Fzd7 (Frizzled-7) Expressed by Endothelial Cells Controls Blood Vessel Formation Through Wnt/β-Catenin Canonical Signaling

Claire Peghaire, Marie Lise Bats, Raj Sewduth, Sylvie Jeanningros, Beatrice Jaspard, Thierry Couffinhal, Cécile Duplàa, Pascale Dufourcq

Objective—Vessel formation requires precise orchestration of a series of morphometric and molecular events controlled by a multitude of angiogenic factors and morphogens. Wnt/frizzled signaling is required for proper vascular formation. In this study, we investigated the role of the Fzd7 (frizzled-7) receptor in retinal vascular development and its relationship with the Wnt/β-catenin canonical pathway and Notch signaling.

Approach and Results—Using transgenic mice, we demonstrated that Fzd7 is required for postnatal vascular formation. Endothelial cell (EC) deletion of fzd7 (fzd7<sup>−/−</sup>) delayed retinal plexus formation because of an impairment in tip cell phenotype and a decrease in stalk cell proliferation. Dvl (dishevelled) proteins are a main component of Wnt signaling and play a functionally redundant role. We found that Dvl3 depletion in dvl<sup>−/−</sup> mice mimicked the fzd7<sup>−/−</sup> vascular phenotype and demonstrated that Fzd7 acted via β-catenin activation by showing that LiCl treatment rescued impairment in tip and stalk cell phenotypes induced in fzd7 mutants. Deletion of fzd7 or Dvl1/3 induced a strong decrease in Wnt canonical genes and Notch partners’ expression. Genetic and pharmacological rescue strategies demonstrated that Fzd7 acted via β-catenin activation, upstream of Notch signaling to control Dll4 and Jagged1 EC expression.

Conclusions—Fzd7 expressed by EC drives postnatal angiogenesis via activation of Dvl/β-catenin signaling and can control the integrative interaction of Wnt and Notch signaling during postnatal angiogenesis. (Arterioscler Thromb Vasc Biol. 2016;36:2369-2380. DOI: 10.1161/ATVBAHA.116.307926.)

Key Words: cardiovascular diseases ■ endothelial cells ■ frizzled receptor ■ neovascularization ■ transgenic mice

Understanding development and vessel formation in an organism is important in the context of developmental biology, oncology, regenerative medicine, and cardiovascular diseases. A majority of newly extended vessel networks are formed via angiogenesis, a process by which new blood vessels are generated from existing blood vessels.
development, as well as in stem cell renewal and differentiation. Fzd7 is the most commonly upregulated Wnt receptor in a variety of cancers, has been shown to promote cancer development and progression, and has emerged as a target for cancer therapy. In this study, we investigated the role of the Fzd7 receptor in retinal vascular development. We reported that EC deletion of fzd7 delays retinal plexus formation with an impairment in tip cell phenotype and a decrease in stalk cell proliferation. Retinal knockdown of dvl3, in null mutant mice deficient in dvl1, mimicked the defects induced by fzd7 deletion. Pharmacological and genetic rescue strategies demonstrated that Fzd7 controls angiogenesis and contributes to retinal vascular development through the Wnt canonical pathway, acting upstream of Notch signaling via the regulation of Jagged1 and Dll4 expression.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Fzd7 Is Expressed in EC During Retinal Vascular Formation
Using a model of postnatal retinal angiogenesis, we investigated the expression of Fzd7 during blood vessel formation. Analysis of total retinal extract by quantitative polymerase chain reaction (qPCR) showed an increase in fzd7 expression from postnatal day (P) 5 to P15 before returning to baseline levels at P21 (Figure 1A). Temporally, this increase occurred during the period of primary (P5–P7) and deep (P15) vascular plexus formation. To analyze the localization of Fzd7, confocal analysis of P7 retinal sections, stained with CD31 and Fzd7, was performed (Figure 1B and 1C). Fzd7 was observed in CD31+ cells in the primary vascular plexus while its expression was not restricted to EC (Figure 1B and 1C).

EC Deletion of fzd7 Delays Retinal Vascular Formation
To investigate the specific role of fzd7 in EC during retinal vascular development, we generated different transgenic mice: fzd7+ECKO with constitutive (Tie2-Cre) and fzd7−ECKO with inducible (Pdgfb-iCre) EC deletion (Figure IA in the online-only Data Supplement). In fzd7−ECKO mice, we induced recombination by tamoxifen injection at the P1, P2, and P3 postnatal stages. The efficiency of Cre expression in the retina was verified using mtG reporter mice for both lines (Figure IB and IC in the online-only Data Supplement). qPCR of whole retinal extracts showed a reduction of fzd7 expression in fzd7−ECKO and fzd7+ECKO mice, as compared with their respective controls (Figure ID and IE in the online-only Data Supplement). Specific deletion was demonstrated by a decrease of Fzd7 mRNA in CD31+ EC isolated from lung (Figure IF in the online-only Data Supplement) and the absence of EC-specific Fzd7 staining in the mutant retina (Figure IG in the online-only Data Supplement). It is worth noting that both fzd7−ECKO and tamoxifen-treated fzd7+ECKO mice did not differ in weight when compared with their respective controls (Figure IH in the online-only Data Supplement).

