Rab11a Mediates Vascular Endothelial-Cadherin Recycling and Controls Endothelial Barrier Function

Zhibo Yan, Zhen-Guo Wang, Nava Segev, Sanyuan Hu, Richard D. Minshall, Randal O. Dull, Meihong Zhang, Asrar B. Malik, Guochang Hu

Objective—Vascular endothelial (VE)-cadherin is the predominant component of endothelial adherens junctions essential for cell–cell adhesion and formation of the vascular barrier. Endocytic recycling is an important mechanism for maintaining the expression of cell surface membrane proteins. However, little is known about the molecular mechanism of VE-cadherin recycling and its role in maintenance of vascular integrity.

Approach and Results—Using calcium-switch assay, confocal imaging, cell surface biotinylation, and flow cytometry, we showed that VE-cadherin recycling required Ras-related proteins in brain (Rab)11a and Rab11 family-interacting protein 2. Yeast 2-hybrid assay and coimmunoprecipitation demonstrated that direct interaction of VE-cadherin with family-interacting protein 2 (at aa 453–484) formed a ternary complex with Rab11a in human endothelial cells. Silencing of Rab11a or Rab11 family-interacting protein 2 in endothelial cells prevented VE-cadherin recycling and VE-cadherin expression at endothelial plasma membrane. Furthermore, inactivation of Rab11a signaling blocked junctional reannealing after vascular inflammation. Selective knockdown of Rab11a in pulmonary microvessels markedly increased vascular leakage in mice challenged with lipopolysaccharide or polymicrobial sepsis.


Key Words: adherens junctions ■ inflammation ■ membrane protein ■ permeability ■ sepsis

Vascular leakage and formation of protein-rich edema resulting from breach of the endothelial barrier is the hallmark of inflammation implicated in the pathophysiology of many disease states, including acute respiratory distress syndrome, ischemia–reperfusion injury, atherosclerosis, and diabetes mellitus.1,2 Vascular endothelial (VE)-cadherin–mediated homotypic cell–cell adhesion is a primary determinant of vascular barrier integrity, whereas dysregulation of VE-cadherin homeostasis is thought to play a crucial role in microvascular hyperpermeability.3,4 Constitutive trafficking of VE-cadherin between intracellular compartments and the plasma membrane allows for dynamic remodeling of adherens junctions (AJs), which is thought to be essential for endothelial cells to sprout, migrate, and repair a dysfunctional barrier after vascular injury.4,5

Expression of cell surface membrane proteins is maintained by the balance between endocytosis, by which membrane proteins are selectively removed from the cell surface, and endosomal recycling pathways that traffic endocytosed or newly formed proteins to the plasma membrane.6 Once membrane proteins are internalized, the endosomal sorting machinery regulates their postendocytic fates. The expression level of membrane proteins on the cell surface depends on whether they are sorted to the lysosome for degradation or recycled back to the cell surface.9,10 Membrane protein recycling is minimal in confluent monolayer of cells, but is markedly increased on disruption of intercellular contacts,5,11 suggesting recycling may be involved in the repair process. VE-cadherin is internalized through both clathrin-mediated10,12,13 and clathrin-independent pathways, including caveolae-mediated uptake in some cell types.14 However, mechanisms of redirecting internalized VE-cadherin back home to the endothelial cell surface to reanneal AJs remain unknown.

The mammalian Ras-related proteins in brain (Rab) GTPases localize to specific subcellular compartments where they orchestrate membrane trafficking, cargo selection, vesicle budding, moving, tethering, docking, and targeting.15 Rab11 subfamily members, comprising Rab11a, Rab11b, and Rab25,15 facilitate recycling of proteins from endosomes to the plasma membrane in polarized epithelial cells.16–18 Rab11a is expressed ubiquitously,19 whereas...
Rab11b is enriched in brain, heart, and testis, and Rab25 expression is restricted to epithelial cells. Rab11a and Rab11b isoforms share 89% amino acid homology, with least similarity found in membrane-binding hypervariable C-terminal domains. Although Rab11a and Rab11b share high sequence homology and localize to pericentriolar apical regions, they seem to mark distinct vesicle populations. Five members of this family (FIP1-5) have been identified that share a conserved carboxyl-terminal Rab11-binding domain. In polarized epithelial cells, Rab11a localized to the pericentriolar, microtubule-associated apical recycling endosomes, where it regulated apical recycling and insertion of membrane proteins in the plasma membrane. Rab11 family-interacting proteins (Rab11-FIPs, henceforth FIPs), link Rab11 to cytoskeletal components, and they are responsible for directing vesicular movement throughout the recycling pathway. Five members of this family (FIP1-5) have been identified that share a conserved carboxyl-terminal Rab11-binding domain (BD).

In this study, we show that VE-cadherin recycling to endothelial cell plasma membrane requires active Rab11a and Rab11a effector FIP2. Depletion of Rab11a drastically reduced recovery of endothelial barrier function at the level of AJs. In mouse models of lung vascular injury induced by endotoxemia and polymicrobial septicemia via cecal ligation and puncture (CLP), depletion of Rab11a induced persistent lung vascular leakage. Our results suggest that Rab11a-mediated VE-cadherin recycling is essential for maintenance and restoration of VE barrier function through assembly of VE-cadherin at cell junctions.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Rab11a Is Required for Plasmalemmal Trafficking of VE-Cadherin

To address the role of Rab11a in VE-cadherin recycling in endothelial cells, we first studied the effects of depletion of Rab11a using small interfering RNA (siRNA) in human lung microvascular endothelial cells (HLMVECs; Figure 1A). VE-cadherin-dependent cell–cell junctions were manipulated using the Ca²⁺ switch procedure, which involves disrupting AJs by removal of extracellular Ca²⁺ followed by re-establishment of VE-cadherin–mediated cell–cell contacts on restoration of physiological levels of extracellular Ca²⁺. In control cells, VE-cadherin was internalized and colocalized with Rab11a after Ca²⁺ depletion (Figure 1A) but recycled back to the junctions after restoration of extracellular Ca²⁺. Depletion of Rab11a severely impaired VE-cadherin recycling although VE-cadherin internalization was not affected (Figure 1A). We also observed that the level of membrane-associated VE-cadherin at baseline (before removal of extracellular Ca²⁺) was significantly decreased in Rab11a-depleted cells (Figure 1A).

To delineate whether active Rab11a is required for VE-cadherin recycling and membrane localization, we transfected HLMVECs with Discosoma sp. red fluorescent protein tagged wild-type Rab11a (Rab11a-WT) or GDP-locked dominant-negative Rab11a-S25N. Ca²⁺ depletion induced VE-cadherin internalization in a similar manner in both Rab11a-WT-transfected and Rab11a-S25N–transfected cells, whereas VE-cadherin pools accumulated intracellularly and cell surface VE-cadherin was notably reduced in Rab11a-S25N–transfected cells after repletion of extracellular Ca²⁺ (Figure 1B), thus suggesting defective recycling of internalized VE-cadherin.

We also used membrane protein biotinylation to study recycling of internalized VE-cadherin back to the cell surface. Here biotinylated cell surface proteins (Figure 1C, lanes 1 and 6) were precipitated using streptavidin–agarose beads, and VE-cadherin immunoreactivity was assessed by Western blot analysis. After 1 hour in media with reduced concentration of extracellular Ca²⁺, ~80% of total biotinylated VE-cadherin was detected in the cytoplasm (Figure 1C, lanes 3 and 8; Figure IA in the online-only Data Supplement), indicating that surface VE-cadherin was substantially internalized and hence protected from stripping. Endocytosis rates of VE-cadherin in Rab11a siRNA–treated and scrambled siRNA–treated cells were similar after Ca²⁺ depletion (Figure 1C; Figure IB in the online-only Data Supplement). After Ca²⁺ repletion, however, the majority of internalized VE-cadherin recycled back to the cell surface within 1 hour in scrambled siRNA–treated cells, whereas 10% of the biotinylated VE-cadherin remaining in the cytosol. In contrast, in Rab11a siRNA–treated cells, nearly all internalized VE-cadherin was retained in the cytosol 1 hour after Ca²⁺ repletion (Figure 1C; Figure IA in the online-only Data Supplement).

VE-cadherin recycling was further quantified using flow cytometry to measure the amount of immunoreactive VE-cadherin present on the plasma membrane. We observed that cell surface VE-cadherin expression was markedly reduced after Ca²⁺ depletion but almost completely restored in scrambled siRNA–treated cells, whereas Rab11a siRNA–transfected cells exhibited minimal recovery of VE-cadherin at the plasma membrane after Ca²⁺ repletion (Figure 1D).

