Development 140, 3051-3061 (2013) doi:10.1242/dev.093351

Notch controls retinal blood vessel maturation and quiescence

Manuel Ehling, Susanne Adams, Rui Benedito* and Ralf H. Adams‡

SUMMARY

Blood vessels form a hierarchically organized network of arteries, capillaries and veins, which develops through a series of growth, pruning and maturation processes. In contrast to the rapidly increasing insight into the processes controlling vascular growth and, in particular, endothelial sprouting and proliferation, the conversion of immature vessels into a fully functional, quiescent vasculature remains little understood. Here we used inducible, cell type-specific genetic approaches to show that endothelial Notch signaling is crucial for the remodeling of veins and the perivenous capillary plexus, which occurs after the completion of the initial angiogenic growth phase in the retina of adolescent mice. Mutant vessels showed ectopic proliferation and sprouting, defective recruitment of supporting mural cells, and failed to downregulate the expression of VEGF receptors. Surprisingly, by contrast Notch was dispensable in the endothelium of remodeling postnatal arteries. Taken together, our results identify key processes contributing to vessel remodeling, maturation and the acquisition of a quiescent phenotype in the final stage of developmental angiogenesis.

KEY WORDS: Vascular development, Angiogenesis, Endothelial cells, Notch, Mouse

INTRODUCTION

The vertebrate body contains an extensive tubular system of blood vessels lined by a monolayer of endothelial cells (ECs). This network grows substantially during development, wound healing and in certain pathological conditions (Chung and Ferrara, 2011; Potente et al., 2011). With the exception of the first vascular structures that are directly assembled by endothelial progenitor cells in the early embryo, most or all vessel growth is mediated by angiogenesis, i.e. the expansion of existing vessel beds (Risau and Flamme, 1995). Angiogenesis is a highly complex growth program that involves highly diverse processes, which need to be tightly coordinated to allow extensive morphological changes while preserving essential blood flow.

The key processes contributing to angiogenic blood vessel growth can be readily observed in the retinal vasculature of the mouse, which has become a highly useful and well-established model system (Dorrell et al., 2002; Gerhardt et al., 2003). The vascularization of the murine retina occurs after birth, which facilitates genetic or pharmacological studies involving inducers or inhibitors. As the vasculature in the rest of the body is already much more advanced at this stage, indirect, secondary alterations due to defects in other organ systems are less likely than in embryos. Flat-mounted retinas allow the imaging of the complete vascular network at high resolution without the inevitable loss of tissue material or of three-dimensional information caused by tissue sectioning. During the radial outgrowth of vessels from the optic head in the central retina into the avascular periphery, different steps of the angiogenic growth program can be studied over time or, to some extent, even within the same sample (Dorrell et al., 2002; Gerhardt et al., 2003).

Max Planck Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, and University of Münster, Faculty of Medicine, D-48149 Münster, Germany.

*Present address: Centro Nacional de Investigaciones Cardiovasculares (CNIC), Molecular Genetics of Angiogenesis Group, Cardiovascular Developmental Biology Program, Madrid 28029, Spain
‡Author for correspondence (ralf.adams@mpi-muenster.mpg.de)

Accepted 10 May 2013

In the early phase of vascular growth visible at the leading edge of the growing vessel front, the formation, extension and anastomosis of endothelial sprouts lead to the generation of an immature plexus, which is strongly controlled by Notch and vascular endothelial growth factor (VEGF) signaling. VegfA, presented in a gradient by astrocytes in the avascular region, binds the receptor tyrosine kinase Vegfr2 (also known as Kdr or Flk1) on ECs, promotes endothelial sprouting and proliferation, and guides sprouts into the periphery (Gerhardt et al., 2003; Ruhrberg et al., 2002; Stone et al., 1995). Vegfr3, the receptor for VegfC (also known as Flt4), is also expressed by growing blood vessels and controls angiogenesis (Hogan et al., 2009; Siekmann and Lawson, 2007; Tammela et al., 2011; Tammela et al., 2008). Notch receptors and the ligands delta-like 4 (Dll4) and jagged 1 (Jag1) regulate the dynamic and transient selection of filopodia-extending tip cells, which lead and guide sprouts. Dll4 expression in tip cells leads to the activation of Notch in adjacent ECs, and suppresses tip cell behavior and thereby induces a stalk-like phenotype characteristic of cells at the base of sprouts (Hellström et al., 2007; Leslie et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Dll4-Notch interactions also suppress EC proliferation in the angiogenic vasculature. Accordingly, reduced Dll4 expression or inhibition of Notch triggers the excessive formation of tip cells, increased sprouting and branching, and endothelial hyperproliferation (Hellström et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Interestingly, our recent work indicates that dysregulated vessel growth after loss of endothelial Notch activity is mediated by Vegfr3 and does not require VegfA/Vegfr2 signaling in the postnatal retina (Benedito et al., 2012). The growth-inhibiting activity of Dll4-Notch is opposed by Jag1, which has pro-angiogenic activity and is highly expressed by stalk cells and in the immature vessel plexus (Benedito et al., 2009).

Endothelial growth at the angiogenic front is accompanied by the proliferation and migration of pericytes, one of the (mural) support cell types that are thought to stabilize vessels (Armulik et al., 2011; Bergers and Song, 2005). As the extension of the vessel plexus into the periphery progresses, the vasculature of the central retina

undergoes remodeling into a hierarchically organized structure, which involves the pruning and reshaping of vessels, the formation of arteries and veins, presumably closer interactions with pericytes, and the recruitment of vascular smooth muscle cells to larger caliber vessels. Although it is widely assumed that angiogenic remodeling generates a more efficient, fully functional, mature and quiescent network devoid of growth features, the underlying cellular and molecular processes remain poorly understood.

MATERIALS AND METHODS

Mice

For gene targeting in the endothelium, mice carrying loxP-flanked (floxed) alleles of *Dll4*^{flox} (Koch et al., 2008), *Rbpj*^{flox} (Han et al., 2002), *Vegfr2*^{flox} (Haigh et al., 2003), *Fbxw7*^{flox} (Hoeck et al., 2010) or *Gt(ROSA)26-Sor*$^{tm1(Notch1)Dam/J}$ (Murtaugh et al., 2003) were combined with *Cdh5(PAC)-CreERT2* (Wang et al., 2010) or *Pdgfb-iCre* transgenics (Claxton et al., 2008), which express tamoxifen-inducible Cre recombinase under the control of endothelial promoters.

Arterial EC-specific *Bmx(PAC)-CreERT2* transgenics were generated as follows. The PAC library RPCI21 (Geneservice) containing 129/SvevTACfBr mouse spleen genomic DNA in a pPAC4 vector (Osoegawa et al., 2000) was screened by filter hybridization with radioactive *Bmx* mouse cDNA probes. A cDNA encoding tamoxifen-inducible Cre recombinase (Feil et al., 1997) followed by a polyadenylation signal sequence and an FRT-flanked ampicillin resistance cassette were introduced by recombineering (Copeland et al., 2001) into the start codon of *Bmx* in clone 557-G1. Following Flp-mediated excision of the ampicillin resistance cassette in bacteria, the resulting constructs were validated by restriction digest and PCR analysis and used in circular form for pronuclear injection into fertilized mouse oocytes. Founders, identified by PCR genotyping, were screened by timed matings with *ROSA26R* Cre reporter animals (Soriano, 1999). Recombination specificity and efficiency were further confirmed with *Rosa26-EYFP* Cre reporter mice (Srinivas et al., 2001). Genotyping was performed as previously described (Pitulescu et al., 2010).

To trigger gene inactivation in postnatal mice, intraperitoneal injections of 200 μg tamoxifen (Sigma T5648; dissolved in 5 μl ethanol and 95 μl peanut oil) were given daily from P10 to P13 unless indicated otherwise. For the generation of mosaic *Dll4* mutants, animals carrying the loxP-flanked *Dll4* gene (Koch et al., 2008) in the Rosa26-mT/mG Cre reporter background (Muzumdar et al., 2007) were interbred with *Cdh5(PAC)-CreERT2* transgenics (Wang et al., 2010). At P12, pups received a single dose of 50 μg tamoxifen (dissolved in 1.25 μl ethanol and 48.75 μl peanut oil) and were analyzed 2 days later at P14. The specificity and efficiency of Cre activity were verified with reporter lines and analysis of the mutant vasculature by immunohistochemistry.

All animal procedures were carried out in accordance with national and regional laws and regulations, and were approved by animal ethics committees of the Federal State of North Rhine-Westphalia (Germany). All efforts were made to minimize suffering of the mice.

Processing and analysis of tissue samples

Dissection and whole-mount staining of postnatal retinas at the indicated stages were performed as previously described (Pitulescu et al., 2010). Retinas were prefixed for 1 hour in 4% paraformaldehyde (PFA) and then stored in methanol at −20°C. Groups of collected samples were processed on the same day and under the same experimental conditions.

