# SUPPLEMENTAL MATERIALS

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

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# Methods

### Plasmids:

The *Fli1* reporter plasmid was generated essentially as described previously[1-3]. Briefly, the full-length (FL) 0.7-kb promoter fragment of the *Fli1* gene was PCR-amplified from mouse tail genomic DNA using the following (forward-TTTTTACGCGTTTGACTCCGGCTGGAAAAAGC) and (reverse-TTTTTCTCGAGTTGGCCAAGTCTGCAGCCGAGC) set of primers, cloned into Mlu1 and XhoI sites of the pGL3 reporter plasmid (Promega) and sequence confirmed. The deletion mutant-1 (Dm-1) was generated as described for FL except that the following forward (ATGCTACGCGTGAGTGATGCGAAAAGCAGGGC) primer was used. The Dm-1 reporter was treated with Mlu1 and Apa1, blunted and forced ligated to generate Dm-3. Generation of Dm-2 harboring WT and mutated Ets-binding sites (EBSs) were described previously[4]. The 2.1-kb upstream promoter fragment of the *Tie2* gene harboring WT or mutated EBSs were both previously described[3]. The plasmid pCMV-HA-Fli1 was generated by in-frame fusion of an HA tag to the N-terminus of a FL murine Fli1 cDNA and sequence verified. Expression plasmids for Etv2[3], Ets1[5] and Elf1 and Elf2[6] were described. Etv2 mutant plasmid, lacking the DNA-binding ETS domain, was generated by serial PCR amplification of upstream N-terminus and downstream C-terminus region of Etv2-ETS domain, cloned into Not I and Xho I sites and sequence confirmed as described[3].

### Mouse and embryo isolations:

Generation of *Etv2*[3] and *Fli1*[7] knockout and Tie2-GFP transgenic[8] mice was described previously. Heterozygous male and female mice were intercrossed to isolate embryos at distinct developmental stages[3, 9]. The staging of embryos was performed by counting the presence of vaginal plug as day 0.5 after conception and by counting the number of somites. Genomic DNA, extracted from yolk sacs was used for genotyping as described[3, 9]. All embryos were harvested in ice-cold PBS, fixed in 4% paraformadehyde (PFA) overnight at 4°C and washed in PBS. The embryos were photographed under identical conditions and magnification before or after fixation as described[3, 9, 10], except that embryos at different developmental stages were photographed at different magnifications to fill the frame. All mice were maintained in the animal facility at University of Texas Southwestern Medical Center according to the guidelines of Institutional Animal Care and Use Committee and the Animal Resource Center.

### Histological and Immunohistochemical analyses:

Histological and immunostaining analyses of transverse and saggital sections of paraffin-embedded embryos were described previously[3, 9]. Immunostaining analyses for Fli1, α-endomucin, Ki67 and Tie2 were described previously[3, 9]. Briefly, sections were deparaffinized before permeabilization with Triton (α-endomucin), citrate based microwave (Ki67) and trypsin (Tie2 and Fli1) for antigen retrieval, quenched for endogenous peroxidase, blocked with normal serum, and incubated overnight at 4°C with primary antibody. Bound α-endomucin, Ki67 and Tie2 primary antibodies were detected using species-specific biotinylated secondary antibodies, peroxidase-streptavidin, and 3,3'-diaminobenzidine (DAB) chromagen and fluorescein-avidin DCS, respectively.

**TUNEL analyses:**

Apoptotic cell death in WT and *Fli1*-null embryos was analyzed using the Promega DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer's instructions as previously described[3, 9]. Briefly, sections were deparaffinized, equilibrated in PBS, permeabilized with proteinase K, postfixed in 4% PFA, and incubated in TdT reaction mixture for 1 h at 37 °C in the dark. Slides were then washed in 2xSSC, counterstained with propidium iodide, and coverslipped with Vectashield mounting medium.

**Cell culture and treatment:**

COS1 and C2C12 cells were cultured as described previously[3, 9]. Primary human aortic endothelial cells (HAECs) (kindly provided by the Shaul lab of UT Southwestern Medical Center) were purchased and cultured according to the manufacturer's instructions (Lonza). All siRNAs were purchased from Sigma and dissolved in opti-MEM (Invitrogen). A total of 100 pmol siRNA was used for each well of a 12-well plate, and transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen). Gene expression in HAECs was analyzed after 48 hrs.

