Supplemental data

Lysophosphatidic acid-induced YAP/TAZ activation promotes developmental angiogenesis by repressing Notch ligand Dll4

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It includes;

1. Supplemental Methods and their references
2. Supplemental Figures 1-17 and their legends
3. Supplemental Tables 1-9

SUPPLEMENTAL METHODS

Reagents.

LPA (1-oleoyl-LPA) was purchased from Avanti Polar Lipids (Alabaster, AL) and stored at -30 °C (10 mM stock in 50% ethanol). S1P was purchased from Cayman Chemical (Ann Arbor, MI) and stored at -30 °C (1 mM stock in methanol). ATP (10 mM stock in water stored at -30 °C), Ki16425 (10 mM stock in water stored at -30 °C), Y27632 (10 mM stock in water stored at -30 °C), and latrunculin A (10 mM stock in dimethyl sulfoxide [DMSO] stored at -30 °C) were from Wako (Osaka, Japan). Rho inhibitor I (0.1 μg/μl stock in water stored at 4 °C) was from Cytoskeleton (Denver, CO).

Mouse breeding and genotyping.

Mice were housed under specific pathogen-free conditions in an air-conditioned room and fed standard laboratory chow *ad libitum* (CE-2; CLEA Japan, Tokyo, Japan), in accordance with institutional guidelines. During the mating of female mice (more than 8 weeks old), presence of the vaginal plug, as evidence of copulation, was checked every morning. Embryonic day (E) 0.5 was defined as noon of the day when the vaginal plug was detected. Pups were weaned at 3-4 weeks old. Mouse genotypes were determined by PCR analysis using genomic DNA samples from the tails and ExTaq DNA polymerase (Takara Bio, Ohtsu, Japan). The primer sequences were listed in Supplemental Table 8.

Macroscopic observation and histological analysis.

Yolk sac and embryo proper were surgically removed from pregnant females at the embryonic days indicated. After taking photographs for macroscopic observations, they were fixed in 10% formalin (Mildform 10N; Wako) and embedded in paraffin. Sections were either stained with hematoxylin and eosin or used for immunohistochemical analysis conducted by incubating the sections with rat monoclonal primary antibody directed against mouse PECAM-1 overnight at 4 °C (diluted 1:50; clone SZ31, Dianova, Hamburg, Germany). As a secondary antibody, peroxidase-conjugated goat anti-rat IgG (diluted 1:100; Nichirei Biosciences, Tokyo, Japan) was used at room temperature for 30 min.

Whole-mount immunohistochemical staining.

Whole-mount embryo immunostaining was performed according to the method previously reported (1). Briefly, E10.5 yolk sac and embryo proper were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4 °C, dehydrated through a graded methanol series, incubated with 5% hydrogen peroxide in methanol for 1 h at 4 °C, and rehydrated through a graded methanol series into PBS. Samples were blocked with PBS containing 3% skim milk and 0.1% Triton X-100 at room temperature for 1 h and incubated in blocking buffer with a rat monoclonal primary antibody directed against mouse PECAM-1 (diluted 1:200; clone MEC13.3, BD Biosciences, San Jose, CA) overnight at 4 °C. After washing with PBS, samples were then incubated with peroxidase-conjugated goat anti-rat IgG (diluted 1:100; Sigma-Aldrich, St. Louis, MO) in blocking buffer at room temperature for 1 h and stained with 3,3′-diaminobenzidine (Tokyo Chemical Industry, Tokyo, Japan) as a peroxidase substrate.

Cell culture and transfection.

HUVECs purchased from Lonza (Walkersville, MD) were cultured in collagen-coated 100-mm dishes at 37 °C in 5% CO_2 with EGM-2 BulletKit (Lonza) containing fetal bovine serum (FBS) at a concentration of 2% (in experiments of Figure 3 and 8, and Supplemental Figure 3, 7, and 9) or 20% (in experiments of Figure 4, 6, 7, 10, and 11, and Supplemental Figure 4-6, 8, 10, and 11). The cells were used for assays between passages 4 and 7. For the RNAi experiments, SMARTpool ON-TARGETplus siRNAs targeting human LPA4, LPA6, Gα12, Gα13, YAP, and TAZ were purchased from GE Healthcare (Piscataway, NJ). These siRNAs as well as control siRNA (ON-TARGETplus Non-targeting Pool) were used at 12.5 nM. Silencer Select siRNAs targeting TEAD1, TEAD2, TEAD3, TEAD4, DLL4, β-catenin, and negative control No.1 were purchased from Invitrogen and used at 5 nM. Transfection of siRNAs was performed using DharmaFECT 1 or Lipofectamine RNAiMAX (both from Thermo Scientific) according to the manufacturer's instructions. For the overexpression experiments, the expression vectors for human LPA1-6 were described previously (2). Transfection of these expression vectors was performed using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer's instructions.

qRT-PCR analysis.

For the preparation of cDNA templates, total RNA was isolated by using QIAzol lysis reagent and an RNeasy mini kit (both from Qiagen, Valencia, CA). cDNA was synthesized from 500 ng

of total RNA using a PrimeScript RT Reagent Kit (TaKaRa Bio) with random hexamers. Quantitative PCR was performed using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) with a KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Wilmington, MA). The cycling conditions were as follows: initial denaturation at 95 °C for 30 sec, followed by 50 cycles of 95 °C for 5 sec, 60 °C for 20 sec, and 65 °C for 15 sec. The mRNA levels of the target genes were normalized to the standard housekeeping gene β-actin (*ACTB)* or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) in HUVEC samples and the endothelial marker gene *Pecam1* in mouse tissue samples. The primer sequences used to detect gene expression for LPA receptors and angiogenesis factors are listed in Supplemental Table 9.

Luciferase reporter assay.

HUVECs (2×10^4) were seeded onto collagen-coated 24-well plates. Immediately after the medium change from EGM-2 BulletKit containing 2% FBS to EGM-2 BulletKit containing 0.1% BSA, the cells were transfected with serum response factor response element (SRF-RE)-firefly luciferase-pGL4.34 (Promega, Madison, WI) and SV40-*Renilla* luciferase-pRL-SV40 (Promega) using Lipofectamine 2000. After a 4-h incubation at 37 °C, cells were treated with 10 μM LPA for 6 h and lysed using passive lysis buffer for the Dual Luciferase assay (Promega). In some experiments, cells were treated with 10 μM Y27632 or Ki16425 for 10 min before LPA stimulation. Firefly and *Renilla* luciferase activities in the cell extracts were determined with a MiniLumat LB9506 luminometer (Berthold, Bad Wildbad, Germany). Firefly luciferase values were standardized to *Renilla* ones.

Intracellular calcium influx measurements.

HUVECs (3×10^6) were seeded onto 10-cm collagen-coated dishes, cultured for 24 h in EGM-2 BulletKit containing 2% FBS, and serum-starved for 8 h in EGM-2 BulletKit containing 0.1% BSA. Cells were detached with PBS containing 2 mM EDTA, washed with buffer A (Hanks' balanced salt solution containing 25 mM HEPES-NaOH [pH 7.4], 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% BSA), and loaded with 3 μM Fura-2 AM (Dojindo, Kumamoto, Japan) in buffer A with 0.01% pluronic acid (Molecular Probes, Eugene, OR) at 37 °C for 1 h. Then, cells were washed with buffer A and re-suspended in buffer A at a density of 1×10^6 cells/ml. The cell suspension (0.5 ml) was applied to a CAF-110 spectrofluorometer (Jasco, Tokyo, Japan). Upon adding 5 μl of 100 × ligand solution, the intracellular Ca^{2+} concentration was measured by

determining the ratio of emissions at 500 nm after being excited by 340- and 380-nm light, as previously described (3).

Measurement of cAMP production.

HUVECs (1×10^4) were seeded onto collagen-coated 96-well plates, cultured for 24 h in EGM-2 BulletKit containing 2% FBS, and serum-starved for 16 h in EGM-2 BulletKit containing 0.1% BSA. Cells were washed with buffer A and incubated in 50 µl of buffer A with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 15 min at room temperature. Reactions were initiated by adding 50 µl of 2 × ligand solution with or without 40 µM forskolin (Sigma-Aldrich; from a 10 mM stock in DMSO stored at -30 °C) for Gαi or Gαs protein activation, respectively. After a 30-min incubation at room temperature, reactions were terminated by adding 10 µl of 10% Tween-20, followed by overnight storage at 4 °C. The cAMP concentration in the cell lysate was determined using an AlphaScreen cAMP assay kit (PerkinElmer, Waltham, MA) as recommended by the manufacturer.

Protein extraction and western blotting.

HUVECs were lysed in lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium dodecyl sulfate [SDS], and 1% sodium deoxycholate) containing phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF) and a protease inhibitor cocktail (cOmplete; Roche Diagnostics). Lysates were centrifuged at 10,000 × *g* for 5 min, and the supernatant was collected. Protein concentrations were determined by BCA assay (Thermo Scientific). The resultant protein samples were diluted in sample buffer (25 mM Tris HCl [pH 6.5], 1% SDS, 5% glycerol, 0.05% bromophenol blue, and 5% 2-mercaptoethanol). Equal amounts of total proteins were electrophoresed on 8% SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. Gels containing phos-tag (Wako) were prepared according to manufacturer's instructions. YAP proteins can be separated into multiple bands in the presence of phos-tag depending on differential phosphorylation levels, with phosphorylated proteins migrating more slowly. After blocking with 5% skim milk in TBS-T (20 mM Tris-buffered saline [pH 7.6] and 0.1% [v/v] Tween 20), the blots were incubated overnight at 4 °C with one of the following primary antibodies: Gα12 (#sc-409), Gα13 (#sc-410), YAP (#sc-15407) (Santa Cruz Biotechnology, Santa Cruz, CA), TAZ (#4883), phospho-YAP (Ser127; #4911), Dll4 (#2589), β-catenin (#8480), pan-TEAD (#13295), and β-actin (#4967) (Cell Signaling Technology). The membranes were then washed with TBS-T

and incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The proteins were visualized using ImmunoStar LD (Wako) and a C-DiGit blot scanner (LI-COR Biotechnology, Lincoln, NE). To evaluate phosphorylation of Akt, HUVECs transfected with YAP/TAZ siRNA or control siRNA were starved for 8 h, and stimulated with 50 ng/ml VEGF-A（PeproTech, Rocky Hill, NJ）, 400 ng/ml Ang-1 (R&D Systems), or 10% FBS for 10 min. Cell were then washed with ice cold PBS and lysed in lysis buffer containing phosphatase inhibitors and a protease inhibitor cocktail. Aliquots of cell lysate were subjected to SDS-PAGE and western blotting with pan-Akt (#4691) and phospho-Akt (Ser473; #4060) (Cell Signaling Technology).

Isolation of mouse lung ECs.

Lungs were excised from 6-week-old male mice, minced well with a razor blade, and digested with 50 U/ml DNase I (Sigma-Aldrich) and 0.26 U/ml Liberase (Roche Diagnostics) in DMEM for 45 min at 37°C. Digested tissue was filtered through a 40-μm cell strainer (BD Biosciences). Lung ECs were purified from the cell suspension for 15 min at room temperature using positive selection with rat anti-mouse PECAM-1 antibody (#553370, BD Biosciences) that was preconjugated to sheep anti-rat IgG Dynabeads (Thermo Scientific) overnight at 4°C. Lung ECs were cultured on 0.2% gelatin-coated tissue culture dishes until they became confluent in DMEM supplemented with 20% FBS and Endothelial Mitogen (Biomedical Technologies, Stoughton, MA). Then, cells were further purified after trypsinization using the Dynabeads coupled to anti-PECAM-1 antibody.

Flow cytometry.

Cells were incubated with 2 μg/ml phycoerythrin-conjugated anti-mouse PECAM-1 antibody (clone MEC 13.3; BD Biosciences) in PBS containing 2% goat serum for 1 h at room temperature. After washing with PBS, cells were analyzed with a flow cytometer (BD Accuri C6, BD Biosciences).

Immunofluorescence staining of YAP.

HUVECs and mouse lung ECs were grown to confluence at 37 °C under 5% CO_2 on collagen-coated glass-bottom dishes (Mat Tek Corporation, Ashland, MA), washed with PBS, and fixed with 4% PFA at room temperature for 10 min. To determine whether YAP was localized with internal compartments, cells were permeabilized with 20% permeabilization

buffer (IntraPrep; Beckman Coulter, Fullerton, CA) at room temperature for 10 min, washed with PBS, and incubated with anti-YAP primary antibody (diluted 1:100; sc-15407, Santa Cruz Biotechnology) at room temperature for 1 h. After washing with PBS, cells were then incubated with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (diluted 1:200; Abcam) at room temperature for 1 h. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; diluted 1:100,000; BioLegend, San Diego, CA). Fluorescent images of labeled cells were acquired using a laser scanning confocal microscope (LSM 510 META; Carl Zeiss). More than five microscopic fields were randomly chosen, and cells displaying preferential nuclear YAP localization, even distribution of YAP in nucleus and cytoplasm, or cytoplasmic YAP localization were counted.

Proliferation assay.

Forty-eight h after transfection with LPA4 and LPA6 siRNAs, HUVECs were trypsinized and seeded onto 96-well plates at 5.0×10^3 cells per well. Cell proliferation was analyzed using thiazolyl blue tetrazolium bromide (Sigma-Aldrich) according to the manufacturer's instructions.

