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

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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 D_3) 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.

Keywords

Stroke; Cerebrovascular Disorders; Endothelium; Genetics; Hemorrhage

Introduction

There are at least 7,000 diseases designated as rare in the United States[1]. Taken as a whole at least 25 million Americans are affected by a rare disease[1, 2]. Disease-causing mutations in a single gene account for as many as 5,000 rare diseases, the largest subset[1, 2]. 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[3]. However, more than 95% of rare diseases still have no approved treatment[1].

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[4–6]. 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[7]. 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[8, 9]. 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[10]. The only treatment for CCM is neurosurgical resection[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[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, *KRIT1*, *CCM2*, or *PDCD10*[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[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[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 4×10^4 cell/well onto a 96-well ECIS plate (96W10E+, Applied Biophysics). Plates were monitored using an ECIS Zθ 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[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[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[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[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^{T2})[42]. Strikingly, these mice develop CCM lesions that reproduce all of the pathologic aspects of human CCM disease[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[43]. 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)[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)[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)[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)[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 it's 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*$^{f/-}$; +/Tg(*Pdgfb-iCreER*^{T2}), 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[43]. 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)[51–57]. 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[65]; 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-β, 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-β signaling and endMT, offering a potential mechanistic link[67]. Additionally, metabolites of cholecalciferol have also been shown to strongly inhibit TGF-β 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[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 $25(OH)D_3$ 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[38, 43]. 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[72, 73]. 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[74]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Field MJ, Boat TF. Institute of medicine (us) committee on accelerating rare diseases research and orphan product development. Rare Diseases and Orphan Products: Accelerating Research and Development. 2010
2. Rinaldi A. Adopting an orphan. EMBO Rep. 2005; 6:507–510. [PubMed: 15940282]
3. Meekings KN, Williams CS, Arrowsmith JE. Orphan drug development: An economically viable strategy for biopharma r&d. Drug Discov Today. 2012; 17:660–664. [PubMed: 22366309]
4. Garnier JP. Rebuilding the r&d engine in big pharma. Harv Bus Rev. 2008; 86:68–70. 72–66, 128. [PubMed: 18543809]
5. Brown MS, Kovanen PT, Goldstein JL. Evolution of the ldl receptor concept-from cultured cells to intact animals. Ann N Y Acad Sci. 1980; 348:48–68. [PubMed: 6994567]
6. Sardana D, Zhu C, Zhang M, Gudivada RC, Yang L, Jegga AG. Drug repositioning for orphan diseases. Brief Bioinform. 2011; 12:346–356. [PubMed: 21504985]
7. Ghofrani HA, Osterloh IH, Grimminger F. Sildenafil: From angina to erectile dysfunction to pulmonary hypertension and beyond. Nat Rev Drug Discov. 2006; 5:689–702. [PubMed: 16883306]
8. Melnikova I. Rare diseases and orphan drugs. Nat Rev Drug Discov. 2012; 11:267–268. [PubMed: 22460117]
9. Boguski MS, Mandl KD, Sukhatme VP. Drug discovery. Repurposing with a difference. Science. 2009; 324:1394–1395. [PubMed: 19520944]
10. Gault J, Sarin H, Awadallah NA, Shenkar R, Awad IA. Pathobiology of human cerebrovascular malformations: Basic mechanisms and clinical relevance. Neurosurgery. 2004; 55:1–16. discussion 16–17. [PubMed: 15214969]
11. Batra S, Lin D, Recinos PF, Zhang J, Rigamonti D. Cavernous malformations: Natural history, diagnosis and treatment. Nat Rev Neurol. 2009; 5:659–670. [PubMed: 19953116]
12. Otten PPG, Rilliet B, Berney J. A propos de 131 cas d'angiomes caverneux (cavernomes) du s.N.C. Reperes par l'analyse retrospective de 24535 autopsies. Neurochirurgie. 1989; 35:82–83. [PubMed: 2674753]
13. Vernooij MW, Ikram MA, Tanghe HL, Vincent AJ, Hofman A, Krestin GP, Niessen WJ, Breteler MM, van der Lugt A. Incidental findings on brain mri in the general population. N Engl J Med. 2007; 357:1821–1828. [PubMed: 17978290]
14. Riant F, Bergametti F, Ayrignac X, Boulday G, Tournier-Lasserve E. Recent insights into cerebral cavernous malformations: The molecular genetics of ccm. FEBS J. 2010; 277:1070–1075. [PubMed: 20096038]
15. Akers AL, Johnson E, Steinberg GK, Zabramski JM, Marchuk DA. Biallelic somatic and germline mutations in cerebral cavernous malformations (ccms): Evidence for a two-hit mechanism of ccm pathogenesis. Hum Mol Genet. 2009; 18:919–930. [PubMed: 19088123]
16. Bergametti F, Denier C, Labauge P, Arnoult M, Boetto S, Clanet M, Coubes P, Echenne B, Ibrahim R, Irthum B, Jacquet G, Lonjon M, Moreau JJ, Neau JP, Parker F, Tremoulet M, Tournier-Lasserve E, Societe Francaise de N. Mutations within the programmed cell death 10 gene cause cerebral cavernous malformations. Am J Hum Genet. 2005; 76:42–51. [PubMed: 15543491]
17. Cave-Riant F, Denier C, Labauge P, Cecillon M, Maciazek J, Joutel A, Laberge-Le Couteulx S, Tournier-Lasserve E. Spectrum and expression analysis of krit1 mutations in 121 consecutive and unrelated patients with cerebral cavernous malformations. Eur J Hum Genet. 2002; 10:733–740. [PubMed: 12404106]
18. Denier C, Goutagny S, Labauge P, Krivosic V, Arnoult M, Cousin A, Benabid AL, Comoy J, Frerebeau P, Gilbert B, Houtteville JP, Jan M, Lapierre F, Loiseau H, Menei P, Mercier P, Moreau JJ, Nivelon-Chevallier A, Parker F, Redondo AM, Scarabin JM, Tremoulet M, Zerah M, Maciazek