The retina is avascular at birth, and outgrowth of the first vascular plexus, the superficial retinal vascular plexus, progresses from the center to the periphery from P1 until P8. Retina from both mutant mice had delayed vascularization in the superficial retinal vascular plexus at P5 and P7, with a reduction in the extent of vascular coverage and radial expansion from the optic nerve to the periphery (Figure 2A through 2C). Because fzd7 was strongly induced in the retina at P15, we next investigated whether secondary and tertiary layers of the retinal vasculature could be formed in fzd7 mutant mice. Interestingly, at P11, the deep capillary plexus was significantly reduced in retina from fzd7−ECKO and fzd7+ECKO mice (Figure 2D), demonstrating that Fzd7 is required for the formation of different vascular plexus. It has been previously proposed that Fzd7 is expressed in myeloid cells. To exclude a possible contribution of these cells to the vascular phenotype in fzd7−/−; Tie2-Cre mice, we conditionally deleted fzd7 in myeloid cells using the M lysozyme-Cre line (LysM-Cre). fzd7−LysM-Cre(+) mice developed a retinal vascular plexus similar to control mice, indicating that myeloid Fzd7 is not required for retinal vascularization (Figure II in the online-only Data Supplement). Collectively, these results support the hypothesis that EC-dependent Fzd7 signaling is critical for retinal vascular development.

fzd7 Deletion in EC Impaired Tip Cell Phenotype and Stalk Cell Proliferation
To better understand vascular formation and remodeling changes associated with an EC-specific fzd7 deletion, we quantified morphometric and angiogenic parameters in fzd7−ECKO mice (Figure 3). Remarkably, at the vascular front, a significant increase in tip cell numbers was observed in fzd7−/−deficient vessels at P7 (Figure 3A). Filopodia mediate endothelial tip cell migration to guide vascular sprout formation and anastomosis. High-resolution analysis of tip cells identified an increase in filopodia protrusions. Moreover, the majority of filopodia (80%) showed an angle profile between 0 and 45° in controls, whereas in retina from fzd7−/−ECKO mice, filopodia were randomly positioned with a wider angle profile (Figure 3A). Finally, the number of branch points was quantified in different zones across the vascular plexus and revealed a significant increase in vascular density in the venous region of retina from fzd7−ECKO mice but no significant modification in the arterial zone (Figure 3D). All these data suggest that tip cell function and the process of vessel sprouting is impaired in fzd7−ECKO mice.

EC proliferation at P7 is observed in stalk cells at the leading edge of growing plexus, in veins and to a lesser extent in arteries. We found significantly reduced proliferation rates of isoleucin B4+ ECs in fzd7−ECKO retina at P7 (Figure 3B) in the stalk cell area, whereas no modification in proliferation was observed in the vein or artery (data not shown). In contrast,
vascular remodeling across the plexus was analyzed using retinas stained with collagen IV and isolectin B4 and did not reveal significant impairment in vessel regression in \textit{fzd7}-deficient vessels (Figure 3C).

**Silencing \textit{dvl3} in \textit{dvl1}^{-/-} Mice Mimicked the Defects Induced by \textit{fzd7} Deletion**

Our group has previously demonstrated that Fzd7 can recruit Dvl1 and 3 at the cell membrane to activate EC signaling\cite{25}; therefore, we sought to compare retina from \textit{dvl1}^{-/-} mice with those from \textit{fzd7}^{iECKO} mice. We observed a significant delay and incomplete outgrowth of the superficial retinal vascular plexus with reduced vascular area (Figure 4A) and radial expansion at P5 (Figure 4B) and P7 (Figure 4C). Moreover, increased tip cell number at the leading edge at P5 and P7 was observed in \textit{dvl1}^{-/-} mice (Figure 4B and 4C). There was no modification in body weight between \textit{dvl1}^{-/-} and \textit{dvl1}^{+/+} mice (Figure IIIA in the online-only Data Supplement). Because \textit{dvl1}^{-/-} retinal phenotype was similar but less severe than that of \textit{fzd7}^{iECKO} mice, we then asked whether this mild effect might be linked to a...
Figure 2. Effects of conditional endothelial cell (EC) deletion of fzd7 during retinal vascular formation. A–D, Representative whole mount retina from fzd7F/F, Tie2-Cre(−) or Cre(+) and fzd7F/F, Pdgfb-iCre(−) or Cre(+) mice stained with CD31 to visualize vessels at postnatal day (P) 5 (A), P7 (B–C), and P11 (D). Quantification of the percentage of vascularization and spreading showed a strong delay in vascular formation in fzd7 mutant mice. Scale bar=400 or 200 μm. n=10 for each genotype. ** P<0.005; *** P<0.0001.
Figure 3. Fzd7 (frizzled-7) deletion impaired tip cell phenotype and stalk cell proliferation. A, Retinal whole mounts of fzd7ECWT and fzd7ECKO mice stained with isolectin B4 (IB4) or CD31 antibody at postnatal day (P) 7. Number of tip cells per 100 μm quantified after IB4 staining at P7 was increased in fzd7 mutants: fzd7ECWT (n=8) vs fzd7ECKO (n=10). Scale bar=100 μm. High magnification of tip cell (Continued)
compensatory redundant Dvl isoform function. We analyzed expression rates of dvl isoforms in dvl1−/− retina and found a significant increase in dvl3 without modification of dvl2 transcripts (Fig IIIB in the online-only Data Supplement), suggesting that Dvl1 and Dvl3 could have redundant functions. To test this, we investigated the vascular phenotype of dvl1−/− mice injected with siRNA targeting dvl3 or control siRNA in the contralateral eye of the same animal. In control mice, we found that si dvl3 injection efficiently reduced the level of dvl3 mRNA (≈60%), whereas no modification in dvl1 or dvl2 was observed (Figure IIIC in the online-only Data Supplement). In dvl1+/− littermates, loss of dvl3 did not modify the vascular area; however, it did significantly increase the number of tip cells (Figure 4C). When dvl3 was silenced in dvl1+/− mice, double deletion significantly delayed the vascular outgrowth and enhanced the increase in tip cell number (Figure 4C). These data demonstrate that dvl1/3 deletion mimicked the vascular phenotype induced by fz7 deletion.

