Notch controls retinal blood vessel maturation and quiescence
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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).

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; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Dll4-Notch interactions also suppress EC proliferation in the angiogenic vasculature. Accordingly, reducedDll4 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; Berger 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 Dil\textsuperscript{crx} (Koch et al., 2008), Rhbp\textsuperscript{flox} (Han et al., 2002), Vegfr\textsuperscript{flox} (Haigh et al., 2003), Fbxw7\textsuperscript{flox} (Hoecck et al., 2010) or Gt(RosA26)Syp\textsuperscript{flox} (Murtagh et al., 2003) were combined with Gdh5(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/SvEvTACBr 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 recombinering (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 ROSA26\textsuperscript{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 Dil\textsuperscript{crx} mutants, animals carrying the loxP-flanked Dil\textsuperscript{crx} (Koch et al., 2008) in the Rosa26-mT/mG Cre reporter background (Muzumdar et al., 2007) were interbred with Rosa26-Fth1(Bmp)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 alleles of Dll4\textsuperscript{flox} (Koch et al., 2008), Vegfr\textsuperscript{flox} (Han et al., 2002), Fbxw7\textsuperscript{flox} (Haigh et al., 2003), Pdgfb-iCre (Claxton et al., 2008) or Pdgfb-iCre transgenics (Wang et al., 2010). 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

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
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
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...
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.
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Notch signalling was no longer required in the Bmx+ 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. S5,C,D and Fig. S6A-C). Rbpjkie mice showed significantly enlarged and contained more and, based on the ratio of Erg1+ 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. S5,C,D and Fig. S6C,D). The organization of the Rbpjkie 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 vasculogenesis before P14.

Next, we analyzed the defects in the P28 Rbpjkie 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 Rbpjkie vascular network 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 Rbpjkie mutants. In line with this interpretation, ECs in the Rbpjkie perivenous plexus expressed the tip cell marker Esm1, which was absent from the equivalent region in control mice (Fig. 4A,B). Furthermore, Rbpjkie mutant vessels retained strong Isolectin B4 staining.
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\[EC] mutants (Fig. 4A,B).

As Notch signaling has not previously been associated with vein formation and patterning, we also investigated the distribution ofDll4 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\[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

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**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.
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\(\text{i}^\Delta\)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 (\(\text{NICD}^{\text{EC-OE}}\)) 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 \(\text{NICD}^{\text{EC-OE}}\) mutants, Vegfr3 staining in perivenous capillaries and, in particular, in distal veins was

**Fig. 6. Downregulation of VEGF receptors during maturation requires Notch.** (A) Regions with vascular defects in \(\text{Rbpj}^{\Delta\text{EC}}\) 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 \(\text{Rbpj}^{\Delta\text{EC}}\) (B) and \(\text{Pdgfb}\text{-iCre} \times \text{Rosa26-NICD (NICD}^{\text{GOF}}\) (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 \(\text{NICD}^{\text{EC-OE}}\) mutants lowered residual Vegfr3 (blue, arrows) expression in veins at P28. a, artery; v, vein.
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. 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 VEGF 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<sup>+</sup> 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; Nicolì 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 VEGF/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.

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**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**


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