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The role of Wnt signaling in embryonic development

Úloha Wnt signalizace v embryonální vývoj

Ph.D. thesis

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ABSTRACT

Wnt signaling plays important roles in multiple developmental processes. The binding of Wnt ligands to their receptors and coreceptors activates three main downstream pathways: canonical Wnt/β-catenin signaling, which results in the activation of β-catenin/Tcf mediated gene expression and noncanonical Wnt/PCP and Wnt/Ca^{2+} pathways. In this thesis, we aimed at studying the role of Wnt/β-catenin signaling during embryonic development, especially in the telencephalon and the eye.

Wnt/β-catenin signaling is essential for the maintenance of proliferation of neuronal progenitor cells and dorso-ventral specification during the telencephalon development. To provide further insights, we studied transcriptional targets of canonical Wnt signaling. We show that the ectopic activation of Wnt/β-catenin signaling results in the up-regulation of *Sp5* gene, which encodes a member of the Sp1 transcription factor family. A proximal promoter of *Sp5* gene contains five Tcf/Lef binding sites that mediate direct regulation of *Sp5* expression by canonical Wnt signaling. We further provide evidence that Sp5 works as a transcriptional repressor. Finally, our data strongly suggest that Sp5 has the same DNA binding specificity as Sp1 and represses Sp1 target genes such as *p21*. We conclude that Sp5 transcription factor mediates the downstream responses to Wnt/β-catenin signaling by directly repressing Sp1 target genes.

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 conditionally inactivated or ectopically activated Wnt/β-catenin signaling in the RPE. Inactivation of Wnt/β-catenin signaling results in transdifferentiation of the RPE to the neural retina. 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. We further provide evidence that *Otx2* and RPE-specific isoforms of *Mitf* are direct transcriptional targets of Wnt/β-catenin signaling. Combined, our data suggests that Wnt/β-catenin signaling plays an essential role in development of RPE by maintaining or inducing expression of *Mitf* and *Otx2*.

Wnt/β-catenin signaling is required to suppress lens formation in the periocular ectoderm during eye development and inhibition of the signaling in the presumptive lens placode is one of the prerequisites for lens development. But its exact mechanism is unknown. We show that Pax6 directly controls expression of several Wnt inhibitors such as *Sfrp1*, *Sfrp2*, and *Dkk1* in the presumptive lens. In accordance, absence of Pax6 function leads to aberrant canonical Wnt activity in the presumptive lens, which subsequently impairs lens development. Thus Pax6 is required for down-regulation of canonical Wnt/β-catenin signaling in the presumptive lens ectoderm.

ABSTRAKT

Wnt signalizace hraje důležitou roli v mnoha vývojových procesech. Vazba ligandu Wnt na příslušné receptory následně aktivuje tři hlavní dráhy: kanonickou Wnt/β-catenin signalizaci, která má za následek aktivaci β-catenin/Tcf zprostředkované genové exprese a nekanonické Wnt/PCP a Wnt/Ca^{2+} dráhy. V této práci jsem se zaměřila na studium úlohy Wnt/β-catenin signalizace během embryonálního vývoje, zejména v koncovém mozku (telencefalonu) a očích.

Wnt/β-catenin signalizace je nezbytná pro zachování proliferace neurálních progenitorových buněk a pro dorzo-ventrální specifikaci koncového mozku. V této práci jsme se zabývali transkripčními cíly kanonické Wnt signalizace během tohoto procesu. Ukázali jsme, že výsledkem ektopické aktivace Wnt/β-catenin signalizace je zvýšená exprese genu *Sp5*, který kóduje protein Sp5 z rodiny Sp1 transkripčních faktorů. Proximální promotor genu *Sp5* obsahuje pět Tcf/Lef vazebných míst, která zprostředkují přímou regulaci *Sp5* v důsledku kanonické Wnt signalizace. Dále jsme prokázali, že Sp5 funguje jako transkripční represor, mající stejnou DNA vazebnou specifitu jako Sp1, a tudíž potlačuje expresi Sp1 cílových genů, jako je *p21*. Došli jsme k závěru, že transkripční faktor Sp5 zprostředkovává efekt Wnt/β-cateninové signalizace přímým potlačením Sp1 cílových genů.

Wnt/β-catenin signalizace je velmi aktivní v dorzální oblasti pigmentového epitelu sítnice (RPE) během vývoje oka. Roli Wnt/β-catenin signalizace ve vývoji RPE jsme studovali pomocí podmíněné inaktivace nebo ektopické aktivace Wnt/β-catenin signalizace v RPE. Inaktivace Wnt/β-catenin signalizace vedla k transdiferenciaci RPE na nervovou sítnici (neuroretina). Naproti tomu ektopická aktivace Wnt/β-catenin signalizace vede k narušení patterningu RPE, což naznačuje, že přesná prostorová a časová regulace Wnt/β-catenin signalizace je nezbytná pro normální vývoj RPE. Dále jsme prokázali, že *Otx2* a RPE-specifické izoformy *Mitf* jsou přímé transkripční cíle Wnt/β-catenin signalizace, což vysvětluje zásadní roli Wnt/β-catenin signalizace ve vývoji RPE zachováním nebo potlačením exprese genů *Mitf* a *Otx2*.

Dále jsme studovali roli Wnt/beta-cat signalizace během časného vývoje čočky. Jedním z předpokladů pro její normální vývoj je, potlačení Wnt/β-catenin signalizace v periokulárním ektodermu. Přesný mechanismus této inhibice není znám. Ukazujeme, že Pax6 přímo řídí expresi několika Wnt inhibitorů jako *Sfrp1*, *Sfrp2*, a *Dkk1* při formaci čočky. V souladu s tímto tvrzením se ukázalo, že absence genu *Pax6* vede k abnormální aktivitě kanonické Wnt signalizace v budoucí čočce, která následně brání jejímu normálnímu vývoji. Z uvedeného vyplývá, že Pax6 je nezbytný pro potlačení kanonické Wnt signalizace při formování čočkového ektodermu.

ABBREVIATIONS

APC	adenomatosis polyposis coli
BMP	bone morphogenetic protein
CK1	casein kinase 1
Chx10	cer-10 homeodomain containing homolog
CtBP	C-terminal binding protein
Dkk	dikkopf
Dvl	dishevelled
E10.0	embryonic day 10.0
EMSA	electrophoretic mobility shift assay
FGF	fibroblast growth factor
GSK3β	glycogen synthase kinase 3β
HDAC	histone deacetylase
Lef	lymphocyte enhancer factor
LGE	lateral ganglionic eminence
LRP	low-density lipoprotein receptor-related protein
MGE	medial ganglionic eminence
Mitf	microphthalmia-associated transcription factor
NR	neural retina
Otx2	orthodenticle homeobox 2
Pax6	paired box 6
PCP	planar cell polarity
RPE	retinal pigment epithelium
Rx	retinal homeobox
Sfrp	secreted Frizzled related protein
Tcf	T-cell factor
TGFβ	transforming growth factor β
Trp1	tyrosinase-related protein 1

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1. INTRODUCTION

1.1. Overview of Wnt signaling pathway

Wnt signaling pathway plays fundamental roles in embryonic development by controlling cell proliferation, cell fate decisions, cell movements and the establishment of tissue polarity (Klaus and Birchmeier, 2008). The signaling is initiated by the interaction of Wnt ligands with their receptors and coreceptors, which elicits a variety of intracellular responses. There are three main branches: canonical Wnt/β-catenin signaling, noncanonical Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+} pathways. In canonical Wnt/β-catenin signaling, the Wnt-receptor interaction results in the activation of β-catenin/Tcf mediated gene expression, whereas noncanonical pathways are β-catenin-independent and implicated in the activation of other intracellular messengers (Logan and Nusse, 2004). In Wnt/PCP pathway, the interaction activates the small GTPase Rho and Rac and directs asymmetric cytoskeletal organization and coordinated polarization of cells within the plane of the tissue, such that cells organize themselves in a particular orientation with respect to a common body axis. Wnt/Ca^{2+} pathway leads to release of intracellular calcium. This results in the activation of phospholipase C and protein kinase C (Veeman et al., 2003). Interestingly, it has been shown that these β-catenin independent signaling antagonizes the Wnt/β-catenin signaling in vertebrate (Torres et al., 1996).

1.1.1. Canonical Wnt/β-catenin signaling

The key component of Wnt/β-catenin signaling pathway is β-catenin that has two main functions in the cell (Fig. 1) (Willert and Jones, 2006; Heuberger and Birchmeier, 2010). First, it acts as an essential component of the cell adherens junction. It binds to the transmembrane protein cadherin and regulates actin filament assembly through α-catenin (Perez-Moreno et al., 2003). Second, as mentioned above it acts as a co-activator of Tcf/Lef transcription factors. In the absence of Wnt ligands, cytoplasmic β-catenin is constantly degraded by β-catenin destruction complex, which is composed of Axin, Adenomatosis polyposis coli (APC), Glycogen synthase kinase 3β (GSK3β), and Casein kinase 1 (CK1) (Farr et al., 2000; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998; Salic et al., 2000). CK1 and GSK3β phosphorylate a set of conserved Serine and Threonine residues in the amino terminal region of β-catenin (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). The phosphorylation creates a binding site for E3 ubiquitin ligase β-Trcp, leading to β-catenin ubiquitination and degradation by the proteasome (Aberle et al., 1997). This continual elimination of β-catenin prevents it from reaching the nucleus and Wnt target genes are repressed by Tcf/Lef transcription factors bound to co-repressor Groucho and CtBP (Billin et

al., 2000; Brannon et al., 1999; Brantjes et al., 2001; Cavallo et al., 1998; Roose et al., 1998; Valenta et al., 2003). Binding of Wnt ligands to Frizzled receptor and coreceptor Lrp5/6 triggers GSK3β and CK1-mediated phosphorylation of Lrp5/6 (Davidson et al., 2005; Zeng et al., 2005). The intracellular domains of the phosphorylated Lrp5/6 act as docking sites for Axin (Tamai et al., 2004). This inactivates the destruction complex, as a result, β-catenin is stabilized and translocated into the nucleus (Fig. 1). In the nucleus, β-catenin displaces Groucho from Tcf/Lef, initiating expression of Wnt target genes such as *c-Myc*, *Axin2* and *Lef1* (He et al., 1998; Hovanes et al., 2001; Jho et al., 2002).

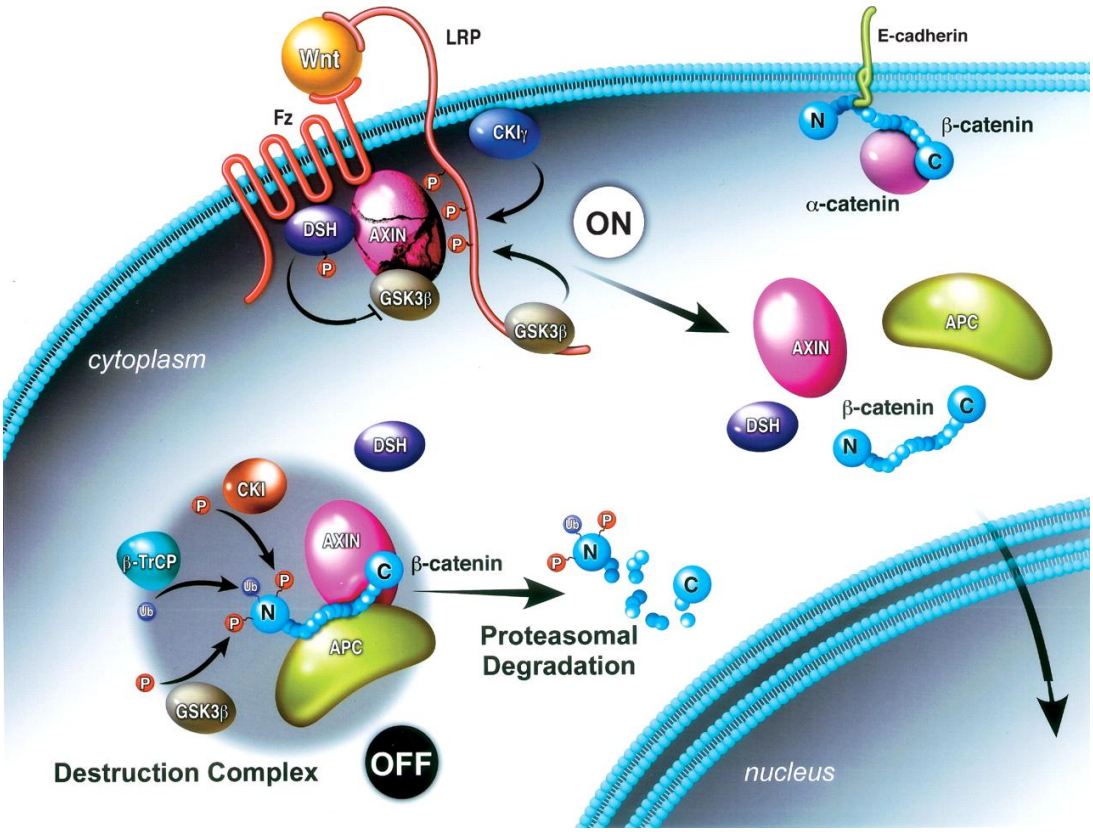

Fig. 1. Wnt/β-catenin signaling.

In the absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated in the destruction complex composed of Axin, APC, GSK3β, and CK1. Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenin for proteosomal degradation. In the nucleus, Wnt target genes are repressed by Tcf/Groucho complex. In the presence of Wnt ligands, they bind to the Frizzled/LRP receptor complexes. Dishevelled is recruited by the receptor complex, which leads to the phosphorylation of LRP. The phosphorylated LRP recruits Axin to the plasma membrane, which results in decay of the β-catenin destruction complex. This allows β-catenin to accumulate and translocate into the nucleus. In the nucleus, β-catenin serves as a co-activator for Tcf to activate Wnt target genes (Adapted from Willert and Jones, 2006).

1.1.2. Wnt/β-catenin signaling in the nucleus

In the absence of β-catenin, Tcf/Lef is bound to Groucho and CtBP in the nucleus (Billin et al., 2000; Brannon et al., 1999; Brantjes et al., 2001; Cavallo et al., 1998; Roose et al., 1998; Valenta et al., 2003). Both Groucho family proteins and CtBP proteins function as general transcriptional corepressors by interacting with histone deacetylases (HDAC) (Chen et al., 1999). β-catenin displaces Groucho from Tcf/Lef by binding to a second, low-affinity binding site on Tcf/Lef that overlaps the Groucho binding site (Daniels and Weis, 2005). On the contrary, it is not clear how CtBP corepressors are released from Tcf/Lef by Wnt signaling. Additionally, β-catenin functions as a scaffold to link Tcf/Lef to specific chromatin remodeling complexes such as Brg1 and the Wnt coactivator, Pygopus and Bcl-9 (Barker et al., 2001; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002).

A core region of β-catenin is composed of 12 copies of a 42 amino acid sequence motif called as an armadillo repeat that is involved in protein-protein interaction. The N-terminal armadillo repeat interacts directly with Bcl-9 that can mediate nuclear import of β-catenin by targeting it to the nuclear Pygopus protein (Brembeck et al., 2004; Townsley et al., 2004). Bcl-9 and Pygopus are also implicated in transcription. Pygopus contains conserved PHD finger domain and N-terminal homology domain (NHD). NHD binds to the Med12 and Med13 subunits of the mediator complex that is essential for Wnt-regulated transcriptional activation (Carrera et al., 2008). Furthermore, PHD finger associates with HD1 domain of Bcl-9 to bind specifically to the histone H3 tail methylated at lysine 4 that is associated with gene activation (Fiedler et al., 2008). In addition, Pygopus is associated constitutively with Wnt response elements by binding to Tcf/Lef and proposed to function as an anti-repressor by overcoming the transcriptional repression imposed on Tcf/Lef target genes by Groucho (de la Roche and Bienz, 2007; Mieszczanek et al., 2008). The region from the C-terminal to the armadillo repeats contains a very strong activation domain that interacts to multiple chromatin remodeling complexes and histone modifying complexes. These include TRRAP/TIP60 (Sierra et al., 2006), mixed-lineage-leukemia (MLL1/MLL2) SET1-type chromatin-modifying complexes (Sierra et al., 2006), CBP/p300 (Hecht et al., 2000; Takemaru and Moon, 2000), Brg-1 (Barker et al., 2001), and TATA binding protein (Hecht et al., 1999). Indeed, upon Wnt stimulation, histones are rapidly acetylated over a wide region surrounding the Tcf/Lef binding sites. This acetylation is dependent upon CBP and is correlated with activation of target gene expression (Parker et al., 2008).

There are several factors that contribute to Tcf/β-catenin mediated transcription. Chibby and ICAT bind to the C-terminal region of β-catenin and prevent Tcf/β-catenin interaction (Tago et al., 2000; Takemaru et al., 2003). Sox9 competes with Tcf/Lef for β-catenin interaction (Akiyama et al., 2004). APC antagonizes the transcriptional activity of β-

catenin in three ways. The best known function is to reduce the level of cytoplasmic β-catenin in the destruction complex as mentioned above. Additionally, APC shuttles between the cytoplasm and the nucleus and promotes the nuclear export and destruction of β-catenin (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). APC also acts as an adaptor between β-catenin and CtBP. CtBP sequesters nuclear APC/β-catenin complexes and antagonizes Tcf/Lef-mediated transcription by lowering the availability of free β-catenin for binding to Tcf/Lef (Hamada and Bienz, 2004). Nemo-like kinase NLK phosphorylates Tcf/Lef and inhibits the interaction of the β-catenin/Tcf complex with DNA (Ishitani et al., 1999). On the contrary, transducin β-like protein 1 (TBL1) and its highly related family member TBLR1 facilitate Wnt/β-catenin mediated transcription (Li and Wang, 2008). Wnt signaling induces the interaction between β-catenin and TBL1/TBLR1 in the nucleus, as well as their binding to the Wnt target genes (Li and Wang, 2008). Since TBL1/TBLR1 preferentially bind to the hypoacetylated histones and target the co-repressor complexes to chromatin (Yoon et al., 2005), it is suggested that β-catenin use them in order to bind to the hypoacetylated region of the Wnt response elements (Li and Wang, 2008). Moreover, nuclear Dishevelled and c-Jun transcription factor form a complex with β-catenin and Tcf/Lef on the Wnt target genes, which stabilize β-catenin/Tcf interaction (Gan et al., 2008).

Tcf/Lef transcription factors recognize specific DNA sequence through their HMG domains. In mammals, the Tcf/Lef family consists of four genes: *Tcf1*, *Lef1*, *Tcf3*, and *Tcf4*. Tcf1, Lef1 and Tcf4 act as both repressors and activators whereas Tcf3 seems to act as a repressor (Arce et al., 2006). The zebrafish *headless* mutants show severe head defects due to a mutation in *Tcf3* (Kim et al., 2000). This mutant zebrafish is rescued by an N-terminally truncated dominant negative Tcf3 lacking its β-catenin binding domain (Kim et al., 2000). Furthermore, Tcf3 represses whereas Lef1 stimulates Wnt-reporter TOPFLASH activity in primary cultured keratinocytes (Merrill et al., 2001). Their consensus sequence is CTTTGA/TA/T, however Tcf/Lef can also bind motifs differing from this sequence (Arce et al., 2006). An additional DNA-binding domain was recently shown to provide greater DNA-binding specificity. Alternatively spliced isoforms of Tcf1 and Tcf4 contain an additional DNA interaction motif called cysteine clamp. This motif recognizes an additional GC element downstream of their consensus, which allows regulation of specific Wnt target genes with lower affinity Wnt response elements (Atcha et al., 2007). Indeed, some Wnt response elements locating in *Lef1* promoter require the additional GC element for stable binding and ß-catenin activation (Atcha et al., 2007). Alternative promoters in mammalian *Lef1* gene result in the use of alternative downstream translation start sites to produce short Lef1 isoforms that lack β-catenin interaction domain. This acts as a dominant negative isoform by competing for binding to the Wnt response elements (Hovanes et al., 2001). Another inhibitory isoform is Tcf-4N isolated from developing pituitary. This contains the N-terminal interaction domain for β-catenin but lacks the DNA binding domain. Tcf-4N inhibits Tcf/Lef-

mediated transcription by sequestering β-catenin (Kennell et al., 2003). Interestingly, Tcf-4N and β-catenin synergize to activate transcription from non-Tcf-responsive promoters such as *α-inhibin* promoter by interacting with the orphan nuclear receptor steroidogenic factor 1 (Kennell et al., 2003). In addition to Tcf/Lef, several other transcription factors such as Pitx2, Mitf, and FoxO can recruit β-catenin to their target genes (Essers et al., 2005; Kioussi et al., 2002; Schepsky et al., 2006).

1.1.3. Noncanonical Wnt signaling

In vertebrates, noncanonical Wnt signaling pathways are involved in diverse processes, such as convergent extension cell movements during gastrulation, inner ear cell polarity, dorsoventral patterning, tissue separation, neuronal migration and cancer (Veeman et al., 2003). For instance, *Wnt11* zebrafish mutants have defects in convergence extension movements and the phenotype can be rescued by a truncated Dishevelled (Dvl) which dose not signal through the canonical signaling (Heisenberg et al., 2000). Wnt5a is essential for PCP regulated processes in the inner ear and in the neural tube (Qian et al., 2007). In the Wnt/PCP pathway, the binding activates the recruitment of cytoplasmic scaffold protein Dvl to the plasma membrane. This results in the activation of the small GTPase Rho and Rac to regulate the actin cytoskeleton (Habas et al., 2003; Strutt et al., 1997). In the Rho pathway, Dvl-associated activator of morphogenesis 1 (DAAM1) binds to Dvl by its carboxyl terminus and Rho by its amino terminus, which results in the Wnt-induced Dvl-Rho complex formation (Habas et al., 2001). This leads to the activation of the Rho-associated kinase ROCK, which mediates cytoskeletal re-organization (Marlow et al., 2002; Winter et al., 2001). On the other hand, the activation of Rac stimulates Jun N-terminal kinase (JNK) -mediated actin remodelling (Boutros et al., 1998; Habas et al., 2003; Yamanaka et al., 2002). The Wnt/Ca^{2+} pathway promotes the intracellular increase in Ca^{2+} concentration by stimulating the phosphatidylinositol signaling pathway via heterotrimeric G-protein subunits (Slusarski et al., 1997) and then activates Ca^{2+}-sensitive enzymes such as protein kinase C, calcium/calmodulin-dependent kinase II, and nuclear factor of activated T cells (Kühl et al., 2000; Saneyoshi et al., 2002; Sheldahl et al., 1999). Interestingly, these β-catenin independent signaling antagonizes the Wnt/β-catenin signaling in vertebrate (Saneyoshi et al., 2002; Torres et al., 1996). For instance, overexpression of Wnt5a can block secondary axis induction by Wnt8 in *Xenopus* embryos (Torres et al., 1996). In addition, Wnt/β-catenin signaling is ectopically activated in the distal part of the limb of *Wnt5a* knockout mice (Topol et al., 2003).

1.1.4. Wnts, their antagonists and agonists

Wnt ligands constitute a large family of cysteine-rich secreted proteins. In humans and mice, at least 19 Wnt members have been shown to be present and different family members exhibit unique expression patterns and distinct functions during development (Willert and Jones, 2006). They are divided into two classes, canonical Wnts such as Wnt1, Wnt3a and Wnt8 and noncanonical Wnts such as Wnt5a and Wnt11 (Veeman et al., 2003). However, recent studies propose that receptor context dictates Wnt signaling output rather than Wnt ligands (Mikels and Nusse, 2006). For instance, Wnt5a can also activate β-catenin signaling in the presence of the appropriate Frizzled receptor, Frizzled 4 (Mikels and Nusse, 2006). In *Xenopus* embryos, Wnt1 induces axis duplication, whereas Wnt5a does not (McMahon and Moon, 1989; Moon et al., 1993). However, Wnt5a induces axis duplication and an ectopic Spemann organizer in the presence of Frizzled 5 (He et al., 1997). Additionally, maternal Wnt11 activates Wnt/β-catenin signaling and is required for axis formation in *Xenopus* embryos (Tao et al., 2005).

A number of secreted protein families antagonize or modulate Wnt signaling. Secreted Frizzled related proteins (Sfrp) and Wnt-inhibitory protein (Wif) bind to Wnt ligands through a region related to the cysteine-rich domain of Frizzled, and thereby function as Wnt antagonists for both canonical and noncanonical pathway (Hsieh et al., 1999; Leyns et al., 1997; Wang et al., 1997). Another Wnt inhibitor is the Dickkopf (Dkk) family. Dkk1 is a head inducer secreted from the vertebrate head organizer and induces anterior development by antagonizing Wnt signaling (Semënov et al., 2001). It is Lrp6 ligand, and thereby inhibits Wnt signaling by preventing Wnt-induced Frizzled/Lrp6 complex formation (Semënov et al., 2001). The secreted protein Wise is a context-depend activator and inhibitor of Wnt signaling. This protein interacts with Lrp6 and competes with Wnt for binding to Lrp6 (Itasaki et al., 2003). Wise activates Wnt/β-catenin signaling in animal caps by mimicking some of the effects of Wnt ligands, on the contrary it antagonizes the axis-inducing ability of Wnt8 in an assay for secondary axis induction (Itasaki et al., 2003). In addition, SOST is also suggested to act as a Wnt antagonist by disrupting Wnt-induced Frizzled/LRP complex formation (Semënov et al., 2005).

Shisa represents a distinct family of Wnt antagonists. This protein physically interacts with immature forms of Frizzled receptors within the endoplasmic reticulum and inhibits their posttranslational maturation and trafficking to the cell surface (Yamamoto et al., 2005). Shisa is specifically expressed in the prospective head ectoderm and the Spemann organizer of *Xenopus*. Loss of Shisa function suppresses head formation during gastrulation. Shisa also antagonizes FGF signaling in the same way (Yamamoto et al., 2005). Other Wnt antagonists with multivalent activities are Cerberus and Insulin-like growth-factor-binding protein-4 (IGFBP-4) (Piccolo et al., 1999; Zhu et al., 2008). Cerberus binds to Nodal, BMP and Wnt

proteins via independent sites in the extracellular space to inhibit these signaling (Piccolo et al., 1999). IGFBP-4 physically interacts with Frizzled 8 and Lrp6 and inhibits the binding of Wnt3a while it binds to and modulates the actions of IGFs (Zhu et al., 2008).

Norrin and R-spondin proteins are two families of agonists for Wnt/β-catenin signaling. Norrin is a specific ligand for Frizzled 4/Lrp5 complex and activates Wnt/β-catenin signaling. Norrin plays a central role in vascular development in the retina and inner ear (Xu et al., 2004). The R-spondin family composed of 4 members and their expression is often co-expressed that of Wnt ligands during mouse development. R-spondin 2 is essential for limb morphogenesis (Aoki et al., 2008; Bell et al., 2008). R-spondin 3 promotes angioblast and vascular development and is also essential for mouse placental development (Aoki et al., 2007; Kazanskaya et al., 2008). Each contains an N-terminal signal peptide, two furin-like domains, one thrombospondin type 1 domain, and C-terminal low complexity region enriched with positively charged amino acids (Kazanskaya et al., 2004). Although all four R-Spondin proteins activate the canonical Wnt pathway, R-Spondin 2 and 3 are more potent than R-Spondin 1, whereas R-Spondin 4 is relatively inactive (Kim et al., 2008). All members require Wnt ligands and Lrp6 for activity and amplify Wnt/β-catenin signaling by Wnt3a, Wnt1, and Wnt7a. It has been suggested that R-spondins antagonize Dkk1 activity by interfering with it, causing the amplification of Wnt signaling (Kim et al., 2008).

