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Fli1 Acts Downstream of Etv2 to Govern Cell Survival and Vascular Homeostasis via Positive Autoregulation

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Abstract

Rationale—Cardiovascular health depends on proper development and integrity of blood vessels. Etv2 (Ets variant 2), a member of the E26 transforming specific (ETS) family of transcription factors, is essential to initiate a transcriptional program leading to vascular morphogenesis in early mouse embryos. However, endothelial expression of the *Etv2* gene ceases at mid-gestation; therefore, vascular development past this stage must continue independent of Etv2.

Objective—To identify molecular mechanisms underlying transcriptional regulation of vascular morphogenesis and homeostasis in the absence of Etv2.

Methods and Results—Utilizing loss- and gain-of-function strategies and a series of molecular techniques, we identify *Fli1* (Friend leukemia integration 1), another ETS family transcription factor, as a downstream target of Etv2. We demonstrate that Etv2 binds to conserved Ets-binding sites (EBSs) within the promoter region of the *Fli1* gene and governs *Fli1* expression. Importantly, in the absence of Etv2 at mid-gestation, binding of Etv2 at EBSs in the *Fli1* promoter is replaced by Fli1 protein itself, sustaining expression of *Fli1* as well as selective Etv2-regulated endothelial genes to promote endothelial cell survival and vascular integrity. Consistent with this, we report that Fli1 binds to the conserved EBSs within promoter and enhancer regions of other Etv2-regulated endothelial genes, including *Tie2*, to control their expression at and beyond mid-gestation.

Conclusions—We have identified a novel positive feed-forward regulatory loop in which Etv2 activates expression of genes involved in vasculogenesis, including *Fli1*. Once the program is activated in early embryos, Fli1 then takes over to sustain the process in the absence of Etv2.

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DISCLOSURES
None

Keywords

Transcription factors; autoregulation; vasculogenesis; gene expression; embryogenesis; apoptosis; developmental biology; embryonic development

INTRODUCTION

Endothelial dysfunction is a cardinal feature of several human cardiovascular diseases[1]. During embryogenesis, growth and survival of the vertebrate embryo relies critically on proper morphogenesis and homeostasis of the organs, tissues, and cell types that comprise the circulatory and cardiovascular systems[2]. Endothelial and blood progenitor cells of the circulatory system derive from a common ancestor known as the hemangioblast[3, 4]. Progenitor cell survival, specification, and differentiation require precisely orchestrated interacting action of numerous transcription factors. However, details of the transcriptional network that governs endothelial cell (EC) function and homeostasis during embryogenesis are still being defined. Elegant work using gene disruption and mutation strategies have demonstrated that diverse transcription factors are involved in vascular development[5]. Among them, the importance of the E26 transforming specific (ETS) family of transcription factors has been highlighted[5].

The ETS family of transcription factors is conserved in metazoans and first identified in avian erythroblastosis virus, E26[6, 7]. They are highly conserved in their DNA-binding ETS domain and govern a plethora of biological processes including, but not limited to, development, cellular growth, differentiation, cell-cell contact and viability[5, 8]. To date, 29 different *Ets* genes have been identified in mammals. Each binds to a core “GGAA/T” DNA-binding element[6, 7], suggesting that aspects of their functions overlap. Global inactivation or mutation of numerous *Ets* genes either in mouse or zebrafish indicates that despite high conservation of the DNA-binding motif and recognition of a similar *cis*-regulatory element, certain ETS factors play an essential role in vascular morphogenesis and homeostasis which cannot be compensated by other family members[5].

Recently, we and others have reported that the ETS family transcription factor Etv2 (also called Etsrp71/ER71) is essential for the genesis of hemato-endothelial progenitor cells in mice[9–11]. Mice lacking Etv2 die *in utero* around embryonic (E) day 9.5 with complete loss of vasculature[9, 11]. Similarly, knockdown of the Etv2 ortholog Ets-related protein (Etsrp) in zebrafish provokes a profound impairment of vasculogenesis[12]. Furthermore, recent studies demonstrate that loss of the Etv2/Etsrp in mice and zebrafish redirects endothelial progenitor cells to a myogenic fate[13, 14], highlighting a central role for this transcription factor in endothelial specification and vascular development in the early embryo. Intriguingly, endothelial expression of *Etv2* in wild type (WT) mice is undetectable at mid-gestation[9–11], thus raising two major questions: 1) how are vascular morphogenesis and integrity maintained through the remainder of gestation? and 2) what controls the continued expression of known Etv2 target genes, such as *Tie2* (also known as *Tek*)[9], in the absence of Etv2?

Genome-wide sequence analyses identified Ets-binding sites (EBSs) in the promoter and enhancer regions of many endothelial-expressed genes, including ones encoding other ETS proteins[5, 15]. Therefore, we postulated that once expression of an *Ets* gene is activated by Etv2 early in development, an Etv2-targeted *Ets* gene may function to maintain its own expression as well as that of additional endothelial genes involved in EC survival, vascular morphogenesis and homeostasis. Recently published studies demonstrate that compared with WT embryos and embryonic stem cells (ESCs), transcript levels of several transcription factors involved in hematopoietic and vascular development during early embryogenesis are attenuated in *Etv2* knockout embryos and ESCs[11, 16, 17]. However, the identity of a specific Etv2 target and its role in vascular morphogenesis in the absence of *Etv2* remain elusive.

The primary purpose of this study was to identify a specific Etv2 target which can activate a positive autoregulatory feedback mechanism that persists beyond mid-gestation (and in the absence of Etv2) to govern critical elements of embryonic vasculogenesis. We demonstrate *Fli1* (Friend leukemia integration 1) has such properties, being dependent on Etv2 to initiate embryonic expression early in development, and then acting to regulate its own expression, as well as that of additional Etv2-target endothelial genes involved in EC survival, vascular morphogenesis and homeostasis, at and beyond mid-gestation.

METHODS

Lentivirus production, purification, and infection

HA-tagged mouse Fli1 cDNA was subcloned into a lentiviral expression vector (Clontech) and co-transfected with pCD and VSVG constructs into HEK293T cells according to the manufacturer's instructions (Clontech). Lentivirus was harvested from the culture supernatant and concentrated using ultracentrifugation for 2 hours at 22k rpm using SW28 rotor. Expanded protocols for viral infection, gene expression and histological and immunohistochemical (IHC) analyses utilizing isolated mouse embryos are provided in the Supplementary Data section.

RESULTS

Excessive endothelial cell death leads to hemorrhage in Fli1-null embryos at mid-gestation

We and others have reported that mice lacking Fli1 die between E12.0–E12.5 due to widespread hemorrhage at mid-gestation[18, 19]. However, the underlying cause of this perturbation is unknown. To gain insights into mechanisms underlying vascular leakage in *Fli1*-null embryos, we mated *Fli1* heterozygous mice[19] and isolated WT and *Fli1*-null embryos from timed pregnant females at distinct developmental stages. Consistent with previously reported studies[18, 19], *Fli1*-null embryos isolated at E10.5 (Figure 1A) and E11.5 (Figure 1B) manifested hemorrhage within the embryo proper as well as in the extra-embryonic (e.g. yolk sac) vasculature (data not shown) culminating ultimately in lethality by E12.0 (Supplemental Fig. IA). This was further supported by the absence of erythrocytes within the vasculature (e.g. the dorsal aorta and cardinal vein) and hemorrhage in the canal of the neural tube of *Fli1*-null, but not WT, embryos (Figure 1A). In addition, *Fli1*-null

embryos also revealed diminished endocardial cushion formation (Supplemental Fig. IB), supporting a role of Fli1 in endocardial endothelium.

