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Flt-1 (Vascular Endothelial Growth Factor Receptor-1) Is Essential for the Vascular Endothelial Growth Factor–Notch Feedback Loop During Angiogenesis

John C. Chappell, Kevin P. Mouillessaux, Victoria L. Bautch

Objective—Vascular endothelial growth factor (VEGF) signaling induces Notch signaling during angiogenesis. Flt-1/VEGF receptor-1 negatively modulates VEGF signaling. Therefore, we tested the hypothesis that disrupted Flt-1 regulation of VEGF signaling causes Notch pathway defects that contribute to dysmorphogenesis of Flt-1 mutant vessels.

Approach and Results—Wild-type and *flt-1*^{-/-} mouse embryonic stem cell–derived vessels were exposed to pharmacological and protein-based Notch inhibitors with and without added VEGF. Vessel morphology, endothelial cell proliferation, and Notch target gene expression levels were assessed. Similar pathway manipulations were performed in developing vessels of zebrafish embryos. Notch inhibition reduced *flt-1*^{-/-} embryonic stem cell–derived vessel branching dysmorphogenesis and endothelial hyperproliferation, and rescue of *flt-1*^{-/-} vessels was accompanied by a reduction in elevated Notch targets. Surprisingly, wild-type vessel morphogenesis and proliferation were unaffected by Notch suppression, Notch targets in wild-type endothelium were unchanged, and Notch suppression perturbed zebrafish intersegmental vessels but not caudal vein plexuses. In contrast, exogenous VEGF caused wild-type embryonic stem cell–derived vessel and zebrafish intersegmental vessel dysmorphogenesis that was rescued by Notch blockade.

Conclusions—Elevated Notch signaling downstream of perturbed VEGF signaling contributes to aberrant *flt-1*^{-/-} blood vessel formation. Notch signaling may be dispensable for blood vessel formation when VEGF signaling is below a critical threshold. (*Arterioscler Thromb Vasc Biol.* 2013;33:1952-1959.)

Key Words: angiogenesis ■ embryonic stem cells ■ Flt-1 protein, mouse ■ Notch receptors
■ vascular endothelial growth factor A ■ zebrafish

Oxygen and nutrient delivery in developing embryos depends on the formation of vascular networks, and many pathologies, including solid tumor growth, also involve the development and remodeling of blood vessels.¹ Growth factors released from nutrient-deprived tissues initiate angiogenic sprouting from preexisting vessels. Endothelial cells emerge from parent vessels and begin migrating outward using local guidance cues to ensure proper extension.² As the sprout lengthens, extrinsic patterning cues provided by other cell types and the extracellular matrix guide the sprout toward other vessels or sprouts.^{3,4} A connection forms between the nascent sprout and its target, and this newly formed branch acquires a patent lumen for blood flow.⁵ A range of molecular mechanisms, including the vascular endothelial growth factor (VEGF) and Notch pathways, regulate these cellular processes for vascular network expansion.

VEGF-A induces and directs endothelial cell sprouting. Binding of VEGF-A to the tyrosine kinase receptor Flk-1 (VEGF receptor-2) initiates signaling in endothelial cells to promote migration, proliferation, and survival.⁶ Flt-1 (VEGF receptor-1) binds VEGF-A with 10-fold higher affinity than

Flk-1 but acts primarily as a ligand sink, limiting the amount of VEGF-A that can access the Flk-1 receptors on the endothelial cell surface.⁷ Both membrane-bound Flt-1 and soluble Flt-1 modulate endothelial cell proliferation,⁸ but soluble Flt-1 uniquely regulates vessel branching by contributing to a local sprout guidance mechanism.² Expression of both VEGF receptors is regulated during sprouting angiogenesis as part of a dynamic competition among endothelial cells to lead the extending sprout,⁹ and the Notch pathway is important in the competition for tip cell position.

The Notch pathway facilitates cell–cell communication in many contexts, and it is important for lateral inhibition.¹⁰ As one cell acquires a particular role or fate, the Notch pathway is used to restrict neighboring cells from acquiring the same fate or phenotype, as seen in *Drosophila* trachea development,¹¹ and epidermal differentiation.¹² Endothelial cells express the Notch1 and Notch4 receptors, as well as the ligands delta-like 1 (Dll1), Dll4, Jagged1, and Jagged2.¹³ Ligand binding of Notch receptors leads to a series of enzymatic cleavages that result in release of the intracellular domain. The Notch intracellular domain translocates into the nucleus and forms a

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complex that activates the transcription of target genes, such as Hes and Hey. Notch coordinates vessel sprouting such that suppression of Notch signaling yields increased vessel sprouting.^{9,14} The Notch pathway also negatively modulates endothelial cell division, and reduced Notch signaling promotes endothelial cell proliferation.¹⁵

Crosstalk between the VEGF and Notch pathways is important for orchestrating endothelial cell behaviors during angiogenesis.^{16,17} In response to VEGF stimulation, some endothelial cells initiate new sprouts and emerge as tip cells, whereas other cells follow as stalk cells and contribute to vessel expansion through proliferation.¹⁸ To accomplish this coordination, VEGF signals through Flk-1 to increase Dll4 expression on emerging tip cells. Tip cell Dll4 ligands engage Notch receptors on adjacent stalk cells to reduce their sensitivity to VEGF through increased expression of Flt-1^{19,20} and reduced expression of Flk-1 and Flt-4.^{21–24} Here, we directly test the hypothesis that Flt-1 is critical to VEGF–Notch crosstalk in developing blood vessels. We show that Flt-1 is upstream of Notch signaling through regulation of VEGF signaling and thus mediates an important feedback loop in VEGF–Notch pathway crosstalk during blood vessel formation.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Notch Inhibition Rescues Branching and Proliferation Defects in *flt-1*^{-/-} Vessels

