NIH Public Access
Author Manuscript
Development. Author manuscript; available in PMC 2009 May 1.

Published in final edited form as:
Development. 2008 November ; 135(22): 3755–3764. doi:10.1242/dev.022475.

Artery and vein size is balanced by Notch and ephrin-B2/EphB4 during angiogenesis

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Summary

A mutual coordination of size between developing arteries and veins is essential for establishing proper connections between these vessels and, ultimately, a functional vasculature; however, the cellular and molecular regulation of this parity is not understood. Here, we demonstrate that the size of the developing dorsal aorta and cardinal vein is reciprocally balanced. Mouse embryos carrying gain-of-function *Notch* alleles show enlarged aortae and underdeveloped cardinal veins, whereas those with loss-of-function mutations show small aortae and large cardinal veins. *Notch* does not affect the overall number of endothelial cells but balances the proportion of arterial to venous endothelial cells thereby modulating the relative sizes of both vessel types. Loss of *ephrin-B2* or its receptor *EphB4* also leads to enlarged aortae and underdeveloped cardinal veins; however, endothelial cells with venous identity are mislocalized in the aorta, suggesting that ephrin-B2/EphB4 signaling functions distinctly from Notch by sorting arterial and venous endothelial cells into their respective vessels. Our findings provide mechanistic insight into the processes underlying artery and vein size equilibration during angiogenesis.

Keywords

Angiogenesis; Vascular morphogenesis; Notch; Ephrin-B2/EphB4; Mouse embryo; Arterial-venous differentiation

Introduction

Angiogenesis, or new blood vessel growth, is a principal biological process in embryonic development, cancer progression, tissue regeneration, ischemic recovery, and many other physiological and pathological conditions (Carmeliet 2005; Coultas et al. 2005; Folkman 2007). New vessel segments are generated by the well-described process of capillary sprouting from pre-existing vessels. Coordination between the sizes of developing arteries and veins is crucial in establishing an interface between these vessels and for a functional vasculature; however, the cellular and molecular regulation of this parity is unknown (Jones et al. 2006; Gridley 2007). Elucidating the cellular and molecular basis of arterial and venous specification and endothelial cell (EC) distribution would provide a conceptual advance in our understanding of angiogenesis.

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In this study we examined the first artery and vein to develop in the body: the dorsal aorta (DA) and the cardinal vein (CV), respectively. Given that initial DA and CV development involves ECs and not adjacent mural cells, this model provides an experimental system in which to study the role of EC signaling in arteriovenous morphogenesis. The DA emerges prior to the CV, and its morphogenesis begins with the assembly of ECs into the DA primordium, a transient capillary plexus (Sabin 1917; Coffin et al. 1991). Remodeling of this primitive network generates a lumenized vessel, which subsequently matures into the body's major artery. The CV emerges slightly later, at which stage transient capillary channels develop between the DA and CV (Sabin 1917; Gerety and Anderson 2002) (Supplementary Fig. 1), suggesting that the two vessels may interact to establish the proper circulatory system.

The discovery of *ephrin-B2*, a gene encoding a transmembrane signaling molecule specifically expressed in arterial ECs prior to the onset of circulation, unveiled a genetic program of arteriovenous differentiation (Wang et al. 1998; Adams et al. 1999). These studies demonstrate that the ephrin-B2 ligand and its venous-specific EphB4 tyrosine kinase receptor (Wang et al. 1998; Gerety et al. 1999) are important for vascular remodeling of primitive capillary networks into distinct arteries and veins. Despite its distinctive arterial expression, ephrin-B2 does not determine arterial specification in ECs (Wang et al. 1998). The precise cellular mechanism underlying ephrin-B2 function in ECs is unknown. Ephrin/Eph signaling mediates cellular behavior such as repulsion, adhesion, and motility in neuronal, bone, and other tissue types (Klein 2004; Poliakov et al. 2004; Kuijper et al. 2007), raising the possibility that ephrin-B2/EphB4 signaling functions in a similar fashion in ECs. Notch receptors and their ligands are transmembrane proteins that are primarily expressed in arteries and not veins (Villa et al. 2001).

Notch signaling influences bi-potential cell fate decisions through cell-cell communication (Artavanis-Tsakonas et al. 1999). Studies in zebrafish and mice show that Notch activation promotes arterial characteristics in ECs (Lawson et al. 2001; Zhong et al. 2001; Carlson et al. 2005). Gain- and loss-of-function mutations in the Notch pathway lead to abnormal vascular development in mice (Krebs et al. 2000; Uyttendaele et al. 2001; Duarte et al. 2004; Fischer et al. 2004; Gale et al. 2004; Krebs et al. 2004). We have shown that expression of constitutively active *Notch4* in a subset of ECs can cause prompt and massive arteriovenous malformations in adults (Carlson et al. 2005). In addition to its ability to promote arterial characteristics, Notch signaling also restricts capillary sprouting in normal and tumor angiogenesis (Noguera-Troise et al. 2006; Ridgway et al. 2006; Hellstrom et al. 2007; Siekmann and Lawson 2007; Suchting et al. 2007). However, the precise cellular function of Notch signaling in the establishment of arteriovenous distinction remains unknown.

We have combined mouse genetics and in vivo analysis to concurrently examine the effects of these pathways on DA and CV development and have found that the size of the developing DA and CV is coordinated. ECs are distributed between the DA and CV, and both Notch and ephrin-B2/EphB4 signaling pathways are crucial for this coordination during vascular morphogenesis. Notch controls the proportion of ECs in the DA and CV by promoting arterial specification, thereby modulating their respective lumen size. The ephrin-B2/EphB4 signaling pathway segregates arterial and venous ECs into their respective vessel. Our work suggests that the growth of arteries and veins during angiogenesis is inversely coordinated, and that the Notch and ephrin-B2/EphB4 pathways are essential for balanced arteriovenous development during blood vessel formation.

Materials and Methods

Mice

The *Tie2-tTA*, *TRE-int3*, and *TRE-LacZ* transgenic mice have been described (Carlson et al. 2005), as have the *ephrin-B2-tauLacZ* (Wang et al. 1998), *ephrin-B2-H2BGFP* (Davy and Soriano 2006), *EphB4-tauLacZ* (Gerety et al. 1999), *ephrin-B2*$^{flox/flox}$ (Gerety and Anderson 2002), *Notch1*$^{+/-}$ (Conlon et al. 1995), *Notch1*$^{flox/flox}$ (Radtke et al. 1999), and *Tie2-LacZ* (Schlaeger et al. 1997) mice. Embryos were genotyped as described previously (Braren et al. 2006). All animals were treated in accordance with the guidelines of the UCSF Institutional Animal Care and Use Committee.

Immunofluorescence

Immunofluorescence was performed according to a previously described protocol. (Braren et al. 2006). Goat anti-EphB4 (1:50) was from R&D Systems, Inc. (Minneapolis, MN), rabbit anti-βGal (1:200) was from MP Biomedicals (Irvine, CA), and Alexa 488 donkey anti-goat (1:1000) was from Invitrogen (Carlsbad, CA). Cy5 donkey anti-rabbit (1:500) was from Jackson Immunoresearch Laboratories (Baltimore, MD).

EC Counting

To quantitatively assess the distribution of ECs, we counted ECs in serial cross-sections of the trunk region between the otic vesicle and the heart of e8.75 embryos. ECs were identified by CD31 immunofluorescent staining, and total ECs included those in the DA, primordial anterior CV, and capillaries in the vicinity. For *Notch4* gain-of-function analysis, 5 pairs of controls and mutants at 15–16ss were used, and between eight and twelve 10 μm frozen sections per embryos were analyzed. For *Notch1* loss-of-function, 4 pairs of controls and mutants at 12–15ss were used. Depending on the quality of the sections, two, six, nine and ten 10 μm frozen sections per embryo were analyzed. For *ephrin-B2* loss-of-function, 3 pairs of controls and mutants at 15–17ss were used, with thirteen 5 μm paraffin sections per embryo being analyzed. The number of sections analyzed between mutant and somite stage-matched littermate control was equal. The sum of ECs per mutant embryo (Supplementary Table 3) was normalized against that of its control, with controls expressed as 100%. Primordial CV compartment includes all ECs except those in the DA. The ratio of DA and primordial CV ECs was calculated over the total EC number.

Whole-mount LacZ Staining, Histology, and Immunohistochemistry

LacZ staining, tissue embedding, histology, and immunohistochemistry were performed as described (Carpenter et al. 2005), with modifications in fixation duration for LacZ staining: 40 minutes (e9.0), 45 minutes (e9.5), or 2 hours (e12.5) at 4°C. For imaging, e12.5 and e9.5 LacZ-stained embryos were cleared in benzyl alcohol and benzyl benzoate (1:2 ratio) after serial dehydration in 25, 50, 75, and 100% methanol, in 20 minutes intervals. Section positions were identified according to Kaufman (Kaufman 1992).

In situ Hybridization

A 2.7 kb *Dll4* anti-sense probe was used at a final concentration of 1 μg/ml (probe plasmid kindly provided by D. Pleasure). After fixation in 4% PFA, followed by dehydration in methanol and rehydration in PBS, 0.1% Tween-20, e9.0 embryos were digested with 10μg/ml Proteinase K for 3 minutes on ice. AP-conjugated Digoxygenin-labeled RNA probes were prepared according to manufacturer's instructions (Roche, Indianapolis, IN), hybridized at 65°C overnight under stringent conditions (1.3X SSC, 50% formamide, 0.2% Tween-20, 5 mM EDTA, pH 8.0, 50 μg/ml Yeast RNA and 100 μg/ml heparin) and stained with BM purple (Roche). Stained embryos were embedded in paraffin, and cross-sectioned (10 μm).

