Capillary malformation-arteriovenous malformation (CM-AVM) is a blood vascular anomaly caused by inherited loss of function mutations in \textit{RASA1} or \textit{EPHB4} genes that encode p120 Ras GTPase-activating protein (p120 RasGAP/RASA1) and Ephrin receptor B4 (EPHB4) respectively. However, whether RASA1 and EPHB4 function in the same molecular signaling pathway to regulate the blood vasculature is uncertain. Here, we show that induced endothelial cell (EC)-specific disruption of \textit{Ephb4} in mice results in accumulation of collagen IV in the EC endoplasmic reticulum leading to EC apoptotic death and defective developmental, neonatal and pathological angiogenesis, as reported previously in induced EC-specific RASA1-deficient mice. Moreover, defects in angiogenic responses in EPHB4-deficient mice can be rescued by drugs that inhibit signaling through the Ras pathway and drugs that promote collagen IV export from the ER. However, EPHB4 mutant mice that express a form of EPHB4 that is unable to physically engage RASA1 but retains protein tyrosine kinase activity show normal angiogenic responses. These findings provide strong evidence that RASA1 and EPHB4 function in the same signaling pathway to protect against the development of CM-AVM independent of physical interaction and have important implications with regards possible means of treatment of this disease.
Angiogenesis depends upon EPHB4-mediated export of collagen IV from vascular endothelial cells

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Capillary malformation-arteriovenous malformation (CM-AVM) is a blood vascular anomaly caused by inherited loss of function mutations in \textit{RASA1} or \textit{EPHB4} genes that encode p120 Ras GTPase-activating protein (p120 RasGAP/RASA1) and Ephrin receptor B4 (EPHB4) respectively. However, whether RASA1 and EPHB4 function in the same molecular signaling pathway to regulate the blood vasculature is uncertain. Here, we show that induced endothelial cell (EC)-specific disruption of \textit{Ephb4} in mice results in accumulation of collagen IV in the EC endoplasmic reticulum leading to EC apoptotic death and defective developmental, neonatal and pathological angiogenesis, as reported previously in induced EC-specific RASA1-deficient mice. Moreover, defects in angiogenic responses in EPHB4-deficient mice can be rescued by drugs that inhibit signaling through the Ras pathway and drugs that promote collagen IV export from the ER. However, EPHB4 mutant mice that express a form of EPHB4 that is unable to physically engage RASA1 but retains protein tyrosine kinase activity show normal angiogenic responses. These findings provide strong evidence that RASA1 and EPHB4 function in the same signaling pathway to protect against the development of CM-AVM independent of physical interaction and have important implications with regards possible means of treatment of this disease.
Introduction

Capillary malformation-arteriovenous malformation (CM-AVM) is an inherited autosomal dominant blood vascular disorder in humans that affects 1:10,000 to 1:100,000 individuals (1-4). The pathognomonic feature of CM-AVM is the presence of one more cutaneous CM. However, in approximately one third of patients, there are additional life-threatening fast flow blood vascular lesions that include AVM and arteriovenous fistulas. Lymphatic vascular abnormalities including abnormal lymphatic flow, chylothorax, chylous ascites and lymphedema have also been described in some CM-AVM patients (2, 3, 5-8).

CM-AVM1 and CM-AVM2 represent two different forms of CM-AVM that are caused by mutations in different genes. CM-AVM1, which accounts for approximately 50% of cases, is caused by mutations in the RASA1 gene that encodes the RASA1 protein also known as p120 Ras GTPase-activating protein (p120 RasGAP) (1-3). In growth factor receptor (GFR) signaling pathways, RASA1 interacts with the active GTP-bound form of the Ras small GTP-binding protein (9, 10). This interaction increases the ability of Ras to hydrolyze bound GTP to GDP by several orders of magnitude resulting in the conversion of Ras to its inactive GDP-bound state. As such, RASA1 acts as a negative regulator of GFR-induced Ras activation and downstream signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway that couple cell surface GFR-ligand recognition events to cellular outcomes (9, 10).

The vast majority of inherited RASA1 mutations in CM-AVM1 are nonsense mutations, frameshift mutations or splice substitutions that result in premature translation termination codons (2, 3). Thus, mutations are thought to be inactivating as transcripts are likely rapidly
degraded by nonsense-mediated RNA decay. However, inheritance of one null *RASA1* allele is considered insufficient for lesion development. Instead, development of lesions requires the acquisition of an additional somatic second hit mutation in the wild-type *RASA1* allele in endothelial cells (EC) during development (11, 12). This second hit mutation, together with the germline *RASA1* mutation, renders RASA1 null EC that are that are thought to give rise to lesions.

CM-AVM2 accounts for approximately 30% of CM-AVM cases and is phenotypically similar to CM-AVM1 with the addition of telangiectasias in CM-AVM2 (4). The affected gene in CM-AVM2 is *EPHB4* that encodes the growth factor receptor, Ephrin receptor B4 (EPHB4). Approximately 50% of *EPHB4* mutations are predicted to result in null alleles as a result of nonsense-mediated RNA decay. The remaining *EPHB4* mutations are missense mutations located mostly in codons that encode amino acids contained in the extracellular domain or the intracellular protein tyrosine kinase domain (4). Although not yet demonstrated, it is likely that development of vascular lesions in CM-AVM2 is also dependent upon acquisition of somatic second hit mutations in EC during development, in this instance in the inherited wild-type *EPHB4* allele.

The occurrence of both *RASA1* and *EPHB4* mutations in CM-AVM suggests that RASA1 and EPHB4 function in the same signaling pathway to regulate vascular development. Consistent with this is the finding that global knockout mice that constitutively lack RASA1, EPHB4 or the EPHB4 ligand, Ephrin B2, all die in mid-gestation as a consequence of failed vascular development (13-15). Specifically, primitive vascular plexuses formed as a result of
vasculogenesis fail to become remodeled through the process of developmental angiogenesis into hierarchical arterial-capillary-venous networks. Studies of conditional RASA1- and EPHB4-deficient mice are also consistent with the notion of a close functional relationship between RASA1 and EPHB4 in the vasculature. Thus, vascular-specific disruption of Rasa1 and Ephb4 blocks the development of each of venous valves, lymphatic vessel (LV) valves and lymphovenous valves (16-20).

In its role as a GFR, EPHB4 activates the Ras-MAPK signaling pathway (21). In contrast, RASA1 inhibits Ras-MAPK signaling (10). Therefore, the finding that disruption of Ephb4 and Rasa1 in the vasculature in mice results in the same phenotype seems counterintuitive, at least from the perspective that they function in the same molecular signaling pathway in EC. However, whereas EPHB4 can promote Ras-MAPK signaling in some cell types, in human umbilical vein endothelial cells (HUVEC), EPHB4 functions as an inhibitor of Ras-MAPK signaling initiated through other growth factor receptors such as vascular endothelial cell growth factor receptor (VEGFR) and Tie2 (21, 22). Moreover, an ability of EPHB4 to dampen Ras-MAPK signaling through these other GFR is dependent upon expression of RASA1 in HUVEC (21). During EPHB4 signal transduction, RASA1 interacts physically with EPHB4 (22-24). Ephrin B2 binding to EPHB4 induces the kinase activity EPHB4 resulting in the phosphorylation of multiple EPHB4 tyrosine residues including Y590 and Y596 in the juxtamembrane (JM) region of the intracellular domain. Phosphorylated Y590 and Y596 are then recognized by two Src-homology-2 (SH2) domains contained in RASA1 which allow binding of RASA1 to EPHB4 (23, 24). Based on these findings, one straightforward model to account for the role of EPHB4 and RASA1 in vascular development is that in EC, EPHB4 functions as an inhibitory receptor
that serves primarily to recruit RASA1 to the plasma membrane allowing its juxtaposition to Ras-GTP, an event necessary for Ras inactivation. To test this model, Kawasaki et al generated zebrafish that expressed a Y590F/Y596F double point mutant of EPHB4 that is unable to bind RASA1 (24). These zebrafish demonstrated the same defect in vascularization of the caudal end of the tail as observed in zebrafish that lack EPHB4 or RASA1 completely. However, it is of note that phosphorylation of these tyrosine residues in other members of the Ephrin receptor family (EPHB2 and EPHA2) is required to switch the kinase domain from a restrained inactive conformation to an open active conformation with full kinase activity (25-27). Therefore, it is not possible to conclude from these studies that a physical association between RASA1 and EPHB4 underlies any apparent functional relationship in vascular development.

