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Dynamic regulation of the endothelial adherens junction

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Abstract

Dynamic regulation of the endothelial adherens junction

By Benjamin A. Nanes

Endothelial cells, which line the interior surfaces of blood vessels, must join together to form barriers that are stable enough to resist vascular leak, yet also flexible enough to support the dynamic rearrangements necessary for vascular development and function. This dissertation explores the basic cellular mechanisms allowing endothelial cells to establish cell–cell contacts that properly balance stability and plasticity. Endothelial cell–cell adhesion is mediated through the adherens junction complex, and the strength of these junctions depends on the balance of trafficking of adhesion molecules to and from the membrane.

Vascular endothelial (VE)-cadherin, the principal intercellular adhesion molecule in the endothelium, undergoes constitutive endocytosis and recycling, conferring plasticity to cell–cell junctions. This dissertation identifies a dual-function motif in the VE-cadherin cytoplasmic tail as the key mediator of constitutive VE-cadherin endocytosis. The motif alternately serves as a binding site for p120-catenin (p120), stabilizing the cadherin, or as an endocytic signal, driving internalization of the cadherin. This mechanism allows constitutive VE-cadherin endocytosis to contribute to adherens junction dynamics without causing junction disassembly. Mutation of the constitutive endocytic motif results in a cadherin variant that is both p120-uncoupled and resistant to endocytosis. This mutant potently suppresses a key component of angiogenesis, the collective migration of endothelial cells in response to vascular endothelial growth factor, revealing the importance of constitutive VE-cadherin endocytosis to endothelial function.

While constitutive endocytosis of VE-cadherin is required for junction plasticity, inappropriate loss of endothelial adhesion contributes to disease. This dissertation also identifies a distinct motif that drives induced VE-cadherin endocytosis and pathological junction disassembly associated with the endothelial-derived tumor Kaposi sarcoma. Human herpesvirus 8, which causes Kaposi sarcoma, expresses a ubiquitin ligase, K5, which targets VE-cadherin for ubiquitination at sites within the p120-binding region. K5-mediated ubiquitination of VE-cadherin displaces p120 and drives endocytosis and down-regulation of the cadherin. However, K5-induced VE-cadherin endocytosis does not require the constitutive endocytic motif. Thus, multiple context-dependent signals drive VE-cadherin endocytosis in physiologic and disease states, but p120 binding to the cadherin acts as a master regulator guarding cadherin stability.

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Chapter 1

Finding the balance between stability and plasticity

1.1 Introduction

Assembled end to end, the blood vessels and capillaries of the human vascular system would stretch some 60,000 miles (Jones, 1969). These vessels form an intricate tree, precisely patterned to deliver nutrients and remove waste from every tissue in the body. More remarkable still, the vascular tree responds dynamically to physiologic events, sprouting new vessels in response to hypoxia and delivering immune cells to sites of infection. Many different tissues and cell types combine to form the complete vascular system, but all vessels, large and small, are lined with a single layer of thin cells called the endothelium. These endothelial cells define the barrier between the vasculature and the surround tissues, ensuring a closed system in which blood can flow, while simultaneously allowing access to the tissues which the vascular system must supply. Thus, endothelial cells must join together to form a barrier that is stable enough to resist vascular leak, yet also flexible enough to support appropriate transfer of cells and nutrients to and from the vasculature, as well as the dynamic rearrangements necessary for vessel development and function.

This dissertation addresses several fundamental questions in mammalian biology. How do endothelial cells establish cell–cell contacts which appropriately balance stability and flexibility? How do these contacts respond to physiologic events requiring dynamic changes in the vasculature? And, how are these processes disrupted in disease? In order to address these basic questions, this dissertation focuses on the cellular mechanisms respon-

sible for mediating endothelial cell–cell adhesion, and in particular on the principal adhesion molecule in endothelial cells, vascular endothelial (VE)-cadherin. Modulation of the amount of cadherin available at cell–cell junctions to form adhesive contacts can be an important mechanism for regulating the strength and stability of cell–cell adhesion, and one way to control the amount of cadherin available at the cell surface is through the balance of trafficking of cadherin to and from the membrane. Internalization of cadherin from the cell surface by endocytosis can rapidly deplete the pool of cadherin available to mediate adhesion or, if combined with recycling of the cadherin back to the cell surface, confer plasticity to the cell–cell junction.

This chapter discusses the dynamic nature of the endothelium. It considers the dramatic rearrangements undergone by the endothelium during vascular development (Section 1.2), the signaling pathways guiding that development (Section 1.3), the cellular components which mediate endothelial cell–cell adhesion (Section 1.4), how those components respond to dynamic signals (Section 1.5), and how disrupted regulation of endothelial cell–cell adhesion contributes to disease (Section 1.6).

Chapter 2 looks more broadly at the role of endocytosis in the regulation of cadherin-based adhesive interactions, both in the endothelium and in other tissues. It considers the role of cadherin endocytosis in development and disease (Section 2.2), the major trafficking pathways used to transport cadherins (Section 2.3), and the factors regulating those trafficking pathways, including cadherin-binding proteins (Section 2.4), post-translational modification of cadherins by ubiquitination (Section 2.5), growth factor signaling pathways (Section 2.6), and proteolysis of cadherins (Section 2.7).

Chapter 3 presents original research identifying an important mechanism regulating VE-cadherin endocytosis. This mechanism relies on a novel dual-function motif in the VE-cadherin cytoplasmic tail. The motif alternately serves as a binding site for p120-catenin, which stabilizes the cadherin, or as an endocytic signal, which drives removal of VE-cadherin from the cell surface by internalization. Furthermore, constitutive endocytosis of VE-cadherin through this pathway is required for endothelial cell migration, an important component of angiogenesis.

Chapter 4 presents further work determining how normal cellular regulation of VE-

cadherin endocytosis is disrupted in Kaposi Sarcoma, an endothelial-derived tumor characterized by abnormal, leaky vasculature. A ubiquitin ligase expressed by the herpesevirus that causes Kaposi Sarcoma targets VE-cadherin for ubiquitination, disrupts the binding of p120-catenin, and triggers VE-cadherin endocytosis and down-regulation.

Finally, Chapter 5 reviews the conclusions of these studies, and discusses some of the remaining questions limiting our understanding the dynamic regulation of endothelial cell–cell adhesion.

1.2 Development of the vascular system

Perhaps the most dramatic illustration of the dynamic nature of endothelial adhesion comes from the development of the vascular system itself. Once the embryo grows beyond a few layers of cells, areas that would otherwise become inaccessible through diffusion rely on the vascular system for the supply of nutrients and the removal of waste. The vascular system must therefore become functional very early in development. In fact, beating of the cardiac tube in the chick embryo is visible by light microscopy at the ten-somite stage (Sabin, 1920). Because the vasculature begins to form so early in development, *in situ* assembly of the mature vascular tree is not possible. Instead, vascular development occurs through a series of dynamic and highly coordinated steps, beginning with the formation of a primitive vascular plexus and continuing through progressive stages of growth and remodeling into a mature vascular system as the organism develops.

1.2.1 Vasculogenesis

Vasculogenesis, the initial assembly of a primitive vascular plexus, begins soon after gastrulation. Mesoderm-derived vascular progenitor cells, originally named angioblasts, aggregate along the endodermal surface, both within the embryo and along the yolk sac (His, 1900). These aggregations, called blood islands in the yolk sac, organize into lumenal structures that join together to form the vascular plexus. Blood islands also generate the first erythrocytes to populate the vascular system. Studies of the chick embryo determined that these early blood cells share a common origin with the cells lining the developing vascu-

lar plexus, and that angioblasts are more properly named hemangioblasts (Murray, 1932; Sabin, 1917). As it forms, the plexus undergoes continuous remodeling. Thoma reported the first observations of vascular remodeling in the yolk sac of the chick embryo, and proposed that differences in blood flow, pressure, and tension promoted the growth of certain vessels over others (1893).[1] In the chick embryo itself, Evans used dye injections to visualize the early vascular plexus, and found that even the largest vessels in the embryo, the dorsal arotae and cardinal veins, arose through plexus remodeling, rather than simple extension at the vessel ends (1909). The development of electron microscopy in the latter half of the twentieth century confirmed these findings, and corrected a few misconceptions. For example, while the same hemangioblasts do give rise to both the lining and contents of the yolk sac blood islands, their lumens are formed not by "liquefaction degeneration of central cells," but by cellular rearrangements and the secretion of proteinaceous fluid (Gonzalez-Crussi, 1971).

1.2.2 Angiogenesis

After the primitive vascular plexus forms, repeated sprouting and pruning of vessels develops the mature vascular tree. Angiogenesis, the formation of a new blood vessel by sprouting from an existing vessel, plays a key role in vascular development. In a remarkable observational study using nothing more than transmitted-light microscopy with a 100 Watt "frosted-glass bulb" for illumination, Clark and Clark tracked vessel formation by angiogenesis in the tails of frog larvae over periods of eight days (1925). They observed not only "blood-vessel endothelium extend[ing] by the process of sprouting," but also the stabilization of new capillary loops and the recruitment of pericytes.[2] Clark and Clark noted that there was initially no blood flow in the newly-formed capillaries, but more advanced technology, *in vivo* light microscopy with correlated electron microscopy, established that angiogenic sprouts initially form without a lumen (Cliff, 1963).[3] Electron microscopy also

[1] In an early attempt to test Thoma's hypothesis, Chapman dissected hearts from chick embryos (1918). Removal of the embryonic heart limited vascular development, providing early support for the "histo-mechanical laws."

[2] Pericytes were first described by Rouget (1873), but the name "pericyte" is credited to Zimmermann (1923).

[3] The advent of photographic technology also facilitated a change in the customary observation period from twelve hours of careful drawing each day to single observations every other day, and every third day over weekends (Clark and Clark, 1925; Cliff, 1963).

revealed fine structural details of angiogenic sprouts, including "psuedopodia-like cytoplasmic protrusions" at the tips (Bär and Wolff, 1972). Of particular note for this discussion of endothelial dynamics, endothelial cells within the sprouts sometimes appeared to "slide past one another," and the "junctions between cells were frequently loosened" (Ausprunk and Folkman, 1976). Even as seen with the limited resolution of simple light microscopes or through the static view of electron microscopy, the dynamic nature of endothelial cells during vascular development was apparent.

1.3 Signals guiding vascular patterning

1.3.1 Inducing permeability and driving growth

An in-depth understanding of endothelial cell dynamics requires not only intricate observations of sprouting angiogenesis and vessel stabilization, but also consideration of the molecular mechanisms guiding vessel development and the machinery linking endothelial cells together. In 1983, Senger *et al.* purified a substance from both the ascites fluid and culture supernatant of a guinea pig hepatocellular carcinoma which rapidly induced vascular permeability. They named the 34–42 kD protein "line 10 permeability factor." Further investigation found several human tumor cell lines which secreted remarkably similar "vascular permeability factors" (Senger et al., 1986). All of the factors could be isolated using similar purification schemes, and antibodies raised against the guinea pig factor successfully neutralized the permeability-inducing activity of the human tumor factors. Notably, vascular permeability induced by the factors was rapidly reversible and did not prompt an inflammatory response, hinting at the potential role of this factor as a key regulator of endothelial cell dynamics (Senger et al., 1983, 1986).

Senger *et al.*'s vascular permeability factor was ultimately isolated from non-diseased organisms as well, and renamed vascular endothelial growth factor (VEGF) in recognition of its ability to induce angiogenic sprouting in addition to permeability (Ferrara and Henzel, 1989). In fact, VEGF plays a critical role in vascular development. Inactivation of even a single VEGF allele is lethal. Mice heterozygous for VEGF die between embryonic days ten and eleven with marked vascular defects (Carmeliet et al., 1996; Ferrara

et al., 1996). Vascular development is guided not only by the genetic does of VEGF, but also by the precise distribution of VEGF concentration gradients in tissues. Transgenic mouse models demonstrated that VEGF gradients, and in particular concentration of the heparin-binding VEGF-A isoform, guide vessel branching and angiogenic sprouting (Ruhrberg et al., 2002). In mice engineered with a VEGF-A mutant defective in heparin binding, VEGF gradients in developing embryos were altered. As a result, developing vessels tended not to sprout new vessels, but to simply increase in diameter.

Additional work determined that VEGF induced two coordinated phenotypes in the endothelial cells of angiogenic sprouts. Cells at the tip of the sprout developed filopodial extensions and migrated in the direction of the VEGF gradient, while endothelial cells in the stalk began to proliferate and follow the tip cell (Gerhardt et al., 2003). This pattern fit well with earlier studies of an inflammation-induced vascular sprouting model, which observed angiogenic sprouts initiating through endothelial cell migration, with endothelial proliferation occurring later (Sholley et al., 1984). That the same growth factor can both direct endothelial cell migration and induce vascular permeability underscores the importance of balance in endothelial cell–cell adhesion. Loosening cell–cell contacts facilitates migration, a key component of angiogenesis, but it can also lead to vascular leak, potentially harmful if uncontrolled.

1.3.2 Receptors tune the growth factor response

Not surprisingly for a signaling molecule with such an important role in vascular patterning, expression of several different receptors can tune the endothelial cell response to VEGF. The first two VEGF receptors, VEGF receptor 1 (VEGFR-1; also known as fms-related tyrosine kinase 1, Flt-1) and VEGF receptor 2 (VEGFR-2; also known as fetal liver kinase 1, Flk-1; and kinase insert domain receptor, KDR), were originally identified based on their sequence homology to other receptor tyrosine kinases, and retain alternate names based on those protein families (de Vries et al., 1992; Matthews et al., 1991). The first hint that these receptors might play a role in vascular development came from *Xenopus laevis* oocytes expressing VEGFR-1, which released calcium in response to VEGF treatment (de Vries et al., 1992). Deletion of either VEGFR-1 or VEGFR-2 in mouse models caused lethal vascular

defects as early as embryonic day nine (Fong et al., 1995; Shalaby et al., 1995). However, each deletion caused specific effects with subtle differences.

Deletion of VEGFR-2 inhibited angioblast differentiation and the formation of yolk sac blood islands (Shalaby et al., 1995). In contrast, deletion of VEGFR-1 did not prevent endothelial cell differentiation, but the endothelial cells formed abnormal vascular channels (Fong et al., 1995). Furthermore, examination of embryonic stem cell cultures found that VEGFR-1 loss induced endothelial cell hyperproliferation, suggesting that VEGFR-1 and VEGFR-2 might mediate opposing responses to VEGF signaling (Kearney et al., 2002). The earlier identification of a soluble VEGFR-1 isoform lacking kinase activity, proposed to function as a VEGF sink to dampen endothelial activation by signaling through VEGFR-2, further supported this model (Kendall and Thomas, 1993). Soluble VEGFR-1 is now understood to be an important modulator of local VEGF concentrations, with an essential influence on vessel patterning (Chappell et al., 2009; Kappas et al., 2008).

A third receptor, VEGF receptor 3 (VEGFR-3; also known as fms-related tyrosine kinase 4, Flt-4), serves a less clearly established role. VEGFR-3 is expressed by endothelial cells during development, but becomes restricted to the lymphatic system in the adult. Interestingly, VEGFR-3 expression is transiently increased in angiogenic sprouts, and suppression of VEGFR-3 inhibits sprout formation (Tammela et al., 2008). The existence of three different VEGF receptors with different expression patterns and different responses to VEGF signaling highlights the intricacy of the growth factor signaling networks underlying vascular development.

1.3.3 Establishment of sprout morphology

Even with precise local concentration gradients and response tunning by three separate receptors, signaling by a single growth factor is clearly insufficient for establishing angiogenic sprout morphology. In particular, some signal must define the different phenotypes of the tip cell, which leads sprout formation through directed migration, and the stalk cells following behind to form the nascent vessel. This specification is mediated by the delta–notch signaling system.

Tip cells express delta-like ligand 4 (Dll-4), while Dll-4 expression is inhibited in stalk

cells. Notch-1 follows the opposite pattern—it is primarily expressed in stalk cells and inhibited in tip cells. Dll-4 binds to Notch-1 at the tip cell–stalk cell interface and triggers signaling cascades in both cells which reinforce their respective Dll-4 and Notch-1 expression patterns, supporting the specific tip cell or stalk cell phenotypes (Hellström et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Initial specification of tip cells is likely stochastic, but Dll-4–Notch-1 signaling establishes a positive feedback loop supporting the pattern.

Recent work has increasingly recognized the dynamic nature of tip cell and stalk cell specification. In fact, live-cell imaging has shown endothelial cells "competing" for tip cell position through changes in VEGFR-1 and VEGFR-2 expression levels (Jakobsson et al., 2010). Mathematical models of angiogenic sprouts support this concept, and further suggest that varying endothelial cell–cell adhesion strength facilitates sprout migration (Bentley et al., 2014). Additionally, the Wnt–β-catenin signaling pathway may dynamically influence Dll4 transcription (Corada et al., 2010). Thus, angiogenic sprout formation is supported both by concentration gradients of extracellular growth factors like VEGF and by endothelial cell–cell contact interactions.

1.3.4 Steering vessel growth

Once the general structure of the angiogenic sprout has been specified, a further diverse set of signals guides its extension. Interestingly, many of the signaling systems guiding vascular development strongly resemble those guiding neuronal development (reviewed and compared in Adams and Eichmann, 2010; Carmeliet and Tessier-Lavigne, 2005). These systems operate as repulsive guidance cues that steer the developing vessel.

One such system is the Roundabout (Robo) axon guidance receptor family. Most Robo family members are expressed only in neurons. However, expression of Robo-4 is endothelial-specific. Activation of Robo-4 by Slit-2 has been found to block angiogenic signaling by inhibiting VEGF-mediated Src activation (Jones et al., 2008). However, whether Slit-2 can actually bind Robo-4 remains somewhat controversial. Cell culture based assays suggest Slit-2 and Robo-4 interact (Park et al., 2003; Zhang et al., 2009), while binding affinity measurements using surface plasmon resonance and structural predictions based

on sequence differences between Robo-4 and other members of the Robo family cast doubt on the feasibility of direct binding (Morlot et al., 2007; Suchting et al., 2005). Whether or not Slit-2 binds Robo-4 directly, activation of Robo-4 has also been implicated in VEGF signaling blockade through the vascular Netrin receptor Unc5b (Koch et al., 2011; Larrivée et al., 2007).

Other repulsive guidance systems operate on nascent vessels as well. For example, semaphorin-3E can serve as a repulsive signal for endothelial cells expressing plexin-D1 (Gitler et al., 2004; Gu et al., 2005). Eph–Ephrin signaling has also been proposed as a cue for vessel guidance and artery or vein specification. *In vivo*, expression of Ephrin-B2 is specific to arteries, while expression of Eph-B4, which can bind to Ephrin-B2 on an adjacent cell, is specific to veins (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). At least in principle, Eph-B4–Ephrin-B2 signaling could create a positive-feedback loop leading to artery–vein segregation, much like how delta–Notch signaling reinforces differentiation between tip cell and stalk cell phenotypes. However, since arterial endothelial cells are not typically in direct contact with venous endothelial cells, additional signals for artery and vein specification may be required.

1.3.5 Promoting vessel stability

Once nascent vessels have been guided into their proper positions, they are stabilized by the recruitment of pericytes to line the endothelium and the deposition of basement membrane. In capillaries and small vessels, mural cells contact endothelial cells directly, although they may not form a completely enclosing sheath. In larger vessels, vascular smooth muscle cells surround the vessel, separated from the endothelium by a layer of extracellular matrix, and confer stiffness and elasticity to the vessel. Several signaling pathways support vessel stabilization and pericyte recruitment.

Platlet-derived growth factor (PDGF) B is particularly important. PDGF-null mouse embryos fail to recruit pericytes, which express PDGF receptor β (PDGF-Rβ), to developing vessels (Lindahl et al., 1997). PDGF signaling from angiogenic sprouts induces pericyte recruitment, replication, and co-migration with the sprout, and eventual stabilization of the nascent vessel (Hellström et al., 1999). Interestingly, PDGF transcription is increased

by laminar shear stress, a potential stabilizing influence in mature vessels (Resnick et al., 1993). Other growth factors which may play a role in pericyte recruitment and vessel stabilization include transforming growth factor β and fibroblast growth factor (Murakami et al., 2008; Pardali et al., 2010).

Even after new vessels have been stabilized by pericyte recruitment and matrix deposition, they often remain dependent on growth factor signaling for their continued survival. For early vessels, withdrawal of VEGF signaling leads to apoptosis of endothelial cells and vessel regression, and pruning of newly sprouted vessels likely plays as important a role in vascular patterning as angiogenesis itself (Benjamin et al., 1999). Furthermore, an additional signaling system promotes vessel maturation and stabilization. Characterization of this system began with the discovery of two novel tyrosine kinase receptors, Tie-1 and Tie-2 (also known as Tek), expressed in endothelial cells (Dumont et al., 1992; Iwama et al., 1993; Partanen et al., 1992; Sato et al., 1993). The ligands of these receptors were originally unknown. However, deletion of either gene in mouse models caused fatal defects in angiogenesis (Dumont et al., 1994; Sato et al., 1995). Identification of two Tie-2 ligands lent considerable insight to the mechanisms behind the vascular defects observed in Tie-null mice. Angiopoietin 1 (Ang-1) acts as a Tie-2 agonist (Davis et al., 1996), and angiopoietin 2 (Ang-2) acts as a Tie-2 antagonist (Maisonpierre et al., 1997). Increased expression of Ang-1 greatly reduces vascular leak in response to inflammatory signals, such as VEGF and histamine (Thurston et al., 1999). In contrast, Ang-2 inhibits periciyte recruitment in a revascularization after ischemic injury model (Reiss et al., 2007). Thus, Ang-1–Tie-2 signaling supports endothelial cell quiescence and vessel maturation. In fact, mural cell secretion of Ang-1 plays a key role in stabilizing the endothelial cells they surround (Augustin et al., 2009).

Recent work has begun to elucidate the mechanism by which Tie-2 activation promotes endothelial cell quiescence. Ang-1 binding to Tie-2 activates RhoA, which induces the association of mDia with Src, preventing VEGF-mediated Src activation and disassembly of endothelial cell–cell junctions (Gavard et al., 2008). Ultimately, VEGF signaling and Ang-1 signaling may operate as opposing influences on the same effector, the stability of endothelial cell–cell adhesion.

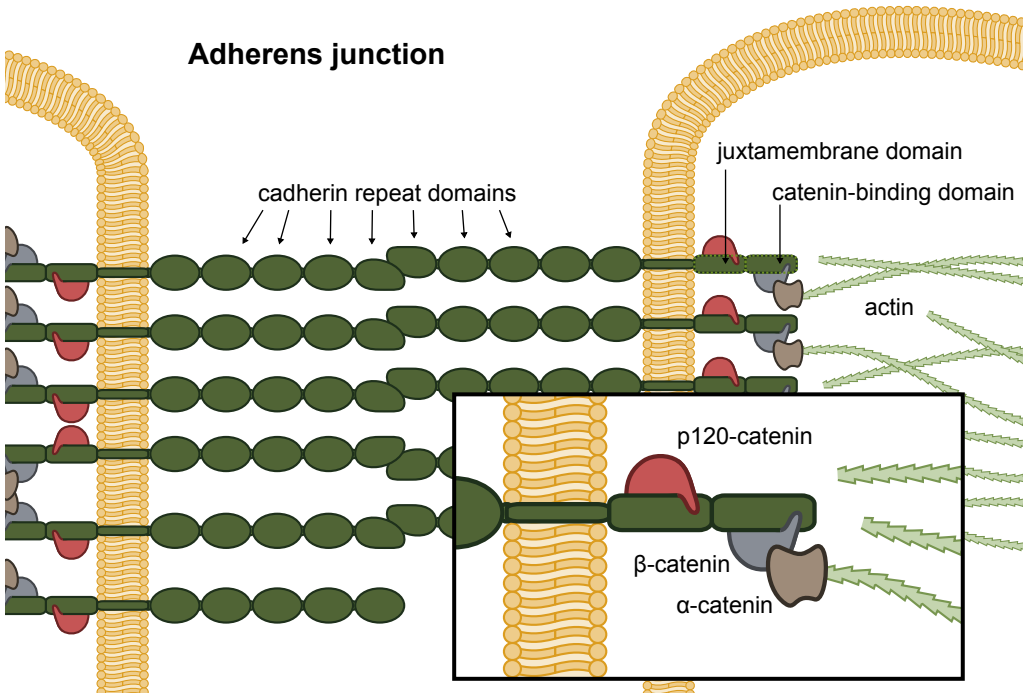

Figure 1.1: *Main components and organization of the adherens junction.*

1.4 The adherens junction

As with all tissues in multicellular organisms, the endothelium relies on intercellular junction protein complexes to link its constituent cells together. Understanding the dynamics of endothelial cell–cell adhesion requires consideration of the structures mediating that adhesion. Electron microscopy studies of columnar epithelia originally identified three distinct junctional complexes, the *zonula occludens* (tight junction), *zonula adhaerens* (adherens junction), and the *macula adhaerens* (desmosome) (Farquhar and Palade, 1963). Tight junctions form barriers between the apical and basal surfaces of the epithelium, while adherens junctions and desmosomes mediate adhesion. Adherens junctions associate with circumferential actin bundles and desmosomes associate with intermediate filaments, thus linking the cytoskeletons of adjacent cells (Hirokawa et al., 1983). Endothelial cells, which are considerably flatter than typical columnar epithelial cells, lack the classical tripartate junction organization. Instead, endothelial cell tight junction components intermingle with endothelial adherens junctions rather than organizing separately. In addition, endothelial cells do not form desmosomes.