For the analysis of retina cryosections, whole mouse eyes were fixed overnight in 4% PFA at 4°C before the sclera and the vitreous body were removed. Following dehydration steps in 15% and 30% sucrose solutions dissolved in PBS, retinas were embedded in Tissue Freezing Medium (Leica) and sectioned using a Leica CM-3050-S cryostat. Sections were immunostained on coverslips using the same concentrations and conditions as for retina whole-mounts (Benedito et al., 2012; Pitulescu et al., 2010). Primary antibodies were rabbit anti-Erg (1:100; Lifespan Biosciences, LS-C127740), goat anti-Esm1 (1:100; R&D Systems, AF1999), rabbit anti-collagen IV (1:100; Serotec, 2150-1470), goat anti-Dll4 (1:100; R&D Systems, AF1389), rabbit anti-desmin (1:100; Abcam, ab15200), mouse anti-SMA-Cy3 (1:200; Sigma, C6198), rabbit anti-GFP-488 (1:200; Invitrogen, A21311), rat anti-VE-cadherin (1:100; BD Biosciences, 555289), rat anti-Vegfr2 (1:100; BD Pharmingen, 555307) and goat anti-Vegfr3 (1:100; R&D Systems, AF743). ECs were labeled with biotinylated Isolectin B4 from *Griffonia simplicifolia* (1:50; Vector Labs, B-1205) and cell nuclei with DAPI (1:500; Sigma, D9542) or TOPRO3 (1:500; Invitrogen, T3605). For detection, suitable species-specific Alexa Fluor-coupled secondary antibodies (1:500; Invitrogen), donkey anti-rat Cy3 (1:500; Jackson ImmunoResearch, 712-165-150) or Alexa Fluor-coupled streptavidin (1:200; Invitrogen, S11223 and S11225) were used.

Quantitative PCR analysis on lung lysates was performed as previously described (Benedito et al., 2012). Transcript expression was normalized to *Pecam1* to account for increased EC numbers in *Rbpj*$^{i\Delta EC}$ samples.

EdU labeling of proliferating cells

For detection of proliferating cells *in vivo*, a stock of 1 mg 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen) dissolved in 10 μl DMSO plus 90 μl PBS was generated; 10 μl of this stock solution per gram of body weight were injected intraperitoneally 4 hours before the animals were humanely sacrificed. Retinas were isolated and processed as described (Pitulescu et al., 2010). EdU-positive cells were detected with the Click-iT EdU Alexa Fluor-647 Imaging Kit (Invitrogen, C10340).

Microscopy and image analysis

Samples were analyzed using a stereomicroscope (Leica MZ16F) for low-magnification images, or a confocal microscope (Leica SP5). For overview pictures, a 10× (HC PL APO) lens was used to capture sample areas of 2.4 mm^2. A 40× (HCX PL APO) oil-immersion lens covering a sample area of 0.15 μm^2 was used for high-resolution images and quantitation purposes. Visual data sets were analyzed using Volocity software (Improvision, version 6.0.1) and managed in Photoshop CS4/CS5 (Adobe Systems).

All analysis is based on a minimum of four mutant and four control animals for each time point and experimental condition. Arteries and veins were identified based on morphological criteria. In particular, arteries and distal arterioles are surrounded by capillary-free zones, are straight and have a smaller caliber, contain a small number of branch points and acquire α-SMA coverage much earlier during development than veins (Benjamin, 1998). Arterial branch points were counted starting at the optical nerve head in the central retina and for 1500 μm [*Bmx(PAC)-CreERT2*] or for full artery length (Pdgfb-iCre). Staining intensities were measured using four representative areas of veins or, when indicated, other vascular beds per sample area. The results were normalized to the background staining of non-endothelial areas. For the measurement of venous EC number and size, 15 stacks covering 7 μm of the most distal area of veins were taken. The stacks were projected in two dimensions and the ratio of total vein area divided by the length of the measured veins was determined. The number of ECs per vein area was quantified by counting Erg-positive nuclei and normalized to 100 μm of vessel length. The size of ECs was calculated by dividing a defined area of vein by the number of ECs that it contains. Pericyte coverage was quantified as the desmin-positive area among the total Isolectin B4-positive area. All graphs show s.e.m. and significance levels were calculated using Student's *t*-test or ANOVA combined with Tukey's post-hoc test as indicated.

RESULTS

Vessel remodeling and the cessation of endothelial proliferation

To visualize changes in the organization of the vascular network and EC behavior during postnatal development of the retinal vasculature, we monitored vessel architecture and cell proliferation in retina whole-mounts and sections between postnatal day (P) 6 and 18. At P6, the growing superficial vessel plexus had not yet reached the periphery of the retina and numerous sprouts were visible at the angiogenic growth front (Fig. 1A). As an indication of high levels of endothelial proliferation, substantial EdU incorporation was observed in the Isolectin B4-labeled vessels (a

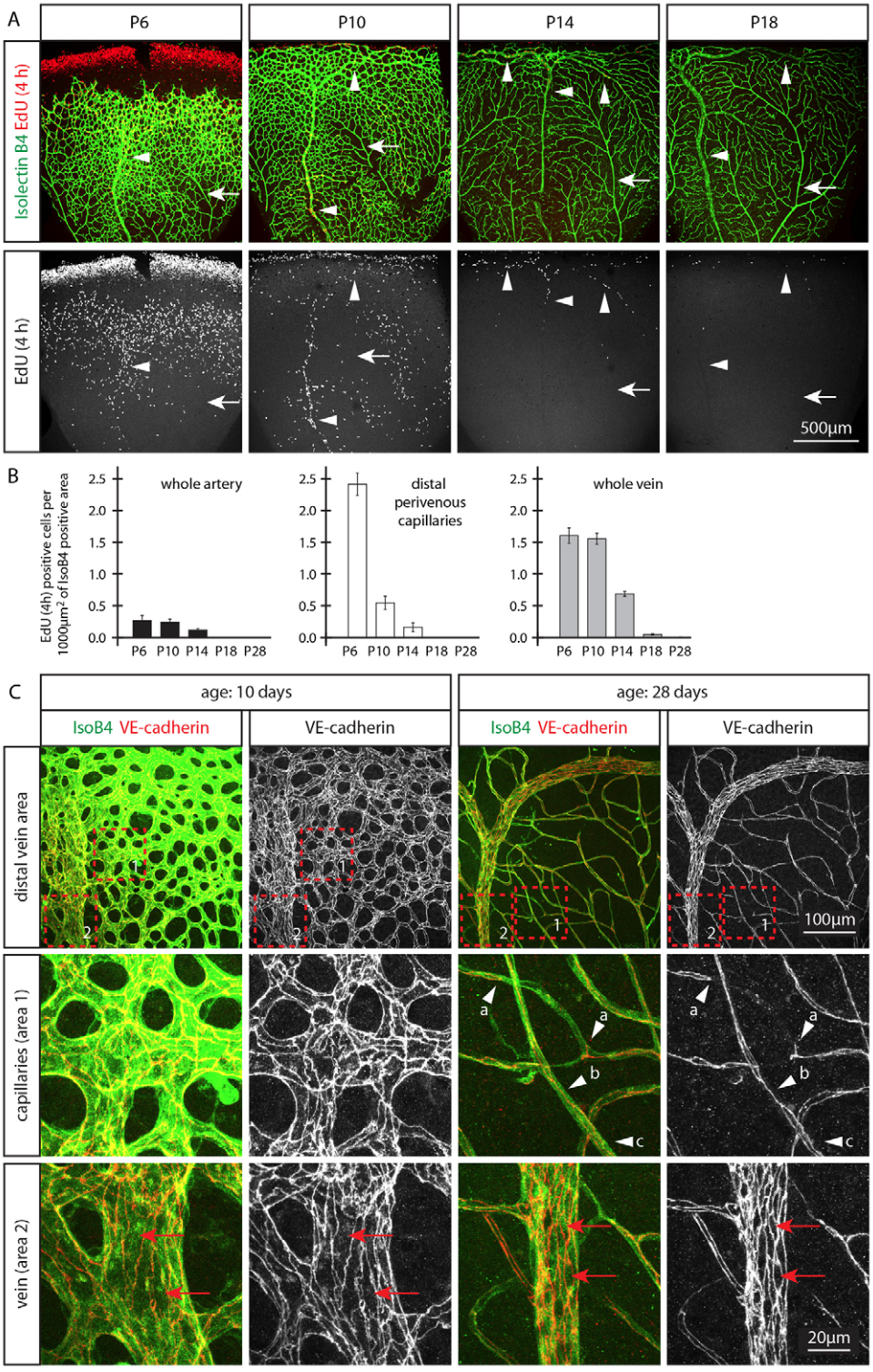

Fig. 1. Remodeling and maturation of the mouse retinal vasculature. (**A**) Confocal images showing proliferation (4 hours of EdU incorporation, red) together with Isolectin B4 staining (green) in the retina at the indicated stages. The bottom row shows EdU signals only. Note that proliferation is absent in arteries (arrows) already at P6, but persists in veins (arrowheads) until P14. (**B**) Quantitative analysis of relative endothelial proliferation at the indicated stages measured by EdU incorporation into retinal arteries, perivenous capillaries and veins as defined by Isolectin B4 staining and morphological criteria. Error bars indicate s.e.m. (**C**) Whole-mounted P10 and P28 retinas labeled with anti-VE-cadherin antibody (red) and Isolectin B4 (IsoB4, green). The center and bottom rows show higher magnifications of boxed areas 1 and 2, respectively, in the top row. Note the extensive changes in the caliber and endothelial cell (EC) number of vessels (area 1). In contrast to the many ECs found within P10 capillaries, remodeled capillaries in the P28 retina (center row) contained tubules formed by single, hollowed ECs (a, arrowheads mark junctions at both ends), single ECs with autocellular junctions (b), or two ECs with two adherens junctions (c). ECs in veins (bottom row) elongate and align along the flow direction between P10 and P28 (red arrows).

