Recurrent PTPRB and PLCG1 mutations in angiosarcoma

Sam Behjati1,2,17, Patrick S Tarpey1,17, Helen Sheldon3,17, Inigo Martincorena1, Peter Van Loo1,4, Gunes Gundem1, David C Wedge1, Manasa Ramakrishna1, Susanna I Cooke1, Nischalan Pillay5,6, Hans Kristian M Vollan1,7,8, Elli Papaemmanuil1, Hans Koss9,10, Tom D Bunney9, Claire Hardy1, Olivia R Joseph1, Sancha Martin1, Laura Mudie1, Adam Butler1, Jon W Teague1, Meena Patil11, Graham Steers11, Yu Cao12, Curtis Gumbs12, Davis Ingram12, Alexander J Lazar12, Latasha Little12, Harshad Mahadeshwar12, Alexei Protopopov12, Ghadah A Al Sannaa12, Sahil Seth12, Xingzhi Song12, Jiabin Tang12, Jianhua Zhang12, Vinod Ravi12, Keila E Torres12, Bhavisha Khatri5, Dina Halai5, Ioannis Roxanis11, Daniel Baumhoer13, Roberto Tirabosco5, M Fernanda Amary5, Chris Boshoff6,14, Ultan McDermott1, Matilda Katan9, Michael R Stratton1, P Andrew Futreal1,12, Adrienne M Flanagan5,6, Adrian Harris3,11 & Peter J Campbell1,15,16

Angiosarcoma is an aggressive malignancy that arises spontaneously or secondarily to ionizing radiation or chronic lymphoedema1. Previous work has identified aberrant angiogenesis, including occasional somatic mutations in angiogenesis signaling genes, as a key driver of angiosarcoma1. Here we employed whole-genome, whole-exome and targeted sequencing to study the somatic changes underspinning primary and secondary angiosarcoma. We identified recurrent mutations in two genes, PTPRB and PLCG1, which are intimately linked to angiogenesis. The endothelial phosphatase PTPRB, a negative regulator of vascular growth factor tyrosine kinases, harbored predominantly truncating mutations in 10 of 39 tumors (26%). PLCG1, a signal transducer of tyrosine kinases, encoded a recurrent, likely activating p.Arg707Gln missense variant in 3 of 34 cases (9%). Overall, 13 of 39 tumors (38%) harbored at least one driver mutation in angiogenesis signaling genes. Our findings inform and reinforce current therapeutic efforts to target angiogenesis signaling in angiosarcoma.

We performed whole-genome sequencing of DNA from three angiosarcomas, along with paired normal DNA from the same cases. The somatic mutation burden of the three cases varied from 0.7–2.2 substitutions per megabase and 0.1–0.2 indels per megabase (Supplementary Tables 1–5). Remarkably, in two of the three angiosarcomas, we identified truncating mutations in the PTPRB (VE-PTP) gene, encoding a tyrosine phosphatase specific to the vascular endothelium that inhibits angiogenesis2. One tumor had a nonsense alteration (p.Glu1444*), and the other had both a nonsense (p.Cys1693*) and a missense (p.Tyr309Cys) alteration.

To explore this observation further, we extended our investigation to 36 angiosarcomas that we studied by whole-exome sequencing (n = 8) or by targeted sequencing of 360 cancer-related genes (n = 4; Supplementary Table 6) or 28 angiogenesis-related genes (n = 24; Supplementary Table 7). The entire footprint of PTPRB was sequenced to enable the identification of structural rearrangements in addition to coding point mutations. We also sequenced angiogenesis-related genes in eight epithelioid hemangiendotheliomas, nine Kaposi sarcomas and two hemangiomas.

