Cell

The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis

Rui Benedito,[1,2] Cristina Roca,[1] Inga Sörensen,[3] Susanne Adams,[1,2] Achim Gossler,[3] Marcus Fruttiger,[4] and Ralf H. Adams[1,2,*]

[1]Cancer Research UK London Research Institute, Vascular Development Laboratory, London WC2A 3PX, UK
[2]Max Planck Institute for Molecular Biomedicine, Department Tissue Morphogenesis and University of Münster, Faculty of Medicine, Münster 48149, Germany
[3]Institute for Molecular Biology OE5250, Medizinische Hochschule Hannover, Hannover D-30625, Germany
[4]Institute of Ophthalmology, University College London, London EC1V 9EL, UK
*Correspondence: ralf.adams@mpi-muenster.mpg.de
DOI 10.1016/j.cell.2009.03.025

SUMMARY

The Notch pathway is a highly conserved signaling system that controls a diversity of growth, differentiation, and patterning processes. In growing blood vessels, sprouting of endothelial tip cells is inhibited by Notch signaling, which is activated by binding of the Notch receptor to its ligand Delta-like 4 (Dll4). Here, we show that the Notch ligand Jagged1 is a potent proangiogenic regulator in mice that antagonizes Dll4-Notch signaling in cells expressing Fringe family glycosyltransferases. Upon glycosylation of Notch, Dll4-Notch signaling is enhanced, whereas Jagged1 has weak signaling capacity and competes with Dll4. Our findings establish that the equilibrium between two Notch ligands with distinct spatial expression patterns and opposing functional roles regulates angiogenesis, a mechanism that might also apply to other Notch-controlled biological processes.

INTRODUCTION

During development, growth, or regeneration, the local blood vessel network expands through angiogenic sprouting into areas that have demand for nutrients and oxygen. This process is strongly controlled by hypoxia-dependent, tissue-derived proangiogenic signals, such as vascular endothelial growth factor (VEGF), which binds to cognate receptors in the endothelium (Carmeliet, 2005; Liao and Johnson, 2007). However, the regulation of endothelial cell (EC) sprouting and proliferation also involves intrinsic signaling interactions between ECs that are, for example, mediated by the Notch pathway (Bray, 2006; Roca and Adams, 2007). The ligand Dll4, a transmembrane protein, is upregulated by VEGF in the angiogenic vasculature (Hainaud et al., 2006; Hellstrom et al., 2007; Lobov et al., 2007; Noguera-Troise et al., 2006; Suchting et al., 2007). High expression of Dll4 in filopodia-rich endothelial tip cells, which lead and guide new sprouts, is thought to activate Notch and suppress the tip phenotype in adjacent (stalk) ECs. Thus, a sufficient number of ECs maintains vascular integrity and tissue perfusion. This activity of Notch is at least partially mediated by downregulation of VEGF receptor expression, a process that will dampen down the response to VEGF and suppress sprouting by stalk ECs (Harrington et al., 2008; Hellstrom et al., 2007; Ridgway et al., 2006; Suchting et al., 2007). The delicate nature of this balance is uncovered in mice lacking a single *Dll4* allele or by interfering with Dll4 or Notch function in mice (Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007), zebrafish (Leslie et al., 2007; Siekmann and Lawson, 2007), or in experimental tumors (Li et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006). In all these models, more ECs extend filopodial protrusions and sprout toward the angiogenic stimulus. In experimental tumors, Dll4 inhibition leads to excessive sprouting from intratumor blood vessels, which compromises tumor perfusion and growth (Li et al., 2007; Noguera-Troise et al., 2006).

Here, we have investigated the role of the Notch ligand Jagged1, a member of the Jagged/Serrate family, in angiogenesis. Delta-like or Jagged ligand binding to Notch receptors triggers the separation of the Notch extracellular domain by proteases of the ADAM family. Subsequent γ-secretase processing releases the Notch intracellular domain (NICD), which translocates into the nucleus and regulates downstream gene expression (Nichols et al., 2007; Schweisguth, 2004).

Although the important role of Dll4-Notch signaling in the cardiovascular system is already widely appreciated, we now show that Jagged1 is a critical positive regulator of tip cell formation and sprouting because of its ability to modulate Dll4-Notch signaling in the angiogenic endothelium.

RESULTS

Jagged1 Controls Angiogenesis in the Embryo

Because the global inactivation of the *Jagged1* gene (*Jag1*) leads to early embryonic lethality (Xue et al., 1999), we have used EC-specific and inducible genetic approaches in mice. For loss-of-function studies, mice carrying a loxP-flanked *Jag1* gene ($Jag1^{flox/flox}$) (Brooker et al., 2006) were intercrossed with *Tie1*-Cre (Gustafsson et al., 2001) or *Pdgfb*-iCreER (Claxton et al., 2008) transgenics, which express in ECs constitutive or

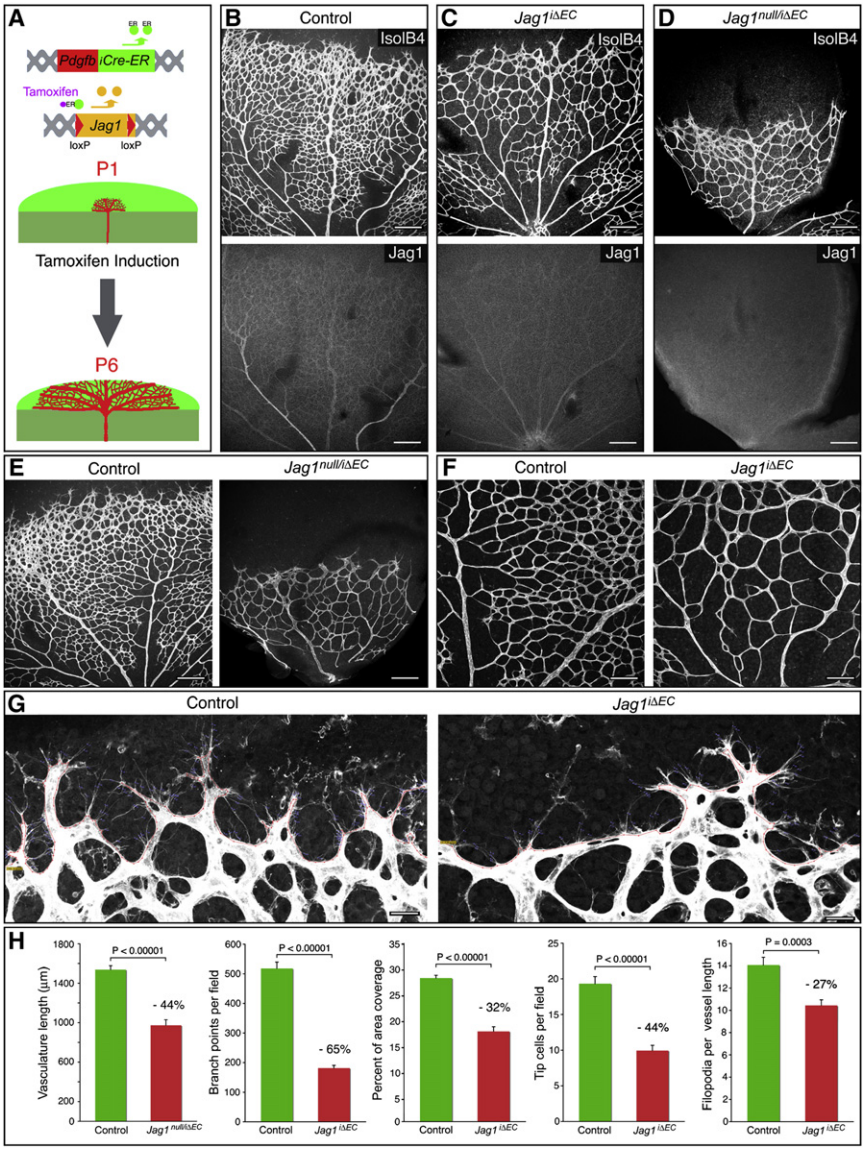

Figure 1. Retinal Angiogenesis in EC-Specific Jagged1 Loss-of-Function Mutants

(A) Diagram illustrating the inducible gene deletion in retinal ECs. Following tamoxifen administration from P1 to P3, retinas were analyzed by isolectin B4 (B–G) and Jagged1 (Jag1) (B–D) whole-mount immunostaining at P6.

(B, C, and F) Confocal images show decreased vessel branching and low EC coverage in $Jag1^{i\Delta EC}$ retinas. $Jag1^{i\Delta EC}$ mutants have some residual Jagged1 protein (~10%–20% of control levels).

(D and E) Compromised branching and growth of the retinal vasculature in $Jag1^{null/i\Delta EC}$ mutants. Anti-Jagged1 staining is absent in mutant ECs.

(G) Decreased tip cell and filopodia extension at the $Jag1^{i\Delta EC}$ angiogenic front.

(H) Quantitation of vascular parameters in the control and mutant retina as indicated. Error bars represent s.e.m.; P values are indicated. Scale bars: B–E: 200 μm; F: 100 μm; G: 25 μm.

tamoxifen-inducible Cre recombinase, respectively. As previously shown (Enge et al., 2002), *Tie1*-Cre mice yielded variable levels of gene inactivation. The most severely affected $Jag1^{flox/flox}$ *Tie1*-Cre ($Jag1^{\Delta EC}$) mutants died as early as embryonic day 10.5 (E10.5) and phenocopied a published EC-specific *Jag1* knockout (High et al., 2008), while the others succumbed later. Despite of this variability, $Jag1^{\Delta EC}$ E17.5 embryos show reduced branching in dermal vasculature (see Figure S1A available online), suggesting that Jagged1, like Dll4, controls angiogenesis. We also generated an inducible and EC-specific gain-of-function model consisting of mice carrying a murine *Jag1* cDNA under control of a tetracycline-regulated minimal promoter (tetO-*Jag1*) and *VE-Cadherin*-tTA transgenics, which express the tetracyclin-controlled transactivator (tTA) (Sun et al., 2005). Without tTA transactivator repression, the resulting mutants ($Jag1^{GOF}$) die before E16.5. Freshly isolated $Jag1^{GOF}$ embryos at E15.5 show mild growth retardation and extensive hemorrhaging in the skin. Opposite to the phenotype of $Jag1^{\Delta EC}$ loss-of-function mutants, vessel branching and EC density are increased in the $Jag1^{GOF}$ dermal vasculature (Figure S1B).

Reduced Sprouting Angiogenesis in *Jag1* Loss-of-Function Mutants

Next, we investigated Jagged1 function in the retinal vasculature. The retina is avascular at birth, and a single, superficial layer of blood vessels grows progressively from the center toward the periphery from postnatal day (P) 1 until P7 (Figure 1A). *Pdgfb*-iCreER transgenics, which target retinal ECs, including tip and stalk cells (Claxton et al., 2008) (Figure S2), were bred into the $Jag1^{flox/flox}$ background. Following postnatal tamoxifen administration, retinas with induced endothelial *Jag1* deletion ($Jag1^{i\Delta EC}$) display significantly decreased vascular branching and EC coverage at P6 (Figures 1A–1C, 1F, and 1H). As Jagged1 is strongly diminished but not fully absent in $Jag1^{i\Delta EC}$ ECs (Figure 1C), we also combined loxP-flanked and constitutive null alleles. *Pdgfb*-iCreER-mediated *Jag1* deletion is more efficient in this $Jag1^{null/i\Delta EC}$ background, and residual protein is absent (Figure 1D). Growth of the $Jag1^{null/i\Delta EC}$ retinal vasculature is strongly inhibited, resulting in reduced branching and delayed extension toward the periphery (Figures 1D, 1E, and 1H). Consistent with a general role of Jagged1 in postnatal angiogenesis, $Jag1^{null/i\Delta EC}$ pups are also generally smaller than tamoxifen-treated control littermates (data not shown).

One hallmark of angiogenesis is the emergence of filopodia-extending endothelial tip cells, which indicate that ECs have acquired an exploratory, motile mode (Gerhardt et al., 2003). The loss of Jagged1 in ECs leads to a significant decrease in the number of tips and filopodia (Figures 1G and 1H). Moreover,

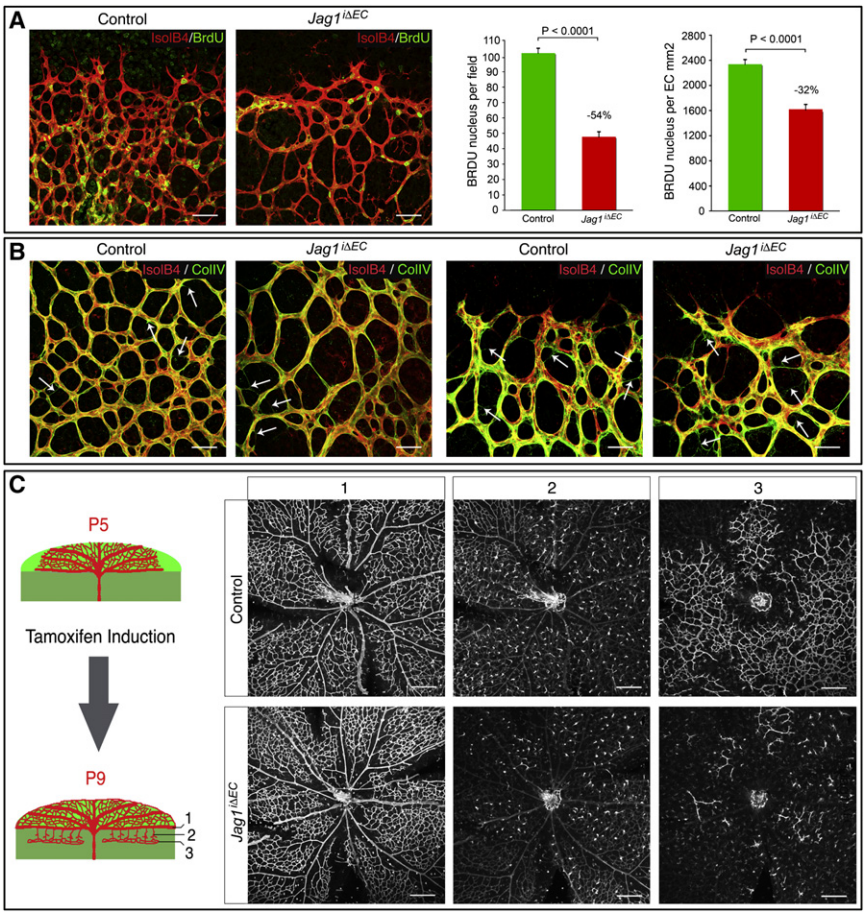

Figure 2. *Jag1* Deletion Compromises EC Proliferation but Not the Pattern of Established Vessels

(A) Isolectin B4 (red) and BrdU labeling (green) of whole-mount control and *Jag1*$^{i\Delta EC}$ P6 retinas (left). Quantitation of BrdU-positive cells shows reduced EC proliferation in *Jag1*$^{i\Delta EC}$ mutants (right). Error bars represent s.e.m.; P values are indicated.