Rab4 is implicated in the fast recycling pathway of endosomes. Rab6 localizes to the Golgi where it regulates retrograde traffic between endosomes, the Golgi, and endoplasmic reticulum as well as exocytic traffic to the plasma membrane. Rab11b was shown to regulate apical recycling of the cystic fibrosis transmembrane conductance regulator. Interestingly, depletion of Rab4 (Figure IIA in the online-only Data Supplement), Rab6 (Figure IIB in the online-only Data Supplement), or Rab11b (Figure IIC in the online-only Data Supplement) with siRNAs did not alter VE-cadherin recycling
or membrane expression. Together, these data demonstrate the crucial role for Rab11a in mediating recycling and membrane localization of VE-cadherin and thereby promoting AJ assembly.

Rab11a Stabilizes VE-Cadherin by Avoiding Lysosomal Targeting

On the basis of the finding that the basal levels of VE-cadherin expression at the cell membrane were decreased in...
Rab11a-depleted cells (Figure 1), we sought to determine the role of Rab11a in regulating VE-cadherin expression through its ability to avoid degradation in lysosomes. In endothelial cells transfected with Rab11a siRNA, total VE-cadherin protein expression decreased within 48 to 72 hours, and then gradually returned to baseline level matching the pattern of Rab11a expression (Figure 2A). Reduced VE-cadherin expression after Rab11a depletion seemed to be specific because protein expression of Rab11b and 2 other AJ-associated proteins, p120-catenin and β-catenin, remained unchanged (Figure IIIA in the online-only Data Supplement). To extend this observation, we determined whether Rab11a expression regulated the synthesis of VE-cadherin. Here, we examined the effects of Rab11a depletion on VE-cadherin mRNA expression and observed no significant change (Figure IIIB in the online-only Data Supplement). Furthermore, depletion of Rab4, Rab6, or Rab11b did not affect expression of VE-cadherin (Figure IIC in the online-only Data Supplement). These results indicate that Rab11a specifically contributes to the stabilization of VE-cadherin protein expression by avoiding degradation pathways.

The intracellular fate of internalized VE-cadherin through sorting to degradation or recycling pathways dictates whether VE-cadherin is available to reanneal AJJs.12,29 We next assessed endosomal trafficking of VE-cadherin to examine the role of Rab11a in regulating the fate of VE-cadherin. Here, we observed that trafficking of VE-cadherin to lysosomes as suggested by colocalization (Figure 2B) of VE-cadherin with the lysosome marker lysosomal-associated membrane protein 1 was increased in Rab11a-knockdown cells. To delineate whether enhanced degradation of VE-cadherin after Rab11a depletion was because of enhanced trafficking of VE-cadherin to lysosomes, HLMVECs were treated with lysosomal inhibitor chloroquine. As shown in Figure 2C and 2D, chloroquine restored total and surface-associated VE-cadherin, whereas the proteasome inhibitor MG132 had no effect on VE-cadherin expression. Thus, blockade of normal Rab11a-mediated VE-cadherin recycling resulted in VE-cadherin trafficking, by default, to lysosomes for degradation.

**Direct Interaction of VE-Cadherin With FIP2 Forms a Ternary Complex With Rab11a**

We next investigated the mechanism of Rab11a-mediated VE-cadherin trafficking by assessing the possible interaction of Rab11a with VE-cadherin. Using purified recombinant Histagged Rab11a along with purified recombinant glutathione-S-transferase-VE-cadherin cytoplasmic tail (CT), we failed to observe direct binding of VE-cadherin with Rab11a (data not shown). Furthermore, yeast 2-hybrid assay confirmed that FIP2 interacted with Rab11a as opposed to VE-cadherin (Figure 3A).

We assessed whether VE-cadherin directly binds to FIPs. Among the 5 FIPs, FIP2 was found to interact with VE-cadherin as evidenced by the ability of yeast colonies to grow on synthetic defined media without adenine and histidine when yeast were cotransformed with plasmids bearing full-length FIP2 (aa 1–512) fused to Gal4 DNA-BD and human VE-cadherin CT fused to Gal4 activation domain (Figure 3B). We thus focused on the role of FIP2 in the endocytic recycling compartment mediating the interaction between Rab11a and VE-cadherin. To determine which fragment of FIP2 interacted with VE-cadherin, different FIP2 fragments were fused to Gal4 DNA-BD and their ability to bind VE-cadherin CT was assessed. We found that the C2 domain (aa 1–129), the center part containing the myosin Vb-BD (aa 1–413/aa 324–326, aa 406–408, and aa 440–442),30 did not interact with VE-cadherin. However, the C terminus of FIP2 (aa 453–512) containing the coiled-coil (aa 453–491) and Rab11-BD (aa 477–498)30 interacted with VE-cadherin (Figure 3B). Studies employing sequential FIP2 truncations revealed that...
The I481E mutation with Rab11a was not observed. However, this mutant still interacted with VE-cadherin (Figure 3D), implying that VE-cadherin–binding residues in FIP2 differ from those for Rab11a.

To address whether VE-cadherin association with Rab11a and FIP2 in endothelial cells was required for VE-cadherin trafficking and formation of AJs, we carried out VE-cadherin coimmunoprecipitation studies in HLMVECs. We observed Rab11a and FIP2 in the coimmunoprecipitation with an anti–VE-cadherin antibody, Rab11a and VE-cadherin with an anti-FIP2 antibody, and FIP2 and VE-cadherin with an anti-Rab11a antibody (Figure IVA in the online-only Data Supplement) consistent with formation of a ternary complex in endothelial cells. This complex was also observed on expression of exogenous VE-cadherin (Figure IVB in the online-only Data Supplement). The complex of Rab11a, FIP2, and VE-cadherin was found to primarily localize in a perinuclear pattern and along cell–cell contacts of confluent endothelial cells (Figure VIC in the online-only Data Supplement). On depletion of extracellular Ca²⁺, VE-cadherin dispersed from the lateral membrane to perinuclear sites where it colocalized with Rab11a and FIP2 (Figure IVC in the online-only Data Supplement). The same changes were evident in HLMVECs expressing exogenous WT Rab11a (Figure IVD in the online-only Data Supplement). These data together suggest that Rab11a, FIP2, and VE-cadherin form a complex and that Ca²⁺ depletion enhanced complex formation in perinuclear endosomal compartments.

**Functional Dissection of the Role of Rab11a-FIP2-VE-Cadherin Complex Formation in VE-Cadherin Recycling**

Next, we determined the role of FIP2 in VE-cadherin recycling in HLMVECs using a cell surface biotinylation assay.²,²⁶ We observed that FIP2 depletion inhibited recycling of internalized VE-cadherin back to the cell surface after repletion of extracellular Ca²⁺ (Figure 4A), indicating that FIP2 was required for VE-cadherin recycling and formation of AJs. To further address the role of Rab11a and FIP2 interaction in the regulation of VE-cadherin recycling, we determined the effect of FIP2 I481E mutant in HLMVECs. After knockdown of endogenous FIP2, we introduced WT CFP-FIP2 or CFP-FIP2 I481E (Figure 4B). We observed much reduced return of VE-cadherin to the plasma membrane in CFP-FIP2 I481E–transfected cells when compared with WT CFP-FIP2–transfected cells (Figure 4B), suggesting that binding of Rab11a with FIP2 is required for VE-cadherin recycling. Interestingly, Rab11a knockdown increased the colocalization of FIP2 and VE-cadherin (Figure 4C). Expression of dominant-negative Rab11a-S25N or depletion of Rab11a with siRNA increased the association of FIP2 and VE-cadherin (Figure 4D), suggesting that FIP2 binding to VE-cadherin competes with Rab11a binding to FIP2.