1.4.1 Cadherins

The principal adhesion molecules in the adherens junction are cadherins, so named because they mediate adhesion only in the presence of calcium ions (Figure 1.1). Several parallel approaches led to the independent discovery of cell adhesion molecules that were only later recognized as the founding member of the cadherin family, epithelial (E)-cadherin (Takeichi, 1988). The first approach relied on recognition of distinct calcium-dependent and calcium-independent adhesive mechanisms in embryonal carcinoma cell lines (Takeichi, 1977). The calcium-dependent adhesive mechanism was associated with an increase in the contact area between cells, suggesting a more physiological mechanism than the calcium-independent adhesive mechanism, which appeared to be simple clumping. The relative contribution of the two adhesive mechanisms varied between cells, and importantly, expression of a certain cell surface protein correlated with strong calcium-dependent adhesion. A series of further studies developed antibodies against this protein, with a molecular weight of approximately 140 kD (Takeichi et al., 1981; Yoshida and Takeichi, 1982; Yoshida-Noro et al., 1984). Finally, the gene coding this calcium-dependent adhesion protein, named E-cadherin, was cloned, and its expression was shown to be sufficient to confer calcium-dependent adhesiveness to typically non-adherent L fibroblasts (Nagafuchi et al., 1987).

Meanwhile, a second set of studies focused on proteins associated with mouse embryo compaction. A screen of antibodies raised against cell surface antigens identified one antibody that could prevent compaction of the morula (Kemler et al., 1977). This antibody was used to isolate first an 84 kD protein fragment, followed by an approximately 120 kD protein named uvomorulin (Hyafil et al., 1980; Peyriéras et al., 1983). Interestingly, uvomorulin underwent conformational changes in the presence or absence of divalent cations, correlating with the calcium-dependence of morula compaction (Hyafil et al., 1981). Immunohistochemistry and electron microscopy studies eventually showed that uvomorulin localized to epithelial adherens junctions (Boller et al., 1985). Finally, peptide sequencing confirmed that uvomorulin and E-cadherin were the same protein (Ringwald et al., 1987). Similar approaches in other models also succeeded in identifying E-cadherin or its

homologs, including liver cell adhesion molecule (L-CAM) from chick embryos (Brackenbury et al., 1981; Gallin et al., 1987), Cell-CAM120/80 from mouse mammary tumor epithelial cells (Damsky et al., 1981, 1983), and Arc-1 from Madin-Darby canine kidney cells (Behrens et al., 1985).

Along with E-cadherin, early studies of calcium-dependent adhesion identified two additional members of the cadherin family. Antibodies raised against E-cadherin labeled a wide variety of tissues, but not brain. Instead, antibody screening identified a different protein expressed in neurons, glial cells, and several other cell types, that also appeared to mediate calcium-dependent cell–cell adhesion. This antibody disrupted neuronal cell adhesion in mouse embryos, analogous to antibodies against E-cadherin, but with different tissue specificity (Hatta et al., 1985). Peptide sequencing found that this protein, named neuronal (N)-cadherin, was similar, but not identical, to E-cadherin (Shirayoshi et al., 1986). Like E-cadherin, expressing N-cadherin in L fibroblasts was sufficient to confer calcium-dependent adhesiveness (Hatta et al., 1988). A third member of the cadherin family, named placental (P)-cadherin, was cloned from placental tissue (Nose et al., 1987). Importantly, in mixed populations of cells expressing different cadherins, cells rearranged to preferentially associate with other cells expressing the same cadherin (Nose et al., 1988). Thus, expression of different cadherins in different cell types serves as an important mechanism for tissue organization.

1.4.2 Structural basis of adhesion

Structural studies have contributed significantly to understanding the adhesive mechanism of cadherins. Cadherin extracellular regions contain five cadherin repeat domains. Magnetic resonance spectroscopy identified calcium binding sites between the cadherin repeat domains, suggesting that the calcium-dependence of cadherin-mediated adhesion derives from calcium stabilization of the cadherin tertiary structure (Overduin et al., 1995). Point mutations in E- and P-cadherins revealed that the amino-terminal cadherin domain contains sites responsible for determining the specificity of cadherin-based adhesive interactions (Nose et al., 1990). Additionally, antibodies capable of blocking those interactions recognized the same domain.

A crystal structure of N-cadherin amino-terminal fragment dimers further revealed the molecular basis for cadherin-mediated adhesion. Reciprocal insertion of a conserved tryptophan residue of one cadherin into a hydrophobic pocket in its binding partner forms a "strand swap" which anchors the interaction (Shapiro et al., 1995). Another structure of whole extracellular domains of the *Xenopus laevis* cadherin C-cadherin places this intereaction in its broader context. Cadherin extracellular domains form extended curved conformations supporting both reciprocal *trans* interactions with a cadherin on the adjacent cell through "strand swap" dimers and *cis* interactions with cadherins on the same cell (Boggon et al., 2002). Combined, cadherin *trans* and *cis* interactions facilitate formation of the organized adhesive structures comprising adherens junctions (Harrison et al., 2011).

Of course, since cell–cell adhesion is not a static process, the cadherin interactions mediating cell adhesion are themselves dynamic. Cadherins form an alternate *trans* interacting conformation, the "X dimer," with very different binding kinetics from the "strand swap" conformation (Harrison et al., 2010). Cadherin "X dimers" likely form as intermediates during junction disassembly (Hong et al., 2011), and switching between the two conformations may regulate adherens junction dynamics.

1.4.3 Catenins

In addition to mediating cell–cell adhesive interactions, cadherins also interact with components inside the cell. Proteins called catenins bind to the cadherin cytoplasmic domain, link the cadherin to the actin cytoskeleton, and stabilize the cadherin at the junction. The catenins, labeled with the greek letters alpha, beta, and gamma, were initially recognized as unidentified proteins which immunoprecipitated with E-cadherin (Ozawa et al., 1989).

β-catenin, which binds the carboxy-terminal catenin-binding domain of cadherins, was identified as a homolog of *Drosophila* armadillo, and initial evidence suggested it participated in linking E-cadherin to actin (McCrea et al., 1991). β-catenin interacts with cadherins through its twelve central armadillo (Arm) domain repeats, with its amino- and carboxy-terminal tails free to participate in other regulatory interactions (Huber and Weis, 2001). E-cadherin and β-catenin bind in an antiparallel orientation, with the amino-terminal of β-catenin oriented toward the carboxy-terminal cytoplasmic end of E-cadherin,

and the carboxy-terminal of β-catenin oriented toward the plasma membrane. γ-catenin was identified as plakoglobin, which shares significant homology with β-catenin and armadillo. In fact, plakoglobin binds to a similar location on the cadherin cytoplasmic tail as β-catenin, but while β-catenin preferentially associates with the classical cadherins of adherens junctions, plakoglobin preferentially associates with desmosomal cadherins (Peifer et al., 1992).

α-catenin was identified as a homolog of vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991). Mammals express three principal versions of α-catenin, αE-catenin, expressed in most epithelial tissues, αN-catenin, primarily expressed in neurons, and αT-catenin, primarily expressed in cardiac muscle, but all three proteins have similar functions (Hirano et al., 1992; Janssens et al., 2001). α-catenin does not bind to cadherins directly, but binds to the first Arm domain of β-catenin, placing it near the carboxy-terminal end of the cadherin (Aberle et al., 1996). This interaction occurs through the amino-terminal region of α-catenin (Huber et al., 1997; Koslov et al., 1997; Nieset et al., 1997; Obama and Ozawa, 1997).

1.4.4 Links to the cytoskeleton

In addition to binding to β-catenin, α-catenin also binds to filamentous actin (Rimm et al., 1995). This led to a model where α-catenin links the E-cadherin–β-catenin complex with the actin cytoskeleton. However, biochemical evidence challenged this model. The same amino-terminal region through which α-catenin interacts with β-catenin can alternately mediate homodimerization of soluble α-catenin (Koslov et al., 1997; Pokutta and Weis, 2000). Compared with monomeric α-catenin, α-catenin dimers have a much higher affinity for actin. Furthermore, a β-catenin–α-catenin–actin ternary complex could not be assembled *in vitro*, suggesting that α-catenin could not simultaneously bind β-catenin and actin (Drees et al., 2005; Yamada et al., 2005). If the same was true *in vivo*, α-catenin could not serve as a direct link between cadherins and the actin cytoskeleton. An alternative model was proposed in which dynamic α-catenin binding to β-catenin at adherens junctions locally enriched the concentration of soluble α-catenin. This pool of soluble α-catenin could dimerize and bind actin, forming an indirect link between the adherens junction and the cy-

toskeleton. In yet other alternative models, molecules such as vinculin or epithelial protein lost in neoplasm (EPLIN) served as intermediates in the cadherin–actin connection, relieving α-catenin of the responsibility for binding β-catenin and actin simultaneously (Abe and Takeichi, 2008; le Duc et al., 2010; Taguchi et al., 2011; Yonemura et al., 2010).

However, several functional and *in vivo* studies continued to support a direct link through α-catenin. First, cadherins are subject to constitutive actin and myosin generated tension, indicating that the link between the cadherin and the cytoskeleton cannot be transient (Borghi et al., 2012; Smutny et al., 2010). Second, the *C. elegans* α-catenin homolog HMP-1 must contain intact β-catenin and actin binding sites in order to function *in vivo*, even though the β-catenin–α-catenin–actin ternary complex could not be isolated *in vitro* (Kwiatkowski et al., 2010). Furthermore, studies in *Drosophila* demonstrated that functional adherens junctions require persistent linkages both between α-catenin and β-catenin, and between α-catenin and actin, while the cytoplasmic pool of α-catenin is dispensable for functional adherens junctions (Desai et al., 2013). While they do not directly demonstrate α-catenin linking β-catenin and actin, these studies do suggest that *in vitro* binding experiments do not fully capture the function of α-catenin in living organisms.

Recent crystal structures of near–full-length α-catenin may finally hint at an explanation for the contradictory evidence. In solution, α-catenin forms an auto-inhibited conformation with the actin binding site masked (Ishiyama et al., 2013; Rangarajan and Izard, 2013). A conformational change, perhaps induced by mechanical tension on the adherens junction, could relieve the inhibition, allowing α-catenin to function as a direct link after all.

1.4.5 Regulating cadherin stability

An additional protein binds to the juxtamembrane domain of cadherins. p120-catenin (p120) was originally identified as a membrane-associated Src substrate, and only later recognized as a member of the adherens junction complex (Reynolds et al., 1994, 1989). A member of an armadillo family subgroup with nine Arm repeat domains, p120 binds directly to E-cadherin near the membrane (Daniel and Reynolds, 1995; Ishiyama et al., 2010). p120 has several roles in the adherens junction, including supporting lateral cadherin clus-

tering (Yap et al., 1998) and local modulation of Rho family GTPases (Oas et al., 2013; Oldenburg and de Rooij, 2014). Importantly, p120 also serves as a key regulator of cadherin stability at the junction. As will be discussed in detail in subsequent chapters, p120 binding stabilizes the cadherin at the membrane; without p120, cadherins undergo rapid endocytosis and degradation. This mechanism is particularly important for the dynamic modulation of endothelial cell–cell adhesion.

1.5 Cell–cell adhesion in the endothelium

Two key pieces of evidence suggested a potential role for cadherins in endothelial cell–cell adhesion. First, heparin-binding growth factors that could disrupt endothelial adhesion in a high-calcium environment had no effect at low calcium levels, indicating an adhesive process that required the presence of calcium (Bavisotto et al., 1990). Second, calcium-dependent endothelial cell adhesion could also be disrupted by a monoclonal antibody specific to an unidentified 130 kD protein (Heimark et al., 1990). Cloning of N-cadherin and P-cadherin from bovine aortic endothelial cells fueled speculation that they might be the mediators of calcium-dependent endothelial cell adhesion (Liaw et al., 1990). However, further work revealed a previously unidentified member of the cadherin family to be the true endothelial cell–cell adhesion molecule.

First cloned from rat brain and retinal preparations, cadherin-5 was not initially associated with the endothelium (Suzuki et al., 1991). Lampugnani *et al.* identified cadherin-5 as an endothelial-specific cadherin through a screen for antibodies recognizing antigens localized to endothelial cell–cell junctions. One antibody they identified immunoprecipitated a 140 kD protein with a peptide sequence matching that of the protein coded by the cadherin-5 gene (Lampugnani et al., 1992). Thus, cadherin-5 was renamed vascular endothelial (VE)-cadherin.

1.5.1 VE-cadherin in development

After its identification as an endothelial-specific adhesion protein, the importance of VE-cadherin to vascular development quickly became clear. VE-cadherin expression is de-

tected from the earliest stages of vasculogenesis in mouse embryos (Breier et al., 1996), and embryoid bodies lacking VE-cadherin fail to organize vascular-like structures (Vittet et al., 1997). Suprisingly, deletion of VE-cadherin in a mouse model does not prevent initial formation of the vascular plexus. However, subsequent maturation and remodeling of the vascular tree is defective, and the embryos do not survive past day 9.5 (Carmeliet et al., 1999; Gory-Fauré et al., 1999). Studies of mouse allantois explants also support vessel stabilization, rather than formation, as the primary functional role for VE-cadherin. Vascular tube formation in the explants does not require VE-cadherin, but VE-cadherin is required for stability of the vessels (Crosby et al., 2005). Interestingly, the temporal occurrence of vessel defects in the VE-cadherin–null explant model followed the order of vessel formation, with the earliest defects appearing in the vessels first to form.

Other developmental processes which have revealed a VE-cadherin requirement for vessel maturation include lumen formation in the dorsal aortea (Strilić et al., 2009) and vessel anastamosis in zebrafish embryos (Lenard et al., 2013). In the zebrafish model, VE-cadherin–null endothelial cells at the tips of angiogenic sprouts did form cell–cell contacts, but the contacts did not stabilize, and the contacting tip cells continued to create lamellipodea (Lenard et al., 2013). Even in the mature vascular system, VE-cadherin–mediated adhesion remains essential for vessel stability. Intravenous injection of a VE-cadherin–blocking antibody induced a rapid increase in vascular permeability in the hearts and lungs of mice (Corada et al., 1999). Additionally, postnatal deletion of VE-cadherin destabilizes vessels and increased agiogenic sprouting (Gaengel et al., 2012). Both during development and in the adult, VE-cadherin–mediated endothelial cell–cell adhesion is a key regulator of vessel stability.

Although VE-cadherin plays a key role in mediating endothelial cell–cell adhesion, it is not the only cadherin expressed in endothelial cells. Endothelial cells also express significant levels of N-cadherin, which, like VE-cadherin, is required for vascular development. Deletion of N-cadherin in a mouse model is lethal by embryonic day 10 (Radice et al., 1997). However, while N-cadherin can localize to cell–cell junctions when expressed in other cell types, it does not localize to junctions in endothelial cells and adopts a diffuse cytoplasmic localization pattern instead (Salomon et al., 1992). In endothelial cells, VE-cadherin

excludes N-cadherin from junctions by competing for p120-catenin binding (discussed in detail in Section 2.4; Ferreri et al., 2008; Navarro et al., 1998). Nonetheless, some evidence suggests that N-cadherin may predominate in the early stages of vascular development, guiding the early formation of endothelial adherens junctions until increased VE-cadherin expression displaces it as the principal mediator of endothelial adhesion (Luo and Radice, 2005). While most endothelial cell–cell adhesive interactions occur through VE-cadherin, other adhesion molecules clearly have important regulatory roles.

1.5.2 Response to permeability signals

The endothelial adherens junction is not a static structure, and a variety of mechanisms have been proposed to regulate the dynamics of endothelial adhesion at the molecular level. In the following chapters, we will consider the role of cadherin endocytosis in detail (Chapter 2), along with p120-mediated stabilization of VE-cadherin (Chapter 3), and the disruption of that regulatory mechanism by the viral ubiquitin ligase K5 (Chapter 4). First, we will conclude this chapter with a more general overview of VE-cadherin dynamics in response to permeability signals and how disruption of endothelial adhesion contributes to disease.

Long prior to the identification of VE-cadherin, studies of the effect of inflammatory signals, such as histamine, noted that they induced the formation of gaps between endothelial cells (Majno and Palade, 1961). Thus, disruption of endothelial adhesion is a key effector of inflammation. Many efforts to understand the disruption of endothelial cell–cell adhesion in response to inflammatory signals have focused on the post-translational modification of VE-cadherin, particularly by phosphorylation. Both histamine and VEGF trigger VE-cadherin phosphorylation at multiple sites (Andriopoulou et al., 1999; Esser et al., 1998). Furthermore, in a mouse model of metastasis, VEGF potentiated extravasation of tumor cells circulating in the vasculature, but not in mice lacking the kinases Src and Yes (Weis et al., 2004). Antibodies against VE-cadherin could overcome resistance to tumor cell extravasation in the kinase-deficient mice, hinting that VEGF-induced permeability involved Src-mediated phosphorylation and disruption of VE-cadherin–based junctions.

More direct evidence of a role for Src in VE-cadherin phosphorylation came from another study which identified Src-mediated phosphorylation of VE-cadherin at two specific

tyrosine residues, 658 and 331. Phosphomimetic substitutions at those sites disrupted binding of p120 and β-catenin, and induced endothelial permeability (Potter et al., 2005). In another study using VE-cadherin mutants, a phosphomimetic substitution at tyrosine 658 disrupted p120 binding to the extent that N-cadherin, which is typically excluded from endothelial cell–cell junctions by VE-cadherin, localized preferentially to cell borders (Hatanaka et al., 2011). However, a third study identified Src-mediated phosphorylation of VE-cadherin at a different tyrosine residue, 685 (Wallez et al., 2007). Further complicating the picture, a fourth study found that activation of Src through expression of a dominant-negative mutant of the Src regulator Csk did result in VE-cadherin phosphorylation at tyrosines 658, 685, and 731, but did not disrupt endothelial adherens junctions or induce increased permeability (Adam et al., 2010). In contrast, expression of a constitutively active Src mutant did decrease barrier function, but that effect was temporally disconnected from Src-mediated VE-cadherin phosphorylation. Two factors might explain the different results of these studies. First, cadherin mutants intended to mimic phosphorylation might behave differently from cadherin that has actually been phosphorylated. While the mutations used, tyrosine to aspartic acid, do mimic the negative charge of phosphorylation, they also introduce other structural changes, such as loss of the tyrosine aromatic ring, which might affect cadherin–catenin binding interactions. Second, as suggested by the results of Adam et al. (2010), increased expression of kinases may drive phosphorylation of targets other than cadherins. In particular, activation of cytoskeletal regulatory proteins might drive changes in cell architecture that disrupt cell–cell junctions, and cadherin phosphorylation could be an incidental finding.

Despite the confusion, several studies have identified possible mechanisms by which VE-cadherin phosphorylation could affect endothelial permeability. One involves the VEGF-triggered Src-dependent phosphorylation of the guanine nucleotide-exchange factor Vav2, leading to activation of the small GTPase Rac. Rac then triggers the p21-activated kinase (PAK)-mediated phosphorylation of VE-cadherin. Phosphorylated VE-cadherin is bound by β-arrestin, triggering VE-cadherin internalization, disruption of the adherens junction, and induction of endothelial permeability (Gavard and Gutkind, 2006; Hebda et al., 2013). Another possible mechanism involves VEGF and Src-mediated activation

of focal adhesion kinase (FAK). FAK phosphorylates VE-cadherin at tyrosine 658 and β-catenin at tyrosine 142, disrupting endothelial adhesion and increasing tumor cell transmigration (Chen et al., 2012; Jean et al., 2014). Yet a third possible mechanism involves Gα13 binding to VE-cadherin, recruiting Src to phosphorylate VE-cadherin at tyrosine 658 (Gong et al., 2014). The particular roles of these different pathways in different physiological contexts remain unclear.

Of course, if phosphorylation of VE-cadherin has physiological importance, it is likely to be a highly regulated and reversible process. Studies of mouse models lacking vascular endothelial phosphotyrosine phosphatase (VE-PTP) suggest that is in fact the case. VE-PTP is an endothelial-specific receptor tyrosine phosphatase that interacts with VE-cadherin (Nawroth et al., 2002). A variety of inflammatory signals, including VEGF and leukocyte binding to endothelial cells, trigger dissociation of VE-PTP from VE-cadherin and phosphorylation of VE-cadherin and β-catenin (Nottebaum et al., 2008). Mice lacking VE-PTP die by embryonic day ten with severe vascular malformations reminiscent of those seen in mice lacking VE-cadherin (Bäumer et al., 2006). Also similar to VE-cadherin–null mice, VE-PTP-null mice undergo seemingly normal vasculogenesis, but do not remodel the primitive vascular plexus into a mature vasculature (Dominguez et al., 2007). Thus, both kinases and phosphatases regulate the dynamics of endothelial cell–cell adhesion.

One clue to the potential physiological role of VE-cadherin phosphorylation comes from *in vivo* studies of mouse vasculature which identified VE-cadherin phosphorylation on tyrosine residues 658 and 685 in veins, but not in arteries. VE-cadherin phosphorylation, attributed to shear-stress–induced activation of Src in veins, was also associated with bradykinin-induced vascular permeability and VE-cadherin ubiquitination, endocytosis, and degradation (Orsenigo et al., 2012). Phosphorylation of VE-cadherin also plays a role in leukocyte transmigration. Adhesion of leukocytes to endothelial cells induces phosphorylation of VE-cadherin at tyrosine residues 645, 731, and 733, and expression of non-phosphorylatable VE-cadherin mutants partially blocks leukocyte transmigration through paracellular, but not transcellular, routes (Turowski et al., 2008). Interestingly, phosphorylation of VE-cadherin at different sites may influence different endothelial processes. Mice expressing a VE-cadherin mutant with a non-phosphorylatable substitution at

tyrosine 685 have impaired induction of permeability in response to inflammatory stimuli, but normal leukocyte transmigration. In contrast, mice expressing a VE-cadherin mutant with a non-phosphorylatable substitution at tyrosine 731 had impaired leukocute transmigration, but normal induction of vascular permeability (Wessel et al., 2014). These studies strongly suggest that multiple distinct mechanisms tune the balance between stability and flexibility in endothelial cell–cell adhesion, and that different pathways are activated in different contexts.

1.6 Disruption of endothelial adhesion in disease

Vascular development requires dynamic and flexible endothelial adhesion to support the cellular rearrangements necessary for angiogenesis and maturation of the vascular tree. However, inappropriate loss of endothelial cell–cell adhesion contributes to disease. Disruption of the balance between stability and flexibility of endothelial adhesion occurs in a wide variety conditions, facilitating both excessive inflammation, as well as tumor growth and metastasis.

1.6.1 Inflammation

Decreased endothelial cell–cell adhesion results in fluid leak from the vasculature into the surrounding tissue, causing swelling, and the recruitment and extravisation of immune cells, propagating the inflammatory response. When properly controlled, both processes play important roles in defense against pathogens and the wound healing process. In contrast, excessive induction of vascular permeability can result in tissue damage and compromise organ function. One example of the harmful consequences of excessive vascular permeability is the acute respiratory distress syndrome (ARDS), where loss of barrier function in lung capillaries leads to fluid accumulation that blocks gas exchange in the alveoli (Maniatis et al., 2008). Osmolality measurements first established that pulminary edema in ARDS was due to increased permeability, in contrast to the pressure-induced pulmonary edema resulting from heart failure (Fein et al., 1979). ARDS is a significant and dangerous complication associated with a variety of pulmonary insults, including lung injury and in-

fection, with few treatments available other than supportive therapy through mechanical ventilation. Another dangerous condition caused by excessive vascular permeability is septic shock, in which a systemic infection triggers generalized loss of vessel integrity (Angus and van der Poll, 2013). This results in a sharp drop in blood pressure refractory to fluid resuscitation, often causing organ damage.

Disrupted endothelial adhesion also contributes to diseases involving ischemic insults, such as myocardial infarction and stroke. In both diseases, tissue damage caused by ischemia is worsened by the development of an inflammatory response following reperfusion, a phenomenon named ischemia reperfusion injury. In myocardial infarction, reperfusion leads both to direct damage of cardiac myocytes due to oxidative stress, calcium overload, and rapid pH changes, and to endothelial dysfunction, including loss of capillary barrier function causing swelling (Hausenloy and Yellon, 2013; Turer and Hill, 2010). In ischemic stroke, reperfusion can lead to inflammation, swelling, and breakdown of the blood–brain barrier. Reperfusion injury causes significant tissue damage, often exceeding the damage caused directly by the infarct itself (Khatri et al., 2012; Pundik et al., 2012).

In addition to its response to inflammatory cytokines such as histamine (Section 1.5.2), VE-cadherin has been directly implicated in the inflammatory processes of several diseases. Neutrophil adhesion to endothelial cells triggers the removal of VE-cadherin from cell–cell contacts, suggesting that contact signals as well as soluble signals can affect junction organization and induce endothelial permeability (Del Maschio et al., 1996). Interestingly, the malaria-causing parasite *Plasmodium falciparum* was also found to disrupt VE-cadherin and endothelial barrier function in a Src-dependent manner (Gillrie et al., 2007). Disruption of endothelial cell–cell junctions has additionally been linked to ischemic acute renal failure. In a rodent model of kidney injury, a sudden ischemic insult caused loss of VE-cadherin from junctions and increased vascular permeability (Sutton et al., 2003). Another study found that stabilization of VE-cadherin at the cell surface using a VE-cadherin-binding fibrin fragment protected against kidney injury in a similar ischemia model (Urbschat et al., 2014). Finally, recruitment of VE-cadherin to endothelial cell–cell junctions increased survival in rodent models of bacterial endotoxin exposure, polymicrobial sepsis, and H5N1 influenze (London et al., 2010). VE-cadherin was recruited to junctions through

the activation of Slit–Robo signaling (Section 1.3.4), reducing their susceptibility to permeability. While no therapies reinforcing endothelial cell–cell adhesion against vascular leak are currently available in the clinic, these studies clearly indicate the potential of such an approach.

