SUPPLEMENTAL MATERIAL

Table SI. Sequences of the Oligonucleotides.

Bisulfite sequencing		
-802 to +16	forward	5'-GATGGGATGGGTTTAAAAAGAAATATAT-3'
	reverse	5'-ACCCATAACTACTCTCAACCCTATATC-3'
-1259 to -817	forward	5'-ATTTTTATTTGGTAGGTTGTTAGTTTGTGGTTG-3'
	reverse	5'-TTTTAAACCCATCCCATCACTAAAACTATTAAA-3'
-2297 to -1252	forward	5'-GTAGATTGTTGATAGTGATATTTTTGATAAGTTG-3'
	reverse	5'-AACCACAAACTAACAACCTACCAAA-3'
-3030 to -2347	forward	5'-AGGGGGAGTTAGAAAATATAAAATAT-3'
	reverse	5'-ACATTAATTTTAAAAACACTAATTAAATAC-3'
EMSA (Methylated oligonucleotides were used for the methylated probes.)		
-153 SP1 probe	sense	5'-GTTTCT**CG**CCTCTGGTCTCCTCCCAGTTCTCCAAG-3'
	antisense	5'-CTTGGAGAACTGGGAGGAGACCAGAGG**CG**AGAAAC-3'
-119 ETS probe	sense	5'-GGGCCAGGCAGGAAGCAT**CG**GTTTC-3'
	antisense	5'-GAAAC**CG**ATGCTTCCTGCCTGGCCC-3'
-42 SP1 probe	sense	5'-CCACCAC**CG**GCCCACCC**CG**CCCCTCCTTCCC-3'
	antisense	5'-GGGAAGGAGGGG**CG**GGGTGGGC**CG**GTGGTGG-3'
Real-time PCR		
Robo4	forward	5'-TTATGGCTCCCTCATCGCTG-3'
	reverse	5'-GAGGCTGTCTGAGCTGGAAC-3'
GAPDH	forward	5'-TGCACCACCAACTGCTTAGC-3'
	reverse	5'-GGCATGGACTGTGGTCATGAG-3'
KLF2	forward	5'-CTTTCGCCAGCCCGTGCCGCG-3'
	reverse	5'-AAGTCCAGCACGCTGTTGAGG-3'
DNase hypersensitivity assay		
-149 to -10	forward	5'-TCTGGTCTCCTCCCAGTTCTCCAAG-3'
	reverse	5'-CGAGCACTTTGTCCTGCTGCTCTG-3'
-1337 to -1217	forward	5'-GTTTGTAGAGACCATGGTGTTTC-3'
	reverse	5'-CTTCGGTGCCAGCCACAGAC-3'
-2387 to -2242	forward	5'-CTAGCGTCTTTCTGGATTGTGGAG-3'
	reverse	5'-CAAAGCCTCCAAGACTGTCTGACTC-3'
-2990 to -2857	forward	5'-CTAGGGATGAAGGAAGGCACTG-3'
	reverse	5'-CACAAACTAAGGAAGAGCCGAC-3'

