Strategy for Identifying Repurposed Drugs for the Treatment of Cerebral Cavernous Malformation

Running title: Gibson et al.; Strategy for Identifying CCM Treatments

Christopher C. Gibson, PhD1,2,3,4; Weiquan Zhu, PhD1,5; Chadwick T. Davis, PhD1,5; Jay A. Bowman-Kirigin, BS1; Aubrey C. Chan, MD, PhD1,6; Jing Ling, BA1; Ashley E. Walker, PhD7; Luca Goitre, PhD8; Simona Delle Monache, PhD9; Saverio Francesco Retta, PhD8; Yan-Ting E. Shiu, PhD8,10; Allie H. Grossmann, MD, PhD1,11; Kirk R. Thomas, PhD1,3; Anthony J. Donato, PhD7,12; Lisa A. Lesniewski, PhD7,12; Kevin J. Whitehead, MD1,13,15; Dean Y. Li, MD, PhD1,3,4,6,13,14,15

1Program in Molecular Medicine, University of Utah, Salt Lake City, UT; 2Dept of Bioengineering, University of Utah, Salt Lake City, UT; 3Dept of Medicine, University of Utah, Salt Lake City, UT; 4Recursion Pharmaceuticals, LLC, Salt Lake City, UT; 5Dept of Human Genetics, University of Utah, Salt Lake City, UT; 6Dept of Oncological Sciences, University of Utah, Salt Lake City, UT; 7Division of Geriatrics, Dept of Medicine, University of Utah, Salt Lake City, UT; 8CCM Italia, Dept of Clinical and Biological Sciences, University of Torino, Orbassano, Torino, Italy; 9CCM Italia, Dept of Biotechnological and Applied Clinical Sciences, University of L’Aquila, L’Aquila, Italy; 10Division of Nephrology and Hypertension, Dept of Medicine, University of Utah, Salt Lake City, UT; 11Dept of Pathology, University of Utah, Salt Lake City, UT; 12Geriatrics Research Education and Clinical Center, Veteran’s Affairs Medical Center, Salt Lake City, UT; 13Division of Cardiology, Dept of Medicine, University of Utah, Salt Lake City, UT; 14The Key Laboratory for Human Disease Gene Study of Sichuan Province, Institute of Laboratory Medicine, Sichuan Academy of Medical Sciences & Sichuan Provincial People’s Hospital, Chengdu, Sichuan, China; 15Cardiology Section, VA Salt Lake City Health Care System, Salt Lake City, UT

Address for Correspondence:
Dean Y. Li, MD, PhD
University of Utah, Salt Lake City
Building 533 Room 4220, 15 N 2030 E
Salt Lake City, Utah, 84112
Tel: 801-587-8294
Fax: 801-585-0701
E-mail: dean.li@u2m2.utah.edu

Abstract

Background—Cerebral cavernous malformation (CCM) is a hemorrhagic stroke disease affecting up to 0.5% of North Americans with no approved non-surgical treatment. A subset of patients have a hereditary form of the disease due primarily to loss-of-function mutations in KRIT1, CCM2, or PDCD10. We sought to identify known drugs that could be repurposed to treat CCM.

Methods and Results—We developed an unbiased screening platform based on both cellular and animal models of loss-of-function of CCM2. Our discovery strategy consisted of four steps: an automated immunofluorescence and machine-learning-based primary screen of structural phenotypes in human endothelial cells deficient in CCM2; a secondary screen of functional changes in endothelial stability in these same cells; a rapid in vivo tertiary screen of dermal microvascular leak in mice lacking endothelial Ccm2; and finally a quaternary screen of CCM lesion burden in these same mice. We screened 2,100 known drugs and bioactive compounds, and identified two candidates for further study, cholecalciferol (Vitamin D3) and tempol (a scavenger of superoxide). Each drug decreased lesion burden in a mouse model of CCM vascular disease by approximately 50%.

Conclusions—By identifying known drugs as potential therapeutics for CCM, we have decreased the time, cost, and risk of bringing treatments to patients. Each drug also prompts additional exploration of biomarkers of CCM disease. We further suggest that the structure-function screening platform presented here may be adapted and scaled to facilitate drug discovery for diverse loss-of-function genetic vascular disease.

