Supplemental Material

Supplemental Methods:

Reagents.

Primary human adult dermal microvascular endothelial cells (HMVEC-D) and cell culture reagents were obtained from Lonza (Basel, Switzerland). Small interfering RNA (siRNA) were obtained from Dharmacon Thermo Scientific (Waltham, MA). The Spectrum Collection compound library was obtained from MicroSource Discovery Systems (Gaylordsville, CT), via the University of Utah Drug Screening Resource. All remaining reagents were obtained from Tocris Biosciences (Bristol, U.K.) unless otherwise stated.

Cell culture.

Four individual lots of Adult Dermal Human Microvascular Endothelial Cells (HMVEC-D) were pooled at passage 0 and expanded according to manufacturer's instructions in EBM-2 media (Lonza) supplemented with EGM-2 MV Bulletkits (Lonza). After two expansions, cells were frozen in identical lots at $1x10^6$ cells/mL for use in all screening experiments. All *in vitro* experiments were completed using endothelial cells between passage 3 and 6. The continuous human endothelial cell line EA-hy926 (Fig. 6F,G) was maintained according to the protocol provided by the distributor (ATCC cat. CRL 2922, Manassas, Virginia, USA). Specifically, cells were cultured in DMEM medium with 25 mM glucose (HG) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 2% HAT supplement.

Endothelial transfection.

A well validated ON-TARGETplus siRNA targeting CCM2 (5' GGAAUUGUCUCGCCAUUUA 3') and a scrambled control was obtained from Dharmacon.

Endothelial cell transfection with siRNA was carried out as previously described[7]. Briefly, cells were passaged into 25nM siRNA with 1:166 HiPerFect reagent (Qiagen) in 4:1 EGM-2 MV to EBM-2 respectively and plated. After an overnight incubation in the transfection mix, cells were washed and fed with EGM-2 MV. After an additional 48 hours, the transfection process was repeated to achieve more complete knockdown. During the second transfection, cells were seeded into assay plates as described below. Cells were again fed with EGM-2 MV after overnight incubation with the transfection mix. After an additional 48-72 hours, cells were subjected to experimental conditions.

EA-hy926 transfection.

EA-hy926 endothelial cells ($1x10^6$) were plated in 10 cm culture dishes in 8 ml antibiotic-free standard growth medium supplemented with FBS. Cells were grown to 60% confluence and then transfected for 5 h at 37°C with either CCM2, CCM3 or control siRNAs (final concentration: 100 nmol/L). Specifically, silencing experiments were performed using CCM2 and CCM3 siRNAs from Dharmacon (Dharmacon ON Target Plus J-014728-05-0050 and J-004436-05-0050, respectively). Cell transfections were performed using INTERFERin kit (Polyplus transfection, New York, NY, USA) according to the manufacturer's protocol. Cells were cultured with siRNAs for 24 h before treatments and analysis.

Immunofluorescence imaging.

96-well imaging plates (BD Falcon) were used for immunofluorescent imaging screening. Cells were fixed with 4% formaldehyde in PBS for 10 minutes, washed 3x in ultrasaline (Lonza), then

incubated overnight with mouse anti-human CD144 antibody (BD Biosciences). Cells were washed 3x with ultrasaline, then a mixture of Hoechst 33342, Alexa-Fluor 488 Phalloidin (Invitrogen), and Alexa-fluor 594 goat anti-mouse antibody in ultrasaline was added for 6 hours. After an additional 3 washes, 200 µl ultrasaline was added to each well, and then the plates were covered with adhesive foil and immediately imaged. 96-well imaging plates were imaged using a high-throughput BD Pathway 855 Bioimager. A custom macro was written using the Attovision software (BD) encompassing imaging autofocus at each well, followed by acquisition of each channel. Each image consisted of a 4x4 montage using a 40x objective, covering a total area of 810 x 600 µm in each well. Images were automatically saved in files appended with plate and well name meta-data.

Drug treatment.

