

Vascular morphogenesis: tales of two syndromes

Douglas A. Marchuk*, Sudha Srinivasan, Teresa L. Squire and Jon S. Zawistowski

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Box 3175,
Durham, NC 27710, USA

Received January 31, 2003; Revised and Accepted February 14, 2003

Advances in our understanding of fundamental biological processes can be made by the analysis of defects manifested in inherited diseases. The genes responsible for these genetic syndromes often encode proteins that act at critical points of the pathways that control biological processes such as cell proliferation, cell–cell communication, cellular differentiation, and cell death. This approach has led to the discovery of novel gene products and/or biochemical pathways involved in disease, genes that in turn play a fundamental role in normal biological processes. This forward genetic approach, focusing on Mendelian disorders of vascular anomalies, has been particularly fruitful for the study of genetic regulation of angiogenesis. This review summarizes the ongoing saga of two genetic syndromes involving disruption of normal vascular morphogenesis. Each inherited disorder involves the focal development of a distinct vascular anomaly. In hereditary hemorrhagic telangiectasia (HHT), the hallmark vascular lesion is termed an arteriovenous malformation, which involves the direct communication of an artery with a vein (arteriovenous shunt), without an intervening capillary bed. For cerebral cavernous malformations (CCM), the lesions are grossly-dilated, closely-packed, capillary-like sinusoidal chambers. The autosomal dominant mode of inheritance of each of these distinct syndromes suggested that the underlying genes might regulate critical aspects of vascular morphogenesis. Emerging but intriguing tales are being told by the genes (and their protein products) mutated in these disorders.

TALE ONE: HEREDITARY HEMORRHAGIC TELANGIECTASIA AND THE ANGIOGENIC SWITCH

Hereditary hemorrhagic telangiectasia (HHT), or Osler–Rendu–Weber syndrome, is an autosomal dominant condition characterized by multi-systemic vascular malformations and hemorrhage from the associated vascular lesions. Patients with HHT display several classic symptoms, including recurrent epistaxis, gastrointestinal hemorrhage, cutaneous and mucocutaneous telangiectases, and arteriovenous malformations (AVM) in the pulmonary, cerebral, or hepatic vasculature (1).

Vascular lesions in HHT patients are present as telangiectases on the skin and inner linings of the mouth, nose and gastrointestinal tract, and as direct arteriovenous shunts primarily in the lungs, brain and liver. A systematic electron microscopic study of biopsied lesions taken from HHT patients has provided an overview of the development of these vascular malformations (2). Cutaneous lesions of different sizes (≤ 2 mm) were analyzed and distinct changes were identified

in the vascular bed beginning with the dilatation of postcapillary venules beneath the skin's surface. At this earliest developmental stage, a mononuclear infiltrate composed mostly of lymphocytes surrounded these dilated venules and remained throughout the later stages. Expansion of the lumen in the postcapillary venules was accompanied by an increase in vessel wall size resulting from increased recruitment of pericytes. Dilated arterioles were observed next, yet were still connected to the enlarged venules via an intervening capillary bed. In the final stages of lesion development, the enlarged venules had become increasingly dilated and elongated and were spread throughout the entire dermis in a tortuous pattern. The capillary bed was no longer present and had been replaced by two to four direct arteriovenous connections. The arteriole and venule portions of each arteriovenous connection maintained features distinctive of their origins. An exception that was noted in the venule portion, however, was an excessive number of smooth muscle cell layers. In addition, although stress fibers were found in the pericytes along the dilated venules, no other abnormalities in the ultrastructure of the individual cellular components were reported (2).

*To whom correspondence should be addressed. Email: march004@mc.duke.edu

Molecular genetics of HHT

Linkage studies revealed that mutations in two distinct genes cause HHT. The HHT type 1 locus was mapped to human chromosome 9q33-34 (3-5) and encodes the endoglin (*ENG*) gene (6). The HHT type 2 locus was mapped to human chromosome 12q13 (7,8) and encodes the activin A receptor, type II-like kinase 1 or activin receptor-like kinase 1 (*ACVRL1* or *ALK1*) gene (9). Both endoglin and ALK1 are expressed predominantly in endothelial cells (10,11) and play distinct roles in the transforming growth factor-beta (TGF- β) signaling pathway. The range of mutations in both HHT type 1 and HHT type 2 patients includes nonsense and missense mutations, as well as insertions and deletions resulting in frameshifts (9,12-27) and are present throughout the coding region. The nature and location of these mutations suggest that HHT arises from loss-of-function mutations. Expression analyses of the mutant proteins have confirmed this indication (17,28-31).

The existence of a third locus for HHT has also been proposed based on exclusion of the known loci in two HHT families (32,33). However, the family described by Piantanida and colleagues (27) was recently shown to harbor an *ACVRL1* mutation, and there has been no subsequent report of a novel map position for the locus in the second family. Thus, an elusive third locus for HHT remains unidentified.

Overview of TGF- β signaling

In vertebrates, the TGF- β superfamily of ligands comprises several members including the activins, bone morphogenetic proteins (BMP), growth and differentiation factors (GDF), TGF- β , nodal and dorsalin. These members are structurally related polypeptides that play roles in a number of different biological processes including cell cycle control, embryogenesis, growth, development, differentiation of several cell types, immunosuppression and embryonic patterning (34).

TGF- β is a pleiotropic cytokine that exhibits diverse effects on endothelial cell function and morphology. In mammals, three isoforms of TGF- β exist: - β 1, - β 2, and - β 3. *In vivo* data suggest that these ligand isoforms are potent angiogenic factors and mediators of vascular remodeling. The diverse effects of TGF- β are mediated by three types of cell surface receptors—type I (RI), type II (RII), and type III (RIII)—that differ in their structural and functional properties. The type I receptors contain an intracellular serine/threonine kinase domain, but do not bind ligand unless co-expressed with a type II receptor. The type II receptors are constitutively active serine/threonine kinases that undergo autophosphorylation upon serine residues (35). These can also phosphorylate the type I receptor in the cytoplasmic juxtamembrane GS (glycine-serine rich) domain of the protein (36-38). Heteromeric complex formation between type I and type II receptors is required for ligand binding to the type I receptor and subsequent signaling (11,36,39). The type III receptors, which include betaglycan and endoglin, have relatively short cytoplasmic domains with no known signaling capacity and are proposed to act instead as modulators of this signaling process (40-42).

A general model for TGF- β signaling has been proposed by several investigators (34,37,43). TGF- β binds the constitutively phosphorylated type II receptor. Ligand binding enables

subsequent recruitment of the type I receptor into this complex. The intracellular kinase domain of the type II receptor then phosphorylates the type I receptor on serine and threonine residues in its cytoplasmic juxtamembrane GS-domain (38). Once activated, the type I receptor phosphorylates a member(s) of the Smad family, which transduces the signal into the nucleus and acts as a transcription factor (44-46).

Distinct Smad proteins are activated by different TGF- β family type I receptors and ligands. The T β RI receptor, upon TGF- β stimulation, specifically activates the receptor (R-) Smad proteins, Smad 2 (47,48) and Smad 3 (48-50). In contrast, constitutively activated ALK1 specifically phosphorylates and activates R-Smad proteins Smad 1 (51,52) and Smad 5 (52,53). The R-Smads associate with Smad 4 and translocate to the nucleus to activate transcription (49,54) (Fig. 1).