In a recent study, we investigated the mechanism by which loss of RASA1 in mouse embryos impairs developmental angiogenesis (28). We determined that RASA1-deficient embryonic EC fail to export the extracellular matrix protein, collagen IV, for deposition in nascent vascular basement membranes. Consequently, EC undergo apoptosis, either as a result of an inability to attach to the basement membrane or as a result of ER stress. Available data are consistent with a model in which dysregulated Ras-MAPK signaling in RASA1-deficient embryonic EC results in an increased abundance of proline and lysine hydroxylases within the EC endoplasmic reticulum (ER). The increased abundance of these enzymes results in excessive hydroxylation of collagen IV monomers that impairs their folding and assembly into trimeric collagen IV protomers that are normally exported from the ER via the coat polymer II (COPII) secretory mechanism. Consequently, drugs that inhibit MAPK signaling, promote collagen IV folding in the ER, or inhibit the activity of collagen proline and lysine hydroxylases, can each rescue the
developmental angiogenesis defect in EC-specific RASA1-deficient embryos (28). In contrast to developmental angiogenesis in the embryo, RASA1 is not required for the maintenance of the blood vasculature in adults (16, 29). However, RASA1 is required for retinal angiogenesis in neonates and pathological angiogenesis to solid tumors in adults (28). These findings are consistent with a requirement for de novo EC synthesis of collagen IV in these responses.

To obtain further evidence that EPHB4 and RASA1 function in the same signaling pathway to regulate the blood vasculature, we examined the basis of a requirement for EPHB4 for developmental angiogenesis in mice. We report that loss of EPHB4 in EC during developmental angiogenesis results in their inability to export collagen IV from the EC ER which leads to their apoptotic death. We also show that like RASA1, EPHB4 is necessary for retinal angiogenesis in newborns and pathological angiogenesis in adults. However, as revealed with the use of a novel EPHB4 knockin model, physical association of EPHB4 with RASA1 is not required for EPHB4 to promote any of these angiogenic responses.
**Results**

Disruption of Ephb4 during developmental angiogenesis results in apoptotic death of EC. To understand the role of EPHB4 in developmental angiogenesis, mice with a conditional allele of Ephb4 in which exons 2 and 3 were flanked by loxP sites (Ephb4\(^{fl}\)) were crossed with ubiquitin promoter-driven ert2cre (Ub\(^{ert2cre}\)) transgenic mice to generate Ephb4\(^{fl/\beta}\) Ub\(^{ert2cre}\) and control Ephb4\(^{fl/\beta}\) cre-negative littermate embryos. Pregnant dams carrying embryos of both genotypes were administered tamoxifen (TM) at E13.5 and embryos were harvested at E18.5. By E13.5 vasculogenesis is complete and from E13.5-18.5 developmental angiogenesis predominates (30). When examined at E18.5, cre-positive embryos exhibited severe cutaneous hemorrhage that manifest visibly across most of the surface of the embryo (Supplemental Figure 1A, Table 1). Staining of tissue sections with H&E and anti-CD31 and LYVE-1 antibodies confirmed hemorrhage that was associated with damaged blood vessels (BV) and a near absence of LV at this time point consistent with an edematous appearance of embryos (Supplemental Figure 1A and B). The same hemorrhagic and edematous phenotype was observed in TM-treated Ephb4\(^{fl/\beta}\) Ub\(^{ert2cre}\) embryos that carried distinct Ephb4\(^{fl}\) alleles in which exon 1 was flanked by loxP sites (Supplemental Figure 2A, Table 1). To determine if hemorrhage was associated with the apoptotic death of EC, tissue sections from Ephb4\(^{fl/\beta}\) Ub\(^{ert2cre}\) embryos were stained with antibodies that detect the activated form of caspase 3. In both strains of Ephb4\(^{fl/\beta}\) Ub\(^{ert2cre}\) mice, apoptotic EC were readily identified in BV of skin (Supplemental Figure 1 C and D and Supplemental Figure 2 C and D). In all subsequent studies with Ephb4\(^{fl}\) mice, we used the exon 2-3 loxP-flanked allele.
We next examined if EC apoptosis and hemorrhage resulted from loss of EPHB4 within EC. For this purpose, we generated Ephb4\textsuperscript{fl/fl} embryos with an EC-specific Cdh5-ert2cre driver. Administration of TM to pregnant dams at E13.5 resulted in the same cutaneous hemorrhagic phenotype and near absence of LV associated with apoptotic death of EC when examined at E18.5 (Figure 1, Table 1). Therefore, embryonic vascular phenotypes observed upon induced global loss of EPHB4 are consequent to loss of EPHB4 in EC specifically.

Loss of EPHB4 in EC during developmental angiogenesis results in accumulation of collagen IV within the EC ER. Apoptotic death of induced RASA1-deficient EC during developmental angiogenesis is secondary to the inability of EC to export collagen IV for deposition in vascular basement membranes (28). To investigate if induced loss of EPHB4 in EC during developmental angiogenesis also resulted in intracellular accumulation of collagen IV in EC, tissue sections from E18.5 Ephb4\textsuperscript{fl/fl} \textit{U}\textsuperscript{ert2cre} and Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} embryos administered TM at E13.5 were stained with anti-collagen IV antibodies. Intracellular accumulation of collagen IV was readily apparent in EC of skin of Ephb4\textsuperscript{fl/fl} \textit{U}\textsuperscript{ert2cre} and Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} embryos but not in EC of corresponding littermate cre-negative embryos (Figure 2 A-D). Similarly, intracellular accumulation of collagen IV was observed in EC of TM-treated exon 1 loxP-flanked Ephb4\textsuperscript{fl/fl} \textit{U}\textsuperscript{ert2cre} embryos (Supplemental Figure 3). To determine the subcellular location of the intracellular collagen IV, sections were additionally stained with antibodies to identify different cell organelles. Within EC of Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} embryos, punctae of collagen IV were identified that were surrounded by rings of calnexin, a transmembrane resident ER protein (Figure 3). In addition, colocalization of collagen IV punctae with calreticulin, a luminal ER protein, was observed (Supplemental Figure 4). In contrast, no colocalization of collagen IV with
markers that identify the ER-Golgi intermediate compartment (ERGIC) (LMNA1), the Golgi (TGN46), or lysosomes (LAMP-1) was apparent (Supplemental Figure 4). Thus, loss of EPHB4 in EC during developmental angiogenesis leads to the accumulation of collagen IV within the EC ER.

Partial rescue of vascular phenotypes in induced EPHB4-deficient embryos by 4PBA. In induced RASA1-deficient embryos, collagen IV is retained within the EC ER because it is improperly folded. Evidence for this is derived from the observation that the small molecular chaperone, 4-phenylbutyric acid (4PBA) that promotes collagen IV folding, rescues EC collagen IV export, EC apoptosis and hemorrhage in induced RASA1-deficient embryos (28, 31, 32). To examine if 4PBA could also rescue vascular phenotypes in induced EPHB4-deficient embryos, 4PBA was administered to Ephb4fl/fl and Ephb4fl/fl Cdh5ert2cre embryos at the same time as TM at E13.5 and for each day of development thereafter until embryo harvest at E18.5. Cotreatment of embryos with 4PBA partially rescued hemorrhage as evidenced by the absence of hemorrhage or much reduced hemorrhage (mild hemorrhage) in most embryos (Figure 4, Table 1). Immunostaining of skin sections of embryos with mild hemorrhage revealed mostly intact BV and a normal number of LV (Figure 4 A and B). Apoptotic EC could not be identified in BV of these embryos and collagen IV export was mostly normal (Figure 4 C-F). These findings are consistent with the notion that impaired folding of collagen IV in EPHB4-deficient EC during developmental angiogenesis is responsible for EC apoptosis, reduced LV density and hemorrhage.

Partial rescue of vascular phenotypes in induced EPHB4-deficient embryos by 2,4PDCA. Induced loss of RASA1 during developmental angiogenesis results in an increased abundance of
collagen IV proline and lysine hydroxylases in EC (28). These enzymes belong to a family of enzymes known as 2-oxoglutarate (2OG)-dependent oxygenases (33). The increased abundance of hydroxylases is thought to result in over hydroxylation of collagen IV on prolines and lysines that could impact proper folding. Consistent with this possibility is the finding that drugs that inhibit 2OG-dependent oxygenases also rescue EC collagen IV export, EC apoptosis and hemorrhage resulting from loss of RASA1 during developmental angiogenesis (28). To examine if inhibition of 2OG-dependent oxygenases could also rescue vascular phenotypes in induced EPHB4-deficient embryos, we tested the effect of the 2OG-dependent oxygenase inhibitor, 2,4 pyridinedicarboxylic acid (2,4PDCA) (33). Similar to 4PBA treatment, administration of 2,4PDCA to Ephb4fl/fl Cdh5ert2cre embryos at the same time as TM at E13.5 and for all subsequent days up to embryo harvest at E18.5 partially rescued EC collagen IV export, EC apoptosis and hemorrhage (Figure 5 and Table 1). This finding provides evidence of a role for 2OG-dependent oxygenases in collagen IV misfolding in EPHB4-deficient EC and is further supportive of a functional link between RASA1 and EPHB4 in the regulation of vascular development.

