *Pax6*<sup>-/-</sup> OV expresses Pax2 strongly (p), expression in the medial region is at a lower level (m) and the distal region is Pax2 negative (d). (F, inset) In the *Pax6*<sup>lacZ/lacZ</sup> OV, the

$\beta$ -gal expression is restricted to the distal and the medial regions, which reflects the distribution of  $\beta$ -gal expression in the RPE and NR in *Pax6*<sup>lacZ/+</sup> eyes (B, inset). Broken lines in E-H indicate the borders between d, m and p regions. The comparison of the patterning of E10.5 wild-type (I-L) and *Pax2*<sup>-/-</sup> eyes (M-P) revealed in both genotypes the unchanged expression of Mitf (I and M) in the RPE and optic stalk, of Otx2 (J and N), which is found in both phenotypes in the RPE and optic stalk, gradually in the NR and some cells of the optic stalk, as well as the unchanged expression of Chx10 in the NR (K and O) and of Pax6 throughout all eye components (L and P). (I, inset) Expression of Pax2 in the wild-type optic disc and the missing Pax2 expression in the *Pax2*<sup>-/-</sup> eye (M, inset). le, lens; os, optic stalk; nr, neural retina; rpe, retinal pigmented epithelium.

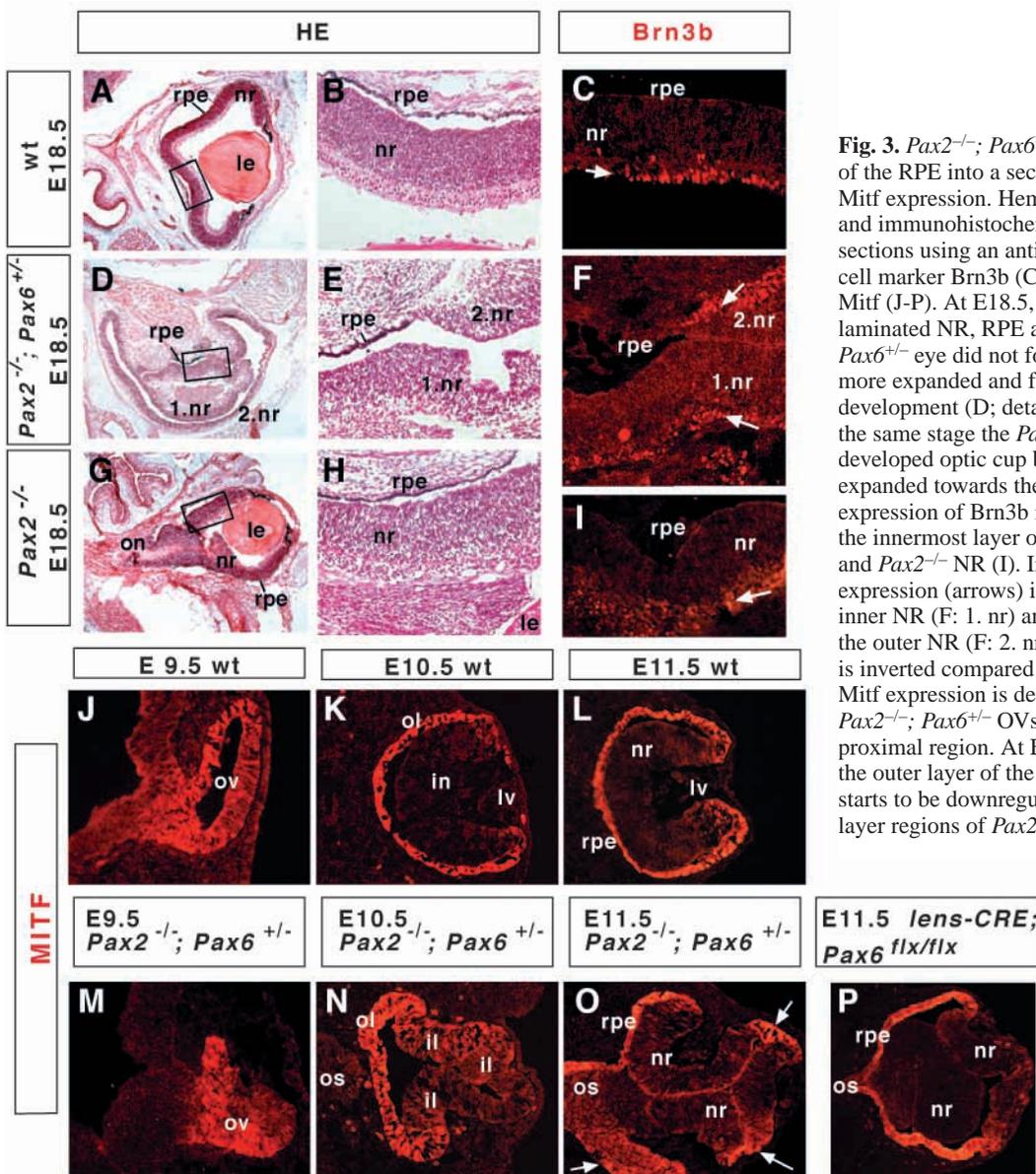


differentiation is initiated in *Pax6*-null mutants (Grindley et al., 1995; data not shown). At E12.5, the Mitf-positive domain was at the very distal tip of the *Pax6*<sup>-/-</sup> OV (Fig. 2E), just adjacent to a more proximal Chx10-positive domain (Fig. 2G). Similar to the wild type, in the mutant eye virtually no overlap could be detected between Chx10 and Mitf-positive areas (Fig. 2A,C). At this stage, Otx2 was expressed at a high level in the RPE and the distal-most NR, and decreased sharply towards the proximal half of the NR (Fig. 2B) (see also Martinez-Morales et al., 2001). In addition Otx2 was expressed in the surface ectoderm (Fig. 2B). The immunoreactivity in the dorsal optic stalk might be due to an, as yet, unpublished cross-reactivity with Otx1 (Baas et al., 2000) because optic-stalk expression has been reported for Otx1, but not for Otx2 (Fig. 2B) (Martinez-Morales et al., 2001). Surface ectoderm of *Pax6* mutant failed to express Otx2 (Fig. 2F), possibly reflecting the failure of the lens ectoderm specification in *Pax6*-null embryos (Grindley et al., 1995). However, the distal, Mitf-positive domain in *Pax6*<sup>-/-</sup> OV co-expressed high levels of Otx2 (Fig. 2F), indicating that this region corresponds to the wild-type Mitf-positive, Otx2-positive, RPE domain. In addition, in the *Pax6*<sup>-/-</sup> Ovs, a distal (high)-proximal (low) gradient of Otx2

activity was observed in the Chx10-positive NR domain (Fig. 2F-G), which matched the Otx2 expression characteristics in wild-type NR (Fig. 2B).

