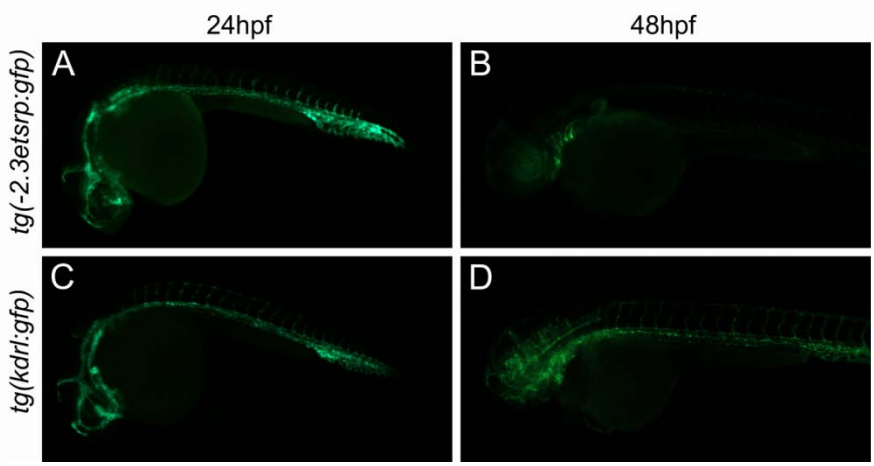
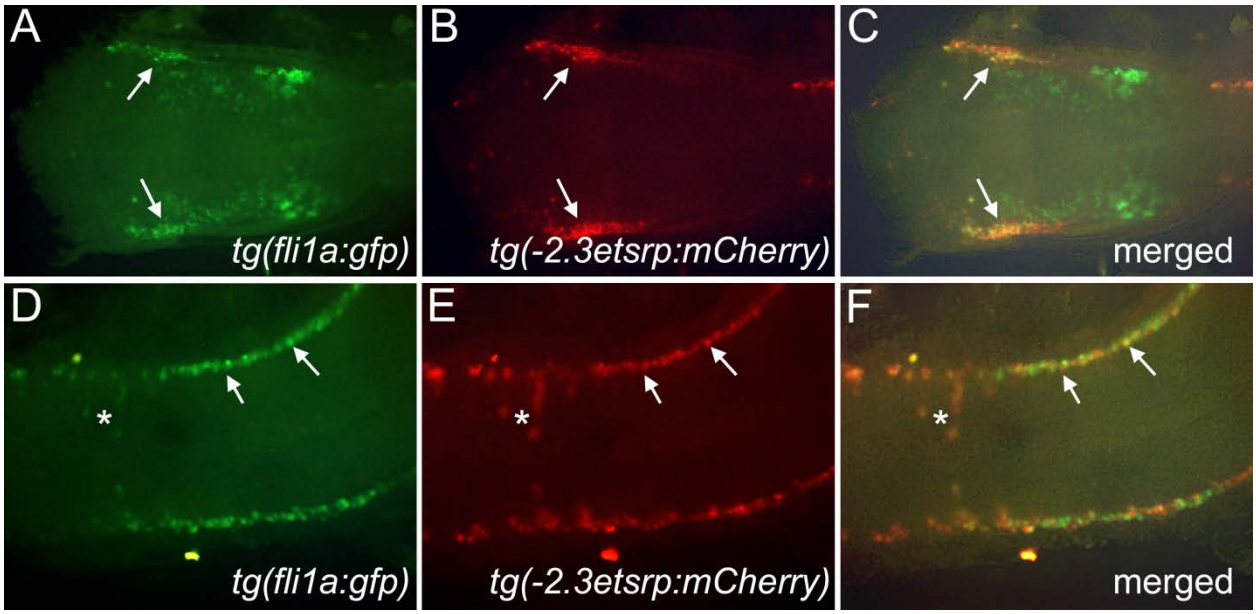
SUPPLEMENTAL MATERIAL

