Supporting Information

Lee et al. 10.1073/pnas.1408472111

SI Materials and Methods

Mouse Lung Endothelial Cell Isolation. Mouse lung endothelial cells (MLECs) were isolated from three to four pairs of lungs dissected from WT, Akt1$^{-/-}$, and Akt2$^{-/-}$ mice between 3 and 4 wk of age. Freshly isolated lung tissue was minced with scissors and allowed to digest at 37 °C in Type I collagenase (SIGMA, at 2 mg/mL, ~175 U/mg) for 1 h. Lung tissue was further subjected to mechanical disruption by passage 20–30 times through a 14-gauge needle and filtration through fine steel mesh (130–150 μm; BELLCO). Cells were washed once with culture media consisting of 20% (vol/vol) heat-inactivated FBS, 40% (vol/vol) low-glucose DMEM, 40% (vol/vol) F-12 HAM, 2 mM L-glutamine, penicillin (10 international units/mL)/streptomycin (10 μg/mL), 100 μg/mL heparin, and 50 μg/mL endothelial cell (EC) growth supplement (Collaborative Research). Cells were plated onto a 0.1% gelatin-coated T175 flask overnight. Blood cells were washed off the next day and replaced with media harvested from an Eco-Pack2-mT (ClonTech) retroviral packaging cell line containing polyoma middle T-antigen and 8 μg/mL Polybrene (Sigma) to immortalize lung cell homogenates. Lung cells were infected three to four times with either 100% (vol/vol) Eco-Pack2-mT retroviral supernatant for 6–8 h or 50% (vol/vol) culture media and 50% (vol/vol) Eco-Pack2-mT retroviral supernatant overnight over the course of 2–3 d. When cells were 70–90% confluent with visible EC clusters, ECs were immunoisolated using 107 sheep anti-rat IgG–coated magnetic beads (Dynal) precomplexed with 12.5 μg of rat α-platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody (Pharmingen) and 6.25 μg of rat α-ICAM-2 antibody (Pharmingen) per three to four lung homogenates. Immunoisolated cells were washed in culture media three times, plated onto a 0.1% gelatin-coated T75 flask, and cultured in endothelial basal medium (EBM-2)/endothelial growth medium micro vasculature as described above. When cells became 100% confluent, they were immunoisolated again to purify the EC population further.

Immunoprecipitation and in Vitro Kinase Assays. Confluent mouse embryonic fibroblasts (MEFs) were serum-starved for 16 h and then either left untreated or stimulated with PDGF (40 ng/mL for 15 min). Cells were washed twice with ice-cold PBS and lysed on ice with lysis buffer: 50 mM Tris·HCl (pH 7.4), 1.5 mM $MgCl_2$, 137 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% (vol/vol) glycerol, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 25 mM sodium β-glycerophosphate, 1 mM Pefabloc SC (Roche), and 2 mg/mL protease inhibitor mixture (Roche Diagnostics). Insoluble proteins were removed by centrifugation at 17,000 × *g* for 10 min at 4 °C. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad), after which lysates were diluted to a protein concentration of 1 mg/mL. Next, 600 μL of cell lysates was precleared with Protein G Sepharose beads [30 μL of 50% (vol/vol) solution; Sigma–Aldrich] for 1 h at 4 °C with end-to-end rotation. Protein G Sepharose was pelleted by centrifugation, and 500 μL of precleared lysates was transferred into new microfuge tubes, followed by addition of 4 μg of either rat anti-HA antibody (Roche) or anti-rat IgG control antibody (Santa Cruz Biotechnology). Samples were rotated at 4 °C for 2 h, and Protein G Sepharose beads [50 μL of a 50% (vol/vol) slurry] were then added to each sample and rotated for an additional 1 h at 4 °C, followed by centrifugation at 8,000 × *g* for 1 min. In vitro kinase assays were performed using a commercial kit (Nonradioactive Akt Kinase Assay Kit; Cell Signaling Technology). Immunoprecipitates were washed twice with lysis buffer and twice with the Akt kinase buffer: 25 mM Tris (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, and 1 mM Na_3VO_4. Pellets were resuspended in 50 μL of kinase buffer supplemented with 1 μL of 10 mM ATP and 1 μg of glycogen synthase kinase 3 (GSK-3) fusion protein (Cell Signaling Technology) or 1 μg of purified recombinant full-length bovine endothelial nitric oxide synthase (eNOS). The reaction mixtures were incubated at 30 °C for 30 min and terminated by the addition of 25 μL of 3× SDS sample buffer. After vortexing and microcentrifugation for 30 s at 14,000 × *g*, reaction products were resolved by SDS/PAGE and analyzed by Western blotting.

