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Endogenous endothelial cell signaling systems maintain vascular stability

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Abstract

The function of the endothelium is to provide a network to allow delivery of oxygen and nutrients to tissues throughout the body. This network is comprised of adjacent endothelial cells which utilize adherens junction proteins such as vascular endothelial cadherin (VE-cadherin) to maintain the appropriate level of vascular permeability. The disruption of VE-cadherin interactions during pathologic settings can lead to excessive vascular leak with adverse effects. Endogenous cell signaling systems have been defined that help to maintain the proper level of vascular stability. Perhaps the best described system is Angiopoietin-1 (Ang-1). Ang-1 acting through its receptor Tie2 generates a well described set of signaling events ultimately leading to enhanced vascular stability. In this review we will focus on what is known about additional endogenous cell signaling systems that stabilize the vasculature, and using Ang-1/Tie2 as a model, we will address where our understanding of these additional systems is lacking.

Keywords

CCM; Permeability; Robo4; Vascular Stability; VE-cadherin

Introduction

The vascular system provides an essential network allowing blood to flow throughout the body. At the interface between the blood and various organs is the endothelial cell which lines all blood vessels. This endothelial cell barrier forms a semi-permeable membrane that regulates the passage of fluid, nutrients, and leukocytes out of the blood and into the interstitial space [1,2]. In order to regulate barrier function, adjacent endothelial cells utilize adherens and tight junctions to maintain strong cell-cell contacts [3]. Further maturation and stabilization of the capillary occurs through tight endothelial associations with pericytes [4]. Under normal conditions, the endothelium of a mature capillary is quiescent, stable, and limits vascular leak. Many pathologic conditions cause a de-stabilization of the vascular network resulting in endothelial hyper-permeability, excessive vascular sprouting, and angiogenesis. These pathologic settings include tumor angiogenesis, proliferative diabetic retinopathy, age-related macular degeneration (AMD), and cerebral cavernous malformations (CCM) [5-7].

Destabilization of vascular barrier function occurs when interactions between inter-endothelial junction proteins are disrupted. In the endothelium, these critical vascular stabilizing interactions are provided by the adherens junction protein vascular endothelial cadherin (VE-cadherin) (Fig 1a) [2,8]. This is supported by evidence that multiple antibodies directed to the extracellular domain of VE-cadherin can disrupt endothelial barrier function *in vitro* [9]. Furthermore, a VE-cadherin blocking antibody can cause a concentration and time dependent increase in permeability in the lung and heart *in vivo* [10]. In the cell, breakdown of VE-cadherin interactions can be mediated by phosphorylation (Fig 1b). Interestingly, phosphomimetic mutations at Y658 and Y731 were sufficient to cause decreased barrier function in CHO cells [11]. Furthermore, multiple permeability inducing factors cause phosphorylation of VE-cadherin including histamine [12], lipopolysaccharides [13], and vascular endothelial growth factor (VEGF) [14]. Kinases are necessary to propagate these signals and phosphorylate the cytoplasmic tail of VE-cadherin. VEGF, for example, activates Src, a non-receptor kinase necessary for the permeability inducing effects of VEGF. The necessity of Src has been demonstrated as VEGF-induced permeability in the dermis and brain was inhibited in *Src*^{-/-} mice [15]. Phosphorylation of the cytoplasmic tail of VE-cadherin is also regulated by phosphatases such as vascular endothelial protein tyrosine phosphatase (VE-PTP). In fact, over-expression of VE-PTP inhibited VEGF receptor 2 (VEGFR2)-mediated phosphorylation of VE-cadherin resulting in enhanced barrier integrity [16].

Phosphorylation of VE-cadherin not only disrupts homophilic interactions, but can also result in endocytosis of VE-cadherin and removal from the cell surface. For example, VEGF causes Src-dependent activation of the Rac pathway, ultimately resulting in phosphorylation of a serine residue on the cytoplasmic tail of VE-cadherin [17]. This phosphorylation recruits β -arrestin which then mediates VE-cadherin endocytosis in a clathrin-dependent manner. Cadherin endocytosis and turnover is also regulated through cytoplasmic binding partners such as p120-catenin (Fig 1c) [18]. This has been demonstrated as a direct p120-catenin interaction inhibits VE-cadherin endocytosis [19]. Furthermore, this interaction has been demonstrated to be necessary for maintaining endothelial barrier function [20].

