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RASA1-dependent cellular export of collagen IV controls blood and lymphatic vascular development

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Combined germline and somatic second-hit inactivating mutations of the *RASA1* gene, which encodes a negative regulator of the Ras signaling pathway, cause blood and lymphatic vascular lesions in the human autosomal-dominant vascular disorder capillary malformation–arteriovenous malformation (CM-AVM). How *RASA1* mutations in endothelial cells (ECs) result in vascular lesions in CM-AVM is unknown. Here, using different murine models of *RASA1* deficiency, we found that *RASA1* was essential for the survival of ECs during developmental angiogenesis, in which primitive vascular plexuses are remodeled into hierarchical vascular networks. *RASA1* was required for EC survival during developmental angiogenesis, because it was necessary for export of collagen IV from ECs and deposition in vascular basement membranes. In the absence of *RASA1*, dysregulated Ras/MAPK signal transduction in ECs resulted in impaired folding of collagen IV and its retention in the endoplasmic reticulum (ER), leading to EC death. Remarkably, the chemical chaperone 4-phenylbutyric acid and small-molecule inhibitors of MAPK and 2-oxoglutarate-dependent collagen IV-modifying enzymes rescued ER retention of collagen IV and EC apoptosis and resulted in normal developmental angiogenesis. These findings have important implications for a better understanding of the molecular pathogenesis of CM-AVM and possible means of treatment.

Introduction

Capillary malformation–arteriovenous malformation (CM-AVM) is an autosomal-dominant inherited vascular disease that is characterized by 1 or more cutaneous CMs together with fast-flow vascular lesions in one-third of patients (1–3). Fast-flow lesions, which include AVMs and arteriovenous fistulas (AFs), occur in different anatomical locations and can be life threatening. Lymphatic vessel (LV) abnormalities that result in lymphedema, chylothorax, and chylous ascites have also been identified in a minority of patients with CM-AVM (2–7). In the majority of CM-AVM cases, blood vessel (BV) and LV lesions are present at birth, although they can also develop throughout childhood and up to early adulthood.

Inactivating germline mutations of the *RASA1* gene are responsible for approximately 50% of CM-AVM cases (1–3). *RASA1* encodes p120 Ras GTPase-activating protein (p120 RasGAP or RASA1), a negative regulator of the Ras small GTP-binding protein that promotes cell growth, proliferation, and differentiation (8–10). In quiescent cells, Ras exists predominantly in an inactive GDP-bound state. Growth factors promote the conversion of Ras to an active GTP-bound state that results in the triggering of downstream signaling pathways including the MAPK and PI3K pathways that drive cellular responses. *RASA1* inhibits Ras signal transduction by augmenting the ability of Ras to hydrolyze bound GTP, resulting in its conversion to the inactive GDP-bound form (8). Vascular lesions in patients with CM-AVM with germline *RASA1* mutations arise as a

consequence of somatic inactivating mutation of the inherited WT *RASA1* allele in endothelial cells (ECs) or their precursors (6, 11). Loss of *RASA1* in these ECs would be expected to result in dysregulated Ras signal transduction that could drive lesion development.

Recently, it has been shown that inactivating germline mutations of *EPHB4*, which encodes the ephrin receptor B4, are responsible for the majority of CM-AVM cases that are not explained by the mutation of *RASA1* (12). Accordingly, CM-AVM resulting from *RASA1* mutation has been renamed CM-AVM1, and CM-AVM resulting from *EPHB4* mutation has been named CM-AVM2. Clinically, CM-AVM1 and CM-AVM2 are almost indistinguishable except for the additional occurrence of telangiectasias in CM-AVM2 (12). These findings raise the possibility that lesion development in CM-AVM results from loss of an *EPHB4*/*RASA1* negative-regulatory axis in ECs, in which *EPHB4* serves to recruit *RASA1* to the inner leaflet of the cell membrane, allowing its juxtaposition to Ras-GTP (12, 13). It is likely that second-hit mutations of *EPHB4* are required for the development of lesions in CM-AVM2, although this has yet to be demonstrated.

Studies of genetically engineered mutant mice have the potential to provide information on the pathogenesis of diseases such as CM-AVM that could not otherwise be obtained from human studies alone. Concerning *RASA1* and CM-AVM1, constitutive loss of *Rasa1* in mice results in mid-gestation lethality at E10.5 as a consequence of impaired vascular development (14, 15). Developmental angiogenesis, in which primitive vascular plexuses are remodeled into hierarchical vascular networks, is abnormal in these embryos. This is evident in the yolk sac, for example, where ECs initially assemble into a vascular plexus but then fail to organize into a vascular network that supplies blood to the developing embryo. Some defects in vasculogenesis are also evident in *RASA1*-defi-

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Table 1. Embryonic disruption of *Rasa1*

Littermate group	<i>Rasa1</i>	<i>Ert2Cre</i> driver	TM day	Drug ^A	Harvest	Embryonic vascular phenotypes	Percentage of embryos with phenotype (affected vs. total)
1	<i>fl/fl</i>	<i>Ub</i>	E12.5	None	E18.5	Hemorrhage/lymphedema; EC apoptosis ^C	100 (11 of 11)
	<i>fl/fl</i>	None	E12.5	None	E18.5	None	0 (0 of 5)
2	<i>fl/fl</i>	<i>Ub</i>	E13.5	None	E18.5	Hemorrhage/lymphedema; EC apoptosis ^C	91 (10 of 11)
	<i>fl/fl</i>	None	E13.5	None	E18.5	None	0 (0 of 8)
3	<i>fl/fl</i>	<i>Ub</i>	E13.5	4PBA ^A	E18.5	None	0 (0 of 7)
	<i>fl/fl</i>	None	E13.5	4PBA ^A	E18.5	None	0 (0 of 5)
4	<i>fl/fl</i>	<i>Ub</i>	E13.5	EDHB ^A	E18.5	None	0 (0 of 5)
	<i>fl/fl</i>	None	E13.5	EDHB ^A	E18.5	None	0 (0 of 7)
5	<i>fl/fl</i>	<i>Ub</i>	E13.5	2,4PDCA ^A	E18.5	None	0 (0 of 3)
	<i>fl/fl</i>	None	E13.5	2,4PDCA ^A	E18.5	None	0 (0 of 6)
6	<i>fl/fl</i>	<i>Ub</i>	E13.5	AZD6244 ^B	E18.5	None	0 (0 of 7)
	<i>fl/fl</i>	None	E13.5	AZD6244 ^A	E18.5	None	0 (0 of 8)
7	<i>fl/fl</i>	<i>Ub</i>	E14.5	None	E19.5	Hemorrhage/lymphedema; EC apoptosis ^C	100 (1 of 1)
	<i>fl/fl</i>	None	E14.5	None	E19.5	None	0 (0 of 2)
8	<i>fl/fl</i>	<i>Ub</i>	E15.5	None	E17.5	Valve LEC apoptosis; impaired LV valve development ^C	100 (6 of 6) Ref. 17
	<i>fl/fl</i>	None	E15.5	None	E17.5	None	Ref. 17
9	<i>fl/fl</i>	<i>Cdh5</i>	E13.5	None	E18.5	Hemorrhage (local) ^C	83 (10 of 12)
	<i>fl/fl</i>	None	E13.5	None	E18.5	None	0 (0 of 6)
10	<i>fl/fl</i>	<i>Cdh5</i>	E13.5	None	E19.5	Hemorrhage/lymphedema; EC apoptosis ^C	100 (7 of 7)
	<i>fl/fl</i>	None	E13.5	None	E19.5	None	0 (0 of 5)
11	<i>fl/R780Q</i>	<i>Ub</i>	E12.5	None	E18.5	Hemorrhage/lymphedema; EC apoptosis ^C	86 (6 of 7)
	<i>fl/R780Q</i>	None	E12.5	None	E18.5	None	0 (0 of 6)

^AAdministered at the same time as TM and everyday thereafter until E18.5; ^Badministered at the same time as TM and on the following 2 days;

^Cphenotypes not apparent prior to this time.

cient embryos. In contrast to this, in adult mice, induced global disruption of *Rasa1* does not result in any spontaneous BV abnormalities (16). Instead, the mice develop LV hyperplasia and leakage that result in chylous ascites and chylothorax (16). Recently, we demonstrated that RASA1 is essential for the development and maintenance of valves in collecting LVs, which accounts for LV leakage in the absence of RASA1 (17).

To further understand the role of RASA1 in the BV and LV systems and how its loss may contribute to the vascular phenotypes observed in CM-AVM1, in the current study, we examined the influence of embryonic loss of RASA1 after E10.5. By E10.5, vasculogenesis is largely complete, and the remainder of vascular development is devoted to remodeling of the vascular network by angiogenic processes (18). RASA1 was found to be essential for continued vascular development during this period by promoting the survival of ECs. Unexpectedly, the prosurvival function of RASA1 in ECs during developmental angiogenesis could be explained on the grounds that RASA1 is required for the proper folding and export from ECs and vascular smooth muscle cells (VSMCs) of collagen IV, a major constituent of vascular basement membranes (BMs). We further show that RASA1 is required for normal retinal angiogenesis in newborns and pathological angiogenesis in adults and that this is again most likely explained by its role in the export of collagen IV for deposition in the BM. These findings reveal a previously unappreciated role for RASA1 in vascular biology and are of relevance to the understanding of the pathogenesis and treatment of CM-AVM.

Results

Global disruption of *Rasa1* during developmental angiogenesis results in hemorrhage, edema, and EC apoptosis. To examine the influence of global RASA1 loss upon developmental angiogenesis, pregnant *Rasa1^{fl/fl}* mice carrying *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at the E12.5–E14.5 stage were administered tamoxifen (TM). We found that administration of TM to *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at this time point resulted in visible cutaneous hemorrhage and an edematous appearance at E18.5 to E19.5 (Table 1 and Figure 1A). Histological analysis of embryos revealed extravasated erythrocytes in skin associated with damaged cutaneous BVs and a vastly reduced number of cutaneous LVs (Figure 1A). We did not observe the same phenotypes in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos that were not administered TM (Supplemental Figure 1A). Administration of TM to *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at E15.5 and later also did not result in hemorrhage or other spontaneous embryonic BV abnormalities, although TM administration at this embryonic stage does result in failed LV valve development, as we reported previously (17).

