

Title: Somatic Activating Mutations in *Pik3ca* Cause Sporadic Venous Malformations in Mice and Human

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One Sentence Summary: Mutant *Pik3ca* gives rise to venous malformations

Abstract

Venous malformations (VMs) are painful and deforming vascular lesions composed of dilated vascular channels, present from birth. Mutations in the *TEK* gene, encoding the tyrosine kinase receptor TIE2, are found in approximately half of sporadic (non-familial) VMs, with the cause of the remaining cases unknown. Sclerotherapy, widely accepted as first-line treatment, is not fully

efficient and targeted therapy for this disease remains underexplored. In this study, we have generated a mouse model that faithfully mirrors human VM, through mosaic expression in the embryonic mesoderm of *Pik3ca*^{H1047R}, a constitutively active mutant of the p110 α isoform of PI 3-kinase (PI3K). Endothelial expression of *Pik3ca*^{H1047R} led to hyperproliferation, reduction in pericyte cell coverage of vessels and decreased expression of arteriovenous specification markers. PI3K pathway inhibition with rapamycin normalized endothelial cell (EC) hyperproliferation and pericyte cell coverage in postnatal retinas and led to VM regression *in vivo*. In line with the mouse data, we also report the presence of activating *PIK3CA* mutations in human VMs, mutually exclusive with *TEK* mutations. Our data demonstrate a causal relationship between activating *Pik3ca* mutation and the genesis of VMs, provide the first genetic model that faithfully mirrors the normal etiology and development of this human disease and establish proof-of-principle for the use of PI3K-targeted therapies in VMs.

Introduction

The PI3K family of enzymes signal downstream of a variety of cell surface receptors to regulate multiple cellular functions, including growth and proliferation (1). The p110 α isoform of phosphoinositide 3-kinase (PI3K), encoded by the *PIK3CA* (OMIM 171834) gene, is frequently activated through mutation in cancer (2). Similar activating mutations in *PIK3CA* have recently been linked to non-cancerous disease conditions, such as a spectrum of regional overgrowth disorders (3-6) (which have now been designated under the umbrella term PIK3CA-Related Overgrowth Spectrum (PROS) disease (7)) and isolated (i.e. not associated with overgrowth) lymphatic malformations (LMs) (8). In these conditions, the *PIK3CA* mutations are present from birth and occur in a mosaic pattern, mainly in tissues of mesodermal origin. This contrasts with somatic *PIK3CA* mutations in cancer, which are almost exclusively present in epithelial tissues. The *PIK3CA* mutations present in PROS and LMs are similar to those found in solid tumors, with

the H1047R mutation in the catalytic domain being the most prevalent (8,9). In this study, we report the presence of somatic activating *PIK3CA* mutations in another human disease condition of mesodermal origin, namely isolated VMs. In addition, we created a genetically-modified mouse model that displays mutant *Pik3ca*-driven congenital vascular malformations with features pathognomonic for human VMs.

VMs are the most common vascular malformations, with an overall incidence of 1/5000 (10). VMs are usually congenital lesions that consist of dilated venous channels with scarce mural cell coverage. These VMs can be of different sizes and be present in any tissue, such as subcutaneous tissue or internal organs. VMs are painful and disfiguring, many lead to bleeding and obstruction of organs, and in some cases to localized intravascular coagulopathy and pulmonary embolism (11,12). Treatment of VMs mainly consists of alleviation of symptoms using compression garments when possible, but curative treatment is seldom possible. At present, sclerotherapy is the mainstay therapy to diminish the volume of VMs, but it causes several side effects and is not fully efficient (12).

Mutations in the *TEK* gene, which codes for the endothelial tyrosine kinase receptor TIE2, are known to cause about half of sporadic VMs (13), the cause of the other half remaining unknown. Here, in a cohort of patients diagnosed with sporadic VMs, without any associated overgrowth or other predominant vascular component, we found *PIK3CA*-activating mutations in a mutually exclusive manner to previously identified mutations in *TEK* (14). This was corroborated by a study in the accompanying paper by Castel *et al.* (15) in which *PIK3CA* mutations were found in 25% of the VMs analysed, again in a mutually exclusive manner to *TEK* mutations. The finding that activating mutation in *PIK3CA* plays a key role in the pathogenesis of VMs provides guidance for refined diagnosis for this disease and a possible targeted therapeutic avenue using PI3K pathway inhibitors.

Results

Mosaic *Pik3ca*^{H1047R} expression in embryonic mesoderm leads to VMs in mice

In this study, we set out to investigate the effects of *Pik3ca* mutation in mesodermal tissue in mice. Given that all known disease conditions of mesodermal origin currently associated with *PIK3CA* mutation are congenital or early childhood onset, we induced expression of mutant *Pik3ca* in the embryonic mesoderm. To more precisely model the human disease context, mutant *Pik3ca* expression was induced in a mosaic fashion, in the heterozygous state and from its endogenous promoter (16). We reasoned that such tissue-restricted mosaic induction could also avoid the detrimental effect of ubiquitous expression of mutant *Pik3ca* during embryonic mouse development (17). The mutant used for these experiments is *Pik3ca*^{H1047R} which encodes the H1047R hot-spot mutation found in cancer (2), LMs (8) and PROS (9).

For these studies, we generated a mouse line (referred to as *T-CreER*^{T2}) by introducing a transgene (**Supplementary Fig. 1A**) that drives the expression of a 4-hydroxytamoxifen (4-OHT)-inducible Cre recombinase under the control of the previously characterized promoter of the *T* gene (18). The *T* gene encodes the Brachyury transcription factor that is transiently expressed in the nascent embryonic mesoderm (19). To evaluate Cre activity, we crossed *T-CreER*^{T2} mice with *Rosa26-lacZ* reporter mice (20) that ubiquitously express the floxed *lacZ* gene expression cassette from the *Rosa26* locus (**Supplementary Fig. 1B**), and stained for lacZ expression following treatment of mice with 4-OHT. We administered 4-OHT to pregnant females between embryonic day (E) 7.5 and 10.5. In line with the known temporal expression of the *T* gene (19), induction of Cre led to a widespread lacZ expression in the mesoderm, and was highest at E7.5 (**Supplementary Fig. 1C**). Cre activity at E7.5 was dependent on the 4-OHT dose used and present in a mosaic pattern, except at the higher doses tested (**Supplementary Fig. 1D**).

We next crossed *T-CreER*^{T2} mice to mice that are heterozygous for a Cre-inducible knock-in allele of *Pik3ca*^{H1047R} (Ref. (16)), followed by mosaic *Pik3ca*^{H1047R} induction at E7.5 using different

doses of 4-OHT (**Fig. 1A**). Interestingly, several of these mice [hereafter referred to as MosMes-*Pik3ca*^{H1047R} (Mosaic Mesodermal-*Pik3ca*^{H1047R}) mice] but not *T-CreER*^{T2};*Pik3ca*^{WT} (hereafter referred to as WT) littermate mice, were born with subcutaneous vascular malformations in different body sites (**Fig. 1B**). No apparent overgrowth was observed in other tissues, nor alterations in body weight, organ size or tissue histology (tested up to 6 months of age), compared to WT littermate mice that had also been treated with 4-OHT (**Supplementary Fig. 2; Supplementary Table S1**). This phenotype was not fully penetrant at lower 4-OHT doses, but increased in a dose-dependent manner (**Supplementary Table S2**). The lower doses of 4-OHT tested gave rise to localized vascular lesions, whereas the higher doses, possibly due to the higher probability of targeting multiple EC progenitors, induced multifocal, diffuse and more severe malformations (**Fig. 1B**). High 4-OHT doses also led to a lower than expected Mendelian distribution of mutant mice (**Supplementary Table S3**), with some of the mutant embryos displaying vascular developmental defects (**Supplementary Fig. 3**). Our observation on the lethality and vascular defects in MosMes-*Pik3ca*^{H1047R} embryos are similar to the previously reported phenotypes of ubiquitous or EC-specific expression of *Pik3ca*^{H1047R} in the developing embryo (17).