Role of ALK1 and endoglin in TGF- β signaling and angiogenesis

Both ALK1 and endoglin are expressed primarily in endothelial cells (11,55). ALK1 is a type I receptor, which can partner with TGF- β receptor type II (T β RII), bind TGF- β 1 or TGF- β 3, and signal through Smads 1 or 5 and possibly 8 (11,29,51-53, 56,57). Because T β RI is also expressed in endothelial cells (52,57), this overlapping ligand and receptor type II specificity would place ALK1 in direct competition with T β RI. T β RI, however, signals via Smad 2 and Smad 3 (47,48,51,52), so the profile of affected genes downstream of each pathway would probably vary, as would the physiological impact of signaling through each receptor. Endoglin, is a type III receptor and has no innate signaling capacity, but is rather thought to modulate signaling. Like ALK1, endoglin can bind TGF- β 1 and TGF- β 3 (58,59), and endoglin can associate with T β RI/T β RII and ALK1/T β RII heteromeric complexes (29,59).

How might endoglin and ALK1 influence the process of angiogenesis? Angiogenesis can be viewed as two separate, but balanced phases: activation and resolution (60,61). In the activation phase, endothelial cell exposure to an angiogenic stimulus results in increased permeability in the endothelial cell layer and fibrin deposition into the extravascular space. The basement membrane is degraded, and the formation of the endothelial sprout begins as endothelial cells migrate into the surrounding matrix. Cell division behind this migrating front enables further invasion into this space. Lumen formation then proceeds from the proximal region of the sprout. Resolution requires that the above processes be terminated and that vessel maturation be completed. Termination involves a halt of endothelial cell proliferation and migration. The basement membrane, which was degraded initially, is reconstructed around the new vessel, and the endothelial junctional complexes are reformed. The shift between the two phases is known as the angiogenic switch.

A number of investigators (29,52,57,62) have proposed that ALK1, T β RI, and endoglin are critical components in the angiogenic switch. One model would suggest that ALK1 and T β RI have distinct and opposing roles in the activation and resolution phases of angiogenesis. Unfortunately, there is conflicting data on their respective roles during the events of angiogenesis. Oh *et al.* (52) examined the expression of a number of angiogenic factors in *Acvrl1* knockout embryos. The

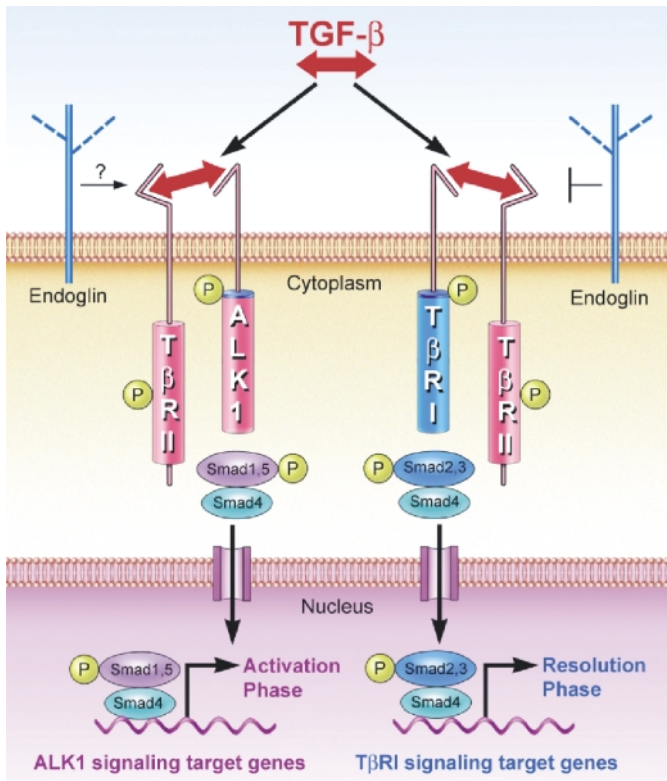


Figure 1. A model for the role of ALK1 and endoglin in the angiogenic switch during angiogenesis. TGF-β can signal via two distinct receptor-mediated pathways in endothelial cells. ALK1 signals through a pathway that includes Smad 1 or Smad 5, which may lead to transcriptional activation of genes involved in the activation phase of angiogenesis. TβRI activates a pathway that includes Smad 2 and Smad 3, which may lead to transcriptional activation of genes involved in the resolution phase of angiogenesis. Endoglin is a negative modulator of TGF-β signaling through the TβRI receptor, although its role in signaling via the ALK1 receptor is less clear. Mutation of either endoglin or ALK1 would favor signaling through TβRI, and favor the resolution phase.

embryos exhibited increased mRNA levels for the following genes: vascular endothelial growth factor (VEGF), Ang-2, tissue type plasminogen activator (t-PA), urokinase-type PA (u-PA) and PA inhibitor-1 (PAI-1). The products of these genes play distinct roles in the activation phase of angiogenesis, namely increased permeability in the endothelial cell layer and migration of the endothelial cells. Therefore they concluded that ALK1 acts in the resolution phase of angiogenesis. Lamouille *et al.* (62) examined the effects of constitutively activated ALK1 on cell proliferation, migration, adhesion and cell spreading using a human microvascular endothelial cell line. Activated ALK1 inhibited proliferation and migration and decreased adhesion and spreading of these cells grown in 2-dimensional culture. Thus they also concluded that ALK1 acts in the resolution phase of angiogenesis.

Goumans *et al.* (57), on the other hand, came to the opposite conclusion. Migration of endothelial cells in culture exhibited a biphasic dose response to TGF-β3 ligand. At low TGF-β3 concentrations (0.25–0.5 ng/ml), endothelial cell migration was enhanced. Above this concentration, migration was inhibited. To evaluate the respective contributions of ALK1 and TβRI, they determined the effects of TGF-β (at the low dose range

favoring cell migration) on endothelial cell migration, after antisense inhibition of either receptor. Antisense inhibition of ALK1 expression resulted in a marked decrease in cell migration. Antisense inhibition of TβRI resulted in an increase in cell migration. Finally, using mouse embryonic endothelial cells, they showed that constitutively activated ALK1 enhanced cell migration, whereas constitutively activated TβRI decreased migration. These results are consistent with ALK1 playing a role in the activation phase of angiogenesis, and TβRI in the resolution phase. These results are also consistent with the biphasic effects of TGF-β concentration of angiogenesis. A model based on this view is shown in Figure 1.

This model makes some predictions about the effects of mutations in *ENG* and *ACVRL1* on TGF-β signaling in HHT. Mutations in the *ACVRL1* gene would reduce signaling through this receptor, thus favoring signaling through the TβRI receptor, and the resolution phase of angiogenesis. Mutations in the *ENG* gene would have a similar effect, reducing its negative modulation of TβRI signaling, again favoring signaling through TβRI, and the resolution phase. This model might predict that endoglin is a positive modulator of ALK1 receptor-mediated signaling, although data to support this are currently lacking.

Animal models

Despite the identification of the genes mutated in HHT patients, questions pertaining to vascular lesion formation in HHT remain largely uninvestigated. *ENG* and *ACVRL1* are expressed in all vascular endothelial cells. Thus, heterozygosity of either of these two genes should result in the reduced expression of the corresponding protein throughout the vasculature. However, the vascular lesions in HHT develop in discrete locations, and often increase in number and severity with age. The focal nature of these lesions and their age-dependent progression suggest that critical factors initiate lesion development. The severity of the disease is also highly variable, even among family members who possess the same mutant allele, suggesting that modifying factors play an important role in disease progression. The investigation of genetic and environmental factors that initiate lesion formation in HHT and those that influence disease progression require animal models that recapitulate the disease pathology.

