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ETS factors regulate Vegf-dependent arterial specification

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Summary

Vegf signaling specifies arterial fate during early vascular development by inducing the transcription of *Delta-like 4* (*Dll4*), the earliest Notch ligand gene expressed in arterial precursor cells (aPCs). *Dll4* expression precedes that of Notch receptors in arteries, and factors that direct its arterial-specific expression are not known. To identify the transcriptional program that initiates arterial *Dll4* expression we characterized an arterial-specific and Vegf-responsive enhancer of *Dll4*. Our findings demonstrate that Notch signaling is not required for initiation of *Dll4* expression in arteries, and suggest that Notch instead functions as a maintenance factor. Importantly, we find that Vegf signaling activates MAP kinase (MAPK)-dependent ETS factors in the arterial endothelium to drive expression of *Dll4*, as well as *Notch4*. These findings identify a Vegf/MAPK-dependent transcriptional pathway that specifies arterial identity by activating Notch signaling components, and illustrate how signaling cascades can modulate broadly expressed transcription factors to achieve tissue-specific transcriptional outputs.

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Introduction

Arterial and venous blood vessels are anatomically, functionally and molecularly distinct. Establishing and maintaining these separate endothelial cell fates is critical to the proper function of circulatory networks in the embryo and the mature adult (Marchuk, 1998). In mice, angioblasts are specified in the lateral plate mesoderm during gastrulation. Bilateral streaks of VEGFR2/Flk1^{+}-pre-aortic angioblasts, or arterial precursor cells (aPCs), form the dorsal aortae, followed by the formation of the embryonic veins by a distinct group of angioblasts (Chong et al., 2011). Arterio-venous (AV) specification is genetically determined before hemodynamic forces come into play. Hedgehog signaling induces expression of Vegf in a dorsal to ventral gradient in the early embryo (Lawson et al., 2002). Arterial cells are thought to receive a higher concentration of Vegf, which activates p42/p44 MAPK signaling (Hong et al., 2006) and induces the expression and activation of Notch signaling pathway components exclusively in the arterial endothelium (Lawson et al., 2002). Once activated, the intracellular domain of the cleaved Notch receptor (NICD) interacts with its transcriptional co-factor, RBPJk, in the nucleus to induce expression of *Hey1/2*, *Hes1*, EphrinB2 (*Efnb2*) and other downstream arterial genes (Yamamizu et al., 2010). The molecular events following Notch activation that maintain arterial identity are well understood (Swift and Weinstein, 2009), yet the initial transcriptional cues that function downstream of Vegf activation to induce the expression of Notch signaling components are unknown.

Dll4 is the first Notch ligand gene expressed in the arterial endothelium (Chong et al., 2011), and its expression is induced by Vegf (Lawson et al., 2001). Expression of *Dll4* is initiated in the dorsal aorta before genes encoding its cognate receptors, *Notch1* and *Notch4*, making *Dll4* one of the earliest markers of the arterial lineage (Chong et al., 2011). Significantly, loss of only one copy of *Dll4* produces AV specification defects and embryonic lethality in mice (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). Defining the transcriptional program responsible for early *Dll4* expression will therefore provide key insights into arterial specification.

We describe the isolation of an arterial-specific enhancer of *Dll4*, which labels aPCs and differentiated arterial cells. We find that this enhancer is responsive to Vegf/MAPK signaling, and through deletion analysis identify a previously unappreciated role for ETS transcription factors in mediating the Vegf-responsiveness and arterial-specificity of this enhancer and endogenous *Dll4*. This Vegf/MAPK/ETS pathway also regulates the expression of *Notch4*, which encodes an arterial-specific receptor of Dll4. These studies provide mechanistic insight into the transcriptional program downstream of the Vegf receptor that mediates arterial specification through the induction of Notch signaling components.

Results

The genomic region 5′ of Dll4 does not drive arterial-specific expression

To understand the transcriptional basis of AV specification, we searched for evolutionarily conserved non-coding regions (ECRs) of the *Dll4* locus. The region proximal to the *Dll4* transcriptional start-site is regulated by β-catenin signaling through TCF/LEF sites and can also be activated by FOXC1/2 and RBPJk in vitro (Caolo et al., 2010; Corada et al., 2010; Seo et al., 2006). We cloned this 5-kb region (fragment 1, F1), and placed it upstream of a promoter-less β-galactosidase reporter (Figure 1A and S1A). The activity of this reporter construct was tested in transient transgenic mouse embryos, where it failed to direct any arterial expression (Figure 1A, S1A,B). This suggests that this region is not sufficient to mediate the arterial-specific expression of *Dll4*.

Wnt signaling is not required for early Dll4 expression or artery specification

Endothelial-specific deletion of *Ctnnb1*, the gene encoding β-catenin, results in embryonic lethality at E12.5 (Cattelino et al., 2003). However, whether AV specification occurs normally in *Ctnnb1* loss-of-function mice has not been previously assessed. Over-expression of a dominant-active allele of *Ctnnb1* induces *Dll4* expression (Corada et al., 2010), leading to the suggestion that Wnt/β-catenin plays an instructive role in arterial specification by inducing Notch signaling. However, we were unable to detect active canonical Wnt signaling in the arterial endothelium at E8.5 or E9.5 using multiple established Wnt reporter lines (*BAT-gal*, *Axin2-d2EGFP*, *Axin2*^{lacZ}) (Figure S1C–E). To determine if Wnt signaling regulates early *Dll4* expression, we ablated *Ctnnb1* specifically in the endothelium. The dorsal aortae and cardinal vein were morphologically normal at E8.5 (data not shown) and E9.5 (Figure S1F), expression of *Dll4* mRNA was unchanged (Figure S1G), and we did not observe any arteriovenous malformations (AVMs) in *Ctnnb1* mutants at E9.5 (Figure S1H). Collectively, these results demonstrate that Wnt/β-catenin signaling in the endothelium is dispensable for early artery formation and early *Dll4* expression, and that the DNA region upstream of the promoter of *Dll4* is not sufficient for artery-specific expression.

Identification of a Dll4 enhancer with activity in the developing arterial endothelium and endocardium

Another well-conserved ECR (fragment 2, F2) is located within the third intron of *Dll4* (Figure 1A). This region can respond to FOXN4 (Luo et al., 2012), RBPJk/NICD, and β-catenin (Yamamizu et al., 2010) in both in ex-vivo and in vitro reporter analyses, but its in vivo activity has not been assessed. In transient transgenic embryos, F2 drove robust activation of a minimal promoter-*lacZ* reporter (*hsp68-lacZ*) in the arterial endothelium and endocardium, similar to endogenous *Dll4* mRNA expression and to the β-galactosidase activity of *Dll4*$^{lacZ/+}$ embryos (Figure 1B). Analysis of embryos at E7.5–7.75 from multiple stable transgenic founder lines demonstrated that this enhancer labeled aPCs prior to their coalescence into the cord-like structures of the dorsal aorta (Figure 1B,C). Examination of transverse sections confirmed the arterial-specificity of the enhancer from E8.5 through E10.5 (Figure 1D). F2 also drove strong expression in the endocardium, another tissue where *Dll4* mRNA is observed (Figure 1B), suggesting that this enhancer recapitulates the entire developmental endothelial expression pattern of endogenous *Dll4*. F2, like endogenous *Dll4*, was active in the arteries of the post-natal retina, but it was not active in the vasculature of the adult retina, suggesting that F2 is a developmentally-regulated enhancer (data not shown). To determine whether this enhancer's functionality was conserved in other vertebrate species, we injected an F2:*GFP* transgene into zebrafish embryos, and established stable transgenic lines. Expression of GFP was specific to the dorsal aorta, cardinal artery and intersomitic vessels, with very little expression detectable in the posterior cardinal vein or caudal vein plexus (Figure 1E,F). This intronic *Dll4* F2 element is therefore a *bona fide* arterial-specific enhancer.

A 36-bp fragment necessary and sufficient for arterial-specific enhancer activity is regulated by ETS and RBPJk

Comparative genomic analysis revealed that the full-length F2 enhancer contains regions of high conservation among distantly-related mammals (Figure 2A). Using deletion analyses of the 802-bp F2 enhancer, we identified a 350-bp (F2-2) and a 225-bp fragment (F2-4) that both labeled the arterial endothelium, as well as a weak endocardial enhancer (F2-3) that failed to label the arterial endothelium in mice (Figure 2A,B). The proximal 176 bp of the F2 enhancer (F2-5), which is contained in F2 and F2-2, but absent in F2-4, was not sufficient to drive arterial expression (Figure 2A,B). F2, F2-2, and F2-4 share a common 100-bp region that, when deleted in the context of the entire 802-bp enhancer (F2Δ1), abolished arterial activity (Figure 2A,B). A well-conserved 36-bp element within this

common region was necessary (F2Δ2) and, when concatamerized head-to-tail in triplicate (F2-6(3X)), was sufficient to direct arterial and endocardial expression (Figure 2A–C). Further characterization revealed that this minimal 36-bp enhancer (F2-6(3X)) was capable of directing robust and reproducible arterial expression at early stages (i.e. E8.5) of vascular development (Figure 2D). Importantly, transient transgenesis revealed that this 36-bp enhancer was also able to drive strong arterial-specific expression in the dorsal aorta and intersomitic vessels (Figure 2E), as well as the endocardium (data not shown), of zebrafish embryos. Venous expression was not observed in embryonic mice or zebrafish (Figure 2C,E).

