Supporting Information

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SI Materials and Methods

Genotyping. PCR genotyping of *Brg1*-floxed embryos and mice was performed as described (1). *Tie2-Cre* transgenic mice and embryos were genotyped using a gene-specific forward primer (5′-GGGAAGTCGCAAAGTTGTGAGTTG-3′) and a Cre-specific reverse primer (5′-TCCATGAGTGAACGAACCTGGTCG-3′) that amplify a 533-bp product. The PCR was performed at an annealing temperature of 60 °C. *BAT-gal* transgenic mice and embryos were genotyped using a forward primer from the *Xenopus laevis Siamois* promoter (5′-CATTTCCCCCAAAACACATC-3′) and a reverse primer specific to *LacZ* (5′-GTTTTCCCAGTCACGACGTT-3′) that amplify a 166-bp product. The PCR was performed at an annealing temperature of 55 °C. Control primers amplifying a 324-bp product from the *IL2* gene were used as a template control for *Tie2-Cre* and *BAT-gal* amplification reactions: forward (5′-CTAGGCCACAGAATTGAAAGATCT-3′) and reverse (5′-GTAGGTGGAAATTCTAGCATCATCC-3′).

Real-Time Quantitative PCR (qPCR) Analysis. The relative fold change in transcription was determined using the comparative C_t method and the housekeeping gene *Gapdh* as the internal control. Data from four independent C166 siRNA transfection experiments or from four to six independent primary endothelial cell isolation experiments were combined and presented as the mean ±SEM. Statistical differences between NS- and *Brg1*-siRNA–transfected or littermate controls and mutants were detected using a two-tailed Student's *t* test.

qPCR Primers. The following qPCR primers were used: *Brg1* (5′-CAGTGGCTCAAGGCTATCG-3′ and 5′-TGTCTCGCTTACGCTTACG-3′); *Fzd1* (5′-CAAGGTTTACGGGCTCATGT-3′ and 5′-GTAACAGCCGGACAGGAAAA-3′); *Fzd2* (5′-CCGTCTCTGGATCCTCACAT-3′ and 5′-TAGCAGCCGGACAGAAAGAT-3′); *Fzd3* (5′-CTAACCACTGAGCCATTCC-3′ and 5′-ACATCAACCTATACCTACTTCC-3′); *Fzd4* (5′-AACCTCGGCTACAACGTGAC-3′ and 5′-TGGCACATAAACCGAACAAA-3′); *Fzd5* (5′-CCGCATACCACAAGCAAG-3′ and 5′-GCATCAGCACCAAGAAGG-3′); *Fzd6* (5′-GCTATGAATAAGGTTGAAGGAGAC-3′ and 5′-AGGCACAGAGGCAGAAGG-3′); *Fzd7* (5′-CCATCCTCTTCATGGTGCTT-3′ and 5′-ATGGCCAAAATGGTGATTGT-3′); *Fzd8* (5′-CAGAGCCTTGACAACCTACG-3′ and 5′-GGAACAGCGACACGAAGC-3′); *Axin2* (5′-CTCCCCACCTTGAATGAAGA-3′ and 5′-ACTGGGTCGCTTCTCTTGAA-3′); *T* (5′-TCCCGAGACCCAGTTCATAG-3′ and 5′-TTCTTTGGCATCAAGGAAGG-3′); *CyclinD1* (5′-GCGTACCCTGACACCAATCT-3′ and 5′-CTCTTCGCACTTCTGCTCCT-3′); *Myc* (5′-TCCTGTACCTCGTCCGATTC-3′ and 5′-GGTTTGCCTCTTCTCCACAG-3′); *Wisp1* (5′-TGATGATGACGCAAGGAGAC-3′ and 5′-CGGGCATTGACGTTAGAGAT-3′); *β-catenin* (5′-TGGCAGCAGCAGTCTTAC-3′ and 5′-GAGGTGTCAACATCTTCTTCC-3′); *VEGFR-1* (5′-TGAGGAGCTTTCACCGAACT-3′ and 5′-TATCTTCATGGAGGCCTTGG-3′); *VEGFR-2* (5′-GGGTCGATTTCAAACCTCAATGT-3′ and 5′-AGAGTAAAGCCTATCTCGCTGT-3′); *VE-cadherin* (5′-AGAGTCCATCGCAGAGTC-3′ and 5′-CAGCCAGCATCTTGAACC-3′); and *Gapdh* (5′-TCAACGGCACAGTCAAGG-3′ and 5′-ACTCCACGACATACTCAGC-3′).