Western Blot Analysis. For analysis of basal protein phosphorylation, MLECs were cultured for 72–96 h before growth arrest in serum-free EBM-2 medium for 48 h. In experiments with MEFs, cells were growth-arrested in serum-free DMEM for 12–16 h and then treated with 20 ng/mL PDGF (Calbiochem) for 15 min. Following the appropriate treatment, cells were washed twice with ice-cold PBS and lysed on ice with lysis buffer: 50 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 25 mM sodium β-glycerophosphate, 1 mM Pefabloc SC, and 2 mg/mL protease inhibitor mixture (Roche Diagnostics). Protein concentrations were determined using the DC Protein Assay Kit. Lysates (containing 20–50 μg of protein) were analyzed by SDS/PAGE and immunoblotting. Primary antibodies used include the following: Akt1 mAb (1:2,500 dilution, no. 06-558; Upstate Biotechnology), Akt2 polyclonal antibody (pAb) (1:3,000 dilution) (1), Akt3 pAb (1:1,000 dilution; Cell Signaling Technology), anti-HA mAb (1:1,000 dilution; Roche Diagnostics), PECAM-1 pAb (1:500 dilution; Santa Cruz Biotechnology), pAkt-S473 mAb (1:500 dilution; Cell Signaling Technology), pAkt-T308 mAb (1:500 dilution; Cell Signaling Technology), Hsp90 mAb (1:500 dilution; BD Transduction Laboratories), β-actin mAb (1:5,000 dilution; Sigma–Aldrich), PDGF receptor β pAb (1:500 dilution; Santa Cruz Biotechnology), phosphorylated (p)-eNOS-S1179 pAb (1:500 dilution; Zymed), peNOS-S617 pAb (1:500 dilution; Upstate Biotechnology), p-eNOS-S116 pAb (1:200 dilution; Upstate Biotechnology), eNOS mAb (1:1,000 dilution; BD Transduction Laboratories), pFKHR-S256 pAb (1:250 dilution; Cell Signaling Technology), phosphorylated GSK3β-S9 pAb (1:500 dilution; Cell Signaling Technology), GSK3α/β mAb (1:1,000 dilution; Upstate Biotechnology). Secondary antibodies were conjugated directly to IR fluorescent dyes (IRDye680 and IRDye800, 1:10,000 dilution; LI-COR Biotechnology). Bands were visualized using the Odyssey IR imaging system (LI-COR Biotechnology).

Phosphoproteomic Analysis of RxRxxS*/T* Substrates. For each experimental condition ($n = 3$), 2×10^8 MLECs were grown to 70–80% confluency, washed twice in PBS, and lysed in urea lysis buffer [20 mM Hepes (pH 8.0), 9.0 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate). The Akt isoform-specific phosphoproteome of MLEC lysates was characterized using PTMScan Technology (Cell Signaling Technology) based on LC/tandem MS (MS/MS), as described previously (2). Briefly, cellular proteins were reduced with 4.1 mM DTT, alkylated with 8.3 mM iodoacetamide, and digested overnight with 1 mg/mL GluC in 20 mM Hepes (pH 8.0; Worthington Diagnostics), 1.5 units per 20 mg of protein of LysC

(Roche Penzberg), and 2 mg/mL of chymotrypsin in 20 mM Hepes (pH 8.0; Worthington Diagnostics). The resulting peptides were subjected to immunoaffinity purification using a PTMScan (RxRssS/T*) motif antibody conjugated to protein A agarose beads (Cell Signaling Technology). Unbound peptides were removed through washing, and the captured posttranslationally modified (PTM)-containing peptides were further digested with trypsin (Worthington Diagnostics). The digested peptides were extracted using Sep-Pak C18 Solid Phase Extraction Column (Waters). After drying in a SpeedVac (Thermo Scientific), the peptides were redissolved in 5 μL of 5% (vol/vol) MeCN and 0.1% TFA and loaded onto a PicoFrit capillary column (New Objective) packed with Magic C18 AQ reversed-phase resin (Michrom Bioresources). The chromatographic column was developed with a 72-min linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nL/min. MS/MS spectra were obtained using an LTQ-Orbitrap-Elite system using collision-induced dissociation (Thermo Fisher Scientific), and the label-free quantification of the phosphoproteome was evaluated and normalized using SEQEST 3G (Thermo Fisher Scientific) and the Sorcerer 2 platform (Sage-N Research) (3). Searches were performed against the National Center for Biotechnology Information *Mus musculus* database with a mass accuracy of ±50 ppm for precursor ions and 1 Da for product ions. Results were filtered with mass accuracy of ±5 ppm on precursor ions and presence of the intended motif.

1. Calera MR, et al. (1998) Insulin increases the association of Akt-2 with Glut4-containing vesicles. *J Biol Chem* 273(13):7201–7204.
2. Moritz A, et al. (2010) Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Sci Signal* 3(136):ra64.
3. Lundgren DH, Martinez H, Wright ME, Han DK (2009) Protein identification using Sorcerer 2 and SEQUEST. *Curr Protoc Bioinformatics* Chapter 13:Unit 13.3.

