Induction of hematopoietic and endothelial cell program orchestrated by ETS transcription factor ER71/ETV2

Fang Liu¹, Daofeng Li², Yik Yeung Lawrence Yu¹, Inyoung Kang¹, Min-Ji Cha¹,†, Ju Young Kim³, Changwon Park³, Dennis K Watson⁴, Ting Wang²,*, & Kyunghee Choi³,5,**

Abstract

The ETS factor ETV2 (aka ER71) is essential for the generation of the blood and vascular system, as ETV2 deficiency leads to a complete block in blood and endothelial cell formation and embryonic lethality in the mouse. However, the ETV2-mediated gene regulatory network and signaling governing hematopoietic and endothelial cell development are poorly understood. Here, we map ETV2 global binding sites and carry out in vivo differentiation of embryonic stem cells, and germ line and conditional knockout mouse studies to uncover mechanisms involved in the hematogenic fate commitment from mesoderm. We show that ETV2 binds to enhancers that specify hematopoietic and endothelial cell lineages. We find that the hematangiogenic progenitor population in the developing embryo can be identified as FLK¹⁺⁺PDGFRα⁺⁻. Notably, these hematangiogenic progenitors are exclusively sensitive to ETV2-dependent FLK1 signaling. Importantly, ETV2 turns on other Ets genes, thereby establishing an ETS hierarchy. Consequently, the hematopoietic and endothelial cell program initiated by ETV2 is maintained partly by other ETS factors through an ETS switching mechanism. These findings highlight the critical role that transient ETV2 expression plays in the regulation of hematopoietic and endothelial cell lineage specification and stability.

Keywords ChIP-Seq; ER71/ETV2; ETS hierarchy; ETS switching; hematangioblast; VEGFR2/Flk1

Subject Categories Development & Differentiation; Transcription; Chromatin, Epigenetics, Genomics & Functional Genomics

DOI 10.15252/embor.201439939 | Received 2 December 2014 | Revised 24 February 2015 | Accepted 26 February 2015 | Published online 23 March 2015


Introduction

Functional blood and its conduit vascular system are the first to form during embryogenesis. Blood cells in the developing embryo are generated in close association with endothelial cells. For example, yolk sac blood islands composed of centrally located embryonic blood cells and an outer luminal layer of endothelial cells of the embryo are generated from the extra-embryonic mesoderm presumably through a hemangioblast intermediary [1–4]. While the hemangioblast is believed to be a common hematopoietic and endothelial cell progenitor, a recent study suggests that primitive erythroid cells in the yolk sac are developmentally distinct from the yolk sac endothelium [5]. It has also been suggested that hemangioblast is not a progenitor, but represents a competent cell state that can generate either blood or endothelium depending on the surrounding signals [6]. Regardless, it is now widely accepted that hemogenic endothelium within the dorsal aorta of the embryo is the cell origin of the definitive hematopoietic system [7–10]. Later in adult life, hematopoietic system is maintained partly through the vascular system. As such, sinusoidal endothelial cells form an important component of the niche in which hematopoietic stem cells reside [11–13]. It is thus noticeable that many transcription factors and signaling pathways are largely shared between blood and endothelial cells. Gene-targeting studies have shown that mutations in any of the shared genes often affect both cell lineages, supporting the notion of common genetic pathways regulating hematopoietic and endothelial cell lineage development and function. Remarkably, Vegfa- or Vegfr2 (Flk1)-deficient animals completely fail to generate blood and blood vessels and die early in embryogenesis, indicating that precise VEGFA signaling via FLK1 is critical for the proper formation of the blood and vascular systems [14–16]. Clearly, molecular mechanisms by which blood and vessel lineages are specified in the developing embryo need to be better elucidated. Such information in turn would be greatly useful for future applications for blood and
vascular repair and regeneration as well as for obtaining hematopoietic and endothelial cells from pluripotent stem cells.

ETS transcription factors have emerged as critical regulators of hematopoietic and vascular development [17–19]. The ETS domain, which is composed of a winged helix-turn-helix motif, binds a consensus sequence (GGAA/T) to regulate target gene expression. Many ETS factors are redundantly expressed in blood and endothelial cells. Consistently, mice or zebrafish deficient in Ets factors display differing levels of hematopoietic and vascular defects [20–23]. Distinct from other ETS factors, Etv2 is transiently expressed in the primitive streak, yolk sac blood islands, and large vessels including the dorsal aorta during embryogenesis [24]. Remarkably, Etv2-deficient animals display a complete block in blood and blood vessel formation, indicating that ETV2 performs a non-redundant and indispensable function in hematopoietic and vessel development [24–27]. As such, Etv2 inactivation leads to similar hematopoietic and vascular defects to those of Vegfa or Flk1 deficiency. Herein, we characterized germ line and conditional Etv2 knockout mice and performed genomewide ChIP-Seq of ETV2 using in vitro differentiated embryonic stem (ES) cells to better understand how ETV2 can achieve such a non-redundant predominant role in hematopoietic and endothelial cell development. We discover that specification of the hemangiogenic program requires ETV2 activation of the blood and endothelial cell development. We discuss that Etv2-deficient animals display a complete block in blood and blood vessel formation, indicating that ETV2 performs a non-redundant and indispensable function in hematopoietic and vessel development [24–27]. As such, Etv2 inactivation leads to similar hematopoietic and vascular defects to those of Vegfa or Flk1 deficiency. Herein, we characterized germ line and conditional Etv2 knockout mice and performed genomewide ChIP-Seq of ETV2 using in vitro differentiated embryonic stem (ES) cells to better understand how ETV2 can achieve such a non-redundant predominant role in hematopoietic and endothelial cell development.

Our discovery of the hemangiogenic program requires ETV2 activation of the blood and endothelial cell lineage-specifying genes and VEGF signaling. Moreover, ETV2 establishes an ETS hierarchy by directly activating other Ets genes, which then maintain blood and endothelial cell program initiated by ETV2 through an ETS switching mechanism. Collectively, we provide molecular and cellular basis by which ETV2 establishes the hematopoietic and endothelial cell program.