In total, we identified 14 PTPRB mutations in 10 of 39 angiosarcomas (26%), comprising 8 nonsense variants, 2 essential splice-site variants, 1 frameshift insertion and 3 missense variants (Fig. 1). No large deletions or rearrangements were identified, although the presence of small intragenic deletions cannot be excluded. All truncating mutations disrupted the coding sequence of PTPRB before or within the sequence encoding the tyrosine phosphatase domain. Two of the missense mutations (encoding p.Tyr309Cys and p.Trp130Arg substitutions) lie within the portion of PTPRB encoding the extracellular domain, the inhibition of which has been shown to disrupt PTPRB function3. The third missense mutation, encoding p.Pro1996Leu, affected the tyrosine phosphatase domain. The SIFT in silico prediction tool of variant effects ascribed

1Cancer Genome Project, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, UK. 2Department of Paediatrics, University of Cambridge, Cambridge, UK. 3Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK. 4Human Genome Laboratory, Department of Human Genetics, VIB and KU Leuven, Leuven, Belgium. 5Histopathology, Royal National Orthopaedic Hospital National Health Service (NHS) Trust, Stanmore, UK. 6University College London Cancer Institute, London, UK. 7Department of Oncology, Oslo University Hospital, Oslo, Norway. 8KG Jebsen Center for Breast Cancer Research, University of Oslo, Oslo, Norway. 9Institute of Structural and Molecular Biology, Division of Biosciences, University College London, London, UK. 10Division of Molecular Structure, Medical Research Council (MRC) National Institute for Medical Research, London, UK. 11Department of Pathology, John Radcliffe Hospital, Oxford, UK. 12Department of Genomic Medicine, MD Anderson Cancer Center, University of Texas, Houston, Texas, USA. 13Bone Tumour Reference Centre, Institute of Pathology, University Hospital Basel, Institute for Applied Cancer Science, Basel, Switzerland. 14Pfizer Oncology, La Jolla, California, USA. 15Department of Haematology, Addenbrooke’s Hospital, Cambridge, UK. 16Department of Haematology, University of Cambridge, Cambridge, UK. 17These authors contributed equally to this work.

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deleterious consequences to these missense variants. No PTPRB mutations were identified in hemangioendothelioma, Kaposi sarcoma or hemangioma. Inactivating PTPRB mutations are also rare in other cancer types, as documented in the Catalogue of Somatic Mutations in Cancer (COSMIC)\(^4\), suggesting that PTPRB disruption is largely specific to angiosarcoma. Statistical analysis demonstrated that these truncating PTPRB mutations were extremely unlikely to have accumulated by chance in angiosarcoma \((q = 1 \times 10^{-9})\), suggesting that inactivating PTPRB mutations are driver events. Notably, all PTPRB mutations were identified in tumors that were either known to be secondary and/or had MYC amplification, a biomarker of radiation-associated secondary angiosarcoma\(^3\) \((P = 0.005)\). In this group, the prevalence of PTPRB mutations was 45\% (10/22 cases).

Four angiosarcomas harbored two different nonsynonymous PTPRB mutations each, including at least one truncating variant in each case. In two of these two cases, both mutations were truncating, consistent with biallelic inactivation and suggesting that PTPRB operates as a recessive cancer gene. In six angiosarcomas, there was a single heterozygous PTPRB variant, five truncating and one missense, without evidence of loss of heterozygosity (LOH). The presence of a single detectable nonsynonymous mutation in 60\% of tumors is not unusual for tumor suppressor genes (Supplementary Fig. 2). We analyzed published catalogs of somatic mutations in 4,073 tumors to determine the frequency of a second mutation, including LOH, co-occurring with a truncating mutation in established tumor suppressor genes. This analysis indicated that the pattern of mutation we observed in PTPRB is compatible with a recessive driver mechanism (Supplementary Fig. 2). Nevertheless, we cannot exclude the possibility that other mechanisms, such as haploinsufficiency or dominant-negative effects, are operative.