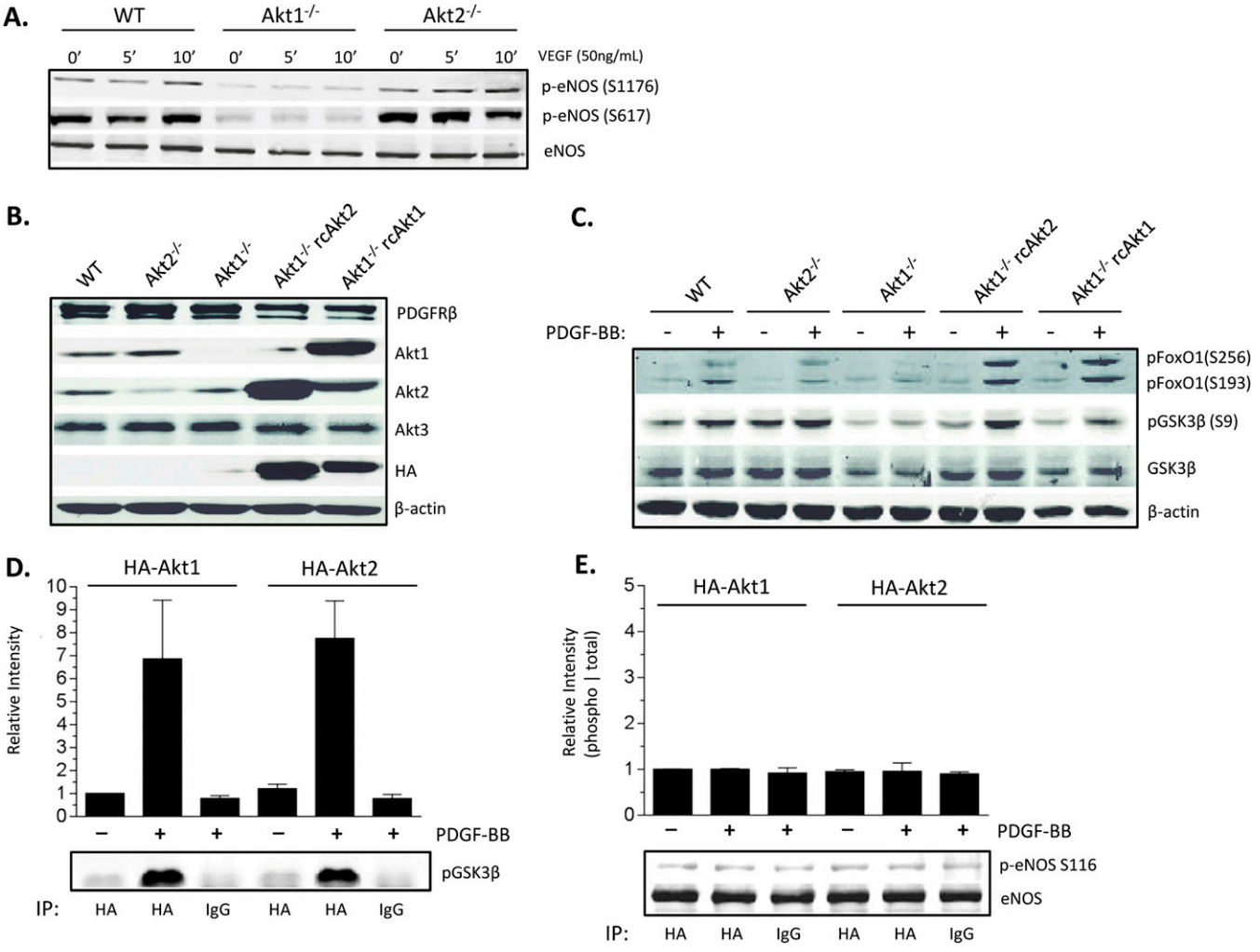

Fig. S1. (*A*) Serum-starved MLECs were stimulated with VEGF (50 ng/mL) for subsequent p-eNOS immunoblotting. (*B*) Fibroblasts isolated from WT, Akt1$^{-/-}$, or Akt2$^{-/-}$ embryos were reconstituted with adenoviral HA-tagged Akt1 or Akt2. (*C*) Isolated MEFs were serum-starved for 16 h before a 15-min PDGF-BB (20 ng/mL) stimulation. Protein lysates were analyzed for the indicated phosphorylation events. (*D* and *E*) Akt1$^{-/-}$ MEFs were infected with adenoviral HA-tagged Akt1 or Akt2. Cells were serum-starved (16 h) and stimulated with PDGF-BB (40 ng/mL) for 15 min. Cell lysates were collected for immunoprecipitation with either anti-HA or a control IgG antibody (Fig. 1*C*). Immunoprecipitates (IPs) were incubated with a GSK3 peptide for in vitro kinase assays. Reaction products were then analyzed by SDS/PAGE and immunoblotting using antibodies specific for p-GSK3 (*D*) or p-eNOS S116 (*E*). Dll4, Delta-like 4. Representative experiments are shown.