Results

ETV2 ChiP-Seq and target gene identification

To understand ETV2-mediated genetic program regulating hematopoietic and endothelial cell lineage development, we performed ETV2 ChiP-Seq analysis using in vitro differentiated embryonic stem (ES) cells. We previously described A2 ES cells expressing ETV2-V5 in a doxycycline (DOX)-inducible manner [24,27]. DOX addition from day 2 to 3.5, a time frame when Etv2 is normally expressed, in these cells robustly induced hemangioblast formation. To facilitate ETV2 target identification, we additionally generated polyclonal antibodies against ETV2 using in vitro differentiated embryonic stem (ES) cells to better understand how ETV2 can achieve such a non-redundant predominant role in hematopoietic and endothelial cell development. We discover that specification of the hemangiogenic program requires ETV2 activation of the blood and endothelial cell lineage-specifying genes and VEGF signaling.

Moreover, ETV2 establishes an ETS hierarchy by directly activating other Ets genes, which then maintain blood and endothelial cell program initiated by ETV2 through an ETS switching mechanism. Collectively, we provide molecular and cellular basis by which ETV2 establishes the hematopoietic and endothelial cell program.

protein. Moreover, focusing on reproducible ChiP-seq peaks based on independent antibodies ensures a higher specificity. Indeed, several initial quality assurance analyses suggested that these ETV2 binding peaks had high quality and were connected to ETV2 biology, supporting that they were bona fide biological target sites of ETV2. First, raw read densities within these ETV2 peaks were highly reproducible between experiments (cc = 0.974 for ETV2-polyAbs and 0.969 for V5 between replicates, cc = 0.993 between the two antibodies, Supplementary Fig S1A–C). ChiP-Seq signals were highly enriched in peak centers across samples when compared to surrounding genomic regions or control (Fig 1A and B). We also identified the most significant sequence motif associated with the ChiP-Seq peaks (Fig 1C). This motif, which represented ~85% of the peaks, matched perfectly with the known binding specificity of other ETS factors, FLI1, and ERG [30]. We additionally identified GATA, SOX, or E-box motifs to be frequently associated with the ETV2 peaks (Supplementary Fig S1D). Genomewide distribution of these binding peaks was far from random expectation, with ~14% significant enrichment in the promoter regions and ~70% in introns or intergenic regions, suggesting that ETV2 functions by interacting with both gene promoters and distal enhancers (Fig 1D and Supplementary Fig S1F). We next subjected these ETV2 binding peaks to a GREAT analysis [31] to understand the overall ETV2-mediated biological function and found that they were strongly associated with endothelial and hematopoietic cell lineage development and differentiation (Fig 1E, for complete result see Supplementary Table S2A). Finally, we examined evolutionary conservation of sequences associated with ETV2 peaks and found that they are much more conserved than their neighboring sequences (Fig 1F). Indeed, 2,231 (56.7%) peaks overlapped with conserved elements determined based on 30-way vertebrate alignment [32] from the UCSC Genome Browser (P-value < 2.2e−16, binomial test), suggesting that the identified peaks were strongly enriched for functionally constrained sequences. As the majority of the ETV2 binding peaks were outside gene promoters, we reasoned that some of them could serve as distal enhancers. Connecting enhancers to their target genes is an extremely challenging problem, because enhancers do not necessarily regulate the nearest genes, nor do they necessarily regulate one single target genes. Therefore, we combined GREAT analysis with gene expression analysis to optimize sensitivity and specificity.

Using GREAT default parameters, we defined a “basal regulatory domain” for each gene (Methods), and we associated an ETV2 ChiP-seq peak with a target gene if the peak was within the basal regulatory domain of the gene. This approach resulted in 4,580 ETV2 peak-associated genes.

To narrow down to a more confident list of direct target genes of ETV2, we integrated our ChiP-Seq data with gene expression pattern of FLK1+ mesoderm isolated from control and iETV2 EB cells that were generated with DOX (from day 2–3.5) as well as FLK1+ mesoderm sorted from Etv2+/− and Etv2−/− day 3.5 EBs [27]. ETV2 peak-associated genes were significantly enriched for genes that exhibited increased expression in the ETV2 overexpression system and/or reduced expression in the ETV2 knockout system (Fig 2A, P-value < 2.2E−16, hypergeometric test). On the other hand, there was no significant enrichment of genes with the opposite expression pattern (i.e., upregulated in ETV2 knockout and downregulated in ETV2 overexpression, Supplementary Fig S2A). This expression pattern suggested that ETV2 primarily functions as a transcriptional
activator. We identified 425 ETV2 peak-associated genes that exhibiting the expected expression difference. They constitute a group of high confidence, direct target genes of ETV2. Functional enrichment analysis revealed that this group of genes was considerably enriched for hematopoietic and endothelial cell lineage development and differentiation, with the enrichment level markedly improved from genes identified by ChIP-seq peaks alone (Supplementary Table S2B). Many genes that exhibited expression changes upon either ETV2 overexpression or ETV2 knockout did not associate with ETV2 binding peaks. They are potentially downstream but not direct targets of ETV2. Additionally, many ETV2 peak-associated genes did not show expression change, even though as a group they are strongly enriched for endothelial and hematopoietic cell relevant functions. These binding peaks could potentially be false positives, but could also reveal functions of ETV2 other than directly activating target genes. One such potential function could be to modulate chromatin structure to a permissive state that allows genes to express at future developmental stages. We thus examined the epigenetic landscape of ETV2 binding sites across several cell and tissue types. We took advantage of publicly available whole-genome bisulfite sequencing data of mouse embryonic stem cells, neural progenitor cells, as well as adult tissues [33,34] to examine epigenetic changes co-occurring with ETV2 binding, focusing on distal sites that potentially function as lineage-specific enhancers.
Intriguingly, these ETV2 binding sites were largely methylated in ES cells. The relatively high DNA methylation levels were maintained in neuronal progenitor cells and cerebellum, but reduced in heart and bone marrow (Fig 2B and Supplementary Fig S2B).