RT-PCR

Total mRNA was extracted from snap-frozen, pooled e9.5 embryos and yolk sacs using PolyATtract System (Promega, Madison, WI), and reverse-transcribed using oligo dT primers according to the manufacturer's instructions (Superscript III RT, Invitrogen). The *int3* cDNA was amplified with transgene-specific primers, CGGAGGGAAGGTGTATGCTC (sense) and GGGTCCATGGTGATACAAGG (anti-sense), at 60°C annealing temperature. Primer sequences for *gapdh* were AGCTTGTCATCAACGGGAAG (sense), and GGATGCAGGGATGATGTTCT (anti-sense), and for *β-actin* were ATGAAGATCCTGACCGAGCG (sense) and TACTTGCGCTCAGGAGGAGC (anti-sense). For e8.5 embryos and yolk sacs, total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) for cDNA synthesis.

Ink Injection Analysis

Black ink (Staedtler, Nuernberg, Germany) diluted 1:4 in PBS, 0.1% Tween-20, was injected into the outflow tract of e9.5 embryos still attached to the yolk sac, using a micro-needle. Embryos were subsequently fixed in 4% PFA.

In vivo EC Proliferation Assay

Proliferating cells were labeled 2 hours before embryo collection by intra-peritoneal injection of BrdU (Sigma, 100 μg/g body weight) into pregnant females 9 days post-coitum. EC proliferation was detected by double immunofluorescent staining for CD31 and BrdU in frozen cross-sections (10 μm) using a BrdU staining kit (Zymed Laboratories, South San Francisco, CA) in combination with fluorescent secondary antibodies. In each section, DA ECs were counted over an area spanning the otic vesicle to the heart, and the proportion of BrdU-positive ECs calculated.

Statistical Analyses

Data bars represent the mean values and error bars the standard deviation. All cell counts were analyzed using two-tailed *t*-test.

Results

Tet-Regulatable Endothelial-Specific Expression of Constitutively Active *Notch4*

To determine the effect of gain-of-function *Notch* on the developing vasculature, we used transgenic mice, in which a constitutively active form of *Notch4* (*int3*) is expressed specifically in ECs. *Int3* is driven by a Tetracycline (Tet) response element (TRE) (*TRE-int3*), which is activated by a Tet transactivator (tTA) that is, in turn, driven by the EC-specific *Tie2* promoter (*Tie2-tTA*) (Carlson et al. 2005). We verified that tTA was active specifically in the ECs by examining β-galactosidase (β-gal) activity in embryos carrying both *Tie2-tTA* and *TRE-LacZ* reporter genes. β-gal activity was detected specifically in the vasculature of yolk sacs by e9.5 and more strongly at e12.5 (Supplementary Fig. 2A, C), and as shown in embryo cross-sections, was restricted to a subset of the ECs of DA and CV at e9.5 while more uniformly at e12.5 (Supplementary Fig. 2B, D).

We verified *int3* expression by RT-PCR in pooled embryos and yolk sacs using transgene-specific primers that do not amplify the endogenous *Notch4* gene. The *int3* mRNA was detected in the *Tie2-tTA;TRE-int3* mutant at e8.5 (9–12 somite stage (ss)) and e9.5 (22–26ss), at higher than the low, basal level seen in *TRE-int3* tissues, and was not detected in *Tie2-tTA* tissues (Supplementary Fig. 2E). The *Tie2-tTA;TRE-int3* embryos exhibited severe vascular abnormalities by e9.5 and ultimately died by e11.5. Characterization of the gross phenotype of the mutant embryos is described in Supplementary Data (Supplementary tables 1, 2, and

Supplementary Fig. 2F–H). Since no obvious abnormalities were detected in either the *TRE-int3* or *Tie2-tTA* embryos, they, along with the wild-type, were included as controls.

Constitutively Active *Notch* Elicits Enlarged DA and Underdeveloped Anterior CV

To examine the development of DA and CV, we performed CD31 immunostaining and found that the defects first appeared at e9.0 (15–19ss) with larger DA and aortic arch arteries in all mutants compared to the controls (Fig. 1A, B). In addition, the mutant anterior CVs were less elaborate. *Ephrin-B2-tauLacZ* and *EphB4-tauLacZ* reporter assays (Wang et al. 1998; Gerety et al. 1999) verified that *int3* results in an enlarged anterior DA and an underdeveloped CV (Fig. 2A–D). We confirmed with serial cross-sections at e9.5 the enlargement of mutant DA and the underdeveloped mutant CVs displaying a primitive capillary structure lacking the well-defined lumen seen in controls (Fig. 1C, D). The morphological defects in the mutant DA and CV were accompanied by the development of arteriovenous shunting at e9.5, demonstrated by ink injection (Fig. 2F).

To determine if alterations in smooth muscle cell (SMC) recruitment were involved in the DA enlargement, we performed CD31 and smooth muscle α-actin double staining in e9.0 (18ss) embryos (Fig. 2G, H). At this stage, no SMCs were associated with either the control or mutant DA, yet the mutant DA was enlarged (Fig. 2H). This result suggests that enlargement of the DA occurred before, thus independent of, the recruitment of SMCs.

int3 Does not Affect Absolute EC Number

To investigate the cellular mechanism underlying the reciprocal DA and CV size, we tested if increased EC proliferation is associated with the enlarged DA. We performed in vivo BrdU-labeling combined by CD31 staining in embryos at e8.75 (13–15ss), prior to apparent gross mutant abnormalities. CD31-positive and BrdU-positive proliferating ECs were counted in cross-sections of the DA (Fig. 3A). The mutant DA exhibited a 14.5% (± 15.2) increase in EC number compared to controls, indicating the enlargement of DA. However, the number of proliferating ECs was indistinguishable between mutant and control at approximately 12% (± 2.9, control vs. ± 3.2, mutant; Fig. 3B). This result suggests that the increase in DA size was not due to an increase in EC proliferation.

We also tested if EC death was decreased in the mutant but did not detect any apoptotic ECs in either control or mutant DA by TUNEL assay and CD31 staining (data not shown), thus we could not evaluate the effect of *int3* on EC apoptosis directly. We then counted the total ECs, including in the DA, CV and capillaries in the vicinity, from the cross section of anterior e8.75 (15–16ss) embryos labeled by immunofluorescent CD31 staining, and found no significant change in the absolute number of ECs between the mutant and control (0.3% ± 9.4 increase in the mutant over the control; p=0.94, n=5). Because both total EC number and EC proliferation were not significantly affected, these results also suggest that *int3* did not affect EC survival.

int3 Increases the Ratio of Arterial to Venous ECs

To quantitatively assess the distribution of ECs between DA and CVs, we counted ECs in serial cross-sections of the anterior trunk e8.75 (15–16ss). The proportion of DA ECs increased from 34.8% in controls (Fig. 1E) to 49.5% in mutants (p=0.02, n=5; Fig. 1F), reflecting the enlarged mutant DA. Conversely, EC proportion in the mutant CV including capillaries in the vicinity was reduced from 65.2% in controls, to 50.5% in mutants, confirming the underdevelopment of CVs. These data show that *int3* leads to an increase in the number of arterial ECs with a concomitant reduction in the number of venous ECs.

To analyze EC identity, we examined the expression of the arterial markers ephrin-B2-tauLacZ and Dll4 and showed that ephrin-B2- or Dll4-positive ECs were detected in the DA and not

CVs in the control, however, they were ectopically present in the mutant CVs (Fig. 3C–H). Furthermore, we found ephrin-B2-tauLacZ and EphB4-double positive ECs in mutant but not control CVs (Fig. 3E, F). These data show at single cell resolution that *int3* promoted arterial identity, even in venous ECs. Taken together, our results indicate that *int3* leads to an increase in the number of arterial ECs at the expense of venous ECs, thus increasing the allocation of ECs in arteries over veins, without affecting the absolute EC number.

int3 also Elicits Enlarged Arteries and Underdeveloped Veins in the Head

To verify whether the reciprocal changes in arterial and venous size occurred at other locations, we analyzed the development of the head arteries and veins. The optimal time to analyze these vessels is approximately e10.5, when *Tie2-tTA;TRE-int3* embryos were severely retarded from *int3* expression. We thus optimized the timing of *int3* expression by treating the pregnant females with tetracycline in water until day 7.5 of gestation, as we described previously (Carpenter et al. 2005). Embryos were collected at e10.5. Under these conditions, only a subset of mutant embryos was affected (51.2%; 22 out of 43 mutants), and 4 mutants analyzed for head vessels exhibited enlarged internal carotid arteries, which were often accompanied by smaller head veins (Fig. 1G, H). This finding suggests that *int3* can also induce enlarged arteries along with underdeveloped veins in other organs.

The CV Primordium is Expanded, While DA is Smaller in *Notch1*$^{-/-}$ Embryos

It has been previously reported that the *Notch1*$^{-/-}$ DA is smaller than wild type DA (Krebs et al. 2000), and we have confirmed this finding (Supplementary Fig. 3B). To analyze the CV structure in *Notch1*$^{-/-}$ mutants, we stained the embryos for both EphB4 and CD31 at e9.0 (15ss) when the mutant embryos were affected. At this stage, when the control CV was still composed of capillary plexus, the *Notch1*$^{-/-}$ primordial CV was expanded (Fig. 4B arrowheads). This phenotype is reciprocal to that of the *Notch4* gain-of-function mutant.

To quantitatively assess the DA and CV sizes, we counted ECs in serial cross-sections of anterior e8.75 (12–15ss) embryos. Total EC numbers, including those in the DA and primordial CV were comparable in mutants and controls (4.2% ± 11.7 increase in the mutant over the control; p=0.52, n=4). However, the proportion of ECs in the DA was reduced in *Notch1*$^{-/-}$ (21.1%, compared to 47.7% in controls, p=0.0007; Fig. 4C, D). Concomitantly, the proportion of ECs in the CV region was significantly increased. These findings further suggest that the reduced DA size is accompanied by an increase in the CV size in the *Notch1*$^{-/-}$ embryos, reciprocal to that of the *Notch4* gain-of-function mutant.

