CCM1 regulates vascular-lumen organization by inducing endothelial polarity

Maria Grazia Lampugnani[1,2,*], Fabrizio Orsenigo[1], Noemi Rudini[1], Luigi Maddaluno[1], Gwénola Boulday[3], Francoise Chapon[4] and Elisabetta Dejana[1,5,*]

[1]IFOM, FIRC Institute of Molecular Oncology Foundation, Via Adamello 16, 20139 Milan, Italy
[2]Mario Negri Institute of Pharmacology, 20156 Milan, Italy
[3]Université de Medeciné Paris Diderot - Paris 7, 75005 Paris, France
[4]Faculté de Mèdeciné, CHU Cote de Nacre, 14000 Caen, France
[5]Universita' degli Studi di Milano, 20100 Milan, Italy
*Authors for correspondence (mariagrazia.lampugnani@ifom-ieo-campus.it; elisabetta.dejana@ifom-ieo-campus.it)

Accepted 20 January 2010
Journal of Cell Science 123, 1073-1080

doi:10.1242/jcs.059329

Summary

Little is known about the molecular mechanisms that regulate the organization of vascular lumen. In this paper we show that lumen formation correlates with endothelial polarization. Adherens junctions (AJs) and VE-cadherin (VEC, encoded by *CDH5*) are required for endothelial apicobasal polarity in vitro and during embryonic development. Silencing of *CDH5* gene expression leads to abrogation of endothelial polarity accompanied by strong alterations in lumenal structure. VEC co-distributes with members of the Par polarity complex (Par3 and PKCζ) and is needed for activation of PKCζ. CCM1 is encoded by the *CCM1* gene, which is mutated in 60% of patients affected by cerebral cavernous malformation (CCM). The protein interacts with VEC and directs AJ organization and AJ association with the polarity complex, both in cell-culture models and in human CCM1 lesions. Both VEC and CCM1 control Rap1 concentration at cell-cell junctions. We propose that VEC, CCM1 and Rap1 form a signaling complex. In the absence of any of these proteins, AJs are dismantled, cell polarity is lost and vascular lumenal structure is severely altered.

Key words: Adherens junctions, Cell polarity, Endothelium, Vascular lumen

Introduction

The human disease cerebral cavernous malformation (CCM) is characterized by abnormal vascular structures mostly localized in the brain. Germline mutation in any of three independent genes, *CCM1*, *CCM2* or *CCM3*, causes the inherited form of the disease (Plummer et al., 2005; Labauge et al., 2007), whereas local somatic mutation of these genes might induce the sporadic form (Limaye et al., 2009). The most dangerous clinical symptom in CCM is hemorrhagic stroke. Additionally, hemosiderin deposits in the tissue adjacent to lesions strongly suggest continuous blood leakage from the abnormal vessels, which could account for epilepsy and other neurological symptoms (Rigamonti et al., 1988).

Histological and ultrastructural analyses reveal that malformations consist of clusters of dilated vascular sacs (Wong et al., 2000), with endothelial cells (ECs) lacking both tight junctions (TJs) (Clatterbuck et al., 2001) and the support of astrocyte foot processes at the ablumenal aspect of vessels (Clatterbuck et al., 2001). Fibronectin, growth factors (VEGF, bFGF, TGFα), and smooth muscle actin are abundant in the ECs of lesions, whereas laminin and collagen IV are reduced (Hallmann et al., 2005), reminiscent of an immature or angiogenic situation (Kilic et al., 2000). Together, these observations strongly suggest that defects in lumen formation and vascular fragility are key features of CCM lesions.

Regulation of vascular permeability by CCM1 and CCM2 has been analyzed both in vitro and in vivo, and is associated with the organization of cell-cell junctions (Glading et al., 2007; Whitehead et al., 2009). In particular, CCM1 acts as an effector of the small GTPase Rap1, by enhancing the stability of endothelial adherens junctions (AJs).

The presence of vascular malformations might also contribute to vascular fragility. Abnormal blood flow generates turbulence and alterations in shear stress that can induce vascular rupture. The mechanism by which the absence of CCM proteins produces vascular malformation remains largely unexplored (Whitehead et al., 2004), possibly because of the gaps that still remain to be filled in our understanding of the pathways that direct vascular lumen formation (Iruela-Arispe and Davis, 2009). Kamei and co-workers (Kamei et al., 2006) showed, in cultured ECs and in zebrafish, that lumen formation requires the intercellular fusion of intracellular vesicles. However, other mechanisms, such as cavitation of endothelial cords, are also possible (Lubarsky and Krasnow, 2003; Iruela-Arispe and Davis, 2009; Blum et al., 2008; Kleaveland et al., 2009).

Although different mechanisms might regulate lumen formation in different types of vessels, the establishment of endothelial apical-basal polarity is expected to be an absolute requirement for all of them. In epithelial cells, apical-basal polarity is a prerequisite of correct lumen organization (Martin-Belmonte and Mostov, 2008; Jaffe et al., 2008). Since the partitioning defective (Par) polarity complex is a key determinant of cell polarity (Goldstein and Macara, 2007), it is likely to be important in the organization of vascular lumen. The specific localization and activity of this complex is determined, at least in part, by the small GTPases Cdc42 (Martin-Belmonte et al., 2007) and Rac (Chen and Macara, 2005), the Rac-specific GEF Tiam (Mertens et al., 2005), the adaptor and regulatory molecules Par3 and Par6, and the effector PKCζ (Goldstein and Macara, 2007; Iden and Collard, 2008). Cdc42 and Par3 have been shown to regulate lumen formation of in three-dimensional (3D) cultures of ECs (Koh et al., 2008). In addition, the small GTPase

Rap1 controls lumen formation (Itoh et al., 2007) and anteroposterior polarity (Gerard et al., 2007; Schwamborn and Puschel, 2004). Excessive Rap1 activity leads to altered epithelial acinar polarity (Itoh et al., 2007).

Furthermore, indirect data support the concept that AJs are involved in regulating EC polarity and vascular lumen formation. VE-cadherin (VEC) promotes endothelial cell-cell adhesion at AJs. In the early murine embryo, a homozygous null mutation of the gene encoding VEC (*Cdh5*) results in a reduction in lumenal size of the aorta and of cardinal veins, although the lumens of other vessels, such as cephalic vessels, become enlarged (Carmeliet et al., 1999; Gory-Faure et al., 1999). Consistent with this data, impaired lumen formation has been reported in zebrafish upon knockdown of expression of the gene encoding VEC (Montero-Balaguer et al., 2009). VEC can directly bind Par3, which is an element of the polarity complex (Iden et al., 2006), and it is required for junctional organization of Tiam (Lampugnani et al., 2002); however, the actual pathway through which VEC regulates endothelial polarity remains unclear.

Here, we present data indicating that VEC is needed for the correct localization and to promote activation of the polarity complex, which, in turn, establishes endothelial apical-basal polarity. CCM1 stabilizes VEC at AJs and thus promotes the concentration of the polarity complex at intercellular junctions. In the absence of CCM1, apical-basal polarity of ECs is altered, and the size and organization of vascular lumens is severely affected. These data support the concept that CCM1 acts in concert with VEC and the members of the polarity complex to modulate the vascular lumen and offer a mechanistic explanation for the vascular malformations observed in CCM disease.

