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Investigation of Frizzled-5 During Embryonic Neural Development in Mouse

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Recent studies revealed that the Wnt receptor Frizzled-5 (Fzd5) is required for eye and retina development in zebrafish and *Xenopus*, however, its role during mammalian eye development is unknown. In the mouse embryo, Fzd5 is prominently expressed in the pituitary, distal optic vesicle, and optic stalk, then later in the progenitor zone of the developing retina. To elucidate the role of Fzd5 during eye development, we analyzed embryos with a germline disruption of the *Fzd5* gene at E10.25, just before embryos die due to defects in yolk sac angiogenesis. We observed severe defects in optic cup morphogenesis and lens development. However, in embryos with conditional inactivation of Fzd5 using Six3-Cre, we observed no obvious early eye defects. Analysis of Axin2 mRNA expression and TCF/LEF-responsive reporter activation demonstrate that Fzd5 does not regulate the Wnt/β-catenin pathway in the eye. Thus, the function of Fzd5 during eye development appears to be species-dependent. *Developmental Dynamics 237:1614–1626, 2008.* © 2008 Wiley-Liss, Inc.

Key words: mouse; eye; optic cup; morphogenesis; retina; lens; wnt; Frizzled; pituitary

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INTRODUCTION

Cellular and tissue–tissue interactions regulate development of the central nervous system (CNS) and a paramount question is what is the nature of the signals involved in these interactions. The vertebrate eye represents an excellent and challenging CNS model because it contains multiple cell and tissue types that must coordinate their development to form a functional unit. Eye development becomes apparent when the ventral diencephalic neuroepithelium evaginates to form the optic vesicles. The neural retina and retinal pigment epithelium become patterned in the distal and proximal domains of the optic vesicle, respectively. Adjacent extraocular tissues such as the surrounding mesenchyme, the overlying surface ectoderm and the ventral diencephalon regulate these patterning events. Interaction between the distal optic vesicle and surface (lens) ectoderm leads to invagination, formation of the optic cup, and subsequent development of the lens. Several genes have been shown to control optic cup morphogenesis and differentiation of ocular tissues (for reviews, see: Fuhrmann et al., 2000; Chow and Lang, 2001; Lang, 2004; Yang, 2004; Donner et al., 2006; Adler and Canto-Soler, 2007; Medina-Martinez and Jamrich, 2007).

The Wnt family of secreted glycoproteins (approximately 19 genes in mammals) regulates key developmental processes in the CNS such as proliferation, apoptosis, stem cell maintenance, lineage decision, differentiation, and axon guidance. Several Wnt pathway components are expressed in developing ocular tissues and modulation of Wnt signaling has

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revealed the importance of these signals at multiple stages of eye development (Wang et al., 1996; Borello et al., 1999; Rasmussen et al., 2001; Jin et al., 2002; Fuhrmann et al., 2003; see below and for reviews, see Van Raay and Vetter, 2004; Fuhrmann, 2008). Wnt proteins bind to surface receptors and act by means of three major pathways, all of which signal by means of Frizzled (Fzd) receptors, and approximately 10 Fzds are known in vertebrates to date. However, the specificity of Wnt and Fzd receptors for these pathways is often dependent on the cellular context and species. The best-characterized pathway is the Wnt/β-catenin (canonical) pathway, in which low-density lipoprotein receptor-related proteins (LRP) act as co-receptors and β-catenin represents the key player to activate transcription of target genes. Upon activation, β-catenin is stabilized and translocates into the nucleus where it interacts with high mobility group (HMG) box transcription factors such as TCF or LEF forming a transcriptional activator complex. (For details, see: http://www.stanford.edu/~rnusse/wntwindow.html) Alternatively, Wnt/Fzd signaling can stimulate intracellular Ca^{2+} release or activate the planar cell polarity pathway that involves activation of Jun- and MAP-kinases. Wnt/Fzd signaling is modulated by extracellular signals such as secreted frizzled-related proteins (SFRPs), Dickkopf proteins (Dkk), or Wnt-inhibitory factor (WIF).

Recent studies in different vertebrates demonstrate that both canonical and noncanonical Wnt pathways regulate events during early eye development. Noncanonical Wnt/Fzd signaling is required during eye field formation in frog and zebrafish (Cavodeassi et al., 2005; Maurus et al., 2005). Overexpression of Fzd3 in *Xenopus* embryos leads to formation of ectopic eyes and the Wnt pathway modulator SFRP1 is required for normal development of the eye field in medaka fish (Rasmussen et al., 2001; Esteve et al., 2004). However, it is not clear from these studies whether Fzd3 and SFRP1 regulate canonical or noncanonical Wnt/Fzd signaling. Support for a role of Wnt/β-catenin signaling is evident from mice with a homozygous deletion of the co-receptor LRP6 that exhibit severe eye defects such as microphthalmia and coloboma (Pinson et al., 2000; Stump et al., 2003). In addition, analysis of transgenic LEF/TCF-dependent reporter lines in zebrafish, frog, and mice suggest that Wnt/β-catenin signaling is active in developing ocular tissues (Dorsky et al., 2002; Liu et al., 2003, 2006; Maretto et al., 2003), and that in *Xenopus* it regulates Sox2 expression and retinal neurogenesis (Van Raay et al., 2005). Early conditional disruption of the canonical Wnt pathway, however, revealed that β-catenin is necessary for correct lamination but dispensable for retinal specification and cell cycle exit in mouse (Fu et al., 2006). Thus, these studies suggest that both canonical and noncanonical Wnt signaling control different aspect of eye development and that the actual role of these pathways can differ among vertebrate species. Interestingly, Wnt/β-catenin signaling needs to be suppressed in the developing lens ectoderm to ensure normal morphogenesis of lens and eye (Smith et al., 2005; Miller et al., 2006).

Fzd5 is unique because it is almost exclusively expressed in the eye during early embryonic development in frog, zebrafish, chick, and mouse suggesting a specific, nonredundant role in the regulation of early eye development (Borello et al., 1999; Sumanas and Ekker, 2001; Fuhrmann et al., 2003; Cavodeassi et al., 2005; Van Raay et al., 2005). Surprisingly, recent studies suggest that Fzd5 can activate either noncanonical Wnt signaling in zebrafish or the Wnt/β-catenin pathway in frog and, in addition, exerts different functions in both species during eye development. In zebrafish, Fzd5 mediates noncanonical Wnt-11 signaling and promotes eye field formation (Cavodeassi et al., 2005). In frog, Fzd5 is strongly expressed in the optic vesicle and controls the neural potential of retinal progenitors by regulating the expression of the competence factor Sox2 (Sumanas and Ekker, 2001; Van Raay et al., 2005). Thus, these studies indicate that Fzd5 function during eye development appears to be dependent on the cellular context and on the species. The question arises, therefore, how Fzd5 functions in mammals, specifically in mouse.

Here, we analyze the expression of Fzd5 and its role during mouse retinal development using mice with a targeted deletion of *Fzd5* (Ishikawa et al., 2001). In *Fzd5*−/− embryos, early eye patterning appears to be largely normal; however, germline deletion of *Fzd5* results in a failure of optic cup morphogenesis and loss of gene expression in retina and lens at embryonic day 10.5 (E10.5) just before the embryos die due to defects in yolk sac angiogenesis. These eye defects, however, are likely secondary and result from aberrations caused by an earlier requirement for *Fzd5* in nonocular tissues, because conditional inactivation of a LoxP-flanked allele of *Fzd5* using Six3-Cre results in the formation of normal optic cups with normal gene expression. Surprisingly, analysis of mice transgenic for a TCF/LEF reporter and Axin2 expression reveal that Fzd5 does not activate Wnt/β-catenin signaling in the developing mouse eye.

