Supporting Information

Ferdous *et al.* 10.1073/pnas.0807583106

SI Materials and Methods

Plasmids. A 2-kb upstream promoter fragment of the *Etsrp71* gene was cloned into Mlu1 and XhoI sites of pGLT-luc reporter plasmid, containing the core promoter sequence (TATA box) of SV40 promoter into HindIII and NcoI sites of pGL3 (Promega). A 2.1-kb upstream promoter fragment of the *Tie2* gene was cloned into Mlu1 and HindIII sites of pGL3 reporter plasmid (Promega). The Nkx2–5 response element (NKE) in *Etsrp71* and Ets-binding elements in *Tie2* promoter were mutated by serial PCR amplification of the promoter fragments using mutant primer pairs. Mutation of the transcription factor binding sites was sequence confirmed. To generate a (HA)-tagged expression vector, the cDNA sequence of Etsrp71 (amino acids 23–358) (generously provided by R. Janknecht, Mayo Clinic, Rochester, MN) (1) was PCR amplified, cloned into NotI and XhoI sites of pIRES-hrGFP-2a (Clontech), and sequence verified. Myc-tagged Nkx2–5 expression plasmid was used to engineer the Nkx2–5 mutant that lacked the homeodomain.

Transcriptome Analyses. Combinatorial matings of Nkx2–5 transgenic (2) and heterozygous (3) mice were used to isolate EYFP-positive WT and Nkx2–5 null embryos at distinct developmental stages. RNA was extracted from EYFP-positive cells and amplified. Oligonucleotide array hybridizations were carried out according to the Affymetrix protocol as previously described (2, 4). Detailed procedures for the isolation of EYFP-positive cardiac progenitor cells, RNA extraction, and amplification for GeneChIP analysis were performed as previously described (2, 5). The array data were analyzed using the MAS5.0 software package and Dchip to determine significant transcript expression and to determine common and unique expression profiles associated with the respective samples.

In Situ Hybridization. In situ hybridization techniques were performed as previously described (6). Briefly, ^{35}S-labeled sense and anti-sense mRNA riboprobes of 636-bp *Etsrp71* cDNA were prepared as described (2). The cDNA (corresponding to amino acids 24–235) was PCR amplified and cloned into pCR4 according to the manufacturer's protocols. Sequence verification and orientation of the cloned cDNA were performed using conventional techniques. Radiolabeled sense and anti-sense probes were prepared using T7 and T3 polymerases, respectively, according to the manufacturer's protocol (Ambion).

Semiquantitative RT-PCR and Quantitative RT-PCR Analyses. RNA was extracted from EYFP$^+$ cardiac progenitor, Tie2-GFP$^+$, WT, and Etsrp71 null hearts and C2C12 cells using Tripure (Roche) according to the manufacturer's instructions. RNA isolated from cardiac progenitors and Tie2-GFP$^+$ cells was subjected to two rounds of linear amplification and cDNA was used for RT-PCR and semiquantitative and quantitative RT-PCR (qRT-PCR) analyses as described previously (2, 7).

TUNEL Assay. The Promega DeadEnd Fluorometric TUNEL system (Promega) was performed according to the manufacturer's instructions. Briefly, sections were deparaffinized, equilibrated in PBS, permeabilized with proteinase K, postfixed in 4% paraformaldehyde, and incubated in TdT reaction mix for 1 h at 37 °C in the dark. Slides were then washed in 2× SSC, counterstained with propidium iodide, and coverslipped with vectashield mounting medium.

Immunohistochemical Analysis. Immunohistochemical analysis for Ki67, a cell-cycle-associated protein expressed from G1 through the end of M phase, was performed to evaluate active cellular proliferation. Sections were deparaffinized before citra-buffer microwave antigen retrieval, quenched for autofluorescence, and blocked with Mouse-On-Mouse (MOM) IgG Blocking Reagent (Vector Laboratories). Antigenic epitopes were equilibrated in MOM Diluent and then incubated overnight at 4 °C with Novocastra (Vector) anti-Ki67 serum. Bound primary antibody was detected with MOM biotinylated anti-mouse IgG and fluorescein–avidin DCS. Specimens were subsequently counterstained with propidium iodide and coverslipped with vectashield mounting medium. The staging of embryos was performed by counting the presence of a vaginal plug as day 0.5 after conception and by counting the number of somites. Immunostaining for endomucin, an endothelial-specific sialomucin, was performed using rat anti-endomucin antibody (clone V.7C7, gift from Dietmar Vestweber) according to previously published methods. Bound primary antibody was detected with biotinylated rabbit ani-rat IgG, peroxidase-streptavidin, and DAB chromagen.