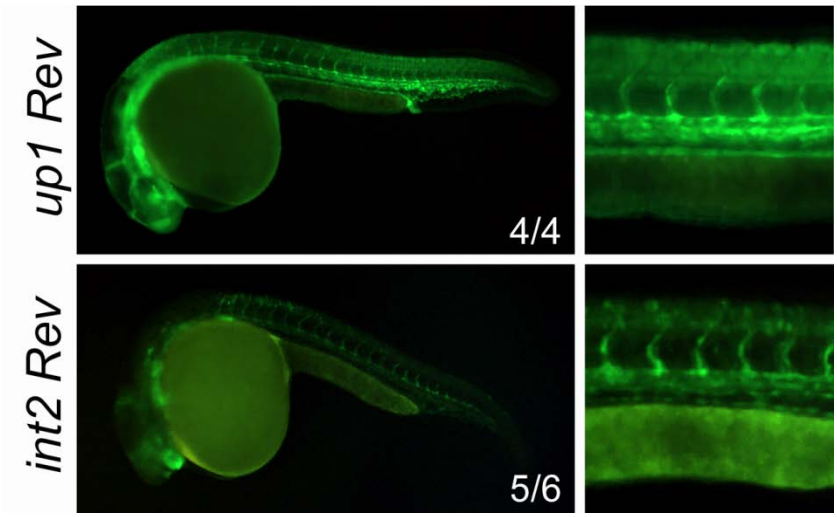
Online Figures and Figure Legends



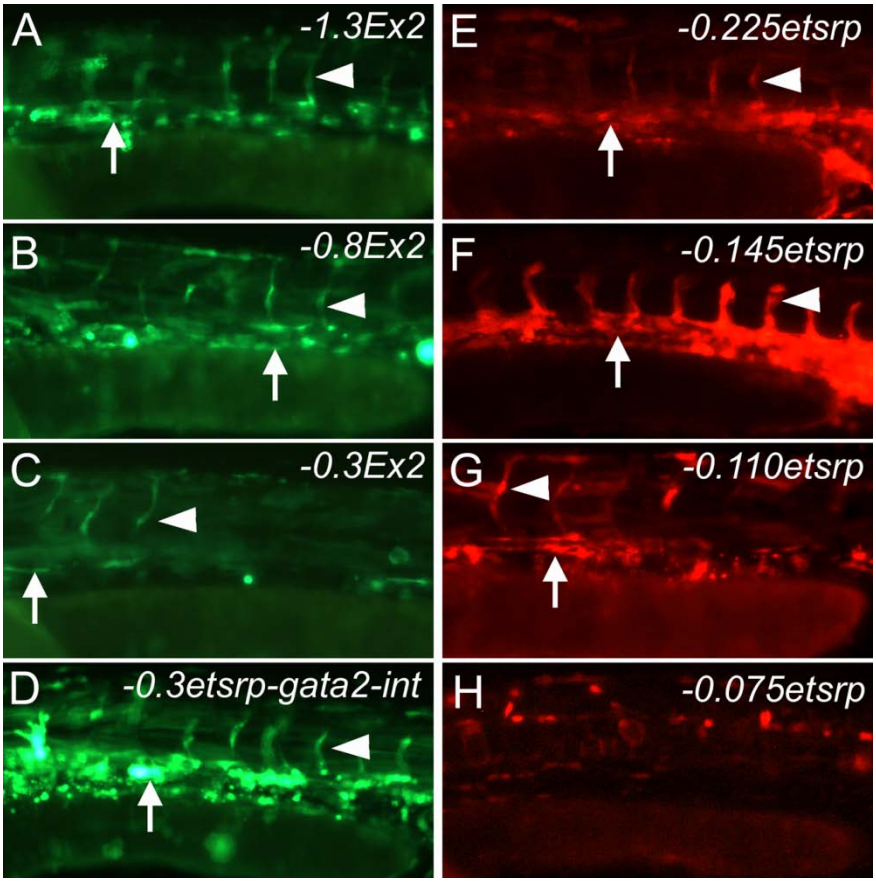
Online Figure I. ***Tg(-2.3etsrp:gfp)*** **expression is similar to** ***tg(kdrl:gfp)*** **at 24 hpf but very different at 48hpf.** (A and B) *Tg(-2.3etsrp:gfp)* is expressed throughout the vascuature at 24hpf but is downregulated by 48hpf with strong expression limited to the aortic arches. Some ectopic 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.