PTPRB, a negative regulator of angiogenesis, is expressed exclusively in vascular endothelium, both during development and in adult tissues\(^2-9\). The encoded protein inhibits vascular endothelial growth factor receptor 2 (VEGFR2), vascular endothelial (VE)-cadherin and angiopoietin signaling, thus acting as an integral modifier of angiogenesis\(^3,6-15\). In in vitro models of angiogenesis, PTPRB inhibition increases angiogenesis\(^12\). Ptprb-null mice die in utero and display severe vascular malformations\(^6\). Although the role of PTPRB as a negative regulator of angiogenesis is well established, it is not known whether PTPRB-driven angiogenesis can be inhibited through pharmacological VEGF inhibition. Consequently, we investigated the effects of knockdown of PTPRB on primary cultures of human umbilical vein endothelial cells (HUVECs). Silencing of PTPRB via small interfering RNA (siRNA) induced features of angiogenesis such as spheroid sprouting after 24 h and spindle-like morphology. In the presence of sunitinib or vatalanib, inhibitors of the VEGFR2 kinase, these features were abolished (Fig. 2 and Supplementary Fig. 3). These findings in HUVECs, a model of the vascular endothelium, provide a rationale for exploring whether PTPRB mutation status correlates with treatment response to anti-angiogenic agents.

To explore the contribution of other genes in angiosarcoma, we analyzed variant data from 15 angiosarcomas interrogated by whole-genome, whole-exome or targeted cancer gene sequencing (Fig. 3). Cancer-related genes mutated in more than one tumor included TP53 (3/15 cases; 20\%), KDM6A (2/15 cases; 13\%) and MYC (6/15 cases; 40\%). Strikingly, we also identified a recurrent missense variant, encoding p.Arg707Gln, in PLCG1 in 3 of 15 cases (20\%). PLCG1 encodes phospholipase C\(\gamma\)1 (PLC\(\gamma\)1), a tyrosine kinase signal transducer in the phosphoinositide signaling pathway. Statistical analysis showed that enrichment of the mutation encoding p.Arg707Gln in angiosarcoma was highly significant \((q = 0.000002)\). Capillary sequencing of an additional 15 cases of angiosarcoma indicated that the overall prevalence of mutations encoding p.Arg707Gln was 9\% (3/34 cases). No PLCG1 mutations were found in any of the other tumor types investigated here. Notably, all three PLCG1 mutations encoding p.Arg707Gln co-occurred with PTPRB mutations.

The presence of a single recurrent p.Arg707Gln missense variant suggests that PLC\(\gamma\)1 is activated in angiosarcoma. Arg707 lies within the autoinhibitory Src homology 2 (cSH2) domain of PLC\(\gamma\)1 (refs. 16-19) and provides structural support to this domain. In silico modeling of the mutated protein predicted that the substituted glutamine destabilizes the cSH2 domain, which may result in overactive PLC\(\gamma\)1 (Supplementary Fig. 4). Disruption of the autoinhibitory domain.
likely driver variants are indicated by colored squares. Truncating variants include nonsense variants, essential splice-site variants and frameshift indels. Secondary angiosarcomas are either clinically classified as secondary or are unclassified with MYC amplification.

cSH2 domain has been shown to cause immune disorders in mice and humans through constitutive activation of PLCγ enzymes. Interestingly, forward genetic screening of zebrafish has identified plcγ1 as a non-redundant regulator of arterial angiogenesis that transduces activation of VEGF signaling, and plcγ1-deficient mice exhibit reduced vasculogenesis. PLCG1 is ubiquitously expressed in normal tissue, and total-RNA sequencing of four angiosarcomas, including two positive for PLCγ1 p.Arg707Gln, demonstrated PLCG1 expression in each case. In the context of existing knowledge about PLCG1, our observations therefore lend support to the hypothesis that activated PLCγ1 drives angiogenesis, downstream of receptor tyrosine kinases, through constitutive activation of angiogenesis signaling. The effects of PLCG1 mutations on the response to therapeutics targeting tyrosine kinases will be an important future investigation.

