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CCM2 Molecular Signaling Pathway

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CCM2 MOLECULAR SIGNALING PATHWAY

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Cerebral cavernous malformations (CCM) are a central nervous system vascular malformation often responsible for hemorrhagic strokes. Molecular genetic studies have identified three genes (CCM1 KRIT1, CCM2 Malcavernin, and CCM3 PDCD10) and four possible loci responsible for the pathogenesis of these lesions. CCM1 functions through integrin signaling and regulation of RAC1 activity and may be involved in the MAPK and JNK signaling cascades. We hypothesized that CCM2 likely functioned through the same pathways and that CCM3 expression is regulated by these stress-induced signaling cascades.

We showed that CCM2 likely functions through the MAPK pathway as the mouse homolog, osmosensing scaffold protein for MEKK3 (OSM), has been shown to interact in the p38 mitogen activated protein kinase (p38 MAPK) signaling pathway regulated by RAC1. We confirmed that CCM1 and CCM2 signal as a complex since co-immunoprecipitation indicates joint expression. We also characterized the role of CCM3 in the MAPK pathway by identifying interacting serine threonine kinases (STK) and KIAA0826 based on yeast two-hybrid data. This was further examined through immunohistochemical analysis showing CCM3 is expressed in a variety of human organs especially arterial vascular endothelium in a similar pattern to CCM2. The yeast two-hybrid data supports current theories that there is a link between CCM pathogenesis and the ERK-MAPK cascade. These findings correlate with previous studies and further elucidate the signaling pathways involved in CCM pathogenesis which may in turn be helpful in future therapeutic advances.

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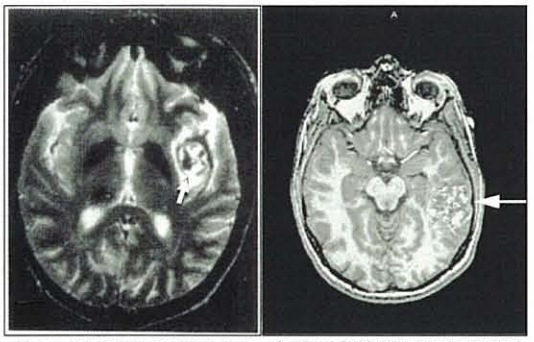
INTRODUCTION

Stroke is the third leading cause of death and a leading cause of disability in the US. The majority of cerebral infarctions are ischemic in nature but hemorrhagic infarctions account for 20% of cerebral vascular accidents. The primary risk factors for hemorrhagic stroke include hypertension, coagulation disorders and cerebral vascular malformations. These factors are implicated in 5-7% of intracranial hemorrhages[1]. Vascular malformations are described by their size and location with the four most common categories being capillary telangectasias, cavernous malformations, venous malformations, and arteriovenous malformations (AVM)[2].

Clinical Aspects of Cerebral Cavernous Malformations

Cavernous malformations are the most commonly identified cerebral vascular malformation accounting for 8-15% of central nervous system vascular malformations[2]. An estimated 2 million Americans have CCMs and have a 50-70% risk of hemorrhage, epilepsy or neurological deficit during their lifetime[4]. Cerebral cavernous malformations (CCM) are found in 0.5% of the general population as sporadic (15-33% of patients) or familial (73%) lesions[3]. In general, sporadic CCMs are single lesions while the autosomal dominant form presents with multiple lesions. The sporadic CCM form tends to be asymptomatic, while the familial form exhibits both phenotypic and genetic heterogenecity[5].

CCMs are often an incidental finding on MRI appearing as a raspberry-like angioma or popcorn lesion[2] (Fig 1). CCMs are small hemorrhages isolated



Günel et al. PNAS. 1995; 92(14):6620–6624. Günel et al. PNAS 2002; 99(16):10677–10682.

Fig 1. MRI images with classic popcorn-like appearance of cerebral cavernous malformations. Arrows point to lesions.

to the location of the lesion with hemoglobin degradation products such as methemoglobin, hemosiderin and ferritin present[6]. These lesions are often dynamic in nature and the mechanism of growth is thought to be due to repeated microhemorrhages or recanalization after intraluminal thrombus[7]. Functional MRI imaging may become essential in the evaluation of CCM lesions[6, 8].

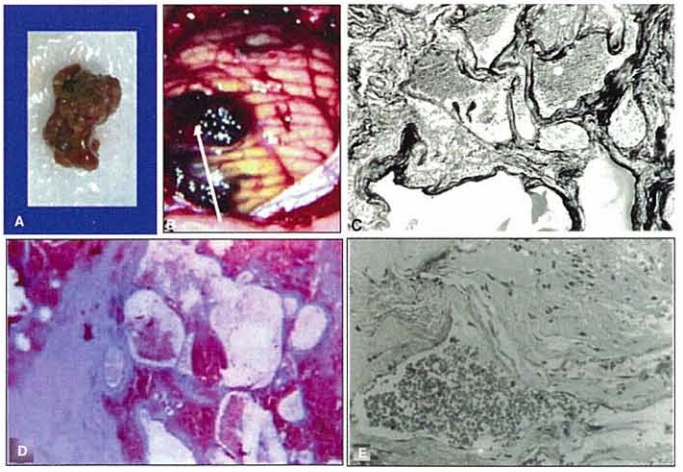


Fig 2. Histological images characterized by well circumscribed clusters of dilated thin-walled vessels that are surrounded by a gliotic rim. (A) Surgical specimen (http://www.brain-aneurysm.com/cm.html) (B) Intraoperative image of CCM marked by arrow with corresponding histology (D) (Neurosurg Focus 2006 American Assoctn Neurological Surgeons) (C), (E) Histology of CCM (Mazza et al. Child's Nerv Syst (1991) 7:139 146)

On histology, CCMs are 2-3mm to several centimeters in diameter and appear as clusters of well circumscribed, abnormally dilated thin-walled vessels surrounded by a gliotic ring (Fig 2). Clusters of sinusoidal vessels are often found outside these capsules[9]. The walls of CCMs lack elastin and smooth muscle cells and are lined by a single layer of endothelial cells[10]. The pathology develops as the vessel walls become distended because they lack elastin, thus slowing blood flow through the vascular space[3].

Cavernous malformations are located in the brain, spinal cord, skin, retinal vasculature, liver, and kidney. Cerebral cavernous malformations (CCMs) are dynamic and change in size over time ranging from 0.1 to 9cm and are usually located in the cortex. CCMs are most commonly found in the frontal and temporal lobes with 80-90% being supratentorial usually in the deep cerebral white matter, corticomedullary junction or basal ganglia. The most common posterior fossa sites include the pons and the cerebellar hemispheres[11].

Cerebral cavernomas bleed at a rate of 0.7-1.1% per year. There has not been significant data correlating CCM size with the risk of bleeding or neurological deficits. In approximately 8-44% of patients, CCMs are associated with other vascular malformations such as capillary telangiectasias[11]. The presence of more than one type of malformation is associated with increased risk of hemorrhage. CCM lesions prone to hemorrhage may have two hits, making them biallelic and creating two nonfunctional copies of a CCM gene[12, 13].

The clinical symptoms of CCM include recurrent headache, intractable seizures (40-50%), and hemorrhagic stroke (10-25%) which may lead to neurological deficits (20% of patients) without surgical resection of a symptomatic lesion[14]. Current treatment is resection of the symptomatic lesion and careful monitoring of small asymptomatic lesions[15]. Clinical studies have shown that patient age, gender, symptoms, localization and size of the cavernous malformations are important factors in determining timing of surgical resection[16, 17]. Preoperative imaging is necessary to determine the vascularity of the surrounding tissue. Cavernous malformations do not respond to conventional radiotherapy or radiosurgery and necessitate microsurgical resection[18].

Cavernous malformations provide an excellent genetic model for the study of familial stroke syndromes. There is significant evidence for autosomal dominant inheritance among monozygotic twins. Studies show that 10-20% of Caucasian CCM patients have a first degree relative with a cavernoma. A Dutch study showed that 10% of monozygotic twins were likely to suffer a fatal stroke as opposed to 5% of dizygotic twins[15]. Hispanic-Americans have a higher prevalence of CCMs than the average population, suggesting a founder effect[17, 19]. Affected individuals may share a common ancestor in Sorona County, Mexico and there are other "Italian-American" and French families who exhibit the similar linkages[20, 21]. The mechanism through which gene mutations produce CCMs is not clear. However, suggested models include a two-hit mechanism in which one

mutation occurs in somatic cells and one is inherited or two somatic mutations may occur[15].

Familial cases are due to mutations in one of three loci: CCM1 on chromosome 7q21.2, CCM2 on 7p15-p13, or CCM3 on 3q25.2-q27. These chromosomal abnormalities are associated with the genes KRIT1 or Krev1/Rap1A Interaction Trapped 1 (CCM1), MGC4607 or Malcavernin (CCM2), and PDCD10 or Programmed Cell Death 10 gene (CCM3). Normal levels of KRIT1, Malcav, and PDCD10 expression are necessary for angiogenesis. The three characterized CCM gene mutations are frameshift or nonsense mutations which result in premature truncation of protein products. CCM1 mutations are responsible for 40% of familial cavernous angiomas. CCM2 mutations account for 20% of cavernomas. The remaining 40% of CCM patients have lesions associated with CCM3, a fourth CCM mutation, or a combination of the above mutations[22].

Background and Expression of CCM1

The CCM1 gene KRIT1 was identified using yeast two-hybrid screening looking for proteins interacting with the bait Krev1/Rap1A mutations in Hispanic families[23]. The majority of Hispanic-American cases and 40% of total familial CCM cases are attributable to CCM1 mutations[24]. However recent evidence suggests that external factors in addition to the CCM1 germline mutation may be involved in phenotypic manifestation of the CCM pathology[25]. The fact that KRIT1

is expressed in many tissues throughout the body but CCMs are a pathology found only in the central nervous system further confirms this theory[26]. Approximately half of symptomatic CCM1 patients hemorrhage in comparison to 87% of patients with CCM2 or CCM3 familial lesions[23].

