Regulation of retinal progenitor expansion by Frizzled receptors: 
Implications for microphthalmia and retinal coloboma

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Abstract

Nineteen Wnt ligands and ten Frizzled (Fz) receptors mediate multiple distinct cellular events during neuronal development. However, their precise roles in cell type specification and organogenesis are poorly delineated because of overlapping functions and expression profiles. Here, we have explored the role of two closely related Frizzled receptors, Fz5 and Fz8, in mouse retinal development. We previously showed that $Fz5^{-/-}$ mice exhibit mild coloboma and microphthalmia at ~50% penetrance. Fz8 expression overlaps with Fz5 in the neural retina and optic fissure/disc. Mice lacking Fz8 show minimal eye and retinal defects. The embryos lacking both $Fz5$ and $Fz8$ die early in development, but a majority of triallelic $Fz5^{-/-};Fz8^{+/-}$ mutants survive until birth. The triallelic mutant develops severe retinal coloboma and microphthalmia with full penetrance. At the cellular level, impaired neurogenesis is indicated by increased early-born retinal neurons that result from accelerated cell cycle exit of progenitors. Deficiency of apical retinal neuroepithelium is indicated by altered localization of apical junction markers, such as aPKC, RhoA and $\beta$-catenin. $Hes1$ expression, which is critical for retinal progenitor expansion, is down-regulated in the triallelic mutant mouse. Furthermore, blocking Frizzled receptors in cultured retinal explants led to basally-shifted divisions of retinal progenitors. Together, our studies suggest a dose-dependent regulation of signaling by Fz5 and Fz8 in optic fissure/disc formation and progenitor expansion.
Introduction

How diverse neurons are produced in precise numbers during development is poorly understood. Stringent control mechanisms appear to dictate the production of neuronal diversity and eliminate unwanted cells by apoptosis (1-3). Control of cell cycle length and time of exit are expected to modulate the nature and extent of the neural progenitors. Intrinsic genetic programs and extracellular signaling cues contribute to the expansion of neural progenitor pool (4-6). During retinal development, six major types of neurons and one type of glia are produced in a conserved and overlapping order (7-8). Transcriptional factors provide key intrinsic control, qualitatively directing cell fate specification and differentiation. For example, Pax6 is required for the multipotent state of the retinal progenitors (9); Math5, Brn3 proteins and Islet-1 control ganglion cell production (10-12); and OTX2, RORβ, NRL and CRX control rod and cone photoreceptor differentiation (13). The major signaling pathways, including Notch, Hedgehog and Wnt, mediate the responses to extrinsic cues and modulate the plasticity of specific pools or proportion of retinal progenitors (14), thereby exerting a spatiotemporal regulation by defining the number of each cell type produced along the window of retinal neurogenesis.

Signaling pathways coordinate the spatiotemporal regulation of neurogenesis by integrating microenvironment to intrinsic genetic program. Unlike transcription regulatory factors, the impact of extrinsic cues on retinal progenitor fate is permissive and quantitative rather than instructive. As a result, imbalance in signaling dose can lead to disproportionate production of cell types, consequently affecting retinal size, architecture and/or function. For instance, disruption of Notch pathway by targeted deletion of
Notch1 receptor, its mediator Rbpj, or its effector Hes-1 can cause microphthalmia and increased production of early born cones and/or ganglion cells at the expense of retinal progenitors (15-17). Conditional deletion of Shh signaling leads to overproduction of retinal ganglion cells as a result of precocious cell cycle exit of the retinal progenitors (18).

The signaling dosage for retinal neurogenesis is coordinated, at least in part, by the polarized neuroepithelium, providing an environment for fine-tuning the neuroblast fate decision(s). This seems to be a dogma for the whole CNS neurogenesis where, for example, in zebrafish neural tube, the progenitors produced apical daughters (based on cleavage plane) biased to make neurons (19). Cell division and fate choice coincide with an apical-basal gradient of Notch activity in the retinal neuroepithelium (20). Insufficient interkinetic nuclear movement of neuroblasts causes early cell cycle exit due to reduced exposure to high dose of Notch at the apical retina (20, 21). Similarly, ganglion cell-derived Shh (18) is present at a high dosage at the basal retina and contributes to the maintenance of neural progenitors. Interestingly, Notch and Shh pathways share a downstream target, Hes1, during retinal neurogenesis (22); this observation has led to the hypothesis that different signaling pathways might converge and coordinate the signaling dosage for progenitor proliferation/expansion.

Multiple components of Wnt pathway are expressed in early embryonic mouse retina (23) indicating their role in retinogenesis. Notably, Wnt2b is critical for peripheral retinal fate in chick (24, 25), and Wnt3a promotes adult retinal neurogenesis upon injury in mouse (26). Canonical Wnt signaling appears to be active only in the ciliary marginal zone (CMZ) of developing mouse retina, as revealed by Tcf-β-gal reporter analysis and
loss- or gain- of-function studies of β-catenin (23, 27-29). Additionally, conditional ablation of β-catenin, a downstream target of canonical Wnt pathway, in the developing retina affects retinal lamination but not neurogenesis (25), while inactivation of β-catenin in retinal pigment epithelium (RPE) cells causing transformation of RPE to neural retina (30). Finally, in Xenopus, Frizzled 5 mediated canonical pathway acts through Sox2 to regulate neuronal potential of the progenitor (31), though this does not apply to mouse knockout models (28, 32).

To further explore the role of Wnt-Frizzled signaling in retinal neurogenesis and its contribution to retinal diseases, we have focused on the major Wnt receptors, Frizzled (Fz), which localize to several regions of the CNS and mediate both canonical and non-canonical Wnt pathways (33). The major cellular processes modulated by Fz receptors include planar cell polarity (PCP) in the inner-ear sensory neurons (34), neural tube closure (34), axon pathfinding (35-37), retinal vasculature development and regression (32, 38, 39), retinal optic fissure closure (32), and survival of thalamic neurons (40).

Previously, we and others have shown that Fz5 was expressed in developing mouse retina (27, 28, 32), brain ventricles and thalamic nuclei. Mutation in Fz5 in mouse caused retina vitreous persistent fetal vasculature (PFV) (28, 32), microphthalmia, retinal coloboma (32), and missing thalamic parafascicular nucleus (40). As Fz8 and Fz5 have the highest similarity and are activated by Wnt9b (40, 41), we examined whether Fz8 and Fz5 receptors function in a dose-dependent manner during retinal neurogenesis. Here, we demonstrate that Fz8 and Fz5 together maintain the
organization of retinal neural epithelia, thereby regulating the retinal progenitor pool and neurogenesis.