Western Blot Analyses. Western blot analyses were carried out as described previously (7). Nuclear extracts (NE) from C2C12 myoblast cells with or without overexpression of myc-tagged Nkx2–5 were separated on a 10% SDS-PAGE gel and the proteins were transferred to a nylon membrane. The membrane was initially probed with anti-Nkx2–5 serum (1:1000, Santa Cruz). After stripping, the membrane was reprobed with anti-myc serum (1:3000, Sigma) and analyzed as previously described (7).

Electrophoretic Mobility Shift Assay (EMSA). EMSAs were performed as described previously (7). Briefly, the reaction mixture (20 μl) containing the radiolabeled probe and poly(dI)-(dC) (1 μg) was incubated for 20–30 min at room temperature in the presence or absence of 8 μg NE of C2C12 myoblast cells or 4 μg NE of C2C12 cells containing myc-tagged Nkx2–5, anti-myc or anti-Nkx2–5 sera, and excess of unlabeled probe containing either WT or mutant NKE. The reaction mixture was separated on a 5% native polyacrylamide gel at 4 °C using 0.5× TGE as running buffer. In selected reactions, heat-denatured (10 min at 95 °C) antibodies were added.

Reporter Gene Assays. Transcriptional assays were performed as described with several modifications (7). In a 6-well plate, C2C12 myoblast cells were transiently transfected with control (pGLT-Luc/pGL3-Luc) and 2.0-kb *Etsrp71* or 2.1-kb Tie2 promoter containing WT or mutated Nkx2–5-binding elements (NKE) or Ets-binding elements fused to the luciferase reporter with increasing amounts (0.01–1.2 μg) of myc-tagged Nkx2–5 or HA-tagged Etsrp71 expressing vectors. Total amount of DNA (1–2 μg) was adjusted with a control plasmid. Twenty-four hours after transfection, cells were washed twice with PBS, lysed, and used for luciferase activity. β-Galactosidase activity was used to normalize transfection efficiency and all fold inductions in luciferase activity were normalized to the vector alone. Each assay was performed in triplicate and repeated 3 times.

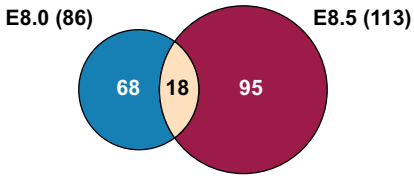
Generation of an Inducible ES/EB System for Nkx2–5 Overexpression. Doxycycline-inducible Nkx2–5 overexpressing ES cells were generated from A2Lox ES cells, which express rtTA from

Rosa26 and carry a doxycycline-inducible locus on the X chromosome. These cells are an improved version of previously described Ainv15 ES cells (8) in which the inducible locus can be targeted by Cre-mediated cassette exchange recombination. To generate the inducible cell line, cDNA of myc-tagged Nkx2–5 was subcloned into p2Lox, the targeting vector. Cre/Lox recombination to insert p2Lox into the inducible locus was accomplished by cotransfecting Cre recombinase as previously described (8). Embryoid bodies (EBs) were prepared using the hanging drop technique and then cultured in suspension on a rotating plate beginning on day 2. Doxycycline (0.05 mg/ml) was added in culture for 24 h to induce protein expression and EBs were collected for RT/qRT-PCR analyses.

Generation and Analysis of Etsrp71 Chimera Mice. Germline transmission of 1 copy of the potentially trapped *Etsrp71* allele was confirmed by PCR analysis using a common reverse primer from intron 5 (5′-AGAAAGTCAGTTCCGTCCTCTCAC-3′) and 2 different forward primers. One forward primer was from exon 5 (5′-AGAGCCACATTGACTCGCTACTCC-3′) and the other from the trapping construct (5′-GACAGACACAGATAAGTTGCTGGC-3′).