(B) Whole-mount Isolectin B4 (red) and collagen IV (green) staining of P6 retinas. The number of empty (ColIV+ IsolB4−) sleeves (arrows) in the proximal and distal capillary plexus is similar in control and *Jag1*$^{i\Delta EC}$ animals.

(C) Diagram of experiments during late retina vascular development (left). Following tamoxifen administration at P5 and P6, retinas were analyzed by isolectin B4 immunostaining at P9. Confocal images of three different levels (1, 2, 3) within the whole–mount retinas are shown (right), as indicated. The preestablished superficial vascular plexus (1) is not affected, while the extension of perpendicular endothelial sprouts (2) and vascularization of the deeper retina (3) are impaired in *Jag1*$^{i\Delta EC}$ mutants. Scale bars: A and B: 50 μm; C: 200 μm.

in vivo labeling with 5-bromodeoxyuridine (BrdU) shows that Jagged1 also positively controls EC proliferation in addition to sprouting (Figure 2A).

Vessel Stability and Mural Cell Coverage in *Jag1*$^{i\Delta EC}$ Mutants

Besides angiogenic defects, decreased vessel stability might contribute to the reduced vessel density in *Jag1*$^{i\Delta EC}$ mutants. Regressing ECs leave empty sleeves of matrix deposits rich in collagen IV (Baluk et al., 2003). However, we found no overt change in the number of empty (collV+ isolectin B4−) sleeves in the *Jag1*$^{i\Delta EC}$ retina (Figure 2B). Furthermore, *Jag1* inactivation by tamoxifen administration between P5 and P9 confirmed that the stability of established vessels in the superficial capillary plexus does not require Jagged1. In contrast, perpendicular sprouting and neovascularization of the deeper retina, which occur from P6 onward, are strongly compromised in *Jag1*$^{i\Delta EC}$ mutants (Figure 2C).

Since it was shown that endothelial Jagged1 is important for the recruitment of vascular smooth muscle cells (vSMCs) in the early embryo (High et al., 2008), we analyzed the coverage of blood vessels by mural cells, namely vSMCs and pericytes. Analysis of α-smooth muscle actin-stained P6 retinas revealed decreased vSMC coverage of *Jag1*$^{i\Delta EC}$ arteries (Figures 3A–3F), which is, however, unlikely to cause sprouting defects. Pericytes, which cover capillary beds, make direct cell-cell contact to ECs and may well affect sprouting, show no apparent change in the mutant vasculature (Figures 3G–3L).

Jag1 Overexpression Promotes Sprouting Angiogenesis

Next, we addressed whether Jagged1 overexpression enhances angiogenesis in the neonatal retina. To extend the survival of *Jag1*^{iGOF} mutants to postnatal stages, tTA activity was suppressed by administering tetracycline to pregnant females until E14.5. Like in the embryonic dermis, upregulated *Jag1* expression in the postnatal endothelium increases vessel branching, EC density, and proliferation (Figures 4A–4E). Likewise, tip cells and filopodia are significantly more abundant at the *Jag1*^{iGOF} vascular front (Figures 4C and 4D).

Immunohistochemistry uncovered notable differences in the spatial distribution pattern of Jagged1 among ECs. Although the ligand is readily detectable in stalk cells, expression in tip cells is low or absent (Figure 4A). This difference is maintained in the *Jag1*^{iGOF} vasculature despite elevated Jagged1 levels (Figure 4B).

Jagged1 Inhibits Dll4-Notch Signaling in ECs

Our experimental results suggest that Jagged1 and Dll4 have opposite effects on sprouting angiogenesis. This could be explained by Jagged1-mediated inhibition of Dll4-Notch signaling, even though Jagged1 is known to activate Notch in many cell types. If Jagged1 actually acts as an antagonist, inactivation of the gene would upregulate Dll4-Notch signaling in the endothelium. Indeed, the transcriptional repressor Hey1 (Fischer and Gessler, 2007), a downstream target that is positively regulated by Notch, is strongly upregulated in the *Jag1*$^{i\Delta EC}$ endothelium (Figure 5A). Expression in tips is lower and nuclear Hey1 signal

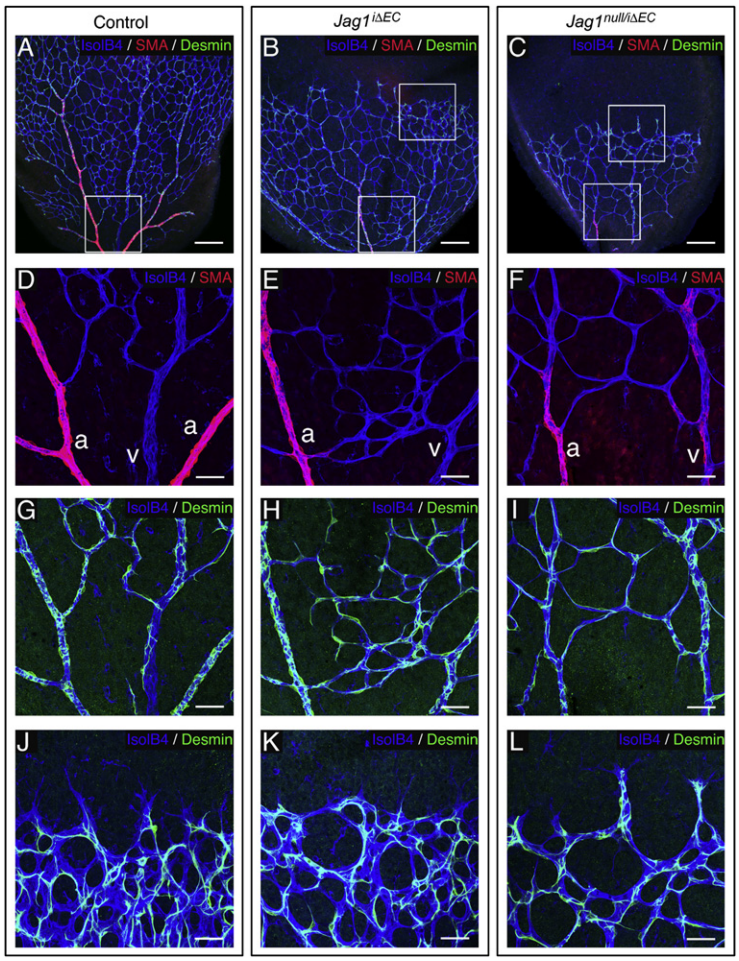

Figure 3. Mural Cell Coverage of *Jag1* Mutant Vessels

(A–C) Analysis of the control and *Jag1* mutant retinal vasculature at P6 by whole-mount staining for isolectin B4 (blue), α-smooth muscle actin (SMA, red), and Desmin (green).

(D–F) Higher magnification of arterioles and venules (bottom insets in A–C) showing decreased arterial (a) smooth muscle cell coverage (red fluorescence) in $Jag1^{i\Delta EC}$ mutants. In $Jag1^{null/i\Delta EC}$ retinas (F), smooth muscle cell coverage of developing arteries is further reduced, whereas more SMA+ cells are visible on venules (v).

(G–L) Higher magnification of insets in A–C showing no obvious alterations in the number and association of Desmin+ pericytes (green) in proximal, established (G–I) and distal, angiogenic (J–L) capillaries of *Jag1* mutants. Scale bars: A–C: 200 μm; D–L: 50 μm.

appears confined to stalks and established vessels (Figure 5B). Thus, even in the $Jag1^{i\Delta EC}$ context, ECs with lower levels of Notch signaling are sorted to the front, whereas strong Notch activation (nuclear Hey1) correlates with exclusion from the tip position.

The related transcriptional repressor Hes1, another Notch target, is expressed in endothelial, perivascular, and nonvascular cells (Figures 5C and 5D). Separation of these expression domains with image processing software uncovers an increase in Hes1-expressing $Jag1^{i\Delta EC}$ ECs, whereas Hes1+ perivascular cells are reduced (Figures 5C, 5D, and S3). Dll4 is also positively regulated by Notch (Ridgway et al., 2006; Shawber et al., 2003; Figures 6F and 7C), and, accordingly, expression is increased in $Jag1^{i\Delta EC}$ ECs. Although microvascular expression of Dll4 is normally higher at the angiogenic front, high levels of the ligand are found throughout the Jagged1-deficient endothelium (Figure 5E).

Because all three Notch targets tested are upregulated in $Jag1^{i\Delta EC}$ ECs, we analyzed whether Notch inhibition would restore a wild-type-like response in mutants. The administration of the γ-secretase inhibitor DAPT, which blocks Notch cleavage and signaling, to control animals induces excessive sprouting, increased EC proliferation, and growth of highly branched network in the peripheral portion of the retinal vasculature (Hellstrom et al., 2007; Suchting et al., 2007; Figure 5F). Despite the lower density of $Jag1^{i\Delta EC}$ blood vessels, DAPT triggers a strong sprouting response that is similar to that in DAPT-treated littermate controls (Figure 5F). In more mature blood vessels of the central retina, where DAPT is able to promote filopodia formation but not sprouting, DAPT-treated $Jag1^{i\Delta EC}$ ECs also extend numerous filopodia, comparable to controls (Figure S4). Thus, the chemical inhibition of Notch confirms that the reduced angiogenic growth of $Jag1^{i\Delta EC}$ blood vessels is indeed a consequence of increased Notch signaling.

Expression and Function of Fringe Genes in the Vasculature

Notch signaling can be modulated by various posttranslational modifications of the receptors, such as the addition of fucose residues by protein O-fucosyltransferase 1 (POFUT1) to the extracellular EGF-like repeats, which can be further modified by Fringe family β-1,3-N-acetyl-glucosaminyltransferases. Fringe enhances the activation of Notch in response to Delta-like ligands, but has the opposite effect for Serrate/Jagged ligands (Yang et al., 2005). We hypothesized that Fringe-mediated Notch modification might explain the opposite functional roles of Dll4 and Jagged1 in ECs, and found that all three mammalian Fringe genes are expressed in the developing vasculature. In the E10.5 embryo, *Manic Fringe* (*Mfng*) transcripts are abundant throughout the vasculature, while *Lunatic Fringe* (*Lfng*) signals label the dorsal aorta and cardinal vein (Figure S5). In the postnatal retina, *Mfng* expression is found in arteries, veins, and capillary ECs, and strong signals highlight some stalk as well as established and newly emerging tip ECs (Figures 6A–6C). Moreover, qPCR on FACS-sorted GFP-expressing *Pdgfb*-iCreER (Claxton et al., 2008) retinal ECs uncovered expression of *Radical Fringe* (*Rfng*) in addition to *Lfng* and *Mfng* (Figure 6D). EC markers such as *PECAM1* and *Dll4* were enriched 573-fold and 100-fold in these isolates.

Lfng, *Mfng*, or *Rfng* single knockout mice are viable, suggesting redundancy and raising questions about the role of Fringe genes in the vasculature (Ryan et al., 2008; Zhang et al., 2002). Nevertheless, our examination of the $Lfng-/-$ retinal vasculature uncovered significantly enhanced sprouting and an increase in the vascular area (Figure 6E), which indicates reduced Notch signaling even in the absence of a single Fringe gene.