tetrameric lectin from *Griffonia simplicifolia* that binds to α-D-galactosyl residues in glycoproteins), which was particularly prominent for the immature vasculature found within 300-400 μm of the leading edge of the growing plexus (Fig. 1A). By P10, the expanding network had reached the outer retinal perimeter and sprouts were no longer visible in the superficial vessel plexus. Endothelial proliferation was decreased relative to that at P6 and restricted to veins, the surrounding perivenous vessel plexus and capillaries of the deeper layers (Fig. 1A,B; supplementary material Fig. S1A). In the P14 retina, EdU-labeled ECs were almost exclusively found in the most distal, peripheral sections of veins, and, by P18, only very few and isolated proliferating cells were detectable (Fig. 1A,B).

Vessels and cells in the superficial plexus underwent significant remodeling and shape changes from P10 onwards (Fig. 1C; supplementary material Fig. S1B). In particular, the density and branching of the capillary network decreased (Fig. 1A), which involved the pruning of side branches visible by the emergence of

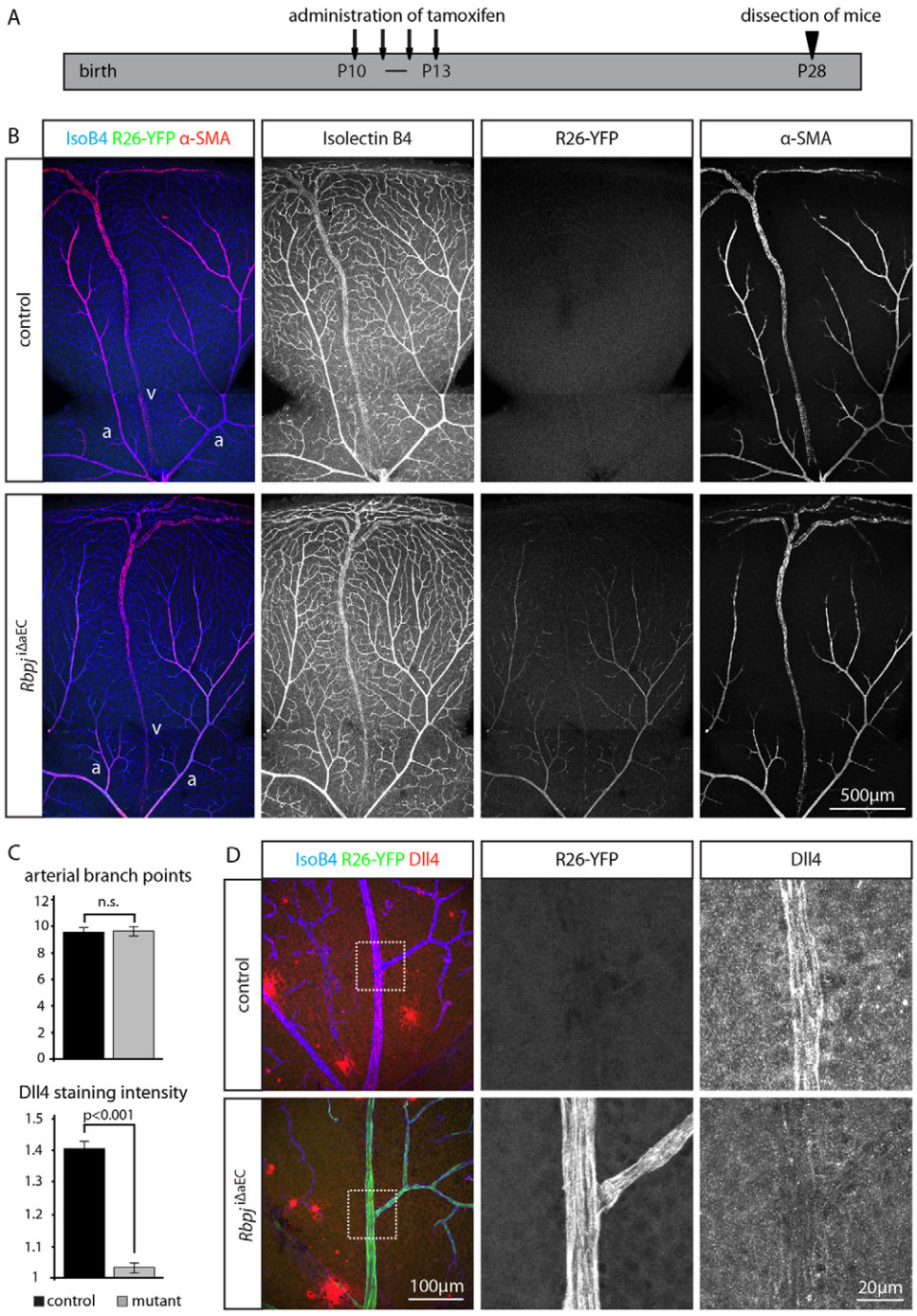

Fig. 2. Arterial Notch signaling is not required for artery patterning. (**A**) Timeline of the experimental procedure. (**B**) Maximum intensity projections of Isolectin B4 (blue) and α-SMA (red) stained retinal whole-mounts of *Bmx(PAC)-CreERT2* × *Rbpj*$^{flox/flox}$ (*Rbpj*$^{i\Delta aEC}$) mutants and littermate controls at P28. Artery-specific Cre activity in the mutant was visualized by incorporation of a *Rosa26-YFP* reporter allele (green). Note the normal vessel patterning in the absence of arterial *Rbpj*. a, artery; v, vein. (**C**) Analysis of arterial branch points (top) showed no significant (n.s.) differences by Student's *t*-test, whereas arterial Dll4 protein (bottom) was significantly reduced. Error bars indicate s.e.m. (**D**) Anti-Dll4 immunofluorescence (red) was strongly reduced in P28 *Rbpj*$^{i\Delta aEC}$ mutants carrying a *Rosa26-YFP* allele (green). ECs are labeled by Isolectin B4 (blue).

thin (collapsed) and 'empty' (Isolectin B4-negative and collagen IV-positive) matrix sleeves (supplementary material Fig. S1B). This vessel remodeling was accompanied by extensive changes in EC morphology and arrangement. Whereas P10 capillaries contained many cells and therefore a large number of cell-cell junctions, anti-VE-cadherin (cadherin 5) staining indicated that each of the slender, remodeled capillaries at P28 contained only one or two ECs per transverse section (Fig. 1C). Likewise, venous ECs were visibly smaller and aligned along the direction of blood flow at P28 but not at P10 (Fig. 1C). The intensity of signals obtained with Isolectin B4 also decreased during postnatal life and were much weaker at P28 than at earlier stages (supplementary material Fig. S2A).

The remodeling of blood vessels involved substantial changes in the mural cell population. Desmin^{+} pericytes displayed an elongated, stretched morphology at P28, but were more abundant, dense and irregularly organized at P6-14 (supplementary material Fig. S2A,B). Vein remodeling and maturation was accompanied by the increasing presence of vascular smooth muscle cells [immunopositive for alpha smooth muscle actin (α-SMA, also known as Acta2)] during later postnatal stages. α-SMA^{+} cells were also transiently seen in perivenous capillaries at P10 and P14 (supplementary material Fig. S2A,C,D).

