Development 134, 2709-2718 (2007) doi:10.1242/dev.004184

Notch signaling in vascular development and physiology

Thomas Gridley

Notch signaling is an ancient intercellular signaling mechanism that plays myriad roles during vascular development and physiology in vertebrates. These roles include regulation of artery/vein differentiation in endothelial and vascular smooth muscle cells, regulation of blood vessel sprouting and branching during both normal development and tumor angiogenesis, and the differentiation and physiological responses of vascular smooth muscle cells. Defects in Notch signaling also cause inherited vascular and cardiovascular diseases. In this review, I summarize recent findings and discuss the growing relevance of Notch pathway modulation for therapeutic applications in disease.

Introduction

The Notch signaling pathway is an evolutionarily conserved, intercellular signaling mechanism essential for proper embryonic development in all metazoan organisms in the Animal kingdom. Notch signaling frequently plays a crucial role in precursor cells making binary cell fate decisions. However, Notch signaling also regulates boundary formation between cell populations, cell proliferation and cell death. In addition, perturbations in Notch signaling contribute to the pathogenesis of several inherited human diseases and cancers. In this review, I will highlight the multiple roles that the Notch signaling pathway plays during vascular development and physiology in vertebrates.

Core components of the Notch signaling pathway

Notch family receptors are large single-pass type I transmembrane proteins (Fig. 1). In mammals, four Notch family receptors have been described: NOTCH1 through to NOTCH4. The extracellular domain of Notch family proteins contains up to 36 tandemly repeated copies of an epidermal growth factor (EGF)-like motif. Each Notch family receptor exists at the cell surface as a proteolytically cleaved heterodimer comprising a large ectodomain and a membrane-tethered intracellular domain. Notch receptors interact with single-pass type I transmembrane ligands expressed on neighboring cells. This restricts the Notch pathway to regulating short-range intercellular interactions. In mammals, the Notch ligands are encoded by the Jagged (*JAG1* and *JAG2*) and Delta-like (*DLL1*, *DLL3* and *DLL4*) gene families.

Upon ligand binding, a signal is transmitted intracellularly by a process involving the proteolytic cleavage of the receptor and the subsequent nuclear translocation of the Notch intracellular domain (NICD). The receptor-ligand interaction induces two additional proteolytic cleavages in the membrane-tethered fragment of the Notch heterodimer. The final cleavage, catalyzed by the γ-secretase complex, frees the NICD from the cell membrane. This cleaved fragment translocates to the nucleus because of the presence of nuclear localization signals located in the NICD. Once in the nucleus, the NICD forms a complex with the recombination signal binding protein for immunoglobulin kappa J region (RBPJ) protein – a sequence-specific DNA-binding protein. In the absence of the NICD, the RBPJ protein binds to specific DNA sequences in the regulatory elements of various target genes and represses transcription of these genes by recruiting histone deacetylases and other components to form a co-repressor complex. The nuclear translocation of the NICD displaces the histone deacetylase–co-repressor complex from the RBPJ protein. The NICD-RBPJ complex recruits other proteins, such as the mastermind-like 1 (MAML1) protein and histone acetyltransferases, leading to the transcriptional activation of Notch target genes. Among the most commonly induced Notch target genes are the basic helix-loop-helix (bHLH) transcriptional repressors of the hairy and enhancer of split/hairy and enhancer of split related with YRPW motif (Hes/Hey) family (Kageyama et al., 2007). Further details on the biochemistry of the Notch signaling pathway can be found in several recent reviews (Bray, 2006; Ehebauer et al., 2006a; Ehebauer et al., 2006b; Ilagan and Kopan, 2007; Kageyama et al., 2007; Le Borgne, 2006).

Artery-vein differentiation

A role for the Notch pathway in regulating vascular development was initially suggested based on findings from the analysis of several targeted mouse mutants in Notch pathway components. Mouse mutants for which targeted mutagenesis and transgenic studies have demonstrated a role in embryonic vascular development include the receptors Notch1 (Huppert et al., 2000; Krebs et al., 2000; Limbourg et al., 2005) and Notch4 (Carlson et al., 2005; Krebs et al., 2000; Uyttendaele et al., 2001); the ligands Jag1 (Xue et al., 1999) and Dll4 (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004); the Notch transcriptional regulator Rbpj (Krebs et al., 2004); the E3 ubiquitin ligase Mib1 (Barsi et al., 2005; Koo et al., 2005); components of the γ-secretase complex, such as nicastrin (Li et al., 2003), presenilin 1 and presenilin 2 (Herreman et al., 1999); and the Notch pathway downstream effector bHLH proteins Hey1 and Hey2 (Fischer et al., 2004; Kokubo et al., 2005). Most of these mutants exhibit a similar phenotype characterized by the absence of angiogenic vascular remodeling in the extraembryonic yolk sac, placenta and embryo proper (Fig. 2). However, an analysis of zebrafish embryos with reduced Notch signaling gave the first clues that a primary function of the Notch pathway during vascular development was to regulate the specification of arterial fate in endothelial cells.

It had long been believed that the primary factor that regulates the differentiation of arteries and veins was blood flow. The endothelial cells that line arteries experience higher blood pressures, higher rates of hemodynamic flow and higher oxygen tensions than do the endothelial cells that line veins. However, as described below, recent work has established that genetic pre-patterning, largely mediated by the Notch pathway, plays a primary role in regulating arteriovenous differentiation. This genetically determined pre-pattern is established prior to the initiation of blood flow, but endothelial cells at this stage are not yet committed to an arterial or venous cell fate (Jones et al., 2006). Indeed, recent work has

The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA.

e-mail: tom.gridley@jax.org

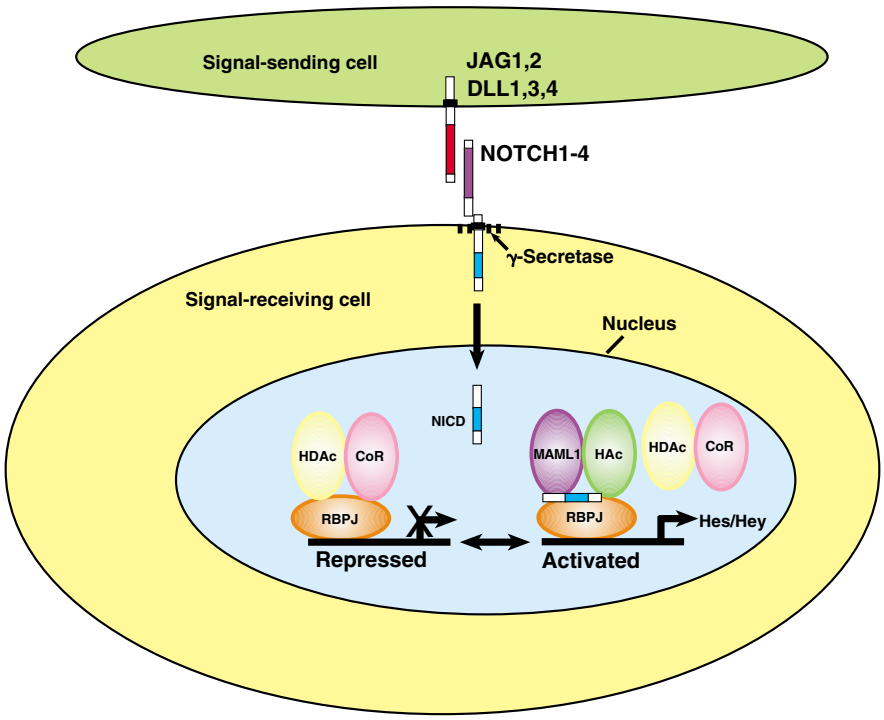

Fig. 1. Core components of the canonical Notch signaling pathway. Ligands of the Jagged (JAG1 and JAG2) and Delta-like (DLL1, DLL3, DLL4) families (upper cell, shown in green) interact with Notch family receptors (NOTCH1 through to NOTCH4) on an adjacent cell (lower cell, shown in yellow). The Notch receptor exists at the cell surface as a proteolytically cleaved heterodimer consisting of a large ectodomain and a membrane-tethered intracellular domain. The receptor-ligand interaction induces two additional proteolytic cleavages that free the Notch intracellular domain (NICD) from the cell membrane. The NICD translocates to the nucleus (blue), where it forms a complex with the RBPJ protein, displacing a histone deacetylase (HDAc)–co-repressor (CoR) complex from the RBPJ protein. Components of an activation complex, such as MAML1 and histone acetyltransferases (HAc), are recruited to the NICD-RBPJ complex, leading to the transcriptional activation of Notch target genes.

established that, in zebrafish, a single hemangioblast, the bipotential precursor of a subset of hematopoietic and endothelial cells, can give rise to endothelial cell progeny that populate both arteries and veins (Vogeli et al., 2006).

