Supporting Information

Supporting Methods

Molecular biology: The primers used in this study are provided below. The human *robo4* promoter construct (Dr. William Aird, BIDMC, Harvard) was used as template for the generation of the human Robo4 promoter-Sox^{M}-luciferase construct, which was generated using the Stratagene mutagenesis kit with primers included in the Table (supplemental material). All mutations were confirmed by sequencing. For siRNA Synthesis the human *robo4* (1324 bp to 1732 bp), human *sox18* (441 bp to 863 bp) and human *sox7* (474 bp to 880 bp) regions were amplified using the primers mentioned in the Table. SiRNA was generated according to BLOCK-iT Complete Dicer RNAi Kit (Invitrogen) recommendations. HUVEC were grown to 70-80% confluence and transiently transfected with 250 and 500 ng of individual siRNA per dish using Lipofectamine 2000 Reagent. All transfections were carried for 36 h followed by isolation of the transfected cells for end-point assays. All primers used in this study are listed below:

Sequence Name	**Sequence 5'-3'**
h Sox18^{PM1} Forward	CCAGGAACAATTGGGAATAAAGGCTGGATACTCTTCCG
h Sox18^{PM1} Reverse	CGGAAGAGTATCCAGCCTTTATTCCCAATTGTTCCTGG
h Sox18^{PM2} Forward	CCAGGAACAATTGGGAACTAAGGCTGGATACTCTTCCG
h Sox18^{PM2} Reverse	CGGAAGAGTATCCAGCCTTAGTTCCCAATTGTTCCTGG
h Sox18^{PM3} Forward	CCAGGAACAATTGGGAACAGAGGCTGGATACTCTTCCG
h Sox18^{PM3} Reverse	CGGAAGAGTATCCAGCCTCTGTTCCCAATTGTTCCTGG
h Sox18^{PM4} Forward	CCAGGAACAATTGGGAATTGAGGCTGGATACTCTTCCG
h Sox18^{PM4} Reverse	CGGAAGAGTATCCAGCCTCAATTCCCAATTGTTCCTGG
zf Sox18 Forward	GGGGTACCCACCCATGAATATATCTGAGTCTAGTTGCTGTC
zf Sox18 Reverse	CGGAATTCTCCTGTAATGCAGGCGCTGTAATAG
zf Sox7 Forward	GGGGTACCCACCCATGGCGGCTCTGATAAGTGCGTAT
zf Sox7 Reverse	CGGAATTCTGAAATGCTGTAGTTGTTGTAGTAGGC
SiRNA: hSox18 Forward	CGACCACCCCAACTACAAG
SiRNA: hSox18 Reverse	CCCAGGGTGCCGTAGTACA
SiRNA: hSox7 Forward	GAAGGAGGACAGGGGTGAGT
SiRNA: hSox7 Reverse	AGTGGAGTGGGTGGTAGGTG
WTSox18bind-F	CAG AGA AGC TGG CCA GGA ACA ATT GGG AAC AAA GGC TGG ATA CTC TTC CG
WTSox18bind-R	CGG AAG AGT ATC CAG CCT TTG TTC CCA ATT GTT CCT GGC CAG CTT CTC TG

Mutant1 Sox18bind-F	[$]CAC AGA AGC TGG CCA GGA ACA ATT GGG AAT TGA GGC TGG ATA CTC TTC CG
Mutant1 Sox18bind-R	[$]CGG AAG AGT ATC CAG CCT CAA TTC CCA ATT GTT CCT GGC CAG CTT CTG TG
Mutant 2 Sox18bind-F	[$]CAC AGA AGC TGG CCA GGA ATT GAC GGG AAT TGA GGC TGG ATA CTC TTC CG
Mutant 2 Sox 18bind-R	[$]CGG AAG AGT ATC CAG CCT CAA TTC CCG TCA ATT CCT GGC CAG CTT CTG TG
Real Time zf Robo4 Forward	TGA CTA TGG GCT CTG TGA TG
Real time zf Robo4 Reverse	TGT CCC AGA ATG CCT CAT
Real Time zf Robo1 Forward	AAG TGC CGT CTA CCT GTT TC
Real Time zf Robo1 Reverse	ACA TCT CCC AGT TCC CTC T
Real Time zf β-Actin Forward	AAT CCC AAA GCC AAC AGA GA
Real Time zf β-Actin Reverse	CAC ACC ATC ACC AGA GTC CA

[$]Underlined sequence indicate mutation site, h: human, zf: zebrafish, WT: wild type

In vitro transient transfections: Transient siRNA transfections were performed using Lipofectamine 2000 reagent (Invitrogen) using manufacturer's protocol. Briefly siRNA transfections were performed in HUVEC using 250-500 ng of the individual siRNA. For co-transfection experiments, equal amounts of the two siRNAs were used. All transfections were conducted for 36 h in antibiotic free medium.