By E12.5, the expression of *Pax2* in the wild-type eye was confined to the optic stalk and the ventral NR (Fig. 1J; Fig. 2D; see also Fig. 4C). In the *Pax6*<sup>-/-</sup> OV at E12.5, Pax2 expression was detected in the medial Chx10-positive, Otx2 (low), NR domain (Fig. 2F-H, 'm'). Furthermore, higher levels of Pax2 activity were localized in the proximal region of the OV, the presumptive optic stalk (Fig. 2H, 'p'). A similar distribution of Mitf, Chx10, Otx2 and Pax2 in wild-type and *Pax6*<sup>-/-</sup> embryos was also observed in E9.5 and E10.5 OVs (data not shown). Together these results indicate that in the mutant OV, the domains 'd' and 'm' represent the anlagen of the RPE and the NR, respectively.

We next examined whether the distribution of Pax6 expression itself was affected in the OV of *Pax6*-null mutants. Recently, we found that the  $\beta$ -galactosidase ( $\beta$ -gal) activity in the transgenic *Pax6*<sup>lacZ/+</sup> knock-in line mainly reflects the endogenous expression pattern of *Pax6* (St-Onge et al., 1997; Bäumer et al., 2002). At E12.5 in *Pax6*<sup>lacZ/+</sup> eyes,  $\beta$ -gal was expressed in the NR, anterior RPE, lens and corneal ectoderm,



**Fig. 3.** *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes show transdifferentiation of the RPE into a second, inverted NR and reduced *Mitf* expression. Hematoxylin-Eosin (A,B,D,E,G,H) and immunohistochemical staining of serial, 6 μm sections using an antibody against the retinal ganglion cell marker *Brn3b* (C,F,I) and the RPE determinant *Mitf* (J-P). At E18.5, the wild-type eye differentiated to laminated NR, RPE and lens (A,B). The *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eye did not form a lens, but the NR was much more expanded and folded at the expense of RPE development (D; detailed view in E). By contrast, at the same stage the *Pax2*<sup>-/-</sup> mutant eye displays a fully developed optic cup but the NR and the RPE are expanded towards the optic stalk (G,H). The expression of *Brn3b* in retinal ganglion cells marked the innermost layer of the NR in wild-type (arrow, C) and *Pax2*<sup>-/-</sup> NR (I). In the *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eye, *Brn3b* expression (arrows) is confined to the inner layer of the inner NR (F: 1. nr) and to the outermost cell layer in the outer NR (F: 2. nr), indicating that the second NR is inverted compared to the inner NR. (J-P) At E9.5, *Mitf* expression is detected in the wild-type (J) and *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> OV (M), predominantly in the proximal region. At E10.5, *Mitf* activity is confined to the outer layer of the optic cup in wild type (K, ol), but starts to be downregulated in the folded distal inner layer regions of *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes (N, il). *Mitf* is still expressed in the forming RPE in the wild-type E11.5 eye (L), whereas in the *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> optic cup, it is only found in regions between the newly formed, folded, NR regions and in the proximal RPE remnant (O, arrows). In the *lens-CRE/Pax6*<sup>flx/flx</sup> eye, *Mitf*-positive RPE surrounds the folded NRs, similar to wild-type RPE (P).

but was largely absent from the optic stalk (Fig. 2B, inset). The E12.5 *Pax6*<sup>lacZ/lacZ</sup> (*Pax6*<sup>-/-</sup>) OV had high levels of β-gal expression in the distal and the medial ('RPE' and 'NR'), but much lower levels in the proximal 'optic-stalk' domain (Fig. 2F, inset), which is comparable to the β-gal expression in *Pax6*<sup>lacZ/+</sup> eyes (Fig. 2B, inset). In situ hybridization with a *Pax6* riboprobe revealed essentially the same localization of *Pax6* transcripts in the distal OV of *Pax6*<sup>Sey/Sey</sup> embryos (Grindley et al., 1995; data not shown). Therefore, the distribution of *Pax6* transcripts detected in *Pax6*-null mutants reflects the largely undisturbed proximo-distal patterning of the OV.

*Pax2*<sup>-/-</sup> eyes display retinal and optic nerve coloboma, which are visible by E12.5 (Torres et al., 1996) (data not shown). Morphologically, these eyes can be identified after this stage by elongation of the NR towards the optic stalk (Torres et al., 1996; Schwarz et al., 2000) (Fig. 3G; data not shown). We further studied the putative function of early *Pax2* expression

during OV regionalization of *Pax2*<sup>-/-</sup> OV and eyes from E9.5 to E12.5 (Fig. 2I-P; data not shown). Interestingly, the expression domain of *Mitf* in the RPE (Fig. 2I,M), *Otx2* expression in the RPE, NR and a subpopulation of optic-stalk cells (Fig. 2J,N), and *Chx10* in the NR (Fig. 2K,O) were identical in wild-type and *Pax2*<sup>-/-</sup> OV. *Pax6* expression in mutant eyes was comparable to wild type up to stage E11.5 (Fig. 2L,P; data not shown). However, at stage E12.5, unlike wild type, the *Pax6* activity was maintained in the optic stalk region (Schwarz et al., 2000) (data not shown), suggesting that the late *Pax2* expression is required for the downregulation of *Pax6* in the optic nerve. Together, these results indicate that the general subdivision of the OV along the distal-proximal axis into distinct RPE, NR and optic-stalk-progenitor domains is independent of *Pax6* and of *Pax2* activity, suggesting a redundant function of *Pax2* and *Pax6* during these early events of OV development.

### ***Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes transdifferentiate RPE into a second, inverted NR and display reduced *Mitf* expression**

To study the putative redundant function of *Pax2* and *Pax6* during early eye determination, we examined the patterning of developing eyes in *Pax2*; *Pax6* compound-mutant embryos. Interestingly, in absence of *Pax2*, reduction of *Pax6* activity led to a severe optic cup phenotype. At stages E10.5 and E11.5, the *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> optic cups were not an ordered, two-layered structure (Fig. 3N,O). Instead the inner layer (the presumptive NR) appeared folded, whereas the outer layer (the presumptive RPE) seemed to be reduced (compare Fig. 3K-L with Fig. 3N-O). Additionally, the lens vesicle was either reduced in size or completely absent (Fig. 3N,O; data not shown). Interestingly, at E18.5 the RPE in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes was severely reduced compared to wild type (Fig. 3A,D), and, in the region of the outer layer, a second, multilayered NR had transdifferentiated, which is not found in the *Pax2*<sup>-/-</sup> eye (Fig. 3B,F,G-I). This second NR in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes contained all the neural retina cell types and progenitors associated with the inner NR and the wild-type and *Pax2*<sup>-/-</sup> NR (Fig. 3C,F,I; data not shown). However, cell layering in the newly transdifferentiated NR was inverted, as indicated by the expression of the ganglion-cell marker *Brn3b* in the outer part of the mutant NR rather than inner-most layer of the wild-type NR (Fig. 3C,F,I, arrows).

