Endothelial Akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates


The PI3K/Akt pathway is necessary for several key endothelial cell (EC) functions, including cell growth, migration, survival, and vascular tone. However, existing literature supports the idea that Akt can be either pro- or antiangiogenic, possibly due to compensation by multiple isoforms in the EC when a single isoform is deleted. Thus, biochemical, genetic, and proteomic studies were conducted to examine isoform-substrate specificity for Akt1 vs. Akt2. In vitro, Akt1 preferentially phosphorylates endothelial nitric oxide synthase (eNOS) and promotes NO release, whereas non-physiological overexpression of Akt2 can bypass the loss of Akt1. Conditional deletion of Akt1 in the EC, in the absence or presence of Akt2, retards retinal angiogenesis, implying that Akt1 exerts a nonredundant function during physiological angiogenesis. Finally, proteomic analysis of Akt substrates isolated from Akt1- or Akt2-deficient ECs documents that phosphorylation of multiple Akt substrates regulating angiogenic signaling is reduced in Akt1-deficient, but not Akt2-deficient, ECs, including eNOS and Forkhead box proteins. Therefore, Akt1 promotes angiogenesis largely due to phosphorylation and regulation of important downstream effectors that promote aspects of angiogenic signaling.

Significance

The expression of multiple isoforms of a protein kinase in cells raises the question of which substrates are preferentially phosphorylated by each isoform. Endothelial cells (ECs) that line all blood vessels express the protein kinases, Akt1 and Akt2, and we demonstrate here that endothelial nitric oxide synthase (eNOS) is a preferential Akt1 substrate and supra-physiological overexpression of Akt2 is needed to phosphorylate eNOS and promote NO release. In mice, the endothelial loss of Akt1, but not Akt2, retards vessel growth, and quantitative assessment of the phosphoproteomes in ECs isolated from these mice shows that Akt1 uniquely phosphorylates several important proteins regulating vascular function. Thus, despite the presence of both isoforms of Akt in the endothelium, substrate phosphorylation by Akt1 is nonredundant.


The authors declare no conflict of interest.

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Akt. Previous work from our laboratory demonstrated that VEGF stimulates Akt-mediated endothelial nitric oxide synthase (eNOS) phosphorylation at S1176 (bovine S1179), thus activating eNOS to promote NO release (3, 11). NO is an important mediator of many processes, including blood flow, vascular remodeling, and angiogenesis. Furthermore, the in vivo role of Akt1-eNOS kinase-substrate specificity is clear, where a phosphomimetic knock-in, but not a loss-of-function mutation at S1176, rescues pathologically induced angiogenesis in Akt1−/− mice (12). Given the relative EC-selective expression pattern of eNOS, we isolated mouse lung ECs (MLECs) from WT, Akt1−/−, and Akt2−/− mice to determine if Akt2 could compensate for the loss of Akt1 in the context of eNOS activation and NO release.

MLECs were cultured and stimulated with VEGF for 5 and 10 min. Examination of eNOS phosphorylation at the Akt phosphorylation site (S1176 or S614) showed a marked reduction in Akt1−/− cells compared with WT. Diminished phosphorylated p-eNOS levels were also seen under basal conditions in Akt1−/− cells, and the loss of Akt2 did not affect eNOS phosphorylation after VEGF stimulation (Fig. S1A). To assess the role of Akt1 vs. Akt2 on eNOS phosphorylation further, ECs were reconstituted via retroviral expression of HA-tagged versions of Akt1 or Akt2. Akt1−/− ECs demonstrated reduced Akt phosphorylation on S473 and T308, whereby reconstitution with either Akt1 or Akt2 restored Akt phosphorylation to levels comparable to that of WT (Fig. 1). Assessment of eNOS S1176 and glycogen synthase kinase 3β (GSK3β) serine 9 (S9) phosphorylation showed reduced levels in Akt1−/− ECs, where reconstitution with a retroviral vector expressing HA-tagged Akt1 in Akt1−/− ECs restored both p-eNOS and p-GSK3β levels (Fig. 1A, Lower). Reconstitution with Akt2 partially restored p-GSK3β levels, but not p-eNOS levels, to those of WT (Fig. 1B). These observations are consistent with those seen in PDGF-BB–stimulated primary lung fibroblasts (Fig. S1B and C), suggesting that both Akt1 and Akt2 can reconstitute S9 phosphorylation on GSK3β.

