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Review

Angiogenesis: A Team Effort Coordinated by Notch

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The past two decades of angiogenesis research have identified a wealth of pro- and antiangiogenic signals originating from the tissue environment, which control blood vessel density and function. Understanding when and how blood vessels respond to the combination of signals they encounter to achieve a balanced cellular response is a major challenge for the field of developmental and tumor angiogenesis. This review focuses on how endothelial cell-cell communication via the Notch pathway contributes to this signal integration and is essential for functional vessel patterning.

Blood vessel formation supports tissue growth and organ function in development, physiology, and disease. An insufficient supply of nutrients and oxygen prompts the formation of new vessels from the walls of existing vessels in a process termed angiogenic sprouting. Hypoxic tissues secrete growth factors and chemokines that stimulate the endothelial cells to break out of their stable position in the vessel wall and jointly coordinate sprouting, branching, and new lumenized network formation, until supply meets demand and quiescence can be re-established (see also Fraisl et al., 2009 [this issue of *Developmental Cell*]). Once it is initiated by environmental growth factor signals, the sprouting process is spearheaded by leading endothelial tip cells (Gerhardt et al., 2003) (Figure 1). Stimulated by vascular endothelial growth factor-A (VEGF-A), these cells produce long, dynamic filopodia studded with the tyrosine kinase receptor VEGFR2/KDR/Flk-1 and other receptors, which they use to probe the environment for directional cues. The endothelial cells that follow the tip cells, termed stalk cells, produce fewer filopodia and instead proliferate when stimulated with VEGF-A (Gerhardt et al., 2003). They also form the vascular lumen (see Iruela-Arispe and Davis, 2009 [this issue of *Developmental Cell*]), and they establish adherens and tight junctions to maintain the integrity of the new sprout (see Dejana et al., 2009 [this issue of *Developmental Cell*]) and to establish luminal/abluminal polarity, which, in turn, leads to basal lamina deposition and mural cell recruitment/attachment. Endothelial tip and stalk cells also differ in their gene expression profile; tip cells express *Pdgfb*, *Dll4*, *Unc5b*, *Kdr*, and *Flt4* more strongly than stalk cells (Claxton and Fruttiger, 2004; Gerhardt et al., 2003; Lu et al., 2004; Siekmann and Lawson, 2007; Tammela et al., 2008). These differences are quantitative and most prominent at the mRNA level. It is important to note that no single gene identified thus far can serve as a unique marker of tip cells. Nevertheless, these quantitative differences in gene expression support the idea that tip and stalk cells have specialized functions during sprouting angiogenesis. Each new sprout eventually connects with adjacent sprouts via the tip cell to form a continuous lumen and thus establish flow in the new vascular loop (Blum et al., 2008; Leslie et al., 2007). Establishment of flow and basement membrane and mural cell recruitment all contribute to the remodeling and maturation of the new vascular connection (Jain, 2003; Jones et al., 2006; le Noble et al., 2004; Lucitti et al., 2007). Flow-dependent tissue oxygenation finally downregulates paracrine VEGF-A production, and thus helps establish a quiescent state for the new vessels.

Not all connections remain stable, however, as extensive remodeling reshapes the primitive plexus (Fruttiger, 2007). Vascular remodeling, involving the regression of branches, some new sprouting, and vessel splitting by intussusceptive growth, is regulated by hemodynamic forces and crosstalk between the endothelial cells, mural cells, and the tissue environment (Djonov et al., 2002; Jain, 2003; le Noble et al., 2005; Lucitti et al., 2007). Regression of vessel branches, the so-called "pruning" process, involves the disassembly of junctions, followed by cell retraction and, to a variable extent, also endothelial cell apoptosis (Baffert et al., 2006; Hughes and Chang-Ling, 2000).

What controls the specification of endothelial tip and stalk cells, how is tip cell migration and protrusive activity regulated, what controls the number of stalk cells and their proliferation, how do tip cells communicate during the formation of new connections, and what determines which connections will regress during pruning? All of these processes are under the influence of environmental signals, but to achieve an organized and functional vessel network, individual endothelial cell responses need to be coordinated into a team effort. The list of factors capable of eliciting an angiogenic response is steadily growing; some of these factors take a center stage position, whereas others play more auxiliary roles. The "principal dancer" is VEGF-A, which promotes endothelial cell differentiation, migration, and proliferation; controls endothelial cell-cell junctions; suppresses apoptosis, and more. Many other pathways, including Angiopoietin/Tie2, Notch, Wnt, TGFβ/Alk1, FGF, S1P/Edg1, Slit/robo, Semaphorin/Plexin, Netrin/Unc5b, cell matrix/integrin signaling, and others, regulate the angiogenic response. Over the past few years, it has become clear that the Notch signaling pathway plays a key role in coordinating multiple aspects of endothelial behavior during vessel patterning and thus in shaping the formation and remodeling of the vascular network.

The Notch Pathway in Endothelial Cells

The Notch pathway is an evolutionarily conserved signaling system that is required for normal embryonic development, the regulation of tissue homeostasis, and the maintenance of stem cells in adults (Artavanis-Tsakonas et al., 1999; Gridley, 1997) (Figure 2). The pathway was originally identified in *Drosophila*,

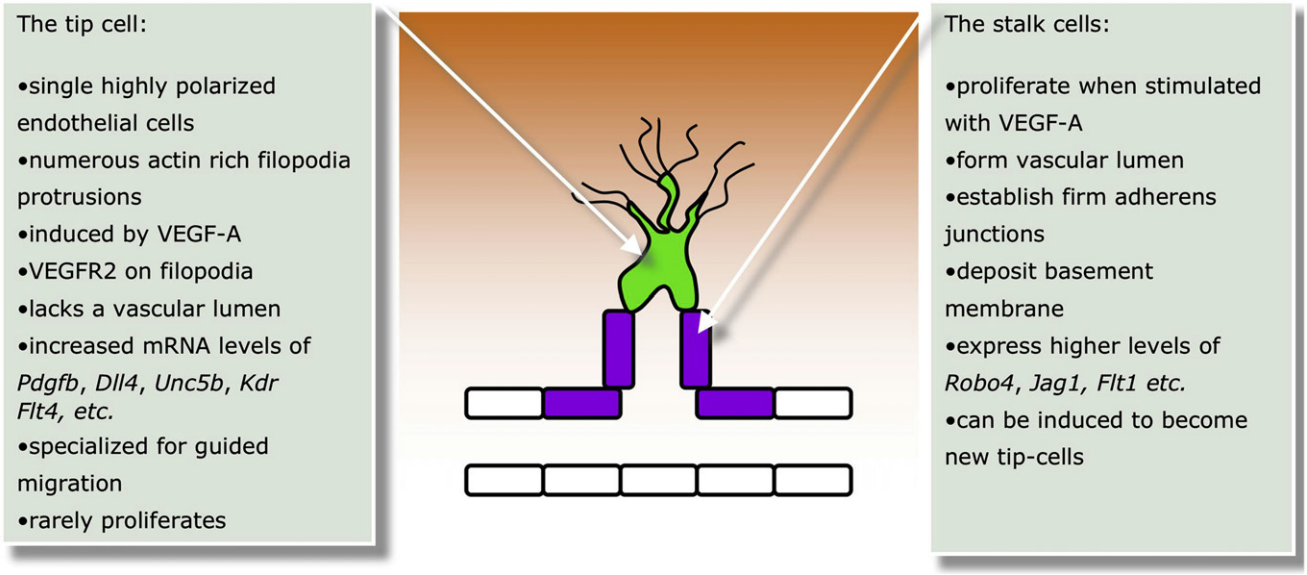

Figure 1. Phenotypic and Molecular Differences between Endothelial Tip and Stalk Cells
Tip cells (green) head each vascular sprout stimulated by an extracellular VEGF-A gradient (orange), and the following endothelial cells (purple) form the lumenized stalk.

in which the first mutant allele gave rise to a notched wing. Since then, proteins of the Notch pathway have been discovered in virtually all metazoans and have been studied extensively in flies, worms, and vertebrates. These studies have unraveled the multiple roles of Notch signaling in cell fate specification, tissue patterning, and morphogenesis through effects on differentiation, proliferation, survival, and apoptosis (Bray, 2006; Fiuza and Arias, 2007).