J, Tournier-Lasserve E, Societe Francaise de N. Mutations within the mgc4607 gene cause cerebral cavernous malformations. Am J Hum Genet. 2004; 74:326–337. [PubMed: 14740320]

19. Guclu B, Ozturk AK, Pricola KL, Bilguvar K, Shin D, O'Roak BJ, Gunel M. Mutations in apoptosis-related gene, pdcd10, cause cerebral cavernous malformation 3. Neurosurgery. 2005; 57:1008–1013. [PubMed: 16284570]
20. Gunel M, Awad IA, Finberg K, Anson JA, Steinberg GK, Batjer HH, Kopitnik TA, Morrison L, Giannotta SL, Nelson-Williams C, Lifton RP. A founder mutation as a cause of cerebral cavernous malformation in hispanic americans. N Engl J Med. 1996; 334:946–951. [PubMed: 8596595]
21. Laberge-le Couteulx S, Jung HH, Labauge P, Houtteville JP, Lescoat C, Cecillon M, Marechal E, Joutel A, Bach JF, Tournier-Lasserve E. Truncating mutations in ccm1, encoding krit1, cause hereditary cavernous angiomas. Nat Genet. 1999; 23:189–193. [PubMed: 10508515]
22. Laurans MS, DiLuna ML, Shin D, Niazi F, Voorhees JR, Nelson-Williams C, Johnson EW, Siegel AM, Steinberg GK, Berg MJ, Scott RM, Tedeschi G, Enevoldson TP, Anson J, Rouleau GA, Ogilvy C, Awad IA, Lifton RP, Gunel M. Mutational analysis of 206 families with cavernous malformations. J Neurosurg. 2003; 99:38–43. [PubMed: 12854741]
23. Liquori CL, Berg MJ, Siegel AM, Huang E, Zawistowski JS, Stoffer T, Verlaan D, Balogun F, Hughes L, Leedom TP, Plummer NW, Cannella M, Maglione V, Squitieri F, Johnson EW, Rouleau GA, Ptacek L, Marchuk DA. Mutations in a gene encoding a novel protein containing a phosphotyrosine-binding domain cause type 2 cerebral cavernous malformations. Am J Hum Genet. 2003; 73:1459–1464. [PubMed: 14624391]
24. Liquori CL, Berg MJ, Squitieri F, Leedom TP, Ptacek L, Johnson EW, Marchuk DA. Deletions in ccm2 are a common cause of cerebral cavernous malformations. Am J Hum Genet. 2007; 80:69–75. [PubMed: 17160895]
25. Liquori CL, Berg MJ, Squitieri F, Ottenbacher M, Sorlie M, Leedom TP, Cannella M, Maglione V, Ptacek L, Johnson EW, Marchuk DA. Low frequency of pdcd10 mutations in a panel of ccm3 probands: Potential for a fourth ccm locus. Human mutation. 2006; 27:118. [PubMed: 16329096]
26. Ortiz L, Costa AF, Bellido ML, Solano F, Garcia-Moreno JM, Gamero MA, Izquierdo G, Chadli A, Falcao F, Ferro J, Salas J, Alvarez-Cermeno JC, Montori M, Ramos-Arroyo MA, Palomino A, Pintado E, Lucas M. Study of cerebral cavernous malformation in spain and portugal: High prevalence of a 14 bp deletion in exon 5 of mgc4607 (ccm2 gene). J Neurol. 2007; 254:322–326. [PubMed: 17345049]
27. Sahoo T, Johnson EW, Thomas JW, Kuehl PM, Jones TL, Dokken CG, Touchman JW, Gallione CJ, Lee-Lin SQ, Kosofsky B, Kurth JH, Louis DN, Mettler G, Morrison L, Gil-Nagel A, Rich SS, Zabramski JM, Boguski MS, Green ED, Marchuk DA. Mutations in the gene encoding krit1, a krev-1/rap1a binding protein, cause cerebral cavernous malformations (ccm1). Hum Mol Genet. 1999; 8:2325–2333. [PubMed: 10545614]
28. Pagenstecher A, Stahl S, Sure U, Felbor U. A two-hit mechanism causes cerebral cavernous malformations: Complete inactivation of ccm1, ccm2 or ccm3 in affected endothelial cells. Hum Mol Genet. 2009; 18:911–918. [PubMed: 19088124]
29. Plummer NW, Gallione CJ, Srinivasan S, Zawistowski JS, Louis DN, Marchuk DA. Loss of p53 sensitizes mice with a mutation in ccm1 (krit1) to development of cerebral vascular malformations. Am J Pathol. 2004; 165:1509–1518. [PubMed: 15509522]
30. Li DY, Whitehead KJ. Evaluating strategies for the treatment of cerebral cavernous malformations. Stroke. 2010; 41:S92–S94. [PubMed: 20876517]
31. McDonald DA, Shi C, Shenkar R, Gallione CJ, Akers AL, Li S, De Castro N, Berg MJ, Corcoran DL, Awad IA, Marchuk DA. Lesions from patients with sporadic cerebral cavernous malformations harbor somatic mutations in the ccm genes: Evidence for a common biochemical pathway for ccm pathogenesis. Hum Mol Genet. 2014; 23:4357–4370. [PubMed: 24698976]
32. McDonald DA, Shenkar R, Shi C, Stockton RA, Akers AL, Kucherlapati MH, Kucherlapati R, Brainer J, Ginsberg MH, Awad IA, Marchuk DA. A novel mouse model of cerebral cavernous malformations based on the two-hit mutation hypothesis recapitulates the human disease. Hum Mol Genet. 2011; 20:211–222. [PubMed: 20940147]
33. Gault J, Awad IA, Recksiek P, Shenkar R, Breeze R, Handler M, Kleinschmidt-DeMasters BK. Cerebral cavernous malformations: Somatic mutations in vascular endothelial cells. Neurosurgery. 2009; 65:138–144. discussion 144-135. [PubMed: 19574835]