**Chromatin immunopreciptation (ChIP) assay:**

ChIP assays evaluating *Fli1* and *Tie2* promoter binding of Etv2 and Fli1 were performed as previously described[1, 3, 9, 11], except that the C2C12 myoblasts were transfected with HA-tagged Etv2 and Fli1 expression plasmids in a 15cm culture dish. Formaldehyde (1% final concentration) was added 24 hours post transfection and chromatin solution was prepared by sonication. Immunoprecipitation (IP) of diluted chromatin solution was carried out using anti-HA (Roche) and control IgG sera. Promoter-specific occupancy of Etv2 and Fli1 was analyzed by amplifying the DNA fragment corresponding to *Fli1* promoter and intronic regions using specific primer sets. The following forward (a) gagtgatgcgaaaagcagggc and reverse (b) cctgtgcacgtttgttg ttgtagc primers were used to amplify 257-bp of mouse *Fli1* promoter region, while forward (c) agcatgaccagcacatgaag and reverse (d) tttgaatgcccagagtcaca primers were used to amplify 229-bp of first intronic region (as control) of the *Fli1* gene. On the other hand, forward-gtgtgatgcgaa aagcaggac and same reverse (b) set of primers were used to amplify 271-bp of human *Fli1* promoter region. The binding of Fli1 of *Cdh5* and *Tie2* upstream promoter and *Tie2* intronic enhancer regions was analyzed as described previously[3, 12]. ChIP assays with isolated embryos were carried out as described[13, 14], expect that we isolated WT embryos in DMEM supplemented with 10% fetal bovine serum, minced the embryos before fixation for 15 min at room temperature. After washing with PBS, we used a Dounce homogenizer to disrupt the cell membrane. Protein-DNA complexes were IP'd using anti-Fli1 (Santa Cruz) and anti-Erg (Santa Cruz) (kindly provided by the Cleaver lab of UT Southwestern Medical Center) or control trinitrophenal (TNP) (BD Pharmingen)[1] sera. Genomic DNA isolated before IP was diluted 100-fold and used (1-3µL) as input, and the undiluted IP'd DNA (1-3µL) were used for the PCR reaction, while 1µL was used for quantitative PCR analyses using SYBER master mixture (Roche).

**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 hrs at 22k rpm using SW28 rotor. The viral titer

was determined by qRT-PCR (Clontech) and stored at -80°C until use. To infect HAECs, an MOI of 3-10 of GFP (as control) and Fli1-expressing lentivirus was added in culture medium containing polybrene (8μg/ml). RNA was extracted for gene expression analyses after 48 hrs post-infection.

**Semi-quantitative and quantitative RT-PCR analyses:**

Total RNA from C2C12 myoblasts and WT and *Etv2*- and *Fli1*-null embryos (n=3-4) was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions as previously described[3, 9, 10]. Equal amounts (0.3-1.0μg) of RNA were used to prepare cDNA using iScript cDNA synthesis kit according to manufacturer's instructions (Bio-Rad). All cDNA was diluted 5-10 fold and employed for quantitative RT-PCR (qRT-PCR) analyses for *Ets* and endothelial-specific genes using gene-specific Taqman probes where 18S or *Gapdh* RNA was used as a loading control as described previously[3, 9]. Transcript levels of mouse endothelial specific *Erg* isomers, *Gata2, Cdh5* and *Etv6* in WT and null embryos were analyzed using SYBER green master mix (Roche) as described[14, 15]. To prepare cDNA from endothelial cells, we extracted RNA from Tie2-GFP$^+$ cells isolated from E9.0 and E12.0 Tie2-GFP embryo by FACS (fluorescence activated cell sorting), amplified according to the manufacturer's instructions (Ambion) and employed for semi-quantitative and qRT-PCR analyses as previously described[3, 9]. In addition, cDNA of WT and null embryos and C2C12 cells was used for semi-quantitative RT-PCR analysis of *Etv2* using the following set of primers (forward-ATCACCAAGGCCATCGA GAGC) and (reverse- ACTGTTGTTGTCCATGGACGC) as described previously[3]. Transcript levels of human *Fli1, Tie2,* and *Cdh5* were analyzed as described previously[12]. To assess activation of endogenous *Fli1* promoter activity, we used (forward -GCTCGGCTGCAGACTTGG C) and (reverse-GCTTGACATTGACTCTCACTGGC) primers to analyze mouse endogenous *Fli1* transcript levels spanning exon 1 UTR and exon 2 in C2C12 myoblasts and embryos.