Computed Tomography Angiography (CT-A) analysis of adult MosMes-*Pik3ca*^{H1047R} mice confirmed the presence of subcutaneous vascular malformations and revealed additional internal vascular malformations in the mesentery (not shown) and urogenital area (**Fig. 1C**). Some of these mice displayed rectal bleeding and phlebectasias of the portal vein and inferior *vena cava* (**Fig. 1C**), similar to those observed in patients with venous vascular malformations (reviewed in Ref. (21)).

Additional scanning of blood flow in the vascular malformation by power Doppler ultrasound indicated that the contribution of flow was primarily venous in origin (slow flow) (**Supplementary Fig. 4**). This was corroborated by histological analysis which showed that the vascular lesions in MosMes-*Pik3ca*^{H1047R} mice were poorly circumscribed, non-encapsulated lesions composed of

blood-filled, predominantly thin-walled, irregular and variably sized blood vessels interposed between normal tissues (**Fig. 1D**). Some blood vessels contained organising fibrin thrombi, with focal interstitial haemorrhage present. Immunostaining of these vascular lesions for lymphatic markers (LYVE-1, PROX-1) revealed the presence of malformed lymphatic vessels (**Supplementary Fig. 5**), however, the lymphatic component was much less prominent than the aberrant blood vessels. This is similar with what is seen in human, where purely vascular malformations are rare and usually contain a mixture of vessel types, with some types being pathologically prominent i.e. blood vessels in the case of VMs (22).

Altogether, these observations point to a diagnosis of VMs in *MosMes-Pik3ca^{H1047R}* mice, with a pathology and disease burden remarkably similar to those observed in humans with VMs.

***PIK3CA* is mutated in human VMs**

Our findings of a causative role of activating *Pik3ca* mutation in VMs in mice led us to explore the presence of *PIK3CA* mutations in human VMs. We analysed *PIK3CA* and *TEK* genes by deep sequencing (mean coverage of 2000x) in human sporadic VMs that had no associated overgrowth or other predominant vascular malformation component. Among the VMs sequenced, 3/13 (23%) were mutant for *PIK3CA* and 6/13 (46%) for *TEK*, with an allelic frequency of 4-13% (**Table 1, Fig 2**). Interestingly, mutations in *PIK3CA* and *TEK* were mutually exclusive. The observed *PIK3CA* mutations are hot-spot mutations in cancer (2), PROS (9) and LMs (8), with the observed *TEK* mutations having previously been described for VMs (23). In line with our observations, Castel *et al.* in the accompanying manuscript (15) found that *PIK3CA* was mutated in 25% of VM cases. Taken together, these findings indicate that *PIK3CA* mutation status could be a relevant diagnostic marker for human VMs and corroborate a causal role of *PIK3CA* mutation in VM generation.

Endothelial activation of *Pik3ca* promotes hyperproliferation in ECs, impairs pericyte coverage and downregulates markers of arteriovenous identity

ECs, being the main cell type in the microvasculature, are thought to be important in VM development. Given the important role for *Pik3ca* in EC biology (24-28), we decided to selectively test the impact of *Pik3ca*^{H1047R} expression in ECs using postnatal retina as model for angiogenesis. To this end, we generated EC-*Pik3ca*^{H1047R} mice by crossing conditional heterozygous *Pik3ca*^{WT/H1047R} mice onto *Pdgfb-iCreER* mice that express 4-OHT-inducible Cre specifically in ECs (Ref. (29)) (**Supplementary Fig. 6**). 4-OHT was administered to EC-*Pik3ca*^{H1047R} pups on postnatal day 1 (P1) and retinal angiogenesis was analyzed five days later (**Fig. 3A**). Staining of P6 retinas with isolectin-B₄ (IB4; which binds specifically to the EC plasma membrane) revealed that endothelial expression of *Pik3ca*^{H1047R} resulted in dramatic hyperplasia, with individual vessels no longer being discernible, correlating with increased numbers and proliferation of ECs (**Fig. 3B, C**, **Supplementary Fig. 7**). Radial expansion of the vascular bed was also decreased upon endothelial expression of *Pik3ca*^{H1047R}, possibly due to EC hyperplasia (**Fig. 3D, E**), whereas sprouting angiogenesis, as measured by number and length of sprouts, was not affected (**Fig. 3D, F**). In addition, at P9 when mouse retinas have started vascular remodeling, EC-*Pik3ca*^{H1047R}-driven hyperplasia persisted and there was no difference in apoptosis (as assessed by expression of cleaved caspase-3) between EC-*Pik3ca*^{H1047R} and *Pik3ca*^{WT} retinas (**Supplementary Fig. 8**), indicating that there was no compensation of *Pik3ca*^{H1047R}-driven hyperplasia by enhanced apoptosis during the remodeling process.

A distinctive feature of human VMs is the poor presence, or even absence, of mural cells (30). Proper homeostasis of vessels requires a tight interplay between ECs and their supporting mural cells, which are perivascular cells represented by two cell types, vascular smooth muscle cells (vSMCs) and pericytes. Both cell types are differently distributed over the vasculature, with vSMCs typically present in large vessels and pericytes mainly localized in the microvasculature (31). In

EC-*Pik3ca*^{H1047R} retinas at P6, pericytes (NG2-positive cells) were conspicuously absent from the vascular front and very sparse in the plexus where they were poorly associated with the vessels, in contrast with *Pik3ca*^{WT} retinas that showed a typical attachment of pericytes to the EC surface with multiple extensions (**Fig. 3G, H**). Defective mural cell coverage was also observed in the VMs from *MosMes-Pik3ca*^{H1047R} mice (**Fig. 1D**).

Pdgfb, which is produced by ECs, is the major attractant for pericytes (32) and is present at lower levels in human *TEK*-mutant VMs (33). The Foxo1 transcription factor, which is negatively regulated by PI3K activity, stimulates Pdgfb expression. In line with this, analysis of total mRNA isolated from retinas and lungs (a tissue highly enriched in ECs) from postnatal P6 mice showed a decrease in Pdgfb expression in EC-*Pik3ca*^{H1047R} mice compared to *Pik3ca*^{WT} mice (**Fig. 3I; Supplementary Fig. 9A**). The pericyte phenotype observed in EC-*Pik3ca*^{H1047R} retinas is reminiscent of that observed in the so-called Pdgfb-retention motif knock-out mice, in which Pdgfb produced by the ECs is not retained at the cell surface (34).

Throughout development and in adult life, arteriovenous specification in ECs is associated with selective expression of specific genes (35). Interestingly, the mRNA expression levels of three genes known to be key in this process, namely COUP-TFII (encoded by the *Nr2f2* gene) and Eph-B4 (encoded by the *Ephb4* gene), which are expressed in venous ECs, and Ephrin-B2 (encoded by the *Efnb2* gene), which is selectively expressed in arterial ECs, were reduced in EC-*Pik3ca*^{H1047R} postnatal retinas and lungs compared to those in *Pik3ca*^{WT} mice (**Fig. 3K; Supplementary 9B**). This finding indicates that endothelial expression of *Pik3ca*^{H1047R} compromises arteriovenous identity of ECs.

