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Spatial and temporal regulation of Wnt/β-catenin signaling is essential for development of the retinal pigment epithelium

Naoko Fujimura [a], Makoto M. Taketo [b], Mikiro Mori [c], Vladimir Korinek [a], Zbynek Kozmik [a,*]

[a] Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic
[b] Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, 606-8501, Japan
[c] Department of Ophthalmology, Jichi Medical School, Minami-kawachi, Tochigi, 329-0498, Japan

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ABSTRACT

Wnt/β-catenin signaling is highly active in the dorsal retinal pigment epithelium (RPE) during eye development. To study the role of Wnt/β-catenin signaling in the RPE development we used a conditional Cre/loxP system in mice to inactivate or ectopically activate Wnt/β-catenin signaling in the RPE. Inactivation of Wnt/β-catenin signaling results in transdifferentiation of RPE to neural retina (NR) as documented by downregulation of RPE-specific markers *Mitf* and *Otx2* and ectopic expression of NR-specific markers *Chx10* and *Rx*, respectively. In contrast, ectopic activation of Wnt/β-catenin signaling results in the disruption of the RPE patterning, indicating that precise spatial and temporal regulation of Wnt/β-catenin signaling is required for normal RPE development. Using chromatin immunoprecipitation (ChIP) and reporter gene assays we provide evidence that *Otx2* and RPE-specific isoform of *Mitf*, *Mitf-H*, are direct transcriptional targets of Wnt/β-catenin signaling. Combined, our data suggest that Wnt/β-catenin signaling plays an essential role in development of RPE by maintaining or inducing expression of *Mitf* and *Otx2*.

Introduction

The first indication of the vertebrate eye development is evagination of the diencephalon towards the surface ectoderm to form the optic vesicle (Chow and Lang, 2001). Lens-competent head ectoderm responds to signals from the optic vesicle, which induces columnar thickening of the surface epithelium to form the lens placode (Grainger et al., 1997). As the optic vesicle comes into contact with the surface ectoderm, it becomes partitioned into three territories: a distal territory, a proximal territory and a dorsal territory, which give rise to the neural retina (NR), the optic stalk and the retinal pigment epithelium (RPE), respectively. Coordinated invagination of the optic vesicle and the lens placode leads to formation of the double-layered optic cup and the lens vesicle. The inner layer and the outer layer of the optic cup give rise to the NR and RPE, respectively. The process of the invagination generates the optic fissure that runs from the ventral-most region of the NR and along the ventral aspect of the optic stalk. The optic fissure gradually becomes closed and the NR is completely surrounded by the RPE. The transition part between the NR and the RPE called the ciliary margin gives rise to the ciliary body and the iris (Bharti et al., 2006; Chow and Lang, 2001; Martinez-Morales et al., 2004).

Although little is known about the RPE development, several transcription factors have been shown to be involved in the process. *Mitf*, *Otx1*, and *Otx2* are essential for the RPE development, while *Chx10* prevents RPE development in the presumptive NR (Horsford et al., 2005; Martinez-Morales et al., 2004; Rowan et al., 2004). *Mitf* encodes a member of the basic helix–loop–helix leucine zipper family of transcription factors (Hodgkinson et al., 1993) and consists of nine isoforms with distinct amino-termini (Hallsson et al., 2007; Steingrimsson et al., 2004). Each isoform shows a unique expression pattern (Goding, 2000; Steingrimsson et al., 2004). For example, *Mitf-A*, *-J*, *-H* and *-D* are all expressed in the RPE, whereas expression of *Mitf-M* is restricted to the neural crest-derived melanocytes (Amae et al., 1998; Bharti et al., 2008; Hershey and Fisher, 2005; Takeda et al., 2002). Mitf regulates pigment cell-specific transcription of genes encoding melanogenic enzymes such as tyrosinase (*Tyr*), and tyrosinase-related protein 1 and 2 (Aksan and Goding, 1998; Hemesath et al., 1994; Yasumoto et al., 1994, 1997). During the vertebrate eye development *Mitf* is expressed in the entire optic vesicle, whereas later the expression is restricted to the RPE, the ciliary body and the iris (Baumer et al., 2003; Horsford et al., 2005; Nguyen and Arnheiter, 2000). The RPE of *Mitf* null mutants loses the expression of RPE-specific genes and transdifferentiates into the NR (Nguyen and Arnheiter, 2000). *Otx1* and *Otx2* encode members of the bicoid subfamily of homeodomain-containing transcription factors (Simeone et al., 1992). Similarly as *Mitf*, *Otx1* and *Otx2* are expressed in the entire optic vesicle and later expression is restricted to the presumptive RPE (Baumer et al., 2003; Martinez-Morales et al., 2001). Otx2 cooperates with Mitf to regulate expression of melanogenic enzymes (Martinez-Morales et al., 2003). *Otx1* and *2* double-deficient mice show severe

* Corresponding author. Fax: +420 241063125.
E-mail address: kozmik@img.cas.cz (Z. Kozmik).

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ocular malformation in the lens, the NR, the optic stalk and the RPE. Notably, the presumptive RPE loses expression of *Mitf* and gives rise to the NR-like tissue (Martinez-Morales et al., 2001). The *Chx10* gene encodes a member of paired-type homeodomain-containing transcription factor (Burmeister et al., 1996). *Chx10* is expressed in the distal optic vesicle and at later stages restricted to the NR progenitor cells (Baumer et al., 2003; Burmeister et al., 1996; Chen and Cepko, 2000; Rowan et al., 2004). Chx10 represses expression of photoreceptor genes such as rod arrestin (Dorval et al., 2006). *Chx10* null mutant mice show expansion of the peripheral RPE into NR and ectopic expression of *Mitf* in the entire NR (Horsford et al., 2005). Furthermore, misexpression of *Chx10* in the developing RPE in chick results in significant downregulation of *Mitf* and tyrosinase-related protein 2, although transdifferentiation of the RPE does not occur (Rowan et al., 2004).

It has been proposed that in addition to these transcription factors, secreted molecules from the extraocular mesenchyme are required for RPE development to inhibit the NR development in the presumptive RPE (Fuhrmann et al., 2000). In the absence of the extraocular mesenchyme, explanted chick optic vesicles show downregulation of RPE-specific genes and ectopic expression of NR-specific genes. Activin A, a member of the TGFβ superfamily, can substitute for the extraocular mesenchyme (Fuhrmann et al., 2000). Bone morphogenetic proteins (BMP), other members of the TGFβ superfamily, are essential for RPE development in chick embryos. The presumptive NR develops into the RPE by overexpression of BMPs, while inhibition of BMP results in abrogation of RPE development and in the induction of expression of the NR-specific genes (Muller et al., 2007).

In multi-cellular organisms the Wnt signaling pathway represents one of the key mechanisms controlling cell-fate decisions during embryonic development and also in adult tissues (Klaus and Birchmeier, 2008). The signaling is initiated by the interaction of extracellular Wnt ligands with the transmembrane Frizzled/LRP receptor complex. The activation of the receptor results in the stabilization of β-catenin, a key mediator of canonical Wnt signaling. The protein accumulates both in the cytoplasm and the nucleus, with the nuclear form able to act as a coactivator of Tcf/Lef transcription factors (Logan and Nusse, 2004). During eye development the Wnt pathway has been implicated in the formation of RPE. Several components of the canonical, i.e. β-catenin-dependent, Wnt signaling pathway, including *Wnt2b*, are expressed in the presumptive avian or mammalian RPE (Fuhrmann et al., 2000; Cho and Cepko, 2006; Jasoni et al., 1999; Jin et al., 2002; Liu et al., 2003; Zakin et al., 1998). In the mouse, Wnt/β-catenin signaling is highly active in the developing RPE at the stage of the optic cup formation and its activity is subsequently restricted to the ciliary margin (Kreslova et al., 2007; Liu et al., 2006, 2007; Maretto et al., 2003; Miller et al., 2006; Zhou et al., 2008). Moreover, recent studies have shown that Wnt/β-catenin-mediated signals are essential for the ciliary margin development (Cho and Cepko, 2006; Kubo et al., 2003; Liu et al., 2007). Aberrant activation of the Wnt pathway in the peripheral NR leads to the expansion of the ciliary margin at the expense of the NR; on the contrary, conditional inactivation of the signaling attenuates the ciliary margin development (Liu et al., 2007). Interestingly, β-catenin is a polypeptide with dual roles and besides Wnt signaling also participates in cell adhesion (Grigoryan et al., 2008). β-catenin directly binds to the cytoplasmic tail of cadherins and associates with α-catenin, which links cadherin/catenin complexes to the actin cytoskeleton (Perez-Moreno et al., 2003). Although conditional knockout of *β-catenin* results in a failure of cell adhesion and morphogenesis in several tissues such as the presumptive lens and the forebrain (Junghans et al., 2005; Smith et al., 2005), the embryonic lethality upon complete inactivation of the *β-catenin* gene is more likely related to the deficiency in the β-catenin signaling function (Huelsken et al., 2000). *β-catenin*-null mutant embryos preserve intact adherens junctions since γ-catenin, a β-catenin paralog, possibly substitutes β-catenin in cell adhesion complexes in early stages of embryonic development (Huelsken et al., 2000). A similar phenomenon has been observed in the epidermis and cardiomyocytes (Posthaus et al., 2002; Zhou et al., 2007).

In this study we have investigated the role of the canonical Wnt signaling pathway in the RPE development by inactivating or activating its key component, β-catenin. Deletion of the *β-catenin* gene results in transdifferentiation of the RPE to NR. On the other hand, the ectopic activation of Wnt/β-catenin signaling inhibits formation of the RPE. We suggest that spatial and temporal regulation of Wnt/β-catenin signaling is essential for the RPE development.

Materials and methods

Mice

The following genetically modified mice were used in this study: *Trp1-Cre* (Mori et al., 2002), β-catenin conditional loss-of-function $Catnb^{lox(ex2-6)}$ (Brault et al., 2001), β-catenin gain-of-function $Catnb^{lox(ex3)}$ (Harada et al., 1999), Wnt/β-catenin reporter BAT-gal (Maretto et al., 2003) and Cre recombinase reporter ROSA26R (Soriano, 1999) (Jackson Laboratory, stock #0033069). The age of mouse embryos was determined by timed pregnancy. The noon of the day on which the vaginal plug was observed was counted as embryonic day 0.5 (E0.5).