The role of the Notch pathway in regulating early embryonic vascular development is intertwined with that of another major regulator of vascular development and physiology, the vascular endothelial growth factor A (VEGFA) pathway. VEGFA is a secreted glycoprotein that is a potent inducer of angiogenesis that also regulates multiple other aspects of blood vessel homeostasis (Byrne et al., 2005; Coultas et al., 2005; Shibuya and Claesson-Welsh, 2006). The roles and interdependence of the Notch and VEGFA pathways in regulating the formation of the large axial blood vessels of the trunk – the dorsal aorta and the posterior cardinal vein – was studied first in zebrafish (Lawson et al., 2001; Lawson et al., 2002). Notch signaling-deficient embryos exhibit a poorly formed dorsal aorta and posterior cardinal vein with accompanying arteriovenous malformations (the fusion of arteries and veins without an intervening capillary bed). These embryos also exhibited a loss of expression of arterial markers such as ephrin B2 from arterial vessels with an accompanying expansion of venous markers into normally arterial domains. Embryos in which Notch signaling had been ectopically activated exhibited the reverse phenotype: suppression of vein-specific markers with ectopic expression of arterial markers in venous vessels (Lawson et al., 2001). A similar phenotype was observed in embryos mutant for certain Notch target genes, such as the bHLH transcriptional repressor *hey2* (also referred to in zebrafish as the *gridlock* gene) (Zhong et al., 2001; Zhong et al., 2000).

This analysis of the formation of the major trunk vessels in the zebrafish embryo revealed a signaling cascade that is responsible for determining arterial and venous cell fates in these vessels (Lawson et al., 2002) (Fig. 3). A reduction in *vegfa* activity results in a loss of arterial marker expression from the dorsal aorta and in the ectopic arterial expression of vein markers. Conversely, the injection of *vegfa* mRNA induces ectopic expression of the arterial marker *ephrin B2* in the posterior cardinal vein. *vegfa* expression is regulated by the expression of the secreted morphogen *sonic hedgehog a* (*shha*) along the axial midline. Similar to what is observed in *vegfa*-deficient embryos, *shha* mutant zebrafish embryos also exhibit a loss of arterial differentiation, whereas injection of *shha* mRNA causes the ectopic expression of arterial markers. *shha* acts upstream of *vegfa*, because the injection of *vegfa* mRNA into *shha* mutant embryos restores normal arterial differentiation. This work also demonstrated that, in this setting, the Notch pathway acts downstream of the Vegfa pathway. Whereas injection of *vegfa* mRNA into Notch signaling-deficient zebrafish embryos could not rescue arterial marker gene expression, the expression of an activated *notch1a* transgene in *vegfa*-deficient embryos could rescue the expression of arterial markers (Lawson et al., 2002).

Studies in mammalian cell culture have also placed the Notch pathway downstream of the Vegfa pathway. VEGFA administration induces *NOTCH1* and *DLL4* expression in human arterial endothelial cells, but not in venous endothelial cells (Liu et al., 2003). Targeted-mutagenesis studies in mice have also demonstrated that Vegfa is essential for vascular development. Mouse embryos heterozygous for a *Vegfa* targeted mutation exhibit lethal haploinsufficiency (Carmeliet et al., 1996; Ferrara et al., 1996). Blood vessels, despite forming in these embryos, are severely constricted or atretic. It is not known whether artery-vein differentiation is compromised in $Vegfa^{+/-}$ mouse embryos. However, other gain-of-function transgenic experiments have demonstrated a role of *Vegfa* in regulating arterial endothelial cell differentiation in mice. Alternative splicing of the mouse *Vegfa* gene results in the production of several different protein isoforms (Vegfa 120, Vegfa 164 and Vegfa 188). Genetically engineered mice that express only the Vegfa 164 isoform display normal retinal vascular development. However, mice that express only Vegfa 120 exhibit severe defects in retinal vascular outgrowth, whereas mice expressing only Vegfa 188 exhibit impaired retinal arterial

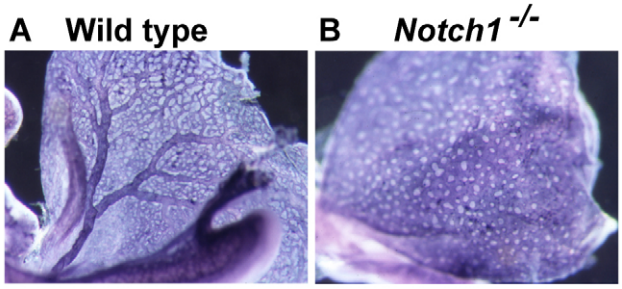

Fig. 2. Vascular defects in a *Notch1*-null mouse embryo. (**A**) Extraembryonic yolk sac from a wild-type mouse embryo exhibits remodeling of the yolk sac vasculature to generate vessels of different sizes. (**B**) Yolk sac from a *Notch1*$^{-/-}$ mutant mouse embryo. The yolk sac has arrested at the primitive vascular plexus stage, and has not undergone any angiogenic vascular remodeling. Both yolk sacs have been immunostained with an antibody to a protein expressed on vascular endothelial cells. Reproduced with permission from Krebs et al. (Krebs et al., 2000). Copyright (2000) Cold Spring Harbor Laboratory Press.

development, but normal venous development (Stalmans et al., 2002). Vegfa 164 overexpression in cardiac muscle increased the number of ephrin B2 (Efnb2)-positive capillaries in the mouse heart while reducing the number of ephrin receptor B4 (EphB4)-positive venules (Visconti et al., 2002). Vegfa could induce ephrin B2 gene expression in mouse primary embryonic endothelial cells, and Vegfa derived from sensory neurons, motor neurons and Schwann cells is required for arterial differentiation of small-diameter nerve-associated vessels in mice (Mukouyama et al., 2005; Mukouyama et al., 2002).

In mice, Dll4 is the primary Notch ligand required for vascular development. Similarly to *Vegfa*$^{+/-}$ heterozygous mouse embryos, *Dll4*$^{+/-}$ heterozygous embryos on inbred genetic backgrounds exhibit embryonic lethal haploinsufficiency due to vascular defects (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). However, some *Dll4*$^{+/-}$ mice are viable on an outbred background, permitting the examination of *Dll4*$^{-/-}$ embryos. The phenotype of *Dll4*$^{-/-}$ homozygotes was similar, although more severe, than that of *Dll4*$^{+/-}$ heterozygous embryos (Duarte et al., 2004; Gale et al., 2004). Similar to that which is observed in Notch signaling-deficient zebrafish embryos, both *Dll4*-deficient embryos and other types of Notch signaling-deficient mouse embryos, such as *Rbpj* mutant and *Hey1; Hey2* double-mutant embryos, do not express arterial markers (Duarte et al., 2004; Fischer et al., 2004; Gale et al., 2004; Kokubo et al., 2005; Krebs et al., 2004). In support of a direct role for Notch signaling in regulating the expression of important arterially expressed genes, Dll4-mediated Notch signaling induces ephrin B2 gene expression in cultured endothelial cells (Iso et al., 2006), and the ephrin B2 gene has recently been shown to be a direct Notch target (Grego-Bessa et al., 2007).

Little is known about the transcriptional regulation of genes that exhibit arterially restricted expression in early embryos. The forkhead (Fox; also known as winged helix) proteins are a large family of evolutionarily conserved transcription factors (Kaestner et al., 2000). Mouse embryos with compound mutations of the *Foxc1* and *Foxc2* genes, two related Fox family transcription factors, display defects in vascular remodeling in the yolk sac and embryo (Kume et al., 2001), accompanied by reduced or absent expression of arterial markers and the occurrence of arteriovenous malformations (Seo et al., 2006). This failure of arterial specification is likely to be due to disrupted regulation of *Dll4* transcription. The Foxc1 and Foxc2 proteins directly activate *Dll4* transcription through a Foxc-binding element in the upstream region of the *Dll4* gene. These results demonstrate that the Foxc proteins are key transcriptional regulators that act upstream of the Notch pathway during arteriovenous differentiation (Seo et al., 2006). However, it is not known whether *Foxc1* and *Foxc2* expression is downstream, or independent of, Vegfa signaling.