Compounds were obtained in 100% DMSO at 2 mM concentration in 96-well polypropylene assay plates from the University of Utah Drug Screening Resource Core and kept frozen at -20˚C. Libraries acquired included the Microsource Spectrum Library of exactly 2000 known drugs and bioactive compounds as well as 100 additional known drugs or bioactive compounds available to us in our own or a collaborator's laboratory. At the time of treatment, drugs were added to 96-well imaging plates containing confluent siCCM2 treated HMVEC-D endothelial monolayers using a 12-channel electronic pipette (Eppendorf Xplorer) to a final concentration of 10uM, 0.5% DMSO (vehicle) in starvation media (EBM-2 + 0.2% bovine serum albumin) and gently mixed. Positive (siCCM2) and negative (siCTRL) control wells received DMSO to 0.5% in starvation media without any compound treatment. Follow-up experiments were performed using the highest available grade of reagent from Tocris or Sigma.

Automated image analysis.

Images were analyzed using CellProfiler and CellProfiler Analyst[35-37]. A custom CellProfiler pipeline imported all images, applied a light correction filter, and down-sampled the size of the images by a factor of 4 to reduce processing time. Loss of authenticity of each image was guaranteed by extracting meta-data from all original files including both plate and well names, and connecting this data digitally to the MySQL output database created by CellProfiler. The Hoechst channel was first utilized to identify nuclei using an Otsu Global thresholding method. The VE-cadherin channel was utilized next to identify cell-cell borders again using a Global thresholding method minimizing entropy. A threshold correction factor of 0.7 and a regularization factor of 0.001 were determined based on trial and error to give the best cell segmentation. Cells not completely enclosed within the image were automatically excluded from analysis. Several hundred parameters were calculated and recorded for each cell including co-localization of each channel, radial localization of actin and VE-cadherin, texture of actin and VE-cadherin, and nuclear and cell shape and size characteristics. The hundreds of quantifications calculated for each cell in every image were stored in a database accessible by CellProfiler Analyst. CellProfiler Analyst was subsequently used to open 100 control images (siCTRL or siCCM2 treated). Using a random cell selection tool available in CellProfiler Analyst software, approximately 20 cells were selected from each control image and added to categorization bins if appearing qualitatively to be representative of the typical positive or negative control cells. Cells that appeared to be dead or dying (characterized by a small, rounded cell not adhering to its neighbors), for example, were not included into sorting bins. Subsequently, rules were automatically developed using the GentleBoosting machine-learning

algorithm applied to regression stumps (as built into the CellProfiler Analyst software). The rules generated were then used to classify images of cells from additional control images, and the author manually corrected any obvious errors in classification (e.g. a prototypic siCCM2 treated cell in a positive control image being classified as siCTRL-treated). This process was repeated until approximately 2,000 cells comprised each bin; positive (siCCM2) and negative (siCTRL). The rules generated were not further tuned by hand. Previously unused control images (siCTRL or siCCM2 treated), as well as images of siCCM2 treated wells treated with individual drugs, were then evaluated using the rules. To directly compare the capability of qualitative human analysis, in which 38 compounds were identified, and automated machine-analysis, we selected the top 38 compounds identified using CellProfiler Analyst for further study without a specific statistical cut-off.

Qualitative image analysis

Two reviewers familiar with immunofluorescence imaging were shown dozens of images of labeled positive and negative controls (siCCM2- and siCTRL-treated, respectively), and were asked to 'learn' the phenotype. When each reviewer felt comfortable with identifying the positive and negative controls (with no more specific instructions given about what aspect of the phenotype to focus on), they were then asked to look at each image (blinded to treatment) from all 2,100 compound treatments for both independent replicates of the treatment (4,200 total images). They were instructed to mark any image in which the phenotype associated with siCCM2 treatment seemed to revert back (even partially) to siCTRL (or 'healthy'). No rating or ranking was given – reviewers were asked to identify any image which appeared 'improved'. Those images which were identified by both reviewers in both replicates as 'improved', were

then noted as primary hits and prioritized for subsequent follow-up in secondary assays (38 compounds).