EPHB4 functions as a negative-regulator of Ras-MAPK signaling in endothelial cells and an inhibitor of Ras-MAPK signaling ameliorates blood vascular phenotypes resulting from loss of EPHB4 during developmental angiogenesis. Since loss of EPHB4 and RASA1 during developmental angiogenesis resulted in the same vascular phenotypes, we next asked if EPHB4, like RASA1, functions as a negative regulator of Ras-MAPK signaling in EC in vivo, as has been reported in vitro (21, 22). To examine this, skin sections from Ephb4fl/f and Ephb4fl/fl Cdh5ert2cre E15.5 embryos treated with TM on E13.5 were stained with antibodies against
phosphorylated activated forms of MAPK. Constitutive activation of MAPK was observed in EC in the majority of skin BV in Ephb4fl/fl Cdh5ert2cre E15.5 embryos at E18.5, whereas very little MAPK activation was observed in EC in skin of Ephb4fl/fl embryos at this time (Figure 6 A and B). To confirm this finding, we examined MAPK activation by Western blotting of liver lysates of E18.5 Ephb4fl/fl and Ephb4fl/fl Cdh5ert2cre E15.5 embryos treated with TM on E13.5 (Figure 6 C). In these experiments, constitutive MAPK activation was consistently observed in the Ephb4fl/fl Cdh5ert2cre embryos.

We next asked if inhibition of Ras-MAPK signaling could rescue developmental EC phenotypes following loss of EPHB4. For this purpose, mice were administered the MEK inhibitor, AZD6244, at the same time as TM at E13.5 and on all subsequent days until embryo harvest at E18.5. AZD6244 completely rescued or reduced hemorrhage in most Ephb4fl/fl Cdh5ert2cre embryos (Figure 7 A and B and Table 1). In embryos with reduced hemorrhage, LV density in skin was restored, there were much fewer BV with apoptotic EC and EC export of collagen IV was mostly normal (Figure 7 A-F). These findings are in accord with the notion that dysregulated Ras-MAPK signaling in EC is responsible for the vascular phenotypes that result from loss of EPHB4 during developmental angiogenesis.

**EPHB4 is required for retinal angiogenesis in newborns.** With few exceptions, induced disruption of Rasa1 past E15.5 does not result in spontaneous BV abnormalities (16, 18, 29, 34). Collagens are considered some of the most stable proteins in the animal kingdom (35). Hence, a lack of requirement of RASA1 for maintenance of the blood vasculature post E15.5 could reflect a much-reduced need of EC to continue to engage in high-rate collagen IV synthesis to remain
attached to the basement membrane. However, it is expected that different forms of neoangiogenesis such as retinal angiogenesis in newborns would require de novo synthesis of collagen IV by EC for BV growth. Accordingly, we showed previously that RASA1 is required for retinal angiogenesis in newborns (28).

To investigate if EPHB4 is also required for retinal angiogenesis, Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} mice were administered TM at P1 and retinas were harvested at P6. Retinas were then examined by whole mount staining using IB4 lectin to identify BV. These analyses revealed a reduced density of BV in retinas from Ephb4^{fl/fl} Cdh5^{ert2cre} mice compared to Ephb4^{fl/fl} controls (Figure 8 A and B). In addition, the number of filopodia at the angiogenic front was reduced in the Ephb4^{fl/fl} Cdh5^{ert2cre} retinas (Figure 8 A and C). Collagen IV accumulation within EC of Ephb4^{fl/fl} Cdh5^{ert2cre} retinas was not readily observed. However, an increased number of collagen IV “empty selves” comprising of a collagen IV basement membrane without EC was apparent in Ephb4^{fl/fl} Cdh5^{ert2cre} retinas (Figure 8 D and E). This finding is consistent with increased loss of EC as a result of apoptosis.

**EPHB4 is required for pathological angiogenesis.** Pathological angiogenesis in response to tumor growth is another form of neoangiogenesis that is inhibited following induced loss of RASA1 in adult mice (28). To determine if pathological angiogenesis is also impaired in the absence of EPHB4, Ephb4^{fl/fl} and Ephb4^{fl/fl} Ub^{ert2cre} mice were administered TM and were subsequently injected subcutaneously with B16 melanoma cells. Growth of injected B16 melanoma cells is dependent upon host BV angiogenesis. As determined 13 d later, B16 tumor growth was substantially reduced in Ephb4^{fl/fl} Ub^{ert2cre} hosts compared to Ephb4^{fl/fl} hosts (Figure
9 A and B). Furthermore, reduced tumor growth was associated with a much-reduced BV density in tumor masses explanted from Ephb4−/− Ubert2cre hosts (Figure 9 C and D). Notably, administration of 4PBA to Ephb4−/− Ubert2cre hosts at the same time as TM and for all subsequent days up to the point of tumor harvest rescued tumor growth and this was associated with an increased BV density in tumors (Figure 9 A-D). These findings are consistent with the notion that impaired pathological angiogenesis in the absence of EPHB4 is consequent to EC retention of collagen IV.

Mice that express RASA1 binding-deficient catalytically active EPHB4 show normal vascular development. The similarities of vascular phenotype resulting from loss of EPHB4 and RASA1 during embryogenesis prompted us to examine if physical association between RASA1 and EPHB4 is necessary for normal developmental angiogenesis. RASA1 interacts with EPHB4 via SH2 domain-mediated recognition of two phosphorylated tyrosine residues, Y590 and Y596, present in the JM region that is highly conserved among Ephrin receptors (Figure 10 A) (23, 24). As shown in structural studies of EPHB2 and EPHA4, the JM region normally adopts a helical conformation that interacts with the kinase domain and restrains the kinase in an inactive state (25-27). However, phosphorylation of the analogous JM tyrosine residues in EPHB2 and EPHA4, disrupts the conformation of the JM region, releasing the kinase domain from autoinhibition resulting in a fully active kinase. With these considerations, simple mutation of Y590 and Y596 to F590 and F596 as a means of specifically disrupting RASA1 interaction with EPHB4 would not represent an informative approach with which to examine a putative role for physical interaction between EPHB4 and RASA1 in vascular development since these mutations would be expected to abrogate EPHB4 kinase activity. To confirm this, wild-type EPHB4 and
EPHB4 with Y590F and Y596F mutations were transfected into Cos-7 cells. Cells were then stimulated with Ephrin B2 ligand before immunoprecipitation of transfected EPHB4 followed by Western blotting to detect coimmunoprecipitated RASA1 as well as EPHB4 phosphotyrosine content as a measure of EPHB4 kinase activity. As expected, mutation of both EPHB4 tyrosines resulted in loss of physical interaction between EPHB4 and RASA1. However, the same mutations also resulted in a dead kinase devoid of an ability to mediate EPHB4 tyrosine phosphorylation (Figure 10 B and C). Therefore, to circumvent this problem and generate an EPHB4 receptor that is unable to engage RASA1 but retains kinase activity we introduced two additional mutations, P593G and P599G, into EPHB4 with Y590F and Y596F mutations to generate a quadruple Y590F/P593G/Y596F/P599G EPHB4 mutant (EPHB4 2YP). In EPHB2 and EPHA4, additional mutation to glycine of the first of these prolines in receptors that contain double tyrosine to phenylalanine mutations restores the kinase activity of these receptors, most likely because of a critical role of this proline residue in stabilizing the helical structure of the JM region necessary for its auto-inhibitory activity (27). Similarly, the second of these prolines is predicted to stabilize the helical structure of the JM region (27). In Cos-7 transfection experiments, we confirmed that EPHB4 2YP was unable to bind RASA1 yet retained kinase activity (Figure 10 B and C).

To examine the impact of EPHB4 2YP upon vascular development, we used CRISPR/Cas9 gene-targeting to generate an Ephb4^{2YP} allele in mice. Unexpectedly, at E10.5, homozygous Ephb4^{2YP/2YP} mice showed entirely normal vascular development and disrupted developmental angiogenesis was not evident as it is in constitutive EPHB4-deficient or RASA1-deficient mice (Figure 10 D, Table 1). By cross-breeding, we also generated littermate Ephb4^{fl/fl} and Ephb4^{fl/2YP}
embryos carrying \textit{U}b\textit{ert2Cre} transgenes. Administration of TM to pregnant dams at E13.5 resulted in EC accumulation of collagen IV and hemorrhage in \textit{Ephb4}^{fl/fl} \textit{U}b\textit{ert2Cre} embryos at E18.5 as noted before (Figure 11, Table 1). However, neither collagen IV accumulation nor hemorrhage was observed in \textit{Ephb4}^{fl/2YP} \textit{U}b\textit{ert2Cre} embryos at E18.5 (Figure 11, Table 1). These findings show that physical interaction between EPHB4 and RASA1 is not required for normal developmental angiogenesis.