By contrast, *Fli1*-null embryos isolated at E8.5 (data not shown) and E9.5 (Supplemental Fig. IIA) are indistinguishable from WT littermates in terms of overall morphology, growth and cardiovascular structures. The presence of erythrocytes within the dorsal aorta of *Fli1*-null embryos and IHC analyses for the endothelial/endocardial marker α-endomucin[9] supported the notion that embryonic vascular structures were normal in early (between E8.5–E9.5) WT and *Fli1*-null littermates (Supplemental Figs. IIA and IIB). This conclusion was further supported by similar transcript levels of two endothelial genes in both genotypes at E9.5 (Supplemental Fig. IIC). Collectively, these data support an essential role for Fli1 in vascular morphogenesis and homeostasis at mid-gestation.

Next, we analyzed whether vascular leakage in *Fli1*-null mice is associated with perturbation of endothelial proliferation and/or viability. IHC analyses for Ki67 demonstrated normal cellular proliferation in *Fli1*-null and WT littermates at E10.5 (Supplemental Fig. IB), suggesting that disruption of vascular integrity was not due to lack of endothelial proliferation. Utilizing terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and IHC analyses for activated caspase-3 and endomucin, we tested for evidence of apoptotic cell death in WT and *Fli1*-null embryos. Compared with WT embryos, *Fli1*-null embryos isolated at mid-gestation manifested excessive EC death (Figures 1A and 1B). It is worth noting that elevated levels of cell death within the blood and cells of the neuropil were also evident in *Fli1*-null embryos (Figure 1 and Supplemental Fig. IB). Taken together, we conclude that Fli1 is essential for EC viability and homeostasis at and beyond mid-gestation.

Etv2 is an essential upstream regulator of the Fli1 gene

Having identified an essential role for Fli1 in ECs at and beyond mid-gestation, we sought to determine molecular mechanisms governing embryonic expression of the *Fli1* gene. Embryonic expression of *Fli1* occurring after *Etv2* expression[16], coupled with significant attenuation of *Fli1* transcript levels in *Etv2*-null ESCs[16, 17] and ECs[13], together imply that Etv2 governs *Fli1* expression in the early embryo. To test this concept, we purified RNA from E8.0 WT and *Etv2*-null littermates[9] and analyzed transcript levels of numerous *Ets* genes. Quantitative RT-PCR (qRT-PCR) analyses showed that compared with WT, transcript levels of *Fli1* and a closely-related Fli1 family member *Erg*[5], were significantly attenuated in *Etv2*-null embryos (Figure 2A). By contrast, the transcript levels of several other *Ets* genes, including *Ets1*, *Ets2*, *Elf1*, *Elf2*, and *Etv6*, were unaffected (Figure 2A). Transcript levels of the *Tie2* gene, an established downstream target of Etv2 in early embryo[9], were also significantly decreased in mutant embryos (Figure 2A), which suggests that attenuation of *Fli1* expression in the *Etv2*-null embryos was specific to the absence of Etv2. Based on these data, we hypothesized that *Fli1* is a downstream target of Etv2 in the early mouse embryo.

It has been demonstrated that synthesis of two major isoforms of the Fli1 protein initiates from two distinct translation initiation sites (ATG) present within exon 1 and exon 2, respectively (Figure 2B)[20]. To confirm that Etv2-dependent *Fli1* gene transcription initiates

from exon 1, we utilized semi-quantitative RT-PCR (sqRT-PCR) techniques to analyze *Fli1* transcripts using primers spanning the exon 1 untranslated region (UTR) and exon 2 (Figure 2B). We noted that *Fli1* transcripts detected in WT embryos were significantly attenuated in *Etv2*-null embryos (Figure 2B). These data suggest that marked reduction of *Fli1* transcripts in the *Etv2*-null embryos was associated with Etv2-dependent regulation of *Fli1* gene transcription from exon 1.

We, and others, have reported that endothelial expression of the *Etv2* gene stops at mid-gestation (E10.5–E11.5)[9–11]. This observation raised the possibility that expression of the *Fli1* gene in whole embryo and/or in ECs will also be undetectable or decline at mid-gestation. Using sq- and qRT-PCR, we analyzed *Etv2* and *Fli1* gene expression in whole embryo and in green fluorescence protein (GFP)-positive ECs isolated from Tie2-GFP mice at distinct developmental stages. Consistent with our previously published study[9], the *Etv2* gene was expressed in the early embryos (E8.5–E9.5) (Supplemental Fig. IIIA) and ECs (Figure 2C), but its expression was undetectable at mid-gestation. In contrast, *Fli1* transcripts were detected at all developmental stages and increased with age in whole embryos (Supplemental Fig. IIIA) and ECs (Figure 2C). Taken together, we conclude that Etv2 is required for endothelial *Fli1* expression in the early embryo, while *Fli1* expression at and beyond mid-gestation becomes Etv2-independent.

To complement our in vivo Etv2 loss-of-function data, we undertook in vitro approaches to validate *Fli1* as a downstream target gene of Etv2. We fused a 0.7-kb *Fli1* promoter region upstream of exon 1 harboring numerous conserved EBSs (Supplemental Fig. IIIB)[21, 22] to a luciferase reporter cassette and conducted transcriptional analyses. Co-transfection of the reporter plasmid with increasing amounts of hemagglutinin (HA)-tagged Etv2 expression plasmid[9] in primary human aortic endothelial cells (HAECs) resulted in significant activation of luciferase activity (Figure 2D). Since expression of numerous endothelial genes can be regulated by co-operative action of several ETS family transcription factors, including Etv2[9–11, 15], we repeated transcriptional assays in non-endothelial cells. Co-transfection of the reporter plasmid with Etv2 expression plasmid in C2C12 (Supplemental Fig. IIIC) or COS1 (Figure 2E) cells resulted in significant and dose-dependent activation of luciferase activity. However, co-transfection of COS1 cells with a mutant Etv2 that lacks the DNA-binding ETS domain failed to activate luciferase activity (Figure 2E), suggesting that DNA-binding by Etv2 is essential for the transcriptional activation of the *Fli1* gene.

To test for the presence of EBSs in the *Fli1* promoter critical for Etv2 activity, we generated a series of deletion constructs of the 0.7-kb promoter fragment (Supplemental Fig. IVA). In transcriptional assays utilizing each reporter plasmid in the presence or absence of Etv2, we identified three conserved EBSs, residing between 200 to 250-bp upstream of the ATG were essential and sufficient for Etv2-dependent activation of luciferase activity (Figure 2F and Supplemental Fig. IVA). Mutation of these EBSs disrupted Etv2-dependent activation of luciferase activity (Figure 2G and Supplemental Fig. IIIB). Utilizing quantitative analyses of chromatin immunoprecipitation (ChIP-qPCR) assays in C2C12 cells expressing HA-tagged Etv2, we confirmed specific occupancy of Etv2 at this *Fli1* promoter region, and that Etv2 occupancy was not detected of an intronic region of the *Fli1* gene which does not harbor a conserved EBS (Figure 2H and Supplemental Fig. IVB). These data suggest that the binding

of Etv2 to these conserved EBSs is essential for activation of *Fli1* expression. Indeed, ectopic *Etv2* expression in C2C12 cells induced endogenous *Fli1* transcript levels in a dose-dependent manner (Supplemental Fig. IVC). Collectively, these data suggest that Etv2 is an important upstream regulator of the *Fli1* gene, and that ectopic *Etv2* expression will activate *Fli1* expression in a cell autonomous manner.