**ETV2 induces hematopoietic and endothelial cell lineage-specifying genes**

ETV2 targets can be broadly categorized into hematopoietic and endothelial cell lineage-specifying genes, VEGF, Notch, Rho-GTPase, and MAP kinase pathway and *Ets* factors (Fig 2C). Specifically, Flk1, Fli1, Erg, Gata2, Scl, Meis1, Lmo2, Tie2, VE-cadherin, Dll4, and Notch were among the 425 genes, which play critical roles in hematopoietic and endothelial cell development (Figs 2C and D, 3A and 5A). While some of these peaks occur on previously identified regulatory regions, such as VE-cadherin [27], currently identified ChIP-Seq peaks represented unique peaks that have not been reported yet. We additionally compared transcriptional profiling among FLK1+ cells generated by enforced Etv2 expression, Etv2-deficient FLK1+ cells [27], and genes immediately upregulated by Etv2 expression [35]. There was a significant enrichment in genes involved in the VEGF and Notch signaling pathways, suggesting the

---

**Figure 2. Identification of ETV2 downstream target genes and regulatory network.**

A Venn diagram showing the overlap between ChIP-Seq peak-associated genes and ETV2 target genes identified by microarray data analysis. UP (+DOX): upregulated genes by ETV2 overexpression; DN (ETV2 KO): downregulated genes in ETV2 knockout.

B DNA methylation profile of ETV2 distal binding peaks. Averaged DNA methylation level was calculated using publicly available whole-genome bisulfite sequencing data from multiple cell and tissue types and plotted for a 6-kb region flanking peak centers.

C ETV2 gene regulatory network showing Notch/MAPK signaling, VEGF signaling/lineage specification, Rho-GTPase, and ETS transcription factor as downstream targets.

D Genomic snapshots depicting the ETV2 binding regions at the indicated genomic loci.
involvement of these pathways in hemangiogenic lineage development (Supplementary Fig S2C).

Previous studies have implicated the core gene regulatory network played by the ETS, GATA, and E-box motifs in hematopoietic and endothelial cell development [36]. *Etv2*, *Gata2*, and *Scl* can independently modulate hemangioblast development [37–39]. Moreover, coexpression of *Etv2*, *Gata2*, and *Scl* during the time of hemangioblast formation stage can robustly induce hemangioblast cell population [39]. Notably, GATA and E-box motifs were frequently associated with *Etv2* peaks (Supplementary Fig S1D and E). Thus, we determined whether sequences representing binding sites of these factors occur in *Etv2* peaks. We utilized the ChIP-Seq data of GATA2 and SCL from Wilson et al [30], which allowed us to train a positional weight matrix for each of these factors. GATA2 and SCL motifs were significantly enriched within the 3,933 peaks: 2,945, 609, and 484 co-occurrences of *Etv2*-SCL motifs (8-fold enrichment over random expectation, *P* = 0 from binomial test), *Etv2*-GATA2 motifs (14-fold enrichment, *P* < 1.1E-35), and *Etv2*-SCL-GATA2 motifs (15-fold enrichment, *P* = 0) (Supplementary Fig S2D). This observation is highly non-random, suggesting that *Etv2* and these factors may interact or cobind to some of these sites. Indeed, *Etv2* and GATA2 have been recently reported to form a complex to regulate hematopoietic and endothelial cell gene expression [40]. *Etv2* presumably specifies the hemangiogenic cell fate by collectively turning on hematopoietic and endothelial cell lineage-specifying genes. The ETS, GATA, and E-box gene regulatory network is integral to this process.

**ETV2 enhances VEGF signaling**

ChIP-Seq analysis revealed *Etv2* binding to VEGF receptors and downstream signaling pathway genes including MAPK. Specifically, *Etv2* targets include *Flk1*, *Flt1*, *Flt4*, *Nrp1*, *Nrp2*, and *Mapk3* genes (Fig 3A). Rho-GTPases and adhesion molecules were also identified as potential *Etv2* direct targets. We selected 15 peak regions associated with *Flk1*, *Flt1*, *Nrp1*, and *Nrp2* genes occupied by *Etv2*, of which 14 were evolutionarily conserved (Fig 3A and Supplementary Table S3), and validated *Etv2* binding in day 3.5 *Etv2* EBs using ChIP-qPCR. A significant mean enrichment was observed for *Etv2* binding at these genomic locations with chromatin pulled down by V5 antibody or endogenous *Etv2* antibody (*Etv2*-polyAbs) in i*Etv2* EB cells (Dox added on day 2) (Fig 3B). Importantly, *Etv2* binding at these genomic locations was confirmed in R1 wild-type EB cells using *Etv2*-polyAbs, validating that these are bona fide *Etv2* targets (Supplementary Fig S3A). To assess the functional significance of the *Etv2* binding, we tested these regions for the response to *Etv2* using the luciferase reporter assay. We found that *Etv2* could activate the luciferase constructs tested, approximately 5- to 100-folds, compared to the pGL4 vector control (Fig 3C). We further selectively deleted the *Etv2* binding motif in several reporter vectors and found that the luciferase activity was greatly impaired when *Etv2* binding motif was deleted (Fig 3C). Collectively, these regions may act as *Etv2* cis-regulatory elements for VEGF receptor gene expression. Future *in vivo* transgenic reporter system would further solidify such notion.