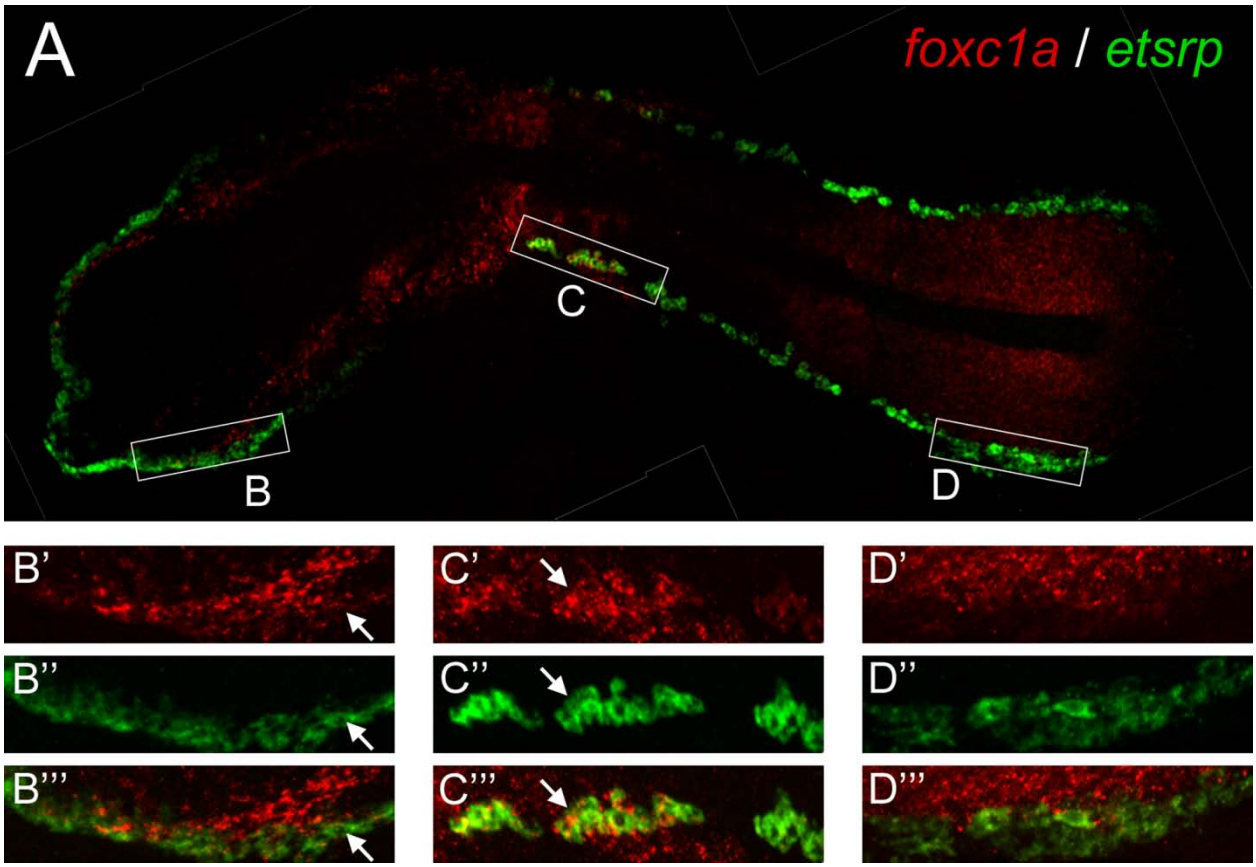
Online Figure II. The -*2.3etsrp* promoter drives expression in early angioblasts co-expressing *tg(fli1a:gfp)*. *Tg(fli1a:gfp)* is a well established transgenic line that expresses gfp in the early angioblasts of the ALPM and PLPM. To confirm that the -*2.3etsrp* promoter is driving expression in angioblasts *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.



