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COUP-TFs regulate eye development by controlling factors essential for optic vesicle morphogenesis

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SUMMARY

Transcriptional networks, which are initiated by secreted proteins, cooperate with each other to orchestrate eye development. The establishment of dorsal/ventral polarity, especially dorsal specification in the optic vesicle, is poorly understood at a molecular and cellular level. Here, we show that COUP-TFI (Nr2f1) and COUP-TFII (Nr2f2) are highly expressed in the progenitor cells in the developing murine eye. Phenotype analysis of *COUP-TFI* and *COUP-TFII* single-gene conditional knockout mouse models suggests that COUP-TFs compensate for each other to maintain morphogenesis of the eye. However, in eye-specific *COUP-TFI/TFII* double-knockout mice, progenitor cells at the dorso-distal optic vesicle fail to differentiate appropriately, causing the retinal pigmented epithelium cells to adopt a neural retina fate and abnormal differentiation of the dorsal optic stalk; the development of proximo-ventral identities, neural retina and ventral optic stalk is also compromised. These cellular defects in turn lead to congenital ocular colobomata and microphthalmia. Immunohistochemical and in situ hybridization assays reveal that the expression of several regulatory genes essential for early optic vesicle development, including *Pax6*, *Otx2*, *Mitf*, *Pax2* and *Vax1/2*, is altered in the corresponding compartments of the mutant eye. Using ChIP assay, siRNA treatment and transient transfection in ARPE-19 cells in vitro, we demonstrate that *Pax6* and *Otx2* are directly regulated by COUP-TFs. Taken together, our findings reveal novel and distinct cell-intrinsic mechanisms mediated by COUP-TF genes to direct the specification and differentiation of progenitor cells, and that COUP-TFs are crucial for dorsalization of the eye.

KEY WORDS: COUP-TF, Eye development, Optic vesicle, Mouse

INTRODUCTION

The development of the murine eye initiates at around embryonic day (E) 8.0, with the evagination of the optic pit from the presumptive ventral diencephalon. Optic primordia continue evaginating to form the early optic vesicle at ~E9.5. The early optic vesicle can be divided into four domains along the dorso-distal and the ventro-proximal axes: presumptive dorsal optic stalk (pdOS), presumptive retinal pigmented epithelium (pRPE), presumptive neural retina (pNR) and presumptive ventral optic stalk (pvOS). Following evagination, invagination takes place at E9.5 to form the optic cup at ~E10.5, which consists of three major components: neural retina (NR), retinal pigmented epithelium (RPE) and optic stalk (OS) (Chow and Lang, 2001). These morphological changes reflect the progress of the cell-specification and differentiation programs.

Retina progenitor cells, as well as RPE and OS cells, originate from early progenitor cells of the optic primordia. Recent evidence indicates that, similar to the neural progenitor/stem cells in other regions of the central nervous system, the pluripotency of the early progenitor cells in the eye field is also gradually restricted due to the influence of various morphogens and effectors. The early progenitor cells in the optic primordia are morphologically and molecularly indistinguishable from each other (Chow and Lang, 2001; Martinez-Morales et al., 2004). Midline Hedgehog signaling promotes the expression of *Pax2* and *Vax1/2* to specify the ventral optic vesicle (Chow and Lang, 2001; Torres et al., 1996; Bertuzzi et al., 1999; Hallonet et al., 1999; Mui et al., 2000; Mui et al., 2005). FGF signals from the surface ectoderm may trigger the expression of Chx10 (Vsx2 – Mouse Genome Informatics), a homeodomain protein, in the distal optic vesicle to define NR identity (Nguyen and Arnheiter, 2000; Rowan et al., 2004; Horsford et al., 2005). At the dorso-distal optic vesicle, BMP signals from extraocular mesenchymal cells activate *Mitf*, a basic helix-loop-helix zipper (bHLH-Zip) transcription factor, to mediate the differentiation of RPE cells (Muller et al., 2007; Bumsted et al., 2000; Nguyen and Armheiter, 2000). Nonetheless, at present, how the dorsal/ventral polarity in the optic vesicle is established remains poorly understood at a molecular and cellular level.

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are orphan receptors of the steroid/thyroid hormone receptor superfamily (Tsai and Tsai, 1997). In the mouse, *COUP-TFI* and *COUP-TFII* (*Nr2f1* and *Nr2f2*) are essential for early neural development and organogenesis (Qiu et al., 1997; Zhou et al., 1999; 2001; Pereira et al., 1999; You et al., 2005a; You et al., 2005b; Li et al., 2009; Kurihara et al., 2007; Petit et al., 2007; Takamoto et al., 2005a; Takamoto et al., 2005b). However, the physiological function of COUP-TFs in the mammalian eye has not been described.

In this study, the expression profiles of COUP-TFI and COUP-TFII in the developing mouse eye were examined in detail. Results obtained from *COUP-TFI* and *COUP-TFII* single-gene conditional knockout mice suggest that these two genes can compensate for each other during eye morphogenesis. In eye-specific *COUP-TFI/TFII* double-knockout mice, the progenitor cells at the dorso-distal optic vesicle failed to differentiate appropriately, resulting in the conversion of RPE into NR and the abnormal differentiation of the dorsal optic stalk (dOS) cells; the development of the ventro-

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proximal identities, NR and vOS was also compromised. These cellular defects in turn led to bilateral ocular coloboma and microphthalmia. We further demonstrated that COUP-TFs directly regulate the transcription of *Pax6* and *Otx2* during morphogenesis of the eye.

MATERIALS AND METHODS

Animals

Generation of floxed *COUP-TFII* mice, *COUP-TFII-lacZ* knock-in mice and *Rx*-Cre mice has been previously described (Takamoto et al., 2005b; Swindell et al., 2006). Floxed *COUP-TFI* mice were generated in this study (see Fig. 1M). Mice used in this study were of mixed background. All animal protocols were approved by the Animal Center for Comparative Medicine at Baylor College of Medicine. Only littermates were used for comparison. At least three to four animals were used in each experiment.

Immunohistochemistry, antibodies and in situ hybridization

Immunohistochemical procedures were performed as reported previously (You et al., 2005a). Antibodies used were: COUP-TFI (R&D, 1:5000), COUP-TFII (R&D, 1:5000), Pax2 (Covance, 1:500), Pax6 (Upstate, 1:500), Chx10 (Upstate, 1:500), Mitf (1:400), Otx2 (Upstate, 1:1000), Vax1 (1:300) and Vax2 (1:1600). Non-radioactive in situ hybridization was conducted as described previously (Bramblett et al., 2004).

Cell line, transfection and immunocytochemistry

The human originated RPE cell line, ARPE-19, was purchased from ATCC, and cells were grown as recommended by the supplier. ARPE-19 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Immunocytochemical procedures were performed as reported previously (Tang et al., 2002).

RNA interference and real-time PCR

siRNA oligonucleotides were purchased from Thermo Fisher Scientific or Applied Biosystems/Ambion. For each RNA interference experiment, cells were prepared at ~40-50% confluence on the day of transfection. siRNAs (50 nM) were delivered into cells with Oligofectamine (Invitrogen) following the manufacturer's protocol. The preparation of total RNA and reverse-transcribed cDNA, and real-time PCR were performed as reported previously (Li et al., 2009). Means for mRNA levels in *siCON*-treated and *siCOUP-TFI/TFII*-treated cells were compared using Student's *t*-test.

