SUPPLEMENTARY MATERIALS AND METHODS

Subjects

Patients were enrolled based on diagnosis of CCM and genotypic demonstration of heterozygous mutation in the *PDCD10* gene. Human subject protocols were approved by the University of Chicago Institutional Review Board to allow imaging and analysis of the participants' clinical information for research purposes. Written informed consent was obtained from each subject (or their legal guardian) prior to inclusion in the study.

Subjects were prospectively enrolled after being evaluated by University of Chicago Neurovascular Surgery clinic, including facilitated referral of all known *PDCD10* cases in the United States who are registered with Angioma Alliance, the patient support and advocacy group dedicated to CCM (www.angioma.org). The cohort included 13 probands with confirmed *PDCD10* mutations, and 18 individual subjects.

CCM lesion specimens for ROCK assays were obtained from The University of Chicago pathology archives for *KRIT1*, *CCM2* and sporadic subjects, and from the Angioma Alliance biobank for *PDCD10* subjects.

Cell culture

HUVECs (Cambrex) were cultured in EGM2 medium (Cambrex), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). Primary human brain microvascular endothelial cells (hbmvEC) were acquired from ScienCell (Carlsbad, CA) at passage #1 and grown on gelatin –coated polystyrene flasks in ScienCell EC Medium supplemented with 5%

FBS and endothelial cell growth supplement, also from ScienCell. Cells were used for experiments between passages 3 and 12.

Pdcd10 heterozygous murine model

Animal procedures were approved by the Duke University Institutional Animal Care and Use Committee (IACUC). *Pdcd10*$^{+/-}$ mice were obtained from Murat Gunel at Yale University[1]. Animals bred in sensitized backgrounds (with loss of *Msh2* or *Trp53*) designed to promote second-hit somatic mutations[2, 3] were examined, as were non-sensitized heterozygotes. B6.129S2-*Trp53*^{tm1Tyi} mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model was development by a similar strategy as was done for the *Krit1*$^{+/-}$ *Trp53*$^{-/-}$ model as previously reported [3]. The *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ model was development by a similar strategy as was done for the *Krit1*$^{+/-}$*Msh2*$^{-/-}$ model as previously reported[2]. The animals were euthanized at about 4 months of age. The brains were removed, immersed in 10% formalin and sent to the University of Chicago for lesion burden assessment and immunohistochemistry. Fifteen *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice (nine male, six female), one *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ (male) mouse and fifteen *Pdcd10*$^{+/-}$ mice (eight male, seven female) were included in the analysis. Heterozygous *Pdcd10*$^{+/-}$ murine models were assessed for lesion burden (single cavern stage 1 lesions and mature, multicavernous stage 2 lesions) at age 4-5 months, and the area of stage 2 lesions on serial histologic sections [2, 4].

Seven *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice, two *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ mice, and no *Pdcd10*$^{+/-}$ mice were excluded from analysis because of attrition. Two *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice died before euthanasia from unknown causes. Five *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice were euthanized before 120 days of age, to prevent suffering as determined by the IACUC, including three mice harboring tumors, one

mouse exhibiting lethargy and weight loss from unknown causes and one mouse with an injury. Two *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ mice were euthanized before 120 days of age, including one mouse with pneumonia and one mouse for unknown reasons. Postmortem analysis of the brains revealed the presence of CCM lesions, but no tumors.

Genetic Testing of Subjects

Probands in families 4, 6, 7, 8, 10, 11, and 12 received clinical diagnostic genetic testing with a positive result for mutation in *PDCD10* as a part of medical care through their personal physicians. For all other probands, genetic testing was performed for research purposes. Coding exons of *PDCD10* were amplified using primers described previously[5] and exons were sequenced using the ABI PRISM Big Dye Terminator chemistry (Applied Biosystems). *PDCD10* gene mutations in families 1, 2, 5 and 9 were identified at Duke University, probands 2, 5 and 9 were originally described by Liquori *et al*, 2006[5]. Patients were consented to allow analysis of their DNA for research purposes.

