Etv2 and Fli1b Function Together as Key Regulators of Vasculogenesis and Angiogenesis

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**Objective**—The E26 transformation-specific domain transcription factor Etv2/Etsrp/ER71 is a master regulator of vascular endothelial differentiation during vasculogenesis, although its later role in sprouting angiogenesis remains unknown. Here, we investigated in the zebrafish model a role for Etv2 and related E26 transformation-specific factors, Fli1a and Fli1b in developmental angiogenesis.

**Approach and Results**—Zebrafish fi1a and fi1b mutants were obtained using transposon-mediated gene trap approach. Individual fi1a and fi1b homozygous mutant embryos display normal vascular patterning, yet the angiogenic recovery observed in older etv2 mutant embryos does not occur in embryos lacking both etv2 and fi1b. Etv2 and fi1b double-deficient embryos fail to form any angiogenic sprouts and show greatly increased apoptosis throughout the axial vasculature. In contrast, fi1a mutation did not affect the recovery of etv2 mutant phenotype. Overexpression analyses indicate that both etv2 and fi1b, but not fi1a, induce the expression of multiple vascular markers and of each other. Temporal inhibition of Etv2 function using photoactivatable morpholinos indicates that the function of Etv2 and Fli1b during angiogenesis is independent from the early requirement of Etv2 during vasculogenesis. RNA-Seq analysis and chromatin immunoprecipitation suggest that Etv2 and Fli1b share the same transcriptional targets and bind to the same E26 transformation-specific sites.

**Conclusions**—Our data argue that there are 2 phases of early vascular development with distinct requirements of E26 transformation-specific transcription factors. Etv2 alone is required for early vasculogenesis, whereas Etv2 and Fli1b function redundantly during late vasculogenesis and early embryonic angiogenesis. *(Arterioscler Thromb Vasc Biol. 2015;35:865-876. DOI: 10.1161/ATVBHA.114.304768.)*

**Key Words:** angiogenesis ■ ETS transcription factor ■ vasculogenesis ■ zebrafish

The vertebrate vasculature forms early in development to support the metabolic needs of the developing embryo. The first blood vessels arise de novo from mesodermally derived endothelial precursors called angioblasts through vasculogenesis. Growth, expansion, and remodeling of the primary vasculature into a mature vascular network occur through the sprouting or longitudinal division of pre-existing vessels, termed angiogenesis. Angiogenesis remains important throughout adulthood during tissue repair, estrus and pregnancy, and during muscle growth with exercise. Effective therapeutic control of angiogenic potential remains a critical unmet goal in the treatment of pathologies of hyper-vascularization (eg, cancer and macular degeneration) and in situations requiring increased blood flow to ischemic tissues (eg, coronary heart disease, peripheral arterial disease, and wound healing disorders). A key difficulty associated with antiangiogenic strategy design is that although many of the individual molecules required for the vertebrate vascular development and maintenance have been identified, the signaling cascades underlying these phenomena remain poorly understood because of a high degree of functional redundancy and overlapping target specificities. The functional overlap known to exist among the E26 transformation-specific (ETS) family of transcription factors is of particular interest because several vascular-specific ETS factors sit atop transcriptional hierarchies and thus represent potential therapeutic targets.

The ETS domain transcription factor Etv2/Etsrp has been identified as a critical regulator of embryonic vasculogenesis, with functional orthologs identified in multiple vertebrates, including zebrafish, Xenopus, mice (ER71/Etv2), and humans (ETV2). Like other ETS factors, Etv2/Etsrp contains a conserved 85-amino acid ETS domain that binds to a core GGA(A/T) motif to transcriptionally activate downstream target genes. Murine Etv2 binds synergistically with...
FoxC2 to activate the expression of a variety of endothelial genes (eg, Vegfr2/flk1, tie2, and cdc5).28 In zebrafish and in mice, etv2 is expressed during early somitogenesis in the lateral plate mesoderm (LPM) and later restricted to the vascular endothelium. *Etv2* null mice and zebrafish lack a formed vasculature, do not achieve circulation, and die during embryogenesis.25,26,29

A related vascular endothelial–specific ETS factor, FLI1, has received significant research focus because translocations between the ETS domain of FLI1 and the transactivation domain of exchange web services generate an aberrant transcriptional activator leading to Ewing sarcoma.30,31 In *Xenopus* embryos, loss of Fli1 results in a substantial reduction in the number of hemangiblasts,32 suggesting that Fli1 is required for early hemangiblast specification and the subsequent formation of the blood and endothelial lineages. Mutant mice similarly display a deficit in the number of both blood and endothelial cells, and although the vasculature initially seems normal in these mutants, they hemorrhage from the dorsal aorta and ventricles of the brain and die by E12.5 because of endothelial cell apoptosis.33,34 *Fli1a* is induced by *Etv2* during early mouse embryogenesis and subsequently maintains its own expression and the expression of other vascular markers through a positive autoregulatory feedback mechanism.34 However, the role of Fli1 during vasculogenesis and the initial stages of angiogenesis have not been established.

The zebrafish (*Danio rerio*) has emerged as an important vertebrate system for studying vasculogenesis and angiogenesis because of the optical transparency, rapid external embryonic development, and high fecundity. Zebrafish have 2 closely related FLI1 paralogs, *fli1a* and *fli1b*. Fli1a and Fli1b share 55% identity and thus exhibit greater sequence homology with each other than either does with Etv2 (which shares 14% identity with Fli1b and 16% with Fli1a; http://uswestensembl.org). Both are initially expressed in the LPM and later restricted to the vascular endothelium.35–37 Overexpression of wild-type (WT) zebrafish Fli1a did not induce ectopic vascular marker expression, whereas a constitutively active VP16-fused form of Fli1a was shown to induce several hemangiblast markers, including *etv2* and *kdr.*32 Far less is known about the role played by fli1b; although it contains a predicted ETS-binding domain and thus potentially regulates a similar subset of ETS-target genes. Fli1b is located ≈4.2 kb downstream of *etv2* on chromosome 16 and is transcribed in the opposite direction, mirroring the arrangement of *ets1* and *fli1a* on zebrafish chromosome 18 and of Ets1 and Fli1 in mice and humans.22,37 Interestingly, related fish species including stickleback and medaka also share similar arrangement of *etv2* and *fli1b* genes, whereas in most other vertebrates, a single Fli1 homolog is located adjacent to Ets1 gene and not *Etv2.*23,38

Previously, combined morpholino (MO) knockdown of all 4 of these ETS factors (*fli1a*, *fli1b*, *etv2*, and *ets1*) in zebrafish was shown to result in a complete absence of angiogenic sprouting.29 By contrast, knockdown of *fli1a* or *fli1b* individually resulted in minor sprouting defects, suggesting that ≥1 of these ETS factors have redundant function. However, only limited phenotypic analysis of vascular defects has been performed, and the specific role of each ETS factor remains unclear. Furthermore, specificity of the observed phenotypes (except for *etv2* MO) has not been demonstrated by RNA rescue or mutant confirmation. Recent studies have called into question many MO-based phenotypes, including Ets1 knockdown, which were not recapitulated in genetic mutants.39

In this study, we isolated genetic *fli1a* and *fli1b* zebrafish mutants that were homozygous viable and displayed no apparent angiogenic defects, suggesting that at least some of the previously reported defects29 were caused by MO off-target effects. We demonstrate that *Etv2* and *Fli1b* function together as regulators of vasculogenesis and angiogenesis. Combined loss of *Etv2* and *Fli1b*, but not *Etv2* and *Fli1a*, blocks the angiogenic recovery otherwise observed in *etv2* mutants and results in increased endothelial cell apoptosis throughout the LPM. Overexpression analyses indicate that both *etv2* and *fli1b* induce the expression of multiple vascular endothelial markers and of each other. Finally, RNA-Seq, chromatin immunoprecipitation, and fluorescent reporter assays indicate that *Fli1b*, similar to *Etv2*, is capable of regulating the expression of multiple vascular endothelial markers and that *Fli1a* is a potential direct transcriptional target of *Fli1b*. Taken together, our data indicate that *fli1b* is induced by *Etv2* during early vasculogenesis and later acts redundantly with *Etv2* to support late vasculogenesis and early developmental angiogenesis. Furthermore, our data provide critical mechanistic insight into the combinatorial roles played by ETS factors in vascularization of the developing embryo.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

