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The Norrin/Frizzled4 signaling pathway in retinal vascular development and disease

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Abstract

Disorders of retinal vascular growth and function are responsible for vision loss in a variety of diseases, including diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, and retinal artery or vein occlusion. Over the past decade, a new signaling pathway that controls retinal vascular development has emerged from the study of inherited disorders - in both humans and mice - that are characterized by retinal hypovascularization. This pathway utilizes a glial-derived extracellular ligand, Norrin, that acts on a transmembrane receptor, Frizzled4, a coreceptor, Lrp5, and an auxiliary membrane protein, Tspan12, on the surface of developing endothelial cells. The resulting signal controls a transcriptional program that regulates endothelial growth and maturation. It will be of great interest to determine whether modulating this pathway could represent a therapeutic approach to human retinal vascular disease.

The retinal vasculature

The vertebrate retina is a thin layer of neural tissue that lines the back of the eye. It is responsible for sensing visual stimuli, and it is the first station for visual information processing (Box 1). The vasculature that supplies the retina has been an object of intense interest for over a century both because of its involvement in retinal disease and because, with the aid of an ophthalmoscope, it can be observed directly. With its relatively flat architecture and stereotyped development, the retinal vasculature is also one of the most experimentally attractive systems for visualizing and investigating basic mechanisms of vascular growth and differentiation.

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The retina is highly active metabolically: per gram, it has the highest oxygen consumption rate of any organ in the body (Figure 1a)[1]. Photoreceptors consume the vast majority of this oxygen. In contrast to most sensory neurons, which increase ionic currents and neurotransmitter release in response to their preferred stimuli, photoreceptors have a high basal ion flux that decreases in response to light [2]. As a result, a large amount of metabolic energy is consumed by the photoreceptor Na/K ATPase to maintain plasma membrane Na⁺ and K⁺ gradients. To support its high metabolic activity, the retina is served by two separate vascular beds: the choroidal vasculature, which resides immediately beyond the RPE, and the retinal vasculature (Figure 1b and c). The choroidal vasculature supports the RPE and photoreceptors, and is characterized by high flow and low oxygen extraction. The retinal vasculature supports the inner retina, and is characterized by low flow and high oxygen extraction [3]. The retinal vasculature appears to be essential for the oxygenation of relatively thick retinas. A few mammals, such as guinea pigs, have relatively thin retinas that are avascular and rely completely on the diffusion of oxygen and nutrients from the choroidal circulation [4]. Both the choroidal and retinal vasculatures are likely to be operating close to the limits imposed by the demands of the cells that they serve: direct measurements using oxygen-sensitive microelectrodes show a resting tissue pO₂ of only 5-10 mm of mercury throughout the dark-adapted cat retina compared to an arterial pO₂ of ~100 mm of mercury (Figure 1b)[5].

The retinal vasculature has a stereotyped architecture composed of three parallel planar vascular plexuses, with the primary arteries and veins residing on the vitreal (inner) surface of the retina, and a pair of intraretinal capillary beds flanking the INL (Figure 1c). The primary arteries and veins are oriented radially, from optic disc to retinal periphery. Among mammals, the primate retina further evolved to have a central avascular zone centered on the fovea, the region that mediates high acuity vision. This avascularity minimizes light scattering that would otherwise degrade the image.

Retinal vascular development

In humans and other large mammals, the retinal vasculature is formed mainly by angiogenesis (formation of new vasculature from a pre-existing vascular network), with vasculogenesis (*de novo* differentiation and assembly of the vasculature from endothelial cell precursors) making a partial contribution to the formation of the superficial layer [6]. In rodents, the retinal vasculature is formed entirely via angiogenesis. In keeping with its distinctive architecture, the retinal vasculature develops in a highly stereotyped manner (Figure 2a-c) [7,8]. The retina initially develops avascularly, and obtains nutrients and oxygen from the choroidal vasculature and the hyaloid vasculature, a transient vascular bed between the lens and the retina. As the retina expands and thickens, diffusion from these two circulations falls short in meeting its demands.