Together, the above findings indicate that the retinal vasculature is undergoing extensive remodeling from P10 onwards, whereas

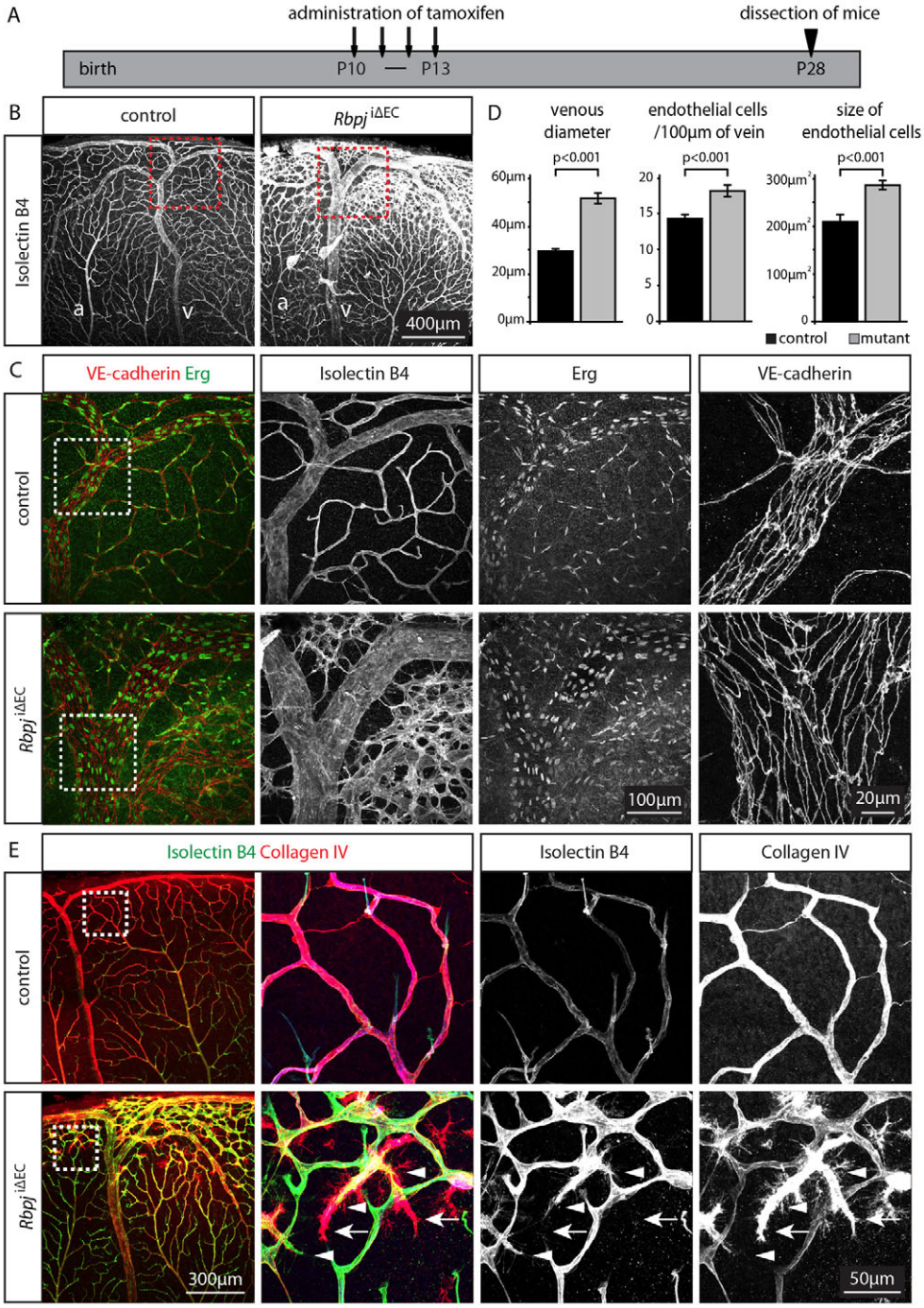

Fig. 3. Targeting of endothelial *Rbpj* during vessel remodeling. (**A**) Timeline of the experimental procedure. (**B**) Confocal images of whole-mount P28 *Pdgfb-iCre* × *Rbpj*$^{flox/flox}$ (*Rbpj*$^{i\Delta EC}$) mutants and control littermates treated with tamoxifen from P10 onwards (P10+). Note the altered morphology of *Rbpj*$^{i\Delta EC}$ distal vein and perivenous plexus. Boxed regions are shown at higher magnification in C. a, artery; v, vein. (**C**) Immunostaining showing junctional VE-cadherin (red) and nuclear Erg (green) together with Isolectin B4 in P28 *Rbpj*$^{i\Delta EC}$ and control retinas. Mutant veins were enlarged and contained more Erg^{+} ECs. *Rbpj*$^{i\Delta EC}$ perivenous capillaries showed high-density and numerous thin protrusions. (**D**) Analysis of vein caliber, venous EC number and size. *P*-values by Student's *t*-test. Error bars indicate s.e.m. (**E**) Visualization of empty matrix sleeves by Isolectin B4 (green) and collagen IV (red) staining. Sprouts and filopodia are indicated by arrowheads, empty matrix sleeves by arrows.

endothelial proliferation and sprouting are largely completed at this stage.

Arterial Notch signaling is dispensable for postnatal artery formation

Previous work has identified Notch signaling as an important regulator of arterial differentiation in the early embryonic vasculature (Lawson et al., 2001). Given the numerous roles of Notch in tissue morphogenesis and in the regulation of (capillary) EC behavior, we employed an inducible approach that enables selective gene targeting in the endothelium of arteries without affecting other vessel beds. For this purpose, *Bmx(PAC)-CreERT2* transgenic mice were generated by recombineering (Copeland et al., 2001), which involved the insertion of a cDNA cassette encoding tamoxifen-inducible Cre recombinase (CreERT2) into a large genomic DNA fragment of the gene encoding the non-receptor tyrosine kinase Bmx (see Materials and methods). The resulting transgenic mice showed inducible artery-specific Cre expression and activity (Fig. 2A,B; supplementary material Fig. S3A,B), similar to what was previously reported for a *lacZ* reporter inserted into the murine *Bmx* gene (Rajantie et al., 2001).

To study the role of Notch signaling in postnatal arteries, *Bmx(PAC)-CreERT2* transgenics were combined with mice carrying a conditional (loxP-flanked) allele of the *Rbpj* gene, which encodes the transcription factor RBP-Jκ, an essential regulator of Notch-induced gene expression within and outside the vasculature

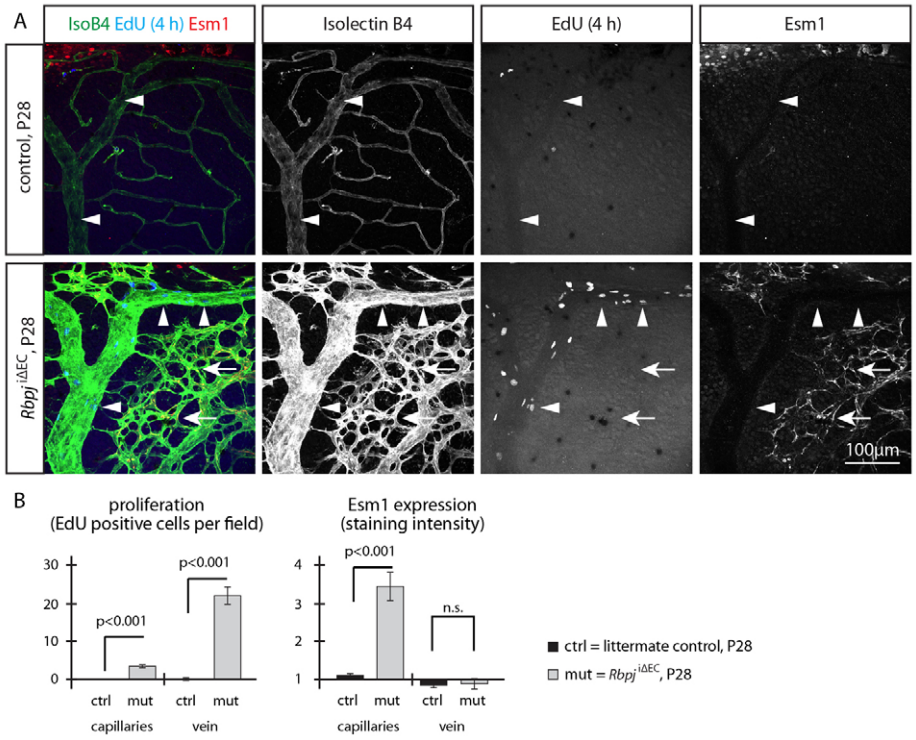

Fig. 4. Ectopic sprouting and proliferation in *Rbpj* veins. (**A**) Maximum intensity projections of P28 *Rbpj*$^{i\Delta EC}$ mutant and control vein regions stained with Isolectin B4 (green) and for the tip cell marker Esm1 (red). Proliferating cells were labeled with EdU (blue). Note EdU^{+} nuclei in the enlarged *Rbpj*$^{i\Delta EC}$ mutant but not control veins (arrowheads) and the abundance of Esm1-expressing cells in the mutant perivenous plexus (arrows). (**B**) Analysis of EdU incorporation (proliferation) and Esm1 immunostaining in control and mutant vessels. The Esm1 immunostaining intensity in P28 veins was below the background of the surrounding tissue. *P*-values by Student's *t*-test. Error bars indicate s.e.m.