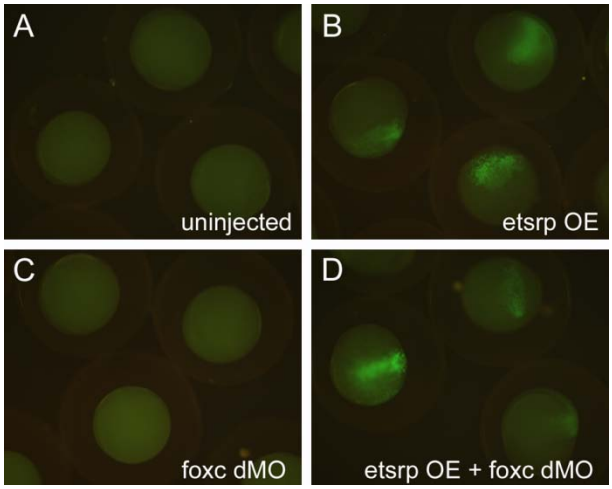
Online Figure III. Both *up1* and *int2* are functional enhancers when placed in the reverse orientation upstream of the *gata2* minimal promoter driving GFP expression. Left panels are whole mount embryos and right panels are close-up images of the trunk above the yolk extension. Vascular expression 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.



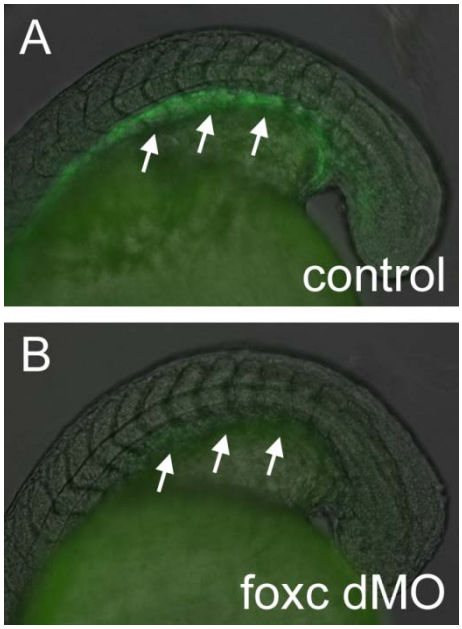
Online Figure IV. Transient transgenic deletion analysis of the *etsrp* promoter localizes an enhancer between -0.110kb and -0.075kb. (A-D) GFP reporter expression is present in the axial vessels (arrows) and intersomitic vessels (arrowheads) of 24hpf embryos injected with the indicated promoter:gfp 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.



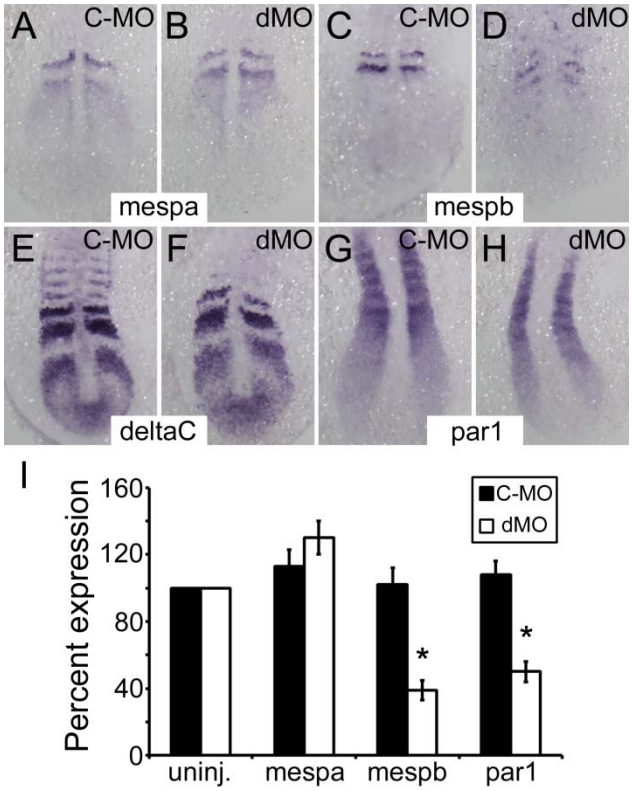
Online Figure V. *Foxc1a* and *etsrp* are co-expressed in the angioblast. Double fluorescent in situ hybridization was performed on 8-10 somite stage embryos for *etsrp* (green) and *foxc1a* (red) mRNA expression. A composite image of a flat mounted embryo (A) demonstrates strong expression of *etsrp* 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 overexression to induce expression of *tg(kdrl:gfp)*.