Similar defects have been described following loss of function of *Mitf* in eyes of the *mi/mi* mice (Scholtz and Chan, 1987; Mochii et al., 1998; Bumsted and Burnstable, 2000). Therefore, we examined the expression of *Mitf* in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> embryos. At E9.5, immunoreactivity of *Mitf* in the *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> and wild-type OV was comparable. Notably, at E10.5 and E11.5, the outer layer of the wild-type optic cup was *Mitf*-positive (Fig. 3K-L), but *Mitf* expression was reduced in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> optic cups (Fig. 3N-O).

To determine whether the RPE transdifferentiation observed in the *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> mutants reflected a secondary effect of either loss or reduction of the lens (West-Mays et al., 1999; Ashery-Padan et al., 2000), we studied *Mitf* expression in *lens-CRE/Pax6*<sup>flx/flx</sup> mutants, in which the lens is genetically ablated by specific inactivation of *Pax6* in the surface ectoderm via the Cre/lox-approach (Ashery-Padan et al., 2000). However, although both *lens-CRE/Pax6*<sup>flx/flx</sup> and *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> mutant eyes show multiple NR domains (Ashery-Padan et al., 2000), the position of the RPE and the level of *Mitf* expression at E11.5 were comparable to wild type (compare Fig. 3L with Fig. 3P). Together, these results indicate that *Mitf* expression is initiated but not maintained at a sufficient level in the optic cups of the *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> embryos, which leads to a transdifferentiation of a second NR at the expense of RPE.

### ***Mitf* expression and OV regionalization depend on cooperative *Pax2* and *Pax6* activity**

The reduced levels of *Mitf* expression in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes could be caused by remaining *Pax6* activity. Likewise, the initiation of *Mitf* expression in *Pax6*-null mutants could result from functional compensation by *Pax2*.

To examine these two possibilities, we studied *Mitf* expression in the complete absence of *Pax2* and *Pax6*. Remarkably, although expression of the early OV markers *Lhx2* and *Six3* was still detectable in the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs (data not shown), *Mitf* expression was not initiated (Fig. 4F,H).

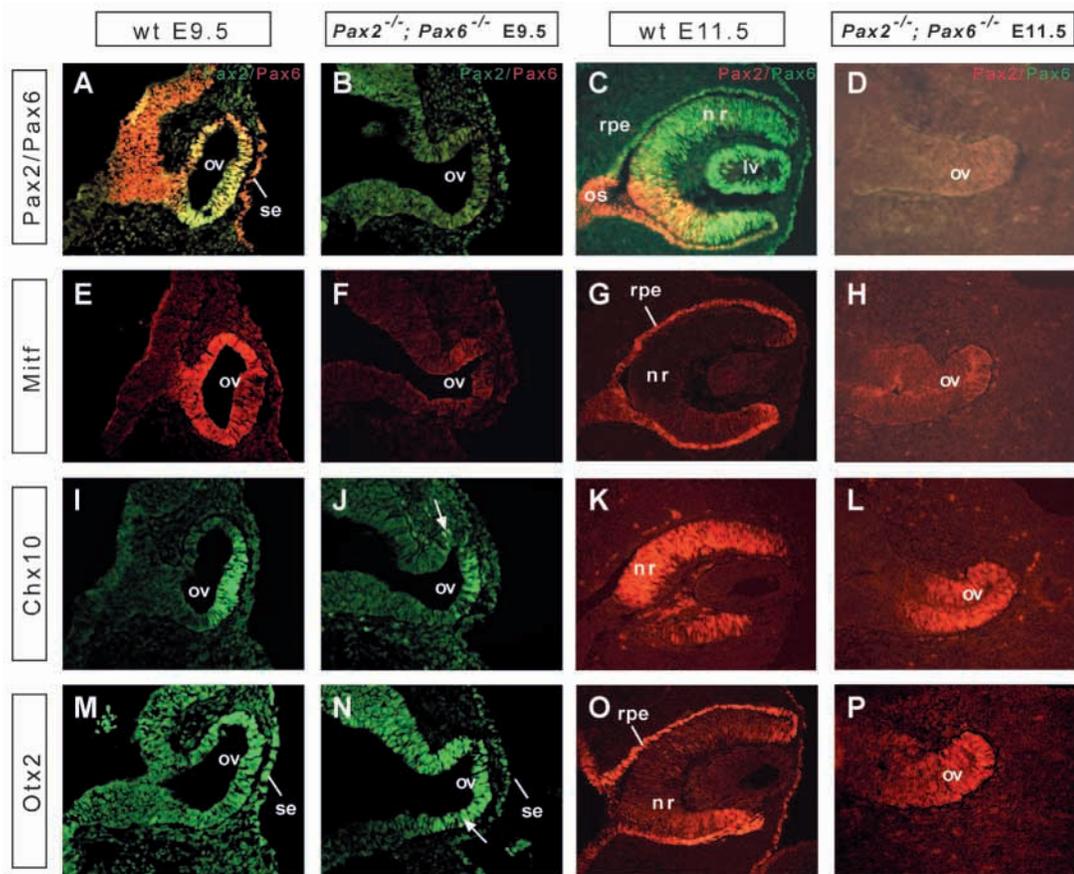
Expression of *Chx10* and *Otx2* were also initiated in mutant eyes (Fig. 4J,N,L,P). Therefore, we conclude, that the lack of *Mitf* expression in the OV of the double-null mutants is a specific defect rather than a general failure of OV development. Similar to the wild type (Fig. 4I), the expression of *Chx10* at E9.5 was mainly confined to the distal portion of the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV (Fig. 4J). Some additional *Chx10*-positive cells were also detected in the more proximal part of the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV that is usually occupied by the *Mitf*-positive RPE domain (Fig. 4J, arrow). Although in the absence of *Pax6*, *Chx10* expression was regionalized mostly within the 'm' domain of the OV remnant (Fig. 2G), in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV, *Chx10* immunoreactivity was confined to the very distal tip (region 'd') of the vesicle, occupying the normally *Mitf*-positive progenitor domain of the RPE. By contrast, expression of *Otx2* was detected first at E9.5 in both wild-type and *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs (Fig. 4M,N) possibly slightly expanded proximally in the mutant OV (Fig. 4N, arrow). At E11.5, wild-type RPE expressed high levels and NR low levels of *Otx2* (Fig. 4O). At this stage, the distal-most region of the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV was *Otx2* positive (Fig. 4P), which indicates that the distal tip of the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV co-expressed the NR marker *Chx10* and the RPE marker *Otx2*.