As ChIP-Seq analysis suggested that *Etv2* could elevate FLK1 signaling activity, we determined whether *Etv2* could directly modulate VEGF signaling. To this end, we performed VEGF signaling and Cell Motility PCR array, which included Rho-GTPases, adhesion and integrin genes, using RNA obtained from *Etv2* EBs (differentiated ES cells) with DOX [27]. Strikingly, VEGF signaling pathway genes were upregulated by *Etv2* overexpression, which were reciprocally downregulated in *Etv2*−/−embryos as well as *Flk1−/−embryos (Fig 3D and E, Supplementary Table S4). Additionally, while Rho-GTPase and its activating protein genes were upregulated by *Etv2* overexpression, they were greatly downregulated in E8.5 *Flk1−/−embryos* [41] (Fig 3F and G, Supplementary Table S5). These studies support the notion that hemangiogenic program specification requires *Etv2*-mediated FLK1 signaling activation.

**ETV2 is required for the formation and expansion of FLK1+ hemangiogenic progenitors**

Consistent with the data that *Etv2* enhances VEGF signaling, previous findings suggested that blood and endothelial cell progenitors express high levels of FLK1 (FLK1high) compared to other FLK1-expressing cardiac or muscle progenitors (FLK1low) [42]. To determine whether we can identify differential FLK1 activity associated with hemangiogenic cell population development in the embryo, we subjected embryos to FLK1 and PDGFRα expression analysis. As FLK1+PDGFRα− cells isolated from differentiating ES cells were enriched for the hemangioblast [27] and as FLK1+PDGFRα− cells in the developing embryo have not been characterized yet, we initially analyzed FLK1 and PDGFRα expression kinetics in developing embryos. In wild-type embryos, FLK1+PDGFRα− hemangiogenic progenitors were already detected around embryonic day (E) 7.5 and progressively expanded with time during the course of E7.5–E8.5 (Fig 4A). Notably, the mean fluorescence intensity of the FLK1 staining within the FLK1+PDGFRα− cell population became greater as embryos develop, suggesting an elevated FLK1 signaling activity within FLK1+PDGFRα− cells compared to FLK1+PDGFRα+ cells (Fig 4A). Remarkably, FLK1highPDGFRα− cell population was exclusively missing in *Etv2*−/−embryos, indicating that *Etv2* was specifically required for the stage of FLK1highPDGFRα− cell generation in the embryo (Fig 4A, Supplementary Fig S3B and C).

*Etv2* is transiently expressed in developing embryos and ES/EBs [24]. To determine whether *Etv2* was still required once FLK1+ hemangioblast was formed, we conditionally deleted *Etv2* within *Flk1*-expressing cells, *Flk1Cre;Etv2* CKO, by crossing *Flk1Cre;Etv2*−/+ (or *Flk1Cre;Etv2*−/−) and *Etv2*Δf/+ mice to generate *Flk1Cre;Etv2*−/+ (or *Flk1Cre;Etv2*−/−) and *Etv2Δf*/+ mice. No live *Flk1Cre;Etv2* CKO mice were obtained at weaning. Timed matings between *Flk1Cre;Etv2*−/− and *Etv2*Δf/+ mice were performed, and embryos were analyzed at different time points. *Flk1Cre;Etv2* CKO embryos died around E10.5, later than *Etv2*−/− mice that die around E9.5, and exhibited wrinkled yolk sacs with dispersed blood (Fig 4B). At E8.5, FLK1+PDGFRα− cells were detected but at much reduced levels within E8.5 *Flk1Cre;Etv2* CKO embryos (Fig 4C, Supplementary Fig S3D). Consequently, the embryo proper of the mutants was relatively pale and small and showed fluid and blood accumulation in the pericardial cavities. Whole-mount PECAM1/Cd31 staining revealed disorganized vasculatures in the mutants (Fig 4D). E8.5 mutant yolk sacs contained fewer hematopoietic progenitor cells (Fig 4E). Cd31-expressing cells also appeared to be reduced in E9.5 yolk sacs (Supplementary Fig S3E). Consistent with the phenotypic defects, expression of hematopoietic and endothelial genes in *Flk1Cre;Etv2* CKO yolk sacs was also greatly reduced (Fig 4F).
ER71/ETV2 initiates hemangiogenic program

Figure 3.
and G). Collectively, we conclude that ETV2 is required until sufficient FLK1\textsuperscript{high}PDGFR\textsuperscript{−} cells are generated to guarantee proper blood and endothelial cell development.

**ETS genes are ETV2 direct targets**

Many ETS factors are potential ETV2 direct targets (Fig 5A and Supplementary Table S3). Importantly, Etv2 was also identified as a target, suggesting that there is a positive auto-feedback regulation involving Etv2 expression. We selected 15 peak regions associated with Fli1, Ets1, Erg and Ets2 genes, of which 12 were conserved (Supplementary Table S3), and validated ETV2 binding on these genes (Fig 5B). We next constructed luciferase reporters using the validated peak regions of the Ets genes and found that all the tested fragments enhanced the luciferase activity by ETV2 (Fig 5C). Importantly, deletion of the respective ETV2 binding motifs resulted in substantially reduced luciferase activities (Fig 5D). In addition, we observed cooperative action among the potential ETV2 motifs. For example, while the deletion of the two binding sites (mt1) in the Fli1 regulatory region (peak 7 in Fig 5A) still conferred the luciferase activity in response to ETV2, deletion of all three ETV2 binding motifs (mt2 or mt3) in this region almost completely abrogated ETV2 responsiveness in the luciferase activity (Fig 5D). Collectively, we conclude that ETV2 positively regulates its own expression and that of other Ets genes.