Failed LV valve development in embryos administered TM at E15.5 is explained by apoptosis of LV endothelial cells (LECs) in developing LV valve leaflets (17). Therefore, we asked whether disruption of *Rasa1* in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos before E15.5 induced apoptosis of BV endothelial cells (BECs) and LECs in BV and LV walls. As revealed by immunostaining for activated caspase 3, we identified apoptotic BECs in the vast majority of cutaneous BVs of *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos that were administered TM between E12.5 and E14.5, as determined at E18.5 to

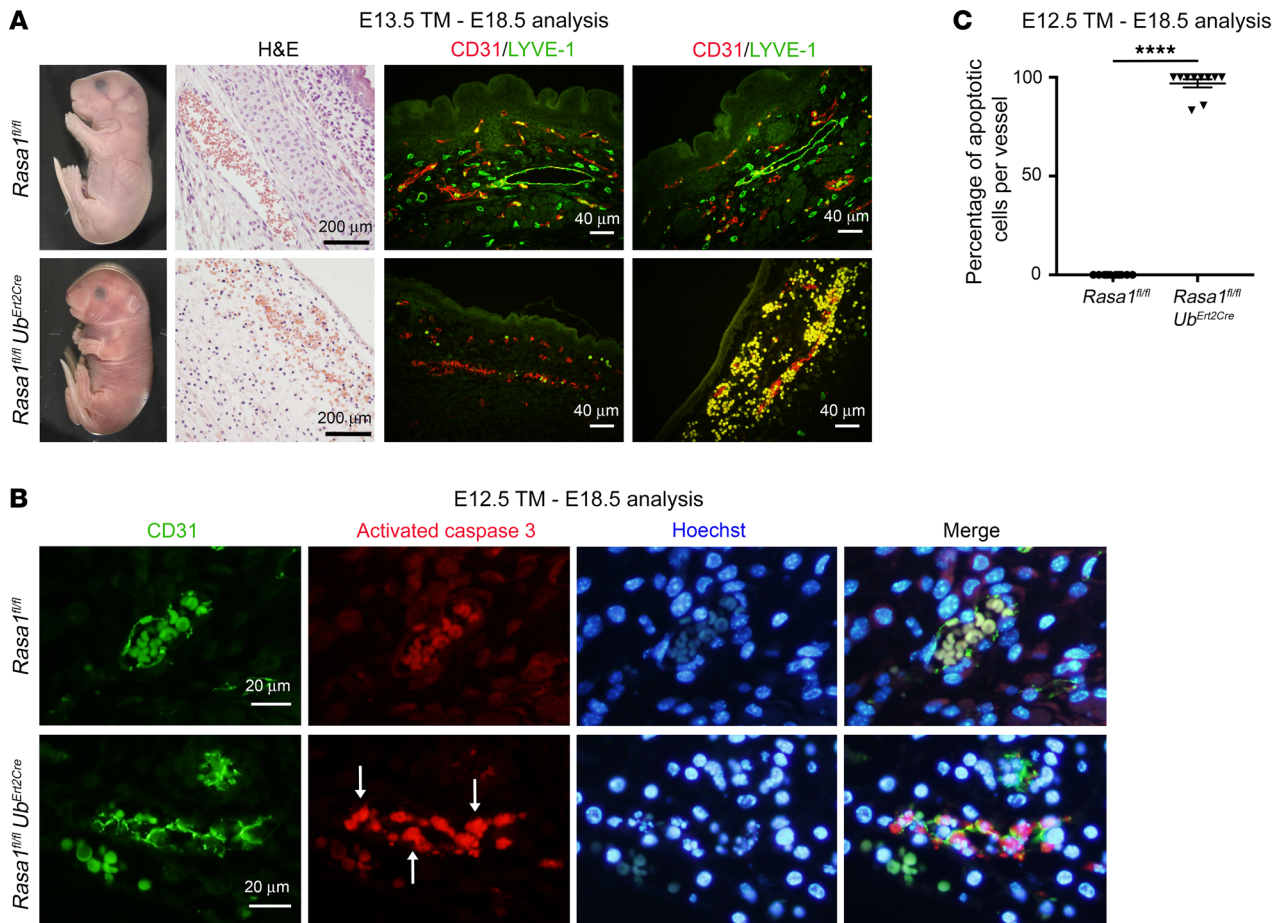


Figure 1. Hemorrhage, edema, and EC apoptosis following global disruption of *Rasa1* during developmental angiogenesis. (A) TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at E13.5, and embryos were harvested at E18.5. *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos show extensive cutaneous hemorrhage that was confirmed by staining of skin sections with H&E. Sections were additionally stained with antibodies against CD31 and LYVE-1 to identify BVs and LVs, respectively. Note the abundant BVs and LVs in skin of control *Rasa1^{fl/fl}* embryos (separate representative fields are shown) and damaged BVs and the absence of LVs in skin of *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos (separate fields show areas with and without extravasated autofluorescent erythrocytes in yellow). Scale bars: 200 μ m and 40 μ m. (B) TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at E12.5, and embryos were harvested at E18.5. Skin sections were stained with Hoechst and antibodies against CD31 and activated caspase 3. Note the activated caspase 3 (arrows) surrounding fragmented nuclei of apoptotic BECs of *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos. Scale bars: 20 μ m. (C) Quantitation of BEC apoptosis in skin BVs of *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos administered TM at E12.5 and harvested at E18.5. Data show the mean \pm 1 SEM of the percentage of activated caspase 3⁺ BECs per BV (n = 10 BVs for each genotype). **** P < 0.0001, by Student's 2-sample t test.

E19.5 (Figure 1, B and C) but not in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos that did not receive TM (Supplemental Figure 1B). Likewise, within the few LVs that could be identified in these embryos at these time points, we frequently observed apoptotic LECs (Supplemental Figure 2). Apoptosis of BECs and LECs, therefore, is likely to contribute to hemorrhage and edema in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos administered TM between E12.5 and E14.5.

Disruption of *Rasa1* specifically within ECs is sufficient for EC apoptosis during developmental angiogenesis. To determine whether the vascular abnormalities observed in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos treated with TM between E12.5 and E14.5 were a consequence of loss of RASA1 within ECs themselves, we performed similar experiments using an EC-specific *Cdh5^{Ert2Cre}* driver (19). Pregnant *Rasa1^{fl/fl}* mice carrying *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos were administered TM at E13.5, and the embryos were harvested at E18.5 or E19.5, i.e., 5 or 6 days later, respectively. At E18.5, we observed cutaneous hemorrhage in TM-treated, but not untreated,

Rasa1^{fl/fl} Cdh5^{Ert2Cre} embryos, which was confirmed by histological analysis (Table 1, Figure 2A and Supplemental Figure 3A). However, hemorrhage was more localized in *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos than in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at this time point (compare with Figure 1A). In contrast, at E19.5, we observed extensive hemorrhage and edema in *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos (Table 1 and Figure 2B). Furthermore, apoptotic BECs were readily observed in BVs of *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos at E19.5 (Figure 2C) but not at E18.5 (see below). As shown by real-time quantitative PCR (qPCR) analysis of sorted skin BECs from TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* and *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos, deletion efficiency of the *Rasa1* gene in BECs was comparable using the 2 different types of *ErtCre* drivers at E18.5 (Supplemental Figure 4). Therefore, differences in the time of onset of the phenotypes cannot be explained by differences in *Rasa1* gene deletion efficiency. In conclusion, disruption of *Rasa1* within ECs during developmental angiogenesis was sufficient for the development of vascular abnormalities, including EC

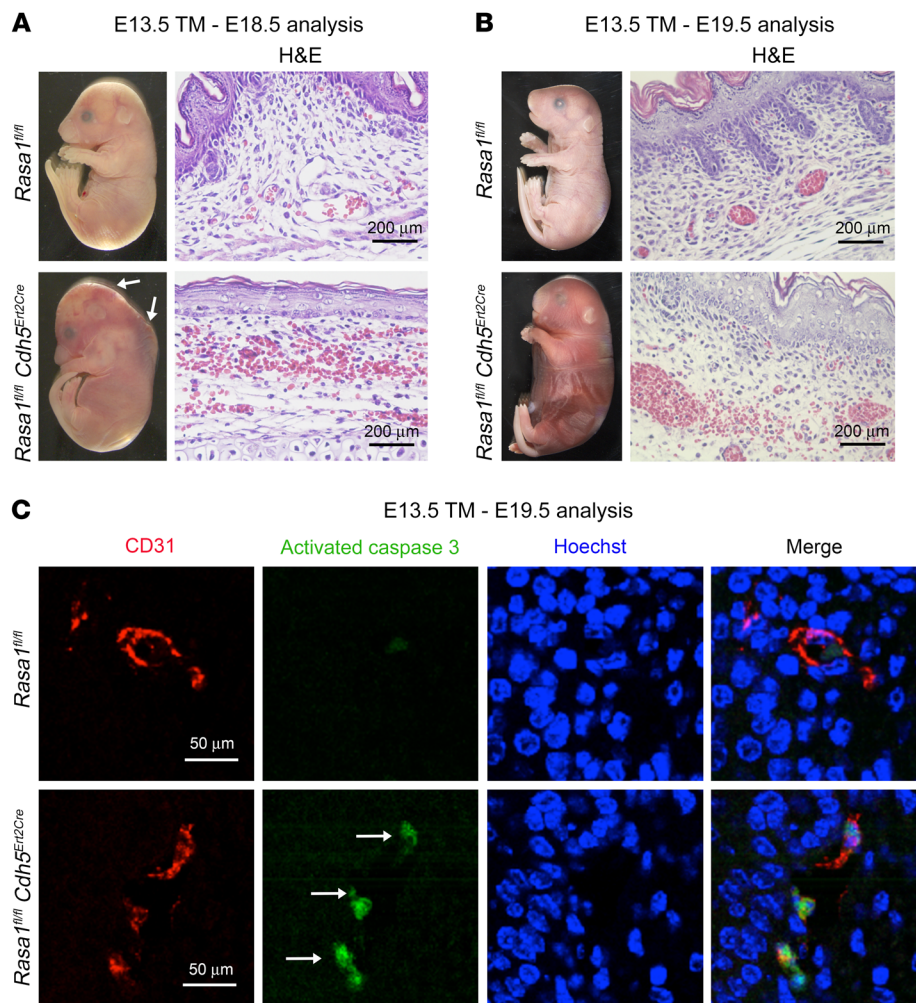


Figure 2. BV abnormalities following disruption of *Rasa1* specifically within ECs during developmental angiogenesis. TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos at E13.5. (**A** and **B**) Embryos were harvested at E18.5 or E19.5, and skin sections were stained with H&E. Note the localized hemorrhage in *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos at E18.5 (arrows) and the more extensive hemorrhage and edema at E19.5. Note the extravasated erythrocytes in skin sections from E18.5 and E19.5 embryos. The section from the E18.5 *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryo is from an area of skin with visible hemorrhage. Scale bars: 200 μ m. (**C**) Embryos were harvested at E19.5, and skin sections were stained with Hoechst and antibodies against CD31 and activated caspase 3. Note the apoptotic BECs in *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos (arrows). Scale bars: 50 μ m.

apoptosis, although the full manifestation of vascular phenotypes was slightly delayed.

Accumulation of collagen IV within BECs of embryos with induced EC-specific RASA1-deficiency. Since induced loss of RASA1 during developmental angiogenesis results in hemorrhage, we also examined the integrity of vascular BMs. Vascular BMs are predominantly composed of collagen IV and laminins that are produced mostly by ECs but also VSMCs during developmental angiogenesis (20). Apoptotic death of BECs would be expected to result in reduced deposition of BM that would result in reduced barrier function that could contribute to hemorrhage. To examine this, we stained skin sections from *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos that had been administered TM between E12.5 and E14.5 (or not) with antibodies against collagen IV (Supplemental Figure 1C and Supplemental Figure 5). As predicted, at E18.5 to 19.5, BV BMs in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos stained less intensely with collagen IV antibodies than did BV BMs in *Rasa1^{fl/fl}* littermates (Supplemental Figure 5). In addition, in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos, the collagen IV staining of BMs was frequently discontinuous. Unexpected, however, was the finding that BECs in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos frequently contained intracellular accumulations of collagen IV in discrete foci (Supplemental Figure 5). These abnormalities of collagen IV distribution were not observed in the absence of TM treatment (Supplemental Figure 1C).

To determine whether intracellular collagen IV accumulation was a consequence of or independent of BEC apoptosis, we stained skin sections of E18.5 *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos administered TM at E13.5 with collagen IV antibodies (Figure 3). BEC apoptosis was observed infrequently in *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos at this time point (Supplemental Figure 6). Nonetheless, BECs in these embryos (but not BECs in E18.5 *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos that were not treated with TM) showed intracellular accumulation of collagen IV associated with a reduced density of collagen IV in the BM (Figure 3 and Supplemental Figures 3 and 6). Therefore, intracellular accumulation of collagen IV occurred independently of BEC apoptosis. In contrast to collagen IV, laminin α 4 was deposited normally in BV BMs in *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos at E18.5 (Supplemental Figure 7). These findings indicate that impaired export of collagen IV from BECs is a contributing factor to the paucity of collagen IV in BV BMs, independent of BEC apoptosis. We also observed less intensity of collagen IV staining in BMs of LVs in TM-treated E18.5 *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos compared with controls (Supplemental Figure 8). Furthermore, this was associated with intracellular accumulation of collagen IV within LECs (Supplemental Figure 8). Therefore, as with BVs, intracellular accumulation of collagen IV in LECs probably contributes to the reduced density of collagen IV in LV BM following loss of RASA1.