In addition to breaking down vascular barrier function, pro-angiogenic cues such as VEGF destabilize the vascular network by causing sprouting of new blood vessels. This occurs by enhanced endothelial cell proliferation, breakdown of the endothelial barrier, and directional migration towards a signal gradient [21]. At the forefront of the vascular sprout is the endothelial tip cell. Tip cells utilize multiple filopodial extensions to sample the environment and follow chemotactic gradients, thus determining the direction the sprout will grow [22]. In contrast to tip cells, the endothelial cells that trail behind the tip cells, known as stalk cells, form the lumenized vascular network. These cells perform similar functions as the quiescent mature capillary. In this review, we will address newly identified endogenous endothelial cell signaling systems that enhance vascular stabilization.

Ang1-Tie2

Angiopoietin-1 (Ang-1) like VEGF is a potent pro-angiogenic factor, but conversely stabilizes the vasculature against vascular leak. The receptor for Ang-1 is the endothelial-specific receptor Tie-2. When over-expressed in the dermis, Ang-1 induces enhanced neovascularization. When combined with VEGF over-expression, Ang-1 had an additive effect on neovascularization but stabilized these vessels as evidenced by decreased Evans Blue extravasation in the mouse ear (Fig 2) [23]. Furthermore, Ang-1 can inhibit permeability induced by additional factors such as mustard oil [24].

How Ang-1 specifically inhibits the permeability inducing effects of VEGF has recently been described. While Ang-1 administration did not affect VEGF-induced signaling events

necessary for endothelial proliferation, Ang-1 inhibited VEGF-induced activation of Src [25]. Interestingly, Ang-1 activates Rho and both Rho siRNA knockdown and a Rho inhibitor blocked the ability of Ang-1 to inhibit VEGF-induced permeability *in vitro*. A Rho inhibitor also blocked Ang-1 *in vivo* as assessed by Evans Blue extravasation in the mouse dermis. This demonstrates that Ang-1 inhibits VEGF-induced permeability through a Rho-dependent mechanism. Furthermore, Ang-1 stimulates the dimerization of mDia1 and mDia2, downstream targets of Rho signaling. Ang-1 also stimulates the binding of mDia to Src, suggesting that Ang-1 inhibits VEGF-induced permeability through mDia mediated sequestration of Src. Knockdown of either mDia1 or mDia2 using siRNA inhibited the activity of Ang-1; further demonstrating that mDia1 and mDia2 are indeed necessary for Ang-1 mediated signaling [25]. These data demonstrate that Ang-1 specifically stabilizes the endothelium against VEGF-induced permeability by inhibiting Src through a Rho/mDia dependent pathway (Fig 3a, b). Furthermore, defining these signaling events has linked the ligand and receptor to a central mechanism regulating VE-cadherin stability.

Vascular instability can also be manifest in the form of vascular malformations. Activating mutations in the kinase domain of Tie2 result in a rare hereditary form of mucocutaneous venous malformation [26], and somatic mutations of Tie2 have recently been described in a high percentage of sporadic venous malformations [27]. Recently, additional ligand receptor signaling pathways have been hypothesized to promote junctional stability of the endothelium, though a detailed understanding of their mechanistic pathway does not match that of Ang-Tie2.

Dll4-Notch

The necessity of Delta-like 4 (Dll4)-Notch1 signaling in the endothelium has been well established as loss of a single copy of Dll4 or deletion of Notch1 causes vascular defects and embryonic lethality [28,29]. When Dll4 activates Notch1, a portion of the intracellular domain of Notch1 is cleaved through a γ -secretase dependent mechanism. The resulting fragment known as the Notch1 intracellular domain (ICD) translocates to the nucleus where gene expression changes are enacted [30]. Notch signaling plays a well defined role in cell fate decisions through a process known as lateral inhibition. A classic example in *Drosophila* is neural-epidermal determination where cells that have adopted a neural cell fate signal to adjacent cells to adopt an epidermal cell fate. When Notch signaling is removed, all cells adopt a pro-neural cell fate [31].

