LETTERS

Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis

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In sprouting angiogenesis, specialized endothelial tip cells lead the outgrowth of blood-vessel sprouts towards gradients of vascular endothelial growth factor (VEGF)-A[1,2]. VEGF-A is also essential for the induction of endothelial tip cells[2], but it is not known how single tip cells are selected to lead each vessel sprout, and how tip-cell numbers are determined. Here we present evidence that delta-like 4 (Dll4)–Notch1 signalling regulates the formation of appropriate numbers of tip cells to control vessel sprouting and branching in the mouse retina. We show that inhibition of Notch signalling using γ-secretase inhibitors, genetic inactivation of one allele of the endothelial Notch ligand *Dll4*, or endothelial-specific genetic deletion of *Notch1*, all promote increased numbers of tip cells. Conversely, activation of Notch by a soluble jagged1 peptide leads to fewer tip cells and vessel branches. Dll4 and reporters of Notch signalling are distributed in a mosaic pattern among endothelial cells of actively sprouting retinal vessels. At this location, Notch1-deleted endothelial cells preferentially assume tip-cell characteristics. Together, our results suggest that Dll4–Notch1 signalling between the endothelial cells within the angiogenic sprout serves to restrict tip-cell formation in response to VEGF, thereby establishing the adequate ratio between tip and stalk cells required for correct sprouting and branching patterns. This model offers an explanation for the dose-dependency and haploinsufficiency of the *Dll4* gene[3–5], and indicates that modulators of Dll4 or Notch signalling, such as γ-secretase inhibitors developed for Alzheimer's disease, might find usage as pharmacological regulators of angiogenesis.

Sprouting angiogenesis shows morphological and molecular similarities to epithelial tubulogenesis[6] and axon guidance[7]. Through dynamic filopodial protrusions, the sprout tip cells migrate, sense and respond to guidance cues provided by soluble, cell- or matrix-bound ligands, such as VEGF(s), netrin(s) and semaphorin(s)[2,8,9]. During tracheal sprouting in *Drosophila melanogaster*, Notch signalling participates in the selection of cells involved in branch fusion and