Key words: Cerebral Cavernous Malformation, Vascular Malformation, Vitamin D3, Screening, stroke, cerebrovascular disorders, endothelium, genetics, hemorrhage
Introduction

There are at least 7,000 diseases designated as rare in the United States. Taken as a whole at least 25 million Americans are affected by a rare disease. Disease-causing mutations in a single gene account for as many as 5,000 rare diseases, the largest subset. The often arduous quest for a proper diagnosis and treatment by highly specialized practitioners is costly to our healthcare system and for families faced with a rare disease. Further, the high cost of drug development combined with relatively small markets exerts a negative effect on commercial interest in treatment of these diseases. The Orphan Drug Act of 1983 and its subsequent amendments have created regulatory and economic incentives for drug development in the rare disease space, and there have been major successes both for pharmaceutical companies and for rare-disease patients. However, more than 95% of rare diseases still have no approved treatment.

An area of current excitement and challenge in the pharmaceutical industry is drug repurposing (sometimes referred to as drug rediscovery or drug repositioning), whereby existing drugs are used for additional or alternative indications from those for which they were originally designed or intended. The classic example of drug repurposing is that of sildenafil (Viagra), intended originally to treat angina, but attaining ‘blockbuster’ status for the treatment of erectile dysfunction. The vast majority of drug repurposing successes can be attributed to serendipity or to a reasoned approach based on a deep understanding of a specific disease mechanism. While these reasoned approaches often work for well-studied diseases, a comprehensive picture of the molecular mechanisms underlying many rare diseases is lacking. A scalable platform for drug repurposing across many rare diseases would thus have considerable impact.

Over the past decade we have studied a monogenic loss-of-function stroke disease,
Cerebral Cavernous Malformation (CCM), in an attempt to learn more about the molecular mechanisms underlying vascular stability and with an emphasis on translational research. CCM is a hemorrhagic stroke syndrome characterized by vascular malformations in the central nervous system. CCM lesions are leaky and unstable, with chronic and acute bleeding leading to inflammation and stroke respectively\(^\text{10}\). The only treatment for CCM is neurosurgical resection\(^\text{11}\). CCM occurs in two forms: sporadic and familial, which together affect as many as 1 in 200 to 500 individuals in the United States\(^\text{12, 13}\). The familial form of CCM accounts for 20% of cases, and is most often associated with heterozygous loss-of-function mutations in one of three genes, \textit{KRIT1}, \textit{CCM2}, or \textit{PDCD10}\(^\text{14}\). Though a very limited number of missense mutations have been reported, more than 90% of the mutations identified in all CCM genes are null (loss-of-function) frameshift, deletion, or splicing mutations\(^\text{15-27}\). A somatic second-hit loss-of-heterozygosity mechanism has been proposed, and limited experimental results from human samples are so far consistent with that model\(^\text{28-34}\). We have previously reported a reductionist, target-based, hypothesis-driven, drug repurposing strategy for CCM, and we show here results that caused us to re-evaluate that general approach. As a result, we developed an unbiased drug discovery platform for identification of an effective therapeutic for CCM. Here we report that this platform has identified two known drugs, cholecalciferol (vitamin D\(_3\)) and tempol (a superoxide scavenger), as investigational treatments for CCM. We demonstrate that both of these compounds successfully reduce CCM lesions in a mouse model of human CCM disease.

**Materials and Methods**

The complete detailed methods are included in the supplemental material.

**Primary Screen**

Primary human adult dermal microvascular endothelial cells and cell culture reagents were from
Lonza (Basel, Switzerland); Small interfering RNA (siRNA) from Dharmacon Thermo Scientific (Waltham, MA), and the Spectrum Collection compound library from MicroSource Discovery Systems (Gaylordsville, CT). Cells were subjected to two rounds of transfection (48 hrs apart) with siRNA targeting CCM2 or a scrambled control, and seeded into 96-well plates. After 72 hours, cells were fixed, stained with probes, and imaged using a high-throughput microscope. For screening, compounds were added to each at a final concentration of 10 μM in 0.5% DMSO for 24 hours.

**Secondary Screen (Electric cell substrate impedance sensing)**

Following the second siRNA transfection, HMVEC-D were seeded at 4x10^4 cell/well onto a 96-well ECIS plate (96W10E+, Applied Biophysics). Plates were monitored using an ECIS Z0 system 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. 72 hours after the second siRNA transfection, compounds were added to each well at a final concentration of 10 μM in 0.5% DMSO.