Accordingly, we conclude that, in absence of both *Pax2* and *Pax6* function, the bipotent, early OV cells could not be further specified into the determined NR and RPE domains, a process that possibly involves a direct control on *Mitf*-gene activity.

### ***Pax2* and *Pax6* can bind and activate a *MITF* RPE promoter in vitro**

The specific loss of *Mitf* expression in *Pax2/Pax6* double-null mutants raised the question of whether *Mitf* was a direct target gene of *Pax2* and *Pax6*. The *Mitf* gene produces at least four different splice variants (see Fig. 5A). Three of them, *Mitf*-A, *Mitf*-H and *Mitf*-D are expressed in RPE cells (Hallsson et al., 2000; Udono et al., 2000; Takeda et al., 2002). The recently identified *MITF*-A promoter drives expression in RPE and melanocyte cell lines (as expected by the expression pattern of this splice variant) as well as in HeLa cells, which might indicate a more widespread activity caused by a missing repressor element in this promoter fragment (Udono et al., 2000). However, our results suggested a role of *Pax2* and *Pax6* in activation, rather than in repression of *Mitf*. Therefore, we studied the effect of the binding of *Pax2* and *Pax6* proteins on the human *MITF*-A promoter. The 2.2 kb *MITF*-A promoter sequence is 66.3% identical to the homologous mouse sequence, in some regions 79-91%, compared with sequence information by Celera (data not shown). The *MITF*-A promoter, which is located upstream of the first exon, was fused to the luciferase reporter gene (Fig. 5A) (Udono et al., 2000).

Using the consensus sequence of optimal *Pax2* and *Pax6* binding to DNA established by Epstein and co-workers (1994) (Fig. 5B), five potential binding sites, A1-A5, were identified (Fig. 5B). Bandshift assays using in vitro overexpressed *Pax2* and *Pax6* proteins and <sup>32</sup>P-labelled oligonucleotides (for the sequences see Fig. 5B) revealed that A5 represented a *MITF*-A promoter sequence that can bind both *Pax2* and *Pax6* (Fig. 5C, red arrow). The other four oligonucleotides only bound unspecific components of the assay, as shown by controls without overexpressed proteins (Fig. 5C, -Pax). The binding specificity of the A5 site was verified by preincubation of *Pax2*



**Fig. 4.** *Mitf* expression and OV regionalization are lost in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> embryos. Immunohistochemical staining of serial, 6  $\mu$ m cryosections of E9.5 (A-B,E-F,I-J,M-N) and E11.5 (C,D,G,H,K,L,O,P) using antibodies against Pax2 and Pax6 (A-D), *Mitf* (E-H), Chx10 (I-L) and Otx2 (M-P). (A) Pax2 (green) and Pax6 (red) are expressed throughout the wild-type E9.5 OV. (C) At E11.5, Pax6 expression (green) is found in all compartments of the eye, but Pax2 (red) is mainly restricted to the ventral NR and RPE and the optic stalk. Both Pax2 and Pax6 are absent in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs at both stages (B and D). The E9.5 wild-type OV expresses *Mitf* predominantly in the proximal regions (E). At E11.5, *Mitf* expression is confined to the RPE (G). *Mitf* fails to be expressed in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs at both stages (F,H). The presumptive NR is marked by the Chx10-positive distal domain at E9.5 in both the wild-type (I) and the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs (J), but seems to be expanded dorsally in the mutant OV (J, arrow). At E11.5, Chx10 is confined to the NR region in the wild type (K) and to the distal OV in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OVs (L). The proximal expression of Otx2 in the wild type at E9.5 (M) appears expanded ventrally in the mutant OV (N, arrow). The Otx2 expression becomes restricted to the RPE domain at E11.5 in wild type (O), but remains co-expressed with the Chx10 positive distal OV in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> mutants (P). Iv, lens vesicle; ov, optic vesicle; os, optic stalk; nr, neural retina; rpe, retinal pigmented epithelium; se, surface ectoderm

and the Pax6 proteins with specific antibodies, which impaired formation of the binding complex (Fig. 5D, lanes 2 and 5). By contrast, addition of the Pax6 antibody to the Pax2 protein, and vice versa, did not perturb the binding (Fig. 5D, lanes 3 and 6). Therefore, we concluded that the A5 sequence in the *MITF-A* promoter represents a specific binding site for Pax2 and Pax6.

To examine the potential transactivation of this promoter element by Pax2 and/or Pax6, we co-transfected Cos-7 cells with the luciferase-coupled *MITF-A* promoter, CMV-*Pax2*-cDNA and/or CMV-*Pax6*-cDNA. CMV-*Pax1*-cDNA was used as a control (Fig. 5E). These co-transfections showed that the basal level of luciferase activity driven by the *MITF-A* promoter was increased ~13-fold by the addition of Pax6, ~40-fold by addition of Pax2, and ~12-fold by an equimolar mixture of Pax2 and Pax6 (Fig. 5E). Pax1 increased the luciferase activity only fivefold (Fig. 5E).