**Rab11a Regulates Assembly of AJs Secondary to VE-Cadherin Plasmaemmal Positioning**

We further investigated the role of Rab11a in VE-cadherin trafficking during inflammation. The mediator thrombin,
which is generated at the site of infection during sepsis and intravascular coagulation,\textsuperscript{32} proteolytically activates protein-activated receptor-1 eliciting an increase in vascular permeability.\textsuperscript{33,34} In HLMVECs challenged with thrombin, we observed Rab11a activation without alteration in Rab11a expression (Figure 5A). Confocal images showed internalization of VE-cadherin and formation of the ternary complex of VE-cadherin with FIP2 and Rab11a (Figure 5B; Figure VA in the online-only Data Supplement) as described above within 30 minutes after thrombin stimulation. At this time, VE-cadherin dispersed from the membrane pool via endocytosis as evident by internalized VE-cadherin appearing as VE-cadherin dispersed from the membrane pool via endocytosis. At this time, intracellular VE-cadherin puncta remained abundant in Rab11a siRNA–treated cells, whereas in contrast, scrambled siRNA–treated cells showed accumulation of VE-cadherin at AJs at 2 hours when compared with Rab11a-depleted cells (Figure 5C). In addition, the majority of surface-biotinylated, internalized VE-cadherin in control siRNA–treated cells recycled to the junctions within 2 hours after thrombin challenge, whereas VE-cadherin remained in the intracellular pool in Rab11a-depleted HLMVECs (Figure 5D). We did not detect any biotinylated VE-cadherin in the intracellular pool at 30 or 120 minutes after vehicle treatment (data not shown). The same phenotype was evident in FIP2-depleted HLMVECs (Figure 5E). Depletion of Rab11a also increased the colocalization of VE-cadherin and lysosomal-associated membrane protein in both control and thrombin-challenged cells (Figure VB in the online-only Data Supplement). Thus, Rab11a promoted the restoration of junctional VE-cadherin localization via recycling to the plasmalemma after thrombin challenge.

**Rab11a Is Required for Endothelial Barrier Restoration**

To test the hypothesis that Rab11a depletion impairs endothelial barrier repair, endothelial monolayer integrity at the level of AJs was monitored in real time by transendothelial electrical resistance (TER) measurements. TER decreased in HLMVECs treated with scrambled siRNA after thrombin challenge, indicating thrombin-induced formation of interendothelial gaps, and TER returned to baseline within 3 hours after thrombin challenge, indicating reannealing of AJs (Figure 6A). In Rab11a-depleted cells, however, basal resistance was lower, indicating higher basal junctional permeability when compared with control or scrambled siRNA–treated HLMVECs. Addition of thrombin to Rab11a-depleted cells further reduced TER but the response did not recover to baseline values (Figure 6A). Thus, Rab11a depletion induced persistent loss of AJ integrity resulting in sustained endothelial barrier dysfunction.
To address the role of Rab11a in transendothelial permeability to macromolecules, we assessed the effects of thrombin on fluorescein isothiocyanate-dextran transendothelial permeability in confluent endothelial monolayers. Rab11a-depleted monolayers showed higher basal permeability when compared to control siRNA-treated cells (Figure 6B). After thrombin exposure, we observed significantly greater fluorescein isothiocyanate-dextran permeability, which peaked at 30 minutes and remained elevated for 2 hours in Rab11a-depleted cells, whereas the permeability response to thrombin was transient in control cells (Figure 6B). Thus, Rab11a is an important regulator of endothelial barrier function.

To elucidate the role of Rab11a activation in regulating endothelial barrier integrity, HLMVECs were transfected with GFP-tagged Rab11a-WT, constitutively active Rab11a-Q70L, or dominant-negative Rab11a-S25N (Figure 6C, right). Endothelial cells expressing GFP-Rab11a-WT and GFP-Rab11a-Q70L showed faster recovery of TER after
thrombin stimulation when compared with the vector control group. In contrast, overexpression of GFP-Rab11a-S25N severely impaired recovery of TER after thrombin stimulation (Figure 6C, left). Thus, active Rab11a was required for restoration of endothelial monolayer integrity.

Loss of Rab11a Causes a Drastic Increase in Lung Vascular Leakage and Mortality in Septic Mice

Sepsis in humans is characterized by diffuse microvascular leak and tissue edema.35 To test if Rab11a plays a potential role in vascular leakage during sepsis, we first examined Rab11a expression in mouse lungs in preclinical sepsis models. Rab11a protein expression in mouse lung dramatically decreased after lipopolysaccharide challenge (Figure 7A) and CLP (Figure 7B). It seems that the loss of Rab11a in this CLP model is not as great as that seen for lipopolysaccharide (Figure 7A and 7B). Immunostaining further showed loss of Rab11a (Figure 7C) and VE-cadherin (Figure 7D) in pulmonary microvessel endothelial cells in lipopolysaccharide-treated mice. To determine whether a decrease in Rab11a protein expression after lipopolysaccharide exposure or CLP was functionally relevant, we studied the effects of Rab11a depletion in pulmonary vessel endothelia. After liposome-based delivery of Rab11a siRNA,36–38 Rab11a protein expression in pulmonary vascular endothelium was reduced by 90% shown by immunostaining of lung sections (Figure 7E) and Western blot analysis of isolated lung endothelial cells (data not shown). We observed extensive pulmonary vascular leakage as indicated by a significant increase in lung weight/dry ratio (Figure 7F and 7G) in the Rab11a-depleted mouse lungs after lipopolysaccharide challenge or CLP when compared with scrambled siRNA–treated control mouse lungs. Downregulation of Rab11a expression in pulmonary vascular endothelium increased mortality in mice challenged with endotoxemia (Figure 7H) and CLP (Figure 7I), which was associated with fulminant pulmonary edema. Consistent with our findings from cultured cells, knockdown of FIP2 in pulmonary vasculature (Figure VIA in the online-only Data Supplement) also enhanced lipopolysaccharide-induced lung edema (Figure VIB in the online-only Data Supplement). These results suggest the fundamental role of Rab11a/FIP2-mediated VE-cadherin recycling in regulating vascular leakage during sepsis.

Discussion

This study demonstrates the requisite role of Rab11a in mediating VE-cadherin recycling to the plasma membrane and thereby in maintaining and restoring endothelial barrier integrity. Rab11a functioned by interacting with VE-cadherin through the Rab11a-binding protein FIP2. The formation of the Rab11a-FIP2-VE-cadherin complex was required for recycling VE-cadherin and stabilizing AJs. Our findings thus establish the link between Rab11a-mediated VE-cadherin recycling and regulation of VE permeability and identify Rab11a as a key regulator of AJ assembly and endothelial barrier homeostasis.

 Trafficking pathways regulated by Rab GTPases have emerged as key transport mechanisms mediating delivery of cadherins to their designated sites.39 In the context of formation of intercellular junctions in polarized cells, Rab5 was shown to regulate endocytic transport of E- and N-cadherin, and resultant formation of cell–cell adhesions during vertebrate gastrulation and brain development.40,41 Rab11-mediated trafficking was shown to increase basolateral epithelial cadherin localization in epithelial cells.42 Also Rab35 facilitated N- and M-cadherin recruitment to cell–cell contacts in myoblasts and HeLa cells.43 Here, we for the first time demonstrated the central role of Rab11a and its effector FIP2 in mediating recycling of endocytosed VE-cadherin to reassemble AJs in endothelial cells, and hence delineate an essential mechanism for restoring vascular integrity after the endothelial barrier dysfunction during inflammation. We observed that VE-cadherin trafficking to the plasmalemma was a function of Rab11a activity and that disruption of AJs induced Rab11a activation, suggesting that Rab11a activation itself was regulated by loss of AJ integrity. We also observed that decreased Rab11a expression or loss of Rab11a activity inhibited VE-cadherin recycling to the nascent AJs, and by default induced VE-cadherin targeting to the lysosomal degradation pathway. The mechanism of
regulation of Rab11a activation (GTP-Rab11a) is not clear in our model. It is likely that P2XA-mediated decrease in intracellular Ca\(^{2+}\), causes inhibition of Rab GAP, which results in activation of Rab11a.44 The Rab11a-mediated VE-cadherin recycling machinery required interaction with VE-cadherin through FIP2 as opposed to Rab11a alone. Using the yeast 2-hybrid system, we identified that the major BD on FIP2 for VE-cadherin resides in the C-terminal α-helix (aa 453–484). This region consisted of 2 components (aa 453–474 and aa 475–484) with each capable of binding VE-cadherin. The distal region of the VE-cadherin–BD (aa 475–484) on FIP2 overlapped with the Rab11a-BD (aa 477–498).38 Mutation of hydrophobic isoleucine-481 to glutamate did not affect FIP2–VE-cadherin interaction, but it abrogated FIP2 binding to Rab11a and prevented trafficking of VE-cadherin. Thus, VE-cadherin and Rab11a directly interact with FIP2 via different residues in this region.