**In vivo screens**

The CCM2 mouse model, dermal permeability and MRI analyses were performed as previously described. Endothelial specific CCM2 knockout mice were fed standard chow, chow enhanced with vitamin D3, or water enhanced with tempol, for five months prior to MRI-analysis. 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.
Results

Motivation for a new approach

We and others have identified dysregulation of the small GTPase, RHOA, in cells lacking either KRIT1 or CCM2 as a possible pathogenic mechanism of CCM disease. This identification was based on reasoned probing of the molecular mechanisms associated with the disease and a reductionist methodology to target identification\textsuperscript{38-40}. Because of that approach, we suggested that statins, which inhibit RHOA activation through antagonistic activity on HMG-CoA reductase, might be repurposed to treat CCM disease\textsuperscript{30}. Though missense mutations have been described in KRIT1 and CCM2 patients, the vast majority of patients with disease harbor frameshift, deletion, or splicing mutations in a single CCM allele\textsuperscript{15-27}. Though the exact mechanism by which heterozygosity for a null allele dramatically increases predisposition to disease is incompletely understood, the limited evidence available in humans and from animal models supports a Knudsonian second-hit loss-of-heterozygosity mechanism\textsuperscript{14, 28, 29, 31-34, 41}. Thus, we created mice that are compound heterozygous for a loss of function allele in Ccm2 with the cognate allele flanked by loxp sites permitting its post-natal, endothelial-specific, deletion (PDGFb-iCreER\textsuperscript{T2})\textsuperscript{42}. Strikingly, these mice develop CCM lesions that reproduce all of the pathologic aspects of human CCM disease\textsuperscript{43}. To our disappointment, four months of simvastatin treatment in this model did not result in a decreased burden of disease, as measured by small animal MRI (Supplementary Fig. 1). Potential explanations for this result are myriad and do not exclude a role of RHO activation in CCM pathophysiology, and certainly do not exclude the use of statins or other modulators of RHO activity as an effective treatment of CCM disease. However, the effect of this result was to prompt us to critically reexamine the reductionist, target-centric approach to drug identification we had employed.
Development, evaluation, and execution of the screen

Endothelial cells deficient in CCM2 have obvious structural and functional phenotypes. We hypothesized that we could use these phenotypes for unbiased drug discovery. Our strategy was to develop a multi-stage screen taking advantage of in vitro structural phenotypes (primary screen), in vitro functional phenotypes (secondary screen), acute in vivo functional phenotypes (tertiary screen), and chronic in vivo disease phenotypes (quaternary screen) (Fig. 1). We chose fluorescent microscopy and automated cellular quantification and profiling as our primary screen, transendothelial resistance as our secondary screen, measurement of the leakiness of dermal vasculature in a CCM mouse model as our tertiary screen, and measurement of lesion burden using small animal magnetic resonance imaging (MRI) as our quaternary and final screen. The specific assays were chosen based upon throughput and the quantity and quality of data provided. In general, each successive step in our platform exchanged decreased throughput and increased associated time and effort with increased predictive ability. Another important aspect of our strategy was to use a library of 2100 small molecules composed of known drugs and bioactive compounds based upon our hypothesis that hits from this library could more quickly be translated to the bedside.

Primary imaging screen

A primary imaging screen was chosen because of the richness of data available relative to the ease with which an academic laboratory can perform such medium- or high-throughput assays. As the vast majority of patients with CCM disease have mutations hypothesized to result in loss-of-function or amounts of CCM protein, we chose to model disease in human cells using well-validated siRNA to knock-down CCM2. Human Dermal Microvascular Endothelial Cells (HMVEC-D) were treated with well-validated CCM2 mRNA-targeting siRNA or a scrambled
control, and then seeded into 96-well imaging plates (Fig. 2A)\textsuperscript{38-40}. Large immunofluorescence images composed of 16 adjacent fields of view stitched together automatically were captured from each well of a 96-well plate in three channels sufficient to give an impression of the cell structure including the nucleus, actin stress fibers, and VE-cadherin cell-cell junctions (Fig. 2B). A high-throughput microscope developed for phenotypic drug discovery allowed automated imaging of an entire 96-well plate in about 60 minutes. We used CellProfiler, an open-source high-content imaging analysis tool developed and overseen by Dr. Anne Carpenter of the Broad Institute, to import images, identify the borders of each cell, and create a database of a multitude of mathematical descriptors of every cell in every image collected (Fig. 2C, Supplementary Fig. 2A-B)\textsuperscript{35-37,44}. We then used CellProfiler Analyst, a machine-learning tool, to develop rules that could be used to distinguish whether each cell in an image was more likely to have been treated with scrambled control siRNA or siCCM2 (Supplementary Table 1)\textsuperscript{36,37}. The software was able to accurately categorize images (based on the proportion of individual cells in each image scored as siCTRL or siCCM2), as ‘siCTRL-treated’ or ‘siCCM2-treated’ as calculated by a Z’ of 0.7, a statistical test for evaluating assays for high-throughput screening for which any value between 0.5 and 1 is considered amenable to high-throughput screening (Fig. 2D)\textsuperscript{45}.

