Etsrp/Etv2 Is Directly Regulated by Foxc1a/b in the Zebrafish Angioblast

Matthew B. Veldman, Shuo Lin

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 placed in front of a heterologous promoter. The deletion of all 3 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 reverse transcriptase–polymerase chain reaction and in situ hybridization. Decreased expression of primitive erythrocyte genes scl and gata1 was also observed, whereas 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. (Circ Res. 2012; 110:220-229.)

Key Words: angioblast ■ etsrp ■ foxc1a ■ scl ■ zebrafish

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

Original received June 24, 2011; revision received November 21, 2011; accepted November 23, 2011. In October 2011, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15 days.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.111.251298

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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,15 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 3 enhancer regions that are sufficient to drive green fluorescent protein (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. Transgenic plasmids were generated using Tol2Kit plasmids20 and the Multiple Gateway System (Invitrogen). Zebrafish embryos 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 reverse transcriptase–polymerase chain reaction (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 Axiostar 2 plus microscope (Zeiss) or a Stem2000-C (Zeiss) using 5× or 10× objectives with an AxioCam camera and Openlab 4.0 software (Improvision). Adobe Photoshop was used to adjust brightness and contrast and assemble composite images. The Student t test was used to determine significance with P<0.05 for quantitative RT-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 kb of sequence between human, mouse, Xenopus, pufferfish, and zebrafish found very little homology outside of the exonic sequences (unpublished data). However, 2 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 2, was called int2. We generated a transgene, Tg(–2.3etsrp:gfp), that encompassed these 2 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 through 1G). Similarly, at the 18-somite stage, the expression of GFP and etsrp correlate almost identically (Figure 1H through 1M). At 24 hpf, GFP is highly expressed in both the cranial vasculature (Figure 1N through 1Q) and the axial and intersomitic vessels of the trunk (Figure 1R through 1S). By 36 hpf, endogenous etsrp is significantly reduced in the vasculature with the exception of

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ALPM</td>
<td>anterior lateral plate mesoderm</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>DA</td>
<td>dorsal aorta</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<td>FOX</td>
<td>forkhead box</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>hpf</td>
<td>hours post fertilization</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>PAE</td>
<td>porcine aortic endothelial cell</td>
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<td>PCV</td>
<td>posterior cardinal vein</td>
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<td>PLPM</td>
<td>posterior lateral plate mesoderm</td>
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<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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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 germ-line transgenics (Figure 2A and 2B). 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 nonconserved 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 through 3C). Deletion of 35 base pairs from the 5′-end completely abolished vascular specific expression (Figure 3D through 3F). However, the remaining 75 base pair promoter was still capable of driving nonvascular 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 through 3I), suggesting that the combination of these 3 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, whereas deletion of these enhancers resulted in PCV expression greater than DA (Figure 2A through 2C). A summary of the relative transgene expres-