Determining EC identity, we found that EphB4-positive ECs were exclusively located in the control CV primordium, and not in the DA (Fig. 4A). In contrast, EphB4-positive ECs clustered at the smaller, atretic DA in addition to the CV primordium (Fig. 4B). Quantitative analysis showed that the ratio of EphB4-postive to negative ECs in the DA region (as DAs were small and atretic in the mutant) was increased from 0.01 in the control to 0.15 in *Notch1*$^{-/-}$ (data not shown). As previously demonstrated (Fischer et al. 2004), we observed that the mutant DA ECs were devoid of ephrin-B2 expression (data not shown). In addition, in situ hybridization revealed that the DA ECs express Dll4 in the control but not in *Notch1*$^{-/-}$ embryo (Supplementary Fig. 3C, D). These findings demonstrate that *Notch1*$^{-/-}$ DA may lose arterial identity, but harbor ECs with venous identity.

To determine whether lack of *Notch1* in ECs is responsible for such defects, we used conditional mutants, in which the *Notch1*$^{flox/flox}$ allele (Radtke et al. 1999) was excised in ECs by Cre recombinase under the control of *Tie1* promoter, *Tie1-Cre* (Gustafsson et al. 2001). We have shown that *Tie1-Cre* is active in about 80% ECs and a minority of hematopoietic cells (unpublished data). CD31 staining reveals that these mutant embryos displayed similarly smaller, atretic DAs and enlarged CV primordia, at a similar developmental stage as *Notch1*$^{-/-}$ embryos (Fig. 4F). These results suggest that *Notch1* in ECs is essential for the balanced growth of the DA and the CV. In summary, these data suggest that *Notch* loss- and gain-of-function mutants elicit reciprocal effects balancing DA and CV morphogenesis.

Enlarged DA and Underdeveloped CV in *ephrin-B2*$^{-/-}$ Embryos

The balanced distribution of ECs between the DA and CV led us to hypothesize that a cell sorting mechanism would be involved. The ephrin-B2/EphB4system is known to mark these specific venous and arterial compartments, and has the potential to affect cell sorting. Twenty out of 29 *ephrin-B2*$^{-/-}$ embryos (average 17.2ss) developed enlarged DA on both sides, which were accompanied by reduced CV primordial capillaries (Fig. 5B, F). The remaining 9 mutants, at a later stage (average 19.6ss) with more severe developmental defects, exhibited an enlarged left-anterior DA that was still accompanied by a reduction in number of CV capillaries. But the right-anterior DA was smaller and coincided with an increase in number of CV primordial capillaries (data not shown).

To quantitatively assess the vessel defects in these mutants, we counted ECs in serial cross-sections through the trunk region of e8.75 (15–17ss) embryos. Total EC numbers, including those in the DA, primordial CV, and capillaries in the vicinity, were reduced in *ephrin-B2*$^{-/-}$ mutants by 20% compared to controls (19.5% ± 5.6 decrease in the mutant over the control; p=0.02, n=3), suggesting that loss of *ephrin-B2* may have affected EC proliferation and/or survival. Nevertheless, the proportion of DA ECs increased to 47.9% in mutants (p=0.02, n=3; Fig. 5D) from 31.2% in controls (Fig. 5C). Therefore, *ephrin-B2*$^{-/-}$ embryos with enlarged DA and reduced CVs primarily resemble *Tie2-tTA;TRE-int3* and not *Notch1*$^{-/-}$ embryos.

To determine the arterial-venous identity of the ECs, we stained cross-sections for EphB4 and CD31 and demonstrated that in controls, EphB4-positive cells were present only in the veins at e8.75 (Fig. 5E). In *ephrin-B2*$^{-/-}$ mutants, however, EphB4-positive ECs were also present in the enlarged DA (Fig. 5F). The expression of another arterial marker, Dll4, absent in the *Notch1*$^{-/-}$ mutant, was unchanged in the *ephrin-B2*$^{-/-}$ mutant (data not shown), suggesting that lack of *ephrin-B2* did not affect overall EC identity. This data indicates that venous ECs may mislocalize to the DA when the embryo lacks *ephrin-B2*.

To examine whether *ephrin-B2* in ECs is responsible for DA and CV development, we analyzed EC-specific conditional knockouts, using *Tie1-Cre* lines described above and the *ephrin-B2*$^{flox/flox}$ allele (Gerety and Anderson 2002). The conditional mutant embryos developed similar phenotypes to the *ephrin-B2*$^{-/-}$ embryos at the same stage, suggesting that loss of *ephrin-B2* in ECs is responsible for the vascular defects (Fig. 5H). In summary, these results imply that ephrin-B2 signaling within the ECs is responsible for the coordinated sizes of the developing DA and CV, in a manner similar to but distinct from Notch signaling.

EphB4$^{-/-}$ Embryos Also Exhibit Enlarged DA and Underdeveloped Anterior CV

Because EphB4 is a putative receptor for ephrin-B2, and *EphB4*$^{-/-}$ embryos exhibit similar vascular phenotypes to the *ephrin-B2*$^{-/-}$ mutants (Gerety et al. 1999), we examined the *EphB4*$^{-/-}$ DA and CV. *EphB4*$^{-/-}$ embryos indeed developed enlarged DA along with underdeveloped anterior CV around e9.25 (20ss) (Fig. 6B, D, F). In addition, the enlarged DA harbored ephrin-B2-negative ECs (Fig. 6C, D) and EphB4-positive ECs as judged by *EphB4-tauLacZ* promoter activity (Fig. 6E, F) not seen in the controls. These data demonstrate that *EphB4* deficiency led to similar DA enlargement and CV underdevelopment as with *ephrin-B2* deficiency, and that the enlarged mutant DA contained mislocalized EphB4-positive, ephrin-B2-negative, and thus likely venous, ECs. In summary, our results demonstrate that both *ephrin-B2* and *EphB4* deficiency led to DA enlargement and CV reduction, with the enlarged DA containing mislocalized ECs expressing EphB4. Together, our findings suggest that ephrin-B2/EphB4 signalling is required to sort ECs with differential identities into their respective vessels to coordinate artery and vein sizes.

Discussion

To understand the molecular basis of arterial-venous growth, we conducted concurrent analysis of DA and CV morphogenesis in mouse *Notch*, *ephrin-B2*, and *EphB4* mutants (summarized in Fig. 7A). Our findings lead us to propose that the sizes of the DA and CV are balanced through the reciprocal regulation of vessel growth (Fig. 7B). By promoting arterial differentiation, Notch balances the proportion of arterial to venous ECs without affecting their absolute number, thus regulating both artery and vein size. Ephrin-B2/EphB4 signaling functions distinctly from Notch by sorting differentiated arterial or venous ECs into their respective vessels.

Coordinated Arterial and Venous Growth is Achieved through a Reciprocal Balance

Developing arteries and veins must coordinate both the number and size of their branches to generate a proper circulatory system. The cellular and molecular mechanisms underlying this regulation are poorly understood. One potential mechanism to achieve such equilibrium is interdependent vessel growth. In support of this hypothesis, we have provided quantitative evidence showing that an increase or decrease in DA size leads to a reciprocal change in CV size.

An expansion of the CV region with a concomitant loss of DA segments has been observed in zebrafish. In a subset of zebrafish embryos, inhibition of Notch signaling through high dose antisense constructs targeting the *Notch* downstream gene *gridlock* (*grl*), was shown to increase CV length or region but not lumen size, with loss of DA segments (Zhong et al. 2001). However, this phenotype did not occur in the majority of embryos injected with the high dose construct nor in embryos injected with low dose antisense DNA. In addition, even in the most severe zebrafish *mindbomb* mutant, a putative *Notch* loss-of-function mutant, the DA remained normal, although the expression of arterial markers was diminished (Lawson et al. 2001). Conversely, in gain-of-function mutants, induced by over-expression of *grl* or expression of *Notch ICD*, the size of the DA was not affected, despite increased ephrin-B2 expression (Lawson et al. 2001; Zhong et al. 2001). Therefore, while these earlier studies show that Notch activity is necessary and sufficient for arterial marker expression, a role for Notch in balancing DA and CV lumen size has not been established. Our findings in both *Notch* gain- and loss-of-function mouse mutants suggest that Notch is critical in equilibrating both arterial and venous lumen size.

Consistent with our findings, prior studies have shown that the DA is small and atretic in *Notch* loss-of-function mutants (Krebs et al. 2000; Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004). However, these previous reports did not elaborate on CV development. Enlarged and reduced vessel sizes have been reported in *Notch4* gain-of-function mutants (Uyttendaele et al. 2001), further suggesting that Notch controls vessel size. However, this earlier study did not specify coordinated changes in arteries and veins. We have combined mouse genetics and in vivo imaging to examine the effects of Notch on both the DA and the CV concurrently. We report here that Notch signaling regulates coordinated growth of both DA and CV in mice by balancing the ratio of arterial versus venous ECs.

It is unclear at present whether this balanced regulation is a universal mechanism during angiogenesis. Our evidence from the carotid arteries and the head veins supports the notion that it occurs in other developing arteries and veins. Furthermore, VEGF, a molecule genetically upstream of Notch (Lawson et al. 2002; Mukouyama et al. 2002), dictates the ratio of arterial and venous blood vessel types during angiogenesis in cardiac muscle (Visconti et al. 2002), suggesting that this mechanism of angiogenesis may be universal. In this study, approximately 50% of capillaries in control animals were ephrin-B2-positive. In the VEGF over-expressing mutant, nearly 90% of capillaries were ephrin-B2-positive and less than 10% of capillaries were EphB4-positive. Similarly, a recent study reports that the *Tie2-Cre* conditional deletion of *Smad4*, a component of TGF-β signaling, yields a small DA and an enlarged CV at e9.5 (Lan et al. 2007). This result also lends support to the reciprocal regulation of arterial and venous size, which, together with our findings, suggests that the reciprocal relationship between growing arteries and veins may be a general process.