Results

VEC regulates the formation of a polarized vascular lumen

HUVECs cultured in collagen gel rapidly organize into a network of hollow structures (supplementary material Fig. S1) (Bayless and Davis, 2002). Using podocalyxin (Podxl) and collagen IV (Coll IV) as markers of the apical and basal surfaces, respectively (Horvat et al., 1986; Hallmann et al., 2005), we observed that cells forming these vascular-like structures were correctly polarized (Fig. 1A). Knockdown of VEC protein expression using siRNA against *CDH5* resulted in the formation of several small lumens lined with ECs (supplementary material Fig. S1A) that were not regularly polarized (Fig. 1A). This effect was not due to reduced expression of polarity markers (supplementary material Fig. S2C). Consistently, in wild-

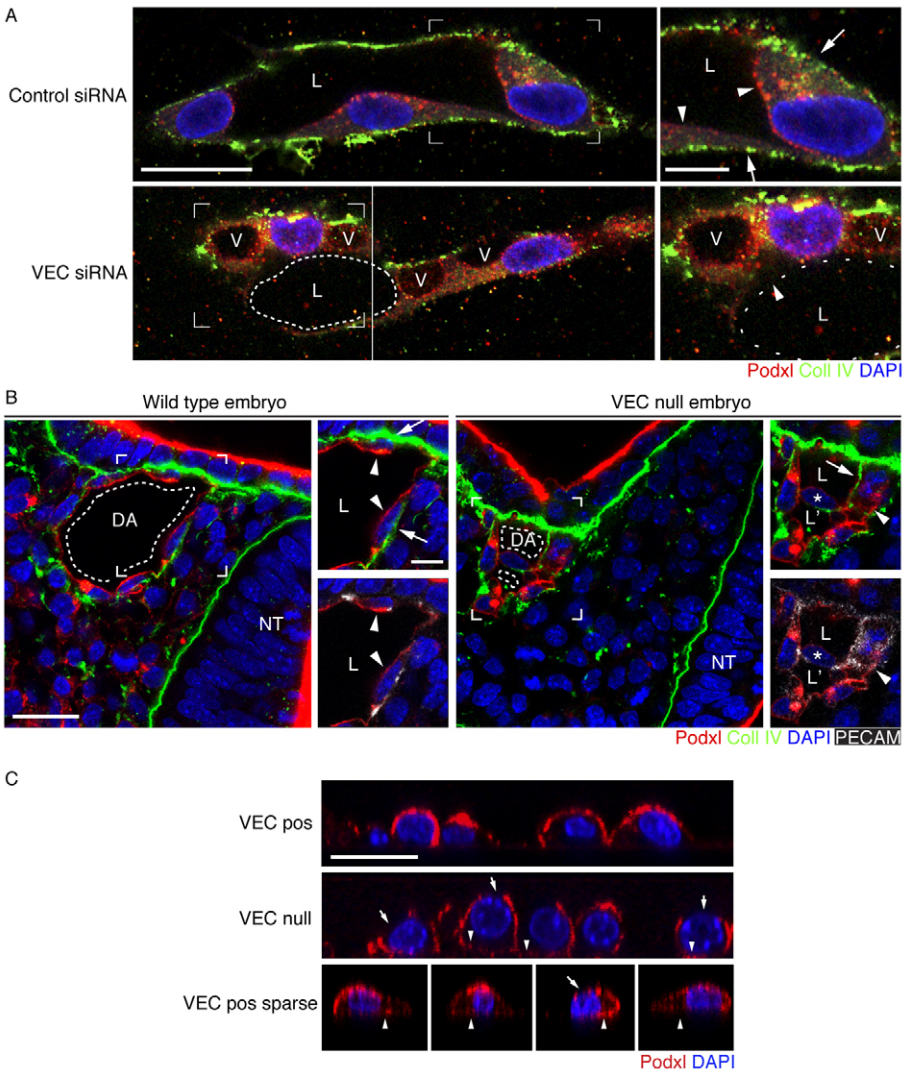

Fig. 1. VEC is required for the formation of a polarized endothelial lumen in vitro and in vivo. (**A**) HUVECs after control siRNA in 3D collagen gel form vascular structures with a central lumen (L) lined with a monolayer with apical Podxl and basal Coll IV (arrowheads and arrows, respectively, boxed magnification). By contrast, *CDH5*-silenced (VEC siRNA) ECs show abnormal lumen maturation: the lumen is smaller, lacks apical Podxl and intracellular vacuoles (V) that are typical of early phases of lumen organization (Kamei et al., 2006) are frequently present. Dashed line outlines the lumen. Confocal sections at 24 hours. Scale bars, 50 µm and 10 µm (in magnification). (**B**) Confocal sections, of one dorsal aorta (DA) of wild-type (wt) and VEC-null mouse embryos before the onset of embryonic circulation (5 somites). The lumen (L) in the wild-type embryo is wide and lined with polarized ECs (Podxl apical and Coll IV basal, arrowheads and arrows, red and green channels, respectively, see magnification of boxed area). In VEC-null embryos, the lumen is restricted and ECs can be found inside forming a septum (asterisk), which defines two lumens (L and L′). Both Podxl and Coll IV show ectopic basal and apical distribution, arrowheads and arrows, respectively (red and green channels, respectively, see magnification of boxed areas). Dashed lines outline the lumen. ECs are positive for PECAM (white channel) and Podxl (red channel) in both wild-type and VEC-null embryos (see magnification) (Carmeliet at al., 1999). NT, neural tube. Scale bars: 50 µm and 10 µm (in magnification). (**C**) In 2D culture, Podxl is often distributed to the basal (arrowheads) instead of the apical surface (arrows) in VEC-null ECs, or in sparse VEC-positive cells. Confocal sections, *z*-axis. Scale bar: 25 µm.

type embryos at the 5-somite stage, ECs in the dorsal aorta (Fig. 1B) were polarized and expressed Podxl and Coll IV at the apical and basal sides, respectively. Conversely, in *Cdh5*-null embryos, the vascular lumen was strongly altered and ECs were abnormally polarized (Fig. 1B). In addition, the absence of VEC protein (in VEC-null cells) or the lack of its engagement in cell-cell contacts (in sparse VEC-positive cells, which were genetically modified to express human *CDH5*) impaired the apical distribution of Podxl in 2D culture (Fig. 1C).

We then tested whether VEC could determine the localization and activate the Par polarity complex that has a crucial role in the establishment and maintenance of apical-basal polarity (Goldstein and Macara, 2007; Iden and Collard, 2008). We first observed that Par3, phosphorylated Thr410 PKCζ and Tiam (all of which are components of the Par polarity complex) required the presence of VEC for their correct localization at cell-cell junctions (supplementary material Fig. S3A). In addition, phosphorylated PKCζ expression levels were higher in ECs expressing VEC than in VEC-null cells (Fig. 4C). Higher levels of GTP-bound Rac and Cdc42 were also observed in control cells than in VEC-null cells (supplementary material Fig. S3B). Finally, the knockdown (KD) of components of the polarity complex, Tiam and Par3, inhibited lumen formation in HUVECs (supplementary material Fig. S1A), but did not affect the expression and distribution of VEC (supplementary material Fig. S2B and M.G.L., unpublished results), suggesting a role for these molecules as effectors, rather than activators, of the VEC complex.

CCM1 acts in concert with VEC to establish and maintain correct EC polarity and vascular lumen

When the expression of *CCM1* was downregulated in HUVECs by siRNA (which knocked down CCM1 protein levels by some 70%, independently of the oligos used, supplementary material Fig. S2A), the lumen of vascular-like structures in 3D collagen gel was highly abnormal, both at early and late stages of culture (Fig. 2A; supplementary material Fig. S1A). *CCM1*-knockdown cells lining the lumen were poorly polarized. In several regions, Coll IV presented apical distribution (Fig. 2A) and, although the expression levels of Podxl were not reduced by *CCM1* gene silencing (supplementary material Fig. S2C), it did not show the normal distinct apical polarization (Fig. 2A).

Podxl was also depolarized in the endothelium of CCM lesions from a patient with mutated *CCM1* (Fig. 2B). In the areas of tortuous, lace-like vessels, Podxl was often lacking from the lumenal surface and was instead concentrated at the basal aspect of ECs. Podxl appeared normally polarized in peri-lesion vessels (Fig. 2B).

