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Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinB2 and EphB4 mutants

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Summary

Notch signaling is essential for embryonic vascular development in mammals and other vertebrates. Here we show that mouse embryos with conditional activation of the *Notch1* gene in endothelial cells (*Notch1* gain of function embryos) exhibit defects in vascular remodeling, increased diameter of the dorsal aortae, and form arteriovenous malformations. Conversely, embryos with either constitutive or endothelial cell-specific *Notch1* gene deletion also have vascular defects, but exhibit decreased diameter of the dorsal aortae and form arteriovenous malformations distinctly different from the *Notch1* gain of function mutants. Surprisingly, embryos homozygous for mutations of the ephrinB/EphB pathway genes *Efnb2* and *Ephb4* exhibit vascular defects and arteriovenous malformations that phenocopy the *Notch1* gain of function mutants. These results suggest that formation of arteriovenous malformations in *Notch1* gain of function mutants and ephrinB/EphB pathway loss of function mutant embryos occurs by different mechanisms.

Keywords

angiogenesis; arteriovenous malformation; EphrinB2; EphB4; Notch signaling pathway; vascular morphogenesis

The Notch signaling pathway is an evolutionarily-conserved intercellular signaling mechanism, and mutations in Notch pathway components disrupt embryonic development in diverse multicellular organisms (Bray, 2006; Ehebauer *et al.*, 2006). In mammals, four genes encode Notch family receptors (*Notch1-Notch4*), while five genes encode Notch ligands (*Jag1*, *Jag2*, *Dll1*, *Dll3*, *Dll4*). The signal induced by ligand binding is transmitted intracellularly by a process involving proteolytic cleavage of the receptor, followed by nuclear translocation of the intracellular domain of the Notch family protein (Notch-IC). Once in the nucleus, Notch-IC forms a complex with the RBPJ protein, a sequence-specific DNA binding protein that is the primary transcriptional mediator of Notch signaling. The Notch-IC/RBPJ complex then activates transcription of downstream target genes.

The Notch pathway is one of several conserved signaling pathways whose function is critically important for embryonic vascular development in mammals and other vertebrates (Gridley, 2007; Hofmann and Iruela-Arispe, 2007; Phng and Gerhardt, 2009; Roca and Adams, 2007). For example, mouse embryos with endothelial cell-specific deletion of the *Rbpj* gene (Krebs *et al.*, 2004) or with loss of function mutations of the *Dll4* gene (Duarte *et al.*, 2004; Gale *et*

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al., 2004; Krebs *et al.*, 2004) exhibit vascular remodeling defects that are accompanied by the presence of arteriovenous malformations, the aberrant fusion of arteries and veins without an intervening capillary bed.

The ephrinB/EphB receptor pathway is also essential for vascular development. While loss of function mutants of the ephrinB2 (*Efnb2*) (Wang *et al.*, 1998) and EphB4 (*Ephb4*) (Gerety *et al.*, 1999) genes exhibit embryonic vascular remodeling defects very similar to those exhibited by Notch pathway mutants, it had not been determined whether these ephrin pathway mutants form arteriovenous malformations. Similarly, it had been shown that ectopic activation of the *Notch4* gene (*Notch4*^{GOF}, for *Notch4* gain of function) in endothelial cells and their progenitors leads to embryonic vascular defects (Uyttendaele *et al.*, 2001). However, it had not been established whether ectopic activation of other Notch pathway receptors such as *Notch1* could lead to similar vascular remodeling defects. Here we report that ectopic *Notch1* activation in endothelial cells (*Notch1*^{GOF}) leads to embryonic vascular remodeling defects and arteriovenous malformations that are distinct from those exhibited by Notch pathway loss of function mutants. We also show that *Efnb2* and *Ephb4* loss of function mutants form arteriovenous malformations, and that these arteriovenous malformations phenocopy the arteriovenous malformations present in *Notch1*^{GOF} embryos.