As mentioned previously, Notch signaling-deficient zebrafish embryos form arteriovenous malformations. Arteries normally connect to veins only through an intervening capillary bed. An aberrant direct communication between an artery and vein is termed an arteriovenous malformation. One mechanism that might explain the formation of arteriovenous malformations is the failure to establish or maintain distinct arterial and venous vascular beds. In mouse embryos, injection of ink into the heart is an effective way to visualize the presence of arteriovenous malformations (Sorensen et al., 2003). In Notch signaling-deficient mouse embryos (e.g. *Dll4*$^{+/-}$ or *Notch1*$^{-/-}$ embryos) arteriovenous malformations that are also detectable by histological analysis (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004) form (Fig. 4) (Krebs et al., 2004). Interestingly, the inducible expression of an activated *Notch4* transgene in adult mice results in vessel arterialization, such as the induction of venous expression of the ephrin B2 gene, and causes arteriovenous malformations in several organs, including liver, uterus and skin (Carlson et al., 2005). Surprisingly, these malformations are reversible if activated *Notch4* transgene expression is repressed. These studies demonstrate that the ability of Notch signaling to arterialize blood vessels is not confined to the embryonic period. Although gain-of-function in vivo assays using the expression of an activated *Notch4* transgene can cause mutant vascular phenotypes (Carlson et al., 2005; Uyttendaele et al., 2001), it should be noted that no obvious phenotype is observed in *Notch4*$^{-/-}$ mice (Krebs et al., 2000).

In addition to regulating arterial specification of endothelial cells, Notch signaling also regulates arterial specification of vascular smooth muscle cells. The *Notch3* gene is expressed in vascular smooth muscle cells of arteries, but not in those of veins. Marked arterial defects occur in *Notch3*$^{-/-}$ mice, including enlarged arteries with a thinner vascular smooth muscle cell coat than is found in wild-type arteries (Domenga et al., 2004). These defects arise postnatally, because arterial vessels fail to mature. Morphologically, arterial vascular smooth muscle cells of *Notch3*$^{-/-}$ mice resemble those surrounding veins in wild-type mice. Only a few markers are known to be expressed predominantly in arterial vascular smooth muscle cells and not in venous ones. These include smoothelin (van der Loop et al., 1997) and a transgenic line expressing the β-galactosidase protein from arterial-specific regulatory elements of the SM22α promoter (Moessler et al., 1996). The expression of both of these markers is markedly downregulated in arteries of *Notch3*$^{-/-}$ mice (Fig. 5). Combined with the morphological data, this indicates that vascular smooth muscle cells that surround arteries in *Notch3*$^{-/-}$ mice have acquired a venous fate. Notably, in arteries of *Notch3*$^{-/-}$ mice, which do not express arterial markers for vascular smooth muscle cells, normal expression of several endothelial cell arterial markers occurs, including that of ephrin B2, connexin 40 (also known as Gja5 – Mouse Genome Informatics), Hes1, Hey1, Hey2 and Heyl (Domenga et al., 2004). These results demonstrate that the arterial identity of endothelial cells, and of the vascular smooth muscle cells surrounding them, is specified independently.

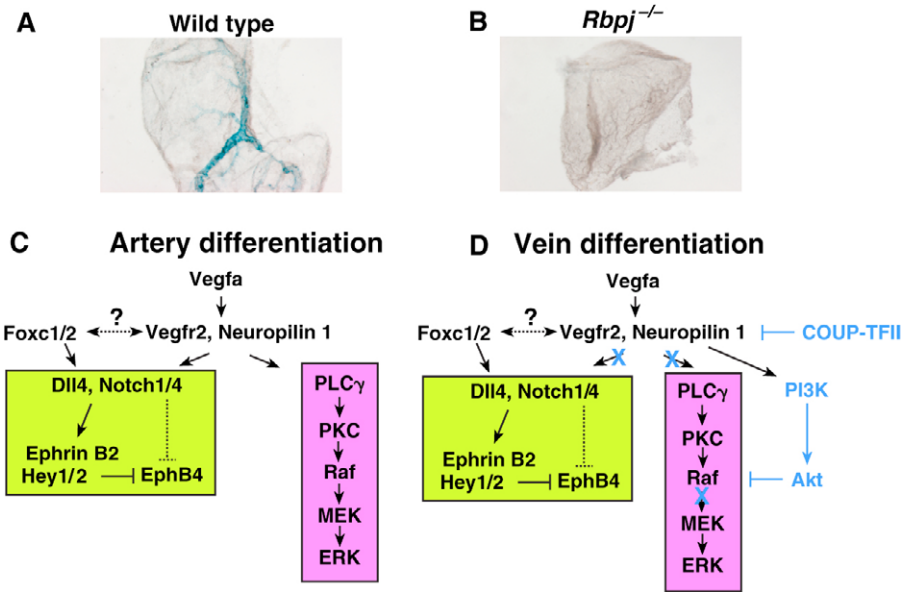

Fig. 3. Artery-vein differentiation. (**A**) Yolk sac from a wild-type (*Rbpj*$^{+/+}$) mouse embryo, which expresses an ephrin B2-*lacZ* transgene, a marker of arterial differentiation. (**B**) Yolk sac from an *Rbpj*$^{-/-}$ mutant mouse embryo, which does not express the ephrin B2-*lacZ* gene. (**C**,**D**) Model for genetic regulation of artery-vein differentiation. (C) During artery differentiation, two primary signaling pathways operate downstream of Vegfa: the Notch pathway (green box) and the PLCγ/MAPK pathway (pink box) (Lamont and Childs, 2006). The transcription factors Foxc1 and Foxc2 induce *Dll4* gene expression, but it is unknown whether *Foxc1* and *Foxc2* expression is regulated by Vegfa. (D) During vein differentiation, two different mechanisms inhibit artery differentiation (blue text). The orphan nuclear receptor COUP-TFII (Nr2f2) suppresses neuropilin 1 expression, thereby suppressing reception of the Vegfa signal and activation of Notch signaling. In addition, the activation of PI3K/Akt signaling antagonizes the promotion of arterial cell differentiation by blocking (blue cross) ERK activation. A and B are reproduced with permission from Krebs et al. (Krebs et al., 2004). Copyright (2004) Cold Spring Harbor Laboratory Press.

Endothelial tip cell differentiation

During angiogenesis, new capillaries sprout from existing blood vessels. Tip cells are specialized endothelial cells situated at the tips of vascular sprouts that extend filopodia in response to cues within the local extracellular environment, guiding the growth of these sprouts along Vegfa gradients (Gerhardt et al., 2003; Gerhardt et al., 2004). Recent work has identified a primary role for the Notch pathway in regulating the formation and function of these endothelial tip cells, a role that was first described several years ago (Sainson et al., 2005). In an in vitro angiogenesis culture system using human umbilical vein endothelial cells (HUVECs), Notch signaling inhibits branching at the tip of developing angiogenic sprouts. The suppression of Notch signaling led to tip cell division, with both daughter cells being specified as tip cells. This subsequently led to increased branching as a result of vessel bifurcation.

Numerous recent papers have confirmed and extended our understanding of the role of Notch signaling in tip cell formation. DLL4/Notch signaling has now been demonstrated to regulate tip cell numbers, filopodia extension in tip cells and the branching of angiogenic sprouts in HUVECs, as well as in several additional model systems: the mouse retina and hindbrain (Hellstrom et al., 2007; Lobov et al., 2007; Ridgway et al., 2006; Suchting et al., 2007); the zebrafish embryo (Leslie et al., 2007; Siekmann and Lawson, 2007); and xenograft tumor models (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). A finding common to all of these studies is the increased sprouting and branching of blood vessels when Notch signaling is inhibited. The Notch pathway regulates sprouting and branching behaviors in these vessels by influencing the differentiation, migration and proliferation of vascular tip cells; reduced Notch signaling leads to increases in tip cell numbers, filopodia extension and vessel branching. Suppression of tip cell formation and angiogenic sprouting by Notch signaling is downstream of the VEGFA signal, because pharmacological or genetic manipulations that block VEGFA function reduce both *DLL4* expression and blood vessel sprouting.