Eng knockout mice. Mice lacking functional alleles for *Eng* have been generated by gene targeting by three groups (63–65). While one of these *Eng*^{−/−} mouse strains was generated from ES cells derived from mice with 129/SVJ background (63), the other two *Eng*^{−/−} strains were generated using ES cells derived from 129/Ola mice (64,65). The *Eng*^{−/−} mice died during mid-gestation (E10.5–11.5) due to impaired cardiovascular development. The overall development of the embryo and the organization of endothelial cells were normal in *Eng*^{−/−} embryos until E8.5. However, the endothelial cells in the primary capillary plexus failed to remodel into mature networks of vessels and contained reduced number of red blood cells, presumably due to defective hematopoiesis. Yolk sac hemorrhage had occurred frequently. A striking feature of the *Eng*^{−/−} embryos was the failure of the endothelial tubes to recruit vascular smooth muscle cells (vsmc), a

phenomenon that might contribute to pathogenesis in HHT. The defect in vsmc recruitment preceded that in endothelial remodeling. These three studies established that endoglin is not required for vasculogenesis (with the exception of formation of cephalic capillary bed in certain cases), but is essential for angiogenesis or the development of new blood vessels from pre-existing ones in the developing embryo.

The *Eng*^{-/-} mice also exhibited impaired cardiac development. At least half of the embryos contained enlarged ventricles and hyper-dilated outflow tracts. The endothelial surface of the truncal cushions failed to organize in the embryos and there was abnormal cardiac looping. The embryos also exhibited pericardial diffusion. Importantly, the cardiac defects in the *Eng*^{-/-} embryos develop only after defects in angiogenesis appear.

Developmental expression of *Eng* was examined using β -galactosidase assays on *Eng*^{+/-} mouse embryos in which the disrupted *Eng* allele contained a *LacZ* insertion (66). The pattern of *Eng* expression was very similar to that of *Acvrl1* (67). *Eng* expression was first detected in the embryo at E6.5 in the extra-embryonic ectoderm and subsequently in the amnion, the allantois and the cardiogenic plate. By E8.5, the primitive endothelial cells of the yolk sac expressed *Eng*. By E9.5, *Eng* was expressed in endothelial cells throughout the developing vasculature and in the endocardium. High levels of *Eng* expression were found in the capillary plexus surrounding the brain and in vessels surrounding optic vesicles. In the trunk, *Eng* was expressed in developing blood vessels and capillaries, with levels being highest in the capillaries, lowest in veins and intermediate in arteries. Between E12.5 and E13.5, *Eng* was expressed in endothelial cells irrespective of whether they were derived by vasculogenesis or angiogenesis.

Interestingly, heterozygous *Eng* mice derived from mice generated using the 129/Ola ES cells exhibited vascular phenotypes similar to that in HHT (64,65) while no such phenotype was reported in the heterozygotes derived from another strain (63) suggesting that there might be strain-specific effects of *Eng* mutations in mice. In the former category, one model showed the presence of extremely dilated, tortuous vessels. Upon histological examination, these vessels were found to be weak-walled, with disorganized and sparsely distributed elastin fibers and smooth muscle cells, that might contribute to vessel fragility (65). A second model demonstrated the occurrence of the clinically important phenotype of recurrent nosebleeds in the heterozygous mice, in addition to telangiectases (64). An elaborate study of the phenotypes of *Eng*^{+/-} mice in various genetic backgrounds revealed clinically relevant vascular pathology in multiple organs and a strong strain-specific nature of the *Eng*^{+/-} phenotype (68,69). This study established the *Eng*^{+/-} mice in the 129/Ola background as an animal model for HHT type 1 and a potential role for modifier genes in HHT in the mouse.

***Acvrl1* knockout mice.** Mice with loss-of-function mutations in *Acvrl1* were generated by two groups (52,70). The *Acvrl1*^{-/-} embryos exhibit defective endothelial remodeling and die during mid-gestation, as do *Eng*^{-/-} mice. Vasculogenesis proceeds normally in these embryos, as in *Eng*^{-/-} embryos, but capillaries are few and display arteriovenous shunts by E8.5. Recruitment of

vascular smooth muscle cells around the arteries is also disrupted. In order to understand the basis of angiogenesis defects in the *Acvrl1*^{-/-} mice, Oh *et al.* (52) analyzed the expression levels of members of the plasminogen-plasmin system, enzymes implicated in proteolysis of perivascular matrix during embryogenesis. The *Acvrl1*^{-/-} embryos expressed elevated transcript levels for t-PA, u-PA and PAI-1. Transcript levels of VEGF and Ang-2 were also elevated in the *Acvrl1*^{-/-} embryos (52). To explore the formation of arteriovenous shunts in the *Acvrl1*^{-/-} embryos, Urness *et al.* (70) analyzed the expression of *Ephrin-B2* (*Efnb2*) in the *Acvrl1*^{-/-} embryos. *Efnb2* is expressed in the arterial but not venous endothelial cells, and is the only such marker known to date. *Efnb2* expression was reduced or undetectable in various regions of the *Acvrl1*^{-/-} embryos compared to the wild-type embryos. Similarly, intravascular hematopoiesis in the *Acvrl1*^{-/-} embryos was not confined to arteries (70). These findings suggested that loss of ALK1 might lead to loss of cues that determine structural and functional arteriovenous identity in the developing vasculature.

Developmental expression of *Acvrl1* in mice has been analyzed by RT-PCR and *in situ* hybridization from one-cell zygote to E12.5 (67). Similar to *Eng* (66), *Acvrl1* expression was first detected at E6.5. Between E7.5 and E8.5, *Acvrl1* expression was highest at sites of vasculogenesis in the conceptus, in trophoblast giant cells, and in the endothelial lining of blood vessels in the decidua. At later stages (E9.5–12.5), *Acvrl1* was expressed in several tissues and organs, but highest levels were found in blood vessels, mesenchyme of the lung, submucosal layer of the stomach and intestines and at specific sites of epithelial-mesenchymal interactions.

Heterozygous *Acvrl1* mice developed age-dependent vascular lesions in the skin, extremities, oral cavity and in the internal organs including the lung, liver, intestine, spleen and brain, organs similar to those affected in HHT patients (71). Vascular defects were noted in 40% of the mice, although this is only a minimal penetrance estimate. Major histopathological features of the lesions included thin-walled dilated vessels in close proximity to each other, hemorrhage and fibrosis. In a similar way to HHT patients, the mice also exhibited gastrointestinal bleeding, as evidenced by visible hemorrhage in addition to positive fecal occult blood tests.

The consequences of liver involvement in HHT patients range from asymptomatic to life-threatening. In the latter case, liver transplantation is sometimes the only treatment (72). Notable amongst the vascular defects seen in the *Acvrl1*^{+/-} mice was the preponderance of liver lesions and the associated morbidity (71). The range of gross liver lesions in the *Acvrl1*^{+/-} mice included focal lesions that were about 1–2 mm in diameter, severe sinusoidal dilation, organ enlargement and hemorrhage. These lesions were reminiscent of pinpoint telangiectases, nodular hyperplasia of the liver and pseudocirrhosis that occur in HHT patients. Histology of the lesions in the *Acvrl1*^{+/-} mice demonstrated presence of multiple vessels that were extremely dilated and juxtaposed to each other, and of chronic hemorrhage and fibrosis. Recently, it has been suggested that HHT type 2 patients exhibit a high degree of liver involvement (27). In fact, an HHT family with an unusually high hepatic involvement that was previously thought to be a distinct type of HHT (32), has now

been identified as HHT type 2 (27). In the light of these observations, the prominence of hepatic phenotype in the *Acvrl1*^{+/-} mice is of particular interest.

