

# Notch signaling in vascular morphogenesis

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## Purpose of review

This review highlights recent developments in the role of the Notch signaling pathway during vascular morphogenesis, angiogenesis, and vessel homeostasis.

## Recent findings

Studies conducted over the past 4 years have significantly advanced the understanding of the effect of Notch signaling on vascular development. Major breakthroughs have elucidated the role of Notch in arterial *versus* venular specification and have placed this pathway downstream of vascular endothelial growth factor.

## Summary

An emerging hallmark of the Notch signaling pathway is its nearly ubiquitous participation in cell fate decisions that affect several tissues, including epithelial, neuronal, hematopoietic, and muscle. The vascular compartment has been the latest addition to the list of tissues known to be regulated by Notch. Unraveling the contribution of Notch signaling to blood vessel formation has resulted principally from gain-of-function and loss-of-function experiments in mouse and zebrafish. During the past 4 years, these mechanistic studies have revealed that Notch is required for the successful completion of several steps during vascular morphogenesis and differentiation. In addition, the findings that Notch mutations are linked to some late-onset hereditary vascular pathologic conditions suggest the added contribution of this signaling pathway to vascular homeostasis.

## Keywords

angiogenesis, artery specification, endothelial cells, Notch, vascular development

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## Abbreviations

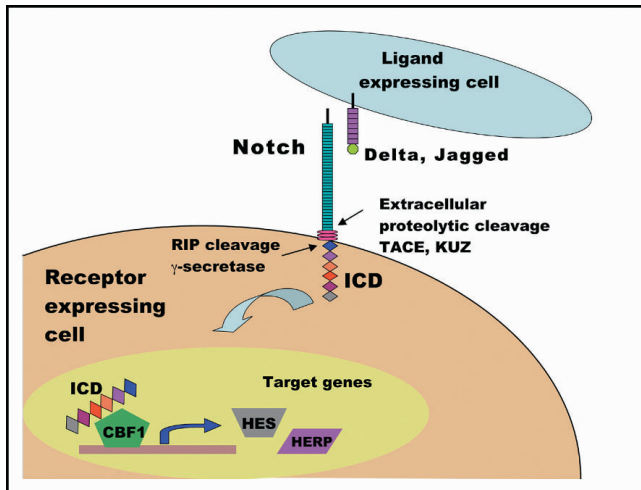
<b>CADASIL</b>	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
<b>Dll</b>	Delta-like
<b>Fbw7</b>	F box and WD containing gene
<b>Grl</b>	Gridlock
<b>HES</b>	Hairy enhancer of split
<b>HERP</b>	HES-related repressor protein
<b>VEGF</b>	vascular endothelial growth factor

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## Introduction

Notch encodes a 300-kDa transmembrane receptor protein characterized by extracellular epidermal growth factor repeats and an intracellular domain that consists of a RAM motif, six ankyrin repeats, and a transactivation domain. Four mammalian Notch receptors (Notch 1–4) have been cloned and characterized in mammals. These bind to five ligands (Jagged 1 and 2 and Delta-like (Dll) 1, 3, and 4). Because both Notch receptors and ligands contain transmembrane domains, signaling occurs between closely associated cells. The interaction between ligand and receptor leads to proteolytic cleavage and shedding of the extracellular portion of the Notch receptor. This is followed by a second cleavage event via a regulated membrane proteolysis that releases the intracellular Notch from the cell membrane. The resulting intracellular Notch translocates to the nucleus, where in some cases it associates with the transcription factor recombination signal sequence-binding protein (also known as CBF-1/RBk in mammals and Su[H] in *Drosophila*) [1–3]. Thus, Notch distinguishes itself from other signaling receptors because it acts as a receptor and a direct signaling molecule (Fig. 1). Ligand-receptor affinity can be modulated by the stage of glycosylation of the extracellular domain of Notch. The glycosyltransferase Fringe has been shown to either potentiate or inhibit Notch signaling in a cell-autonomous fashion depending on the developmental context in which it functions [4,5]. Notch downstream effectors include the hairy enhancer of split (HES) and HES-related repressor protein (HERP) family of helix-loop-helix transcriptional repressors [6].