**Reporter gene assays**

Transcriptional assays using *Fli1* and *Tie2* reporter constructs, harboring WT or mutated Ets-binding sites (EBSs), were performed as described previously[3, 9, 16], except that Fugene-HD (Roche), Lipofectamine plus and Lipofectamine 2000 (Invitrogen) were used to transfect DNA into COS1, C2C12 and HAE cells, respectively. Luciferase activity was measured using the dual-luciferase kit (Promega), and luciferase activity of pTK-renilla (control) was used to normalize firefly luciferase activity. Each assay was performed in triplicate and repeated 2-3 times. Luciferase activity in the absence of Etv2 and Fli1 was normalized to one to determine Etv2- and Fli1-dependent fold activation of luciferase activity. Error bars represent mean ± SD, and $p$ values were calculated by Student's $t$ test.

**Western blot analyses:**

Western blot analyses were carried out as described previously[3, 9, 16], except that whole cell extracts from HAECs, control C2C12 myoblast cells or cell expressing HA-tagged Etv2 and Fli1 were separated on a 10% SDS-PAGE gel and the proteins were transferred to a nylon membrane. The membrane was incubated with anti-HA (1:3000, Roche) and anti-GAPDH (1:7500, Santa Cruz), washed and then processed as described[17, 18].

**Statistical analyses:**

Error bars represent mean ± SD, and *p* values, calculated by Student's *t* test, less than 0.05 were considered to be statistically significant.