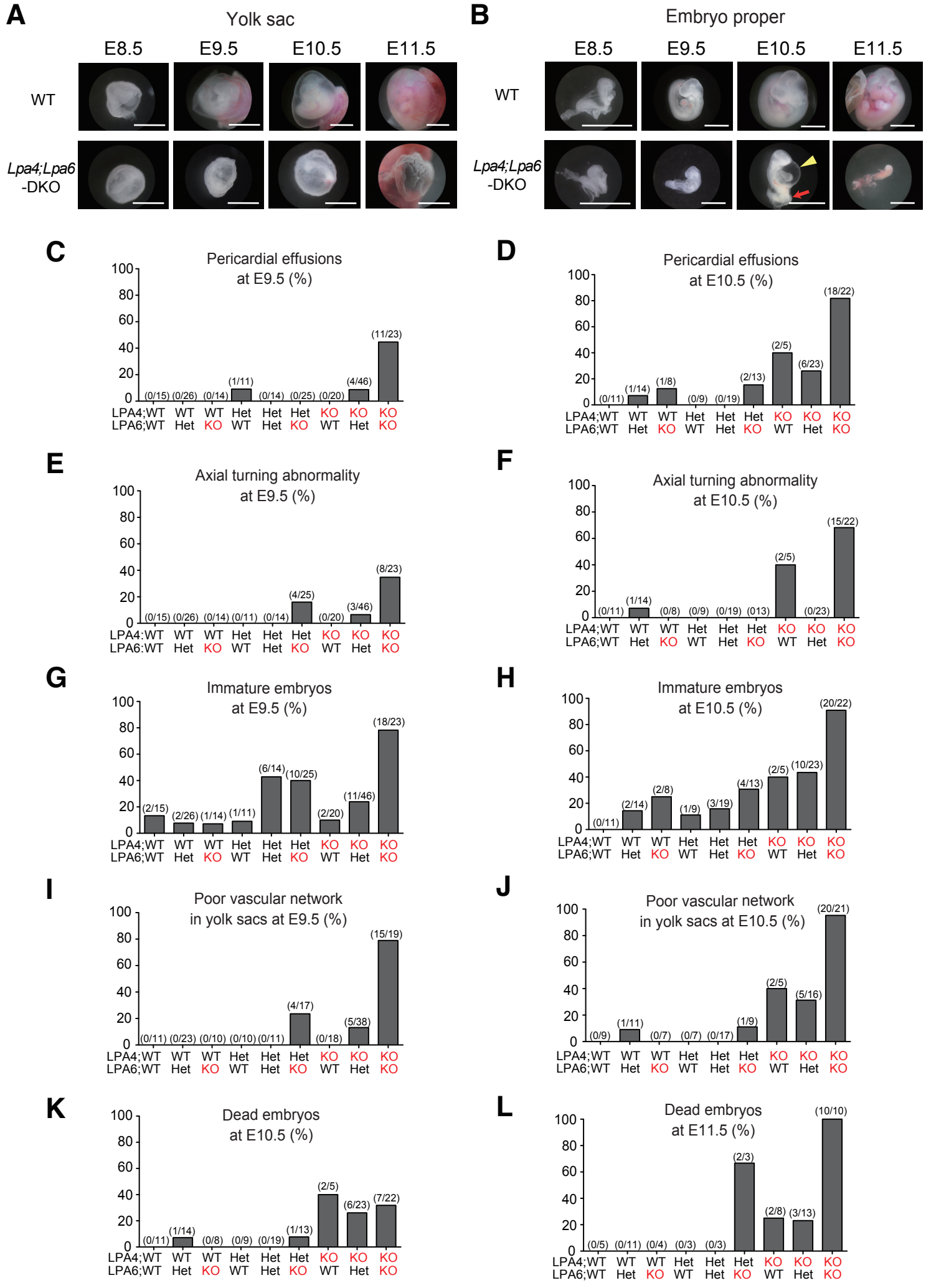
Retrovirus infection.

Retroviruses were produced by co-transfecting 293gp packaging cells (RIKEN BRC) with pCMV-VSV-G-RSV-Rev vector (RIKEN BRC) and either pQCXIH-empty vector (TaKaRa Bio), pQCXIH-Myc-YAP (Addgene), pQCXIH-Myc-YAP-5SA (Addgene), or pQCXIH-Myc-YAP-S94A (Addgene) vector using Lipofectamine 2000. Forty-eight hours after transfection, retroviral supernatant was collected and filtered through a 0.45-μm filter. The viral particles were precipitated by centrifugation with PEG-it virus precipitation solution (SBI System Biosciences, Mountain View, CA) for concentration. HUVECs were spin-infected with the concentrated retrovirus at $800 \times g$ for 1 h in the presence of 10 μg/ml polybrene (Santa Cruz Biotechnology). After 2 days of recovery, infected cells were selected with 100 μg/ml hygromycin B (Wako), and the entire surviving population of cells was used.

References for supplemental methods

1. Kono M, et al. The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. *J Biol Chem.* 2004;279(28):29367-29373.
2. Yanagida K, et al. Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6. *J Biol Chem.* 2009;284(26):17731-17741.
3. Yasuda D, et al. Helix 8 of leukotriene B4 type-2 receptor is required for the folding to pass the quality control in the endoplasmic reticulum. *FASEB J.* 2009;23(5):1470-1481.

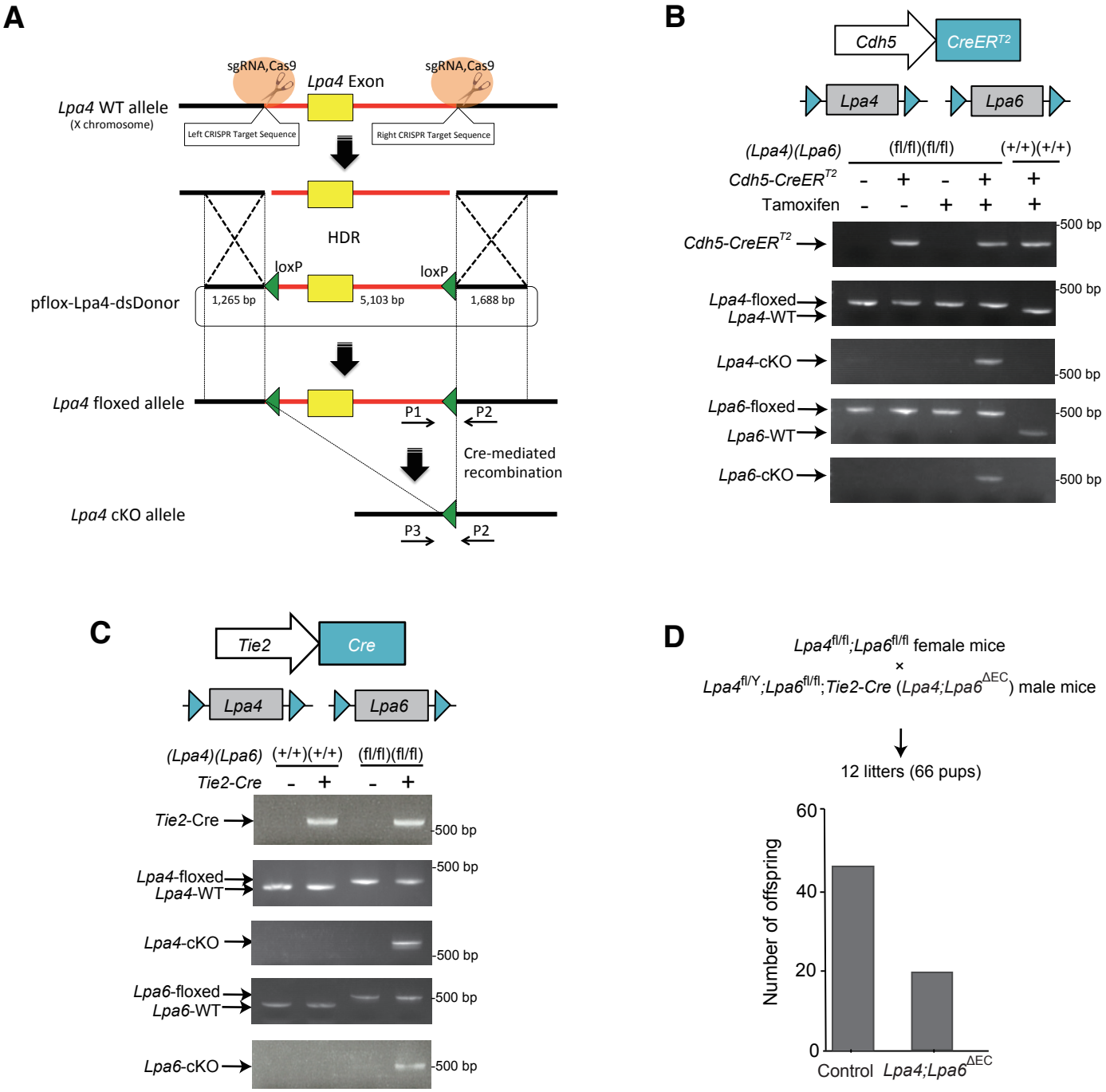
SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 1 (Continued)

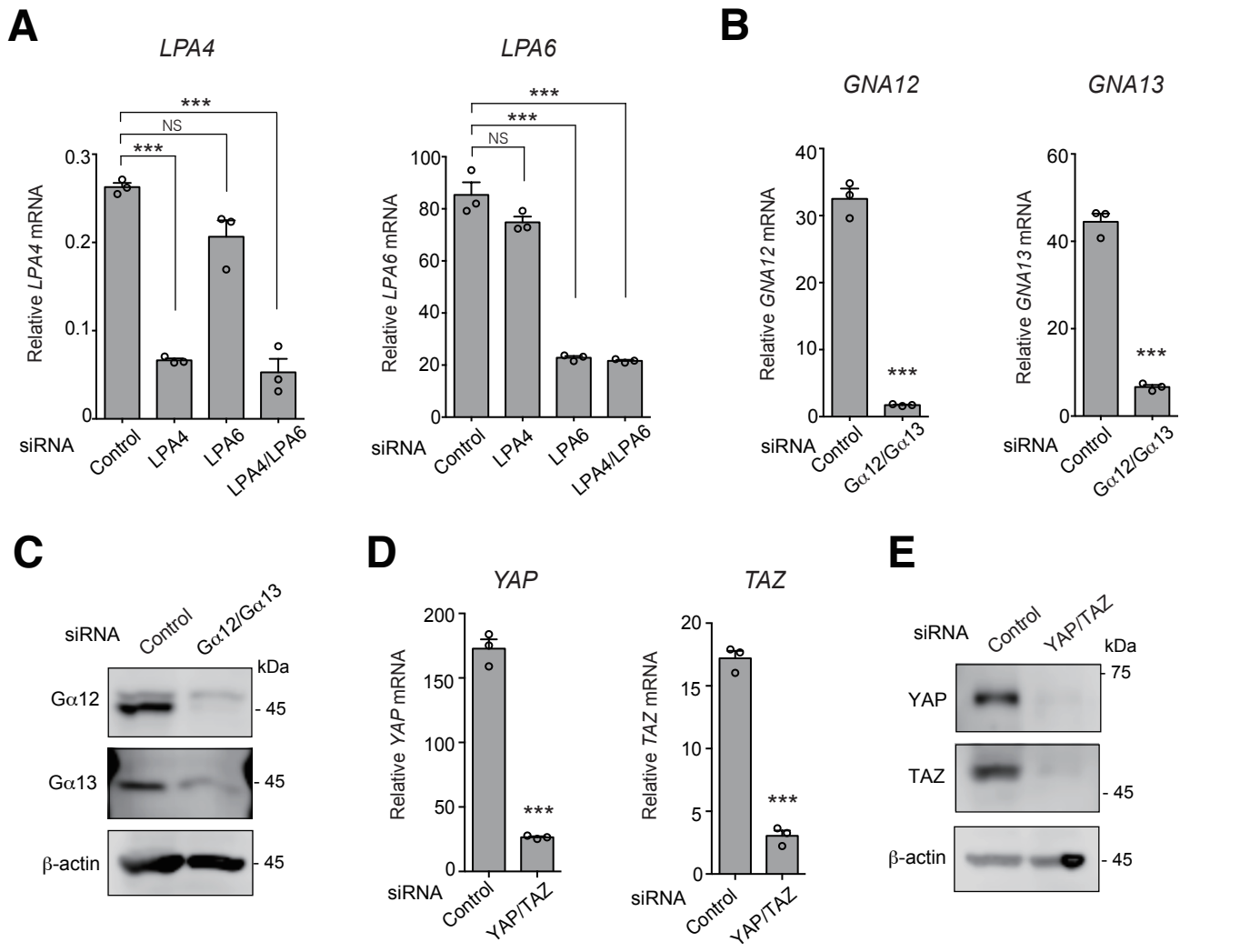
Supplemental Figure 1. Vascular abnormalities and lethality by E11.5 of *Lpa4*;*Lpa6* DKO yolk sac and embryo proper. (**A** and **B**) Gross morphologies of WT and *Lpa4*;*Lpa6* DKO yolk sac (**A**) and embryo proper (**B**) from E8.5 to E11.5. At E8.5, *Lpa4*;*Lpa6* DKO embryo proper appeared normal. However, at E9.5 and E10.5, some vascular abnormalities, such as pericardial effusion (yellow arrowhead), axial turning abnormality (red arrow), and developmental delay appeared. Scale bars: 1 mm. (**C–L**) Ratios of abnormal yolk sac and embryo proper. Figures in parentheses represent ratios of embryos with each abnormality to observed samples.