Immunohistochemistry

Mouse embryos were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded and frozen in OCT (Tissue Tek; Sakura Finetek). Frontal frozen sections were prepared at 10 μm thickness. The cryosections were re-fixed by 4% paraformaldehyde for 10 min, washed by PBS and permeabilized with 0.1% Tween 20/PBS (PBT) for 15 min. The sections were blocked by 10% BSA/PBT and incubated with primary antibodies in 1% BSA/PBT overnight. The following day the sections were washed with PBS, incubated with secondary antibodies in 1% BSA/PBT for 30 min, washed with PBS, stained with DAPI for 5 min and mounted in glycerol. Immunohistochemistry was repeated at least three times for each antibody. The following antibodies were used in this study: anti-β-catenin (1:1000, Sigma, C2206), anti-Mitf (1:250, a gift from Dr. Arnheiter), anti-Otx2 (1:1000, a gift from Dr. Vaccarino), anti-Chx10 (1:200, Chemicon), anti-Pax6 (1:500, Covance), anti-Pax2 (1:300, Zymed), anti-Nr2f1 (1:250, Perseus Proteomics), anti-Nr2f2 (1:250, Perseus Proteomics), anti-Sox2 (1:100, Santa Cruz Biotechnology), anti-N-cadherin (1:500, BD Transduction Laboratories), anti-α-catenin (1/1000, Sigma), anti-γ-catenin (1:100, BD Transduction Laboratories), anti-P-cadherin (1/50, R&D Systems), anti-Lef1 (1/1000, Cell Signaling), anti-rabbit Alexa 594, anti-rabbit Alexa 488, anti-sheep Alexa 594, anti-mouse Alexa 594 and anti-goat Alexa 594 (all 1:500, Molecular Probes). Phalloidin-Alexa 488 conjugate (1:150, Molecular Probes) was used for visualization of actin filaments. The sections were analyzed by either Leica TCS Sp5 confocal microscope or Nikon DIAPHOT 300 inverted fluorescence microscope.

The β-galactosidase assay

The β-galactosidase assay was carried out as described previously and repeated at least three times for each genotype (Kreslova et al., 2007). Briefly, mouse embryos were fixed in 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), 2 mM $MgCl_2$ and 5 mM EGTA on ice, washed by rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris–HCl pH 7.3, 0.01% sodium deoxycholate and 0.02% Nonidet P20) and incubated in staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal). The stained embryos were re-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded and frozen in OCT. Frontal frozen sections were prepared at 12 μm thickness.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed according to standard protocols and repeated at least three times for each probe. Plasmid carrying mouse Rx (Open Biosystems) or Vax2 (a gift from Dr. Lemke) cDNA was linearized with an appropriate restriction enzyme. The antisense riboprobe was synthesized using the DIG RNA labeling kit (Roche). Mouse embryos were fixed in 4% paraformaldehyde, bleached by methanol/30% H_2O_2 (4:1) for 20 min and treated with 20 μg/ml of Proteinase K/PBT for 15 min. The reaction was stopped with 2 mg/ml glycine/PBT. The embryos were washed with PBT, re-fixed with fix solution (0.2% glutaraldehyde/4% paraformaldehyde in PBS) for 20 min and washed with PBT. The embryos were incubated with pre-warmed prehybridization solution (50% formamide, 5× SSC pH4.5, 5 mM EDTA, 0.1% Tween 20 and 50 μl/ml Heparin) for 60 min at 70 °C, then incubated with hybridization solution (prehybridization solution supplemented with 1 μg of DIG-labeled riboprobe, 25 μg/ml tRNA and 25 μg/ml herring sperm DNA) overnight at 70 °C. The following day the embryos were washed with solution I (50% formamide, 4× SSC pH 4.5 and 1% SDS) at 70 °C, solution I/II (solution II: 0.5 M NaCl, 10 mM Tris–HCl pH 7.5 and 0.1% Tween20) at 70 °C, and solution II at RT. After the washing steps, embryos were treated with 100 μg/ml RNase I at 37 °C, washed with pre-warmed solution III (50% formamide, 2× SSC pH4.5) at 65 °C, then with TBST (5 mM Levamisole, 130 mM NaCl, 2.7 mM KCl, 25 mM Tris–HCl and 0.1% Tween20) at RT. The embryos were blocked with 10% sheep serum/TBST for 2 h and incubated with anti-DIG alkaline phosphatase (1:1000, Roche) in 10% sheep serum/TBST overnight at 4 °C. Then, they were washed with TBST at RT, equilibrated with NTMT solution (100 mM NaCl, 100 mM Tris–HCl pH9.5, 50 mM $MgCl_2$ and 0.1% Tween20) and stained with NBT/BCIP (Roche) in NTMT. The stained embryos were re-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded and frozen in OCT. Frontal frozen sections were prepared at 12 μm thickness.

Chromatin immunoprecipitation (ChIP)

The chromatin immunoprecipitation assay was performed as described previously (Fujimura et al., 2007). Briefly, embryos at E10.5 were homogenized and cross-linked in 1% formaldehyde in PBS for 10 min at room temperature. The cross-linking reaction was stopped by adding glycine. Cross-linked cells were washed with PBS containing proteinase inhibitors and sonicated in 2× SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0). Approximately 30 μg of sonicated chromatin were diluted ten times with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl) containing protein inhibitors, then precleared with 50 μl of protein A agarose slurry (Upstate Biotech) for 3 h. The precleared chromatin was incubated either with 20 μg of anti-β-catenin rabbit serum, anti-Tcf/Lef rabbit serum (Valenta et al., 2006) or with a control IgG overnight at 4 °C (Valenta et al., 2006). Protein A agarose slurry (20 μl) was added and incubated for 3 h at 4 °C. The samples were washed three times in low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, 150 mM NaCl), three times in high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, 500 mM NaCl), four times in LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0) and twice in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). After washing, the immunocomplex was eluted twice using 100 μl of elution buffer (1% SDS, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0) for 20 min at room temperature. Immunoprecipitated DNA was de-cross-linked overnight at 65 °C in the presence of proteinase K (Roche) and 250 mM NaCl. The samples were purified using MinElute reaction cleanup kit (Qiagen) with a final elution volume of 50 μl. One μl of the purified sample was used for PCR. The ChIP assay was repeated three times. The primers used were as follows (written in the 5′ to 3′ direction): Mitf-H, GGCTAAACTCCCTGGCTGAC and CCTTGGCGAAGAGTGAAAGT; Mitf-D, TTGCGCTATCCCCTTTAGAA and GCTTCATGCTTCAACCACAA; Nr2f2-pro, AGGCATGAAATAGGGGAACC and TCCTCTCACCCTGGATCAAA; Nr2f2-UTR, AGTGTGCAGGCTTTTCCAAC and CTTTAGTTTGGCGGGTGAAA; Nr2f2-ECR1, TTGGATTTTAGGCAGCAAGG and AGAGAAGGGGCTTCCATTGT; Nr2f2-ECR2, GCTAAGTTGCAGCAGTCGTG and GGGGAGAAACGGAGAGAAAG; Nr2f2-ECR3, GAGGGGAAAGAAACAGCTCA and TACAGTGTGCATGGGGATTG; Otx2-FM2, CTCTCACTACCCCCACGAGA and TCACCGTTCGGAGATAATCC.

Cell culture, transient transfection, and luciferase reporter assay

ARPE19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 Ham mixture [supplemented with 10% fetal bovine serum (PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma)]. Transient transfection of ARPE19 cells was performed in triplicates using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. Cells were plated in 24-well plates 24 h prior to transfection. Typically, the total amount of DNA transfected per well was 300 ng and was adjusted with pUC 18 when necessary. A *Renilla* luciferase expression plasmid was cotransfected to normalize the transfection efficiency. Two days after transfection, the cells were lysed in 100 μl of passive lysis buffer and luciferase reporter assays were performed using Dual-Luciferase Reporter assay system (Promega).

The mouse *Mitf-H* promoter, *Mitf-D* promoter, *Otx2* FM2 enhancer, *Nr2f2* promoter *Nr2f2* 5′ UTR, and *Nr2f2* ECR1 were amplified by PCR using C57BL/6J mouse genomic DNA as a template. Promoter and enhancer fragments were cloned into pGL3 basic or pGL4.26 vector, respectively. Each luciferase reporter construct (200 ng) was cotransfected with the stabilized form of β-catenin (β-cat) and Lef1 (50 ng each) or an empty vector (100 ng) into ARPE19 cells.

Results

Conditional inactivation of Wnt/β-catenin signaling in the presumptive retinal pigment epithelium

Mouse strains with the integrated Wnt/β-catenin-responsive reporters represent a valuable tool to monitor the activity of canonical Wnt signaling during embryogenesis and in adult tissues (DasGupta and Fuchs, 1999; Maretto et al., 2003; Mohamed et al., 2004). Using such "reporter mice" we and others have previously shown that Wnt signaling is active during eye development (Kreslova et al., 2007; Liu et al., 2006; Maretto et al., 2003; Smith et al., 2005). In this study, we assessed the role of the canonical Wnt pathway in development of the RPE using transgenic mice containing the *lacZ* gene regulated by a synthetic enhancer element composed of seven optimal Tcf/Lef binding DNA motifs (BAT-gal) (Maretto et al., 2003). As indicated by the lacZ expression, Wnt/β-catenin signaling is highly active in the presumptive RPE at E10.5, and subsequently, the lacZ activity is restricted to the distal RPE (Figs. 1A, C) (Kreslova et al., 2007; Liu et al., 2006). Importantly, *Axin2* and *Lef1*, direct target genes of Wnt/β-catenin signaling, and *Wnt2b* are expressed in the distal RPE (Supplementary Figs. 1A, B) (Burns et al., 2008; Filali et al., 2002; Hovanes et al., 2001; Jho et al., 2002; Liu et al., 2006; Yan et al., 2001; Zakin et al., 1998). Based on these data, we hypothesized that Wnt/β-catenin signaling plays a role in the RPE development. To test this hypothesis, we manipulated the Wnt pathway in the RPE by conditional inactivation or activation of β-catenin using the Cre/loxP system in mice. We utilized the *Trp1-Cre* transgenic mice as a "deleter" (Mori et al., 2002). In these mice Cre recombinase is active in the RPE from E10.0 and its expression is also detected in the optic stalk and the ciliary margin at a later stage (Mori et al., 2002) (Supplementary Figs. 2A, B). To achieve conditional inactivation of Wnt/β-catenin signaling, *Trp1-Cre* was combined with a conditional allele of β-