The minimal enhancer contains conserved predicted binding sites for ETS, MEF2, E2F and RBPJk transcription factors (Figure 2F). We tested which sites were required for activity in vivo by separately mutating each DNA-binding site in the context of the F2-6(3X):*GFP* reporter and performing transient transgenesis assays in embryonic zebrafish. At 24 hours post fertilization (hpf), ~75% of embryos injected with the wild-type F2-6(3X) enhancer displayed arterial expression of GFP. Mutation of the E2F element did not affect enhancer activity, but mutation of the MEF2 element resulted in a modest, but significant reduction in arterial expression (Figure 2G). In mice, *Mef2a* and *Mef2c* are expressed within the endothelium (Lin et al., 1998; Wang et al., 2003) and loss of *Mef2c* leads to early embryonic lethality (~E9.5) and produces a range of vascular defects, including an absent or disorganized dorsal aorta (Bi et al., 1999; Lin et al., 1998). Although recombinant MEF2C protein bound to the MEF2 site of F2-6(3X) (Figure S2A), *Mef2c* is not required for either F2 activity or endogenous *Dll4* expression (Figure S2B–C). Conversely, only 5% of zebrafish embryos injected with an ETS mutant F2-6(3X):*GFP* construct had arterial expression and mutation of the RBPJk-binding site abolished arterial expression (Figure 2G). These results suggest that ETS and RBPJk play a critical role in regulating F2-6(3X) enhancer activity in vivo, and imply that they may regulate endogenous *Dll4*.

Neither the RBPJk site in the F2 full-length enhancer nor Notch signaling are required for initiation of Dll4 expression

It has been proposed that activated Notch signaling can induce *Dll4* gene expression via RBPJk-mediated transcriptional regulation through a feed-forward mechanism (Caolo et al., 2010). Given that the activity of the minimal F2-6(3X) enhancer was dependent upon a putative RBJPk-binding site, we sought to determine if Notch signaling is necessary or sufficient to regulate the activity of the full-length F2 enhancer. Forced induction of Notch signaling throughout the vasculature promotes the arterialization of veins (Krebs et al., 2010). Indeed, endothelial-specific over-expression of the intracellular domain of Notch1 produced AVMs in mouse embryos and expanded F2 reporter activity into the venous endothelium (Figure S2D), suggesting that the enhancer is Notch responsive. However, although RBPJk bound to the F2 enhancer (Figure S2E), mutation of the RBPJk site in the context of the full-length F2 enhancer failed to diminish arterial-specific expression in transient transgenesis analyses in zebrafish or mice (Figure A, B). We next assessed whether Notch signaling is required for F2 enhancer activity. *Rbpjk* knockdown reduced late (i.e. 26 hpf), but not early (i.e. 20 somites) enhancer activity (Figure 3C), consistent with a role for Notch signaling in the maintenance, but not the establishment of arterial *Dll4* enhancer activity. In mice, global loss of *Rbpjk,* which leads to defects in somitogenesis and neural tube formation (Oka et al., 1995), diminished but failed to abolish F2 reporter activity at E8.25 or E8.5 (Figure 3D). Additionally, in embryos where *Rbpjk* was ablated specifically in the endothelium – bypassing global morphogenetic defects – the activity of the F2 reporter was unaffected at E8.5 or E9.5 (Figure 3E), demonstrating that RBPJk does not play a cell-autonomous role in initiating *Dll4* enhancer activity. Finally, *Dll4* mRNA was only moderately reduced in $Rbpjk^{-/-}$ mice compared to wild-type littermates at E8.5 (Figure 3F).

Together, these results indicate that Notch signaling is not required for the early arterial expression of *Dll4*, but is essential for its maintenance.

ETS factors regulate the activity of the Dll4 enhancer

ClustalW analysis revealed the presence of nine perfectly conserved minimal ETS elements (ETS sites A–G), including the site within F2-6(3X) (ETS-B) (Figure S3A). This was intriguing since several ETS family members are key regulators of endothelial development (De Val and Black, 2009). EMSA confirmed that six of these sites competed for binding of the ETS1 DNA binding domain (DBD) to a known ETS element (Figure S3B). Among these six sites, ETS site B (ETS-B) is present in all arterial-positive constructs (F2, F2-2, F2-4, and F2-6), and corresponds to a canonical ETS-1 binding site (cETS-1). ETS-B also directly and specifically bound recombinant ETS1-DBD (Figure S3C), and ETV2, an ETS family member (Figure S3D).

To determine the functional requirement of the ETS binding sites in vivo we generated transient transgenic zebrafish harboring the full-length F2 enhancer driving GFP in which ETS-B or all six of the ETS1 DBD-binding ETS sites were mutated. Mutation of the six ETS binding sites abolished F2 enhancer activity, and mutation of the ETS-B site alone severely reduced enhancer activity in arteries (Figure 4A); confirming the importance of this single ETS element. Given the necessity of ETS binding sites for enhancer activity, we wondered whether ETS factors were sufficient to induce *Dll4* expression. Thus, we next determined whether over-expression of ETS factors was sufficient to induce expression of endogenous *DLL4* in human umbilical vein endothelial cells (HUVEC), a venous cell type that expresses low levels of *DLL4.* Indeed, over-expression of two different endothelial-enriched ETS family members, *ETV2* or *ERG*, induced endogenous *DLL4* expression (Figure 4B). Induction of *DLL4* was accompanied by binding of V5-ERG to the endogenous region of *DLL4* corresponding to F2 (i.e. intron 3), but not to a distal region of the *DLL4* locus, as assessed by ChIP (Figure 4C). Furthermore, we detected high levels of endogenous ERG occupancy at intron 3, but not a distal site in the *Dll4* locus, in an arterial cell line (bovine aortic endothelial cells (BAECs)) (Figure 4D). By comparison, NICD was only modestly enriched at intron 3 in BAECs (Figure 4D). Collectively, these results demonstrate that ETS factors can induce expression of endogenous *DLL4* mRNA, and that ETS factors directly occupy the endogenous *DLL4* genomic region corresponding to F2 in arterial cells.

To further define the role of ETS factors in the regulation of *Dll4* expression we performed loss- and gain-of-function experiments in mouse and zebrafish. Fli1a and Erg are closely related ETS factors that play redundant roles in regulating angiogenesis during zebrafish vascular development (Liu and Patient, 2008). Combined morpholino knock-down of these two ETS factors decreased F2:GFP expression at both early and late stages of vascular development (Figure 4E), and endogenous *dll4*, *efnb2a* and *hey2* expression were also diminished (Figure 4F). However, *kdrl*:GFP expression (a pan-endothelial marker) was unaffected (Figure 4E), as was *kdr* mRNA (Figure 4F). Conversely, injection of *ERG* mRNA increased F2:GFP expression within the dorsal aorta and in what appeared to be the forming posterior cardinal vein at early stages of arterial specification (i.e 20 somite-stage), and also promoted premature sprouting of intersomitic vessels (Figure S4A). However, expanded enhancer activity in the vein was not observed at later stages of vascular development (i.e. 24 hpf; not shown). Since Notch target genes (i.e. *efnb2a, hey2*) were decreased in *erg/fli1a* morphants, we assessed Notch transcriptional activity using a Notch reporter line (*Tp1:GFP*) (Parsons et al., 2009) in an *erg/fli1a* loss-of-function setting. Strong *Tp1*:GFP expression in the dorsal aorta and intersomitic vessels was observed in 94% of control embryos (n=83) compared to only 67% of *erg/fli1a* morphants (n=165), suggesting that arterial Notch signaling was compromised in a proportion of ETS morphants (Figure 4G).

Erg splice variants in mice, driven from a translational start site in Exon 4, are specifically expressed in the endothelium, and *Erg Exon 4* null mouse embryos ($Erg^{\Delta Ex4/\Delta Ex4}$) die early in development (E10.5–11.5) with severe vascular remodeling defects in the yolk sac and impaired vascularization of the central nervous system (Vijayaraj et al., 2012). We found that the dorsal aortae formed normally in $Erg^{\Delta Ex4/\Delta Ex4}$ embryos (Figure S4B), but that *Dll4* expression was modestly reduced at E8.5 (Figure 4H) and substantially down regulated at E9.5 (Figure 4I), confirming a role for this ETS family member in regulating *Dll4* mRNA expression. Interestingly, several other arterial markers were also reduced in $Erg^{\Delta Ex4/\Delta Ex4}$ embryos at E8.5 (Figure 4H), and levels of *Efnb2* were modestly reduced at E8.5 (Figure 4H; Figure S4C) and E9.5 (Figure 4J), but were severely altered at E10.5 (data not shown). However, we did not find evidence of gross morphological AVM defects in $Erg^{\Delta Ex4/\Delta Ex4}$ embryos at E9.5 (Figure S4B,D), and only rarely observed shunts in *erg/fli1a* morphant zebrafish embryos (data not shown). We take these results to indicate that ETS factor redundancy may functionally compensate for the loss of ERG or Erg/Fli1a within the endothelium. Together these data define a role for ETS factors in the regulation of the arterial-specific expression of *Dll4*, and indicate that ETS factors are involved in the specification of arterial fate.