SUPPLEMENTAL FIGURE 2



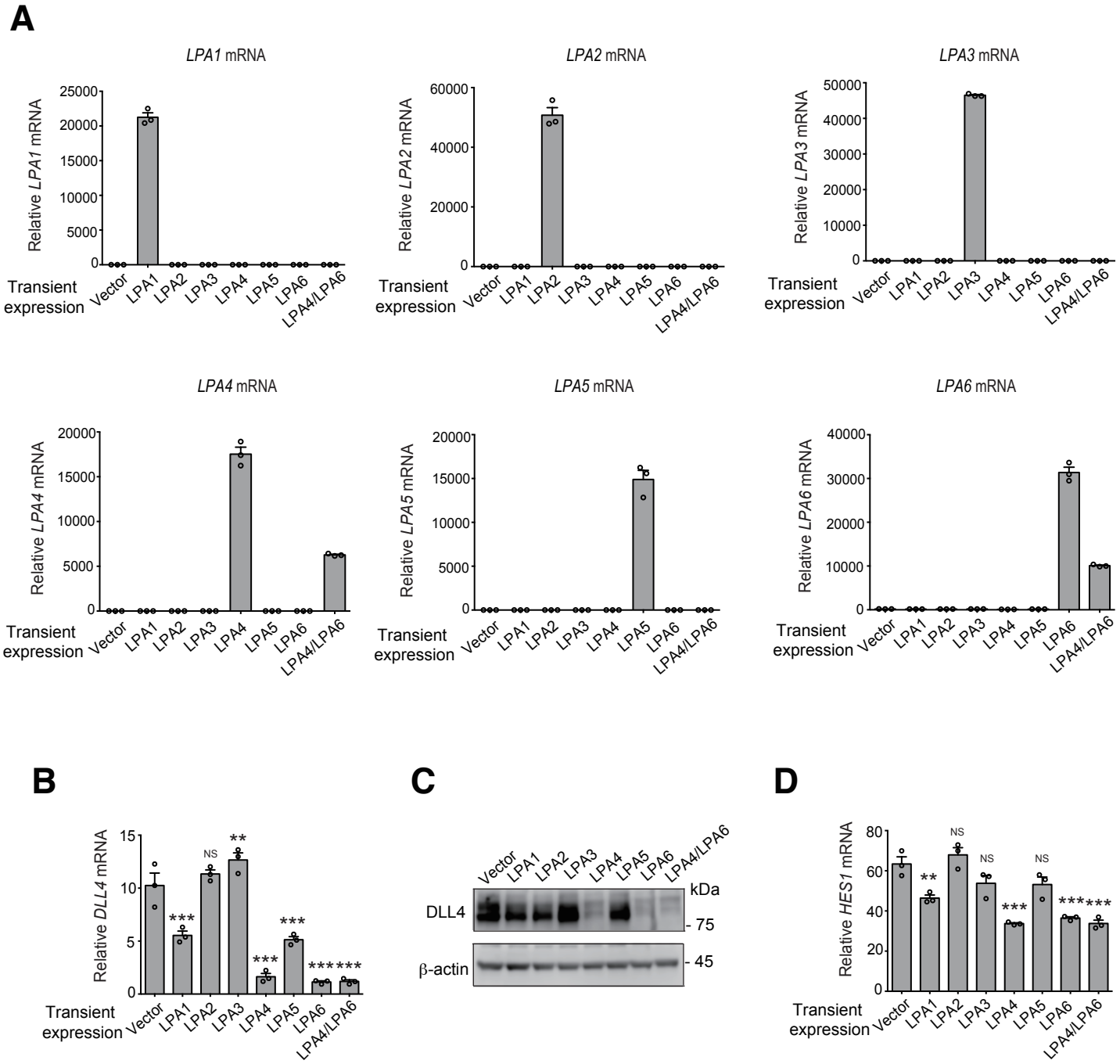
Supplemental Figure 2. Generation of endothelial-specific *Lpa4*;*Lpa6* DKO mice. (**A**) Schematic diagram of the construction of *Lpa4* conditional knockout (cKO) allele. The exon of *Lpa4* gene containing the entire open reading frame (yellow box), loxP sites (green triangles), and three PCR primers (arrows) are shown. HDR, homology-directed repair; sgRNA, single-guide RNA. (**B**) Generation of tamoxifen-inducible endothelial-specific *Lpa4*;*Lpa6* DKO (*Lpa4;Lpa6*$^{i\Delta EC}$) mice. PCR genotyping using neonatal tail DNA was performed for detecting *Lpa4* WT, floxed and cKO alleles. Primers used were forward P1 and reverse P2 for the *Lpa4* WT and floxed alleles, and forward P3 and reverse P2 for cKO allele. PCR genotyping for detecting *Lpa6* WT, floxed and cKO alleles were performed as previously described (15). PCR primers for detecting *Cdh5-CreER*^{T2} allele are shown in Supplemental Table 8. (**C**) Generation of endothelial-specific *Lpa4*;*Lpa6* DKO (*Lpa4*;*Lpa6*$^{\Delta EC}$) mice. PCR genotyping for detecting *Tie2-Cre* allele were performed as previously described (75). (**D**) Numbers of *Lpa4*;*Lpa6*$^{\Delta EC}$ mice at 4 weeks of age were about 50% less than the values expected by Mendelian ratios. Unprocessed original scans of PCR genotyping are shown in Supplemental Figure 16.

SUPPLEMENTAL FIGURE 3



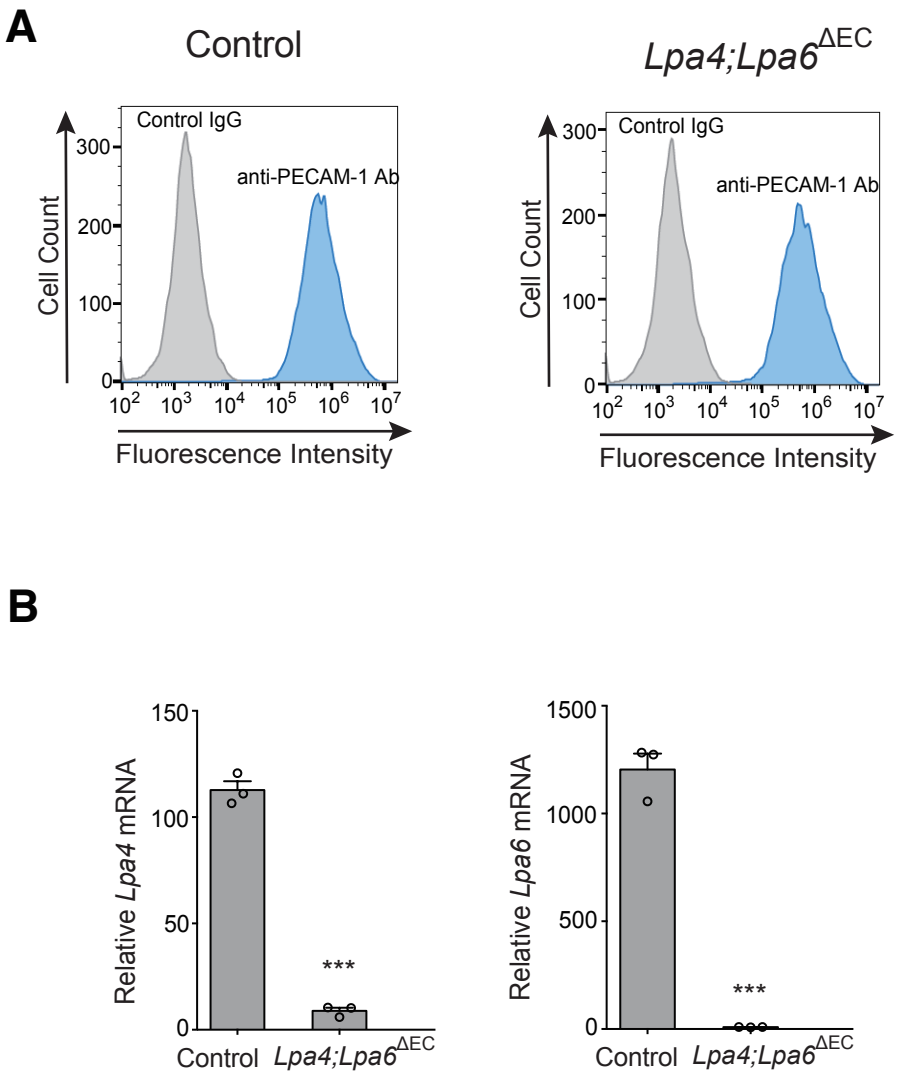
Supplemental Figure 3. Reduction of mRNA expression by gene-specific siRNA treatment in HUVECs. Effects of LPA4 and/or LPA6 siRNAs (**A**), Gα12/Gα13 siRNAs (**B** and **C**), and YAP/TAZ siRNAs (**D** and **E**) on mRNA and protein expression are shown. HUVECs were transfected with siRNAs for 96 h. Data are mean ± s.e.m. of triplicates. ***$P < 0.001$, one-way ANOVA followed by Tukey's multiple comparisons test (**A**), or two-tailed unpaired Student's *t*-test (**B, D**). NS, not significant. Unprocessed original scans of western blots are shown in Supplemental Figure 17.

SUPPLEMENTAL FIGURE 4



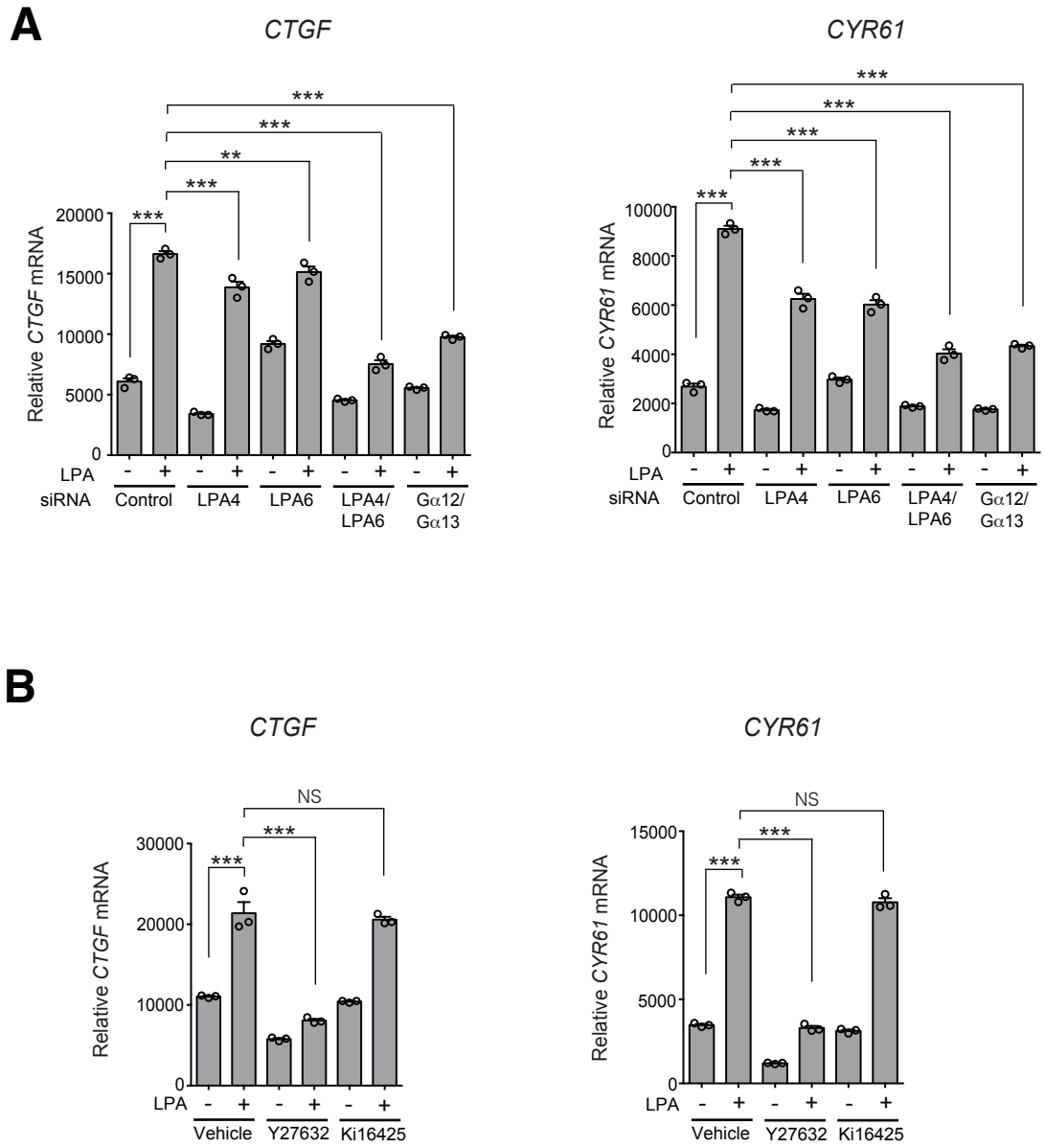
Supplemental Figure 4. Transient expression of LPA4 and/or LPA6 effectively suppressed DLL4 expression. (**A**) Increases of *LPA1-6* mRNA expression were confirmed in HUVECs 24 h after transient transfection of expression vectors. (**B**-**D**) Expression levels of DLL4 mRNA (**B**) and protein (**C**) and also *HES1* mRNA (**D**) were significantly reduced after the transfection of LPA4 and/or LPA6. Data represent mean ± s.e.m. of triplicates. $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's (**B** and **D**). NS, not significant. Unprocessed original scans of western blots are shown in Supplemental Figure 17.

SUPPLEMENTAL FIGURE 5



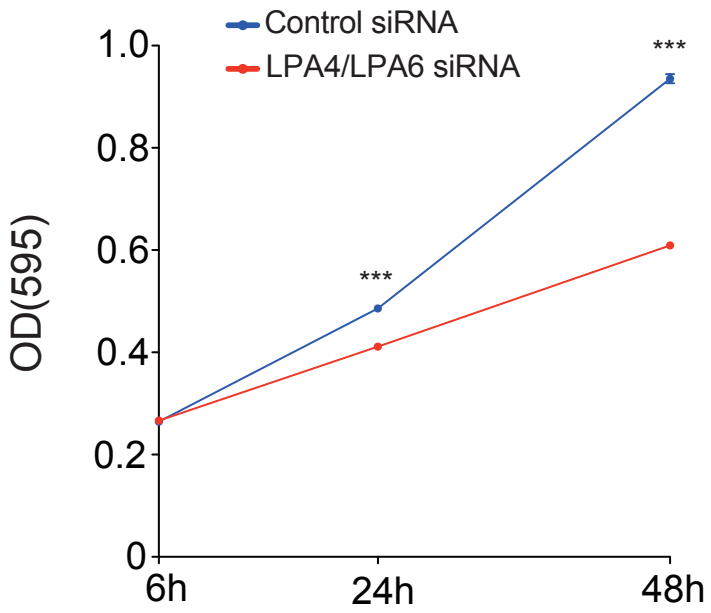
Supplemental Figure 5. Isolation of lung ECs from *Lpa4*;*Lpa6*$^{\Delta EC}$ mice. (**A**) Expression of Pecam-1 protein in lung ECs isolated from control and *Lpa4;Lpa6*$^{\Delta EC}$ mice was examined by flow cytometry. (**B**) mRNA expression levels of *Lpa4* and *Lpa6* in lung ECs isolated from control and *Lpa4;Lpa6*$^{\Delta EC}$ mice are shown. Data are mean ± s.e.m. of triplicates. ***$P < 0.001$, two-tailed unpaired Student's *t*-test.