1.1.5. Wnt/β-catenin signaling during embryonic development

Wnt/β-catenin signaling is essential for multiple developmental processes. For instance, it plays central roles during several stages of hair morphogenesis. Wnt family members are expressed in distinct patterns and stages in the developing skin (Reddy et al., 2001). It has been shown that Wnt/β-catenin signaling is crucial for the generation of the hair follicles using conditional deletion of *β-catenin* in mice (Huelsken et al., 2001). Deletion of the gene prevents hair placode formation and results in a lack of hair (Huelsken et al., 2001). Moreover, transgenic mice expressing dominant negative Lef1 also block hair cell differentiation (Merrill et al., 2001; Niemann et al., 2002). A similar phenotype is observed in transgenic mice which ectopically express *Dkk1* in the skin (Andl et al., 2002). On the other hand, accelerated and excessive formation of hair placodes is observed *β-catenin* gain-of-function mutant mice in the skin. However, most of the developing hair follicles eventually fail to produce hair (Närhi et al., 2008). Transgenic mice which overexpress *Wnt3a* or *Dishevelled 2* in the skin show a short-hair phenotype due to disturbed differentiation of hair shaft precursor cells (Millar et al., 1999). In addition, Lef1/β-catenin complex directly regulates expression of *movo1* and *jagged 1* which are required for the proper formation of hair (Estrach et al., 2006; Li et al., 2002).

Wnt/β-catenin pathway is required for the neural crest induction and differentiation. Co-expression of Wnt1 or Wnt3a with either of the neural inducers noggin and chordin in *Xenopus* animal cap explants results in generation of the neural crest, as determined by the ectopic expression of the neural crest markers *Slug*, *Ap-2* and *Krox20* (Saint-Jeannet et al., 1997). Overexpression of a dominant negative Wnt8 in *Xenopus* embryos inhibits the expression of the neural crest markers (LaBonne and Bronner-Fraser, 1998). In *Xenopus* embryos, Lrp6 overexpression significantly expands neural crest progenitors and dominant negative Lrp6 inhibits neural crest formation, as determined by the expression of *Slug* (Tamai et al., 2000). Indeed, Lef1/β-catenin complex binds to *Xenopus Slug* promoter and regulates its expression (Vallin et al., 2001). Mouse embryos which lack both *Wnt1* and *Wnt3a* show a marked deficiency in the neural crest derivatives and pronounced reduction in dorso-lateral neural precursors within the neural tube itself, suggesting Wnt signaling regulates expansion of the dorsal neural precursors (Ikeya et al., 1997). Conditional loss-of-function mutations of the *β-catenin* gene using *Wnt1Cre*, which express Cre recombinase in the dorsal neural tube and all neural crest cells, also resulted in dramatic defects in neural crest derivatives (Brault et al., 2001; Hari et al., 2002). Although several neural crest-derived structures develop normally, the *Wnt1Cre/β-catenin* mutants lack melanocytes and sensory neural cells in dorsal root ganglia (Hari et al., 2002). The formation of a further neural crest cell derivative, the heart outflow tract, is also affected in the conditional *Wnt1Cre/β-catenin* mutant (Kioussi et al., 2002). Additionally, Wnt/β-catenin signaling directly regulates expression of *Mitf-M* which is essential for the melanocyte differentiation and *Gbx2* which is required for the neural crest induction by recruiting Tcf/β-catenin to its promoter (Dorsky et al., 2000; Li et al., 2009; Saito et al., 2002).

The central nervous system development requires Wnt/β-catenin signaling. For instance, the midbrain and the cerebellum fail to develop in mouse embryos homozygous for *Wnt1*-null allele (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Inactivation of *β-catenin* by *Wnt1Cre* results in absence of part of the midbrain and the entire cerebellum, which is reminiscent of *Wnt1* knockout mice (Brault et al., 2001). This indicates Wnt1 acts through canonical β-catenin signaling in midbrain and hindbrain development (Brault et al., 2001). A key role of Wnt1 signaling is to maintain *Engrailed-1* expression that is essential for the midbrain development in mice (Danielian and McMahon, 1996). In addition, *Engrailed-2* gene is a direct target of Wnt/β-catenin signaling pathway in *Xenopus* (McGrew et al., 1999). Wnt signaling also plays an essential role in neuronal specification of the dorsal spinal cord. *Wnt1* and *Wnt3a* are expressed in overlapping regions within the central nervous system, predominantly along the dorsal midline from the diencephalon to the spinal cord (McMahon and Bradley, 1990; Parr et al., 1993; Roelink and Nusse, 1991; Wilkinson et al., 1987). Mouse embryos lacking both *Wnt1* and *Wnt3a* are defective in generation of D1 and D2 classes of dorsal interneurons during the spinal cord development (Muroyama et al., 2002).

Furthermore, expression of D1 marker *LH2A* and D2 marker *Islet1* are activated by Wnt3a condition medium in the explants from the medial region of avian neural plate (Muroyama et al., 2002). Indeed, it has been shown that Wnt/β-catenin directly regulates expression of *Islet1* through its promoter in the cardiac progenitors (Lin et al., 2007). In addition, Wnt/β-catenin signaling is essential for the maintenance of proliferation of neuronal progenitors and the decision of neuronal progenitors to proliferate or to differentiate in the spinal cord (Zechner et al., 2003). The tissue mass of the spinal cord is reduced in *β-catenin* loss-of-function mutants because of lower numbers of progenitors and higher numbers of differentiated neurons. On the contrary, the spinal cord of *β-catenin* gain-of-function mutants is enlarged in mass since the neuronal precursor population is increased in size (Zechner et al., 2003).

Wnt/β-catenin pathway also provides a key signal for determining cell lineages during pituitary development. Multiple members of the canonical Wnt pathway, including *Wnt4*, *Frizzled 2*, *Tcf4*, *β-catenin* and *APC*, are expressed in the developing pituitary (Douglas et al., 2001; Olson et al., 2006). Wnt4 is implicated as a necessary element in anterior pituitary precursor cell expansion (Treier et al., 1998). Mice deficient in *Wnt5a* display abnormal morphology in the dorsal part of the developing pituitary (Cha et al., 2004). *Tcf4* null mutant embryos exhibit severe pituitary overgrowth (Brinkmeier et al., 2003). Canonical Wnt signaling directly regulates expression of the transcription factor *Pitx2* which is required at multiple stages of pituitary development (Kioussi et al., 2002). Wnt/β-catenin signaling is required for specific cell-lineage determination in the anterior pituitary gland (Olson et al., 2006) Transcription factors *Pit1* and *Prop1* are required for the pituitary gland development (Hermesz et al., 1996; Li et al., 1990; Sornson et al., 1996). β-catenin directly associates with Prop1 to activate *Pit1* expression. Furthermore, the Prop1/β-catenin complex simultaneously represses expression of the *Hesx1* which negatively regulates expression of *Pit1* via recruitment of Groucho, HDACs, and Reptin (Olson et al., 2006).

There are multiple evidences that Wnt/β-catenin signaling plays a role in tooth development. Several Wnt genes such as *Wnt10a* and *Lef1* are expressed in the tooth germs (Kratochwil et al., 2002). Canonical Wnt signaling is active at multiple stages of tooth morphogenesis (Liu et al., 2008). Constitutive activation of the Wnt/β-catenin pathway in the dental epithelium by either expression of stabilized form of β-catenin or conditional deletion of *Apc* results in supernumerary tooth formation (Järvinen et al., 2006; Kuraguchi et al., 2006; Liu et al., 2008; Wang et al., 2009). In mice the inhibition of canonical Wnt signaling either by deleting *Lef1* function or ectopically expressing the Wnt inhibitor *Dkk1* arrests tooth morphogenesis at an early stage (Andl et al., 2002; Kratochwil et al., 2002; van Genderen et al., 1994). In addition, Lef1 directly regulates expression of *Fgf4* gene and beads soaked with recombinant Fgf4 protein can rescue the developmental arrest in *Lef1* null mutant tooth germs (Kratochwil et al., 2002).

1.2. Overview of vertebrate eye development

The first indication of the vertebrate eye development is evagination of the diencephalon towards the surface ectoderm to form the optic vesicle (Fig. 2A) (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 (Fig. 2B). 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 (Fig. 2C). 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; Martínez-Morales et al., 2004). The inner layer of the optic cup will give rise to the different layers of the mature retina. The pool of retinal progenitor cells expands by proliferation and will subsequently generate the Müller glia cells and the six types of neurons, retinal ganglion cells, amacrine cells, horizontal cells, bipolar cells, and rods and cones photoreceptor cells (Graw, 2003).

1.2.1. Development of the ocular lens

As the lens placode detaches from the surface ectoderm, it forms the lens cup and subsequently the lens vesicle which is nearly spherical with a large central cavity (Fig. 2D). The cells at the posterior portion of the lens vesicle elongate till they reach the anterior epithelial cells and fill the entire lens vesicle (Fig. 2E). These elongated cells are referred as primary lens fiber cells. The cells at the anterior portion of the lens vesicle remain as epithelial cells. The lens epithelial cells are mitotically active and provide a continuous source of undifferentiated cells that migrate toward the equator of the lens where the cells initiate differentiation into secondary fiber cells that form concentric layer overlying the core of primary fiber cells. The terminally differentiated lens fiber cells lose their organelles including the mitochondria, nucleus, and endoplasmic reticulum. The lens continues to develop throughout life, although at a slower rate, with new fibers successively added and retained for the entire life of the organism (Cvekl and Piatigorsky, 1996; Chow and Lang, 2001). Fiber cell differentiation is characterized by a dramatic increase in the expression of crystallins and other lens-specific proteins such as MIP26 and connexin 43. Crystallins are a

large family of proteins that constitute 90% of the water-soluble proteins in the lens fiber cells. In mammals, the lens contains three major classes of crystallin, designated as α-, β-, and γ-crystallin. Each crystallin is expressed in the lens with spatial and temporal expression patterns characteristic for each class (Graw, 2003). αA-crystallin is detected in the mouse lens pit and later becomes abundant in the lens fiber cells (Robinson and Overbeek, 1996). On the other hand, γ-crystallins are highly expressed in the developing lens fiber cells and later in the secondary fiber cells, but not in the lens epithelium cells (Santhiya et al., 1995).

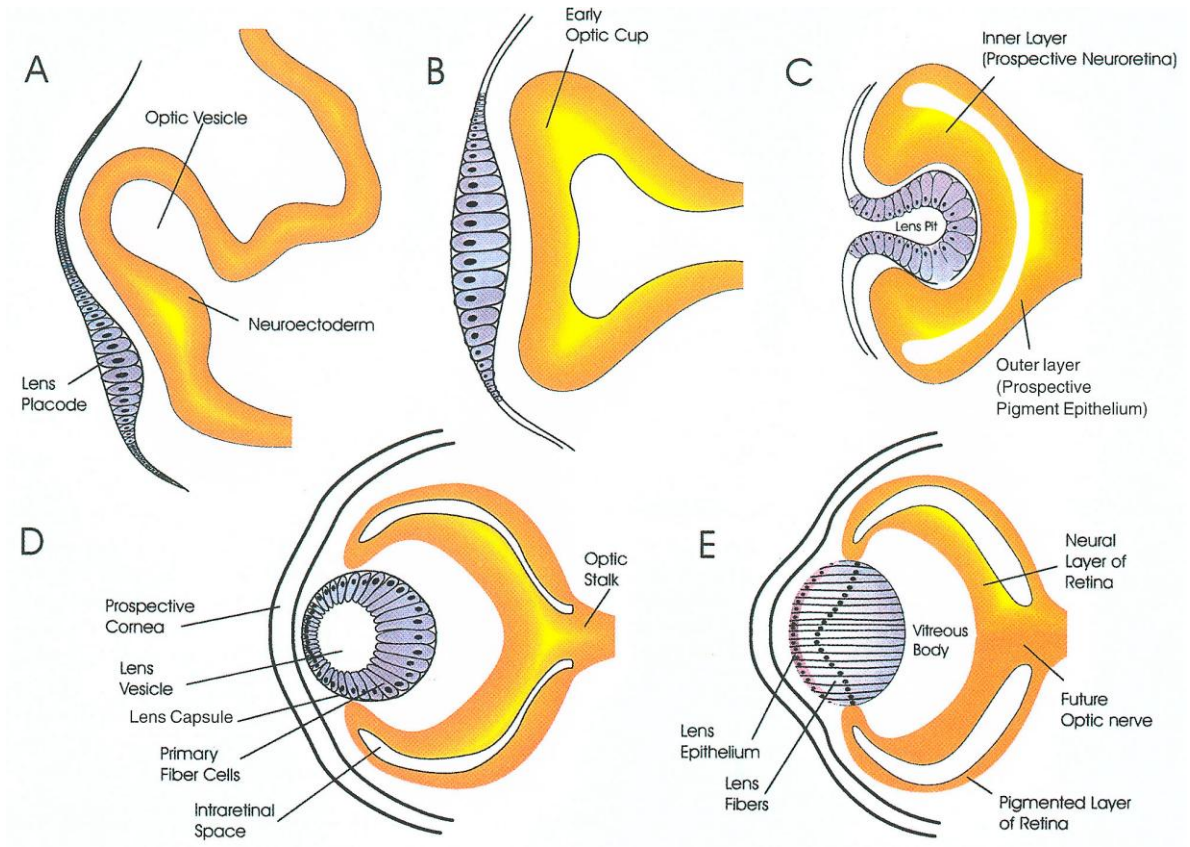

Fig. 2. Schematic view of a developing eye in mice.

(A) At E9.0-E9.5, the diencephalon evaginates towards the surface ectoderm to form the optic vesicle. When the optic vesicle meets the head surface ectoderm, it induces the formation of the lens placode. **(B)** At E9.5-10.0, the lens placodes becomes thickened. **(C)** At 10.5, coordinated invagination of the optic vesicle and the lens placode leads to formation of the double-layered optic cup and the lens pit. The outer layer of the optic cup gives rise to the retinal pigmented epithelium (RPE), whereas the inner layer differentiates into the neural retina. **(D)** At E11.5, the lens pit closes to form the lens vesicle, the prospective cornea becomes visible and the retina begins to differentiate. Once the lens vesicle is formed, the primary lens fibers elongate from the posterior epithelium of the lens vesicle and gradually fill its entire lumen. **(E)** At E13.5, the lens contains three regions: an anterior zone of dividing epithelial cells, an equatorial zone of cellular elongation, and a posterior and central zone of crystallin-containing fiber cells. The inner layer of the optic cup subsequently generate the Müller glia cells and the six types of neurons, retinal ganglion cells, amacrine cells, horizontal cells, bipolar cells, and rods and cones photoreceptor cells. (Adapted from Cvekl and Piatigorsky 1996)

1.2.2. Transcriptional regulation of the lens development

Transcription factor *Pax6* is essential for lens development (Willians et al., 1998, Dimanlig et al., 2001, Ashery-Padan et al., 2000). *Pax6* encodes a paired box and a paired-like homeobox gene and contains two DNA-binding domains called a paired domain and a homeobox domain. *Pax6* is expressed in both the lens-inducing optic vesicle and in the lens forming surface ectoderm (Grindley et al., 1995). At later stage, the anterior lens epithelial cells retain high expression of *Pax6* while the posterior lens vesicle cells that differentiate into primary lens fiber cells lose *Pax6* expression. In the fetal and postnatal stages, *Pax6* expression is maintained in the lens epithelium and in the equatorial transitional zone (Grindley et al., 1995). The lens inductive signals from the optic vesicle trigger a thickening of the surface ectoderm known as the lens placode and *Pax6* is needed for the surface ectoderm to respond to the signal (Fujiwara et al., 1994). Conditional deletion of *Pax6* in the lens surface ectoderm has confirmed the requirement of *Pax6* in the lens development. Although lens induction occurs in the mutant, further development of the lens was arrested (Ashery-Padan et al., 2000). Pax6 is involved in the activation of crystallins such as *αB-*, *αA-*, *δ1- crystallins* expressed in the lens epithelium and in the repression of the fiber cell-specific *βB1-crystallin* (Cvekl et al., 1994, 1995; Duncan et al., 1998; Richardson et al., 1995). In addition to crystallin genes, Pax6 directly controls expression of several integrin genes that are required for lens differentiation through their promoters (Duncan et al., 2000). Additionally, Pax6 also regulates expression of *Shroom3* that is necessary for apical constriction during lens placode invagination (Plageman et al., 2010).

Members of the group B1 *Sox* family members are also involved in the lens development. *Sox* genes encode transcription factors containing a sex-determining factor-related HMG box (Kamachi et al., 1998). *Sox1*, *Sox2* and *Sox3* are all expressed in the developing lens. *Sox1* is first expressed during the invagination of the lens placode and later is predominantly expressed in lens fiber cells. On the other hand, *Sox2* is expressed during early eye development in the lens placode and in the presumptive lens vesicle (Kamachi et al., 1998). *Sox2* expression in the early lens placode is dependent on the optic vesicle and this implies that it is responsive to the lens induction signals (Kamachi et al., 1998). Conditional deletion of *Sox2* in the lens placode arrests lens development at the lens pit stage (Smith et al., 2009). Sox2 is involved in the lens development through its regulation of the *δ 1-crystallin* gene in the chick (Kamachi et al., 2001), and of *N-cadherin* (Matsumata et al., 2005), an adhesion molecule known to be required for normal lens morphogenesis and the differentiation of lens fiber cells (Leonard et al., 2011; Pontoriero et al., 2009). Expression of *Sox2* gene is regulated by Sox2 itself in combination with Pax6 through the N-3 enhancer that is active in the presumptive lens (Inoue et al., 2007). Additionally, recent study has shown that *Sox2* expression is dependent on Pax6 at the lens pit stage using conditional *Pax6* loss-of-

function mutant (Smith et al., 2009).

Members of the TALE (three amino acid loop extension) family of homeodomain-containing transcription factors play important roles during development. This family is composed of five members: *Meis1*, *Meis2*, *Meis3*, *Prep1*, and *Prep2* (Berthelsen et al., 1998; Fognani et al., 2002; Moskow et al., 1995; Nakamura et al., 1996). These proteins all contain an N-terminal Meis domain that mediates the interaction with Pbx transcription factor and a C-terminal homeodomain (Zhang et al., 2006). *Meis1*-deficient mice develop small lenses (Hisa et al., 2004). Meis1 and Meis2 bind a specific sequence in the *Pax6* lens placode enhancer that is required for its activity (Zhang et al., 2002). In *Prep1* mutant mice, eye development arrests by E10.5 and lens tissue is absent (Ferretti et al., 2006; Rowan et al., 2010). Furthermore, Prep1 regulates *Pax6* expression by binding to its lens enhancer (Rowan et al., 2010).

Pitx3 encodes a paired-like class of homeobox transcription factors. It is expressed first in the lens vesicle, and later in the anterior epithelium and the lens equator. *Pitx3*-deficient *Aphakia* mice show the abnormal lens phenotype (Rieger et al., 2001; Semina et al., 2000). In this mutant, the lens begins to form, but lens development is eventually arrested and the lens disappears (Grimm et al., 1998; Varnum and Stevens, 1968; Zwaan, 1975; Zwaan and Kirkland, 1975).

Several members of the *Maf* family of basic-leucine zipper transcription factors are implicated in the lens induction and differentiation. *c-Maf* plays an essential role in lens differentiation. *c-Maf* is first expressed in the early lens placode and later in the elongating lens fiber cells. *c-Maf* null mutant mice are microphthalmic secondary to defective lens formation and the expression of crystallin genes is severely impaired (Kim et al., 1999; Ring et al., 2000). Indeed chromatin immunoprecipitation experiments have shown binding of c-Maf to *αA-crystallin* locus (Yang et al., 2006). *L-Maf* is first expressed in the thickened surface ectoderm before the lens placode formation and is later expressed by both epithelial and fiber cells (Ishibashi and Yasuda, 2001; Ogino and Yasuda, 1998). Ectopic expression of *L-Maf* induces lens differentiation in the neural retina cultures and chick embryos (Ogino and Yasuda, 1998). In addition, L-Maf binds and transactivates through lens-specific enhancer elements of crystallin genes in co-transfection experiment (Ogino and Yasuda, 1998).

Six3, a homologue of the *Drosophila sine oculis* encodes a homeodomain transcription factor. *Six3* expression first appears during the formation of the lens placode and is later restricted to the lens epithelium (Oliver et al., 1995). Conditional deletion of mouse *Six3* in the presumptive lens ectoderm disrupts lens formation. In the most severe cases, lens induction and specification are defective and the lens placode and lens are absent. In this mutant, *Pax6* is downregulated and *Sox2* is absent in the lens placode (Liu et al., 2006).

Additionally, it has been shown that Six3 directly activates *Pax6* and *Sox2* expression (Liu et al., 2006). Furthermore, human *SIX3* promoter is positively regulated by PAX6 and PROX1 and negatively by its own feedback loop (Lengler and Graw, 2001).

Prox1, a vertebrate homologue of *Drosophila prospero*, encodes a divergent homeodomain protein (Oliver et al., 1993). *Prox1* is first detected in the posterior portion of the lens pit (Duncan et al., 2002). Prox1 is critical for lens fiber cell elongation and differentiation. In *Prox1* null mutant mice, although the initial induction of the lens vesicle is unaffected, lens fibers do not elongate properly and a hollow lens is formed. (Wigle et al., 1999). Prox1, Pax6 and Mafs cooperatively activate the chicken *βB1-crystallin* promoter by binding one of functionally important cis elements (Cui et al., 2004). In addition, Prox1 also regulates expression *γF-crystallin* by binding to the promoter (Lengler et al., 2001).

Transcription factor *Forkhead* box E3 (*FoxE3*) plays critical role in vertebrate lens formation. In mice, *FoxE3* is initially expressed in the undifferentiated lens placode, and later its expression persists in the anterior lens epithelium, but not in the differentiating lens fiber cells (Blixt et al., 2000; Brownell et al., 2000). *FoxE3* expression is lost both in *Pax6* null mutants and *Rx* null mutants (Brownell et al., 2000). In addition, early *FoxE3* expression is highly sensitive to *Pax6* gene dosage, suggesting Pax6 is required for *FoxE3* expression (Blixt et al., 2007). In mice with a targeted deletion of both alleles of *FoxE3*, the lens is smaller and the anterior lens epithelium fails to separate from the cornea. Furthermore the differentiating fiber cells do not lose their nuclei and also do not elongate properly (Medina-Martinez et al., 2005).

BMP-mediated signaling in early lens development is essential for lens formation, particularly involving two members of this protein family of morphogens, Bmp4 and Bmp7 (Dudley et al., 1995; Furuta and Hogan, 1998; Wawersik et al., 1999). In the mouse, *Bmp4* is expressed strongly in the optic vesicle and weakly in the surrounding mesenchyme and surface ectoderm (Furuta and Hogan, 1998). Lens induction is absent in *Bmp4* homozygous null mutant embryos (Furuta and Hogan, 1998). *Sox2* fails to be induced in the mutant surface ectoderm while expression of *Pax6* and *Six3* is not affected (Furuta and Hogan, 1998). *Bmp7* is a critical for the lens placode formation. Before appearance of the lens placode, *Bmp7* localizes to the head surface ectoderm and the directly adjacent mesenchymal cells (Dudley et al., 1995; Wawersik et al., 1999). Bmp7 protein is detected in the head surface ectoderm and the invaginating lens placode at the optic cup stage. At later stage, it is exclusively present in the head surface ectoderm immediately dorsal to the lens vesicle (Wawersik et al., 1999). *Bmp7* mutant embryos exhibit defects in lens development, resulting from a failure in the lens placode development. Prior to the lens placode formation, the developing eye appears to be normal in *Bmp7* mutant embryos, however, later no placodal structure is visible in the *Bmp7* mutant head surface ectoderm (Dudley et al., 1995; Wawersik et al., 1999). In addition, *Pax6*

expression is lost in the presumptive lens placode. Therefore, it is suggested that *Bmp7* functions upstream of *Pax6* and regulates lens placode induction (Wawersik et al., 1999). In addition, recent study has shown that the type I BMP receptors, *Bmpr1a* and *Acvr1* are required for lens formation (Rajagopal et al., 2009).

FGF signaling is implicated to play an important role for the lens placode formation and later for lens development and differentiation. The developing lens expresses all four genes encoding the FGF receptors, *Fgfr1-4* (Lea et al., 2009) and *Fgf1*, *Fgf2* (de Iongh and McAvoy, 1993), *Fgf8* (Kurose et al., 2005) and *Fgf15* (Kurose et al., 2004, 2005). The developing optic vesicle expresses *Fgf8*, *Fgf9* and *Fgf15* (Kurose et al., 2004, 2005; McWhirter et al., 1997; Vogel-Höpker et al., 2000). Transgenic mice that express a dominant-negative FGF receptor exclusively in the presumptive lens ectoderm show defects in formation of the lens placode with reduced levels of expression for markers of lens induction such as *Pax6*, *Sox2* and *FoxE3* (Faber et al., 2001). In addition, disruption of FGF receptor substrate 2 alpha (FRS2alpha), that plays a critical role in FGF-induced MAP kinase stimulation and PI-3 kinase activation, can result in defective induction of the lens and retina (Gotoh et al., 2004). Later stages of the lens development also require FGF signaling. Lens fiber cell differentiation can be suppressed with dominant-negative FGF receptors (Chow et al., 1995; Robinson et al., 1995). Furthermore, fiber cell differentiation can be enhanced by FGF ligands either in culture or in vivo (McAvoy and Chamberlain, 1989; Robinson et al., 1995). Mice lacking *Fgfr1*, *Fgfr2*, and *Fgfr3* in the lens displayed profound defects, including lack of fiber cell elongation, abnormal proliferation in prospective lens fiber cells, and reduced expression of lens specific markers such as *Prox1* (Zhao et al., 2008).

1.2.3. Molecular mechanisms of the retinal pigment epithelium development

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 neural retina (NR) (Horsford et al., 2005; Martínez-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; Steingrímsson et al., 2004). Each isoform shows a unique expression pattern (Goding, 2000; Steingrímsson 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., 2000). Mitf regulates pigment cell-specific transcription of genes encoding melanogenic enzymes such as tyrosinase (*Tyr*), and tyrosinase-related protein 1 and 2 (*Trp1* and *Trp2*)

(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 (Bäumer 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* sub-family 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 (Bäumer et al., 2003; Martinez-Morales et al., 2001). Otx2 cooperates with Mitf to regulate expression of melanogenic enzymes (Martínez-Morales et al., 2003). *Otx1* and *2* double-deficient mice show severe 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 (Bäumer 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 *Trp2*, although transdifferentiation of the RPE does not occur (Rowan et al., 2004).

Pax6 and *Pax2* are also 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). 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; Torres et al., 1996). A null mutation of *Pax2* results in extension of the RPR 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).

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). 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 (Müller et al., 2007).

1.2.4. Wnt signaling in the eye development

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/β-catenin 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).

Wnt signaling is implicated to play important roles in the lens epithelium differentiation. Multiple Wnt ligands, Frizzled receptors, LRP coreceptors, and Wnt antagonists such as *Sfrp1* and *Dkk1* are expressed in the developing lens (Stump et al., 2003). Furthermore, it has been shown that Wnt/β-catenin is active in the anterior lens epithelium by using reporter mouse line (Liu et al., 2003, 2006). *Lrp6* null mutants exhibit defects in the lens epithelium and disruption of lens fiber cell differentiation (Stump et al., 2003). Consistently, analysis of *β-catenin* loss-of-function mutants revealed that β-catenin is required for proliferation and differentiation of the lens epithelium cells (Cain et al., 2008).

Analysis of *β-catenin* loss-of-function and gain-of-function mutants indicate that canonical Wnt/β-catenin signaling is required to suppress lens formation in the periocular ectoderm (Kreslova et al., 2007; Smith et al., 2005). Conditional deletion of the *β-catenin* gene in the presumptive lens and surrounding head surface ectoderm results in the ectopic formation of Prox-1 and β-crystallin-positive ectopic lentoid bodies in periocular ectoderm (Kreslova et al., 2007; Smith et al., 2005). On the other hand, *β-catenin* gain-of-function resulted in the complete absence of lens formation (Smith et al., 2005). *Rx*-deficient embryos do not form a lens, despite the fact that *Rx* is not expressed in the surface ectoderm (Mathers et al., 1997; Zhang et al., 2000). Interestingly, recent paper has shown that lens formation can be restored in *Rx*-deficient embryos by the elimination of *β-catenin* expression in the head

surface ectoderm (Swindell et al., 2008).