**Etv2/etsrp Mutant and Morphant Embryos Exhibit Late-Stage, Aberrant Sprouting Angiogenesis**

Although *etsrp/etv2* null mutants (henceforth referred to as *etv2*−/−) and morphants exhibit strong defects in vasculogenesis before 24 hours post fertilization (hpf),22,29 they undergo a partial recovery of the intersegmental vessels (ISVs) by sprouting angiogenesis at later stages. To understand what promotes this recovery, we analyzed vascular development in *etv2*−/− embryos ranging in age from the 9-somite stage (13.5 hpf) through 72 hpf (Figure 1). *Etv2*−/− embryos lacked detectable *fli1a-GFP* expression in the anterior or posterior LPM at the 9-somite stage (Figure 1A and 1B), consistent with previous reports, indicating that *etv2* is both necessary and sufficient for initiating early vascular endothelial marker expression (eg, *fli1a*).22 The first indication of vascular...
Figure 1. *Etv2*<sup>−/−</sup> mutants undergo a partial recovery of vasculogenesis and aberrant sprouting angiogenesis by 72 hpf. A–L, Lateral views of wild-type (WT) and *etv2*<sup>−/−</sup> mutant embryos in the Tg(*flia*:GFP) vascular reporter line. *Flia*:GFP expression was evident at the 9-somite stage (13.5 hpf) in WT embryos (A) and localized to the bilateral angioblasts of the anterior and posterior lateral plate mesoderm (ALPM and PLPM, respectively). Endothelial *flia*:GFP was not observed in *etv2*<sup>−/−</sup> mutant embryos at this stage, but an additional nonendothelial population of *flia*:GFP cells located in the presumptive pharyngeal endoderm was retained in *etv2*<sup>−/−</sup> mutant embryos (asterisks, A and B). The first sign of vascular recovery in *etv2*<sup>−/−</sup> mutant embryos was observed at 15-somites (17 hpf, arrow, D). Uniform pairs of intersegmental vessels (ISVs) were observed sprouting from fully lumensized dorsal aorta (DA) vessels at 24 hpf in WT embryos (E), whereas vasculogenesis remained absent and ISVs absent in age-matched *etv2*<sup>−/−</sup> mutants (F). Fully formed ISVs could be seen extending from the DA and posterior cardinal vein (PCV) and branching at their dorsal terminus to form the dorsal longitudinal anastomotic vessel (DLAV) by 36 hpf in WT embryos (G), whereas ISV sprouts in age-matched *etv2*<sup>−/−</sup> mutants were just starting to form (arrowheads, H). The regularly patterned ISVs observed in 48-hpf WT embryos (I) were disrupted in *etv2*<sup>−/−</sup> mutants, with a majority mispatterned (arrowheads, J). Continued recovery was observed at 72 hpf (L). Fractions indicate the number of embryos with the observed pattern of green fluorescent protein (GFP) expression (numerator) and the total evaluated (denominator).

Angiogenic Recovery in *etv2*-Deficient Embryos Is Mediated by Existing Vascular Endothelial Cells Rather Than a Latent Population of Progenitor Cells

To properly interpret the basis of the recovery of *etv2*-deficient embryos, the source of cells contributing to this recovery needed to be determined. We used the photoconvertible TgBAC(*etv2*:Kaede) line that specifically labels angioblasts and their daughter (ie, differentiated) vascular endothelial cells (VECs) to identify populations of VECs arising before and after key stages of the observed recovery. Using this approach, VECs present before photoconversion exhibit both red and green fluorescence, whereas those arising later seem green only. *Etv2* morpholino MO1 inhibits translation of *Etv2* protein but is designed to not affect *etv2*:Kaede reporter expression, which allows observation of vascular endothelial progenitors in *etv2* morphant embryos. *Etv2*:Kaede embryos injected with *etv2* morpholino exhibited a partial impairment of the axial vasculature at 24 hpf and absence of ISVs and a lack of blood circulation (not shown), indicating that *etv2* morpholino knockdown was successful. Embryos were photoconverted at the 20-somite stage (19 hpf) to enable the assessment of the endothelial contribution to ISVs during the recovery period (Figure 2). Photoconversion at the 20-somite stage in control embryos resulted in 97% (n=91 of 95) of trunk ISVs exhibiting both red and green fluorescence (Figure 2A), indicating that a majority of cells contributing to the ISVs in WT embryos were specified before the 20-somite stage, as we have previously reported. Morphants showed a partial recovery of axial and intersegmental vasculature by 36 hpf, but the average number of fluorescent ISVs per fish was significantly reduced relative to WT controls (2.1±0.3 in morphants versus 7.3±0.3 in controls, Figure 2B). Because vascular expression in *etv2*:Kaede embryos was mosaic, only a fraction of the total number of ISVs exhibited fluorescence. Ninety percent of the ISVs in the morphant embryos were both red and green (n=40 of 46), arguing that the angiogenic recovery in control and morphant embryos was also largely mediated by VECs present before the photoconversion period. Occasionally, green only cells were detected in the vascular plexus of both WT and morphant embryos at 36 hpf, providing confirmation that new VECs had formed, but these new cells did not significantly contribute to the observed recovery. It is likely that red fluorescence was not apparent in some of the green cells because of faint fluorescence and turnover of the photoconverted Kaede...
protein. Photoconversions performed before the 20-somite stage were uninformative because converted Kaede expression was not detectable through the target angiogenic recovery period because of weak etv2::Kaede expression during early stages (data not shown).

**Loss of fli1b Exacerbates the etv2 Vascular Phenotype**

We hypothesized that other ETS transcription factors function redundantly with Etv2 during the later stages of vascular development to mediate the vascular recovery observed in etv2 mutants and morphants. Three related ETS factors Ets1, Fli1a and Fli1b have been previously implicated as having a redundant function with Etv2.29 However, the previous study used a month-only approach without stringent controls, such as RNA rescue. Furthermore, the individual roles of each of these transcription factors have not been determined, and only limited phenotypic analysis has been previously performed. We focused our analysis on the 2 Fli1 homologs, Fli1a and Fli1b. As previously reported, strong expression of fli1a and fli1b continues in vascular endothelium past 24 hpf, whereas etv2 expression becomes greatly reduced29,36 (Figure IA through IK in the online-only Data Supplement). Furthermore, fli1b expression was significantly reduced but detectable by whole-mount in situ hybridization in etv2 mutants from 24 hpf through 36 hpf (Figure IL and IM in the online-only Data Supplement), suggesting that fli1b expression occurs in an etv2-independent manner throughout the recovery period of etv2-deficient embryos.

Fli1a and fli1b mutant lines were generated in Tol2-mediated insertional mutagenesis screens. The fli1a\textsuperscript{Tol2\textsuperscript{10G}} mutant line contains a gene trap vector insertion in the third intron of fli1a gene with a splice acceptor site followed by enhanced green fluorescent protein (GFP) and polyA sequence (Figure IIA in the online-only Data Supplement). The insertion causes a truncation of the C-terminal ETS DNA-binding domain (Figure IIB in the online-only Data Supplement). The insertion resulted in 21.3-fold reduction in expression of full-length message as determined by reverse transcription polymerase chain reaction (Figure III in the online-only Data Supplement) and whole-mount in situ hybridization using an antisense probe against the 3’ end of the message containing the ETS-binding domain (Figure III and IJ in the online-only Data Supplement). Fluorescence of the truncated fli1a\textsuperscript{Tol2\textsuperscript{10G}}-GFP fusion protein was evident in the vascular endothelium, detectable by the 10-somite stage (14 hpf), and vascular-restricted expression was observed throughout embryogenesis (Figure IJK in the online-only Data Supplement and data not shown). A gene-trap fli1b\textsuperscript{pre\textsuperscript{50G}} mutant line was generated in a Tol2-mediated insertional mutagenesis screen using GBT-B4 gene trap vector (C. Seiler et al, unpublished data, 2015) similar to the recently published GBT-B1.42–44 The gene trap transposon (Figure IIE in the online-only Data Supplement) integrated into the first intron and near the start of the coding sequence resulting in a Gal4-VP16 fusion protein lacking the ETS DNA-binding domain (Figure IIF through III in the online-only Data Supplement); therefore, the mutation is expected to be null or severe hypomorph.45 Consistent with this view, the gene-trap disruption resulted in an 18.5-fold (5.4±2.0%) reduction in full-length fli1b message as determined by reverse transcription polymerase chain reaction and whole-mount in situ hybridization (Figure IIJ, IIN, and IIP in the online-only Data Supplement). RNA-Seq also confirmed significant reduction of terminal exon 9 reads of fli1b (2.4±2.6%, n=2 samples of 20 embryos, age 24 hpf, read at a depth of 20 million reads per sample). Gene trap embryos exhibited high levels of vascular endothelial GFP reporter expression beginning at the 12-somite stage (15 hpf), vascular restricted at 48 hpf (Figure IIO in the online-only Data Supplement), and maintained into adulthood (data not shown). In stark contrast to the previous study using morpholinos,29 fli1a\textsuperscript{Tol2\textsuperscript{10G}} or fli1b\textsuperscript{pre\textsuperscript{50G}} mutant embryos had no apparent defects in vascular development (Figure IJK and IIO in the online-only Data Supplement) or vascular marker expression (Figure III in the online-only Data Supplement).
Supplement). Homozygous flia<sup>−/−</sup> and flib<sup>−/−</sup> mutants (referred to as flia<sup>−/−</sup> and flib<sup>−/−</sup>) developed normally into adulthood with no overt effect on growth or fecundity.