Transfection

HbmvEC were transfected using Amaxa Nucleoporator II with siRNA targeting human *KRIT1, CCM2* or *PDCD10* mRNA 5'UTR sequences (Life Technologies), with or without wild-type cDNA encoding wild type human *KRIT1, CCM2* or *PDCD10* in either pEGFP or pcDNA3.1 vectors. HUVECs were transfected with *PDCD10* siRNA or control siRNA. Cells were harvested and lysed using Qiagen RNeasy kit lysis buffer, and total RNA purified per kit directions. RNA quality was evaluated by NanoDrop and 1 μg was subjected to reverse transcription using BioRad iScript Advanced RTPCR reagents. Equal amounts of DNA were

assayed by qPCR using a BioRad CFX96-Touch thermocycler. Expression in hbmvEc used the following primers:

KRIT1: Forward TGCCACCAGCCCCTGAGAGAC

Reverse TGGGACAGTCACCGTGGCTATGAAA

CCM2: Forward GGCAAGAAGGGCAAGAAGCCTGGA

Reverse GAGGGCGCCTCTCTGTCACC

PDCD10: Forward TCCCCGTAGCTCTGCACCGA

Reverse AGTGCTCCTCTTCCGCCCGTA

GAPDH: Forward GTCGCCAGCCGAGCCACATC

Reverse CCAGGCGCCCAATACGACCA

PDCD10 expression in HUVECs used human-specific exon-spanning 100 bp amplicon primers detecting all *PDCD10* isoforms, or control *GAPDH* primers. Gene expression data was quantitated using BioRad CFX Manager software, and data from three experiments reported as fold-expression of control HUVECs transfected with control siRNA. In hbmvEC, expression was normalized to *GAPDH* content of each sample and reported as fold-increase or decrease of control treatment.

Immunofluorescence

HUVECs were transfected with siRNA against *PDCD10* or control siRNA per above, plated onto gelatin-coated glass cover slips and grown for 24 hours. Indicated cells were treated with H-1152 or vehicle for 4 hours in growth medium. Cells on slips were fixed for 30 min in 3.7% methanol-free formaldehyde, permeabilized for 10 min with 0.15% TX100 buffer, blocked for 30 minutes with 1% bovine serum albumen in Tris buffered saline tween (TBST), then

incubated for 18 hours with rabbit anti-*PDCD10* antibody (Abnova), or rabbit anti-phosphoMLC antibody (Cell Signaling Technology) in TBST with 10% goat serum, at 4°C. Cells were washed then incubated with goat anti-rabbit IgG-Alexa 488 plus Phalloidin-Alexa 568 (Invitrogen) for 2 hours at room temp. Slips were washed then mounted using Prolong Gold medium plus DAPI (Molecular Probes). Probed cells were photographed using an EVOS fluorescence microscope with the same gain and acquisition settings for all micrographs having same primary antibody.

Western Blotting

HUVECs treated with *PDCD10* or control siRNA + H1152 per above were harvested by scraping in lysis buffer (25 uM Tris-HCl, 135 mM NaCl, 0.5% NP40), with protease and phosphatase inhibitor cocktails (Sigma-Aldrich) added. Cells were ruptured by rapid aspiration of lysates through a 20 gauge needle multiple times, and then centrifuged at 13000 x g for 10 minutes. Protein assay of supernatants was performed (BioRad) and 50 μg total protein each sample loaded on a 4-20% reducing gel (BioRad) and electrophoresed. Proteins were transferred to nitrocellulose, blots blocked with 5% milk in Tris buffered saline and probed overnight with 1:200 rabbit anti-pMLC (Cell Signaling Technology) and mouse anti-GAPDH. Blots were washed and probed with goat anti-rabbit IgG IR700 and donkey anti-mouse IR800 antibodies for 1 hr at room temp, washed and imaged using a BioRad Gel Doc.