Embryonic Blood Quantification. E10.5 embryos were dissected away from maternal tissue in room temperature PBS, and embryonic heart beats were confirmed. The placenta was removed, umbilical vessels were severed, and the vitelline vein was detached before placing each embryo and yolk sac into 1 mL warm DMEM on a rocking platform for 20 min at room temperature. Embryos and yolk sacs were removed for genotyping, and blood cells were collected by centrifugation (5 min at 800 × *g*, 4 °C). Blood cells were resuspended in 0.07% Trypan blue in PBS, and viable cells were counted on a hemocytometer.

Chromatin Immunoprecipitation. Subconfluent C166 yolk sac endothelial cells were treated with 0.4% formaldehyde to cross-link endogenous protein–protein and protein–DNA interactions. Addition of 0.125 M glycine stopped the cross-linking reaction. Cells were washed, harvested in PBS, and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) with 1× protease inhibitors (Sigma). The cell lysate was sonicated to shear the chromatin and diluted fivefold in chromatin immunoprecipitation (ChIP) buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl) with 1× protease inhibitors (Sigma). Protein A:G Sepharose (Calbiochem) was added to preclear the chromatin for 1 h with rotation at 4 °C. A mixture of BRG1-specific (07-478, Millipore; ab4081, Abcam) or AcH3-specific (06-599, Millipore) antibodies was used for immunoprecipitation of protein–DNA complexes. The isotype-matched polyhistidine epitope tag (His) antibody (600-401-382, Rockland) was used for a negative control. Protein A:G Sepharose was added, and immunoprecipitated complexes were washed once with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 150 mM NaCl), twice with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl), and twice with TE (10 mM Tris, pH 7.4, 1 mM EDTA). Complexes were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$), and cross-links were reversed with addition of 0.2 M NaCl and incubation at 65 °C for 4 h. Protein was digested with 10 mM EDTA, 40 mM Tris, pH 7.6, and 60 μg/mL Proteinase K (Thermo Scientific) at 45 °C for 2 h. DNA was extracted, precipitated, and resuspended. Real-time quantitative PCR using SYBR Green PCR master mix (Applied Biosystems) and the ABI7000 thermocycler (ABI) was performed with gene-specific primers. Data from four independent experiments were combined and presented as the percentage of input ±SEM. Statistical differences were detected using a two-tailed Student's *t* test.

ChIP qPCR Primers. PCR primers flanking the *Fzd1* promoter (5′-CGCAGCACTCAAAGTAGCAG-3′ and 5′-GACAGGCTAGGTCGCTTTTG-3′), *Fzd4* promoter (5′-TTTGCTCAAGGTCACACAGC-3′ and 5′-ACATCTATGCCCCTTTCACG-3′), *Fzd5* promoter (5′-CTTTAAACAAGCGCCAGACC-3′ and 5′-TAGTGGACCGGGAAGTTACG-3′), *Fzd6* promoter (5′-TCTGAAGTGATGGTGGCAGA-3′ and 5′-CGGGGATCTGCTCTGTAGTG-3′), *Fzd8* promoter (5′-CTTGACTGAGGCCAGAAAGG-3′ and 5′-CAATTCCACAGGGTCTGTCC-3′), the *Wisp1* promoter (5′-GGTGGGGAGACAGTGAAGAA-3′ and 5′-GTCCCAGACTCCACAGGGTA-3′), the *ADAMTS1* promoter (5′-CACTCTTTTCCCCAGAGCAG-3′ and 5′-CGCCCCTTTATAGCCACATA-3′), and a region upstream of the *Fzd5* promoter (5′-GGTGACTTAGGGCAAAACCA-3′ and 5′-AGGCCACCATACCAGGTTCT-3′) were used for real-time quantitative PCR.

Wnt Signaling Array. Total RNA from siRNA-transfected C166 cells or from platelet endothelial cell adhesion molecule-1 (PECAM)-isolated yolk sac endothelial cells was isolated using

TRIzol (Invitrogen) according to the manufacturer's instructions. The DNA-*free* kit (Ambion) was used to digest any contaminating DNA, and cDNA was prepared using the RT^2 First Strand kit (SABiosciences). Three independent experiments were performed using Mouse Wnt Signaling Pathway RT^2 *Profiler* PCR Arrays (SABiosciences), and four independent experiments were performed using Wnt Signaling Target Gene RT^2 *Profiler* PCR Arrays (custom made by SABiosciences to contain 28 direct Wnt target genes selected from the Wnt home page: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/) according to the manufacturer's instructions. Data analysis and statistical determinations were performed using the web-based PCR array data analysis tool available through the SABiosciences website.