CCM1 has been shown to associate indirectly with AJs (Glading et al., 2007). As reported above, VEC is important in endothelial polarization and lumen formation. Furthermore, the 3D phenotype observed in *CDH5*-knockdown cells resembles that of *CCM1*-knockdown cells. CCM1 might, therefore, act in concert with VEC to promote cell polarity and regular lumen formation. As reported in Fig. 3A, we found that CCM1 associated with VEC. This was further supported by the observation that CCM1 was lost from junctions when *CDH5* gene

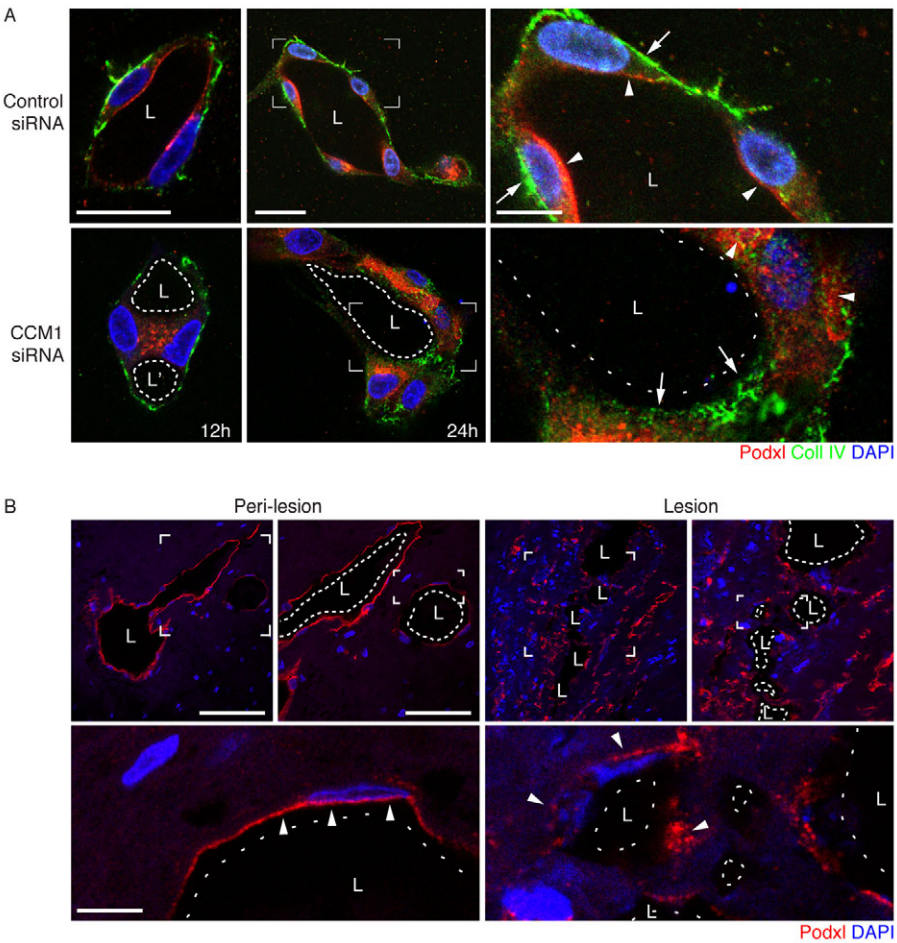

Fig. 2. Endothelial lumen and polarity are abnormal in *CCM1*-silenced HUVECs and in human CCM1 vascular lesions. (**A**) HUVEC vascular structures in 3D culture. At early stages (12 hours) a single central lumen (L) is formed with control siRNA, whereas two eccentric lumens (L and L′) are typically observed with *CCM1* siRNA. At later stages (24 hours), *CCM1*-silenced ECs form an irregular lumen with cell aggregates at the wall. Podxl (arrowheads) is cytoplasmic or ablumenal whereas collagen IV appears to be lumenal (arrows) (see boxed magnification). Dashed lines outline the lumen. Scale bars: 50 µm (left), 50 µm (central) and 10 µm (magnified areas). (**B**) Vascular lesion in a patient with heterozygous mutation of *CCM1*. In vessels at the periphery of the lesion (Peri-lesion) the lumen (L) is correctly polarized, with apical Podxl (arrowheads). By contrast, within the lesion, tortuous and multiple lumens (L) often lack apical Podxl, which appears to be basal or displays a diffuse pattern of expression (arrowheads). Dashed lines outline the lumens. Scale bars: 115 µm (top), 60 µm (bottom left) and 10 µm (bottom right).

expression was silenced (Fig. 3B), indicating that VEC is needed for CCM1 organization at AJs.

We then examined the effects of CCM1 on junction organization and localization of components of the polarity complex. Silencing of *CCM1* expression strongly affected junctional clustering of VEC and β-catenin. Both proteins relocalized from lateral junctions towards large areas flanking the junctions (Fig. 3D). VEC and β-catenin were often distributed toward the apical membrane (Fig. 3C, top panels, *z*-axis) and toward the basal aspect of HUVECs in 3D culture (Fig. 3C, bottom panels). Similarly to VEC, junctional staining of components of the Par polarity complex (Par3 and phosphorylated PKCζ and Tiam) was strongly altered after knockdown of *CCM1* (Fig. 4A). Comparable effects were observed using RNA-silencing oligos that targeted independent sequences of *CCM1* mRNA (our unpublished results), that were as effective at silencing the expression of *CCM1* as the oligos used for experiments described in Figs 3 and 4 (see also supplementary material Fig. S2A). Most importantly, ablation of the *Ccm1* gene in ECs isolated from *Ccm1* fl/fl mice produced effects comparable with those described after siRNA treatment (Fig. 3E, Fig. 4B, and supplementary material Fig. S2A).

Together, these data show that VEC and CCM1 interact at AJs, and that both are required for junctional localization of the polarity complex. However, VEC was needed for phosphorylation of PKCζ at Thr410 (Fig. 4C), whereas CCM1 controlled the junctional localization of this molecule. Analysis of lesions from a patient with mutated *CCM1* confirmed that both VEC (Fig. 3F) and phosphorylated PKCζ (Fig. 4D) are strongly delocalized from endothelial junctions in these areas: their focal junctional distribution is lost, but is maintained in the endothelium of peri-lesion vessels.

Finally, we observed that Rap1 was also important in EC polarity. Indeed, localization of the components of the Par polarity complex phosphorylated PKCζ (Fig. 5A), as well as Par3 and Tiam (supplementary material Fig. S3C) were strongly reduced at junctions upon treatment of ECs with *RAP1B* siRNA. Consistent with this observation, *RAP1B* siRNA altered endothelial cell polarity and lumen formation (Fig. 5C). Dismantling AJs by silencing either *CCM1* (Fig. 5D) or *CDH5* (Gore et al., 2008) led to loss of Rap1b