Loss of Flt-1 leads to vessel overgrowth and branching dysmorphogenesis through elevated VEGF signaling.^{7,8,25} Because Notch signaling is activated by VEGF signaling,¹⁷ we hypothesized that elevated VEGF signaling as a result of genetic loss of *flt-1* increases Notch signaling and contributes to vessel

branching defects. To test this hypothesis, we used differentiation of mouse embryonic stem (ES) cells in vitro to form primitive lumenized vessel networks in the context of other embryonic cell types.²⁶ Although these vessels lack blood flow, their development in vitro mimics in vivo development of primitive vessel networks.²⁷ First, we manipulated Notch signaling during ES cell differentiation by incubation with the Notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) during the angiogenic phase (days 6–8). Although wild-type (WT) tip cell numbers increased with Notch inhibition (Figure I in online-only Data Supplement), vessel branching and proliferation, as well as vessel area and diameter, were not significantly different from controls (Figure 1A–1C and 1G–1I; Figure IIA in online-only Data Supplement). Interestingly, loss of *flt-1* (*flt-1*^{-/-}) also led to increased tip cell numbers, despite an overall reduction in vessel branching (Figure I in online-only Data Supplement; Figure 1D–1G), suggesting the existence of multiple control points for successful branch formation. In contrast to Notch-inhibited WT vessels, the reduced vessel branching of ES cell-derived vessels lacking Flt-1 was rescued with Notch inhibition (Figure 1D–1G), despite no change in tip cell numbers with DAPT treatment (Figure I in online-only Data Supplement). Notch blockade also unexpectedly reduced the excessive endothelial proliferation characteristic of *flt-1*^{-/-} ES cell-derived vessels (Figure 1H). However, the increased vessel area and diameter of *flt-1*^{-/-} vessels were not rescued by Notch blockade (Figure 1I; Figure IIA in online-only Data Supplement).

To further investigate Flt-1 interactions with Notch, we disrupted Notch signaling with Dll4-Fc, a competitive inhibitor of Notch–Dll4 interactions.²⁸ Similar to Notch inhibition with DAPT, WT ES cell-derived vessel branching, area, and endothelial cell mitotic index were unaffected by Dll4-Fc treatment (Figure 2A–2C and 2G–2I). However, the reduced vessel branching and elevated endothelial cell mitotic index of

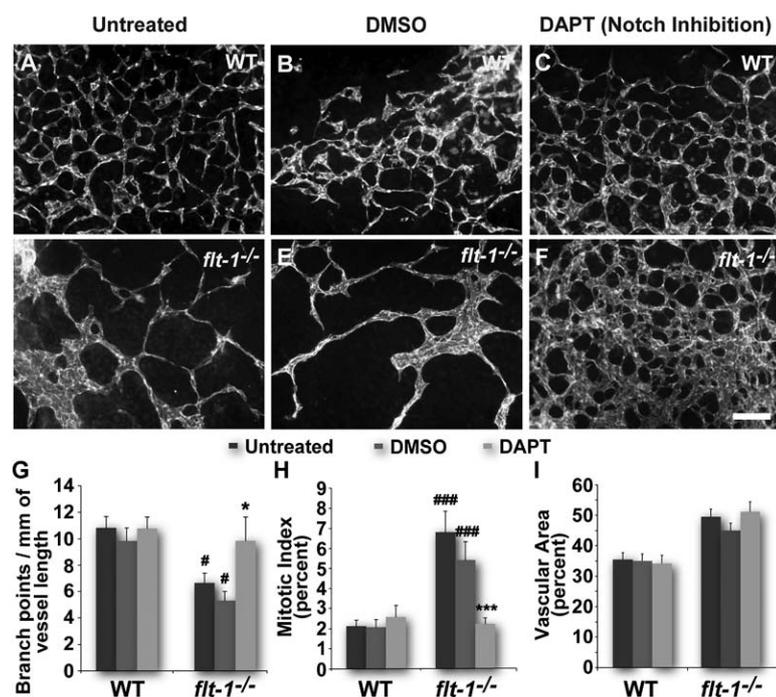


Figure 1. Notch inhibition by N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) rescues the dysmorphogenesis of *flt-1*^{-/-} blood vessels. Wild-type (WT; A–C) and *flt-1*^{-/-} (D–F) day 8 embryonic stem (ES) cell-derived vessels stained for platelet endothelial cell adhesion molecule (PECAM)-1. Scale bar, 100 μ m. Day 8 vessel networks assessed for branch points per vessel length (G). [#] $P \leq 0.05$ vs WT of same treatment group; ^{*} $P \leq 0.05$ vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/dimethyl sulfoxide (DMSO). Day 7 vessel mitotic indices were quantified by counting phospho-histone H3+/PECAM-1+ cells and normalizing to total PECAM-1+ cells and normalizing to total PECAM-1+ cells (H). ^{###} $P \leq 0.0005$ vs WT of same treatment group. ^{***} $P \leq 0.0005$ vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/DMSO. Vessel area relative to total area for day 8 ES cell-derived blood vessels (I). Values are averages \pm SEM.

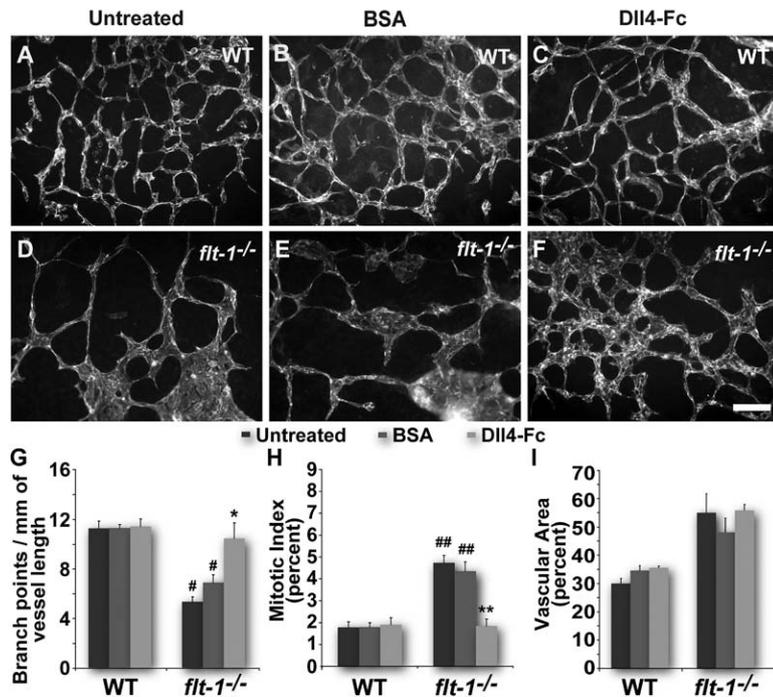


Figure 2. *Flt-1*^{-/-} blood vessel dysmorphogenesis is rescued by delta-like 4 (Dll4)-Fc treatment. Wild-type (WT; **A–C**) and *flt-1*^{-/-} mutant (**D–F**) day 8 embryonic stem (ES) cell-derived vessels stained for platelet endothelial cell adhesion molecule-1. Scale bar, 100 μ m. Day 8 branch points were counted and normalized to vessel length (**G**). # P < 0.05 vs WT of same treatment group; * P < 0.05 vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/BSA. Mitotic indices calculated for day 7 vessels (**H**). ## P < 0.005 vs WT of same treatment group. ** P < 0.005 vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/BSA. Day 8 ES cell-derived vessels assessed for vascular area (**I**). Values are averages \pm SEM.

