

# Regulation of endothelial barrier function by p120-catenin-VE-cadherin interaction

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**ABSTRACT** Endothelial p120-catenin (p120) maintains the level of vascular endothelial cadherin (VE-Cad) by inhibiting VE-Cad endocytosis. Loss of p120 results in a decrease in VE-Cad levels, leading to the formation of monolayers with decreased barrier function (as assessed by transendothelial electrical resistance [TEER]), whereas overexpression of p120 increases VE-Cad levels and promotes a more restrictive monolayer. To test whether reduced endocytosis mediated by p120 is required for VE-Cad formation of a restrictive barrier, we restored VE-Cad levels using an endocytic-defective VE-Cad mutant. This endocytic-defective mutant was unable to rescue the loss of TEER associated with p120 or VE-Cad depletion. In contrast, the endocytic-defective mutant was able to prevent sprout formation in a fibrin bead assay, suggesting that p120-VE-Cad interaction regulates barrier function and angiogenic sprouting through different mechanisms. Further investigation found that depletion of p120 increases Src activity and that loss of p120 binding results in increased VE-Cad phosphorylation. In addition, expression of a Y658F-VE-Cad mutant or an endocytic-defective Y658F-VE-Cad double mutant were both able to rescue TEER independently of p120 binding. Our results show that in addition to regulating endocytosis, p120 also allows the phosphorylated form of VE-Cad to participate in the formation of a restrictive monolayer.

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## INTRODUCTION

The endothelium is a monolayer of cells lining the inside of all blood and lymph vasculature. One important function of the endothelium is to regulate the movement of fluid, macromolecules, and white blood cells between the vasculature and the interstitial tissue. This is mediated, in part, by the ability of endothelial cells to form strong cell-cell contacts by using a number of transmembrane junctional proteins. Vascular endothelial-cadherin (VE-Cad) spans the plasma membrane, binding homotypically through its extracellular domain to VE-Cad molecules on adjacent endothelial cells (Vincent *et al.*, 2004). Through its cytoplasmic domain, VE-Cad binds to p120-catenin (p120),

$\beta$ -catenin, plakoglobin, and indirectly to  $\alpha$ -catenin, forming a complex known as the adherens junction (Tanihara *et al.*, 1994; Peifer *et al.*, 1994).  $\beta$ -Catenin and plakoglobin bind in a mutually exclusive manner to the C-terminal cytoplasmic catenin-binding domain (CBD) of VE-Cad.  $\alpha$ -Catenin binds to  $\beta$ -catenin/plakoglobin and to actin filaments, thereby linking the adherens junction complex to the actin cytoskeleton. The cytoplasmic tail of VE-Cad also contains a membrane-proximal region called the juxtamembrane domain (JMD), which binds to p120 (Yap *et al.*, 1998; Ferber *et al.*, 2002). The binding of p120 to the VE-Cad JMD is known to maintain VE-Cad levels in endothelial cells by preventing the endocytosis and subsequent degradation of VE-Cad (Xiao *et al.*, 2003, 2005). A loss of VE-Cad attachment to the actin cytoskeleton through a decrease in VE-Cad binding to  $\beta$ - and  $\alpha$ -catenin and/or a decrease in VE-Cad levels due to loss of p120 binding have been implicated in regulating endothelial barrier function (Iyer *et al.*, 2004; Guo *et al.*, 2008; Herron *et al.*, 2011).

A role for p120 in the regulation of endothelial barrier function has been well established. Indeed, p120 is necessary for the formation of a restrictive monolayer, as depleting p120 from human dermal microvascular endothelial cells (HDMECs) before seeding leads to monolayers with decreased barrier function as well as decreased

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Abbreviations used: p120, p120-catenin; TEER, transendothelial electrical resistance; VE-Cad, vascular endothelial cadherin.

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expression of adherens junction proteins VE-Cad,  $\beta$ -catenin,  $\alpha$ -catenin, and plakoglobin (Herron *et al.*, 2011). Restoring p120 levels rescued barrier function and adherens junction protein expression through a RhoA-independent mechanism (Herron *et al.*, 2011). In addition, forced overexpression of p120 in confluent human umbilical vein endothelial cell (HUVEC) monolayers results in an increase in VE-Cad levels that is associated with enhanced barrier function and attenuation of transendothelial migration (TEM) of leukocytes across monolayers (Xiao *et al.*, 2003; Alcaide *et al.*, 2008). In contrast, forced expression of VE-Cad in HUVEC will not attenuate TEM (Alcaide *et al.*, 2012). In addition, just restoring VE-Cad levels in HDMEC depleted of p120 does not rescue the loss barrier function (Herron *et al.*, 2011; Alcaide *et al.*, 2012). Taken together, these studies suggest that p120 plays a second role in mediating endothelial barrier function in addition to regulating VE-Cad level through the regulation of endocytosis. The underlying mechanism for VE-Cad endocytosis was demonstrated in a series of studies from the Kowalczyk laboratory. These studies demonstrated that a loss of p120 binding to the JMD of VE-Cad reveals a site that binds AP-2 and clathrin, resulting in VE-Cad endocytosis and degradation (Xiao *et al.*, 2005; Ishiyama *et al.*, 2010; Nanes *et al.*, 2012). The JMD region contains a series of nine amino acid residues (646-DEEGGGEMD-654) that are required for p120 binding. Using a recombinant form of VE-Cad in which the first three amino acids of this sequence were changed to AAA (DEE-VE-Cad), Nanes *et al.* (2012) showed that these amino acids not only are required for p120 binding, but also act as an endocytic signal that is required for subsequent VE-Cad endocytosis. Thus DEE-VE-Cad is not endocytosed despite the loss of p120 binding.

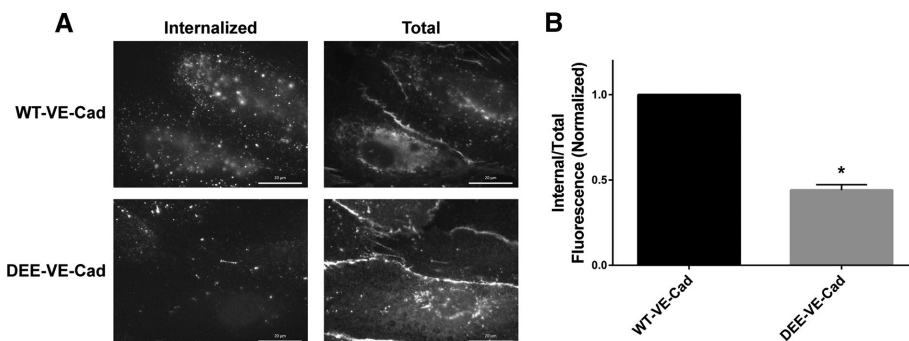
Disruption of p120 binding to the JMD region of VE-Cad by phosphorylation at Y658 has been implicated as a mechanism of mediator-induced increases in endothelial permeability by regulating VE-Cad levels. In this model, Src-induced phosphorylation of VE-Cad at Y658 is believed to cause a decrease in p120 binding, leading to an increase in VE-Cad endocytosis (Gong *et al.*, 2014). The cytoplasmic domain of VE-Cad contains nine potential phosphotyrosine sites, three of which (Y658, Y685, and Y731) participate in the regulation of barrier function (Adam, 2015). Several studies demonstrated an association of VE-Cad tyrosine phosphorylation with a loss of barrier

function. For instance, vascular endothelial growth factor (VEGF) treatment results in phosphorylation of VE-Cad by Src and FAK, a decrease in p120 and  $\beta$ -catenin binding to VE-Cad, and a decrease in VE-Cad levels, all of which are associated with a loss of barrier function (Weis *et al.*, 2004; Potter *et al.*, 2005; Chen *et al.*, 2012; Orsenigo *et al.*, 2012). Mechanistic studies using the expression of nonphosphorylatable mutants of VE-Cad further provided support for VE-Cad tyrosine phosphorylation in regulating endothelial monolayer integrity. Indeed, *in vitro* expression of a nonphosphorylatable Y658F-VE-Cad mutant attenuates the loss of barrier function resulting from inflammatory mediators such as VEGF and bradykinin (Monaghan-Benson and Burrige, 2009; Orsenigo *et al.*, 2012). In addition, *in vivo* studies that substitute Y685F-VE-Cad for endogenous VE-Cad show this site is also important for regulating VEGF and histamine-induced increase in endothelial permeability (Wessel *et al.*, 2014). Use of nonphosphorylatable VE-Cad mutants also demonstrated the importance of VE-Cad tyrosine phosphorylation in the regulation of TEM of leukocytes (Allingham *et al.*, 2007; Alcaide *et al.*, 2008; Wessel *et al.*, 2014). Although it is widely accepted that tyrosine phosphorylation of VE-Cad is critical to controlling the barrier function of the endothelium, the mechanism by which tyrosine phosphorylation contributes to endothelial barrier function has been elusive. In fact, p120 can still be bound to VE-Cad that is tyrosine phosphorylated, and both molecules colocalize at cell-cell junctions (Adam *et al.*, 2010; Orsenigo *et al.*, 2012). In the studies presented here, we used an endocytic-defective mutant of VE-Cad (DEE-VE-Cad) to determine the role of VE-Cad endocytosis in the regulation of barrier function. We provide evidence that maintaining VE-cad levels by preventing VE-Cad endocytosis by p120 is not sufficient for establishing a restrictive barrier. Instead, we find that in addition to regulating endocytosis, p120 also allows the phosphorylated form of VE-Cad to participate in the formation of a restrictive monolayer.