Our results firmly establish the essential role of Rab11a in mediating recycling of VE-cadherin in a unidirectional manner to the plasma membrane and in forming AJs. The reversible assembly of multiprotein complexes on distinct vesicle membranes is required for the function of Rab GTPases in regulating protein trafficking.38 We observed that both Rab11a and FIP2 were indispensable for VE-cadherin recycling because knockdown of either Rab11a or FIP2 similarly reduced VE-cadherin localization at cell junctions. Inhibition of FIP2 binding to Rab11a by the expression of FIP2 I481E mutant in FIP2-depleted endothelial cells also prevented VE-cadherin recycling and formation of junctions.

Interestingly, expression of the dominant-negative Rab11-S25N mutant, which increased the binding of VE-cadherin to FIP2 without affecting FIP2 binding to Rab11a, also reduced cell surface VE-cadherin levels, suggesting a crucial role for Rab11a activation in the regulation of VE-cadherin recycling. FIP2 serves as a Rab11 scaffolding protein, which regulates Rab11 localization and it is also involved in the recruitment of various cellular factors to different endocytic compartments.45 Thus, our results support a model whereby recruitment of FIP2 to the recycling compartment by GTP-bound Rab11a46 initially leads to the formation of the GTP-Rab11a-FIP2 complex. VE-cadherin in turn is recruited by FIP2 to form the Rab11a/FIP2/VE-cadherin ternary complex, which mediates delivery of VE-cadherin to reanneal AJ junctions.

We showed that Rab11a maintained expression of VE-cadherin at the junctions not only by trafficking VE-cadherin but also by avoiding VE-cadherin sorting to lysosomes for degradation. VE-cadherin internalization is critical for regulation of cell surface VE-cadherin localization and represents a primary mechanism of the loss of endothelial barrier integrity.4 Internalized cadherin can be routed from early endosomes to late endosomes and lysosomes for degradation, to the trans-Golgi network, or to recycling endosomal carriers to restore expression of cadherin and form stable junctions.10 We observed that downregulation of cell surface and total VE-cadherin expression in Rab11a siRNA–treated endothelial cells were rescued by an inhibitor of lysosomal proteases. Similarly, in human dermal microvascular endothelial cells, the lysosomal inhibitor chloroquine also dramatically...
inhibited the downregulation of VE-cadherin. Interestingly, lysosomotropic agent chloroquine not only rescued steady-state levels of VE-cadherin in Rab11a-knockdown cells but also restored the localization of VE-cadherin at junctions. The potential mechanisms involved in the effects of chloroquine on cell surface VE-cadherin expression remain to be clarified. Chloroquine has been reported to increase cell surface localization of bone morphogenetic protein receptor type-II independent of transcription.44 In our study, it is likely that the proportion of VE-cadherin in the recycling pool increases because of accumulation of total VE-cadherin by inhibition of its degradation. An increased intracellular pool of VE-cadherin drives recycling to the surface via an unidentified Rab11-independent route. It is worth mentioning that in calcium switch and throbmin experiments, no further degradation of VE-cadherin beyond that induced by Rab11a silencing could be detected during the 2-hour period that elapsed, indicating the validity of our biotinylation approach. Taken together, our data highlight the pivotal role for Rab11a in determining the fate of internalized VE-cadherin through balancing VE-cadherin recycling and degradation.

We provide novel insights into the role of Rab11a-mediated VE-cadherin recycling in the recovery of AJs after inflammation-induced loss of endothelial barrier function. Depletion of Rab11a prevented VE-cadherin from returning to the cell surface to form AJs, thus increasing basal junctional permeability (as evidenced by increased TER) and also markedly delaying recovery of AJ integrity after disassembly of AJs with thrombin. Furthermore, restoration of AJs by overexpression of WT Rab11a was faster in thrombin-stimulated cells. In contrast, overexpression of dominant-negative GDP-bound Rab11a delayed restoration of endothelial barrier function. These findings reinforce our hypothesis that Rab11a has a homeostatic role in VE-cadherin localization, assembly of AJs, and regulation of endothelial permeability. In this regard, Rab11a might serve as a new therapeutic target to repair leaky microvessels for the treatment of inflammatory diseases, such as acute lung injury.

We observed that decreased expression of Rab11a and VE-cadherin in endothelial cells was a feature of acute lung injury in lungs of septic mice, although the exact mechanisms involved remain unclear. Selective depletion of Rab11a in lung endothelia induced massive pulmonary edema, reflecting disruption of pulmonary endothelial AJ barrier of mice challenged with endotoxemia and polymicrobial sepsis. These results suggest that failure to recycle VE-cadherin to AJs because of loss of Rab11a contributes to persistent microvascular leakage during sepsis. In support of our findings, anthrax toxin was shown to block Rab11/Sec15-dependent endocytic recycling resulting in disruption of cadherin-dependent adhesion at AJs of Drosophila and mice.45

In summary, we identified the critical role of Rab11a-mediated VE-cadherin recycling in regulating endothelial AJ barrier integrity. On disassembly of interendothelial contacts, internalized VE-cadherin forms a complex with Rab11a via direct binding to FIP2 in the endosomal recycling compartment. VE-cadherin recycling to the plasmalemma induces the formation of AJs through homotypic VE-cadherin interaction to promote recovery of endothelial barrier integrity. Loss of Rab11a impairs VE-cadherin recycling, thus resulting in persistent disruption of the AJ barrier manifested as endothelial hyperpermeability.

Acknowledgments
We thank Maricela Castellon for technical assistance.

Sources of Funding
This work was supported by National Institutes of Health Grant HL104092 (to G. Hu).

Disclosures
None.

References

Significance

Vascular endothelial (VE)-cadherin is the predominant component of endothelial adherens junctions essential for cell-cell adhesion and formation of the vascular barrier. However, little is known about the molecular mechanism of VE-cadherin recycling and its role in maintenance of vascular integrity. We for the first time demonstrate that VE-cadherin recycling required Ras-related proteins in brain (Rab11a and Rab11 family-interacting protein 2). Direct interaction of VE-cadherin with family-interacting protein 2 (at aa 453–484) formed a ternary complex with Rab11a in human endothelial cells. Rab11a/family-interacting protein 2–mediated VE-cadherin recycling is required for formation of adherens junctions and restoration of vascular endothelial barrier integrity during inflammation, and hence a potential target for clinical intervention in inflammatory disease.
Rab11a Mediates Vascular Endothelial-Cadherin Recycling and Controls Endothelial Barrier Function
Zhibo Yan, Zhen-Guo Wang, Nava Segev, Sanyuan Hu, Richard D. Minshall, Randal O. Dull, Meihong Zhang, Asrar B. Malik and Guochang Hu

Arterioscler Thromb Vasc Biol. 2016;36:339-349; originally published online December 10, 2015;
doi: 10.1161/ATVBAHA.115.306549

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/2/339

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2015/12/10/ATVBAHA.115.306549.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Detailed Materials and Methods

Reagents

Endothelial growth medium EGM-2 MV Bullet Kit, HUVECs Nucleofector kit and Amaxa electroporation system were obtained from Lonza (Walkersville, USA). HEK293 cells were obtained from American Type Cell Culture (ATCC). Dulbecco's modified Eagle's medium (DMEM), Eagle minimal essential medium (EMEM), fetal bovine serum (FBS), anti-Alexa Fluor-568, -488 and -633 antibodies, DAPI, and ProLong Gold antifade, and transfection reagent Lipofectamine 2000 were from Invitrogen. Human α-thrombin was obtained from Enzyme Research Laboratories. ECIS plates for transendothelial resistance measurements were obtained from Applied Biosciences. Transwell plates with 0.4-μm polyester membranes were purchased from Fisher Scientific. Chloroquine, cycloheximide and 2-Mercaptoethanesulfonic acid (MESNA) were purchased from Sigma. Cell Surface Protein Isolation Kit was purchased from Peirce. Anti-Rab11a (sc-166523), anti-Rab11b (sc-26591), anti-Rab6 (sc-310), anti-Rab4 (sc-28569), anti-VE-cadherin (sc-9989, sc-52751, and sc-31017), anti-p120-catenin (sc-13957), anti-LAMP1 (sc-8098), anti-β-catenin (sc-1496), anti-HA (sc-805), anti-GFP (sc-5385), anti-GST (sc-459), and protein A/G agarose beads were purchased from Santa Cruz Biotechnology Inc. Anti-GAPDH (#2118), anti-β-actin (#4970) and mouse anti-rabbit IgG (Conformation specific) HRP secondary antibodies (#5127) were purchased from Cell Signaling Technology. Anti-VE-Cadherin Alexa Fluor® 488 antibody (#53-1449) was obtained from eBioscience. Yeast growth media YPDA, SD-Trp-Leu, SD-Trp-Leu-Ade-His were bought from Clontech. IgG1 heavy chain HRP secondary antibody was obtained from Lifespan biosciences. pDsRed-C1- or GFP-Rab11a dominant negative (Addgene Plasmid 12680), pDsRed-C1- or GFP-Rab11a wild type (Addgene Plasmid 12679) were obtained from Addgene. pGFP-C1-Rab11a Q70L was a gift from Dr. Wei Guo (University of Pennsylvania, Philadelphia, PA). GFP-tagged VE-cadherin was a gift from Dr. Yulia Komarova (University of Illinois, Chicago, IL). Chloroquine was purchased from Sigma and used at 100 μM, and the proteasome inhibitor MG132 was obtained from Calbiochem (San Diego, CA) and used at 24 μg/ml.

