Cerebral Cavernous Malformations: From Two-Hit Mechanism to Developing a Targeted Therapy

by

David Andrew McDonald

University Program in Genetics & Genomics
Duke University

Date: ________________

Approved:

___________________________
Douglas A. Marchuk, Supervisor

___________________________
Gerard C. Blobe

___________________________
Blanche Capel

___________________________
Michael A. Hauser

___________________________
Beth A. Sullivan

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics & Genomics in the Graduate School of Duke University

2013
ABSTRACT

Cerebral Cavernous Malformations: From Two-Hit Mechanism to Developing a Targeted Therapy

by

David Andrew McDonald

University Program in Genetics & Genomics
Duke University

Date: ______________

Approved:

___________________________
Douglas A. Marchuk, Supervisor

___________________________
Gerard C. Blobe

___________________________
Blanche Capel

___________________________
Michael A. Hauser

___________________________
Beth A. Sullivan

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics & Genomics in the Graduate School of Duke University

2013
Abstract

Cerebral cavernous malformations (CCMs) are multicavernous vascular lesions affecting the central nervous system. Affected individuals have a lifetime risk of recurrent headaches, focal neurological deficits, seizures, and intracerebral hemorrhage leading to stroke. Patients tend to fall into two classes: familial cases with a known family history and multiple lesions, and; sporadic cases with no family history and single lesions. This epidemiological pattern suggests a two-hit mutational mechanism for CCM. While somatic mutations have been identified in lesions from familial patients, it is unknown if sporadic cases follow the same genetic mechanism. Using a next-generation sequencing strategy, I have identified somatic mutations from sporadic CCM lesions in the three known CCM genes, including one lesion bearing two independent mutations in CCM1. These data support a two-hit mutation mechanism in CCM for sporadic patients.

The mechanism of CCM pathogenesis (how mutations in one of the three CCM genes causes lesions to form and develop) is currently unknown. We developed mouse models that recapitulate the human disease. We have further shown that inhibition of Rho Kinase decreases the number of late-stage, multicavernous lesions. This is the first potential therapeutic strategy to specifically treat CCM, and suggests that the RhoA pathway is a central player in CCM pathogenesis.
Contents

Abstract ..............................................................................................................................................iv

List of Tables .......................................................................................................................................viii

List of Figures .....................................................................................................................................ix

Acknowledgements ............................................................................................................................ xiii

1. Cerebral Cavernous Malformations (CCM): Etiology and Genetics ........................................... 1
   1.1 Disease Overview ...................................................................................................................... 1
       1.1.1 Clinical Summary ............................................................................................................. 2
       1.1.2 Diagnosis ......................................................................................................................... 3
       1.1.3 Identification of Three CCM Genes ............................................................................... 4
           1.1.3.1 CCM1/KRIT1 ........................................................................................................... 5
           1.1.3.2 CCM2 ...................................................................................................................... 6
           1.1.3.3 CCM3/PDCD10 ....................................................................................................... 8
       1.1.4 Molecular Genetics of CCM .......................................................................................... 9
   1.2 CCM Pathogenesis ................................................................................................................... 11
       1.2.1 Two-Hit Mutation Mechanism ...................................................................................... 11
       1.2.2 The RhoA Pathway in CCM Pathogenesis .................................................................. 13
       1.2.3 Other Genes in the CCM Pathway ............................................................................... 15
   1.3 Proposal of Research .............................................................................................................. 17

2. A Two-Hit Genetic Mechanism Causes Sporadic CCM .............................................................. 19
   2.1 Introduction ............................................................................................................................... 19
2.2 Results ........................................................................................................................................... 20
  2.2.1 Sporadic CCM Lesion Containing Two Somatic Mutations ............................................... 22
  2.2.2 Sporadic CCM Lesions with a Single Somatic Mutation ................................................. 25
  2.2.3 Evidence of Trans-heterozygosity in a CCM Lesion ....................................................... 27
  2.3 Summary and Discussion ........................................................................................................... 30
  2.4 Future Directions ...................................................................................................................... 32
  2.5 Materials and Methods ............................................................................................................ 34
    2.5.1 CCM Samples ..................................................................................................................... 34
    2.5.2 Next-Generation Sequencing .......................................................................................... 34
    2.5.3 SNaPshot Analysis ............................................................................................................ 35
    2.5.4 Sanger Sequencing ........................................................................................................... 35

3. Inhibition of Rho Kinase Decreases Lesion Burden in Mouse Models of CCM ................. 36
  3.1 Introduction .................................................................................................................................. 36
  3.2 Results ......................................................................................................................................... 38
    3.2.1 Genetically sensitized CCM mice exhibit CCM lesions at a high frequency ................ 38
    3.2.2 Loss of KRIT1 protein in lesions from \textit{Ccm1}^{+/}\textit{Msh2}^{-/-} mice .................................. 44
    3.2.3 Phenotypic maturation of CCM lesions ........................................................................... 46
    3.2.4 Ultrastructural changes in CCM lesions by electron microscopy .............................. 50
    3.2.5 ROCK activation in background vessels and CCM lesions ........................................ 53
    3.2.6 Fasudil decreases lesion burden in CCM mouse models ............................................. 57
  3.3 Summary and Discussion ............................................................................................................ 64
    3.3.1 A Novel Mouse Model of CCM ..................................................................................... 64
3.3.2 The Knudsonian Two-Hit Mechanism..........................................................68

3.3.3 ROCK Inhibition Is the First Potential, Targeted Therapy for CCM............70

3.4 Future Directions..................................................................................................71

3.5 Materials and Methods .......................................................................................72

3.5.1 Mice ..................................................................................................................72

3.5.2 MRI Protocol .....................................................................................................72

3.5.3 Iron Staining and Immunohistochemistry.......................................................72

3.5.4 Transmission electron microscopy.....................................................................75

3.5.5 Fasudil Treatment ............................................................................................75

4. Discussion & Future Directions...............................................................................77

4.1 CCM Genes Converge on RhoA Pathway .........................................................77

4.2 Non-genetic “Hits” Involved in CCM Pathogenesis........................................82

4.3 CCM, Cancer, and Radiation...............................................................................85

4.4 CCM as a Cell-Cell Junction Disease ................................................................87

References ..................................................................................................................89

Biography ....................................................................................................................105
List of Tables

Table 1: Summary of Germline and Somatic Mutations Identified in Sporadic CCM Lesions by Next-Generation Sequencing.................................................................21

Table 2: Allele frequencies for CCM1 mutations found in sporadic lesion sample 4392 assayed by SNaPshot..................................................................................................24

Table 3: Allele frequencies for the CCM1 somatic mutation (exon 12, c.1492_1521indel) found in sporadic lesion sample 4386, as assayed by SNaPshot..............................................27

Table 4: Allele frequencies for the CCM3 somatic mutation (exon 9, c.475-2A>G) found in sporadic lesion sample 4386, as assayed by SNaPshot.................................................28

Table 5: Penetrance of CCM lesions in the three mouse models with and without long-term fasudil treatment.................................................................................................59
List of Figures

Figure 1: Appearance of human CCM lesions. A. MRI image showing a CCM lesion (circled in yellow), which appears dark due to the deposition of hemosiderin. B. Histology of a multicavernous CCM lesion showing clustered, dilated capillaries. ........... 1

Figure 2: Mutant alleles appear in lesion sample 4392 DNA (red arrows) but not in controls. Mutant allele frequencies were analyzed by SNaPshot. A. CCM1, exon 8, c.993T>G, Y331X (wild-type green peak, mutant black peak). B. CCM1, exon 9, c.1159C>T, Q387X (wild-type blue peak, mutant green peak). ................................................. 24

Figure 3: Sporadic lesion 4386 contains a CCM1 somatic mutation (exon 12, c.1492_1521indel). A. Comparison of wild-type (WT) and mutant (Mut) nucleotide and amino acid sequences (codons indicated with alternating black and green text). The mutant sequence causes a frameshift and premature stop after five amino acid residues (red X). B. Mutant alleles (black peak indicated by red arrow, compared to the wild-type red peak) appear in lesion sample 4386 but not in controls, as assayed by SNaPshot. C. Agarose gel of a PCR amplicon specific for the mutant allele shows that the mutation is found in sample 4386 lesion DNA, but not in controls. ......................................................... 26

Figure 4: Trans-heterozygosity in a CCM lesion. Lesion sample 4384 contains a germline mutation in CCM2, exon 4, c.472+1G>T (panel A, Sanger sequencing electropherogram, mutation indicated by red arrow), and a somatic mutation in CCM3, exon 9, c.475-2A>G (panel B, SNaPshot, mutant allele blue peak indicated by red arrow, compared to wild-type green peak). ......................................................................................... 28

Figure 5: Breeding scheme to create Ccm1+/−/Msh2+/− mice. A two-generation cross was used to produce the Msh2 knockout allele (box). The first cross generated the Msh2 knockout allele using CRE-lox technology and the second cross aimed at breeding out the CRE transgene by back-crossing mice to C57BL/6J. From that point, a three-generation cross produces Ccm1+/−/Msh2+/− mice. First, Msh2 heterozygotes without the CRE transgene were crossed with mice heterozygous for Ccm1. Double heterozygotes from this cross were mated with each other in the fourth generation to produce Ccm1+/−/Msh2+/− mice as well as littermate controls. A similar breeding scheme was used to generate the other sensitized mouse models in these experiments. ................................................................. 40

Figure 6: Characterization of lesions in Ccm1+/−/Msh2+/− mice. Both early-stage (isolated dilated vessels) and late-stage (clusters of dilated vessels) are found in the brains of the mice. Images are shown of coronal sections of brains from gradient recalled echo MRI (left) and H&E staining (middle and right). The white boxes in the middle panels denote
the area represented under higher magnification in the right panels. In the middle row, black arrows denote representative early-stage lesions and white arrows denote a late-stage lesion. Scale bars are 1 mm (left), 0.5 mm (center) and 0.1 mm (right).

Figure 7: CCMs in Ccm3+/−Trp53−/− mice tend to be late-stage, multicavernous lesions. Representative images are shown of CCM lesions on the external brain surface (A) and coronal sections of brains from gradient recalled echo MRI (B) and H&E staining (C and D). The black box in the panel B denotes the area represented under higher magnification in panel C. Scale bars are 200 µm (A), 1 mm (B and C) and 0.5 mm (D).

Figure 8: Reduced KRIT1 expression in the endothelium of mouse CCM lesions. Capillaries in Msh2−/− control brains (first row) showed prominent brown staining for both KRIT1 (left) and CCM2 (right). Consistent with the genotype of the mice, KRIT1 staining was reduced, while CCM2 staining remained at control levels in normal capillaries of the Ccm1+/−Msh2−/− mouse brain (second row). Endothelial cells lining two Stage 1 CCM lesions (third row) and two caverns of a Stage 2 CCM lesion (fourth row) showed normal staining for CCM2 (right), but either reduced (white arrow) or no staining (black arrow) for KRIT1 (left). Scale bar is 50 µm.

Figure 9: Phenotypic maturation in early-stage vs. late-stage lesions. Late-stage lesions (right panels) in brains from Ccm1+/−Msh2−/− mice harbor B cells (brown B220), proliferating endothelium (brown Ki67, highlighted by arrows) and iron (blue Perl stain). These are not visible in early-stage lesions (left panels). Images are shown at 3X higher magnification in the respective insets (yellow boxes). Circles indicate blue iron particles in late-stage, multicavernous lesions (bottom right panel). Scale bar is 100 µm.

Figure 10: Electron microscopy reveals abnormal ultrastructure of CCM lesion endothelium. (A) Three CCM lesions (arrows) in a Ccm1+/−Msh2−/− mouse brain shown by Toluidine Blue staining (40X, scale bar 25 µm). (B) Intact tight junctions (arrows) and basal lamina (arrowheads) are present within a normal capillary in a control C57BL/6J mouse (4600X, scale bar 1 µm). Intact tight junctions (arrows) and basal lamina (arrowheads) are also present within a normal capillary (C, 35000X, scale bar 200 nm) and a CCM lesion (D, 35000X, scale bar 200 nm) in a Ccm1+/−Msh2−/− mouse brain. (E) In the same lesion filopodia (arrows) are present (4060X, scale bar 2 µm). (F) A tight junction and the basal lamina (arrowhead) are both broken in this lesion and erythrocyte extravasation is visible (8260X, scale bar 1 µm). RBC = red blood cell; NU = endothelial nucleus; BL = basal lamina.
Figure 11: Tight junctions are disrupted between endothelial cells in murine CCM lesions. Tight junction complexes (arrows) are in close proximity between two brain capillary endothelial cells from a wild-type C57BL/6J mouse (A), but the complexes are impaired and remain farther apart between endothelial cells lining mouse CCM lesions. Red lines indicate tight junction distance measured.

Figure 12: ROCK activation in mouse CCM lesions. ROCK activity was assessed by pMLC immunohistochemistry (dark brown staining, left panels). Normal capillaries (arrowheads) from an Msh2−/− control mouse (top row) do not show evidence of ROCK activation. By contrast, in Ccm1+/−Msh2−/− mice, endothelial cells (ECs) lining early-stage lesions (arrows) stain weakly for pMLC (middle row) and ECs lining three caverns (asterisks) of a late-stage lesion show stronger pMLC staining. Corresponding serial sections show no staining with an isotype control (right panels). All tissue sections were counterstained blue with hematoxylin. Scale bar is 100μm.

Figure 13: ROCK activation increases as CCM lesions grow. (A) The relative intensities of pMLC staining were compared between normal capillaries, early-stage CCM lesions and late-stage lesions. The intensity of pMLC staining increases with the size and complexity of the lesions in Ccm1+/−Msh2−/− mouse brains with CCMs (regression coefficient=0.7655, p<0.0001; common odds ratio=2.150). (B) The pMLC staining intensities of normal capillaries were compared between the genotypes and MRI phenotypes of the mice. Normal capillaries in brains from Ccm1+/−Msh2−/− mice with CCM lesions show stronger pMLC staining than those from mice with the same genotype without CCM lesions, which in turn show stronger pMLC staining than those from Msh2−/− control mice (p<0.0001, exact Mantel-Haenszel test). Post-hoc Bonferroni-adjusted p-values were < 0.001 between two groups in all three comparisons.

Figure 14: Fasudil treatment reduces late-stage lesion burden in CCM mouse models. Bars represent the average total number of lesions per mouse, and are divided into early-stage (gray) and late-stage (black) lesions, with error bars representing SEM. *significantly decreased late-stage lesions in fasudil-treated mice compared to the placebo group (p = 0.008). †significantly reduced total number of lesions in fasudil-treated mice compared to the placebo group (p = 0.04).

Figure 15: The appearance of late-stage lesion phenotypes in Ccm1+/−Msh2−/− mouse brains was reduced in fasudil-treated mice (right) as compared with placebo (left). Iron deposits (blue patches identified by Perls’ Prussian blue), inflammatory cell infiltration (B cells shown by brown immunostaining), endothelial cell proliferation (arrows identify Ki67 positive cells), pMLC, and pMBS staining (biomarkers of ROCK activation, brown
immunostaining) were all reduced in lesions from mice treated with fasudil. Scale bars are 100 µm.

Figure 16: Fasudil reduces ROCK activity in Ccm1+/Msh2± lesion endothelial cells. The prevalence of ROCK-positive endothelial cells significantly decreased with administration of fasudil. CCM lesion sections were immunostained for pMLC and scored with a none/weak/strong scale.

Figure 17: Attrition/mortality of CCM mice during fasudil/placebo treatment. Ccm1+/Msh2± mice treated with fasudil had a significantly lower rate of attrition compared to controls (p<0.05), whereas the same trend was not observed in Ccm2+/Trp53± mice or Ccm3+/Trp53± mice.