Taken together, *Pik3ca*^{H1047R} expression in ECs results in EC hyperproliferation, defective pericyte coverage and loss of arteriovenous identity.

Rapamycin reduces *Pik3ca*^{H1047R} EC hyperproliferation, prevents loss of pericyte coverage and induces regression of *Pik3ca*^{H1047R}-driven VMs *in vivo*

At the moment, there are no targeted therapies for VMs. Rapamycin and its analogs interfere with signaling downstream of PI3K by inhibition of mTOR (36) and are approved for compassionate use in human therapy of vascular anomalies (37-43). To test the effect of rapamycin on *Pik3ca*^{H1047R} ECs *in vivo*, we treated EC-*Pik3ca*^{H1047R} pups with rapamycin first at P1 (administered together with 4-OHT) followed by a second injection at P5 (8 h prior to analysis of retinal angiogenesis; **Fig. 4A**). Drug treatment prevented retinal vascular hyperplasia, reduced the number of ECs and proliferation (**Fig. 4B, C**), and normalised pericyte coverage in both the vascular front and plexus of EC-*Pik3ca*^{H1047R} retinas (**Fig. 4D**). Rapamycin also diminished vessel area and number of ECs in *Pik3ca*^{WT} retinas, with a borderline reduction in EC proliferation (**Fig. 4B, C**), in line with its previously documented anti-angiogenic effects (44).

To take these observations closer to the disease context, adult MosMes-*Pik3ca*^{H1047R} mice with VMs were treated with rapamycin (**Table 2**). The rapamycin dose chosen for this study (4 mg/kg every other day) was based on previous studies using long-term rapamycin treatment (45,46). In WT mice, this dose was found to have no detectable impact on overall health, weight and behavior (**Table 2**). Due to different degrees of severity of the VMs, only mice that were deemed to be sufficiently fit to tolerate the treatment were included. The mice recruited to this therapeutic experiment showed subcutaneous VMs that were heterogeneous in size and location, in some cases accompanied with phlebectasias of main internal veins (inferior *vena cava*, portal vein). Two weeks of rapamycin treatment led to a ~25% reduction in the volume of the VMs, as assessed by CT-A (**Supplementary Fig. 10C**), with a cessation of bleeding from the VM or anus shortly after the start of the treatment with rapamycin. Continued treatment showed progressive regression of the VMs (**Table 2, Fig. 5, Supplementary Fig. 10**). Overall, these observations are in line with data from a study on VM patients with a wide range of lesions, in which a heterogeneous response to

rapamycin treatment was observed, with reduction in VM volume between 10-20%, resulting in symptom improvement and clinical benefit (43).

Overall, our data suggest that PI3K pathway intervention might be an effective therapeutic option for human *PIK3CA*-driven VMs.

Discussion

Here we report on the presence of activating *PIK3CA* mutations in human VMs and on a genetic mouse model for this disease. We demonstrate that somatic, mosaic expression of an activating mutation (H1047R) in *Pik3ca* in the embryonic mesoderm of mice can induce and maintain VMs. Previous mouse models of VM have used transgenic expression of a murine viral oncogene (47) or xenotransplantation into nude mice of either human vascular endothelial cells (HUVECs) transduced with mutant *TEK* (43) or the MS1 immortalized endothelial cell line expressing mutant *Akt1* (48). Xenograft models do not take into account the developmental origin and natural progression of the human disease nor the complex admixture of vessel and stromal cells of human VM lesions. They also show full regression upon rapamycin treatment, which is not observed in our mouse models in which regression was very variable after long-term treatment, in line with observations in human VM patients (43). These xenograft models may therefore be a less predictive model for the human disease. Yet, our mosaic mesodermal VM mouse model shows some limitations for use in standardized uniform cohort therapeutic experiments, namely some degree of embryo lethality, a variable penetrance of the VM phenotype and a wide heterogeneity of VMs in terms of size, localization and expansion. However, these limitations of our mouse model adequately reflect the characteristics of this human disease, with every patient showing a distinct disease pattern, progression and response to therapy, most likely due to the spatially and temporally distinct and mosaic acquisition of the mutation leading to the disease. Of note, activation of PI3K signaling in adult mice, either by ubiquitous expression of mutant *Pik3ca* from its endogenous

promoter (49) or EC-specific expression of transgenic activated *Akt1* (50) leads to increased blood vessel size without inducing VMs. This underscores the concept that VMs arise as a result of genetic errors during embryonic development.

Mechanistically, our data suggest that VMs are likely to develop as a result of *Pik3ca*^{H1047R}-induced enhancement of EC proliferation during embryonic vasculogenesis. Indeed, EC hyperproliferation during vasculogenesis has previously been shown to give rise to vessel hyperfusion, and to result in dilated, dysfunctional vessels, similar to those found in VMs (51,52). Another typical feature of VMs is poor mural cell coverage (30). We also show that *Pik3ca*^{H1047R} expression in ECs inhibits pericyte attachment likely due to the decreased expression of *Pdgfb*, which is necessary for pericyte coverage (53). This might therefore also contribute to the genesis of VMs upon *Pik3ca* mutation. Lastly, *Pik3ca*^{H1047R} might also contribute to the formation of VMs by preventing arteriovenous differentiation, as seen by decreased expression of arteriovenous differentiation markers (54). It is therefore tempting to speculate that VMs could be the result of hyperproliferation of ECs and lack of maturation of primitive capillary beds as a consequence of PI3K pathway overactivation.

At present, it is not clear why widespread mosaic induction of *Pik3ca*^{H1047R} in the mouse mesoderm gives rise to malformations solely in the vasculature and does not induce obvious overgrowth or malformation in other tissue compartments. This might be due to the chosen strategy (timing and tissue location) of *Pik3ca*^{H1047R} gene induction. However, Castel *et al.* in the accompanying manuscript (15) have made similar observations in their models. One explanation could be the previously reported exceptional sensitivity of ECs to PI3K pathway deregulation (24-28).

Human genetic data presented here and in the accompanying manuscript (15) reveal the presence of *PIK3CA* mutations in VMs, mutually exclusive with mutations in the *TEK* gene that were discovered over 20 years ago (30). Our studies thus uncover and document the potential for a

new targeted therapy in human VMs, and the potential for repurposing of p110 α inhibitors currently being developed for cancer treatment. An important consideration is how diagnosis of *TEK* and *PIK3CA* mutations in VMs can influence the treatment of this disease using targeted agents. The wild-type TIE2 receptor tyrosine kinase is known to signal through both the PI3K and MAPK signaling pathways in ECs (55,56). Both pathways have been implicated in signaling downstream mutant TIE2 in ECs, with their relative contribution to biological outputs not being clear at present (14,33,43). *TEK*-mutant cells surprisingly do not respond to TIE2 kinase inhibitors (43), with one explanation put forward that the mutant TIE2 kinase is not very responsive to drugs that were developed against the wild-type TIE2. *TEK*-mutant cells show chronic activation of the PI3K and MAPK pathways and respond very well to inhibition of each of these pathways (14). Our studies identify a subpopulation of patients with *PIK3CA* mutant VMs, which will most likely be non-responsive to TIE2 or MAPK inhibitors. However, like the *TEK*-mutant VMs (43), *PIK3CA*-mutant VMs are expected to respond to PI3K inhibitors or rapamycin. It is also likely that *TEK* mutant VMs will benefit from treatment with PI3K inhibitors in combination with MAPK inhibitors.