In mammals, there are five canonical DSL (Delta, Serrate, LAG-2) ligands: Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4

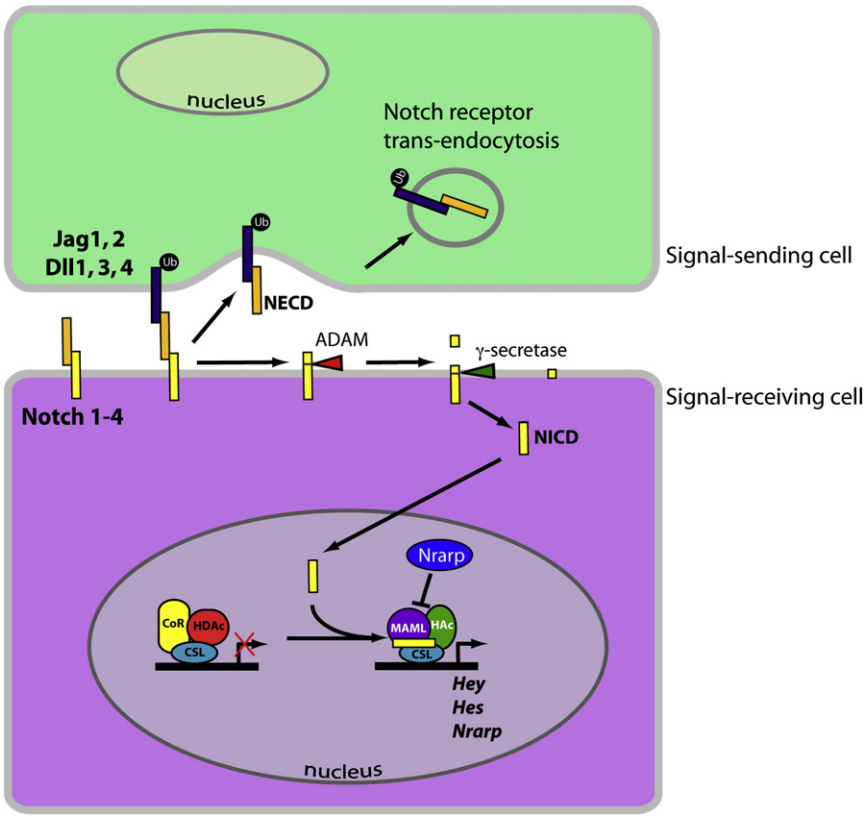

Figure 2. The Notch Signaling Pathway
The Notch receptor is expressed on the cell surface as a heterodimeric receptor. The extracellular and membrane-bound intracellular fragments of Notch are held together through noncovalent interactions. Upon ligand binding, DSL ligand-mediated endocytosis nonenzymatically dissociates the Notch heterodimer (Nichols et al., 2007). The Notch extracellular domain is transendocytosed into the signal-sending cell, exposing the remaining membrane-bound receptor to ADAM and γ-secretase proteolysis for release of the NICD. The NICD translocates to the nucleus to trigger transcriptional activation of Notch target genes. CSL, CBF, Suppressor of hairless, LAG-1; DSL, Delta, Serrate, LAG-2; HDAc, Histone deacetylase; MAML, Mastermind-like; HAc, Histone acetyltransferase; NECD, Notch extracellular domain; NICD, Notch intracellular domain; ADAM, a disintegrin and metalloprotease; Ub, ubiquitin.

(Dll4), Jagged-1 (Jag1), and Jagged-2 (Jag2).(Figure 2) These ligands are type 1 cell-surface proteins with multiple tandem epidermal growth factor (EGF) repeats in their extracellular domains (ECDs). DSL ligands bind to Notch receptors, which are large (300 kDa), single-pass, type I transmembrane receptors. In mammals, there are four Notch receptors, Notch1 to Notch4. Binding of a DSL ligand to the ECD of Notch receptor (NECD) triggers a series of proteolytic cleavages of Notch, first by a member of the disintegrin and metalloproteases (ADAM) family within the juxtamembrane region, followed by γ-secretase within the transmembrane domain. The final cleavage releases the Notch intracellular domain (NICD) from the cell membrane, which translocates to the nucleus and directly interacts with the transcription factor CSL (named after mammalian CBF1, *Drosophila* Su(H), and *Caenorhabditis elegans* LAG1), which binds to the sequence 5′-TTCCCAC-3′. In the absence of NICD, CSL represses transcription through interactions with a corepressor complex that contains a histone deacetylase (Kao et al., 1998). Binding of the NICD to CSL displaces the corepressor complex and replaces it with a transcriptional activation complex that includes the NICD, Mastermind-like (Maml, a transcriptional coactivator for Notch receptors), and histone acetyltransferases such as p300 to turn on the expression of Notch target genes such as the basic helix-loop-helix (bHLH) proteins *Hairy/Enhancer of Split* (*Hes*), *Hes-related proteins* (*Hey/HRT/HERP*), and *Notch-regulated ankyrin repeat protein* (*Nrarp*, discussed in more detail in the section "Notch Signaling and Vessel Stability"). Proteins encoded by the *Hes* and *Hey* genes are, in turn, transcriptional repressors of both their own expression and further downstream genes.

Several Notch receptors, ligands, and signaling pathway components have been identified in endothelial cells in vitro and in vivo, during development and tumor angiogenesis (for details, see the recent comprehensive reviews [Gridley, 2007; Hofmann and Iruela-Arispe, 2007; Roca and Adams, 2007]). Of the Notch receptors, Notch1 and Notch4 are expressed by endothelial cells; among the DSL ligands, Dll1, Dll4, Jag1, and Jag2 are expressed by endothelial cells (Claxton and Fruttiger, 2004; Favre et al., 2003; Hofmann and Iruela-Arispe, 2007; Villa et al., 2001). Key signaling components expressed in endothelial cells include Rbpj (Dou et al., 2008), Hey1, Hey2 (Fischer et al., 2004; Iso et al., 2002; Taylor et al., 2002), Maml1 (Liu et al., 2006), Numb (Favre et al., 2003), and Nrarp (Krebs et al., 2001; Phng et al., 2009). Functional studies using gene targeting in mice, mutagenesis and knockdown in zebrafish, and biochemical analysis in cultured endothelial cells have demonstrated that Notch signaling plays a fundamental role in many aspects of endothelial cell biology during angiogenesis (see Table 1).

Notch Signaling and Endothelial Cell Specification

Endothelial cells are heterogeneous in morphology, function, and gene expression. Depending on their state of activation, their position in the vascular bed, and the organ context, endothelial cells are specified toward particular roles (Aird, 2007). Lineage tracing, grafting, flow rerouting, and cell culture experiments suggest that heterogeneous endothelial cell specification is likely dynamic, and that differentiated cells retain a surprising degree of plasticity (Aitsebaomo et al., 2008; Kudo et al., 2007; Lacorre et al., 2004; le Noble et al., 2005; Moyon et al., 2001).