In the mouse, as in other mammals, including humans, retinal vascularization begins with endothelial sprouts from the optic nerve head. These sprouts expand centrifugally along the vitreal surface of the retina, giving rise to a primary vascular plexus that extends to the retinal periphery. This phase of retinal vascularization is accomplished just before birth in humans but spans the first postnatal week in mice. The superficial plexus then gives rise to vertical sprouts that penetrate into the retina, forming the two intraretinal capillary beds, with the outer layer established first. In mice, the retinal vasculature is essentially mature by postnatal day (P) 14, roughly the time of eye-opening, and is accompanied by concomitant regression of the hyaloid vasculature (Figure 2a-c).

The precise patterning of the retinal vasculature is controlled by inductive signals and guidance cues from the surrounding environment. As is generally the case in vascular development, vascular endothelial growth factor (VEGF) serves as a critical regulator. Tissue hypoxia

induces a peripheral-to-central VEGF gradient that is sensed by the tip cells, specialized endothelial cells (ECs) at the front of the growing vasculature, that send out numerous filopodia similar to those on axonal growth cones [9] (Figure 2d). Tip cells migrate along a pre-existing astrocyte network and are trailed by stalk cells that respond to VEGF by proliferating (Figure 2e). Delta-like 4, a Notch receptor ligand, regulates tip cell identity, and is needed for proper retinal vascularization [10]. In addition to astrocytes, retinal ganglion cells (RGCs) also play a role during retinal vascularization. One recent study indicates that signaling in RGCs via GPR91, a receptor for extracellular succinate, induces vascular sprouting, thus coupling vascularization to tissue metabolism [11].

Like the radial expansion of the vascular plexus on the retinal surface, vertical sprouts that penetrate into the retina are also composed of navigating tip cells and trailing stalk cells. At present, the cellular and molecular mechanisms that control lamination of the intraretinal capillary beds remain elusive. The overlap in timing with the formation of precisely laminated neuronal arbors in the outer plexiform layer (OPL) and inner plexiform layer (IPL) suggests that neuronal and vascular lamination could share some of the same molecular guidance cues.

Once in place, the primary vascular plexus undergoes maturation, a process that includes specification of arteries, veins, and capillaries, pruning of the nascent network, mural cell (MC) recruitment, and formation of the blood-retina barrier (BRB). Among the maturation processes, recruitment of MCs - vascular smooth muscle cells and pericytes - is of great importance to the differentiation and stabilization of the newly formed vessels. In the mature retina, the ratio of MCs to ECs is approximately 1:1, making it the tissue with the highest relative density of MCs [12]. This extensive EC-MC interaction is largely mediated by platelet-derived growth factor signaling from ECs to MCs. EC-MC interactions continue to contribute to the maintenance of vascular quiescence and the BRB in adulthood. Loss of MCs is the first sign of vascular changes in diabetic retinopathy, and is correlated with neovascularization [13].

Clinical characteristics of inherited retinal vascular diseases

Disruption of retinal vascular development owing either to genetic or nongenetic causes can lead to severe visual dysfunction. Tissue hypoxia is the initial consequence of retinal hypovascularization. In response, hypoxia-induced VEGF promotes vascular sprouting, leading to the growth of new blood vessels (neovascularization), and an increase in vascular permeability, leading to BRB breakdown [7,14]. Neovascularization eventually leads to intraocular hemorrhage, scarring, and retraction of the retina.

Norrie disease (ND; gene name, *Norrie Disease Protein* or *NDP*; MIM #310600), a severe retinal hypovascularization disease, was first described in 1927, and later shown to be an X-linked recessive trait [15]. The disease is apparent soon after birth and generally leads to blindness. Most affected individuals develop bilateral retinal scarring and retraction, accompanied by persistence of the hyaloid vasculature. The onset of these abnormalities is probably later than gestational week 11, as histological analysis of the retina from an 11-week-old fetus that carries an *NDP* mutation revealed normal development of the retina [16]. In addition to the ocular phenotypes, many ND patients exhibit some degree of mental retardation and/or develop sensorineural hearing loss later in life.