In conclusion, our findings in mice have led us to discover the presence of *PIK3CA* mutations in a new human disease. VMs join the growing list of diseases where the presence of activating *PIK3CA* mutations, commonly associated with cancer, leads to tissue malformation, without leading to malignancy (8,9). Our finding that mutation in *PIK3CA* plays a key role in the pathogenesis and maintenance of VMs provides guidance for improved diagnosis for this disease and a possible new targeted therapeutic avenue using PI3K pathway inhibitors.

Materials and Methods

Study design

This study was designed to test the effect of *Pik3ca*^{H1047R} expression in the embryonic mesoderm of mice. We obtained sufficient data on mice to determine that this leads to VMs. To translate these findings to the human context, we tested *PIK3CA* mutation in human VMs and found that this gene is mutated in this disease. For this, well-characterized patients with confirmed VMs and without any associated overgrowth or malformation were recruited at Great Ormond Street Hospital, London, UK. All patients provided informed consent. In addition, to better understand the mechanisms underlying the role of *Pik3ca*^{H1047R} in ECs, we performed mouse retinal angiogenesis studies as a vascular biology read-out. For these experiments, power analysis on preliminary data was performed, suggesting a minimal cohort size of n=4-6 to observe a medium difference with 80% power, depending on the type of experiment. The sample size for each experiment is indicated in the corresponding figure legend. Given that n was <10, non-parametric tests were applied. All mechanistic studies were performed without randomization or blinding. Exclusion criteria were only applied to data shown in Fig. 4 (rapamycin treatment of mouse harbouring VMs): only mice that were deemed to be sufficiently fit to tolerate the treatment were included in the experiment. This is in line with UK Home Office regulations.

Subjects

This study was approved by the UK National Research Ethics Committee. Written informed consent was obtained from all participants or their parents. Tissues from individuals diagnosed as having sporadic VMs, not associated with other syndromes, were investigated. Genomic DNA was extracted from lesions using standard procedures.

Next generation sequencing with preceding target enrichment

Sequencing of *TEK* and *PIK3CA* was performed using Next Generation Sequencing with preceding target enrichment. All equipment and materials were purchased from Life Technologies, ThermoFisher Scientific. In brief, DNA was quantified using Qubit® and libraries were prepared using the Ion AmpliSeq Library 96 Kit 2.0 with 10 ng of genomic DNA in each reaction. This involved an initial PCR reaction to amplify genomic targets using a custom-designed primer pool which provides coverage of all coding regions of *PIK3CA* and *TEK*. Samples were then partially digested prior to ligation of barcode adaptors and emulsion PCR. Enrichment steps were carried out using the Ion OneTouch 200 Template Kit v2 and the amplicon libraries were sequenced on an Ion Torrent Personal Genome Machine system using 318 chips, and bar-coding was applied with an Ion Xpress Barcode Adapters 1–16 Kit. Ion Reporter® software was used to analyse data, or alternatively Bam files were uploaded and viewed in the Integrative Genomics Viewer, Broad Institute (<http://www.broadinstitute.org/igv/>). The mean depth of coverage for sequencing was 2000X.

Reagents

Primary antibodies to the following proteins were used: Erg (ab92513; Abcam), NG2 (AB5320; Merck Millipore), endomucin (sc-65495; Santa Cruz Biotechnology), LYVE-1 (AF2125; R&D Systems), PROX-1 (PRB-238C; Covance) and Cleaved caspase-3 (9664S; Cell Signalling Technology). Secondary antibodies conjugated to Cy3, Cy5 or AlexaFluor 647 were from Jackson ImmunoResearch Laboratories. For immunohistochemistry (IHC), biotinylated antibodies were from Vector Laboratories. Isolectin GS-IB₄ conjugated to AlexaFluor 488 (I21411) or AlexaFluor 568 (I21412) (further referred to as IB₄) were from Life Technologies. Immu-Mount mounting media was from Thermo Scientific. AuroVist 15 nm gold nanoparticles were from Nanoprobes Inc.

Rapamycin used for therapeutic studies was obtained from Merck Millipore. All chemicals, unless otherwise stated, were from Sigma-Aldrich.

Mice

Mice were housed in individually-ventilated cages and cared according to UK Home Office guidelines and legislation, with procedures accepted by the Ethics Committees of University College London and Queen Mary University of London. All mice used were backcrossed on a C57BL/6 background for more than 12 generations. *Pik3ca*^{WT/H1047R} mice harbour a germline *Pik3ca* allele with a conditional H1047R mutation (16). In these mice, *LoxP* sites flank exon 20 of *Pik3ca*, which, upon Cre recombination, is replaced by a downstream copy of exon 20 containing a CAT to AGG change in codon 1047. *Pdgfb-iCreER* mice (29) express an inducible iCreER recombinase from the endogenous *Pdgfb* locus.

Generation of the *T-CreER*^{T2} mouse line

The pTcreER^{T2} vector **Supplementary Fig. S1** comprising the *T* regulatory sequence from -1050 bp to +140 bp (Ref.(18)) was inserted upstream of a *CreER*^{T2} sequence. To eliminate uninduced background activity of Cre in the absence of 4-OHT, the *nls* sequence at the 5' of *CreER*^{T2} was removed. This plasmid was *FspI*-linearized and electroporated into E14TG2a ES cells as described previously (57). After 10 days of selection with Hygromycin (140 µg/ml), resistant clones were isolated, expanded and screened by Southern blot to verify integration of the transgene and to identify ES clones with single and multiple insertions of the transgene. Clones were tested in parallel by *in situ* hybridisation for Cre expression in differentiating ES cells. Four clones (three with a single integration and one with two integrations) that showed the characteristic spatial pattern of *T* expression in differentiating ES cell monolayers (as explained in Ref. (58)) were selected for further analysis. Absence of uninduced background recombination and 4-OHT-induced

Cre recombination in these four clones were tested *in vitro* by transient transfection of a Cre-conditional Egfp transgene (pPHCAG-C2-egfp, a gift from Austin G. Smith, Wellcome Trust, Medical Research Council Cambridge Stem Cell Institute, UK). Clones with single *T-CreER^{T2}* integration showed efficient recombination upon treatment with 0.2 μ M or 1 μ M of 4-OHT, with virtually no background of uninduced recombination. These clones were injected into C57BL/6 host blastocysts and chimeric male offspring mated to C57BL/6 females. F1 heterozygotes were identified by coat color and PCR amplification of the Cre sequence. Offspring were backcrossed for >5 generations to C57BL/6 mice before generating a homozygote *T-CreER^{T2}* line by intercrossing. Following further characterization by crosses with *Rosa26-lacZ* reporter mice (20), one *T-CreER^{T2}* mouse line (number 53) was maintained and established in the homozygote state.

Mesoderm- and EC-specific *Pik3ca^{H1047R}* expression in mice

Mesoderm-specific mosaic expression of *Pik3ca^{H1047R}* (MosMes-*Pik3ca^{H1047R}* mice) was achieved by crossing *Pik3ca^{WT/H1047R}* mice with *T-CreER^{T2}* mice. To induce mosaic Cre activation, pregnant females were given varying doses of 4-OHT (12.5 to 250 μ g, from a stock solution of 10 mg/ml, diluted in 1:1 absolute ethanol-Kolliphor®EL) with 200 μ l of PBS added for intraperitoneal injection at E7.5 (E0.5 relates to the female presence of a vaginal plug indicating that the mating occurred the night before). Progesterone (dissolved in Kolliphor®EL) was co-administered at half the total dose of 4-OHT given, in order to avoid undesirable oestrogen agonist effects of 4-OHT, which can result in late fetal abortions in pregnant mice. Postnatal EC-specific expression of *Pik3ca^{H1047R}* was achieved by crossing *Pik3ca^{WT/H1047R}* with *Pdgfb-iCreER* mice. To induce Cre activation, newborn pups (P1) were injected intragastrically with 5 μ g 4-OHT (dissolved in absolute ethanol).