Electric cell substrate impedance sensing (ECIS).

A 96-well ECIS plate (96W10E+, Applied Biophysics) was used. At the second siRNA transfection, HMVEC-D were seeded at $4x10^4$ cell/well. Plates were monitored using an ECIS Zθ system (Applied Biophysics) to measure resistance of an alternating current (4000 Hz) across the cell monolayer. Resistance was normalized for each well to just before treatment, and was plotted in real time. Cells were fed according to the same schedule as in the transfection protocol. 72 hours after the second siRNA transfection, compounds were added to each well at a final concentration of 10 µM in 0.5% DMSO.

Mouse strains.

Mice used have been previously reported[38, 39]. Tamoxifen (Sigma-Aldrich) was re-suspended in corn oil (Sigma-Aldrich) to a concentration of 1 mg/ml, and 40 µl was injected into the peritoneum of pups on perinatal day 1.

Dermal permeability.

We performed a modification of a previously described Miles Assay[40]. Male mice between 2 and 4 months of age were anesthetized with isoflurane and their backs gently shaved and depilated using a hair-removal cream (Nair). The mice were then gently washed with warm water and allowed to recover. The following day, the mice were again anesthetized with isoflurane and 20 µL intradermal injections of treatments (50 µM in 0.5% DMSO in sterile

saline) in various positions on the back (the drugs were added to syringes by a person not performing the assay and labeled “a”, “b”, etc. therefore blinding the primary experimenter. 90 minutes later, Evans blue dye (100 µL of 1% dye in sterile saline, Sigma) was injected into the tail vein. After 30 minutes, mice were sacrificed and equal-sized skin biopsies from each injection site were collected and placed in formamide (Invitrogen) overnight at 60˚C. Absorbance at 620 nm (measurement of blue dye) and 740 nm (to correct for any blood in the sample) was used to quantify the amount of Evans blue dye in each sample (reading at 620 nm – reading at 740 nm) and the results tabulated. The absorbance is reported with the standard error of the mean.

Murine CCM lesion formation and MRI analysis.

5 days after birth (P5), litters were assigned to a standard chow (Harlan 2018, 1.5 IU/g D_3), a standard chow plus tempol in drinking water (1mM), or a cholecalciferol-enhanced chow (Harlan 2018 + 25 IU/g D_3). The chow was provided to the mother of each litter until the mice were weaned at P21. Mice from each litter continued on their respective diets until 5 months of age. At 5 months of age, mice were sacrificed by exsanguination (blood was collected for later analysis), and subsequent perfusion with saline and then 4% formaldehyde. Brains were dissected from the skull, and postmortem MRI scanning was performed. A gradient recalled echo sequence was used to acquire coronal slices spanning the whole brain. Sequence parameters were as follows: repetition time, 328 ms; echo time, 5.4 ms; flip-angle, 40°; 12 averages, in-plane-resolution, 125 µm × 125 µm; and slice thickness, 0.5 mm. For a representative subset of brains (for use in 3D reconstructions), high-resolution 3D gradient echo was acquired using the following parameters: isotropic voxel size of 78 µm × 78 µm × 78 µm over 9 hours. Other

sequence parameters were as follows: repetition time, 250 ms; echo time, 7.5 ms; flip angle, 30°; and 2 averages. Lesion area and number were quantified by multiple blind reviewers using ImageJ and Osirix software. Specifically, each reviewer was provided with all MRIs which had been relabeled randomly. Each reviewer then used software to circle all 'lesions' of any size in every slice of every MRI from all mice. Contiguous lesions were outlined as one large lesion. The results of all reviewers were tabulated. 3D reconstructions were assembled using Osirix software by a blinded reviewer. For murine simvastatin treatment analysis (Supplementary Figure 1), mice were fed a standard diet (Harlan 8640) or a simvastatin-enhanced diet starting at weaning (Harlan 8640, 311 mg/kg simvastatin). Lesions were imaged in live mice on a monthly basis (using the same parameters for non high-resolution MRI as described above). Images from mice at 4.5 or 5 months were scored by blinded reviewers as described as above.