RESULTS

Frizzled-5 Is Expressed in the Optic Vesicle and in the Developing Pituitary

Previous studies revealed that mouse Fzd5 is expressed in the eye at embryonic day (E) 9.5 (Borello et al., 1999; Ishikawa et al., 2001). To obtain a more detailed analysis of the spatial and temporal expression pattern of Fzd5, we performed in situ hybridization at different developmental stages. At E8.0 and E9.0 (6–8 and 12–14 somites, respectively), whole-mount in situ hybridization showed that Fzd5 is expressed broadly within the anterior neural plate encompassing the eye anlage and becomes restricted to the anterior edge of the neural plate as well as the ventral diencephalon (Fig. 1A,B). At E9.0 and E10.5, Fzd5 is expressed in the ventral forebrain, in the presumptive retina and optic stalk of the optic primordia (Figs. 1B–D, 2A). Analysis of sections revealed that neither the overlying lens ectoderm, lens vesicle nor the presumptive retinal pigment epithelium (RPE) express Fzd5 (Fig. 2A–D). At E13.5, Fzd5 expression is reduced within the regions of neuronal differentiation such as the ganglion cell layer but is maintained within the undifferentiated region of the neural retina (Fig. 2C). In addition, expression along the dorsal optic

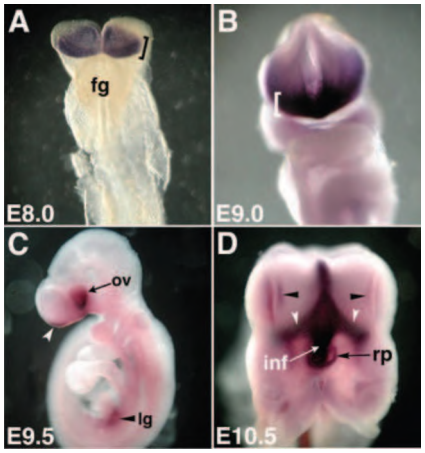

Fig. 1. Fzd5 expression pattern in ventral forebrain structures. Whole-mount in situ hybridization of Fzd5 mRNA expression showing frontal (A,B), lateral (C), and ventral views (D) of the embryonic mouse head. **A:** Fzd5 is expressed in the anterior neural plate at embryonic day (E) 8 (bracket, six to eight somites; fg, foregut). **B:** At E9, Fzd5 expression is detectable in the anterior forebrain (bracket). **C:** At E9.5, Fzd5 is expressed in the optic vesicle (ov) and optic stalk as well as in the olfactory placode (white arrowhead) and lung buds (black arrowhead). **D:** By E10.5, Fzd5 is expressed in the ventral forebrain, Rathke's pouch (rp), and infundibulum (inf). Fzd5 expression was also detected in the olfactory placode (black arrowheads) and along the optic stalks (white arrowheads). E, embryonic day; Lg, lung.

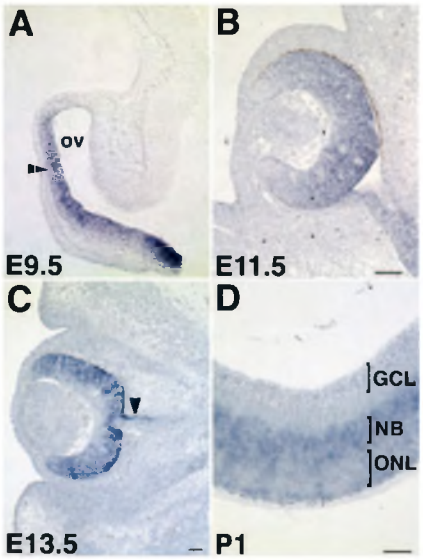

Fig. 2. Fzd5 expression in the embryonic and perinatal mouse eye. **A:** Fzd5 mRNA is expressed in the presumptive neural retina (arrowhead) and optic stalk of the optic vesicle (ov) at embryonic day (E) 9.5 but not in the future retinal pigment epithelium (RPE) or overlying lens ectoderm. **B:** Fzd5 expression is maintained in the neural retina and optic stalk at E11.5. **C:** By E13.5, Fzd5 expression is decreased in the presumptive ganglion cell layer (GCL), but maintained in the progenitor zone. In addition, some Fzd5 expression is present in the dorsal optic stalk (arrowhead). **D:** Subsequently, Fzd5 becomes restricted to the neuroblastic layer (NBL) in the P1 retina and is down-regulated in the future outer nuclear layer (ONL). P: postnatal day. Scale bars = 100 μm.

nerve was observed at this stage (Fig. 2C; arrowhead). At E15.5 and postnatal day (P) 1, Fzd5 expression remained in the neuroblastic layer (Fig. 2D; not shown). In addition, the presumptive outer nuclear layer (ONL), which contains fewer dividing cells at this age, exhibits a mottled pattern of Fzd5 (Fig. 2D).

During pituitary organogenesis, Fzd5 is broadly expressed in the anterior neural plate at E8 encompassing the region that will give rise to the telencephalon, olfactory epithelium, anterior hypothalamus, and pituitary (six to eight somites; Fig. 3A). At E9, Fzd5 expression is detectable in the ventral forebrain as well as the oral ectoderm (Fig. 3B,C), which will produce the placode that forms Rathke's pouch (Baker and Bronner-Fraser, 2001; Scully and Rosenfeld, 2002). By E9.5 and E10.5, Fzd5 is expressed in the ventral forebrain, Rathke's pouch and infundibulum (Figs. 1D, 3D,E; not shown). Fzd5 expression is also detectable in the medial olfactory placode (Fig. 3F, arrowhead) and along the midline of the ventral forebrain (Fig. 3C,E). At E11.5, low levels of expression were detected in the developing infundibulum; however, expression in Rathke's pouch appeared to be down-regulated as well as in the ventral forebrain and medial olfactory epithelium (not shown). Our data demonstrate that Fzd5 is expressed in both the neural and non-neural anlage of the developing pituitary from the early open neural plate stages of mouse development through E10.5. This expression pattern corresponds to early specification, commitment, and patterning of oral ectoderm and ventral diencephalon to the pituitary fate.

Fzd5 Expression Does Not Coincide With Regions of Wnt/β-catenin Pathway Activity in the Optic Vesicle and Ventral Forebrain

In mouse, Fzd5 activates the Wnt/β-catenin (canonical) pathway during yolk sac angiogenesis and maturation of intestinal Paneth cells (Ishikawa et al., 2001; Van Es et al., 2005), and in *Xenopus* Fzd5 regulates Wnt/β-catenin signaling in the optic vesicle. To determine whether canonical signaling is active in the mouse optic vesicle and in the ventral forebrain, we analyzed mice transgenic for a LEF/TCF-responsive promoter that drives expression of *LacZ* (TOPGAL; DasGupta and Fuchs, 1999). Activation of this reporter is first detected in the developing eye at E9.5, but this activity is restricted to the dorsal most portion of the optic vesicle (Fig. 4A) as previously shown by Maretto et al. (2003) in a different reporter line. Of interest, Fzd5 expression and the pattern of canonical Wnt activity detected by the TOPGAL reporter mice are mutually exclusive at the optic vesicle stage (compare Fig. 2A with Fig. 4A). In addition, expression of the canonical target gene Axin2 starts in the dorsal optic vesicle at E9.75 (Fig. 4C). This expression pattern overlaps with the TOPGAL reporter, but not with the Fzd5 domain of the optic vesicle. Furthermore, the ventral forebrain, Rathke's pouch and infundibulum did not show TOPGAL reporter expression between E9.5 and E11.5 in regions that overlapped with Fzd5 (not shown). Therefore, our observations suggest that Fzd5 does not activate the β-catenin–dependent Wnt pathway during early eye and pituitary development in mouse.