Whole-Mount Yolk Sac and Placental Staining. For whole-mount yolk sac immunostaining, embryos with attached yolk sacs were dissected from maternal tissue, fixed for 15 min in 4% paraformaldehyde, permeabilized for 30 min in 0.02% Triton X-100/PBS, and blocked overnight (4 °C) in 5% donkey sera (Jackson ImmunoResearch)/3% BSA (Rockland)/PBS. Samples were then incubated overnight in 1:100 anti-PECAM1 (557355, BD Pharmingen) diluted in blocking solution. After washing 5 × 1 h in blocking solution, samples were incubated overnight (at 4 °C) in 1:250 donkey-anti–rat-Cy3 (712-165-153, Jackson ImmunoResearch). Tissues were washed as above followed by a 20-min wash in PBS and postfixed for 5 min in 4% paraformaldehyde, and yolk sacs were dissected away from their attached embryos and flat-mounted on glass slides for microscopic examination. Embryonic tissue was subsequently digested for genotyping. Whole-mount β-galactosidase staining was performed on embryos attached to their yolk sacs and placentas; tissues were immersed for 48 h in X-gal staining solution at room temperature as described (1). Stained yolk sacs and placentas were subsequently dissected apart, cryoembedded as described (1), cryosectioned (8 μm), and counterstained with eosin.

Immunohistochemistry. *Brg1* mutants and littermate controls were cryoembedded with maternal decidual tissue intact; genotypes were confirmed after cryosectioning (8 μm) by scraping embryonic tissue into DEXPAT solution (TaKaRa) for extraction before PCR. Double immunostaining for phospho-histone H3 and PECAM was performed as follows: cryosections were thawed and blocked in 5% normal goat serum/5% normal donkey serum/0.1% Triton X-100/PBS for 2 h at room temperature. Primary antibodies were diluted (1:100) in the blocking solution, and sections were incubated overnight at 4 °C. The primary antibodies used were anti–phospho-histone H3 (Millipore, no.06–570) and anti-PECAM (BD Pharmingen, no.553370). Sections were washed three times (3 min each) in 0.1% Triton X-100/PBS. Secondary antibodies were diluted (1:500) in the blocking solution along with 20 μg/mL Hoechst stain, and sections were incubated for 1 h at room temperature. Secondary antibodies used were Cy3-donkey anti-rat IgG for PECAM (Jackson ImmunoResearch, no.712–165-153) and Alexa488-goat anti-rabbit IgG for phospho-histone H3 (Invitrogen, A11034). Sections were washed three times (3 min each) in 0.1% Triton X-100/PBS and coverslipped with 2.5% DABCO/90% glycerol/PBS, pH 8.6. Immunostaining for PECAM in conjunction with TUNEL staining was performed as follows: Cryosections were thawed and incubated in permeabilization buffer (0.1% Triton X-100/0.1% sodium citrate) for 2 min on ice. Sections were washed for 5 min in PBS and incubated in TUNEL label (Roche, no.11684795910) for 1 h at 37 °C. Following TUNEL staining, sections were washed three times (5 min each) and incubated in blocking solution (5% normal donkey serum/0.1% Triton X-100/PBS) for 2 h at room temperature. Sections were washed three times (3 min each) in 0.1% Triton X-100/PBS and incubated overnight at 4 °C in anti-PECAM antibody diluted 1:100 in blocking solution. The following day, sections were washed three times (3 min each) and incubated in Cy3-donkey anti-rat IgG (Jackson ImmunoResearch, no. 712–165-153) diluted 1:500 in blocking solution along with 20 μg/mL Hoechst stain for 1 h at room temperature. Sections were washed three times (3 min each) and incubated in 0.05% Sudan black/70% ethanol for 10 min at room temperature to quench background fluorescence. Sections were washed three times (5 min each) and coverslipped with 2.5% DABCO/90% glycerol/PBS, pH 8.6.