Figure 18: No significant difference in body weight gain between fasudil- and placebo-treated mice. Mice were weighed at 2 months of age and at sacrifice. The change in weight was calculated as weight at sacrifice minus weight at 2 months. While all groups gained weight (p<0.05), there were no significant differences in change in body weight for fasudil treatment compared to placebo for Ccm1+/Msh2±, Ccm2+/Trp53±, or Ccm3+/Trp53± mice. Error bars represent SEM.

Figure 19: Diagram of the CCM pathway.
Acknowledgements

I would like to acknowledge Angioma Alliance, particularly the efforts of Amy Akers and Connie Lee, for all the support they have provided to our lab and the CCM community. I would also like to acknowledge our long-term collaborations with Issam Awad’s group (especially Robert Shenkar and Changbin Shi) and Mark Ginsberg’s group (including Rebecca Stockton). Our extensive and multifaceted research projects are the direct result of these interdisciplinary cooperative efforts.

Furthermore, I want to acknowledge the support of the Marchuk Lab. My advisor, Doug Marchuk, has been an incredible resource and mentor throughout my graduate school career. The other members of the lab and our departmental staff (past and present), Chris Bennett, Kasey Carroll, Pei-lun Chu, Chris Clayton, Nick De Castro, Esther DeMarco, Carol Gallione, Sehoon Keum, Han Kyu Lee, Vicki Michael, Rachel Mullis, and Hao Tang, have also greatly helped me with their knowledge and their friendship.

Lastly, I would like to thank the American Heart Association and the National Institute of Neurological Disorders and Stroke for awarding me pre-doctoral fellowships to complete the work described in this dissertation.
1. Cerebral Cavernous Malformations (CCM): Etiology and Genetics

1.1 Disease Overview

Cerebral cavernous malformations (CCMs) are vascular lesions affecting the central nervous system. CCM lesions consist of grossly enlarged, multicavernous, capillary-like structures that lack intervening parenchyma or connective tissue. Lesions can be found in approximately 0.5% of the general population (Rigamonti et al., 1988, Otten et al., 1989, Del Curling et al., 1991, Robinson et al., 1991), and affected individuals have a lifetime risk of recurrent headaches, focal neurological deficits, seizures, and intracerebral hemorrhage leading to stroke. Age of onset varies greatly, but most patients present with clinical symptoms between their third and fifth decades of life.

Figure 1: Appearance of human CCM lesions. A. MRI image showing a CCM lesion (circled in yellow), which appears dark due to the deposition of hemosiderin. B. Histology of a multicavernous CCM lesion showing clustered, dilated capillaries.
1.1.1 Clinical Summary

Patients with CCM present with vascular lesions primarily located in the central nervous system. CCM lesions appear as grossly dilated, capillary-like structures consisting of multiple, blood-filled caverns (Challa et al., 1995, Clatterbuck et al., 2001, Gault et al., 2004). Lesions are composed of endothelial cells and are devoid of astrocytic foot processes and perivasacular cell types, such as pericytes and smooth muscle cells. The intracellular matrix surrounding the vessels, the basal lamina, is often disrupted around CCM lesions. Endothelial cells of CCM lesions show a paucity of tight junctions, and adjacent endothelial cells can be separated by gaps of almost a micron (Wong et al., 2000, Clatterbuck et al., 2001, Tu et al., 2005). Such alterations impair the blood-brain barrier, and allow substrates from the blood to move into central nervous system tissues. For instance, red blood cells extravasate through the defective lesion endothelium, leaving hemosiderin deposits in surrounding tissue that are evident by magnetic resonance imaging (MRI).

Outside of the central nervous system, CCMs can occur at lower frequencies in the retina and on the skin. Retinal CCMs appear as clustered, saccular aneurisms in about 5% of CCM patients (Labauge et al., 2006). Cutaneous CCMs are irregularly shaped skin lesions with a hyperkeratotic epidermal layer and are composed of abnormal capillaries. While there appears to be no correlation between retinal lesions
and a particular CCM gene, cutaneous CCMs only occur in patients with germline mutations in \textit{KRIT1} and at a frequency of 9\% (Eerola et al., 2000, Sirvente et al., 2009).

\subsection*{1.1.2 Diagnosis}

CCM patients can present with a variety of symptoms including stroke, seizures, headaches, and focal neurological deficits (Aiba et al., 1995, Moriarity et al., 1999, Denier et al., 2004, Batra et al., 2009), all of which are likely due to intracranial hemorrhage of CCM lesions. The type of symptoms may depend on the location of the lesion within the central nervous system, and the distribution of symptoms across patients has been recently reviewed (Washington et al., 2010).

MRI remains the primary test for diagnosing CCM, and newer, more sensitive MRI techniques are improving our ability to identify lesions. The standard MRI sequence for clinical diagnosis and observation of CCM is the T2-weighted gradient echo (T2*GRE) MRI (Zabramski et al., 1994, Lehnhardt et al., 2005), which is more sensitive at showing the presence of CCMs than lower power T1- or T2-weighted MRI sequences (Atlas et al., 1988, Bradley, 1993, Kim et al., 1993, Haque et al., 2003). A recent study showed that using a high-field MRI (7 Tesla compared with the standard 1.5 Tesla) can improve the rate of detection of CCM lesions (Schlamann et al., 2010).

Another MRI technique called susceptibility weighted imaging (SWI) takes advantage of tissues’ different degrees of magnetization in response to a magnetic field. SWI has been shown to dramatically increase the sensitivity of MRI in identifying CCM lesions (de
Souza et al., 2008). In this study, the average numbers of lesions detected per patient were 5.7 by T2-weighted imaging, 26.3 by T2*GRE, and 45.6 by SWI. These more sensitive imaging techniques have revealed that patients with multiple CCMs (almost always inherited cases) tend to have a much higher lesion burden than previously thought.

Treatment strategies for CCM fall into two categories – surgical removal and symptom management (Kivelev et al., 2012). If a particular CCM lesion has hemorrhaged and is severely impacting the health of the patient, then the lesion can be removed by surgical resection. Stereotactic radiosurgery shows promise in removing CCMs with a lowered risk of rebleeding (Lunsford et al., 2010), but a significant amount of morbidity (Pham et al., 2009) makes this surgical technique controversial. Depending on the location of the lesion, surgical removal can present a great risk of complications. The alternative treatment is to manage symptoms using medications and physical therapy. Many smaller lesions may remain radiologically stable over long periods of time, and as such only require routine monitoring by MRI.

1.1.3 Identification of Three CCM Genes

CCMs can develop sporadically or in an inherited, autosomal dominant manner. In families affected by CCM, linkage analysis identified three loci, CCM1 mapping to chromosome 7q (Dubovsky et al., 1995, Gunel et al., 1995, Marchuk et al., 1995), CCM2
on chromosome 7p (Craig et al., 1998), and CCM3 on chromosome 3q (Craig et al., 1998). Genes have been identified for all three loci that, when mutated, cause CCM disease.

1.1.3.1 CCM1/KRIT1

Mutations in the gene KRIT1 cause CCM1 (Laberge-le Couteulx et al., 1999, Sahoo et al., 1999). KRIT1 (Krev-1/Rap1 interaction trapped) was originally identified in a screen of proteins binding to Rap1 (Serebriiskii et al., 1997). The full-length KRIT1 protein contains 736 amino acid residues and has a molecular weight of 84 kDa (Eerola et al., 2001, Sahoo et al., 2001). The protein contains an N-terminal NPxY/F motif-rich region (asparagine-proline-any amino acid-tyrosine/phenylalanine), three ankyrin repeats, and a FERM domain (protein 4.1/ezrin/radixin/moesin), all of which could mediate protein-protein interactions. Through separate NPxY/F motifs, KRIT1 binds to the PTB domains of both ICAP1α (Zawistowski et al., 2002, Liu et al., 2013) and CCM2 (Zawistowski et al., 2005). The KRIT1 FERM domain also interacts with activated, GTP-bound Rap1 (Liu et al., 2011, Li et al., 2012) and may act as a Rap1 effector by regulating cell junctions (Glading et al., 2007).

KRIT1 is primarily expressed in vascular endothelial cells, chiefly in arterioles and capillaries, of various organs as well as in astrocytes and pyramidal neurons (Guzeloglu-Kayisli et al., 2004). During development, KRIT1 expression appears ubiquitous throughout the brain and other organs (Denier et al., 2002, Petit et al., 2006). When KRIT1 and ICAP1α are co-expressed, both localize to the cell nucleus.
(Zawistowski et al., 2005). When co-expressed with CCM2, KRIT1 localizes to the cytoplasm (Zawistowski et al., 2005) with enrichment at the plasma membrane and cell-cell junctions (Glading et al., 2007, Glading and Ginsberg, 2010). While the functional significance of nuclear KRIT1 is yet to be determined, the junctional co-localization of KRIT1 and CCM2 potentially implicates defective cell-cell junctions in CCM pathogenesis.

In vivo studies indicate that KRIT1 is involved in angiogenesis and vascular development. In mice (Whitehead et al., 2004) and zebrafish (Mably et al., 2006, Hogan et al., 2008), deletion of Ccm1 prevents proper vascular development and patterning, causing embryonic lethality. During in vivo development and in cell culture, KRIT1 deficiency increases endothelial cell proliferation (Whitehead et al., 2004), but nascent vessels fail to form proper lumens (Lampugnani et al., 2010, Wustehube et al., 2010). Other studies have connected a loss of KRIT1 to an increase in levels of reactive oxygen species, modulated through SOD2 and the transcription factor FoxO1 (Goitre et al., 2010). The nematode C. elegans has an ortholog of KRIT1, kri-1, that is required for germ cell apoptosis (Ito et al., 2010).

1.1.3.2 CCM2

Mutations in the previously uncharacterized gene MGC4607 cause CCM2 (Liquori et al., 2003, Denier et al., 2004). CCM2 encodes a 444-amino-acid protein with a molecular weight of 49 kDa. The CCM2 protein contains a single phosphotyrosine-
binding (PTB) domain, which can interact with NPxY/F motifs, leading to the discovery that CCM2 can bind to KRIT1 (Zawistowski et al., 2005). Proteomic analysis also found PDCD10 in complex with KRIT1 and CCM2 (Hilder et al., 2007), demonstrating a direct connection between the proteins produced by all three CCM genes.

Other than the CCM proteins, CCM2 has been found in a complex with p38 MAPK pathway proteins MKK3 (a mitogen-activated protein kinase kinase) and MEKK3 (a STE11-like kinase), leading some groups to rename CCM2 as OSM (osmosensing scaffold for MEKK3) (Uhlik et al., 2003). These interactions occur at sites of dynamic actin polymerization, and they may modulate MEKK3 signaling in response to hyperosmotic shock. Immunoprecipitation experiments found that CCM2 also binds RhoA and \textit{in vitro} depletion of CCM2 transcript increases levels of active, GTP-bound RhoA (Whitehead et al., 2009). CCM2 also binds to the ubiquitin ligase Smurf1 to mediate degradation of RhoA (Crose et al., 2009).

The expression pattern of CCM2 is similar to that of KRIT1: arterial vascular endothelium of most organs and, in the central nervous system, neurons and astrocytes (Seker et al., 2005). Interestingly, CCM2 is expressed in parenchymal cerebral vessels of mice at postnatal day 8 (P8) whereas KRIT1 is not, potentially indicating a role for CCM2 in active angiogenesis (Petit et al., 2006). CCM2 localizes to the plasma membrane (Crose et al., 2009), specifically at cell junctions (Stockton et al., 2010).
1.1.3.3 CCM3/PDCD10

Mutations in the gene *PDCD10* cause CCM3 (Bergametti et al., 2005). *PDCD10* encodes a 212-amino-acid protein with a molecular weight of 25 kDa. *PDCD10* shows a similar expression pattern to *Ccm2*, chiefly appearing in neuronal layers of the central nervous system as well as in brain blood vessels (Petit et al., 2006). The PDCD10 protein contains a six alpha-helical bundle with a C-terminal focal adhesion targeting (FAT)-domain, which mediates binding to CCM2 and paxillin (Li et al., 2010). PDCD10 can also form homodimers through an N-terminal dimerization domain (Ding et al., 2010, Li et al., 2010).

The most well studied family of PDCD10 interactors is the GCKIII family of Ste20 kinases including STK24, STK25, and MST4. PDCD10 co-immunoprecipitates with STK25 and MST4 (Voss et al., 2009, Fidalgo et al., 2010, Zheng et al., 2010). In a proteomics study, all three of these proteins were found in a large, striatin-binding complex called STRIPAK, composed of 20 proteins including kinases and phosphatases (Goudreault et al., 2009). Biochemically, STK25 phosphorylates PDCD10, and another interactor, the non-receptor protein tyrosine phosphatase FAP-1, dephosphorylates PDCD10 (Voss et al., 2007). These interactions are important during development, as shown by a recent study in *Drosophila*. Flies have a single ortholog of *PDCD10* and a single GCKIII gene. Tracheal-specific knockdown of *PDCD10* causes trachea to branch properly but they fail to lumenize; this phenotype is rescued by expression of wild-type
*PDCD10*, but not by an allele of *PDCD10* that is unable to bind to GCKIII (Chan et al., 2011). These *in vivo* data validate the protein interactions identified in the proteomics data, but the role of the GCKIII pathway in CCM biology is still unknown.

*PDCD10* may participate in other signaling pathways. It binds to various phosphatidylinositol species, including phosphatidylinositol-3,4,5-triphosphate (Dibble et al., 2010) and inositol-1,3,4,5-tetrakisphosphate (Ding et al., 2010). *PDCD10* also interacts with vascular endothelial growth factor (VEGF) receptor 2, an interaction which is enhanced by the presence of VEGF (He et al., 2010).

*PDCD10* was originally identified in a screen for programmed cell death mutants (Wang, 1999). Overexpression of *PDCD10* in cell culture induces apoptosis in some studies (Chen et al., 2009, Lin et al., 2010), while others have reported increased cell survival (Schleider et al., 2011). *In vitro* knockdown (Lin et al., 2010) or *in vivo* knockout (Louvi et al., 2011) of *PDCD10* has been found to inhibit apoptosis, but another group has shown that this increases apoptosis (He et al., 2010). How *PDCD10* affects apoptosis, and if this plays a role in CCM pathobiology, remains unclear.

### 1.1.4 Molecular Genetics of CCM

CCMs occur in approximately 0.5% of the general population (Rigamonti et al., 1988, Otten et al., 1989, Del Curling et al., 1991, Robinson et al., 1991) in sporadic and familial (inherited) forms. The familial form of CCM follows an autosomal dominant genetic inheritance pattern. Germline mutations can be found in approximately 75% of
apparently familial cases (Labauge et al., 2009), though this number is likely an underestimate due to limitations in DNA sequencing. Of these mutation-positive patients, 65% had a mutation in \textit{KRIT1}, 20% had a mutation in \textit{CCM2}, and 15% had a mutation in \textit{PDCD10}. A publicly available mutation database is maintained by the patient advocacy group Angioma Alliance on their website (angiomaalliance.org). The vast majority of CCM mutations severely alter the transcripts or proteins through deletions, frameshifts, disrupted splicing, or premature stop codons. The lack of missense mutations in this disease adds support to the hypothesis that the CCM proteins have no enzymatic activity and that they function as scaffolds for signaling proteins.

While the sporadic form of CCM accounts for the majority of cases (Pozzati et al., 1996), some populations have an increased number of familial cases. For instance, in the Hispanic population of the United States, approximately 72% of familial CCM patients bear the same germline mutation, a single-base substitution in exon 7 that converts glutamine 455 into a premature stop codon (Sahoo et al., 1999, Sahoo et al., 2001, Laurans et al., 2003), an apparent founder mutation in this population. More recently, another founder mutation in \textit{CCM2} has been reported in the Ashkenazi Jewish population (Gallione et al., 2011).