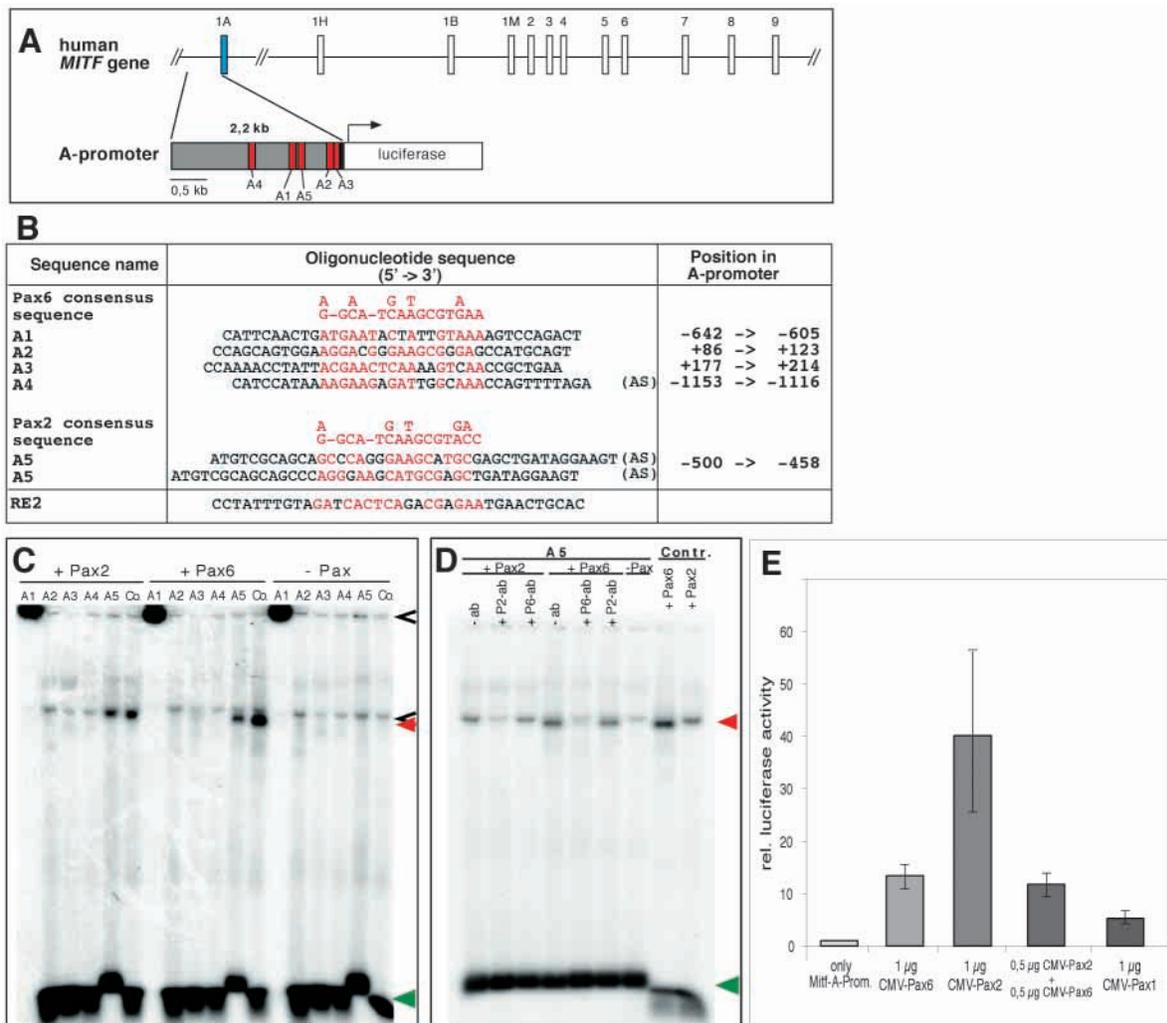
These results demonstrate that both Pax2 and Pax6 can bind to and activate the *MITF-A* promoter. The fact that the mixture

of Pax2 and Pax6 did not surpass the activation obtained by either Pax2 and Pax6 alone accords with the single binding site for both factors identified in this promoter sequence (Fig. 5D). It is likely that both factors compete for this binding site and that the double-mutant phenotypes result from the requirement of a specific concentration of either protein for sufficient activation of *Mitf*.

A similar competitive relationship has been reported for the segmentation genes *kreisler* (*Mafb* – Mouse Genome Informatics) and *Krox20*, which control the expression of the *Hoxb3* gene in rhombomere 5 (Manzanares et al., 2002).

#### Ectopic expression of Pax6 in the Pax2-positive optic nerve results in the development of ectopic, *Mitf*-expressing RPE

To test if Pax2 and Pax6 can direct the expression *Mitf* in vivo, we took advantage of a previously generated transgenic mouse line (*pPax2<sup>Pax6</sup>*) that expresses *Pax6* under the control of a *Pax2*-upstream promoter fragment. This drives expression of



**Fig. 5.** Pax2 and Pax6 directly bind to and activate the *MITF*-A promoter in vitro. (A) Organization of the human *MITF*-gene (after Hallsson et al., 2000), position upstream promoter of exon A and of the predicted Pax2 and/or Pax6 binding sites (red boxes) in the *MITF*-A promoter construct, which is coupled to the luciferase reporter gene (Udono et al., 2000). (B) Sequences of the oligonucleotides used in the EMSAs in C and D. Red letters indicate the Pax6 or Pax2 consensus sequences (Epstein et al., 1994) in the *Mitf* promoter sequences. RE2 represents the Pax2 and Pax6 binding site (Schwarz et al., 2000). (C) EMSA with the sequences A1-A5 from (B), the control sequence RE2 and Pax2, Pax6 and 'null' proteins (+Pax2, +Pax6, -Pax). Arrowheads indicate nonspecific binding. (D) The specificity of Pax2 and Pax6 binding to sequence A5 (lane 1 and 4, -ab) is confirmed by the addition of anti-Pax2 antibody (lane 2, +P2-ab) and anti-Pax6 antibody (lane 5, +P6-ab), which inhibits the formation of the complex. The addition of the reciprocal antibodies did not inhibit the binding (lanes 3 and 6). Without an overexpressed protein, only a faint band appears (lane 7, -Pax). The red arrowhead indicates the binding of Pax2 and of Pax6 by oligo A5. The green arrowhead marks the unbound oligos. (E) Pax2 and/or Pax6 activate the *MITF*-A promoter in cell culture experiments. Co-transfection of Cos-7 cells with the luciferase-coupled human *MITF*-A promoter and CMV-*Pax2*-cDNA and/or CMV-*Pax6*-cDNA lead to a strong increase in luciferase activity. CMV-*Pax1*-cDNA activates the *MITF*-A promoter only moderately.

*Pax6* in the optic stalk (Schwarz et al., 2000) (Fig. 6A). At E13.5, *Mitf* expression was confined to the developing iris (Fig. 6C,G, ir) and to some cells in the RPE (Fig. 6E, arrows).

Interestingly, *pPax2<sup>Pax6</sup>* mice displayed ectopic RPE in the region of the distal optic nerve after E13.5, possibly caused by prolonged Pax6 activity in the Pax2-positive optic stalk (compare Fig. 6F,H with Fig. 2D). The appearance of ectopic RPE was accompanied by elevated *Mitf* immunoreactivity in the optic nerve itself, which was even stronger than *Mitf* immunoreactivity in the RPE in the normal optic cup (compare Fig. 6E,I, arrows).