Arterial-Venous Differentiation, not Cell Proliferation, is Crucial for the Balanced Growth of the DA and CV

Our data demonstrate that the cellular mechanism underlying the interdependence between arterial and venous size is a balanced allocation of ECs between these vessels. Balanced differentiation of one cell type at the expense of another by Notch during cell fate decisions has been observed in *C. elegans* ventral uterine precursor/anchor cells in the gonad, *Drosophila* neural versus epidermal precursor cells in the ventral ectoderm (Artavanis-Tsakonas et al. 1999), and T versus B cells in the mouse immune system (Pear and Radtke 2003). Our quantitative data at cellular resolution suggest that Notch's role in the balance between two cell types seems to extend into the mouse vasculature, where it similarly regulates the balance between arterial and venous ECs.

Although changes in cell proliferation could lead to differential size, we demonstrate that the proliferation of ECs was not affected by *int3*. Thus, Notch regulates EC allocation by dictating arterial specification, thereby controlling the ratio of arterial to venous ECs.

Coincident with defective DA and CV size is evidence of abnormal vascular perfusion and arteriovenous shunting. We show that ink injected into the heart leaks from the DA into the CV compartment in *Notch* gain-of-function mutants. Others have similarly demonstrated DA and CV shunting in embryos lacking Notch1 (Gridley 2007). These studies suggest the importance of proper EC allocation between arteries and veins in the establishment of a functional circulatory system.

The Reciprocal Size Changes Between the Mutant DA and CVs are Unlikely Results of Aberrant Blood Flow

It is well established that increase in blood flow induces enlargement, while a decrease leads to reduction in vessel diameter (Korshunov and Berk 2003). Such observations raise the question of whether the reciprocal DA and CV size changes are secondary to hemodynamic changes. Since it is currently not feasible to measure blood flow changes in early mouse embryos, it is difficult to address this question empirically. However, evidence suggests that the reciprocal DA and CV sizes are likely to be primary effects of genetic perturbation and not of blood flow changes. First, the phenotypes were apparent at e8.75–e9.0, shortly after e8.5, when blood pressure is irregular and minimal, and unlikely to cause such defects (Jones et al. 2004). We have intentionally analyzed the defects early to avoid flow influence, and mutants were compared with size and somite-stage matched littermate controls. Second, the inverse size change does not fit the well-established flow theory. If the observed size changes in the DA were due to changes in flow, then CV size would coincide, as opposed to the reciprocal phenotype we observed. In contrast, both arteries and veins were reduced in a *c-myc* mutant specifically harboring flow defects, as predicted by the flow theory (He et al. 2008). In this mutant, *c-myc* was deleted only in the hematopoietic lineage, affecting blood cells and blood viscosity, therein changing flow as well. Therefore, while variation in blood flow may contribute to vascular defects, it is unlikely to be the cause of reciprocal DA and CV size changes seen in the *Notch* and *ephrin-B2* mutants.

Coordination of DA and CV Sizes May Involve Proper EC Allocation

The presence of EphB4-positive ECs in the enlarged DA of both EphB4 and ephrin-B2 null mutants is of great interest. EphB4-positive cells, as single cells and in small clusters, have been observed in the vitelline artery of wild type mouse embryos (Gerety et al. 1999) and in the DA of adult mice (Shin et al. 2001). It is unknown why these venous cells reside in arteries and where their ultimate destination may be. Our data suggest that venous ECs transiently inhabit the DA, and that ephrin-B2/EphB4 signaling may be responsible for the proper distribution of these ECs from DA to CV.

The ephrin/Eph pathway mediates both forward signaling in the Eph receptor expressing cell and reverse signaling in the ephrin ligand expressing cell. Forward signaling is crucial for embryonic vascular development, as mice capable of forward but not reverse signaling, survive through birth without the apparent embryonic vascular defects seen in the complete null mutant (Cowan et al. 2004). One characteristic outcome of forward signaling is cell repulsion, specifically the repulsion of the Eph-positive cell away from the ephrin-B2-positive cell (Pasquale et al. 2008, Kuijper et al. 2007). Ephrin-B2/EphB4 signaling results in such repulsion in ECs, where EphB4-positive ECs retract from ephrin-B2 positive ECs in culture (Marston et al. 2003). We found that without ephrin-B2/EphB4 signaling, the aberrant intermingling of EphB4-positive ECs in the enlarged DA may reflect a failure in the repulsion of EphB4-positive ECs from ephrin-B2-positive ECs in the DA *in vivo*. Considering the concurrent enlarged DA and diminished CV size, these mislocalized EphB4-positive ECs may normally originate in the DA and subsequently contribute to CV formation. The lack of an enlarged CV and an underdeveloped DA as well as the absence of ephrin-B2 positive ECs in the CV of either ephrin-B2 or EphB4 null mutants suggests that ephrin-B2/EphB4 signaling functions to repel EphB4-positive ECs away from ephrin-B2-positive ECs, and from the DA to the CV, not *vice-versa*.

Sabin proposed that the DA extends diverticula to form the CV, based on her observations in living chick embryos and prior studies (Sabin 1917). However, later electron microscopy studies failed to detect such aortic protrusions, thus questioning her model (Hirakow and Hiruma 1981; Poole and Coffin 1988). Our molecular evidence supports Sabin’s theory. More recent support for this model includes in vivo real time imaging of zebrafish vascular development, which demonstrated that segments of DA extensions become an integral part of veins (Isogai et al. 2003). In addition, arterial segments have been shown to incorporate into the vitelline vein during yolk sac vascular remodeling in the chick (le Noble et al. 2004). Thus, it is likely that ECs may migrate from arteries to veins.

Although we have not directly observed EC migration in the developing mouse embryo, we and others have detected lateral capillary channels linking the DA and CV (Supplementary Fig. 1) (Gerety and Anderson 2002). These structures may be physical bridges between the developing DA and CV. Thus, we propose a model where Notch promotes arterial differentiation therein regulating EC allocation, and ephrin-B2/EphB4 forward signaling segregates venous ECs, transiently residing in the DA, to the CV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Drs. D. Srivastava and J. Reiter for helpful comments of the manuscript; Drs. F. Chanut and C. Munkittrick for editorial advice. We also thank members of our laboratory for helpful discussions and the UCSF Liver Center Morphology Core supported by NIH P30-DK26743. This work was supported by a generous grant from the Foundation for Accelerated Vascular Research (formerly the Pacific Vascular Research Foundation), HHMI UCSF BRSP, NIH R01 HL075033, and Pfizer Atorvastatin Award to R.A.W.