HHT patients with hepatic AVM exhibit secondary phenotypes such as high-output cardiac failure, portal hypertension, pulmonary hypertension and portosystemic encephalopathy (73–75). High-output cardiac failure due to a hyperdynamic state of circulation resulting from a hepatic arteriovenous shunt (75–77) is sometimes the first diagnosed symptom (78) or even the primary cause of death (79) in HHT patients. An *Acvrl1*^{+/-} mouse with profound liver involvement also displayed a secondary cardiac phenotype, similar to that observed in human patients (71). The similarity of affected organs, age-dependent penetrance, variable expressivity, histological similarity of the lesions, the recapitulation of a secondary phenotype and the occurrence of life-threatening defects suggest that the *Acvrl1*^{+/-} mice can be used for the identification of additional genetic and environmental factors that modulate disease expressivity in HHT type 2 patients.

The lack of survival of the *Eng*^{-/-} or *Acvrl1*^{-/-} mutant embryos to term in the mouse is consistent with the apparent absence of human patients homozygous for inactivating mutations in either of these genes. A complete absence of either of these two proteins is highly unlikely to be compatible with birth, although the possibility of survival of individuals with two hypomorphic mutant alleles cannot be excluded. Recently, a naturally occurring mutation *violet beaugarde* (*vbg*) in zebrafish was identified as a loss-of-function mutation in the homolog of mammalian *Acvrl1* (80). Homozygous *vbg* mutations result in embryonic lethality in zebrafish, as do *Acvrl1*^{-/-} mutations in mice. The zebrafish homozygous mutant embryos exhibit a defective pattern of circulation due to increased number of endothelial cells in specific cranial vessels. Together, these studies demonstrate an essential role for ALK1 homologs in blood vessel development in vertebrates.

Potential triggers for lesion development

In HHT patients, formation of telangiectases in the skin or mucous membranes, or formation of the larger AVM in the internal organs, involves remodeling of the existing vasculature in discrete locations. The focal nature of these changes is intriguing because they occur on what appears to be a homogeneous genetic background. As an autosomal dominant disorder, a single mutated allele of *ACVRL1* or *ENG* is sufficient for development of the phenotype. Against this genotype, however, the lesions form only at specific sites. This pattern of lesion development suggests that, in addition to the mutated allele, an event or trigger is necessary to upset the balance of regulatory factors that maintains the cells in their quiescent state.

A number of hypotheses for this trigger have surfaced over the years. The focal nature of the vascular lesions might suggest a two-hit model, where somatic mutation of the remaining wild-type allele initiates the development of vascular lesions. In a proliferative lesion such as a solid tumor, clonal expansion of the mutant cells would typically result in loss-of-heterozygosity (LOH) and the absence of detectable protein. HHT type 1-associated vascular lesions do not show an absence of endoglin staining in the endothelial cells (68). However, the

vascular lesions in HHT may result primarily from vascular remodeling, without excessive proliferation. Therefore, the vascular lesions might not be expected to show LOH, even if triggered by a somatic mutation of the wild-type allele. Thus, other approaches may be required to unequivocally exclude this hypothesis.

Other hypotheses relate to local inflammation of the tissue or endothelial cell injury as the trigger. A more recent hypothesis, hypoxia, is based on new data on the regulation of endoglin gene expression. Hypoxia causes an increase in expression of endoglin mRNA and protein in human endothelial and monocytic cell lines (81). In addition, when these same cell lines are treated with TGF- β and cultured under hypoxic conditions, a synergistic effect on endoglin expression is observed (81). Because hypoxia and TGF- β 1 have a similar effect on VEGF expression in human endothelial cells (82) and because VEGF plays an important role in the promotion of angiogenesis (61), hypoxia appears to be a reasonable candidate for the trigger of vascular remodeling that occurs in HHT patients at the site of lesion development. Although the event or trigger for the formation of telangiectases in HHT patients is yet to be identified, the availability of animal models of HHT opens new avenues for testing these hypotheses, as well as in working towards therapeutic approaches.

Other roles of endoglin and ALK1 in normal physiology and pathology

The roles of endoglin and ALK1 in the maintenance of normal adult vascular architecture, including arteriovenous identity, and their roles in embryonic vascular development were established by the discovery that mutations in these genes cause HHT in humans, and from the analyses of the embryonic phenotypes of the respective null mice. However, endoglin and ALK1 may have less-recognized functions in normal and pathological processes related to, or even unrelated to, angiogenesis. Although vascular endothelium is the major site of endoglin expression, endoglin is also expressed in certain other cell types such as smooth muscle cells, activated monocytes, tissue macrophages, hematopoietic cells and trophoblasts (83–89). Endoglin might be important for implantation and placental development, as suggested by both its expression in the extravillous cytotrophoblasts, syncytiotrophoblasts and placental blood vessels (10,83,86,89) and by its ability to modulate trophoblast invasion (90). Recently, endoglin expression in arterial endothelial cells of the uterus was found to be regulated through the various phases of menstrual cycle (91). These are not surprising in view of the fact that the female reproductive system is the only site of new blood vessel formation in the adult organism in the absence of an associated pathology, and that members of the TGF- β superfamily play extensive roles in the female reproductive function (92), including placental growth and differentiation (93).

Similarly, there is emerging evidence that endoglin might be important for the development of various cell lineages derived from the hematopoietic stem cells (94–96). The embryonic lethality of the *Eng*^{-/-} mice precludes the examination of the *in vivo* implications of these phenomena. Nevertheless, these findings indicate an important therapeutic potential for endoglin in disorders involving defects in hematopoiesis, and those in

which altering the state of hematopoiesis is a therapeutic alternative.

Formation of new blood vessels is essential for growth and survival of tumors (97). Because endoglin is important for angiogenesis, and because increased levels of endoglin are expressed in tumor vasculature as compared to quiescent vessels (98–100), experiments were conducted to determine whether endoglin is important for tumor angiogenesis. Several lines of evidence indicate that this is indeed true (99,101,102). In this light, the recent report of a combination therapy of anti-human endoglin monoclonal antibody and cyclophosphamide in a SCID mouse model with human skin and tumor grafts is of particular interest (103). This treatment resulted in complete regression of established tumors and suppression of human tumor vessels in a few mice. However, as in other therapies, the fact that endoglin is also expressed in quiescent vasculature entails that this approach be used with caution. Similarly, the recent finding that ALK1 upregulates the expression of Id protein (57), a tumor marker, suggests that ALK1 might be important for tumor angiogenesis.

HHT was the first to be identified in the increasing list of disorders now known to be caused by mutations that perturb the TGF- β signaling pathway (6). A natural corollary of the identification of *ENG* and *ACVRL1* as the genes mutated in HHT was the question of whether mutations in other members of TGF- β pathway are the underlying causes of other vascular disorders, and conversely, whether mutations in *ENG* or *ACVRL1* would be involved in other diseases. Primary pulmonary hypertension (PPH) is a vascular disorder characterized by sustained elevation of pulmonary arterial pressure. PPH involves uncontrolled remodeling of pulmonary arteries and formation of characteristic plexiform lesions, resulting in their obstruction leading to heart failure and death. The existence of PPH-like symptoms in certain HHT patients has intrigued scientists for a long time. Recently, germline mutations in the gene encoding bone morphogenetic protein receptor type II (*BMPR2*), a receptor for the TGF- β superfamily of ligands, were found to cause autosomal dominant PPH (104,105). Germline *BMPR2* mutations were also found in a large number of apparently sporadic cases of PPH (106). Thus, it is now evident that members of TGF- β superfamily have an important role in the maintenance of normal vascular architecture in humans, and that mutations in these genes can result in clinically important disorders. Interestingly, mutations in *ACVRL1* were found to be the cause of combined HHT and PPH in several families (26). Similarly, an intronic polymorphism in *ENG* has been identified as a risk factor for sporadic intracerebral hemorrhage (107). More recently, the same *ENG* polymorphism in the homozygous state was found to be associated with intracranial aneurysms in a Japanese population (108), although this association may be population-specific (109). In the Japanese population, individuals with intracranial aneurysms who were homozygous for the polymorphism included a relatively large percentage of patients with hypertension and multiple aneurysms (108). Thus, endoglin and ALK1 might have multiple functions in the normal human adult vascular system. The specific symptoms manifested in a patient might be a reflection of the combination of mutations or polymorphisms in either *ENG* or *ACVRL1*, those in other genes, and of environmental influences.