Having confirmed that Ets genes are ETV2 targets, we validated the kinetics of the Ets gene expression in developing EBs and embryos. Indeed, Etv2 expression precedes that of other Ets genes in differentiating ES cells. Specifically, Fli1 expression was detected shortly after Etv2, followed by Erg, Elk3, Ets1, and Ets2 (Fig 6A). Moreover, in the DOX-inducible Etv2 expression system (iEtv2 ES), where Etv2 expression becomes visible within 6 h of DOX addition, expression of Fli1, Erg, and Elk3 was prominently induced by 12–24 h after DOX addition (Fig 6B). Ets1 and Ets2 were also induced, although at much modest levels (Fig 6B). Importantly, expression of Fli1, Erg, and Elk3 was greatly reduced in Etv2-null embryos (Fig 6C). Expression of Ets1 and Ets2 was also reduced in Etv2-null embryos (Fig 6C). If Fli1 is truly a direct target of ETV2 in hematopoietic and endothelial cell development, it was expected that Etv2 expression would be unaffected by Fli1 manipulation. Indeed, expression of Etv2, Erg, and Elk3 was not impaired in Fli1-null ES cells differentiated in culture (Fig 6D) or in embryos (Fig 6E). Moreover, enforced Fli1 expression in differentiating ES cells (iFli1 ES cells) did not alter Etv2 expression, although other Ets genes were upregulated (Fig 6F).

**Hierarchical ETS function in hemangioblast, hematopoietic, and endothelial cell lineage development**

One possibility for the non-redundant role of ETV2 in the FLK1\textsuperscript{high}PDGFR\textsuperscript{−} hemangioblast commitment could be the timing of the ETS factor availability. As such, ETV2 happens to be expressed before the other ETS factors and thus carries out such a robust function. To determine whether ETS factors are inherently redundant or unique in their function in the hematopoietic and endothelial cell specification, we analyzed the hemangiogenic output by Fli1, the immediate ETV2 target. Specifically, we determined whether \textsuperscript{2015}Fli1\textsuperscript{−/−} embryos showed defects in the FLK1\textsuperscript{1}PDGFR\textsuperscript{−} hemangiogenic progenitor generation. As shown, FLK1\textsuperscript{1}PDGFR\textsuperscript{−} progenitor cell population still developed in the Fli1-null embryos and ES cells, similar to wild-type control levels, suggesting that hemangioblast formation does not require Fli1 function (Fig 7A and B, Supplementary Fig S4A and B). However, enforced Fli1 expression was able to skew mesoderm, although at a lesser degree than ETV2, into the hemangiogenic fate in iFli1 ES cells (Fig 7C and Supplementary Fig S4C). Ets1 or Ets2 cannot skew mesoderm into the hemangioblast [39]. This supports the notion that Fli1 is not necessary, but sufficient for hemangioblast generation. Ets1 or Ets2 cannot induce hemangioblast, even if overexpressed at the same time frame as Etv2.

To further elicit functional hierarchy among ETS factors, we additionally determined the hemangioblast skewing effect by different ETS factors in the context of Gata2 and Scl coexpression. We previously reported that Etv2, when coexpressed with Gata2 and Scl, robustly skewed mesoderm toward the hemangioblast while suppressing the cardiac output from ES cells [39]. Thus, we compared the coexpression effect of Ets genes with Gata2 and Scl on the FLK1\textsuperscript{1}PDGFR\textsuperscript{−} hemangioblast formation. We specifically generated doxycycline-inducible ES cells coexpressing Fli1-Gata2-Scl, Erg-Gata2-Scl, or Ets1-Gata2-Scl. As previously reported [39], Etv2-Gata2-Scl coexpression during mesoderm formation stage robustly induced FLK1\textsuperscript{1}PDGFR\textsuperscript{−} hemangioblast from ES cells. While FLK1\textsuperscript{1}PDGFR\textsuperscript{−} hemangioblast population was increased by Fli1-Gata2-Scl or Erg-Gata2-Scl, the level of the FLK1\textsuperscript{1}PDGFR\textsuperscript{−}...
cells generated by Fli1-Gata2-Scl or Erg-Gata2-Scl was not as robust as that by the Etv2-Gata2-Scl (Fig 7D, Supplementary Fig S4D). Importantly, there was incomplete skewing of mesoderm toward FLK1+PDGFRα+ hemangioblast by Fli1-Gata2-Scl or Erg-Gata2-Scl, as judged by FLK1+PDGFRα+ cardiac mesoderm production (Fig 7D, Supplementary Fig S4D). Ets1-Gata2-Scl was
Figure 5. Ets genes are direct targets of ETV2.

A Genome browser views of the ETV2 binding peaks associated with Ets loci. Numbers (1–15) indicate ETV2 peaks.

B ETV2 enrichment at ETV2 peaks (1–15) by ChIP-qPCR analysis (mean ± SD, n = 4 biological replicates). PCR primers and genomic locations are provided in Supplementary Table S3.

C Luciferase reporter assay for 1–15 ETV2 peak regions from (A). Similar to Fig 3C, the graph shows relative luciferase activity over reporter construct alone in the presence or absence of ETV2 expression plasmids (pMSCV-Etv2). Data are represented as mean ± SEM. n = 4. *P < 0.05; **P < 0.01; ***P < 0.001, two-tailed Student’s t-test.

D Luciferase reporter assay for the deletion mutants of the ETV2 motif in the selected peak regions (6, 7, and 12) is shown on the left (see Supplementary Table S3). Deletions of 2 (mt1, mt2) or 3 (mt3) ETV2 motifs within the peak 7 are shown on the right. Error bars represent the SEM obtained from four biological replicates.
Figure 6. ETV2 functions at the top of the ETS hierarchy in development.

A Kinetic analysis of Brachyury and Ets gene expression in wild-type EBs, day 0 to day 5, is shown. Gene expression was normalized to Gapdh. Error bars represent the SD from four independent experiments.

B Inducible Etv2 ES cells were differentiated in serum-free conditions with DOX added on day 2. Ets gene expression was analyzed at indicated times by qRT–PCR. Mean ± SD, n = 4 biological experiments. Genes were normalized to Gapdh, and the ratio of the +DOX gene quantity to −DOX gene quantity was determined to yield fold changes shown on the y-axis.