SUPPLEMENTAL FIGURE 6



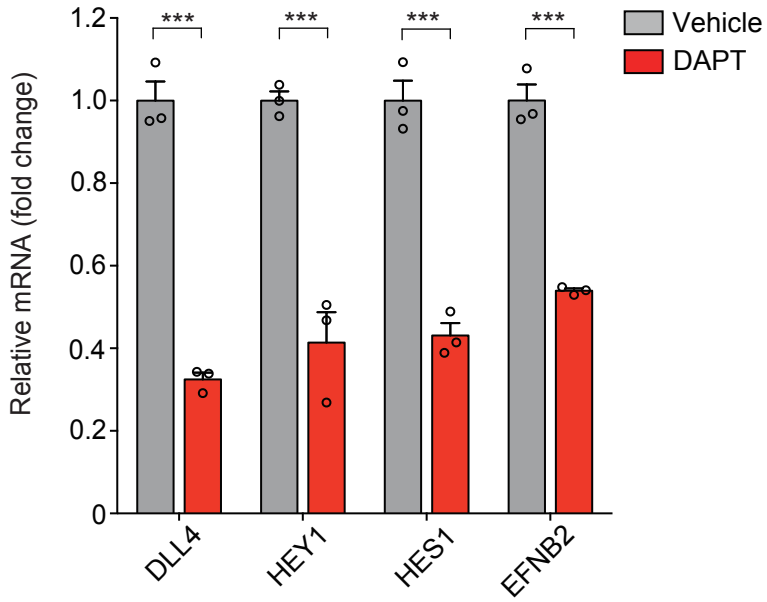
Supplemental Figure 6. Attenuation of the LPA-induced YAP target gene induction in HUVECs by LPA4/LPA6 signaling blockade. (**A**) Effects of LPA4/LPA6 or Gα12/Gα13 siRNAs (96 h pre-treatment) on LPA (10 μM, 1 h)-induced mRNA expression of *CTGF* and *CYR61*. (**B**) Effects of Y27632 (10 μM, 10 min pre-treatment) or Ki16425 (10 μM, 10 min pre-treatment) on LPA (10 μM, 1 h)-induced mRNA expression of *CTGF* and *CYR61*. Data represent mean ± s.e.m. of triplicates. ***P* < 0.01, ****P* < 0.001, one-way ANOVA followed by Tukey's multiple comparisons test. NS, not significant.

SUPPLEMENTAL FIGURE 7



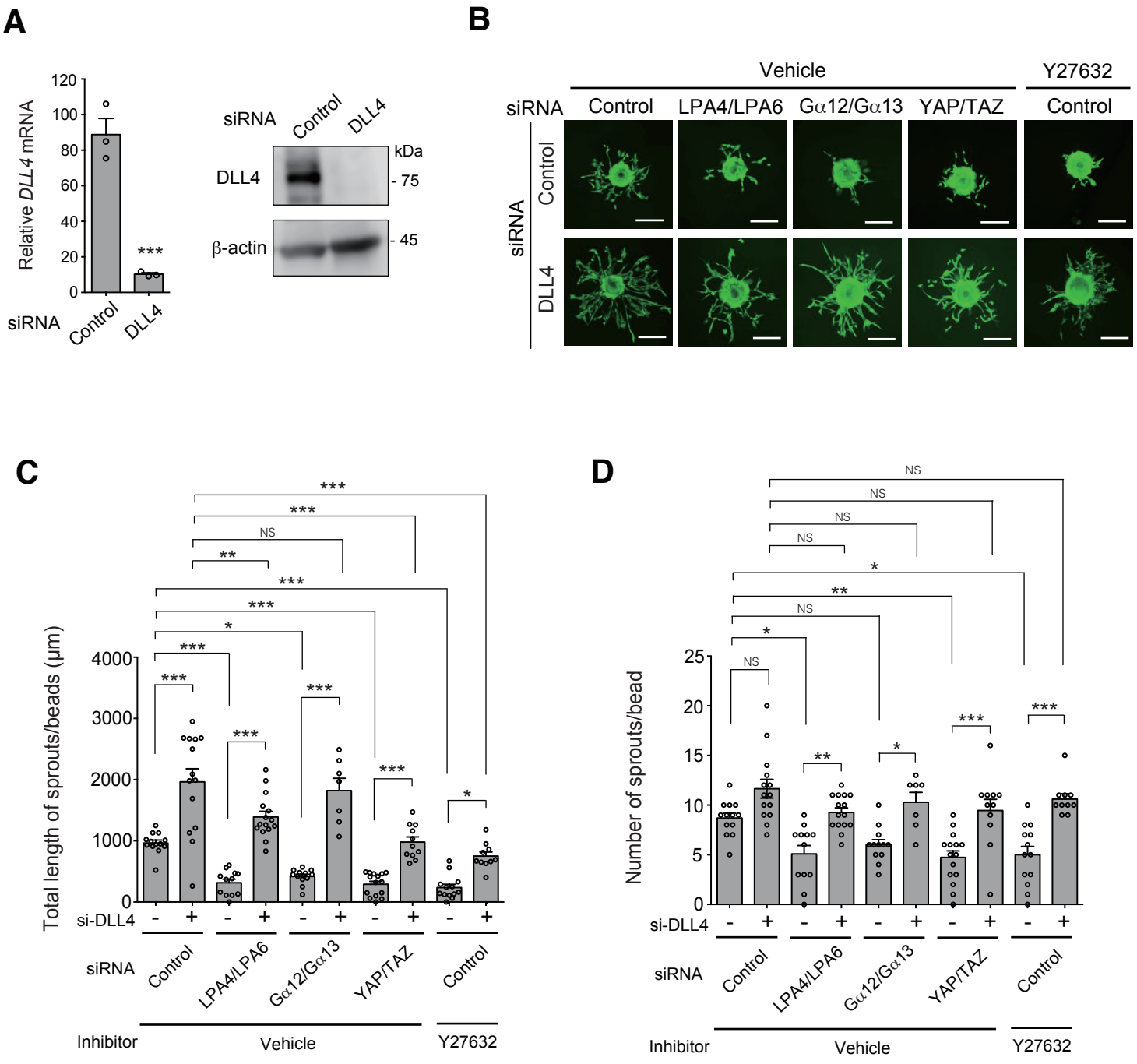
Supplemental Figure 7. Reduced proliferation of HUVECs by LPA4/LPA6 knockdown. Data represent mean ± s.e.m. of triplicates. ****P* < 0.001, two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test.

SUPPLEMENTAL FIGURE 8



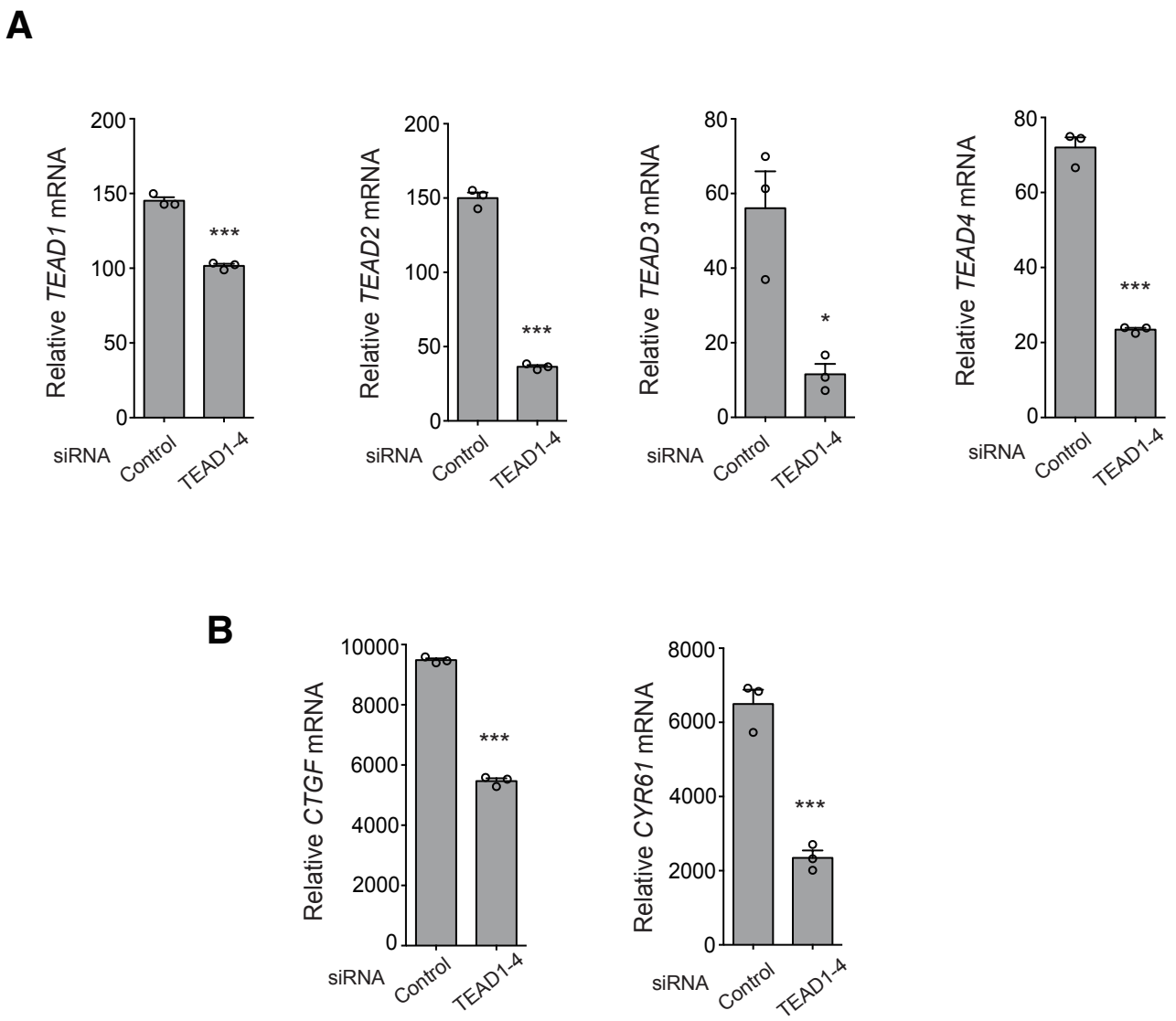
Supplemental Figure 8. Reduced mRNA expression of *DLL4* and Notch target genes by DAPT in HUVECs. Cells were treated with 10 μM DAPT for 2 h. Data represent mean ± s.e.m. of triplicates. ****P* < 0.001, two-tailed unpaired Student's *t*-test.

SUPPLEMENTAL FIGURE 9



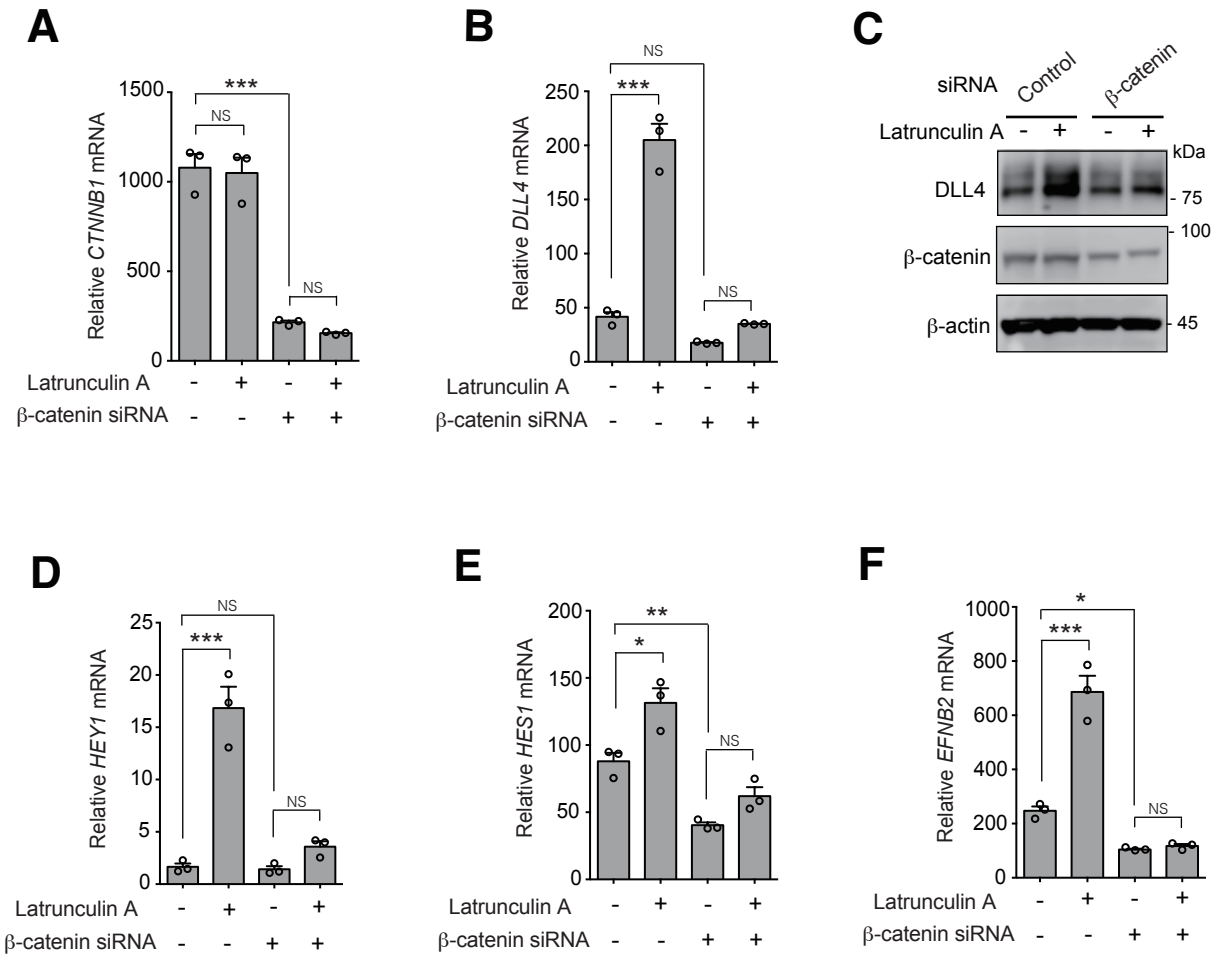
Supplemental Figure 9. Impaired EC sprouting by LPA4/LPA6-Gα12/Gα13-TAP/TAZ signaling blockade is ameliorated by DLL4 knockdown. (**A**) Blockade of DLL4 expression by specific siRNA was confirmed in HUVECs. Data are mean ± s.e.m. of triplicates. ***P < 0.001, two-tailed unpaired Student's t-test. (**B**-**D**) siRNAs for LPA4/LPA6, Gα12/Gα13, or YAP/TAZ, or Y27632 (10 μM) suppressed the length and number of sprouts from the HUVEC-coated beads. DLL4 siRNA significantly ameliorated the sprouting defects caused by these treatments. Representative fluorescence images of sprouting are shown in (**B**). HUVECs are stained green with calcein. Scale bars, 100 μm. Quantitative analyses of total length (**C**) and number (**D**) of sprouts were performed. Data are mean ± s.e.m (n = 7-15 beads). *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Tukey's multiple comparisons test. NS, not significant. Unprocessed original scans of western blots are shown in Supplemental Figure 17.