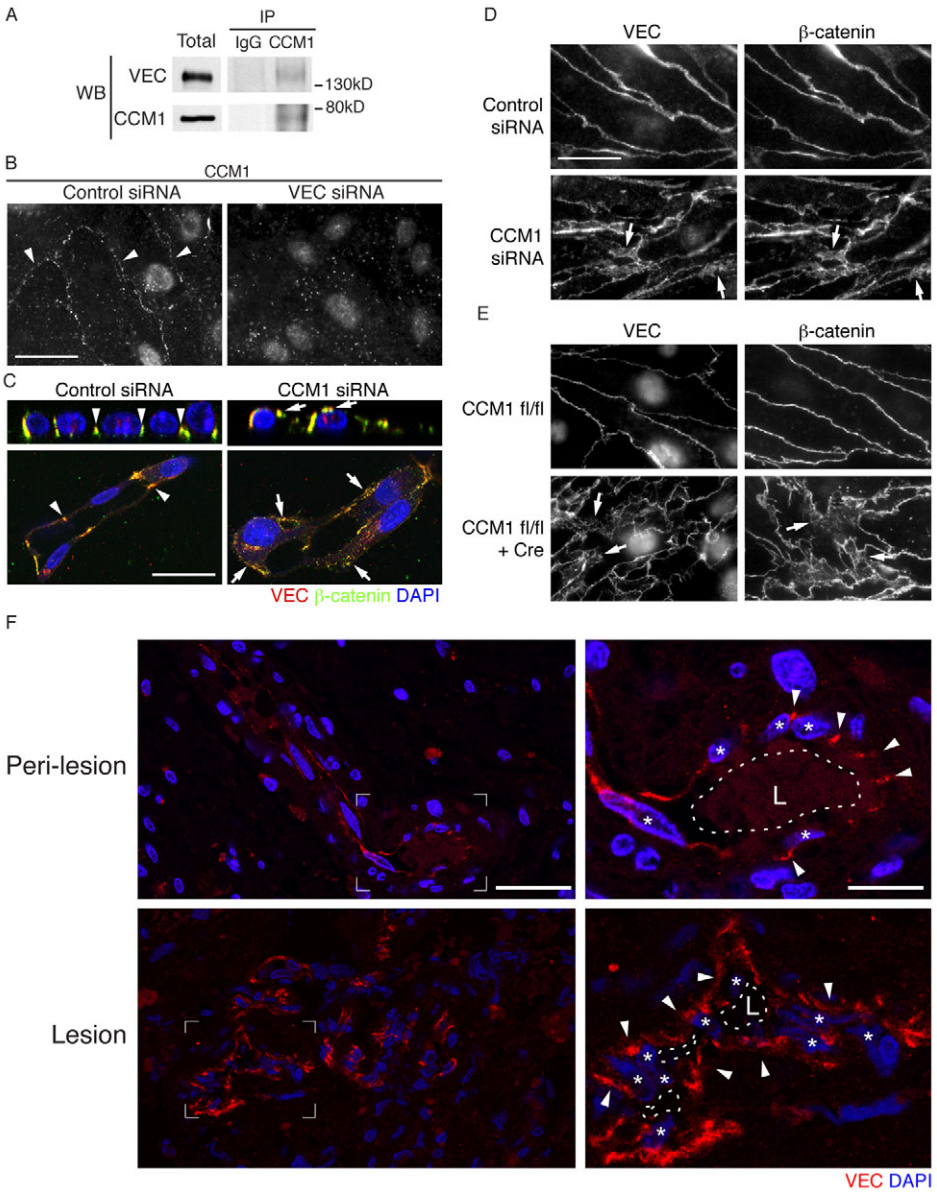

Fig. 3. VEC and CCM1 form a complex and reciprocally regulate their localization to endothelial cell-cell junctions both in cultured ECs and in human CCM1 vascular lesions. (**A**) VEC and CCM1 are coimmunoprecipitated in VEC-positive ECs. The complex is detected after immunoprecipitation (IP) with antibodies against CCM1. IP with irrelevant immunoglobulins (IgG) and IP-input extracts (Total) were also probed. The blots are representative of three independent experiments. (**B**) In *CDH5*-silenced (VEC siRNA) HUVECs, CCM1 disappears from cell-cell contacts (compare with arrowheads in control siRNA). Scale bar: 20 μm. (**C**,**D**) In *Ccm1*-silenced VEC-positive cells (2D culture, C top panels and D) or *CCM1*-silenced HUVECs (3D culture; C, bottom panels) VEC and β-catenin expression is lost at junctions and the proteins are redistributed apically or basally (C, arrows and arrowheads). Four independent oligos and smart pooled oligo against *CCM1* and *Ccm1* were used in silencing experiments with comparable results (see supplementary material Fig. S2). (**E**) Lung-derived ECs isolated from *Ccm1* fl/fl mice and treated with TAT-Cre (CCM1 fl/fl+Cre) to inactivate *Ccm1* gene in vitro (see supplementary material Fig. S2) show diffuse VEC and β-catenin expression patterns that are similar to those observed with *Ccm1* siRNA (arrows in D). (**F**) Vascular lesion in a patient with heterozygous mutation of *CCM1* gene. VEC appears to be diffusely expressed outside the junctions in ECs of the lesion (arrowheads, magnification of boxed area). By contrast, in vessels at the periphery of the lesion (Peri-lesion) VEC is focally concentrated at cell-cell contacts in ECs (arrowheads, magnification of boxed area). The dashed lines outline the lumens (L). Scale bars: 50 μm (C), 20 μm (D,E); 50 μm and 10 μm (F).

from intercellular contacts and to its relocalization to focal contacts, as previously reported in other cell types (Balzac et al., 2005). The active form of Rap, Rap1 G12V, was observed at junctions in a CCM1-dependent manner (Fig. 5E), although knockdown of VEC abrogated Rap1b activation (Fig. 5B).

Discussion

In the present paper, we have shown that VEC and CCM1 form a complex and act in concert to determine the correct junctional localization of the Par polarity complex that is required for endothelial polarization and formation of vascular lumen. This conclusion is supported by the observation that cadherin proteins might act as scaffolds for the construction of polarized structures (Capaldo and Macara, 2007). We did not perform detailed studies on the molecular basis of the interaction of CCM1 with VEC, but Glading and colleagues (Glading et al., 2007) reported that CCM1 could bind β-catenin directly. It is therefore likely that CCM1 associates indirectly with VEC, via binding to β-catenin. These authors also found that CCM1 is an effector of Rap1. The active state of this small GTPase induces a conformational change in CCM1 that is required for CCM1 binding to β-catenin. Rap1, which also concentrates at intercellular contacts, is known to stabilize AJs, thus reducing vascular fragility (Kooistra et al., 2005). CCM1 has therefore been proposed to act downstream of Rap1 in the stabilization of AJs. We show here that Rap1b, which is recruited to the complex formed by VEC and CCM1, contributes to polarization and lumen formation by stabilizing AJs. The need for VEC homophilic interactions to permit Rap1b activity has been previously reported. Clustering of VEC would allow junctional localization of guanosine-exchange factors such as C3G or PDZ-GEF to promote local activation of Rap1 (Kooistra et al., 2007; Sakurai et al., 2006). VEC and CCM1 exert a different type of control on PKCζ, the effector component of the polarity complex. Both VEC and CCM1 direct junctional localization of PKCζ. However, only VEC is required for the phosphorylation of PKCζ at Thr410, which is needed for expression of the catalytic activity of the enzyme (Standaert et al., 1999). Nevertheless, segregation of PKCζ is fundamental to allow phosphorylation of the correct molecular targets in the appropriate subcellular compartment (Wirtz-Peitz et al., 2008). Loss of segregation of the Par polarity complex and PKCζ can have a strong impact on their signaling and functional outcome (Goldstein and Macara, 2007; Martin-Belmonte et al., 2007). This has recently been shown in vivo during the organization of tubular structures in the morphogenesis of the mammary gland (McCaffrey and Macara, 2009).