flt-1^{-/-} mutant vessels were normalized by Dll4-Fc exposure (Figure 2D–2H). Similar to DAPT-mediated Notch reduction, the vascular area of *flt-1*^{-/-} ES cell-derived vessels was unchanged by Dll4-Fc (Figure 2D–2F and 2I). Taken together, these results indicate that although reduced Notch signaling increased WT tip cells, this did not affect WT vessel branching; in contrast, vessels lacking *flt-1* function were phenotypically rescued by Notch blockade.

Because WT ES cell-derived vessels were unexpectedly phenotypically unaffected by Notch blockade, we asked whether this was a model-specific effect or evidence that Notch effects are also context dependent in vivo. To test this idea, we analyzed the developing vessels in the zebrafish embryo, an established model of blood vessel formation that occurs in the context of blood flow.²⁹ Notch manipulations in zebrafish are reported to affect vessel formation in certain scenarios,^{30,31} but not all situations of vessel growth.³² Furthermore, the caudal vein plexus does not exhibit detectable Notch activation via Notch reporter readout (Wiley et al,³³ in revision). Therefore, we subjected zebrafish embryos to Notch inhibition via DAPT treatment and analyzed them for vascular defects. We found perturbed intersegmental vessel (ISV) development in Notch-inhibited embryos (Figure 3A–3C), similar to previous reports.^{30,31} However, in these same embryos, the caudal vein plexuses were unaffected, as determined by the presence of multiple lumenized vessels conducting blood flow. (Figure 3A–3C). These observations demonstrate that effects of Notch inhibition on blood vessel formation in vivo are also context dependent.

VEGF-A–Disrupted Vessel Morphology Is Affected by Notch Blockade

Because loss of *flt-1* elevates VEGF-A–mediated signaling,⁸ we reasoned that the differences in response to Notch blockade between WT and *flt-1*^{-/-} ES cell-derived vessels might result from the amount of VEGF signaling experienced by the vessels.

Thus, we hypothesized that Notch inhibition would elicit changes in WT vessels exposed to ectopic VEGF-A. To test this idea, we inhibited Notch signaling in WT and *flt-1*^{-/-} vessels with and without addition of exogenous VEGF-A. The added VEGF-A caused a significant decrease in WT vessel branching and an increase in endothelial proliferation and vessel area, suggesting that added VEGF-A recapitulates, although not fully, the loss of *flt-1* (Figure 4A–4C and 4G–4I). Notch inhibition of VEGF-A–treated WT vessels partially normalized these changes (Figure 4A–4C and 4G–4I). VEGF-A treatment of *flt-1*^{-/-} ES cell-derived vessels had no effect on vessel branching, area, or endothelial mitotic index, consistent with the idea that loss of Flt-1 elevates VEGF signaling independent of additional ligand (Figure 4D–4I). Exposure to ectopic VEGF-A and Notch blockade rescued *flt-1*^{-/-} vessel branching dysmorphogenesis and endothelial mitotic index without vessel area rescue, similar to Notch blockade alone (Figure 4D–4I). These results indicate that WT vessels are not intrinsically defective in Notch-mediated responses, but rather that Notch responsiveness depends on the level of VEGF signaling.

We next manipulated VEGF and Notch signaling in zebrafish embryos to further explore the influence of VEGF signaling levels on the Notch responsiveness of developing blood vessels. Zebrafish ISVs are more sensitive to VEGF manipulations than the caudal vein plexus.³³ For this reason, we focused on ISV defects in Notch-inhibited embryos with and without the overexpression of *Vegfaa* via heat-shock induction of the *Tg(hsp70l:vegfaa)* transgene. Increased *Vegfaa* induced significant morphological perturbations in the ISVs of developing zebrafish (Figure 5A, 5C, and 5E). Notch blockade in embryos overexpressing *Vegfaa* led to an additional and significant increase in ISV defects (Figure 5B and 5D–5F). Although zebrafish vessels exposed to Notch blockade, in conjunction with increased VEGF signaling, exhibited a distinct phenotypic outcome from ES cell-derived vessels,

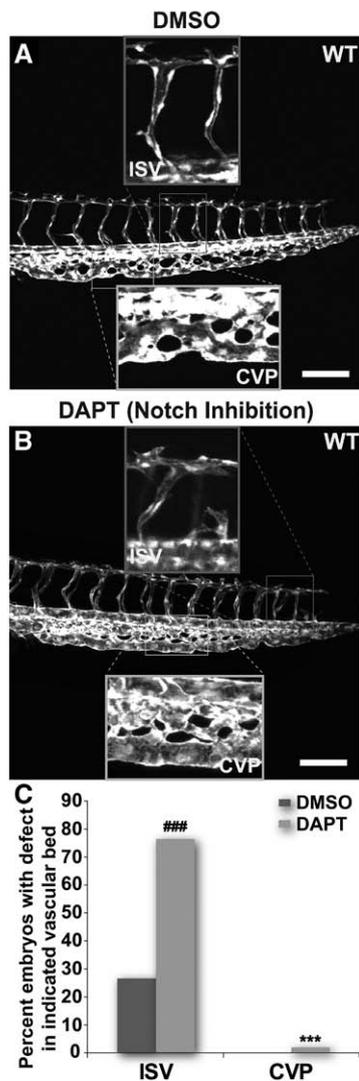


Figure 3. Notch inhibition by N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) disrupts zebrafish intersegmental vessel (ISV) formation but has no effect on the developing caudal vein plexus (CVP). Dimethyl sulfoxide (DMSO)-treated (A) and DAPT-treated (B) 48 hpf *Tg(kdr1:GFP)* zebrafish embryos. Scale bars, 100 μ m. Embryos with normal (top inset, A) and defective ISVs (top inset, B), as well as normal (bottom inset, A and B) and defective CVPs, were quantified (C). ### $P \leq 0.0001$ vs ISV/DMSO; *** $P \leq 0.0001$ vs ISV/DAPT. Values are percentages.

the interaction between the VEGF and Notch pathways was consistent between the 2 models as seen by the increase in defective zebrafish ISVs. Taken together, these observations indicate that endothelial cells vary in their responsiveness to Notch, depending on VEGF signaling levels.