1.6.2 Cancer

In addition to its role in inflammation, disruption of the balance between stability and flexibility of endothelial cell–cell adhesion also contributes to tumor formation and cancer metastasis (Le Guelte et al., 2011). Many tumors induce the growth of their own vascular supply, and tumor-induced angiogenesis has long been recognized as an important pathomechanism in cancer (Folkman, 1971). Inhibition of angiogenic VEGF signaling is a common cancer treatment strategy (Jain et al., 2006). Increasingly, inflammation is recognized as a cancer hallmark as well, highlighting the relevance of endothelial adhesion to understanding cancer biology and developing cancer therapies (Mantovani et al., 2008). Loss of cell adhesion proteins occurs in many tumors, so an association between loss of VE-cadherin and increased growth of vascular tumors such as angiosarcomas hardly comes as a surprise (Zanetta et al., 2005). However, several non-endothelial tumors can induce the loss of VE-cadherin in adjacent vasculature, an effect which has been demonstrated in culture both with breast cancer cell lines (Cai et al., 1999) and with pancreatic carcinoma cell lines (Nakai et al., 2005). In some cases, tumor cells themselves can differentiate into endothelial-like cells expressing VE-cadherin and form tumor-derived vessels with abnormally increased permeability (Ricci-Vitiani et al., 2010; Wang et al., 2010). Tumor-associated vascular abnormalities are an important potential target for new anti-cancer therapies. At present, however, the molecular mechanisms underlying the disruption of endothelial cell–cell adhesion in cancer remain incompletely understood.

1.7 Conclusion

Endothelial cells maintain delicately balanced adhesive contacts, stable enough to resist vascular leak, yet also flexible enough to allow dynamic remodeling of the vasculature.

Flexibility of endothelial cell–cell adhesion is thought to be particularly important for migration and rearrangement of endothelial cells during angiogenesis. The iterative process of forming and remodeling the vascular tree during development underscores the importance of this flexibility. However, inappropriate regulation of endothelial cell–cell adhesion flexibility can contribute to disease by causing excessive inflammation and facilitating cancer metastasis. Thus, understanding the basic cellular mechanisms controlling the strength and flexibility of endothelial cell–cell adhesion has important implications for human health and disease.

Considerable progress has been made toward understanding adherens junctions, the principal adhesion complexes in endothelial cells, from a static perspective. The core protein components of the adherens junction have been identified, and their interactions are increasingly well understood. In particular, structural data from X-ray crystallography and other studies reveal a detailed picture of the adhesive interactions mediated by cadherins. However, considerably less is known about how these adhesive complexes are regulated dynamically, and of particular importance to endothelial adherens junctions, how these junctions develop plasticity.

The remainder of this dissertation focuses on the question of flexibility in the endothelial adherens junction. The next chapter reviews the regulation of adherens junctions by cadherin endocytosis, and the evidence that balanced trafficking of cadherins to and from the cell surface serves as a mediator of adherens junction dynamics. Chapters 3 and 4 present evidence that constitutive endocytosis of VE-cadherin confers plasticity to endothelial cell–cell junctions which is required for endothelial cell migration, and that disruption of this tightly regulated process is associated with disease.

Chapter 2

Adherens junction turnover: regulating adhesion through cadherin endocytosis, degradation, and recycling

Abstract

Adherens junctions are important mediators of intercellular adhesion, but they are not static structures. They are regularly formed, broken, and rearranged in a variety of situations, requiring changes in the amount of cadherins, the main adhesion molecule in adherens junctions, present at the cell surface. Thus, endocytosis, degradation, and recycling of cadherins are crucial for dynamic regulation of adherens junctions and control of intercellular adhesion. In this chapter, we review the involvement of cadherin endocytosis in development and disease. We discuss the various endocytic pathways available to cadherins, the adaptors involved, and the sorting of internalized cadherin for recycling or lysosomal degradation. In addition, we review the regulatory pathways controlling cadherin endocytosis and degradation, including regulation of cadherin endocytosis by catenins, cadherin ubiquitination, and growth factor receptor signaling pathways. Lastly, we discuss the proteolytic cleavage of cadherins at the plasma membrane.

This chapter is adapted from:

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2.1 Introduction

Cell contacts are not static structures. They are regularly formed, broken, and rearranged both during normal physiological processes and in disease states. In order to allow for dynamic changes in cell contact strength, adherens junctions must themselves be plastic. A key mechanism for modulating adhesion strength is the adjustment of the amount of cadherin, the main adhesion molecule in adherens junctions, present at the plasma membrane.[1] Cadherin levels are determined by the balance between endocytosis and degradation, which remove cadherin from the plasma membrane, and synthesis and recycling, which increase the amount of cadherin available. Transcriptional regulation of cadherins also plays an important role in development and disease (Peinado et al., 2004). However, because the metabolic half-life of cadherins is long, approximately five to ten hours in cultured cells (McCrea and Gumbiner, 1991; Shore and Nelson, 1991), transcriptional regulation cannot account for more rapid changes in adhesion strength. As we discuss in this chapter, endocytosis, degradation, and recycling of cadherins are crucial for dynamic regulation of adherens junctions and control of intercellular adhesion.

Cadherins are named for their calcium-dependent adhesion. Depletion of extracellular calcium disrupts adherens junctions (Kartenbeck et al., 1982), and it was this process that provided the first evidence that cadherin turnover might play a role in the dynamic control of cell adhesion. Classic electron microscopy and immunofluorescence studies demonstrated that, subsequent to calcium depletion, cadherins are removed from cell junctions by endocytosis (Kartenbeck et al., 1991; Mattey and Garrod, 1986). Cadherin endocytosis plays a role in physiological processes as well. For example, cells undergoing mitosis often appear to adopt a rounded morphology, suggesting that they have become detached from their neighbors. Cadherin endocytosis was found to accompany mitosis-related cell rounding, decreasing the junctional pool of cadherin to allow for decreased adhesion, even as the total amount of cadherin expression remained constant (Bauer et al., 1998). More recent work suggests that cadherin endocytosis is a particularly important mechanism for the disassembly of cadherin-based adhesive contacts (Troyanovsky et al., 2006). The significance

[1] Unless otherwise noted, we use 'cadherin' to mean classical cadherins, the cadherin subfamily which forms adherens junctions.

of cadherin internalization to the dynamic regulation of cell–cell adhesion is now well established. Cadherin endocytosis has been observed in a large variety of developmental and disease processes, and in recent years, tremendous progress has been made toward understanding the molecular mechanisms involved in cadherin internalization and degradation.

In this chapter, we review the evidence for the involvement of cadherin endocytosis during development and its misregulation in disease. We also discuss the rapidly accumulating body of work detailing the trafficking pathways involved in cadherin endocytosis. Both clathrin-dependent and clathrin-independent pathways have been implicated, and several endocytic adaptors which interact with cadherins have been identified. In addition, we consider the process of sorting internalized cadherin for recycling or degradation and how the regulation of cadherin recycling may be used to control adherens junction turnover. Regulation of cadherin endocytosis by catenins is also important, and we review the effects of catenins on cadherin internalization. p120-catenin in particular has gained prominence as a "set-point" for cadherin levels, but α- and β-catenins may have important roles as well. We also review the evidence for cadherin ubiquitination as a signal for adherens junction turnover and the ubiquitin ligases which have been found to target cadherins and affect cadherin trafficking. In order to further consider the regulation of cadherin internalization, we discuss the many growth factor signaling pathways that affect cadherin trafficking. Interestingly, in some cases the connection is bidirectional, with growth factor signaling altering cadherin trafficking and cadherins modulating growth factor receptor signaling. Finally, we briefly discuss another important mechanism for adherens junction turnover, the proteolytic degradation of cadherins at the plasma membrane.

2.2 Cadherin endocytosis in development and disease

Perhaps the best examples of the importance of cadherin endocytosis and the dynamic regulation of adherens junctions come from tissue patterning and development. Initially, cadherins were observed to control tissue patterning by facilitating cell sorting based on the type of cadherin expressed (Nose et al., 1988). However, Steinberg and Takeichi also demonstrated that varying the expression level of a single cadherin could also be used as

a mechanism for cell sorting (1994). Thus, the prominent role of cadherin endocytosis in development should come as no surprise. For example, during epithelial–mesenchymal transitions, cells decrease the expression level of cadherins through a process involving cadherin internalization (Miller and McClay, 1997). Cadherin internalization has also been reported during gastrulation in a variety of organisms (Oda et al., 1998; Ogata et al., 2007), where it may be controlled by Wnt signaling (Ulrich et al., 2005). Other developmental processes where cadherin internalization is important include nervous system development, where both the Rab-5–dependent endocytosis and Rab-11–mediated recycling of N-cadherin are required for neuronal patterning (Kawauchi et al., 2010). Two lines of investigation also demonstrate the importance of cadherin endocytosis for developmental processes involving planar cell polarity. First, convergent extension in *Xenopus* embryos typically involves the coordinated down-regulation of C-cadherin in response to mesoderm-inducing signals (Brieher and Gumbiner, 1994; Zhong et al., 1999). Inhibiting dynamin in *Xenopus* embryos blocks C-cadherin endocytosis, disrupting convergent extension (Jarrett et al., 2002). Second, in *Drosophila*, planar-polarized endocytosis of DE-cadherin mediates cell intercalation necessary for germ band extension, and blocking cadherin endocytosis prevents this critical developmental process (Levayer et al., 2011). Thus, cadherin internalization plays a key role in a variety of developmental processes.

Of course, processes which play important roles in development often contribute to disease when they are activated inappropriately. Cadherin internalization is no exception, and loss of cell adhesion is a key requirement for cancer metastasis. Loss of adhesion in many types of cancer is often attributed to decreased E-cadherin expression (Hirohashi, 1998). While this is most often due to decreased synthesis, there is some evidence that increased cadherin endocytosis may also play a role. One recent study found that a non-junctional, presumably internalized, E-cadherin expression pattern was associated with poor survival in nasopharyngeal cancer (Xie et al., 2010). Another found Src-dependent E-cadherin internalization with shear stress in an oropharyngeal cancer cell line (Lawler et al., 2009). Increased E-cadherin internalization has also been found in a mouse model of UV-irradiation-induced squamous cell carcinoma (Brouxhon et al., 2007). As discussed below, there is also considerable evidence for the involvement of cancer-associated signaling molecules, such

as receptor tyrosine kinases and v-Src, in cadherin internalization.

Cadherin endocytosis may play a role in other disease processes as well. For example, internalization of E-cadherin by pancreatic acinar cells was found to be increased in an experimental model of acute pancreatitis (Lerch et al., 1997). Acute pancreatitis is classically associated with significant pancreatic edema, and increased cadherin endocytosis leading to loss of epithelial integrity is an attractive pathophysiological mechanism. Another disease process in which cadherin endocytosis has been implicated is the autoimmune blistering disease pemphigus vulgaris. Auto-antibodies from pemphigus patients cause increased internalization of the desmosomal cadherin desmoglein 3, which may contribute to loss of epithelial integrity and blister formation (Calkins et al., 2006; Delva et al., 2008). Intriguingly, cadherin endocytosis may also be involved in infectious processes. The bacterium *Listeria monocytogenes* appears to hijack a constitutive cadherin endocytic pathway in order to gain entry to cells, a key contributor to the pathogen's virulence (Veiga and Cossart, 2005). The potential involvement of cadherin endocytosis in such a variety of diseases makes it a tempting target for new therapies, though it remains to be seen whether aberrant cadherin internalization in disease can be inhibited without affecting cadherin endocytosis necessary for normal biological processes. Turning these discoveries into a new generation of anti-cancer drugs will certainly require a better understanding of the molecular mechanisms and regulation of adherens junction turnover.

2.3 Cadherin trafficking pathways

Understanding the pathways cadherins use to move in and out of adherens junctions has been a major research focus over the past decade (Chiasson and Kowalczyk, 2008). This work has significantly increased our understanding of how cadherins are internalized and how they are selected for degradation or for recycling back to the plasma membrane. Trafficking pathways essentially control the rate of cadherin turnover; the higher the rate of cadherin endocytosis and the higher the proportion of endocytosed cadherin selected for degradation rather than recycling, the lower the amount of cadherin that will be available to form adherens junctions. We review the clathrin-dependent endocytosis of cadherins and

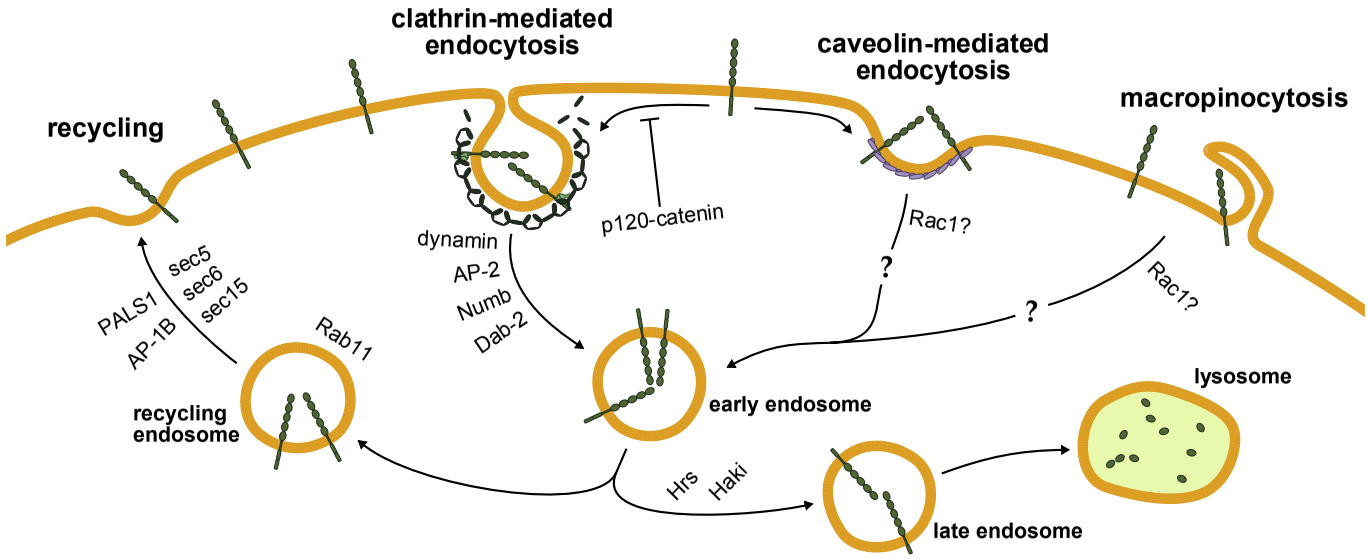

Figure 2.1: *Cadherin trafficking pathways.* Cadherin internalization can occur through either clathrin-mediated, caveolin-mediated, or macropinocytosis-like pathways. Internalized cadherin is then sorted either for lysosomal degradation or recycling back to the plasma membrane.

the adaptor proteins involved, as well as several clathrin-independent endocytic pathways and pathways involved in the recycling of internalized cadherin (Figure 2.1).

2.3.1 Clathrin-mediated endocytosis

Cadherin internalization occurs through several distinct endocytic pathways. Of them, most work has focused on clathrin-mediated endocytosis, which is also the endocytic pathway understood in the greatest detail (Bonifacino and Traub, 2003). Proteins are targeted for clathrin-mediated endocytosis by the binding of adaptor protein complexes. Once bound, adaptor proteins recruit other components of the endocytic machinery and cluster into clathrin-coated pits. Clathrin-coated pits containing proteins targeted for endocytosis then undergo dynamin-mediated scission from the plasma membrane, budding off to form endocytic vesicles. Internalized proteins can be sorted for recycling back to the plasma membrane or sorted to the lysosome for degradation.

Cadherin was first recognized to undergo clathrin-mediated endocytosis by Le and colleagues, who observed constitutive clathrin-mediated endocytosis and recycling of E-cadherin in MDCK cells (Le et al., 1999). We also found that endocytosis of VE-cadherin in endothelial cells occurs through a clathrin-mediated pathway ultimately resulting in degradation of the cadherin by the lysosome (Xiao et al., 2003b). Furthermore, clathrin-mediated endocytosis appears to be responsible for two types of growth factor-induced cadherin internalization, FGF-mediated internalization of E-cadherin (Bryant et al., 2005) and VEGF-mediated internalization of VE-cadherin (Gavard and Gutkind, 2006). Interestingly, clathrin-mediated endocytosis of E-cadherin may be related to the cadherin's adhesive state. Izumi and colleagues isolated adherens junction-containing membrane from rat liver and, using a reconstitution system, observed budding of E-cadherin into clathrin-coated vesicles with electron microscopy and biochemical fractionation. Adding antibody against the extracellular domain of E-cadherin, which blocks trans interactions, to the reconstitution system increased the amount of cadherin which entered clathrin-coated vesicles, while adding E-cadherin extracellular domain fragments decreased recruitment of cadherin to clathrin-coated vesicles. They also found that *trans* interaction-mediated inhibition of cadherin endocytosis involved activation of the small G-proteins Rac and Cdc42,

as well as the actin-binding protein IQGAP1 (Izumi et al., 2004). In addition, exposing an intestinal epithelial cell line to low-calcium conditions, which disrupts cadherin *trans* interactions, results in the clathrin-mediated endocytosis of E-cadherin, along with other adherens junction and tight junction components, into a unique syntaxin-4–positive compartment (Ivanov et al., 2004). Thus, clathrin-mediated endocytosis appears to modulate cadherin function in a variety of biological contexts.

2.3.2 Endocytic adaptors

Clathrin-mediated endocytosis depends on adaptor proteins to recognize proteins targeted for internalization and to recruit other components of the endocytic machinery. Identifying clathrin-mediated endocytosis as a pathway for cadherin internalization raises the question of what endocytic adaptors might recognize cadherins. One likely candidate is the adaptor protein complex AP-2, which commonly recognizes cargo proteins with a tyrosine- or dileucine-based motif (Traub, 2003). E-cadherin contains a putative dileucine-based AP-2 binding motif in its cytoplasmic tail, and mutating those residues disrupts the normal basolateral localization of E-cadherin (Miranda et al., 2001) and prevents E-cadherin clathrin-mediated endocytosis (Miyashita and Ozawa, 2007b). This motif is also present in many other classical cadherins, including N- and P-cadherins. It is not, however, present in VE-cadherin or in *Drosophila* DE-cadherin. Nonetheless, the VE-cadherin cytoplasmic tail is sufficient to mediate clathrin-dependent endocytosis when attached to an unrelated transmembrane protein, strongly suggesting that cadherins may contain other endocytic adaptor binding sequences as well (Xiao et al., 2005). In recent years, more direct evidence for the involvement of AP-2 in the clathrin-mediated endocytosis of cadherins has begun to accumulate. We found that internalization of VE-cadherin is clathrin-, dynamin-, and AP-2–dependent and that AP-2 both co-localizes with VE-cadherin and co-immunoprecipitates with the VE-cadherin cytoplasmic tail (Chiasson et al., 2009). An AP-2 subunit was also found to co-immunoprecipitate with the E-cadherin cytoplasmic tail (Sato et al., 2011). Interestingly, Levayer and colleagues also found that AP-2– and clathrin-mediated endocytosis of DE-cadherin is crucial for the establishment of planar cell polarity in germ band extension. Polarized distributions of Dia and Myosin-II induce planar DE-

cadherin clustering in junctions perpendicular to the developing long axis of the germ band. DE-cadherin clustering recruits AP-2 and clathrin to these junctions, leading to the preferential endocytosis of DE-cadherin from perpendicular junctions and the relative accumulation of DE-cadherin in junctions parallel to the germ band axis (Levayer et al., 2011).

However, the question of what endocytic adaptors are important for cadherin endocytosis remains incompletely resolved. It is not yet clear that AP-2 interacts directly with cadherins. It is also possible that other endocytic adaptors may be involved depending on the biological context. Mice null for Dab-2, another adaptor protein associated with clathrin-mediated endocytosis, support this possibility. They exhibit loss of apical-basal polarized distribution of E-cadherin, as well as the LDL receptor-related protein megalin, in the developing endoderm (Yang et al., 2007). Several reports also suggest a role for the endocytic adaptor Numb in cadherin internalization. In radial glial cells, Numb co-immunoprecipitates with cadherins, and Numb depletion disrupts adherens junctions (Rasin et al., 2007). Numb also binds to E-cadherin in epithelial cell lines and mediates endocytosis of cadherins specifically from the apical surface, contributing to the lateral localization of cadherins in adherens junctions (Lau and McGlade, 2011; Wang et al., 2009). This polarization is due to localized phosphorylation and inactivation of Numb at lateral membranes by the PAR polarity complex member aPKC (Sato et al., 2011). Consequently, the role of adaptor proteins in cadherin endocytosis remains an exciting area for future discovery.

2.3.3 Clathrin-independent endocytic pathways

Cadherin turnover has also been associated with clathrin-independent endocytic pathways, though considerably less work has been done in this area compared to clathrin-mediated cadherin endocytosis. Studies have suggested that cadherin endocytosis may occur through both caveolin-mediated and macropinocytosis-like pathways. Akhtar and colleagues found that a dominant-active form of the small GTPase Rac1 could disrupt cell–cell adhesion in keratinocytes. This was associated with the endocytosis of E-cadherin through a pathway that appeared to be distinct from the uptake of transferrin, which is clathrin-mediated, and through structures that co-localized with caveolin (Akhtar and Hotchin, 2001). Further

evidence for caveolin-mediated cadherin endocytosis was provided by Lu and colleagues, who demonstrated that EGF signaling could disrupt cell–cell adhesion by triggering the caveolin-mediated internalization of E-cadherin, a mechanism which may be relevant to epithelial-to-mesenchymal transition in cancers (Lu et al., 2003). In contrast, Bryant and colleagues characterized the EGF-induced internalization of E-cadherin in a breast carcinoma cell line, in which E-cadherin was internalized along with the cadherin-binding proteins p120 and β-catenin, as Rac1-modulated macropinocytosis, rather than caveolin-mediated (Bryant et al., 2007). It is not clear if the EGF-related mechanisms described by Lu and Bryant are in fact different and, if they are, how they can be reconciled. However, Paterson and colleagues have observed E-cadherin endocytosis that is both clathrin- and caveolin-independent, but dynamin-dependent. This pathway, which they identify as similar to macropinocytosis, appears to affect cadherin that is not engaged in *trans* interactions in an adherens junction (Paterson et al., 2003). Lastly, the desmosomal cadherin desmoglein 3 undergoes lipid-raft-mediated endocytosis, though it is unclear if this pathway is available to classical cadherins as well (Delva et al., 2008). Though some of the specific details of the clathrin-independent pathways remain unclear, it appears that both clathrin-dependent and clathrin-independent endocytic pathways play a role in cadherin turnover.

2.3.4 Recycling pathways

Not all molecules that enter an endocytic pathway face immediate degradation in the lysosome. Some are sorted and recycled back to the plasma membrane. Recycling pathways are particularly important for cadherins, and the choice between degradation and recycling can help fine-tune the amount of cadherin present at adherens junctions and the strength of cell–cell adhesion. The first suggestion of the importance of a recycling pathway to cadherin trafficking came from the discovery that E-cadherin does not travel directly from the Golgi complex to the cell surface, but transits first through Rab11-positive recycling endosomes (Lock and Stow, 2005). Interestingly, while expressing dominant-negative Rab11 blocked delivery of wild type E-cadherin to the plasma membrane, an E-cadherin mutant lacking the dileucine motif important for clathrin-mediated endocytosis traffics to the plasma mem-

brane without impediment, though it is mislocalized to the apical surface (Lock and Stow, 2005; Miranda et al., 2001). In contrast, *Drosophila* DE-cadherin traffics through Rab11-positive endosomes and inhibiting Rab-11 disrupts the integrity of the embryonic ectoderm, even though DE-cadherin lacks the dileucine motif (Roeth et al., 2009). In addition to acting as way stations for newly synthesized cadherin on its way to the plasma membrane, Rab11-positive recycling endosomes can also sort internalized cadherin for recycling back to the cell surface. In fact, Classen and colleagues found that Rab11 recycling of cadherin mediates the rearrangements in cell–cell contacts seen in the hexagonal packing of *Drosophila* wing disk cells (Classen et al., 2005). Desclozeaux and colleagues also found that cadherin recycling is necessary for maintaining adherens junctions and epithelial polarity and that disrupting the recycling endosome with dominant-negative Rab11 prevented MDCK cells from forming cysts when grown in three-dimensional culture (Desclozeaux et al., 2008).