Prior to the optic vesicle formation, the group of cells that will give rise to the eyes exists as a single bilateral domain called the eye field (Chuang and Raymond, 2002). Both canonical and noncanonical Wnt signaling are involved early stages in eye field development. During late gastrulation, several Wnt ligands *Wnt1*, *Wnt10b*, *Wnt8b*, and *Wnt11* are expressed in the prospective diencephalon close to the border with the eye field in zebrafish (Cavodeassi et al., 2005). It has been shown that Wnt/β-catenin signaling antagonizes eye specification by the activity of Wnt8b and Frizzled 8a, while noncanonical Wnt11 and Frizzled 5 promote eye field development through local antagonism of Wnt/β-catenin signaling in zebrafish (Cavodeassi et al., 2005). Additionally, Wnt11 regulates the behavior of eye field cells, promoting their cohesion. Furthermore, noncanonical Wnt4 signaling is required for eye development in *Xenopus* (Maurus et al., 2005). Interestingly, it was demonstrated that *Eaf2* that encodes a cofactor of the RNA polymerase II elongation factor ELL is a target of Wnt4 signaling and Eaf2 regulates expression of transcription factor *Rx* (Maurus et al., 2005). In the zebrafish *masterblind* mutants, both telencephalon and eye are converted into diencephalon. It was shown that this mutant has a mutation in the GSK3β-binding domain of Axin1 which results in disruption of the binding of Axin1 to GSK3β and Tcf/Lef dependent transcription (Heisenberg et al., 2001). The zebrafish *headless* mutants are characterized by complete loss of eyes, forebrain and part of the midbrain due to a mutation in Tcf3 that represses Wnt target genes (Kim et al., 2000). In addition, overexpression of the Wnt receptor Frizzled 3 induces ectopic eye formation by activating noncanonical signaling in *Xenopus* embryos (Rasmussen et al., 2001).

Wnt antagonists Sfrps are essential for eye development. *Sfrp1* is needed for a proper establishment of the eye field within the forebrain and for retinal neurogenesis (Esteve et al., 2003, 2004; Rodriguez et al., 2005). Morpholino-based interference with *Sfrp1* expression in medaka results in a reduced eye field (Esteve et al., 2004). Overexpression of *Sfrp1* in chick retinal progenitors increases the number of retinal ganglion cells and cone photoreceptors while decreases the number of amacrine cells (Esteve et al., 2003). Sfrp1 also regulates the growth of retinal ganglion cell axons through the Frizzled2 receptor (Rodriguez et al., 2005).

Norrin activates canonical β-catenin signaling through Frizzled-4 and Lrp5 and is essential for controlling vascularization of the retina during development (Xu et al., 2004). The primary site of Frizzled 4 and signaling is in the endothelial cells and Muller glia cells are the principal source of Norrin (Ye et al., 2009). *Sox17* is upregulated by Norrin/Frizzled4 signaling and plays a central role in inducing the angiogenic program (Ye et al., 2009). Sox17 overexpression rescues defects in capillary formation by cultured endothelial cells lacking *Frizzled 4* (Ye et al., 2009). Recent study has shown that the tetraspanin Tspan12 is required for Norrin/β-catenin signaling in retinal endothelial cells (Junge et al., 2009). Tspan12

associates with the Norrin-receptor complex. Furthermore, it significantly increases Norrin/β-catenin signaling by modulating Frizzled 4 multimerization, but not Wnt/β-catenin signaling (Junge et al., 2009).

1.3. Overview of the telencephalon development

The telencephalon derives from cells at the anterior end of the neural plate. The anterior neural plate gives rise to the prosencephalon, which is subsequently subdivided into the anterior telencephalon and the more caudal diencephalon. The developing telencephalon is subdivided into two main territories, the dorsal telencephalon called the pallium and the ventral telencephalon called the subpallium (Fig. 3A). In mammals, the pallium further is split into two territories: the cerebral cortex which mainly gives rise to the neocortex and the hippocampus; and the dorsal midline which gives rise to the choroid plexus and the cortical hem. The subpallium primarily consists of the medial (MGE) and lateral ganglionic eminences (LGE), which later form the striatal and pallidal components of the basal ganglia (Fig. 3B) (Marín and Rubenstein, 2003). The pallium primary produces the excitatory glutamatergic projection neurons of the cerebral cortex, which sequentially reach the different layers of the cortex by radial migration. Progenitors of the subpallium generate GABA-ergic inhibitory neurons, including basal ganglia neurons, as well as interneurons that migrate tangentially to contribute to the formation of the cortex. (Hébert and Fishell, 2008). The initial subdivision that specify dorsal and ventral domains is regulated by the dorsalizing effects of *Gli3* expression (Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000) and the ventralizing influence of *Shh* expression (Ericson et al., 1995; Chiang et al., 1996).

During the telencephalon development, neural progenitor cells pass through expansion and neurogenesis phases. Before neurogenesis, neuroepithelial progenitor cells expand their population by symmetric cell division that produces two neuroepithelial progenitor cells (Götz and Huttner, 2005). After the onset of the neurogenesis, neuroepithelial progenitor cells give rise to radial glial cells. These cells undergo asymmetric division, generating one self-renewing radial glial cell and one daughter cell that become a neuron or another type of a neuronal progenitor cell called as basal progenitor cell (Götz and Huttner, 2005; Miyata et al., 2001; Noctor et al., 2001, 2004). The timing of the onset of the neurogenesis greatly affect the neuronal number, since it regulates the initial pool size of neural progenitor cells (Miyata et al., 2010). The basal progenitor cells subsequently divide symmetrically to produce two basal progenitor cells or two neurons (Haubensak et al., 2004; Noctor et al., 2004). Radial glial cells primary differentiate into oligodendrocytes, astrocytes and neurons (Anthony et al., 2004; Noctor et al., 2001) while basal progenitor cells seem to be restricted to producing neurons or glias (Haubensak et al., 2004; Noctor et al., 2004).

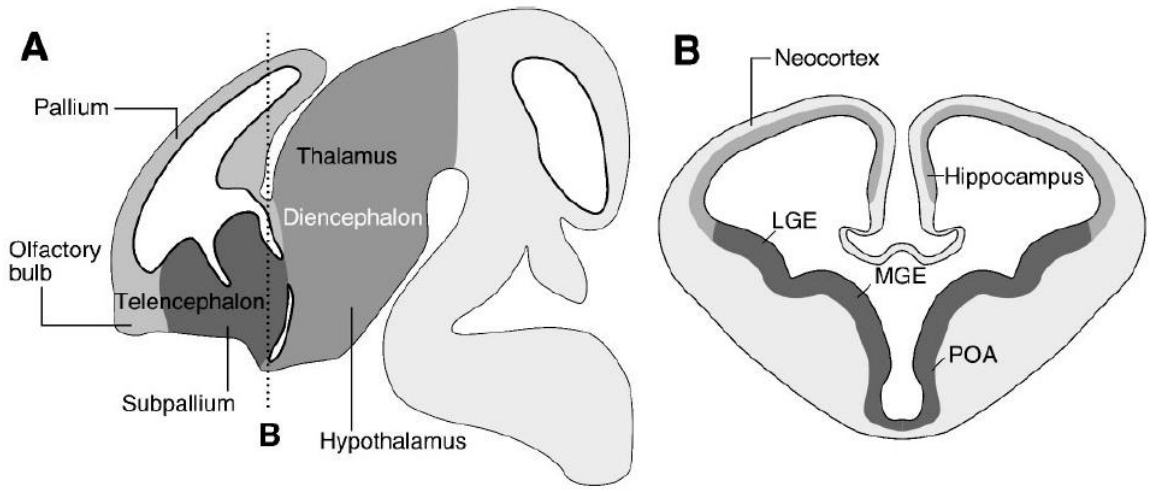

Fig. 3. Anatomical organization of the developing forebrain. (A) Schematic representation of a sagittal section through the brain of an E12.5 mouse showing the main subdivisions of the forebrain, the diencephalon and the telencephalon. In the telencephalon, the pallium (the dorsal telencephalon) is depicted in lighter gray than the subpallium (the ventral telencephalon). (B) Schematic representation of a transversal section through the telencephalon of an E12.5 mouse embryo, indicating some its main subdivisions. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, anterior preotic area. (Adapted from Marin and Rubenstein 2003)

Growth factors such as Fgf2 and Notch1 inhibit neuronal differentiation and promote the self-renewal capacity of the neural progenitor cells (Panchision and McKay, 2002). The neural progenitor cells are depleted in the early embryonic brains of *Notch1* null mutant mice (Hitoshi et al., 2002). Both neuronal and glial differentiation *in vitro* are enhanced by attenuation of Notch signaling and suppressed by expressing an active form of Notch1 (Hitoshi et al., 2002). Furthermore, transient ectopic expression of the transcription factor *Hes1* and *Hes5* that mediates Notch signaling keeps embryonal telencephalic cells undifferentiated (Ohtsuka et al., 2001). Fgf2 drives proliferation of the neural progenitor cells *in vivo*, and loss of *Fgf2* gene results in decreased cell number (Vaccarino et al., 1999). On the other hand, *Fgf10* promotes the radial glia differentiation. *Fgf10* is transiently expressed in the neuroepithelial cells coincident with the transition period of neuroepithelial cell differentiation into radial glial cells (Sahara and O'Leary, 2009). Targeted deletion of *Fgf10* delays radial glial cell differentiation, whereas overexpression has opposing effects (Sahara and O'Leary, 2009).

1.3.1. Wnt signaling in the telencephalon development

A number of Wnt genes are expressed during the telencephalon development (Grove

et al., 1998; Lee et al., 2000). *Wnt3a* and *Lef1* are required for mammalian hippocampus (Galceran et al., 2000; Lee et al., 2000). In the *Wnt3a* mutants, the hippocampus is missing or severely reduced in size because of loss of hippocampal progenitor pool (Lee et al., 2000). *Lef1*-deficient embryo lack dentate gyrus granule cells in the hippocampus. Furthermore, mice carrying a mutant allele of *Lef1* that is not only deficient in DNA binding but also impairs β-catenin-mediated transcription by other Tcf/Lef proteins lack the entire hippocampus complex (Galceran et al., 2000). Indeed, Wnt/β-catenin signaling regulates expression of *Emx2*, which is essential for growth for the hippocampus (Theil et al., 2002). Wnt/β-catenin signaling is essential for the maintenance of proliferation of neuronal progenitor cells by controlling the size of the progenitor pool. Transgenic mice expressing a stabilized β-catenin in neural precursors develop enlarged brains with increased cerebral cortical surface area due to an expansion of the progenitor population (Chenn and Walsh, 2002; Zechner et al., 2003). Additionally, Wnt/β-catenin signaling maintains progenitor proliferation in the ventral telencephalon. Targeted deletion of *β-catenin* in the MGE severely impairs proliferation due to premature cell cycle exit without grossly altering differentiated fate (Gulacsi and Anderson, 2008). Wnt/β-catenin signaling inhibits the self-renewal capacity of cortical neural precursor cells, and directs their neuronal differentiation only at late stages of cortical development (Hirabayashi et al., 2004). Overexpression of Wnt7a or of a stabilized form of β-catenin in mouse cortical neural precursor cell cultures induces neuronal differentiation even in the presence of Fgf2 which act as a self-renewal-promoting factor. On the contrary, inhibition of Wnt/β-catenin signaling suppresses neuronal differentiation *in vitro* and in the developing mouse neocortex (Hirabayashi et al., 2004). In addition, β-catenin/Tcf complex directly regulates the promoter of *neurogenin 1*, a gene implicated in cortical neuronal differentiation (Hirabayashi et al., 2004). Wnt/β-catenin signaling is required for dorso-ventral specification of the mouse telencephalon prior to the neurogenic period. Wnt/β-catenin signaling is highly active in the dorsal telencephalon. Inactivation of the signaling in the telencephalon results in downregulated expression of the dorsal telencephalic markers such as *Emx1* while ectopic activation leads to a repression of ventral telencephalic cell identities (Backman et al., 2005). Consistently, it has been shown that Wnt signals suppress ventral cell fates and that sequential Wnt and FGF signaling induces dorsal telencephalic character in the chick telencephalon (Gunhaga et al., 2003).

2. HYPOTHESES AND AIMS OF THE STUDY

Wnt signaling plays important roles in multiple developmental processes and has a profound effect on cell proliferation, cell polarity and cell fate determination. The signaling is initiated by the interaction of Wnt ligands with the Frizzled/LRP receptor complex. Wnt signal can be mediated through three main intracellular branches, canonical Wnt/β-catenin signaling, noncanonical Wnt/PCP, and Wnt/Ca^{2+} pathways. This thesis focuses on studying the role of the Wnt/β-catenin signaling during embryonic development, especially, the telencephalon and the eye development. Specific aims of my Ph.D. thesis are as follows.

1. During the telencephalon development, multiple Wnt genes are expressed, including *Wnt3a*, *Wnt7a*, *Wnt7b*, and *Wnt8b*. Wnt/β-catenin signaling is active in the pallium and is important for dorso-ventral specification of the telencephalon. Furthermore, transgenic mice which express a stabilized form of β-catenin in neural progenitor cells develop enlarged brains. These reports indicate critical roles of canonical Wnt signaling in the telencephalon development. However, the molecular mechanisms that contribute to the phenotype are largely unknown. In this thesis we aimed at identifying transcriptional targets of Wnt/β-catenin signaling in the developing telencephalon to provide further insights.

2. Wnt/β-catenin signaling pathway has been implicated in the formation of the retinal pigment epithelium (RPE). Several components of the signaling such as *Wnt2b* are expressed in the presumptive RPE. Additionally, Wnt/β-catenin signaling is highly active in the developing RPE at the stage of the optic cup formation. Therefore, we studied the role of the canonical Wnt signaling pathway in the RPE development by inactivating or activating its key component, β-catenin.

3. Wnt/β-catenin signaling is implicated to suppress lens fate. Conditional deletion of *β-catenin* genes in the presumptive lens and periocular ectoderm results in the formation of ectopic lentoid bodies in the periocular region. Interestingly, it has been shown that Wnt/β-catenin signaling is active in the periocular ectoderm. On the contrary, ectopic activation of the signaling abrogates lens development. These observations indicate that inhibition of Wnt/β-catenin signaling within the lens placode and its activation in the periocular ectoderm are required for the proper lens formation and patterning. Thus we investigated the molecular mechanisms responsible for the inhibition of Wnt/β-catenin signaling in the lens development.

3. MATERIALS AND METHODS

Histology

Immunohistochemistry, *in situ* hybridization, Hematoxylin and eosin staining, and X-gal staining.

Gene expression and transcriptional regulation

Chromatin immunoprecipitation (ChIP), Luciferase reporter assay, Electrophoretic Mobility Shift Assay (EMSA), and Microarray

Protein-protein interaction

Co-immunoprecipitation and GST-pull-down Assay

4. RESULTS AND DISCUSSIONS

4.1. Comments on presented publications

Wnt/β-catenin signaling is essential for the maintenance of proliferation of neuronal progenitor cells and dorso-ventral specification in the telencephalon. During the eye development Wnt/β-catenin signaling has been implicated in the formation of the RPE. In addition, it is required to suppress lens formation in the periocular ectoderm. However, the molecular mechanisms how Wnt/β-catenin signaling controls developmental processes in the telencephalon and the eye and how the signaling is regulated in these tissues remain largely unknown. In the following three research papers, I studied the role of Wnt/β-catenin signaling during the telencephalon and the eye development using *β-catenin* gain-of-function and loss-of-function mutant mice.

Research paper I

Fujimura N., Vacik T., Machon O., Vlcek C., Scalabrin S., Speth M., Diep D., Krauss S., Kozmik Z.: Wnt-mediated down-regulation of Sp1 target genes by a transcriptional repressor Sp5.

J Biol Chem. 2007 Jan 12;282(2):1225-37.

To identify target genes of Wnt/β-catenin signaling during CNS development, two lines of mice were interbred to activate Wnt/β-catenin signaling. *Catnb*$^{lox(ex3)}$, in which exon 3 of *β-catenin* gene is floxed by loxP sites (Harada et al., 1999), was mated to *Nes11Cre*, a transgenic mouse line expressing Cre recombinase in neural progenitor cells (Tronche et al., 1999). Exon 3 of *β-catenin* gene encodes phosphorylation sites necessary for β-catenin degradation (Liu et al., 2002). Therefore, Cre recombinase-mediated deletion of exon 3 of *β-catenin* gene results in the expression of a stabilized form of β-catenin, which leads to the constitutive activation of Wnt/β-catenin signaling. RNA was isolated from the telencephalon at E13.5, and overall gene expression was analyzed by Affymetrix microarray. We found that *Sp5*, a member of *Sp1* family, was up-regulated 32-fold in the Affymetrix data, and strong gene activation was confirmed by *in situ* hybridization.

We next examined whether *Sp5* is regulated by Wnt/β-catenin signaling directly. Computer analysis revealed that the *Sp5* proximal promoter contains five Tcf/Lef consensus sites. Luciferase reporter assay showed that the *Sp5* promoter is responsive to Wnt/β-catenin signaling. We further provided evidence that Tcf/Lef transcription factors bind to their consensus sites in the promoter by chromatin immunoprecipitation and EMSA. We therefore conclude that *Sp5* is a direct target gene of Wnt/β-catenin signaling.

To our surprise, many genes were down-regulated in the telencephalon of *Nes11Cre/Catnb*$^{lox(ex3)}$ mice as compared with control mice. We hypothesized that down-regulation of at least some of the genes could be mediated by Sp5, because Sp5 itself is highly induced in *Nes11Cre/Catnb*$^{lox(ex3)}$, and several Sp1 family members are known to act as repressors (Kaczynski et al., 2003). We therefore examined the transcriptional properties of Sp5. Gal4 reporter assay revealed that Sp5 works as a repressor. We further investigated the mechanism(s) that control the transcriptional properties of Sp5. We found a core mSin3a-interacting domain (Zhang et al., 2001) and confirmed the interaction of Sp5 with mSin3a by coimmunoprecipitation and GST-pull-down assay. Our results suggest that the transcriptional repression activity is mediated through the interaction with mSin3a corepressor.

Sp5 belongs to the large family of *Sp1*-like transcription factors. Intrigued by the fact that the founding member, Sp1, acts as an activator, whereas Sp5 acts as a repressor, we next examined whether Sp5 down-regulates Sp1 target genes. To examine whether Sp5 has the same DNA binding specificity as Sp1, EMSA was performed. Our data revealed that Sp5 has a very similar if not identical DNA binding specificity as Sp1. We next examined whether Sp5 represses a well characterized Sp1 target gene *p21* (Koutsodontis et al., 2002). Using luciferase reporter assay and EMSA, we showed that Sp5 represses p21 promoter by binding to the Sp1 binding sites within the *p21* promoter.

To obtain further evidence that Sp5 represses Sp1 target genes *in vivo*, and to identify additional Sp5 targets in neural cells, we have established primary neurosphere cultures over-expressing Sp5. We then profiled gene expression of Sp5-overexpressing neurospheres by Affymetrix microarray analysis. We found that 107 genes were down-regulated >2-fold in Sp5-infected neurospheres. Notably, 90 genes were Sp1 target genes or genes that contain canonical Sp1 binding sites in the proximal promoter (-500/+1) and 5′-untranslated region. In conclusion, our results show that Sp5 represses Sp1 target genes.

Research paper II

Fujimura N., Taketo MM., Mori M., Korinek V., Kozmik Z.: Spatial and temporal regulation of Wnt/β-catenin signaling is essential for development of the retinal pigment epithelium.

Dev Biol. 2009 Oct 1;334(1):31-45.

Wnt/β-catenin signaling is highly active in the presumptive RPE at E10.5, and subsequently restricted to the distal RPE. 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.

To achieve conditional inactivation of Wnt/β-catenin signaling, *Trp1-Cre* which

expresses Cre recombinase in the RPE (Mori et al., 2002) was combined with a conditional allele of *β-catenin Catnb*$^{lox(ex2\text{-}6)}$ in which exons 2-6 are flanked by loxP sites (Brault et al., 2001). The prospective RPE of the wild-type mice was thin and the cells formed a single layer; on the contrary, the analogous tissue of the mutants was thicker. 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. Interestingly, expression of Mitf and Otx2 was significantly downregulated in the thickened RPE of mutant. The absence of Mitf results in transdifferentiation of the RPE into the neural retina (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 (Bäumer et al., 2003; Burmeister et al., 1996; Mathers et al., 1997). In contrast to the wild-type mice, the expression of Chx10 and *Rx* in the 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.

To achieve the ectopic activation of Wnt/β-catenin signaling in the entire RPE, *Trp1-Cre* mice were crossed with *Catnb*$^{lox(ex3)}$ mice. Interestingly, in the mutant, the proximal part of the RPE was significantly thickened while the distal part of the RPE remained thin by E11.5. 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; Martínez-Morales et al., 2004). By E11.5, the expression of *Mitf* and *Otx2* was downregulated in the presumptive RPE of the 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. On the contrary, there was no significant difference in the expression pattern of the NR markers *Chx10* and *Rx*. This suggests that the abnormal thickness of the proximal RPE is not caused by transdifferentiation to NR. 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.

The inactivation of Wnt/β-catenin signaling results in downregulation of *Mitf* and *Otx2*, indicating that they may be directly regulated by Wnt/β-catenin signaling. Indeed, it has been indicated that the expression of *Otx2* is directly regulated by Wnt/β-catenin signaling through FM2 enhancer (Kurokawa et al., 2004). To investigate possible direct regulation by Wnt/β-catenin signaling we searched for Tcf/Lef binding sites within the promoters of *Mitf-D*, and *Mitf-H* and found that the promoters of *Mitf-D* and *Mitf-H* contain potential Tcf/Lef binding sites. Chromatin immunoprecipitation assay showed that Tcf/Lef/β-catenin complex binds to these sites *in vivo*. The luciferase reporter gene assay further showed Lef1/β-catenin activated the *Mitf-H* promoter and *Otx2* FM2 enhancer. These results indicate that Wnt/β-catenin signaling directly regulates expression of *Mitf-H* and *Otx2*, and possibly, of *Mitf-D*. Taken

together, our data suggest that Wnt/β-catenin signaling regulates the RPE development by inducing expression of the key differentiation genes.

Research paper III

Machon O., Kreslova J., Ruzickova J., Vacik T., Klimova L., **Fujimura N.**, Lachova J., Kozmik Z.: Lens morphogenesis is dependent on Pax6-mediated inhibition of the canonical Wnt/β-catenin signaling in the lens surface ectoderm.

Genesis. 2010 Feb;48(2):86-95.

Wnt/β-catenin signaling is required to suppress lens fate in the periocular ectoderm and inhibition of the signaling in the presumptive lens placode is essential for proper lens development (Kreslova et al., 2007; Smith et al., 2005). To provide further evidence, we performed conditional activation of Wnt/β-catenin signaling in the presumptive lens and retina. Consistent with the previous study (Smith et al., 2005), the ectopic activation of the signaling resulted in loss of lens structure.

We next explored the molecular mechanisms underlying Wnt inhibition in the presumptive lens. One of the candidates is *Pax6*. It is essential for the lens formation (Ashery-Padan et al., 2000). In the developing telencephalon, *Pax6* is required for the expression of Wnt antagonist *Sfrp2* (Kim et al., 2001) and recently, a similar dependence of *Sfrp2* on *Pax6* was reported in the embryonic eye (Duparc et al., 2006). We therefore performed whole mount *in situ* hybridization of *Sfrp1* and *Sfrp2* mRNA on wild type and Small eye (*Sey/Sey*; *Pax6*-null mutant) embryos (Hill et al., 1991). Both *Sfrp1* and *Sfrp2* were not detected in the optic cup at E9.5 and in the eye at E10.5 of *Sey/Sey* embryos. To examine whether Pax6 regulates expression of Wnt inhibitors directly, we searched for Pax6 binding sites in regulatory elements of several known Wnt inhibitor genes. Computer analysis revealed three putative Pax6 binding sites at positions 240, 28, and 22 kb upstream of the *Sfrp2* transcription start site. EMSA documented that Pax6 binds to respective binding sites in the *Sfrp2* locus *in vitro*. Furthermore, chromatin immunoprecipitation assays showed Pax6 binding to the sites *in vivo*. Additionally, we confirmed a 250-bp fragment including Pax6 binding site at position 28 kb upstream from the *Sfrp2* start site is Pax6 responsive by luciferase reporter assay. Analysis of other Wnt inhibitor genes showed that the *Dkk1* locus contains a Pax6 binding site 338 bp upstream of the transcription start. EMSA and chromatin immunoprecipitation assay showed that Pax6 binds to this site both *in vitro* and *in vivo*. Taken together, these findings suggest that Pax6 may directly control transcription of several Wnt inhibitor genes in the developing eye.

Since our data indicates that Pax6 inhibits Wnt/β-catenin signaling during eye development, inactivation of Pax6 could cause ectopic activation of Wnt signaling. We

examined this possibility by crossing *BAT-gal* Wnt reporter mouse (Maretto et al., 2003) into *Sey/Sey* mutants. The optic cup was negative for the Wnt activity in control animals at E9.5. In *BATgal; Sey/Sey* littermates, however, many lens placodal cells as well as retinal progenitors within the eye field expressed β-galactosidase. In wild-type at E10.5, both the retina and the lens were again negative for β-galactosidase activity while in the *Sey/Sey* mutants strong activation of Wnt reporter gene was seen in the eye field.

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

4.2. Presented publications

4.2.1. **Fujimura N.**, Vacik T., Machon O., Vlcek C., Scalabrin S., Speth M., Diep D., Krauss S., Kozmik Z.: *Wnt-mediated down-regulation of Sp1 target genes by a transcriptional repressor Sp5.* **J Biol Chem. 2007 Jan 12;282(2):1225-37.**

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Wnt-mediated Down-regulation of Sp1 Target Genes by a Transcriptional Repressor Sp5*

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Wnt/β-catenin signaling regulates many processes during vertebrate development. To study transcriptional targets of canonical Wnt signaling, we used the conditional Cre/loxP system in mouse to ectopically activate β-catenin during central nervous system development. We show that the activation of Wnt/β-catenin signaling in the embryonic mouse telencephalon results in the up-regulation of *Sp5* gene, which encodes a member of the Sp1 transcription factor family. A proximal promoter of *Sp5* gene is highly evolutionarily conserved and contains five TCF/LEF binding sites that mediate direct regulation of *Sp5* expression by canonical Wnt signaling. We provide evidence that Sp5 works as a transcriptional repressor and has three independent repressor domains, called R1, R2, and R3, respectively. Furthermore, we show that the repression activity of R1 domain is mediated through direct interaction with a transcriptional corepressor mSin3a. Finally, our data strongly suggest that Sp5 has the same DNA binding specificity as Sp1 and represses Sp1 target genes such as *p21*. We conclude that Sp5 transcription factor mediates the downstream responses to Wnt/β-catenin signaling by directly repressing Sp1 target genes.

Wnt/β-catenin signaling plays important roles in multiple developmental processes and has a profound effect on cell proliferation, cell polarity, and cell fate determination (1). Wnt molecules are secreted glycoproteins that work as signaling molecules. Wnt molecules bind with Frizzled receptors and low density lipoprotein receptor-related protein coreceptors at the cell surface to initiate the signaling. In the absence of Wnt/β-catenin signaling, the level of cytoplasmic β-catenin, the key mediator of Wnt/β-catenin signaling, is kept low. β-Catenin is recruited to a destruction complex containing the tumor suppressors adenomatous polyposis coli, axin, casein kinase 1, and glycogen synthase kinase 3β, respectively, and is constitutively phosphorylated. The phosphorylated β-catenin protein is degraded by the ubiquitin pathway. Members of the TCF/LEF transcription factor family bind corepressor Groucho and repress Wnt target genes in the nucleus. The binding of Wnt molecules to the receptors and the coreceptors results in the inactivation of the kinase activity of the destruction complex. As a consequence, β-catenin protein is not phosphorylated, begins to accumulate in the cytoplasm, and is then translocated to the nucleus where it binds to TCF/LEF transcription factors. The binding converts TCF/LEF into an activator that initiates the transcription of Wnt target genes, including c-*myc*, *Axin2*, and *Lef1* (2–4).