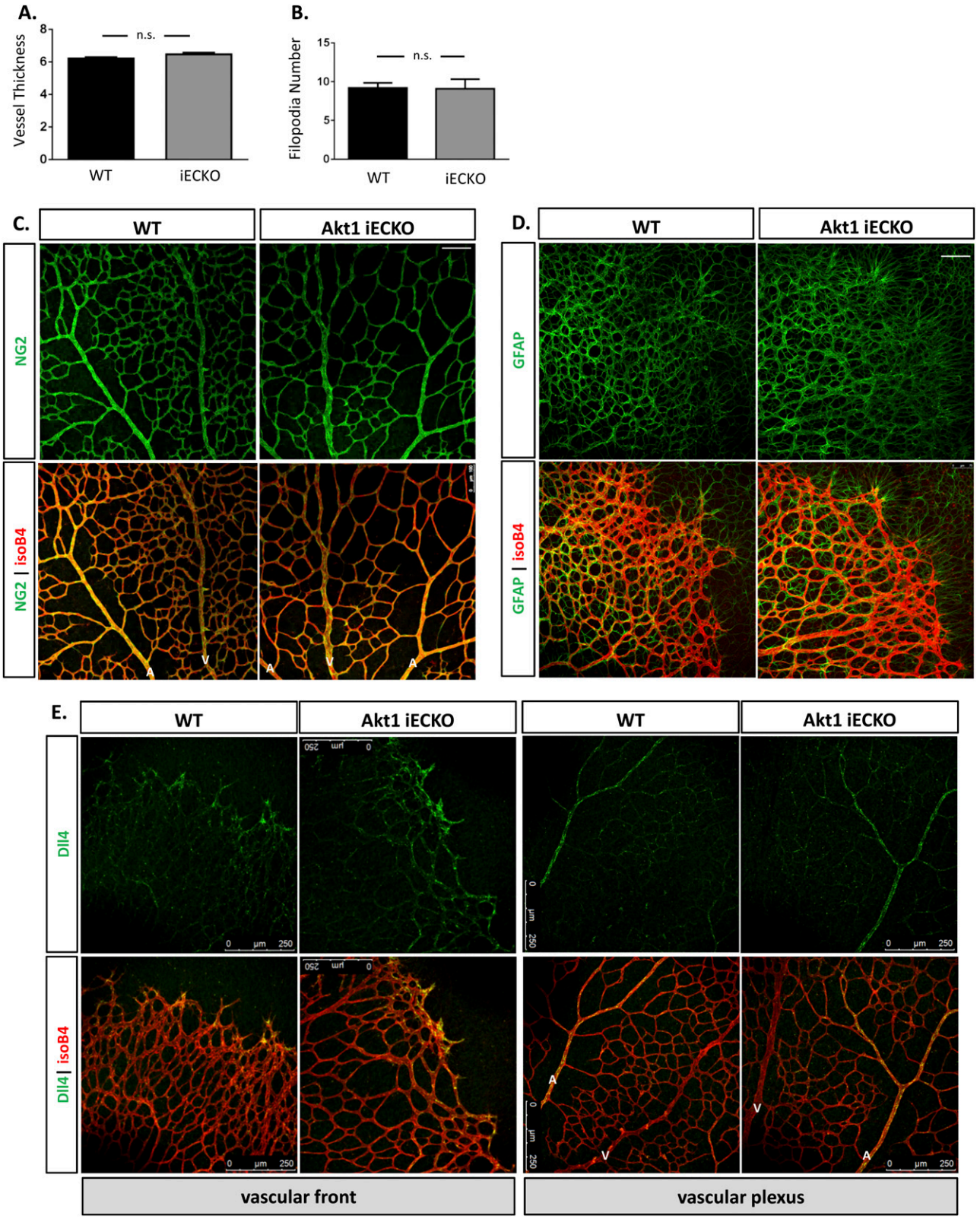

Fig. S2. Loss of endothelial Akt1 does not significantly affect vessel thickness (*A*) or filopodia number (*B*) [WT, $n = 6$; Akt1-inducible EC KO (iECKO), $n = 8$]. NG2-positive pericyte (vascular plexus; *C*) and GFAP-positive astrocyte coverage (vascular front; *D*) are not overtly affected by Akt1 loss in ECs. (*E*) Loss of endothelial Akt1 does not drastically affect tip/stalk cell identity or arterial/venous specification patterns. n.s., not significant. Representative images are shown on postnatal day 6. A, artery; IsoB4, isolectin B4; NG2, neuron-glial antigen 2; V, vein. (Magnification: *C*–*E*, 200×.)