In the endothelium, VEGF induces endothelial cells to adopt a tip cell phenotype. However, the cell signaling systems that keep all endothelial cells from becoming tip cells have just recently been described. These studies have demonstrated that Dll4 from the endothelial tip cell signals through Notch on adjacent stalk cells to become stalk cells rather than tip cells. During vascular patterning, this allows for the correct number of endothelial tip cells and vascular sprouts. Using a γ -secretase inhibitor to inhibit Notch signaling, Hellstrom et al. [32] found increased filopodial protrusions in endothelial tip cells of the mouse retina. When given over an extended period of time, Notch inhibition resulted in increased vascular sprouting, density, and disrupted normal vascular patterning. In a separate experiment, inhibition of Notch1 signaling by endothelial-specific genetic deletion, or using *Dll4*^{+/-} mice also caused enhanced tip cell filopodial extensions and vascular sprouting. Furthermore, activation of Notch signaling reduced tip cell filopodia and vascular density [32].

The role of Dll4-Notch signaling not only applies to developmental angiogenesis, but also applies to pathologic neovascularization. Mice undergoing oxygen-induced retinopathy (OIR) and given a γ -secretase inhibitor displayed enhanced pathologic angiogenesis [32]. Additionally, in tumors over-expressing Dll4, a marked decrease in tumor angiogenesis was observed [33]. Interestingly, tumors over-expressing soluble Dll4, which acts as an inhibitor

of Notch signaling, caused enhanced angiogenesis. Taken together, these studies demonstrate the importance of Dll4-Notch signaling in inducing a stalk cell fate decision and the effect this has on developmental and pathologic neovascularization (Fig 2). In addition to its differentiation state, cell-cell contacts distinguish stalk cells from tip cells and play an important role in vascular stability. While the Dll4-Notch ligand receptor interaction appears to utilize the canonical Notch downstream signaling pathway, how the nuclear translocation of the Notch ICD distinguishes tip cell from stalk at the level of cell-cell interactions remains to be explored (Fig 3c).

Slit-Robo4

After a decision has been made to be a stalk cell instead of a tip cell, there must be additional cues that help the stalk cells maintain a quiescent, stabilized phenotype. Recent data has demonstrated that Slit-Robo4 signaling may be this signal. The Roundabout (Robo) family of single-pass transmembrane receptors is comprised of four members, Robo1-4, with members of the Slit family as ligands. Robo1-3 are expressed in the nervous system and have a well established role in axon guidance [34]. Conversely, Robo4 expression is limited to the endothelium [35,36]. Because the neural and vascular systems share similar patterning mechanisms, this suggests a role for Robo4 in vascular guidance. However, in studying this question *in vivo*, an unexpected role for Robo4 in vascular stability was uncovered.

Robo4^{-/-} mice are viable, fertile, and show no vascular patterning defects in the cephalic or intersomitic vessels [37]. This demonstrates that Robo4 expression is not necessary for vascular guidance during mouse development. Interestingly, Robo4 expression in the mouse retina was found in the endothelial stalk cells but was often absent in the tip cells. As previously mentioned, endothelial tip cells are important for vascular guidance. The lack of Robo4 expression in the tip cell could perhaps explain why Robo4 does not play a role in vascular guidance *in vivo*. It would be interesting to see if Dll4 from the tip cell was responsible for inducing stalk cell specific expression of Robo4. While endothelial tip cells are important for vascular guidance, stalk cells are similar to a mature, lumenized vascular tube that is important for regulating fluid leak and vascular stability. Vascular barrier function can be modeled *in vitro* by measuring flux of a reporter across an endothelial monolayer. By using mouse lung endothelial cells from *Robo4*^{+/+} and *Robo4*^{-/-} mice in this system, Jones et al. [37] found that Slit2 inhibited VEGF-induced permeability through a Robo4-dependent mechanism. Slit2 also inhibited VEGF-induced permeability *in vivo* in the mouse dermis and retina as measured by Evans Blue dye extravasation. The effect of Slit2 was lost in *Robo4*^{-/-} mice, further demonstrating that Robo4 is necessary for the effect of Slit2 *in vivo*. As previously mentioned, VEGF acting through its receptor VEGFR2, activates Src which is necessary for VEGF-induced vascular destabilization. Although Slit2 did not inhibit VEGF-induced autophosphorylation of VEGFR2, Slit2 inhibited VEGF-induced Src activation. This demonstrates that Slit2 signaling acts downstream of VEGFR2 activation to inhibit Src signaling. Furthermore, Slit3 also inhibited VEGF-induced Src activation and retinal permeability *in vivo*, demonstrating that additional Slit family members also enhance vascular stability. Additionally, *Robo4*^{-/-} mice demonstrated an increased level of permeability in the retina under basal conditions. Taken together, these data demonstrate a role for Slit-Robo4 signaling in inhibiting vascular permeability and enhancing vascular stability (Fig 2).