Luciferase assays: Luciferase assays were performed according to the instructions mentioned in the Dual-Luciferase® Reporter Assay System (Promega, WI, USA). Briefly, cells were transiently transfected with the respective constructs for 36-48 h using Lipofectamine 2000 reagent® or electroporated with the respective constructs using microporator (Neon Transfection System, Invitrogen). *Renilla* luciferase (100ng per well) was used as an internal control. Post transfection, cells were lysed 1X Passive Lysis Buffer (Promega) for 15 mins at RT. Lysates were stored at -80°C and were freeze-thawed once before use and cleared by centrifugation. *Firefly* and *Renilla* luciferase activity was measured by AutoLumat LB953 luminometer (Berthold Technologies, Wildbad, Germany). In vivo luciferase assays were performed similarly. Briefly, embryos were microinjected with linearized luciferase constructs at one-cell stage and 25 embryos were lysed in 1X Passive Lysis Buffer (Promega) for 15 mins at RT.

Nuclear protein preparation: HUVEC cells (P3 passage) were grown in 100 mm culture plates (3 per experiment). All buffers except PBS contained 0.5 mM DTT and 1% Protease Inhibitor Cocktail (Sigma),

and all centrifugations were performed at 4°C. Nuclear proteins were prepared according to our established protocol (1). Briefly, cells were washed in PBS, centrifuged, and the Packed Cell Volume (PCV) was estimated. The cell pellets were then re-suspended in hypotonic buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$] in volumes of 5 times the PCV and centrifuged. Cells were then re-suspended in hypotonic buffer in volumes of 3 times the PCV, incubated on ice for 10 min, and then homogenized. After centrifugation at 3,300 xg, the supernatant containing cytoplasmic proteins was collected, concentrated, quantified, and stored at -80°C. The pellet was used to prepare nuclear proteins. The Packed Nuclear Volume (PNV) was estimated. Next, half the volume of PNV of each low-salt [20 mM Hepes (pH 7.9), 20 mM KCl, 1.5 mM $MgCl_2$, 25% glycerol] and high-salt [20 mM Hepes (pH 7.9), 1 M KCl, 1.5 mM $MgCl_2$, 25% glycerol] buffer were sequentially added. Following 30 min incubation at 4°C, centrifugation at 18,000 xg was performed. Next, soluble fraction was collected and dialyzed against at least 50 times volumes of dialysis buffer [20 mM Hepes (pH 7.9), 100 mM KCl, 20% glycerol]. After centrifugation at 18,000 xg, the supernatants were collected, quantified, and stored at -80 ºC.

Western blot: As control, expression of transcription factors Sox18 and Sp1 was examined by western blot analysis (data not shown). Briefly, 9 µg of nuclear or cytoplasmic proteins were loaded on 10% gel (Invitrogen). After electrophoresis, proteins were blotted on to PVDF membranes and blots were incubated with either 1:1000 dilution of SOX18 antibody (Santacruz) or 1:1000 dilution of antibody to Sp1 (sc-14027x) (Santa Cruz Biotechnology; Santa Cruz, CA). Following overnight incubation at 4°C, primary antibodies were removed and blots were incubated for 1 h at RT with 1:10000 dilution of HRP-conjugated goat anti-rabbit IgG secondary antibody (Cell Signal). Finally, chemiluminescent detection was carried out and was visualized by X-ray film exposure.

