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Supplementary Materials for

Somatic *PIK3CA* mutations as a driver of sporadic venous malformations

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Published 30 March 2016, *Sci. Transl. Med.* **8**, 332ra42 (2016)
DOI: 10.1126/scitranslmed.aaf1164

The PDF file includes:

Supplementary Materials and Methods

Study design

This study was designed to confirm the effect of *PIK3CA* H1047R expression in the genesis of VM, as suggested by our serendipitous findings. We characterized the prevalence of *PIK3CA* mutations in human specimens of VM using targeted next-generation sequencing. Our cohort of patients was obtained from Memorial Sloan-Kettering Cancer Center (US) and from the Hospital de la Santa Creu i Sant Pau (Spain) and were reviewed by a board-certified pathologist (C.R.A). All patients provided informed consent. We further confirmed our findings using different mouse models that express the *PIK3CA* transgene in all cells and consistently develop VM. For these experiments we used cohorts of n=45 mice. Littermates were used as controls. For efficacy studies with different inhibitors, animals were randomized with at least n=8-10 tumors/arm.

Mice

The following mouse strains were obtained from The Jackson Laboratories: R26-LSL-*PIK3CA*^{H1047R} (016977), CAG-CreER (017595), Tie2-Cre (004128), and UBC-CreER (008085). The Sprr2f-Cre strain (01XNA) was acquired from the National Cancer Institute (NCI) Mouse Repository and was previously described (*23*). The R26-LSL-LacZ reporter strain was available at the Mouse Transgenic Core of MSKCC.
Mice were housed and maintained in a controlled environment at the Research Animal Resource Center (RARC) of MSKCC, and all procedures were performed in accordance

with Institutional Guidelines under the protocol number 12-10-019. Tamoxifen (Harlan; TD.130856) was administered through the food chow at approximately 40 mg × kg^{-1}. In all the experiments using mice, WT littermates were used as a control.

For allograft studies, vascular lesions isolated from *PIK3CA*$^{CAG\text{-}CreER}$ mice were rinsed with ice-cold PBS, minced, resuspended with cold 1:1 DMEM/Matrigel, and injected subcutaneously into six-week-old female athymic *Foxn1*^{nu} nude mice. Once VM reached a volume of 250-350 mm^3, mice were treated with the PI3Kα inhibitor BYL719 (Chem Express; 25 mg × kg^{-1} in 0.5% carboxymethylcellulose (Sigma), daily p.o.), everolimus (Stand Up to Cancer pharmacy; 10 mg × kg^{-1} in PBS, daily p.o.), or propranolol (Sigma; 40 mg × kg^{-1} in PBS, daily p.o.), over 7 days. Propranolol dose was chosen on the basis of FDA guidelines for the conversion of animal doses to Human Equivalent Doses (HED), using the dose previously described for Infantile Hemangioma (*28*) (6 mg × kg^{-1}) as a reference. The following formula was applied:

$$HED(mg\ \times\ kg^{-1}) = mouse\ dose\ (mg\ \times\ kg^{-1}) \times \left(\frac{mouse\ weight\ (kg)}{human\ weight\ (kg)}\right)^{0.33}$$

After one week of treatment, VM were measured and harvested for further analysis. VM volume was calculated using the following formula:

$$VM\ volume\ (mm^3) = \frac{width\ (mm)^2\ \times\ lenght\ (mm)}{2}$$

For the treatment of pregnant *PIK3CA*$^{Tie2\text{-}Cre}$ mice, BYL719 was administered orally three times at E7.5, E8.5, and E9.5. Two hours after the last treatment, embryos were harvested and characterized.

For the formulation of topical preparations, we used the cream base Versatile (Fargon), which allows the incorporation of active principles and is rapidly absorbed into the skin of the mice. We prepared two different formulations using BYL719 at 1% (w/w) that were:

- Free BYL719: This formulation was generated by mixing BYL719 powder with the base cream and homogenized using an Ultra-Turrax to achieve a uniform distribution of the active principle.
- Soluble BYL719: For this formula, we dissolved BYL719 powder in a small volume of DMSO (400 mg × mL^{-1}). We incorporated this concentrated solution into the base cream with gentle agitation, achieving a homogeneous distribution of the active principle.

The topical formulations were applied into the mouse lesions on a daily basis, and VM growth was assessed as described above.

Histology and IHC

Mice and human tissue was fixed, dehydrated, paraffin-embedded, sectioned at 5 microns, and H&E-stained using standard histology protocols.

Prussian blue staining was performed by incubating deparaffinized tissue sections in a mixture of 20% hydrochloric acid aqueous solution and 10% aqueous solution of potassium ferrocyanide over 20 minutes. Slides were washed three times using distilled water and counterstained using Nuclear Fast Red staining.

For LacZ staining, mouse tissue was fixed using a solution containing 0.2% glutaraldehyde, 1% formaldehyde, and 0.02% NP-40 in PBS for 1 hour at 4°C. After washing, tissue was incubated in X-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$, 1 mg/ml X-gal in PBS) overnight at room temperature. For

histology, LacZ stained tissues were fixed in 3.7% formaldehyde-PBS and embedded in paraffin, sectioned, and counterstained with Nuclear Fast Red staining.

IHC and IF staining was performed using Discovery XT processor (Ventana Medical Systems) at the Molecular Cytology Core (MSKCC).

Tissue sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), and antigen retrieval was performed with CC1 buffer (Ventana Medical Systems). Sections were blocked for 30 minutes with Background Buster solution (Innovex) or 10% normal rabbit serum (for LYVE-1 antibody) followed by avidin/biotin blocking for 8 minutes. Sections were stained with the following antibodies:

- **CD31**: (Dianova, DIA-310, 1 $\mu g \times mL^{-1}$) for 5 hours, followed by 60 minutes of incubation with biotinylated goat anti-rat IgG (Vector Laboratories, PK-4004) at 1:200 dilution. The detection was performed with Streptavidin-HRP D (Ventana Medical Systems), followed by incubation with Alexa Fluor 488 (Invitrogen, T20922).
- **BrdU**: sections were pretreated with Protease K (5 $\mu g \times mL^{-1}$) and incubated with anti-BrdU (Roche, 1170376, 1 $\mu g \times mL^{-1}$) for 5 hours, followed by a 60-minute incubation with biotinylated horse anti-mouse IgG (Vector Laboratories, MKB-22258). The detection was performed with Streptavidin-HRP D (Ventana Medical Systems), followed by incubation with Alexa Fluor 594 (Invitrogen, T20935). When two markers were used, staining was performed consecutively according to the procedure described above. After staining, slides were counterstained with DAPI (Sigma, D9542, 5 $\mu g \times mL^{-1}$) for 10 min and coverslipped with Mowiol.
- **GLUT-1:** Sections were incubated with anti-GLUT-1 antibody (Chemicon, AB1340, 0.5 $\mu g \times mL^{-1}$) for 4 hours, followed by 60 minutes of incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories, PK6101) at 1:200 dilution.

- **LYVE-1:** Sections were incubated with anti-LYVE-1 antibody (R&D Systems, AF2125, 1 $\mu g \times mL^{-1}$) for 3 hours, followed by 60 minutes of incubation with biotinylated rabbit anti-goat IgG (Vector Laboratories, BA-5000) at 1:200 dilution.
- **WT-1:** Sections were incubated with anti-WT-1 antibody (Abcam, Ab89901, 1:50 dilution) for 1 hour, followed by 60 minutes of incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories, PK6101) at 1:200 dilution.
- **Prox-1:** Sections were incubated with anti-Prox-1 antibody (Covance, cat#PRB-238C, 1ug/ml) for 5 hours, followed by 60 minutes of incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories, PK6101) at 1:200 dilution.
- **Ki67:** Sections were incubated with anti-Ki-67 antibody (Ventana, clone 30-9; catalog# 790-4286, prediluted) for 1 hour, followed by 60 minutes of incubation with biotinylated rabbit anti-goat IgG (Vector Laboratories, BA-5000) at 1:200 dilution. For GLUT-1, LYVE-1, WT-1, Prox-1, and Ki-67 IHC, detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer instructions, followed by counterstaining with hematoxylin (Ventana Medical Systems) and coverslipping with Permount (Fisher Scientific).