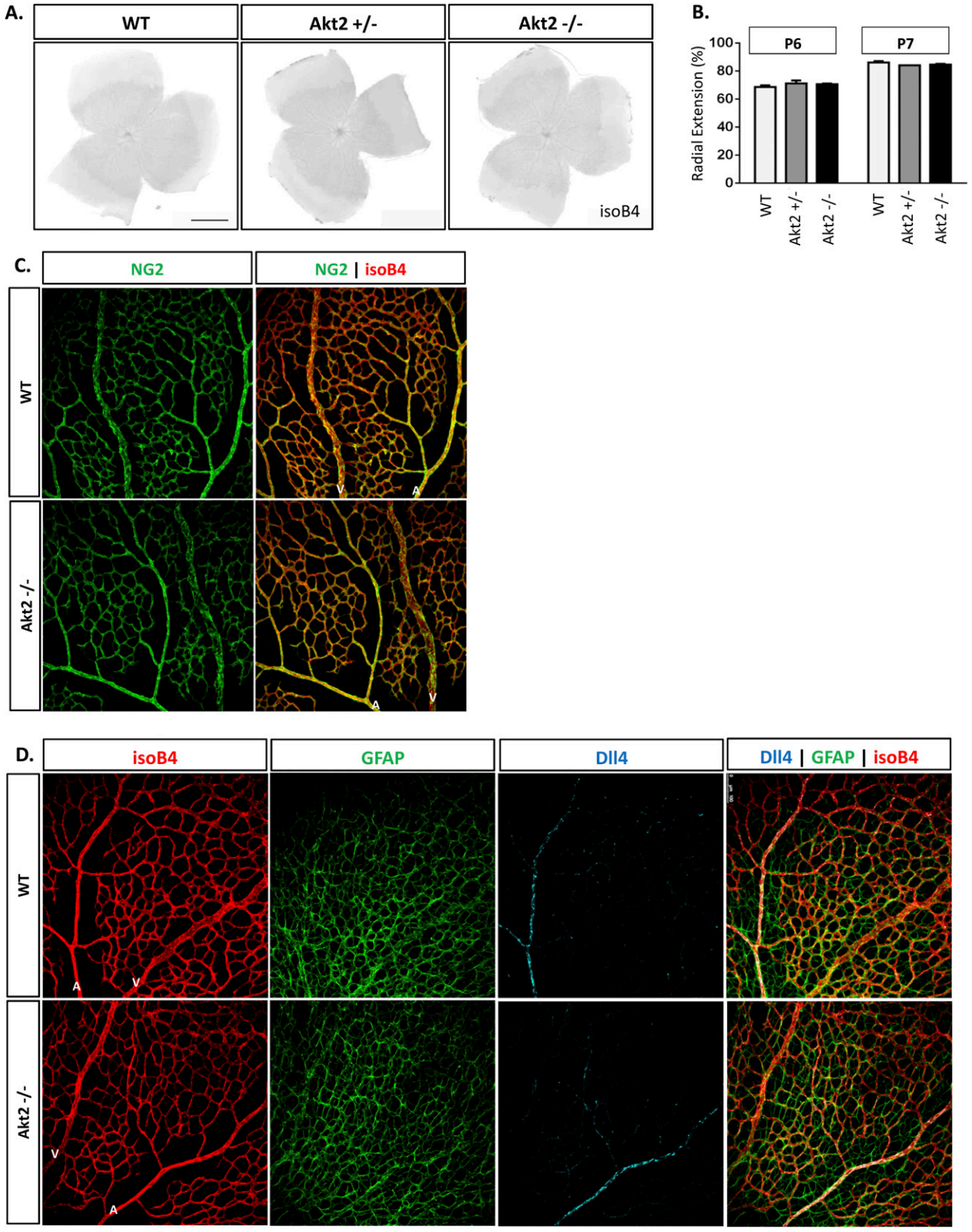

Fig. S3. (Continued)

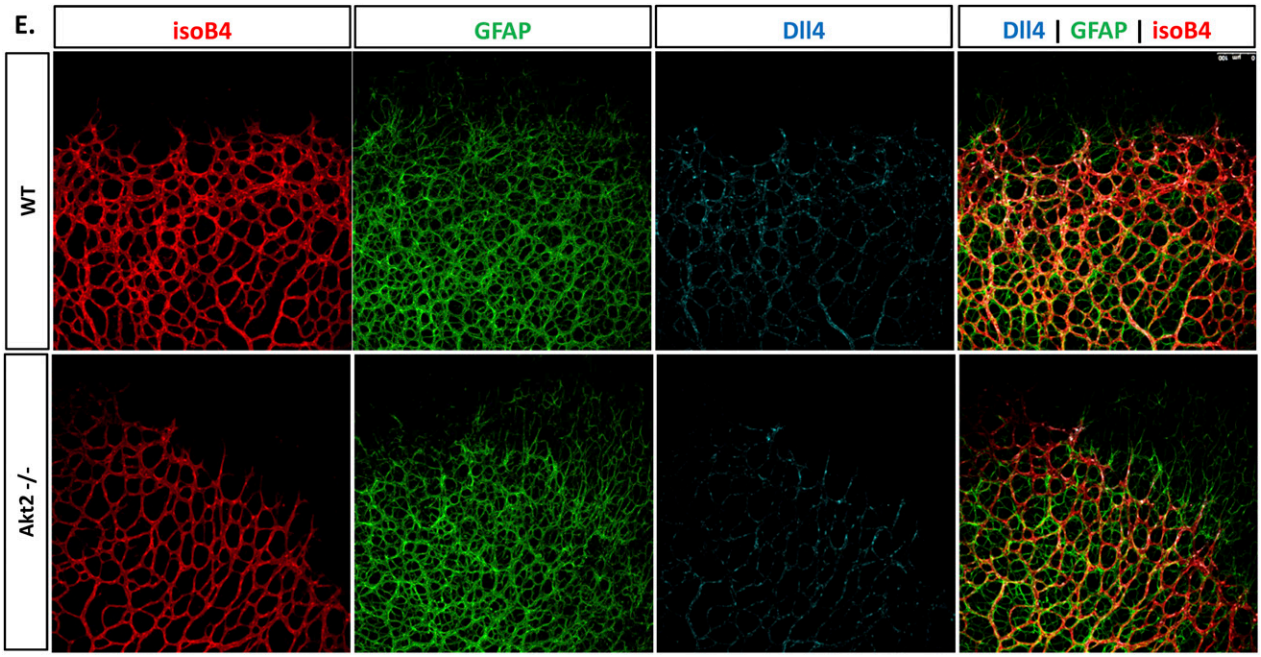

Fig. S3. (*A*) Global Akt2 deletion does not impair retinal outgrowth as depicted by littermate comparisons (postnatal day 6). (*B*) Global Akt2 deletion does not affect radial extension on either postnatal day 6 or postnatal day 7. (*C*) NG2-positive pericyte coverage appears normal in Akt2$^{-/-}$ retinas. (*D* and *E*) GFAP-positive astrocyte coverage in Akt2$^{-/-}$ retinas is comparable to that in WT. Artery/venous specification and tip/stalk cell specification are maintained in Akt2$^{-/-}$ mice, as assessed through dll4 staining. Representative images are shown. (Scale bar: *A*, 5 mm. Magnification: *C*–*E*, 200×.)