Results

**Fz8 expression in developing mouse retina**

To investigate *Fz8* expression, we used a knock-in β-gal reporter at the endogenous locus in *Fz8*+/lacZ mice (42) that show no distinct phenotype compared to the wild type mice. *Fz8* is expressed in the forebrain and edge of the eye field during early embryogenesis (Fig. 1, A-D). At embryonic day 13.5 (E13.5), strong staining was observed in optic disc and retinal proliferating marginal zone (PMZ), while weaker signal was detected in neural retina (Supplemental Fig.1). At E14.5 and E16.5, in bisected whole mount retina, *Fz8* expression was observed in optic disc, PMZ and neural retina with a preference to the ventral (Fig. 1E-G). In 4-week old retina, *Fz8* is expressed in all retina layers with less staining in the outer nuclear layer (ONL)(Fig. 1H). Immunofluorescence labeling of dissociated cells from adult *Fz8*+/− retina (Fig. 1I) revealed *Fz8* expression in ganglion cells (marked by Brn3a and NFL), Müller glia (marked by Sox9) and Calbindin positive cells. *Fz8* expression in optic disc region and neural retina overlaps with the *Fz5* during the early development (32) (Supplemental Fig.1A-C)(27). *Fz8* expression in Müller glia in adult retina also overlaps with *Fz5*, and its expression in Calbindin positive cells potentially overlaps with *Fz5* expression in amacrine cells (compare Fig.1I and Supplemental Fig.1 D-E).
Ocular phenotype in the *Fz8*−/− mice

We then examined the retinal phenotypes of *Fz8*−/− mice. The major cell types such as cones, rods, ganglion cells, amacrine cells and bipolar cells are developed correctly in *Fz8*−/− retina (Fig. 1 J-M). However, a fraction of the mutant retina showed a modest increase in GFAP staining of astrocytes (Fig. 1N, O) with an ectopic pigment stalk sitting on the optic nerve head (Fig. 1P), which has been well described as persistent fetal vasculature (PFV) (32) and resembles the human eye disease called persistent hyperplastic primary vitreous (PHPV)(28). The phenotype, though weaker and with incomplete penetrance (6 out 11 mice examined), was broadly similar to the optic disc defects in *Fz5*−/− mutant retina (28, 32).

Severe retinal coloboma, microphthalmia, astrocytic gliosis and axon sprouting in *Fz5* and *Fz8* compound mutant retina

The phenotypic similarity of Fz5 and Fz8 mutants in optic disc development led us to hypothesize that the two receptors work together during retinal neurogenesis since their expression also overlaps in neural retina. We therefore generated *Fz5* and *Fz8* compound mutant mice. Mice carrying double null alleles of *Fz5* and *Fz8* died before E13.5 likely due to systemic angiogenesis defects, making it difficult to analyze the retinal phenotype. However, adding one *Fz8* WT allele to the double null (*Fz5*−/−;*Fz8*+/-) allowed these mice to survive until E17.5 or birth. Occasional pups survived to one month but had significantly reduced body weight and more severe eye defects (Fig. 2, and data not shown). In contrast, *Fz5*+/−;*Fz8*+/− mice did not show apparent abnormalities and are therefore indistinguishable from the wild type. Henceforth *Fz5*−/−;*Fz8*+/− and *Fz5*+/−.
;Fz8^+/- mice are referred as compound mutant/mutant and wild type/control, respectively. We observed that optic fissure failed to close in all Fz5^-/-;Fz8^+/- retinas at all stages examined. Retinal coloboma was readily visible at E15.5 (Fig. 2A), with a lack of ventral retinal tissue in the young adult (Fig. 2F). Pax2 expression in the mutant retina expanded laterally (Fig. 2B, C). Overgrown embryonic fetal vasculature packed behind the embryonic lens (Fig. 2D, E). Increased neural fiber thickness and abnormal axon routing was also consistently observed by staining of neural filament using anti-NFL-70 (Fig. 2D, E).

Occasionally, compound mutant survived to 4-week of age developed severe microphthalmia and retinal coloboma (Fig. 2F). Persistent fetal vasculature (PFV) was also evident (Fig. 2G) with highly pigmented mesenchymal cells intermingled with GFAP stained astrocytes (not shown). Astrocytic gliosis is apparent along the surface of open optic fissure by GFAP staining (Fig. 2H). Axonal bundles sprout in both neural fiber and outer plexiform layer (OPL), as revealed by NFL staining (Fig. 2I, J). The axonal sprouting phenotype was also observed in Fz5^-/- single mutant, which occurred later at 6-month of age and was relatively milder (Supplemental Fig. 2). Despite the whole retina being thinner, INL was more affected compared to the ONL (Fig. 2J, M). Müller glia and/or astrocytes were activated in vast regions of the retina with thickened fiber reaching through the INL (Fig. 2 K, M).

The more severe and complete penetrance of ocular defects in Fz5^-/-;Fz8^+/- mice stands in contrast to those of either Fz8^-/- or Fz5^-/-, alone, suggesting that Fz5 and Fz8 function together dose-dependently during retinal development. We have also examined
the $Fz5^{+/-};Fz8^{-/-}$ triallelic combination and found the retina developed normally in this mutant. Therefore, $Fz5$ provides more genetic load than $Fz8$ during retinal development.

**Increased early born retinal neurons and accelerated neurogenesis in $Fz5$ and $Fz8$ compound mutant retina**

Despite the smaller eye size, the ganglion cell layer labeled by Brn3a is thicker in the compound mutant retina at E15.5 (Fig. 3A, B). Similarly, Islet-1 labeled early born neurons including ganglion cell and amacrine cells were also increased when examined at E17.5 (Fig. 3C, D, E). We therefore hypothesized that early born neurons were generated over a broader window of time during early neurogenesis in compound mutant retina. To examine this possibility, we re-evaluated neurogenesis in E13.5 mutant retina. Indeed, we observed advanced and broad areas of neurogenesis occurring as early as E13.5 in the mutant retina using Tuj1 as a marker of postmitotic neuronal precursors (Fig. 3F,H). Segmentation of the retina showed that the advancing area (AA) for retinal neurogenesis is more expanded in the mutants,(Fig. 3G, I), suggesting a faster spreading of retinal neurogenesis. Quantitative analysis revealed a 6-10 percent increase in the neurogenesis area of the mutant retina.