Mouse experiments.

All mouse experiments were approved by the University of Utah Institutional Animal Care and Use Committee or the George E. Wahlen Department of Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Middle cerebral artery endothelial function.

Endothelial function assays are based upon previously published methods[41]. Middle cerebral artery endothelial function was performed as previously described. Mice (N=7-14/group) were euthanized via exsanguinations by cardiac puncture while under isoflurane anesthesia. Right and left middle cerebral arteries approximately 100-120 micrometers in luminal diameter were excised from the brain and placed in myograph chambers (DMT A/S, Aarhus, Denmark)

containing EDTA-buffered physiological saline solution (PSS), cannulated onto glass micropipettes and secured with nylon (11–0) suture. Once cannulated, the middle cerebral arteries were warmed to 37˚C and pressurized to 60 mmHg intraluminal pressure and allowed to equilibrate for 1 h. All arteries were then submaximally preconstricted with phenylephrine (2 μM). Increases in luminal diameter in response to increasing concentrations of the endothelium-dependent dilator acetylcholine (Ach; 1×10^{-9} to 1×10^{-4} M) in the absence or presence of the nitric oxide synthase inhibitor, L-NAME (0.1 mmol/L, 30 min) or the superoxide scavenger, TEMPOL (1 mmol/L, 60 min) was assessed. Endothelium-independent dilation to sodium nitroprusside (SNP: 1x10-10 to 1x10-4 mol/L) also was determined (5). Arterial segments were imaged and diameters measured by an automated edge detection; VAS software (DMT A/S, Aarhus, Denmark). All dose response data are presented as a percent of possible dilation after phenylephrine preconstriction.

Non-invasive blood pressure measurements in conscious mice.

Murine blood pressures were non-invasively measured by determining the tail blood volume with a volume pressure recording (VPR) sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT). Blood pressure and heart rate recordings were made in a quiet and warm (24 C) environment. Mice were placed in restrainers on a heating unit and given 15-20 minutes to acclimate and reach a steady body temperature (30-35 C). Each session consisted of 5-10 acclimatization measurements; if those measures were stable, they were followed by 20 experimental measures. Measures with aberrant movement/behavior or inadequate volume pressure measures were treated as outliers. Averages from each session were used for systolic

blood pressure, diastolic blood pressure, mean blood pressure and heart rate for each individual mouse.

Biochemical assays.

Rabbit polyclonal antibody against CCM2 was from Sigma (Rabbit HPA020273). α-actinin antibody (clone H-2) was from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were from Jackson Immunoresearch. Western blots were developed using the ECL Plus Western blotting reagent (GE Healthcare) and Kodak Biomax MR film. Wild-type, siCTRL or siCCM2 treated HMVEC-D cells were incubated with either 100 nM or 10μM cholecalciferol (Tocris), 7-DHC (Sigma) or vehicle (0.5% DMSO) for 60 min (pMLC, ARF6, RAC, CDC42, RRAS) or 24 hours (RHOA), unless otherwise indicated. After treatment, the cells were washed with ice-cold PBS and lysed in 50mM Tris pH 7.4, 150mM NaCl, 10mM MgCl2, 10% Glycerol, 1% NP-40, 1X protease inhibitors, and 1X phosphatase inhibitors. For RhoA, ARF6, Rac1 /cdc42 and R-Ras activation assays, crude total cell lysate were generated and GTP-RhoA, ARF6, Rac1/ cdc42 and R-Ras were precipitated with Rhotekin-RBD (Millipore), GGA3-PBD (Cell Biolabs), PAK-1-PBD (Millipore) and Raf-1 RBD respectively. Following three washes with lysis buffer, bound proteins were eluted with 2X sample buffer. RhoA, ARF6, Rac1 /cdc42 and R-Ras was detected by western blotting with antibodies (RhoA, Rac1 and R-Ras antibody are from Cell Signaling, ARF6 and cad42 antibody are from Millipore). Each blot is representative of at least three independent experiments, for which quantification is shown.