To determine whether Fzd5 function is required for eye development, we analyzed embryos with a germline deletion of *Fzd5* (Ishikawa et al., 2001). Homozygous embryos die at E10.75 due angiogenesis defects in yolk sac and placenta (Ishikawa et al., 2001); thus, we were limited in our analysis to the optic vesicle and optic cup stages. If Fzd5 does not signal through activation of TCF/LEF, it may use one of the alternative Wnt/Fz signaling pathways and, in addition, may repress canonical Wnt signaling as shown for Fzd5 in zebrafish and for other Fzd receptors (Westfall et al., 2003; Roman-Roman et al., 2004). However, *Fzd5*−/− embryos carrying the TOPGAL transgene did not show ectopic activation of the reporter in the distal and ventral optic vesicle, suggesting that Fzd5 does not inhibit the canonical pathway (Fig. 4B).

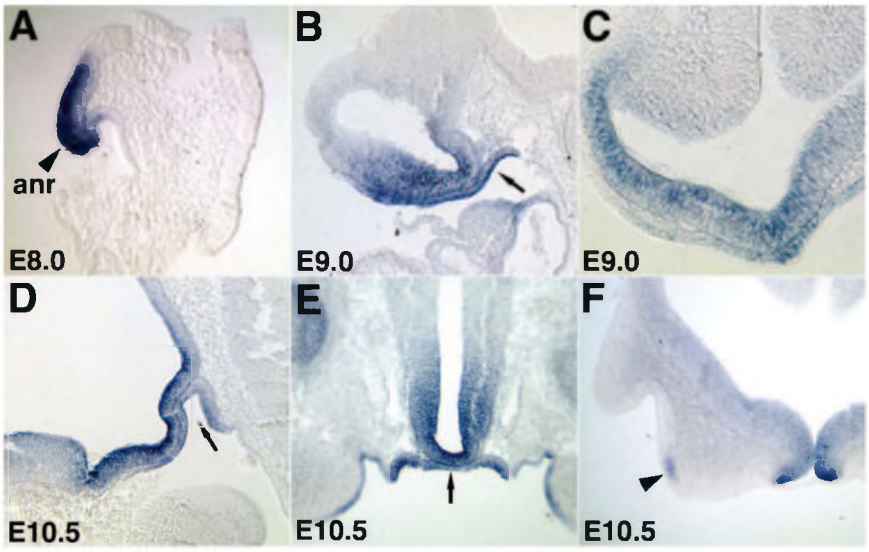

Fig. 3. Expression of Fzd5 mRNA in the developing pituitary. **A:** During pituitary organogenesis, Fzd5 is expressed in the anterior neural plate (anr) at embryonic day (E) 8 (sagittal section; six to eight somites). **B,C:** At E9, Fzd5 expression is detectable in the ventral forebrain (sagittal section in B, coronal section in C) as well as the oral ectoderm (B, arrow). **D,E:** By E10.5, Fzd5 is expressed in the ventral forebrain (sagittal section in D, coronal section in E), oral ectoderm (arrow in D), and Rathke's pouch (arrow in E). **F:** Fzd5 expression was also detected in the olfactory placode (black arrowhead, coronal section).

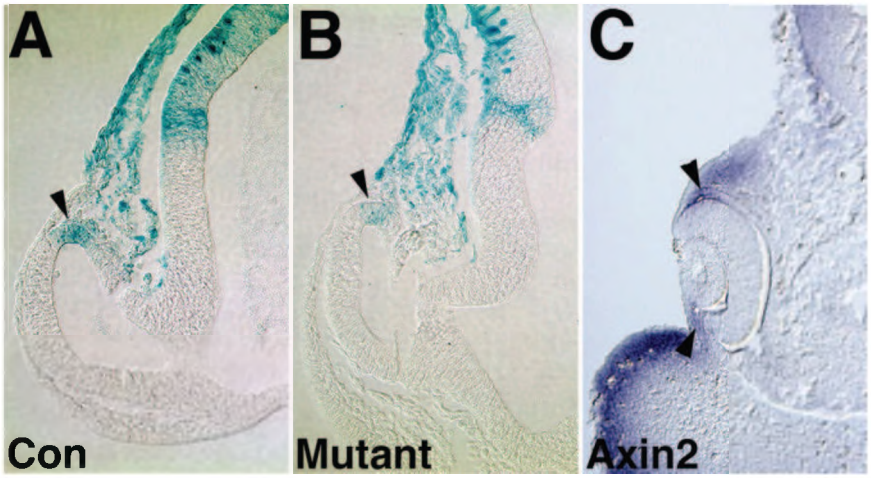

Fig. 4. Fzd5 does not appear to inhibit or activate the canonical pathway. **A,B:** Control (A) or *Fzd5*−/− embryos (B) carrying the TOPGAL transgene were analyzed by Xgal staining. Activation of the TOPGAL reporter is restricted to the dorsal optic vesicle in *Fzd5*−/− embryos at embryonic day (E) 9.5, similar to control embryos (A,B; arrowheads). **C:** Expression of Axin2 at 30 somites is restricted to the dorsal optic cup (arrowhead).

Frizzled-5 Mutant Mice Exhibit Abnormal Eye Development, Increased Cell Death, and Decreased Retinal Proliferation by the Optic Cup Stage

Histological analysis of *Fzd5*−/− embryos between E9.75 and E10.75 revealed a severe defect in optic cup morphogenesis compared with control littermates (Fig. 5C,D). Mutant optic vesicles fail to invaginate and, instead, a vesicle-like structure remains, in which the outer portion is partially thickened, reminiscent of the developing neural retina. In addition, lens development does not proceed beyond a rudimentary lens pit (Fig. 5D, inset). This finding suggests that Fzd5 may be required for morphogenesis of the optic cup and lens. We performed additional experiments to further analyze the *Fzd5*−/− phenotype in more detail. The loss of Fzd5 function could cause cells to undergo apoptosis and/or prematurely exit the cell cycle and differentiate, because Wnt/Fzd signaling is known to regulate both proliferation and cell survival within the developing nervous system (Chen et al., 2001; Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003). While at E9.5, few apoptotic cells in the distal optic vesicle of mutant embryos are detectable (not shown), significantly more terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) -labeled cells are present by E10.25, compared with controls (Fig. 5E,F; arrows). The increase of the number of TUNEL-labeled cells in the presumptive retina of the optic vesicle is significant (*Fzd5*−/−: 41.14 ± 5.84; control: 5.05 ± 1.09; $P < 0.00002$; n = 4 optic vesicles, 2 embryos per genotype analyzed) and extends far beyond normal cell death observed in the developing optic vesicle (Laemle et al., 1999). At E10.5, an increase of apoptosis was also observed in regions that do not express Fzd5 such as the dorsal diencephalon and spinal cord reflecting compromised development of the whole embryo due to defects in angiogenesis of yolk sac and placenta (not shown; Ishikawa et al., 2001). Furthermore, premature differentiation in mutant optic vesicles did not occur because Tuj-1–positive cells were not observed in either the mutant or control embryos at E9.5 or E10.0, which is consistent with previous studies (not shown; Philips et al., 2005). Another explanation for the observed defects in *Fzd5*−/− optic vesicles could be a decrease in proliferation of retinal progenitor cells. Using bromodeoxyuridine (BrdU) incorporation to determine effects on proliferation, we observed that at E10, the neural retina domain in the mutant optic vesicle exhibited changes in proliferation compared with controls; mutant optic vesicles showed a significant decrease in the number of proliferating cells in the distal and ventral portion but not in the dorsal diencephalon, which does not express Fzd5 (Fig. 5G; Supplementary Figure S1, which can be viewed at http://www.interscience.