1. De Haro L, Janknecht R (2005) Cloning of the murine ER71 gene (Etsrp71) and initial characterization of its promoter. *Genomics* 85:493–502.
2. Masino AM, *et al* (2004) Transcriptional regulation of cardiac progenitor cell populations. *Circ Res* 95:389–397.
3. Lyons I, *et al*. (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2–5. *Genes Dev* 9:1654–1666.
4. Goetsch SC, Hawke TJ, Gallardo TD, Richardson JA, Garry DJ (2003) Transcriptional profiling and regulation of the extracellular matrix during muscle regeneration. *Physiol Genomics* 14:261–271.
5. Gallardo TD, Hammer RE, Garry DJ (2003) RNA amplification and transcriptional profiling for analysis of stem cell populations. *Genesis* 37:57–63.
6. Shelton JM, Lee MH, Richardson JA, Patel SB (2000) Microsomal triglyceride transfer protein expression during mouse development. *J Lipid Res* 41:532–537.
7. Martin CM, *et al* (2008) Hypoxia-inducible factor-2α transactivates Abcg2 and promotes cytoprotection in cardiac side population cells. *Circ Res* 102:1075–1081.
8. Kyba M, Perlingeiro RC, Daley GQ (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109:29–37.

A



B

Differential gene expression values (Nkx2-5-/- vs. Nkx2-5+/+)

Title	Symbol	E8.0	E8.5
Natriuretic peptide precursor type A	Nppa	-4.9	-5.7
Natriuretic peptide precursor type B	Nppb	-5.6	-5.6
Small muscle protein, X-linked	Smpx	-2.0	-6.1
Ets related protein 71	Etsrp71	-1.9	-2.1
Acid phosphatase 1, soluable	Acp1	-2.0	-1.9
Aldo-keto reductase family1, E1	Akr1e1	-1.9	-1.41
Cardiac responsive adriamycin protein	Carp	-2.18	-2.46
Creatin kinase, brain	Ckb	-1.52	-1.9
RIKEN 1700029I01 gene	EST	-9.9	-3.03
BG070258	EST	-2.64	-1.52
RIKEN 2700050F09	EST	-2.0	-2.14
RIKEN 0610033H09	EST	-1.9	-4.0
Homeobox a9	Hox a9	-2.0	-1.41
Myosin light chain, cardiac ventricles	Mylpc	-4.0	-11.3
Proviral integration site 1	Pim1	-7.46	-2.14
Rap guanine nuclear exchange factor 5	Rapgef5	-2.0	-2.30
RIO kinase 2	Riok2	-2.83	-2.63
BG066277	EST	-2.64	-1.52

Fig. S1. Downregulation of *Etsrp71* in Nkx2–5 null cardiac progenitors. (*A*) Venn diagram of significantly downregulated transcripts in 6-kb Nxk2–5-EYFP Tg:Nkx2–5 null vs. 6-kb Nxk2–5-EYFP Tg:WT cardiac progenitor cells at E8.0 and E8.5 stages. The total number of significant downregulated transcripts is indicated with parentheses at each developmental stage. Note 18 transcripts are significantly downregulated at both the E8.0 and the E8.5 stages. (*B*) List of 18 transcripts that were significantly downregulated at defined developmental stages in the Nkx2–5 null background compared to the age-matched WT cardiac progenitor cells. Note the statistically significant downregulation of Anf (Nppa), Bnp (Nppb), Smpx, and Etsrp71.