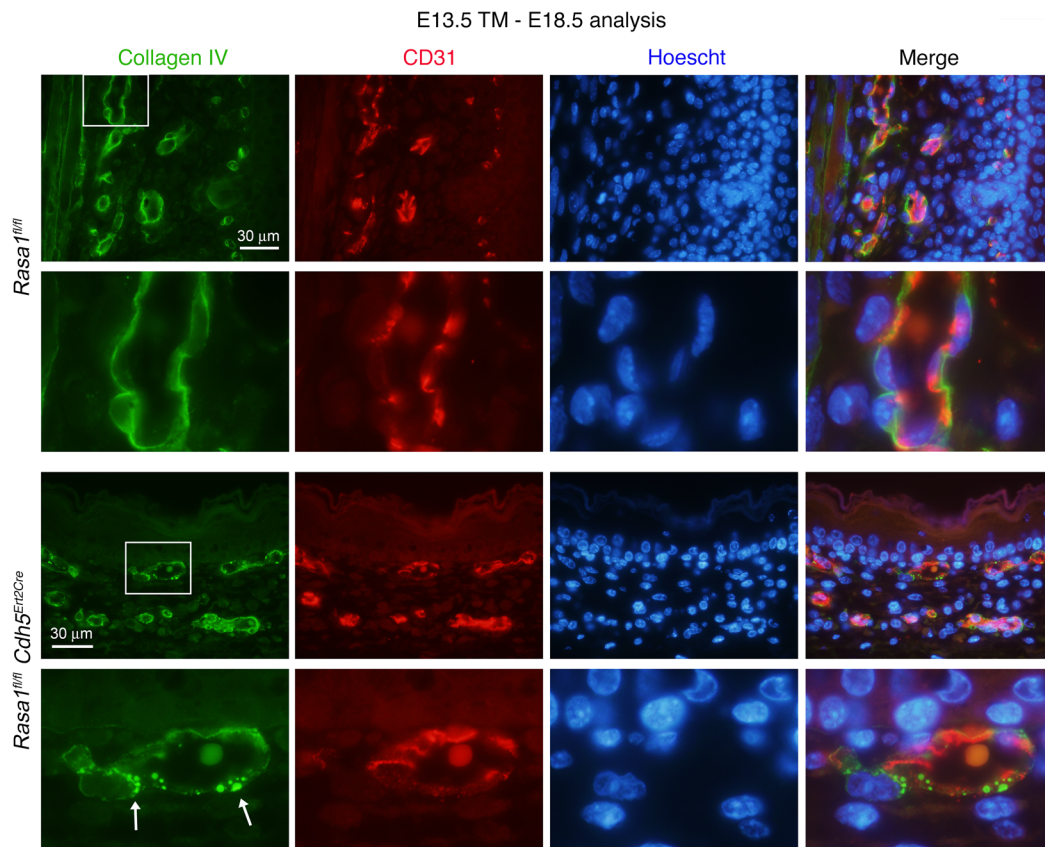


Figure 3. EC-specific disruption of *Rasa1* during developmental angiogenesis results in retention of collagen IV within BECs. TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos at E13.5. Embryos were harvested at E18.5 and skin sections were stained with Hoechst and antibodies against CD31 and collagen IV. Lower-power images are shown in the top rows. Higher-power images of boxed areas are shown below. Note the accumulation of collagen IV within BECs of *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos (arrows). Scale bars: 30 μ m. Magnification: $\times 1000$.

Collagen IV is retained within the endoplasmic reticulum of ECs of embryos with induced EC-specific RASA1-deficiency. Newly synthesized collagen IV in the endoplasmic reticulum (ER) is packaged into coat protein II-coated (COPII-coated) vesicles that deliver collagen IV to the Golgi apparatus via the ER Golgi intermediate compartment (ERGIC). From the Golgi apparatus, collagen IV is further packaged into secretory vesicles for export to the extracellular space (21). Potentially, therefore, intracellular accumulation of collagen IV in RASA1-deficient ECs could be a result of retention in any of the ER, ERGIC, or the Golgi itself. It is also theoretically possible that intracellular collagen IV reflects not impaired secretion, but ingestion of collagen IV through an endocytic process. To examine this, skin sections from E18.5 *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos treated with TM at E13.5 were costained with antibodies against collagen IV and organelle-specific antibodies (Figure 4). We observed no colocalization of collagen IV with ERGIC, Golgi, or lysosomal markers. In contrast, both of 2 different ER markers colocalized with collagen IV. Calnexin, a transmembrane ER chaperone that is highly restricted to the ER, encircled discrete collagen IV punctae (22). In contrast, calreticulin, an ER luminal chaperone, was coincident with the majority of collagen IV punctae (Figure 4). Thus, intracellular collagen IV accumulation in RASA1-deficient ECs is explained by impaired export of collagen IV from the ER.

Mechanism of EC death upon loss of RASA1 during developmental angiogenesis. Blocked export of collagen IV from vascular cells could contribute to EC death during developmental angiogenesis in 2 distinct ways. First, blocked export could result in detachment or failed attachment of ECs to the vascular BM, thereby resulting in apoptotic death by anoikis (23). In this regard, we frequently observed ECs with accumulated collagen IV in the process of detachment from the underlying BM following induced loss of RASA1 (Supplemental Figure 9). Furthermore, the notion that anoikis contributes to EC death in the absence of RASA1 is supported by the observation of an earlier EC apoptotic response in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* compared with *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos (Figure 1, Figure 2, and Table 1). Vascular BM collagen IV is synthesized both by ECs and VSMCs (20). In TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos, collagen IV export from both types of cells could be affected, resulting in less collagen IV deposition in BM compared with TM-treated *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos, in which export of collagen IV only from ECs would be affected. To address this possibility, we examined whether VSMCs in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos also accumulated collagen IV following administration of TM. As predicted, intracellular accumulation of collagen IV was readily identified in the VSMCs of these embryos (Supplemental Figure 10). Furthermore, apoptotic VSMCs were occasionally identified (Supplemental Figure 11).

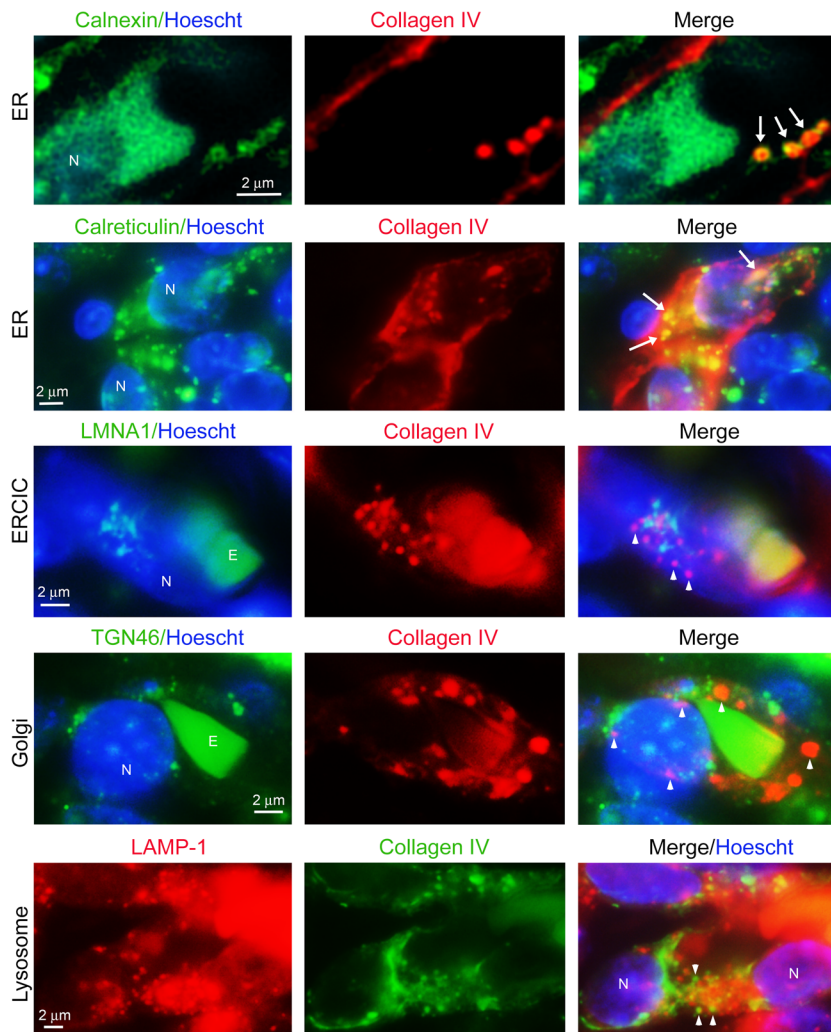


Figure 4. Collagen IV is trapped within the ER of BECs following disruption of *Rasa1* during developmental angiogenesis. Skin sections from E18.5 *Rasa1^{fl/fl}* *Cdh5^{Ert2Cre}* embryos administered TM at E13.5 were stained with Hoechst and antibodies against collagen IV, together with antibodies against calnexin (ER), calreticulin (ER), LMNA1 (ERCIC), TGN46 (Golgi), or LAMP-1 (lysosome). Note the encircling of collagen IV punctae with calnexin (arrows) and colocalization with calreticulin (arrows) but the absence of colocalization of collagen IV punctae with other organelle markers (arrowheads). N, nucleus; E, erythrocyte. Scale bars: 2 μ m

A second mechanism through which accumulated intracellular collagen IV could induce EC apoptosis is through induction of ER stress, resulting in an unfolded protein response (UPR) (24, 25). The purpose of the UPR is to assist the cell with the folding of unfolded and misfolded proteins in the ER. However, in circumstances in which there remains an excess of unfolded protein, the UPR triggers apoptosis. In humans and mice, point-mutated collagen IV variants induce cell apoptosis via this mechanism (26–29). In addition, in mice deficient in the TANGO1 protein that is involved in export of collagen IV from the ER and in mice that are deficient in the hsp47 chaperone that assists with collagen IV folding, accumulating WT collagen IV in the ER induces a UPR and BEC apoptosis (30, 31). The principal sensor of unfolded protein in the ER is BiP (also known as GRP78), whose expression is increased during the course of a UPR. Therefore, to determine whether a UPR is induced in BECs upon loss of *RASA1* during developmental angiogenesis, we examined BiP expression. We found that the amounts of BiP were sharply increased in ECs of E18.5 *Rasa1^{fl/fl}* *Cdh5^{Ert2Cre}* embryos treated with TM at E13.5 compared with ECs of E18.5 *Rasa1^{fl/fl}* controls and ECs of E18.5 *Rasa1^{fl/fl}* *Cdh5^{Ert2Cre}* embryos not treated with TM (Figure 5 and Supplemental Figure 12). These findings are consistent with the induction of a UPR in ECs upon loss of *RASA1*.