Several of these recent studies have assessed the effects of modulating Notch signaling on the differentiation of the developing retinal vasculature in mice (Hellstrom et al., 2007; Lobov et al., 2007; Ridgway et al., 2006; Suchting et al., 2007). Compared with other organs, the mouse retina possesses several distinct advantages for the analysis of developmental angiogenesis (Dorrell and Friedlander, 2006; Gariano and Gardner, 2005; Uemura et al., 2006). The mouse retinal vascular system develops postnatally in a highly reproducible spatial and temporal pattern. The retinal vascular system emerges first in the region of the optic nerve head, and then grows radially towards the periphery. The primitive vascular plexus that forms initially is then remodeled into large and small arterial and venous vessels. During these stages, the retinal vasculature is accessible both for observation and for the experimental administration of exogenous agents.

The *Dll4* gene is highly expressed in the developing retinal vasculature, and reduced Dll4/Notch signaling leads to striking defects in the early postnatal retinal vasculature. The observed defects are concordant whether Dll4/Notch signaling is reduced genetically, by assessing *Dll4*$^{+/-}$ heterozygous mice (Hellstrom et al., 2007; Lobov et al., 2007; Suchting et al., 2007) or mice with temporally-regulated *Notch1* deletion in the retinal vasculature (Hellstrom et al., 2007), or by administering anti-Dll4 blocking reagents (Lobov et al., 2007; Ridgway et al., 2006) or γ-secretase

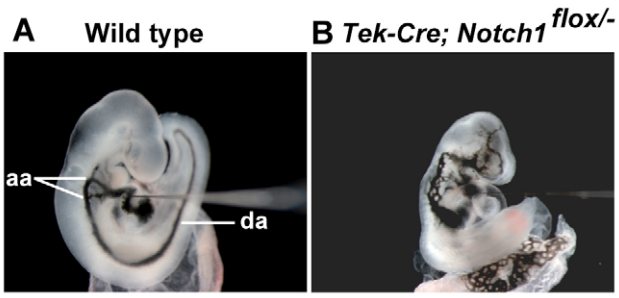

Fig. 4. Arteriovenous malformations in Notch pathway mutant mice. (**A**) In a wild-type embryo, India ink is injected into the heart to visualize blood flow as it exits anteriorly through the aortic arch arteries (aa) and enters the descending dorsal aorta (da). (**B**) In an embryo with an endothelial cell-specific deletion of the *Notch1* gene (*Tek-Cre/+; Notch1*$^{flox/-}$), arteriovenous malformations permit the injected ink to leak directly into the venous system. Original images provided by Luke Krebs in my laboratory; reproduced with permission from Weinmaster and Kopan (Weinmaster and Kopan, 2006).

inhibitors (Hellstrom et al., 2007; Suchting et al., 2007). The retinal vasculature in these mice displays severe patterning defects (Fig. 6). The vascular plexus has an increased capillary density and diameter, with increased filopodial extensions both at the growing vascular front, and in the interior of the plexus. Furthermore, portions of the vascular plexus fuse to form syncytial sinuses. Markers specific for tip cells, such as platelet derived growth factor receptor beta (*Pdgfrb*) and unc-5 homolog B (*Unc5b*), are also upregulated in mice with reduced Dll4/Notch signaling. These data indicate that Dll4/Notch signaling restricts the acquisition of an endothelial tip cell fate in angiogenic sprouts.

Additional insights into endothelial tip cell formation, migration and behavior have been obtained from the analysis of the vasculature of zebrafish embryos (Leslie et al., 2007; Siekmann and Lawson, 2007). The optical clarity of these embryos and the availability of several transgenic lines that express fluorescent proteins in endothelial cells makes this animal model an ideal system for high-resolution fluorescent microscopy, including live time-lapse confocal microscopy. Mosaic analysis has revealed that transplanted cells that lack *rbpj* function do not contribute to the dorsal aorta, but become located preferentially in the posterior cardinal vein or the most dorsal position of the segmental arteries. Conversely, transplanted cells that express activated Notch1 become located preferentially in the dorsal aorta or the base of developing sprouts (Siekmann and Lawson, 2007). These results indicate that Notch signaling is required cell-autonomously to specify endothelial cell fate in segmental artery sprouts. The use of time-lapse confocal microscopy on living embryos revealed that, in both *dll4* morphant and *rbpj* morphant embryos, segmental artery sprouts contain more cells than in controls. These additional cells are incorporated via both the increased migration of endothelial cells into the initial sprout, and by the proliferation of normally quiescent stalk cells. Interestingly, vascular defects in *rbpj* morphant embryos are more severe than those in *dll4* morphant embryos, suggesting that additional Notch ligands play important roles during early vascular development (Leslie et al., 2007; Siekmann and Lawson, 2007). Blocking Vegfa signaling with a small molecule inhibitor prevents both normal endothelial sprouting and the ectopic sprouting observed in *dll4* morphant embryos (Leslie et al., 2007). In addition, the reduction of Vegf

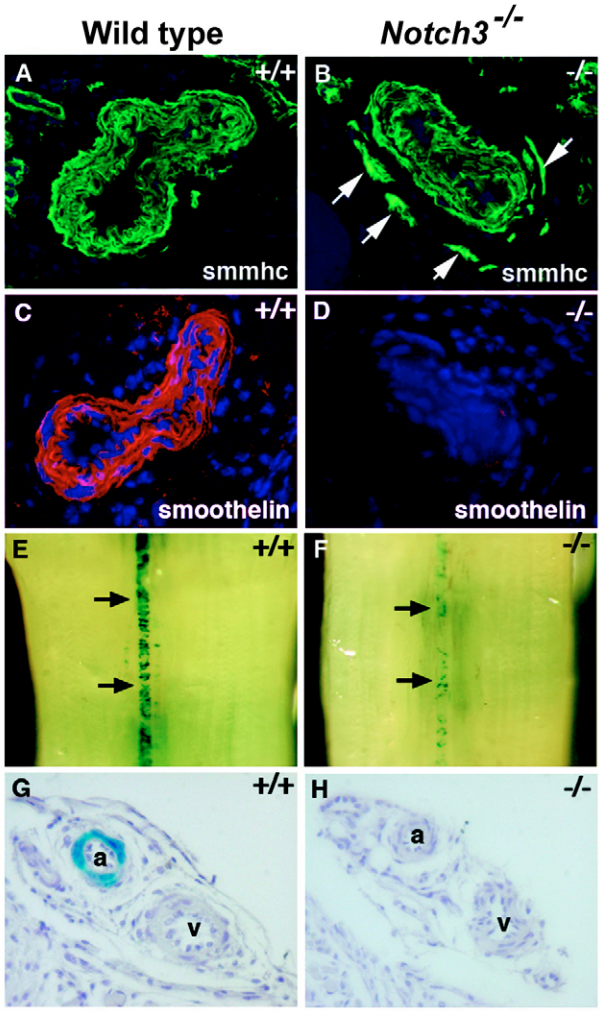

Fig. 5. Notch signaling specifies arterial differentiation of vascular smooth muscle cells. (**A**,**B**) Smooth muscle myosin heavy chain (smmhc, green) expression in wild-type and *Notch3*$^{-/-}$ tail arteries. (B) Arteries of the *Notch3*$^{-/-}$ mouse exhibit normal smmhc expression levels. Arrows highlight ectopic vascular smooth muscle cells expressing smmhc in the *Notch3*$^{-/-}$ mutant artery. (**C**,**D**) Smoothelin (red) (cell nuclei are stained with DAPI, blue) expression in wild-type and *Notch3*$^{-/-}$ tail arteries. Smoothelin expression levels are markedly reduced in the *Notch3*$^{-/-}$ mutant artery (D). (**E-H**) β-galactosidase staining (blue) of tails from wild-type and *Notch3*$^{-/-}$ mice heterozygous for a *SM22α-lacZ* transgene. (E,F) Whole-mount view of caudal artery (arrows) in the tail and (G,H) microscopic view through artery (a) and vein (v) demonstrate that β-galactosidase staining is restricted to arterial vascular smooth muscle cells in control mice and is markedly reduced in *Notch3*$^{-/-}$ arteries. Reproduced with permission from Domenga et al. (Domenga et al., 2004). Copyright (2004) Cold Spring Harbor Laboratory Press.

receptor 3 (also known as Flt4 – Zebrafish Information Network) levels in *rbpj* morphant embryos partially rescues the *rbpj*-knockdown phenotype, suggesting that Notch activation might normally repress Vegf receptor 3 and thus limit angiogenic cell behavior in developing segmental artery sprouts (Siekmann and Lawson, 2007). Taken together, the studies in both the mouse retina and the zebrafish embryo indicate that Notch signaling acts as a negative regulator of Vegfa-induced angiogenesis, and is essential for proper vascular morphogenesis.