Plasmid construction

Plasmid construction used in this study is summarized in Supplemental Table I. Supplemental Table II shows 23 oligonucleotide primers used in the PCR. Rab11a bait vector pGBKTK7/Rab11a was constructed by inserting Ncol and EcoRI digested PCR fragment (using primers 1, 2 and PGEX-4T3-rab11a as template) into Ncol and EcoRI site of pGBKTK7. Rab11a prey vector pGADT7/Rab11a was constructed by cloning EcoRI and BamHI digested PCR fragment (using primers 3, 4 and pGBKTK7/Rab11a as template) in EcoRI and BamHI site of pGADT7. Human VE-cadherin prey vector pGADT7/VE-cad-ter was constructed by inserting BamHI and Xhol digested PCR fragment (using primers 5, 6, template pEGFPN1-VEcadherin-wt) in BamHI and Xhol site of pGADT7AD. This construct contains VE-cadherin coding sequence from codon 609 to codon 784 (last codon). PCR4/FIP2 was bought from Open biosystems. FIP2 bait vector pGBKTK7/FIP2 was constructed by inserting EcoRI and BamHI-digested PCR fragment (using primers 7, 8, PCR4/FIP2 as template) in EcoRI and BamHI site of
pGBK7. FIP2 prey vector pGADT7/FIP2 was obtained by inserting the same fragment in EcoRI and BamHI digested pGADT7. pGBK7/FIP2 (1-129) was constructed by cloning EcoRI and BamHI digested PCR fragment (using primers 7 and 9, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (1-413) was obtained by inserting EcoRI and BamHI cut PCR fragment (using primers 7 and 10, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (1-129) was constructed by cloning EcoRI and BamHI digested PCR fragment (using primers 7 and 9, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (297-512) was constructed by inserting EcoRI and BamHI cut PCR fragment (using primers 11 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (412-512) was constructed by inserting EcoRI and BamHI cut PCR fragment (using primers 12 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. Plasmid pGBK7/FIP2 (453-512) was constructed by inserting EcoRI and BamHI cut PCR fragment (using primers 13 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (467-512) was constructed by inserting EcoRI and BamHI cut PCR fragment (using primers 14 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (475-512) was obtained by inserting EcoRI and BamHI cut PCR fragment (using primers 13 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (485-512) was constructed by inserting EcoRI and BamHI digested PCR fragment (using primers 16 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. pGBK7/FIP2 (488-512) was obtained by cloning EcoRI and BamHI cut PCR fragment (using primers 21 and 8, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. Plasmid pGBK7/FIP2 (453-474) was constructed by cloning EcoRI- and BamHI-digested PCR fragment (using primers 13 and 22, PCR4/FIP2 as template) in EcoRI and BamHI site of pGBK7. Plasmid pGBK7/FIP2 (453-512) I481E bearing isoleucine-481 to glutamate point mutation was constructed by overlap PCR in two steps. First, two different PCR fragments were obtained by using primer pair 18 and 8 and pGBK7/FIP2 (453-512) as template or primer pair 19, 13 and pGBK7/FIP2 (453-512) as template. Second, the two PCR fragments were mixed and served as template for a new PCR reaction using primers 23. This construct contains cDNA for human VE-cadherin residues 623-784. Plasmid pACYCDuet/FIP2 (297-512) was constructed by cloning Ncol-BamHI fragment from pGBK7/FIP2 (297-512) in Ncol and BamHI site of pACYCDuet-1. pACYCDuet/FIP2 (412-512) was obtained by inserting Ncol-BamHI fragment from pGBK7/FIP2 (412-512) in Ncol and BamHI site of pACYCDuet-1. pECFP-C1/FIP2 was constructed by cloning PCR fragment (BamHI cut) (using primers 23 and 8 and pGBK7/FIP2 as template) in modified vector pECFP-C1 (BglII cut). In this plasmid N-terminal Myc-tagged FIP2 was fused to the C-terminal end of fluorescent ECFP protein. pECFP-C1/FIP2 (I481E) was constructed as follows. First, the two different PCR fragments were obtained from using primers 23 and 19 and pGBK7/FIP2 as template for PCR fragment 1 and primers 18 and 8 and pGBK7/FIP2 (453-512) as template for PCR fragment 2. The two PCR fragments were mixed and served as template for a new PCR reaction using primers 23.
and 8. The obtained PCR fragment was digested with BamHI and inserted in the modified vector pECFP-C1 (BglII cut). In this plasmid N-terminal Myc-tagged FIP2 with Isoleucine-481 mutated to glutamate was fused to the C-terminal end of fluorescent ECFP protein.

**Mice**

C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME), housed in a specific pathogen–free barrier facility, and used in experiments at 6–8 weeks of age. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

**Cell culture, RNA interference, and cDNA transfection**

HLMVECs (Lonza) were transfected with Rab11a siRNA or cDNAs using DharmaFECT transfection reagents (Thermo Scientific) or by electroporation (Lonza). The siRNA duplexes were based on human sequences and were purchased from Qiagen (HPP grade siRNA) or from Thermo Fisher Scientific (standard A4 grade). The human Rab11a siRNA target sequence is 5′-AATGTCAGACAGACGCGAAAA-3′. Sequences of the sense strands are as follows: Rab11a-1 (5′-UGUCAGACAGACGCGAAAA-3′), Rab11a-2 (5′-GGCAUUGUAGAGAUCCUGAATT-3′). Negative control siRNA contains at least four mismatches to any human genes, as previously determined by the manufacture using Microarray. The sequence for the Control-siRNA is 5′-UAAGGCUAUGAAGAGAUAC-3′. The target sequences for other siRNAs used in this study are: Rab4 (5′-AATGCAGGAACTGGCAAATCT-3′), Rab6 (5′-GAGAAGAUAUGAUUGACAU-3′), Rab11b (5′-AAGCACCTGACCTATGAGAAC-3′), and Rab11-FIP2 (5′-GGUCCCUGGUGGGUCUGGAUAAUU-3′). Endothelial cells were transfected with FIP2 cDNA using X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN). For adenoviral vector-mediated gene transduction, HEK293T cells were grown to 90% confluent and infected with the Ad-control vector or Ad-target protein vector for 48 h. The plasmids were delivered into HEK293T cells using Lipofectamine 2000 (Invitrogen).

**Western blot analysis and co-immunoprecipitation**

Protein extraction, Western blotting, and co-immunoprecipitation procedures were carried out as described previously. For Western blot analysis, equal amounts of proteins were loaded for PAGE (10–15%) and transferred onto nitrocellulose membranes. For co-immunoprecipitation assay, samples were precleared with 1 mg normal rabbit IgG overnight at 4°C, followed by addition of 30 μl protein A/G PLUS-agarose beads. Immunoprecipitates were dissolved in sample buffer for electrophoresis and immunoblot analysis. To avoid the obscure bands caused by IgG light chain or heavy chain for co-immunoprecipitation, we used conformation specific secondary HRP antibody (Cell Signaling Technology, Danvers, MA).