All slides were scanned using Mirax Midi Slide Scanner (Zeiss). For immunofluorescence staining, confocal images were acquired using the Leica SP8 confocal microscope. An expert sarcoma pathologist (C.R.A.) blindly reviewed the histology for mice and humans.

microCT scan

For microCT scan, mice were injected with gold nanoparticles (1115, AuroVist) in the tail vein. Two hours after injection, mice were anesthetized with isoflurane. The cone beam microCT scans were acquired on a Nano SPECT/CT Plus system (Mediso). Each scan averaged approximately 5 minutes using 240 projections with an exposure time of 1,000

ms and angular increment of 1 degree. The X-ray tube voltage and current were 55 kVp and 145 mA, respectively. The reconstructed voxel dimensions were 73x73x73 mm. Images were reconstructed and analyzed using the InVivoScope software provided on the Nano SPECT/CT Plus.

D-dimer measurement

Blood was collected from control animals or mice bearing VM grafts through cardiac terminal puncture. Plasma was obtained by centrifugation in EDTA-treated tubes, and 50 µL were used for the quantification of D-dimers using the mouse-specific ELISA kit from MyBiosource (MBS2022411) according to the manufacturer's protocol.

Cell-based assays

Human dermal ECs were derived from healthy face skin tissue and were a gift from Dr. Joyce E. Bischoff (Boston Children's Hospital). HUVEC cells were a gift from Dr. Valiente (CNIO). ECs were maintained in supplemented EBM-2 medium (CC-3162) following the manufacturer's protocol. ECs were transduced with the retroviruses pBabe-EV, pBabe-*PIK3CA* (WT), and pBabe-*PIK3CA* (H1047R), obtained from Addgene (ID: 1764, 12523, and 12524, respectively). Briefly, GP2-293 (Clontech) cells were transiently co-transfected with retroviral pBabe and CMV-VSV-G plasmids. 48 hours after transfection, supernatants were harvested, supplemented with 8 $\mu g \times mL^{-1}$ of polybrene, and used to infect ECs. 48 hours after infection, ECs were selected with 0.5 $\mu g \times mL^{-1}$ of puromycin.

Proteins were extracted using RIPA buffer, and Western blots were performed using standard methods. Antibodies for Western blots were: pAKT (S473) (Cell Signaling; 4060), pAKT (T308) (Cell Signaling; 2965), AKT (Cell Signaling; 9272), pS6K (T389) (Cell Signaling; 9205), S6K (Cell Signaling; 2708), pS6 (S240/4) (Cell Signaling; 5364),

pS6 (S235/6) (Cell Signaling; 4858), S6 (Cell Signaling; 2217), actin (Cell Signaling; 4970). Tube formation assay was performed using Matrigel-coated 48 multiwells as a substrate. 10^4 ECs were seeded in EBM-2 medium without FBS and analyzed after 6 hours for tube formation.

For EdU incorporation, cells were serum-starved overnight and labeled for 4 hours using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (C-10425) following the manufacturer's instructions. Flow cytometry was performed using FACSCalibur (BD Bioscience).

Proteome Profiler Human Angiogenesis Antibody Array (R&D; ARY007) was used to assess the differences in the expression of angiogenesis-related cytokines in our EC models as described by the manufacturer's protocol. All the expression experiments were carried out after overnight serum starvation. ANG-2 quantification was validated by RT-PCR and ELISA.

For validation of mRNA expression, we prepared cDNA using the iSpcript Bio-Rad cDNA synthesis kit (1708891). cDNA was amplified by quantitative PCR using SYBR Select Master Mix (Applied Biosystems) with the ViiA 7 Real-Time PCR system. ANGPT-2 expression was normalized to the expression of actin (ACTB). Primers used for mRNA expression were:

ANGPT2: Fw- CTCAGCTAAGGACCCCACTG; Rv- CATCCTCACGTCGCTGAATA

ACTB: Fw-CGTCTTCCCCTCCATCGT; Rv-GAAGGTGTGGTGCCAGATTT

For the quantification of secreted ANG-2, medium was collected from the different ECs and assayed using the Human Angiopoietin-2 Quantikine ELISA Kit (R&D; DANG20).

Inhibitors used in the in vitro experiment were: BYL719 (Chem Express; 1 μM), MK2206 (Selleckchem; 1 μM), and everolimus (S2UC; 50 nM).

Patients

Thirty-two archival samples from sporadic VM patients were collected for the sequencing study. Age, gender, and localization of the lesions are detailed in Table S1. All patients provided informed consent and had their samples sequenced under the MSKCC IRB protocol number 02-060. When required, samples were macrodissected to increase the cellular content. All samples were GLUT-1 and WT-1 negative by IHC. DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen).

Targeted exome sequencing (MSK-IMPACT).

MSK-IMPACT was performed as previously described (*37*). Briefly, DNA derived from 32 venous malformation patients was further subjected to deep coverage targeted sequencing of 341 key cancer-associated genes. MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets) involves hybridization of barcoded libraries to custom oligonucleotides (Nimblegen SeqCap) designed to capture all protein-coding exons and select introns of 341 commonly implicated oncogenes, tumor suppressor genes, and members of pathways deemed actionable by targeted therapies. Barcoded sequence libraries were prepared using 100 - 250 ng genomic DNA (Kapa Biosystems) and combined into equimolar pools of 13-21 samples. The captured pools were subsequently sequenced on an Illumina HiSeq 2000 as paired-end 100-base pair reads, producing a median of 588-fold coverage per tumor.

Sequence data were demultiplexed using CASAVA, and reads were aligned to the reference human genome (hg19) using BWA and post-processed using the Genome Analysis Toolkit (GATK) according to GATK best practices.

MuTect and GATK were used to call single-nucleotide variants and small indels, respectively. Candidate mutations were manually reviewed using the Integrative Genomics Viewer (IGV) to eliminate likely false positive calls. Because matched normal DNA was not available, tumors were compared to a pool of 10 unmatched normal

samples to eliminate common polymorphisms and systematic sequencing artifacts. Additional sequence variants detected in the 1000 Genomes Project in >1% of individuals were flagged as likely germline. We observed that all variants detected at known somatic mutation hotspots (such as *PIK3CA* H1047R) had mutant allele fractions between 3 and 20%, supporting the observation that the tumor purities were relatively low. Consequently, we speculated that new sequence variants observed in >45% of reads were possibly germline mutations.

Because *TEK* is not included in our MSK-IMPACT assay, we recaptured the libraries from MSK-IMPACT using specific probes targeted against *TEK* (Integrated DNA Technologies). Bait sequences can be found in Table S2. Captured pools were sequenced and analyzed as described above.

FISH

FISH analysis was performed on whole paraffin sections and tissue microarray (TMA) using a three-color probe mix as described:

PIK3CA (3q26.32) (Red; clone RP11-682A21, RP11-737O18,RP11-959N23).

PTEN (10q23) (Orange; clone RP11-380G5, RP11-165M8).