Fzd7 Deletion Decreases Wnt Canonical Target Genes and Notch Partner Expression

We next analyzed whether Fzd7/Dvl could control vascular development via Wnt canonical signaling, as previously reported in cultured EC.13 Quantitative analysis by Western blot showed a decrease of the activated form of β-catenin in lysate from fz7iECKO retina (Figure 5A) and by RT-qPCR a downregulation of Axin2 and Lef1 genes, 2 main Wnt canonical target genes (Figure 5B). Interestingly, we obtained a similar effect in dvl1+− retina extracts on Axin2 and Lef1 gene expression (Figure 5C).

Because Fzd4/LRP5/Norrin pathway has been reported to regulate postnatal angiogenesis via the β-catenin pathway,6,20 we investigated mRNA expression of fzd4, LRP5, and Norrin in total retina. No modification of these transcripts was observed in tissue from fz7iECKO versus fz7iECWT mice (Figure 5D), suggesting that the vascular defect in fz7 mutant is not because of Fzd4/LRP5/Norrin signaling impairment.

The Notch pathway is crucial for vascular development in postnatal retina and has been shown to be regulated by β-catenin transactivation activity in embryonic EC, during tumoral angiogenesis or in non-EC.10,12,30 We, therefore, hypothesized that fz7 deletion may impair Notch signaling. qPCR performed on retina extracts consistently demonstrated that EC deletion of fz7 strongly decreased Notch pathway gene expression (Figure 6A). Expression of Notch ligands, Dll4 and Jag1, were downregulated by >80%, whereas Notch1 and 4, Hey1, and Nrarp transcripts were downregulated by 60% to 70% in fz7iECKO retina (Figure 6A). Impairment in Notch gene expression was confirmed in EC isolated from fz7iECKO or fz7iECWT mouse lung (Figure 6B). Moreover, in fz7-deficient retinal vasculature, we found that EC had significantly lower levels of Dll4 and Jagged1 protein in tip and stalk cells, respectively, as compared with their control littermate (Figure 4VA and IVB in the online-only Data Supplement). Because loss of expression of EphrinB2 and an increase EphB4 were also observed in mice with targeted deletion of Notch signaling partners,31 we next investigated whether Fzd7 signaling could alter the EphrinB2/EphB4 balance. We found a significant decrease in the arterial marker Ephrin-B2 and an increase in the venous marker EphB4 expression at the mRNA (Figure 6C) and protein level (Figure 6D) in fz7iECKO mouse retina.

We found that dvl1 deletion mirrored the effects of fz7 deletion on some Notch pathway genes, decreasing Dll4 and Hey2 transcript expression in dvl1−/− extracts (Figure 6E). Because deletion of Dvl1 was not specific to EC, the direct effect of dvl deletion on gene expression was next examined in cultured EC treated with different siRNA. Depletion of dvl1 or dvl3 induced a significant decrease in Dll4, Notch1, and Hey2 mRNA expression in cultured EC, whereas dvl2 siRNA had no effect (Figure 6F). We noted that this mild impact on Notch genes may be associated with the intermediate phenotype observed in dvl1−/− retina. Cotreatment of EC by dvl1 and dvl3 siRNA had a synergic effect for Dll4 and Hey2 expression (Figure 6F). These results, taken together, suggest that Fzd7 might control angiogenesis by β-catenin signaling acting upstream of Notch signaling.

Fzd7 Controls Blood Vessel Formation via the Wnt-Canonical Pathway

fzd7iECKO and fz7iECKO mice were treated with LiCl, to determine whether this treatment rescued the fz7−/−-induced retinal phenotype. Activation of the Wnt-signaling canonical pathway by LiCl treatment was confirmed by quantification of Lef1 gene expression in control and fz7−/− mutant retina (Figure 7B). LiCl treatment in fz7−/− littermates did not modify tip cell number or vascular plexus spreading while EC proliferation was slightly increased (Figure 7A). In fz7−/− mice, LiCl treatment rescued the vascular outgrowth, impairment of tip and filopodia number, and stalk cell proliferation (Figure 7A). These results demonstrated that activation of the Wnt-canonical pathway completely rescued the fz7−/−-induced phenotype and suggests that Fzd7 activates canonical Wnt signaling via Dvl1/3 to control retinal vascular development.