Elevated Notch Target Gene Expression in *flt-1*^{-/-} Vessels Is Rescued by Notch Blockade

To determine whether Notch pathway transcriptional targets are elevated in *flt-1*^{-/-} mutant vessels, we dissociated WT and *flt-1*^{-/-} ES cell cultures and used magnetic bead-assisted cell sorting to enrich for endothelial cells. Real-time quantitative polymerase chain reaction was used to assess RNA levels of the Notch targets *hey1*, *dll4*, and *nrarp*. As expected, Flt-1 RNA

levels were reduced in enriched endothelial cell preparations from *flt-1*^{-/-} vessels, whereas all 3 Notch targets were increased ≥ 5 -fold (Figure 6A). Interestingly, Notch target gene RNA levels in WT-enriched endothelial cell preparations showed no significant changes with Notch blockade (Figure 6Aii–6Aiv). In contrast, the elevated expression of Notch targets in *flt-1*^{-/-} mutant preparations was rescued back down toward WT levels with Notch blockade (Figure 6Aii–6Aiv).

We next evaluated protein levels of Notch pathway components in WT and *flt-1*^{-/-} endothelial cell-enriched preparations exposed to Notch blockade. Protein levels of the transcription factor Hey1 and the Notch1 ligand Dll4, which are also Notch targets, were also highly elevated in the *flt-1*^{-/-} EC-enriched preparations (Figure 6B). These elevated levels of Notch targets were partially rescued with Notch blockade. However, Notch targets were unchanged in WT EC-enriched preparations exposed to Notch blockade (Figure 6B). The lack of change in Notch target gene expression in the WT scenario supports the finding that Notch blockade does not affect the overall morphology of WT ES cell-derived vessels, whereas the elevation with loss of *flt-1* and partial rescue with Notch blockade suggest that Notch is a required effector downstream of elevated VEGF signaling.

Discussion

The rescue of *flt-1*^{-/-} ES cell-derived vessel branching dysmorphogenesis by Notch blockade demonstrates that Flt-1 regulation of VEGF signaling upstream of the Notch pathway is critical for normal vascular development. In addition, VEGF overexpression in zebrafish impaired the ability of Flt-1 to modulate VEGF activity and induced ISV defects that were further affected by Notch suppression. Previous studies showed that Flt-1 expression was upregulated downstream of Notch signaling, but did not critically test *flt-1* function in the crosstalk.^{9,20,22,34,35} Our data support an additional requirement for *flt-1* upstream of Notch via modulation of VEGF signaling. Thus, Flt-1 mediates a critical component of the feedback loop that governs coordination of endothelial cell behavior during vascular development (Figure 6C).

We propose that Flt-1 mediates crosstalk between the VEGF and Notch pathways by keeping VEGF signaling at appropriate levels to effectively use Notch for lateral inhibition (Figure 6Cii). Furthermore, Flt-1 completes the VEGF–Notch feedback loop by further reinforcing the differential responsiveness of endothelial cells to the oncoming VEGF. Loss of Flt-1 modulation of VEGF signaling results in excessively high Notch signaling, undermining the VEGF–Notch feedback loop and disrupting coordination of endothelial cell phenotypes (Figure 6Ciii). Thus, *flt-1*^{-/-} endothelial cells are predicted to experience excessive lateral inhibition via Notch signaling. Consistent with this model, we found that the reduced branching and elevated endothelial proliferation in *flt-1*^{-/-} blood vessel networks^{25,36} were rescued by lowering elevated levels of Notch signaling through Notch blockade. Notch blockade in zebrafish ISVs exposed to ectopic VEGF elicited additional changes in vessel morphology, suggesting that VEGF-mediated effects on vessel formation are influenced by Notch manipulation. RNA and protein levels of Notch targets in ES cell-derived endothelial cells are