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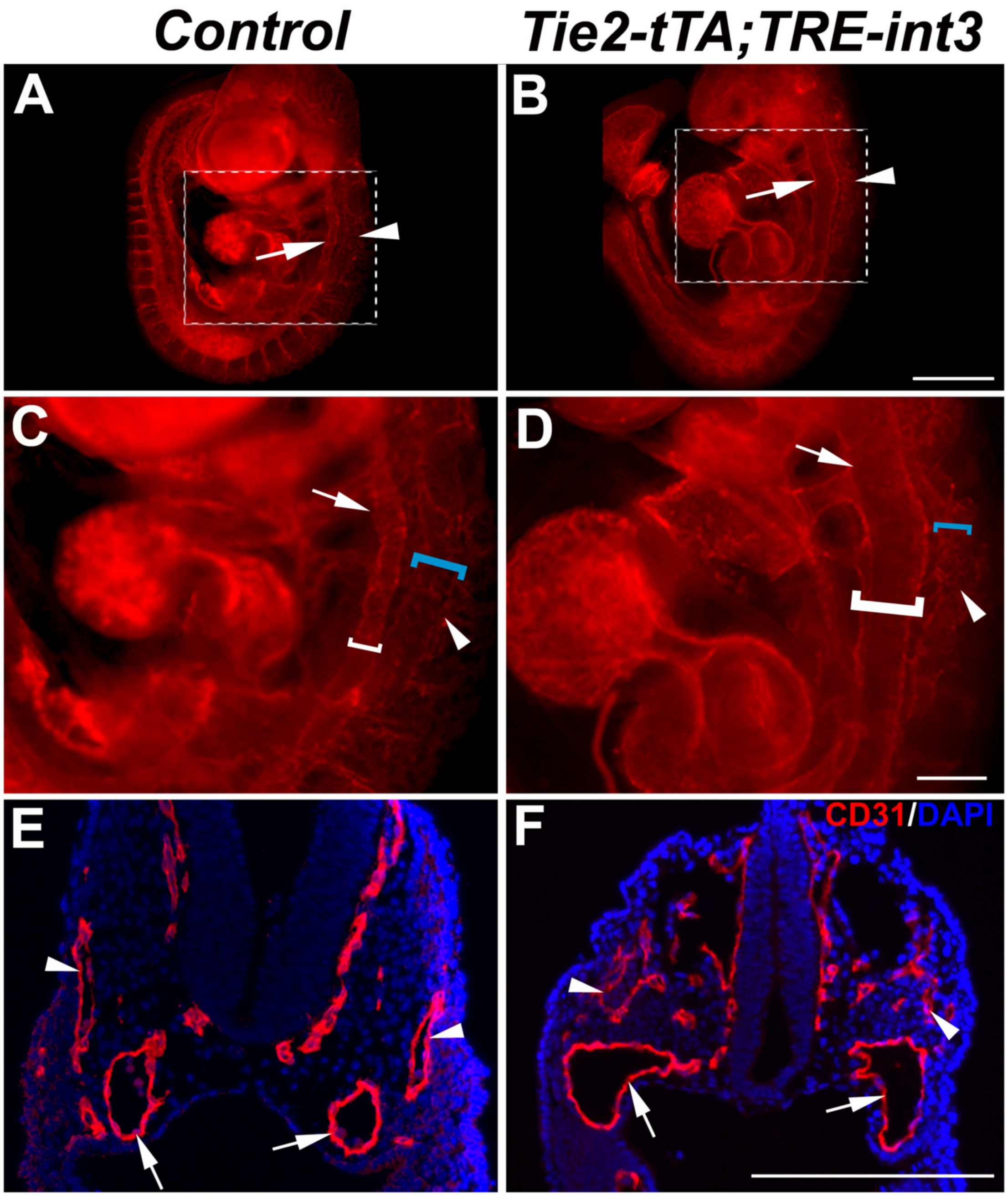
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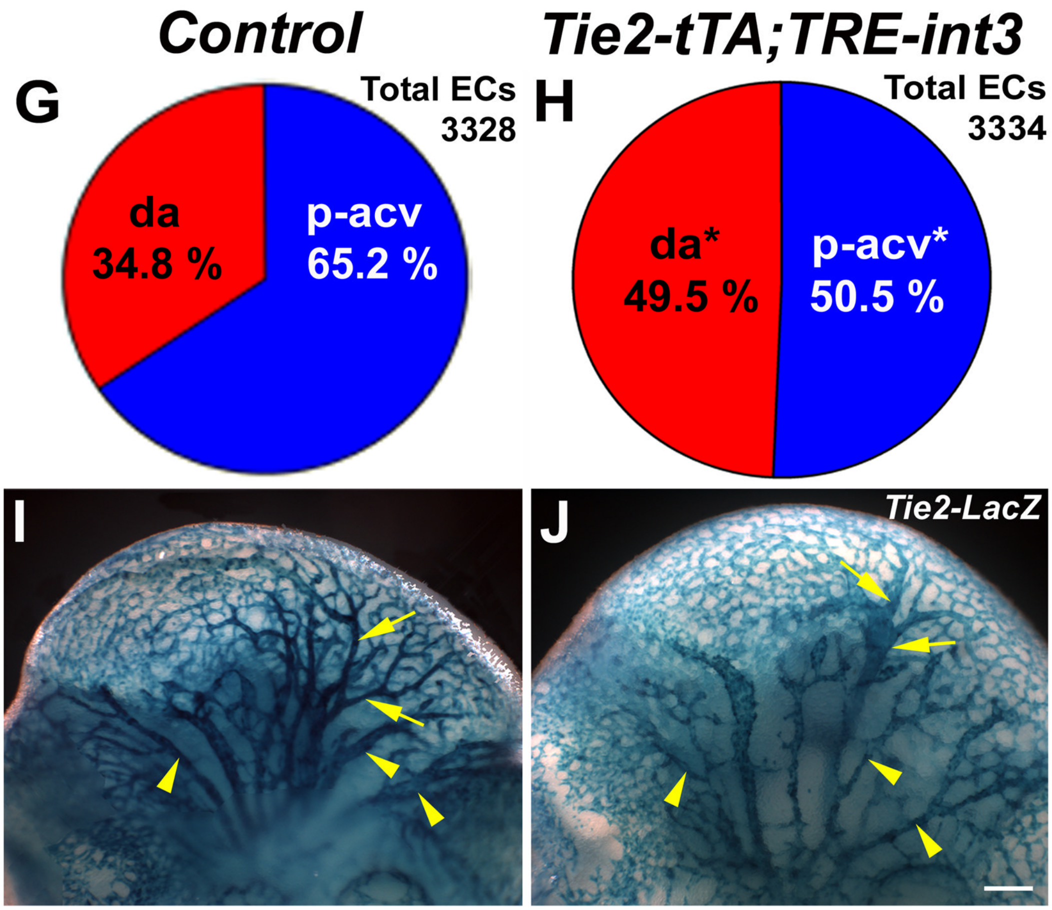


Figure 1. EC-specific gain-of-function allele of *Notch4* elicits DA enlargement and CV underdevelopment

A, B, Whole-mount CD31 staining shows enlarged DA and underdeveloped CV in the trunk region of embryos expressing int3 in ECs at e9.0 (18ss). Arrows, DA; arrowheads, anterior CV (ACV). **C, D,** Higher magnifications of panels **A**, **B**, respectively. Arrows and white brackets, DA; arrowheads and blue brackets, ACV. **E, F,** CD31 staining (red) of cross-sections of **A, B,** respectively, confirms enlarged DA and underdeveloped CV in embryos expressing EC-specific int3. Arrows, DA; arrowheads, ACV. **G, H,** Quantitative analysis of EC distribution. Total ECs, including those in the DA, primordial CV, and capillaries, were counted from cross sections of the anterior region of e8.75 (15–16ss) embryos. A total of 3328 and 3334 ECs were counted in control and mutant embryos, respectively. Total EC number between mutant and control is comparable (N=5, p=0.94). The proportion of ECs in DA (da, red) to primordial ACV including capillaries (p-acv, blue) is significantly increased (N=5; *, p=0.02) in mutants (**H**), as compared to controls (**G**). **I, J,** Whole-mount LacZ staining of the Tie2-LacZ reporter identifies head vessels at e10.5 females were treated with Tetracycline water (500 μg/ml) until e7.5, and embryos were collected at e10.5. Internal carotid arteries (yellow arrows) are enlarged, and head veins are reduced (yellow arrowheads) in embryos expressing EC-specific int3 (**J**). Scale bars, 600μm (B) 200 μm (D, F, J).

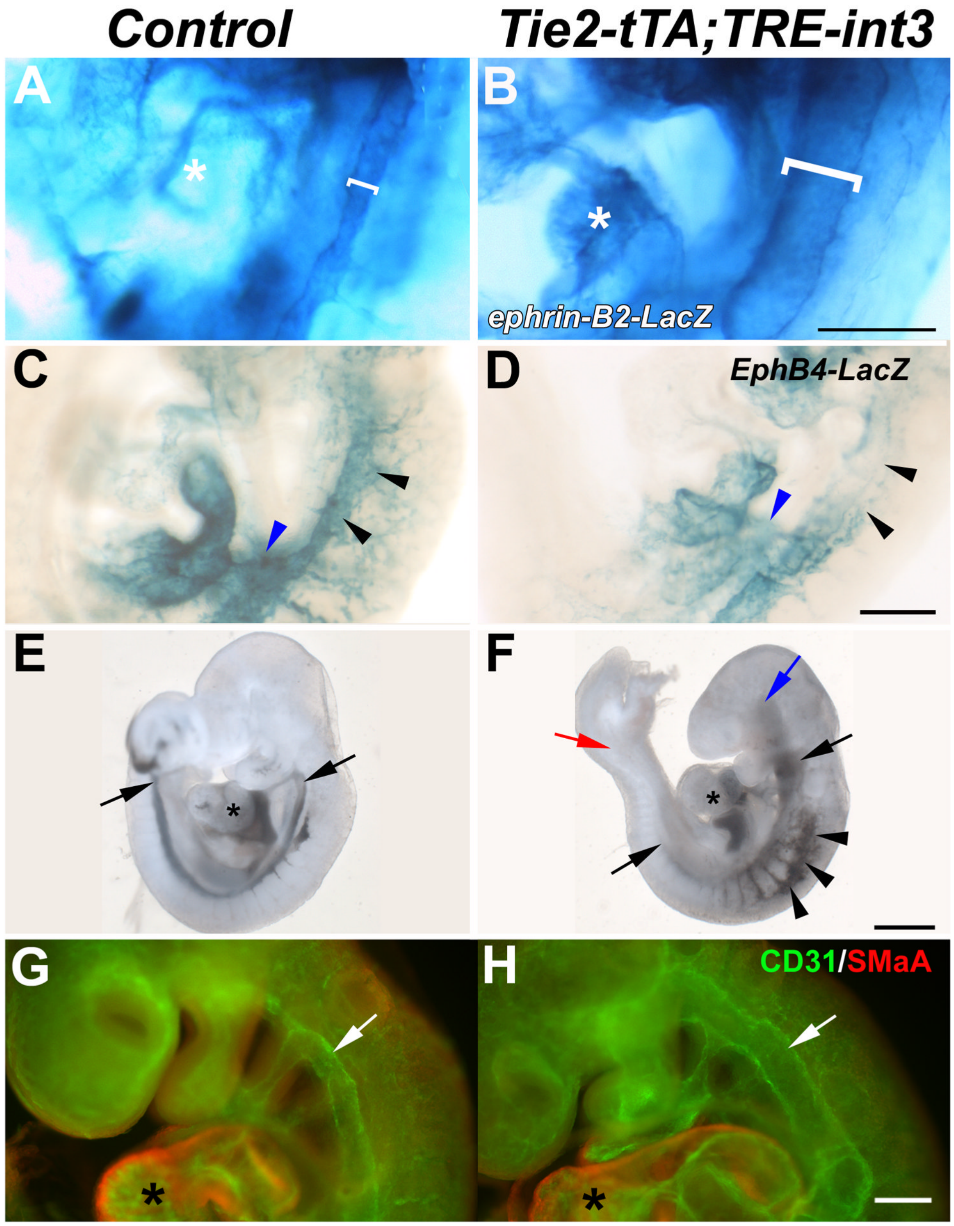


Figure 2. *Notch4* gain-of-function mutation causes enlargement of DA prior to SMC recruitment and leads to vascular shunting

A, B, Whole-mount LacZ staining for the arterial marker ephrin-B2-tauLacZ in e9.5 embryos reveals an enlarged DA (bracket) in the mutant (**B**). **C, D,** Whole-mount LacZ staining for the venous marker EphB4-tauLacZ in e9.5 embryos shows reduced staining in the CV (black arrowheads) in the mutant (**D**). Common CV (CCV), blue arrowheads. **A**–**D,** Anterior is up, dorsal is right. **E, F,** Vasculature of e9.5 (24ss) embryos as revealed by ink injection. In the control (**E**), ink filled the DA (black arrows) evenly from the heart (*) to the tip of the tail. In the mutant (**F**), ink leaked into head vessels (blue arrow) and the venous compartment (arrowheads), and failed to reach the tip of the tail (red arrow). **G, H,** CD31 (green) and SMαA (red) staining of e9.0 (18ss) embryos. DA enlargement in the mutant (**H**) occurs before recruitment of SMCs to the vessel walls. Scale bars, 200 μm (**B, D, H**); 800 μm (**F**).