**Normal mitotic division rate, increased BrdU-retained early born neurons and cell death**

The accelerated neurogenesis indicates cell cycle defects of the proliferating progenitors. One possibility of the cell cycle defects is gross reduction of proliferation of the progenitors. This should be reflected by expression of proliferating antigens, for
example, Ki67. However, no obvious changes were detected in either Ki67 expression territory or intensity at E13.5 (Fig. 4A) and E17.5 (Fig. 4F-H), indicating a grossly normal proliferation in the mutant retina. We then examined whether mitotic rate has slowed down in the mutant retina because of the microphthalmia phenotype. Quantitative analysis of pH3 labeled dividing cells (Fig. 4B, C) on retinal apical surface and single pulse of 1 hr BrdU labeled proliferating cells (not shown) showed no significant difference between wild type and mutant retinas. Another possibility was that although progenitor cells divided at a normal rate in the mutant apical retina, more daughter cells exited cell cycle by asymmetrical divisions. To test this possibility, we delivered a single pulse of BrdU at E13 and monitored BrdU retention after 60 hours, at which time the progenitors would have gone through three cell cycles. Bright and round BrdU+ cells are predicted to be newly post-mitotic with higher labeling since BrdU was not diluted in these cells. We observed an increase in BrdU-positive cells in both inner neuroblast layer (INBL) and apical outer neuroblast layer (ONBL) in all sections at E15.5 (Fig. 4D, E). The BrdU-positive cells in apical ONBL likely corresponded to early-born cones though this was not tested directly. Similar result of BrdU retention was also seen after 16 hr pulse (Supplemental Fig. 3A-B). We then immunostained the retina for cyclin D kinase inhibitor p27kip1, which promotes the exit of retinal progenitors from cell cycle (43). We observed a stronger staining of p27Kip1 at the apical mutant retina, and a reduction of INBL staining (Supplemental Fig. 4A), suggesting a role for p27Kip1 in maintaining cell morphology and in cell migration (44, 45). Interestingly, total p27Kip1 protein was slightly but consistently reduced, whereas cyclin D1 remained unchanged (Supplemental Fig. 4B). Together, our data suggest that accelerated neurogenesis is
likely due to an early retreat of progenitors from the cell cycle, probably caused by increased asymmetrical divisions rather than the rate of cell division.

We also detected increased cell death in the INBL at E17.5 (Fig. 4F-H). The increased cell death appears to be a secondary event in post-mitotic cells since Fz5 is not expressed in the differentiated neurons in INBL, and Fz8 alone does not cause retinal neuronal cell death.

Deficiency of retinal apical junctions and down-regulation of RhoA and β–catenin

We hypothesized that early cell cycle exit of the mutant progenitors might result from defects in apicobasal polarity of the neural epithelia. Consistent with this hypothesis, Frizzled receptors are shown to be central players of PCP pathway that is involved in apical-basal polarity of the embryonic epiblasts during gastrulation (46, 47). We first examined retinal apical neuroepithelium by staining for β-catenin, an important apical junction marker that is critical for retinal lamination and its loss causes disorganized retinal neural epithelium (25). We detected a significant reduction of β-catenin on apical surface of the mutant retina at E15.5 (Fig. 5A). F-actin that is enriched at the apical surface was also reduced at apical junctions (Fig. 5B). Upstream of actin assembly, RhoA-GTPase, an important player in PCP pathway and enriched in apical junction, was also mislocalized (Fig. 5C). The localizations of other junction proteins, aPKC and N–cadherin (Fig. 5D, E), were also compromised at the apical junctions, indicating perturbation of retinal apicobasal polarity. Interestingly, at the total protein level, we observed that both RhoA and β-catenin were down-regulated significantly in the mutant retina by western blots (Fig. 5F, G). We also examined the expression of
laminin, an ECM protein that is affected by PCP pathways (46). Laminin deposition was reduced and shifted more to the periphery in the mutant retina at E13.5 (supplemental Fig. 5). Together, these results show a deficiency in apical junctions of retinal neuroepithelium, which might lead to aberrant neurogenesis.

Cell-autonomous retraction of neuroblast apical processes and ectopic progenitor divisions upon blocking Frizzled receptors in vitro

To further elucidate the effect of apical junction deficiency, we generated mosaic Frizzled mutant neuroblast cells by injection of 4HT to control Cre-ER activity in excising \( Fz5^{ckoAP} \) allele (32). The apical processes of many mutant neuroblasts retracted from retinal surface (Fig. 6A-B). In concordance, we also observed that \( \gamma \)-Tubulin-labeled centrioles in retinal progenitors of the compound mutant retina delocalized from the retinal apical surface at E13.5 and E15.5 (Fig. 6C-D). Thus, deficiency at apical junctions might cause the retraction of apical processes of retinal neuroblasts. Our data also indicated that Frizzled receptors function primarily in a cell-autonomous manner in neural retina.

Apical niche of the neuroepithelium is where the progenitors renew and generate neurons. In many cases, disruption of apicobasal polarity can result in ectopic progenitor divisions; however, this is barely observed in \( Fz5^{-/-}; Fz8^{+/-} \) retina. Nevertheless, we detected ectopic progenitors’ divisions along the retinal neuroepithelium by blocking Frizzled receptor function in cultured retinal explants, using a secreted form of Fz8 cystine rich domain (Fz8Ig-CRD) (Fig. 6E-F). Fz8 CRD is highly conserved among Frizzled members (40) and widely inhibits Frizzled receptor-mediated canonical Wnt
pathway. Fz8Ig-CRD was added to cultured E13.5 retinal explants. After 42 hr incubation, more than 30% of the dividing progenitors in treated retinas divided ectopically or shifted basally along the retinal epithelium (Fig. 6E-G); however, γ-Tubulin-marked neuroblast apical centrioles were roughly in continuum (Fig. 6E-F), indicating the ectopic divisions are not artifacts of distortion or folding of the cultured retinal explants. Interestingly, a significant increase of dividing rate of the progenitors (labeled by pH3) was also observed (Fig. 6E-G), which has not been seen in Fz5−/−; Fz8+/− retina. One interpretation for these outcomes is that the remained 50% of Fz8 dose in the triallelic mutant retina can be completely blocked by applying Fz8Ig-CRD to retinal explants in vitro culture system; alternatively, other Frizzled receptors which may compensate for Fz5/8 function loss in compound mutant retina could also be further blocked in vitro. In any case, more severe retinal neurogenesis defects were caused.

Altered gene expression of signaling pathways required for neurogenesis in the mutant retina

To address more directly the cause of earlier cell cycle exit in the mutant retina, we evaluated Notch and Shh signaling pathways by in situ hybridization and q-PCR. While Notch1 receptor expression appeared unaffected in the mutant retina (Fig. 7A, C, Supplemental table 1, P=0.18), its downstream target, Hes1, was reduced significantly in most area of the retina at E13.5 (Fig. 7B, C, Supplemental table 1, P=0.0067). Hes5 expression seems slightly increased on the other hand in the mutant retina, but with a P value of 0.14 (Supplemental table1). Since it has been shown that Hes1 is also a major target of Shh in retina independent of Notch signaling (22), we examined expression of
Shh downstream effectors Gli2 and Gli3. Although Gli2 is required for Hes1 activation in developing retina (22), it remains unchanged in the mutant by q-PCR assay, so does Gli3. Therefore, the compromised Hes1 expression in Fz5 and Fz8 triallelic mutant retina is likely mediated through Notch signaling.