Immunocytochemistry. For immunocytochemistry, confluent C166 cells grown on 22- × 22-mm glass coverslips or PECAM-isolated primary yolk sac endothelial cells grown on poly-D-lysine–coated 12-mm round glass coverslips (354086, BD Biosciences) were fixed for 5 min in 4% paraformaldehyde (on ice), washed in PBS/1 mM $CaCl_2$, and permeabilized with ice-cold methanol for 30 s. Cells were then washed three times (5 min each) with 1% nonfat dried milk/150 mM sodium acetate, pH 7/PBS, followed by three times (5 min each) with 1% nonfat dried milk/PBS. Anti–PAN-β-catenin (610153, BD Biosciences) or anti–active-β-catenin (05-665, Millipore) was diluted in the second wash buffer at a concentration of 1:100. The primary antibody incubations occurred for 1 h at room temperature. Cells were then washed three times (5 min each) with 1% nonfat dried milk/PBS and incubated with 1:500 goat-anti–mouse-Alexa488 (A11001, Invitrogen) and 20 μg/mL Hoechst for 1 h at room temperature. The coverslips were washed five times with PBS (5 min each) and mounted on glass slides for microscopic examination.

Microscopy and Image Acquisition. Gross embryonic images were obtained with a Nikon SMZ800 stereomicroscope and Nikon DS-Fi1 camera and monitor. Bright-field histological images were obtained with a Nikon Eclipse 80i microscope using a 40× (NA 0.75) objective and a Nikon DS-Fi1 camera. Fluorescent images were obtained with a Nikon Eclipse 80i microscope using 10× (NA 0.3), 20× (NA 0.5), and 40× (NA 0.75) objectives, an X-cite 120Q light source, and a Nikon DS-Qi1Mc camera. NIS-Elements AR3.0 (Nikon) software was used for used for all bright-field and fluorescent image acquisition and assembly. Histological analysis and transmission electron microscopy were performed as described (1).

1. Griffin CT, Brennan J, Magnuson T (2008) The chromatin-remodeling enzyme BRG1 plays an essential role in primitive erythropoiesis and vascular development. *Development* 135:493–500.

