Published in final edited form as:
Circ Res. 2012 January 20; 110(2): 220–229. doi:10.1161/CIRCRESAHA.111.251298.

Etsrp/etv2 is directly regulated by *foxc1a/b* in the zebrafish angioblast

Matthew B. Veldman, Ph.D. and **Shuo Lin, Ph.D.**
Department of Molecular, Cellular, and Developmental Biology, University of California-Los Angeles, CA, USA

Abstract

Rationale—Endothelial cells are developmentally derived from angioblasts specified in the mesodermal germ cell layer. The transcription factor *etsrp/etv2* is at the top of the known genetic hierarchy for angioblast development. The transcriptional events that induce *etsrp* expression and angioblast specification are not well understood.

Objective—We generated *etsrp:gfp* transgenic zebrafish and used them to identify regulatory regions and transcription factors critical for *etsrp* expression and angioblast specification from mesoderm.

Methods and Results—To investigate the mechanisms that initiate angioblast cell transcription during embryogenesis, we have performed promoter analysis of the *etsrp* locus in zebrafish. We describe three enhancer elements sufficient for endothelial gene expression when place in front of a heterologous promoter. The deletion of all three regulatory regions led to a near complete loss of endothelial expression from the *etsrp* promoter. One of the enhancers, located 2.3 kb upstream of *etsrp* contains a consensus FOX binding site that binds Foxc1a and Foxc1b in vitro by EMSA and in vivo using ChIP. Combined knockdown of *foxc1a/b*, using morpholinos, led to a significant decrease in *etsrp* expression at early developmental stages as measured by quantitative RT-PCR and in situ hybridization. Decreased expression of primitive erythrocyte genes *scl* and *gata1* was also observed while pronephric gene *pax2a* was relatively normal in expression level and pattern.

Conclusions—These findings identify mesodermal *foxc1a/b* as a direct upstream regulator of *etsrp* in angioblasts. This establishes a new molecular link in the process of mesoderm specification into angioblast.

Keywords

angioblast; *etsrp*; *foxc1a*; *scl*; zebrafish

Introduction

Endothelial cells are developmentally derived from precursor cells termed angioblasts. These cells initially appear in the mesoderm and coalesce to form the primary vessels

Corresponding author: Shuo Lin, Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, 621 Charles E. Young Drive South, Los Angeles, CA 90095-1606, shuolin@ucla.edu., Fax: 310-267-4971, Telephone: 310-267-4970.

Disclosures
None.

through a process known as vasculogenesis. From these primary vessels the rest of the vasculature spreads throughout the embryo through the process of angiogenesis. The morphological events that occur during these processes are well defined; however, the molecular mechanisms driving these processes are still unclear.

The zebrafish embryo has been a valuable tool for studying the molecular and genetic events occurring during vascular development. For example, the transcription factor Etsrp was first identified in a microarray screen for gene expression changes in the *cloche* mutant embryo. [1] *Cloche* embryos lack blood and vascular cells but have normal development of other organ systems. [2] Etsrp overexpression is sufficient to rescue expression of vascular and primitive myeloid genes in *cloche* embryos. [3] Additionally, overexpression of Etsrp in wild-type embryos ectopically induces the expression of hundreds of vascular and myeloid genes, whereas morpholino knockdown or mutation of Etsrp disrupts vasculogenesis as well as angiogenesis [4-6] Epistasis experiments in zebrafish embryos have demonstrated that *etsrp* is at the top of the angioblast transcriptional hierarchy, placing it above *scl, fli1a*, and *kdrl*. [6-8]

The mammalian homolog of *etsrp, Etv2* (formerly *ER71* or *Etsrp71*), is expressed in mesodermal tissues of the early mouse embryo, including vascular and hematopoietic lineages. [9-11] *Etv2* knockout mice are embryonic lethal by E11.0 with severe defects in hematopoietic and vascular development. [10, 11] In embryonic stem cells, Etv2 directly regulates *Kdr (Flk1)* expression and can increase the derivation of blood and endothelial cells when overexpressed. [10] Interestingly, *Scl* and *Kdr* were shown to function downstream of *Etv2* in mice as was found in zebrafish. [10, 12] In fact, human or mouse Etv2 protein overexpression in zebrafish embryos was sufficient to induce the ectopic expression of *scl* and *kdrl*, [8] suggesting that *etsrp* and *Etv2* are homologous genes that have conserved functions in vertebrate vascular development and hematopoiesis.

Although much effort has been made to study the genes downstream of *etsrp/Etv2*, little is known about its upstream regulators. In mouse, the transcription factor Nkx2-5 has been suggested to regulate *Etv2* expression in the endocardium. [11] However, *Nkx2-5* expression is limited to cardiac and endocardial lineages implying that this regulatory interaction is limited to the developing heart. [13, 14] Additionally, the zebrafish *Nkx2-5* homolog *nkx2.5* is expressed in the cardiac mesoderm where it is discretely segregated from the *etsrp* expression domain in the anterior and posterior lateral plate mesoderm, suggesting that a direct positive interaction does not occur in zebrafish. [15, 16] Combined morpholino knockdown of *gata4, gata5*, and *gata6* can delay the expression of *etsrp* and other vascular and cardiac genes in the anterior lateral plate. [17] However, angioblasts in the posterior lateral plate are unaffected and a direct interaction between these factors and *etsrp* has not been established. The *cloche* mutant locus is upstream of *etsrp*, but the specific genetic lesion in this mutant has not been conclusively identified. Xiong et. al. suggested that the *lycat* gene, a predicted lipid acetyltransferase, is responsible for the *cloche* phenotype. [18] Although knockdown of *lycat* blocks the expression of *etsrp*[18], it is unlikely that *lycat* directly regulates the transcription of *etsrp*. Therefore a significant gap exists in our knowledge of angioblast specification from mesodermal tissue at the level of the *etsrp* transcription factor.

To identify upstream regulators of *etsrp* gene expression we have studied the regulatory regions of the *etsrp* locus. Using transgenic zebrafish, we identify three enhancer regions that are sufficient to drive GFP expression similar to the endogenous pattern. We identify Foxc1a/b as a direct upstream regulator of *etsrp* and demonstrate its involvement in angioblast specification.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Zebrafish embryos were maintained and staged as described. [19] The University of California, Los Angeles Animal Care and Use Committee approved all protocols used in this study. Transgene plasmids were generated using Tol2Kit plasmids[20] and the Multisite Gateway System (Invitrogen). Zebrafish embryos were microinjected at the one cell stage with DNA, mRNA, or morpholinos as previously described. [21] Electrophoretic Mobility Shift Assay (EMSA) was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's suggested protocol. Nuclear protein extracts from Porcine Aortic Endothelial (PAE) and Human Umbilical Vein Endothelial Cells (HUVEC) were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) as recommended. In vitro synthesized Foxc1a, Foxc1b, and mCherry protein was created using the TnT in vitro transcription/translation kit (Promega). Chromatin Immunoprecipitation (ChIP) and quantitative RT-PCR methods are available in the online supplement. Whole mount in situ hybridization was performed as described[22] using DIG labeled riboprobes (Roche). Images were captured on an Axioskop 2 plus microscope (Zeiss) or a Stemi2000-C (Zeiss) using 5x or 10x objectives with an AxioCam camera and Openlab 4.0 software (Improvision). Adobe Photoshop was used to adjust brightness and contrast and assemble composite images. Students *t*-test was used to determine significance with $p<0.05$ for qRT-PCR experiments.

Results

Tg(−2.3etsrp:gfp) transgene recapitulates the endogenous expression pattern of *etsrp*

To begin dissecting the regulatory mechanisms of *etsrp* expression, we undertook a bioinformatic analysis of the *etsrp/Etv2* locus in multiple species using Multi-Pipmaker analysis (http://pipmaker.bx.psu.edu/pipmaker) [23] and the web-based ECR Browser (http://ecrbrowser.dcode.org/).[24] Comparison of approximately 200 kilobases of sequence between human, mouse, *Xenopus*, pufferfish, and zebrafish found very little homology outside of the exonic sequences (unpublished data). However, two peaks of conserved sequence were identified near the transcription start site of *etsrp* between zebrafish and pufferfish (Figure 1A). One of the conserved peaks, located 2.3 kb upstream of the *etsrp* transcription start site, was called *up1*. The second region of conservation, located in *etsrp* intron two, was called *int2*. We generated a transgene, *tg(−2.3etsrp:gfp)*, that encompassed these two conserved sequences (Figure 1A). Transgenic embryos exhibited strong vascular specific expression at 24 hours post fertilization (hpf) suggesting that the conserved regions may be relevant to the endogenous gene's expression.