Fzd7/β-Catenin Signaling Controls the Notch Pathway During Postnatal Angiogenesis

Next, we investigated whether Fzd7 regulates Notch signaling via the Wnt canonical pathway. qPCR of retina extracts showed that GSK3β inhibition by LiCl treatment activated the Notch pathway in fz7iECKO mice as demonstrated by an increase in Dll4, Jag1, and Notch1 receptors Hey2 and Nrarp.
Figure 4. Deletion of dvl1 (dishevelled-1) and knockdown of dvl3 expression mimics the fzd7iECKO phenotype. A and B, Representative retinal whole mount or peripheral retinal flat mounts from dvl1+/+ and dvl1−/− mice stained with CD31 antibody at postnatal day (P) 5. Quantification of the % vascularization (A) and vascular spreading (B) showed a significant delay in vascular plexus formation (Continued)
mRNA expression (Figure 7C). Similarly, LiCl treatment rescued Notch pathway component expression in fz7ΔECXO mice retina to a similar level (Figure 7C). Because LiCl could lead to indirect effects on other cells in the retina, in vitro experiments were performed using an siRNA strategy on MS1 cells.13 In cultured EC (MS1), fz7 silencing decreased expression of Dll4, Jag1, Notch1, Hey2, and Nrarp. LiCl treatment prevented Notch pathway impairment under siRNA fz7 conditions (Figure 7D). As expected, when ECs were infected with lentivirus encoding for active β-catenin, active β-catenin strongly induced mRNA expression of Axin2, a downstream target of β-catenin, and Dll4, Jag1, and Hey2 Notch partners, in control EC (Figure 7D). Interestingly, in cultured fz7 knockout ECs, downregulation of these genes was rescued by activated β-catenin (Figure 7D). These results demonstrate that Fzd7, via β-catenin transcriptional activity, controls the expression of both Dll4 and Jag1 in addition to downstream Notch targets.

We next explored the potential rescue of the fz7 phenotype by Dll4 or Jagged1 activation in vitro because Dll4 and Jag1 signaling enhanced EphrinB2 and EC proliferation, respectively.31,32 In accordance with our in vivo results, fz7 knockout significantly decreased EC proliferation in vitro (Figure 7E). Stimulation with immobilized Jag1 protein induced, as expected, a significant increase in EC proliferation. Interestingly, after Jagged1 activation, the decrease in EC proliferation became less potent after fz7 knockout, suggesting that Fzd7 may control proliferation partially via Jag1 signaling (Figure 7E). Under Dll4 activation, EphrinB2 expression was totally rescued in cells depleted of fz7, suggesting that Fzd7/β-catenin can control EphrinB2 expression via Dll4.

Discussion

We provide evidence that Fzd7 expressed by EC drives angiogenesis via the Wnt canonical pathway and contributes to retinal vascular development. We further demonstrate that Fzd7 signaling occurs predominantly via activation of Dvlβ-catenin canonical Wnt signaling acting upstream of Notch signaling.

Using both constitutive and the inducible EC-specific deletion approaches, we show that fz7 deficiency results in a delay of primary retinal vascular plexus outgrowth, impairment in the tip cell phenotype, decreased stalk cell proliferation, and induced hyperbranching morphology in venous areas. Moreover, deletion of fz7 in EC impaired deep plexus formation with a decrease in the vascular area of the tertiary plexus. In cell culture, ECs have been shown to express almost all of the Fzd receptors, and they have been shown to regulate EC survival, proliferation, and gene expression. In vivo, only 3 Fzd receptors have been demonstrated to be involved in angiogenesis by genetic deletion or soluble receptor strategies. Fzd5 is essential for yolk sac and placental angiogenesis. In the retina, Fzd5 has been implicated in eye formation and hyaloid vasculature regression. A soluble Fzd-8 ectodomain, used to bind extracellular Wnt ligands, leads to severe central nervous system–specific angiogenesis defects. Global or EC-specific fz4−/− mutants present a strong inhibition of different vascular plexus formation and hyaloid regression during postnatal angiogenesis. Our data provide a novel insight into the biology of Wnt/Fzd signaling during blood vessel morphogenesis and provide evidence of EC-autonomous effects of Fzd7 to control postnatal angiogenic processes.

Depending on the context, Fzd7 can activate either the Wnt-canonical β-catenin pathway or the noncanonical Wnt pathway via Dvl. Dvl is considered to be a central hub for Wnt/
Fzd signaling to relay cellular information. Genetic studies have reported that the different orthologs have unique but also overlapping functions. However, evidence for their functional specialization in distinct organs and cellular events remain largely unexplored. Previous work has demonstrated that Dvl1 and 3 can be recruited at the cell membrane by Fzd7 in EC to activate cell signaling. Our results indicate that mice deficient in dvl1 display a similar but relatively milder retinal vascular phenotype, compared with the phenotype observed in mice deficient in EC-specific fzd7. Moreover, a worsening of the vascular phenotype was observed after injection of dvl3 siRNA in dvl1−/− mice. Using this strategy to target expression of dvl1/3 in the retina, we circumvented early embryonic lethality of dvl1−/−/dvl3−/− double mutants and have been able to identify Dvl1/Dvl3 pathway dependence for angiogenic events in vivo.

In the present study, a decrease of Wnt-canonical activation and target genes expression, specifically Axin2 and lef1, was observed in fzd7 iECKO retinas and dvl1−/− retinas. Our data are in line with results from our recent study demonstrating that Fzd7 activates the Wnt canonical pathway in EC. We then demonstrated that retinal vascular defects in fzd7 iECKO were rescued by treatment with LiCl. Although we cannot rule out non-EC effects of LiCl in vivo, these experiments strongly support the notion that Fzd7 expressed by EC controls vascular retinal development through Wnt canonical signaling. These results are in accordance with the literature reporting a major role of Wnt/β-catenin for retinal vessel development and blood–retinal barrier integrity.