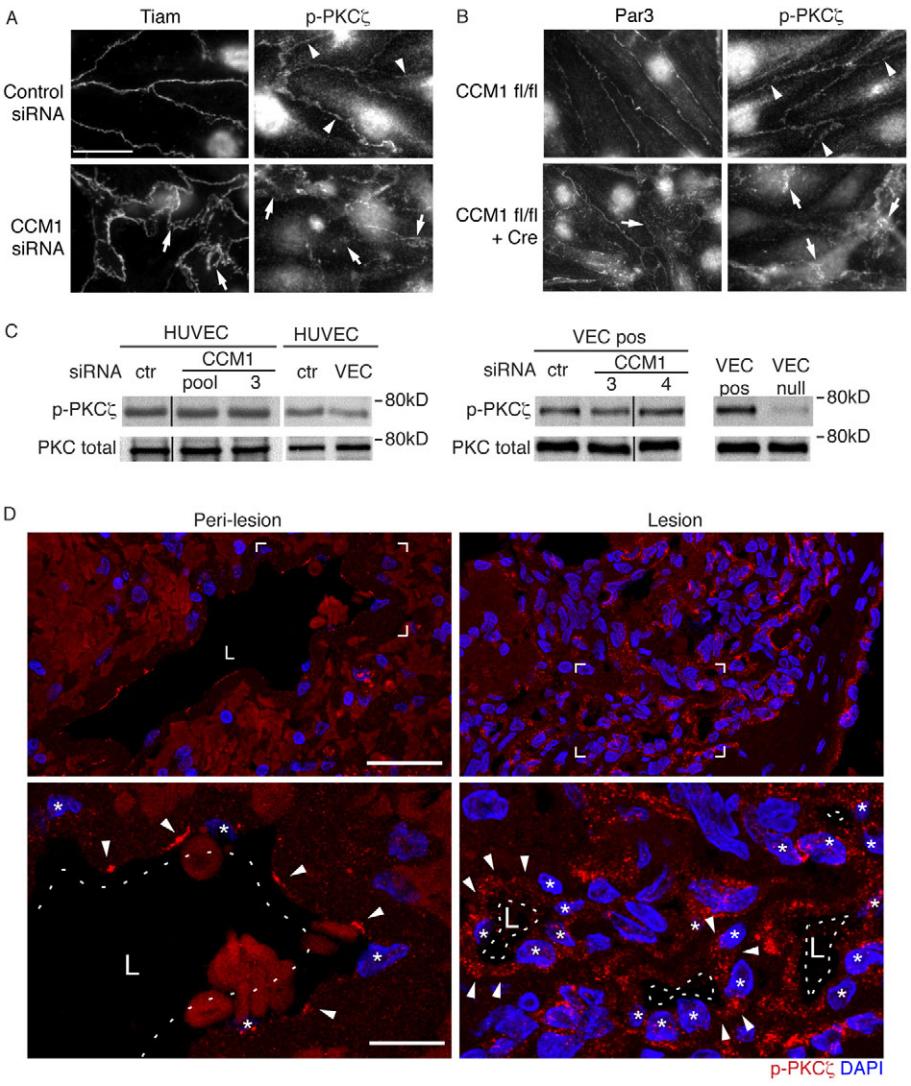

Fig. 4. CCM1 is required for junctional localization of the components of the polarity complex Tiam, Par3 and phosphorylated PKCζ in cultured ECs and in human CCM1 vascular lesion. (**A**,**B**) Junctional staining of Tiam, Par3 and phosphorylated PKCζ, in both *Ccm1*-silenced VEC-positive cells and in *Ccm1* fl/fl+Cre cells, appears convoluted and fragmented (arrowheads). This pattern is reminiscent of VEC and β-catenin distribution in *Ccm1*-silenced ECs (Fig. 3D,E). Scale bar: 20 µm. (**C**) Phosphorylation of PKCζ at Thr410 (p-PKCζ) is downregulated by 40% and 80%, respectively, in *CDH5*-silenced HUVECs and in VEC-null cells. Phosphorylation of PKCζ is not modified after *CCM1* or *Ccm1* siRNA treatment. Data shown are representative of three independent experiments. Vertical bars indicate that the bands, from the same blot, do not come from contiguous lanes. (**D**) Brain vascular lesion of a patient with heterozygous mutation of *CCM1* gene. Phosphorylated PKCζ expression is diffuse over large areas in the endothelium of the vascular mulberry lesion (arrowheads, magnification of boxed area). It is instead concentrated to interendothelial junctions in peri-lesion vessels (arrowheads, magnification of boxed area). Scale bars: 50 µm and 10 µm (in magnifications). Asterisks indicate the nuclei of ECs lining the lumen (dashed line).

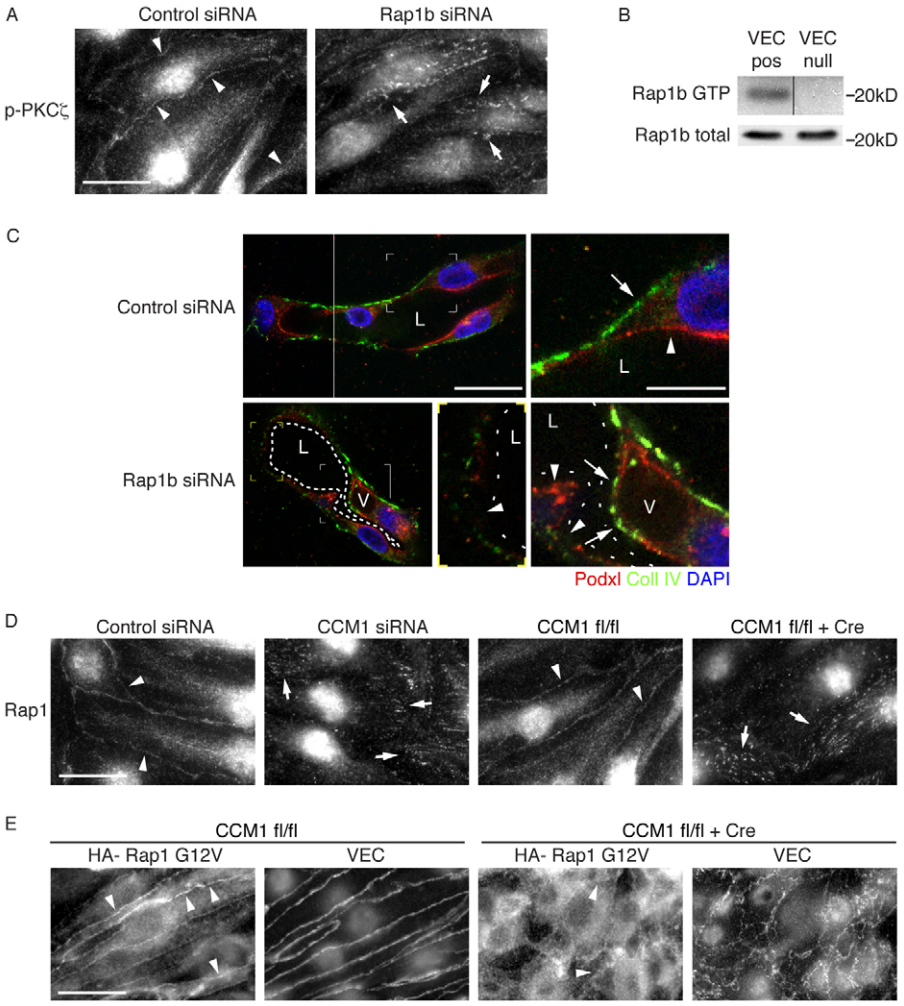

Fig. 5. Rap1b contributes to the polarizing activity of VEC and CCM1. (**A**) Rap1b regulates the junctional localization of phosphorylated PKCζ. In *RAP1B*-silenced HUVECs, phosphorylated PKCζ loses its linear junctional expression pattern (arrowheads), which instead becomes fragmented (arrows). Scale bar: 20 µm. (**B**) Rap1b requires VEC expression for its activation. Pull-down of GTP-Rap1was followed by western blotting for Rap1b. Two additional experiments gave comparable results. (**C**) *RAP1B*-silenced HUVECs show abnormal lumen (L) maturation (24 hours). Podxl and Coll IV apical or basal localization, respectively, is lost (arrowheads and arrows). The dashed lines outline the lumen. Scale bars: 50 µm and 10 µm (magnifications). (**D**) Rap1 disappears from cell-cell contacts in *Ccm1* silenced or *Ccm1* fl/fl+Cre cells and often concentrates at focal contacts (arrows and arrowheads). (**E**) Active Rap1 (HA-Rap1 GV12) concentrates to cell-cell junctions with VEC, in control cells (*Ccm1* fl/fl) (arrows). The junctional localization is strongly reduced or lost (arrows) in parallel with diffusion of VEC in *Ccm1*-silenced (*Ccm1* fl/fl+Cre) ECs. Immunofluorescence staining with an antibody against the HA tag present in Rap1 GV12. Scale bar: 20 µm (D,E).