34. Gault J, Shenkar R, Recksiek P, Awad IA. Biallelic somatic and germ line ccm1 truncating mutations in a cerebral cavernous malformation lesion. Stroke. 2005; 36:872–874. [PubMed: 15718512]
35. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM. Cellprofiler: Image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 2006; 7:R100. [PubMed: 17076895]
36. Jones TR, Carpenter AE, Lamprecht MR, Moffat J, Silver SJ, Grenier JK, Castoreno AB, Eggert US, Root DE, Golland P, Sabatini DM. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. Proc Natl Acad Sci U S A. 2009; 106:1826–1831. [PubMed: 19188593]
37. Jones TR, Kang IH, Wheeler DB, Lindquist RA, Papallo A, Sabatini DM, Golland P, Carpenter AE. Cellprofiler analyst: Data exploration and analysis software for complex image-based screens. BMC Bioinformatics. 2008; 9:482. [PubMed: 19014601]
38. Whitehead KJ, Chan AC, Navankasattusas S, Koh W, London NR, Ling J, Mayo AH, Drakos SG, Jones CA, Zhu W, Marchuk DA, Davis GE, Li DY. The cerebral cavernous malformation signaling pathway promotes vascular integrity via rho gtpases. Nat Med. 2009; 15:177–184. [PubMed: 19151728]
39. Stockton RA, Shenkar R, Awad IA, Ginsberg MH. Cerebral cavernous malformations proteins inhibit rho kinase to stabilize vascular integrity. J Exp Med. 2010; 207:881–896. [PubMed: 20308363]
40. Borikova AL, Dibble CF, Sciaky N, Welch CM, Abell AN, Bencharit S, Johnson GL. Rho kinase inhibition rescues the endothelial cell cerebral cavernous malformation phenotype. J Biol Chem. 2010; 285:11760–11764. [PubMed: 20181950]
41. Mleynek TM, Chan AC, Redd M, Gibson CC, Davis CT, Shi DS, Chen T, Carter KL, Ling J, Blanco R, Gerhardt H, Whitehead K, Li DY. Lack of ccm1 induces hypersprouting and impairs response to flow. Hum Mol Genet. 2014 Jul 2. pii: ddu342. [Epub ahead of print].
42. Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible cre-recombinase activation in vascular endothelium. Genesis. 2008; 46:74–80. [PubMed: 18257043]
43. Chan AC, Drakos SG, Ruiz OE, Smith AC, Gibson CC, Ling J, Passi SF, Stratman AN, Sacharidou A, Revelo MP, Grossmann AH, Diakos NA, Davis GE, Metzstein MM, Whitehead KJ, Li DY. Mutations in 2 distinct genetic pathways result in cerebral cavernous malformations in mice. J Clin Invest. 2011; 121:1871–1881. [PubMed: 21490399]
44. Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, Ljosa V, Rueden C, Eliceiri KW, Carpenter AE. Improved structure, function and compatibility for cellprofiler: Modular high-throughput image analysis software. Bioinformatics. 2011; 27:1179–1180. [PubMed: 21349861]
45. Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen. 1999; 4:67–73. [PubMed: 10838414]
46. Wegener J, Keese CR, Giaever I. Electric cell-substrate impedance sensing (ecis) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces. Exp Cell Res. 2000; 259:158–166. [PubMed: 10942588]
47. Hilder T, Malone M, Bencharit S, Colicelli J, Haystead T, Johnson G, Wu C. Proteomic identification of the cerebral cavernous malformation signaling complex. J Proteome Res. 2007; 6:4343–4355. [PubMed: 17900104]
48. Goitre L, Balzac F, Degani S, Degan P, Marchi S, Pinton P, Retta SF. Krit1 regulates the homeostasis of intracellular reactive oxygen species. PLoS One. 2010; 5:e11786. [PubMed: 20668652]
49. Fidalgo M, Guerrero A, Fraile M, Iglesias C, Pombo CM, Zalvide, Fro J. The adaptor protein cerebral cavernous malformation 3 (ccm3) mediates phosphorylation of the cytoskeletal proteins ezrin/radixin/moesin by mammalian ste20-4 to protect cells from oxidative stress. Biol Chem. 2012; 287:11556–11565. Epub 2012 Jan 30.
50. Moschovi M, Alexiou GA, Stefanaki K, Tourkantoni N, Prodromou N. Propranolol treatment for a giant infantile brain cavernoma. J Child Neurol. 2010; 25:653–655. [PubMed: 20413807]