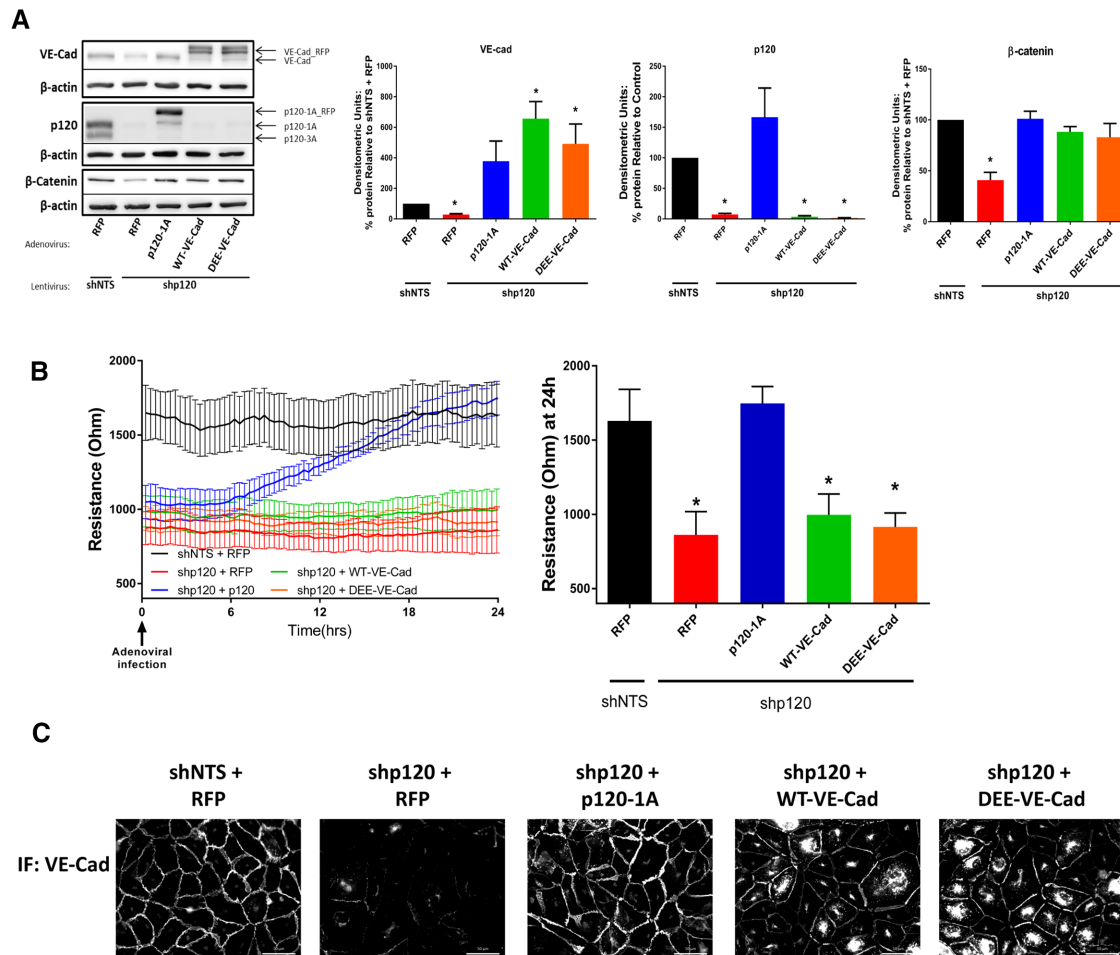
## RESULTS

### An endocytic-defective VE-Cad mutant cannot restore transendothelial electrical resistance after p120 knockdown

A number of studies have suggested a model in which the endocytosis of VE-Cad after a loss of p120 binding is important in regulating endothelial integrity (Vandenbroucke St Amant *et al.*, 2012; Gong *et al.*, 2014), leading us to hypothesize that preventing VE-Cad endocytosis is required to develop a restrictive barrier. To test this hypothesis, we sought to use the p120 binding- and endocytosis-defective VE-Cad mutant in which the amino acids 646DEE648 were replaced with alanines (DEE-VE-Cad; Nanes *et al.*, 2012). We first confirmed that DEE-VE-Cad was not subject to normal endocytosis in our HUVEC model system. In this experiment, we expressed wild-type (WT) VE-Cad or DEE-VE-Cad in HUVECs that had been depleted of endogenous VE-Cad and performed an immunofluorescence-based internalization assay (Xiao *et al.*, 2003). Consistent with the report of Nanes *et al.* (2012), we found that DEE-VE-Cad was endocytosed significantly less than WT VE-Cad, as shown by a decrease in the number of cytoplasmic vesicles containing DEE-VE-Cad (Figure 1). Thus the mechanism regulating VE-Cad internalization is similar in HUVECs and HDMECs.



**FIGURE 1:** Triple-alanine substitution of 646DEE648 in VE-Cad decreases VE-Cad endocytosis in HUVECs. HUVECs were transfected with siVE-Cad and seeded on glass-bottomed plates (Ibidi). After 48 h, monolayers were infected with adenovirus containing WT VE-Cad<sub>RFP</sub> or DEE-VE-Cad<sub>RFP</sub>, and 24 h was allowed for protein expression. Cells were incubated with an antibody against the extracellular domain of VE-cadherin, and an internalization period was allowed. After the internalization period, surface-bound antibody was removed with a low-pH wash. Cells were then fixed, and immunofluorescence was performed using a second VE-Cad antibody (cytoplasmic) to stain for total VE-Cad. (A) Representative images of internalized VE-cad or total VE-cad in cells expressing either WT VE-cad or DEE-VE-cad. Scale bar, 20  $\mu$ m. (B) The ratio of internalized VE-Cad/total VE-Cad was calculated and plotted on a bar graph as means  $\pm$  SEM. \* $p < 0.05$ . Three experiments.



**FIGURE 2:** Restoration of VE-Cad levels using the endocytic-defective VE-Cad mutant (DEE-VE-Cad) does not restore TEER in p120-depleted HUVECs. HUVECs expressing shNTS or shp120 were seeded at confluence and allowed to mature for 48 h. Monolayers were infected 48 h postseeding with adenovirus to induce expression of RFP, p120-1A\_RFP, WT VE-Cad\_RFP, or DEE-VE-Cad\_RFP. Cells were either monitored for 24 h using ECIS or processed at 24 h for immunoblot analysis or IF microscopy. (A) Cells were lysed into sample buffer 24 h after adenoviral infection, and immunoblot analysis was performed for VE-Cad, p120, and β-catenin as described in *Materials and Methods*. Densitometric analysis was performed to quantify bands, and data are presented as mean ± SEM; \*  $p < 0.05$  compared with shNTS + RFP (four experiments). (B) TEER was assessed and plotted at 15-min intervals as mean ± SEM for the 24-h time period after adenoviral infection (left). The average TEER at 24 h after adenoviral infection was plotted as mean ± SEM (right), and statistical analysis was performed. \* $p < 0.05$  compared with shNTS + RFP (four experiments). (C) Cells were fixed 24 h after adenoviral infection and immunostained for VE-Cad. Scale bar, 50 μm. Representative result of four independent experiments.

We previously reported that p120 is necessary for maintaining VE-Cad levels and for the formation of a restrictive HDMEC monolayer as assessed by transendothelial electrical resistance (TEER; Herron *et al.*, 2011). Consistent with results using HDMECs, knockdown of p120 using lentiviral-delivered short hairpin RNA (shRNA) directed against p120 resulted in the formation of confluent monolayers that have lower TEER (Figure 2B and Supplemental Figure S1C) in conjunction with a decrease in VE-Cad levels (Figure 2A and Supplemental Figure S1A). Because p120 is involved in localizing cadherins to the cell junctions, we determined whether loss of p120 binding to VE-Cad changed the localization of VE-Cad at the cell-cell junctions. To do so, we quantified the amount of junctional VE-Cad immunofluorescence in all experiments in which p120 was depleted into a single table (Table 1A). Thus, as shown in Figure 2C and Table 1A, a loss of p120 decreases the junctional localization of VE-cad by ~70%. Restoring p120 to control levels using an adenovirus to express p120-1A reestablished VE-Cad levels (Figure 2A), localization of VE-Cad at cell-cell junctions (Figure 2C and Table 1A),

and TEER (Figure 2B). In contrast to p120 reexpression, restoring VE-Cad levels using adenoviral delivery of WT VE-Cad in p120-depleted HUVEC monolayers was unable to rescue TEER to control levels (Figure 2B), despite the fact that reexpressed VE-Cad levels were higher than that of control and restoration of β-catenin to control levels (Figure 2A). Although VE-Cad levels were restored and localization at the cell-cell junction increased, they did not reach control levels (control =  $1.00 \pm 0.05$  vs. WT VE-Cad =  $0.77 \pm 0.07$ ).

To determine whether endocytosis of VE-Cad contributed to the inability of VE-Cad to restore barrier function in the absence of p120, we restored VE-cad levels by expressing the endocytic-defective mutant DEE-VE-Cad. Expression of DEE-VE-Cad was similarly unable to rescue TEER to control levels (Figure 2B). Similar to WT VE-Cad, DEE-VE-cad showed only a partial restoration of its localization at the cell-cell junction (Figure 2C and Table 1A), despite the presence of similar levels of VE-Cad as for the p120 rescue (Figure 2A). These results suggest that p120 participates in regulating barrier function by an additional mechanism other than the prevention of VE-Cad endocytosis.

A							
Knockdown Reexpress	shNTS		shp120				
	RFP	RFP	p120-1A	WT VE-Cad	DEE-VE-Cad	Y658F-VE-Cad	DEE-Y658F-VE-Cad
Junctional VE-Cad (AU)	1.00 ± 0.05	0.31 ± 0.06	1.35 ± 0.21	0.77 ± 0.07	0.62 ± 0.09	1.10 ± 0.19	1.13 ± 0.26
	n = 8	n = 8	n = 3	n = 8	n = 7	n = 4	n = 4
		<0.05	NS	<0.05	<0.05	NS	NS
B							
Knockdown Reexpress	siNTS		siVE-cad				
	RFP	RFP	WT VE-Cad	DEE-VE-Cad	Y658F-VE-Cad	DEE-Y658F-VE-Cad	p120-1A
Junctional VE-Cad (AU)	1.00 ± 0.06	0.2 ± 0.06	0.92 ± 0.13	0.53 ± 0.11	0.87 ± 0.13	1.15 ± 0.22	0.13 ± 0.09
	n = 6	n = 6	n = 6	n = 6	n = 3	n = 3	n = 3
		<0.05	NS	<0.05	NS	NS	<0.05
Junctional p120 (AU)	1.00 ± 0.07	0.25 ± 0.05	0.87 ± 0.08	0.28 ± 0.05	0.87 ± 0.09	0.29 ± 0.05	0.53 ± 0.09
	n = 7	n = 7	n = 7	n = 7	n = 3	n = 3	n = 4
		<0.05	NS	<0.05	NS	<0.05	NS

AU, arbitrary units; n, number of experiments; NS, not significant.