Although the contribution of Notch in cell fate determination of neuronal, hematopoietic, and muscle cells has long been acknowledged, its impact on the cardiovascular system was recognized only recently. Deletion of several Notch receptors and ligands by homologous recombination in mice results in embryonic lethality caused by vascular and cardiac defects. In addition, several vascular anomalies have been linked to Notch receptors and ligands. Together, these results indicate that the Notch signaling pathway affects the differentiation and specification of the vasculature during development and that it is required for homeostasis of vessels in the adult. Here we review the recent genetic evidence that demonstrates an essential role for Notch in vascular morphogenesis,

**Figure 1. Schematic overview of the Notch signaling pathway**

The interaction between Delta or Jagged family ligands with Notch receptors on adjacent cells promote receptor proteolytic cleavage. An initial extracellular cleavage is mediated by members of the ADAM metalloproteinase family, TACE and KUZ. A subsequent RIP cleavage via the  $\gamma$ -secretase/presenilin complex results in the release of the Notch intracellular domain (ICD). The ICD then translocates to the nucleus, where it interacts with CBF1 to activate transcription of target genes.

with specific emphasis on its contribution to the specification of arteries *versus* veins.

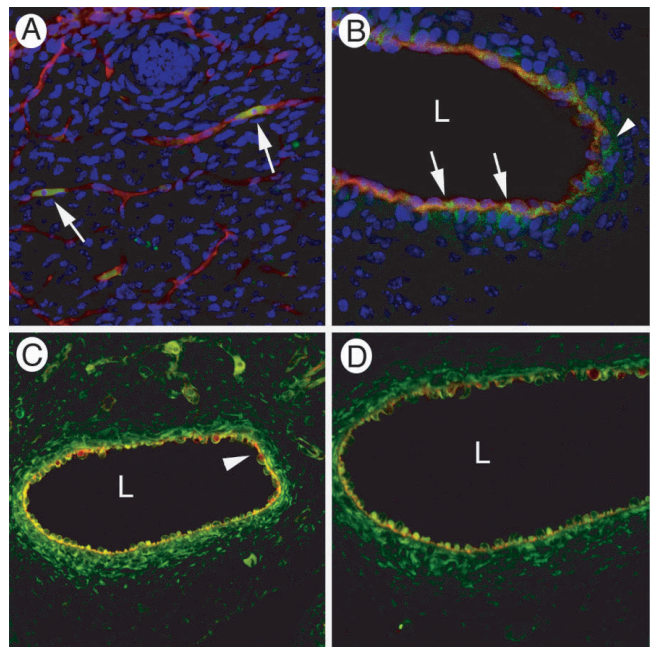
### Notch is required for vascular remodeling during development

The importance of the Notch signaling pathway in vascular development surfaced with loss-of-function studies. Mice deficient for the Notch 1 gene die between E9.5 and E10 with multiple developmental abnormalities that affect somites and the heart and blood vessels (Fig. 2) [7,8,9•]. Mutant embryos and yolk sacs are able to form the primary vascular plexus, indicating that Notch function is not necessary for vasculogenesis or initial differentiation of mesenchymal progenitors into hemangioblasts. However, the yolk sacs of Notch-deficient mice lack remodeling of the primitive plexus into the typical branching architecture of larger to smaller vessels. This absence of remodeling is also evident in other regions of Notch 1 null mice, such as in the cerebral vascular plexus. A lack of vascular hierarchy suggests possible contributions to the recruitment of mural cells and to secondary sprouting, or angiogenesis. Furthermore, in these mutant embryos, the dorsal aortae and cardinal vein form, but are smaller than, the wild-type vessels, and appear collapsed. Notch 1 null mice also display highly disorganized intersomitic vessels.

Gene inactivation has also been performed for Notch 2, 3, and 4. Alone, none of these mutants mimic the effects noted by removal of Notch 1. Combination mutants have been done for Notch 1 and 4. Mice deficient in Notch 4 are normal. However, 50% of mice deficient in Notch 1

and 4 have a more severe vascular phenotype than the Notch 1 knockout mice alone, suggesting that Notch 1 and 4 have partially redundant roles [9•]. Mice deficient in Notch 2 display milder vascular anomalies, whereas Notch 3 null mice do not exhibit overt vascular defects during development [10,11•].