Expression of the intracellular domain of the NOTCH4 protein in endothelial cell precursors under the control of the *Vegfr2* promoter has previously been shown to cause lethal embryonic vascular remodeling defects (Uyttendaele *et al.*, 2001). To determine whether endothelial cell-specific activation of the *Notch1* gene would cause similar vascular defects, we crossed *Tek-Cre* (also known as *Tie2-Cre*) mice to *Notch1-IC*^{cond} mice, a transgenic mouse line in which expression of the NOTCH1 intracellular domain (NOTCH1-IC) is activated by Cre recombinase. We compared the *Tek-Cre; Notch1-IC*^{cond} (referred to hereafter as *Notch1*^{GOF}, for *Notch1* gain of function) embryos to mutant embryos with either constitutive (*Notch1*$^{-/-}$) or endothelial cell-specific (*Tek-Cre; Notch1*$^{flox/-}$) loss of function mutations in the *Notch1* gene.

Notch1^{GOF} embryos exhibited phenotypes characteristic of defective vascular remodeling. Visualization of the vascular network by whole mount immunostaining with a monoclonal antibody to platelet endothelial cell adhesion molecule-1 (PECAM-1) demonstrated that, similarly to *Notch1*$^{-/-}$ (Figure 1b, f), *Tek-Cre; Notch1*$^{flox/-}$ (Figure 1c, g), and other Notch pathway loss of function mutant embryos (Krebs *et al.*, 2004; Krebs *et al.*, 2000), *Notch1*^{GOF} embryos (Figure 1d, h) exhibited vascular defects in the embryo proper and failed to remodel the primary vascular plexus of the extraembryonic yolk sac to form the large and small vessels of the mature wild type yolk sac (Figure1 a, e). Histological analyses of the PECAM-1-stained embryos demonstrated that while the paired dorsal aortae were reduced in diameter or atretic in the two *Notch1* loss of function mutant embryos (Figure 1j, k), the diameter of the dorsal aortae was increased in *Notch1*^{GOF} embryos (Figure1l).

We have demonstrated previously that Notch pathway loss of function mutants, such as *Dll4*$^{+/-}$ embryos or embryos with endothelial cell-specific deletion of the *Rbpj* gene, form arteriovenous malformations (AVMs) (Krebs *et al.*, 2004). We examined *Notch1*^{GOF} embryos for the presence of AVMs by intracardiac ink injection at E9.5, and compared them to wild type embryos and embryos with loss of function *Notch1* mutations. In wild type control embryos, ink injected into the proximal outflow tract of the heart entered the aortic sac and exited through the paired branchial arch arteries. The ink then entered the paired dorsal aortae and traversed the entire length of the embryo to exit through the umbilical artery (Figure 2a, Supporting Information Movie S1). All *Notch1*^{GOF} mutant embryos exhibited the presence of AVMs. In *Notch1*^{GOF} embryos (Figure 2d), injected ink exited the distal outflow tract via the aortic sac. Ink then entered an enlarged branchial arch artery, entered the descending dorsal

aorta, and was shunted back into the heart via fusion of the dorsal aorta with the common cardinal vein (Figure 2d, Supporting Information Movie S2). By contrast, embryos with either constitutive (*Notch1*$^{-/-}$) or conditional (*Tek-Cre; Notch1*$^{flox/-}$) loss of function mutations in the *Notch1* gene exhibit AVMs of a distinctly different type. In these embryos, ink injected into the proximal outflow tract of the heart exited via the aortic sac, then entered the venous circulation in the head of the embryo via small diameter anastamoses with the anterior cardinal vein (Figure 2b, c, Supporting Information Movie S3). The AVMs exhibited by *Notch1*$^{-/-}$ and *Tek-Cre; Notch1*$^{flox/-}$ embryos were identical to those we had observed previously in other Notch pathway loss of function mutants, such as *Dll4*$^{+/-}$ and *Tek-Cre; Rbpj*$^{flox/-}$ embryos (Krebs *et al.*, 2004). Both the loss of function (Figure 2f, g) and gain of function (Figure 2h) *Notch1* mutant embryos had an AVM caudal to the heart caused by fusion of the dorsal aorta with the common cardinal vein, shunting blood back into the heart through the sinus venosus. We have observed this type of AVM previously in another Notch pathway loss of function mutant, the *Rbpj*$^{-/-}$ embryo (Krebs *et al.*, 2004). However, the distinct type of AVM in the anterior region of the embryo clearly distinguishes the Notch pathway gain of function mutants from the Notch loss of function mutants. While this work was in progress, two other groups also characterized vascular defects in different loss or gain of function Notch pathway mutants (Benedito *et al.*, 2008; Kim *et al.*, 2008; Trindade *et al.*, 2008). Our results are concordant with the results of these other groups.

