RESEARCH COMMUNICATION

Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants

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The Notch signaling pathway is essential for embryonic vascular development in vertebrates. Here we show that mouse embryos heterozygous for a targeted mutation in the gene encoding the DLL4 ligand exhibit haploinsufficient lethality because of defects in vascular remodeling. We also describe vascular defects in embryos homozygous for a mutation in the *Rbpsuh* gene, which encodes the primary transcriptional mediator of Notch signaling. Conditional inactivation of *Rpbsuh* function demonstrates that Notch activation is essential in the endothelial cell lineage. Notch pathway mutant embryos exhibit defects in arterial specification of nascent blood vessels and develop arteriovenous malformations. These results demonstrate that vascular remodeling in the mouse embryo is sensitive to *Dll4* gene dosage and that Notch activation in endothelial cells is essential for embryonic vascular remodeling.

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The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism. Mutations in Notch pathway components disrupt embryonic development in diverse multicellular organisms and cause inherited disease syndromes in humans (Gridley 2003; Lai 2004; Schweisguth 2004). Genes of the Notch family encode large transmembrane receptors that interact with membrane-bound ligands encoded by genes of the Delta and Serrate/Jagged families. The signal induced by ligand binding is transmitted intracellularly by a process involving proteolytic cleavage of the receptor and nuclear translocation of the intracellular domain of the Notch family protein (Notch-IC). Once in the nucleus, Notch-IC forms a complex with the RBP-J protein, which is encoded by the *Rbpsuh* gene. The RBP-J protein is a sequence-specific DNA-binding protein that is the primary transcriptional mediator of Notch signaling. The Notch-IC/RBP-J complex then activates transcription of downstream target genes.

Recent work has shown that Notch signaling is essential for embryonic vascular development in vertebrates (Lawson and Weinstein 2002; Rossant and Howard 2002; Shawber and Kitajewski 2004). Here we demonstrate that mouse embryos heterozygous for a targeted mutation in the gene encoding the DLL4 ligand exhibit haploinsufficient lethality because of defects in vascular remodeling. We describe vascular defects in embryos homozygous for a mutation in the *Rbpsuh* gene and show that *Rbpsuh*-null (–/–) embryos do not express several arterial-specific endothelial cell markers. Conditional inactivation of *Rpbsuh* function demonstrates that Notch activation is essential in the endothelial cell lineage. Notch pathway mutant embryos also exhibit arteriovenous malformations, likely as a consequence of an inability to establish and maintain distinct arterial–venous vascular beds. These results demonstrate that the *Dll4* gene encodes the predominant Notch ligand for early vascular development, that vascular remodeling in the mouse embryo is sensitive to the *Dll4* gene dosage, and that Notch activation in endothelial cells is required for embryonic vascular remodeling.

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Results and Discussion

Haploinsufficient lethality of Dll4$^{+/-}$ *embryos*

Previous gene-expression analysis suggested that the *Dll4* gene encoded the Notch ligand most likely signaling to the NOTCH1 and NOTCH4 receptors during early vascular development in mice (Krebs et al. 2000; Shutter et al. 2000). To analyze whether the *Dll4* gene is required for vascular development, we created a *Dll4* deletion allele by gene targeting (Supplementary Fig. 1). Three independent *Dll4*$^{+/-}$ embryonic stem (ES) cell clones were generated and were used to make chimeric mice, which then were mated to outbred Black Swiss or inbred C57BL/6J mice. Despite germ-line transmission of the ES cell genome (assessed by inheritance of agouti coat color), only a single viable *Dll4*$^{+/-}$ heterozygote, from a mating to Black Swiss mice, was obtained in 180 adult agouti progeny. This included 115 progeny in California from matings to Black Swiss mice and 65 progeny in Bar Harbor from matings to C57BL/6J mice. Normally, 50% of the agouti progeny (~90 mice) should be *Dll4*$^{+/-}$ heterozygotes. Although the single *Dll4*$^{+/-}$ heterozygous male mouse was runted, it was at least partially fertile, yielding 12 wild-type but no *Dll4*$^{+/-}$ heterozygous progeny in matings to Black Swiss females. This pattern of transmission is suggestive of haploinsufficient lethality (Carmeliet et al. 1996; Ferrara et al. 1996), so we isolated embryos at different gestational ages from matings of the chimeras to wild-type C57BL/6J mice. At embryonic days 9.5 and 10.5 (E9.5 and E10.5), *Dll4*$^{+/-}$ embryos exhibited characteristic vascular remodeling defects, such as a mottled avascular yolk sac, growth retardation, and pericardial effusions (Supplementary Fig. 2). Staining of endothelial cells with anti-PECAM-1 antibody revealed a complete absence of vascular remodeling in the *Dll4*$^{+/-}$ yolk sacs at E9.5, similar to what we observed previously