Vegf signaling regulates the expression of Dll4 through the induction and activation of ETS factors

Arterial specification is largely Hh- and Vegf-dependent (Lawson et al., 2002; Vokes et al., 2004), and *Dll4* expression is regulated by Vegf levels (Coultas et al., 2010; Liu et al., 2003). We therefore assessed whether F2 enhancer activity was Vegf-responsive. *Vegfa* knockdown in zebrafish dramatically reduced F2 arterial enhancer activity (Figure 5A,B), similar to endogenous *dll4* mRNA (Figure 5C). However, the expression of a pan-endothelial marker (*kdrl*:GFP) in the axial vessels and levels of *kdr* and *kdrl* mRNA were largely unaffected (Figure 5A–C); confirming that *vegfa* does not regulate vasculogenesis in zebrafish. Global over-expression of Vegfa, achieved by co-injection of mRNAs encoding $Vegfa_{121}$ and $Vegfa_{165}$, dramatically increased F2 activity in arterial cells (Figure 5D). In addition, F2 activity was expanded into the vein, confirming that Vegf levels regulate the arterial-specific expression of *Dll4*. Additionally, we examined F2-*lacZ* activity in two mutant mouse backgrounds that affect Vegf signaling. Hh-mediated activation of Smoothened (SMO) is required for expression of *Vegf* in the rostral somites at E8.5 and loss of *Smo* leads to disorganized or collapsed anterior (but not posterior) dorsal aortae by E8.5; although $Flk1^{+}$ cells are specified and present in normal numbers (Vokes et al., 2004). Significantly, *Smo* mutants have reduced *Dll4* mRNA in the anterior, but not posterior, dorsal aorta at E8.5 (Coultas et al., 2010). Correspondingly, we found that F2 enhancer activity was reduced in the anterior, but not the posterior dorsal aorta of *Smo* mutants at E8.25, and activity was abolished in this region of the dorsal aorta by E8.5 (Figure S5A). Additionally, enhancer activity was absent in $Vegfr2^{-/-}$ embryos (Figure S5A), although this is likely the result of reduced vasculogenesis (Shalaby et al., 1995).

To determine which cis elements within F2 mediate this Vegf responsiveness we performed luciferase assays in cultured arterial endothelial cells treated with VEGF or the VEGF receptor inhibitor, SU5416. Endogenous *DLL4* mRNA was elevated in cells treated with VEGF compared to cells treated with SU5416 (Figure 5E). While wild-type F2-*luciferase* activity was enhanced upon VEGF-treatment, mutation of ETS-B abrogated Vegf responsiveness and this reduction was only slightly more pronounced in the ETS-6x mutant (Figure 5F). While mutation of the RBPJk site modestly reduced enhancer activity, this reduction was not as severe as mutation of the ETS elements. This suggests that the Vegf responsiveness of the *Dll4* enhancer is largely ETS-dependent.

Control of ETS activity by Vegf signaling could be mediated by alterations in the expression and/or activity of ETS factors. We first assessed whether the recruitment of ETS factors to the enhancer at the endogenous *DLL4* locus was dependent on Vegf signaling. RNA Polymerase II occupancy at the *DLL4* enhancer in cultured arterial cells was enhanced in VEGF-treated cells compared to cells treated with VEGF inhibitor (Figure 5G), a finding consistent with transcriptional regulation of *DLL4*. Importantly, ERG occupancy of the enhancer was also increased in the presence of Vegf signaling (Figure 5G). However, Vegf treatment did not alter ERG protein levels (Figure 5H), or ERG subcellular localization in arterial cells (Figure 5I and Figure S5B). Interestingly, ERG protein levels were elevated in lysates from cells of arterial origin (human umbilical artery endothelial cells (HUAEC)) compared to corresponding venous cells (HUVEC) (Figure 5J), and expression of ERG was moderately enriched in the dorsal aorta compared to the cardinal vein in vivo (Figure S5C), suggesting that ERG is enriched in arterial cells. Although ERG levels were not affected by short-term inhibition of Vegf signaling in cultured arterial cells, the expression of several ETS factors are Vegf-responsive in vitro (Ghosh et al., 2012; Heo et al., 2010). Therefore, we examined the levels of endothelial-enriched ETS factors (Liu and Patient, 2008) to determine whether Vegf regulated their expression during vascular development. Compared to controls, qRT-PCR analyses revealed that levels of *elf2a*, *elf2b, elk4, erg, ets1a* and *fli1a* were significantly reduced in *vegfa* morphants (Figure S5D,E). In contrast, the expression of *etv2*, which acts early in the gastrulation-stage mesoderm to specify endothelial cells (Lee et al., 2008), was unaffected by Vegf inhibition (Figure S5D,E). Thus, expression of several endothelial ETS factors is modulated by Vegf signaling in vivo, and their decreased expression coincides with a reduction in *dll4* expression.

To determine if ETS factors function downstream of Vegf signaling to induce *Dll4* expression, we over-expressed ERG in *vegfa* morphants. Co-injection of *ERG* mRNA partially rescued *Dll4* F2 enhancer activity, sprouting of ISVs, and endogenous *dll4* expression in *vegfa* morphant embryos (Figure 6A,B). Interestingly, F2 enhancer activity was expanded into the vein in ERG over-expressing embryos. Conversely, knock-down of *erg* and *fli1a* (using a sub-phenotypic dose of morpholino) prevented the Vegf-mediated induction of F2:GFP in both the artery and the vein (Figure 6C). Increased expression of endogenous *dll4* (Figure 6D) and *efnb2a* (Figure S6A) induced by Vegf over-expression was also blunted in *erg/fli1a* morphants. Collectively, our results show that Vegf regulates ETS factors in arterial endothelial cells by inducing their expression and recruitment to DNA. Vegf-regulated ETS factors in turn control F2 enhancer activity, endogenous *Dll4* expression and contribute to arterial specification.

The Vegf/MAPK pathway activates an arterial transcriptional program through activation of ETS factors

Vegf-mediated activation of the p42/p44 MAPK and PI3K signaling pathways are known to promote or antagonize arterial specification, respectively (Deng et al., 2013; Hong et al., 2006; Ren et al., 2010). Pharmacological inhibition of p42/p44 MAPK signaling in arterial cells in vitro decreased *DLL4* expression (Figure 7A). An arterial-enriched Notch receptor gene, *NOTCH4*, was also reduced, but *NOTCH1* and venous gene expression (*EPHB4* and *COUP-TFII*), were unchanged (Figure 7A). Consistent with decreased Notch ligand (ie. *DLL4*) and receptor (i.e. *NOTCH4*) levels, expression of a Notch-activated transcription factor gene, *HES1*, and a Notch-dependent arterial marker, *EFNB2*, were also reduced (Figure 7A). These data suggest that MAPK signaling is required for the maintenance of Notch signaling in arterial endothelium. In contrast, inhibition of PI3K activity with the small molecule, LY29004, enhanced expression of *DLL4, NOTCH4, HES1* and *EFNB2* (data not shown). Since *NOTCH4* was regulated similarly to *DLL4* we examined ECRs within the *NOTCH4* locus for conserved ETS elements. An ECR within intron 1 of

NOTCH4 contained multiple conserved ETS sites, and this region had high ERG occupancy in arterial cells, as assessed by ChIP (Figure 7B). Over-expression of ERG in HUVEC induced *NOTCH4* mRNA, suggesting that *NOTCH4* is an ETS factor-regulated gene (Figure 7C). Similar to *DLL4, NOTCH4* expression was down-regulated upon Vegf inhibition (Figure 7D), and this was accompanied by a decrease in ERG recruitment to intron 1 (Figure 7E). We also found that ERG recruitment to *DLL4* intron 3 and *NOTCH4* intron 1 was reduced in cells treated with a MAPK inhibitor (Figure 7F), suggesting that ERG recruitment to these enhancers is MAPK-dependent. Accordingly, Vegf-induced *Dll4* F2-luciferase activity was suppressed by MAPK inhibition in arterial cells and mutation of the ETS-B site largely abrogated this responsiveness (Figure 7G). In contrast, inhibition of PI3K signaling enhanced Vegf-induction of F2-luciferase activity, and this was also dependent on the ETS-B site (Figure S6B), suggesting that PI3K inhibition may enhance ETS activity. To determine whether MAPK regulates *Dll4* in vivo, we inhibited MAPK signaling beginning at the 10-somite stage and found a significant decrease in F2:GFP expression at 28 hpf (Figure S6C,D). Expression of endogenous *dll4* and *hey2* mRNA was also reduced, while *efnb2a* levels were modestly decreased (Figure S6E). However, *kdrl*:GFP and *kdr* expression were unaffected (Figure S6D,E). In agreement with the MAPK inhibitor data, over-expression of dominant active MEKK in endothelial cells resulted in ectopic expression of F2:GFP within venous cells in vivo (Figure S6F), implying a cell-autonomous role for MAPK signaling in the regulation of arterial *Dll4* expression.