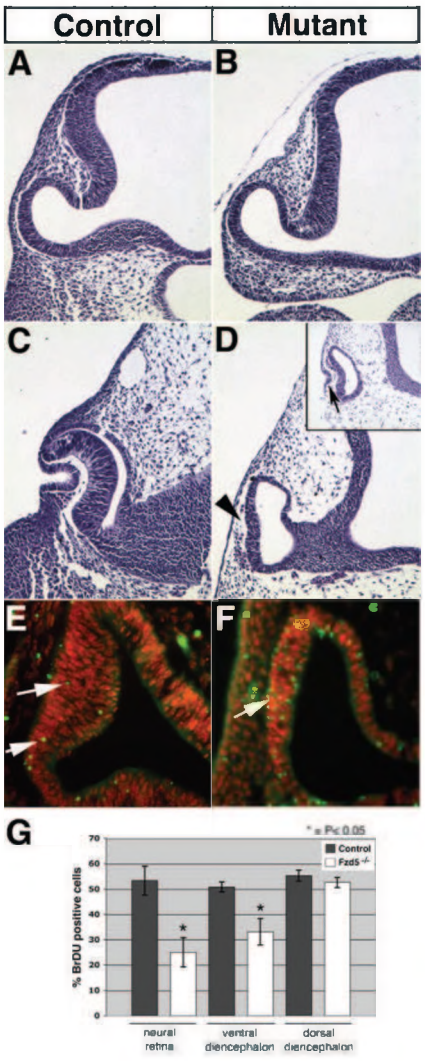

Fig. 5.

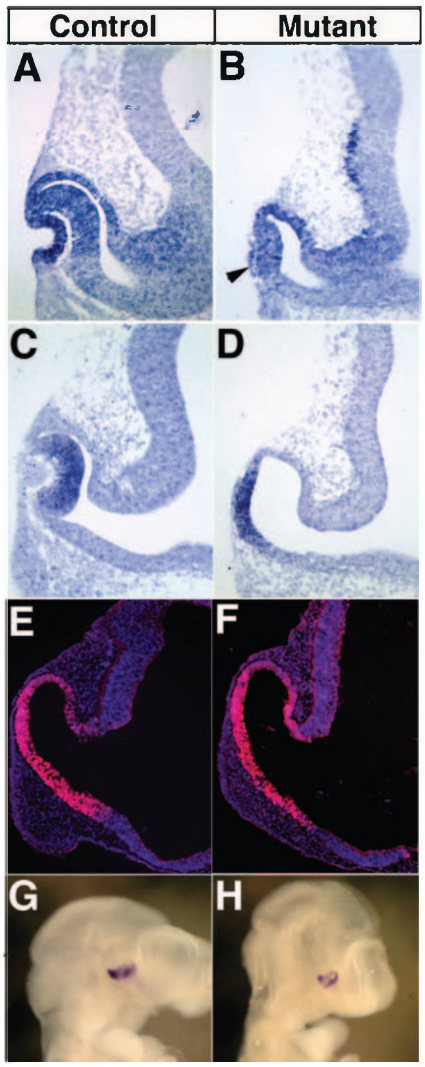

Fig. 6.

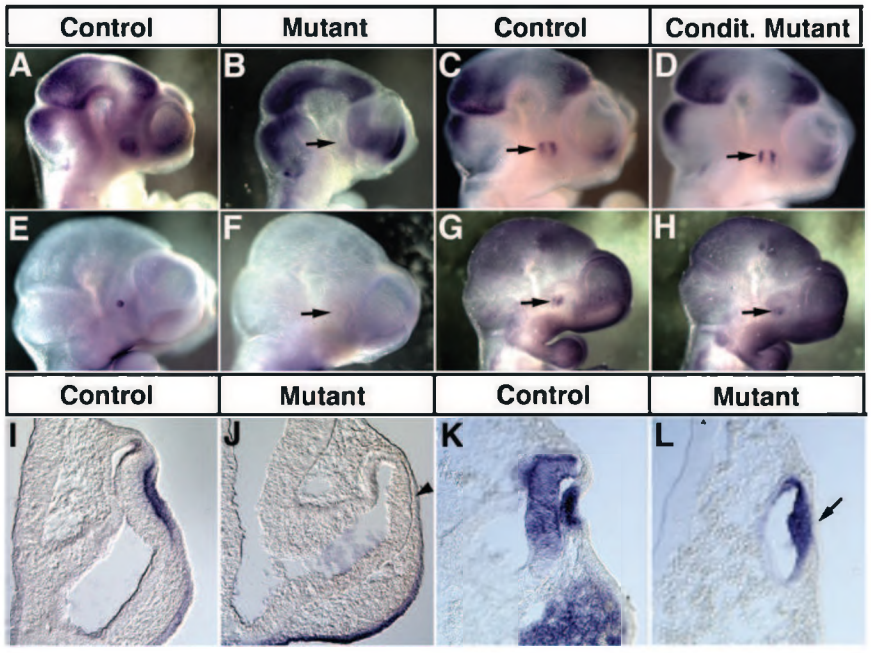

Fig. 7.

Fig. 5. *Fzd5−/−* embryos fail to develop an optic cup. **A–D:** Morphology of control (wild-types, A,C) and *Fzd5−/−* embryos (B,D) was analyzed by hematoxylin and eosin staining using frontal sections of paraffin-embedded tissue; dorsal is toward the top in all panels. *Fzd5−/−* and control embryos develop a morphologically normal optic vesicle at embryonic day (E) 9.5 (compare A and B). By E10.75, the *Fzd5−/−* eye has failed to form the bilayered optic cup, unlike the control littermates (compare C and D; arrowhead). The lens placode does invaginate to some extent in *Fzd5−/−* embryos (inset in D; arrow). **E,F:** Control (E) or mutant embryos (F) were analyzed for TUNEL labeling (green; DAPI, red). At E10 (30 somites), *Fzd5−/−* embryos showed increased TUNEL labeling in the presumptive neural retina compared with controls (F; arrow). **G:** To determine effects on proliferation, bromodeoxyuridine (BrdU) incorporation was determined at E10. The proportion of BrdU-positive cells was significantly reduced in the distal (25.03 ± 14.14) and ventral optic vesicle (33.1 ± 12.67) in *Fzd5−/−* embryos in comparison to controls (distal, 53.35 ± 5.67; ventral, 50.80 ± 5.10; n = 3 embryos). In contrast, proliferation did not significantly change in the dorsal diencephalon where Fzd5 is not expressed (55.27 ± 5.27 in controls versus 52.62 ± 5.05 in *Fzd−/−* embryos; n = 2 embryos). Solid bars, control; open bars, *Fzd−/−* embryos.