**TABLE 1: Immunofluorescence quantitations.**

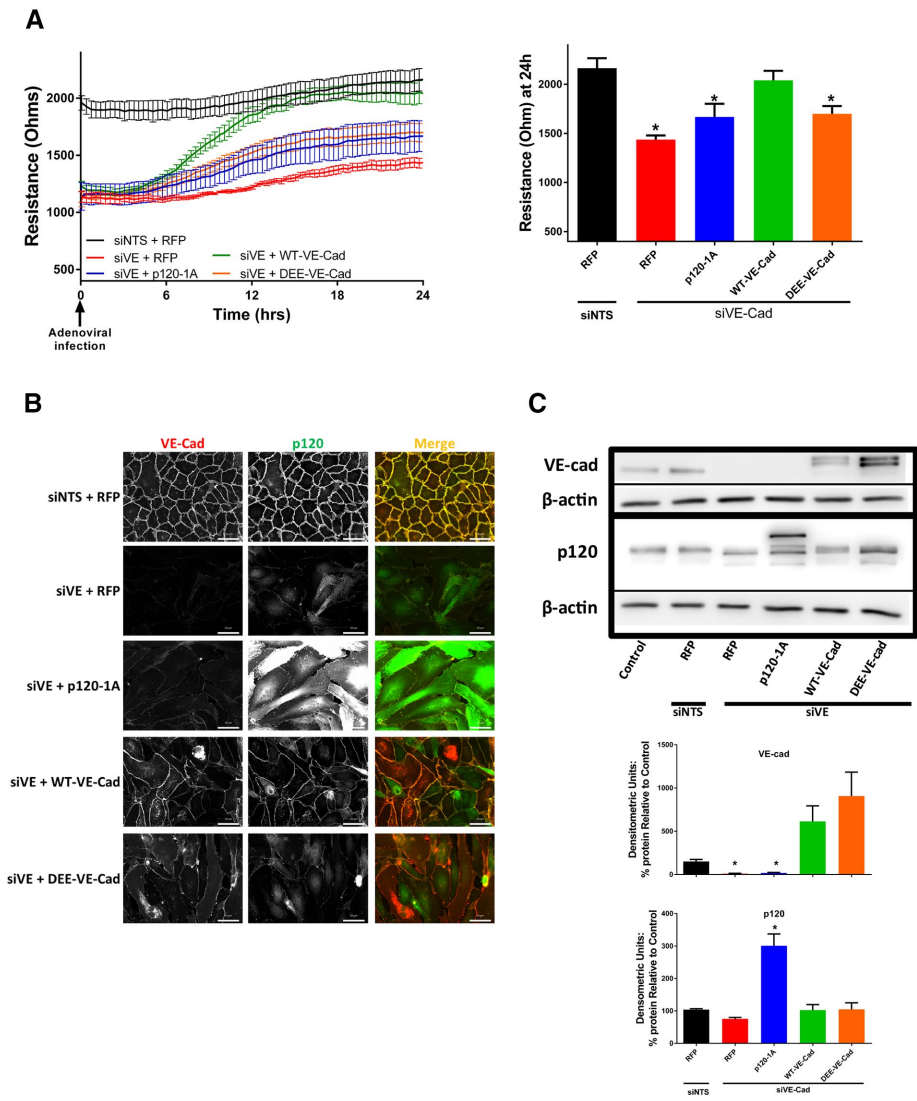
### The endocytic-defective VE-Cad mutant that cannot bind p120 does not restore TEER in VE-Cad-depleted HUVECs

We next sought to determine whether the interaction of p120 with VE-Cad was required for restoration of TEER even when both proteins were maintained at normal levels. To test this, we first depleted VE-Cad from HUVECs by transfecting siRNA targeted against a portion of the 3' untranslated region of VE-Cad mRNA (siVE). This resulted in an ~40–50% decrease in TEER, which was associated with 90% loss of endogenous VE-Cad (Figure 3, A and C). Overall p120 levels did not significantly drop below that of control (Figure 3C). We quantified the amount of junctional VE-Cad immunofluorescence in all experiments in which VE-Cad was depleted and compiled the results into a single table (Table 1B). Upon VE-Cad depletion, p120 localization shifted away from the cell–cell junctions and into the cytoplasm (Figure 3B and Table 1B). As expected, the decrease in TEER observed in VE-Cad-depleted cells could be restored to control levels by reexpressing WT VE-Cad (Figure 3A). Consistent with the restoration of TEER after reexpression of WT VE-Cad, both VE-Cad and p120 were observed at the cell–cell borders, similar to control levels (Figure 3B and Table 1B). In contrast, reexpression of DEE-VE-Cad did not restore TEER (Figure 3A). In addition, even though DEE-VE-Cad was restored at the cell–cell junctions (Figure 3B), the level localized at the junction with DEE-VE-Cad (0.53 ± 0.11) was lower than that of WT VE-Cad (0.92 ± 0.13). As anticipated, p120 did not localize to the cell–cell junction, as it cannot bind to DEE-VE-Cad (Figure 3B and Table 1B). Furthermore, increasing p120 levels to above that of control does not restore TEER after VE-Cad knockdown (Figure 3A). The results of these experiments support the hypothesis that the p120•VE-Cad interaction, and not just the levels of p120 and VE-cad, is essential for the formation of a restrictive barrier. Taking these together with the results in Figure 2, one can further conclude that p120 is not just maintaining VE-Cad levels as a means to regulate the formation of a restrictive barrier, but it is also performing another important function when bound to VE-Cad at the cell–cell junction.

### Preventing VE-Cad endocytosis is not sufficient to increase basal barrier function or block thrombin- or interleukin-1β-induced loss of TEER

Overexpression of p120 increases VE-Cad levels and TEER and causes a decrease in TEM, whereas overexpression of VE-Cad is not sufficient to decrease TEM or increase TEER (Xiao *et al.*, 2003; Alcaide *et al.*, 2008, 2012). Consistent with its inability to rescue barrier function, we found that overexpression of either WT VE-cad or DEE-VE-Cad was unable to increase TEER (Figure 4A). Of note, VE-Cad overexpression did not increase the levels of endogenous p120 (Figure 4B), demonstrating that VE-Cad is not a limiting factor in regulating p120 levels. We then hypothesized that although the expression of DEE-VE-Cad might not be sufficient to form a more restrictive, less permeable barrier, it might still be able to prevent the disassembly of intact cell–cell junctions. To test this hypothesis, we used two inflammatory mediators known to decrease barrier function of a confluent endothelial monolayer: thrombin and interleukin 1β (IL-1β). Thrombin-induced loss of TEER is believed to require disassociation of p120 from VE-Cad and subsequent VE-Cad endocytosis (Vandenbroucke St Amant *et al.*, 2012). Consistent with previous findings, addition of thrombin resulted in a decrease in TEER by ~50%, which returned to control within 90 min after treatment (Supplemental Figure S2A). Overexpression of p120-1A, WT VE-Cad, or DEE-VE-Cad did not protect against the loss of TEER. IL-1β causes a more gradual and sustained decrease in TEER, and enhanced endocytosis of VE-Cad has also been implicated in the IL-1β-induced decrease in barrier function (Zhu *et al.*, 2012). In control cells, IL-1β resulted in loss of TEER, which began ~4 h after treatment and dropped to ~75% of control within 12 h (Supplemental Figure S2B). Increasing cellular VE-Cad levels by overexpressing p120-1A, WT-VE-Cad, or DEE-VE-Cad did not attenuate loss of TEER. Taken together, the experiments show that inhibition of VE-Cad endocytosis is not sufficient to increase basal TEER or prevent the decrease in TEER produced in response to thrombin or IL-1β.





**FIGURE 3:** The endocytic-defective VE-Cad mutant does not restore TEER in VE-Cad-depleted HUVECs. HUVECs were transfected with either siNTS or siVE-Cad and seeded at confluence. Monolayers were infected with adenovirus 48 h after seeding to induce expression of RFP, p120-1A-RFP, WT VE-Cad-RFP, or DEE-VE-Cad-RFP. (A) TEER was determined and plotted as mean  $\pm$  SEM at 15-min intervals for the 24-h period after adenoviral infection (left). The average TEER at 24 h after adenovirus infection was plotted as mean  $\pm$  SEM (right), and statistical analysis was performed. \* $p < 0.05$  compared with shNTS + RFP. (B) Cells were fixed 24 h after adenoviral infection and IF performed for VE-Cad and p120. Scale bar, 50  $\mu$ m. (C) Representative Western blots (top) of VE-Cad, p120, and  $\beta$ -actin (loading control), where cells were lysed 24 h after adenoviral infection. Densitometric analysis was performed to quantify bands, and data are presented as mean  $\pm$  SEM (middle and bottom); \* $p < 0.05$  compared with shNTS + RFP (four experiments).