Chromatin immunoprecipitation (ChIP) and PCR

ChIP assays were carried out with an EZ ChIP Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer's protocol. Monoclonal mouse COUP-TFII antibody, polyclonal rabbit Sp1 antibody and corresponding control IgG antibodies were used in the assays. ARPE-19 cells were treated with *siCON*, *siCOUP-TFII* or *siSp1*. PCR assays were performed with HotStar Taq DNA polymerase (Qiagen). The PCR conditions for each primer pair were optimized, and PCR cycles performed were in the linear range. Primers a to h were as follows: (a) *Pax6*-Con-forward, 5′-CAAATCCTGTAACTCACACC-3′; (b) *Pax6*-Con-reverse, 5′-TTCAGTCTTGGCTAAGCCCA-3′; (c) *Pax6*-DR1-forward, 5′-GTCTTCCCTAGAAATCCTCA-3′; (d) *Pax6*-DR1-reverse, 5′-GATGCACAAAATGATTGGAC-3′; (e) *Otx2*-Con-forward, 5′-AGGGAGGAAGAAACGTGCCA-3′; (f) *Otx2*-Con-reverse, 5′-CCCTCCATCCTAGAGCTTCG-3′; (g) *Otx2*-Sp1-forward, 5′-GGCAAACAGTGCTTCCAGCA-3′; (h) *Otx2*-Sp1-reverse, 5′-GGGGAGAGCATTGGTAGGCT-3′.

Luciferase assays

Three copies of the DR1 element in the *Pax6* gene were cloned into *pGL4-minP-Luc*. ARPE-19 cells treated with siRNAs were transfected with 200 ng *pGL4-DR1X3-minP-luc* or *pGL4-minP-luc*, using FuGene 6 according to the manufacturer's instructions (Roche). Firefly luciferase activities were measured 48 hours post-transfection with the Luciferase Assay System (Promega) and a luminometer (Berthold Technologies). Data are presented as the mean ± s.e.m. of three separate experiments.

RESULTS

Expression of COUP-TFI and COUP-TFII during early eye development

In order to determine whether COUP-TFs have a role in murine eye development, their expression was characterized by immunohistochemical assays. At E9.5, both COUP-TFI and COUP-TFII were highly expressed in the dorso-distal optic vesicle, where the pdOS and pRPE are situated, and COUP-TFI was expressed at a relatively low level in the proximo-ventral optic vesicle (Fig. 1A,B). In the pRPE region, the expression of COUP-TF proteins generates a 'ventral high-dorsal low' gradient (Fig. 1A,B). The specificity of immunostaining was evident in that the signals largely disappeared from the optic vesicle in a *COUP-TFI/TFII* eye-specific double conditional knockout mutant at E9.5 (Fig. 1C,D). At the same stage, immunostaining of sagittal sections showed that at the distal plate, the expression of COUP-TFI was stronger at the temporal optic vesicle (Fig. 1E, arrow), whereas the expression of COUP-TFII was localized in the dorsal optic vesicle (Fig. 1F, arrowhead). At the proximal plate (optic stalk area), the expression of COUP-TFI was distributed throughout the presumptive optic stalk (pOS) (Fig. 1G), whereas COUP-TFII was clearly expressed in the dorsal, but not in the ventral, pOS (Fig. 1H).

As an alternative method to analyze COUP-TFII expression, *COUP-TFII-lacZ* knock-in mice were assayed, and the *lacZ* signal found to mirror COUP-TFII expression (see Fig. S1A-C in the supplementary material). COUP-TFI and COUP-TFII were co-expressed in the progenitor cells at the pOS (the green COUP-TFI immunostain co-localized with the red signal from *lacZ* staining; see Fig. S1D-F in the supplementary material). Their expression patterns in the OS remained unchanged throughout E10.5 (see Fig. S1G,H in the supplementary material). In frontal sections at E10.5, COUP-TFI expression was readily detected in the retina progenitor cells and in the ventral differentiating RPE cells (see Fig. S1I in the supplementary material), whereas COUP-TFII was clearly expressed throughout the entire RPE region (see Fig. S1J in the supplementary material). In the proximal OS area, the expression of COUP-TFI was clearly apparent in the vOS and ventral dOS at E11.5 (Fig. 1I). COUP-TFII expression was low in the dOS and hardly detectable in the vOS cells (Fig. 1J). In the distal plate at this stage, COUP-TFI was mainly expressed in the NR (Fig. 1K), whereas COUP-TFII was exclusively expressed in the RPE (Fig. 1L).

Generation of single and double conditional COUP-TF knockout mouse models

Both *COUP-TFI*-null and *COUP-TFII*-null mutant mice die early during development (Qiu et al., 1997; Pereira et al., 1999). To circumvent the lethality due to ablation of these genes, *COUP-TFI* (Fig. 1M) and *COUP-TFII* (Takamoto et al., 2005b) floxed mice were created.

To investigate the potential function of COUP-TF genes in eye development, *COUP-TFI* and *COUP-TFII* single conditional knockout mice were generated in an *Rx-Cre* background (*Rx* is also known as *Rax* – Mouse Genome Informatics), in which the Cre protein is specifically expressed in the developing eye and ventral forebrain (Swindell et al., 2006). In the *COUP-TFI* mutant mouse (*COUP-TFI*$^{\Delta/\Delta}$) at E11.5, whereas COUP-TFI expression was no longer detectable (Fig. 1N), expression of COUP-TFII was increased and distributed evenly along the NR at low level, and its expression remained high in the RPE (Fig. 1O). In the *COUP-TFII* mutant (*COUP-TFII*$^{\Delta/\Delta}$) at E11.5, COUP-TFII expression was lost in the RPE as expected (Fig. 1Q), and, interestingly, COUP-TFI was