Few clinical features differ between patients with mutations in different CCM genes. As previously mentioned, individuals with germline mutations in \textit{KRIT1} are more likely to show cutaneous CCM lesions (Eerola et al., 2000, Sirvente et al., 2009).
Furthermore, germline mutations in *PDCD10* may predispose patients to a more severe form of CCM disease. A comparison of the three CCM genes revealed that CCM3 patients have an increased risk of hemorrhage, particularly during childhood (Denier et al., 2006, Gault J., 2006).

### 1.2 CCM Pathogenesis

#### 1.2.1 Two-Hit Mutation Mechanism

CCM cases tend to fall into two categories: familial (inherited) cases where patients have a family history of the disease, and; sporadic cases where patients have no known family history. Familial cases have a greater risk of acute hemorrhage than sporadic cases (Robinson et al., 1991, Labauge et al., 2000), and familial cases tend to present with multiple CCM lesions whereas sporadic cases tend to show a single lesion (Petersen et al., 2010). The differences in disease severity and the number of lesions in sporadic versus inherited cases are similar to those observed by Knudson in retinoblastoma patients (Knudson, 1971). Knudson proposed a two-hit mutation hypothesis where each of the two copies of a particular gene must be inactivated by mutation to cause disease pathogenesis. Familial patients have a germline mutation of a particular gene, and a single somatic mutation inactivating the wild-type copy of the gene initiates disease. Sporadic disease, then, is caused by two independent, biallelic, somatic mutations occurring within the same cell.
Somatic mutations have been found in CCM lesion samples by a number of groups (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009). These researchers estimate that the somatic mutations are found in 6-20% of the cells within the lesion, most likely within the endothelial cells (Akers et al., 2009, Gault et al., 2009). It is clear that, unlike a two-hit mechanism for cancer, cells within the CCM do not proliferate by a purely clonal expansion, leading to fewer than 50% of cells within the lesions bearing the somatic mutation. Another study found a loss of CCM protein immunostaining in the endothelial cells of CCM lesions, indirect evidence of a two-hit mechanism (Pagenstecher et al., 2009).

While it is evident that two genetic hits are necessary to cause CCM pathogenesis, it is unclear if they are sufficient or if other non-genetic factors are required. The presence of a brain developmental vascular anomaly (DVA) may increase the risk of sporadic lesion growth and subsequent hemorrhage (Aboian et al., 2009, Petersen et al., 2010). The overall incidence of DVAs in the general population is approximately 2.5% (Ostertun and Solymosi, 1993), while CCMs are found in 8-33% of DVA cases (Hong et al., 2010). Patients with a DVA alone have a minimal increase in hemorrhage rate, while patients with CCMs alone have a 38% rate of hemorrhage, and patients with both a DVA and an associated CCM have a 62-93% rate of hemorrhage (Aboian et al., 2009). Clearly, a clinical connection exists between a DVA and CCM lesion genesis, but the specific mechanism remains unknown.
An immune response may play a role in the development and progression of CCM. In addition to the infiltration of immune cells around CCM lesions in animal models (McDonald et al., 2011), there is increasing evidence of a CCM-specific immune response in humans. Antibody B lymphocytes and plasma cells have been found in human CCM samples (Shenkar et al., 2007). Further analysis revealed an antigen-directed, oligoclonal pattern of immunoglobulin G (IgG) within the lesions (Shi et al., 2007, Shi et al., 2009). While it is clear that the immune system is mounting a specific response to the CCM lesion, the role of this immune response in CCM pathogenesis requires further investigation.

1.2.2 The RhoA Pathway in CCM Pathogenesis

After identification of the three CCM genes, all three CCM proteins were found in a ternary complex with KRIT1 and PDCD10 each binding to CCM2 (Zawistowski et al., 2005, Hilder et al., 2007, Voss et al., 2007, Zhang et al., 2007). This was the first evidence of a clear molecular connection between the three CCM genes. Recent research suggests that all three CCM genes converge on the RhoA pathway. Knockdown of CCM1, CCM2, or CCM3 in cell culture causes an increase in actin stress fiber formation (Glading et al., 2007, Crose et al., 2009, Whitehead et al., 2009, Stockton et al., 2010). The same phenotype occurs when the small GTPase RhoA is activated (Kimura et al., 1996). Knockdown of any one of the CCM genes causes an increase in RhoA expression and activity (Crose et al., 2009, Whitehead et al., 2009, Borikova et al., 2010). Additionally,
when a CCM protein is knocked down in endothelial cell culture, vessel-like tubes form
that fail to lumenize (Whitehead et al., 2009, Borikova et al., 2010, Chan et al., 2011). A
downstream effector of the RhoA pathway, Rho Kinase (ROCK) is inhibited by multiple
compounds including Y-27632, H-1152, fasudil, and simvastatin. Treatment of cells in
culture with Y-27632 or H-1152, or shRNA knockdown of ROCK, abrogated the stress
fiber formation and tube formation phenotypes (Borikova et al., 2010).

The connections between PDCD10 and the RhoA pathway are tenuous but still
compelling. While knockdown of KRIT1 or CCM2 in cell culture clearly causes actin
stress fiber formation (Glading et al., 2007, Crose et al., 2009, Whitehead et al., 2009,
Stockton et al., 2010), the effect of PDCD10 knockdown on stress fibers is more
controversial: some groups report stress fibers in PDCD10-depleted HMVEC (primary
human microvascular endothelial cell) culture (Zheng et al., 2010); others report that
PDCD10 depletion in SaOS2 cells (sarcoma osteogenic cell line) causes no stress fiber
formation (Fidalgo et al., 2010). This discrepancy may be due to differences in technique
and in vitro cell lines used in the experiment. PDCD10 also has binding partners distinct
from KRIT1 and CCM2, including members of the GCKIII family of kinases.

Knockdown of GCKIII family proteins in vitro causes actin stress fiber formation (Zheng
et al., 2010). It is unclear if the three CCM proteins function in a single pathway with
RhoA, separate pathways that converge on RhoA, or separate, parallel pathways that do
not converge. The resolution of this mystery will determine the effectiveness of potential therapies for CCM based on inhibiting the RhoA pathway.

The RhoA pathway regulates endothelial cell function and cell shape. Activation of this pathway leads to increased actomyosin contractility, loss of cell-cell contacts, and increased permeability. Changes in permeability are particularly of note for CCM disease as permeabilization of the blood-brain barrier is thought to cause symptoms and clinical sequelae. Increased permeability has been observed in KRIT1-depleted endothelial cell cultures and in the blood vessels of multiple organs from Krit1+/- and Ccm2+/- mice (Stockton et al., 2010). In both of these systems, normal vascular permeability was restored by treatment with the ROCK inhibitors H-1152 or fasudil. Another study found that Krit1+/- mice had an enhanced edema response to particular inflammatory stimuli (Corr et al., 2012). These findings indicate that the connection between the CCM genes and the RhoA pathway may be key to disease pathogenesis. Furthermore, these data imply that familial CCM patients with germline mutations in one of the CCM genes may also be at risk for increased vascular leakage and impaired blood-brain barrier function.

1.2.3 Other Genes in the CCM Pathway

While the mutations in CCM patients have only been found in either KRIT1, CCM2, or PDCD10, other genes have been identified in the CCM pathway. Zebrafish mutants of KRIT1 (santa) and CCM2 (valentine) are characterized by enlarged cardiac
chambers during development which lead to heart failure (Mably et al., 2006). Similarly, zebrafish displayed similar cardiac defects when both of the zebrafish orthologs of \textit{PDCD10} (\textit{ccm3a} and \textit{ccm3b}) were simultaneously knocked down by morpholinos (Zheng et al., 2010). This enlarged heart phenotype was also observed in a separate zebrafish mutant \textit{heart of glass (heg)} (Kleaveland et al., 2009). \textit{Heg} encodes a type I transmembrane receptor. During mouse development, \textit{Heg1} (the mouse ortholog of \textit{heg}) is expressed at embryonic day 10.5 (E10.5) in the endothelial cells of the heart and aorta of the developing neural tube. By E14.5, expression of \textit{Heg1} appears in the brain vasculature, arterial endothelium, heart endocardium, and smooth muscle. In human cells, multiple lines of evidence show that HEG1 binds to the KRIT1 FERM domain, effectively anchoring KRIT1 to the plasma membrane (Kleaveland et al., 2009, Gingras et al., 2012). Multiple labs have sequenced CCM patient blood DNA samples for mutations in \textit{HEG1}, but to date, none have been identified (unpublished data). The ligand that binds to the HEG1 receptor and whether HEG1 affects CCM pathogenesis are currently unknown.

\textit{PTEN} (phosphate and tension homolog deleted on chromosome 10) acts as a tumor suppressor and regulates various cellular functions. Various studies have found roles for \textit{PTEN} regulating angiogenesis in normal vessels and tumors. Hypermethylation of the \textit{PTEN} promoter activates angiogenesis in tumors. In human CCM samples, there is evidence of \textit{PTEN} promoter methylation, especially in familial cases with multiple lesions (Zhu et al., 2009). Overall, 16% of CCM samples contained
hypermethylated *PTEN* promoters. How these observations fit into CCM pathogenesis and if these epigenetic alterations are heritable are currently unknown.

Two recent studies found a novel paralog of CCM2 through a BLAST search and named it CCM2-like or CCM2L (Zheng et al., 2012, Rosen et al., 2013). CCM2L is expressed in the vasculature, particularly during development and angiogenesis. Whereas CCM2 binds both KRIT1 and PDCD10, CCM2L only binds KRIT1 and appears to compete with CCM2 in this binding. In contrast to CCM2, overexpression of CCM2L increases RhoA activity. CCM2L also appears to have novel regulatory roles in controlling the expression of endocardial growth factors *Fgf9* and *Fgf12*. The relationship of CCM2L to CCM pathogenesis remains untested. Along with HEG1 and *PTEN*, CCM2L is a new addition to the CCM pathway that merits further study.

### 1.3 Proposal of Research

Most of the research in CCM focuses on inherited cases of the disease; however, sporadic patients (individuals with single CCM lesions and no apparent family history) account for the majority of the cases of CCM (Pozzati et al., 1996). Various groups have reported finding somatic mutations in inherited cases of CCM, evidence of a two-hit mutation mechanism. It is unknown if sporadic cases similarly follow the two-hit mechanism where each CCM lesion is caused by two independent, biallelic, somatic mutations in one of the three CCM genes. The presence of a genetic underpinning of sporadic CCM is not only important for understanding the disease mechanism, but it
will also determine how effective targeted therapeutics are in this population of patients.

If sporadic CCM does not follow a two-hit mutation mechanism, then therapies developed for familial patients may not show efficacy in sporadic patients. For my thesis research, I first proposed to investigate the two-hit mutation mechanism in sporadic cases of CCM using a next-generation sequencing approach targeted to the three CCM genes (Aim 1).

Given the recent success in generating mouse models of CCM and the elucidation of multiple downstream signaling pathways, we were able to test the first potential therapeutics specifically for treating CCM. While each of the three CCM genes appears to have unique cellular functions, multiple lines of evidence connect the CCM genes to the RhoA pathway. I proposed to investigate the centrality of the RhoA pathway in CCM lesion pathogenesis using our mouse models (Aim 2).
2. A Two-Hit Genetic Mechanism Causes Sporadic CCM

2.1 Introduction

CCM epidemiology shows that patients tend to have either a familial (inherited) form of the disease or a sporadic form. This pattern fits a Knudson two-hit mutation mechanism where both copies of a particular gene must be mutated in order for disease to occur. Lesions in familial cases would result from an inherited germline mutation and biallelic somatic mutations. Lesions in sporadic cases would result from two independent, biallelic, somatic mutations occurring in the same cell.

Our lab and others have identified biallelic somatic mutations in lesions from familial cases (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009). There is a single report of somatic mutations identified from sporadic cases of CCM (Kehrer-Sawatzki et al., 2002), but the somatic mutations presented differ from the vast majority of reported CCM mutations. Whereas nearly all CCM mutations cause a premature stop codon, insertion, or deletion, all of which would create a null allele, the mutations reported by Kehrer-Sawatzki, et al, are missense mutations – K97S and K569E. No functional analysis was performed on these mutations, so their effects on the KRIT1 protein function are unclear. The sporadic patient in this report also had the lesion removed at the age of 23 years old, much earlier than most sporadic patients. This report of a single sporadic lesion, then, does not present conclusive results on the genetics of sporadic CCM.
Furthermore, most of the literature focuses on the inherited form of CCM, but the majority of CCM patients treated clinically are sporadic cases (Pozzati et al., 1996). Based on the two-hit genetic mechanism, I hypothesize that sporadic CCM lesions are caused by two independent, biallelic, somatic mutations. I will investigate this hypothesis through next-generation sequencing of DNA from the lesions of sporadic CCM patients.

2.2 Results

Previous work to examine CCM lesions for somatic mutations relied on methods to separate mutant alleles from wild-type alleles (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009). Mutant alleles in these samples were present at frequencies between 5% and 20%. If a single mutant cell became a CCM lesion by clonal expansion (such as that seen in cancerous tumors), then the frequency of mutant alleles within the samples would be nearly 50%. Thus, CCM lesions do not grow from a purely clonal expansion. The low frequencies of mutations within the lesions also demonstrate the need for using techniques such as high pressure liquid chromatography or molecular cloning in order to separate mutant alleles from the wild-type sequences.

In examining sporadic CCM lesions for two somatic mutations, both of which are likely to be at frequencies below 20%, a next-generation sequencing strategy was used. The coding exons of all three CCM genes were amplified by PCR, pooled and submitted
to the Duke Sequencing and Analysis Core Resource for sequencing on a Roche 454 GS-FLX Titanium platform.

Eight lesion samples were analyzed in this manner. A total of 122,208 reads aligned with the human reference sequence, with an average of 395 reads per amplicon, though this value had a range from 0 to 3003. Since the vast majority of CCM mutations that have been identified severely alter protein function (premature stop codons, splice-site mutations, insertions/deletions leading to frameshifts, etc.), the sequences from these sporadic CCM lesions were analyzed for these types of mutations. The results are summarized in Table 1.

**Table 1: Summary of Germline and Somatic Mutations Identified in Sporadic CCM Lesions by Next-Generation Sequencing.**

<table>
<thead>
<tr>
<th>CCM Sample #</th>
<th>Germline Mutation (% reads)</th>
<th>Somatic Mutation (% reads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2049*</td>
<td>CCM1, exon 10, c.1363C&gt;T, Q455X (42%)</td>
<td>CCM1, exon 10, c.1270_1273 delTATA (4.3%)</td>
</tr>
<tr>
<td>4382</td>
<td>None found</td>
<td>None found</td>
</tr>
<tr>
<td>4384</td>
<td>CCM2, exon 4, c.472+1G&gt;T (52%)</td>
<td>CCM3, exon 9, c.475-2A&gt;G (1.4%)</td>
</tr>
<tr>
<td>4386</td>
<td>None found</td>
<td>CCM1, exon 12, c.1492_1521indel (0.4%)</td>
</tr>
<tr>
<td>4388</td>
<td>None found</td>
<td>CCM2, exon 9, c.916-1G&gt;A (5.6%)</td>
</tr>
<tr>
<td>4390</td>
<td>None found</td>
<td>None found</td>
</tr>
<tr>
<td>4392</td>
<td>None found</td>
<td>CCM1, exon 8, c.993T&gt;G, Y331X (7.2%); CCM1, exon 9, c. 1159C&gt;T, Q387X (6.1%)</td>
</tr>
<tr>
<td>4394</td>
<td>None found</td>
<td>None found</td>
</tr>
</tbody>
</table>

*Familial CCM lesion control.*
Of the eight CCM samples analyzed, seven were from patients with no apparent family history, a single CCM lesion, and a later age of onset of symptoms. The remaining CCM lesion is a sample previously analyzed by our lab (Akers et al., 2009). This sample, number 2049, had a known germline mutation in CCM1 (exon 10, c.1363C>T, Q455X), and our lab identified a biallelic, somatic mutation in CCM1 (exon 10, c.1270_1273delTATA) by cloning and Sanger sequencing, occurring at 4.6% frequency. Using next-generation sequencing, the germline mutation was found in 39/94 reads (42% frequency) and the somatic mutation was found in 4/94 reads (4.3% frequency). Previously, the somatic mutation was identified by amplifying the coding exons of CCM1, cloning the amplicons, and sequencing at least 42 clones per amplicon. This positive control sample shows as proof of principle that next-generation sequencing is sensitive enough to detect this previously reported somatic mutation.