Figure 1. Tg(−2.3etsrp:gfp) contains 2 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 through S, Fluorescent images of Tg(−2.3etsrp:gfp): B through S, 10-somite stage; H through M, 18-somite stage; N through S, 24 hours after fertilization (hpf).
sion 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 4 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, 4B, 4F, and 4G). 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, whereas fragments C and D were dispensable (Figure 4A and 4D through 4I). 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 5 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 3 distinct protein bands in HUVEC extracts, whereas 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 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, whereas the others could not (Figure 6A). To determine if the zebrafish homologs of FoxC1/2, Foxc1a and Foxc1b, could bind to Up1–1, we synthesized these proteins in vitro and performed EMSA with the Up1–1 probe. Both Foxc1a and Foxc1b were able to bind to Up1–1, whereas 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. Because 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 gastrulation 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- to 2-somite stage.3 We performed double fluorescent in situ hybridization to examine colocalization of foxc1a and etsrp. At the 8- to 10-somites stage we noted colabeling 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 cofactors 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 foxc1alb played a larger role in regulating etsrp, we tested the effect of their knockdown on tg(−2.3etsrp:gfp) expression and endogenous etsrp expression by in situ hybridization at the 6- to 8-somite stage. Morpholino knockdown of foxc1alb significantly reduced the expression of both tg(−2.3etsrp:gfp) and etsrp, most notably in the PLPM (bracketed in Figure 7A through 7D). 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; in primitive erythroid cells, fli1a, scl, and gata1 function independent of etsrp. Fli1a, scl, and gata1 expression are all reduced in foxc1alb morphants (Figure 7E through 7J). To test whether other mesoderm or nonmesodermal 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 foxc1alb morphants, with the exception of the pronephric primordium (Figure 7K through 7L).30 Addi-
ional paraxial mesoderm genes mespa, mespb, deltaC, and par1 were examined in foxc1a/b morphants and behaved as previously reported (Online Figure VIII). 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 is 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 2 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 2 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 toward 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 3 enhancers present. Although we have found that 
tg(-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 have not been described. 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, whereas deletion of up1 had relatively little effect on transgene expression. One possible explanation for this finding is that foxc1a/b binds at nonconserved sites or is recruited to the promoter through nondirect 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 Env2 null phenotype; although FoxC1/FoxC2 double null mice have visible blood, Env2 null mice are completely anemic. The genetic
The 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. In fact, a mammalian Mef2c enhancer containing this double binding site is capable of driving expression in zebrafish vasculature. 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. Although this site probably drives expression in angio blasts, 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. 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. 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 because FOXC1 is linked to Axenfeld-Rieger anomaly and glaucoma, whereas FOXC2 is linked to lymphedema-distichiasis syndrome both diseases are associated with circulatory defects. Recently, FOXC2 has been suggested to be an important mediator of tumor angiogenesis. The link between FOXC1, FOXC2, and
ETV2 in these diseases may be a clinically important avenue of study in the future.

Acknowledgments

We thank Anqi Liu for maintenance of zebrafish lines, the UCLA Orthopedic Hospital Research Center for the use of their real-time quantitative PCR machine, Andrew Harmon for helping generate the tgf(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 grants NIH-T32 HL08634 and NIH-T32 HL69766. S.L. was supported by grant NIH-5R01DK054508-13.

Disclosures

None.

References

Novelty and Significance

What Is Known?
- Etsrp/etv2 is a member of the ETS family of transcription factors that regulates 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 nonendothelial cells.

What New Information Does This Article Contribute?
- A new transgenic zebrafish line was developed using the etsrp/etv2 promoter to drive GFP expression in the developing vasculature. Within the promoter sequence we 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 expression of etsrp/etv2 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. We report that foxc1a/b is upstream of etsrp/etv2 in the genetic hierarchy of endothelial cell development. This provides a direct genetic link between the mesoderm to endothelial cells. Previous work has demonstrated that the BMP, Notch, and Wnt signaling pathways regulate etsrp/etv2 in embryonic stem cells. It will be of interest in future studies to determine whether the identified regulatory regions respond to these pathways through Foxc1a/b or other transcription factors in vivo.