(Benedito et al., 2012; Han et al., 2002; Tanigaki and Honjo, 2010). Inactivation of arterial *Rbpj* from P10 onwards led to the expected reduction in expression of Dll4, a Notch target and arterial marker, but not to appreciable defects in arterial patterning or vascular smooth muscle cell recruitment at P28 (Fig. 2A-D). Only weak defects in arterial smooth muscle cell alignment, without significant alterations in branching, were seen after tamoxifen-induced inactivation of *Rbpj* or *Dll4* in the arterial endothelium during earlier postnatal development (supplementary material Fig. S4A-E; data not shown). By contrast, manipulation of Notch signaling with *Pdgfb-iCre* transgenics (Claxton et al., 2008) throughout the postnatal vasculature (in the ECs of arteries, capillaries and veins) substantially altered the pattern of retinal arterialization. Inactivation of endothelial *Dll4* during vessel remodeling led to shorter arteries with fewer α-SMA-covered side branches (supplementary material Fig. S5A,B). Conversely, EC-specific overexpression of the active Notch intracellular domain (NICD) increased the length and branching of retinal arteries in a dose-dependent fashion, which was accompanied by enhanced pruning of periarterial capillaries (supplementary material Fig. S5A,C).

These findings indicate that Dll4 and Notch signaling in ECs, most likely in the endothelium of periarterial capillaries, control the formation and patterning of retinal arteries. By contrast, endothelial Notch signaling was no longer required in the Bmx^{+} arterial endothelium.

Notch-controlled remodeling of the perivenous vessel plexus

Although Notch activity in the vasculature has been frequently linked to arterial growth and differentiation (Gridley, 2007; Shawber and Kitajewski, 2004; Swift and Weinstein, 2009), there is little evidence for a role of the pathway in vein formation. Surprisingly, our analysis of *Rbpj* function in the remodeling retinal vasculature uncovered striking alterations predominantly in veins and perivenous capillaries, whereas capillaries in other regions appeared comparably unaffected and similar to those in control littermates (Fig. 3A,B; supplementary material Fig. S5C,D and Fig. S6A-C). *Rbpj*$^{i\Delta EC}$ veins were significantly enlarged and contained more and, based on the ratio of Erg^{+} endothelial nuclei and the Isolectin B4^{+} vessel area as well as the analysis of junctional contacts, larger and less elongated ECs (Fig. 3B-D). Likewise, the density of desmin^{+} perivascular cells was strongly enhanced in P28 mutant perivenous capillaries, whereas the coverage of veins by α-SMA^{+} cells was strongly reduced (supplementary material Fig. S5C,D and Fig. S6C,D). The organization of the *Rbpj*$^{i\Delta EC}$ retinal vasculature at P14 (i.e. shortly after tamoxifen administration at P10-13) was comparable to that of control littermates, and no significant differences in the desmin^{+} or α-SMA^{+} mural cell populations were observed (supplementary material Fig. S6C,D). This further supports the conclusion that the phenotypic alterations at P28 were the result of defective vessel remodeling and were not caused by dysregulated endothelial sprouting, proliferation or other processes occurring in the early phase of retinal vascularization before P14.

Next, we analyzed the defects in the P28 *Rbpj*$^{i\Delta EC}$ perivenous capillary plexus. The appearance of numerous thin Isolectin B4-positive connections and endothelial protrusions suggested endothelial sprouting and/or the retraction of unstable vessels (Fig. 3B,C; supplementary material Fig. S6C). Indeed, collagen IV^{+} Isolectin B4-stained structures representing empty matrix sleeves were abundant in the *Rbpj*$^{i\Delta EC}$ vasculature and decorated blind-ended vessels or sprouts. This, together with the spiky appearance of ECs and collagen IV sleeves (Fig. 3E) suggested a high level of ectopic and unstable sprouting activity in P28 *Rbpj*$^{i\Delta EC}$ mutants. In line with this interpretation, ECs in the *Rbpj*$^{i\Delta EC}$ perivenous plexus expressed the tip cell marker Esm1, which was absent from the equivalent region in control mice (Fig. 4A,B). Furthermore, *Rbpj*$^{i\Delta EC}$ mutant vessels retained strong Isolectin B4 staining

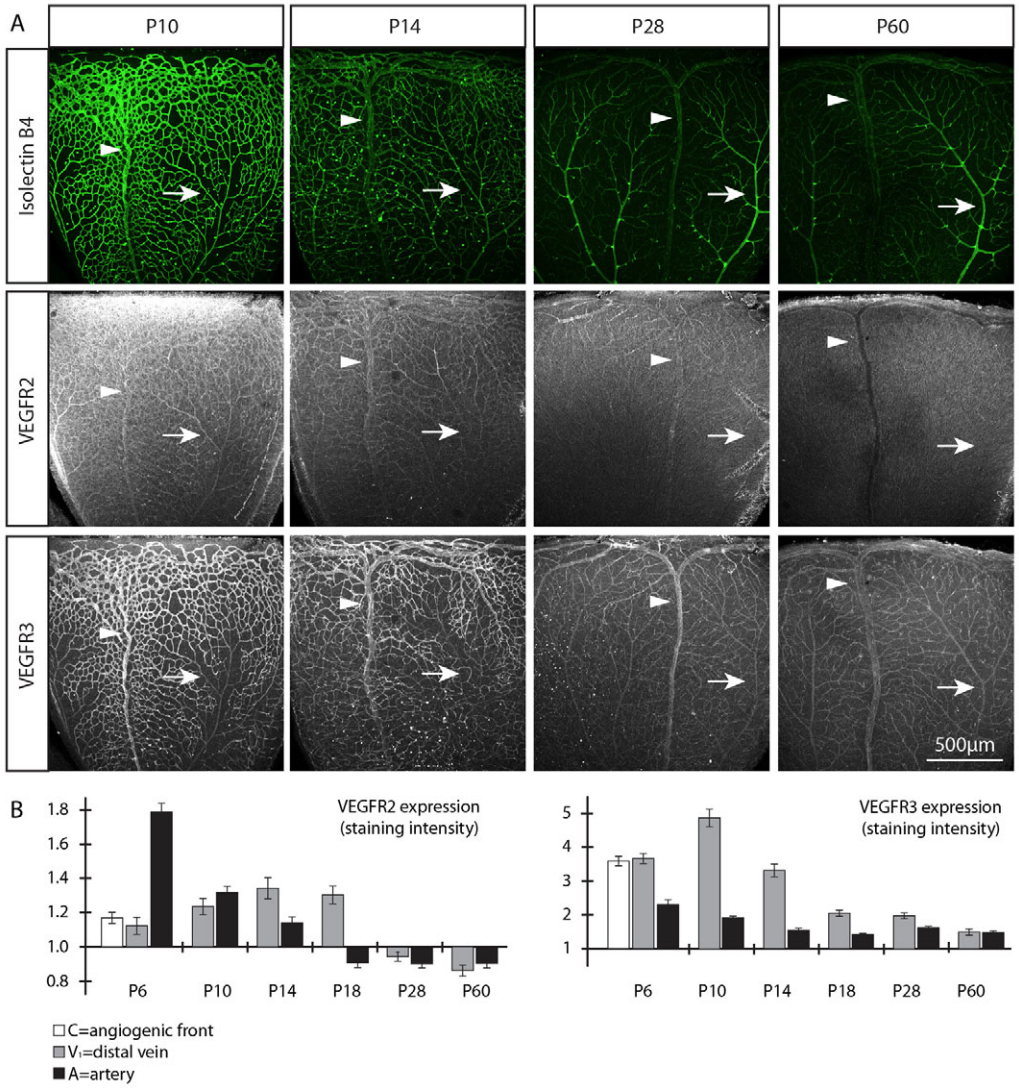

Fig. 5. VEGF receptor downregulation during vascular maturation. (**A**) Immunostaining for Vegfr2 and Vegfr3 at the indicated postnatal stages. ECs were labeled with Isolectin B4. Arteries (arrows) and veins (arrowheads) are indicated. Anti-Vegfr2 staining of neural cells in the deeper retina is visible as background. (**B**) Analysis of Vegfr2 and Vegfr3 expression in capillaries at the angiogenic front, arteries and distal veins. Negative values for Vegfr2 at P18-60 reflect very low vascular expression (less than neural background). Error bars indicate s.e.m.

reminiscent of the actively growing, angiogenic vasculature in younger (control) animals (supplementary material Fig. S2A). Whereas no incorporation of EdU was observed in the P28 control veins and surrounding capillaries, endothelial proliferation was significantly enhanced in *Rbpj*$^{i\Delta EC}$ mutants (Fig. 4A,B).

As Notch signaling has not previously been associated with vein formation and patterning, we also investigated the distribution of Dll4 in control retinas at P14, a stage at which strong remodeling activity can be observed in veins. In addition to the pronounced arterial signal, we observed Dll4 staining within distal veins and surrounding capillaries (supplementary material Fig. S7). The specificity of this anti-Dll4 immunostaining was validated with mosaic *Dll4*$^{i\Delta EC}$ mutants (supplementary material Fig. S8), which were generated in the Rosa26-mT/mG Cre reporter background (Muzumdar et al., 2007) by administering a single, low dose of tamoxifen (see Materials and methods). Furthermore, inducible inactivation of *Fbxw7*, which encodes the substrate-recognizing subunit of an E3 ubiquitin ligase that mediates the degradation of active NICD (Izumi et al., 2012; Tetzlaff et al., 2004; Tsunematsu et al., 2004), led to increased Dll4 expression in perivenous capillaries and enhanced vein remodeling at P14 relative to that in littermate controls (supplementary material Fig. S7A,B). These findings support the conclusion that endogenous Notch activity is present in distal veins where it controls vein patterning and remodeling.