To assess the potential interaction between ev2 and the flia and flib ETS factors, we used ev2 morpholino knockdown in the corresponding mutant lines. Flia mutant embryos were injected with ev2 MO at the 1-cell stage (0.2 hpf) and imaged at stages from 24 to 72 hpf. Double flia<sup>−/−</sup>:ev2 MO embryos were indistinguishable from ev2 morphant embryos (Figure IV in the online-only Data Supplement), thus suggesting that the loss of Flia did not significantly enhance on the ev2 morphant phenotype. In contrast, the vascular phenotype associated with ev2 morpholino knockdown alone (Figure 3A–3D) was clearly exacerbated in flib<sup>−/−</sup> embryos (Figure 3E–3H) and further impaired in flib<sup>−/−</sup> embryos (Figure 3I–3L). Flib<sup>−/−</sup> and flib<sup>−/−</sup> embryos with ev2 morpholino knockdown were easily distinguishable from ev2 morphant embryos by 24 hpf because of a reduction in the number of GFP-positive VECs in these embryos when compared with ev2 morphant embryos (Figure 3E and 3I). Although endothelial cells in ev2 morphants coalesced to form the axial vasculature by 24 hpf, angioblasts in ev2<sup>−/−</sup>flib<sup>−/−</sup>-deficient embryos remained dispersed. At 36 hpf, both ev2<sup>−/−</sup>MO:flib<sup>−/−</sup> and ev2<sup>−/−</sup>MO:flib<sup>−/−</sup> embryos completely lacked intersegmental sprouts and showed a reduced number of GFP-positive cells relative to ev2 morphants. At 48 hpf, a few intersegmental sprouts were observed in the anterior trunk of ev2<sup>−/−</sup>MO:flib<sup>−/−</sup> (Figure 3G) but not in ev2<sup>−/−</sup>MO:flib<sup>−/−</sup> embryos (Figure 3K), which we interpret as a dose–effect resulting from the loss of functional flib protein. By 72 hpf, ev2 morphants exhibited a significant recovery in both the axial and ISVs (Figure 3D), whereas embryos also heterozygous for the flib gene trap displayed only a moderate recovery of the axial vessels and sporadic ISVs in the anterior trunk region (Figure 3). Finally, ev2<sup>−/−</sup>MO:flib<sup>−/−</sup> embryos showed a persistent gap in the axial vessels (Figure 3J–3L, arrow), indicating further impaired vasculogenesis. These embryos did not undergo angiogenic sprouting (Figure 3L), and their vasculature remained largely unchanged until they died at 5 dpf (data not shown). Because of the close proximity of ev2 and flib genes within the zebrafish genome, it was not possible to obtain the double ev2:flib<sup>−/−</sup> mutants using a simple cross because of the expected low frequency of recombination between the 2 genes. Nevertheless, ev2 MO has been validated in multiple previous studies and results in the same vascular phenotype as ev2<sup>−/−</sup> null mutants (Figures 1 and 3). We also tested whether double heterozygous ev2<sup>−/+</sup>:flib<sup>−/+</sup> mutants showed any vascular defects. Ev2<sup>−/+</sup>:flib<sup>−/+</sup> double heterozygous embryos were indistinguishable from WT embryos and did not display any defects in vascular patterning (Figure VA in the online-only Data Supplement).

We and others have previously reported a strong loss of multiple vascular endothelial marker expression observed in ev2<sup>−/−</sup> and mutant embryos at 24 hpf<sup>22,29</sup> By 36 hpf, a partial recovery of the endothelial marker expression was observed in ev2<sup>−/−</sup> and morphant embryos (Figure 4), consistent with the partial recovery of ISV sprouting observed in ev2 morphants. However, no significant vascular marker recovery was observed in embryos deficient in both ev2 and flib, and expression of the cdh5, flia, fltb, and kdrl vascular markers was largely undetectable through 36 hpf. Interestingly, a bilateral population of ev2<sup>−/−</sup> cells located in the trunk was retained in double-knockdown embryos, potentially representing a population of nondifferentiated ev2<sup>−/−</sup> vascular endothelial progenitors. Taken together, these data argue that Flib is capable of supporting vascular development in the absence of ev2 and that it participates in the angiogenic recovery of ev2-deficient embryos.

We also tested whether double flia<sup>−/−</sup>:flib<sup>−/−</sup> mutants showed any vascular defects. However, vascular patterning in...
fli1a−/−;fli1b−/− mutant embryos was normal (Figure VB in the online-only Data Supplement). fli1a−/−;fli1b−/− embryos exhibited impaired circulation at 32 hpf (not shown) and pericardial edema at 48 hpf, and the vessels appeared to fail to lumenize. Although the primary cause of this phenotype needs further investigation, these results show that the initial vasculogenesis and angiogenesis proceed normally in the absence of fli1a and fli1b functions.

**Etv2 and fli1b Function in a Redundant Manner to Support Angiogenesis**

The complete lack of angiogenic recovery observed in etv2 MO;fli1b−/− mutant embryos could be attributed to a direct requirement for etv2 and fli1b in angiogenesis or an indirect consequence of the failed vasculogenesis observed in etv2 MO;fli1b−/− mutant embryos. To determine whether etv2 and fli1b contribute combinatorially to sprouting angiogenesis, we used a previously validated photoactivatable (ie, caged) etv2 morpholino to block endogenous etv2 function at selected stages. Early photoactivation at 50% epiboly (5.3 hpf) confirmed effective uncaging and morpholino function as evidenced by a complete absence of angiogenesis at 24 hpf (data not shown).

Fli1a:GFP expression at 42 hpf was robust in control embryos (Figure 5A), whereas embryos which were injected with the caged MO and uncaged at the 50% epiboly stage exhibited a partial recovery of the ISVs (Figure 5B), consistent with standard morpholino results (Figure 3B). Etv2-caged MO–injected embryos uncaged at the 18-somite stage (18 hpf) appeared grossly normal (Figure 5C) and were indistinguishable from control embryos that were injected with caged etv2 MO and never uncaged (Figure 5D). GFP fluorescence in 42

![Figure 5](image_url). Etv2 and fli1b are required for angiogenesis independently from Etv2 early requirement in vasculogenesis. A–D, Caged etv2 morpholino (MO) analyses in 42-hpf wild-type (WT) embryos in a Tg(fli1a:GFP) background (lateral views, anterior to the left). A, Vascular patterning was normal and all intersegmental vessels (ISVs) were complete in control (uninjected) WT embryos that underwent the 30-minute UV exposure. B, All ISVs were incomplete in embryos injected with the caged etv2 MO and uncaged at 50% epiboly (5.3 hpf). Embryos uncaged at this stage showed truncated ISVs, typical for similarly staged morphants (compare with Figure 3B), thus confirming efficient morpholino uncaging. WT embryos uncaged at the 18-somite stage (C) were indistinguishable from the never uncaged embryos (D) indicating etv2 knockdown alone at 18-somites (18 hpf) did not block angiogenic sprouting. E–H, Caged etv2 MO analyses in 42-hpf fli1b−/− embryos visualized using the green fluorescent protein (GFP) expressed in the gene trap line. E, Fli1b mutants had a normally appearing vasculature. Uncaging at 50% epiboly in the fli1b mutant background again resulted in a more significant impairment of both vasculogenesis and angiogenesis (F) than observed in WT background (B). G, Uncaging of the etv2 MO at 18-somites in a fli1b mutant background resulted in truncated ISV sprouts (with no full-length sprouts observed). D and H, Embryos which were injected with caged MO and never uncaged had normally patterned ISVs (ie, <2 mispatterned ISVs), thus confirming effective caging. Fractions indicate the number of embryos with the >15 full ISVs (numerator) and the total evaluated (denominator).
Embryos Deficient in Both Etv2 and Fli1b Have an Expanded Zone of Apoptosis During Vasculogenesis

Because embryos deficient in both Etv2 and Fli1b exhibited a dramatic reduction in the number of VECs, particularly in the axial vasculature, we sought to determine whether this deficiency was because of increased apoptosis. Minimal caspase 3 staining was observed in WT embryos (Figure 6A), whereas etv2 morphant embryos showed a marked increase in apoptosis all along the axial vasculature (Figure 6B), similar to previous reports. Apoptotic staining in fli1b−/− embryos was significantly different from WT embryos (Figure 6C). Staining was significantly expanded in etv2 MO/fli1b−/− embryos into the trunk axial vasculature (Figure 6D), suggesting that the observed reduction in VEC number was because of apoptotic cell death, either as a direct result of combined Etv2 and Fli1b loss or as an indirect consequence of impaired VEC differentiation. Cyclopamine induction of apoptosis was used to confirm caspase 3 staining, and a no antibody control was used to confirm a lack of nonspecific staining (data not shown). However, little overlap between endothelial GFP and caspase staining was detected. To rule out the possibility that the caspase-positive cells were primitive erythrocytes that originate in the adjacent LPM, we performed heme staining (data not shown) and whole-mount in situ analysis for hematopoietic markers hbbe3 and gata1 that were not changed in etv2 MO/fli1b−/− embryos (Figure 6A′−D′ and A−D′), thus indicating that primitive erythropoiesis is normal in etv2 MO/fli1b−/− embryos. We repeated caspase 3 staining at earlier 10-somite (14 hpf) and 20-somite (19 hpf) stages. In both cases, caspase 3 staining was observed in the LPM, but no costaining with fli1a:GFP was observed (data not shown). We thus conclude that the vascular endothelial progenitors undergo apoptosis before they can initiate fli1a:GFP expression.

Etv2 and fli1b Can Independently Induce Vascular Endothelial Marker Expression

Previous studies have shown that etv2 overexpression is sufficient for the specification of endothelial and myeloid but not the erythroid lineages. To determine whether these functional roles also hold true for fli1a and fli1b, sense mRNA was injected into WT embryos. Overexpression of fli1a had no effect on the expression of vascular endothelial markers kdrl, fli1b, or etv2, hemangioblast marker scl, or erythroid marker gata1 expression (Figure VI in the online-only Data Supplement). In contrast, overexpression of fli1b mRNA induced ectopic expression of the vascular endothelial kdrl:GFP reporter and fli1a marker (Figure 7B and 7D). Also consistent with the published function of etv2, fli1b overexpression induced the early hemangioblast marker scl (Figure 7F) but had no effect on the erythroid marker gata1 (Figure 7H), thus arguing that fli1b overexpression is sufficient to induce vascular endothelial differentiation, similar to Etv2. To determine the relationship between etv2 and fli1b, a series of epistatic interaction analyses were performed. Overexpression of etv2 caused ectopic expression of fli1b (Figure 7I), and conversely, overexpression of fli1b was sufficient to induce expression of etv2 (Figure 7L), suggesting that a positive feedback relationship potentially exists between the 2 transcription factors.