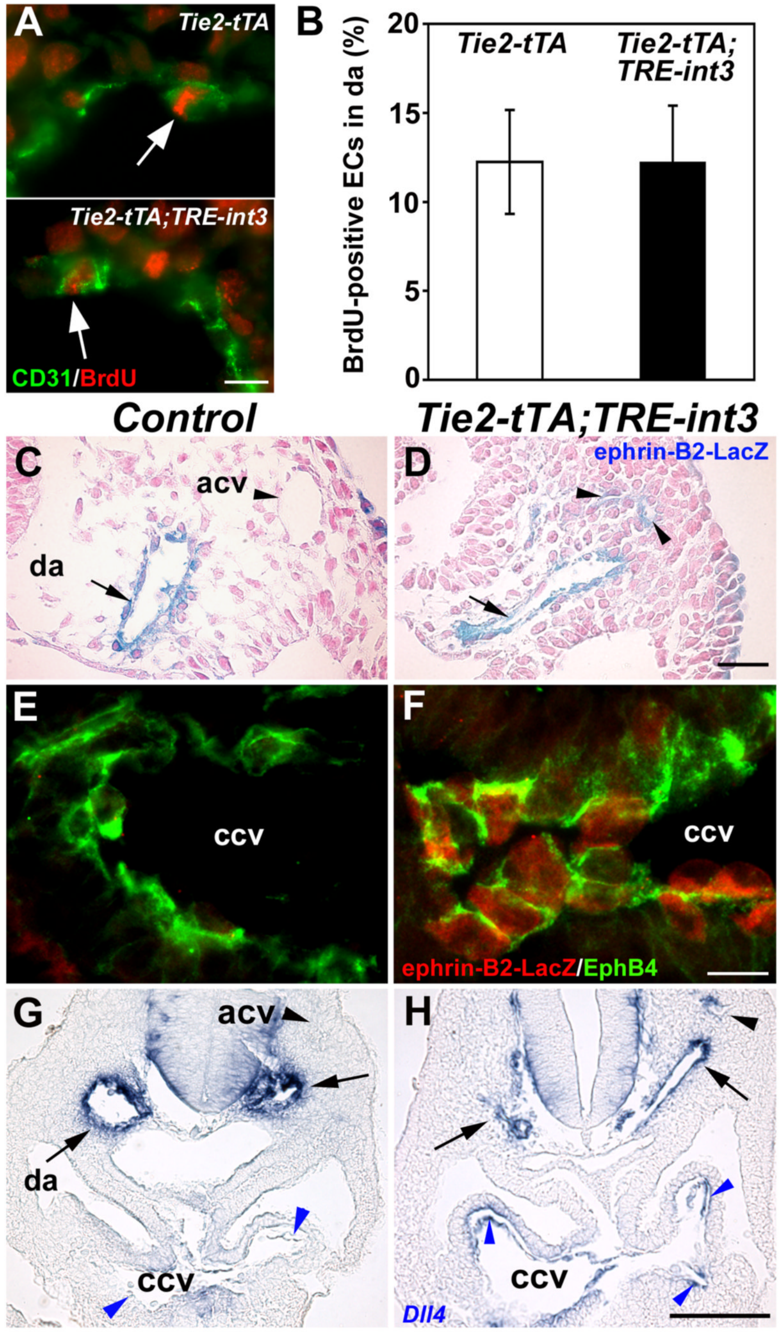


Figure 3. *Notch4* gain-of-function mutation promotes arterial marker expression in venous ECs
A, CD31 (green) and BrdU (red) staining of DA cross-sections in e9.0 (13–15ss) embryos prior to any apparent gross abnormalities in the mutant. Arrows, BrdU-labeled ECs. **B,** Rate of BrdU incorporation in DA ECs at 13–15ss suggests int3 does not affect proliferation in the enlarged DA. Data were analyzed by *t*-test and results are reported as mean±SD (*N*=3). A total of 2320 and 2074 ECs were counted in control and mutant embryos, respectively. **C, D,** Cross-sections of e9.0 (16ss) embryos expressing *ephrin-B2-tauLacZ*. LacZ-stained sections (blue) through the heart were counterstained with eosin (pink). *Ephrin-B2* is expressed in the ACV (arrowheads) in the mutant (**D**) but not in the control (**C**). Arrows, DA. **E, F,** CCV of 15ss embryos stained for expression of *ephrin-B2-tauLacZ* (red) and EphB4 (green). Both markers

are co-expressed in the mutant EC (**F**). **G, H,** Cross-sections of 15ss embryos after *Dll4* in situ hybridization staining. *Dll4* is expressed in the ACV (black arrowheads) and the CCV (blue arrowheads) in the mutant (**H**). Note the atretic anterior DA on the right side of the mutant, one of the occasional cases where the DA on one side was small. Scale bars, 10 μm (**A**); 25 μm (**D, F**); 100 μm (**H**).

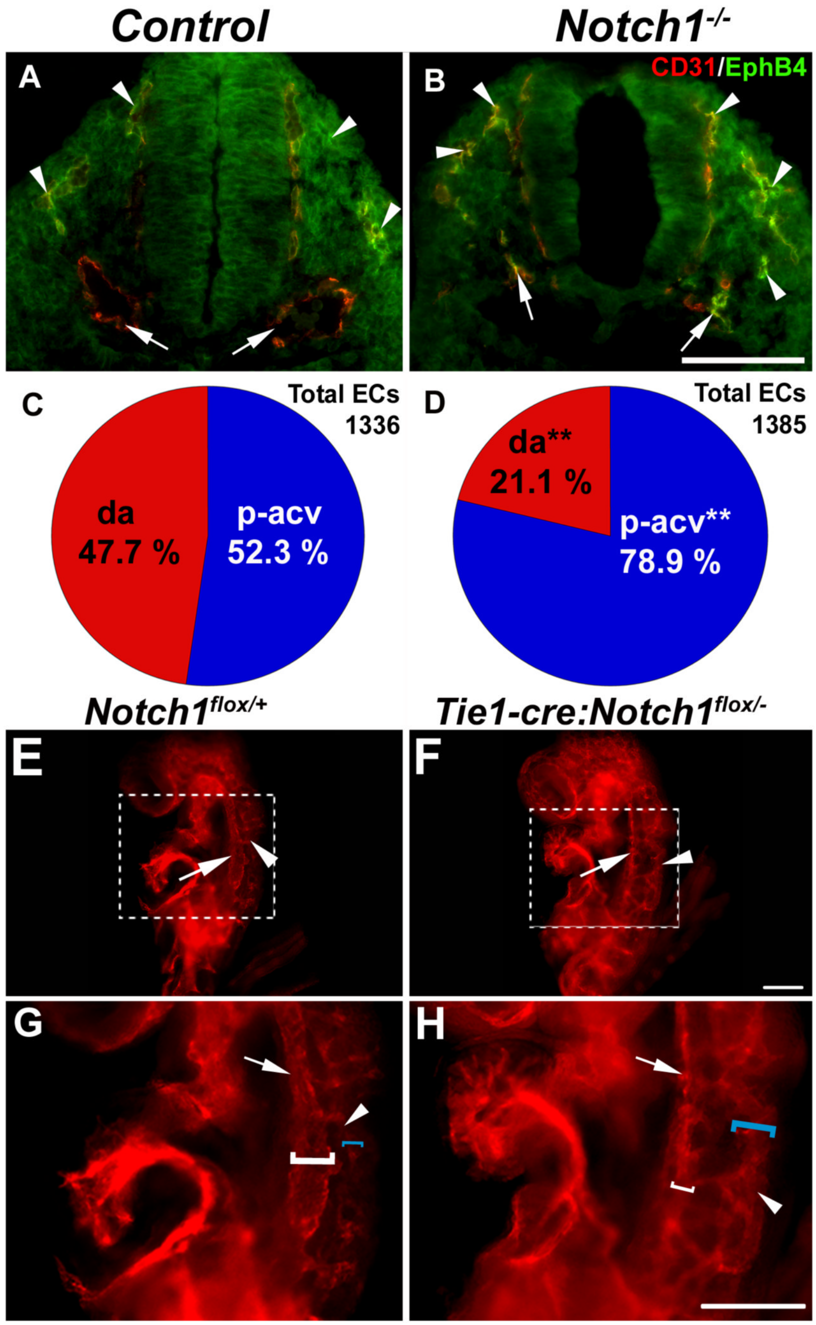
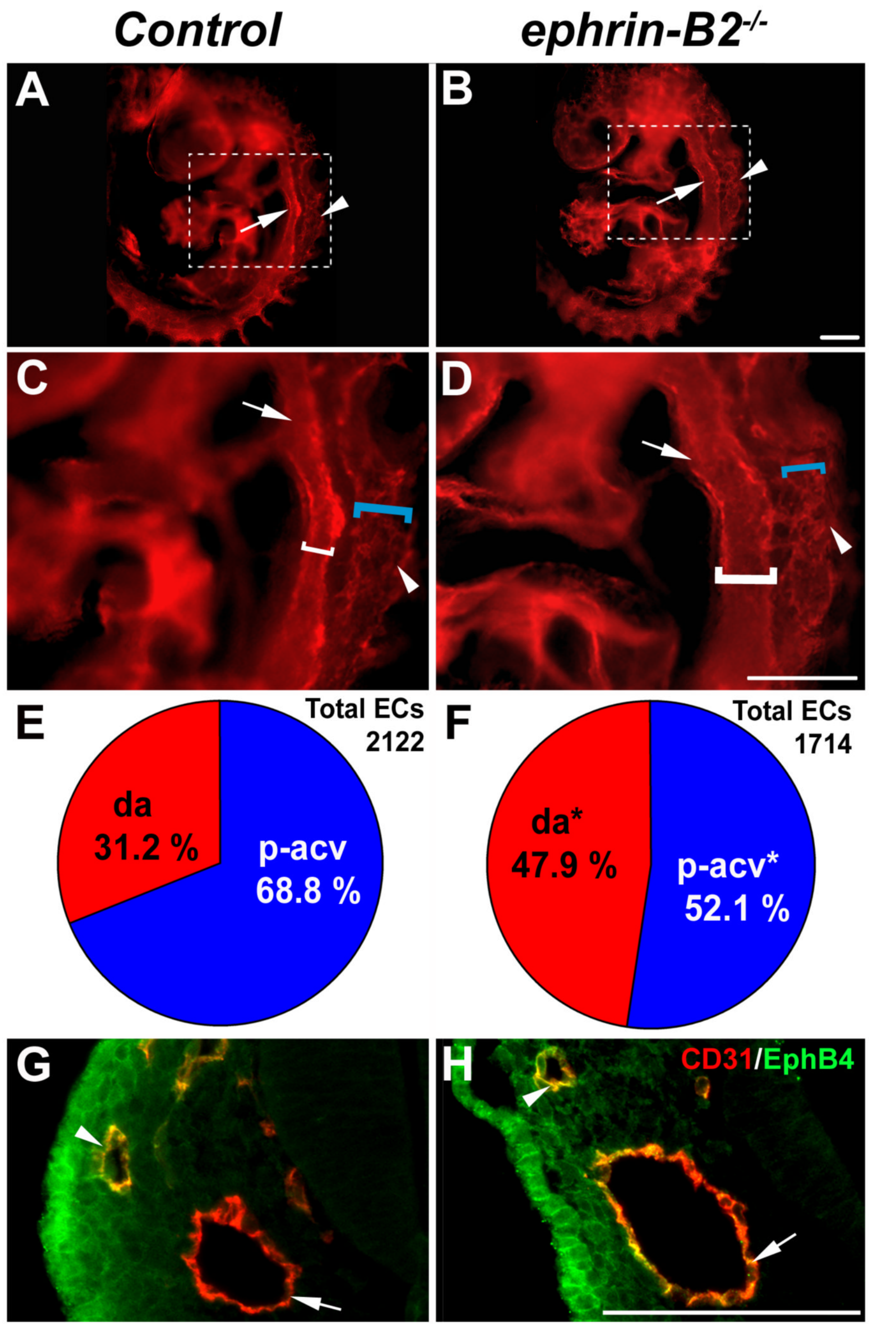