In addition to screening 15 angiosarcomas for driver mutations, we performed a focused screen for mutations in angiogenesis-related genes in a further 24 angiosarcomas (Supplementary Table 6). Considering both cohorts together, 15 of 39 angiosarcomas harbored at least one mutation in an angiogenesis-related gene, highlighting aberrant angiogenesis as a common driver in a subset of tumors. Mutated genes included HRAS, KRAS and NRAS (5/39 cases), PIK3CA (1/39 cases) and FLT4 (1/39 cases). We did not find variants in KDR (VEGFR2), which have previously been reported (Supplementary Table 1). Among other vascular tumors, we identified one Kaposi sarcoma (n = 9) with a PIK3CA driver mutation and one epithelioid hemangioendothelioma (n = 8) with a PTEN driver mutation (Supplementary Table 1). Interestingly, in our series, aberrant angiogenesis was most frequent among secondary and/or MYC-amplified angiosarcomas (12/22 cases), although this observation requires investigation in larger series. There was no evidence of mutual exclusivity of mutations in angiogenesis-related genes (Fig. 3 and Supplementary Table 1). In four angiosarcomas, we found more than one angiogenesis-related gene mutated, indicating that targeting treatment exclusively to the tyrosine kinase level might not suffice to overcome aberrant angiogenesis.

As angiosarcoma is a rare tumor, combined efforts to curate larger case series are required to further explore the somatic changes that underpin its pathogenesis. This study, however, provides a first comprehensive insight into the somatic variation in angiosarcoma and identifies frequent mutations in angiogenesis-related genes in a subset of tumors. The next challenge will be to functionally explore these findings in appropriate angiosarcoma models that accommodate the complexity of the driver mutation landscape we report here. It is now indicated to determine the clinical usefulness of PTPRB and PLCG1 as possible biomarkers of secondary disease and as novel therapeutic targets in angiosarcoma.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes

Sequencing data have been deposited in the European Genome-phenome Archive (EGA), which is hosted by the European Bioinformatics Institute (EBI), under accession EGAD00001000735.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.B. and P.S.T. performed analyses of sequencing data. H.S. performed in vitro experiments. P.V.L. performed copy number analysis. D.C.W. and I.M. performed statistical analyses. S.L.C. performed rearrangement analysis. G.G., N.P., M.R., H.K.M.V and E.P. contributed to data analysis. H.K., T.D.B. and M.K. contributed structural analyses. C.B., V.R., K.E.T., D.I., A.J.L., G.A.A.S. and A.H. provided samples and clinical data. P.J.C., M.R.S., A.H., P.A.F., U.M. and A.M.F. directed the research. M.R.S., P.J.C., S.B. and P.S.T. wrote the manuscript, with contributions from C.H., O.R.J., L.M., H.M., A.P., J.T., L.L., Y.C. and C.G. coordinated sample processing and technical investigations. B.K., D.H., D.B., M.P., G.S., I.R., R.T., M.F.A., A.M.F., and C.G. contributed to data analysis. H.K., T.D.B. and M.K. performed statistical analyses. S.L.C. performed rearrangement analysis. A.H., R.L., S.S. and J.Z. provided samples and clinical data. P.J.C., M.R.S., A.H., P.A.F., U.M. and A.M.F. directed the research. M.R.S., P.J.C., S.B. and P.S.T. wrote the manuscript, with contributions from A.H., A.M.F. and P.A.F.

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The authors declare competing financial interests: details are available in the online version of the paper.

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5. Guo, T. et al. Consistent MYC and FLT4 gene amplification in radiation-induced angiosarcoma but not in other radiation-associated atypical vascular lesions. Genes Chromosom. Cancer 50, 25–33 (2011).

6. Dominguez, M.G. et al. Vascular endothelial tyrosine phosphatase (VE-PTP)-null mice undergo vasculogenesis but die embryonically because of defects in angiogenesis. Proc. Natl. Acad. Sci. USA 104, 3243–3248 (2007).