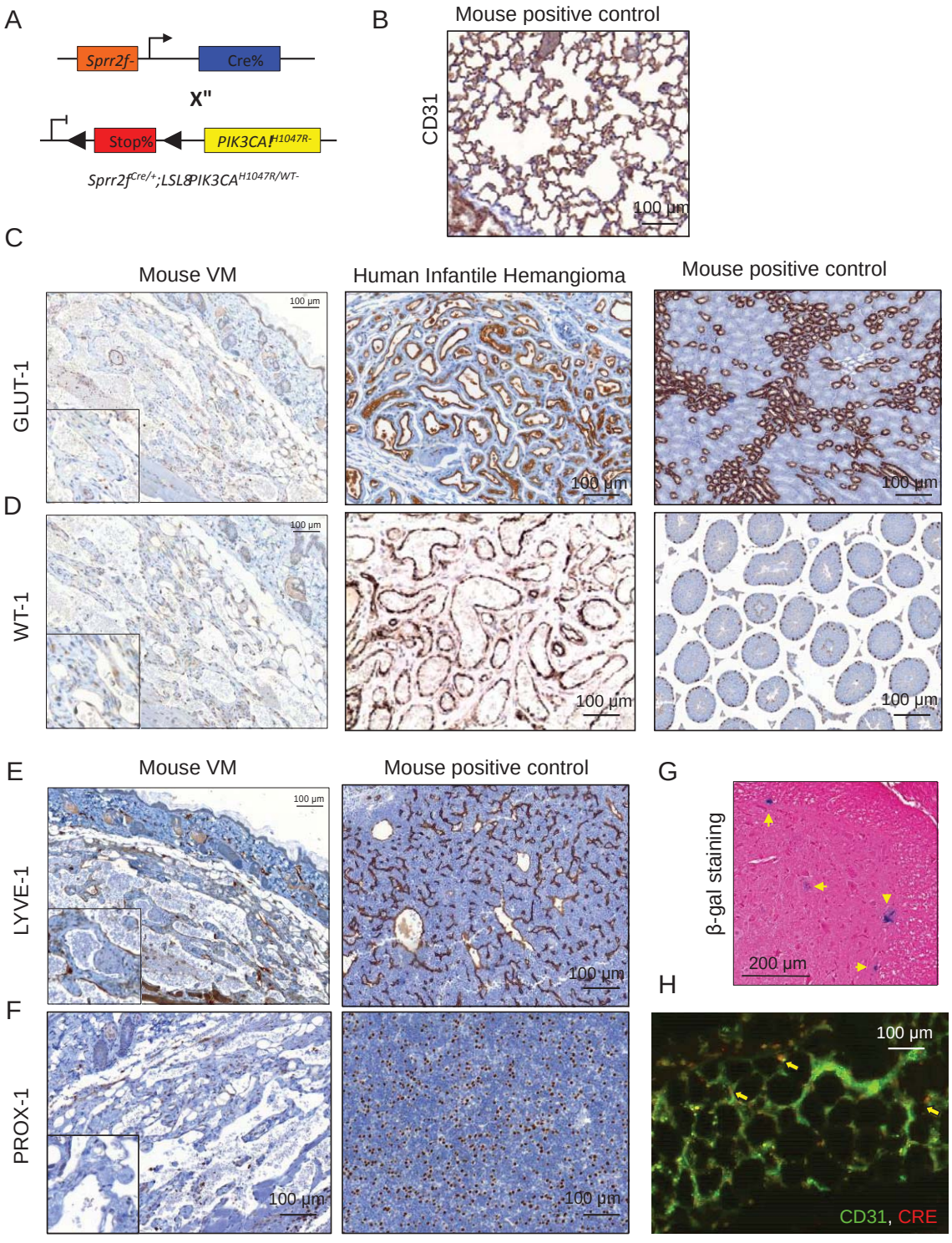
Control (3p11-12) (Green; clone RP11-312H1, RP11-81P15).

Clone DNA was labeled by nick translation using fluorochrome-conjugated dUTPs (Enzo Life), supplied by Abbott Molecular Inc. Hybridization, post-hybridization washing, and fluorescence detection were performed according to standard procedures. Slides were scanned using a Zeiss Axioplan 2i epifluorescence microscope equipped with a megapixel CCD camera (CV-M4+CL, JAI) controlled by Isis 5.2 imaging software (Metasystems). The entire section was scanned under a 63X objective to assess the quality of signal hybridization, and representative regions were imaged through the depth of the tissue (compressed/merged stack of 12 z-section images taken at 0.5 μm

intervals under the red, green, or orange filter). For each case/core, a minimum of 2-6 captured image fields (>50 cells) were selected and signals enumerated. To obtain copy number (mean signal) per cell, the total number of signals for each gene/locus was divided by the total number of cells within the field(s). Only intact cells and cells with at least one signal each for 2/3 loci were selected. Cut-off values for copy number gain and loss were established from the control samples (normal hepatic tissue and placental tissue). Amplification of *PIK3CA* was defined as *PIK3CA*: control ratio of ≥2.0 or >10 *PIK3CA* copies independent of control locus. Cells with ~3-5 copies and ~6-10 copies were considered to be polysomic and high-polysomic, respectively. A mean signal of ≤1.0 copies/cell was considered as true loss of *PTEN* or *PIK3CA*.

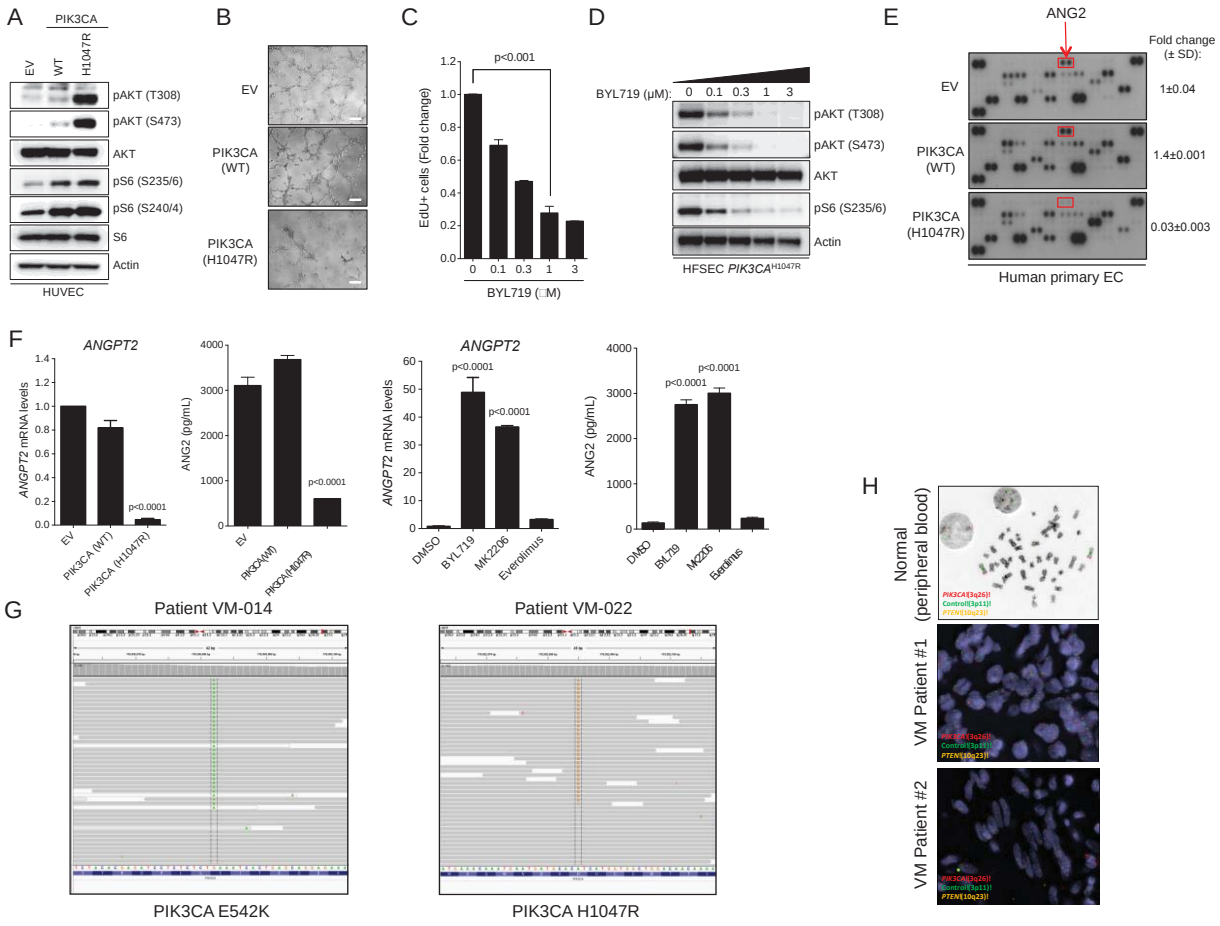
Statistics

Student's t test was used to assess the statistical differences between the treatments and control arms and the effects of the *PIK3CA* mutation in the phenotype in Figures 2C, 3J, 3K, 4B-F. Disease-free survival plots were analyzed using the Mantel-Cox log-rank test.