Figure 4. *Notch1* loss-of-function mutation elicits smaller DA and enlarged CV regions

A, B, CD31 (red) and EphB4 (green) staining of cross-sections of e9.0 (15ss) embryos shows remnant DA (arrows) and expanded primordial ACV areas (arrowheads) in the mutant (**B**). Note the increase in EphB4-expressing ECs in the primordial ACVs and DA areas (**B**). **C, D,** Quantitative analysis of EC distribution. Total ECs, including those in the DA, CV, and capillaries, were counted from the cross sections of the anterior region of e8.75 (12–15ss) embryos. A total of 1336 and 1385 ECs were counted in control and mutant embryos, respectively. Total EC number between mutants and controls is comparable (N=4, p=0.52). The proportion of ECs in the DA (da, red) over primordial ACVs including capillaries (p-acv, blue) is significantly decreased (N=4; **, p=0.0007) in the mutants (**D**), compared to the controls (**C**). **E, F,** Whole-mount CD31 staining shows reduced DA (arrows) and enlarged CV (arrowheads) in e8.75 (13ss) embryos with Tie1-cre-mediated deletion of *Notch1*. **G, H,** Higher magnifications of panels **E, F,** respectively. Arrows and white brackets, DA; arrowheads and blue brackets, ACV. Scale bars, 200 μm.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ephrin-B2-/-
A
B
C
D
E
Total ECs
2122
da
31.2 %
p-acv
68.8 %
F
Total ECs
1714
da*
47.9 %
p-acv*
52.1 %
G
H
CD31/EphB4

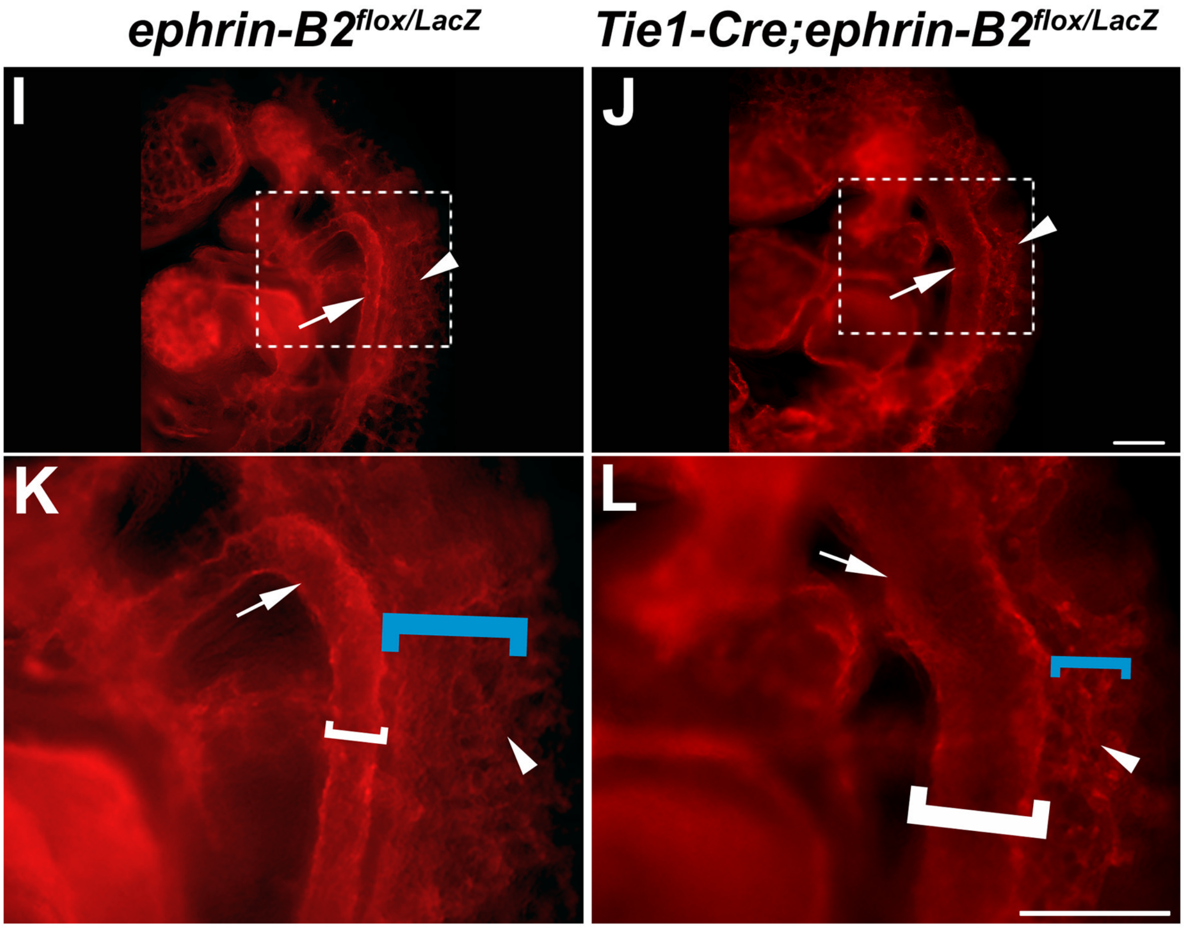

Figure 5. *ephrin-B2* loss-of-function mutation elicits enlarged DA and underdeveloped CV, resembling *Notch* gain-of-function mutant morphology

A, B, Whole-mount CD31 staining of e9.0 (17ss) embryos. Arrows, DA; arrowheads, CV. **C, D,** Higher magnifications of panels **A**, **B**, respectively. Note the enlarged DA (arrows and white brackets) and underdeveloped CV (arrowheads and blue brackets) in the *ephrin-B2* deficient embryo (**B, D**). **E, F,** Quantitative analysis of EC distribution. Total ECs, including those in the DA, CV, and capillaries, were counted from the cross sections of the anterior region of e8.75 (15–17ss) embryos. A total of 2122 and 1714 ECs were counted in control and mutant embryos, respectively. Total EC number between mutants and controls is decreased (N=3, p=0.02). The proportion of ECs in DA (da, red) over primordial ACVs (p-acv, blue) is significantly increased (N=3; *, p=0.02) in mutants (**F**), as compared to controls (**E**). **G, H,** CD31 (red) and EphB4 (green) staining of cross-sections of e8.75 (15ss) embryos. EphB4^{+} ECs are present in the DA (arrow) of the enlarged *ephrin-B2* deficient DA (**H**). **I, J,** Whole-mount CD31 staining shows enlarged DA and reduced CV in e8.75 (16ss) embryos with Tie1-cre-mediated deletion of *ephrin-B2*. **K, L,** Higher magnifications of panels **I, J,** respectively. Arrows and white brackets, DA; Arrowheads and blue brackets, ACV. Scale bars, 200 μm.