We then screened 2,100 known drugs to identify those that could rescue the structural phenotype associated with loss of CCM2. We analyzed the resulting images to identify rescue using CellProfiler and CellProfiler Analyst as well as using qualitative scoring by two blinded reviewers as a comparison. The two reviewers who performed qualitative analysis identified 38 compounds in common that when added to siCCM2-treated cells resulted in what they perceived was rescue of structural phenotypes. We simultaneously used the CellProfiler software system to prioritize compounds, and we selected the top 38 compounds so as to provide a direct numerical
comparison of the performance of qualitative analysis (38 compounds) and our automated analysis. Interestingly, there was no overlap between the compounds selected by human analysis and those selected by the automated computational scoring system.

**Secondary trans-cellular resistance screen**

To validate our hits and prioritize future analysis, we developed a secondary orthogonal screen using trans-cellular resistance based on the functional defect in monolayer stability in cells deficient in CCM2 (Fig 3A)\(^{38}\). Trans-cellular resistance was chosen due to its relatively high-throughput, its real-time nature, the quality and quantity of data generated, and its label-free, functional output which is highly orthogonal to the primary imaging screen. This assay employs real-time measurements of the resistance encountered when an electrical current is passed between electrodes upon which a monolayer of cells are growing, and we used it to simultaneously screen the two sets of 38 compounds identified in the primary screen by our manual and automated image analysis\(^{46}\). Of the 38 compounds selected by human analysis, only one compound (simvastatin) showed rescue of the defect in monolayer stability of cells treated with siCCM2 (Fig. 3B, Supplementary Fig. 3A). However, of the 38 compounds identified using CellProfiler’s automated machine-learning analysis, seven showed full or partial rescue of the functional phenotype (Fig. 3B). The seven compounds selected from our automated analysis and validated by our secondary screen include compounds from classes previously connected to CCM disease, as well as compounds without any previously described association with the disease (Supplementary Table 2, Supplementary Fig. 3B-H)\(^{47-50}\).

**Compound prioritization in an acute animal model of CCM**

Due to the time and cost associated with chronic treatment trials in our CCM mouse model, we next took advantage of a relatively high throughput microvascular leak assay in our inducible
endothelial-specific Ccm2 knockout mice (Ccm2<sup>f5</sup>; +/Tg(Pdgfb-iCreER<sup>T2</sup>), hereafter referred to as Ccm2 ecKO, as a tertiary screen to prioritize hits for chronic treatment models. When we injected small intradermal wheals of a subset of our hit compounds into these mice, we found that both tempol and cholecalciferol significantly reduced peri-injection microvascular leakiness (multiplicity adjusted comparisons, p=0.04 and p=0.04, respectively) (Fig. 4A,B). We do not exclude the possibility that the other compounds could be relevant therapeutic candidates for the treatment of CCM disease, but this assay allowed us to immediately prioritize tempol and cholecalciferol for further study.

**Compound validation in a chronic animal model of CCM**

Next, we performed chronic treatment studies of the effects of tempol and cholecalciferol in our Ccm2 ecKO mice<sup>43</sup>. These studies serve not just to evaluate the potential of these treatments for CCM disease, but as a proof of principle for the potential usefulness of our screening platform. Nursing mothers were treated with either standard chow (1.5 IU/g cholecalciferol), an identical chow enhanced with cholecalciferol (25 IU/g), or standard chow plus tempol dissolved in drinking water (1mM) starting 5 days after delivery of their pups. After weaning, mice were fed the same diet as their mother until 5 months of age, a point at which 100% of untreated endothelial specific Ccm2 knockout mice have cerebrovascular lesions detectable by MRI (Fig. 5A). As progression of human disease is followed by MRI, mice were also evaluated for lesion status by MRI. Two reviewers with experience reading murine and human MRI were provided MRI files from all mice in the study, but were totally blinded to treatment. The reviewers manually outlined each lesion in every MRI, and their results were tabulated. Mice receiving the diet enriched with cholecalciferol had a significant reduction in lesions compared to those receiving standard chow, while mice receiving tempol had a marginally significant reduction in
lesions (P=0.052) (Fig. 5B). When lesion size distributions were compared, cholecalciferol appeared to significantly reduce the number of small lesions and tempol appeared to marginally reduce the number of small lesions, while for both treatments there was a trend toward reduction of most lesion sizes (Fig. 5C). The effect of cholecalciferol and tempol supplementation was qualitatively obvious when comparing MRI-based three dimensional reconstructions of mouse brains (the brain with the median number of lesions from each treatment group is shown) (Fig. 5D).

