Dynamic regulation of VEGF-inducible genes by an ERK/ERG/p300 transcriptional network

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ABSTRACT

The transcriptional pathways activated downstream of vascular endothelial growth factor (VEGF) signaling during angiogenesis remain incompletely characterized. By assessing the signals responsible for induction of the Notch ligand delta-like 4 (DLL4) in endothelial cells, we find that activation of the MAPK/ERK pathway mirrors the rapid and dynamic induction of DLL4 transcription and that this pathway is required for DLL4 expression. Furthermore, VEGF/ERK signaling induces phosphorylation and activation of the ETS transcription factor ERG, a prerequisite for DLL4 induction. Transcription of DLL4 coincides with dynamic ERG-dependent recruitment of the transcriptional co-activator p300. Genome-wide gene expression profiling identified a network of VEGF-responsive and ERG-dependent genes, and ERG chromatin immunoprecipitation (ChIP)-seq revealed the presence of conserved ERG-bound putative ERG-dependent genes, and ERG chromatin immunoprecipitation (ChIP)-seq revealed the presence of conserved ERG-bound putative enhancer elements near these target genes. Functional experiments performed in vitro and in vivo confirm that this network of genes requires ERK, ERG and p300 activity. Finally, genome-editing and transgenic approaches demonstrate that a highly conserved ERG-bound enhancer located upstream of HLX (which encodes a transcription factor implicated in sprouting angiogenesis) is required for its VEGF-mediated induction. Collectively, these findings elucidate a novel transcriptional pathway contributing to VEGF-dependent angiogenesis.

KEY WORDS: Endothelial cell, Transcription, Enhancer, Angiogenesis, Genome editing, ETS factor, Zebrafish, Mouse, Human

INTRODUCTION

The growth of new blood vessels is requisite for tissue repair and homeostasis and contributes to the pathogenesis of several diseases, including cancer and diabetic retinopathy. A comprehensive understanding of the signaling pathways and downstream transcriptional networks that control angiogenesis could be leveraged to identify novel therapeutic targets to either promote or inhibit vascular growth. The central mechanism responsible for the majority of vascular growth is angiogenesis. Angiogenesis is a highly coordinated process that requires the interaction of several intracellular and intercellular signaling pathways that ultimately converge on a network of transcriptional pathways to elicit cellular behaviors (Herbert and Stainier, 2011). Vascular endothelial growth factor (VEGF), one of the central drivers of angiogenesis, is required for blood vessel development during embryogenesis (Carmeliet et al., 1996; Ferrara et al., 1996) and contributes to vascular homeostasis, as well as physiological and pathological postnatal vascular growth (Kim et al., 1993; Lee et al., 2007). VEGF activates a number of signal transduction pathways in endothelial cells (ECs) that modulate cytoskeletal dynamics and gene expression (Olsson et al., 2006), resulting in a suite of angiogenic cell behaviors, including directed, polarized cell migration. Although some of the transcriptional networks involved in VEGF signaling have been identified (Herbert and Stainier, 2011), much remains to be discovered regarding the mechanisms by which VEGF coordinates new vessel growth.

ECs receiving a threshold of VEGF stimulation initiate a signal transduction pathway that culminates in the transcription of the Notch ligand delta-like 4 (DLL4) (Lobov et al., 2007), as well as a network of other angiogenic genes (Liu et al., 2008). Phenotypic changes occur in the VEGF receiving cell, endowing it with ‘tip’ cell characteristics, including acquisition of numerous filopodial projections, increased migratory behavior, and elevated VEGF receptor 2 (VEGFR2; also known as KDR) expression (Blanco and Gerhardt, 2013). DLL4 on the surface of a tip cell binds to, and activates, Notch receptors on adjacent stalk cells. Notch activity in stalk cells induces the transcription of Notch-dependent genes, such as those encoding members of the basic helix-loop-helix transcription factor families HEY and HES, and suppresses filopodia formation and cell migration, while also dampening VEGFR2 expression. Importantly, tip and stalk cell phenotypes are dynamic, and in time a stalk cell can become a tip cell, and vice versa (Jakobsson et al., 2010). Coordinating these dynamic cellular behaviors is essential for an effective angiogenic response. The molecular mechanisms responsible for the maintenance and conversion between these phenotypes are only partially understood, and include oscillations in DLL4 induction in the tip cell (Lobov et al., 2007; Ubezio et al., 2016), as well as tight control of Notch signal duration in neighboring stalk cells (Guarani et al., 2011). How VEGF-regulated transcriptional programs control the dynamic expression of DLL4 and other angiogenic genes during sprouting angiogenesis remains poorly understood.
We previously identified a highly conserved enhancer element located within intron 3 of murine DLL4 that directs expression in arteries and angiogenic vessels (Wythe et al., 2013). Activity of this enhancer in arteries is VEGF responsive, and this is at least in part dependent on ETS transcription factors, including ETS-related gene (ERG) (Wythe et al., 2013). The ETS family of transcription factors play crucial roles in multiple stages of vascular development, including angiogenesis (Randi et al., 2009). More than a dozen ETS factors are expressed in ECs, and several of these [e.g. ETV2, TEL (ETV6), ETS1, ETS2, FLI1, ERG] have been implicated in vascular growth (Liu and Patient, 2008; Pham et al., 2007). ETS factors bind to a consensus 5′-GG(A/T)-3′ sequence in the genome through a highly conserved ~85 amino acid ETS domain (Sharrocks, 2001). Several of the family members also contain additional functional domains, such as the pointed (PNT) domain, a docking site for the serine/threonine kinase extracellular regulated kinase-2 (ERK2; MAPK1), which phosphorylates ETS1 and ETS2 in response to mitogen-activated protein kinase (MAPK) activation (Seidel and Graves, 2002). Phosphorylation of ETS1 and ETS2 enhances their activity through the recruitment of the transcriptional co-activator proteins p300 (EP300) and Cred-binding protein (CBP; CREBBP) (Foulds et al., 2004). Modulation of ETS factor activity by signal transduction pathways is not unique to ERK2, as other MAPK signaling pathways, such as p38 (MAPK14) and JNK (MAPK8) have been documented (Wasylk et al., 1998; Yordy and Muise-Helmericks, 2000).

The specificity of MAPK pathways for particular ETS family members has recently been explored in prostate cancer cells in vitro. Interestingly, of the three MAPKs analyzed (p38, JNK and ERK2), only ERK2 phosphorylates ERG (yet multiple MAPK members act on ETS1/2) (Selvaraj et al., 2015). ERK2 primarily phosphorylates three residues on ERG: S96 (amino terminal to the PNT domain), S215 and S276 (both carboxy terminal to the PNT domain). Crucially, mutation of S215 to alanine, an amino acid refractory to phosphorylation (a so-called ‘phospho mutant’), abolishes ERG function in prostate cancer cells (Selvaraj et al., 2015). Although several studies have implicated ERG as a mediator of EC survival, proliferation, motility and vascular integrity (Birdsey et al., 2009; Pham et al., 2007; Liu and Patient, 2008; Yuan et al., 2011), whether ERG acts as a hub, integrating signals downstream of VEGF to control these diverse EC behaviors is not known.

Here, we explore the signaling and transcriptional pathways activated downstream of VEGF signaling in ECs. We find that the dynamic induction of MAPK/ERK activity controls DLL4 transcription in human ECs and that MAPK/ERK is required for angiogenesis in zebrafish in vivo. Furthermore, we demonstrate that MAPK/ERK activity leads to phosphorylation of ERG, and that ERG is required for the induction of DLL4 and a network of other angiogenic genes in human, mouse and zebrafish ECs. Mechanistically, we show that ERG recruits p300 to enhance elements to coordinate angiogenic gene expression. These findings provide new insight into the molecular mechanisms of VEGF-mediated angiogenesis, and suggest that MAPK/ERK activation of ERG/p300 might represent a novel therapeutic target for modulating vascular growth.

RESULTS

Dynamic MAPK/ERK signaling regulates gene induction in response to VEGF stimulation

DLL4 is dynamically expressed in tip cells during sprouting angiogenesis (Hellström et al., 2007; Jakobsson et al., 2010; Suchting et al., 2007; Ubezio et al., 2016). We first delineated the kinetics of VEGF-dependent DLL4 transcription in vitro. We assayed DLL4 unspliced pre-mRNA (as a surrogate of transcription) and mature mRNA transcript levels in VEGF-stimulated serum- and growth factor-starved human microvascular ECs (MVECs) or human umbilical vein ECs (HUVECs). In both cell types, DLL4 transcription responded dynamically to VEGF stimulation, peaking at 15-30 min (15-30’) post addition of VEGF, and returning to baseline levels by 2 h (Fig. S1A; Fig. 1A). The expression of spliced DLL4 mRNA was also transient and dynamic, with expression peaking at 1 h and returning to near baseline levels by 2 h (Fig. S1A; Fig. 1A).

As VEGF engagement of its principal angiogenic receptor, VEGFR2, can activate multiple downstream signaling pathways, we employed a panel of pharmacological cell signaling inhibitors to define the pathway(s) responsible for the rapid and transient induction of DLL4 transcription. Inhibition of the MAPK/ERK signaling pathway [using inhibitors of either MEK or protein kinase C (PKC)] abrogated DLL4 induction in response to VEGF (Fig. S1B; Fig. 1B).

Immunofluorescent staining of VEGF-stimulated HUVECs revealed the presence of phosphorylated ERK (pERK) in both the nucleus and cytoplasm 15-30’ after treatment, with levels returning to baseline after 1 h (Fig. 1C). Measurement of pERK by western blotting mirrored the rapid and dynamic MAPK/ERK activation observed in immunofluorescence experiments, as pERK levels returned to near baseline levels by 1 h after stimulation (Fig. 1D). The kinetics of MAPK/ERK activation therefore parallels that of DLL4 transcription in response to VEGF treatment.

Several VEGF/MAPK/ERK-responsive genes have been characterized, including the immediate early gene early growth response 3 (EGR3) (Liu et al., 2008) and the ERK phosphatase dual specificity phosphatase 5 (DUSP5) (Bellou et al., 2009; Kucharska et al., 2009). The transcriptional induction of EGR3 and DUSP5 (as measured by qRT-PCR analysis of unspliced pre-mRNA) largely mirrored that of MAPK/ERK activation and DLL4 transcription (Fig. 1E). As expected, the induction of DLL4, EGR3 and DUSP5 mRNA was completely inhibited in the presence of the highly selective small molecule MEK inhibitor U0126 (Fig. 1F). In addition, the induction of these genes by VEGF stimulation was attenuated in HUVECs in which ERK1 (MAPK3) and ERK2 (MAPK1) were knocked down by siRNA (Fig. S1C). To determine whether MAPK/ERK activity in the absence of VEGF signaling was sufficient to induce expression of these genes, we treated serum-starved HUVECs with a PKC activator (and therefore an activator of MEK/ERK signaling), phorbol-ester myristate acetate (PMA) (Franklin et al., 1994; Schultz et al., 1997). PMA treatment markedly elevated DLL4, EGR3 and DUSP5 mRNA levels, and this response was blocked by pre-treatment with U0126, demonstrating that the MEK/ERK pathway is necessary and sufficient to activate transcription of a subset of angiogenic genes (Fig. 1G,H).

We further assessed the physiological relevance of MAPK/ERK signaling during sprouting angiogenesis in vivo. In agreement with recent reports (Costa et al., 2016; Shin et al., 2016), pERK was enriched in angiogenic sprouts (i.e. intersomitic vessels) in developing zebrafish embryos, indicative of active MAPK/ERK signaling (Fig. 2A; Fig. S2A). Importantly, inhibition of MAPK/ERK signaling using the MEK inhibitor SL327 completely abrogated the pERK signal throughout the embryo, including in the sprouting vessels (Fig. 2A; Fig. S2A). Inhibition of MEK signaling had a functional effect on angiogenesis, as sprout length (Fig. 2B) and the number of ECs per sprout (Fig. S2B) were decreased, as demonstrated previously (Shin et al., 2016). At this dose, SL327 did not cause developmental delay or necrosis (Fig. S2C). Inhibition of MAPK/ERK signaling diminished the
expression of dll4 mRNA, as determined by qRT-PCR (Fig. 2C). Furthermore, time-lapse microscopy using a Notch biosensor revealed that attenuation of MEK activity reduced Notch signaling within the developing vasculature in vivo, and these results were confirmed by static confocal microscopy of a conventional Notch reporter (Fig. 2D,E; Fig. S3; Movies 1 and 2).

**ERG activity is controlled by VEGF/MAPK/ERK signaling**

To determine whether ERG is required for the dynamic induction of DLL4 downstream of VEGF, we knocked down ERG using siRNA in HUVECs. ERG knockdown reduced the basal levels of DLL4 and completely abrogated the induction of DLL4 in response to VEGF stimulation (Fig. 3A,B). Furthermore, activation of MAPK/ERK with PMA stimulation failed to elevate DLL4 transcription in ERG knockdown cells, confirming that ERG functions downstream of VEGF and MAPK/ERK (Fig. 3C).