The mechanism through which VEC influences endothelial polarity has not been investigated, as yet. In epithelial cells, cadherins promote the organization of TJs. In turn, TJs determine cell polarity, and act as a barrier to the redistribution of proteins and lipids between the apical and basolateral surfaces (Martin-Belmonte et al., 2007; Nejsum and Nelson, 2007; Mertens et al., 2006). However, early phases of epithelial polarization occur also in the absence of TJs (Umeda et al., 2006; Qin et al., 2005). TJs are either absent or very poorly organized in cultured ECs or in vascular endothelium at early phases of embryo development (Liebner et al., 2008; Carmeliet et al., 1999), which are the experimental models used in the present study. VEC and AJs could therefore serve to control early phases of polarity and lumen formation in the absence of TJs (Yu et al., 2005). As both phosphorylation at Thr410 (Standaert et al., 1999), and GTP-Cdc42 (Martin-Belmonte et al., 2007) or GTP-Rac (Chen and Macara, 2005; Mertens et al., 2005) are required for full activation and correct positioning of PKCζ, our data strongly suggest that VEC induces the early steps of cell polarization by permitting the correct localization and activation of the polarity complex. However, since junctional clustering of VEC precedes and triggers TJ organization (Taddei et al., 2008), it is possible that when junctions are fully stabilized, the polarity complex re-localizes from AJs to TJs (Mertens et al., 2006) to maintain cell polarity (Capaldo and Macara, 2007).

CCM1 can form a complex with CCM2 and CCM3 (Zawistowski et al., 2005; Voss et al., 2007). Although CCM proteins are structurally distinct, gene inactivation of any one of them leads to a comparable clinical outcome (Plummer et al., 2005), suggesting that these proteins act in concert to promote similar cell functions. CCM2 limits Rho activation and, as a consequence, reduces endothelial cell contractility and vascular permeability (Whitehead et al., 2009). The organization of the actin cytoskeleton might also be implicated in cell polarity (Iden and Collard, 2008). It is therefore possible that CCM2 also contributes indirectly to the establishment of cell polarity through a reshaping of the cell cytoskeleton (Whitehead et al., 2009).

In conclusion, our data show that the presence of VEC and CCM1 is needed at AJs to establish cell polarity and lumen formation. These effects are mediated by recruitment and activation of the polarity complex and Rap1b. In the absence of any of these proteins, AJs are not correctly organized, cell polarity is lost and vascular lumen formation is severely altered. These observations suggest that the various proteins implicated in inducing cell polarity do not function independently, but interact with one another in a signaling network.

Materials and Methods

Cells and 2D culture

Human umbilical vein endothelial (HUVEC) cells and murine endothelial cells genetically ablated for *Cdh5* (VEC-null) or genetically modified to express human

CDH5 (VEC-positive) were obtained and cultured as described (Lampugnani et al., 2002). Lung endothelial cells from a mouse (57/BL/6N) in which exons 4-5 of *Ccm1* gene are flanked by *loxP* sites (*Ccm1* fl/fl) (produced by Artemis Taconis, Cologne, Germany) were isolated and immortalized as described (Balconi et al., 2000). Deletion of exons 4-5 by Cre-mediated recombination produces loss of function by removing the exons encoding part of the p53-like DNA-binding domain and generating a frame-shift in downstream exons. *Ccm1* was inactivated treating cells in culture with TAT-Cre recombinase (Liebner et al., 2008). For TAT-Cre treatment: *Ccm1* fl/fl ECs were treated with 100 μg/ml TAT-Cre for 60 minutes in HyClone ADCF-MAb medium (Thermo Scientific) and with 100 μM chloroquine for further 60 minutes (Wadia et al., 2004). Cells (*Ccm1* fl/fl + Cre) were used for experiments after at least two in vitro passages from TAT-Cre treatment.

For two-dimensional (2D) cultures, cells were seeded in 35 mm Petri dishes or in 0.2 cm^2 wells created with flexiPERM frames (Greiner Bio-One, Germany) on polystyrene cell culture slides (NUNC, Thermo Fisher, Denmark). In both cases, the coating was with gelatin (Difco Gelatin, BD, 0.1% for murine cells and 1.5% for HUVECs). Cells were cultured for 72-96 hours. The splitting ratio was such that confluence was reached overnight after seeding. HA-Rap1 G12V (a gift from Giorgio Scita, IFOM, Milano, Italy) and HA-Rap GAP (a gift from Johannes Bos, University Medical Center Utrecht, Utrecht, The Netherlands) were introduced in lentiviral plasmids to produce lentiviral vectors, as described (Taddei et al., 2008).

RNA interference

For small RNA interference (siRNA) the following oligos were used: anti-CCM1, human-specific (pool, L-003825-00) (ON-TARGET plus SMART pool, Dharmacon); murine-specific oligo 2 (KRIT1MSS234457) and oligo 4 (KRIT1MSS294386); human- and murine-specific oligo 3 (KRIT1MSS234455) were all stealth oligo, Invitrogen. Anti-humanVE-cadherin: (L-003641-00); Anti-Rap1b: human-specific (L-010364-00) and murine-specific (L-062638-01); anti-human Tiam (L-003932-01); anti-human Par3 (L-015602-00) were all ON-TARGET plus SMART pool, Dharmacon. Control oligos were ON-TARGET plus siControl (D-001810-10, Dharmacon) and Medium G-C control oligo (12935-300, Invitrogen) when Dharmacon SMART pool or Invitrogen oligos were used, respectively. For oligo transfection, cells were seeded at a density of $10^4/cm^2$. 20 hours later, they were transfected with 40 nM of small interfering oligo for 5 hours in Optimem (Invitrogen) using Lipofectamine 2000 (Invitrogen). The effects of silencing were tested 72-96 hours after transfection.

Antibodies

For immunofluorescence (IF), western blot (WB) and immunoprecipitation, the following antibodies were used: VE-cadherin (C-19, sc-6458, goat, Santa Cruz), β-catenin (mouse, BD Transduction Laboratories), CCM1 (residues 1-207 of the human sequence and crossreacting with mouse protein, rabbit, gifts from Elisabeth Tournier-Lasserve, Université de Médicine Paris Diderot, Paris, France and Francesco Retta, University of Torino, Torino, Italy) and (KRIT1, K-16, sc-23997, goat, Santa Cruz), phosphorylated PKCζ (rabbit, Cell Signaling), total a-PKCζ (C-20, sc-216, rabbit, Santa Cruz), Rap1 (121, sc-65, rabbit, Santa Cruz, for IF), Rap1b (rabbit, Cell Signaling, for WB), Tiam 1 (C-16, sc-872, rabbit, Santa Cruz), Podxl (murine-specific and human-specific, R&D), Coll IV (murine-specific and human-specific, AbD Serotec), Par3 (rabbit, Millipore), Pecam (clone 13.3, rat anti-mouse), Flag, (M2, mouse, Sigma), rac (mouse, BD Transduction Laboratories), cdc42 (rabbit, Cell Signaling), HA (clone 11, mouse, Covance). For in vivo treatment and three-dimensional (3D) culture, a murine anti-human VEC (clone BV9, home made), which inactivates VEC (Corada et al., 2001) was used as specified in supplementary material Fig. S1. Secondary antibodies for IF were donkey antibodies to the appropriate species conjugated with Alexa Fluor 488, 555 or 647 (Molecular Probes).

Immunofluorescence microscopy

Cells in 2D culture were fixed either with 3% picric-acid-paraformaldehyde (PAF) in PBS (followed by permeabilization with 0.5% Triton X-100 for 5 minutes) or with 1% PAF in triethanolamine, pH 7.5, containing 0.1% Triton X-100 and 0.1% NP-40. Blocking (1 hour), primary (1 hour) and secondary (50 minutes) antibodies were in PBS with 1% BSA or 0.7% fish skin gelatin (FSG). For some antibodies (Rap1, phosphorylated PKCζ), the first antibody was incubated overnight at 4°C. Primary antibody contained also 5% donkey serum, because all the secondary antibodies used were donkey IgG.

