The VE-cadherin cytoplasmic domain undergoes proteolytic processing during endocytosis

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ABSTRACT VE-cadherin trafficking to and from the plasma membrane has emerged as a critical mechanism for regulating cadherin surface levels and adhesion strength. In addition, proteolytic processing of cadherin extracellular and cytoplasmic domains has been reported to regulate cadherin adhesion and signaling. Here we provide evidence that VE-cadherin is cleaved by calpain upon entry into clathrin-enriched domains. This cleavage event occurs between the β-catenin and p120-binding domains within the cadherin cytoplasmic tail. Of interest, VE-cadherin mutants that are resistant to endocytosis are similarly resistant to cleavage. Furthermore, p120-catenin overexpression blocks cadherin internalization and cleavage, coupling entry into the endocytic pathway with proteolytic processing. Of importance, the cleavage of the VE-cadherin tail alters the postendocytic trafficking itinerary of the cadherin, resulting in a higher turnover rate due to decreased recycling and increased degradation. In conclusion, this study identifies a novel proteolytic event that regulates the trafficking of VE-cadherin after endocytosis.

INTRODUCTION

Vascular endothelial cells form a lining on the interior surface of blood vessels and play important roles in thrombosis, vascular permeability, and inflammation (Boulanger, 2016). Endothelial cell–cell adhesion is essential for normal endothelial barrier function and immune responses (Dejana and Orsenigo, 2013; Gavard, 2014). Adherens junctions are the major adhesive cell–cell junctions in endothelial cells and are critical for endothelial barrier properties and for angiogenesis during development, wound healing, and tumor growth. Vascular endothelial cadherin (VE-cadherin) is the major adhesion molecule in endothelial adherens junction (Dejana and Orsenigo, 2013; Lagendijk and Hogan, 2015). As a member of the classical cadherin family, VE-cadherin mediates homophilic adhesion through cadherin repeats in the extracellular domain, while the cytoplasmic domain associates with linker molecules that stabilize the cadherin and couple the adhesion molecule to the actin cytoskeleton (Gavard, 2014; Cadwell et al., 2016b). Cytoplasmic binding partners for VE-cadherin include p120-catenin, which binds to the juxta-membrane domain of the cadherin cytoplasmic tail, and β-catenin, which binds to the membrane-distal, carboxyl-terminal domain of VE-cadherin (Gavard, 2014; Cadwell et al., 2016b). Previous studies showed that p120 binding to the cadherin tail stabilizes the cadherin by preventing endocytosis (Davis et al., 2003; Xiao et al., 2003a; Chiasson et al., 2009; Nanes et al., 2012; Kourtidis et al., 2013), whereas β-catenin binding functions as a linker to actin-binding proteins such as α-catenin (Buckley et al., 2014; Bianchini et al., 2015).

VE-cadherin dynamics at the plasma membrane is believed to be essential in modulating endothelial adhesion strength and adherens junction plasticity (Cadwell et al., 2016b). The level of cell surface VE-cadherin is regulated in part by endocytosis. Previous work found that VE-cadherin undergoes clathrin-mediated endocytosis (Xiao et al., 2005; Chiasson et al., 2009; Kowalczyk and Nanes, 2012; Semina et al., 2014; Zhang et al., 2014; West and Harris, 2016). This process is inhibited by p120-catenin, which binds to the cadherin tail and masks an endocytic motif in the juxtamembrane domain (Chiasson et al., 2009; Nanes et al., 2012). This regulatory mechanism is believed to be a major control point for cadherin expression levels on the plasma membrane in a variety of mammalian cell types and tissues. In addition to endocytosis, cadherin cell...
surface levels can also be regulated by proteolysis. For example, metalloproteinases cleave the VE-cadherin extracellular domain, leading to reduced cell–cell adhesion strength (Drey Mueller et al., 2012; Flemming et al., 2015). Both γ-secretase and caspase-3 cleave cadherins to promote disassembly of adherens junctions and reduce adhesion (Hunter et al., 2001; Steinhusen et al., 2001; Mrambaud et al., 2002). Calpain, a calcium-dependent cysteine protease, also has been shown to cleave several classical cadherins and thereby down-regulate cell–cell adhesion (Jang et al., 2009; Miyazaki et al., 2011; Ye et al., 2013; Kudo-Sakamoto et al., 2014; Trillsch et al., 2016). Despite the growing evidence of a role for calpain in modulating the activity of cadherins and other adhesion receptors, the subcellular localization of cadherin cleavage by calpain and the relationship of this processing to VE-cadherin endocytosis have not been established. Here we provide evidence that VE-cadherin is proteolytically processed upon entry into clathrin-enriched membrane domains during the process of endocytosis. This cleavage event, which removes the catenin-binding domain of the cadherin tail, is mediated by calpain and fates the cadherin for a degradative rather than recycling pathway. These findings reveal a novel mechanism for how proteolytic processing could modulate cadherin surface levels by altering the itinerary of the cadherin during endocytosis.