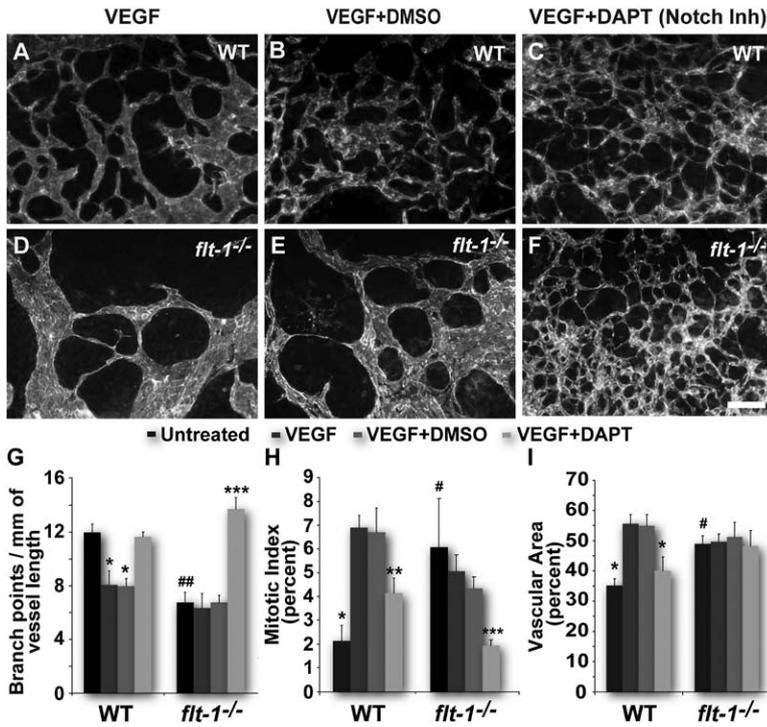


Figure 4. Notch blockade rescues vessel defects induced by added vascular endothelial growth factor (VEGF). VEGF-treated wild-type (WT; **A–C**) and *flt-1*^{-/-} (**D–F**) day 8 embryonic stem (ES) cell-derived vessels stained for platelet endothelial cell adhesion molecule-1. Scale bar, 100 μ m. Day 8 vessels evaluated for branch points per vessel length (**G**). * $P \leq 0.05$ vs WT/untreated or WT/VEGF+N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT); ## $P \leq 0.002$ vs WT/untreated; *** $P \leq 0.008$ vs *flt-1*^{-/-}/untreated, *flt-1*^{-/-}/VEGF, or *flt-1*^{-/-}/VEGF+dimethyl sulfoxide (DMSO). Mitotic indices of day 7 ES cell-derived vessels (**H**). * $P \leq 0.05$ vs WT/VEGF; ** $P \leq 0.01$ vs WT/VEGF or WT/VEGF+DMSO; # $P \leq 0.05$ vs WT/untreated; *** $P \leq 0.006$ vs *flt-1*^{-/-}/untreated, *flt-1*^{-/-}/VEGF, or *flt-1*^{-/-}/VEGF+DMSO. Day 8 vascular area (**I**). * $P \leq 0.05$ vs WT/VEGF or WT/VEGF+DMSO; # $P \leq 0.002$ vs WT/untreated. Values are averages \pm SEM.

consistent with the idea that loss of Flt-1 modulation of VEGF signaling leads to Notch hyperactivation. In this way, Notch signaling downstream of VEGF is required for the defects in *flt-1*^{-/-} blood vessel formation. Bentley et al³⁷ developed a computational model of VEGF and Notch signaling interactions during vessel branching, and their simulation results suggested a need for Notch signaling (ie, lateral inhibition) to be turned down in situations of high VEGF signaling. The current study provides experimental evidence that Flt-1 regulates the feedback loop between VEGF and Notch signaling to effectively turn down signaling levels of both pathways and thus supports proper coordination of endothelial cell behaviors.

Excessive *flt-1*^{-/-} endothelial cell proliferation is reduced with Notch inhibition, suggesting a unique relationship between upstream Flt-1 regulation of VEGF signaling and the downstream Notch pathway in modulating endothelial proliferation. Increased Notch signaling causes endothelial cells to adopt a stalk cell phenotype¹⁴ but is also known to suppress endothelial cell proliferation.^{17,19,38–40} However, stalk cells are presumed to undergo division more frequently than tip cells for sprout elongation,¹⁸ which is seemingly incongruent with stalk cells experiencing elevated Notch signaling.¹⁴ Interestingly, *flt-1* mutant endothelial cells overproliferate, despite having elevated levels of Notch signaling, and both elevated Notch target levels and elevated

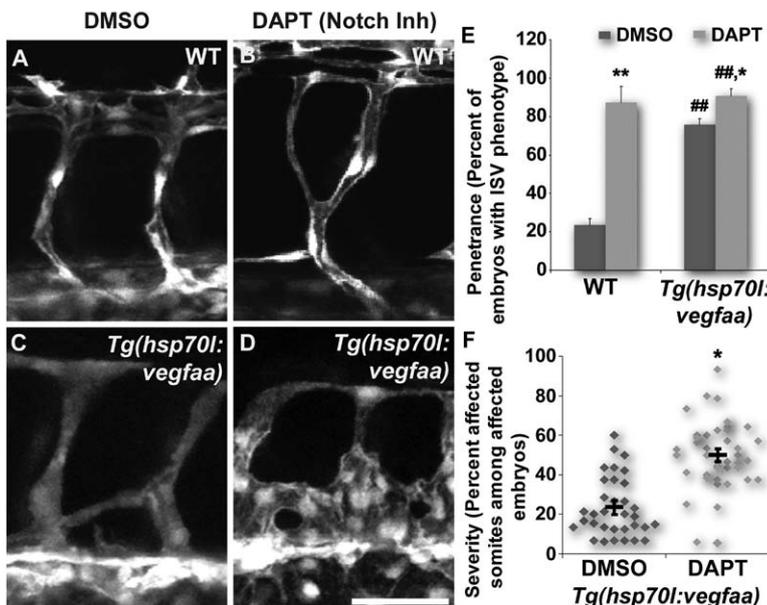


Figure 5. Notch inhibition by N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) exacerbates vascular endothelial growth factor (VEGF)-A-mediated zebrafish intersegmental vessel (ISV) defects. ISVs from dimethyl sulfoxide (DMSO)- and DAPT-treated wild-type (WT; **A** and **B**) and *Tg(hsp70l:vegfaa)*; **C** and **D**) zebrafish embryos at 48 hpf visualized by endothelial expression of green fluorescent protein [*Tg(kdrl:GFP)*]. Scale bar, 50 μ m. Embryos with affected ISVs (**B–D**) were quantified, and penetrance was determined as the percent of embryos with an ISV phenotype (**E**). ** $P \leq 0.005$ vs WT/DMSO; ## $P \leq 0.007$ vs WT/DMSO; * $P \leq 0.016$ vs *Tg(hsp70l:vegfaa)*/DMSO. Values are averages \pm SEM. Of the *Tg(hsp70l:vegfaa)* embryos with an ISV phenotype, the percent of somites with affected ISVs was determined (**F**). * $P \leq 0.0001$ for DMSO vs DAPT. Severities for individual zebrafish are shown as diamonds, with bars representing averages \pm SEM.