Histology and IHC

Mouse tissues and VMs were fixed in 4% PFA and embedded in paraffin. 5 µm sections were stained with haematoxylin-eosin using standard histology procedures. Immunostaining was performed using the automated Discovery XT slide staining system (Ventana Medical Systems) at the Molecular Cytology Core in Memorial Sloan Kettering Cancer Center (New York, USA). Briefly, tissue sections were deparaffinised with EZPrep buffer and antigen retrieval was performed with CC1 buffer (Ventana Medical Systems). Sections were blocked for 30 min. with Background Buster solution (Innovex) (for PROX-1 antibody) or 10% rabbit serum (for LYVE-1 antibody) followed by endogenous Avidin/Biotin blocking for 8 min. Then, sections were incubated with specific antibodies for 3 h, followed by incubation with biotinylated secondary antibodies. All slides were scanned using Zeiss Mirax Midi digital slide scanner.

Whole-mount embryo X-gal staining

Freshly isolated E12.5 embryos were fixed in 4% paraformaldehyde on ice for 30 min, washed (3 x 10 min) in PBT (PBS with 0.1% Tween) and stained for 48 h at 30°C in X-gal staining solution (4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM MgCl₂, 1 mg/ml X-gal in PBT). After staining, embryos were washed (3 x 10 min) in PBS, post-fixed in 4% paraformaldehyde for ≥ 1 h and mounted.

Whole-mount embryo immunofluorescence

Freshly isolated E9.5 embryos were fixed in 4% paraformaldehyde overnight at 4°C. After washing in PBT, embryos were dehydrated in increasing concentrations of methanol (50%, 80% methanol/PBT, 100% methanol), rehydrated in decreasing concentrations of methanol, followed by a 30 min wash in Pblec buffer (0.2 mM CaCl₂, 0.2 mM MgCl₂, 0.2 mM MnCl₂ and 2% Triton X-100 in PBS) and overnight incubation at 4°C with antibody to endomucin, diluted 1:20 in Pblec.

After five washes in PBT, embryos were incubated with Cy5-labeled secondary antibody diluted 1:100 in PBS, 0.5% BSA, 0.25% Tween-20. Finally, embryos were washed three times in PBS, post-fixed for 1 min in 4% paraformaldehyde and mounted.

Mouse imaging by CT-A

Mice were anesthetized with an isoflurane/O₂ mix and tail vein cannulated for the delivery of approximately 0.1 ml of gold nanoparticles per 25 g of mouse weight. Animal temperature was maintained at 37°C and physiological monitoring was recorded. Whole body CT scans were acquired with mice placed in the supine position 4 h post injection of gold nanoparticles using a nanoScanPET/CT scanner (Mediso, Hungary) with a 50 kVP X-ray source, 300 ms exposure time in 720 projections with an acquisition time of 15 min. CT images were reconstructed in voxel size 68 x 68 x 68 µm using Nucline (Mediso, Hungary) software. Image analysis and 3D visualization was performed using VivoQuant (inviCRO version 1.23patch3) software.

Mouse imaging by Doppler ultrasound

A Vevo 2100 imaging station (VisualSonics Inc., Toronto, ON, Canada) was used for ultrasound imaging. A VisualSonics MS-550D (central frequency 40 MHz, axial resolution 40 µm, lateral resolution 90 µm, image depth 15 mm, image width 14 mm) was used for data acquisition. Mice were anesthetized with an isoflurane/O₂ mix and placed on a heated imaging stage maintained at 37°C. The body temperature, heart rate and respiratory rate were closely monitored throughout each imaging session. Abdominal hair was removed using a depilatory cream (Veet, Reckitt Benckiser, UK) and rinsed with warm water. Pre-warmed ultrasound gel (UltrasoundGel.co.uk) was then placed on the abdomen. Flow velocity waveforms of vascular lesions were obtained by locating with color Doppler, power Doppler and then placing the pulsed wave Doppler sample gate within the vessels and at the appropriate angle relative to flow direction. 2D-guided M-mode images were

obtained to measure maximum vessel diameter. Image analysis was performed using the VisualSonics Vevo 2100 software package.

Retinal angiogenesis

Newborn pups (P1) were injected intragastrically with 5 µg 4-OHT (dissolved in absolute ethanol). P6 eyes were harvested and fixed 1 h in 4% PFA at 4°C and washed in PBS. Retinas were dissected, stained with IB4 and flat-mounted, or processed for immunostaining (59). For immunostaining, fixed retinas were blocked and permeabilized using blocking buffer (1% BSA, 0.3% Triton X-100 in PBS) for 4 h at 4°C, then incubated overnight at 4°C with the corresponding primary antibodies in blocking buffer and washed in PBT (3 x 10 min) (anti-Erg (1/300), anti-NG2 (1/200), anti-Cleaved-caspase 3 (1/50). After 30 min incubation in Pblec, retinas were incubated for 2 h with the corresponding labelled secondary antibody and IB4 in Pblec, washed with PBT (3 x 10 min), post-fixed with 4% paraformaldehyde for 1 min and flat-mounted.

***In vivo* EC proliferation**

Pups were injected intraperitoneally with 50 µl of EdU (diluted to 2 mM in 1:1 DMSO-PBS) 2 h before harvesting of the eyes. Detection of EdU was done using Click IT® EdU Alexa Fluor®647 (Life Technologies) according to the manufacturer's instructions. Retinas were then labelled with the corresponding antibodies and IB4 as described above. Retinas were imaged with a Zeiss LSM 700 confocal microscope. EdU-positive nuclei were counted as proliferating ECs in a 100 µm² region at the angiogenic front or plexus using the ImageJ software.

RNA extraction and quantitative PCR

Total RNA was extracted from P6 mouse retinas and lungs using the RNeasy Plus MiniKit (#74134, QIAGEN) according to the manufacturer's protocol. Samples were quality-controlled and

quantified using a Nanodrop ND-100 spectrophotometer. Reverse transcription of total RNA was performed using a High-Capacity cDNA Reserve Transcription Kit (#4368814, Applied Biosystems™). Real-time quantitative PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) and the proprietary TaqMan Gene Expression assay FAM/TAMRA primers (Applied Biosystems) for *Pdgfb* (Mm00440678_m1), *Ephb4* (Mm00438750_m1), *Efnb2* (Mm01215897_m1), *Nr2f2* (Mm00772789_m1), *Cadh5* (Mm00486938_m1), and *Hprt* (Mm00446968_m1). *Pdgfb*, *Ephb4*, *Efnb2*, *Nr2f2*, mRNA expression in retinas was normalized to the mRNA of the EC-specific *Cdh5* (VE-Cadherin) housekeeping gene.

***In vivo* rapamycin treatment**

Rapamycin was dissolved at 10 mg/ml in absolute ethanol and used as such for intraperitoneal injection in pups at P1 and P6 at 4 mg per kg. For adult mice, the 10 mg/ml stock of rapamycin in absolute ethanol was dissolved further in 5% polyethylene glycol (PEG-400) and 5% Tween-80 in PBS to 0.5 mg/ml. Mice were dosed with 4 mg rapamycin per kg by intraperitoneal injection every other day for a total of 28 days. This dose was based on previous studies using long-term rapamycin treatment (45,46).