\textit{Physical interaction between EPHB4 and RASA1 is not required for retinal or pathological angiogenesis.} Consistent with a lack of physical interaction between EPHB4 and RASA1 for developmental angiogenesis, homozygous \textit{Ephb4}^{2YP/2YP} mice survive to adulthood and do not show any spontaneous abnormalities. To examine if physical interaction is required for neonatal angiogenesis, we compared retinal angiogenesis in \textit{Ephb4}^{fl/fl}, \textit{Ephb4}^{fl/2YP} and \textit{Ephb4}^{2YP/2YP} mice. At P6, no differences in the percentage of EC coverage or the number of filopodia at the angiogenic front was apparent between strains (Figure 12 A-C). We also examined pathological angiogenesis in \textit{Ephb4}^{fl/fl}, \textit{Ephb4}^{fl/2YP} and \textit{Ephb4}^{2YP/2YP} mice using the B16 melanoma model. Growth of tumors was comparable between the different strains in this model (Figure 12 D and E). In addition, no differences in the density of BV within tumors was noted between the strains (Figure 12 F and G). We conclude that physical interaction between RASA1 and EPHB4 is not necessary for retinal or pathological angiogenesis.
Discussion

Accumulating evidence from human studies and animal models indicates that EPHB4 and RASA1 function in the same molecular signaling pathway to regulate blood and lymphatic vessel development and function. In humans, foremost is the finding that inherited inactivating mutations of \textit{EPHB4} and \textit{RASA1} both result in CM-AVM (2-4). In addition, inherited inactivating mutations of \textit{EPHB4} are responsible for the development of Vein of Galen Malformation in humans, a type of brain AVM that has also been described in CM-AVM (2, 3, 36-38). Inherited inactivating mutations in \textit{EPHB4} and \textit{RASA1} are also responsible for the development of lymphatic vascular abnormalities in man, including lymphedema, chylous ascites and chylothorax, lymphatic-related hydrops fetalis, central conducting lymphatic anomaly, and abnormal lymphatic flow (2, 3, 5-8, 17, 39, 40). In mice, constitutive disruption of \textit{Ephb4} and \textit{Rasa1} both result in impaired development of the cardiovascular system and death at E10.5.

Furthermore, additional gene-targeting and other studies in mice have revealed a required role for EPHB4 and RASA1 in the development of LV valves, lymphovenous valves, and venous valves and in the maintenance of the adult lymphatic vasculature (16-20, 29, 41).

In this study, we provide further evidence of a functional link between EPHB4 and RASA1 as regulators of the blood vasculature. Specifically, we show that EPHB4, like RASA1, is required for each of developmental angiogenesis in mid to late gestation, retinal angiogenesis in newborns and pathological angiogenesis in adults. With regards, developmental angiogenesis, most significantly, we show that loss of EPHB4 in EC leads to collagen IV accumulation within the ER and EC apoptotic death. Similarly, loss of RASA1 in EC during developmental angiogenesis results in accumulation of collagen IV in the ER of EC and EC apoptotic death (28). In both
cases, an inability to export collagen IV for deposition in the nascent basement membrane is mostly likely a consequence of collagen IV misfolding. This is supported by the observation that 4PBA, a small molecular chaperone that facilitates collagen IV folding promotes collagen IV export from both EPHB4-deficient and RASA1-deficient EC. Moreover, 4PBA rescues EPHB4-deficient and RASA1-deficient EC from apoptotic death, thus providing evidence that an inability to export collagen IV is the cause of EC death. Accumulation of collagen IV within the ER could result in EC apoptotic death in two distinct ways. First, the misfolded protein could trigger an unfolded protein response (UPR) that if unable to effectively promote collagen IV folding would lead to apoptosis (31, 42-45). In support of this mechanism, we observed increased abundance of the BIP ER stress protein in EC of induced EPHB4-deficient embryos (Supplemental Figure 5). Another possibility is that the paucity of collagen IV in basement membranes following loss of EPHB4 would lead to EC detachment and a default form of apoptosis known as anoikis (46). In our earlier studies of induced RASA1-deficient embryos, both mechanisms were shown to contribute to EC apoptotic death (28).

Previous in vitro studies indicated that in EC specifically, EPHB4 functions to inhibit Ras-MAPK signaling triggered through other GFR such as VEGFR and Tie2 (21, 22). However, whether EPHB4 performs a similar function in EC in vivo has not been demonstrated beforehand to our knowledge. Therefore, the finding in this study that MAPK are constitutively active in EC of induced EPHB4-deficient embryos provides the first demonstration of a role for EPHB4 as an inhibitor of Ras-MAPK signaling in EC in vivo. As in induced RASA1-deficient embryos, a MEK inhibitor was able to partially rescue developmental vascular phenotypes in induced EPHB4-deficient embryos. This finding is consistent with a central role for dysregulated Ras-
MAPK signaling in EC collagen IV accumulation and downstream sequelae. In the absence of RASA1, dysregulated Ras-MAPK signaling is associated with increased expression of proline and lysine hydroxylases in the ER that are predicted to cause excessive hydroxylation of collagen IV that could account for collagen IV misfolding (28). We propose that a similar Ras-MAPK-driven increased abundance of collagen IV-modifying proline and lysine hydroxylases is responsible for collagen IV misfolding and accumulation in the absence of EPHB4. Hence, 2,4PDCA, a broad inhibitor of the family of 2OG-dependent oxygenases to which collagen IV proline and lysine hydroxylases belong, also promotes collagen IV export in induced EPHB4-deficient embryos as it does in induced RASA1-deficient embryos.

Further suggestive of a functional relationship between EPHB4 and RASA1 is the known physical association between RASA1 and EPHB4 mediated by RASA1 SH2 domain recognition of two phosphorylated tyrosine residues located in the JM segment of EPHB4 (22-24). To address the significance of this interaction for vascular development and function, we generated knockin mice that expressed a 2YP mutant of EPHB4 that is unable to bind RASA1 but retains PTK activity. Surprisingly, homozygous EPHB4 2YP mice showed entirely normal developmental, neonatal and pathological angiogenesis. Thus, a model in which EPHB4 functions as a docking receptor to recruit RASA1 to the plasma membrane whereupon it might become juxtaposed to Ras permitting Ras inactivation is likely incorrect. How EPHB4 and RASA1 cooperate to negatively regulate Ras in EC remains to be determined. In CM-AVM1, missense RASA1 mutations have been reported that are located mostly within the pleckstrin homology (PH) and C2 homology domains of RASA1 (2, 3). These domains coordinate binding to phospholipids suggesting that membrane targeting of RASA1 may be mediated primarily
through lipid recognition rather than receptor interaction (47). Potentially, EPHB4 maybe uniquely involved in the generation of phospholipid ligands of RASA1.

A requirement for EPHB4 and RASA1 for BV angiogenesis but not maintenance of BV in adults is mostly likely explained by the stability of collagens (35). In this regard, sufficient collagen IV maybe deposited in basement membranes during developmental angiogenesis that obviates a requirement thereafter for BEC to continue to engage in high-rate synthesis of collagen IV to remain attached to vessel walls. Accordingly, loss of EPHB4 or RASA1 would not affect BV past the stage of developmental angiogenesis except where de novo synthesis of collagen IV is required, e.g. retinal angiogenesis and pathological angiogenesis. The finding that RASA1 and EPHB4 are required for the maintenance of venous valves and LV valves in adults is also consistent with this hypothesis (16, 20, 28). Valvular EC would be subject to higher shear stress forces than lumenal wall EC that might necessitate their continued high-rate synthesis of collagen IV for them to remain attached to valve leaflets (48, 49).