SUPPLEMENTAL FIGURE 10



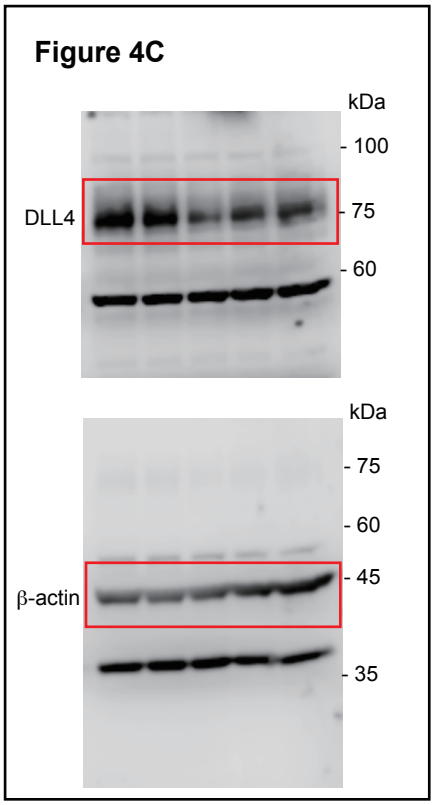
Supplemental Figure 10. Reduction of YAP/TAZ target gene expression by TEAD1-4 siRNAs in HUVECs. (**A**) Blockade of *TEAD1-4* mRNA expression by specific siRNAs was confirmed. HUVECs were transfected with TEAD1-4 siRNAs for 48 h. (**B)** TEAD1-4 knockdown significantly reduced the expression of *CTGF* and *CYR61* mRNA. Data are mean ± s.e.m. of triplicates. **P* < 0.05, ****P* < 0.001, two-tailed unpaired Student's *t*-test.

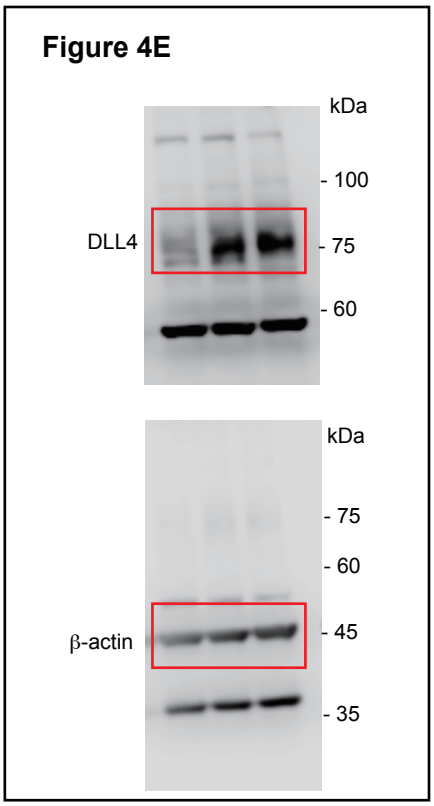
SUPPLEMENTAL FIGURE 11

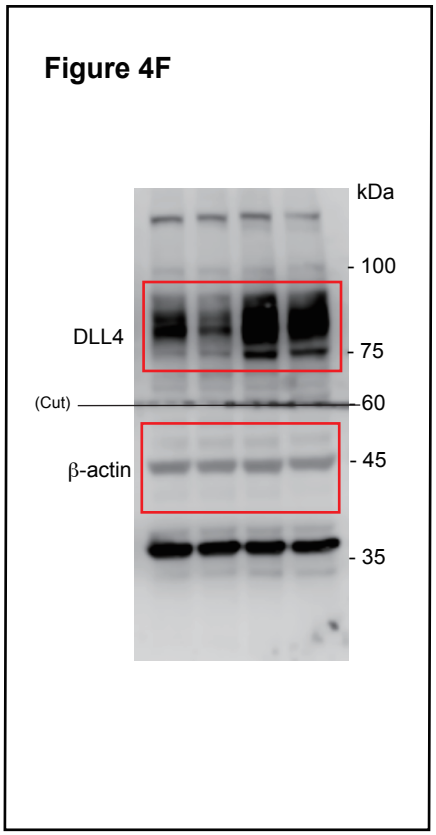


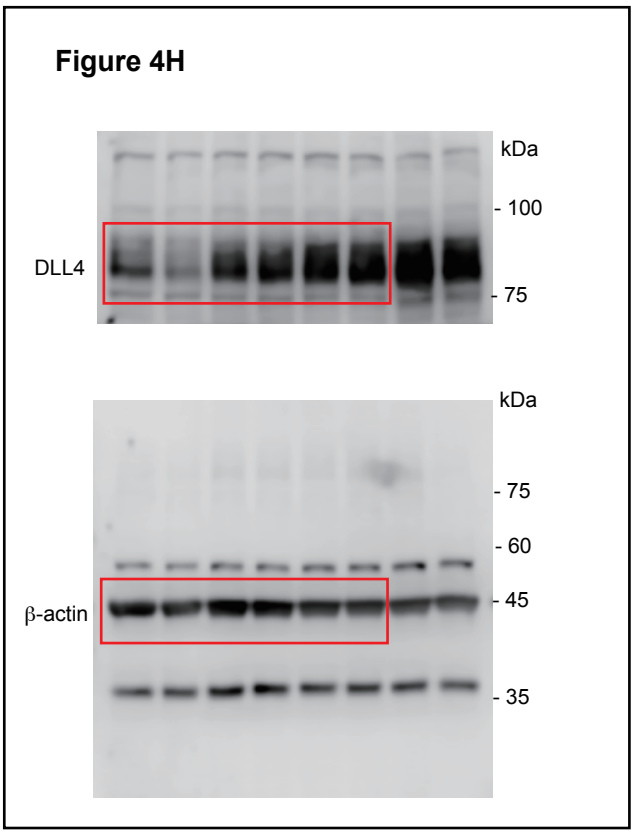
Supplemental Figure 11. β-catenin knockdown attenuated latrunculin A-induced DLL4 expression. (**A**) Blockade of *CTNNB1* mRNA expression by specific siRNA was confirmed. (**B** and **C**) β-catenin siRNA attenuated latrunculin A (1 μM, 3 h)-induced mRNA (**B**) and protein (**C**) expression of DLL4. (**D**-**F**) β-catenin siRNA attenuated latrunculin A (1 μM, 3 h)-induced mRNA of Notch target genes. Data are mean ± s.e.m. of triplicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, one-way ANOVA followed by Tukey's multiple comparisons test. NS, not significant. Unprocessed original scans of western blots are shown in Supplemental Figure 17.

SUPPLEMENTAL FIGURE 12



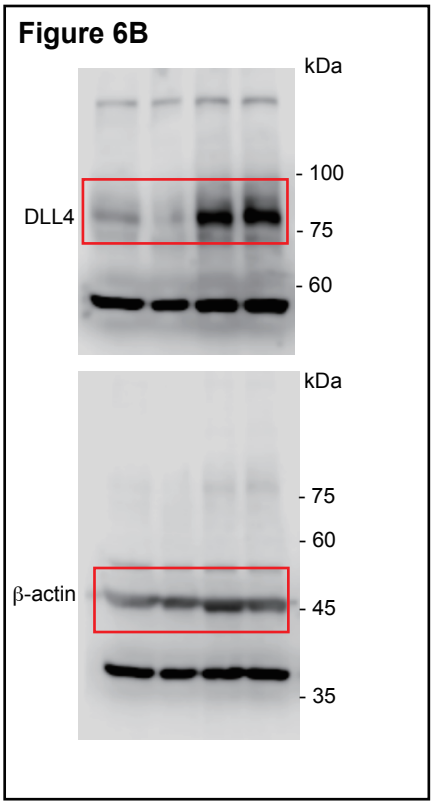


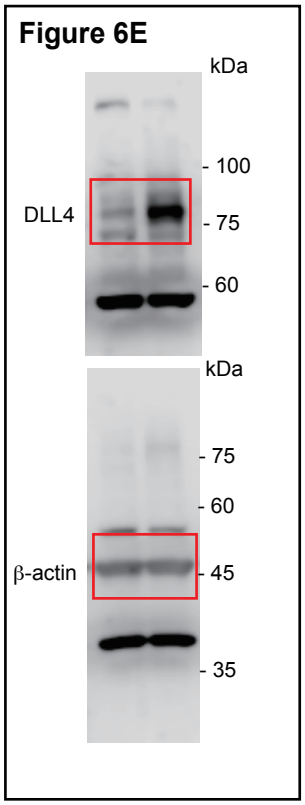


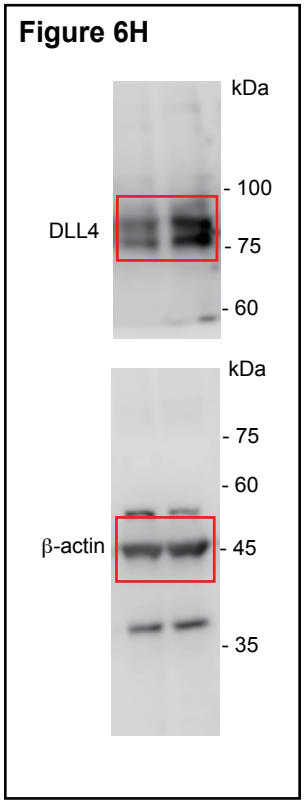


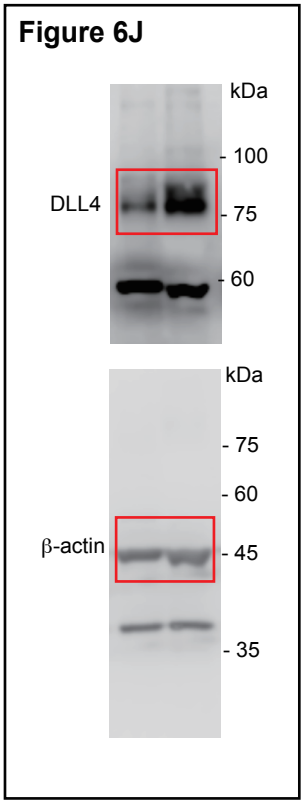
Supplemental Figure 12. Unprocessed original scans of western blots shown in Figure 4.

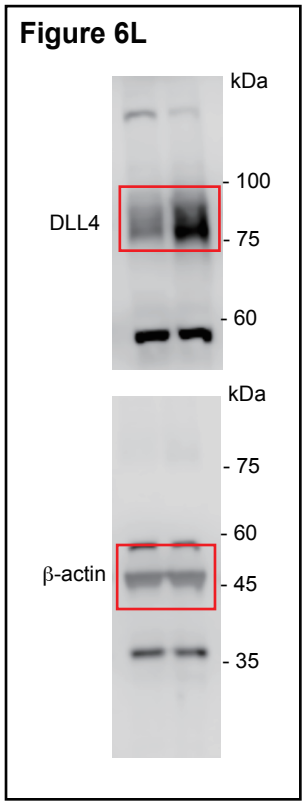
SUPPLEMENTAL FIGURE 13

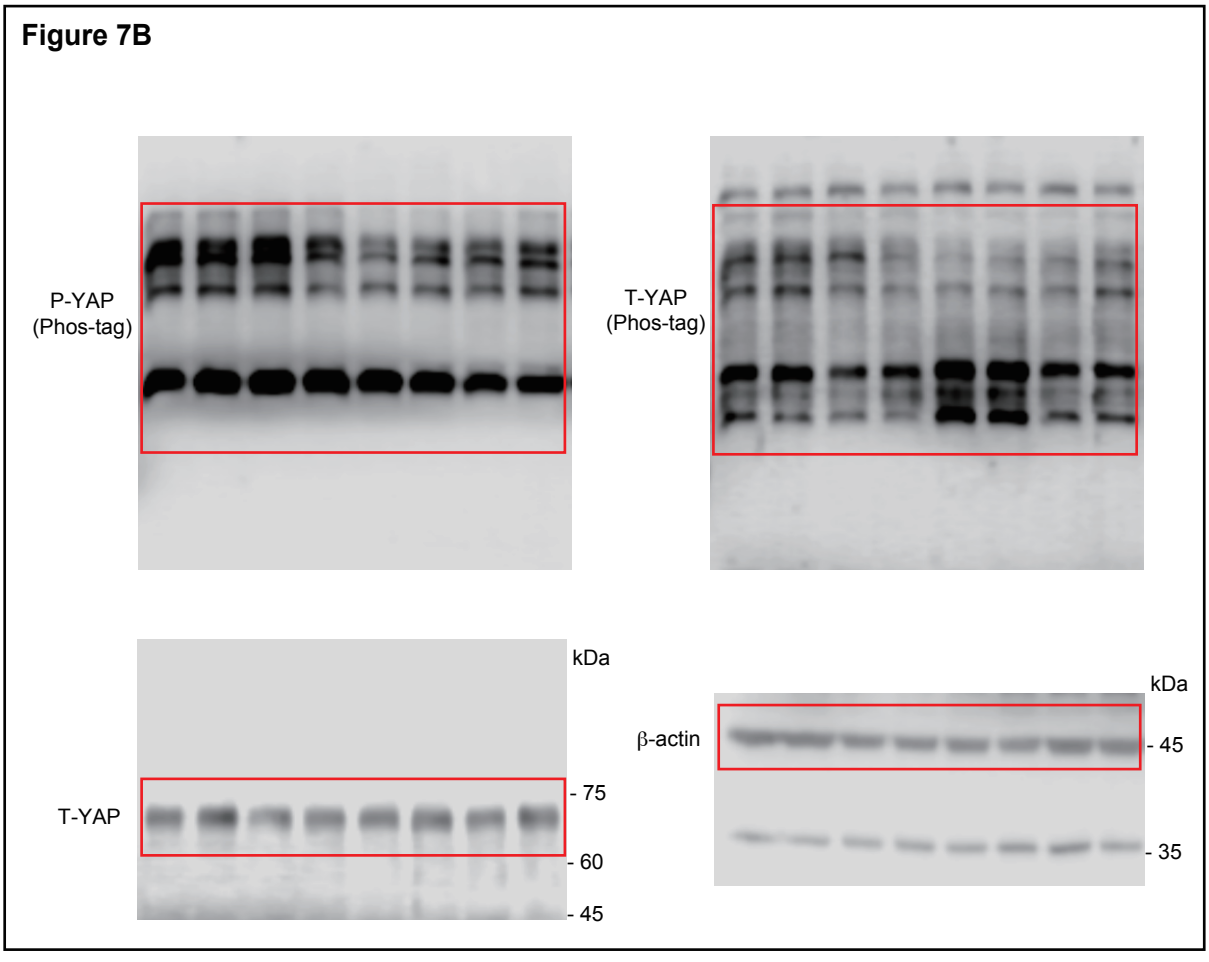












Supplemental Figure 13. Unprocessed original scans of western blots shown in Figure 6 and 7.