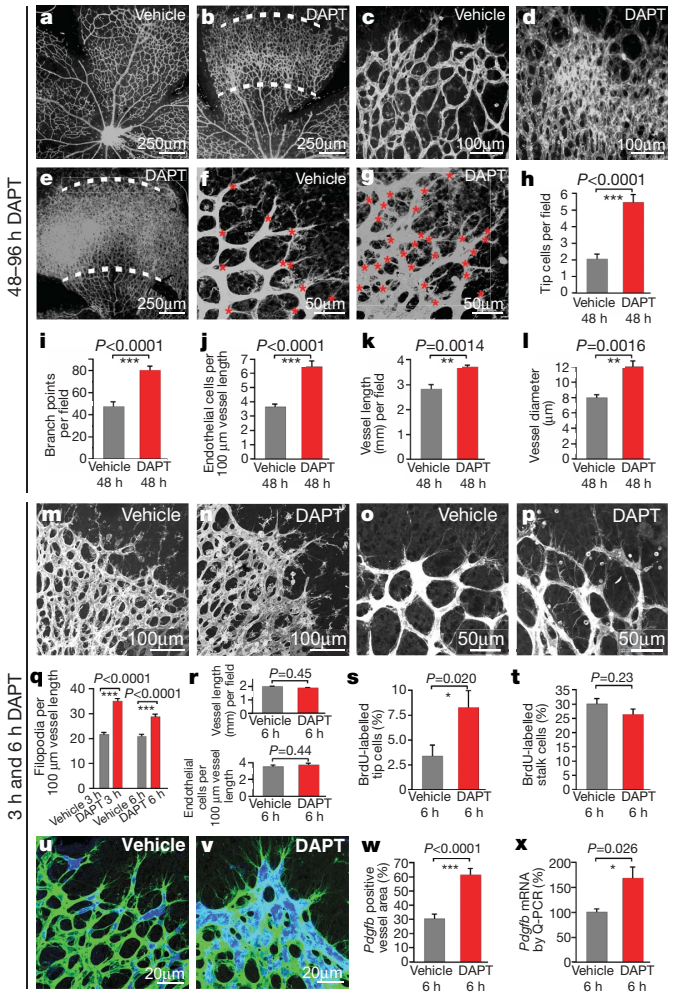

Figure 1 | γ-Secretase inhibitor experiments *in vivo*. Isolectin B4 (white in **a**–**g**, **m**, **n**; green in **u**, **v**) or endomucin staining (white in **o**, **p**) of whole-mounted P4–6 retinas. DAPT treatment lasted for 48 h (**a**–**d**, **f**–**l**), 96 h (**e**), 6 h (**m**–**p**, **r**–**x**) or 3 h (**q**). The affected region (between dashed lines in **b** and **e**) correlates with length of treatment period. **c**, **d**, **f**, **g**, Views of vessel plexus margins. Note the DAPT-induced increase in filopodial protrusions (red asterisks in **f**, **g**). **h**–**l**, Quantifications of densities of tip cells (**h**), branch points (**i**), endothelial cells (**j**), as well as length (**k**) and diameter (**l**) of vessels. **m**–**p**, DAPT-induced increase in filopodia (**o**, **p**), vessel branching (**m**, **n**) and *pdgfb* mRNA expression (blue in **u**, **v**) at the plexus margin. **q**–**t**, Quantifications of the densities of filopodia (**q**), vessels (**r**, top) and endothelial cells (**r**, bottom), as well as of BrdU labelling in tip (**s**) and stalk (**t**) cells. **w**, **x**, Proportion of *pdgfb*-positive endothelium (**w**) and levels of *pdgfb* mRNA (**x**). All error bars represent s.e.m.

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terminal ramification[10–12]. Because many Notch pathway components are expressed and have critical but poorly defined roles during vascular development[13], we investigated whether Notch signalling might have a specific function in tip-cell formation during sprouting angiogenesis.

To study the importance of Notch during sprouting angiogenesis *in vivo*, we focused on developmental retinal angiogenesis during the first post-natal week in mice. Here, systemic administration of the γ-secretase inhibitors (GSIs) DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butylester) or compound X (*S*-3-[*N*′-(3,5-difluorophenyl-α-hydroxy-acetyl)-L-alanilyl]amino-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one), which potently inhibit Notch receptor cleavage and signalling[14,15], led to increased vascular density and vessel diameter associated with elevated numbers of endothelial cells and vascular sprouts in the peripheral part of the developing retinal vascular plexus (Fig. 1a–l). DAPT and compound X also increased filopodial protrusions from endothelial cells both at the sprouting front of the plexus, where filopodia are normally prevalent as they extend from the tip cells[2] (Fig. 1f–h), and proximally in the plexus where filopodial protrusions are normally rare (data not shown). JLK-6 (7-amino-4-chloro-3-methoxyisocoumarin), a GSI that does not inhibit Notch, lacked any of these effects (Supplementary Fig. 1). Prolonged treatment with the Notch-inhibitory GSIs augmented the severity of the vessel abnormalities, leading, in the most extreme cases, to vessel coalescence into large, flat sinuses (Fig. 1e). A similar increase in sprout density and change in vascular patterning was observed when DAPT was administered in conjunction with pathological angiogenesis induced in a mouse model of retinopathy of prematurity (ROP) (Supplementary Fig. 2).

The width of the affected zone correlated with the expected plexus extension during the period of treatment, suggesting that GSIs affect developing (distal), but not already formed (proximal), vessels (Fig. 1b, e). Accordingly, short-term (3–6 h) DAPT exposure led to increased filopodial protrusions and vessel diameter only at the margin of the plexus (Fig. 1m–q) and lacked effects on endothelial cell number and vascular density (Fig. 1r). DAPT exposure for 6 h also increased the frequency of endothelial cells positive for the tip-cell marker platelet-derived growth factor-B (*Pdgfb*)[2] at the plexus margin (Fig. 1u–x). Thus, both morphological and gene expression changes suggested that Notch-inhibitory GSIs raise tip-cell numbers, leading to excessive vascular sprouting and fusion. To address whether proliferation might underlie the increase in tip cells, we performed 5-bromodeoxyuridine (BrdU) labelling experiments. DAPT exposure for 6 h did not affect BrdU labelling of stalk cells (Fig. 1t) but raised the proportion of BrdU-positive tip cells (Fig. 1s and Supplementary Fig. 3). This proliferative response in tip cells is, however, too small to explain the much