Notch signaling controls multiple aspects of endothelial cell specification, such as early specification of a subset of angioblasts from the lateral mesoderm during formation of the dorsal aorta in chicken embryos (Sato et al., 2008) and angioblast specification, migration, and maintenance through snrk-1 and gridlock in zebrafish (Chun et al., 2008; Zhong et al., 2001). Dll4/Notch signaling also controls subsequent endothelial cell specification toward the arterial or venous phenotype in zebrafish and mouse(Carlson et al., 2005; Duarte et al., 2004; Kim et al., 2008; Lawson et al., 2001, 2002; Zhong et al., 2001). Targeted deletion of Dll1, Dll4, Notch1, Notch4, Rbpj, and Hey1 and Hey2 in the mouse (Carlson et al., 2005; Duarte et al., 2004; Fischer et al., 2004; Gale et al., 2004; Kim et al., 2008; Krebs et al., 2000, 2004; Limbourg et al., 2007; Trindade et al., 2008) and knockdown of notch3, gridlock, rbpja, and mind bomb in the zebrafish (Lawson et al., 2001; Siekmann and Lawson, 2007; Zhong et al., 2001) result in the deregulation of arterial and venous specification of endothelial cells as well as in the deformation of arteries and veins. In this process, Notch signaling interacts in a genetically defined pathway with VEGFR2, PLC-γ1, MAPK, and EphrinB2/EphB4 signaling to balance the number and proper assembly of arterial and venous endothelial cells into distinct vascular tubes with an adequate diameter (Hong et al., 2006). EphrinB2, which marks arterial identity, is a direct transcriptional target of Notch (Grego-Bessa et al., 2007). These studies also suggested that the venous phenotype is effectively a default choice, and an arterial phenotype is acquired through active Notch signaling. However, the venous transcription factor COUP-TFII appears to actively repress the arterial marker Neuropilin-1 (Nrp-1) and Notch signaling. Accordingly, loss of COUP-TFII leads to expansion of the arterial cell fate during early mouse embryonic development, challenging the idea of a venous identity by default (You et al., 2005).

Recent studies in the mouse retina, in zebrafish intersegmental vessels, in tumor angiogenesis, and in 3D endothelial cell culture sprouting assays demonstrate that the specification of endothelial cells into tip and stalk cells is regulated by Dll4/Notch signaling (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006; Sainson et al., 2005; Siekmann and Lawson, 2007; Suchting et al., 2007) (Figure 3). Dll4 is most prominently expressed in tip cells (Claxton and Fruttiger, 2004; Hellstrom et al., 2007), whereas the strongest Notch signaling activity is regularly observed in the stalk cells (Hellstrom et al., 2007; Hofmann and Iruela-Arispe, 2007). Suppression of Notch signaling by γ-secretase inhibitor (GSI) treatment or genetic deletion of one *Dll4* allele in the mouse dramatically augments sprouting, branching, and hyperfusion of the capillary network as a result of excessive tip cell formation. Increased and widespread expression of *Pdgfb*, *Unc5b*, *Kdr*, *Flt4*, i.e., genes that are highly expressed in tip cells, and widespread filopodia formation, the morphological hallmark of tip cells, provide evidence of the increased tip cell formation (Hellstrom et al., 2007; Suchting et al., 2007; Tammela et al., 2008). In zebrafish, GSI treatment, Dll4 protein knockdown by morpholino oligonucleotide, or genetic deletion of *Dll4* causes excessive vessel sprouting and branching during the development of intersegmental vessels and DLAVs (Leslie et al., 2007; Siekmann and Lawson, 2007).

Table 1. Endothelial Functions of Notch Components

Endothelial Function	Notch Component(s) Involved	Reference
Cell specification:	Dll4	Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007
• tip versus stalk	Notch1	Hellstrom et al., 2007
	Rbpja (zebrafish)	Siekmann and Lawson, 2007
• arterial versus venous	Dll1	Limbourg et al., 2007
	Dll4	Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004; Trindade et al., 2008
	Notch1	Fischer et al., 2004; Kim et al., 2008; Krebs et al., 2000
	Notch3 (zebrafish)	Lawson et al., 2001
	Notch4	Carlson et al., 2005; Kim et al., 2008
	Hey1 and Hey2/Gridlock (zebrafish)[a]	Fischer et al., 2004; Zhong et al., 2001
	mind bomb (zebrafish)	Lawson et al., 2001
	Rbpj/Rbpja (zebrafish)	Krebs et al., 2004; Siekmann and Lawson, 2007
Proliferation	Dll4	Benedito et al., 2008; Liu et al., 2006; Lobov et al., 2007; Suchting et al., 2007; Trindade et al., 2008
	Notch1	Liu et al., 2006
	Notch4	Noseda et al., 2004
	Rbpj/Rbpja (zebrafish)	Dou et al., 2008; Siekmann and Lawson, 2007
	Mam1	Liu et al., 2006
	Hes1	Liu et al., 2006
Motility	Dll4	Leslie et al., 2007; Trindade et al., 2008
	rbpja (zebrafish)	Siekmann and Lawson, 2007
Filopodia protrusion	Dll4	Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Suchting et al., 2007
	Notch1b (zebrafish)	Leslie et al., 2007
Matrix production/assembly and cell adhesion	Dll4	Benedito et al., 2008; Harrington et al., 2008; Hodkinson et al., 2007; Trindade et al., 2008
	Notch1	Hodkinson et al., 2007
	Notch4	Leong et al., 2002
Vessel stability	Nrarp	Phng et al., 2009

Dll4, Delta-like 4; Nrarp, Notch-regulated ankyrin repeat protein; Rbpja, recombinant signal binding protein for immunoglobulin kappa J region a.
[a] Targeted deletion of *Hey2* alone in the mouse does not lead to defects in arterial-venous specification. Arterial differentiation, however, requires the function of both Hey1 and Hey2 since endothelial cells from mice deficient in both genes fail to establish arterial identity.

Excessive branching also occurred when *notch1b* (Leslie et al., 2007) and the zebrafish CSL protein rbpja were knocked down (Siekmann and Lawson, 2007), illustrating that Dll4 signals through notch1b in an rbpja-dependent manner to limit the number of endothelial tip cells formed.

Mosaic analysis of endothelial cells deficient in Notch signaling in mouse and zebrafish demonstrated that Notch is cell autonomously required for stalk cell specification by actively suppressing the tip cell phenotype. This cell-autonomous function of Notch was previously observed in 3D endothelial cell sprouting assays in vitro (Sainson et al., 2005). In mouse, mosaic endothelial Cre recombination of a floxed Notch1 allele showed that the majority of Notch-deficient endothelial cells adopt tip cell characteristics (Hellstrom et al., 2007). In zebrafish, *rbpja*-deficient *Tg(fli1:EGFP)*^{y1} cells transplanted into wild-type zebrafish embryos show an increased propensity to occupy the tip cell position of intersegmental vessels, but have reduced base cell localization when compared to transplanted wild-type cells (Siekmann and Lawson, 2007). Conversely, ectopic activation of Notch signaling in the mouse retina by injection of the Jag1 peptide leads to reduced tip cell formation and filopodia extension (Hellstrom et al., 2007), and endothelial cell clones carrying a constitutive active NICD are excluded from the tip cell position in zebrafish (Siekmann and Lawson, 2007).