51. Broman MT, Kouklis P, Gao X, Ramchandran R, Neamu RF, Minshall RD, Malik AB. Cdc42 regulates adherens junction stability and endothelial permeability by inducing alpha-catenin interaction with the vascular endothelial cadherin complex. Circ Res. 2006; 98:73–80. [PubMed: 16322481]
52. Broman MT, Mehta D, Malik AB. Cdc42 regulates the restoration of endothelial adherens junctions and permeability. Trends Cardiovasc Med. 2007; 17:151–156. [PubMed: 17574122]
53. Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for src kinases during vegf-induced angiogenesis and vascular permeability. Mol Cell. 1999; 4:915–924. [PubMed: 10635317]
54. Sawada J, Urakami T, Li F, Urakami A, Zhu W, Fukuda M, Li DY, Ruoslahti E, Komatsu M. Small gtpase r-ras regulates integrity and functionality of tumor blood vessels. Cancer cell. 2012; 22:235–249. [PubMed: 22897853]
55. Weis S, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by vegf-mediated src activity potentiates tumor cell extravasation and metastasis. J Cell Biol. 2004; 167:223–229. [PubMed: 15504909]
56. Wojciak-Stothard B, Ridley AJ. Rho gtpases and the regulation of endothelial permeability. Vasc Pharmacol. 2002; 39:187–199.
57. Zhu W, London NR, Gibson CC, Davis CT, Tong Z, Sorensen LK, Shi DS, Guo J, Smith MC, Grossmann AH, Thomas KR, Li DY. Interleukin receptor activates a myd88-arno-arf6 cascade to disrupt vascular stability. Nature. 2012; 492:252–255. [PubMed: 23143332]
58. Schnackenberg CG, Welch WJ, Wilcox CS. Normalization of blood pressure and renal vascular resistance in shr with a membrane-permeable superoxide dismutase mimetic: Role of nitric oxide. Hypertension. 1998; 32:59–64. [PubMed: 9674638]
59. Guazzi P, Goitre L, Ferro E, Cutano V, Martino C, Trabalzini L, Retta SF. Identification of the kelch family protein nd1-l as a novel molecular interactor of krit1. PLoS One. 2012; 7:e44705. [PubMed: 22970292]
60. Fidalgo M, Fraile M, Pires A, Force T, Pombo C, Zalvide J. Ccm3/pdcd10 stabilizes gckiii proteins to promote golgi assembly and cell orientation. J Cell Sci. 2010; 123:1274–1284. [PubMed: 20332113]
61. Corr M, Lerman I, Keubel JM, Ronacher L, Misra R, Lund F, Sarelius IH, Glading AJ. Decreased krev interaction-trapped 1 expression leads to increased vascular permeability and modifies inflammatory responses in vivo. Arterioscler Thromb Vasc Biol. 2012; 32:2702–2710. [PubMed: 22922958]
62. Goitre L, De Luca E, Braggion S, Trapani E, Guglielmotto M, Biasi F, Forni M, Moglia A, Trabalzini L, Retta SF. Krit1 loss of function causes a ros-dependent upregulation of c-jun. Free Radic Biol Med. 2014; 68:134–147. [PubMed: 24291398]
63. Durrant JR, Seals DR, Connell ML, Russell MJ, Lawson BR, Folian BJ, Donato AJ, Lesniewski LA. Voluntary wheel running restores endothelial function in conduit arteries of old mice: Direct evidence for reduced oxidative stress, increased superoxide dismutase activity and down-regulation of nadph oxidase. J Physiol. 2009; 587:3271–3285. [PubMed: 19417091]
64. Rippe C, Lesniewski L, Connell M, LaRocca T, Donato A, Seals D. Short-term calorie restriction reverses vascular endothelial dysfunction in old mice by increasing nitric oxide and reducing oxidative stress. Aging Cell. 2010; 9:304–312. [PubMed: 20121721]
65. Simons DJ. Attentional capture and inattentional blindness. Trends Cogn Sci. 2000; 4:147–155. [PubMed: 10740279]
66. Maddaluno L, Rudini N, Cuttano R, Bravi L, Giampietro C, Corada M, Ferrarini L, Orsenigo F, Papa E, Boulday G, Tournier-Lasserve E, Chapon F, Richichi C, Retta SF, Lampugnani MG, Dejana E. Endmt contributes to the onset and progression of cerebral cavernous malformations. Nature. 2013; 498:492–496. [PubMed: 23748444]
67. Liu RM, Gaston Pravia KA. Oxidative stress and glutathione in tgf-beta-mediated fibrogenesis. Free Radic Biol Med. 2010; 48:1–15. [PubMed: 19800967]
68. Aschenbrenner JK, Sollinger HW, Becker BN, Hullett DA. 1,25-(oh(2))d(3) alters the transforming growth factor beta signaling pathway in renal tissue. J Surg Res. 2001; 100:171–175. [PubMed: 11592788]

69. Ramirez AM, Wongtrakool C, Welch T, Steinmeyer A, Zugel U, Roman J. Vitamin d inhibition of pro-fibrotic effects of transforming growth factor beta1 in lung fibroblasts and epithelial cells. J Steroid Biochem Mol Biol. 2010; 118:142–150. [PubMed: 19931390]

70. University of New Mexico NIoNDaSN. Permeability mri in cerebral cavernous malformation type 1 in new mexico: Effects of statins; 2013. p. 2014http://clinicaltrials.gov/show/NCT01764451.

71. McDonald DA, Shi C, Shenkar R, Stockton RA, Liu F, Ginsberg MH, Marchuk DA, Awad IA. Fasudil decreases lesion burden in a murine model of cerebral cavernous malformation disease. Stroke. 2012; 43:571–574. [PubMed: 22034008]

72. Li DY, Brooke B, Davis EC, Mecham RP, Sorensen LK, Boak BB, Eichwald E, Keating MT. Elastin is an essential determinant of arterial morphogenesis. Nature. 1998; 393:276–280. [PubMed: 9607766]

73. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP. Defective angiogenesis in mice lacking endoglin. Science. 1999; 284:1534–1537. [PubMed: 10348742]

74. Fuchs F, Pau G, Kranz D, Sklyar O, Budjan C, Steinbrink S, Horn T, Pedal A, Huber W, Boutros M. Clustering phenotype populations by genome-wide rnai and multiparametric imaging. Mol Syst Biol. 2010; 6:370. [PubMed: 20531400]