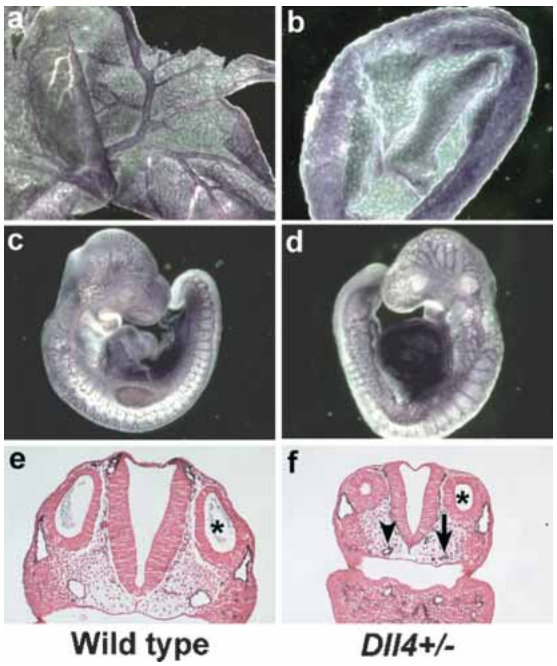

Figure 1. Vascular defects in *Dll4*$^{+/-}$ embryos. PECAM-1-stained yolk sacs (*a*,*b*) and embryos (*c*,*d*) at E9.5. (*b*) The *Dll4*$^{+/-}$ mutant yolk sac has failed to remodel the primary vascular plexus to form the large vitelline blood vessels. (*d*) The vascular network in the *Dll4*$^{+/-}$ embryo appears less intricate and more primitive than the capillary network of the control littermate. (*e*,*f*) Histological sections of PECAM-1-stained E9.5 embryos at the level of the otic vesicle (asterisk). (*f*) The dorsal aorta of this *Dll4*$^{+/-}$ embryo is reduced in diameter (arrowhead) on one side and is atretic (i.e., contains no lumen) on the other side (arrow).

in *Notch1*$^{-/-}$ mutant and *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ double mutant yolk sacs (Krebs et al. 2000). The *Dll4*$^{+/-}$ mutants failed to remodel the yolk sac primary vascular plexus to form the large and small blood vessels of the mature yolk sac (Fig. 1a,b). In *Dll4*$^{+/-}$ embryos (Fig. 1d), the capillary network was less extensive and more primitive than the capillary network of wild-type control littermates. Some vessels in the *Dll4*$^{+/-}$ embryos were reduced in diameter or were atretic (Fig. 1d,f). In contrast to the yolk sacs, vascular remodeling defects in *Dll4*$^{+/-}$ embryos in general were less severe than those observed in *Notch1*$^{-/-}$ and *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ mutant embryos. This may reflect the fact that the *Dll4*$^{+/-}$ embryos retain a functional copy of the *Dll4* gene, but may also reflect a differential sensitivity to *Dll4* gene dosage between the yolk sac and embryo, or a role for other Notch ligands such as JAG1 (Xue et al. 1999) or DLL1 (Hrábe de Angelis et al. 1997) during vascular development in the embryo.