Developmental downregulation of VEGF receptor expression

To gain further insight into the molecular processes contributing to vessel maturation in the postnatal retina, we investigated the expression of the receptors Vegfr2 and Vegfr3, which are important regulators of angiogenic EC sprouting and proliferation (Benedito et al., 2012; Gerhardt et al., 2003; Tammela et al., 2011; Tammela et al., 2008), between P6 and adulthood. Whereas anti-Vegfr2 immunofluorescence labeled arteries, veins and capillaries in the P6 and P10 vasculature, these signals were strongly decreased at

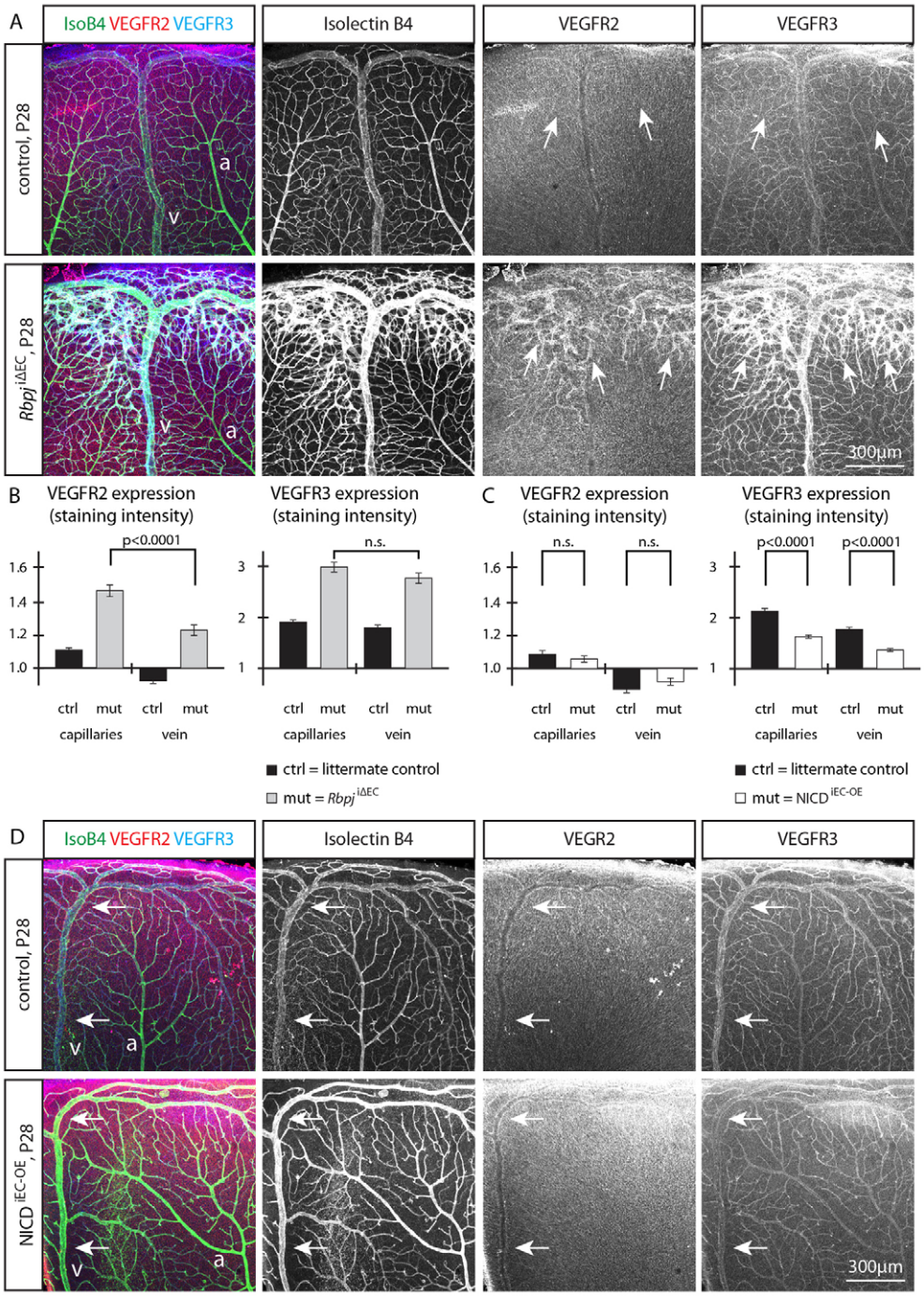

Fig. 6. Downregulation of VEGF receptors during maturation requires Notch. (**A**) Regions with vascular defects in *Rbpj*$^{i\Delta EC}$ mutants at P28 show ectopically high expression of Vegfr2 and Vegfr3 (arrows). ECs are marked by Isolectin B4 (green/white). (**B**,**C**) Analysis of Vegfr2 and Vegfr3 expression in *Rbpj*$^{i\Delta EC}$ (B) and *Pdgfb-iCre* × *Rosa26-NICD* (*NICD*^{iGOF}) (C) capillaries and veins relative to corresponding controls. *P*-values by ANOVA. Error bars indicate s.e.m. (**D**) Inducible overexpression of active Notch (NICD) in *NICD*^{iGOF} mutants lowered residual Vegfr3 (blue, arrows) expression in veins at P28. a, artery; v, vein.

P14/P28 and absent at P60 (Fig. 5A,B; data not shown). The specificity of this staining was validated in EC-specific, inducible *Vegfr2* mutants (supplementary material Fig. S9A). Similar to Vegfr2, Vegfr3 protein, which was much more abundant on veins and capillaries compared with arteries, gradually decreased during maturation and only weak staining was visible at P28 and P60 (Fig. 5A,B; supplementary material Fig. S9A-C). As mentioned above, signals obtained by Isolectin B4 staining also decreased in maturing vessels and were much weaker at P28/P60 than at P10 (Fig. 5A,B).

The developmental downregulation of VEGF receptor expression in the maturation phase between P10 and P28 was compromised in *Rbpj*$^{i\Delta EC}$ mutant retinas. In addition to enhanced Isolectin B4 binding, strongly increased Vegfr2 and Vegfr3 immunostaining was observed in distal veins and perivenous capillaries, which are the sites of ectopic endothelial sprouting and proliferation (Fig. 6A,C). Further indicating a role of Notch in the vessel maturation program, the downregulation of Vegfr3 expression was enhanced after EC-specific NICD overexpression (*NICD*$^{iEC\text{-}OE}$) between P10 and P18 (supplementary material Fig. S10A-D). This was accompanied by accelerated vein remodeling, pruning of side branches and decreased vessel density in the distal perivenous plexus (supplementary material Fig. S10A). Likewise, even at P28, when Vegfr2 levels were already very low in control retinas and comparable to levels in *NICD*$^{iEC\text{-}OE}$ mutants, Vegfr3 staining in perivenous capillaries and, in particular, in distal veins was

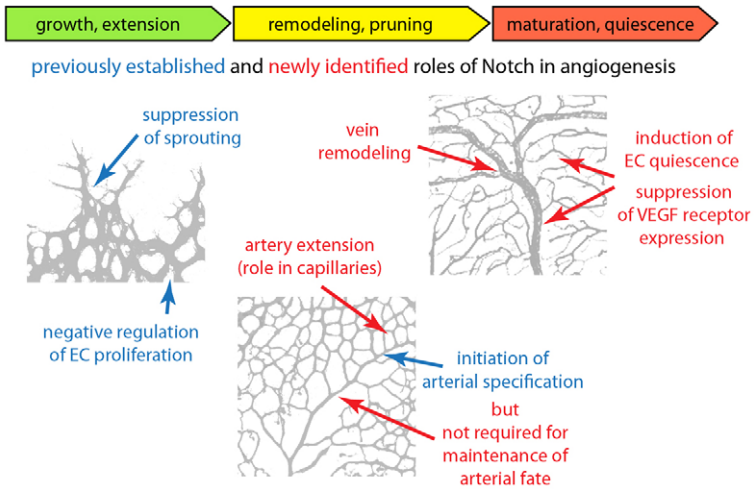

Fig. 7. Notch functions in angiogenesis. Previously known (blue) and newly identified (red) roles of Notch signaling in EC sprouting, proliferation, arterial differentiation, artery extension, vein remodeling, and the transition to a mature, quiescent network. Surprisingly, Notch activity is not required in the Bmx-positive arterial endothelium.

significantly decreased after expression of active Notch from P10 onwards (Fig. 6D). Suggesting that some of this regulation is transcriptional, *Vegfr3* mRNA levels were upregulated in *Rbpj*$^{i\Delta EC}$ lung lysates (supplementary material Fig. S10E,F). These findings identify Notch as a key regulator of vessel maturation and quiescence in the retinal vasculature (Fig. 7).