2.2.1 Sporadic CCM Lesion Containing Two Somatic Mutations

Of the seven sporadic CCM lesion samples analyzed by next-generation sequencing, five somatic mutations were identified in four samples. Sample number 4392 had two somatic mutations in CCM1. The first somatic mutation was a premature stop codon in exon 8 (c.993T>G, Y331X) occurring in 44/610 reads (7.2% frequency). The second somatic mutation was a different premature stop codon in exon 9 (c.1159C>T, Q387X) occurring in 35/578 reads (6.1% frequency).
A single-base extension assay (SNaPshot, Applied Biosystems) was used to validate these mutations. First, primers anneal adjacent to the base of interest, and then an extension reaction adds a single nucleotide to the primer. The resulting fragments are separated by size, and different alleles of the same amplicon appear as adjacent peaks. Occasionally, for peaks with a particularly strong signal, pull-up can occur where smaller peaks appear underneath the larger peak; however, these pull-up peaks do not reflect the biology of the sample and appear in wild-type control samples as well, so they can be ignored. One of the major benefits of this assay is that it can be used to quantify the frequency of alleles at a particular DNA base.

By SNaPshot, the exon 8 and exon 9 mutant alleles appeared in the original lesion DNA sample (4392) at frequencies of 5% and 2%, respectively (Table 2, Figure 2). A second, separate PCR amplification and a PCR amplification from another DNA preparation from the same lesion also replicated these results. Mutant alleles were not apparent in a matched blood DNA sample, the familial lesion sample 2049, or a control DNA sample (non-CCM case).
Figure 2: Mutant alleles appear in lesion sample 4392 DNA (red arrows) but not in controls. Mutant allele frequencies were analyzed by SNaPshot. A. *CCM1*, exon 8, c.993T>G, Y331X (wild-type green peak, mutant black peak). B. *CCM1*, exon 9, c.1159C>T, Q387X (wild-type blue peak, mutant green peak).

Table 2: Allele frequencies for *CCM1* mutations found in sporadic lesion sample 4392 assayed by SNaPshot.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CCM1, exon 8, c.993T&gt;G, Y331X</th>
<th>CCM1, exon 9, c.1159C&gt;T, Q387X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial Lesion (2049)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Control DNA</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>
2.2.2 Sporadic CCM Lesions with a Single Somatic Mutation

Two sporadic lesion samples (4386 and 4388) contained one identifiable somatic mutation each. Sample 4386 bore an insertion-deletion mutation in CCM1 exon 12 (c.1492_1521del) in 3/702 reads (0.4%), causing a frameshift and a premature stop codon after 5 altered amino acid residues (Figure 3A). This mutation was validated by SNaPshot (Table 3, Figure 3B), showing that the mutant allele only appears in sample 4386 and not in control samples or other sporadic lesions. For further confirmation of this insertion/deletion mutation, PCR primers were designed to specifically amplify the mutant sequence. Based on this strategy, PCR bands were only apparent for sample 4386 and not for control samples (Figure 3C).
Figure 3: Sporadic lesion 4386 contains a \textit{CCM1} somatic mutation (exon 12, c.1492_1521indel). A. Comparison of wild-type (WT) and mutant (Mut) nucleotide and amino acid sequences (codons indicated with alternating black and green text). The mutant sequence causes a frameshift and premature stop after five amino acid residues (red X). B. Mutant alleles (black peak indicated by red arrow, compared to the wild-type red peak) appear in lesion sample 4386 but not in controls, as assayed by SNaPshot. C. Agarose gel of a PCR amplicon specific for the mutant allele shows that the mutation is found in sample 4386 lesion DNA, but not in controls.
Table 3: Allele frequencies for the CCM1 somatic mutation (exon 12, c.1492_1521indel) found in sporadic lesion sample 4386, as assayed by SNaPshot.

<table>
<thead>
<tr>
<th>Sample</th>
<th>WT Allele (T) Freq.</th>
<th>Mut Allele (G) Freq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial Lesion (2049)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Sporadic Lesion (4382)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Sporadic Lesion (4384)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Sporadic Lesion (4386)</td>
<td>97%</td>
<td>3%</td>
</tr>
<tr>
<td>4386 (2nd amplification)</td>
<td>96%</td>
<td>4%</td>
</tr>
</tbody>
</table>

WT – wild-type, Mut – mutant.

Sample 4388 also contained a single somatic mutation, a splice site mutation in CCM2 (c.916-1G>A) in 3/54 reads (5.6%). This splice mutation occurs at the invariant splice acceptor site and is predicted to alter the normal splicing of the mRNA transcript. Further validation of this mutation by SNaPshot is required.

2.2.3 Evidence of Trans-heterozygosity in a CCM Lesion

A germline mutation was found in lesion 4384, a splice-site mutation in CCM2 (exon 4, c.472+1G>T, splice donor site) occurring in 52% of the reads (43/82). This heterozygous mutation was verified by traditional Sanger sequencing (Figure 4A). While this patient had similar clinical features to those of a sporadic patient (late age of onset of symptoms, single lesion, and no apparent family history), it is clear that this is a familial case.

In addition to this germline mutation, a somatic mutation was found in this same lesion DNA sample in 2/148 reads (1.4%) – a splice-site mutation in CCM3 (exon 9, c.475-2A>G). The somatic mutation occurs at an invariant splice acceptor site and is
predicted to alter the normal splicing of the mRNA transcript. SNaPshot analysis showed that this somatic mutation was only found in lesion sample 4384 and not in control samples (Figure 4B, Table 4).

![Figure 4: Trans-heterozygosity in a CCM lesion. Lesion sample 4384 contains a germline mutation in CCM2, exon 4, c.472+1G>T (panel A, Sanger sequencing electropherogram, mutation indicated by red arrow), and a somatic mutation in CCM3, exon 9, c.475-2A>G (panel B, SNaPshot, mutant allele blue peak indicated by red arrow, compared to wild-type green peak).](image)

Table 4: Allele frequencies for the CCM3 somatic mutation (exon 9, c.475-2A>G) found in sporadic lesion sample 4386, as assayed by SNaPshot.

<table>
<thead>
<tr>
<th>Sample</th>
<th>WT Allele (T) Freq.</th>
<th>Mut Allele (G) Freq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DNA</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Sporadic Lesion (4386)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Sporadic Lesion (4384)</td>
<td>82%</td>
<td>18%</td>
</tr>
<tr>
<td>4384 (2nd amplification)</td>
<td>92%</td>
<td>8%</td>
</tr>
</tbody>
</table>

WT – wild-type, Mut – mutant.
Epigenetic alterations within CCM lesions are poorly understood. Hypermethylation of promoter CpG dinucleotides functions to silence particular genes, and aberrant hypermethylation is often found in cancerous tumors (Jin and Robertson, 2013). Our lab has previously tested for hypermethylation in the promoter region upstream of CCM2 in CCM lesions from patients with a germline deletion of CCM2 exons 2 through 10, but the results were inconsistent (Amy Akers and Douglas Marchuk, unpublished data). Individual alleles showed one or two methylated cytosines, but none of the alleles showed the expected widespread hypermethylation across multiple CpG dinucleotides.

In addition to the coding regions of the three CCM genes analyzed by next-generation sequencing in this project, CpG island regions upstream of each of the three genes (3 for CCM1, 3 for CCM2, and 2 for CCM3) were examined for evidence of promoter hypermethylation. DNA samples were treated with bisulphite to convert unmethylated cytosines to thymines (methylated cytosines are unaffected). After treating the sporadic CCM lesion sample DNA with bisulfite, CpG island regions were amplified by PCR and analyzed by next-generation sequencing.

Only one of the lesion samples, 4384 with identified germline and somatic mutations, showed consistent results. In a CpG island upstream of CCM1 in this sample, a stretch of 28bp featured 6 methylated cytosines at a frequency of 5% (7/130) to 12% (12/100), depending on the base. Many of these reads contained multiple
methylated cytosines, indicating that all six methylated bases occurred on a single allele (data not shown).

2.3 Summary and Discussion

Four CCM lesion samples were found to contain a total of five somatic mutations and one germline mutation. One sample, 4392, had two somatic mutations in the same gene, as was predicted by the Knudson two-hit mutation hypothesis. Two other samples, 4386 and 4388, each contained a single somatic mutation. While analyzing additional sporadic CCM lesions for somatic mutations is warranted, it is clear from these three lesion samples that there is a genetic component to sporadic CCM disease. Novel therapeutic strategies are currently being tested in animal models of CCM based on the genetics of the inherited form of the disease (see Chapter 3). Since the sporadic form of CCM also follows an underlying genetic mechanism, it is likely that these novel therapies will be effective for the larger sporadic patient population as well.

Our methods did not find two somatic mutations in each of the samples, but this does not indicate that these lesions contain only one or no somatic mutations. It is likely that our next-generation sequencing approach was able to identify only sequenceable mutations (single-base substitutions and small insertions/deletions) while other types of mutations went undetected. For example, loss of heterozygosity (LOH) events such as large deletions of one or more exons would not be found using our method. The major limitation in detecting somatic mutations within CCM lesions is that mutant alleles are
only found at 5-20% frequency. As the proportion of affected cells drops below 10%, it is more difficult to measure allelic imbalance in these samples. I have previously attempted to examine a mouse CCM lesion for evidence of LOH by measuring allele frequencies using SNaPshot. The results of this experiment were inconsistent (unpublished data), because I was looking for a subtle change in allele frequency as low as 5% which is within the margin of error for this assay. Our next-generation sequencing analysis of these sporadic CCM lesions, then, is limited to a particular class of mutations and does not exclude the possibility of other types of somatic mutations.

Sample 4384 is the first example of trans-heterozygosity found for CCM. This disease mechanism has been reported for other diseases such as autosomal dominant polycystic kidney disease (Koptides et al., 2000), where samples from patients with germline mutations in PDK1 showed somatic mutations in either PDK1 or PDK2. Somatic mutations do not necessarily have to be biallelic to the germline mutations in order for disease pathogenesis to initiate. Since there are three CCM genes, it is unclear if all combinations of trans-heterozygosity would cause disease. Multiple lines of evidence show that CCM3 has a different role from CCM1 and CCM2, and one possibility is that only mutation combinations involving CCM3 would cause CCM pathogenesis to occur. Further examination of trans-heterozygosity as a mechanism of CCM disease is certainly warranted.
2.4 Future Directions

It is important to analyze the somatic mutations found in sporadic lesion 4392 to see if they occurred on the two separate alleles. This may not be easily accomplished by sequencing cDNA (reverse transcribed from RNA) from the lesion as both of these somatic mutations cause premature stop codons, meaning the transcripts are likely to undergo nonsense-mediated decay. The mutations are separated by 867 bp, so it may be possible to amplify this region by PCR followed by cloning to separate the alleles and subsequent Sanger sequencing.

One caveat of the two-hit mutation hypothesis is that two distinct lesions from the same (likely familial) patient should contain different somatic mutations. This caveat could be addressed by using this next-generation sequencing strategy on matched lesion samples. Obtaining two separate lesions samples from the same patient is exceedingly rare, though, which limits a thorough investigation into this class of lesions.

The next-generation sequencing strategy employed in the experiments I have performed is limited in scope to point changes and small insertions/deletions within the coding regions of the three CCM genes. For a more complete picture of sporadic CCM somatic mutations, it would be helpful to examine these lesions for LOH (larger indels and rearrangements). Currently, it is difficult to accomplish this due to the low
frequency of somatic mutations within the lesions which masks LOH, but as technologies improve this may become more feasible.

The final evidence of a two-hit mechanism in sporadic CCM lesions would be to show the presence of two independent somatic mutations occurring within the same cell. While this is a technically challenging experiment, it may be possible to use laser-capture microdissection to isolate single cells from the lesion endothelium. From there, whole genome amplification could be used to provide sufficient DNA for sequencing to confirm the presence of somatic mutations previously identified by next-generation sequencing. A similar strategy was used in our lab for familial lesions (Akers et al., 2009), and the major limitation would be obtaining a sufficient quantity and quality of DNA for analysis.

Since there has been little study of CpG hypermethylation in CCM, it is difficult to draw clear conclusions from the single sample showing a stretch of methylated cytosines upstream of CCM1. Analysis of further sporadic CCM lesions for hypermethylation would demonstrate the prevalence of this phenomenon. In vitro functional analyses could help determine if this methylation pattern is sufficient to transcriptionally silence CCM1.

Sequencing additional sporadic CCM samples, to find more evidence of biallelic mutations and trans-heterozygosity, would also help to lend weight to the two-hit mutation hypothesis. To further examine trans-heterozygosity as a disease mechanism
in CCM, trans-heterozygous mouse models could be created. Experiments are currently underway to study the phenotype of mice heterozygous for two CCM genes (i.e. Ccm1+/−/Ccm3+/−); however, with a limited number of brains examined from these mice, there is no obvious difference in lesion burden. While trans-heterozygosity may be a disease mechanism, it is not a strong genetic trigger.

2.5 Materials and Methods

2.5.1 CCM Samples

All CCM samples were obtained from the Angioma Alliance Tissue Bank in accordance with Institutional Review Board standards. Lesions were bisected and tissue samples were removed from the center of the lesion. DNA was isolated using the Puregene tissue protocol (Gentra).

2.5.2 Next-Generation Sequencing

PCR primers (IDT) were designed to amplify the coding exons of all three CCM genes. During primer design, 5′ overhangs were created in accordance to 454 sequencing protocols (Roche), including the addition of a 4bp identification sequence used for multiplexing samples (4 samples per run). Regions of interest were amplified by PCR and a small aliquot was checked for quality control by gel electrophoresis. Where necessary, PCR products of interest were analyzed by gel electrophoresis and isolated using the GeneClean Turbo kit (MP Biomedicals). For CpG island amplicons,
DNA samples were treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research).

PCR products were pooled and submitted to the Duke Genome Sequencing and Analysis Core for quality control and sequencing on a Roche 454 GS-FLX Titanium platform. Data were analyzed using Swap454 (Broad Institute) (Brockman et al., 2008) and Geneious software (Biomatters) (Drummond, 2010).

### 2.5.3 SNaPshot Analysis

PCR products with potential mutations were analyzed by SNaPshot (Applied Biosystems). Primers were designed adjacent to the base of interest, and SNaPshot was performed according to Applied Biosystems protocols. Results were visualized and quantified using GeneMapper software (Applied Biosystems). Allele frequencies were calculated by dividing the area under the peak of a particular allele by the total area under both peaks.

### 2.5.4 Sanger Sequencing

For lesion sample 4384, the germline mutation was validated by Sanger sequencing using the BigDye reaction kit (Applied Biosystems) on the 3130 Genetic Analyzer (Applied Biosystems).
3. Inhibition of Rho Kinase Decreases Lesion Burden in Mouse Models of CCM

A portion of Chapter 3 was modified from two published manuscripts: (McDonald et al., 2011) and (McDonald et al., 2012). Data on the Ccm3 mouse model are currently unpublished.