We next performed a series of in vitro kinase assays to investigate Akt isoform-substrate selectivity. Akt1−/− mouse embryonic fibroblasts (MEFs) were transduced with retroviral HA-tagged Akt1 or Akt2. Following serum starvation, infected MEFs were either left unstimulated or stimulated with PDGF-BB (40 ng/mL) for 15 min. Lysates were immunoprecipitated for HA-tagged Akt1 or Akt2, and HA immunoblotting showed comparable levels of isolated Akt1 and Akt2 (Fig. 1C). PDGF-BB stimulation robustly increased Akt phosphorylation levels (S473 and T308) for both Akt1 and Akt2 (Fig. 1C). The immune-isolated kinase was then incubated with substrates, either a GSK3 fusion protein or recombinant bovine eNOS, for 30 min in kinase reactions. SDS/PAGE and immunoblotting for GSK3 phosphorylation levels demonstrated that Akt activity increased six- to sevenfold higher as a result of PDGF stimulation, regardless of the recombinant Akt isoform in the MEFs (Fig. S1D). Similar analyses for eNOS phosphorylation show that activated Akt1 induced close to a 15-fold increase in eNOS S1179 phosphorylation but lower phosphorylation (approximately fivefold) with Akt2 (Fig. 1D). These isoform-substrate differences are further exemplified when evaluating other Akt-directed eNOS phosphorylation sites, because Akt1 more efficiently phosphorylates eNOS S617 compared with Akt2 (Fig. 1E). Additionally, phosphorylation of a non-Akt phosphorylation site, S116, does not occur (Fig. S1E), suggesting that at relatively equal levels of Akt1 or Akt2 activity, eNOS is preferentially phosphorylated by Akt1.

Next, we assessed the functional consequence of eNOS phosphorylation in Akt1−/− ECs via measurement of NO release. As seen in Fig. 1F, defective NO release in Akt1−/− cells was restored to WT levels with reconstitution of Akt1. Reconstitution of Akt2 to near-endogenous levels of total Akt (as seen with HA and pAkt T308 immunoblotting), however, only slightly increases basal NO accumulation. Furthermore, marked overexpression of Akt2 to nonphysiological levels (~10-fold greater) restores NO release similar to that of WT (Fig. 1G). Therefore, Akt1 more efficiently activates eNOS compared with Akt2 when expressed at equal levels, and this preference is overcome by Akt2 overexpression.

**Endothelial Akt1 Drives Developmental Retinal Angiogenesis.** To study the functional importance of Akt1 in vivo, we inactivated Akt1 specifically in the endothelium using Cre/loxP technology. Briefly, Akt1floxed mice (13) were bred to Cdhh5-CreERT2 (14) mice to generate inducible, endothelial-specific Akt1 KO mice. To account for any tamoxifen-induced variability, all neonates were administered tamoxifen, and only those expressing the Cre allele underwent loxP-mediated excision. WT denotes Cre-negative, floxed littermates that received tamoxifen. WD denotes Cre-negative, floxed littermates that received tamoxifen. These mice pose an advantage over current Akt1 mouse models in that our inducible deletion strategy minimizes the possibility for compensation by other Akt isoforms and/or AGC-family kinases during in utero deletion.

Postnatal deletion of Akt1 in the endothelium [initiated at postnatal day (P) 1 and analyzed at P6–P7, Akt1-inducible EC KO (Akt1iECKO)] impairs retinal angiogenesis, as shown by reduced endothelial coverage and delayed radial outgrowth (Fig. 2A and B). There are also no apparent weight differences between WT and Akt1iECKO pups, thereby eliminating the possibility of...
GFAP-positive astrocyte patterning (Fig. S2). An PI3K inhibitor, was injected acutely into established rat retinas. The observation is similar to previous reports, where wortmannin, an inhibitor of Akt, increased collagen IV-positive sleeves (Fig. 2A). Deletion of Akt1 promotes vessel regression, as seen through in-vivo imaging in Akt1-flox/flox;Akt2−/− mice. Postnatal deletion of Akt2, however, does not result in any significant changes in vessel thickness or tip cell/filopodia number (Fig. S2). To examine if Akt2 compensates for the EC-specific loss of Akt1, Akt1-flox/flox;Akt2−/− females were bred to Akt1-flox/flox; Akt2−/−; Chdh-Cre males, and resulting progeny were similarly injected with tamoxifen for P1–P3. Using this breeding strategy, littermate Akt2−/− mice were used as controls to determine the effect of dual Akt1 and Akt2 deletion in the endothelium on ensuing retinal development. As shown in Fig. 3A, dual loss of Akt1/2 in ECs results in impaired retinal outgrowth compared with control littermates (quantified in Fig. 3B) but does not significantly affect body weight (Fig. 3C). Furthermore, the retinal defects seen in Akt1eECKO mice are not worsened by the additional loss of Akt2. These observations imply that Akt1 is the predominant Akt isoform in ECs necessary for proper retinal vascular growth, because Akt2 does not compensate for its loss (Fig. S3). Quantification of vascular density at the vascular plexus regions indicates fewer branch points, holes, and segments and shorter vessel length in Akt1eECKO; Akt2−/− mice (Fig. 3D–H), whereas vessel thickness and filopodia number were not significantly affected (Fig. S4A and B). The dual loss of Akt1/2 also does not overtly affect NG2-positive pericyte coverage or tip/stalk and artery/vein specification [Fig. S4C (vascular plexus) and D (vascular plexus)]. Vessel regression was, however, significantly increased, as measured by increased collagen IV deposition (Fig. 3I and J). The observed disturbance in vascular network formation thereby phenocopies the conditional loss of Akt1 in ECs, as seen in Fig. 2, implying that Akt1 is the dominant functional Akt isoform in the endothelium.