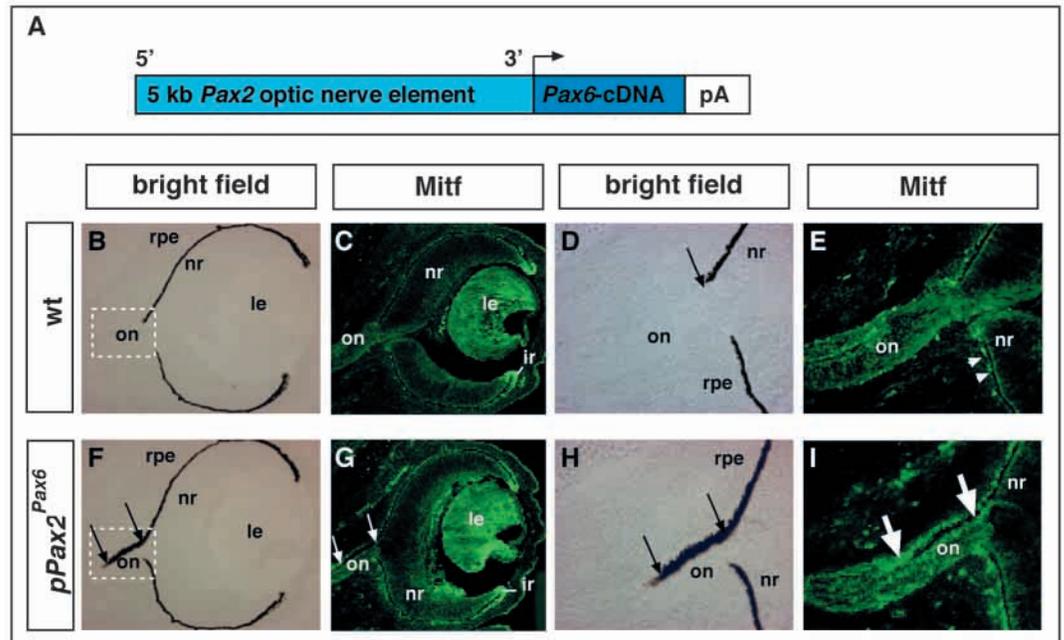
In summary, these results indicate that (1) the expression of

*Mitf* is not initiated in the absence of both *Pax2* and *Pax6*, (2) both *Pax2* and *Pax6* can bind to and activate the *MITF*-A promoter in vitro, and (3) the forced co-expression of *Pax2* and *Pax6* in vivo leads to RPE development and ectopic expression of *Mitf*.

## DISCUSSION

### Determination of distinct progenitor domains in the retinal primordium

The cells of the early OV are bipotent neuroepithelial cells



**Fig. 6.** *pPax2<sup>Pax6</sup>* transgenic mice develop ectopic *Mitf*-positive RPE. (A) Construct used to establish the transgenic mouse line *pPax2<sup>Pax6</sup>*. A *Pax2*-genomic sequence (5 kb) driving expression in the optic nerve was coupled to full-length *Pax6* cDNA (Schwarz et al., 2000). Bright-field photos (B,D,F,H) and immunohistochemical detection of *Mitf* (C,E,G,I) of 12  $\mu$ m cryosections of E14.5 wild-type (B-E, wt) and transgenic (F-I, *pPax2<sup>Pax6</sup>*) eyes. *Mitf* is confined mainly to the iris in the wild-type (C, ir) and transgenic eye (G). Higher magnifications reveal that the ectopic RPE on the transgenic optic nerve is *Mitf* positive (I, arrows). Arrows in G indicate the ectopic *Mitf*<sup>+</sup> RPE on the optic nerve; arrowheads in E indicate the endogenous *Mitf* expression in the RPE. ir, iris; le, lens; nr, neural retina; on, optic nerve; rpe, retinal pigmented epithelium.

from which both NR and RPE can differentiate. Classical embryological experiments indicate that the microenvironment of the developing eye determines the decision of these bipotent cells to enter one of these two fates (reviewed by Chow and Lang, 2001). The surface ectoderm is thought to be one of the major sources of signaling factors, such as fibroblast growth factor 1 (FGF1) and FGF2, that are necessary for regionalization of the eye (Guillemot and Cepko, 1992; Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Araki et al., 2002).

Probably because of these signaling events, the neuroepithelium of the distal OV becomes patterned into distinct NR-progenitor and RPE-progenitor domains, as indicated by the segregation of the expression domains of *Chx10* and *Mitf* (see Fig. 2). Cross-repressive interactions between *Chx10* and *Mitf* are thought to mediate the sharpening and stabilization of the boundary between NR-progenitor and RPE-progenitor domains (Nguyen and Arnheiter, 2000), which might, therefore, mark a switch from exogenous to OV-autonomous patterning mechanisms.

It was unclear how the expression of progenitor factors such as *Chx10* and *Mitf* is initiated in the OV neuroepithelium because both factors are activated, even in the absence of the surface ectoderm (see below) (Nguyen and Arnheiter, 2000). Recently, we demonstrated that the expression of the naso-temporal axis markers *BF1* (*Foxg1* – Mouse Genome Informatics) and *BF2* (*Foxd1* – Mouse Genome Informatics) is dependent on Pax6 activity (Bäumer et al., 2002). This provides a link between broadly expressed retinal determinants and retinal-axial patterning. In *Pax6*-null mutants, however, proximo-distal patterning of the OV into distinct NR, RPE and optic-stalk-progenitor domains appears to be unaffected (Fig. 2E-H). This rules out a ‘master’ function of Pax6 during the specification of murine retinal identity. The remarkable

inverted orientation of the *Pax6*-mutant OV, in which the presumptive RPE domain faces the surface, might contribute to the failure of specification of the mutant surface ectoderm, and strongly resembles the results of ablation experiments (Nguyen and Arnheiter, 2000). Therefore, the surface ectoderm in *Pax6* mutants might lack signaling factors that are required for the activation of either RPE-repressing or NR-activating factors.

In the present study we provide evidence that the combined action of Pax2 and Pax6 directly mediates the initiation of *Mitf* expression in the OV and, thereby, determines the RPE-progenitor domain. Subsequently, the extraocular and OV-autonomous patterning events mentioned above (Araki et al., 2002) restrict the initial, broad *Mitf*-expression domain to the future RPE-progenitor domain, thereby assuring that RPE differentiation is confined to the future outer layer of the optic cup.

### Redundant and distinct functions of Pax2 and Pax6 in regionalization of the OV

In contrast to the distinct regionalization of *Pax2*<sup>-/-</sup> and *Pax6*<sup>-/-</sup> OVs into NR and RPE-progenitor domains, the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV is incorrectly patterned in this respect. The RPE marker *Mitf* fails to be expressed in the *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV and a second RPE marker, *Otx2*, largely colocalizes with *Chx10*, a NR marker (Fig. 4). The expansion of *Chx10* into the region usually occupied by the RPE-progenitor domain probably reflects the failure to express the NR repressor *Mitf* in this region. However, co-localization of *Otx2* and *Chx10* indicates that the normal determination of the NR domain is also affected, which indicates additional, redundant roles of Pax2 and Pax6 in establishing this progenitor domain. Therefore, the determination of both NR and RPE from bipotent OV cells appears to be dependent on redundant Pax2 and Pax6 function.