TALE TWO: CEREBRAL CAVERNOUS MALFORMATIONS AND INTEGRIN-MEDIATED CELL ADHESION

Cavernous malformations are sporadic or inherited anomalies of the vasculature located predominantly in the brain. Histopathologically, the lesions are defined by grossly-dilated, closely-packed, capillary-like sinusoid chambers lined by a single layer of endothelium. Lack of intervening brain parenchyma characterizes the lesions which also are devoid of mature vessel elements including smooth muscle and elastic tissue (110). In addition to the cerebrovasculature, cavernous malformations may be found in the spinal cord (111); while cutaneous (112), retinal (113), hepatic (114) and vertebral (115) lesions have been occasionally observed in patients concurrently with the cerebrovascular lesions that typify inherited cerebral cavernous malformation (CCM). Individuals affected with CCM usually present clinically near the third decade of life with intractable seizures, recurrent headaches and focal neurological deficits (110,116–118). The lesions may also hemorrhage resulting in stroke.

Molecular genetics of CCM

Linkage studies have identified autosomal dominant CCM loci mapping to chromosome bands 7q21-22 (*CCM1*), 7p13-15 (*CCM2*) and 3q25.2-27 (*CCM3*) (118–121). Mutations have been found in the *KRIT1* gene in *CCM1*-linked families, the only CCM disease gene identified thus far (122,123). All mutations identified to date are putative loss-of-function mutations including frameshifts, nonsense and invariant splice site sequence mutations (112–114,124–129). No missense mutations have been identified in *CCM1*-affected individuals.

The *KRIT1* gene encodes a protein of unknown function. Investigations have begun to elucidate the cellular function of the *KRIT1* protein, and importantly, the molecular mechanisms that lead to cavernous malformation lesion formation. Initial insight into understanding *CCM1* pathogenesis has been provided by reports studying *KRIT1* protein binding partners, subcellular localization and expression profile.

KRIT1 functional motifs

Preceding functional investigations of *KRIT1*, the presence of two characterized functional domains provided initial suggestion as to the role *KRIT1* plays in the cell (Fig. 2). *KRIT1* possesses a C-terminal FERM (Four-point one, Ezrin, Radixin, Moesin) motif, a domain known to function at the interface of signal transduction and the cytoskeleton. The ERM family of proteins are known to influence the structure and function specific regions of the cell cortex by regulating the linkage between cortical filamentous (F)-actin and integral membrane proteins. This linkage may be direct—FERM domain binding to the cytoplasmic tail of integral membrane proteins—or indirect—via scaffold proteins (130).

The functional activation of ERM proteins is intriguing. An intramolecular interaction between the amino- and carboxy-terminal domains of the ERM proteins masks sites of protein interaction. Studies with the ERM protein ezrin provide a compelling example of this phenomenon. The FERM domain

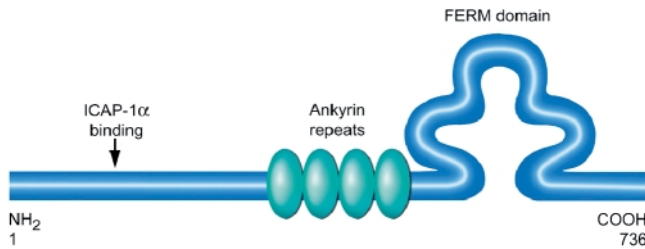


Figure 2. KRIT1 functional motifs. The domain organization of the KRIT1 protein is shown. A C-terminal FERM domain, a motif known to regulate the linkage between cortical actin and integral membrane proteins, is shown schematically as a clover leaf to illustrate the composite nature of the domain (130). Just preceding the FERM domain is a series of ankyrin repeats, known to mediate protein-protein interactions in a multitude of proteins with diverse functions. The site of ICAP-1 α binding, an NPXY amino acid motif within the N-terminal region of KRIT1, is indicated by the arrow.

and C-terminal domain of ezrin interact and, consequently, the F-actin binding site is masked (131–133). Ezrin functions dependent on F-actin binding therefore require the active disassociation of the intramolecular interaction between the two domains—thought to occur in ERM proteins via phosphorylation (134) and lipid-dependent events (135,136). It remains to be determined if the KRIT1 molecule undergoes analogous intramolecular masking, preventing a protein–protein interaction due to the presence of its FERM domain. Such a mechanism might be critical during CCM lesion formation, particularly if some amount of KRIT1 message escapes nonsense-mediated decay in patients and a truncated, potentially dominant negative form of the KRIT1 protein remains.

The second functional domain within the KRIT1 molecule is an ankyrin repeat domain just preceding the FERM domain. Ankyrin repeats are sites of protein–protein interaction found in a large, functionally diverse group of molecules that comprise nuclear, cytoplasmic and extracellular proteins (137). This combination of the ankyrin repeat domain and FERM domain is apparently unique to KRIT1.

Taken together, these motifs suggest that KRIT1 might be a bridging molecule, potentially linking F-actin and integral membrane protein-associated molecules both in terms of structure and signal transduction. Loss of KRIT1 in *CCM1* patients might contribute to lesion formation by loss of regulation of cell shape and adhesion, both crucial in angiogenesis. The identification of protein binding partners specific for these functional domains will shed light on more specific functions of these KRIT1 domains.

KRIT1 as a regulator of RAP1A-dependent cell proliferation

Prior to its identification as the causative gene for CCM, the KRIT1 protein was identified as a yeast interaction trap binding partner for the Ras-like guanine nucleotide binding protein, Krev1(RAP1A)—therefore named *Krev Interaction Trapped-1* (138). Thus, initial hypotheses for mechanisms of *CCM1* pathogenesis centered on KRIT1 as a regulator of RAP1A-dependent cell proliferation pathways. RAP1A was originally shown to revert the transformed phenotype of Ras-overexpressing murine fibroblasts (139), and KRIT1 was

shown to be transcriptionally activated during chemically-induced reverse transformation of CHO-K1 cells (140). Extending this molecular theme, due to the potential for Ras and RAP1A to compete for the same effector molecules (141), one model is that RAP1A functions as a Ras antagonist and loss of KRIT1 in *CCM1* patients perturbs this molecular relationship. More recently, this model has been questioned within the T cell lineage by a study using transgenic mice expressing active RAP1A (142). Utilizing readouts for Ras-MAPK signaling pathways, the expression of active RAP1A activated rather than suppressed these pathways both *in vivo* and *in vitro*.

Taking these models into account, could KRIT1 be a tumor suppressor? Although still a hypothesis, there is some precedent for it based on a related protein. Biallelic loss of tuberlin, a GTPase activating protein (GAP) for RAP1A, results in the accumulation of the GTP-bound active state of RAP1A, and hamartomas develop as a consequence of the uncontrolled RAP1A-regulated cell proliferation (143,144). Although there is no indication of potential KRIT1 GAP activity for RAP1A by amino acid sequence, it is possible that its association with RAP1A sequesters RAP1A from the action of GAP molecules. Accordingly, one model of *CCM1* lesion formation would be KRIT1 serves as a tumor suppressor in the context of RAP1A, and loss-of-function mutations in KRIT1 lead to unregulated endothelial proliferation. Furthermore, this model is more plausible in the context of a two-hit mechanism versus a proposed model of haploinsufficiency. Is the model of unregulated endothelial proliferation plausible when examining the nature of the CCM lesion? Although possible, the closely packed, dilated vessels of the lesion may reflect a defect in endothelial organization or adhesion rather than proliferation.