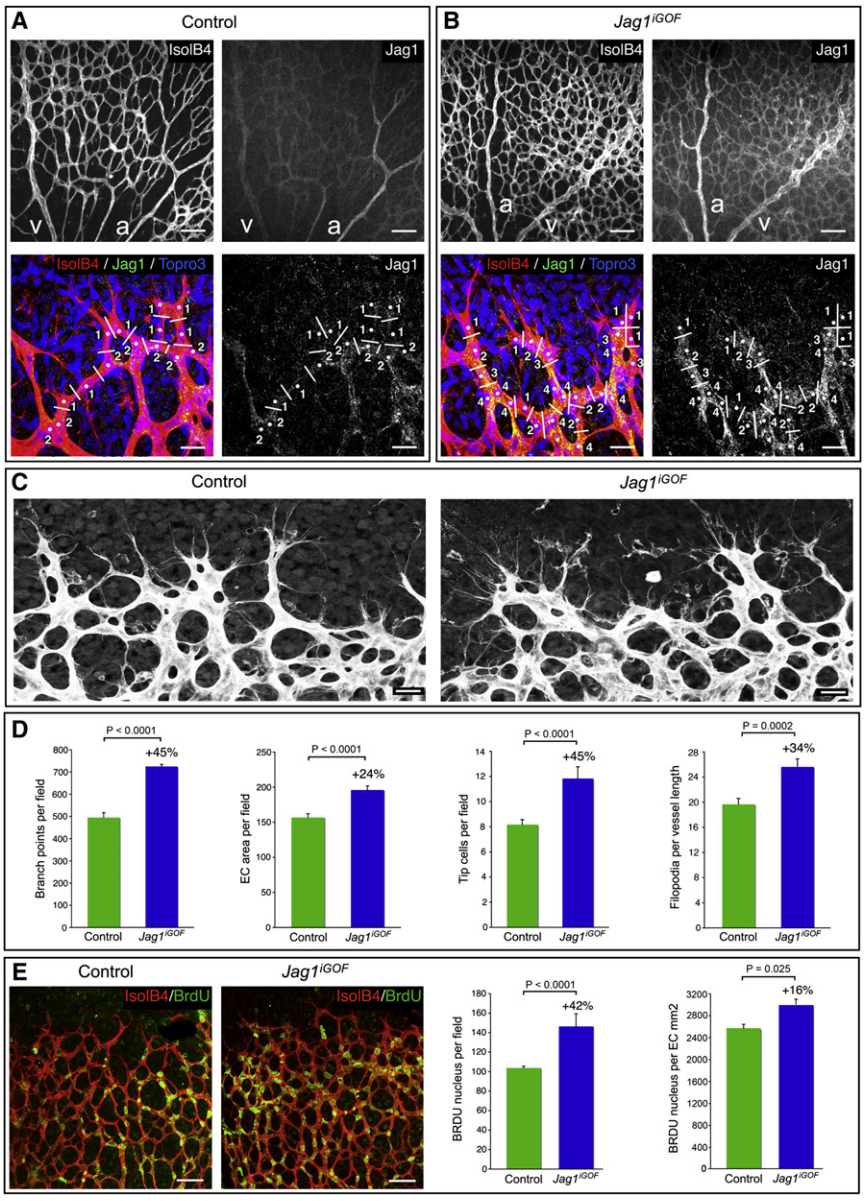

Figure 4. Endothelial Jagged1 Promotes Sprouting Angiogenesis

(A and B) Retinas from control (tetO-*Jag1*) and *Jag1* gain-of-function (*Jag1*^{iGOF}, i.e., tetO-*Jag1* × *Vecad-tTA*) P6 pups were stained for isolectin B4, Jagged1, and Topro3 as indicated. Increased Jagged1 expression in retinal capillaries (B) enhances vessel branching and EC density. Arterioles (a) and venules (v) are indicated. Bottom panels show Jagged1 distribution in single ECs, which were identified by IsolB4 (red) and nuclear Topro3 staining (blue or pink). Individual ECs were separated by white lines and nuclei marked with white dots for analysis. Numbers indicate average intensity level of anti-Jagged1 staining per EC. Tip cells have low or no Jagged1, while stalk cell expression is higher in control and *Jag1*^{iGOF} retinas.

(C) Isolectin B4 immunofluorescence showing increased tip cell and filopodia extension at the *Jag1*^{iGOF} angiogenic front.

(D) Quantitation of branch points, EC area, tip cell and filopodia numbers in control and *Jag1*^{iGOF} retinas.

(E) Isolectin B4 (red) and BrdU labeling (green) of control and *Jag1*^{iGOF} P6 retinas (left) and quantitation of BrdU+ ECs (right) per field or normalized relative to EC coverage, as indicated. Error bars represent s.e.m.; P values are indicated. Scale bars: A and B upper panel: 80 μm; A and B lower panel: 25 μm; C: 17 μm; E: 50 μm.

Fringe Modulates Notch Signaling in a Ligand-Dependent Fashion

Next, we investigated the effect of Fringe (Mfng) modification on Dll4 and Jagged1-mediated signaling in cultured mouse ECs, for which qPCR shows low baseline *Fringe* expression. Stimulation with immobilized Dll4 or Jagged1 proteins, which can activate Notch in a cell contact-independent fashion (Li et al., 2007), leads to the upregulation of the target genes *Hes1*, *Hey1*, and *Dll4*, although to different extent (Figure 6F). In response to *Mfng* overexpression, Dll4 becomes more potent in activating Notch targets, consistent with previous findings for the related ligand Delta-like 1 in other cell types (Yang et al., 2005). In *Mfng*-overexpressing ECs, Jagged1 can induce only weak responses barely above the background of control-treated cells. Combining the recombinant Dll4 and Jagged1 used for the individual stimulation experiments leads to an intermediate response, which confirms that Jagged1 can antagonistically interfere with Dll4-Notch interactions (Figure 6F).

We also investigated the activity of Dll4 and Jagged1 in coculture assays by mixing signal-sending (ligand-presenting) and Notch1 reporter cell lines, only the latter of which contain a $(Rbpj)_6$-luciferase Notch reporter construct (Geffers et al., 2007). Expression of Mfng in Notch1 cells strongly enhances reporter activation by Dll4 cells, whereas Mfng expression has little effect in ligand-presenting cells (Figure 6G, columns 1–6). Similar to the trans-inhibition observed with immobilized Jagged1 and Dll4 proteins, coexpression of Jagged1 and Dll4 in ligand cells strongly reduces the activation of $(Rbpj)_6$-luciferase in adjacent Mfng-expressing Notch1 reporter cells compared to Dll4 alone (Figure 6G, columns 6 and 7). Expression of the two ligands in separate cell populations (i.e., Dll4 in signal-sending and Jagged1 in Mfng+ Notch1 reporter cells) also reduces luciferase expression (Figure 6G, columns 6 and 8). While this effect might hint at Jagged1-mediated *cis*-inhibition of Notch in this assay, $(Rbpj)_6$-luciferase activation was enhanced and not inhibited by Jagged1 when Notch1 cells lacked Mfng (Figure 6G, compare columns 1 and 9). Both in vitro assays indicate that Jagged1 is a productive (agonistic) ligand when Notch1 is not Fringe-modified, but is rendered into an antagonist that

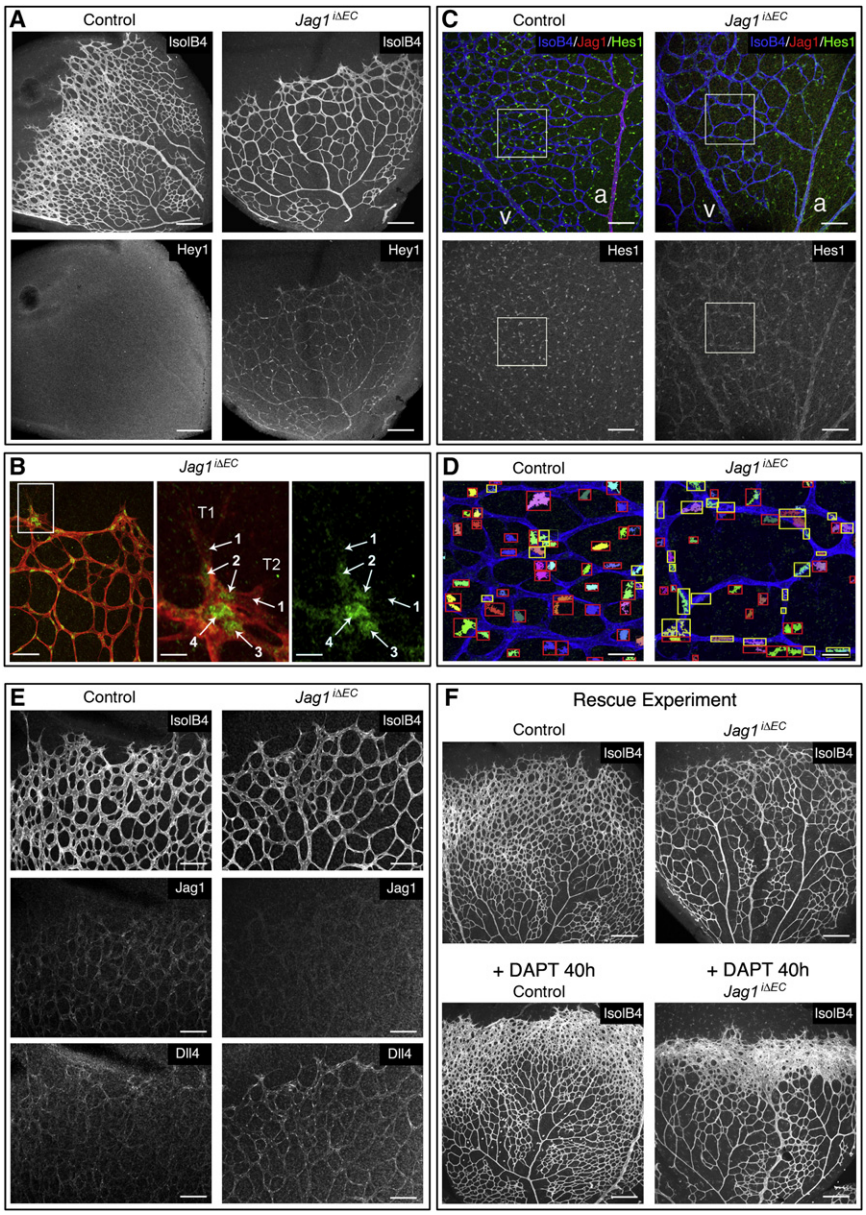

Figure 5. Jagged1 Inhibits Dll4-Notch Signaling in Endothelial Cells

(A) Whole-mount immunofluorescence showing upregulated Hey1 protein in the P6 *Jag1*$^{i\Delta EC}$ retinal vasculature.

(B) Hey1 localization (green) in the angiogenic *Jag1*$^{i\Delta EC}$ retinal endothelium (IsolB4, red). Higher magnification of inset (center) and corresponding channel with Hey1 signal (right) are shown. Note pronounced expression and nuclear localization (arrows) of Hey1 in stalk ECs (numbers indicate signal intensity level), whereas specific signal is weak or absent in filopodia-extending tip cells (T1, T2).

(C) Whole-mount triple immunofluorescence (IsolB4, blue; Jagged1, red; Hes1, green) of P6 control and *Jag1*$^{i\Delta EC}$ retinas.

(D) Analysis of Hes1 expression in higher magnification pictures of insets in (C). Cells with high anti-Hes1 signal are boxed in yellow for IsolB4+ ECs (blue) and in red for non-ECs. The number of Hes1+ ECs is increased in *Jag1*$^{i\Delta EC}$ mutants, whereas fewer Hes1+ perivascular and nonvascular cells can be seen.

(E) Whole-mount triple immunofluorescence for the indicated antigens showing upregulated Dll4 expression in the *Jag1*$^{i\Delta EC}$ endothelium.

(F) Isolectin B4–stained P7 retinas from control and *Jag1*$^{i\Delta EC}$ littermates. Injection of vehicle (upper panels) does not affect branching. Administration of DAPT for 40 hr before dissection (lower panels) triggers enhanced EC sprouting and formation of a dense, hyperfused plexus in the distal control and *Jag1*$^{i\Delta EC}$ retinal endothelium. Scale bars: A and F: 200 μm; B: 70 μm and 18 μm; D: 25 μm; E: 50 μm.

competes with the more signaling-competent Dll4 ligand when Notch is glucosaminylated. Accordingly, (partial) siRNA-mediated knockdown of *Fringe* gene expression in cultured human ECs reduces Dll4-mediated Notch signaling, whereas Jagged1 becomes a more potent activator (Figure S5).

Jag1 and Dll4 in the Angiogenic Growth Program

To understand the interplay between the two ligands during EC sprouting, we have analyzed the spatial distribution of Jagged1 and Dll4 in the retina. As previously shown, Dll4 is enriched at the angiogenic front and labels tip ECs as well as a fraction of stalk cells (Claxton and Fruttiger, 2004; Hellstrom et al., 2007; Hofmann and Luisa Iruela-Arispe, 2007; Lobov et al., 2007). In contrast, anti-Jagged1 immunostaining is weak or absent in tip cells but very prominent in adjacent stalk ECs (Figures 7A and 7B). Since our findings have established that the *Jag1* and *Dll4* genes have complementary functional roles, one would predict that angiogenesis can be modulated by upstream signals that regulate these two ligands differentially. For example, cell contact–mediated Notch activation upregulates the expression of *Dll4* in cultured mouse or human ECs but has no effect on *Jag1* transcript levels (Figures 7C and S6A). Conversely, tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, reduces *Dll4* but increases *Jag1* expression (Sainson et al., 2008; Figure 7C).

Previous work has uncovered that Notch activation downregulates the expression of proangiogenic VEGF receptors, namely VEGFR-2 and VEGFR-3 (Hellstrom et al., 2007; Suchting et al., 2007; Tammela et al., 2008), which could help to dampen down tip-like sprouting activity in stalk cells. We observed that *Jag1* inactivation in postnatal ECs leads to strongly reduced expression of VEGFR-3 at the angiogenic front in the retina (Figure 7D), which provides a direct mechanistic explanation for impaired sprouting and EC proliferation in *Jag1*$^{i\Delta EC}$ mutants.