To determine whether fli1b was capable of inducing vascular marker expression in the absence of functional etv2, fli1b sense mRNA was injected into embryos from an etv2−/− Tg(fli1a:GFP) carrier in-cross. Robust expression of the downstream vascular marker fli1a was observed in the endothelial progenitor cell pools of WT embryos (Figure 7M) but was

![Figure 6. Apoptosis of vascular endothelial cells is expanded in double Etv2/Fli1b-deficient embryos. A–D. Whole-mount immunohistochemical staining of 48-hpf embryos for caspase 3 and green fluorescent protein (GFP) expression. Lateral views shown with anterior to the left. No distinct zones of apoptosis were noted in wild-type (A) and fli1b−/− embryos (C). By contrast, etv2 morphant embryos showed a narrow zone of apoptosis in the vascular plexus (B). D. This zone of apoptosis was expanded in embryos deficient in both fli1b and etv2 (arrow), Hbbe3 (A−D) and gata1 (A−D) levels determined by whole-mount in situ hybridization show normal erythrocyte staining in all groups indicating the observed apoptosis was not because of a loss of blood cells. Fractions indicate the number of embryos with the observed pattern of marker expression (numerator) and the total evaluated (denominator).](http://atvb.ahajournals.org/)

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restricted to nonendothelial group of cells in the presumptive pharyngeal endoderm region in 12-somite (15 hpf) etv2 homozygous mutant embryos (Figure 7N). Embryos obtained from the etv2−/− carrier in-cross and injected with fli1b mRNA showed strong ectopic expression of fli1a:GFP (Figure 7O), thereby arguing that fli1b is capable of inducing vascular fli1a in the absence of etv2. P–R, Fli1b mRNA induces ectopic kdrl expression in cloche mutant embryos. An in-cross of cloche heterozygous adults yielded the expected Mendelian ratio of 10-somite stage (14 hpf) WT (cloche−/−) and hematopoietic lineages, 45 and 46%, respectively. The number of embryos overexpression (numerator) and the total number evaluated (denominator) lower left panel, the age and injected mRNA in the bottom left, and gene probed by in situ in the upper right. A–H, Effect of fli1b overexpression on vascular and hematopoietic markers during the indicated stages. Fli1b overexpression is sufficient to induce expression of vascular endothelial markers in wild-type (WT), etv2−/− and cloche mutant embryos. A, C, E, G, I, and K, Uninjected controls. Whole-mount in situ analysis of embryos is shown in lateral view with anterior of embryo oriented left. A–H, Effect of fli1b overexpression on vascular and hematopoietic markers during the indicated stages. Fli1b induces kdrl:GFP (B), fli1a (D), and scl (F), but not gata1 (H). I–L, Lateral views of WT embryos injected with fli1b and etv2 mRNA. J, Fli1b overexpression induces ectopic etv2 in WT embryos. L, Conversely, fli1b overexpression induces ectopic etv2. The number of embryos with ectopic expression (numerator) and the total number evaluated (denominator) lower left panel, the age and injected mRNA in the bottom left, and gene probed by in situ in the upper right. M–O, Effect of fli1b overexpression in etv2−/− mutant embryos. An in-cross of etv2−/− Tg(fli1a:GFP) adults yielded the expected Mendelian ratio of phenotypically WT (M) and etv2−/− embryos (N) as determined by fli1a:GFP expression. O, 99% embryos injected with fli1b mRNA exhibited ectopic expression of fli1a, indicating that fli1b is capable of inducing vascular fli1a in the absence of etv2. P–R, Fli1b mRNA induces ectopic kdrl expression in cloche mutant embryos. An in-cross of cloche heterozygous adults yielded the expected Mendelian ratio of 10-somite stage (14 hpf) WT (P) and mutant (Q) embryos, based on the presence or absence of kdrl expression. Injection of fli1b mRNA induced ectopic expression of kdrl (R) indicating that Fli1b acts downstream of cloche.

Figure 7. Fli1b overexpression is sufficient to induce expression of vascular endothelial markers in wild-type (WT), etv2−/− and cloche mutant embryos. A, C, E, G, I and K, Uninjected controls. Whole-mount in situ analysis of embryos is shown in lateral view with anterior of embryo oriented left. A–H, Effect of fli1b overexpression on vascular and hematopoietic markers during the indicated stages. Fli1b induces kdrl:GFP (B), fli1a (D), and scl (F), but not gata1 (H). I–L, Lateral views of WT embryos injected with fli1b and etv2 mRNA. J, Fli1b overexpression induces ectopic etv2 in WT embryos. L, Conversely, fli1b overexpression induces ectopic etv2. The number of embryos with ectopic expression (numerator) and the total number evaluated (denominator) lower left panel, the age and injected mRNA in the bottom left, and gene probed by in situ in the upper right. M–O, Effect of fli1b overexpression in etv2−/− mutant embryos. An in-cross of etv2−/− Tg(fli1a:GFP) adults yielded the expected Mendelian ratio of phenotypically WT (M) and etv2−/− embryos (N) as determined by fli1a:GFP expression. O, 99% embryos injected with fli1b mRNA exhibited ectopic expression of fli1a, indicating that fli1b is capable of inducing vascular fli1a in the absence of etv2. P–R, Fli1b mRNA induces ectopic kdrl expression in cloche mutant embryos. An in-cross of cloche heterozygous adults yielded the expected Mendelian ratio of 10-somite stage (14 hpf) WT (P) and mutant (Q) embryos, based on the presence or absence of kdrl expression. Injection of fli1b mRNA induced ectopic expression of kdrl (R) indicating that Fli1b acts downstream of cloche.

Etv2 and Fli1b Have a Combinatorial Effect on Multiple Vascular Endothelial Markers

RNA-Seq analysis was performed on 24- and 48-hpf embryos to profile gene expression changes in etv2 morphant, fli1b−/−, and etv2 MO;fli1b−/− embryos. As expected, the expression of multiple vascular endothelial markers was significantly reduced in etv2 morphant embryos at 24 hpf and partially recovered at 48 hpf, consistent with the partial angiogenic recovery observed in morphant embryos (Table). Vascular markers were largely normal in fli1b mutant embryos, as expected, because these embryos develop a normally appearing vasculature. Vascular markers were further reduced in double-deficient embryos (Table), consistent with the severe vascular deficit in these embryos. Interestingly, most of the endothelial markers partially recovered by 48 hpf in the double-deficient embryos (as observed in the etv2 morphant embryos), despite the lack of an angiogenic recovery in these embryos. Of note, etv2 was significantly increased at 24 hpf in etv2 morphant embryos and double-deficient embryos (confirming in situ analysis shown in Figure 4). Etv2 levels were further increased in morphants during the angiogenic recovery occurring at 48 hpf, but no further increase was observed in double-deficient embryos, consistent with the failed angiogenic recovery observed in these embryos.

Further analysis of our RNA-Seq data (Table) provided additional insight into the role played by fli1b relative to etv2 and fli1a. Fli1a message was reduced 1.3-fold in etv2 morphants and further reduced (down 2.45-fold) in etv2 MO;fli1b−/− embryos at 24 hpf, suggesting that Etv2 and Fli1b are involved in regulating fli1a expression.

Fli1b Directly Induces Fli1a Expression

Etv2 binds together with FoxC homologs to the FOX-ETS motif present within the promoters of multiple vascular
endothelial–specific genes. Evolutionarily conserved ETS sites have been identified within the proximal mouse Fli1 and zebrafish Fli1a promoters with direct binding of Etv2 to these sites shown. Putative ETS-binding sites corresponding to the functional murine ETS sites were identified in the zebrafish fli1a promoter (Figure VII in the online-only Data Supplement). In addition, a conserved FOX-ETS motif with several adjacent ETS motifs was identified within the first intron of fli1a gene (Figure VII in the online-only Data Supplement). All of these identified sites are present within the fli1a[ep] enhancer–promoter region, which has been previously demonstrated to be necessary and sufficient for vascular endothelial reporter expression. To determine whether Fli1b directly binds to the DNA fragments containing these consensus binding sites, we performed chromatin immunoprecipitation analysis on zebrafish embryos injected with FLAG-fli1b RNA. Overexpression of FLAG-tagged fli1b, similar to the overexpression of untagged fli1b, resulted in specific induction of vascular endothelial markers, including fli1a:GFP. Embryos were fixed at the tailbud stage and subjected to chromatin immunoprecipitation using FLAG antibody. Specific enrichment for 2 fragments, containing the previously described ETS sites within the proximal fli1a promoter (2.27±0.80 fold enrichment) and a consensus FOX-ETS site within the first intron of fli1a (4.62±1.64 fold enrichment), was observed. Multiple other genomic regions tested showed no enrichment in the pull down fraction (data not shown), which argues for the specificity of the observed binding.

Functional activity of the predicted ETS-binding sites was verified by cloning the fli1a[ep] enhancer–promoter region upstream of an enhanced GFP reporter and evaluating the ability of fli1b mRNA to induce reporter expression (Figure VIIIA in the online-only Data Supplement). All predicted ETS-binding sites within the fli1a[ep] promoter–enhancer were mutated to test their functional requirement (Figure IX in the online-only Data Supplement). Control injections of the reporter fli1a[ep]:GFP construct with etv2 mRNA yielded ectopic GFP expression at the 8-somite stage (13 hrp) in 44% of embryos (n=192). Mutation of the ETS-binding sites in the fli1a[ep]:GFP reporter construct blocked its induction by etv2, thus indicating that etv2 induced expression in an ETS-binding site-dependent manner. Coinjection of the reporter construct with fli1b mRNA induced GFP reporter expression in 61% of embryos (n=184). Because we had already demonstrated that Fli1b injection induces etv2 expression, we used etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly. When the fli1a[ep]:GFP construct was coinjected with etv2 MO to inhibit Etv2 function and to test fli1b activity directly.