Vascular remodeling defects in Rbpsuh$^{-/-}$ *embryos*

The RBP-J protein, encoded by the *Rbpsuh* gene, is the primary transcriptional mediator of the Notch signal (Kato et al. 1997; for reviews, see Gridley 2003; Lai 2004; Schweisguth 2004). Although the vascular defects present in embryos mutant for Notch family receptors (Krebs et al. 2000), ligands (Hrábe de Angelis et al. 1997; Xue et al. 1999), and downstream effectors (Fischer et al. 2004) have been described, vascular defects of *Rbpsuh*-null mutant embryos have not been reported. The vascular defects of *Rbpsuh*$^{-/-}$ embryos (Fig. 2b,d) were more severe than those of *Dll4*$^{+/-}$ embryos and were similar to defects observed previously in *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ double mutant embryos (Krebs et al. 2000). Histological analysis of the placentas of the *Rbpsuh*$^{-/-}$ embryos also revealed that, similar to *Notch1*$^{-/-}$ *Notch4*$^{-/-}$ embryos (Krebs et al. 2000), *Rbpsuh*$^{-/-}$ mutant blood vessels did not efficiently penetrate the labyrinthine layer of the placenta. However, the histological analysis also suggested that there may be a reduction of the spongiotrophoblast layer

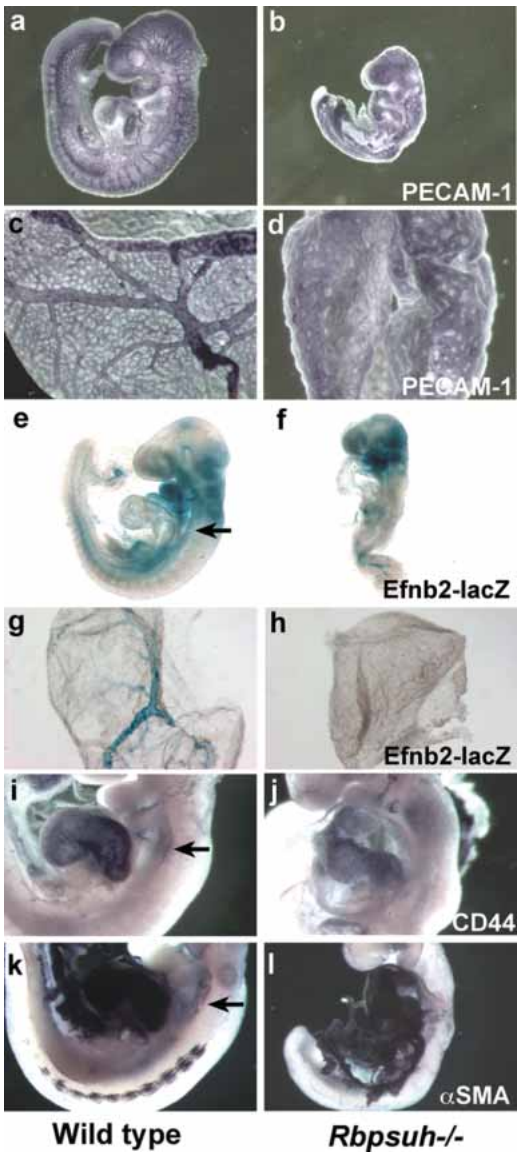

Figure 2. Vascular defects and loss of arterial marker expression in *Rbpsuh*$^{-/-}$ embryos. (*a*–*d*) PECAM-1-stained E9.5 embryos and yolk sacs. (*b*) The *Rbpsuh*$^{-/-}$ embryo exhibits severe growth retardation and a very primitive vascular network. (*d*) The *Rbpsuh*$^{-/-}$ yolk sac exhibits no vascular remodeling. (*e*–*h*) *Efnb2-tau-lacZ* expression in E9.5 embryos and yolk sacs. In the wild-type embryo, *Efnb2-tau-lacZ* is expressed in the dorsal aorta (arrow) and intersomitic arteries of the embryo (*e*) and the vitelline arteries of the yolk sac (*g*). Expression is also observed in somites, nephrogenic mesoderm, branchial arches and hindbrain. Arterial *Efnb2-tau-lacZ* expression is lost in the *Rbpsuh*$^{-/-}$ embryo (*f*) and yolk sac (*h*). (*i*–*l*) Whole mount immunohistochemistry of E9.5 embryos. CD44 (*i*,*j*) and αSMA (*k*,*l*) expression in the dorsal aorta (arrow) of wild-type embryos is downregulated in *Rbpsuh*$^{-/-}$ embryos.