The identity of the ligand(s) that interacts with Notch 1 during early stages of mouse development is currently unknown, but likely candidates are Jagged 1 and Delta 4. Genetic inactivation of Jagged 1 in mice results in lethality between E11.5 and 12.5 from vascular defects [12•]. Jagged 1 mutant yolk sacs form a vascular plexus but are unable to remodel this plexus into large and small vascular channels. The carotid vessels in the head form, but they appear collapsed. Overall, these vascular abnormalities are similar to the defects seen in Notch 1 mutants, supporting the idea that Jagged-mediated activation of the Notch pathway promotes developmental angiogenesis and contributes to remodeling of the initial vascular plexus. However, lethality in Jagged 1-deficient mice occurs 1 to 2 days after that in the Notch 1 knockout mice, suggesting that Jagged 1 may not be the sole ligand required for Notch activation during this developmental stage. Studies by Krebs *et al.* [9•] and Shutter *et al.* [13•] showed that Dll4 is expressed in the vasculature at E9.5. Furthermore, at later stages, Dll4, like Notch 1, is pre-

**Figure 2. Expression of Notch 1 in the primary vascular plexus and in the endothelium of arteries**

(A) Immunolocalization of Notch 1 (green) and PECAM (red) in the skin vasculature of E9.5 mouse embryos. Arrows point to endothelial cells. (B) Expression of Notch (green) in the endothelium (arrows) and smooth muscle (arrowhead) of the dorsal aorta at E10.5. The endothelium was also positive for PECAM (red) immunostaining. (C) Notch expression in the aorta of E13.5. Endothelial cells are also stained with PECAM (red). (D) Higher magnification of C.

dominately expressed in arterial endothelial cells. The characterization of mice deficient for Dll4 will most likely clarify the relative contribution of this ligand versus Jagged 1 during the early stages of embryogenesis.

Gain-of-function experiments have been a complementary approach used to elucidate the effects of Notch signaling in vascular morphogenesis. Expression of an activated Notch 4 in the endothelium by virtue of the VEGFR2 promoter results in embryonic lethality at E10.5 [14•]. The mutant yolk sacs form a vascular plexus, but lack remodeling of the plexus into larger vessels. Unlike in the Notch 1 knockout, these mutants form a dorsal aorta, cardinal vein, and intersomitic vessels. Yet, the vascular networks are highly disorganized, particularly in the cranial region. Although these gain-of-function studies have confirmed the importance of Notch signaling in vascular remodeling, they have yielded little mechanistic insight into the specific effects of Notch signaling during vascular development.

It is interesting to note that both loss-of-function and gain-of-function studies display similar phenotypes, *ie*, arrest of vascular development at the primitive vascular plexus. How can one explain these similarities? The initial rudimentary vascular network that is organized during E8.5 to 9.5 is later remodeled to form arteries, veins, and capillaries in a process that includes fusion, apoptosis, sprouting of new vessels (angiogenesis), and recruitment of mural cells. These events take place during E9.5 to 11.5 and result in typical hierarchic vascular networks. Disruption of any step involved in the remodeling phase results in developmental arrest of the vascular plexus with subsequent lethality. If Notch were to function in a cyclic or intermittent fashion to regulate discrete stages associated with remodeling of the vascular plexus, either constant excess or lack of expression could result in a similar phenotype.

During somitogenesis, both loss and gain of Notch function result in delay and disorganization of somites. The accumulation of experimental evidence suggests that Notch signaling is required for synchronization of the segmentation clock in somites by cycles of activation and deactivation [15]. Is this cyclic signaling of Notch mirrored in the vasculature? This question remains to be answered. However, some important insights of Notch protein turnover have emerged recently. F box and WD containing gene [Fbw7]/SEL10, a component of an SCF-type ubiquitin ligase, binds to Notch and promotes protein turnover, resulting in Notch signal inactivation [16–18]. Mice that lack Fbw7 succumb to embryonic lethality at E10.5 to 11.5, with significant abnormalities in vascular development. Mutant Fbw7 mice showed impaired vascular remodeling in the brain and yolk sac, and lack of several major veins. Interestingly, Fbw7 mice had notably increased levels in Notch 4 and HERP 2 [19•].

Another group has also inactivated Fbw7 with similar results (*ie*, lethality at E10.5 due to vascular anomalies) [20•]. However, these authors found that the lack of Fbw7 results in the upregulation of Notch 1, in addition to Notch 4. These data imply that degradation of Notch is necessary for correct vascular assembly.

### **Notch expression reveals arterial prevalence**

Consistent with the phenotypes displayed by several Notch members, *in situ* hybridization analyses have revealed Notch expression as early as E9.5 either in or around the developing vasculature. Transcripts for Notch 1 and 4 and for Dll4 are detected in the primitive vascular plexus and persist in a subset of venous and arterial endothelial cells concurrently by developmental stage E11.5 in the mouse [21]. Nonetheless, by E12.5, expression becomes progressively restricted to arteries and/or capillaries. A detailed analysis of Notch 1 and 4, Delta 4, and Jagged 1 and 2 in E13.5 embryos demonstrated that all these components were expressed specifically in the arterial endothelium and were not detected in veins. Notch 1, and particularly Dll4, were also expressed in capillaries. Arterial smooth muscle also displayed low levels of Notch 1 and high expression of Notch 3 and Jagged 1 [22].