To explore further the relationship between ETS factors and MAPK activity, we tested whether MAPK/ERK signaling modulates ETS factor activity by creating a luciferase reporter construct under the control of a concatemer (eight tandem copies) of ETS binding sites.
the ETS-DNA binding site within the intron 3 enhancer of murine DLL4 [identified by Wythe et al. (2013)]. ETS reporter activity in bovine aortic ECs (BAECs) was attenuated by both MEK and PKC inhibition (Fig. 3D). This suggests that ETS factor transactivation is controlled by MAPK/ERK signaling.

Selvaraj et al. recently demonstrated that ERK2 preferentially bound and phosphorylated ERG at serines 96 (S96), 215 (S215) and 276 (S276), and that S215 phosphorylation was required for ERG activity in prostate cancer cells (Selvaraj et al., 2015). We found that S215 was dynamically phosphorylated in ECs in response to VEGF stimulation, with peak phosphorylation occurring at 15-30′, which coincides with increased MAPK/ERK activity following VEGF treatment (Fig. 3E). Pretreatment with a MEK inhibitor abolished S215 phosphorylation (Fig. 3F). To determine the functional importance of ERK-mediated phosphorylation of ERG, we eliminated endogenous ERG using an siRNA directed to the 3′ UTR of ERG and then reintroduced wild-type or phospho-mutant ERG. Expression of wild-type ERG restored DLL4 transcription, whereas expression of ERG containing a mutation of one phosphorylation site (S215A) had less activity, and ERG containing mutations in all three ERK-phosphorylated residues (S96A, S215A, S276A) failed to rescue DLL4 transcription (Fig. 3G). This suggests that ERK phosphorylation is functionally important in dictating ERG activity.

To further test the functional importance of ERG phosphorylation, transplantation experiments were performed in zebrafish. Wild-type or mutant (S96A, S215A, S276A) ERG mRNA was injected into kdrl:nls-EGFP donor embryos, followed by transplantation of these cells into kdrl:mCherry recipient hosts at sphere stage. The location of the donor cells within the trunk vasculature was scored at 28-30 hours post-fertilization (hpf) to determine whether expression of mutant ERG affects the ability of these cells to contribute to angiogenesis. There appeared to be no overt phenotypic consequence following mosaic overexpression of wild-type or mutant ERG. However, the percentage of ERG mutant-expressing cells contributing to intersegmental vessels (ISVs) (but not other vascular structures) was significantly reduced compared with wild-type ERG-expressing cells (Fig. 3H).

ERG coordinates dynamic co-activator recruitment to the DLL4 intronic enhancer

p300 is recruited to VEGF-dependent enhancers and is required for regulating the expression of many angiogenic genes (Zhang et al., 2013). As the earliest time-point previously examined was 1 h post-
Fig. 3. VEGF/MAPK signaling stimulates ERG transcriptional activity to induce DLL4 expression. (A) VEGF induction of DLL4 transcription (as assessed by qRT-PCR measurement of DLL4 pre-mRNA) and mature DLL4 mRNA expression in HUVECs requires ERG (n=4). (B) VEGF induction of DLL4 protein expression requires ERG. Representative experiment of three. (C) Induction of DLL4 transcription by PMA, an activator of MAPK/ERK signaling, requires ERG (n=4). (D) ETS activity (as assessed by activity of an 8× concatamer of an ETS element driving luciferase expression) in BAECs is suppressed by MEK or PKC inhibition. Triplicate determinations from a representative experiment of three. (E) ERG is phosphorylated at S215 in response to VEGF stimulation (15-30′) in HUVECs. Representative experiment of two. (F) VEGF-induced phosphorylation of ERG requires MEK activity. Representative experiment of two. (G) ERG was knocked down using siRNAs directed to the 3′ UTR, followed by overexpression of Flag-tagged wild-type (WT) or mutant (S215A or S96A;S215A;S276A, indicated as 3xS→A) ERG. ERG western blot indicates restoration of expression using electroporated constructs (a representative experiment of three is shown). DLL4 expression as assessed by qRT-PCR of pre-mRNA after 1 h of VEGF treatment (n=3). (H) Representative images of transplanted cells from Tg(fli1a:nls-GFP) embryos injected with wild-type or mutant ERG mRNA into Tg(kdrl:mCherry) embryos. Arrows and asterisks indicate endothelial cells that are donor derived. Quantification of cellular position is shown below (n=248 cells from 11 embryos for wild type and n=340 cells from 14 embryos for mutant). Scale bar: 50 µm. DA, dorsal aorta (asterisks); DLAV, dorsal longitudinal anastomotic vessel (arrowheads); ISV, intersomitic vessel (arrows); NS, non-stimulated; PCV, posterior cardinal vein.
VEGF treatment, we sought to define the dynamic nature of p300 recruitment to the DLL4 enhancer by ChIP assays in VEGF-stimulated HUVECs. ERG recruitment was modestly enhanced by VEGF treatment (Fig. 4A), but there is high basal ERG occupancy at this enhancer (Wythe et al., 2013). Strikingly, p300 was transiently recruited to the intronic DLL4 enhancer 15-30′ after VEGF stimulation, mirroring the robust and transient increase in MEK/ERK activity and ERG phosphorylation (Fig. 4A). Of note, p300 recruitment did not coincide with increased acetylation of K27 of histone H3 (H3K27ac), although acetylation is already high at this region in ECs (Wythe et al., 2013). Importantly, we found that p300 recruitment to the intronic DLL4 enhancer required ERG (Fig. 4B). In addition, co-immunoprecipitation in ECs demonstrated that ERG and p300 physically interacted following VEGF stimulation (Fig. 4C), and that this interaction was lost in cells expressing a phospho-mutant ERG (S96A, S215A, S276A) protein (Fig. 4D). To determine the functional importance of p300 in DLL4 induction, we utilized a small molecule inhibitor of p300 and CBP histone acetyltransferase activity, c646 (Bowers et al., 2010). Inhibition of p300/CBP activity in HUVECs in vitro did not affect basal levels of DLL4, but completely blocked VEGF induction of DLL4 mRNA (Fig. 4E). Furthermore, inhibition of p300/CBP in zebrafish suppressed elongation of intersomitic vessels (Fig. 4F), but did not result in other gross developmental defects (Fig. S2C).

**ERG regulates a network of constitutive and VEGF-inducible genes**
To determine the extent of the genetic network regulated by ERG, we transfected HUVECs with control or ERG siRNAs and performed microarray analysis of gene expression in serum-starved and VEGF-stimulated cells. We focused on transcripts induced at early stages of VEGF stimulation (i.e. within 1 h) to identify genes directly regulated by VEGF/ERK/ERG. Knockdown of ERG resulted in the downregulation of 202 genes, including CLDN5, RASIP1 and ARHGAP28 in serum-starved cells (Fig. S4A), consistent with previous studies (Birdsey et al., 2012; Yuan et al., 2012), and the upregulation of 68 genes. Gene ontology (GO) analysis revealed that the most frequent functional categories altered following loss of ERG were: response to wounding, inflammation, cell migration, cell motility and cell adhesion (Fig. S4B). VEGF treatment increased the expression of 160 genes and downregulated only four genes (Fig. S5A). Analysis of the genes modulated by VEGF revealed GO terms associated with transcriptional regulation and gene expression, cell proliferation and vascular development.

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**Fig. 4. p300 is dynamically recruited to the DLL4 enhancer and regulates sprouting angiogenesis.** (A) Recruitment of p300 to the DLL4 enhancer located in intron 3 15-30′ after VEGF stimulation, as assessed by ChIP (n=5 for ERG and p300 ChIP, n=3 for H3K27ac ChIP). (B) p300 recruitment in response to VEGF stimulation requires ERG. Shown is a representative experiment of two with triplicate determinations. The extent of ERG knockdown as assessed by western blot is shown to the right. (C) Endogenous p300 and ERG physically interact by co-immunoprecipitation in HUVECs stimulated with VEGF. Shown is a representative experiment of three. (D) Exogenous Myc-p300 interacts with wild-type Flag-ERG in VAECs, but does not interact with phospho-mutant (S96A,S215A,S276A) Flag-ERG by co-immunoprecipitation. Shown is a representative experiment of three. (E) p300 activity is required for DLL4 mRNA induction in HUVECs in response to VEGF stimulation (n=5). c646 is a potent inhibitor of p300/CBP activity. (F) Inhibition of p300 activity suppresses ISV elongation in zebrafish. Quantification is shown to the right. Note: Quantification of the DMSO control is the same as that shown in Fig. 2B, as both inhibitors were used in the same experiment. Scale bars: 50 μm. NC, negative control (V5 antibody).
Of the VEGF-induced genes, 30 (representing \( \sim 19\% \) of all VEGF-inducible genes) were attenuated in ERG knockdown cells, and 14 genes (\( \sim 9\% \) of all VEGF-inducible genes) were further elevated in ERG knockdown cells (Fig. 5A,B). GO analysis revealed that these genes (e.g. NRARP, HLX, DUSP5, EGR3 and PIK3RI; Bellou et al., 2009; Herbert et al., 2012; Liu et al., 2008;...
Nicoli et al., 2012; Phng et al., 2009) are implicated in angiogenesis, blood vessel morphogenesis and development, homotypic cell-cell adhesion, cell proliferation and differentiation (Fig. 5C).

Examining the kinetics of a subset of the ERG-dependent, VEGF-induced genes revealed that their transcription is increased transiently by VEGF stimulation, with peak transcription occurring between 15′ and 1 h (Fig. S6). To query the requirement of MAPK/ERK signaling and p300 activity in this response, we measured the induction of these genes in the presence of MEK or p300/CBP inhibitors. We found that 12 of 16 (75%) were MEK dependent, and all required p300/CBP activity (Fig. 5D,E).

To probe the relevance of this pathway in vivo, we assessed the expression of several of the identified genes by in situ hybridization in zebrafish embryos treated with inhibitors of VEGF, MEK or p300/CBP. Importantly, dll4, hlx1 and dusp5 were regulated by this pathway within ISVs (Fig. 6A,B). In addition, flt4, which is regulated by MAPK/ERK signaling during sprouting angiogenesis (Shin et al., 2016), was also dependent on p300/CBP and MEK (Fig. 6A,B). To induce ectopic activation of the MAPK/ERK pathway in a VEGF-independent manner, kdr-GFP zebrafish embryos were exposed to PMA for 2 h (until 24 hpf). PMA treatment induced the phosphorylation of ERK1/2 in a MEK-dependent manner (Fig. 6C) and led to the induction of dll4, hlx1, dusp5 and egr3 expression in the endothelium, as determined by qRT-PCR from fluorescence-activated cell sorting (FACS)-isolated ECs (Fig. 6D). Importantly, pre-treatment of the embryos with c646 inhibited the PMA-induced induction of dll4, hlx1 and egr3, whereas dusp5 was refractory to c646 inhibition (Fig. 6E).

To assess further the role of ERG in angiogenesis in vivo, we generated a novel Erg knockout/lacZ knock-in mouse line. Deletion of Erg resulted in embryonic lethality by embryonic day (E) 11.5-E12.5, similar to previous reports (Birdsey et al., 2015; Vijayaraj et al., 2012) (Fig. S7A-F). Following loss of ERG protein (Fig. 7A-B'), we observed major defects in vascular integrity and angiogenesis during embryogenesis, within both the cranial and the trunk vasculature (Fig. 7C-F). Conditional deletion of Erg (Erglcko) using an EC-specific CreERT2 driver (Cdh5(PAC)-CreERT2) (Wang et al., 2010) led to defects in physiological angiogenesis, as determined by examination of angiogenesis within the postnatal retina (Fig. 7G-J; Fig. S7G-K). These data, combined with the embryonic lethality, hemorrhage and reduced angiogenesis all demonstrate a requirement for ERG in physiological angiogenesis.

To determine whether the candidate genes identified by our in vitro screen are downstream of Erg in vivo, we isolated ECs from wild-type or Erg mutant mouse embryos and performed qRT-PCR for several of the ERG- or VEGF/ERG-dependent genes (Fig. 7K,L). We found that several ERG-dependent, VEGF-independent genes identified in our screen (e.g. Rasip1, Sox18) or by others [e.g. Cdh5 (Birdsey et al., 2008; Gory et al., 1998) and Cldh5 (Yuan et al., 2012)] were downregulated in Erg loss-of-function embryos (Fig. 7K). Similarly, we observed a significant reduction in a typical VEGF-induced, ERG-dependent transcript, dll4, in agreement with previous results (Wythe et al., 2013) (Fig. 7L). Additional candidates in this category, which showed robust sensitivity to MAPK and P300 activity in vitro, were substantially downregulated in vivo (e.g. Fjx1, Ptk3r1, Sd211, Nnarp). Collectively, these findings demonstrate that a VEGF/MAPK/ERG/p300 cascade is a crucial regulator of angiogenesis in vitro and in vivo.