During central nervous system (CNS)[2] development, multiple *Wnt* genes are expressed, including *Wnt3a*, *Wnt7a*, *Wnt7b*, and *Wnt8b* (5). Transgenic mice, which express a stabilized form of β-catenin in neural progenitor cells, develop enlarged brains (6). In *Wnt3a* mutant mice as well as in *Lef1* mutant mice, the hippocampus is missing (5, 7). These reports indicate critical roles of canonical Wnt signaling in CNS development.

To study targets of Wnt/β-catenin signaling, we used the conditional Cre/loxP system in mice to ectopically activate Wnt/β-catenin signaling during CNS development. Activation of Wnt/β-catenin signaling is achieved by a deletion of exon 3 of the *β-catenin* gene that encodes phosphorylation sites necessary for β-catenin degradation (8). To activate canonical Wnt signaling during CNS development, *Nes11Cre* mice were crossed to $Catnb^{lox(ex3)}$ mice. Mutant animals Nes11Cre/$Catnb^{lox(ex3)}$ display hyperplasia in the telencephalon that resembles the phenotype of the mouse mutants in which activated β-catenin is directly coupled to the *nestin* enhancer (6). We show that the constitutive activation of Wnt/β-catenin signaling results in the up-regulation of the *Sp5* gene in the mouse telencephalon. The *Sp5* gene encodes a member of Sp1 transcription factor family (9). The proximal promoter of the *Sp5* gene is highly evolutionarily conserved and has five TCF/LEF binding sites that mediate direct regulation of *Sp5* expression by Wnt/β-catenin signaling. Sp5 appears to work as a transcriptional repressor at least in part by directly interacting with a corepressor mSin3a. We show that Sp5 has the same DNA binding specificity as Sp1 and represses Sp1 target genes such as *p21*. In conclusion, our report suggests that the Sp5 transcription factor mediates the downstream responses to Wnt/beta-catenin signaling by directly repressing Sp1 target genes.

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[2] The abbreviations used are: CNS, central nervous system; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; dnTCF4, N-terminally truncated TCF4; aa, amino acid(s); SID, mSin3a-interacting domain.

EXPERIMENTAL PROCEDURES

Mouse Lines—Analysis of Cre-mediated recombination pattern in *Nes11-Cre* (10) was performed by mating to the ROSA26R reporter line as described previously (11). The ROSA26R mice (stock #003309) and *Nes11-Cre* mice (stock #003771) were purchased from Jackson Laboratory. *D6Cre* transgenic mice express Cre recombinase under the control of *Dach1* enhancer, which is active in the telencephalon (12). Mice with a conditional "floxed" allele of *β-catenin*, *Catnb*$^{lox(ex3)}$, were kindly provided by Dr. M. M. Taketo (8).

Plasmids—The mouse *Sp5* promoter and truncated promoters were amplified by PCR using *C57BL/6J* mouse genomic DNA (kindly provided by J. Forejt) as a template. PCR products were cloned to pCR4-TOPO (Invitrogen) and sequenced. The resulting plasmids were digested with EcoRI (New England Biolabs), blunted with T4 DNA polymerase (New England Biolabs), and cloned into SmaI-digested pGL3 basic (Promega) vector. Mouse *Sp5* enhancers were amplified by PCR using primers with XbaI recognition sites. PCR products were digested by XbaI and cloned into a NheI site upstream of the minimal TK promoter cloned in the pGL3 vector. For Gal4-Sp5, the full-length mouse Sp5 cDNA was excised from pBS-KX-Sp5 (kindly provided by D. Houzelstein) and cloned into a Gal4 expression plasmid. To generate Gal4 fusion constructs with individual domains of Sp5, the corresponding regions of mouse Sp5 cDNA were amplified by PCR and cloned into the Gal4 expression plasmid. To generate 6xHis-Sp5, the coding sequence of Sp5 was cloned into the procaryotic expression vector pETH2α. For Sp5-FLAG, the Sp5 cDNA was amplified by PCR and cloned into pKW-FLAG in-frame with the FLAG coding sequence located at the N terminus. For retroviral infection of neurosphere cultures, Sp5 cDNA was inserted into pNIT retroviral vector (provided by F. Gage). To generate GST fusions with a Sp5 R1 domain, the corresponding region was amplified by PCR and cloned into pET42a(+) (Novagen). For GST-Sp5R1A3P and Gal4-Sp5A3P, the R1 region was amplified by PCR using primers that contained the corresponding point mutation and cloned into pET42a(+) or Gal4 expression plasmid. All constructs were verified by sequencing. A luciferase reporter plasmid containing the *p21* promoter (p21-Luc) was kindly provided by E. Sancho. For p21GC-Luc, the p21-Luc plasmid was digested with PstI and BglII (−198/+12), blunted, and cloned into pGL3 basic. For p21ΔGC-Luc, a *p21* promoter fragment (−2326/−197) was cut with PstI/HindIII and fused to the minimal *p21* promoter (−30/+12) located in pGL3 basic.

Microarray Experiment—RNA was isolated from the dissected telencephalon of E13.5 mouse embryos (*Nes11-Cre*/*Catnb*$^{lox(ex3)}$ or *Catnb*$^{lox(ex3)}$) using an Ambion kit and subjected to hybridization on Affymetrix MOE 430A GeneChip. Neurospheres were cultured in neurobasal-A medium with B27 supplement (both Invitrogen) and with epidermal growth factor (20 ng/ml) and basic fibroblast growth factor (8 ng/ml, both R&D Systems). Cells were passaged every 3 days. The Sp5 retrovirus was produced in a Phoenix packaging cell line (provided by G. Nollan) by transient transfection of pNIT-Sp5, and neurosphere cells were infected as described in a previous study (13). Three days after infection, selection with G418 antibiotics was started (250 μg/ml), and pools of cell clones were maintained in the selection media. RNA was isolated from three separate plates of Sp5 virus or mock infected neurospheres using an Ambion kit and used for hybridization on Affymetrix MOE 430A. Microarray data were analyzed by Affymetrix Suite 5.1 software.

Cell Culture, Transient Transfection, and Luciferase Reporter Assay—293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (PAA Laboratories), 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). Neurospheres were cultured in neurobasal-A medium with B27 supplement (both Invitrogen), epidermal growth factor (20 ng/ml), and basic fibroblast growth factor (8 ng/ml, both R&D Systems). Cells were passaged every 3 days and maintained at 37 °C in an atmosphere of humidified air with 5% CO_2. Transient transfection of 293T cells was performed 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 pUC18 when necessary. A β-galactosidase expression plasmid was cotransfected to normalize the transfection efficiency. Triplicate assays were performed to obtain standard deviations. Two days after transfection, the cells were lysed in 100 μl of 1× passive lysis buffer (Promega, Madison, WI). Luciferase reporter assays were performed using Luciferase Reporter assay kit (Promega). β-Galactosidase was detected with Galacto-Star system (Applied Biosystems, Foster City, CA).

Chromatin Immunoprecipitation Assay—A chromatin immunoprecipitation assay was performed according to the manufacturer's protocol (Upstate Biotech) with modifications. The cortical parts of *D6Cre*/*Catnb*$^{lox(ex3)}$ brains were harvested at E18.5, homogenized in 1% formaldehyde in phosphate-buffered saline and cross-linked at 37 °C for 15 min. Cross-linking was stopped by adding glycine (0.125 M) and incubating at room temperature for 5 min. Cross-linked cells were washed twice with cold phosphate-buffered saline containing fresh protease inhibitors, pelleted, and resuspended in 2 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) with protease inhibitors. Samples were incubated on ice for 10 min, and lysates were sonicated on an ice water bath to produce 150–500 bp of DNA fragments. Cell debris was removed by centrifugation for 10 min at 14,000 rpm at 4 °C, and the supernatant was diluted ten times with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) containing protease inhibitors. 30 μg of sonicated chromatin was precleared with 50 μl of protein A(G)/agarose slurry (Upstate Biotech) for 1 h at 4 °C. Beads were pelleted by centrifugation for 5 min at 3,000 rpm at 4 °C. The supernatant was incubated either with 5 μg of antibody or with no antibody (no antibody control) overnight at 4 °C. The following antibodies were used: anti-β-catenin (E-5, sc-7963, Santa Cruz Biotechnology), anti-Lef1 (N-17, sc-8591, Santa Cruz Biotechnology), and anti-Tcf4 (a gift from V. Korinek). 30 μl of protein A(G)/agarose slurry (Upstate Biotech) was added, and samples were rocked at 4 °C for 1 h. After washing for 5 min at 4 °C twice in low salt buffer (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM

Tris-HCl, pH 8.0, 150 mM NaCl), twice in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), four times in LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate,1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), immunocomplexes were eluted twice with 100 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS) for 15 min at room temperature. Immunoprecipitated DNA was de-cross-linked overnight at 65 °C in the presence of proteinase K (0.06 unit/μl, Roche Applied Science) and 250 mM NaCl. Samples were purified using a MinElute reaction cleanup kit (Qiagen), and 1/20th of eluate was used for PCR. PCR was performed as follows: 95 °C 2 min for 1 cycle; then 95 °C 30 s, 60 °C 30 s, and 72 °C 30 s for 40 cycles; and finally 72 °C 5 min. The primers used were as follows: Sp5D-H_F, CCTAGAGATAACAAAGACACT; Sp5D-H_R, AGTCAGAGGAAAGATTTATGG; Sp5-2kb_F, TGGCTGCTTAATTGCCTAAAGAG; Sp5-2kb_R, CAGGGGTTTGAGTGCTGTGGA; Sp5 + 6kb_F, AACGGAAGCTGAGTGTAAATTAG; and Sp5 + 6kb_R, GTAACTAAGACAGACGCCTAAAC.

Electrophoretic Mobility Shift Assay—The following double-stranded oligonucleotides derived from the *Sp5* promoter were used in EMSA (only the top strand is shown for simplicity): Sp5A, ATTGAAGAAACAAAGTTTGATCT; Sp5B, CACTCATCAACAAAGGAAAGCCC; Sp5C, GGATACCTCTTTGAACTGACCCC; Sp5D, CTAGAGATAACAAAGACACTTTG; Sp5E, AAGGCCCCCTTTGATCAGGAAAA; Sp5F, TTTGTGGATTCAAAGGATTTGCT; Sp5G, CCGCTATTCTTTGATGATTGGGT; and Sp5H, CGGCAAACTTCAAAGCCATAAAT. The following double-stranded oligonucleotides derived from the p21 promoter were used: I + II, GAATTCTGAGGCGGGCCCGGGCGGGGCGGTTGGAATTC; III + IV, GAATTCCGAGCGCGGGTCCCGCCTCCGAATTC; and V + VI, GAATTCGGAGGGCGGTCCCGGGCGGCGCGAATTC. The following double-stranded oligonucleotides representing consensus (wt) and multiple versions of the Sp1 binding site were used: WT, ATTCGATCGGGGCGGGGCGAGC; M1, ATTCGATCGGTTCGGGGCGAGC; M2, ATTCGATCGGGGAGGGGCGAGC; M3, ATTCGATCGGGGTGGGGCGAGC; M4, ATTCGATCGAGGCGGGGCGAGC; M5, ATTCGATCGGGGCGGAGCGAGC; SA1, GTGCGGAGGCGTGGTTAGAG; AX2, CGGGCGGCGGGGGAGGCGGGGTC; XN2, CGGCGGGGAGGTGGGGCGAGGAGAG; and BTE, AGCTTGAGAAGGAGGCGTGGCCAACGCATG.

Double-stranded oligonucleotides containing TCF/LEF or Sp1/Sp5 binding sites were radioactively labeled at the 5′-ends with [γ-^{32}P]dATP using polynucleotide kinase (Roche Applied Science) and purified on microspin columns (Amersham Biosciences). The ^{32}P-labeled oligonucleotides were incubated with *in vitro* synthesized LEF1 (TnT Quick, Promega), bacterially purified 6xHis-Sp5 (Qiagen), or Sp1 (Promega) in binding buffer (10 mM HEPES at pH 7.7, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.5% bovine serum albumin, and 0.1 mg/ml poly(dIdC)) on ice for 15 min. For supershifts, ^{32}P-labeled oligonucleotides were preincubated on ice for 10 min with 1 μg of anti-Lef antibody. Samples were analyzed by 6% polyacrylamide gel electrophoresis and autoradiography.

Immunoprecipitation and Western Blotting—293T cells were plated in 10-cm dishes 24 h prior to transfection. Myc-mSin3a plasmid (2 μg, kindly provided by C. Laherty) was cotransfected with FLAG-Sp5 expression plasmid (3 μg) or empty FLAG expression plasmid (3 μg) into 293T cells. Two days after transfection, 293T cells were washed with phosphate-buffered saline and lysed in lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Cell debris was pelleted by centrifugation at 12,000 × *g* for 10 min. An aliquot of this whole cell lysate was boiled with 2× SDS sample buffer for 5 min. For immunoprecipitation, 500 ml of the whole cell lysate was incubated with 40 μl of anti-FLAG M2 affinity beads (Sigma) overnight at 4 °C. The beads were washed with 1× Wash buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA) five times and boiled with 2× SDS sample buffer for 5 min.

Samples were separated by 8% or 12% SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Myc-tagged mSin3a was detected by anti-Myc antibody (Roche Applied Science), and FLAG-tagged Sp5 was detected by anti-FLAG M2 (Sigma). Detection was performed using polyclonal rabbit anti-mouse immunoglobulins/horseradish peroxidase (DakoCytomation) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

GST-pull-down Assay—Myc-tagged mSin3a was prepared by TnT Quick Coupled Transcription/Translation Systems according to the manufacturer's protocol (Promega). GST fusion expression plasmids were transformed into BL21 CodonPlus (DE3)-RIPL cells (Stratagene). A single colony from the transformation was cultured in 2 ml of LB medium containing 50 μg/ml chloramphenicol and 30 ng/ml kanamycin overnight at 37 °C. The cultures were transferred to 100 ml of LB without antibiotics. The expression of the fusion construct was induced by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 2 mM for 2 h. The cells were harvested by centrifugation and resuspended in 5 ml of NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Lysozyme was added to a final concentration of 0.1 mg/ml. The lysates were incubated on ice for 20 min, sonicated, and centrifuged to remove the cell debris. The supernatant was incubated with 200 μl of glutathione-Sepharose slurry beads (BD Bioscience) for 1 h at 4 °C. The beads were washed three times with 5 ml of Binding buffer (20 mM Tris, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol) containing 0.1% Nonidet P-40. GST fusion proteins bound to the beads were checked by SDS-PAGE. Beads containing normalized amounts of fusion proteins were blocked by Binding buffer containing 0.05% of Nonidet P-40 and 5 mg/ml bovine serum albumin for 2 h at 4 °C and resuspended in 150 μl of Binding buffer containing 0.05% Nonidet P-40, 1 mg/ml bovine serum albumin, and 100 μg/ml ethidium bromide. The beads were incubated overnight at 4 °C with 3 μl of Myc-tagged mSin3a produced by TnT. The beads were washed three times with 500 μl of Binding buffer containing 0.05% Nonidet P-40 and boiled with SDS sample buffer. Myc-tagged mSin3a was detected by Western blotting using an anti-Myc antibody.

In Situ Hybridization—*In situ* hybridization on cryosections was carried out as described previously (14). Plasmids carrying

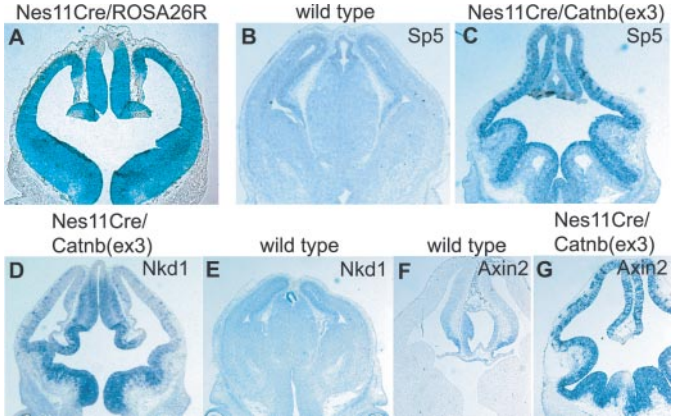


FIGURE 1. **Sp5 is regulated by Wnt/β-catenin signaling.** *A*, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining was performed on coronal brain sections of Nes11Cre/ROSA26R mice at E12.5. *B–G*, *in situ* hybridization was performed on coronal sections of wild-type and Nes11Cre/Catnb$^{lox(ex3)}$ mice using Sp5 (*B* and *C*)-, Nkd1 (*E* and *D*)-, or Axin2 (*F* and *G*)-specific probes.

mouse Sp5, Axin2, and Nkd1 cDNA were linearized with an appropriate restriction enzyme, and an antisense Riboprobe was synthesized using the DIG RNA labeling kit (Roche Applied Science).

RESULTS

Sp5 Is a Target Gene of Wnt/β-Catenin Signaling—To identify target genes of Wnt/β-catenin signaling during CNS development, two lines of mice were interbred to activate Wnt/β-catenin signaling. *Catnb$^{lox(ex3)}$*, in which exon 3 of *β-catenin* gene is floxed by loxP sites (8), was mated to *Nes11Cre*, a transgenic mouse line expressing Cre recombinase under the control of *nestin* regulatory elements in neural progenitor cells (10). Exon 3 of *β-catenin* gene encodes phosphorylation sites necessary for β-catenin degradation (15). Cre recombinase-mediated deletion of exon 3 of *β-catenin* gene results in the expression of a stabilized form of β-catenin, which leads to the constitutive activation of Wnt/β-catenin signaling. To map the area in which Cre recombinase is active in the *Nes11Cre* mice, *Nes11Cre* mice were crossed with a reporter mouse line, ROSA26R (R26R) (11). Within the telencephalon, Cre recombinase activity was detected in the neural progenitor cells of the pallium and the subpallium (Fig. 1*A*). To activate canonical Wnt signaling during CNS development, *Nes11Cre* mice were crossed to *Catnb$^{lox(ex3)}$* mice. Mutant animals Nes11Cre/Catnb$^{lox(ex3)}$ displayed hyperplasia in the telencephalon that resembles the phenotype of the mouse mutants in which activated β-catenin is directly coupled to the *nestin* enhancer (6). Further, the dorso-ventral patterning in the mutant telencephalon is impaired such that genes normally expressed in the dorsal pallium expand into the ventral areas, whereas ventrally expressed genes are down-regulated (69). To identify target genes of Wnt/β-catenin signaling, RNA was isolated from the telencephalon at E13.5, and overall gene expression was analyzed by Affymetrix microarray. We noticed that the expression levels of several known targets of Wnt/β-catenin were up-regulated, as follows: *Axin2* (3.4×), *Nkd1* (9.5×), *Dkk1* (5×), and *Pitx2* (7×) (3, 16, 17). On the other hand, ventrally expressed genes such as *Dlx2*, *Dlx1*, *Lhx6*, or *Mash1* were down-regulated 10.5-, 5.1-, 15.1-, and 7.2-fold, respectively. The expression of several genes was verified by *in situ* hybridization on coronal sections of *Nes11Cre*/*Catnb$^{lox(ex3)}$* mice and wild-type mice at E13.5 (Fig. 1, *D–G*, see also Ref. 69). Interestingly, we found that *Sp5*, a member of *Sp1* family, was up-regulated 32-fold in the Affymetrix data, and strong gene activation was confirmed by *in situ* hybridization (Fig. 1, *B* and *C*). In wild-type mice, *Sp5* is expressed weakly in the hippocampal primordium (Fig. 1*B*). In *Nes11Cre*/*Catnb$^{lox(ex3)}$* mice, *Sp5* is strongly expressed in the pallium and the subpallium, *i.e.* in the area of Cre-mediated recombination (Fig. 1*C*). These results suggest that Wnt/β-catenin signaling positively regulates *Sp5*.

Sp5 Is a Direct Target Gene of Wnt/β-Catenin Signaling—We next examined whether *Sp5* is regulated by Wnt/β-catenin signaling directly. To find important transcriptional regulatory elements, we compared the upstream sequences of *Sp5* of *Mus musculus*, *Homo sapiens*, *Gallus gallus*, *Danio rerio*, and *Xenopus tropicalis*, because the important transcriptional regulatory elements are often evolutionarily conserved. We found three evolutionarily conserved regions containing TCF/LEF consensus sites located at positions −200 bp/+200 bp, −2.9 kbp/−2.7 kbp, and −3.9 kbp/−3.4 kbp, referred to as proximal promoter, ECR2, and ECR1, respectively. ECR2 contains two conserved TCF/LEF consensus sites named B and C. ECR1 contains one conserved TCF/LEF consensus site named A. The *Sp5* proximal promoter contains five TCF/LEF consensus sites, named D, E, F, G, and H, respectively. Sites E, G, and H in the *Sp5* promoter were evolutionarily conserved among all five vertebrate species. Site F was not conserved in *D. rerio*, and site D was conserved only between *M. musculus* and *H. sapiens* (Fig. 2*A*).

To examine if the *Sp5* promoter is responsive to Wnt/β-catenin signaling, a mouse *Sp5* promoter (−1536/+200) was cloned into the luciferase reporter plasmid and transiently transfected into 293T cells. Cotransfection of the promoter with Lef1 and N-terminally truncated β-catenin (β-cateninΔN), which is constitutively stabilized and able to bind with TCF/LEF transcription factors (18), stimulated reporter gene expression ~15-fold. Conversely, cotransfection with N-terminally truncated TCF4 (dnTCF4), which does not bind to β-catenin and acts as a potent inhibitor of the β-catenin/TCF complexes (19), repressed the activity of the promoter construct 4.9-fold (Fig. 2*B*). These results suggest that the *Sp5* promoter is directly responsive to Wnt/β-catenin signaling.

To identify functional TCF/LEF elements within the *Sp5* promoter, three reporter plasmids containing different regions of the promoter cloned upstream of the luciferase reporter gene were constructed (Fig. 2*B*). Luciferase reporter plasmids containing −206/+200, −27/+200, and −1536/+3 of the *Sp5* promoter were named D1, D2, and D3, respectively. Each plasmid was cotransfected in 293T cells with β-cateninΔN/Lef1 or dnTCF4. D1 and D2 were stimulated 10-fold and 6-fold by β-cateninΔN/Lef1, respectively, and repressed 2.9-fold and 9.4-fold by dnTCF4, respectively. In contrast, D3 was not affected by either β-cateninΔN/Lef 1 or dnTCF4 (Fig. 2*B*). These results suggest that sites F, G, and H play a critical role in mediating Wnt/β-catenin signaling and site E supports site F, G, and H to give further activation.

To examine whether ECR1 and ECR2 are also responsive to Wnt/β-catenin signaling, ECR1 and ECR2 were cloned upstream of the minimal TK promoter driving luciferase reporter gene expression. Each of the constructs was cotransfected in 293T cells with β-cateninΔN/Lef1 or dnTCF4. ECR1, ECR2, and TK were stimulated 1.4-, 4.0-, and 1.8-fold by β-cateninΔN/Lef1, respectively, and repressed 2.4-, 2.3-, and 1.5-fold by dnTCF4 (Fig. 2*C*). These results suggest that ECR2 is an additional Wnt-responsive regulatory element.

To examine whether TCF/LEF binds putative A–H binding sites within the *Sp5* proximal promoter, ECR1, and ECR2 (Fig. 3*B*), EMSA was performed. Oligonucleotides containing sites A–H were incubated with *in vitro* translated LEF1 and were analyzed by electrophoresis. As shown in Fig. 3*A*, all sites were bound by LEF1. The identity of the LEF1 protein in the complex was verified by supershifts using LEF1 antibody. This result suggests that TCF/LEF can bind with TCF/LEF binding sites within the *Sp5* proximal promoter, ECR1, and ECR2.

We next examined whether LEF/TCF transcription factors and β-catenin are associated with the *Sp5* promoter *in vivo*. Chromatin immunoprecipitation was performed using antibodies against LEF1, TCF4, and β-catenin using cortical part of brain from *D6Cre/Catnb*$^{lox(ex3)}$ mice at E18.5. *D6Cre* is a transgenic line expressing Cre recombinase in the telencephalon using *Dach1* enhancer (12). Chromatin immunoprecipitation data show that LEF/TCF/β-catenin complexes are present on the proximal *Sp5* promoter (Fig. 3*C*). This result suggests that LEF/TCF/β-catenin complex binds the *Sp5* promoter *in vivo* to regulate transcription. We therefore conclude that *Sp5* is a direct target gene of Wnt/β-catenin signaling.

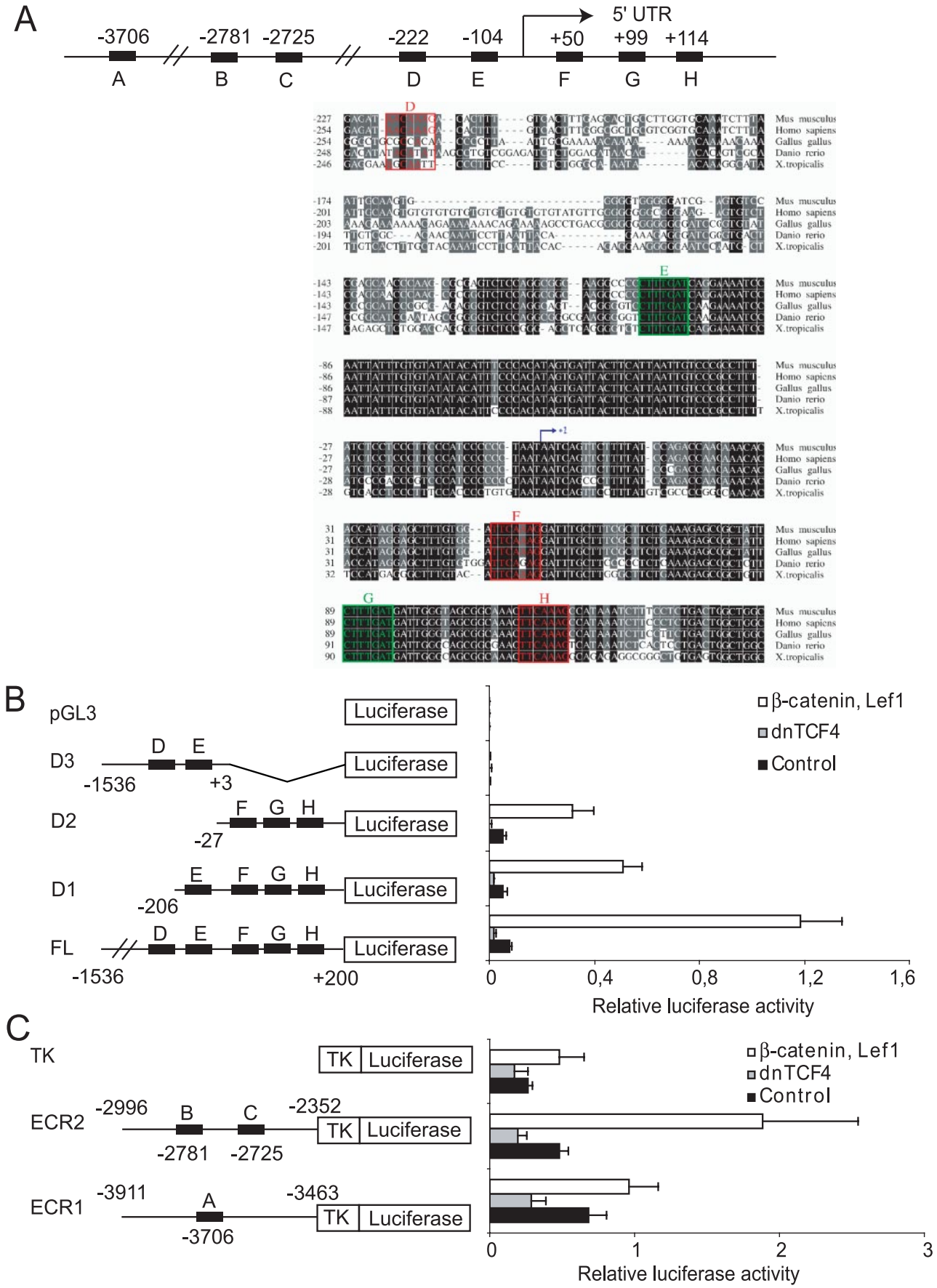
Sp5 Is a Potent Transcriptional Repressor—To our surprise, many genes were down-regulated in the telencephalon of *Nes11Cre/Catnb*$^{lox(ex3)}$ mice as compared with control mice. We hypothesized that down-regulation of at least some of the genes could be mediated by Sp5, because Sp5 itself is highly induced in *Nes11Cre/Catnb*$^{lox(ex3)}$ mice, and several Sp1 family members are known to act as repressors (20). To examine the transcriptional properties of Sp5, a Gal4 reporter assay was employed. Plasmids encoding Gal4, Gal4 fusion with Sp5 (Gal4-Sp5), or Gal4 fusion with Dach1 (Gal4-Dach1), a known repressor (21), were cotransfected with a Gal4-dependent reporter plasmid driving luciferase gene expression. Both Gal4-Sp5 and Gal4-Dach1 repressed transcription 7.8- and 1.9-fold, respectively (Fig. 4*A*). This result indicates that Sp5 acts as a transcriptional repressor.