A
Sprr2f-
Cre%
X"
Stop%
PIK3CA!H1047R-
Sprr2f Cre/+;LSL8PIK3CA H1047R/WT-
B
Mouse positive control
CD31
100 µm
C
Mouse VM
Human Infantile Hemangioma
Mouse positive control
GLUT-1
100 µm
D
WT-1
100 µm
E
Mouse VM
Mouse positive control
LYVE-1
100 µm
F
PROX-1
100 µm
G
β-gal staining
200 µm
H
100 µm
CD31, CRE

Supplementary Figure 1. Histologic characterization of *PIK3CA*$^{Sprr2f\text{-}Cre}$ mice

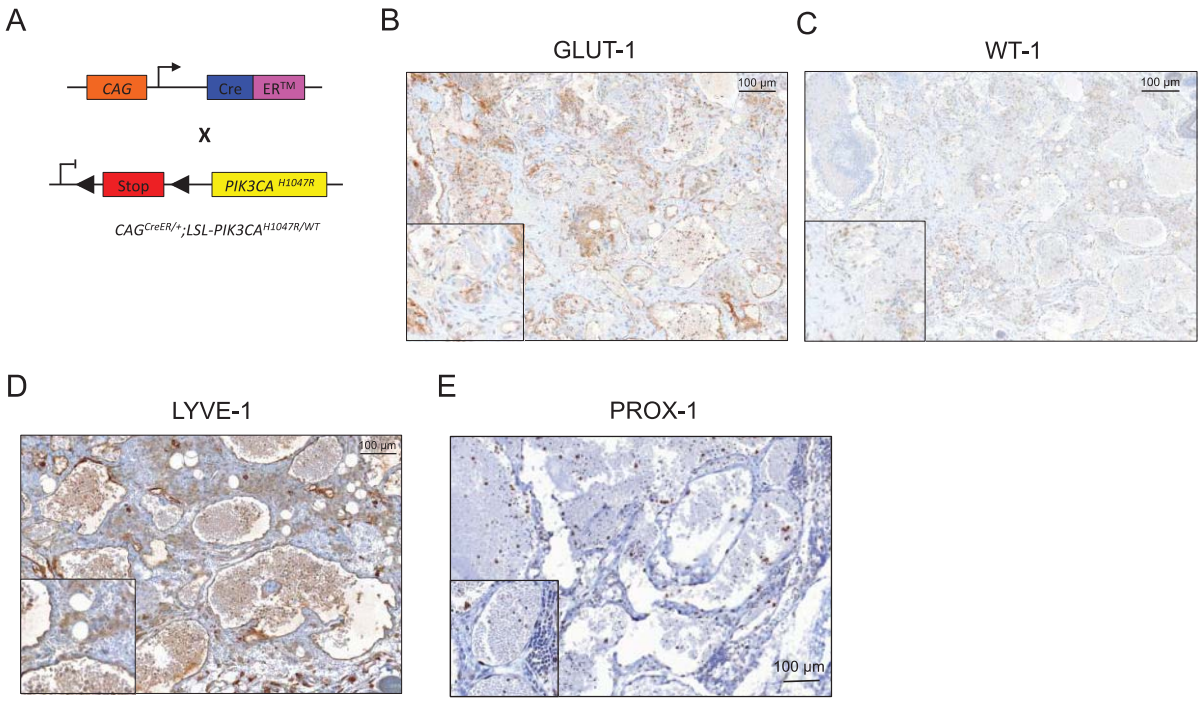
A. Schematic representation of the genetic strategy used to generate *PIK3CA*^{Sprr2fCRE} mice. **B.** Lung tissue from mouse serves as a positive control for CD31 IHC. **C.** GLUT1 IHC for a *PIK3CA*^{Sprr2fCRE} mouse skin lesion (left panel) and a human infantile hemangioma (middle panel). Note the specific staining in the ECs lining the lumen. Mouse positive control for GLUT-1 IHC is shown (kidney; right panel). **D.** WT1 IHC for a *PIK3CA*^{Sprr2fCRE} mouse skin lesion (left panel) and a human infantile hemangioma (middle panel). Note the specific staining in the ECs lining the lumen. Mouse positive control for WT-1 IHC is shown (Sertoli cells of the testis; right panel). **E.** LYVE-1 IHC for a *PIK3CA*^{Sprr2fCRE} mouse skin lesion (left panel). Mouse positive control for LYVE-1 IHC is shown (embryonic liver; right panel). Note that inflammatory cells stain positive for LYVE-1. **F.** PROX-1 IHC for a *PIK3CA*^{Sprr2fCRE} mouse skin lesion (left panel). Mouse positive control for PROX-1 IHC is shown (embryonic liver; right panel). Note that inflammatory cells stain positive for PROX-1. Insets (C-F) represent 20X magnification. **G.** β-galactosidase staining in a spinal section of the LacZ$^{Sprr2f\text{-}Cre}$ mice. Arrows indicate cells with positivity for β-gal staining. **H.** Double immunofluorescence for CD31 and Cre in a VM derived from the *PIK3CA*$^{Sprr2f\text{-}Cre}$ mice. Arrows indicate nuclei that are positive for Cre and CD31 staining.



Supplementary Figure 2. *PIK3CA* mutation in ECs

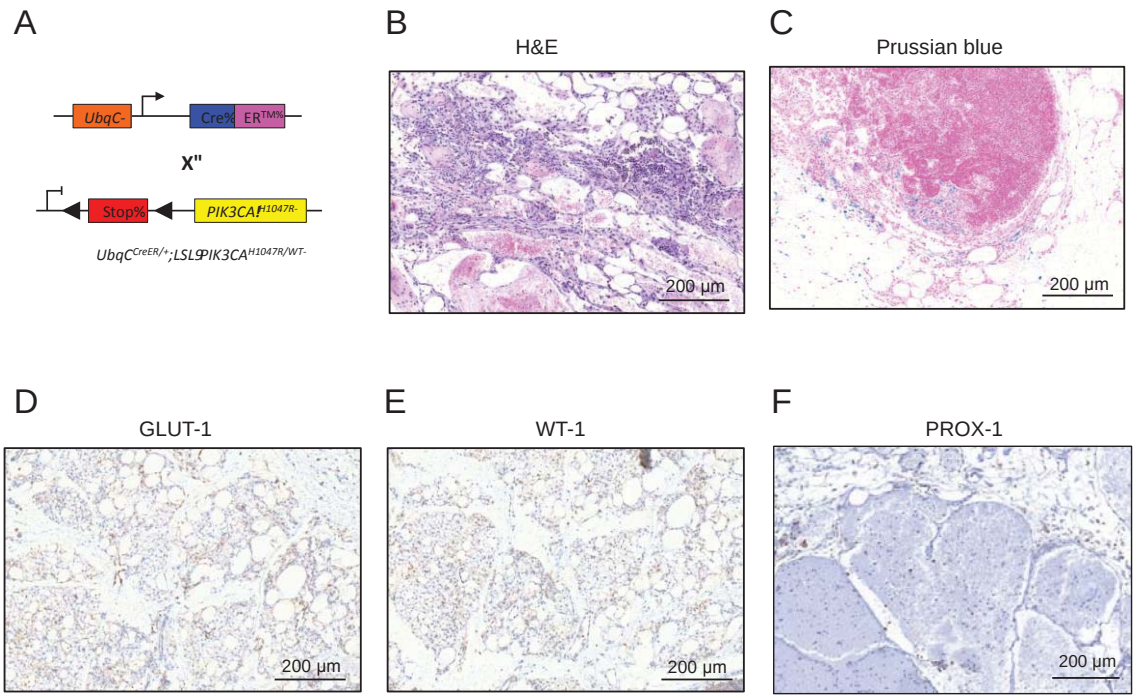
A. Western blot of human HUVECs infected with empty vector (EV), *PIK3CA* WT, or H1047R mutation and probed with the indicated antibodies. Cells were serum-starved overnight before lysis. **B**. Representative images from the tube formation assays of HUVECs infected with empty vector (EV), *PIK3CA* WT, or H1047R mutation and serum-starved overnight before seeding. Photos were taken 8 hours after seeding. Note the reticular network formed in the EV and *PIK3CA* WT cells that fails to form in the PIK3CA H1047R mutant cells. **C.** BYL719 dose-response EdU incorporation assay in primary ECs transduced with the PIK3CA H1047R mutation. P-value was calculated using Student's t-test at 1 μM. Graph indicates fold change ± SD. N=2 biological replicates. **D.**