A stabilized, quiescent phenotype not only applies to inhibiting excessive vascular hyperpermeability, but also is important in inhibiting new vascular sprouting and angiogenesis. This occurs during pathologic conditions such as proliferative diabetic retinopathy and AMD. These pathologic conditions can be modeled in the mouse by oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV) respectively. Slit2 inhibited neovascularization in both these models, demonstrating that Slit2 can inhibit angiogenesis in

a pathologic setting. Interestingly, the effect of Slit2 was lost in *Robo4*^{-/-} mice, again demonstrating that Robo4 is necessary for the effect of Slit. Furthermore, *Robo4*^{-/-} mice demonstrated enhanced angiogenesis in OIR, further exemplifying the importance of Robo4 in limiting excessive vascular destabilization in pathologic settings. Taken together, these data suggest that the role of Robo4 *in vivo* is to maintain vascular stability. Although vascular guidance may appear similar to neural guidance, it is important to remember that while an axon arises from a single cell, blood vessels are multi-cellular systems. This may explain why a neural repulsive cue such as Slit may translate into a vascular stability cue when applied to the multi-cellular blood vessel.

In contrast to studies demonstrating a repulsive effect of Slit [37-40], other studies suggest a pro-angiogenic role for Slit [41,42]. These studies show that Slit increases endothelial cell migration and tube formation *in vitro*. One report suggests that the receptor responsible for this effect is Robo1 while the other proposes a heterodimer complex between Robo1 and Robo4 [41,42]. Of special interest is data that Slit might stimulate migration of Robo1 expressing cells, in contradistinction to the original report that described many of the core reagents used in these subsequent studies and that demonstrated Slit inhibited Robo1 expressing non-neural cells [42,43]. Other studies have favored a Slit independent role for Robo4 and suggested this role is proangiogenic [40,44]. These studies propose that Robo4 plays no part in a receptor complex that mediates Slit signaling and either functions in a ligand independent fashion similar to adhesion proteins or mediates the signaling of a yet unknown set of ligands. Clearly work is needed to reconcile these differences, with an emphasis on relating *in vitro* and cell culture findings to animal models lacking Robo receptors. Such an approach is important as consistent functional readouts that span cell biology and animal models are required to confirm the relevance of any potential downstream mechanism. Given that we are at such an early stage, we remain cautious of any strongly held convictions as the literature is replete with ligands having unexpected receptors [45], receptors having unexpected ligands [46], and transmembrane proteins participating as both adhesion proteins and ligand mediated signaling complexes [47].

While the ligand-receptor system for Ang1-Tie2 and Dll4-Notch have been well defined, little is known about how Slit might activate Robo4. Studies conducted in our laboratory using *Robo4*^{-/-} mice, and lung endothelial cells derived from these mice, have demonstrated that Robo4 is necessary for the effect of Slit *in vitro* and *in vivo*. Our lab has also shown previously that Slit binds to the surface of Robo4 expressing but not control HEK cells [38]. A second recent report has corroborated this finding by demonstrating that Slit no longer binds to endothelial cells when endogenous Robo4 expression is knocked down by siRNA [40]. However, others have found using BiaCore that Slit1-3 do not interact with Robo4, suggesting that Slit does not directly bind to Robo4 [44]. One potential explanation is that Robo4 forms a complex with a co-receptor. Such a model has been suggested for other Robo receptors as heparan sulfate proteoglycans are necessary for repulsive guidance activities of Slit2 *in vitro* [48]. In this study, removal of cell surface heparan sulfate using heparinase III resulted in the loss of Slit2 repulsive activity for olfactory interneuron precursors and olfactory bulb axons.