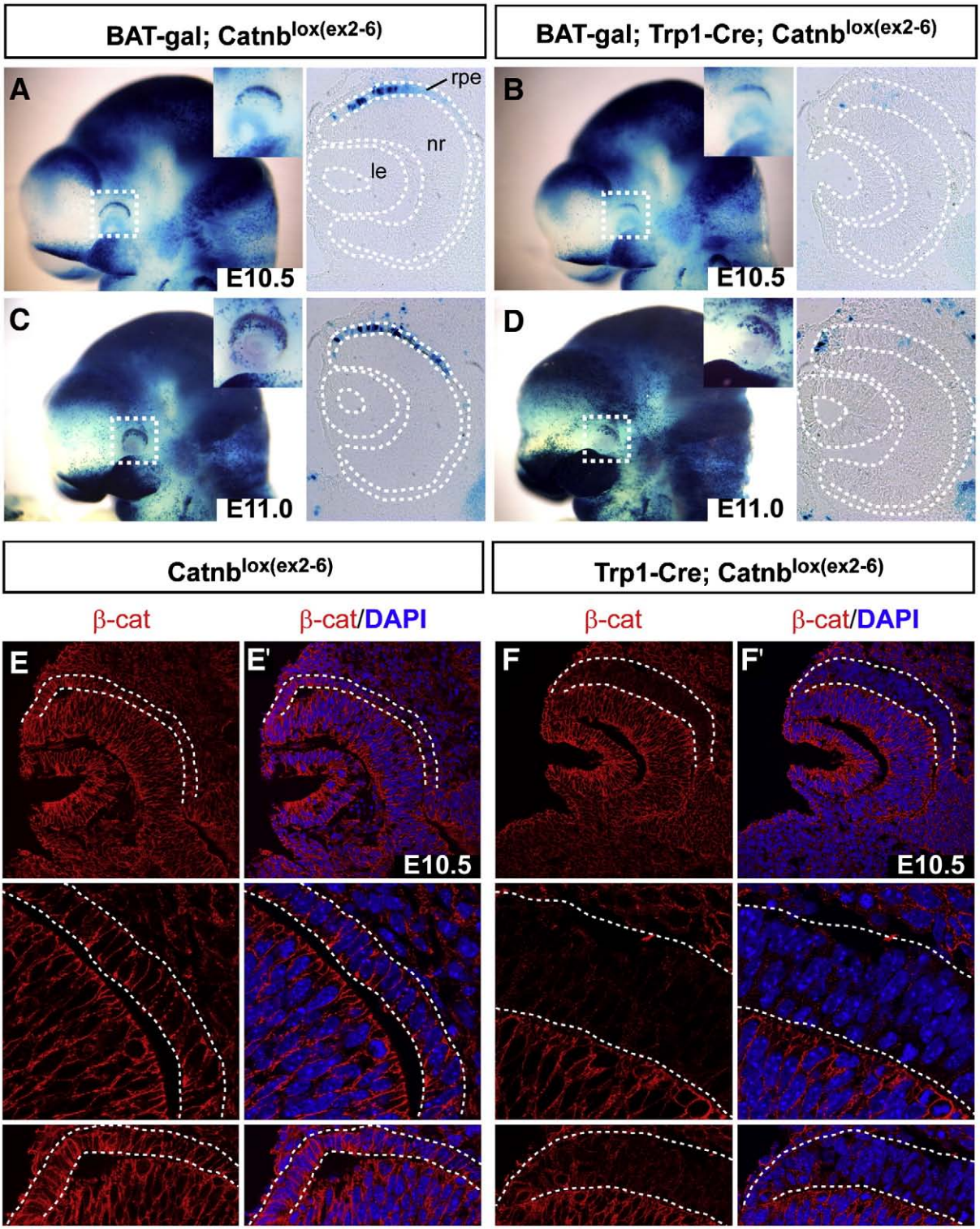

Fig. 1. Conditional inactivation of Wnt/β-catenin signaling in the RPE. The activity of Wnt/β-catenin signaling was assessed using Wnt/β-catenin signaling reporter line BAT-gal. Whole-mount or frontal cryosections of wild-type (A, C) and loss-of-function mutant (B, D) were stained with X-gal at the indicated embryonic stage. Confocal images of frontal cryosections of wild-type (E, E′) and loss-of-function mutant (F, F′) showing expression of β-catenin (red) and DAPI nuclear labeling (blue) at E10.5. Small areas of RPE in panels (E, E′) and (F, F′) are shown magnified below the corresponding panel. The RPE is indicated by a dashed line. Abbreviations used in this and subsequent figures are as follows: rpe, retinal pigment epithelium (RPE); le, lens; nr, neural retina (NR).

catenin Catnb$^{lox(ex2-6)}$ in which exons 2–6 are flanked by loxP sites (Brault et al., 2001). Since β-catenin acts as a coactivator of Tcf/Lef transcription factors (Logan and Nusse, 2004), Cre-mediated conditional deletion of *β-catenin* results in inactivation of Wnt/β-catenin signaling. The genotype of each embryo used in this study is shown in the corresponding figure panel; however, for the sake of simplicity, we refer only to *Catnb*$^{lox(ex2-6)/lox(ex2-6)}$ as wild-type and *Trp1-Cre; Catnb*$^{lox(ex2-6)/lox(ex2-6)}$ as loss-of-function mutants in the text.

We assessed the β-catenin levels in the RPE of loss-of-function mutant using immunohistochemical staining and confirmed that the amounts of β-catenin protein in the RPE was reduced at E10.5 (Figs. 1E, E′, F, F′). We further investigated the status of Wnt/β-catenin

signaling in the presumptive RPE of loss-of-function mutants hemizygous for the BAT-gal reporter. At E10.5 and E11.0, the reporter activity was detected in the presumptive RPE of wild-type (Figs. 1A, C); on the contrary, in the loss-of-function mutants the lacZ reporter activity was significantly decreased (Figs. 1B, D). In addition, *Lef1* was downregulated in the presumptive RPE upon loss of the *β-catenin* gene (Supplementary Fig. 1C). These results imply that Wnt/β-catenin signaling is active in the dorsal RPE and becomes inactivated in the loss-of-function mutants.

Wnt/β-catenin signaling is required for the RPE development

The phenotypic consequences of the inactivation of Wnt/β-catenin signaling in the RPE were further investigated using histochemistry. Abnormal development of the RPE was observed at E10.5. The prospective RPE of the wild-type mice was thin and the cells formed a single layer (Fig. 2A); on the contrary, the analogous tissue of loss-of-function mutants was thicker (Fig. 2B). Moreover, the RPE was hypopigmented and the ventral NR was shortened (Figs. 2E–J). Furthermore, the thickening of the ventral RPE was also observed during later stages of the embryonic development (Figs. 2E–H).

It is well established that β-catenin functions in both cell adhesion and Wnt/β-catenin signaling. Therefore, we tested the possibility that the malformations of the RPE are connected with the reduced or absent cell-to-cell contacts. We performed immunohistochemical analysis of the adhesion-related proteins N-cadherin, α-catenin and γ-catenin. In addition, phalloidin staining was used to detect possible abnormalities of actin filaments. At E10.5, N-cadherin is expressed in all retinal cells (Xu et al., 2002). γ-catenin is known to substitute for β-catenin in adherens junctions under conditions when β-catenin is absent or modified (Huelsken et al., 2000; Posthaus et al., 2002; Zhou et al., 2007). Confocal microscopy images showed identical staining pattern of F-actin, N-cadherin, γ-catenin and α-catenin in the wild-type and β-catenin-deficient RPE (Figs. 3A–L, Supplementary Figs. 3A, B), suggesting that cellular adhesion is not affected by the loss of β-catenin. Altogether, our data suggest that cell adhesion is not grossly affected in the absence of β-catenin at E10.5. Thus, the defect in the RPE development is likely to be caused by the absence of the β-catenin signaling function.

To characterize the phenotype caused by inactivation of Wnt/β-catenin signaling we first examined the expression of *Mitf* and *Otx2*, i.e. the genes that are essential for the RPE development (Bharti et al., 2006; Goding, 2000; Hodgkinson et al., 1993; Martinez-Morales et al., 2004; Martinez-Morales et al., 2001). *Mitf* and *Otx2* are initially expressed throughout the optic vesicle; subsequently their expression is restricted to the dorsal optic vesicle, which gives rise to the RPE (Martinez-Morales et al., 2001; Nguyen and Arnheiter, 2000). At E10.5, both Mitf and Otx2 were detected in the wild-type RPE (Figs. 4A, C).