RhoA Activation Assay

HbmvEC were transfected as indicated with siRNA against *KRIT1, CCM2* or *PDCD10* with or without cDNA encoding the wildtype human gene corresponding to the knockdown target. Cells were incubated for 14 hours, serum-starved for 6 hours (0.5% FBS) then treated

with either 3 nM H-1152 or vehicle (Hank's Balanced Salt Solution) for 4 hours. Cells were collected by scraping in lysis buffer on ice and assayed for GTP-RhoA (activated) content, using the G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton Inc) according to manufacturer's direction. In brief, equal amount of lysate protein from each sample were applied to triplicate wells of a G-LISA assay plate. GTP-bound RhoA adheres to immobilized antibody, while GDP-RhoA is washed out. An added colorimetric substrate to detect binding was read at 490 nm absorbance; absorbance is linearly proportional to GTP-RhoA content. Means of triplicate wells were normalized to subtracted background and assessed as a percentage of control siRNA-treated hbmvEC without H1152. The experiment was repeated three times.

Permeability Assay *in vitro*

HUVECs at passage 3 (ScienCell) were transfected with 100 nM siRNA against human *PDCD10* or control siRNA (Qiagen) by nucleoporation using an Amaxa Nucleoporator II with standard HUVEC program and buffer. Equal numbers of cells were plated in gelatin-coated Transwell filter wells (Costar, NY; 3 micron pore size) and in parallel in additional 6 well plates without filters, all grown 24 hours in Endothelial Medium (ScienCell). Indicated cells were treated for 4 or 24 hours with 0.5 uM H-1152 (Cayman Chemical) to inhibit ROCK 1/2 activity, or equal volume vehicle for control, in growth medium. For the final 2 hours of incubation, growth medium in the upper filters was replaced with 200 µl phenol free Dulbecco's minimal essential medium (DMEM) + H1152 or vehicle, with 2 µM FITC-dextran. Lower wells had 1.0 ml phenol-free DMEM. At termination, lower well medium was collected and assayed for fluorescence using a BioTek Synergy H4 multi-Mode plate reader. Fluorescence units were

normalized as percent fluorescence of control cells transfected with control siRNA without H1152.

Sample Preparation and Histology

Mouse brains were cut with a rodent brain matrix into fourteen 1-mm thick coronal slices form the olfactory bulbs at the frontal rostrum to the most caudal region at the cerebellar hindbrain. The slices were embedded in paraffin, cut to 5-μm thick sections with a microtome and stained with hematoxylin and eosin. Lesion burden was independently assessed by two observers (RS and CS), with the finding adjudicated by a third observer (IAA). Stage 1 lesions were defined as an isolated dilated capillary with a maximum diameter of at least 100 μm. Stage 2 CCM lesions were defined as clusters of two or more contiguous caverns as reported previously[2, 4, 6]. Areas of lesions were determined by using software included with a Dp21 microscope digital camera (Olympus).

Immunohistochemistry

Sections were stained with rabbit polyclonal pMLC [Thr^{180}/Ser^{19}] (Cell Signaling Technology) at 1/60 dilution for mouse coronal brain sections and 1/250 dilution for human CCM sections and rabbit polyclonal pMBS [Thr^{853}] (Sigma-Aldrich) at 1/4000 dilution for mouse sections and 1/5000 dilution for human CCM sections, by methods reported previously[2, 4] to assess for ROCK activity in lesions and background brain. The Thr853 of MYPT1 site is known to be specific for only ROCK and not for other serine/threonine kinases[7, 8]. Caverns in CCM lesions were considered positively stained for ROCK activity if at least one endothelial cell lining the caverns was positively stained.