Retinal hypovascularization is also observed in patients with Osteoporosis-Pseudoglioma Syndrome (OPPG; MIM #259770) and Familial Exudative Vitreoretinopathy (FEVR; MIM #133780). OPPG is a rare autosomal recessive disorder, with ocular symptoms that resemble those of ND [17]. "Pseudoglioma" refers to the appearance of intraocular scar tissue. By contrast, in FEVR, retinal hypovascularization is generally restricted to the peripheral retina. Disease progression is often gradual, and includes vitreoretinal hemorrhage, persistence of

hyaloid vasculature, and retinal folds, tears, or detachment (Figure 3) [18,19]. In contrast to ND and OPPG, FEVR is genetically heterogeneous, and can be inherited in autosomal dominant (adFEVR), autosomal recessive (arFEVR) and X-linked recessive modes. In accordance with its broad genetic spectrum, FEVR symptoms are highly variable, ranging from no visual impairment to severe vision loss.

In addition to these rare genetic disorders, abnormal retinal vascular development is also seen in retinopathy of prematurity (ROP). A leading cause of childhood blindness, ROP affects about 14,000-16,000 infants each year in the United States, and blinds about 400-600 of them (<http://www.nei.nih.gov/health/rop/rop.asp#2>). It is caused by exposure to high oxygen, a necessary treatment for preterm infants with immature lungs. The elevated oxygen level disrupts the physiological hypoxia that normally drives retinal vascular development. The resulting retinal hypovascularization leads to tissue hypoxia and VEGF overproduction when the infant is later moved to atmospheric oxygen, a response that induces neovascularization and its sequelae, as described above. Although ROP is generally nongenetic, a small fraction of ROP cases are associated with *NDP* mutations, suggesting a genetic contribution to ROP susceptibility and/or progression [20].

ROP can be readily induced in laboratory animals, including mice, by raising the pups in a high oxygen environment during the second week of retinal vascular development. The disorganized growth of new retinal vessels that follows transfer to atmospheric oxygen has been used not only as an ROP model but also as a surrogate model for the neovascularization that accompanies diabetic retinopathy [7,8,14].

Norrin/Fz4 signaling in retinal vascular disease

The X-linked *NDP* gene was isolated by positional cloning in 1992. It encodes a small secreted protein, Norrin, that is predicted to form a homodimer and adopt a cysteine-knot structure as seen in the transforming growth factor- β protein family. Over 100 different *NDP* mutations have been identified, including deletions and point mutations [15,21]. In addition to causing ND, *NDP* mutations have been identified in patients with X-linked FEVR.

Two autosomal FEVR genes were identified in 2004: the Frizzled-4 (*FZD4/Fz4*) gene, mutated in adFEVR, and the Low-density lipoprotein receptor-related protein 5 (*LRP5*) gene, mutated in adFEVR, arFEVR and OPPG [21-26]. Both genes are key players in the evolutionarily conserved canonical Wnt signaling pathway (Box 2). Fz4 is a member of the Frizzled family of proteins, which serve as the principal receptors for the Wnt family of glycoprotein ligands. Lrp5 and its close relative Lrp6 serve as the Wnt coreceptor (Figure 4a).

The involvement of Norrin, FZD4, and LRP5 in related genetic diseases suggests a connection between Norrin and the canonical Wnt pathway. This idea is further supported by the observation that mice carrying a loss-of-function mutation (a “knockout” or “KO”) in any of these three genes have strikingly similar retinal vascular defects: the two intraretinal capillary beds that flank the INL are absent, the primary arteries and veins are tortuous and dilated, intraocular hemorrhage is common, and regression of the hyaloid vessels is delayed (Figure 4b and c) [27-31].