CCM1 encodes a 529aa protein with four ankryin domain which interact with RAP1A's carboxyterminal region[27]. RAP1A was isolated through its interaction with DRAS3 which is a *Drosophila melangogaster* homologue[28]. Ras signaling is thought to participate in angiogenesis as suggested by abnormal vascular development in mouse models[29, 30] and its interaction with B-Raf which, when deficient, initiates endothelial apoptosis in mice[30]. RAP1A also participates in morphogenesis and cell differentiation[31]. Recent studies have confirmed that RAP1A signal transduction pathway is involved in vasculogenesis or angiogenesis which also links CCM1 to this cellular function[32].

KRIT1 contains an ankyrin and FERM domain which implies a link between integrins and the actin cytoskeleton[3]. The interaction between KRIT1 and RAP1A supports a loss of function hypothesis for CCM development and a phenotypic 2-hit model. KRIT1 splice variants expressed early in angiogenesis interact with RasGTPases and microtubules. Integrin signaling plays an important role in CCM pathophysiolgy suggesting that KRIT1 regulates bidirectional signaling between the extracellular matrix and cellular cytoskeleton.

KRIT1 may also be involved in the communication between astrocytes and endothelial cells. The CCM phenotype is characterized by leaky CNS vessels, a fact which is supported by knowledge that GTPase KRIT1 is expressed in endothelial cells and localizes to astrocytic foot processes, suggesting a defect in blood brain barrier (BBB) formation[35].

Many signaling pathways have been recognized as potentially important in CCM pathogenesis. KRIT1 contains a NPXY domain which binds Integrin Cytoplasmic Domain Associated Protein-1α (ICAP-1α), which in turn is a binding partner of β-1 integrin[36]. Examination of the molecular function of at least one KRIT1 splice variant indicates expression during early angiogenesis and identified interaction with RasGTPases and microtubules. The Krev1/Rap1a gene ~ GTPase Ras KRIT1 interacts with ICAP1 which binds β-1 integrin and Rac1. The longer KRIT1 splice variant also interacts with Integrin Cytoplasmic Domain Associated Protein-1α (ICAP-1α), which is a binding partner of β-1 integrin. These observations implicate integrin signaling in CCM pathophysiology and further suggest that KRIT1 could mediate signaling between the extracellular matrix and the cellular cytoskeleton[24, 37]. This supports integrin signaling in CCM pathophysiology, implying that KRIT1 mediates signaling between the extracellular matrix and cellular cytoskeleton.

A KRIT1 -/- knock-out mouse model revealed that loss of the gene results in dilation of large vessels[33]. This deletion led to death in many of the knock-out

animals however it has been shown that mice heterozygous for KRIT1 did not develop the vascular lesions[26, 33]. Knock-out mice died at embryonic day 8.5 from closure of the dorsal aorta due to endothelial proliferation[34]. KRIT1 may function in Notch4-mediated arterial development, regulation of RAC1 activity and integrin signaling[3].

CCM1 exhibits predominately nuclear expression in response to stress and is found in the arterial endothelium of mammalian tissues[10]. Within the cerebral cortex, CCM1 is evident in the arterial endothelium as well as the astrocytic foot processes[24]. One clinical study found a family with several patients documenting a CCM1 vertebral lesion with cutaneous lesions present[22]. This is unusual as CCMs are predominately a CNS lesion.

Background and Expression of CCM2

The CCM2 gene MGC4607 has been correlated with 20% of familial CCM cases[38]. The murine homologue of CCM2, osmosensing scaffold protein (OSM), was identified as an interacting partner of Mitogen-activated protein kinase 3 (MEKK3) which activates ERK, JNK, and p38 MAPK signaling depending on the cell context and stimulus. There are two primary splice variants at 49 kDa and 84kDa[39].

CCM2 shares CCM1's expression pattern in mammalian tissues. Previous data has shown expression in brain, heart, lung, kidney, liver and spleen. More specifically, CCM2 is expressed in the arterial endothelium of the vasculature of these organs and is predominately cytoplasmic[40]. In the lungs, CCM2 is expressed in the capillary lining of the alveolar walls, bronchioles, columnar epithelium and epithelial cilia but not in the venous endothelium. Aortic endothelium expresses CCM2 as well. The myocardial and pericardial layers of the heart exhibit CCM2 expression in the arterial endothelium as well as the coronary arteries. Within the liver, CCM2 expression is evident in the hepatic artery and bile duct endothelium, as well as in hepatocytes. CCM2 is expressed in the endothelial lining of the blood-thymus barrier and splenic endothelium. Renal interconnected glomerular capillaries express CCM2 as well. Within the cerebral cortex, CCM2 is expressed in the cytoplasm of the pyramidal neurons and glial cells especially in the astrocytic foot processes[40].

The MGC4607 gene is responsible for CCM2 lesions. A yeast two-hybrid screen of T-cell cDNA library using the CCM2 murine homologue, OSM, as bait identified Mitogen-activated protein kinase 3 (MEKK3) as a binding partner. MEKK3 activates the ERK, JNK, and p38 MAPK signaling pathways. The activation of a specific pathway by MEKK3 is dependent of the cell line and stimulus used. Following hyperosmotic shock, OSM induces p38 expression through the MAPK signaling pathway as a scaffold protein.

CCM2 also interacts with ICAP-1 which interacts with the RhoGTPases and β1integrin which are important in cytoskeleton. CCM2 and ICAP1 bound to CCM1 via PTB domains may influence the subcellular localization of CCM1 Further, recent evidence suggests that CCM2 and CCM1 interacts with MEKK3 in a complex[41]. This suggests that CCM1 and CCM2 may interact as a complex regulating RAC1 which activates the MAPK cascade.

Background and Expression of CCM3

The CCM3 gene PDCD10 was discovered through examination of a large deletion in a CCM family. The PDCD10 gene expression was found to be up-regulated in TF-1 premyeloid cell line after growth factor deprivation and a fibroblast cell line after induction of apoptosis[42, 43]. PDCD10 is highly conserved in vertebrates and invertebrates[43]. PDCD10 is upregulated during apoptosis in a human premyeloid cell line and may function in regulation of cell proliferation and apoptosis[3]. Knockdown of the *C. elegans* homolog of PDCD10 (2K806) results in increased embryonic lethality although the biological functions of PDCD10 have not been fully characterized. Knockdown *C.elegans* survivors exhibit a shorter and fatter phenotype.

In contrast to some studies which have shown that up to 50% of CCM1 lesions may be asymptomatic, CCM3 lesions are highly symptomatic[26]. Patients with CCM3 lesions present with symptoms at a younger age and exhibit a higher risk

of cerebral hemorrhage, especially during childhood[44]. Several families have been identified with this mutation and the cellular function of PDCD10 in relation to angiogenesis and cerebral vessel remodeling is still being investigated[43].

Background and Expression of CCM4

The remaining 40% of CCM mutations were formerly all associated with CCM3 mutations, however more recently a fourth locus was identified on chromosome 3q26.3-27.2 that appears to be unique from CCM3 on 3q25.2-q27[22, 45, 46]. The proximity of these two loci to one another may make it difficult to link this new locus to a novel protein. The K2015 family has a strong link to the PDCD10 locus. A recent study has shown a distinct recombination event between D353053 and GATAB1H05 which excludes the PDCD10 gene indicating the presence of a fourth mutation[46].

MAPK Pathway and Serine Threonine Kinases

Although the mechanisms of cerebral angiogenesis are not understood, the MAPK and JNK pathways have been implicated in cerebral angiogenesis. The molecular mechanism behind CCM pathology has not been identified although three transcripts and protein products have been characterized.

The MAPK signaling cascade has been shown to be activated by environmental stressors such as osmotic changes which can result in various pathologies depending upon the organ system involved. In the eye, corneal epithelium exposed to hypertonic states results in decreased cell proliferation[47]. Osmotic stress has also been implicated in the activation of the TAK1-JNK pathway which is mediated by NF-κB activation[48]. The CCM proteins have been shown to interact with the highly conserved Ras proteins that act as central regulators for several pathways in response to stress[49].

Mitogen activated protein kinase (MAPK) family members are responsible for the maintenance of cells. MAPK regulates apoptosis in response to external stress signals[50]. The MAPK cascades are critical in using extracellular stimuli for intracellular signaling. There are three well known MAPK subfamilies, the extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and the p38 mitogen activated protein kinase (MAPK) signaling cascades. ERK signaling acts against Rho-kinases to promote endothelial cell survival and sprouting during angiogenesis[51]. RAS-RAF-MEK-ERK signaling is thought to be important in tumor angiogenesis or cell proliferation in pathologies such as CCMs[52]. JNK and p38 MAPK are the primary stress-induced cellular responses best documented through osmotic cellular changes. These two pathways are also responsible for apoptosis and the removal of dysfunctional cells. NF-κB is an activator of cell survival signaling[53]. Proinflammatory cytokines such as TNF activate both NF-κB and the JNK pathways through TAK1 which is a

transforming growth factor-β-activated kinase thought to be regulated by osmotic stress[54, 55].