Prior work had demonstrated that mouse embryos homozygous for null mutations of the ephrinB/EphB pathway genes *Efnb2* or *Ephb4* died by E9.5 from vascular defects (Gerety *et al.*, 1999; Wang *et al.*, 1998). We compared in detail the vascular defects exhibited by *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ embryos with the defects exhibited by *Notch1*^{GOF}, *Notch1*$^{-/-}$, and *Tek-Cre; Notch1*$^{flox/-}$ mutant embryos. Previous studies had demonstrated that the *Efnb2* gene is a direct Notch target whose expression is activated by Notch signal reception (Grego-Bessa *et al.*, 2007). We therefore expected that, if ephrinB2/EphB4-mediated signaling functions downstream of Notch signaling during embryonic vascular development in mice, the mutant phenotypes of the *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ embryos would resemble the vascular phenotypes exhibited by Notch pathway loss of function mutants. To our surprise, the phenotypes of the *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mutants were virtually identical to the vascular phenotype exhibited by *Notch1*^{GOF} embryos. *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mutant embryos exhibited vascular remodeling defects in the embryo proper (Figure 3a, b), and did not remodel the yolk sac vascular plexus (Figure 3e, f). *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ embryos were examined for AVMs by intracardiac ink injection at E9.5, and all *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mutant embryos exhibited AVMs of the same type exhibited by *Notch1*^{GOF} embryos (Figure 3c, d, Supporting Information Movie S4). Histological analyses of PECAM-1-stained embryos revealed that, similarly to *Notch1*^{GOF} embryos, *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ embryos exhibited dilated dorsal aortae (not shown) and the caudal AVM resulting from fusion of the dorsal aorta with the common cardinal vein (arrows in Figure 3g, h).

The work described here, as well as that of others (Benedito *et al.*, 2008; Kim *et al.*, 2008; Trindade *et al.*, 2008), demonstrates that both loss and gain of function mutations of multiple Notch pathway components, as well as loss of function mutations in the ephrinB/EphB pathway genes *Efnb2* or *Ephb4*, result in formation of AVMs in the mutant embryos. Formation of these large AVMs during embryonic development is likely due to an inability of the vascular beds to maintain distinct arterial and venous identities in these mutants. It was a surprising finding that *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ embryos exhibit vascular defects and AVMs similar to those exhibited by Notch pathway gain of function mutants, rather than Notch loss of function mutants. Similar findings were made by Kim and colleagues (Kim et al., 2008). Several studies have demonstrated upregulation of *Efnb2* expression in a Notch signaling-dependent manner (Hainaud *et al.*, 2006; Iso *et al.*, 2006; Masumura *et al.*, 2009; Yamanda *et al.*, 2009). In the context of studying the role of Notch signaling during ventricular chamber development of the

heart, Grego-Bessa and colleagues provided evidence that the *Efnb2* gene is a direct Notch target (Grego-Bessa *et al.*, 2007). Two evolutionarily conserved binding sites for the RBPJ protein, the transcriptional mediator of Notch signaling, are present in introns 1 and 2 of the *Efnb2* gene, and these sites are functionally active in mouse embryos and in a human microvascular endothelial cell line (Grego-Bessa *et al.*, 2007). However, the finding that $Efnb2^{-/-}$ and $Ephb4^{-/-}$ embryos exhibit vascular defects and AVMs similar to those exhibited by Notch pathway gain of function mutants, rather than Notch loss of function mutants, is contrary to the results expected if the ephrinB/EphB pathway simply acts in a linear pathway downstream of Notch signal reception. These data suggest independent mechanisms for formation of AVMs in $Notch1^{GOF}$ embryos and in $Efnb2^{-/-}$ and $Ephb4^{-/-}$ mutant embryos.