C qRT–PCR analysis of Ets gene expression in E8.5 embryos of Etv2+/+ (n = 10), Etv2+/− (n = 18) and Etv2−/− (n = 9) (embryos were obtained from 4 to 5 pregnant mice). Gene expression was normalized to Gapdh. Mean values are shown ± SD. Student’s t-test *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

D Fli1+/+ and Fli1−/− ES cells were differentiated on OP9 feeder cells and the expression of Etv2, Erg, and Elk3 genes was analyzed from day 0 to day 6. Mean ± SD, n = 4 experiments.

E Analysis of Etv2 expression in E8.5 embryos of Fli1+/+ (n = 10), Fli1+/− (n = 14) and Fli1−/− (n = 8) (embryos were obtained from 4 to 5 pregnant mice). Gene expression was normalized to b-actin. Mean values are shown ± SD.

F Expression of Ets genes was analyzed on day 3 Fli1 EB cells with DOX added on day 2 (mean ± SD, n = 4). Student’s t-test *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 7. ETS switching mechanism in maintaining hematopoietic and endothelial cell program.

A–C  A representative FACS profile of FLK1 and PDGFRα expression of E8.5 Fli1+/+ (n = 9), Fli1+/- (n = 11), and Fli1-/- (n = 6) embryos (A) (embryos were obtained from 4 to 5 pregnant mice); Fli1+/+ and Fli1+/- EBs (B, four independent biological samples); and iFli1 EBs (± DOX) (C, four independent biological samples) at indicated times. Numbers indicate the percentage within each quadrant. Representative results from four independent biological samples are shown.

D  The enrichment of ETV2 and FLI1 binding at the indicated gene loci by ChIP-qPCR analysis in wild-type EBs is shown. qPCR primers and genomic locations are provided in Supplementary Table S3. Error bars represent SD, n = 4. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's two-tailed t-test.

F  A model showing ETV2 regulation of the hematopoietic and endothelial cell program.
least effective in inducing FLK1\(^{\text{hi}}\)PDGF\(\alpha\)-hemangioblast from ES cells (Fig 7D, Supplementary Fig S4D). Collectively, ETV2 carries out a uniquely distinct role in the hemangioblast generation. No other ETS factors can replace ETV2 in the hemangioblast formation.

Hematopoietic and endothelial cell program is maintained by an ETS switching mechanism

ETV2 ChIP-Seq identified key hematopoietic and endothelial cell genes as ETV2 direct targets. However, the mechanism that allows these genes to be continuously expressed in mature hematopoietic and endothelial cells even when Etv2 is no longer expressed remains to be elucidated. We postulated that ETV2 target gene expression could be maintained within mature hematopoietic and endothelial cells by other ETS factors, which were induced by ETV2. To this end, we analyzed key target genes, Lmo2, Gata2, Cdh5, Tie2/Tek, and Scl/Tal1 for the ETS factor occupancy using day 3 and day 4–5 EB cells. Specifically, we hypothesized that while the ETS sites of the target genes would be occupied by ETV2 in days 2–3 EBs, when ETV2 expression is robust, the same target sites would be occupied by other ETS factors when ETV2 is no longer expressed, that is, days 4–5. To test this idea, we collected wild-type EB cells at day 3, 4, and 5 and performed ChIP-qPCR using ETV2-polyAbs and FLI1 Ab. As shown, while the evolutionary conserved potential ETS binding sites of these genes were occupied by ETV2 in day 3 EBs, the same regions were occupied by FLI1 in later developmental stages, that is, days 4 and 5. FLI1 occupancy on these genes was minimal in day 3 EBs (Fig 7E). Collectively, ETV2-initiated blood and endothelial cell program is maintained by other ETS factors through an ETS switching mechanism.

Discussion

Studies so far have established a non-redundant and essential function of ETV2 in blood and vessel formation. However, the mechanistic understanding of how ETV2 performs such a profound function in blood and vessel formation has been lacking. We present here, ETV2 initiates hemangiogenic program by maintaining a hematopoietic and endothelial cell program through an ETS switching mechanism [26,43]. Studies so far have established a non-redundant and essential function of ETV2 in blood and vessel formation. However, the mechanistic understanding of how ETV2 performs such a profound function in blood and vessel formation has been lacking. We present here, ETV2 initiates hemangiogenic program by maintaining a hematopoietic and endothelial cell program through an ETS switching mechanism [26,43].

As ETV2 function is transiently required, there must be a mechanism maintaining the hematopoietic and endothelial cell program that was initiated by ETV2. We propose that ETV2 drives lineage-specific epigenetic landscape in blood and vascular systems. As ETV2 target loci remained unmethylated in the heart and bone marrow (Fig 2B and Supplementary Fig S1G), ETV2 binding could directly result in demethylation of its binding sites, thereby shaping the lineage-specific epigenome [33]. Hypomethylation of these binding sites is maintained in blood and vascular systems as an epigenetic memory [45], potentially serving as the future tissue-specific enhancer elements of ETV2 downstream factors. We provide ETS switching mechanism as one such mode. Similar to the GATA switching mechanism controlling the erythroid cell lineage differentiation [46,47], ETV2 induces expression of other Ets genes, thereby creating an ETS hierarchy. Specifically, we show that hematopoietic and endothelial cell program is initiated by ETV2, but is maintained by other ETS factors (Fig 7F). However, timing of different Ets factor expression alone cannot explain the non-redundant ETV2 function, as we demonstrated that FLI1, ERG, or ETS1 could not replace ETV2 function even if they were expressed at the same time frame as ETV2, in the hemangioblast induction from ES cells. To this end, structure and function of ETV2 in relationship to other ETS factors may elucidate the unique role of ETV2 in the hematopoietic and endothelial cell development. Additionally, ETV2 may require a unique cofactor(s). Recent finding of OVOL2 as ETV2, not ETS1 or ETS2, cofactor further suggests such non-redundant function played by ETV2 [48].