Etsrp/etv2 is a critical regulator of blood vessel development; however, little is known about how this gene is itself regulated.
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Circ Res. 2012;110:220-229; originally published online December 1, 2011;
doi: 10.1161/CIRCRESAHA.111.251298
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Online Figure I. *Tg(-2.3etsrp:gfp)* expression is similar to *tg(kdrl:gfp)* at 24 hpf but very different at 48hpf. (A and B) *Tg(-2.3etsrp:gfp)* is expressed throughout the vasculature at 24hpf but is downregulated by 48hpf with strong expression limited to the aortic arches. Some ectopic expression is visible in the retina and spinal cord at 48 hpf (B) probably due to insertional effects. (C and D) *Tg(kdrl:gfp)* is strongly expressed in the vasculature at both 24hpf and 48hpf. The differences in expression between these transgenes are reflective of the endogenous expression pattern.
Online Figure II. The -2.3etsrp promoter drives expression in early angioblasts co-expressing tg(fli1a:gfp). Tg(fli1a:gfp) is a well established transgenic line that expresses gfp in the early angioblasts of the ALPM and PLPM. To confirm that the -2.3etsrp promoter is driving expression in angioblasts tg(-2.3etsrp:mCherry) was crossed to tg(fli1a:gfp) and expression was examined at 10-12 somite stage. Embryos were de-yolked and flatmounted with anterior to the left. Co-labeling, indicated by white arrows, was identified in both the ALPM (A-C) and PLPM (D-F). Asterisk in D-F labels double positive angioblasts beginning to migrate medially to form the axial vessels.
Online Figure III. Both up1 and int2 are functional enhancers when placed in the reverse orientation upstream of the gata2 minimal promoter driving GFP expression. Left panels are whole mount embryos and right panels are close-up images of the trunk above the yolk extension. Vascular expression is visible throughout the embryo including the dorsal aorta, caudal vein, and intersomitic vessels. The ratios represent the number of vascular positive germlines over the total number of GFP positive germlines observed.
Online Figure IV. Transient transgenic deletion analysis of the etsrp promoter localizes an enhancer between -0.110kb and -0.075kb. (A-D) GFP reporter expression is present in the axial vessels (arrows) and intersomitic vessels (arrowheads) of 24hpf embryos injected with the indicated promoter:gfp transgene. (D) The first intron of etsrp was replaced by the intron of the minimal gata2 promoter. (E-H) mCherry reporter expression is present in both the axial vessels and intersomitic vessels of transgene injected embryos except the -0.075etsrp promoter transgene in panel H. This suggests an endothelial cell specific enhancer is present between -0.110kb and -0.075kb at the etsrp promoter.
Online Figure V. *Foxc1a* and *etsrp* are co-expressed in the angioblast. Double fluorescent in situ hybridization was performed on 8-10 somite stage embryos for *etsrp* (green) and *foxc1a* (red) mRNA expression. A composite image of a flat mounted embryo (A) demonstrates strong expression of *etsrp* in the ALPM and PLPM and *foxc1a* expression in the presomitic mesoderm, somitic mesoderm, and other anterior tissues as previously reported. Boxed areas in A (B, C, and D) are shown at higher magnification. Closer examination of areas of *etsrp* expression identified regions of overlapping expression in the ALPM (arrows in B'-B'''') and rostral PLPM (arrows in C'-C'''''). Weak to no overlap in expression was seen in the caudal PLPM (D'-D'''''). Interestingly the identified areas of co-expression are the same areas most affected in *foxc1a/b* morphants (see Figure 7). Images were collected on a Zeiss LSM510 confocal microscope using 10X (A) and 20X with 2X optical zoom (B'-D'''''). B'-D''''' are projections of ~10 µm stacks which are the approximate diameter of a single cell.
Online Figure VI. FoxC1a and FoxC1b are not necessary for ectopic induction of \(tg(kdrl:gfp)\) by Etsrp overexpression. (A) Uninjected \(tg(kdrl:gfp)\) embryos are GFP negative at \(~75\%\) epiboly. (B) Overexpression of Etsrp induces ectopic expression of \(tg(kdrl:gfp)\). (C) FoxC1a/FoxC1b double morpholino does not affect \(tg(kdrl:gfp)\) at \(~75\%\) epiboly. (D) Morpholino knockdown of foxc1a and foxc1b does not affect the ability of etsrp overexpression to induce expression of \(tg(kdrl:gfp)\).
Online Figure VII. \textit{Tg(up1-gata2:gfp)} expression is lost when \textit{foxc1a} and \textit{foxc1b} are knocked down. (A) GFP is expressed in the vascular cord, the precursor of the axial vessels (arrows), in \textit{tg(up1-gata2:gfp)} transgenic fish at \textasciitilde 16 somite stage. (B) Knockdown of \textit{foxc1a} and \textit{foxc1b} decreases GFP expression driven by the \textit{etsrp up1} enhancer region.
Online Figure VIII. Morpholino knockdown of *foxc1a/b* causes specific paraxial defects as previously reported. To control for morpholino specificity and toxicity, previously described changes in *mespa* (A and B), *mespb* (C and D), *deltaC* (E and F), and *par1* (G and H) were examined by in situ hybridization in control morpholino (C-MO) (A, C, E, and G) or *foxc1a/b* double morpholino injected (dMO) (B, D, F, and H) embryos at ~6 somite stage. Images show flatmounts of the posterior half of each embryo with anterior up. *Mespa* is unaffected while *mespb* and *par1* are significantly reduced. *DeltaC* is absent in the somitic mesoderm but remains in presomitic mesoderm. Reduce expression of *mespb* and *par1* was supported by quantitative RT-PCR results (I). *DeltaC* was not included in the quantitative RT-PCR analysis because the strong presomitic mesoderm expression would likely mask the relatively small change in the somitic mesoderm. These results are in agreement with those previously published by Topczewska et al., (2001).1
Online Tables