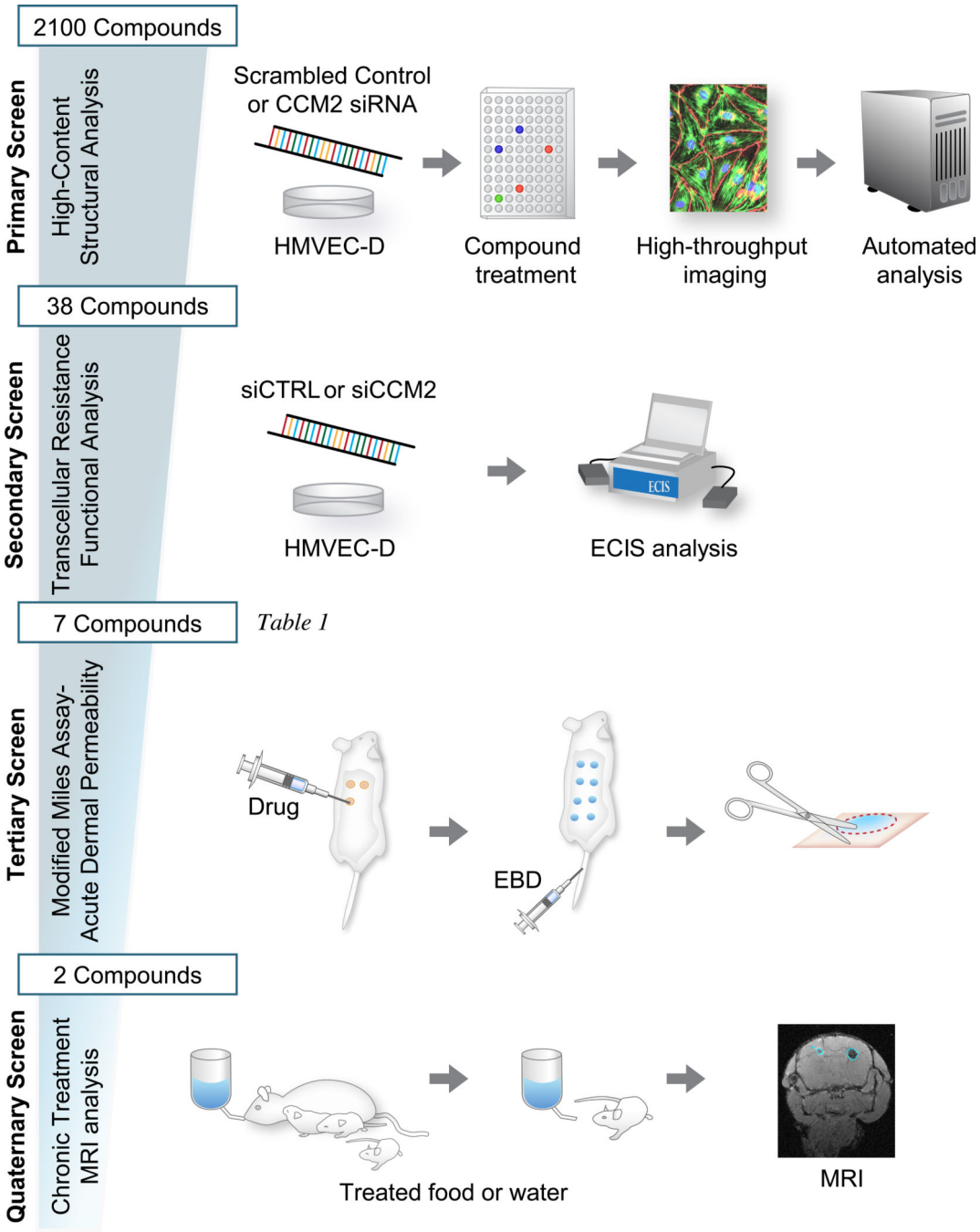

Figure 1.

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

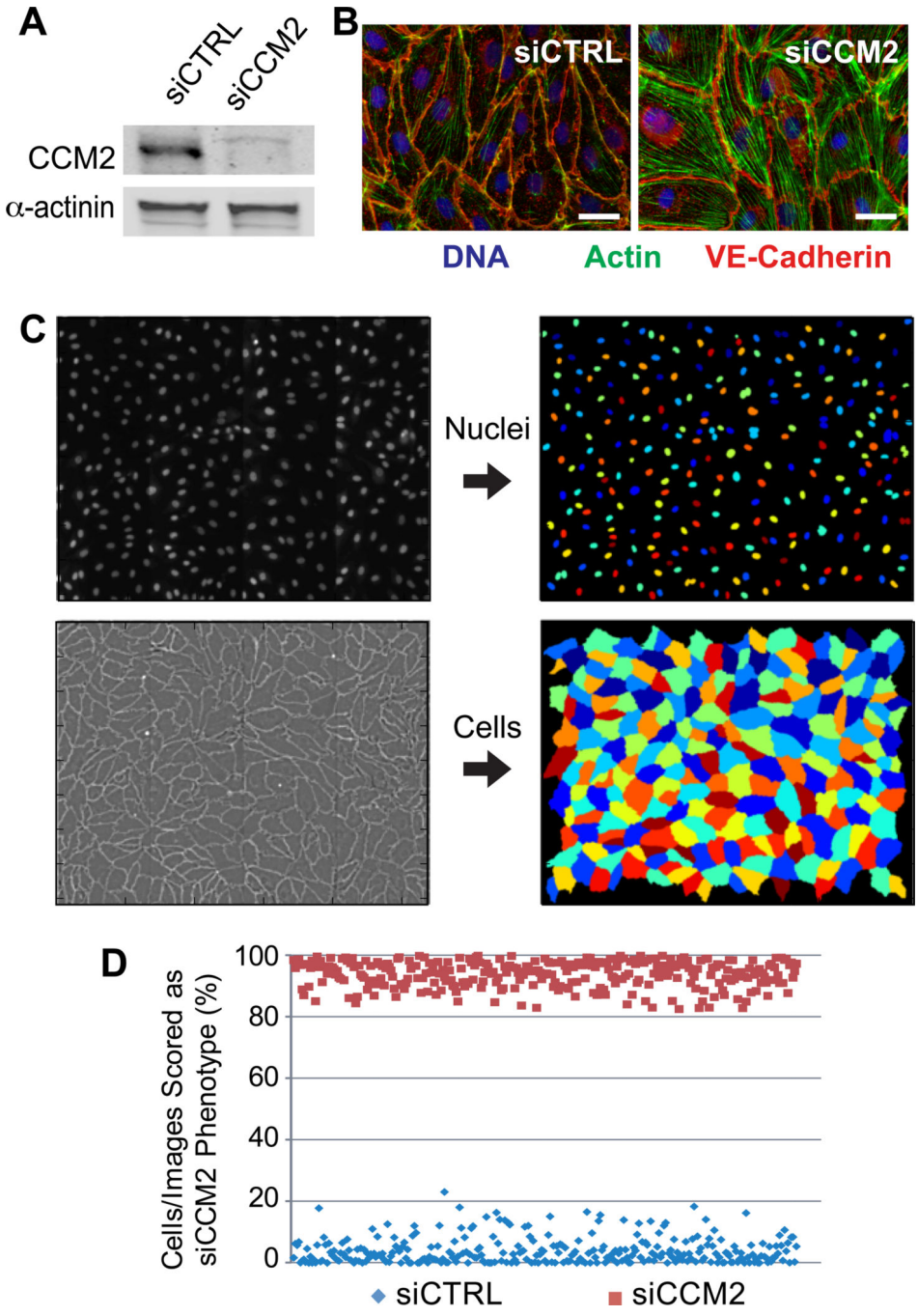

Figure 2.