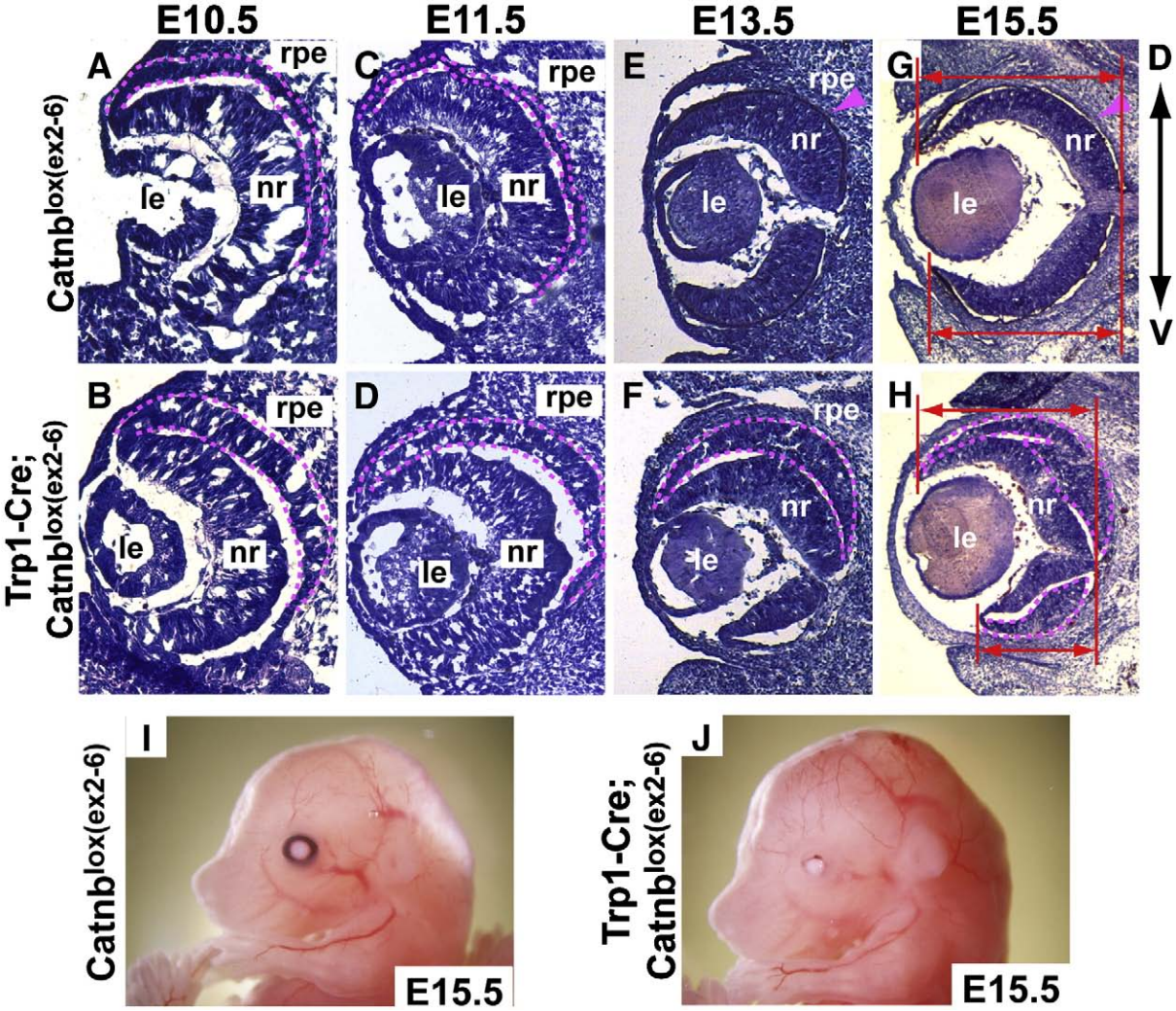

Fig. 2. Phenotypic consequences in the loss-of-function β-catenin mutant. Frontal cryosections of wild-type (A, C, E, and G) and loss-of-function mutant (B, D, F, and H) were stained by hematoxylin and eosin at the indicated embryonic stage. Red lines indicate the size of the ventral and dorsal NR, respectively. The presumptive RPE is indicated by a dashed line or arrowheads (in E, G). The embryo heads show the loss of pigmentation in the loss-of-function mutant (I, J).

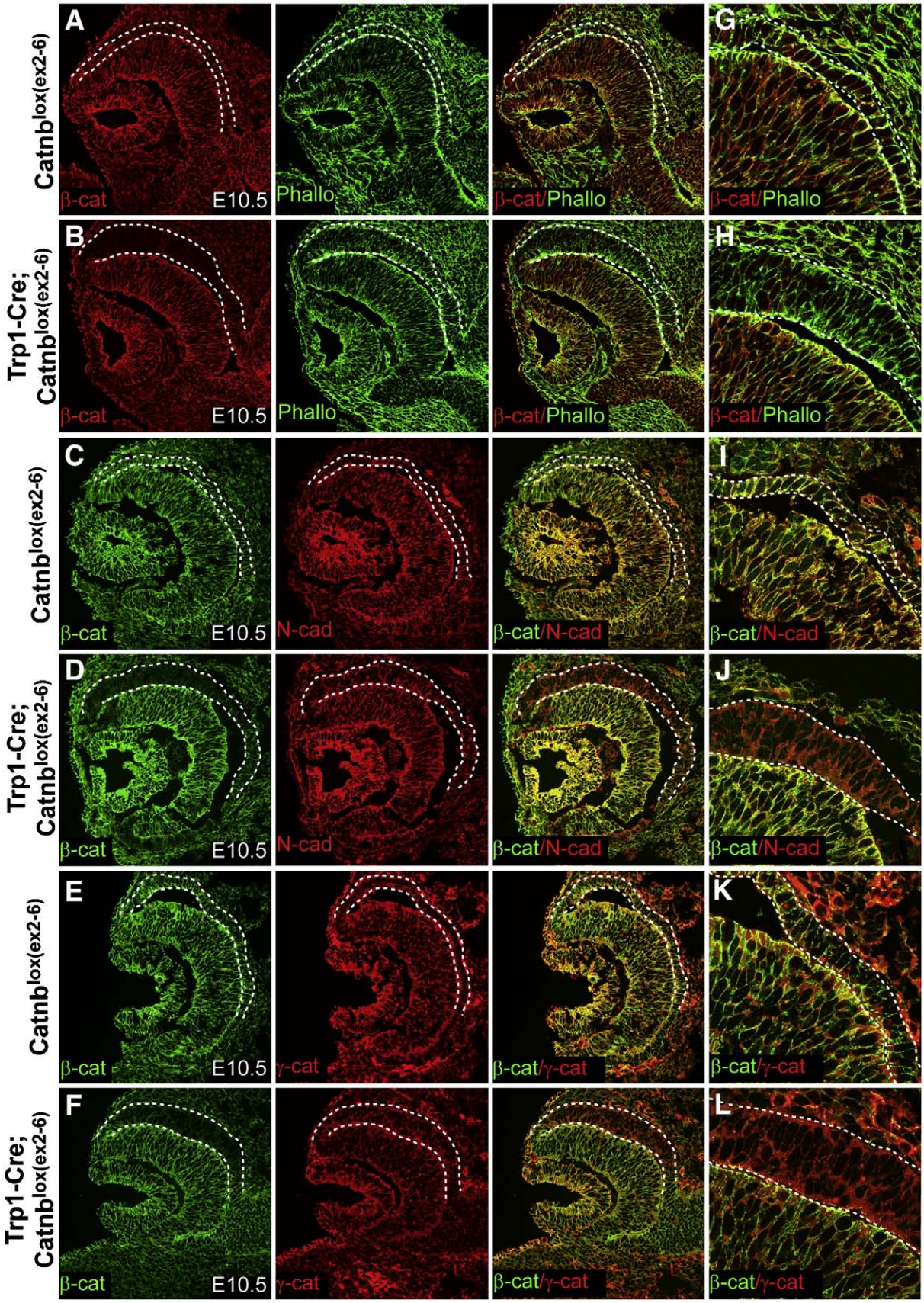

Fig. 3. Conditional inactivation of β-catenin in the RPE does not affect cell adhesion. Frontal cryosections of wild-type (A, C, and E) and loss-of-function mutant (B, D, and F) were immunostained with β-catenin and phalloidin (A, B), β-catenin and N-cadherin (C, D), or β-catenin and γ-catenin (E, F) antibodies. Magnified views of RPE in panels (A–F) are shown next to the corresponding panel (G–L). The images were obtained by confocal microscope analysis. The RPE is indicated by a dashed line.

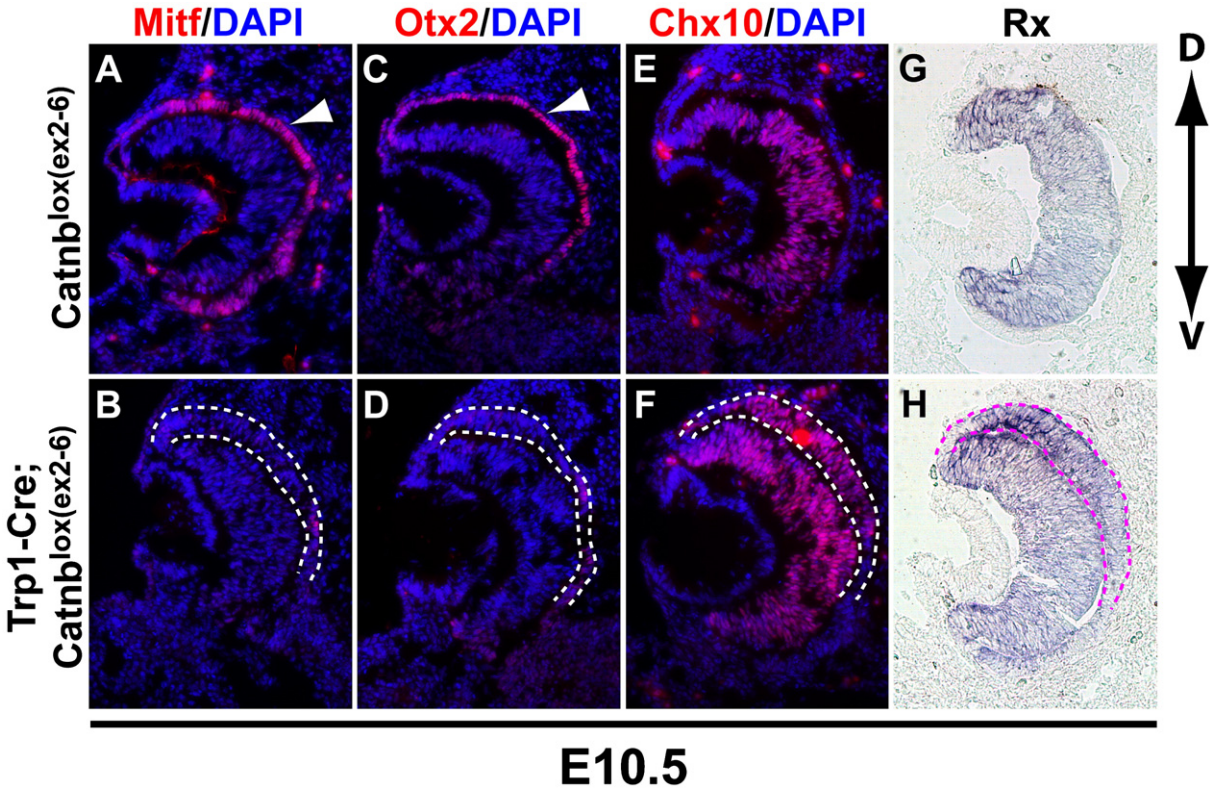

Fig. 4. Conditional inactivation of Wnt/β-catenin signaling results in transdifferentiation of RPE to NR. Frontal cryosections of wild-type (A, C, E and G) and loss-of-function mutant (B, D, F and H) at E10.5 were immunostained with Mitf (A, B), Otx2 (C, D) and Chx10 (E, F) antibodies or hybridized with an antisense probe against *Rx* (G, H). The RPE is indicated by a dashed line or an arrowhead. Note that the RPE-specific markers Mitf and Otx2 are significantly downregulated and the NR-specific markers Chx10 and *Rx* are ectopically expressed in the thickened presumptive RPE of the loss-of-function mutant.