Additional work has begun to illuminate the molecular mechanisms responsible for cadherin recycling. In particular, components of the exocyst complex appear to be critical. Sec5, sec6, and sec15 are all required for DE-cadherin trafficking from recycling endosomes to the plasma membrane (Langevin et al., 2005). Depletion of the scaffolding protein PALS1 also causes the mislocalization of the exocyst complex and disrupts recycling of E-cadherin (Wang et al., 2007). Recently, Guichard and colleagues identified Rab11- and exocyst complex–mediated recycling of cadherins as a target of the pathogen *Bacillus anthracis*, highlighting its pathophysiological importance. *B. anthracis*, the causative agent of anthrax, produces two different toxins, lethal factor and edema factor, which both inhibit the exocyst complex through independent mechanisms. This results in the loss of cadherin from adherens junctions, potentially contributing to the toxin-mediated epithelial and vascular disruption which occurs with *B. anthracis* infection (Guichard et al., 2010). In addition to the exocyst complex, another potential mediator of cadherin recycling is the adaptor protein complex AP-1B, which usually mediates recycling of basolaterally targeted proteins. Ling and colleagues found that AP-1B interacts with E-cadherin through phosphatidylinositol-4-phosphate 5-kinase type Iγ (PIPKIγ), which binds directly to the E-cadherin cytoplasmic tail near the β-catenin binding site (Ling et al., 2007). Interestingly, an E-cadherin mutation at the PIPKIγ binding site is associated with familial diffuse gastric

cancer (Yabuta et al., 2002).

Our understanding of cadherin recycling remains incomplete. Though many of the important components of the cadherin recycling pathway have been identified, the list is likely to grow further. Furthermore, although we review below some evidence that ubiquitination may trigger the selection of cadherin for degradation rather than recycling (Palacios et al., 2005), the regulation of the cadherin recycling pathways remains, for now, only partially elucidated.

2.4 Regulation of cadherin endocytosis by catenins

Given the importance of cadherin endocytosis for the proper maintenance and dynamic regulation of cell–cell adhesion, identifying the regulatory mechanisms controlling cadherin internalization and recycling has become a significant research focus. Much attention has been paid to the catenins, the cytoplasmic binding partners of cadherins, which stabilize adherens junctions and link them to the actin cytoskeleton (Delva and Kowalczyk, 2009). These include α-catenin, β-catenin, and p120-catenin. β-catenin binds to the C-terminal catenin-binding domain of cadherins and, along with α-catenin, helps link the cadherin to the actin cytoskeleton. p120-catenin binds to the juxtamembrane domain, N-terminal to the β-catenin binding site, and stabilizes cadherin at the adherens junction. All three catenins contribute to the regulation of adherens junctions.

2.4.1 p120-catenin

p120-catenin (p120) plays a key role as an inhibitor of cadherin turnover and as a "set-point" for cadherin expression levels (Figure 2.2). A member of the armadillo family of proteins, p120 binds to the juxtamembrane domain of cadherins (Reynolds, 2007). Ireton and colleagues discovered that epithelial morphology in a colon carcinoma cell line lacking p120 could be restored with exogenous p120 expression. Furthermore, p120 rescue of epithelial morphology required p120 binding to E-cadherin. The mechanism of this activity involved increased E-cadherin protein levels and half-life without changes to E-cadherin mRNA levels (Ireton et al., 2002). Those results, which strongly suggested that

p120 binding to cadherin is necessary to prevent rapid cadherin turnover, were confirmed by studies directly demonstrating that loss of p120 results in cadherin endocytosis (Davis et al., 2003; Xiao et al., 2003a). Importantly, p120 acts not only as an inhibitor of cadherin endocytosis, but as a "set-point" for cadherin expression (Figure 2.2 A). Expressing cadherin mutants which compete for p120 binding results in the endocytosis of endogenous cadherin, while cadherin mutants which cannot bind to p120 lack this activity (Xiao et al., 2003a, 2005). This raises the interesting possibility that p120 might serve as a master regulator of cadherin levels in cells. For example, increased expression of one cadherin might, through competition for p120 binding, cause increased turnover and down-regulation of other cadherins in the cell. Exactly this dynamic has been reported to occur in two studies of cells expressing multiple cadherin types. In A431 cells, exogenously expressing R-cadherin caused the endocytosis and down-regulation of endogenous E- and P-cadherins (Maeda et al., 2006). Similarly, in endothelial cells, which express both VE- and N-cadherins, but which rely primarily on VE-cadherin to form adherens junctions, altering expression levels of one cadherin inversely affects protein levels of the other cadherin (Ferreri et al., 2008).

Multiple mechanisms have been proposed to explain how p120 regulates cadherin turnover. Cadherin internalization mediated by p120 loss is clathrin-dependent, as discussed above in more detail (Xiao et al., 2005). Clathrin-dependent endocytosis requires an adaptor protein to bind to cargo and recruit other components of the endocytic machinery. p120 binding to the cadherin cytoplasmic domain could potentially mask the binding site of such an endocytic adaptor. Alternatively, p120 could regulate cadherin turnover by locally modifying actin dynamics through its well-described role as an inhibitor of the small GTPase RhoA (Anastasiadis, 2007). For example, cells exogenously expressing high levels of p120 display increased actin branching and the formation of long dendritic spines (Anastasiadis et al., 2000; Noren et al., 2000; Reynolds et al., 1996). It has become increasingly clear however, that p120 binding to cadherins, not p120 inhibition of RhoA, is the mechanism of p120-mediated cadherin stabilization (Figure 2.2 B). First, our lab and others have shown that p120 binding to cadherin is an absolute requirement for p120-mediated cadherin stabilization (Ireton et al., 2002; Miyashita and Ozawa, 2007b; Xiao et al., 2005). We also demonstrated that inhibition of RhoA signaling is insufficient to block cadherin

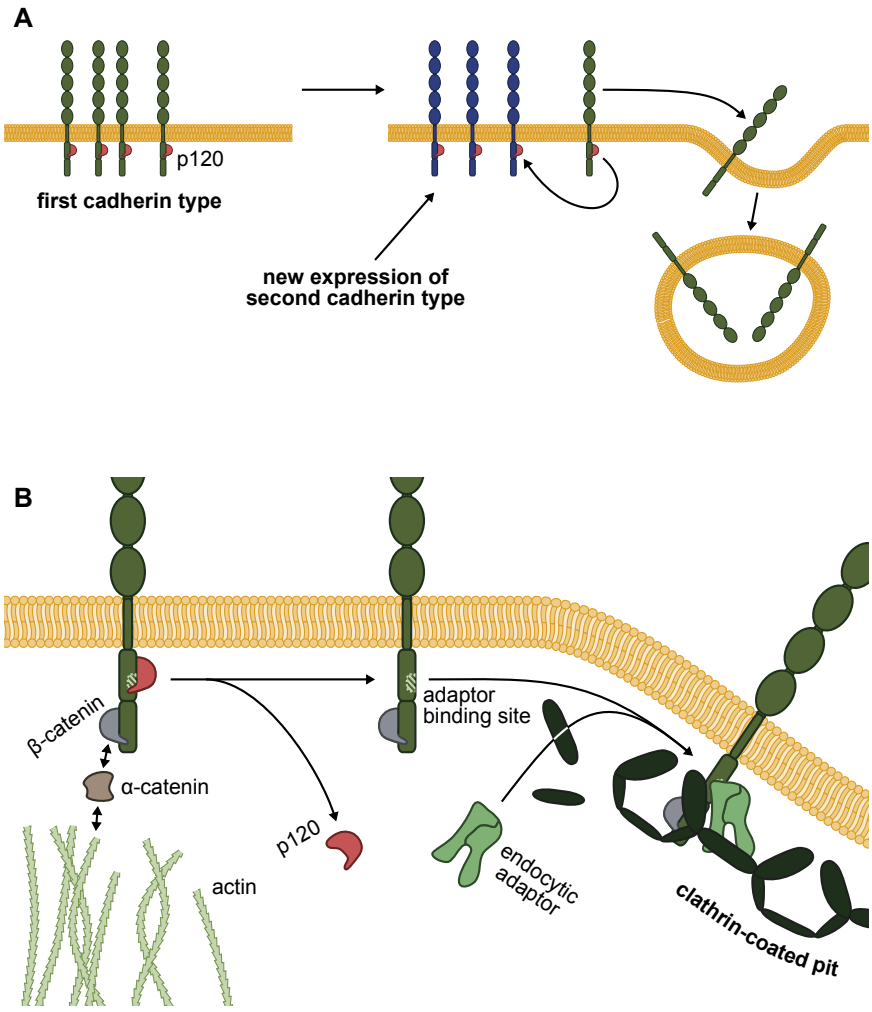

Figure 2.2: *p120-catenin regulates cadherin endocytosis.* (A) p120 acts as a "set-point" for cadherin levels. Increased expression of a second cadherin type competes for p120 binding, causing the internalization of the first cadherin type. This activity allows p120 to serve as a master regulator of cadherin expression in cells (Ferreri et al., 2008; Maeda et al., 2006; Xiao et al., 2003a, 2005). (B) p120 binds to cadherins and masks an endocytic adaptor binding site. When p120 dissociates from the cadherin, the adaptor binding site is exposed, allowing the endocytic adaptor to bind to the cadherin, triggering cadherin endocytosis (Nanes et al., 2012).

Table 2.1: Tissue-specific p120-null mouse models display phenotypes characteristic of decreased cadherin levels and impaired intercellular adhesion.

Tissue / Cell Type	Phenotype	Reference
Salivary gland	E-cadherin levels reduced; acinar development blocked	Davis and Reynolds, 2006
Skin	Reduced levels of cadherins and other adherens junction proteins; chronic inflammation due to NFκB activation	Perez-Moreno et al., 2006
Hippocampal neurons	Decreased cadherin levels; fewer synapses	Elia et al., 2006
Endothelium	VE-cadherin and N-cadherin levels reduced; vascular patterning defects and hemorrhaging	Oas et al., 2010
Intestinal epithelium	Down-regulation of adheres junction proteins; compromised barrier function	Smalley-Freed et al., 2010
Oropharyngeal epithelium	Decreased E-cadherin expression; development of invasive squamous cell carcinoma	Stairs et al., 2011
Kidney	Decreased cadherin levels; impaired tubule morphogenesis; development of cystic kidney disease	Marciano et al., 2011
Cochlea	Decreased cadherin levels; convergent extension defects	Chacon-Heszele et al., 2012

endocytosis and that cadherin can also be stabilized by a p120 mutant unable to inhibit Rho (Chiasson et al., 2009). Neither of these observations support a role for RhoA in p120 regulation of cadherin endocytosis. Lastly, our observation that p120 prevents VE-cadherin from clustering into AP-2– and clathrin-enriched membrane domains directly supports the hypothesis that p120 masks an endocytic adaptor binding site on the cadherin cytoplasmic tail (Chiasson et al., 2009). This model received additional support from the recently published crystal structure of a portion of the E-cadherin cytoplasmic domain in complex with p120. The E-cadherin–p120 interface contains both static and dynamic binding regions, an interaction which could support binding competition or regulated exchange with an endocytic adaptor protein (Ishiyama et al., 2010). In fact, Chapter 3 presents new evidence that the VE-cadherin juxtamembrane domain contains a novel dual-function motif, which alternately serves as a binding site for p120 or as an endocytic signal (Nanes et al., 2012).

Numerous studies of animal models have underscored the physiological importance of

p120 to adherens junction regulation, at least in mammals. p120 binding to cadherin is apparently dispensable in *Drosophila* and *C. elegans* (Myster et al., 2003; Pacquelet et al., 2003; Pettitt et al., 2003). However, p120 binding is critical for adherens junction stability in mice. Numerous tissue-specific p120-null mouse models have been developed, and all of them display disrupted cadherin-mediated cell adhesion (summarized in Table 2.1). The reasons for the different requirements for p120 in mammals and invertebrates remains unknown. Though, as outlined above, cadherin trafficking pathways in *Drosophila* appear similar to those in mammalian systems, there may be significant differences in their regulation. Interestingly, the p120 sub-family of catenins is considerably larger in vertebrates than in invertebrates, with additional members including p0071, δ-catenin/NPRAP, ARVCF, and the plakophilins (Carnahan et al., 2010; Hatzfeld, 2005). These observations suggest that vertebrate tissue patterning requires additional levels of control over cadherin trafficking, with both the expanded role of vertebrate p120 and the expanded size of the vertebrate p120 sub-family serving as points of regulation not present in simpler organisms.

2.4.2 β-catenin and α-catenin

Another cytoplasmic binding partner of cadherins is β-catenin, which binds to the C-terminal portion of the cadherin cytoplasmic tail, termed the catenin-binding domain. β-catenin plays an important role in adherens junction structure, contributing to the link between cadherins and the actin cytoskeleton (Hartsock and Nelson, 2008). β-catenin binding to cadherins is clearly important for its ability to recruit α-catenin, which, through a mechanism that is not fully understood, links cadherins to actin (Yamada et al., 2005). In fact, this may be the primary role of β-catenin in adherens junctions, since mutant cadherin which cannot bind to β-catenin but is fused to α-catenin forms junctions that are apparently normal (Nagafuchi et al., 1994; Pacquelet and Rørth, 2005). Further support for the hypothesis that β-catenin stabilizes adherens junctions through the recruitment of α-catenin comes from a knock-in mouse model recently created by Schulte and colleagues with a mutant VE-cadherin which does not bind to β-catenin but is fused to α-catenin replacing the wild-type VE-cadherin gene. The mutant mice are viable, though they are not born at mendelian frequencies, and are resistant to inflammatory stimuli that trigger in-

creased vascular permeability in wild-type mice, suggesting supra-physiological stabilization of their endothelial adherens junctions (Schulte et al., 2011).

Though β-catenin clearly has an important role in adherens junction regulation, its role in cadherin trafficking is far from clear. One report does suggest that β-catenin is required for proper cadherin localization and that disrupting β-catenin binding to cadherins results in cadherin accumulation in intracellular compartments (Chen et al., 1999). However, other studies have yielded conflicting results, though several studies have found at least circumstantial evidence for a β-catenin role in cadherin trafficking. First, Dupre-Crochet and colleagues found that casein kinase 1 (CK1) inhibition stabilizes adherens junctions, while CK1 over-expression disrupts adherens junctions. CK1 phosphorylates E-cadherin, primarily on a serine residue within the catenin binding domain. They also found that a phosphomimetic mutation at that site weakens β-catenin binding to E-cadherin and increases E-cadherin internalization (Dupre-Crochet et al., 2007). Second, Tai and colleagues report that in cultured hippocampal neurons, NMDA inhibits N-cadherin turnover and causes β-catenin to accumulate in dendritic spines. Both effects are related to β-catenin phosphorylation (Tai et al., 2007). Lastly, Sharma and colleagues report that β-catenin is internalized by macropinocytosis in cultured fibroblasts, and that internalized β-catenin co-localizes with N-cadherin. This process appears to be mediated by IQGAP1 binding to β-catenin (Sharma and Henderson, 2007). These three accounts are somewhat contradictory. The first two suggest that β-catenin binding to cadherin inhibits its endocytosis, while the last one suggests that β-catenin binding has a role in mediating cadherin endocytosis. Complicating things further, Miyashita and Ozawa report that, while β-catenin binding to E-cadherin may affect E-cadherin localization, the mechanism is unrelated to cadherin turnover. They find that an E-cadherin mutant which cannot bind to β-catenin is mislocalized to an intracellular compartment. However, this mislocalization occurs even with the co-expression of dominant-negative dynamin, which blocks all dynamin-mediated endocytosis. Interestingly, mislocalization of the non-β-catenin-binding mutant cadherin is dependent on the dileucine motif important for clathrin-mediated internalization of E-cadherin; mutant cadherin which cannot bind β-catenin and lacks the dileucine motif traffics to the plasma membrane and does not accumulate intracellularly (Miyashita and

Ozawa, 2007a). Given the conflicting evidence, more work is needed to understand how β- and α-catenin-mediated cytoskeletal linkages might affect cadherin endocytosis, as well as any other effects that β-catenin binding to cadherins might have on cadherin trafficking.

2.5 Regulation of cadherin endocytosis and degradation by ubiquitination

Cadherin ubiquitination also plays an important role in regulating cadherin turnover. Proteins are selected for ubiquitination through interaction with E3 ubiquitin ligase proteins which recruit E2 ubiquitin conjugating enzymes charged with ubiquitin and catalyze the transfer of ubiquitin to the target molecule, usually on lysine residues. Ubiquitin molecules can be attached singly or linked together to form a poly-ubiquitin chain. While poly-ubiquitination is usually associated with targeting intracellular proteins for degradation by the 26S proteasome, mono-ubiquitination can also trigger the endocytosis and lysosomal degradation of membrane proteins (Clague and Urbé, 2010). Because of its association with endocytosis and degradation, cadherin ubiquitination has been an attractive candidate process for regulating cadherin turnover. Additionally, as a posttranslational modification, cadherin ubiquitination could potentially be influenced by a variety of signaling pathways, ensuring ample control points for the modulation of cadherin endocytosis and degradation. Circumstantial support for a role for ubiquitination in cadherin turnover comes from studies showing that proteasome inhibitors such as MG-132 can block cadherin endocytosis, though the mechanism of this effect remains unclear (Xiao et al., 2003b). In fact, a significant body of work has now developed to establish the importance of ubiquitination in cadherin turnover.

The first ubiquitin ligase identified to target cadherin was Hakai, a c-Cbl–like protein with phosphotyrosine-binding, RING finger, and proline-rich domains characterized by Fujita and colleagues. Hakai associates with and ubiquitinates E-cadherin, causing its internalization. Interestingly, this function is dependent on Src-mediated phosphorylation of E-cadherin at two specific tyrosine residues in the juxtamembrane domain (Fujita et al., 2002). This both explains the previously reported ability of v-Src to transform cultured

epithelial cells to a fibroblastic phenotype (Behrens et al., 1993) and provides a potential explanation for the ability of p120 to inhibit cadherin internalization, since p120 binding could mask or prevent the phosphorylation of the E-cadherin tyrosine residues required for Hakai binding. However, these tyrosine residues are not conserved in all classical cadherins. P-cadherin contains only one of the two tyrosine residues, and N- and VE-cadherins lack both of them. Hakai-mediated down-regulation of cadherins therefore may not play a role at all adherens junctions.

Further work by Palacios and colleagues has clarified the mechanism of Hakai-induced E-cadherin turnover. Hakai-mediated ubiquitination of E-cadherin may not directly trigger E-cadherin internalization, since an E-cadherin mutant that cannot interact with Hakai can still be internalized. However, Hakai-mediated ubiquitination of E-cadherin changes the destination of E-cadherin once it has been internalized, redirecting it from a recycling pathway to degradation in the lysosome (Palacios et al., 2005). This redirection requires Hrs, a ubiquitin-interacting protein with a role in shuttling mono-ubiquitinated cargo to the lysosome (Palacios et al., 2005; Toyoshima et al., 2007). Studies have also linked Hakai to developmental and disease processes. Hakai is essential for the maintenance of epithelial integrity in *Drosophila*, though its interaction with DE-cadherin is considerably different than the interaction of mammalian Hakai with E-cadherin. *Drosophila* Hakai can interact with DE-cadherin based on the extracellular and transmembrane portions of the cadherin without the intracellular portion (Kaido et al., 2009). Because Hakai is a cytoplasmic protein, it is not clear how this interaction can occur without the assistance of another protein. Hakai has also been linked to disease in some human colorectal carcinomas, where elevated Slit–Robo signaling induces an epithelial to mesenchymal transformation by recruiting Hakai to ubiquitinate E-cadherin, causing its down-regulation. Elevated Slit–Robo signaling is also associated with increased risk of metastasis and decreased survival (Zhou et al., 2011), although this would seem to conflict with another study which found that Slit–Robo signaling recruited VE-cadherin to cell–cell junctions and was associated with increased survival in rodent sepsis models (London et al., 2010). Though the function of Hakai may be limited to only a subset of adherens junctions, it clearly plays an important role.

Hakai is not the only ubiquitin ligase that has been connected to adherens junction

turnover. The ubiquitin ligase MDM2 also ubiquitinates and causes the degradation of E-cadherin, and in human breast carcinoma specimens, increased MDM2 expression was associated with decreased E-cadherin protein levels (Yang et al., 2006). A third ubiquitin ligase, the viral protein K5, has also been shown to target VE-cadherin (Chapter 4; Mansouri et al., 2008). K5 is expressed by human herpesvirus-8 (HHV-8), which causes the angioproliferative neoplasm Kaposi sarcoma. K5 is thought to play a role in the virus's ability to evade the host immune response by ubiquitinating and causing the internalization of immune recognition components such as the class I major histocompatibility complex. The increased vascular permeability associated with Kaposi sarcoma may be due to a similar mechanism inducing the endocytosis and down-regulation of VE-cadherin (Qian et al., 2008). In fact, as discussed in Chapter 4, K5-mediated ubiquitination of VE-cadherin displaces p120 and induces cadherin endocytosis. Because K5 is a member of the membrane-associated RING-CH (MARCH) family of ubiquitin ligases, which includes several human proteins expressed in a variety of tissues (Nathan and Lehner, 2009), it is possible that HHV-8 may be appropriating a more generally important cellular mechanism for cadherin regulation involving endogenous MARCH proteins.

2.6 Growth factor signaling and cadherin endocytosis

Cell–cell junctions are fundamental links between a cell and its environment. It is not a surprise then, that adherens junctions are not regulated only by intracellular processes, but also by intercellular cues. A variety of growth factor signaling pathways have been tied to the dynamic regulation of cadherin endocytosis, including hepatocyte growth factor (HGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor β (TGFβ). Many of these pathways affect cadherin trafficking and catenin binding, which are discussed above in more detail.

The first growth factor receptor associated with cadherin endocytosis was the HGF receptor, c-Met (Figure 2.3 A). HGF is also called scatter factor for its ability to stimulate epithelial cell motility. Treatment of cultured cells with HGF or a small molecule HGF receptor agonist causes the co-endocytosis of the HGF receptor and associated E-cadherin

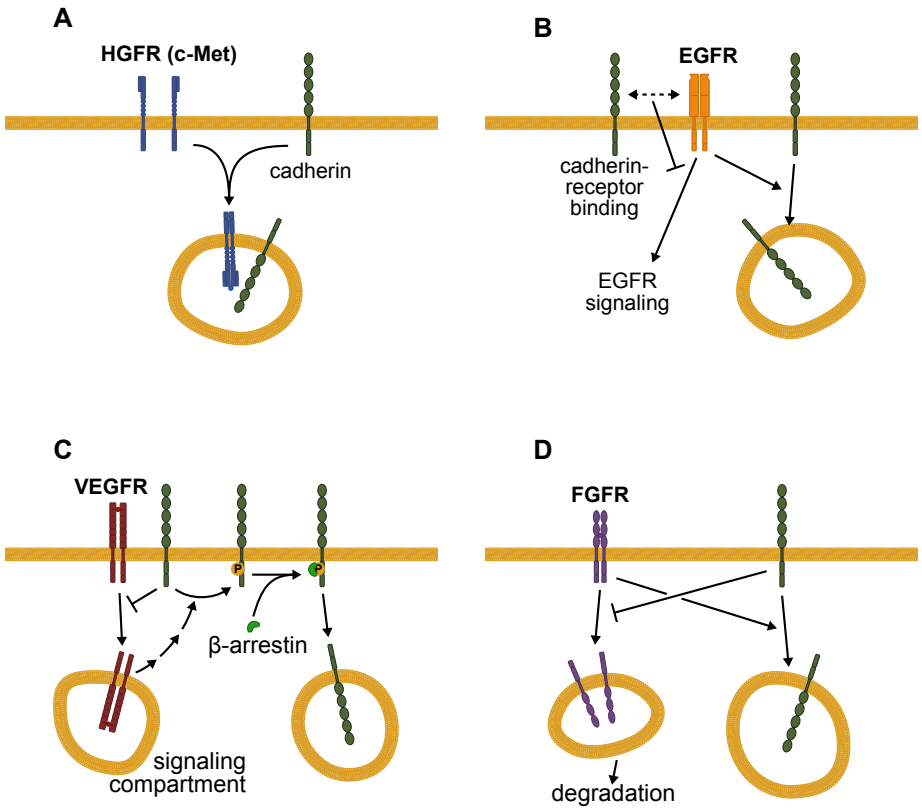

Figure 2.3: *Growth factor signaling pathways influence cadherin endocytosis.* (A) HGF activation of c-Met causes co-endocytosis of the receptor with E-cadherin (Kamei et al., 1999; Palacios et al., 2001). (B) EGFR over-expression induces E-cadherin endocytosis and E-cadherin binding to the receptor inhibits EGFR signaling (Bremm et al., 2008; Bryant et al., 2007; Lu et al., 2003). (C) VEGFR activation triggers the phosphorylation of VE-cadherin through a Src, Vav2, Rac, and PAK signaling cascade. Phosphorylated VE-cadherin recruits β-arrestin and triggers clathrin-mediated endocytosis of the cadherin (Gavard and Gutkind, 2006; Gavard et al., 2008). Conversely, VE-cadherin inhibits the internalization of VEGFR into signaling compartments upon ligand binding (Lampugnani et al., 2006). (D) FGFR activation induces E-cadherin endocytosis, and cadherins inhibit the endocytosis and degradation of FGFR (Bryant et al., 2007, 2005; Suyama et al., 2002).