RESULTS

VE-cadherin is cleaved during endocytosis

Our previous studies demonstrated that a fragment of VE-cadherin missing the β-catenin-binding domain accumulates in endothelial cells treated with chloroquine, which inhibits lysosomal degradation (Xiao et al., 2003b). Data shown in Figure 1A confirm that chloroquine treatment results in the accumulation of an ∼95-kDa fragment of VE-cadherin. This processed form of VE-cadherin can be detected using antibodies to the VE-cadherin extracellular domain but not with antibodies directed against the catenin-binding domain of the cadherin tail (Xiao et al., 2003b). To determine whether this fragment was generated during cadherin internalization, we labeled the cell surface pool of VE-cadherin using an antibody directed against the extracellular domain (BV6) and followed the fate of the cadherin during endocytosis over a 3-h period in the presence of chloroquine. To distinguish cell surface from internalized pools of cadherin, we removed BV6 bound to the surface pool of VE-cadherin using a low-pH wash. The presence of the cytoplasmic catenin-binding domain was monitored using C19, an antibody directed against the VE-cadherin carboxy-terminal domain (Figure 1B). Colocalization of the antibodies directed against the VE-cadherin extracellular domain (BV6) and carboxy-terminal tail (C19) was measured at cell–cell borders and in endosomal compartments. As expected, BV6 and C19 exhibited extensive colocalization at cell–cell borders (Figure 1, C, top, and D). In contrast, the internalized pool of VE-cadherin remained labeled with BV6, but the majority of this internalized cadherin failed to label with C19 (Figure 1, C, bottom, and D). Similar results were obtained in COS7 cells exogenously expressing VE-cadherin labeled with a carboxy-terminal red fluorescent protein (RFP) tag (Supplemental Figure S2). These data were further confirmed by the absence of β-catenin colocalization with internalized pools of VE-cadherin. Whereas β-catenin exhibited high levels of colocalization with the VE-cadherin extracellular domain at cell–cell borders, very little colocalization was observed at endosomes (Figure 2). Collectively these data indicate that the carboxy-terminal tail, including the β-catenin–binding domain of VE-cadherin, is removed during endocytosis or subsequent trafficking of the cadherin through the endosomal system.

Endocytosis is required for VE-cadherin cleavage

To determine whether endocytosis is required for VE-cadherin processing, we used several approaches to inhibit VE-cadherin internalization from the plasma membrane. First, we used a VE-cadherin endo cytotic mutant in which the DEE amino acid residues within the juxtamembrane domain were mutated to alanines. Mutation of the juxtamembrane domain (VE-cad DEE mutant) exhibited dramatically reduced fragmentation of VE-cadherin. To determine whether endocytosis is required for VE-cadherin cleavage, we used several approaches to inhibit VE-cadherin internalization from the plasma membrane. First, we used a VE-cadherin endocytotic mutant in which the DEE amino acid residues within the juxtamembrane domain were mutated to alanines. Mutation of the juxtamembrane domain (VE-cad DEE mutant) exhibited dramatically reduced fragmentation of VE-cadherin.

![FIGURE 1: VE-cadherin is cleaved during endocytosis.](image)
VE-cadherin is proteolytically processed in clathrin-enriched domains, and the cleavage is completed before VE-cadherin reaches the early endosome.