Many questions about the mechanism of Robo4 signaling remain (Fig 3d). To address these questions, the following experiments must be done. The cytoplasmic domain necessary for Robo4 activity must be mapped and its binding partners determined. To confirm that this interaction is real, the domain of this binding partner necessary to bind Robo4 must also be mapped. Additionally, this interaction should be greatly enhanced upon stimulation with Slit and this interaction must also be shown to be necessary for Slit/Robo4 signaling. This could be achieved using siRNA to knockdown expression of the endogenous binding partner and reconstituting using a mutant that can no longer bind Robo4. In this setting, the functional *in vitro* effect of Slit should be lost. Furthermore, small molecules that target signaling effectors

downstream of Robo4 might mimic the effect of Slit *in vitro* and *in vivo*. Such a broad based approach utilizing biochemical, cell culture and animal model studies would help to instill confidence that any mechanistic insights obtained have true biologic relevance and in the process might offer a new therapeutic approach for preventing pathologic angiogenesis or vascular leakage.

Cerebral Cavernous Malformations

Observations relating to the Ang-1/Tie2 pathway suggest that disruption of vascular stability pathways can lead to vascular malformation syndromes [26,27]. Recent human genetic and developmental genetics studies of a different vascular malformation syndrome have led to a new signaling pathway promoting vascular stability. The *heart of glass (HEG)* cell surface receptor interacts with a complex of intracellular proteins identified on the basis of causal relationships with cerebral cavernous malformations (CCM). Mutations in any one of three identified proteins result in cavernous angiomas. Cerebral cavernous malformation is a common vascular malformation syndrome with a prevalence of 1 in 200 [49,50]. Affected individuals develop vascular lesions of the central nervous system and systemic vasculature [51]. Lesions of the CNS are most commonly described and consist of dilated vascular caverns lined by endothelium and lacking supporting smooth muscle cells. Abnormal cell-cell junctions have been observed on ultrastructural studies [52]. The universal finding of hemosiderin (a blood break down product) in association with lesions, even in the absence of overt hemorrhage is further testament to the unstable, leaky endothelium of the cavernous angioma. Thus one hypothesis for the basis of CCM is the presence of focally destabilized endothelium. These proteins act to promote stability of the endothelium.

A large proportion of CCM cases are familial, following an autosomal dominant inheritance pattern. Human genetics studies have identified mutations in three proteins from families with this disease. Mutations were first identified in *KREVI (RAP1A) interaction trapped-1 (KRIT1)* [53,54], also known as *CCM1*. These studies were soon followed by identification of *CCM2* [55,56] (also known as *Malcavernin* [56], or *Osmosensing scaffold for MEKK3* [57]) and *Programmed cell death 10* [58] (*PDCD10*, also known as *CCM3*) in families with cavernous angiomas. All three identified genes are intracellular proteins without identifiable enzymatic function, and are thus predicted to be adaptor or scaffold proteins [59]. Studies with tagged over-expression constructs have found all three proteins to interact with each other and a large number of additional proteins [59,60]. While there is a growing body of biochemical data regarding the complex of CCM proteins, prior to the human genetic studies linking these proteins to CCM there was little evidence to suggest a role in vascular stability.

Human genetic studies have provided the link between CCM disease and *KRIT1*, *CCM2*, and *PDCD10*, but developmental genetics and animal models have greatly expanded our understanding of the *in vivo* roles of these proteins, and have highlighted additional proteins that function in a common genetic pathway to achieve vascular stability. An initial controversy regarding the *in vivo* function of these genes revolved around the cellular autonomy of protein function. The expression patterns of all three CCM proteins are similar [61], with ubiquitous expression in embryonic development and predominantly neural and epithelial expression in adult mice [62]. Although endothelial expression can be detected, the strong neural expression evoked the possibility of a neurally autonomous function for the CCM proteins. Such a mechanism is not without precedent as had been shown with *alpha-V integrin* deficient mice [63]. Such mice die with vascular instability and brain hemorrhage with protein deficiency in neural rather than endothelial cells. Recent studies using mice with conditional mutations in *Ccm2* confirm an essential role for *Ccm2* in the endothelium for vascular development and vascular stability [64]. Profound angiogenesis defects result in failure of circulation and embryonic death in mice lacking *Ccm2* in the endothelium. This phenotype is shared with mice

lacking *Krit1* [65]. In adult mice with heterozygous mutations of *Ccm2* there is increased vascular permeability, whether such mutations are inherited through the germline, or induced by recombination only within the endothelium.