Online Table I. Relative transgene expression level in the axial vasculature at 24 hpf.

<table>
<thead>
<tr>
<th>Transgene promoter</th>
<th>DA &gt; PCV</th>
<th>DA = PCV</th>
<th>DA &lt; PCV</th>
<th>Total lines examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.3etsrp</td>
<td>1 (25%)</td>
<td>2 (50%)</td>
<td>1 (25%)</td>
<td>4</td>
</tr>
<tr>
<td>-1.8etsrp</td>
<td>1 (25%)</td>
<td>2 (50%)</td>
<td>1 (25%)</td>
<td>4</td>
</tr>
<tr>
<td>-1.8Ex2</td>
<td>0</td>
<td>3 (43%)</td>
<td>4 (57%)</td>
<td>7</td>
</tr>
<tr>
<td>-0.110etsrp</td>
<td>0</td>
<td>0</td>
<td>3 (100%)</td>
<td>3</td>
</tr>
<tr>
<td>up1</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>AB</td>
<td>4 (67%)</td>
<td>2 (33%)</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>int2</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Germline transgenic embryos were observed at 24 hpf for GFP fluorescence levels in the axial vasculature above the yolk extension. Deletion of up1 and int2 in -1.8Ex2 and -0.110etsrp results in stronger expression in the PCV while isolated enhancers up1, AB (a sub-sequence of up1), and int2 drive stronger expression in the DA. Dorsal aorta, DA; posterior cardinal vein, PCV.
Online Table II. Primers used to generate transgene vectors.

<table>
<thead>
<tr>
<th>Transgene vector</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.3etsrp</td>
<td>B4F 5’-ggggacaacttttgataagaaatggtcagtaagcagactccttcacaatc-3’</td>
</tr>
<tr>
<td></td>
<td>B1R 5’-ggggactgttttttgataacacttgctgcggatctgttggt-3’</td>
</tr>
<tr>
<td>-1.8Ex2</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagactccttcacaatc-3’</td>
</tr>
<tr>
<td></td>
<td>B1R 5’-ggggactgttttttgataacacttgctgcggatctgttggt-3’</td>
</tr>
<tr>
<td>-1.3Ex2</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagacggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>-0.8Ex2</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagaccttgggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>-0.3Ex2</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagaccttgggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>-0.3etsrp-gata2int</td>
<td>Gata2int F 5’-ataaagactcagtaacatttaaggtgagtaacctccgttagttatttg-3’</td>
</tr>
<tr>
<td></td>
<td>Gata2int R 5’-caaaataactccgggaagactcactcatttaaagttgtcagttacttat-3’</td>
</tr>
<tr>
<td></td>
<td>Gata2int B1R 5’-ggggactgttttttgataacacttgctgcggatctgttggt-3’</td>
</tr>
<tr>
<td>-0.225etsrp</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagaccttgggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>-0.145etsrp</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagaccttgggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>-0.110etsrp</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagaccttgggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>-0.075etsrp</td>
<td>B4F 5’-ggggacaactttgatagaaatggtcagtaagaccttgggtgacgtgaaccgagtc-3’</td>
</tr>
<tr>
<td>up1</td>
<td>F 5’-gagagactcagtcagtcagacagttggaagact-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td>int2</td>
<td>F 5’-gagagactcagtcagacagttggaagact-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td>A</td>
<td>F 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td>B</td>
<td>F 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td>C</td>
<td>F 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td>D</td>
<td>F 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-gagagactcagtcagacagttggaagactcctcc-3’</td>
</tr>
</tbody>
</table>