**Novel treatments inform CCM pathophysiology**

A major advantage of screening a library of known drugs and bioactive factors is that a large body of research is generally available regarding the effects of any one of the compounds. Such was the case with both cholecalciferol and tempol. With knowledge of the various biologic roles of cholecalciferol and other forms of vitamin D at hand, we assessed the timing of the effects of cholecalciferol on the endothelium. We evaluated the effect of knockdown of CCM2, and subsequent treatment with cholecalciferol, on a panel of signaling pathways commonly associated with endothelial instability, including ADP-ribosylation factor 6 (ARF6), cell division control protein 42 homolog (CDC42), transforming protein RhoA (RHOA), phosphorylation of myosin light chain (pMLC), Ras-related C3 botulinum toxin substrate 1 (RAC1), or Ras-related protein R-Ras (RRAS) (Fig. 6A-C, Supplementary Fig. 4A-C). Treatment of monolayers with cholecalciferol, even at a physiologic dose 100 times lower than what was used in the screen (100 nM), inhibited the CCM2 knockdown-induced activation of ARF6, RHOA, and pMLC (Fig. 6A-C). Knockdown of CCM2 did not affect activation of CDC42, RAC1, or RRAS; nor did treatment of up to 10 μM cholecalciferol basally inhibit activation of these markers (Supplementary Fig. 4A-C). Because of a strong role for ARF6 as a central modulator of
endothelial permeability, we further examined the timing of the effects of cholecalciferol on ARF6 activation, and found inhibition to occur within 5 minutes (Fig. 6D-E). Taken together, these data suggest that cholecalciferol, even at physiologic doses, can rapidly and directly inhibit multiple key intracellular signaling pathways that play a role in endothelial activation and stability in the context of mutation-induced destabilization. These data shed light on CCM, and also inform the study of cholecalciferol, which has not previously been shown to have a direct and immediate stabilizing effect on the endothelium.

Similarly, identification of tempol due to our use of a library of known drugs and bioactive molecules enabled us to immediately investigate the known target of tempol, superoxide^{58}. Indeed, a role of oxidative stress in the pathophysiology of CCM disease due to two other causative genes, KRIT1 and PDCD10, has been reported^{48, 59-62}. In cell culture, loss of CCM2 induced increased ROS and decreased FOX01 expression, suggesting a potential common mechanism of CCM pathophysiology as has been proposed for KRIT1 and PDCD10 (Fig. 6F,G)^{48}. These results, and previous work in the field, led us to hypothesize that oxidative stress may be a driving force in CCM at the level of individual cells and may also have broader effects on the physiology of small vessels through effects on endothelial vasodilation^{63}. To test this hypothesis, we isolated murine middle cerebral arteries from our endothelial-specific CCM2 knockout mice and measured both endothelial-dependent and endothelial-independent vasodilation using acetylcholine and sodium nitroprusside, respectively^{64}. We found that endothelial specific loss of CCM2 resulted in a significant defect in endothelial-dependent vasodilation in our model, which could be completely rescued by tempol (Fig. 7A-D).

Additionally, both systolic and diastolic blood pressure trended toward an increase in the endothelial knockout mice (Fig. 7E-G), consistent with constricted, non-responsive vessels.
Interestingly, heart rate of the endothelial knockouts trended lower than in control mice (Fig. 7H). These data suggest that oxidative stress may play an even more critical role in CCM disease than previously described due to systemic effects.

Discussion

We have conceived and implemented a high-content screen querying both structural and functional cellular phenotypes to discover novel factors active in the CCM2 pathway. We combined high-content imaging, machine-learning software, as well as trans-cellular electrical resistance measurements as efficient and powerful tools for drug screening. We specifically focused on known drugs and bioactive compounds because we believe such drugs have a much higher chance of making a rapid impact on patient outcomes. By focusing on such molecules, our high-tech screening method, which combines advances in molecular biology, imaging, and computing, has led to a low-tech solution: the potential for a modulatory role of two known drugs on CCM disease. Chief among these is cholecalciferol, which is inexpensive and widely available in supplement form, freely available outdoors to those with exposed skin, and has a wide safety margin.