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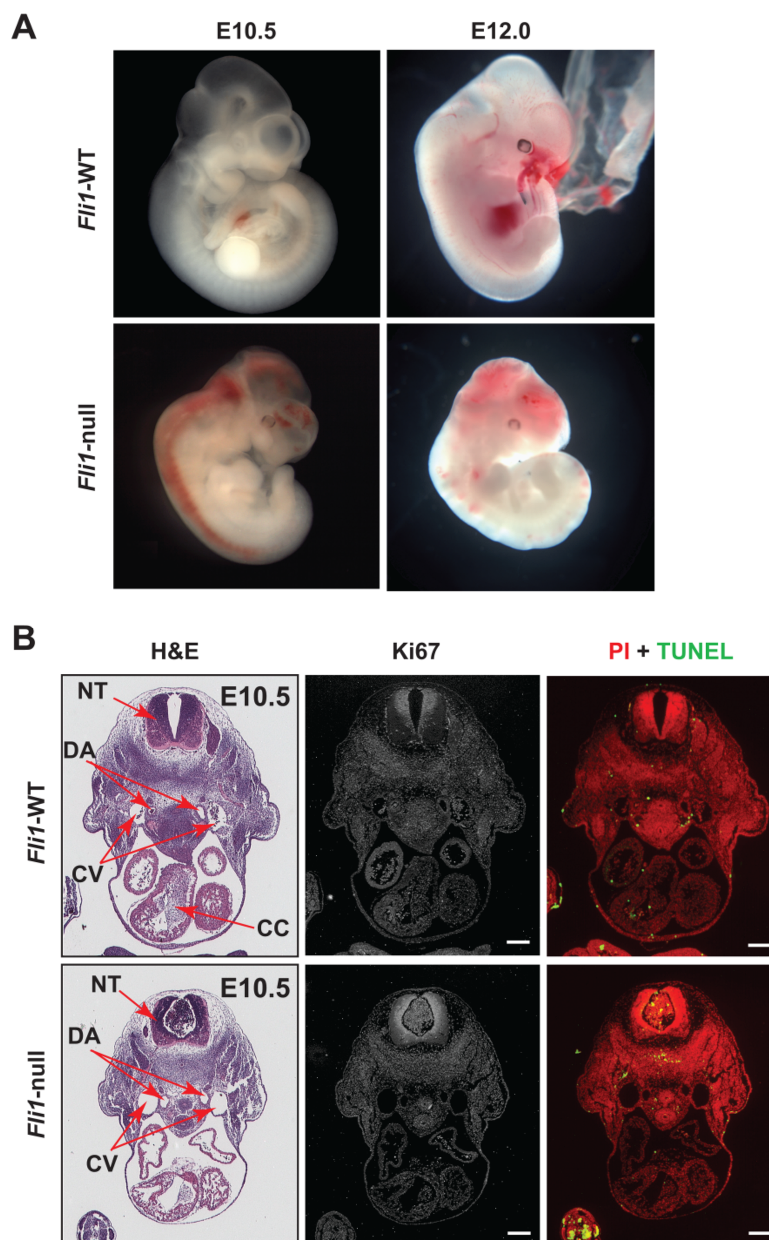