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.

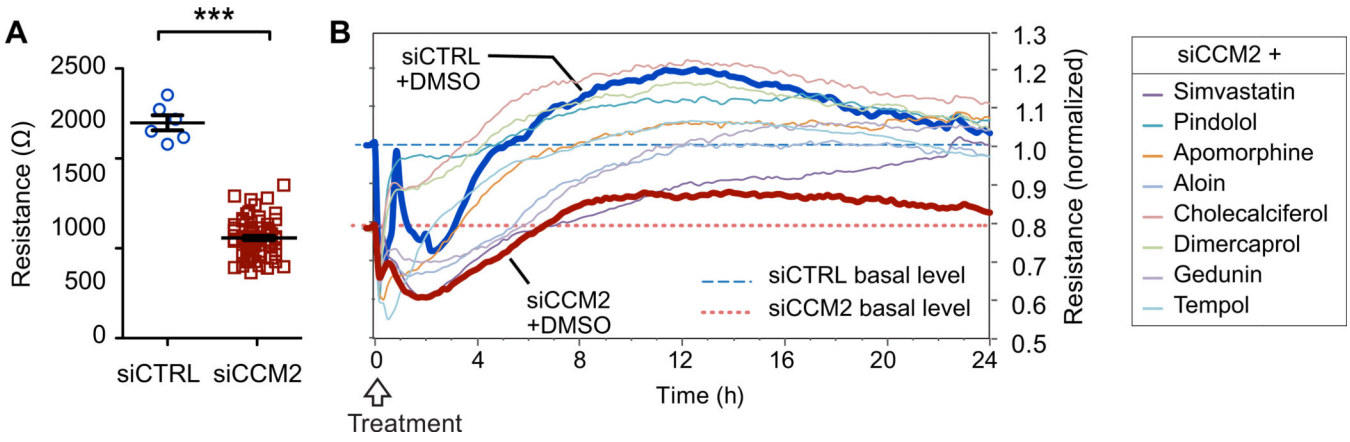

Figure 3.

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.

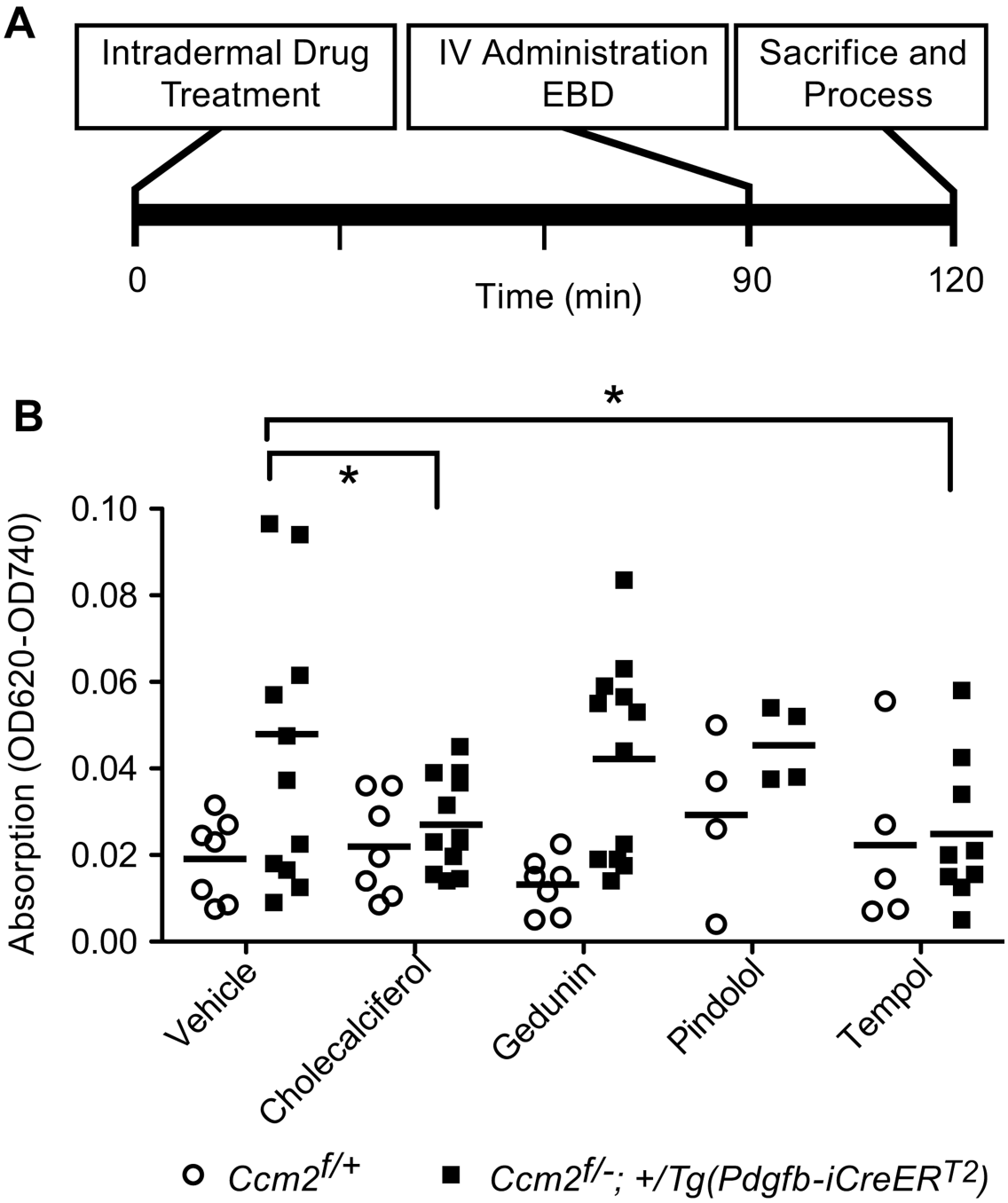

Figure 4.