One of the most exciting aspects of these data was that the drug candidates chosen using automated software analysis outperformed those chosen by human analysis in our secondary screen. We hypothesize that this is due to a well-described human shortcoming called inattentional blindness in which humans often fail to notice an unexpected stimulus when other attention-demanding tasks are being performed \(^6^5\); the actin stress fiber phenotype, which was previously quantified and published by our lab, and known to both qualitative reviewers, may have overwhelmed or obscured more subtle or unexpected aspects of the phenotype that may
have as much or more biological relevance to the disease. Quantitative software analysis, however, is not unduly weighted by the stress-fibers, so that rescue of many subtle changes becomes important. We believe it is likely that well-tuned automated analysis will have the most significant gains when compared to reviewers who are highly informed or educated, as they are likely to be most susceptible to the preconceived notions and inattentional blindness. This is a major benefit of the automated software analysis as demonstrated in its considerable success compared to the human analysis in this screen. We would be interested in whether other future high-content screening studies that directly compare human and automated analysis support our findings.

Also of particular excitement to us are recent advances by other groups who have expanded our understanding of the pathophysiology of CCM disease and who have, in some cases, put forth potential treatments for the disease. Recent evidence suggests that endothelial to mesenchymal transition (endMT) plays a critical role in the pathophysiology of CCM disease\(^{66}\). Through inhibition of TGF-\(\beta\), a key driver of endMT, reduction in severity of disease in a mouse model of CCM disease was identified. There is evidence that the target of tempol, oxidative stress, drives, and is driven by, increased TGF-\(\beta\) signaling and endMT, offering a potential mechanistic link\(^{67}\). Additionally, metabolites of cholecalciferol have also been shown to strongly inhibit TGF-\(\beta\) signaling and epithelial-mesenchymal transition (EMT)\(^{68,69}\). Our laboratory and others have also proposed a critical role for RHOA activation in CCM disease, which has served as the basis of a recently initiated phase zero clinical trial of the effects of simvastatin patients with familial CCM due to mutations in \(KRIT1\)\(^{70}\). Though our attempt at RHOA inhibition in our murine CCM model using simvastatin failed, we await with excitement the results of a clinical trial currently underway and note that others have had success using a downstream inhibitor of
RHOA activation, fasudil\textsuperscript{71}. We have also shown here that RHOA activation in combination with ARF6 and PMLC activation are inhibited by cholecalciferol. Taken together, these studies suggest that both compounds identified in an unbiased manner in our screen could intersect in unique combinations with pathways previously shown to be critical for CCM pathogenesis. The accessibility of cholecalciferol at trivial cost to patients, and the various clinical trials underway in the United States for tempol in a variety of conditions suggest that one or both of these drugs could be rapidly applied to the treatment of CCM disease. No study has yet shown a complete inhibition of CCM lesion formation in mice with any treatment, and our work along with the work of others, opens the possibility that multiple treatments could be used synergistically to treat patients with CCM vascular disease. Moreover, identification of these new treatments provides an avenue toward discovery of additional molecular mechanisms relevant to CCM disease.

Future work will determine whether our findings, obtained in both human endothelial cells and a mouse model of CCM disease, will translate to humans with CCM disease. Our findings underline major priorities upon which the field must agree. Specifically, success of any clinical trial, especially in rare disease research, hinges upon well-studied natural history and clinically meaningful endpoints. The long-term sustainability of treatment often also relies on establishment of relevant biomarkers. In advance of, or in conjunction with, human clinical trials for cholecalciferol or tempol treatment, our work strongly supports immediate amendment of research protocols on human patients with CCM to include measurements of both 25(OH)D\textsubscript{3} and an agreeable marker of oxidative stress. There has even been anecdotal evidence among CCM patients and clinicians that CCM-related symptoms increase during the winter months, which would be consistent with seasonal variation in Vitamin D or the increased oxidative stress
associated with inflammation due to typical winter infections such as influenza. We must also
develop widely-acceptable standards for tracking the natural history of CCM including specific
imaging modalities, lesion evaluation, and symptom assessment. We must use these standards as
a platform by which to perform both observational and interventional studies of CCM disease
and any proposed treatment including simvastatin, fasudil, tempol, cholecalciferol, or others. As
with any rare condition, the probability of obtaining meaningful results for patients is
exponentially enhanced by agreement and adherence of the field to specific protocols so that data
can be aggregated across clinical sites.