Tg(−2.3etsrp:gfp) fish exhibited GFP expression initially in the anterior (ALPM) and posterior lateral plate mesoderm (PLPM) at ~4 somite stage. This is identical in pattern to endogenous *etsrp*, but slightly delayed, most likely due to the time necessary for GFP to mature. By the 10 somite stage, strong GFP expression is present in the ALPM and PLPM in a pattern identical to the endogenous gene (Figure 1B-G). Similarly, at the 18 somite stage, the expression of GFP and *etsrp* correlate almost identically (Figure 1H-M). At 24 hpf, GFP is highly expressed in both the cranial vasculature (Figure 1N-Q) and the axial and intersomitic vessels of the trunk (Figure 1R-S). By 36 hpf, endogenous *etsrp* is significantly reduced in the vasculature with the exception of the aortic arches.[3] *Tg(−2.3etsrp:gfp)* follows this pattern of expression (Online Figure I). In comparison to the well characterized *tg(kdrl:gfp)* [25] zebrafish, *tg(−2.3etsrp:gfp)* expression appears earlier in angioblasts but disappears as the vasculature matures while *tg(kdrl:gfp)* maintains expression throughout

development and into adulthood (Online Figure I). Additionally, *tg(−2.3etsrp:mcherry)* co-localizes with *tg(fli1a:gfp)*[26] in the ALPM and PLPM at 10 somite stage demonstrating the promoter drives expression in angioblasts at this early stage (Online Figure II). Overall, *tg(−2.3etsrp:gfp)* faithfully recapitulates the endogenous expression of *etsrp* both temporally and spatially.

Three enhancers drive angioblast expression in *etsrp* transgenic fish

To test whether the evolutionarily conserved regions have enhancer activity, we placed them in front of the minimal *gata2* promoter driving GFP. Cloning of either *up1* or *int2* into the reporter was sufficient to drive GFP expression in the developing vasculature both transiently and in germline transgenics (Figure 2A-B). Additionally, these regions functioned when placed in the reverse orientation, demonstrating that they are true enhancer regions and not cryptic promoters (Online Figure III). Given that we could not identify any other conserved regions within the promoter, we hypothesized that deletion of *up1* and *int2* would abolish expression of the *etsrp* transgene. However, deletion of each region separately or simultaneously did not significantly disrupt transgene expression (Figure 2C). This suggests that non-conserved regulatory sequences are present in the −1.8 kb region of the promoter.

In an attempt to map the remaining regulatory elements we did a linear deletion analysis of the −1.8 kb promoter sequence (Online Figure IV). 110 base-pairs of *etsrp* promoter sequence were found to be sufficient for vascular expression at 24 hpf (Figure 3A-C). Deletion of 35 base-pairs from the 5′-end completely abolished vascular specific expression (Figure 3D-F). However, the remaining 75 base-pair promoter was still capable of driving non-vascular expression in several lines; presumably due to enhancer trapping effects (unpublished data). This suggests that basal promoter function had been preserved and the −0.110 kb to −0.075 kb region of the promoter was acting as an enhancer. To determine the importance of the enhancer, we deleted it along with *up1* and *int2* from *tg(−2.3etsrp:gfp)*. These fish demonstrated minimal expression in the developing vasculature (Figure 3G-I) suggesting that the combination of these three enhancers drives strong *etsrp* expression during development.

We also noticed that some of the transgenes had stronger or weaker expression in the dorsal aorta (DA) versus the posterior cardinal vein (PCV). Specifically, the *up1* and *int2* enhancers exhibited stronger or equal expression in the DA versus the PCV while deletion of these enhancers resulted in PCV expression greater than DA (Figure 2A-C). A summary of the relative transgene expression in the axial vasculature for each transgene line is provided in Online Table I.

The upstream enhancer, *up1*, contains multiple evolutionarily conserved protein binding sites

To better define the sequences necessary for enhancer activity, we studied *up1* in more detail. The ~500 base-pair element was broken into four overlapping fragments of ~150 base-pairs, termed *A, B, C*, and *D*, and cloned in front of the minimal *gata2* promoter transgene. Unfortunately, none of these sequences was sufficient for expression in the developing vasculature (Figure 4A, B, F, and G). Therefore, different combinations of *A, B, C*, and *D* were tested for enhancer activity. We found that the combination of *A* and *B* was sufficient for vascular expression of the reporter, while fragments *C* and *D* were dispensable (Figure 4A, and D-I). This narrowed the enhancer region to 252 base-pairs. Comparison of this sequence between fish species including zebrafish, stickleback, tetraodon, fugu, and medaka identified several regions of evolutionary conservation (Figure 5A).

The *AB* sequence was divided into five overlapping fragments termed Up1-(1-5) that were used as probes in EMSA (Figure 5). Because no zebrafish endothelial cell lines exist and zebrafish whole embryo extract proved too complex to resolve individual binding complexes, nuclear extracts from HUVEC and PAE cells were used for this analysis. We found that specific protein binding to probes Up1-1, -2, -4, and -5 could be detected (Figure 5B). Most binding activity was present in both arterial and venous cell types. However, Up1-5 bound to three distinct protein bands in HUVEC extracts, while only the lowest band was present in PAE cells (Figure 5B). In fact, *up1* drives expression more strongly in arterial cells (Figure 2A) and the difference in protein binding may be relevant to A/V specific expression levels.

Foxc1a and Foxc1b bind to *up1* in vitro and in vivo

Using the TRANSFAC database[27] we screened the sequence of *up1* for consensus transcription factor binding sites. Four candidates were identified, FoxC1/2, Cebpα, Gata, and Evi1. To test if the binding activity identified by EMSA corresponded to any of these consensus sites, oligos for each were prepared and used as unlabeled competitors. For the Up1-1 probe, we found that the FoxC1/2 consensus site could compete for binding with HUVEC protein extracts while the others could not (Figure 6A). To determine if the zebrafish homologs of FoxC1/2, Foxc1a and Foxc1b, could bind to Up1-1, we in vitro synthesized these proteins and performed EMSA with the Up1-1 probe. Both Foxc1a and Foxc1b were able to bind to Up1-1 while a negative control protein, in vitro synthesized mCherry, could not (Figure 6B).

We next wanted to test if FoxC proteins were able to interact with the *etsrp* promoter in vivo using ChIP. Since no antibody to zebrafish FoxC proteins is available, we generated a C-terminal myc-tagged version of Foxc1a to use for immunoprecipitation. mRNA for *foxc1a-myc* was injected into single cell embryos resulting in ubiquitous expression of the tagged protein. Embryos were processed for ChIP at 50% epiboly due to gastulation defects and death that occurred at later stages. We could detect significant enrichment of the *up1* genomic locus in *foxc1a-myc* mRNA injected embryos but not in uninjected control embryos (Figure 6C). The *rhodopsin* promoter region used as a negative control was not enriched in either uninjected or *foxc1a-myc* injected samples demonstrating that Foxc1a-myc binding to the *up1* site is specific (Figure 6C). Comparison of the FoxC1 consensus binding site to the sequence found in Up1-1 showed perfect alignment (Figure 6D). These results suggest that forkhead transcription factors can bind to the *etsrp* promoter at a conserved site within a functional enhancer.