These data are explained by the observation that Norrin functions as a Fz4 ligand, despite its complete lack of sequence homology with the Wnt family of Frizzled ligands [28]. Norrin binds to the Fz4 receptor with nanomolar affinity and potently activates the canonical Wnt pathway when cotransfected with Fz4 and Lrp coreceptor into reporter cell lines. The interaction between Norrin and Fz4 is highly specific: among the ten mammalian Frizzled receptors, only Fz4 binds Norrin [32]. Norrin-Fz4 binding occurs within the extracellular amino-terminal cysteine-rich domain of Fz4, a compact domain that is also the site of Wnt

binding. Norrin does not detectably bind Lrp5, and there does not appear to be any preference for Lrp5 over Lrp6 in the canonical signaling assay in cell culture.

Recently, a new component of the Norrin/Fz4/Lrp5 pathway, Tetraspanin-12 (Tspan12), was identified by screening a large collection of knockout mouse lines for phenotypes that resemble the retinal vascular defects of *Ndp*, *Fz4*, and *Lrp5* mutant mice [33]. Tspan12 is one of approximately 35 mammalian members of the ancient tetraspanin protein family, so named because each member possesses four transmembrane segments. Members of this family participate in diverse cellular processes, and several of them can organize microdomains in the plasma membrane [34]. *Tspan12* interacts genetically with *Ndp* and *Lrp5* in mice, as transheterozygotes of *Tspan12* and either *Ndp* or *Lrp5* exhibit a modest retinal vascular defect. Moreover, Tspan12 binds to Fz4 and enhances Norrin/Fz4/Lrp5 signaling, perhaps by inducing receptor clustering. The importance of these findings is highlighted by the discovery of *TSPAN12* mutations in adFEVR patients [35,36]. For brevity, we will refer hereafter to Norrin/Fz4/Lrp5/Tspan12 signaling simply as Norrin/Fz4 signaling.

As additional mutations have been identified in patients with FEVR, ND, ROP, or the related syndrome of persistent fetal (*i.e.* vitreous) vasculature, it is becoming clear that disease severity varies substantially between subjects with different mutations in the same gene and even between subjects with the same mutation [21,26,37]. The latter variability presumably reflects differences in genetic background. As expected, disease severity also depends on whether one or both copies of the gene are mutated [38]. The observation that heterozygosity for presumptive loss-of-function *TSPAN12*, *FZD4*, or *LRP5* mutations cause retinal hypovascularization in adFEVR indicates that this signaling pathway is highly sensitive to gene dosage. Additionally, some *FZD4* alleles might act in a dominant negative fashion [39].

Norrin/Fz4 signaling in retinal vascular development

The Norrin/Fz4 pathway functions throughout the course of retinal vascular development [27-31,33,40]. Loss of pathway function in mice results in retarded and disorganized centrifugal vascular growth. The two intraretinal capillary beds are even more severely affected: in *Fz4*, *Ndp*, *Lrp5*, or *Tspan12* mutant mice, the vertical sprouts that give rise to these vessels are stunted, developing as ball-like clusters of ECs in the IPL. Despite the widespread expression of *Fz4* in both neuronal and vascular cells of the retina, these defects reflect a requirement for Norrin/Fz4 signaling specifically in ECs, as determined by eliminating *Fz4* from ECs using EC-specific Cre-LoxP gene deletion [31]. Moreover, EC-specific *Fz4* KO mice display the same severe visual dysfunction as *Fz4* and *Ndp* KO animals, as judged by behavioral and electrophysiology assays. The conclusion that this pathway is primarily required in ECs is further strengthened by the observations that (i) in the retina, Tspan12 is specifically expressed by ECs [33], and (ii) wild-type (WT) but not *Fz4* mutant retinal ECs (RECs) can form capillary-like structures when cultured on Matrigel [31]. These data indicate that the primary defects in the orthologous human retinal vascular diseases - ND, OPPG, and FEVR - arise in the vasculature and that pathologic changes in retinal structure and function are secondary consequences of vascular defects.