Methods

Mutant Mice

Previously described alleles used in these studies include targeted null mutations of the *Notch1* (Swiatek *et al.*, 1994), ephrinB2 (*Efnb2*) (Wang *et al.*, 1998), and EphB4 (*Ephb4*) (Gerety *et al.*, 1999) genes, a conditional null *Notch1* allele (Yang *et al.*, 2004), and the *Tek-Cre* transgenic line (Koni *et al.*, 2001). $Notch1\text{-}IC^{cond}$ mice were supplied by author AVC. For construction *of* $Notch1\text{-}IC^{cond}$ mice, in which expression of the NOTCH1 intracellular domain (NOTCH1-IC) is activated by Cre recombinase, *Notch1-IC* sequences from plasmid p12 Notch1-IC were cloned behind the *loxP*-flanked βgeo/3X SV40pA cassette in the pCALL vector (Lobe *et al.*, 1999). After Cre-mediated excision, *Notch1-IC* expression is driven by the chicken β-actin promoter with a cytomegalovirus enhancer (Niwa *et al.*, 1991). The linearized construct was electroporated into embryonic stem cells, and βgeo-expressing clones were selected in G418. Germline transmission was obtained for two independent clones that behaved similarly. Animal maintenance and experimental procedures were performed in accordance with the NIH Guidelines for Animal Care and Use, and were approved by the Institutional Animal Care and Use Committee of the Jackson Laboratory.

Histology, Immunohistochemistry and Intracardiac Ink Injections

Histological analysis, immunohistochemistry for PECAM (CD31; BD Pharmingen) and intracardiac ink injections were performed as described previously (Krebs *et al.*, 2004; Krebs *et al.*, 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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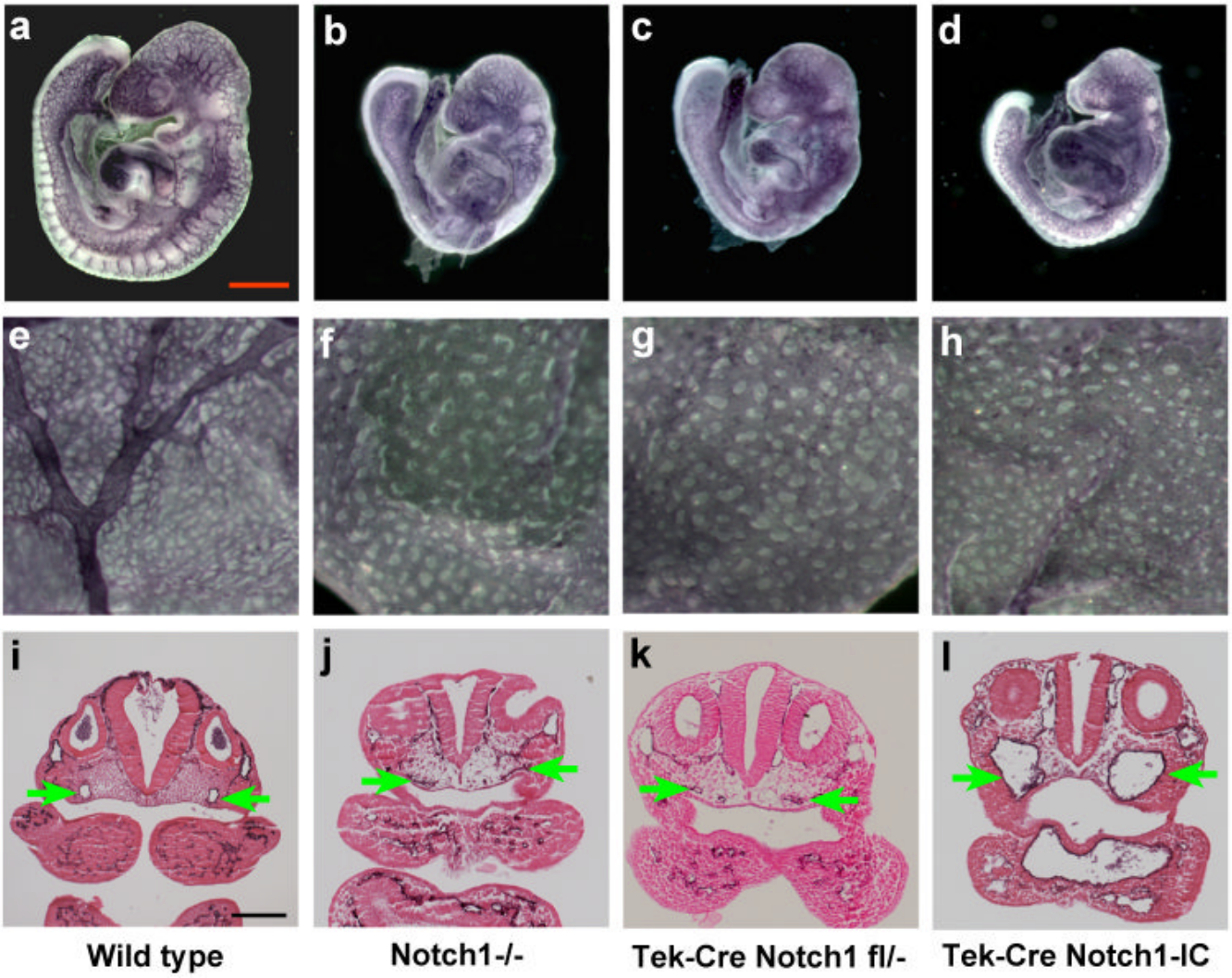