We next sought to identify the enhancers/promoters that ERG might directly act upon to regulate this gene network. We previously found that conserved orthologous transcription factor binding can reveal functional enhancers (Ballester et al., 2014). To identify evolutionarily conserved, epigenetically modified enhancers for further functional analyses, we performed ERG ChIP-seq experiments in both human (HUVECs) and bovine (BAECs) ECs cultured in complete media (i.e. containing VEGF) (Fig. 8). We identified 31,175 ERG ChIP-seq peaks in HUVECs and 34,773 peaks in BAECs, and found that 8337 of the human peaks were conserved in cow (Tables S3 and S4). We also performed H3K27ac ChIP-seq and found that 94% of conserved ERG peaks overlapped H3K27ac-enriched regions, supporting their association with active enhancers. We found that the DLL4 locus contains multiple conserved ERG-bound enhancers, including regions ∼12 kb upstream of the transcriptional start site (TSS) and within intron 3 (Fig. 8A), both of which were previously shown to have artery-specific activity in vivo (Sacilotto et al., 2013; Wythe et al., 2013). We also identified an ERG-bound enhancer, conserved in cows and humans, ∼3.0 kb upstream of the gene H2.0-like homeobox (HLX) (Fig. 8B; Fig. 8E). HLX, a homeobox transcription factor, expression of which is induced by VEGF in vitro (Schweighofer et al., 2009), has been implicated in controlling angiogenic sprouting of human cells in vitro (Prahs et al., 2014; Testori et al., 2011), and in ISV formation in zebrafish (Herbert et al., 2012), as well as yolk sac vascular remodeling in the mouse (Prahs et al., 2014). Further analysis of our ChIP-seq data revealed that the majority of ERG-VEGF-regulated genes (25 of 44) had an ERG ChIP-seq peak within 10 kb of the TSS, and ERG binding was conserved in cow for 16 of these genes (Fig. 8C). This is suggestive of direct regulation of these genes by ERG. Furthermore, ERG binding was significantly enriched near ERG- and ERG/VEGF-regulated genes (Fig. 8D).

HLX transcription is transiently induced in response to VEGF stimulation, similar to DLL4 (Fig. 9A). We found that p300 was dynamically recruited to this evolutionarily conserved non-coding region (Fig. 9B) in an ERG-dependent manner (Fig. 9C). We cloned this conserved H3K27ac- and ERG-enriched −3 kb 5′ putative regulatory region (HLX-3a, 1565 bp fragment) upstream of a minimal promoter (SV40) driving a luciferase reporter, and found that it was VEGF responsive, and that the basal and VEGF-induced activity of this enhancer required ETS DNA-binding sequences (Fig. 9D). Furthermore, inhibition of MEK activity abrogated the VEGF responsiveness of this regulatory region (Fig. 9E). Although the full HLX-3a regulatory region failed to drive endothelial expression in vivo (data not shown; n=75), refinement of the element to the region bound by ERG (which was highly conserved across vertebrates; Fig. 8B) and the 3′ acetylated region (HLX-3b, 435 bp fragment) drove robust EGFP reporter activity in the vasculature of the embryonic zebrafish (Fig. 9F). EGFP reporter expression was preferentially observed in the ECs of the ISV's which form by angiogenesis) compared with the axial vessels (which form by vasculogenesis), and reporter activity was ETS element dependent (Fig. 9F). To test further the functional importance of this enhancer, we utilized clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) genome editing to delete a portion (1201 bp; see Fig. 8B for schematic) of the H3K27ac-enriched, ERG-bound region upstream of HLX in TeloHAECs, an immortalized human aortic EC line. Several clonal lines (HLH15, HLH17 and HLH21) heterozygous for deletion of this region were generated and confirmed by PCR and DNA sequencing (data not shown). Comparison was made with a clonal line generated following transfection of scrambled control gRNAs (Scr3). Although the basal expression of HLX appeared to be unaffected in the deletion lines, the VEGF-
Fig. 6. Regulation of VEGF/ERK/ERG/p300-dependent genes in vivo. (A) In situ hybridization using probes for dll4, hlx1, dusp5 and flt4 at 26 hpf. Embryos were treated with inhibitors of MEK (SL327), p300/CBP (c646) or VEGF (SU5416) starting at the 20-somite stage. Expression of each of these genes is MEK, p300 and VEGF dependent. Arrows indicate ISVs expressing the indicated genes. Representative images are shown. (B) Quantification of in situ hybridization experiments. The number of embryos analyzed is indicated. (C) pERK western blot in embryos pre-treated with SL327 for 1 h, followed by addition of PMA for 2 h. Coomassie staining was used to assess loading. Representative experiment of two. (D) qRT-PCR analysis of endothelial or non-endothelial cells isolated from kdrl:GFP embryos exposed to PMA for 2 h (starting at 24 hpf). All of these genes are induced in the endothelium in response to ectopic MEK activation (n=3). (E) qRT-PCR of whole individual embryos that were exposed to DMSO or c646 for 1 h, prior to stimulation with PMA for 2 h at 24 hpf. The induction of dll4, hlx and egr3 by PMA is p300 dependent (n=6).
dependent induction of \textit{HLX} appeared to be diminished (Fig. 9G). In contrast, \textit{DLL4} induction was unaffected. Furthermore, knockdown of \textit{ERG} appeared to attenuate the induction of \textit{HLX} to a greater extent in the control line compared with the deletion lines, implying that ERG acts through the deleted enhancer region. Collectively, these findings demonstrate the requirement of a highly conserved ERG-bound regulatory element in the VEGF responsiveness of the angiogenic gene \textit{HLX}.

**Fig. 7.** ERG loss of function alters angiogenesis \textit{in vivo}. (A-B') Confocal microscopy following staining for ERG and CD31 on mouse embryo cryosections. Magnified views (A',B') of the dorsal aorta (boxed areas) reveal loss of ERG, and decreased CD31 (PECAM1), in \textit{ErgKO/KO} embryos compared with wild-type littermate controls. Nuclei are stained with DAPI (blue), H, heart; NT, neural tube. (C-D') Representative whole-mount bright-field images of E10.5 \textit{Erg\textsuperscript{+/+}} (C,C') and \textit{Erg\textsuperscript{KO/KO}} (D,D') yolk sacs and embryos. Arrows indicate hemorrhage. (E,F) Representative light-sheet microscopy images of endomucin-stained blood vessels in E10.5 \textit{Erg\textsuperscript{+/+}} (E) and \textit{Erg\textsuperscript{KO/KO}} (F) embryos. Boxed areas 1, 2 and 3 are shown below the whole-mount images at a higher magnification. Arrows in box 1 denote remodeled, larger caliber vessels, which are smaller in \textit{Erg\textsuperscript{KO/KO}} animals, and asterisks denote remodeled areas devoid of vessels, which are reduced in \textit{Erg\textsuperscript{KO/KO}} embryos compared with \textit{Erg\textsuperscript{+/+}}. In box 2, the anterior cardinal vein (ACV), although present in the knockouts, showed a decreased diameter and the major large caliber vessels sprouting from it (denoted by arrows) were also smaller and more tortuous. In box 3, the remodeled ISVs are denoted by arrows, and the sprouting front (dorsal-most edge) is denoted by arrowheads. The vascular front appears less uniform in knockouts and the ISVs appear less organized compared with wild-type littermates. (G-H') Representative images of the total retinal vasculature (G,H) and magnified view of the proximal region (G,H) stained with IB4 in \textit{Erg\textsuperscript{+/+}} (G,G') and \textit{Erg\textsuperscript{KO/KO}} (H,H') retinas at P8 following tamoxifen administration at P1 and P3. The arrow in H indicates an avascular area in \textit{Erg\textsuperscript{KO/KO}} retina. (I) Quantification of radial expansion of the IB4\textsuperscript{+} vasculature within the P8 retina (n=5 for both genotypes). (J) Vascular density as determined by quantification of IB4\textsuperscript{+} branches in the proximal retinal vascular plexus at P8 (n=5 for each genotype). (K,L) ECs were isolated by FACS from \textit{Erg\textsuperscript{+/+}} or \textit{Erg\textsuperscript{KO/KO}} embryos at E10.5. qRT-PCR was performed on the indicated VEGF-independent, ERG-dependent (K) and VEGF-responsive, ERG-dependent (L) genes (n=14 (\textit{Erg\textsuperscript{+/+}}) and 9 (\textit{Erg\textsuperscript{KO/KO}})). Scale bars: 50 µm (A-B); 1000 µm (C-D'); 500 µm (E,F); 100 µm (E1-E3,F1-F3,G' H').
DISCUSSION

Dynamic control of gene expression and the resultant cellular outputs in tip cells and adjacent stalk cells are central to the growth of nascent angiogenic sprouts (Blanco and Gerhardt, 2013; Lobov et al., 2007). The pathways that regulate the temporal VEGF-dependent expression of DLL4 in tip cells are of particular importance...
considering the central role of this ligand, and its receptor, Notch, in directing tip and stalk cell behaviors (Hellström et al., 2007; Jakobsson et al., 2010; Suchting et al., 2007; Ubezio et al., 2016). Here, we identify one potential mechanism for the transient VEGF-dependent induction of DLL4 transcription. VEGF stimulation initiates a rapid and transient burst of MAPK/ERK activity, with similar kinetics to VEGF induction of DLL4 transcription. Downstream target proteins modulated by ERK kinase activity are also dynamically modified, as illustrated by the transient phosphorylation of ERG at serine 215. Additionally, we find that the co-activator p300 is recruited to angiogenic enhancers in an ERG-dependent manner, with kinetics mirroring ERG phosphorylation. This VEGF/ERK/ERG/p300 transcriptional pathway also dynamically regulates a network of genes shown to positively (e.g. HLX, FJX1, EGR3, APOLD1, ADRB2, EPHA2, FUT1, MAP2K3, NDRG1) and negatively (e.g. DUSP5, NRARP) regulate angiogenesis (Fig. 9H) (Al-Greene et al., 2013; Bellou et al., 2009; Herbert et al., 2012; Iaccarino et al., 2005; Liu et al., 2008; Mirza et al., 2013; Moehler et al., 2008; Phng et al., 2009; Pin et al., 2012; Prahl et al., 2014; Toffoli et al., 2009; Zhou et al., 2011).

The ETS family of transcription factors has previously been implicated as signal-dependent effectors (Wasylyk et al., 1998), but how ETS factors act downstream of VEGF has not been explored in detail. Interestingly, we find that DLL4 induction by VEGF signaling requires MAPK/ERK signaling, as well as ERG expression. Previous studies in cancer cells revealed that phosphorylation of ERG at S96, S215 and S276 is mediated by ERK2 (Selvaraj et al., 2015). We find that VEGF signaling leads to dynamic ERK-dependent

Fig. 9. A conserved enhancer upstream of HLX is regulated by ETS factors and is required for VEGF induction. (A) Transcription of HLX (as assessed by qRT-PCR of HLX pre-mRNA) reveals dynamic transcription, peaking at 15-30′ post-VEGF stimulation in HUVECs (n=3). (B) p300 is transiently recruited to a putative HLX enhancer element during VEGF stimulation, as assessed by ChIP assay (n=3). (C) p300 ChIP was performed in control and ERG knockdown cells. Shown are triplicate measures of a representative experiment of two. (D) Luciferase analysis of the HLX enhancer (HLX-3a), demonstrating that it is regulated by VEGF and ETS factors. ETS-binding sites were mutated in the HLX enhancer (HLX-3aMut, see Materials and Methods). A representative experiment (of three) with triplicate determinations is shown. (E) Luciferase analysis of the HLX enhancer (HLX-3b), demonstrating that it is regulated by MAPK/ERK activity. A representative experiment (of two) with triplicate determinations is shown. (F) The human HLX enhancer (HLX-3b) is functional in ISVs in zebrafish during sprouting angiogenesis. Activity is lost when the ETS sites in the enhancer are mutated. Shown are representative images of embryos at 42 hpf. Quantification of enhancer activity is shown to the right. (G) CRISPR/Cas9-mediated deletion of a highly conserved enhancer upstream of HLX inhibits VEGF-mediated induction. Shown are a clonal scrambled-control line (Scr3) and three heterozygous deletion lines (ΔHLX15, ΔHLX17, ΔHLX21). Induction of DLL4 is included as a control. Knockdown of ERG affects the induction of HLX in the control line to a greater extent than in the deletion lines. n=2. (H) Schematic of the VEGF/MEK/ERK/ERG/p300 transcriptional pathway identified in this study.

departments
phosphorylation of ERG at S215, and that S96, S215 and S276 are required for maximal ERG activity. The ability of ERG to drive expression of VEGF target genes appears to be p300 dependent, as VEGF initiates a physical interaction between ERG and p300, and ERG is required for p300 recruitment to DLL4 and HLY enhancer elements. Furthermore, p300/CBP inhibition abolishes VEGF/ERG-dependent gene expression. Mutation of ERK-phosphorylated residues in ERG prevents its interaction with p300, suggesting a role for ERG phosphorylation in recruitment of p300 to target genes. Furthermore, the termination of p300 recruitment temporally coincides with loss of ERG phosphorylation, implying a functional role for these phosphorylation events. It will be of interest to determine whether all DNA-localized ERG, or only those molecules involved in VEGF signaling output, become phosphorylated in response to VEGF signaling. It will also be of interest to determine how diverse activators of MAPK/ERK signaling, which have distinct effects on angiogenesis (e.g. ANG1/TIE2 (ANGPT1/TEK) signaling), might differentially activate ERG. Answering these questions will be vital for the development of targeted therapeutics to suppress angiogenesis. Because ERG regulates vascular integrity (presumably in cells lacking active ERK), it is possible that ERG functions to maintain vascular stability in an ERK-independent manner, suggesting the possibility of selectively blocking angiogenesis through targeting ERG phosphorylation, while maintaining vascular stability. Furthermore, ERG is known to function as an oncogenic fusion protein (e.g. TMPRSS2-ERG) in prostate cancer (Adamo and Ladomery, 2016). Of note, the amino terminus of ERG (included in many of these fusion proteins) appears to contain the same serine residues phosphorylated by ERK2. It will be of interest to determine how upstream signaling pathways (e.g. activated RAS/MAPK/ERK) influence ERG transcriptional activity in cancer. Perhaps targeting ERG phosphorylation could be of interest to quell ERG oncogenic activity.