In the mouse, close examination of the retinal vasculature following Cre-LoxP-mediated deletion of *Fz4* from a subset of RECs at the onset of retinal vascular development has revealed additional interesting features of this pathway [31]. In zones populated exclusively by mutant RECs, there are aborted intraretinal sprouts terminating in clusters of RECs, as is seen in the *Fz4* KO retina. In regions with a mixture of WT and *Fz4* KO RECs, most of the mutant cells are restricted to the vitreal face of the retina and are under-represented in the intraretinal vascular plexus. Thus, the defects exhibited by *Fz4* mutant RECs in mosaic retinas closely resemble those seen in mice with ubiquitous or panendothelial deletion of *Fz4*, and this

resemblance holds even when the RECs populate only a small sector of the retinal vascular tree. These observations indicate that the Norrin/Fz4 pathway acts locally, thus ruling out alternate models in which suppression of intraretinal vascular growth is secondary to a panretinal influence such as excessive oxygenation from an enlarged fetal vasculature. Although Norrin/Fz4 signaling appears to function in a largely cell-autonomous manner in the developing retina, when *Fz4* KO RECs are studied in matrigel cultures, they are able to interact with neighboring WT RECs (but not with themselves) to form capillarylike networks, suggesting that the mutant RECs can respond to some of the “social” interactions that are part of network formation. In addition to promoting vascular growth, the Norrin/Fz4 pathway also contributes to vascular stabilization and maturation by promoting the establishment and maintenance EC-MC interactions, especially along the venules and capillaries. This function requires pathway activation in both RECs and MCs.

Quantitative assessment of the vascular phenotype in *Fz4* and *Ndp* KO retinas reveals indistinguishable defects, implying that, in the context of retinal vascular development, Norrin is likely to be the principal Fz4 ligand, and Fz4 is likely to be the principal Norrin receptor. In contrast, *Lrp5* KO mice consistently manifest a milder retinal vascular phenotype, with the occasional formation of small capillary networks in the INL. This suggests partial compensation from the closely related coreceptor *Lrp6*.

A central question in Norrin/Fz4 signaling is the source of the Norrin ligand and its spatial distribution. Wingless, the best-studied Wnt family member, functions in *Drosophila* as a morphogen, and the Wingless concentration gradient is critical for its role in tissue patterning [41]. By contrast, Norrin is produced by Muller glia in the developing retina with no apparent spatial gradient, as revealed by the expression pattern of an alkaline phosphatase reporter gene knocked-in at the *Ndp* locus [31]. Moreover, high-level production of Norrin within the lens, using a lens-specific Norrin transgene, fully rescues the retinal vascular defects in *Ndp* KO mice, presumably by bathing all intraocular tissues - including the retina - with Norrin [42]. Taken together, these observations indicate that (i) Norrin does not form a spatial concentration gradient, and (ii) retinal vascular development simply requires the presence of sufficient quantities of Norrin throughout the retina to activate canonical signaling via Fz4, *Lrp5*, and *Tspan12*. Thus, Norrin does not act as a morphogen, but instead activates a competence program in RECs and MCs.

What are the molecular components of this developmental competence program? Although much work remains to be done, initial microarray-based transcription profiling has revealed an abundance of changes in several hundred transcripts in cultured WT versus *Fz4* KO RECs [31]. Among these transcripts, the transcription factor *Sox17* is consistently upregulated by Norrin/Fz4 signaling in several different contexts. *Sox17* is part of the Sox F family, members of which play important roles in embryonic angiogenesis and lymphangiogenesis [43-46]. Interestingly, restoration of *Sox17* expression restores the ability of *Fz4* KO RECs to form capillary-like networks in Matrigel. Although these cell culture experiments implicate *Sox17* as a mediator of the Norrin/Fz4 signal, the extent to which *Sox17* regulates retinal vascularization *in vivo* remains to be determined.