Previous work has demonstrated that during angiogenesis, the proper balance of tips/stalk cells is highly dynamic and is
Figure 7. Fzd7 (frizzled-7) controls postnatal angiogenesis and Notch signaling via the canonical Wnt/β-catenin pathway. A, Representative retinal whole mounts and peripheral retinal flat mounts 48 h after injection of LiCl or PBS from postnatal day (P) 7 fzd7ECWT and fzd7ECKO mice. LiCl treatment rescues vascular delay and tip cell number impairment. Isolectin B4 (IB4)/BrdU (bromo-desoxy-uridin; (Continued)
mediated by a balanced expression and interaction of the Notch1 receptor with its ligands Dll4 and Jag1. Dll4 in tip cells activates Notch in adjacent stalk cells to laterally inhibit tip cell selection and maintain the hierarchical organization of sprouting EC. In contrast, Jag1 in stalk cells impedes Notch signaling in adjacent tip cell. Interestingly, although the control of Notch ligand by β-catenin is still not clear in postnatal retinal vascularization, cross talk between β-catenin and Notch signaling has been reported in different conditions. Dll4 was shown to be a direct transcriptional target of Wnt/β-catenin in EC. β-Catenin may form a complex with NICD/RBP-J on the Dll4 promoter, thereby potentiating the Dll4/Notch signal, leading to vascular stabilization. Other studies report that β-catenin activates Jag1 transcription leading to Notch activation in epithelial cells of the skin and cancer cells. 

Figure 7 Continued. right) staining showed that LiCl rescues endothelial cell (EC) proliferation impaired in fzd7 mutant mice. Quantification of % vascularization, number of tip cells per 100 μm, and proliferation of EC: n=5 mice for each condition. B, qRT-PCR was performed in whole retina from P7 fzd7+/CWT and fzd7+/DWT mice treated with LiCl or PBS. LiCl injection increased left expression in both control and mutant mice. The expression level of all genes was normalized against the housekeeping gene β-actin. C, qRT-PCR was performed in retina from P7 fzd7+/CWT and fzd7+/DWT mice treated with LiCl or PBS. LiCl treatment rescues Notch gene expression in fzd7 mutant mice. D, qRT-PCR was performed in siRNA control (siCont) or siRNA fzd7 (siFzd7) treated MS1 after 24 h of LiCl activation or control medium. Knockdown of fzd7 in EC decreases Notch gene expression. Lici treatment rescues decreased expression induced by siFzd7 treatment. Data represent the mean of 3 to 6 experiments performed in triplicate. MS1 cells were transfected with siCont or siFzd7 and transduced with lentivirus encoding for active β-catenin. qRT-PCR showed that activated β-catenin rescues Notch expression impairment induced by silencing of fzd7. Expression level of all genes was normalized against the housekeeping gene β-actin. Data represent the mean of 3 to 4 experiments performed in triplicate. E, HUVEC (human umbilical vein endothelial cell) were treated with siCont or siFzd7 for 48 h then plated either on vehicle or Jag1-coated dishes. After 24 h, EC count was quantified in each conditions (n=13). Unpaired t test on the average values obtained in 3 independent experiments. F, siCont- or siFzd7-treated HUVEC were plated on duplicate either on vehicle or Dil4-coated dishes. qRT-PCR was performed after 24 h activation. The expression level of EphrinB2 was normalized against the housekeeping gene β-actin (n=3). *P<0.01; **P<0.05; ***P<0.001.

In summary, we identified Fzd7 as a relevant Fzd receptor that contributes to retinal vascular plexus development and provides novel molecular insight into the reciprocal interaction of the Wnt/Fzd canonical and Notch pathways in regulating vessel formation.

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Disclosures
None.

References
• Specific Fzd7 (frizzled-7) deletion in endothelial cells inhibits postnatal angiogenesis by controlling tip cell phenotype and stalk cell proliferation.

• Dvl3 downregulation expression in Dvl1-deficient mice phenocopies Fzd7 phenotype.

• Fzd7 acts via the Wnt canonical pathway to control vessel formation.

• Fzd7/β-catenin acts upstream of Notch signaling.


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Supplementary Data

Methods

Mutant mice

Floxed Fzd7 mice were generated previously by Ferreira et al. The neomycin cassette was subsequently removed via flippase-mediated recombination by breeding with Flp mice. Floxed/F+ mice were subsequently backcrossed on C57BL6/J background. Interbred, homozygous fzd7/F+ mice exhibit apparently normal development, and are viable and fertile. For EC-specific deletion, two lines of Cre mice, Tie2-Cre and Pdgfb-iCre transgenics, were bred with fzd7/F+ female to generate fzd7/F+,Tie2-Cre (fzd7ECKO) and fzd7/F+,Pdgfb-iCre (fzd7ECKO), respectively. For Pdgfb-iCre, tamoxifen was administrated by intraperitoneal injection (50mg/kg) from P1 to P3.

We generated transgenic mice with fzd7 deletion in monocytes/macrophages and granulocyte using LysMCre-mediated recombination as we have previously describe. LysM-driven cre recombinase mice (LysMcre) were bread with fzd7/F+ mice to generate double transgenic mice fzd7lox/F+;LysM-Cre(−) and fzd7/F+;LysM-Cre(+).

For Dvl1 mutant mice, animals heterozygous for Dishevelled1 were backcrossed on C57BL6/J background and then mated to generate dvl1 KO mice (dvl1+/−) and their control littermates (dvl1+/+). The phenotype of the mutant was analyzed at the reported time. The number of backcrosses were > than 10. All mice used in this study were bred and maintained at the institute. This study was conducted in accordance with Bordeaux University institutional committee guidelines (committee CEEA50) and those in force in the European community for experimental animal use (L358-86/609/EEC).