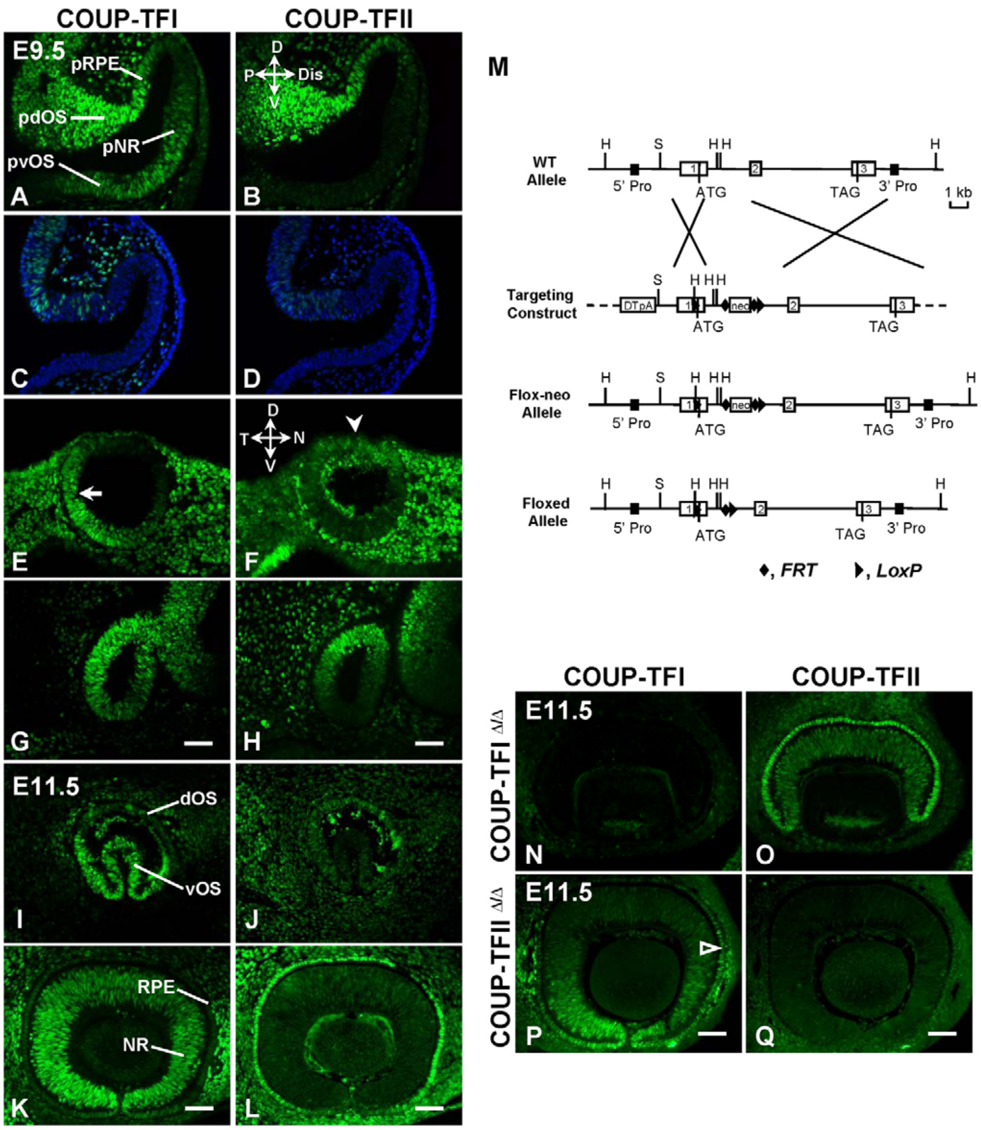

Fig. 1. Expression of COUP-TFI and COUP-TFII in the developing mouse eye and generation of the *COUP-TFI*-floxed mouse. (**A**,**B**) The expression of COUP-TFI and COUP-TFII in the optic vesicle at E9.5. Frontal sections. (**C**,**D**) In the COUP-TF eye-specific double-mutant mouse at E9.5, the expression of COUP-TFI and COUP-TFII is drastically reduced. Frontal sections. (**E-H**) Expression of COUP-TFI and COUP-TFII in the optic vesicle at E9.5. Sagittal sections. (E,F) The distal plate. (G,H) The proximal plate. Arrow, temporal optic vesicle. Arrowhead, dorsal optic vesicle. (**I-L**) The expression of COUP-TFI and COUP-TFII in the optic cup at E11.5. Sagittal sections. (I,J) The proximal OS region. (K,L) The distal NR and RPE regions. (**M**) Generation of the *COUP-TFI*-floxed mouse. H, *Hin*dIII; S, *Sau*3AI. (**N-Q**) Expression of COUP-TFI and COUP-TFII in the eye of *COUP-TFI* (*COUP-TFI*$^{\Delta/\Delta}$) and *COUP-TFII* (*COUP-TFI*$^{\Delta/\Delta}$) single-mutant mice at E11.5. Sagittal sections. Arrowhead, COUP-TFI expression in RPE cells. In A-L and N-Q dorsal is to the top. Scale bars: 50 μm.

now expressed in the RPE cells (Fig. 1P, arrowhead). Therefore, COUP-TFI and COUP-TFII may compensate for each other in the developing eye. Owing to this compensation, neither *COUP-TFI* nor *COUP-TFII* single-knockout mice developed major eye abnormalities. In *COUP-TFI*$^{\Delta/\Delta}$, the expression of *Sox2*, *Otx2*, *Mitf* and *Pax6*, representing several RPE-related genes, was not altered in the RPE at E11.5 (data not shown) and E14.5 (see Fig. S2 in the supplementary material). To overcome the redundancy of COUP-TFs during eye development, a *COUP-TFI/TFII* double conditional knockout mouse, *RxCre*/+;*COUP-TFI*$^{f/f}$;*COUP-TFII*$^{f/f}$, was generated.

Coloboma, microphthalmia and abnormal optic cup in the COUP-TF double-mutant mouse

During invagination, the optic cup becomes shaped at ~E10.5, and the optic fissure at the ventral side of the optic cup closes completely by E13.5. When this process fails, a coloboma phenotype ensues (Chow and Lang, 2001). The experimental strategy we used generated two kinds of three-allele-deleted mutant mice: *RxCre*/+;*COUP-TFI*$^{f/f}$;*COUP-TFII*$^{f/+}$ and *RxCre*/+;*COUP-TFI*$^{f/+}$;*COUP-TFII*$^{f/f}$. Both of these compound mutant mice displayed an open optic fissure bilaterally at E14.5, indicating a coloboma phenotype (Fig. 2B,C). When all four alleles of the *COUP-TFI* and *COUP-TFII* genes were deleted, the mutant mouse displayed the most severe coloboma and microphthalmia (Fig. 2D), which persisted in double-mutant mice after birth (compare Fig. 2F with 2E). The present study mainly focuses on the phenotypes exhibited by the four-allele-deleted *COUP-TFI/TFII* double-knockout mice, which we refer to as 'double-mutant' or simply 'mutant' mice.

Hematoxylin and Eosin (H&E) staining was used to examine morphological differences between control and mutant mice at E14.5. In the control, pigmented RPE developed into a single cell layer encasing the NR (Fig. 2G,I). By contrast, in the mutant a NR-like structure that occupied the proximal and ventral prospective RPE area was observed (Fig. 2H,J). In the control eye, a sharp boundary was established at the optic disc (OD), separating vOS and NR, while vOS served as the link between NR and the midline neural tube (Fig. 2G,I). Neither OD nor vOS was observed in the eyes of the double mutant; instead, the NR-like structure extended all the way to connect directly with the diencephalon (Fig. 2H,J).

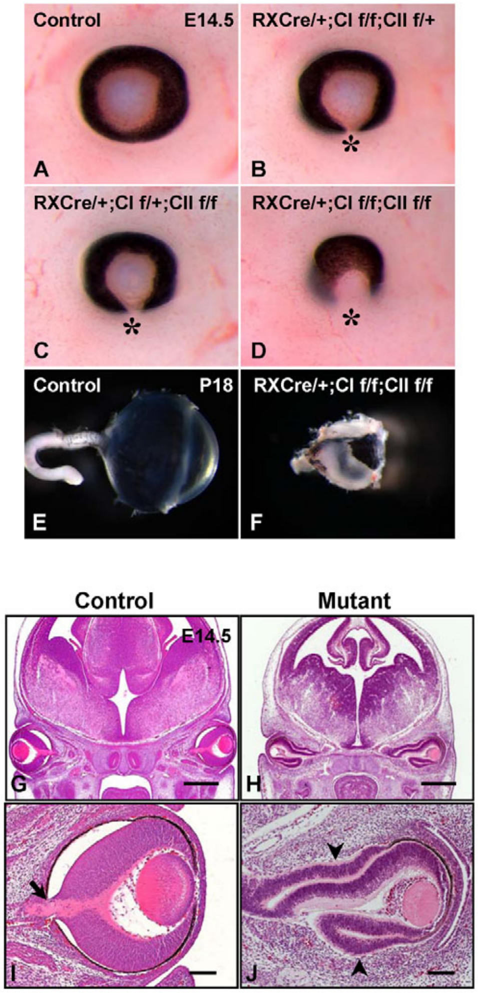

Fig. 2. Coloboma, microphthalmia, secondary neural retina and extended neural retina in the *COUP-TFI/TFII* double-mutant mouse. (**A**) The optic fissure is closed completely at E14.5 in the control. (**B**,**C**) Three-allele-deleted compound mutants display the open optic fissure (asterisk) characteristic of a coloboma phenotype at E14.5. (**D**) The 'complete' double-mutant mouse exhibits a severe coloboma phenotype at E14.5. (**E**) Eye from a control mouse at post-natal day (P) 18. (**F**) Microphthalmia in a double-mutant mouse at P18. (**G**,**I**) H&E staining of the control at E14.5. Arrow indicates the OD. (**H**,**J**) H&E staining of the double mutant at E14.5. Arrowheads indicate the ectopic NR-like structure in the ventral and proximal-dorsal prospective RPE region. G-J are frontal sections. Scale bars: 500 μm in G,H; 50 μm in I,J.

COUP-TFI and *COUP-TFII* are required for proper differentiation of the neural retina and ventral optic stalk

Morphologically, vOS appeared to be absent in the double-mutant embryo at E14.5. To confirm whether the vOS was indeed missing, we carried out immunostaining with a specific antibody against Pax2, a vOS cell marker. Surprisingly, the Pax2 signal in the double mutant was clearly localized in cells at a more proximal region, indicating that the vOS identity was maintained (Fig. 3B). In the control, the signal of Pax6, a neuroretina marker, was mainly localized in the NR (Fig. 3C), and the Pax6-positive NR was segregated from Pax2-positive vOS at the OD (Fig. 3E). In the double mutant, the spatial patterning of Pax6-positive and Pax2-positive domains was maintained (Fig. 3F), although the boundary between the two domains had shifted proximally (Fig. 3F, arrowhead; the dashed line indicates where the junction should be located).