SUPPLEMENTAL FIGURE 14

Figure 10B

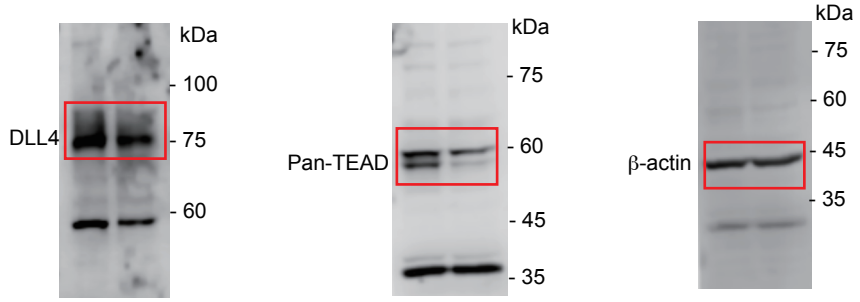


Figure 10D

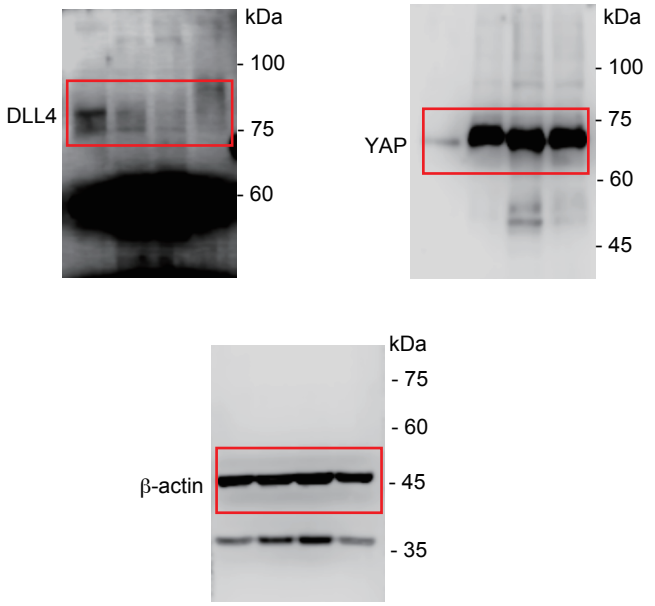
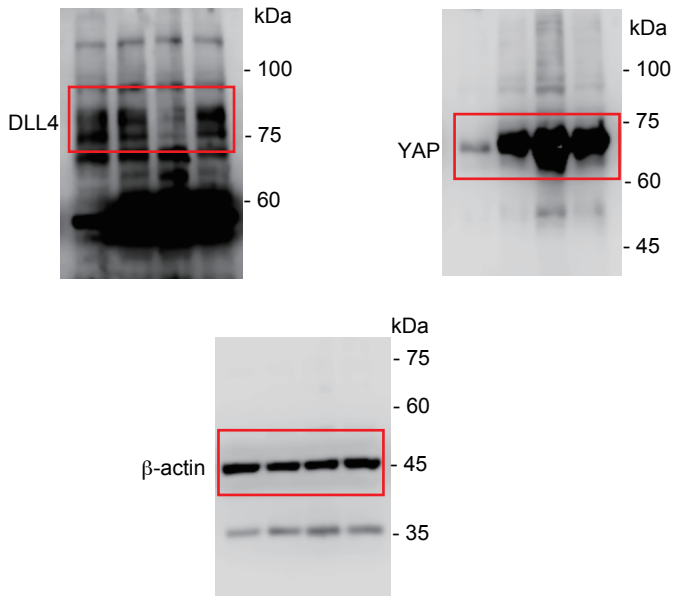
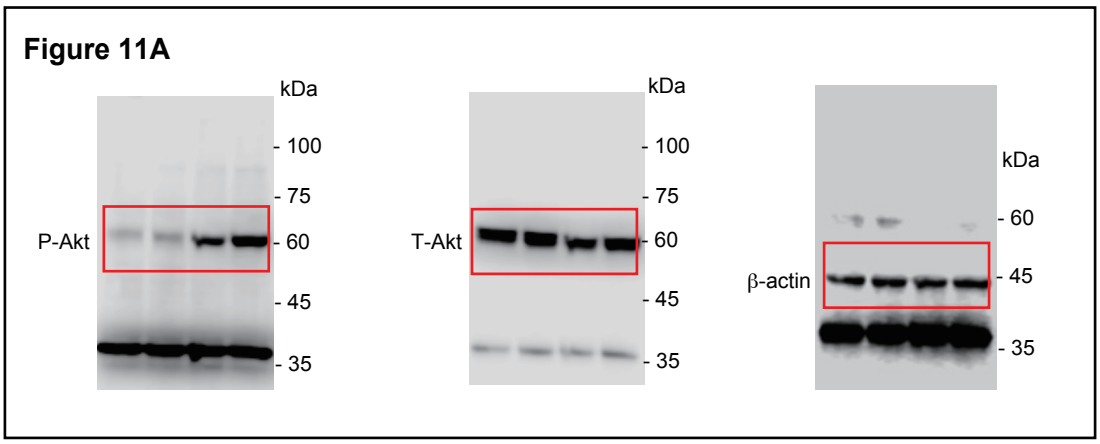


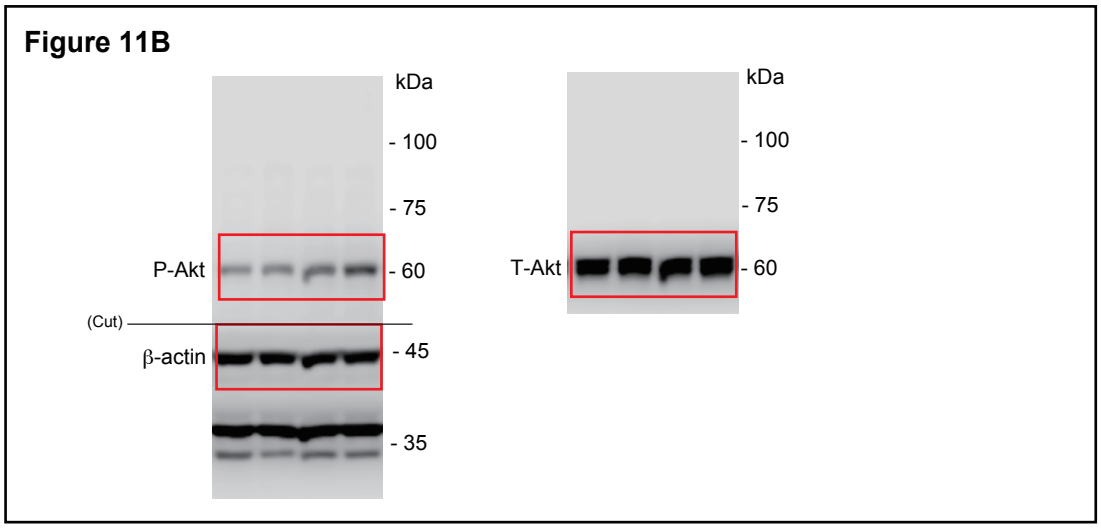
Figure 10F

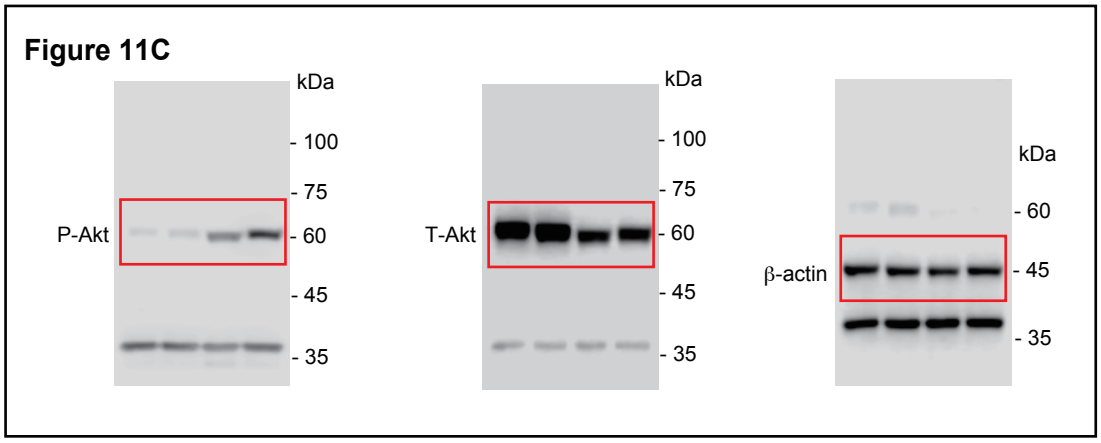


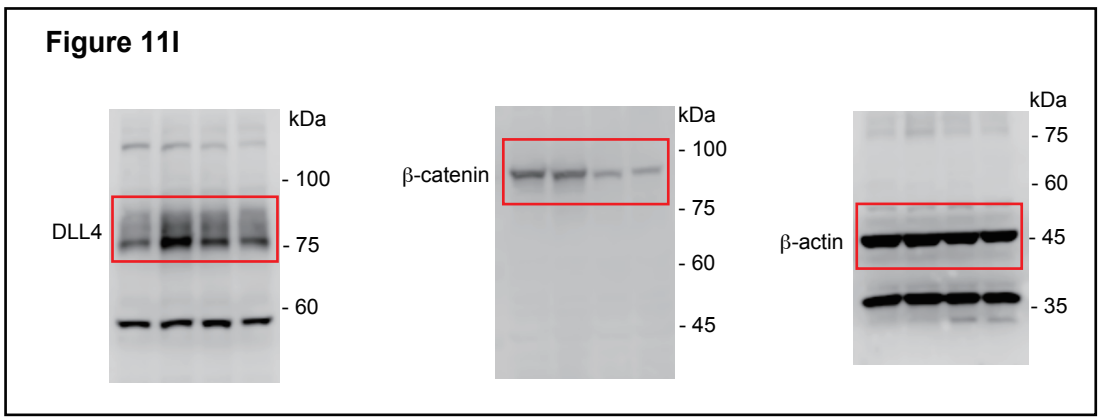
Supplemental Figure 14. Unprocessed original scans of western blots shown in Figure 10.