Fluorimetric intracellular ROS detection.

(Fig. 4f) Briefly, EA-hy926 cells grown to confluence in complete medium were washed twice with PBS, incubated with 2',7'-dichlorfluorescein-diacetate (DCFH-DA) at a final concentration of 5μM in PBS at 37°C for 30 min and analysed by Tali image based cytometer (Invitrogen). Raw data were elaborated by Flowing software (v. 2.5.0, by Perttu Terho, University of Turku, Finland).

Statistics.

Data presented are mean ± SEM unless otherwise indicated. Statistical significance is indicated generally by a single symbol (i.e. *) to indicate $P < 0.05$, two symbols (i.e. **) to indicate $P < 0.01$, three symbols (i.e. ***) to indicate $P < 0.001$. $P<0.01$ was considered marginal significance.

Figure 3A: Individual data, mean and SEM are shown for N = 6-75 samples per group. A Student's t-test was used to evaluate significance.

Figure 4B: Individual data and mean are shown for N = 4-12 samples per group. For dermal permeability analysis, where we screened four compounds to determine which, if any, could inhibit vascular leak in endothelial specific knockout mice, we performed a mixed effects linear model with Absorption (OD) as the outcome and a single predictor variable (drug treatment), with the Vehicle group as the reference category. The mixed model included a random effect for the mice. The wild-type control mice (open circles) were shown descriptively for interested readers but not included in the statistical analysis. This provided four comparisons to the control. The mixed effects model accounted for clustering due to testing multiple treatments within the same mouse. Given that this was a screening experiment, for multiplicity adjustment we used the Benjamini-Hochberg procedure.

Figure 5B: Individual data, mean and SEM are shown for N = 8-12 samples per group. For chronic treatment in mice, as evaluated by MRI, we compared the number of lesions as shown in the dot plot using a one-way ANOVA with Dunnett's multiple comparison test. The data were sufficiently normally distributed to allow for parametric analysis with the given sample sizes.

Figure 5C: The number of lesions within each mouse was subdivided into successive intervals reflecting 2-fold increases (starting with the minimum resolution of the MRI analysis) to define a sequence of bins defined on the horizontal axis of the figure. For each of the resulting bins, the number of lesions within the designated size range was computed for each mouse, and compared between diet groups using separate exact Wilcoxon Mann-Whitney tests to account for non-normality and differences in the amount of variability for the numbers of lesions between different bins. P-values were adjusted to account for 2 comparisons within each bin. Medians are shown for N = 8-12 samples per group.

Figures 6A-C: Individual data, mean and SEM are shown for N = 3 samples per group. For evaluation of the effect of D3 on activation of various biochemical factors, ordinary one-way ANOVA was utilized with Dunnett's multiple comparison analysis.

Figure 6E: Individual data, mean and SEM are shown for N = 5-8 samples per group. The effect of either D3 or 7DHC were evaluated on the timing of inhibition of the activation of ARF6 in cells deficient in CCM2. Control RNAi-treated cells with 7-DHC are shown as a control at t=1 and t=60 minutes. Multiple t-tests were used to evaluate significance with the Sidak-Bonferroni method of multiple comparison

Figures 7A and D: Individual data, mean and SEM are shown for N = 9-19 samples per group. For evaluation of vasodilation, we utilized a two-way ANOVA with one factor being genotype

and the second factor being acetylcholine dose as a repeated measures factor with Sidak's multiple comparison test.

Figures 7E-H: Individual data, medians and interquartile ranges are shown for N = 5-10 samples per group. The exact Wilcoxon Mann-Whitney test was used due to the presence of apparent outliers.