The results of these experiments also provide an indication that tip and stalk cells do not represent stable cell fates, but instead are specified in a dynamic fashion in a process best described as a ''tug-of-war.'' Endothelial cells stimulated by VEGF-A compete for the tip cell position via Dll4/Notch signaling (Figure 3). The cell that produces more Dll4 than its neighbor will eventually remain the tip, because it can effectively suppress the same response in competing neighbors via activation of Notch signaling. This ''social'' behavior has previously been described in *Drosophila* tracheal development, where the epithelial cells that form the tracheal sprout compete for the tip position using FGFR (Breathless) levels and Delta/Notch signaling (Ghabrial and Krasnow, 2006). It seems that the tip cell phenotype is the default acquired in the absence of Notch signaling, whereas

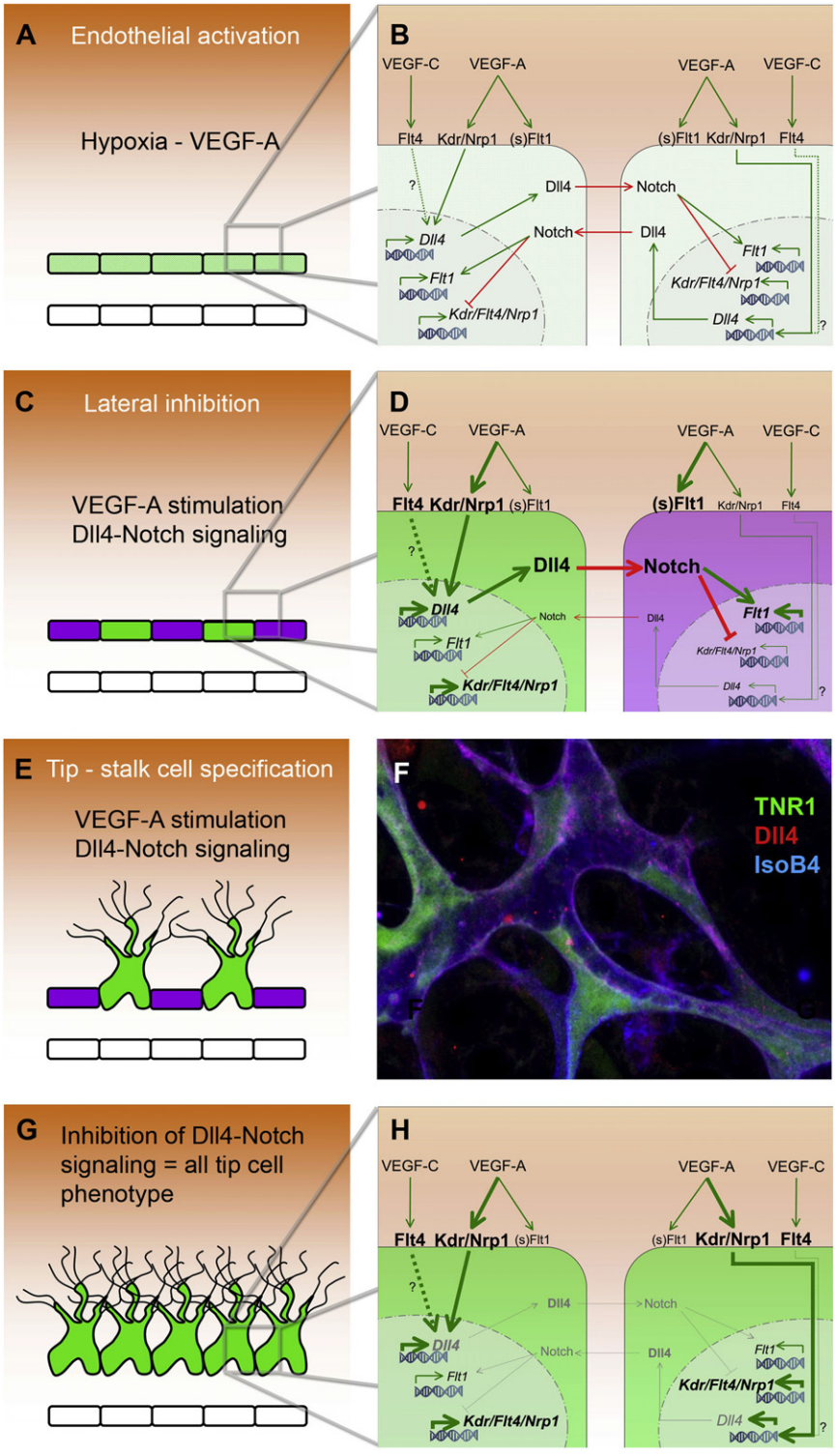

Figure 3. Notch Signaling Regulates Endothelial Tip/Stalk Cell Specification

(A and B) In regions of hypoxia, all endothelial cells become activated by VEGF-A stimulation to express the Notch ligand Dll4.

(B) Endothelial cells compete via bilateral Dll4-Notch signaling involving a VEGF-Notch feedback loop for tip cell specification.

(C) Notch signaling induces lateral inhibition and gives rise to a nonuniform population of endothelial cells in the presence of VEGF-A stimulation.

(D) Schematic illustration of the VEGF-A-Notch feedback loop controlling tip-stalk specification: purple stalk cells receive high Notch signal, which represses transcription of the VEGF receptors Kdr, Nrp1, and Flt4, while stimulating expression of the decoy receptor (s)Flt1; green tip cells receive low Notch signal, allowing for high Kdr, Nrp1, and Flt4 expression, but low (s)Flt1 expression.

(E) Notch signaling induces the acquisition of an endothelial stalk cell, whereas a cell that is low in Notch activity becomes an endothelial tip cell.

(F) Fluorescent laser scanning micrograph of sprouting retinal blood vessels of a transgenic Notch reporter mouse (TNR1). Cells with Notch signaling are detected by GFP expression (green). In blood vessels, endothelial cells (Isolectin-B4, blue) express membranous Dll4 (red) and are heteregeneous in Notch activity.

(G and H) In the absence of Notch signaling, stalk cells are no longer specified; instead, all endothelial cells become tip cells.

the stalk cell phenotype is acquired by Notch signaling (Hellstrom et al., 2007; Roca and Adams, 2007).

Studies in several mouse tumor models illustrated that the principle of tip-stalk specification by Notch signaling is not restricted to developmental angiogenesis, but also controls the branching frequency of tumor blood vessels (Noguera-Troise et al., 2006; Ridgway et al., 2006). Transplantable tumors in Dll4 heterozygous hosts show vastly increased sprouting angiogenesis. Inhibition of Notch signaling by GSI or selective antibody-based blocking of Dll4 leads to similar effects. Analysis of tumor growth provided an intriguing insight into the functional consequences of excessive tip cell formation during sprouting angiogenesis: the increased vascularization after Dll4/Notch inactivation paradoxically causes reduced tumor growth, indicating that unrestrained angiogenesis is unproductive (Thurston et al., 2007). Tracer perfusion experiments demonstrate that the excessive tumor vessels are poorly perfused, causing increased tumor hypoxia and reduced tumor growth. In a converse experiment, increased endothelial Notch signaling triggered by Dll4-expressing tumor cells led to reduced vascular branching and density, but to enhanced vessel diameter, perfusion, and therefore augmented tumor growth (Li et al., 2007; Noguera-Troise et al., 2006). Together, the developmental and tumor angiogenesis studies support the emerging concept that effective vascular patterning and function require a balance of tip and stalk cell numbers coordinated by Notch.