Tumor necrosis factor receptor-associated factors (TRAF) are multifunctional signal adaptors which activate kinase cascades such as c-Jun N-terminal kinase, p38 mitogen activated protein kinase and the transcription factor NF-κB[56]. There are six identified TRAFs[57]. The JNK pathway is initiated by mediators such as TNF- α and IL-1 interacting with TRAF-6. TRAFs act as protein scaffolds and link the TRAF domain to the IL-1 receptor pathways in order to regulate apoptosis and cytokine signaling[58]. Serine/threonine kinases are known to regulate the NF-κB pathway after TNF-α and IL-1 cytokine activation[57, 59]. One study examined astrocytes infected with *Escherichia coli*, which causes inflammation such as meningitis or an abscess. Examination of proinflammatory mediators indicated that TNF- α and other participates in the NF-κB pathway may contribute to inflammation in the brain[55]. Another study found that the NF-κB pathway in epithelial and lymphocytic cells was suppressed with adenosine which is an endogenous immunomodulator that has anti-inflammatory and immunosuppressive properties[60].

The six TRAF proteins each have a different role in cellular responses such as the activation of apoptosis or promotion of cell survival. TRAF-1 is associated with lipid rafts which participate in apoptosis in the traumatized brain and neurogenerative diseases[61]. TRAF-2 activates the NF-κB pathway in order to

promote cell survival[53, 62, 63]. TRAF-2 has a splice variant with an extended RING finger domain which inhibits this activation as well thus providing its own direct feedback[64]. TRAF-3 also participates in apoptosis[65]. TRAF-4 expression has been shown to be critical to the ontogenesis of the CNS and PNS during mouse embryogenesis and is expressed intracellularly in the adult hippocampus and olfactory bulb which are active in neoneurogenesis[66, 67]. TRAF-6 mediates signaling from the amino-terminal domain of CD40 signaling. TRAF-6 activates NF-κB and function independently of the other TRAF family proteins[68]. Thus TRAF-6 directly induces angiogenesis by activating NF-κB and c-Jun N-terminal kinase[69]. It is interesting to note that lipopolysaccharide (LPS) initiates TRAF-6 mediated endothelial survival[70].

There are 28 members of the Ste20 kinase group in humans with homologues in drosophila and *Caenorhabditis elegans*[71]. The Ste20 kinases respond to osmotic stress to initiate signaling cascades[72]. The biological function of mammalian Ste20 kinases (putative yeast mitogen-activated protein kinases kinases kinase kinase, or MAP4k) is largely unknown but they are known to be germinal center kinases that are involved in mating pathways[73]. Ste20 kinases are critical to regulation of the cell cycle, apoptosis, and stress responses. Recent data suggests that Ste20 kinases also participate in cell volume sensing and Cl- transport regulation[74]. As upstream activators of the MAPK cascade the Ste20 family regulates apoptosis, morphogenesis and cytoskeletal rearrangements and thus may be implicated in pathophysiology of many human diseases[71].

Ste20 is a PAK and germinal center kinase (GCK) family member[74]. Ste20 is also a p21-activated kinase (PAK) family member which is a MAP4K for three yeast MAPK cascades. Ste20 group kinases are upstream activators of MAPK which serve to regulate transcription. The Ste20 N-terminus noncatalytic domain binds to and inactivates its kinase domain. Once GTP Cdc42 binds to the CRIB domain of the N-terminus the Ste20 kinase is activated[75]. Deletion of the N-terminal domain is a dominant gain-of-function allele[76].

C. *elegans* is a good model for studying the stress activated signaling cascades responsible for apoptosis and cell survival. The loss of GLA-3 in results in increased apoptosis. GLA-3 has been shown to participate in the MAPK signaling pathway by interacting with MPK-1 which is a meiotic regulator. Thus GLA-3 acts as a negative regulator of the MAPK pathway. MPK-1 is the C. *elegans* homolog of the mammalian ERK/2 and serine/threonine kinases and is required for germline development. Thus C. *elegans* organisms lacking MPK-1 exhibit sterility and increased germ cell apoptosis[77].

The yeast two-hybrid assay is an effective tool for recognizing protein-protein interactions. The mating pathway in the *Saccharomyces cerevisiae* is one of the best understood eukaryotic signal transduction pathways. The mating signal is carried from the plasma membrane to the nucleus via G-protein coupled receptors and the mitogen-activated protein kinase (MAPK) signaling cascade.

The MAPK pathway is regulated by scaffold proteins and the dephosphorylation of kinases which control the pathway via negative feedback based on concentrations. Once the G-protein is activated, it initiates the MAPK cascade by conveying a signal to effectors in the transmembrane scaffold protein Ste5 (serine/threonine kinase) which activates Ste11 (MAPKKK), Ste7 (MAPKK), and phosphorylates Fus3 (MAPK). MAPK voyages to the nucleus and delivers the signal to downstream effectors which arrest the cell cycle in the G1 phase to allow for mating. Thus the yeast mating system is itself a model for the MAPK cascade and mating yeast strains which hold different proteins allows the researcher to determine whether the two proteins interact[78].

The Cre/lox system allows for inducible gene targeting for the expression or ablation of any gene to any tissue at a defined time in transgenic mice. This allows one to build novel chromosomes and further explore mammalian development[79]. While C.*elegans* and yeast provide models for identification of mutant genes and protein interactions, the mouse offers a valuable mammalian genetic model[80].

Based on the recent observation that the mouse homolog of CCM2, osmosensing scaffold protein for MEKK3 (OSM), participates in the p38 mitogen activated protein kinase (p38 MAPK) signaling pathway, it has been hypothesized that human CCM molecules might signal through this pathway as well. Both CCM1 and CCM2 have been shown to signal through the p38 MAPK

as well as the c-Jun NH2-terminal kinase (JNK). Together with the confirmation of MAPK involvement in CCM signaling in humans, Ras GTPase activating proteins may also play a role in CCM pathogenesis by modulating the MAPK signaling cascades through direct association with CCM2. We sought to prove this interaction using a yeast two-hybrid assay. The RasGAP proteins identified as binding partners of CCM2 are predominantly expressed in the CNS which may explain why CCM pathology is restricted to the CNS although the proteins are expressed throughout mammalian tissues.

CCM1 has been shown to regulate β1 integrin-mediated cell adhesion through ICAP1. CCM1 may regulate RAC1 activity through ICAP1 guanine dissociation inhibitor activity and CCM2 acts as a scaffold for RAC1/MEKK3/MKK3 which activates p38 through phosphorylation of MAPK. This relationship suggests the possibility that CCM1 and CCM2 may act together as a complex[3]. There is evidence that interaction between CCM1/CCM2 is prevented by a CCM2 missense mutation. This supports theories that CCM lesion pathogenesis may be linked to the interaction between CCM1 and CCM2 [41].

The aim of this project was to elucidate the relationship between CCM1 and CCM2 in the activation of the p38 MAPK signaling cascade and to further characterize CCM3 by analyzing expression in extracerebral and cerebral tissues in relation to CCM1 and CCM2. The molecular mechanisms underlying CCM pathophysiology are largely unknown. We sought to demonstrate that CCM1

and CCM2 function as a complex to activate the p38 MAPK and c-Jun NH2-terminal kinase signaling pathways and that CCM3 may signal through the p38 MAPK cascade. We also sought to confirm the interaction between CCM3 and serine threonine kinase binding partners identified through the yeast two-hybrid assay in order to understand the role of CCM3 in the activation of molecular pathways responsible for CCM pathology.

MATERIALS AND METHODS

In Vitro Cultures

Human umbilical vein endothelial cells (HUEVC) were cultured in M199 media supplemented with 20% FBS, 200mmol/mL L-Glutamine, 1% Penstrep (Invitrogen), and endothelial cell growth supplement.

Neuro2A (N2A) cells were cultured in MEM with Earle's BSS and supplemented with 10% FBS, L-glutamine, sodium pyruvate and non-essential amino acids. N2A cells were purchased from ATCC

COS7 cells were grown in low glucose DMEM (GIBCO/BRL) with 10% FBS and 1% antibiotic-antimycotic. Cells were grown to confluence on 100x 15mm Petri dishes and harvested for protein for Western blot analysis and co-immunoprecipitation.

Pre-designed and annealed siRNAs (CCM1 ID#15655; CCM2 ID#33399; CCM3 ID# 19312; Negative Control ID#4611) were purchased from Ambio. siRNAs were transfected into cells using Oligofectamine (Invitrogen) at a final concentration of 100nM in OptiMEM (Invitrogen) per the manufacurer's instructions. Following initial transfection, cells were cultured in normal serum media for 4 hours and re-transfected with siRNA at 100nM concentration and then cultured for an additional 24 hours prior to harvesting for subsequent analysis.

Co-immunoprecipitation

COS7 cells were transfected at 90% confluence with 4ug of CCM1, CCM2 cDNA in pDEST52 vectors following the lipofectamine 2000 protocol. COS7 cells were also co-transfected with 2ug of CCM1 and CCM2. Cells were incubated 36 hours at 37°C and cell lysates were collected using 1X Igepal CA-630 Co-IP buffer. Lysates were precleared with Protein A-Agarose beads (Pierce) and spun down at 4000rpm for 4min at 4°C. The supernatant was incubated on a nutator overnight at 4°C. This product was combined with Protein A-Agarose beads at 4°C for 1 hour and centrifuged at 4000rpm. The supernatant was washed with 1X Co-IP lysis buffer, boiled and fractionated on a SDS-PAGE and transferred. via western blot.

CCM3 Antibody characterization

A CCM3 specific antibody was synthesized and affinity purified by Zymed Laboratories Inc. (San Francisco, CA). The specificity of CCM3 antibody was tested by transfecting COS7 cells with an expression vector encoding a CCM3-GFP fusion protein. Full length CCM3 gene was cloned into pcDNA-DEST53 plamsid (Invitrogen, Carisbad, CA) and COS7 cells were transfected with either this construct as the positive control or Lipofectamine (Invitrogen, Carisbad, CA) as the negative control.