Foxc1a and *foxc1b* act upstream of *etsrp* in angioblast development

It was previously reported that *foxc1a* and *foxc1b* knockdown affects artery-vein specification and vascular morphology and integrity in developing zebrafish embryos.[12, 28] These defects are reminiscent of *etsrp* mutants and morphants. [3, 6, 7] De Val et.al. demonstrated an epistatic relationship between *foxc1a* and *etsrp* and suggested that Etsrp and Foxc1a directly interact to activate downstream vascular genes. [12] However, an alternate explanation is that *foxc1a* and *etsrp* have a linear relationship with one factor directly regulating the other. *Foxc1a* and *foxc1b* expression initially appear in the involuting mesendoderm at the shield stage and is then maintained in paraxial mesoderm and other mesodermally derived tissues including the vasculature.[12, 28, 29] This expression precedes *etsrp* which is first induced in the lateral plate mesoderm at the 1-2 somite stage[3]. We performed double fluorescent in situ hybridization to examine co-localization of *foxc1a* and *etsrp*. At 8-10 somites stage we noted co-labeling in a subset of cells in the ALPM and anterior PLPM (Online Figure V). This timing of expression suggests that *foxc1a* and *foxc1b* may act upstream to *etsrp*.

To determine the epistatic relationship of *etsrp* and *foxc1a/b* in vivo we performed morpholino gene knockdown studies using previously published morpholinos that had been shown to be specific and free of off-target effects.[28, 30] It has been suggested that *etsrp* and *foxc1a/b* function in a complex to induce the expression of *Kdr* (*Flk1*).[12] In zebrafish embryos, overexpression of *etsrp* is sufficient to induce robust ectopic expression of *tg(kdrl:gfp)*.[3] To determine if *foxc1a/b* are necessary co-factors for this induction, we blocked their expression in *tg(kdrl:gfp)* embryos while simultaneously overexpressing *etsrp*. Under these conditions, *foxc1a/b* are not necessary for *etsrp* to induce ectopic expression of *tg(kdrl:gfp)* (Online Figure VI). This suggests that *etsrp* function is independent of, or downstream to, *foxc1a/b* at this early developmental stage.

To determine if the *etsrp* promoter is regulated by *foxc1a* and *foxc1b* at the *up1* enhancer, morpholinos were injected into *tg(up1-gata2:gfp)* fish to see if the loss of these factors affects transgene expression. By the 16 somite stage, transgene expression is visible in the axial vessels of control embryos but not *foxc1a/b* morphant embryos (Online Figure VII), demonstrating that the loss of *foxc1a/b* decreases the activity of the *up1* enhancer in vivo.

To see if *foxc1a/b* played a larger role in regulating *etsrp*, we tested the affect of their knockdown on *tg(−2.3etsrp:gfp)* expression and endogenous *etsrp* expression by in situ hybridization at the 6-8 somite stage. Morphlino knockdown of *foxc1a/b* significantly reduced the expression of both *tg(−2.3etsrp:gfp)* and *etsrp*, most notably in the PLPM (bracketed in Figure 7A-D). Three other PLPM markers, *fli1a, scl*, and *gata1*, were also examined. *Fli1a* and *scl* are important transcription factors that functions downstream of *etsrp* in vascular lineages [7]; in primitive erythroid cells, *fli1a, scl*, and *gata1* function independent of *etsrp*.[7] *Fli1a, scl*, and *gata1* expression are all reduced in *foxc1a/b* morphants (Figure 7E-J). To test whether other mesoderm or non-mesodermal tissues were disrupted, expression of *pax2a*, a gene expressed in the intermediate mesoderm and central nervous system, was examined. As previously reported, *pax2a* expression is largely normal in the nervous system and intermediate mesoderm in *foxc1a/b* morphants, with the exception of the pronephric primordium (Figure 7K-L).[30] Additional paraxial mesoderm genes *mespa, mespb, deltaC*, and *par1* were examined in *foxc1a/b* morphants and behaved as previously reported (Online Figure VIII).[30] Quantitative RT-PCR results support the in situ hybridization data, demonstrating significant decreases in *etsrp, fli1a, scl*, and *gata1* expression in *foxc1a/b* morphants (Figure 7M). These results demonstrate a significant decrease in the early expression of primitive erythroid and angioblast genes when *foxc1a/b* are knocked down.

Discussion

Etsrp is the most upstream transcription factor in the regulatory hierarchy of angioblasts. To identify the signals and factors that induce angioblasts from mesoderm we have analyzed regulatory regions in the *etsrp* promoter. Bioinformatic analysis identified two evolutionarily conserved, noncoding sequences near the *etsrp* locus. One region, *up1*, is located 2.3 kb upstream of the transcription start site of *etsrp*. The second, *int2*, is within the second intron of *etsrp*. We tested and confirmed that both of these sequences function as enhancers for angioblast gene expression. Surprisingly deletion of these two conserved enhancers did not abolish the expression of the *etsrp:gfp* transgene. We then mapped a very short, 35 base-pair, proximal enhancer that was responsible for the remaining *etsrp* promoter expression.

Multiple enhancers for a single gene are common. Sometimes the enhancers drive partial spatial or temporal expression that when summed with other enhancers give the full gene expression. In the case of *etsrp*, we have identified three enhancers that drive expression in angioblasts and endothelial cells. There is some bias towards elevated artery or vein

expression from each enhancer suggesting different signaling pathways may converge on each enhancer to drive robust *etsrp* expression during development. When each enhancer is isolated, expression is much weaker than in the context of the *tg(−2.3etsrp:gfp)* with all three enhancers present. Although we have found that t*g(−2.3etsrp:gfp)* is sufficient for strong expression in angioblasts, a recently published BAC transgenic line appears to be even more robust.[32] It may be possible that other distant "shadow" enhancers are able to drive *etsrp* expression; however our bioinformatic analysis suggests that they are not evolutionarily conserved if they do exist.

To identify the transcription factors regulating *etsrp* expression, we focused on the *up1* enhancer. By breaking down the *up1* sequence into multiple overlapping EMSA probes we demonstrated that several protein complexes from endothelial cells bind to *up1*. One protein binding site was identified as a FOX consensus site and zebrafish Foxc1a and Foxc1b were found to bind by both EMSA and ChIP assays. In zebrafish, *foxc1a/b* has established functions in mesodermal, vascular, and mesenchymal development.[12, 28, 30] Although vascular anomalies have been previously reported in *foxc1a/b* morphant embryos, the effects of *foxc1a/b* knockdown on early angioblast gene expression has not been described. Here, we have demonstrated that the early expression of *etsrp, fli1a*, and *scl* are disrupted when *foxc1a/b* are knocked down. Reduced expression of primitive erythrocyte gene *gata1* suggests that both blood and vascular lineages are affected by loss of *foxc1a/b*. In addition, a previously reported disturbance in *pax2a* expression in the anterior pronephric region was confirmed.

It was surprising that knockdown of *foxc1a/b* had a large effect on *etsrp* expression, while deletion of *up1* had relatively little effect on transgene expression. One possible explanation for this finding is that *foxc1a/b* binds at non-conserved sites or is recruited to the promoter through non-direct DNA binding interactions. Another possibility is that *foxc1a/b* has an indirect function in inducing angioblasts, possibly by generating signals in the paraxial mesoderm which is also defective in *foxc1a/b* morphants. In either case, our results highlight the importance of *foxc1a/b* in multiple mesodermal cell lineages and suggest that Foxc1a/b functions directly at the *etsrp* promoter.

In mammals, *FoxC1* and *FoxC2* are the homologs of zebrafish *foxc1a/b*. Null mice generated for each gene display significantly overlapping phenotypes. Both null mice have skeletal, eye, kidney, and cardiovascular problems.[33, 34] The cardiovascular defects of *FoxC1/C2* double null mice are reminiscent of the *Etv2* null phenotype; although *FoxC1/FoxC2* double null mice have visible blood while *Etv2* null mice are completely anemic. The genetic relationship between these genes has yet to be studied in mouse models. However, it has been reported that *FoxC2* and *Etv2* have common downstream targets at a conserved FOX:ETS enhancer binding site.[12] In fact, a mammalian *Mef2c* enhancer containing this double binding site is capable of driving expression in zebrafish vasculature.[12] Our results suggest that *FoxC1/2* may function upstream of *Etv2* in addition to the established shared downstream function.