A caveat for the proposed RAP1A models of *CCM1* pathogenesis is that the KRIT1/RAP1A interaction has not been confirmed by approaches exclusive of the yeast two-hybrid methodology. We have not been able to verify the interaction by GST affinity precipitation and others have reported being unable to verify the interaction using co-immunoprecipitation (145). In addition, using yeast two-hybrid assays, others have reported only a weak interaction between KRIT1 and RAP1A (145) while we have observed that reciprocal bait-prey swapping fails to yield interaction phenotypes. Even though additional experimentation will be required to confirm the authenticity of the interaction, there are other important functions for RAP1A and links to pathways suggested by recent KRIT1 investigations (see ‘Integrating KRIT1 molecular paradigms’ below), and these additional pathways may be more influential in the pathogenesis of *CCM1* than a model of endothelial cell proliferation.

KRIT1 and integrin signaling

Since the identification of KRIT1 by virtue of its association with RAP1A, the KRIT1 coding sequence has been computationally and experimentally verified to encode an additional 207 amino acids than initially predicted (126,146,147). This N-terminal region lacks similarity to any known protein motifs and, importantly, this region has been shown to harbor *CCM1* patient mutations (114,126,127,129,148). Two groups have subsequently identified integrin cytoplasmic domain-associated

protein alpha (ICAP-1 α) as a KRIT1 binding partner for this unique region by two-hybrid screening and have confirmed the interaction by co-immunoprecipitation and GST affinity precipitation, respectively (145,149).

ICAP-1 α is a modulator of β_1 integrin signal transduction, a means whereby mammalian cells sense and respond to the extracellular matrix (ECM) as well as to other cells. ICAP-1 α is a 200 amino acid protein containing a phospho-tyrosine binding (PTB) motif (150), identified by its ability to bind the cytoplasmic tail of the transmembrane β_1 integrin molecule at an NPXY amino acid motif within the integrin tail (150–153). ICAP-1 α was subsequently shown to be required for integrin-mediated cell spreading and proliferation (151–154), suggesting that ICAP-1 α is involved in the relay of information from the cell surface to the cytoplasm, or perhaps, in the reciprocal direction.

As in the β_1 integrin tail, an NPXY amino acid motif within KRIT1 has also shown to be critical for ICAP-1 α binding (145,149). Due to a common critical binding region, it is possible that there is a molecular competition for ICAP-1 α between β_1 integrins and KRIT1 (145). A normal cellular role for KRIT1 may be to sequester ICAP-1 α from β_1 integrins, thus modulating β_1 integrin-mediated cell adhesion. ICAP-1 α involvement in β_1 integrin signal transduction has been expanded by experiments identifying the Rho family GTPases CDC42 and RAC1 as binding partners for ICAP-1 α (153). In addition to demonstrating ICAP-1 α binding to the Rho members in this study, ICAP-1 α expression was shown to interfere with activation of these GTPases during an integrin-mediated cell adhesion assay. Furthermore, the study showed that ICAP-1 α prevents the dissociation of GDP from CDC42, thereby suggesting a mechanism of the prevention of activation of the Rho family GTPase molecules and the inhibition of cell spreading. Purified ICAP-1 α was also able to induce the dissociation of membrane-bound CDC42, providing a potential additional level of regulation of the Rho GTPase by ICAP-1 α . Additionally, ICAP-1 α has been shown to interact with NM23-H2 (also called NDP kinase B), a protein with nucleoside diphosphate kinase activity that has been linked to a variety of cellular activities including suppression of metastasis (155). This report also demonstrated that ICAP-1 α and NM23-H2 co-localize at cell adhesion sites upon integrin ligation.

What might be the consequences of such a competition model in *CCMI* patients? A reduction in the level of KRIT1 may allow a larger pool of free ICAP-1 α to influence β_1 integrin-mediated cell adhesion by directly binding to the integrin cytoplasmic tails. Alternatively, the predominant effect of reduction of KRIT1 levels when considering a competition model may be at the level of the Rho GTPases CDC42 and RAC1. A larger pool of free ICAP-1 α would be predicted to prevent the activation of CDC42 and RAC1 and consequently interfere with integrin-ECM adhesion, as well as potentially affect NM23-H2 associated pathways. Such a model would be consistent with both a haploinsufficiency model and a two-hit model of *CCMI* pathogenesis, although a two-hit model would be predicted to have a more pronounced effect.

Each of these mechanisms predicts a perturbation in cell adhesion processes carried out by integrin molecules. In what context are integrins relevant to *CCMI* pathogenesis? In a vascular context, integrins are crucial players in the regulation

of endothelial cell adhesion and migration during angiogenesis. Integrins are crucial during vessel lumen formation and influence vascular tone and permeability (156). The effect of losing β_1 integrin on the vasculature cannot be determined using knockout mice, as these embryos die shortly after implantation (157,158). Other studies, however, have shed light on the role of β_1 integrin in angiogenesis. Capillary formation is blocked by antibodies to $\alpha_6\beta_1$ integrin (159,160). Additionally, β_1 null cells fail to form blood vessels when tumors are induced in mice, implying this integrin is essential for angiogenesis (161). It has been shown that maturation of blood vessels in the CNS is accompanied by marked upregulation of β_1 integrin expression, suggesting an importance of β_1 integrin not limited to angiogenesis, but in the maintenance of endothelial function in the adult CNS (162). Of particular interest to *CCMI* pathology, mice lacking α_v integrins develop intracerebral hemorrhage (163). Electron microscopy in this study revealed defective associations between cerebral microvessels and the surrounding brain parenchyma. The capillary-like vessels in *CCMI* lesions that lack intervening brain parenchyma may be due to an analogous integrin-mediated perturbation of the parenchyma/vessel interaction via the KRIT1/ICAP-1 α interaction.

In a neuronal context, it is interesting that the murine ortholog of ICAP-1 α , *bodenin*, exhibits spatially restricted patterns of expression in the brain (164). In the developing embryo, a diffuse pattern of *bodenin* expression becomes pronounced in the cranial ganglia at E9.5 and *bodenin* expression shows staining at multiple structures within the forebrain and cerebellum in the adult mouse. As binding partners, KRIT1 may work in concert with *bodenin* at these sites in the brain to influence neuronal development or maintain neural integrity. In support of this notion, recent studies looking at KRIT1 expression in the developing mouse by *in situ* analysis have shown predominant expression in neurons and epithelia both in embryo and adult, suggesting that the role of KRIT1 in development and *CCMI* pathogenesis is not restricted to endothelium of blood vessels (165,166).

Integrins are crucial for neural development and remodeling. Neurite processes probe the ECM for integrin ligands, and once the integrin molecule binds the appropriate ECM ligand, the neuron can migrate due to the tethering of the integrin complex to the actin cytoskeleton via adaptor proteins (167). The role of integrins in the adult CNS, however, is less understood. Studies have demonstrated integrin expression in the adult rat and human brain, β_1 integrin expression in the rat spinal cord (168) and integrins in reactive microglia of Alzheimer's patients (169). An intriguing role for integrins in the CNS is the possible involvement in the physiological plasticity involved in learning and memory (170), although this remains to be tested.