Interestingly, expression of Mitf and Otx2 was significantly downregulated in the thickened RPE of the loss-of-function mutant (Figs. 4B, D). Likewise, the expression of RPE-specific P-cadherin (Xu et al., 2002) was lost in the thickened RPE of the loss-of-function mutant (Supplementary Figs. 3C, D). The absence of *Mitf* results in transdifferentiation of the RPE into the NR (Nguyen and Arnheiter, 2000). Thus, we investigated whether the RPE in the loss-of-function animals expresses *Chx10* and *Rx* that are essential for the NR development (Baumer et al., 2003; Burmeister et al., 1996; Mathers et al., 1997). As shown in Figs. 4E–H, in contrast to the wild-type mice the expression of Chx10 and *Rx* in the loss-of function mutants was not limited to the NR but also extended to the presumptive RPE. These results indicate that Wnt/β-catenin signaling controls the cell-fate decision in the developing RPE and in its absence the tissue transdifferentiates into the NR.

Ectopic activation of Wnt/ β-catenin signaling in the RPE

As evidenced by the activity of the BAT-gal reporter and the expression pattern of the *Axin2* and *Lef1* genes the canonical Wnt pathway is active in the dorsal part of the presumptive RPE at E10.5 (Fig. 1, Supplementary Fig. 1). Subsequently, its activity is restricted to the peripheral RPE (Kreslova et al., 2007). Based on these data, we hypothesized that the restricted pattern of Wnt/β-catenin signaling might be required and that ectopic activation of the signaling in the entire RPE could impair the RPE development. To achieve the ectopic activation of Wnt/β-catenin signaling in the entire RPE, *Trp1-Cre* mice were crossed with *Catnb*$^{lox(ex3)}$ mice in which exon 3 of the *β-catenin* gene is flanked by loxP sites (Harada et al., 1999). Exon 3 encodes Ser/Thr residues that are phosphorylated in the absence of Wnt signaling and the entire protein is subsequently degraded by proteasome. Cre-mediated recombination results in the production of a stabilized form of β-catenin protein. This protein variant accumulates in the cells and constitutively activates Wnt signaling. The genotype of each embryo used in the analysis is shown in the corresponding figure panel; however, for the sake of simplicity, we refer only to *Catnb*$^{lox(ex3)/+}$ as wild-type and *Trp1-Cre; Catnb*$^{lox(ex3)/+}$ as gain-of-function mutant in the text.

In order to assess the level of β-catenin in the RPE of gain-of-function mutants, we performed immunohistochemistry using β-catenin antibody recognizing the C-terminus of the protein. In contrast to the wild-type mice high levels of β-catenin were detected in the entire RPE at E11.5 and in the proximal part of the disorganized retina at E13.5 (Figs. 5E–H). Of note, nuclear β-catenin was detected at both stages (Figs. 5K, L). The activity of the BAT-gal reporter was clearly detected in the cells with increased β-catenin levels. Moreover, the area in the RPE that showed the reporter activity expanded ventrally in the gain-of-function mutant embryos at E10.5 and E11.5 (Figs. 5A–D).

Ectopic activation of Wnt/β-catenin signaling disrupts development of the RPE

At E10.5 the entire RPE of the gain-of-function mutant became thicker compared to that of wild-type (Figs. 6A, B). Interestingly, the proximal part of the RPE was significantly thickened while the distal part of the RPE remained thin by E11.5 (Figs. 6C, D). At later stages, the entire retina was disorganized (Figs. 6E–H) and the embryos showed a complete loss of pigmentation (Figs. 6I, J).

To assess the differentiation of RPE and NR, we examined expression of RPE-specific markers Otx2 and Mitf, and NR-specific markers Chx10 and *Rx* (Bharti et al., 2006; Chow and Lang, 2001; Martinez-Morales et al., 2004). At E10.5 the expression of Otx2, Mitf, Chx10 and *Rx* was not changed (data not shown) despite the morpho-

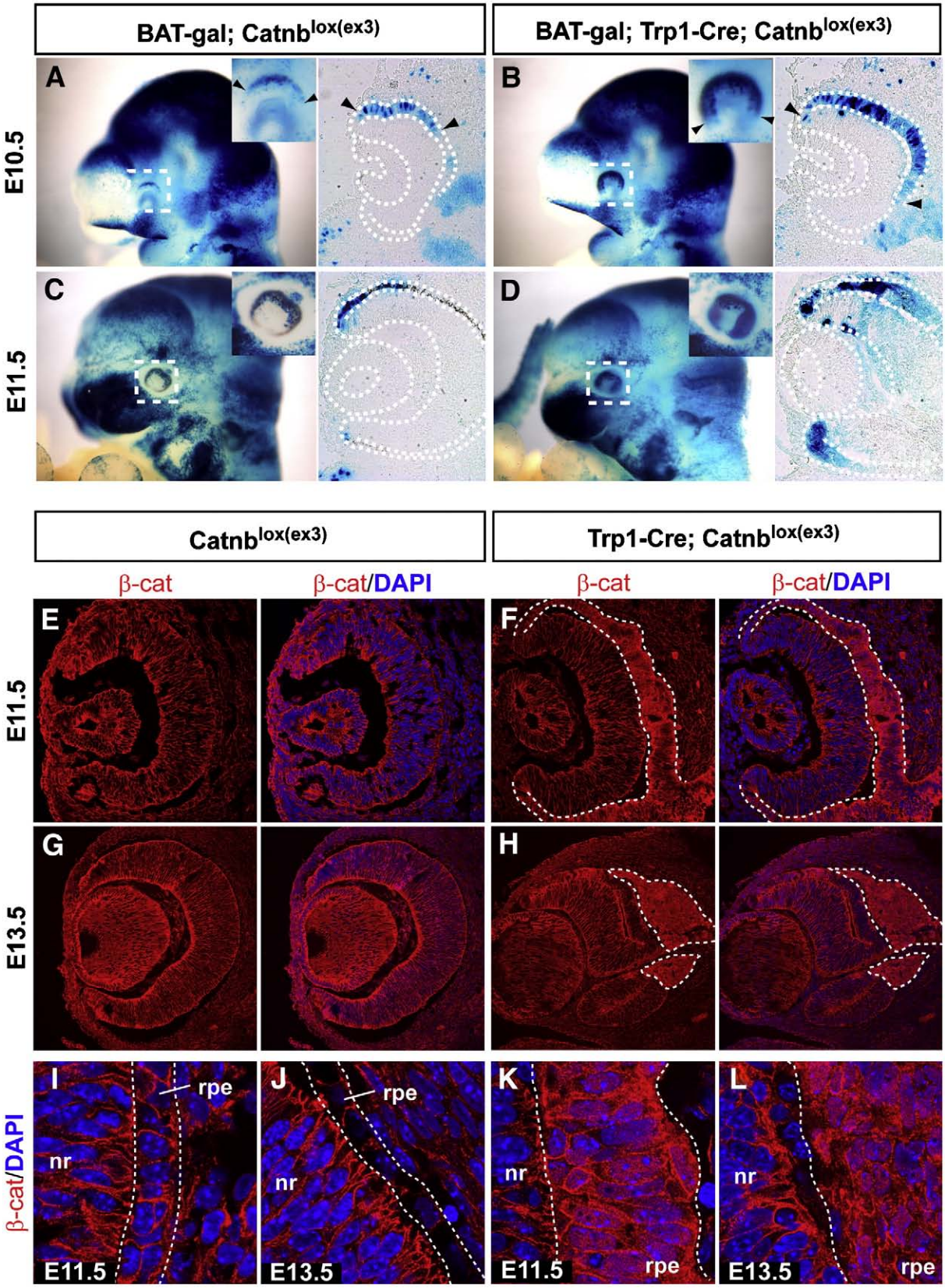

Fig. 5. Ectopic activation of Wnt/β-catenin signaling in the RPE. The activity of Wnt/β-catenin signaling was assessed using Wnt/β-catenin signaling reporter line BAT-gal. Whole-mount or frontal cryosections of wild-type (A, C) and gain-of-function mutant (B, D) were stained with X-gal to assess the activity of Wnt/β-catenin signaling at the indicated embryonic stage. The extent of the RPE area in which Wnt/β-catenin signaling is active is marked by arrowheads. Confocal images of frontal cryosections from wild-type (E, G) and gain-of-function mutant (F, H) showing expression of β-catenin (red) and DAPI nuclear labeling (blue) at E10.5. Small areas of RPE in panels (E, G) and (F, H) are shown magnified in panels (I, J) and (K, L), respectively. The RPE layer of wild-type and the presumptive RPE tissue of the mutant (marked by the increased level of stabilized β-catenin) are indicated by a dashed line.