3D cultures were fixed with 3% PAF for 35 minutes, quenched with 75 mM NH_4Cl and 20 mM glycine in PBS, pH 8, for 10 minutes and blocked with 0.7% FSG and 0.3% Triton X-100 PBS (blocking buffer) for 30 minutes. Primary and secondary antibodies were incubated overnight at 4°C. Primary antibody contained 5% donkey serum. Washes in blocking buffer were performed over the course of a day at room temperature. Nuclei were stained with DAPI for 30 minutes, and samples post-fixed with 3% PAF for 2 minutes. Embryos were stained in whole mount essentially as for 3D cultures (see above). They were then included in low-melting-point agarose and cut in 100-μm-thick slides using a vibratome. Slides were mounted within two coverslips to allow confocal examination on the two sides of the section.

Paraffin sections were deparaffinized according to standard procedures and stained as 3D cultures, except that the secondary antibody was for 1 hour at room temperature. Samples were mounted in Vectashield containing DAPI. 2D culture images were obtained by epifluorescence and confocal microscopy. Images of 3D cultures, embryo sections and tissue sections were obtained by confocal microscopy. Figures were assembled using Adobe Photoshop and Adobe Illustrator.

3D culture in collagen gels

HUVECs were cultured in 3D collagen gel as described (Bayless and Davis, 2002). Final cell concentration in collagen (3.5 mg/ml final concentration collagen type1 from rat tail, High Concentration, BD Biosciences) was 5×10^5 cells/ml. Culture medium was 199 with 1% FCS, Insulin-Transferrin-Selenium supplement (Life Technologies), 50 ng/ml PMA, 50 μg/ml ascorbic acid, 40 ng/ml VEGF, 40 ng/ml bFGF. When indicated, 8-pCPT-2′OMe-cAMP (007, 100 μM, Biolog) was added to the culture medium. For experiments with siRNA-silenced cells, cells were transfected with RNA oligos in 50 cm^2 Petri dishes 48 hours before the beginning of 3D culture. 50 μl cell suspension in collagen was used for each well of a 96-well plate for transmission microscopy. For confocal microscopy, 190 μl cell suspension in collagen was used for each microwell (μ-slide 80826, Ibidi, Germany).

Isolation of mouse embryos

Mouse embryos were dissected from VEC heterozygous females after breeding with VEC heterozygous males). Dissection was at day 8-8.5 of pregnancy. A description of the production and features of this strain is described (Carmeliet et al., 1999). Embryos were fixed in 4% PAF overnight before staining as described below.

Pull down of GTP-bound small GTPases

GTP-bound Rac and cdc42 were pulled-down as described (Lampugnani et al., 2002). The pull-down sample was then loaded in separate lanes and anti-Rac and anti-cdc42 antibodies were used in western blots to specifically recognize each GTPase. Rap-GTP was separated using a GST-RalGDS-RBD probe as described (Cera et al., 2009). Specific antibodies to Rap1b were used in western blotting to recognize this isoform of Rap1 that is the most abundant in ECs (Gore et al., 2008).

Western blot and immunoprecipitation

Total extracts were obtained using 2× Laemmli sample buffer. After 10 minutes of boiling, protein content was quantified (BCA, Pierce). Samples were then reduced by adding DTT (50 mM). Extraction for immunoprecipitation was as described (Rudini et al., 2008). Bands on films revealed through ECL were quantified through ImageJ (freely available at the NIH website). Several exposure times were used for each blot and values in the linear range of detection were used for quantification.

This work was supported by the Fondation Leducq Transatlantic Network of Excellence, Associazione Italiana per la Ricerca sul Cancro, Association for International Cancer Research UK (07-0068), the European Community (Integrated Project Contract No LSHG-CT-2004-503573; EUSTROKE contract 202213, OPTISTEM contract 223098, ANGIOSCAFF NMP3-LA-2008-214402 and ENDOSTEMCELLS Networks), Istituto Superiore di Sanita, Italian Ministry of Health, CARIPLO Foundation contract 2008.2463.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/7/1073/DC1

References

Balconi, G., Spagnuolo, R. and Dejana, E. (2000). Development of endothelial cell lines from embryonic stem cells: A tool for studying genetically manipulated endothelial cells in vitro. *Arterioscler. Thromb. Vasc. Biol.* **20**, 1443-1451.

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J. V. and Retta, S. F. (2005). E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. *J. Cell Sci.* **118**, 4765-4783.

Bayless, K. J. and Davis, G. E. (2002). The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices. *J. Cell Sci.* **115**, 1123-1136.

Blum, Y., Belting, H. G., Ellertsdottir, E., Herwig, L., Luders, F. and Affolter, M. (2008). Complex cell rearrangements during intersegmental vessel sprouting and vessel fusion in the zebrafish embryo. *Dev. Biol.* **316**, 312-322.

Capaldo, C. T. and Macara, I. G. (2007). Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. *Mol. Biol. Cell* **18**, 189-200.

Carmeliet, P., Lampugnani, M. G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M. et al. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147-157.

Cera, M. R., Fabbri, M., Molendini, C., Corada, M., Orsenigo, F., Rehberg, M., Reichel, C. A., Krombach, F., Pardi, R. and Dejana, E. (2009). JAM-A promotes neutrophil chemotaxis by controlling integrin internalization and recycling. *J. Cell Sci.* **122**, 268-277.