The reduced expression of primitive erythrocyte marker *gata1* in *foxc1a/b* morphant embryos was somewhat surprising given that blood cells have been reported to be present in both null mice and morphant zebrafish. However, a FOX:ETS binding site has been reported at the *SCL/TAL* locus and *Scl* is directly upstream of *Gata1*.[12, 35] Although this site likely drives expression in angioblasts, it is possible the site also functions in primitive erythrocytes in conjunction with *FoxC2* and non-*Etv2* ETS proteins. Another possibility is that non-ETS dependent FOX binding sites are present in the multiple enhancers driving *Scl* expression.[36] A third possibility is an indirect effect of *foxc1a/b* knockdown on *scl* expression. The paraxial mesoderm is defective in *foxc1a/b* morphants and this tissue

directly regulates the specification of primitive erythrocytes in zebrafish.[37] Thus, the defect in *scl* and *gata1* expression may be due to missing signals from the developing somites. This idea awaits further study.

In conclusion, *foxc1a/b* functions directly upstream of *etsrp* and upstream of *scl* in zebrafish mesoderm to specify angioblasts and primitive erythrocytes. This finding bridges the knowledge gap in molecular events underlying the mesoderm to angioblast transition. It may also have clinical implications as *FOXC1* is linked to Axenfeld-Rieger anomaly and glaucoma[38] while *FOXC2* is linked to lymphedema-distichiasis syndrome[39, 40] both diseases are associated with circulatory defects. Recently *FOXC2* has been suggested to be an important mediator of tumor angiogenesis.[41, 42] The link between *FOXC1, FOXC2*, and *ETV2* in these diseases may be a clinically important avenue of study in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Anqi Liu for maintenance of zebrafish lines, the UCLA Orthopedic Hospital Research Center for the use of their real-time qPCR machine, Andrew Harmon for helping generate the *tg(int2-gata2:gfp)* lines and screening for founder fish, and Yesenia Rios, Zahra Tehrani, and Michaela Patterson for feedback on the manuscript.

Sources of Funding

M.B.V. was supported by NIH-T32 HL08634 and NIH-T32 HL69766. S.L. was supported by NIH-5R01DK054508-13.

Non-standard Abbreviations and Acronyms

ALPM	Anterior lateral plate mesoderm
ChIP	Chromatin immunoprecipitation
DA	Dorsal aorta
EMSA	Electrophoretic mobility shift assay
FOX	Forkhead box
GFP	Green fluorescent protein
hpf	Hours post fertilization
HUVEC	Human umbilical vein endothelial cell
PAE	Porcine aortic endothelial cell
PCV	Posterior cardinal vein
PLPM	Posterior lateral plate mesoderm
RT-PCR	Reverse transcriptase polymerase chain reaction

References

1. Sumanas S, Jorniak T, Lin S. Identification of novel vascular endothelial-specific genes by the microarray analysis of the zebrafish cloche mutants. Blood. 2005; 106:534–541. [PubMed: 15802528]

2. Stainier DY, Weinstein BM, Detrich HW 3rd, Zon LI, Fishman MC. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. Development. 1995; 121:3141–3150. [PubMed: 7588049]

3. Sumanas S, Lin S. Ets1-related protein is a key regulator of vasculogenesis in zebrafish. PLoS Biol. 2006; 4:e10. [PubMed: 16336046]

4. Wong KS, Proulx K, Rost MS, Sumanas S. Identification of vasculature-specific genes by microarray analysis of etsrp/etv2 overexpressing zebrafish embryos. Dev Dyn. 2009; 238:1836–1850. [PubMed: 19504456]

5. Gomez GA, Veldman MB, Zhao Y, Burgess S, Lin S. Discovery and characterization of novel vascular and hematopoietic genes downstream of etsrp in zebrafish. PLoS One. 2009; 4:e4994. [PubMed: 19308258]

6. Pham VN, Lawson ND, Mugford JW, Dye L, Castranova D, Lo B, Weinstein BM. Combinatorial function of ets transcription factors in the developing vasculature. Dev Biol. 2007; 303:772–783. [PubMed: 17125762]

7. Ren X, Gomez GA, Zhang B, Lin S. Scl isoforms act downstream of etsrp to specify angioblasts and definitive hematopoietic stem cells. Blood. 2010; 115:5338–5346. [PubMed: 20185582]

8. Sumanas S, Gomez G, Zhao Y, Park C, Choi K, Lin S. Interplay among etsrp/er71, scl, and alk8 signaling controls endothelial and myeloid cell formation. Blood. 2008; 111:4500–4510. [PubMed: 18270322]

9. De Haro L, Janknecht R. Functional analysis of the transcription factor er71 and its activation of the matrix metalloproteinase-1 promoter. Nucleic Acids Res. 2002; 30:2972–2979. [PubMed: 12087183]

10. Lee D, Park C, Lee H, Lugus JJ, Kim SH, Arentson E, Chung YS, Gomez G, Kyba M, Lin S, Janknecht R, Lim DS, Choi K. Er71 acts downstream of bmp, notch, and wnt signaling in blood and vessel progenitor specification. Cell Stem Cell. 2008; 2:497–507. [PubMed: 18462699]

11. Ferdous A, Caprioli A, Iacovino M, Martin CM, Morris J, Richardson JA, Latif S, Hammer RE, Harvey RP, Olson EN, Kyba M, Garry DJ. Nkx2-5 transactivates the ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. Proc Natl Acad Sci U S A. 2009; 106:814–819. [PubMed: 19129488]

12. De Val S, Chi NC, Meadows SM, Minovitsky S, Anderson JP, Harris IS, Ehlers ML, Agarwal P, Visel A, Xu SM, Pennacchio LA, Dubchak I, Krieg PA, Stainier DY, Black BL. Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. Cell. 2008; 135:1053–1064. [PubMed: 19070576]

13. Komuro I, Izumo S. Csx: A murine homeobox-containing gene specifically expressed in the developing heart. Proc Natl Acad Sci U S A. 1993; 90:8145–8149. [PubMed: 7690144]

14. Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene nkx2-5. Genes Dev. 1995; 9:1654–1666. [PubMed: 7628699]

15. Chen JN, Fishman MC. Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. Development. 1996; 122:3809–3816. [PubMed: 9012502]

16. Schoenebeck JJ, Keegan BR, Yelon D. Vessel and blood specification override cardiac potential in anterior mesoderm. Dev Cell. 2007; 13:254–267. [PubMed: 17681136]

17. Peterkin T, Gibson A, Patient R. Common genetic control of haemangioblast and cardiac development in zebrafish. Development. 2009; 136:1465–1474. [PubMed: 19297410]

18. Xiong JW, Yu Q, Zhang J, Mably JD. An acyltransferase controls the generation of hematopoietic and endothelial lineages in zebrafish. Circ Res. 2008; 102:1057–1064. [PubMed: 18388326]

19. Westerfield, M. The zebrafish book: A guide for the laboratory use of zebrafish (brachydanio rerio). M. Westerfield; Eugene, OR: 1993.

20. Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB. The tol2kit: A multisite gateway-based construction kit for tol2 transposon transgenesis constructs. Dev Dyn. 2007; 236:3088–3099. [PubMed: 17937395]

21. Meng A, Jessen JR, Lin S. Transgenesis. Methods Cell Biol. 1999; 60:133–148. [PubMed: 9891334]

22. Jowett T. Analysis of protein and gene expression. Methods Cell Biol. 1999; 59:63–85. [PubMed: 9891356]

23. Schwartz S, Zhang Z, Frazer KA, Smit A, Riemer C, Bouck J, Gibbs R, Hardison R, Miller W. Pipmaker--a web server for aligning two genomic dna sequences. Genome Res. 2000; 10:577–586. [PubMed: 10779500]

24. Ovcharenko I, Nobrega MA, Loots GG, Stubbs L. Ecr browser: A tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. Nucleic Acids Res. 2004; 32:W280–286. [PubMed: 15215395]

25. Choi J, Dong L, Ahn J, Dao D, Hammerschmidt M, Chen JN. Foxh1 negatively modulates flk1 gene expression and vascular formation in zebrafish. Dev Biol. 2007; 304:735–744. [PubMed: 17306248]

26. Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol. 2002; 248:307–318. [PubMed: 12167406]

27. Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K, Voss N, Stegmaier P, Lewicki-Potapov B, Saxel H, Kel AE, Wingender E. Transfac and its module transcompel: Transcriptional gene regulation in eukaryotes. Nucleic Acids Res. 2006; 34:D108–110. [PubMed: 16381825]

28. Skarie JM, Link BA. Foxc1 is essential for vascular basement membrane integrity and hyaloid vessel morphogenesis. Invest Ophthalmol Vis Sci. 2009; 50:5026–5034. [PubMed: 19458328]

29. Topczewska JM, Topczewski J, Solnica-Krezel L, Hogan BL. Sequence and expression of zebrafish foxc1a and foxc1b, encoding conserved forkhead/winged helix transcription factors. Mech Dev. 2001; 100:343–347. [PubMed: 11165495]

30. Topczewska JM, Topczewski J, Shostak A, Kume T, Solnica-Krezel L, Hogan BL. The winged helix transcription factor foxc1a is essential for somitogenesis in zebrafish. Genes Dev. 2001; 15:2483–2493. [PubMed: 11562356]

31. Detrich HW 3rd, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, Pratt S, Ransom D, Zon LI. Intraembryonic hematopoietic cell migration during vertebrate development. Proc Natl Acad Sci U S A. 1995; 92:10713–10717. [PubMed: 7479870]

32. Proulx K, Lu A, Sumanas S. Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis. Dev Biol. 2010

33. Kume T, Deng K, Hogan BL. Murine forkhead/winged helix genes foxc1 (mf1) and foxc2 (mfh1) are required for the early organogenesis of the kidney and urinary tract. Development. 2000; 127:1387–1395. [PubMed: 10704385]

34. Kume T, Jiang H, Topczewska JM, Hogan BL. The murine winged helix transcription factors, foxc1 and foxc2, are both required for cardiovascular development and somitogenesis. Genes Dev. 2001; 15:2470–2482. [PubMed: 11562355]

35. Vyas P, McDevitt MA, Cantor AB, Katz SG, Fujiwara Y, Orkin SH. Different sequence requirements for expression in erythroid and megakaryocytic cells within a regulatory element upstream of the gata-1 gene. Development. 1999; 126:2799–2811. [PubMed: 10331989]

36. Dhami P, Bruce AW, Jim JH, Dillon SC, Hall A, Cooper JL, Bonhoure N, Chiang K, Ellis PD, Langford C, Andrews RM, Vetrie D. Genomic approaches uncover increasing complexities in the regulatory landscape at the human scl (tal1) locus. PLoS One. 2010; 5:e9059. [PubMed: 20140202]

37. Rohde LA, Oates AC, Ho RK. A crucial interaction between embryonic red blood cell progenitors and paraxial mesoderm revealed in spadetail embryos. Dev Cell. 2004; 7:251–262. [PubMed: 15296721]

38. Tumer Z, Bach-Holm D. Axenfeld-rieger syndrome and spectrum of pitx2 and foxc1 mutations. Eur J Hum Genet. 2009; 17:1527–1539. [PubMed: 19513095]

39. Nishimura DY, Swiderski RE, Alward WL, Searby CC, Patil SR, Bennet SR, Kanis AB, Gastier JM, Stone EM, Sheffield VC. The forkhead transcription factor gene fkhl7 is responsible for glaucoma phenotypes which map to 6p25. Nat Genet. 1998; 19:140–147. [PubMed: 9620769]

40. Mears AJ, Jordan T, Mirzayans F, Dubois S, Kume T, Parlee M, Ritch R, Koop B, Kuo WL, Collins C, Marshall J, Gould DB, Pearce W, Carlsson P, Enerback S, Morissette J, Bhattacharya S, Hogan B, Raymond V, Walter MA. Mutations of the forkhead/winged-helix gene, fkhl7, in

patients with axenfeld-rieger anomaly. Am J Hum Genet. 1998; 63:1316–1328. [PubMed: 9792859]

41. Hayashi H, Sano H, Seo S, Kume T. The foxc2 transcription factor regulates angiogenesis via induction of integrin beta3 expression. J Biol Chem. 2008; 283:23791–23800. [PubMed: 18579532]
42. Sano H, Leboeuf JP, Novitskiy SV, Seo S, Zaja-Milatovic S, Dikov MM, Kume T. The foxc2 transcription factor regulates tumor angiogenesis. Biochem Biophys Res Commun. 2010; 392:201–206. [PubMed: 20060810]

Novelty and Significance

What is known?

- Etsrp/etv2 is an ETS family transcription factor that is a key regulator of endothelial cell development in embryos.
- Loss of Etsrp/etv2 function results in embryonic lethality due to cardiovascular defects.
- Ectopic expression of Etsrp/etv2 results in the induction of endothelial genes in non-endothelial cells.

What new information does this article contribute?

- A new transgenic zebrafish line was developed using the *etsrp/etv2* promoter to express GFP in the early endothelial cells.
- Three regulatory regions were identified within the promoter to contribute to proper *etsrp/etv2* expression.
- The Foxc1a/b transcription factors were found to regulate *etsrp/etv2* espression in the early endothelial cells through one of the regulatory regions.

Summary

Etsrp/etv2 is a critical regulator of blood vessel development, however, little is known about how this gene is itself regulated. To identifiy upstream regulators we have generated transgenic zebrafish using the *etsrp/etv2* promoter to drive GFP expression in the developing vasculature. Within the promoter sequence we have identified three regions necessary for robust expression. One regulatory region contains a forkhead transcription factor consensus binding site. We demonstrate that Foxc1a/b can bind to this site in vitro and in vivo and knockdown of these proteins results in decreased *etsrp/etv2* expression as well as other blood and blood vessel genes. Previously, Foxc1a/b was found to cooperate with Etsrp/etv2 to regulate many important vascular genes. Here we find that *foxc1a/b* is also upstream of *etsrp/etv2* in the genetic hierarchy of endothelial cell development. This provides a direct genetic link from mesoderm to endothelial cell. Previous work has demonstrated that the BMP, Notch, and Wnt signaling pathways regulate *etsrp/etv2* in embryonic stem cells. It will be interesting to determine in future studies if the identified regulatory regions respond to these pathways through Foxc1a/b or other transcription factors in vivo.

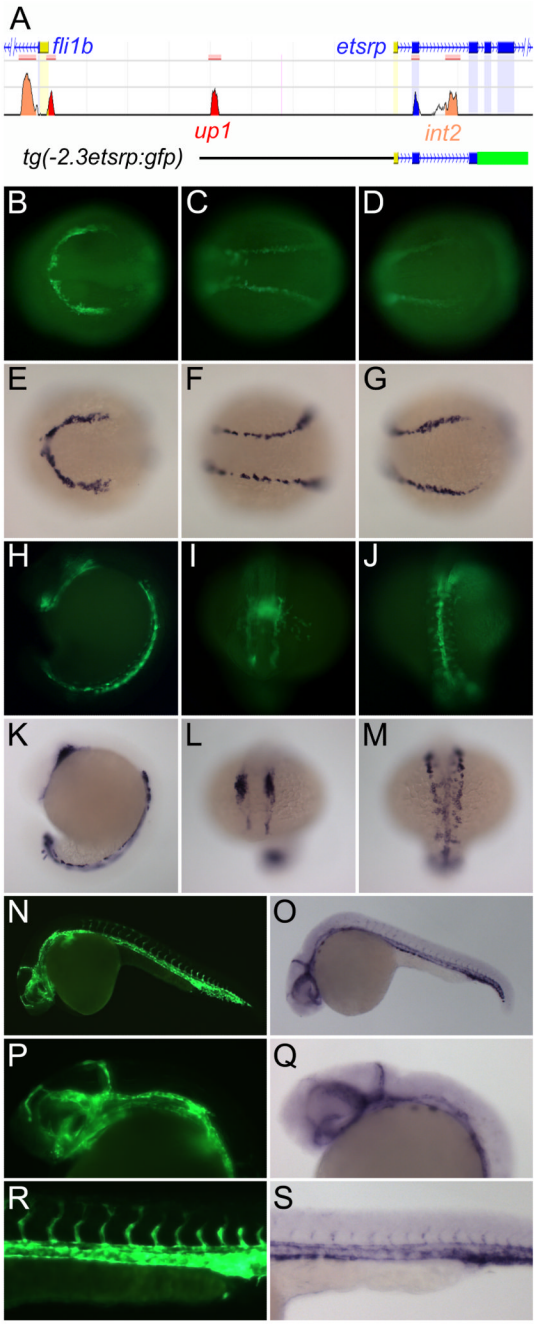

Figure 1. *Tg(−2.3etsrp:gfp)* contains two evolutionarily conserved regions and faithfully recapitulates the endogenous expression pattern of *etsrp*