Interestingly, we found an upregulated RhoA (P=0.02) and unchanged \( \beta \)-catenin expression in contrast to the down-regulation of both proteins detected by western blot in Figure 5. This seemingly paradox could be interpreted as possible alternations of protein stability of RhoA and \( \beta \)-catenin in the mutant retina, which could be regulated by both canonical and/or noncanonical Wnt-Frizzled pathways.

Discussion

Here, we demonstrate that Wnt receptors, Fz5 and Fz8, are critical for modulating progenitor cell cycle exit in mammalian retina, thereby contributing to retinal progenitor expansion. We also show that Frizzled receptors play a role in maintaining retinal neuroblast apical junctions, which in turn may affect reception of Notch signaling along the retinal epithelium. Our data provide insight for the relationship of Frizzled receptors mediated retinal neural epithelial integrity with signaling pathways critical for retinal neurogenesis. We propose a working model for Frizzled receptors’ function in retinal neuroblasts based on our findings (shown in Figure 7D).
Fz5 and Fz8 coordinate optic fissure/disc formation and axon integrity in dose-dependent manner

The complexity of Wnt-Frizzled signaling is compounded by the existence of multiple members in both Wnt and Frizzled families (33). For example, Fz3 and Fz6 function redundantly in the inner ear, yet have distinct roles in the brain and hair follicles (34, 36). In our studies, mutations in Fz5 or Fz8 cause varying degrees of optic fissure/disc abnormalities. The two genes do not fully compensate for each other in retina, as mutation in each of them causes phenotypes. However, mutation in both genes causes more severe retinal phenotypes with full penetrance, indicating Fz5 and Fz8 function in dose-dependent manner in retina. In the Fz5<sup>+/−</sup>;Fz8<sup>+/−</sup> triallelic combination, retinal development proceeds as in Fz8<sup>−/−</sup> mutant. Thus, the ocular defect is most pronounced in the Fz5<sup>−/−</sup>;Fz8<sup>+/−</sup> mutant followed by the Fz5<sup>−/−</sup> single mutant, whereas in the Fz5<sup>−/−</sup>;Fz8<sup>−/−</sup> compound and the Fz8<sup>−/−</sup> single mutant the retinal defect is minimal. These phenotypic variations suggest that Fz5 and Fz8 receptors are signaling similar cellular events in the developing retina, with Fz5 being the dominant contributor by far. As such, the variable severity in ocular defects would simply reflect the varying levels of combined functional Fz5/Fz8 dosage among these mutants.

The dosage effect is also reflected by the aberrant axons’ sprouting in Fz5<sup>−/−</sup>;Fz8<sup>−/−</sup> mutant retina, which is clearly evident in 4-wk old mice yet absent in the Fz5<sup>−/−</sup> mutant mice until 6-month age. Additionally, broadly blocking Frizzled receptors in vitro to further reduce Frizzled receptors dosage produced a qualitatively similar, but even more severe defect in retinal progenitors mitotic divisions, further support this notion.
Role of Frizzled receptors in maintaining retinal progenitor pools and implications for microphthalmia and coloboma diseases

We propose that microphthalmia, coloboma, and excessive production of early neurons in the \( Fz5^{-/-}; Fz8^{+/-} \) embryonic retina are due to altered homeostasis of retinal progenitor expansion. Similar phenotypes of abnormal CNS neurogenesis are caused by mutations in Shh and Notch signaling pathways. We saw no significant changes in mitotic division rate in the \( Fz5^{-/-};Fz8^{+/-} \) mutant retinal progenitors. Rather, we identified a moderate acceleration in cell cycle exit of retinal progenitors. Three lines of evidence support this conclusion. First, Brn3a labeled RGCs and Islet-1-labeled postmitotic neurons are increased in embryonic mutant retinas. Second, Tuj1-labeled early postmitotic neurons increased and expanded more peripherally in E13.5 mutant retina. And finally, BrdU pulse at E13 showed a significant increase in postmitotic labeling in the mutant retina. The increased apoptosis in INBL of \( Fz5^{-/-}; Fz8^{+/-} \) retina is likely to be secondary to the prematurity of excessive early-born neurons populated in the INBL. This is because \( Fz5 \) is not expressed in INBL and \( Fz8 \) mutation alone does not confer the early cell death. Therefore, accelerated cell cycle exit leading to the depletion of retinal progenitors, in combination with the INBL apoptosis, appear to be the major cause of microphthalmia, coloboma. Whether the accelerated cell cycle exit is because of a moderate shift from symmetric proliferative to asymmetric neuronal divisions (one progenitor +one neuron) requires further investigation.

A more direct cause of accelerated neurogenesis of RGC could be explained by gross reduction of \( Hes1 \) expression in the mutant retina. \( Hes1 \) is an important target of both Notch and Shh signaling pathway (22). Shh is also critical for optic fissure/disc
formation (48) (49) and astrocytes development (50), RGC production (18) (51), and retinal lamination (52). Hes1 mutant also show a retinal morphogenesis defects caused wide open of the ventral retinal at E15.5 and over production of RGCs (52) (17). The reduction of Hes1 in retina and optic fissure caused by loss of Fz5 and Fz8 suggest that cross-talks between Frizzled and other signaling pathways is required for in retinal neurogenesis and structural development. Furthermore, the enhancement of Fz8, Fz5 and Hes1 (not shown) expression in optic fissure along with the severer optic fissure phenotype implies a higher dosage Frizzled signaling is required to maintain this structure.

**Role of Fz8 and Fz5 in retinal neuroblast apical junctions**

The apical-basal polarity of the embryonic retina is crucial for maintenance of the retinal progenitor homeostasis and differentiation. Several events take place asymmetrically along the apicobasal axis: (1) mitotic divisions occurring on the apical surface of the retinal neuroblasts (53); (2) Notch signaling gradient along the apical to basal axis of the neuroblast being critical for the maintenance of retinal progenitors’ properties (20); and (3) an efficient interkinetic nuclear migration along the apical-basal axis driving the maturation of the neuroblast(s) for cell division (21, 54-56). Little is known about signaling pathways participating in establishment of retinal neuroblast apical junctions and neurogenesis niche at the apical surface. Non-canonical Wnt-Frizzled signaling is reportedly involved in multiple aspects of cell or tissue polarity (57), where the cytoskeleton reassembly is an important downstream event (46, 47, 58). We observed that two PCP targets, F-actin and RhoA, which normally are enriched at the
apical adherens junctions, were reduced in the mutant retina. Partial loss of β-catenin, aPKCλ, and N-cadherin staining from the apical domain in the mutant is also indicative of diminished apical junctional complexes. Furthermore, by western blotting both β-catenin and RhoA levels are much lower in the mutant. However, we detected increased expression of RhoA but no change in β-catenin RNA by q-PCR analysis, suggesting that reduced β-catenin and RhoA protein levels likely result from post-transcriptional mechanisms. For instance, reduction of β-catenin could be explained by insufficiency of Frizzled signaling, which then leads to an overly active β-catenin destruction complex.