The chemical chaperone 4-phenylbutyrate rescues blood vascular phenotypes in embryos with *RASA1*-deficiency. ER retention of collagen IV in *RASA1*-deficient BECs could be a direct consequence of impaired collagen IV folding in the ER or may instead be a result of altered expression or function of proteins involved in COPII-mediated protein secretion (32–34). To address this, we tested whether a chemical chaperone, 4-phenylbutyrate (4PBA), could ameliorate vascular phenotypes that result from loss of *RASA1*. Previously, it was demonstrated that 4PBA rescued the blocked export of misfolded point-mutant collagen IV variants from human and mouse ECs in vitro and reversed intracerebral hemorrhage in mouse models that express these mutants (27, 35). Pregnant *Rasa1^{fl/fl}* mice carrying *Rasa1^{fl/fl}* and *Rasa1^{fl/fl}* *Ubp^{Ert2Cre}* embryos were administered TM with 4PBA at E13.5 followed by 4PBA every day thereafter until embryo harvesting at E18.5. Administration of 4PBA in these experiments completely rescued EC export of collagen IV, EC apoptosis, and blood vascular hemorrhage (Table 1 and Figure 6, compare with Figure 1). In contrast, 4PBA had no influence on vascular development when administered alone to embryos in the absence of TM (Supplemental Figure 13). As determined by real-time qPCR of tail genomic DNA, 4PBA did not affect the ability of TM to disrupt the *Rasa1* gene in *Rasa1^{fl/fl}* *Ubp^{Ert2Cre}* embryos (Supplemental Figure 14). *RASA1* siRNA-mediated knockdown of *RASA1* in human umbilical vein endothelial cells (HUVECs) also resulted in intracellular accumulation of collagen IV, which could be rescued by 4PBA treatment (Figure 7). These findings provide strong evidence that blocked export of collagen IV from *RASA1*-deficient ECs is a consequence of impaired collagen IV folding in the ER rather than a defect in COPII-mediated secretion.

Loss of *RASA1* during developmental angiogenesis results in an increased abundance of collagen IV-modifying enzymes in ECs. Heterotrimerization of 2 collagen IV α -1 monomers and 1 α -2 monomer and folding to form the mature protomer in the ER (collagen α -1 and α -2 are the predominant forms of collagen IV in ECs) is a complex process that is regulated by different collagen IV-modifying enzymes and molecular chaperones that include protein disulfide isomerase A1 (PDIA1), peptidyl proline isomer-

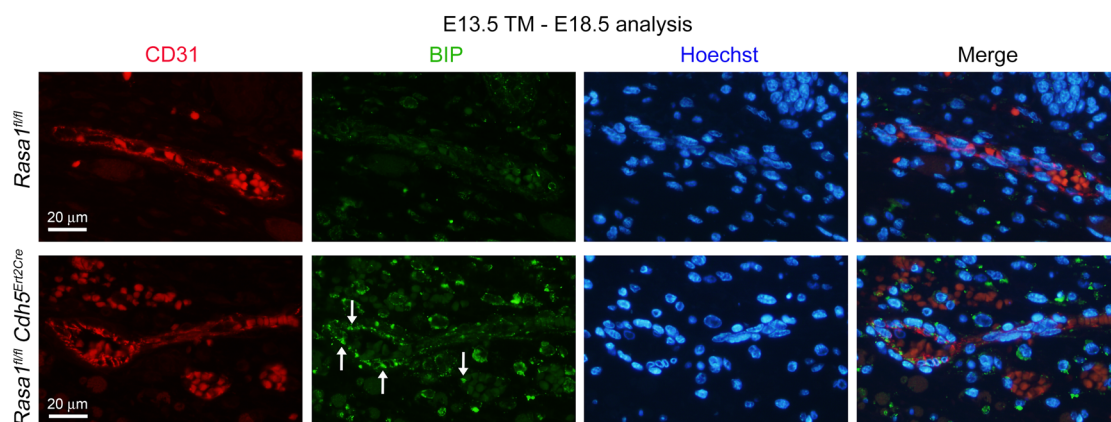


Figure 5. Increased amounts of BIP in BECs following disruption of *Rasa1* during developmental angiogenesis. Skin sections from E18.5 *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos administered TM at E13.5 were stained with Hoechst and antibodies against CD31 and BIP. Note the increase in BIP in BECs from the *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* embryos, indicative of an ongoing UPR (arrows). Scale bars: 20 μ m.

ases (PPIs), proline-4 and proline-3 hydroxylases (P4HA1-3 and P3H1-3, respectively), lysine hydroxylases (LH1-3) also known as procollagen lysine, 2-oxoglutarate 5-dioxygenase enzymes (PLOD1-3) and hsp47. Potentially, therefore, an increased or decreased abundance of collagen IV-modifying enzymes or chap-

erones in RASA1-deficient BECs could affect collagen IV folding (36–40). To examine whether loss of RASA1 results in changes in the amounts of collagen IV-modifying enzymes or chaperones in embryonic BECs during developmental angiogenesis, we performed proteomic analyses. Pregnant *Rasa1^{fl/fl}* mice carrying

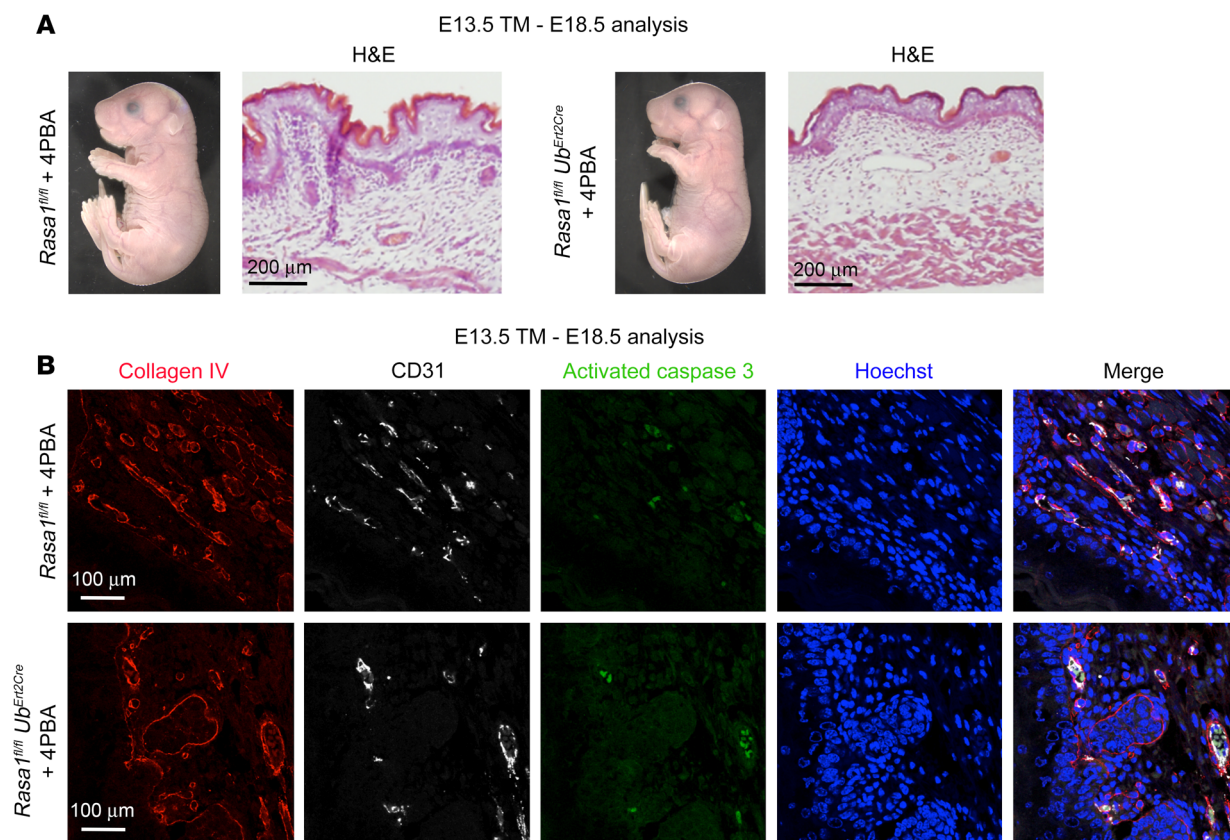


Figure 6. Rescue of developmental angiogenesis defects with the chemical chaperone 4PBA in mice with induced RASA1 deficiency. TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at E13.5. 4PBA was coadministered with TM and was also administered to embryos on consecutive days thereafter until embryo harvesting at E18.5. **(A)** Gross appearance of embryos. Note absence of hemorrhage and edema in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos that was confirmed by H&E staining of skin sections. Scale bars: 200 μ m. **(B)** Skin sections were stained with Hoechst and antibodies against collagen IV, CD31, and activated caspase 3. Note the normal deposition of collagen IV in vascular BM and the absence of BEC apoptosis in the *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos. Scale bars: 100 μ m.

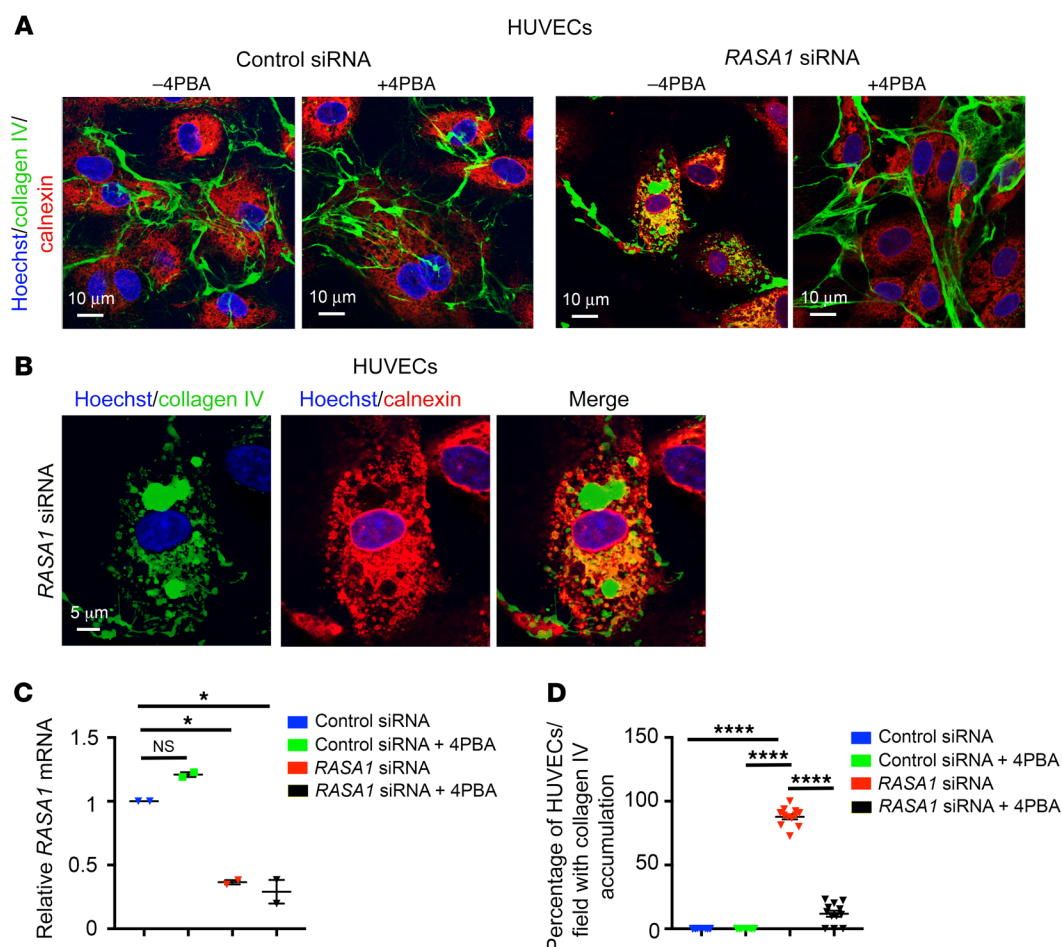


Figure 7. RASA1 knockdown in HUVECs results in collagen IV accumulation in the ER that can be rescued by 4PBA. HUVECs were transfected with control or RASA1 siRNA and cultured for 24 hours in the presence or absence of 4PBA. **(A)** Cells were stained with Hoechst and antibodies against collagen IV and calnexin. Representative images are shown. Note the intracellular accumulation of collagen IV in RASA1 siRNA-treated cells and its rescue by 4PBA treatment. Scale bars: 10 μ m. **(B)** Higher-magnification images of RASA1 siRNA-treated cells in **A** show large intracellular accumulations of collagen IV surrounded by calnexin. Scale bar: 5 μ m. **(C)** Knockdown of RASA1 was confirmed by reverse transcriptase qPCR (RT-qPCR). Data represent the mean \pm 1 SEM of the amount of RASA1 mRNA normalized to RASA1 mRNA levels in control siRNA-treated HUVECs in the same experiment ($n = 2$). **(D)** Data show the mean \pm 1 SEM of the percentage of HUVECs per field with evidence of intracellular collagen IV accumulation ($n = 12$). * $P < 0.05$ and **** $P < 0.0001$, by 1-way ANOVA with Dunnett's multiple comparisons post hoc test.