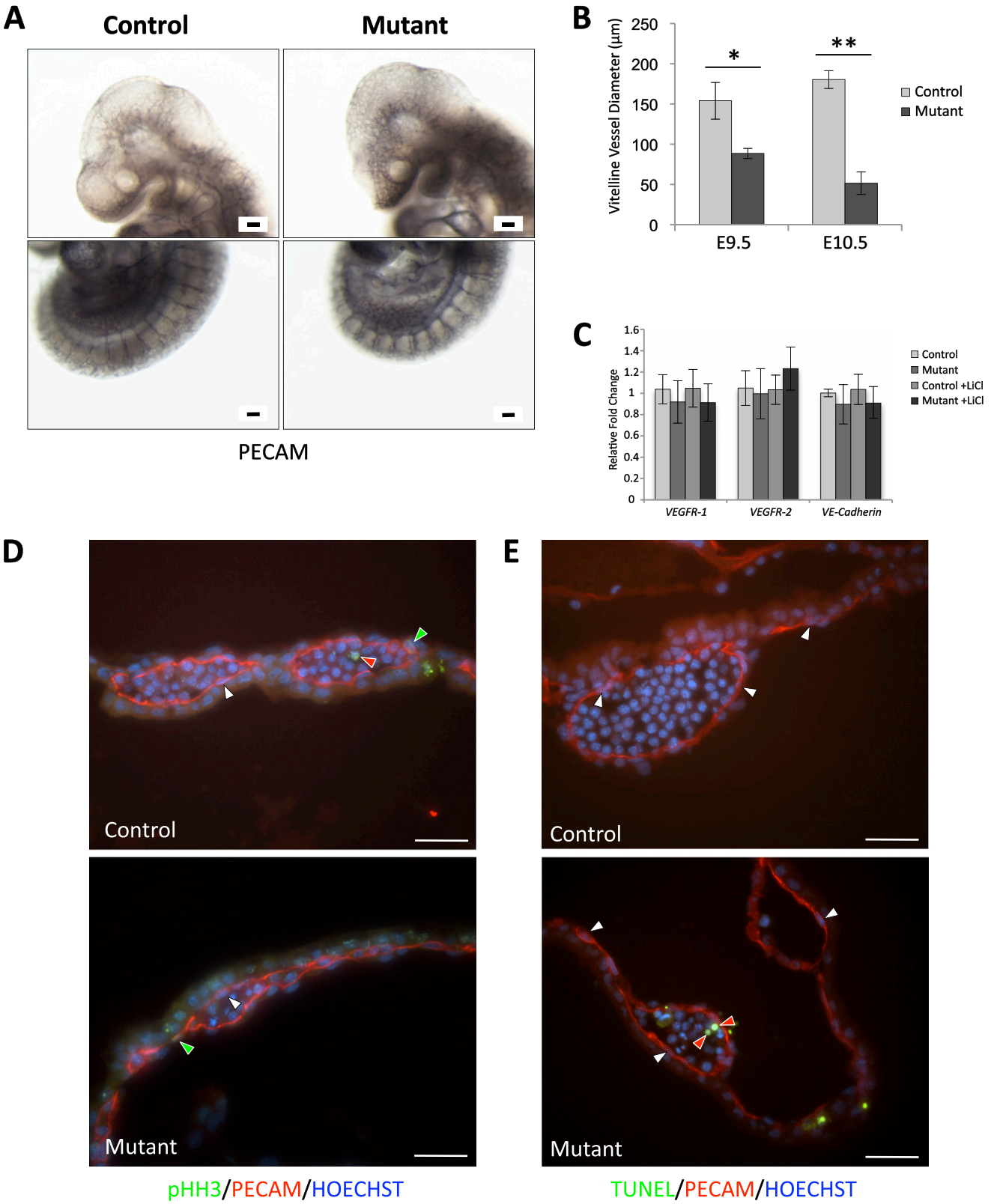

Fig. S1. Phenotypic analysis. (*A*) Whole-mount anti-PECAM1 immunostaining on E9.5 littermate control *Brg1*$^{fl/fl}$ and mutant *Brg1*$^{fl/fl}$;*Tie2-Cre*$^{+/0}$ embryos demonstrates that mutant blood vessels are patterned normally in the embryo as opposed to the yolk sac (Fig. 1). (Scale bars: 100 mm.) (*B*) Mean vitelline vessel diameter measured from four control and four mutant whole-mount yolk sacs at E9.5 and from seven control and four mutant whole-mount yolk sacs at E10.5 demonstrates significant thinning in *Brg1* mutants. Errors were calculated as ±SEM, and a two-tailed Student's *t* test was used to detect statistical differences in controls versus mutants (*$P < 0.05$; **$P < 0.005$). (*C*) Endothelial cells from control and mutant yolk sacs from untreated or lithium chloride (LiCl)-treated litters were isolated, RNA was purified, cDNA was synthesized, and qPCR was carried out for endothelial cell markers (*VEGFR-1*, *VEGFR-2*, and *VE-cadherin*). Errors represent ±SEM from four independent experiments. (*D*) Cryosections from control versus *Brg1* mutant yolk sacs were coimmunostained for the proliferation

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marker phospho-histone H3 (green) and the endothelial marker PECAM1 (red). Green arrowheads, phospho-histone H3–positive endothelial cells; red arrowheads, phospho-histone H3–positive blood cells; white arrowheads, phospho-histone H3–negative endothelial cells. Five of 51 endothelial cells from two control yolk sacs and 10 of 67 endothelial cells from two mutant yolk sacs stained positively for phospho-histone H3. Nuclei were stained with Hoechst (blue). (Scale bars: 50 μm.) (*E*) Cryosections from control versus *Brg1* mutant yolk sacs were costained for TUNEL (green) and the endothelial cell marker PECAM1 (red). White arrowheads, TUNEL-negative endothelial cells; red arrowheads, TUNEL-positive blood cells. Zero of 96 endothelial cells from two control yolk sacs and one of 98 endothelial cells from two mutant yolk sacs stained positively for TUNEL. Nuclei were stained with Hoechst (blue). (Scale bars: 50 μm.)