The transfected cells were lysed and products fractionated with SDS/PAGE. The protein samples were run on 10% Tris-HCL gels (BIO-RAD, Hercules, CA) for

fractionation by electrophoresis followed by transfer to PVDF membrane. Blots were blocked in 5% nonfat dried milk in PBS with 0.05% Tween 20. Blots were then incubated with a 1:1000 dilution of primary antibody overnight at 4°C followed by secondary incubation with horseradish perioxidase conjugated affinity purified donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc, West Grove, PA). Protein bands were visualized using electrochemical luminescence reagents (Amersham Biosciences, Piscataway, NJ). For peptide competition assays, the primary antibody was incubated with twofold molar excess of the immunizing peptide prior to the primary incubation.

Analysis of CCM3 Expression in Mouse Tissue

In order to analyze CCM3 and CCM2 protein expression in various tissues, organs from a CD-1 mouse were homogenized and 15μg of the resulting protein lysates were analyzed by Western blot. Protein concentrations were determined using BSA standards and a Bradford Coomassie Protein Assay Kit from Pierce (Rockford, Il). Primary antibodies for Western blots were used at a 1:1000 dilution. Membranes were treated as stated above.

Tissue Collection

Sections of human organ tissue were obtained from the Department of Pathology at Yale University School of Medicine (Human Investigations Committee Protocol No, 7680). The tissues were fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned 0.5mm thick.

Immunohistochemical Analysis

Sections were incubated for two hours in a 60°C oven to deparaffinize and incubated in Xylene for 10 minutes followed by short incubations through an alcohol gradient: 100%, 90% and 70%. In order to optimize CCM3 antigen exposure, the slides were placed in 10mmol/mL citric acid solution and microwaved. A hydrophobic barrier was drawn around the tissue with ImmEdge Pen (Vector Laboratories, Inc, Burlingame, CA). Sections were then rinsed with 1xPBS, blocked in H_2O_2 for 20 minutes to minimize nonspecific staining, and rinsed again in 1xPBS. A visualization system kit from Lab Vision Corporation (Fremont, CA) was then utilized. The sections were incubated in Ultra V block solution for 10 minutes and without rinsing, a drop of primary antibody was added. The tissue was stained with the CCM3 antibody customized from Zymed and characterized as mentioned above. As a negative control, some sections were incubated in CCM1 or CCM2 antibody. After primary incubation the slides were washed in PBS and then incubated for 30 minutes with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories Inc, Burlingame, CA). The sections were then put through a series of PBS washes and strepdavidin was applied for 10 minutes followed by further PBS washes. The chromogenic reaction was induced with DAB substrate from Vector Laboratories, Inc. The reaction was terminated with tap water and the slides were counterstained with hematoxylin. The slides were then dehydrated, and mounted permanently and viewed under a light microscope.

Microarray Screening and Data Anaylsis

Total RNA from siRNA treated cells were extracted using QIA shredder followed by RNeasy kit per manufacturer's instructions (Qiagen). Isolated mRNA was processed for hybridization using 3DNA Array 900 kit (Genisphere) and applied to Operon OHU21K chips containing 21,000 70mer oligos. The results were read using image analysis software GenePix 5.0 and imported into GeneSpring 6.2 (Silicon Genetics, Redwood City, CA) for gene expression data analysis. To reduce the effect of outliers, the median values instead of the mean were used to calculate both Cy5 and Cy3 channel intensities. The intensity values were calculated by subtracting the local median background value from the median foreground value for each spot. LOWESS normalization using 20% of data for smoothing was performed to eliminate dye-related artifacts. The ratio of the normalized intensity (Cy5/Cy3) calculated by GeneSpring was used for expression analysis. Genes with average intensity values less than 1000 for both channels, or Cy5/Cy3 ratio greater than 0.67 but less than 1.5 (i.e. less than 50% regulated), were excluded in the subsequent statistical analysis. A one-sample t test using p-value cutoff 0.05 with multiple test correction (Benjamini and Hochberg false positive rate) adjustment to the p-value was applied to the distribution of natural logs of the ratios for each gene to determine if the gene was statistically differentially expressed.

Immunofluorescent Staining

CCM1 full length mouse cDNA sequence was cloned into pEGFP_N3 expression vector (Clontech) by inserting between HindIII(5') and XhoI(3') sites. A full length (1828bp) human CCM2 cDNA was cloned into the pOTB7 vector from Open Biosystems (MHS1011-60266) between the attB1 and attB2 site-specific recombinases. A mammalian expression vector containing full-length CCM2 cDNA in frame with the GFP gene to its N-terminus was then created using the Gateway Technology from Invitrogen with the use of an attR-containing pcDEST53 vector. The GFP-tagged constructs were transfected into mouse neuroblastoma (N2A) cells with Lipofectamine 2000 (Invitrogen) and cultured in OPTI-MEME for 24 hours.

The cells were treated as detailed with 0.2M Sorbitol or 10ng/ml TNFα (R&D Systems Inc) for 15 minutes at 37˚C and fixed in 4% PFA and blocked in 10% normal goat serum followed by incubation with primary antibody (p-p38 or pJNK) overnight. Cells were then washed in 1X PBS and incubated with Alexa Fluor 555-conjugated rabbit secondary antibody and washed in 1X PBS before mounting with DAPI-containing mounting medium (Vector). The fixed cells were visualized using a ZEISS Axioplan microscope and the images captured using the Axiovision software program.

Yeast Two-hybrid Screening

The ProQuest yeast two-hybrid system was used for this assay. For the CCM3 screening, the interaction was confirmed by cloning full length CCM3 cDNA sequence into a Clontech pGADT7 bait vector between the Xho and BamH1 sites. STK24, STK25 and KIAA0826 were cloned into a prey pGBKT7 vector between Not1 and Sal1 sites. The pGBKT7 constructs were transformed into AH109 yeast and the pGADT7 construct was transformed into Y187. These were confirmed via western blot and checked for self activation on appropriate dropout plates. The prey and bait were then mated overnight at 37°C. Constructs were plated onto –Leu, -Trp, -Ade/Leu/Trp, and –His/Leu/Trp/Ade drop out plates and incubated at 37°C for 3 to 4 days. Clones were selected on plates lacking leucine, tryptophan, and histidine in the presence of 50mM 3AT. 2% 5-fluoorotic acid plates were used for negative selection. Mated cultures were confirmed via PCR and sent for sequencing to KECK to confirm that successful mating.

In order to verify the protein interactions isolated by the yeast-two hybrid a reversal of prey and bait vectors was done. By cloning 10ng of full-length human CCM3 cDNA including 5' and 3' UTRs (Open Biosystem BC002506 MHS1011-59014) into a pDEST32 yeast expression pGADT7 bait vector (ProQuest yeast two-hybrid system-Gateway Technology, Invitrogen) between the attR1 and attR2 sites. This was set against a human fetal brain cDNA library (Invitrogen) which was inserted into the pEXP-AD502 prey vector between the attB1 and atttB2 sites. Clones were selected on plates lacking leucine, tryptophan, and

histidine in the presence of 50mM 3AT. For negative selection, 2% 5-fluoorotic acid plates were used. For the re-transformation assay, 10ng of CCM3 cDNA was co-transformed with 10ng of test DNA in the MaV203 host yeast strain (Invitrogen). Full-length CCM3 cDNA sequence was cloned into a Clontech pGADT7 "prey" vector. STK24 and STK25 were cloned into a pGBKT7 "bait" vector. The STK24 and STK25 plasmids were transformed into the yeast strain AH109 and the CCM3 prey was transformed in Y187 yeast strain. These two yeast strains were then mated in a confirmation yeast two hybrid reaction and plated on plates lacking leucine, tryptophan and histidine. For negative selection, combination plates were used lacking all three amino acids.

For the CCM2 yeast two-hybrid analysis the Proquest yeast two-hybrid system with Gateway technology was purchased from Invitrogen. The bait was a full length CCM2 cDNA sequence (IMAGE biosystems) including 5' and 3' UTRs was cloned into pDEST32 between attR1 and attR2 sites. A human fetal brain cDNA library (Invitrogen) was inserted into the pEXP-AD502 vector between the attB1 and attB2 sites. These clones were selected on plates lacking –His/-Leu/-Trp/-Ura in the presence of 50mM 3AT. A 0.2% 5Fluoorotic acid plate was used for negative selection. CCM2-AD was used as a negative control for the selection plate due to co-transformation of full length CCM2-DB with an empty vector containing GAL4AD domain. For the retransformation assay, 10ng of CCM2 cDNA was co-transformed with 10ng of test DNA in MaV203 host yeast strain (Invitrogen).

PCR of CCM3 and STK24, STK25 and KIAA0826

Yeast colonies were picked and grown up in YPD broth overnight and presence of the desired construct was tested by digesting the construct. The CCM3 constructs in the pGADT7 vector were digested with BamH1 and Xho1 while the STK24, STK25 and KIAA constructs in the pGBKT7 vector were digested with NOT1 and SAL1 overnight at 60˚. These products were run out on a 1% agarose gel and to check for bands at 1.2bp for STK24 and STK25 and 1.3bp for KIAA0826. These bands were excised from the gel and purified using gateway technology gel extraction and the KIAA0826, STK24 and STK25 yeast colonies were amplified using BDAdvantage enzyme in a 50ul reaction with matched primers and using KIAA0826 primers with sal1-tag forward (CCGGTCGACTACCAGAGCCTCTAGCTCCTGA) and not-tag reverse (CGGGCGGCCGCGTTTCTTTCCTAAAGGCCTGA), STK24 primers with sal1-tag forward (ccggtcgactgatggctcactccccggtgcagt) and not-tag reverse (CGGGCGGCCGCCAAATGCCAAAGGAATTTCA) and STK25 primers with sal1-tag forward (gcgccgtcgactagctcacctccggggattt) and not-tag reverse (GGCGCGGCCGCTTCCGTCCCCTATCTGAACA). CCM3 primers used bam-tag forward (ccgggatccGGATGACAATGGAAGAGATGAA) and xho-tag reverse (CGGCTCGAGCTTTCAGGCCACAGTTTTGA). Product was run out on 2% agarose gel with 100 basepair ladder. These products were sent to KECK for sequencing.