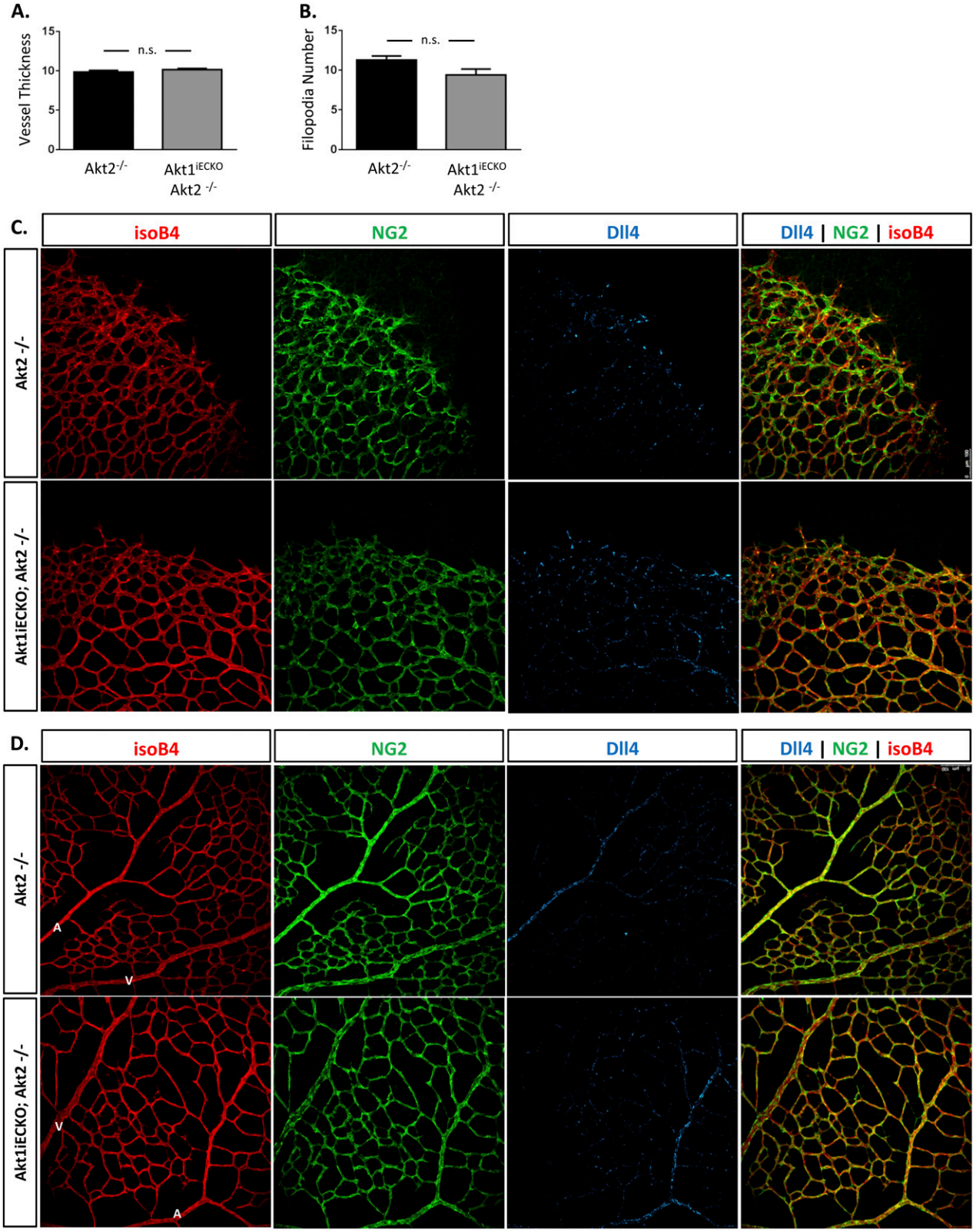

Fig. S4. (*A* and *B*) Endothelial-specific Akt1 deletion on an Akt2-null global background does not significantly affect vessel thickness or filopodia number ($Akt2^{-/-}$, $n > 7$; Akt1iECKO;$Akt2^{-/-}$, $n > 6$). (*C* and *D*) Dual loss of endothelial Akt1/2 does not overtly affect NG2-positive pericyte coverage. Tip cell identity and arterial specification also appear maintained, as seen through Dll4 staining. Representative images are shown on postnatal day 6. (Magnification: *C* and *D*, 200×.)