Retinal vascular development was also assessed in global Akt1-null mice to examine the relative contribution of endothelial Akt1 to the retinal phenotype. Postnatal Akt1−/− mice weigh significantly less than Akt1+/− littermates (Fig. S5A), thereby confirming previous reports of general growth retardation in Akt1-null mice (8–10). More importantly, the global loss of Akt1 phenocopies the endothelial-specific, acute postnatal deletion of Akt1 described herein (Fig. S5B–D), and may be arguably worse than those in Akt1eECKO mice. This could, however, be attributed to the synergistic effects of Akt1 loss in other cell types critical for proper angiogenesis or incomplete deletion of the floxed allele. Nonetheless, the observation that acute, endothelial-specific Akt1 deletion yields retinal defects similar to global deletion suggests that developmental retinal angiogenesis depends largely on functional Akt1 in ECs.

Akt-Selective Phosphoproteomics Reveals a Larger Population of Akt1-Specific Substrates in ECs. Although Akt1 and Akt2 exhibit strong similarities in sequence and function, there are subtle differences between the two isoforms. Mouse models reflecting global Akt1 or global Akt2 loss manifest in unique phenotypes (8, 9, 17). To appreciate Akt1 retinal phenotypes at the molecular level fully, a phosphoproteomics screen was performed in
proteins were down-regulated in both Akt1 and Akt2, respectively (A). There were no statistically significant differences in Akt1 and Akt2 phosphorylation levels in the starting material. Biological duplicates of protein extracts were isolated in urea and digested into peptides, and isolated peptides were subjected to MS analysis and label-free quantification, as previously described (19), to identify Akt1- and Akt2-dependent substrates (i.e., decreased phosphopeptides in Akt1iECKO/ Akt2−/− mice relative to WT ECs). To identify those phosphopeptides that are most likely Akt substrates, we considered only phosphopeptides with the following conditions as being significantly lower expressed: (i) they contain the Akt phosphorylation motif (Fig. 4B) and (ii) they exceeded a 2.5-fold directional cutoff (Materials and Methods). The resultant phosphopeptides from this analysis were depicted as a heat map in Fig. 4C and detailed in Dataset S1, where 59 unique phosphosites in 58 different proteins (HMOX1 was identified to have two distinct phosphosites) were identified. Among these sites, 26 phosphopeptides were down-regulated in both Akt1−/− and Akt2−/− cells (red highlighted text in Dataset S1), and seven phosphopeptides were decreased solely in the Akt2−/− cells (blue text in Dataset S1). The heat map clustering portrays a larger population of Akt1-sensitive substrates in ECs, which likely contributes to the observed EC phenotypes. Furthermore, the identification of mutually exclusive substrates indicates Akt isoform-substrate specificity, and hence distinct downstream functions.

Phosphoproteomic Pathway Analysis Indicates Akt1-Associated Substrates, Not Akt2-Associated Substrates, Are Significantly Correlated with Cardiovascular Development and Function. In an effort to understand the biological relevance of the phosphoscreen, the generated lists were subjected to pathway analysis using the Ingenuity Pathway Analysis (IPA; Ingenuity) database. The phosphoprotomic results were compared against global molecular networks to identify associated diseases/biological function and canonical pathways affected by loss of either Akt1 or Akt2 activity. The absence of the “lower expressed” Akt2 isoform significantly affects broad cellular function, namely, “cell signaling” and “cell function and maintenance” (Fig. S6A and Table S1). Ablation of Akt1, however, is associated with nine additional biological functions. More importantly, the changes in protein phosphorylation due to the loss of Akt1 significantly correlate with “cardiovascular system development and function,” as represented by over one-third (nine of 26 proteins) of the identified mutually exclusive Akt1-specific substrates (Fig. 4D). Furthermore, the nine identified proteins are implicated in angiogenesis and/or endothelial function.