**Cell surface biotinylation**
Surface biotinylation experiments were performed as previously described.\textsuperscript{4,5} HLMVECs were incubated with 1.5 mg/ml sulfosuccinimidyld(2-(biotinamido)ethyl-dithiopropionate (sulfo-NHS-SS-biotin) (Pierce) for 30 min at 4°C to block membrane trafficking. Free sulfo-NHS-SS-biotin was quenched by sulfo-NHS-SS-biotin blocking reagent (50 mmol/L NH\textsubscript{4}Cl in PBS containing 1 mmol/L MgCl\textsubscript{2} and 0.1 mmol/L CaCl\textsubscript{2}). Cells were incubated three times for 10 min each at 4°C with 100 mmol/L sodium 2-mercaptoethanesulfonate (MESNA) in TBS buffer (50 mmol/L Tris-HCl, pH 8.6, 100 mmol/L NaCl, 2.5 mmol/L CaCl\textsubscript{2}) to remove biotin from the sulfo-NHS-SS-biotin-labeled proteins on the cell surface. Cell extracts were centrifuged to obtain a detergent-insoluble pellet and a detergent-soluble supernatant which was incubated with NeutrAvidin beads to collect bound, biotinylated proteins.

Endocytosis and recycling assays

Endocytosis and recycling of VE-cadherin was induced by Ca\textsuperscript{2+} switch experiments.\textsuperscript{4,6,7} For VE-cadherin endocytosis assay, confluent biotinylated HLMVECs were cultured in low Ca\textsuperscript{2+} (0.06 mmol/L) Eagle’s minimal essential medium (EMEM) at 37°C for 2 h to induce VE-cadherin internalization. The cells were then washed twice at 4°C with MESNA to remove the noninternalized VE-cadherin, lysed in RIPA buffer, precipitated with streptavidin-conjugated agarose beads, and subjected to Western blot with an anti–VE-cadherin antibody. VE-cadherin recycling was measured biochemically by the loss of internalized VE-cadherin labeled with biotin. Following endocytosis, cells were cooled to 4°C to stop membrane trafficking and remaining surface biotin was removed with MESNA. Low Ca\textsuperscript{2+} medium was then removed and replaced with endothelial growth medium containing 1.8 mmol/L Ca\textsuperscript{2+} at 37°C for 0.5 or 1 h to allow biotinylated proteins to recycle back the plasma membrane from endocytic vesicles before cell surface biotinylated VE-cadherin was washed twice at 4°C with MESNA. The cells were then lysed, precipitated with streptavidin-conjugated agarose beads, and subjected to Western blot with an anti-VE-cadherin antibody for determining residual biotinylated (internalized) VE-cadherin. The rate of disappearance of biotinylated VE-cadherin provides a measure of VE-cadherin recycling rate. Recycling rate was calculated as follows: (internalized VE-cadherin–non-recycled VE-cadherin)/ internalized VE-cadherin. To visualize VE-cadherin endocytosis and recycling, cells were labeled by anti–VE-cadherin extracellular domain antibody, fixed, and washed with acid buffer (2 mmol/L PBS-glycine, pH 2.0, 2 min twice) to remove cell surface-bound antibody\textsuperscript{8} and images acquired using a Zeiss LSM510 META confocal microscope. To exclude newly synthesized VE-cadherin from consideration, cells were treated with cycloheximide (10 \(\mu\)mol/L) in all relevant experiments.\textsuperscript{4} In some experiments, HLMVECs were stimulated with 25 nM thrombin for the indicated times.

Flow cytometry

Recycling of VE-cadherin back to the plasma membrane was estimated by assaying the recovery of immunoreactive VE-cadherin accessible at the cell surface to anti-VE-cadherin monoclonal antibody recognizing the extracellular extracellular domain. Membrane expression and recycling of VE-cadherin by flow cytometry analysis was
performed as previously described. HLMVECs were non-enzymatically harvested using Cell Stripper solution (Cellgro, VA). A unicellular suspension of cells was then incubated with Alexa Fluor-conjugated mouse anti-human VE-cadherin or Alexa Fluor-mouse IgG1 (eBioscience, CA) for 30 min on ice, followed by washing 3 times with ice cold FACS Buffer (PBS, 1% BSA, 0.1% NaN\textsubscript{3} sodium azide). Fluorescence of 10\textsuperscript{4} cells was detected using a Becton-Dickinson FACSCalibur flow cytometer (San Jose, CA) and FlowJo software.

**Immunofluorescence microscopy and image analysis**

Cells seeded onto 0.2% gelatin coated glass coverslips were fixed with 2% paraformaldehyde at 37°C and subsequently permeabilized with 0.1% Triton X-100 in HBSS for 3 min. Epifluorescence imaging of fixed samples was conducted on a LSM 510 confocal microscope with 63×, 1.2 NA water immersion objectives (Carl Zeiss, Inc). For quantitative analysis of VE-cadherin at cell junctions, 30 cells for each condition were randomly chosen and images were acquired using the same parameters. For the quantification and analysis of colocalization by Pearson correlation coefficient, 50 cells imaged for each condition were randomly chosen. Based on the cell boundary and on the VE-cadherin labeling, the regions of interest were chosen using Image Pro-Plus software (Media Cybernetics, MD), and the overlapping pixels between the two or three structures were calculated.

**Endothelial barrier function**

TER was measured as an index of endothelial barrier function using an impedance sensor system (Applied Biophysics). Endothelial cells were grown to confluence on fibronectin (5 μg/ml) coated 8-well gold-plated electrodes. Data were plotted versus time and normalized as the ratio of measured resistance to baseline resistance or expressed as specific electrical resistance (Ω·cm\textsuperscript{2}).

**Endothelial monolayer permeability**

Endothelial cells were grown on 0.2% gelatin coated 12 mm-diameter Transwell filters (0.4 μm pore size; Corning). FITC-dextran (1 mg/ml, MW 70 kDa, Sigma) was added into the upper chamber. The medium from lower chamber was collected at different time points and fluorescence measured in a spectrofluorimeter (λ\textsubscript{EX} 485 nm; λ\textsubscript{EM} 525 nm). The permeability coefficient was determined by Pc=Js/SΔC. Where S is the surface area of the Transwell membrane (1.12 cm\textsuperscript{2}), and ΔC is the concentration differential of FITC-dextran across the monolayer (taken as the constant luminal concentration which is much greater than the abluminal concentration). The transendothelial FITC-dextran flux (Js) was determined for each monolayer from the rate of change of the fluorescent-labeled dextran concentration in the abluminal medium, ΔCA/Δt, as follows: Js = (ΔCA/Δt) (VA), where VA is the volume of the abluminal medium.

**Rab11a activity assay**
Activation of Rab11a was determined according to the manufacturer’s instructions (Rab11 Activation Assay Kit, Neweast, PA). In brief, cell lysates (1 mg) were mixed with 1 μl anti-active Rab11a monoclonal antibody. Resuspended protein A/G Agarose bead slurry (20 μl) was added and incubated at 4°C for 1 h, followed by washing the beads for 3 times with assay/lysis buffer (0.5 ml). The supernatant was removed and the bead pellet resuspended in SDS-PAGE sample buffer, boiled and centrifuged. Fifteen μL/well of pull-down supernatant was loaded to a polyacrylamide gel (17%) for Western blot analysis.

Quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted from HLMVEC cells using TRizol reagent (Life Technologies) according to the manufacturer’s instructions. The High Capacity cDNA Reverse Transcription Kits (Invitrogen) was used to synthesize single-stranded cDNA from 2.0 μg total RNA. The cDNA was utilized for the amplification of VE-cadherin and the endogenous controls GAPDH by PCR, which was performed using TaqMan® Universal PCR Master Mix (Life Technologies) on an ABI 7300HT real-time PCR system (Applied Biosystems, Foster City, CA). The PCR primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA), and the sequences used in this study were as follows: VE-cadherin forward, 5'-CCA CAT TCA GGG AAA TGC TT-3' and reverse 5'-GACCAG GTG AGGCAG AGAAG-3', TaqMan® TAMRA™ Probes sequence: 5’-GATGTTCCCCCCAGATCAGAA-3’; GAPDH forward, 5’-CCACCC ATGGCAATTCC-3’ and reverse 5’- TGGGATTTCCATTGATGACCAG-3’, TaqMan® TAMRA™ Probes sequence: 5’-TGGCACCCTCAAGGCTGAGAAGC-3’.

Results were normalized to expression of GAPDH, and relative quantification of gene expression was calculated by using the $2^{\Delta\Delta\text{comparative threshold}}$ equation.16

Yeast 2-hybrid assay

Clontech Matchmaker Gold Yeast Two-Hybrid System was used for co-transformation of prey and bait vectors and yeast transformants were selected by growth at 30°C on synthetic defined (SD) media without tryptophan and leucine. Yeast colonies grown on SD medium lacking tryptophan, leucine, adenine and histidine for 6 days at 30°C indicate protein-protein interactions. Plasmid construction used in this study is summarized in Supplemental Table 1. Twenty-three oligonucleotide primers used in the PCR is shown in Supplemental Table 2.