Western blot of primary ECs transduced with the PIK3CA H1047R mutation and probed with the indicated antibodies. Cells were treated for 4 hours with the indicated doses of BYL719. **E.** Antibody arrays including angiogenesis-related cytokine probes were hybridized with lysates (500 µg) from human primary ECs infected with empty vector (EV), *PIK3CA* WT, or H1047R mutation. Densitometry quantification was performed with ImageJ software and is indicated as a fold change. **F.** Angiopoietin-2 mRNA (*ANGPT2*) and protein (ANG2) concentrations in human primary ECs infected with empty vector (EV), *PIK3CA* WT, or H1047R mutation, and then serum-starved overnight and analyzed by RT-qPCR or ELISA, respectively (left 2 graphs). P-value was calculated using Student's t-test. Graphs indicate mean concentrations (ELISA) and fold change (mRNA) ± SEM. N=3 biological replicates. Angiopoietin-2 mRNA (*ANGPT2*) and protein (ANG2) concentrations in human primary ECs infected with *PIK3CA* H1047R mutation, serum-starved, and treated overnight with BYL719 (1 µM), MK2206 (1 µM), or everolimus (50 nM), then analyzed by RT-qPCR or ELISA, respectively (right 2 graphs). P-value was calculated using Student's t-test. Graph indicates mean concentrations (ELISA) and fold change (mRNA) ± SEM. N=3 biological replicates. **G.** Integrated Genome Viewer (IGV) snapshot of the representative *PIK3CA* hotspot mutations in the helical (E545K) and kinase (H1047R) domains from two different patients. Note the presence of WT and mutant reads, which in our patients yielded an allele frequency of 5-10%. **H.** Representative microscopic images of FISH for *PIK3CA* (red) and *PTEN* (orange) from normal peripheral blood (control) and two VM patients. *CEP3* (green) is used as a control probe.



Supplementary Figure 3. Histologic characterization of *PIK3CA*$^{\text{CAG-CreER}}$ mice

A. Schematic representation of the genetic strategy used to generate *PIK3CA*$^{\text{CAG-CreER}}$ mice. **B.** GLUT-1 IHC for a *PIK3CA*$^{\text{CAG-CreER}}$ mouse skin lesion. **C.** WT-1 IHC for a *PIK3CA*$^{\text{CAG-CreER}}$ mouse skin lesion. Note that these stains (GLUT-1 and WT-1) are negative as compared to the infantile hemangioma samples shown in fig. S1. The specificity of these antibodies in mouse tissue is shown in fig. S1. **D.** LYVE-1 IHC for a *PIK3CA*$^{\text{CAG-CreER}}$ mouse skin lesion. **E.** PROX-1 IHC for a *PIK3CA*$^{\text{CAG-CreER}}$ mouse skin lesion. Note that these stains (LYVE-1 and PROX-1) are negative as compared to the positive controls shown in fig. S1 and that inflammatory cells stain positive for these markers. Insets (B-E) represent 20X magnification.

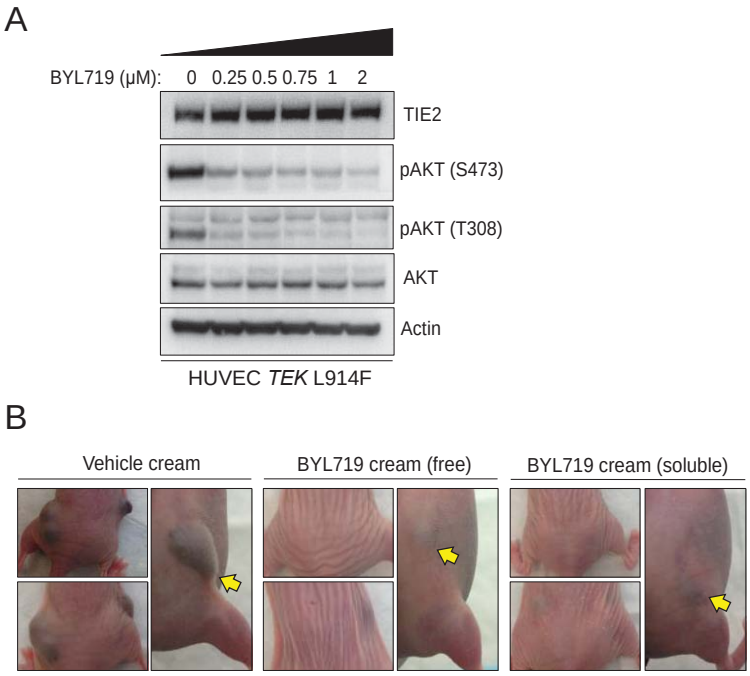


Supplementary Figure 4. Histologic characterization of *PIK3CA*$^{\text{UBC-CreER}}$ mice

A. Schematic representation of the genetic strategy used to generate *PIK3CA*$^{\text{UbqC-CreER}}$ mice. **B.** Representative H&E staining for a skin VM lesion. **C.** Prussian blue staining for a VM lesion from a *PIK3CA*$^{\text{UbqC-CreER}}$ mouse. **D.** GLUT-1 IHC for a *PIK3CA*$^{\text{UbqC-CreER}}$ mouse skin lesion. **E.** WT-1 IHC for a *PIK3CA*$^{\text{UbqC-CreER}}$ mouse skin lesion. **F.** PROX-1 IHC for a *PIK3CA*$^{\text{UbqC-CreER}}$ mouse skin lesion. Note that these stains (GLUT-1, WT-1, and PROX-1) are negative as compared to positive controls shown in fig. S1 and that inflammatory cells stain positive for these markers.