Fig. 6. Patterning of the ventral and distal optic vesicle occurs normally in *Fzd5−/−* embryos. **A,B:** At embryonic day (E) 10.5, Pax6 is expressed in the developing neural retina, lens (arrowhead in B) and presumptive RPE of control (A) and *Fzd5−/−* embryos (B). **C,D:** Similarly, Chx10 is present in the neural retina of control (C) and in the distal region of the optic vesicle in mutant embryos (D). **E,F:** Pax2 protein is present in the optic vesicle and stalk at 27 somites in mutant embryos (E) similar to the expression observed in control embryos (F). Ventral expression of Vax2 is also normal in *Fzd5−/−* optic vesicles at 30 somites (compare **G, H**).

Fig. 7. Effects of germline and conditional inactivation of *Fzd5* on FGF15, FoxE3, and Mab21L1 expression in the mouse optic vesicle. **A–H:** Whole-mount in situ hybridization for FGF15 (A,B, 33 somites; C,D, 35 somites) and FoxE3 (E,F, 33 somites; G,H, 30 somites) showing lateral views. **I–L:** Coronal sections of FoxE3 (I,J, 30 somites) and Mab21L1 expression (K,L, 30 somites) are shown. Controls (A,C,E,G,I,K), embryos with a germline mutation (B,F,J,L), or a conditional disruption of *Fzd5* (D,H) are presented. Germline disruption of *Fzd5* leads to an absence of FGF15 (arrow in B), FoxE3 (arrow in F, arrowhead in J), and Mab21L1 expression (L, arrow) in the optic vesicle or lens placode. Embryos with conditional disruption of *Fzd5* were generated using Six3-Cre heterozygous for the *Fzd5* null allele and one floxed *Fzd5* allele. Conditional *Fzd5−/−* embryos exhibit a normal expression pattern of FGF15 and FoxE3 in the optic vesicle (arrows in D,H, respectively).

wiley.com/jpages/1058-8388/suppmat). Thus, at this age, disruption of *Fzd5* affects proliferation in the Fzd5 expression domains of the optic vesicle specifically. Therefore, it is likely that an increase in cell death and a concomitant decrease of proliferation contribute to formation of defective optic cups in *Fzd5−/−* embryos.

General Patterning of the Optic Cup Is Normal in Frizzled-5 Mutant Mice

To determine whether defects in optic cup morphogenesis in *Fzd5−/−* embryos are caused by abnormal specification of ocular tissues, we analyzed expression of more than 20 different genes expressed in the eye between E9.5 and E10.5 (see list in the Experimental Procedures section). The paired homeodomain transcription factor Pax6 is required for optic cup formation, proliferation, and multipotency of retinal progenitor cells (Hogan et al., 1986; Hill et al., 1991; Walther and Gruss, 1991; Ashery-Padan et al., 2000; Ashery-Padan and Gruss, 2001; Marquardt et al., 2001). In *Fzd5−/−* optic cups, Pax6 expression is normal in the neural retina, future RPE, and lens ectoderm, suggesting that initial specification of the eye occurs normally (Fig. 6B). Furthermore, the homeodomain transcription factor Chx10 is exclusively expressed in retinal progenitors and regulates proliferation (Liu et al., 1994; Burmeister et al., 1996). Although proliferation can be decreased in the mutant optic vesicle (Fig. 5G,H), Chx10 expression was normal compared with control embryos (Fig. 6C,D). Recently, functional analyzes in frog revealed that Fzd5 regulates expression of Sox2 and Xath5 in the developing retina (Van Raay et al., 2005). To test whether Sox2 and Math5 are similarly dependent on Fzd5 function in mouse, we analyzed expression of both genes in *Fzd5−/−* embryos. In *Fzd5−/−* mice, Sox2 expression is present in the presumptive retina between E9.5 and E10.5 (n = 7, not shown). Because *Fzd5−/−* embryos die approximately 1 day before onset of Math5 expression, we examined optic vesicle explants grown for 3 days in culture. However, Math5 expression was detectable in both control optic vesicle explants (4/10 explants) and mutant explants (3/10 explants; Supplementary Figure S2). Because a similar proportion of explants expressed Math5 for both controls and mutants (no significant difference, $P = 1.0$), Fzd5 does not appear to be required for Math5 expression in mouse. The function of Fzd5 to promote Math5 expression during eye development, therefore, is not evolutionary conserved and appears to be species-dependent.

Because Fzd5 is expressed in the optic stalk, we next addressed whether ventral patterning is altered in *Fzd5−/−* eyes. We analyzed the expression of Pax2, a transcription factor that is initially expressed in the developing neural retina and optic stalk during early eye development (Otteson et al., 1998; Schwarz et al., 2000). We observed normal Pax2 protein expression in the optic vesicle, with stronger expression detected in the ventral region of the neural retina and optic stalk (Fig. 6F). The homeodomain protein Vax2 is expressed in the ventral optic vesicle and is required for ventral patterning (Barbieri et al., 1999; Mui et al., 2005). Similarly to Pax2, we found that Vax2 expression in *Fzd5−/−* optic cups is normal at E10–E10.5 (Fig. 6H). In addition, analysis of FGF8 expression confirmed that patterning of the optic stalk is not disturbed (n = 2, not shown). Analysis of expression of the basic helix–loop–helix (bHLH transcription factor Mitf revealed that the presumptive RPE is specified normally (n = 2, not shown). Therefore, patterning of the proximodistal and dorsoventral axes in the optic vesicle appear to be normal without Fzd5 function. No changes of expression of BMP7, Hes1, Hes5, and TBX5 were detectable in *Fzd5−/−* eyes (n = 2–4, not shown). However, we observed changes in expression of FGF15, which is one of the most abundant FGFs expressed in the distal optic vesicle (McWhirter et al., 1997, Wright et al., 2004). FGF15 is initially expressed in the *Fzd5−/−* optic vesicle but is decreased at 30 somites (not shown) and is absent at 33 somites (Fig. 7B).

Lens Development Is Altered in *Fzd5−/−* Embryos

Invagination of the optic vesicle and formation of the lens vesicle require tight interaction between lens ectoderm and optic vesicle as well proper gene expression in the lens ectoderm such as Pax6, Six3, Sox2, and Mab21L1 (for reviews, see Lang, 2004; Medina-Martinez and Jamrich, 2007). In *Fzd5−/−* eyes, the lens placode does form only rudimentarily as shown in Figure 5D (inset); however, several genes required for lens placode formation are expressed normally. For example, Pax6 mRNA and protein expression is detectable in the lens placode up to E10.5 (Fig. 6B, not shown). Other lens placodal markers such as Six3 and Sox2 are expressed in *Fzd5−/−* lens ectoderm at E10 (not shown). Furthermore, genes expressed in the distal optic vesicle and shown to be necessary for lens formation such as Pax6, BMP4, and Mab21L2 are present in the presumptive retina of mutant embryos (n = 2–4, Fig. 6B, not shown). Thus, early stages of induction and specification of the lens placode appear to be normal in *Fzd5−/−* embryos (Lang, 2004; Donner et al., 2006; Medina-Martinez and Jamrich, 2007). However, later stages of lens development are affected in *Fzd5−/−* eyes. Mab21L1, a member of the Mab gene family, is essential for lens placode formation in mouse (Yamada et al., 2003). We observed that at E9.5 (25 somites, not shown), Mab21L1 is initially present in the lens placode but is undetectable at E10 (30 somites; Fig. 7L). Of interest, Mab21L1 expression in the distal optic vesicle appears to be normal, thus, its expression is differentially affected in the presumptive retina and lens placode in *Fzd5−/−* eyes. The forkhead transcription factor FoxE3 regulates lens vesicle closure and separation and is dependent on Mab21L1 function (Blixt et al., 2000; Brownell et al., 2000; Yamada et al., 2003). In *Fzd5−/−* eyes, expression of FoxE3 was not induced in the lens ectoderm between E9.5 and E10.25 (n = 3; Fig. 7F,J) consistent with the down-regulation of Mab21L1 expression. FoxE3 negatively regulates expression of the homeodomain protein Prox-1 in the lens (Blixt et al., 2000; Medina-Martinez et al., 2005). However, we did not observe premature expression of Prox-1 in the lens ectoderm of *Fzd5−/−* eyes up to 35 somites (n = 2; not shown).