Forward genetic screening in zebrafish identified a phenotype characterized by an enlarged heart without circulation. Three separate mutants with this phenotype were termed *santa*, *valentine*, and *heart of glass* [66,67]. As these genes were mapped it was found that *santa* resulted from mutations in the zebrafish orthologue for *KRIT1*, and *valentine* from *CCM2*. *Heart of glass* (*heg*) had not previously been associated with CCM. While no CCM patients have yet been found with mutations in *HEG*, mice lacking *Heg* die in late embryonic and perinatal stages [68]. Unlike zebrafish, this phenotype does not closely resemble *Krit1* or *Ccm2* mutants. However, heterozygosity for *Ccm2* on a *Heg* null background is sufficient to phenocopy *Ccm2* or *Krit1* null mouse embryos, confirming a genetic interaction between this cell surface protein and the CCM genes. Analogous experiments in zebrafish have used morpholino knockdown of gene expression to explore genetic interactions between *krit1* and putative interaction partners [67,69]. Such studies also suggest that *rap1b* interacts genetically with the CCM proteins *in vivo* [69].

A number of studies in a variety of cell types have identified binding partners of the CCM proteins. Beginning with the initial identification of KRIT1 as a binding partner of KREV-1, this work began before the human genetics or developmental genetics studies, but has accelerated in pace recently as the human disease and *in vivo* functional links have been appreciated. Literally hundreds of potential binding partners have been identified through a combination of yeast two-hybrid, co-immunoprecipitation, FRET, immunofluorescence and proteomics approaches [57,59,60,70-75]. The list includes such a dizzying array of different proteins that it is difficult to synthesize into an organized working model. While the role of each specific partner cannot easily be described, some general themes emerge. First, the three CCM proteins, KRIT1, CCM2 and PDCD10 interact with each other, and appear to form a complex. Second, the complex is implicated in both GTPase and MAPK signaling. Third, interactions between the complex and cytoskeletal proteins have often been noted. When viewed in isolation, these protein interaction studies are limited by a lack of functional information from either *in vitro* or *in vivo* models.

While our understanding of the CCM proteins remains incomplete, four recent studies have provided much needed functional relevance to an emerging model of CCM signaling (Fig. 4). Concurrent with the genetic identification of mutations in *CCM2*, the same protein was identified by Uhlik et al. [57] in a screen of binding partners for MEKK3, an upstream kinase in the activation of p38 MAPK in response to osmotic stress. The authors named this protein Osmosensing scaffold for MEKK3 (or OSM). This work indicated a role for CCM2 (OSM) in mediating signaling from the GTPase Rac1 to MAP kinases with a stabilizing role in osmotically stressed cells. By demonstrating that CCM2 is able to mediate activation of MKK3 and p38 MAPK in a variety of non-endothelial cell types in response to osmotic stress, the authors provided initial evidence for the CCM protein complex in defending cell shape against environmental stress.

Further work by Glading et al. [74] has addressed CCM protein function in an endothelial context. Using a combination of siRNA and over-expression constructs of *KRIT1*, and by developing and validating a monoclonal antibody directed against KRIT1, the authors demonstrate that KRIT1 is an effector for the small GTPase RAP1. They show that KRIT1 binds to β -catenin under the control of RAP1, and that RAP1 stabilizes endothelial cell junctions in a KRIT1-dependent manner. The loss of KRIT1 leads to increased endothelial permeability. Endothelial cell junction stability is dependent upon KRIT1.

Recent work by Whitehead et al. [64] demonstrated the endothelial requirement for *Ccm2*. In the same work the authors find that CCM2 binding to the small GTPase RHOA in endothelial cells leads to suppression of RHOA activity. Depletion of *CCM2* by siRNA leads to an increase in activated RHOA. These *CCM2* depleted cells have both increased actin stress fibers and greater monolayer permeability. These two papers show that when the CCM signaling complex is deficient, cell junctions are affected and the vasculature becomes more permeable.

Heterozygous mice are the genotype equivalent of CCM patients. While such mice are viable and fertile and do not spontaneously develop cavernous angiomas, defects in endothelial barrier function are demonstrated [64]. The permeability response to stressors such as VEGF is exaggerated. As with *CCM2* deficient cells, these defects in permeability can be rescued by suppressing RhoA signaling. Thus the vascular stability defects found *in vitro* are also observed *in vivo* and a potential therapeutic strategy begins to emerge for patients with CCM.

By interacting with small GTPases, MAP kinases, cell junctions and the cytoskeleton, the CCM signaling complex is poised to mediate key aspects of vascular stability. For the CCM proteins to respond to external stressors, there must be communication with proteins at the cell surface. The known interactions of CCM proteins point to a variety of possible candidates, including integrins [71,73] and cadherins [74] among others [59]. The heart of glass (HEG) cell surface receptor is expressed by endothelium [68] and endocardium [66], shares a common mutant phenotype in zebrafish with *krit1* and *ccm2* [67], and interacts genetically with both [67]. Kleaveland et al. [68] recently characterized the interaction between HEG and the CCM proteins. The authors show that HEG binds to KRIT1, which is required for binding with *CCM2*. Thus *CCM2* requires input from *HEG* via *KRIT1* in the endothelium.

Stability is achieved in endothelial cells by balancing a series of stabilizing signals in opposition to the wide variety destabilizing inputs. We have briefly reviewed several such stabilizing cell surface input pathways. In contrast to the Ang-Tie, Delta-Notch, and Slit-Robo systems of ligands and cell surface receptors, the CCM complex is not an extracellular ligand-receptor system with uncertain intracellular effectors, but rather an intracellular signaling complex that regulates cell surface events. In endothelial cells, this complex interacts with Heg at the cell surface, yet Heg deficiency cannot account for all CCM signaling. Thus the CCM complex is expected to receive further inputs from different cell surface receptors. While CCM protein interactions may point to some of these inputs, it is possible that the CCM protein complex is involved in mediating the stabilizing signals from Tie, Notch, or Robo cell surface receptors. Future studies to define potential interactions between these receptors and the CCM signaling complex will be needed to determine whether they function in common pathways, or diverse but parallel pathways.

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Figure 1. VE-cadherin interactions are disrupted by phosphorylation and endocytosis
(a) Under normal conditions, VE-cadherin forms tight interactions that inhibit excessive vascular leak. **(b)** Kinases such as Src can phosphorylate the cytoplasmic tail of VE-cadherin, resulting in the disruption of VE-cadherin interactions and increased vascular permeability. **(c)** VE-cadherin interactions are also disrupted through endocytosis, resulting in increased vascular leak. The binding of p-120 catenin to the cytoplasmic tail of VE-cadherin inhibits internalization resulting in enhanced vascular stability.

Figure 2. Endogenous cell signaling systems decrease vascular hyperpermeability
(top) VEGF disrupts the barrier function of the endothelium resulting in enhanced vascular leak. **(bottom)** Cell signaling systems such as Slit-Robo4, Ang-1-Tie2, and DLL4-Notch stabilize the vasculature.

Figure 3. Endogenous cell signaling systems enhance vascular stability

(a) VEGF disrupts VE-cadherin interactions through activation of Src resulting in the phosphorylation of the cytoplasmic tail of VE-cadherin. (b) Ang-1 through its receptor Tie2 activates Rho. This causes the activation of mDia, which sequesters Src resulting in the inhibition of Src and stabilization of VE-cadherin. (c) Dll4 binds Notch resulting in the cleavage of the NICD and its transfer to the nucleus. The resulting signaling events that affect endothelial cell junctional stability are unknown. (d) Slit through a Robo4 dependent mechanism inhibits VEGF-induced activation of Src. The signaling events from Robo4 to Src are unknown.

Figure 4. The CCM genes define an intracellular protein complex promoting vascular stability
 The three genes associated with cerebral cavernous malformations (KRIT1, CCM2, and PDCD10) interact with each other and a variety of other intracellular and cell surface proteins to enhance the stability of endothelial cell junctions and the cellular cytoskeleton. As described in the text, such interactions include the Heart of glass (HEG) cell surface receptor, β-catenin (β-cat), small GTPases including RAP1 and Rho family GTPases, and proteins of MAP kinase signaling cascades.