Figure 1. Vascular defects in *Notch1*$^{-/-}$, *Tek-Cre; Notch1*$^{flox/-}$ and *Notch1*^{GOF} mutant embryos

(a-d) PECAM-1 stained embryos. In the Notch pathway loss of function mutant embryos *Notch1*$^{-/-}$ (b), *Tek-Cre; Notch1*$^{flox/-}$ (c), and the *Notch1*^{GOF} (*Tek-Cre; Notch1-IC*) embryo (d), the capillary network appears less intricate and more primitive than that of the wild type control (a). (e-h) PECAM-1 stained yolk sacs. Both the Notch pathway loss of function mutant embryos (f, g) and the *Notch1*^{GOF} embryo (h) have failed to remodel the primary vascular plexus to form the large vitelline blood vessels observed in the wild type embryo (e). (i-l) Histological sections of PECAM-1-stained embryos at the level of the otic vesicle. In the wild type embryo (i), both dorsal aortae (green arrows) have open lumens and normal morphology. In the two Notch pathway loss of function mutant embryos *Notch1*$^{-/-}$ (j) and *Tek-Cre; Notch1*$^{flox/-}$ (k), the dorsal aortae are either reduced in diameter or atretic (i.e., contains no lumen). In contrast, in the *Notch1*^{GOF} embryo (l) the lumens of the dorsal aortae are increased in diameter. All embryos shown are at E9.5.