To identify functional domains within Sp5 that mediate transcriptional activity, Gal4 fusion constructs with different regions of Sp5 were cotransfected together with the Gal4 reporter plasmid. The Gal4 fusion proteins containing amino acids (aa) 1–76, 1–297, and 1–297 plus 379–398 and 379–398 of Sp5 repressed transcription 5.5-, 11-, 16-, and 7.1-fold, respectively. However, the Gal4 fusion proteins containing aa 1–151 and 1–222 of Sp5 did not exert any significant effect on transcription (Fig. 4*B*). These results suggest that aa 1–76, 223–297, and 379–398 of Sp5 contain repressor domains and that the region between aa 77 and 222 of Sp5 might contain an activation domain. To examine our hypothesis, Gal4 fusions with aa 223–297, 379–398, 77–222, or 152–222 were cotransfected with a Gal4 reporter plasmid. Gal4 fusions with aa 223–297 and 379–398 repressed transcription 10- and 7.1-fold, respectively (Fig. 4*C*). Gal4 fusions with aa 77–222 and 77–151 activated transcription 21- and 15-fold, respectively (Fig. 4*D*). These results suggest that, overall, Sp5 acts as a repressor and has three separable and independent repressor domains located within aa 1–76, 223–297, and 379–398. In addition, there is a potential transcriptional activation domain located within residues 152–222 of Sp5. In the following text, we refer to the repressor domains located within aa 1–76, 223–297, and 379–398 as R1, R2, and R3, respectively.

Corepressor mSin3a Interacts with the R1 Domain of Sp5 and Regulates Its Transcriptional Activity—We next examined the mechanism(s) that control the transcriptional properties of the repressor domains. We found a core mSin3a-interacting domain (SID), A(A/V)*XX*L (22), within the R1 domain of Sp5. Corepressor mSin3a is known to interact with Class I histone deacetylases and a number of transcription factors containing α-helical structure harboring SID (23, 24). We found that the R1 domain is predicted to form α-helical structure. We therefore examined whether the putative SID within Sp5 is responsible for the transcriptional repression function of the R1 domain. The Gal4 fusion constructs with the R1 domain containing wild-type (Gal4-Sp5R1) or a mutated SID (Gal4-Sp5R1A3P), in which alanine is changed to proline to disrupt the formation of α-helical structure, were cotransfected with the Gal4 reporter plasmid. Interestingly, in contrast to the wild-type Gal4-Sp5R1, which acts as a potent repressor, the Gal4-Sp5R1A3P acted as an activator (Fig. 5*A*). In addition, another Gal4 fusion construct with the R1 domain lacking a SID (Gal4-Sp5R1Δ3–7) also worked as an activator.[3] Combined, these results suggest that the SID is crucial for the repressive activity of the R1 domain.

To examine whether Sp5 interacts with mSin3a directly through a SID, GST-pull-down assays were performed with the wild-type Sp5 R1 domain (GST-R1) and the SID mutated R1 domain (GST-R1A3P). GST-R1 pulled down *in vitro* translated mSin3a. In contrast, neither GST nor GST-R1A3P were able to interact with mSin3a (Fig. 5*B*). In accordance with the fact that we have not been able to detect any potential SID motifs within R2 and R2, GST-Sp5R2 and GST-Sp5R3 domain fusions did not pull down *in vitro* translated mSin3a (data not shown). These results suggest that mSin3a interacts with Sp5 directly through the SID located within R1. To provide further evidence that mSin3a interacts with Sp5 *in vivo*, coimmunoprecipitation was performed. FLAG-tagged Sp5 expression plasmid (Sp5-FLAG) was cotransfected with Myc-tagged mSin3a expression plasmid (Myc-mSin3a) into 293T cells, and the total cell lysate was precipitated using FLAG antibody beads. We found that Sp5-FLAG was immunoprecipitated with Myc-mSin3a (Fig. 5*C*) providing evidence that Sp5 can interact with mSin3a *in vivo*. In summary, our results suggest that the transcriptional repression activity of R1 domain is mediated through the interaction with mSin3a corepressor.

[3] N. Fujimura and Z. Kozmik, data not shown.



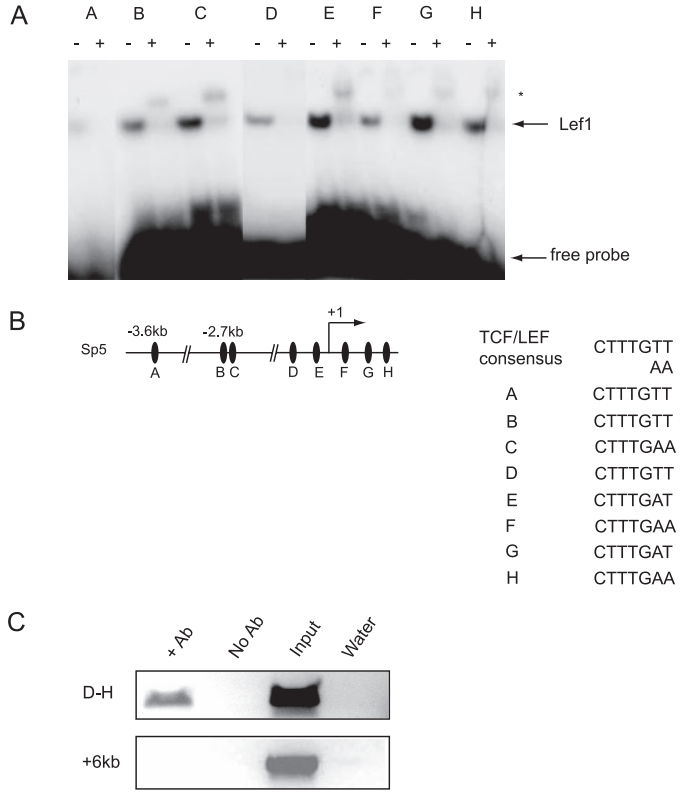


FIGURE 3. **TCF and LEF proteins bind to Sp5 regulatory sequences.** *A*, EMSAs demonstrated *in vitro* binding of LEF1 protein to putative binding sites *A–H* in the *Sp5* locus as depicted in *B*. LEF1 protein binds all of the sites (− *lanes*), and its binding specificity is demonstrated by the addition of anti-Lef1 antibody (+ *lanes*) that results in the formation of a supershifted complex (*asterisk*). *B*, the map of *Sp5* locus with putative TCF/LEF binding sites highlighted (*black circles*). *C*, chromatin immunoprecipitation assay was used to detect the presence of TCF/LEF/β-catenin complex on *Sp5* regulatory elements. TCF/LEF/β-catenin associated DNA in *D6Cre/*Catnb$^{lox(ex3)}$ cortical parts (E18.5) was analyzed by PCR with primers spanning sites *D–H*. The downstream (+6 kb) region was used as a negative control.

Sp5 Binds Sp1 Target Sequences and Attenuates Sp1-regulated Transcription—Sp5 belongs to the large family of Sp1-like transcription factors. Intrigued by the fact that the founding member, Sp1, acts as an activator, whereas Sp5 acts as a repressor, we next examined whether Sp5 down-regulates Sp1 target genes. The zinc finger domain of Sp1 family members conforms to the Cys_2-His_2 zinc finger consensus sequence. The similarity of the zinc finger between Sp1 and Sp5 is 92.6% (20). The amino acids predicted to make contact with the DNA are conserved between Sp5 and Sp1. Furthermore, it is shown that Sp5 binds to a canonical Sp1 consensus site (GGGCGG) *in vitro* by EMSA (9). To examine whether Sp5 has the same DNA binding specificity as Sp1, EMSA was performed using bacterially purified Sp5 and Sp1 proteins on a large panel of binding sites. Oligonucleotides containing the canonical Sp1 binding site (WT), mutated Sp1 binding sites (M1, M2, M3, M4, and M5), Sp1 binding regions within the proximal promoter of *TGF-βRI* gene (SA1, AX2, and XN2), or the BTE (basic transcription element) site (BTE), a well characterized GC-rich element (25, 26), were tested. Binding site M1 has a mutation that abolishes Sp1 binding (27). As shown in Fig. 6*A*, Sp5 and Sp1 bound to WT, M2, M3, M4, M5, XN2, SA1, AX2, and BTE with similar affinities. Consistent with Sp1 data, Sp5 did not bind to M1 (Fig. 6*A*). This result suggests that Sp5 has a very similar if not identical DNA binding specificity as Sp1. We next examined whether Sp5 represses Sp1 target genes. First of all, we have investigated *p21* as a well characterized Sp1 target gene. It was shown previously that the proximal promoter of *p21* gene contains six Sp1 binding sites (I–VI) and that it is positively regulated by Sp1 through these binding sites (28). To examine whether Sp5 binds to the Sp1 binding sites within the *p21* promoter, EMSA was performed using bacterially purified Sp5 or Sp1 and oligonucleotides containing sites I + II, III + IV, and V + VI. As shown in Fig. 6*B*, Sp5 bound strongly with the oligonucleotides in the same manner as did Sp1. To examine whether Sp5 has the ability to repress *p21* gene promoter, the luciferase reporter assay was performed. Reporter genes containing 2.3 kb of the *p21* promoter (p21-Luc), the proximal *p21* promoter (p21GC-Luc), or the promoter lacking the six Sp1 binding sites (p21ΔGC-Luc) were cotransfected with or without the Sp5 expression plasmid into 293T cells. As shown in Fig. 6*C*, both p21-Luc and p21GC-Luc were repressed by Sp5 (13- and 3.3-fold, respectively). We were unable to see any effect of Sp5 on p21ΔGC-Luc, because the basal level of p21ΔGC-Luc was even lower than that of the parental plasmid pGL3. Our results suggest that Sp5 can repress *p21* promoter, most likely due to its ability to compete with Sp1 (or with related activator, Sp3) for promoter binding. Because full-length *p21* promoter and truncated *p21* promoter were repressed 13- and 3.3-fold, respectively, there may be additional Sp5-responsive elements upstream of the proximal *p21* promoter. These results suggest that Sp5 binds *p21* gene regulatory elements and represses its promoter.

To obtain further evidence that Sp5 represses Sp1 target genes *in vivo*, and to identify additional Sp5 targets in neural cells, we have established primary neurosphere cultures overexpressing Sp5. Neurospheres represent cultured neural stem cells that divide *in vitro* and yield major neural lineages upon differentiation. pNIT retroviral vector carrying *Sp5* coding sequence as well as G418 resistance was used to infect neurosphere cells isolated from the mouse telencephalon at E12.5. Neurospheres were grown in medium containing G418, and the pool of G418-resistant clones was used for isolation of RNA. Real-time reverse transcription-PCR revealed that neurosphere cells infected with the Sp5 retrovirus manifested 107.6-fold

FIGURE 2. **Sp5 is a direct target of Wnt/β-catenin signaling.** *A*, localization of putative TCF/LEF binding sites, *A–H*, within the regulatory region of the mouse Sp5 gene. Sp5 promoter sequences from *M. musculus*, *H. sapiens*, *G. gallus*, *D. rerio*, and *X. tropicalis* were compared. Evolutionarily conserved, putative TCF/LEF binding sites are *boxed*. *B* and *C*, the indicated regions of the Sp5 promoter and enhancers were cloned into the pGL3 plasmid. The luciferase reporter plasmids (100 ng) were cotransfected with N-terminally truncated β-catenin (β-cateninΔN) and LEF1 (50 ng each) or N-terminally truncated TCF4 (dnTCF4, 100 ng) into 293T cells. β-Galactosidase expression plasmid (5 ng) was cotransfected to normalize for transfection efficiency. Luciferase reporter assay and β-galactosidase assay were performed as described under "Experimental Procedures."

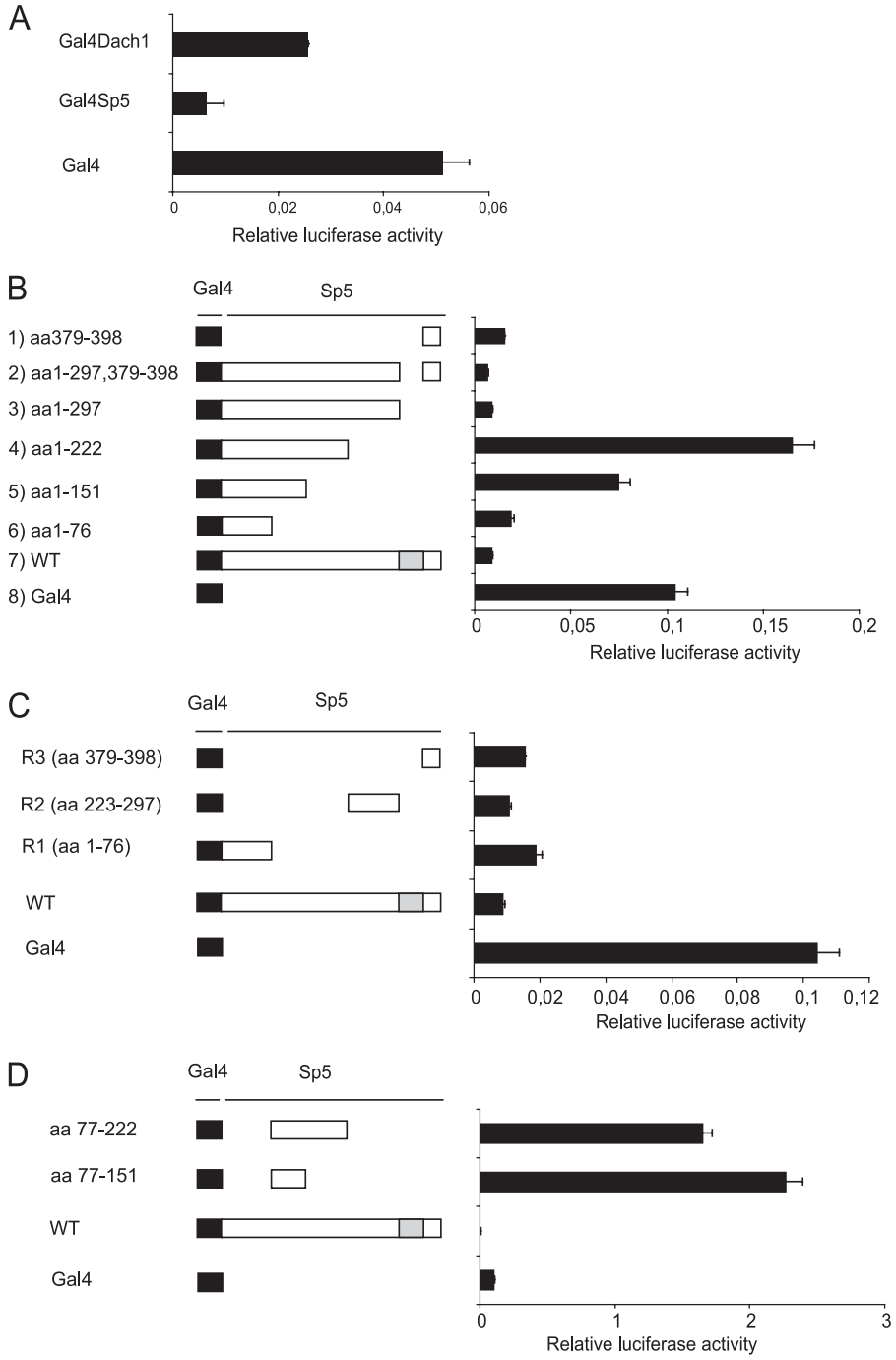


FIGURE 4. **Mapping of transcriptional regulatory domains within Sp5.** *A*, the expression plasmids encoding Gal4, Gal4-Sp5, or Gal4-Dach1 (100 ng) were cotransfected with the Gal4 reporter plasmid (100 ng) into 293T cells. A β-galactosidase expression plasmid (5 ng) was cotransfected to normalize for transfection efficiency. Luciferase reporter assays were performed as described under "Experimental Procedures." *B–D*, the expression plasmids encoding Gal4 fusions with various regions of Sp5 (100 ng) were cotransfected with the Gal4 reporter plasmid (100 ng) into 293T cells. The β-galactosidase expression plasmid (5 ng) was cotransfected to normalize for transfection efficiency.

induction of Sp5 mRNA as compared with mock infected cells.[4] We then profiled gene expression of Sp5-overexpressing neurospheres by Affymetrix microarray analysis. We found that 107 genes were down-regulated >2-fold in Sp5-infected neurospheres. Notably, 90 genes were Sp1 target genes or genes that contain canonical Sp1 binding sites in the proximal promoter (−500/+1) and 5′-untranslated region (Table 1) (29–52). Of a special interest is the gene encoding solute carrier family 12, member 2 (*scl12a2*, NKCC1), which contains canonical Sp1 binding sites and becomes down-regulated by Wnt/β-catenin signaling (53). In conclusion, our results show that Sp5 represses Sp1 target genes.

DISCUSSION

In this study we have shown that the *Sp5* gene is a direct target of Wnt/β-catenin signaling and that Sp5 acts as a transcriptional repressor and represses Sp1-regulated target genes. Because the induction of *Sp5* by Wnt/β-catenin signaling is very high, *Sp5* might be useful as a new marker for Wnt/β-catenin signaling. It is known that Wnt/β-catenin signaling represses the transcription of several genes (54–56). Our report may give some insight into Wnt/β-catenin signaling-dependent repression.

We have shown that *Sp5* is regulated by Wnt/β-catenin signaling directly and predominantly through the proximal promoter, which has evolutionarily conserved TCF/LEF binding sites. We have shown that *Sp5* promoter and ECRs are evolutionarily conserved between mouse and zebrafish. A recent report has shown elevated expression of *Sp5* in colon cancer tissues in which Wnt/β-catenin signaling is constitutively active (57). Previous reports and results presented here suggest that *Sp5* is directly regulated by Wnt/β-catenin signaling. In zebrafish embryos, *Sp5* expression is induced by Wnt8 and repressed by dominant-negative TCF (58). Both ECR1 and ECR2 identified in our study are highly evolutionarily conserved and have TCF/LEF binding sites. Although neither ECR1 nor ECR2 mediated a strong response to Wnt/β-catenin signaling in our cell transfection assays, we cannot rule out the possibility that ECR1 and ECR2 represent genuine regulatory elements under Wnt/β-catenin control. Interestingly, FGF8, which activates MEF2 and ATF1 transcription factor (59, 60), can also induce *Sp5* expression in zebrafish (61). Interestingly, ECR1 and ECR2 contain putative

[4] T. Vacik, data not shown.

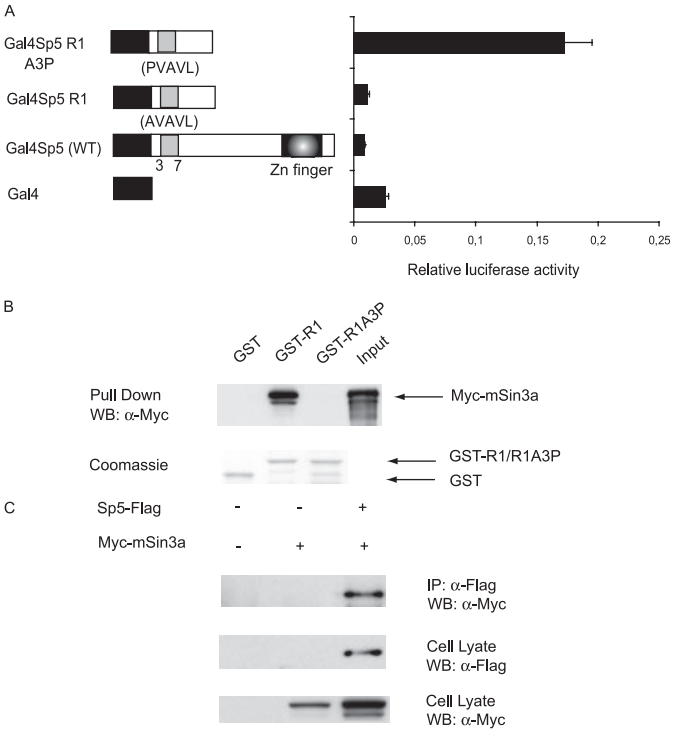


FIGURE 5. **Sp5 interacts with corepressor mSin3a.** *A*, the expression plasmids encoding Gal4 fusion with the R1 domain (Gal4Sp5R1) or mutated R1 domains (Gal4Sp5R1A3P) (100 ng) were cotransfected with the Gal4 reporter plasmid (100 ng). The β-galactosidase expression plasmid (5 ng) was cotransfected to normalize for transfection efficiency. *B*, GST-pull-down assays were performed with GST, GST-Sp5R1, and GST-Sp5R1A3P. An *in vitro* translated, Myc-tagged mSin3a was incubated with the indicated GST fusions bound to the glutathione-Sepharose beads. Western blotting was performed with an anti-Myc antibody to detect Myc-tagged mSin3a (*upper panel*). The normalized amounts of the GST proteins used in the pull-down assay are shown by Coomassie-stained gel (*bottom panel*). *C*, Myc-tagged mSin3a expression plasmid (Myc-mSin3a) was cotransfected with FLAG-tagged Sp5 expression plasmid (Sp5-FLAG) or empty expression plasmid into 293T cells. Cells were harvested 2 days later. Immunoprecipitation was performed with an anti-FLAG M2 affinity beads, and Western blotting was performed using anti-FLAG or anti-Myc antibodies.

AFT1 binding sites and ECR2 contains a putative MEF2 binding site. ECR1 and ECR2 may thus be responsive to FGF8. Further, we have been unable to fully recapitulate the expression pattern of endogenous Sp5 in medaka using a transgene containing the mouse proximal Sp5 promoter fused to enhanced green fluorescent protein reporter despite extremely high sequence conservation between mouse and fish, suggesting a requirement for additional regulatory elements.[5]

We show here that Sp5 is a potent transcriptional repressor and has three autonomous repressor domains. Because mSin3a interacts with Sp5, and deletion and mutation of SID made R1 domain an activator, the transcriptional activity of the R1 domain is regulated by the interaction with corepressor mSin3a. We also examined the mechanisms that control the transcriptional activity of R2 and R3 domains. R2 domain has a polyalanine tract that is often found associated with repressor domains (62). However, a deletion of the polyalanine tract did not change the transcriptional activity of the R2 domain.[3] The R3 domain has an evolutionarily conserved sumoylation consensus ψKXE (63). Sumoylation consensus is often found in repressor domains and inhibitory domains of activators, and sumoylation facilitates transcriptional repression activity (64, 65). One of the consequences of sumoylation is to promote the interaction of transcription factors with corepressors (66). Mutation in sumoylation consensus within the R3 domain of Sp5 had only a modest effect on the repression activity of R3 in our transient reporter assays in 293T cells.[3] However, we cannot exclude the possibility that the transcriptional activity of R3 might be affected by sumoylation *in vivo*. To identify corepressors mediating the activity of R2 and R3 domain, we also tested the effect of common corepressors, CtBP and Groucho, on the transcriptional activity of Sp5. Examining Sp5 protein sequence did not reveal a well defined binding motif for either CtBP or Groucho (67, 68). In accordance with this, cotransfection with either CtBP or Groucho did not have any effect on the transcriptional property of R2 and R3.[3] Combined, our results indicate that the transcriptional property of R1 is mediated by mSin3a, whereas the repressor domains R2 and R3 interact with an, as yet, unidentified corepressor.

Wnt/β-catenin signaling is active in the pallium and is important for dorso-ventral specification of the telencephalon (69). In the telencephalon of Nes11Cre/Catnb$^{lox(ex3)}$ mice, the expression level of subpallial markers, *Nkx2.1*, *Mash1*, *Gsh2*, *Olig2*, and *Dlx2* were significantly decreased. Interestingly *Nkx2.1* is regulated by Sp1 and Sp3 directly (70). In the telencephalon of Nes11Cre/Catnb$^{lox(ex3)}$ mice, the expression level of *Nkx2.1* is significantly reduced in the subpallium where *Sp5* is ectopically expressed (69). In addition, other subpallial markers have putative canonical Sp1 binding sites and its related sequence in their proximal promoters. Furthermore we noticed that *Mash1* was down-regulated in Sp5-infected neurospheres by microarray and real-time reverse transcription-PCR.[6] Our results and previous reports suggest that Wnt/β-catenin signaling induces *Sp5* and represses subpallial markers to establish dorso-ventral specification. Wnt/β-catenin signaling is also essential for the maintenance of proliferation of neural progenitors (6). Wnt/β-catenin signaling induces *Cyclin D1* and c-*myc*, which affect cell proliferation (2, 71). It has been shown that Sp1 and other Sp1 family members have an effect on proliferation and apoptosis (20). In addition, the expression level of several genes that affect cell proliferation, differentiation, and apoptosis were changed in Sp5-infected neurosphere culture and in MCF7-transformed cell line (Table 1) (72). Because *Sp5* gene is induced by Wnt/β-catenin signaling, the maintenance of proliferation of neural precursors might be partially regulated by Sp5.

We attempted to correlate a profound CNS phenotype observed *in vivo* (Ref. 6 and this study) with any discernable phenotype in neurospheres cultured *in vitro*. To this end, we have isolated neurospheres from Nes11Cre/Catnb$^{lox(ex3)}$ telencephalon and compared them to Sp5-infected and mock-infected neurospheres. However, neurospheres overexpressing Sp5 or activated β-catenin (isolated from Nes11Cre/Catnb$^{lox(ex3)}$ telencephalons) did not show any significant

[5] J. Ruzickova and Z. Kozmik, unpublished data.

[6] O. Machon and T. Vacik, data not shown.