7. Baumer, S. et al. Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. Blood 107, 4754–4762 (2006).

8. Broermann, A. et al. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. J. Exp. Med. 208, 2393–2401 (2011).

9. Carra, S. et al. Ve-ptp modulates vascular integrity by promoting adherens junction maturation. PLoS ONE 7, e51245 (2012).

10. Hayashi, M. et al. VE-PTP regulates VEGFR2 activity in stalk cells to establish endothelial cell polarity and lumen formation. Nat. Commun. 4, 1672 (2013).

11. Li, Z. et al. Embryonic stem cell tumor model reveals role of vascular endothelial receptor tyrosine phosphatase in regulating Tie2 pathway in tumor angiogenesis. Proc. Natl. Acad. Sci. USA 106, 22399–22404 (2009).

12. Mellberg, S. et al. Transcriptional profiling reveals a critical role for tyrosine phosphatase VE-PTP in regulation of VEGFR2 activity and endothelial cell morphogenesis. FASEB J. 23, 1490–1502 (2009).

13. Nawroth, R. et al. VE-PTP and VE-cadherin ectodomains interact to facilitate regulation of phosphorylation and cell contacts. EMBD J. 21, 4885–4895 (2002).

14. Nottebaum, A.F. et al. VE-PTP maintains the endothelial barrier via plakoglobin and becomes dissociated from VE-cadherin by leukocytes and by VEGF. J. Exp. Med. 205, 2929–2945 (2008).

15. Saharinen, P., Eklund, L., Pulkki, K., Bono, P. & Alitalo, K. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. Trends Mol. Med. 17, 347–362 (2011).

16. Zhou, Q. et al. A hypermorphic missense mutation in PLCG2, encoding phospholipase Cγ2, causes a dominantly inherited autoimmune disease with immunodeficiency. Am. J. Hum. Genet. 91, 713–720 (2012).

17. Everett, K.L. et al. Characterization of phospholipase Cγ enzymes with gain-of-function mutations. J. Biol. Chem. 284, 23083–23093 (2009).

18. Ombrello, M.J. et al. Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. N. Engl. J. Med. 366, 330–338 (2012).

19. Burney, T.D. et al. Structural and functional integration of the PLCγ interaction domains critical for regulatory mechanisms and signaling deregulation. Structure 20, 2062–2075 (2012).

20. Covassin, L.D. et al. A genetic screen for vascular mutants in zebrafish reveals dynamic roles for Vegf/Plcg1 signaling during artery development. Dev. Biol. 329, 212–226 (2009).

21. Lawson, N.D., Mugford, J.W., Diamond, B.A. & Weinstein, B.M. Phospholipase Cγ-1 is required downstream of vascular endothelial growth factor during arterial development. Genes Dev. 17, 1346–1351 (2003).

22. Liao, H.J. et al. Absence of erythrogenesis and vasculogenesis in Plcg1-deficient mice. J. Biol. Chem. 277, 9335–9341 (2002).

23. Antonescu, C.R. et al. KDR activating mutations in human angiosarcomas are sensitive to specific kinase inhibitors. Cancer Res. 69, 7175–7179 (2009).
Whole-genome, whole-exome and targeted cancer gene sequencing. DNA was extracted from 11 angiosarcomas as well as from matched normal tissue derived from the same individuals. Whole-genome sequencing of three cases was performed to average depths of at least 40× and 30× for tumor and normal DNA, respectively, as previously described24. Whole-exome sequencing was performed on eight cases as previously described25, and at least 70% of the coding sequence was covered by 30×. DNA extracted from an additional 4 tumors that did not have matched normal-tissue DNA was subjected to targeted sequencing of 360 established and putative cancer-related genes using a custom-made bait set (Agilent Technologies) for target enrichment (Supplementary Table 7). Paired-end sequencing was performed on Illumina HiSeq 2000 or 2500 analyzers. Reads were aligned to the reference human genome (NCBI37) using Burrows-Wheeler Aligner (BWA) on default settings26. Reads that were unmapped or were PCR-derived duplicates were excluded from analysis.