### Role of p120-VE-Cad interaction in angiogenesis

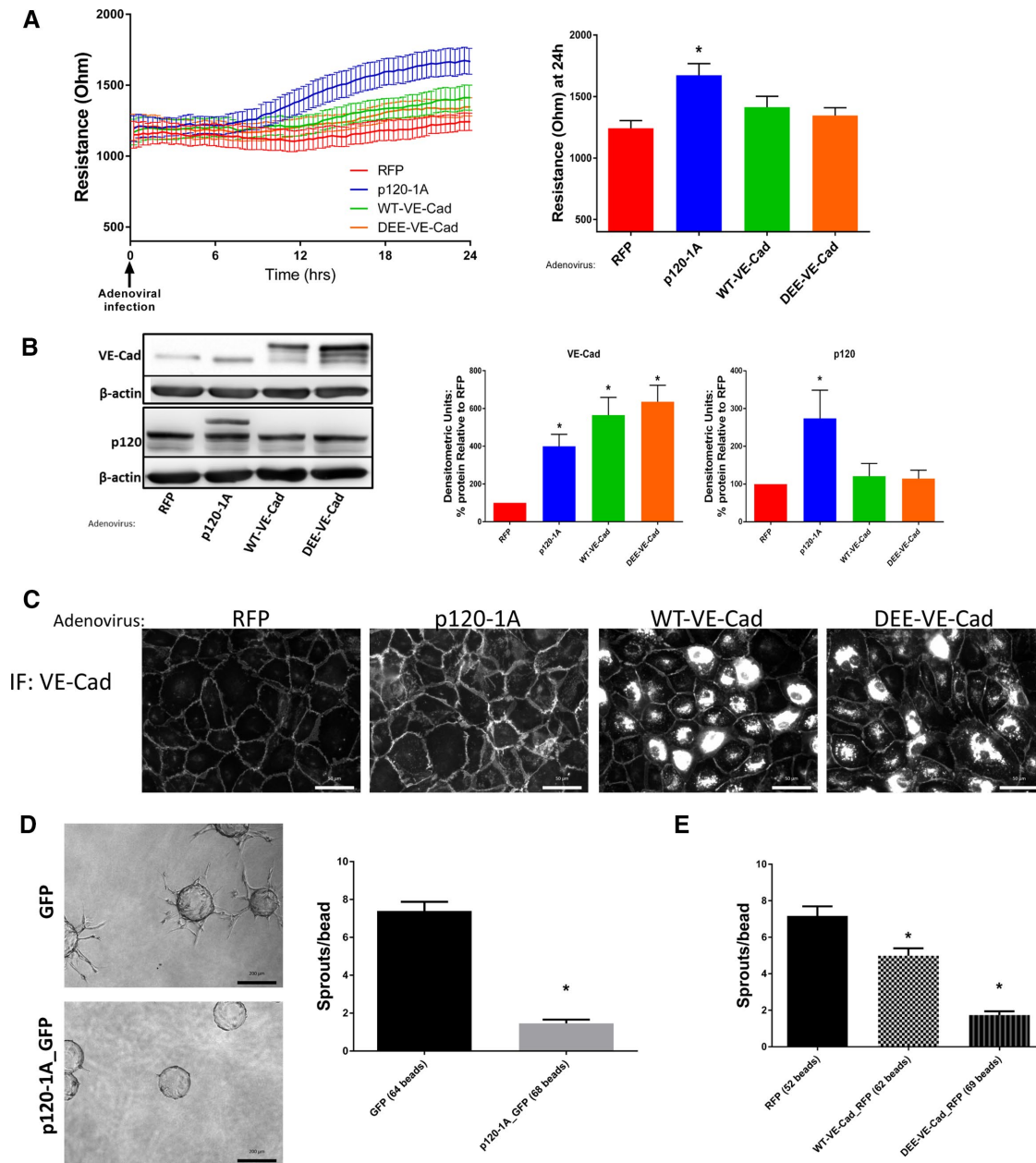
Previous work demonstrated that overexpression of DEE-VE-Cad would decrease wound closure in a scratch wound assay, a process believed to require rapid assembly and disassembly of cell-cell junctions (Nanes et al., 2012). Angiogenesis is highly dependent on the ability of endothelial cells to assemble and disassemble the adherens junction (Waliez and Huber, 2008). Thus we sought to determine the role of VE-Cad endocytosis in angiogenesis in a fibrin assay. In the first set of experiments, HUVECs were stably infected with lentivirus to overexpress p120-1A. These cells were then coated on beads, embedded in a fibrin matrix with fibroblasts on the top surface, and allowed to form sprouts over 12 d. Beads coated with

HUVECs overexpressing p120-1A had a significant reduction in sprouts emanating from the beads (Figure 4D, bottom) compared with HUVECs infected with lentivirus to express green fluorescent protein (GFP; Figure 4D, top). In a second set of experiments, HUVECs were infected with lentivirus to overexpress red fluorescent protein (RFP), WT VE-Cad, or DEE-VE-Cad. Overexpression of WT VE-Cad produced a small but statistically significant decrease in the number of sprouts (Figure 4E). Overexpression of DEE-VE-Cad, however, drastically inhibited sprout formation to a degree similar to that observed with p120 overexpression. These results support a model in which p120 plays an important role in regulating migration and angiogenesis by preventing VE-Cad endocytosis.

### VE-Cad phosphorylation regulates TEER independently of p120 binding

Overexpression of p120 leads to an inhibition of TEM that is associated with reduced VE-Cad binding to Src and lower levels of phosphorylation at Y658 (Alcaide et al., 2008, 2012). This led us to hypothesize that p120 could be working through a similar mechanism to regulate TEER. Consistently, loss of p120 increased the ratio of pY416-Src to total Src (Figure 5A). We then sought to determine whether loss of p120 binding to VE-Cad resulted in an increase in phosphorylation of VE-Cad. HUVEC monolayers were depleted of VE-Cad through transfection of siRNA to VE-Cad, and WT VE-Cad or DEE VE-Cad was then expressed to restore the VE-Cad levels. The reexpressed cadherin was then immunoprecipitated using antibodies to the RFP tag. Immunoblotting with anti-phosphotyrosine antibody (4G10) showed that DEE-VE-Cad had a greater amount of tyrosine phosphorylation than did WT VE-Cad, suggesting that p120 binding was protecting against VE-Cad from being phosphorylated (Figure 5B). However, dual infection with dominant-negative C-terminal Src kinase (DN-CSK; a potent activator of SFK) along with WT VE-Cad adenovirus resulted in robust VE-Cad phosphorylation. Previously we showed that p120

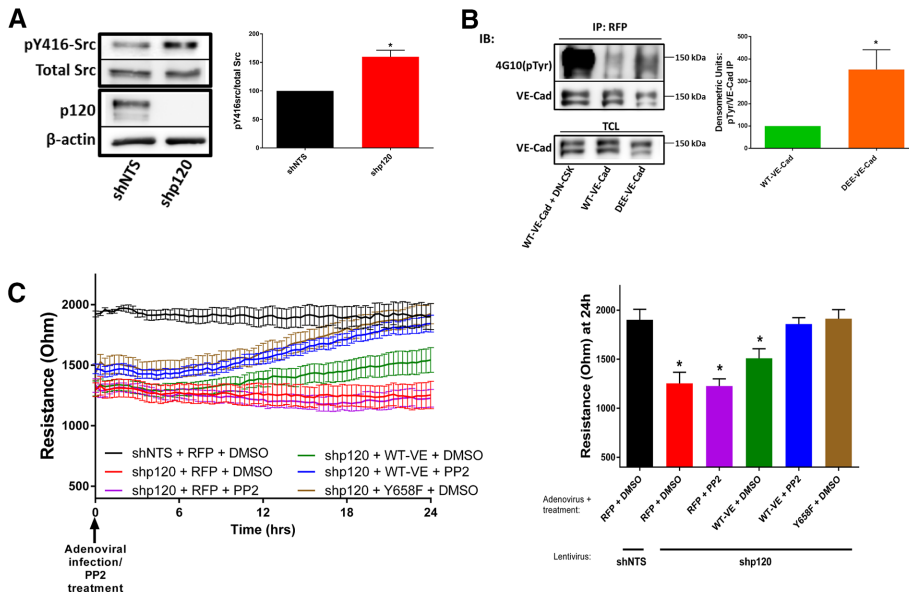
continues to interact with VE-cad when VE-cad is phosphorylated after Src activation with DN-CSK, suggesting that p120 may not protect VE-cad from being phosphorylated in the context of increased Src activation through other mechanisms (Adam et al., 2010). To determine whether increased Src activity played a role in regulating barrier function in the absence of p120, we expressed WT VE-Cad in shp120 HUVECs in the presence of the Src-family kinase (SFK) inhibitor PP2. After SFK inactivation, WT VE-Cad expression was sufficient to restore TEER to control levels in the absence of p120 (Figure 5C). Treating shp120 cells with PP2 but without increasing VE-Cad levels was not sufficient to restore TEER, showing the need for VE-Cad, not just Src inhibition, to restore the restrictive barrier (Figure 5C).



**FIGURE 4:** Overexpression of endocytic-defective VE-Cad mutant does not enhance TEER but suppresses angiogenic sprouting. Confluent HUVEC monolayers were infected with adenovirus to express RFP, p120-1A\_RFP, WT VE-Cad\_RFP, or DEE-VE-Cad\_RFP (at zero time point) over the next 24 h. (A) TEER was determined and plotted as mean  $\pm$  SEM at 15-min intervals for the 24-h period after adenoviral infection (left). The average TEER at 24 h after adenovirus infection was plotted as mean  $\pm$  SEM (right), and statistical analysis was performed. \* $p < 0.05$  compared with shNTS + RFP (five experiments). (B) Cells were lysed 24 h after adenoviral infection, and immunoblotting was performed for VE-Cad and p120. Values are means  $\pm$  SEM. \* $p < 0.05$  compared with RFP (five experiments). (C) Cells were fixed 24 h after adenoviral infection, and IF was performed probing for VE-Cad. (D) HUVECs were stably infected with lentivirus to express GFP or p120-1A\_GFP and were coated onto Cytodex 3 microcarriers and embedded into fibrin matrices. Pictures were taken 12 d postembedding, and sprouts per bead were counted and graphed. Scale bar, 200  $\mu$ m. Data are means  $\pm$  SEM. \* $p < 0.05$  compared with GFP. (E) HUVECs were stably infected with lentivirus to express RFP, WT VE-Cad\_RFP, or DEE-VE-Cad\_RFP, and experiments were performed as described in D. Data (sprouts/bead) are mean  $\pm$  SEM (four experiments).