Rasa1^{fl/fl} and *Rasa1*^{fl/fl} *Ubp^{Er2Cre}* embryos were given TM at E14.5, and the embryos were harvested at E18.5, i.e., prior to BEC apoptosis and hemorrhage (Table 1). Subsequently, BECs from skin of individual embryos were purified, pooled according to genotype, and lysed. Tryptic digests of lysates were then analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). With this approach, we obtained data on the relative abundance of nearly 4000 BEC proteins. Of these, approximately 250 proteins were increased by at least 2-fold, and 200 were decreased by at least 2-fold in RASA1-deficient BECs compared with control BECs (Supplemental Table 1). Strikingly, several of the enzymes involved in collagen IV posttranslational modification were increased in abundance, including FKBP9, P3H1, P4HA2, LH2/PLOD2, and LH3/PLOD3 (Table 2). In contrast, other ER-resident proteins implicated in collagen folding, including PDIA1 and hsp47, showed no or only modest changes in abundance. Furthermore, no significant changes in the abundance of any COPII secretory pathway proteins were apparent, consistent with the

notion that impaired export of collagen IV is not a result of defects in the COPII secretion mechanism.

Inhibitors of 2-oxoglutarate-dependent oxygenases rescue blood vascular phenotypes in embryos with induced RASA1-deficiency. P4HA2, P3H1, PLOD2, and PLOD3 all belong to the same family of enzymes known as 2-oxoglutarate-dependent (2OG-dependent) oxygenases, so called because of their dependency on 2OG for catalysis (41). Consequently, drugs are available that generically inhibit all members of this family. One such drug is the catechol ethyl-3,4-dihydroxybenzoic acid (EDHB), which has been used in vitro and in vivo to block the activity of collagen proline and lysine hydroxylases (41, 42). Therefore, to determine whether increased abundance of any or all of these enzymes is responsible for collagen IV accumulation in BECs during developmental angiogenesis, we examined the ability of EDHB to rescue blocked collagen IV export and vascular phenotypes in induced RASA1-deficient embryos. Pregnant *Rasa1*^{fl/fl} mice carrying *Rasa1*^{fl/fl} and *Rasa1*^{fl/fl} *Ubp^{Er2Cre}* embryos were administered TM at E13.5 together with

Table 2. Abundance of collagen IV-modifying enzymes in RASA1-deficient embryonic BECs^A

Protein	Function	Cre ⁺ /Cre ⁻
PDIA1	PDI	1.4
PPIB	PPI (ER)	0.8
FKBP2	PPI (ER)	0.8
FKBP7	PPI (ER)	0.7
FKBP9^B	PPI (ER)	2.1
FKBP10	PPI (ER)	1.2
P3H1	P3H	2.1
P3H3	P3H	>5 ^C
P4HA1	P4H	1.7
P4HA2	P4H	3.4
PLOD1	PLOD	1.6
PLOD2	PLOD	5.1
PLOD3	PLOD	2
hsp47	hsp	1.2

^AAbundance of all detectable known collagen IV-modifying enzymes and chaperones are shown; ^Bproteins with 2-fold or greater changes in abundance are indicated with bold; ^CP3H3 was undetectable in control cells, and thus the fold increase is of uncertain significance.

EDHB, which was additionally administered to mice every day thereafter until embryos were harvested, at E18.5. Administration of EDHB in these experiments completely rescued EC export of collagen IV, EC apoptosis, and blood vascular hemorrhage (Table 1 and Figure 8, compare with Figure 1). In contrast, when administered to embryos in the absence of TM, EDHB did not affect vascular development (Supplemental Figure 15). As with 4PBA, EDHB did not affect *Rasa1* gene disruption induced by TM in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos (Supplemental Figure 14). The same results were obtained using another generic 2OG-dependent oxygenase inhibitor, 2,4 pyridinedicarboxylic acid (2,4PDCA) (Table 1, Supplemental Figure 14 and Supplemental Figure 16). These findings are consistent with observations from a model in which loss of RASA1 in BECs during developmental angiogenesis results in increased amounts of collagen IV-modifying, 2OG-dependent oxygenases in BECs that account for collagen IV retention in the ER and downstream vascular phenotypes.

Dysregulated Ras/MAPK signaling is responsible for the development of BV phenotypes following RASA1 loss during developmental angiogenesis. RASA1 may participate in certain signaling pathways independently of its ability to regulate Ras (10). Therefore, to address whether vascular phenotypes that result from induced loss of RASA1 during developmental angiogenesis result from dysregulated Ras signaling or to perturbation of a distinct signaling pathway, we examined *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* embryos that we have described previously (15, 17). Administration of TM to these embryos resulted in the expression of *Rasa1^{R780Q}* alone, which encodes a catalytically inactive form of RASA1, in which all putative alternative functions of RASA1 are predicted to remain intact.

Pregnant *Rasa1^{fl/fl}* mice carrying *Rasa1^{fl/fl}* and *Rasa1^{fl/R780Q}* embryos with and without *Ub^{Ert2Cre}* were administered TM at E12.5, and the embryos were harvested at E18.5. Like the *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos (Figure 1), *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* embryos showed extensive

hemorrhage and edema at E18.5 (Table 1 and Figure 9A). Furthermore, this was associated with accumulation of collagen IV in ECs and EC apoptosis (Figure 9, B and C). In contrast, the same vascular phenotypes were not apparent in E18.5 *Rasa1^{fl/+} Ub^{Ert2Cre}* embryos induced to lose 1 *Rasa1* gene copy by administration of TM at E13.5 (Supplemental Figure 17). Thus, vascular phenotypes that result following loss of RASA1 during developmental angiogenesis are consequent to the loss of an ability of RASA1 to regulate Ras and not the loss of a Ras-independent function for this molecule.

Two well-characterized signaling pathways downstream of activated Ras are the MAPK and PI3K pathways. Potentially, therefore, augmented activation of either or both pathways could be responsible for BV phenotypes upon loss of RASA1 during developmental angiogenesis. To address this, *Rasa1^{fl/fl}* mice carrying *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos were administered TM at E13.5 together with a MAPK pathway inhibitor (AZD6244) (43, 44) or a PI3K inhibitor (PX-866) (45), either of which was also administered to mice on each of the 2 days following the TM injection. As assessed at E18.5, the MAPK pathway inhibitor rescued the blockage of collagen IV export from BECs and prevented development of the hemorrhaging in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos that was observed after TM treatment alone (Table 1 and Figure 10, compare with Figure 1). AZD6244 did not affect TM-induced *Rasa1* gene deletion efficiency in *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos (Supplemental Figure 14), and, by itself, AZD6244 did not induce any vascular abnormalities (Supplemental Figure 18). In contrast, the PI3K inhibitor was unable to rescue BEC export of collagen IV or apoptosis, and extensive cutaneous hemorrhage was evident at E18.5 (data not shown). These findings show that dysregulated Ras/MAPK signaling rather than dysregulated Ras/PI3K signaling drives BV phenotypes upon loss of RASA1 during developmental angiogenesis.

RASA1 is required for normal retinal angiogenesis in newborns. No spontaneous BV abnormalities have been noted in mice in which the *Rasa1* gene is disrupted after E15.5 (16, 17). We hypothesize that this is because the majority of the collagen IV in vascular BM is deposited during developmental angiogenesis. Collagen IV is recognized to be one of the most stable proteins in the animal kingdom (38). Thus, in postnatal life, a continued high rate of collagen IV synthesis would be unnecessary for BECs to remain attached to BM. Nonetheless, in situations where de novo deposition of BM is required, abnormalities of BV function might be expected in RASA1-deficient mice. Two such situations are retinal angiogenesis in newborns and pathological angiogenesis in adult mice. To examine retinal angiogenesis, we administered TM to littermate *Rasa1^{fl/fl} Ub^{Ert2Cre}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* mice and Cre-negative controls at P1 and examined the retinal vasculature at P4. The extent of new vessel growth in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* mice was significantly less than in *Rasa1^{fl/fl}* controls as assessed by the number of vessel branch points and the percentage of coverage of the retina with BECs (Figure 11, A and D). Furthermore, the number of BEC filopodia, which are a feature of sprouting angiogenesis, at the periphery of the vascular coverage area was reduced in the *Rasa1^{fl/fl} Ub^{Ert2Cre}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* mice (Figure 11, B and D). Intracellular accumulation of collagen IV could be detected in retinal BECs of *Rasa1^{fl/fl} Ub^{Ert2Cre}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* mice but not *Rasa1^{fl/fl}* control mice at P4 (Figure 11C). In addition, in *Rasa1^{fl/fl} Ub^{Ert2Cre}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}*

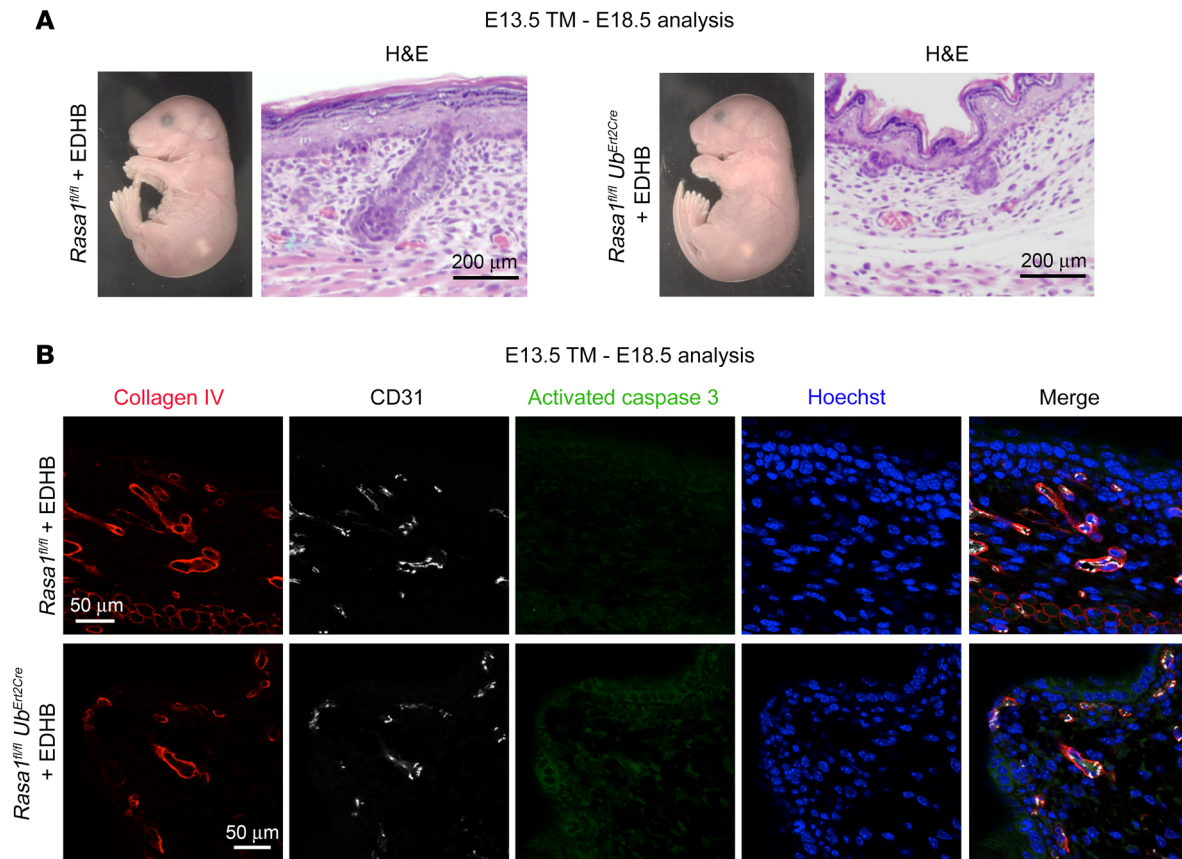


Figure 8. Rescue of developmental angiogenesis defects in mice with induced RASA1 deficiency using the 2OG-dependent oxygenase inhibitor EDHB.

TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at E13.5. EDHB was coadministered with TM and was also administered to embryos on consecutive days thereafter until embryo harvesting at E18.5. **(A)** Gross appearance of embryos. Note the absence of hemorrhage and edema in the *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos, which was confirmed by H&E staining of skin sections. Scale bars: 200 μ m. **(B)** Skin sections were stained with Hoechst and antibodies against collagen IV, CD31, and activated caspase 3. Note the normal deposition of collagen IV in vascular BM and the absence of BEC apoptosis in the *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos. Scale bars: 50 μ m.

Ub^{Ert2Cre} retinas, the number of “empty sleeves” that comprised a thin tube of collagen IV with no BECs was substantially increased compared with controls (Figure 11, C and D). This latter observation is consistent with impaired deposition of collagen IV into BM and BEC death during retinal angiogenesis in the absence of catalytically active RASA1. We also observed decreased angiogenesis in retinas of *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* mice administered TM at P1 and analyzed at P6 (Supplemental Figure 19), confirming an EC-intrinsic role for RASA1 in retinal angiogenesis. In *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice administered TM at P3, we observed areas of hemorrhage in retinas at P10 (Supplemental Figure 20).

Rasa1 is required for pathological angiogenesis in adult mice. To examine pathological angiogenesis, we initially used an ID8 ovarian tumor model (46). Adult littermate female *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice were administered TM and 1 week later were injected in the flanks with ID8 tumor cells. Growth of injected ID8 tumor cells in female recipients is strictly dependent on host BV angiogenesis (46). Six weeks after injection, we found that the ID8 tumors were substantially smaller in *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice compared with those in controls (Supplemental Figure 21, A and B). Upon histological analysis, the density of BVs in tumors from *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice was found to be substantially less than in

tumors from control mice, indicating that reduced tumor growth in the former was a result of impaired BV tumor angiogenesis (Supplemental Figure 21C). Further analysis of BVs in tumors from *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice revealed intracellular accumulation of collagen IV in BECs and BEC apoptosis (Supplemental Figure 21C and Supplemental Figure 22). Thus, blocked export of collagen IV from BECs and BEC apoptosis likely account for an impaired pathological angiogenesis response in the absence of RASA1.

To examine this further, we switched to a B16 melanoma model (47). B16 cells grow more rapidly than do ID8 cells in vivo, thus permitting a more ready analysis of the effect of drugs such as 4PBA that promote collagen IV folding. B16 cells were injected into the flanks of littermate TM-treated *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice. Some mice were also injected with 4PBA at the same time that tumor cells were injected, and additional 4PBA was administered to mice every day thereafter. After 13 days, we assessed tumor growth and angiogenesis (Figure 12). As with ID8 tumor growth, we found that B16 growth was inhibited in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice compared with *Rasa1^{fl/fl}* controls (Figure 12, A and B). In addition, the reduced growth of B16 tumors was also associated with impaired intratumoral BV angiogenesis and collagen IV accumulation in BECs (Figure 12, C–E). 4PBA restored the growth of

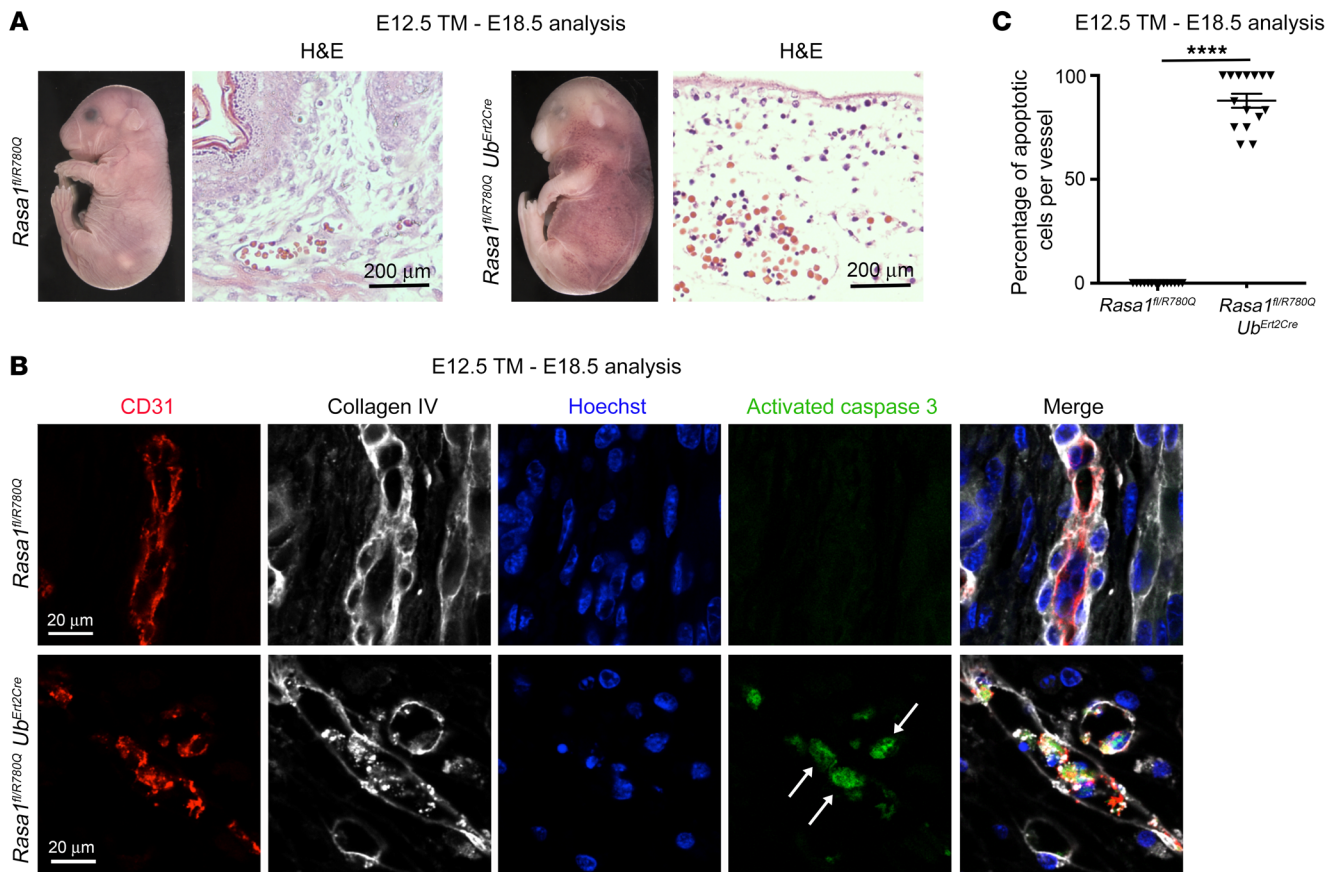


Figure 9. BV abnormalities in embryos induced to express RASA1 R780Q alone during developmental angiogenesis. TM was administered to litter-mate *Rasa1^{fl/R780Q}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* embryos at E12.5 and embryos were harvested at E18.5. **(A)** Gross appearance of embryos. Note the cutaneous hemorrhage in *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* embryos, which was confirmed by H&E staining of skin sections. Scale bars: 200 μ m. **(B)** Skin sections were stained with Hoechst and antibodies against collagen IV, CD31, and activated caspase 3. Note the discontinuous distribution of collagen IV in BV BM, the accumulation of collagen IV in BECs, and the presence of activated caspase 3 in BEC nuclei (arrows) of *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* embryos. Scale bars: 20 μ m. **(C)** Quantitation of BEC apoptosis in skin BVs of *Rasa1^{fl/R780Q}* and *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* embryos administered TM at E12.5 and harvested at E18.5. Data represent the mean \pm 1 SEM of the percentage of activated caspase 3⁺ BECs per BV (n = 15 BVs for each genotype). **** P < 0.0001, by Student's 2-sample t test.

B16 cells in TM-treated *Rasa1^{fl/fl} Ub^{Ert2Cre}* recipients, and this was associated with normal export of collagen IV from BECs and BV angiogenesis (Figure 12). In contrast, 4PBA had no influence on B16 cell growth in TM-treated *Rasa1^{fl/fl}* mice (Figure 12) or when administered alone to *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice that had not previously been injected with TM (Supplemental Figure 23). These findings are consistent with the notion that impaired pathological angiogenesis and tumor growth in RASA1-deficient adult mice is also a consequence of an inability of BECs to export collagen IV for deposition in newly forming BMs.

Discussion

In this study we show that RASA1 has what we believe to be a previously unappreciated critical function in the export of collagen IV from ECs during developmental angiogenesis. In the absence of RASA1, collagen IV is retained in the ER of ECs, leading to their apoptotic death as a result of ER stress and anoikis. The chemical chaperone 4PBA rescued ER retention of collagen IV, EC apoptosis, and BV hemorrhage in induced RASA1-deficient embryos. This finding strongly supports the notion that retention of collagen IV in the ER in the absence of RASA1 is a consequence of

impaired protein folding. Further mechanistic studies indicated that loss of RASA1 within ECs leads to dysregulated Ras/MAPK signaling, which results in an increased abundance of several ER-resident enzymes that carry out posttranslational modifications of collagen IV that are known to regulate folding and ER export of this protein (36, 37). Most notable among these are P3H, P4H, and PLOD enzymes, of which there are 3 different isoforms each in mammals. Of the 7 isoforms of a total of 9 that could be detected in BECs by LC-MS/MS, all were increased in abundance in RASA1-deficient BECs. Increased abundance of these enzymes could lead to excessive posttranslational modification of collagen IV that would explain impaired folding and ER retention of this protein (36–40). Consistent with this, generic inhibitors of this class of enzymes, EDHB and 2,4PDCA, rescued ER collagen IV retention, EC apoptosis, and hemorrhage in induced RASA1-deficient mice. This finding demonstrates that, although the abundance of numerous other proteins was also altered by more than 2-fold in RASA1-deficient BECs (Supplemental Table 1), an increased abundance of these collagen IV-modifying enzymes specifically is responsible for the development of vascular phenotypes in the absence of RASA1.