(A) The *etsrp* gene locus with conserved regions *up1* and *int2* along with the region corresponding to *tg(−2.3etsrp:gfp)* highlighted. The *up1* region is approximately equidistant from the transcription start sites of *etsrp* and the adjacent *fli1b* gene and *int2* is located within intron 2 of *etsrp*. Conserved regions between zebrafish and pufferfish were identified using the ECR Browser (http://ecrbrowser.dcode.org) and the locus image is adapted from this website. (B-S) Fluorescent images of *tg(−2.3etsrp:gfp)* panels B-D, H-J, N, P, and R and in situ hybridization for *etsrp* panels E-G, K-M, O, Q, and S demonstrating near perfect correlation of expression between the transgene and endogenous gene at different

developmental stages. (B-G) 10-somite stage; (H-M) 18-somite stage; (N-S) 24 hours post fertilization (hpf).

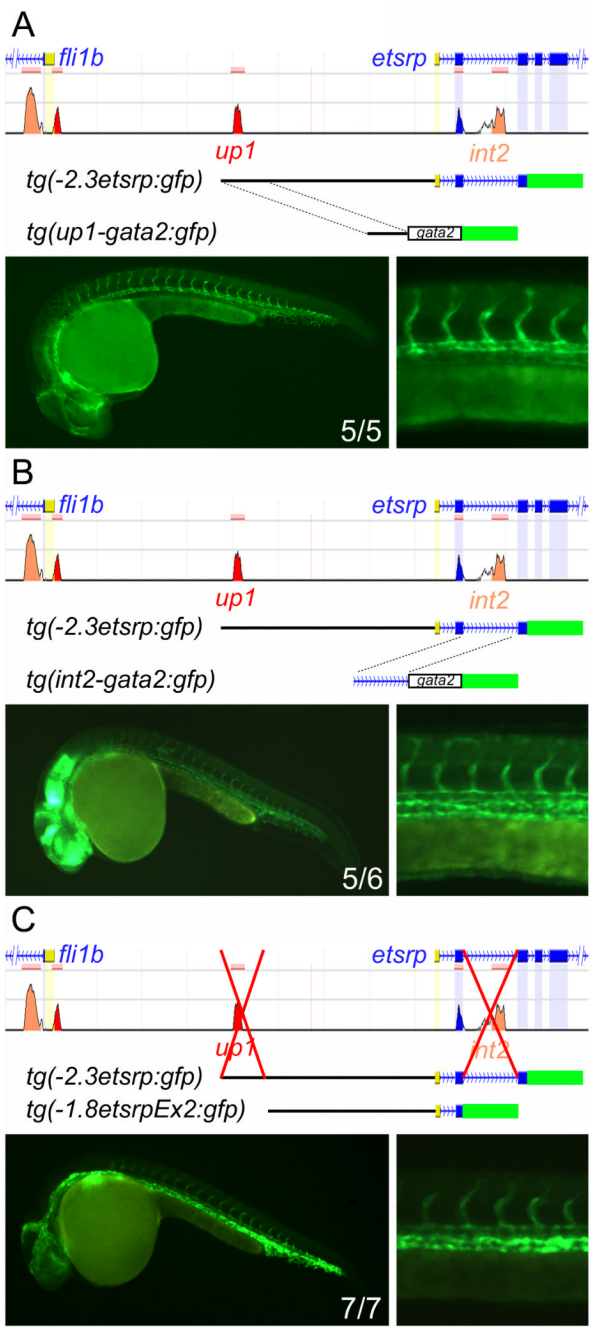

Figure 2. Evolutionarily conserved regions *up1* and *int2* are sufficient but not necessary for *tg(−2.3etsrp:gfp)* expression in endothelial cells

(A and B) The conserved region *up1* (A) or *int2* (B) was placed in front of the minimal *gata2* promoter driving GFP expression and germline transgenics generated. Both regions are sufficient to drive GFP expression in the developing vasculature as demonstrated by fluorescent images of whole embryo and trunk vasculature at 24hpf. (C) *Up1* and *int2* are not necessary for strong vascular expression from the *etsrp* promoter transgene. Deletion of both *up1* and *int2* from the GFP reporter lines does not eliminate vascular expression as demonstrated by *tg(−1.8etsrpEx2:gfp)*, suggesting that more regulatory elements are present. The numbers in the whole embryo image represents the number of germline transgenic fish

lines expressing vascular GFP versus the total number of lines examined. Note that *up1* drives stronger expression in the dorsal aorta and its deletion results in decreased relative expression in the dorsal aorta (A and C).

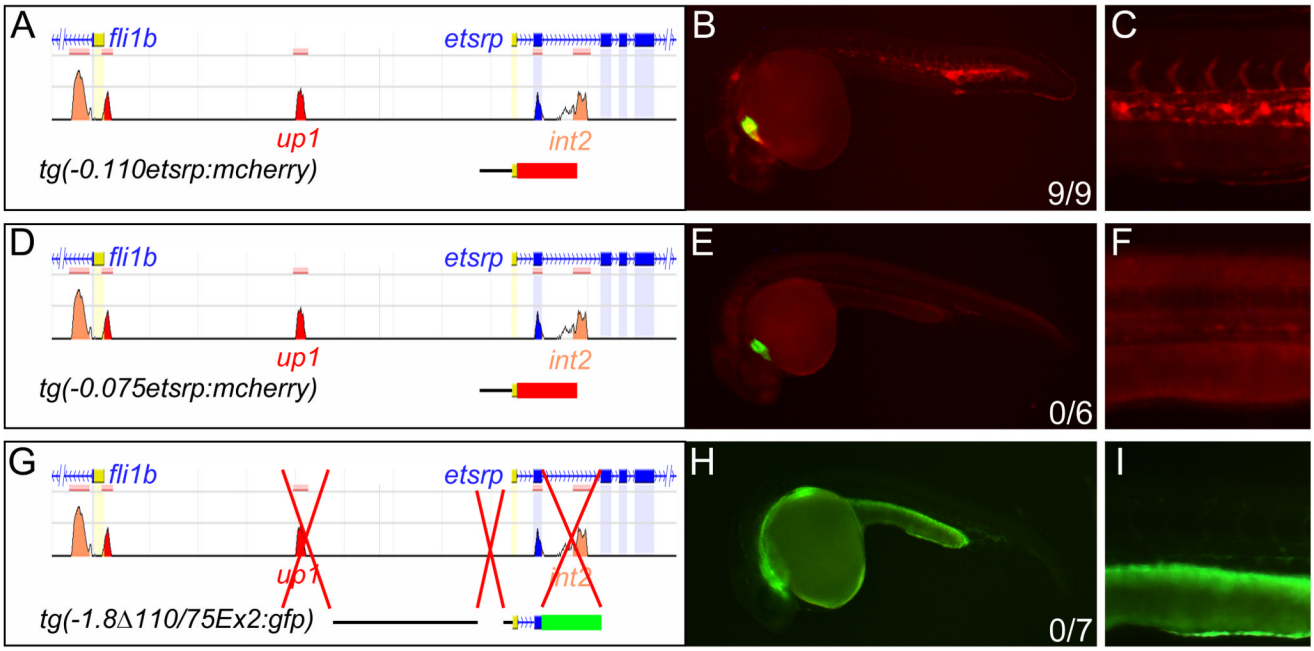

Figure 3. An unconserved region at −110 to −75 accounts for the majority of the remaining promoter activity in endothelial cells

(A-C) *Tg(−0.110etsrp:mcherry)* containing only 110 base pairs of the *etsrp* promoter is sufficient for vascular expression at 24hpf. (D-F) Deletion of 35 base pairs from this site *tg(−0.075etsrp:mcherry)* abolishes expression in the vasculature. (A-F) mCherry was used as a reporter for the element being tested and GFP was driven by a constitutive cardiac promoter in the same transgene to identify transgenic germlines independently of the element being tested. (G-I) Deletion of *up1, int2*, and *−0.110/−0.075, tg(−1.8Δ110/75Ex2:gfp)*, almost completely abolishes expression from the *etsrp* promoter, compare expression in H and I to Figure 2C, suggesting these three regions are critical for high levels of *etsrp* expression in the developing vasculature.