How exactly Notch suppresses tip cell formation is not fully resolved. Accumulating evidence suggests a model in which

Notch signaling modulates the output of VEGF-VEGFR signaling in endothelial cells at least in part through the transcriptional regulation of *Flt1*, *Kdr*, *Nrp1*, and *Flt4* (reviewed in Siekmann et al., 2008) (Figure 3). Collectively, data gathered from in vitro and in vivo studies demonstrate that Notch negatively regulates *Kdr* expression (Harrington et al., 2008; Henderson et al., 2001; Li et al., 2006; Suchting et al., 2007; Williams et al., 2006), likely through the direct binding of Hey1 to the Kdr promoter (Henderson et al., 2001; Holderfield et al., 2006). *Nrp1* is also repressed by Notch, whereas the VEGF-A decoy receptor *Flt1*, which can quantitatively and spatially modulate Kdr signaling (Kappas et al., 2008), is upregulated by Dll4/Notch signaling. In addition, the soluble splice variant of Flt1 (sFlt1) is upregulated upon Notch activation in endothelial cells (Harrington et al., 2008), suggesting that active Notch signaling could potentially reduce or spatially restrict the overall response of endothelial cells to VEGF-A through the sequestration of extracellular VEGF-A.

In the adult organism, *Flt4* expression is largely confined to the lymphatic endothelium, where it acts as a signaling receptor for VEGF-C. However, during embryonic development, Flt4 plays an important role in VEGF signaling in the blood vasculature, possibly by forming heterodimers with Kdr. Flt4 is strongly expressed in zebrafish intersegmental vessels (Siekmann and Lawson, 2007), at the tips of ISVs in mouse embryos, at the sprouting front in mouse retina, and in sprouting tumor vessels (Tammela et al., 2008). Intriguingly, loss of Notch signaling leads to widespread Flt4 expression, and blocking antibodies against Flt4 partially restored normal sprouting. Although these studies suggest that Flt4 is downregulated in the endothelial stalk by Notch signaling activity, a study in cultured endothelial cells demonstrated direct activation of the *Flt4* promoter by the NICD/CSL complex (Shawber et al., 2007). More work is required to clarify exactly how and under which conditions Notch signaling regulates the different VEGF receptors.

The complexity of the relationship between Notch and VEGF signaling pathways is also illustrated by the discovery that VEGF induces *Dll4* expression through Kdr in the retina (Liu et al., 2003; Lobov et al., 2007; Suchting et al., 2007) and endothelial cultures (Liu et al., 2003). Mechanistically, at least in arterial endothelium, VEGF signaling activates *Dll4* transcription through phosphoinositide 3 kinase (PI3K) and Erk signaling in collaboration with Foxc transcription factors (Hayashi and Kume, 2008; Seo et al., 2006). VEGF and Notch pathways thus operate a negative-feedback loop in which (i) VEGF-A triggers endothelial *Dll4* expression, and (ii) Dll4 activates Notch signaling in adjacent cells, leading (iii) to the downregulation of VEGF receptors and thus of the VEGF response (Figure 3). Computational modeling of tip cell selection illustrated that this feedback loop is sufficient to pattern a row of endothelial cells stimulated by adequate concentrations of VEGF-A into alternating tip and stalk cells (Bentley et al., 2008). This study also suggested that VEGF-A gradients and filopodia formation confer robustness to VEGF/Dll4/Notch-dependent tip-stalk specification.

Notch Signaling and Endothelial Proliferation

The formation of a new vessel requires not only the selection of an endothelial tip cell, but also endothelial cell proliferation to enable sprout growth in length and diameter. There is ample evidence that the Notch pathway inhibits proliferation in endothelial cells. Suppression of Notch signaling results in increased endothelial cell proliferation in 3D sprouting assays in vitro (Sainson et al., 2005), in mouse and zebrafish development in vivo (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007), in the adult mouse (Dou et al., 2008), and during tumor angiogenesis (Noguera-Troise et al., 2006; Ridgway et al., 2006). In mouse, increased endothelial cell proliferation of both tip and stalk cells may contribute to increased vessel diameter and branching after GSI treatment (Hellstrom et al., 2007), after neutralization of Dll4 activity by Dll4-Fc (Lobov et al., 2007), and in $Dll4^{+/-}$ mutants (Suchting et al., 2007). In zebrafish, the increase in proliferation and the aberrant migratory behavior of endothelial cells resulted in an increased number of endothelial cells in intersegmental vessels in rbpja-deficient zebrafish (Siekmann and Lawson, 2007). Conversely, stimulation of Notch signaling in endothelial cells in vitro (Liu et al., 2006; Noseda et al., 2004) and in mouse decreased endothelial cell proliferation (Harrington et al., 2008; Trindade et al., 2008).

Studies in endothelial cell cultures suggest that the inhibitory effect of Notch signaling on endothelial cell proliferation is mediated by the transcriptional regulation of downstream targets of the NICD/CSL/MAML complex (Liu et al., 2006). The inhibition may be caused by a decrease in the activation of the MAPK and PI3K/Akt signaling pathways involving MAML1-mediated transcription of target genes. Liu et al. proposed that Notch signaling regulates MAPK and PI3K/Akt indirectly through differential regulation of Flt1 and Kdr, thereby reducing Kdr-mediated MAPK and PI3K/Akt signaling. Notch signaling also regulates the expression of the cyclin-dependent kinase inhibitor $p21^{CIP1}$ in endothelial cells (Noseda et al., 2004). $p21^{CIP1}$ expression is downregulated by Notch1 and Notch4 activity, resulting in a reduction in the nuclear translocation of cyclin D and cdk4, in the downregulation of cyclin D-cdk4-mediated Rb phosphorylation, and, consequently, in cell cycle arrest (Dou et al., 2008; Noseda et al., 2004). Conversely, endothelial deletion of RBP-J in adult mice induced $p21^{CIP1}$ and endothelial cell proliferation (Dou et al., 2008).

The regulation of cell proliferation by Notch signaling shows cell-type-dependent differences. For example, in cardiomyocytes (Collesi et al., 2008; Campa et al., 2008) and keratinocytes (Rangarajan et al., 2001), Notch signaling promotes cell cycle progression. Also, the regulatory role of $p21^{CIP1}$ in cell cycle progression is cell type dependent since Notch signaling induces the transcription of $p21^{CIP1}$ in an RBP-J-dependent manner to promote cell proliferation of keratinocytes (Rangarajan et al., 2001).

Notch Signaling and Cell Motility/Filopodia Formation

Filopodia are involved in a number of cellular processes such as adhesion to extracellular matrices, guidance toward chemoattractants, and cell migration. A prominent phenotype that arises from the suppression or overactivation of Notch signaling in endothelial cells is an increase or decrease in filopodia protrusion, respectively, suggesting a role of Notch in regulating endothelial cell motility (Figure 4). Overactivation of Notch signaling reduces the migratory behavior of endothelial cells. In mice overexpressing *Dll4*, there is decreased endothelial cell migration and sprouting from the dorsal aorta to form intersomitic vessels

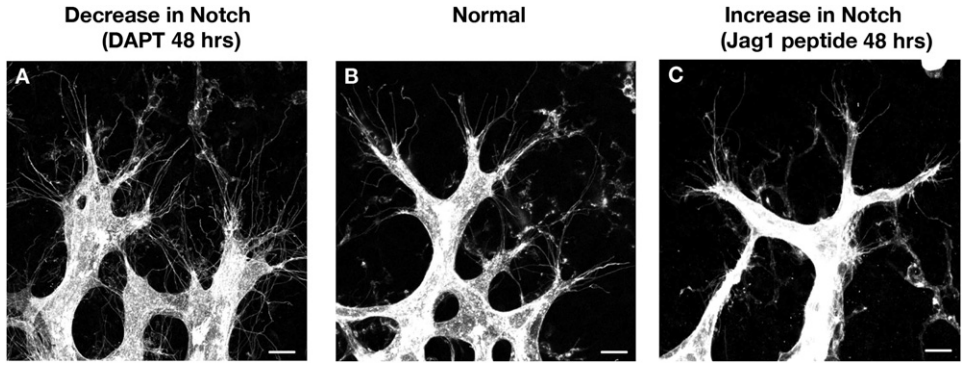

Figure 4. Notch Signaling Regulates Filopodia Protrusion

(A–C) (A) Suppression of Notch signaling leads to a significant increase in filopodia protrusion by endothelial tip cells, whereas an ectopic increase of Notch signaling results in (C) a decrease in filopodia formed when compared to (B) normal vessels. Early postnatal mice were treated with the γ-secretase inhibitor DAPT to inhibit Notch signaling or the Jag1 peptide, which corresponds to the DSL domain of the human Jag1 protein, to activate Notch receptor. Retinas have been stained with Isolectin-B4. Scale bars represent 10 μm.