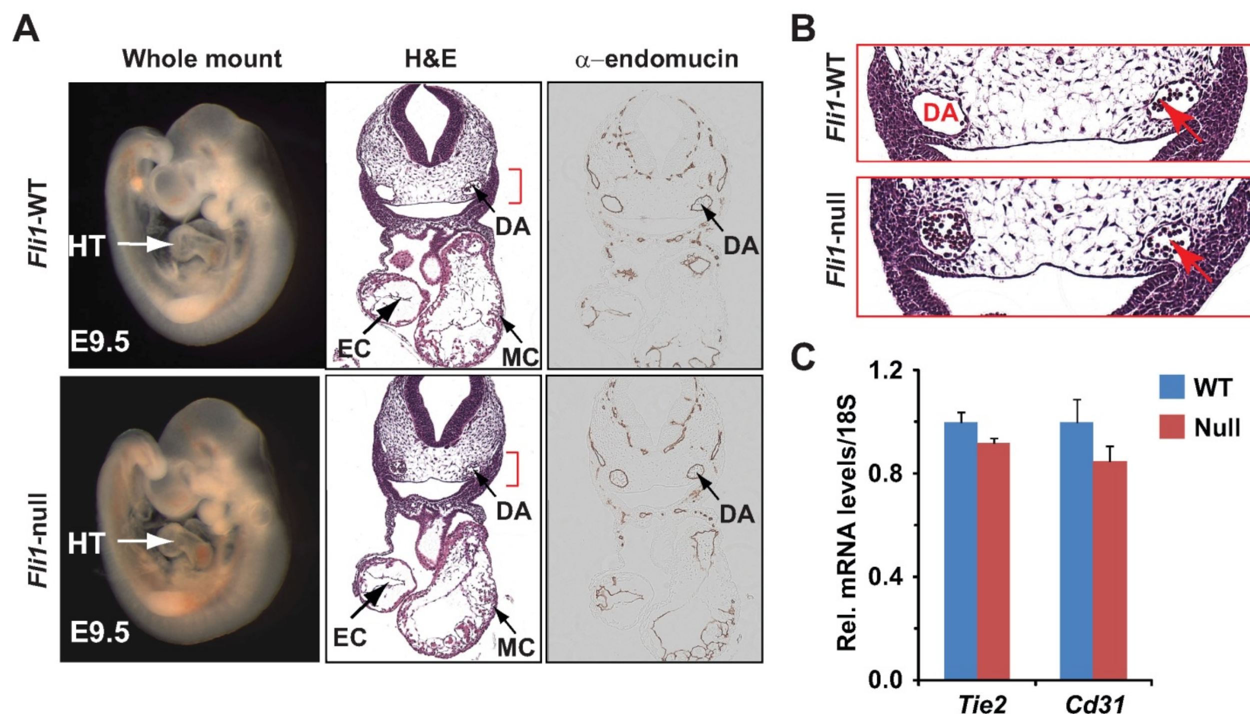
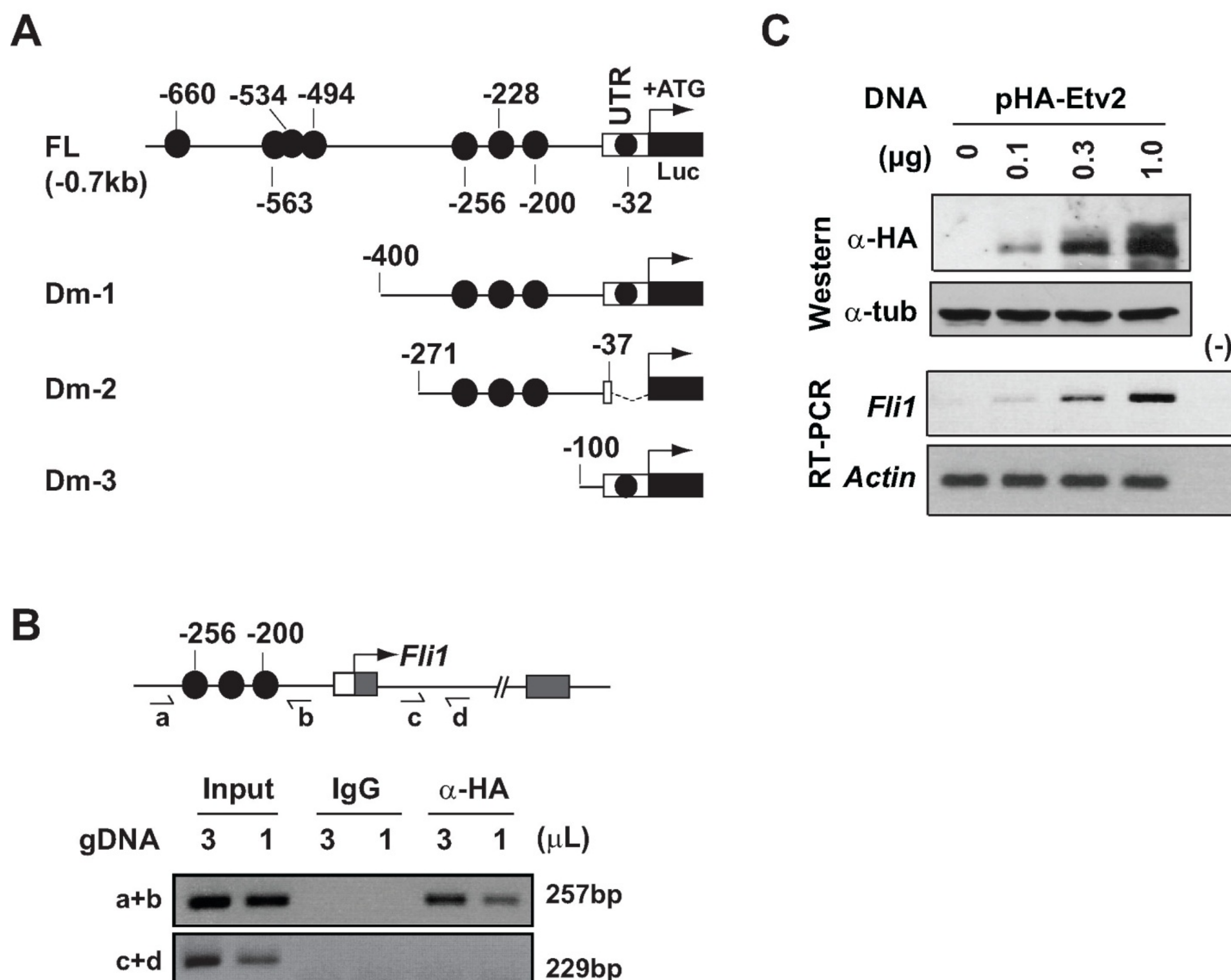