AFFECTED CANONICAL PATHWAYS WERE ALSO MODELLED USING THE IPA DATABASE TO CLARIFY THE PHENOTYPIC DIFFERENCES BETWEEN AKT1- AND AKT2-NULL CELLS. FURTHER, THE LOSS OF AKT2 DID NOT SIGNIFICANTLY INFLUENCE CARDIOVASCULAR PATHWAY SIGNALING, WHEREAS THE LOSS OF AKT1 IS ASSOCIATED WITH SEVERAL KNOWN PATHWAYS, ALL OF WHICH ARE NECESSARY FOR DEVELOPMENTAL ANGIOGENESIS (Fig. S6B). THE PARALLEL OUTPUT BETWEEN CANONICAL PATHWAYS AND BIOLOGICAL FUNCTION SUGGESTS AKT1-SPECIFIC SUBSTRATES Dictate GROWTH AND DEVELOPMENT IN THE ENDOTHELIUM, WHEREAS AKT2 LIKELY PLAYS A MINOR ROLE.

**Discussion**

Our study identifies Akt1 as the predominant Akt isoform responsible for eNOS phosphorylation and developmental angiogenesis in the endothelium. The in vivo conditional models paired with an unbiased, high-throughput phosphoproteomics analysis demonstrate that endothelial Akt1, as opposed to Akt2, influences multiple signaling networks critical for vascular development and function. Interestingly, we identified common and isoform-specific Akt substrates via proteomics, providing evidence for isoform selectivity in vivo. These data reporting the consequential developmental vascular phenotypes with conditional deletion of Akt1 in ECs and characterization of the substrates preferred by Akt1 in ECs are consistent with the importance of Akt downstream of VEGF signaling (2).

Previous studies investigating the consequence of Akt1 loss used global KO mice. Global Akt1 deletion results in sub-Mendelian birth ratios and increased neonatal mortality, where surviving Akt1−/− mice are viable despite growth retardation. Although these global-null studies have been instrumental in moving the field forward, the ubiquitous nature of Akt limits the ability to study isoform-specific effects in particular cell types. Additionally, the viability of Akt1−/− mice suggests compensation from other Akt isoforms. Several groups have generated double-null and even triple-null Akt mouse models to address the possibility of functional overlap. Interestingly, both Akt1−/−;Akt2−/−;DKO and Akt1−/−;Akt3−/−;DKO result in postnatal and embryonic lethality, respectively (20, 21). Furthermore, triple Akt1−/−;Akt2−/−;Akt3−/− mice are viable with a severe growth impairment, suggesting a single Akt1 allele
is sufficient for successful embryonic development and postnatal survival (22). Akt1 is critical for postnatal angiogenesis, because Akt1 is downstream of several proangiogenic and survival factors, including VEGF (23, 24), angiopoietins (25), and sphingosine 1-phosphate (26). The global loss of Akt1 in mice, however, reduces lymphangiogenesis (27) and can reduce (3, 5, 6) or increase (4) angiogenesis depending on the context and model used, thereby supporting the possibility that remaining Akt isoforms compensate for Akt1 loss by phosphorylating its substrates. Therefore, we used an inducible, endothelial-targeted Akt1 deletion mouse model in an effort to minimize long-term noncanonical pathways. Substrate differences, however, may reflect the fact that the MLECs used for the analysis express higher levels of Akt1 than Akt2. Nonetheless, despite these expression differences, exclusive Akt1 vs. Akt2 substrates were identified. Collectively, these data indicate that Akt2 activity is negligible for vascular retinal development.

Several of the Akt phosphosites identified from our screen as Akt1-dependent and cardiovascular-associated have previously been described as substrates, thereby validating the proteomic methodology. The phosphoscreen detected eNOS S1176, a well-characterized Akt1-specific substrate, as an Akt1-specific substrate (11, 31), and eNOS is the predominant Akt1 substrate necessary for angiogenesis, blood flow, and tissue repair (12). These in vivo data are supported by in vitro data, where Akt1, but not Akt2, optimally phosphorylates eNOS and promotes NO release. However, massive overexpression of Akt2 can rescue the loss of Akt1, implying that the levels of Akt1 are critical for the fidelity of eNOS phosphorylation and NO release. PI3K/Akt-mediated phosphorylation of the Forkhead box (FOXO) subcellular transcription factors has been described to inactivate all 4 FOXO family members, three of which were detected in our screen (FOXO1A, FOXO3A, and FOXO4). Dephosphorylation and subsequent activation of FOXO-dependent transcription stimulates several key cellular aspects, including apoptosis, cell-cycle arrest, and oxidative-stress resistance. FOXO1 activity is critical for development and viability, because global deletion of FOXO1 results in vasculogenesis defects and embryonic lethality (32). Analysis of the FOXO transcriptome suggests FOXO regulates the expression of genes related to angiogenesis and vascular remodeling, including angiopoietins and matrix metalloproteinases (33). FOXO1 has also been studied as a transcriptional effector of angiopoietin1/Akt signaling and related vessel stability, further supporting the role of Akt (via FOXO) in vessel remodeling (34).