Previous studies identified a role for another ETS factor, TEL (ETV6), in the repression, rather than the activation, of DLL4 (Roukens et al., 2010). In this case, TEL bound to the DLL4 promoter under basal conditions to recruit a co-repressor protein, CTBP. Addition of VEGF led to the rapid disassembly of this repressive complex. The kinetics of this repressive TEL/CTBP complex disassembly are comparable to the assembly of the activating ERG/p300 complex that we report here, suggesting that TEL and ERG dynamically control co-activator/co-repressor recruitment. Recently, VEGF has also been shown to stimulate dynamic exchange of co-repressors for co-activators bound to MEF2 transcription factors (Sacilotto et al., 2016), suggesting that several families of transcription factors may coordinate VEGF-dependent sprouting angiogenesis. Indeed, ETS proteins interact with multiple transcription factor families (Carrère et al., 1998; De Val et al., 2008). Of note, we have identified a number of transcription factor binding motifs that are enriched under ERG ChIP-seq peaks in the vicinity of ERG- and VEGF/VEG-dependent genes that might functionally interact with ERG to control gene expression (Table S3). Although a subset of VEGF inducible genes are regulated by ERG, it is equally important to note that many VEGF-dependent genes are ERG independent. This could be attributable to redundancy of ETS factors, but could also imply that additional transcriptional pathways responsible for angiogenic gene regulation remain to be uncovered.

In summary, our study has identified a VEGF/MAPK/ERK/ERG/p300 network that is required for the induction of a subset of VEGF-inducible genes in ECs, allowing us to propose a model for how transient activation of an angiogenic program might be regulated to orchestrate sprouting (Fig. 9H).

MATERIALS AND METHODS

Zebrafish experiments

Zebrafish protocols were approved by the Animal Care Committee at the University Health Network, the University of Texas MD Anderson Cancer Center and Baylor College of Medicine. The following transgenic lines were utilized: Tg(kdrl:mCherry)joy1 (Proulx et al., 2010), Tg(kdrl:GFP)h945 (Jin et al., 2005), Tg(fli1a:nls-EGFP)u41 (Roman et al., 2002), Tg(TP1bglob:EGFP)pm14 (Parsons et al., 2009) and Tg(TP1bglob:VenusPEST)5900 (Nimov et al., 2012).

Inhibitor treatments

Embryos were dechorionated, then treated from 18-20 hpf until 26-28 hpf (unless noted otherwise) with the following inhibitors: SU5416 (VEGFR2 inhibitor, 5 μM, LC Laboratories), SL327 (MEK inhibitor, 30 μM, Sigma) or c646 (p300 inhibitor, 3 μM, Sigma), with all inhibitors prepared as 1000× stocks in DMSO, and embryos treated in E3 supplemented with PTU to prevent pigmentation. PMA (Bioshop) was used at a concentration of 1 μM. DMSO (0.1%) was used as a vehicle control. Of note, repeated freeze thaw of c646 stocks diminished efficacy and higher doses of c646 produced serious developmental delay and growth defects (data not shown).

Imaging

See supplementary Materials and Methods for details regarding confocal imaging.

pERK immunofluorescence

Treated Tg(kdrl:GFP)h945 embryos were processed following the protocol of Inoue and Wittbrodt (2011), with the modifications suggested in Le Guen et al. (2014) (see supplementary Materials and Methods for details).

Time-lapse microscopy

Tg(TP1bglob:VenusPEST); Tg(kdrl:mCherry) animals at 18-20 somites were mounted in 1% low-melt agarose on a four-compartment glass-bottom cell culture dish (Cellview, #627975), treated with PTU and tricaine in E3, along with the same concentration indicated above for either DMSO (vehicle control) or SL327 (MEK inhibitor) (see supplementary Materials and Methods for details).

Isolation of ECs by FACs

Embryos were washed with PBS (without calcium/magnesium) and 1 ml of pre-warmed 0.25% Trypsin was added to the embryos. Embryos were incubated at 28°C and gently pipetted up and down every 5 min until digestion was complete. After digestion, 100 μl of fetal bovine serum (FBS; 100%) was added to stop digestion. The cells were spun at 1100 rpm (300 g) for 5 min at 4°C and the supernatant was removed and the cell pellet was re-suspended in 500 μl of FACs solution (450 μl PBS+50 μl 10% bovine serum albumin). Sytox Red (0.5 μl) was added and the samples were incubated at room temperature for 15 min and then passed through a cell sieve. FACs was performed by the UHN Flow Cytometry Facility using a low differential pressure (20 psi). Cells were sorted directly into RLT buffer (Qiagen) for RNA extraction using the RNeasy Micro Kit (Qiagen).

In situ hybridization

Experiments were performed as described previously (Wythe et al., 2011). Riboprobe, with the exception of flt4, were amplified by PCR with primers containing SP6 and T7 overhangs and sense and digoxigenin-labeled antisense probes were synthesized from the PCR template. The hxl1 template was provided by Dr Saulius Sumanas (Cincinnati Children’s Medical Center, OH, USA), dll4 in pGEM-T was from Dr Jiandong Liu (University of North Carolina, NC, USA), dusp5 was from GE Dharmacon (Clone ID: 4199935), and flt4 was from Dr Jeffery Essner (Iowa State University, IA, USA) (digested with EcoRI, transcribed with T7). See supplementary Materials and Methods for primers used.

Enhancer transgenesis

pTo12 enhancer injections were performed as previously published (Wythe et al., 2011). Briefly, an injection mixture consisting of 100 ng of DNA, 125 ng of To12 transposase mRNA (Kawakami et al., 2004), 1 μl 0.8% Phenol Red/0.1 M KCl, pH 7.0, and ddH2O in 10 μl total volume was
combined and 1 nl injected directly into the cell of one-cell stage Tg(kdr:: mCherry) zebrafish embryos. Embryos were then maintained at 28.5°C and scored at 42 hpf for enhancer activity within and outside of the vasculature (mCherry).

Transplantation experiments
Sense-stranded mRNA was transcribed using the mMessage mMachine kit (Ambion) from either a pCS2-3xFlag-ERG or pCS2-3xFlag-ERG-3xS-A template (see ‘Cloning’ in supplementary Materials and Methods for details). mRNA (5 µl of 125 ng/µl) was mixed with 1 µl of 2 mg/ml Alexa 647 10,000, MW, Anionic, Fixable Dextran (Life Technologies, D22914) as a lineage tracer, then 1 nl of this mixture was injected into one-cell stage Tg(kfla:: nls-EGFP) donor embryos. Dividers were pulled 1-2 h later for Tg(kdr:: mCherry) host embryos. Animals were dechorionated on agarose dishes using pronase in E2 media supplemented with penicillin and streptomycin, as suggested by Westerfield (2007). When donor embryos reached approximately sphere stage to 30% epiboly (about 4 hpf), they were transferred to agarose wells (Adaptive Science Tools, PT-1) in E2 plus antibiotics, and 20-40 cells from the lateral margin were transferred from donor to host embryos (at a similar location). Embryos were reared at 28.5°C in 1.5% agarose dishes in E2 supplemented with antibiotics until their fixation in 4% paraformaldehyde (PFA) at 28-30 hpf. See supplementary Materials and Methods for details regarding imaging. The percentage contribution of donor-derived cells contributing to a location in the host trunk vasculature was quantified as the number of donor-derived cells within the structure divided by the total number of donor-derived cells within the entire vasculature in the region of interest.

Erg murine knockout experiments
Generation of Erg murine knockout mice was carried out by the KOMP consortium (project ID: 48771; Ergtm1a(KOMP)Wtsi). Cryopreserved sperm were received and in vitro fertilization was performed at the Genetically Engineered Mouse (GEM) core at Baylor College of Medicine. In this Erg<sup>mull</sup> allele, insertion of a splice acceptor-IRE-LS2-stop, human beta actin promoter driving Neomycin between exon 5 and 6 acts as a null mutation (i.e. a gene trap) (Fig. S7A). For these studies, a global gene trap. The resulting animals, with two null alleles, were crossed to Tg(ACTB::Cre)ΔNEO/+, Tg(kdrl::EGFP) females to generate db null mice (aka Erg<sup>mull</sup>/mull females were crossed to Erg<sup>ΔNEO</sup> males and 30 µl of tamoxifen was administered by subcutaneous injection at a concentration of 10 mg/ml (≥30 µg total per mouse) to their progeny at postnatal days (P) 1 and 3. See supplementary Materials and Methods for details regarding quantification of vascular branching and radial expansion in the retina.

Cell culture
The following cells were utilized for experiments: human umbilical vein endothelial cells (HUVECs, ScienCell), telomerase-immortalized aortic endothelial cells (TeloHAECs, ATCC), dermal microvascular endothelial cells (MVECs, Life Technologies) and bovine aortic endothelial cells (BAECs, Lonza). Cells were cultured according to manufacturer’s recommendations. ECs were serum-starved in basic EC media (ScienCell) containing 0.1% FBS and no growth factors for at least 6 h (typically overnight) prior to stimulation with VEGF or PMA. Pathway inhibitors were added 1 h prior to stimulation with VEGF-165 (50 ng/ml, recombinant human protein, R&D Systems or Thermo Fisher Scientific), except for c646, which was added 20 min prior to stimulation. The following inhibitors were used: U0126 (MEK inhibitor, 20 μM, Tocris Bioscience), LY294002 (PI3K inhibitor, 10 μM, Sigma). All drugs were dissolved in DMSO, and a comparison was made with vehicle (i.e. DMSO, 0.1%) treated controls. PMA was from BioShop and was used at a concentration of 100 nM.

siRNA experiments
HUVECs were transfected at 30-50% confluency with 40 nM siRNA (Silencer Select, Thermo) targeting the coding region of ERG (assay ID: s4813), ERK1 (MAPK3) (assay ID: S230180) or ERK2 (MAPK1) (assay ID: S11138) using RNAiMax (Invitrogen), and cellular assays were performed 48-72 h later. Western blotting and qRT-PCR were used to assess ERG, ERK1 and ERK2 knockdown. Comparison was made with cells transfected with 40 nM Silencer Select negative control #1. For ERG rescue experiments, an independent siRNA recognizing the 3’ untranslated region of ERG (40 nM, Silencer Select, custom synthesis) was utilized. After 48-72 h, cells were electroporated using the P5 Primary Cell 4D Nucleofector kit and a Lonza 4D Nucleofector; ~0.5×10<sup>6</sup> cells were electroporated with 2.5 µg of pCS2 control or pCS2-Flag-ERG expression constructs (wild type and phospho-mutants; see ‘Cloning’ in supplementary Materials and Methods), and 0.2 µg of pmaxGFP (to assess electroporation efficiency). After 18 h, cells were serum-starved for 6 h prior to VEGF stimulation (1 h) and harvested for RNA/protein analyses.

Cloning
For details regarding cloning of ETS concatamer reporter constructs, H LX enhancer reporter constructs and wild-type and mutant ERG expression constructs, please see the supplementary Materials and Methods.
Luciferase experiments

For luciferase assays, BAECs (80% confluent) were transfected with 0.5 μg of luciferase construct (ETS reporter, HLX enhancer, see ‘Cloning’ in supplementary Materials and Methods) and 0.1 μg of pRenilla construct using Lipofectamine 2000 (Invitrogen) (2 μl per 1 μg of plasmid) in 12-well dishes. Cells were treated with cellular signaling pathway inhibitors for 18 h (as above). In some experiments, VEGF (50 ng/ml) was added to OptiMEM medium for 18 h. After 24 h, dual luciferase (Renilla and firefly) was measured using a GloMax20/20 Luminometer (Promega).