Fli1 promotes its own expression in the absence of Etv2

Having established *Fli1* as an essential downstream target of Etv2 in early embryos, we set out to unveil molecular mechanisms underlying Etv2-independent transcriptional regulation of *Fli1* expression at and beyond mid-gestation. Normal transcript levels of selected *Ets* genes in *Etv2*-null embryos (Figure 2A) along with the absence of vascular abnormalities at mid-gestation in mice lacking these factors[5] suggest that they are not required for *Fli1* expression at mid-gestation. Moreover, significant attenuation of transcript levels of Etv2 target genes, *Erg* and *Gata2*, in *Fli1*-null embryos at E10.5 (Supplemental Fig. VA) suggests that they are also not involved in *Fli1* expression at mid-gestation. Based on these data, we hypothesized that Fli1 regulates its own promoter activity in the absence of Etv2.

To test this hypothesis, we analyzed the N-terminal transcript levels of *Fli1* in *Fli1*-null embryos which express very little Fli1 protein that lacks the C-terminal activation domain encoded by the exon 9[19]. We detected *Fli1* transcripts spanning the exon 1 UTR and exon 2 in E9.5, but not in E10.5 and E11.5, *Fli1*-null embryos (Figure 3A), while *Fli1* transcripts at these developmental stages were readily detected in WT embryos (Supplemental Fig. IIIA). These data suggest that Fli1 is dispensable for *Fli1* expression in early embryos but required for sustained expression at mid-gestation. To complement this observation, we performed ChIP assays using WT embryos isolated at distinct developmental stages and confirmed increased Fli1 occupancy of its own promoter in embryos isolated at E11.5 (Figures 3B and 3C). Surprisingly, such occupancy was not detected in embryos isolated at E9.5 or in the *Fli1* intronic region in embryos isolated at E11.5 (Figures 3B and 3C). Collectively, these data lend strong support to our hypothesis that Fli1 occupancy of its own promoter region was associated with sustained endothelial *Fli1* expression at mid-gestation in the absence of Etv2 (Figure 2C).

To complement these in vivo data, we performed transcriptional assays using a *Fli1* reporter construct. In COS1 cells, we detected robust and dose-dependent activation of luciferase activity by Fli1 from a *Fli1* reporter plasmid harboring WT (Figure 3D), but not mutated (Figure 3E), EBSs. Utilizing ChIP-qPCR, Western blot and RT-PCR analyses using primers designed to amplify only endogenous *Fli1* transcripts, we further demonstrated that Fli1 occupancy of its own promoter region in C2C12 cells can induce endogenous *Fli1*, but not *Etv2*, transcript levels (Supplemental Figs. VB-D). These data further support our hypothesis that Fli1 acts downstream of Etv2 and can activate its own promoter activity. Interestingly, activation of *Fli1* reporter activity by Etv2 or Fli1 was indistinguishable from that of other ETS factors, including Elf1 and Elf2 (Figure 3E). However, we found that unlike *Etv2* and *Fli1*, ectopic expression of *Elf1* and *Elf2* in C2C12 cells did not induce endogenous *Fli1* expression, and that expression of *Ets1* showed only a modest effect (Figure 3F). Together, these data suggest that the failure of Elf1/Elf2 to induce endogenous *Fli1* gene expression

was secondary to an inability to access the EBSs sites in native chromatin but not due to an inability to recognize the EBSs in the *Fli1* reporter plasmid. Based on our in vitro and in vivo data, we conclude that in the absence of Etv2 at mid-gestation, Fli1 binds to conserved EBSs within the *Fli1* promoter to induce its own expression via positive autoregulation.

Fli1 is required for endothelial Fli1 expression for the remainder of gestation

To determine whether feed-forward autoregulation of *Fli1* is essential for sustained *Fli1* expression during the remainder of gestation, we undertook Fli1 loss- and gain-of-function strategies in adult primary HAECs. Consistent with our recent findings of *Fli1* expression in adult mouse ECs[23], we detected marked reduction of *Fli1* transcript (Supplemental Fig. VE) and protein (Figure 4A) levels in HAECs using two independent Fli1-specific siRNAs. We also noted that compared with COS1 cells, baseline *Fli1* reporter activity was significantly higher in HAECs (data not shown), suggesting that endogenous Fli1 might activate its own reporter activity. To test this concept, we analyzed *Fli1* reporter activity in HAECs and found that knockdown of Fli1 significantly attenuated *Fli1* reporter activity (Figure 4B). Utilizing ChIP-qPCR, we detected Fli1, but not Erg, occupancy of the *Fli1* promoter region in HAECs (Figure 4C). Taken together, we conclude that Fli1 is required to maintain *Fli1* expression in adult ECs.

To further test our model, we undertook Fli1 gain-of-function approaches finding that co-transfection of a *Fli1* reporter construct and HA-tagged Fli1 expression plasmids in HAECs resulted in significant and dose-dependent induction of luciferase activity (Figure 4D). Furthermore, lentivirus-mediated overexpression of mouse *Fli1* in HAECs resulted in marked induction of endogenous *Fli1* transcript levels (Figure 4E). Collectively, these data lend additional support to our hypothesis that *Fli1* is essential for sustained *Fli1* expression at and beyond mid-gestation.

Fli1 is an upstream regulator of selected Etv2-regulated endothelial genes in developing and adult endothelium

Finally, we set out to investigate whether Fli1 is essential for sustained expression of selected Etv2 target endothelial genes involved in EC viability and vascular homeostasis. For example, mice lacking Tie2, an endothelial receptor tyrosine kinase[24, 25] and vascular endothelial cadherin 5 (VE-Cdh5)[26, 27] manifest excessive EC death, abnormal vascular remodeling, hemorrhage and embryonic lethality. Consistent with previously reported studies[18, 23], transcript levels of *Tie2, Cdh5 and Cd31*, but not *Vegf-A* and *Flk1*/*Vegfr2*, genes were significantly attenuated in *Fli1*-null embryos at E10.5 (Figure 5A). Furthermore, similar transcript levels of the myogenic transcription factor myogenin in both genotypes at E10.5 suggest that attenuation of transcript levels of endothelial genes, including *Tie2* was specific to the loss of Fli1. IHC analyses for Tie2 and Fli1 also supported the notion that significant attenuation of Tie2 protein levels in vascular ECs of *Fli1*-null littermates was associated with EC-specific loss of Fli1 (Supplementary Fig. VIA). Given that *Tie2* transcripts in *Fli1*-null embryos declined to a similar extent in *Etv2*-null embryos (Figure 2A), we reasoned that the regulation of endothelial genes abundance in mid-gestational endothelium occurred at the level of gene transcription.

It has been reported that an upstream promoter fragment (UPF) and a distal first intronic enhancer fragment (IEF) are essential for sustained endothelial *Tie2* expression at mid- and late gestation[28]. Moreover, both the UPF and IEF have been reported to harbor several conserved EBSs[9]. Therefore, we examined whether immunoprecipitation of Fli1-DNA complexes from embryos (Figure 3B) harvested regulatory elements of *Tie2* and a known Fli1 downstream target *Cdh5*[23]. Consistent with our previous report[23], we detected Fli1 occupancy of the *Cdh5* promoter in vivo (Figure 5B). In fact, we also noted Fli1 occupancy of conserved EBSs within the UPF (Figure 5B) and IEF (Supplemental Fig. VIB) of the *Tie2* gene in embryos isolated at E11.5, but not at E9.5. These data suggest that Fli1 binds to the conserved EBSs within the promoter of selected Etv2 target endothelial genes, including *Tie2*, to govern their expression at and beyond mid-gestation.