(Kamei et al., 1999). This effect requires the activation of the small GTPase Arf6 (Palacios et al., 2001). Additionally, HGF signaling causes Numb, an endocytic adaptor which may play a role in establishing the lateral localization of cadherins by facilitating their specific endocytosis from the apical surface, to decouple from E-cadherin and associate with aPKC and Par6 instead, disrupting cell polarity (Wang et al., 2009). Thus, HGF appears to cause both general down-regulation of cadherin and disruption of adherens junction polarity. Both effects are consistent with the ability of HGF to induce a fibroblast-like phenotype. However, the cause of HGF-mediated cell scattering remains in dispute, since, in MDCK cells, HGF enhances integrin-mediated interactions with the extracellular matrix which pull the cells apart, but does not appear to disrupt E-cadherin mediated adhesion (de Rooij et al., 2005). More work will be needed to understand the functional importance and precise mechanism of HGF-mediated cadherin endocytosis.

EGF signaling has also been tied to cadherin endocytosis (Figure 2.3 B). The effect of EGF receptor signaling is notable because it causes cadherin internalization through a clathrin-independent pathway. As discussed above, however, beyond clathrin independence, there is disagreement over which endocytic pathway is involved. Lu and colleagues reported that EGF receptor over-expression caused E-cadherin internalization through a caveolin-mediated pathway (Lu et al., 2003). In contrast, Bryant and colleagues reported that EGF induced E-cadherin internalization through macropinocytosis (Bryant et al., 2007). More work will need to be done to sort out these conflicting findings. Interestingly, the relationship between the EGF receptor and cadherins appears to be bidirectional. Certain mutations in the extracellular domain of E-cadherin are associated with decreased formation of E-cadherin–EGF receptor complexes, resulting in increased EGF receptor signaling in both cultured cells and human gastric carcinoma samples (Bremm et al., 2008). This finding suggests that while EGF signaling can cause E-cadherin endocytosis, E-cadherin can inhibit EGF signaling. Clearly, adherens junctions are not simply acted upon by signaling pathways, but are active participants in them as well.

A third growth factor associated with cadherin internalization is VEGF, an important growth factor in vasculogenesis and angiogenesis, which increases vascular permeability by disrupting endothelial cell–cell junctions (Figure 2.3 C). Gavard and Gutkind demon-

strated that VEGF signaling causes the Src-mediated phosphorylation of VE-cadherin, resulting in the recruitment of β-arrestin and the subsequent clathrin-mediated endocytosis of VE-cadherin (Gavard and Gutkind, 2006). The pathway is interrupted by angiopoietin-1, which strengthens vascular integrity and decreases permeability. Angiopoietin-1 inhibits Src activation by the VEGF receptor, counteracting VEGF-mediated cadherin internalization (Gavard et al., 2008). As with E-cadherin and the EGF receptor, the relationship between VE-cadherin and the VEGF receptor is bidirectional. In cell culture, confluent endothelial cells are resistant to the effects of VEGF, an effect which requires both VE-cadherin and β-catenin (Lampugnani et al., 2003). VE-cadherin association with the VEGF receptor prevents VEGF receptor internalization in response to VEGF binding. When internalized in response to VEGF binding, the VEGF receptor is not degraded. Rather, it enters an endosomal signaling compartment where it activates the MAP kinase pathway. Thus, by preventing VEGF receptor endocytosis, VE-cadherin can inhibit VEGF signaling (Lampugnani et al., 2006).

A similar two-way interaction also occurs between cadherins and the FGF receptor (Figure 2.3 D). FGF activation of the FGF receptor induces macropinocytosis of E-cadherin (Bryant et al., 2007, 2005). Conversely, increased expression of E- or N-cadherin inhibits internalization of the FGF receptor (Bryant et al., 2005; Suyama et al., 2002). In contrast to the VEGF receptor, however, internalization of ligand-bound FGF receptor serves to shut off FGF signaling, primarily through subsequent degradation of the receptor. Thus, FGF signaling down-regulates cadherins and cadherins support FGF signaling, essentially forming a negative-feedback loop. Lastly, cadherin trafficking can be affected by TGFβ signaling. TGFβ and Raf-1 synergistically induce E-cadherin endocytosis and epithelial to mesenchymal transition in mammary epithelial cells (Janda et al., 2006). Interestingly, TGFβ- and Raf-1–induced cadherin internalization is associated with cadherin ubiquitination.

The large variety of growth factor signaling pathways affecting cadherin endocytosis clearly indicates the importance of the dynamic and coordinated regulation of cadherin internalization and intercellular adhesion. More work is needed, however, to understand how these disparate pathways are interrelated in different biological contexts. The potential for two-way communication between growth factor receptors and adherens junctions is

particularly intriguing, and the full potential of these mechanisms has yet to be explored.

2.7 Cadherin shedding

In this chapter, we have focused mainly on down-regulation of adherens junctions through the removal of cadherin from the cell surface. However, this is not the only mechanism available for reducing the amount of cadherin available to form adhesive contacts. In some situations, cadherins may be proteolytically cleaved while they remain at the plasma membrane. This process, often termed cadherin "shedding," can lead to the release of cadherin extracellular domains from the cell or fragments of the cadherin cytoplasmic tail into the cytoplasm, with potential effects beyond loss of adhesion.

Released fragments of cadherin extracellular domains were first identified as factors that inhibited cell adhesion in conditioned medium from a breast cancer cell line (Damsky et al., 1983; Wheelock et al., 1987). Inducing E-cadherin shedding in cell culture can also promote cell invasion into a collagen substrate (Noë et al., 2001). Consequently, there has been considerable excitement for the possible involvement of cadherin shedding in the loss of intercellular adhesion in cancer and the use of cadherin extracellular domain fragments as tumor biomarkers. However, results from observational studies have been mixed (reviewed in De Wever et al., 2007). While serum levels of E-cadherin extracellular domains are elevated approximately three-fold in patients with several types of cancer, there is no correlation with disease progression. It is also possible that increased cadherin shedding detected in these studies is related to general inflammatory processes rather than to the tumor specifically (Pittard et al., 1996). In addition to possible roles in cancer and inflammation, cadherin shedding appears to be involved in several developmental processes. N-cadherin is cleaved during chick retinal development, where, counter-intuitively, the truncated product promotes cell adhesion and neurite development (Paradies and Grunwald, 1993). N-cadherin shedding has also been reported in neural crest delamination and in adult neurons (Marambaud et al., 2003; Shoval et al., 2007). Lastly, in response to Eph–ephrin signaling, E-cadherin shedding plays a role in cell sorting (Solanas et al., 2011). Given the variety of processes in which it has been implicated, cadherin shedding

appears to have an important role in development. However, more work will need to be done to understand the role cadherin shedding in more detail and in additional developmental processes.

Many of the proteases responsible for cadherin shedding have been identified. Members of the "a disintegrin and metalloprotease" (ADAM) family, and ADAM10 in particular, appear to be an important generators of free E-cadherin and N-cadherin extracellular domain fragments (Maretzky et al., 2005; Reiss et al., 2005). Interestingly, EGFR-mediated down-regulation of desmosomal cadherins appears to occur, at least in part, through ADAM proteases, a result suggesting how cadherin shedding might be connected to signaling pathways (Klessner et al., 2009). A variety of other proteases have also been implicated in cadherin shedding, including matrix metalloproteinases and kallikreins (Klucky et al., 2007; McGuire et al., 2003; Noë et al., 2001). Still other proteases, including caspases and presenilin, can cleave cadherins intracellularly, releasing a soluble cadherin fragment into the cytoplasm (Marambaud et al., 2002). Interestingly, these intracellular fragments can traffic to the nucleus, potentially affecting a variety of transcription factors (Ferber et al., 2008). The relationship of intracellular cadherin proteolysis to extracellular cadherin shedding is not yet understood, but, in addition to modulating intercellular adhesion, these mechanisms have the potential to integrate adherens junctions with cell signaling networks.

2.8 Summary and future perspectives

Cadherin endocytosis and degradation play crucial roles in the dynamic control of intercellular adhesion. By adjusting the rate of cadherin internalization, cells are able to quickly modify the strength of their adherens junctions, rearranging their relationship with their environment. This process is absolutely critical during development, and, as we have seen, cadherin endocytosis and degradation have been linked to a growing number of developmental processes in a variety of species. A particularly exciting area of current research focuses on planar-cell-polarized endocytosis of cadherin as a mechanism for the establishment of planar polarization of an epithelial layer. The role of cadherin endocytosis during development may turn out to be more complicated—and more important—than simply

allowing cells to switch between epithelial and mesenchymal phenotypes. The misregulation of cadherin endocytosis also appears to be increasingly important in disease processes, and, consequently, as a possible therapeutic target. However, our understanding remains incomplete, and devising a new generation of anti-cancer drugs targeting cadherin endocytosis will require further work.

In addition to contributing to our understanding of the role of cadherin internalization in development and disease, recent work has also advanced our understanding of the molecular mechanisms underlying cadherin endocytosis. In particular, we have learned a great deal about clathrin-mediated cadherin endocytosis and its contribution to adherens junction dynamics. However, more needs to be done in order to characterize the clathrin-independent endocytic pathways that cadherins can enter, as well as to better understand which pathways are active in different biological contexts. Furthermore, while several endocytic adaptors have been associated with adherens junction turnover, the nature of the interactions between these adaptors and cadherins remains largely unknown. In order to unwind the pathways regulating cadherin endocytosis, it will be necessary to more precisely identify the cadherin domains which drive their removal from the cell membrane. Do cadherin endocytic signals overlap with the p120 binding site, allowing p120 to compete with endocytic adaptors for cadherin binding, thus stabilizing cadherins at the cell membrane? Furthermore, how does cadherin shedding relate to cadherin internalization? The first question will be addressed in Chapter 3, but an answer to the second must await further investigation.

In addition to better understanding the molecular mechanisms of cadherin endocytosis, another important focus of future research will be the signaling pathways that allow for its dynamic regulation. One possibility is raised by studies supporting the role of α- and β-catenins in cadherin regulation. Since α- and β-catenins link cadherins to the actin cytoskeleton, might this link play some role in cadherin trafficking? For now, the evidence is unclear. A second possibility is that cadherin ubiquitination may be used as signal to promote cadherin endocytosis. Several ubiquitin ligases have been found to mediate the ubiquitination and down-regulation of cadherins. However, based on what is known so far, the scope of each of the pathways identified remains limited to specific biological con-

texts. Further research will be needed to determine whether cadherin ubiquitination is a broadly applicable mechanism that regulates cell–cell adhesion. Finally, the many growth factor signaling pathways implicated in cadherin endocytosis suggest several opportunities to link intercellular contacts to intercellular signaling. The possibility that this relationship might be bidirectional, allowing growth factors to affect cadherin endocytosis and cadherins to affect growth factor signaling pathways, is particularly exciting. Still, it will take more work to integrate the disparate pathways that have been identified.

Though our understanding of cadherin internalization and degradation and the mechanisms that regulate them is far from complete, much has been learned in the decades since cadherin endocytosis was first observed in response to calcium depletion. Cadherin endocytosis is now recognized as an important factor in the dynamic control of intercellular adhesion. It remains an active area of research, with the promise to further our understanding of the ever-changing adhesive interactions between cells and the implications of adherens junction dynamics for development and disease.

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Chapter 3

p120-catenin binding masks an endocytic signal conserved in classical cadherins

Abstract

p120-catenin (p120) binds to the cytoplasmic tails of classical cadherins and inhibits cadherin endocytosis. Although p120 regulation of cadherin internalization is thought to be important for adhesive junction dynamics, the mechanism by which p120 modulates cadherin endocytosis is unknown. Here, we identify a dual-function motif in classical cadherins consisting of three highly conserved acidic residues that alternately serve as a p120 binding interface and an endocytic signal. Mutation of this motif resulted in a cadherin variant that was both p120-uncoupled and resistant to endocytosis. In endothelial cells, where dynamic changes in adhesion are important components of angiogenesis and inflammation, a vascular endothelial (VE)-cadherin mutant defective in endocytosis assembled normally into cell–cell junctions but potently suppressed cell migration in response to vascular endothelial growth factor (VEGF). These results reveal the mechanistic basis by which p120 stabilizes cadherins and demonstrate that VE-cadherin endocytosis is crucial for endothelial cell migration in response to an angiogenic growth factor.

This chapter is adapted from:

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3.1 Introduction

Dynamic and coordinated changes in cell adhesion are essential for cell migration, tissue patterning, and wound healing. Adherens junctions and their principal cell–cell adhesion molecules, the classical cadherins, are well described (Harris and Tepass, 2010; Saito et al., 2012). However, adherens junction regulation remains poorly understood, limiting our ability to relate static models of cadherin-based adhesion to dynamic biological processes. Cadherins mediate cell adhesion through their extracellular domains, which form calcium-dependent trans interactions with cadherin molecules on adjacent cells. The cadherin cytoplasmic tail couples cadherins to the actin cytoskeleton and engages in a variety of signaling and membrane trafficking activities. The cytoplasmic tail can be divided into two regions, the catenin-binding domain at the C-terminus of the molecule and the more N-terminal juxtamembrane domain. Each of these domains binds to members of the armadillo family of proteins. The catenin-binding domain binds to β-catenin, which links cadherins to the actin cytoskeleton through a mechanism that is not completely understood (Drees et al., 2005; Taguchi et al., 2011; Yamada et al., 2005). The juxtamembrane domain binds to p120-catenin (p120), an important regulator of adherens junction stability. In the absence of p120, cadherins are rapidly internalized from the cell surface and degraded in the lysosome (Davis et al., 2003; Miyashita and Ozawa, 2007b; Xiao et al., 2003a,b). Because modulation of cadherin availability at the cell surface has emerged as a key factor determining adhesion strength, and because cadherin endocytosis can drive junction disassembly (Troyanovsky et al., 2006), understanding how p120 controls cadherin endocytosis is necessary to understand dynamic regulation of cell adhesion.

In the endothelium, dynamic changes in vascular endothelial (VE)-cadherin–mediated adhesion are important components of angiogenesis and inflammation, and improper regulation of endothelial cell adhesion can facilitate cancer metastasis (Dejana et al., 2008; Vincent et al., 2004). Similarly to other classical cadherins (Le et al., 1999), VE-cadherin undergoes clathrin-mediated endocytosis (Chiasson et al., 2009; Xiao et al., 2005). In endothelial cells, p120 serves as a master regulator of cadherin expression, balancing cellular levels of VE-cadherin with N-cadherin, which is also expressed in endothelial cells,

but typically does not contribute significantly to adherens junctions (Ferreri et al., 2008). Endothelial-specific knockout of p120 causes hemorrhaging, defects in vessel patterning, and embryonic lethality, underscoring the importance of p120 regulation of cadherins for endothelial barrier function and vascular development (Oas et al., 2010). Similar results have been reported in a variety of other conditional knockout models (Chacon-Heszele et al., 2012; Davis and Reynolds, 2006; Elia et al., 2006; Kurley et al., 2012; Marciano et al., 2011; Perez-Moreno et al., 2006; Smalley-Freed et al., 2010; Stairs et al., 2011). These findings demonstrate that p120 inhibition of cadherin endocytosis represents a fundamental cellular mechanism that controls cadherin cell surface levels in most cell types.

While previous studies have highlighted the importance of p120 in regulating cadherin cell surface levels, the precise mechanism by which p120 inhibits cadherin endocytosis has remained elusive (Nanes and Kowalczyk, 2012). This lack of mechanistic insight underlies two significant gaps in our understanding of the role of cadherin endocytosis in development and disease. First, it has been difficult to uncouple p120 binding to the cadherin cytoplasmic tail from control of cadherin endocytosis. Disrupting p120 binding triggers cadherin endocytosis, masking other potential effects. As a result, determining whether cadherins recruit p120 for any purpose other than stabilization of the junction has proven difficult. Second, the contribution of cadherin endocytic trafficking to cell behavior and tissue patterning has thus far been studied through broad perturbations of endocytic pathways (de Beco et al., 2009; Jarrett et al., 2002; Kawauchi et al., 2010; Levayer et al., 2011). These strategies also impact trafficking of other membrane proteins, thereby limiting their specificity and complicating interpretations. Thus, identification of cadherin motifs that mediate endocytosis, allowing for selective perturbation of cadherin internalization and permitting loss-of-function experiments in various model systems, has been an important goal.

The results presented here demonstrate that the core p120-binding region of classical cadherins, which mediates the strongest interactions with p120, comprises a highly conserved endocytic signal, and that p120 inhibits cadherin endocytosis by physically occupying this motif. Furthermore, mutation of a three–amino acid acidic cluster uncouples VE-cadherin from p120 while simultaneously preventing endocytosis and stabilizing the

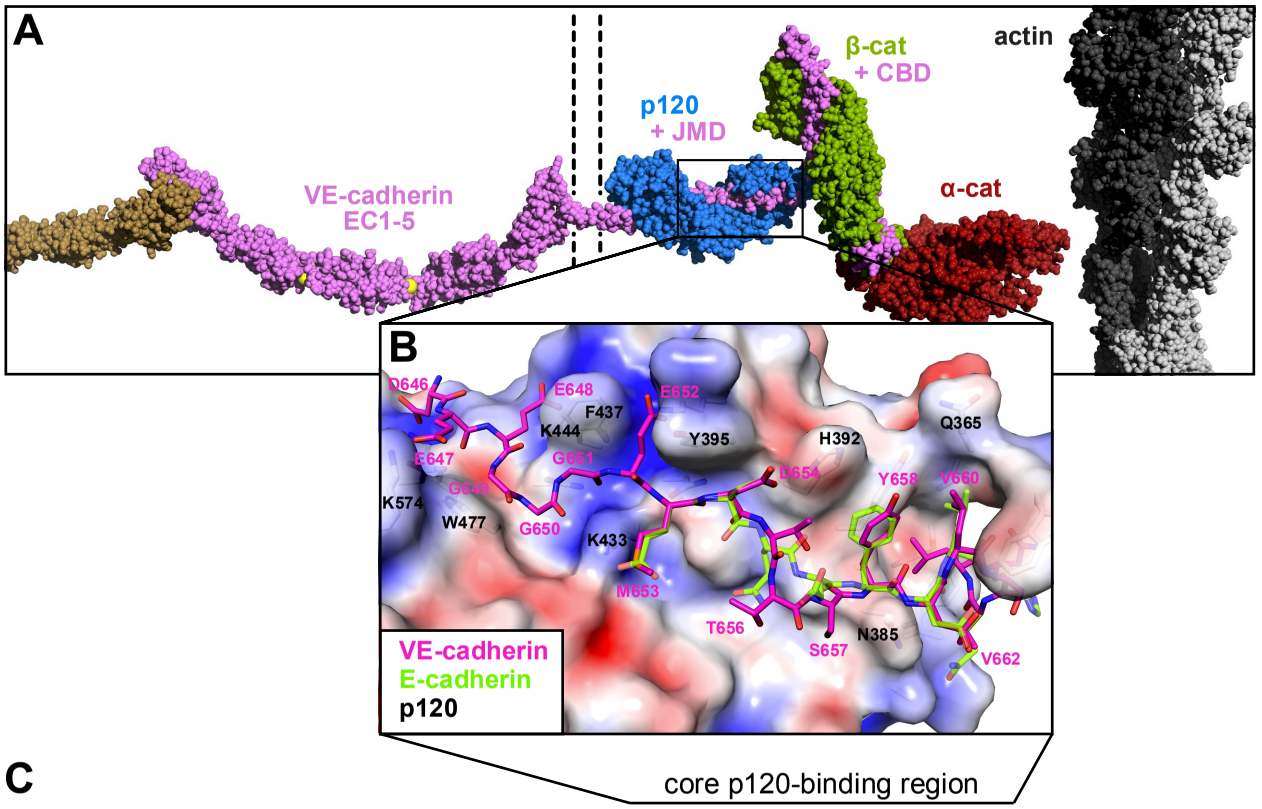
cadherin at the cell surface. Functional analysis of this VE-cadherin mutant reveals a critical role for cadherin internalization in endothelial cell migration in response to the angiogenic agent vascular endothelial growth factor (VEGF). These results demonstrate that the core p120-binding region of classical cadherins is a dual-function motif, which mediates both p120 binding and cadherin endocytosis, and reveal a key role for endocytic processing of cadherins in cell migration.

3.2 Results

3.2.1 The core p120-binding region of classical cadherins is well conserved

p120 binding to the cadherin juxtamembrane domain regulates cadherin cell surface levels by preventing cadherin endocytosis. This regulatory activity requires p120 association with the cadherin tail, but does not require p120 inhibition of RhoA (Chiasson et al., 2009; Ireton et al., 2002; Miyashita and Ozawa, 2007b; Xiao et al., 2003a, 2005). These findings support a model where the cadherin juxtamembrane domain contains an endocytic signal, and p120 binding to the cadherin physically masks that signal (Chiasson et al., 2009; Xiao et al., 2003a). Structural studies have revealed that the interaction between E-cadherin

Figure 3.1 ***(facing page)*****:** *The core p120-binding region of classical cadherins is well conserved.* (A) Schematic illustration of the cadherin–catenin complex. Extracellular cadherin domains mediate adhesion through *trans* interactions with cadherins on the adjacent cell. The cadherin juxtamembrane domain binds to p120 (blue) and the catenin-binding domain interacts with β-catenin (green). β-catenin and α-catenin (red) link the cadherin with the actin cytoskeleton (grey) through a mechanism that is not fully understood. (B) Predicted molecular interface between VE-cadherin and p120 catenin. A simulated three-dimensional model of VE-cadherin juxtamembrane domain residues 646–664 (magenta) bound to the armadillo-repeat domain of p120 (surface electrostatic potential: blue, positive; red, negative) was constructed based on the crystal structure of the E-cadherin juxtamembrane domain (green) bound to p120 (PDB ID: 3L6X). Selected residues of VE-cadherin and p120 are labeled in magenta and black, respectively. (C) Multiple sequence alignment of classical cadherins from human (hm), mouse (ms), and *Drosophila* (dr). Conserved residues are highlighted in green. The core p120-binding region (residues 644–664), which mediates the strongest interactions between the cadherin and p120, is indicated. Other notable features are marked below the alignment: 1, E-cadherin dileucine endocytic signal; 2, E-cadherin Y753 and Y754 Src phosphorylation sites required for Hakai-mediated ubiquitination of E-cadherin; 3–5, VE-cadherin mutations used in Figures 3.4–3.15; Δ644 and Δ657, location of VE-cadherin truncation mutations used in Figure 3.2.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actin
β-cat
+ CBD
p120
+ JMD
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VE-cadherin
EC1-5
B
D646
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F437
E652
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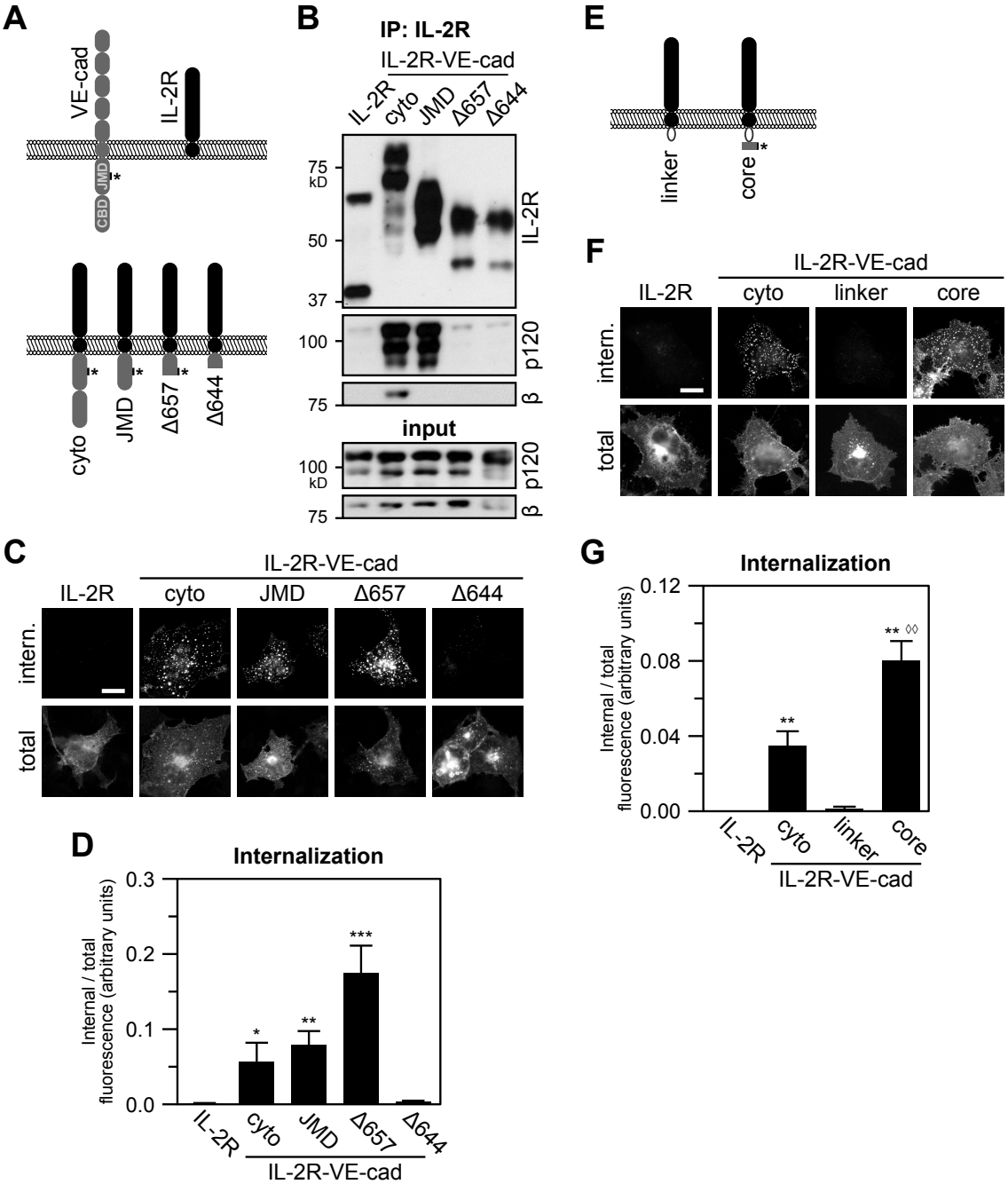
Y395
H392
Q365
E647
G651
D654
Y658
V660
G649
K574
W477
G650
K433
M653
T656
S657
N385
V662
VE-cadherin
E-cadherin
p120
core p120-binding region
C
VE-cad (hm) 623 RLRKQARA-HGKSVPEIHEQLVTYDEEGGGEMDTTSYDVSVLNSVRRGGAKPP 674
VE-cad (ms) 623 RIRKQAHA-HSKSALEIHEQLVTYDEEGGGEMDTTSYDVSVLNSVRGGSTKPL 674
E-cad (hm) 732 RRRAVVKEPLLPPEDDTRDNVYYYDEEGGGEEDQD-FDLSQLHRGLDARPEVT 783
E-cad (ms) 734 RRRTVVKEPLLPPDDDTRDNVYYYDEEGGGEEDQD-FDLSQLHRGLDARPEVT 785
N-cad (hm) 753 ---RQAKQLLIDPEDDVRDNILKYDEEGGGEEDQD-YDLSQLQQPDTVEPDAI 801
P-cad (hm) 678 RKKRKIKEPLLLPEDDTRDNVFYYGEEGGGEEDQD-YDITQLHRGLEARPEVV 729
cad-6 (hm) 636 LRRQRKKEPLIISKEDIRDNIVSYNDEGGGEEDTQAFDIGTLRNPEAIEDNKL 688
DE-cad (dr) 1351 KKQKNG-W-HEKDIDDIRETIINYEDEGGGERDTD-YDLNVLRTQPFYEEKLY 1400
①
②
Δ644
③
④
⑤
Δ657

and p120 contains both a static binding site, with strong interactions between the cadherin and p120, and a dynamic binding site, with weaker transient interactions (Ishiyama et al., 2010). An important endocytic signal in E-cadherin, a putative AP-2–binding dileucine motif (Miyashita and Ozawa, 2007b), falls within the dynamic binding site, suggesting that p120 binding could interfere with this motif, thereby inhibiting E-cadherin endocytosis (Ishiyama et al., 2010). A simulated model of the p120-VE-cadherin complex derived from the p120-E-cadherin crystal structure shows that the interactions of both cadherins with p120 are broadly similar. The important electrostatic and hydrophobic interactions, as well as the general position of the cadherin core p120-binding region along a groove across p120, are conserved in the p120–VE-cadherin model (Figure 3.1 A and B). However, VE-cadherin lacks the dileucine endocytic signal present in E-cadherin (Figure 3.1 C). VE-cadherin does contain a putative tyrosine-based endocytic signal at Y685, though it is located C-terminal to the p120 binding site, making it unlikely to be subject to regulation by p120 binding. Therefore, we hypothesized that the VE-cadherin cytoplasmic tail contains an additional uncharacterized endocytic signal within the p120-binding region.