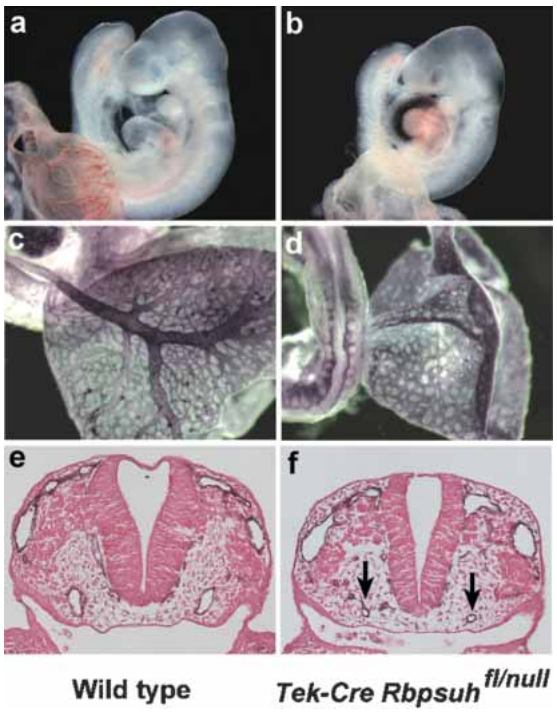

Figure 3. Notch signal activation is required in endothelial cells. The *Rbpsuh* gene was conditionally inactivated in the endothelial cell lineage by crossing mice containing a floxed *Rbpsuh* allele to mice expressing Cre recombinase under control of the *Tek* promoter. (*a*,*b*) Whole mounts of E9.5 embryos. (*b*) The *Tek-Cre Rbpsuh*$^{fl/null}$ embryo exhibits growth retardation and pericardial effusion. (*c*,*d*) PECAM-1-stained E9.5 embryos and yolk sacs. (*d*) The *Tek-Cre Rbpsuh*$^{fl/null}$ mutant yolk sac has failed to remodel the primary vascular plexus to form large vitelline blood vessels. (*e*,*f*) Sections of PECAM-1-stained E9.5 embryos. The dorsal aortae (arrows) of the *Tek-Cre Rbpsuh*$^{fl/null}$ mutant are reduced in diameter.

of the placenta in the *Rbpsuh*$^{-/-}$ embryos (data not shown). This may be due to a role for Notch signaling during placental development that is independent of its role in the vasculature, as previous work has shown that the *Notch2* gene is expressed in the spongiotrophoblast and trophoblast giant cell layers of the developing placenta (Nakayama et al. 1997). In addition to the observed defects in vascular remodeling, *Rbpsuh*$^{-/-}$ embryos exhibit multiple additional defects that have been described previously. These include defects in somite formation (del Barco Barrantes et al. 1999) and in heart looping morphogenesis, caused by Notch pathway regulation of left–right asymmetry determination (Krebs et al. 2003; Raya et al. 2003).

Loss of arterial marker expression in Rbpsuh$^{-/-}$ *embryos*

Recent studies have revealed that endothelial tubes are specified as arteries or veins at the earliest stages of vessel formation, prior to the onset of blood flow (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999; for reviews, see Lawson and Weinstein 2002; Rossant and Howard 2002; Shawber and Kitajewski 2004). In addition, studies in zebrafish have indicated that Notch signaling regulates arterial–venous specification (Lawson et al. 2001, 2002; Zhong et al. 2001). We therefore examined expression of several arterial markers in *Rbpsuh*$^{-/-}$ embryos. EphrinB2 (*Efnb2*), a ligand for the EphB family of tyrosine kinase receptors, is one of the earliest arterial-specific endothelial cell markers. To most sensitively examine *Efnb2* expression, we crossed an *Efnb2-tau-lacZ* knockin allele (Wang et al. 1998) into the *Rbpsuh* null background. In *Rbpsuh*$^{+/+}$ or *Rbpsuh*$^{+/-}$ embryos heterozygous for the *Efnb2-tau-lacZ* allele, whole mount staining for β-galactosidase enzymatic activity revealed *Efnb2-tau-lacZ* expression in arterial endothelium of embryos and their yolk sacs, as well as in the somites, nephrogenic mesoderm, and hindbrain (Fig. 2e,g). In *Rbpsuh*$^{-/-}$ embryos heterozygous for the *Efnb2-tau-lacZ* allele, β-galactosidase expression in the arterial endothelium was lost whereas expression in all other tissues was retained (Fig. 2f,h). CD44 protein expression in the dorsal aorta (Wheatley et al. 1993), another arterial marker, was down-regulated in *Rbpsuh*$^{-/-}$ embryos (Fig. 2j). Recruitment of pericytes and smooth muscle cells to support the walls of nascent blood vessels occurs earlier in arteries than in veins. We therefore examined expression of α smooth muscle actin (αSMA) around the dorsal aorta in *Rbpsuh*$^{-/-}$ embryos and found that αSMA protein expression was down-regulated (Fig. 2l). Examination of the expression of these arterial markers indicates that, as in zebrafish (Lawson et al. 2001, 2002; Zhong et al. 2001), loss of Notch signaling leads to loss of arterial specification of the forming blood vessels.