In the early optic vesicle, the reciprocal repression between Pax2 and Pax6 gradually establishes the sharp boundary between the NR and vOS (Chow and Lang, 2001). Since the boundary had shifted in the COUP-TF double mutant, the expression of Pax2 and Pax6 was examined in detail. At E11.5 in the control, Pax2 was highly expressed at the vOS, dOS, future OD region and in the ventral NR (Fig. 3G). In the double mutant, Pax2 expression was reduced in vOS and was barely detectable around the OD region and dOS (Fig. 3H). In the control, Pax6 was highly expressed in RPE and dorsal NR, but at a lower level in the OD (Fig. 3I). By contrast, Pax6 expression was extended ventrally into the vOS in the double mutant (Fig. 3J, arrowhead). Pax2 and Pax6 double-positive cells were mainly localized in the future OD and ventral NR region in the control mouse (Fig. 3K, yellow). However, in the double-mutant mouse, yellow cells were observed in the prospective vOS (Fig. 3L). This result strongly suggests that the progenitor cells at the distal vOS are gaining NR identity.

At E10.5 in the control, the morphology of the OS resembled that of the early optic vesicle at E9.5. Pax2 expression showed a ventral high-dorsal low gradient along the OS area (Fig. 3M,Q), whereas the expression of Pax6 was complimentary to that of Pax2, with a dorsal high-ventral low gradient (Fig. 3O,Q). In the mutant, the expression of Pax2 was reduced (Fig. 3N,R), whereas the expression of Pax6 was enhanced throughout the entire OS region (Fig. 3P,R). It has been reported that Vax1 and Vax2 repress *Pax6* gene expression to ventralize the mouse eye (Mui et al., 2005). At E10.5, Vax1 was expressed throughout the OS in the control (Fig. 3S), but its expression was greatly reduced in the mutant (Fig. 3T). Vax2 was expressed at the ventral NR in the control at E10.5 (Fig. 3U); by contrast, its expression was barely detectable in the mutant (Fig. 3V). Our results suggest that COUP-TF genes are required to maintain the proper expression of genes that are important for establishing the boundary between NR and vOS.

Differentiation of the dorsal optic stalk is compromised in the COUP-TF double-mutant mouse

H&E staining on sagittal sections revealed that in the control, dOS cells had already developed into a single cell sheet surrounding the vOS at E12.5 (Fig. 4A,B). At E14.5, the optic nerve from retina ganglion cells appeared between the vOS and dOS cells (Fig. 4D,E). In the double-mutant mouse, however, the OS was still open ventrally at both stages (Fig. 4C,F, arrowheads), and two or three layers of cells at the prospective dorsal and ventral OS were present. At E11.5, Pax2, a marker of the OS, was differentially expressed in the OS along the distal-proximal axis. In the control, at the distal plate Pax2 was detected in vOS cells and differentiating cells at the ventral dOS, but not in the differentiated cells at the dorsal dOS (Fig. 4G, arrow). At the proximal plate, Pax2 was distributed uniformly in both dOS and vOS (Fig. 4I). At E12.5 and E14.5, its expression was only apparent in vOS cells and was undetectable in dOS cells (Fig. 4K,M,O, arrowhead). However, in the mutant, Pax2 expression was consistently observed in the cells at the dOS at E11.5 and E12.5 (Fig. 4H,J,L, arrowhead). Even at E14.5, the cells in the prospective dOS were still positive for Pax2 (Fig. 4N,P and Fig. 3B), but negative for Pax6 and Chx10, two markers for neuroretina cells (data not shown). These results indicate that differentiation of dOS

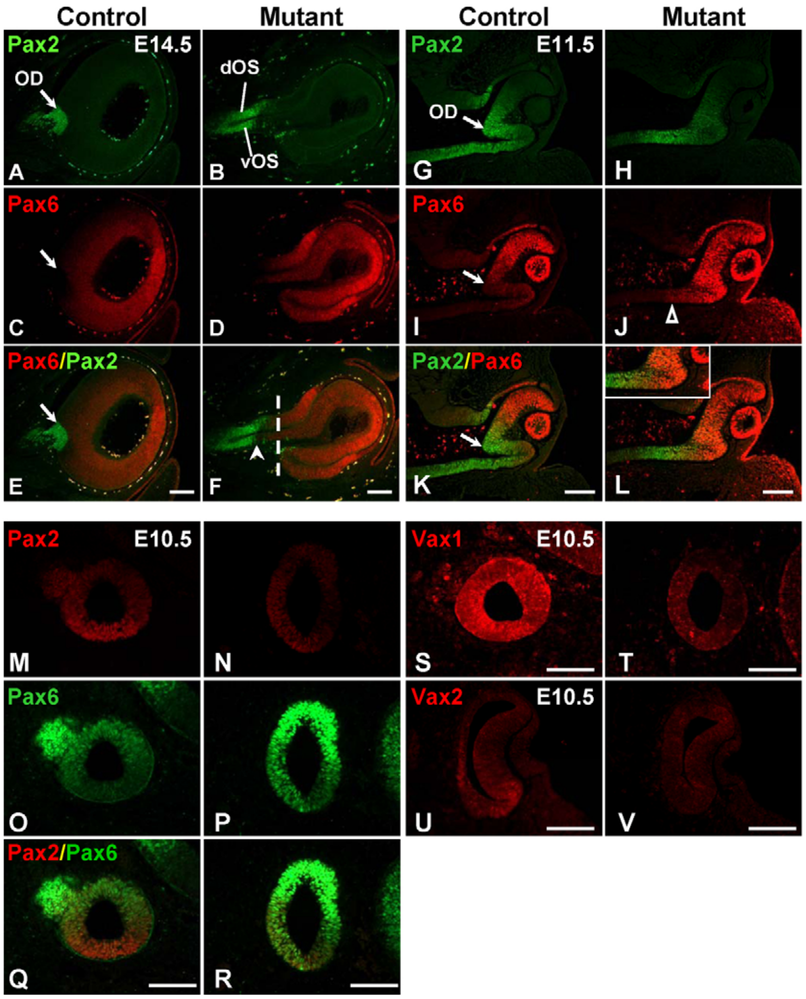

Fig. 3. Extension of the NR into vOS territory and abnormal expression of Pax2 and Pax6 in the *COUP-TFI/TFII* double mutant. (**A-F**) The expression of Pax2 and Pax6 in the eye of control and *COUP-TFI/TFII* double mutant at E14.5. Arrow indicates the OD in the control. Arrowhead indicates the boundary between Pax2-positive and Pax6-positive domains in the mutant. The dashed line indicates the location at which the boundary would normally be. (**G-L**) The expression of Pax2 and Pax6 in the eye of the control and mutant at E11.5. Arrowhead indicates Pax6 expression in the vOS in the mutant. The inset in L shows Pax2/Pax6 double-positive cells in the vOS. (**M-R**) The expression of Pax2 and Pax6 at the OS in the control and mutant at E10.5. (**S**,**T**) Expression of Vax1 at the OS in the control and mutant at E10.5. (**U**,**V**) Expression of Vax2 in the optic cup in the control and mutant at E10.5. A-L and U,V are frontal sections, M-T sagittal sections. Scale bars: 100 μm.

cells is impaired in the COUP-TF double-mutant mouse, suggesting that COUP-TFs in the dOS are essential for downregulating Pax2 and that downregulation of Pax2, in turn, is required for the differentiation of dOS cells.