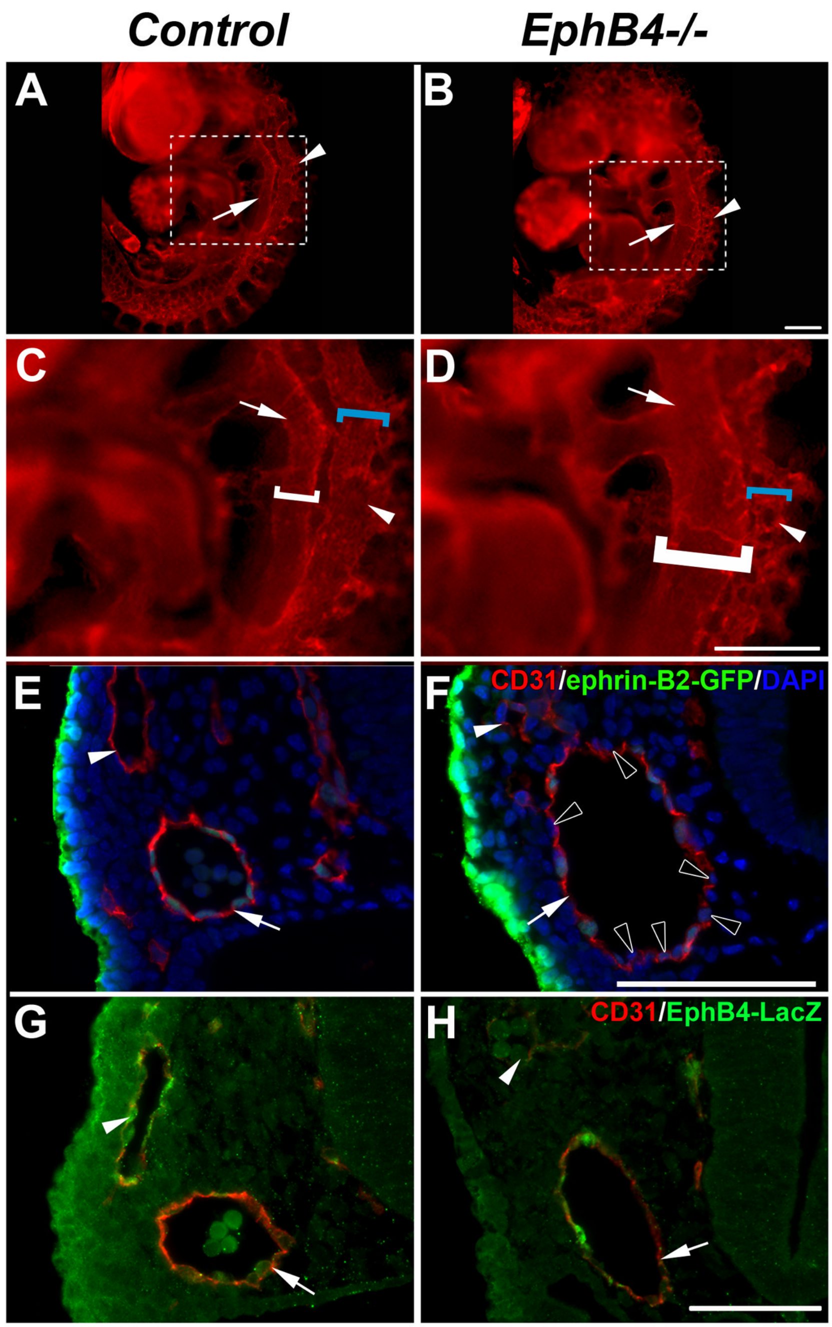

Figure 6. *EphB4* loss-of-function mutation elicits DA enlargement and CV underdevelopment resembling *ephrin-B2* loss-of-function and *Notch* gain-of-function mutant morphology

A, B, Whole-mount CD31 staining of e9.5 (20ss) embryos. **C, D,** Higher magnifications of panels **A**, **B**, respectively. Note the enlarged DA (arrows and white brackets) and underdeveloped ACV (arrowheads and blue brackets) in the *EphB4* deficient embryo (**B, D**). **E, F,** CD31 (red) and ephrin-B2-H2BGFP (green) staining of e9.5 (22ss) embryo cross-sections. The enlarged mutant DA contains ephrin-B2^{-} ECs (**F**, open arrowheads) not seen in the control (**E**). Nuclei were stained with DAPI (blue). **G, H** CD31 (red) and EphB4-tauLacZ (green) staining of e9.5 (20ss) embryo cross-sections. Note that the enlarged mutant DA (arrow) contains EphB4-tauLacZ$^+$ ECs (**H**) not seen in the control (**G**). Scale bars, 200 μm (**B, D**); 100 μm (**F, H**).

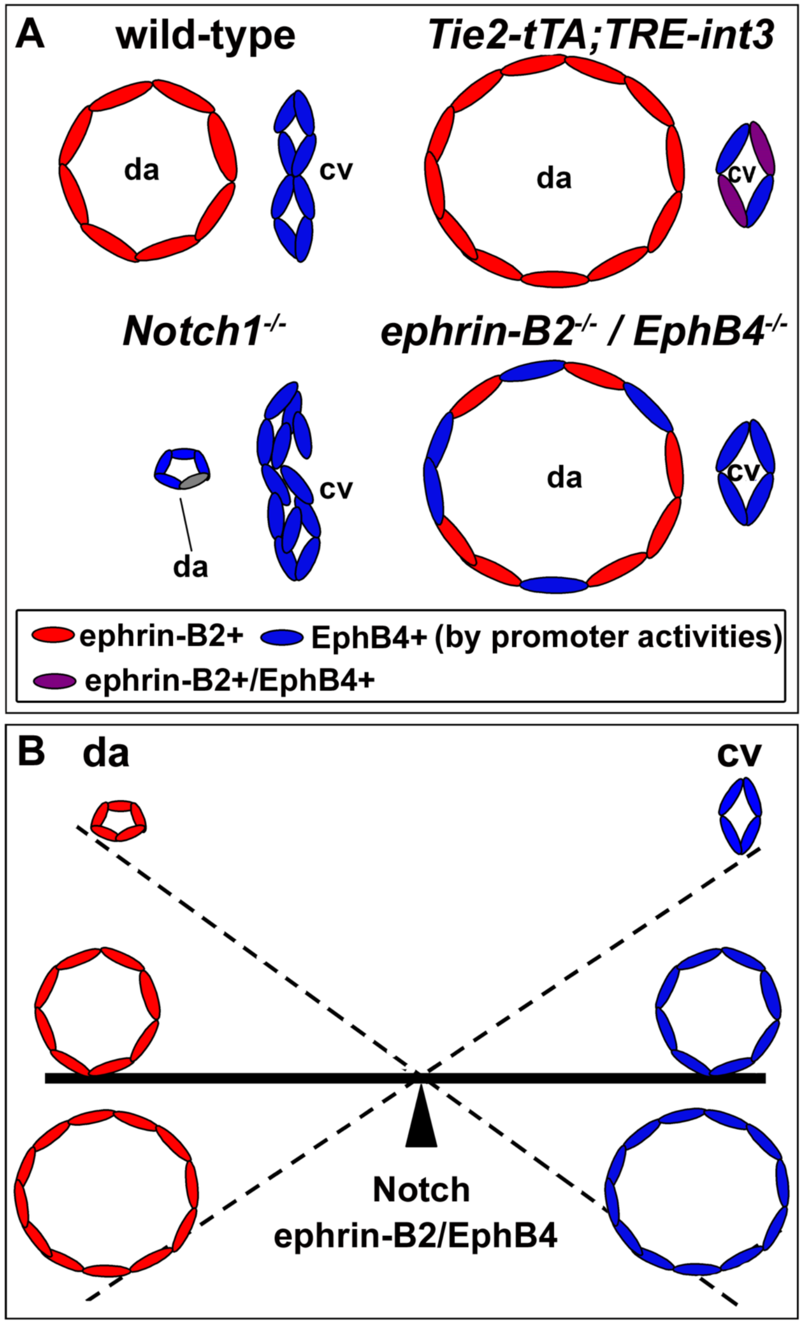

Figure 7. Notch and ephrin-B2/EphB4 pathways regulate the balanced anterior DA and CV morphogenesis

A, Summary of DA and CV phenotypes at approximately e9.0. In wild-type, all ECs in the DA express ephrin-B2 (red) and all ECs in the CV express EphB4 (blue). In the gain-of-function *Notch* mutants, the DA is enlarged while the CV is reduced, and cells in the CV, in addition to the DA, express ephrin-B2. Some CV cells co-express ephrin-B2 and EphB4 (purple). The ratio of arterial to venous ECs is increased. In the loss-of-function *Notch* mutant, the DA is reduced while the CV is enlarged, and some ECs in the DA, in addition to the CV, express EphB4. The ratio of arterial to venous ECs is reduced. In both loss-of-function *ephrin-B2* and *EphB4* mutants, the DA is enlarged, while the CV is reduced. The enlarged DA bears some

ECs with venous identity, ephrin-B2^{-} and EphB4^{+} (blue). The CV size is reduced and its ECs express EphB4. **B,** Proposed model depicts the Notch and ephrin-B2/EphB4 pathways as molecular regulators in the balanced growth of the DA and CV. Alterations in the size of one type of vessel are accompanied by reciprocal changes in the other. Notch signaling controls this equilibrium by promoting arterial differentiation thereby dictating the ratio of arterial to venous ECs. ephrin-B2/EphB4 signaling regulates this balance by sorting differential ECs into the respective vessels.