Notch activation is essential in the endothelial cell lineage

Gain-of-function experiments expressing an activated form of the NOTCH4 receptor have shown that constitutive activation of Notch signaling in the endothelial cell lineage leads to embryonic vascular defects (Uyttendaele et al. 2001). However, a requirement for Notch signaling in endothelial cells has not been demonstrated by loss-of-function analysis. To test this, we deleted the *Rbpsuh* gene in endothelial cells by crossing mice con-

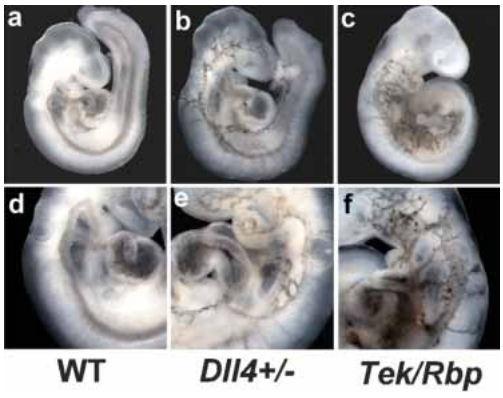

Figure 4. Arteriovenous malformations in Notch pathway mutant embryos. India ink was injected into the proximal outflow tract of the heart in order to visualize blood flow and arteriovenous malformations. (*a*,*d*) In wild-type embryos, ink injected into the heart exited through the branchial arch arteries, entered the paired dorsal aortae, and traversed the entire length of the embryo. In *Dll4*$^{+/-}$ (*b*,*e*) and *Tek-Cre Rbpsuh*$^{fl/null}$ (*c*,*f*) embryos, injected ink exited the distal outflow tract, then entered the venous circulation via small-diameter anastamoses with the anterior cardinal vein. The *Dll4*$^{+/-}$ and *Tek-Cre Rbpsuh*$^{fl/null}$ embryos shown in *b* and *c* are different embryos than those shown in *e* and *f*. (*a–c*) Low-magnification views. (*d–f*) High-magnification views.

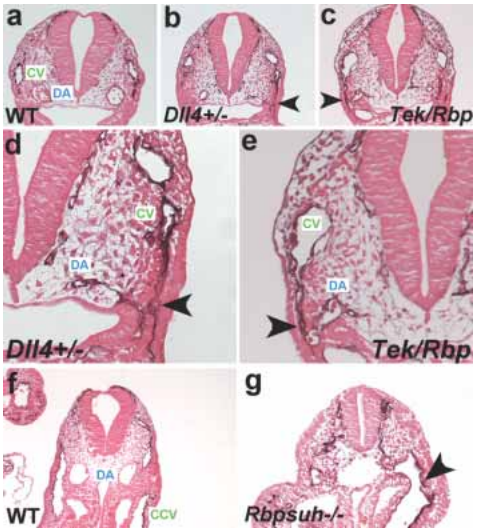

Figure 5. Histological analysis of arteriovenous malformations in Notch pathway mutants. (*a–c*) Sections of wild-type, *Dll4*$^{+/-}$ and *Tek-Cre Rbpsuh*$^{fl/null}$ PECAM-1-stained embryos at E9.5. (*d,e*) Higher-magnification views of the embryos in *b* and *c*. In each mutant, an arteriovenous malformation (arrowhead) consisting of a small-diameter anastomosis connecting the cardinal vein (CV) with the dorsal aorta (DA) can be observed. (*f,g*) Sections of wild-type and *Rbpsuh*$^{-/-}$ PECAM-1-stained embryos at E9.5. (*g*) The mutant embryo exhibits an arteriovenous malformation (arrowhead) caused by the fusion of the dorsal aorta with the common cardinal vein (CCV).