Discussion

Hh/Vegf-dependent activation of *Dll4* expression and Notch/RBPJk activity is a prerequisite for early artery specification and maintenance in the developing vertebrate embryo (Swift and Weinstein, 2009). The MAPK pathway activates the arterial program downstream of VEGFR2, while PI3K signaling antagonizes the MAPK pathway and arterial fate (Deng et al., 2013; Hong et al., 2006; Ren et al., 2010). However, the transcriptional mediators that are activated downstream of VEGFR2/MAPK that induce *Dll4* expression, and hence Notch activation, remain unknown. Herein we report the identification of an arterial-specific enhancer (F2) of *Dll4*, one of the earliest markers of the coalescing aPCs that form the dorsal aortae. The activity of the *Dll4* F2 enhancer described here recapitulates endogenous *Dll4* expression in the arterial and endocardial endothelium of the developing embryo. Functional analysis of this enhancer reveals that a minimal 36-bp DNA element, when concatenated, can drive arterial-specific expression, and through systematic mutation of the cis elements in this enhancer, we identify a previously undescribed role for the ETS family of transcription factors in the Vegf- and MAPK-dependent initiation of *Dll4* expression (Figure 7H). Critically, *Notch4* is also regulated by this Vegf/MAPK/ETS pathway, suggesting that this genetic network may coordinately activate expression of Notch signaling components to initiate Notch signaling in the early artery.

Previous work implicated FOXC1/2 and β-catenin in the regulation of *Dll4* expression (Corada et al., 2010; Seo et al., 2006; Yamamizu et al., 2010). However, our results argue against these factors directing the arterial-specific expression of *Dll4*. In the case of *Foxc1/2*-dependent regulation of *Dll4*, the conclusions rely largely upon the phenotype of $Foxc1^{-/-};Foxc2^{-/-}$ mice, which have AVMs and reduced arterial gene expression. However, these embryos also completely lack somites 1–8 at E8.5 (Seo et al., 2006), which are a critical source of Hh-induced Vegf that is required for artery specification (Coultas et al., 2010). Similarly, while prior studies suggested that canonical Wnt/β-catenin signaling is active in early arteries (i.e. E9.5), and that β-catenin drives *Dll4* promoter activity (Corada et al., 2010), we find no evidence of active canonical Wnt/β-catenin signaling in early arteries (i.e. E8.5 and E9.5) and *Dll4* expression in the dorsal aorta is intact when β-*catenin* is deleted from the endothelium, suggesting that β-catenin is dispensable for early artery

specification. Finally, the genomic fragment that is located just upstream of *Dll4* – where β-*catenin*/TCF/LEF and Foxc1/2 have been suggested to function – does not direct arterial-specific expression.

Vegf-mediated activation of Notch signaling, via NICD/RBPJk transcriptional activity is necessary and sufficient to induce downstream transcription factors, such as *Hey2* and other arterial markers, such as *Efnb2* (Swift and Weinstein, 2009). However, we postulate that Notch signaling is not required for the initiation of *Dll4* expression and primitive dorsal aortae formation. Dll4 is the first Notch ligand expressed in the forming arteries in the mouse, and *Dll4* expression (first detected at E8.0) precedes that of the genes encoding arterial Notch receptors, *Notch1* and *Notch4* (detected at E8.25) (Chong et al., 2011); calling into question how endothelial cell-autonomous Notch signaling could initiate *Dll4* expression. Indeed, $Notch1^{-/-};Notch4^{-/-}$ mice have normal levels of *Dll4* at E9.5 (albeit in an abnormal expression pattern), and the dorsal aortae are still formed (Krebs et al., 2000). Similarly, dorsal aortae are present when *Rbpjk* is deleted specifically in the endothelium (Krebs et al., 2004). While the arterial enhancer of *Dll4* that we isolated (F2) contains a conserved functional binding site for RBPJk, we provide several lines of evidence demonstrating that Notch signaling is not required for the initial arterial expression of *Dll4*. First, mutation of the RBPJk site in the context of the full-length enhancer does not affect its arterial-specificity in mice or zebrafish embryos. Second, global loss of *Rbpjk* in embryonic mice or zebrafish fails to abolish enhancer activity early in arterial development. Third, early arterial expression of endogenous *Dll4* mRNA is present, albeit at reduced levels, in $Rbpjk^{-/-}$ embryos. However, endothelial-specific ablation of Notch signaling does not diminish F2 activity at E8.5 or E9.5, suggesting a possible non-endothelial role for Notch signaling in regulating *Dll4.* Interestingly, *Dll4* enhancer activity in the dorsal aorta is greatly reduced in later-stage *rbpjk* morphant zebrafish embryos, suggesting that Notch signaling maintains *Dll4* expression. Collectively, our findings demonstrate that initiation of *Dll4* expression during artery specification is Notch-independent. We propose that *Dll4* expression is initiated during vasculogenesis (through Notch-independent mechanisms; see below), and that Notch signaling subsequently becomes activated by a Vegf/MAPK/ETS pathway that regulates both *Dll4* and *Notch4* (Figure 7H). Subsequently, *Dll4* expression is maintained through a Notch-dependent positive feedback loop to sustain artery specification, as has been previously suggested (Caolo et al., 2010).

A highly conserved ETS site (i.e. ETS-B) is responsible for the majority of the activity of the arterial enhancer of *Dll4*, including its responsiveness to Vegf/MAPK signaling. How then do ETS factors, many of which are uniformly expressed in the vasculature, regulate the arterial specificity of *Dll4*? The *Dll4* enhancer can be bound (Figure S3D) and activated (data not shown) by the angioblast-enriched ETS family member ETV2 (Lee et al., 2008). Interestingly, the first angioblasts to arise during murine development appear to be arterial (Chong et al., 2011). We therefore posit that ETV2 initiates *Dll4* expression during vasculogenesis (Figure 7H). Our data further suggests that a second wave of Vegf-dependent ETS factors appears to reinforce *Dll4* expression in the forming dorsal aortae (Figure 7H). We find that Vegf regulates the expression of several zebrafish ETS factors in the endothelium, including *elf2a*, *elf2b*, *elk4*, *erg*, *ets1* and *fli1a*. Furthermore, ERG appears to be enriched in arterial cells, implying that some ETS factors may be arterial-enriched. More importantly, we discover that the recruitment of ERG to the *Dll4* F2 enhancer and an ECR within *Notch4* is induced by Vegf/MAPK signaling. In this case, differential ERG occupancy is not mediated by changes in total ERG levels or subcellular localization, suggesting that Vegf/MAPK signaling enhances the DNA binding activity of ERG. We have also observed Vegf-dependent recruitment of ETS1 to these same enhancers (unpublished results mined from data in (Zhang et al., 2013)), suggesting that additional ETS family members may be regulated similarly to ERG, and co-operatively control arterial

specification. Importantly, the enhanced activation of the *Dll4* enhancer and endogenous *dll4* in response to *vegfa* over-expression can be attenuated by knock-down of *erg* and *fli1a*, suggesting that ETS factors are necessary for the Vegf-induced expression of *Dll4*. We also find reduced *Dll4* expression in the dorsal aorta of $Erg^{\Delta Ex4/\Delta Ex4}$ mice and in *erg/fli1a* morphant zebrafish embryos, but failed to observe AVMs, suggesting that other ETS factors compensate in the specification of the early artery.

Vegf is known to selectively activate MAPK signaling in arteries compared to veins during embryogenesis (Corson et al., 2003; Hong et al., 2006). Interestingly, several ETS family members are phosphorylated by MAPKs (Hill et al., 1993; Murakami et al., 2011; Petrovic et al., 2003), and these modifications are known to affect their interaction with other transcription factors as well as their binding to DNA (Hollenhorst et al., 2011). Future experiments will examine ETS factor phosphorylation downstream of Vegf/MAPK signaling, and whether ETS factors functionally interact with other transcription factor families to establish arterial fate. Finally, we also demonstrate that a Vegf/MAPK/ETS pathway regulates *Notch4*, suggesting that this pathway lies upstream of induction of Notch signaling in the arterial endothelium. Indeed, we observe reduced Notch-regulated gene expression and Notch-dependent reporter activity in embryos with reduced ETS activity. Interestingly, *Notch4* is not as sensitive as *Dll4* to ERG over-expression, which may in part explain the delay in *Notch4* expression during artery formation (Chong et al., 2011).

In summary, we have uncovered a genetic pathway that integrates Vegf signaling, ETS-dependent transcriptional regulation, and the induction of one of the earliest, essential ligands for artery specification and Notch pathway activation, *Dll4*. This same pathway also regulates the expression of Notch4, an arterial-specific receptor for Dll4, and appears to initiate Notch signaling in the artery. These findings may provide insight into congenital defects in AV specification and maintenance and suggest novel approaches to direct endothelial cells towards the arterial lineage.

Experimental Procedures

Bioinformatic analyses, cloning, mutagenesis, generation of transgenic mice and mouse lines used

See Supplemental Experimental Procedures for details.

Mouse experiments

All mouse protocols were approved by the Institutional Animal Care and Use Committee at UCSF and Harvard Medical School. Histology and in situ hybridization (Dodou et al., 2003; Wythe et al., 2011), ink injections (Krebs et al., 2004) and CD31 immunofluorescence (Coultas et al., 2010) were performed as described elsewhere.

Electrophoretic mobility shift assay

DNA binding assays were performed as described previously (Dodou et al., 2003). See Supplemental Experimental Procedures for details.