Consequently, in *Pax6* mutants *Pax2* activity is sufficient to direct the formation of the NR-progenitor and RPE-progenitor domains, but *Pax2* activity alone is not sufficient to induce optic-cup formation (Fig. 2) (Grindley et al., 1995). Conversely, *Pax6* activity is sufficient to direct optic-cup formation in *Pax2*<sup>-/-</sup> mutants, but not to maintain the sharp border between optic cup and optic nerve after E12.5, and the differentiation of the optic nerve itself (Fig. 3G) (Torres et al., 1996). These observations imply distinct, nonredundant functions of these two Pax proteins at later stages of OV development. Interestingly, *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> mutants have reduced RPE differentiation as well as more severe optic-nerve defects than *Pax2*<sup>-/-</sup> mutants (Fig. 3D), which might indicate additional functions of *Pax6* in optic-nerve formation.

The involvement of *Pax2* and *Pax6* in the regionalization of other tissues has already been implied. For example, *Pax6* can restrict the expression of *Pax2* at the boundary between the diencephalon and mesencephalon in chick embryos (Matsunaga et al., 2000) and this border is affected in absence of *Pax6* in mice (Stoykova et al., 1996; Mastick et al., 1997; Pratt et al., 2000). *Pax6* is, furthermore, required for the specification of ventral-progenitor-cell identity in the spinal cord and hindbrain (Ericson et al., 1997; Takahashi and Osumi, 2002), and the lack of *Pax6* function causes a prominent ventralization of the molecular patterning and morphogenesis of the embryonic forebrain (Stoykova et al., 2000; Yun et al., 2001).

In the OV, after E10, the expression of *Pax6* and *Pax2* increasingly segregates and becomes mutually exclusive after E12.5. During these later stages both factors might acquire distinct functions in the further specification of the optic cup and the optic nerve domains, where they might now repress each other (Macdonald et al., 1995; Schwarz et al., 2000). Taken together, these observations suggest that, during eye development, *Pax2* and *Pax6* initially function redundantly in the OV during the determination and patterning of the RPE, NR and, possibly, optic-stalk-progenitor domains. During later stages, a switch appears to occur that brings out the distinct functions of both factors, so that they now mediate the differentiation of discrete tissue compartments of the eye in a mutually exclusive manner (Macdonald et al., 1995; Schwarz et al., 2000). Interestingly, redundant as well as distinct functions have been implied for other Pax-family members, such as *Pax3* and *Pax7* in spinal cord and somite development (Borycki et al., 1999; Mansouri and Gruss, 1998), *Pax1* and *Pax9* in sclerotome development (Neubüser et al., 1996; Peters et al., 1999), and *Pax2* and *Pax5* in different developing organs (Schwarz et al., 1997; Urbanek et al., 1997; Bouchard et al., 2000). The recruitment of the same factor to drive distinct processes during sequential stages in the development of the same tissue or organ is starting to become a recurring theme in developmental biology (reviewed by Marquardt and Pfaff, 2001).

Based on observations that members of the same subgroup of the Hox-gene family can, to a large extent, substitute for each other, it has been proposed recently that the quantity rather than the quality of a required factor might be decisive for some developmental mechanisms (Duboule, 2000; Greer et al., 2000). The dose-dependence of the eye phenotype on the *Pax6* concentration in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> compound mice, as shown in this study, corroborates this idea. Moreover, the *Pax2*

dose dependence of *Mitf* expression can be observed in OVs of *Pax6*<sup>-/-</sup>; *Pax2*<sup>+/-</sup> embryos that are phenotypically identical to *Pax6*<sup>-/-</sup> OVs. These *Pax6*<sup>-/-</sup>; *Pax2*<sup>+/-</sup> OVs express less *Mitf* in the distal presumptive RPE region than the *Pax6*<sup>-/-</sup> OVs, although the medial presumptive NR region is highly *Chx10* positive in both genotypes (data not shown; Fig. 2).

### ***Mitf* as a putative target gene of *Pax2* and *Pax6***

*Mitf* was the only OV determinant identified that failed to be expressed in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV (Fig. 4). Maintained expression of another RPE marker, *Otx2*, in mutant OVs implied a specific loss of *Mitf* activity rather than a complete failure in the specification of RPE characteristics. As *Otx2* is co-expressed with the NR marker *Chx10*, we assume that the bipotent character of the OV cells is maintained, possibly due to the absence of *Mitf*.

Transdifferentiation of the RPE in *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes closely resembles the ocular phenotype of *Mitf*-deficient mice (Scholtz and Chan, 1987; Bumsted and Burnstable, 2000; Nguyen and Arnheiter, 2000). Because RPE differentiation appears to be normal in the lens-ablated *lens-CRE*; *Pax6*<sup>flx/flx</sup> mutants (Ashery-Padan et al., 2000) (this study) this defect is unlikely to be due to the partial loss of lens tissue in these mutants.

Interestingly, our results indicate that both *Pax2* and *Pax6* can specifically bind to and activate the *MITF-A* promoter in vitro (see Fig. 5). This might indicate that one aspect of the complicated regulation of the *Mitf* gene might involve direct binding of *Pax2* and *Pax6*, although the in vitro results are indirect because a transgenic approach to study the in vivo function of the binding has not yet been examined. However, the fact that the pan-specific *Mitf*-antibody did not detect *Mitf* activity in *Pax2*<sup>-/-</sup>; *Pax6*<sup>-/-</sup> OV might indicate a general requirement of *Pax2* and *Pax6* function for the expression of all *Mitf* isoforms in the RPE. To follow this hypothesis, it will be necessary to further characterize *Mitf* regulatory elements, such as the *MITF-H*, *MITF-D* and *MITF-A* promoters. Although the H-form of *Mitf* also occurs at low level in the RPE, we were unable to identify binding sites for *Pax2* and *Pax6* in the *MITF-H* promoter (Udono et al., 2000), and did not detect activation of this promoter in cell-culture experiments (data not shown). Because the promoter region of *MITF-H* is less well conserved than the *MITF-A* promoter between humans and mice (data not shown), it is also possible that the *MITF-H* promoter did not adequately represent the mouse promoter in the in vitro analysis. Alternatively, the *MITF-A* promoter region could act as an enhancer to control the expression of the other isoforms. Furthermore, because expression driven by the *MITF-A* promoter is more widespread than wild-type *Mitf* expression (Udono et al., 2000) (N.B. and D.S., unpublished), the existence of other regulatory elements that restrict *Mitf* expression, is likely. This issue requires further intensive studies, including mutational analysis of potential transcription-factor binding sites.