3.1 Introduction

Current understanding of the CCM vascular phenotype is based largely on the pathologic study of surgically resected lesions. The multicavernous structures are lined by a single layer of endothelium, and are separated by a loose, collagenous matrix lacking mature vessel wall structure. The endothelial layer lining the caverns manifests gross breaches, including vacuoles and apparently defective inter-endothelial junctions, and seems to lack both a well-developed basement membrane and the structural integrity of the blood-brain barrier (Wong et al., 2000, Clatterbuck et al., 2001, Tu et al., 2005). The disruption of the blood-brain barrier and brittle structure of the vascular wall are thought to account for repetitive hemorrhages and reactive changes in adjacent brain.

Sporadic cases of CCM are characterized by an isolated lesion whereas familial cases usually exhibit multiple lesions in random distribution throughout the brain with increasing prevalence of lesions during the patients’ lifetimes (Labauge et al., 2000, de Champfleur et al., 2010). Based on these observations, we and others hypothesized that CCM lesion genesis follows a two-hit mutation mechanism, where both copies of a
single gene must be inactivated in the same cell to initiate lesion genesis. There is growing evidence for a two-hit mechanism in human CCM lesion samples as multiple studies have identified somatic mutations (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009) as well as loss of CCM protein immunostaining (Pagenstecher et al., 2009) in human lesions from familial and sporadic patients (see Chapter 2).

Despite the knowledge gained thus far, analysis of mature, late-stage CCM lesions may not reflect the earliest pathologic changes associated with lesion genesis. Magnetic resonance imaging (MRI) data can show the appearance of new lesions and growth of existing lesions over time (de Souza et al., 2008). Although the earliest stages of lesion development have been increasingly described with MRI techniques (de Souza et al., 2008, de Champfleur et al., 2010), these early lesions do not cause clinical symptoms and, as such, they are not readily available for pathologic examination. A major gap is the ability to study lesion genesis and progression in vivo.

To address these questions, we have created mouse models of CCM. The development of knockout alleles of the Ccm1 (Whitehead et al., 2004), Ccm2 (Plummer et al., 2006), and Ccm3 (He et al., 2010) have been described previously. Mice homozygous for these knockout alleles die mid-gestation and heterozygous animals have not shown lesions at an appreciable frequency (Whitehead et al., 2004, Plummer et al., 2006, Whitehead et al., 2009). Based on the two-hit mechanism, we hypothesized that lesion penetrance of the heterozygous CCM mice could be increased by crossing these
knockout alleles into a genetic background with elevated genetic instability.

Homozygous knockout of \( Trp53 \), a tumor suppressor gene, and \( Msh2 \), a mismatch repair complex gene, increase the overall rate of somatic mutation (Shao et al., 2000, Hegen et al., 2006). Specifically, homozygous knockout of \( Msh2 \) increases the rate of single-base mutations and short (1-4 bp) insertions and deletions (Modrich and Lahue, 1996, Bacon et al., 2001). Knockout of \( Trp53 \), on the other hand, increases the rate of chromosomal abnormalities such as aneuploidy, large deletions, duplications, and homologous recombination (Shao et al., 2000). The \( Msh2 \)-null background was first used to sensitize \( Apc^{+/-} \) mice to generate a more robust and penetrant intestinal neoplasia phenotype (Reitmair et al., 1996). Owing to their more penetrant phenotype, the compound mutant \( Apc^{+/-}Msh2^{-/-} \) mice have been used for studies of therapeutic interventions. In a similar fashion, we have used both \( Trp53^{-/-} \) and \( Msh2^{-/-} \) genotypes as genetic sensitizers to increase the probability of somatic mutation and, thereby, lesion burden of the Ccm heterozygous mice.

### 3.2 Results

#### 3.2.1 Genetically sensitized CCM mice exhibit CCM lesions at a high frequency

An \( Msh2 \) knockout allele was generated by crossing mice with an allele of \( Msh2 \) flanked by loxP sites (Kucherlapati et al., 2010) with a mouse strain bearing the Cre recombinase transgene under control of the ubiquitous promoter EIIa (The Jackson Laboratory, stock number 003724). After a stably transmitting \( Msh2 \)-null allele was
generated, the Cre recombinase was bred out of the mice to prevent unforeseen effects of the transgene. The resulting $\text{Msh2}^{+/\cdot}$ animals were then crossed with mice heterozygous for $\text{Ccm1}$, $\text{Ccm2}$, or $\text{Ccm3}$ to produce double heterozygotes (ex. $\text{Ccm1}^{+/-}\text{Msh2}^{+/-}$). These mice were then intercrossed to generate genetically sensitized Ccm heterozygotes (ex. $\text{Ccm1}^{+/-}\text{Msh2}^{-/-}$) as well as littermate controls. A summary of these crosses is shown in Figure 5. A similar breeding scheme was used with the $\text{Trp53}$ sensitizer (The Jackson Laboratory, stock number 002101). Importantly, all mouse lines have been maintained by back-crossing with C57BL/6J mice (at least 18 generations for $\text{Ccm1}$ and $\text{Ccm2}$ and at least 5 generations for $\text{Ccm3}$). Random mice from each line were selected for single nucleotide polymorphism (SNP) genotyping (Illumina GoldenGate 377 mouse SNP panel) to measure the purity of these strains genetic backgrounds. SNP genotyping revealed that the genomes of genetically sensitized $\text{Ccm1}$ and $\text{Ccm2}$ mice were uniformly derived from C57BL/6J except for the regions surrounding the knockout alleles, which were originally derived from 129X1Sv/J (for $\text{Ccm1}$), 129P2/OlaHsd (for $\text{Ccm2}$), 129S2 (for $\text{Trp53}$) or WW6 (for $\text{Msh2}$). For $\text{Ccm3}$ mice, SNP genotyping showed that the genetic background was at least 93% C57BL/6J (data not shown), which should be sufficient for these studies.
Figure 5: Breeding scheme to create $\text{Ccm1}^{+/-}\text{Msh2}^{-/-}$ mice. A two-generation cross was used to produce the $\text{Msh2}$ knockout allele (box). The first cross generated the $\text{Msh2}$ knockout allele using CRE-lox technology and the second cross aimed at breeding out the CRE transgene by back-crossing mice to C57BL/6J. From that point, a three-generation cross produces $\text{Ccm1}^{+/-}\text{Msh2}^{-/-}$ mice. First, $\text{Msh2}$ heterozygotes without the CRE transgene were crossed with mice heterozygous for $\text{Ccm1}$. Double heterozygotes from this cross were mated with each other in the fourth generation to produce $\text{Ccm1}^{+/-}\text{Msh2}^{-/-}$ mice as well as littermate controls. A similar breeding scheme was used to generate the other sensitized mouse models in these experiments.

Homozygous knockout of $\text{Trp53}$ or $\text{Msh2}$ will create genetic backgrounds of increased somatic mutation. However, $\text{Trp53}^{-/-}$ mice tend to develop tumors around five months of age and $\text{Msh2}^{-/-}$ mice are prone to lymphoma and small intestinal tumors by 6 months of age (de Wind et al., 1995, Reitmair et al., 1995, Kucherlapati et al., 2010). In order to avoid cancer-induced morbidity and mortality in these CCM models, we sacrificed the animals at 4 months of age ($4.1 \pm 0.1$ months) for $\text{Trp53}$-sensitized mice and 5 months of age ($5.2 \pm 0.1$ months) for $\text{Msh2}$-sensitized mice. At sacrifice, brains were removed, fixed in formalin, and screened for CCM lesions using gradient echo $\text{ex vivo}$ high-field MRI. Subsequently, 2mm serial coronal sections of the brains were surveyed.
histologically by hematoxylin and eosin (H&E) staining (Shenkar et al., 2008). Although Msh2-null mice have not been reported to develop cerebral vascular disease, we examined six Msh2−/− mouse brains and found neither tumors nor CCMs (data not shown).

In these mouse models, lesions were found at varying stages of development – from smaller, early-stage, isolated caverns to late-stage, multicavernous lesions. To differentiate between these stages, we defined “early-stage” lesions as dilated capillaries having a diameter of at least 100µm and not joined to any other lesion. “Late-stage” lesions were defined as multicavernous structures composed of the confluence of two or more caverns (Shenkar et al., 2008).
Figure 6: Characterization of lesions in Ccm1<sup>+/−</sup>Msh2<sup>+/−</sup> mice. Both early-stage (isolated dilated vessels) and late-stage (clusters of dilated vessels) are found in the brains of the mice. Images are shown of coronal sections of brains from gradient recalled echo MRI (left) and H&E staining (middle and right). The white boxes in the middle panels denote the area represented under higher magnification in the right panels. In the middle row, black arrows denote representative early-stage lesions and white arrows denote a late-stage lesion. Scale bars are 1 mm (left), 0.5 mm (center) and 0.1 mm (right).

Using these definitions, 11 out of 16 (69%) Ccm1<sup>+/−</sup>Msh2<sup>+/−</sup> mice, 13 out of 18 (72%) Ccm2<sup>+/−</sup>Trp53<sup>+/−</sup> mice, and 6 out of 6 (100%) Ccm3<sup>+/−</sup>Trp53<sup>+/−</sup> mice exhibited one or more lesions. These percentages of animals with CCMs were higher than the previously published 40% penetrance for Ccm1<sup>+/−</sup>Trp53<sup>+/−</sup> and Ccm2<sup>+/−</sup>Trp53<sup>+/−</sup> mice (Plummer et al.,
Representative images of MRI and H&E staining are shown in Figure 6.

Compared to the $\text{Ccm1}^{+/\cdot}\text{Msh2}^{-/-}$ and $\text{Ccm2}^{+/\cdot}\text{Trp53}^{-/-}$ models, $\text{Ccm3}^{+/\cdot}\text{Trp53}^{-/-}$ mice appear to have an increased penetrance of lesions (100%) and, on average, $\text{Ccm3}^{+/\cdot}\text{Trp53}^{-/-}$ mice show an eight- to nine-fold increase in lesion burden (Figures 7 and 14), the majority of which are late-stage, multicavernous CCMs. Out of 11 $\text{Ccm2}^{+/\cdot}\text{Msh2}^{-/-}$ mouse brains examined, no CCM lesions were found. Lastly, the $\text{Ccm3}^{+/\cdot}\text{Msh2}^{-/-}$ mice are currently breeding and experiments are underway.

**Figure 7:** CCMs in $\text{Ccm3}^{+/\cdot}\text{Trp53}^{-/-}$ mice tend to be late-stage, multicavernous lesions. Representative images are shown of CCM lesions on the external brain surface (A) and coronal sections of brains from gradient recalled echo MRI (B) and H&E staining (C and D). The black box in the panel B denotes the area represented under higher magnification in panel C. Scale bars are 200 µm (A), 1 mm (B and C) and 0.5 mm (D).
3.2.2 Loss of KRIT1 protein in lesions from \textit{Ccm1^{+/−} Msh2^{−/−}} mice

In support of a two-hit mutation mechanism in CCM, two groups have independently shown a loss of particular CCM protein expression in the endothelial cells surrounding human CCM caverns (Pagenstecher et al., 2009, Stockton et al., 2010). We sought to confirm these results in our mouse model to show that it faithfully recapitulates the human disease. Coronal sections from brains removed from one \textit{Msh2^{−/−}} control, one \textit{Ccm2^{+/-} Msh2^{−/−}} and four \textit{Ccm1^{+/-} Msh2^{−/−}} mice were stained with anti-KRIT1 and anti-CCM2 primary antibodies for analysis by immunohistochemistry (Figure 8).
Figure 8: Reduced KRIT1 expression in the endothelium of mouse CCM lesions. Capillaries in Msh2⁻/⁻ control brains (first row) showed prominent brown staining for both KRIT1 (left) and CCM2 (right). Consistent with the genotype of the mice, KRIT1 staining was reduced, while CCM2 staining remained at control levels in normal capillaries of the Ccm1⁺/-Msh2⁻/- mouse brain (second row). Endothelial cells lining two Stage 1 CCM lesions (third row) and two caverns of a Stage 2 CCM lesion (fourth row) showed normal staining for CCM2 (right), but either reduced (white arrow) or no staining (black arrow) for KRIT1 (left). Scale bar is 50 µm.
Capillary endothelial cells in Msh2+/ control brains showed robust expression of KRIT1 and CCM2. Normal capillaries in Ccm2+/Msh2− brain also showed robust KRIT1 endothelial cell expression, but reduced expression of CCM2 as expected in animals heterozygous for Ccm2 (data not shown). Normal capillaries in the four Ccm1+/Msh2− brains had normal levels of CCM2 and reduced levels of KRIT1 due to Ccm1 heterozygosity. In early- or late-stage lesions from these four Ccm1+/Msh2− mice, KRIT1 was either reduced or absent in the endothelial cells lining the caverns. Haploinsufficiency would reduce, but not eliminate, KRIT1 expression in all cells expressing the transcript. However, loss of heterozygosity caused by a somatic mutation in a subset of endothelial cells would result in a Ccm1-null genotype, with the complete absence of KRIT1 protein expression in these cells. These phenomena produce a mosaic expression pattern in the CCM lesions. From these data, it is clear that in lesions from Ccm1+/Msh2− animals, the KRIT1 protein is not expressed in a subset of cells lining the cavern. The mosaic nature of this loss of expression also suggests a somatic mutation has occurred to inactivate the wild-type copy of Ccm1 within this subset of endothelial cells.

3.2.3 Phenotypic maturation of CCM lesions

The ability to visualize CCM lesions on MRI in humans depends on the accumulation of hemosiderin deposits around the lesion, a sign of a chronic
cerebrovascular bleed. Iron deposits around the mouse CCM lesions, identified by Perls’ staining, were found in four of the five late-stage lesions examined (Figure 9), and the intensity of iron deposits showed remarkable variation (from a few iron particles per lesion to a high density of iron surrounding the lesion). By contrast, there was no iron deposition in any of the approximately forty early-stage lesions examined ($p = 0.048$, two-tailed Fisher’s exact test). Thus, this mouse model of CCM provides a means of studying late-stage lesions that behave similarly to those seen in the multicavernous human lesion samples. Additionally, the model allows the study of CCM lesions at an earlier stage of development before hemosiderin deposits form, and presumably before they would cause clinical symptoms.
Figure 9: Phenotypic maturation in early-stage vs. late-stage lesions. Late-stage lesions (right panels) in brains from Ccm1+/Msh2−/− mice harbor B cells (brown B220), proliferating endothelium (brown Ki67, highlighted by arrows) and iron (blue Perl stain). These are not visible in early-stage lesions (left panels). Images are shown at 3X higher magnification in the respective insets (yellow boxes). Circles indicate blue iron particles in late-stage, multicavernous lesions (bottom right panel). Scale bar is 100 μm.
Gene expression data from surgically resected human CCM lesions suggest a role for an immune response in CCM pathology (Shenkar et al., 2003). Furthermore, the prevalence of immune cells surrounding CCM lesions has been studied and an immune response may occur against an antigen specific to CCM lesions (Shi et al., 2007, Shi et al., 2009). As in late-stage human lesions, infiltration of immunocompetent cells, including B cells, plasma cells, and macrophages, was observed in all three of the late-stage Ccm1+/Msh2−/+ lesions examined (Figure 9). However, only a small subset of early-stage lesions showed evidence of immune cell infiltration. The number of cells per total caverns counted for early-stage vs. late-stage lesions was, respectively, 0.61 vs. 3.1 for B lymphocytes, 0.52 vs. 1.5 for plasma cells, and 0.05 vs. 0.2 for macrophages. There was a trend for significant differences in B cell density between early-stage and late-stage lesions ($p = 0.06$, Wilcoxon two-sample test). Based on these results, this mouse model recapitulates what is seen for the immune response in late-stage, resected human CCM lesions.