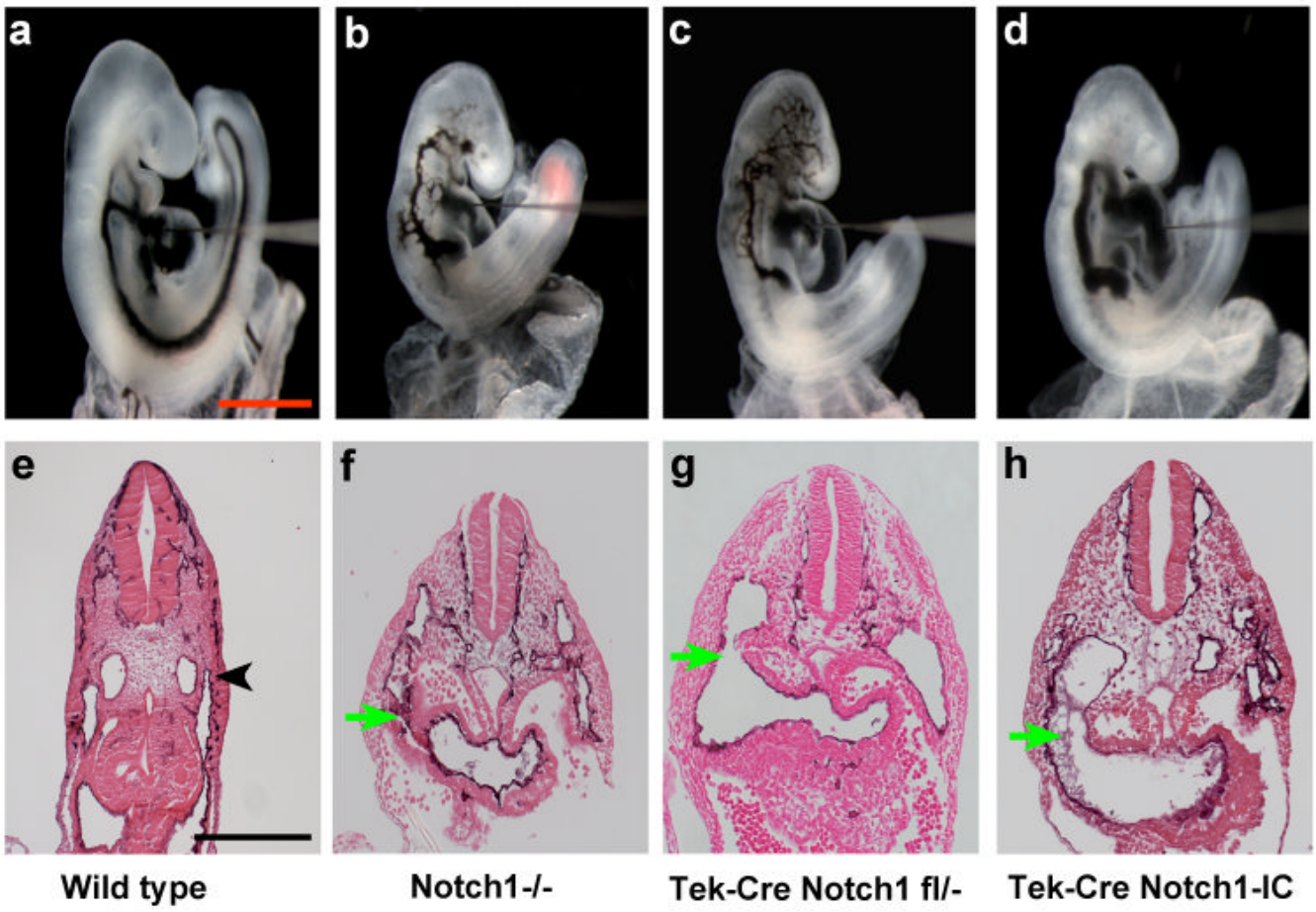

Figure 2. Arteriovenous malformations in *Notch1*^{GOF} mutant embryos are distinct from those of Notch pathway loss of function mutant embryos

(a-d) India ink injected embryos. India ink was injected into the proximal outflow tract of the heart in order to visualize blood flow and arteriovenous malformations. (a) In the wild type embryo, ink injected into the heart exited through the branchial arch arteries, entered the paired dorsal aortae and traversed caudally the entire length of the embryo. (b, c) In the Notch pathway loss of function mutant embryos *Notch1*$^{-/-}$ (b) and *Tek-Cre; Notch1*$^{flox/-}$ (c), injected ink exited the distal outflow tract, then entered the venous circulation in the anterior of the embryo via small diameter anastamoses with the anterior cardinal vein. Caudally, injected ink was shunted back into the heart via fusion of the descending dorsal aorta with the common cardinal vein. (d) In the *Notch1*^{GOF} (*Tek-Cre; Notch1-IC*) embryo, injected ink exited via an enlarged branchial arch artery, entered the descending dorsal aorta, and was shunted back into the heart via fusion of the dorsal aorta with the common cardinal vein. (e-f) Sections of PECAM-1 stained embryos just caudal to the heart. (e) In the wild type embryo, the dorsal aorta and common cardinal vein (black arrowhead) are distinct. (f-h) In both Notch loss of function and gain of function mutant embryos, the dorsal aorta has fused with the common cardinal vein (green arrow), shunting blood back into the heart through the sinus venosus. All embryos shown are at E9.5.