How may perturbation of integrin-mediated cell adhesion via the KRIT1/ICAP-1 α interaction contribute to *CCMI* lesions? The abnormally dilated and packed vessels of a *CCMI* lesion may be the result of impaired capillary formation or maintenance due to failure of β_1 integrin to respond to the appropriate ECM ligand present in the endothelial basement membrane on the abluminal face of the vessel. Alternatively, lack of intervening brain parenchyma within the cavernous malformation may be a reflection of a perturbation of neuronal and/or endothelial migration mediated by β_1 integrins. In addition to

responding to the ECM, cytoplasmic signaling events at integrin tails are known to influence ECM secretion, as shown for the integrin ligand fibronectin (171). It is possible that perturbation of the KRIT1/ICAP-1 α interaction alters proper ECM secretion, in turn affecting neuronal and/or endothelial migration through the matrix. This phenomenon may contribute to the buildup of ECM within the cavernous malformation and prevent neuronal and smooth muscle infiltration. Finally, although not yet proven *in vivo*, apoptosis is known to occur *in vitro* in response to cells expressing unligated integrins—a phenomenon known as integrin-mediated-death (IMD) (172–175). Perhaps perturbation of the KRIT1/ICAP-1 α interaction leads to an over-abundance of β_1 integrins resulting in localized neuronal IMD, in turn influencing capillary morphology at the site of the lesion.

Finally, the theme of cell adhesion brought to attention by interaction with ICAP-1 α may also be relevant for *CCM1* pathogenesis in terms of integrin-mediated or tight junction cell–cell adhesion. Ultrastructural studies of cavernous malformations excised from patients showed that the lesions lacked endothelial tight junctions—structures necessary for endothelial integrity and maintenance of the blood-brain barrier (176).

KRIT1 and the cytoskeleton

Based on the presence of the FERM domain within KRIT1 and a role in integrin signaling, a role of cytoskeleton in KRIT1 signal transduction is plausible, specifically the actin cytoskeleton. KRIT1 may serve as a bridging protein linking the actin cytoskeleton and integral membrane proteins or via adaptors. This model remains to be tested.

A recent study has implicated another cytoskeletal component, microtubules, in the KRIT1 pathway by studies with anti-KRIT1 peptide antibodies (177). By immunofluorescence in bovine aortic endothelial (BAEC) cells, KRIT1 mirrors microtubule localization throughout the cell cycle: fluorescence is along the length of the microtubules in interphase, at the mitotic spindle and spindle pole bodies in metaphase and in telophase fluorescence is consistent with the migrating (+) end of the microtubules which exhibits dynamic instability. KRIT1 and β -tubulin co-immunoprecipitate, although it is possible that KRIT1 may not directly bind microtubules but do so via another protein. It is interesting that endogenous KRIT1 detected by the peptide antibodies used in the study reveals a 58 kDa band, instead of the 83 kDa predicted molecular weight. The significance of this discrepancy remains to be determined.

As KRIT1 localizes to the plus end of microtubules, the authors propose that KRIT1 may help guide endothelial shape much as a plus-end-tracking protein (178), and loss of KRIT1 may lead to abnormal endothelial tubulogenesis and thus abnormal capillary development seen in *CCM1* lesions (177).

Integrating KRIT1 molecular paradigms

Considering KRIT1 association with RAP1A, ICAP-1 α and microtubules, one can postulate distinct mechanisms of lesion formation: tumor suppression, cell adhesion or cell morphology, respectively. Molecular links between each molecular paradigm exist, thus, a more comprehensive molecular model can be made due to more players in the story. The probable

mechanism would thus involve crosstalk or a coordinated effort between the pathways.

RAP1 and integrin signaling. Multiple studies have linked RAP1A and integrin-mediated cell adhesion. Overexpression of the RAP1 GTPase activating protein (GAP) SPA-1 prevents RAP1 activation and reduces cell adhesion on human fibronectin—an integrin ligand (179). In human T-cell and murine pre-B cell leukemia lines, RAP1 stimulates strong $\alpha_L\beta_2$ -mediated adhesion (180,181). Furthermore, in a study using Mn^{2+} and integrin-specific antibodies to functionally activate integrins, it was found that this activation is blocked by expression of proteins which block RAP1 function (182). Another group generated transgenic mice expressing constitutively active RAP1A (V12RAP1A) in T-lymphocytes, resulting in strong activation of β_1 integrins in a thymocyte adhesion assay to fibronectin (142). In a similar fibronectin adhesion assay, RAP1A regulated β_1 integrin-mediated cell adhesion in the hematopoietic cell line 32D (183).

These studies and others have established a link between RAP1 and integrin signaling. KRIT1 may mediate integrin-mediated cell adhesion by communicating in concert with both RAP1A and ICAP-1 α . KRIT1 interaction with RAP1A may still have a duality of function: perturbation of the interaction in *CCM1* patients may alter integrin-mediated signaling and/or cell proliferation pathways.

RAP1A and microtubules. The RAP1A ortholog in *Saccharomyces cerevisiae* is *Bud1/RSR1*, a protein required for selection of the budding site. This process involves targeting of microtubules (184), and one model for *CCM1* pathogenesis would be that loss of KRIT1 would alter microtubule targeting via the RAP1A interaction (177)—consequently inhibiting endothelial tube formation.

Microtubules and ICAP-1 α . In endothelial cell directional migration, the microtubule organizing center (MTOC) reorients toward the leading edge of migration (185). The Rho-family GTPase CDC42, inhibited by ICAP-1 α , was shown to be both necessary and sufficient for the serum lipid LPA-(lysophosphatidic acid) stimulated MTOC reorientation in NIH3T3 fibroblasts (185). As LPA plus adhesion activate CDC42-GTP formation (186), this study showed that LPA increased CDC42-GTP levels, thus triggering MTOC reorientation in wounded monolayers of NIH3T3 cells (185). In addition, this process was shown to be Rho and Rac independent, and blocked by dominant negative N17CDC42. A reduction in KRIT1 level in *CCM1* patients may affect microtubule targeting and thus endothelial tubulogenesis at several levels: KRIT1 microtubule association (177), the KRIT1/RAP1A interaction and CDC42-controlled MTOC reorientation via ICAP-1 α .

In terms of linking molecular paradigms of *CCM1* pathogenesis, there are connections between the microtubule cytoskeleton and actin cytoskeleton at the site of integrin focal adhesion complexes. This may be influential in *CCM1* lesion formation because of potential actin connections for KRIT1 via the FERM domain and actin tethering complexes at integrin tails, while microtubule connections exist as described above. As an example, the protein vinculin is enriched at integrin focal