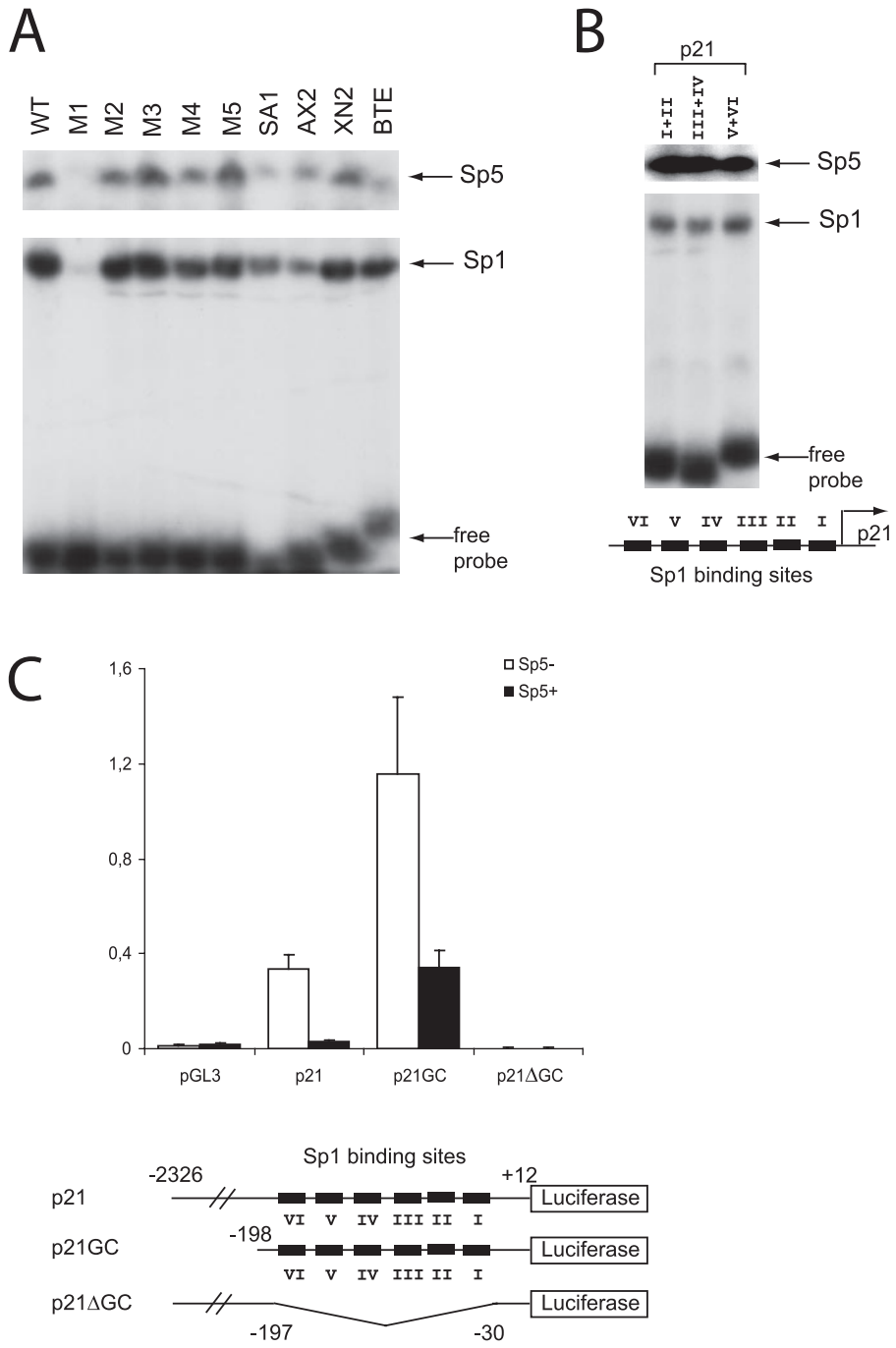


FIGURE 6. **Sp5 binds the Sp1 recognition sequences and regulates the Sp1 target gene, *p21*.** *A*, EMSA was performed using a consensus Sp1 binding site (*WT*) and various modifications (mutants *M1–M5*). *SA1*, *AX2*, and *XN2* represent Sp1 regulatory elements from the *TGF-βRI* gene (25). The BTE binding site is a target sequence of the closely related transcription factor BTEB3 (26). Sequences of the oligonucleotides are described under "Experimental Procedures." *B*, Sp5 binds Sp1 regulatory elements from the human *p21* gene promoter in EMSA. *C*, mapping of the Sp5-responsive elements in *p21* promoter. An expression plasmid encoding Sp5 or an empty expression plasmid (100 ng) were cotransfected with the indicated luciferase reporter plasmids (100 ng) into 293T cells. β-Galactosidase expression plasmid (5 ng) was cotransfected to normalize for transfection efficiency.

changes in cell growth and differentiation into various neural lineages when compared with wild-type neurospheres.[7] This is most likely due to the dominant effect of epidermal growth factor/basic fibroblast growth factor growth factors necessary to propagate neurospheres *in vitro*. We have recently shown that the original dorsal telencephalon cell fate is lost in neurosphere cultures grown in the presence of epidermal growth factor/basic fibroblast growth factor and that the expression profile is specifically changed in cultured cells in just three passages (77).

A more than 100-fold up-regulation of Sp5 expression in Sp5-infected neurospheres lead to only a 2- to 3-fold down-regulation of most known Sp1 target genes. There are at least two potential reasons to explain this apparent discrepancy. First of all, the level of Sp5 protein as a repressor has to reach the level at which it can overcome the activator function of ubiquitously expressed Sp1-family members such as Sp1 and Sp3. Therefore, even 100-fold up-regulation of Sp5 mRNA may not represent a sufficient amount of Sp5 protein to observe stronger repression of known Sp1 target genes in our experimental system. In addition, Sp5 expression was determined across the whole neurosphere population at the mRNA level using quantitative reverse transcription-PCR. To achieve a widespread overexpression of Sp5, the infected cells carrying the Sp5 retrovirus were selected using G418. Nevertheless, the selected cell pools might contain some proportion of G418-resistent cells not expressing Sp5 protein. No commercial Sp5 antibodies are currently available to allow analysis of Sp5 protein expression at the single-cell level.

Intriguingly, it was argued that zebrafish Sp5 might work as a transcriptional activator. First of all, Sp5 partially rescued *Drosophila* embryos mutated in *buttonhead* (Btd), one of *Drosophila* Sp1 homologues known to act as an activator (61). Furthermore, zebrafish Sp5 induced pax2.1 expression in the midbrain-hindbrain boundary (61). However, this latter result could be explained by an indirect effect: by Sp5 repressing a repressor of pax2.1. In fact all three repressor domains R1, R2, and R3 are highly conserved between mouse and zebrafish, 71%, 63 and 56%, respectively. In addition mSin3a core consensus site and small ubiquitin-

[7] O. Machon, S. Krauss, and Z. Kozmik, unpublished data.

TABLE 1

List of genes down-regulated in Sp5-overexpressing primary neurospheres

From a total of 107 genes downregulated >2-fold, only those containing Sp1 binding sites in their regulatory regions (−500/+1 and 5′ UTR) are shown. References indicate previous studies of the genes with respect to Sp1 regulation.

Gene Symbol	Gene	Fold change (Sp5+ / Sp5-)	reference
Rpl14	ribosomal protein L14	0,38	
Gpiap1	GPI-anchored membrane protein 1	0,42	
Ccnd1	cyclin D1	0,47	43
Sc4mol	sterol-C4-methyl oxidase-like	0,5	
2610209M04Rik	RIKEN cDNA 2610209M04 gene	0,33	
Cd24a	CD24a antigen	0,43	51
Poldip3	polymerase (DNA-directed), delta interacting protein 3	0,45	
Trim27	tripartite motif protein 27	0,44	39
Rab8a	RAB8A, member RAS oncogene family	0,46	
Mtap2	Microtubule-associated protein 2	0,37	
Msn	moesin	0,26	52
Ranbp9	RAN binding protein 9	0,45	
Hnrpab	heterogeneous nuclear ribonucleoprotein A/B	0,46	
Tde1	Tumor differentially expressed 1	0,48	
Hip1	huntingtin interacting protein 1	0,44	
Atp1b2	ATPase, Na+/K+ transporting, beta 2 polypeptide	0,35	31
Klf3	Kruppel-like factor 3 (basic)	0,32	
Mtx1	metaxin 1	0,5	36
1110001A07Rik	RIKEN cDNA 1110001A07 gene	0,5	
Tfrc	transferrin receptor	0,46	42
Fscn1	fascin homolog 1, actin bundling protein (Strongylocentrotus) purpuratus)	0,4	34
Eif2s3x	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	0,48	
Pdlim5	PDZ and LIM domain 5	0,41	
Vps35	vacuolar protein sorting 35	0,48	
Git2	G protein-coupled receptor kinase-interactor 2	0,47	
Ptprs	protein tyrosine phosphatase, receptor type, S	0,23	
Stard4	StAR-related lipid transfer (START) domain containing 4	0,48	47
Nes	nestin	0,38	35
Pafah1b2	platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit	0,46	
Cbx5	chromobox homolog 5 (Drosophila HP1a)	0,45	
Tagln2	transgelin 2	0,47	
Scd1	stearoyl-Coenzyme A desaturase 1	0,32	45
Kif2a	kinesin family member 2A	0,36	
Myo10	myosin X	0,22	
Slc41a3	solute carrier family 41, member 3	0,35	
Slc12a2	solute carrier family 12, member 2	0,29	46
Ctcf	CCCTC-binding factor	0,37	40
Epb4.1l4a	erythrocyte protein band 4.1-like 4a	0,48	
Ctdsp2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2	0,45	
Kif11	kinesin family member 11	0,39	
Coro1c	coronin, actin binding protein 1C	0,5	
Skp2	S-phase kinase-associated protein 2 (p45)	0,38	38
Ndn	necdin	0,48	49
Map2k4	mitogen activated protein kinase kinase 4	0,32	
Eif4g1	eukaryotic translation initiation factor 4, gamma 1	0,15	
D2Ertd435e	DNA segment, Chr 2, ERATO Doi 435, expressed	0,43	
Syncrip	synaptotagmin binding, cytoplasmic RNA interacting protein	0,37	
Fzd2	frizzled homolog 2 (Drosophila)	0,47	
Trip4	thyroid hormone receptor interactor 4	0,3	
Ddx6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	0,4	
Mthfd2	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	0,39	32
Smarcc1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	0,45	29
Trp53inp1	transformation related protein 53 inducible nuclear protein 1	0,4	
Clcn6	chloride channel 6	0,49	
AW557805	expressed sequence AW557805	0,41	
Mdm2	transformed mouse 3T3 cell double minute 2	0,48	33
Brd3	bromodomain containing 3	0,46	
Wnt7b	wingless-related MMTV integration site 7B	0,49	50
Spry4	sprouty homolog 4 (Drosophila)	0,38	37
Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0,34	
Csen	calsenilin, presenilin binding protein, EF hand transcription factor	0,49	
Baz1b	bromodomain adjacent to zinc finger domain, 1B	0,42	
Usp1	ubiquitin specific protease 1	0,45	
Sort1	sortilin 1	0,44	
D030056L22	hypothetical protein D030056L22	0,3	
Shoc2	soc-2 (suppressor of clear) homolog (C. elegans)	0,44	
Lman1	lectin, mannose-binding, 1	0,44	48
Kif1b	kinesin family member 1B	0,41	
Ccnd2	cyclin D2	0,4	41
Rab3b	RAB3B, member RAS oncogene family	0,42	
Adcyap1r1	adenylate cyclase activating polypeptide 1 receptor 1	0,34	30
Rrbp1	Ribosome binding protein 1	0,49	
Arhgef1	Rho guanine nucleotide exchange factor (GEF) 1	0,48	
2610507B11Rik	RIKEN cDNA 2610507B11 gene	0,44	
Ugcg	UDP-glucose ceramide glucosyltransferase	0,36	
Adcyap1r1	adenylate cyclase activating polypeptide 1 receptor 1	0,4	
Xpr1	xenotropic and polytropic retrovirus receptor 1	0,42	
BC010304	cDNA sequence BC010304	0,23	
Smad5	MAD homolog 5 (Drosophila)	0,33	
Tbl1xr1	transducin (beta)-like 1X-linked receptor 1	0,46	
Kif1a	kinesin family member 1A	0,48	
Narg1	NMDA receptor-regulated gene 1	0,5	
Mbtps1	membrane-bound transcription factor protease, site 1	0,48	44
C1galt1	core 1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase	0,4	
2610005L07Rik	RIKEN cDNA 2610005L07 gene	0,2	
Dia1	diaphorase 1 (NADH)	0,17	
Rev3l	REV3-like, catalytic subunit of DNA polymerase zeta RAD54 like (S. cerevisiae)	0,34	
Fmr1	fragile X mental retardation syndrome 1 homolog	0,33	
Smc1l1	SMC (structural maintenance of chromosomes 1)-like 1 (S. cerevisiae)	0,37	

related modifier (SUMO) modification site are evolutionarily conserved. According to the high sequence similarity within the repressor domains, zebrafish Sp5 has the potential to act as a repressor. Although in the full-length context Sp5 acts as a potent repressor, Sp5 has a cryptic transactivation domain between amino acids 77 and 151. Furthermore, the R1 domain with a mutation in the SID that abrogates mSin3a binding acted as transactivation domain. The phosphorylation of TIEG2 at Thr/Ser adjacent to SID by Erk2 results in the disruption of TIEG2-mSin3a interaction (73). Sp5 also contains an Erk2 consensus site, (S/T)P (74), adjacent to the SID. Previous reports and our results indicate that Sp5 may act as an activator in some contexts.

Sp8 is another member of *Sp1* family (75). The sequence similarity of the zinc finger domain between Sp5 and Sp8 is 93.8%. The expression pattern of *Sp8* and *Sp5* in mouse is quite similar, for example during CNS development (76). *Sp8* knock-out mice die at birth and manifest severe phenotypes in the CNS (76). On the other hand, *Sp5* knock-out mice show no obvious phenotype (9). Furthermore, Wnt/β-catenin signaling induces *Sp8*, although it is not known whether *Sp8* is directly regulated by TCF/LEF/β-catenin transcription complex (75, 76). Sp8 acts as an activator during limb development (75). However, Sp8 has the potential to act as a repressor, because Sp8 has several mSin3a core consensus sequences and polyalanine tracts.[8] Sp8 is likely to bind to most Sp1 binding sites due to the high sequence similarity of its zinc finger domain to that of Sp1. It may be *Sp8* that compensates for *Sp5* in the *Sp5* knock-out mice. Our results and previous reports suggest that Wnt/β-catenin signaling regulates Sp1 target genes by inducing *Sp5* and potentially *Sp8*.

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

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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

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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-

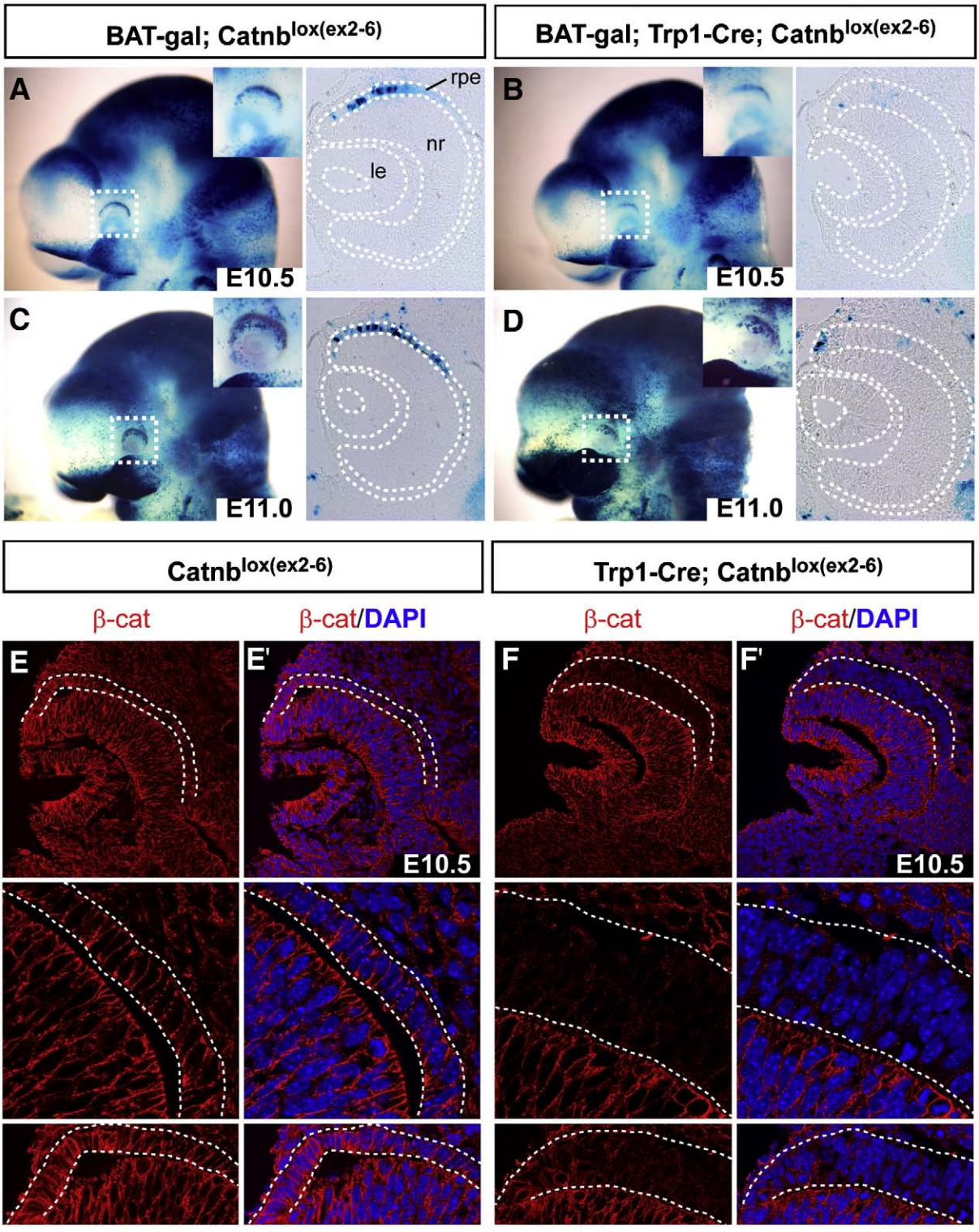

Fig. 1. Conditional inactivation of Wnt/β-catenin signaling in the RPE. The activity of Wnt/β-catenin signaling was assessed using Wnt/β-catenin signaling reporter line BAT-gal. Whole-mount or frontal cryosections of wild-type (A, C) and loss-of-function mutant (B, D) were stained with X-gal at the indicated 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).

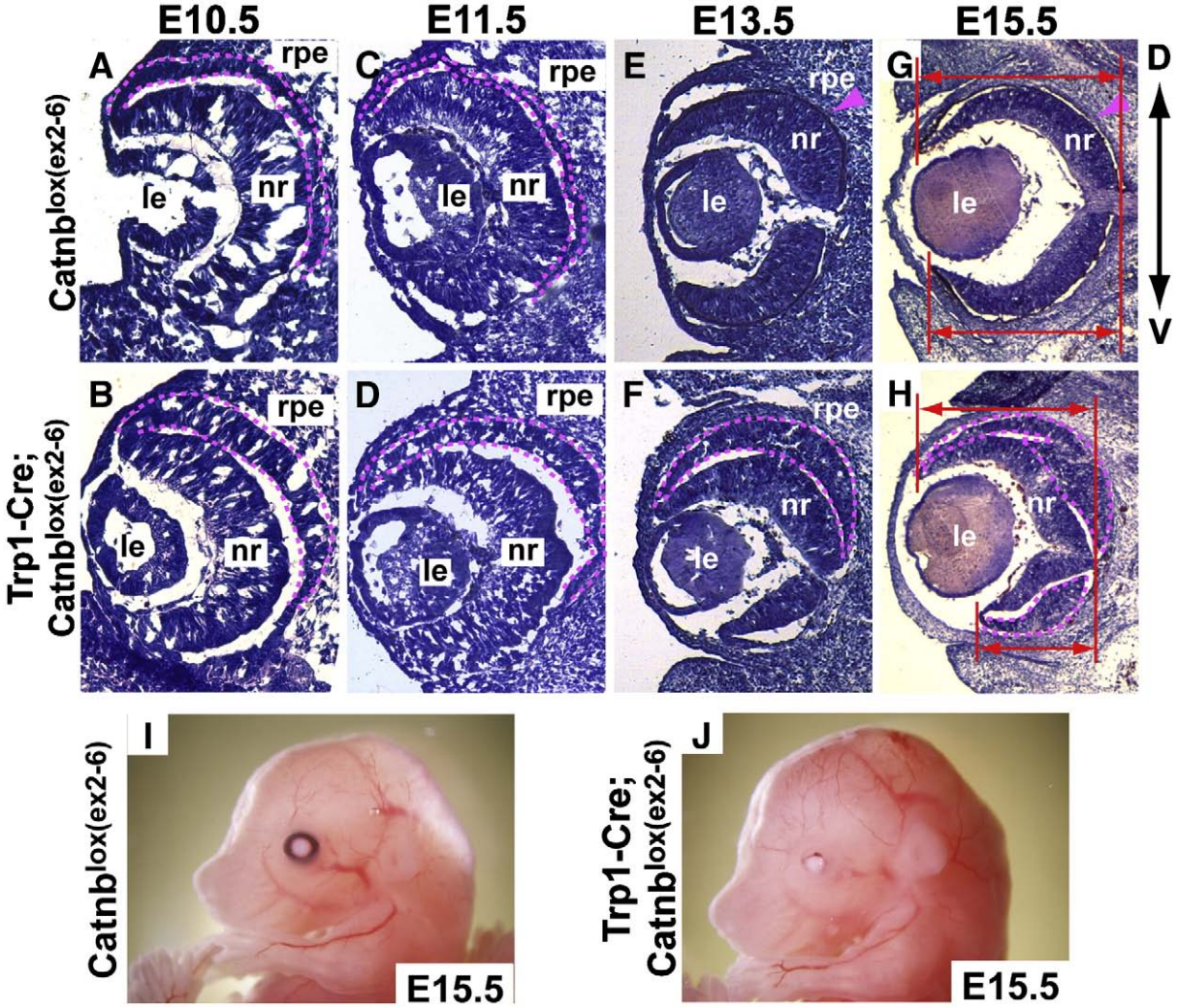

Fig. 2. Phenotypic consequences in the loss-of-function β-catenin mutant. Frontal cryosections of wild-type (A, C, E, and G) and loss-of-function mutant (B, D, F, and H) were stained by hematoxylin and eosin at the indicated embryonic stage. Red lines indicate the size of the ventral and dorsal NR, respectively. 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).

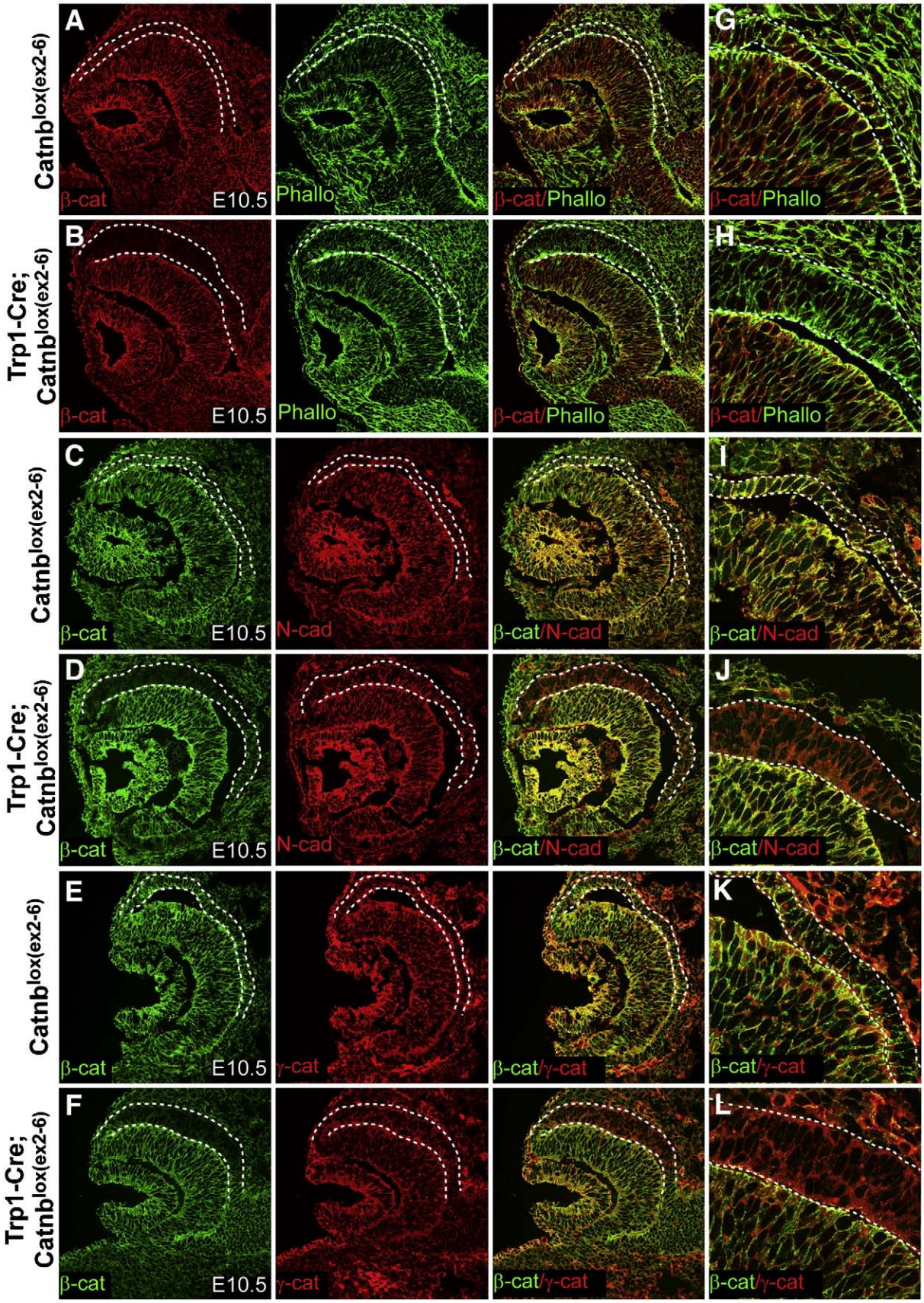

Fig. 3. Conditional inactivation of β-catenin in the RPE does not affect cell adhesion. Frontal cryosections of wild-type (A, C, and E) and loss-of-function mutant (B, D, and F) were immunostained with β-catenin and phalloidin (A, B), β-catenin and N-cadherin (C, D), or β-catenin and γ-catenin (E, F) 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.

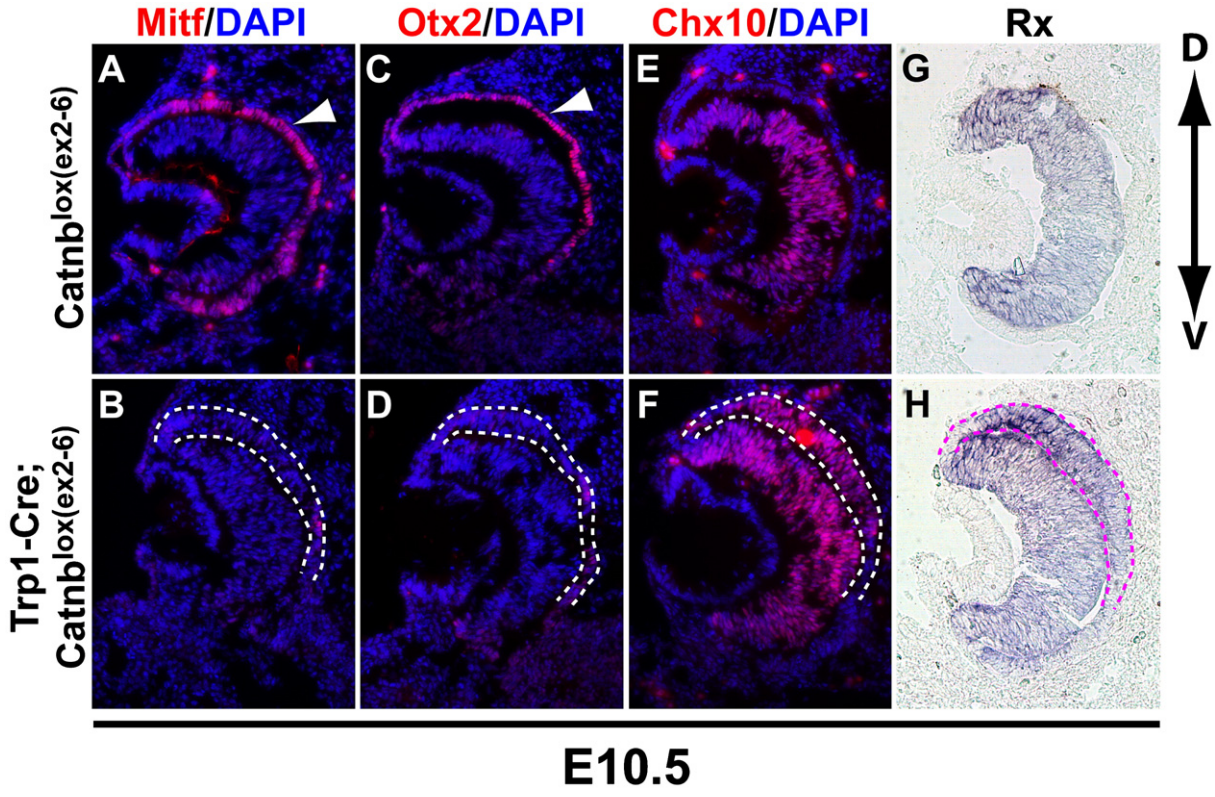

Fig. 4. Conditional inactivation of Wnt/β-catenin signaling results in transdifferentiation of RPE to NR. Frontal cryosections of wild-type (A, C, E and G) and loss-of-function mutant (B, D, F and H) at E10.5 were immunostained with Mitf (A, B), Otx2 (C, D) and Chx10 (E, F) antibodies or hybridized with 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-

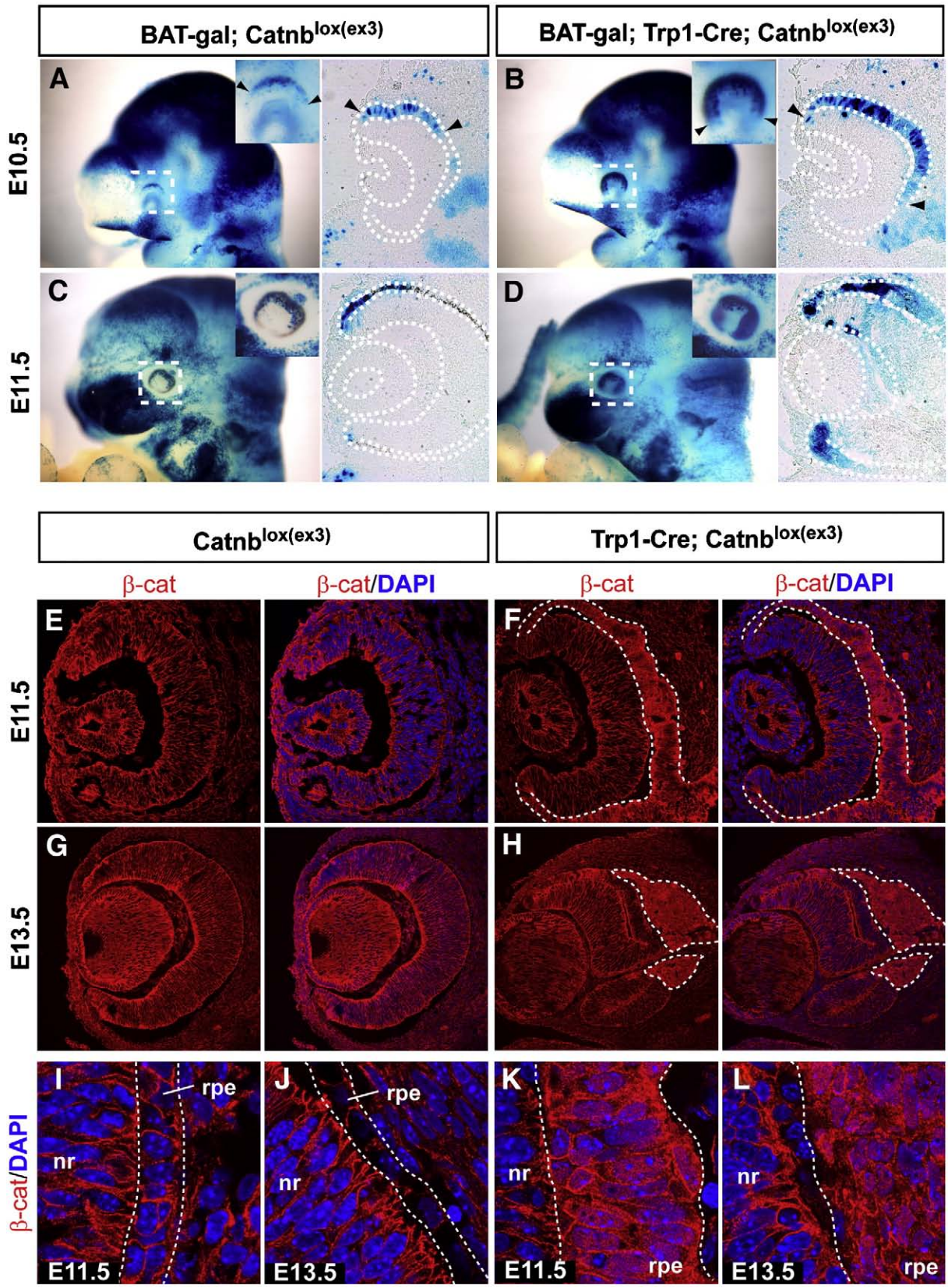

Fig. 5. Ectopic activation of Wnt/β-catenin signaling in the RPE. The activity of Wnt/β-catenin signaling was assessed using Wnt/β-catenin signaling reporter line BAT-gal. Whole-mount or frontal cryosections of wild-type (A, C) and gain-of-function mutant (B, D) were stained with X-gal to assess the 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.

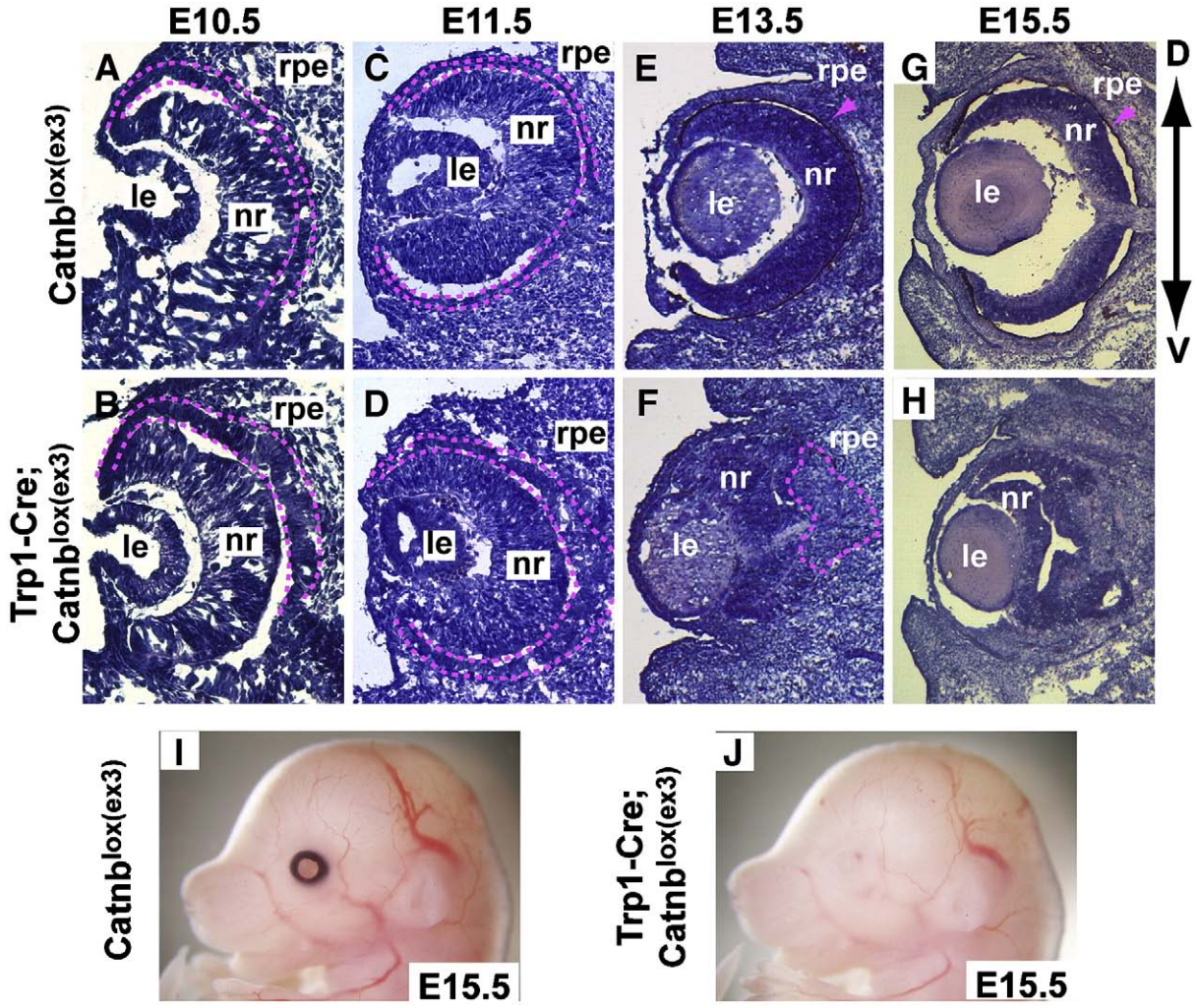

Fig. 6. Phenotypic consequences in the gain-of-function β-catenin mutant. Frontal cryosections of wild-type (A, C, E and G) and gain-of-function mutant (B, D, F and H) were stained by hematoxylin and eosin at the indicated embryonic stage. The RPE is indicated by a dashed line or an arrowhead. The embryo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

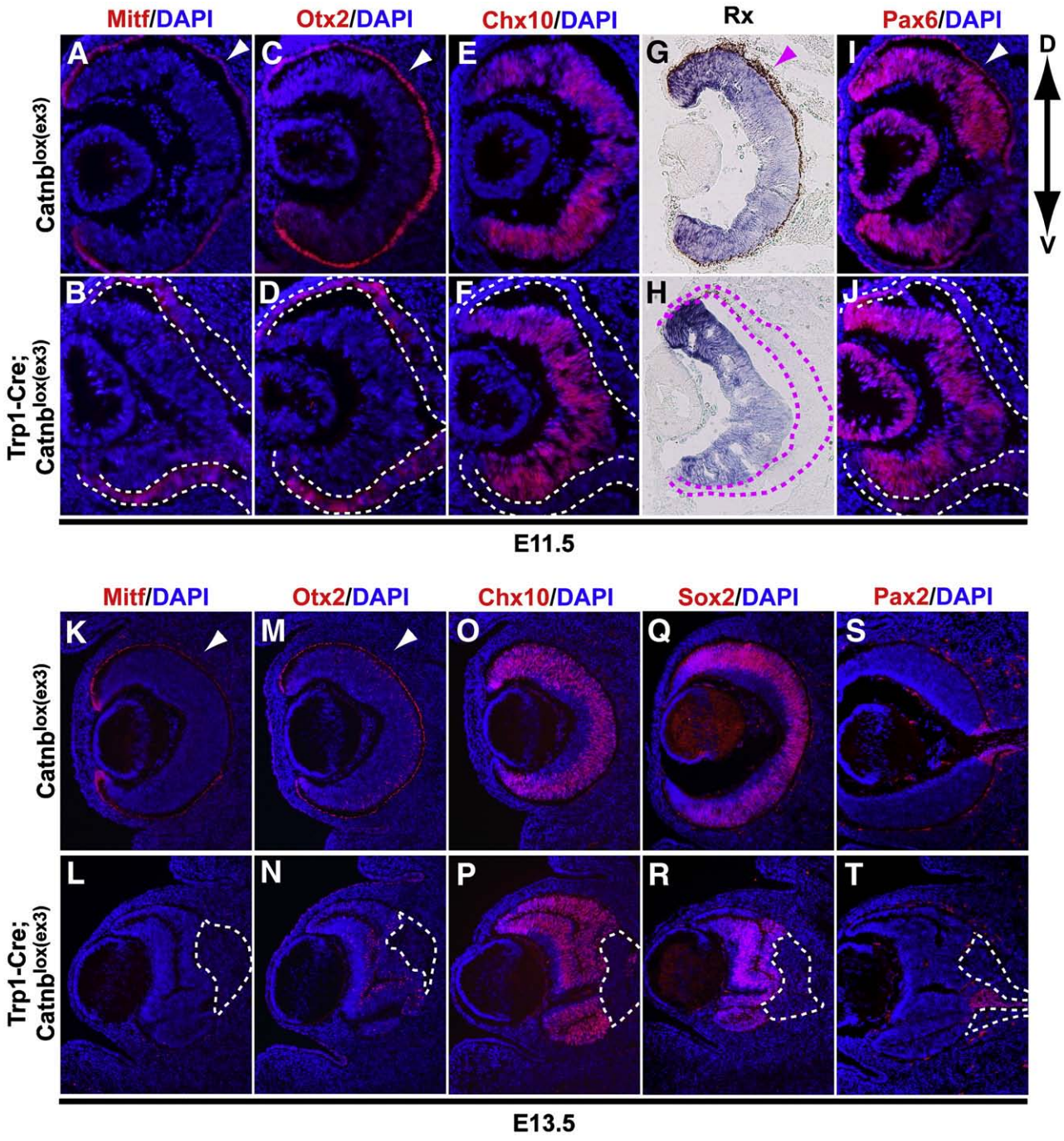

Fig. 7. Ectopic activation of Wnt/β-catenin signaling results in the abrogation of the RPE development. Frontal cryosections of wild-type (A, C, E, G, I, K, M, O, Q, and S) and gain-of-function mutant (B, D, F, H, J, L, N, P, R, and T) at E11.5 or E13.5 were immunostained with Mitf (A, B, K, and L), 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

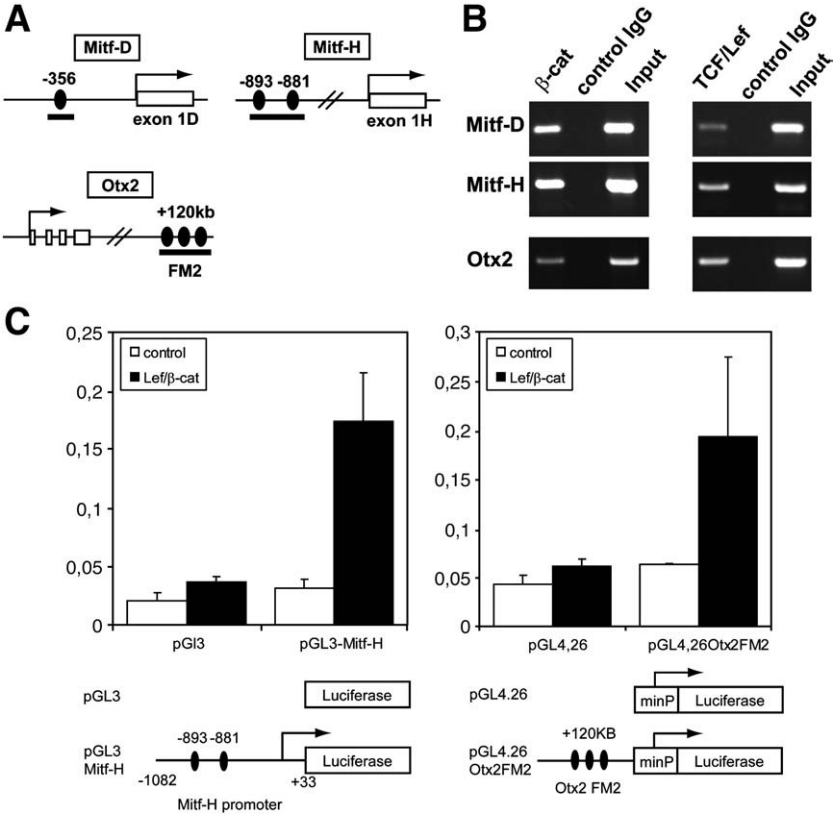

Fig. 8. Wnt/β-catenin signaling directly regulates *Mitf* and *Otx2* genes. (A) Map of the *Mitf-D*, *Mitf-H*, and *Otx2* (Kurokawa et al., 2004) locus with putative Tcf/Lef binding sites depicted by black ovals. (B) Chromatin immunoprecipitation was performed with wild-type embryos at E10.5 using Tcf/Lef, 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

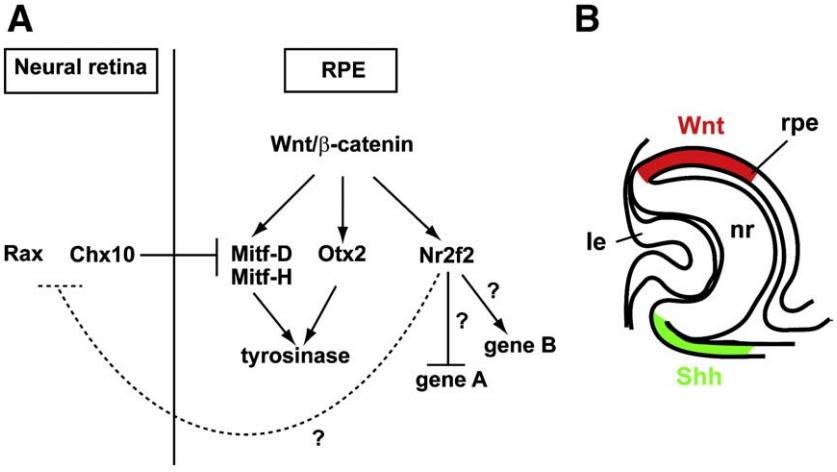

Fig. 9. A model summarizing the role of Wnt/β-catenin signaling in RPE development. (A) Transcriptional network downstream of Wnt/β-catenin signaling in RPE. Wnt/β-catenin signaling in the RPE directly maintains or induces expression of *Mitf-H*, *Mitf-D*, *Otx2* and *Nr2f2*. *Mitf-D*, Mitf-H and Otx2 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.

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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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Supplementary material

Supplementary Fig. 1. Previously identified direct target genes of Wnt/β-catenin signaling, *Axin2* and *Lef1*, are expressed in dorsal RPE. Frontal cryosections of E10.5 embryo hybridized with *Axin2* antisense riboprobe (A). Frontal cryosections of E10.5 wild type (B) and loss-of-function mutant (C) with immunostaining for Lef1 (red) and DAPI nuclear labeling (blue). Magnified view of the RPE is shown below the corresponding panels. The RPE is indicated by a dashed line and the arrowheads demarcate the area of staining. Please note that Lef1 is downregulated in the RPE of the loss-of-function mutant. Abbreviations used in this and in subsequent figures are as follows: rpe, retinal pigment epithelium (RPE); le, lens; nr, neural retina (NR).

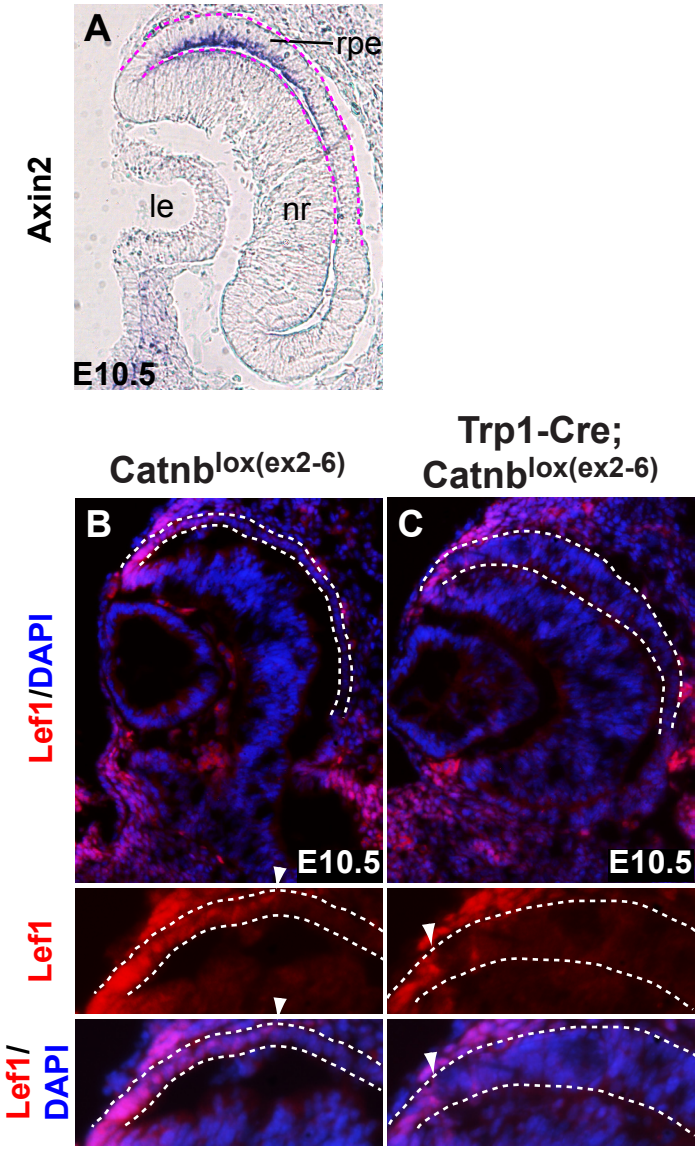
Supplementary Fig. 2. Cre recombinase activity of *Trp1-Cre* line. Cre recombinase activity was assessed using *ROSA26R* Cre recombinase reporter mice at E10.0 (A) and E11.5 (B). Whole mount or frontal cryosections were stained with X-gal. The RPE is indicated by a dashed line.

Supplementary Fig. 3. Expression of α-catenin and P-cadherin in wild type and loss-of-function mutant. Frontal cryosections of wild type (A, C) and loss-of-function mutant (B, D) were immunostained with α-catenin (A, B), or double-immunostained with β-catenin (green) and P-cadherin (red) (C, D) at E10.5. Please note that the absence of β-catenin does not affect the staining pattern of α-catenin. A magnified view of the RPE is shown below the corresponding panels. The RPE is indicated by a dashed line and DAPI is used for nuclear labeling (blue). The images were obtained by confocal microscope analysis.

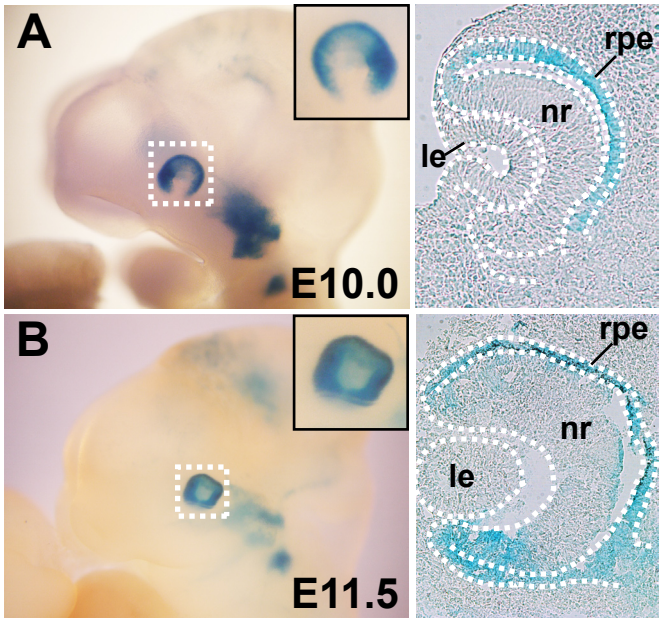
Supplementary Fig. 4. Ectopic activation of Wnt/β-catenin signaling results in the abrogation of the RPE development. Horizontal cryosections of wild type (A, C, E, G, and I) and gain-of-function mutant (B, D, F, H, and J) at E11.5 were immunostained with Mitf (A, B), Otx2 (C, D), Chx10 (E, F), Pax6 (G and H), and Pax2 (I and J) antibodies. The RPE is indicated by a dashed line and DAPI is used for nuclear labeling (blue).

Supplementary Fig. 5. Tcf/Lef and β-catenin bind to *Nr2f2* regulatory regions *in vivo*. (A-J) Frontal cryosections of wild type (A, C, E, G and I) and loss-of-function mutant (B, D, F, H,

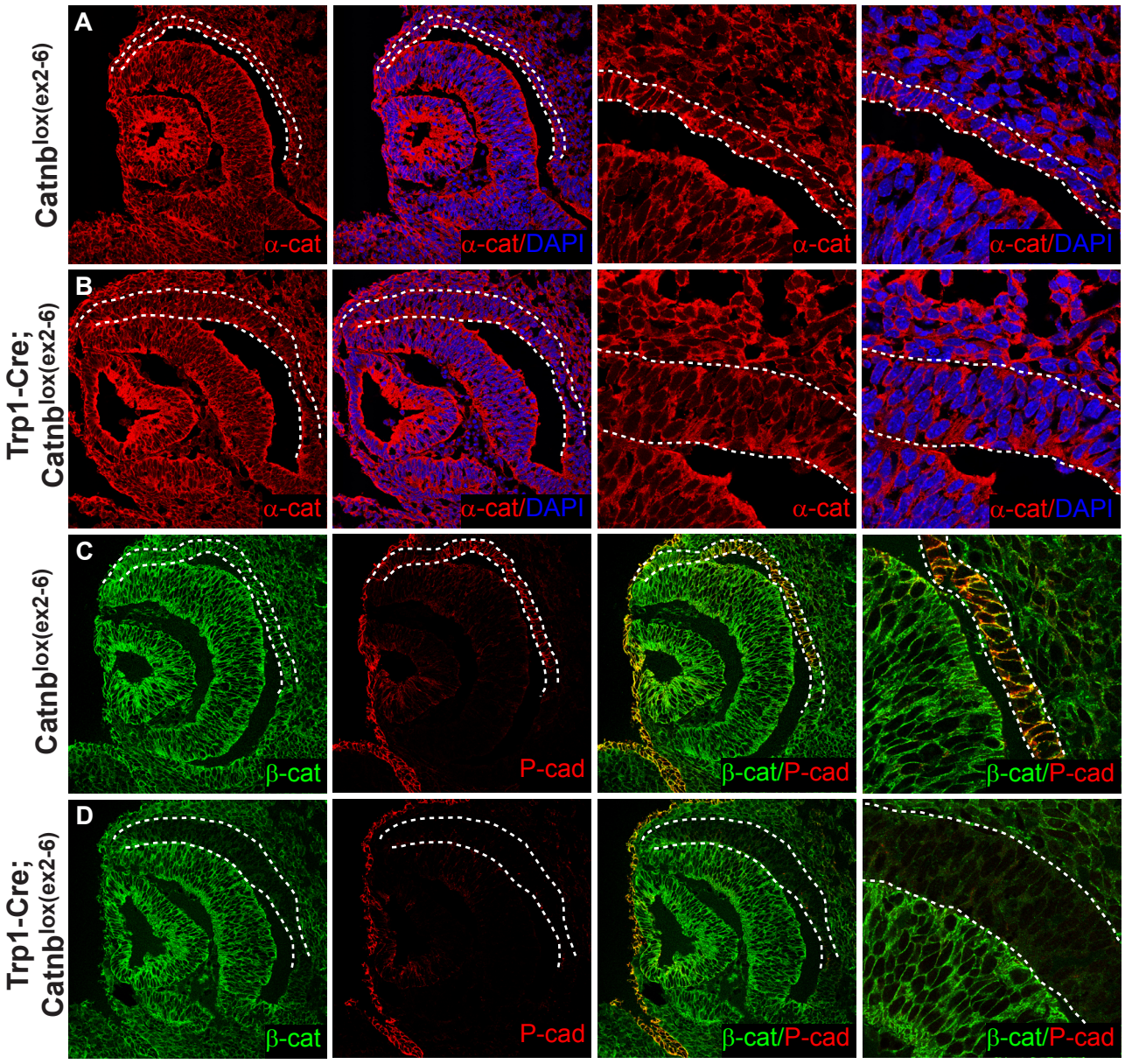
and J) at E10.5 were immunostained with Pax6 (A and B), Pax2 (C and D), Nr2f1 (E and F) and Nr2f2 (G and H) antibodies or hybridized with an antisense probe against *Vax2* (I and J). (K) Map of the *Nr2f2* locus with putative Tcf/Lef binding sites depicted by black ovals. (L) Chromatin immunoprecipitation was performed with wild type embryos at E10.5 using β-catenin, Tcf/Lef, or control antiserum. PCR was carried out with specific primers detecting the indicated transcriptional regulatory elements depicted in panel (K).



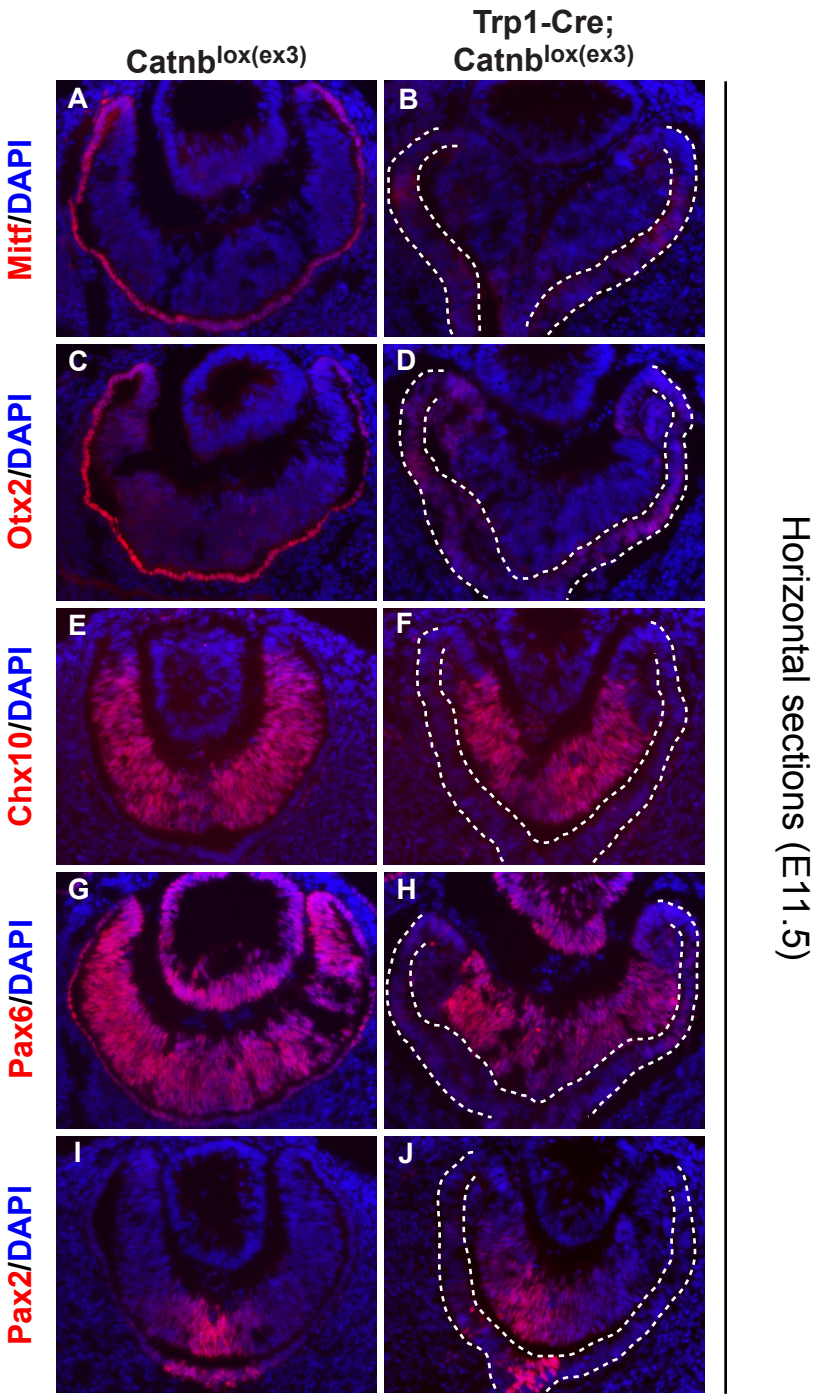
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3



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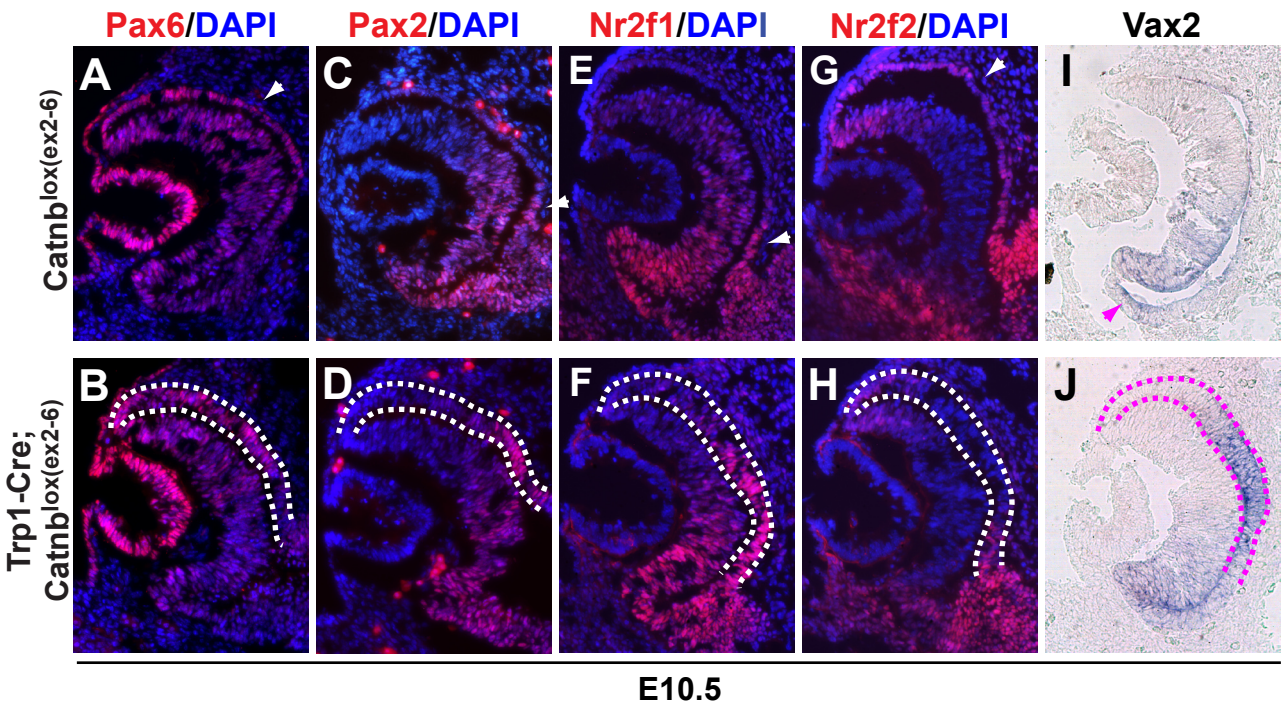

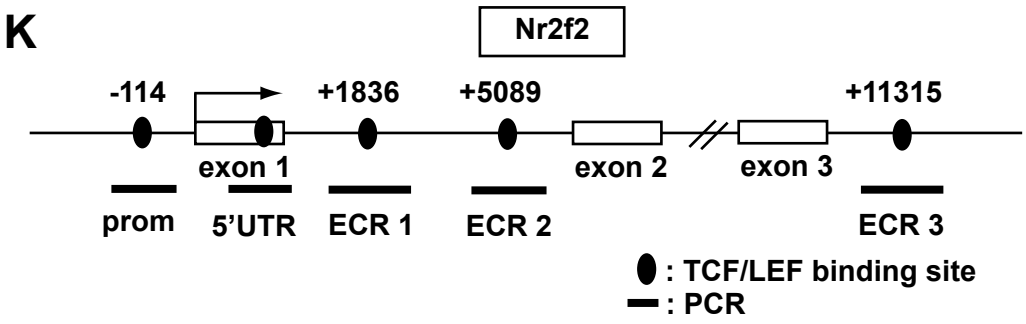

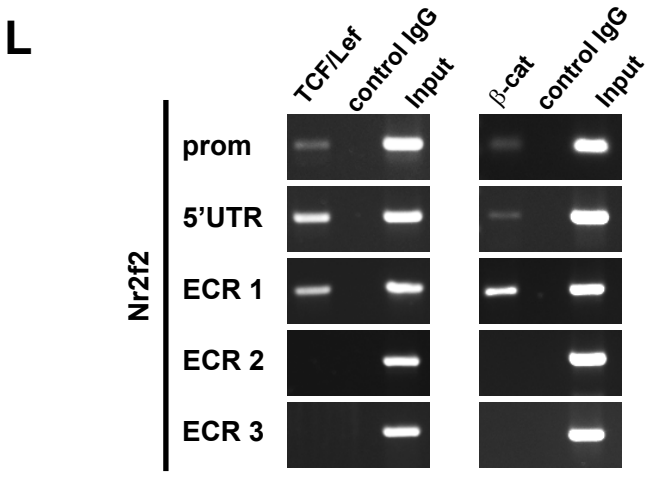

Supplementary Fig. 5

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

ARTICLE

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

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

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

INTRODUCTION

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

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

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

RESULTS

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

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

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

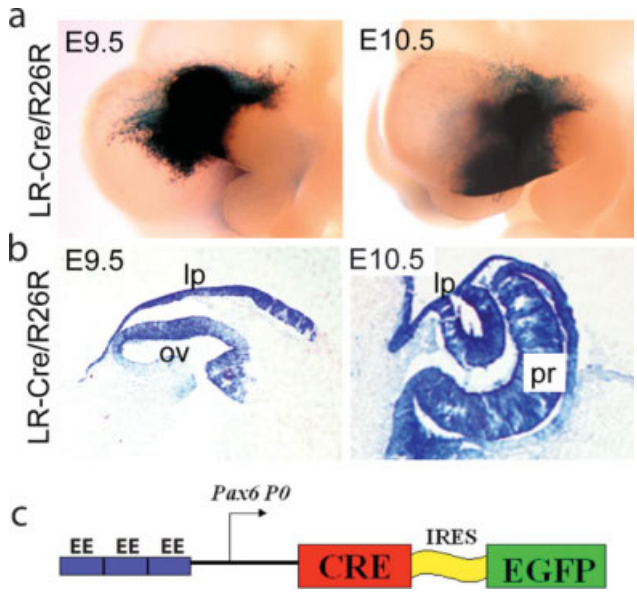

FIG. 1. Cre activity in the LR-Cre transgenic mouse. (**a**) The LR-Cre activity was monitored in the R26R reporter mouse crossed to LR-Cre with a strong staining in the lens ectoderm and the surrounding face ectoderm. (**b**) Sections of LR-Cre; R26R eyes showing the Cre activity in developing lens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

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

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

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

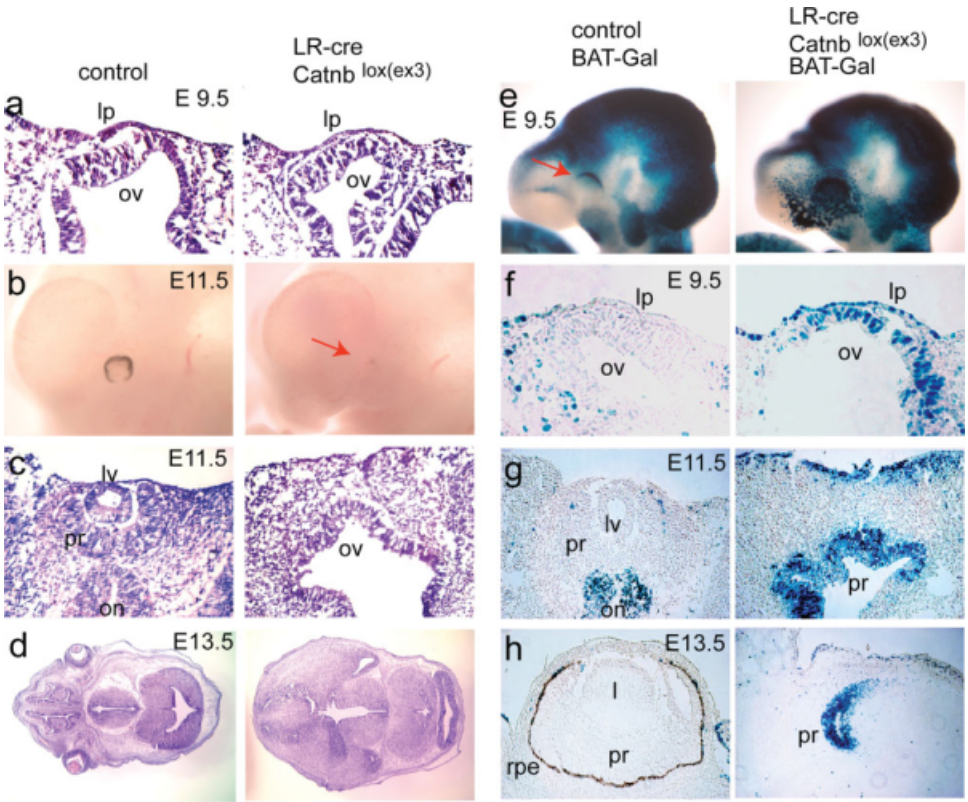

FIG. 2. Aberrant canonical Wnt/β-catenin signaling inhibits proper lens development. (**a**) Hematoxylin-eosin staining of the lens placode in controls and LR-Cre;Catnb$^{lox(ex3)}$ mutants showing thinner placodal layer at E9.5. (**b**) Missing lens in LR-Cre;Catnb$^{lox(ex3)}$ mutants at E11.5 with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

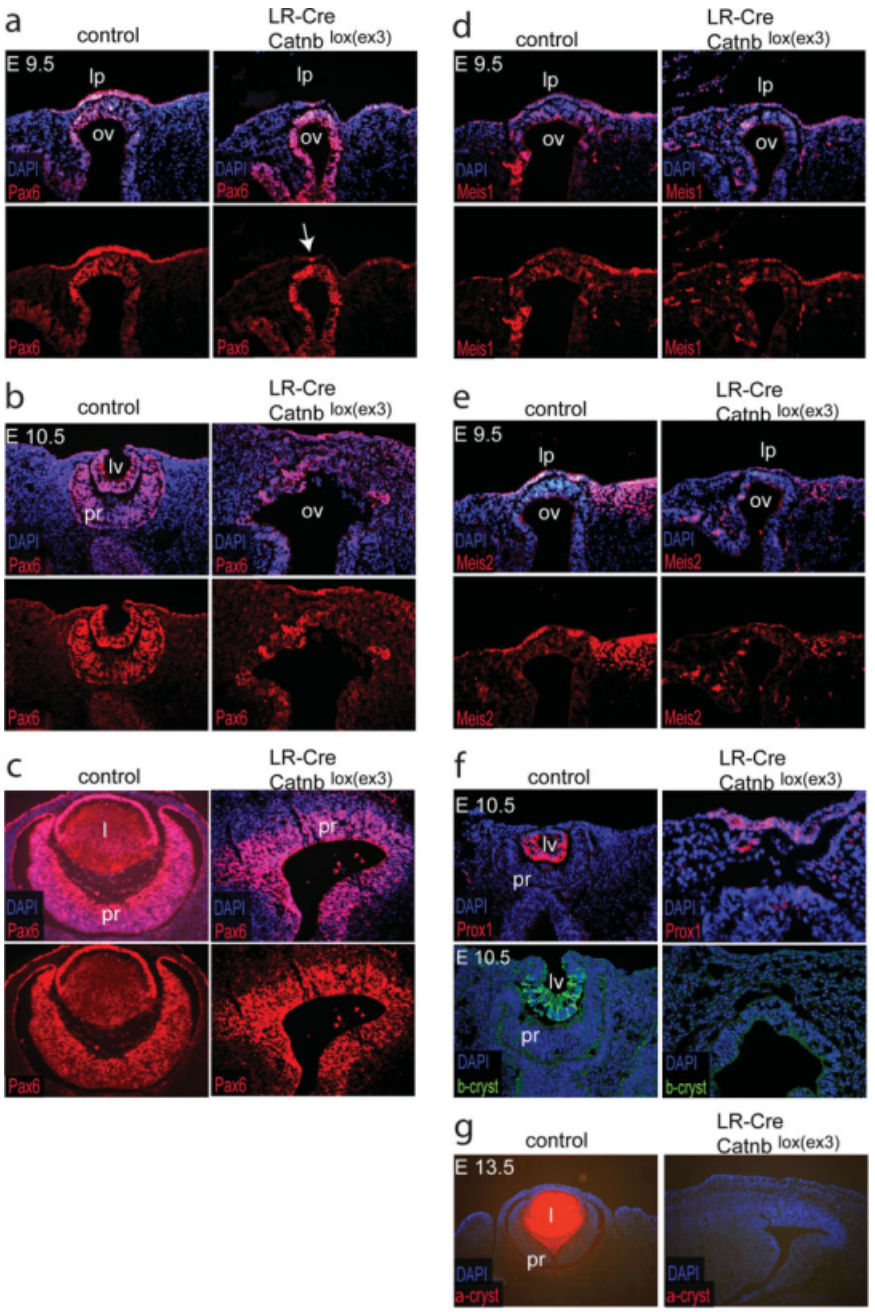

FIG. 3. Aberrant canonical Wnt/β-catenin signaling inhibits lens cell fate. Fluorescent immunostaining of various lens markers (in red or green) in LR-Cre;Catnb$^{lox(ex3)}$ mutants and controls with parallel DAPI counterstaining (in blue). (**a**) Pax6 expression at E9.5, at E10.5 (**b**) and at E13.5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

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

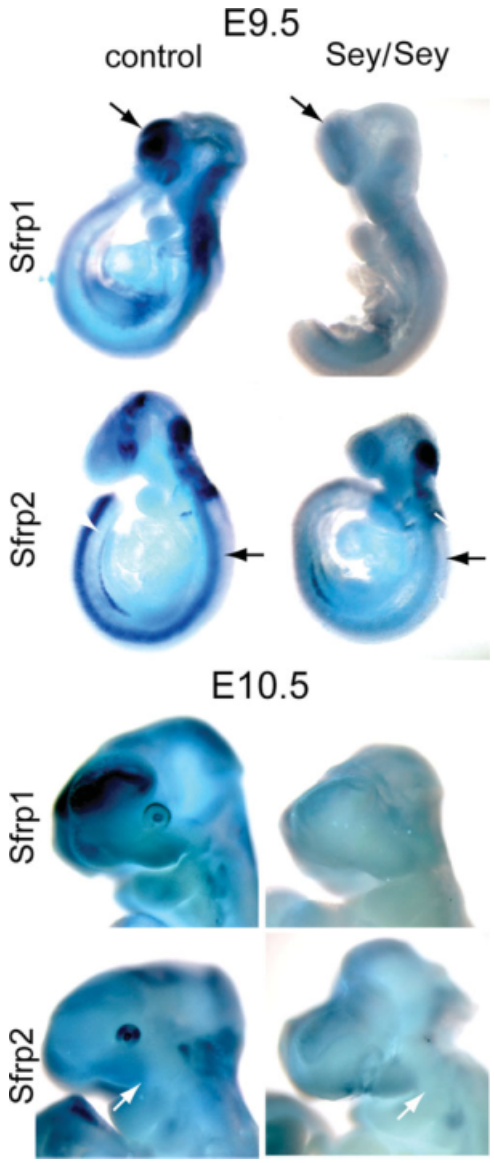

FIG. 4. Expression of *Sfrp1* and *Sfrp2* in wild type and Sey/Sey (Pax6-null) embryos by whole mount in situ hybridization. *Sfrp1* mRNA at E9.5 and E10.5 is normally seen in the optic vesicle and in the telencephalon of wild type embryos while in Sey/Sey embryos the signal disappears in both tissues.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

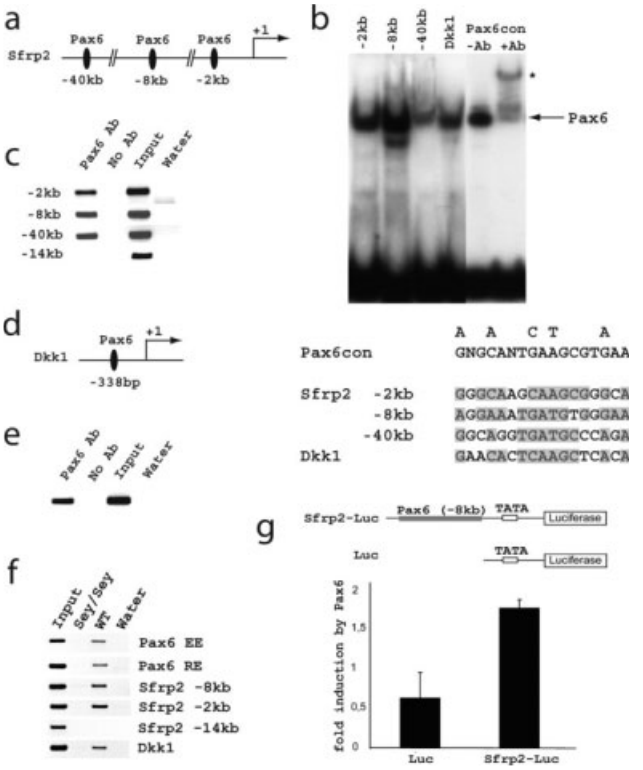

FIG. 5. Pax6 regulates transcription of *Sfrp2* and *Dkk1*. (**a**) Three putative binding sites for Pax6 reside in the *Sfrp2* gene. (**b**) Electrophoretic mobility shift assay showing that Flag-Pax6 binds to all three binding sites in the *Sfrp2* gene and to a binding site in *Dkk1* gene. Supershift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

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

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

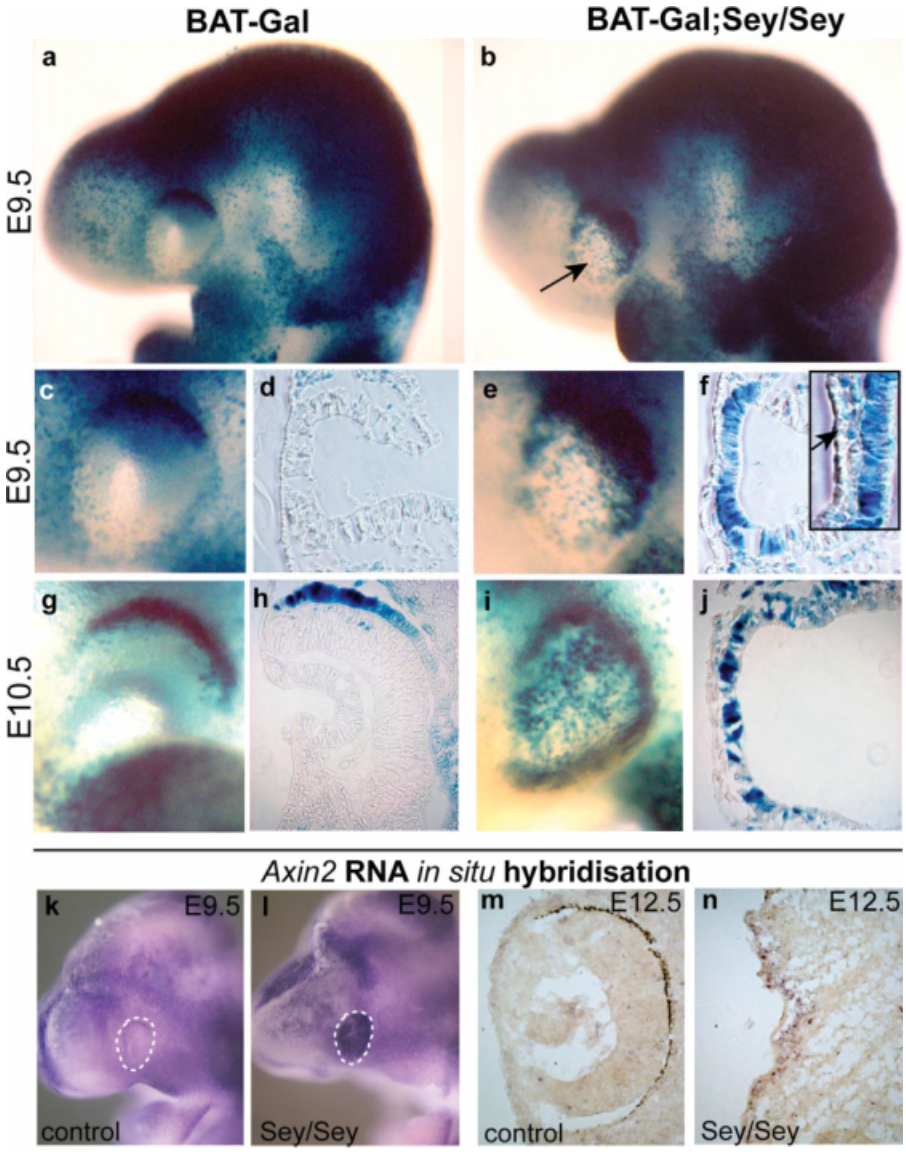

FIG. 6. Pax6 is involved in down-regulation of canonical Wnt//β-catenin signaling in the eye field. (**a–j**) Canonical Wnt activity was monitored using BAT-gal transgenic mice. (a,b) Whole-mount β-galactosidase staining of BAT-gal embryos at E9.5 in controls (a) and BAT-gal;Sey/Sey (b). (c,e) Detail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

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

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

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

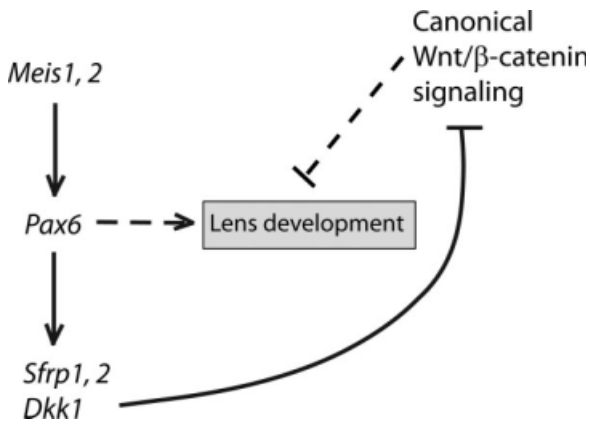

FIG. 7. Schematic diagram of the relationship between Pax6 transcriptional network and Wnt/β-catenin signaling depicting their positive and negative roles in lens development.

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

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

DISCUSSION

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

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

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

EXPERIMENTAL PROCEDURES

Mouse Lines

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

Tissue Collections and Histology

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

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

X-Gal Staining

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

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

Immunohistochemistry

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

In Situ Hybridization

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

Luciferase Assay

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

Electroforetic Mobility Shift Assay (EMSA)

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

Chromatin immunoprecipitation

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

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

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

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5. CONCLUSIONS

1. *Sp5* gene is a direct target of Wnt/β-catenin signaling and that Sp5 acts as a transcriptional repressor and represses Sp1-regulated target genes. Because the induction of *Sp5* by Wnt/β-catenin signaling is very high, *Sp5* might be useful as a new marker for Wnt/β-catenin signaling. It is known that Wnt/β-catenin signaling represses the transcription of several genes. Our report may give some insight into Wnt/β-catenin signaling-dependent repression.

2. 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.

3. Pax6 is required for down-regulation of canonical Wnt signaling in the presumptive lens ectoderm. Pax6 directly controls expression of several Wnt inhibitors such as *Sfrp1*, *Sfrp2*, and *Dkk1* in the presumptive lens. In accordance, absence of Pax6 function leads to aberrant canonical Wnt activity in the presumptive lens that subsequently impairs lens development.

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