Figure 3.2 ***(facing page)*****:** *The core p120-binding region of VE-cadherin functions as an endocytic signal.* (A) Portions of the VE-cadherin cytoplasmic tail (grey) were fused to the extracellular and transmembrane domains of the interleukin-2 receptor alpha chain (IL-2R, black) to create chimeric proteins. The core p120-binding region is indicated by an asterisk. CBD, catenin-binding domain; JMD, juxtamembrane domain; cyto, entire VE-cadherin cytoplasmic tail; Δ657 and Δ644, VE-cadherin cytoplasmic tail truncated at residues 657 and 644. (B) IL-2R–VE-cadherin chimeras were expressed in COS cells and isolated by immunoprecipitation of IL-2R. β-catenin (β) co-precipitates only with the chimera containing the catenin-binding domain and p120 co-precipitates only with the chimeras containing the entire juxtamembrane domain. (C) Fluorescence-based internalization assay of IL-2R–VE-cadherin chimeras expressed in COS cells. Internalized chimera (top row) was identified by antibody-labeling of surface IL-2R, followed by a 10-minute incubation to allow internalization and a low-pH wash to remove antibody remaining at the cell surface. Cells were then fixed and stained for total IL-2R (bottom row). (D) Quantification of the ratio of internalized to total chimera. Mean ± SEM (n = 8–16 cells per group); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to IL-2R and Δ644. (E) The VE-cadherin core p120-binding region (core) was fused to IL-2R with a short linker peptide (linker, white oval) used to maintain spacing from the plasma membrane. (F and G) Fluorescence-based internalization assay with a 10-minute internalization period. Mean ± SEM (n = 8–11 cells per group); **, $P < 0.01$ compared to IL-2R; ◊◊, $P < 0.01$ compared to linker. Scale bars: 20 μm.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cyto
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Δ644
75
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IL-2R
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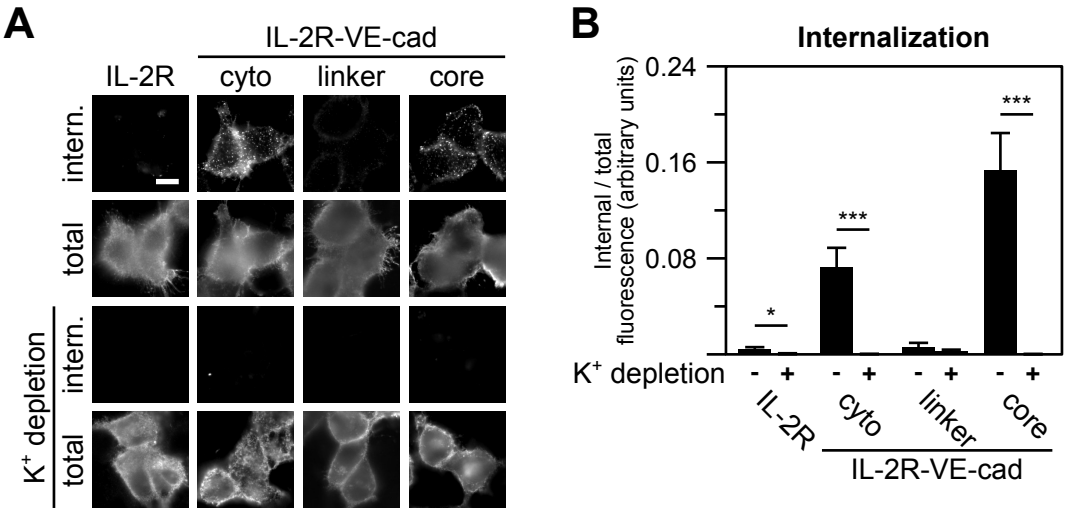


Figure 3.3: *The VE-cadherin core p120-binding region mediates internalization through a clathrin-dependent mechanism.* The IL-2R or chimeras containing the full VE-cadherin cytoplasmic tail (cyto), the VE-cadherin core p120-binding region and a short linker peptide (core), or the linker peptide alone (linker) joined to the IL-2R were expressed in HeLa cells, and their endocytosis was measured using a fluorescence-based internalization assay with a 10-minute internalization period. Prior to internalization, cells were either left untreated or incubated in a potassium depletion buffer to inhibit clathrin-mediated endocytosis (K^+ depletion). Mean ± SEM (n = 15–20 cells per group); *, $P < 0.05$; ***, $P < 0.001$. Scale bar: 10 μm.

3.2.2 The core p120-binding region of VE-cadherin harbors an endocytic signal

A gain-of-function approach was adopted to define the roles of different portions of the VE-cadherin cytoplasmic tail in cadherin internalization. Portions of the VE-cadherin cytoplasmic tail were joined to the extracellular and transmembrane domains of the interleukin-2 receptor alpha chain (IL-2R), a transmembrane protein that does not mediate cell adhesion and is not rapidly endocytosed (Xiao et al., 2003a). Sequential deletions from the intracellular (C-terminal) end of the resulting chimera allowed us to test the requirements for different portions of the VE-cadherin cytoplasmic tail for catenin binding and internalization. As expected, the VE-cadherin catenin-binding domain was required for co-immunoprecipitation of β-catenin with the IL-2R–VE-cadherin chimera, and the VE-cadherin juxtamembrane domain was required for co-immunoprecipitation of p120. Truncating the VE-cadherin cytoplasmic tail at residues 657 or 644, resulting in the removal of part or all of the core p120-binding region respectively, prevented p120 co-

immunoprecipitation (Figure 3.2 A and B).

Using a fluorescence-based internalization assay, we tested which portions of the VE-cadherin cytoplasmic tail were required to mediate endocytosis. The full VE-cadherin cytoplasmic tail and the juxtamembrane domain alone were sufficient to mediate chimera internalization. The 657 truncation chimera was also internalized, even though it lacked the ability to bind p120 (Figure 3.2 C and D). In fact, internalization mediated by the 657 truncation was greater than internalization mediated by the juxtamembrane domain or full cytoplasmic tail, consistent with our model that p120 binding masks an endocytic signal to inhibit internalization. In contrast, the 644 truncation chimera was not internalized, indicating that a significant portion, though not all, of the core p120-binding region is necessary for VE-cadherin endocytosis (Figure 3.2 C and D).

The VE-cadherin core p120-binding region is not only necessary, but also sufficient to mediate endocytosis. Chimeras containing only the VE-cadherin core p120-binding region (residues 644 to 664) joined to the IL-2R by a short linker peptide were internalized, indicating that the core p120-binding region itself doubles as an endocytic signal (Figure 3.2 E–G). To verify that the core p120-binding region mediates endocytosis in a clathrin-dependent manner similar to the full-length VE-cadherin cytoplasmic tail, potassium depletion was used to block clathrin-mediated endocytosis. Internalization mediated by both the full VE-cadherin cytoplasmic tail and the core p120-binding region alone was inhibited (Figure 3.3), indicating that the core p120-binding region functions similarly to the full cytoplasmic tail to mediate endocytosis. Together, these results demonstrate that the core p120-binding region of VE-cadherin also functions as an endocytic signal.

3.2.3 p120 binding can be uncoupled from control of cadherin endocytosis

While there are substantial differences in the juxtamembrane domains of classical cadherins, the core p120-binding regions are well conserved, especially between VE-cadherin residues 644 and 657 (Figure 3.1 C). We mutated three consecutive sets of highly conserved amino acids in the core p120-binding region, DEE 646–648, GGG 649–651, and EMD 652–654 to alanines. Each of these mutations prevented co-immunoprecipitation of p120 with the IL-2R–VE-cadherin chimera (Figure 3.4 A), consistent with the important roles

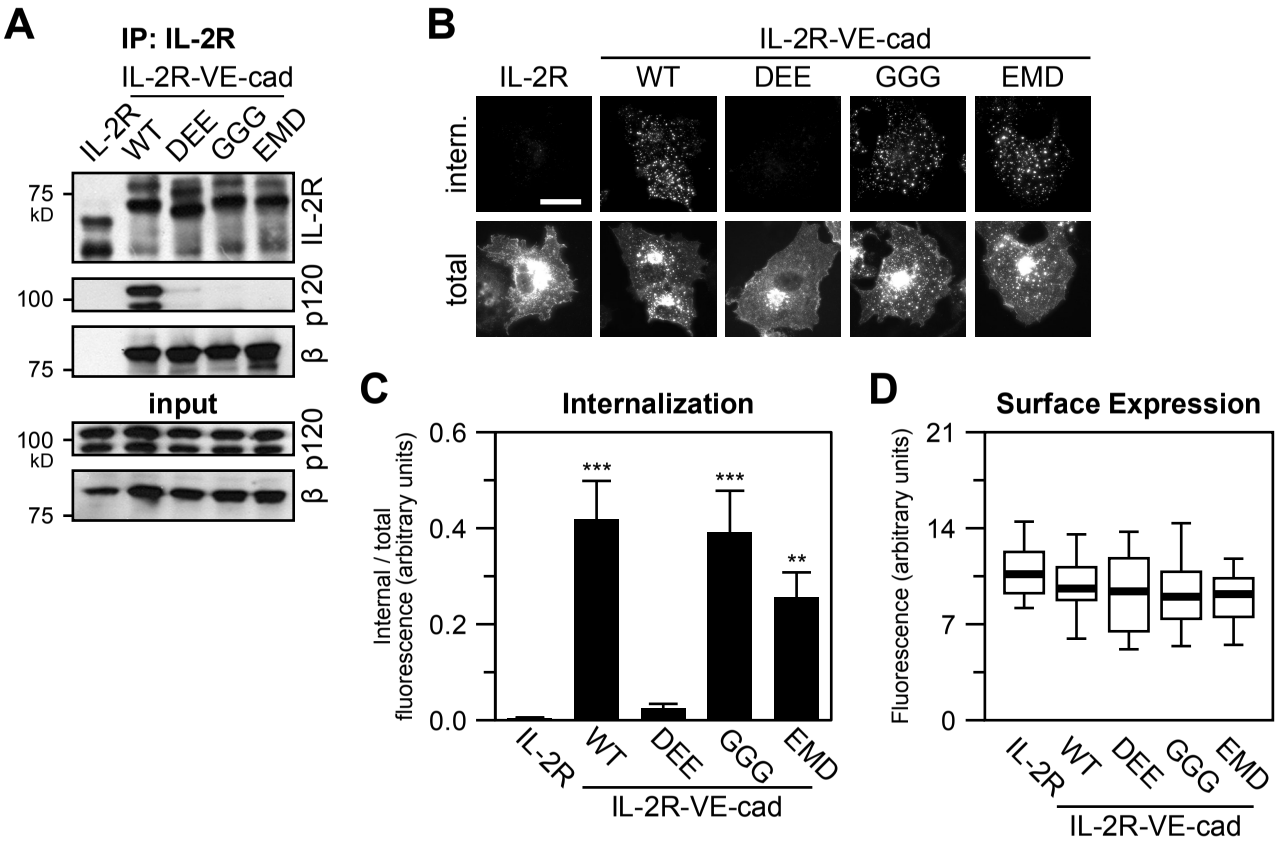


Figure 3.4: *p120 binding can be uncoupled from control of cadherin endocytosis.* Three consecutive sets of amino acids in the core p120-binding region, DEE 646–648, GGG 649–651, and EMD 652–654 (also see Figure 3.1 C), were mutated to alanines in IL-2R–VE-cadherin cytoplasmic tail chimeras and expressed in COS cells. (A) Chimeras were isolated by immunoprecipitation of IL-2R, and co-precipitation of p120 and β-catenin (β) was determined by Western blot. (B and C) Endocytosis of the chimeras was measured using a fluorescence-based internalization assay with a 10-minute internalization period. While each mutation prevented pull-down of p120, only the DEE mutation significantly inhibited endocytosis. Mean ± SEM (n = 15 cells per group); **, $P < 0.01$; ***, $P < 0.001$ compared to IL-2R and DEE mutation. (D) Surface expression of the chimeras in COS cells was quantified by antibody labeling of surface IL-2R and immunofluorescence microscopy. Thick line, median (n = 17–21 cells per group); box, interquartile range; whiskers, 90% range. Scale bar: 20 μm.

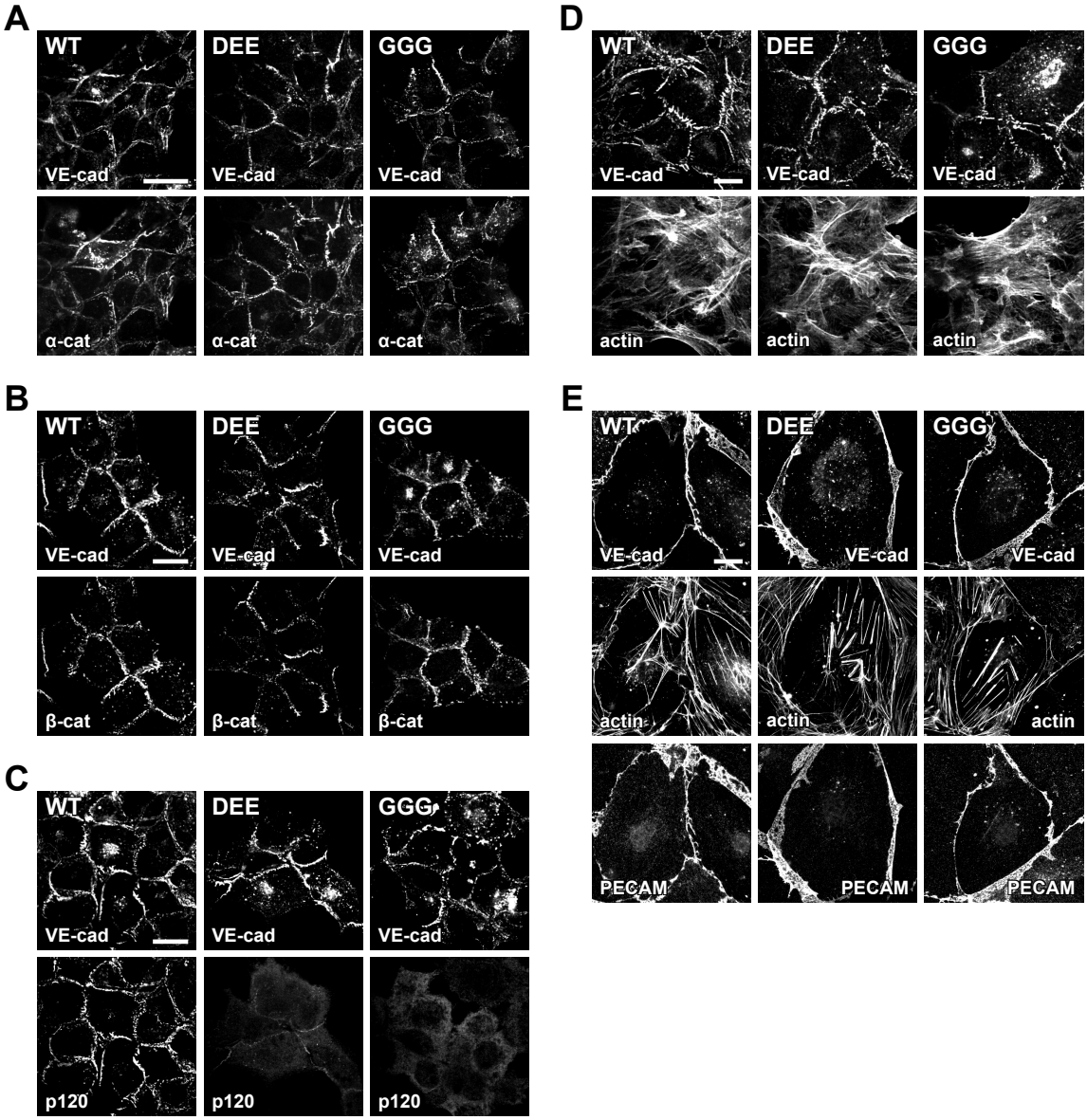


Figure 3.5: *p120 binding is not required for adherens junction assembly.* (A–D) Wild-type (WT), DEE-mutant, or GGG-mutant VE-cadherin with a C-terminal RFP tag was expressed in A-431D cells, which lack endogenous cadherins, using an adenoviral system. Cells were fixed and stained for VE-cadherin and α-catenin (A), β-catenin (B), p120 (C), or actin (D). (E) Wild-type or mutant VE-cadherin–RFP was expressed in primary human microvascular endothelial cells. Cells were fixed and stained for RFP (VE-cad), actin, and PECAM-1. Scale bars: (A–D) 5 μm; (E) 20 μm.

of these amino acids in the VE-cadherin–p120 binding interaction (Figure 3.1 B; Thoreson et al., 2000). However, these three mutations had very different effects on chimera internalization. Mutation of the DEE residues, but not the GGG or EMD residues, almost completely eliminated chimera endocytosis (Figure 3.4 B and C). The EMD mutation resulted in a small decrease in internalization which, while reproducible, did not reach statistical significance, and the GGG mutation had no effect (Figure 3.4 B and C). Control experiments confirmed that the steady-state surface expression levels of each chimera were similar (Figure 3.4 D).

We next verified these results in the context of full-length VE-cadherin. When expressed in A-431D cells, an epithelial cell line that lacks endogenous cadherin expression, wild-type, DEE-mutant, and GGG-mutant VE-cadherin all localized to cell–cell contacts. In addition, wild-type VE-cadherin, as well as both mutants, recruited α- and β-catenins to junctions, while only wild-type VE-cadherin recruited p120 (Figure 3.5 A–C). Neither mutant altered the actin cytoskeleton in A-431D cells (Figure 3.5 D) or in primary human microvascular endothelial cells, where PECAM-1 localization was also unaffected (Figure 3.5 E). Furthermore, the DEE mutation almost completely eliminated full-length VE-cadherin internalization while the GGG mutation did not (Figure 3.6 A and B). Interestingly, GGG-mutant full-length VE-cadherin was internalized more rapidly than wild-type (Figure 3.6 A and B), consistent with its inability to bind p120. Note that this effect was not observed with the GGG-mutant IL-2R–VE-cadherin chimera (Figure 3.4 B and C), probably because the chimeras were expressed in excess of available cellular p120, so even wild-type chimera was mostly unbound by p120 and, as a result, was internalized at a rate similar to the p120-uncoupled GGG-mutant chimera. Further confirming the ability of the DEE mutation to alter cadherin dynamics, time-lapse imaging of VE-cadherin-RFP expressed in primary human microvascular endothelial cells showed that junctions containing wild-type VE-cadherin were dynamic, with visible endocytic events, while junctions containing DEE-mutant VE-cadherin lacked visible endocytic events (Figure 3.6 C). Thus, while both the DEE and GGG mutations disrupt p120 binding, only the DEE mutation inhibits internalization, demonstrating that p120 binding to the cadherin can be uncoupled from control of cadherin endocytosis.

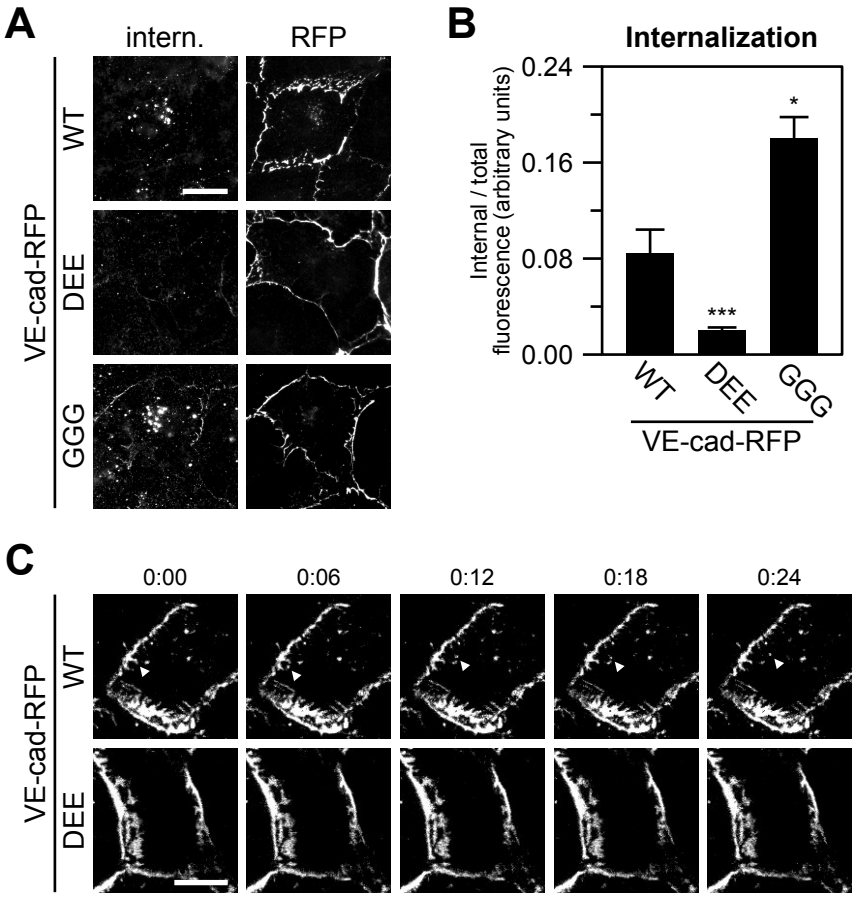


Figure 3.6: *A conserved cluster of three acidic residues is required for full-length VE-cadherin endocytosis.* (A and B) Wild-type or mutant VE-cadherin–RFP was expressed in COS cells, and endocytosis was measured using a fluorescence-based internalization assay with an internalization period of 30 minutes. Antibody labeling and low-pH wash was used to identify internalized cadherin (A, left column), and the RFP tag was used to identify total cadherin (A, right column). Mean ± SEM (n = 20–24 cells per group); ***, $P < 0.001$ compared to WT and GGG mutation; *, $P < 0.05$ compared to WT. (C) Time-lapse images of wild-type and DEE-mutant VE-cadherin–RFP expressed in primary human microvascular endothelial cells. Scale bars: (A) 20 μm; (C) 10 μm.