Increased cell proliferation has been proposed as a mechanism of growth of the CCM lesions, and previous studies examining this process in late-stage human CCM lesion tissue have found evidence of proliferating endothelial cells (Sure et al., 2001, Shenkar et al., 2005). Expression of Ki67, a proliferation-associated nuclear protein, around mouse CCM lesions was evident in the endothelial cells within late-stage lesions, but not in early-stage lesions (Figure 9). The percentage of caverns with Ki67 positive
endothelial cells was 0% for early-stage lesions and 50% for late stage lesions \((p<0.001,\) Fisher’s exact test). Comparing the percentage of Ki67 positive endothelial cells per total number of endothelial cells showed similar results \((0% \text{ vs. } 11\%, \text{ respectively, Fisher’s exact test})\). From these data, cell proliferation appears only in larger multicavernous CCM lesions, but not at the earliest stages of pathogenesis.

### 3.2.4 Ultrastructural changes in CCM lesions by electron microscopy

Electron micrographs from human CCM lesion samples have revealed large gaps in the endothelial cell layer and other defects in blood-brain barrier ultrastructure (Wong et al., 2000, Clatterbuck et al., 2001, Tu et al., 2005). If the CCM lesions in this mouse model follow the same development and pathobiology as those in humans, we would expect to document similar phenotypes by electron microscopy. Transmission electron microscopy demonstrated capillary endothelial cells with flat ovoid nuclei in wild-type C57BL/6J control mice; all tight junctions between endothelial cells were intact, with surrounding pericytes, a defined basement membrane layer, and astrocytic foot processes (Figure 10B). Normal brain capillaries in the \(Ccm1^{+/Msh2^{+-}}\) and \(Ccm2^{+/Msh2^{+-}}\) mice had similar features, including grossly normal morphology of endothelial cells, tight junctions, pericytes, and basement membrane (Figure 10C). Several CCM lesions in \(Ccm1^{+/Msh2^{+-}}\) mice included blood-filled caverns of varying sizes (Figure 10A). The shape of endothelial cells lining these caverns and their nuclei appeared the same as in control capillaries, but there were no typical pericytes or
basement membrane surrounding the endothelial cell layer in the CCM lesions. The majority of tight junctions between endothelial cells lining the caverns appeared morphologically normal (Figure 10D). However, we observed filopodia in endothelial cells of the larger caverns and several gaps in endothelial cells (Figure 10E), with instances of grossly extravasated erythrocytes (Figure 10F). These features resemble those previously reported for human CCM lesions (Wong et al., 2000, Clatterbuck et al., 2001, Tu et al., 2005), so this model allows us to begin to study more carefully the nature and extent of ultrastructural defects associated with lesions at various stages of development.
Figure 10: Electron microscopy reveals abnormal ultrastructure of CCM lesion endothelium. (A) Three CCM lesions (arrows) in a Ccm1<sup>+/−</sup>Msh2<sup>−/−</sup> mouse brain shown by Toluidine Blue staining (40X, scale bar 25 µm). (B) Intact tight junctions (arrows) and basal lamina (arrowheads) are present within a normal capillary in a control C57BL/6J mouse (4600X, scale bar 1 µm). Intact tight junctions (arrows) and basal lamina (arrowheads) are also present within a normal capillary (C, 35000X, scale bar 200 nm) and a CCM lesion (D, 35000X, scale bar 200 nm) in a Ccm1<sup>+/−</sup>Msh2<sup>−/−</sup> mouse brain. (E) In the same lesion filopodia (arrows) are present (4060X, scale bar 2 µm). (F) A tight junction and the basal lamina (arrowhead) are both broken in this lesion and erythrocyte extravasation is visible (8260X, scale bar 1 µm). RBC = red blood cell; NU = endothelial nucleus; BL = basal lamina.

In order to quantify differences in blood-brain barrier integrity, the distance between tight junctions was measured from the electron micrographs (Figure 11). The width of the inner cleft between the tight junction complexes was measured as the
widest space between tight junction complexes (shown in Figure 11 as red lines). There was no significant difference in tight junction cleft width between normal capillaries in wild-type mice compared to similar capillaries in Ccm1\(^{-/}\)Msh2\(^{-/-}\) mice (p = 0.68, Figure 11A). However, while the tight junction complexes appear intact, there was a significant increase in tight junction cleft width in CCM lesions compared to wild-type controls (p = 0.001, Figure 11B). The disruptions in the blood-brain barrier seen in CCM may be attributable to this loss of tight junction integrity.

![Image of tight junctions in murine CCM lesions](image)

**Figure 11**: Tight junctions are disrupted between endothelial cells in murine CCM lesions. Tight junction complexes (arrows) are in close proximity between two brain capillary endothelial cells from a wild-type C57BL/6J mouse (A), but the complexes are impaired and remain farther apart between endothelial cells lining mouse CCM lesions. Red lines indicate tight junction distance measured.

### 3.2.5 ROCK activation in background vessels and CCM lesions

Recent studies have found multiple connections between the CCM genes and the RhoA pathway, particularly through Rho kinase (ROCK), the downstream RhoA
effector (see Chapter 1 – 1.2.2). We measured ROCK activity by phosphorylation of myosin light chain (pMLC) within the endothelial cells (Crose et al., 2009, Borikova et al., 2010, Stockton et al., 2010) of early- and late-stage lesions from \( Ccm1^{+/}\text{-}Msh2^{-/-} \) mice as well as \( Msh2^{-/-} \) control mouse brain specimens (Figure 12).

**Figure 12:** ROCK activation in mouse CCM lesions. ROCK activity was assessed by pMLC immunohistochemistry (dark brown staining, left panels). Normal capillaries (arrowheads) from an \( Msh2^{-/-} \) control mouse (top row) do not show evidence of ROCK activation. By contrast, in \( Ccm1^{+/}\text{-}Msh2^{-/-} \) mice, endothelial cells (ECs) lining early-stage lesions (arrows) stain weakly for pMLC (middle row) and ECs lining three caverns (asterisks) of a late-stage lesion show stronger pMLC staining. Corresponding serial sections show no staining with an isotype control (right panels). All tissue sections were counterstained blue with hematoxylin. Scale bar is 100\( \mu \)m.
We quantified the relative pMLC staining intensity of the lesions in order to analyze the data for statistical significance. Each vessel or lesion was assessed for staining independently by two investigators using the following categories: “none” (completely absent of pMLC staining), “weak” (definite, but diffuse staining) and “strong” (intense and confluent staining). Using a scale (0/1/2 for none/weak/strong), we found that the intensity of pMLC staining increased significantly with the size and complexity of lesions (capillaries < early-stage lesions < late-stage lesions) in mouse brains with CCM lesions (Figure 13A). Interestingly, normal capillaries in brains from Ccm1+/Msh2−/− mice with CCM lesions had stronger pMLC staining than normal capillaries from Ccm1+/Msh2−/− mice without CCM lesions, which in turn had stronger pMLC staining than those from Msh2−/− control mice (Figure 13B). From these data, it is apparent that this mouse model validates the previous ROCK activation findings in late-stage human CCM lesions and cell culture. Our mouse models may suggest that the presence of a CCM lesion activates ROCK in apparently normal brain capillaries.
Figure 13: ROCK activation increases as CCM lesions grow. (A) The relative intensities of pMLC staining were compared between normal capillaries, early-stage CCM lesions and late-stage lesions. The intensity of pMLC staining increases with the size and complexity of the lesions in Ccm1+/Msh2−/− mouse brains with CCMs (regression coefficient=0.7655, p<0.0001; common odds ratio=2.150). (B) The pMLC staining intensities of normal capillaries were compared between the genotypes and MRI phenotypes of the mice. Normal capillaries in brains from Ccm1+/Msh2−/− mice with CCM lesions show stronger pMLC staining than those from mice with the same genotype without CCM lesions, which in turn show stronger pMLC staining than those from Msh2−/− control mice (p<0.0001, exact Mantel-Haenszel test). Post-hoc Bonferroni-adjusted p-values were < 0.001 between two groups in all three comparisons.
3.2.6 Fasudil decreases lesion burden in CCM mouse models

Of the lesion phenotypes examined, only ROCK activity linearly correlates with lesion development. Normal capillaries have the least amount of ROCK activity, early-stage lesions have moderately increased ROCK activity, and late-stage lesions have the highest levels of ROCK activity. This progression implicates increased ROCK activity in CCM pathogenesis. Inhibiting ROCK, then, may inhibit CCM lesion growth.

Figure 14: Fasudil treatment reduces late-stage lesion burden in CCM mouse models. Bars represent the average total number of lesions per mouse, and are divided into early-stage (gray) and late-stage (black) lesions, with error bars representing SEM. *significantly decreased late-stage lesions in fasudil-treated mice compared to the placebo group (p = 0.008). †significantly reduced total number of lesions in fasudil-treated mice compared to the placebo group (p = 0.04).
Fasudil, a specific ROCK inhibitor (Davies et al., 2000, Yamaguchi et al., 2006), was given to Ccm1+/Msh2+/-, Ccm2+/Trp53+/-, and Ccm3+/Trp53+/- mice orally in their drinking water (100 mg/kg/day) from weaning (21 days) until 4-5 months of age. Mice treated with fasudil tended to have fewer late-stage lesions than placebo-treated mice (Figure 14, p=0.008 for Ccm1+/Msh2+/- mice, p=0.18 for Ccm2+/Trp53+/- mice, and p=0.08 for Ccm3+/Trp53+/- mice). While not all of these groups reached statistical significance, fasudil-treated Ccm1+/Msh2+/- mice had significantly fewer late-stage lesions (1 lesion per mouse in the placebo group vs. 0.3 late-stage lesions per mouse in the fasudil-treated group), and fasudil-treated Ccm3+/Trp53+/- mice showed a trend toward fewer late-stage lesions but failed to reach significance (7.8 late-stage lesions per mouse in the placebo group vs. 2.6 late-stage lesions in the fasudil-treated group). The Ccm3+/Trp53+/- mice treated with fasudil, however, had fewer total lesions per mouse than the placebo group (10.7 lesions per mouse when treated with placebo vs. 7 lesions per mouse when treated with fasudil, p=0.04). There was also a significant difference in the types of CCM lesions seen in Ccm1+/Msh2+/- mice (6 early-stage and 16 late-stage lesions in the placebo-treated mice vs. 11 early-stage lesions and 6 late-stage lesions in the fasudil-treated mice, p=0.03, Fisher’s exact test). Fasudil reduced the penetrance of CCM lesions in Ccm1+/Msh2+/- mice with a large reduction in penetrance of late-stage lesions (Table 5). While there was no difference in total CCM lesion penetrance in the Ccm3+/Trp53+/- mice, modest reductions in the numbers of early-stage and late-stage lesions were observed. Together,
these data indicate that inhibition of ROCK by fasudil reduces the development of late-stage CCM lesions.

**Table 5: Penetrance of CCM lesions in the three mouse models with and without long-term fasudil treatment.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ccm1+/Msh2+</th>
<th>Ccm2+/Trp53+</th>
<th>Ccm3+/Trp53+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Fasudil</td>
<td>Placebo</td>
</tr>
<tr>
<td>Mice with lesions</td>
<td>69%</td>
<td>45%</td>
<td>72%</td>
</tr>
<tr>
<td>Early-stage</td>
<td>25%</td>
<td>32%</td>
<td>56%</td>
</tr>
<tr>
<td>Late-stage</td>
<td>56%</td>
<td>23%</td>
<td>22%</td>
</tr>
<tr>
<td>Early-stage &amp; late-stage</td>
<td>13%</td>
<td>9%</td>
<td>6%</td>
</tr>
<tr>
<td>Mice without lesions</td>
<td>31%</td>
<td>55%</td>
<td>28%</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>22</td>
<td>18</td>
</tr>
</tbody>
</table>
Figure 15: The appearance of late-stage lesion phenotypes in Ccm1+/−Msh2−/− mouse brains was reduced in fasudil-treated mice (right) as compared with placebo (left). Iron deposits (blue patches identified by Perls’ Prussian blue), inflammatory cell infiltration (B cells shown by brown immunostaining), endothelial cell proliferation (arrows identify Ki67 positive cells), pMLC, and pMBS staining (biomarkers of ROCK activation, brown immunostaining) were all reduced in lesions from mice treated with fasudil. Scale bars are 100 µm.
Phenotypic features of lesions in the fasudil- and placebo-treated groups are presented in Figure 15. Extravascular iron deposits, indicative of chronic hemorrhage, were significantly reduced by fasudil treatment \((p=0.03)\), as was B cell infiltration \((p=0.03)\), and the number of Ki67-positive proliferative endothelial cells \((p=0.05)\). ROCK activity was also reduced by fasudil treatment (Figure 16), as shown by antibody staining for pMLC \((p=0.000006)\) and another indicator of ROCK activity, phosphorylated myosin binding subunit \((pMBS, p=0.00002)\). When ROCK activity was assessed using a none/weak/strong scale (see section 3.2.5), fasudil treatment significantly reduced the number of ROCK-positive endothelial cells within the lesion caverns. These data show that fasudil treatment inhibited lesion ROCK activity and prevented CCM lesions from developing the late-stage phenotypes that may cause clinical symptoms.
Figure 16: Fasudil reduces ROCK activity in Ccm1+/Msh2−− lesion endothelial cells. The prevalence of ROCK-positive endothelial cells significantly decreased with administration of fasudil. CCM lesion sections were immunostained for pMLC and scored with a none/weak/strong scale.

The RhoA pathway has a central role in development and basic cellular processes such as cytoskeletal structure. One concern we had was that inhibiting ROCK with fasudil starting at weaning may adversely affect the mice. Fasudil is only in use clinically in Japan where it is prescribed to treat acute cerebrovasospasm following a stroke, so there is a paucity of clinical information on long-term fasudil treatment such as we used with our mice. In order to monitor the safety of fasudil treatment, we examined the rates of attrition and mortality in our mice (Figure 17). No adverse reactions were seen in the fasudil-treated group. On the contrary, Ccm1+/Msh2−− mice treated with fasudil had a lower rate of attrition compared to the placebo group (p<0.05). Ccm2+/Trp53−− mice and Ccm3+/Trp53−− mice both showed equivalent rates of attrition in
the fasudil- and placebo-treated groups. All mice gained weight significantly between two months of age and sacrifice at 4-5 months of age (p<0.05), but there was no significant difference in weight gain between the fasudil- and placebo-treated groups (Figure 18).

Figure 17: Attrition/mortality of CCM mice during fasudil/placebo treatment. Ccm1+/Msh2− mice treated with fasudil had a significantly lower rate of attrition compared to controls (p<0.05), whereas the same trend was not observed in Ccm2+/ Trp53− mice or Ccm3+/Trp53− mice.
Figure 18: No significant difference in body weight gain between fasudil- and placebo-treated mice. Mice were weighed at 2 months of age and at sacrifice. The change in weight was calculated as weight at sacrifice minus weight at 2 months. While all groups gained weight (p<0.05), there were no significant differences in change in body weight for fasudil treatment compared to placebo for \(Ccm1^{+/+}Msh2^{-/-}\), \(Ccm2^{+/+}Trp53^{-/-}\), or \(Ccm3^{+/+}Trp53^{-/-}\) mice. Error bars represent SEM.

3.3 Summary and Discussion

3.3.1 A Novel Mouse Model of CCM

Like other mouse models of inherited human diseases, the \(Ccm1\) and \(Ccm2\) heterozygous mice do not faithfully recapitulate the human CCM disease phenotype. Autosomal dominant diseases that have been shown to follow a two-hit mutational mechanism are often difficult to model in mice. Mice have a much shorter lifespan than
humans, and the number of relevant target cells is often much less, leading to a greatly reduced chance for a random, biallelic somatic mutation to occur during the lifetime of the animal. In such cases, moving the orthologous gene mutation into a different genetic background can often generate a more faithful animal model while also revealing important aspects of disease pathogenesis.