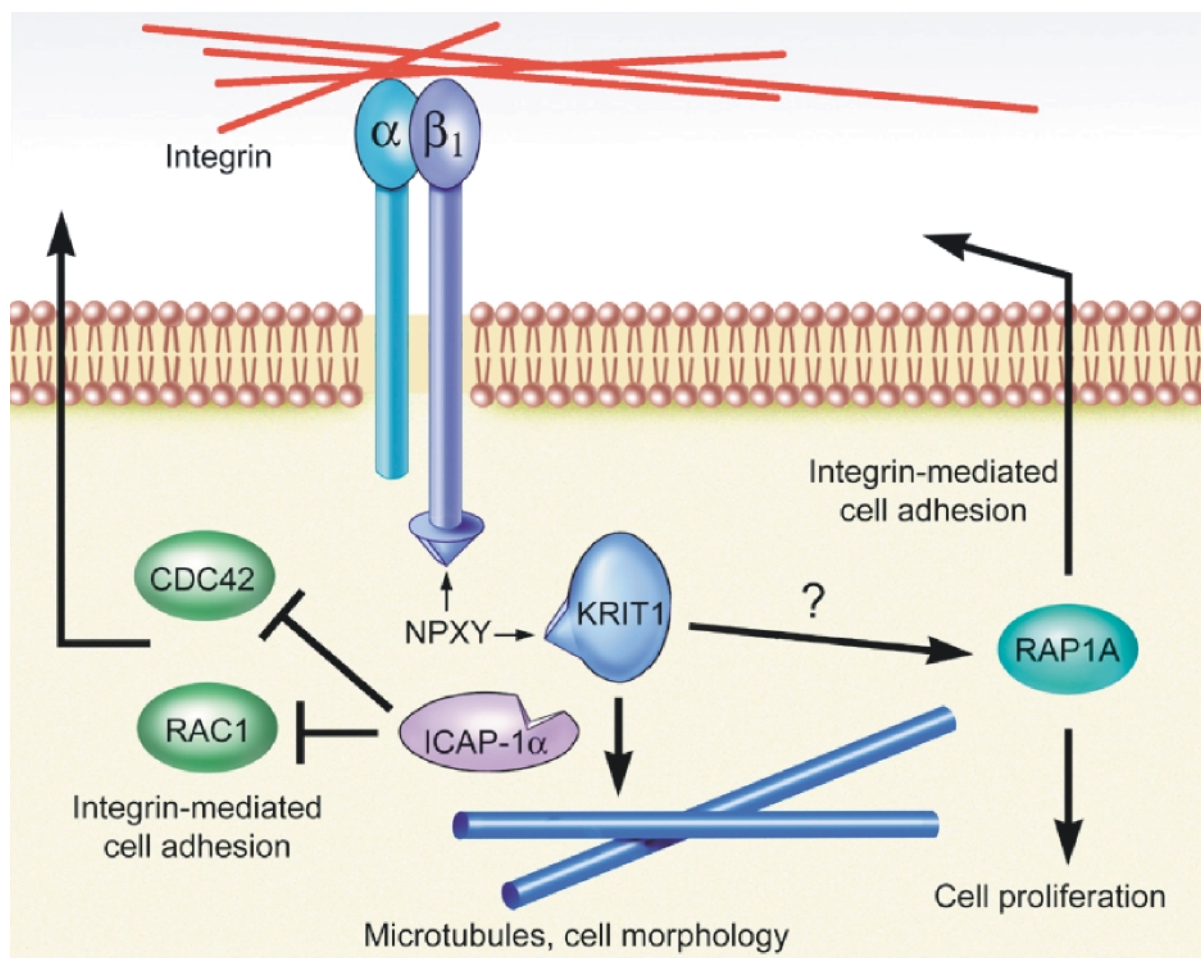


Figure 3. Model of KRIT1 in integrin signal transduction. Integrins are heterodimeric receptors that serve as a primary mammalian cellular mechanism for sensing and responding to the extracellular matrix environment as well as to other cells. The integrin-binding protein ICAP-1 α binds to β_1 integrin cytoplasmic tails as well as to KRIT1 via a common amino acid motif (NPXY). KRIT1 may be competing for ICAP-1 α binding with β_1 integrin cytoplasmic tails via its NPXY motif, thus influencing integrin-mediated cell adhesion by sequestering ICAP-1 α from β_1 integrin cytoplasmic tails and/or from RAC1/CDC42. Alternatively, KRIT1 may be influencing integrin-mediated signaling via its association with RAP1A. Loss of function KRIT1 mutations in *CCM1* patients may perturb this potential regulation of cell adhesion via integrin signal transduction and cell morphology via KRIT1 microtubule association and consequently result in vascular lesions.

contacts, and is capable of binding both actin microfilaments and microtubules simultaneously while tethered to integrin adaptor molecules such as talin (187). KRIT1 may be involved in driving endothelial tubulogenesis by affecting both the actin and microtubule cytoskeleton.

A comprehensive picture?

The molecular links and the themes suggested by the binding partners identified hitherto and localization of KRIT1 are intriguing. A working model for *CCM1* pathogenesis based on studies so far and analysis may be modulation of angiogenesis by integrin-mediated cell–ECM or cell–cell adhesion and cell migration/morphology mediated by microtubules (Fig. 3). As details of KRIT1 signal transduction continue to be elucidated, and when *CCM2* and *CCM3* are identified, the molecular interconnections will allow the development of a more comprehensive model of pathogenesis.

Unanswered questions regarding CCM lesion formation

As our molecular knowledge of KRIT1 and associated pathways develops, there remain fundamental unanswered questions. Namely, do *CCM1* lesions result as a result of KRIT1 haploinsufficiency or biallelic loss? Although the molecular models proposed in this review could in principle result from haploinsufficiency, the focal nature of the lesions suggests a somatic event at the wild-type KRIT1 allele and localized expansion of the cell with biallelic loss. A recent paper describing vertebral lesions in *CCM1* patients is particularly intriguing with regard to a two-hit hypothesis (115). In the patient presenting with the vertebral *CCM1* lesion in addition to cerebrovascular lesions, the patient also presents with a cutaneous lesion directly over the site of the vertebral lesion—implying a two-hit event was transmitted segmentally during development. The two-hit mechanism would also not be limited to a tumor suppression model with RAP1A, perhaps biallelic loss of KRIT1 is pathogenic with regard to ICAP-1 α

or localization to microtubules, or a non-tumor suppression function of RAP1A (integrin signaling).

The identification of binding partners specific for KRIT1 ankyrin repeats and FERM domain will be paramount in elucidating the mechanisms of *CCMI* pathogenesis. It is interesting that no partners have been identified for these domains thus far, as there is strong precedent for these domains as candidates to have multiple binding partners.

Although *in situ* studies during murine embryonic development have implicated KRIT1 in neurons and epithelia (165,166), we still do not know whether loss of KRIT1 has its primary pathogenic effect in neurons or endothelial cells, or whether both contribute. Answers to this and the previous questions will be facilitated by animal models of *CCMI* and continued probing of KRIT1 function using cell culture.

CONCLUSION: THE TALES THAT GENES TELL

This review began with two intriguing vascular anomalies which in addition to occurring sporadically, were the hallmark lesions of autosomal dominant syndromes. The inherited nature of these phenotypes suggested that a forward genetic approach toward gene identification might shed new light on genetic control of angiogenesis. The tales told by the genes identified for these syndromes have not disappointed us.

For HHT, a single phenotype was found to be caused by mutations in either of two distinct loci. Subsequent investigation revealed mutations in two previously-described genes, *ENG* and *ACVRL1*, each of which exhibited a predominantly endothelial-specific expression pattern which might have foretold their importance in angiogenesis. Sequence homology suggested that they might be receptors for the TGF- β superfamily. Their involvement in HHT revealed a crucial role for both in vascular morphogenesis. Molecular and biochemical analyses have suggested that both receptors are important in the switch from the activation phase of angiogenesis to the resolution phase. Although the endoglin-ALK1 story is far from complete, it is now clear that an angiogenesis goldmine lay beneath the phenotype of this rare vascular syndrome.

For CCM, a single phenotype was found to be linked to three distinct loci. Only one causative gene has been identified to date. KRIT1 was virtually an unknown protein when disease-causing mutations were identified in *CCMI* kindreds. There was no hint of its involvement in angiogenesis. Indeed, the acronym KRIT1 implies a role in RAP1A signal transduction which subsequent biochemical analysis suggests may be incorrect. However, the importance of this protein to cerebrovascular disease sparked interest in its functional analysis. Although the KRIT1 story is just beginning to unfold, the introductory chapters suggest a role in integrin-mediated signaling, and in cell morphology and adhesion. As an added bonus, two more CCM genes have yet to be identified. Hopefully, the stories told by these yet unidentified genes will be equally intriguing, and the gene products equally important for the regulation of angiogenesis.

ACKNOWLEDGEMENTS

The authors were supported by NIH grants HL-49171 and NS-43543, March of Dimes grant 1-FY02-19, American Heart Association Bugher Foundation Award for the Investigation of Stroke 0070028N to D.A.M., and an American Heart Association predoctoral fellowship award 01-10078U to J.S.Z.

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