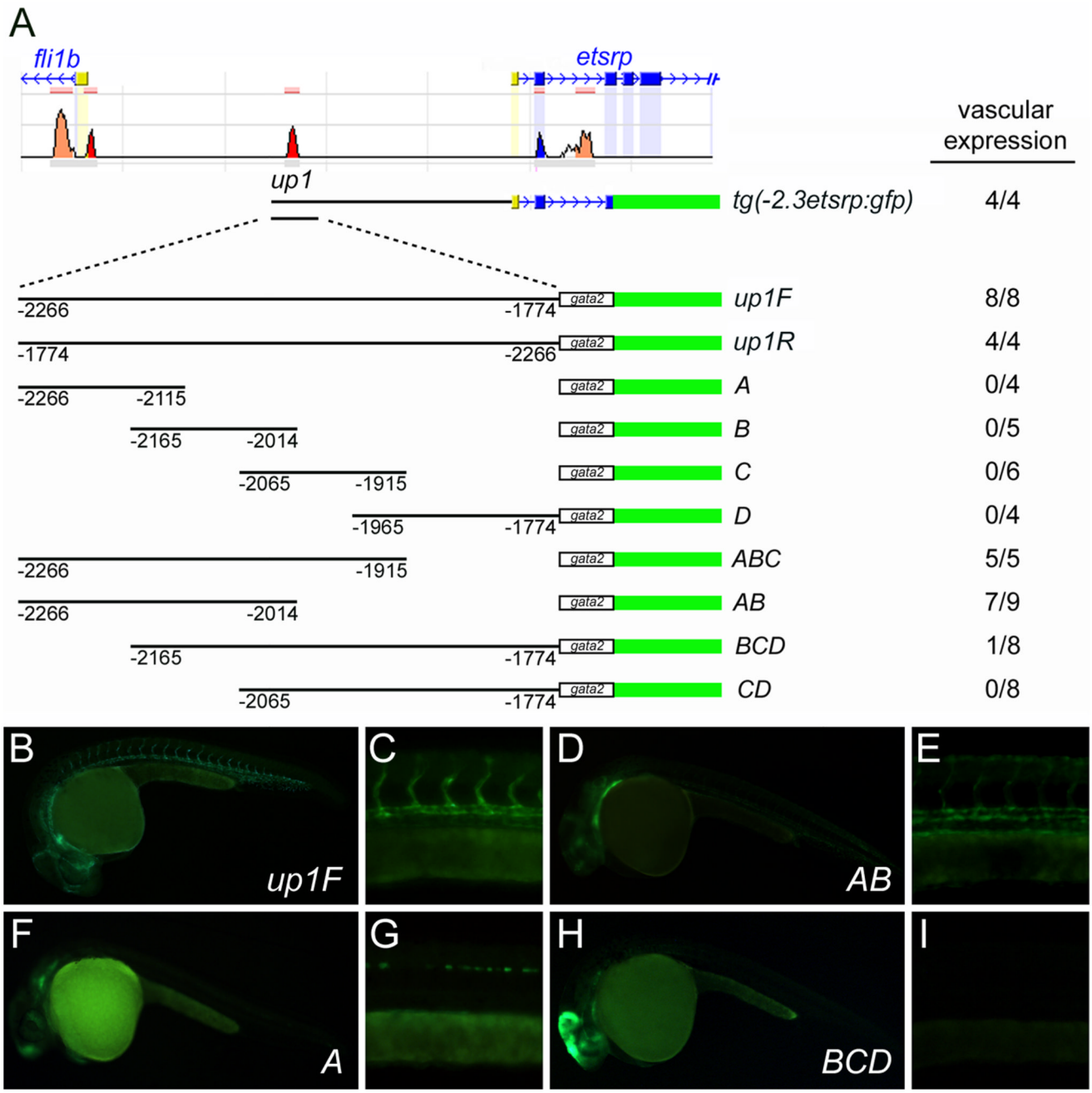

Figure 4. *Up1* enhancer activity is present at −2266 to −2014

(A) Schematic of the *up1* fragments tested for enhancer activity using the *gata2* minimal promoter and GFP reporter. Number of lines demonstrating vascular expression out of total lines examined is noted. (B-I) Fluorescent images demonstrating that fragment *AB* (−2266 to −2014) is the minimal region necessary for vascular expression from the *up1* enhancer. Fragments *A* (F and G) and *BCD* (H and I) exhibit some nonvascular expression presumably due to insertional enhancer trapping effects.

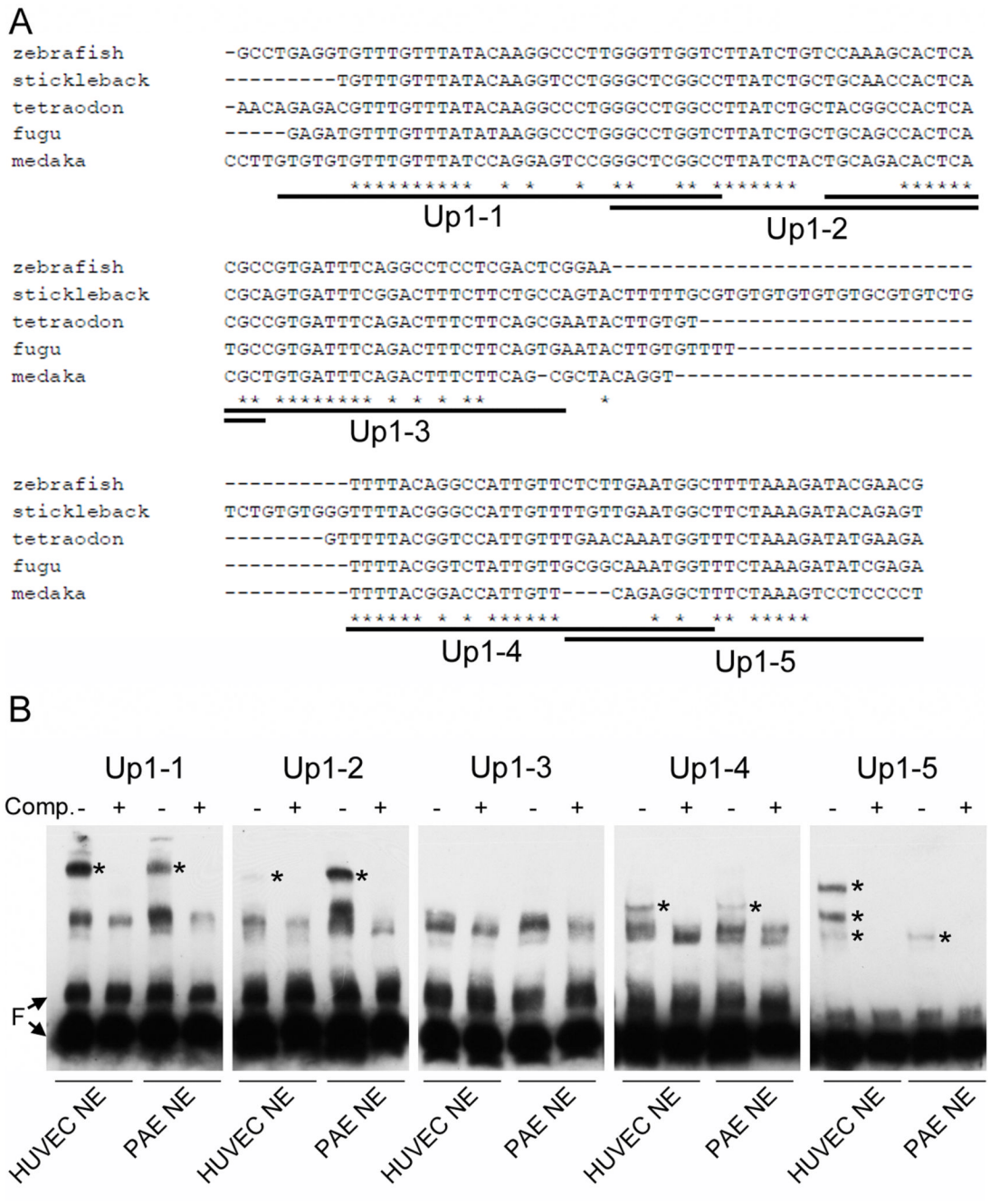

Figure 5. Multiple protein binding sites are present in the *AB* region of the *up1* enhancer