Low or absent Notch activity leads to upregulated VEGF receptor expression, and, accordingly, Notch1- or Rbpsuh-deficient

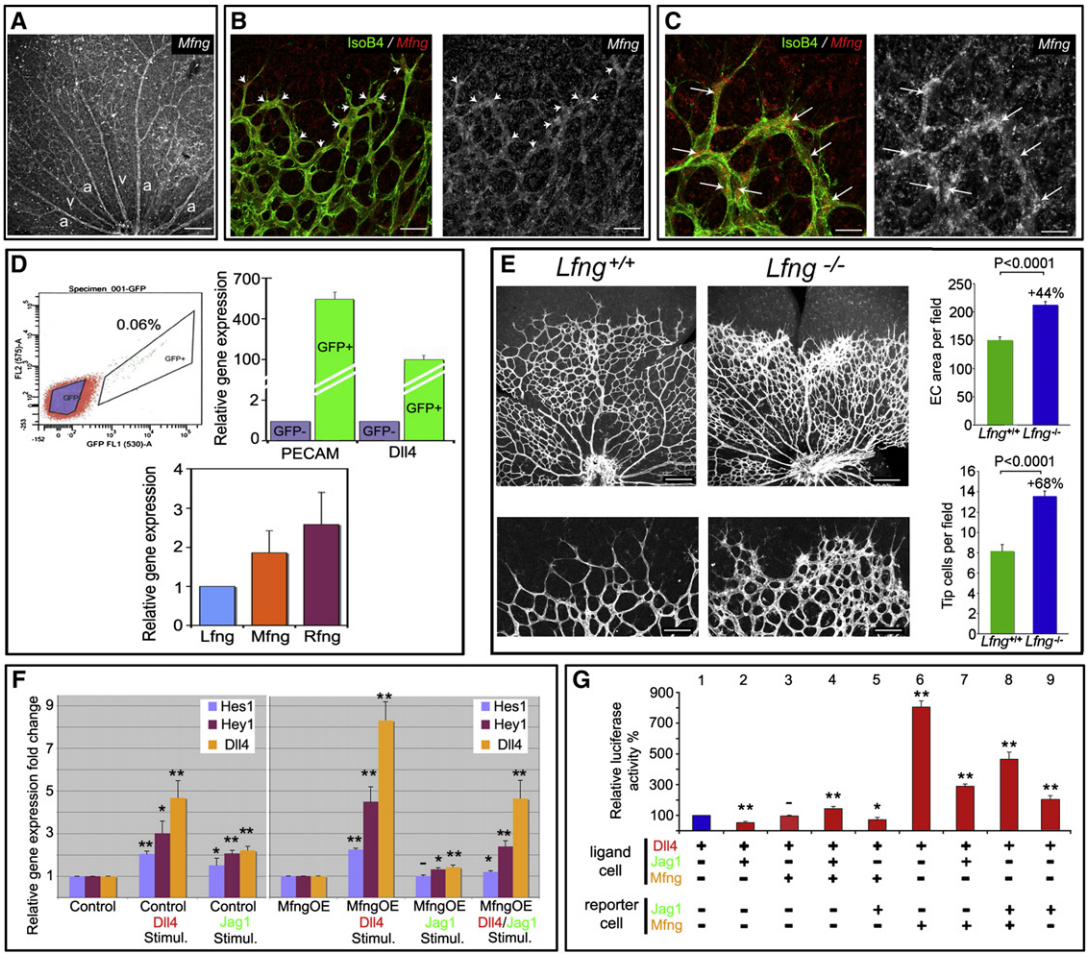

Figure 6. *Mfng* Modulates Endothelial Notch Signaling by Jag1 and Dll4

(A–C) Whole-mount immunofluorescence for Isolectin B4 in the P6 retina in combination with fluorescent in situ hybridization for *Mfng* as indicated. (A) *Mfng* is expressed in arterioles (a), venules (v), and a subset of capillary ECs. (B and C) *Mfng* transcripts in the angiogenic front tip (arrows in B). Strongest staining highlights subsets of both stalk and tip cells (arrows in C).

(D) qRT-PCR analysis of *PECAM1*, *Dll4*, *Lfng*, *Rfng*, and *Mfng* expression in FACS-sorted ECs (0.06% of total cells) from the P9 retina.

(E) Confocal images showing increased vessel branching and high EC coverage in isolectin B4–stained *Lfng*-deficient P4 retinas (left panels) and quantitation of tip cell number and EC area in comparison to wild-type littermates (right panels).

(F) Quantitative RT-PCR analysis of *Hes1*, *Hey1*, and *Dll4* mRNAs from cultured control (left) or *Mfng*-overexpressing mouse ECs (right) following stimulation with immobilized recombinant Dll4, Jagged1, or a mixture (1:1) of both ligands. Expression of mouse *Gapdh* was used as control and changes were calculated relative to unstimulated cells.

(G) Notch coculture assay. Modulation of $(Rbpj)_6$-luciferase activity in Notch1 (reporter) cells mixed with signal-sending (ligand) cells stably expressing Dll4. Effects of transient expression of Mfng or Jagged1 in reporter or ligands cells are shown, as indicated (n = 5). Equal expression levels of Jagged1 were confirmed by Western blot analysis. Scale bars: A: 100 μm; B: 50 μm; C: 22 μm; E: 200 μm and 77 μm. p values are < 0.05 (*), < 0.01 (**), or not significant (-).

ECs preferentially formed tip cells when mosaic experiments were performed in mouse or zebrafish (Hellstrom et al., 2007; Siekmann and Lawson, 2007). Consistent with the concept that coexpression of Jagged1 in tips would impair the ability of Dll4 to signal to stalk cells, *Jag1*-deficient ECs expressing a fluorescent protein Cre reporter are preferentially found at the tip position in mosaic mutants induced with a low dose of tamoxifen (Figure 7E). On the other hand, ECs with higher Jagged1 levels are preferentially sorted to the stalk in the $Jag1^{iGOF}$ retinal vasculature (Figures 4A and 4B). The sum of all our results delineates a molecular pathway of tip cell selection that is controlled by the interplay of two Notch ligands, Dll4 and Jagged1.

DISCUSSION

Dll4 and Jagged1 Control Tip Cell Selection

Angiogenesis requires a tightly coordinated balance between EC sprouting and the maintenance of existing vascular tubes. Previous work has shown that this equilibrium can be controlled by Dll4 expression in endothelial tip cells, which activates Notch signaling and thereby suppresses sprouting in adjacent ECs. Accordingly, impaired Dll4/Notch expression or function leads to excessive but nonproductive sprouting because too many ECs respond to proangiogenic growth factors such as VEGF. However, the spatial expression pattern of Dll4 is not fully

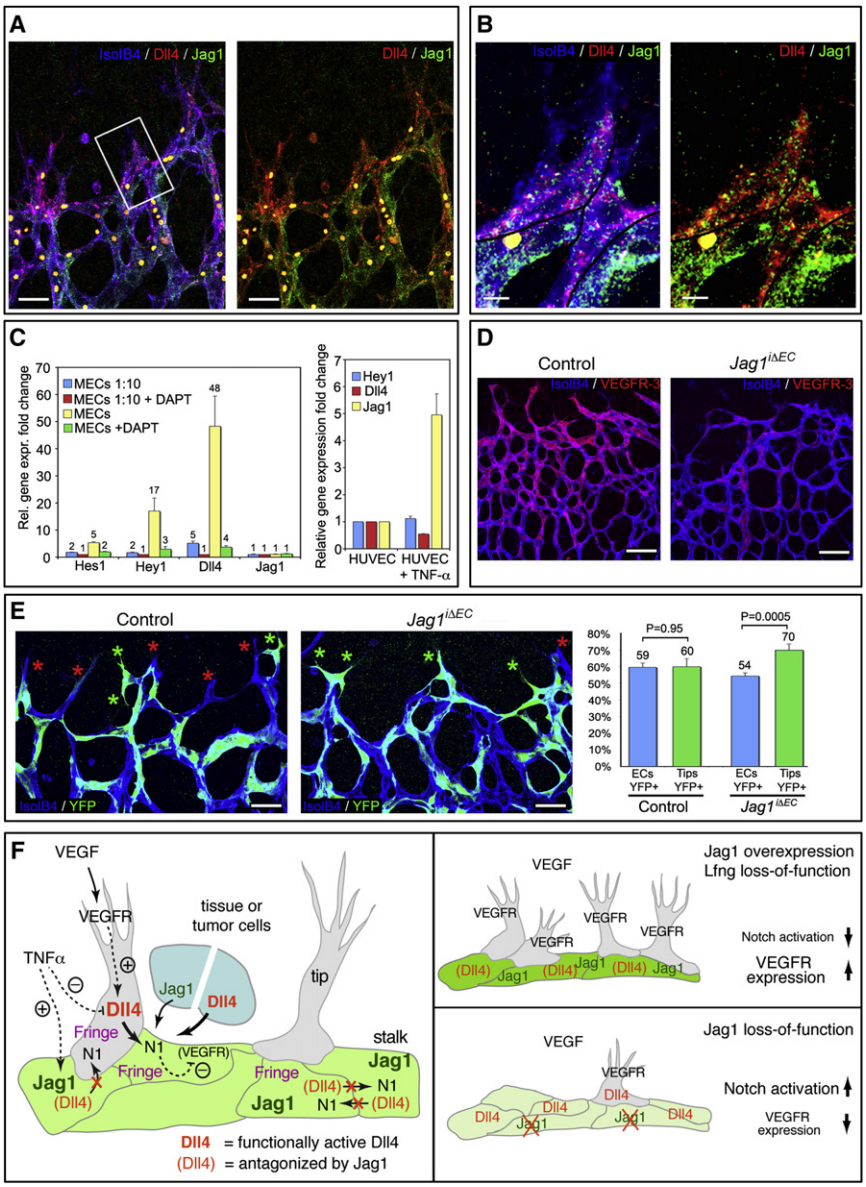

Figure 7. Regulation of Sprouting Angiogenesis by Dll4 and Jagged1

(A and B) Triple whole-mount immunofluorescence for Dll4 (red), Jagged1 (green), and isolectin B4 (blue) at the angiogenic front of P6 retinas. (B) Higher magnification of the inset in (A). Individual channels and merged images are shown as indicated. While Dll4 expression is high in tip cells and also visible in adjacent stalk ECs at the edge of the growing plexus, Jagged1 expression is low/absent in tips but abundant in adjacent stalk cells and capillaries. Yellow dots are autofluorescent blood cells.

(C) qRT-PCR analyses of *Hes1*, *Hey1*, *Dll4*, and *Jag1* mRNAs in mouse ECs (MECs) cultured at low density (1:10) or in confluent conditions in the presence or absence of DAPT (left). Changes in *Hey1*, *Dll4*, and *Jag1* transcripts following stimulation of human ECs (HUVECs) with TNF-α for 6 hr (right).

(D) Whole-mount isolectin B4 (blue) and VEGFR-3 (red) staining of P6 control and $Jag1^{i\Delta EC}$ retinas.

(E) Mosaic analysis showing correlation between Cre reporter (green, YFP) activation and EC sorting. YFP+ (green asterisk) and YFP− (red asterisk) tips are indicated. Quantitation (right) shows that *Jag1*-deficient but not control ECs more often become tips relative to their total proportion.

(F) Proposed model for the modulation of Dll4-Notch signaling by Jagged1 (left) and alterations in the *Jag1* and *Lfng* mutant vasculature (right). VEGF signaling in tip cells induces (+) the expression of Dll4. Fringe modification of Notch (most probably Notch1; N1) in stalk ECs enhances Notch signaling by Dll4-presenting tip cells, which reduces VEGF receptor expression and maintains the stalk phenotype. Dll4 is antagonized by Jagged1, which promotes angiogenesis and increases tip cell numbers by lowering Notch activation levels, while VEGF signaling is enhanced. Angiogenic sprouting might be positively (+) or negatively (−) modulated by differential regulation of Jagged1 and Dll4 in endothelial cells as well as in adjacent nonendothelial cells, such as tumor cells. TNF-α upregulates (+) *Jag1* but lowers (−) *Dll4* transcript levels. *Dll4* but not *Jag1* is induced by Notch signaling. Jagged1 in stalk cells prevents that coexpressed Dll4 can activate Notch in neighboring (stalk or tip) ECs. This activity of Jagged1 depends on Fringe, which reduces Notch activation by Jagged1 and thereby leads to competition between a strong agonist (Dll4) and antagonistically acting Jagged1. All these processes are presumably highly dynamic. Scale bars: A: 25 μm; B: 6 μm; D: 65 μm; E: 45 μm.

compatible with a simple tip-to-stalk signaling interaction. Our study identifies Jagged1 as a further critical component in the process of tip cell selection. In contrast to Dll4, Jagged1 is proangiogenic and functions by downregulating Dll4-Notch signaling. We propose that this is of particular importance in stalk cells, where Jagged1 levels are high and will therefore efficiently antagonize the more potent Dll4 ligand (Figure 7F). As a consequence, stalk cells should have little ability to activate Notch in adjacent tip cells. Jagged1 also counteracts Dll4-Notch signaling interactions between stalk ECs, which helps to sustain elevated VEGF receptor expression in the freshly formed and therefore immature vascular plexus at the angiogenic front. Thus, ECs in this region can still respond to VEGF, which, in turn, promotes proliferation as well as the dynamic emergence of new tip cells.

The Role of Fringe Glycosyltransferases

Our data suggest that the expression of Fringe glucosaminyltransferases is highly relevant in the growing vasculature. Loss of *Lfng* enhances angiogenic sprouting in the retinal endothelium despite the expression of *Rfng* and *Mfng*. We propose that Fringe-mediated modification of Notch critically controls tip cell selection in at least two different ways. First, Notch activation in response to Dll4 binding is enhanced, which will amplify the

ability of tip cells to signal to adjacent stalk ECs. In addition, Fringe modification will reduce Notch activation upon Jagged1 binding so that Jagged1 effectively acts as an antagonist, which competes with Dll4 and thereby locally enhances angiogenic growth. Although Notch ligands can be also modified by Fringe glycosyltransferases (Panin et al., 2002), our coculture assays indicate that receptor modification is critical, whereas coexpression of Mfng and Notch ligands has relatively minor effects on Notch activation (Figure 6G).

Given the important role of Fringe molecules in the regulation of angiogenesis, understanding the upstream signals controlling Mfng, Lfng, and Rfng expression and activity should be of great importance.

Alternative Mechanisms of Jagged1 Activity?

Despite the evidence that Jagged1 predominantly acts by blocking Dll4-Notch interactions, it is useful to consider putative alternative mechanisms. For example, Jagged1 might trigger so-called "noncanonical" Notch signaling that does not involve RBP-J, the key downstream signaling partner of Notch (Le Gall et al., 2008), and therefore might not affect the normal Notch target genes. However, the upregulation of Notch targets in the *Jag1*$^{i\Delta EC}$ endothelium and the reversal of the mutant phenotype by Notch inhibition show that Jagged1 strongly modulates "canonical" Dll4-Notch signaling.

Several studies have proposed that Delta-like/Delta and Jagged/Serrate ligands can inhibit signaling by coexpressed Notch in a cell-autonomous fashion, termed *cis*-inhibition (Glittenberg et al., 2006). Although Notch *cis*-inhibition may occur in ECs, our in vitro signaling assays and the expression of Jagged1 in stalk cells argue that the ligand predominantly inhibits Dll4-Notch signaling in a non-cell-autonomous fashion (i.e., by binding to Notch receptors on adjacent cells). *Cis*-inhibition of Notch by Jagged1 in stalk cells would impair the effect of Dll4 presented by tip cells and compromise essential tip-to-stalk signaling. For the same reason, Jagged1 reverse signaling, a process that would involve ligand cleavage and release of a cytoplasmic, Notch-inhibiting fragment (LaVoie and Selkoe, 2003; Six et al., 2003) cannot explain our findings. Stimulation of cultured ECs with immobilized, recombinant Notch1 fusion protein also has no appreciable effect on Notch target genes (Figure S6).