DISCUSSION

Vessel remodeling and maturation

The angiogenic expansion of the blood vessel network involves a series of distinct but strongly coordinated steps including proliferation, sprouting, anastomosis, pruning, remodeling and the acquisition of quiescence (Eilken and Adams, 2010; Potente et al., 2011). Some of these processes occur simultaneously, whereas others are spatially or temporally separated (Fig. 7). For example, while endothelial sprouting, proliferation and vessel anastomosis contribute to the expansion of the vasculature at the angiogenic front in the peripheral retina, the more central, previously formed vessels have already reorganized into hierarchically organized arterial and venous trees with connecting capillaries. The majority of the remodeling and maturation processes in the retinal vasculature occur after the growth phase (i.e. when ECs have stopped sprouting and proliferating) and extend over a period of least 2 weeks (Fig. 1A). This lengthy time course and the extensive reorganization of the retinal vasculature between P10 and adulthood suggest that the underlying regulation is likely to be complex and multifactorial. Certain vessel connections are pruned away, whereas others are presumably enforced. One can expect that factors such as blood flow, laminar shear stress, EC-EC junctional contacts, cell-matrix adhesion, and interactions with perivascular cells become enhanced. As we show here, the expression of VEGF receptors is gradually downregulated, which is likely to reduce the ability of ECs to respond to pro-angiogenic VEGF family growth factors. The transcriptional profile of the vascular cells and many other cellular parameters are likely to change substantially. Thus, although we identify the Notch pathway as an important regulator of vascular remodeling and conversion to quiescence, numerous other pathways are likely to contribute.

Are the findings in the retinal vasculature, which was chosen as a model system because it develops later than most other vessel beds and can be imaged in whole-mounts at high resolution, relevant for remodeling processes in other organs? Several examples suggest that this might be the case. The vascularization of the brain occurs at mid-gestation in the mouse embryo, whereas the sealing of EC-EC contacts and the formation of the blood-brain barrier are only completed in the postnatal animal (Nitta et al., 2003; Ruhrberg et al., 2002). Vessel sprouting can be observed in the dermis of the mouse embryo (Wang et al., 2010), but this process is largely completed before birth despite the extensive growth of the organism during postnatal life. A dense and primitive plexus of vessels has formed around the trachea of the mouse by embryonic day 16.5, and this collapses soon after birth and regrows, adopting a hierarchical, ladder-like pattern. In this system, capillary loops and blind-ended sprouts/projections continue to be resolved until the animals have reached adulthood (Ni et al., 2010). The lymphatic vasculature, as the second tubular system assembled by ECs, is formed and extends substantially during the second half of embryonic development, but it is only in postnatal life/adolescence that morphological changes are completed and the vessels acquire resistance to VegfC withdrawal (Karpanen et al., 2006; Norrmén et al., 2009). Taken together, vessel remodeling and maturation appear to involve similarly time-consuming and complex processes in different tissues and vascular beds, which, in turn, indicates the wider relevance of findings made in the retina model.

Notch function in the growing vasculature

The functional roles of Notch signaling and its molecular crosstalk with other pathways appear increasingly complicated. Previous work has established that the pathway is a key regulator of arterial differentiation. Loss of Notch signaling in early mouse and zebrafish embryos led to compromised arterial-venous (AV) differentiation, the loss of arterial markers and the ectopic expression of venous markers within the dorsal aorta (Duarte et al., 2004; Krebs et al., 2004; Lawson et al., 2001; Zhong et al., 2001). Conversely, ectopic activation of Notch signaling repressed venous markers and triggered the formation of AV shunts (Carlson et al., 2005; Lawson et al., 2001; Murphy et al., 2012). Although the continued expression of Notch pathway components in the arterial endothelium might suggest a role in the maintenance or stabilization of arteries throughout life, our findings show that this is actually not the case in the retina. Nevertheless, our genetic experiments affecting all retinal ECs confirm that Notch activity controls the patterning and extension of growing arteries, which presumably reflects a role in remodeling periarterial capillaries and not in Bmx^{+} arterial ECs.

Another well-established function of the Notch pathway is the regulation of EC behavior in angiogenic growth. Published work has shown that the pathway suppresses endothelial proliferation and sprouting and helps to coordinate the dynamic behavior of tip and stalk cells (Hellström et al., 2007; Jakobsson et al., 2010; Lobov et al., 2007; Suchting et al., 2007). These activities have been linked to the modulation of VEGF receptor expression and, in zebrafish, the microRNA miR-221 (Benedito et al., 2012; Hellström et al., 2007; Nicoli et al., 2012; Suchting et al., 2007). By contrast, much less is known about the function of Notch in vessel remodeling, stabilization and maturation. Lobov et al. (Lobov et al., 2011) have shown that Dll4/Notch inhibition can prevent retinal capillary occlusion and regression in the oxygen-induced retinopathy model. This was attributed to upregulated expression of the vasodilator adrenomedullin and suppression of the vasoconstrictor angiotensinogen. The effect of angiotensin II, a potent vasoconstrictor that can induce the collapse and regression of immature vessels, was significantly attenuated 24 hours after the administration of soluble Dll4-Fc fusion protein, which inhibits Dll4-Notch signaling (Lobov et al., 2011). The same study also suggested that global inactivation of a single *Dll4* allele or Dll4-Fc administration can reduce vessel pruning without affecting sprouting or proliferation between P7 and P10. Our characterization of genetic models, however, strongly indicates that Notch promotes physiological remodeling of the retinal vasculature and thereby enhances activities such as pruning. Moreover, we find that Dll4-Notch signaling is necessary to impose a quiescent endothelial phenotype devoid of activities such as sprouting and proliferation at stages up to P28. Unexpectedly, this important role of Dll4-Notch signaling was most prominently required in veins and perivenous capillaries, whereas previous work has linked the pathway to the growth and differentiation of arteries (Gridley, 2007; Shawber and Kitajewski, 2004; Swift and Weinstein, 2009).

Vascular remodeling in pathological settings

Pathological angiogenesis in diseases such as cancer, diabetic retinopathy or the wet form of age-related macular degeneration is frequently associated with excessive expression of VEGFA and other pro-angiogenic factors, which leads to structural abnormalities, excessive proliferation and sprouting, high permeability and defective patterning (Carmeliet and Jain, 2011; Ferrara, 2005). In tumors, the administration of VEGFA/VEGFR2 inhibitors can affect many of these features and induce pronounced regression of the unstable vasculature (Carmeliet and Jain, 2011; Jain, 2003; Mancuso et al., 2006). The residual tumor vasculature surviving such treatments often displays a ‘normalized’ morphology resembling many aspects of mature vessels in healthy tissues.

As the cellular and molecular processes contributing to vessel normalization remain incompletely understood, better insight into the events mediating physiological vessel remodeling and maturation is likely to improve our understanding of the defects in pathological angiogenesis and might clear the path to new therapeutic opportunities. This might also benefit therapeutic neoangiogenesis in wound healing and tissue regeneration, as we currently lack clear strategies for the local generation of new vessel beds. Thus, insights gained in the remodeling of retinal vasculature might well provide valuable clues for the development of therapeutic regimes for the generation of fully functional and stable blood vessels during tissue repair processes.

Acknowledgements
We thank A. Duarte and F. Radtke for generously providing *Dll4* conditional mice, G. Breier and T. Honjo for floxed *Vegfr2* and *Rbpj* strains, and M. Wöste for help with qPCR experiments.

Funding
Funding was provided by the Max Planck Society and the University of Münster.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
M.E., R.B. and R.H.A. designed experiments and interpreted results. M.E. and R.H.A. wrote the manuscript. M.E., R.B. and S.A. generated mouse mutant lines. M.E. performed all experiments, analyzed the results and processed the image data.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.093351/-/DC1