**Supplemental Fig. I: Embryonic hemorrhage and lethality of *Fli1*-null mice are associated with increased cell death. (A)** Morphological appearances of WT and *Fli1*-null littermates isolated at the indicated developmental stages. Vascular leakage within the embryo proper is evident only in *Fli1*-null embryos. Initiation of tissue disintegration and absence of heart beat (i.e. embryonic lethality) were noted in *Fli1*-null embryos at E12.0. **(B)** Routine histology (H&E), IHC analyses for proliferative marker, ki67 and TUNEL assays revealed hemorrhage in neural tube of null embryos and significant increased cell death in endothelial and blood cells as well as cells of the neural tube (also see Figure 1), but cellular proliferation was comparatively normal (bar=200μM). Neural tube (NT), dorsal aorta (DA), cardiac cushion (CC) and cardinal vein (CV) are indicated.

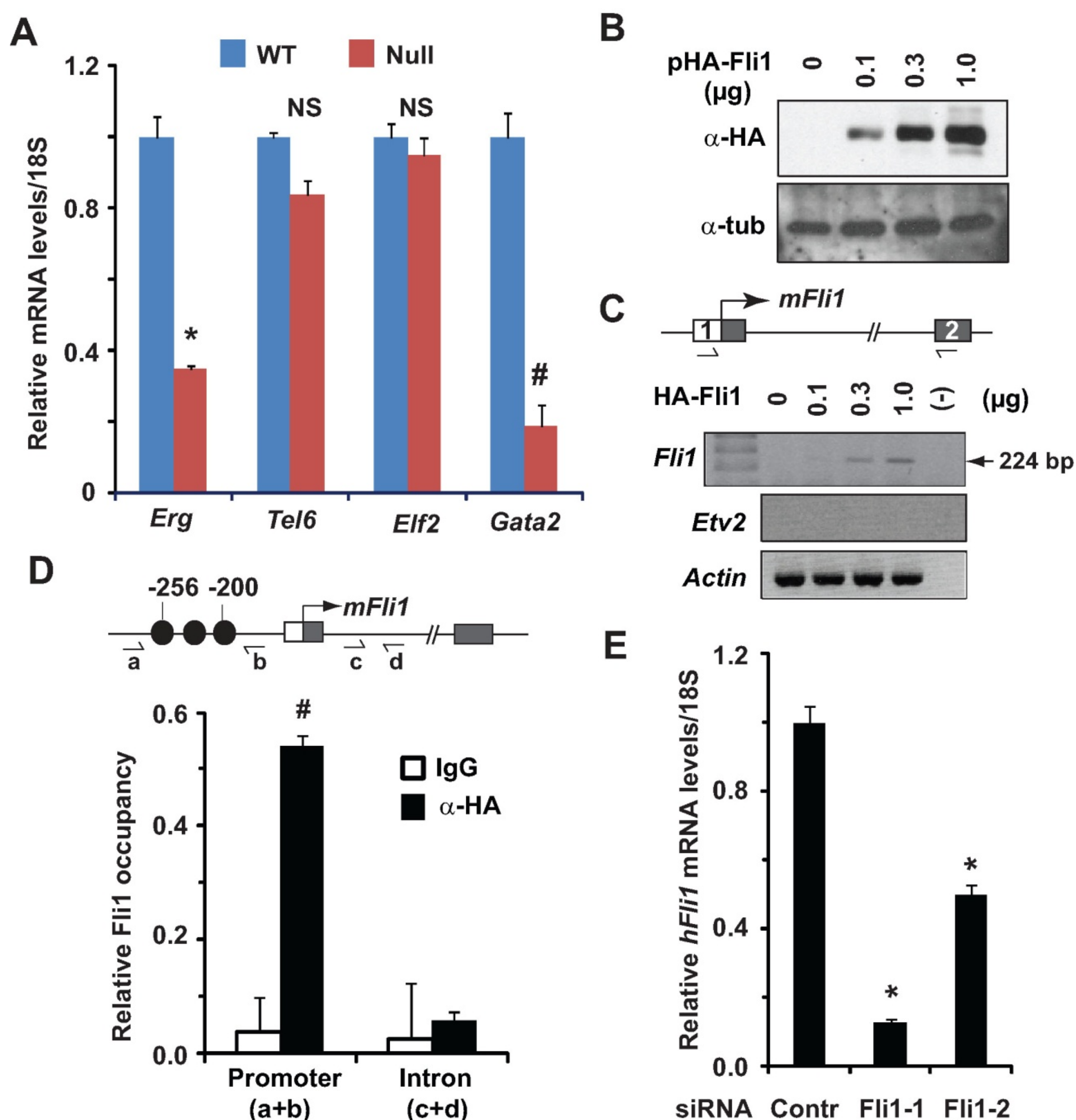



**Supplemental Fig. II: Vascular morphogenesis is normal in early *Fli1*-null embryos. (A)** Morphological appearances of WT and *Fli1*-null littermates are grossly indistinguishable at E9.5. Histologic (H&E) and IHC (α-endomucin) analyses revealed normal vascular and cardiovascular development in both WT and *Fli1*-null littermates. HT (heart), DA, PHV (primary head vein), EC (endocardium), MC (myocardium). **(B)** H&E close-ups of DA clearly illustrate blood within the DA of *Fli1*-null mice (red arrow). **(C)** qRT-PCR analyses of the indicated endothelial genes in WT and *Fli1*-null embryos. Note that expression of both genes is essentially similar in WT and *Fli1*-null littermates (n=3).