Differential Regulation of Dll4 and Jagged1

Given that Dll4 and Jagged1 have opposing roles in endothelial sprouting, upstream signals controlling the expression of one or the other ligand might modulate angiogenesis positively or negatively. While VEGF has been shown to induce the expression of *Dll4* in ECs (Hainaud et al., 2006; Lobov et al., 2007; Noguera-Troise et al., 2006), Jagged1 is absent in tip cells, which are exposed to the highest levels of VEGF, suggesting that the two ligands might be regulated differentially. Notch signaling is another positive regulator of Dll4 but not of Jagged1, whereas the inflammatory (and, in some settings, proangiogenic) cytokine TNF-α upregulates *Jag1* but reduces *Dll4* transcript levels. Thus, these and other signals might modulate angiogenesis by changing the ratio of Jagged1 and Dll4 expression. The existence of two Notch ligands with opposing roles and differential regulation allows the integration of different pro- or antiangiogenic signals into a single biological process, the selection of endothelial tip cells. Moreover, Notch pathway components such as Delta ligands and Lfng can be expressed in an oscillatory manner (Kageyama et al., 2007), which could provide an appealing explanation for the regulation of dynamic and repetitive processes such as endothelial sprouting.

Jagged1 and Notch Signaling in Other Tissues

Delta-like/Delta and Jagged/Serrate ligands have different biological roles in many organs and tissues, for example in the developing nervous and immune systems (Amsen et al., 2004; Brooker et al., 2006), which has been attributed to spatiotemporal differences in ligand expression or the cellular context of Notch activation. Because Fringe proteins are well-established modulators of Notch signaling (Bray, 2006; Kageyama et al., 2007), Jagged/Serrate ligands might act more frequently as antagonists of Fringe-amplified Delta-Notch signaling. Thus, our findings may have much broader relevance for the many other cell types, tissues, and biological processes that are regulated by the Notch pathway.

Therapeutic Potential of Dll4 and Jagged1 Inhibition

Several studies have found that the inhibition of Dll4 leads to enhanced but nonproductive endothelial sprouting, poor perfusion, and reduced growth of experimental tumors, which might prove particularly useful for tumors that are resistant against anti-VEGF therapy (Li et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006). Conversely, expression of Dll4 in tumor cells can block sprouting from tumor blood vessels, presumably by suppressing emerging tip cells. The remaining vasculature, however, lacks the fragility and leakiness of typical tumor blood vessels and gives good access to oxygen (Li et al., 2007; Noguera-Troise et al., 2006). Because of the links between Dll4-Notch signaling and the VEGF pathway, Dll4-expressing cancers might be more resistant to anti-VEGF therapy.

Little is known about the role of Jagged1 in tumor vessels, but overexpression of the ligand in cancer cells has been shown to promote neovascularization and the growth of experimental tumors in mice (Zeng et al., 2005). These results are consistent with the proangiogenic activity of Jagged1 during developmental angiogenesis. If intratumor ECs express Fringe molecules, Jagged1 might reduce Notch signaling and thereby enhance responses to growth factors such as VEGF. It is feasible that such tumors are more susceptible to VEGF inhibition or other antiangiogenic treatments. In other pathological situations where angiogenesis is deregulated, selective inhibition of Dll4 or Jagged1 might allow the transient enhancement or suppression of blood vessel growth. The benefit of such treatments will have to be explored in future work.

EXPERIMENTAL PROCEDURES

Mutant Mice and Inducible Genetic Experiments

To delete *Jag1* in ECs, *Tie1*-Cre transgenics (Gustafsson et al., 2001) were bred into a background of *Jag1*$^{floxed/floxed}$ mice (Brooker et al., 2006), and embryos were analyzed at different stages. For postnatal EC-specific loss-of-function experiments, *Pdgfb*-iCreER (Claxton et al., 2008) *Jag1*$^{floxed/floxed}$ or *Pdgfb*-iCreER *Jag1*$^{KOnull/floxed}$ males were mated with *Jag1*$^{floxed/floxed}$ females. Gene

inactivation in pups was triggered by intraperitoneal injection of 50 μl of tamoxifen solution (Sigma, T5648; 1 mg/ml; generated by diluting a 10 mg/ml tamoxifen stock solution in 1:4 ethanol:peanut oil with peanut oil) once daily at P1, P2, and P3 or at P5 and P6, respectively.

For the overexpression of *Jag1* in ECs, a single copy of the full-length murine *Jag1* cDNA coupled to a tetracycline-responsive minimal promoter (Gossen and Bujard, 1992) was introduced into embryonic stem (ES) cells by targeted integration into the X-linked HPRT locus. Blastocyst injection of validated ES cell clones yielded tetO-*Jag1* transgenic mice, which were bred to VE-Cadherin-tTA transgenics (Sun et al., 2005). To avoid lethality triggered by Jagged1 overexpression in the embryonic endothelium, pregnant females were given tetracycline (1 mg/ml) in the drinking water from E1.5 to E14.5.

Notch signaling was inhibited in some pups injected with tamoxifen from P1 until P3 by intraperitoneal injection of 0.2 mg/g N-[N-(3,5–Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT; Calbiochem) dissolved in 10% ethanol and 90% peanut oil. DAPT solution was injected twice at P5 and P6, and retinas were collected 40 hr later at P7. Control mice were injected with vehicle only.

For mosaic deletion of Jagged1 in the retina, *Jag1*$^{floxed/floxed}$ were combined with VE-Cadherin(PAC)-CreERT2 (R.H.A., unpublished data) and Rosa26 EYFP Cre reporter (Srinivas et al., 2001) transgenes. P4 *Jag1* mutant and control CreERT2+ and Rosa26 EYFP+ pups received one intraperitoneal injection of 25 μl of tamoxifen solution (1 mg/ml), and retinas were collected 48 hr later for analysis.

To study the role of the *Lfng* gene in the retinal vascular development, *Lfng*+/− mice (Zhang and Gridley, 1998) were interbred. *Lfng*−/− and +/+ retinas were isolated and analyzed at P4. Animal experiments comply with the relevant laws and were approved by local animal ethics committees.

Immunohistochemistry and In Situ Hybridization

Embryos were dissected in PBS and fixed in 4% paraformaldehyde (PFA) overnight, and skin from the head region was used for whole-mount immunostaining, as described previously (Foo et al., 2006). Retinas for double or triple whole-mount immunohistochemistry were fixed in 4% PFA overnight at 4°C, 2 hr on ice in PFA 2%, or in MeOH at −20°C. After fixation, retinas were incubated in 1% BSA and 0.3% Triton, washed three times in Pblec buffer (1% Triton X-100, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2 in PBS [pH 6.8]), and incubated overnight in Pblec containing biotinylated isolectin B4 (1:25, VectorLabs). The following primary antibodies were diluted in 1% BSA and 0.3% Triton and incubated overnight: PECAM1 (1:100, PharMingen), GFP (1:500, Molecular Probes), Hey1 (1:200, Chemicon), Hes1 (1:200, Santa Cruz), Dll4 (1:200, R&D Systems), Jagged1 H114 (1:200, Santa–Cruz), collagen IV (1:200, Chemicon), VEGFR-3 (1:100, R&D Systems), αSMA-CY3 (Sigma), and desmin (1:500, Abcam). For secondary detection, Alexa Fluor streptavidin conjugates (Molecular Probes, 1:100) or species-specific Alexa Fluor–coupled secondary antibodies (1:500) were used. Cell nuclei were visualized with TO-PRO 3 (Molecular Probes, 1:1000).

For labeling of proliferating cells, 300 μg of BrdU per pup was injected intraperitoneally 2 hr before sacrifice. Following isolectin B4 staining, retinas were fixed for 30 min in 4% PFA, washed 3 times with PBS, incubated for 1 hr in 6 M HCl and 0.1% Triton X-100, washed 5 times in PBS plus 0.1% Triton X-100, blocked, and incubated overnight with anti-BrdU antibody (1:50, BD PharMingen). Secondary detection was performed with Alexa Fluor-coupled secondary antibodies.

Whole-mount in situ hybridization was performed as published previously (Claxton and Fruttiger, 2004; Myat et al., 1996). A linearized murine *Mfng* cDNA was used for the transcription of a digoxigenin-labeled anti-sense riboprobe. Whole eyes were fixed for 2 hr on ice in 4% PFA and stored in Methanol at −20°C. Bound riboprobes were visualized with anti-digoxigenin antibody conjugated to alkaline phosphatase (1:2000, Roche) and Fast Red tablets (Roche). Afterward, retinas were fixed for 1 hr and immunostained with biotinylated isolectin B4.

Stained and flat mounted retinas were analyzed with a Leica TCS SP5 confocal microscope. In situ hybridization for the detection of *Mfng* or *Lfng* transcripts in tissue cryosections was performed as described previously (Myat et al., 1996), but with Fast Red tablets (Roche) for the visualization of digoxigenin-labeled riboprobes.

EC Isolation, Culture, and qRT-PCR

For the stable overexpression of murine Mfng, the full-length cDNA was inserted into the pBABE-puro retroviral construct. pBABE-puro (control) or the pBABE-puro-Mfng constructs were transfected into Phoenix packaging cells, and the virus-containing supernatant of these cells was used to infect immortalized mouse ECs (see Supplemental Experimental Procedures). Stable expressing clones were obtained by selection with 12.5 μg/ml puromycin. qRT-PCR revealed a 125-fold upregulation of Mfng transcripts, compared with the low baseline expression in control cells.

For Notch stimulation with recombinant ligands, 6-well plates were incubated with anti-Fc (Jackson), anti-His (Zymed), or a 1:1 mixture of both antibodies (6.48 μg/ml) for 30 min at 37°C. Plates were washed with PBS and blocked for 1 hr at 37°C with 10% FCS in DMEM. Recombinant Notch1-Fc (R&D, #1057-TK), Dll4 (mouse Dll4-His R&D; #1389-D4), or Jagged1 (rat Jag1-Fc, R&D #599–JG), diluted in PBS to a concentration of 18 nM, or a 1:1 mixture of both ligands were added (700 μl/well) and incubated for 2 hr at 37°C and washed; 3.5 × 10^5 cells/well control or Mfng-overexpressing ECs were plated and left for 8 hr at 37°C.

TNF-α stimulations of HUVECs were done with 2 ng/ml for 6 hr. To inhibit Notch signaling in vitro, MECs or HUVECs were treated with 10 μM DAPT.

To block Fringe expression in cultured cells, transfection with combined 100 nM smartpool siRNAs for Mfng, Rfng, and Lfng (Dharmacon) or 300 nM control siRNA was performed according to the manufacturer's instructions. After 48 hr, cells were detached by trypsinization and stimulated with recombinant Dll4 or Jagged1 ligands as described above.

For the analysis of gene expression, total RNA was isolated with the RNAeasy mini kit (QIAGEN), and 500 ng/reaction were used to generate cDNA with the SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. qPCR was performed in triplicate by using an ABI PRISM 7900HT and Power SYBR green Master Mix (Applied Biosystems). Gene expression was normalized to Glyceraldehyde 3-phosphate dehydrogenase (Gapdh). The relative expression differences represent the average of 3 independent stimulation experiments. Two separate qPCRs with triplicate reactions for each gene and condition were performed (see Supplemental Data for primer details).

For the isolation of retinal ECs, retinas from P9 Pdgfb-iCreER (Claxton et al., 2008) pups were dissected and dissociated with the Papain dissociation system (Worthington). GFP + and GFP− cells were separated with the FACSAria Cell-Sorting System (BD Biosciences) and collected in lysis buffer, and total RNA was extracted with the RNAeasy Mini Kit (QIAGEN).

Transactivation (Coculture) Notch Assay

CHO cells stably expressing Dll4 were transiently transfected with Jag1 and or Mfng expression plasmids using jetPEI DNA transfection reagent following the manufacturer's instructions. HeLa cells stably expressing Notch1 (Minoguchi et al., 1997) were transiently transfected with Jag1 and/or Mfng expression plasmids and the RBP–luciferase reporter (Minoguchi et al., 1997) construct; 1 × 10^6 transfected HeLa reporter cells were cocultivated with 1 × 10^6 CHO ligand cells in 6-well plates for 24 hr. Luciferase activity was measured using the Dual-Luciferase reporter Assay System (Promega). Firefly luciferase activity was normalized to cotransfected Renilla luciferase (phRL-TK, Promega). Equal levels of transient Jagged1 expression were validated by western blot. Reporter activation by untransfected parental CHO cells was taken as baseline and subtracted from each data point.

Statistics and Image Processing

Volocity (Improvision), Photoshop CS, and Illustrator CS (Adobe) software were used for image processing in compliance with the "Cell Press Data Processing Policy." Data are based on a minimum of three independent experiments or three mutant and control animals for each stage and result shown. Methods for the quantitative analysis of the retinal vasculature are provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00324-9.

ACKNOWLEDGMENTS

We thank L. Benjamin, T. Gridley, and J. Lewis for providing VE-Cadherin-tTA, *Lfng*, and floxed *Jag1* mice; K. Serth and K. Schuster-Gossler for isolating *Lfng* eyes; and M. Stehling for the FACS isolation of ECs. This study was funded by Cancer Research UK, the Max Planck Society, the University of Muenster, and the Deutsche Forschungsgemeinschaft for the Cluster of Excellence REBIRTH.

Received: August 21, 2008
Revised: December 22, 2008
Accepted: March 16, 2009
Published: June 11, 2009

REFERENCES

Amsen, D., Blander, J.M., Lee, G.R., Tanigaki, K., Honjo, T., and Flavell, R.A. (2004). Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell *117*, 515–526.