Statistics

GraphPad Prism 6 was used for all statistical analysis. Means were compared between two groups using the nonparametric Mann–Whitney U test. All statistical tests and sample size have been indicated in the figure legends.

List of Supplemental Materials

Supplemental Data include 10 figures and 3 tables.

Figure S1. Cre-mediated mosaic recombination in the *T-CreER^{T2}* mouse line.

Figure S2. Body weight over time and organ size at 6-month-old of WT and *MosMes-Pik3ca^{H1047R}* mice.

Figure S3. Whole-mount endomucin-stained E9.5 embryos dosed with 170 µg 4-OHT at E7.5.

Figure S4. *MosMes-Pik3ca^{H1047R}* mouse with a subcutaneous vascular malformation and dilated vein.

Figure S5. Representative images of immunostaining for lymphatic markers (PROX-1, LYVE-1) in *MosMes-Pik3ca^{H1047R}* mouse VMs.

Figure S6. Genetic strategy to activate *Pik3ca^{H1047R}* in ECs.

Figure S7. Expression of VE-Cadherin in P6 EC-*Pik3ca^{H1047R}* retinas.

Figure S8. Apoptosis in P9 EC-*Pik3ca^{H1047R}* retinas.

Figure S9. Expression of *Pdgfb* and arteriovenous markers in EC-*Pik3ca^{H1047R}* lungs.

Figure S10. Rapamycin treatment of *MosMes-Pik3ca^{H1047R}* mice.

Supplementary Table 1. List of organs and tissues subjected to histological examination (H&E staining) in WT and *MosMes-Pik3ca^{H1047R}* mice.

Supplementary Table 2. Percentage of *MosMes-Pik3ca^{H1047R}* mice with VMs after dosing pregnant females with different doses of 4-OHT.

Supplementary Table 3. Percentage of live WT and *MosMes-Pik3ca^{H1047R}* offspring obtained after administration of different doses of 4-OHT to pregnant females.

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Note added in proof

While this work was under review, the presence of *PIK3CA* mutations in human VMs has been reported: N. Limaye, J. Kangas, A. Mendola, C. Godfraind, M.J. Schlöge, R. Helaers, L. Eklund, L.M. Boon, M. Vikkula, Somatic activating *PIK3CA* mutations cause venous malformation. *American Journal of Human Genetics* **97**, 914–921 (2015).

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Figures

Figure 1

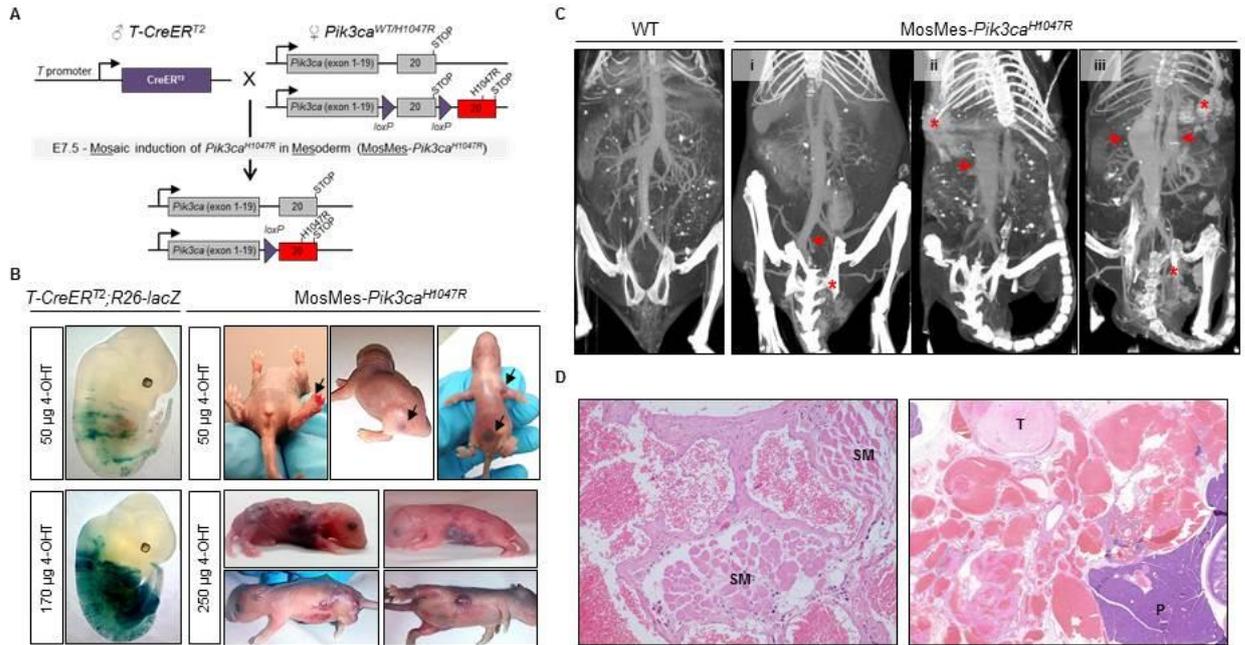


Figure 1. Mosaic expression of *Pik3ca^{H1047R}* in embryonic mouse mesoderm induces vascular malformations in offspring. **(A)** Genetic strategy for mosaic *Pik3ca^{H1047R}* induction in the embryonic mesoderm. *T-CreER^{T2}* mice were crossed with *Pik3ca^{WT/H1047R}* mice that have a germline *Pik3ca* allele with a conditional H1047R mutation in exon 20. Mosaic recombination in the mesoderm is induced by a single intra-peritoneal injection of a low dose of 4-OHT to pregnant mice at E7.5. **(B)** Left, E12.5 *T-CreER^{T2};R26-lacZ* mouse embryos from pregnant mice injected with the indicated dose of 4-OHT at E7.5 and stained for β -galactosidase (β -gal) activity. Right, Representative photographs of P1-P5 *MosMes-Pik3ca^{H1047R}* pups with congenital vascular malformations (arrowed in the top panel), born to pregnant mice injected with the indicated 4-OHT

dose at E7.5. (C) CT-A scans of adult mice four hours after intravenous injection of gold nanoparticles. The WT mouse (left) shows normal vascular anatomy, whereas *MosMes-Pik3ca^{H1047R}* mice display dilatation of the left common iliac vein (arrow) and VM in the urogenital area (asterisk) (mouse #1); subcutaneous VM (asterisk) and dilatation of the inferior *vena cava* (arrow) (mouse #2); subcutaneous and urogenital VMs (asterisks) and dilatation of the inferior *vena cava* and portal vein (arrows) (mouse #3). (D) Representative haematoxylin and eosin (H&E)-stained sections of a subcutaneous (left) and a deep, mesenteric (right) VMs in *MosMes-Pik3ca^{H1047R}* mice, showing abnormal, enlarged and irregular vascular channels, most containing blood and organising fibrin thrombi (T), some interposing between skeletal muscle (SM) and other tissue structures. No cytological atypia was observed. (Original magnifications x20-x200; P, pancreas).

Figure 2



Figure 2. Clinical photographs of patients with VMs mutant for *PIK3CA*. (A) Patient 9 showing VMs (*PIK3CA*^{H1047L}) in the lower lip and tongue. (B) Patient 13 with VM (*PIK3CA*^{E545K}) in the left hand.