The catenin-binding domain regulates VE-cadherin turnover rates

Previous studies suggested that the catenin-binding domain of classical cadherins regulates cadherin transport to the plasma membrane (Chen et al., 1999). Therefore we hypothesized that VE-cadherin cleavage, which was shown here (Figures 1 and 2) and previously to remove the catenin-binding domain (Xiao et al., 2003b), alters the trafficking dynamics of VE-cadherin and leads to cadherin degradation instead of recycling. To determine how loss of the catenin-binding domain alters the trafficking dynamics of cell surface cadherin, we examined the turnover rate of VE-cadherin and a VE-cadherin truncation mutant lacking the catenin-binding domain (VE-cadherin ΔCBD; Figure 5A). The VE-cadherin ΔCBD comigrates with the VE-cadherin cleavage fragment observed in chloroquine-treated cells (Xiao et al., 2003b) and therefore was used to mimic the N-terminal cleaved fragment of VE-cadherin. Cell surface biotinylation and pulse-chase analysis were used to monitor VE-cadherin turnover rates. Of interest, deletion of the catenin binding reduced the half-life of cell surface VE-cadherin from around 7.5 to ∼3.8 h (Figure 5, B and C). The rate constant, k, of VE-cadherin turnover increased from −0.09 (wild type) to −0.18 (ΔCBD).

To determine whether the increased rates of cadherin turnover were due to increased endocytosis or alterations in postendocytic trafficking of the cadherin, we measured endocytosis rates and the subcellular localization of internalized full-length VE-cadherin and the ΔCBD mutant cadherin. To avoid misinterpretation due to the cleavage of full-length VE-cadherin, we used another VE-cadherin extracellular domain antibody (Cad-5, 610252; BD TransLab) in combination with an antibody to visualize the total pool of VE-cadherin. We were unable to detect any significant difference in endocytosis rates when we compared full-length VE-cadherin to VE-cadherin ΔCBD (Figure 6, A and B). However, using both transferrin (Figure 6, C and D) and Rab11 (Figure 6, E and F) as markers for recycling compartments (Lock and Stow, 2005; Mayle et al., 2012; Yan et al., 2016), we observed significantly less colocalization of internalized ΔCBD VE-cadherin with recycling markers than we did for the full-length cadherin. On the other hand, internalized VE-cadherin ΔCBD exhibited more colocalization with the lysosomal marker CD63 than the full-length VE-cadherin (Figure 6, G and I). Together our data suggest that loss of the catenin-binding domain alters the VE-cadherin postendocytic trafficking itinerary, resulting in a higher turnover rate due to less recycling and more degradation.

VE-cadherin is cleaved by calpain

Several previous studies showed that calpain cleaves the cytoplasmic domain of cadherins (Jang et al., 2009; Miyazaki et al., 2011; Ye et al., 2013; Kudo-Sakamoto et al., 2014; Trillsch et al., 2016). To determine whether calpain activity is required for VE-cadherin overexpression system was complemented by a loss-of-function approach in which VE-cadherin fragmentation was monitored in a p120-null background in which cadherin internalization rates are increased (Oas et al., 2010). p120-null endothelial cells (Oas et al., 2010) were treated with chloroquine overnight, and VE-cadherin fragmentation was monitored by Western blot analysis. Increased VE-cadherin fragmentation was observed in p120-null cells compared to controls (Figure 3, E and F). Collectively these observations indicate that endocytosis is required for VE-cadherin cleavage and removal of the catenin-binding domain.

VE-cadherin is cleaved at the plasma membrane upon entry into clathrin-enriched domains

To determine the subcellular location and endocytic step in which the cadherin cytoplasmic tail is processed, we performed three-channel colocalization analysis for the VE-cadherin extracellular domain, the VE-cadherin carboxyl-terminal domain, and various endocytic markers. For these experiments, we expressed VE-cadherin with a C-terminal RFP tag and used the BV6 antibody against the cadherin extracellular domain as illustrated in Figure 4A. Cell surface cadherin was labeled with BV6 at 4°C, followed by incubation at 37°C for various amounts of time, depending on the marker (5 min for clathrin and 30 min for EEA1). As expected, BV6 and the carboxyl-terminal RFP tag exhibited extensive colocalization at cell–cell junctions, indicating the presence of intact, full-length cadherin. However, colocalization is reduced as the cadherin enters clathrin-enriched membrane domains (Figure 4B) and is further reduced by the time the cadherin enters early endosomes (Figure 4C). These findings, together with the data demonstrating that endocytosis is required for cleavage (Figure 3), suggest that inhibited VE-cadherin fragment formation (Figure 3, C and D).
Proteolytic processing of VE-cadherin

DISCUSSION

The results of this study identify a proteolytic processing event that occurs during endocytosis of VE-cadherin. Our findings suggest that VE-cadherin is cleaved by calpain upon entry into clathrin-enriched membrane domains during endocytosis and that this cleavage event removes the β-catenin–binding domain of the cadherin. This cleavage event appears to fate the cadherin for degradation rather than recycling, suggesting that the calpain-mediated cleavage of VE-cadherin influences the trafficking itinerary of the cadherin after endocytosis. These findings reveal a novel means by which cadherin endocytosis and recycling are regulated.