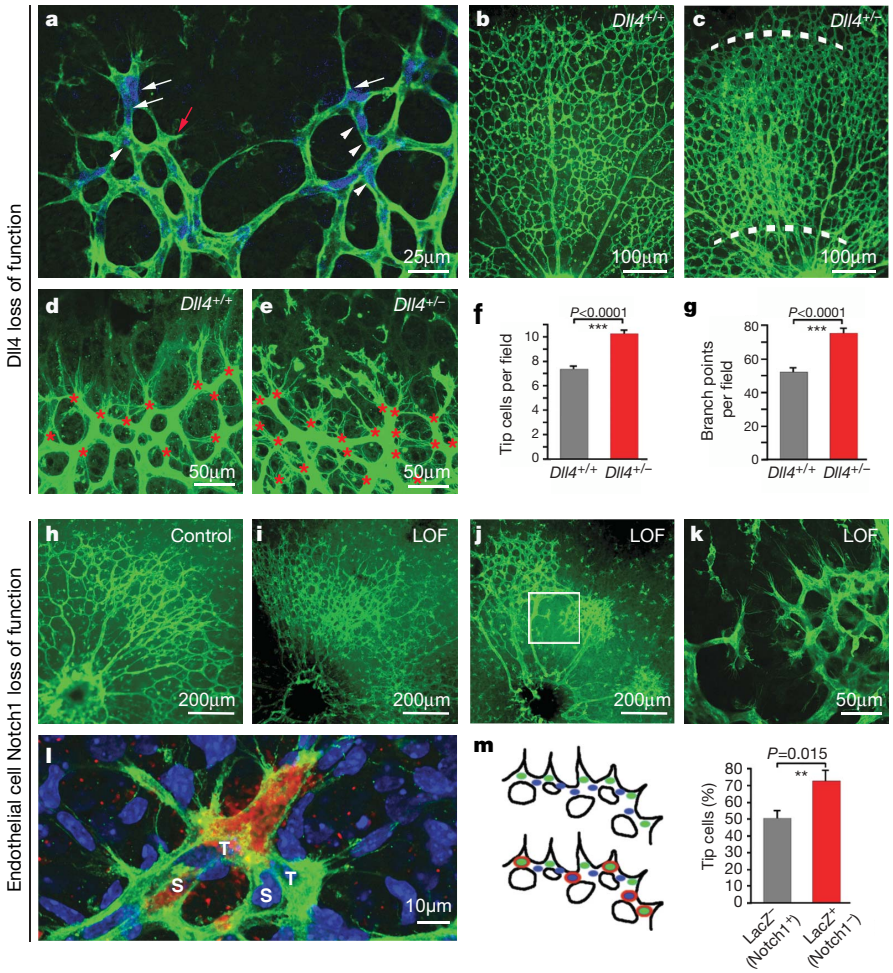

Figure 2 | Dll4 and Notch1 loss-of-function analyses *in vivo*. Isolectin B4 staining (green) of whole-mounted retinas. **a**, mRNA *in situ* hybridization for *Dll4* (blue) on wild-type P5 retina. *Dll4*-positive tip cells (white arrows) and non-tip cells, (white arrowheads), as well as *Dll4*-negative tip cells (red arrow), are indicated. **b**–**g**, Increased vessel density (**b**, **c**, **g**) and filopodia protrusions (**d**–**f**) (red asterisks) in $Dll4^{+/-}$ retinas at P4. **h**–**k**, P5 retinas from control (**h**) and two tamoxifen-treated (LOF) VEcad-$CreER^{T2}$/R26R/$Notch1^{floxed/floxed}$ mice with a different degree of vascular abnormality (**i**, **j**; the box in **j** is magnified in **k**). **l**, Example of an image used to score LacZ-positive (red and green) and LacZ-negative (green) tip cells (T) and stalk cells (S). Nuclei (blue) are stained by TOPRO-3. **m**, Schematic illustration of the scoring principle: retinas with mosaic recombination were analysed at the sprouting plexus margin for tip cells (green) and stalk cells (blue) that were either LacZ positive (red circle) or LacZ negative (no red circle). The graph shows the percentage of tip cells among LacZ-positive and LacZ-negative cells. All error bars represent s.e.m.

larger increases in filopodia extension (Fig. 1q) and *Pdgfb* expression (Fig. 1u–x) observed after short-term DAPT administration.

The Notch-inhibitory activity of DAPT was confirmed by the downregulated retinal messenger RNA levels of the known Notch target gene Notch-regulated ankyrin-repeat protein (*Nrarp*) after 6 h (Supplementary Fig. 4) and an expected increase in the number of intestinal Goblet cells after 4 days[16] (Supplementary Fig. 5). However, because γ-secretase has substrates in addition to Notch that may be involved in angiogenesis[17], we sought specific genetic evidence for a role of Notch signalling in retinal angiogenesis. The restricted vascular expression of the Notch ligand *Dll4* (ref. 18) and the previously described vascular defects of *Dll4* mouse mutants prompted us to study retinal angiogenesis in $Dll4^{+/-}$ mice[3–5]. First, we confirmed the previously reported expression of *Dll4* mRNA in arteries[19] and in a proportion of the tip cells[18], but noticed expression also in a proportion of the endothelial stalk at the sprouting front (Fig. 2a). $Dll4^{+/-}$ mice displayed a similar retinal phenotype compared to long-term GSI-treated pups (Fig. 2b, c). Increased filopodial protrusions, vessel branching and numbers of *Pdgfb*-expressing cells were observed (Fig. 2d–g and Supplementary Fig. 6), consistent with an increased number of tip cells. $Dll4^{+/-}$ retinas displayed morphologically distinguishable arteries and veins (Fig. 2b, c), and normal mural cell abundance and distribution, as revealed by α-smooth muscle actin- and chondroitin sulphate proteoglycan NG2 staining (Supplementary Fig. 7). Thus, whereas previous work demonstrated a role for Notch in arterial specification of both endothelial and mural cells[20,21], the phenotypes observed in GSI-treated and $Dll4^{+/-}$ retinas appeared to reflect mainly an increased formation of tip cells during angiogenic sprouting.