The findings reported herein for EPHB4 and RASA1 previously are relevant to an understanding of the pathogenesis of fast flow lesions in CM-AVM. One possibility is that somatic second hit mutation of \textit{EPHB4} or \textit{RASA1} in patients with germline \textit{EPHB4} or \textit{RASA1} mutations respectively occurs in isolated EC during vasculogenesis such that the majority EC within a single vessel within a primitive vascular plexus are EPHB4 or RASA1 null. As such, remodeling of that vessel during developmental angiogenesis would not be possible since this would be dependent upon an ability of EC within that vessel to deposit collagen IV in a nascent basement membrane. The result would be a direct connection between arteries and veins without an
intervening capillary bed that constitutes the quintessential feature of an AVM. However, another possibility is that EPHB4 or RASA1 null EC generated by second hit mutation during vasculogenesis or developmental angiogenesis are rescued from apoptosis by neighboring EC that have not acquired second hit mutations and continue to express EPHB4 or RASA1. These neighboring EC could provide sufficient local collagen IV in basement membranes that could rescue adjacent EPHB4- or RASA1-deficient EC from anoikis. In this model, dysregulated Ras-MAPK signaling in EPHB4 or RASA1 null EC could drive AVM formation through a distinct mechanism. Which of these models is correct will have bearing upon the ability of drugs that inhibit Ras-MAPK signaling or promote collagen IV folding to prevent the development of and to treat vascular lesions in this disease.
Methods

Mice. Mice carrying exon 2-exon 3 floxed alleles of Ephb4 and exon 1 floxed alleles of Ephb4 have been described (17, 50). Mice were crossed with Ub\textsuperscript{ert2cre} and Cdh\textsuperscript{5ert2cre} transgenic mice to generate littermate Ephb4\textsuperscript{fl/fl} progeny with and without either cre transgene. Mice were on mixed 129S6/SvEv X C57BL/6 genetic backgrounds.

Mice carrying a knockin Ephb4 \textit{Y590F/P593G/Y596F/P599G} (Ephb4 2YP) allele (numbering based on transcript variant 2, NM_010144.6; isoform b, NP_034274.4) were generated using CRISPR/Cas9 technology. Exon 11 of Ensembl gene model Transcript Ephb4-203 (ENSMUST00000111055.9) includes codons Y599, P602, U605, and P608. The CRISPOR algorithm (51) was used to identify specific single guide RNAs (sgRNA). sgRNA predicted to cut the chromosome near codon 608 were tested to determine if they cause chromosome breaks. sgRNAs were chemically synthesized with phosphorothioate modifications by MilliporeSigma.com (52, 53). Enhanced specificity Streptomyces pyogenes Cas9 endonuclease protein (ESPCAS9) (54) was obtained from MilliporeSigma. sgRNAs (30 ng/ul) was complexed with ESPCAS9 (50 ng/ul) and individually tested to determine if the ribonucleoprotein (RNP) complexes cause chromosome breaks in mouse zygotes. RNPs were microinjected into fertilized mouse eggs. Eggs were placed in culture until they developed into blastocysts. DNA was extracted from individual blastocysts for analysis. PCR with primers spanning the predicted cut site was used to generate amplicons for Sanger sequencing. The process is essentially as described (55). sgRNA candidates were tested with Forward primer 5’ GTATGACTCAGTTTGCCTTTTGCTTCTTT 3’ and Reverse Primer 5’TTTTCAGTAATTAGTTCTCTCCTCCAGC 3’, 788bp amplicon. Sequence chromatograms of amplicons from individual blastocysts were evaluated to determine if small
insertions/deletions caused by non-homologous endjoining (NHEJ) repair of chromosome breaks were present. sgRNA C248G2 was found to induce chromosome breaks. It targets the sequence 5’ TACTTACGAAGACCCTAATG (PAM=AGG) 3’ (CFD score of 86) (56).

After determining that C248G2 caused chromosome breaks, RNPs were combined with 10ng/ul single stranded oligonucleotide DNA donor (IDTDNA.com). The sequence of the single stranded oligonucleotide DNA donor was: CAGAGGAATTTACTTCTGGTTAATGGG-CTCCTGTGTGACTCCTTAGGTACCAAGGTCTcATTGAtggcTTTTACTTtCGAAGACggeAATGAGGCAGTGAGGGAATTGCCAAAGAGATCGATGTCTCCTATGTCAAGATTGAAGAG (lower case letters indicate coding changes to the wild type exon, exon 11 is underlined).

The CRISPR reagents were microinjected into fertilized mouse eggs produced by mating superovulated B6SJLF1 female mice (Jackson Laboratory stock no. 100012) to B6SJLF1 male mice as described (57). CRISPR/Cas9 microinjection of 325 B6SJLF1 zygotes produced 79 potential founder mice. Five generation zero founder (G0) pups were identified by Sanger sequencing of amplicons spanning exon 11. G0 founders were mated with wild type C57BL/6J mice to obtain germline transmission of the Eph4b mutant. Following germline transmission, correct targeting and an absence of incorrect targeting at several other possible loci identified by CRISPOR (Epha2, Epha4, Ephb1, Ephb2, Nfat5, PYKFyve2, and Plxna2) was confirmed by Sanger sequencing (58). Heterozygote Ephb4^{2YPG} mice were crossed with Ephb4^fl exon 2-3 mice and Ubyert2cre mice for studies.
Developmental angiogenesis. To induce embryonic disruption of EphB4, pregnant mice carrying embryos with Ephb4\textsuperscript{fl} alleles were given 3 i.p. injections of TM (Sigma; 0.05 mg/g body weight per injection, dissolved in corn oil) on consecutive days. The MEK inhibitor, AZD6244 (Selleckchem; 0.05 mg/g body weight per injection), the chemical chaperone, 4PBA (Sigma; 0.25 mg/g body weight per injection), and the 2OG dependent oxygenase inhibitor, 2,4PDCA (Sigma; 0.1 mg/g body weight per injection) were injected i.p. into mice at the same time as TM and on all subsequent days of gestation until embryo harvest. Embryos were fixed in 3.75% formaldehyde overnight and embedded in paraffin. Five micrometer sections were dehydrated and antigen retrieval was performed with a Diva de-cloaking kit (Biocare Medical). Sections were blocked in PBS/10% donkey serum/0.3% Triton-X100 and incubated overnight with the following primary antibodies in PBS in 10% donkey serum: rat anti-CD31 (SZ31, Dianova), rabbit anti-active caspase 3 (AF835, R&D Systems), goat anti-collagen IV (1340-01, Southern Biotech), rabbit anti-BIP (3177, Cell Signaling Technology), rabbit anti-TGN46 (ab16059, Abcam), rabbit anti-LMNA1 (ab125006, Abcam), rabbit anti-calnexin (ab22595, Abcam), rabbit anti-LYVE-1 (ab14917, Abcam), rabbit anti-calreticulin (D3E6, Cell Signaling Technology), rat anti-LAMP1 (1D4B, Thermo Scientific), and rabbit anti-phospho-ERK (D13.14.4E, Abcam). Secondary antibodies used were species-specific anti-immunoglobulin donkey F(ab)\textsubscript{2} fragments coupled to Alexa Fluor 488, 594 or 647 (Jackson Immunoresearch) and were incubated with tissues in PBS for 2 hours. Sections were stained with Hoechst (Invitrogen) to identify nuclei before mounting and viewing on a Leica SP5 X confocal microscope (Leica Microsystems).

For whole mount staining of E10.5 embryos from Ephb4\textsuperscript{fl}2YP intercrosses, embryos were fixed in 1% paraformaldehyde for 1 hour, blocked in 3% donkey serum/0.3% Triton X-100 in PBS
overnight and stained with anti-CD31 antibody (MEC13.3, eBioscience) in 0.3% Triton X-100 in PBS overnight. Embryos were then incubated with Alexa Flour 488 donkey anti-rat Ig overnight before mounting and viewing on a BX60 upright fluorescence microscope (Nikon).

Retinal angiogenesis. Newborn pups were injected with TM (0.05 mg/g body weight per injection) on two consecutive days from P1-P2 and retinas were harvested at P6. Retinas from pups of Ephb4^fl/2YP intercrosses were also harvested at P6. Retinas were fixed in 4% paraformaldehyde for 2 hours, were blocked in PBS/10% donkey serum and incubated overnight with IB4-FITC (Sigma) and goat anti-collagen IV in PBS/10% donkey serum. Retinas were subsequently incubated for 2 hours with a secondary anti-goat donkey F(ab')2 coupled to Alexa Fluor 594 in PBS to detect collagen IV. Whole mounts were viewed on a BX60 upright fluorescence microscope. The percentage EC coverage and number of empty sleeves per field was determined in randomly chosen 50 µm x 50 µm areas behind the angiogenic front in a region between an artery and a vein. The number of filopodia at the angiogenic front was determined for randomly chosen 300 µm regions.