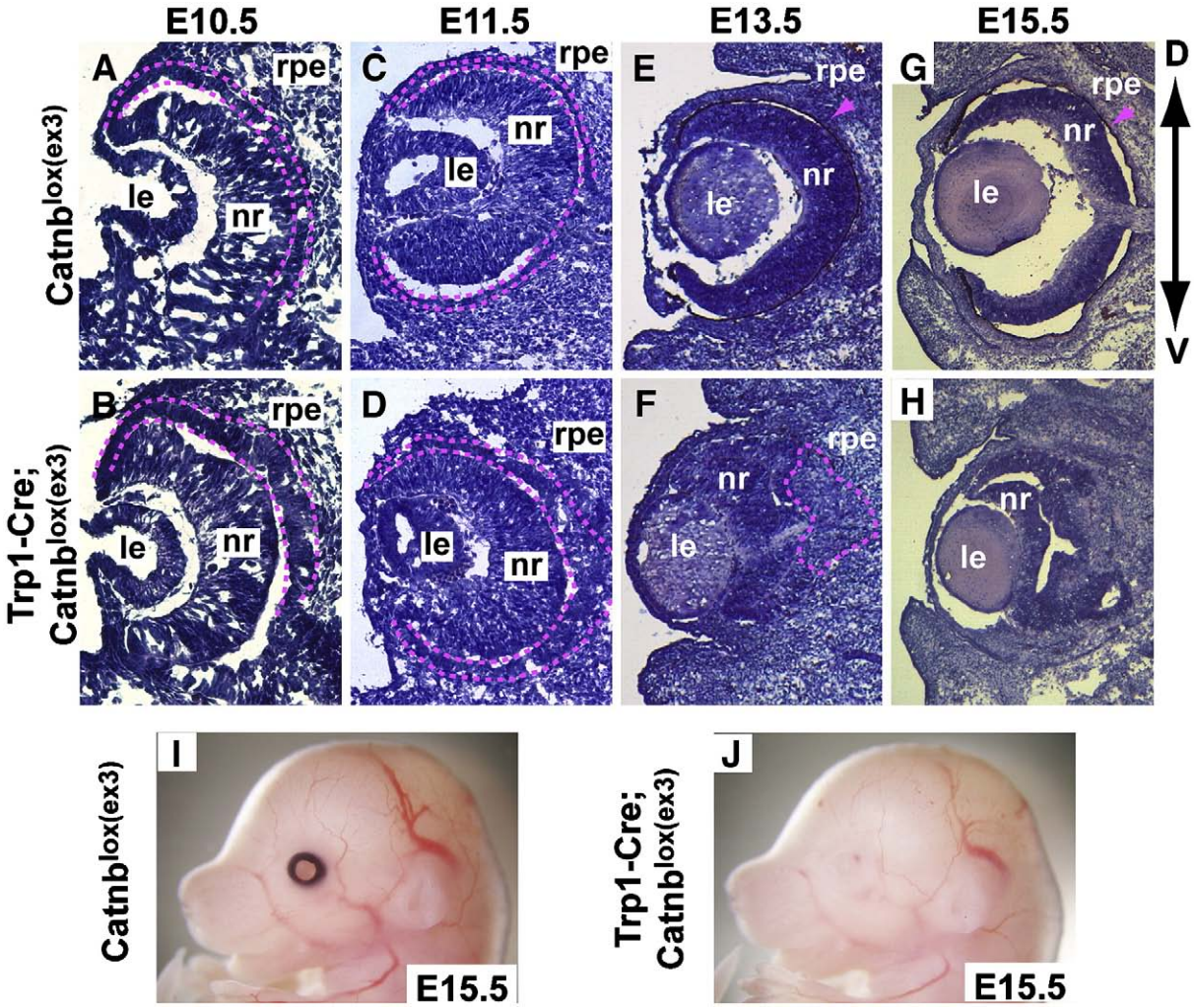

Fig. 6. Phenotypic consequences in the gain-of-function β-catenin mutant. Frontal cryosections of wild-type (A, C, E and G) and gain-of-function mutant (B, D, F and H) were stained by hematoxylin and eosin at the indicated embryonic stage. The RPE is indicated by a dashed line or an arrowhead. The embryo heads show the loss of pigmentation in the gain-of-function mutant (I, J).

logical differences in the RPE of the gain-of-function mice (Figs. 6A, B). By E11.5, the expression of Mitf and Otx2 was downregulated in the presumptive RPE of gain-of-function mutant; expression of both genes appeared to be only detected in the distal part of the RPE and notably absent in the proximal part, in which Wnt/β-catenin signaling is ectopically activated (Figs. 7A–D). On the contrary, there was no significant difference in the expression pattern of the NR markers Chx10 and *Rx* (Figs. 7E–H). This suggests that in contrast to the phenotypic changes in the loss-of-function mice the abnormal thickness of the proximal RPE is not caused by transdifferentiation to NR. Next we examined the expression pattern of Pax6 and Pax2, which are involved in the RPE development (Baumer et al., 2003). Pax6 is initially detected throughout the optic vesicle and its expression is later maintained in both NR and RPE (Ashery-Padan et al., 2000; Baumer et al., 2003; Marquardt et al., 2001). Null mutations of *Pax6* in mice result in failure of the optic vesicle formation (Grindley et al., 1995). Ectopic expression of *Pax6* in the mouse optic stalk under the control of *Pax2* promoter results in expansion of the RPE, suggesting that *Pax6* is a positive regulator of RPE (Baumer et al., 2003). Pax2 is initially detected in the entire optic vesicle; subsequently the expression is restricted to the ventral optic cup and the optic stalk (Baumer et al., 2003; Nornes et al., 1990; Schwarz et al., 2000; Torres et al., 1996). A null mutation of *Pax2* results in extension of the RPE into the optic stalk, suggesting that *Pax2* is essential for formation of the RPE/optic stalk boundary by suppressing the RPE development at the boundary (Torres et al., 1996). As shown in Fig. 7, Pax6 was downregulated in the proximal RPE of gain-of-function mutant at E11.5 (compare panels 7I and 7J). In order to obtain more information about the effects of activated Wnt/β-catenin signaling on the distal/proximal axis of RPE we performed immunohistochemistry with horizontal sections at E11.5. There was no significant difference in the Pax2 expression pattern in wild-type and gain-of-function mutant embryos at E11.5 and E13.5, indicating that the optic stalk development was not affected by ectopic activation of Wnt signaling (Supplementary Figs. 4I, J and Figs. 7S, T). The expression of Pax6, Mitf and Otx2 was downregulated in the entire RPE of the gain-of-function mutants, while the expression of Chx10, a marker of the NR, virtually did not differ from the wild embryos (Supplementary Fig. 4). At E13.5, the expression of Mitf and Otx2 remained undetectable in the gain-of-function mutants (Figs. 7K–N), whereas a production of the neural retina markers Sox2 and Chx10 was clearly localized to the abnormally folded neural retina but not to the presumptive RPE (Figs. 7O–R). Thus, none of the relevant markers was detected in cells expressing stabilized β-catenin. Taken together, our results indicate that the spatial and temporal activation or restriction of Wnt/β-catenin signaling is essential for proper development of the RPE. Nevertheless, the developmental fate of the cells resulting from aberrant Wnt signaling remains elusive.

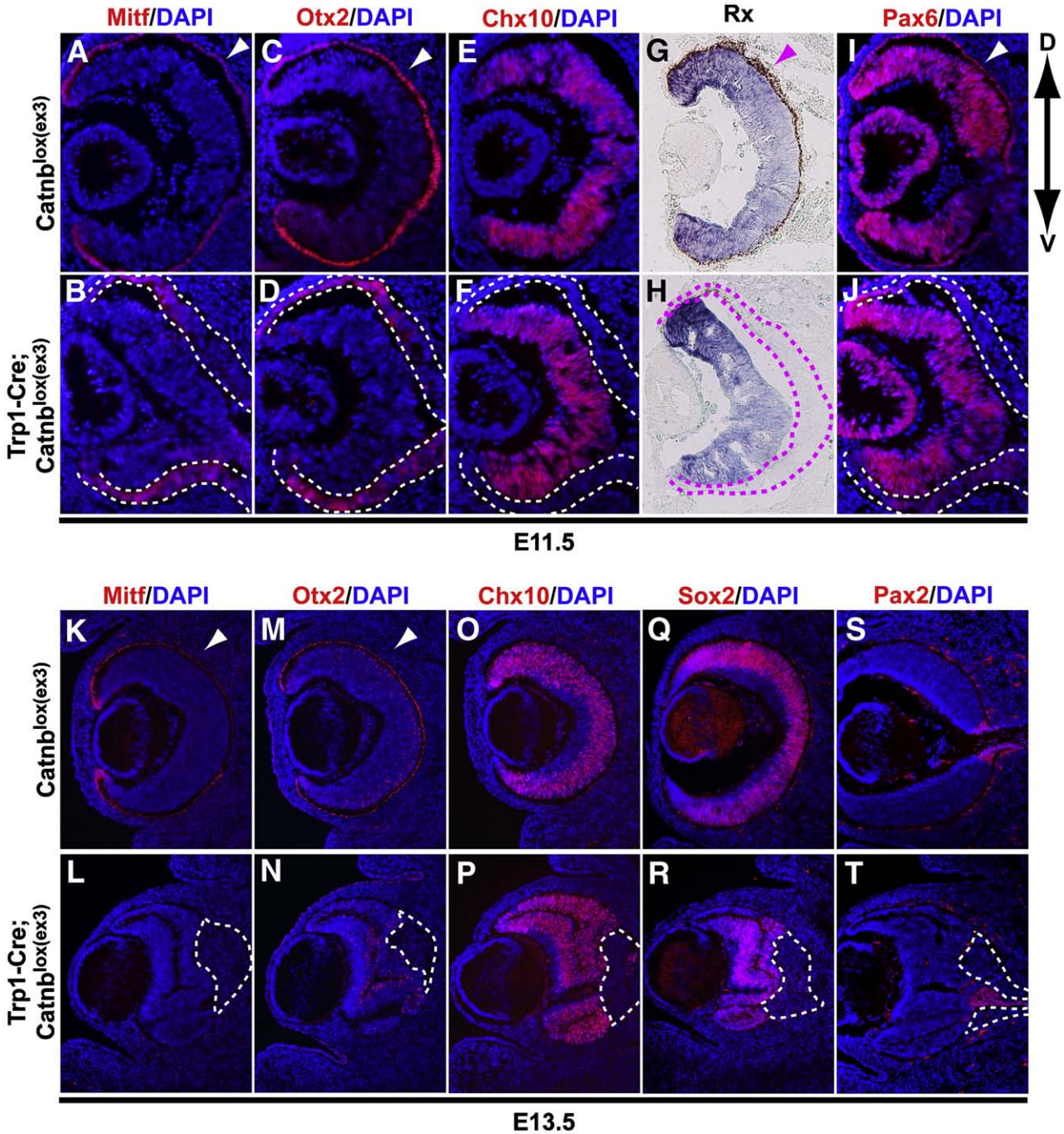

Fig. 7. Ectopic activation of Wnt/β-catenin signaling results in the abrogation of the RPE development. Frontal cryosections of wild-type (A, C, E, G, I, K, M, O, Q, and S) and gain-of-function mutant (B, D, F, H, J, L, N, P, R, and T) at E11.5 or E13.5 were immunostained with Mitf (A, B, K, and L), Otx2 (C, D, M, and N), Chx10 (E, F, O, and P), Pax6 (I, J), Sox2 (Q, R), Pax2 (S, T) antibodies or hybridized with an antisense probe against *Rx* (G and H). The RPE is indicated by a dashed line or an arrowhead. Note that Mitf and Otx2 are downregulated specifically in the proximal RPE of the gain-of-function mutant at E11.5 and E13.5.