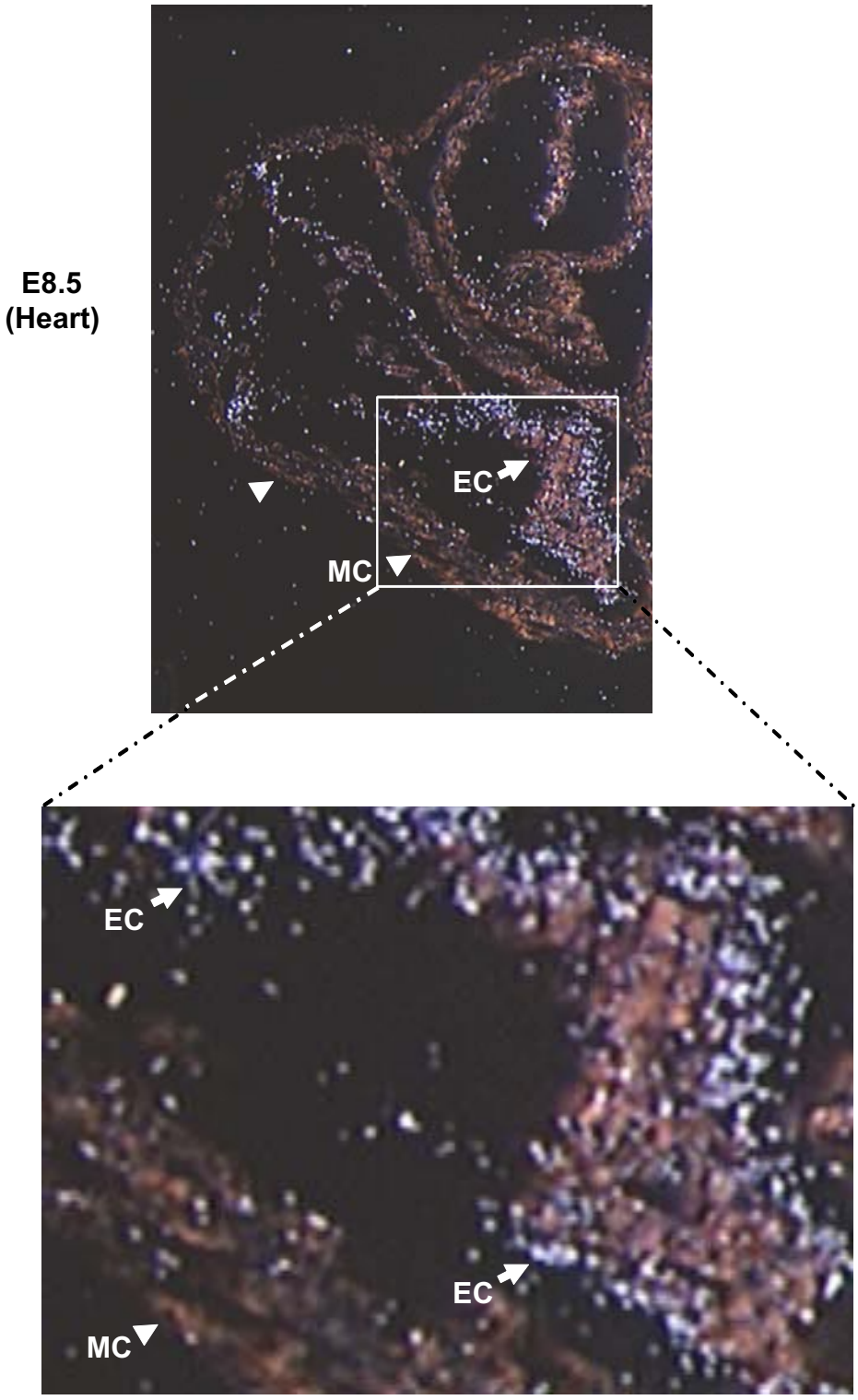


Fig. S2. Transient expression of Etsrp71 in the endothelial/endocardial lineage of the developing embryo. In situ hybridization techniques were used to analyze endogenous expression of Etsrp71 in developing heart (also see Fig. 1*C*). The transverse section of an E8.5 heart (*Upper*) and an enlarged view of the boxed area (*Lower*) are shown. Note that Etsrp71 expression was observed only in the endocardium (EC)/endothelium (arrow) but not in myocardium (MC, arrowheads). Using these techniques, no myocardial expression was observed. Endocardial/endothelial expression of Etsrp71 was also observed at E9.5 (data not shown) and no signal was detected with a sense probe at either of these early developmental stages (data not shown).