To complement our in vivo data, we undertook Fli1 loss- and gain-of-function strategies and analyzed transcript levels of *Tie2* and *Cdh5* in vitro. We found that siRNA-mediated knockdown of *Fli1* in HAECs resulted in marked reduction of transcripts levels of *Tie2* and *Cdh5* (Figure 5C). Furthermore, co-transfection of the *Tie2* reporter with increasing amounts of Fli1 expression plasmid in COS1 cells revealed marked and dose-dependent induction of luciferase activity, while mutation of the EBSs in a 2.1-kb upstream promoter fragment of the *Tie2* gene[9] significantly attenuated the induction of reporter activity (Figure 5D). Importantly, ectopic *Fli1* expression in C2C12 cells (Supplementary Fig. VIC) and HAECs (Figure 5E) resulted in significant induction of endogenous *Tie2* expression. Thus, we conclude that in the absence of Etv2 at mid-gestation, Fli1 governs EC survival and vascular integrity by regulating expression of selected Etv2 target transcription (Supplementary Fig. VA) and signaling (e.g. Tie2) factors at and beyond mid-gestation.

DISCUSSION

The overall goal of this study was to decipher the Etv2-mediated transcriptional network that integrates vascular morphogenesis from early to late gestation in the developing mouse embryo. Our study reports three important findings. First, using a combination of molecular and Etv2 loss- and gain-of-function experiments, we have identified Fli1 as a downstream target of Etv2 in the early embryo and defined the underlying regulatory mechanisms. Second, we have uncovered a previously unrecognized positive autoregulatory mechanism controlling *Fli1* gene expression at and beyond mid-gestation, thereby providing insight into mechanisms governing vascular morphogenesis and homeostasis in the absence of Etv2. We demonstrate that when *Etv2* expression is extinguished, Fli1 acts to regulate its own expression, as well as that of selected other Etv2 target genes, at and beyond mid-gestation. In so doing, it governs EC viability and vascular integrity, which accounts for the vascular leakage and ultimate embryonic lethality reported in *Fli1* mutant mice[18, 19]. Third, we have identified Fli1 as an upstream regulator of *Tie2* and demonstrated that Fli1 binds to the conserved EBS within the UPF and IEF of the *Tie2* gene to regulate endothelial *Tie2* expression at and beyond mid-gestation. Collectively, our findings identify Etv2 and Fli1 as a classic example of a feed-forward autoregulatory feedback loop that initiates and maintains vascular morphogenesis, homeostasis and subsequent fetal growth and survival during development.

Since the discovery of Etv2 as an essential regulator of endothelial fate of progenitor cells, and its temporal and spatial expression pattern in the EC of early mouse embryo[9–11], the general consensus has been that Etv2 activates a downstream transcriptional network in early embryo, which in turn regulates EC homeostasis and vascular morphogenesis in the absence of Etv2. Indeed, compared with WT ESCs[16, 17] and ECs[13, 16], transcript levels of several transcription factors, including *Fli1*, were significantly attenuated in *Etv2*-null cells. Reciprocally, ectopic expression of Etv2 in zebrafish embryo[29] and differentiating ESCs[30] induces endogenous *Fli1* transcript levels. Although these data are consistent with our hypothesis that Etv2 can induce *Fli1* expression in a cell autonomous manner, the specificity of this response and the underlying mechanisms of *Fli1* gene expression regulation by Etv2 were incompletely understood. A recently published study describes modest activation of the *Fli1* gene by Etv2 through binding to a conserved EBS located 11.4-kb downstream of the *Fli1* translation initiation site in exon 1, but mutation of that site did not significantly attenuate *Fli1* reporter activity[16], highlighting the physiological significance of *cis*-regulatory motifs within the *Fli1* promoter region for transcriptional regulation of *Fli1* expression by Etv2.

Although all ETS factors recognize a similar *cis*-regulatory element[6, 7], attenuation of *Fli1* expression in *Etv2*-null mice was not complemented by several other ETS factors, which are known to be expressed in endothelial cells[21, 22]. However, all of them, including Elf1 and Elf2, induced *Fli1* reporter activity in vitro. These data suggest that the binding specificity of ETS factors to a *cis*-regulatory element is uniquely regulated in native chromatin, which is often indistinguishable in naked DNA. It is also plausible that the transcriptional regulation of *Fli1* and *Tie2* genes by other ETS factors in endothelial and non-endothelial cells is context-dependent or dependent on their cooperative action with other family members. Indeed, cooperative actions among numerous ETS factors, including Etv2, Fli1 and Erg, and other transcription factors, are known to play essential roles in endothelial gene expression during development[10, 31]. Consistent with these studies, a cooperative action between Fli1 and Erg is required for hematopoiesis in mice[32] and mice lacking Fli1 and endothelial isoforms of Erg (isoforms 5–7)[33] manifest vascular as well as cardiac malformations at mid-gestation. Therefore, it is conceivable that a cooperative action between Fli1 and other ETS factors governs endothelial genes expression and vascular morphogenesis in the absence of Etv2.

Autoregulation of gene expression is a common theme among transcription factors that govern expression of a large number of genes during embryogenesis[34]. Although autoregulatory mechanisms can be positive or negative, the significance of autoregulation relates to maintenance of cell fate and cellular homeostasis. Positive autoregulation has been described for numerous developmental transcription factors including, but not limited to, paired box protein 6 (Pax6)[35], homeobox (Hox) proteins and several myogenic factors, MyoD and myocyte enhancer factor 2 (Mef2)[34, 3637], which are essential for cardiac and skeletal myogenesis. For example, the basic helix-loop-helix transcription factor Twist, which governs mesodermal specification and myogenesis[36] and Mef2 proteins are co-expressed during early phases of mesodermal development. However, *Twist* expression is extinguished before muscle cell differentiation, while *Mef2* expression persists throughout

mesodermal development and subsequent muscle differentiation[34]. Of interest is that positive autoregulatory mechanisms related to Twist and Mef2 in skeletal myogenesis parallel those reported here for Etv2 and Fli1 in vascular morphogenesis.

Our model suggests that in the early embryo, Etv2 activates a transcriptional network[16, 17] involving *Fli1* and *Tie2*[9] to govern the hemato-endothelial fate of progenitor cells and subsequent fetal growth and survival (Figure 6). At mid-gestation, when *Etv2* expression turns off, Fli1 autoregulates its own expression as well as that of selected Etv2 target genes (such as *Erg, Cdh5* and *Tie2*) for the remainder of gestation to govern EC survival and vascular integrity (Figure 6). Consistent with our model, endothelial expression of a *lacZ* reporter driven by the native *Fli1* promoter is significantly diminished at mid-gestation and undetectable at E14.5 in *Fli1*-null embryos, while reporter expression in ECs persists in *Fli1*-heterozygous embryos during these developmental stages[18, 19]. By contrast, endothelial expression of a *lacZ* reporter driven by the native *Erg* promoter is not reduced in *Erg*-null embryo at mid-gestation[33], suggesting that Fli1, but not Erg, is required to govern its own promoter activity at mid-gestation. Although embryonic lethality of *Etv2*-null mice is not associated with excessive EC death[9], our data along with previously published studies[24–27] support a model in which attenuation of endothelial *Tie2* and *Cdh5* expression is associated with excessive EC death in *Fli1*-null embryos. However, we cannot rule out the possibility that additional factors might be involved in this process. Previously, we demonstrated that Etv2 governs *Tie2* expression in early embryonic ECs[9], yet the molecular mechanisms underlying transcriptional regulation of *Tie2* expression at and beyond mid-gestation were unknown. Our study is the first to report that Fli1 is an important regulator of endothelial *Tie2* expression at and beyond mid-gestation.