Underlined sequences are gateway system recombination sites or Bam HI or Sal I restriction enzyme sites for cloning. -0.3etsrp-gata2int was generated by fusion PCR using -0.3Ex2 B4F, gata2int F, gata2int R, and gata2int B1R primers. -0.225etsrp to -0.075etsrp were generated using -0.3etsrp-gata2int as a template and the B4F primers with Gata2int B1R and the resulting product was recombined into pDONR P4-P1R.
Online Table III. EMSAs probe oligos.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Probe sequence</th>
</tr>
</thead>
</table>
| Up1-1      | F 5'-'gaggttgttgttatataacagagcccttgcttggtg-3'  
             | R 5'-'accaaccaagggccttgatataacaccc-3'         |
| Up1-2      | F 5'-'gggttgctttatcgttctcaacagactcaagcc-3'    
             | R 5'-'ggcctgctggcttggacagatataaccc-3'         |
| Up1-3      | F 5'-'caagccagctacgctgtgtgtcctgct-3'          
             | R 5'-'gtcagagagctggctaatcaaggctgtggtt-3'      |
| Up1-4      | F 5'-'acctgtggctgttatggtg-3'                  
             | R 5'-'gccatcagaaagctaagcc-3'                  |
| Up1-5      | F 5'-'ctctgtgatggtttaaagataaggacg-3'         
             | R 5'-'gtttcggcttgtttaaagccattcaagaga-3'      |
| FoxC1/2    | F 5'-'ggggagagccgctttgctggctgagatctgc-3'     
             | R 5'-'gcacagatggccaaacacaggtgctgccctc-3'      |
| Cebpα      | F 5'-'gatccatcccttgtgcccaataaggctaaagac-3'    
             | R 5'-'gtcctgttcctgcttgatgaaggtgatc-3'         |
| Gata       | F 5'-'gatcgggcaactgataaggctaaagc-3'          
             | R 5'-'tgagatctcttcatgccctggtgccctg-3'         |
| Evi1       | F 5'-'gatctgtgccaagaaggtccgttc-3'            
             | R 5'-'tccagtctctctttgccagccatc-3'            |
**Online Table IV. qPCR primers.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
</table>
| **β-actin** | F 5’-tgttttccctccattgttg-3’  
R 5’-acatacatggeagggtt-3’ |
| etsrp | F 5’-gaggaattctggaaggatgg-3’  
R 5’-tggtgtttaagccacctagc-3’ |
| scl | F 5’-ggagatgccgaacagatgg-3’  
R 5’-gaaggcacctgcctcatttc-3’ |
| gata1 | F 5’-atggagaactctctgagctctc-3’  
R 5’-tttccagaattgagatgag-3’ |
| pax2a | F 5’-ggcagctacccacctct-3’  
R 5’-tccttcagctggcgtccc-3’ |
| mespa | F 5’-ctcgagctggctgaagat-3’  
R 5’-cgcttcgctgggaatga-3’ |
| mespb | F 5’-gggagccgtatgagagggtt-3’  
R 5’-cgcccccccgttttggg-3’ |
Online Table V. ChIP primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| up 1        | F 5’-ggcgtgagtgctttggac-3’  
              | R 5’-aagcctcgcacagtttgg-3’ |
| rhodopsin   | F 5’-gactccacacaatcgcaacat-3’  
              | R 5’-accacctaagtaagaaacca-3’ |