Tails of pups were genotyped by PCR using the P1/P2 primer set for the Tie2-Cre allele and the P3/P4 primer set for the Pdgfb-iCre allele: P1: 5'- TAAAGATATCTCTCAGTACTGACGGTG -3'; P2: 5’- TCTCTGACCAGAGTCATCCTAGC -3'; P3: 5’ – CCAGCCGCGTCGCAACT – 3’; P4: 5’- GCCGCGGGCCACATCTTCGC – 3’. Cre-negative littermates were used as controls. To verify efficiency of Cre recombination and specificity, transgenic mice expressing Cre recombinase were mated with the reporter transgenic line mTmG ((ROSA)26Sortm4ACTB-tdTomato,EGFP)Luo/J, Jackson Lab).

Tissue immunofluorescence

Immunostaining in whole-mount retina conducted on eyes from P5, P7, and P11 pups as previously described. Retinas were stained with: anti CD31 (BMA), Isolectin-B4-FITC (Sigma), anti Collagen IV (Santa cruz), anti-Jag1 (R&D), anti-Dll4 (R&D) antibodies before being flat mounted. Retinal vasculatures were analyzed for the percent of vascularization, spreading of vascular plexus, number of branch points, vessel proliferation, number of tip cells and filopodia, and vessel regression after specific labelling as describe by. For in vivo proliferation assays, intraperitoneal (IP) injection of 50µl of 5-Bromo-2’-desoxyuridine (BrdU) (Sigma) at 10mg/ml was performed and mice sacrificed after 6 hours at P7. After PFA fixation and dissection, anti BrdU (Oxford Biotechno) and IB4 staining was performed. For cryosection immunostaining, tissue from P7 pups was fixed in 4% PFA-PBS, washed in PBS, embedded in OCT and frozen. Immunofluorescence staining was performed on cryosections with anti CD31 (BMA), anti Fzd7 (R&D) antibodies or Control IgG (Sigma). Images were acquired using an Olympus confocal microscope (Olympus FV 1000).
Mouse retina analysis
Retinal vasculatures were imaged with a fluorescent microscope (Axio-observer, Zeiss). Percent of vascularization, spreading of primary vascular plexus (4 fields per retina), and number of branch points per field (8 fields per retina), were all examined. Tip cells and filopodia were imaged with a confocal microscope (Olympus FV 1000) and analyzed using Imaris (Bitplane) software. Angles between each filopodia and the tip cell migration axis (10 fields per retina) were measured with Axiovision software (Zeiss) and the mean vector and circular variance were quantified with Oriana 3.2 software (Kovack computing Services). To assess vessel proliferation, the ratio of BrdU and IB4 positive cells/ percentage of vascularized area was analyzed using Image J software (8 fields per retina). To evaluate vessel regression, the number of IB4 negative and collagen IV positive branch points per field was quantified using Axiovision software (4 fields per retina). Immunofluorescence intensity was quantified in tip cell or stalk cell areas using Image J software. Each intensity was normalized to IB4+ area.

Mouse intravitreal injection and treatment
For intravitreal injection, mice were anesthetized with isoflurane (Aerrane®, Baxter), 0.5µl delivered with UltraMicroPomp III was used to inject either siRNA Dvl3 or siRNA control. For intravitreal injection, mice were injected at P4 with 0.5µg of dvl3 siRNA. Control siRNA was injected in the contralateral eye of the same animal to account for biological variation of retinal vascular development, and animals were sacrificed at P7 for analysis. Oligonucleotides were designed by Ambion for m-dvl3 (NM_008057): sense–GGA GAA UCU GGA CAA UGA C55; antisense– GUC AUU GUC CAG AUU C56.

Lithium chloride (LiCl), 50mg/kg or PBS, was injected IP at P5 and P6 and sacrificed at P7 for analysis.

Isolation of primary EC from mouse lung
Lungs were removed after sacrifice, placed in PBS-containing CaCl2 and MgCl2 (Gibco), minced in slices and incubated for 45 min at 37 °C in a solution of PBS Ca / Mg containing collagenase type I (Sigma). The suspensions were triturated with a cannula, then filtered with a 70 microns (BD) and a 30 microns filter (Miltenyi). After centrifugation, cells were seeded in Petri dishes in MEM medium supplemented with D-valine. Five days later, the cells were trypsinized and incubated with two antibodies: anti-CD31 (Pharmingen) and anti-endoglin (Santacruz) for 30 min at room temperature. The cell suspension was then placed in the presence of microbeads coupled with rat IgG (Miltenyi) for 15 min at 4 °C. After several washes with Ca / Mg PBS, the cell suspension was separated by passing it through a magnetic column (MACS MS columns, Miltenyi) according to the protocol described and cells were used directly. The purity of the selected EC was verified by immunostaining with anti-CD31 antibody and by RT-PCR.