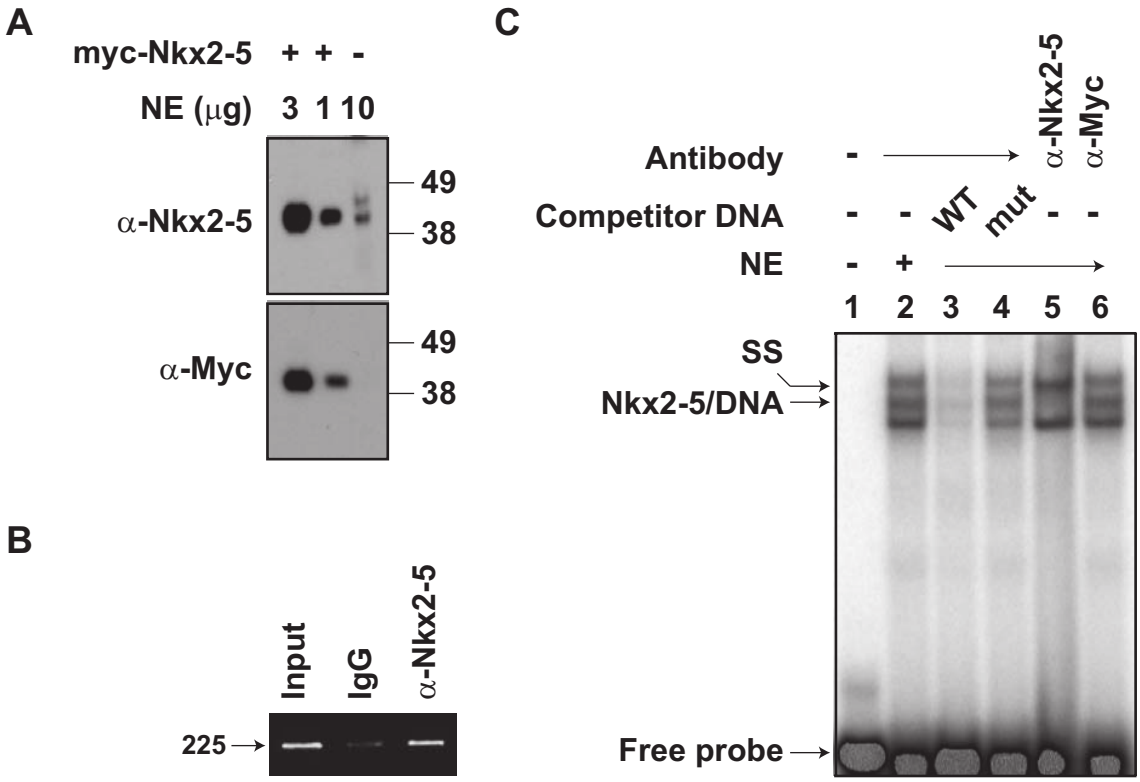


Fig. S3. Endogenous Nkx2–5 binds to the *Etsrp71* promoter. (*A*) Nkx2–5 is expressed in C2C12 myoblast cells. The indicated amount (μg) of NE of C2C12 cells with or without overexpression of myc-tagged Nkx2–5 was separated on a 10% SDS-PAGE to detect the endogenous Nkx2–5 in C2C12 myoblasts cells, using Western blot analyses. Note that endogenous Nkx2–5 was detected only with anti-Nkx2–5 serum. (*B*) ChIP assay for in vivo binding of endogenous Nkx2–5 to the *Etsrp71* promoter. Anti-Nkx2–5 and control IgG sera were used to immunoprecipitate (IP) Nkx2–5/DNA complexes and DNA were purified to PCR amplify a 225-bp DNA fragment harboring the NKE. DNA purified before IP was used as input. Note that the *Etsrp71* promoter harboring the NKE was enriched only following IP with anti-Nkx2–5 serum. (*C*) EMSA revealing the formation of a stable endogenous Nkx2–5-DNA complex (lane 2) that could be competed with WT but not with mutant (mut) probe (lanes 2–4) and supershifted (SS) (lane 5). Note that anti-myc serum is unable to supershift the complex (lane 6)