Transformation of RPE into neural retina in the COUP-TF double-mutant mouse

The ectopic NR-like structure was observed at the prospective RPE region in the COUP-TF double mutant at E14.5 (Fig. 2H,J). Similar to the expression of Pax6 shown in Fig. 3D, Chx10, another neuroretina marker, was also expressed in the ectopic NR-like structure, whereas Mitf, an RPE marker, was only detected in the distal-dorsal cells with the characteristic morphology of RPE cells (Fig. 5B,D). The above findings indicate that proximo-ventral RPE cells possess NR identity in the absence of COUP-TFs.

The induction of RPE, as well as of NR and OS, takes place during early invagination, and the cells in dorsal compartments progress earlier. H&E staining in the control revealed that RPE had differentiated into a structure with a single cell layer (Fig. 5E) along the dorsal-ventral axis at E10.5. This layer of cells was positive for both Otx2 (Fig. 5G) and Mitf (Fig. 5I). In the mutant, only the most dorsal RPE area contained a monolayer of cells (Fig. 5F), which was positive for Otx2 and Mitf (Fig. 5H,J), but there were two or three cell layers in the ventral region (Fig. 5F), in which the expression of Otx2 and Mitf was hardly detectable (Fig. 5H,J). Chx10 was only detectable in the NR in the control (Fig. 5K). Chx10 was expressed in the NR in the mutant as expected, but it was also detected in a few cells at the prospective ventral RPE region (Fig. 5L, arrowheads). Merged images from Mitf and Chx10 double staining revealed that there was no overlap between the two domains in both control and mutant mice (Fig. 5M,N, arrow). One day later, at E11.5, the expression of Chx10 was readily detected in cells at the ventral and proximo-dorsal RPE in the mutant (Fig. 5T, arrowheads), suggesting acquisition of NR identity. In the control, Pax6 expression was highest in the lens and considerably lower in the RPE and NR (Fig. 5O). By contrast, the expression of both Pax6 protein and transcript in the mutant mouse was greatly enhanced in the RPE and NR to a level higher than, or similar to, that of the lens (Fig. 5P,R), suggesting that the upregulation might be transcriptional. Taken together, these results indicate that COUP-TFs suppress the expression of *Pax6* and enhance the expression of *Mitf* and *Otx2* to specify the RPE fate along the dorsal/ventral axis.

Since COUP-TFs were differentially expressed along the nasal/temporal axis, with higher expression in the temporal optic vesicle at E9.5 (Fig. 1E,F), we asked whether the RPE also had a differential defect along this axis. At E11.5, H&E staining on sagittal sections revealed that in the mutant there was an NR-like structure localized at the prospective temporal RPE, which was Chx10 positive, suggesting a change of RPE identity to NR identity (see Fig. S3B,D in the supplementary material, arrowheads). Next, we examined whether those regulatory genes, the expression of which was affected along the dorsal/ventral axis by mutation of COUP-TFs, were also

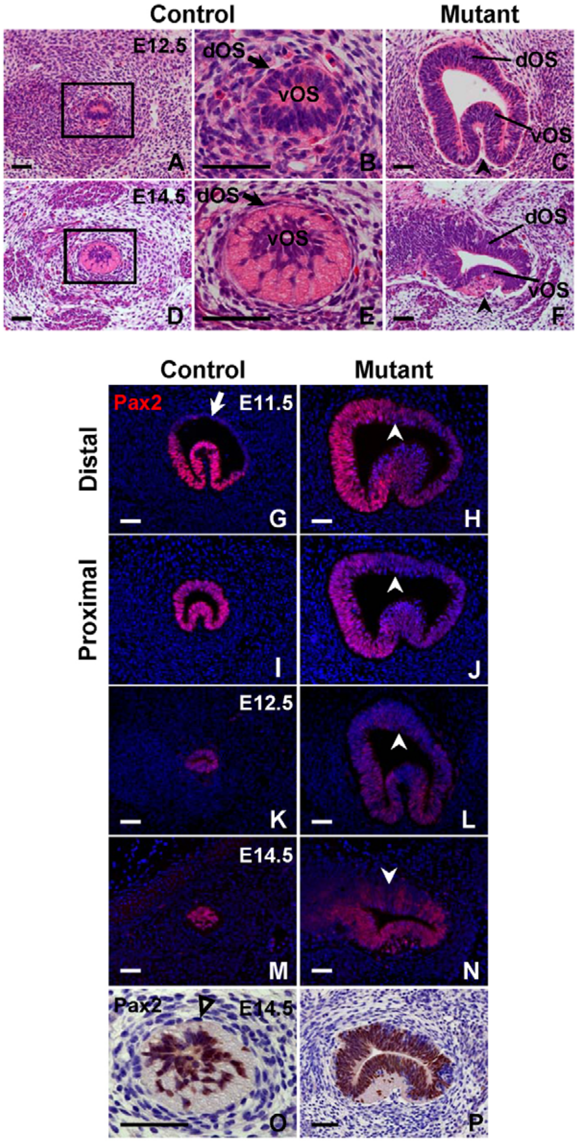

Fig. 4. Dorsal optic stalk cells fail to differentiate properly in the *COUP-TFI/TFII* double-knockout mouse. (**A**,**B**,**D**,**E**) H&E staining of control mice at E12.5 and E14.5. (**C**,**F**) H&E staining of the double-mutant at E12.5 and E14.5. Arrowheads indicate the opened optic fissure in the mutant. (**G**,**I**,**K**,**M**,**O**) The expression of Pax2 at the OS in the control at E11.5, E12.5 and E14.5. The arrow in G indicates the dOS. (**H**,**J**,**L**,**N**,**P**) The expression of Pax2 at the OS in the double mutant at E11.5, E12.5 and E14.5. Arrowhead indicates the prospective dOS in the mutants. A-P are frontal sections. Scale bars: 50 µm.

modulated along the nasal/temporal axis. As expected, the expression of Mitf, Otx2 and Dct (TRP2), a downstream target of the *Mitf* and *Otx2* genes (Martinez-Morales et al., 2004), was greatly reduced in the prospective temporal RPE in the mutant (see Fig. S3H,J,L in the supplementary material, asterisk), whereas their expression was distributed uniformly in the RPE of the control (see Fig. S3G,I,K in the supplementary material). At the same time, in the mutant the expression of Pax6 at the temporal RPE was slightly higher than at the nasal RPE (see Fig. S3F in the supplementary material). Taken together, these results suggest that in the absence of COUP-TFs, the temporal-ventral RPE cells gain NR cell characteristics, while the nasal-dorsal RPE cells maintain their RPE identity.

COUP-TF proteins directly regulate the expression of *Pax6* and *Otx2*

Given that the expression of *Pax6* was greatly enhanced in the differentiating cells at the prospective RPE in the double mutants, we asked whether *COUP-TFI* and *COUP-TFII* directly regulate *Pax6* gene expression. The ARPE-19 cell line, which originated from human RPE, was chosen for this analysis. Western blotting and immunocytochemical assays revealed that high COUP-TFII and low COUP-TFI expression profiles were maintained in the ARPE-19 cells, resembling their in vivo expression patterns in the mouse RPE (see Fig. S4 in the supplementary material; data not shown).

To characterize COUP-TF-mediated regulation of the genes expressed in the RPE, we knocked down *COUP-TFI* and/or *COUP-TFII* expression in ARPE-19 cells by siRNA treatment. The expression levels of COUP-TFs and other RPE marker genes were assessed by western blot and real-time PCR. As expected, the expression of *Pax6* was increased when COUP-TFs were knocked down (see Fig. S4 in the supplementary material; Fig. 6A). Expression levels of the key RPE genes, *Otx2* and *Mitf*, were significantly lower when COUP-TFs were knocked down and so was the expression of *Vax2*, a negative regulator of the *Pax6* gene (Fig. 6A). These observations strongly suggest that the COUP-TFs repress *Pax6* expression but activate *Mitf*, *Otx2* and *Vax2* expression in this cell line, in agreement with what we observed in vivo. Thus, the ARPE-19 cell line is suitable for analyzing COUP-TF regulation of their target genes.