In conclusion, we have uncovered a unique and previously unrecognized positive autoregulatory transcriptional circuit whereby coordinated transcriptional activity of Etv2 and Fli1 regulates vascular morphogenesis and homeostasis at distinct stages of embryogenesis. Given the essential role of Fli1 in cardiovascular morphogenesis and vascular inflammation[6, 7], it is conceivable that Fli1 may be intimately involved in vascular and cardiovascular diseases. Moreover, Fli1 is associated with thymus development and cancer pathogenesis[38–40]. Looking to the future, development of small molecules that positively or negatively modulate Fli1 activity will provide molecular insights into a novel mechanism with potential clinical relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ETS	E26 transforming specific
E	Embryonic
EC	Endothelial cell
ChIP	Chromatin immunoprecipitation
UPF	Upstream promoter fragment
IEF	Intronic enhancer fragment
EBS	Ets-binding site
sqRT-PCR	semi-quantitative RT-PCR
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Novelty and Significance

What Is Known?

- The E26 transforming specific (ETS) family transcription factor Etv2 governs endothelial fate of the progenitor cells and vascular morphogenesis in early development.
- In early mouse embryo, the Etv2 activates numerous endothelial genes, including the ETS family transcription factor Fli1 gene.
- Endothelial expression of the *Etv2* gene stops at mid-gestation; hence, molecular mechanisms underlying transcriptional regulation of vascular morphogenesis and homeostasis beyond this stage remain incompletely understood.

What New Information Does This Article Contribute?

- We have identified the mechanism underlying Etv2-dependent regulation of the *Fli1* gene expression in early mouse embryo.
- At mid-gestation, when *Etv2* expression turns off, Fli1 regulates its own expression as well as that of selected Etv2 target endothelial genes at and beyond mid-gestation.
- This positive autoregulatory mechanism of *Fli1* gene expression is critical for endothelial cell (EC) survival and vascular integrity.

Mice lacking Etv2 die *in utero* around embryonic day 9.5 with complete loss of vasculature. Etv2 is an essential upstream regulator of numerous endothelial genes and governs endothelial fate of the progenitor cells. Intriguingly, in mice endothelial expression of the *Etv2* gene ceases at mid-gestation. This study was designed to identify a specific Etv2 target that controls its own expression as well as that of additional Etv2-regulated genes and maintains vascular morphogenesis and integrity through the remainder of gestation. We demonstrate that *Fli1* has such properties, being dependent on Etv2 to initiate embryonic expression early in development, and then acting to regulate its own expression as well as that of selected Etv2 target endothelial genes. In so doing, Fli1 governs EC viability and vascular integrity for the remainder of gestation critical for fetal growth and survival. Our study identifies the Etv2 and Fli1 axis as a classic example of a feed-forward autoregulatory feedback loop in the endothelium that initiates and maintains cardiovascular health at distinct developmental stages.

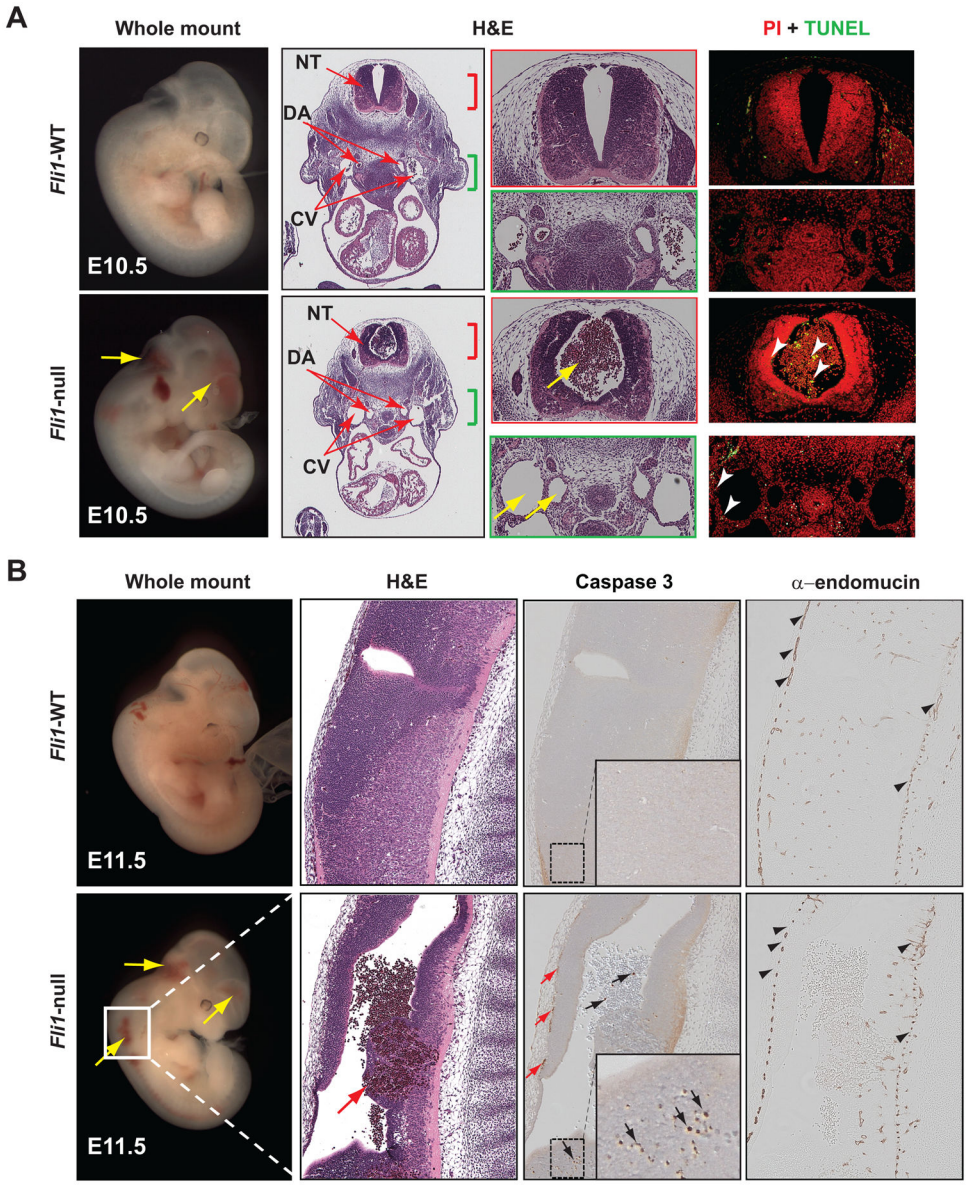

Figure 1. Vascular leakage in *Fli1*-null embryos at mid-gestation is due to excessive ECs death

(A) Gross (whole-mount) and hematoxylin and eosin (H&E) sections of E10.5 WT and *Fli1*-null littermates are shown. Blood in the canal of neural tube (NT) but absent in the vasculature, dorsal aorta (DA) and cardinal vein (CV), due to hemorrhage (yellow arrows) is evident in null mice. Histology corresponding to NT (red bracket) and vasculatures (green bracket) are shown to demonstrate increased apoptotic cell death (TUNEL) of ECs, blood and neuropil (arrowheads) in null mice. **(B)** Gross and histologic (H&E) sections of E11.5 WT and *Fli1*-null littermates are shown. Higher magnification H&E images of NT illustrate hemorrhage in *Fli1*-null mice (red arrow). IHC analyses for activated caspase 3 and endomucin revealed increased apoptotic EC death (small red arrows), blood (black arrows) and cells of the neuropil (arrows in inset).