Western blotting and co-immunoprecipitation

Western blotting was performed as before (Fish et al., 2011) using the following antibodies: anti-pERK1/2 (Thr202/Tyr204, rabbit polyclonal, Cell Signaling, #9101; 1:1000), anti-ERK2 (mouse monoclonal, Santa Cruz, D-2; 1:500), anti-ERK1/2 (rabbit monoclonal, Cell Signaling, clone 137F5; 1:500), anti-DLL4 (rabbit polyclonal, Cell Signaling, #2589; 1:1000), anti-GAPDH (mouse monoclonal, Santa Cruz, #0411; 1:500), anti-ERG (rabbit polyclonal, Santa Cruz, C-20 or mouse monoclonal antibody, BioCare Medical, 9FY; 1:1000), anti-pERG5215 [rabbit polyclonal, a kind gift from Peter Hollenhorst, Indiana University, IN, USA (Selvaraj et al., 2015); 1:500], anti-p300 (rabbit polyclonal, Santa Cruz, C-20; 1:200). All antibodies have been previously validated. See supplementary Materials and Methods for details regarding pERG western blots and co-immunoprecipitation experiments.

Immunofluorescence on cultured cells

HUVECs were plated on Permanox eight-well chamber slides. Following stimulation with VEGF, cells were fixed with 4% PFA followed by permeabilization with 0.25% Triton X-100. Staining with anti-pERK (rabbit polyclonal, Cell Signaling, #9101, 1:500) was performed overnight at 4°C, followed by addition of a secondary antibody (anti-rabbit Alexa Fluor647, Cell Signaling #4414). Slides were mounted using Vectashield mounting medium with DAPI (Vector Labs H-1200) and imaged using an Olympus FV1000 confocal microscope.

RNA isolation, reverse transcription and quantitative PCR

RNA was isolated from cells and zebrafish using Trizol and reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using a Roche Lightcycler 480 with LC 480 SYBR Green I Master Mix (Roche). Data were normalized to TATA-box binding protein (Gapdh) using the ΔΔCT method. For further details regarding primer sequences, see Table S2.

Gene expression array

HUVECs were transfected with control or ERG siRNA and after 48 h the cells were serum-starved overnight and cells were then left unstimulated or were treated with 50 ng/ml VEGF for 1 h. RNA was isolated from four independent experiments using Trizol and analyzed on Agilent microarray, performed at the Princess Margaret Genomics Centre. See supplementary Materials and Methods for details regarding microarray processing and analysis.

Gene ontology analysis

Differentially expressed genes were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (https://david-d.ncifcrf.gov/home.jsp) to be classified into gene ontology (GO) annotation groups (Ashburner et al., 2000; Huang et al., 2009). Fisher’s exact test was applied to identify significant GO categories. Select representative GO categories are included in figures.

ChIP-qPCR experiments

ChIP was performed as before (Wythe et al., 2013), using the Imprint ChIP kit (Sigma) or the Magna ChIP A/G kit (Millipore). HUVECs were serum-starved overnight prior to stimulation with 50 ng/ml VEGF for 15’, 30’ or 2 h. Following fixation and shearing of chromatin, immunoprecipitation was performed overnight at 4°C using 1 μg (or 1 μl) of antibodies to ERG (rabbit polyclonal, Santa Cruz, C-20), p300 (rabbit polyclonal, Santa Cruz, C-20) or H3K27ac (rabbit polyclonal, Abcam, ab4729). Mouse IgG (Sigma) was used as a non-specific background control. qPCR was performed using primers that amplified the Dll4 intron 3 enhancer or an enhancer upstream of HLX (see Table S2 for primer sequences). IP DNA was calculated by subtracting the IgG value from the specific antibody value and dividing by a diluted input sample. In some experiments, control or ERG siRNAs were transfected into HUVECs 48 h prior to stimulation with VEGF (15’), followed by p300 ChIP.

ChIP-seq experiments and analysis

Primary HUVECs and BAEC cells were grown in supplier-recommended EC Growth Media (ScienCell) and cultured at 37°C in a 5% CO2 humidified incubator. Approximately 20 million cells were used for the ERG and ~3 million cells for the H3K27ac ChIPs. ChIP experiments were conducted as previously described (Ballester et al., 2014). Antibodies used for ChIP were mouse anti-H3K27ac (Millipore, 05-1334 monoclonal) and rabbit anti-ERG 1/2/3 (Santa Cruz Biotechnology, sc353 polyclonal). Two replicates were performed. See supplementary Materials and Methods for details regarding processing and analysis of ChIP-seq experiments.

CRISPR/Cas9-mediated HLX enhancer deletion

The HLX enhancer region to be targeted for deletion was defined by H3K27ac ChIP-seq enhancer marks (~1200 bp). The MIT CRISPR/Cas9 design tool (http://crispr.mit.edu/) was used to generate gRNAs targeting the 5′ and 3′ boundaries of the HLX enhancer. Two scrambled sequence gRNAs were used as controls. For further details, see the supplementary Materials and Methods.

Statistical analyses

Unless otherwise stated, all experiments were performed a minimum of three times and data represent the mean±s.e.m. Statistical analyses were performed using a Student’s t-test (for two groups) or ANOVA (for more than two groups), followed by the Newman–Keuls post-hoc test. P<0.05 was considered statistically significant. In all figures, *P<0.05, **P<0.01 and ***P<0.001.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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Data availability
All microarray and ChIP-seq data have been made publicly available. Microarray data were submitted to ArrayExpress (accession number: E-MTAB-5207). ERG and H3K27ac ChIP-seq data from HUEVC and ERG Chip-seq data from BAEC were submitted to ArrayExpress (accession number: E-MTAB-5148). We also utilized HUEVC H3K4me3 ChIP-seq data (poled signal from biological replicates, ENCODE accession numbers: ENCF000007TS, ENCF0000BLTL and HUEVC H3K4me3 ChIP-seq data (poled signal from biological replicates, ENCODE accession numbers: ENCF000007TD, ENCF0000BSY, ENCF0000BSX) from the Encyclopedia of DNA Elements Consortium (ENCODE Project Consortium, 2012). Vertebrate conservation across 100 genomes was extracted from UCSC Genome Browser.

Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.146050.supplemental

References
Supplementary Materials and Methods:

Zebrafish Experiments:

*Imaging inhibitor-treated embryos* − 28-30 hpf embryos were fixed overnight, then mounted in low melt agarose the following day, and somites 7-12 were imaged (20X objective, NA=0.8, laser power=20%, pinhole = 182 μm, 32 μm step size, 16 total slices) using a LSM780 microscope and ZenPro imaging software. Intersomitic vessel length was measured using the Distance Tracking function in ZenPro.

*pERK immunostaining −* Briefly, embryos were fixed in freshly made 4% PFA / PBS / 0.1% Tween-20 overnight at 4°C, then dehydrated to absolute methanol at 4°C. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ / 97% methanol at 4°C for 1 h, then embryos were washed extensively in methanol and stored for at least 2 days at -20°C. Embryos were then rehydrated to PBS / 0.1% Tween-20 (PBST), and cryoprotected in PBST / 30% sucrose overnight at 4°C. The following day, embryos were equilibrated for 5 min in 150 mM Tris-HCl (pH 9.0) at room temperature (RT), then this was replaced with 70°C 150 mM Tris-HCl (pH 9.0) and the embryos were maintained at this temperature for 15 min for antigen retrieval. After cooling to RT, embryos were washed in PBST, rinsed multiple times in ddH₂O, then incubated in acetonitrile at -20°C for 20 min. Embryos were then washed in PBST at RT, transferred to TBS / 0.1% Tween-20 / 0.1% Triton X-100 (TBSTx), then blocked (TBST / 0.8% Triton X-100 / 1% BSA / 10% goat serum) overnight at 4°C. The next day embryos were incubated with anti-phospho ERK antibody (phospho-p44/42 MAPK (Thr202/Tyr204) XP rabbit monoclonal antibody, Cell Signaling Technologies, #4370) (1:250) in TBST / 0.8% Triton X-100 / 1% BSA / 1% goat serum overnight at 4°C for 3 days. Embryos were then washed extensively in TBSTx, then washed in maleic acid buffer (150 mM maleic acid (pH 7.4) / 100 mM NaCl / 0.001% Tween-20) (MABT), then incubated in HRP-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111-035-144) (1:1,000) in 2% Blocking Reagent (Sigma,
Embryos were washed in 2% blocking reagent / MABT at RT, washed in MABT, transferred to PBS, then incubated (upright, rocking) in 150 μL of Alexa-568 TSA reagent (Life Technologies, T20950) (1:50) for 3 h in the dark at RT. Embryos were washed extensively in TBSTx, then blocked in TBSTx / 10% goat serum / 1% BSA for 1 h at RT, and incubated with rat anti-GFP (Chromotek, 3h9) (1:500) overnight 4°C in TBST / 0.5% Triton X-100 / 1% goat serum / 1% BSA, washed in TBST / 0.5% Triton X-100, washed in TBSTx, then incubated with Alexa-488 conjugated goat anti-Rat IgG (Life Technologies, A11006) (1:250) overnight at 4°C. Embryos were then washed extensively, briefly post-fixed in 4% PFA, washed in PBS, then mounted in low-melt agarose for confocal analysis. Embryos without primary pERK antibody were always included as a negative control for tyramide amplification. Images were collected at a step size of 1.5 μm, for 43 total slices, using a Plan Apochromat 20X/0.8 objective, laser power=2.20% (488 nm), 5.50% (561 nm), pinhole=57 μm, on a LSM800 confocal laser scanning microscope using ZenBlack imaging software. Stacks were exported to ImageJ where individual cells were counted and the stack was compressed to a Maximum Intensity Projection (MIP). Vessels were imaged as above (for sprout length) and GFP* vessels with pERK staining were scored as positive.

Time-lapse confocal microscopy – 2 embryos were analyzed per experiment, and the movies shown are representative of these results (DMSO=6 embryos, SL327=6 embryos). Images were collected every 10 minutes, at a 5 μm step size, with a total stack size of 205 μm, using a Plan Apochromat 20X objective, laser power=20.8% (488), 56.4% (546), NA=0.8, WD=0.55 mm, pinhole=57 μm, on a LSM800 confocal laser scanning microscope using ZenBlack imaging software. Images were compressed to a maximum intensity projection (MIP), then stitched together as a movie in Zen and exported as an AVI (with no compression).

Imaging transplantation experiments: Embryos were embedded in low melt agarose, then imaged using a LSM 780 confocal microscope (Zeiss) with the following conditions: Plan-Apochromat 20x/0.8 M27 objective, 488 laser power at 4.5%, 594 laser power at 4.7%, 647 laser power at 5%. A total tack of 96 μm was collected, scanning every 2 μm (averaging set to 8). The pinhole was set to 126 μm (488), 55 μm (561), 113 μm (640). Stacks were exported to ImageJ where individual cells were counted and the stack was compressed to a Maximum Intensity Projection (MIP).

In situ hybridization primers –
JDW 436 (T7 drhlx FWD): 5’-GCAAATTAATACGACTCCTATAGGGAGAGCGGTGGGATAGCATGTAAGAAAC
Mouse experiments:

**Immunohistochemistry on sections** – For frozen sections, after equilibration at -20°C, 10 µm sections were collected on Superfrost Plus slides (Fisher, #12-550-15) using a Leica CM1850 cryotome. Sections were stored at -80°C until ready for processing. For IHC, slides were dried for 10 minutes at 42°C, washed 3X in PBS, permeabilized in 0.3% Triton-X100 / 1X PBS, washed in PBS, blocked in TNB (0.1 M Tris-HCl (pH 7.5) / 0.15 M NaCl / 0.5% Blocking Reagent (Roche, #10447200)) for 1 h at room temperature, then incubated with anti-ERG1/2/3 (Santa Cruz Biotechnology, SC-353) (1:200) overnight at 4°C. Tissue was washed in PBS, incubated with Alex 594 conjugated goat anti-rabbit (Life Technologies, A11034) (1:150) for 1.5 h at room temperature, washed in PBS, then stained for CD31 (BD Biosciences, #550274) for 2 h at room temperature, washed in PBS, incubated in Alexa 488 conjugated goat anti-rat (Life Technologies, A11006) (1:150) for 1.5 h at room temperature, washed in PBS, counterstained in DAPI (300 nM) (Life Technologies, #D3571) for 10 min at room temperature, washed in PBS, coverslipped in aqueous mounting media (Vector Labs, #H-5501), cured, then sealed with nail polish. Images were acquired using a EC Plan-Neofluar 10x/0.30 M27 objective with laser power 6.5% (405 nm); 11% (488 nm); 6% (561 nm); or a 40x/1.4 oil DIC M27 objective with laser power 0.8% (405 nm); 4% (488 nm); 4% (561 nm) using a Zeiss LSM 780 confocal microscope. Confocal stacks of 12.25 µm were collected with a step size of 1.75 µm. Stacks were compressed to a maximum intensity projection using ZenBlue (Zeiss), and images were exported to Adobe Photoshop and Illustrator.