Frizzled signaling and vascular development beyond the retina

As Norrin/Fz4 signaling appears to regulate the developmental competence of RECs, it is of interest to ask whether this pathway or related ones function in angiogenesis beyond the retina. Interestingly, mouse genetic studies have revealed a prominent role for Wnt signaling in angiogenesis and blood-brain barrier (BBB) development in the embryonic brain and spinal cord [47,48]. Simultaneous deletion of *Wnt7a* and *Wnt7b* in mice disrupts vascular development throughout the embryonic central nervous system (CNS). Furthermore, neural

epithelium-specific deletion of *Wnt7a* and *Wnt7b* or EC-specific deletion of β -catenin phenocopies the *Wnt7a/Wnt7b* double KO phenotype, indicating that neural epithelium-derived *Wnt7a/Wnt7b* activates the canonical Wnt pathway in ECs to control CNS vascular development. Intriguingly, in these *Wnt7a/Wnt7b* and β -catenin KO embryos, the endothelial sprouts invading the CNS parenchyma fail to elaborate capillary structures, instead terminating in disorganized EC clusters. These EC clusters are reminiscent of the abortive intraretinal vascular sprouts seen in *Ndp*, *Fz4*, *Lrp5*, and *Tspan12* KO retinas.

Another link between Frizzled signaling and the BBB comes from the observation that panendothelial deletion of *Fz4* in mice causes BBB breakdown in the cerebellum, which leads to progressive neuronal degeneration and ataxia [31,49]. This phenotype is not seen in *Ndp* KO mice, and is therefore presumed to reflect the activation of *Fz4* by one or more of the many Wnts expressed in the brain. Consistent with these observations, canonical Wnt signaling can induce BBB features in cultured mouse brain ECs [50]. Taken together, these data indicate that the retinal and embryonic CNS vasculatures use a variety of ligand-receptor combinations to activate similar competence programs via the canonical Wnt pathway.

In mice, *Fz4*, as well as other Frizzled receptors, are widely expressed in the embryonic and adult vasculature, and Wnt and Norrin signaling play a role in vascular development outside of the CNS. In particular, *Fz5*, *Wnt2*, and Norrin are required in placental angiogenesis [51-53], and Norrin and *Fz4* are required for the maintenance of the capillaries in the stria vascularis of the inner ear, the rich vascular plexus that supplies the central chamber of the cochlea [27,28]. *Fz4* is also required for corpora lutea formation and female fertility, but it is not known whether this defect involves the vasculature [54]. Excessive activation of *Fz4* during embryonic angiogenesis disrupts vascular remodeling throughout the embryo and yolk sac, perhaps by overstabilizing the nascent vascular network [31].

In humans, ND and OPPG also exhibit phenotypes that point to Wnt and/or Norrin signaling beyond the retina. As its name implies, OPPG is characterized by osteoporosis. In addition to the *LRP5* loss-of-function mutations found in OPPG, *LRP5* gain-of-function mutations have been found in individuals with unusually strong bones [55]. Thus, both gain and loss of *Lrp5* function affect bone development. The progressive sensorineural hearing loss associated with ND likely arises from the same degeneration of the stria vascularis seen in *Ndp* and *Fz4* KO mice. Less well understood is an association between *NDP* mutations and peripheral venous insufficiency [56,57]. Taken together, these extraocular defects suggest additional roles for Wnt and/or Norrin signaling relevant to human health and disease.