Tumor angiogenesis. TM was administered to male and female mice at 2 months of age. After 2 weeks, mice were injected s.c. in the flank with 0.75 x 10^6 B16F10 cells (provided by Weiping Zou, Michigan Medicine) suspended in 100 µl matrigel (Corning). 4PBA was administered to some mice at the same time as tumor cells and for each day until tumor harvest at day 13 (0.4 mg/g body weight per injection). Tumors were fixed in 4% paraformaldehyde overnight and 5 µm sections were stained with Hoechst and antibodies against CD31. Sections were viewed on a
BX60 upright fluorescence microscope or a Leica SP5 X confocal microscope. BV density was assessed within random 200 µm x 200 µm regions of tumor sections.

*EPHB4 physical association with RASA1 and EPHB4 kinase activity.* c-myc-tagged murine Ephb4 cDNA in pCMV6 was purchased from Origene. Double Y590F/Y596F (2Y) and P593G/P599G (2P) and quadruple Y590F/P593G/Y596F/P599G (2YP) EPHB4 mutations were introduced by site-directed mutagenesis using a QuickChange Lightning Multi Site-Directed Mutagenesis kit (Agilent) according to the manufacturer’s instructions. Cos-7 cells (ATCC) at 80% confluency in 10 cm culture dishes were transfected with 10 µg of plasmids using Lipofectamine (Thermo) in Opti-MEM medium (Thermo). After 48 hours, cells were serum-starved in DMEM medium for 16 hours before stimulation with Ephrin B2 Fc chimeric protein (1 µg/ml; R&D Systems) for 15 minutes. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1mM EDTA) and lysates were incubated with anti-c-myc antibody (9E10; Sigma) overnight followed by rotation with protein A/G PLUS-agarose beads (Santa Cruz) for 2 hours. After washing, beads were boiled in 1X SDS sample buffer and eluates were run on 10% SDS-PAGE gels. Co-immunoprecipitated RASA1 was detected by Western blotting using an anti-RASA1 antibody (B4F8; Santa Cruz) and secondary goat anti-mouse IgG linked to horse radish peroxidase (GAM-HRP; Cell Signaling Technology). Phosphotyrosine content of immunoprecipitated EPHB4 was determined by Western blotting using an anti-phosphotyrosine antibody (4G10; Sigma) and GAM-HRP. Equivalent transfection of Cos-7 cells was shown by Western blotting of immunoprecipitates and whole cell lysates using 9E10 and GAM-HRP.
Western blot analysis of MAPK activation. Fetal liver was harvested from E18.5 embryos and whole cell lysates were prepared in RIPA buffer. MAPK activation was determined by Western blotting using an anti-phospho-ERK antibody (D13.14.E, Cell Signaling Technology) and GAM-HRP. Total ERK content was determined by Western blotting using an anti-ERK antibody (137F5, Cell Signaling Technology) and goat anti-rabbit IgG-HRP (Cell Signaling Technology).

Statistics. For quantitative assessment of embryonic vascular development, replicate determinations were obtained from at least 2 embryos for each genotype and condition. For quantitative assessment of retina angiogenesis, replicate determinations were obtained from all examined retinas (Figure 8) or 6 examined retinas of each genotype (figure 12). For quantitative assessment of tumor angiogenesis, replicate determinations were obtained from all examined tumors (Figures 8 and 12). Statistical analysis was performed using Mann Whittney non-parametric tests, Student’s 2-sample t tests and One way ANOVA tests with Tukey correction as indicated. A P value less than 0.05 was considered significant.

Study approval. All experiments performed with mice were in compliance with University of Michigan guidelines and were approved by the university committee on the use and care of animals.
Author contributions

DC and PDK contributed to the design of studies. JW, MHV and TM provided Ephb4 conditional mouse models, EDH and TLS assisted with the generation of Ephb4 knockin mice. DC performed experiments. The manuscript was written by DC and PDK.

Acknowledgements

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References


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<sup>a</sup> ex2-3, loxP sites upstream of exon 2 and downstream of exon 3; ex1, loxP sites flank exon 1

<sup>b</sup> Administered at same time as TM and everyday thereafter until E18.5

<sup>c</sup> Percent of hemorrhaged skin categorized as none (0%), mild (<10%), moderate (25-75%), or severe (>75%)

<sup>d</sup> For mice in littermate groups 9 and 10 the fl ex2-3 allele was used

<sup>e</sup> NS, not shown
Figure legends

Figure 1. Hemorrhage, EC apoptosis and paucity of lymphatic vessels following induced EC-specific disruption of Ephb4 during developmental angiogenesis. TM was administered to Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos at E13.5 and embryos were harvested at E18.5. (A) Images at left show extensive cutaneous hemorrhage and an edematous appearance of Ephb4^{fl/fl} Cdh5^{ert2cre} embryos. H&E staining of skin sections confirmed vascular hemorrhage and combined anti-CD31 and anti-LYVE-1 antibody staining of sections revealed a reduced number of intact CD31^{lo}LYVE-1+ initial LV in Ephb4^{fl/fl} Cdh5^{ert2cre} embryos (shown with arrowheads in images of Ephb4^{fl/fl} sections). (B) Plot shows the number of identified CD31^{lo}LYVE-1+ LV in randomly selected 200 µm x 200 µm areas of skin. Bars show the mean +/- 1 SEM of LV/field (Ephb4^{fl/fl}, n=5; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=10). (C) Skin sections were stained with anti-CD31 and anti-activated caspase 3 antibodies and Hoechst to identify apoptotic EC. Examples of activated caspase 3-positive EC with fragmented nuclei in images of Ephb4^{fl/fl} Cdh5^{ert2cre} sections are indicated with arrowheads. (D) Plot shows the percentage of apoptotic EC in individual CD31+ BV in skin selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage apoptotic EC per vessel (Ephb4^{fl/fl}, n=14; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=20). **, P<0.01; ***, P<0.001; Mann Whittney test.
Figure 2

A

CD31

Collagen IV

Merge/Hoechst

Ephb4^{fl/fl}

Ephb4^{fl/fl}_\text{ert2cre}

50 \mu m

B

% EC per vessel with trapped collagen IV

Ephb4^{fl/fl}

Ephb4^{fl/fl}_\text{ert2cre}

D

CD31

Collagen IV

Merge/Hoechst

Ephb4^{fl/fl}

Ephb4^{fl/fl}_\text{ert2cre}

Cdh5

15 \mu m

% EC per vessel with trapped collagen IV

Ephb4^{fl/fl}

Ephb4^{fl/fl}_\text{ert2cre}

Cdh5

E

E

E

E

E

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Figure 2. Collagen IV accumulation in EC of induced EPHB4-deficient embryos. TM was administered to Ephb4^{fl/fl} Ubert2cre, Ephb4^{fl/fl} Cdh5^{ert2cre} and corresponding littermate Ephb4^{fl/fl} embryos at E13.5. Embryos were harvested at E18.5 and skin sections were stained with anti-CD31 and anti-collagen IV antibodies and Hoechst. Note intracellular accumulation of collagen IV in EC of Ephb4^{fl/fl} Ubert2cre embryos (A) and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos (C) (examples highlighted with arrowheads) and relative paucity of collagen IV in basement membranes (asterisks). E, erythrocyte. (B and D) Plots shows the percentage of EC with intracellular collagen IV punctae in individual CD31+ BV in skin of embryos selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage EC with collagen IV accumulation (B, Ephb4^{fl/fl}, n=13; Ephb4^{fl/fl} Ubert2cre, n=20), (C, Ephb4^{fl/fl}, n=13; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=20). ***, P<0.0001; Mann Whitney test.
Figure 3

Collagen IV  Calnexin  Merge/Hoechst

Ephb4 flox/flox  5 µm  5 µm  5 µm

Ephb4 flox/flox; Cdh5 er2cre

Ephb4 flox/flox  5 µm  5 µm  5 µm

5 µm
Figure 3. Collagen IV is retained within the ER of induced EPHB4-deficient EC during developmental angiogenesis. TM was administered to Ephb4<sup>fl/fl</sup> and Ephb4<sup>fl/fl</sup> Cdh5<sup>ert2cre</sup> embryos at E13.5 and embryos were harvested at E18.5. Skin sections were stained with anti-collagen IV antibodies and antibodies against the calnexin to identify the ER. Representative images of individual BV are shown. Note collagen IV punctae surrounded by rings of calnexin in Ephb4<sup>fl/fl</sup> Cdh5<sup>ert2cre</sup> embryos (asterisks).
Figure 4