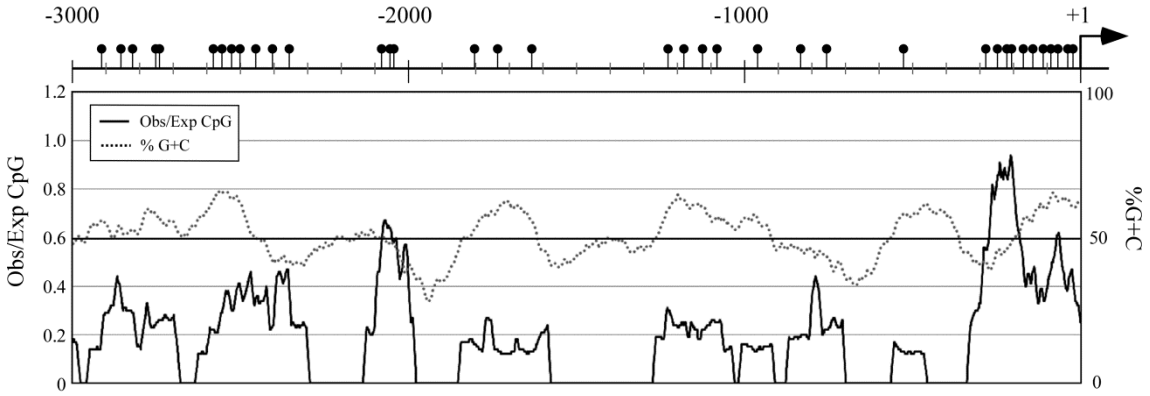

Figure SI. Analysis of the distribution of CpG sites in the Robo4 promoter. In the top panel, locations of the CpG sites are indicated with lollypops. In the bottom panel, the moving average of the observed/expected (Obs/Exp) CpG and %G+C were calculated as described in a previous report.[1] Each point on the graph represents the average values for 10 adjacent 100-bp windows. Values for Obs/Exp CpG and %G+C are plotted as a continuous line and a broken line, respectively. The typical definition of a CpG island is a region in which the Obs/Exp CpG and %G+C are greater than 0.6 and 50, respectively.