Gamze Tanriover and Arianne Boylan were both responsible for the immunohistochemical data. Dana Shin performed the CCM2 yeast two-hybrid and microarray assays. Arianne Boylan was responsible for all other data presented here.

RESULTS

Recent studies have shown that CCM1 and CCM2 activate p38 MAPK and JNK signaling pathways due to cellular and environmental stressors. We sought to confirm that CCM1 and CCM2 act as a complex to initiate p38 MAPK signaling through a co-immunoprecipitation. In order to address the role of CCM3 in the MAPK signaling and JNK cascades we used the yeast-two hybrid system to isolate proteins interacting with CCM3.

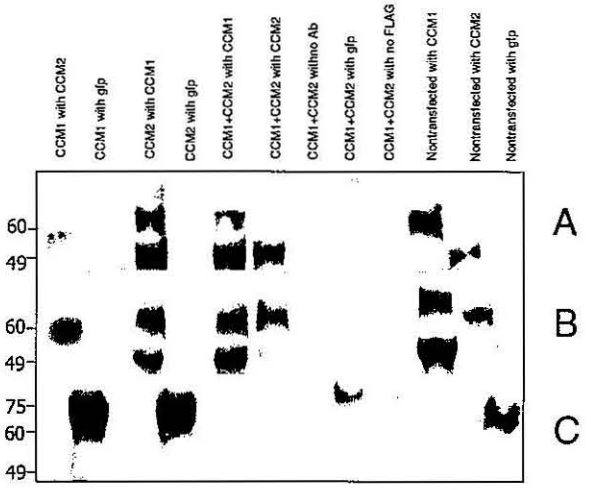


Fig 3. Coimmunoprecipitation with CCM1 and CCM2 transfected in COS7 cells indicates that the proteins are endogenously expressed in this cell line and express as a construct. GFP and FLAG antibodies were used as controls as GFP is endogenously expressed. CCM1 produces the expected band at 62kDa and CCM2 at 49KDa. The GFP bands were expected to be 75kDa. Blot A represents CCM1 primary antibody with an antirabbit secondary. Blot B represents CCM2 primary antibody with antirabbit secondary. Blot C represents antiGFP primary antibody with antimouse secondary.

CCM1 and CCM2 act as a complex in initiating p38MAPK cascade

The interaction between CCM1 and CCM2 was analyzed through a co-immunoprecipitation between the two proteins. COS7 cells were transfected with CCM1, CCM2 or co-transfected with both and exposed to either a CCM1 antibody, CCM2 antibody, or to no antibody. CCM constructs tagged with gfp and FLAG were used as controls in order to determine if the proteins were

endogenously expressed in this cell line (Fig 3). The COS7 cells incubated with CCM1 primary and anti-rabbit secondary antibodies produced the expected results. Under these conditions, COS7 cells transfected with CCM1 and pulled down with CCM2 antibody produced a band at 49kDa. Cells which were transfected with CCM1, CCM2, the CCM1/CCM2 co-transfection, or non-transfected cells were pulled down with gfp antibody did not produce a band. Cells transfected with CCM2 and pulled down with CCM1 antibody produced the expected band at 62kDa.

Non-transfected cells pulled down with CCM1 produced a band at 62kDa and non-transfected cells pulled down with CCM2 produced a band at 49kDa. Similar results were found for lysates which were incubated in a CCM2 primary and anti-rabbit secondary antibody. Lysates were incubated in an anti-gfp primary and anti-mouse secondary as a control. Only those cells which were pulled down with gfp produced a band at the expected band width of 75kDa (Fig 3). The lysates which were incubated with the anti-gfp and anti-mouse antibodies produce a band corresponding to gfp and not CCM1 or CCM2 showing that the co-immunoprecipitation accurately reflects interaction between the CCM protein and antibody.

CCM1 and CCM2 are involved in p38MAPK and JNK signaling

Microarray analysis of HUVEC cells after CCM1 and CCM2 knockdown indicated that CCM1 and CCM2 but not CCM3 are involved in the p38 MAPK and JNK signaling cascades. CCM1 and CCM2 silencing affected expression of several genes which have been associated with these stress-responsive signaling pathways. However, CCM3 knockdown did not affect the genes. This implies that CCM3 does not activate these signaling cascades (Table 1).

Accession #	CCM1	CCM2	CCM3	Gene	Description	MAPK
BC003525		2.24		MAX	MYC associated factor X	p38
NM_001540		-2.17		HSP27	Heat shock 27kD protein 1	p38
L08895	-2.05	2.16		MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	p38
NM_024117		-3.12		MAPKAP1	SAPK interacting protein 1	JNK
NM_053056	-3.55	-2.06		CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	JNK
NM_004672		2.99		MAP3K6	Mitogen-activated protein kinase kinase kinase 6 (ASK2)	JNK, p38
NM_002577		-2.40		PAK2	P21/Cdc42/Rac1-activated kinase 2	JNK, p38
NM_015716	-2.37	-2.77		MINK	Misshapen/NIK-related kinase	JNK, p38
NM_004759		-2.44		MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2	ERK1/2, p38

Table 1. Genes in the MAPK signaling pathways affected by CCM1 and CCM2 silencing.

Table above illustrates the effects of CCM1 and CCM2 on p38 MAPK as well as JNK signaling pathways (Shin, D. unpublished data).

CCM2 interacts with RasGAP proteins

GTPase Ras and other small proteins in this family are upstream signaling molecules which regulate the MAPK signaling cascade[81]. CCM1 was initially cloned as a binding partner of a small GTPase Ras-related protein (Rap1A) in a yeast two-hybrid assay[82]. Thus CCM1 and CCM2 might regulate MAPK signaling

via Ras molecules. A yeast two-hybrid assay using CCM2 as bait against a human fetal brain library, isolated three Ras GTPase activating proteins (RasGap) (Fig 5a). Of 37 independent clones, 11 were Synaptic GAP (SynGAP), four were Ras activating protein like-2 (RASAL2) and two were ASK1 interacting protein 1 (AIP1 or DAB21P). The CCM2 yeast two-hybrid screen indicated that CCM2 interacts with KIF5A (Fig 5).

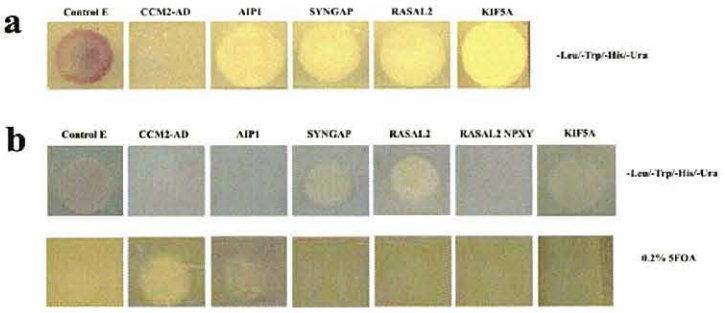


Fig 5. Identification of RasGAP protens as CCM2 binding partners. (a) Yeast two-hybrid screening identified SYNGAP; RASAL2 as potential interactors with CCM2. Control E confers a strong interaction phenotype.CCM2-AD is a negative control for the selection plate due to co-transformation of full length CCM2-DB with an empty vector containing GAL4AD domain. (b) Assessment of their interaction by re-transformation with clones obtained from primary screening above. Growth of AIP1 as well as RASAL2-NPXY mutant is abrogated. SYNGAP and RASAL2 show strong interaction phenotype on the same selection plate used as above. 0.2% 5FOA is a negative selection plate and shows a complementary growth pattern (Shin, D. unpublished data).

The RASAL2 proteins contain a NPXY motif which binds the phosphotyrosine binding (PTB) domain of several proteins[83]. CCM2 contains a PTB domain so we wanted to determine whether this interaction specificity depended upon NPXY-PTB binding. The interaction between CCM2 and RASAL2 disappeared when the amino acid residue asparagines (N783) on the NPXY domain of RASAL2 was mutagenized to histidine (Fig 5b).