**X-gal staining on sections** – 10 µm cryosections (processed as detailed above) were dried for 10 min at room temperature, washed in PBS, post-fixed (2% PFA / 0.2% glutaraldehyde / 1X PBS / 0.02% sodium deoxycholate / 0.01% IGEPAL CA-630) for 5 min at room temperature, washed in
Permeabilization Buffer (1X PBS / 0.02% sodium deoxycholate / 0.01% IGEPAL CA-630) at room temperature, then incubated in Staining Buffer (1 mg/mL X-gal / 5 mM postassium ferricyanide / 5 potassium ferrocyanide / 2 mM MgCl₂ in Permeabilization Buffer) overnight at 37°C, washed in PBS, fixed, washed, then counter-stained with nuclear fast red (Vector Labs, #H-3404) for 10 min, washed in dH₂O, dehydrated to absolute ethanol, washed in xylene, and mounted in Entellan New (Electron Microscopy Sciences, #14800). Slides were imaged on a Zeiss Axio Zoom.V16 stereoscope using Zen Blue software. Images were exported in Adobe Photoshop.

Collecting ECs from mouse embryos by FACS – Following collagenase digestion, cells were washed and blocked in HBSS++ (HBSS without calcium, magnesium, or phenol red (Gibco, Cat#14175095) / 2% FBS (HyClone, Cat #SH30910.03) / 1% penicillin-streptomycin (Lonza, Cat #12001-324) / 1% 1M HEPES (Gibco, Cat #15630080). Cells were then incubated in HBSS++ with BV421 Rat anti-mouse CD31 (BD Biosciences, Cat #562939) (1:100) for 90 min at 4 °C, washed in HBSS++, then stained in TO-PRO-3 Iodide (Life Technologies, Cat # T3605) (1:1,000) for 15 min at 4°C, all while rocking gently. Cells were then washed and filtered through a 35 µm cell strainer (Falcon, Cat#352235). The single cell suspension was sorted at the Cytometry and Cell Sorting Core (CCSC) (BCM) on a FACSAria II (BD Biosciences) using the following gating parameters: Sort set up to 85 µm, precision set to purity. Events were recorded until 25,000 live endothelial cells per embryo were collected into 350 µL of RLT Buffer (Qiagen) for downstream processing. Embryos with EC viability lower than 70% were not used for downstream analysis.

Analysis of retinal vasculature – Following administration of tamoxifen at P1 and P3, pups were euthanized at P8 by CO₂ asphyxiation, tail tissue was acquired for DNA genotyping, and eyes were enucleated and placed in 4% PFA at 4°C overnight. The following morning, retinas were isolated, then partially cut into 4 quadrants (i.e. leaflets) to allow for flat mounting. Isolated retinas were first blocked and permeabilized overnight with gentle shaking at 4°C in retina blocking buffer (1% BSA / 0.5% Triton-X100 / 1X PBS / pH 7.2). The following morning, retinas were washed 3 times for 5 minutes each wash in 1X PBS. Retinas were then equilibrated to room temperature in Pblec solution (1X PBS / 1 mM CaCl₂ / 1 mM MgCl₂ / 0.1 mM MnCl₂ / 1% Triton-X100 / pH 6.8) 3 times, for 20 minutes each time. Retinas were then incubated overnight with gentle shaking at 4°C with biotinylated Griffonia simplicifolia Isolectin B4 (Vector Labs, B-1205) at a concentration of 1:50 prepared in Pblec solution. Retinas were rinsed twice with 1X PBS, followed by 3X 10-minute washes in retina wash buffer (retina blocking buffer diluted 1:1 in...
1X PBS), then incubated in DyLight 594 Streptavidin (Vector Labs, SA-5594) prepared in retina blocking buffer for 2 h at room temperature with gentle agitation. After incubation, retinas were rinsed twice with 1X PBS, followed by 3X 10-minute washes in retina wash buffer. Retinas were mounted on glass slides and coverslipped using Fluoromount-G mounting medium (SouthernBiotech, 0100-01). For P6 retinal harvests, pups were given a single, 50 µL dose (10 mg/mL) of tamoxifen (equivalent to 50 µg per mouse) by subcutaneous injection at P1, and harvested eyes were fixed in 4% PFA and then retinas were collected after 2 hours and processed identically as P8 retinas.

To quantify vascular branching in the retina, a single 20X image was taken from a middle point of the central plexus for each of the 4 quadrants per retina (i.e. 4 images per retina examined). All branch points were tallied using ImageJ software within each full 20X field of view and averaged across the 4 quadrants for a single averaged measurement of vascular branching per biological sample. This was performed from 1 retina per animal, from 5 different animals for both control (ERGfl/+), and experimental (ERGiECKO) groups at P8, and 3 animals for control (ERGfl/+), and 2 animals for the experimental (ERGiECKO) groups at P6. Data was graphed using GraphPad Prism software and shown as an average ± SEM. Comparisons were made using a two-way, unpaired Student’s T-test for the P8 retinas. Images for both ages were obtained on a Leica TCS SPE confocal microscope with a 20X objective lens (1,024 x 1,024 pixels) at 15% power (561 nm).

Radial expansion of the retinal vasculature was quantified using the integrated length calculator function in the Zen Pro software suite (Zeiss). Images were taken on a Zeiss Axio Zoom.V16 fluorescent stereoscope at a 65X magnification. At this magnification, the entirety of a retinal quadrant to the optic nerve could be collected in one image. Images were taken for each quadrant (4 per retina), and a single line was drawn from the center of the optic nerve, through the center of the quadrant, to the vascular front. Total length was recorded for each of the 4 quadrants for a single retina and the average determined. This was performed for all retinas from both control and experimental groups. Radial expansion was graphed using GraphPad Prism software and shown as an average ± SEM. Comparisons were made using a two-way, unpaired Student’s T-test for the P8 retina data.

To quantify sprouting within the P6 retina, a single 40X image (1,024 x 1,024 pixels) was acquired from each leaflet (i.e. 4 images per retina). Major sprouting vessels at the vascular front were tallied within each full 40X field of view and averaged across the four leaflets to yield a single measurement per animal. One retina per animal was analyzed, and for P6 3 control (ERGfl/+) and 2 experimental (ERGiECKO) animals were quantified. Data was graphed using
GraphPad Prism software and shown as an average ± SEM. Images were taken on a Leica TCS SPE confocal microscope using a 20X objective with a 2X zoom magnification at 22% laser power (561 nm).

Cloning:

ETS concatemer – Oligos containing the previously EMSA-validated ETS site B (site #2, GCGTTTTCCTGCGGG) of the minimal 30 bp murine Dll4 intron 3 arterial enhancer (F2-6) (Wythe et al., 2013) were synthesized as a multimer (8X), and the duplex was annealed and cloned into pDONR221 to generate JDW 295 (8X WT) and JDW 242 (8X MT, GCGTTTTtTGCGGG) constructs. Subsequently, this was recombined into pGL3-Pro-DV (JDW 250/ETS WT; JDW 241/ETS MT).

HLX enhancers – Nucleotides of the gene regulatory element HLX-3a, spanning 1,565 bp on human chromosome 1, beginning approximately 3 kb upstream of the 5’ UTR of HLX, were directly synthesized as a gene block (IDT) with all ETS sites intact, or all ETS sites mutated as follows: ETS A: TTCC>TTaa; ETS B: GGAA>aaAA; ETS C: TTCC>TTtt; ETS D: TTCC>TTtt; ETS E: GGAA>aaAA; ETS F: GGAA>ttaA; ETS G: GGAA>aaAA; ETS H: TTAA>TTtt; ETS I: GGAA>GGgg; ETS J: GGAA>aaAA; ELK1: TCCG>TttG; ETS K: TTCC>TTtt; ETS L: GGAA>ctct; ETS M: GGAA>aaAA; ETS N: GGAA>aaAA; ETS O: TTCC>ccCC; ETS P: GGAA>aaAA; ETS Q: GGAA>aaAA

Subsequently, primers containing flanking attB1/B2 sites and BamHI and NotI restriction sites (5’ and 3’, respectively) were designed to amplify the entire region (HLX-3a) or the smaller fragment (HLX-3b), from both wild-type and mutant templates, and were directly cloned into pDONR221 via a BP clonase reaction.

HLX Enhancer Activity Assays – Following sequence verification, HLX-3a/3b donor clones were recombined with a destination vector, pGL4.23[luc2 minP]-DV, for in vitro luciferase analysis. pGL4.23[luc2 minP]-DV was created by digesting the parental vector (pGL4.23[luc2 minP]) with Nhel and inserting a PCR-amplified gateway RFA cassette via Cold Fusion (SBI). The RFA insert was amplified using the following primers, JDW 356 (FWD): 5’-ACTGGCCGGTGACCTGAGCTCACAAGTTT GTACAAAAAGC; JDW 357 (REV): 5’-CAGATCTTGTGATATCTCGAGCCACTTTTTG TACAAGAAAGCT. For in vivo analyses, the HLX pDONR clones were recombined with a modified pTol2-E1b-EGFP destination vector. Briefly, to stabilize reporter activity, pT2KKXIGQ-DV (Smith et al., 2013) (a kind gift of Nadav Ahituv, UCSF), which has a gateway RFA cassette inserted between Xhol and BgIII, and is a variant of
pT2KXIGQ (Li et al., 2010), originally derived from pT2KXIG (Kawakami et al., 2004), was digested with ClaI and a WPRE element was amplified by PCR and placed downstream of EGFP, upstream of the poly(A), using Cold Fusion (SBI).

JDW 348 (FWD): 5’-GGTGGAGCTCGAATTAATTCATCGAATCAACCTCTGGATTACAA AATTTGTG; JDW 360 (REV): 5’-CTTATCATGCTGGATCATCATCGATCGATTGAG GTCGAGGTGACGATAT. This construct was validated using a myh6 (aka cmlc2) promoter (data not shown).

Wild-type and mutant ERG constructs – The ORF of human ERG was amplified by PCR, adding a 3X-FLAG sequence and a Gly-Gly-Ala-Gly-Gly flexible linker (Sabourin et al., 2007) at the amino terminus, with two stop sequences and an XbaI site at the carboxy terminus. JDW 497 (FWD) (ERG homology is underlined, linker is in bold): GCCACCATGGACTAC AAGACCATGACGTTGATTATAAGATCATGATATCGATTACAAGGATGACGAT GACAAGGGGTGGTGCTGGGTGTATGGCCAGCAGCTATTAAGGAAGCC
JDW 498 (with an attB2 site):

CCCACCCACTTTTGACAAAGAAAGCTGGGTCTAGATTATATAGTAAGTGCCAGGATGAGA AGGC

Then an attB1 site was added at the 5’ end by PCR, using the same REV primer as above:

JDW 499 (FWD):

GGGGACAAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGACTACAAAGACCATGGTGGTGCTGGTGGT ATGGCCAGCACTATTAAGGAAGCC

This PCR product was cloned via a BP reaction directly into pDONR221, and then used as the template for site directed mutagenesis. The primers are listed below:

JDW 500 (S215A FWD; TCT>GcC): gatgttgataaagccttacaaaacgccccacggttaatgcatgctatgct,
JDW 501 (S215A REV; TCT>GcC): tagcatgcattaaccgtggggcgttttgtaaggctttattacaatc.
JDW 448 (S96A FWD; AGC>gGC): 5’-ggaagatggtgggctcccccagacacccggt, JDW 449 (S96A REV; AGC>gGC): 5’- caacggtgctgccccccagcacatctttc
JDW 450 (S276A FWD; TCT>gCT) 5’-gaagctggctcaacaccagcctctctcagtgcc
JDW 451 (S276A REV; TCT>gCT) 5’-ggcacgatggaggggggtgacgagctctttc

All constructs were then recombined by a LR clonase reaction into pCS2-DEST (Addgene # 22423) (Villefranc et al., 2007) for in vitro validation by immunofluorescence and western blot (using by anti-Myc and anti-Flag antibodies).

pERG western blots and co-immunoprecipitation experiments:

For pERG western blots, ERG was immunoprecipitated from cell lysates (in RIPA buffer). 25 µL of Dynabeads M-280 sheep anti-mouse IgG was mixed overnight at 4°C with 5 µL of anti-ERG
antibody (BioCare) in 500 µl ChIP dilution buffer. 1 mg of cell lysate in RIPA was added to the mixture and rotated overnight at 4°C. The protein complexes bound to the beads were collected using a magnetic separator, and Western blotting was performed on SDS-PAGE gels. Blots were probed with anti-pERG$^{215}$ [provided by Dr. Peter Hollenhost, (Selvaraj et al., 2015)] and then stripped and reprobed with anti-ERG (BioCare). The specificity of this antibody was determined by performing western blots on wild-type and S215A mutant ERG. No signal was observed for S215A ERG. Similar procedures to those described above were used for co-immunoprecipitation experiments in HUVEC, except non-denaturing buffer was used (20 mM Tris-HCl (pH 8.0) / 137 mM NaCl / 1% NP-40 / 2 mM EDTA). Anti-V5 antibody (mouse monoclonal, Thermo Fisher Scientific) was used as a negative control for co-immunoprecipitation experiments. Blots were probed with anti-p300 (Santa Cruz Biotechnology) and anti-ERG (BioCare). Co-immunoprecipitation experiments were performed in BAECs to assess the interaction between Myc-p300 (a gift from Dr. Tso-Pang Yao, Addgene plasmid #30489) and either wild-type or mutant Flag-ERG (cloning described below). Briefly, BAECs on a 10 cm plate were transfected with 6 µg of Myc-p300 and 6 µg of Flag-ERG constructs using Lipofectamine 2000. After 48 h, cells were treated with VEGF (50 ng/mL) for 1 h. Flag-ERG was precipitated from two 10 cm plates of BAEC (~ 1 mg of total input protein per IP) overnight at 4°C using 5 µL of Flag antibody (Cat. #F1804, Sigma) and Dynabeads (Sacilotto et al.). V5 antibody was used as a negative control (as above). The samples were lysed in Non-denaturing lysis buffer (20 mM Tris HCl (pH 8) /137 mM NaCl / 1% Nonidet P-40 / 2 mM EDTA) and run on a 4% SDS-PAGE gel, transferred using a semi-dry blotter (15V, 1 h) and then blotted with anti-Flag (1:1,000) or anti-Myc antibody (Cat. #sc-4084) (1:500). The signals were detected using standard chemiluminescence.