Detailed Materials and Methods

Zebrafish husbandry and strains

Zebrafish embryos were maintained and staged as described. Wild-type strain zebrafish were originally purchased from Scientific Hatcher. The University of California, Los Angeles Animal Care and Use Committee approved all protocols used in this study.

Molecular cloning of transgene vectors

Transgene plasmids were generated using Tol2Kit plasmids and the Multisite Gateway System (Invitrogen). 5’-entry vectors of etsrp promoter sequences were generated by recombining PCR products of the desired genomic sequence flanked by B4-Fwd and B1-Rev sequences with the pDONR P4-P1R vector in a BP reaction. The resulting 5’-entry vectors were recombined with pME-EGFP, p3E-pA, and pDestT2pA in the LR reaction to generate EGFP reporter transgenes flanked by Tol2 sequences for genomic integration. Proximal promoter analysis was performed using a mCherry reporter from the pME-mCherry vector recombined into the pDestT2CG2 vector, which contains a constitutively expressed cardiac EGFP marker, with the desired 5’ promoter entry vector and p3E-pA. For testing potential enhancers with a heterologous promoter, a vector was generated encoding a multiple cloning site (MCS) upstream of the gata2 minimal promoter driving EGFP expression enabling the cloning of candidate enhancer regions upstream of the EGFP reporter. A middle entry vector was generated harboring the gata2 minimal promoter and this vector was recombined with p5E-MCS, p3E-EGFPpA, and pDestT2pA to generate a Tol2 vector with unique Bam HI and Sal I restriction enzyme sites available for cloning. Putative enhancer sequences were PCR amplified with flanking Bam HI and Sal I sites, digested, and ligated into the reporter vector. A list of vectors and primers used is available in the Online Table II.

Microinjection of transgenes, mopholinos, and mRNAs

Zebrafish embryos were microinjected at the one cell stage using a Picolnjector PLI-90 (Harvard Apparatus). To generate transgenic germlines, 20 pg of vector was co-injected with 20 pg of Tol2 transposase mRNA per embryo in a final volume of 2 nL. Generally 50 embryos were injected and raised for each transgene with ~50% of those resulting in founders. Founders were identified by visual inspection of EGFP fluorescence at 24 hpf. Screening for founders stopped after >5 independent lines had been identified. mRNA for injection was generated using the mMessage mMachine Kit (Ambion) according to the manufacturer’s suggested protocol. Morpholino oligos were obtained from GeneTools LLC. Morpholino’s used include the standard control morpholino (5’-CCTCTTACCTACGTTACAATTTATA3’), foxc1a MO2 (5’-CCTGCACTGCTCTTCAAACCGG-3’), and foxc1b MO1 (5’-GCATCGTACCCCCCCGGTACA-3’). These morpholinos have been previously reported to be specific and effective. Combined foxc1a MO2/foxc1b MO1 injections were at a volume of 2 nL and 4 ng of each per embryo. Control morpholino was injected at 8 ng per embryo.

Electrophoretic Mobility Shift Assay (EMSA)
EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s suggested protocol. Nuclear protein extracts from PAE and HUVEC cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) as recommended. Protein concentration was assayed using the BCA protein assay (Pierce) and 5 ug was used in each reaction. In vitro synthesized Foxc1a, Foxc1b, and mCherry protein was created using the TnT in vitro transcription/translation kit (Promega) with plasmids pCS2mCherry, pCS2foxc1a, and pCS2foxc1b. For each EMSA reaction 25 fmol of biotinylated oligo probe, labeled with the Biotin 3’ End DNA Labeling Kit (Pierce), was used and 5 pmol of unlabeled competitor oligo was used. Oligos were supplied by Integrated DNA Technologies (Online Table III).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed on chromatin isolated from 100 50% epiboly embryos injected with 100 pg of myc-tagged foxc1a mRNA or uninjected control embryos. ChIP was repeated on a second clutch of embryos independent of the first with similar results. Primers for ChIP PCR are contained in Online Table V.