(ISVs) (Trindade et al., 2008). In vitro, human umbilical vein endothelial cells (HUVECs) expressing full-length Dll4 exhibit decreased motility in the presence of exogenous VEGF (Trindade et al., 2008). Similarly, in zebrafish, overexpression of the NICD inhibited the migration and filopodia activity of endothelial cells (Leslie et al., 2007; Siekmann and Lawson, 2007). Furthermore, when *Tg(fli1:EGFP)*^{y1} cells with activated Notch were transplanted into wild-type zebrafish embryos, these cells showed increased incorporation at the base of the ISV, but did not occupy positions in the dorsal longitudinal anastomotic vessels (DLAV) (Siekmann and Lawson, 2007).

How does Notch signaling regulate endothelial cell motility? As Notch signaling downregulates Kdr, Flt4, and Nrp1 levels, it could potentially reduce endothelial cell motility by making cells less responsive to VEGF-A. However, it is not yet clear whether the levels of VEGF receptors could directly influence endothelial cell motility. The levels of the ligand, VEGF-A, appear to be rate limiting for endothelial cell proliferation, in part through quantitative activation of Erk1/2 (Gille et al., 2001), but endothelial migration is not likely to depend on absolute levels of VEGF-A. Endothelial cell migration is regulated by several signaling pathways, including PI3K, which are activated by the phosphorylation of distinct VEGFR2 tyrosine residues (for an overview, see Olsson et al., 2006). PI3K inhibitors quantitatively suppress endothelial cell migration (Gille et al., 2001; Graupera et al., 2008), and genetic inactivation of the p110α isoform of the class IA PI3K illustrated that p110α is selectively required for endothelial migration, but not proliferation, downstream of VEGFR activation (Graupera et al., 2008). P110α regulates endothelial cell migration by activation of the small GTPase RhoA. Notably, blocking of the Semaphorin and VEGF co-receptor Nrp-1 by antibodies selectively abrogates VEGF-induced endothelial migration, but not proliferation (Pan et al., 2007), and the C-terminal domain of Nrp-1 stimulates endothelial migration via PI3K, Akt, and RhoA (Wang et al., 2003). Nrp1 knockout mice show defects in endothelial cell migration and endothelial tip cell guidance, but not proliferation (Gerhardt et al., 2004; Jones et al., 2008). Nrp1 is strongly downregulated by Notch signaling (Harrington et al., 2008; Williams et al., 2006), suggesting that Notch could suppress endothelial motility by modulating coreceptors for VEGF-A, which play a particular role in endothelial migration.

Alternatively—and not yet tested in endothelial cells—Notch could regulate cell motility more directly in a CSL-independent manner. In *Drosophila*, Notch receptors can activate the Abl tyrosine kinase to stimulate neuronal migration and axonal guidance (Crowner et al., 2003; Le Gall et al., 2008). Abl performs its functions in cooperation with a range of proteins, such as Disabled and the guanine nucleotide-exchange factor Trio, which alleviate or enhance the severity of defects observed in *Drosophila Abl* mutants. Notch binds directly to Disabled and Trio to regulate Abl signaling. Similarly, the mouse homolog Disabled 1 binds to the intracellular domain of Notch1 in embryonic brain lysates (Hashimoto-Torii et al., 2008). In *Drosophila*, specific deletion of the Disabled-binding domain in Notch resulted in defects in embryonic axon patterning, a process that requires axon guidance and migration, with little or no effect on neurogenesis (Le Gall et al., 2008). In addition, the canonical Notch signaling pathway appeared to be dispensable for axonal function. Therefore, although axon patterning and neurogenesis are both dependent on Notch signaling, the underlying mechanisms seem to be distinct. In the former case, a CSL-independent pathway that involves Disabled and Abl is required, whereas in the latter case, the canonical CSL-dependent pathway is important. Given the functional and molecular similarities between axonal guidance and endothelial tip cell guidance, it is tempting to speculate that a similar CSL-independent pathway involving Disabled and Abl may regulate endothelial cell motility downstream of Notch.

Notch Signaling and Cell Adhesion

In vertebrates, Notch receptor activation can also be modulated by non-DSL ligands. Of particular interest is microfibril-associated glycoprotein (MAGP)-2, a small glycoprotein that specifically associates with fibrillin-containing microfibrils (Gibson et al., 1996) and promotes cell adhesion in a variety of cell types, including bovine arterial endothelial cells, by binding to $\alpha_v\beta_3$ integrin (Gibson et al., 1999). More recently, MAGP-2 has been shown to bind directly to Jag1, Jag2, and Dll1 (Nehring et al., 2005) and also to Notch1 (Miyamoto et al., 2006). Binding of

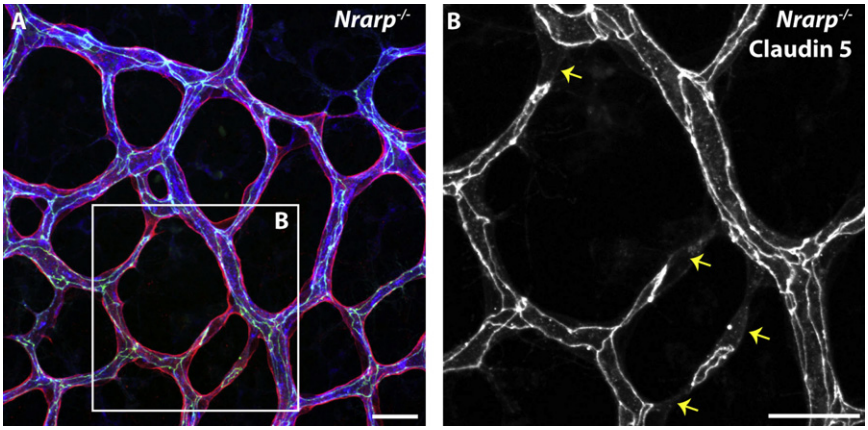

Figure 5. Notch Confers Vessel Stability through Nrarp
(A and B) Loss of Nrarp leads to excessive segregation of endothelial junctions (arrows) and vessel instability. Postnatal day 5 retinas were stained for Claudin 5 (green), Collagen IV (red), and Isolectin-B4 (blue). Scale bars represent 25 μm.