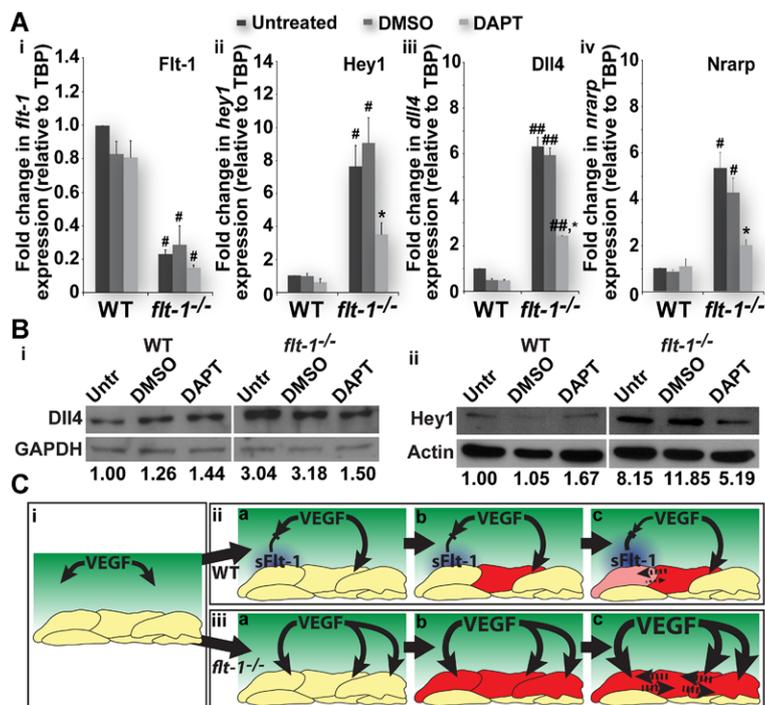


Figure 6. Loss of endothelial *flt-1* upregulates the Notch pathway. *Flt-1*^{-/-} endothelial cell-enriched preparations increases Notch target RNAs (A). Real-time quantitative polymerase chain reaction of Flt-1 (Ai) and Notch pathway components Hey1 (Aii), delta-like 4 (Dll4; Aiii), and Nrarp (Aiv) from untreated, vehicle control-treated, and N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)-treated wild-type (WT) and *flt-1*^{-/-} endothelial cell-enriched preparations.

Ai, #*P*≤0.05 vs WT of the same treatment group. **Aii**, #*P*≤0.05 vs WT of the same treatment group; **P*≤0.05 vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/dimethyl sulfoxide (DMSO). **Aiii**, ##*P*≤0.008 vs WT of the same treatment group; **P*≤0.01 vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/DMSO. **Aiv**, #*P*≤0.05 vs WT of the same treatment group; **P*≤0.05 vs *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/DMSO. Values are averages+SEM. *Flt-1*^{-/-} endothelial cell-enriched preparations have elevated Notch target proteins (B). Representative Western blots for Dll4 (75 kDa) and Hey1 (34 kDa), as well as GAPDH (36 kDa) and actin (45 kDa; for normalization), from untreated, vehicle control-treated, and DAPT-treated WT and *flt-1*^{-/-} embryonic stem cell-derived endothelial cell-enriched preparations. Dll4 signal intensities were normalized to those for corresponding GAPDH control bands, and untreated WT levels were set to 1 for comparison (B). Hey1 levels were also compared across treatment groups and cell types using actin control

bands, just as described for Dll4 and GAPDH (Bii). Model of Flt-1-mediated crosstalk between the vascular endothelial growth factor (VEGF) and Notch pathways (C). The model illustrates how Flt-1 (blue), and soluble Flt-1 (sFlt-1) in particular (iia–iic), modulates the concentration of available VEGF (green, i–iii) that induces Dll4 expression in endothelial cells (red and pink cells, iia–iic). Notch signaling between adjacent cells (dotted lines in iic) then reinforces competition dynamics for sprouting (iic), which completes the Flt-1-mediated feedback loop between VEGF and Notch signaling pathways (iic). In the absence of Flt-1 activity (iia–iic), VEGF induces widespread activation of Dll4 (red cells, iia–iic), and thus Notch signaling is elevated, and normal competition dynamics among endothelial cells are disrupted (dotted lines in iic). In addition, without Flt-1-mediated feedback, VEGF signaling is unchecked (iic), exacerbating the excessive Notch signaling and further undermining normal sprouting and proliferation.

endothelial cell division were rescued by Notch blockade. In one model consistent with these observations, *flt-1*^{-/-} endothelial cells have elevated lateral inhibition (Figure 6Ciii), and Notch blockade releases some endothelial cells from this lateral inhibition, allowing them to contribute more to branching and less to vessel expansion via proliferation. Nevertheless, further investigation will be required to elucidate how Flt-1 integrates VEGF and Notch signals to regulate endothelial cell division.

WT ES cell-derived vessels and zebrafish embryo caudal vein plexus exposed to Notch blockade showed no obvious changes in overall vessel morphology or endothelial cell proliferation, despite an increase in tip cell numbers, and Notch blockade did not affect Notch target gene expression levels in WT endothelial cells. In contrast, Notch blockade in the postnatal retina, tumors, and wound healing models increases vessel density and branching, although these increases do not necessarily result in more lumenized conduits.^{14,22,41–43} Thus, an increase in tip cells may not inherently result in more patent vessel branches, as seen in the current study. Furthermore, not all Notch perturbations affect vessel branching, as previous observations of embryonic and yolk sac vessels in Notch-manipulated mice revealed defects in network remodeling and arteriovenous specification rather than plexus formation.^{44–46} These data and our results suggest that non-Notch pathways may act in parallel or in place of Notch to regulate vessel branching in certain situations. We hypothesized that the level of VEGF signaling might determine the involvement of Notch

signaling in endothelial cells and thus their response to Notch blockade. Indeed, we found that adding VEGF ligand to ES cell-derived vessels or developing zebrafish ISVs affected vessel formation, and Notch blockade had additional effects on these vessels. These results are consistent with previous studies showing that endothelial cells respond to Notch inhibition more strongly with added VEGF.^{32,47,48} Thus, Notch-based therapies will need to be developed with consideration of the treatment context.

Pathological conditions, such as cancer and diabetes mellitus, have as hallmarks misregulated angiogenesis associated with aberrant VEGF signaling. Antiangiogenic therapies, particularly those targeting the VEGF pathway, have had limited success because of acquired resistance and suboptimal efficacy.⁴⁹ Notch perturbations in mouse tumor and hind-limb ischemia models increase the formation of poorly perfused vessels.^{41–43} This undermines recovery after ischemia,⁴¹ but for solid tumors it reduces tumor burden,^{42,43} supporting the potential for Notch-based cancer therapies. Thus, understanding the systemic effects of disrupted Notch signaling⁵⁰ and how Notch intersects with other pathways will be essential for development of effective treatments. In the present study, we found that Flt-1 is important in VEGF–Notch signaling cross-talk and that loss of *flt-1* disrupts VEGF signaling, which in turn perturbs the Notch pathway and contributes to *flt-1*^{-/-} vessel dysmorphogenesis.