The severe vascular abnormalities and embryonic lethality of both ubiquitous[22] and endothelium-specific[23] Notch1 homozygous null mutants suggest a critical vascular function for Notch1 but precludes postnatal analysis of these mice. To study the role of endothelial Notch1 in retinal angiogenesis we therefore used VEcad-CreER^{T2}/Notch1$^{floxed/floxed}$ mice, in which tamoxifen-inducible Cre enzyme in endothelial cells[24] deletes critical *Notch1* sequences flanked by *loxP* sites[25]. We first analysed the efficiency of tamoxifen-induced recombination in VEcad-CreER^{T2}/R26R retinas after drug administration at postnatal days (P)0–4 and subsequent reporter (LacZ) staining at P5. A mosaic endothelium-specific LacZ expression was observed, ranging between 10–30% in different individuals (Supplementary Fig. 8). The same tamoxifen administration regimen for VEcad-CreER^{T2}/R26R/Notch1$^{floxed/floxed}$ mice resulted in regionally increased sprout and filopodia densities in the retinal vasculature (Fig. 2h–k). We took advantage of individual VEcad-CreER^{T2}/R26R/Notch1$^{floxed/floxed}$ mice that exhibited the lowest degree of recombination resulting in a few scattered LacZ-positive endothelial cells in an otherwise normal retina. Analysis of endothelial cells at the peripheral sprouting front (Fig. 2l) of those mice revealed that a high proportion (73%) of the LacZ-positive cells displayed morphological tip-cell characteristics (Fig. 2m). In contrast, LacZ-negative cells were distributed evenly among tip and stalk cells (50:50) (Fig. 2m). The correlation between Cre expression (and thereby Notch1 inactivation) and acquirement of tip-cell