GST pull-down assay

*Escherichia coli* BL21 (DE3) (Invitrogen) was co-transformed with pGEX-4T1 or pGEX-4T1/VE-cadherin together with pACYCDuet/FIP2 (453-512). Cells grown at 30°C were induced to express exogenous proteins by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), lysed and sonicated in Buffer A (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA, 1mmol/L PMSF, pH7.4). The lysates were incubated with glutathione-agarose beads (Sigma) in a rocker at 4°C for 30 min, washed with PBS six times. The purified beads were incubated with SDS-Laemmli buffer (Bio-Rad) for SDS-PAGE.
Depletion of Rab11a in mouse lung vascular endothelia using liposomes

A well-established approach was used to selectively deplete Rab11a in mouse pulmonary vasculature by intravascular injection of cationic liposome-siRNA complexes. Liposome was made using a mixture of dimethyldioctadecyl-ammonium bromide and cholesterol in chloroform. Successful depletion of Rab11a was confirmed by Western blot of lung homogenates and immunohistochemistry.

Animal models of endotoxemia and polymicrobial sepsis

Endotoxemia was induced in mice by intraperitoneal injection of bacterial endotoxin lipopolysaccharide (LPS 5 mg/kg). CLP was performed to induce polymicrobial sepsis. Briefly, mice were anesthetized with a mixture of Ketamine/xylazine (20 mg/kg, 4 mg/kg, respectively i.p.). After ligation of the distal half of the caecum, the caecum was subjected to three punctures with a 20-gauge needle. In sham control animals, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. Analgesia was provided by a subcutaneous injection of buprenorphine (0.1 mg/kg). For the survival study, mice were monitored every 4 h and scarified when moribund or after 96 h when the observations were terminated.

Immunohistochemistry

Frozen sections of mouse lung tissues (4 μm thickness) were immunostained with anti-VE-cadherin, Rab11a or CD31 antibodies using a standard protocol. Images of lung sections were obtained using a LSM 510META confocal microscope with 63×, 1.2 NA water immersion objective (Carl Zeiss, Inc).

Pulmonary vascular permeability assay in mice

Lung wet/dry weight ratio was performed to assess the pulmonary vascular permeability. At the end of the experiment, lungs were weighed, dried to a constant weight in an oven at 60°C for 48 h and reweighed.

Statistical analysis

Comparisons between experimental groups were made by one-way ANOVA and Student’s Newman-Keuls test for post hoc comparisons. Parameter changes between different groups over time were evaluated by a two-way ANOVA with repeated measures. Differences between survival curves following LPS challenge and CLP were determined by the Log-rank test. Data were expressed as mean ± SD. A value of \( P<0.05 \) was considered statistically significant.
References


Supplemental Figure I. Effects of depletion of Rab11a on VE-cadherin internalization and recycling. A, Densitometric analysis of VE-cadherin in HLMVECs as shown in Figure 1 C. The density of proteins in biotin group was used as a standard (1 arbitrary unit) to compare relative densities in the other groups. \( n = 4 \). *, \( P < 0.05 \) vs. Biotin groups; †, \( P < 0.05 \), vs. corresponding scrambled (Sc) siRNA groups; ‡, \( P < 0.05 \), vs. siRab11a Biotin group. B, Effects of depletion of Rab11a on VE-cadherin internalization rate. The rate of VE-cadherin internalization is quantified and calculated by the density of proteins (Figure 1 C).
**Supplemental Figure II. Effects of depletion of Rab4, Rab6 or Rab11b on VE-cadherin recycling.** HLMVECs were transfected with a scrambled (si Sc), Rab4 (si Rab4), Rab6 (si Rab6) or Rab11b (si Rab11b) siRNA. At 48 h post-transfection, cycloheximide-treated cells were surface-biotinylated and then sequentially treated with low Ca^{2+} (0.06 mM) EMEM for 2 h to induce VE-cadherin internalization. Low Ca^{2+} EMEM was replaced with EGM-2 containing 1.8 mM Ca^{2+} to initiate VE-cadherin recycling and restoration of cell-cell junctions. At each time point during the “chase” phase, biotin groups were removed from endocytosed proteins as they returned to the cell surface. The remaining non-recycled biotinylated proteins were recovered and immunoblotted for presence of VE-cadherin. Data shown are representative of three independent experiments. B = biotinylated; W = washing out (control). A, Effects of depletion of Rab4 on VE-cadherin recycling. B, Effects of depletion of Rab6 on VE-cadherin recycling. C, Effects of depletion of Rab11b on VE-cadherin recycling.
Supplemental Figure III. Effects of depletion of Rab11a, Rab4, Rab6 or Rab11b on total VE-cadherin expression. HLMVECs were transfected with a scrambled, Rab11a-1, Rab11a-2, Rab4, Rab6 or Rab11b siRNA. At 48 h post-transfection, Rab11b, p120 catenin (p120), β-catenin and VE-cadherin (VE-Cad) protein expression was determined by Western blot analysis. A, Representative of Western blot of total Rab11a-1/2, Rab11b, p120 and β-catenin protein expression level. B, Effects of depletion of Rab11a on VE-cadherin mRNA expression. At 48 h post-transfection, mRNA was extracted as described in “Materials and methods.” Real time (RT)-PCR data are means ± SD of 3 separate experiments performed in triplicate and are expressed as relative amounts using control cell lysate as reference value. Top, quantitative RT-PCR analysis of VE-cadherin mRNA. Bottom, Rab11a protein expression. CON = Control. C, Representative of Western blot of total VE-cadherin protein expression level 48 h after Rab4, Rab6 or Rab11b siRNA transfection. Data are representative of three independent experiments.
Supplemental Figure IV Rab11a/FIP2/VE-cadherin form a complex. A, Endogenous VE-cadherin (VE-Cad) associates with Rab11a and FIP2 in HLMVECs. Rab11a, FIP2 or VE-cadherin was immunoprecipitated (IP) from cell lysates and co-immunoprecipitated proteins detected by immunoblotting, as indicated. TCL = total cell lysates. B, Exogenous VE-cadherin associates with Rab11a and FIP2 in HLMVECs. Rab11a was immunoprecipitated from the lysates of cells expressing GFP-VE-cadherin, and co-immunoprecipitation detected by immunoblotting (IB). C, Colocalization of VE-cadherin, Rab11a and FIP2 in HLMVECs. Left panel, confocal images showing the colocalization of VE-cadherin, Rab11a, and FIP2. Bars, 10 µm. Right panel, quantification of VE-cadherin/Rab11a/FIP2 colocalization. *P<0.001. D, Colocalization of VE-cadherin, exogenous DsRed-Rab11a and FIP2 in HLMVECs. Left panel, confocal images of cells showing the colocalization of VE-cadherin, DsRed-Rab11a, and FIP2. Bars, 10 µm. Right panel, quantification of VE-cadherin/DsRed-Rab11a/FIP2 colocalization. *P<0.001.
Supplemental Figure V. Effects of depletion of Rab11a on VE-cadherin recycling following thrombin stimulation. 

**A,** Effects of thrombin on VE-cadherin (blue)/FIP2 (green)/exogenous Rab11a (red) complex formation. Monolayers of HLMVECs expressing DsRed-Rab11a-WT were treated with thrombin for 30 min. *Left panel,* representative confocal images showing colocalization of VE-cadherin, exogenous Rab11a and FIP2. Bars, 10 µm. *Right panel,* quantification of VE-cadherin/exogenous Rab11a/FIP2 colocalization. n = 3. *P* < 0.001.