Nuclear CCM1 and CCM2 translocate to cytoplasm in response to stress

CCM1 and CCM2 have potential nuclear localization signal sequences. Based upon PSORTII software analysis, we found that CCM1 has two putative nuclear

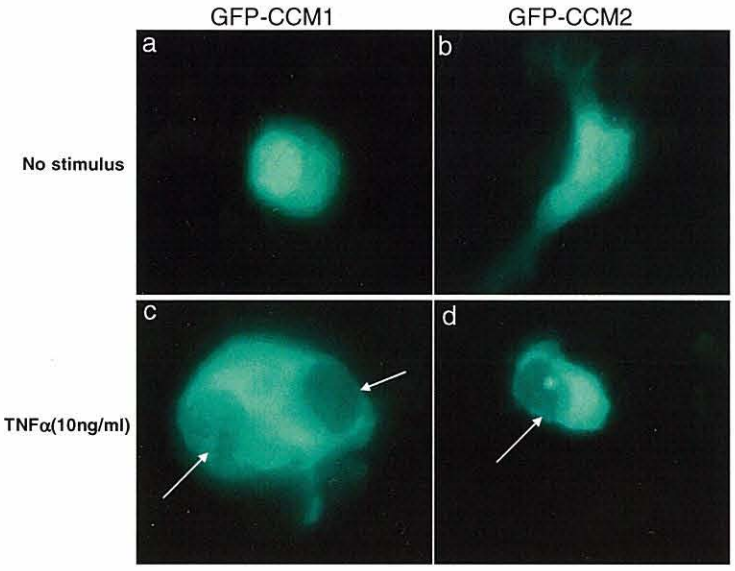


Figure 6. Nuclear CCM1 and CCM2 translocate to the cytoplasm following TNFα treatment in N2A cells. As predicted by the PSORTII program, CCM1 protein overexpressed in N2A (a) shows a predominant nuclear localization, while CCM2 is overexpressed equally in both cytoplasm and the nucleus (b). When the cells were treated with TNFα for 15 minutes, nuclear CCM1 as well as CCM2 proteins no longer remain nuclear, but translocate to the cytoplasm (c,d). White arrows in c and d indicate the unoccupied nuclei after TNFα treatment. (Shin, D. unpublished data).

localization signals: KKKRKK (46-51) and KKHK (569-572). According to this analysis the CCM1 protein has a 69% chance of being found in the nucleus. CCM2 has one nuclear localization signal, RHRR (387-390) with a 52% chance of finding CCM2 in the nucleus.

To determine the subcellular localization of CCM1 and CCM2, mouse N2A cells were transfected with GFP-tagged constructs containing each protein after TNFα or sorbitol stimulation. CCM1 was expressed mostly in the nucleus with weak cytoplasmic expression after over expression and in the absence of stimuli.

CCM2 was expressed equally in the cytoplasm and nucleus (Fig 6 a,b). The subcellular localization of CCM1 and CCM2 changed to mostly cytoplasmic after sorbitol or TNFα treatment for 15 minutes (Fig 6 c,d). CCM3 was localized to both the nucleus and cytoplasm both before and after treatment (data not shown).

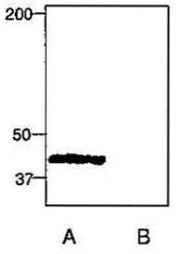


Fig 7. CCM3 [1:500] antibody was used to stain Western blots of protein lysate extracted from homogenized mouse tissue. Staining detected a protein of the expected band size (43kDa) for CCM3 in brain (A) but not in the negative control aortic tissue(B).

Characterization of CCM3 antibody

Antibody specificity was determined for CCM3 by comparison of protein lysates from homogenized mouse aortic and brain tissue and stained with CCM3 antibody. Western blot stained a protein of expected band size of 43kDa (Fig 7).

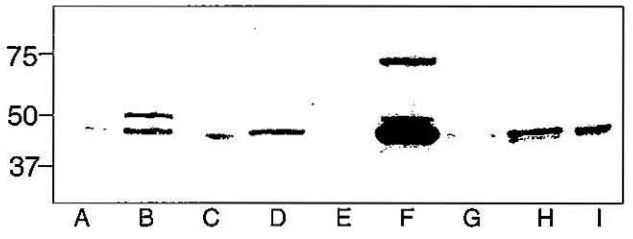


Fig 8. CCM3 was expressed across all tissues except aorta using CCM3 antibody staining on Western blot of protein lysate from homogenized mouse tissue. The expected band size of 43kDa appeared Across all tissues except for the aorta. A. Heart B. Lung C. Pancreas D. Liver E. Aorta F. Brain G. Muscle H. Spleen I. Kidney. Multiple bands were seen in brain, pancreatic and lung tissue.

CCM3 expression parallels CCM1 and CCM2 expression in mouse tissue

In order to evaluate the pattern of CCM3 expression in different tissues lysates of various mouse organs were analyzed by Western blot. Sections of human tissue from those organs which expressed CCM3 endogenously were stained with CCM3 antibody to analyze expression in human organs. Protein extracted from homogenized mouse tissue showed that CCM3 is expressed in lung, pancreas, brain, muscle, kidney, liver and heart but not in the aorta (Fig 8). Immunohistochemical staining of human tissue showed a similar pattern of expression with venous but not aortic CCM3 expression in the aorta (Fig 8). This correlates with the pattern of expression of CCM2 in tissues (Fig 4).

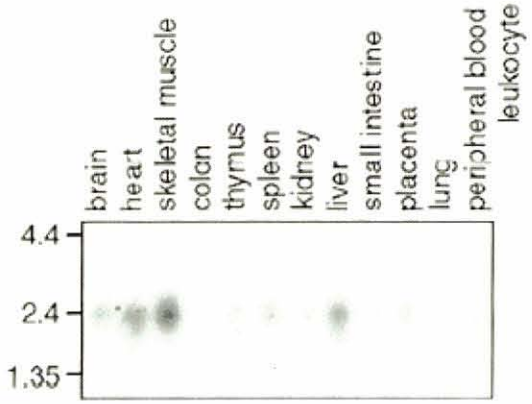


Fig 4. Western blot analysis of MGC4607 expression in 12 human tissues. Bands are expected size of 2.4kb for CCM2. (Seker et al. *Stroke.* 2006;37:518.)

CCM2 is expressed in brain, heart, spleen, skeletal muscle, thymus, kidney, liver, small intestines, placenta, lung, colon and peripheral blood leukocytes (Fig 4).

CCM3 parallels CCM2 expression in Arterial Endothelial Cells

The distribution pattern of CCM3 expression was determined by immunohistochemical staining of paraffin-embedded human tissue. CCM3 was

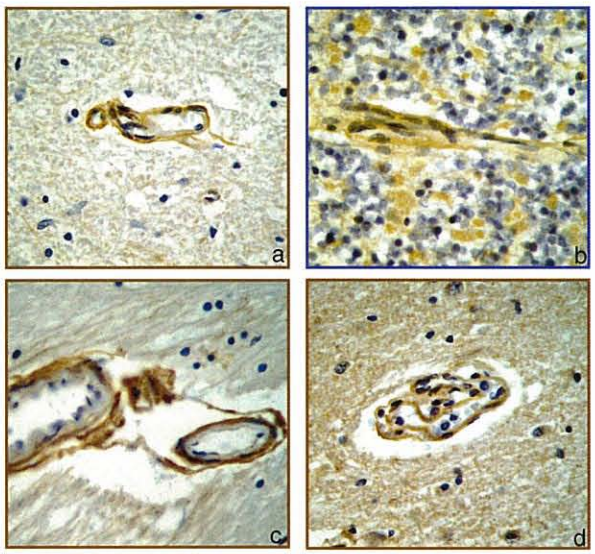


Fig 9. Staining with CCM3-specific polyclonal antibody illustrates specific expression pattern in cerebrovascular structures. CCM3 is expressed in arterial endothelial cells in a similar pattern to CCM2 in a. medulla b. cerebellum c.thalamus d.cortex.

expressed in the perinuclear regions above the arterial endothelium in the medulla, cortex, cerebellum and thalamus (Fig 9).

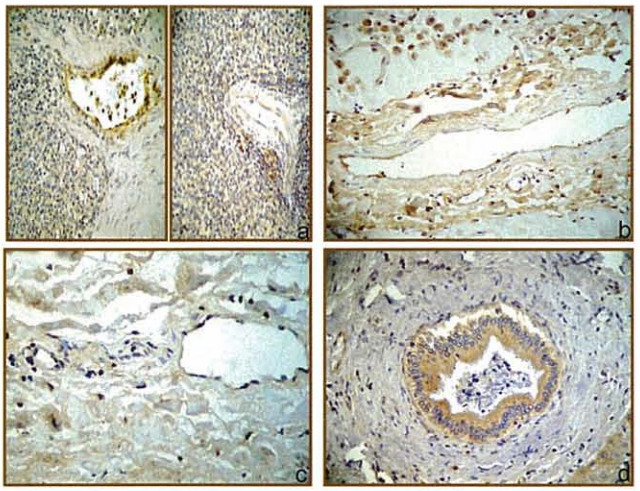


Fig 10. CCM3 localizes to various epithelia in the extracerebral organs. a. CCM3 is expressed in the splenic central arteries and some venous endothelium of the red pulp on the left. CCM3 is expressed in the arterial endothelium of the white pulp of the spleen on the right. b. CCM3 is strongly expressed in the arterial endothelium lung but low expression of cytoplasmic or perinuclear staining c.There is low venous CCM3 expression in the aorta. d. CCM3 Is expressed in the ductus and hepatocyte cytoplasm of the liver

Extracerebral Tissue Expression

Spleen:

The CCM3 antibody is expressed in the central arterial endothelium but not in the venous endothelium in the white pulp but this pattern was not replicated in the red pulp of the spleen where CCM3 was expressed in some veins. On higher magnification CCM3 was shown to have nuclear expression in the spleen (Fig 10a).

.Aorta:

CCM3 was shown to be expressed in the endothelium or perinuclear region of the aorta (Fig 10b).

Lung:

Lung tissue showed intense arterial endothelial CCM3 staining but venous endothelium had very low levels of expression (Fig 10c).

Liver:

CCM3 was expressed in the ductus and hepatocyte cytoplasm of the liver but there was no venous endothelial staining (Fig 10d).

Nuclear Renal Expression of CCM3

Immunofluorescence of COS7 renal cell line indicated nuclear CCM3 expression in the kidney. CCM3-HA tagged constructs were transfected into COS7 cells. Cells transfected with CCM3-HA were incubated with Cy3, Dapi and gfp. A CCM1-GFP tagged construct was used as a positive control and nontransfected COS7 cells were used as a negative control. The Dapi staining indicates that the Immunofluorescent staining was successful in that the majority of cells stained with CY3 also stained with the nuclear stain Dapi. Overlap of the Cy3 and Dapi shows significant nuclear expression of CCM3. CCM1 was previously known to have nuclear expression (data not shown).