Wnt/β-catenin signaling directly regulates expression of the genes that are essential for the RPE development

The loss-of-function mutant exhibited transdifferentiation of RPE to NR, suggesting that Wnt/β-catenin signaling could suppress neurogenesis in the RPE. In accordance with this hypothesis it has been shown that ectopic activation of Wnt/β-catenin signaling in the central retina results in loss of NR identity (Fu et al., 2006). One of the possible explanations is that Wnt signaling induces expression of transcriptional repressors or corepressors to inhibit transcription of the NR markers in the RPE. The identity of such repressive factors is unknown. Several RPE-expressed factors such as Pax6, Pax2, Vax2, Nr2f1 (COUP-TFI) and Nr2f2 (COUP-TFII) were previously shown to act as transcriptional repressors (Cai et al., 2003; Duncan et al., 1998; Mui et al., 2005; Naka et al., 2008). *Vax2* encodes a homeobox-containing transcriptional repressor and is expressed in the ventral optic cup (Barbieri et al., 1999; Kim and Lemke, 2006; Mui et al., 2005; Schulte et al., 1999). In *Vax1* and *Vax2* double-knockout mice, the dorsal RPE expands into the dorsal optic stalk and development of the ventral RPE is disrupted (Mui et al., 2005). *Nr2f1* and *Nr2f2* belongs to orphan members of the steroid/thyroid hormone receptor superfamily (Cooney et al., 1992; Pereira et al., 2000). *Nr2f2* is expressed in the RPE, the dorsal NR, and the optic stalk during eye development (McCaffery et al., 1999). Despite its expression in the developing eye

the function of *Nr2f2* during eye development is not known since the inactivation of the *Nr2f2* gene in the mouse is lethal around E10.0 (Pereira et al., 1999). Nr2f1 and Nr2f2 have been shown to antagonize the nuclear receptor signaling pathway (Beland and Lohnes, 2005; Butler and Parker, 1995; Cooney et al., 1992).

We examined the expression of the aforementioned transcriptional factors in loss-of-function mutants at E10.5 using *in situ* hybridization and immunohistochemistry. There was no obvious difference in the expression pattern of Pax2 and Pax6 between the wild-type and loss-of-function animals (Supplementary Figs. 5A–D). However, we observed changes in the expression of the *Vax2* gene. While in the wild-type embryos *Vax2* mRNA was detected in the ventral parts of the NR and RPE, in the transdifferentiated RPE the area of *Vax2* expression extended proximally (Supplementary Figs. 5I, J). We hypothesized that the ectopic *Vax2* expression could be due to the disturbed dorsoventral polarity in the transdifferentiated RPE. Nr2f1 was detected in the ventral NR, the ventral RPE, and the optic stalk of the wild-type mice (Supplementary Fig. 5E). In the transdifferentiated RPE of the loss-of-function mutants the expression of Nr2f1 expanded distally (Supplementary Fig. 5F), presumably for the same reason as that of *Vax2*. Nr2f2 was expressed in the distal NR, the optic stalk, and the RPE (Supplementary Fig. 5G). Interestingly, Nr2f2 was downregulated in the transdifferentiated RPE of loss-of-function mutants (Supplementary Fig. 5H), suggesting that *Nr2f2* is regulated by Wnt/β-catenin signaling and could be involved in inhibition of the neural differentiation in the RPE.

As shown above, Mitf and Otx2 were downregulated in the transdifferentiated RPE of loss-of-function mutants, indicating that *Mitf* and *Otx2* may be directly regulated by Wnt/β-catenin signaling. Wnt/β-catenin signaling-mediated regulation of *Otx2* and *Mitf* has been investigated (Dorsky et al., 2000; Kurokawa et al., 2004; Takeda et al., 2000). The *Otx2* enhancer, which contains multiple Tcf/Lef binding sites, was found to be active in RPE as well as in forebrain and midbrain; mutations in Tcf/Lef sites nearly abolish the enhancer activity, indicating that the expression of *Otx2* is directly regulated by Wnt/β-catenin signaling (Kurokawa et al., 2004). Among *Mitf* isoforms, *Mitf-M* is known to be directly regulated by Wnt/β-catenin signaling (Takeda et al., 2000). However, *Mitf-M* is melanocyte-specific and is not expressed in RPE (Bharti et al., 2008). Recent study has shown that two other isoforms, *Mitf-H* and *Mitf-D*, play a key role in the RPE development (Bharti et al., 2008). Interestingly, the expression of *Mitf-D* and *Mitf-H* is initiated in the presumptive RPE at E10.5 (Bharti et al., 2008), which coincides with initiation of Wnt/β-catenin signaling in the presumptive RPE (Kreslova et al., 2007). To investigate possible direct regulation by Wnt/β-catenin signaling we searched for Tcf/Lef binding sites within the promoters of *Mitf-D*, *Mitf-H*, and *Nr2f2*. We found that the promoters of *Mitf-D*, *Mitf-H*, and *Nr2f2* as well as 5′ UTR of *Nr2f2* contain potential Tcf/Lef binding sites (Fig. 8A and Supplementary Fig. 5K). Additionally, we compared sequences of *Nr2f2* introns of frogs, chickens, humans and mice in an attempt to find other potential transcriptional regulatory elements since these elements are often evolutionarily conserved. We found three evolutionarily conserved regions containing potential Tcf/Lef binding sites that are located 1.8 kb, 5.1 kb, and 11 kb downstream of the *Nr2f2* promoter, referred to as ECR1, ECR2, and ECR3, respectively (Supplementary Fig. 5K). In order to examine whether these binding sites are responsible for mediating Wnt/β-catenin signaling through a direct interaction with the Tcf/Lef/β-catenin complex, chromatin immunoprecipitation was performed with β-catenin and Tcf/Lef antibody using wild-type embryos at E10.5. The previously described

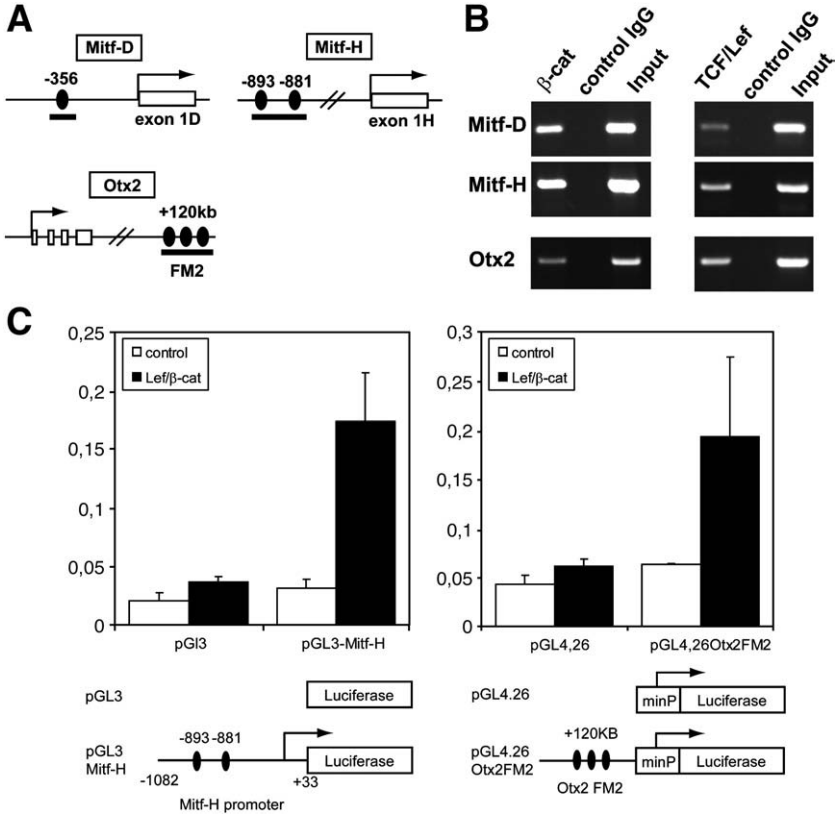

Fig. 8. Wnt/β-catenin signaling directly regulates *Mitf* and *Otx2* genes. (A) Map of the *Mitf-D*, *Mitf-H*, and *Otx2* (Kurokawa et al., 2004) locus with putative Tcf/Lef binding sites depicted by black ovals. (B) Chromatin immunoprecipitation was performed with wild-type embryos at E10.5 using Tcf/Lef, β-catenin or control antiserum. PCR was carried out with specific primers detecting the indicated transcriptional regulatory elements described in panel (A). The presence of β-catenin and Tcf/Lef was confirmed on the promoters of *Mitf-H*, *Mitf-D*, and the enhancer of *Otx2*. (C) The indicated luciferase reporter plasmids (200 ng) were cotransfected with the stabilized form of β-catenin (β-cat) and LEF1 (50 ng each) or an empty expression plasmid (100 ng) into ARPE19 cells. *Renilla* luciferase expression plasmid (10 ng) was cotransfected to normalize for transfection efficiency.

Otx2 enhancer (Kurokawa et al., 2004) was also analyzed by chromatin immunoprecipitation to confirm that the enhancer is regulated by Wnt/β-catenin signaling. We found that β-catenin and Tcf/Lef were present on the potential Wnt/β-catenin signaling-responsive elements of *Nr2f2*, *Mitf-D* and *Mitf-H*, with the exception of ECR2 and ECR3 (Fig. 8B, Supplementary Fig. 5). We further investigated whether these elements are Wnt/β-catenin signaling-responsive by the luciferase reporter gene assay. Lef1/β-catenin activated the *Mitf-H* promoter and *Otx2* FM2 enhancer (Fig. 8C), but not the other putative regulatory elements (data not shown). These results indicate that Wnt/β-catenin signaling directly regulates expression of *Mitf-H* and *Otx2*, and possibly, of *Mitf-D* and *Nr2f2*. Taken together, our data suggest that Wnt/β-catenin signaling regulates the RPE development by inducing expression of the key differentiation genes.

Discussion

In this study we have investigated the role of Wnt/β-catenin signaling in the RPE development using conditional loss- and gain-of-function *β-catenin* alleles. Intriguingly, elimination of β-catenin in the RPE resulted in transdifferentiation of the dorsal RPE to NR as evidenced by downregulation of the RPE-specific markers *Mitf* and *Otx2* and ectopic expression of the NR-specific genes *Chx10* and *Rx*. Given the dual role of β-catenin in transcription activation and in cell adhesion we first assessed which of these molecular functions contribute to the observed phenotype.