**B,** VE-cadherin colocalized with LAMP1 following thrombin stimulation. HLMVECS transfected with si Sc or si Rab11a were treated with vehicle or human α thrombin for 30 min and then fixed, permeabilized, stained for VE-cadherin (red) and LAMP1 (green) expression. The nucleus (blue) was stained with DAPI. Bars, 10 µm. *Left panel,* representative confocal images showing colocalization of VE-cadherin and LAMP1. *Right panel,* quantification of VE-cadherin and LAMP1 colocalization. *, P < 0.05; **, P < 0.01.
Supplemental Figure VI. FIP2 is required to restore lung vascular integrity in mice. FIP2 expression in pulmonary vasculature was down-regulated with a specific FIP2 siRNA (si). Mice were injected intravenously with liposomes containing Scrambled (Sc) or FIP2 siRNA. After 48 hour, mice were challenged with vehicle or LPS (5 mg/kg, i.p.) for 24 h. A, Western blots show endothelial FIP2 expression in the lung. Lung microvascular endothelial cells were isolated from mice transfected with Sc or FIP2 siRNA. n = 3. B, Lung edema formation measured by wet/dry lung weight ratio following LPS challenge. n = 6 in each group. *P<0.05 vs. Sc siRNA alone groups, †P<0.05 vs. Sc siRNA+LPS.
<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Encoded product</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBKT7/Rab11a</td>
<td>Gal4 BD-Rab11a fusion protein</td>
</tr>
<tr>
<td>pGADT7/Rab11a</td>
<td>Gal4 AD-Rab11a fusion protein</td>
</tr>
<tr>
<td>pGADT7/VE-cad-cte</td>
<td>Gal4 AD-VE-cadherin fragment (Leu$^{609}$-Tyr$^{784}$) fusion protein</td>
</tr>
<tr>
<td>PCR4/Rab11-FIP2</td>
<td>FIP2</td>
</tr>
<tr>
<td>pGBKT7/Rab11-FIP2</td>
<td>Gal4 BD-FIP2 fusion protein</td>
</tr>
<tr>
<td>pGADT7/Rab11-FIP2</td>
<td>Gal4 AD-FIP2 fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(1-129)</td>
<td>Gal4 BD-FIP2 fragment (Met$^1$-Arg$^{129}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(1-413)</td>
<td>Gal4 BD-FIP2 fragment (Met$^1$-Arg$^{413}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(297-512)</td>
<td>Gal4 BD-FIP2 fragment (Phe$^{297}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(412-512)</td>
<td>Gal4 BD-FIP2 fragment (Phe$^{412}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(453-512)</td>
<td>Gal4 BD-FIP2 fragment (Tyr$^{453}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(467-512)</td>
<td>Gal4 BD-FIP2 fragment (Leu$^{467}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(475-512)</td>
<td>Gal4 BD-FIP2 fragment (Arg$^{475}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(485-512)</td>
<td>Gal4 BD-FIP2 fragment (Leu$^{485}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(488-512)</td>
<td>Gal4 BD-FIP2 fragment (Val$^{488}$-Ser$^{512}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(453-474)</td>
<td>Gal4 BD-FIP2 fragment (Tyr$^{453}$-Ile$^{474}$)fusion protein</td>
</tr>
<tr>
<td>pGBKT7/FIP2(453-512)I481E</td>
<td>Gal4 BD-FIP2 fragment (Tyr$^{453}$-Ser$^{512}$)Ile$^{481}$Glu fusion protein</td>
</tr>
<tr>
<td>pGEX-4T1/VE-cad-cte</td>
<td>GST-VE-cadherin fragment (Arg$^{623}$-Tyr$^{784}$) fusion protein</td>
</tr>
<tr>
<td>pEGFPN1/hVE-cadherin-wt</td>
<td>EGFP-VE-cadherin fusion protein</td>
</tr>
<tr>
<td>pACYCDuet/FIP2(297-512)</td>
<td>FIP2 fragment (Phe$^{297}$-Ser$^{512}$)</td>
</tr>
<tr>
<td>pACYCDuet/FIP2(412-512)</td>
<td>FIP2 fragment (Phe$^{412}$-Ser$^{512}$)</td>
</tr>
<tr>
<td>pACYCDuet/FIP2(453-512)</td>
<td>FIP2 fragment (Tyr$^{453}$-Ser$^{512}$)</td>
</tr>
<tr>
<td>pECFPC1/FIP2</td>
<td>ECFP-FIP2 fusion protein</td>
</tr>
<tr>
<td>pECFPC1/FIP2(I481E)</td>
<td>ECFP-FIP2(Ile$^{481}$Glu)fusion protein</td>
</tr>
</tbody>
</table>
### Supplemental Table II. Oligonucleotide primers used in the PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Gene location*</th>
<th>Restriction sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>aaccATGGGCACCCGACGACGACGA</td>
<td>1 to 20</td>
<td>NcoI</td>
</tr>
<tr>
<td>2</td>
<td>aagaattcAGATGTTCTGACACAGCCTCCTT</td>
<td>625 to 649</td>
<td>EcoRI</td>
</tr>
<tr>
<td>3</td>
<td>aagaattcATGGGCACCCGACGACGACGA</td>
<td>1 to 20</td>
<td>EcoRI</td>
</tr>
<tr>
<td>4</td>
<td>aaggatccTTAGATGTTCTGACAGCCTCCTT</td>
<td>625 to 651</td>
<td>BamHI</td>
</tr>
<tr>
<td>5</td>
<td>aaggATCTCTACCCACGCTACGACACCC</td>
<td>1822 to 1845</td>
<td>BamHI</td>
</tr>
<tr>
<td>6</td>
<td>aactcGCGCCTAAATACAGCAGCCTCCTC</td>
<td>2337 to 2358</td>
<td>XhoI</td>
</tr>
<tr>
<td>7</td>
<td>aagaattcATGATGCTGTCACAGCAGCAGGCA</td>
<td>1 to 23</td>
<td>EcoRI</td>
</tr>
<tr>
<td>8</td>
<td>aaggatccTTAACTGTTAGAATTTGCCAGCTTCTC</td>
<td>1509 to 1539</td>
<td>BamHI</td>
</tr>
<tr>
<td>9</td>
<td>tggatcCGTTTTTCTTGTTTGATCTAATCT</td>
<td>361 to 386</td>
<td>BamHI</td>
</tr>
<tr>
<td>10</td>
<td>aaggatcCTGAAATTGGCTGTAATGGATGCTC</td>
<td>1211 to 1239</td>
<td>BamHI</td>
</tr>
<tr>
<td>11</td>
<td>aagAAATTCAGGGCTTCAAATATAATGCCA</td>
<td>888 to 914</td>
<td>EcoRI</td>
</tr>
<tr>
<td>12</td>
<td>aagAAATTGCAGGCTTCAAATATAATGCCA</td>
<td>1232 to 1258</td>
<td>EcoRI</td>
</tr>
<tr>
<td>13</td>
<td>aagaattcTATGAAGGGTCTACTACAGGAGCT</td>
<td>1356 to 1380</td>
<td>EcoRI</td>
</tr>
<tr>
<td>14</td>
<td>CAAAGAAATCCTGTTAGGAGGAAGAC</td>
<td>1387 to 1413</td>
<td>EcoRI</td>
</tr>
<tr>
<td>15</td>
<td>aagaattcTCCGGAAAATCACGAGGTACGATACAT</td>
<td>1421 to 1442</td>
<td>EcoRI</td>
</tr>
<tr>
<td>16</td>
<td>aagaattcTCTTGTAAATGGTAATGGAAGAC</td>
<td>1451 to 1476</td>
<td>EcoRI</td>
</tr>
<tr>
<td>17</td>
<td>aaggatcCGAGGACTCGAGAATATCTGGGCGT</td>
<td>1474 to 1499</td>
<td>BamHI</td>
</tr>
<tr>
<td>18</td>
<td>aaggatcCTACAGGGCTAATACGAGGAGGAGCT</td>
<td>1431 to 1460</td>
<td>EcoRI</td>
</tr>
<tr>
<td>19</td>
<td>GAGGTTGTCGCGTACTCGAGTGTC</td>
<td>1426 to 1452</td>
<td>EcoRI</td>
</tr>
<tr>
<td>20</td>
<td>aaggatcCCGGCTGGAAGAGAGGAGC</td>
<td>1867 to 1884</td>
<td>BamHI</td>
</tr>
<tr>
<td>21</td>
<td>aagaattcGTAATGGAAGAGAAGCCCTAGTATTCTC</td>
<td>1462 to 1488</td>
<td>EcoRI</td>
</tr>
<tr>
<td>22</td>
<td>aagaattcGTAATGGAAGAGAAGCCCTAGTATTCTC</td>
<td>1399 to 1422</td>
<td>BamHI</td>
</tr>
<tr>
<td>23</td>
<td>aaggatcGCTCCCGAACGAGGAGGCTCGGCGTACGACTCAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* human Rab11a, Rab11-FIP2 or VE-cadherin gene

- The bases in lowercase letters differ from the wild type gene sequence.
- Assume “A” in initiator (ATG) was the first nucleotide in sequence numbering.