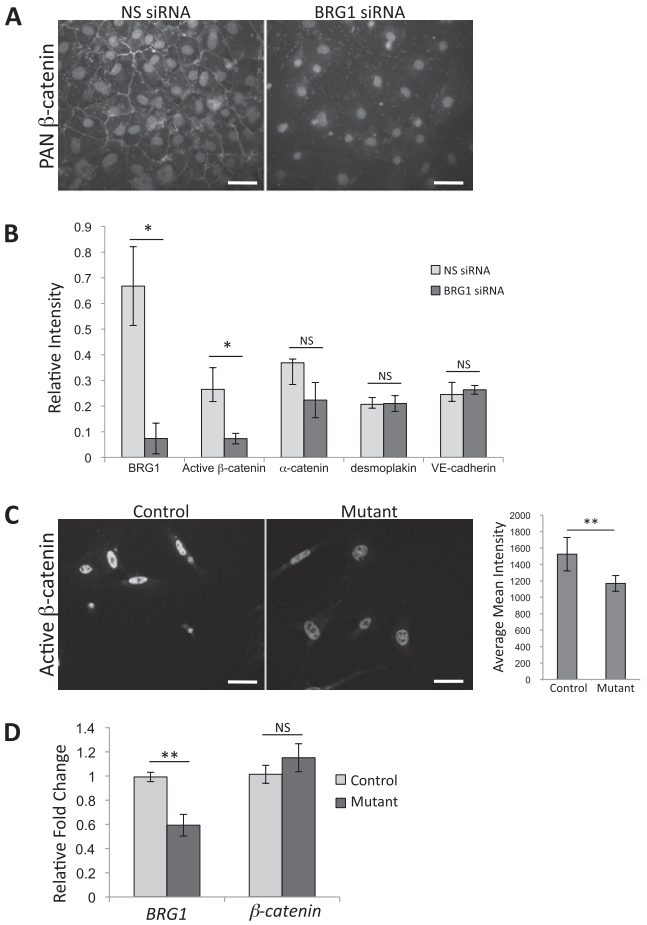

Fig. S2. Analysis of adherens junction proteins in *Brg1* mutants. (*A* and *B*) C166 cells were transfected with nonspecific (NS) or *Brg1*-specific siRNA for 48 h. (*A*) Adherent cells were immunostained with an anti–PAN-β-catenin antibody. (Scale bars: 50 μm.) (*B*) Western blot analysis was performed using antibodies that recognize BRG1, active β-catenin, α-catenin, desmoplakin, VE-cadherin, and GAPDH. Intensity was determined for each protein and normalized to the intensity of GAPDH. Data are presented as the mean ±SEM from two to five independent experiments, and significant differences between NS or *Brg1*-specific siRNA-transfected cells were determined using a two-tailed Student's *t* test (**P* < 0.05). (*C*) Endothelial cells from littermate control and mutant yolk sacs were isolated and cultured. Adherent cells were immunostained with an anti–active-β-catenin antibody. (Scale bars: 50 μm.) Average mean intensity was compiled from mean intensity readings for eight control and nine mutant fields. Error bars represent ±SD, and a two-tailed Student's *t* test was used to detect statistical differences in controls versus mutants (***P* < 0.005). (*D*) Endothelial cells from littermate control and mutant yolk sacs were isolated, RNA was purified and qPCR for *Brg1* or *β-catenin* was performed. Errors represent ±SEM from six independent experiments, and significant differences between littermate controls and mutants were calculated using a two-tailed Student's *t* test (***P* < 0.005).