Cell Culture, Immunofluorescence, Luciferase Analysis and ChIP

Human umbilical vein endothelial cells (HUVECs) (ScienCell) and Bovine arterial endothelial cells (BAECs) (Lonza) were grown in endothelial media (ScienCell). BAECs were treated with 50 ng/ml recombinant Vegf-A_{165} (R&D Systems), 0.5 μM SU5416 (Sigma), 20 μM U0126 (Invivogen), or 10 μM LY29004 (Cell Signaling) for 24 h. Immunofluorescence of ERG and phalloidin was performed as described (Fish et al., 2011) using ERG antibody (Santa Cruz, C-20, 1:100 dilution). pCS2-6xMYC-Etv2 (De Val et al.,

2008) has been described and pCMV-Sport6-ERG was from Open Biosystems (IMAGE clone 6052140). Cloning of pCS2-V5-ERG is described in Supplemental Experimental Procedures. Luciferase experiments were performed as described (Cheng et al., 2013). Electroporation of HUVEC was performed using a Lonza 4D Nucleofector with the P5 Primary Cell Kit with 2.5 μg of expression construct and 0.5 μg of pmaxGFP. ChIP was performed using the Imprint Kit (Sigma) with 1 μg of antibody: RNA Pol II (mouse monoclonal, Sigma), ERG (rabbit polyclonal, Santa Cruz, C-20), V5 (mouse monoclonal, Invitrogen), or NICD (rabbit polyclonal, ChIP grade, Abcam). IgG (mouse, Sigma) was used as a negative control. Fold enrichment was calculated by determining the fold-change of qPCR values for the specific antibody compared to IgG control. See Table S1 for primer sequences.

Zebrafish experiments

Zebrafish protocols were approved by the Animal Care Committee at the University Health Network and UCSF. The following transgenic lines were utilized: *Tg(kdrl:ras-mCherry)*^{s896} (Chi et al., 2008), *Tg(kdrl:GFP)*^{s843} (Jin et al., 2005), *Tg(EPV.Tp1-MmuHbb:EGFP)*^{um14} (Parsons et al., 2009). See Supplemental Experimental Procedures for generation of *Tg(Dll4-F2-E1b:GFP)* and experimental details.

Quantitative reverse-transcriptase real-time PCR (qRT-PCR)

qRT-PCR was performed as before (Fish et al, 2008). See Supplemental Experimental Procedures for details and Table S1 for primers sequences.

Western blot

Western blotting was performed as described previously (Fish et al., 2011) using ERG (rabbit polyclonal, Santa Cruz, C-20), Sin3a (Santa Cruz, AK-11), V5 (mouse monoclonal, Invitrogen) or GAPDH (mouse monoclonal, Santa Cruz) (mouse monoclonal, Abcam) antibodies. Cells were starved overnight, and treated with 50 ng/ml recombinant Vegf-A_{165} (R&D Systems) for 20 minutes before harvesting for sub-cellular fractionation, as previously described (Wythe et al., 2011). HUVEC and HUAEC lysates were purchased from Sciencell.

Statistics

All graphs depict the mean ± standard error of the mean (SEM) of at least 3 independent experiments, unless otherwise indicated. Statistical significance was performed using a Student's T-test, ANOVA or Chi-squared test as appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Identification of a Vegf/MAPK-dependent arterial-specific enhancer of *Dll4*
- Enhancer activity requires ETS transcription factors
- Vegf/MAPK signaling drives recruitment of ETS factors to *Dll4* and *Notch4* enhancers
- Notch signaling is required for maintenance, not specification, of arterial identity

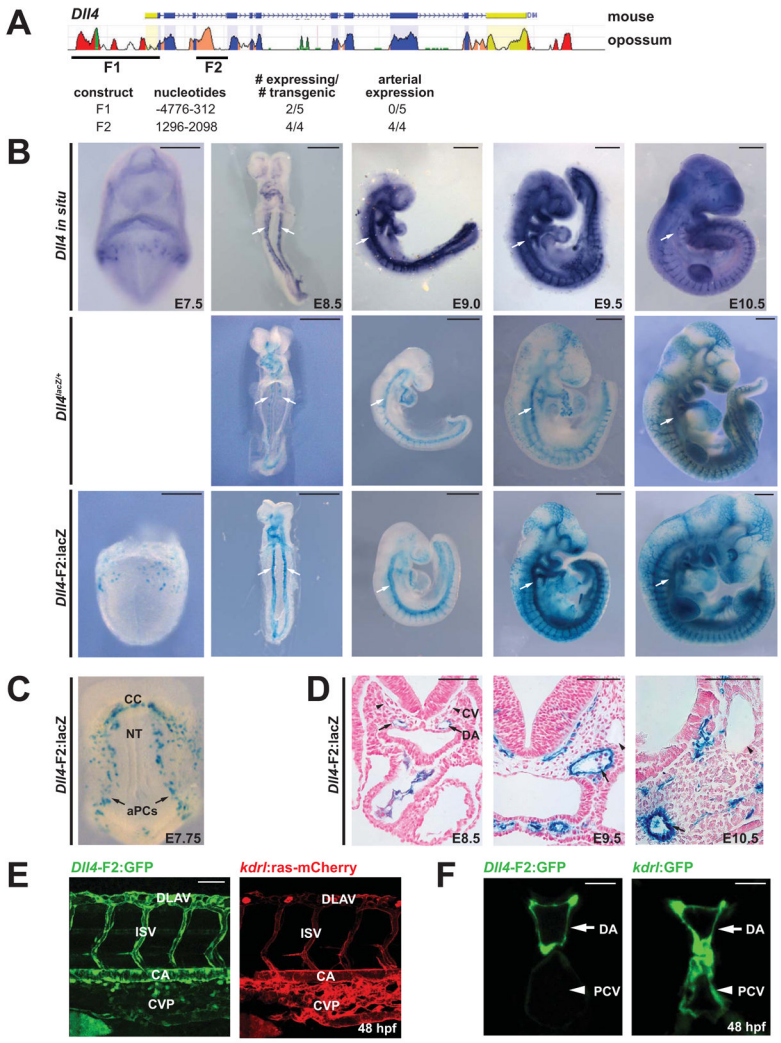

Figure 1. Identification of an intronic enhancer of *Dll4* that drives arterial-specific expression

(**A**) Conservation between murine and opossum *Dll4* with location of fragment 1 (F1) and F2 indicated. Transgenic analysis of F1-*lacZ* and F2-*hsp68-lacZ* (E9.5) is below. Further analysis of F1-*lacZ* is shown in Figure S1A,B. (**B**) In situ hybridization of endogenous *Dll4* (top) and expression in $Dll4^{lacZ/+}$ (middle) and a stable *Dll4*-F2-*hps68-lacZ* (F2) reporter line (bottom). Dorsal aorta (arrows). (**C**) F2 expression in early arterial precursors (aPCs) and in cardiac crescent (CC). NT, neural tube. (**D**) Transverse sections of F2 expression. DA, dorsal aorta (arrow); CV, cardinal vein (caret). (**E**) A stable *Dll4*-F2-*E1b*:GFP transgenic zebrafish line demonstrates arterial-specific expression. *kdrl*:ras-mCherry marks all blood vessels. CA, caudal artery; CVP, caudal vein plexus; ISV, intersomitic vessel; DLAV, dorsal longitudinal anastomotic vessel. (**F**) Cross-section of axial vasculature of F2:*GFP* zebrafish. PCV, posterior cardinal vein. Scale bars: 500 μm (B), 100 μm (D), 50 μm (E), 10 μm (F). See also Figure S1.

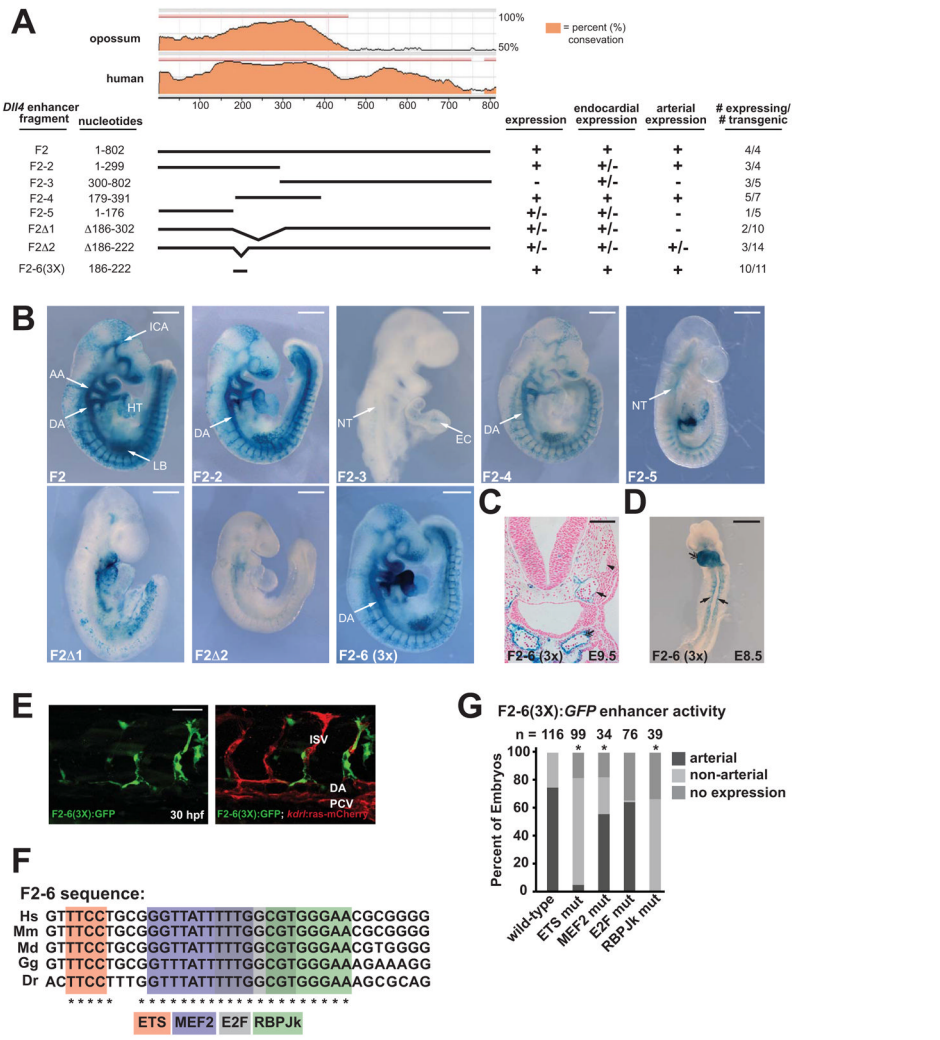

Figure 2. Isolation of a minimal *Dll4* enhancer element that drives arterial-specific expression