Supplemental Figures

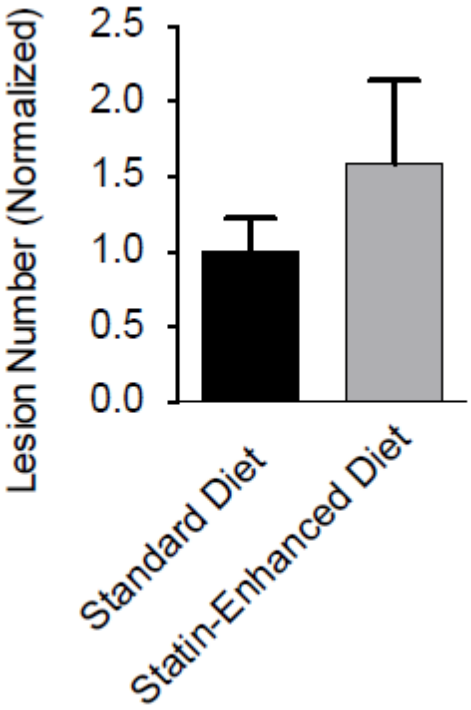


Fig. S1: Simvastatin treatment has no effect on lesion burden in a murine model of CCM. Mice were treated with a diet enhanced with simvastatin for 4 months. At 5 months of age, the lesions were quantified by blinded reviewers using MRI. These data were normalized due to variations in the analysis software used by the reviewers.

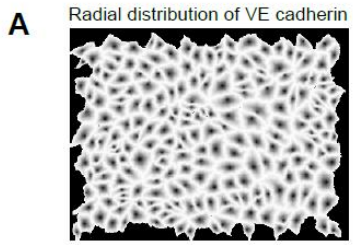


Image	Objects	Bin #	Bin count	Fraction	Intensity	COV
VECADHERIN	Cells	1	8	0.0053	0.9295	0.0
VECADHERIN	Cells	2	8	0,0207	0,9229	0,0002
VECADHERIN	Cells	3	8	0.0453	0.9282	0.0017
VECADHERIN	Cells	4	8	0.0764	0.9394	0.0018
VECADHERIN	Cells	5	8	0.1147	0.938	0.0018
VECADHERIN	Cells	6	8	0,1659	0,9367	0,0078
VECADHERIN	Cells	7	8	0,2472	0,9648	0,0243
VECADHERIN	Cells	8	8	0,3245	1,1331	0,057

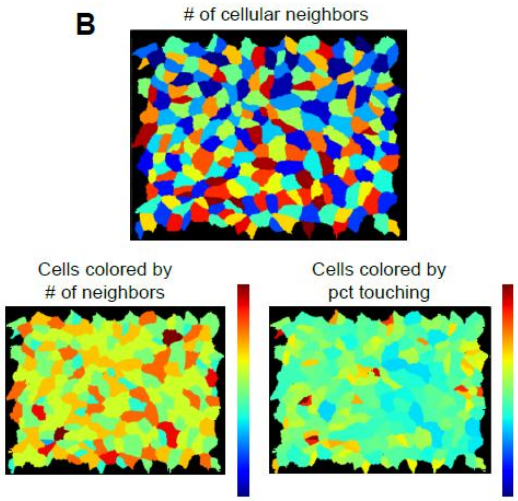


Fig. S2: Software analysis of immunofluorescence images. Calculations are made for each cell in each image for a wide variety of mathematical descriptors including, for example, (**A**) the radial distribution of VE-cadherin, and (**B**) the number of neighbors and percent of cell borders touching other cells.