A number of studies have demonstrated that cadherins are targeted by extracellular and cytoplasmic proteases, including metalloproteinases, elastase, and cathepsin G (Dejana et al., 2008; Dreymueller et al., 2012). One study suggested that the VE-cadherin cytoplasmic tail is cleaved by m-calpain in response to inflammatory mediators during atherosclerosis (Miyazaki et al., 2011). In the present study, we found that VE-cadherin is cleaved by calpain during endocytosis. This interpretation is based on the fact that inhibiting endocytosis prevents cleavage (Figure 2) and on imaging data suggesting that the cadherin tail is removed as cell surface cadherin is recruited into clathrin-enriched membrane domains (Figure 4). This is also supported by our data showing that the VE-cadherin DEE mutant, which fails to enter clathrin-enriched domains (Nanes et al., 2012), does not get cleaved (Figure 3). Of interest, binding to phospholipids at the plasma membrane is known to facilitate calpain activation (Kuboki et al., 1992; Tompa et al., 2001; Shao et al., 2006), and calpain is a component of clathrin-coated vesicles (Sato et al., 1995). These findings suggest that calpain may cleave the cadherin upon recruitment into clathrin-coated pits and/or early in endocytic trafficking and before the clathrin coat dissociates from endocytic vesicles. Additional studies will be needed to define with higher spatial and temporal resolution precisely where and when the cadherin tail is cleaved by calpain.

The observation that VE-cadherin is cleaved into only two fragments is consistent with the fact that calpain only partially digests processing and the removal of the catenin-binding domain, we pretreated cells with the calpain inhibitor Calpeptin and then analyzed colocalization between the cadherin extracellular domain and carboxyl-terminal tail after a 30-min internalization period. Immunofluorescence data show that Calpeptin treatment reduced the loss of the cadherin tail during endocytosis (Figure 7, A and B). Finally, we directly tested whether VE-cadherin can be cleaved by calpain in vitro. Cell lysates from adenovirus-infected COS7 cells expressing VE-cadherin-RFP were treated with purified active calpain 1 large domain.
Calpain has been shown to cleave a variety of classical cadherins (Jang et al., 2009; Ye et al., 2013; Kudo-Sakamoto et al., 2014; Trillsch et al., 2016), suggesting that the mechanism of cadherin regulation reported here may apply to other cadherins in a variety of cell types. For example, it has been shown that E-cadherin cleavage by calpain is involved in tumor progression (Ye et al., 2013; Trillsch et al., 2016) and that calpain cleavage of N-cadherin reduces cell–cell adhesion (Jang et al., 2009; Kudo-Sakamoto et al., 2014). VE-cadherin cleavage by calpain has also been shown to result in disorganization of adherens junctions and hyperpermeability of vascular endothelial cells (Miyazaki et al., 2011). It is possible that the alteration in cadherin adhesive function observed in these other model systems and cell types could reflect an underlying mechanism of calpain-mediated regulation of adherens junctions through altered cadherin trafficking. Calpain is a calcium-dependent protease that is activated by intracellular calcium (Campbell and Davies, 2012; Ono and Sorimachi, 2012). Thus increases in intracellular calcium downstream of inflammatory or angiogenic signaling could result in increased calpain activity and altered endothelial cell–cell