(**A**) Sequence conservation of the F2 enhancer and deletion constructs used in transgenic analyses. Endocardial and arterial expression is indicated. (**B**) Representative transgenic embryos from panel (A) at E9.5. F2, F2-2, and F2-4 directed strong arterial-specific expression. F2-3 directed weak endocardial (EC) expression. Deletion of a highly conserved 100- and 36-bp region (F2Δ1 and F2Δ2, respectively) abrogated arterial activity of the F2 enhancer. The 36-bp region, F2-6, was sufficient, when arrayed in triplicate, F2-6(3X), to direct arterial expression. DA, dorsal aorta; AA, aortic arch; NT, neural tube; ICA, internal cerebral artery; HT, heart. Scale bar = 500 μm. (**C**) Transverse section of X-gal stained F2-6(3X)-*lacZ* embryo at E9.5. DA (arrow); cardinal vein (caret); EC (double arrow). Scale bar = 100 μm. (**D**) Whole-mount image of F2-6(3X)-*lacZ* embryo at E8.5. Scale bar = 500 μm. (**E**) Mosaic expression of F2-6(3x):GFP in the DA and intersomitic vessels (ISVs) of a transient transgenic 30 hpf zebrafish embryo. PCV, posterior cardinal vein. Scale bar = 50 μm. **(F**) Sequence comparison of F2-6 in human (Hs), mouse (Mm), opossum (Md), chicken (Gg) and zebrafish (Dr). *Cis* elements are indicated. (**G**) Each *cis* element was mutated in F2-6(3X):*GFP* and transient transgenics were assessed for arterial expression at 24 hpf. The percentage of embryos with expression in arteries (i.e. DA and/or ISV), expression elsewhere, and no expression, are indicated. * indicates a significant difference in arterial expression compared to wild-type (Chi-squared test). See also Figure S2.

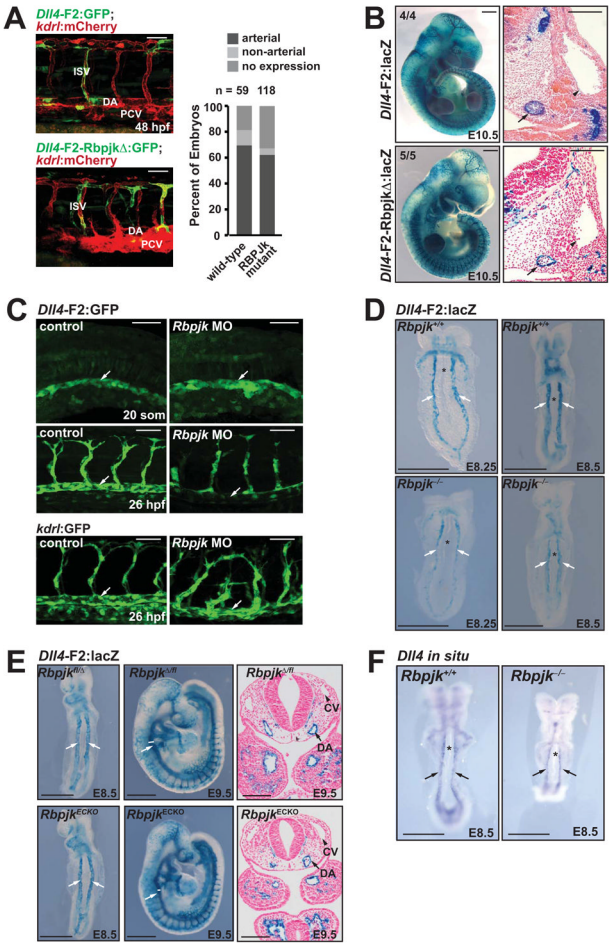

Figure 3. Notch signaling is not required for early arterial expression of *Dll4*

(**A**)Transient transgenesis of wild-type F2:*GFP* compared to a RBPJk-mutant construct (F2ΔRBPJk:*GFP*). DA, dorsal aorta; PCV, posterior cardinal vein; ISV, intersomitic vessel. (**B**) Whole-mount images (left), and transverse sections (right) of wild-type and RBPJk-mutated F2-*lacZ* transgenic embryos. (**C**) Expression of F2:GFP in control and *rbpjk* morphant (MO) embryos, demonstrating normal enhancer activity in the DA (arrow) at 20 somites, but decreased expression at 26 hpf. Expression of *kdrl*:GFP in the DA was unaffected. (**D**) F2-lacZ expression was reduced but present in the DA of $Rpbjk^{-/-}$ embryos at E8.5 ($Rpbjk^{+/+}$, n=25; $Rpbjk^{-/-}$, n=5) and E8.25 ($Rpbjk^{+/+}$, n=14; $Rpbjk^{-/-}$, n=4). (**E**) F2-lacZ expression was normal at E8.5 (left), and at E9.5 in whole-mount (center) and transverse sections (right) from embryos with endothelial cell-specific loss (ECKO) of *Rbpjk*, although mutants had a reduced DA diameter (bar) at E9.5, as previously reported (Krebs et al., 2004). E9.5, $Rpbjk^{del/fl}$, n=6; $Rpbjk^{ECKO}$, n=4; E8.5, $Rpbjk^{del/fl}$, n=8; $Rpbjk^{ECKO}$, n=3. (**F**) In situ hybridization shows that *Rbpjk* is not absolutely required for *Dll4* expression at E8.5 (n=3 for each genotype). DA, arrows; asterisk, neural tube. Scale bars: 50 μm (A,C), 500 μm for whole-mounts and 100 μm for sections (B,D,E,F). See also Figure S2.