Online Figure VII. *Tg(up1-gata2:gfp)* expression is lost when *foxc1a* and *foxc1b* are knocked down. (A) GFP is expressed in the vascular cord, the precursor of the axial vessels (arrows), in *tg(up1-gata2:gfp)* transgenic fish at ~16 somite stage. (B) Knockdown of *foxc1a* and *foxc1b* decreases GFP expression driven by the *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 quatitative 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.

Transgene promoter	DA > PCV	DA = PCV	DA < PCV	Total lines examined
-2.3etsrp	1 (25%)	2 (50%)	1 (25%)	4
-1.8etsrp	1 (25%)	2 (50%)	1 (25%)	4
-1.8Ex2	0	3 (43%)	4 (57%)	7
-0.110etsrp	0	0	3 (100%)	3
up1	2 (50%)	2 (50%)	0	4
AB	4 (67%)	2 (33%)	0	6
int2	4 (80%)	1 (20%)	0	5

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.

Transgene vector	Primers
-2.3etsrp	B4F 5'-ggggacaactttgtatagaaaagttgttcagtaagcagactccttcaatc-3' B1R 5'-ggggactgctttttttgtacaaacttgcttcggcatactgctgttgg-3'
-1.8Ex2	B4F 5'-ggggacaactttgtatagaaaagttgagtcatcaagcataaaaaacgttg-3' B1R 5'-ggggactgctttttttgtacaaacttgcatatgaactgaagtcgaaaccagc-3'
-1.3Ex2	B4F 5'-ggggacaactttgtatagaaaagttgcggcctataattaatatcgtt-3'
-0.8Ex2	B4F 5'-ggggacaactttgtatagaaaagttgatgtttagaaactataagaa-3'
-0.3Ex2	B4F 5'-ggggacaactttgtatagaaaagttgctctattatttgctgaaaacat-3'
-0.3etsrp-gata2int	Gata2int F 5'-ataaggactcagtgaaaactttaaggtgagtacttcccggtagttatttg-3' Gata2int R 5'-caaataactaccgggaagtactcaccttaaagttttcactgagtccttat-3' Gata2int B1R 5'-ggggactgctttttttgtacaaacttgctcaagtgtccgcgcttagaaaatgc-3'
-0.225etsrp	B4F 5'-ggggacaactttgtatagaaaagttggaacgtgtctgtcaaatctg-3'
-0.145etsrp	B4F 5'-ggggacaactttgtatagaaaagttgggtcgtgactcctgtctgac-3'
-0.110etsrp	B4F 5'-ggggacaactttgtatagaaaagttgaggaggggaagcgcagtcacg-3'
-0.075etsrp	B4F 5'-ggggacaactttgtatagaaaagttgaagacgcgcggacacgcc-3'
up1	F 5'-gagagactcgagtcgcacagtttggcaactta-3' R 5'-gagagactcgagtagcaacgtttttttatgcttgatg-3'
int2	F 5'-gagagagtcgacgtaatatcttatttcaacat-3' R 5'-gagagagtcgacctggaggtacattatc-3'
A	F 5'-gagagagtcgactcgcacagtttggcaacttttcc-3' R 5'-gagagaggatccttccgagtcgaggaggcctga-3'
B	F 5'-gagagagtcgactatctgtccaaagcactcacg-3' R 5'-gagagaggatccactggaagttagaaacaagatc-3'
C	F 5'-gagagagtcgac-attaaagctaaagttacttgttctg-3' R 5'-gagagaggatcctgataggctgaacctaagttatc-3'
D	F 5'-gagagagtcgaccaaattatcatttaaggctac-3' R 5'-gagagaggatcctagcaacgtttttttatgcttg-3'

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.