The transition of Notch expression from primitive vessels to differentiated arteries supports a role for Notch in the regulation of arterial/venous endothelial cell specification or in the maintenance of the arterial phenotype. Genetic experimentation in zebrafish has demonstrated that this is in fact the case.

### **The latest “top-Notch” result: arterial/venous specification**

A role for Notch in arterial/venous specification was originally suggested when Gridlock (Grl), the zebrafish homolog of HERP, was found to be required for development of the dorsal aorta [23]. Mutations in Grl resulted in frequent shunts between arteries and veins. It was subsequently shown that the loss of Grl resulted in vein expansion, pointing to a role for Grl in arterial/venous specification [24••]. In an elegant and concise body of work, Lawson *et al.* [27••] demonstrated that the Notch signaling pathway lies upstream of Eph-B4/ephrin-B2 in arterial/venous specification. In zebrafish, Notch is expressed solely in arteries, and lack of Notch signaling results in the loss of ephrin-B2 expression in the arterial tree. Notch signaling was also required for the repression of the venous-specific genes Flt4 and Rtk5 (Eph-B4 zebrafish homolog) in arteries. However, Grl expression was not affected in zebrafish embryos lacking Notch signaling, thus challenging the notion that Grl is the downstream effector of Notch signaling in zebrafish vascular development. This series of experiments demonstrated the requirement of Notch signaling for the expression of artery-

specific genes and arterial repression of venous-specific genes.

The landmark study by Wang *et al.* [25] provided the first genetic evidence that arterial and venous specification is imprinted molecularly. This group showed that ephrin-B2 is expressed on arteries, whereas Eph-B4 is primarily expressed on vein endothelial cells. Mice lacking ephrin-B2 die at approximately E11.5, a few days later than Notch 1-deficient mice, and display several vascular abnormalities. Although ephrin-B2 is expressed in arteries, ephrin-B2-deficient mice have defects in both arteries and veins. Similar to Notch 1-deficient animals, the primary vascular plexus fails to undergo remodeling and form larger vessels. Furthermore, the mutants appear to have fused vessels. Eph-B4-deficient mice have a similar phenotype as ephrin-B2-deficient mice [26]. These mice die at embryonic day 10.5, with fusion of cranial vessels, intersomitic vessels, and the anterior cardinal vein. The lumen of the anterior cardinal vein was reduced and appeared split into multiple branches. The dorsal aorta did not appear affected. These genetic studies suggest that the interaction between ephrin-B2 and Eph-B4 is required for proper remodeling of the vasculature, and it appears that this signaling pathway may define the borders between arteries and veins. Although it has been shown that Notch 1 is upstream of ephrin-B2 in zebrafish, there are a few differences in the phenotypes of the Notch 1- and ephrin-B2-deficient mice, suggesting that Notch 1 may have multiple roles in vessel morphogenesis, in addition to arterial/venous specification.

In 2002, Lawson *et al.* [27••] provided genetic evidence demonstrating that the specification of arteries and veins in zebrafish is initiated by the morphogen sonic hedgehog. In their model, somites secrete sonic hedgehog adjacent to the developing aorta, which signals to neighboring cells to secrete vascular endothelial growth factor (VEGF). The endothelial cells that receive the VEGF signal upregulate Notch expression, which will subsequently specify the arterial fate. This has been a landmark contribution that linked VEGF to Notch signaling.

### Insights from cell culture studies

*In vitro* studies using human arterial endothelial cells further support the role of VEGF as an upstream regulator of the Notch signaling pathway [28•]. Liu *et al.* [28•] found that VEGF was able to increase the expression of Notch 1 in human arterial endothelial cells, as well as its ligand Dll4. In these cells, VEGF acts via the phosphatidylinositol 3-kinase/Akt pathways to activate Notch 1 expression. Expression of activated Notch 1 stabilized network formation, and suppression of Notch partially inhibited vessel formation. These results are consistent with a role for Notch in remodeling of the vasculature.

*In vitro* gain-of-function studies have also suggested a role for Notch 4 in the inhibition of angiogenesis [29•]. Expression of a constitutively active Notch 4 in human dermal microvascular endothelial cells inhibited endothelial sprouting. The mechanism responsible for the inhibition of endothelial migration was the activation of  $\beta$ -1 integrins on Notch 4-expressing cells. The authors speculate that increased integrin activation leads to endothelial cell-matrix adherence and therefore decreased endothelial cell migration/sprouting [29•]. The mechanism of Notch activation leading to changes in integrin activation state remains to be elucidated. However, these results support the view that constitutive high levels of Notch 4 suppress vessel formation—a concept that was also supported by the Fbw7-deficient mouse.