### Discussion

In this study, we describe a novel critical function for 2 related ETS transcription factors Etv2 and Fli1b in initiating angiogenesis and completing vasculogenesis during early embryonic development. The data presented herein are consistent with a model of embryonic vascularization in which etv2 initiates the vascular endothelial program (including expression of fli1a, fli1b, and related markers) during early vasculogenesis, and later etv2 and fli1b are redundantly required during late vasculogenesis and early sprouting angiogenesis (Figure 8). During early stages of vascular development (1–15 somite stages), zebrafish etv2 single mutants display essentially

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**Table. Fold Changes in Vascular Gene Expression in etv2 Morphants, fli1b−/− Mutants, and Double etv2 MO;fli1b−/− Mutant Embryos When Compared With Uninjected Wild-Type Control Embryos Measured by RNA-Seq**

<table>
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<tr>
<th>Gene Symbol</th>
<th>24hpf etv2 MO</th>
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<th>48hpf etv2 MO</th>
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<td>−3.9†</td>
<td>−9.7*†</td>
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All genes listed were altered >1.5-fold in 24-hpf morphants relative to 24-hpf wild-type controls (P<0.1; with the exclusion of fli1a which has been included for reference purposes). Note that etv2 expression was significantly increased in morphant embryos likely because of compensatory feedback in the absence of functional protein. Most of the vascular genes (18/20) showed less reduction in 48-hpf etv2 morphants than in 24-hpf etv2 morphants. Vascular genes were largely unaffected in fli1b mutants consistent with the lack of a vascular phenotype in mutant embryos. Vascular marker expression in double-deficient embryos was reduced beyond that of the age-matched embryos in 15/20 (75%) instances in 24-hpf embryos and in 17/20 instances (85%) in 48-hpf embryos, with the exacerbated phenotype in double-deficient embryos.

*Significant expression changes (P<0.1).
†Fold changes which are enhanced in age-matched double-deficient embryos.
complete lack of VECs and have little if any vascular endothelial marker expression; therefore, Etv2 seems to be the single major master regulator of early vasculogenesis. The lack of a vascular phenotype in \textit{fli1a} and \textit{fli1b} double-mutant embryos suggests that both \textit{Fli1a} and \textit{Fli1b} are largely dispensable for early vasculogenesis in WT embryos. However, \textit{etv2} mutants do form vascular endothelial progenitors at later stages and initiate angiogenesis after 36 hpf. Only double-deficient \textit{etv2;fli1b} embryos show persistent loss of vascular endothelial progenitors within the axial vasculature and complete absence of angiogenesis during 24 to 72 hpf. This argues for the functional redundancy between Etv2 and Fli1b during these stages of late vasculogenesis and early angiogenesis (Figure 8).

In support of this model, the reduced endothelial cell numbers and gaps in the axial vasculature at later stages are only observed in double \textit{etv2;fli1b} knockdown embryos, which argues that Etv2 and Fli1b function redundantly and that Fli1b is responsible for mediating the angiogenic recovery observed in \textit{etv2} mutants and morphants. The compensatory supporting function of \textit{fli1b} in \textit{etv2}-deficient embryos is further supported by the induction of multiple vascular markers by Fli1b overexpression, including direct induction of \textit{fli1a} by binding to ETS sites in \textit{fli1a} promoter–enhancer region.

Whole-mount in situ hybridization and reverse transcription polymerase chain reaction data presented here and elsewhere indicate that \textit{fli1a} is expressed at low levels beginning at the 8-somite stage (13 hpf) and then increases to a sustained plateau of high-level expression at around the 20-somite stage (19 hpf) when \textit{etv2} expression is waning.29 Because \textit{fli1b} expression exceeds that of \textit{etv2} during the angiogenic recovery of both \textit{etv2} morphants and mutants (ie, after 24 hpf) and because Fli1b is capable of inducing the same vascular target genes as Etv2, it is likely that the angiogenic recovery observed in \textit{etv2}-deficient embryos occurs through the compensatory action of \textit{fli1b}. In this scenario, loss of \textit{Fli1b} in \textit{etv2} mutants would result in a failure of angiogenic sprouting altogether, as we observed. Our data do not exclude potential contribution from other vascular ETS factors (eg, \textit{Erg} or \textit{Ets-1}), as suggested previously,29 but the normal profile of vascular markers in \textit{fli1a} mutants combined with the lack of vascular marker induction in \textit{fli1a} injected embryos suggests that \textit{fli1a} is not mediating the observed angiogenic recovery and thus serves a functional role, which is distinct from \textit{fli1b}. It is possible that such a functional redundancy has evolved as a means of protecting the embryo from vascular development defects and that additional ETS factors have the capacity to support vascularization in the absence of the normally dominant \textit{etv2}.

It has been previously reported that \textit{fli1a}−/− and \textit{fli1b}−/− single-MO knockdown embryos exhibit defects in ISV sprouting.29 Despite the profound loss of target gene expression in the \textit{fli1a} and \textit{fli1b} gene trap lines, we did not observe any apparent vascular patterning defects in either mutant line. Therefore, it is likely that some of the previously reported defects were caused by MO off-target effects. However, we cannot rule out the possibility that small amounts of WT transcript that remained in \textit{fli1a}−/− and \textit{fli1b}−/− lines were sufficient to allow normal development. An increased incidence of hemorrhages attributed to a loss of vessel integrity has been previously reported in \textit{fli1a} MO knockdown zebrafish embryos,37 and in \textit{Fli1} mutant mice,38 but hemorrhages were not observed in \textit{fli1a}−/− or \textit{fli1b}−/− embryos.

Our observation that the failed angiogenic recovery in embryos deficient in \textit{Fli1b} and \textit{Etv2} correlated with an elevated level of apoptosis and a reduction in the number of VECs (relative to \textit{Etv2}-deficiency alone) is consistent with other studies which have implicated ETS factors as modulators of apoptosis. Overexpression of human \textit{Fli1} and \textit{Erg} inhibit apoptosis in vitro, thus providing critical insight into the progression of Ewing sarcoma.49 Further, exogenous expression of both \textit{Ets}-2 and \textit{PU.1} increase \textit{Bcl-x} activation to inhibit apoptosis in macrophages.50 While it is possible that Etv2 and Fli1b directly serve to inhibit apoptosis in vascular progenitor cells, our data cannot rule out an indirect initiation of apoptosis as a mechanism to remove undifferentiated vascular progenitor cells which fail to differentiate in the double \textit{Etv2;Fli1b} knockdown embryos.

Previous studies have demonstrated that Etv2 directly binds to multiple endothelial enhancers and acts as a potent transcriptional regulator.28 We and others have also demonstrated that Etv2 overexpression results in strong upregulation of multiple vascular endothelial–specific genes.27–29 In this study, we report that Fli1b overexpression upregulates a subset of vascular endothelial and early hematopoietic markers in the same manner as Etv2. Furthermore, Fli1b is sufficient to induce vascular endothelial marker expression in Etv2 null and \textit{cloche} mutant embryos. These results suggest that Fli1b and Etv2 bind to a similar set of transcriptional targets. RNA-Seq analysis seems to support this contention because double-knockdown embryos show a marked reduction in vascular markers compared with \textit{etv2} MO alone. Our analysis also indicates that \textit{fli1b} directly induces \textit{fli1a} expression in an ETS-binding site–dependent manner by binding to known Etv2-target ETS sites within the \textit{fli1a} promoter and first intron. Further testing is required to identify additional direct transcriptional targets of Fli1b and to determine the extent of transcriptional target overlap with Etv2 in both vasculogenesis and angiogenesis.

Previous reports have shown that \textit{etv2} is significantly posttranscriptionally repressed by the \textit{let-7} family of micro-RNAs during the formation of the peripheral vasculature and that protein levels are drastically reduced shortly thereafter, as determined by immunostaining in WT embryos.46 By contrast, Fli1a and Fli1b are not predicted targets of the \textit{let-7} family,
which could explain the persistent expression of both in later development. Despite the reduced expression of etv2 during angiogenesis, our data suggest that etv2 functions beyond its generally recognized role in vasculogenesis and acts in combination with fli1b in these later stages of angiogenesis (ie, 24–72 hpf). This later role is supported by caged morpholino knockdown of etv2 in fli1b mutant background, which caused impaired angiogenic sprouting in embryos with intact vasculogenesis (Figure 5). It is important to point out that limited ISV sprouting was observed in fli1b mutant embryos injected with caged etv2 MO and photoactivated at the 18-somite (18 hpf) stage, whereas earlier inhibition of Etv2 function in fli1b–/– mutant background resulted in the complete absence of ISVs. The partial sprouting observed in caged etv2 MO/fli1b–/– embryos after photoactivation at the 18-somite stage could be explained by the contribution of other vascular ETS factors that may also serve a compensatory role and support angiogenesis in the absence of etv2 function.