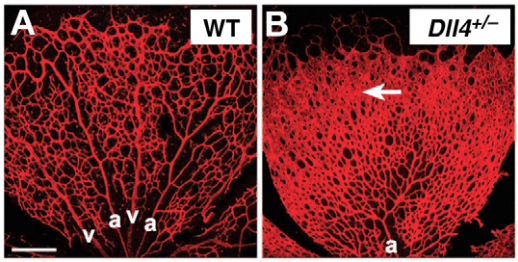

Fig. 6. Vascular defects in *Dll4*$^{+/-}$ retinas. Retinal vasculature at postnatal day 5 from (**A**) wild-type (WT) and (**B**) *Dll4*$^{+/-}$ mice. Note the areas of vessel fusion (arrow) and increased sprouting and branching at the leading edge (top) in the *Dll4*$^{+/-}$ retina. a, artery; v, vein. Reproduced with permission from Suchting et al. (Suchting et al., 2007). Copyright (2007) National Academy of Sciences, USA.

Tumor angiogenesis

The maintenance, growth and metastasis of solid tumors require the recruitment of host blood vessels into the tumor. Many solid tumors express VEGFA, and therapies that use anti-Vegfa antibodies or other blocking reagents effectively inhibit solid tumor growth in preclinical rodent models (Ferrara and Kerbel, 2005; Jain et al., 2006). Given the prominent role of the Notch pathway in regulating vascular development, protein components of the Notch pathway might provide novel drug targets during tumor angiogenesis. Dll4 is expressed at high levels in tumor vasculature (Gale et al., 2004; Hainaud et al., 2006; Mailhos et al., 2001; Patel et al., 2005), and several recent studies have identified the Dll4 protein as just such a therapeutic target (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). The systemic administration of neutralizing anti-Dll4 antibodies (Noguera-Troise et al., 2006; Ridgway et al., 2006), and the systemic (Noguera-Troise et al., 2006) or localized (Scehnet et al., 2007) administration of recombinant forms of the Dll4 protein that have been modified to block Dll4/Notch signaling, inhibit the growth of several different solid tumors in mice. Similar to the findings in zebrafish embryos and mouse retinas, anti-Dll4 treatment increases blood vessel sprouting and branching, and leads to a marked increase in tumor blood vessel density in the treated tumors. Paradoxically, tumor growth was inhibited in these mice despite the increased blood vessel density. Analysis of the vascular network in the anti-Dll4-treated tumors by perfusion assays with fluorescent lectins or by assessing hypoxic regions in these tumors revealed that the newly induced vessels function inefficiently. Many of these vessels are not connected to the vascular network in the tumors, leading to poor perfusion, increased hypoxia and an overall inhibition of tumor growth. Importantly, anti-Dll4 therapies are effective against tumors that were resistant to anti-VEGFA treatments, and could provide synergistic effects against certain tumors when combined with anti-Vegfa therapies (Noguera-Troise et al., 2006; Ridgway et al., 2006).

Although the results from the studies described above are very promising, many issues remain to be resolved before anti-DLL4 treatments reach the clinic. For example, despite the efficacy of anti-VEGFA therapies in the treatment of xenograft tumor models in rodents, in clinical trials, anti-VEGFA antibody treatment of several cancer types has been found to only provide an overall survival benefit for patients when it is combined with conventional chemotherapy treatment (Ferrara and Kerbel, 2005; Jain et al., 2006). Similar issues might arise as anti-DLL4 treatments progress from preclinical models into human clinical trials. However, these promising studies present a novel therapeutic approach for cancer treatment, particularly for tumors that are unresponsive to anti-VEGFA therapies.

Vascular smooth muscle cell differentiation and physiology

It is clear that Notch signaling plays an important role in the differentiation, physiology and function of vascular smooth muscle cells. However, contradictory results suggest that its role might be context-, time- or cell line-dependent. Several groups have described a role for Notch signaling in repressing smooth muscle cell differentiation during in vitro cell culture, and that this repressive effect is likely to be mediated via the induction of the HEY2 protein (Doi et al., 2005; Morrow et al., 2005a; Proweller et al., 2005). However, more-recent studies have indicated that Notch signaling induces smooth muscle cell differentiation (Doi et al., 2006; High et al., 2007); JAG1-mediated Notch signaling is reported to promote smooth muscle cell differentiation both in human aortic smooth muscle cells and in a murine embryonic fibroblast cell line (Doi et al., 2006). Both smooth muscle myosin heavy chain (Doi et al., 2006) and smooth muscle α-actin (Noseda et al., 2006) were demonstrated to be direct Notch target genes. Furthermore, in vivo studies in which Notch signaling was inactivated specifically in mouse neural crest cells reveals that Notch signaling plays an essential role in the differentiation of cardiac neural crest cells into smooth muscle cells (High et al., 2007).

Several studies have characterized the expression of Notch pathway genes during the response to vascular injury (Campos et al., 2002; Doi et al., 2005; Lindner et al., 2001; Wang et al., 2002). The expression of several Notch pathway components, including Notch1, Notch3, Jag1, Jag2, Hey1 and Hey2, is modulated after experimentally induced vascular injury. The expression of the genes encoding these proteins is downregulated within the first 2 days following vascular injury, but is upregulated in comparison to uninjured contralateral control vessels at 7-14 days after injury. In support of a functional role for the modulation of Notch pathway components during the response to vascular injury, formation of the neointima (a thickened layer of vascular smooth muscle cells) after vascular injury was significantly decreased in *Hey2*$^{-/-}$ mice (Sakata et al., 2004). The culture of primary aortic vascular smooth muscle cells from *Hey2*$^{-/-}$ mice has revealed that these mutant cells proliferate at a reduced rate compared with wild-type cells. The overexpression of *Hey1* (Wang et al., 2003) or *Hey2* (Havrda et al., 2006) in vascular smooth muscle cells led to increased vascular smooth muscle cell proliferation associated with reduced levels of the cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ (*Cdkn1a*) (Wang et al., 2003) or $p27^{kip1}$ (*Cdkn1b*) (Havrda et al., 2006). The Hey2 protein directly interacts with the $p27^{kip1}$ promoter to repress transcription (Havrda et al., 2006).

Mechanical forces are one of several factors implicated in regulating vascular smooth muscle cell differentiation and physiology. Adult vascular smooth muscle cells are not terminally differentiated and can exhibit substantial plasticity in their phenotype in response to local environmental changes. The exposure of primary human or rat vascular smooth muscle cells to cyclic mechanical strain during in vitro culture causes a significant reduction in NOTCH1 and NOTCH3 receptor expression, concomitant with an increase in the expression of vascular smooth muscle cell differentiation markers (Morrow et al., 2005a; Morrow et al., 2005b). Vascular smooth muscle cells that are exposed to mechanical strain also exhibit reduced proliferation and increased

apoptosis. Overexpression of the NOTCH1 or NOTCH3 intracellular domains in vascular smooth muscle cells exposed to such mechanical strain restored the percentages of proliferative or apoptotic cells to the levels observed in unstrained cells. These results indicate that cyclic mechanical strain inhibits vascular smooth muscle cell growth while increasing apoptosis, and that these effects are mediated, at least in part, via the modulation of Notch signaling.

Vascular smooth muscle cell physiology: CADASIL

The importance of Notch signaling in vascular development is highlighted by the finding that mutations in several Notch pathway components cause inherited vascular or cardiovascular diseases. The vascular defects associated with two of these inherited diseases, Alagille syndrome and inherited bicuspid aortic valve, primarily affect the development and function of the heart (Box 1). A third disease, which is caused by mutations in the *NOTCH3* gene (Joutel et al., 1996), is an inherited degenerative vascular disease that affects vascular smooth muscle cells. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, termed CADASIL, is the most common genetic form of stroke and vascular dementia (Kalaria et al., 2004). Affected individuals exhibit a variety of symptoms, including migraines, mood disorders, recurrent subcortical ischemic strokes, progressive cognitive decline, dementia and premature death. CADASIL is characterized by the progressive degeneration of vascular smooth muscle cells and the accumulation of granular osmiophilic material (GOM) within the smooth muscle cell basement membrane (Kalaria et al., 2004). GOM accumulation in vascular smooth muscle cells is one of the most distinguishing features of CADASIL.