Using Msh2-null and Trp53-null sensitized backgrounds for Ccm heterozygous mice, we have created mouse models of CCM that faithfully recapitulate the phenotype of mature CCM lesions, as well as generate lesions at earlier stages of development. These mice develop CCM lesions with a penetrance between 69% and 100%. The development of CCM lesions occurred specifically in sensitized heterozygotes and not in any of the control mice (Ccm heterozygotes, Trp53\(^{-}\) mice, or Msh2\(^{-}\) mice). The stochastic nature of the murine CCM lesions, both in penetrance and localization, also replicates what is observed in humans, particularly the increased penetrance and lesion burden in the CCM3 mouse model (Denier et al., 2006, Gault J., 2006). It is unlikely that the results of our model are confounded by the cancer predisposition per se, as vascular malformations have not been reported in humans with MSH2\(^{-}\) germline mutations (Lynch Syndrome) (Jarvinen et al., 2009). While cancer phenotypes are a concern with these genetic sensitizers, these mice rarely develop brain tumors and animal attrition rates are favorably low. Finding both early- and late-stage lesions in these studies has
shown that these mouse models of CCM enable the study of the natural history of lesions.

Unlike the Ccm1\textsuperscript{+/-}Msh2\textsuperscript{+/-} mice that exhibit both early- and late-stage CCM lesions, we did not observe cerebrovascular lesions in the 11 Ccm2\textsuperscript{+/-}Msh2\textsuperscript{+/-} mice examined. By contrast, biallelic somatic mutations have been found in mature CCM lesions from patients harboring CCM1, CCM2, or CCM3 germline mutations (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009), suggesting that all forms of inherited CCM follow a two-hit mutational mechanism. Since the murine Ccm1 coding region (2211 nucleotides) is nearly twice the size of that of murine Ccm2 (1362 nucleotides), the stochastic nature of somatic mutagenesis in the mismatch repair-deficient sensitized mice may favor lesion genesis in the Ccm1 model harboring the larger target. However, this size difference alone would not be expected to account for the apparent lack of penetrance in the Ccm2\textsuperscript{+/-}Msh2\textsuperscript{+/-} model. We do not believe that this invalidates the two-hit hypothesis for Ccm2 pathogenesis, as this mechanism is supported by the lesions found in the Ccm2\textsuperscript{+/-}Trp53\textsuperscript{+/-} mouse model. To date we cannot explain why Trp53 deficiency sensitizes Ccm2\textsuperscript{+/-} mice to develop CCM lesions while Msh2 deficiency does not. This difference requires further study and may reflect key differences between CCM1 and CCM2 pathogenesis.

From multiple lines of evidence, the murine lesions observed in this model appear identical to those seen in CCM patients. The integrity of the endothelial cell
layer and other blood-brain barrier ultrastructure are often compromised in human CCM lesions, leaving gaps of almost a micron between endothelial cells (Wong et al., 2000, Clatterbuck et al., 2001, Tu et al., 2005). This has been proposed to be the etiology of the vascular leakage and hemorrhage associated with clinical symptoms of CCM disease. *In vitro* work has shown that while knockdown of KRIT1 by siRNA had no effect on tight junction protein expression, it did cause an increase in permeability of *in vitro* endothelial cell monolayers (Glading et al., 2007). Other *in vitro* and *in vivo* studies have supported an increase in permeability after loss of CCM protein expression (Whitehead et al., 2009, Stockton et al., 2010). The electron microscopy data provide clear evidence that the CCM lesions in this mouse model manifest the same ultrastructural defects in blood-brain barrier as human lesions, including gross gaps in endothelial layer integrity that account for increased permeability. But these preliminary results demonstrate apparently normal tight junctions between most endothelial cells in the cavern wall, consistent with normal *in vitro* tight junction protein expression.

This new model of CCM is the first means of studying the molecular mechanisms of lesion genesis and progression *in vivo*. Studies of early lesions have been limited in humans because typical MRI using gradient-echo or fast spin-echo protocols can only detect the largest lesions. More recently, susceptibility-weighted imaging (SWI) has been used to identify much smaller lesions and potential pre-lesions in sharp detail (de
Souza et al., 2008). Based on our work with this novel mouse model, it appears that the earliest lesions manifest far less iron deposit, cell proliferation, and inflammatory infiltrate, yet they already appear as grossly dilated capillaries with cavernous structure.

While late-stage CCM lesions from humans and mice show an increase in pMLC levels (Stockton et al., 2010), an indicator of ROCK activity, a modest but significant increase in pMLC is also observed in the early-stage lesion. The pMLC expression was significantly stronger in normal capillaries of animals harboring CCM lesions than in animals of the same genotype without lesions. We speculate that aberrant ROCK signaling in the CCM lesion may activate this pathway in apparently normal brain vasculature, potentially through cell-cell contacts or a secreted paracrine signaling factor.

### 3.3.2 The Knudsonian Two-Hit Mechanism

The two-hit mutational mechanism for CCM pathogenesis is supported by numerous clinical and molecular observations. Clinically, sporadic cases are characterized by solitary lesions, whereas autosomal dominant cases are characterized by multiple lesions (Rigamonti et al., 1988). The two-hit mechanism is also supported by clinical observations of increasing lesion burden throughout the life of humans with heterozygous germline mutations (Labauge et al., 2000, de Champfleur et al., 2010). In further support of this hypothesis, biallelic somatic mutations have been recently found in surgically-resected, mature lesion tissue from CCM patients (Gault et al., 2005, Akers
et al., 2009, Gault et al., 2009), as well as in sporadic lesions (see Chapter 2). We have made repeated attempts to identify somatic mutations in the murine lesions using the same approaches used in human familial CCM lesions (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009) and in human sporadic CCM lesions (see Chapter 2). Unfortunately, we have reached a technical limit in the analysis of the murine lesions, which are much smaller than the human lesions, resulting in a paucity of cellular material for molecular analysis. With increased rounds of amplification to generate sufficient DNA for the above sequencing techniques, distinguishing bona fide somatic mutations from PCR-induced errors became a serious problem. This remained true even when using next-generation DNA sequencing, as with deeper coverage the signal-to-noise distinction became even more problematic.

In lieu of DNA sequence data, the KRIT1 immunohistochemistry data show a mosaic lack of staining in the murine lesions as direct evidence of loss of expression, which indirectly suggests that somatic mutation occurred (Pagenstecher et al., 2009). The mosaic absence of KRIT1 protein from the early-stage lesions of Ccm1+/−Msh2−/− animals strongly suggests that lesion growth is initiated by loss of functional CCM protein. While this evidence is indirect, it supports the two-hit hypothesis as the genetic mechanism of the initial stages of CCM lesion genesis.
3.3.3 ROCK Inhibition Is the First Potential, Targeted Therapy for CCM

At present, there are no therapies specific for treating CCM. Treatments currently focus on mitigating patients’ symptoms, such as prescribing anti-epileptic drugs for seizures and pain medications for recurrent headaches. What is needed is a therapeutic strategy to stop, or even reverse, the growth of CCM lesions.

By targeting the ROCK pathway in these experiments, we have found the first potential therapy specifically for CCM. Fasudil treatment appears to reduce lesion maturation in our mouse models, as judged by significantly fewer late-stage CCM lesions in treated animals. The CCM lesions in fasudil-treated mice were smaller in size and lacked features of hemorrhage, B-cell infiltration, and endothelial cell proliferation present in more mature CCM lesions. The inhibition of pMLC and pMBS staining indicated effective blocking of ROCK activity by fasudil in the disease-target ECs lining CCM lesions. Fasudil treatment was well tolerated with both groups exhibiting comparable weights. The reduction in attrition in the Ccm1+/Msh2-/- group treated with fasudil indicates that some of the morbidity and mortality observed in these mice was due to the development of CCMs. The CCM2 and CCM3 mouse models did not show the same trend, but this may be due to higher rates of attrition of the Trp53-/- sensitizer mutation masking any beneficial effect of fasudil treatment.
These data represent the first report of therapeutic benefit of pharmacological intervention in CCM lesion growth \textit{in vivo} and indicate that ROCK inhibition is a potential therapy for CCM disease.

\section*{3.4 Future Directions}

Using these mouse models, it will be possible to study how apparently normal vessels can, over time, develop into large, multicavernous lesions that are the hallmark of CCM. Longitudinal studies with these mice can utilize \textit{in vivo} MRI techniques to track lesion dynamics. It will also be possible to identify mice with CCM lesions, treat them with ROCK inhibitors such as fasudil, and examine the effect of the drug on extant multicavernous lesions.

Other factors are undoubtedly involved in CCM pathogenesis. Multiple different signaling pathways have been identified \textit{in vitro} and \textit{in vivo}. While continuing to study ROCK inhibition therapy, it would be worthwhile to examine how inhibiting these other pathways affects lesion genesis and growth. Moving forward, it is uncertain if a single treatment would be sufficient to fully treat CCM or if multiple pharmacologic agents may be necessary. For instance, inhibition of ROCK or other pathways may be effective at early stages of disease development while anti-angiogenic, anti-proliferative, or anti-inflammatory strategies may be more effective at later stages of lesion maturation.
3.5 Materials and Methods

3.5.1 Mice

All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee. Mice were genotyped by PCR for Ccm1 (Whitehead et al., 2004), Ccm2 (Plummer et al., 2006), Ccm3 (He et al., 2010), Msh2 (Kucherlapati et al., 2010), Trp53 (Jackson Laboratory protocol), and Cre recombinase (Jackson Laboratory protocol). All alleles were maintained in a C57BL/6J background. Unless otherwise noted, upon sacrifice, the brain of each mouse was carefully removed and immediately immersed in 10% formalin for fixation.

3.5.2 MRI Protocol

MRI was conducted on formalin-fixed murine brains using either a 14.1T (600 MHz) or 9.4T (400 MHz) Bruker Avance imaging spectrometer. Three-dimensional-gradient recalled echo (T2*-weighted) images were acquired ex vivo as described previously (Shenkar et al., 2008). Analysis was blinded to treatment status.

3.5.3 Iron Staining and Immunohistochemistry

Chemical detection of iron deposits in mice with CCM lesions was performed by Perls’ Prussian stain method. Five millimeter sections of mouse brain were treated by a fresh solution of 20% hydrochloric acid and 10% potassium ferrocyanide, and counterstained with nuclear fast red. Adjacent sections were stained by immunohistochemistry for macrophages, B cells, plasma cells, and proliferation-
associated nuclear protein Ki67. The sections were treated for antigen retrieval using DAKO buffer (pH 6.0). Endogenous peroxidase was extinguished with 3% hydrogen peroxide. Staining was performed with the avidin-biotin complex technique using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The primary antibodies used were mouse monoclonal anti-CD11b (macrophage) antibody (eBioscience, rat IgG, 14-0112-81, 1:500), mouse monoclonal anti-B220 (B-cells) antibody (BD Biosciences, rat IgG, 550286, 1:500), mouse monoclonal anti-CD138 (plasma cells) antibody (BD Biosciences, rat IgG, 553712, 1:500), or mouse monoclonal anti-Ki67 antibody (DAKO, rat IgG, clone TEC-3, M7249, 1:75). A biotinylated rabbit anti-rat secondary antibody (Vector Labs, BA 4000. 1:50) was then administered. Negative controls were performed throughout the entire immunohistochemistry procedure.

Paraffin-embedded 5µm tissue sections of mouse cerebral lesion specimens were deparaffinized by serial washing first in xylene and then in ethanol dilutions (100, 95, and 70%). Endogenous peroxidase was neutralized by 3% hydrogen peroxide. Antigen retrieval was performed using citrate-buffered antigen retrieval solution (Vector Laboratories, Burlingame, CA). Sections were blocked using phosphate-buffered saline supplemented with 0.5% fish skin gelatin (PBS-FSGO, Sigma-Aldrich, St. Louis, MO) and 5% goat serum (Invitrogen, Carlsbad, CA), and then biotin-blocked (Vector Laboratories, Burlingame, CA). Slides were probed with rabbit polyclonal anti-KRIT1 6382 (Ginsberg Lab, UCSD; 1 mg/mL affinity purified, immunogen GST-tagged
recombinant protein corresponding to human KRIT1 FERM domain) at 1:250 in PBS-FSGO; rabbit polyclonal anti-CCM2 (Abnova, Taipei City, Taiwan; immunogen corresponds to full-length human CCM2 protein) at 1:250; rabbit polyclonal anti-pMLC (Thr^{180}/Ser^{19}, Cell Signaling Technology, Massachusetts) at 1:250, as previously described (Stockton et al., 2010), or; rabbit polyclonal anti-pMBS (MYPT1, Thr^{696}) at 1:250. Control sections were treated with 1.5 µg/mL of rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All primary antibody incubations were performed overnight at 4°C, covered, in a humidified chamber. Slides were then washed and probed with biotinylated goat anti-rabbit IgG (1:1000, Vector Laboratories, Burlingame, CA) overnight at 4°C in a humidified chamber, washed in PBS-FSGO, and incubated with Elite ABC kit reagents (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Color was developed using DAB substrate (3,3’-diaminobenzidine) for 5 minutes. Slides were washed and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA) for 1 minute, washed, sequentially dehydrated with absolute ethanol, then xylenes, mounted with Vectashield, and photographed using a bright-field microscope (Leica, Bannockburn, IL) with digital camera (Diagnostic Instruments, Sterling Heights, MI).

pMLC staining was assessed as “none” (completely absent of staining), “weak” (definite, but diffuse staining), or “strong” (intense and confluent staining) independently by two investigators. In order to analyze the pMLC staining data, a
Poisson distribution was assumed to construct an ordinal log linear model and a log link function. The significance of a linear-by-linear association was assessed by the Chi-square test using SAS 9.2 PROC GENMOD.

3.5.4 Transmission electron microscopy

Brains were carefully removed from the mice and immediately immersed in freshly prepared 4% paraformaldehyde. After 24 hours, the brains were sliced into 1mm coronal sections and observed for possible lesions under a dissecting microscope. Regions that contained suspected lesions were cut into 1mm cubes and placed into PBS at 4°C. Specimens were postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Representative areas of interest were identified by light microscopy after staining with Toluidine Blue. Sections were then stained with uranyl acetate and lead citrate, viewed on a Philips CM 10 transmission electron microscope, and images were captured using a Gatan ES1000W digital camera. Morphological changes were observed independently by two investigators.

3.5.5 Fasudil Treatment

Mice were treated with 100 mg/kg/day fasudil dissolved in their drinking water from weaning (21 days of age) until sacrifice (4-5 months of age). To determine the daily dose of fasudil consumed by the mice, the volume of fasudil solution that was consumed was measured over the course of a 24-hour period on multiple days. Male mice consumed 4.0mL ± 0.9mL and female mice consumed 4.0mL ± 0.5mL (no significant
difference). A subset of mice was also weighed at various time points during treatment. From these data, we calculated that the mice received an average dose of 109 mg/kg/day, very close to our attempt to provide 100 mg/kg/day.
4. Discussion & Future Directions

4.1 CCM Genes Converge on RhoA Pathway

The discovery of all three CCM proteins in a ternary complex (Zawistowski et al., 2005, Hilder et al., 2007, Voss et al., 2007, Zhang et al., 2007) was the first clear connection between the CCM genes. Aside from this interaction, each of the three CCM genes has its own unique set of interactors and pathways (summarized in Figure 19). CCM1 interacts with ICAP1 (Zawistowski et al., 2002, Liu et al., 2013), giving CCM1 a potential role in integrin and RAP1 signaling. CCM1 is also the only CCM protein to shuttle between the cytoplasm and the nucleus (Zawistowski et al., 2005), though any nuclear function has yet to be reported. CCM2 is the anchor of the CCM complex, binding to both CCM1 and CCM3 (Hilder et al., 2007). Meanwhile, CCM2 may also participate in a MAP kinase cascade through MEKK3 (Uhlik et al., 2003). CCM3 interacts with various GCKIII family kinases and may regulate striatin (Goudreault et al., 2009, Voss et al., 2009, Fidalgo et al., 2010, Zheng et al., 2010). The aggressive nature of disease in patients with CCM3 mutations (Denier et al., 2006, Gault J., 2006) possibly indicates that CCM3 has functions distinct from interacting with CCM1 and CCM2.