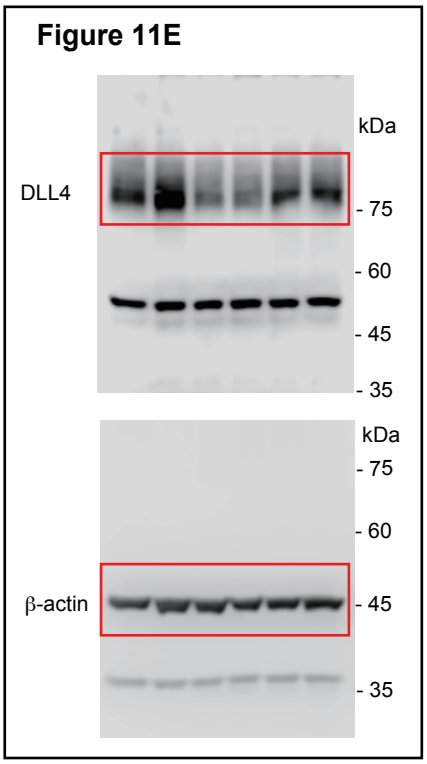
SUPPLEMENTAL FIGURE 15

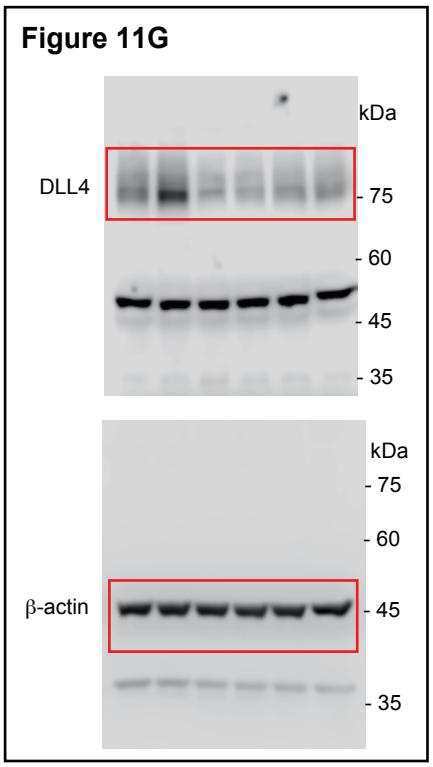






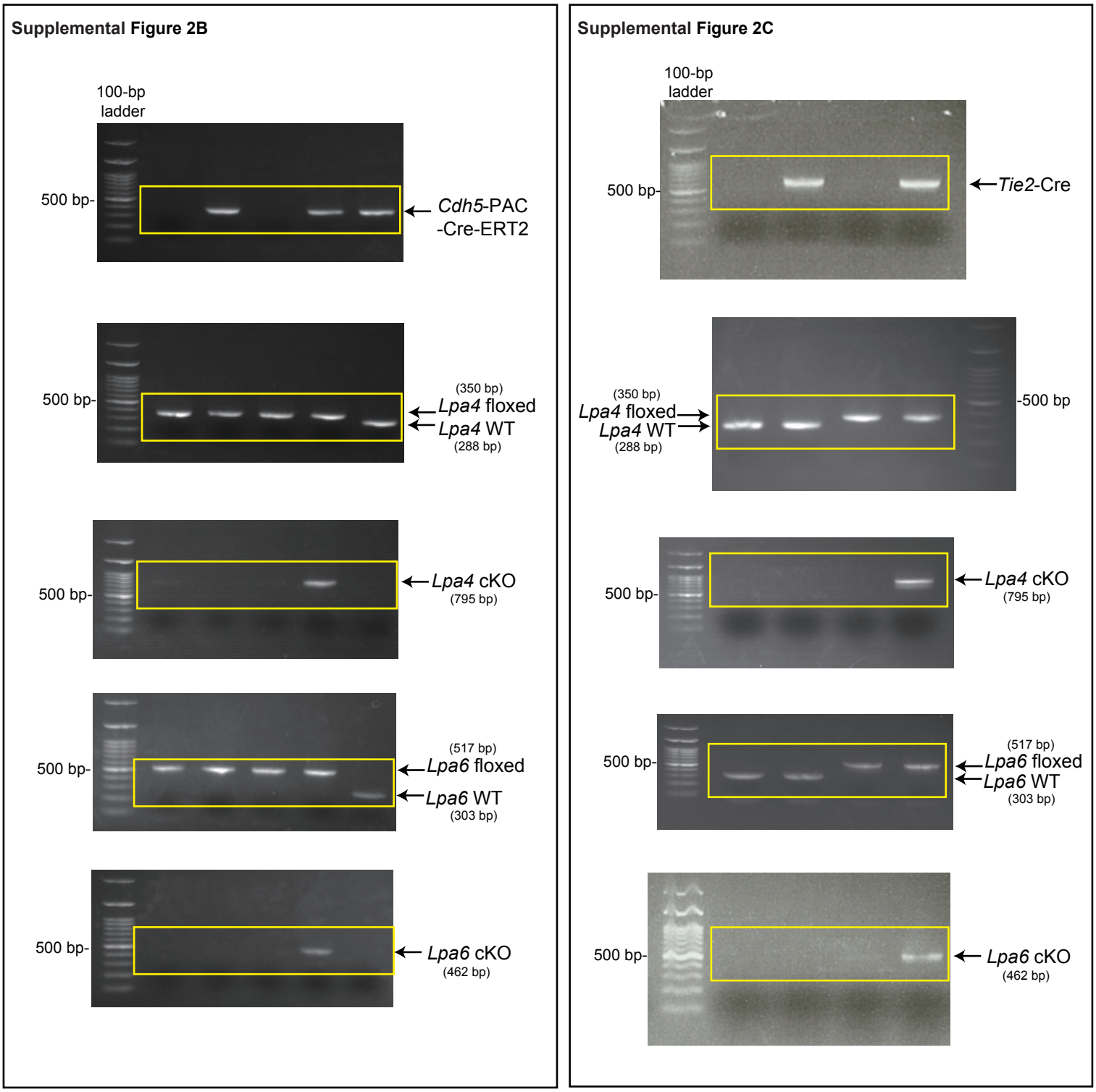






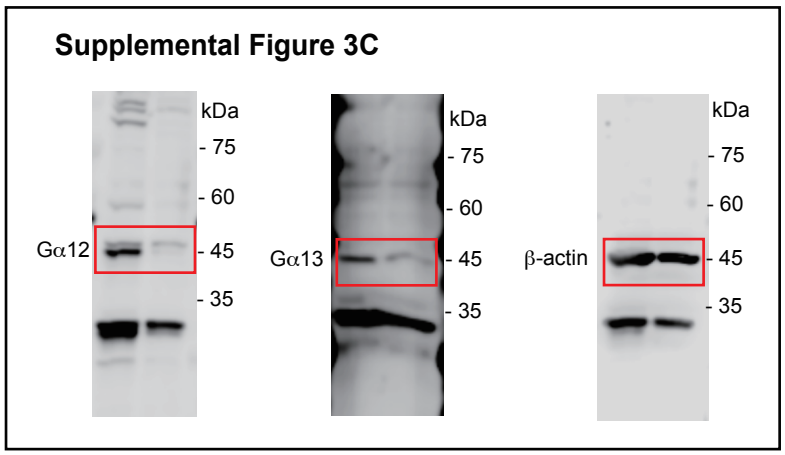
Supplemental Figure 15. Unprocessed original scans of western blots shown in Figure 11.

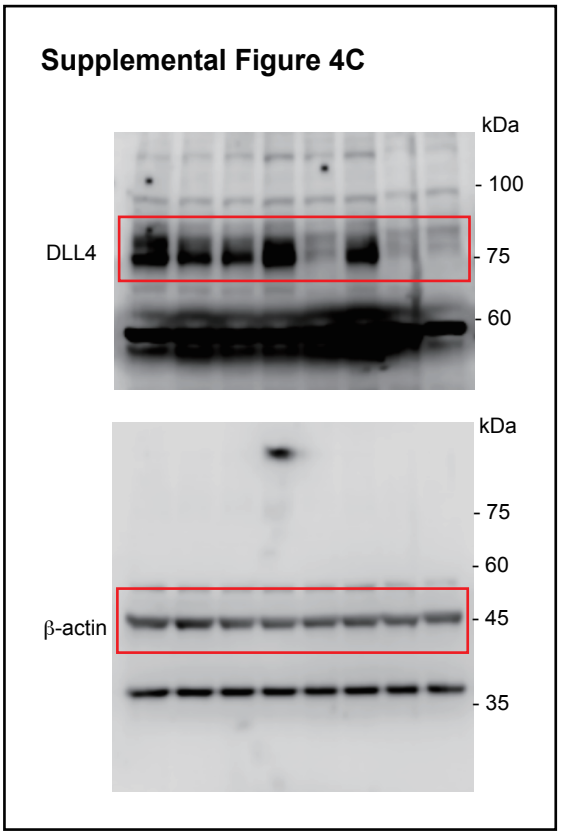
SUPPLEMENTAL FIGURE 16

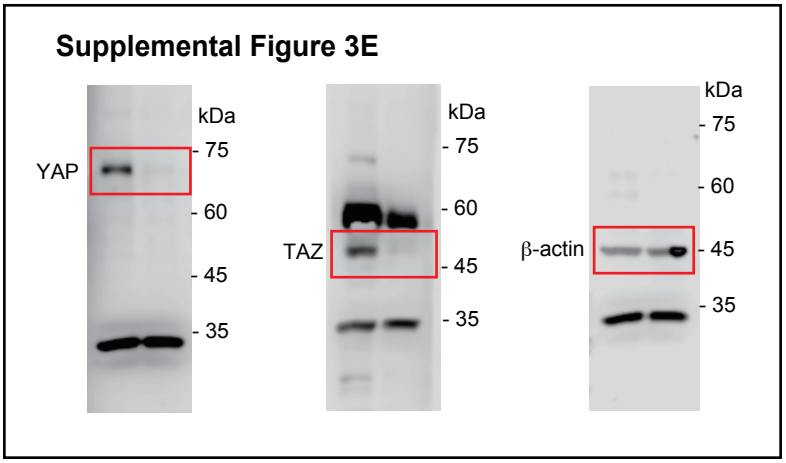


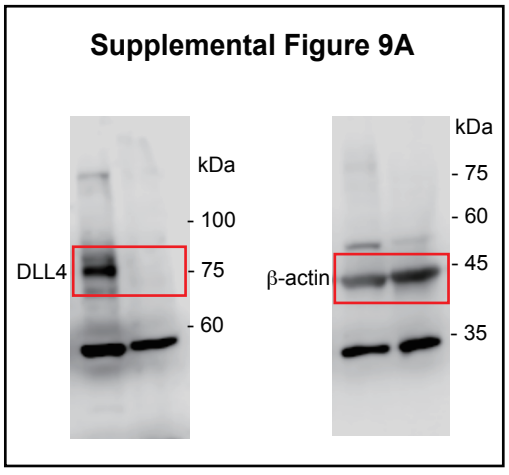
Supplemental Figure 16. Unprocessed original scans of PCR genotyping shown in Supplemental Figure 2.

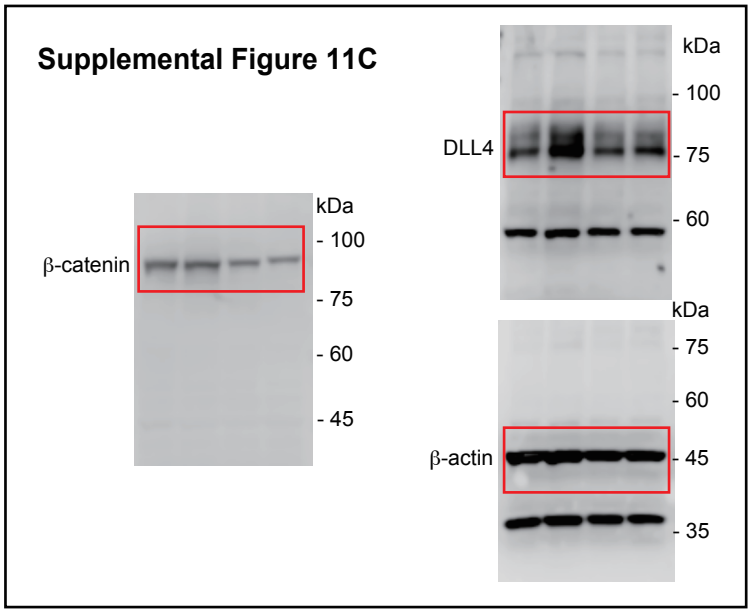
SUPPLEMENTAL FIGURE 17











Supplemental Figure 17. Unprocessed original scans of western blots shown in Supplemental Figure 3, 4, 9, and 11.

Supplemental Table 1

Genotype distributions of offspring of *Lpa6* Het intercrosses (*Lpa4*$^{+/y}$;*Lpa6*$^{+/-}$ male × *Lpa4*$^{+/+}$;*Lpa6*$^{+/-}$ female).

Male		Female	
Genotype	Number	Genotype	Number
Lpa4$^{+/y}$;*Lpa6*$^{+/+}$	41	*Lpa4*$^{+/+}$;*Lpa6*$^{+/+}$	37
Lpa4$^{+/y}$;*Lpa6*$^{+/-}$	76	*Lpa4*$^{+/+}$;*Lpa6*$^{+/-}$	73
Lpa4$^{+/y}$;*Lpa6*$^{-/-}$	38	*Lpa4*$^{+/+}$;*Lpa6*$^{-/-}$	41

Numbers of offspring of each genotype at 4 weeks of age are shown. A total of 306 offspring of 44 litters were analyzed. *Lpa6* KO mice were born at the expected Mendelian ratios.

Supplemental Table 2

Genotype distributions of offspring of *Lpa4* KO;*Lpa6* Het intercrosses (*Lpa4*$^{-/y}$;*Lpa6*$^{+/-}$ male × *Lpa4*$^{-/-}$;*Lpa6*$^{+/-}$ female).

Male		Female	
Genotype	Number	Genotype	Number
Lpa4$^{-/y}$;*Lpa6*$^{+/+}$	29	*Lpa4*$^{-/-}$;*Lpa6*$^{+/+}$	31
Lpa4$^{-/y}$;*Lpa6*$^{+/-}$	48	*Lpa4*$^{-/-}$;*Lpa6*$^{+/-}$	33
Lpa4$^{-/y}$;*Lpa6*$^{-/-}$	0	*Lpa4*$^{-/-}$;*Lpa6*$^{-/-}$	0

Numbers of offspring of each genotype at 4 weeks of age are shown. A total of 141 offspring of 40 litters were analyzed. No *Lpa4*;*Lpa6* DKO mice were obtained.

Supplemental Table 3

Genotype distributions of offspring of *Lpa4* KO;*Lpa6* Het male and *Lpa4* Het;*Lpa6* Het female intercrosses (*Lpa4*$^{-/y}$;*Lpa6*$^{+/-}$ male × *Lpa4*$^{+/-}$;*Lpa6*$^{+/-}$ female).

Male		Female	
Genotype	Number	Genotype	Number
Lpa4$^{+/y}$;*Lpa6*$^{+/+}$	51	*Lpa4*$^{+/-}$;*Lpa6*$^{+/+}$	49
Lpa4$^{+/y}$;*Lpa6*$^{+/-}$	114	*Lpa4*$^{+/-}$;*Lpa6*$^{+/-}$	94
Lpa4$^{+/y}$;*Lpa6*$^{-/-}$	54	*Lpa4*$^{+/-}$;*Lpa6*$^{-/-}$	31
Lpa4$^{-/y}$;*Lpa6*$^{+/+}$	42	*Lpa4*$^{-/-}$;*Lpa6*$^{+/+}$	33
Lpa4$^{-/y}$;*Lpa6*$^{+/-}$	38	*Lpa4*$^{-/-}$;*Lpa6*$^{+/-}$	34
Lpa4$^{-/y}$;*Lpa6*$^{-/-}$	0	*Lpa4*$^{-/-}$;*Lpa6*$^{-/-}$	0

Numbers of offspring of each genotype at 4 weeks of age are shown. A total of 540 offspring of 86 litters were analyzed. Numbers of *Lpa4* KO;*Lpa6* Het mice and *Lpa4* Het;*Lpa6* KO mice were 50% less than the values expected by Mendelian ratios. No *Lpa4*;*Lpa6* DKO mice were obtained.