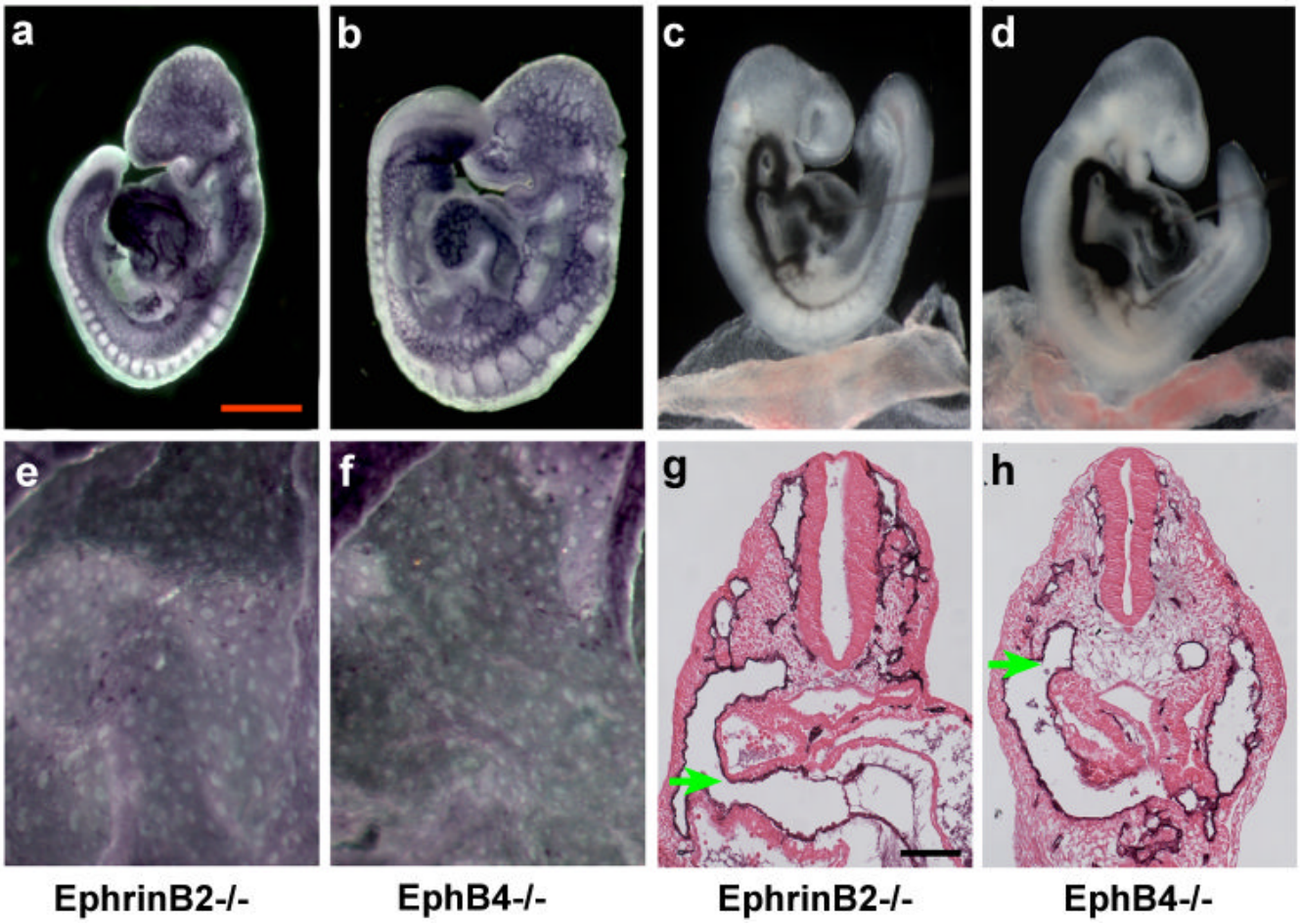

Figure 3. Vascular defects and arteriovenous malformations in *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mutant embryos phenocopy those of *Notch1*^{GOF} embryos

Efnb2$^{-/-}$ (a, c, e, g) and *Ephb4*$^{-/-}$ (b, d, f, h) mutant embryos at E9.5. (a, b) PECAM-1 stained embryos. (c, d) India ink injected embryos revealing arteriovenous malformations. (e, f) PECAM-1 stained yolk sacs exhibiting the absence of vascular remodeling. (g, h) Histological sections of PECAM-1-stained embryos. Vascular defects in both the *Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$ mutant embryos phenocopy those of *Notch1*^{GOF} embryos.