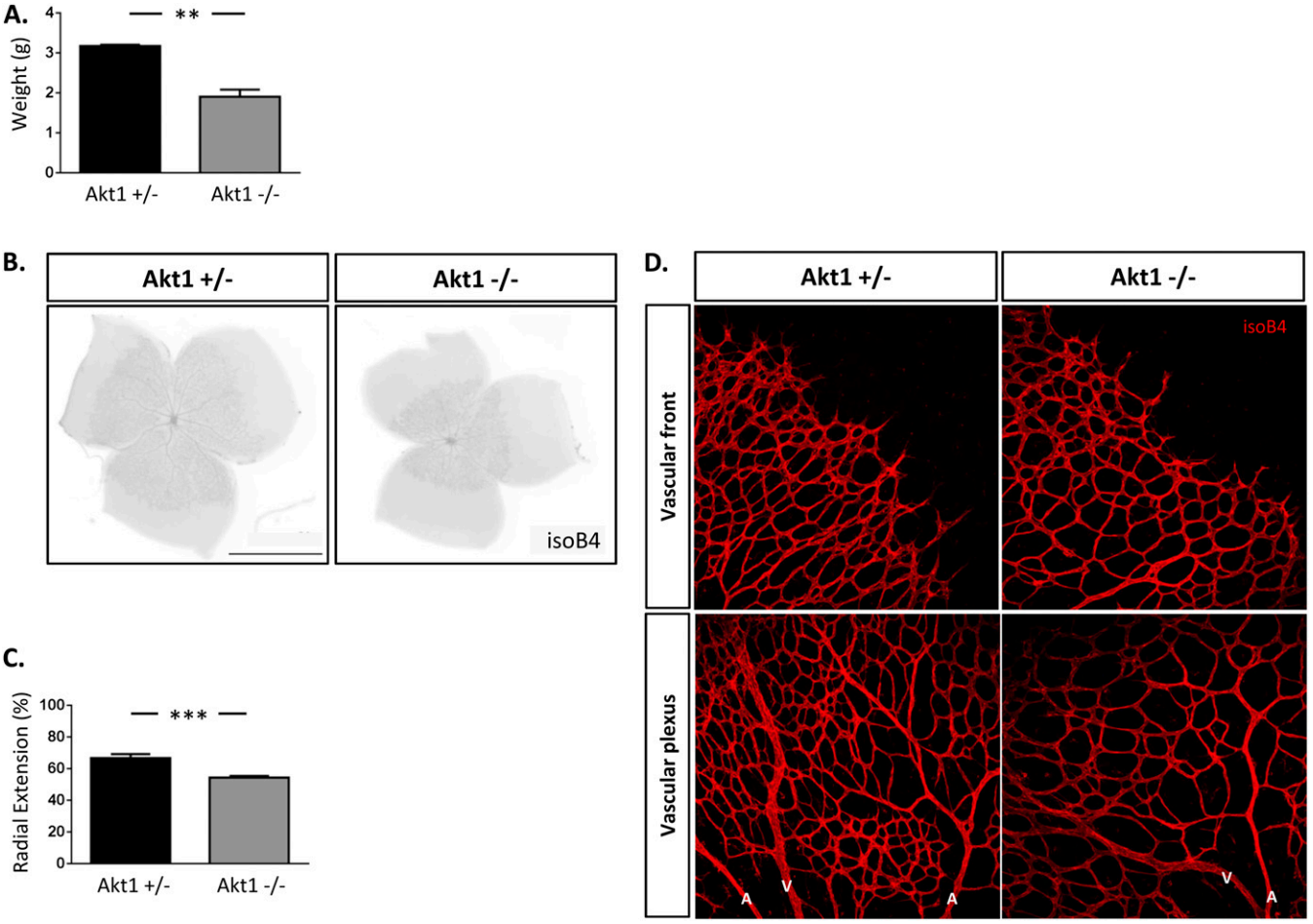

Fig. S5. (*A*) Global Akt1 deletion significantly affects overall growth (postnatal day 7, $Akt1^{+/-}$, $n = 3$; $Akt1^{-/-}$, $n = 6$). (*B–D*) Global Akt1 deletion impairs radial outgrowth and vascular patterning, similar to acute, endothelial-specific deletion. $^{**}P < 0.01$; $^{***}P < 0.001$. Representative images are shown. (Scale bar: *B*, 5 mm. Magnification: *D*, 200×.)