Our success in identifying candidates for future study in CCM disease was based not only
on an unbiased screen centered on structural and functional phenotypes, but also on the
generation and characterization of animal models that faithfully recapitulate the genotype and
phenotype most often found in the human disease. The adaptation of clinical imaging
technology used in the human disease for these mouse models allowed us to evaluate lesions in
much the same way as would be done in a clinical setting. The success of our approach has led
us to wonder whether we could apply this same methodology to other vascular diseases for
which a genetic basis is known, such as supravalvular aortic stenosis or hereditary hemorrhagic
telangiectasia, or even for a broader set of all genetic loss-of-function diseases. Other
analyses of structural changes in a whole genome screen using similar imaging and analysis
methods to those we have reported here, found that at least 10% of all gene knockdowns resulted
in a quantifiable structural phenotype. Taken together, the aforementioned whole genome
study and our drug-repurposing study highlight the possibility that there may be a substantial
number of phenotypes associated with human disease-related genes that are amenable to
chemical suppressor screening in a systematic and automated manner. Thus, there may be
additional opportunity for the rapid and efficient identification of candidate compounds for the treatment of other human genetic diseases.

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**Figure Legends:**

**Figure 1.** Project work-flow. An overview of the four different screening assays used to identify two promising compounds from among 2100 initial candidates.

**Figure 2.** Primary screen - rescue of structural phenotypes associated with loss of CCM2. (A) Western blot analysis of siCCM2 knockdown. (B) Immunofluorescence images of endothelial cells treated with siCTRL or siCCM2 stained for DNA (blue), actin (green), and VE-cadherin (red). (C) DNA (top) and VE-cadherin (bottom) raw images segmented into nuclei and cell objects, respectively. (D) Result of scoring positive and negative control images using rules generated by machine-learning algorithms in CellProfiler Analyst software. Scale bars = 50 µm.

**Figure 3.** Secondary screen - rescue of functional phenotypes associated with loss of CCM2. (A) Baseline transendothelial resistance of unperturbed siCTRL and siCCM2 monolayers. N=6 (siCTRL) and N=75 (siCCM2). Graph depicts each value, the mean, and SEM. *** indicates
P<0.001 as evaluated by t-test. (B) When added to siCCM2-treated cell monolayers, seven compounds identified using automated analysis in the primary screen partially or fully rescued resistance measures after 24-hours of treatment. Each compound was tested individually with two replicates. A representative replicate is plotted.

**Figure 4.** Tertiary screen - rescue of dermal leak phenotype associated with a murine model of CCM. (A) Timeline of dermal permeability experiment. Drugs were administered into intradermal wheals in the shaved backs of either Ccm2 control mice (*Ccm2*^+/^) or endothelial cell knockout mice (*Ccm2*^−/−^; */Pdgfb-iCreERT2^Tg^). 90 minutes later, Evans blue dye (EBD) was administered systemically by tail vein injection. After an additional 30 minutes, skin from the site of each drug injection was removed and the amount of EBD assayed. (B) Quantification of dermal permeability assay described in (A). N=4-12 mice per group were tested. Graph depicts each value and the mean. * indicates P<0.05 as evaluated by mixed effects linear regression with Benjamini-Hochberg adjusted significance test.

**Figure 5.** Quaternary screen – rescue of lesion burden in a murine model of CCM. (A) Timeline of treatment and analysis of cholecalciferol or tempol in *Ccm2* ecKO mice. (B) The number of CCM lesions as measured by MRI in ecKO mice. Experiment represents N=8-12 mice per group. Graph depicts each value, the mean, and SEM. * indicates P<0.05 as evaluated by one-way ANOVA and Dunnett’s multiple comparison test, comparing each active diet against the control diet. An effect of Tempol achieved marginal significance compared to control, denoted by # (P=0.0518). (C) For each mouse, the count of lesions within the designated size range (horizontal axis) was computed and compared between treatment groups using exact Wilcoxon
Mann-Whitney tests, with p-values adjusted for 2 comparisons within each bin. The median count of lesions of each size among the mice for each treatment is depicted. * denotes P<0.05, and # denotes P<0.1 after multiplicity adjustment. (D) Three-dimensional reconstruction of the brain (grey/cream) and lesions (red) are shown for a representative brain from each treatment arm (the mouse with the median number of lesions for each treatment group is shown). The top, middle, and bottom brains are from mice treated with standard, VD3-enhanced, or tempol enhanced diets, respectively.