Insights from *in vitro* studies have also shown that HERP family members are targets of Notch signaling in endothelial and smooth muscle cells. HERP1 is absent in migrating and proliferating endothelial cells, whereas it is upregulated during tubular formation [30]. Furthermore, expression of HERP in these cells results in the downregulation of VEGF receptor 2. Because HERP is expressed primarily on arterial endothelial cells, the data imply that activation of Notch in arteries leads to the upregulation of HERP. HERP would then decrease the expression of VEGF receptor 2 on the arterial endothelial cell surface, suppressing proliferative and migratory signals and enabling arterial differentiation. The Notch signaling pathway could then provide a mechanism for controlling arterial morphogenesis independently from that of veins. Because it has been shown that VEGF is upstream of Notch, the decrease in expression of VEGF receptor 2 can also function as a negative feedback loop to decrease VEGF signaling. Shawber *et al.* [31] expressed an activated Notch 4 in human dermal microvascular endothelial cells and reported upregulation in Dll4, HES1, HERP1, and HERP2. Additional *in vitro* experiments have demonstrated that the intracellular domain (ICD) of Notch 1 ICD can induce expression of HERP1 in A10 smooth muscle cells derived from thoracic aorta [30]. Together, these studies support that HERP1 and HES are downstream effectors of Notch in the vasculature similarly as in other tissues. Elucidation of the HERP downstream target genes in endothelial and smooth muscle cells will lend insight into the specific contribution of the Notch signaling in angiogenesis.

### The Notch signaling pathway is required for vascular homeostasis

The importance of Notch signaling in adult blood vessels was initially suggested when mutations in Notch 3 were found to cause the human genetic disease cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [32]. CADASIL is an adult-onset vascular dementia in which affected persons are at an increased risk of stroke and coronary

occlusion. These persons have an accumulation of Notch 3 and progressive degeneration of the smooth muscle cell layer. Recent studies have revealed that the specific mutations in CADASIL patients have a negative effect on Notch 3 downstream signaling via recombination signal sequence-binding protein [33••]. Depending on the mutated Notch 3 proteins, the mutation either prevents ligand binding or does not allow proper presentation of the receptor. *In vitro* studies have shown that in smooth muscle cells, the transcriptional repressors HERP1 and HERP2 are downstream of Notch 3 [34]. Furthermore, platelet-derived growth factor, which participates in smooth muscle cell recruitment, leads to the downregulation of Notch 3 and its effectors. Studies by Chin *et al.* [35] demonstrated that HERP1 and HERP2 may contribute to the terminal differentiation of smooth muscle cells. Taken together, these studies suggest that Notch 3 signaling may upregulate the HRT proteins to repress the downstream target genes necessary for smooth muscle cell differentiation. Once the cells have taken on a smooth muscle cell fate, platelet-derived growth factor would then signal to decrease Notch 3 signaling and recruit the cells to angiogenic vessels.

In addition to Notch 3, genetic mutations in Jagged 1 cause Alagille syndrome in humans [36,37]. The Alagille syndrome is an early-onset autosomal dominant disease characterized by abnormal development of the heart, liver, skeleton, and kidney. Cardiovascular abnormalities include congenital heart defects, pulmonic stenosis, and coarctation of the aorta. Consistent with Jagged mutations leading to abnormalities of the pulmonary circulation, during mouse embryogenesis Jagged 1 is expressed in the endothelium on large and fine vessels of the lung [38].

## Conclusion

Genetic studies on several components of the Notch signaling pathway have elucidated an unexpected role for Notch in vascular morphogenesis and specification. These studies have also revealed that the contribution of Notch affects several steps during vascular development. Thus, intact Notch signaling is required for remodeling the primary plexus into the hierarchy of mature vascular beds and maintaining arterial fate, and it is essential for the homeostatic functions of fully differentiated arteries. Whereas the past 4 years have elucidated many aspects of Notch effects on blood vessels, developmental studies of Notch have also expanded the number of questions. In the next few years, further *in vitro* analysis and cell-specific knockouts combined with temporal control for loss-of-function and gain-of-function will likely shed more light on the contribution of Notch signaling in vascular development and function. Clearly, this will continue to be a very exciting and “top-Notch” area of research in the field of endothelial biology.

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