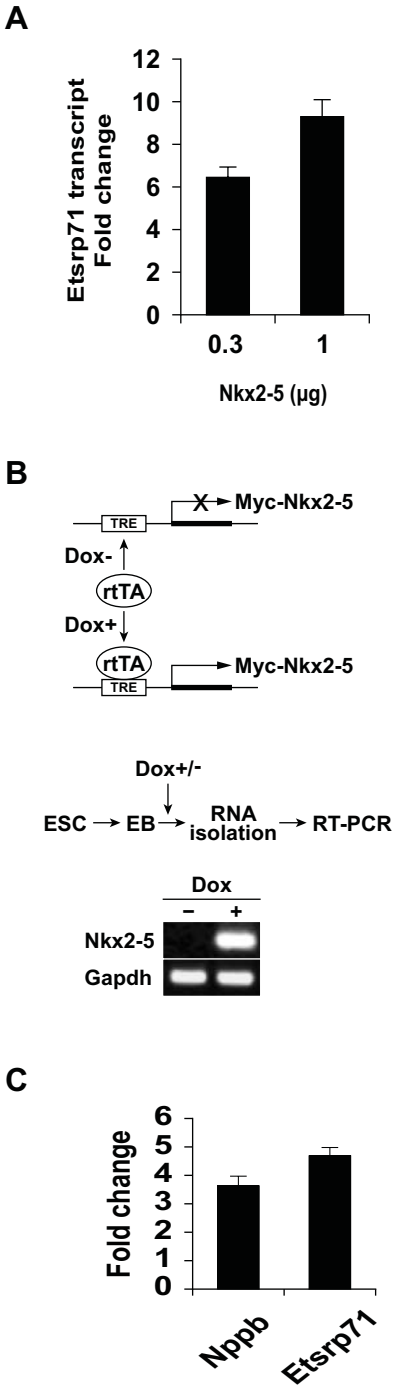


Fig. S4. Induction of endogenous *Etsrp71* gene expression by Nkx2–5. (*A*) Nkx2–5 induces *Etsrp71* gene expression in C2C12 cells. C2C12 myoblasts were transfected with control or Nkx2–5-expressing plasmids. After 16 h of transfection, RNA was extracted and analyzed for Etsrp71 transcript expression using qRT-PCR. (*B*) Nkx2–5 activates endogenous *Etsrp71* gene expression in an inducible ES cell line. (*Upper*) Schematic of an inducible Nkx2–5 ES cell line that was generated in this study by placing the myc-tagged Nkx2–5 cDNA under the control of the tetracycline responsive element (TRE) to induce Nkx2–5 expression only in the presence of doxycycline (+Dox). (*Lower*) Schematic of an ES/EB system for transcript analysis using RT-PCR. ES cells were cultured to form embryoid bodies (EBs). RNA was extracted from doxycycline-treated (+Dox) and untreated (−Dox) EBs for transcript analysis of myc-tagged Nkx2–5 by RT-PCR using a specific primer pair for myc-Nkx2–5 RNA. (*C*) Extracted RNA was used for transcript analysis of selected genes using qRT-PCR. Note that overexpression of Nkx2–5 results in induction of Nppb (a known downstream target of Nkx2–5) and Etsrp71.

A

Genotypic analyses of the Etsrp71+/- progeny

Stage	Total number of embryos/pups	Genotype		
		WT	Het	Null
E8.0	30	9	14	7
E8.5	20	6	10	4
E9.5	21	5	10	6*
E10.5	17	6	7	3*
P21	28	9	19	0