The fact that the absence of canonical Wnt signaling upon loss of β-catenin was the primary mechanism of the aberrant development of RPE was concluded from the following observations. We and others have shown that during mouse eye development Wnt signaling is initially detected in the distal part of the presumptive RPE. In the later developmental stages the active Wnt pathway is restricted to the peripheral area of the RPE [Fig. 1 and results in references (Kreslova et al., 2007; Liu et al., 2006)]. As all these studies used two well-established but nevertheless synthetic Wnt/β-catenin signaling reporter genes, along with the β-galactosidase assay we performed hybridization of mRNA *in situ*. This analysis clearly showed that the activity of the reporters in the RPE correlates well with the expression pattern of two endogenous target genes of canonical Wnt signaling, *Axin2* and *Lef1* (Supplementary Figs. 1A, B). In addition, active Wnt/β-catenin signaling is detected in the presumptive RPE not only in mice but also in chickens, fishes, and frogs (Dorsky et al., 2002; Cho and Cepko, 2006; Kreslova et al., 2007; Liu et al., 2006; Van Raay et al., 2005; Veien et al., 2008). Available data thus suggest an ancient and prominent role for Wnt/β-catenin signaling in the control of RPE development. The expression of both BAT-gal reporter and *Lef1* genes was significantly downregulated in the presumptive RPE of the loss-of-function β-catenin animals (Figs. 1B, D and Supplementary Fig. 1C), indicating that Wnt signaling is indeed disrupted in the mutant RPE. In contrast to Wnt signaling, the loss of β-catenin did not influence cell adhesion in the affected tissues as evidenced by the overall cell morphology and unchanged expression and localization of actin filaments and membrane N-cadherin (Fig. 3). We concluded that the lack of β-catenin is compensated for by its paralog γ-catenin as observed previously in various developmental contexts (Huelsken et al., 2000; Posthaus et al., 2002; Zhou et al., 2007). Intriguingly, in the NR β-catenin does not regulate cell differentiation but is essential for the normal migration. In *β-catenin*-null neural retinas Fu et al. detected retinal lamination defects possibly related to disorganized localization of N-cadherin and F-actin and decreased cell adhesion (Fu et al., 2006). Why β-catenin is differentially involved in signaling (RPE) or adhesion (NR) in the different parts of retina and why the β-catenin function in the NR cannot be compensated by γ-catenin remains elusive. The RPE-to-NR transdifferentiation is reminiscent of the phenotype described in *Mitf*-deficient and *Otx1*/*Otx2* double-deficient mice (Martinez-Morales et al., 2001; Nguyen and Arnheiter, 2000). Interestingly, *Otx2* is directly regulated by Wnt/β-catenin signaling via its remote enhancer [Fig. 8 and see also reference (Kurokawa et al., 2004)] and furthermore, the promoter of *Mitf-H*, the RPE-enriched isoform of the *Mitf* gene, responds to the Lef/β-catenin stimulation (Fig. 8). Altogether, the published data and experimental evidence presented in this study strongly support the idea that Wnt/β-catenin signaling is required for the proper RPE specification. We suggest that canonical Wnt signaling induces or maintains the expression of the RPE-specific transcription factors and simultaneously inhibits the NR differentiation program in the developing RPE.

It is worth noting that the expression of melanogenic genes such as *Tyr* is induced at E10.5 (Beermann et al., 1992), which coincides with initiation of Wnt/β-catenin signaling in the presumptive RPE. Expression of melanogenic genes is directly regulated by the transcription factors Mitf and Otx2 (Aksan and Goding, 1998; Hemesath et al., 1994; Martinez-Morales et al., 2003; Yasumoto et al., 1994, 1997) (Fig. 9A). Even though *Mitf* and *Otx2* are expressed in the entire optic vesicle at E9.5 (Baumer et al., 2003; Martinez-Morales

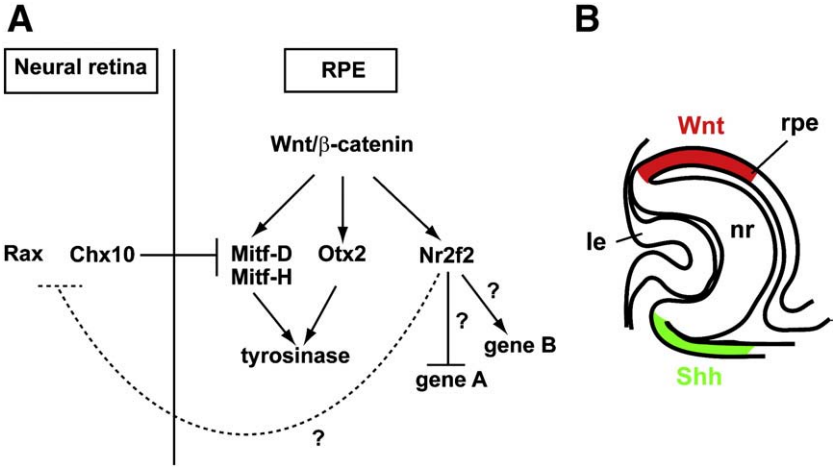

Fig. 9. A model summarizing the role of Wnt/β-catenin signaling in RPE development. (A) Transcriptional network downstream of Wnt/β-catenin signaling in RPE. Wnt/β-catenin signaling in the RPE directly maintains or induces expression of *Mitf-H*, *Mitf-D*, *Otx2* and *Nr2f2*. *Mitf-D*, Mitf-H and Otx2 regulate expression of the melanogenic genes such as tyrosinase (Aksan and Goding, 1998; Martinez-Morales et al., 2003). Nr2f2 may positively regulate expression of genes that are involved in RPE development or inhibit expression of NR-specific genes. In NR the expression of *Chx10* and *Rx* is maintained, and Chx10 directly inhibits expression of *Mitf-D* and *Mitf-H* (Bharti et al., 2008; Horsford et al., 2005; Rowan et al., 2004). Dashed lines indicate indirect regulation, whereas solid lines indicate direct regulation. (B) Signaling pathways in developing RPE. The distal part of the dorsal RPE is regulated by Wnt/β-catenin signaling. On the contrary, the ventral RPE is regulated by Sonic hedgehog (Shh) signaling (Zhang and Yang, 2001).

et al., 2001; Nguyen and Arnheiter, 2000), the optic vesicle of *Mitf*- and *Otx1*/*Otx2*-deficient mice is normal, and their target genes such as *Tyr* are not expressed (Martinez-Morales et al., 2001; Nguyen and Arnheiter, 2000). This indicates that their transcriptional activity might be regulated by an RPE-specific signal. Interestingly, recent studies have indicated that Wnt/β-catenin signaling modifies transcriptional properties of the Mitf protein. First of all, Mitf directly interacts with β-catenin and recruits β-catenin on the its target promoters as a transcriptional activator in melanoma cells (Schepsky et al., 2006). Another study has suggested that the transcriptional activity of Mitf is attenuated until Pax3-mediated repression is relieved by Wnt/β-catenin signaling in melanocyte stem cells (Lang et al., 2005). Thus, it is possible that Wnt/β-catenin signaling plays a role as a differentiation signal within the RPE by boosting the transcriptional activity of Mitf (and possibly Otx2) in a similar manner as in melanocyte stem cells (Fig. 9A).

Wnt-mediated inhibition of neural differentiation has been implicated in the ciliary margin development (Cho and Cepko, 2006; Liu et al., 2007). Ectopic activation of Wnt/β-catenin signaling in the peripheral NR results in expansion of the ciliary margin toward the NR and inhibition of neurogenesis (Liu et al., 2007). On the contrary, conditional inactivation of the signaling in the peripheral NR leads to downregulation of the ciliary margin markers *Otx1* and *Msx1* (Liu et al., 2007). Similarly, ectopic activation of Wnt/β-catenin signaling in the chick eye inhibits the NR differentiation and induces the ciliary margin markers (Cho and Cepko, 2006). It is therefore possible that one of the roles of Wnt/β-catenin signaling in the RPE is to inhibit neurogenesis in a similar manner as in the ciliary margin.

Interestingly, in contrast to the dorsal RPE, the ventral RPE remained thin and contained a subset of pigmented cells until E13.5 in the loss-of-function β-catenin mutant. This may suggest that the dorsal and the ventral RPE are regulated by two distinct mechanisms. Supporting this idea, in *AP2α*-deficient mice only the dorsal but not the ventral RPE transdifferentiates into NR (West-Mays et al., 1999). In contrast, only the ventral RPE-to-NR transdifferentiation occurs in null mutant mice of *Gas1*, which positively regulates Sonic hedgehog (Shh) signaling (Allen et al., 2007; Lee et al., 2001; Martinelli and Fan, 2007). Shh signaling is important for the ventral specification of the eye (Chow and Lang, 2001; Mui et al., 2005). For instance, Shh signaling regulates the activity of Vax2, which ventralizes the eye by controlling its subcellular localization (Kim and Lemke, 2006). Additionally, anti-Shh antibody treatment results in transdifferentiation of exclusively the ventral RPE to NR in chicken (Zhang and Yang, 2001). In summary, it seems that the dorsal RPE development is regulated by Wnt/β-catenin signaling, whereas the ventral RPE development is regulated by other signaling pathways such as Shh (Fig. 9B).

We have shown that ectopic activation of Wnt/β-catenin signaling results in the disruption of normal RPE development. At E10.5 Wnt/β-catenin signaling was activated in the entire RPE of the gain-of-function mutant and the presumptive RPE appeared to be morphologically different compared to that of the wild-type. However, the basic patterning of RPE and NR was still normal according to the expression of NR- and RPE-specific markers. Only by E11.5 *Mitf* and *Otx2* became downregulated in the proximal RPE. However, in contrast to the loss-of-function mutant, the expression of *Chx10* and *Rx* remained restricted to NR. The expression pattern of these key transcription factors in the gain-of-function mutant indicates that the proximal RPE development was more seriously disrupted. Apart from Mitf and Otx2, transcription factor Pax6 was downregulated in the proximal RPE, while its expression in the distal RPE remained unchanged in the gain-of-function mutant. The downregulation of the *Pax6* gene is particularly interesting because Pax6 together with Pax2 is essential for the RPE development and was previously shown to bind to *Mitf-A* promoter and activate its expression *in vitro* (Baumer et al., 2003). Furthermore, ectopic expression of *Pax6* in the optic stalk results in formation of Mitf-positive RPE (Baumer et al., 2003). It was recently shown that two other isoforms, *Mitf-H* and *Mitf-D*, play a key role in the RPE development (Bharti et al., 2008). No data is available about the possible role of Pax6 in the activation of *Mitf-H* and *Mitf-D*. It is nevertheless intriguing to think that the downregulation of *Mitf* expression could be caused, at least in part, by *Pax6* downregulation in the proximal RPE.

Taken together, spatial and temporal regulation of Wnt/β-catenin signaling is essential for the proper RPE development in mice. We suggest that the two distinct roles of Wnt/β-catenin signaling in the RPE development could be to regulate differentiation of the RPE by initiating or maintaining expression of *Mitf-D*, *Mitf-H* and *Otx2*, and to protect the RPE from NR-specifying signals.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.07.002.

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