All *NOTCH3* mutations associated with CADASIL result in a gain or loss of a cysteine residue in one of the 34 EGF-like repeats in the extracellular domain of the NOTCH3 receptor. The characteristic nature of these mutations, in addition to the absence of any examples in CADASIL patients of mutations or deletions of the *NOTCH3* gene that are obviously inactivating, strongly suggests that mutations that cause CADASIL are not *NOTCH3*-null alleles. In CADASIL patients, the ectodomain of the NOTCH3 protein accumulates in the cerebral microvasculature at the cytoplasmic membrane of vascular smooth muscle cells (Joutel et al., 2000), although it is controversial whether the NOTCH3 ectodomain constitutes part or all of the GOM deposits (Ishiko et al., 2006; Joutel et al., 2000).

Two different mouse models that express NOTCH3 proteins containing mutations found in CADASIL patients have been developed. In one model, an Arg142Cys knock-in mutation was introduced into the endogenous mouse *Notch3* gene (Lundkvist et al., 2005). These mice do not exhibit any CADASIL-like morphological or behavioral phenotypes, even when homozygous for this mutation. The second model more successfully recapitulates the early, preclinical phase of CADASIL. To create this model, transgenic mice were generated that express a human *NOTCH3* cDNA that contains a different CADASIL mutation, the Arg90Cys mutation, in vascular smooth muscle cells (Ruchoux et al., 2003). An age-dependent accumulation of the NOTCH3 ectodomain and of GOM deposits in vascular smooth muscle cells of both cerebral and peripheral arterioles is observed in these mice. However, despite GOM accumulation, no evidence of damage to the brain parenchyma is seen. Physiological studies of these NOTCH3 Arg90Cys transgenic mice revealed an impaired cerebral vasoreactivity that suggests either decreased relaxation or increased resistance of cerebral blood vessels (Lacombe et al., 2005). Isolated caudal arteries from the tails of these mice exhibit increased pressure-induced contraction and decreased flow-induced dilation (Dubroca et al., 2005). Transgenic mice that express either the wild-type human NOTCH3 protein or the human NOTCH3 Arg90Cys mutation were equally effective in rescuing the arterial defects of *Notch3*$^{-/-}$ mice. Furthermore, the expression of the mutant NOTCH3 Arg90Cys protein correctly regulates the in vivo expression of a Notch signaling reporter (Monet et al., 2007). These data suggest that novel pathogenic roles for CADASIL mutant proteins, rather than compromised NOTCH3 signaling activity, underlie the etiology of CADASIL. Further analysis and development of the NOTCH3 Arg90Cys mouse model should lead to valuable insights into the onset and progression of CADASIL, particularly during its early, preclinical stages.

Box 1. Notch signaling and inherited cardiovascular disease

Alagille syndrome is an autosomal dominant disorder characterized by developmental abnormalities of the liver, heart, eye, skeleton and, at lower penetrance, several other organs (Gridley, 2003). Most cases of Alagille syndrome are caused by *JAG1* mutations (Warthen et al., 2006), although a small number of Alagille syndrome patients with *NOTCH2* mutations have been identified (McDaniell et al., 2006). The cardiac defects associated with Alagille syndrome include pulmonary artery stenosis and hypoplasia, pulmonic valve stenosis, and tetralogy of Fallot. These defects are likely to be due to a requirement for Notch signaling-mediated differentiation of cardiac neural crest cells into smooth muscle cells, which has been demonstrated in a mouse model (High et al., 2007).

Bicuspid aortic valve affects 1-2% of the population, making it the most common congenital cardiac malformation (Braverman et al., 2005; Garg, 2006). Bicuspid aortic valve predisposes one to aortic valve calcification, which can impair blood flow through the valve. Aortic valve calcification was linked to Notch regulation of the transcription factor RUNX2 (Garg, 2006; Garg et al., 2005). Heterozygous mutations in the *NOTCH1* gene were found in two families with autosomal-dominant aortic valve disease (Garg et al., 2005). *NOTCH1* mutations are also found in 4% of sporadic bicuspid aortic valve patients (Mohamed et al., 2006). The formation of bicuspid aortic valve might reflect the role of Notch signaling in regulating the epithelial-mesenchymal transition required for the generation of the heart valves (Fischer et al., 2007; Noseda et al., 2004; Timmerman et al., 2004).

Conclusion

As can be seen by the recent flurry of publications that focus on the role of Notch signaling in regulating endothelial tip cell formation and the resulting implications for tumor angiogenesis (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007), we still have much to learn about the roles that the Notch pathway plays in the vasculature. How are the different roles that the Notch pathway plays in, for example, arteriovenous patterning, tip cell differentiation, and vessel wall formation, integrated during vascular development and physiology? Are these roles related mechanistically, or do they represent different aspects of Notch pathway function? What is the contribution of Notch signaling during normal vascular physiology in adults, and how do perturbations in Notch signaling contribute to vascular pathology and disease?

Areas in which there will certainly be significant advances in upcoming years include the development of anti-DLL4 therapies (and possibly other types of 'anti-Notch' therapies) that target tumor angiogenesis as well as other vascular diseases characterized by pathological angiogenesis, such as neovascular age-related macular degeneration and diabetic retinopathy. We are also likely to see the development and characterization of better animal models for CADASIL. Because CADASIL causes the most common form of inherited stroke and vascular dementia in humans, animal models that better recapitulate the pathological and behavioral abnormalities exhibited by CADASIL patients will permit the development and evaluation of therapeutic treatment regimens both for CADASIL and for sporadic cases of vascular dementia. Significant advances in our understanding of the mechanisms for cross-talk between the Notch pathway and other signaling pathways, such as the TGFβ, Wnt, ephrin/Eph receptor and PI3K/Akt pathways, and in the developmental and physiological decisions in which such cross-talk is operative, are also likely to occur in the near future. The answers to these and other questions will keep Notch pathway researchers occupied for many years to come.

I apologize to colleagues whose work could not be cited directly due to space constraints. I thank Anne Joutel, Anne Eichmann and Luke Krebs for images for some of the figures. Work in my laboratory on the Notch pathway is supported by the National Institutes of Health.

References

Barsi, J. C., Rajendra, R., Wu, J. I. and Artzt, K. (2005). Mind bomb1 is a ubiquitin ligase essential for mouse embryonic development and Notch signaling. *Mech. Dev.* **122**, 1106-1117.

Braverman, A. C., Guven, H., Beardslee, M. A., Makan, M., Kates, A. M. and Moon, M. R. (2005). The bicuspid aortic valve. *Curr. Probl. Cardiol.* **30**, 470-522.

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* **7**, 678-689.

Byrne, A. M., Bouchier-Hayes, D. J. and Harmey, J. H. (2005). Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). *J. Cell. Mol. Med.* **9**, 777-794.

Campos, A. H., Wang, W., Pollman, M. J. and Gibbons, G. H. (2002). Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation. *Circ. Res.* **91**, 999-1006.

Carlson, T. R., Yan, Y., Wu, X., Lam, M. T., Tang, G. L., Beverly, L. J., Messina, L. M., Capobianco, A. J., Werb, Z. and Wang, R. (2005). Endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. *Proc. Natl. Acad. Sci. USA* **102**, 9884-9889.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

Coultas, L., Chawengsaksophak, K. and Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature* **438**, 937-945.

Doi, H., Iso, T., Yamazaki, M., Akiyama, H., Kanai, H., Sato, H., Kawai-Kowase, K., Tanaka, T., Maeno, T., Okamoto, E. et al. (2005). HERP1 inhibits myocardin-induced vascular smooth muscle cell differentiation by interfering with SRF binding to CArG box. *Arterioscler. Thromb. Vasc. Biol.* **25**, 2328-2334.

Doi, H., Iso, T., Sato, H., Yamazaki, M., Matsui, H., Tanaka, T., Manabe, I., Arai, M., Nagai, R. and Kurabayashi, M. (2006). Jagged1-selective notch signaling induces smooth muscle differentiation via a RBP-Jkappa-dependent pathway. *J. Biol. Chem.* **281**, 28555-28564.

Domenga, V., Fardoux, P., Lacombe, P., Monet, M., Maciazek, J., Krebs, L. T., Klonjkowski, B., Berrou, E., Mericskay, M., Li, Z. et al. (2004). Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev.* **18**, 2730-2735.

Dorrell, M. I. and Friedlander, M. (2006). Mechanisms of endothelial cell guidance and vascular patterning in the developing mouse retina. *Prog. Retin. Eye Res.* **25**, 277-295.