**FIGURE 4:** VE-cadherin cleavage occurs after VE-cadherin enters clathrin-enriched membrane domains but before it reaches the early endosome. (A) Where VE-cadherin cleavage may occur during endocytosis and the labeling approach used for experiments in B and C. (B) Adenovirus-infected COS7 cells expressing VE-cadherin–RFP were labeled with BV6 and then warmed to 37°C for 5 min to visualize surface pools and very early endocytic events. Three-channel colocalization reveals that cleavage occurs while VE-cadherin enters clathrin-enriched domains. Scale bar, 20 μm. (C) COS7 cells were warmed to 37°C for 30 min to induce endocytosis, followed by acid wash to remove cell surface antibodies. Three-channel colocalization reveals cleavage occurs before VE-cadherin reaches the early endosome. Scale bar, 20 μm. (D) Quantification of the results in B and C. Border regions of COS7 cells expressing VE-cadherin–RFP without acid wash were used as positive controls. Colocalization analysis between BV6 and clathrin or BV6 and EEA1 was used to identify cadherin pools at different steps of endocytosis. Colocalization analysis between RFP and VE-cadherin in clathrin-enriched domains or early endosomes was then performed to reveal whether the VE-cadherin carboxyl-terminal domain was present at these endocytic steps. Data are presented as means ± SEM; ≥16 cells in each group. Each condition was conducted in triplicate and represents data from three independent experiments. **p < 0.01, two-tailed t test.

its substrates and is therefore considered to be a regulatory protease (Goll et al., 2003; Ono and Sorimachi, 2012). The VE-cadherin ΔCBD polypeptide, which mimics the N-terminal fragment of VE-cadherin after cleavage (Xiao et al., 2003b), exhibits decreased colocalization with recycling markers and increased colocalization with lysosomal markers compared to full-length VE-cadherin (Figure 6). These findings suggest that the cleavage of VE-cadherin in the cytoplasmic domain results in an altered trafficking and sorting itinerary of VE-cadherin. Thus calpain cleavage appears to be a fate-determining step during VE-cadherin endocytic processing. Previous studies showed that β-catenin facilitates E-cadherin transport to the plasma membrane (Chen et al., 1999). Therefore it is likely that decreased VE-cadherin recycling after cleavage (Figure 6) is due to the removal of the β-catenin–binding domain of VE-cadherin. This altered trafficking dynamics is likely to affect VE-cadherin cell surface levels. Thus calpain activity could be a potential regulatory target for modulating VE-cadherin surface levels during a variety of pathophysiological circumstances.
adhesion. In fact, increases in the intracellular calcium levels have been shown to disrupt endothelial adherens junction (Sandoval et al., 2001; Komarova et al., 2012). Although the exact mechanism of how the adherens junction is disrupted in response to increased intracellular calcium levels is not clear, our findings provide evidence that supports calpain activation as an intermediate step during the disassembly of the adherens junction in response to increased intracellular calcium.

The work presented here and in our previous studies indicates that VE-cadherin is cleaved between the juxtamembrane and the catenin-binding domain (Xiao et al., 2003b). It has also been shown that calpain cleaves E-cadherin and N-cadherin in a similar region and generates similar-sized fragments (~100 kDa; Jang et al., 2009; Ye et al., 2013; Kudo-Sakamoto et al., 2014; Trillsch et al., 2016). Calpain is known to act based on protein tertiary structures instead of specific amino acid sequences and exhibits a preference for interdomain unstructured regions (Stabach et al., 1997; Goll et al., 2003; Ono and Sorimachi, 2012). Based on structural studies of the E-cadherin cytoplasmic domain (Ishiyama et al., 2010), there is a flexible linker region between the juxtamembrane and the catenin-binding domain that is a likely target for calpain cleavage (Figure 8A). A previous study indicated that five amino acids within this linker region in VE-cadherin are required for m-calpain cleavage (Miyazaki et al., 2011). To determine whether this region was also involved in VE-cadherin processing during endocytosis, we generated a similar VE-cadherin cytoplasmic domain (Ishiyama et al., 2011). The African green monkey kidney fibroblast-like (COS7; American Type Culture Collection, Manassas, VA) and HEK QBI-293A cell lines were cultured in DMEM (10-013-CV; Corning, Corning, NY) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (30-004-CI; Corning). Primary mouse endothelial cells were cultured as described previously (Oas et al., 2010).

**FIGURE 5:** Deletion of the VE-cadherin catenin-binding domain increases VE-cadherin turnover rates. (A) VE-cadherin ΔCBD mimics the cleaved fragment of VE-cad. (B) Surface biotinylation reveals faster turnover of VE-cadherin ΔCBD than for full-length VE-cadherin. COS7 cells were transfected with indicated constructs. (C) Quantification of the results in B. Data are presented as means ± SEM. The half-life of each cadherin is labeled with red dotted lines. Rate constant, $k$, on the right in red. Each condition was conducted in triplicate and represents data from three independent experiments.