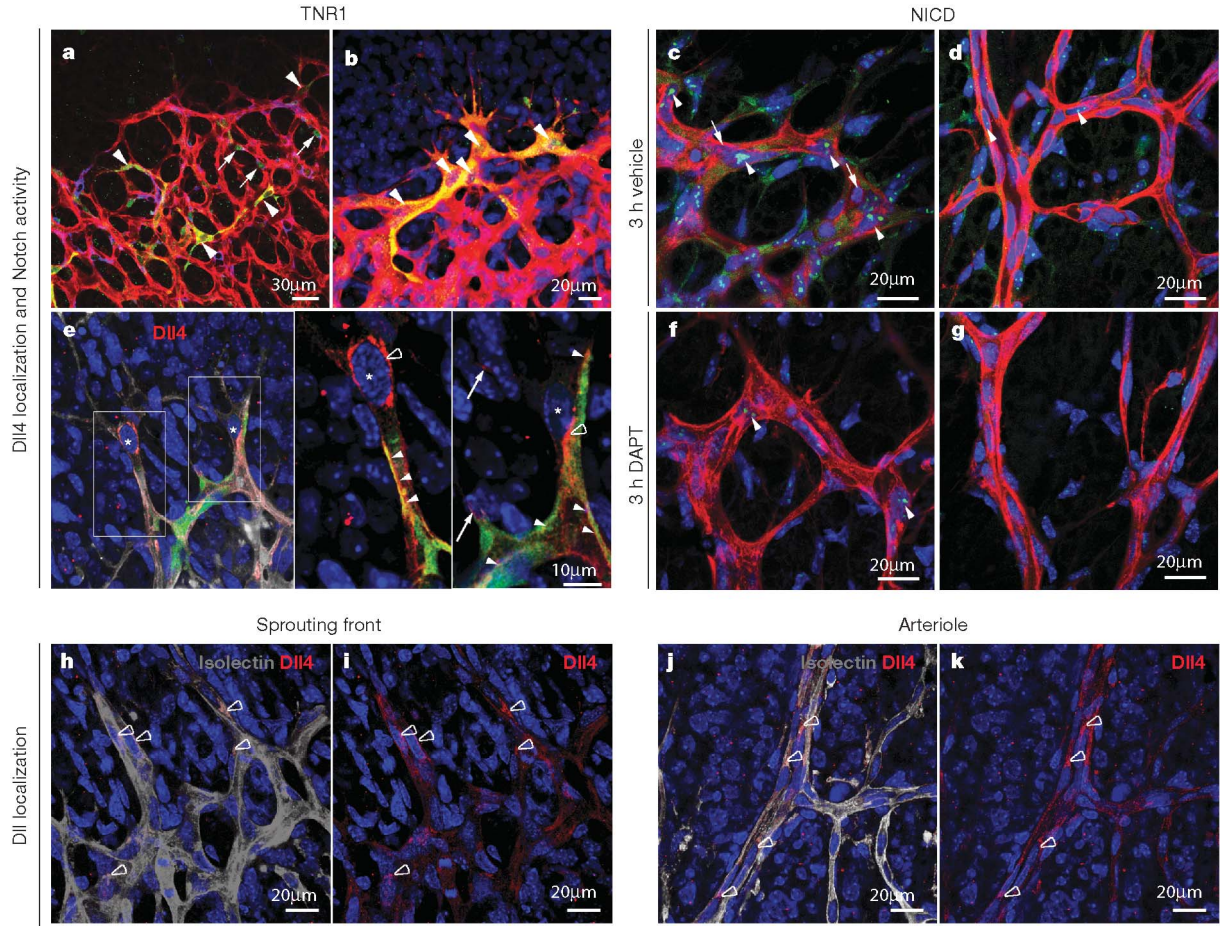

Figure 3 | Endothelial Dll4–Notch signalling *in vivo*. **a–k**, Isolectin B4 (red in **a–d**, **f**, **g**; grey in **e**, **h**, **j**) NG2 (blue in **a**) and DAPI (blue in **b–k**) staining of whole-mounted P4–6 retinas. **a**, **b**, **e**, Notch-reporter activity in TNR1 mouse (GFP; green/yellow) observed in a proportion of endothelial cells localized at the sprouting front (arrowheads). Open arrowheads in **e** indicate typical intracellular Dll4 protein (red) localization in tip cells (asterisks). Left and right boxes in the left panel of **e** are magnified in the middle and right panels, respectively. Filled arrowheads indicate typical membrane Dll4 localization observed in stalk cells and in the plexus. Arrows indicate occasional filopodial Dll4. **c**, **d**, **f**, **g**, Notch1-ICD-positive (turquoise) endothelial nuclei (arrowheads) were abundant at the vascular front (**c**) but scarce in the mature plexus (**d**) and were eliminated at both sites by 3 h of DAPT treatment (**f**, **g**). **h–k**, Internalization of Dll4 (open arrowheads) was detected in tip and stalk cells at the vascular front and in arterioles, but not in other endothelial cells.

characteristics suggests that Notch1 suppresses the tip-cell phenotype in a cell-autonomous manner. This chimaeric analysis also argues against systemic or secondary effects of endothelial Notch1 inhibition on tip-cell formation in the retina.

To demonstrate Notch signalling in sprouting retinal endothelial cells *in vivo*, we used a transgenic Notch-reporter mouse (hereafter called TNR1), which carries a CBF-1 response element and a minimal SV40 promoter followed by an enhanced green fluorescent protein (GFP) sequence[26]. We found GFP expression broadly in the endothelium at the vascular front, and a weak signal in some non-vascular retinal cells, consistent with Notch receptor expression and signalling in several retinal cell types[18]. However, conspicuous GFP expression was only seen in endothelial cells at the sprouting front, distributing in a mosaic pattern involving both tip and stalk cells. Thus, the most active Notch signalling was mainly observed in the vascular region affected by Notch inhibition (Fig. 3a, b). Furthermore, strongly GFP-positive endothelial cells intermingled with Dll4-expressing endothelial cells at the sprouting front (Fig. 3e). Some Dll4-positive cells exhibited intracellular accumulation of Dll4 staining, suggesting previous engagement in Notch activation (Fig. 3e)[27]. Almost all tip cells (48 out of 50 analysed) showed intracellular accumulation of Dll4 staining. Notably, many stalk cells immediately in contact with the tip cells (33 out of 58) also showed intracellular accumulations of Dll4 staining, indicating active bi-directional Dll4 signalling between tip cells and stalk cells at the sprouting front (Fig. 3h, i). Dll4 accumulation was similarly observed in arterioles, a known site of Dll4–Notch signalling (Fig. 3j, k), but endothelial cells in the plexus behind the growing front or adjacent to arterioles showed no or minor intracellular accumulation of Dll4. To demonstrate Notch1 cleavage *in vivo* we used an antibody against Notch1 intracellular domain (ICD). This labelled nuclei of vascular cells, astrocytes and microglia, in agreement with the reported retinal Notch1 expression pattern[18]. As with Notch-reporter activity in TNR1 mice, Notch1-ICD was readily detected in endothelial cells at the sprouting front (Fig. 3c, arrowheads), but rarely and weakly at more proximal locations in the retinal plexus (Fig. 3d, arrowheads). The specificity of the Notch1-ICD signal was shown by its downregulation after DAPT administration (Fig. 3f, g, arrowheads in f show residual signal). Thus, both Notch reporter in TNR1 mice and Notch1-ICD were preferentially located in endothelial cells at sites exposed to VEGF-A[2] (that is, where selection of tip cells occurs).