While ETV2 is a potent inducer of the hemangiogenic program, it is transiently expressed, suggesting that there might be a strong pressure to keep this gene off once hematopoietic and vascular systems are established. Indeed, sustained Etv2 expression during development results in hematopoietic and vessel defects as manifested by dilated yolk sac vessels [49]. Future studies on Etv2 gene regulation are warranted. Presumably, Etv2 reactivation in mature endothelial cells might be a helpful strategy for hematopoietic and endothelial cell regeneration or diseases requiring angiogenesis, such as peripheral arterial disease and wound healing. To this end, it is worth noting that Etv2, alone or in combination with other endothelial cell factors, can reprogram somatic cells to functional
endothelial cells [50,51]. It will also be important to determine whether Etv2 is aberrantly expressed in pathologic conditions, such as tumor angiogenesis. If so, mechanisms involved in Etv2 down-regulation may have a direct efficacy in the pathologic angiogenesis.

Materials and Methods

Antibodies

Antibodies against V5 (ab15828), control rabbit IgG (ab31475), and FLI1 (ab15289) were purchased from Abcam (Cambridge, MA). Polyclonal antibodies against endogenous ETV2 (ETV2-polyAbs) were produced by YenZym Antibodies (South San Francisco, CA). Briefly, rabbits were immunized three times with a synthetic peptide corresponding to the mouse ETV2200-219 (EGHQQPAFPTTPSSKNQ SDR). Polyclonal antibody was affinity purified using ETV2 peptide-conjugated affinity matrix. Antibody specificity was further confirmed by antigen-specific ELISA and immunoprecipitation followed by Western blot.

Knockout and conditional knockout mouse analysis

Heterozygous Etv2+/– or Flit1+/– mice were subjected to brother–sister matings to generate Etv2−/– or Flit1−/– embryos. Flk1Cre;Etv2 CKO mice were generated by crossing Flk1+/Cre;Etv2+/– (or Flk1+/Cre; Etv2+/–) and Etv2+/– mice. The generation of Flk1Cre; Etv2+/–, Etv2–/–, and Flit1–/– mice has been previously described [20,24,52,53]. Information on genotyping primers was previously described [24,27,38,39]. Embryos from E7.5 to E9.5 were dissected and dissociated with 0.1% trypsin for 5 min or 0.25% collagenase for 30 min at 37°C into single cells and then subjected to FACs analysis or RNA extraction using RNeasy micro Kit (Qiagen) for gene expression. All mouse experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis Medical School.

ES cell generation and differentiation

Flit1−/– ES cells were generated from blastocysts obtained from the Flit1+/– brother–sister matings. Inducible ES cell lines (iEtv2 and iEos) were previously described [24,27,37,38]. Inducible Flit1 (iFlit1) ES cells were generated by targeting Flit1 into the tet-responsive locus of A2Lox cells [38]. To generate inducible Flit1-2A-Gata2-2A-Scl (iFlit1-G), Erg-2A-Gata2-2A-Scl (iErg-G), or Ets1-2A-Gata2-2A-Scl (iEts1-G) ES cells, coding sequence of Ets (Flit1, Erg and Ets1), Gata2, and Scl, linked by 2A peptides [54], were targeted into the tet-responsive locus of A2 Lox cells. The correct targeting event was confirmed by a tet-responsive locus/cDNA vector-specific PCR. Doxycycline (DOX) was typically added to the differentiating ES cells from day 2 to induce the genes.

Chromatin immunoprecipitation (ChIP)-sequencing

Wild-type or iEtv2 EBs from day 3–5 were subjected to ChIP assay. Briefly, EBs were disassociated into single cells by trypsin, fixed with 1% formaldehyde at room temperature for 10 min, and quenched by adding glycine to 125 mM final concentration. The cross-linked cell pellets (1 × 107) were resuspended in lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris–HCl [pH 8.1], plus protease inhibitor) and gently rocked at 4°C for 10 min, then sonicated to 200- to 600-bp fragments using an AFA Focused-ultrasonicator or a Diagenode Bioruptor. Sonicated lysates were cleared by pelleting insoluble material at 20,000 × g for 20 min at 4°C. The soluble lysate was diluted by adding 9 volumes of IP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl [pH 8.1], 1× protease inhibitor cocktail). Subsequently, the lysate was precleared with 30 μl of protein A- or G- Sepharose beads for 2 h at 4°C under rocking, followed by incubation with specific antibody overnight at 4°C. Subsequently, 45 μl protein A- or G- Sepharose beads were added and incubated for 2–3 h at 4°C. The beads were then washed three times serially with cold wash buffer TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1) and then TE buffer. Precipitated chromatin complexes were eluted in 100 μl elution buffer (1% SDS, 0.1 M NaHCO3) at room temperature for 10 min and uncross-linked overnight at 65°C, followed by RNase A treatment for 2 h and then proteinase K for 2 h. DNA was extracted with QiaQuick PCR purification kit (Qiagen).

Immunoprecipitated DNA yield was determined via Quant IT fluorescence assay (Invitrogen), and transcription factor enrichment was evaluated by qPCR. Illumina sequencing libraries were generated as following. ChIP DNA was blunt-ended, “A” base was added to 3’ end, and sequencing adapters were ligated to the ends. The fragments were size-selected to 200–600 base pairs and underwent amplification for 15 cycles. The resulting libraries were sequenced using the Illumina HiSeq-2000 as single reads extending 50 bases. The raw data were demultiplexed and aligned to the reference genome using Bowtie. MACS was used to call peaks.

Sequence alignment and peak calling

Sequences reads were submitted to NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under Accession Number GSE59402. Sequence reads were aligned to mouse genome assembly mm9 [28] using BWA aligner [55] for alignment and methylQA [56] for initial process. MACS2 [29] was used for peak calling against IgG control. The raw data were demultiplexed and aligned to the reference genome using Bowtie. MACS was used to call peaks.

GREAT analysis

Peak regions were submitted to GREAT (http://bejerano.stanford.edu/great/public/html/, version 2.0.2) using default “Basal plus extension” settings. Enrichment terms less than FDR threshold 0.05 were regarded as statistical significant.