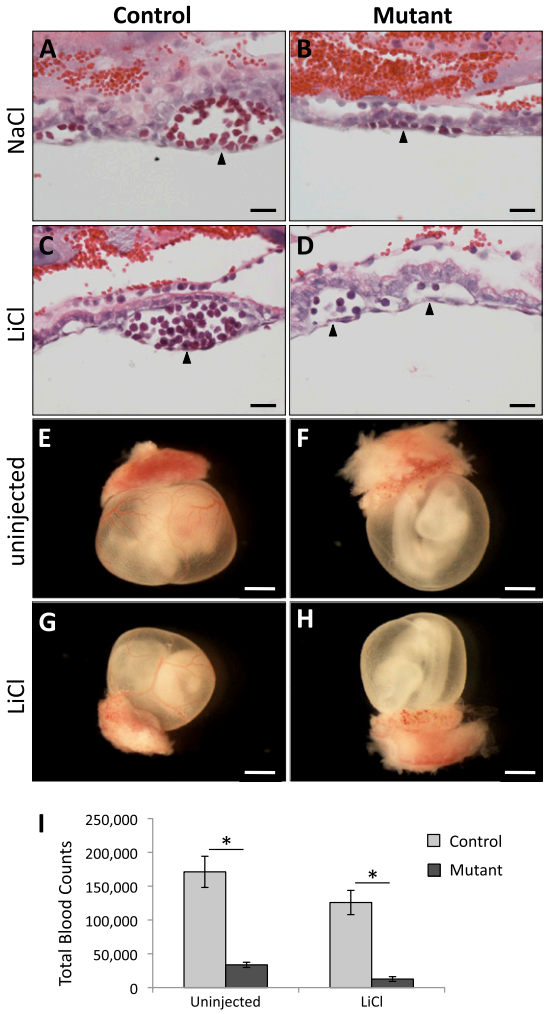

Fig. S3. Inhibition of β-catenin degradation with lithium chloride (LiCl) treatment rescues *Brg1* mutant yolk sac vascular flattening but does not rescue anemia. (*A–D*) Pregnant mice were injected with 400 mg/kg NaCl (*A* and *B*) or LiCl (*C* and *D*) on E8.5 and E9.5. Paraffin sections of E10.5 littermate control $Brg1^{fl/fl}$ (*A* and *C*) and mutant $Brg1^{fl/fl}$;$Tie2\text{-}Cre^{+/0}$ (*B* and *D*) yolk sacs were stained with hematoxylin and eosin. Vascular flattening is partially rescued in LiCl-treated mutant yolk sacs (*D*) compared with NaCl-treated mutants (*B*). Vessels are delineated by arrowheads. (Scale bars: 50 μm.) (*E–H*) Gross photos of E10.5 embryos and yolk sacs without treatment (*E* and *F*) or treated with 400 mg/kg LiCl (*G* and *H*). The mutant $Brg1^{fl/fl}$;$Tie2\text{-}Cre^{+/0}$ specimens (*F* and *H*) are visibly paler than their control $Brg1^{fl/fl}$ (*E* and *G*) littermates. (Scale bars: 1 mm.) (*I*) Total blood counts from untreated and LiCl-treated control and *Brg1* mutant embryos at E10.5. A total of 11 control and 7 mutant uninjected embryos from three separate litters were analyzed; 14 control and 3 mutant LiCl-treated embryos from two separate litters were analyzed. Data are presented as the mean ±SEM, and significant differences between blood counts from control versus mutant embryos were determined using a two-tailed Student's *t* test (*$P < 0.05$).