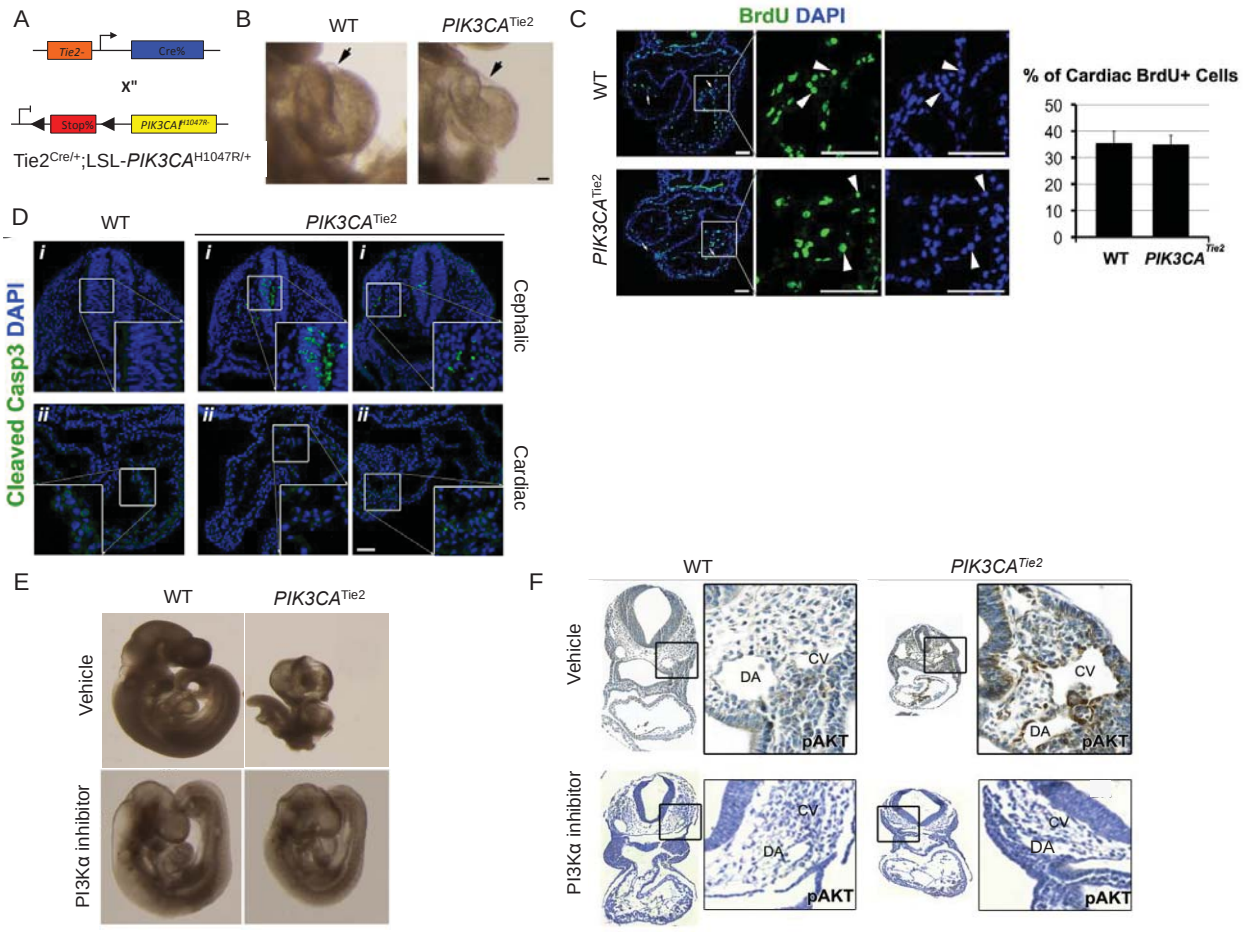
Supplementary Figure 5. Cell proliferation in mouse VM with or without in vivo treatments

A. Ki-67 IHC for a *PIK3CA*$^{CAG\text{-}CreER}$ mouse VM lesion. Arrows indicate positivity in EC nuclei. **B.** Representative images depicting proliferation by Ki67 staining (red; magnified field) in normal and VM vessels stained with CD31 (green). DAPI is shown in blue. **C.** Representative images depicting proliferation by BrdU incorporation (red; magnified field) in the VM treated with vehicle or PI3Kα inhibitor for one week (BYL719, 50 mg × kg^{-1}; daily p.o.). Images were acquired for 10 different biological replicates. **D.** Representative images depicting proliferation by BrdU incorporation (red; magnified field) in the VM treated with vehicle, everolimus (10 mg × kg^{-1}; daily p.o.), or propranolol (40 mg × kg^{-1}; daily p.o.) for one week. Images were acquired for 8 different biological replicates.



Supplementary Figure 6. Treatment of VM with PI3K inhibitors

A. Western blot of HUVEC cells transduced with *TEK* L914F mutation and probed with the indicated antibodies. Cells were treated for 4 hours with the indicated doses of BYL719. **B.** Representative images of the allograft VM treated with the topical formulation of BYL719. Free and soluble refer to the two different preparations of the topical formula. Arrows indicate the area affected by the VM allograft.



Supplementary Figure 7. Histological assessment of *PIK3CA*$^{\text{Tie2-Cre}}$ embryos

A. Schematic representation of the genetic strategy used to generate the *PIK3CA*$^{\text{Tie2-Cre}}$ mice. **B.** Pericardial cavity (arrow) was normal in E9.0 *PIK3CA*$^{\text{Tie2-Cre}}$ embryos, suggesting normal cardiac function at this stage. Scale bar is 100 μm. **C.** BrdU (green) incorporation assay in the heart of WT and *PIK3CA*$^{\text{Tie2-Cre}}$ E9.0 embryos. Quantification is shown as the mean percentage ± SD. (WT = 35.5% ± 4.4, n = 475 from 3 different embryos; *PIK3CA*$^{\text{Tie2-Cre}}$ = 34.9% ± 3.5; n = 413 from 3 embryos; p = ns). Arrowheads indicate representative nuclei from the heart that are positive for BrdU incorporation. Scale bar is 100 μm. DAPI is shown in blue. **D.** Cleaved Caspase 3 (green) expression in cross-sections of E9.5 embryos. At the dorsal region (i), staining is negative in wild-

type embryos (0.4% ± 0.2; n = 821 cells from 3 embryos) but is increased in *PIK3CA*$^{Tie2\text{-}Cre}$ embryos (19% ± 6.0; n = 754 from 3 embryos; $p<0.001$ by Student's t-test). In contrast, cleaved Caspase 3 staining is negative at the cardiac region (ii) (WT = 0.6% ± 0.3; n = 444 from 3 embryos; *PIK3CA*$^{Tie2\text{-}Cre}$ = 0.5% ± 0.3; n = 428 from 3 embryos; p = ns). Values are mean ± SD. Scale bar is 100 μm. DAPI is shown in blue. **E.** Embryonic phenotype of WT and *PIK3CA*$^{Tie2\text{-}Cre}$ embryos treated with PI3Kα inhibitor. For morphological studies upon PI3K inhibition, a minimum of 3 dissections/treatment were performed. **F**. pAKT IHC in sections from WT and *PIK3CA*$^{Tie2\text{-}Cre}$ embryos treated with vehicle or PI3Kα inhibitor. For pAKT histologic studies, we used a minimum of 4 embryos for each condition.

Patient	Sex	Age	Localization	*TEK* mutation	Reads (Mutant/Total)	Allele Frequency (%)	*PIK3CA* mutation	Reads (Mutant/Total)	Allele Frequency (%)	Other mutations	Sequencing Coverage	Notes
VM-003	M	41	Soft tissue, orbit								838.0	No mutations detected
VM-004	F	14	Muscle, buttock	Y897H; R918L	15/208; 12/187	7,21%; 6.03%	I143V	18/115	15.65%	AKT2 (K181M)	243.5	
VM-005	M	72	Subcutaneous, finger							FOXL2 (P257T); BCOR (P326T)	814.0	
VM-006	F	3	Muscle, calf	Y897N; R918C	47/543; 50/560	8.66%; 8.93%				MLL2 (Q2819R)	588.0	
VM-007	F	51	Muscle, thigh							ATM (G1818V)	807.0	
VM-008	F	20	Muscle, thigh				H1047 R	48/889	5.40%		630.0	
VM-009	M	41	Bone, L5							AKT3 (R247C)	586.2	This sample presents an epithelioid morphology not typical of VM.
VM-010	F	52	Bone, skull							MAP3K1 (H468Q)	594.0	
VM-011	F	17	Subcutaneous, elbow							TGFBR2 (S527I); PHOX2B (247_252del)	454.0	
VM-012	M	64	Muscle, thigh							IRS2 (373_377del)	541.0	
VM-013	M	20	Muscle, thigh								484.7	No mutations detected
VM-014	M	50	Muscle, thigh				E542K	18/242	7.74%	MDC1 (C1599G)	343.0	
VM-015	M	51	Muscle, thigh				H1047 R	20/653	3.10%		523.7	
VM-019	F	68	Post Mediastinum							GNAQ (Q209P)	587.4	
VM-020	F	12	Muscle, calf	L914F	34/481	7.07%					637.3	
VM-021	F	18	Muscle, thigh	L914F	80/567	14.11%				TERT (441_442del)	602.1	
VM-022	F	34	Muscle, thigh				H1047 R	81/857	9.45%		634.6	
VM-023	F	48	Muscle, thigh							MAP2K1 (K57N)	596.0	
VM-024	M	43	Muscle, thigh								564.0	No mutations detected
VM-025	M	42	Muscle, deltoid				E542K	28/442	6.34%		588.1	
VM-026	M	32	Muscle, thigh	Y897C; R918H	44/470; 54/442	9.36%; 12.22%					549.7	
VM-027	F	37	Muscle, paraspinal	Y897C; R918H	28/479; 38/465	5.85%; 8.17%				MED12 (Q2113_Q2114ins QQHQ)	533.8	
VM-028	F	36	Muscle, calf								649.5	No mutations detected
VM-029	F	67	Skin, Face	L914F	16/217	7.37%				NF1 (C324S)	451.4	
VM-030	M	6	Skin, Thigh				E542K	22/200	11%		560.6	
VM-031	F	13	Skin, finger	L914F	26/621	4.19%					915.5	
VM-032	F	3	Skin, finger							MED12 (Q2113_Q2114ins QQHQ)	712.3	
VM-033	F	33	Skin, hand				C420 R	30/311	9.60%		691.5	
VM-035	F	7	Lip	L914F	16/400	4.00%				MDC1 (207_214del)	598.4	
VM-036	M	25	Skin, Thorax	L914F	61/408	14.95%					778.3	
VM-037	F	63	Skin, finger								460.0	No mutations detected
VM-042	M	54	Skin, neck, and oral mucosa	L914F	26/256	10.16%					427.0	