Chen, X. and Macara, I. G. (2005). Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nat. Cell Biol.* **7**, 262-269.
Clatterbuck, R. E., Eberhart, C. G., Crain, B. J. and Rigamonti, D. (2001). Ultrastructural and immunocytochemical evidence that an incompetent blood-brain barrier is related to the pathophysiology of cavernous malformations. *J. Neurol. Neurosurg. Psychiatry* **71**, 188-192.
Corada, M., Liao, F., Lindgren, M., Lampugnani, M. G., Breviario, F., Frank, R., Muller, W. A., Hicklin, D. J., Bohlen, P. and Dejana, E. (2001). Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. *Blood* **97**, 1679-1684.
Gerard, A., Mertens, A. E., van der Kammen, R. A. and Collard, J. G. (2007). The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. *J. Cell Biol.* **176**, 863-875.
Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. *J. Cell Biol.* **179**, 247-254.
Goldstein, B. and Macara, I. G. (2007). The PAR proteins: fundamental players in animal cell polarization. *Dev. Cell* **13**, 609-622.
Gore, A. V., Lampugnani, M. G., Dye, L., Dejana, E. and Weinstein, B. M. (2008). Combinatorial interaction between CCM pathway genes precipitates hemorrhagic stroke. *Dis. Model. Mech.* **1**, 275-281.
Gory-Faure, S., Prandini, M. H., Pointu, H., Roullot, V., Pignot-Paintrand, I., Vernet, M. and Huber, P. (1999). Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093-2102.
Hallmann, R., Horn, N., Selg, M., Wendler, O., Pausch, F. and Sorokin, L. M. (2005). Expression and function of laminins in the embryonic and mature vasculature. *Physiol. Rev.* **85**, 979-1000.
Horvat, R., Hovorka, A., Dekan, G., Poczewski, H. and Kerjaschki, D. (1986). Endothelial cell membranes contain podocalyxin-the major sialoprotein of visceral glomerular epithelial cells. *J. Cell Biol.* **102**, 484-491.
Iden, S. and Collard, J. G. (2008). Crosstalk between small GTPases and polarity proteins in cell polarization. *Nat. Rev. Mol. Cell. Biol.* **9**, 846-859.
Iden, S., Rehder, D., August, B., Suzuki, A., Wolburg-Buchholz, K., Wolburg, H., Ohno, S., Behrens, J., Vestweber, D. and Ebnet, K. (2006). A distinct PAR complex associates physically with VE-cadherin in vertebrate endothelial cells. *EMBO Rep.* **7**, 1239-1246.
Iruela-Arispe, M. L. and Davis, G. E. (2009). Cellular and molecular mechanisms of vascular lumen formation. *Dev. Cell* **16**, 222-231.
Itoh, M., Nelson, C. M., Myers, C. A. and Bissell, M. J. (2007). Rap1 integrates tissue polarity, lumen formation, and tumorigenic potential in human breast epithelial cells. *Cancer Res.* **67**, 4759-4766.
Jaffe, A. B., Kaji, N., Durgan, J. and Hall, A. (2008). Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. *J. Cell Biol.* **183**, 625-633.
Kamei, M., Saunders, W. B., Bayless, K. J., Dye, L., Davis, G. E. and Weinstein, B. M. (2006). Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* **442**, 453-456.
Kilic, T., Pamir, M. N., Kullu, S., Eren, F., Ozek, M. M. and Black, P. M. (2000). Expression of structural proteins and angiogenic factors in cerebrovascular anomalies. *Neurosurgery* **46**, 1179-1191.
Kleaveland, B., Zheng, X., Liu, J. J., Blum, Y., Tung, J. J., Zou, Z., Chen, M., Guo, L., Lu, M. M., Zhou, D. et al. (2009). Regulation of cardiovascular development and integrity by the heart of glass-cerebral cavernous malformation protein pathway. *Nat. Med.* **15**, 169-176.
Koh, W., Mahan, R. D. and Davis, G. E. (2008). Cdc42- and Rac1-mediated endothelial lumen formation requires Pak2, Pak4 and Par3, and PKC-dependent signaling. *J. Cell Sci.* **121**, 989-1001.
Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett.* **579**, 4966-4972.
Kooistra, M. R., Dube, N. and Bos, J. L. (2007). Rap1: a key regulator in cell-cell junction formation. *J. Cell Sci.* **120**, 17-22.
Labauge, P., Denier, C., Bergametti, F. and Tournier-Lasserve, E. (2007). Genetics of cavernous angiomas. *Lancet Neurol.* **6**, 237-244.
Lampugnani, M. G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V. and Dejana, E. (2002). VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. *Mol. Biol. Cell* **13**, 1175-1189.
Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupalla, C. J., Reis, M., Felici, A., Wolburg, H., Fruttiger, M. et al. (2008). Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J. Cell Biol.* **183**, 409-417.
Limaye, N., Boon, L. M. and Vikkula. M. (2009). From germline towards somatic mutations in the pathophysiology of vascular abnormalities. *Hum. Mol. Genet.* **18**, R65-R74.
Lubarsky, B. and Krasnow, M. A. (2003). Tube morphogenesis: making and shaping biological tubes. *Cell* **112**, 19-28.
Martin-Belmonte, F. and Mostov, K. (2008). Regulation of cell polarity during epithelial morphogenesis. *Curr. Opin. Cell Biol.* **20**, 227-234.
Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V. and Mostov, K. (2007). PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell* **128**, 383-397.
McCaffrey, L. M. and Macara, I. G. (2009). The Par3/aPKC interaction is essential for end bud remodeling and progenitor differentiation during mammary gland morphogenesis. *Genes Dev.* **23**, 1450-1460.
Mertens, A. E., Rygiel, T. P., Olivo, C., van der Kammen, R. and Collard, J. G. (2005). The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. *J. Cell Biol.* **170**, 1029-1037.
Mertens, A. E., Pegtel, D. M. and Collard, J. G. (2006). Tiam1 takes PARt in cell polarity. *Trends Cell Biol.* **16**, 308-316.
Montero-Balaguer, M., Swirsding, K., Orsenigo, F., Cotelli, F., Mione, M. and Dejana, E. (2009). Stable vascular connections and remodeling require full expression of VE-cadherin in zebrafish embryos. *PLoS One* **4**, e5772.
Nejsum, L. N. and Nelson, W. J. (2007). A molecular mechanism directly linking E-cadherin adhesion to initiation of epithelial cell surface polarity. *J. Cell Biol.* **178**, 323-335.
Plummer, N. W., Zawistowski, J. S. and Marchuk, D. A. (2005). Genetics of cerebral cavernous malformations. *Curr. Neurol. Neurosci. Rep.* **5**, 391-396.
Qin, Y., Capaldo, C., Gumbiner, B. M. and Macara, I. G. (2005). The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *J. Cell Biol.* **171**, 1061-1071.
Rigamonti, D., Hadley, M. N., Drayer, B. P., Johnson, P. C., Hoenig-Rigamonti, K., Knight, J. T. and Spetzler, R. F. (1988). Cerebral cavernous malformations. Incidence and familial occurrence. *N. Engl. J. Med.* **319**, 343-347.
Rudini, N., Felici, A., Giampietro, C., Lampugnani, M., Corada, M., Swirsding, K., Garre, M., Liebner, S., Letarte, M., ten Dijke, P. et al. (2008). VE-cadherin is a critical endothelial regulator of TGF-beta signalling. *EMBO J.* **27**, 993-1004.
Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M., Nakaoka, Y. and Mochizuki, N. (2006). MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. *Mol. Biol. Cell* **17**, 966-976.
Schwamborn, J. C. and Puschel, A. W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.* **7**, 923-929.
Standaert, M. L., Bandyopadhyay, G., Perez, L., Price, D., Galloway, L., Poklepovic, A., Sajan, M. P., Cenni, V., Sirri, A., Moscat, J. et al. (1999). Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J. Biol. Chem.* **274**, 25308-25316.
Taddei, A., Giampietro, C., Conti, A., Orsenigo, F., Breviario, F., Pirazzoli, V., Potente, M., Daly, C., Dimmeler, S. and Dejana, E. (2008). Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat. Cell Biol.* **10**, 923-934.
Umeda, K., Ikenouchi, J., Katahira-Tayama, S., Furuse, K., Sasaki, H., Nakayama, M., Matsui, T., Tsukita, S. and Furuse, M. (2006). ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* **126**, 741-754.
Voss, K., Stahl, S., Schleider, E., Ullrich, S., Nickel, J., Mueller, T. D. and Felbor, U. (2007). CCM3 interacts with CCM2 indicating common pathogenesis for cerebral cavernous malformations. *Neurogenetics* **8**, 249-256.
Wadia, J. S., Stan, R. V. and Dowdy, S. F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **10**, 310-315.
Whitehead, K. J., Plummer, N. W., Adams, J. A., Marchuk, D. A. and Li, D. Y. (2004). Ccm1 is required for arterial morphogenesis: implications for the etiology of human cavernous malformations. *Development* **131**, 1437-1448.
Whitehead, K. J., Chan, A. C., Navankasattusas, S., Koh, W., London, N. R., Ling, J., Mayo, A. H., Drakos, S. G., Jones, C. A., Zhu, W. et al. (2009). The cerebral cavernous malformation signaling pathway promotes vascular integrity via Rho GTPases. *Nat. Med.* **15**, 177-184.
Wirtz-Peitz, F., Nishimura, T. and Knoblich, J. A. (2008). Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. *Cell* **135**, 161-173.
Wong, J. H., Awad, I. A. and Kim, J. H. (2000). Ultrastructural pathological features of cerebrovascular malformations: a preliminary report. *Neurosurgery* **46**, 1454-1459.
Yu, W., Datta, A., Leroy, P., O'Brien, L. E., Mak, G., Jou, T. S., Matlin, K. S., Mostov, K. E. and Zegers, M. M. (2005). Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Mol. Biol. Cell* **16**, 433-445.
Zawistowski, J. S., Stalheim, L., Uhlik, M. T., Abell, A. N., Ancrile, B. B., Johnson, G. L. and Marchuk, D. A. (2005). CCM1 and CCM2 protein interactions in cell signaling: implications for cerebral cavernous malformations pathogenesis. *Hum. Mol. Genet.* **14**, 2521-2531.