Cell culture, transfection and drug treatments
MS1 (mouse pancreatic islet EC line) cells were cultured in DMEM (Dubelco) supplemented with 5% FBS (Hyclone) and penicillin-streptomycin. MS1 were transduced with the different lentivirus as describe by 1, 4. Human umbilical vein endothelial cells (HUVEC) were cultured in EGM-2 media (Lonza, CC-3156) supplemented with EGM-2 Single Quots (Lonza, CC-4176).

siRNA was transfected using Interferin (Polyplus) at a concentration of 30nM as presviously describe 1. Oligonucleotides are listed below. Lentiviruses were transduced at a multiplicity of infection (MOI) of 30 for MS1. Lithium chloride (LiCl) (Merck) was used at 20mM in vitro.
For Dll4 and Jag1 stimulation, recombinant Dll4 (mouse Dll4-His, 500 ng/ml, R&D Systems) and Jag1 (rat Jag1-Fc, 3µg/ml, R&D Systems) were immobilized by coating culture dishes for 18 h at 4°C. HUVEC treated for 48h with siCont or sizd7 were detached by trypsinization and stimulated with immobilized recombinant Dll4 or Jagged1 ligands as described. For EC proliferation, HUVEC were seeded in duplicate in 4-well labtek dishes (10 000/cm²) and the number of cells/field were counted after 24h activation onto vehicle or Jag1 coated dishes. For EphrinB2 expression rescue, HUVEC treated with siCont or sizd7 were seeded in triplicate in 12-well plates (20 000/cm²) coated with vehicle or Dll4. Cells were lysate after 24h for qRT-PCR analysis.

**PCR primers and siRNA**

Primer sequences are as follows: Mouse beta-actin: forward primer (NM_007393) 5'-ATTTCATTTGACTTGCGGGC -3', and reverse primer 5'-GGAGGAGCTGGAAGCAGCC -3'– Mouse Fz7: forward primer (NM_008057) 5'-GCCCGACTTTACAGTCTTC -3', and reverse primer 5'-ATACCGCATTCCTCCCTG -3'– Mouse beta-catenin: forward primer 5'-GTAGCGCACCAGTGAATAAC -3', and reverse primer 5'-TGAGACAGATATATGCAG -3'– Mouse Axin2: forward primer 5'-AACCTATGGCGGTTCTCTCT -3', and reverse primer 5'-CTGGTCAACCAACCAAGGAG -3'– Mouse Lef1: forward primer (NM_010703) 5'-CAGTGACAGCAACCTTACCTGAG -3', and reverse primer 5'-CCGCTTCTTTTGGAAATCGG -3'– Mouse Dll4: forward primer 5'-TGGCAATGTCTCCACGCGGG -3', and reverse primer 5'-TGTCCGCAAATCTTTACCACAGCA -3'– Mouse Jag1: forward primer 5'-AGTGATGTGTCCTCGGTGG -3', and reverse primer 5'-CCGTCCACACAGTGTCGCTA -3'– Mouse Notch1: forward primer (NM_010929.2) 5'-ACACGGATGAGTGCAGCCAGC -3'– Mouse Notch4: forward primer (NM_008714.3) 5'-TGGCAACATGGCGGCTCCTG -3', and reverse primer 5'-GCCGGTGGAATTCCAGGAGG -3'– Mouse Hey1: forward primer (NM_010423) 5'-TCCATGTCCCCAAGCACATC -3', and reverse primer 5'-GCATGTTGACGATTTCCTCAG -3'– Mouse Hey2: forward primer (NC_000076) 5'-CCATTACCGCAACAATCC -3', and reverse primer 5'-TGTTCCGCAATCTTTACCACAGCA -3'– Mouse Dll4: forward primer 5'-AGTGATGTGTCCTCGGTGG -3', and reverse primer 5'-AGTCCGACGTGTCCTACCT -3'– Mouse Jag1: forward primer 5'-AGTGATGTGTCCTCGGTGG -3', and reverse primer 5'-CCGCTTCTTTTGGAAATCGG -3'. The oligonucleotides used were designed by Ambion, mzd7 (NM_008057): sense – GCC AUA UCA CGG CGA GAA Att; antisense – UUU CUC GCC GUG AUA UGG Ctg, for m-dvl3 (NM_007889): sens-GGA GAA UCU GGA CAA UGA Ctt; anti-sens-GUC AUU GUC CAG AUU CUC Ctg and by Qiagen for m-dvl1 (NM_010091): sens-GGG ACG GAA UGG ACA AUG AdTd; UCA UUC AUU CCG UCC CdGd, for m-dvl2 (NM_007888): sens-GCC UUU GGU ACU CUA UUU AdTd; anti-sens-UAA AUA GAG UAA CAA AGG CdGdG and for h-zd7 (NM_003507): sens-CUC CAA UCU UGC AAC ACU AdTd; anti-sens-UAG UGU UGC AAG AUA GGA GdGdG.
**Expression construct**

β-catenin T41A (plasmid from MA Buendia, Institut Pasteur, France) was subcloned into the lentiviral vector pRRLsin-MND-MCS-WPRE. Lentivirus preparations were produced at the Bordeaux University lentivirus platform.

**Western blot (WB)**

Immunoblotting was performed as described previously. In brief, proteins were resolved by SDS-PAGE and blotted with the following antibodies: anti tubulin (Sigma), β-catenin (Sigma), Active β-catenin (Upstate), anti EphB4 (Santa Cruz) and anti ephrin-B2 (Santa Cruz). Binding of antibodies was detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

**RNA preparation and qPCR**

Mouse tissues or cells were homogenized in TRI-REAGENT™ (Euromedex) and RNA extracted according to the manufacturer’s instructions. qPCR was performed as previously. All experiments were performed in triplicate and differences in cDNA input were compensated by normalization to expression of β-actin.