Data published previously and several lines of data presented herein suggest that Etv2 is the primary driver of early expression of the downstream target fli1a, including (1) reduced fli1a-GFP transgenic expression in etv2 morphants and mutants, (2) reduced fli1a expression in etv2-deficient embryos by in situ analyses, (3) failure of fli1a mutant embryos to recapitulate (or even enhance) the etv2 mutant phenotype, and (4) direct evidence of Evt2 binding to fli1a promoter–enhancer in fluorescent reporter assays. However, the observation that fli1a-GFP transgene expression and fli1a message partially recovered by 48 hpf suggests that etv2 is not solely required for inducing fli1a in vivo. Rather, because fli1a is significantly reduced only in etv2 MO/fli1b–/– embryos and ectopic Fli1b induced expression of the fli1a[ep]::GFP reporter construct, it is possible that Etv2 and Fli1b act cooperatively to induce fli1a expression or that both bind to ETS sites within the fli1a[ep] sequence and that the contribution of each ETS factor is based on the temporal concentrations of each. Although beyond the scope of this study, it would be interesting, therefore, to see whether etv2 MO knockdown further exacerbates the fli1a–/–/fli1b–/– mutant phenotype. Also, it is possible that additional transcription factors or cofactors not part of this study are required for sustained expression of fli1a. Interestingly, a recent study demonstrated that in mouse embryos, Etv2 initiates Fli1 expression, which is further maintained by Fli1 autoregulation. Similarly, our results show that zebrafish Etv2 initiates fli1a and fli1b expression, and then Fli1b is further involved in maintaining fli1a and possibly its own expression. Our results do not exclude the possibility that Fli1a also participates in maintaining its own expression. Thus, it seems that transcriptional regulation of Etv2 and Fli1 expression is evolutionarily conserved.

Taken together, our data indicate that Etv2 and Fli1b have redundant roles during late vasculogenesis and early embryonic angiogenesis. It is also intriguing to consider the possibility that they are involved in the remarkably similar processes of adult neoangiogenesis or in pathological hypervascularization. Our findings demonstrate a novel role for ETS transcription factors in angiogenesis and eventually may lead to the design of improved therapeutics to enhance wound healing or block tumor-induced vascularization.

Acknowledgments

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Disclosures

None.

References
differentiation. We expect that our results will be highly significant in elucidating molecular mechanisms of vascular development.

etv2

vasculogenesis. We show that Fli1b, similar to Etv2, directly initiates transcription of multiple endothelial–specific genes and that Etv2 and Fli1b first time that knockdown of 2 E26 transformation-specific factors results in a complete absence of angiogenesis and nearly complete absence of

onic vasculogenesis. Here, we demonstrate that Etv2 has a previously unrecognized role in angiogenesis and late embryonic vasculogenesis.

The E26 transformation-specific transcription factor Etv2/Etsrp has been previously shown to function as a master regulator during early embryonic hematopoiesis.

The role of ets factors in tumor angiogenesis.

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the developing vasculature.

Weinstein BM. Combinatorial function of ETS transcription factors in


Ferdous A. Fli1 acts downstream of Etv2 to govern cell survival and vascular

homeostasis via positive autoregulation.


Etv2 and Fli1b Function Together as Key Regulators of Vasculogenesis and Angiogenesis
Michael P. Craig, Viktorija Grajevskaja, Hsin-Kai Liao, Jorune Balcuniene, Stephen C. Ekker, Joo-Seop Park, Jeffrey J. Essner, Darius Balcuniunas and Saulius Sumanas

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MATERIALS AND METHODS

Fish Lines and Embryos

The following transgenic and mutant lines were used for experiments: Tg(fli1a:EGFP)y1,1 fli1ais10Gt, fli1btpl50Gt, etsrpy11,2 Tg(etv2:Kaeade)c6 3 and AB wild type (Zebrafish International Resource Center). All subsequent references to these lines use the updated gene designations [i.e. etsrpy11 as etv2-/]. Embryos were incubated at 28.5°C and staged as described previously4 and treated with 1-phenyl-2-thiourea (PTU) to inhibit pigment formation for analyses performed beyond 24 hours post fertilization (hpf). Analyses of earlier developmental stages was facilitated by incubating embryos to 23.5°C beginning at 50% epiboly through early somitogenesis. Etv2-/- embryos were identified during somitogenesis by reduced fli1a:GFP fluorescence or at 24 hpf by the absence of intersomitic vessels and defective development of the axial vessels as previously reported2. Mutant identity was again confirmed prior to experimental sampling between 24hpf and 5dpf by lack of peripheral circulation and presence of pericardial edema.

Generation of the Fli1a-is10Gt Gene Trap Line.

The EGFP-containing Tol2<SA-EGFP-p> gene-breaking transposon is derived from GBT-R14 (pDB662) and GBT-R15 (pDB713) vectors 5 by replacing mRFP with eGFP. EGFP was amplified using EGFP-F (5'-CCCGGGTACCGGAAGGTGTGAGCAAGGGCGAGGAGCT-3') and EGFP-R (5'-ATCGATTTCCTACTTGACCTCGTCCA-3') and inserted as an XmaI - ClaI fragment. After Tol2-mediated transgenesis (performed as described below for fli1btpl50Gt), 5'-RACE analysis (Invitrogen) using two primers complementary to GFP (5'-GCCTTCGGGCAATGGGCTTG-3' and 5'-GGCCAGGGCACGGGAGCTG-3') confirmed EGFP Tol2 transposon integration between Exon 3 and Exon 4 of the fli1a gene. Genotyping fli1ais10Gt carriers and mutants was performed using EGFP primers paired with either fli1a E4R (5'-CTGGCTACATGGATGAGAAAT-3') or fli1a E3F (5'-GTGTCGATCTCCTGAAGACC-3') primers using an annealing temperature of 56 oC for 35 cycles.

Fli1b Gene Trap Mutagenesis and Identification of the Trapped Gene in tpl50GT Line

Gene trap mutagenesis was carried out as previously described.6,7 Briefly, GBT-B4 gene trap vector (Sieler et al., submitted) and in vitro transcribed Tol2 transposase mRNA8 were injected into 1-cell zebrafish embryos. About 30% of embryos with brightest GFP expression at 3 days post fertilization were selected for raising, grown to adulthood and screened for germ-line transmission of gene trap events by crossing to Tg(14XUAS:mRFP)tpl2 reporter fish. Embryos containing gene trap events were identified by co-expression of GFP and RFP. The gene trap line tpl50Gt was identified by strong co-expression of RFP and GFP in the vascular system. The F1 generation was again outcrossed to fish, and embryos were collected for molecular identification of gene trap events by inverse PCR6. Genomic DNA was prepared from batches of 20 fluorescence-positive and 20 fluorescence-negative embryos, digested with Taqα1, diluted and self-ligated. Nested inverse PCR was performed using primers Tol2-R3 (5'-ACTGGGCACTACGCAATTCAATTG-3') and B1/3'No1 (5'-CAGGGTAAATACTCCTGATAGCA-3') for the first reaction, and primers Tol2-R4 (5'-ATAATCTAAGAAGACGTTACGGATAG-3') and B1/3'No2 (5'-GCATACTTACGGAAGCAATTCG-3') for the second reaction. After agarose gel electrophoresis, bands present in GFP-positive DNA amplifications but absent in GFP-negative amplifications were sequenced using Tol2-R4 primer. Sequencing results were used in a BLAST search of UCSD genome browser, thus identifying integration into the first intron of Fli1b as the candidate gene trap locus. To confirm that this integration was linked to GFP expression, we performed three-primer PCR reactions with two genomic primers (fli1b-int1-F1 5'-CGTCAACTTCTGCTTCTTCG-3' and fli1b-int1-R1

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5'-TAACCACAGGCACTGTCACG-3') and a Tol2 primer (either Tol2-F10 5'-CCCTAAGTACTTGTACTTTCACTT-3' or Tol2-R4). Two PCR bands were observed in DNA prepared from GFP-positive embryos, but only one band was observed in genomic DNA prepared from GFP-negative embryos, demonstrating linkage of gene trap integration into the first intron of Fli1b to GFP expression. PCR bands obtained with fli1b-int1-F1/Tol2-F10 and with fli1b-int1-R1/Tol2R4 were sequenced, revealing 8 base pair duplication of the target site indicative of Tol2 transposition.

**Morpholino Microinjection**

Etv2 knockdown experiments were performed by injecting 5 ng each of translation-blocking etv2/etsrp MO1 (5'-TTGGTACATTTCCATATCTTAAAGT-3') and MO2 (5'-CAGTGGTCCTTATTCTACTATC-3') into 1- to 2-cell stage embryos as described previously9. Morpholino knockdown in Tg(fli1a:GFP) embryos was verified by fluorescence microscopy as a successful phenocopy of the etv2/− phenotype (as described above).

**qRT-PCR Analyses**

Pools of twenty dechorionated embryos (per treatment or age group) were snap frozen on dry ice, homogenized by passage ten times through a 22-gauge needle, and then used to transcribe total RNA using an RNA-Aqueous 4RT-PCR kit (Life Technologies Corporation). cDNA was prepared from 1 microgram of total RNA using oligo-dT primers and a Superscript III First-strand Synthesis Kit (Life Technologies Corporation). Serial dilutions of each cDNA sample were used for qRT-PCR reactions using Sybr-Green Mix (Life Technologies Corporation) using Opticon Monitor 3 (Life Technologies Corporation) and the following primers: ef1a (5'-TCACCCTGGGAGTGAAACAGC-3' forward, 5'-ACTTGCAGGCGATGTGAGCAG-3' reverse, 693 bp band), etv2 (5'-GAGCTGTTGCGACAAAGGTCA-3' forward, 5'- CAGAGAGGGACGAGGTTCTG-3' reverse, 179 bp band), fli1a (5'-GTGTCGATCTCCTGAAGACCG-3' forward, 5'-ATCAACCCTTGTGTGTGCTCAT-3' reverse, 1021bp), fli1b (5'-CCCAGTGGACTGTAGTGTGAC-3' forward, 5'-TCCGGAGGATTTGAGATGG-3' reverse, 363bp band). Serial dilutions of a 24 hpf sample were used as a standard curve for each primer set and the values were normalized to the 24 hpf sample.