MAGP-2 to Jag1 and Notch1 results in ectodomain shedding, and, in the case of Notch1, MAGP-2 binding induces γ-secretase-dependent NICD generation and CSL-dependent activation of Notch signaling (Albig et al., 2007, 2008; Miyamoto et al., 2006). However, the regulatory role of MAGP-2 on Notch signaling is cell type dependent. In nonendothelial cells such as COS7 (Miyamoto et al., 2006) and various tumor cell lines (Albig et al., 2008), MAGP-2 induces Notch signaling. However, MAGP-2 suppresses Notch signaling in several endothelial cell lines, including HUVEC and MB114 (Albig et al., 2008). Interestingly, MAGP-2 has an angiogenic function; it is able to promote endothelial cell sprouting in vitro by antagonizing Notch signaling (Albig et al., 2007, 2008). However, the expression pattern of MAGP-2 in developing vessels and their extracellular milieu is as yet unknown, and a role in vessel patterning in vivo remains to be shown.

There is also some evidence that Notch signaling regulates the expression of extracellular matrix molecules. For example, there is increased transcription of *fibronectin*, *laminin*, and *collagen* in endothelial cells isolated from mouse embryos overexpressing Dll4 (Trindade et al., 2008). As a result, these mutants show increased deposition of extracellular matrix around the dorsal aorta. Conversely, *Dll4*$^{+/-}$ mouse embryos show decreased expression and irregular deposition of collagen IV and laminin (Benedito et al., 2008). Harris and colleagues also observed significant regulation of integrin expression by Notch signaling in endothelial cells (Harrington et al., 2008). Together, these results illustrate that Notch can influence both matrix production and adhesive properties in the form of integrin receptor expression, although, again, the full in vivo significance remains to be examined. The induction of extracellular matrix components that stabilize the vessel wall is consistent with the arterial specification effect of Notch.

Although most Notch signaling is mediated by the CSL-dependent pathway, there is growing evidence that the activation of Notch receptor also triggers cellular responses through CSL-independent pathways. One such pathway is the activation of β1-integrins by Notch1 (Hodkinson et al., 2007). This mechanism still relies on the production of NICD1 from γ-secretase-mediated cleavage of the Notch receptor. NICD1 specifically activates R-Ras, which antagonizes H-Ras-mediated integrin suppression to increase integrin affinity (Hodkinson et al., 2007). The increase in integrin activity enhances cell adhesion to extracellular matrix proteins. For example, human myeloid cells that have been transfected with NICD1 or that have been activated with recombinant Dll4 exhibit increased adhesion to fibronectin (Hodkinson et al., 2007). In addition, overexpression of the intracellular domain of Notch4 (NICD4) in endothelial cells results in a β1 integrin-mediated increase in adhesion to collagen, and these cells show a reduced sprouting response to VEGF both in vitro and in vivo (Leong et al., 2002). The activation of R-Ras does not require CSL-mediated transcription and may therefore transmit rapid changes in cellular signaling in response to interaction with Notch ligands expressed on adjacent cells (Hodkinson et al., 2007).

Notch Signaling and Vessel Stability

As part of the overall sprouting angiogenesis process, newly generated vessels are stabilized to form a functional vascular network. Once a vascular network is established, endothelial cells become quiescent to prevent excessive vessel sprouting. Recent studies showed that loss of Rbpj in endothelial cells in adults reinitiated vascular outgrowth from existing vessels (Dou et al., 2008), demonstrating that Notch signaling has a regulatory role in maintaining endothelial cell quiescence.

Furthermore, we recently found that a downstream target of the Notch pathway, Nrarp (Krebs et al., 2001; Lamar et al., 2001; Pirot et al., 2004), stabilizes nascent blood vessels during retinal angiogenesis and intersegmental vessel formation in zebrafish (Phng et al., 2009). *Nrarp*$^{-/-}$ mice and zebrafish morphants show reduced endothelial cell proliferation and excessive junctional rearrangement within nascent blood vessels—defects that culminate in ectopic vessel regression during sprouting angiogenesis and thus reduced vessel density (Figure 5). Nrarp is directly induced by Notch signaling, but it functions as a negative regulator by promoting the degradation of NICD (Ishitani et al., 2005; Lamar et al., 2001). Accordingly, loss of Nrarp leads to increased Notch activity, potentially explaining the reduced stalk cell proliferation and the opposing phenotype to that seen with the loss of *Rbpj* function in endothelial cells. Remarkably, however, this increased Notch signaling does not affect endothelial sprouting activity or filopodia formation, probably because it occurs only in the stalk, leaving the tip cells unaffected (Phng et al., 2009). Increased Notch signaling per se does not appear to cause vessel regression or loss of stability. Instead, our study suggests that the stabilization of nascent vessels requires Nrarp-mediated promotion of canonical Wnt signaling via stabilization of the β-catenin cofactor TCF/Lef-1, and that Notch and Wnt signaling are coordinated by Nrarp in stalk cells to control the stability of newly formed vessel connections