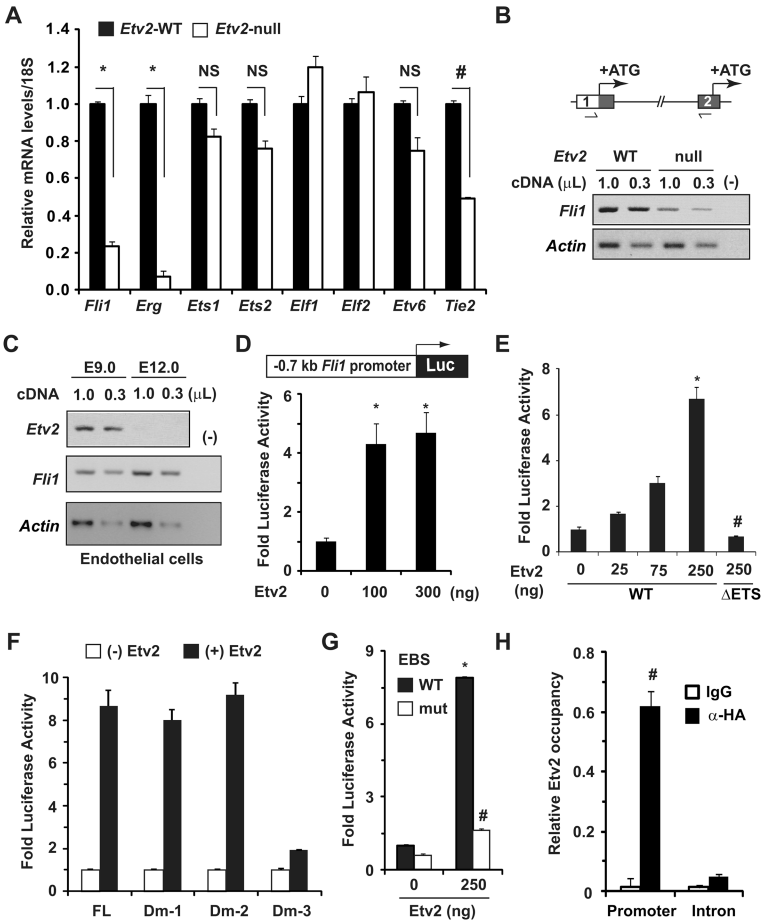

Figure 2. Etv2 governs *Fli1* gene expression in vitro and in vivo

(A) qRT-PCR analyses for transcript levels of the indicated genes in E8.0 WT and *Etv2*-null embryos (n=3–4). Relative gene expression in WT embryos was normalized to 1. Note the significant attenuation of *Fli1*, *Erg* (*p<0.001 vs. WT) and *Tie2* (#p<0.0001 vs. WT) gene expression in *Etv2*-null mice. NS: not significant. **(B)** Semi-quantitative RT-PCR (sqRT-PCR) analyses of *Fli1* transcripts spanning exon 1 UTR and exon 2 (boxes) of the *Fli1* gene (schematized top) in E8.0 WT and *Etv2*-null embryos. Translation initiation sites (ATG) in exon 1 and exon 2 are indicated. PCR loading (α-*actin*) and negative (−) controls are indicated. **(C)** sqRT-PCR analyses of *Etv2* and *Fli1* transcripts in GFP$^+$ ECs isolated at the indicated developmental stages. Note the transient co-expression of the *Etv2* and *Fli1* genes in ECs of early embryos, while expression of the *Fli1* gene persisted beyond mid-gestation. **(D)** Schematic of the *Fli1* promoter region (−0.7kb) fused to a luciferase (Luc) reporter. Co-transfection of reporter and indicated amounts of Etv2 expression plasmids in HAECs resulted in significant induction of luciferase activity (*p<0.008 vs. control). **(E)** Transcriptional assays in COS1 cells elicited dose-dependent activation of luciferase activity by WT, but not mutant (lack of DNA-binding domain, ΔETS) Etv2. **(F and G)** Fold-activation of luciferase activity in the absence (−) and presence (+) of Etv2 (250ng) from FL and indicated Dm reporter plasmids are shown in panel F. Note the EBSs located between 200–250bp upstream of ATG were essential for efficient activation of luciferase activity,

and that activity was significantly abrogated when they were mutated, panel G (*p<0.001 vs. control; # p<0.001 vs. WT). Luciferase activity without Etv2 was normalized to 1. **(H)** Quantitative analyses of Etv2 occupancy of the *Fli1* promoter. Note the specific and significant induction of Etv2 occupancy of the *Fli1* promoter (# p<0.001 vs. control).