**Gene Overexpression**

Etv2 mRNA was generated using an overexpression construct containing the open reading frame of etv2 inserted into the Spel site of pT3TS.9 The etv2-T3TS vector was linearized with XbaI and mRNA was transcribed using T3 mMessage mMachine Kit (Cat# AM1340, Ambion, Austin, Texas, United States). Fli1a mRNA was prepared by KpnI linearization of a fli1a-pCS2+ expression vector followed by SP6 transcription using a mMessage kit. Fli1b mRNA was similarly prepared by NotI linearization of fli1b-pCS2+ (graciously provided by Leonard Zon) and SP6 transcription. At the one- to two-cell stage, 35 pg of either mRNA was injected into zebrafish embryos. Embryos were scored at the 6-10 somite stage, imaged, and fixed in 4% paraformaldehyde overnight at 4°C for in situ hybridization. The initial overexpression studies were performed using a fli1b construct found to include K222R and H376Y sequence variants, both of which lie outside the functional ETS or Pointed domains and may represent either natural sequence polymorphisms or mutations that occurred during PCR and cloning. A second round of overexpression studies was performed using the wild-type fli1b mRNA which confirmed all data generated using the mutated mRNA (embryo scoring data not shown).

**Whole-mount In Situ Hybridization**

DIG-UTP labeled riboprobes were synthesized using T3, T7, or SP6 RNA polymerases (Ambion/Promega). In situ hybridization was performed as described10 using previously
published probes for etv2,11 fl1a,12 flk/kdrl,12 flt4,12 cdh5,11 gata113 and scl14. The DIG-labeled fl1a antisense riboprobe was generated from EcoRV-linearized fl1a-pCS2+ using T7 polymerase. The DIG-labeled fl1b riboprobe was generated from fl1b-pCS2+ by PCR amplification using 5'-ATTAAGGAAGCTCTATCGGTGGTG-3' (forward) and 5'-CTGCATTCTAGTTGTGGTTTGTC-3' (reverse) primers followed by antisense strand transcription with T7 polymerase. Processed zebrafish embryos younger than 22 somites were dehydrated in 100% ethanol and flat mounted in araldite for imaging. Later staged embryos were mounted in glass-bottom dishes in 0.6% low-melting agarose for imaging. Z-stacks of images were captured using an AxioImager compound microscope (Carl Zeiss Inc., USA) equipped with a Plan-Neofluar 10X/0.3 NA microscope objective (Carl Zeiss Inc., USA) and an AxioCam ICC3 color camera (Carl Zeiss Inc., USA). Extended focus images were produced in AxioVision 4.6 software (Carl Zeiss Inc., USA). Image contrast and color balance were adjusted using Adobe Photoshop CS3.

**Apoptosis Assay**

Apoptosis assay was performed largely as described previously.15 Embryos were raised to 24hpf or 48hpf, lightly fixed in 1% PFA for 3 hours at room temperature, dehydrated and stored at -20°C. Rehydrated embryos were washed in 0.2% saponin buffer, blocked in 10% normal goat serum, and detected with rabbit anti-human cleaved caspase 3 antibody (1:400, Becton Dickinson catalog #559565) and a goat anti-rabbit Alexa 594 (1:1200, Invitrogen catalog #A21245). Vascular-GFP was detected using rabbit anti-GFP-Alexa488 (1:400, Invitrogen catalog #A21311). Positive apoptosis controls were prepared for each sample by transferring a portion of the embryos into 100 µM cyclopamine at 50% epiboly stage.

**Photoconversion of Etv2:Kaede**

TgBAC(Etv2:Kaede) embryos were injected with 10 ng of etv2 MO1 at the 1-2 cell stage to induce an early vascular phenotype. Etv2 MO1 only was used for these experiments because MO2 blocks etv2:Kaede fluorescence due to sequence overlap in the 5'UTR region of etv2 mRNA. Photoconversions were performed at the 15-22 somite stage using a wide-field fluorescence Zeiss Axiovert microscope (Zeiss Microsystems, Inc.) and a Zeiss 10X/0.3 plan-apochromat objective lens. Photoconversions were performed by placing embryos in glass depression slides and exposing them to DAPI-filtered (405 nm peak) light for 10 seconds each. Dishes containing test and control embryos were wrapped in aluminum foil to prevent background subsequent photoconversion. All embryos (30-36 hpf) were mounted in 0.6% low melting agarose with 0.004% Tricaine and imaged using a Nikon A1R upright confocal equipped with a 25X/1.1 NA objective. Maximum intensity projection images were generated in Nikon IS Elements (Nikon Instruments Inc.) and processed in Adobe Photoshop (Adobe Inc.).

**Etv2 Caged Morpholino Injections and Photo-activation**

Caged etv2 morpholino solution was prepared as described previously.3 Briefly, 500 µM of caging strand (Supernova Life Sciences, custom product) designed against etv2 MO2 was mixed with 50 µM of etv2 MO2 and diluted in nuclease-free water and protected from ambient light. The mixture was denatured for 30 minutes at 70°C and allowed to anneal overnight at 4°C. A total of 2.5 nanoliters of the caged-MO solution was injected into zebrafish embryos at the 1-2 cell stage. Injections were performed in a room in which all overhead and microscope lights were equipped with yellow filters to prevent premature uncaging of caged etv2 morpholino. Dishes containing injected embryos were wrapped in aluminum foil to minimize background uncaging and subsequently uncaged by exposure to 365 nm UV light for 30 minutes.
RNA-Seq

Total RNA was purified from control (fli1a:GFP) embryos and 3 test populations (etv2 MO, fli1b+/− and etv2-MO; fli1b−/) at 24 and 48 hours of development (20 embryos each, performed in duplicate). cDNA was synthesized from 1 microgram of total RNA from each sample using Superscript III First-Strand Synthesis cDNA kit (Life Technologies Inc., Grand Island, NY, catalog #18050-051) and amplified in a 24-cycle PCR reaction. PCR products were purified by phenol-chloroform extraction and ethanol precipitation followed by sequencing on an Illumina Genome Analyzer II standard 36-cycle single-end protocol (20 million reads per sample). Sequences were aligned to the Danio rerio subset of Ensembl Zv9 reference sequence dataset in Avadis Strand NGS (Strand Life Sciences, San Francisco, CA). Raw reads were filtered to remove reads with more than one match, with average base qualities of less than twenty and those with raw read counts less than fifteen. Differentially expressed genes were identified using a 1.5-fold change minimum and a significance cutoff of p < 0.1. The resulting list of differentially expressed genes was filtered for vascular GO terms. Differential expression was confirmed by RT-PCR for selected genes.

Chromatin Immunoprecipitation Assay for Direct Binding Targets of Fli1b

A 6x-FLAG coding sequence was cloned upstream of etv2 and fli1b, and these were inserted into pCS2+. Sense mRNA was generated with T3 polymerase and either 1 nl of a 1:30 dilution of FLAG-etv2 or 2 nl of a 1:20 dilution of FLAG-fli1b was injected into 1-cell stage embryos. Embryos were dechorionated, collected at tailbud stage and fixed with 1% PFA for 30 minutes. Embryos were rinsed twice in ice cold PBS. Embryos were sonicated in 1 mM EDTA/0.5mMEGTA/Tris pH8.0 buffer and sarkosyl was added to 0.5%. After centrifugation, the supernatant was subjected to immunoprecipitation with magnetic beads conjugated with anti-FLAG M2 antibody. The magnetic beads were washed with RIPA buffer (1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 50mM HEPES-KOH pH7.5, 0.5M LiCl). After the eluted DNA was treated with RNAse A and Proteinase K, phenol:chloroform extraction was performed before ethanol precipitation. The enrichment of putative target DNA was measured by real-time PCR.

Validation of Fli1ep Promoter-Enhancer ETS-site Dependent Binding by Fli1b

The fli1ep minimal enhancer-promoter described in Villefranc et al. was cloned upstream of an eGFP fluorescent reporter sequence by Gateway cloning.16 A fli1a[ep] sequence with mutant ETS-binding sites was synthesized by GenScript was also cloned upstream of eGFP to confirm ETS-site dependence of fli1b binding. The wild-type and mutant fli1a[ep] sequences are provided in Figure IX of the online-only Data Supplement. 50pg of reporter construct was co-injected with tol2 mRNA to facilitate stable genomic integration and 5ng each of etv2 MO1 and etv2 MO2 to knock down endogenous etv2 activity and prevent background induction due by Etv2.17 Injections were made with and without fli1b mRNA in order to determine the ability of fli1b to induce expression of wild-type fli1aep or mutant (no-ETS) fli1[ep] reporter constructs. Embryos were screened at the 8-10 somite stage (13-14 hpf) for ectopic marker expression using an AxioImager compound microscope (Carl Zeiss Inc.,USA).
Supplemental References