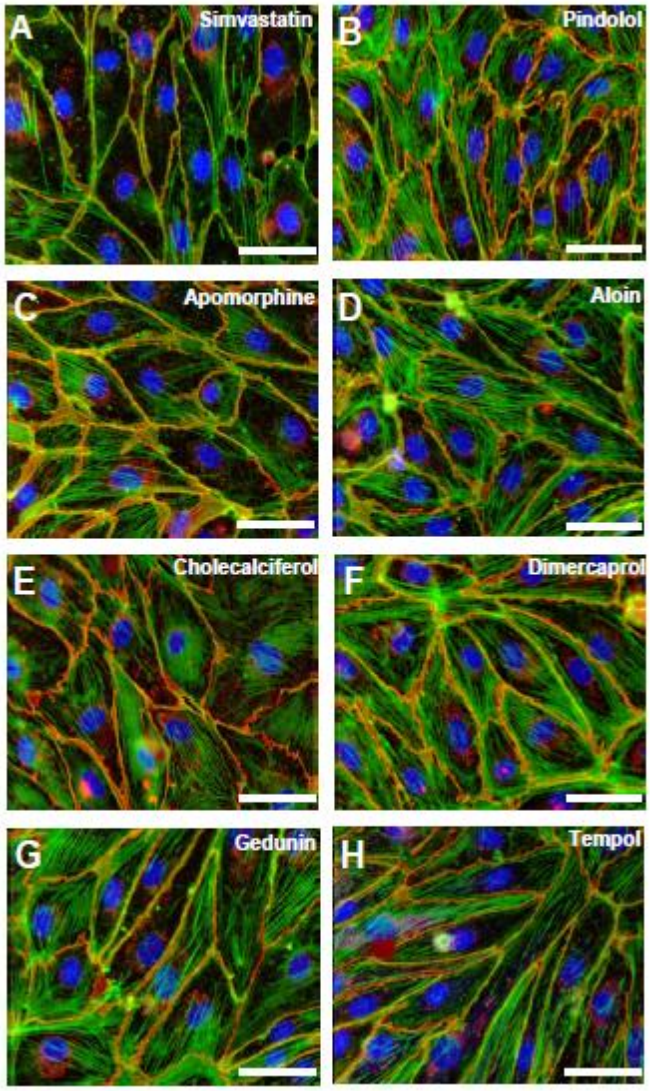


Fig. S3: Compounds of interest after primary and secondary screens. Immunofluorescence images of siCCM2-treated endothelial cells stained for DNA (blue), actin (green), and VE-cadherin (red) after treatment with (**A**) simvastatin, (**B**) pindolol, (**C**) apomorphine hydrochloride, (**D**) aloin, (**E**) cholecalciferol, (**F**) dimercaprol, (**G**) gedunin, or (**H**) tempol. Scale bars represent 50 µm.

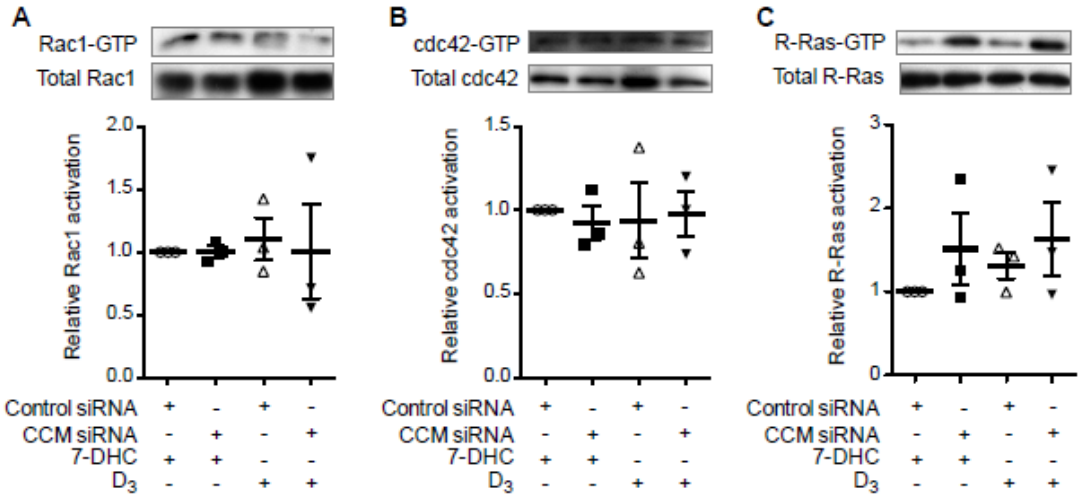
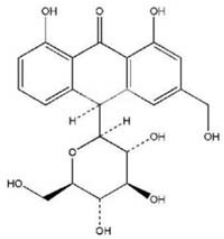
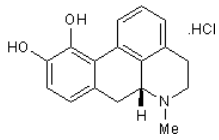
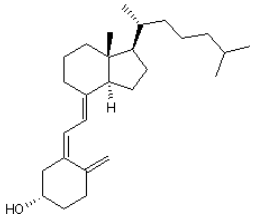
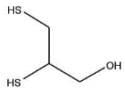
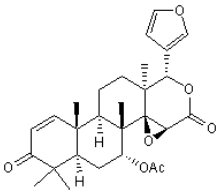
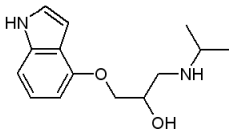
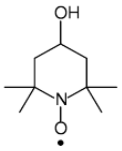