While canonical Wnt/β-catenin signaling does not appear to regulate TCF family of transcription factors during retinal neurogenesis, there is no data indicating the initial step of canonical signaling, namely the stabilization of β-catenin is not operative in the developing retina.

The disturbance of retinal neuroblast apical processes is likely to be a cell-autonomous event as suggested by mosaic analysis. The retraction of processes from the apical surface in single mutant neuroblast may directly lead to apical junction defects when all neuroblasts along the retinal neurogenesis window are considered.

**Interactions among signaling pathways during retinal neurogenesis and optic fissure development**

Attenuated *Hes1* expression indicated that Notch signaling was compromised probably due to insufficient cell-cell interaction of the neuroblasts resulting from their apical deficiency. In this regard, Notch signaling belongs to juxtaposed signaling category, requiring direct cell-cell contact. Even though Notch receptors and ligands remain
largely intact, Notch signaling still could be disturbed by compromised cell-cell contact, eventually causing reduced Hes1 expression. Meanwhile, Shh signaling may not be compromised since Shh is a diffusible morphogen less relying on cell-cell contacts. In accordance, we did not detect altered expression of Gli2, which is required for Shh mediated Hes1 activation (22). In addition, Hes1 seems to suppress RGC fate only (17). Therefore, the increased apical cone-like cells in Frizzled mutant retina might be contributed by other Notch signaling members, for example Hes5 or Rpbj (17), mutations in which also give cone phenotypes. Whether Frizzled receptors receive Wnts signaling in the retina or use other ligands remains to be explored.

Taken together, we propose a model in which Fz8 and Fz5 are required for establishing and/or maintaining retinal neuroblast apical junctions, which are critical for communication of signaling pathways. In Fz5−/−; Fz8+/− mutant retina, disturbance of retinal apical junctions leads to compromised Notch signaling in the defective neuroblasts, biasing retinal progenitors toward premature cell cycle exit, which in turn causes disproportion of retinal progenitor pool size. Alternatively, the perturbed retinal apical junction may also affect the efficiency of interkinetic nuclear migration of the neuroblasts, thereby altering their exposure to the signaling cues in the developing retina. In any event, impaired neurogenesis will result in microphthalmia and/or coloboma.
Materials and Methods

Generation of Fz5 and Fz8 compound alleles

All procedures involving the use of mice were approved by Animal Care and Use Committee of the National Eye Institute. Fz5 straight and conditional knockout mice and Rosa26-CreER: Fz5^{ckoAP/+} mice have been described previously (40). Briefly, Fz5 straight knockout (Fz8^{lacZ}/lacZ) mice are lethal at around E9.5 due to placenta defects; hence, Sox2-Cre (not expressed in placenta; (59)) is combined with Fz5^{ckoAP/+} allele to produce null mutants. Fz8-knockout was created by replacing its coding region with a β-gal reporter (generously provided by Jeremy Nathans; (42)). Multiple intercrosses were used to produce experimental parental lines, designated as Sox2-Cre;Fz5^{lacZ/+}:Fz8^{lacZ}/lacZ, Rosa26-CreER; Fz5^{lacZ/+} and Fz5^{ckoAPckkoAP}:Fz8^{lacZ/+} based on the names of transgenes, endogenous alleles and knock-in reporters. None of the lines individually showed phenotypes pertaining to our analysis described in the results. Crossing the two parental lines generated mice with a range of genotypes, from which Sox2-Cre;Fz5^{lacZ/AP}:Fz8^{lacZ/+}, Sox2-Cre;Fz5^{AP/+}:Fz8^{lacZ/+}, Rosa26-CreER; Fz5^{ckoAP/+}:Fz8^{lacZ/+} and CreER; Fz5^{lacZ/AP}:Fz8^{lacZ/+} mice were used for experiments. Sox2-Cre;Fz5^{lacZ/AP}:Fz8^{lacZ}/lacZ mice, which are equivalent to the double knockout of Fz5 and Fz8, had paler and smaller embryonic size and died before E13.5. Genotyping methods have been described earlier (40).

Mosaic mutant analysis of retinal neuroblasts

Mosaic mutational analysis was performed as described (32). Briefly, 4-Hydroxytamoxifen (4HT) was introduced by intraperitoneal injection into pregnant mice
carrying *Rosa26-CreER; Fz5^{lacZ/ckoAP};Fz8^{lacZ/+}* and *Rosa26-CreER; Fz5^{ckoAP/};Fz8^{lacZ/+}* pups at 5 µg/g body weight at E10. Embryos were harvested at E13 and subjected to alkaline phosphatase staining (32).

Histology and Imaging

Procedures for preparation of flat mount retina, light and electron microscopy sections have been described previously (32). Standard protocols were used for X-gal and X-phos staining, *in situ* hybridization and immunohistochemistry (IHC) (32). Antibodies and dilutions used in this study are: rabbit anti-Sox9, 1:500 (Chemicon AB5535); rabbit ant-NFL, 1:500 (Chemicon MAB5294); mouse anti-Brn3a, 1:2000 (Santa Cruz, sc-8429); rabbit anti-calbindin, 1:1000 (Calbiochem, PC253L); rat anti-β-gal, 1:1000 (a gift from Tom Glaser); mouse anti-GFAP, 1:500 (Millipore, MAB3402); mouse anti-Islet-1, 1:2000 (DSHB, 40.3A4); rabbit anti-phospho-Histone H3, 1:150 (Cell Signaling, 9701); mouse anti-Tuj1, 1:1000 (Covance, MMS-435P); mouse anti-Ki67, 1:200 (abcam, ab8191); rabbit anti-Caspase-3, 1:200 (Cell Signaling, 9661); rabbit anti-laminin, 1:50 (Sigma-Aldrich, L9393); Phalloidin-Alexa 594, (1:200, Invitrogen); mouse anti-γ-tubulin,1:500 (Sigma-Aldrich, T6557); rabbit cone arrestin, 1:500 (Millipore, ab15282); mouse rhodopsin 1D4, 1:200 (ab5417); mouse Glutamine synthetase, 1:200 (laboratories, 610518); rat anti-BrdU, 1:200 (ab6326); anti-PKCλ,λ,3 (cell signaling, 9378); anti-N-cadherin, 1:200 (Invitrogen, 33-3900); anti-RhoA, 1:1000, sigma (SAB1400017); anti- β-catenin (610154, BD transduction laboratories); mouse anti-p27Kip1, 1:1000 (BD transduction, 610241), mouse anti-Ccnd1, 1:250 (Santa Cruz, SC...
Fluorescent images were collected by Olympus epifluorescence microscope, Leica SP2, or Olympus FV1000 confocal microscope.