Previous work showed that tip-cell filopodial extension in the developing retina depends on astrocyte-derived VEGF-A acting directly on tip cells[2]. We therefore investigated whether Notch inhibition affected astrocytes or VEGF-A expression. Glial fibrillary acidic protein (GFAP) staining revealed a normal astrocyte network ahead of the plexus front in GSI-treated and $Dll4^{+/-}$ mice (Supplementary Fig. 9). Retinal VEGF-A mRNA and protein levels as well as the relative levels of the VEGF-A splice isoforms were unchanged in DAPT-treated retinas (Supplementary Fig. 10). *In situ* hybridization also showed that the retinal pattern of VEGF-A expression was normal after 6 h of GSI treatment but shifted proximally to the areas of dense and hyper-fused vasculature after long-term DAPT treatment and in $Dll4^{+/-}$ retinas (Supplementary Fig. 10). Thus, whereas the central retinal vascular abnormalities observed in $Dll4^{+/-}$ mice and after long-term DAPT treatment might, in part, result from changes in the pattern of VEGF-A expression, the short-term effects of GSIs on tip-cell formation at the

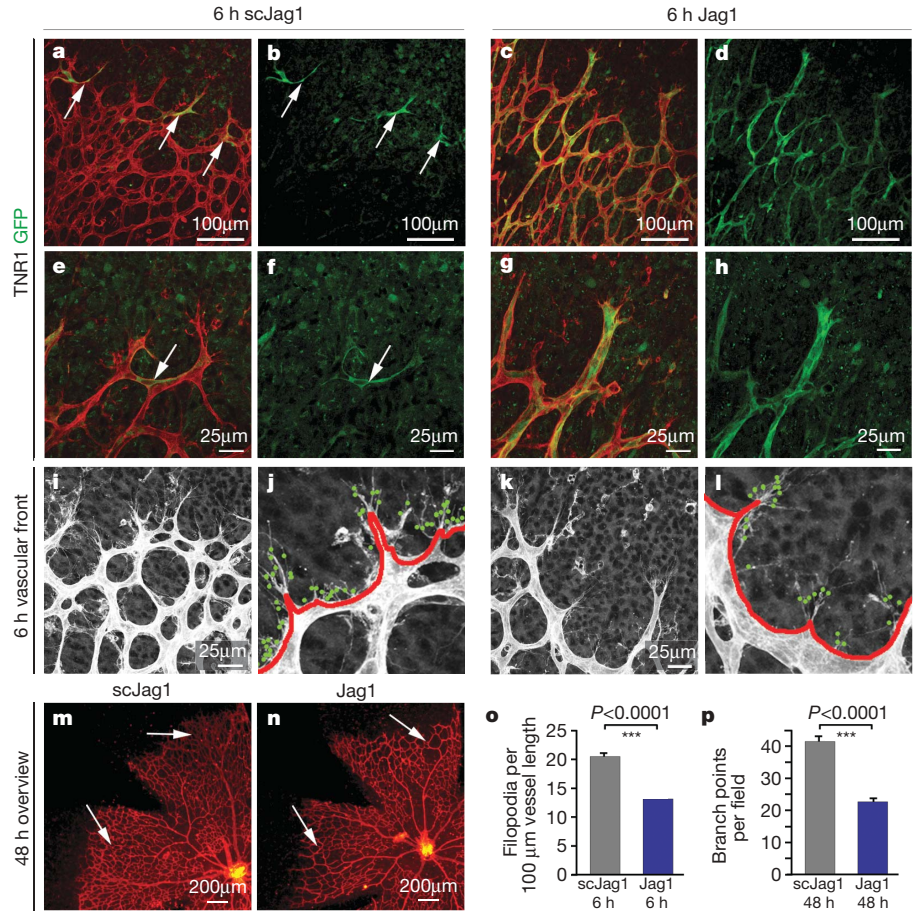

Figure 4 | Notch gain-of-function analysis *in vivo*. Isolectin B4 (red in **a**, **c**, **e**, **g**, **m**, **n**; white in **i**–**l**) staining of whole-mounted P4–5 retinas. **a**–**h**, Broad induction of Notch reporter in TNR1 mouse (GFP; green/yellow) by 6 h treatment with Jag1 (**c**, **d**, **g**, **h**) but not a scrambled peptide (scJag1; **a**, **b**, **e**, **f**; arrows indicate scattered Notch-reporter activity at the front). **i**–**l**, **o**, Six hours of Jag1 treatment reduced tip cells and filopodia at the sprouting front. **j**, **l**, **o**, Quantification of filopodia (green dots) per vessel length (red line) after scJag1 (**j**) and Jag1 (**l**) treatment. **j** and **l** are magnifications of **i** and **k**. Forty-eight hours of Jag1 treatment reduced retinal vessel density in the peripheral vascular plexus (arrows in **m**, **n**; quantification in **p**). All error bars represent s.e.m.

plexus margin are unlikely to be caused by changes in VEGF-A. Short term (6 h) DAPT treatment also did not lead to a significant change in retinal expression of mRNAs encoding the hypoxia marker solute carrier family 2 member 1 (*Slc2a1*) (Supplementary Fig. 10).

Our data suggest that Dll4–Notch1 signalling within the endothelial cell population serves to suppress the tip-cell phenotype. To test this hypothesis in a direct gain-of-function experiment, we used a synthetic peptide corresponding to the δ/serrate/Lag-2 domain of jagged1 (Jag1) that has previously reported Notch agonistic activity[28]. The ability of Jag1 peptide to activate Notch signalling *in vivo* was confirmed in TNR1 mice: systemic administration of Jag1 peptide, but not a scrambled control peptide, induced widespread GFP expression in retinal endothelial cells (Fig. 4a–h; compare panel b to d). It also induced a 35% decrease in filopodia density at the vascular front after 6 h (Fig. 4i–l, o). Repeated Jag1 peptide injections also led to a 45% decrease in vessel density (Fig. 4m, n, p); that is, the opposite effects compared to the different abovementioned situations of Notch inhibition.