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*$^{f/+}$) or endothelial cell knockout mice (*Ccm2*$^{f/-}$; +/Tg(*Pdgfb-iCreER*^{T2}). 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.

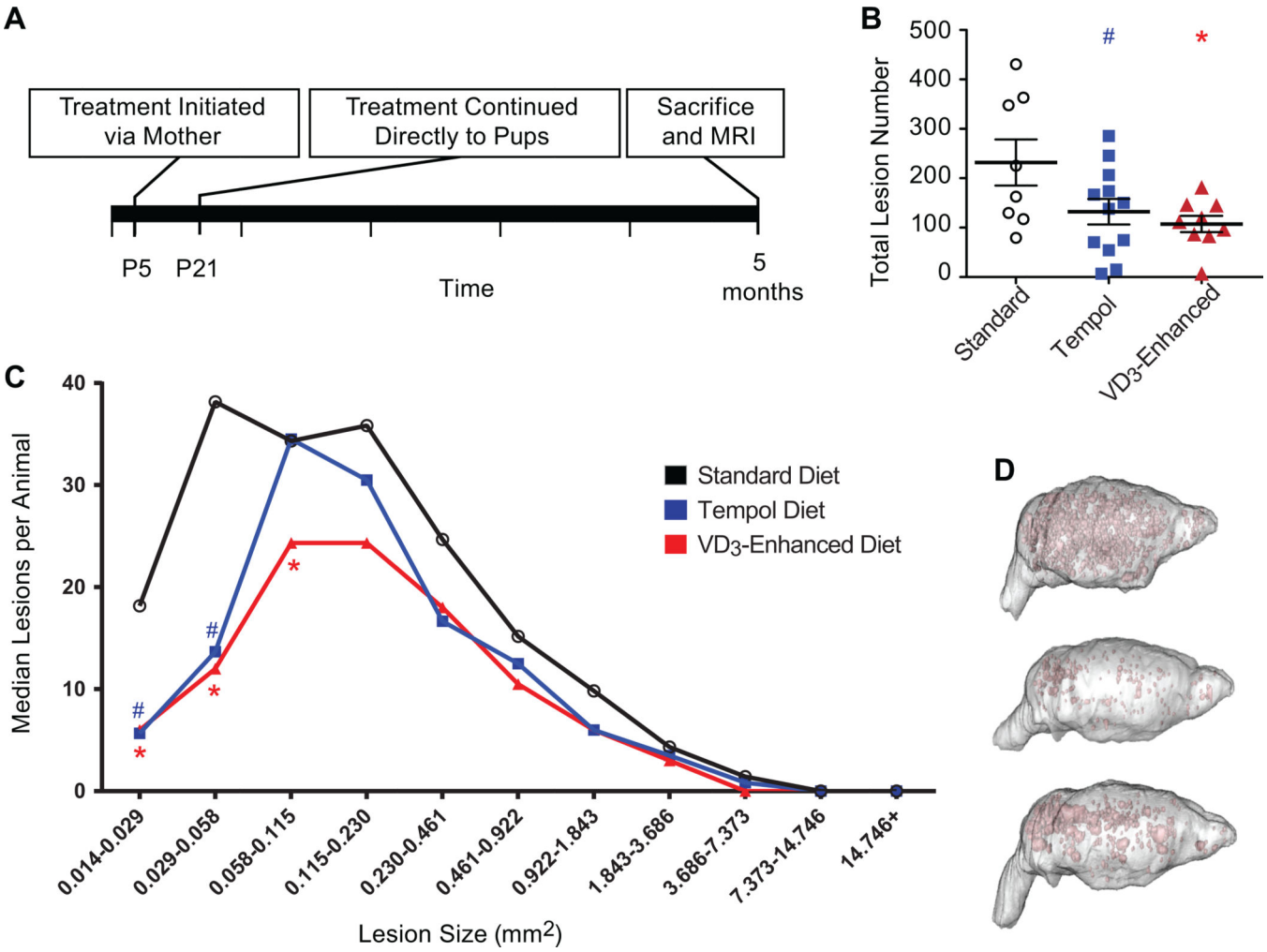

Figure 5.

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.