**Retinal tissue extraction and western blot.**

Embryonic retina were dissected and extracted using RAPI lysis buffer, following vortex for 2 min. Tissue lysates were then centrifuged at 14,000g for 10 min, and the supernatant used for SDS-PAGE. Immunoblots were probed with anti-RhoA, β-catenin, Ccnd1 and p27Kip1 (Antibody resources are listed as above for IHC).

**Retinal cell dissociation and immunofluorescence studies**

Adult retinas were dissected, cut into pieces, and digested in 15 ml conical tubes containing papain solution at 8°C for 30 min. The samples were then shifted to 28°C for another 10 min incubation with intermittent shaking. Digested tissues were mixed using Pasteur pipette, and centrifuged at 150g at 4°C for 5 min. After resuspension in anti-papain solution and another spin, cell pellet was resuspended in DMEM/F12 medium with 10% serum. Cells were then spread on L-lysine coated slides and incubated for 2 hr at 37°C. After rinsing several times with PBS or DMEM/F12 medium, cells were fixed and stored in methanol at -80°C for immunofluorescence labeling, as described for IHC using tissue sections.

**BrdU pulse assay**

BrdU (50 μg/g body weight) was injected in E13-timed pregnant mice intraperitoneally. Mice were sacrificed after 16 or 60 hr of BrdU injection. Retina sections
were subjected to IHC. BrdU positive cells are then counted separately in ONBL and INBL. For 16 hr BrdU incorporation, cell counting is implemented by using ImageJ software to set signal threshold for difficulty of getting single cell resolution. For 60 hr BrdU incorporation study, BrdU positive cells were counted in INBL and apical surface of the ONBL.

**Cell counting**

A group of three sections from each of the Islet-1, phospho-histone 3 (pH3) or BrdU labeled embryonic retina were collected from the central area. Three retinal sections each from three independent animals were subjected to cell counting for each marker. The number of Islet-1 positive cells in ONBL was normalized to the counting area X 1000 using Meta imaging (series 7.5) software. Islet-1 positive cells in INBL are very dense, thus counting was performed by staining area and normalized to total neuroblast layer (NBL) area. Data are presented as percentage. The number of pH3 positive cells was normalized to the retinal apical circumferential length, which is measured by MetaMorph software and given arbitrary unit value. For 16 hr BrdU single pulse, BrdU signal (intensity) in ONBL was detected by NIH ImageJ by setting threshold, while in INBL was counted by cells. Using unconventional counting method for ONBL is because BrdU labeling after 16hr was very intense in ONBL, single cell morphology was barely distinguishable, therefore, software was applied. While in INBL, most of labeled cells are postmitotic and well separated, therefore, suitable for counting by cells. Students’ t-test was used to obtain P values in paired groups of sections.
Production of Fz8Ig-CRD conditional medium and retinal explants culture

HEK293 cells were transfected with Fz8Ig-CRD plasmid, in which Fz8 extracellular cystine rich domain (CRD) was fused with human immunoglobulin G (IgG) heavy chain, or with IgG heavy chain gene alone (as control), driven by a CMV promoter in a pRK5 plasmid backbone (40). The secretion of Fz8IgCRD was tested by immunoblot analysis of the culture medium (data not shown).

Retinas were dissected from E13.5 mice, cultured in a drop of Fz8IgCRD conditioned or DMED/F12 medium on a Nucleopore filter (Whatman) floated in wells filled with cultured medium of a 6-well dish. After 42 hr incubation, retinas were fixed and sectioned for IHC analysis. pH3 positive cells were counted from 3 retinas (3 sections/retina). Statistical analysis was conducted using Microsoft Excel.

RNA preparation and quantitative PCR analysis

Total RNA was prepared from 2-3 retinas using TriPure Isolation Reagent (Roche) and RNeasy Mini Kit (Qiagen). Three independent samples of total RNA from wild type and mutant mice were used for the synthesis of first strand of cDNA using SuperScript® First-Strand synthesis system (Invitrogen). RT-qPCR was then performed using SYBR® Green-Based Detection of gene expression (Applied Biosystem, 7900HT). Data analysis employed Livak \( \Delta\Delta Ct \) method, in which gene expression fold change is calculated as \( 2^{-\Delta\Delta Ct} \). Change ratios of <1 were treated with negative reciprocals for the presentation purpose.
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Conflict of Interest Statement

None declared.
References


Legends to Figures

Figure 1. Fz8 retinal expression during mouse development and phenotypic analysis of Fz8 mutant retina.

A-H, Fz8 expression was visualized by a β-gal reporter from its knock-in endogenous locus. A-C, Fz8 was expressed in the areas of embryonic forebrain (FB), optic vesicle (OV) and otic vesicle (OTV) at early stages of embryonic day 8.5 (E8.5) and E9.5. D, Fz8 expression was continuously seen in forebrain ear and eye regions at E14.5. E-F, Bisected X-gal stained whole-mount retinas at E14.5 (E) and E16.5 (F) showing Fz8 enhanced expression in optic disc (OD, asterisk), proliferating marginal zone (PMZ, arrows) and ventral retina (arrowhead). Dorsal-ventral orientation (d-v) is indicated by up-down arrows in each panel. G, A top view of an X-gal stained E16.5 retina showing a preferential Fz8 expression in optic disc and ventral retina. H, A 4-week-old X-gal stained retinal section shows that Fz8 was expressed through all retinal layers. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer. I, Co-immunostaining using anti-β-gal antibody (red) and other retinal cell markers (green) shows that Fz8 is expressed in Müller cell, ganglion cell (RGC) and Calbindin-positive cells (likely amacrine cells). Merged images show co-labeling of β-gal with Sox9 for Müller cell, NFL and Brn3a for ganglion cell, and Calbindin (Calb) positive cells. DAPI staining (blue) shows number of cells in the pictured field. Boxed areas indicate that same cells are being labeled by Sox9 and β-gal. a, double labeled cells with Sox9 and β-gal; a’, DAPI stained cell nuclei; and aa’, zooming in on merged
channels of Sox9, β-gal and DAPI in a & a’ areas. J-M, Normal development of retinal cell types in Fz8 mutant retinas: No significant differences in immunostained Fz8 heterozygous and mutant retinas for Brn3a and NFL positive RGCs (J, green and red are for RGCs and RGC axons, respectively), Islet-1 positive RGCs, ACs and bipolar cells (K, green), calbindin (Calb) positive ACs and HCs (K, red), rhodopsin-labeled rods (L, green), cone arrestin (Carr)-labeled cones (L, red) and glutamate synthetase (GS)-labeled Müller glia. Non-specific staining (green signals in IPL and OPL) in J and L is from anti-mouse Ig secondary antibody. N-P, Increased GFAP staining of astrocytes dendrites in the mutant retina, and mild optic disc defects. N, Low magnification showing the GFAP stained whole-mount retinas showing enhanced GFAP staining. O, Zoom-in areas roughly correspond to the boxed areas in N, showing individually stained astrocytes dendrites. P, A frequent observation (6/11 mutant mice) of optic disc defects associated with the enhanced GFAP staining, featured by persistent fetal vasculature (PFV) (white arrowhead). The optic disc (OD, black arrowhead) does not seem to close properly in the mutant mice.