CCM3 interacts with Serine/Threonine Kinases

The Y2H screen using CCM3/PDCD10 identified two members of the subfamily of STE20-like kinases: STK24 or Mst3, which was shown to phosphorylate p42MAPK, and STK25 or SOK-1, which is known to be induced by oxidative stress. CCM3 also interacted with KIAA0826 which is a homolog of the sensory axon guidance family member (sax-2) in C.elegans (Fig 11). These interactions were further confirmed by a second yeast two-hybrid analysis using only CCM3

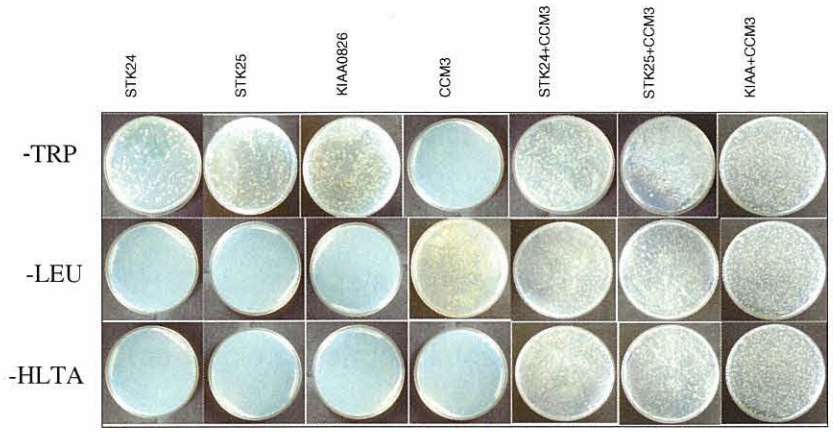


Fig 11. Identification of STK24, STK25, KIAA0826 as CCM3 binding partners. STK24,STK25, KIAA0826 in pGBKT7 Clontech bait vector which is –trp and CCM3 in pGADT7 prey vector which is –leu. The individual clones do not grow on the full drop out plate but the mated constructs grow which indicates that the complex activates the reporter gene. Controls are AH109, bait yeast strain, and YP187, prey yeast strain.

against STK24, STK25 and KIAA0826 with identical yeast two hybrid technology by Invitrogen. The transformation was confirmed via western blot and verified on appropriate dropout plates for self-activation. The prey and bait were then mated overnight at 300°C. Constructs were plated onto -Leu, -Trp, -Ade/Leu/Trp, and -His/Leu/Trp/Ade (-HLTA) drop-out plates and incubated at 300°C for three to four days. Mated cultures were confirmed via PCR using gene specific primers and sent for sequencing to KECK to confirm that successful mating.

Additional yeast-two hybrid done to verify interactions between STK24, STK25, and Kiaa0826. The –Trp plates yielded STK24, STK25, KIAA0826 colonies which corresponds to the pGBKT7 constructs where the vector is –Trp. This indicates activation of the reporter gene and confirms interaction between CCM3 and these proteins. The CCM3 construct in the pGADT7 vector is –Leu and so did not yield colonies. The co-transformed plates with STK24 and CCM3, STK25

and CCM3 as well as KIAA0826 and CCM3 all yielded colonies indicating that the nonCCM3 colonies grew. The –Leu plates yielded CCM3 growth for the individual and co-transformed plates. The most stringent drop out plates –HLTA showed no growth for the individual constructs but yielded colonies for the cotransformed plates indicating the complex activates the reporter gene. The bait yeast strain AH109 served as a control to indicate there was no contamination of the single construct plates.

Fig 12. PCR amplification of bacterial STK25 in pGBKT7 and STK24 in pGBKT7 using matched and reverse primers. Amplification with the appropriate STK24 or STK25 primer yielded the expected band Of 1.3bp. The same colonies did not amplify using the primer set for the other STK protein, indicating that STK24 and STK25 are on different loci although they appear the same size and share significant overlap.

Stk24 and Stk25 both produce a band at 1.3bp and KIAA0816 produces a band at 1.4bp. STK24 (13q31.2-q32.3) and STK25 (2q37.3) have a 73.7% overlap in 408 amino acid residues. Since the serine threonine kinases produce bands of the same size and share significant homology we amplified colonies using matched and reverse primers to confirm that these two proteins were on separate loci. The colonies produced the appropriate size band when amplified with matched primers but did not when amplified with the opposite primer set (Fig 12).

CCM Signaling Links Neural and Endothelial Elements

Based on above data we constructed a hypothetical pathway in which CCM1 and CCM2 link the extracellular matrix to the cellular cytoskeleton and nucleus through integrin/Ras-MAPK signaling[3]. Induction of apoptosis through these signaling cascades leads to CCM3 expression and cellular proliferation which are exhibited in the CCM pathology (Fig 13).

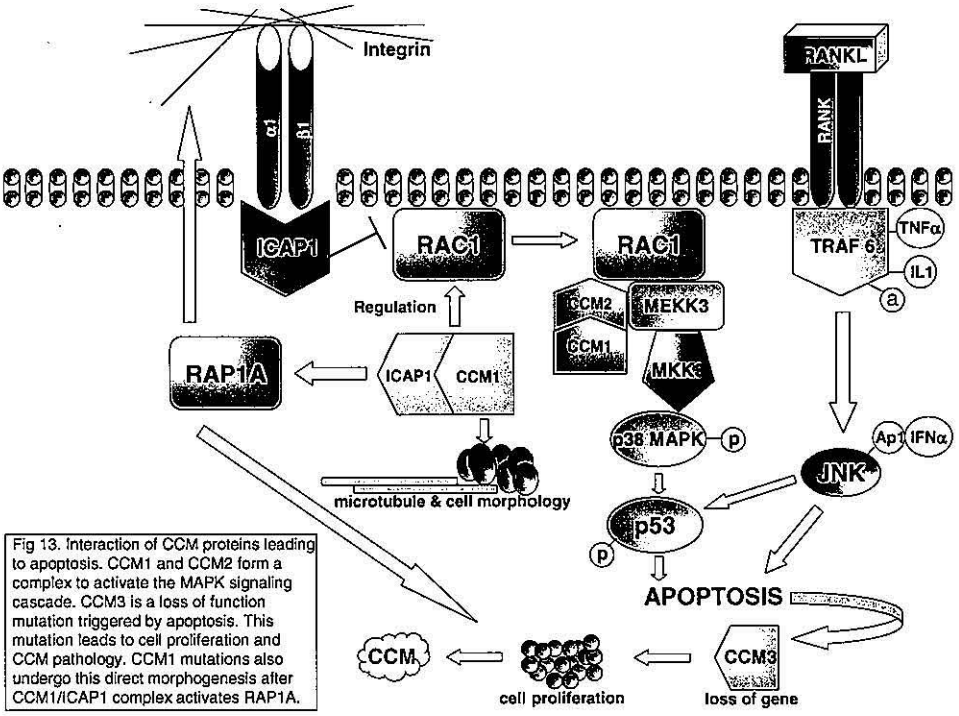


Fig 13. Interaction of CCM proteins leading to apoptosis. CCM1 and CCM2 form a complex to activate the MAPK signaling cascade. CCM3 is a loss of function mutation triggered by apoptosis. This mutation leads to cell proliferation and CCM pathology. CCM1 mutations also undergo this direct morphogenesis after CCM1/ICAP1 complex activates RAP1A.

DISCUSSION

Cerebral cavernous malformations are caused by mutations in three known genes KRIT1, Malcaverinin (MGC4607), PDCD10, and possibly a fourth gene whose locus is close to PDCD10. Although mutations in the three distinct genes have been associated with CCMs they remain identical clinically and pathologically.

The role of p38 MAPK signaling in CCM pathophysiology correlates with evidence that p38 MAPK signaling is induced by integrin activation and may regulate other cellular processes such as actin remodeling and apoptosis[84-88]. Data showing that p38α null mice die early in gestation with significant placenta vascularization defects implicates the MAPK pathway in the regulation of angiogenesis[89].

Our data indicates that CCM1 and CCM2 are necessary for the activation of stress-responsive mitogen activated signaling pathways p38 MAPK and JNK pathways through sorbitol and yeast two-hybrid assays. Further these two molecules appear to act as a complex to initiate the signaling cascade. Although CCM3 does not participate directly in these pathways yeast two hybrid data confirms direct interaction between CCM3 and STKs which are implicated in MAPK signaling. This is also confirmed through siRNA mediated gene silencing and overexpression studies. CCM1, CCM2 and CCM3 appear to be expressed in similar tissue distribution across the body although the CCM pathology is CNS

specific. In addition, the intracellular CCM expression is identical between CCM1 and CCM2 and CCM3 is expressed in both the nucleus and cytoplasm, further supporting theories that CCM1 and CCM2 work as a complex that interacts with CCM3 to yield CCM pathology.

Based on previous data we hypothesized that CCM1 and CCM2 but not CCM3 were implicated in the same molecular genetic pathway responsible for communication between neural and glial elements in the CNS[38]. Inflammatory states impact astrocytes as well as endothelial cells as studies have shown increased CD95-driven apoptosis in response to inflammation factors such as TNF-α, IL-1β and INF-γ[90]. Data presented here supports the theory that CCM1 and CCM2 work as a complex to activate the stress-responsive p38 MAPK, JNK pathways and that CCM3 works independently in conjunction with the other proteins. The CCM1 and CCM2 proteins act as components of the MAPK cascade which activate CCM3 through apoptosis[41]. Signaling pathways do not progress in a linear manner but act as multiple signaling pathways regulating one another to produce biological effects[91-93].