The foregoing results suggested that loss of p120 induces Src activation, leading to VE-Cad phosphorylation, which may prevent VE-Cad from promoting cell-cell adhesion. To test this hypothesis, we reexpressed the nonphosphorylatable mutant Y658F-VE-Cad in endothelial monolayers depleted of p120. Unlike WT VE-Cad, ex-

pression of Y658F-VE-Cad was able to rescue the loss of TEER induced by p120 depletion (Figure 6A). It has been suggested that the affinity between p120 and VE-Cad is increased when VE-Cad is not phosphorylated at Y658 (Hatanaka *et al.*, 2011). Thus, to make sure that Y658F-VE-Cad did not restore TEER because it was better



**FIGURE 5:** Increased Src activity contributes to the inability of VE-cad to rescue TEER in p120-depleted monolayers. (A) HUVECs were stably infected with lentivirus to generate shNTS/shp120 cells. Cells were seeded at confluence (passage 1 or 2 after infection) and lysed 48 h after seeding for immunoblot analysis for pY416-Src, total Src, p120, and  $\beta$ -actin (loading control). Densitometry was performed taking the ratio of pY416-Src:total Src. Data plotted as mean  $\pm$  SEM,  $*p < 0.05$  (four experiments). (B) HUVECs were transfected with siRNA to VE-Cad and seeded at confluence. Forty-eight hours later, monolayers were infected with adenovirus to express WT VE-Cad\_RFP or DEE-VE-Cad\_RFP for 24 h. Cells were then lysed in IP buffer, and IP was performed using antibodies to RFP. IP lysates were run on Western blot and probed for phosphotyrosine using 4G10 (phosphotyrosine) and VE-Cad. The ratio of pTyr:VE-Cad was calculated and plotted as mean  $\pm$  SEM.  $*p < 0.05$  (three experiments). (C) shNTS/shp120 monolayers were infected with indicated adenovirus and treated with 5  $\mu$ M PP2 (or dimethyl sulfoxide [DMSO] control) at the zero time point, and resistance was monitored for 24 h. The average TEER was calculated 24 h after adenovirus infection/PP2 treatment. Values are means  $\pm$  SEM.  $*p < 0.05$  compared with shNTS + RFP + DMSO (four experiments).

able to bind the small amount of p120 remaining after shp120 infection, we generated a VE-Cad double mutant by substituting a phenylalanine for tyrosine 658 and making a triple-alanine substitution at DEE (DEE-Y658F-VE-Cad), thereby preventing p120 binding while also preventing Y658 phosphorylation. Expression of DEE-Y658F-VE-Cad in p120-depleted HUVECs was also able to restore TEER, confirming that unphosphorylated VE-Cad can restore TEER independently of p120 binding (Figure 6A). Furthermore, the ability of the VE-Cad nonphosphorylatable mutants to restore barrier function was not due to differences in expression levels of these constructs (Figure 6B).

The ability of DEE-Y658F-VE-Cad to restore barrier function suggests that the inability of Y658 to be phosphorylated may interfere with the ability of VE-Cad to be endocytosed. As shown in Supplemental Figure S3, however, WT VE-Cad and Y658F-VE-Cad were internalized to a similar extent. Further, the endocytosis-defective DEE-VE-Cad and the double mutant DEE-Y658F-VE-Cad were both internalized to a much lesser degree (Supplemental Figure S3). This shows that VE-Cad phosphorylation at Y658 is not required for VE-Cad endocytosis and that the inability of DEE-VE-Cad to be endocytosed is independent of Y658 phosphorylation.

If the foregoing conclusion is correct, then the double-mutant VE-cad constructs should be able to restore TEER after VE-cad depletion. In fact, as shown in Supplemental Figure S4A, reexpression of WT VE-Cad, Y658F-VE-Cad, or DEE-Y658F-VE-Cad rescued TEER after VE-cad knockdown. As shown before, neither RFP nor

DEE-VE-Cad reestablished TEER in these conditions. Consistent with their ability to bind p120, both WT VE-Cad and Y658F-VE-Cad restored p120 membrane localization, whereas DEE-VE-Cad and DEE-Y658F-VE-Cad were unable to reestablish p120 at the plasma membrane (Table 1B and Supplemental Figure S4B). Together the data shown here support the notion that unphosphorylated VE-Cad can regulate TEER independently of p120 binding.

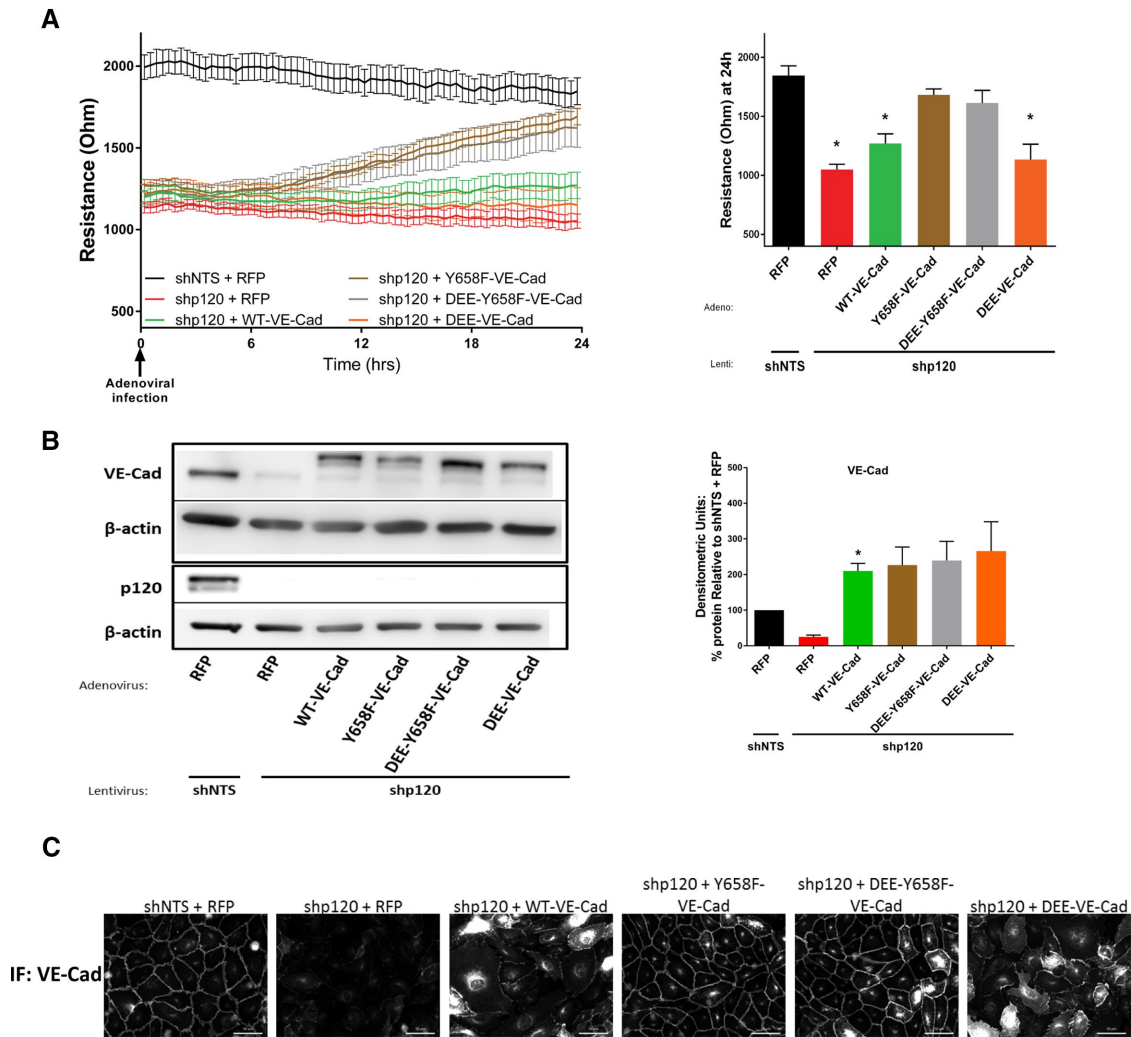
As shown in Figure 4A, forced expression of p120-1A can increase baseline TEER in HUVEC monolayers, whereas forced expression of WT VE-Cad or DEE-VE-Cad does not. To test whether the inability of VE-Cad to enhance barrier function is due to phosphorylation of VE-Cad, we overexpressed either WT VE-Cad or VE-Cad nonphosphorylatable mutants in mature HUVEC monolayers. Forced expression of Y658F-VE-Cad enhanced barrier function (Figure 7A) similarly to p120 overexpression (Figure 4A), whereas, as expected, WT VE-Cad or DEE-VE-Cad did not significantly increase baseline TEER. Further confirming that unphosphorylated VE-cad promotes barrier function independently of p120 binding, expression of DEE-Y658F-VE-Cad significantly increased TEER above control (Figure 7A).

Prolonged tyrosine phosphorylation of adherens junction proteins has been reported to be associated with delayed barrier recovery in response to thrombin (Timmerman *et al.*, 2012), whereas p120-1A overexpression improved recovery after thrombin-induced decreases in permeability (Zebda *et al.*, 2013). Because the design of many of the foregoing experiments assessed the ability of VE-Cad or p120 to restore barrier function, we next sought to test whether p120 or the recombinant forms of VE-Cad would improve the restoration of TEER after thrombin treatment. In this experiment, the decrease in TEER in response to thrombin was not attenuated by overexpression of p120-1A, WT VE-Cad, or any of the VE-Cad mutants (Figure 7B). Of note, there was a decrease in the time of recovery with expression of p120-1A, Y658F-VE-Cad, and DEE-Y658F-VE-Cad (Figure 7B). This suggests that p120 and/or maintaining VE-Cad in an unphosphorylated state are important factors in the restoration of an endothelial barrier after thrombin.