Future Prospects

The Norrin/*Fz4* pathway is a recent addition to a growing list of angiogenic signaling pathways. Its biological importance is clear from the hypovascularization phenotypes of humans and mice with inherited deficiencies in its various components, but its larger clinical implications are still largely unexplored. As the study of the Norrin/*Fz4* pathway is still in its infancy, many questions remain unanswered. What is the structural basis of *Fz4* binding by Norrin, of signal transduction by the *Lrp5* coreceptor, and of signal enhancement by *Tspan12*? How is this pathway regulated? Are there additional extracellular or intracellular proteins involved? How does pathway activation regulate MC function? How does it regulate the BBB? Finally, a question that is of both theoretical and practical importance: is this pathway directly involved in pathological neovascularization in the adult retina? A recent study indicates a role for Norrin in neovascularization in a mouse model of ROP [58]. If these results apply to humans and generalize to other diseases associated with neovascularization, then the Norrin/*Fz4* pathway could be an attractive target for drug treatments aimed at inhibiting the neovascularization of diabetic retinopathy, age-related macular degeneration, or ROP. Importantly, the chimeric

vasculature experiments in the *Fz4* conditional KO mouse suggest that the Norrin/*Fz4* pathway is dispensable in the mature retina, implying that pharmacologic blockade of the Norrin/*Fz4* pathway might have minimal side effects. Currently, the most prevalent treatments for retinal neovascular diseases are intraocular injection of anti-VEGF antibodies and photocoagulation or photodynamic therapy (*i.e.* physically destroying abnormal vessels with intense laser light). Neither therapy is optimal. Addressing the questions outlined above will deepen our understanding of vascular development, and could pave the way for the development of new therapeutic approaches to vascular disease.

Box 1. The Retina

The retina is composed of five major classes of neurons (photoreceptor, horizontal, bipolar, amacrine, and ganglion cells) and two type of glia (Muller cells and astrocytes). Retinal neurons and Muller glia are generated from a monolayer of proliferating neuroepithelial cells that resembles the ventricular zone of the developing brain. During embryonic development, the retina develops as an outgrowth from the brain, and the stalk that connects them becomes the optic nerve.

The nuclei of retinal cells are organized into three layers. Photoreceptors lie in the outermost retinal layer (the layer furthest from the cornea), and their nuclei are densely packed in the outer nuclear layer (ONL). They are primary sensory neurons and they are activated directly by light. Each photoreceptor extends a specialized cilium, the outer segment, beyond the ONL. The outer segment is a membrane-rich structure that harbors the light-capturing visual pigment molecules and the signal transduction machinery. The tips of the outer segments abut the retinal pigment epithelium (RPE), a specialized monolayer of cells that performs a variety of functions including recycling of the visual pigment chromophore and engulfing and degrading the distal tips of the outer segments. Visual information received by photoreceptors is relayed to bipolar cells, which have their cell bodies in the inner nuclear layer (INL). The INL is flanked by two synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL). The cell bodies of the output neurons of the retina, the retinal ganglion cells (RGCs), reside in the innermost layer of the retina and their axons travel radially along the vitreal surface of the retina in the nerve fiber layer (NFL). These axons converge on the optic disc, exit the retina and project to the brain via the optic nerve. In addition to bipolar cells, the INL includes the cell bodies of two other types of interneurons that participate in visual information processing, the horizontal cells and amacrine cells, as well as the Muller glia. The radial processes of the Muller glia span the full thickness of the retina. For more background on the vertebrate retina see <http://webvision.med.utah.edu/>.

Box 2. The canonical Wnt signaling pathway

Wnts are secreted glycoproteins that act on several distinct families of cell surface receptors, including the Frizzled family of receptors. In the best-characterized Wnt-Frizzled signaling pathway, referred to as the canonical Wnt pathway, a complex of Wnt ligand, Frizzled receptor, and Lrp co-receptor produces an intracellular signal that inhibits the degradation of cytosolic β -catenin. The increase in cytosolic β -catenin leads to an increase in nuclear β -catenin, which forms a complex with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors. The β -catenin-LEF/TCF complex binds to target genes and activates their transcription.