(A) Evolutionarily conserved sequence between different fish species identified using clustalW analysis at the *AB* region. Overlapping EMSA probe sequences are underlined and labeled Up1-(1-5). (B) EMSA using the oligonucleotide probes defined in (A) and nuclear protein extracts, NE, from HUVEC or PAE cells. Unlabeled probe competition, Comp. +/-, was used to define specific binding protein complexes denoted with an asterisk (*). All oligos bound specific, well-defined proteins except for Up1-3. F, free probe.

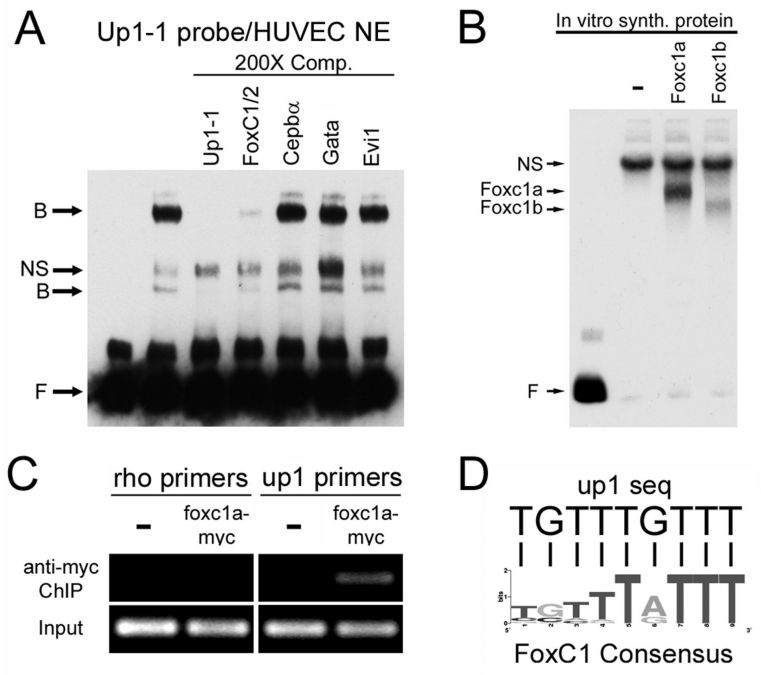

Figure 6. ***Foxc1a/b*** **binds to** ***up1*** **in vitro and in vivo**

(A) EMSA using Up1-1 probe and HUVEC nuclear protein extracts, NE, demonstrates that the FoxC1/2 consensus binding site oligo can compete for binding with the Up1-1 probe while Cepbα, Gata, and Evi1 consensus binding site oligos cannot. B, bound; NS, nonspecific; F, free probe. (B) EMSA demonstrating Up1-1 probe bound to in vitro synthesized Foxc1a and Foxc1b protein. (C) Chromatin immunoprecipitation, ChIP, from wild-type zebrafish embryos (−) or embryos expressing a foxc1a-myc. *Up1* primers detect enrichment in embryos expressing foxc1a-myc while negative control *rhodopsin*, rho, primers do not. (D) The Up1-1 sequence, 5′-TGTTTGTTT-3′, contains a FoxC1 consensus binding site, 5′-(T/G)(G/C)(T/R)(T/Y)T(A/G)TTT-3′.

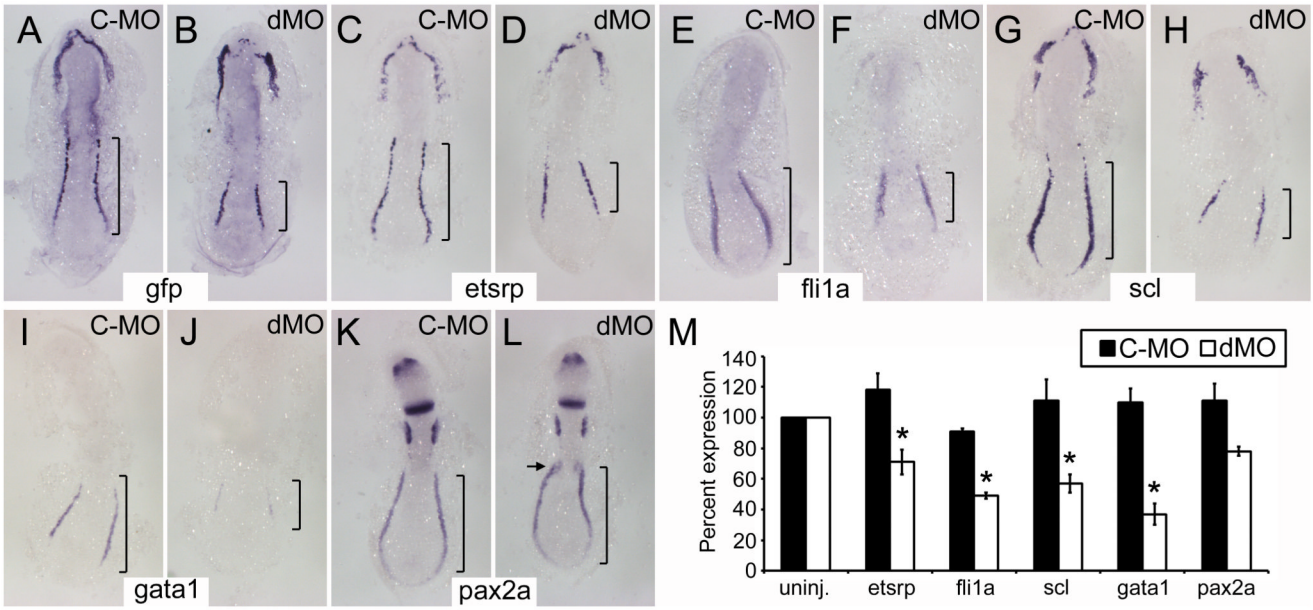

Figure 7. Morpholino knockdown of *foxc1a/b* results in decreased angioblasts and primitive erythrocytes

(A-L) Dorsal view flatmounts, anterior up, of 6-8 somite stage embryos injected with control morpholino, C-MO, or morpholinos to knockdown expression of both *foxc1a* and *foxc1b*, dMO. In situ hybridization for *tg(−2.3etsrp:gfp)* or the genes noted at the center of each pair of panels was performed to assay the affects of *foxc1a/b* knockdown. Representative images of more than 10 embryos per group. Note the decreased expression of *tg(−2.3etsrp:gfp), etsrp, fli1a, scl*, and *gata1* in dMO treated embryos especially at the posterior lateral plate mesoderm (bracketed). Expression of *pax2a* in the intermediate mesoderm is not affected by *foxc1a/b* knockdown except at the pronephric primordia, arrow in L, and nervous system expression is unaffected. (M) Quantitative RT-PCR demonstrates reduced expression of *etsrp, fli1a, scl*, and *gata1* in dMO treated embryos as compared to C-MO treated embryos. All groups were normalized to β-actin and expression in uninjected wildtype embryos was set at 100%. Asterisks (*) denote statistically significant changes as determines with *Students t-test* ($p<0.05$).