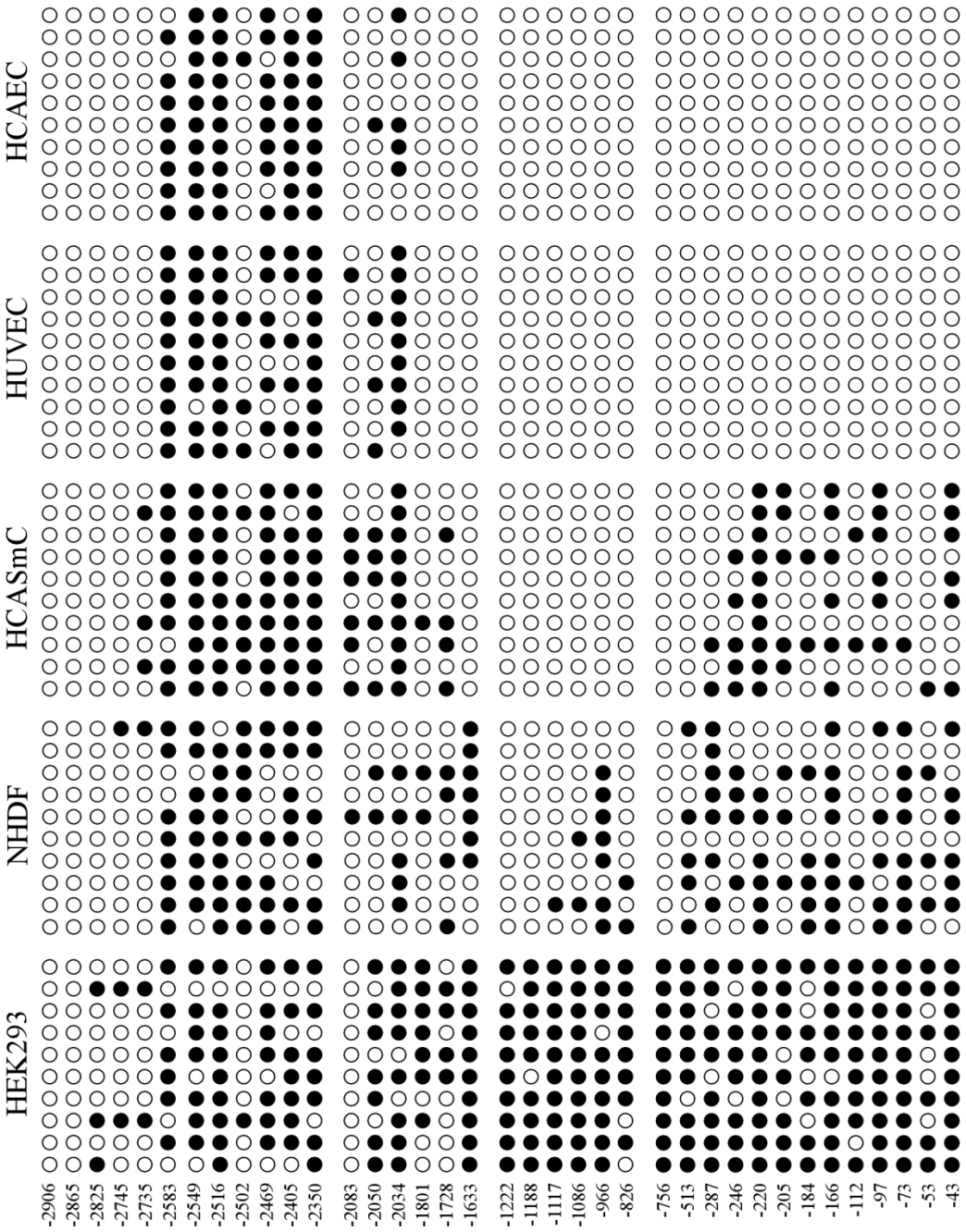

Figure SII. Bisulfite sequencing of the human Robo4 promoter. 3-kb human Robo4 promoter bisulfite sequencing results for 10 cloned PCR products prepared with bisulfite-treated genomic DNA from 2 endothelial cell types (HCAEC and HUVEC) and 3 non-endothelial cell types (HCASmC, NHDF, and HEK293). Open and closed circles indicate non-methylated and methylated CpGs, respectively.

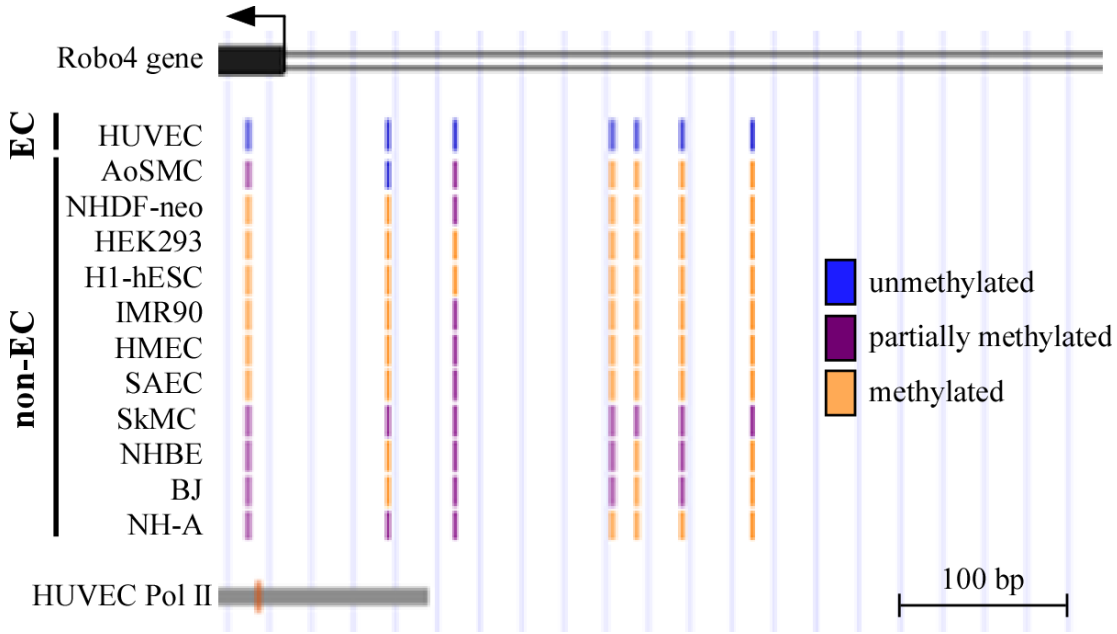

Figure SIII. DNA methylation patterns of the Robo4 proximal promoter in various cell-types. The methylation of the Robo4 proximal promoter and Pol II binding were analyzed using data generated by the ENCODE consortium. The data includes the promoter methylation pattern in ECs (HUVEC) and non-ECs, including aortic smooth muscle cells (AoSMC), neonatal dermal fibroblasts (NHDF-neo), HEK293, human embryonic stem cells (H1-hESC), fetal lung fibroblasts (IMR90), mammary epithelial cells (HMEC), small airway epithelial cells (SAEC), skeletal striated muscle cells (SkMC), bronchial epithelial cells (NHBE), skin fibroblasts (BJ), and astrocytes (NH-A). Levels of methylation are color coded, where blue represents non-methylated, purple represents partial methylation, and orange represents full methylation, as identified by Methyl 450K Bead Arrays. Pol II chromatin immunoprecipitation-seq data in HUVEC is shown at the bottom. A black arrow denotes the transcription start site.