Baluk, P., Morikawa, S., Haskell, A., Mancuso, M., and McDonald, D.M. (2003). Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. *163*, 1801–1815.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. *7*, 678–689.

Brooker, R., Hozumi, K., and Lewis, J. (2006). Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. Development *133*, 1277–1286.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932–936.

Claxton, S., and Fruttiger, M. (2004). Periodic Delta-like 4 expression in developing retinal arteries. Gene Expr. Patterns *5*, 123–127.

Claxton, S., Kostourou, V., Jadeja, S., Chambon, P., Hodivala-Dilke, K., and Fruttiger, M. (2008). Efficient, inducible Cre-recombinase activation in vascular endothelium. Genesis *46*, 74–80.

Enge, M., Bjarnegard, M., Gerhardt, H., Gustafsson, E., Kalen, M., Asker, N., Hammes, H.P., Shani, M., Fassler, R., and Betsholtz, C. (2002). Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. EMBO J. *21*, 4307–4316.

Fischer, A., and Gessler, M. (2007). Delta-Notch–and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res. *35*, 4583–4596.

Foo, S.S., Turner, C.J., Adams, S., Compagni, A., Aubyn, D., Kogata, N., Lindblom, P., Shani, M., Zicha, D., and Adams, R.H. (2006). Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. Cell *124*, 161–173.

Geffers, I., Serth, K., Chapman, G., Jaekel, R., Schuster-Gossler, K., Cordes, R., Sparrow, D.B., Kremmer, E., Dunwoodie, S.L., Klein, T., et al. (2007). Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. J. Cell Biol. *178*, 465–476.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J. Cell Biol. *161*, 1163–1177.

Glittenberg, M., Pitsouli, C., Garvey, C., Delidakis, C., and Bray, S. (2006). Role of conserved intracellular motifs in Serrate signalling, cis-inhibition and endocytosis. EMBO J. *25*, 4697–4706.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA *89*, 5547–5551.

Gustafsson, E., Brakebusch, C., Hietanen, K., and Fassler, R. (2001). Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. J. Cell Sci. *114*, 671–676.

Hainaud, P., Contreres, J.O., Villemain, A., Liu, L.X., Plouet, J., Tobelem, G., and Dupuy, E. (2006). The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-Ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions. Cancer Res. *66*, 8501–8510.

Harrington, L.S., Sainson, R.C., Williams, C.K., Taylor, J.M., Shi, W., Li, J.L., and Harris, A.L. (2008). Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. Microvasc. Res. *75*, 144–154.

Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N., et al. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 722–723.

High, F.A., Lu, M.M., Pear, W.S., Loomes, K.M., Kaestner, K.H., and Epstein, J.A. (2008). Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc. Natl. Acad. Sci. USA *105*, 1955–1959.

Hofmann, J.J., and Luisa Iruela-Arispe, M. (2007). Notch expression patterns in the retina: An eye on receptor-ligand distribution during angiogenesis. Gene Expr. Patterns *7*, 461–470.

Kageyama, R., Masamizu, Y., and Niwa, Y. (2007). Oscillator mechanism of Notch pathway in the segmentation clock. Dev. Dyn. *236*, 1403–1409.

LaVoie, M.J., and Selkoe, D.J. (2003). The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J. Biol. Chem. *278*, 34427–34437.

Le Gall, M., De Mattei, C., and Giniger, E. (2008). Molecular separation of two signaling pathways for the receptor, Notch. Dev. Biol. *313*, 556–567.

Leslie, J.D., Ariza-McNaughton, L., Bermange, A.L., McAdow, R., Johnson, S.L., and Lewis, J. (2007). Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. Development *134*, 839–844.

Li, J.L., Sainson, R.C., Shi, W., Leek, R., Harrington, L.S., Preusser, M., Biswas, S., Turley, H., Heikamp, E., Hainfellner, J.A., et al. (2007). Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. Cancer Res. *67*, 11244–11253.

Liao, D., and Johnson, R.S. (2007). Hypoxia: a key regulator of angiogenesis in cancer. Cancer Metastasis Rev. *26*, 281–290.

Lobov, I.B., Renard, R.A., Papadopoulos, N., Gale, N.W., Thurston, G., Yancopoulos, G.D., and Wiegand, S.J. (2007). Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc. Natl. Acad. Sci. USA *104*, 3219–3224.

Minoguchi, S., Taniguchi, Y., Kato, H., Okazaki, T., Strobl, L.J., Zimber-Strobl, U., Bornkamm, G.W., and Honjo, T. (1997). RBP-L, a transcription factor related to RBP-Jkappa. Mol. Cell. Biol. *17*, 2679–2687.

Myat, A., Henrique, D., Ish-Horowicz, D., and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. Dev. Biol. *174*, 233–247.

Nichols, J.T., Miyamoto, A., Olsen, S.L., D'Souza, B., Yao, C., and Weinmaster, G. (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. J. Cell Biol. *176*, 445–458.

Noguera-Troise, I., Daly, C., Papadopoulos, N.J., Coetzee, S., Boland, P., Gale, N.W., Lin, H.C., Yancopoulos, G.D., and Thurston, G. (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature *444*, 1032–1037.

Panin, V.M., Shao, L., Lei, L., Moloney, D.J., Irvine, K.D., and Haltiwanger, R.S. (2002). Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. J. Biol. Chem. *277*, 29945–29952.

Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W.C., Chanthery, Y., Kowalski, J., Watts, R.J., Callahan, C., Kasman, I., et al. (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature *444*, 1083–1087.

Roca, C., and Adams, R.H. (2007). Regulation of vascular morphogenesis by Notch signaling. Genes Dev. *21*, 2511–2524.

Ryan, M.J., Bales, C., Nelson, A., Gonzalez, D.M., Underkoffler, L., Segalov, M., Wilson-Rawls, J., Cole, S.E., Moran, J.L., Russo, P., et al. (2008). Bile duct proliferation in Jag1/fringe heterozygous mice identifies candidate modifiers of the alagille syndrome hepatic phenotype. Hepatology *48*, 1989–1997.

Sainson, R.C., Johnston, D.A., Chu, H.C., Holderfield, M.T., Nakatsu, M.N., Crampton, S.P., Davis, J., Conn, E., and Hughes, C.C. (2008). TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. Blood *111*, 4997–5007.

Schweisguth, F. (2004). Regulation of notch signaling activity. Curr. Biol. *14*, R129–R138.

Shawber, C.J., Das, I., Francisco, E., and Kitajewski, J. (2003). Notch signaling in primary endothelial cells. Ann. N Y Acad. Sci. *995*, 162–170.

Siekmann, A.F., and Lawson, N.D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. Nature *445*, 781–784.

Six, E., Ndiaye, D., Laabi, Y., Brou, C., Gupta-Rossi, N., Israel, A., and Logeat, F. (2003). The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. Proc. Natl. Acad. Sci. USA *100*, 7638–7643.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., and Eichmann, A. (2007). The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc. Natl. Acad. Sci USA *104*, 3225–3230.

Sun, J.F., Phung, T., Shiojima, I., Felske, T., Upalakalin, J.N., Feng, D., Kornaga, T., Dor, T., Dvorak, A.M., Walsh, K., et al. (2005). Microvascular patterning is controlled by fine-tuning the Akt signal. Proc. Natl. Acad. Sci. USA *102*, 128–133.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., et al. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature *454*, 656–660.

Xue, Y., Gao, X., Lindsell, C.E., Norton, C.R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E.B., Weinmaster, G., and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum. Mol. Genet. *8*, 723–730.

Yang, L.T., Nichols, J.T., Yao, C., Manilay, J.O., Robey, E.A., and Weinmaster, G. (2005). Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. Mol. Biol. Cell *16*, 927–942.

Zeng, Q., Li, S., Chepeha, D.B., Giordano, T.J., Li, J., Zhang, H., Polverini, P.J., Nor, J., Kitajewski, J., and Wang, C.Y. (2005). Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell *8*, 13–23.

Zhang, N., and Gridley, T. (1998). Defects in somite formation in lunatic fringe-deficient mice. Nature *394*, 374–377.

Zhang, N., Norton, C.R., and Gridley, T. (2002). Segmentation defects of Notch pathway mutants and absence of a synergistic phenotype in lunatic fringe/radical fringe double mutant mice. Genesis *33*, 21–28.

Cell, Volume *137*

Supplemental Data

The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis

Rui Benedito, Cristina Roca, Inga Sörensen, Susanne Adams, Achim Gossler, Marcus Fruttiger, and Ralf H. Adams

Supplemental Experimental Procedures

Isolation and culture of immortalized mouse ECs

For the isolation of ECs, lungs and hearts of wild–type mice carrying the 'immorto' transgene (Jat et al., 1991) were treated with 0.25% collagenase (Gibco) at 37°C for 30min and passed through a 100μm cell strainer. The retained tissue was incubated in 0.25% collagenase, 1 U/ml dispase (Gibco) at 37°C for 3hrs and passed through a cell strainer. Cells in this flow–through were collected by centrifugation (5min, 210g), washed in DMEM + 10% FCS, placed in Medium200 (Invitrogen) and cultured for 3 days. ECs were selected using a biotinylated anti–PECAM1 (Becton Dickinson) antibody and the CELLection Biotin Binder Kit (Dynal) according to the manufacturer's instructions. The selected cells were cultured at 33ºC and 5% CO2 in the presence of 50U/ml IFNγ and 5ng/ml VEGF–A165 (PeproTech) in DMEM containing 10% FCS, 3mM L–glutamine, 1x NEAA, 1mM sodium pyruvate and Pen–strep. Staining with antibodies against mouse PECAM1 (Becton Dickinson) confirmed the endothelial identity of the cells.

Quantitative analysis of the retinal vasculature

All quantifications were done with high–resolution confocal images representing a thin z–section of the sample. The number of branchpoints and the area covered by ECs were

calculated with the Volocity (Improvision) software from 24 fields sized 770x770μm, 6 retina samples per group. The number of endothelial tip cells and filopodial extensions were quantified at the angiogenic front. Tip cells were defined as protusive endothelial cells with filopodial extensions. Tip cells and filopodia were counted in 36 fields (sized 385x385μm, 6 retinas per group) of control (*Jag1*$^{flox/flox}$) and *Jag1*$^{i\Delta EC}$ retinas and 24 fields (sized 205x205μm, 6 retinas per group) of control (tetO–*Jag1*) and *Jag1*^{iGOF} retinas. The total number of filopodia was normalized for a standard size (100μm in length) of vessels at the angiogenic front. BrdU–labeled isolectin B4–positive ECs were counted in 20 fields (sized 385x385μm, 5 retinas per group).

Signal intensity for Jagged1 staining was measured with the ImageJ software. Individual cells in Figure 4A, B were delimited by lines between the nucleus. Each dot represents a TO–PRO3 labeled nucleus enclosed by Isolectin B4–labeled endothelial cell membrane. For each cell, the IsolectinB4+ area was delimited and the average Jagged1 signal intensity calculated by the software. Number represents the interval signal level increasing from 1st (lowest) to 4th (highest). Average intensity for Hey1 nuclear staining in the Jag1$^{i\Delta EC}$ retinas (Fig. 5 B) was also measured with the ImageJ software.

Quantitation of total and endothelial Hes1+ cells was done on images representing 16 fields (sized 385x385μm, 4 retinas per group) of isolectin B4 and Hes1 double stained retinas. Volocity software was used to select objects (cell structures) with Hes1 signal above background level. Objects colocalizing with ECs (isolectin B4+) or isolectin B4–negative non–endothelial cells were quantified.

Tip cells were counted in 36 fields (sized 205x205 μM, 6 retinas per group) of *Lfng*+/+ and *Lfng*-/- retinas.

In the mosaic inactivation of the *Jag1* gene in the retina, 24 fields (sized 385x385um, 4 retinas

per group) of each group were analyzed. Total endothelial cell area (IsolectinB4+) and YFP+ endothelial cell area was quantified using Volocity (Improvision).

qPCR primers

Primers were mDll4 (5'–ggaaccttctcactcaacatcc–3'; 5'–ctcgtctgttcgccaaatct–3'), mJag1 (5'–tctctgacccctgccataac–3'; 5'–ttgaatccattcaccagatcc–3'); mMfng (5'–caccctcagctacggtgtct–3'; 5'–gggtgtgtctgggtagagga–3'); mHes1 (5'– acaccggacaaaccaaagac–3'; 5'–cgcctcttctccatgatagg–3'); mHey1 (5'–catgaagagagctcacccaga–3'; 5'–cgccgaactcaagtttcc–3'), and mGapdh (5'–accacagtccatgccatcac–3'; 5'–tccaccaccctgttgctgta–3'). In addition, Taqman gene expression assays (Applied Biosystems) for murine *Gapdh*, *Dll4*, *Hey1*, *Hes1*, *Jag1*, *Mfng*, *Rfng*, *Lfng* and *Pecam1*, and human *GAPDH*, *HEY1*, *DLL4*, *JAG1*, *MFNG*, *RFNG* and *LFNG* were used in combination with Taqman Gene Expression Master Mix.

Administration of a soluble Jagged1 peptide

It has been reported that Notch signaling can be activated by a small, soluble peptide corresponding to a sequence in the Jagged1 DSL domain (Hellstrom et al., 2007; Li et al., 1998; Tammela et al., 2008), which seems incompatible with evidence showing that physical forces (normally produced by ligand endocytosis) are required to extract the Notch extracellular part and release the NICD (Nichols et al., 2007). Nevertheless, administration of the peptide as described previously (Hellstrom et al., 2007) reduces sprouting and upregulates Dll4 expression in $Jag1^{iGOF}$ retinas that were collected after a period of 18hrs (Figure S7). This could reflect inhibition of Jagged1-Notch interactions or, as previously proposed, direct activation of Notch.

Supplemental References

Claxton, S., Kostourou, V., Jadeja, S., Chambon, P., Hodivala-Dilke, K., and Fruttiger, M. (2008). Efficient, inducible Cre-recombinase activation in vascular endothelium. Genesis *46*, 74-80.

Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N., *et al.* (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature *445*, 776-780.

Jat, P.S., Noble, M.D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L., and Kioussis, D. (1991). Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. Proc. Natl. Acad. Sci. U. S. A. *88*, 5096-5100.

Li, L., Milner, L.A., Deng, Y., Iwata, M., Banta, A., Graf, L., Marcovina, S., Friedman, C., Trask, B.J., Hood, L., *et al.* (1998). The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. Immunity *8*, 43-55.

Nichols, J.T., Miyamoto, A., Olsen, S.L., D'Souza, B., Yao, C., and Weinmaster, G. (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. J. Cell Biol. *176*, 445-458.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., *et al.* (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature *454*, 656-660.

Supplemental Figures

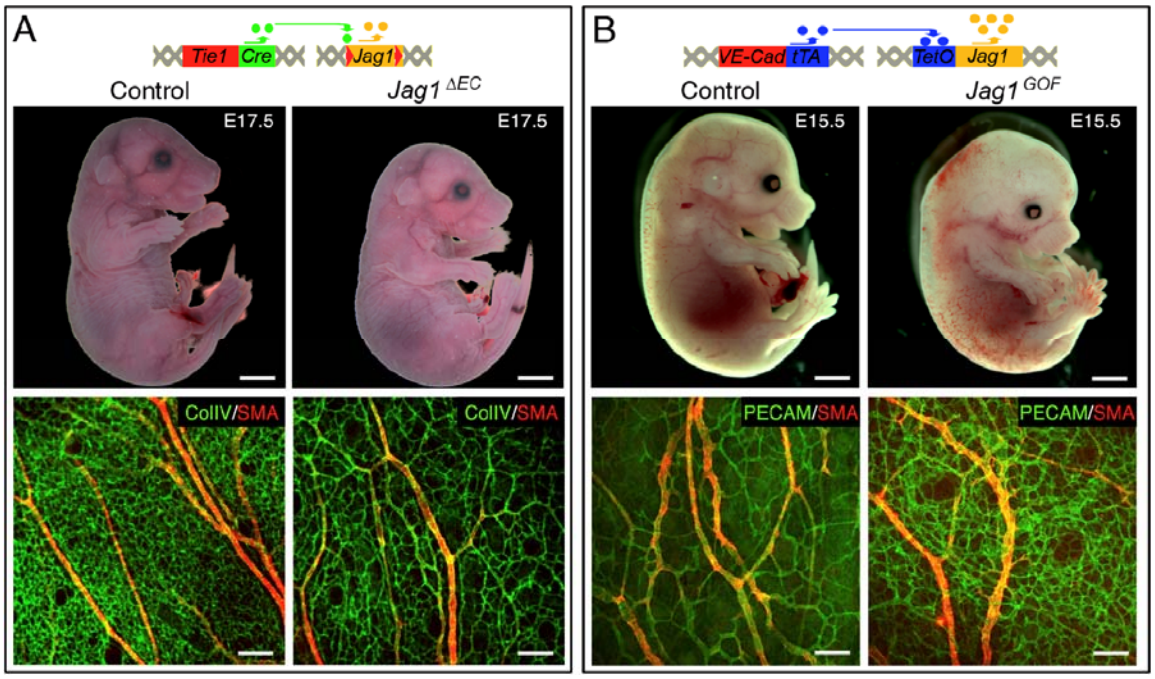

Figure S1. Defects in the *Jag1* mutant dermal vasculature

(A) Images of freshly isolated control and *Jag1*$^{floxed/floxed}$ *Tie1–Cre* (*Jag1*$^{\Delta EC}$) E17.5 embryos showing growth retardation compared to a control littermate (top panels). Whole–mount staining of skin sample from the head region shows decreased branching and density of *Jag1*$^{\Delta EC}$ capillaries compared to control (bottom panels). Primary antibodies were anti collagenIV (ColIV; green) and α–smooth muscle actin (SMA, red).

(B) Appearance of freshly isolated control and *Jag1*^{GOF} (tetO–*Jag1 x Vecad–tTA*) E15.5 embryos showing that Jagged1 overexpression leads to growth retardation and hemorrhaging of the mutant embryos (top panels). The branching and density of *Jag1*^{GOF} capillaries is increased, as shown by whole–mount staining of head skin samples for PECAM1 (green) and α–smooth muscle actin (SMA, red) (bottom panels).

Scale bars: Upper panel in A: 2mm; upper panel in B: 1.2mm. Bottom panels A, B: 200 μm.

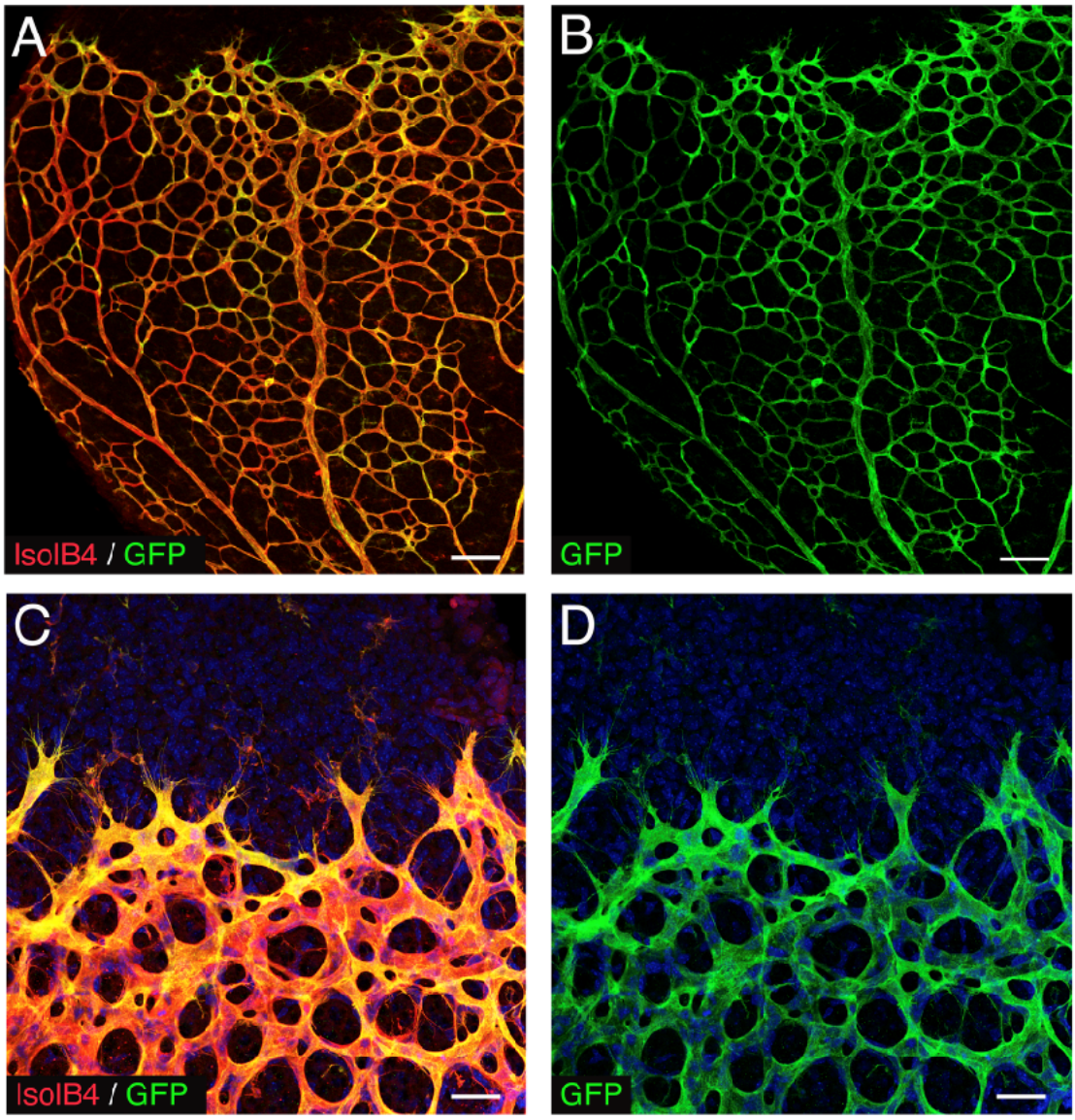

Figure S2. Pattern of *Pdgfb–iCreER* expression in the retinal vasculature

Confocal images of whole–mount P6 retina stained with anti–isolectin B4 (IsolB4, red) and anti–GFP antibodies (green), detecting the expression of an IRES–*GFP* in the *Pdgfb*–iCreER transgene (A, C), or just the isolated green channels (B, D) are shown. Prominent signal is visible throughout the retinal vasculature including arteries, vein, capillaries and tip cells. Nuclei in (C, D) were stained with Topro–3.

Scale bars: A, B: 150μm; C, D: 40μm.

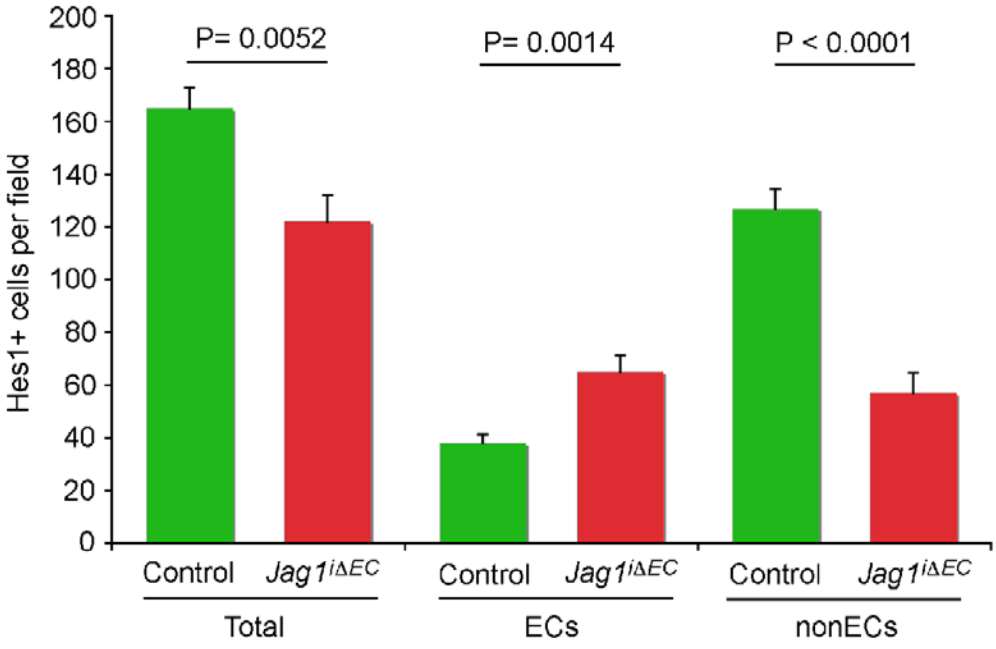

Figure S3. Hes1 expression in the *Jag1*$^{i\Delta EC}$ retina

Quantitation of Hes1+ cell populations in the control and *Jag1*$^{i\Delta EC}$ P6 retina (see Figure 5C and D). Loss of endothelial Jagged1expression leads to an increase of Hes1–expressing ECs but to a strong reduction of Hes1+ non–endothelial (largely perivascular) cells. Error bars represent s.e.m. P values are indicated.

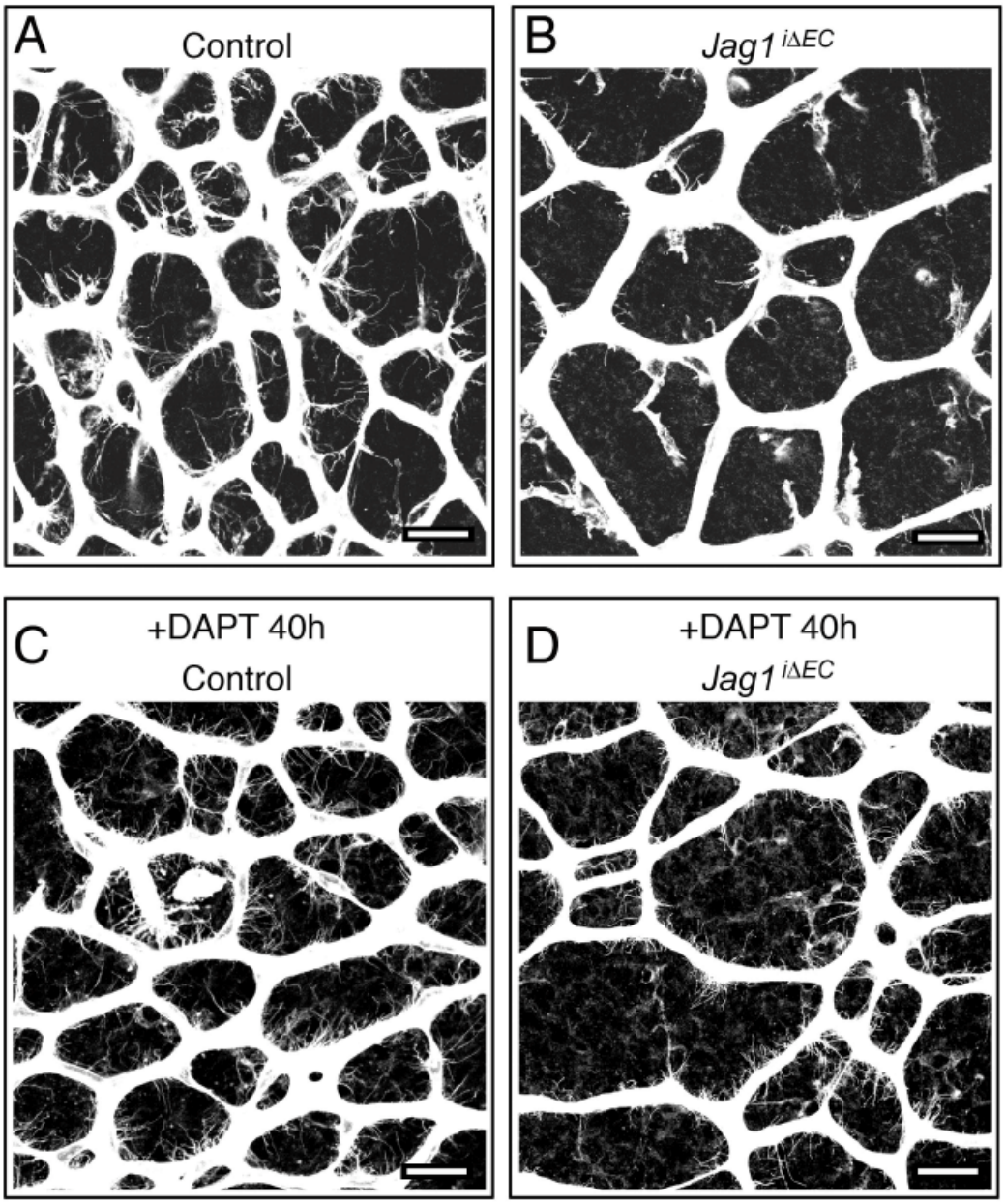

Figure S4. DAPT induces filopodia extension from established control and *Jag1*$^{i\Delta EC}$ blood vessels

(A–D) Confocal images of established, proximal retinal blood vessels stained for isolectin B4. Pups have been treated with vehicle (A, B) or DAPT (C, D) for 40hrs before analysis. Fewer filopodia extend from vehicle–treated *Jag1*$^{i\Delta EC}$ blood vessels (B) compared to control (A). In response to DAPT, a large increase in filopodia numbers can be seen both in control (C) and *Jag1*$^{i\Delta EC}$ retinas (D). Scale bars: A-D: 50μm.