**MATERIALS AND METHODS**

**Cell culture**

Human dermal microvascular endothelial cells (MECs) were cultured in endothelial growth medium 2 (CC-3159; Clonetics, Walkersville, MD) supplemented with EGM-2 MV SingleQuots (CC-4147; Clonetics) on gelatin-coated plates. The African green monkey kidney fibroblast-like (COS7; American Type Culture Collection, Manassas, VA) and HEK QBI-293A cell lines (MP Biomedicals, Santa Ana, CA) were cultured in DMEM (10-013-CV; Corning, Corning, NY) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (30-004-CI; Corning). Primary mouse endothelial cells were cultured as described previously (Oas et al., 2010).

**Virus production**

To generate adenovirus for protein expression in mammalian cells, the gene of interest was cloned into the gateway pAd/CMV/VS-DEST vector (V49320; Invitrogen, Carlsbad, CA). The vector was linearized using PacI and transfected into HEK QBI-293A cells using TransFectin (1703350; Bio-Rad, Hercules, CA) to produce virus. After a second round of infection, cells were lysed and virus was harvested.

**Internalization assay and image analysis**

Cells were infected with adenovirus or transfected with plasmid expressing the protein of interest 48 hours before the experiment. Transfection was conducted using Lipofectamine 2000 (11668-019; ThermoFisher, Hudson, NH) or 3000 (L3000008; ThermoFisher) according to the protocol provided by the manufacturer. Internalization assays were performed as described previously (Xiao et al., 2003a; Chiasson et al., 2009; Cadwell et al., 2016a). Briefly, cultured cells on glass coverslips were incubated with an antibody against the VE-cadherin extracellular domain (BV6; MABT134; Millipore, Billerica, MA) in cell culture medium for 30 min at 4°C. Cells were washed three times with cold phosphate-buffered saline (PBS) containing calcium and magnesium to remove unbound antibody. To allow internalization, cells were incubated in prewarmed medium at 37°C. Cells were then returned to cold medium. A low-pH buffer (100 mM glycine, 20mM magnesium acetate, and 50 mM potassium chloride, pH 2.2) was used to remove any remaining antibody from the cell surface. Cells were then fixed and permeabilized by incubation in 4% paraformaldehyde for 10 min, followed by 0.1% Triton X-100 for 10 min at room temperature. Goat anti–VE-cadherin antibody (C19; sc-6458; Santa Cruz Biotechnology, Dallas, TX) or C-terminal RFP tag was used to visualize the C-terminal domain of VE-cadherin. Mouse anti–VE-cadherin (Cad-5, 610252; BD TransLab, San Jose, CA), an alternative antibody that recognizes the extracellular domain of VE-cadherin, was used to determine the total amount of VE-cadherin. Additional primary antibodies used included mouse anti–clathrin heavy chain (610500; BD TransLab), mouse anti-EEA1 (610457; BD TransLab), and streptavidin pull-down (Supplemental Figure S3). However, biochemical assays indicated that calpain-1 could indeed cleave this VE-cadherin polypeptide in vitro (unpublished data). Although further studies will be needed to map the precise site of calpain cleavage, these studies suggest that the linker sequence between the juxtamembrane and the catenin-binding domain is a potentially important regulatory site that is subject to cleavage by calpain during endocytosis. Finally, additional studies are needed to determine the fate of both β-catenin and the carboxyl-terminal VE-cadherin fragment after cleavage, since these polypeptides may exhibit biological activity upon release from the cadherin during endocytosis.
mouse anti-p120 (610134; BD TransLab), rabbit anti–β-catenin (C2206; Sigma-Aldrich, St. Louis, MO), rabbit anti-Rab11 (71-3500; Invitrogen), and mouse anti-CD63 (H5C6; lowLabs). Secondary antibodies conjugated to fluorescent dyes (Alexa Fluor 488, 555, or 647 nm; Life Technologies, Carlsbad, CA) were used to visualize antibody binding.