Variant detection. The CaVEMan (Cancer Variants through Expectation Maximization) algorithm was used to call single-nucleotide substitutions27. To call insertions and deletions, we used split-read mapping implemented as a modification of the Pindel algorithm27. To call rearrangements, we applied the BRASS (Breakpoint via Assembly) algorithm, which identifies rearrangements by grouping discordant read pairs that point to the same breakpoint event27. Post-processing filters were applied to output to improve specificity. Mutations were annotated to Ensembl version 58.

Variant validation. In whole-genome samples, all coding variants as well as randomly selected mutations—in total, 508 of 15,292 (3.3%) substitutions and 342 of 1,386 (25%) indels—were experimentally validated by whole-exome sequencing or targeted capture with massively parallel sequencing27. The overall precision of the catalog of substitutions and indels was thus determined to be at least 94%. Rearrangements were validated by defining the exact location of the breakpoint at nucleotide-level resolution through extraction of split reads across the breakpoint, algorithmically or manually as previously described25. Variants called in whole-exome samples were confirmed by visual inspection or resequencing of the exomes.

Angiogenesis gene screen. Forty-three tumors were included in this screen: 24 angiosarcomas, 9 Kaposi sarcomas, 8 hemangioidoendotheliomas and 2 hemangiomas. Genes of interest (Supplementary Table 6) and genotyping SNPs were enriched through targeted capture and were sequenced by massively parallel sequencing, as described previously27. The PLCG1 mutation encoding p.Arg707Gln was screened for by capillary sequencing (primer sequences available upon request).

RNA sequencing and analysis. Total RNA was isolated from fresh-frozen tissue from four angiosarcomas using TRizol. Standard Illumina libraries of polyA-selected RNA were sequenced on an Illumina HiSeq 2000 (paired end, 75-bp read length). TopHat28 (version 1.3.3) was used for alignment. Expression values were derived using Cufflinks29 (version 1.0.2).

Detection of copy number variation. Copy number data were derived from whole-genome or whole-exome reads using the ASCAT algorithm (version 2.2) and were validated by SNP6.0 arrays in two cases25. In the whole-exome extension study, amplifications were detected by comparing the coverage of candidate genes to the average coverage across the exome, after normalization using matched germline exome sequencing data. A 1.75-fold increase (corresponding to ≥5 copies in 50% of tumor cells) was reported as an amplification. In the targeted extension study, amplifications were detected by comparing the coverage of candidate genes to the coverage of 96 SNPs in the same sample, both normalized against data from a panel of non-tumor samples. A five-fold increase in relative, normalized coverage in tumors was reported as an amplification. To assess LOH in PTPRB, all SNPs that lie within the footprint of the gene were interrogated, and their allele fraction was assessed for deviations from 0.5.

Cell culture. HUVECs pooled from multiple donors were purchased from Lonza. These cells were routinely cultured in Endothelial Growth Medium 2 (EGM-2; Lonza) up to passage 7 and were cultured in Endothelial Basal Medium 2 (EBM-2; Lonza) during the experiments. Cell cultures were tested for mycoplasma.

RNA interference transfection. Stealth siRNAs targeting PTPRB (HS5108847 and HS5108849) and Stealth RNAi siRNA Negative Control Med GC Duplex 2 were purchased from Life Technologies. siRNA was transfected into HUVECs at a final concentration of 30 nM using Lipofectamine RNAiMAX reagent (Life Technologies). Cells were transfected at 50% confluency in Opti-MEM Reduced-Serum Medium with GlutaMAX I (Life Technologies) and analyzed after 24 h.