Probe name	Probe sequence
Up1-1	F 5'-gaggtgtttgtttatacaaggcccttgggttggt-3' R 5'-accaacccaagggccttgtataaacaaacacctc-3'
Up1-2	F 5'-gggttggtcttatctgtccaaagcactcacgcc-3' R 5'-ggcgtgagtgctttggacagataagaccaaccc-3'
Up1-3	F 5'-caaagcactcacgccgtgatttcaggcctcctcgac-3' R 5'-gtcgaggaggcctgaaatcacggcgtgagtgctttg-3'
Up1-4	F 5'-attttacaggccattgttctcttgaatggc-3' R 5'-gccattcaagagaacaatggcctgtaaaat-3'
Up1-5	F 5'-tctcttgaatggcttttaaagatacgaacg-3' R 5'-cgttcgtatctttaaaagccattcaagaga-3'
FoxC1/2	F 5'-ggggaggagcagcctgtttgttttgccagatctgtgc-3' R 5'-gcacagatctggcaaaacaaacaggctgctcctcccc-3'
Cebpα	F 5'-gatccatatccctgattgcgcaataggctcaaaagatc-3' R 5'-gatcttttgagcctattgcgcaatcagggatatggatc-3'
Gata	F 5'-gatccgggcaactgataaggattccca-3' R 5'-tgggaatccttatcagttgcccggatc-3'
Evi1	F 5'-ggatctccgtgacaagataaggattccctg-3' R 5'-cagggaatccttatcttgtcacggagatcc-3'

Online Table IV. qPCR primers.

Gene	Primers
β-actin	F 5'-tgttttcccctccattgttg-3' R 5'-acatacatggcaggggtgtt-3'
etsrp	F 5'-gaggaattctcgaaggattgg-3' R 5'-tggttttctaaaggcacctagc-3'
scl	F 5'-ggagatgcggaacagtatgg-3' R 5'-gaaggcaccgttcacattct-3'
gata1	F 5'-atggagaactcctctgagccttct-3' R 5'-tttcccagaattgactgagatgag-3'
pax2a	F 5'-ggcagctaccccacctct-3' R 5'-tcataggcagtggcagca-3'
mespa	F 5'-ctcggatgcggtgaagat-3' R 5'-cgtcttgagctgggaatga-3'
mespb	F 5'-gggagcggtatggaggtt-3' R 5'-cgccctcagtttttggtg-3'

Online Table V. ChIP primers.

Primer	Sequence
up1	F 5'-ggcgtgagtgctttggac-3' R 5'-aagcctcgcacagtttgg-3'
rhodopsin	F 5'-gactccacacaatctgcaacat-3' R 5'-accacctacgctaaagaaacca-3'

Detailed Materials and Methods

Zebrafish husbandry and strains

Zebrafish embryos were maintained and staged as described. [2] Wild-type strain zebrafish were originally purchased from Scientific Hatcheries. 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[3] 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 PicoInjector 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′-CCTCTTACCTCAGTTACAATTTATA3′), *foxc1a* MO2 5'-CCTGCATGACTGCTCTCCAAAACGG-3'), and *foxc1b* MO1 (5'-GCATCGTACCCCTTTCTTCGGTACA-3'). These morpholinos have been previously reported to be specific and effective.[1, 4] 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^{o}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^{o}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^{o}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^{o}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^{o}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

<Day4>

Precipitation and PCR

1. 14k rpm, 20 min at 4^{o}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^{o}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[5] 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[6] using DIG labled *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 uninjected controls as previously described.[7] 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

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