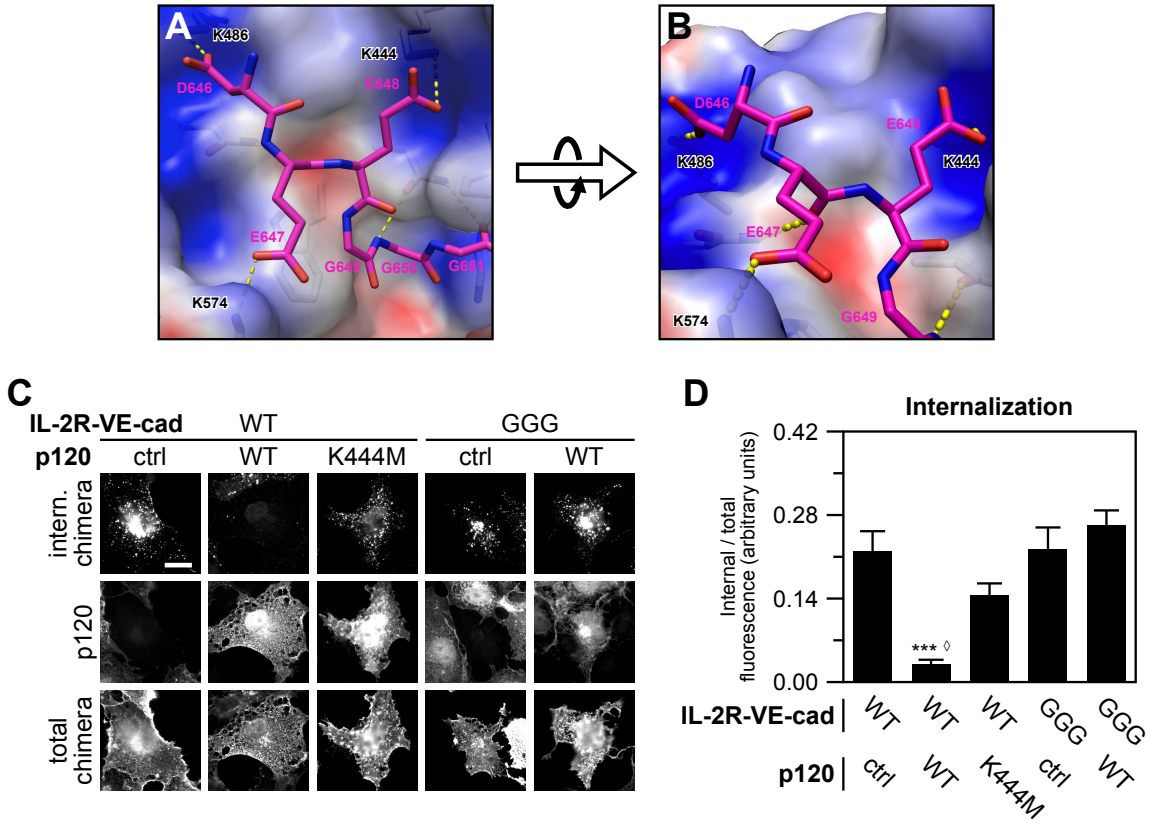


Figure 3.7: *p120 occupies the DEE sequence to prevent cadherin endocytosis.* (A and B) Close views of the predicted molecular interface between VE-cadherin and p120. Selected residues of VE-cadherin and p120 are labeled in magenta and black, respectively. Three negatively charged side-chains of the DEE sequence are enveloped by positively-charged binding pockets (blue) of p120. Hydrogen bonds are indicated by yellow dashes. (C and D) Wild-type (WT) or GGG-mutant IL-2R–VE-cadherin cytoplasmic tail chimeras were expressed in COS cells along with wild-type or K444M mutant fluorescently-tagged p120. Untransfected cells adjacent to cells transfected with the wild-type p120 construct were used as negative controls (ctrl). Endocytosis of the chimeras was measured using a fluorescence-based internalization assay with a 10-minute internalization period. Mutation of p120 K444, which is predicted to interact with the last residue of the DEE endocytic signal (A), disrupts p120-mediated inhibition of IL-2R–VE-cadherin chimera internalization, as does mutation of VE-cad GGG 649–651. Mean ± SEM (n = 12–15 cells per group); ***, $P < 0.001$ compared to no exogenous p120 expression and GGG-mutant chimera; ◊, $P < 0.05$ compared to p120 K444M. Scale bar: 20 μm.

3.2.4 p120 occupies the DEE sequence to prevent cadherin endocytosis

The position of the VE-cadherin DEE endocytic signal within the core p120-binding region strongly suggests that p120 binding inhibits cadherin endocytosis by physically occupying the endocytic signal. Indeed, the structural model identifies important electrostatic interactions between p120 and all three side chains of the DEE sequence (Figure 3.7 A and B). We therefore tested whether a p120 variant mutated at a critical lysine that interacts with the last residue of the DEE sequence (K444M; Ishiyama et al., 2010) was unable to inhibit cadherin endocytosis. Consistent with our model, increased expression of wild-type p120 significantly inhibited IL-2R–VE-cadherin cytoplasmic tail chimera endocytosis, while expression of p120 K444M did not inhibit chimera internalization (Figure 3.7 C and D). Furthermore, increased expression of wild-type p120 did not inhibit internalization of the GGG-mutant chimera (Figure 3.7 C and D), to which p120 is unable to bind (Figure 3.4 A). Therefore, p120 occupation of the DEE endocytic signal is required for the inhibition of cadherin endocytosis.

3.2.5 The core p120-binding region is the primary endocytic signal in VE-cadherin

Given the high degree of homology between the core p120-binding regions of classical cadherins, we tested whether the core p120-binding regions of other cadherins also function as endocytic signals. When fused to the IL-2R, the core p120-binding regions of human E-cadherin and N-cadherin, as well as *Drosophila melanogaster* DE-cadherin, all mediated internalization, although they did so less efficiently than the VE-cadherin core p120-binding region (Figure 3.8 A and B). This result suggests that, while this endocytic signal is conserved across a wide variety of classical cadherins, it is particularly important to VE-cadherin. In fact, VE-cadherin does have a putative tyrosine-based endocytic signal at Y685, but substitution of this residue with an alanine inhibited VE-cadherin chimera internalization by only a modest amount (Figure 3.8 C and D). Furthermore, this site is distal to the p120-binding region, and therefore unlikely to be subject to p120-mediated regulation of VE-cadherin internalization. In contrast, E-cadherin contains a dileucine endocytic

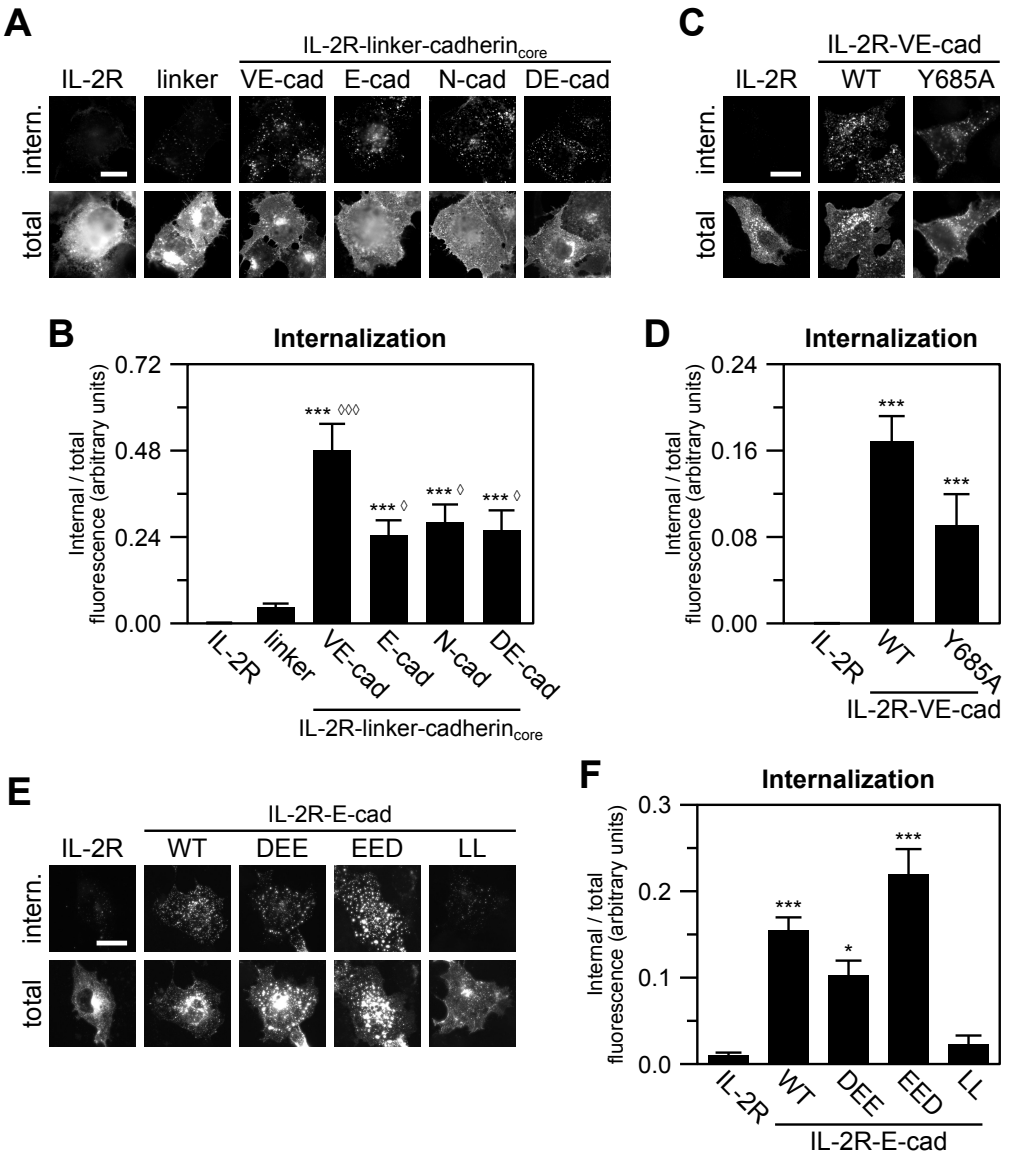


Figure 3.8: *The core p120-binding region is the primary endocytic signal in VE-cadherin.* Fluorescence-based internalization assays with a 10-minute internalization period were used to measure endocytosis of various IL-2R-cadherin chimeras expressed in COS cells. (A and B) Internalization of chimeras containing the core p120-binding regions of human VE-cadherin, E-cadherin, and N-cadherin, and *Drosophila* DE-cadherin, joined to IL-2R by a linker peptide. Mean ± SEM (n = 12–25 cells per group); ***, $P < 0.001$ compared to IL-2R; ◊, $P < 0.05$; ◊◊◊, $P < 0.001$ compared to linker. (C and D) Internalization of chimeras containing the VE-cadherin cytoplasmic tail, wild-type (WT) or with a Y685A mutation, joined to IL-2R. Mean ± SEM (n = 15 cells per group); ***, $P < 0.001$ compared to IL-2R. (E and F) Internalization of chimeras containing the E-cadherin cytoplasmic tail, wild-type (WT), or with DEE 758–760 (corresponding to the VE-cadherin DEE mutation), EED 664–666 (corresponding the VE-cadherin EMD mutation), or LL 743–744 mutated to alanines, joined to IL-2R. Mean ± SEM (n = 12 cells per group); *, $P < 0.05$; ***, $P < 0.001$ compared to IL-2R and LL mutation. Scale bars: 20 μm.

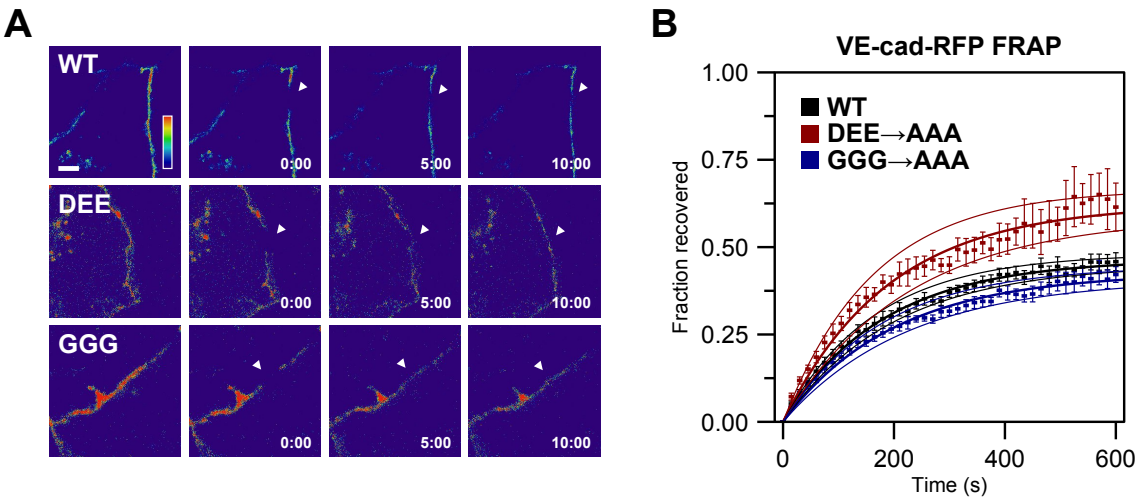


Figure 3.9: *VE-cadherin mobility does not require endocytosis.* RFP-tagged wild-type (WT), DEE-mutant, or GGG-mutant VE-cadherin was expressed in primary human dermal microvascular endothelial cells and used in fluorescence recovery after photobleaching (FRAP) experiments. (A) False-color images of VE-cadherin–RFP at cell junctions before bleaching (left), immediately after bleaching a 5μm long section of the junction (indicated by arrowheads), and at 5 minutes and 10 minutes after bleaching. Scale bar: 5 μm. (B) Quantification of fluorescence recovery. Average fluorescence within the bleach area, corrected for image acquisition–related photobleaching, as a fraction of pre-bleach value. Exponential curves were fit to the data, and their coefficients are given in Table 3.1. Points, mean ± SEM (*n* = 16–18 sequences per group); lines, exponential models ± 95% confidence interval.

signal not present in VE-cadherin. Mutation of these residues in an IL-2R–E-cadherin chimera significantly inhibited internalization, while mutation of the DEE sequence in an IL-2R–E-cadherin chimera only modestly inhibited internalization (Figure 3.8 E and F). Taken together, these results indicate that the core p120-binding region is a conserved endocytic signal in a variety of classical cadherins, although each cadherin appears to contain flanking sequences that may modulate its function or act as alternative endocytic signals.

3.2.6 VE-cadherin mobility does not require endocytosis

Identification of mutations that uncouple p120 binding from control of cadherin endocytosis allows us to test the effects of inhibiting cadherin endocytosis without affecting the internalization of other membrane proteins. Since prior reports have suggested, based on broad disruption of endocytic pathways with dynamin inhibitors, that cadherin mobility within the plasma membrane results primarily from endocytosis and recycling

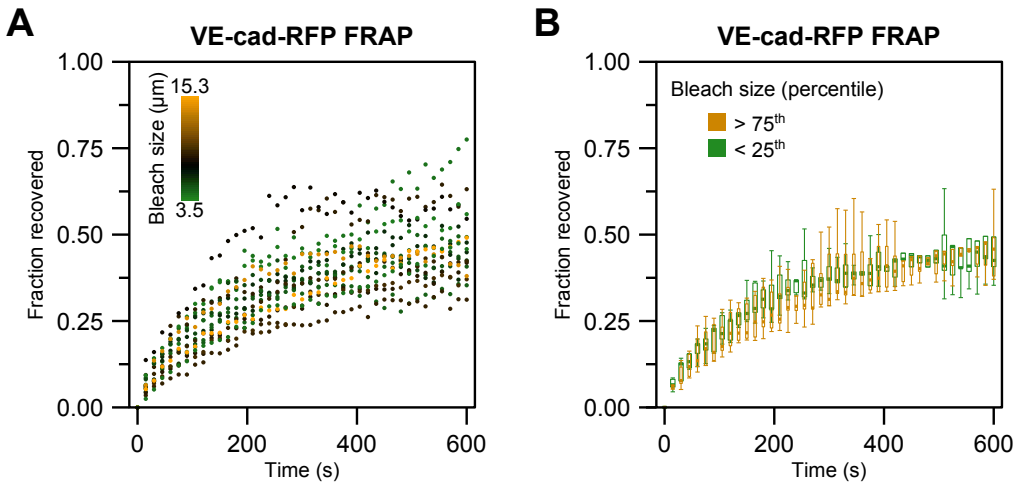


Figure 3.10: *VE-cadherin mobility within the cell membrane is not diffusion-limited.* Endothelial cells expressing fluorescently-tagged VE-cadherin were used for FRAP experiments with the size of the bleach region varying from 3.5 µm to 15.3 µm. (A) Recovery values from individual FRAP sequences are plotted as points with color corresponding to the length of the junction area that was bleached (*n* = 17 sequences). (B) Comparison of the 5 sequences with the largest bleach areas (tan) to the 5 sequences with the smallest bleach areas (green). Boxplots represent the distribution of recovery values at each time point: thick line, median; box, interquartile range; whiskers, full range. Lack of relationship between bleach area size and recovery rate indicates that the diffusible pool of VE-cadherin remains at an equilibrium distribution over the time-course of the experiment.

(de Beco et al., 2009), we used fluorescence recovery after photobleaching (FRAP) to test whether selective inhibition of cadherin endocytosis restricted cadherin mobility at cell borders. Surprisingly, neither the endocytosis-defective DEE mutation nor the GGG mutation altered the half-life of VE-cadherin FRAP compared to wild-type. In fact, the DEE mutation increased the mobile fraction of VE-cadherin, the proportion of cadherin free to diffuse within the membrane, while the GGG mutation slightly decreased the mobile fraction (Figure 3.9 A and B; Table 3.1). Recovery rate was not affected by variation in the size of the bleach region, justifying our use of an exponential, rather than diffusion-limited, model

Table 3.1: *VE-cadherin FRAP model parameters*

Protein	Mobile fraction	Recovery half-life (s)
WT VE-cadherin-RFP	0.465 [0.450 – 0.481]	125 [114 – 137]
VE-cadherin-RFP (DEE→AAA)	0.623 [0.587 – 0.666]	132 [113 – 156]
VE-cadherin-RFP (GGG→AAA)	0.426 [0.409 – 0.446]	138 [124 – 155]

Square brackets indicate 95% confidence interval.

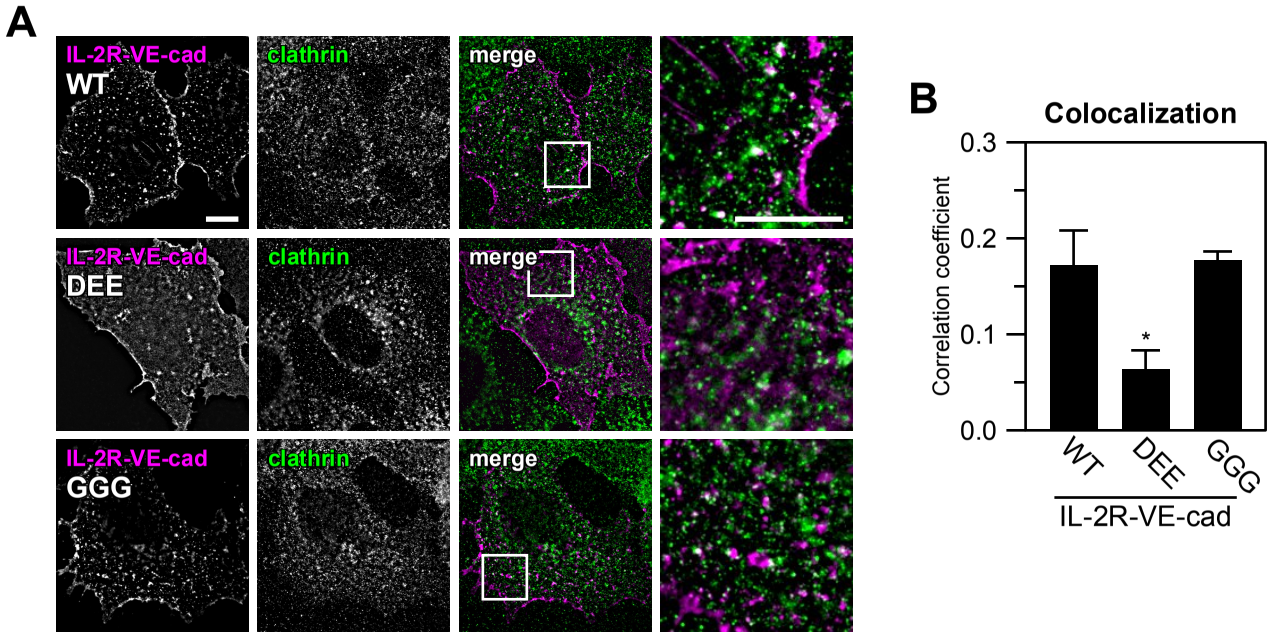


Figure 3.11: *Disrupting the endocytic signal prevents VE-cadherin recruitment into clathrin-enriched membrane domains.* (A) COS cells expressing wild-type (WT), DEE-mutant, or GGG-mutant IL-2R–VE-cadherin cytoplasmic tail chimeras were surface-labeled with antibody against IL-2R. After a 30-minute incubation on ice to allow chimera clustering, the cells were fixed and stained for clathrin heavy chain. Scale bars: 10 µm. (B) Quantification of IL-2R–VE-cadherin chimera and clathrin colocalization. Pearson's correlation coefficient, mean ± SEM (n = 33–34 cells per group); *, $P < 0.05$ compared to WT and GGG mutant.

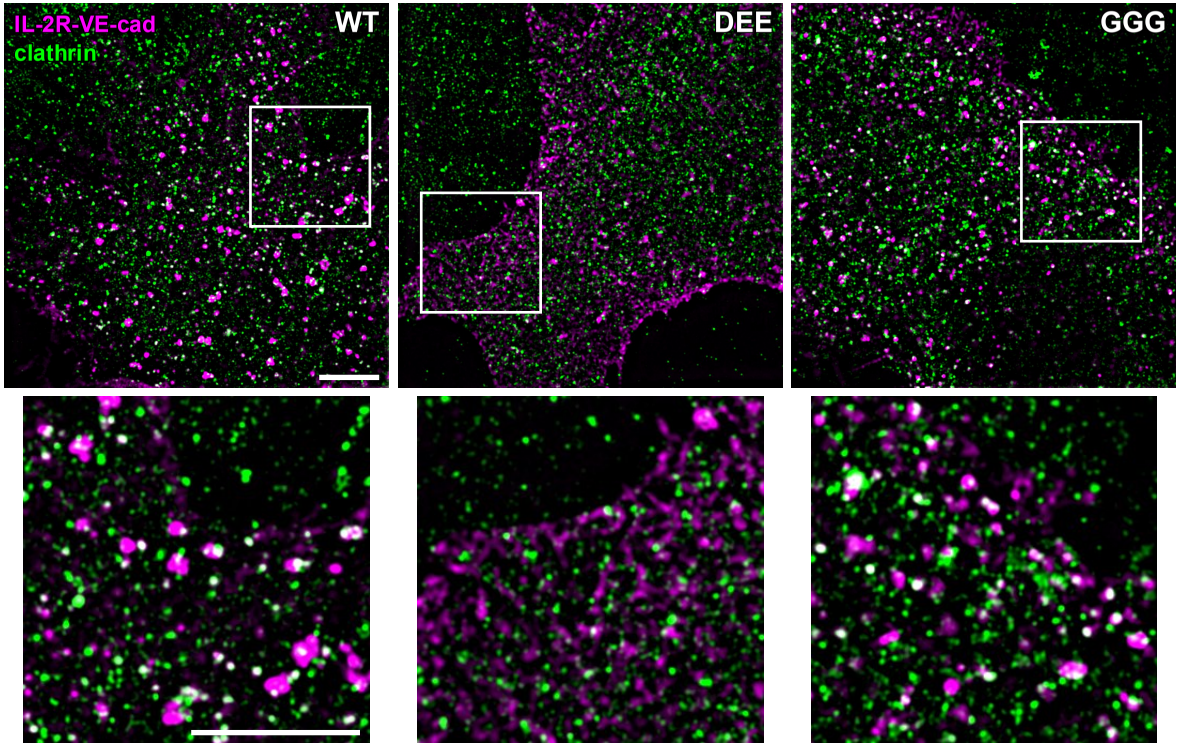


Figure 3.12: *Super-resolution microscopy confirms VE-cadherin cytoplasmic tail recruitment into clathrin-enriched membrane domains.* COS cells expressing wild-type (WT), DEE-mutant, or GGG-mutant IL-2R–VE-cadherin cytoplasmic tail chimeras were surface-labeled with antibody against IL-2R. After a 30-minute incubation on ice to allow chimera clustering, the cells were fixed and stained for clathrin heavy chain and imaged using structured-illumination microscopy for evaluation of colocalization beyond the diffraction limit. Scale bars: 5 μm.