While there was an apparent physical connection between the CCM proteins, initially no common pathways emerged that would explain how mutations in the CCM genes cause CCM lesions.
Multiple investigators have found connections between the CCM proteins and the RhoA pathway. Knockdown of the CCM proteins in endothelial cell culture causes actin stress fibers to form (Glading et al., 2007, Crose et al., 2009, Whitehead et al., 2009, Stockton et al., 2010), similar to activating RhoA in vitro (Kimura et al., 1996). Further experiments showed that knockdown of the CCM proteins increases the activity of RhoA and its effector ROCK (Glading et al., 2007, Crose et al., 2009, Whitehead et al., 2009, Stockton et al., 2010, Zheng et al., 2010). For these reasons, we investigated the activity of ROCK in the CCM lesions from our mouse models. Not only was ROCK
activated in the lesion endothelium, but it appeared that ROCK activity increases with the development and maturation of the lesion (McDonald et al., 2011).

Inhibiting ROCK in our mouse models showed a remarkable decrease in lesion burden, particularly in the late-stage, multicavernous lesions (McDonald et al., 2012). While the penetrance of lesions in our Ccm2+/Trp53-/- model may limit the power of measuring a decrease in lesions, the Ccm1+/Msh2-/- and Ccm3+/Trp53-/- models both showed large decreases in late-stage lesions. These data indicate that while the CCM proteins may have unique functions, they all converge downstream on the RhoA pathway. RhoA and ROCK, then, are the optimum targets for developing the first wave of therapeutic pharmaceuticals. Compounds that inhibit this pathway could feasibly be used in all CCM patients, including both sporadic and familial forms of the disease. The promiscuity of the CCM proteins also bears the potential for developing therapies tailored to familial patients depending on which gene is affected. For instance, in a patient with a CCM1 mutation, the standard therapy may be ROCK inhibition (through fasudil or another compound) plus an agonist/antagonist of the pathways downstream of integrin β1. In a patient with a CCM3 mutation, ROCK inhibition therapy may be combined with a compound affecting GCKIII family kinases. Such treatments require additional insight into CCM pathogenesis and the individual functions of the CCM proteins.
In addition to fasudil and a handful of other compounds, the drug simvastatin also inhibits ROCK activity. Simvastatin is a member of the statin family of drugs that are approved by the Food and Drug Administration to treat dyslipidemia. As such, the drug prevents cholesterol biosynthesis by inhibiting the HMG-CoA reductase enzyme. By blocking this pathway, simvastatin prevents the isoprenylation of ROCK (Park et al., 2002, Zeng et al., 2005), a posttranslational modification that targets proteins to cell membranes. Without an isoprenyl group to anchor ROCK to the membrane, its function is greatly diminished. Simvastatin should be assessed for effectiveness in mouse models of CCM. Since the drug is already in common use in the United States, the approval process for using simvastatin in CCM would be much faster and simpler than starting with a non-approved drug such as fasudil. In addition, there is currently a retrospective study under way to analyze CCM patient records for beneficial effects of statin use. Comparing statin-free patients to patients prescribed statins (for dyslipidemia) will examine the effectiveness of this class of drugs on clinically relevant outcomes such as lesion burden and incidence of stroke. We are currently treating our mouse models with simvastatin to test the efficacy of this drug in reducing lesion burden.

While lesions appear to be seeded by somatic mutations, the mechanism of lesion growth is not well understood. In our mouse models, the normal brain capillaries of mice with lesions showed higher ROCK activity than normal vessels in mice with the same genotype but no lesions. From this observation, we can conclude that increased
ROCK activity in the brain vasculature may be necessary, but not sufficient, to cause CCM lesion pathogenesis.

I further hypothesize that CCM lesions develop in two phases: initial capillary dilation (forming a single cavern) and subsequent remodeling (forming the classic multicavernous structure). The initial capillary dilation is likely a direct result of somatic inactivation of one of the CCM proteins (or potentially a trans-heterozygous loss). The RhoA pathway is initially activated in the affected endothelial cells, causing remodeling of the actin cytoskeleton and growth of the capillary. The remodeling of this capillary into multiple caverns may require a molecular “switch,” such as a particular signaling molecule (e.g. VEGF). Another possibility is evident from the progressive increase in ROCK activity in CCM lesions, which may demonstrate a positive feedback loop in the RhoA pathway upon loss of a CCM protein. RhoA and ROCK activity may reach a tipping point above which endothelial cells form clusters of caverns.

To test this hypothesis, transgenic mice could be developed to inducibly express ROCK in a tissue specific manner. Upon inducing ROCK expression in the cerebral endothelium, I would expect to find no CCM lesions in a wild-type background, but in a Ccm heterozygous background increased ROCK expression would cause more late-stage CCM lesions to form. Treating these mice with fasudil to reduce ROCK activity could be used to confirm the centrality of the RhoA pathway in disease pathogenesis. In the reverse experiment, ROCK knockout alleles could be crossed into our current mouse
models. In these mice, I would expect a reduction in lesion burden. Drawbacks from these experiments would be in the development of these ROCK mouse models: inducible ROCK alleles may prove lethal during development; though ROCK knockout alleles exist, homozygotes experience some morbidity (Thumkeo et al., 2003), and; the breeding scheme to produce Ccm3+/Trp53-/Rock2-/ mice would be complex and costly.

4.2 Non-genetic “Hits” Involved in CCM Pathogenesis

Based on the data presented here of somatic mutations within sporadic CCM lesions as well as previous studies from human familial lesions (Gault et al., 2005, Akers et al., 2009, Gault et al., 2009, Pagenstecher et al., 2009), it is clear that CCM follows a two-hit mutation mechanism. Our mouse models further support this claim because the mice only show CCM lesions when they are genetically sensitized with an increased rate of somatic mutations (Shenkar et al., 2008, McDonald et al., 2011, McDonald et al., 2012).

Despite this extensive evidence, it is still unknown if loss of both functional copies of a CCM gene is sufficient to cause lesions to form or if other factors are involved. In addition to the two-hit mechanism, other non-genetic “hits” may affect lesion pathogenesis. Knowledge of how these factors are involved in lesion genesis or growth would inform the development of therapies to treat and prevent CCMs. As discussed in Section 4.1, ROCK activity may be a major determinant of CCM lesion growth, but other “hits” still require further investigation.
An interesting recent discovery in human CCM lesions is evidence of an oligoclonal immune response where antibodies appear to recognize an antigen specific to the lesion (Shi et al., 2007, Shi et al., 2009). The target antigen is still under investigation, but the fact that the immune system reacts specifically to CCM lesions may indicate that an immune response or inflammation is a non-genetic determinant of CCM pathogenesis. To test this hypothesis, future experiments should focus on inhibiting immune or inflammation pathways through knockout mice, pharmacologic inhibition, or antibody infusion (Lee et al., 2006) to examine the effects of these processes on lesion genesis and maturation.

Conditional knockout mouse models of CCM have been developed using inducible Cre-lox technology (Boulday et al., 2009, He et al., 2010, Boulday et al., 2011, Chan et al., 2011, Cunningham et al., 2011, Louvi et al., 2011). These mice develop CCM lesions with a high penetrance within weeks of inducing Cre recombinase, though in some models the lesions are localized to a particular region of the brain. For example, under control of the Cdh5 (cadherin 5) promoter, Cre-mediated knockout of Ccm2 caused the formation of lesions exclusively in the cerebellum (Boulday et al., 2011). One interesting conclusion from these studies is that the timing of the somatic mutation is important in generating CCM lesions. Mice that are injected with tamoxifen (which induces Cre recombinase expression) starting at P0 (day of birth) generate CCM lesions. After the first few days of life, however, injection with tamoxifen yields no CCM lesions.
During postnatal mouse development, the brain vasculature undergoes extensive angiogenesis. The results with the conditional knockout mice imply that periods of active angiogenesis may be required for CCM lesion growth. One group has found associations between a vascular endothelial growth factor (VEGF) receptor and CCM3 (He et al., 2010), so it is possible that VEGF-induced angiogenesis is involved in lesion pathogenesis.

As lesions grow and remodel, they become more permeable. It is thought that this increased permeability, with various blood components crossing the blood-brain barrier into neural tissue, is the cause of many clinical symptoms such as seizures and focal neurological deficits. The body likely responds to CCM lesions in a manner similar to other damaged blood vessels, such as recruitment of immune cells and inflammation. In a mature, multicavernous CCM lesion, it is difficult to differentiate between factors causing lesion growth (non-genetic “hits”) and the results of vascular hemorrhage. Further mechanistic evaluation of inflammation, immune response, and angiogenesis is required in order to establish their role in CCM lesion pathogenesis.

In sporadic CCM patients, lesions are often observed near a developmental venous anomaly (DVA) (Hong et al., 2010). Whereas the general population incidence of DVA is 0.05-2.56%, the incidence in the CCM population is 8-33%. Not only are CCMs more often associated with a DVA, but these lesions may be prone to increased hemorrhage. The rate of hemorrhage is 38% of CCM patients, but if there is a nearby
DVA, that rate increases to 62-93% (Aboian et al., 2009). Mechanistically, it is not clear if the presence of a DVA increases the somatic mutation rate of the surrounding tissue (increasing lesion genesis), if the DVA sensitizes that region of the brain to CCMs by locally increasing RhoA (increasing lesion growth and maturation), or if the DVA exposes the nearby tissue to increased levels of non-genetic “hits” (also increasing lesion growth and maturation). No reliable mouse models of DVA exist, so it is currently difficult to examine the effects of DVA on lesion pathogenesis, but it is clear that this angioarchitectural anomaly (presumably non-genetic in nature) can directly affect CCM.

### 4.3 CCM, Cancer, and Radiation

CCM bears some similarities to cancer. Chief among these is the abnormal growth of CCM lesions. Additionally, after the surgical resection of a lesion, a new lesion can later appear at the same stereotactic location, potentially seeded by mutant endothelial cells that were not removed. As yet, unlike cancer, there is no evidence of local or distal metastasis.

Other connections between CCM and cancer have arisen from recent clinical case reports. In one report a symptomatic CCM appeared after irradiation of the brain, followed by subsequent growth of a glioma and a meningioma (Kamide et al., 2010). Another study reported an astrocytoma arising after a CCM was removed by gamma-knife radiotherapy (Zhang et al., 2012). Further anecdotal reports note a seemingly high incidence of brain tumors of various types in CCM3 patients.
The potential carcinogenic effect of CCM in these cases is confounded by the use of radiation therapies. While there are no direct reports linking radiation with CCM, it is reasonable that irradiation of the brain would induce CCM gene somatic mutations within endothelial cells. By irradiating Ccm heterozygous mice, it should be possible to test this hypothesis. With what has been learned about the developmental timing of somatic mutations from conditional mouse models, it may be necessary to irradiate neonatal mice to see an effect on CCM lesion burden. We are currently performing these studies on cohorts of Ccm1+/−, Ccm2+/−, and Ccm3+/− mice as well as wild-type littermate controls. If irradiation can seed the growth of CCM lesions, then radiotherapies for CCM or cancerous tumors could, in fact, cause somatic mutations and additional CCM lesions.

Taking this idea a step further, it is possible that any environmental or genetic factor that induces somatic mutations could seed the growth of CCM lesions, particularly in individuals with an inherited germline mutation. One study has found that CCM1 can regulate the levels of intracellular reactive oxygen species (Goitre et al., 2010), agents of oxidative damage to DNA. Even without a direct role in oxidative stress pathways, individuals with germline CCM mutations may benefit from increased antioxidant intake, potentially preventing somatic mutations from occurring and leading to the development of lesions.
4.4 CCM as a Cell-Cell Junction Disease

One of the major unanswered questions in the CCM field is why do lesions chiefly appear in the vasculature of the central nervous system (CNS)? In the CNS, the cell-cell junctions between vascular endothelial cells are less permeable than in other organ systems, creating the blood-brain barrier (BBB). The BBB is formed and maintained through multiple cell-cell junction complexes including adherens junctions and tight junctions. In CCM, the endothelium of lesions is severely disrupted, often showing large gaps between endothelial cells (Wong et al., 2000, Clatterbuck et al., 2001, Tu et al., 2005).

From these observations, we can surmise that the CCM proteins have an important function in maintaining the BBB within the CNS. Somatic mutations occur specifically within endothelial cells (Akers et al., 2009, Gault et al., 2009), implicating that CCM pathogenesis originates in this cell type. While most mouse models have confirmed this endothelial specificity (Boulday et al., 2009, He et al., 2010, Boulday et al., 2011, Chan et al., 2011, Cunningham et al., 2011), one group has found that somatic inactivation of Ccm3 in neurons or glia severely altered brain cytoarchitecture (Louvi et al., 2011). Since most CCM mutant mice (and endothelial-specific knockouts) present with vasculature defects, the brain developmental abnormalities of this Ccm3 mouse model demonstrate a possible role for CCM3 in neurons and astrocytes. Indeed, the vascular unit of the brain is a unique compartment where endothelial cells, neurons,
astrocytes, and pericytes all interact. The specificity of CCM lesions to the CNS may reflect a role for the CCM proteins in cell-cell junctions between these cell types.

Mutations in tight junction proteins have been reported for multiple syndromic diseases, including familial hypercholanemia (Carlton et al., 2003), autosomal recessive deafness (Wilcox et al., 2001, Riazuddin et al., 2006), and Nance-Horan syndrome (Burdon et al., 2003). Each of these maladies affects particular subsets of cells, likely reflecting the unique roles of the particular mutated tight junction gene. Furthermore, homozygous mutations in the tight junction gene JAM3 cause cerebral hemorrhage, leading to destruction of the brain parenchyma (Mochida et al., 2010). Granted, this disease differs from CCM in many ways, but they are similar in the cerebral hemorrhage phenotype. In addition to a brain vascular disease, then, CCM can potentially be viewed as a cell junction or BBB disease. Future work focusing on the dynamics of the BBB cell junction proteins may provide mechanistic insight as to the CCM proteins’ specific functions.
References


Glading, A., J. Han, R.A. Stockton, and M.H. Ginsberg. 2007. KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell junctions. *J. Cell Biol.* 179:247-254.


Petit, N., A. Blecon, C. Denier, and E. Tournier-Lasserve. 2006. Patterns of expression of the three cerebral cavernous malformation (CCM) genes during embryonic and postnatal brain development. *Gene Expr Patterns*


Biography

David Andrew McDonald was born on November 29, 1985, in Monroe, MI. He graduated summa cum laude from the University of Florida in 2008 with a B.S. in biochemistry. In early 2009, he joined Dr. Douglas Marchuk's lab at Duke University. While at Duke, David was selected for the James B. Duke Fellowship as well as two travel awards for the Angioma Alliance Pathobiology of CCM Workshop. In 2011, he received two predoctoral fellowships – one from the American Heart Association and one from the National Institute of Neurological Disorders and Stroke. He received his Ph.D. from the Duke University Program in Genetics & Genomics in March 2013.

David participated in the Preparing Future Faculty program as well as the Duke Scholars in Molecular Medicine program. He is currently a trainee member of the American Society of Human Genetics and the North American Vascular Biology Organization, as well as a reviewer for the science education journal Syllabus.

Publications:


