**A. BACKGROUND AND SIGNIFICANCE**

Cerebral cavernous malformations (CCMs) primarily occur in the central nervous system where CCM-affected capillaries have grossly dilated vascular channels lined by only a single layer of endothelium (reviewed in 1). CCM lesions do not contain any intervening neural parenchyma or identifiable mature vessel wall elements such as smooth muscle or elastin. Furthermore, gaps exist between the endothelial cells, disrupting the tight junctions that would normally create the blood-brain barrier, allowing for the leakage of blood into the surrounding brain parenchyma2. Hemorrhages associated with these lesions lead to headaches, focal neurological defects, seizures and strokes in 20-30% of affected individuals, and the incidence of CCMs has been estimated to be 0.5% of the population. Recurrent seizures or strokes necessitate risky and complex excision of lesions, the only therapeutic option currently available. As lesions can be located in difficult areas to access in the brain, and patients may have multiple lesions (over 100 in more aggressive familial cases), less invasive therapeutic interventions are clearly required for managing this devastating disease3.As only the endpoint of CCMs (vascular dilatation and hemorrhage) is observed in patients, the mechanism that leads up to disease manifestation is currently unclear. Roughly 40% of CCMs are familial in origin, with single-copy germline loss-of-function mutations in one of three genes (here referred to as CCM1-3; see Fig 1 for an extended list) being responsible for the vast majority of these cases4-12. While the histology of CCM3 vascular lesions is indistinguishable from those caused by mutations in CCM1 and 2, the risk of hemorrhage in patients with CCM3 mutations is much higher4. There is a great deal of debate surrounding CCM disease: do additional modifier gene(s) affect the severity of disease12; what is the cause of CCMs in non-familial cases (where CCM1-3 appear normal); is the loss of the second copy of CCM1-3 leading to disease in familial cases13,14; and is a single molecular pathway responsible for all CCMs?

The identification of the three genes mutated in familial CCMs (CCM1-3) has led to research on possible molecular mechanisms underlying the formation of these lesions in patients. CCM1, CCM2 and CCM3 are all mediators of intracellular signaling, predominantly via the small GTPases of the Rho family15-17, though roles in stress signaling, apoptosis, and various other intracellular pathways have been reported, as briefly reviewed below.

***CCM1 and CCM2:*** CCM1 and 2 contain protein interaction domains and motifs that are consistent with roles as signaling scaffolds18 (Fig 2). CCM1 contains a FERM (4.1-Ezrin-Radixin-Moesin) domain at its C-terminus implicated in membrane binding19,20. Rap1A, a small GTPase interacting with CCM1 in yeast two-hybrid assays, contributes to junctional recruitment of CCM120,21. In addition, three NPxY motifs mediate interaction with the phosphotyrosine-binding (PTB) domains of CCM222 and of ICAP1 (Integrin beta Cytoplasmic domain-Associated Protein)23,24. Interestingly, CCM1 binding to ICAP1 and to CCM2 is mediated by different NPxY motifs22, enabling formation of CCM1•CCM2•ICAP1 complexes (preliminary data). CCM1 also contains a series of ankyrin repeats, for which the binding partners are not yet known. Other interaction partners and functions for CCM1 are also likely: for example, the *C. elegans* orthologue of CCM1 (*kri-1*) is required for cross-tissue signaling in promoting longevity and apoptosis25,26(Appendix 2).

While the interaction between CCM1 and CCM2 occurs at high stoichiometry (the protein ICAP1 is also abundant in these complexes), CCM2 establishes additional interactions, including with the CCM3 protein27, although the fraction of CCM3 that co-purifies with CCM1•CCM2 is small in all cell types we have analyzed. We recently demonstrated, together with M. Fainzilber, that the CCM2 Karet domain28 mediates association with CCM3 (preliminary data). Other interactions were also reported for CCM2, including the upstream component of the p38 MAP kinase pathway MEKK322. Regulation of Rho signaling by CCM1, CCM2 and CCM3 may involve degradation of Rho protein, and CCM2 has been shown to associate with the SMURF1 ubiquitin E3 ligase in this process17. Tissue-specific interactions are also likely, as reported for an interaction between CCM2 (PTB domain) and TrkA, which regulates cell death in medulloblastoma and neuroblastoma cells28. While we have not been able to identify an obvious CCM2 gene in *C. elegans* by homology searches, we will utilize genetics and proteomics approaches to search for a functional homologue in Aim 1.

***CCM3***: CCM3 was first identified through a screen for genes expressed during the induction of apoptosis in a premyeloid cell line12. Despite its small size, CCM3 establishes multiple protein-protein interactions, and also homodimerizes via its amino terminus29. However, heterodimerization with members of the germinal center kinase III (GCKIII) family30 via the same region is much favored, as we recently demonstrated (Appendix 4). The interaction between CCM3 and the GCKIII kinases creates a modular unit that can be recruited to different complexes. CCM3 also contains a C-terminal globular domain bearing structural homology to the FAT (focal adhesion targeting) domain of focal adhesion kinase29 which can bind phospholipids31, but also associates, at least *in vitro*, with an acidic peptide on paxillin29. Interaction with CCM229 and the striatin component of STRIPAK (Appendix 4, see below) also occurs via the same molecular contacts, indicating that they are mutually exclusive. Lastly, other interaction partners have been reported for CCM3, most notably the VEGF receptor 2 (VEGFR2), a critical mediator of endothelial cell development that is stabilized by interaction with CCM332, and a protein tyrosine phosphatase called PTPN1333. How these other interaction partners are recruited to CCM3 is not currently known. The *C. elegans* CCM3 gene (*C14A4.11*) has not been characterized until now, but our preliminary data indicate that it is critical for tubulogenesis of excretory cells, which we will use as an *in vivo* model to study CCM function (see below).

***GCKIII kinases***: GCKIII kinases (members of the Sterile 20 kinase family) are critical mediators of CCM3 function. Three members, STK24, STK25 and MST4, are present in human cells that are characterized by a kinase domain followed by a family-defining tail that is used for heterodimerization with CCM3 (Appendix 3). They work positively with CCM3 in cell biology assays in mammalian cells (Appendix 4) and in vascular models. Overexpression of the sole worm GCKIII ortholog can restore truncated excretory canals in *ccm3* mutants to wild type lengths (preliminary data), and overexpression of the GCKIII kinase rescues vascular defects caused by depletion of CCM3 in zebrafish (I Scott, pers comm.). Collectively, these exciting results suggest that the key function of CCM3 is dependent on the kinase activity of GCKIII. How these kinases are activated remains poorly defined, but caspase cleavage has been proposed as a possible mechanism of activation34. In addition, GCKIII localization is affected by oxidative stress34b, activation of the tumor suppressor Lkb1, and depletion of CCM3 or striatin35,36 (Appendix 4). Substrates for these kinases still remain largely unknown, though STK25 was shown to phosphorylate a 14-3-3 protein37, and MST4 the ezrin-radixin-moesin (ERM) proteins34b,35. The GCKIII kinases are also implicated in the regulation of p38, JNK and ERK, although there is no clear consensus as to their exact function. While STK25 was reported to phosphorylate CCM3 *in vitro*33, whether this happens in a cellular context is not known.

***STRIPAK***: We discovered and characterized the large molecular weight complex STRIPAK (striatin interacting phosphatase and kinase) using interaction proteomics and biochemical methods (Appendix 1), and showed that the majority of CCM3 protein resides within this complex in mammalian cells. STRIPAK possesses both kinase and phosphatase activities, and several of its components have been conserved through eukaryotic evolution (Fig 3), including its cytoskeletal functions. In *S. cerevisiae*, depletion of the STRIPAK components suppresses the lethality and actin polarization defects associated with mutations in the Target of Rapamycin complex 2 (TORC2), as we and our collaborators have recently shown38. In filamentous fungi, STRIPAK promotes membrane fusion39,40, a phenotype that appears conserved in mammals41. CCM3 and the GCKIII kinases only appear in metazoa, and both are present in *C. elegans*. We recently demonstrated that, like CCM3, depletion of STRIPAK components cause truncations in excretory canals, indicating CCM3 and STRIPAK are genetically linked in early metazoans. The excretory cell in *C. elegans* will be used here as a model of vascular tube development (see below). In zebrafish embryos, depletion of the GCKIII kinases or of the STRIPAK component striatin produce the same phenotype (a grossly malformed vascular system) as depletion of CCM342 (Ian Scott, pers. comm.). Lastly, experiments in cell lines by our groups and others have revealed additional roles for the STRIPAK and CCM3-associated kinases, for example in Golgi polarization and cell migration. We recently demonstrated that CCM3 and striatin oppose each other in targeting the MST4 kinase to Golgi, where it promotes Golgi polarization towards the leading edge of a wound (Appendix 4). In addition, a recent report demonstrated that PP2A inactivates MST4 via interaction with striatin42b (also see Fig 17), revealing an intricate link between the phosphatase and kinase activities in mediating the biological functions of this complex.

***C. elegans as a model for understanding CCM gene function.*** Homozygous deletion of *ccm1* or *ccm2* in the mouse causes cardiovascular defects and embryonic lethality43-45 but mice lacking *ccm3* have distinct and more severe phenotypes46,47, consistent with patients harbouring a *ccm3* mutation4. While *in vitro* and animal models have provided some mechanistic insight into CCMs, many key questions remain. Firstly, what signaling pathway(s) does CCM3 engage to promote vascular development and integrity? Second, do other CCM genes or modifiers of CCM gene function exist? Since patients with the same mutations present a range of symptoms, it is likely that modifier genes exist, which is one of the key advantages of using *C. elegans* to probe the genetic landscape of CCM genes. The roundworm *C. elegans* is a genetic powerhouse and many of the core signaling pathways that control development and homeostasis are highly conserved in this multicellular organism. Much of what we have learned for these pathways has been derived from unbiased whole-genome genetic screens in *C. elegans*.

*Our central hypothesis is that the CCM3•kinase module may target different substrates when associated with the CCM1•CCM2•ICAP1 complex, with the STRIPAK complex or with alternative protein assemblies. We postulate that the worst outcome for CCM3 patients, as well as the distinct phenotypes for CCM1•CCM2 versus CCM3 depletion, may result from a cumulative role for the CCM3•kinase module across these different complexes. Combining worm genetics and functional proteomics will undoubtedly synergize to provide major insights into this disease and reveal new opportunities for treating CCM patients pharmacologically.*

**B. PRELIMINARY DATA**

Our groups have made substantial contributions to understanding the biochemistry and cellular functions of CCM proteins over the past two years. We have generated high quality protein-protein interaction maps for each of the three CCM proteins, characterized the structural nature of the interactions, and identified several genes that modify the biological function of CCM proteins *in vivo*.

***Protein-protein interactions:*** The Gingras lab has established a very solid expertise in the generation of protein-protein interaction maps using affinity purification coupled to mass spectrometry (AP-MS), which consists of the purification of a protein from a cell or tissue source using an affinity step, followed by identification of all proteins associating with the protein of interest using mass spectrometry. We have developed more sensitive biochemical approaches (see, e.g. 48,49), improved tracking of mass spectrometry data50, and most importantly, led the development and implementation of statistical tools which enable the identification of true interaction partners among a sea of contaminants51-53. In addition, we have been working closely with our industrial partner AB-SCIEX to implement more sensitive mass spectrometry instruments and robust quantification methods, for example using the new TripleTOF 5600 (e.g. 54,55). With these innovations, we now have a very robust platform dedicated to quantitative interaction proteomics (Fig 4), which has enabled us and our collaborators to make several significant discoveries (e.g. 56-60). Importantly, while we have been using this platform most often within the context of purifications from epitope-tagged proteins expressed at low levels in a model human cell (HEK293, which expresses all three CCM proteins at endogenous levels and is now believed to be a neuronally derived cell61), the same approach can be applied to virtually any purification protocol, from tagged or endogenously expressed proteins and in any cell type (or species) we have tried thus far. We determined that the CCM3•STRIPAK interaction occurs endogenously in HEK293 and HeLa cells (Appendices 1 and 3), and in cells of endothelial origin. We performed AP-MS analysis on an allelic series of CCM3 proteins, including point mutants unable to associate with the GCKIII kinases or with STRIPAK. This helped us identify new CCM3 interaction partners, and in many cases to map the interacting regions (Fig 5). We also performed, in collaboration with Mike Fainzilber (Israel), AP-MS analysis with the full-length CCM2 protein, with a truncation mutant expressing only the PTB domain and a truncation mutant expressing only the Karet domain28. This unambiguously defined the PTB domain as the region responsible for interaction with CCM1•ICAP1 and the Karet domain as responsible for the interaction with CCM3. Interestingly, a strong CCM2-kinase interaction (likely mediated by CCM3) was only apparent when the Karet domain was analyzed in isolation. Additional interaction partners for CCM2 were also detected by these approaches, and are listed in Fig 6. Importantly, we have shown that CCM3•GCKIII associates in a mutually exclusive manner with another protein, c10orf47, further expanding the characterization of the range of action of CCM3 and associated kinases (Figs 7-8). We have now also performed AP-MS analysis with CCM1, ICAP1, RAP1A, and several of the new CCM2 and CCM3 interactors; this will be continued in Aim 1.

***Whole organism study of CCM function***. Dr Derry’s lab has developed *C. elegans* as an *in vivo* model to study *ccm1* and *ccm3* function in a multicellular setting, and demonstrated that the powerful genetics of the worm can be used to find new and conserved regulators of CCM signaling. They found that *ccm3* and GCKIII (*gck-1*) mutants recapitulate the human disease phenotype, with extensive truncations and formation of cysts that resemble cavernomas in the excretory cell, a single-celled vasculature structure in this organism (Fig 9). In collaboration, our groups have also provided important mechanistic insights into CCM1-3 function, which are briefly summarized below.

Dr. Derry’s lab first identified *ccm1* (known as *kri-1* in *C. elegans*) in a screen for genes that regulate programmed cell death26 (Appendix 2). Following up from this initial work his lab has found that *ccm1* and *ccm3* have distinct functions *in vivo*. Ablation of *ccm1* by mutation or RNAi causes resistance to radiation-induced apoptosis of germline stem cells26 (Appendix 2) and hypersensitivity to osmotic stress (Fig 10). In contrast, ablation of *ccm3* results in truncation of the excretory cell during development, but like *ccm1*, causes hypersensitivity to osmotic stress (Fig 10 & 11). Importantly, ablation of *ccm1* and *ccm3* together results in synthetic lethality (Fig 14), providing strong evidence that these genes act in parallel signaling pathways that impinge on a common cellular process required for viability. Both the human and worm CCM3 proteins interact with all Ste20-like kinases of the GCKIII family30,62 (Appendix 3; Fig 12) and Dr. Derry’s lab has also shown that ablation of the worm sole GCKIII gene (*gck-1*) causes identical excretory cell truncations as ablating *ccm3* (Fig 13). Furthermore, truncated excretory cells in *ccm3* mutants can be rescued by overexpressing *gck-1* (Fig 13), suggesting that CCM3 activates this kinase to promote excretory canal development/stability. This genetic evidence supports biochemical studies showing that CCM3 and GCKIII function in a common signaling pathway that is distinct from *ccm1*/*ccm2*. To uncover additional components of the *ccm3*/STRIPAK pathway, Dr. Derry’s lab performed a pilot RNAi screen to identify more genes that cause synthetic lethality when knocked down in *ccm1* mutants. After completing a screen of chromosome 1 (~2,700 genes) they identified 35 genes that reproducibly cause synthetic lethality when knocked down in *ccm1* mutants. They refined this list of genes to four, which are required for excretory canal morphology, and thirteen genes that cause hypersensitivity to osmotic stress (Fig 15), similar to *ccm3* mutants. Ablation of the core STRIPAK genes in *C. elegans* resulted in excretory canal truncations that were indistinguishable from *ccm3* or *gck1* mutants (Fig 16), demonstrating a robust conservation of function for this pathway. Genes required for excretory cell development in *C. elegans* that are also conserved in vertebrates (Aim 2) will be prioritized for proteomic analysis in human cells. One interesting candidate from the screen of chromosome 1 is Y71G12B.11, an open reading frame with high homology to mesoderm development candidate (*MESDC1*), an actin-binding protein also detected in our proteomics screen (Fig 5).

***Substrates for the GCKIII kinases***. A) *In vivo* phosphorylation: The CCM3•kinase module associates with alternative assemblies and we postulate that some of its interactors can also be substrates for the kinase(s). We therefore carried out pilot experiments to test the feasibility of performing quantitative phosphoproteomics experiments after affinity purification. By combining our AP-MS approach with phosphopeptide enrichment using Immobilized Metal Enrichment Chromatography (IMAC63, Fig 17), we identified >60 phosphorylation sites on the STRIPAK core components. This allows us to monitor variations in phosphorylation, as we showed by treating cells with okadaic acid, a potent pharmacological inhibitor of PP2A enzymes64, prior to FLAG purification, IMAC and LC-MS. While there was no drastic change in the global phosphorylation pattern of STRIPAK subunits, we found 12 phosphopeptides that were increased by >3-fold, including a phosphopeptide in the activation segment of the GCKIII kinases. Importantly, this preliminary result supports the feasibility of the *in vivo* phosphorylation experiments proposed in Aim 3. Since the previous submission of this proposal we have developed more sensitive mass spectrometry-based quantification methods (including those presented in Appendix 4) as well as MRM-HR and SWATH (Fig 18). B) *In vitro* phosphorylation: the identification of direct substrates for the GCKIII family has been hampered in part by a relatively poor definition of the consensus site(s) favored by these kinases. James Knight (Gingras lab) developed a novel approach for the identification of substrates by *in vitro* phosphorylation from a total cell lysate64b. This method involves the irreversible inactivation of all kinases from the lysate using the ATP analog 5′-4-fluorosulfonylbenzoyladenosine (FSBA) followed by *in vitro* phosphorylation with an exogenously-added kinase. Because of its compatiblity with both 32P labeling and quantitative mass spectrometry, this is an excellent approach for identifying kinase substrates. We have begun applying this approach to the GCKIII kinases, starting with MST4, and identified a potential consensus motif for phosphorylation, as well as several *in vitro* substrates. Interestingly, these include the STRIPAK component MOB3, which we have validated with recombinant proteins (Figs 19-21).

**C. RESEARCH PROPOSAL**

In order to uncover the molecular pathways that lead to CCM pathology, we will utilize the powerful and complimentary methods of proteomics and genetics in mammalian cells and *C. elegans*. Our three Specific Aims, as described in detail below, are to:

Aim 1: Establish physical interaction maps for CCM proteins in mammals and *C. elegans*

Aim 2: Define the global genetic interaction map for CCM1 and CCM3

Aim 3: Identify substrates for GCKIII kinases

***Aim 1. Establish physical interaction maps for CCM proteins in mammals and C. elegans***

***A. Refine and expand the interactome maps for CCM proteins in HEK293 cells.*** As mentioned above, we have generated a high-density interaction map surrounding CCM3 that has led to the identification of the STRIPAK complex into which CCM3 predominantly resides; new interactors for CCM2 and CCM3 have also been uncovered (Figs 5 and 6). ***Methods and expected outcomes****:* Newly discovered interactors from the proteomics screens (in years 1-2), and high confidence interactors from the genetic screens (in years 3-5; see Aim 2) will be cloned and epitope-tagged, expressed in the Flp-In T-REx human HEK293 tetracycline inducible cells (this allows for a single integration event, and tunable expression54,66). These reagents will also be used as baits for AP-MS analysis (many of these proteins have already been cloned and expressed in the Flp-In T-REx cells). We have access at the Lunenfeld to collections of full-length cDNAs that should encompass most of the discovered interactors. This strategy is routinely used in our group, and should not present major difficulties. In the case where expression of a given interaction partner proved difficult, we will employ alternative expression systems which we have done extensively in the past (e.g. 67,68). In addition, as we have done for CCM3 and CCM2, mutagenesis of CCM1 will be performed to eliminate isolated protein domain-mediated interactions and attempt to provide more structural information; when appropriate, the same strategy will be undertaken for the newly identified interactors. Similar mutations will be introduced to the orthologous worm genes and expressed in the appropriate knock out strain to determine which residues are important for their *in vivo* functions. Taken together, this should expand the knowledge of the molecular context into which the CCM proteins and their genetic interactors reside. New physical interacting partners will also be functionally characterized in cells and *C. elegans* (Aim 2).

***B. Investigate the CCM interaction networks in endothelial cells.*** Our preliminary data indicate conservation of the CCM3•kinase•STRIPAK complex across cell types; however, it is possible that we may have missed interaction partners relevant to the disease by performing our initial analyses in HEK293 cells. To address this issue, we will characterize the interaction profiles of CCM1, CCM2 and CCM3 in endothelial cells. We have already demonstrated that antibodies to endogenous proteins can be used to perform AP-Western and AP-MS (Appendices 1 and 4). ***Methods****:* We will use antibodies to endogenous CCM1, CCM2 and CCM3 proteins to identify interaction partners from endothelial model cell lines, including HUVEC, and bovine lymphatic, venous and aortic endothelial cells (all provided generously by D. Dumont), using protocols well established in the Gingras lab (Appendices 1,4)51,55,69. Control samples will include isotype-matched antibodies and antibodies to unrelated proteins to eliminate common contaminants and multiple affinity reagents will be used per protein analyzed. ***Alternative strategy****:* If any endogenous protein cannot be efficiently immunoprecipitated, AP-MS will be performed using recombinant proteins expressed with lentivirus, as we recently have done68. If endogenous antibody cross-reactivity is an issue, AP-MS will be performed after knocking down the bait protein and analyzing the resulting interactions by quantitative proteomics (as in 66). ***Expected outcomes:***we may uncover additional endothelial specific CCM interactors: orthologs in *C. elegans* will be investigated as described in Aim 2. We also note that, while the endothelium is clearly affected in the disease, CCMs may also have a neuronal, and/or smooth muscle defect, as the SMC layer is missing from CCM lesions, and a tissue-specific knock-out of CCM3 in neurons exhibits CCM-like phenotypes47. If no new partner for CCM is detected in endothelial cells, neuronal and smooth muscle interactors will be investigated.

***C. Define the physical CCM interactome of C. elegans.*** We have not been able to identify a CCM2 orthologue in *C. elegans* by sequence searching. Given that we consistently detect strong physical interactions between CCM1 and CCM2 in mammalian cells and that CCM2 is able to bridge CCM1 and CCM3, we hypothesize that a functional orthologue of CCM2 exists in worms and binds CCM1 and/or CCM3 *in vivo*. Several proteins encoded in the *C. elegans* genome that are below the limit of detection by sequence homology have been identified and shown to exhibit structurally homology to mammalian proteins. For example, Dr. Derry was the first to identify the elusive *C. elegans* p53 protein (CEP-1) which has only ~15% sequence identity with human p53 but 74% structural homology with the DNA binding domain of human p5370,71. We propose to use the available GFP-tagged transgenic lines for worm CCM1 and CCM3 (as well as negative controls) to perform AP-MS analysis. Alternatively, we will raise polyclonal antibodies to *C. elegans* CCM1 and CCM3 to use for the purification of the endogenous proteins from cell lysate prepared from *C. elegans*72. Interacting proteins will be identified by mass spectrometry, as described above. ***Alternative strategy and expected outcomes***: Given that our proteomics methods have worked well, we anticipate no major drawbacks in *C. elegans*, and we expect to identify not only a CCM2 ortholog, but also additional binding partners for worm CCM1 and CCM3 (these may or may not be the orthologs of the binding partners identified in mammalian cells). As an alternative strategy to identify CCM2, we will use RNAi to knock down the expression of the thirteen *C. elegans* proteins that contain phosphotyrosine binding (PTB) domains (the most conserved feature of the vertebrate CCM2 protein as well as the ICAP1 protein for which no worm ortholog can be detected, with the possible exception of Y45F10D.10) and determine if they are synthetically lethal with CCM3, or required for CCM1/*kri-1*-dependent apoptosis (see Aim 2). Note that as the genetic screens described in Aim 2 progress, we may also identify a CCM2 ortholog on the basis of genetic interaction with CCM3. If the putative CCM2 ortholog is detected by genetic means, the first step of validation will be to determine whether it can physically associate with worm CCM1 and/or CCM3, both by co-immunoprecipitation/immunoblotting and via the use of recombinant proteins expressed in bacteria (e.g. Appendix 3). If we uncover new partners not detected in mammalian cell lines, interaction between the human orthologs will also be assessed. This work will set the stage for future work testing the therapeutic potential of these proteins for suppressing the phenotypes associated with loss of CCM gene function.

***D. Analyze regulated interactions for the CCM proteins.*** As knowledge of the signaling pathways leading to CCM signaling expands, we will revisit which of the interactions are regulated; this aim will be integrated with the phosphorylation analysis described below. ***Methods***: We already have cell lines expressing tagged CCM1, CCM2, CCM3, ICAP1 and the three GCKIII kinases. Each cell line will be subjected to a number of treatments known to affect CCM signaling. Briefly, the tagged proteins will be purified in parallel, and analyzed either by IP-western using antibodies to the known partners, or by quantitative proteomics using label-free quantification as in Appendix 4. For the characterization by IP-Western, we will establish time-course and dose-dependence curves. Because of the reported role of the GCKIII kinases (and CCM3) in stress pathways, we will first explore oxidative stress (using H2O2 73-75), osmotic stress (400mM sorbitol), chemical anoxia (sodium cyanide and 2-deoxyglucose76) and staurosporine34. In addition, we will investigate whether stimulation of endothelial cells with vascular endothelial growth factor (VEGF)32 or of neurons with nerve growth factor (NGF)28 alters the interactions. ***Alternative strategy:*** In our hands, the label free quantitation has been working well for identifying regulated protein interactions, but if this was not successful here, we would employ the SILAC (stable isotopic labeling with amino acids in cell culture77) approach which has been optimized at our institute78 (see 79 for a review). ***Expected outcomes:*** We expect that one or more of the interactions will be regulated by selected treatments. Post-translational modifications detected by mass spectrometry are also anticipated. Once we identify regulated interactions, we will focus on their characterization: 1) are the interactions regulated by other physiologically relevant treatments? 2) do these interactions require post-translational modifications? 3) what are the consequences of abrogating the interaction, or of generating a constitutive interaction?

***E. Assess the organization of the CCM interaction network.*** As shown in Appendices 3 and 4, we have been able to identify at the molecular level how interactions between STRIPAK, CCM3 and the GCKIII kinases occur. Given the relevance of CCM proteins and their binding partners in CCMs, we will pursue this work with the newly identified physical and genetic interaction partners of CCM proteins. ***Methods***: Truncation analysis followed by immunoblotting or mass spectrometry (as in Appendix 4, Fig 1) will be performed to uncover the regions of each of the proteins mediating interactions (focusing on regulated interactions). We will also, when appropriate, deplete selected proteins by RNA interference and monitor the recovery of binding partners after immunoprecipitation and immunoblotting (or MS), as in Appendix 4. Taken together with quantitative proteomics information, this will help us narrow down direct interactors. As we did in Appendices 3 and 4, we will then express the proteins in bacteria (or the baculovirus expression system), and monitor direct interactions. Lastly, we will refine the mapping of the direct protein-protein interactions by mutagenesis and binding assays, as we are doing routinely e.g. 49,55,80, and by peptide array strategies as in Appendix 4. This work will be performed in a continued collaboration with F. Sicheri (letter attached). ***Expected outcomes:*** We do not anticipate any major conceptual difficulty with this aim, but technical difficulties may arise. Some of the proteins or protein fragments may be difficult to express, or exhibit poor solubility, for example. In this case, we typically adopt different expression systems (e.g. baculovirus or *in vitro* transcription/translation67), or alter the construct boundaries or the species used (e.g. 81). Silencing the expression of a given protein may in itself alter the expression or stability of its binding partners (as we observed in 49), which would complicate the analysis of the silencing experiment. Despite these difficulties, we should be able to provide a much clearer picture of the molecular assembly of the CCM pathway components. As we identify substrates and interactors in Aims 1-3, the information regarding post-translational modifications (and especially phosphorylation) will be interpreted within the context of this binding site mapping.

***Aim 2: Defining the CCM genetic interaction network***

***A. Phenotypic assessment for physical interaction partners and potential substrates:*** As we have done for the STRIPAK components (preliminary data), we will test each of the interaction partners detected in Aim 1 and the high confidence potential substrates from Aim 3 for CCM1- and CCM3-associated functions in human cell lines and in *C. elegans*. ***Method 1***: In *C. elegans*, confirmed binding partners (or orthologs of human partners) will be analyzed for CCM1 (*kri-1*)-related functions by assaying for resistance to germline apoptosis as we have described (i.e. 26,82-84) and hypersensitivity to osmotic stress (Fig 10). To determine CCM3-related functions, we will ablate candidates by RNAi and look for truncations and cyst formation in excretory cells of living animals (Fig 11). ***Method 2:*** Interaction partners and substrates identified above will be knocked down by RNAi to determine their role in a) localizing MST4 at the Golgi and affecting Golgi polarization (Appendix 4), b) modulating the expression of Rho proteins17, c) protecting from or promoting apoptosis85, and d) signaling through MAP kinase pathways30. As new biological readouts become available (including through Aim 1D), new assays will also be implemented. ***Expected outcomes***: The advantage of these functional validation approaches is that they are scalable, enabling us to test dozens of candidates in parallel. While we do not expect all of them to generate a clear phenotype across all readouts, this strategy will help us prioritize hits to follow up in depth.

***B. Genetic modifier screen in C. elegans to uncover novel CCM3 signaling proteins:*** Genes operate in signaling pathways that control all biological processes, and most developmental processes require collaboration between distinct signaling pathways to ensure robustness. The key advantages of using *C. elegans* as model system to understand how genes collaborate during development and in disease states are its amenability to genetic manipulation, the ability to study genes in a multicellular setting, and the high degree of conservation in gene structure and function with humans86. We hypothesize that delineating genetic interactions with *ccm1* and *ccm3* in *C. elegans* will uncover conserved pathways in vertebrates relevant to the pathology of CCMs. ***Methodology*:** *C. elegans* use bacteria as a source of food and growing these animals on bacteria that produce double-stranded RNA to worm genes result in potent and specific knock down of the endogenous gene in these animals and their progeny86b. This RNAi approach allows for rapid, whole-genome screens86c, 86d. We found that knocking down via RNAi the *C. elegans* *ccm3* gene in *ccm1* mutant animals results in synthetic lethality (Fig 14), suggesting that these genes function in parallel signaling pathways. Furthermore, ablation of *ccm3* by RNAi or mutation causes a severe truncation of the excretory cell (Fig 11), the only vasculature structure present in *C. elegans*. We hypothesize that other genes that cause synthetic lethality in *ccm1* mutants will reveal additional components of the *ccm3* pathway. To achieve this goal, we will first systematically knock down worm genes in *ccm1* mutants by the RNAi feeding method as we have previously described82-84. Genes that cause synthetic lethality when knocked down in *ccm1* mutants will be examined for defects in development of the excretory cell. Briefly, transgenic lines that express *pes-6*::GFP in the excretory cell (Fig 9C) will be grown on bacteria producing double-stranded RNA to genes that exhibit synthetic lethal interactions with *ccm1*. The effect of inhibiting these genes on excretory cell length and morphology will be monitored over a period of 1 week and quantified in staged young adult animals. From a pilot RNAi screen of chromosome 1 we identified 35/~2,700 genes that exhibit synthetic lethality in *ccm1* mutants, of which four are required for proper excretory cell development (Fig 14-15). In addition, since we have identified a role for *ccm1* and *ccm3* in osmoregulation we will also evaluate this for candidate genes by growing animals on bacterial RNAi plates containing 400 mM NaCl then quantifying viability (Fig 10). We anticipate that it will take approximately 24 months to complete the rest of the genome. Analysis of excretory cell lengths, osmotic stress, and other phenotypes (i.e., apoptosis) will take an additional 12-24 months, depending on the number of positive hits obtained in the genome-wide screen. Based on the number of genes identified on chromosome 1 we anticipate uncovering 25-50 more genes (5-10 genes per chromosome – *C. elegans* has five autosomes and one sex chromosome) that function in the *ccm3* pathway to regulate excretory cell morphology. Prioritization of potential *ccm3* pathway genes will be as follows: (i) reproducible synthetic lethal interactions with *ccm1*, (ii) defects in excretory cell development (when knocked down alone or in *ccm1* mutants), and (iii) conservation of interacting genes in vertebrates. Genes that fulfill all three of these criteria will be prioritized for future analysis of vascular phenotypes in mouse models with our collaborator Dr. Murat Gunel (see letter of collaboration) and pipelined for identification of physical interaction partners for mammalian orthologs (Aim 1)

***C. Expression patterns and tissue-specific requirements for CCM3 signaling proteins.***

To determine where the novel CCM pathway genes are expressed we will clone their promoters and open reading frames and generate GFP fusions. These constructs will be injected into worms to generate transgenic lines in order to visualize their spatiotemporal expression patterns throughout development, which is routinely carried out in the Derry lab. The expression constructs will also be used to rescue excretory cell defects and other phenotypes in strains carrying loss-of-function mutations in candidate genes. Combined, the *C. elegans* knockout consortium and Million Mutation Project has generated over 9,000 null alleles, and the list is growing (D. Moerman, pers comm.). If a knockout does not exist for the top hits identified in our RNAi screen we will make our own deletion by standard mutagenesis and PCR screening, as we have previously done70. Once we establish the tissues in which these genes are required we will attempt to rescue the various phenotypes (excretory canal truncations, osmotic stress, etc) by cloning the gene into tissue-specific expression vectors. This will be critical for understanding their biological functions in a multicellular setting since CCM proteins have important roles in cross-tissue signaling, as we demonstrated for the worm *ccm1* gene, *kri-1* (Appendix 2). We are currently generating tissue-specific promoters driving *kri-1* or *ccm3* only in the excretory cell (*pes-6* promoter), the intestine (*elt-2* promoter), and other tissues to determine the tissue of focus for excretory cell morphology, apoptosis, and osmotic tolerance. These experiments will set the stage for understanding how CCM proteins collaborate with their binding partners to control their various biological functions through autonomous and/or non-autonomous signaling.

***Aim 3: Identify substrates for GCKIII kinases***

Very few substrates have been uncovered for the GCKIII family members, and a consensus site for phosphorylation is not yet clearly defined. We propose a comprehensive approach to identify *bona fide* substrates using active versions of each kinase (Appendix 3) and cell lines expressing FLAG-tagged versions of each kinase, including point mutants that abolish their catalytic activity.

***A. Candidate approach***. We postulate that one or more of the interaction partners for CCM3•kinase pairs may be a substrate for the GCKIII kinases. Even though kinase-substrate interactions have not been generally regarded as stable enough to withstand biochemical purification, there has been an increasingly high number of interactions pairs confirmed to be enzyme-substrate partners. Re-analysis of our yeast kinome data51 by computational biologists revealed a statistically significant enrichment in the interaction partners for consensus motifs associated with each kinase (P.M. Kim, pers comm.), and preliminary data in Mike Fainzilber’s laboratory has identified CCM2 as a putative target for STK25. We therefore propose to test whether components of the CCM1•CCM2•ICAP1 complex, STRIPAK, or new interaction partners (identified genetically or physically) can be substrates for any of the three kinases. ***Methods***: Active and dead kinases will be incubated with recombinantly expressed proteins in the presence of -32P-ATP and incorporation monitored by autoradiography. We will purify previously reported substrates of these kinases (14-3-3zeta, ezrin) to serve as positive controls. Once a putative substrate is identified, phosphorylation reactions will be performed in the absence of 32P, and sites identified by mass spectrometry. As a final level of confirmation, we will perform alanine substitution and test for phosphorylation, as we have described87-89. ***Caveats and expected outcomes:*** This approach will be limited to those proteins that we can express recombinantly, though expression of our constructs in other systems (e.g. in the transient mammalian T7/vaccinia hybrid expression system90) could also be performed. *In vivo* phosphorylation mapping will be attempted as described below.

***B. Global in vitro/in vivo analysis***. In order to complement the candidate approach described above, we will exploit an unbiased approach for the identification of kinase substrates (Fig 19). ***Methods***: Endothelial cell lysates will be treated with the ATP analog FSBA to inactivate all endogenous kinases, and desalted lysates will be incubated with one of the GCKIII kinases (active or dead). After optimization of the conditions using radioactive assays, we will scale up the reactions and couple them with quantitative mass spectrometry using isotopic based labeling (we have used dimethyl-labeling for the preliminary data showed in Fig 20, but will alternatively employ the widely used stable isotope labeling with amino acids in cell culture; SILAC77,79,78). We will perform biological replicates analysis, permuting the kinases and isotopic labels. After phosphorylation assays, the kinases will be inactivated, lysates subjected to proteolysis and phosphopeptide enrichment with titanium dioxide and IMAC. The phosphopeptides will be identified and quantified using high mass accuracy mass spectrometers. Statistically significant changes in phosphorylation will be analyzed to identify potential phosphorylation motifs. *In vitro* phosphorylation may not be fully representative of the situation *in vivo*, and a modification of the substrate screening approach has been developed to emphasize substrates more likely to be biologically relevant (Fig 19, 21). This approach exploits a parallel mapping of sites dependent on the presence of a kinase *in vivo*, and those which can be directly targeted *in vitro*. When kinase inhibitors are available, they will be used; however, in the case of the GCKIII kinases, we will employ cells in which individual kinases have been depleted by RNAi knock-down. Alternatively, in collaboration with Geoff Hicks (U Manitoba) we are generating mouse ES knockout lines for each of the three kinases as well as a knock-in line in which the kinase binding site of CCM3 has been deleted. Each of these cell lines will be compared to wild-type in phosphorylation mapping experiments. ***Caveats and expected outcomes***: We have performed a preliminary substrate identification analysis for MST4 in HEK293 cells lysate, and found several possible targets, including the STRIPAK component MOB3, which we have validated. As an alternative approach to the FSBA method (in case it does not work as well with STK24 or STK25), we would investigate the use of a gatekeeper-mutated kinase and bulky-ATP analog (i.e. the Shokat kinases91). It is also possible that pre-phosphorylation of the sites in the lysate may occlude the effect of the kinases. If this is the case, we will pre-treat the lysate with a phosphatase prior to adding the kinase or use lysate deficient in the GCKIII kinases. Lastly, while we believe that isotope-based approaches are the simplest for this strategy, if necessary we will consider other quantitative mass spectrometry methods, including SRM92, TRAQ labeling93, or the SWATH technique that we recently implemented in the lab (Fig 18). While we will initially attempt the phosphoproteomics experiments using mouse ES cells, they will be repeated after differentiation into endothelial/neuronal lineages (and in co-cultures).

***C. Validation of the substrates in vitro and in vivo***. ***Methods***: Candidates substrates identified in Aims 3A and 3B will be validated as follows: 1) for substrates for which we already have stable cell lines, quantitative phosphoproteomics will be performed following depletion of individual kinases (or all three kinases, simultaneously) by RNAi and immunoprecipitation of the FLAG-tagged bait protein (similar to what we have done with STRIPAK components following treatment with okadaic acid in Fig 17, using our newer more accurate quantification methods). Phosphorylation events that can be recapitulated in cells will be further investigated (see below). 2) For substrates identified in Aim 3B, we will first validate the phosphorylation *in vitro* as in 3A; validated sites will be analyzed in cells and in *C. elegans*, as above. 3) We will perform site-directed mutagenesis to confirm the identification of the phosphorylation site and the kinase-substrate relationship; these mutants will also be used to address the physiological consequences of phosphorylation. 4) For sites of particular interest (e.g. those in MOB3, CCM2, etc.), we will raise phosphospecific antibodies87,88 to examine in details signaling through the CCM proteins. ***Expected outcomes***: We have already identified a number of phosphorylation targets of MST4, including MOB3 for which we have initiated validation. Other highly modulated putative substrates, and substrates biologically linked to CCM signaling will be pursued for validation studies, as below. Our plan for hit prioritization is depicted in Fig 21.

***Alternative strategies. Forward genetic screen in C. elegans ccm3 mutants.*** While we expect that our RNAi screen may uncover some direct targets of the GCKIII kinases, forward mutagenesis screens have identified kinase substrates in *C. elegans*. One advantage of a mutagenesis screen compared with RNAi-based screening is that rare gain-of-function alleles can be identified, which is important if the target of the kinase becomes activated by phosphorylation. For example, gain-of-function mutations in the *pdk-1* and *akt-1* genes were identified from mutagenesis screen carried out in *age-1*/PI3K loss-of-function mutants97. The *pdk-1* gene is directly downstream of *age-1*/PI3K and the AKT-1 kinase is phosphorylated and activated by PDK-1. There are many more examples of how forward genetics has uncovered kinase substrates and signaling pathways in *C. elegans*, so we expect this to be a fruitful alternative to the proteomics approaches. ***Expected outcomes***: These screens are routinely carried out in the Derry lab and we anticipate no major problems with this method. Top candidates will be prioritized based on how well they restore excretory cell morphology in *ccm3* mutants, then pipelined for kinase assays, as described above.

**D. PERSPECTIVES.**

The proposed studies in this application are the result of collaborative work initiated two years ago between Anne-Claude Gingras and Brent Derry. The serendipitous discoveries of new biological roles and binding partners for CCM proteins presented a unique opportunity to combine our strengths to understand the molecular mechanisms that underlie this devastating disease. The two papers we recently published in JBC are a testament of our commitment to work together in this new and emerging area. We will exploit the complimentary tools of proteomics and genetics to understand how the CCM3/STRIPAK pathway contributes to vascular integrity in *C. elegans* and discover new components of this network. We have assembled a team of outstanding collaborators with expertise in all facets of this project, including Dr. Frank Sicheri (structural biology), Dr. Geoff Hicks (ES knockouts, knock-ins), and Dr. Ian Scott (zebrafish models of CCM). This project represents a new direction of research for Dr. Derry and Dr. Gingras, and is independent of their other CIHR-funded work. While we are still at the discovery phase of the molecular mechanisms of CCMs, the proposed project is performed in close collaboration and consultation with Dr. Murat Gunel, a neurosurgeon and CCM expert, who will follow-up on candidates detected in this study to determine if the corresponding genes are mutated in patient samples. We are also communicating our results to Dr. Issam Awad, another leading CCM clinician who is currently performing pre-clinical trials for ROCK inhibitors in CCM mouse models and is seeking other therapeutic targets. We anticipate that this work will uncover new proteins that can be targeted for the clinical treatment of CCM disease in humans, which will offer hope for patients.

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