## DISCUSSION

p120 is known to regulate the level of VE-Cad in endothelial cells by preventing endocytosis and subsequent degradation of VE-Cad (Xiao *et al.*, 2003, 2005; Chiasson *et al.*, 2009). Clearly, the level of VE-Cad is important in forming and maintaining endothelial barrier function, as depletion of VE-Cad reduces TEER (Figure 3A and Supplemental Figure S3A) and knocking out VE-Cad *in vivo* using an endothelial-specific, tamoxifen-inducible knockout mouse has been shown to cause an increase in protein permeability in the lung (Frye *et al.*, 2015). To further investigate the role of VE-Cad endocytosis in regulating barrier function, we expressed the endocytic-defective DEE-VE-Cad mutant under various conditions. Our





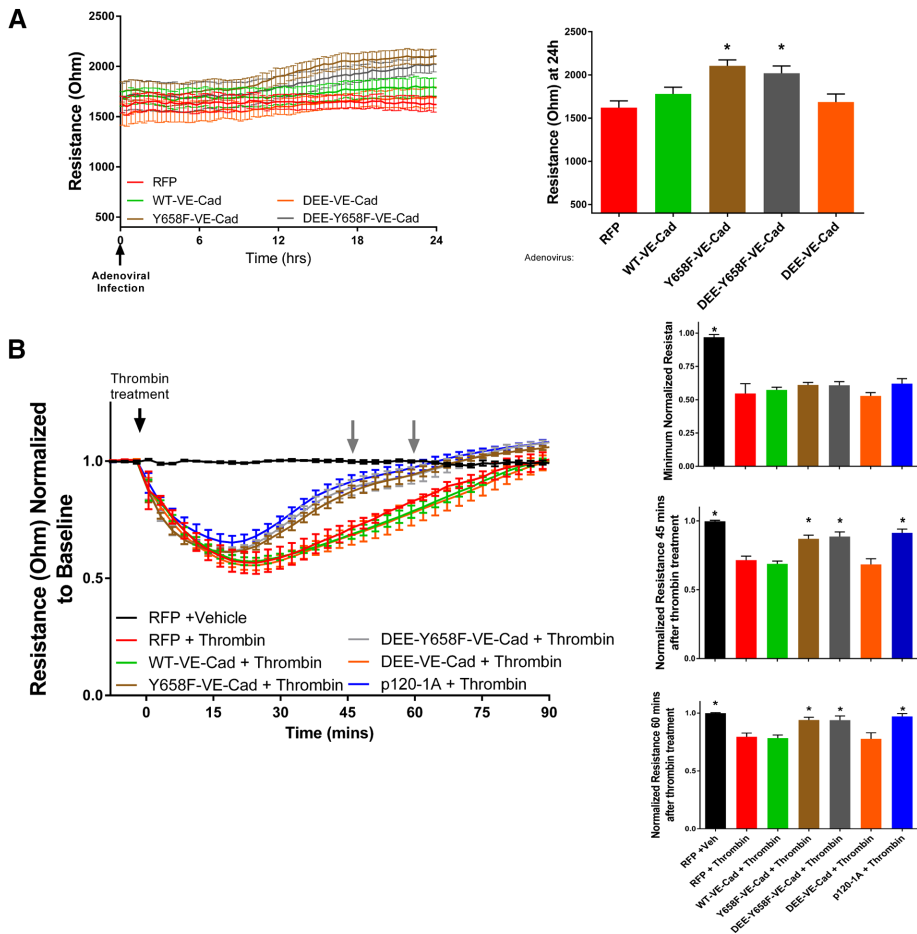
**FIGURE 6:** Expression of the VE-Cad Y658 nonphosphorylatable mutant localizes to cell–cell junctions and restores TEER in the absence of p120. HUVECs expressing shNTS or shp120 were seeded at confluence and allowed to mature for 48 h. Monolayers were infected 48 h postseeding with adenovirus to induce expression of RFP, WT VE-Cad\_RFP, Y658F-VE-Cad\_RFP, DEE-Y658F-VE-Cad\_RFP, or DEE-VE-Cad\_RFP for 24 h. (A) TEER was assessed and plotted at 15-min intervals as mean  $\pm$  SEM for the 24-h time period after adenoviral infection (left). Average TEER at 24 h after adenoviral infection was plotted as mean  $\pm$  SEM (right), and statistical analysis was performed. \* $p < 0.05$  compared with shNTS + RFP (four experiments). (B) At 24 h postinfection, cells were lysed into sample buffer, and immunoblot analysis was performed for VE-Cad and p120 as described in *Materials and Methods*. Densitometric analysis was performed to quantify bands, and data are presented as mean  $\pm$  SEM. \* $p < 0.05$  compared with shNTS + RFP; \* $p < 0.05$  compared with shNTS + RFP. Four experiments. (C) Cells were fixed 24 h after infection, and IF was performed for VE-Cad (four experiments).

results show that p120 does not regulate barrier function solely by regulating VE-Cad levels through the prevention of endocytosis, as expressing the endocytic-defective DEE-VE-Cad mutant cannot restore barrier function in cells depleted of either p120 (Figure 2B) or endogenous VE-Cad (Figure 3A). Our results further suggest that simply maintaining VE-Cad level is not sufficient to prevent mediator-induced changes in permeability. A decrease in VE-Cad has been implicated in contributing to mediator-induced increases in permeability, such as by thrombin (Gong *et al.*, 2014) or interleukin 1 $\beta$  (Zhu *et al.*, 2012), which suggested that maintaining the level of VE-Cad by blocking endocytosis may be sufficient to prevent mediator-induced changes in permeability. The data presented in Supplemental Figure S2 show that this is not a viable option, as maintaining VE-Cad expression level by overexpressing either WT-VE-Cad or blocking endocytosis by expressing DEE-VE-Cad did not prevent the decrease in TEER after

treatment with thrombin or interleukin 1 $\beta$ . In addition, DEE-VE-Cad did not accelerate recovery after thrombin. Taken together, these data show that p120 has a role in addition to maintaining VE-Cad levels when it comes to forming a restrictive endothelial barrier.

On the contrary, the process of endocytosis may play a more important role during angiogenesis. Indeed, overexpressing p120-1A or DEE-VE-Cad resulted in decreased endothelial sprouting, suggesting that constitutive VE-Cad endocytosis may be required for sprouting to occur. Furthermore, expression of WT VE-Cad did not suppress sprouting to the same extent as p120 or DEE-VE-Cad expression, implying that the decrease in sprouting was due not simply to increase in VE-Cad levels but also to inhibition of endocytosis. This is consistent with the findings of Nanes *et al.* (2012), who demonstrated that DEE-VE-Cad expression inhibited VEGF-induced cell migration in a scratch wound assay. In addition,





**FIGURE 7:** Preventing VE-Cad phosphorylation improves barrier recovery after thrombin treatment. (A) Confluent HUVEC monolayers were infected with adenovirus to express RFP, WT VE-Cad, Y658F-VE-Cad\_RFP, DEE-Y658F-VE-Cad\_RFP, or DEE-VE-Cad over the next 24 h. (A) TEER was determined and is plotted as mean  $\pm$  SEM at 15-minute intervals for the 24-h period after adenoviral infection (left). Average TEER at 24 h after adenovirus infection is plotted as mean  $\pm$  SEM (right), and statistical analysis was performed. \* $p < 0.05$  compared with shNTS + RFP for 24 h; \* $p < 0.05$  compared with RFP (three experiments). (B) Confluent HUVEC monolayers were infected with adenovirus to express RFP, WT VE-Cad, Y658F-VE-Cad\_RFP, DEE-Y658F-VE-Cad\_RFP, or DEE-VE-Cad over the next 24 h. At 24 h postinfection, monolayers were treated with 500 mU/ml thrombin or (0.1% BSA vehicle control) and TEER was recorded. TEER was normalized to average TEER obtained over the 30 min before treatment. The lowest normalized TEER during the 1.5-h thrombin treatment was determined and is graphed (top). Average TEER for each group was calculated at 45 and 60 min after thrombin treatment. Data are plotted as mean  $\pm$  SEM. \* $p < 0.05$  compared with RFP + thrombin (three experiments).

exogenous expression of WT VE-Cad did not slow wound closure, showing that inhibition of cell migration is not caused simply by increased cadherin levels. Taken together, these results suggest that p120-VE-Cad interaction regulates barrier function and angiogenic sprouting through different mechanisms.

Lampugnani *et al.* (1997) first implicated tyrosine phosphorylation in the formation of a restrictive barrier by describing a decrease in tyrosine phosphorylation of adherens junction proteins as endothelial monolayers became more confluent. Since that study, a large body of research has highlighted three important tyrosine residues in VE-Cad that participate in the regulation of endothelial permeability according to their phosphorylation state: Y658, Y685, and Y731 (Adam, 2015). Using Y-to-F mutations at each of these sites, a number of investigations have shown that prevention of VE-Cad phosphorylation attenuates leukocyte TEM and changes in perme-

ability induced by a number of mediators (Alcaide *et al.*, 2008; Turowski *et al.*, 2008; Monaghan-Benson and Burrige, 2009; Gong *et al.*, 2014). Furthermore, expression of a VE-Cad phosphomimetic (Y658E-VE-Cad) was not able to restore barrier function in the absence of endogenous VE-Cad (Gong *et al.*, 2014). Consistent with these studies, we also showed that Y658 phosphorylation is important in the formation of a restrictive barrier (Figure 6A and Supplemental Figure S3A). In addition, the studies presented here extend our knowledge of how the phosphorylation of VE-Cad and the interaction of p120 contribute to the regulation of endothelial monolayer integrity. In cells depleted of p120, expression of a mutant VE-Cad with a Y658F substitution, but not WT VE-Cad, is able to restore TEER to control levels (Figure 6A). Of interest, we found that Y658F VE-Cad is internalized to the same extent as WT VE-Cad. This demonstrates that p120 is not needed to form a restrictive barrier as long as VE-Cad Y658 is not phosphorylated, which is independent of VE-Cad internalization. This is further supported by experiments using the DEE-Y658F-VE-Cad double mutant, which cannot be phosphorylated nor bind p120 but can restore barrier function to control levels in cells depleted of either p120 (Figure 6A) or VE-Cad (Supplemental Figure S3A). To our knowledge, this is the first study to demonstrate that VE-Cad can form a restrictive barrier independent of p120 binding, with the caveat that VE-Cad Y658 must be unphosphorylated.