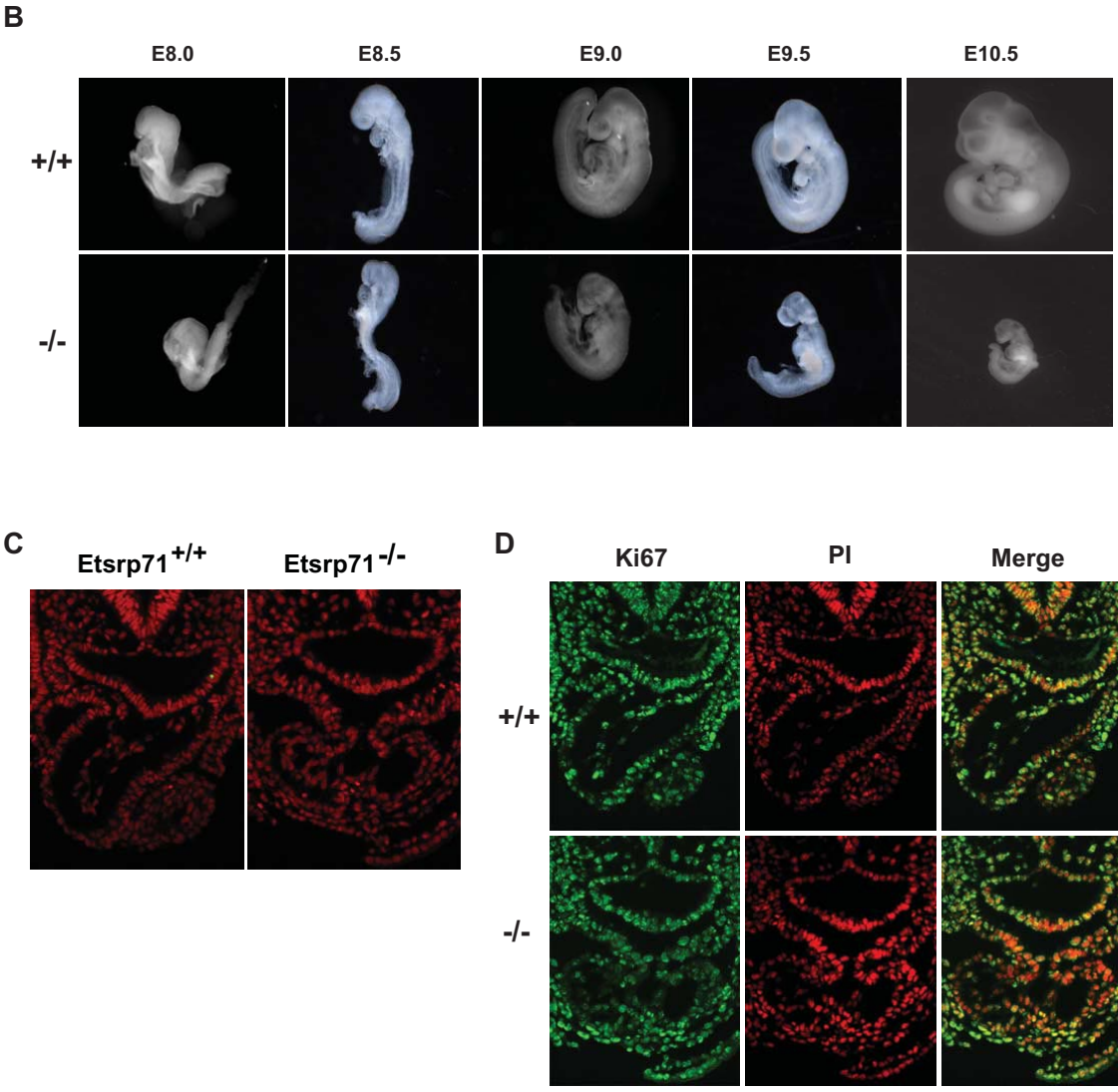
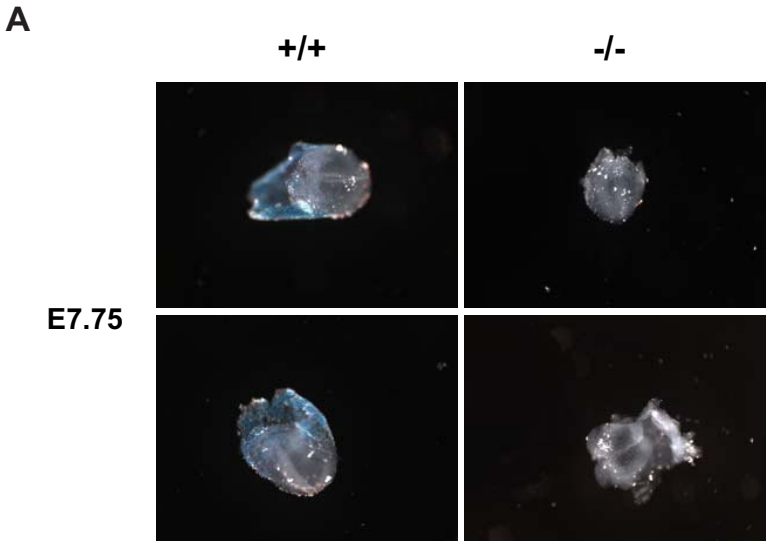


Fig. S5. Etsrp71 homozygous null mice die early during gestation. (*A*) Analyses of the progeny from Etsrp71 heterozygous mating. A database search of the International Gene Trap Consortium, University of Toronto (www.genetrap.org) identified a clone (141.1H7) that contains an insertion of the trapping construct (pGep-SD5) at the exon–intron boundary of exon 5 of *Etsrp71*/*Etv2*. The insertion of the trapping construct was confirmed by sequencing the 3′-RACE products from mRNA isolated from the ES cell lines. Etsrp71 heterozygous male and female mice were mated to generate wild-type (WT), heterozygous (Het), and null embryos. Genotypic analyses of the developing embryos at the indicated developmental stages (E8.0–E10.5) and offspring are shown. Note that no homozygous null offspring (P21) was born and the homozygous null embryos die between E9.0 and E10.5. Absence of a beating heart (*) suggests lethality of the null embryos. (*B*) Morphological appearance of the WT (+/+) and Etsrp71 homozygous null (−/−) embryos. Note that the WT and null embryos are morphologically indistinguishable between E8.0 and E8.5 but the null embryos manifest embryonic lethality and severe growth retardation between E9.0 and E10.5. (*C*) Absence of Etsrp71 did not promote apoptosis. The DeadEnd fluorometric TUNEL system (Promega) was used according to the manufacturer's instructions to compare programmed cell death in WT (+/+) and Etsrp71 homozygous null (−/−) embryos. Note that there are no TUNEL-positive cells in WT and null embryos. (*D*) Active cellular proliferation was not affected in the Etsrp71 homozygous null embryo. Immunohistochemical analyses for Ki67 were used to identify the proliferative cells in WT (+/+) and Etsrp71 homozygous null (−/−) embryos. Note that no proliferative defect was observed in the Etsrp71 null embryo.