**Microarray processing and analysis:**

*Processing* – Low Input Quick Amp Labeling Kit from Agilent (Cat#0006177230) was used to generate fluorescent cyanine 3-labeled complimentary RNA (cRNA) from total RNA (150 ng) for one-color processing. Labeled cRNA was purified using RNeasy Mini Kit (Qiagen, Cat# 74104). For microarray hybridization, 600 ng of cyanine 3-labeled cRNA was fragmented and hybridized on Agilent G3 Human 8x60K microarrays at 65°C for 17 hours at 20 rpm using the Agilent gene expression hybridization kit (part number 5188-5281). The hybridized microarrays were dissembled at room temperature in gene expression wash buffer 1 (part number 5188-5325), then washed in gene expression wash buffer 1 at room temperature for 1 minute. This was followed by a wash for 1 minute in gene expression wash buffer 2 (part number 5188-5326) at
37°C. The processed microarrays were scanned with an Agilent DNA microarray scanner (Scanner Model G2505B-C), and extracted with Agilent feature extraction software (version 10.7.3.1).

Analysis – Data was checked for overall quality using R (v2.15.3) with the Bioconductor framework and the Array Quality Metrics package installed. All samples passed quality control, but two outliers (1 sample from control siRNA, non-stimulated and 1 sample from ERG siRNA, VEGF stimulated) were identified from visualizing correlation coefficient data and were removed from subsequent analysis. Data was imported into GeneSpring v12.6.1 for analysis. During import, the data was normalized using the recommended Agilent spatial detrending method with a “per probe” median centered normalization. All data analysis and visualization were performed on log2 transformed data. Data was first filtered to remove probes that showed low signal and positive control probes were also eliminated from further analysis. Only probes that were above the 20th percentile of the distribution of intensities in 80% of any of the 4 groups were allowed to pass through this filtering. One-way ANOVA with a Benjamini-Hochberg FDR corrected p<0.05 was performed, revealing 498 significantly varying probes. A post-hoc Tukey’s HSD test was used following the ANOVA to identify significant differences between groups. The probes that were significantly different between control siRNA and ERG siRNA in the presence of VEGF stimulation (n=357 probes) were cross-referenced with the list of VEGF regulated genes (control siRNA, no stimulation vs. control siRNA, 1 h VEGF stimulation, n=196 probes) to identify a group of 44 unique genes that were both ERG regulated and VEGF-dependent. Microarray data was submitted to ArrayExpress (accession number: E-MTAB-5207).

ChIP-seq experiments:

Processing – ChIP DNA was prepared for Illumina sequencing by blunt-end repair, dA-tailing, and ligation of Illumina adaptors using a NEBNext DNA library preparation kit (New England Biolabs, catalogue #E6040L). Total ChIP DNA (approximately 200-500 ng) and 220 ng of DNA input (WCE) was end repaired for 30 minutes at room temperature, and then purified using column purification with either DNA Clean and Concentrator (Zymogen, catalogue #D4014) or PCR purification columns (Qiagen, catalogue #28106) as recommended by the manufacturers’ protocols. Blunt-end repaired DNA was dA-tailed for 40 minutes at 37°C, then column purified. dA-tailed DNA was ligated to Illumina adaptors (final concentration 6.67 nM) that have a T-overhang. USER enzyme was used to cleave the uracil hairpin of the Illumina adaptor, and adaptor-ligated DNA was column purified. The library was PCR amplified for 16-18 cycles using a universal primer and a barcoded primer (New England Biolabs, catalogue #E7335L). PCR-
amplified DNA was purified using PCR column purification and eluted in 20 μL of elution buffer for preparation of gel extraction, or 30 μL of TE for preparation of Pippin Prep size selection. Library samples were size selected from 200-350 bp using a 2% agarose dye-free automated size selection cassette from Pippin Prep (Sage Sciences, catalogue #CDF2010).

**Analysis** – Samples were submitted for quality control analysis to the Donnelley Sequencing Center (University of Toronto) or The Center for Applied Genomics (Hospital for Sick Children) for Bioanalyzer analysis and library quantification using KAPA Biosystems. Libraries were sequenced using Illumina HiSeq2500. The flowcells were prepared and processed by the sequencing facility according to the manufacturer's protocol, with 100-bp single-end sequencing for 75 cycles. ChIP-seq and input reads were aligned to hg19 [GRCh37] genome assembly with Burrow-Wheeler Aligner (BWA), using default parameters (Li and Durbin, 2009), and quality control indicators were measured according to the ENCODE Consortium guidelines (Landt et al., 2012). Peaks were called for each sample relative to the WCE input using MACS2 with a cutoff of false-discovery rate (q ≤ 0.05) (Zhang et al., 2008). ChIP-seq data was submitted to ArrayExpress (accession number: E-MTAB-5148). We also utilized HUVEC H3K4me3 (pooled signal from biological replicates, ENCODE accession numbers: ENCF000BTS, ENCF000BTL) and HUVEC H3K4me1 (pooled signal from biological replicates, ENCODE accession numbers: ENCF000BTD, ENCF000BSY, ENCF000BSX) from the Encyclopedia of DNA Elements Consortium (Consortium, 2012). Vertebrate conservation across 100 genomes was extracted from UCSC Genome Browser. Further details regarding quality control of ChIP-seq experiments can be found in the supplementary Materials and Methods (Table S4).

**Motif analysis** – Motif enrichment analysis was performed using the tool peak-motifs (Thomas-Chollier et al., 2012) from the Regulatory Sequence Analysis tools (Medina-Rivera et al., 2015) with parameters: -markov auto -disco oligos, positions -nmotifs 5 -minol 6 -maxol 7 -no_merge_lengths -2str -origin center. peak-motifs builds motifs using over-represented oligos present in the sequence set given as input, the tool is also able to compare the discovered motifs with any given set of known motifs in order to identify the putative transcription factors that could be binding the sequences, for this task we used the none redundant JASPAR motif collection for vertebrates (Castro-Mondragon et al., 2016; Mathelier et al., 2016). Secondary motif sets were discovered by using a control sequence set as background to eliminate motifs enriched in such control set and increase the signal of other motifs with less representation but that still can have biological and statistical significance. Further details regarding motif analysis of ERG target genes can be found in the supplementary Materials and Methods (Table S3).
CRISPR-mediated HLX enhancer deletion:
The gRNAs were ordered as standard DNA oligomers from Integrated DNA Technologies (IDT) (see below for sequences). The oligomers were then annealed, phosphorylated, and cloned into pSpCas9(BB)-2A-GFP (PX458) under control of the U6 promoter (Addgene Plasmid ID 48138). TeloHAECs were transfected with 6 μg of PX458 plasmid containing each 5’ and 3’ gDNA or two scrambled control gRNAs using Lipofectamine 2000 (2 μL/1 μg DNA, Invitrogen) in 100 mm dishes. After 48 h, TeloHAECs were FACS sorted to isolate GFP+ cell populations. Following expansion of the GFP+ cells, they were then seeded as single cell colonies in 96 well plates. Subsequently, the cells were cultured, expanded and genotyped. Genomic DNA was isolated from cell pellets after overnight incubation at 56°C in lysis buffer (100 mM NaCl / 10 mM Tris- HCl (pH 8) / 25 mM EDTA (pH 8) / 0.5% SDS / 0.2 mg/mL ProK) and phenol-chloroform extraction. For genotyping, PCR reactions were performed using Taq Polymerase (Invitrogen) as per manufacturer’s instructions. A forward anchor primer was used along with a reverse primer nested either within the deletion region (wild type allele) or 3’ of the deletion region (deletion allele). The PCR products were separated by gel electrophoresis and visualized using MiniBIS Pro (DNR Bio-Imaging Systems). Furthermore, the PCR products were gel extracted (QIAquick Gel Extraction Kit) and the deletion was confirmed by DNA sequencing.

Guide sequences for CRISPR experiments:

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<tr>
<th>Guide sequence</th>
<th>Forward primer (5’- 3’)</th>
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<tr>
<td>HLX_3’_gRNA_F</td>
<td>5’-CACCGTCCAAGGTTTGCGACGCTCC</td>
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<td>HLX_3’_gRNA_R</td>
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Genotyping primers for HLX CRISPR experiments:

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<th>Genotyping primer</th>
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<tr>
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Table S1: Primers used for murine genotyping

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Table S2: Primers used for quantitative PCR: qPCR primers were designed using Primer3 (http://bioinfo.ut.ee/primer3/), selecting for an amplicon length of 80-140 bp, spanning at least one exon-exon boundary with an intervening intron greater than 400 bp to favor mRNA amplification, with an efficiency of greater than 80% as determined by qPCR dissociation (melting curve), with a single amplicon (confirmed by melt curve dissociation).

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<td>Ccdn1 mRNA</td>
<td>CCAACAACTCCTCTCTGCT</td>
<td>GACTCCAGAAAGGGCTTCAATC</td>
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Table S3: Motif analysis of ERG target genes.

Primary motifs were discovered using peak-motifs in ERG peaks overlapping extended (10 kb) transcriptional start sites (TSSs) of the corresponding gene data sets. Motifs were annotated as belonging to a transcription factor (TF) by similarity to JASPAR vertebrates non-redundant annotate motifs.

Secondary motifs were discovered using peak-motifs in the ERG peaks overlapping extended TSSs for the specified data set using a control background corresponding to ERG peak regions overlapping extended TSS for genes with opposite expression pattern. Motifs were annotated as belonging to a TF by similarity to JASPAR vertebrates non-redundant motifs.

* Indicates data sets where the Secondary Motifs contained one or more TFs not contained in the Primary Motifs set.

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<th>Data Set</th>
<th>Num. Peaks</th>
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<th>Secondary Motifs</th>
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<td>Gabpa, ETV6, ELF5, ETS1, ERG, Gabpa, ELK4, ETS1, NFIC, SPIB, ETV6, NKKX6-1, NKKX6-2, Bsx, Tcf15, ZBTB33, TEAD4, EBF1, MZF1, ERF, SP1, SP2, KLFS, SPI, FOSL1, JUNB, FOSL2, OTX2, Pitx1, Arid3b, FOXD2, ELK3</td>
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<td>ERG target genes (down-regulated following ERG knockdown)</td>
<td>187</td>
<td>REL, RELA, SPIB, EWSR1-FU1, ETV2, SP2, KLFS, SP2, TEAD4, POLR2A, POLR3, TEAD3, Bcl6, Ahr, Aromat; ETS1, ELF5, ERF, FOSL2, FOSL1, JUNB, EWSR1-FU1</td>
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<td>ERG-sensitive VEGF regulated genes (down-regulated following ERG knockdown)</td>
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<td>ERG-sensitive VEGF regulated genes (upregulated following ERG knockdown)</td>
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<td>Nr2e1, FOXP3, FOXD1, FOXD, Nr2e1, Atf3</td>
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Table S4: Summary and quality control analysis of ChIP-seq experiments. A) Sequencing statistics and QC measurements for individual libraries. ENCODE practice (see Landt et al., 2012 and https://genome.ucsc.edu/ENCODE/protocols/dataStandards/ChIP_DNase_FAIRE_DNAme_v2_2011.pdf for a detailed explanation of QC). Normalized strand-cross correlation (NSC) coefficient and the relative strand cross correlation (RSC) are a peak-calling independent means to assess data quality and NSC values above 1.05 and RSC above 0.8 indicate high quality transcription factor ChIP-seq data. PCR bottleneck coefficient (PBC) indicates PCR based artifacts. We observe a low PBC score in the WL375 library however this library is comparable to the better replicate WL379 and so we retained both experiments for analysis. B) Irreproducible discovery rate (IDR) analysis of ChIP-seq replicates. As a rule of thumb, ENCODE recommends that reproducible replicates possess Nt/Np and N1/N2 ratios within a factor of 2 (0.5 ≤ N ≤ 2). C) Summary of number peaks and conserved peaks in HUVEC and BAEC experiments.