Figure 2. Severe retinal coloboma, microphthalmia, retinal gliosis and axon sprouting in Fz5<sup>+/−</sup>;Fz8<sup>−/−</sup> mutant retina.

A, Lacking ventral retinal tissue, severe retinal coloboma and microphthalmia at E15.5 in Fz5<sup>+/−</sup>;Fz8<sup>−/−</sup> compound mutant retina (arrowheads). B, Staining of Pax2 at optic fissure is more restricted in the wild type (arrowheads, left panel) than in the mutant (arrowheads, right panel). Asterisks indicate the optic discs. C, Merged images of Pax2 staining with DAPI indicate the retinal structure at optic disc. D-E, Widened optic fissure with massive
RGC axons routing out the mutant retina at E17.5. Misrouting of axons often happens (white arrow, right panel) with a consistently observed thickened retinal neural fiber layer and malformation of retinal fetal vasculature (dashed lines enclosure). E, DAPI staining of the same sections in D showing the retinal structure of optic disc area (asterisk) at E17.5. Dashed lines indicate the embryonic vasculature tissues. F, A brightfield (BF) picture of 4-week old mutant eyeball showing the server ventral retinal coloboma (arrowhead). The pupil-iris boundary is demarcated by dashed lines. G, Phase contrast image of a 4-week old retinal section showing severely deformed mutant retina and intraocular pigmented persistent fetal vasculature (PFV, arrowhead). H, Boxed area in G showing overwhelming GFAP stained astroglia lining the surface of unclosed optic fissure. I, NFL stained axon sprouting in the 4-week old compound mutant retina in both GCL and OPL (arrows). J, Images from I merged with DAPI showing inner retina thickness (white brackets, INL and IPL) is significantly reduced in the mutant compared to the wild type. Mutant ONL thickness is similar to the wild type at this stage (yellow brackets). K, Abnormal glial activation in the mutant retina. GFAP labeled astrocytes sparsely distribute below the GCL in the wild type retina, while they spread along the neural fiber layer in the mutant retina (arrows) and go up to through the mutant retina. M, DAPI stained retinal nuclear layers merged with GFAP staining shown in K.

Figure 3. Increased early born neuronal production and neurogenesis in \( Fz5^{+/−};Fz8^{+/−} \) mutant retina.

A-B, Two consecutive Brn3a-stained retinal sections at E15.5 showing increased RGC production in the mutant retina (brackets, sectioning orientation is drawn in J, line
through the embryonic eye). C-E, Increased Islet-1 labeled early born neuronal precursors in the Fz5−/−;Fz8+/− retina at E17.5. C, Islet-1 stained inner neuroblast layer (INBL, under dashed lines) of the mutant retina is significantly thicker in the mutant compared to the wild type. Correspondingly, the newly born neuronal precursors in outer neuroblast layer (ONBL, above dashed lines) are also significantly increased (boxed area, also in D). D, A close view of the retina in the boxed regions in C showing the increased Islet-1 cells in both INBL and ONBL. Arrowheads point some individual Islet-1 positive neurons in ONBL. E, Quantification of Islet-1 positive cells in the ONBL: a, Index values from Islet-1 cells in ONBL divided by the counting area; b, Area ratio of INBL/NBL (total neuroblast layer). Students’ t-test was used to obtain P values from the comparison of paired groups of sections between wild type and mutant. F-I, Quantitative analysis of neurogenesis area in wild type and mutant retina at E13.5 based on Tuj1 staining. F, H, Two representative E13.5 retinal sections at consecutive levels of both wild type and mutant (sectioning orientation shown in J) stained with Tuj1 showing advanced neurogenesis in the mutant retina (AA, advancing area; and CA, central area). AA of the mutant retina expanded more than wild type. G, I, Pie chart expression of retinal neurogenesis in comparison of the wild type and the mutant based on Tuj1 staining. Retina sections were segmented using inner retina surface curvature as reference. The segmentation points (G, drawn in green) are the angular vertex of the smallest angle formed by the crossing of lines tangential to the inner retinal surface, where the biggest curvature occurs. A segmentation line was then drawn from a segmental point parallel to the direction of a nearest Tuj1- positive neuroblast. The line is roughly vertical to the tangential outer retinal surface (short green lines).
segmental lines are roughly the boundary between the central (CA) and advancing differentiating areas (AA). The outer boundary of AA area is defined by Tuj1-positive INBL peripheral boundary (red lines in G). A line is drawn following INBL peripheral boundary parallel to the nearest Tuj1-positive neuroblast as AA border. Area counting was conducted using MetaMorph software. Students t-test was used to obtain P values. There are ~7% more Tuj1 positive area in the mutant retina in the sampled region of E13.5 retina. J, Schematic description of sectioning and sampling of retinal tissues. Total 9 (n=3X3) sections from 3 animals were subjected to Tuj1 staining and thereafter statistical analysis.

Figure 4. Increased BrdU-labeled postmitotic neuronal precursors, and cell death during early retinogenesis of Fz5−/−;Fz8+/− mutant retina