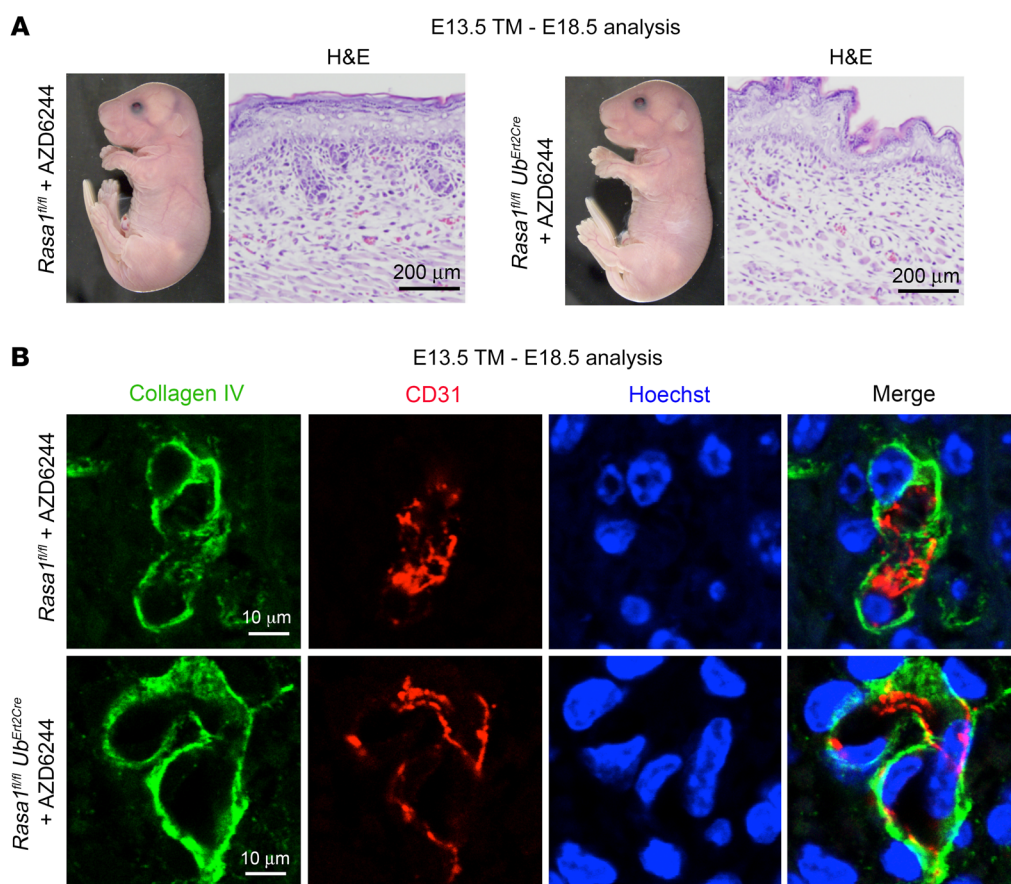


Figure 10. An inhibitor of MAPK signaling blocks the development of BV abnormalities resulting from induced loss of RASA1 during developmental angiogenesis. TM was administered to littermate *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos at E13.5. The MAPK pathway inhibitor AZD6244 was coadministered with TM and was also administered to embryos on the following 2 days afterward. Embryos were harvested at E18.5. **(A)** Gross appearance of embryos. Note the absence of hemorrhage and edema in the *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos, which was confirmed by H&E staining of skin sections. Scale bars: 200 μ m. **(B)** Skin sections were stained with Hoechst and antibodies against collagen IV and CD31. Note the normal deposition of collagen IV in vascular BM in the *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos. Scale bars: 10 μ m.

Dysregulated Ras/MAPK signaling could result in an increased abundance of collagen IV-modifying enzymes as a result of increased gene transcription. MAPK signaling modulates the activity of several different transcription factor complexes including activator protein 1 (AP1) complexes and ternary complex factors (TCFs) with the potential to modify the transcription of collagen IV-modifying genes (48). Alternatively, dysregulated MAPK signaling could affect the abundance of these enzymes at a posttranslational level, for example, by phosphorylation of substrates that affect protein stability. To distinguish between these possibilities, we examined mRNA levels of 2 select enzymes, *Plod2* and *P3h1*, in sorted skin BECs from TM-treated *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* embryos (Supplemental Figure 24). We found that the levels of *Plod2* mRNA were significantly increased in *Rasa1^{fl/fl} Ub^{Ert2Cre}* BECs, which was consistent with increased *Plod2* transcription. In contrast, *P3h1* mRNA levels were not significantly increased in *Rasa1^{fl/fl} Ub^{Ert2Cre}* BECs, suggesting that loss of RASA1 in BECs results in an increased abundance of P3H1 through a posttranslational mechanism.

Whether impaired folding of collagen IV in RASA1-deficient ECs is a result of a collective increase in the abundance of

all 3 classes of collagen-modifying enzymes or an increase in the abundance of select enzymes remains to be determined. The collagenous domains of collagen IV contain multiple repeats of the sequence of Gly-Xaa-Yaa (where Xaa and Yaa are any amino acid). P4Hs hydroxylate prolines in the Yaa position, whereas P3Hs subsequently hydroxylate prolines in the Xaa position of Gly-Xaa-4-hydroxyPro. PLODs hydroxylate lysine residues in the Yaa position of Gly-Xaa-Yaa, and PLOD3 additionally catalyzes glycosylation of hydroxylysine to form galactosylhydroxylysine or galactosylglucosylhydroxylysine. Proline 3 hydroxylation is known to destabilize the collagen triple helix, and, thus, excessive proline 3 hydroxylation of collagen IV is probably an important contributor to impaired folding (38–40). In contrast, proline 4 hydroxylation promotes electrostatic interactions between collagen IV monomers, and lysine hydroxylation and glycosylation are required for collagen IV secretion (36–38). Nonetheless, an increased abundance of P4Hs and PLODs could lead to excessive modification that could also have a negative impact on folding of collagen protomers.

Vasculogenesis commonly results in the formation of a vascular plexus that comprises a primitive vascular network with presumptive arterial inputs and venous outputs. Subsequently,

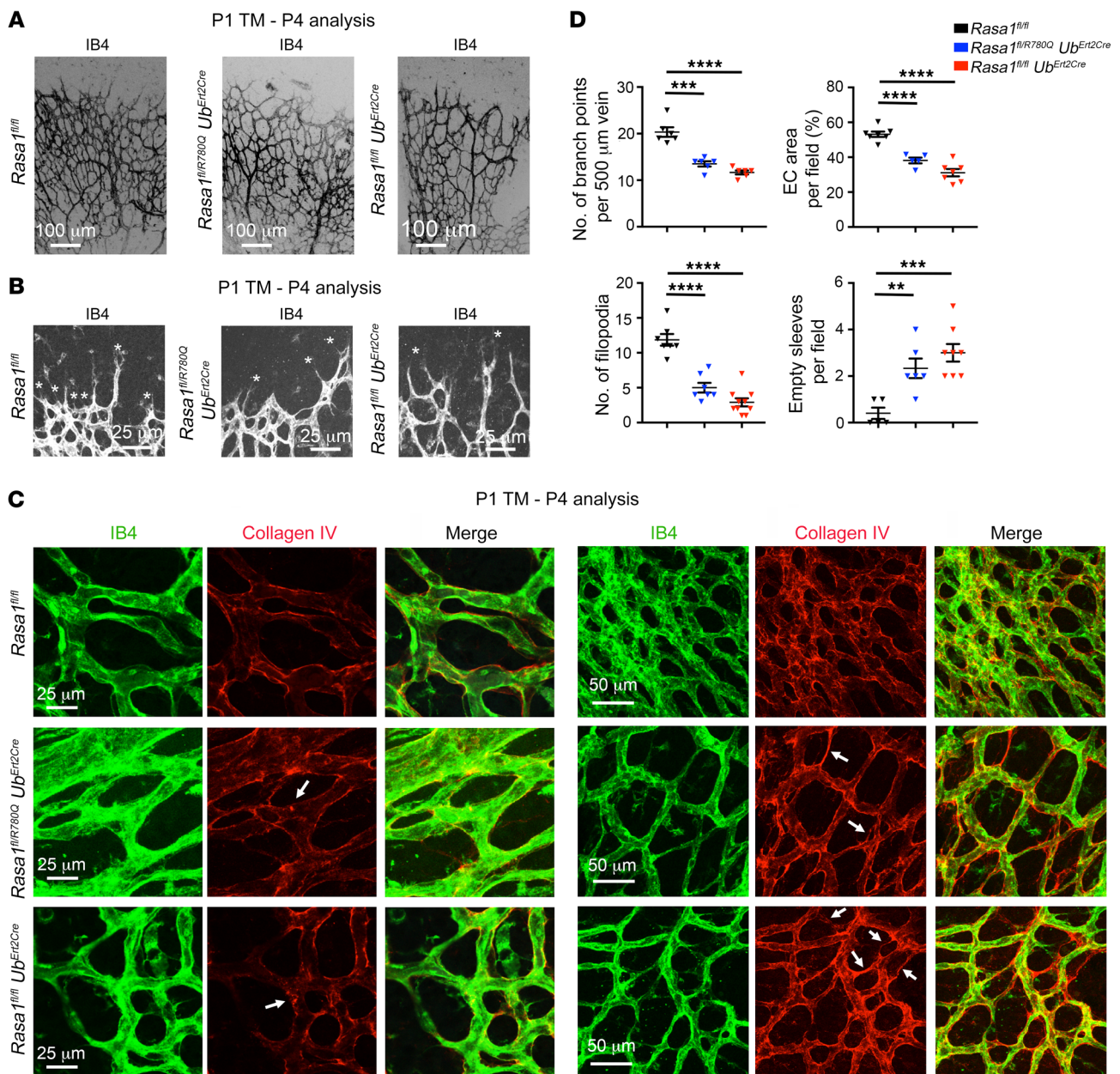


Figure 11. Impaired retinal angiogenesis in neonatal mice with induced RASA1 R780Q and mice with induced RASA1 deficiency. TM was administered to littermate *Rasa1^{fl/fl}*, *Rasa1^{fl/R780Q} Ub^{Ert2Cre}*, and *Rasa1^{fl/fl} Ub^{Ert2Cre}* mice at P1, and retinas were harvested at P4. (A–C) Retinas were stained with isolectin B4 (IB4) to identify BVs and anti-collagen IV (C). (A and B) Representative low-power (A) and high-power (B) images of IB4 staining are shown. Asterisks indicate filopodia at the vascular front. (C) High-power images (left) show collagen IV accumulation in BECs from *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* retinas (arrows), and lower-power images (right) illustrate empty collagen sleeves in *Rasa1^{fl/R780Q} Ub^{Ert2Cre}* and *Rasa1^{fl/fl} Ub^{Ert2Cre}* retinas (arrows). (D) Graphs show the mean \pm 1 SEM of the number of branch points from veins ($n = 6$ retinas/genotype), the percentage of coverage of retinas with BECs per field ($n = 5$ –7 retinas/genotype), the number of filopodia per vascular field ($n = 7$ –10 retinas/genotype), and the number of empty collagen sleeves per field ($n = 5$ –8 retinas/genotype). ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, by 1-way ANOVA test with Dunnett's multiple comparisons post-hoc test. Scale bars: 100 μ m (A), 25 μ m (B and C), and 50 μ m (C).

angiogenic processes that include fusion, intussusception, regression, and sprouting angiogenesis remodel the plexus to yield a hierarchical network of arteries, arterioles, venules, and veins connected by smaller-diameter capillaries (18). Previous studies of global EPHB4-deficient and RASA1-deficient mice have provided evidence that AVMs and AFs in CM-AVM arise as a consequence of impaired angiogenic remodeling of these primitive

vascular plexuses (49). Thus, in EPHB4-deficient embryos and in embryos of mice deficient in the EPHB4 ligand ephrin B2, arteries form near-direct connections with veins through large-diameter vessels, as observed in CM-AVM (50, 51). Similarly, in RASA1-deficient embryos, the same defect in angiogenic remodeling of vascular plexuses has also been noted (14). In light of these observations, findings in this study are directly relevant to an under-