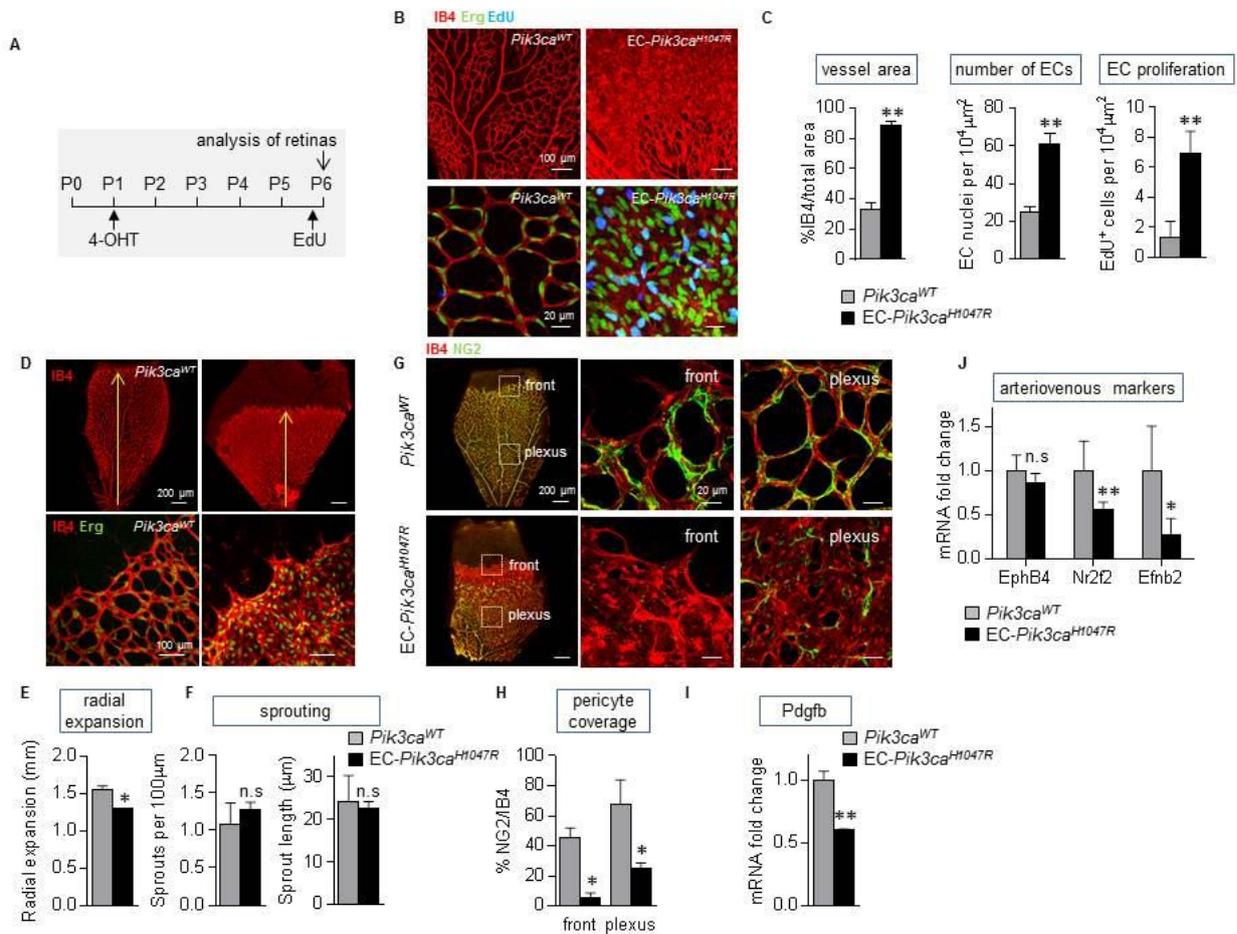
Figure 3

Figure 3. Endothelial activation of *Pik3ca* promotes hyperproliferation in ECs, impairs pericyte coverage and downregulates markers of arteriovenous identity. **(A)** Schematic of the 4-OHT and EdU administration regime used. **(B)** Representative flat-mounted *Pik3ca*^{WT} and *EC-Pik3ca*^{H1047R} P6 retinas stained with IB4 (red, revealing ECs), antibody to the Erg transcription factor (nuclear marker of ECs; green) and labelled with EdU (blue). **(C)** Quantitative analysis of the retina vessel area (assessed by IB4 staining), EC numbers (assessed by staining for Erg) and number of proliferating ECs (cells positive for both EdU and Erg). Data represent mean ± SEM. ***p* ≤ 0.01, (Mann-Whitney U test). *n*=6/genotype. **(D)** Representative flat-mounted control and *EC-Pik3ca*^{H1047R} P6 retinas stained with IB4 and antibody to the Erg transcription factor. **(E)** Quantification of the radial expansion of vasculature in retinas. Data represent mean ± SEM. **p* <

0.05 (Mann-Whitney U test). n=6/genotype. **(F)** Quantification of the number of sprouts at the vascular front per unit length, and the length of sprouts. Data represent mean \pm SEM. n.s., not significant, $p > 0.05$, (Mann-Whitney U test). n=6/genotype. **(G)** Flat-mounted *Pik3ca*^{WT} and EC-*Pik3ca*^{H1047R} retinas showing vasculature (IB4; red) and pericytes (stained for NG2, a membrane proteoglycan found in pericytes; green). **Right**, higher magnification of sections highlighted. **(H)** Quantification of pericyte coverage in the vasculature of retinas (assessed by % of NG2 staining over IB4 staining). Data represent mean \pm SEM. * $p < 0.05$, (Mann-Whitney U test). n=6/genotype. **(I)** *Pdgfb* mRNA expression levels in EC-*Pik3ca*^{H1047R} P6 retinas. Data represent mean \pm SEM. ** $p < 0.01$ (Mann-Whitney U test). n=5/genotype. **(J)** *Ephb4*, *Nr2f2* and *Efnb2* mRNA expression levels in EC-*Pik3ca*^{H1047R} P6 retinas. Data represent mean \pm SEM. n.s., not significant, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, (Mann-Whitney U test). n=5/genotype.