A, Ki67 expression in proliferating retinal progenitors of mutant retina is grossly normal at E13.5. B, Phospho-Histone3 (pH3) labeled dividing cells at the apical surface of the E15.5 wild type and mutant retina. Enlarged views of cornered areas in B are shown below with arrowheads indicating dividing cells. C, Schematic pH3 positive cells’ counting and analysis. The pH3 positive cells are normalized to the circumferential length (arbitrary length unit detected by MetaMorph imaging software). No statistical difference is detected between the wild type and the mutant retina (p=0.46, sectioning and sampling is described in Fig. 3J). D, BrdU was injected at E13 and analyzed 60 hours later, which is around E15.5, by immunohistochemistry (IHC). Bright BrdU+ cells were considered as newly postmitotic since they likely exit cell cycle soon after the BrdU injection. BrdU incorporation in proliferating cells would get diluted within few cell cycles.
Increased BrdU retention in apical cells (arrowheads), presumably early born cones, in ONBL neuroblast and INBL cells indicate an early cell cycle exit of mutant retinal progenitors. BrdU labeling below INBL are retinal embryonic vasculature cells (asterisk). E, Quantitative analysis of BrdU retention in both INBL and apical retina (AR). In these areas, only cells that retained nuclear BrdU-labeling (arrowheads), which could be easily defined by shape and labeling strength were counted as the postmitotic cells at the moment BrdU is pulsed. BrdU retention in ONBL in the mutant retina, though qualitatively more, is more diffused, which probably reflect many progenitors went through additional rounds of cell cycles causing more dilution of BrdU. Therefore, these cells were excluded from counting. A significant increase of BrdU positive cells was detected in both INBL (P<0.001) and AR (P<0.01) by Student's t-Test in the mutant retina. F-H, Grossly normal expression of Ki67 at E17.5 (green) in the mutant retina with an increased cell death primarily in the INBL (red, cleaved form of caspase-3). F, Dorsal half a retinal section from the central region; G, ventral half of a retinal section from the central region; H, Boxed area in G showing apoptotic cells in the INBL. There are barely detectable apoptotic cells in the INBL in the wild-type retina at this age (0.75 cells/section), but more in the mutants (26.7 cells/section). Retinal sections from 3 independent animals of each genotype were counted for caspase-3 positive cells. Note the strong yellow staining on each section adjacent to nerve fiber layer is non-specific staining.

Figure 5. Retinal apical junction is perturbed in developing Fz5−/−;Fz8+/− mutant retina.
A, Mislocalization of β-catenin in the mutant retina. β-catenin localization is enriched on the apical retina (left panel, solid bracket), but became more homogenous in the mutant retina. The DAPI stained apical nuclei in the wild type are well topped by β-catenin enriched apical cytoplasm, while they are seemingly more naked in the mutant (dashed bracket). B, In the wild-type E15.5 retina, phalloidin stained F-actins are enriched on the apical retinal surface (bracket, left panel), while in the mutant retina less staining is seen (right panel). C, Reduction and discontinuity of RhoA enrichment on apical surface of the Fz5/-;Fz8+/- mutant retina (dashed bracket). D, Discontinuity of aPKC on the apical junction of the mutant retina (compare regions above solid and dashed brackets in the mutant retina). E, Discontinuity of N-cadherin staining at the mutant retinal apical junctions. Ectopic N-cadherin staining is also sometimes seen in the subapical area in the mutant retina (arrow in right panel), but not in wild type. F, G, Western blots using whole retina and brain tissue extracts of E15.5 embryos showing significant down-regulation of RhoA and β-catenin at protein level. w, wild type; m, mutant. Retinal sections were cut through the central-ventral area horizontally, where the mutant retinal phenotype is readily obvious under the microscope. Each panel represent 5 projected images at 1µm step. All mutant retinal sections are shown in right panels.

Figure 6. Cell-autonomous neuroblast retraction and ectopic retinal progenitor divisions upon blocking Frizzled receptors in vitro.

A-B, Mosaic mutant analysis shows single or clustered mutant neuroblasts labeled by alkaline phosphatase (AP) reporter. A, An overview of AP-labeled isolated or clustered neuroblasts in E13 retina by pharmacological delivery of 4HT (see materials and
methods). Left panel: wild type neuroblasts; Right panel: mutant neuroblast. Boxed regions are shown in B. Le: lens. B, boxed areas in A show the detached neuroblasts from apical surface of the mutant retina (brackets, right panel). C-D, γ-Tubulin labeled centrosomes on the apical surface of E13.5 and E15.5 retina. In Fz5⁻/⁻;Fz8⁺/⁻ retina (right panels), more centrioles were delocalized to the sub-apical area (short lines), probably because neuroblasts defects were occurring at the apical ends. E-F, Representative retinal explants’ sections stained for γ-Tubulin and pH3. In control (E, F, left panels), most of the dividing cells (pH3 positive) are restricted to the apical surface, while they are ectopic and basally shifted in Fz8Ig-CRD treated retinal explants (right panels). γ-Tubulin staining (red) lined up the apical surface of both control and treated retinal explants. G, a) Increased total pH3 dividing cells in Fz8Ig-CRD treated retinas. pH3 positive cells are counted as an average of 9 sections from 3 retina explants (3 sections from each retina) Students’ t-test obtained a P-value of 0.042. b) Percentage of apical localized pH3 cells calculated from a). About 68% of pH3-positive cells localized apically in Fz8Ig-CRD treated explants, while in the controls, this number is 93% (P<0.01).

**Figure 7. Altered gene expression of signaling pathways.**

A-B, In situ hybridization at E13.5 shows that although Notch1 expression is essentially unaffected (A), its downstream effector, Hes1 is grossly reduced through the mutant retina (B). The dashed lines demarcated the boundary between INBL, ONBL, and persistent fetal vasculature tissue (PFV). C, q-PCR detection of gene expression involving Notch and Shh signaling and apicobasal polarity. Consistent with *in situ* hybridization, Hes1 expression is downregulated in the mutant retina about 3.5 fold
There is a moderate upregulation of $RhoA$ expression ($P=0.02$) and unchanged $\beta$–catenin expression in the mutant retina. The expression of Shh downstream effectors, Gli2 and Gli3, was not altered. Y-axis: fold change of gene expression in mutant retina with respect to control. Positive values indicate upregulation, while negative values indicate downregulation. Value of either ‘-1’ or ‘1’ indicates no changes. There are no values between -1 and 1 since the expression ratios less than 1 were treated with negative reciprocal, and plotted along the downregulation direction.

A schematic model for $Fz5$ and $Fz8$ functions during retinal neurogenesis. During the retinal neurogenesis, $Fz8$ and $Fz5$ are critical for neuroblast apical junction maintainance through a set of apical complex proteins, which include $RhoA$ and $\beta$–catenin. This is important for the neuroblast polarity and organization. In $Fz5^{-/-};Fz8^{+/-}$ mutant retina, the retinal apical deficiency subsequently affect Notch and/or Shh signaling components, of which Hes-1 is an example. This in turn pushes more progenitors to exit cell cycle. In such, the size of proliferating progenitor pool in the mutant retina becomes smaller (see schematic pie charts), which is likely the cause of microphthalmia and retinal coloboma.
Abbreviations

CMZ: Ciliary Marginal Zone
RPE: Retinal Pigment Epithelium
PCP: Planar Cell Polarity
PFV: Persistent Fetal Vasculature
PMZ: Proliferating Marginal Zone
ONL: Outer Nuclear Layer
INL: Inner Nuclear Layer
PHPV: Persistent Hyperplastic Primary Vitreous
INBL: Inner Neuroblast Layer
ONBL: Outer Neuroblast Layer
NBL: Neuroblast Layer
Fz8Ig-CRD: Fz8Ig Cystine Rich Domain
IHC: Immunohistochemistry
OPL: Outer Plexiform Layer
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7