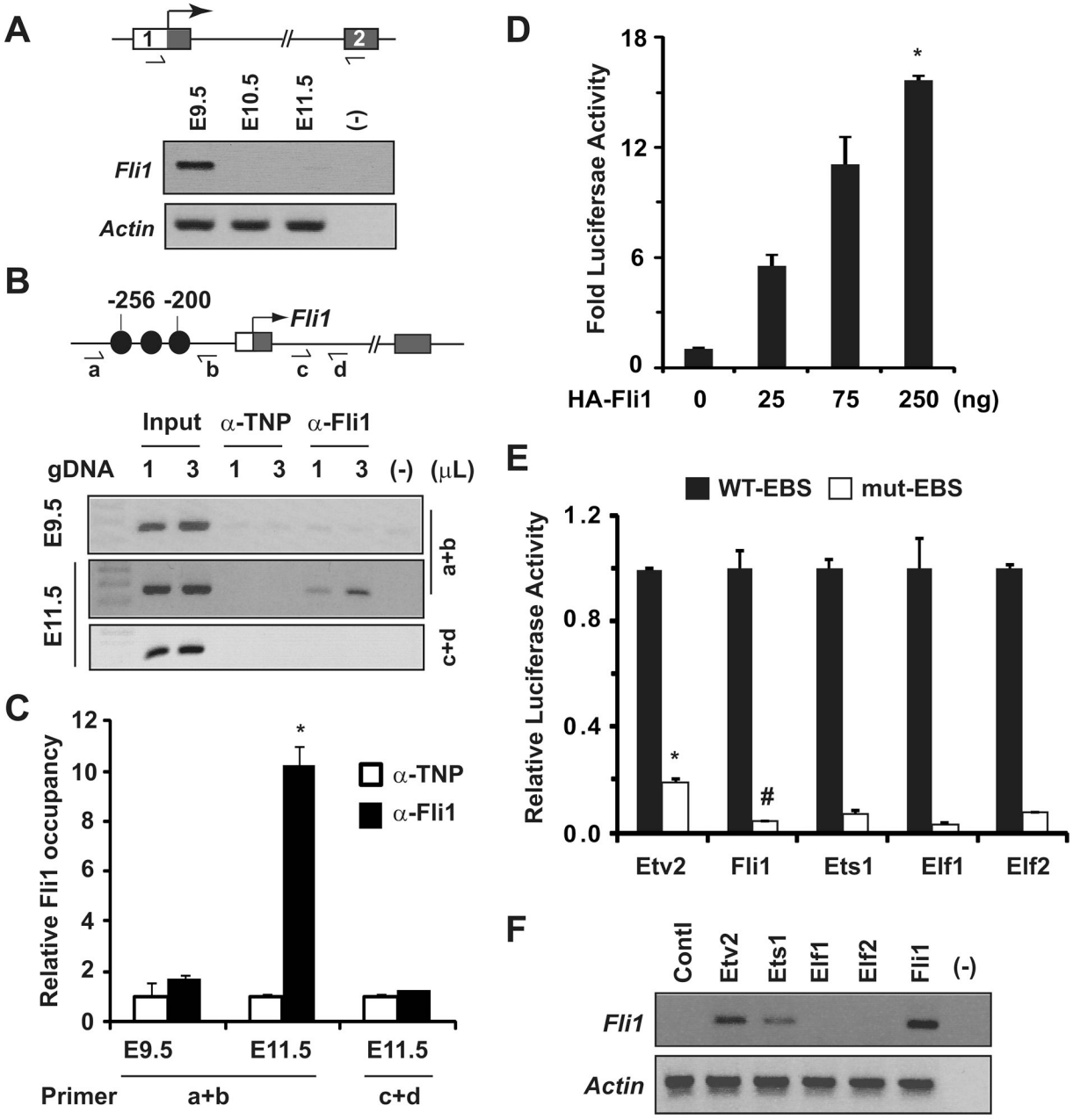

Figure 3. Fli1 governs *Fli1* gene expression via positive autoregulation

(A) Semi-quantitative RT-PCR analyses of *Fli1* expression in *Fli1*-null embryos isolated at the indicated stages. Note the *Fli1* transcripts detected at E9.5 were significantly attenuated at mid-gestation. **(B and C)** ChIP assays (**B**) and quantitative analyses **(C)** for Fli1 occupancy of the *Fli1* promoter (schematized top) in embryos isolated at the indicated stages. Fli1-DNA complexes were immunoprecipitated with anti-Fli1 and anti-TNP (control) sera. Note that promoter-specific Fli1 binding was enriched at E11.5, but not at E9.5, embryos (**p*<0.0001 vs. control). **(D)** Transcriptional assays using Dm-2 reporter and Fli1 expression plasmids in COS1 cells elicited robust and dose-dependent activation of luciferase activity (**p*<0.0001 vs. control). **(E)** Transcriptional assays in the presence of

indicated ETS factors (250ng) and Dm-2 reporter plasmids harboring WT and mutated EBSs are shown. Note that mutation of EBSs significantly attenuated luciferase activity. Luciferase activity for each ETS factor from reporter plasmid harboring WT EBS was normalized to 1. **(F)** RT-PCR analyses of the endogenous *Fli1* transcripts following expression of the indicated ETS factors in C2C12 cells. Note that Etv2 and Fli1, but not Elf1 or Efl2, induced endogenous *Fli1* transcript levels.

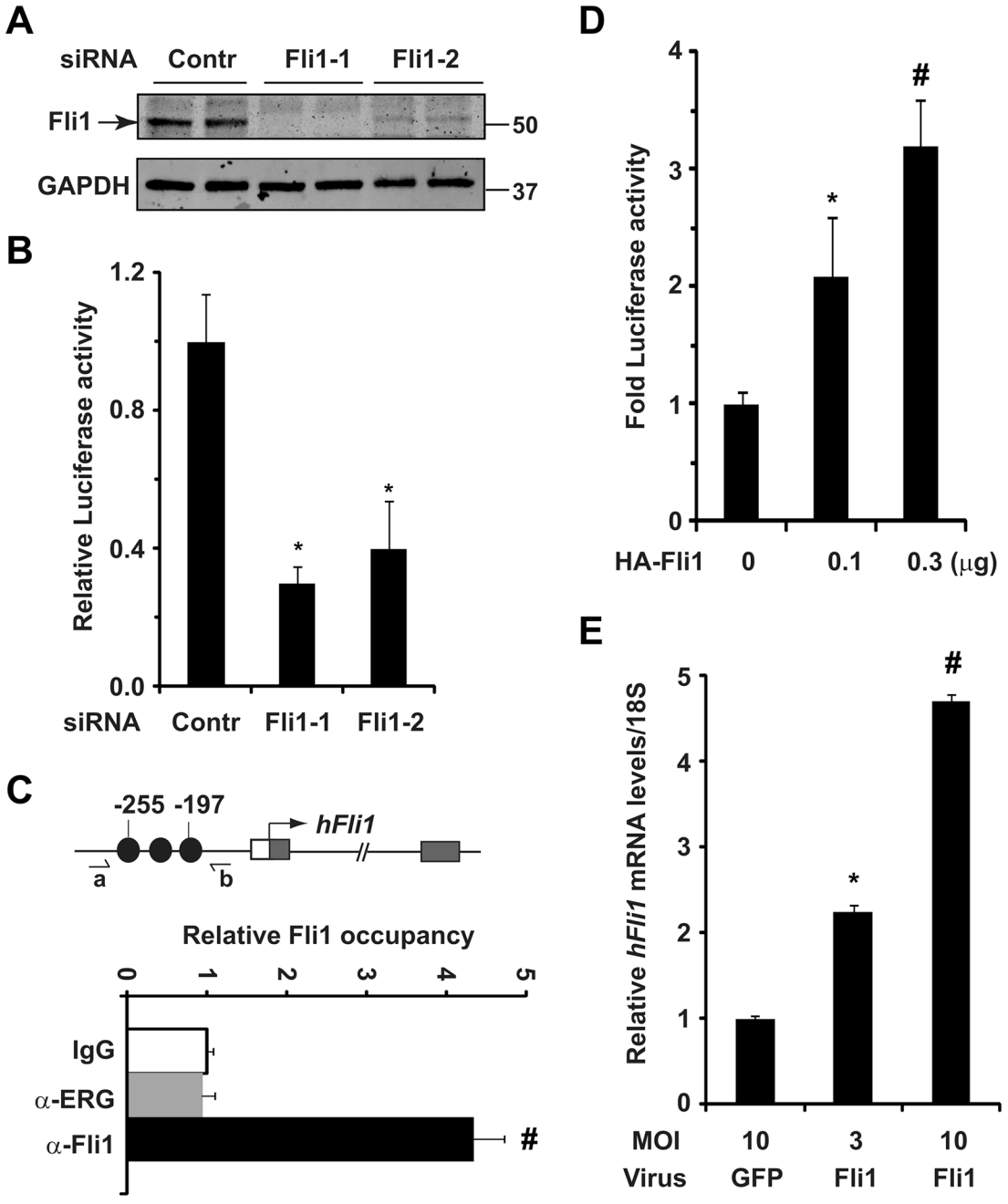

Figure 4. Fli1 is required for sustained *Fli1* expression for the remainder of gestation

(A) Western blot analyses of Fli1 in HAECs transfected with control (Contr) and Fli1-specific siRNAs. Note the marked reduction of Fli1 protein levels in siFli1-treated cells. GAPDH was used as a loading control. Numbers indicates protein molecular markers (kD). **(B)** Baseline Fli1 reporter activity in HAECs transfected with the indicated siRNA ($p<0.05$ vs. control). **(C)** ChIP-qPCR analyses of Fli1 occupancy of the *Fli1* promoter. Note the Fli1, but not Erg, occupancy of *Fli1* promoter (# $p<0.003$ vs. control). **(D)** Transcriptional assays reveal significant and dose-dependent induction of luciferase activity by Fli1 from *Fli1*

reporter plasmid in HAECs (*p<0.001 vs. control). **(E)** qRT-PCR analyses for the endogenous *Fli1* transcripts in HAECs infected with control (GFP) and Fli1-encoding lentivirus (*p<0.003 vs. control, #p<0.0001 vs. control).