The canonical Wnt pathway is found in both vertebrates and invertebrates, and it has been implicated in regulating cell proliferation and differentiation in a wide variety of contexts. Dysregulation of canonical Wnt signaling is one of the most common defects in human

cancers, with oncogenic loss-of-function mutations commonly observed in the adenomatous polyposis coli (APC) protein, a large protein required for β -catenin degradation. Less commonly, oncogenic gain-of-function mutations are found in β -catenin that render it resistant to degradation. Although large gene families code for Wnt ligands and Frizzled receptors - there are 19 Wnt genes and 10 Frizzled genes in mammals - there is only a single β -catenin gene. How canonical Wnt signaling produces such a wide variety of biological responses in different cell types while funneling the intracellular signal through a single intermediate - β -catenin - is not understood [59]. (For more information on Wnt signaling see [60] and <http://www.Stanford.edu/~rnusse/wntwindow.html>.)

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Figure 1.

Retinal oxygen consumption and the microanatomy of the retinal vasculature. (a) Relative oxygen consumption in the rat, per gram of tissue [1]. (b) Oxygen levels at different depths within the dark adapted cat retina [5]. (c) Left, the human eye in cross-section; right, the anatomy of the retinal and choroidal vasculatures (red). The retinal vasculature spans the GCL and INL; the choroidal vasculature is immediately beyond (to the right of) the RPE.

Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; RPE, retinal pigment epithelium.

Figure 2.

Development of the retinal vasculature. (a-c) Three successive stages in ocular development are shown from left to right. In humans, these stages occur *in utero*; in mice, they occur during the first two postnatal weeks. The retinal vasculature grows out from the optic nerve along the vitreal surface of the retina (a) before sending secondary branches into the retina to form the outer and then the inner layers of capillaries (b and c). Regression of the fetal vasculature within the vitreous and on the posterior surface of the lens (b and c) accompanies development of the retinal vasculature. (d) An endothelial tip cell displays numerous long filopodia; this morphology closely resembles that of an axonal growth cone. (e) In the early postnatal mouse retina, ECs (green) migrate along a network of astrocytes (red) as the developing vasculature advances across the vitreal surface of the retina.

Abbreviations: NBL, neuroblast layer.

Figure 3. Ophthalmoscopic images of the human retina. (a) Fundus photograph of a normal retina showing the origin of the retinal vessels at the optic disc (left) and the dorsal and ventral sweep of the major arteries and veins above and below the fovea (right). (b) Fundus photograph of a retina from a FEVR patient showing a horizontal retinal fold (white arrows).

Figure 4.

Norrin/Fz4 signaling in the retinal vasculature. (a) Proteins at the EC membrane that mediate Norrin/Fz4 signaling. The Lrp5 coreceptor (blue) has a large extracellular domain with four tandem copies of the YWTD-propeller and EGF domain (ovals), three tandem copies of the low density lipoprotein receptor domain (rectangles), a single transmembrane domain, and a cytosolic carboxy-terminal domain. Fz4 (red) has an amino-terminal ligand binding cysteine-rich domain (ball) followed by a GPCR-like integral membrane domain with seven transmembrane segments. Tspan12 (green) has four membrane spanning segments. Norrin (brown) is a disulfide-linked dimer. (b) Defects in retinal vascular development in the absence of Norrin/Fz4 signaling. Cross sections of adult mouse retinas of the indicated genotypes, with

endothelial cells (red) visualized with GS lectin staining and nuclei (blue) visualized with DAPI staining. In the WT retina, the vasculature can be seen at the vitreal surface of the retina, and on either side of the INL. In the two mutant retinas, vertical white arrows show two clusters of ECs that have penetrated partway into the retina from the vitreal surface. (c) Flatmounts of adult WT or *Fz4* KO mouse retinas with the blood vessels visualized by intravascular filling with fluorescein-dextran. The major arteries and veins can be seen emerging from the optic disc in the upper left of each image. Vertical white arrows show two of the many clusters of ECs that have penetrated partway into the retina from the vitreal surface. Abbreviations: CC, choricapillaris, i.e. the choroidal vasculature.