We also found that, in cells previously depleted of endogenous VE-Cad, DEE-VE-Cad, which does not bind p120, is highly tyrosine phosphorylated, whereas WT VE-Cad is not (Figure 5B), suggesting that p120 protects Y658 from being phosphorylated. This is consistent with the findings of Alcaide *et al.* (2008), who found that overexpression of p120 prevented basal VE-Cad phosphorylation and also blocked phosphorylation of VE-Cad when Src activity was increased by ICAM activation. Indeed, depletion of p120 is associated with an increase in Src activity (Figure 5A), and WT VE-Cad was able to restore TEER to control levels in the presence of the Src inhibitor PP2 (Figure 5C). However, there may be an alternative interpretation. We previously demonstrated that activation of Src Family kinases using the expression of a DN-CSK produces an increase in Src activity and a robust increase in VE-Cad phosphorylation but does not change endothelial monolayer permeability (Adam *et al.*, 2010). In addition, VE-Cad still binds p120 in cells with active SFKs (Adam *et al.*, 2010). Similar to the results obtained in HDMECs, DN-CSK does not promote a loss of barrier function in HUVEC (Adam *et al.*, 2016). We used the DN-CSK as a positive control here to show that reexpressed VE-Cad can be phosphorylated in these studies. The lack of a change in TEER when VE-Cad is phosphorylated in the presence of p120

suggests that p120 allows the phosphorylated form of VE-Cad to maintain barrier function. This may explain how Orsenigo *et al.* (2012) observed an elevated phosphorylation of VE-Cad in venous endothelial cells in conjunction with very low protein permeability if, in venous cells, p120 allows for the formation of a restrictive barrier even though VE-Cad Y658 is phosphorylated. When bradykinin was added, the permeability increased in the veins of mice (Orsenigo *et al.*, 2012). This would be consistent with p120 being released from VE-Cad due to posttranslational modification of p120 induced by inflammatory mediators. For example, an increase in protein kinase C-induced phosphorylation of p120 has been implicated in the change in endothelial monolayer permeability after lipopolysaccharide challenge in vitro or thrombin challenge in vivo (Vandenbroucke St Amant *et al.*, 2012). The increase in p120 phosphorylation would release the p120, allowing for phosphorylation of VE-Cad to promote a loss of cell–cell junctions and subsequent loss of VE-Cad level due to endocytosis of VE-Cad in the absence of p120 binding. This represents a change in the traditional model of the sequence of events leading to junction disassembly, in which VE-Cad phosphorylation causes a loss of p120 binding, resulting in junction disassembly due to VE-Cad endocytosis.

Why is p120 binding or maintenance of VE-Cad in an unphosphorylated form important for the formation of an endothelial monolayer, which has a more restrictive barrier? The answer may involve trafficking VE-Cad to the cell membrane. Our data suggest that p120 could be regulating barrier function by improving delivery of VE-Cad to the cell–cell junctions. This would be similar to the findings of Chen *et al.* (2003), who demonstrated that the binding of p120 is required for proper transport of N-cadherin to intercellular junction via a mechanism involving kinesin. As shown by the quantification of immunofluorescence data in Table 1, the DEE–VE-Cad mutant was unable to efficiently accumulate at the cell borders. This occurred even though Western blot data showed that the total level of the DEE–VE-Cad was at control levels or higher (Figures 2A and 3C). Data using Y658F–DEE–VE-Cad, however, argue against the requirement for p120 binding to get VE-Cad to the plasma membrane. Expression of Y658–DEE–VE-Cad can rescue TEER in cells depleted of p120, and in cells depleted of VE-Cad, it restores TEER without returning p120 to the cell–cell junction. Indeed, DEE–Y658F–VE-Cad localizes normally at the cell–cell junction, as shown in the quantitation of immunofluorescence (IF; Table 1), without p120 returning to the cell–cell junction (Figures 3B and 6B and Table 1). Thus p120 binding to VE-Cad is not required to get VE-Cad to the plasma membrane if Y658 cannot be phosphorylated.

Alternatively, p120 binding and/or unphosphorylated Y658 may keep VE-Cad at the cell–cell junction by limiting the mobility into and out of the junction. This is supported by the junctional localization of VE-Cad being restored close to control levels with p120 rescue or expression of Y658F mutants in the absence of p120 (Table 1, A and B). Fluorescence recovery after photobleaching (FRAP) experiments suggested that loss of p120 binding increases VE-Cad's ability to move freely throughout the membrane, as DEE–VE-Cad has a higher mobile fraction than WT VE-Cad (Nanes *et al.*, 2012). In contrast, Y658F–VE-Cad has a lower mobile fraction than WT (Orsenigo *et al.*, 2012), suggesting that phosphorylation of Y658 in VE-cad would allow for higher mobility in the plasma membrane. Our data are consistent with these FRAP data, as Y658F–VE-Cad and DEE–Y658F–VE-Cad both localized to the cell–cell junctions in the absence of endogenous p120 to the same extent as VE-Cad in the nontargeting short hairpin RNA (shNTS) control (Table 1), suggesting that Y658 phosphorylation regulates the mobility of VE-cad into and out of the junction in the absence of p120. One could speculate that an

increase in lateral clustering and formation of *cis*-dimers would allow for a high degree of junctional localization, which would then lead to an enhanced barrier function. Indeed, p120 has been shown to play a role in the lateral clustering of cadherins (Yap *et al.*, 1998). How the Y658 phosphorylation state contributes to junctional localization is an open question. One could hypothesize that Y658 phosphorylation itself decreases lateral clustering when p120 is absent due to the increase in negative charge. Alternatively, increased binding of a SH2 domain-containing protein to phosphorylated VE-Cad may be limited in the absence of p120 *cis* clustering and reduce junctional localization. Future experiments will need to be performed to determine how different residues in these molecules regulate kinetics within the junction.

In summary, we showed that regulation of the endocytic process is not the only mechanism by which p120 regulates the formation of a restrictive endothelial monolayer. In addition to regulating endocytosis, p120 also allows the phosphorylated form of VE-Cad to participate in the formation of a restrictive monolayer. If VE-Cad is not phosphorylated at Y658, then VE-Cad will form a restrictive barrier independently of p120. These findings suggest that loss of p120 binding in conjunction with phosphorylation of VE-Cad at Y658 is required to prevent VE-Cad from forming a restrictive monolayer of endothelial cells. However, losses of p120 binding without tyrosine phosphorylation or tyrosine phosphorylation in the presence of p120 binding is not sufficient to prevent formation of a restrictive barrier. Further research will need to be conducted to understand the role that p120 binding and VE-Cad phosphorylation plays in the regulating the kinetics of these two molecules within the endothelial cell–cell junction.

## MATERIALS AND METHODS

### Cell culture

Pooled HUVECs were purchased from Lonza (Walkersville, MD). Cells were expanded for two passages and then frozen and stored in liquid nitrogen. Cells were cultured in EGM2 growth medium (Lonza) containing 2% (vol/vol) fetal bovine serum (FBS). Cells were used between passages 5 and 10. All experiments were performed by seeding at  $8 \times 10^4$  cells/cm<sup>2</sup>, allowing for cells to be confluent immediately after seeding.

### RNA interference (small interfering RNA)

Small interfering RNA (siRNA) targeted to the 3' untranslated region (UTR) of VE-cadherin mRNA (AGUAAUUGCUCUACAGAUUU) was purchased from Dharmacon, Lafayette, CO. The 3'UTR was used as a target to allow reexpression of VE-Cad. siRNA was delivered into HUVECs at 80 pmol per  $1.0 \times 10^5$  cells in suspension using RNAiMax in Opti-Mem (Thermo Scientific, Waltham, MA). Six hours after seeding, an equal amount of EGM2 medium was added. Twenty-hours after seeding, a complete medium change was performed. Knockdown was evaluated by Western blotting at the end of each experiment.

### Inflammatory mediator treatment

HUVECs were seeded at confluence and incubated for 2 d in EGM2 to allow the formation of a mature monolayer. Monolayers were infected with adenovirus to express indicated proteins over a 24-h period. At 24 h postinfection (48 h postseeding), cells were serum starved for 6 h (in EBM-2 with 0.3% FBS with no additional factors). For thrombin, cells were treated with 500 mU/ml thrombin (Sigma-Aldrich, St. Louis, MO) or vehicle only (EBM/0.1% FBS) for 2 h. For IL-1 $\beta$ , cells were treated with 10 ng/ml IL-1 $\beta$  or vehicle only (EBM/0.1% FBS) for 12 h.

## Adenovirus constructs

Murine p120 mutant constructs were made from the p120-1A vector provided by Albert B. Reynolds (Vanderbilt University, Nashville, TN). To make RFP-tagged constructs, we performed PCR using p120-1A\_GFP as a template and forward primers consisting of the start site of the p120-1A isoform with an EcoRI restriction site and Kozak (5'-GAATTCGCCACCATGGACGACTCAGAGGTGGAG-3') along with the reverse primer containing an AgeI site and the COOH terminus of p120 (5'-GCACCCTTGATGCAGAAGATTGCACCGGT-3'). Full-length VE-cadherin\_RFP was a generous gift from Keith Burridge (University of North Carolina, Chapel Hill, NC). The DEE→AAA-VE-cadherin\_RFP was made by mutating amino acids 646–648 to alanine residues using PCR site-directed mutagenesis using VE-cadherin\_RFP as a template (primers: forward, 5'-CTGGTCACCTACGCAGCAGCAGGCGGCGGCGAGATG-3'; reverse 5'-CATCTCGCCGCCGCTGCTGCTGCGTAGGTGACCAG-3'; Nanes *et al.*, 2012). Y658F-VE-Cad and DEE-Y658F-VE-Cad were made by substituting tyrosine with phenylalanine using PCR site-directed mutagenesis using VE-cadherin\_RFP or DEE-VE-Cad, respectively, as templates (primers: forward, 5'-GAGATGGACACCACCAGCTTCGATGTGTCGGTGCTCAAC-3'; reverse, 5'-GTTGAGCACCGACACATCGAAGCTGGTGGTGGTGGTGGTCCATCTC-3'). Dominant-negative CSK adenovirus containing a lysine-to-arginine substitution at position 222 (DN-CSK) was a generous gift from S. Tanaka (Faculty of Medicine, University of Tokyo, Tokyo, Japan; Takayama *et al.*, 1999). p120 and VE-cadherin constructs were cloned into TagRFP-AS-N (Gateway; Evrogen) in-frame with a C-terminal TagRFP, a monomeric RFP, and then shuttled into pAd/CMV/V5-DEST (Gateway Clonase; Life Technologies). Plasmids were digested with *PacI* to expose the viral inverted terminal repeats. Adenoviruses were amplified in QBI-HEK-293A cells (Qbiogene, Carlsbad, CA) and purified using cesium chloride gradients.