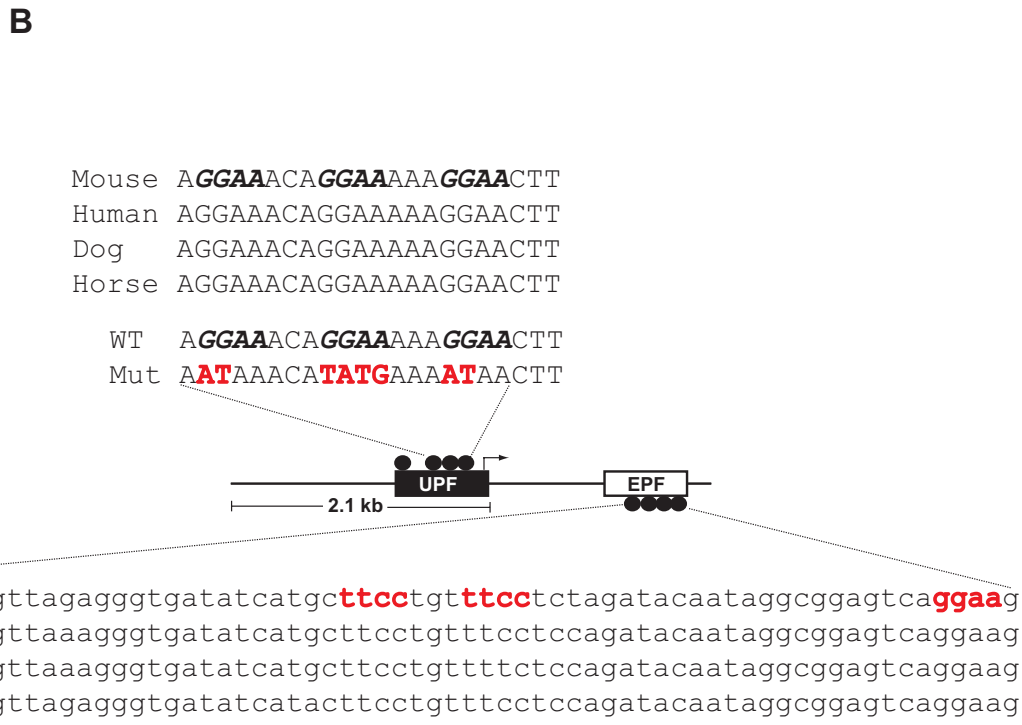


Fig. S6. Etsrp71 regulates *Tie2* gene expression and endothelial specification. (*A*) Etsrp71 is essential to specify endothelial/endocardial progenitors. Tie2-lacZ Tg/Etsrp71$^{+/-}$ and Etsrp71$^{+/-}$ mice were mated to analyze β-galactosidase expression in WT (+/+) and Etsrp71 null (−/−) littermate embryos at E7.75. Note the absence of embryonic and extraembryonic β-galactosidase expression in the Etsrp71 null embryos. (*B*) The *Tie2* gene harbors evolutionarily conserved Ets-binding elements (EBEs). Shown is a schematic alignment of the mouse, human, dog, and horse *Tie2* upstream promoter fragment (UPF) and intronic enhancer promoter fragment (EPF) revealing evolutionarily conserved EBEs (black dots) shown in bold italics (UPF) and red (EPF), respectively. Nucleotide sequences for wild-type (WT) and mutated EBEs, used for transcriptional assays (see Fig. 4*C*), are shown. Mutated nucleotides of EBEs are indicated in red.