Motif analysis

The ETV2 motif was de novo trained from peaks we identified using Homer software [57]. We also used Homer for motif scanning on peak regions. Statistic tests were performed using R environment (http://www.r-project.org/). ETV2 and GATA2 motif came from the de novo training results; SCL (E-box) motif was generated using
seq2profile.pl utility from Homer software using consensus sequence CANNTG. Homer was also used for motif scanning in peaks. Peaks with all 3 motifs occurring were used for calculating the nearest distance of ETV2-SCL and SCL-GATA2 using custom script. The ETV2-SCL-GATA2 logo was generated by WebLogo 3 (http://weblogo.threeplusone.com/create.cgi).

Microarray analysis
We analyzed microarray data following instructions as previously described [58]. Genes shown 1.5-fold downregulated in Etv2 knock out and DOX+ upregulated, or 1.5-fold upregulated in Etv2 knock out and DOX+ downregulated were used to overlap with genes identified from ChIP-Seq experiments.

RNA obtained from controls and FliKCre;Etv2CKO E9.5 yolk sacs was used to gene expression profiling analyses with Illumina MouseWG-6 v2.0 Expression BeadChip Kits at the Northwestern University Genomic Core, Chicago.

Evolutionary analysis
The 30-way vertebrate alignment conservation data were downloaded from UCSC Genome Browser; for each peak, we extend 3 kb from the peak center and split this 6-kb region to 50-bp bins, we assigned each bin with an average conservation score using custom script.

Analysis of whole-genome bisulfite sequencing data
We downloaded published whole-genome bisulfite sequencing (WGBS) data from GEO [33,34] and assigned the CpG sites around 6-kb region centered each peak center with methylation values for these tissues including ESC (GSE30202), heart (GSM1051154), bone marrow (GSM1051150), neuronal progenitors (GSE30202), and cerebellum (GSM1051151). These 6-kb regions were split to 50-bp bins, and methylation values for each bin were averaged from all ChIP-Seq peaks. Profile of peaks, which were distal (> 1 kb distance) to nearest TSS, was plotted, and a generalized linear model was applied to smooth the methylation changes.

Luciferase reporter assay
ETV2 bound sequences (Supplementary Table S3) obtained through MACS peak-calling algorithm were PCR-amplified and cloned into the pGL4.24[luc2P/minP] vector containing the minimal promoter and the luciferase reporter gene luc2P. Selected peak regions containing ETV2 binding motif were deleted as shown in Supplementary Table S3. For luciferase assays, 293T cells cultured in DMEM supplemented with 10% fetal bovine serum were plated onto 24-well plates (3 × 10⁴ cells) and transfected the next day with the pGL4.24[luc2P/minP] vector constructs with or without the ETV2 expression plasmid (pMSCV-Etv2). Renilla luciferase vector (Promega) was cotransfected to normalize transfection efficiency. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were harvested 48 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay kit and GloMax™ Systems (Promega). Reporter gene activities were first normalized to the Renilla luciferase value and further compared to that of the control pGL4.24 reporter. All experiments were repeated four biological replicates.

Flow cytometry
Embryoid bodies (EBs) or mouse embryos were harvested and dissociated into single cells using 0.1% trypsin (EBs) or 0.25% collagenase (embryos) for staining. Primary Abs included biotin-α-FLK1 (1:200, Avas12, BioLegend), APC-α-PDGFRα (1 μg/ml, AP5, ebioscience), and PE-α-CD31 (1 μg/ml, MEC13.3, Biolegend). Streptavidin (SA)/PE (0.5 μg/ml, ebioscience) was used as secondary antibody. Data were acquired on a FACSCompBecton flow cytometer (Becton Dickinson) and analyzed using FlowJo (Treestar) software.

Signaling pathway RT² profiler PCR array
The RT² Profiler PCR Array that profiles the expression of 84 key genes in the mouse VEGF signaling (PAMM-091ZA) and the Cell Motility (PAMM-128ZA) pathway were purchased from SA-Bioscience (Qiagen). Total RNA was isolated from day 3.5 Etv2 EBs (±DOX on day 2–3.5) and a pool of E8.5 Etv2+/− or Etv2−/− embryos, converted to cDNAs, and used for screening by real-time PCR following the manufacturer’s instructions. qRT-PCR assays performed in triplicate, normalized to 4 housekeeping genes (Gusb, Hsp90ab1, Gapdh, and Actb), and analyzed according to the ΔΔCt method with the SA-Biosciences proprietary software.

Quantitative real-time reverse transcription PCR (qRT–PCR)
Total RNA from embryos and EB cells were prepared with RNeasy Micro/Mini Kit, and reverse-transcribed into cDNAs according to manufacturer’s protocol. Expression of genes was measured by quantitative real-time RT–PCR with primers indicated in Supplementary Table S6. Gene expression levels were normalized to Gapdh or β-actin.

Statistical analysis
Student’s t-test (Prism5, GraphPad Software, La Jolla, CA) was used for statistical analysis. A P-value of < 0.05 was considered as significant.

Supplementary information for this article is available online:
http://embor.embopress.org

Acknowledgements
This work was supported by grants from the National Institutes of Health, NHLBI, HL63736, and HL55337 (K.C.); NHGRI, HG007354, HG007175, ES024992 (T.W.); American Cancer Society, RSG-14-049-01-DMC (T.W.); HL119291 (C.P.); American Heart Association, 11SDG7390074 (C.P.); the March of Dimes Foundation, #5-FY12-44 (C.P.).

Author contributions
FL and KC conceived the experiments, analyzed the data, and wrote the manuscript. FL, YY, IK, and MC performed experiments. JK and CP performed Etv2 conditional knockout studies. DW provided FliK knockout mice. DL and TW performed bioinformatics analysis and wrote the manuscript.
Conflict of interest
The authors declare that they have no conflict of interest.

References
shape the mouse methylome at distal regulatory regions. Nature 480: 490 – 495