The competence to become a tip cell is broad, perhaps ubiquitous, within the endothelial population[2]. VEGF-A gradients and point sources occur in tissues as a result of cell-type-specific expression and matrix binding of VEGF-A, and have been shown to be essential for vascular patterning[1,29]. Although the endothelial cells closest to the VEGF-A source would be assumed to stand a higher chance than their neighbours of becoming induced as tip cells, it is nevertheless difficult to conceive how a graded distribution of VEGF-A alone—an 'analogue' signal—would suffice to select singular tip cells to lead each sprout—a 'digital' response. Our loss- and gain-of function analyses together with the localization of the Dll4 signal and Notch activity suggest a model in which Dll4–Notch1 signalling between endothelial cells functions equivalent to an analogue-to-digital converter, establishing cell boundaries and the selection of either tip (low Notch signal) or stalk (high Notch signal) phenotypes within the population of VEGF-A-stimulated endothelial cells (Supplementary Fig. 11). The fundamental importance of the selection of correct numbers of endothelial tip cells, sprouts and branches is consistent with the unique genetic haploinsufficiency for the two critical ligands that regulate the process: VEGF and Dll4. VEGF inhibition is so far the only clinically validated anti-angiogenic strategy, and as such applicable to pathological angiogenesis both in tumours and ocular diseases[30]. Our present data suggest that GSIs or other modulators of Dll4–Notch1 signalling might find similar usage in the clinic.

METHODS

Animal procedures. Pharmacological Notch inhibition or activation was induced by subcutaneously injecting GSIs and Jag1 peptides, respectively, into newborn mice, followed by analysis at different time points during the first postnatal week. Retinal angiogenesis in genetic loss-of-function of Dll4 was studied in postnatally surviving $Dll4^{+/-}$ mice on an ICR background. Inducible endothelium-specific inactivation of Notch1 was obtained using intragastric injections of tamoxifen into VEcad-CreER^{T2}/R26R/Notch1$^{floxed/floxed}$ mice on a congenic C57Bl6J background. Pathological eye angiogenesis was induced by exposure to 80% oxygen during P7–12 followed by a return to normoxia and analysis at P17.

Tissue imaging. Standard protocols and commercially available antibodies were used in most instances to whole-mount-stain fixed and dissected retinas. Combined nuclear- and isolectin B4 labelling was done to allow discrimination between nuclei belonging to endothelial tip and stalk cells. Confocal laser scanning microscopy was performed using Zeiss LSM 510 Meta microscopes.