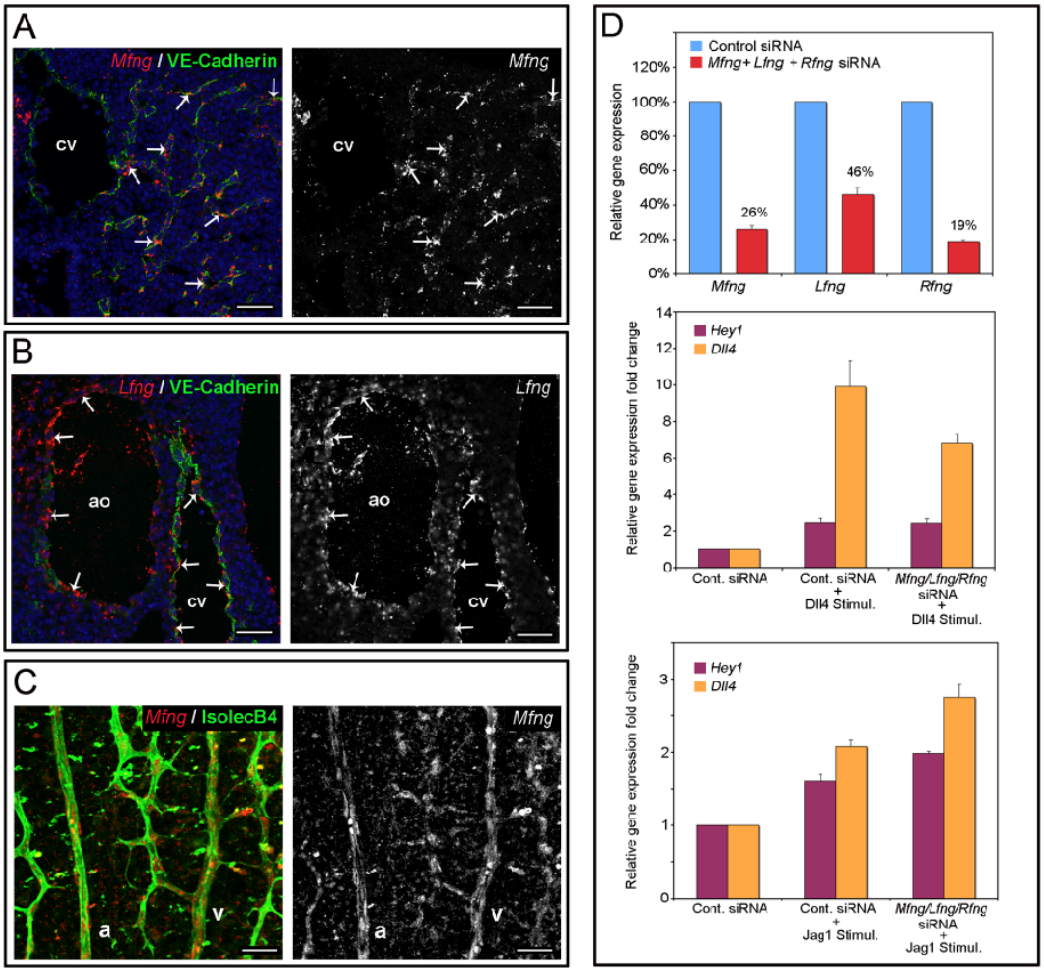
A
Mfng / VE-Cadherin
cv
Mfng
cv
B
Lfng / VE-Cadherin
ao
cv
Lfng
ao
cv
C
Mfng / IsolecB4
a
v
Mfng
a
v
D
Control siRNA
Mfng+ Lfng + Rfng siRNA
Relative gene expression
120%
100%
80%
60%
40%
20%
0%
26%
46%
19%
Mfng
Lfng
Rfng
Hey1
Dll4
Relative gene expression fold change
14
12
10
8
6
4
2
0
Cont. siRNA
Cont. siRNA + Dll4 Stimul.
Mfng/Lfng/Rfng siRNA + Dll4 Stimul.
Hey1
Dll4
Relative gene expression fold change
3
2
1
0
Cont. siRNA
Cont. siRNA + Jag1 Stimul.
Mfng/Lfng/Rfng siRNA + Jag1 Stimul.

Figure S5. Fringe expression and knock–down experiments

(A, B) In situ hybridization for *Mfng* or *Lfng* (red) in combination with anti–VE–Cadherin immunofluorescence (green) and nuclear DAPI staining on transversal cryosection of E10.5 mouse embryos. Arrows indicate Fringe expression in aorto (ao), cardinal vein (cv) or microvessels.

(C) Whole–mount immunofluorescence for Isolectin B4 in the P6 retina in combination with fluorescent *Mfng in situ* hybridization signal. *Mfng* expression labels retinal arterioles (a), venules (v) and capillaries.

(D) Efficiency of siRNA–mediated downregulation of individual Fringe genes (top panel) and effects of combined (but incomplete) *Mfng*/*Lnfg*/*Rfng* knock–down on *Hey1* and *Dll4* mRNA in HUVECs stimulated with immobilized, recombinant Dll4 (center) or Jag1 (bottom), as indicated.

Error bars represent s.e.m. Scale bars: A, B: 25μm; C: 28μm.

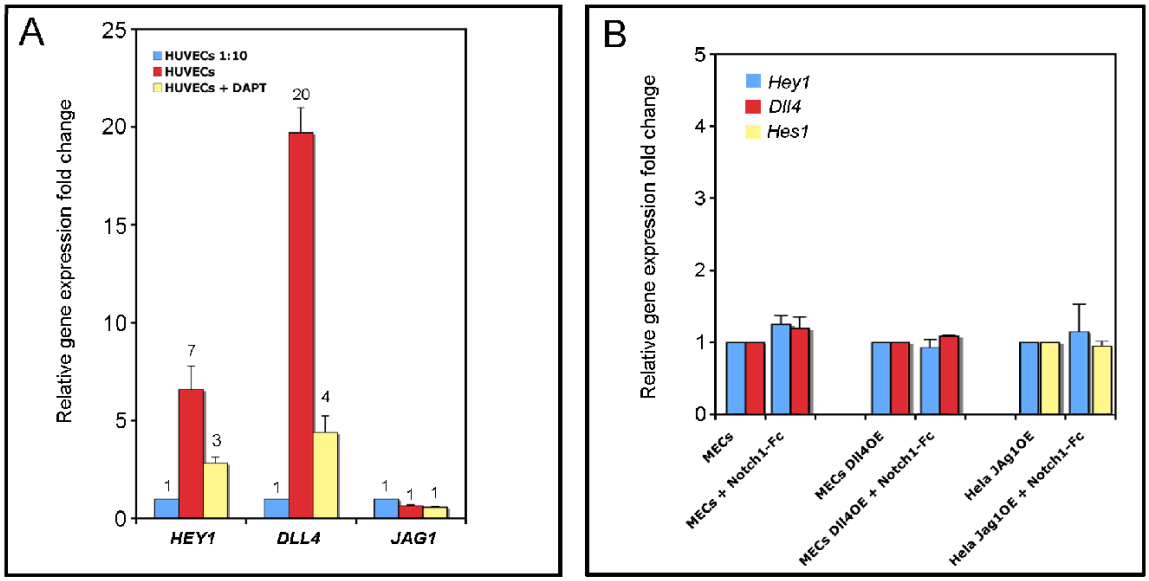

Figure S6. Regulation of Notch pathway genes

(A) qRT–PCR analysis of *HEY1*, *DLL4* and *JAG1* mRNAs in human ECs (HUVECs) cultured at low density (1:10) or in confluent conditions in the presence or absence of DAPT.

(B) Stimulation with immobilized, recombinant Notch1 (Notch1–Fc) protein does not alter *Hey1* or *Dll4* transcripts levels in cultured mouse ECs (MECs) or MECs overexpressing Dll4 (Dll4OE). Likewise, plating on Notch1–Fc does not alter *Hey1* or *Hes1* levels in HeLa cells overexpressing Jagged1 (Jag1OE).

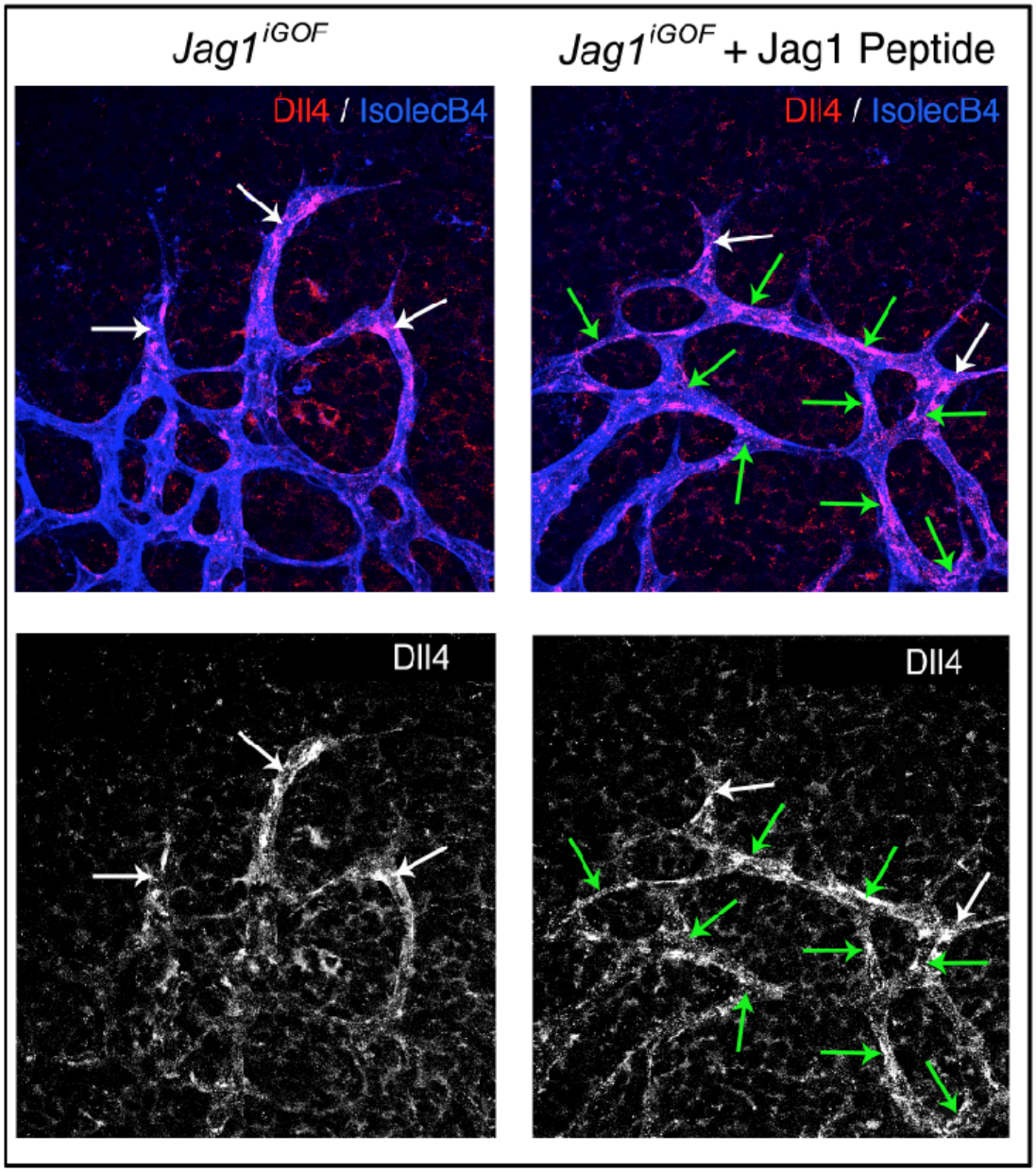

Figure S7. Jag1 peptide administration to *Jag1*^{iGOF} mutants

Confocal images of the whole–mount isolectin B4 (blue) and Dll4 (red) antibody–stained P6 retinal *Jag1*^{iGOF} vasculature. Bottom panels separately show channel with Dll4 signal. Note reduced expression of Dll4 in the *Jag1*^{iGOF} vasculature, which is mostly confined to tip cells (white arrows). Administration of soluble Jag1 peptide (right panels) upregulates Dll4 protein expression in stalk cells (green arrows) despite overexpression of Jagged1 in the endothelium.