Next, we asked whether overexpression of COUP-TFs could suppress the expression of *Pax6*. Plasmids *pCXN2-vector*, *pCXN2-COUP-TFI* and *pCXN2-COUP-TFII* were transiently transfected into ARPE-19 cells and Pax6/COUP-TFI or Pax6/COUP-TFII antibody double-staining assays were performed. In the *pCXN2-vector*-transfected control cells, the expression of COUP-TFI was barely detectable, whereas Pax6 was expressed in every ARPE-19 cell (Fig. 6B, upper panel). COUP-TFII co-localized with Pax6 in the nuclei (data not shown). Pax6 expression was repressed in the nucleus of *COUP-TFI*-overexpressing cells (Fig. 6B, middle panel, arrowhead). A similar repression of Pax6 was observed in *pCXN2-COUP-TFII*-transfected cells (Fig. 6B, bottom panel, arrowhead). The number of COUP-TF-overexpressing cells and the number of cells with greatly reduced Pax6 expression were counted in five fields randomly picked from each experiment. Quantitative data revealed that the expression of Pax6 was greatly reduced in 78.3% of *COUP-TFI*-overexpressing cells, being less or unaffected in the other 21.7%. Among *COUP-TFII*-transfected cells, 76.9% showed drastically reduced Pax6 expression. Taken together, all the data suggest that both COUP-TFI and COUP-TFII can negatively regulate the *Pax6* gene.

We and others have previously shown that COUP-TFI and COUP-TFII function indistinguishably as repressors by binding to direct repeat (DR) elements (Tsai and Tsai, 1997). One such highly evolutionarily conserved DR1 binding site (<u>TGTTCA</u>C<u>AGTCCA</u>) was identified at the 3′-UTR region of the mouse *Pax6* gene (Fig. 6C). To address whether *Pax6* is a direct downstream target of the COUP-TF genes, we conducted ChIP assays in ARPE-19 cells using a COUP-TFII antibody, as the expression of COUP-TFII is much higher than that of COUP-TFI in RPE cells. Two pairs of PCR primers were designed: one pair (c/d) targets the DR1 binding site in the 3′-UTR, whereas the other pair (a/b) targets a region in intron 3 and served as the control (Fig. 6C). As shown in Fig. 6C, with control siRNA (*siCON*) treatment, COUP-TFII was preferentially recruited to the DR1 site (c/d). When the expression of *COUP-TFII* was knocked down with siRNA (*siCOUP-TFII*), the recruitment

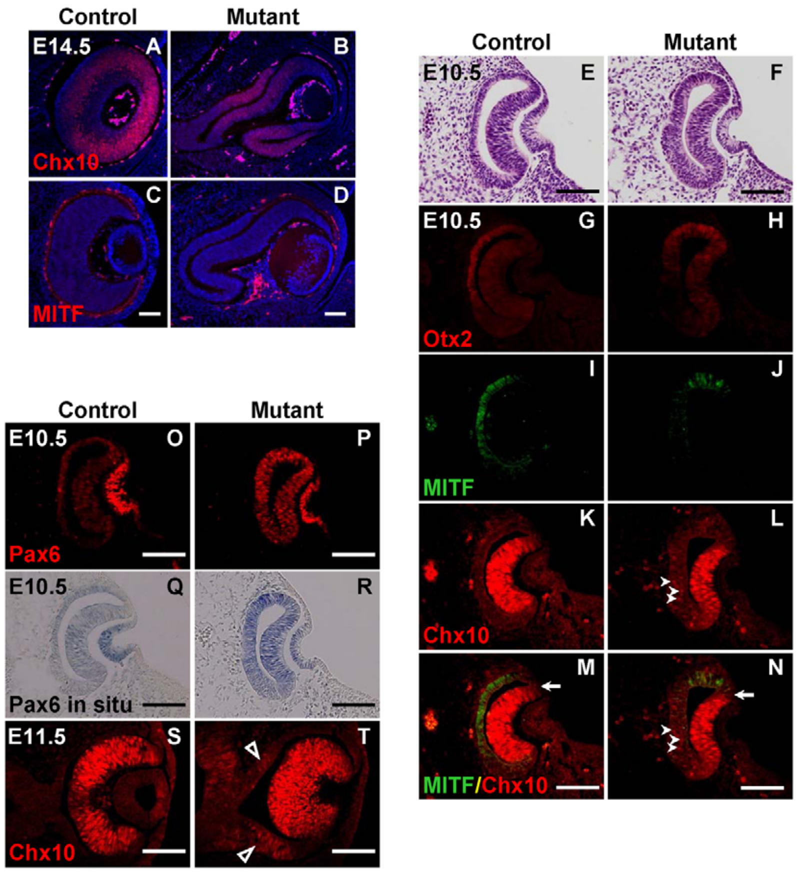

Fig. 5. Transformation of the RPE into neural retina and misexpression of RPE regulatory genes in the *COUP-TFI/TFII* double mutant. (**A-D**) The expression of Chx10 (A,B) and Mitf (C,D) in the eye of the control and mutant at E14.5. (**E**,**F**) H&E staining of the control and mutant at E10.5. (**G**,**H**) The expression of Otx2 in the eye of the control and mutant at E10.5. (**I-N**) The expression of Mitf and Chx10 in the eye of the control and mutant at E10.5. Arrowheads in L and N indicate Chx10-expressing cells in the prospective RPE region in the mutant. The arrow in M and N indicates the junction between Mitf- and Chx10-expressing domains. (**O-R**) Expression of Pax6 protein (O,P) and transcripts (Q,R) in the eye of the control and mutant at E10.5. (**S**,**T**) The expression of Chx10 in the eye of the control and mutant at E11.5. Arrowheads indicate Chx10-expressing cells at the ventral and proximal-dorsal prospective RPE. All images are of frontal sections. Scale bars: 100 μm.

was abolished (lane 6). A more quantitative ChIP assay with real-time PCR confirmed that COUP-TFII is recruited to the regulatory region of the *Pax6* and *Otx2* genes (see Fig. S5 in the supplementary material). In order to demonstrate that both COUP-TFI and COUP-TFII inhibit *Pax6* expression through the DR1 site, three copies of the DR1 element were cloned into the *pGL4-minP-Luc* reporter, and luciferase assays were conducted in ARPE-19 cells in which the expression of *COUP-TFI* and/or *COUP-TFII* was knocked down by siRNAs. Compared with that of the control, the luciferase activity increased by 70% in *COUP-TFI*-knockdown cells, approximately doubled in *COUP-TFII*-knockdown cells, and was enhanced further in cells depleted of both COUP-TFs (Fig. 6E). By contrast, the empty vector, which lacks DR1 sequences, was unaffected by the depletion of COUP-TFs. These results suggest that COUP-TFs repress *Pax6* transcription directly via binding to the DR1 element.

COUP-TFI and COUP-TFII can also function indistinguishably as activators through protein-protein interaction with Sp1 transcription factors (Tsai and Tsai, 1997; Park et al., 2002; Pipaon et al., 1999; Kim et al., 2009). The expression of *Otx2* was decreased in both the double-mutant mouse and in COUP-TF-depleted ARPE-19 cells, revealing that COUP-TFI and COUP-TFII could positively regulate its expression. Indeed, one Sp1 site (AGGGTGGGGG), which is highly evolutionarily conserved, was identified in the third intron of the mouse *Otx2* gene (Fig. 6D). ChIP assays in ARPE-19 cells showed that COUP-TFII was specifically recruited to this Sp1 site (amplified by primers g/h), but not to the control site in the second intron (amplified by primers e/f) that lacks the Sp1 binding element (Fig. 6D). In order to confirm that Sp1 binds to this site, ChIP assays were also performed in combination with siRNA treatments using Sp1 antibody and control IgG antibody. As expected, Sp1 was recruited to the site (Fig. 6D). These results indicate that COUP-TFII directly modulates transcription of *Otx2* through this region containing a conserved Sp1 site. Taken together, COUP-TFs regulate mouse eye development by directly or indirectly controlling cell-intrinsic factors, including Pax6, Otx2, Pax2, Mitf and Vax1/2, that are essential for morphogenesis of the eye.