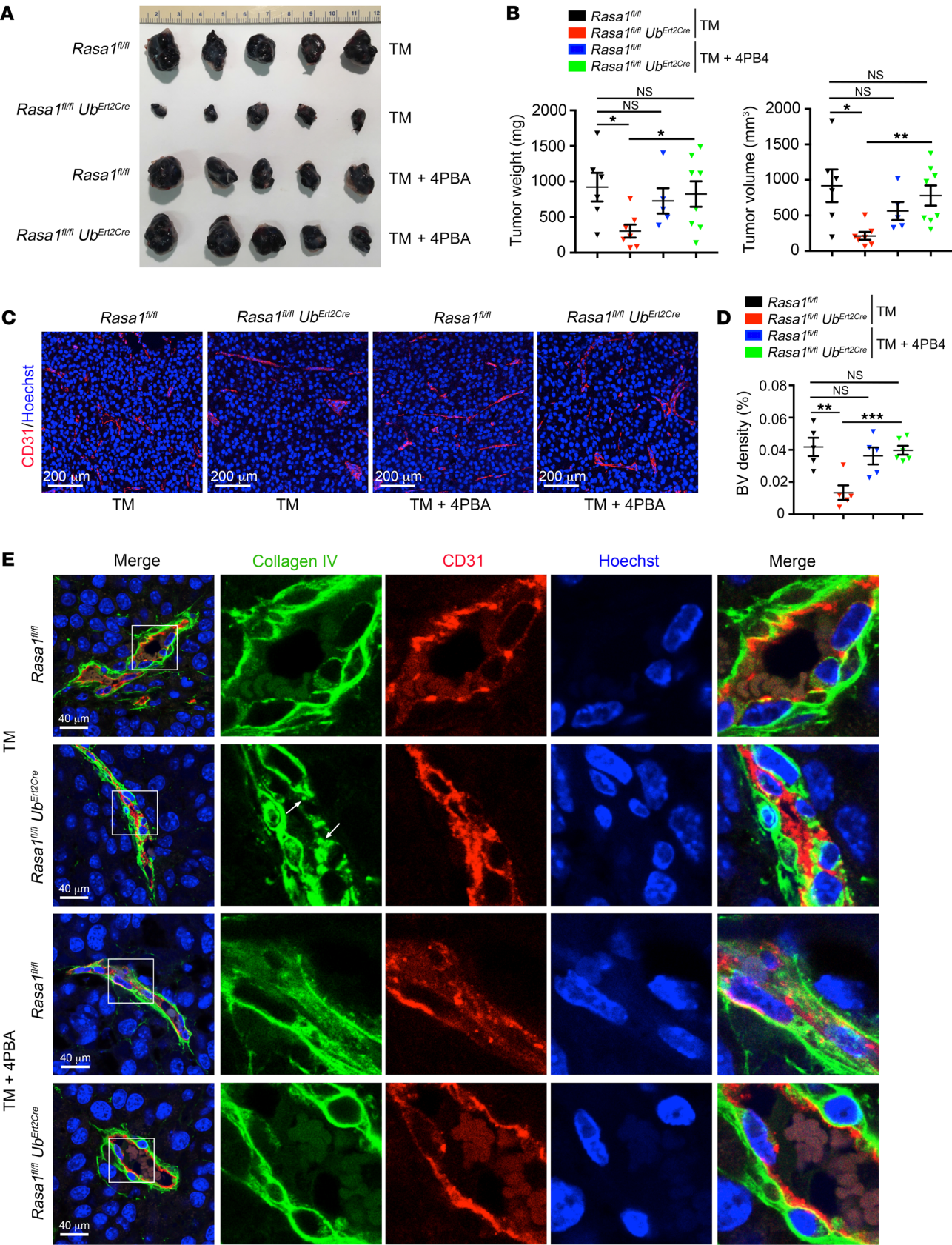


Figure 12. Disruption of *Rasa1* in adult mice inhibits pathological angiogenesis in a B16 melanoma model. Littermate adult *Rasa1^{fl/fl}* and *Rasa1^{fl/fl} U^bErt2^{Cre}* mice were administered TM before subcutaneous injection of B16 melanoma cells into their flanks 1 week later. 4PBA was administered to some mice at the same time as B16 melanoma cells and every day thereafter for the duration of the experiment. After 13 days, the mice were euthanized, and the tumors were harvested. **(A)** Representative gross images of harvested tumors. **(B)** Graphs show the mean \pm 1 SEM of tumor weight and volume ($n = 5$ –8 tumors from mice of each genotype and treatment condition). **(C)** Sections of tumors were stained with Hoechst and CD31 antibodies. Representative images show reduced BV density in tumors from *Rasa1^{fl/fl} U^bErt2^{Cre}* mice treated with TM alone. Scale bars: 200 μ m. **(D)** Graph shows the mean \pm 1 SEM of the percentage of coverage of fields with BVs ($n = 5$ –6 tumors from mice of each genotype and treatment condition). **(E)** Tumor sections were stained with Hoechst and antibodies against collagen IV and CD31. Representative images are shown. Note the accumulation of collagen IV in tumor BECs from *Rasa1^{fl/fl} U^bErt2^{Cre}* mice treated with TM alone (arrows). Scale bars: 40 μ m. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by 1-way ANOVA with Dunnett's multiple comparisons post hoc test.

standing of the pathogenesis of CM-AVM. Acquisition of somatic second-hit mutations in *RASA1* in ECs or their precursors at the time of or prior to vasculogenesis, respectively, could potentially result in the loss of *RASA1* in the majority of ECs in an individual primitive vessel within a vascular plexus. Consequently, sprouting angiogenesis and potentially other forms of angiogenesis from that vessel would be blocked, as these events would require de novo synthesis of collagen IV by ECs in these vessels and deposition of collagen IV in newly forming BM. Thus, an inability of ECs in these vessels to export collagen IV during angiogenic remodeling could account for the development of AVMs and AFs in CM-AVM. Alternatively, acquisition of second-hit mutations in *RASA1* in ECs later in development, after remodeling of vascular plexuses, could result in CM, again as a consequence of blocked export of collagen IV. In either scenario, the ability of chemical chaperones and inhibitors of 2OG-dependent oxygenases to rescue impaired developmental angiogenesis in the absence of *RASA1* suggests possible means of prevention of vascular lesions in human embryos and patients with inherited *RASA1* mutations. Whether such drugs would be effective in the treatment of existing vascular lesions in CM-AVM is far less certain. ECs of CM-AVM lesions would not be expected to be engaged in a high rate synthesis of collagen IV, and, in the absence of angiogenic stimuli, intracellular accumulation of collagen IV in ECs of lesions would not be predicted, nor would it be expected to contribute to lesion pathology. To confirm this, we examined collagen IV accumulation in CM and AVM lesions of multiple patients with CM-AVM1, including 2 patients in whom somatic inactivating second-hit *RASA1* mutations had been identified in lesional tissue. Intracellular collagen IV accumulation was not apparent in ECs of these lesions, again, as predicted (Supplemental Figure 25).

LV abnormalities have also been reported in CM-AVM and may also be explained by impaired angiogenic remodeling of primitive LV plexuses as a consequence of LEC retention of collagen IV (2–7). In addition, *RASA1* is required for the development of LV valves (17). Therefore, second-hit mutations of *RASA1*, if they were to affect a sufficient number of valve-forming LECs at a site of valvulogenesis, could affect leaflet development in that vessel,

which would contribute to lymphatic dysfunction. Formation of LV valves requires coordinated LEC synthesis and deposition of extracellular matrix proteins, including collagen IV, into a leaflet extracellular matrix core to which LECs attach (52, 53). Therefore, an inability of valve-forming LECs to export collagen IV for deposition in this matrix core would provide a logical explanation for failed LV valve development. Furthermore, this would be consistent with the observation that, in the absence of *RASA1*, LECs in valve-forming regions initially upregulate expression of *PROX1*, which is a characteristic of valve-forming LECs, but then undergo apoptosis resulting in failed valve development (17).

The absence of spontaneous BV abnormalities following loss of *RASA1* in mice at any point after birth is consistent with a model in which a continued high rate of collagen IV synthesis by BECs is not necessary for BV function. Exceptions to this would include retinal angiogenesis in newborn mice and pathological angiogenesis in adult mice, in which EC synthesis of collagen IV and deposition in BMs would be necessary. Accordingly, we show here that *RASA1* is required for both processes, and, at least for pathological angiogenesis, our evidence indicates that impaired angiogenesis is, again, consequent to an inability of ECs to export collagen IV. Earlier studies of pathological angiogenesis and retinal angiogenesis in mice indicated that miR-132-mediated downregulation of *Rasa1* mRNA in ECs is required in order for Ras activation and angiogenesis to proceed (54, 55). The current studies do not contradict this notion but instead explore the consequences of complete and permanent loss of *RASA1* in ECs. miR-132-mediated downregulation of *Rasa1* during normal angiogenesis was probably not complete and would be expected to be transient. In contrast, genetic disruption of *Rasa1* would result in chronic uncontrolled activation of Ras in ECs, with distinct downstream consequences. In this regard, it is of note that mice that express constitutively active H-Ras (resistant to RasGAP-mediated inactivation) in ECs develop brain vascular malformations and hemorrhagic stroke (56). Moreover, somatic activating mutations in K-Ras have been identified in the majority of brain AVMs in humans (57). To our knowledge, the effect in mice of induced loss of *RASA1* upon retinal angiogenesis and pathological angiogenesis caused by actively growing tumors had not previously been examined. The current studies, therefore, are the first to our knowledge to address this question.

Notably, *RASA1* was also required for the maintenance of LEC numbers in LV valves in adult mice. We propose that the loss of LECs in adult mouse valves upon *RASA1* loss reflects a requirement for valve leaflet LECs to engage in a high rate of collagen IV synthesis in order to remain attached to leaflets that encounter higher shear stress forces than LV or BV wall ECs (52, 53). Indeed, shear stress has previously been shown to induce collagen IV expression in ECs (58). Whether *RASA1* is also required for the maintenance of BECs in venous valves that would encounter similarly high shear stress forces remains to be determined.

Methods

For a full description of the Methods, see Supplemental Methods.

Mice. *Rasa1^{fl/fl}* and *Rasa1^{fl/fl}/R780Q* mice with and without *U^bErt2^{Cre}* transgenes have been described previously (15–17, 59). *Cdh5^{Ert2Cre}* mice were obtained from Cancer Research UK. *Rasa1^{fl/fl} Cdh5^{Ert2Cre}* mice

were generated through cross-breeding. All mice were on a mixed 129S6/SvEv and C57BL/6 genetic background.

Statistics. Statistical analysis was performed using Student's 1-sample and 2-sample *t* tests or 1-way ANOVA with Dunnett's multiple comparisons post hoc test. A *P* value of less than 0.05 was considered statistically significant.

Study approval. All experiments performed with mice were in compliance with University of Michigan guidelines and were approved by the IACUC of the University of Michigan. All work performed with CM-AVM1 tissue samples was approved by the IRBs at the University of Michigan, Stanford University, and the Medical College of Wisconsin.

Author contributions

DC, PEL, and PDK contributed to the design of the studies. DC performed the majority of experiments, with assistance from PEL.

JT and PN provided CM-AVM1 tissue samples. PN assisted with interpretation of the findings. The manuscript was written by PDK.

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