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Disclosures

None.

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Significance

In the current study, we have shown that the vascular endothelial growth factor receptor Flt-1 plays an important role in the crosstalk between vascular endothelial growth factor and Notch signaling to coordinate endothelial cell dynamics during blood vessel formation. Previous studies showed that Notch signaling upregulates Flt-1 expression. Here, we have found evidence for an additional requirement for Flt-1 in regulating vascular endothelial growth factor signaling upstream of the Notch pathway. Thus, disrupted Flt-1 activity undermines this critical vascular endothelial growth factor–Notch feedback loop and perturbs the coordination of endothelial cells during angiogenesis. Because therapeutic strategies, particularly those treating solid tumors, are being developed to target these pathways, we believe our study addresses the important need for understanding how these pathways intersect and possible systemic effects of disrupted signaling.

MATERIALS AND METHODS

ES Cell Culture and In Vitro Differentiation

Wild-type (WT) and *flt-1*^{-/-} (gift of G.H. Fong) mouse ES cell maintenance and differentiation was as described.¹ The γ -secretase inhibitor IX, DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, Santa Cruz Biotechnology, Inc.), was resuspended in DMSO (Sigma), and added to ES cell cultures at 8 μ M and 12 μ M on dy 5 and 7. DAPT doses above 8 μ M did not increase the overall response of the ES cell cultures (Figure I and IIB in online-only Data Supplement), and in some cases, led to deleterious effects (data not shown). Recombinant human VEGF-A (Peprotech) was reconstituted in PBS/0.1% BSA and added on dy 5 and 7 at 30 ng/ml. The recombinant Dll4-Fc protein inhibitor (Adipogen, mouse extracellular Dll4:human Fc) was resuspended in PBS/0.1% BSA, and added at 1 μ g/ml every other day from dy 3 onward.

ES Cell Differentiation Culture Antibody Staining and Quantitative Analysis

Fixation and staining of dy 7 and 8 ES cell cultures was as described.² Primary antibodies used were: rat anti-mouse PECAM-1 (BD Biosciences) at 1:1000, and rabbit polyclonal anti-phospho-Histone H3 (ser10, EMD Millipore) at 1:500. Secondary antibodies were donkey anti-rat AlexaFluor488 (IgG; H+L) at 1:1000 (Invitrogen), and donkey anti-rabbit AlexaFluor568 (IgG; H+L) at 1:500 (Invitrogen). Incubation with DAPI for 30 min followed all staining.

ES cell cultures were imaged with a confocal microscope (Leica TCS SP5) using a \times 40 objective (HCX PL APO oil), and 6-10 z-axis confocal scans were acquired and flattened. Vessel images were traced using the ArtStudio v5.0 application (Lucky Clan) for an Apple iPad, and traces were processed using ImageJ plugins (BoneJ/Skeletonise 3D, Skeleton/AnalyzeSkeleton (2D/3D)) to generate branch point density values. ImageJ was used to count phospho-Histone H3+ (PH3+) mitotic endothelial cells and total endothelial cells, to yield a mitotic index (PH3+ and PECAM+ cells per total PECAM+ cells) for each image. Vascular area was also measured using ImageJ, and pixel intensity threshold values used to determine total PECAM+ area/total image area. An endothelial tip cell was defined as a cell oriented away from a parent vessel, extending filopodia but not obviously engaged with a target cell, and emerging from a parent vessel with its nucleus clearly beyond the axis of the parent vessel. For dy 7 cultures from each treatment group/dose and cell type, tip cells were counted and normalized to vessel length. Vessel diameter for dy 8 cultures was also measured using ImageJ by determining the midpoint of a vessel between two branch points and measuring the vessel width perpendicular to the vessel axis.

Zebrafish Husbandry and Experimentation

Zebrafish (*Danio rerio*) embryos were bred as previously described.³ Transgenic adults, *Tg(hsp70l:vegfaa,myl7:EGFP,kdrl:GFP)nc2a* [ref. 4, referred to as *Tg(hsp70l:vegfaa)*], were maintained as heterozygotes, and WT and transgenic embryos were genotyped by PCR upon conclusion of each experiment. Heat-shock treatment occurred 30 hr post-fertilization (hpf) for 1 hr at 42°C. DAPT was administered at 14 and 30 hpf at a final concentration of 100 μ M in 1% DMSO.

Zebrafish Imaging and Quantification

Zebrafish vessels expressing GFP were imaged at 48 hpf with a confocal microscope (LSM 5 PASCAL, Carl Zeiss, Inc.) using a 10× objective (Carl Zeiss, Inc.), acquiring 6 μm thick optical slices through the z-axis. For DAPT experiments without VEGF over-expression, the percentage of embryos with abnormal intersegmental vessels (ISVs) or an aberrant caudal vein plexus (CVP) was determined by scoring each embryo as having perturbed vessels or not, and dividing the total number of defective embryos by the total number evaluated. In heat-shock VEGF experiments with Notch inhibition, ISV defect penetrance and severity were determined. Penetrance was calculated as the percent of embryos with irregular ISV formation among all embryos observed. Defect severity was found by determining, for each embryo with affected ISVs, the percent of somites containing malformed ISVs, and the percentages for each embryo were then averaged.

Endothelial Cell Enrichment

Endothelial cells were enriched from ES cell cultures using magnet-assisted cell sorting (MACS) (Miltenyi Biotec). Briefly, cultures were digested in 2 mg/ml collagenase in HBSS for 45 min at 37°C with repeated, vigorous pipetting. Dissociated cells were passed through a 70 μm filter, centrifuged 5 min at 2000 rpm, and re-suspended in autoMACS® Running buffer with a mouse Fc receptor-blocking reagent (Miltenyi Biotec) on ice for 10 min. Rat anti-mouse PECAM-1 linked to phycoerythrin (PE, BD Bioscience) was added for 15 min. Cells were centrifuged 5 min at 2000 rpm and re-suspended in buffer with magnetic beads coated with anti-PE antibodies for 15 min on ice. After another 5 min spin at 2000 rpm, cells were re-suspended in buffer, passed through a 30 μm filter, and put over a magnetized column. Columns were washed 3X, removed from the magnetic field and placed into new tubes; new buffer was added, and the isolated cells collected.