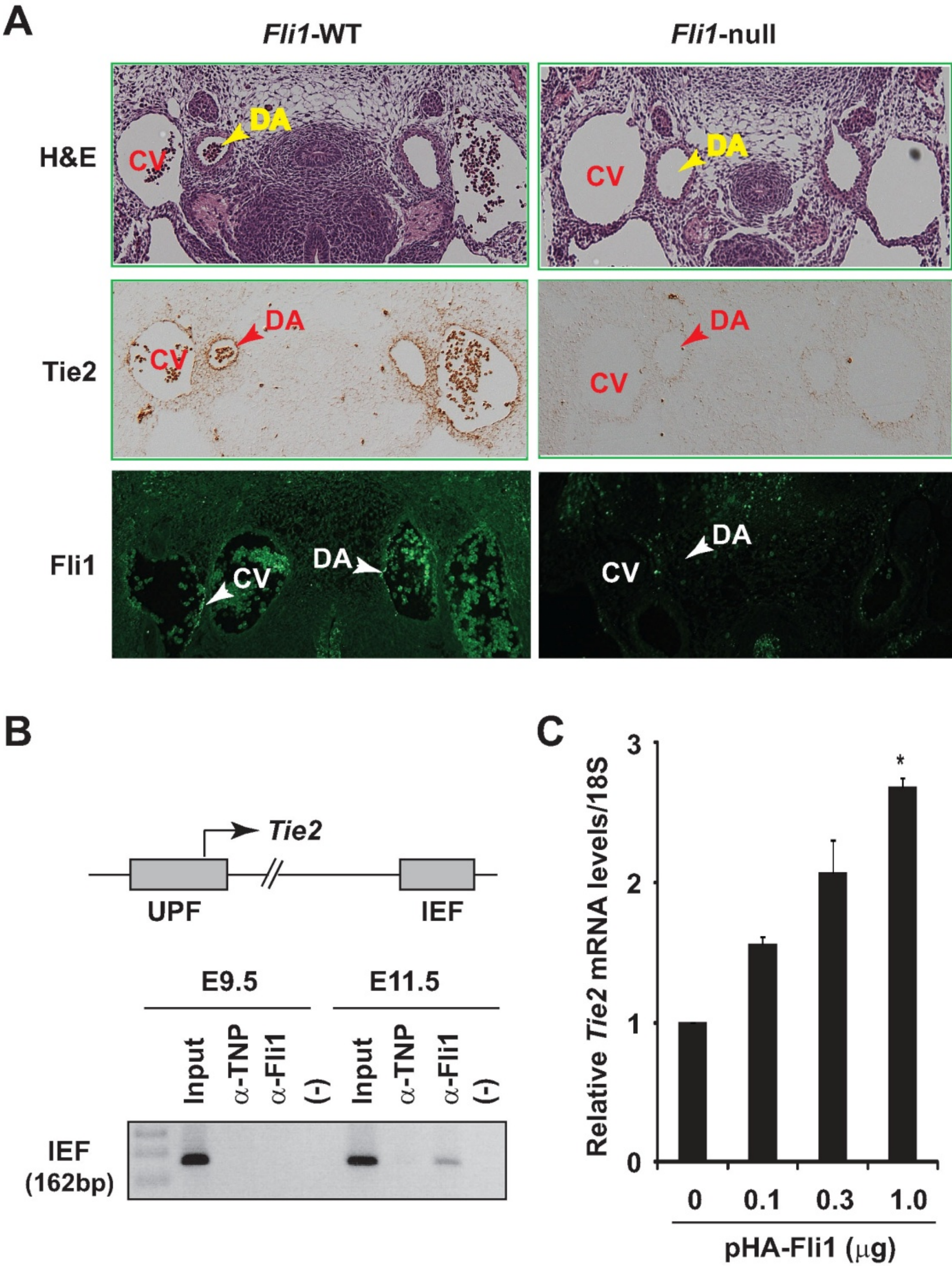
**Supplemental Fig. III: Conserved EBSs within *Fli1* promoter region confer Etv2-mediated *Fli1* gene expression. (A)** Semi-quantitative (left) and qRT-PCR (right) analyses of *Etv2* and *Fli1* transcripts in whole embryo isolated at the indicated developmental stages. Note the transient co-expression of the *Etv2* and *Fli1* genes in early embryos, while that expression of the *Fli1*, but not *Etv2*, gene persisted beyond mid-gestation. Transcript levels of each gene at E8.5 were normalized to 1. **(B)** Nucleotide sequence of 0.7-kb *Fli1* promoter region. Evolutionarily conserved Ets-binding sites (EBSs) (highlighted), 5' untranslated region (UTR) (upper case), translation initiation site (ATG) and primer sequence in 5' UTR (see Figure 2) are indicated. Schematic alignment of nucleotide sequence of the indicated species for three essential EBSs (highlighted green for mouse) in reverse orientation is shown. Mutated nucleotides in the EBSs are indicated blue. **(C)** Transcriptional assays in C2C12 cells reveal that co-transfection of *Fli1* reporter and the indicated amounts of Etv2 expression plasmids resulted in dose-dependent and significant induction of luciferase activity (*$p$<0.002 vs. control).