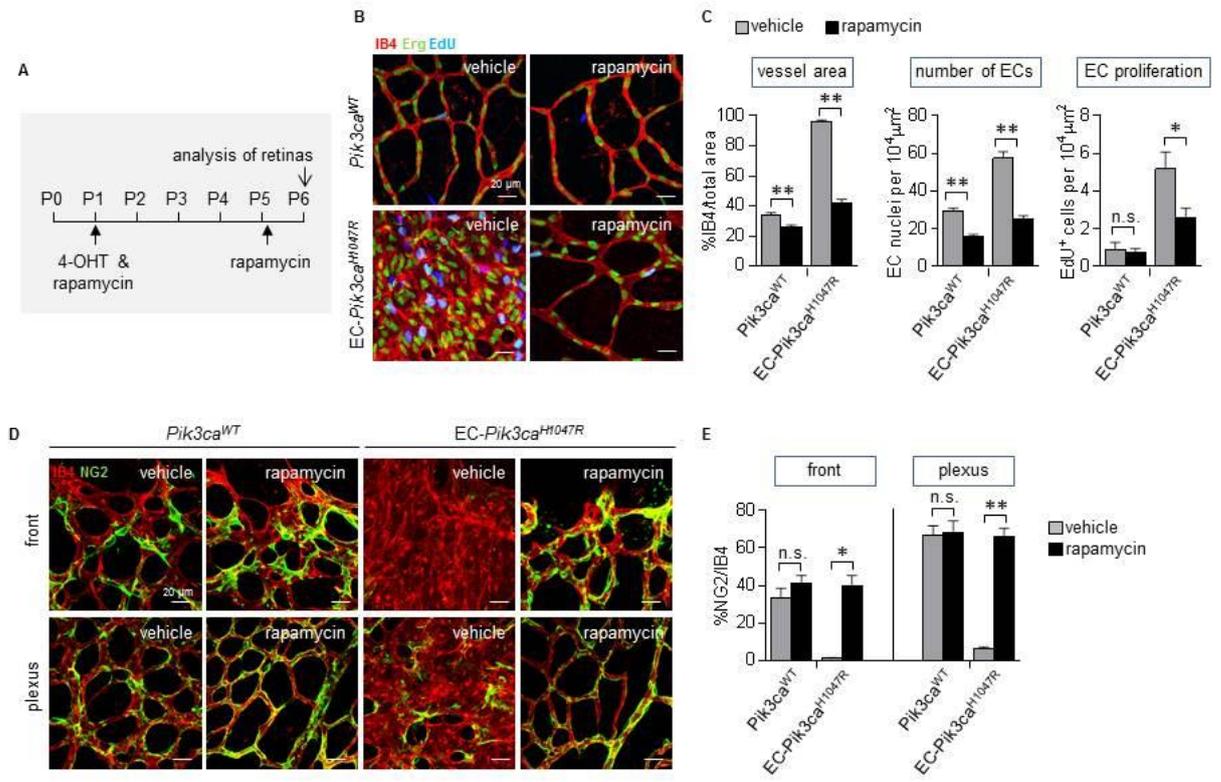
Figure 4

Figure 4. Rapamycin reduces *Pik3ca*^{H1047R} EC hyperproliferation, prevents loss of pericyte coverage in postnatal retinas. **(A)** Schematic of the 4-OHT and rapamycin administration regime used for analysis of retinal angiogenesis. **(B)** Representative flat-mounted P6 retinas from vehicle and rapamycin-treated *Pik3ca*^{WT} and EC-*Pik3ca*^{H1047R} pups. Retinas are stained with IB4 (red), antibody to the Erg transcription factor (green) and labelled with EdU (blue). **(C)** Quantitative analysis of the retina vessel area (assessed by IB4 staining), EC numbers (assessed by staining for Erg) and number of proliferating ECs (cells positive for both EdU and Erg). Data represent mean ± SEM. n.s., not significant, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, (Mann-Whitney U test). $n = 6$ /genotype. **(D)** Representative flat-mounted P6 retinas from untreated *Pik3ca*^{WT} and EC-*Pik3ca*^{H1047R} pups and rapamycin-treated EC-*Pik3ca*^{H1047R} pups showing vasculature (IB4; red) and pericytes (stained for NG2; green). **(E)** Quantitative analysis of pericyte coverage in the vascular front and plexus of

retinas (assessed by % of NG2 staining over IB4 staining). Data represent mean \pm SEM. n.s., not significant, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, (Mann-Whitney U test). n=6/genotype.

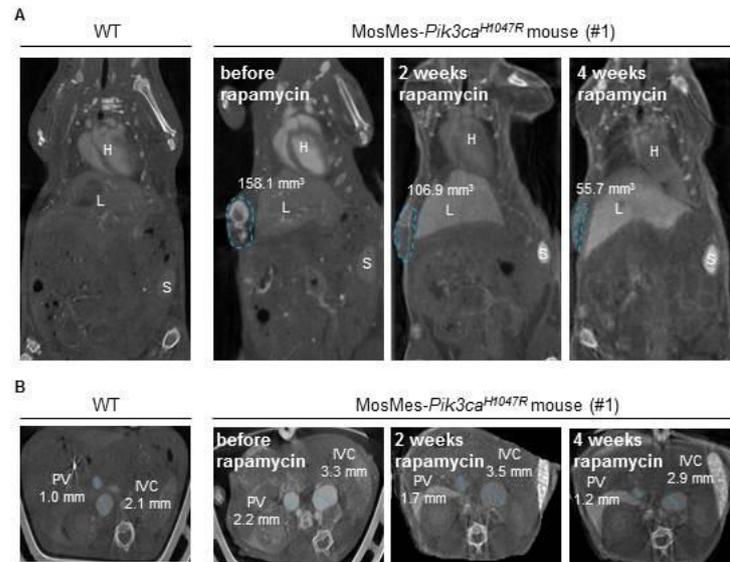
Figure 5

Figure 5. CT-A images of *MosMes-Pik3ca^{H1047R}* mouse #1 (Table 2) upon rapamycin treatment. (A) Measurement of the volume of the subcutaneous VM (circled in blue) from CT-A images, showing the volumes of the VM. H, heart; L, liver; S, spleen. (B) Measurement of the average diameter of the inferior *vena cava* and portal vein from CT-A images of an untreated WT mouse and a *MosMes-Pik3ca^{H1047R}* littermate mouse, before and after 2 and 4 weeks of rapamycin treatment. IVC, inferior *vena cava*; PV, portal vein.

Table 1. *PIK3CA* and *TEK* mutations in human sporadic VMs

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13
Age	4 years	20 years	3 years	15 years	3 years	10 years	9 years	15 years	14 years	10 years	20 years	3 years	6 years
Sex	M	M	M	M	F	F	F	F	F	F	F	M	M
Location of VM	Upper lip	Sole of foot	Scapular area	Arm, chest, extensive	Face	Cheek	Face	Arm	Tongue, lower lip, neck	Tongue, lower lip	Hand	Foot	Hand
<i>PIK3CA</i>	WT	E545K	WT	WT	WT	WT	WT	WT	H1047L	WT	WT	WT	E545K
Percent <i>PIK3CA</i> mutant allele	NA	6%	NA	NA	NA	NA	NA	NA	4%	NA	NA	NA	4%
<i>TEK</i>	G1115X	WT	Y897C, R918H	WT	WT	WT	L914F	L914F	WT	L914F	WT	Y897C, R918S	WT
Percent <i>TEK</i> mutant allele	9%	NA	5%	NA	NA	NA	4%	13%	NA	5%	NA	11%	NA

NA: not applicable

Table 2. Treatment of Mes-*Pik3ca*^{H1047R} mice with rapamycin

Mouse #	1	2	3	4	5	6
Sex	m	m	m	f	m	f
Phenotype	Subcutaneous VM at right flank, phlebectasia of portal vein and inferior <i>vena cava</i>	Subcutaneous VM at left flank, phlebectasia of portal vein and inferior <i>vena cava</i>	Subcutaneous VM at the base of the tail, below left hind limb	Phlebectasia of portal vein and inferior <i>vena cava</i> . No VM detected by CT-A.	WT control	WT control
Age at starting treatment*	8-week-old	8-week-old	10-week-old	5-week-old	10-week-old	5-week-old
Duration of treatment	4 weeks	2 weeks	6 weeks	4 weeks	6 weeks	4 weeks
Outcome	VM volume reduction: 32.4% at 2 weeks; 65% at 4 weeks. Normalization of phlebectasias in portal vein and inferior <i>vena cava</i> . Cessation of bleeding of lesion after 1 week of treatment. VM no longer detected upon necropsy 4 weeks after treatment.	No volume reduction, mouse became ill after 2 weeks and had to be sacrificed in line with UK Home Office regulations.	VM volume reduction: 18.7% at 2 weeks, 28.4% at 4 weeks, 37% at 6 weeks. Cessation of rectal bleeding immediately after treatment	Normalization of portal vein and inferior <i>vena cava</i> phlebectasia	Overall healthy	Overall healthy

* Mice were treated with rapamycin, 4 mg/kg, every other day