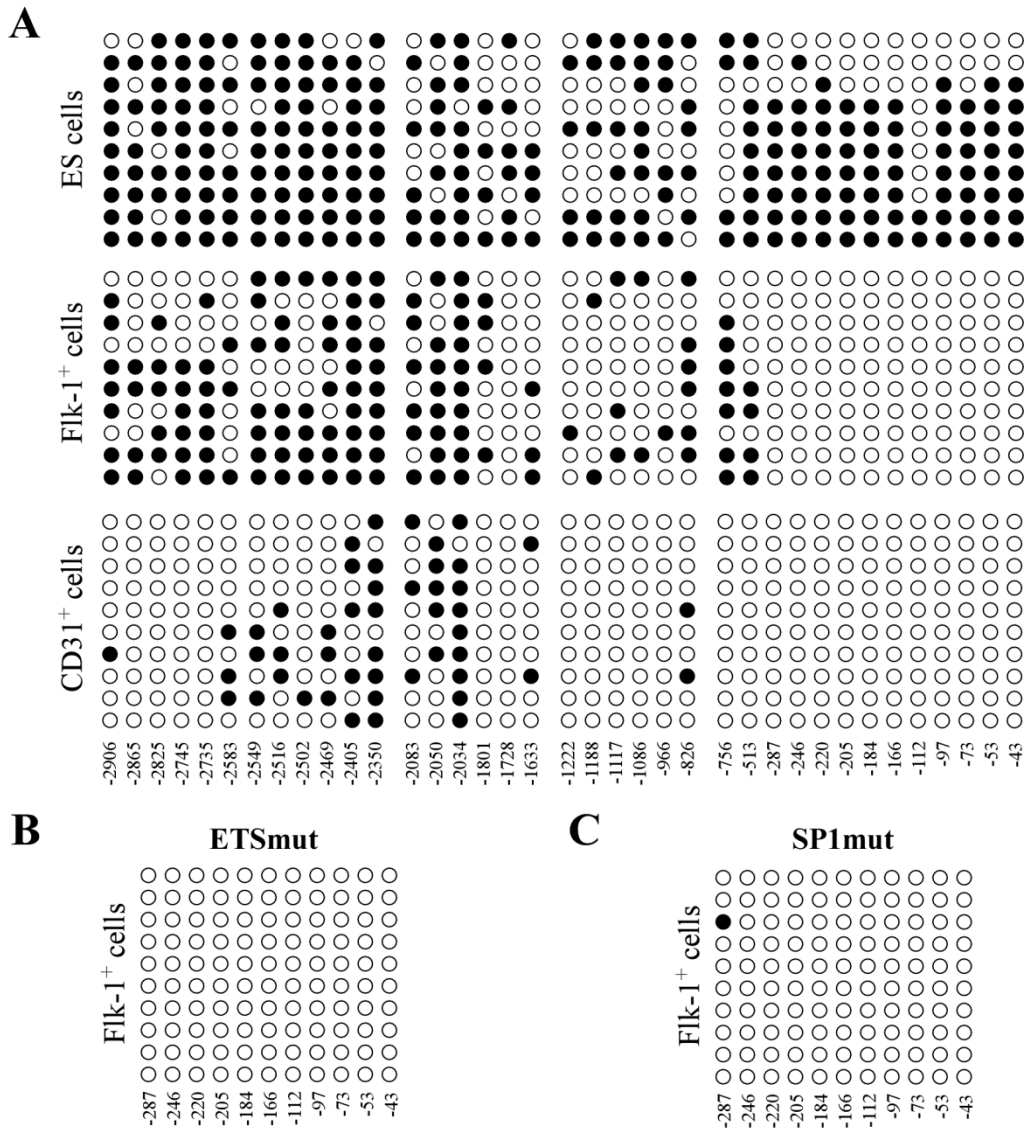

Figure SIV. Bisulfite sequencing of the human Robo4 promoter in ES cells and Flk-1$^+$ cells. (A) The targeted ES cells containing the wild type Robo4 promoter were differentiated, and Flk-1$^+$ and CD31$^+$ cells were separated by MACS. The methylation pattern of the targeted Robo4 promoter in undifferentiated and differentiated cells was analyzed by bisulfite sequencing. Open and closed circles indicate non-methylated and methylated CpG, respectively. (B and C) Targeted ES cells containing the mutated Robo4 promoters with either the -119 ETS mutation (B) or the -42 and -153 SP1 double mutation (C) were differentiated into Flk-1$^+$ cells. The methylation patterns of the targeted Robo4 promoters were analyzed by bisulfite sequencing.