Detailed ChIP protocol.

Dynabeads ChIP protocol adapted from Junji Lin’s protocol from Richard Dorsky’s Lab (University of Utah) found on the ZFIN protocols web page (https://wiki.zfin.org/display/prot/Chromatin+Immunoprecipitation+%28ChIP%29+Protocol+using+Dynabeads).

**Dynabeads ChIP Protocol**

**<Prior to Day1>**
Coating protein G beads
1. Take enough dynabeads slurry (30 µl - 50 µl each sample) (Invitrogen #100-01D)
2. Separate beads and buffer with Magnetic Separation Stand (Promega # Z5332)
3. Remove the supernatant with loading tip
4. Wash three times with 1 mL IP dilution buffer
5. Resuspend beads in 1 mL pre-blocking buffer
6. Incubate at 4°C, greater than 2 hr or O/N
7. Separate beads and buffer with Magnetic Separation Stand and remove the sup.
8. Wash twice with 1 mL IP dilution buffer
9. Add IP dilution buffer back to original volume

**<Day 1>**
Cross-linking of protein and DNA
1. Dechorinate 100 embryos in 1xER (put dechorinated embryos in cold HBSS (Invitrogen #14170-112) on ice)
2. Add 1 mL of 1% formaldehyde in 1xPBS in 1.5 mL tube
3. Rotate 15 min at RT, remove sup.
4. Add immediately 143 µL 1M glycine and spin down. Remove the sup.
5. Add 0.125 M glycine to soak embryos, rotate 10 min at RT
6. Rinse embryos with 1 mL cold 1xPBS twice, centrifuge at 4°C.

Cell lysis and chromatin DNA extraction
1. Add 600 µL Cell lysis buffer (for 100 embryos), on ice 10–20 min
2. Pipet up and down every 5-10 min until no clear tissue can be visible (bone structure might be still there, but most of the tissue should be broken and dissolved)
3. Cfg: 3.5k rpm, 5 min at 4°C, remove the sup.
4. Rinse once more with 600 µL Cell lysis buffer and spin down, remove the sup.
5. Re-suspend the nuclear pellet (white) in 200 µL nuclei lysis buffer
6. Pipet up and down to disrupt clumps
7. On ice 10~20 min (lay the tube on ice to avoid precipitation of SDS in the nuclei lysis buffer)
8. Add 400 µL IP dilution buffer + proteinase inhibitors
9. Freeze at -70 or -80°C

**Sonication**
1. Thaw sample on ice and divide equally into two microfuge tubes, 300 µL each.
2. Sonicate using a Bioruptor as recommended by the manufacturer on high setting for 10 pulses of 30 sec. on 30 sec. off.
3. Take 10 µL for agarose gel electrophoresis to check the size of fragmented DNA after decross-linking
4. Cfg: 14k rpm, 15 min at 4°C
5. Take the supernatant and combine in one tube, add IP dilution buffer to 600 µL per tube.
6. Divide for ChIP and total input control. (For example: total amount after sonication is 600 µl, using 500 µl for ChIP assay and 100 µl for input control for each sample)
7. Store input sample at -20°C.

**Antibody binding**

- **Pre-clear:**
  1. Add 20-30 µL blocked dynabeads (in pre-Day1) to the ChIP sample
  2. Rotate at 4°C, > 2hrs

- **Antibody binding:**
  1. Separate beads and buffer with Magnetic Separation Stand.
  2. Take the supernatant (Do not contaminate with any beads) and divide it equally as antibody and no-antibody control.
  3. Add antibody 5 µg to the sample (Keep the no-antibody control sample at 4°C without adding any antibody) rotate O/N at 4°C.