Supplemental Table 4

Genotype distributions of offspring of *Lpa4* KO;*Lpa5* Het intercrosses (*Lpa4*$^{-/y}$;*Lpa5*$^{+/-}$ male × *Lpa4*$^{-/-}$;*Lpa5*$^{+/-}$ female).

Male		Female	
Genotype	Number	Genotype	Number
Lpa4$^{-/y}$;*Lpa5*$^{+/+}$	18	*Lpa4*$^{-/-}$;*Lpa5*$^{+/+}$	14
Lpa4$^{-/y}$;*Lpa5*$^{+/-}$	32	*Lpa4*$^{-/-}$;*Lpa5*$^{+/-}$	30
Lpa4$^{-/y}$;*Lpa5*$^{-/-}$	16	*Lpa4*$^{-/-}$;*Lpa5*$^{-/-}$	12

Numbers of offspring of each genotype at 4 weeks of age are shown. A total of 122 offspring of 22 litters were analyzed. *Lpa4*;*Lpa5* DKO mice were born at the expected Mendelian ratios and appeared normal.

Supplemental Table 5

Genotype distributions of offspring of *Lpa5* Het;*Lpa6* KO intercrosses (*Lpa5*$^{+/-}$;*Lpa6*$^{-/-}$ male × *Lpa5*$^{+/-}$;*Lpa6*$^{-/-}$ female).

Male		Female	
Genotype	Number	Genotype	Number
Lpa5$^{+/+}$;*Lpa6*$^{-/-}$	16	*Lpa5*$^{+/+}$;*Lpa6*$^{-/-}$	13
Lpa5$^{+/-}$;*Lpa6*$^{-/-}$	35	*Lpa5*$^{+/-}$;*Lpa6*$^{-/-}$	32
Lpa5$^{-/-}$;*Lpa6*$^{-/-}$	19	*Lpa5*$^{-/-}$;*Lpa6*$^{-/-}$	17

Numbers of offspring of each genotype at 4 weeks of age are shown. A total of 132 offspring of 16 litters were analyzed. *Lpa5*;*Lpa6* DKO mice were born at the expected Mendelian ratios and appeared normal.

Supplemental Table 6

Genotype distributions of offspring of *Lpa4* KO;*Lpa6* Het male and *Lpa4* Het;*Lpa6* Het female intercrosses (*Lpa4*$^{-/y}$;*Lpa6*$^{+/-}$ male × *Lpa4*$^{+/-}$;*Lpa6*$^{+/-}$ female) at different stages of development.

Stage of development	Total number of litters	Total number of embryos	Embryo genotype												Number of embryos resorbed
			Lpa4$^{+/y}$; *Lpa6*$^{+/+}$	*Lpa4*$^{+/y}$; *Lpa6*$^{+/-}$	*Lpa4*$^{+/y}$; *Lpa6*$^{-/-}$	*Lpa4*$^{-/y}$; *Lpa6*$^{+/+}$	*Lpa4*$^{-/y}$; *Lpa6*$^{+/-}$	*Lpa4*$^{-/y}$; *Lpa6*$^{-/-}$	*Lpa4*$^{+/-}$; *Lpa6*$^{+/+}$	*Lpa4*$^{+/-}$; *Lpa6*$^{+/-}$	*Lpa4*$^{+/-}$; *Lpa6*$^{-/-}$	*Lpa4*$^{-/-}$; *Lpa6*$^{+/+}$	*Lpa4*$^{-/-}$; *Lpa6*$^{+/-}$	*Lpa4*$^{-/-}$; *Lpa6*$^{-/-}$	
E8.5	3	32	1	5	0	1	4	2	2	7	2	1	5	1	1
E9.5	19	178	12	24	13	16	22	11(2)	11	13	16(3)	5	20	5(1)	4
E10.5	10	93	9	10	6	3(1)	11	2	6(1)	13	7(1)	2	5(1)	4(7)	4
E11.5	5	44	5	8	3	2	3	(4)	0	2	(1)	4(1)	5(1)	(3)	2
E12.5	7	70	5	9	4	7	8	0	4	5	(1)	0	5	(1)	21
E14.5	13	107	6	14	6	4	6	0	6	14	7	2	2(1)	0	39
E18.5	3	25	1	4	0	4	1	0	0	3	0	2	0	0	10

Numbers of embryos of each genotype and resorbed embryos of unknown genotype are shown. Figures in parentheses are numbers found dead. All *Lpa4*;*Lpa6* DKO embryos died by E11.5.

Supplemental Table 7

The nucleotide sequence of the pflox vector.

This vector was constructed based on a plasmid backbone of pBluescript2 KS+. Binding sites of the M13 forward and reverse primers are written in italic with underline and italic, respectively. The sequences of LoxP, FRT, and multi-cloning site are highlighted in blue, green, and grey, respectively.

CACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTA
ACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTT
GTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCG
TCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCG
TAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAA
CGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGG
TCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGC
CATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGG
CGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTT
*GTAAAACGACGGCCAGT*GAATTGTAATACGACTCACTATAGGGCGAATTGgagctccaccgcggtggcggccG
AAGTTCCTATACTTTCTAGAGAATAGGAACTTCtgggatccacgtttaaacATAACTTCGTATAGCATACATTAT
ACGAAGTTATggcgcgccaattcgatatcaagctATAACTTCGTATAGCATACATTATACGAAGTTATgcggccgcgaa
ttcGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCtcgaggggggcccggtaccCAGCTTTTGTTCCCTTTAG
TGAGGGTTAATTGCGCGCTTGGCGTAATCATG*GTCATAGCTGTTTCCTG*TGTGAAATTGTTATCCGCT
CACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGC
TAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGC
ATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT
CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT
ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCC
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AAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCC
TGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTC
CCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTC
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ATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT
TAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACT
AGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTC

TTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGC

AGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA

CTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAA

ATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGT

GAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGAT

AACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCA

CCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCA

ACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAA

TAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTT

CATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTT

AGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGC

AGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAAC

CAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT

ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTC

AAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCAT

CTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATA

AGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGT

TATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACA

TTTCCCCGAAAAGTGC

Supplemental Table 8

Primer sequences used for genotyping.

Allele	Forward primer	Reverse primer
LPA4 WT	ACCTAGCAGGCTCTCTGGGAAAA	CCAGGTTGGTGATGAAAATAGCC
LPA4 KO	ACCTAGCAGGCTCTCTGGGAAAA	AGGTCAAATTCAGACGGCAAAC
LPA5 WT	ATGTTTATCTGTACACCAGACAGCA	TGGTGCACCTCTGCAATCTA
LPA5 KO	ATGTTTATCTGTACACCAGACAGCA	TCTAGGACAAGAGGGCGAGAC
LPA6 WT	GTGACCACATCTGAATAGCAAAGG	AAAAATCCGAAATGGCAAAGTAAA
LPA6 KO	GTGACCACATCTGAATAGCAAAGG	TTCCGTAAACAACATCTCGGTTC
LPA4 floxed	CCCCTACACCTTAAACATGATTGGC	GTACTTTCTTCCCAGCACAACCTCA
LPA4 cKO	GTTTCCTTGGTGTCTTGAAGGGA	GTACTTTCTTCCCAGCACAACCTCA
LPA6 floxed	GTGACCACATCTGAATAGCAAAGG	AAAAATCCGAAATGGCAAAGTAAA
LPA6 cKO	GTGACCACATCTGAATAGCAAAGG	TTCCGTAAACAACATCTCGGTTC
Tie2-Cre	CCCTGTGCTCAGACAGAAATGAGA	CGCATAACCAGTGAAACAGCATTGC
Cdh5-CreERT2	GACTAGCTTACCATTCTGCTGGTGT	GGACAGAAGCATTTTCCAGGTATG

Supplemental Table 9

Primer sequences used for qRT-PCR analysis.

Target gene	Forward primer	Reverse primer
ACTB	CAGGATGCAGAAGGAGATCACTG	TACTCCTGCTTGCTGATCCACAT
GAPDH	CAGGTGGTCTCCTCTGACTTCAA	ACCCTGTTGCTGTAGCCAAATTC
LPA1	GAATCGGGATACCATGATGAGTC	GCACACGTCTAGAAGTAACAAAACC
LPA2	CTGGTCAAGACTGTTGTCATCATCC	AGGACTCACAGCCTAAACCATCC
LPA3	TAGGGGCGTTTGTGGTATGCT	ATGGGGTTCACGACGGAGTT
LPA4	GCAAGCCTGCTACTCTGTCTCAA	TTGCAAATCTTTCCAAAAAGCAA
LPA5	CGTGTCCTGACTACCGACCTACC	CAGCGAGAGGGTGAAGAGCA
LPA6	TCATCTGCGTCCTCAAAGTCC	CCAATTCCGTGTTGTGAAGTAAAA
VEGFA	GATGAGCTTCCTACAGCACAACAA	TTTCGTTTTTGCCCCTTTCC
VEGFR1	GGGACAGTAGAAAGGGCTTCATC	TGGGCGTGGTGTGCTTATTT
VEGFR2	CGCAGAGTGAGGAAGGAGGA	GGATGATGACAAGAAGTAGCCAGAA
VEGFR3	AGAGACTTTGAGCAGCCATTCATC	GTCATCCCACACCACCTCCT
NRP1	GGAAACACCAACCCCACAGA	CATACCCAACATTCCAGAGCAAG
TGFB1	CCTGGCGATACCTCAGCAAC	GCTAAGGCGAAAGCCCTCAA
PECAM1	CAGGACCGCGTTTTATCCTTC	TGATGTGGAACTTGGGTGTAGAGA
CDH5	CGCAATAGACAAGGACATAACACC	CCGGTCAAACTGCCCATACTT
PDGFB	TTGGCTCGTGGAAGAAGGAG	CGTTGGTGCGGTCTATGAGG
TEK	ACCTCTTCACCTCGGCCTTC	TCACACGTCCTTCCCATAAACC
ANGPT1	CAGAAAGCTGACAGATGTTGAGACC	ACTCTTCCTTGTGTTTTCCTTCCA
ANGPT2	GAACCAAACAGCGGAGCAAA	TCGAGAGGGAGTGTTCCAAGAG
DLL1	GAGCGTGGGGAGAAAGTGTG	ACTTGCATTCCCCTGGTTTGT
DLL4	ACCCTCTCCAACTGCCCTTC	TGCTGGTTTGCTCATCCAATAAC
JAG1	CAGATTTCCTTGTTCCCTTGCT	CGTTGTTGGTGGTGTTGTCCT
NOTCH1	CGACAACGCCTACCTCTGCT	ACAGGCACACTCGTAGCCATC
NOTCH4	TCTCGTCCTCCAGCTCATCC	CATCACAACTCCATCCTCATCAAC
RBPJK	TACGAGTGTGGTTTGGGGATG	GTAGGTAAAGGTAAGGCTGGTGGAA
HES1	GCTACCCCAGCCAGTGTCAA	TCTTGCTCTTCGTCTTTTCTCCA
EFNB2	CAAGTCCCTTTGTAAAACCAAATCC	GGCGAGTGCTTCCTGTGTCT
PROX1	GGCTCTCCTTGTCGCTCATAAA	GGAGCTGGGATAACGGGTATAAAAA

NR2F2	AGTGGGCATGAGACGGGAAG	GACAGGTACGAGTGGCAGTTGAG
SOX18	TCATGGTGTGGGCAAAGGAC	GTTCAGCTCCTTCCACGCTTT
FOXC1	GGAGATGTTCGAGTCACAGAGGA	GACGTGCGGTACAGAGACTGG
FOXC2	GAGTCCCAGGTGAGTGGCAAT	ATTTCGTGCAGTCGTAGGAGTAGG
ERG	CCAGCGTCCTCAGTTAGATCCTT	CATCTTGAACTCCCCGTTGGT
ETV2	CGATGCCCCAAAACTAACCA	TAATTCATGCCCGGCTTTCTC
MEF2C	CAGGACAAGGAATGGGAGGA	ACTGACTGAGGGCAGATGGTG
ATX	AGAGCAGAAGGATGGGAGGAAG	TCACAGCGACAATCAGGAGGT
LPP3	CTGCTCATCTGCCTCGACCT	CACAGCGTCATTTATTGTCTCACC
GNA12	AGTTCCGCGACACCATCTTC	AACATCCCATGCTTCTCATTTTC
GNA13	AGTTCCGCCCCACCATCTAC	ACCCTTGTTTCCACCATTCCTT
HEY1	AGGTTCCATGTCCCCAACTACA	TGCAGGATCTCGGCTTTTTC
HEY2	CGTCGGGATCGGATAAATAACA	CAAGAGCGTGTGCGTCAAAG
YAP	CAGGTTGGGAGATGGCAAAG	GGGCTGTGACGTTCATCTGG
TAZ	GTGCTGGAAAAAGAAGAGAGAAAGG	GCAGGATGATGGGGTTGAGA
CTGF	TTCCAAGACCTGTGGGATGG	GGGAGTACGGATGCACTTTTTG
CYR61	TGCCGCCTTGTGAAAGAAAC	CTCAAACATCCAGCGTAAGTAAACC
TEAD1	GATGCTGGGGCTTTTTATGGT	ACATTGGGGAGCGGTTTATTC
TEAD2	CCGAAGGAAATCAAGGGAAA	GAGATGAGCTGGGCAGAGGA
TEAD3	CCCCTAATGCCTTCTTCCTTGT	TCTCCACCTTCTCTACCACCTGTTT
TEAD4	CCAGTATGAGAGCCCCGAGA	CGGTGGATGCGGTAAGAGTAG
CTNNB1	CTTGGACTTGATATTGGTGCCCA	GGCCACCCATCTCATGTTCCATC
Pecam1	CACAGATAAGCCCACCAGAGACA	TTCACAGAGCACCGAAGTACCA
Lpa4	ACGGCTATTTTCATCACCAACCT	ATGGCTAGGAAACGATCCACAC
Lpa6	GGTCATCTTCTGTTTCTGTTTTGTG	TGAGTTCTGAATTGTGTCTGAGGTG
Dll4	CCGGGAACCTTCTCACTCAAC	GCCAAATCTTACCCACAGCAA
Hey1	CCGACGAGACCGAATCAATAAC	GTGCGCGTCAAAATAACCTTTC