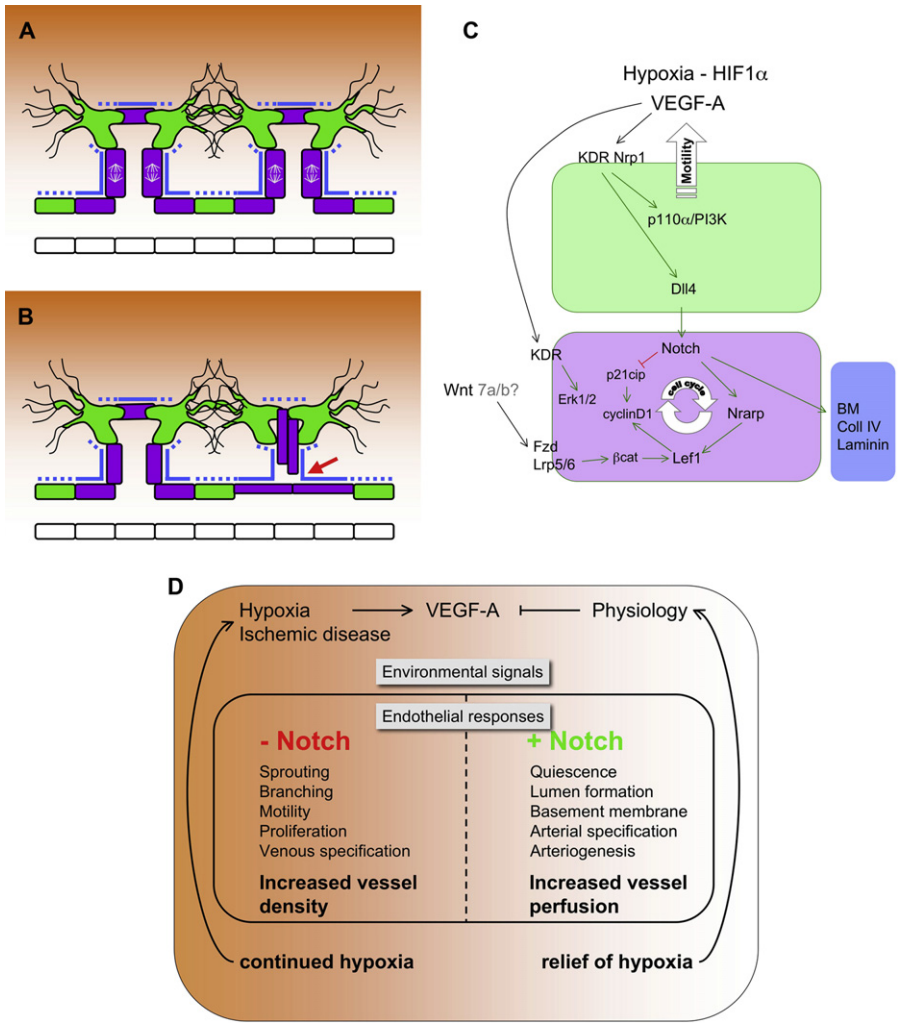

Figure 6. Notch Signaling Promotes Patent Vessels and Perfusion

(A–D) (A and C) Notch signaling induces the expression of basement membrane components such as collagen IV and laminin, which are concentrated along stalk cells. (B) Loss of the downstream Notch target gene Nrarp leads to vessel instability and regression as a result of reduced endothelial cell proliferation and junctional rearrangement, leaving behind empty basement sleeves (arrow). (C) Although Notch signaling represses endothelial cell proliferation in stalk cells, Wnt signaling promotes cell cycle progression by, for example, positively regulating the expression of Cyclin D1. (A and C) As Nrarp expression is high in stalk cells and it enhances Wnt signaling by stabilizing Lef1, stalk cells undergo high proliferation. Tip cells, compared to stalk cells, are highly motile and migrate following a gradient of VEGF-A expression. (C) The migratory activity of tip cells is regulated by KDR-induced p110α activity, whereas the proliferative activity of stalk cells is largely influenced by ERK1/2 activity. (D) Different endothelial cell responses to environmental signals, such as hypoxia and VEGF-A, are coordinated by Notch signaling. The unifying outcome of Notch signaling in endothelial cells is the generation of patent and well-perfused vessels to relieve hypoxia.

(Phng et al., 2009). One important component of the stability program appears to be the control of endothelial cell proliferation through Notch/Nrarp and Wnt crosstalk (Figure 6). In contrast to Notch signaling, activation of Wnt signaling promotes endothelial cell proliferation (Masckauchan et al., 2005, 2006) and the β-catenin/Lef1 complex can directly activate the transcription of *Cyclin D1* to induce cell cycle progression (Shtutman et al., 1999). Whereas Notch signaling appears to regulate the output of the VEGF signaling pathway promoting quiescence, Nrarp modulates the output of Notch and Wnt to allow for sufficient proliferation during vessel stabilization (Figure 6). The Wnt ligands involved in vivo are currently unknown, but Wnt7a and Wnt7b are potential candidates, as their deletion from neuroepithelium results in impaired CNS vascularization (Stenman et al., 2008). The question of how general this crosstalk may be for coordinated angiogenesis in other organ systems or in tumor angiogenesis requires further investigation.

The positive regulation of endothelial basement membrane components by Notch signaling (Benedito et al., 2008; Trindade et al., 2008) will likely also contribute to the stability of nascent vessels (Figure 6). However, regressing vessels leave empty basement membrane sleeves behind (Baffert et al., 2006) (Figure 5), suggesting that the basement membrane alone is not sufficient to promote vessel stability.

Conclusions and Perspectives

Combined information from a wealth of recent studies has led to the conclusion that Notch signaling plays a pivotal role in the control of vascular morphogenesis during development and in tumor angiogenesis. The precise function of Notch is difficult

to pinpoint, however, as Notch signaling clearly influences multiple aspects of endothelial cell biology, which superficially appear to have little in common. The varied demonstrated functional roles, including specification of angioblasts from the lateral mesoderm, specification of arterial and venous endothelial cell populations in the early assembly of dorsal aorta and cardinal vein, and the selection of tip and stalk cells during subsequent angiogenic sprouting, suggest that endothelial cells reiteratively engage Notch signaling at various stages of development, and even require continued Notch activity to maintain quiescence in adult vessels. Also, at the molecular level, Notch appears to regulate and crosstalk with multiple signaling pathways that operate in endothelial cells in a context-dependent manner. Our understanding of defined genetic interactions of Notch in vascular development is most advanced for its role in artery vein formation. The more recent identification of Notch controlling tip versus stalk selection to pattern the sprouting response still lacks detailed mapping of many components, and we expect rapid progress in this area over the next few years.

We also anticipate that investigation of the dynamics of Notch signaling in endothelial cells will receive greater attention, as it may explain how Notch controls the spatiotemporal patterning events in angiogenesis. For example, oscillations of Notch signaling have been observed in several other organ systems, in particular vertebrate somitogenesis, as well as in isolated cells during neurogenesis (Kageyama et al., 2007). Although these oscillations may be tied to oscillations of other pathways, such as Wnt, the intrinsic properties of selective Notch targets, including transcriptional repressors of the Hes family, may be important for the periodicity of the oscillations, which control, for example, the spacing of tissue boundaries during somitogenesis (Jiang et al., 2000; Lewis, 2003). The conspicuous spatial expression pattern of Dll4 in arteries and in sprouting vessels has implications for temporal aspects of endothelial Notch signaling. The alternating stripes of high and low Dll4 in arteries are unlikely to be static over time. Conceptually, Dll4-high endothelial cells should have low Notch activity, whereas their neighbors will have high Notch activity, but low Dll4 expression. As Notch signaling is required for the specification and maintenance of arterial identity, continuously low Notch activity in a subset of endothelial cells along the artery would lead to a loss of identity. Coordinated oscillations, similar to the situation in the presomitic mesoderm, could theoretically produce a wave of Dll4 expression running the length of arteries, providing recurring Notch input in each cell to stabilize the arterial phenotype. Similarly, in static images of sprouting vessels, Dll4 expression is not always found in the tip, and Notch reporter activity is not always confined to the stalk. One intriguing possibility is that negative-feedback loops within the Notch signaling pathway itself are the primary reason for reiterative sprouting and branching during angiogenesis. Dynamic regulation of Notch signaling could therefore be as important for the spatial patterning of branching in angiogenesis as it is in the spatial patterning of somites.

The precise phenotypic outcome of Notch activity in endothelial cells appears to be context dependent, but one common underlying theme may be that Notch signaling promotes cellular responses in endothelial cells that are collectively suited to establish and maintain increased perfusion and hence alleviate hypoxia (Figure 6D). Although this may appear to be the obvious aim of all signaling that promotes angiogenesis, the lessons learned from Notch signaling tell us otherwise. VEGF-A signaling triggers endothelial sprouting, migration, proliferation, cell survival, and thus vessel maintenance, and it is important for artery formation, lumen formation, and more. However, in situations in which Notch signaling is absent or reduced, for example in Dll4 heterozygous animals, no functional vessel patterning and perfusion can occur and hypoxia/ischemia persists. Without Notch signaling, endothelial cells expand, migrate, and produce filopodia to make new connections, i.e., all responses suited to providing more building material for new blood vessels. Notch activity is required to coordinate these efforts so that a functional and perfused network of arteries and capillary tubes can meet the tissue demands for nutrients and oxygen. To this end, Notch interacts with various other pathways (including Wnt) at many levels, and, at this point, we have likely only scratched the surface of the complex signaling network that controls endothelial cell responses in conjunction with Notch.

One important principle of the signaling network emerges: the signals from the tissue in need of new blood vessels, i.e., hypoxia and hypoxia-regulated growth factors such as VEGF-A, induce both the sprouting response and the production of the Notch ligand required to coordinate the response into functional tubular morphogenesis. Mounting evidence from studies in mouse and fly shows that hypoxic signaling cooperates directly with Notch, controlling cellular behavior and metabolism to cope with hypoxic stress (Poellinger and Lendahl, 2008; Zhou et al., 2008) (see also Fraisl et al., 2009). A deeper understanding of these fundamental principles will undoubtedly aid in the development of new avenues for the treatment of blood vessel-related pathologies. The important challenges ahead include deciphering which Notch signaling components function in endothelial cells in vivo during control of the different aspects of endothelial cell biology.

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