References

Armulik, A., Genové, G. and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* **21**, 193-215.
Benedito, R., Roca, C., Sörensen, I., Adams, S., Gossler, A., Fruttiger, M. and Adams, R. H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124-1135.
Benedito, R., Rocha, S. F., Woeste, M., Zamykal, M., Radtke, F., Casanovas, O., Duarte, A., Pytowski, B. and Adams, R. H. (2012). Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. *Nature* **484**, 110-114.
Benjamin, L. E., Hemo, I. and Keshet, E. (1998). A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* **125**, 1591-1598.
Bergers, G. and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro. Oncol.* **7**, 452-464.
Carlson, T. R., Yan, Y., Wu, X., Lam, M. T., Tang, G. L., Beverly, L. J., Messina, L. M., Capobianco, A. J., Werb, Z. and Wang, R. (2005). Endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. *Proc. Natl. Acad. Sci. USA* **102**, 9884-9889.
Carmeliet, P. and Jain, R. K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat. Rev. Drug Discov.* **10**, 417-427.
Chung, A. S. and Ferrara, N. (2011). Developmental and pathological angiogenesis. *Annu. Rev. Cell Dev. Biol.* **27**, 563-584.
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.
Copeland, N. G., Jenkins, N. A. and Court, D. L. (2001). Recombineering: a powerful new tool for mouse functional genomics. *Nat. Rev. Genet.* **2**, 769-779.
Dorrell, M. I., Aguilar, E. and Friedlander, M. (2002). Retinal vascular development is mediated by endothelial filopodia, a preexisting astrocytic template and specific R-cadherin adhesion. *Invest. Ophthalmol. Vis. Sci.* **43**, 3500-3510.
Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D. and Rossant, J. (2004). Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev.* **18**, 2474-2478.
Eilken, H. M. and Adams, R. H. (2010). Dynamics of endothelial cell behavior in sprouting angiogenesis. *Curr. Opin. Cell Biol.* **22**, 617-625.
Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem. Biophys. Res. Commun.* **237**, 752-757.
Ferrara, N. (2005). VEGF as a therapeutic target in cancer. *Oncology* **69 Suppl. 3**, 11-16.
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.
Gridley, T. (2007). Notch signaling in vascular development and physiology. *Development* **134**, 2709-2718.
Haigh, J. J., Morelli, P. I., Gerhardt, H., Haigh, K., Tsien, J., Damert, A., Miquerol, L., Muhlner, U., Klein, R., Ferrara, N. et al. (2003). Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. *Dev. Biol.* **262**, 225-241.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int. Immunol.* **14**, 637-645.
Hellström, 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.
Hoeck, J. D., Jandke, A., Blake, S. M., Nye, E., Spencer-Dene, B., Brandner, S. and Behrens, A. (2010). Fbw7 controls neural stem cell differentiation and progenitor apoptosis via Notch and c-Jun. *Nat. Neurosci.* **13**, 1365-1372.
Hogan, B. M., Herpers, R., Witte, M., Heloterä, H., Alitalo, K., Duckers, H. J. and Schulte-Merker, S. (2009). Vegfc/Flt4 signalling is suppressed by Dll4 in developing zebrafish intersegmental arteries. *Development* **136**, 4001-4009.
Izumi, N., Helker, C., Ehling, M., Behrens, A., Herzog, W. and Adams, R. H. (2012). Fbxw7 controls angiogenesis by regulating endothelial Notch activity. *PLoS ONE* **7**, e41116.
Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat. Med.* **9**, 685-693.
Jakobsson, L., Franco, C. A., Bentley, K., Collins, R. T., Ponsioen, B., Aspalter, I. M., Rosewell, I., Busse, M., Thurston, G., Medvinsky, A. et al. (2010). Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat. Cell Biol.* **12**, 943-953.
Karpanen, T., Wirzenius, M., Mäkinen, T., Veikkola, T., Haisma, H. J., Achen, M. G., Stacker, S. A., Pytowski, B., Ylä-Herttuala, S. and Alitalo, K. (2006). Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. *Am. J. Pathol.* **169**, 708-718.
Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N. R., Duarte, A., Macdonald, H. R. and Radtke, F. (2008). Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J. Exp. Med.* **205**, 2515-2523.
Krebs, L. T., Shutter, J. R., Tanigaki, K., Honjo, T., Stark, K. L. and Gridley, T. (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev.* **18**, 2469-2473.
Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A. and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128**, 3675-3683.
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.
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.
Lobov, I. B., Cheung, E., Wudali, R., Cao, J., Halasz, G., Wei, Y., Economides, A., Lin, H. C., Papadopoulos, N., Yancopoulos, G. D. et al. (2011). The Dll4/Notch pathway controls postangiogenic blood vessel remodeling and regression by modulating vasoconstriction and blood flow. *Blood* **117**, 6728-6737.
Mancuso, M. R., Davis, R., Norberg, S. M., O'Brien, S., Sennino, B., Nakahara, T., Yao, V. J., Inai, T., Brooks, P., Freimark, B. et al. (2006). Rapid vascular regrowth in tumors after reversal of VEGF inhibition. *J. Clin. Invest.* **116**, 2610-2621.
Murphy, P. A., Kim, T. N., Lu, G., Bollen, A. W., Schaffer, C. B. and Wang, R. A. (2012). Notch4 normalization reduces blood vessel size in arteriovenous malformations. *Sci. Transl. Med.* **4**, 117ra118.
Murtaugh, L. C., Stanger, B. Z., Kwan, K. M. and Melton, D. A. (2003). Notch signaling controls multiple steps of pancreatic differentiation. *Proc. Natl. Acad. Sci. USA* **100**, 14920-14925.
Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593-605.
Ni, A., Lashnits, E., Yao, L. C., Baluk, P. and McDonald, D. M. (2010). Rapid remodeling of airway vascular architecture at birth. *Dev. Dyn.* **239**, 2354-2366.
Nicoli, S., Knyphausen, C. P., Zhu, L. J., Lakshmanan, A. and Lawson, N. D. (2012). miR-221 is required for endothelial tip cell behaviors during vascular development. *Dev. Cell* **22**, 418-429.
Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M. and Tsukita, S. (2003). Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J. Cell Biol.* **161**, 653-660.
Norrmén, C., Ivanov, K. I., Cheng, J., Zangger, N., Delorenzi, M., Jaquet, M., Miura, N., Puolakkainen, P., Horsley, V., Hu, J. et al. (2009). FOXC2 controls formation and maturation of lymphatic collecting vessels through cooperation with NFATc1. *J. Cell Biol.* **185**, 439-457.
Osoegawa, K., Tateno, M., Woon, P. Y., Frengen, E., Mammoser, A. G., Catanese, J. J., Hayashizaki, Y. and de Jong, P. J. (2000). Bacterial artificial chromosome libraries for mouse sequencing and functional analysis. *Genome Res.* **10**, 116-128.
Pitulescu, M. E., Schmidt, I., Benedito, R. and Adams, R. H. (2010). Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. *Nat. Protoc.* **5**, 1518-1534.
Potente, M., Gerhardt, H. and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* **146**, 873-887.
Rajantie, I., Ekman, N., Iljin, K., Arighi, E., Gunji, Y., Kaukonen, J., Palotie, A., Dewerchin, M., Carmeliet, P. and Alitalo, K. (2001). Bmx tyrosine kinase has a redundant function downstream of angiopoietin and vascular endothelial growth factor receptors in arterial endothelium. *Mol. Cell. Biol.* **21**, 4647-4655.
Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 73-91.
Ruhrberg, C., Gerhardt, H., Golding, M., Watson, R., Ioannidou, S., Fujisawa, H., Betsholtz, C. and Shima, D. T. (2002). Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev.* **16**, 2684-2698.
Shawber, C. J. and Kitajewski, J. (2004). Notch function in the vasculature: insights from zebrafish, mouse and man. *BioEssays* **26**, 225-234.
Siekmann, A. F. and Lawson, N. D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445**, 781-784.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
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.
Stone, J., Itin, A., Alon, T., Pe'er, J., Gnessin, H., Chan-Ling, T. and Keshet, E. (1995). Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *Neuroscience* **15**, 4738-4747.
Suchting, S., Freitas, C., le Noble, F., Benedito, R., Bréant, 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.
Swift, M. R. and Weinstein, B. M. (2009). Arterial-venous specification during development. *Circ. Res.* **104**, 576-588.
Tammela, T., Zarkada, G., Wallgard, E., Murtomäki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellström, M., Schomber, T., Peltonen, R. et al. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* **454**, 656-660.
Tammela, T., Zarkada, G., Nurmi, H., Jakobsson, L., Heinolainen, K., Tvorogov, D., Zheng, W., Franco, C. A., Murtomäki, A., Aranda, E. et al. (2011). VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nat. Cell Biol.* **13**, 1202-1213.
Tanigaki, K. and Honjo, T. (2010). Two opposing roles of RBP-J in Notch signaling. *Curr. Top. Dev. Biol.* **92**, 231-252.
Tetzlaff, M. T., Yu, W., Li, M., Zhang, P., Finegold, M., Mahon, K., Harper, J. W., Schwartz, R. J. and Elledge, S. J. (2004). Defective cardiovascular development and elevated cyclin E and Notch proteins in mice lacking the Fbw7 F-box protein. *Proc. Natl. Acad. Sci. USA* **101**, 3338-3345.
Tsunematsu, R., Nakayama, K., Oike, Y., Nishiyama, M., Ishida, N., Hatakeyama, S., Bessho, Y., Kageyama, R., Suda, T. and Nakayama, K. I. (2004). Mouse Fbw7/Sel-10/Cdc4 is required for notch degradation during vascular development. *J. Biol. Chem.* **279**, 9417-9423.
Wang, Y., Nakayama, M., Pitulescu, M. E., Schmidt, T. S., Bochenek, M. L., Sakakibara, A., Adams, S., Davy, A., Deutsch, U., Lüthi, U. et al. (2010). Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* **465**, 483-486.
Zhong, T. P., Childs, S., Leu, J. P. and Fishman, M. C. (2001). Gridlock signalling pathway fashions the first embryonic artery. *Nature* **414**, 216-220.