**Statistical analysis**

Results were expressed as mean ± SD or mean ± min to max. Statistical significance was determined by two-tailed Student’s t-test. Multiple group comparison was performed with a one-way ANOVA followed by Bonferroni’s post-test correction. A value of $p<0.05$ was considered to be statistically significant.

**References**


Supplementary figures

Supplementary Fig. I:

A  

B  

Whole retina  

Vein  

Capillary plexus  

Tip/stalk cells  

C  

Whole retina  

Vein  

Capillary plexus  

Tip/stalk cells  

D  

Fold difference in gene expression

E  

Fold difference in gene expression

F  

Fold difference in gene expression

G  

Fzd7<sup>ICGT</sup>  

Fzd7<sup>IECKO</sup>

H  

Weight of mice in grams

- Tail-Cre
- Fzd7<sup>Fz7</sup>
- Fzd7<sup>Fz7</sup>
- Fzd7<sup>Fz7</sup>
- Fzd7<sup>Fz7</sup>
Supplementary Fig. I: Characterization of fzdf7ECKO and fzdf7ECKO mice.

(A) Homozygote floxed fzdf7FF mice were crossed with Tie2-driven Cre-recombinase mice (Tie2-Cre) or tamoxifen-inducible Pdgfb-driven Cre-recombinase mice (Pdgfb-iCre) to generate fzdf7 knockout (KO) mice specifically in the endothelium, respectively fzdf7ECKO and fzdf7ECKO. fzdf7FF, Pdgfb-iCre(+) mice and their control littermates fzdf7FF, Pdgfb-iCre(-) were injected intraperitoneally at P1, P2 and P3 with tamoxifen (50mg/kg) to induce fzdf7 deletion in endothelial cells (EC). (B-C) To control Cre expression in the retina, transgenic mice expressing Cre-recombinase driven by the Tie2 or the Pdgfb tamoxifen-inducible promoter were crossed with mTmG/mTmG reporter mice. Whole mount retina were analyzed by immunofluorescence microscopy. Specific GFP expression was observed demonstrating Cre expression in the vascular plexus, tip and stalk cells. (D-E) Quantification of fzdf7 mRNA was performed by RT-qPCR. (D-F) fzdf7 expression in control and littermate mice retina, for each Cre-driver line. Expression level of fzdf7 was normalized against the housekeeping gene β-actin. (F) Quantification of fzdf7 mRNA by RT-qPCR was performed in CD31+ (EC) cells and CD31- cells isolated from fzdf7FF, Pdgfb-iCre(-) and fzdf7FF, Pdgfb-iCre(+) mice lung after tamoxifen treatment. Error bars represent standard deviation (SD). Data represent the mean of 4 experiments carried out in triplicate. p-values below 0.05 were considered statistically significant (*). (G) Cryosection of P7 retina from fzdf7ECWT and fzdf7ECKO, were stained with Fzd7 antibody followed by conjugated DAB 2nd antibody revelation (in brown). As expected no Fzd7 antibody staining was observed in fzdf7ECKO. CD31 staining (brown) showed EC in the SRVP (Insert). (H) Weight of mice at the various time points was evaluated in the two different mouse Cre-lines and no difference was observed between the fzdf7 mutant and their respective control mice.
Supplementary Fig. II: Vascular phenotype analysis of $fzd7^{LysM}$ mice.

LysMCre-mediated recombination mice were bred with $fzd7^{F/F}$ mice to generate double transgenic mice $fzd7^{F/F};$LysM-Cre(-) and $fzd7^{F/F};$LysM-Cre(+) ($fzd7^{LysM}$). No modification in retinal vascular formation was observed at P7 in $fzd7^{LysM KO}$ versus $fzd7^{LysM WT}$. % of vascularization and spreading were quantified (n=7, p NS).
Supplementary Fig. III:  

(A) Weight of \(dvl^{+/+}\) and \(dvl^{1/-}\) mice at the different time points. (B) Expression of \(dvl2\) and \(dvl3\) by RT-qPCR in control and \(dvl1\) KO mice. (C) Expression of \(dvl1\), \(dvl2\), and \(dvl3\) by RT-qPCR in control mice injected with either PBS or siRNA \(dvl3\). *\(p<0.05\).
Supplementary Fig. IV:

Supplementary Fig. IV: Expression of Dll4 and Jagged1 in retina

Immunofluorescence staining for Dll4 (red), or Jagged1 (red) and IB4 (green) in whole-mount retina from mutant vs control mice at P5. (A) Dll4 staining intensity was quantified specifically in tip cells in fz7ECWT and fz7IECKO retina, and showed a decrease in Dll4 expression in Fzd7 deleted cells (n=35 tip cells/mice, n=3 mice for each condition, * p<0.05). Arrows: tip cells. (B) Jagged1 expression quantified in the stalk cell area showed a reduced expression in the retina from fz7IECKO as compared to their control littermate (n=4 areas/mice; n=3; * p<0.05).
Supplementary Figure V:

The molecular mechanism we propose is that Fzd7 expressed by tip cells can control Dll4 expression via β-catenin activation. Dll4 activates Notch in adjacent stalk cells required for stalk cell specification. Fzd7 in stalk cells, controls proliferation during sprouting through a direct effect of β-catenin activation on EC proliferation via lef1 expression. Moreover, Fzd7/β-catenin, by controlling Jagged1, may contribute to maintaining stalk cell properties at the vascular front. Finally, Fzd7/β-catenin may control EphrinB2 expression, an arterial marker, depending on Dll4 activation.