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<th>N2</th>
<th>N1 / N2</th>
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References:


Supplemental Figures

A. Human microvascular endothelial cells

- Dll4 pre-mRNA
- Dll4 mRNA

B. Dll4 pre-mRNA (30' VEGF)

C. ERK1
- Relative Expression
- siRNA Added: Control, ERK, ERK1, ERK2, ERK1/2

- DLL4
- EGR3
- DUSP5

- Relative Expression
- siRNA Added: Control, ERK, ERK1, ERK2, ERK1/2

Pathway Inhibitor

Control ERK PKC PI3K P38 Notch

ERK1 ERK2

GAPDH
Figure S1: Transcriptional induction of DLL4 is dynamic and requires MAPK/ERK signaling
A) Induction kinetics of DLL4 pre-mRNA and mRNA in dermal microvascular ECs as assessed by qRT-PCR (n=3).
B) MAPK-dependence (MEK and PKC inhibitors) of DLL4 induction in dermal microvascular ECs assessed through the use of small molecule inhibitors of these pathways (n=3).
C) ERK1 and ERK2 were knocked down in HUVEC using siRNA. qRT-PCR reveals that the induction of DLL4, EGR3 and DUSP5 is ERK1/2-dependent. N = 4-5. A representative western blot (of 3) reveals the extent of ERK1/2 knockdown. GAPDH is shown as a loading control.
**A**

- **DMSO**

- **SL327**

  - **kdrl**:GFP
  - **pERK**

**B**

- **kdrl**:mCherry; **fl1a:nls-EGFP**

  - **DMSO**
  - **SL327**

**C**

- **DMSO**
  - **SU5416**
  - **SL327**
  - **c646**
Figure S2: Characterization of sprouting defects in embryos treated with MEK inhibitor (SL327)
A) pERK is enriched in tip cells during ISV formation in zebrafish and this is dependent on MEK signaling as it is negated in MEK-inhibitor (SL327) treated embryos (treated from 18-20 hpf to ~24 hpf). Quantification of the number of ISVs that stained positive for pERK is indicated. 24 hpf. Scale bar = 50 μm.
B) The number of endothelial cells per intersomitic vessel was quantified in Tg(kdrl:mCherry;fl1a:nls-EGFP) embryos exposed to DMSO or SL327 (30 μM). The number of mCherry/EGFP double-positive cells was counted per intersomitic vessel and averaged per embryo. The number of embryos quantified is indicated.
C) Transmitted light images of DMSO, SL327 (30 μM), SU5416 (5 μM) and c646 (3 μM) treated embryos revealing normal overall development and lack of tissue necrosis or developmental delay.
Figure S3: Notch induction in the vasculature is compromised in embryos treated with MEK inhibitor (SL327)

Notch activity is reduced in the vasculature of SL327-treated Tg(kdrl:mCherry); Tg(Tp1bglob:Venus-PEST) embryos. Still images from time-lapse microscopy of a representative experiment are shown. Arrows indicate Notch signaling-positive ISVs, while asterisks indicate Notch-negative ISVs. Scale bar = 20 μm. See Videos S1 and S2.
A

Row z-score

control siRNA

ERG siRNA

1h VEGF

NS

Biological Process # of genes

Response to Wounding 34
Inflammatory Response 22
Regulation of Cytokine Production 21
Cell Migration 31
Cell Motility 33
Positive Regulation of Response to Stimulus 47
Cell Surface Receptor Signaling Pathway 56
Locomotion 41
Circulatory System Development 26
Cell Adhesion 34

B

ERG-dependent genes (non-stimulated)

p-value

10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}

144: doi:10.1242/dev.146050: Supplementary information
Figure S4: A network of genes are regulated by ERG in endothelial cells

A) Heat map depicting hierarchical clustering of significantly regulated genes in control- and ERG siRNA-transfected cells +/- 1h VEGF treatment. NS = not stimulated. Shown are genes that are differentially expressed between ‘Control siRNA-NS’ and ‘ERG siRNA-NS’.

B) Gene Ontology analysis of dysregulated genes (‘Control siRNA-NS’ vs. ‘ERG siRNA-NS’). Selected, representative GO terms are displayed with their associated p-value. The number of genes in each GO category is indicated. The GO terms depicted are: Response to wounding (GO:0009611), Inflammatory response (GO:0006954), Regulation of cytokine production (GO:0001817), Cell migration (GO:0016477), Cell motility (GO:0048870), Positive regulation of response to stimulus (GO:0048584), Cell surface receptor signaling pathway (GO:0007166), Locomotion (GO:0040011), Circulatory system development (GO:0072359), Cell adhesion (GO:0007155).
Figure S5: A network of genes are regulated by VEGF and ERG in endothelial cells
A) Heat map of genes that are differentially expressed between ‘Control siRNA-NS’ and ‘Control siRNA-1h VEGF’. The VEGF-inducible genes that are dysregulated upon ERG knock-down are highlighted in red (activated) or blue (repressed), and indicated to the right.

B) Gene Ontology analysis of VEGF inducible genes (‘Control siRNA-NS’ vs. ‘Control siRNA-1h VEGF’). Representative GO terms are indicated: Regulation of transcription from RNA Polymerase II promoter (GO:0006357), Regulation of RNA biosynthetic processes (GO:2001141), Regulation of cell proliferation (GO:0042127), Negative regulation of signaling (GO:0023057), Regulation of cell death (GO:0010941), Positive regulation of gene expression (GO:0010628), Intracellular signal transduction (GO:0035556), Vasculature development (GO:0001944), Cardiovascular system development (GO:0072358), Positive regulation of biosynthetic process (GO:0009891).
Figure S6: ERG-/VEGF-regulated genes are dynamically expressed

Kinetics of a subset of ERG-/VEGF-regulated genes, as measured by qRT-PCR of pre-mRNA in HUVEC (n=3). Transcriptional induction of these genes is dynamic, with peak transcription between 15'-1h. Note that some of this data is also found in Fig. 1A,E, 7A, but is included here to facilitate direct comparison.
A

Wildtype Allele
Targeting Vector

Knockout First Allele (KO)
KO (post Cre)
Flox (post Flp)

Exon 5

342

lacZ neo

β actPSA IRES pA

FRT FRTloxP loxP loxP pA

Exon 7

440

Exon 6

B

C

Adult (male and female)

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E11.5

predicted 25% 50% 25% 100%
observed 31% 44% 14% 100%
n 6 12 3 21

E10.5

predicted 25% 50% 25% 100%
observed 26% 44.5% 29.5% 100%
n 57 98 65 220

E10.5

phenotype | Erg^{+/+} | Erg^{KO/+} | Erg^{KO/KO} |
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D

Erg^{+/+} Erg^{KO/+} E9.5

E

Erg^{+/+} Erg^{KO/+} E10.5

G

H

G’

H’

I

Radial Expansion (μm)

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J

Sprouts/Field

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K

Branch Frequency

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Figure S7: Deletion of Erg in mouse embryos results in embryonic lethality, accompanied by defects in angiogenesis, vascular remodeling and vascular integrity

A) Strategy for the generation of an Erg knock-out/LacZ knock-in first, conditional ready allele (also known as Erg\textsuperscript{tm1a}). Homologous recombination was used to insert an IRES-LacZ / \( \beta \)-Actin::Neo cassette into intron 5 of Erg. Following Cre-mediated recombination, the FRT-flanked \( \beta \)-actin::NEO selection cassette is deleted, leaving an ERG-ires-LacZ gene-trap (also known as Erg\textsuperscript{KO} or Erg\textsuperscript{tm1c}). Following Flp-mediated recombination of Erg\textsuperscript{tm1a}, loxP sites are left flanking exon 6, generating a conditional loss of function allele (also known as Erg\textsuperscript{lox} or Erg\textsuperscript{tm1b}). Genotyping primers are indicated. PNT=pointed domain; ETS=ETS DNA binding domain.

B) Agarose gel electrophoresis of PCR genotyping products to identify Erg mutants. 100 bp ladder on the far left lane.

C) Expected and observed ratios of genotypes from the progeny of Erg\textsuperscript{+/c} crosses. Deletion of Erg results in embryonic lethality ~E11.5, and increased presence of hemorrhage and smaller embryo size. No defects were observed in heterozygous mutant embryos.

D) Phase images of wholemount, E9.5 Erg\textsuperscript{+/c} and Erg\textsuperscript{KO/+/c} embryos following lacZ staining.

E) Phase images of wholemount, E10.5 Erg\textsuperscript{+/c} and Erg\textsuperscript{KO/+/c} embryos following lacZ staining. Scale bar = 500 \( \mu \)m.

F) Immunohistochemistry for ERG (red), CD31 (green), and DAPI (blue) in the left panel, and LacZ staining in the right panel, from serial sections of an Erg\textsuperscript{KO/+} E10.5 embryo, show overlap of Erg and gene-trap Erg-ires-LacZ activity. Scale bar = 100 \( \mu \)m. Lower panels are magnified views of the boxed area in the upper panels, scale bar = 20 \( \mu \)m. NT, neural tube; DA, dorsal aorta.

G, H) Representative images of the total retinal vasculature (G, H) and magnified view of the distal, vascular front (G', H') and proximal region (G'', H'') stained with IB4 in Erg\textsuperscript{+/+} (G, G', G'') and Erg\textsuperscript{ECKO} (H, H', H'') retinas at P6 following tamoxifen administration at P1. Scale bar = 100 \( \mu \)m. The bar in (G, H) indicates avascular area in each retina. Arrows in (G', H') indicate sprouting vessels at the vascular front. Scale bar = 100 \( \mu \)m.

I) Quantification of radial expansion of the IB4\textsuperscript{*} vasculature within the P6 retina.

J) Vascular sprouting as determined by quantification of IB4\textsuperscript{*} sprouts in the distal retinal vascular plexus at P6.

K) Vascular density as determined by quantification of IB4\textsuperscript{*} branches in the proximal retinal vascular plexus at P6.
**A**

RefSeq Genes

**B**

<table>
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<th>Gene</th>
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**Development 144: doi:10.1242/dev.146050: Supplementary information**
**Figure S8: Comparison of conservation between an upstream human and zebrafish HLX enhancer**

A) Shown are browser screenshots comparing evolutionary conserved regions (ECR) across multiple, distantly-related vertebrates, with the zebrafish, possum, and mouse genomes aligned to the human genome (chr1:221049800-221053860). Peaks indicate sequence conservation. Red denotes intergenic sequence, yellow indicates the 5’ UTR, blue denotes exons, and salmon shows intronic regions. The x-axis denotes position in the base genome and the y-axis represents the percent identity between the base and aligned genomes. The region analyzed in (B) is underlined.

B) ClustalW alignment of a portion of the HLX-3a (and HLX-3b) enhancer regions from human (hg19), mouse (mm10), and zebrafish (danRer7). Asterisks below the nucleotides indicate sequence conservation across all three species. The human HLX-3a region contains 26 ETS consensus sequences (GGA(A/T)) (not shown). The sites are numbered relative to their positive within HLX-3a. As H3K27ac flanked HLX-3a region did not direct expression in the endothelium (see main text and methods), we focused on the smaller, conserved, region identified by ERG ChIP-seq, HLX-3b (denoted by primers). HLX-3b contained 6 deeply conserved ETS sites (ETS-H, ets, ETS-I, ETS-J, ELK1, and ETS-K), as well as 3 deeply conserved MEF2 sites, an SRF site, and a GATA site. All ETS sites tested by mutation analysis are indicated in blue and capital letters, while lowercase and gray coloring indicates sites that were not experimentally validated. The human HLX-3b enhancer contained 9 total ETS sites.
Supplementary Movie 1. Notch signaling is active in the developing vasculature. Representative time-lapse confocal microscopy of a $Tg(kdrl:mCherry); Tg(Tp1:VenusPEST)$ embryo treated with DMSO demonstrates Notch activity within the axial and sprouting vasculature (n=6). Embryos were mounted in E3 with tricaine. Treatment began at 18 ss, and continued, at RT, for 15 hours and 10 minutes. See Fig. S3 for still images.
Supplementary Movie 2. Notch signaling is lost in the vasculature upon MAPK inhibition. Representative time-lapse confocal microscopy of a Tg(kdrl:mCherry); Tg(Tp1:VenusPEST) embryo treated with SL327 (30 uM) demonstrates reduced Notch activity within the vasculature, and decreased sprout elongation (n=6). Treatment began at 18 ss, and continued, at RT, for 15 hours and 10 minutes. See Fig. S3 for still images.