Molecular analyses. The MMV00 mouse VEGF-A ELISA kit (R&D Systems) was used to quantify VEGF-A protein concentration in tissues lysed in NP-40-based buffer. Quantitative real-time PCR was performed on tissues collected in RNA*later* (Qiagen) before and during dissection of retinas. Total retinal RNA was isolated using the RNeasy Mini Kit (Qiagen) and assays were run using TaqMan solutions and gene expression assays specific for individual mouse genes (Applied Biosystems) on an ABI Prism 7700 Sequence Detection System.

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1. Ruhrberg, C. *et al.* Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev.* **16,** 2684–2698 (2002).
2. Gerhardt, H. *et al.* VEGF guides angiogenic sprouting utilizing endothelial tip-cell filopodia. *J. Cell Biol.* **161,** 1163–1177 (2003).
3. Gale, N. W. *et al.* Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl Acad. Sci. USA* **101,** 15949–15954 (2004).
4. Krebs, L. T. *et al.* Haploinsufficienct lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev.* **18,** 2469–2473 (2004).
5. Duarte, A. *et al.* Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev.* **18,** 2474–2478 (2004).
6. Uv, A., Cantera, R. & Samakovlis, C. *Drosophila* tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. *Trends Cell Biol.* **13,** 301–309 (2003).
7. Carmeliet, P. & Tessier-Lavigne, M. Common mechanisms of nerve and blood vessel wiring. *Nature* **436,** 193–200 (2005).
8. Lu, X. *et al.* The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* **432,** 179–186 (2004).
9. Torres-Vazquez, J. *et al.* Semaphorin-plexin signaling guides patterning of the developing vasculature. *Dev. Cell* **7,** 117–123 (2004).
10. Llimargas, M. The Notch pathway helps to pattern the tips of the *Drosophila* tracheal branches by selecting cell fates. *Development* **126,** 2355–2364 (1999).
11. Steneberg, P., Hemphala, J. & Samakovlis, C. Dpp and Notch specify the fusion cell fate in the dorsal branches of the *Drosophila* trachea. *Mech. Dev.* **87,** 153–163 (1999).
12. Ghabrial, A. S. & Krasnow, M. A. Social interactions among epithelial cells during tracheal branching morphogenesis. *Nature* **441,** 746–749 (2006).
13. Shawber, C. J. & Kitajewski, J. Notch function in the vasculature: Insights from zebrafish, mouse and man. *Bioessays* **26,** 225–234 (2004).
14. Dovey, H. F. *et al.* Functional γ-secretase inhibitors reduce β-amyloid peptide levels in brain. *J. Neurochem.* **76,** 173–181 (2001).
15. Searfoss, G. H. *et al.* Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional γ-secretase inhibitor. *J. Biol. Chem.* **278,** 46107–46116 (2003).
16. van Es, J. H. *et al.* Notch/γ-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435,** 959–963 (2005).
17. Kopan, R. & Ilagan, M. X. G. γ-secretases: proteasome of the membrane? *Nature Rev. Mol. Cell Biol.* **5,** 499–504 (2004).
18. Claxton, S. & Fruttiger, M. Periodic Delta-like 4 expression in developing retinal arteries. *Gene Expr. Patterns* **5,** 123–127 (2004).
19. Shutter, J. R. *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.* **14,** 1313–1318 (2000).
20. Lawson, N. D. *et al.* Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128,** 3675–3683 (2001).
21. Domenga, V. *et al.* Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev.* **18,** 2730–2735 (2004).
22. Krebs, L. T. *et al.* Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev.* **14,** 1343–1352 (2000).
23. Limbourg, F. P. *et al.* Essential role of endothelial Notch1 in angiogenesis. *Circulation* **111,** 1826–1832 (2005).
24. Monvoisin, A. *et al.* VE-cadherin-CreER^{T2} transgenic mouse: A model for inducible recombination in the endothelium. *Dev. Dyn.* **235,** 3413–3422 (2006).
25. Wolfer, A. *et al.* Inactivation of Notch1 in immature thymocytes does not perturb CD4 or CD8 T cell development. *Nature Immunol.* **2,** 235–241 (2001).
26. Duncan, A. W. *et al.* Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nature Immunol.* **6,** 314–322 (2005).
27. Itoh, M. *et al.* Mind Bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4,** 67–82 (2003).
28. Weijzen, S. *et al.* The notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic cells. *J. Immunol.* **169,** 4273–4278 (2002).
29. Grunstein, J., Masbad, J. J., Hickey, R., Giordano, F. & Johnson, R. S. Isoforms of vascular endothelial growth factor act in a coordinate fashion to recruit and expand tumor vasculature. *Mol. Cell. Biol.* **20,** 7282–7291 (2000).
30. Ferrara, N. Vascular endothelial growth factor: Basic science and clinical progress. *Endocr. Rev.* **25,** 581–611 (2004).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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