**Supplemental Fig. IV: Etv2 governs *Fli1* gene expression by binding to specific conserved EBSs within the *Fli1* promoter. (A)** Schematic of the full-length (FL) and deletion mutant (Dm) *Fli1* reporter plasmids. Numbers indicate the position of the conserved Ets-binding sites (EBSs) (black circles) upstream from the translation initiation site (ATG). White bar indicates the 5' untranslated region (UTR). **(B)** ChIP assays revealing Etv2 occupancy of the *Fli1* promoter *in vivo*. Chromatin solution of HA-tagged Etv2 expressing C2C12 myoblasts was IP'd with anti-HA and control IgG sera. Genomic DNA (gDNA) purified before IP was diluted 100 fold and used as input. Specific primer sets and indicated volume (µL) of the gDNA were used to PCR amplify the promoter (a+b) and intronic (c+d) region (schematized top) of the *Fli1* gene. Note that DNA sample IP'd with anti-HA, but not IgG, sera amplified only the *Fli1* promoter harboring EBSs. **(C)** Western blot (top) and semi-quantitative RT-PCR (bottom) analyses demonstrating increased Etv2 protein levels in C2C12 myoblasts induces endogenous *Fli1* expression in a dose-dependent manner. PCR negative (-), protein (Tub) and PCR (actin) loading controls are indicated.




**Supplemental Fig. V: Fli1 is required for *Fli1* expression at and beyond mid-gestation.** **(A)** qRT-PCR analyses for transcript levels of the indicated genes using RNA from E10.5 WT and *Fli1*-null embryos**.** Relative gene expression in WT embryo was normalized to 1. Significant attenuation of *Erg* and *Gata2* expression was observed in *Fli1*-null (**p*<0.005 vs. WT and (#*p*<0.005 vs. WT) embryos. NS: not significant. **(B and C)** Western blot (**B**) and RT-PCR (**C**) analyses demonstrate increased Fli1 protein levels and dose-dependent induction of endogenous *Fli1* transcripts by Fli1 in C2C12 cells. Tubulin (**B**) and α-*actin* (**C**) were used as loading control. **(D)** ChIP-qPCR analyses for Fli1 occupancy of the *Fli1* promoter in C2C12 cells were performed as described in panel B of Supplemental Fig. IV, except that HA-tagged Fli1 expression plasmid was transfected. **(E)** Quantitative RT-PCR analyses of *Fli1* transcripts in primary HAECs following transfection of the indicated siRNAs. Note the marked reduction of *Fli1* transcripts by two independent Fli1-specific siRNAs (**p*<0.05 vs. control).




**Supplemental Fig. VI: Fli1 is an upstream regulator of endothelial *Tie2* gene expression at mid-gestation. (A)** Enlarged view of histology (H&E) and IHC analyses for Tie2 and Fli1 corresponding to vasculatures (green bracket) of panel A of Figure 1 are shown. Note the marked reduction of Tie2 levels in *Fli1*-null vascular endothelium, such as dorsal aorta (DA) and cardinal vein (CV), was specific to the loss of Fli1 in vascular endothelium. **(B)** ChIP assays revealing Fli1 occupancy of the intronic enhancer fragment (IEF) of *Tie2* gene (schematized) in mouse embryos isolated at E11.5 but not at E9.5. **(C)** qRT-PCR analyses of RNA from C2C12 cells transfected with indicated amounts of the Fli1 expression plasmid reveal significant and dose-dependent induction of endogenous *Tie2* transcript levels (*$p$<0.002 vs. control).