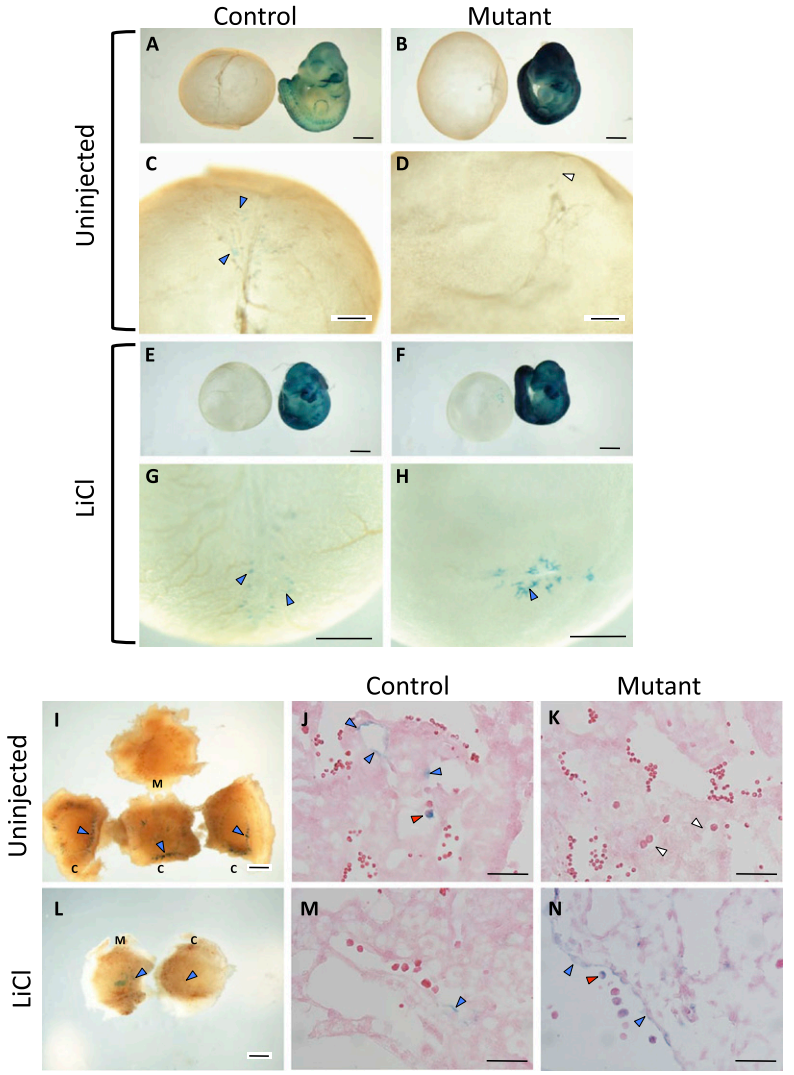

Fig. S4. Lithium chloride (LiCl) treatment rescues Wnt signaling in *Brg1* mutant yolk sac and placental endothelial cells. (*A–H*) E10.5 embryos carrying the *BAT-gal* Wnt-signaling reporter were stained for β-galactosidase activity and photographed with their yolk sacs. Littermate control (*A*, *C*, *E*, *G*) and mutant (*B*, *D*, *F*, *H*) embryos were compared following no treatment (*A–D*) or 2 d of in utero LiCl treatment (*E–H*). Blue arrowheads, yolk sac vessels with reporter activity; white arrowhead, yolk sac vessels with no reporter activity. [Scale bars: 1 mm (*A, B, E, F*); 500 μm (*C, D, G, H*).] (*I–N*) Placentas from untreated versus LiCl-treated E10.5 embryos were removed and photographed after staining for β-galactosidase activity. Cryosections of stained placentas from littermate controls (*J* and *M*) and mutants (*K* and *N*) were examined for reporter-positive cells. Blue arrowheads, placental extraembryonic endothelial cells with reporter activity; red arrowheads, embryonic blood cells with reporter activity; white arrowheads, extraembryonic endothelial cells with no reporter activity. [Scale bars: 1 mm (*I* and *L*); 50 μm (*J, K, M, N*).]

A

gene	fold	
Wisp1	0.13	DOWNREGULATED
Lef1	0.49	
Vcan	0.50	
Jag1	0.51	
CyclinD1	0.54	
T	0.61	
Fn1	0.68	
Fst	0.80	
Nkx2-2	0.80	
Cdx1	0.80	
Fgf4	0.80	
Jun	0.81	
Bglap2	0.81	
Cdkn2a	0.82	
Gja1	0.83	
Sp5	0.84	
Hnf1a	0.87	
Axin2	0.88	
Myc	0.89	
Tnfrsf19	0.90	
Pitx2	1.01	NO CHANGE
Vegfa	1.09	
Fosl1	1.30	UPREGULATED
Ppard	1.33	
Fgf18	1.75	
Id2	1.81	
Cldn1	2.62	
Tcf4	3.17	

B

gene	fold change	*p*-value
Fzd5	-1.65	0.0032
Fzd2	-1.38	0.0131
Fzd3	-2.20	0.0312
Fzd6	-2.61	0.0319

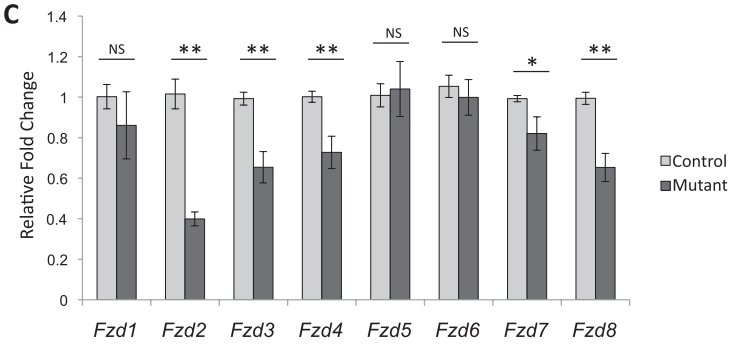

Fig. S5. BRG1 impacts Wnt signaling through regulation of the *Fzd* family of receptors. (*A*) Endothelial cells from littermate control and mutant yolk sacs were isolated, RNA was purified, and cDNA was synthesized. Samples were processed for qPCR using a custom-designed array containing 28 Wnt target genes. Data from four independent experiments were compiled using SABiosciences Excel-based data analysis, and fold changes in gene expression are shown. Shaded boxes indicate target genes for which expression changes were further verified by individual qPCR analyses (Fig. 2*D*). (*B*) C166 cells were transfected with nonspecific (NS) or *Brg1*-specific siRNA for 48 h. RNA was isolated, cDNA was synthesized, and samples were processed for qPCR using a commercially available Wnt signaling array. Data from three independent experiments were compiled using SABiosciences Excel-based data analysis. (*C*) Endothelial cells from littermate control and mutant yolk sacs were isolated, RNA was purified, cDNA was synthesized, and qPCR for *Fzd1-8* was performed. Errors represent ±SEM from six independent experiments, and significant differences between littermate controls and mutants were calculated using a two-tailed Student's *t* test (**P* < 0.05; ***P* < 0.005).