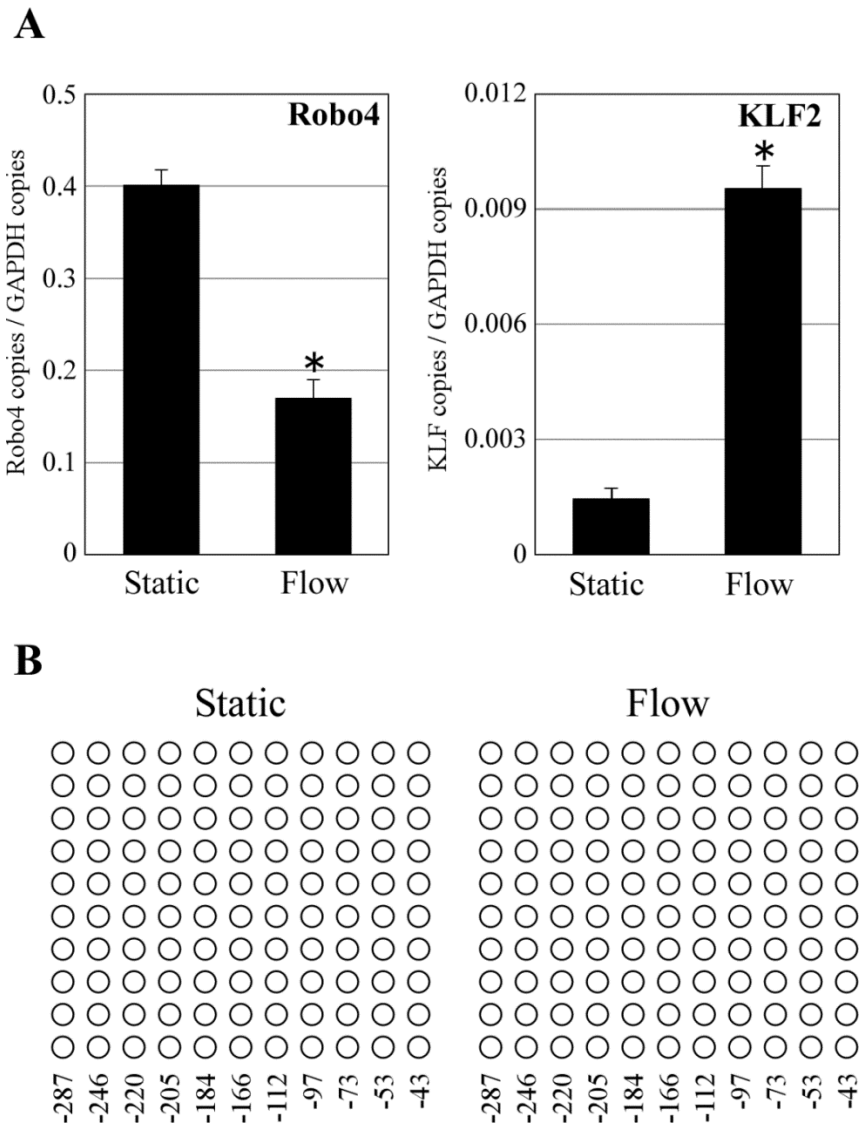

Figure SV. Bisulfite sequencing of the human Robo4 promoter in shear stress treated ECs. (A) The expression of KLF2 and Robo4 mRNA in HUVECs treated with or without shear stress was measured by real-time RT-PCR. The data are presented as the mean ± SE. (n=3, Student's t-test, * $p < 0.05$). (B) DNA methylation patterns of Robo4 proximal promoter in HUVECs treated with or without shear stress were analyzed by bisulfite sequencing.

SUPPLEMENTAL FIGURE SV METHODS

Preparation of genomic DNA and RNA samples from HUVEC exposed to shear stress.

HUVEC were plated to confluence and cultured for 24 h in M199 medium containing 20% fetal calf serum, 1% L-glutamine, 1% endothelial cell growth supplement (Alfa Aesar, Lancashire, United Kingdom), 1% heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were then exposed to constant laminar shear stress (20 dynes/cm2) or left under static (no flow) conditions for 24 h, and subsequently used for preparations of genomic DNA and RNA for bisulfite-sequencing and real-time RT-PCR analyses, respectively. Primer sequences are shown in Table SI.

REFERENCES

1. Gardiner-Garden M, Frommer M. Cpg islands in vertebrate genomes. *J Mol Biol*. 1987;196:261-282