A

H&E

CD31

LYVE-1

Merge/Hoechst

Ephb4^{fl/fl} Cdh5^{ert2cre} + 4PBA

Ephb4^{fl/fl} + 4PBA

B

No. LV/field

C

CD31

Activated caspase 3

Merge/Hoechst

Ephb4^{fl/fl} + 4PBA

Ephb4^{fl/fl} Cdh5^{ert2cre} + 4PBA

D

% Apoptotic ECvessel

Ephb4^{fl/fl} Cdh5^{ert2cre} + 4PBA

Ephb4^{fl/fl} + 4PBA

F

% EC per vessel with trapped collagen IV

Ephb4^{fl/fl} Cdh5^{ert2cre} + 4PBA

Ephb4^{fl/fl} + 4PBA

ns

ns

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**Figure 4.** Partial rescue of developmental angiogenesis in induced EPHB4-deficient mice by 4PBA. TM and 4PBA were administered to Ephb4$^{0/0}$ and Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryos at E13.5 and 4PBA was administered on each subsequent day until embryo harvest at E18.5. (A) At left are shown images of whole embryos. The Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryo is categorized to have mild cutaneous hemorrhage (see Table 1) confirmed by H&E staining of skin sections which revealed a lack of hemorrhage in most areas. Staining of skin sections with anti-CD31 and anti-LYVE-1 antibodies revealed an intact lymphatic vasculature in TM + 4PBA-treated Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryos with mild hemorrhage. (B) Plot shows the number of identified CD31$^+$LYVE-1$^+$ LV in randomly selected 200 μm x 200 μm areas of skin of Ephb4$^{0/0}$ embryos and Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryos with mild hemorrhage treated with TM and 4PBA. For comparison, data from Ephb4$^{0/0}$ and Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryos treated with TM alone is also shown (Figure 1). Bars show the mean +/- 1 SEM of LV/field (Ephb4$^{0/0}$ TM alone, n=5; Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ TM alone, n=10; Ephb4$^{0/0}$ TM + 4PBA, n=8; Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ TM + 4PBA, n=16). (C) Skin sections were stained with anti-CD31 and anti-activated caspase 3 antibodies and Hoechst to identify apoptotic EC. (D) Plot shows the percentage of apoptotic EC in individual CD31$^+$ BV in skin of TM +4PBA-treated Ephb4$^{0/0}$ embryos and Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryos with mild hemorrhage and TM alone-treated Ephb4$^{0/0}$ and Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ embryos (Figure 1) selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage apoptotic EC per vessel (Ephb4$^{0/0}$ TM alone, n=14; Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ TM alone, n=20; Ephb4$^{0/0}$ TM + 4PBA, n=11; Ephb4$^{0/0}$ Cdh5$^{aret2cre}$ TM + 4PBA, n=42). (E) Skin sections were stained with anti-CD31 and anti-collagen IV antibodies and Hoechst to determine the distribution of collagen IV in BV. (F) Plot shows the percentage of EC with intracellular collagen IV punctae in individual CD31$^+$ BV in skin of TM +4PBA-treated
Ephb4\textsuperscript{fl/fl} embryos and Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} embryos with mild hemorrhage and TM alone-treated Ephb4\textsuperscript{fl/fl} and Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} embryos (Figure 1) selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage EC with collagen accumulation (Ephb4\textsuperscript{fl/fl} TM alone, n=13; Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} TM alone, n=20; Ephb4\textsuperscript{fl/fl} TM + 4PBA, n=19; Ephb4\textsuperscript{fl/fl} Cdh5\textsuperscript{ert2cre} TM + 4PBA, n=38). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant; One way ANOVA with Tukey.
Figure 5. Partial rescue of developmental angiogenesis in induced EPHB4-deficient mice by 2,4PDCA. TM and 2,4PDCA were administered to Ephb4<sup>fl/fl</sup> and Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos at E13.5 and 2,4PDCA was administered on each subsequent day until embryo harvest at E18.5.

(A) At left are shown images of whole embryos. The Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryo is categorized to have mild cutaneous hemorrhage (see Table 1) confirmed by H&E staining of skin sections. Staining of skin sections with anti-CD31 and anti-LYVE-1 antibodies revealed an intact lymphatic vasculature in TM + 2,4PDCA-treated Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos with mild hemorrhage. (B) Plot shows the number of identified CD31<sup>lo</sup>LYVE-1<sup>+</sup> LV in randomly selected 200 µm x 200 µm areas of skin of Ephb4<sup>fl/fl</sup> embryos and Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos with mild hemorrhage treated with TM and 2,4PDCA. For comparison, data from Ephb4<sup>fl/fl</sup> and Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos treated with TM alone is also shown (Figure 1). Bars show the mean +/- 1 SEM of LV/field (Ephb4<sup>fl/fl</sup> TM alone, n=5; Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> TM alone, n=10; Ephb4<sup>fl/fl</sup> TM + 2,4PDCA, n=5; Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> TM + 2,4PDCA, n=15). (C) Skin sections were stained with anti-CD31 and anti-activated caspase 3 antibodies and Hoechst to identify apoptotic EC. (D) Plot shows the percentage of apoptotic EC in individual CD31<sup>+</sup> BV in skin of TM +2,4PDCA-treated Ephb4<sup>fl/fl</sup> embryos and Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos with mild hemorrhage and TM alone-treated Ephb4<sup>fl/fl</sup> and Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos (Figure 1) selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage apoptotic EC per vessel (Ephb4<sup>fl/fl</sup> TM alone, n=14; Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> TM alone, n=20; Ephb4<sup>fl/fl</sup> TM + 2,4PDCA, n=16; Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> TM + 2,4PDCA, n=36). (E) Skin sections were stained with anti-CD31 and anti-collagen IV antibodies and Hoechst to determine the distribution of collagen IV in BV. (F) Plot shows the percentage of EC with intracellular collagen IV punctae in individual CD31<sup>+</sup> BV in skin of TM +2,4PDCA-treated Ephb4<sup>fl/fl</sup> embryos and Ephb4<sup>fl/fl</sup> Cdhs<sup>ert2cre</sup> embryos with mild-
hemorrhage and TM alone-treated Ephb4fl/fl and Ephb4fl/fl Cdh5ert2cre embryos (Figure 1) selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage EC with collagen accumulation (Ephb4fl/fl TM alone, n=13; Ephb4fl/fl Cdh5ert2cre TM alone, n=20; Ephb4fl/fl TM + 2,4PDCA, n=19; Ephb4fl/fl Cdh5ert2cre TM + 2,4PDCA, n=36). *, P<0.05; ***, P<0.001; ****, P<0.0001; ns, not significant; One way ANOVA with Tukey.
Figure 6. Activation of MAPK in EPHB4-deficient EC during developmental angiogenesis.

(A) TM was administered to Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos at E13.5 and embryos were harvested at E18.5. (A) Skin sections were stained with anti-CD31 and anti-phospho-ERK MAPK antibodies (pERK) and Hoechst. Note strong activation of MAPK in EC of Ephb4^{fl/fl} Cdh5^{ert2cre} embryos. (B) Plot shows the percentage of pERK+ EC per BV identified in randomly selected areas of skin. Bars show the mean +/- 1 SEM of percentage pERK+ EC per vessel (Ephb4^{fl/fl}, n=11; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=20). ****, P<0.0001, Mann-Whitney test. (C) Liver tissue from individual embryos was analyzed by Western blotting using pERK antibodies. Note constitutive activation of MAPK in Ephb4^{fl/fl} Cdh5^{ert2cre} liver samples.
Figure 7. Partial rescue of developmental angiogenesis in induced EPHB4-deficient mice by AZD6244. TM and AZD6244 were administered to Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos at E13.5 and AZD6244 was administered on each subsequent day until embryo harvest at E18.5.