DISCUSSION

During morphogenesis of the eye, the pluripotency of the early progenitor cells in the optic primordia is gradually restricted to generate diverse types of neural cells. How these progenitor cells are specified and differentiated by various extrinsic and intrinsic factors is largely unknown. The present studies provide the first evidence that *COUP-TFI* and *COUP-TFII* are expressed and required in the early progenitor cells to dorsalize the optic vesicle. Our observations reveal that *COUP-TFI* and *COUP-TFII* compensate for each other in programming the development of the progenitor cells through at least two different mechanisms. First, both COUP-TFI and COUP-TFII are co-expressed in the progenitor cells of the dorso-distal optic vesicle at E9.5 (Fig. 1; see Fig. S1 in the supplementary material). In this scenario, when one COUP-TF gene is deleted by a single-knockout strategy, the other remains functional to maintain the normal progress of the progenitor cells. Second, COUP-TFI and COUP-TFII also display reciprocal expression patterns in the optic cup. At E11.5, COUP-TFI is mainly expressed in the NR cells, whereas COUP-TFII is exclusively expressed in RPE cells (Fig. 1). In the *COUP-TFI*$^{\Delta/\Delta}$ mutant mouse, COUP-TFII expression is increased and distributed uniformly along the NR, whereas the expression of COUP-TFI is readily detected in the RPE cells of the

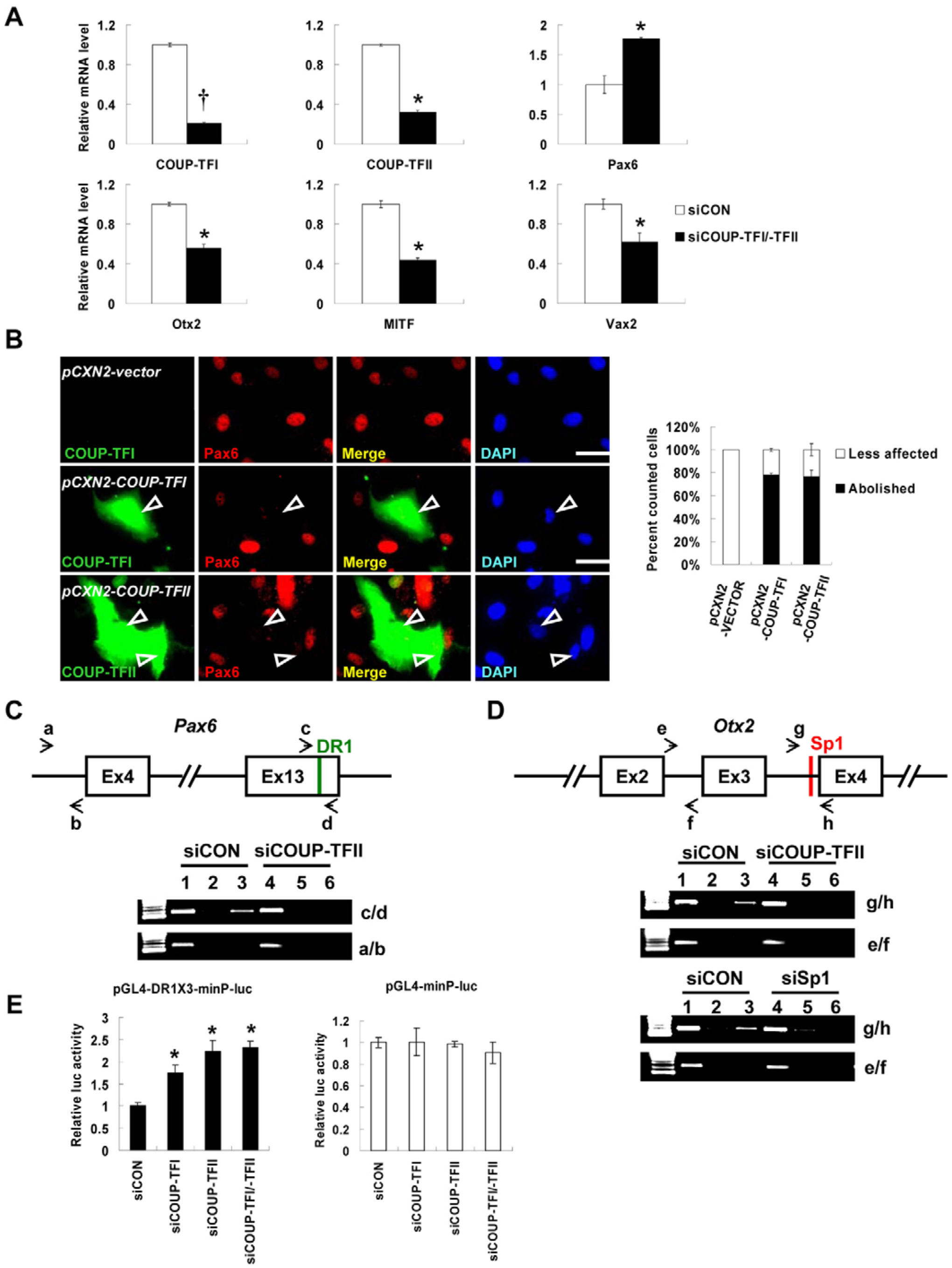

Fig. 6. COUP-TFs directly regulate *Pax6* and *Otx2* transcription. (**A**) Real-time PCR analysis of RPE marker genes in human ARPE-19 cells. RNAs were isolated from *siCON*-treated cells (white) and *siCOUP-TFI/TFII*-treated cells (black). Expression levels of each gene were normalized to that of 18S rRNA. Data indicate mean ± s.e.m. *, $P<0.05$; †, $P<0.001$. (**B**) Immunocytochemical assays with COUP-TFI, COUP-TFII and Pax6 antibodies in ARPE-19 cells transiently transfected with plasmids *pCXN2-vector*, *pCXN2-COUP-TFI* or *pCXN2-COUP-TFII*. When *COUP-TFI* (middle) or *COUP-TFII* (bottom) was overexpressed, the expression of Pax6 was repressed (arrowheads). The bar chart to the right shows the percentage of cells in which Pax6 expression was abolished or less/not affected in *pCXN2-vector*-transfected (367 cells), *COUP-TFI*-transfected (143 cells) or *COUP-TFII*-transfected (268 cells) cells. Error bars indicate s.e.m. (**C**,**D**) ChIP assays in ARPE-19 cells. COUP-TFII is recruited to the COUP-TF DR1 binding site in the 3′-UTR of the *Pax6* gene in *siCON*-treated samples. COUP-TFII and Sp1 are recruited to the Sp1 binding site in the third intron of the *Otx2* gene in *siCON*-treated samples. Lanes 1-3, *siCON* treatment; lanes 4-6, *siCOUP-TFII* or *siSp1* treatment; lanes 1 and 4, input; lanes 2 and 5, control IgG antibody-treated samples; lanes 3 and 6, COUP-TFII antibody- or Sp1 antibody-treated samples. (**E**) Luciferase assays with *pGL4-DR1X3-minP-Luc* and *pGL4-minP-Luc* in siRNA-treated ARPE-19 cells. *, $P<0.05$. Scale bars: 50 μm.

COUP-TFII$^{\Delta/\Delta}$ mutant mouse at E11.5 (Fig. 1). These data indicate that COUP-TF genes repress each other in these compartments during eye development; therefore, while one gene is dysfunctional in a compartment, the expression of the other gene is enhanced in situ to maintain normal development.