**<Day 2>**

**Dynabeads binding, washing, elution, and decross-linking**

1. Add 30 µL blocked dynabeads to each sample (also to no-antibody control tube)
2. Incubate on a rotating wheel/platform at RT 60-90 min.
3. Separate beads and buffer with Magnetic Separation Stand.
4. Remove supnatant (Beads from no-antibody tube could serve as “no Ab” negative control after washing and elution)
5. Wash the beads twice with 1 mL 1x dialysis buffer
6. Add 1 mL buffer
7. Rotate 15 min at RT
8. Separate beads and buffer with Magnetic Separation Stand.
9. Remove the supernatant as much as possible with loading tip
10. Wash the beads twice with 1 mL IP wash buffer (same as above)

(Comment: Washing step could be held at 4°C with longer time; If non-specific binding still occur, wash 3 times for each buffer followed by 1~3 times TE wash)

**Elution and decross-linking**

1. Add 150 µL elution buffer to the beads
2. Incubate in 65°C water bath for 10-15min (vortex every 2-3 min)
3. Separate beads and buffer with Magnetic Separation Stand.
4. Transfer the supernatant to a new tube
5. Repeat elution steps again and combine both elutions (300 µL)
6. Add 30 µL 3 M NaCl and 1 µL 10 mg/mL RNaseA (For input sample, add right amount of NaCl and RNaseA)
7. Incubate 65°C, 4-5 hr or O/N (input control samples also need to be decross-linking at the time)
8. Add 2 µL 5 mg/mL glycogen and 2.5V absolute EtOH, -80°C O/N

<Day 3>
**Proteinase K treatment**
1. Dissolve pellet in TE, and mix with 5x PK buffer PK(10 mg/mL)
   - For ChIP sample 100 µL 25 µL 2 µL
   - For Input sample 200 µL 50 µL 2 µL
   - 45°C, 1-2 hr
2. Add TE to 300 µL
3. Add 300 µL phenol/CHCl3, vortex, 14k rpm, 5 min at RT, take the supernatant
4. Add 300 µL CHCl3, vortex, 14k rpm, 5 min at RT, take the supernatant
5. Add 3 M NaCl 54 µL, 5 mg/mL glycogen 2 µL, 2.5V absolute EtOH
6. -80ºC, O/N

<Day 4>
**Precipitation and PCR**
1. 14k rpm, 20 min at 4°C
2. Dissolve pellet in 10-30 µL TE or ddH2O
3. Further DNA purification can use QIAquick PCR purification Kit if necessary. But it will also lose quite a bit amount of DNA.
4. DNA can be stored at -20°C
5. qPCR with ChIP sample, no-Ab control and total input.

**Whole mount in situ hybridization**
Whole mount in situ hybridization was performed as described using DIG labeled riboprobes (Roche) generated from linearized plasmid. The following probes were used: etsrp, scl, gata1, and pax2a. Fluorescent whole mount in situ hybridization was performed as previously described using DIG labeled etsrp probe and DNP labeled foxc1a probe.

**Imaging**
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. Confocal imaging was done on a Zeiss LSM510 confocal microscope system.

**Quantitative PCR (qPCR)**
Real time qPCR was performed using FastStart SYBR Green Master Mix (Roche) on a Stratagene Mx3005P qPCR system. RNA was isolated using Trizol reagent (Invitrogen) and cDNA generated using Superscript III reverse transcriptase (Invitrogen) with oligo dT primers. Gene expression levels were calculated relative to un.injected controls as previously described. Three independent biological samples were analyzed in triplicate for each experimental and control group. Students t-test was used to determine significance with p<0.05. Gene specific primers are listed in Online Table IV.

**Supplemental References**