We showed that CCM1 and CCM2 are endogenously coexpressed in COS7 cells providing further evidence that these two proteins are involved in the same biochemical pathway (Fig 3). This is an important step towards elucidating the molecular signaling pathways responsible for CCM pathogenesis. Non-

transfected cells pulled down with CCM1 or CCM2 but not the gfp antibody indicates that CCM1 and CCM2 are endogenously expressed.

The theory that CCM1 and CCM2 act as a complex to initiate the p38MAPK cascade,[41] is supported by the fact that the CCM1/CCM2 co-transfected cell lysates pulled down with either the CCM1 and CCM2 antibody produces the corresponding band width. Previous data has shown this activation to be in response to osmotic stress. Our data confirms that CCM1 and CCM2 function via the p38 MAPK and c-Jun NH2-terminal kinase signaling pathways and are expressed as a complex in COS7 cells. This supports previous data of a model where CCM signaling links the extracellular matrix to the cellular cytoskeleton and nucleus through integrin/Ras-MAPK signaling[94].

Evidence of siRNA mediated gene silencing and overexpression studies support this hypothesis (Table 1). Signal transduction of the integrin activated p38 MAPK signaling pathway may mediate cellular processes such as actin remodeling and cell apoptosis as well as vessel formation. The association between angiogenesis and MAPK cascade is suggested by the observation that p38α null mice are embryonic lethal and exhibit severe placental vascularization defects [89].

Although the molecular function of CCM2 is still being established, CCM2's mouse homolog, Osmosensing Scaffold for MEKK3 (OSM), has offered insight into CCM2 function and interaction with CCM1. OSM has a phosphotyrosine

binding domain and is implicated in mechanosensing and osmosensing[83]. OSM regulates p38 MAPK signaling through direct interactions with MEKK3, Rac and actin[83]. The KRIT1 binding partner, ICAP1α inhibits Rac signaling, thus the Rac molecule may be a critical point of interaction between CCM1 and CCM2[37, 95, 96]. Further, yeast two-hybrid data indicates that RasGAP proteins interact directly with CCM2 which supports the theory that this molecule is involved in the MAPK pathway and interacts directly with CCM1 to initiate involvement.

The RasGAP proteins identified as CCM2 binding partners through yeast two-hybrid assays are either predominately or exclusively expressed in the central nervous system (CNS)[55]. RasGAP proteins exhibit a spatial expression profile which may explain why CCM pathology is limited to the CNS although CCM expression is found throughout mammalian tissues (Fig 4; 8). One of the CCM2 binding partners RASAL2 is a close family member to RASAL1 which is mutated in hereditary capillary malformation-arterious malformation (CM-AVM). RASAL1 encodes p120 RasGAP which binds Rap1A which may be a binding partner of CCM1.

Further studies have not supported this interaction possibly because there are two biologically active splice isoforms of CCM1 the other of which binds ICAP1α[97]. Clinically this is intriguing since CCMs occasionally produce cutaneous lesions similar to those of CM-AVMs[98]. This evidence corroborates theories that CCM1 and CCM2 are involved in the Ras-mediated MAPK signaling

pathway. Additional yeast two-hybrid data suggests that CCM3 may signal through the same pathways through interactions with serine threonine kinases which are upstream in the MAPK pathway.

Yeast two-hybrid analysis of CCM2 also indicates that KIF5A interacts with CCM2. The isolation of this protein as directly interacting with CCM2 has interesting clinical implications and further supports theories that CCM1 and CCM2 work in concert. CCM1/KRIT1 knock-out mice have been shown to die early from closure of the dorsal aorta[34]. This supports histological data which shows CCM1 expression is restricted to arterial and microvascular endothelium with neurons and astrocytes[99]. Another CNS specific condition, $\alpha_v$integrin mutants die from intracerebral hemorrhage early in gestation[100]. Histological examination of these brains shows lesions which are phenotypically similar to CCMs. Conditional knock-out $\alpha_v$integrin mutants that survive exhibit hindlimb weakness which is similar to spastic paraplegia (SPG) which is a heterogeneous disease in humans[101, 102]. An autosomal dominant form of the disease, SPG10, is caused by mutations in kinesin 5A (KIF5A) gene which has been shown to interact with CCM2 [101]. This suggests an interaction between CCM signaling and $\alpha_v\beta$8integrin which supports the theory that CCM signaling links neural elements with endothelial cells and is important in cerebrovascular development[100].

CCM1 and CCM2 have been shown to be necessary for activation of the stress-responsive mitogen activated signaling pathways, p38 MAPK and JNK in

response to hyperosmotic shock (sorbitol) or TNFα treatment. CCM1 has been shown to be a positive regulator of the p38 MAPK and JNK signaling pathways and CCM2 interacts with RasGAP proteins to initiate the MAPK cascade.

Based upon these results we conclude that CCM signaling evokes a novel pathway allowing communication between neural and endothelial cells of the neurovasculature[40]. When this pathway is disrupted by a mutation in a CCM protein, the normal CNS angiogenesis is altered and the changed communication between neural and endothelial cells results in the CCM pathology. CCM1, CCM2 and CCM3 show similar patterns of expression in structures critical to the blood brain barrier.

We showed that CCM3 is expressed across all tissues studied including the brain, heart and kidney (Fig 8-10). Although protein lysate from homogenized mouse tissue did not appear to express CCM3 on repeated antibody staining on Western blot, the presence of CCM3 venous expression on immunohistochemical staining of human aortic tissue suggests its presence in the aorta (Fig 8). This correlates with previous data on CCM1 and CCM2 expression (Fig 4) as well as current theories on CCM pathology and arterial morphogenesis[103]. The similar pattern of expression between CCM2 and CCM3 across various extracerebral tissues supports theories that these two proteins may work through the same mechanism or signaling pathway in yielding the CCM pathology.

Unlike CCM2 protein expression, CCM3 appeared to be predominately expressed in the cytoplasm in the medulla. CCM3 was expressed in the purkinje cells of the cerebellum as well as glial cells in the molecular layer of the cerebellum (Fig 9). These glial cells form part of the membrane encapsulating the vessels which establishes the blood-brain barrier[35]. Thus mutations in CCM proteins in these cells may be responsible for the phenotypic leaky blood-brain barrier associated with CCM pathology.

CCM2 is expressed by endothelial cells, specifically arterial and microvascular endothelium in all tissues. In the brain CCM2 is also expressed in pyramidal cells and astrocytes. CCM2 expression has been seen in astrocytes with foot processes which terminate on cerebral blood vessels. The glial membrane which encapsulates the vessel and creates the blood brain barrier is established by these foot processes[35]. CCM lesions lack astrocytic foot processes and tight adherence junctions between endothelial cells creating the appearance of an immature vessel[104]. These lesions may have a proliferating, non-adherent endothelium rich in fibronection and poor in laminin[105].

Previous data from this lab has shown high levels of CCM1 and CCM2 expression in arterial endothelium as well as microstructures important to the integrity of the blood brain barrier. CCM lesions are CNS specific and CCM1 expression has been shown to be isolated to arterial and microvascular endothelium of neurons and astrocytes including foot processes. This is

supported by the clinical phenotype of KRIT1 knock-out mice which die early due to closure of the dorsal aorta[33, 34]. To further support this hypothesis we found CCM3 shares a similar pattern of expression in mouse and human tissue which suggests that it is also implicated in normal angiogenesis and arterial morphogenesis. Data has shown this CCM protein to be important in cerberovascular development and remodeling[43]. CCM3 may also help regulate apoptosis in smooth muscle cells which is mediated by β1-integrin signaling which interacts with CCM1 and CCM2 as well[88]. The interaction between KRIT1 and ICAP1 implicate CCM1 in the β1-integrin signaling pathway.

CCM3 is important in cerebrovascular development and remodeling[43]. CCM3 is caused by mutations in PDCD10 which is a gene involved in the initiation of programmed cell death which is critical to arterial morphogenesis. Some evidence shows that apoptosis in smooth muscle cells is mediated by β1-integrin signaling which interacts with CCM1 and CCM2[88]. Previous yeast two-hybrid analysis of KRIT1/ICAP1 indicate β1-integrin signaling involvement in CCM pathophysiology[36, 37]. Our data confirms that CCM1 and CCM2 are positive regulators of p38 MAPK and JNK signaling pathways and that CCM3 expression is related to these pathways.

In addition, CCM3 yeast two-hybrid data identified serine threonine kinases which are part of the STE20 family which is upstream of the MAPK cascade. This indicates that CCM3 may signal through the p38 MAPK cascade. Since CCM1

and CCM2 are implicated in the p38 MAPK and JNK signaling pathways, their activation of apoptosis may trigger CCM3. Another binding partner of CCM3, KIAA0826 is an osmold scaffolding protein which provides further evidence that the CCM proteins act in the communication between arterial and neuronal elements (Fig 11).

Ste20-like kinases are a germinal center kinase that activate apoptosis through the p38 MAPK signaling cascade. Interestingly, Ste20 expression is increased in renal development and during the recover from acute ischemic renal failure. Overepxression of Ste20 has been documented during anoxia/recovery-induced apoptosis and is activated by homodimerization, signaling through ASK1 and p38 to induce apoptosis [106].

Further yeast two-hybrid assays are underway to find binding partners of STK24, STK25 and KIAA0826 in order to dissect the molecular signaling pathway responsible for CCM3 action.

These findings offer greater insight into the understanding of CCM pathology. We suggest a model where CCM signaling links the extracellular matrix to the cellular cytoskeleton and nucleus through integrin/Ras-MAPK signaling (Fig 13). Identification of the signaling pathway(s) in which the CCM genes participate will not only provide insight into the molecular mechanisms underlying cerebral

angiogenesis and CCM pathology, but may also lead to new therapeutic approaches.

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