Eye Morphogenesis Proceeds Normally in Conditional *Fzd5−/−* Embryos

Because embryos with a homozygous germline disruption of *Fzd5* die at E10.75 due to a defect in yolk sac angiogenesis (Ishikawa et al., 2001), we extended our analysis to embryos with Cre-mediated inactivation of a *LoxP*-flanked *Fzd5* allele (Van Es et al., 2005). To inactivate the *Fzd5* gene specifically in the optic vesicle, we used a Six3-Cre transgenic mouse line heterozygous for the *Fzd5* null allele. Six3-Cre expresses Cre recombinase in the distal and ventral optic vesicle similar to Fzd5 (Furuta et al., 2000). Cre recombinase activity was confirmed by crosses with ROSA-26 reporter mice showing *LacZ* expression in the distal and ventral optic vesicle at E10.5 (Supplementary Figure S3A; Soriano, 1999). In addition, we observed reduced Fzd5 mRNA expression in the eye of conditional *Fzd5−/−* embryos at E9.5 in the distal and ventral optic vesicle where Six3-Cre is expressed (Supplementary Figure S3C). The residual Fzd5 expression in the Six3-Cre expression domain is consistent with the fact that the germline *Fzd5* null allele is an insertional mutant that does not remove the coding region (Ishikawa et al., 2001), and that there may also be transcript for the remaining part of the 3′-untranscribed region of the conditional *Fzd5* allele (Van Es et al., 2005). Furthermore, we cannot exclude that the Six3-Cre line recombines the floxed *Fzd5* gene incompletely in some cells of the distal optic vesicle. However, we found a non–cell-autonomous effect on the hyaloid vasculature in conditional *Fzd5−/−* embryos as early as E14.5 and in the postnatal eye (to be described elsewhere). Therefore, we are confident that Six3-Cre–mediated inactivation of *Fzd5* in the developing retina is successful. However, following conditional inactivation of *Fzd5*, surprisingly, we observed that no defects in optic cup and lens morphogenesis occur and FoxE3 and FGF15 expression are not altered in comparison to control embryos (Fig. 7C,D,G,H; Supplementary Figure S4A,B). In addition, proliferation and total cell number are not affected at E10.25 (Supplementary Figure S4C,D). This finding suggests that Fzd5, although highly expressed in the optic vesicle, is not directly required for optic cup morphogenesis and lens formation in mouse. However, we cannot completely exclude the possibility that unanticipated Fzd5 expression domains before E9 (e.g., mesenchyme or surface ectoderm) are not eliminated by Six3-Cre, allowing eye morphogenesis to proceed normally in conditional *Fzd5−/−* embryos. Finally, it is also possible that eye morphogenesis is highly dependent on proper systemic vascularization and blood circulation of the embryo.

DISCUSSION

We have shown in mouse that Fzd5 is expressed at early stages of eye and pituitary development. TCF/LEF reporter activity and Axin2 expression are not detectable in these domains of Fzd5 expression, suggesting that this receptor does not activate Wnt/β-catenin signaling at the ages examined. We examined the function of Fzd5 more precisely during eye development and observed that a failure of optic cup morphogenesis occurs in *Fzd5−/−* embryonic eyes. However, expression of genes required for retinal neurogenesis such as Math5 and Sox2 are not dependent on Fzd5 function. These observations reveal that Fzd5 is dispensable for early mouse eye development, in contrast to other vertebrates such as zebrafish and frog.

Potential Role of Fzd5 During Pituitary Development

Pituitary organogenesis occurs through a series of signaling events, and the Wnt family of secreted glycoproteins is one of the extrinsic signals thought to normally regulate this process (for review, see Zhu et al., 2007). However, except for confirmation of Fzd2 expression during pituitary development, and in vitro assays that implicate Wnt/β-catenin signaling in transcriptional control of pituitary gene expression, the Frizzled receptor(s) that regulate pituitary organogenesis remain elusive (Treier et al., 1998; Douglas et al., 2001; Kioussi et al., 2002). Our studies identify Fzd5 as a candidate receptor for mediating Wnt signals during early events of pituitary organogenesis in mouse, because Fzd5 is transiently expressed in Rathke's pouch and oral ectoderm between E9 and E10.5. Mouse Fzd5 is most closely related to *Xenopus* Fzd5, human Fzd5, and zebrafish Fzd8c (Kim et al., 1998; Ishikawa et al., 2001; Sumanas and Ekker, 2001). However, recent observations suggest that frog Fzd5 is also expressed in the developing pituitary, ventral diencephalon, and hypothalamus at late neurula/early tail bud stages (personal communication, M.L. Vetter and K.B. Moore). The expression pattern of Fzd5 in zebrafish appears to be very similar in the eye field and diencephalon (Cavodeassi et al., 2005). Thus, the expression of Fzd5 in different vertebrates is evolutionarily conserved across species and is consistent with a potential role of Fzd5 during pituitary development.

In the developing pituitary, Fzd5 function appears to be independent of TCF/LEF transcriptional activity because TCF/LEF reporter activity and expression of the target gene Axin2 are not detectable in the Fzd5 domain between E9 and E10.5 (Fig. 4, not shown; Olson et al., 2006). These observations are in agreement with a previous study revealing that Wnt/β-catenin signaling in the developing pituitary is tightly regulated (Olson et al., 2006). While after E10.5, β-catenin is necessary to control cell determination events, premature activation of Wnt/β-catenin signaling disrupts formation of Rathke's pouch (Olson et al., 2006). The transient expression of Fzd5 before E10.5 could indicate a role in repressing Wnt/β-catenin signaling. However, at E10, we did not observe ectopic Axin2 and TOPGAL reporter expression in the pituitary in *Fzd5−/−* embryos (not shown). Furthermore, in *Fzd5−/−* embryos, we observed no obvious defects in Rathke's pouch formation as well as BMP4, Hes1, or Sox2 expression in transversal sections at E10 (not shown). This finding suggests that initial formation and patterning of the pituitary in mouse occurs independently of Fzd5 and more detailed studies are necessary to determine the precise role of Fzd5.

Function of Fzd5 During Early Eye Development Is Not Evolutionary Conserved

Fzd5 is strongly expressed in the distal and ventral optic vesicle as well as

in retinal progenitors (our study; Wang et al., 1996; Borello et al., 1999; Ishikawa et al., 2001; Kim et al., 2001). This spatial and temporal expression pattern at the optic vesicle stage is very similar to Fzd5 expression in frog and zebrafish (Sumanas and Ekker, 2001; Cavodeassi et al., 2005; Van Raay et al., 2005). In chick, however, expression starts later at the optic cup stage in the dorsal portion and extends subsequently throughout the whole retina (Fuhrmann et al., 2003; Kubo et al., 2003). In the differentiating and adult retina, Fzd5 expression is quite different in these species; in mouse, Fzd5 is present in the inner nuclear layer, whereas in frog, Fzd5 expression becomes restricted to the ciliary margin (Blackshaw et al., 2004; Van Raay et al., 2005). In chick, Fzd5 expression disappears entirely around the time when later born retinal cell types such as rod photoreceptors and Muller glia start to exit the cell cycle (embryonic day 7; Fuhrmann et al., 2003). However, in all species examined, Fzd5 appears to be expressed in the majority of progenitors that reside in the proliferative zone during retinal histogenesis.

In our study, conditional inactivation of *Fzd5* in the optic vesicle shows that it is not directly required for optic cup morphogenesis and lens development. One explanation could be functional redundancy. Recent expression analysis in mouse indicates that several Fzd receptors are expressed in retinal progenitors in the mouse embryo, including Fzd3, Fzd4, Fzd6, and Fzd7 (Liu et al., 2003). Furthermore, previous studies show that Fzd receptors can compensate for each other. In fly, absence of expression of both Fz and *Drosophila* (D)Fz2 produces more severe patterning defects in the embryo than with loss of function of either Fz or DFz2 alone (Bhat, 1998; Kennerdell and Carthew, 1998; Bhanot et al., 1999; Chen and Struhl, 1999). In mouse, only combined deletion of Fzd3 and Fzd6 results in orientation defects of hair bundles in the inner ear (Wang et al., 2006). Therefore, it is possible that loss of Fzd5 function in retinal progenitors in mouse is compensated by other Fzd receptors.

The actual function of Fzd5 during eye development appears to be species-dependent. In chick, Fzd5 appears to be involved in retinotectal pathfinding, but an earlier role of Fzd5 has not been investigated (Schmitt et al., 2006). In zebrafish, Fzd5 is expressed in the eye field and is sufficient to produce ectopic eyes by promoting eye field formation (Cavodeasssi et al., 2005). Conversely, knock-down of Fzd5 results in smaller eye fields and Wnt11 was identified as a good candidate ligand for Fzd5. Of interest, here Wnt11/Fzd5 appears to antagonize the Wnt/β-catenin pathway, which inhibits eye field formation, possibly by promoting posterization of the forebrain (Cavodeassi et al., 2005). In addition, it is postulated that Wnt11/Fzd5 signaling directly regulates morphogenetic movements of cells in the eye field through activation of a noncanonical Wnt pathway. In *Xenopus*, blocking Fzd5 function results in reduced eye size, inhibition of neurogenic genes expression and increased formation of Muller Glia. This effect is due to loss of Sox2 and can be correlated with loss of Wnt/β-catenin signaling. These observations indicate that Fzd5 controls the neurogenic potential of retinal progenitors in the developing *Xenopus* eye. However, our observations in mouse are not consistent with the results in zebrafish and frog. We did not observe a change of expression of eye patterning markers such as Pax6 or of genes required for neurogenesis such as Sox2 or Math5 in *Fzd5*−/− eyes. Loss of function of LRP6 results in variable eye defects but does not lead to changes in expression of Sox2, further suggesting that Sox2 expression in the developing mouse eye is not dependent on Fzd5 (Smith et al., 2005; Stump et al., 2003). Overall, our observations reveal that, although germline deletion of *Fzd5* can cause a defect in optic cup morphogenesis in mouse, the *Fzd5* gene is not directly involved in this process as shown by conditional disruption. It may be that the eye phenotype results indirectly from defective angiogenesis in yolk sac and placenta; however, it is not clear whether these two phenotypes are linked. We conclude that Fzd5 function is highly context-dependent—regulation of eye field formation or retinal neurogenesis—and species-dependent in frog, zebrafish, chick, and mouse.

Fzd5 Does Not Appear to Activate the Canonical Pathway in Ventral Diencephalon Derivatives in Mouse

In the presence of its co-receptor LRP6, Fzd5 binds Wnts such as Wnt5A or Wnt7A and activates the Wnt/β-catenin (canonical) pathway in different in vitro and in vivo systems. Human Fzd5 induces a secondary axis in frog when overexpressed with Wnt5A and the soluble extracellular cysteine-rich domain can inhibit Wnt3A-induced β-catenin accumulation (He et al., 1997; Kemp et al., 2007). In PC12 cells, Fzd5 interacts with LRP6 and Wnt7A to activate the canonical pathway (Caricasole et al., 2003). In mouse, Fzd5 is expressed in neonatal and adult intestinal crypts and mediates differentiation of Paneth cells through nuclear localization of β-catenin and activation of TFC/LEF transcription factors (Van Es et al., 2005). Furthermore, Ishikawa et al. showed that mouse Fzd5 induces a secondary axis with head structures in frog embryos when co-injected into blastomeres with Wnt2 or Wnt5A (Ishikawa et al., 2001). These results demonstrate that mouse Fzd5 can activate Wnt/β-catenin signaling. Surprisingly, in the mouse optic vesicle, we have no evidence that canonical signaling is active where Fzd5 is expressed. Our analysis of TCF/LEF reporter activation in TOPGAL mice rather revealed that reporter expression and Fzd5 expression are mutually exclusive; Fzd5 is expressed in the distal and ventral optic vesicle, whereas the TOPGAL reporter is expressed dorsally. Another TCF/LEF reporter shows a similar dorsal expression domain in the optic vesicle (BATgal; Maretto et al., 2003). Furthermore, Axin2 expression, a very reliable read out for canonical Wnt signaling, is consistent with these results. Our observations suggest that Fzd5 does not activate or suppress the canonical pathway in the embryonic mouse eye. In fact, Wnt/β-catenin signaling might not play a prominent role in retina proliferation and differ-

entiation in mouse (for review, see Fuhrmann, 2008). Conditional ablation of β-catenin in the optic vesicle leads to cell adhesion and lamination defects but does not interfere with optic cup morphogenesis and retinal cell differentiation (Fu et al., 2006). In agreement with these observations in mouse, TCF/LEF reporters are not active in the developing central retina in chick and zebrafish (Dorsky et al., 2002; Cho and Cepko, 2006; Lee et al., 2006). This supports the notion that canonical Wnt signaling does not regulate neurogenesis or proliferation in the central retina of other vertebrates, with the exception in frog. Interestingly, more recent studies demonstrate that Wnt/β-catenin signaling is sufficient and required in controlling differentiation of the peripheral retina into ciliary body and iris (Cho and Cepko, 2006; Liu et al., 2007). Furthermore, Wnt/β-catenin may also function to regulate proliferation of progenitors in the embryonic and adult ciliary margin zone (Ahmad et al., 2000; Tropepe et al., 2000; 2003, 2005; Inoue et al., 2006; Sun et al., 2006; Asami et al., 2007). Because the frog retina grows by generating new cells from the ciliary margin zone, the function of Wnt/β-catenin signaling in peripheral proliferation may indeed be conserved across vertebrates.

Of interest, Weeraratna et al. (2002) showed that the Fzd5 receptor in combination with Wnt5A is required for PKC activation in primary metastatic melanoma cells. In zebrafish, Fzd5 appears to antagonize canonical Wnt signaling possibly by activation a noncanonical pathway and, interestingly, overexpression can also induce formation of a secondary axis (Cavodeassi et al., 2005). Thus, depending on the cellular context, Fzd5 might be able to activate a noncanonical Wnt pathway. Similarly, other Fzd receptors such as Fzd3 and Fzd4 have been shown to activate both canonical and noncanonical Wnt pathways (Sheldahl et al., 1999; Umbhauer et al., 2000; Mikels and Nusse, 2006). Thus, the same Fzd receptor can activate different Wnt pathways in the same species, which is tightly regulated depending on the tissue-specific context.