Since COUP-TFI and COUP-TFII are highly expressed in the optic vesicle, they might be required for appropriate morphogenesis of the eye. Indeed, the COUP-TF double-mutant mouse phenocopies ocular coloboma, a congenital ocular disease in humans. The morphologic alterations seen in COUP-TF mutant mice mirror the changes in cell specification and differentiation. The NR-like structure is observed at the prospective RPE area of the double-mutant mouse (Fig. 2; see Fig. S3 in the supplementary material). These results support the hypothesis that the prospective RPE cells change their fate to NR identity in COUP-TF double-knockout mice. The differentiation of RPE cells and NR progenitor cells has been well studied in recent decades. The progenitor cells in the early optic vesicle are bi-potent, giving rise to either RPE or NR identity depending on the microenvironment. *Pax2/6* or *Otx1/2* genes are required for the expression of *Mitf* in the early optic vesicle (Martinez-Morales et al., 2001; Baumer et al., 2003). The *Mitf* gene plays a crucial role in the differentiation of RPE cells (Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000). Chx10 antagonizes Mitf to determine and maintain the neuroretina fate (Burmeister et al., 1996; Rowan et al., 2004; Horsford et al., 2005). In the COUP-TF double-knockout mouse, the proximal Mitf and distal Chx10 expression domains remain similar to those of the control mouse at E10.5 (Fig. 5); therefore, the boundary between the NR and RPE has not shifted.

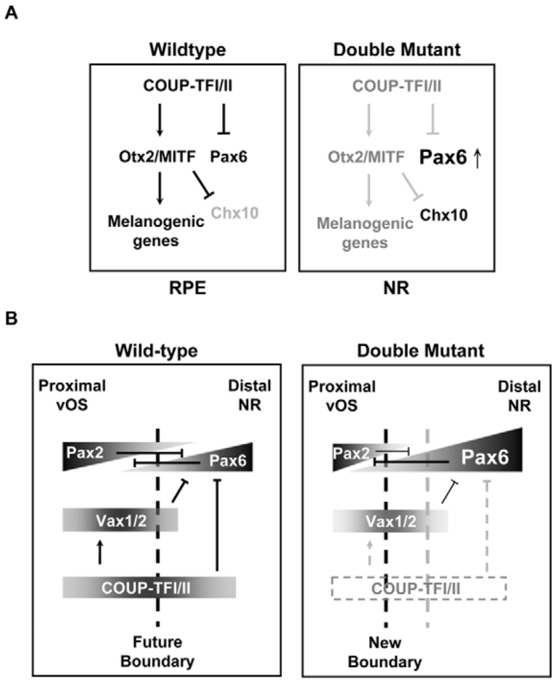

Fig. 7. Cell-intrinsic mechanisms mediated by *COUP-TFI/TFII* genes during morphogenesis of the murine eye. (**A**) *COUP-TFI/TFII* genes regulate RPE versus NR fates. (**B**) *COUP-TFI/TFII* genes program the NR and vOS identities to establish the proper boundary. See text for details.

However, at the prospective ventral RPE region, the expression of both Mitf and Otx2, two important determinants of the RPE, is greatly reduced in the double-mutant mouse. Concomitantly, the expression of Pax6 in the RPE is upregulated to a level as high as that seen in the lens (Fig. 5). High-level expression of Pax6 has also been observed in cells at an abnormal dorsal RPE domain in *Mitf* mutant mice (Nguyen and Arnheiter, 2000; Bharti et al., 2008). In chicken, overexpression of *Pax6* alone is sufficient to cause the transdifferentiation of RPE cells into NR cells (Azuma et al., 2005). Pax6 can also positively regulate the expression of *Mitf* (Baumer et al., 2003). However, in COUP-TF double mutants, expression of the *Mitf* and *Otx2* genes is not detected in the ventral RPE, despite the enhanced Pax6 expression. Consistent with this observation, the expression of *Mitf* and *Otx2* mRNA is also reduced in COUP-TF-depleted ARPE-19 cells in vitro, despite the increased expression of the *Pax6* gene. Therefore, at least in the ventral RPE, COUP-TFs are required to maintain the expression of *Mitf* and *Otx2*, and Pax6 alone is not sufficient to promote their expression. In the COUP-TF double mutants, the differential ability of the dorsal, but not the ventral, precursor cells to maintain RPE identity is interesting. Most likely, it is due to the ventral high-dorsal low COUP-TF expression gradient along the pRPE at E9.5 (Fig. 1). Our in vitro assays demonstrate further that transcription of both *Pax6* and *Otx2* is directly regulated by COUP-TFs (Fig. 6). These results support the notion that the COUP-TF genes are the key intrinsic determinants of the RPE cell fate, limiting the expression of *Pax6*, but maintaining the expression of *Otx2* and *Mitf*, in the progenitor cells that will eventually differentiate with RPE identity (Fig. 7A).

Retinogenesis is regulated at both the dorsal-ventral and nasal-temporal axes. It is known that the differentiation of the RPE proceeds from dorsal to ventral. Retinoid signaling, the Wnt/β-catenin pathway, *AP2*α (*Tcfap2a*) and *Mitf* are involved in restricting the dorsal RPE (Halilagic et al., 2007; Westernskow et al., 2009; West-Mays et al., 1999; Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000), whereas *Gas1* is necessary to maintain ventral RPE identity (Lee et al., 2001). However, up to now, the specification of RPE cells along the nasal-temporal axis has never been addressed. In the COUP-TF double-knockout mouse, we observed that the progenitor cells at the prospective temporal RPE are compromised, but not those cells at the nasal RPE. Our immunohistochemical assays reveal that COUP-TFI expression is high at the temporal presumptive optic vesicle at E9.5 (Fig. 1). Therefore, consistent with the expression profile, COUP-TFs might play an important role in the specification of temporal RPE. Moreover, COUP-TF genes are most highly expressed at the pdOS as well as at the pRPE (Fig. 1). Accompanying the conversion of RPE into NR, the dOS seems to acquire vOS fate in the double-mutant mice (Figs 3, 4). It is clear that the development of both dOS and RPE, which originate from the dorso-distal optic vesicle, is compromised in COUP-TF double-mutant mice, supporting the notion that COUP-TF genes are key factors in dorsalization of the eye.

The expression profiles of COUP-TFs during patterning of the proximo-ventral optic vesicle suggest that they might be involved in the specification of NR and vOS identities. Indeed, the boundary between the NR and vOS shifts more proximally in the absence of COUP-TFs (Fig. 3). It has been demonstrated that *Pax2* and *Pax6* repress each other in the developing eye, and in the *Pax2*-null mouse the NR extends into vOS territory (Schwarz et al., 2000). *Vax1* and *Vax2* genes antagonize *Pax6* to ventralize the optic vesicle (Mui et al., 2005). In the COUP-TF double-mutant mouse, the expression of *Vax1/2* and *Pax2* is reduced. By contrast, the expression of *Pax6* is enhanced (Fig. 3). We hypothesize that COUP-TF genes modulate the transcription network, including the Vax and Pax genes, to specify vOS and NR identities (Fig. 7B). COUP-TF proteins, especially COUP-TFI, may directly limit the expression of *Pax6* in the distal part of the ventral optic vesicle; they might also indirectly inhibit *Pax6* expression through activating Vax genes. Then, distal *Pax6* and proximal *Pax2* antagonize each other in the progenitor cells, which leads to the establishment of a proper boundary between NR and vOS in the wild-type mouse eye. In the absence of COUP-TFs, the inhibition of *Pax6* expression is relieved and Pax6 expression extends proximally, resulting in a shift of the boundary into the prospective vOS territory.

In summary, our studies demonstrate that *COUP-TFI* and *COUP-TFII* are crucial determinants in the eye progenitor cells. They specify the dorso-distal identities in the optic vesicle and generate the proper boundary between NR and vOS through directly or indirectly regulating the expression of a group of key transcription factors, including Pax6, Pax2, Otx2, Mitf and Vax1/2. Our findings reveal novel and distinct cell-intrinsic mechanisms used by the progenitor cells in the early developing eye, and show that COUP-TFs are the crucial regulators in dorsalization of the optic vesicle. The major unanswered question is how the expression of COUP-TF genes is initiated at the time when the eye primordia are induced.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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