taining a floxed *Rbpsuh* allele (*Rbpsuh*^{fl}) (Tanigaki et al. 2002) to mice expressing Cre recombinase under control of the *Tek* (also known as *Tie-2*) promoter (*Tek-Cre*) (Koni et al. 2001). *Tek-Cre Rbpsuh*$^{fl/null}$ embryos isolated at E9.5 exhibited vascular remodeling defects similar to those observed in Notch pathway mutants such as *Notch1*$^{-/-}$, *Notch1*$^{-/-}$ *Notch4*$^{-/-}$, and *Dll4*$^{+/-}$ embryos. The *Tek-Cre Rbpsuh*$^{fl/null}$ mutant embryos exhibited an avascular yolk sac, growth retardation, and pericardial effusion (Fig. 3b). PECAM-1 antibody staining revealed a complete absence of vascular remodeling in the yolk sac (Fig. 3d), and histological analysis of the *Tek-Cre Rbpsuh*$^{fl/null}$ mutant embryos showed reductions in diameter of some vessels, such as the dorsal aortae (Fig. 3f). These data demonstrate that Notch pathway activation in the endothelial cell lineage is essential for embryonic vascular development. Taken together, the vascular defects exhibited by the Notch receptor gain-of-function mutant embryos (Uyttendaele et al. 2001) and the haploinsufficient lethality and vascular remodeling defects observed in *Dll4*$^{+/-}$ embryos indicate that the levels of Notch activation in endothelial precursors of the forming vasculature must be precisely regulated for normal vascular development.

Formation of arteriovenous malformations in Dll4$^{+/-}$ *and* Tek-Cre Rbpsuh$^{fl/null}$ *embryos*

Reduction of Notch signaling in zebrafish embryos results in formation of arteriovenous malformations (AVMs) (Lawson et al. 2001, 2002; Zhong et al. 2001), the fusion of arteries and veins without an intervening capillary bed. We examined *Dll4*$^{+/-}$ and *Tek-Cre Rbpsuh*$^{fl/null}$ embryos for the presence of AVMs by ink injection into the hearts of E9.5 embryos. All mutant embryos exhibited the presence of AVMs. In wild-type embryos (n = 40), ink injected into the proximal outflow tract of the heart exited through the branchial arch arteries, entered the paired dorsal aortae, and traversed the entire length of the embryo (Fig. 4a,d). In *Dll4*$^{+/-}$ (n = 5) (Fig. 4b,e) and *Tek-Cre Rbpsuh*$^{fl/null}$ (n = 8) (Fig. 4c,f) embryos, injected ink exited the distal outflow tract, then entered the venous circulation via small-diameter anastamoses with the anterior cardinal vein. Histological analysis of *Dll4*$^{+/-}$ (Fig. 5b,d) and *Tek-Cre Rbpsuh*$^{fl/null}$ (Fig. 5c,e) embryos confirmed the presence of AVMs. Some *Dll4*$^{+/-}$ and *Tek-Cre Rbpsuh*$^{fl/null}$ embryos also exhibited AVMs more caudally that were caused by fusion of the dorsal aorta with the common cardinal vein. Although the small size and multiple defects present in *Rbpsuh*$^{-/-}$ embryos prevented successful intracardiac ink injections, histological analysis of *Rbpsuh*$^{-/-}$ embryos revealed the presence of AVMs similar to those observed in *Dll4*$^{+/-}$ and *Tek-Cre Rbpsuh*$^{fl/null}$ embryos (Fig. 5g).