EXPERIMENTAL PROCEDURES

Mice

Mice carrying null alleles of the *Frizzled-5* gene (referred to as *Fzd5−/−*) were generated as previously described (Ishikawa et al., 2001). Mice transgenic for the TOPGAL reporter were generously provided by E. Fuchs (The Rockefeller University, New York; DasGupta and Fuchs, 1999). To obtain *Fzd5−/−* embryos harboring the TOPGAL transgene, mice heterozygous for TOPGAL reporter and the *Fzd5* null allele were mated. Noon of the day observing the vaginal plug was considered 0.5 days post coitum. To precisely match mutant embryos with similarly aged control littermates, somites were counted. Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline for 2 hr at room temperature or overnight at 4°C. *Fzd5−/−* mice were genotyped as previously published (Ishikawa et al., 2001) and using the following primer pair to amplify the neomycin resistance cassette: 5′ cgatgaatccagaaaagcgg 3′(forward), 5′ gcttgggtggagaggctatt 3′ (reverse). TOPGAL mice were genotyped using the following primer pair: 5′ cgatgaatccagaaaagcgg 3′ (forward); 5′ gcttgggtggagaggctatt 3′ (reverse).

Mice with floxed *Fzd5* alleles ($Fzd5^{LoxP/LoxP}$) were previously described (Van Es et al., 2005). For Cre-mediated recombination, Yasuhide Furuta (University of Texas) generously provided Six3-Cre transgenic mice (Furuta et al., 2000). Cre recombinase activity was examined using Rosa26R reporter mice and labeling with X-gal substrate (Soriano, 1999). To obtain conditional *Fzd5* mutant embryos, Six3-Cre females heterozygous for the *Fzd5* null allele were generated and crossed with $Fzd5^{LoxP/LoxP}$ males. Littermates from the same litter were used as controls. Genotyping of the floxed *Fzd5* allele was performed using primers complementary to Neo (pn5b: 5′ cta aag cgc atg ctc cag act 3′) and to *Fzd5* downstream of the stopcodon (sj2: 5′ cct tta gca aag agt cct aac 3′) generating a 700-bp PCR product. The wild-type allele was identified using a third primer (f5x: 5′ aga gga ggc ctt ata gag cg 3′), which generates a 250-bp product in combination with sj2 using 31 cycles with an annealing temperature of 55°C. Genotyping of the Six3-Cre transgene was performed as previously described (Furuta et al., 2000).

Whole-Mount and Section In Situ Hybridization

Whole-mount in situ hybridization using digoxigenin-labeled riboprobes was performed as previously described (Henrique et al., 1995). Templates for Axin2 (Zeng et al., 1997), BMP4 (Furuta and Hogan, 1998), BMP7 (Furuta et al., 1997), Chx10 (Green et al., 2003), FGF8 (Moon and Capecchi, 2000), FGF15 (McWhirter et al., 1997), FoxE3 (Blixt et al., 2000), Fzd5 (IMAGE clone ID#445088), Hes1 (Brown et al., 1998), Hes5 (Takebayashi et al., 1995), Mab21L1 (Yamada et al., 2003), Mab21L2 (Yamada et al., 2004), Math5 (Brown et al., 1998), Mitf (Hodgkinson et al., 1993), Pax2 (Nornes et al., 1990), Pax6 (Brown et al., 1998), Prox1 (Oliver et al., 1993), Six3 (Oliver et al., 1995), Sox2 (Avilion et al., 2003), Tbx5 (Chapman et al., 1996), and Vax2 (Barbieri et al., 1999) were used. For each marker, between two and seven mutant embryos were analyzed. In situ hybridization on paraffin sections was performed as previously described (Perron et al., 1998) with riboprobes for Fzd5, Pax6 (Brown et al., 1998), and Chx10 with the following modifications to the hybridization buffer: 1 mg/ml Torula RNA, 1× Denhardts, 0.1% CHAPS; no blocking reagent was added. In some experiments, 5% polyvinyl alcohol (MW-30-70K, Sigma) was added to the reaction buffer. Expression was visualized using NBT/BCIP tablets (Roche) dissolved in 10% polyvinyl alcohol. For Fzd5 in situ hybridization, sense controls confirmed the specificity of the antisense probe (data not shown).

Math5 expression was analyzed using optic vesicle explant cultures. At E9.5, embryos were dissected and optic vesicles including extraocular tissues carefully removed. Single explants were incubated at 37°C and 5% CO_2 for 3 days in four-well dishes (Nunc, Denmark) in serum-free 250 μl DMEM/F12 media (Gibco) with Sigma I-1884 supplement (Rachel et al.,

2002). After fixation, explants were dehydrated and stored at −80°C until processed for whole-mount in situ hybridization as described above. Ten optic vesicle explants of five embryos heterozygous (control) or homozygous for the *Fzd5* null allele were analyzed for Math5 mRNA expression. Statistical analysis was performed using Fisher's exact test.

Cell Death and Proliferation Analysis

Cryostat sections (12–16 μm) were analyzed for apoptotic cell death using the Fluorescein In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. At least 8 embryos for each genotype between E9.75 and E10.25 were examined. For quantification, three 16-μm sections of each optic vesicle of two embryos for each genotype were counterstained with 4,6-diamidino-2-phenylindole (DAPI), imaged, and the percentage of TUNEL-labeled cells was determined in the distal portion of the optic vesicle. Statistical analysis was performed using the *t*-test.

Pregnant females were injected subcutaneously with 75 mg BrdU/kg body weight 30 min before embryo dissection. Paraffin sections of BrdU-labeled embryos were processed for immunohistochemical analysis using a rat anti-BrdU antibody (1:10, Immunologicals Direct; 0BT0030S). For visualization, donkey anti-rat Alexa488-conjugated antibody secondary antibody was used. To visualize cell nuclei, sections were counterstained with DAPI (Roche). For each *Fzd5*−/− and control optic vesicle, at least three embryos were analyzed. For proliferation of the dorsal diencephalon, two embryos were analyzed. To quantify cell numbers, DAPI and BrdU fluorescent images were obtained using a Spot digital camera (Diagnostic Instruments) on a Nikon Eclipse E800 epifluorescent microscope. The central sections at the level of the optic vesicle were determined for the left and right sides of the embryo and images of the six central most, alternating sections of the optic vesicle, ventral diencephalon, and dorsal diencephalon were obtained. Using NIH Image software, the total number of DAPI-positive cells or BrdU-positive cells was counted for each region and the proportion of BrdU-positive cells was determined. No significant difference was detected between the right and the left side of the mutant or wild type embryos; therefore, further statistical analysis (*t*-test) combined the left and right side of each respective region.

For the proliferation analysis of conditional *Fzd5*−/− optic cups, three conditional heterozygous and conditional *Fzd5*−/− embryos were analyzed similar as described above. Four or five alternating sections were obtained (similar to described above for the BrdU analysis) and processed for rabbit anti–phospho-Histone H3 (pHH3; 1:1,000; Upstate, #06-570), DAPI labeling and imaging. The proportion of pHH3-labeled cells was manually determined in the optic cup and analyzed for statistical significance using the *t*-test.

Immunohistochemistry

Antibodies used for immunohistochemical detection on paraffin or cryostat sections were mouse monoclonal anti-Tuj-1 (Covance; MMS-435P), rabbit anti-Pax2 (Zymed, #71-6000), and Pax-6 (Philips et al., 2005). Alexa-conjugates of secondary antibodies (Molecular Probes) in appropriate combination with the primary antibodies were used.

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