Supplemental Figure I: Comparison of etv2 and fli1b expression. Expression of etv2 (A-E) and fli1b (F-J) in the trunk vasculature from the 10-somite stage through 48 hpf (lateral views, anterior to the left). Etv2 expression was restricted to endothelial progenitor cell (EPC) populations at the 10-somite (A) and 20-somite stages (B), and was strongly expressed in the vascular endothelium by 24 hpf (C). Expression of etv2 was reduced, but detectable at 36 hpf (D) and at 48 hpf (E). Fli1b expression was first observed at 10 somites (F) and strongly expressed beginning at 20 somites (G). Note that fli1b expression appeared more intense than etv2 at 36 hpf (D) and at 48 hpf (E). K, Relative expression of etv2 and fli1b as analyzed by RT-PCR from the 10 somite stage to 72 hpf. Data points represent an average of two independent samples of pooled embryos, each run in duplicate, and normalized to the 24 hpf expression level. Error bars represent ± 1 s.d. L-N, Fli1b expression in the trunk vasculature of etv2−/− embryos from 24-48 hpf. Fli1b expression in etv2−/− mutant embryos was low at 24 hpf (L), but increased during the 36 to 48 hpf period corresponding to the angiogenic recovery of etv2−/− embryos (M,N). Fractions indicate the number of embryos with the observed staining pattern (numerator) and the total evaluated (denominator).
Supplemental Figure II: The \textit{fli1a}^{is10Gt} and \textit{fli1b}^{is50Gt} gene-trap lines have lost \textit{fli1a} or \textit{fli1b} expression, respectively, but develop a normally appearing vasculature. Schematic and functional confirmation of the \textit{fli1a} gene trap (A-D; I-L) and \textit{fli1b} gene trap lines (E-H, M-P). (A) The \textit{fli1a}^{is10Gt} gene trap allele contains \textit{Tol2} inverted repeat sequences (black arrows), carp β-
actin splice acceptor (SA) from GBT-R15,\(^1\) enhanced green fluorescent protein (eGFP, green), zebrafish β-actin polyadenylation and transcripational termination signals (zp(A), white) and loxP sites (purple). Flanking exons (blue boxes) of the zebrafish fli1a gene are shown. (B) The fli1a gene trap is inserted between exon 3 and 4 resulting in a 127 AA truncated GFP-fusion protein lacking a functional ETS domain. (C) The DNA sequence flanking the fli1a\(^{s10Gr}\) gene-trap is shown. Note the 8 base pair duplication indicative of Tol2-mediated transposition events (underlined). (D) Sequence of the expected Fli1a-GFP fusion protein. Fli1a is in blue, eGFP is in green. (E) The fli1b\(^{ppl50Gr}\) gene trap allele contains the same carp β-actin splice acceptor (SA), an AUG-less Gal4-VP16 (orange), a zebrafish β-actin 3’ untranslated region and transcriptional termination / polyadenylation cassette [zp(A)], an enhanced green fluorescent protein (eGFP, green) zebrafish β-actin 3’UTR and polyadenylation site (pA, gray) from the GBT-R15 vector,\(^1\) and the hybrid yeast-based UAS (syUAS, light blue) (described in Methods). Tol2 5’ and Tol2 3’ are miniTol2 transposon arms as previously described.\(^2\) (F) The fli1b gene trap is inserted between exon 1 and 2 resulting in 6 AA of Fli1b protein fused with Gal4 domain which transactivates the UAS-GFP reporter. (G) The DNA sequence flanking the fli1b\(^{ppl50Gr}\) gene-trap is shown. Note the 8 base pair duplication indicative of Tol2-mediated transposition events (underlined). (H) The expected Fli1b-Gal4 fusion protein. Fli1b sequence is in blue, AUG-less Gal4 is in orange. (I,J) Fli1a expression is greatly diminished in fli1a\(^{−/−}\) mutants relative to wild-type embryos as confirmed by WISH at 24 hpf. (K) Vascular patterning is normal in fli1a\(^{−/−}\) embryos, as evident by the endothelial GFP expression at 48 hpf. (L) qRT-PCR quantitation of full-length fli1a mRNA levels in fli1a\(^{−/−}\) embryos. (M,N) Fli1b expression was disrupted in 24 hpf fli1b\(^{−/−}\) embryos as detected by WISH. (O) Vascular patterning was normal in 48 hpf fli1b\(^{−/−}\) embryos as observed by endothelial GFP expression. Fractions indicate the number of embryos with the normal pattern of expression (numerator) and the total evaluated (denominator). (P) RT-PCR quantitation of full-length fli1b mRNA levels in fli1b\(^{−/−}\) embryos.
Supplemental Figure III: Vascular endothelial marker expression is normal in 24 hpf fli1a\textsuperscript{-/-} and fli1b\textsuperscript{-/-} embryos as determined by whole-mount ISH. Lateral views with the anterior oriented to the left are shown in panels. Fractions indicate the number of embryos staining positive for the indicated vascular marker (numerator) and the total evaluated (denominator). Note the reduction of fli1a marker expression in fli1a\textsuperscript{-/-} mutants.
Supplemental Figure IV: Loss of Fli1a does not significantly alter the etv2 morphant phenotype. Intersegmental vessel patterning in control 65 hpf wild-type Tg(fli1a:GFP) embryos (A) was disrupted by etv2 MO knockdown, yielding a number of mispatterned but full-length ISVs (B). Fluorescent ISVs labeled with the fli1ais10Gt transgene observed in age-matched fli1a/- embryos (C) were also impaired by etv2 MO knockdown (D), but the vascular phenotype in these double-knockdown embryos was indistinguishable from the etv2 morphant phenotype. Fractions indicate the number of embryos appearing as shown (panels A,C) or the number of embryos with greater than 15 partial or full ISVs at 65 hpf (panels C,D) (numerator) and the total evaluated (denominator).
Supplemental Figure V: Combinatorial mutant phenotypes in 48 hpf embryos. (A) Representative embryos from crosses of \textit{etv2}/- and \textit{fli1b}/- zebrafish which yielded 182 embryos with no observable vascular phenotype. \textit{Etv2}/-;\textit{fli1b}/- embryos (expected to comprise 50\% of the embryos evaluated) were indistinguishable from age-matched wild-type embryos or the remaining \textit{etv2}/+;\textit{fli1b}/+ clutch-mates. (B) In-crosses of \textit{fli1a}/-;\textit{fli1b}/+ double carriers yielded a total of 254 embryos, 14 of which (approximately 1/16th) had mild edema (arrow) and lacked circulation but retained normal vascular patterning, corresponding to the expected ratio of double \textit{fli1a}/-;\textit{fli1b}/+ mutants, while the remaining 240 were phenotypically normal). Fractions indicate the number of embryos appearing as shown (numerator) and the total evaluated (denominator).
Supplemental Figure VI: *Fli1a* overexpression does not induce expression of other vascular endothelial markers. Uninjected controls are shown in panels A, C, E, G, I and K. Whole-mount *in situ* analysis of embryos is shown in lateral view with anterior of embryo oriented left. (A-H) Effect of *fli1a* overexpression on vascular markers during the indicated stages. *Fli1a* mRNA does not induce ectopic marker expression of *kdrl* (B), *scl* (F), *gata 1* (H), *fli1b* (J) or *etv2* (L). Note the intense *Fli1a* staining in *fli1a* mRNA injected embryos (D) relative to uninjected controls (C) indicating successful mRNA injection. The fraction of embryos with ectopic expression is shown in the lower left corner of each panel, the age and injected mRNA in the bottom left, and gene probed by *in situ* in the upper right.
**Supplemental Figure VII: The zebrafish fli1a gene contains multiple ETS-binding sites within the proximal promoter and first intron.**

(A) Putative ETS-binding sites (colored dots) in the proximal promoter and first intron of the zebrafish fli1a gene. Sites previously identified as functional binding targets for etv2 in the mouse Fli1 gene\(^3\) are shown in red, specifically -159, -133, and -107 correspond to murine -200, -228 and -256, respectively. The consensus intronic FOX-ETS site is shown in yellow and adjacent putative ETS sites are shown in green.

(B) Regional sequence of the zebrafish fli1a (NM_131348) showing exonic and intronic regions in caps and lowercase, respectively. Primer sequences used for ChIP are shown in bold. The promoter fragment enriched by ChIP qPCR is underlined once, and the enriched intronic FOX-ETS fragment is underlined twice.

(C) Conservation of the FOX-ETS motif.
Supplemental Figure VIII: *Fli1b* induces expression of *fli1a* in an ETS-binding site dependent manner. (A) Schematic *fli1a*ep:GFP reporter construct showing the 23 putative ETS-binding sites identified within the *fli1a* minimal enhancer-promoter sequence. ETS sites previously identified as sufficient for vascular-specific expression of *fli1a* by Abedin et al.\(^4\) (red highlight) and the putative intronic FOX-ETS site identified herein (yellow highlight). (B) Strong ectopic expression in 8-somite staged wild-type embryos injected with *fli1b* mRNA, *tol2* mRNA, *etv2* MO, and the wild-type *fli1a*ep:GFP construct (left embryo) and the corresponding lack of GFP readout in embryos similarly injected but using the mutated (noETS) *fli1a*ep:GFP construct (right embryo). (C) Percentage of fluorescent embryos injected with different combinations of wild-type (WT) or mutant ETS sites (no ETS) *fli1a*ep:GFP construct, *etv2* MO, *fli1b* or *etv2* mRNA.
Supplemental Figure IX: Sequence comparison of the wild-type *fli1α[ep]* sequence (top rows) containing intact ETS sites (highlighted in yellow and underlined) versus the mutated version with disruption of all 23 putative ETS sites. The intronic region (+2095 through +3100 nt from the transcription start site) is positioned upstream and shown in capital letters, while the core promoter-enhancer region (-1079 to ATG start) is downstream and shown in lowercase letters.
Supplemental Material References