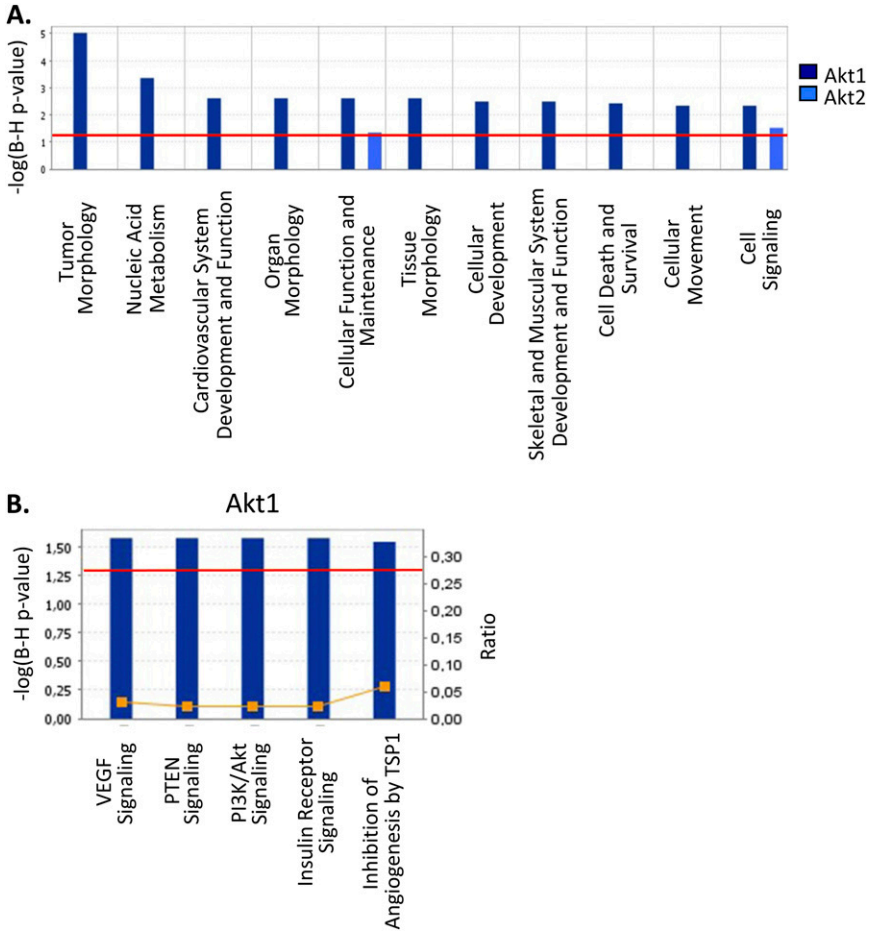

Fig. S6. (*A*) Diseases/biological functions affected by the loss of Akt1 or Akt2 activity in MLECs using the Ingenuity Pathways Knowledge Analysis (IPA) database. Only phosphoproteomic changes that were larger than −2.5-fold were included in the pathway analysis. The red line indicates where the significance value equals 0.05. B-H, Benjamini–Hochberg method. (*B*) Canonical pathway analysis using the IPA database for the Akt1-null condition. The Akt2-null condition did not yield any associated, statistically significant canonical pathways. PTEN, phosphatase and tensin homolog; TSP1, thrombospondin 1.

Table S1. Comparison of disease/biological functions affected by the loss of Akt1 or Akt2 in MLECs

Diseases and biological functions	B-H *P* values*	Proteins included in the disease/biological functions
Tumor morphology (results for Akt1$^{-/-}$)	9.5×10^{-6}	MKK4, HMOX1, FOXO4, TIAM2, FOXO1, CABLES1, FOXO3, NOS3
Nucleic acid metabolism (results for Akt1$^{-/-}$)	4.39×10^{-4}	HMOX1, FOXO4, TIAM2, FOXO1, FOXO3, NOS3
Cardiovascular system development and function (results for Akt1$^{-/-}$)	2.45×10^{-3}	MKK4, AMOTL1, HMOX1, FOXO4, FOXO1, PTPRB, FLNC, FOXO3, NOS3
Organ morphology (results for Akt1$^{-/-}$)	2.45×10^{-3}	MKK4, HMOX1, ITPKB, FOXO1, FLNC, CABLES1, FOXO3, NOS3
Cellular function and maintenance (results for Akt1$^{-/-}$)	2.45×10^{-3}	AMOTL1, MKK4, FOXO4, HMOX1, TIAM2, ITPKB, FOXO1, FOXO3, MARK4, NOS3
Cellular function and maintenance (results for Akt2$^{-/-}$)	4.49×10^{-2}	WDFY3, TBC1D1
Tissue morphology (results for Akt1$^{-/-}$)	2.45×10^{-3}	MKK4, HMOX1, ITPKB, FOXO1, FLNC, FOXO3, NOS3
Cellular development (results for Akt1$^{-/-}$)	3.24×10^{-3}	MKK4, FOXO4, CABLES1, NOS3, Palld, HMOX1, HNRNPA1, TIAM2, FOXO1, ITPKB, FLNC, PTPRB, FOXO3
Skeletal and muscular system development and function (results for Akt1$^{-/-}$)	3.24×10^{-3}	MKK4, FOXO4, HMOX1, FOXO1, FLNC, FOXO3, NOS3
Cell death and survival (results for Akt1$^{-/-}$)	3.76×10^{-3}	AMOTL1, Palld, MKK4, HMOX1, FOXO4, HNRNPA1, ITPKB, FOXO1, CABLES1, FOXO3, CLOCK, NOS3
Cellular movement (results for Akt1$^{-/-}$)	4.6×10^{-3}	AMOTL1, Palld, MKK4, FOXO4, HMOX1, TIAM2, FOXO1, FLNC, FOXO3, ASAP2, NOS3
Cell signaling (results for Akt1$^{-/-}$)	4.62×10^{-3}	MKK4, HMOX1, NOS3
Cell signaling (results for Akt2$^{-/-}$)	3×10^{-2}	NUP93

*Resulting *P* values were adjusted for multiple comparisons using the Benjamini–Hochberg method (B-H).

Other Supporting Information Files

Dataset S1 (XLSX)