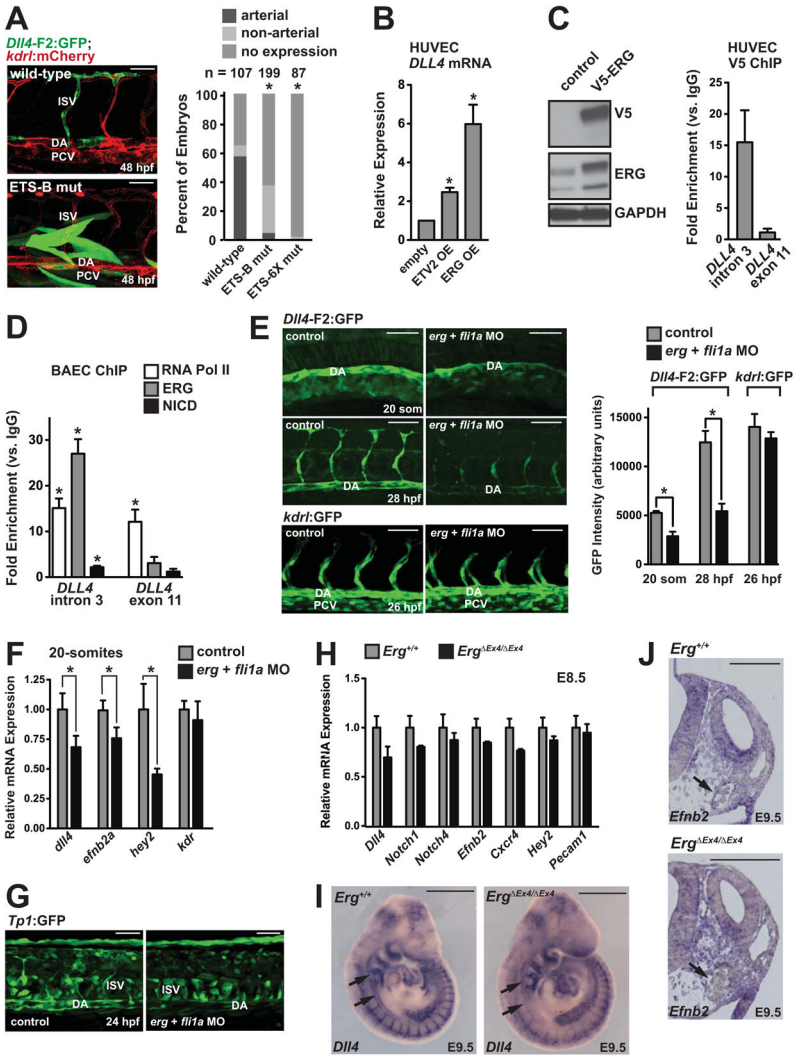

Figure 4. *Dll4* is regulated by ETS factors

(**A**) Representative images (left) and quantification (right) of wild-type, ETS-B and ETS-6x mutant F2:*GFP* embryos at 48 hpf. ISV, intersomitic vessel; DA, dorsal aorta; PCV, posterior cardinal vein. * indicates a significant difference in arterial expression compared to wild-type (Chi-squared test). (**B**) Expression of endogenous *DLL4* in HUVEC electroporated with *ETV2* or *ERG* expression constructs. (n=3, *ETV2*; n=5, *ERG*). (**C**) V5-ERG expression in electroporated HUVEC (left). ChIP in V5-ERG-electroporated HUVEC. Fold enrichment (V5 vs IgG) was measured at the *Dll4* enhancer (intron 3) and *Dll4* exon 11 (n=2). (**D**) ChIP in BAEC for RNA Polymerase II (RNA Pol II), ERG or the Notch intracellular domain (NICD) (n=3). * indicates significant enrichment over IgG control. (**E**) Expression of F2:GFP (arterial) and *kdrl*:GFP (pan-endothelial) in *erg;fli1a* morphants. Representative images (left) and quantification (n=5–10) (right). (**F**) Endogenous *dll4*, *ephrinb2a, hey2* and *kdr* in control and *erg;fli1a* morphants assessed by qPCR (n=5–10 individual embryos). (**G**) Notch activity (*Tp1*:GFP) was diminished in the dorsal aorta of ~30% of *erg/fli1a* morphants. (**H**) Levels of arterial markers and a pan-endothelial marker (*Pecam1*) were quantified by qRT-PCR in E8.5 embryos (n=3 for wild-type, n=2 for $Erg^{\Delta Ex4/\Delta Ex4}$ embryos). (**I**) Endogenous *Dll4* mRNA is down-regulated in the DA of $Erg^{\Delta Ex4/\Delta Ex4}$ embryos (arrows; n=3 embryos per genotype) at E9.5. (**J**) *Efnb2* section in

situ hybridization at 9.5 shows down-regulation of *Efnb2* in the DA (arrows) of $Erg^{\Delta Ex4/\Delta Ex4}$ embryos ($Erg^{+/+}$, n=3; $Erg^{\Delta Ex4/\Delta Ex4}$ n=2). Scale bars: 50 μm (A,E,G), 500 μm (I), 100 μm (J). All data is mean ± SEM. See also Figures S3 and S4.

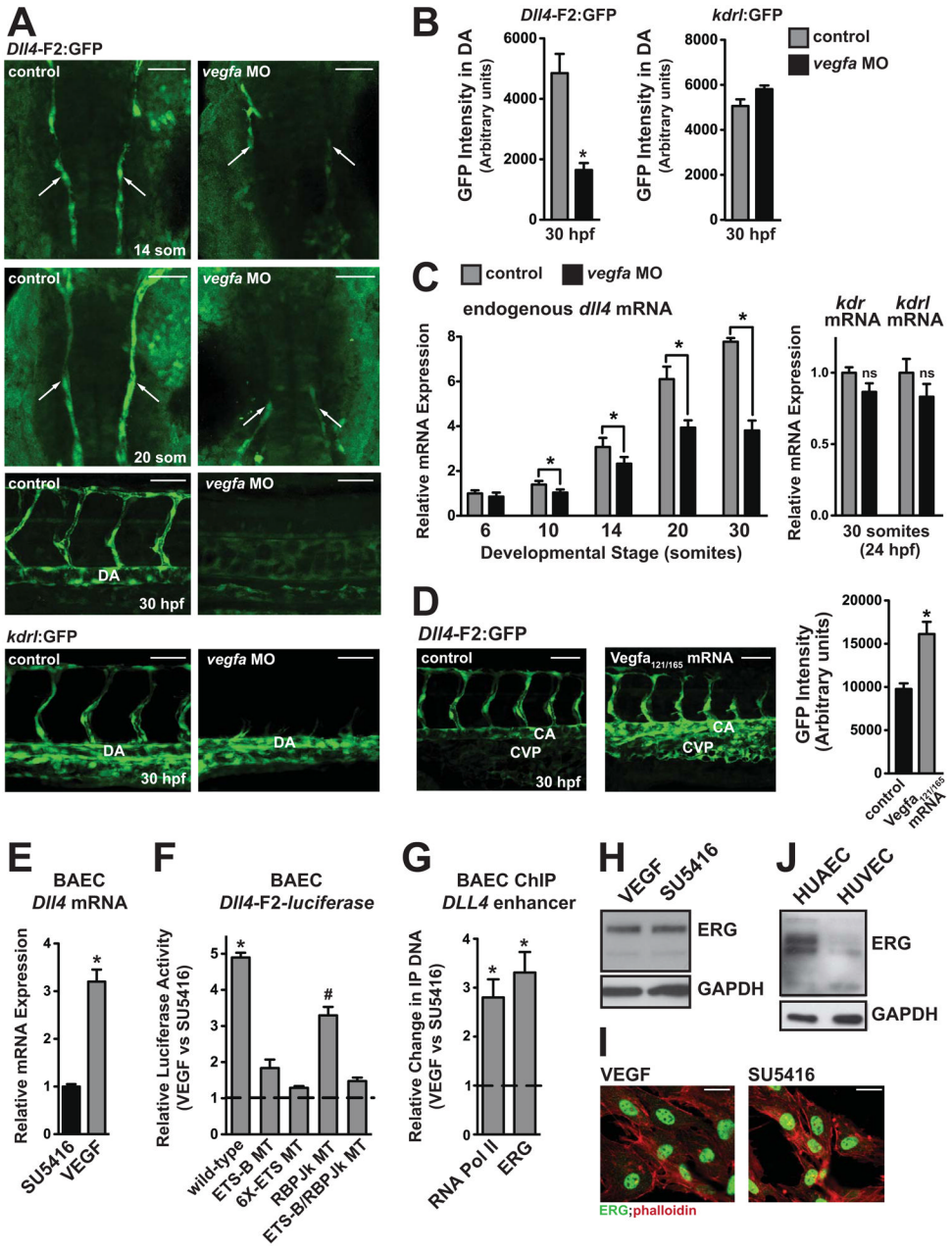

Figure 5. Vegf regulates the *Dll4* enhancer through an ETS element

(**A**) Expression of F2:GFP (arterial) and *kdrl*:GFP (pan-endothelial) in control or *vegfa* morphant embryos. Lateral dorsal aorta (arrows); dorsal aorta (DA). Scale bar = 50 μm. (**B**) Quantification of GFP intensity in the DA (n≥10 per group). (**C**) Expression (qRT-PCR) of *dll4* (arterial), *kdr* and *kdrl* (pan-endothelial) in control and *vegfa* morphant embryos (n≥3 per group). (**D**) Over-expression of Vegfa enhanced expression of F2:GFP in arterial cells and expanded expression to venous cells. CA, caudal artery; CVP, caudal vein plexus. Quantification is shown (n=6–8 embryos per group). Scale bar = 50 μm. (**E**) Expression of *DLL4* (qRT-PCR) in BAECs treated with VEGF or VEGF receptor inhibitor (SU5416) for 24 h (n=3). (**F**) Relative F2:*luciferase* activity in BAEC treated with VEGF or SU5416. A representative experiment with 3 technical replicates is shown. * indicates a significant difference in luciferase activity compared to all other constructs. # indicates a significant

difference between RBPJk MT and ETS-B MT, 6x-ETS MT and RBPJk/ETS-B MT. (**G**) ChIP assays for RNA Pol II and ERG in BAEC treated with VEGF or SU5416 for 24 h (n=3). (**H**) Western blotting demonstrating equal expression of ERG protein in BAECs treated with VEGF or SU5416 for 24 h. (**I**) Intracellular localization of ERG was unchanged by VEGF treatment. Scale bar = 20 μm. (**J**) Expression of ERG was elevated in arterial cells (HUAEC) compared to venous cells (HUVEC). All data is mean ± SEM. See Figure S5.