## Lentiviral production

Construction of pFUGW-dest containing shRNA targeting human p120 (Davis *et al.*, 2003) has been described and used previously by our lab (Herron *et al.*, 2011). p120 and VE-cadherin constructs were cloned into TagRFP-AS-N (Gateway; Evrogen) in-frame with a C-terminal TagGFP or TagRFP, respectively, and then shuttled into pFUW/dest using homologous recombination (Gateway Clonase; Life Technologies). Lentivirus was produced by transfection of 90% confluent HEK-293FT cells with pFUGW-dest, pCMV, and pVSV at a 1:1:1 M ratio using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham MA) and Opti-MEM (Thermo Fisher Scientific) supplemented with 10% FBS 5% penicillin/streptomycin, and 5% L-glutamine. Medium was collected between 24 and 48 h after transfection, and cell debris was cleared by centrifugation at 3000 × g. Lentivirus was then concentrated using an Amicon Ultra Centrifugal Filter (Millipore) and stored at -80°C. To produce p120-depleted HUVECs, cells were seeded at 25% confluence. After 24 h, when cells reached ~50% confluence, HUVECs were infected with lentivirus, and 72 h was allowed for protein knockdown. Cells were then resuspended and plated at confluence for experiments. Cells were used upon first or second passage after lentiviral infection.

## Gel electrophoresis and immunoblotting

At the appropriate times during experimental procedures, cells were lysed in Laemmli buffer containing complete protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) and boiled. Lysates were loaded on SDS-PAGE gels with a stacking buffer and run for 15 min at 75 V, followed by 80 min at 150 V. Proteins were then transferred to nitrocellulose membranes. For immunoblotting,

membranes were blocked with 3% nonfat milk in phosphate-buffered saline (PBS) or 5% bovine serum albumin (BSA) in PBS supplemented with 0.01% Tween-20 (Sigma-Aldrich), followed by overnight incubation with the following antibodies: p120 (sc-1101; Santa Cruz Biotechnology, Santa Cruz, CA), VE-cadherin (sc-6458, Santa Cruz Biotechnology; TA590724, SDIX, Newark, DE), and β-actin (Sigma-Aldrich). Secondary mouse, rabbit, or goat antibodies conjugated with peroxidase (Jackson ImmunoResearch, West Grove, PA) were incubated for 2 h at room temperature, and membranes were developed using SuperSignal West Pico (Pierce, Rockford, IL) and the Fujifilm LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Densitometric analysis was performed using Fujifilm Multigauge 3.0 software.

## Immunofluorescence microscopy

HUVECs were seeded at confluence on Ibidi μ-Slide eight-well culture plates (Ibidi USA, Madison, WI) and fixed with 4% paraformaldehyde (PFA; USB, Cleveland, OH) in PBS+ for 30 min at 4°C and washed three times with Tris-buffered saline (TBS). Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in TBS for 15 min, treated with Image-It FX signal enhancer (Invitrogen, Waltham, MA) for 30 min, and then blocked with 5.5% bovine serum in TBS for 1 h. Primary antibodies (listed earlier) were incubated for 2 h at room temperature. Secondary anti-goat, -rabbit, or -mouse antibodies conjugated with Alexa Fluor 350, 488, 594, or 647 were incubated for 1 h at room temperature (Thermo Fisher Scientific). Images were taken using a Zeiss Observer Z1 microscope with Zeiss Zen Microscope Software (Carl Zeiss Microscopy, Thornwood, NY).

Immunofluorescence data shown in Table 1 are a compilation all IF data from Figures 2, 3, and 6 and Supplemental Figure S3. For quantification of junctional pixel intensity, line plots were drawn perpendicular to junctions using ImageJ software (National Institutes of Health, Bethesda, MD). Five line plots were drawn per image at junctions of five independent cells, and the area under each curve, with the background subtracted out, was determined. The average area under the curve (calculated from the average of the five line plots) was calculated for each image. Each experiment consisted of at least three images per condition. The mean pixel intensity of each image was normalized to the mean of the control images. These experiments were done three times, and the mean of means was determined for each condition. Data were plotted using GraphPad Prism software (GraphPad Software, La Jolla, CA).

## Measurement of TEER to assess barrier function

Endothelial monolayer barrier function was determined by measuring changes in TEER using an electric cell-substrate impedance sensor (ECIS; Applied BioPhysics, Troy, NY; Giaever and Keese, 1991). Monolayers were formed by seeding HUVECs onto 8W10E plates that had been precoated with 0.1% gelatin for 30 min. Impedance was measured every 5 min and used to calculate resistance (TEER) using the manufacturer's software. Electrical resistance across the monolayer was measured in ohms and graphed over time. A higher TEER is indicative of a more restrictive monolayer. Average resistance was calculated and graphed at indicated time points. For normalized ECIS, the average resistance was calculated during the 0.5-h period before inflammatory mediator treatment and referred to as baseline resistance. The resistance at each time point during an experiment was divided by the baseline resistance and graphed.

## Immunoprecipitation

Cells were lysed in 1 ml (per 100-mm plate) of IP lysis buffer (200 mM NaCl, 600 mM sucrose, 2 mM CaCl<sub>2</sub> dihydrate, 6 mM MgCl<sub>2</sub>



hexahydrate, and 20 mM 1,4-piperazinediethanesulfonic acid) containing complete protease/phosphatase, ethylenediaminetetraacetic acid (EDTA)-free inhibitor mixture (Thermo Fisher Scientific) and 0.5 mM pervanadate, and the lysates were centrifuged at  $14,000 \times g$  to clear debris. A portion of the supernatant was lysed in Laemmli buffer and used for total cell lysate. The remaining supernatant was removed, placed into a fresh tube, and incubated at  $4^{\circ}\text{C}$  for 2 h under constant agitation with anti-RFP (AB233; Evrogen, Moscow, Russia) antibody. SureBeads Protein A Magnetic Beads (Bio-Rad, Hercules, CA) were then added to the supernatant/antibody mixture and incubated at  $4^{\circ}\text{C}$  overnight under constant agitation. The beads were washed three times in IP lysis buffer. Bound proteins were removed by lysing in Laemmli buffer and then boiling for 5 min. The samples were resolved using SDS-PAGE and Western blotting probing for VE-cadherin (SDIX) and 4G10 platinum phosphotyrosine (EMD Millipore, Billerica, MA).

### Internalization assay

Assays were performed as described in Nanes *et al.* (2012). Briefly, HUVECs were depleted of endogenous VE-Cad via siRNA transfection, followed by adenoviral expression of WT VE-Cad or DEE-VE-Cad. Cells were incubated with an extracellular antibody toward VE-Cad (BV6 clone [EMD Millipore], representing internalized VE-Cad) at  $4^{\circ}\text{C}$  and then subjected to a 30-min incubation at  $37^{\circ}\text{C}$  to allow for internalization. Cells were returned to  $4^{\circ}\text{C}$ , and the remaining surface-bound antibody was removed with a low-pH wash (PBS with 100 mM glycine, 20 mM magnesium acetate, and 50 mM potassium chloride, pH 2.2). Cells were then fixed in 4% PFA, permeabilized using 0.1% Triton-X, and then incubated with a secondary VE-Cad antibody directed toward the cytoplasmic domain (Santa Cruz Biotechnology; represents total VE-Cad). Images were taken, and internalization was quantified by taking the ratio of fluorescence signals corresponding to the internalized and total cadherin.

### Fibrin bead assay

The fibrin bead assay was performed as previously described (Nakatsu and Hughes, 2008) with slight modifications. Briefly, HUVECs were stably infected with lentivirus containing GFP, p120-1A\_GFP, RFP, WT VE-Cad\_RFP, or DEE-VE-Cad\_RFP and mixed with dextran-coated Cytodex 3 microbeads (GE Healthcare, Piscataway, NJ) at 500 HUVECs/bead in 1.5 ml of EGM2. Beads with cells were shaken gently every 20 min for 4 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . After incubation, cell-coated beads were transferred to a  $25\text{-cm}^2$  tissue culture flask with 5 ml of EGM2 and incubated overnight at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . The next day, cell-coated beads were washed three times with 1 ml of EGM2 and resuspended at a concentration of 200 cell-coated beads/ml in 2.0 mg/ml fibrinogen (Sigma-Aldrich) with 0.15 U/ml aprotinin (Sigma-Aldrich). A 200- $\mu\text{l}$  amount of fibrinogen/bead solution was added to 0.625 U of thrombin (Sigma-Aldrich) into each well of an eight-well Falcon tissue-treated glass plate (BD Biosciences, San Jose, CA). Fibrinogen/bead solution was allowed to clot for 5 min at room temperature and then at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 20 min. We plated  $5.0 \times 10^5$  neonatal human dermal fibroblasts (NHDF)/well on top of the clot in 300  $\mu\text{l}$  of EGM2. Medium was changed every second day and images taken every third day. Bead assays were monitored for at least 12 d after embedding. The number of sprouts per bead was determined at day 12.

### Statistical analysis

Individual bands on immunoblots were quantified using a Fujifilm LAS-3000 imaging system with AxioVision software. Values were normalized first to actin and then normalized to NTS/RFP controls for

individual experiments. Owing to the absence of error around the controls, single-sample *t* tests were performed on immunoblot data, and a Bonferroni correction was applied to correct for multiple comparisons (Figures 2B, 4B, and 6B). For experiments measuring TEER, statistical analysis was performed on the time point corresponding to 24 h after the addition of adenovirus, which was graphed as mean  $\pm$  SEM in each figure. ECIS and IF results (Table 1), which had multiple groups, were analyzed using a one-way analysis of variance, followed by a post hoc Dunnett's multiple comparison test to compare experimental groups to the control (NTS/RFP). A single-sample *t* test was used if only a single comparison was performed (Figures 1B and 5, A and B, and Supplemental Figure S1A). All statistical analysis was performed using GraphPad Prism software. Data are presented as mean, and error bars indicate SEM. A 95% confidence level ( $p < 0.05$ ) was considered statistically significant.

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