Other interesting molecules detected as Akt substrates in the phosphoscreen that may influence vascular function are filamin C (FLNC), vascular endothelial tyrosine phosphatase (PTPRB/VE-PTP) and angiomotin-like protein 1 (AMOTL1). FLNC has been...
shown to interact with several signaling-related proteins, including SHIP-2 and RasGAP, suggesting FLNC may harness functions related to signal transduction and may play a role in EC migration and trafficking events (35, 36). PTEN activity appears to be nonessential for initial blood vessel formation but vital for maintenance and vascular remodeling (37, 38). AMOTL1 governs endothelial tip cell formation during sprouting angiogenesis and aspects of functional stability to mediate paracellular permeability (39, 40). Additional studies characterizing the Akt-dependent phosphorylation of these proteins in the context of growth factor signaling and angiogenic functions are ongoing.

The important roles for each of the identified Akt1-specific substrates highlight the possible contributions of multiple substrates to the observed Akt1ECKO retinal phenotype. Furthermore, although many well-characterized Akt substrates were identified in the phosphoscreen, other known targets did not appear in the final substrate list. This observation can be explained by several reasons. First, our initial analysis used fairly high stringencies, because previously identified substrates (i.e., Sin1, TBC1D4) emerged in the output when we relented the fold-change requirements (from −2.5- to −2.0-fold directional cutoff). In addition, phosphorylation events are dynamic and spatially regulated, because Akt has been shown to have differential effects on substrate phosphorylation depending on its subcellular localization (41, 42). This activity, in turn, is regulated by phosphatases, such as protein phosphatase 2A and PH domain and leucine rich repeat protein phosphatase (30). Considering the phosphoscreen-reflected cells under basal serum-grown conditions, agonist stimulation poststarvation may have yielded a larger cohort of known Akt substrates (2). Collectively, this study clearly shows the critical role of Akt1 in angiogenesis and begins to decipher the complexity of Akt isoform selectivity in the endothelium.

**Materials and Methods**

**Mice.** The Akt1+/−, Akt2−/−, and Akt1flox/flox mice were generated as previously described (9, 13, 17). Conditional pups were injected with 50 μg of tamoxifen per day (P1–P3) via i.p. delivery. All mouse experiments were approved by the Institutional Animal Care Use Committee at the Yale School of Medicine.

**Phosphoproteomic Analysis of Rux5x1/1** **Substrates.** Immortalized MELECs were grown to 70–80% confluency and harvested in appropriate lysis buffer for each experimental condition. The Akt isoform-specific phosphoproteome of MELECs lysates was characterized using PTMscan technology (Cell Signaling Technology) based on LC/tandem MS, as described previously (19).

**Statistics.** Data are expressed as mean ± SEM. Comparisons between groups were made using a two-tailed Student t test or ANOVA with the Bonferroni post hoc test.

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Supporting Information

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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 t-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 T75 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 immunosolated 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 immunosolated 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 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 MgCl2, 5 mM β-glycerophosphate, and 1 mM Na2VO4. Pellets were resuspended in 50 μL of kinase buffer supplemented with 1 μL of 10 mM ATP and 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), p-Akt-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 (IRDy680 and IRDye680, 1:10,000 dilution; LI-COR Biotechnology). Bands were visualized using the Odyssey IR imaging system (LI-COR Biotechnology).

Phosphoproteomic Analysis of Rnx55**T** Substrates. For each experimental condition (n = 3), 2 × 109 MLECs were grown to 70–80% confluency, washed twice in PBS, and lysed in urea lysis buffer [20 mM Hepes (pH 8.0), 90 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate]. 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 μg 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 SEQUEST 3G (Thermo Fisher Scientific) and the Sorcerer 2 platform (Sage-N Research). 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.

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, 200x.)
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, 200x.)
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

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

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

Dataset S1 (XLSX)