Protein blotting and staining. Antibodies for protein blotting were obtained from the following suppliers: β-actin–horseradish peroxidase (HRP) (Sigma, A3854; 1:25,000 dilution), VEGFR2 (Cell Signaling Technology, 2479; 1:1,000 dilution) and phosphorylated VEGFR2 (Cell Signaling Technology, 2478; 1:1,000 dilution). Staining was performed on formalin-fixed cells using antibody to VE-cadherin (Cell Signaling Technology, 2500; 1:100 dilution) and Alexa Fluor 488 anti-rabbit IgG (Life Technologies, A21206; 1:200 dilution).

Hanging drop assays. HUVECs were trypsinized and resuspended in EBM-2 containing 2% FCS at 2.5 × 104 cells/ml. Methylcellulose was added to 0.25%, and 20-µl drops were seeded in a non-adherent dish. After inversion of the plate, suspended cells form a single spheroid containing approximately 500 cells. Treated spheroids were incubated with 1 µM sunitinib (Cell Signaling Technology) or 100 nM vatalanib (Santa Cruz Biotechnology) for 1 h before being embedded in a fibrin gel using 2.5 mg/ml fibrinogen-PBS solution (Sigma) and 0.007 units of thrombin (Sigma). Once clotting occurred, EB2 containing 2% FCS was added with or without 1 µM sunitinib or 100 nM vatalanib. Photographs of spheroids were taken after 24 h using an AMG Eos XL Core digital microscope (Fisher Scientific). Sprouting area was measured using ImageJ64 software, and the results were analyzed using GraphPad Prism version 6 (GraphPad Software).

Statistical analyses. A Fisher’s exact test was used to assess the significance of the association between PTPRB mutations and subtypes of angiosarcoma. For analysis of in vitro findings, one-way ANOVA was performed on the data with a Sidák’s multiple-comparisons test. To determine whether the frequency of an individual mutation class was higher than expected by chance for each gene, we implemented a likelihood model as previously described31. To determine the probability of the identical PLCG1 mutations having occurred by chance, we employed the following approach. Using the rate of each mutation class estimated by the aforementioned method, the neutral rate of such an event assuming uniform mutation rates was determined to be 5.905 × 10−6. The probability of observing this site mutated 3 times in the coding sequences of the 11 samples that were interrogated by unbiased sequencing was therefore approximately 4.57 × 10−14 (cumulative Poisson distribution). Adjusting the P value for the total number of sites in the exome, the q value for this mutation was 1.49 × 10−6.

To analyze the frequency of two hits in known tumor suppressor genes, we downloaded publicly available catalogs of somatic mutations, including copy number data from 4,073 tumors of The Cancer Gene Atlas consortium (as of November 2013). For every gene on a list of established tumor suppressors (Supplementary Fig. 2), we selected all those samples in which the gene had a truncating mutation (nonsense variant, essential splice-site variant or out-of-frame indel) and quantified the frequency of a second mutation, i.e., truncating variant, LOH, missense variant or in-frame indel, in the gene.

24. Behjati, S. et al. Distinct H3F3A and H3F3B driver mutations define chondroblastoma and giant cell tumor of bone. Nat. Genet. 45, 1479–1482 (2013).
25. Tarpey, P. S. et al. Frequent mutation of the major cartilage collagen gene COL2A1 in chondrosarcoma. Nat. Genet. 45, 923–926 (2013).
26. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589–595 (2010).

27. Ye, K., Schulz, M.H., Long, Q., Apweiler, R. & Ning, Z. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. *Bioinformatics* **25**, 2865–2871 (2009).

28. Trapnell, C., Pachter, L. & Salzberg, S.L. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105–1111 (2009).

29. Trapnell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511–515 (2010).

30. Van Loo, P. et al. Allele-specific copy number analysis of tumors. *Proc. Natl. Acad. Sci. USA* **107**, 16910–16915 (2010).

31. Greenman, C., Wooster, R., Futreal, P.A., Stratton, M.R. & Easton, D.F. Statistical analysis of pathogenicity of somatic mutations in cancer. *Genetics* **173**, 2187–2198 (2006).