Real-Time Quantitative PCR Analysis

Endothelial cell-enriched preps were digested in TRIzol (Invitrogen), and RNA was extracted using 1-Bromo-3-chloropropane (Sigma) and ethanol. Following purification using an RNeasy Plus Kit (Qiagen), 0.3 μg of RNA was retro-transcribed using a SuperScript II RT kit (Invitrogen). Real-time quantitative PCR was performed in triplicate using TaqMan Universal Master Mix II (Applied Biosystems) and the Applied Biosystems 7900HT Fast Real-Time PCR System. PCR primers for TATA binding protein (tbp), Flt-1, Hey1, Dll4, and Nrarp were obtained from Applied Biosystems. The tbp values were used for normalization, and the comparative CT method was used to determine expression changes.

Protein Analysis

Western blot analysis was performed as previously described.² Enriched endothelial cell preps were incubated in ProteoJet mammalian cell lysis buffer (Fermentas). Following protein separation on a 10% SDS-polyacrylamide gel, samples were transferred to a polyvinylidene fluoride membrane (GE Healthcare). Primary antibodies used were: rabbit anti-Hey1 (5 μg/ml, Millipore), goat anti-actin (1:5000, Santa Cruz Biotechnology), goat anti-Dll4 (1:500, Abcam), and goat anti-GAPDH (1:5000, Abcam). The horseradish

peroxidase (HRP)-tagged secondary antibodies included anti-rabbit (GE Healthcare) and anti-goat (Santa Cruz Biotechnology), and were visualized with enhanced chemiluminescence (GE Healthcare).

Statistics

Statistical comparison of ES cell-derived vessel branch points, vessel area and diameter, tip cell numbers and endothelial RNA levels was done using the Student's two-tailed t-test, as was zebrafish ISV penetrance and severity. Mitotic index values and DAPT effects on zebrafish ISVs and CVPs were compared using χ^2 analysis.

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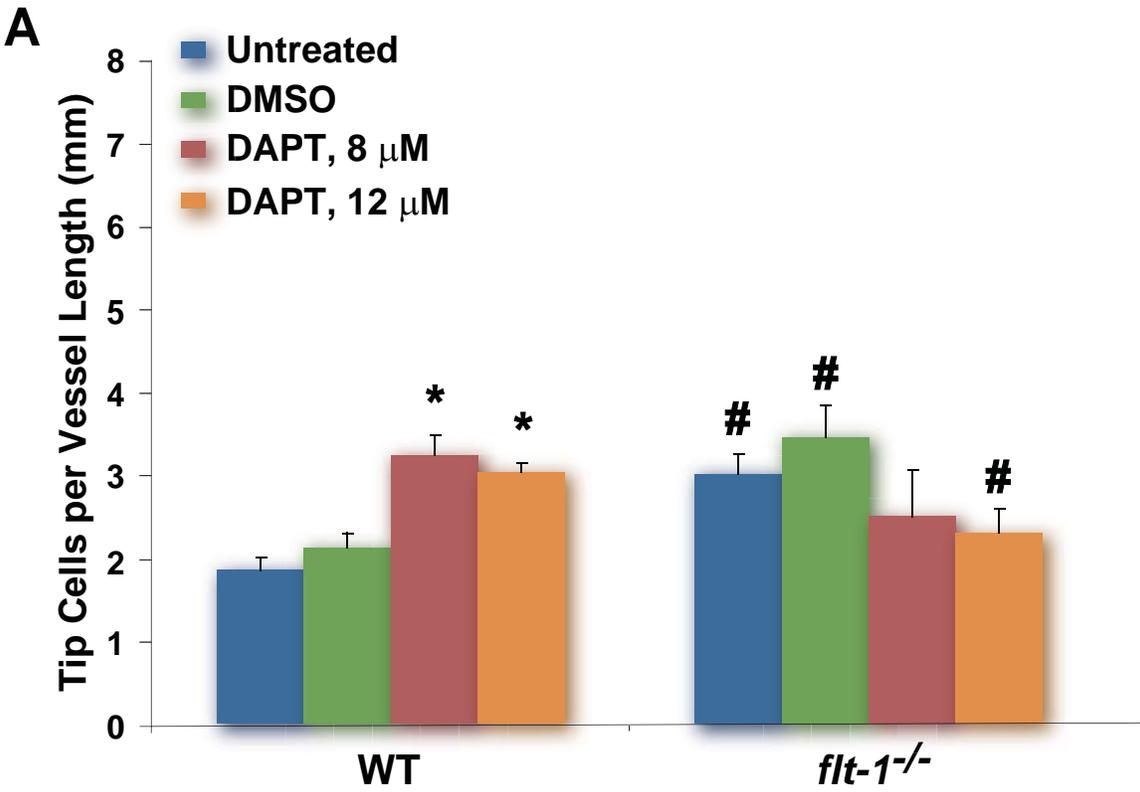
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Supplemental Material

Supplemental Figure I. DAPT treatment induces more tip cells from WT vessels but not *flt-1*^{-/-} vessels, and an increased DAPT concentration does not alter these responses. Dy 7 tip cells per vessel length (A). *, $p \leq 0.004$ vs. WT/untreated or WT/DMSO. #, $p \leq 0.03$ vs. WT of same treatment group. Values are averages +/- standard error of the mean (SEM).

Supplemental Figure II. Vessel diameter is unaffected by Notch inhibition, and endothelial proliferation is unaffected by an increased dose of DAPT. Dy 8 vessel networks assessed for average vessel branch diameter (A). Dy 7 vessel mitotic indexes were quantified by counting PH3+/PECAM-1+ cells and normalizing to total PECAM-1+ cells (B). #, $p \leq 0.04$ vs. WT of same treatment group. *, $p \leq 0.05$ vs. *flt-1*^{-/-}/untreated or *flt-1*^{-/-}/DMSO. Values are averages +/- SEM.

Supplemental Figure I.



Supplemental Figure II.