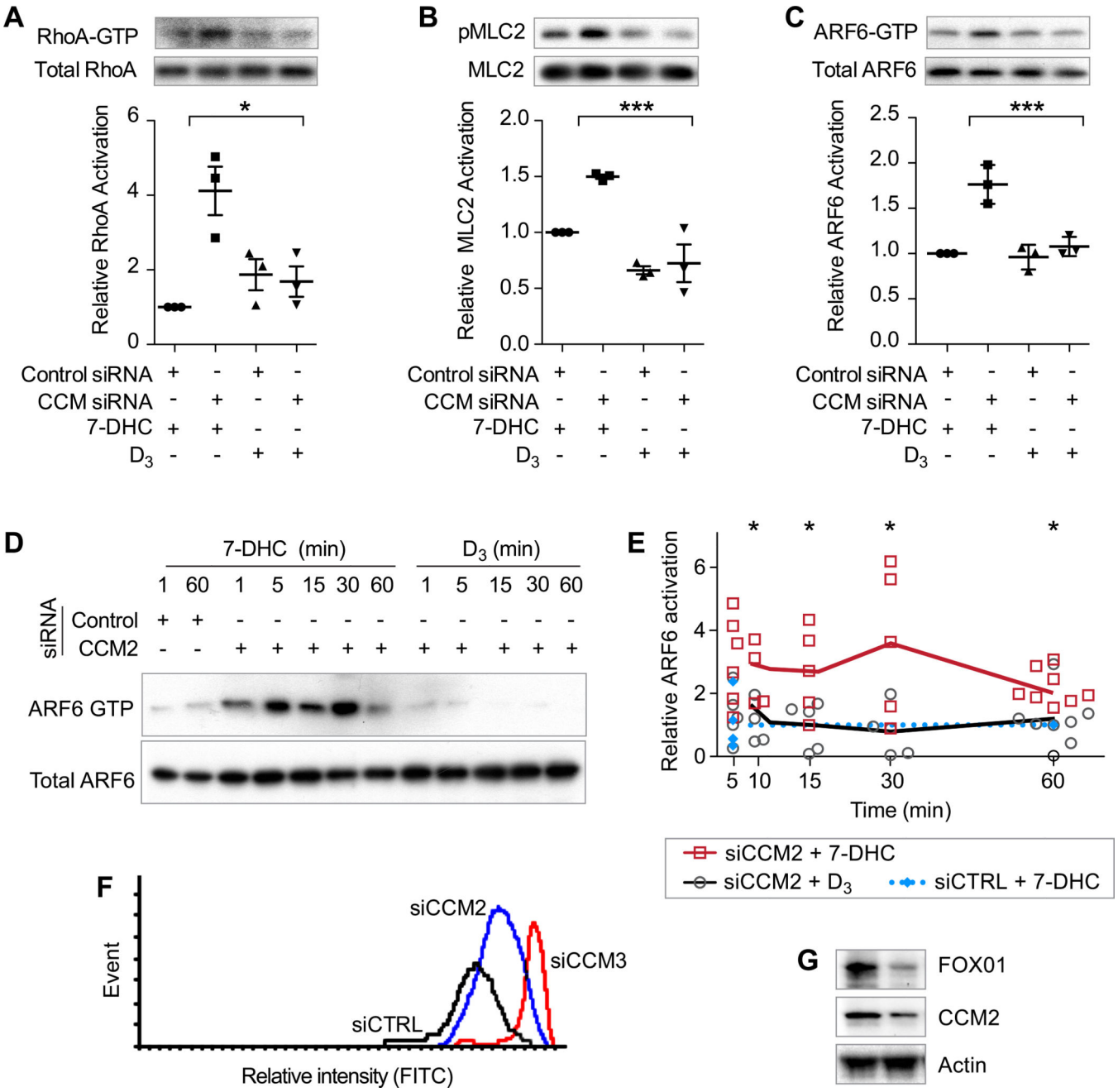

Figure 6.

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.

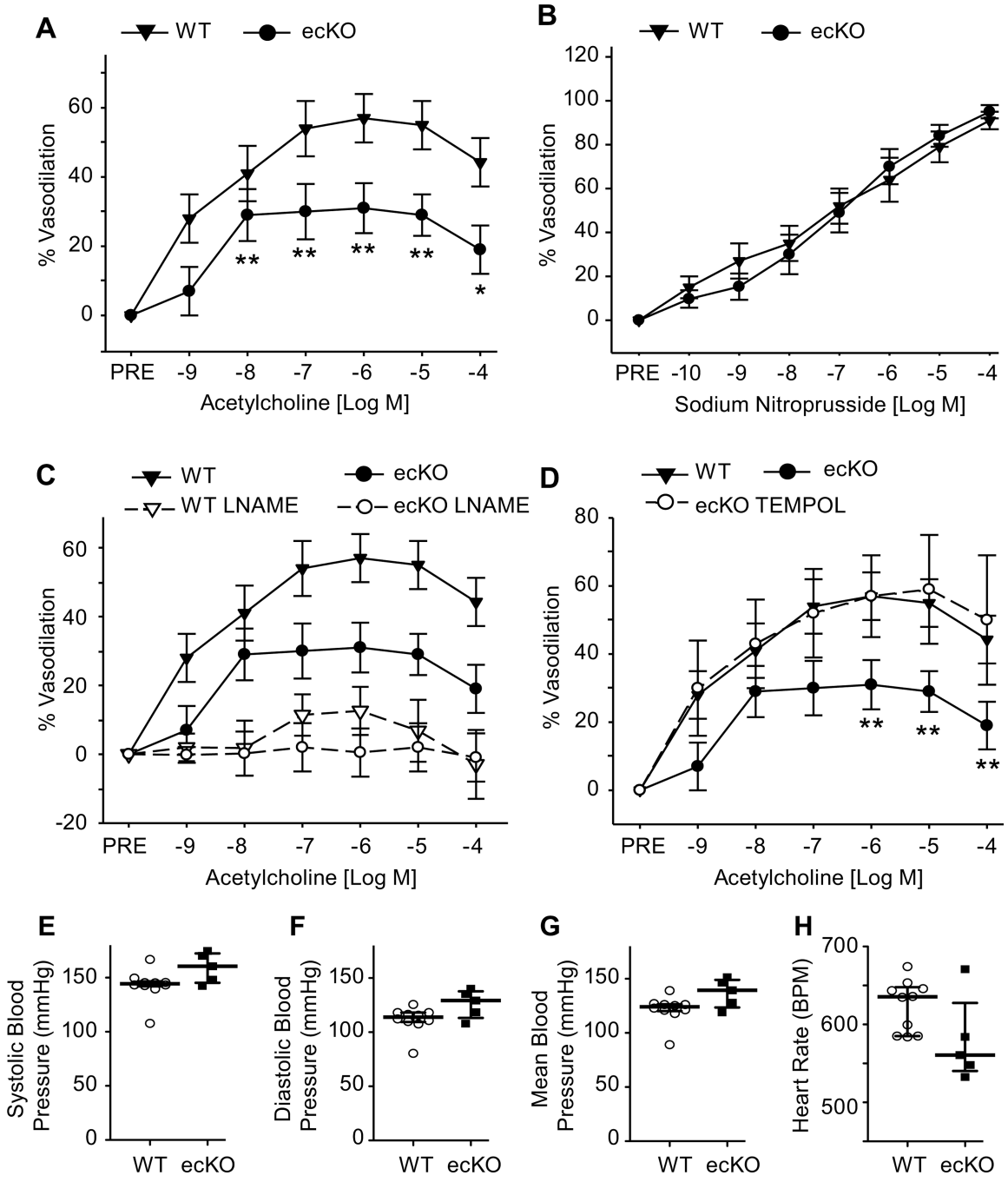

Figure 7.

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.