In summary, our results demonstrate that, as was suggested by our previous gene expression studies (Krebs et al. 2000; Shutter et al. 2000), the *Dll4* gene encodes the predominant Notch ligand during early vascular development in mice. Further, vascular remodeling in the mouse embryo is sensitive to *Dll4* gene dosage, as *Dll4*$^{+/-}$ embryos exhibit haploinsufficient lethality. Notch signaling has been shown to be downstream of VEGF-A signaling in both zebrafish (Lawson et al. 2002) and mammalian endothelial cells in culture (Liu et al. 2003). Strikingly, mouse embryos heterozygous for a targeted null mutation of the *Vegfa* gene also exhibit haploinsufficient lethality (Carmeliet et al. 1996; Ferrara et al. 1996), suggesting that the *Dll4* gene may be a target for regulation by VEGF-A and may be at least part of the mechanism causing the haploinsufficient lethality exhibited by *Vegfa*$^{+/-}$ mouse embryos.

This work also demonstrates that, as in zebrafish, loss of Notch activation in mice leads to defects in arterial specification of endothelial cells forming nascent blood vessels. This finding is supported by recent work demonstrating that expression of arterial markers is downregulated in mouse embryos doubly homozygous for mutations of the *Hey1* and *Hey2* genes (Fischer et al. 2004), which encode basic helix–loop–helix (bHLH) transcription factors that are downstream Notch pathway effectors. As an apparent consequence of the loss of arterial–venous specification, Notch pathway mutant embryos form arterial–venous vascular shunts, or AVMs. Similar AVMs have been observed in Notch pathway mutants in zebrafish (Lawson et al. 2001), as well as in mouse mutants in the Transforming Growth Factor β (TGFβ) pathway components endoglin (*Eng*) and activin receptor-like kinase I (*Acvrl1*) (Sorensen et al. 2003). These AVMs appear to arise as a consequence of an inability to establish and maintain distinct arterial–venous vascular beds. Future work will examine whether there is any cross-talk between the Notch pathway and the TGFβ pathway, or between these pathways and other signaling pathways involved in arterial–venous specification, such as the ephrin/Eph receptor pathway.

Materials and methods

Dll4 *gene targeting and genotyping*

The *Dll4* targeting vector (*Dll4*^{tl}) contained a 4.9-kb genomic fragment subcloned upstream of a PGK-*neo* expression cassette, and a 3.2-kb fragment subcloned downstream. This resulted in the deletion of part of exon 1 and exons 2–6 of the *Dll4* gene, including the predicted translation

initiation codon and the exons encoding the DSL domain of the DLL4 protein. A thymidine kinase cassette was introduced for negative selection. The targeting vector was electroporated into 129x1/SvJ-derived Gsi-1 ES cells (Genome Systems). *Dll4* chimeras were crossed to outbred Black Swiss (Taconic) or inbred C57BL/6J (Jackson Laboratory) mice. Yolk sacs or progeny were genotyped for the $Dll4^{d1}$ allele using the oligonucleotide primers CGACATCCCTAACAAGCAG and TCGCCTTC TATCGCCTTCTTG. The $Rbpsuh^{fl}$ allele was genotyped by Southern blotting as previously described (Tanigaki et al. 2002). The *Rbpsuh* null allele was genotyped using primers TAGACCTTGGTTTGTTGTTTGG and CCATAGGAAAACATCCACAGC, which span the deleted region. *Tek-Cre* mice (Koni et al. 2001) were obtained from the Jackson Laboratory and were genotyped with the primers TGATGAGGTTCG CAAGAACC and CCATGAGTGAACGAACCTGG. *Efnb2-tau-lacZ* mutant mice were genotyped as previously described (Wang et al. 1998).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Krebs et al. 2000). Primary antibodies included rat monoclonal anti-PECAM-1 (BD Biosciences Pharmingen), rat monoclonal anti-CD44 (BD Biosciences Pharmingen), and mouse monoclonal anti-α smooth muscle actin (clone 1A4; Sigma). Horseradish peroxidase-coupled secondary antibodies were from Jackson ImmunoResearch.

Cardiac ink injections

Embryos from timed mating were dissected in phosphate buffered saline (PBS) and immediately injected into the proximal outflow tract of the heart with 1% India ink in PBT (PBS with 0.1% Tween 20). Glass injection needles were made using a Model P-97 Flaming/Brown Micropipette Puller (Sutter Instruments). Embryos were fixed in 4% paraformaldehyde and photographed. For sectioning, embryos were dehydrated, embedded in paraffin, sectioned, and were counterstained with eosin.

Acknowledgments

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