Fig. S4: Cholecalciferol has no effect on the activation of certain destabilizing pathways in the endothelium. 60 minute treatment with 10 µM cholecalciferol had no effect on activation of (**A**) RAC1, (**B**) CDC-42, or (**C**) R-RAS in endothelial cells. All data, mean and SEM are plotted and significance was evaluated using ordinary one-way ANOVA was utilized with Dunnett's multiple comparison analysis

Rules Developed by CellProfiler Analyst Machine-Learning Algorithms
• IF (Cells_Correlation_Correlation_ACTIN_VECADHERIN > 0.313844295602
• IF (Cells_Texture_SumVariance_ACTIN_4 > 3.1764155229000002
• IF (Cells_RadialDistribution_MeanFrac_VECADHERIN_7of8 > 0.94580706432899997
• IF (Cells_Texture_Correlation_ACTIN_4 > 0.48952960487899999
• IF (Cells_RadialDistribution_MeanFrac_ACTIN_1of8 > 0.77109633025199997
• IF (Cells_Neighbors_SecondClosestDistance_Adjacent > 68.268449998600005
• IF (Cells_Texture_AngularSecondMoment_ACTIN_4 > 0.25715311579799999
• IF (Cells_RadialDistribution_FracAtD_ACTIN_7of8 > 0.30024600000000001
• IF (Cells_Number_Object_Number > 163.0
• IF (FilteredNuclei_Correlation_Correlation_VECADHERIN_NUCLEI > -0.33414821391100002
• IF (Cells_RadialDistribution_MeanFrac_ACTIN_6of8 > 0.98140631941000001
• IF (Cells_RadialDistribution_FracAtD_VECADHERIN_3of8 > 0.046892200000000002
• IF (Cells_Granularity_2_ACTIN > 5.9838954003299998
• IF (Cells_RadialDistribution_MeanFrac_VECADHERIN_3of8 > 1.0490967062300001
• IF (Cells_Correlation_Correlation_ACTIN_NUCLEI > -0.30085506399900003

Supplementary Table 1. Rules generated by Machine-Learning Analysis.

Compound	Activity/Target	Structure	In CCM Literature
Aloin	Unknown		
Apomorphine Hydrochloride	Non-selective dopamine agonist		
Cholecalciferol (vitamin D3)			
Dimercaprol	Chelating agent		
Gedunin	Inhibitor of Heat Shock Protein 90		HSP90 selectively binds CCM2[47]
Pindolol	Non-selective beta-adrenergic receptor antagonist		Role for beta blockers in clinical improvement of CCMs and other vascular malformations[50]
Tempol	Superoxide scavenger		Krit1 (CCM1) and PDCD10 (CCM3) linked to oxidative stress[48, 49, 60-62]

Supplementary Table 2. Compounds of interest after primary and secondary screens.