To quantify cleavage, colocalization between the signals from endocytosed VE-cadherin (labeled at the extracellular domain) and C-terminal end of VE-cadherin (labeled at the C-terminal domain) were quantified using the Manders correlation coefficients:

\[ M_R = \frac{\sum R_i,\text{colocal}}{\sum R_i}, \quad M_G = \frac{\sum G_i,\text{colocal}}{\sum G_i} \]

where \( R_i \) denotes the red signal, \( R_i,\text{colocal} \) the red signal that colocalizes with the green signal, \( G_i \) the green signal, and \( G_i,\text{colocal} \) the green signal that colocalizes with the red signal. The Coloc 2 program from ImageJ/Fiji was used for quantification.

To quantify internalization, signals from endocytosed VE-cadherin were divided by signals from the total pool of VE-cadherin labeled with Cad-5, an alternative extracellular domain antibody.

Microscopy was performed using an epifluorescence microscope (DMRXA2; Leica, Buffalo Grove, IL) equipped with a 63× oil immersion objective with apochromatic aberration and flat-field corrections, narrow-bandpass filters, and a digital camera (ORCA-ER C4742-80; Hamamatsu Photonics, Hamamatsu City, Japan). Images were captured using Simple PCI software (Hamamatsu Photonics). Quantification was done in ImageJ/Fiji. Statistical analysis was performed in RStudio.

**Western blot analysis**

Samples were prepared by either scraping cells into Laemmli Sample Buffer (1610737; Bio-Rad) with 5% β-mercaptoethanol (β-ME) or adding the Laemmli Sample Buffer with 5% β-ME directly into the reaction mixture. Samples were then heated at 95°C for 5 min before SDS–PAGE and analyzed by immunoblotting on nitrocellulose membranes (Whatman, Maidstone, United Kingdom). Quantification was done using the gel analysis program in ImageJ/Fiji, and statistical analysis was performed in RStudio. Primary antibodies used were mouse anti–VE-cadherin (Cad-5, 610252; BD TransLab), mouse anti–VE-cadherin (BV6, MABT134; Millipore), rat anti–mouse VE-cadherin (E028599; eBioscience, San Diego, CA), rat anti–mouse VE-cadherin (550548; BD Pharmingen, San Jose, CA), and rabbit anti-p120 (sc-1101; Bio-Rad).
Secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) and ECL Western blot detection reagents (RPN2106; GE Healthcare, Chicago, IL) were used to visualize proteins by Western blot.

Surface biotinylation pulse-chase assay and VE-cadherin half-life determination
COS7 cells were transfected with the plasmid of interest 48 h before the experiment. Cells were pulse-labeled with biotin (21331; ThermoFisher) on ice for 30 min. Excess biotin was quenched by 50 mM ammonium chloride. Cells were then incubated at 37°C for various amounts of time, returned to ice, and then lysed with 1% Triton X-100 in PBS with calcium and magnesium for 30 min. Cell lysates were centrifuged at 13,200 rpm for 30 min at 4°C. The supernatants were added to streptavidin beads (20349; ThermoFisher) and incubated for 1 h at 4°C. Beads were washed four times using ice-cold PBS with 0.1% Tween-20. Biotinylated protein was eluted using Laemmli Sample Buffer with 5% β-ME at 95°C for 5 min, followed by Western blot analysis.

The following equation was used calculate VE-cadherin half-life:

\[ t_{1/2} = \frac{t}{\ln 2 \left( \frac{N_t}{N_0} \right) } \]

with the rate constant

\[ k = \frac{\ln \left( \frac{N_0}{N_t} \right) }{t} \]

where \( t_{1/2} \) is the half-life, \( t \) is the given time, \( N_t \) is the amount of protein at the given time, and \( N_0 \) is the amount of protein at time 0.

In vitro calpain digestion
COS7 cells were infected with adenovirus expressing VE-cadherin RFP 48 h before the experiment. Cells were lysed in M-PER lysis buffer (78501; ThermoFisher) supplemented with 1% protease inhibitors (P8849; Sigma-Aldrich) for 10 min on ice. We added 1 mM CaCl\(_2\), 1 U of active calpain 1 large subunit (C6108; Sigma-Aldrich), 100 μM Calpeptin (03-3-0051; Millipore), and 250 μM ALLM (CAS 136632-32-1; Santa Cruz Biotechnology) separately to the cell lysates. The reaction mixture was incubated at room temperature for 30 min. The reaction was then terminated by adding Laemmli Sample Buffer with 5% β-ME to the mixture, followed by Western blot analysis.

Santa Cruz Biotechnology).
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