Lesion Burden and *in vivo* Brain Permeability in Humans

Magnetic resonance imaging (MRI) was performed at least once in each subject, including susceptibility weighted imaging (SWI) at 3 Tesla. Lesion burden was assessed by cataloging every CCM lesion on conventional MRI T2-weighted sequences, as well as the most sensitive SWI sequences as validated previously[9]. In addition, 33 subjects also underwent dynamic contrast enhanced perfusion studies in conjunction with their MRI. This allowed the determination of lesional and brain permeability indices as per previously published techniques[10, 11].

Lesion burden and clinical features

For each subject we reviewed the past history (including previous medical records provided by the patient or family) and we noted the age at first symptom onset, age at diagnosis, presenting symptom, and each demonstrated bleed meeting the criteria of overt CCM hemorrhage[12] or requiring surgical intervention. Other clinical manifestations were queried in each case, noting the presence or absence of any skin lesions (on physical examination), scoliosis (defined on screening spine radiographs performed in every subject), and brain tumors (on contrast enhanced brain MRI). Cognitive disability was noted if there was an objectively diagnosed learning disorder requiring special schooling (in children), or cognitive symptoms interfering with activities of daily living or employment (in adults). Parental genetic testing was performed whenever possible to identify cases with spontaneous mutation.

Statistical Methods and Control Comparisons

Statistical significance for the RhoA activity assay was assessed by ANOVA using Sigma Plot software. Comparisons for sensitized mice, between *Pdcd10*$^{+/-}$ vs. other genotypes *Krit1*$^{+/-}$ and *Ccm2*$^{+/-}$, on total CCM lesion burden and Stage 2 lesion burden were achieved using negative binomial regression. Wilcoxon signed rank test was applied for the same comparisons on non-sensitized mice between *Pdcd10*$^{+/-}$ and other genotypes, since there was no documented lesions in the non-sensitized *Krit1*$^{+/-}$ or *Ccm2*$^{+/-}$ mice. The difference in lesion area in sensitized mice between *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ and other genotypes was assessed by Student's *t* test, following log transformation. The prevalence of positive pMLC caverns in human CCM lesions was compared between sporadic patients and patients with germline mutations in the three CCM genes using one way ANOVA with Dunnett's post hoc test.

Bleeds were calculated per year of life (age), year since first symptom, and for cases with bleeds, rate of subsequent rebleed. Negative binomial regression was conducted to detect the independent effects of age at first symptom, age at diagnosis, age at first bleed, lesion burden, sex, as well as clinical features on annual bleed rates and bleed rates per lesion per year. This analysis was chosen as an extension of the Poisson regression taking into account of overdispersion, given the fact that the variance of the rates was much larger than mean.

Age at first bleed, lesion burden, and the annual rates of lesion genesis in *PDCD10* cases were compared to a cohort of nine familial CCM cases with non-*PDCD10* mutations (genotyped *KRIT1* or *CCM2* mutations) evaluated in our clinic and undergoing identical imaging, five of whom had repeat scans over time. Permeability indices of white matter regions far from lesions and of lesions themselves in sixteen *PDCD10* cases were compared to indices derived using the same technique in six familial CCM cases with non-*PDCD10* mutations, and in eighteen control cases with sporadic CCM lesions (no germline mutation in any of the CCM genes). For age at

first bleed, we also considered an additional control group of 18 subjects, along with three subjects, with adjudicated hemorrhage among 57 subjects enrolled in the Angioma Alliance DNA & Tissue Bank and nine subjects enrolled in the University of Chicago clinical registry, respectively, with familial non-*PDCD10* cases (confirmed *KRIT1* and *CCM2* mutations). Age at the first bleed for subjects who previously had a bleed was compared using ANOVA. Age distribution of the first bleed for all subjects was compared using the log rank test. For lesion burden and annual rates of lesion genesis, the comparisons were conducted using negative binomial regression. For comparisons between continuous variables, such as permeability, ANOVA with Bonferroni's post hoc test was applied. All statistical analyses were performed using Stata 12 (StataCorp LP, College Station, Texas), all reported *P* values were two sided and were considered to be statistically significant at $*P<0.05$, $**P<0.01$, $***P<0.001$.

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