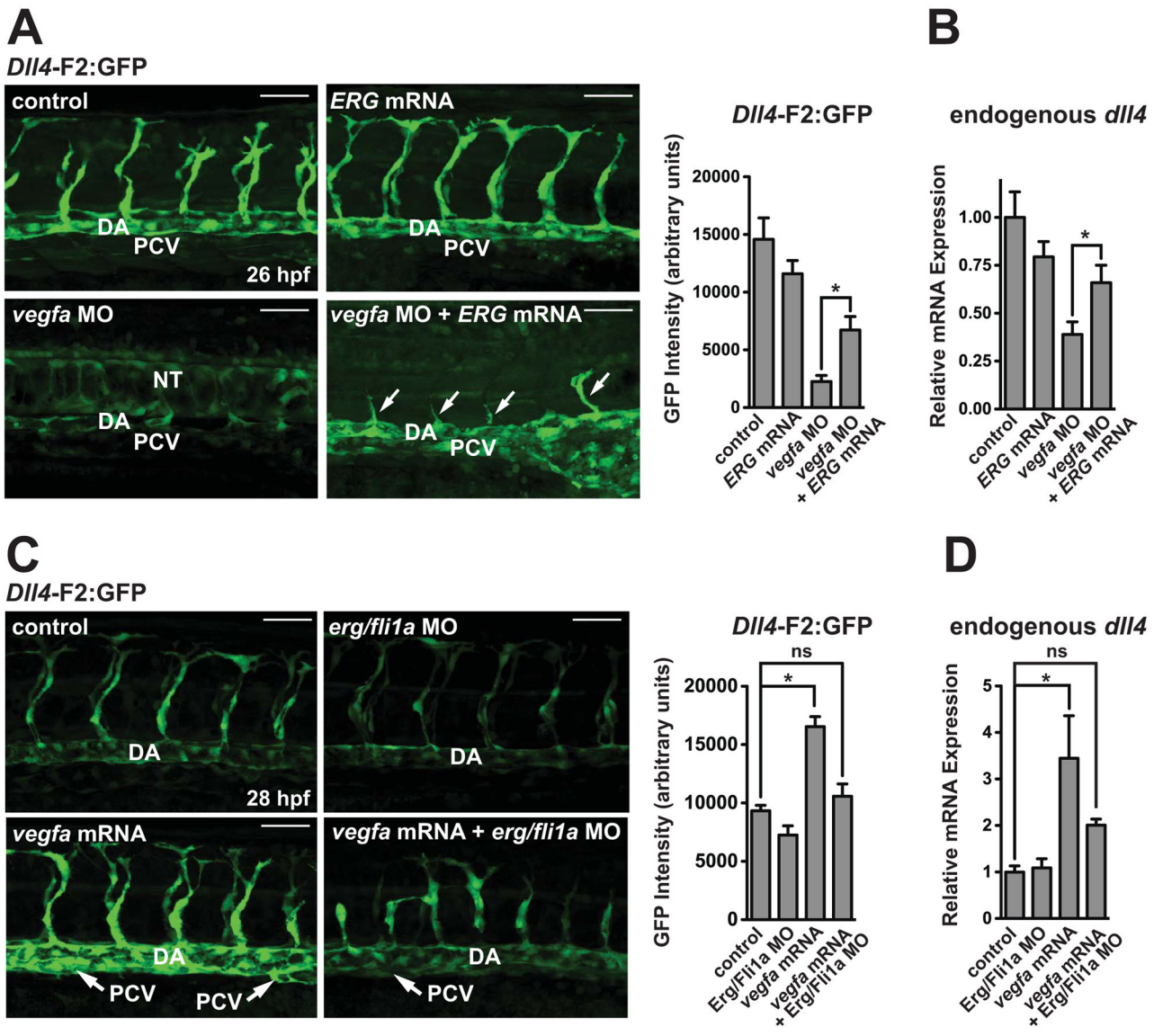

Figure 6. ETS factors act downstream of Vegf to induce *dll4* expression

(**A**) *ERG* mRNA (200 pg) injection partially rescued F2:GFP expression and sprouting (arrows) in *vegfa* morphants at 26 hpf. Representative images (left) and quantification (right, n=10–12). (**B**) *dll4* mRNA expression (normalized to *kdr* expression) was assessed by qRT-PCR in individual 26 hpf embryos injected with *ERG* mRNA and/or *vegfa* morpholino (n=4). (**C**) Partial knock-down of *erg* and *fli1a* with a sub-phenotypic dose of morpholino inhibited the induction of F2:GFP expression in both the dorsal aorta (DA) and posterior cardinal vein (PCV) in *Vegfa*$_{121/165}$ mRNA-injected embryos. Arrows indicate collapsed PCV. Quantification of 10-13 embryos per group is shown. (**D**) Endogenous *dll4* mRNA was assessed by qRT-PCR in individual embryos at 24 hpf (n=5). All data is mean ± SEM. See Figure S5A.

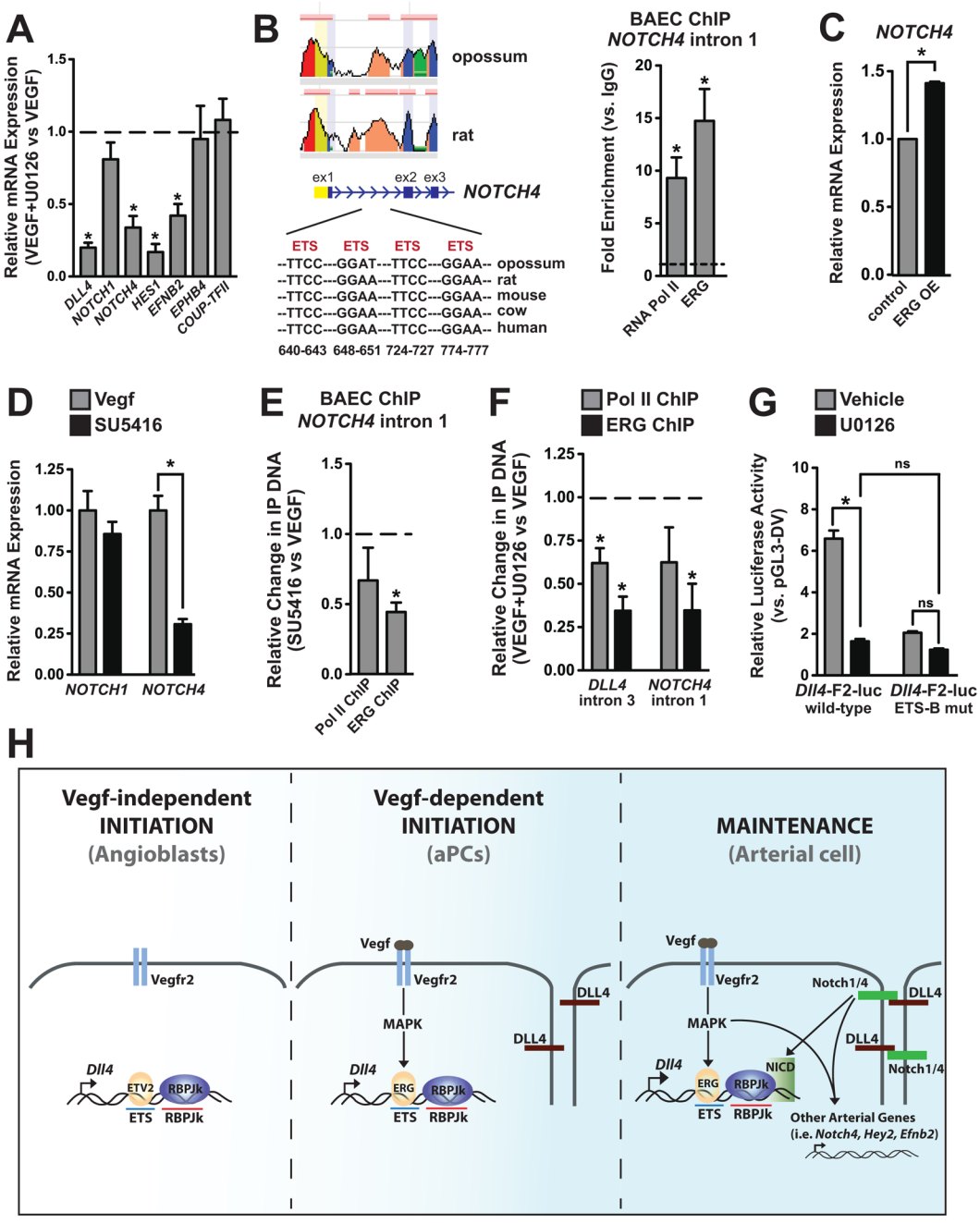

Figure 7. Vegf/MAPK signaling activates arterial genes by promoting ETS factor binding

(**A**) Expression of arterial genes (*DLL4*, *NOTCH4, EFNB2*) and the Notch target gene, *HES1*, were decreased in VEGF-treated BAEC co-treated with the MAPK inhibitor, U0126 (n=4). *NOTCH1* and vein markers were unaffected. (**B**) An ECR (human reference genome) present in intron 1 of *NOTCH4* contains multiple conserved ETS elements and binds ERG, as assessed by ChIP assay (n=6). (**C**) Over-expression of ERG in HUVEC increased *NOTCH4* expression by qRT-PCR (n=4). (**D**) Inhibition of Vegf signaling decreased *NOTCH4* expression, but had no effect on *NOTCH1* (n=3). (**E**) Inhibition of Vegf signaling decreased ERG binding to intron 1 of *NOTCH4* (n=5). (**F**) Inhibition of MAPK signaling decreased ERG binding to *DLL4* intron 3 and *NOTCH1* intron 1 (n=3). (**G**) Inhibition of

MAPK decreased F2:luciferase activity and mutation of the ETS-B site similarly decreased activity and abrogated MAPK-responsiveness. Shown is a representative experiment, triplicate determinations. (**H**) Model of *Dll4* regulation during artery development. At early stages of vasculogenesis (left), ETV2 is expressed independent of Vegf signaling in angioblasts and activates the *Dll4* enhancer. At later stages of development, activity of other ETS factors, such as ERG are induced downstream of Vegf/MAPK signaling in the arterial precursor cells (aPCs) (middle), and expression of *Dll4* and *Notch4* is activated. Once Dll4 and Notch receptors are both expressed, continued Notch signaling maintains *Dll4* enhancer activity and activates and maintains the expression of other arterial genes (right). All data is mean ± SEM. See Figure S6.