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D. and Rossant, J. (2004). Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev.* **18**, 2474-2478.

Dubroca, C., Lacombe, P., Domenga, V., Maciazek, J., Levy, B., Tournier-Lasserve, E., Joutel, A. and Henrion, D. (2005). Impaired vascular mechanotransduction in a transgenic mouse model of CADASIL arteriopathy. *Stroke* **36**, 113-117.

Ehebauer, M., Hayward, P. and Martinez-Arias, A. (2006a). Notch signaling pathway. *Sci. STKE* **2006**, cm7.

Ehebauer, M., Hayward, P. and Martinez-Arias, A. (2006b). Notch, a universal arbiter of cell fate decisions. *Science* **314**, 1414-1415.

Ferrara, N. and Kerbel, R. S. (2005). Angiogenesis as a therapeutic target. *Nature* **438**, 967-974.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.

Fischer, A., Schumacher, N., Maier, M., Sendtner, M. and Gessler, M. (2004). The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev.* **18**, 901-911.

Fischer, A., Steidl, C., Wagner, T. U., Lang, E., Jakob, P. M., Friedl, P., Knobeloch, K. P. and Gessler, M. (2007). Combined loss of Hey1 and HeyL causes congenital heart defects because of impaired epithelial to mesenchymal transition. *Circ. Res.* **100**, 856-863.

Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., Murphy, A. J., Adams, N. C., Lin, H. C., Holash, J. et al. (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl. Acad. Sci. USA* **101**, 15949-15954.

Garg, V. (2006). Molecular genetics of aortic valve disease. *Curr. Opin. Cardiol.* **21**, 180-184.

Garg, V., Muth, A. N., Ransom, J. F., Schluterman, M. K., Barnes, R., King, I. N., Grossfeld, P. D. and Srivastava, D. (2005). Mutations in NOTCH1 cause aortic valve disease. *Nature* **437**, 270-274.

Gariano, R. F. and Gardner, T. W. (2005). Retinal angiogenesis in development and disease. *Nature* **438**, 960-966.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D. et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell Biol.* **161**, 1163-1177.

Gerhardt, H., Ruhrberg, C., Abramsson, A., Fujisawa, H., Shima, D. and Betsholtz, C. (2004). Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. *Dev. Dyn.* **231**, 503-509.

Grego-Bessa, J., Luna-Zurita, L., Del Monte, G., Bolos, V., Melgar, P., Arandilla, A., Garratt, A. N., Zang, H., Mukouyama, Y. S., Chen, H. et al. (2007). Notch signaling is essential for ventricular chamber development. *Dev. Cell* **12**, 415-429.

Gridley, T. (2003). Notch signaling and inherited disease syndromes. *Hum. Mol. Genet.* **12** Spec No 1, R9-R13.

Hainaud, P., Contreres, J. O., Villemain, A., Liu, L. X., Plouet, J., Tobelem, G. and Dupuy, E. (2006). The role of the vascular endothelial growth factor-delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions. *Cancer Res.* **66**, 8501-8510.

Havrda, M. C., Johnson, M. J., O'Neill, C. F. and Liaw, L. (2006). A novel mechanism of transcriptional repression of p27kip1 through Notch/HRT2 signaling in vascular smooth muscle cells. *Thromb. Haemost.* **96**, 361-370.

Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A. K., Karlsson, L., Gaiano, N. et al. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776-780.

Herreman, A., Hartmann, D., Annaert, W., Saftig, P., Craessaerts, K., Serneels, L., Umans, L., Schrijvers, V., Checler, F., Vanderstichele, H. et al. (1999). Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proc. Natl. Acad. Sci. USA* **96**, 11872-11877.

High, F. A., Zhang, M., Proweller, A., Tu, L., Parmacek, M. S., Pear, W. S. and Epstein, J. A. (2007). An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. *J. Clin. Invest.* **117**, 353-363.

Huppert, S. S., Le, A., Schroeter, E. H., Mumm, J. S., Saxena, M. T., Milner, L. A. and Kopan, R. (2000). Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. *Nature* **405**, 966-970.

Ilagan, M. X. and Kopan, R. (2007). SnapShot: notch signaling pathway. *Cell* **128**, 1246.

Ishiko, A., Shimizu, A., Nagata, E., Takahashi, K., Tabira, T. and Suzuki, N. (2006). Notch3 ectodomain is a major component of granular osmiophilic material (GOM) in CADASIL. *Acta Neuropathol.* **112**, 333-339.

Iso, T., Maeno, T., Oike, Y., Yamazaki, M., Doi, H., Arai, M. and Kurabayashi, M. (2006). Dll4-selective Notch signaling induces ephrinB2 gene expression in endothelial cells. *Biochem. Biophys. Res. Commun.* **341**, 708-714.

Jain, R. K., Duda, D. G., Clark, J. W. and Loeffler, J. S. (2006). Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat. Clin. Pract. Oncol.* **3**, 24-40.

Jones, E. A., le Noble, F. and Eichmann, A. (2006). What determines blood vessel structure? Genetic prespecification vs. hemodynamics. *Physiology* **21**, 388-395.

Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E. et al. (1996). Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* **383**, 707-710.

Joutel, A., Andreux, F., Gaulis, S., Domenga, V., Cecillon, M., Battail, N., Piga, N., Chapon, F., Godfrain, C. and Tournier-Lasserve, E. (2000). The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. *J. Clin. Invest.* **105**, 597-605.

Kaestner, K. H., Knochel, W. and Martinez, D. E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev.* **14**, 142-146.

Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development* **134**, 1243-1251.

Kalaria, R. N., Viitanen, M., Kalimo, H., Dichgans, M. and Tabira, T. (2004). The pathogenesis of CADASIL: an update. *J. Neurol. Sci.* **226**, 35-39.

Kokubo, H., Miyagawa-Tomita, S., Nakazawa, M., Saga, Y. and Johnson, R. L. (2005). Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Dev. Biol.* **278**, 301-309.

Koo, B. K., Lim, H. S., Song, R., Yoon, M. J., Yoon, K. J., Moon, J. S., Kim, Y. W., Kwon, M. C., Yoo, K. W., Kong, M. P. et al. (2005). Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development* **132**, 3459-3470.

Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R. et al. (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev.* **14**, 1343-1352.

Krebs, L. T., Shutter, J. R., Tanigaki, K., Honjo, T., Stark, K. L. and Gridley, T. (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev.* **18**, 2469-2473.

Kume, T., Jiang, H., Topczewska, J. M. and Hogan, B. L. (2001). The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev.* **15**, 2470-2482.

Lacombe, P., Oligo, C., Domenga, V., Tournier-Lasserve, E. and Joutel, A. (2005). Impaired cerebral vasoreactivity in a transgenic mouse model of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy arteriopathy. *Stroke* **36**, 1053-1058.

Lamont, R. E. and Childs, S. (2006). MAPping out arteries and veins. *Sci. STKE* **2006**, pe39.

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A. and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128**, 3675-3683.

Lawson, N. D., Vogel, A. M. and Weinstein, B. M. (2002). sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev. Cell* **3**, 127-136.

Le Borgne, R. (2006). Regulation of Notch signalling by endocytosis and endosomal sorting. *Curr. Opin. Cell Biol.* **18**, 213-222.

Leslie, J. D., Ariza-McNaughton, L., Bermange, A. L., McAdow, R., Johnson, S. L. and Lewis, J. (2007). Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development* **134**, 839-844.

Li, T., Ma, G., Cai, H., Price, D. L. and Wong, P. C. (2003). Nicastrin is required for assembly of presenilin/gamma-secretase complexes to mediate Notch signaling and for processing and trafficking of beta-amyloid precursor protein in mammals. *J. Neurosci.* **23**, 3272-3277.

Limbourg, F. P., Takeshita, K., Radtke, F., Bronson, R. T., Chin, M. T. and Liao, J. K. (2005). Essential role of endothelial Notch1 in angiogenesis. *Circulation* **111**, 1826-1832.

Lindner, V., Booth, C., Prudovsky, I., Small, D., Maciag, T. and Liaw, L. (2001). Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. *Am. J. Pathol.* **159**, 875-883.

Liu, Z. J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G. P., Fairman, R. M., Velazquez, O. C. and Herlyn, M. (2003). Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol. Cell. Biol.* **23**, 14-25.

Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D. and Wiegand, S. J. (2007). Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc. Natl. Acad. Sci. USA* **104**, 3219-3224.