**Figure 6.** Treatments illuminate pathophysiology of disease. 60 minute treatment with 100 nM cholecalciferol, but not its precursor 7-DHC, rescues CCM2 knockdown-induced (A) RHOA, (B) pMLC, and (C) ARF6 activation. N=3 for each group in A-C. Graphs depict each value, the mean, and SEM. * indicates P<0.05, ** indicates P<0.01, and *** denotes P<0.001 as evaluated by one-way ANOVA and Dunnett’s Multiple Comparison Test. (D) Cholecalciferol rescues CCM2-induced activation of ARF6 within 5 minutes. (E) Quantification of (D). N=5-8. Graph depicts each value, mean and SEM.—Multiple t-tests were used to evaluate significance with the Sidak-Bonferroni multiple comparison. (F) Knockdown of CCM2 in EA-hy926 endothelial cells induces increased reactive oxygen species. (G) Knockdown of CCM2 in EA-hy926 endothelial cells induces a reduction of FOX01 expression.

**Figure 7.** CCM mice display endothelial-dependent vascular dysfunction. (A) Middle cerebral arteries isolated from endothelial-specific CCM2 knockout mice (KO) subjected to acetylcholine-induced vasodilation. (B) Sodium Nitroprusside treatment (an NO-donor) of middle cerebral arteries of both mice genotypes demonstrates the normal function of the smooth
muscle in both wild-type and knockout mice. (C) L-name, a specific inhibitor of eNOS, eliminates the ability of endothelial cells to produce NO. (D) Tempol completely rescues acetylcholine-induced vasodilation in endothelial-specific CCM2 knockout mice. For A-D, N=9-19. Graphs depict mean and SEM. 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 were used to evaluate significance. (E) Systolic blood pressure (P=0.093)(F) diastolic blood pressure (P=0.051), (G) and mean blood pressure (P=0.052) trended toward an increase in ecKO mice, consistent with decreased vasodilatory function. (H) Heart rate trended toward a reduction (P=0.051) in the endothelial-specific CCM2 knockout mice. For E-H, N=5-10. Graphs depict each value, median and interquartile range. Wilcoxon Mann-Whitney exact analysis was used to evaluate significance.
Figure 1

2100 Compounds
Primary Screen
High-Content Structural Analysis
Scrambled Control or CCM2 siRNA
HMVEC-D → Compound treatment → High-throughput imaging → Automated analysis

38 Compounds
Secondary Screen
Transcellular Resistance Functional Analysis
siCTRL or siCCM2
HMVEC-D → ECIS analysis

7 Compounds
Tertiary Screen
Modified Miles Assay Acute Dermal Permeability
Drug
HMVEC-D → EBD

2 Compounds
Quaternary Screen
Chronic Treatment MRI analysis
Treated food or water

Table 1
Figure 2

A. Western blot analysis showing expression levels of CCM2 and α-actinin in siCTRL and siCCM2 conditions.

B. Immunofluorescence images showing DNA, actin, and VE-Cadherin in siCTRL and siCCM2 conditions.

C. Microscopy images illustrating the change in nuclei and cell morphology from siCTRL to siCCM2 conditions.

D. Scatter plot showing the percentage of cells/images scored as siCCM2 phenotype compared to siCTRL.
Figure 3
Figure 4
Figure 5
Figure 6
Strategy for Identifying Repurposed Drugs for the Treatment of Cerebral Cavernous Malformation

Christopher C. Gibson, Weiquan Zhu, Chadwick T. Davis, Jay A. Bowman-Kirigin, Aubrey C. Chan, Jing Ling, Ashley E. Walker, Luca Goitre, Simona Delle Monache, Saverio Francesco Retta, Yan-Ting E. Shiu, Allie H. Grossmann, Kirk R. Thomas, Anthony J. Donato, Lisa A. Lesniewski, Kevin J. Whitehead and Dean Y. Li

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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. 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 (1x10⁶) 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 10μM, 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. 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 $4 \times 10^4$ 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 D₃), a standard chow plus tempol in drinking water (1mM), or a cholecalciferol-enhanced chow (Harlan 2018 + 25 IU/g D₃). 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. 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, TEMPO (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.
**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.
Supplementary Table 1. Rules generated by Machine-Learning Analysis.

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