(A) At left are shown images of whole embryos. The Ephb4^{fl/fl} Cdh5^{ert2cre} embryo is categorized to have mild cutaneous hemorrhage (see Table 1) confirmed by H&E staining of skin sections which revealed a lack of hemorrhage in most areas. Staining of skin sections with anti-CD31 and anti-LYVE-1 antibodies revealed an intact lymphatic vasculature in TM + AZD6244-treated Ephb4^{fl/fl} Cdh5^{ert2cre} embryos with mild hemorrhage. (B) Plot shows the number of identified CD31^{lo}LYVE-1+ LV in randomly selected 200 µm x 200 µm areas of skin of Ephb4^{fl/fl} embryos and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos with mild hemorrhage treated with TM and AZD6244. For comparison, data from Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos treated with TM alone is also shown (Figure 1). Bars show the mean +/- 1 SEM of LV/field (Ephb4^{fl/fl} TM alone, n=5; Ephb4^{fl/fl} Cdh5^{ert2cre} TM alone, n=10; Ephb4^{fl/fl} TM + AZD6244, n=8; Ephb4^{fl/fl} Cdh5^{ert2cre} TM + AZD6244, n=18). (C) Skin sections were stained with anti-CD31 and anti-activated caspase 3 antibodies and Hoechst to identify apoptotic EC. (D) Plot shows the percentage of apoptotic EC in individual CD31+ BV in skin of TM +AZD6244-treated Ephb4^{fl/fl} embryos and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos with mild hemorrhage and TM alone-treated Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos (Figure 1) selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage apoptotic EC per vessel (Ephb4^{fl/fl} TM alone, n=14; Ephb4^{fl/fl} Cdh5^{ert2cre} TM alone, n=20; Ephb4^{fl/fl} TM + AZD6244, n=16; Ephb4^{fl/fl} Cdh5^{ert2cre} TM + AZD6244, n=37). (E) Skin sections were stained with anti-CD31 and anti-collagen IV antibodies and Hoechst to determine the distribution of collagen IV in BV. (F) Plot shows the percentage of EC with intracellular collagen IV punctae in individual CD31+ BV in skin of TM +AZD6244-
treated Ephb4^{fl/fl} embryos and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos with mild hemorrhage and TM alone-treated Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} embryos (Figure 1) selected from multiple randomly chosen areas. Bars show the mean +/- 1 SEM of percentage EC with collagen accumulation (Ephb4^{fl/fl} TM alone, n=13; Ephb4^{fl/fl} Cdh5^{ert2cre} TM alone, n=20; Ephb4^{fl/fl} TM + 4PBA, n=19; Ephb4^{fl/fl} Cdh5^{ert2cre} TM + 4PBA alone, n=44). ***, P<0.001; ****, P<0.0001; ns, not significant; One way ANOVA with Tukey.
Figure 8. Impaired retinal angiogenesis in induced EC-specific EPHB4-deficient mice. TM was administered to Ephb4^{fl/fl} and Ephb4^{fl/fl} Cdh5^{ert2cre} mice at P1 and P2. Retinas were harvested at P6 and stained with isolectin B4 (IB4) to identify BV and anti-collagen-IV antibodies. (A) Representative low power images of IB4 staining are shown at top. Representative higher power images of IB4 staining at the angiogenic front are shown at bottom. Asterisks indicate filopodia. (B) Plot shows the percentage coverage of retinas with EC. Bars show the mean +/- 1 SEM of percentage EC coverage of individual retinas (Ephb4^{fl/fl}, n=7; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=7). (C) Plot shows the number of filopodia per 300 µm of angiogenic front that were randomly selected. Bars show the mean +/- 1 SEM of filopodia (Ephb4^{fl/fl}, n=10; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=11). (D) Shown are representative images of IB4 and anti-collagen IV staining. Empty collagen sleeves are indicated with asterisks. (E) Plot show the number of empty sleeves in randomly selected 200 µm x 200 µm areas of retinas. Bars show the mean +/- 1 SEM of empty sleeves (Ephb4^{fl/fl}, n=16; Ephb4^{fl/fl} Cdh5^{ert2cre}, n=16). ***, P<0.001; ****, P<0.0001; Student’s 2-sample t-test.
Figure 9. Reduced B16 melanoma growth in adult induced EPHB4-deficient mice associated with impaired tumor angiogenesis. Adult TM-treated Ephb4fl/fl and Ephb4fl/fl Ubert2cre mice were injected in flanks with B16 melanoma cells and tumors were harvested 13 days later. Some mice received 4PBA at the same as the tumor and all on all subsequent days until tumor harvest. (A) Representative images of explanted tumors. (B) Plot shows tumor weights at day 13. Bars represent mean +/- 1 SEM tumor weight (Ephb4fl/fl TM alone, n=14; Ephb4fl/fl Ubert2cre TM alone, n=18; Ephb4fl/fl TM + 4PBA, n=12; Ephb4fl/fl Ubert2cre TM + 4PBA, n=18). (C) Sections of explanted tumors were stained with anti-CD31 antibodies to identify BV. Shown are representative images. (D) Plot shows percentage BV coverage of randomly selected 200 μm x 200 μm areas of tumors. Bars represent mean +/- 1 SEM percentage BV coverage (Ephb4fl/fl TM alone, n=26; Ephb4fl/fl Ubert2cre TM alone, n=27; Ephb4fl/fl TM + 4PBA, n=18; Ephb4fl/fl Ubert2cre TM + 4PBA, n=35). *, P<0.05; **, P<0.01; ****, P<0.0001; ns, not significant; One way ANOVA with Tukey.
**Figure 10. Normal vascular development in homozygous EPHB4 2YP mice.** (A) At left is a schematic representation of EPHB4 showing the ligand-binding domain (LBD), fibronectin domains (FN), juxtamembrane segment (JM), protein tyrosine kinase domain (PTK) and sterile alpha motif domain (SAM). Ext, extracellular; int, intracellular. At right are shown the amino acid sequences of the JM regions of mouse and human EPHB4, and mouse EPHB2 and EPHA4. Numbering of the indicated conserved tyrosine and proline residues (mutated in EPHB4 2YP) is based upon mouse EPHB4 isoform b. Below are shown the secondary structure elements of the JM segment (27). Ex1, extended strand segment; αA’, single turn helix; αB’, four turn helix. Asterisks indicate residues that contact the PTK domain. (B and C) Cos-7 cells were transfected with c-myc-tagged wild-type (WT), Y590F/Y596F (2Y), Y590F/P593G/Y596F/P599G (2YP) or P593G/P599G (2P) EPHB4. Cells were stimulated or not with Ephrin B2 and transfected EPHB4 receptors were immunoprecipitated from lysates using an anti-c-myc antibody. (B) Co-immunoprecipitated RASA1 was detected by Western blotting. (C) Phosphotyrosine content of immunoprecipitated EPHB4 was detected by Western blotting. (D) Heterozygous Ephb4fl/2YP mice were intercrossed and embryos were harvested at E10.5. Development of Ephb4fl/2YP and Ephb42YP/2YP is normal at E10.5 (top). Anti-CD31 antibody staining reveals a normal vasculature in the head region of Ephb4fl/2YP and Ephb42YP/2YP embryos (bottom).
Figure 11. Absence of hemorrhage and EC collagen IV accumulation in EC of induced EPHB4 2YP embryos. TM was administered to littermate embryos of the indicated genotypes at E13.5 and embryos were harvested at E18.5. Hemorrhage that was confirmed by H&E staining of skins sections was observed in Ephb4^{fl/fl} Ubert2cre embryos but not Ephb4^{fl/YP} Ubert2cre embryos or Cre-negative embryos. Skin sections were additionally stained with anti-CD31 and anti-collagen IV antibodies and Hoechst. Note accumulation of collagen IV in EC of Ephb4^{fl/fl} Ubert2cre embryos (arrowheads) but not embryos of other genotypes.
Figure 12. Normal retinal and pathological angiogenesis in EPHB4 2YP mice. (A-C) Retinas were harvested from mice of the indicated littermate mice at P6 and stained with isolectin B4 (IB4) to identify BV. (A) Representative low power images of IB4 staining are shown at top. Representative higher power images of IB4 staining at the angiogenic front are shown at bottom. Asterisks indicate filopodia. (B) Plot shows the percentage coverage of retinas with EC. Bars show the mean +/- 1 SEM of percentage EC coverage of individual retinas ($Ephb4^{fl/fl}$, n=9; $Ephb4^{fl/2YP}$, n=15; $Ephb4^{2YP/2YP}$, n=11). (C) Plot shows the number of filopodia per 300 µm of angiogenic front that were randomly selected. Bars show the mean +/- 1 SEM of filopodia ($Ephb4^{fl/fl}$, n=6; $Ephb4^{fl/2YP}$, n=6; $Ephb4^{2YP/2YP}$, n=6). (D-G) Adult mice of the indicated genotypes were injected in flanks with B16 melanoma cells and tumors were harvested 13 days later. (D) Representative images of explanted tumors. (E) Plot shows tumor weights at day 13. Bars represent mean +/- 1 SEM tumor weight ($Ephb4^{fl/fl}$, n=6; $Ephb4^{fl/2YP}$, n=7; $Ephb4^{2YP/2YP}$, n=5). (E) Sections of explanted tumors were stained with anti-CD31 antibodies and Hoechst to identify BV. Shown are representative images. (F) Sections of explanted tumors were stained with anti-CD31 antibodies to identify BV. Shown are representative images. (G) Plot shows percentage BV coverage of randomly selected 200 µm x 200 µm areas of tumors. Bars represent mean +/- 1 SEM percentage BV coverage ($Ephb4^{fl/fl}$, n=10; $Ephb4^{fl/2YP}$, n=10; $Ephb4^{2YP/2YP}$, n=10). ns, not significant; One way ANOVA with Tukey.