Lundkvist, J., Zhu, S., Hansson, E. M., Schweinhardt, P., Miao, Q., Beatus, P., Dannaeus, K., Karlstrom, H., Johansson, C. B., Viitanen, M. et al. (2005). Mice carrying a R142C Notch 3 knock-in mutation do not develop a CADASIL-like phenotype. *Genesis* **41**, 13-22.

Mailhos, C., Modlich, U., Lewis, J., Harris, A., Bicknell, R. and Ish-Horowicz, D. (2001). Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation* **69**, 135-144.

McDaniell, R., Warthen, D. M., Sanchez-Lara, P. A., Pai, A., Krantz, I. D., Piccoli, D. A. and Spinner, N. B. (2006). NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. *Am. J. Hum. Genet.* **79**, 169-173.

Moessler, H., Mericskay, M., Li, Z., Nagl, S., Paulin, D. and Small, J. V. (1996). The SM 22 promoter directs tissue-specific expression in arterial but not in venous or visceral smooth muscle cells in transgenic mice. *Development* **122**, 2415-2425.

Mohamed, S. A., Aherrahrou, Z., Liptau, H., Erasmi, A. W., Hagemann, C., Wrobel, S., Borzym, K., Schunkert, H., Sievers, H. H. and Erdmann, J. (2006). Novel missense mutations (p.T596M and p.P1797H) in NOTCH1 in patients with bicuspid aortic valve. *Biochem. Biophys. Res. Commun.* **345**, 1460-1465.

Monet, M., Domenga, V., Lemaire, B., Souilhol, C., Langa, F., Babinet, C., Gridley, T., Tournier-Lasserve, E., Cohen-Tannoudji, M. and Joutel, A. (2007). The archetypal R90C CADASIL-NOTCH3 mutation retains NOTCH3 function in vivo. *Hum. Mol. Genet.* **16**, 982-992.

Morrow, D., Scheller, A., Birney, Y. A., Sweeney, C., Guha, S., Cummins, P. M., Murphy, R., Walls, D., Redmond, E. M. and Cahill, P. A. (2005a). Notch-mediated CBF-1/RBP-J{kappa}-dependent regulation of human vascular smooth muscle cell phenotype in vitro. *Am. J. Physiol. Cell Physiol.* **289**, C1188-C1196.

Morrow, D., Sweeney, C., Birney, Y. A., Cummins, P. M., Walls, D., Redmond, E. M. and Cahill, P. A. (2005b). Cyclic strain inhibits Notch receptor signaling in vascular smooth muscle cells in vitro. *Circ. Res.* **96**, 567-575.

Mukouyama, Y. S., Shin, D., Britsch, S., Taniguchi, M. and Anderson, D. J. (2002). Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109**, 693-705.

Mukouyama, Y. S., Gerber, H. P., Ferrara, N., Gu, C. and Anderson, D. J. (2005). Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development* **132**, 941-952.

Noguera-Troise, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., Lin, H. C., Yancopoulos, G. D. and Thurston, G. (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* **444**, 1032-1037.

Noseda, M., McLean, G., Niessen, K., Chang, L., Pollet, I., Montpetit, R., Shahidi, R., Dorovini-Zis, K., Li, L., Beckstead, B. et al. (2004). Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. *Circ. Res.* **94**, 910-917.

Noseda, M., Fu, Y., Niessen, K., Wong, F., Chang, L., McLean, G. and Karsan, A. (2006). Smooth Muscle alpha-actin is a direct target of Notch/CSL. *Circ. Res.* **98**, 1468-1470.

Patel, N. S., Li, J. L., Generali, D., Poulsom, R., Cranston, D. W. and Harris, A. L. (2005). Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res.* **65**, 8690-8697.

Proweller, A., Pear, W. S. and Parmacek, M. S. (2005). Notch signaling represses myocardin-induced smooth muscle cell differentiation. *J. Biol. Chem.* **280**, 8994-9004.

Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chanthery, Y., Kowalski, J., Watts, R. J., Callahan, C., Kasman, I. et al. (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* **444**, 1083-1087.

Ruchoux, M. M., Domenga, V., Brulin, P., Maciazek, J., Limol, S., Tournier-Lasserve, E. and Joutel, A. (2003). Transgenic mice expressing mutant Notch3 develop vascular alterations characteristic of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Am. J. Pathol.* **162**, 329-342.

Sainson, R. C., Aoto, J., Nakatsu, M. N., Holderfield, M., Conn, E., Koller, E. and Hughes, C. C. (2005). Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. *FASEB J.* **19**, 1027-1029.

Sakata, Y., Xiang, F., Chen, Z., Kiriyama, Y., Kamei, C. N., Simon, D. I. and Chin, M. T. (2004). Transcription factor CHF1/Hey2 regulates neointimal formation in vivo and vascular smooth muscle proliferation and migration in vitro. *Arterioscler. Thromb. Vasc. Biol.* **24**, 2069-2074.

Scehnet, J. S., Jiang, W., Kumar, S. R., Krasnoperov, V., Trindade, A., Benedito, R., Djokovic, D., Borges, C., Ley, E. J., Duarte, A. et al. (2007). Inhibition of Dll4 mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood* **109**, 4753-4760.

Seo, S., Fujita, H., Nakano, A., Kang, M., Duarte, A. and Kume, T. (2006). The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Dev. Biol.* **294**, 458-470.

Shibuya, M. and Claesson-Welsh, L. (2006). Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp. Cell Res.* **312**, 549-560.

Siekmann, A. F. and Lawson, N. D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445**, 781-784.

Sorensen, L. K., Brooke, B. S., Li, D. Y. and Urness, L. D. (2003). Loss of distinct arterial and venous boundaries in mice lacking endoglin, a vascular-specific TGFbeta coreceptor. *Dev. Biol.* **261**, 235-250.

Stalmans, I., Ng, Y. S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S. et al. (2002). Arteriolar and

venular patterning in retinas of mice selectively expressing VEGF isoforms. *J. Clin. Invest.* **109**, 327-336.
Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A. and Eichmann, A. (2007). The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc. Natl. Acad. Sci. USA* **104**, 3225-3230.
Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. et al. (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* **18**, 99-115.
Uemura, A., Kusuhara, S., Katsuta, H. and Nishikawa, S. (2006). Angiogenesis in the mouse retina: a model system for experimental manipulation. *Exp. Cell Res.* **312**, 676-683.
Uyttendaele, H., Ho, J., Rossant, J. and Kitajewski, J. (2001). Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proc. Natl. Acad. Sci. USA* **98**, 5643-5648.
van der Loop, F. T., Gabbiani, G., Kohnen, G., Ramaekers, F. C. and van Eys, G. J. (1997). Differentiation of smooth muscle cells in human blood vessels as defined by smoothelin, a novel marker for the contractile phenotype. *Arterioscler. Thromb. Vasc. Biol.* **17**, 665-671.
Visconti, R. P., Richardson, C. D. and Sato, T. N. (2002). Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proc. Natl. Acad. Sci. USA* **99**, 8219-8224.
Vogeli, K. M., Jin, S. W., Martin, G. R. and Stainier, D. Y. (2006). A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature* **443**, 337-339.
Wang, W., Campos, A. H., Prince, C. Z., Mou, Y. and Pollman, M. J. (2002). Coordinate Notch3-hairy-related transcription factor pathway regulation in response to arterial injury. Mediator role of platelet-derived growth factor and ERK. *J. Biol. Chem.* **277**, 23165-23171.
Wang, W., Prince, C. Z., Hu, X. and Pollman, M. J. (2003). HRT1 modulates vascular smooth muscle cell proliferation and apoptosis. *Biochem. Biophys. Res. Commun.* **308**, 596-601.
Warthen, D. M., Moore, E. C., Kamath, B. M., Morrissette, J. J., Sanchez, P., Piccoli, D. A., Krantz, I. D. and Spinner, N. B. (2006). Jagged1 (JAG1) mutations in Alagille syndrome: increasing the mutation detection rate. *Hum. Mutat.* **27**, 436-443.
Weinmaster, G. and Kopan, R. (2006). A garden of Notch-ly delights. *Development* **133**, 3277-3282.
Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum. Mol. Genet.* **8**, 723-730.
Zhong, T. P., Rosenberg, M., Mohideen, M. A., Weinstein, B. and Fishman, M. C. (2000). gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science* **287**, 1820-1824.
Zhong, T. P., Childs, S., Leu, J. P. and Fishman, M. C. (2001). Gridlock signalling pathway fashions the first embryonic artery. *Nature* **414**, 216-220.