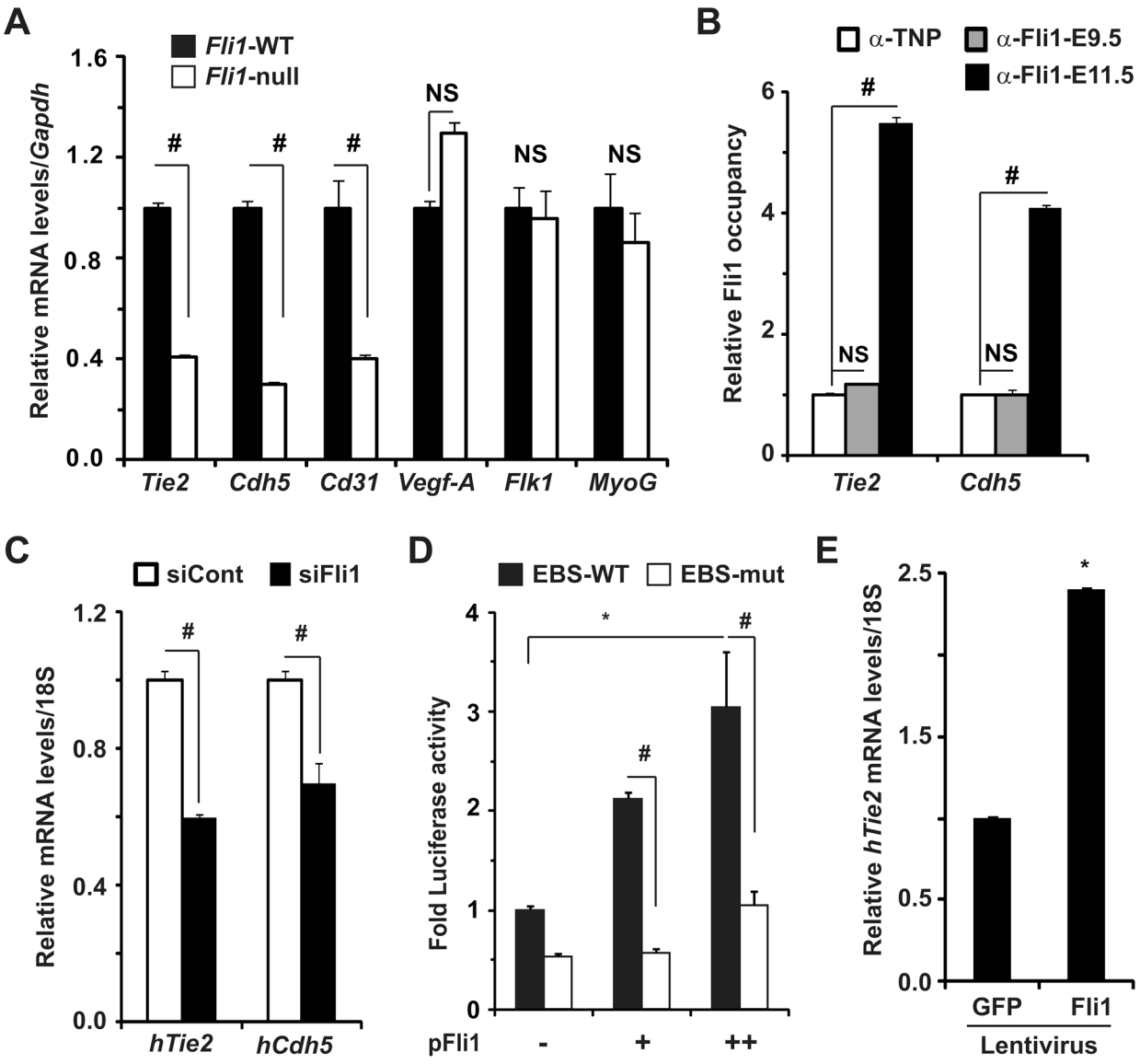

Figure 5. Fli1 governs selective Etv2-regulated endothelial gene expression at and beyond mid-gestation

(A) qRT-PCR analyses for transcript levels of the indicated genes in E10.5 WT and *Fli1*-null embryos (n=3). Relative gene expression in WT mice was normalized to 1 (**p*<0.001 vs. WT). NS: not significant. **(B)** ChIP-qPCR analyses demonstrating Fli1 occupancy of the promoter of indicated endothelial genes in E11.5, but not E9.5, embryos (# *p*<0.001 vs. control). **(C)** qRT-PCR analyses revealing significant attenuation of *Tie2* and *Cdh5* transcript levels in siFli1-treated HAECs (#*p*<0.01 vs. control). **(D)** Transcriptional assays in COS1 cells reveal significant and dose-dependent induction of luciferase activity by Fli1 from *Tie2*-UPF reporter plasmid harboring WT (black), but not mutated (white), EBSs (**p*<0.001 vs. control; # *p*<0.002 vs. WT EBS). **(E)** qRT-PCR analyses for *Tie2* transcript levels in HAECs infected with control (GFP) and Fli1-encoding lentivirus (MOI 10) (**p*<0.001 vs. control).

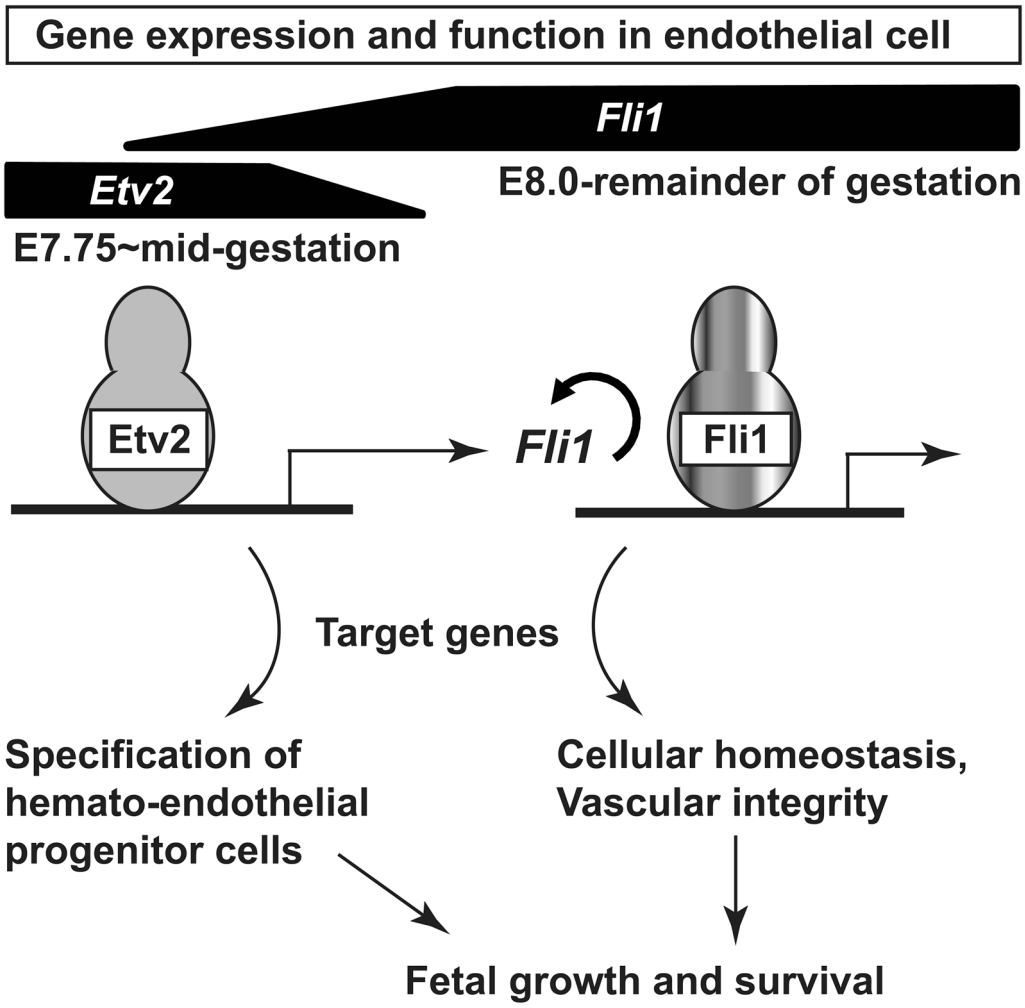

Figure 6.

Working model for positive autoregulation between Etv2 and Fli1 regulates vascular morphogenesis and integrity at early and mid-gestation of mouse embryogenesis. Embryonic expression of *Etv2* and *Fli1* genes at distinct developmental stages and their role in EC are indicated. See text for details.