Quantitative real-time PCR (RT-PCR): Levels of *robo4* mRNA following *sox7/18* knockdown in zebrafish were determined by quantitative RT-PCR in iQ5 (Bio-Rad, Hecules, CA). Briefly, one-cell stage zebrafish embryos were microinjected with *sox7* and *sox18* MO (0.25 pmol) and total RNA was isolated from 25 embryos per group by Trizol method. 1.5 µg of total RNA per group was used for 1st strand cDNA in a final volume of 30 µl according to the manufacture's protocol (Invitrogen). 2 µl of the 1st strand cDNA reaction mixture were amplified with 1X DyNAmo HS SYBR Green qPCR pre-mixture (NEB). RT-PCR was performed using primers mentioned above. All measurements were performed in triplicate. The data were analyzed using iQ2.0 analysis software.

Supporting Figure Legends

Figure S1. A, B and C are *robo4* ISH panels previously published in Bedell et al (2) for comparison purposes with *sox7* and *sox18* ISH panels in Fig. 2. D shows the activity of wild type h*Robo4* promoter in HUVEC. Briefly, HUVEC were transiently transfected with 1.5 μg of the h*Robo4* promoter cDNA fused to luciferase. 36 h post transfection the promoter activity was determined as described in the Materials and Methods. E shows the point mutations created in the Sox18 binding sequence of the h*Robo4* promoter. The numbers in the cartoon indicate the corresponding Sox binding site location in mouse and human *robo4* promoter sequence.

Figure S2. A-C are in situ flourescence images of *sox18* (A, red) and *robo4* (B, green) transcripts and co-localization (merge) of the two transcripts (C, yellow) in the trunk region of 24 hpf zf embryo. D-F are in situ fluorescence images of *sox7* (D, red) and *robo4* (E, green) transcripts and co-localization (merge) of the two transcripts (F, yellow). The embryos are oriented with anterior to the left. G is a three dimensional surface rendered image that shows co-localization of *sox7/sox18* transcripts with *robo4* transcript. H is a snapshot 3D reconstruction of the different *z* slices of the confocal image shown in panel 2Q. I is a western blot with Sox18 specific and actin antibody depicting the knockdown of endogenous Sox18 protein with 120 nM and 240 nM of Sox18 siRNA in HUVECs. Quantitation of the band marked with asterisk is indicated below the blot.

Figure S3. A shows the 50-base oligo design for wild type and mutant (M1 and M2) probe generation for EMSA. The regions on the oligo in red indicate putative Sox18 binding sites. A point mutation (G➔C) on the third base was inadvertently introduced (underlined) during oligo design. However, this does not alter the consensus Sox18 binding sequence on the oligo. B shows the competition assay between the binding of the labeled and unlabelled (excess) probe to the nuclear protein extract during EMSA. In presence of increasing amounts of the unlabeled probe, the binding complexes (1 and 2) show a concentration-dependent decrease in their intensities. C is the quantification of band intensities of the complex 1 and 2 in panel B in relation to those at 1:0 ratio between the labeled and unlabeled probes. D shows the fold change in the *robo4* and *robo1* transcript in 24 hpf zebrafish embryos following *sox18* and *sox7* double knockdown. β-actin levels were used as internal standards. Graph is a representation of 3 independent experiments, *$p<0.05$ (0.0004) and **$p<0.05$ (0.0001).

Figure S4. A-D shows *robo4* ISH expression in 18 hpf zebrafish embryo microinjected with *sox7* (n=34) (B), *sox18* (n=24) (C), and *sox7*+*sox18* (n=37) mRNA (D) (50 pg each) compared to uninjected embryo (n=75) (A). Black asterisks indicate neural tube *robo4* expression and red arrows indicate midbrain hindbrain boundary (MHB). n: notochord, y: yolk. Panel E shows quantitation for embryos that show *robo4*-induced expression in MHB, NT as shown in the figure. Panel F shows the quantitation in terms of number of 24-28 hpf embryos with induced *robo4* expression in ISVs for each sample group [*sox7* (n=29), *sox18* (n=36), *sox7*+*sox18* (n=43) mRNA, uninjected embryo (n=65)].

Supporting References

1. Tabatabai, N. M., Blumenthal, S. S., and Petering, D. H. (2005) *Toxicology* **207**, 369-382
2. Bedell, V. M., Yeo, S. Y., Park, K. W., Chung, J., Seth, P., Shivalingappa, V., Zhao, J., Obara, T., Sukhatme, V. P., Drummond, I. A., Li, D. Y., and Ramchandran, R. (2005) *Proc Natl Acad Sci U S A* **102**, 6373-6378