Supplementary Table 1. Clinical features and genomic findings in VM patients.

This table includes the age, gender, location of VM, mutations identified, and allele frequencies for the *PIK3CA* and *TEK* mutations.

SequenceName	Sequence	Start	Stop
475_439815_7010(TEK)_1a_1	/5Biosg/TTTGTGGAAACTGGATGGAGAGATTTGGGGAAGCATGGACTCTTTAGCCAGCTTAGTTCTCTGTGGAGTCAGCTTGCTCCTTTCTGGTAAGGTTTGGCTTTATTTTTTTTTAATTTAGTAT	27109554	27109674
475_439816_7010(TEK)_2a_1	/5Biosg/ATTATTGTCTCTCTTTCCTTTTAGGAACTGTGGAAGGTGCCATGGACTTGATCTTGATCAATTCCCTACCTCTTGTATCTGATGCTGAAACATCTCTCACCTGCATTGCCTCTGGGTGGC	27157804	27157924
475_439816_7010(TEK)_2a_2	/5Biosg/GCCCCCATGAGCCCATCACCATAGGAAGGGACTTTGAAGCCTTAATGAACCAGCACCAGGATCCGCTGGAAGTTACTCAAAGATGTGACCAGAGAATGGGCTAAAAAAGTTGTTTGGAAGA	27157924	27158044
475_439816_7010(TEK)_2a_3	/5Biosg/GAGAAAAGGCTAGTAAGATCAATGGTGCTTATTTCTGTGAAGGGCGAGTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAAGGTAACATGCCCCTAAGTTTTGGGC	27158044	27158164
475_439817_7010(TEK)_3a_1	/5Biosg/AAAGCTTCCTTCCTACCAGCTACTTTAACTATGACTGTGGACAAGGGAGATAACGTGAACATATCTTTCAAAAAGGTATTGATTAAAGAAGAAGATGCAGTGATTTACAAAAATGGTGAG	27168488	27168608
475_439818_7010(TEK)_4a.1_1	/5Biosg/TGTTTCAGTGTGACCTACGGTTCTTCACTCTTCCCTCTTACTAGGTTCCTTCATCCATTCAGTGCCCCGGCATGAAGTACCTGATATTCTAGAAGTACACCTGCCTCATGCTCAGCCCCA	27169430	27169550
475_439818_7010(TEK)_4a.1_2	/5Biosg/GGATGCTGGAGTGTACTCGGCCAGGTATATAGGAGGAAACCTCTTCACCTCGGCCTTCACCAGGCTGATAGTCCGGAGTAAGTGATGGAGAGGCCACCATTTGTGATGGTGTAGTTGTTA	27169550	27169670
475_439819_7010(TEK)_5a.1_1	/5Biosg/ATGTGTTGAGCGAATGCGCTCTACTCACCACAGCCTTGTTTTCCTTAACAAAAGGATGTGAAGCCCAGAAGTGGGGACCTGAATGCAACCATCTCTGTACTGCTTGTATGAACAATGGTG	27172559	27172679
475_439819_7010(TEK)_5a.1_2	/5Biosg/TCTGCCATGAAGATACTGGAGAATGCATTTGCCCTCCTGGGTTTATGGGAAGGACGTGTGAGAAGGGTAAGTAAAGAGACTTGATAAGTAAGCTGTGGATTTAAAAAGCCATCGTTGCTG	27172679	27172799
475_439820_7010(TEK)_6a_1	/5Biosg/CAAGGGGTTGCATATTTGACTCTGAATCATCTTTTCTTTTCCTCCCAAAGCTTGTGAACTGCACACGTTTGGCAGAACTTGTAAAGAAAGGTGCAGTGGACAAGAGGGATGCAAGTCTTA	27173169	27173289
475_439820_7010(TEK)_6a_2	/5Biosg/TGTGTTCTGTCTCCCTGACCCCTATGGGTGTTCCTGTGCCACAGGCTGGAAGGGTCTGCAGTGCAATGAAGGTATGCACCCAATCACACCCTTGGACAGAGGATGTTCTAGCAGGTATATA	27173289	27173409
475_439821_7010(TEK)_7a_1	/5Biosg/TTAGTTTCCTCTCTTCCCCTGGATTAATACTGGTTTTTTGATGTCTCTGTTTACAGCATGCCACCCTGGTTTTTACGGGCCAGATTGTAAGCTTAGGTGCAGCTGCAACAATGGGGAGAT	27180181	27180301
475_439821_7010(TEK)_7a_2	/5Biosg/GTGTGATCGCTTCCAAGGATGTCTCTGCTCTCCAGGATGGCAGGGGCTCCAGTGTGAGAGAGAAGGTAAAGCAAGGTAACACTGTAGTCAGGGCCATGTTCAGCATGTCTGAACTGAGCT	27180301	27180421
475_439822_7010(TEK)_8a.1_1	/5Biosg/CTCTGTTAAATATTAGATTTCACAGTGCTGTTTTCTTCCTTCAGGCATACAGAGGATGACCCCAAAGATAGTGGATTTGCCAGATCATATAGAAGTAAACAGTGGTAAATTTAATCCCAT	27183412	27183532
475_439822_7010(TEK)_8a.1_2	/5Biosg/TTGCAAAGCTTCTGGCTGGCCGCTACCTACTAATGAAGAAATGACCCTGGTGAAGCCGGATGGGACAGTGCTCCATGTAAGAGCCATTCTTAATTTGCCCTTCTTAAAGCATGAGATGCT	27183532	27183652
475_439823_7010(TEK)_9a_1	/5Biosg/TTTATGCCTCCCTAGAAGTTTTTATTTTTTTGTATTTGACCTTTTCAGCCAAAAGACTTTAACCATACGGATCATTTCTCAGTAGCCATATTCACCATCCACCGGATCCTCCCCCCTGAC	27185434	27185554
475_439823_7010(TEK)_9a_2	/5Biosg/TCAGGAGTTTGGGTCTGCAGTGTGAACACAGTGGCTGGGATGGTGGAAAAGCCCTTCAACATTTCTGTTAAAGGTAAGTTCATTTCCCAGAAAAAGGGATTGTGTCCTTGATGCATTATG	27185554	27185674
475_439824_7010(TEK)_10a_1	/5Biosg/AAGCCGAAGGACTAATCTGCCTTCTGAAATTGTATTTAGTTCTTCCAAAGCCCCTGAATGCCCCAAACGTGATTGACACTGGACATAACTTTGCTGTCATCAACATCAGCTCTGAGCCTT	27190487	27190607
475_439824_7010(TEK)_10a_2	/5Biosg/ACTTTGGGGATGGACCAATCAAATCCAAGAAGCTTCTATACAAACCCGTTAATCACTATGAGGCTTGGCAACATATTCAAGGTAAGCTTTGGACAGGATAGATGCCAGCTGGGGATGTGG	27190607	27190727
475_439825_7010(TEK)_11a.1_1	/5Biosg/AGGAATGTAAGAGAATGCCAACTTAAGTTTCCTGGACGTTTTCTCTTCTCAGTGACAAATGAGATTGTTACACTCAACTATTTGGAACCTCGGACAGAATATGAACTCTGTGTGCAACTG	27192434	27192554