To date, more detailed information is available concerning the regulation of *Mitf* activity during melanocyte development (reviewed by Tachibana, 2000). Mutations in the *MITF* gene in humans cause type II Waardenburg syndrome, a severe disease that specifically affects melanocyte function (Tassabehji et al., 1994; Hodgkinson et al., 1998; Lee et al., 2000). Intriguingly, in melanocyte cell lines the human *MITF*-

*M* promoter is activated by another Pax-family member, *PAX3*, which is also involved in type II Waardenburg syndrome (Watanabe et al., 1998; Potterf et al., 2000).

An essential role of Pax6 in RPE differentiation has been suggested previously. Quinn and coworkers established chimeric mice from *Pax6*-deficient and wild-type ES cells to examine the influence of *Pax6* deficiency in different compartments of the eye. When these mutants had incorporated a high percentage of *Pax6*-deficient cells in the outer layer of the optic cup, the eyes had a disorganized optic cup with a folded NR and reduced RPE differentiation (Quinn et al., 1996). This resembles the phenotype of *Pax2*<sup>-/-</sup>; *Pax6*<sup>+/-</sup> eyes. Further studies, possibly involving tissue-specific inactivation of *Pax6* in the RPE, should specifically address the direct function of Pax6 in RPE differentiation.

Another hint for the possible function of Pax6 in inducing RPE differentiation came from experiments in which *Pax6* was ectopically expressed. In frog embryos, ectopic expression of *Pax6* leads to the formation of complete ectopic eye structures outside the optic system, but after overexpression within the optic system it induces only RPE, not NR, along the (Pax2-positive) optic nerve (Chow et al., 1999). In transgenic mice that express Pax6 under the control of a *Pax2*-regulatory element, differentiation of ectopic RPE (again without NR differentiation) was observed in the distal optic nerve (Schwarz et al., 2000) (this study). Although, initially, we interpreted this phenotype as an indication of the retinal differentiation potential of Pax6 in a region of the optic stalk that expresses lower concentrations of Pax2 (Schwarz et al., 2000), we had to refine this interpretation following our more recent results showing the important role of the redundant function of Pax2 and Pax6 in RPE development (see below). The region of ectopic RPE formation in these mice appears to correlate with the region that endogenously expresses *Otx1* in the dorsal optic stalk (see Fig. 2). This indicates that *Otx1* is required to allow Pax6; Pax2-mediated RPE differentiation. Because *Otx1* and *Otx2* are thought to be essential for RPE development (Martinez-Morales et al., 2001), this would reflect the situation during normal RPE determination. In this case, *Otx2* is co-expressed with Pax2 and Pax6 in the RPE-progenitor domain (see Fig. 2) (Martinez-Morales et al., 2001), and *Otx1*, which can take over various functions of *Otx2* in different tissues (Acampora et al., 1999; Martinez-Morales et al., 2001) in the ectopic situation of the transgenic optic stalk.

In summary, Pax6 activity appears to be sufficient to induce RPE development, whereas both *Pax2* and *Pax6* are necessary and sufficient to activate the RPE determinant *Mitf* in a competent tissue.

### Redundant and divergent roles of Pax2 and Pax6 in eye development

To summarize, initially Pax2 and Pax6 carry out redundant functions in setting up the RPE progenitor domain in the OV neuroepithelium. This shared role is demonstrated clearly by their entirely overlapping expression domains in the OV neuroepithelium and by their redundant function in mediating *Mitf* expression. Later, during optic-cup stages, the distribution of Pax2 and Pax6 segregate to give their well-documented, mutually exclusive patterns in optic stalk and optic cup, respectively. At these stages Pax2 is necessary for

oligodendrocyte differentiation in the optic stalk and for the closure of the choroid fissure (Torres et al., 1997), whereas Pax6 is required for normal retinal neurogenesis (Marquardt et al., 2001) and iris morphogenesis (Glaser et al., 1992). Therefore, the later segregation of Pax2 and Pax6 activities reflects their divergent functions during later eye development and might be necessary for them to carry out their functions in different tissues of the eye.

In this respect it might be significant that the late retinal enhancer of Pax6 ('alpha') (Kammandel et al., 1999; Bäumer et al., 2002), which is repressed by Pax2 (Schwarz et al., 2000), is excluded from the Pax2-positive choroid fissure (see Bäumer et al., 2002). In this light, the original model in which the mutual repression of Pax2 and Pax6 was assumed to direct the spatial segregation of territories in the early eye (Schwarz et al., 2000) might only apply to later aspects of eye development, such as optic-cup morphogenesis. Such mutual repression might assure the spatial exclusion of their diverging functions in optic nerve/choroid fissure and optic cup/retina. It remains to be shown how the switch from coordinate expression and function to divergent activities and mutual exclusion is achieved at the level of gene regulation.

### Pax transcription factors as regulators of bHLH transcription factors in cellular determination

*Mitf* is not the first bHLH transcription factor that is known to be regulated by Pax-family transcription factors. During muscle development, Pax3 is involved in the activation of MyoD, a myogenic bHLH transcription factor (Maroto et al., 1997; Tajbakhsh et al., 1997), although it is unclear whether activation is direct or indirect (Borycki et al., 1999). Similarly, direct activation of the bHLH factors *Myf5* and *MyoD* by Pax7 during the differentiation of pluripotent muscle-stem cells into satellite cells was assumed (Rudnicki et al., 1993; Megeney et al., 1996; Seale et al., 2000) (reviewed by Tajbakhsh and Buckingham, 2000). Recently, a direct requirement of Pax6 activity for the activation of another bHLH factor, *Ngn2*, in the spinal cord (Scardigli et al., 2001) and the developing neuroretina (Marquardt et al., 2001) was demonstrated. We previously found that Pax6 normally binds to and activates *Ngn2*-specific enhancers (Marquardt et al., 2001; Scardigli et al., 2003) that are not activated in *Pax6*-deficient embryos (Scardigli et al., 2001). Furthermore, after specific inactivation of *Pax6* in the distal NR in  $\alpha$ -*Cre/Pax6*<sup>flx/flx</sup> mice, bHLH factors *Mash1* and *Math5* are not expressed (Marquardt et al., 2001). Another bHLH transcription factor, *NeuroD1* (previously *NeuroD*), is still expressed in the distal NR of this *Pax6* mutant (Marquardt et al., 2001), but is absent in *Pax6*<sup>-/-</sup> OV, which might indicate its dependence on early *Pax6* expression (data not shown).

Pax transcription factors are often required for the determination of a specific cell fate from multipotent cells (Nutt et al., 1999; Marquardt et al., 2001; Marquardt and Gruss, 2001; Seale et al., 2000; Borycki et al., 1999) and bHLH transcription factors function in the differentiation of determined progenitor cells (Cepko, 1999; Kageyama et al., 1997). Taken together, several lines of evidence indicate that the presence of particular Pax transcription factors in different progenitor cells might be a general requirement for the initiation of a number of specific differentiation pathways.

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