475_439825_7010(TEK)_11a.1_2	/5Biosg/GTCCGTCGTGGAGAGGGTGGGGAAGGGCATCCTGGACCTGTGAGACGCTTCACAACAGCTTCTATCGGTCAGTGGAAGCCAACAGGCATTTATTCATGAGCTGGGTGGGAGGGGGAGGAA	27192554	27192674
475_439826_7010(TEK)_12a.1_1	/5Biosg/CCATATATAAAAATAATGATTTTTCTGGATTCTCCTAGGACTCCCTCCTCCAAGAGGTCTAAATCTCCTGCCTAAAAGTCAGACCACTCTAAATTTGACCTGGCAACCAATATTTCCAAG	27197274	27197394
475_439826_7010(TEK)_12a.1_2	/5Biosg/CTCGGAAGATGACTTTTATGTTGAAGTGGAGAGAAGGTCTGTGCAAAAAAGTGATCAGCAGAATATTAAAGTTCCAGGCAACTTGACTTCGGTGCTACTTAACAACTTACATCCCAGGGA	27197394	27197514
475_439826_7010(TEK)_12a.1_3	/5Biosg/GCAGTACGTGGTCCGAGCTAGAGTCAACACCAAGGCCCAGGGGGAATGGAGTGAAGATCTCACTGCTTGGACCCTTAGTGACAGTAAGTAATTCATGCTGCTCCAGCCTCATCTGAGCAA	27197514	27197634
475_439827_7010(TEK)_13a_1	/5Biosg/AAGTGAATCTTTTTTCTTTTTAATTTCTAGTTCTTCCTCCTCAACCAGAAAACATCAAGATTTCCAACATTACACACTCCTCAGCTGTGATTTCTTGGACAATATTGGATGGCTATTCTA	27202787	27202907
475_439827_7010(TEK)_13a_2	/5Biosg/TTTCTTCTATTACTATCCGTTACAAGGTTCAAGGCAAGAATGAAGACCAGCACGTTGATGTGAAGATAAAGAATGCCACCATCACTCAGTATCAGCTCAAGGGCCTAGAGCCTGAAACAG	27202907	27203027
475_439827_7010(TEK)_13a_3	/5Biosg/CATACCAGGTGGACATTTTTGCAGAGAACAACATAGGGTCAAGCAACCCAGCCTTTTCTCATGAACTGGTGACCCTCCCAGAATCTCAAGGTTGGTTGAATGGACAAGTATTTACATAGG	27203027	27203147
475_439828_7010(TEK)_14a.1_1	/5Biosg/TGTAAACTAAACTACCTGCTTCACCTCTGTCTTCCTGCACAGCACCAGCGGACCTCGGAGGGGGGAAGATGCTGCTTATAGCCATCCTTGGCTCTGCTGGAATGACCTGCCTGACTGTGC	27204866	27204986
475_439828_7010(TEK)_14a.1_2	/5Biosg/TGTTGGCCTTTCTGATCATATTGCAATTGAAGAGGGCAAATGTGCAAAGGAGAATGGCCCAAGCCTTCCAAAACGTGGTAGTGTCTCATCTTCCTACTAGCTAATAAGGGCAAGTCCAAG	27204986	27205106
475_439829_7010(TEK)_15a_1	/5Biosg/AATTATTTTTCCAGAGGGAAGAACCAGCTGTGCAGTTCAACTCAGGGACTCTGGCCCTAAACAGGAAGGTCAAAAACAACCCAGATCCTACAATTTATCCAGTGCTTGACTGGAATGACA	27206565	27206685
475_439829_7010(TEK)_15a_2	/5Biosg/TCAAATTTCAAGATGTGATTGGGGAGGGCAATTTTGGCCAAGTTCTTAAGGCGCGCATCAAGAAGGATGGGTTACGGATGGATGCTGCCATCAAAAGAATGAAAGGTCAGTGGTTGACCA	27206685	27206805
475_439830_7010(TEK)_16a_1	/5Biosg/CCAGAATATGCCTCCAAAGATGATCACAGGGACTTTGCAGGAGAACTGGAAGTTCTTTGTAAACTTGGACACCATCCAAACATCATCAATCTCTTAGGAGCATGTGAACATCGAGGTAAG	27209114	27209234
475_439831_7010(TEK)_17a_1	/5Biosg/CGATGCTCTCTTCCTTCCCTCCAGGCTACTTGTACCTGGCCATTGAGTACGCGCCCCATGGAAACCTTCTGGACTTCCTTCGCAAGAGCCGTGTGCTGGAGACGGACCCAGCATTTGCCA	27212680	27212800
475_439831_7010(TEK)_17a_2	/5Biosg/TTGCCAATAGCACCGCGTCCACACTGTCCTCCCAGCAGCTCCTTCACTTCGCTGCCGACGTGGCCCGGGGCATGGACTACTTGAGCCAAAAACAGGTTTGTCCGGAGGACTTCGCTTTGG	27212800	27212920
475_439832_7010(TEK)_18a_1	/5Biosg/CAGTTTATCCACAGGGATCTGGCTGCCAGAAACATTTTAGTTGGTGAAAACTATGTGGCAAAAATAGCAGATTTTGGATTGTCCCGAGGTCAAGAGGTGTATGTGAAAAAGACAATGGTA	27213478	27213598
475_439833_7010(TEK)_19a_1	/5Biosg/GAAATCTCACTTTGTTCTCTCCAGGGAAGGCTCCCAGTGCGCTGGATGGCCATCGAGTCACTGAATTACAGTGTGTACACAACCAACAGTGATGTGTGAGTAAACTTCTTATTGCCAAGG	27217661	27217781
475_439834_7010(TEK)_20a_1	/5Biosg/TGTTTGCTGATTGTTGGTTTCACACTTGTCCCTCCTGCAGATGGTCCTATGGTGTGTTACTATGGGAGATTGTTAGCTTAGGTGAGTATCTATGTTTATCTACCAGGTGAGACTCTAGGC	27218734	27218854
475_439835_7010(TEK)_21a_1	/5Biosg/TTTGTCTTCCAGGAGGCACACCCTACTGCGGGATGACTTGTGCAGAACTCTACGAGAAGCTGCCCCAGGGCTACAGACTGGAGAAGCCCCTGAACTGTGATGATGAGGTGTAAGTCAGGC	27220034	27220154
475_439836_7010(TEK)_22a_1	/5Biosg/GCTTTCGAAGGTATGATCTAATGAGACAATGCTGGCGGGAGAAGCCTTATGAGAGGCCATCATTTGCCCAGATATTGGTGTCCTTAAACAGAATGTTAGAGGAGCGAAAGGTAAGTATTA	27228193	27228313
475_439837_7010(TEK)_23a_1	/5Biosg/TGAACCATTTTCATTCTTCCAGACCTACGTGAATACCACGCTTTATGAGAAGTTTACTTATGCAGGAATTGACTGTTCTGCTGAAGAAGCGGCCTAGGACAGAACATCTGTATACCCTCT	27229133	27229253

Supplementary Table 2. Bait sequences used for *TEK* targeted sequencing.

This table includes the DNA sequence for the oligonucleotides used in the targeted TEK sequencing.