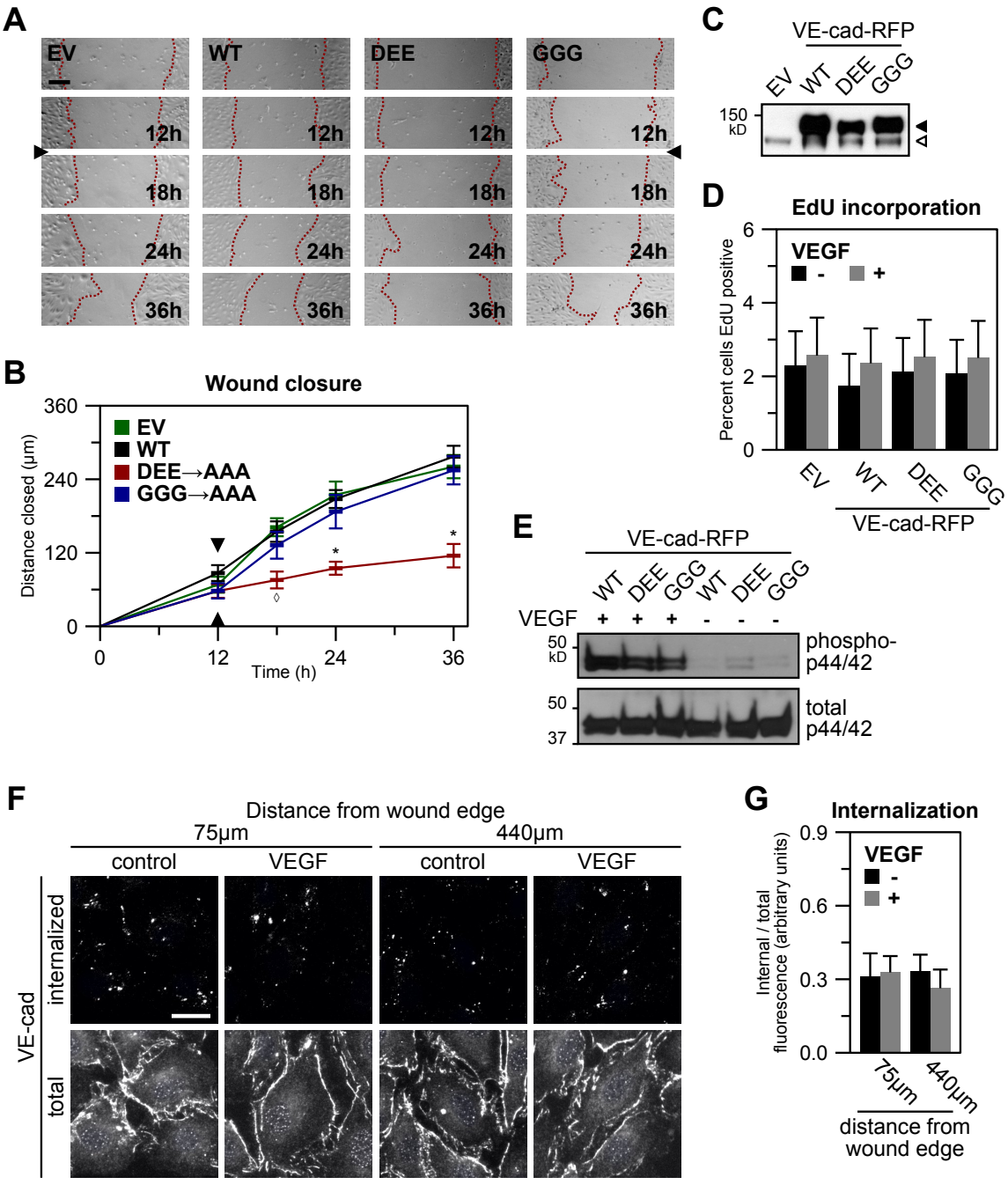
(Figure 3.10; see Appendix A for derivation and comparison of these models). These observations are incompatible with the hypothesis that cadherin FRAP is facilitated by endocytosis and recycling, and suggest instead that the endocytic signal mediates interactions that restrict VE-cadherin diffusion. We previously reported that the VE-cadherin cytoplasmic tail mediates clustering into clathrin-enriched membrane domains (Chiasson et al., 2009). Therefore, we hypothesized that the DEE mutation might disrupt this interaction. Consistent with this hypothesis, the DEE mutation, but not the GGG mutation, prevented IL-2R–VE-cadherin chimera from co-clustering with clathrin, as determined using both conventional (Figure 3.11 A and B) and super-resolution (Figure 3.12) immunofluorescence microscopy. Thus, the ability of the VE-cadherin cytoplasmic tail to cluster into clathrin-enriched membrane domains limits rather than facilitates lateral mobility of the cadherin within the plasma membrane.

3.2.7 VEGF-induced endothelial cell migration requires cadherin endocytosis

Cadherin endocytosis has been implicated in a variety of developmental events and cellular activities (Nanes and Kowalczyk, 2012). Similarly, p120 association with cadherins has been linked to the regulation of cadherin adhesion and cell migration (Xiao et al., 2007). The ability to selectively uncouple the regulation of endocytosis from p120 binding to cadherins through mutation of the DEE endocytic signal provides an opportunity to define the contribution of these activities of p120 to endothelial cell functions. It is likely that dynamic changes in cell adhesion are necessary for effective cell migration, particularly in the context of angiogenesis and re-endothelialization of denuded vessels (Dejana et al., 2009). Using scratch-wound assays, we found that expression of endocytosis-defective DEE-mutant VE-cadherin in primary human microvascular endothelial cells markedly slowed migration in response to VEGF, while expression of wild-type or GGG-mutant VE-cadherin had no effect (Figure 3.13 A and B). Similar results were obtained when constitutive migration of endothelial cells grown in serum was assessed (Figure 3.14 A and B), as well as with cells lacking endogenous cadherin (Figure 3.14 C and D). We also confirmed that DEE-mutant VE-cadherin was not expressed at a higher level than the other cadherin mutants (Figure

3.13 C) and that wild-type, DEE-mutant, and GGG-mutant VE-cadherin expressing cells replicated at the same rate, as measured by EdU uptake (Figure 3.13 D). We performed two experiments to further verify that the observed migration defect was related to cadherin endocytosis. First, we determined that neither wild-type nor mutant VE-cadherin prevented p44/42 MAPK phosphorylation in response to VEGF, indicating that the migration defect caused by endocytosis-defective VE-cadherin was not due to inhibition of VEGF signaling (Figure 3.13 E). Second, we confirmed that inhibition of cadherin endocytosis with a dynamin inhibitor also slowed migration of endothelial cells expressing wild-type or p120-uncoupled VE-cadherin (Figure 3.14 E–H). Additionally, since GGG-mutant VE-cadherin, which does not bind p120 but undergoes internalization normally, did not affect migration (Figure 3.13 A and B), we also conclude that inhibition of migration by the DEE-mutant cadherin resulted specifically from inhibition of cadherin endocytosis, and not from disruption of p120 recruitment to the adherens junction.

Figure 3.13 *(facing page)*: *VEGF-induced endothelial cell migration requires cadherin endocytosis.* (A–E) Wild-type (WT), DEE-mutant, and GGG-mutant VE-cadherin was expressed in monolayers of primary human dermal microvascular endothelial cells using an adenoviral transduction system. Infection with an empty adenovirus (EV) was used as a negative control. (A and B) Endothelial monolayers were serum starved for 1 hour, then scratched with a pipette tip. 12 hours after the scratch, 100 mg/mL VEGF was added to the medium (indicated by arrowheads). Migration of cells into the wound area was tracked over time. Mean distance closed ± SEM (n = 8 wounds per group); *, $P < 0.05$ compared to EV, WT, and GGG; ◊, $P < 0.05$ compared to EV and WT only. (C) VE-cadherin expression was measured by Western blot. Empty arrowhead, endogenous VE-cadherin; filled arrowhead, exogenously expressed VE-cadherin–RFP. (D) Replication was measured by a thymidine analog incorporation assay. Confluent monolayers were serum starved for 12 hours, then either left untreated (black bars) or treated with VEGF (grey bars) for 6 hours, with incubation in EdU during the final hour. Following fixation and labeling, replication rate was estimated by the fraction of infected cells that were EdU-positive. Proportion ± standard error (n = 94–103 cells per group). (E) Activation of VEGF signaling was verified by Western blot for phosphorylated p44/42 MAPK (top) and total p44/42 MAPK (bottom). Cells were serum starved for 12 hours, then treated with VEGF or left untreated for 20 minutes before harvesting. (F and G) Endocytosis of VE-cadherin in endothelial cells migrating into a scratch wound was measured using a fluorescence-based internalization assay. Confluent monolayers of endothelial cells were serum starved for 45 minutes, then scratched with a pipette tip and either left untreated (control, black bars) or treated with VEGF (grey bars) for 45 minutes. VE-cadherin endocytosis was measured over a 1-hour internalization period in cells near to (75±75 μm) or far from (440±75 μm) the wound edge. Mean ± SEM (n = 22–32 cells per group). Scale bars: (A) 100 μm; (F) 20 μm.


A
EV
WT
DEE
GGG
12h
18h
24h
36h
B
Wound closure
EV
WT
DEE→AAA
GGG→AAA
Distance closed (μm)
Time (h)
C
VE-cad-RFP
EV
WT
DEE
GGG
150 kD
D
EdU incorporation
VEGF
Percent cells EdU positive
E
VE-cad-RFP
VEGF
50 kD
phospho-p44/42
total p44/42
F
Distance from wound edge
75μm
440μm
control
VEGF
VE-cad
internalized
total
G
Internalization
VEGF
Internal / total fluorescence (arbitrary units)
distance from wound edge

We next attempted to determine whether inhibition of endothelial cell migration by endocytosis-defective VE-cadherin was related to junction formation. Wild-type, DEE-mutant, and GGG-mutant VE-cadherins exhibited similar distributions at the wound edge in migrating cells (Figure 3.15 A). However, migration of sparsely seeded cells, which could not form cell-cell contacts, was apparently unaffected by expression of the DEE mutant (Figure 3.15 B–E). In order to better understand the relationship between junctions, cadherin endocytosis, endothelial cell migration, and VEGF signaling, we monitored VE-cadherin dynamics and internalization in endothelial cells migrating into a scratch wound. Interestingly, we were unable to observe a difference in VE-cadherin endocytosis with and without VEGF treatment and at different distances from the wound edge (Figure 3.13 F and G; Figure 3.15 F and G). These results suggest that VE-cadherin internalization is not confined to the wound edge and that VEGF does not induce endothelial migration by stimulating cadherin endocytosis. Rather, efficient endothelial migration appears to require adherens junction plasticity derived from the constitutive endocytosis of VE-cadherin. These findings reveal the importance of cadherin endocytosis to the dynamic changes in cell ad-

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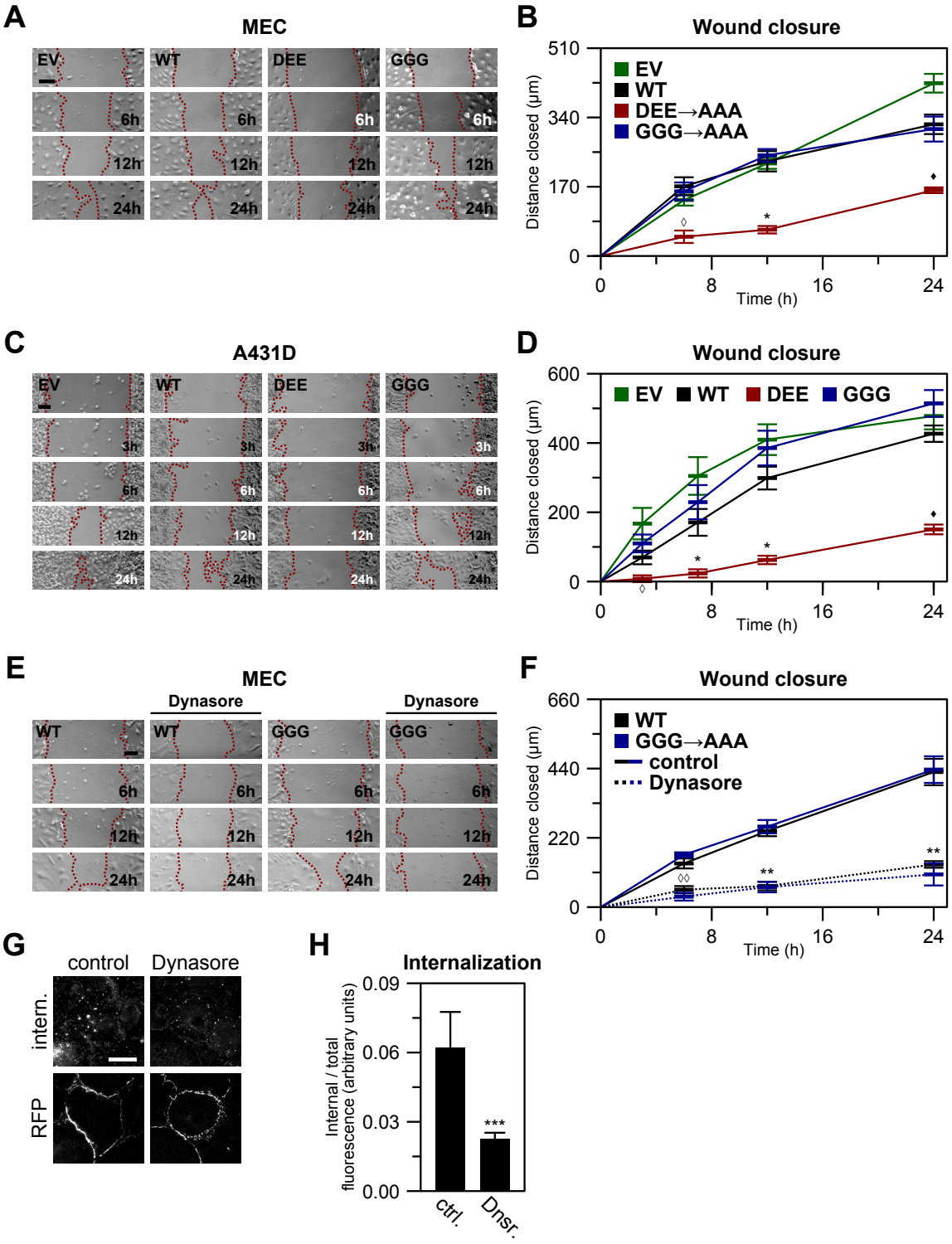
Figure 3.14 ***(facing page)*****:** *Constitutive endothelial cell migration requires VE-cadherin endocytosis.* (A and B) Wild-type (WT), DEE-mutant, and GGG-mutant VE-cadherin was expressed in monolayers of primary human microvascular endothelial cells using an adenoviral transduction system. Infection with an empty adenovirus (EV) was used as a negative control. Cells were not serum starved. Monolayers grown in complete serum-containing medium were scratched with a pipette tip and migration of cells into the wound area was tracked over time. Mean distance closed ± SEM (n = 8 wounds per group); *, $P < 0.05$ compared to all others; ◊, $P < 0.05$ compared to all except EV; ♦, $P < 0.05$ compared to EV only. (C and D) Wild-type or mutant VE-cadherin was expressed in A-431D cells, which lack expression of endogenous cadherin. Monolayers were scratched with a pipette tip and migration of cells into the wound area was tracked over time. Mean distance closed ± SEM (n = 8 wounds per group); *, $P < 0.05$ compared to all others; ◊, $P < 0.05$ compared to all except WT; ♦, $P < 0.05$ compared to all except EV. (E and F) Wild-type or GGG-mutant VE-cadherin was expressed in primary human microvascular endothelial cells, which were not serum starved and were grown in complete medium. Monolayers were scratched with a pipette tip and migration of cells into the wound area was tracked over time in the presence or absence of the dynamin inhibitor Dynasore. Mean distance closed ± SEM (n = 8 wounds per group); **, $P < 0.01$ control compared to Dynasore; ◊◊, $P < 0.01$ GGG only control compared to Dynasore. (G and H) To confirm that Dynasore inhibited VE-cadherin endocytosis, wild-type VE-cadherin–RFP was expressed in COS cells, and its endocytosis was measured using a fluorescence-based internalization assay with a 30-minute internalization period in the presence or absence of Dynasore. Mean ± SEM (n = 47–50 cells per group); ***, $P < 0.001$. Scale bars: (A) 100 μm; (C) 100 μm; (E) 100 μm; (G) 20 μm.


A
MEC
EV
WT
DEE
GGG
6h
12h
24h
B
Wound closure
EV
WT
DEE→AAA
GGG→AAA
Distance closed (µm)
Time (h)
C
A431D
3h
D
Wound closure
E
MEC
Dynasore
F
Wound closure
control
Dynasore
G
control
Dynasore
intern.
RFP
H
Internalization
Internal / total fluorescence (arbitrary units)
ctrl.
Dnsr.

hesion necessary for endothelial cell migration and demonstrate that p120 binding and regulation of cadherin endocytosis can be uncoupled during this process.

3.3 Discussion

The results presented here reveal that p120 inhibits VE-cadherin endocytosis by binding to and physically masking a cluster of acidic residues in the cadherin cytoplasmic tail (DEE 646–648) which functions as an endocytic signal. This signal is contained within the core p120-binding region, the portion of the cadherin cytoplasmic tail which mediates the

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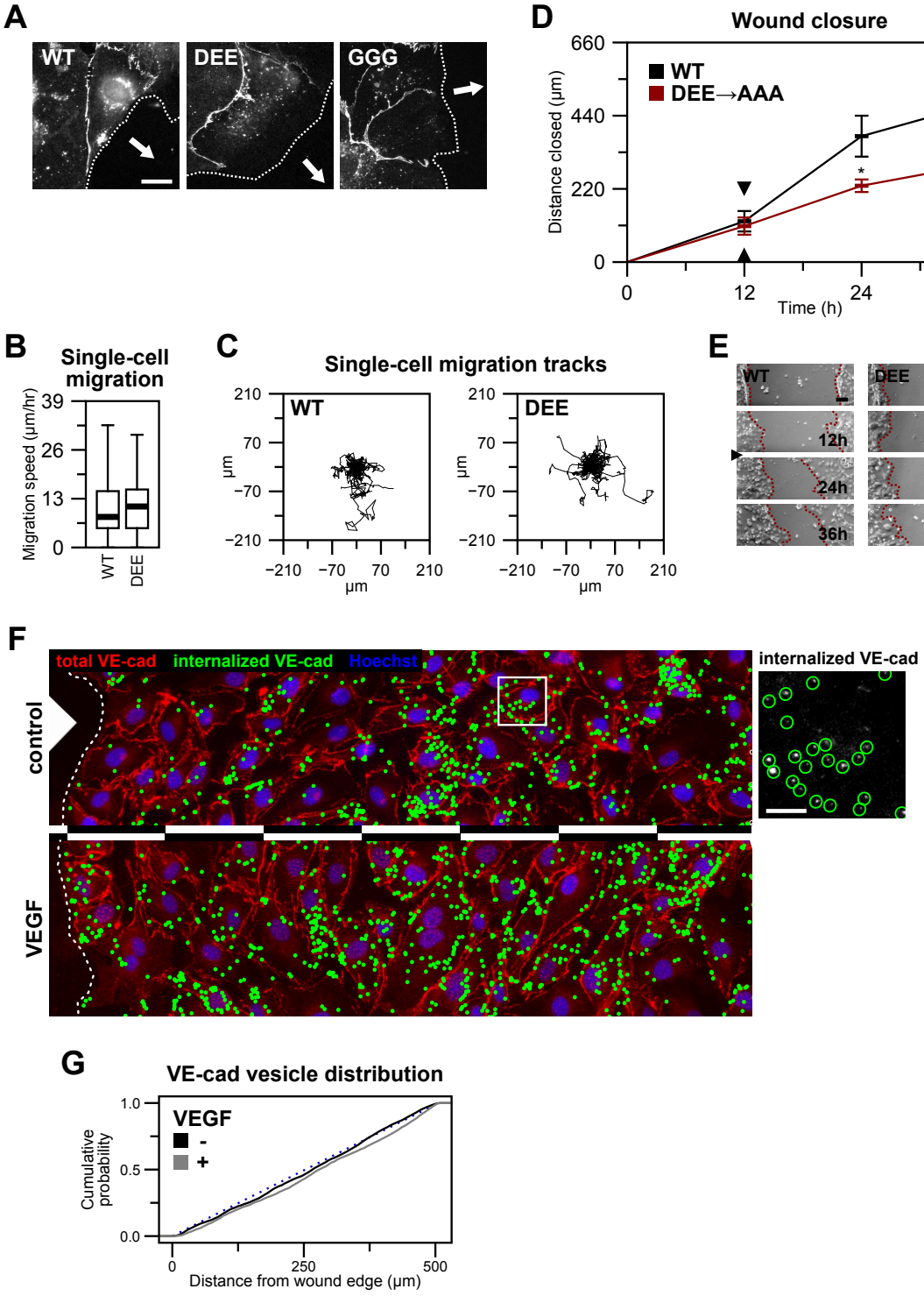
Figure 3.15 ***(facing page)*****:** *Cadherin mutations do not affect distribution at the wound edge or undirected single-cell migration, and cadherin endocytosis is not increased at the wound edge.* (A) Monolayers of primary human microvascular endothelial cells expressing wild-type (WT), DEE-mutant, or GGG-mutant VE-cadherin were scratched with a pipette tip, allowed to migrate into the wound area for two hours, then fixed and processed for immunofluorescence. Dotted line, wound edge; arrows, direction of migration. (B and C) Wild-type or DEE-mutant VE-cadherin was expressed in COS cells, which were then sparsely seeded in fresh serum-containing medium onto new plates so that no contacts formed between neighboring cells. After allowing 150 minutes for cells to adhere to the plates, low-powered images were captured at 30-minute intervals for 15 hours, and cell positions were recorded (n = 54–67 cells per group). (B) Migration speeds were calculated for each cell at each time point. Thick line, median (n = 1,635–1,799 measurements per group); box, interquartile range; whiskers, 90% range. (C) Overlaid traces of single-cell migration tracks, each beginning at the origin. (D and E) To confirm that endocytosis-defective cadherin inhibited migration of COS cell monolayers, monolayers expressing wild-type and DEE-mutant VE-cadherin were scratched with a pipette tip, and migration into the wound area was tracked over time. At 12 hours, serum-depleted medium was replaced with fresh serum-containing medium (indicated by arrowheads). Mean distance closed ± SEM (n = 8 wounds per group); *, $P < 0.05$. (F and G) Endocytosis of VE-cadherin in endothelial cells migrating into a scratch wound was measured using a fluorescence-based internalization assay. Confluent monolayers of endothelial cells were serum starved for 45 minutes, then scratched with a pipette tip and either left untreated or treated with VEGF for 45 minutes, then VE-cadherin endocytosis was measured over a 1-hour internalization period. (F) Partially overlapping fluorescence images of the migrating cells were joined to produce a continuous view extending more than 450 μm from the wound edge. Vesicles of internalized VE-cadherin were identified by computer algorithm, and their locations are marked by green dots. The internalized VE-cadherin fluorescence channel was segmented based on a threshold value, maintained for each image, and individual vesicles were filtered based on size. The inset on the right illustrates the algorithm's accuracy. (G) Cumulative distribution plot of internalized VE-cadherin vesicle distance from the wound edge, normalized based on differences in area sampled at different distances. Distribution of VE-cadherin vesicles is similar with (grey) and without (black) VEGF treatment, and both distributions are similar to a uniform distribution extending over the sample area (blue dotted line). Scale bars: (A) 20 μm; (E) 100 μm; (F) main images, 75 μm; (F) inset, 20 μm.


A
WT
DEE
GGG
D
Wound closure
660
440
220
0
Distance closed (µm)
WT
DEE→AAA
0
12
24
36
Time (h)
B
Single-cell migration
Migration speed (µm/hr)
39
26
13
0
WT
DEE
C
Single-cell migration tracks
WT
DEE
210
70
−70
−210
µm
E
WT
DEE
12h
24h
36h
F
total VE-cad
internalized VE-cad
Hoechst
control
VEGF
internalized VE-cad
G
VE-cad vesicle distribution
VEGF
-
+
1.0
0.5
0.0
Cumulative probability
0
250
500
Distance from wound edge (µm)

strongest interactions with p120 (Figure 3.1), and this sequence is both necessary and sufficient to mediate endocytosis (Figure 3.2). Thus, when p120 is bound to the cadherin juxtamembrane domain, this endocytic signal is masked, and the cadherin is stabilized. p120 dissociation from the cadherin exposes this signal and triggers cadherin endocytosis. Mutations in either the cadherin or p120 which prevent p120 from masking the endocytic signal disrupt this regulatory mechanism (Figures 3.4, 3.6, and 3.7). These findings demonstrate that the core p120-binding region of classical cadherins serves mutually exclusive roles as either a p120 binding site or an endocytic motif. Furthermore, we find that cadherin endocytosis is essential for efficient endothelial cell migration in response to an angiogenic growth factor (Figure 3.13), highlighting the importance of this regulatory mechanism in the context of endothelial cell biology.

The DEE acidic cluster of the core p120-binding region is well conserved among classical cadherins, including E-cadherin and N-cadherin (Figure 3.1 C). The endocytic function of this cadherin domain is also well conserved, although with varying degrees of efficiency, suggesting that additional endocytic signals may play important roles in other cadherins (Figure 3.8 A and B). These additional motifs include the dileucine endocytic signal in E-cadherin and Y685 in VE-cadherin. Interestingly, while the dileucine endocytic signal present in E-cadherin is outside of the core p120-binding region, it is within a region of dynamic binding between p120 and the E-cadherin cytoplasmic tail, so p120 binding may mask this signal as well (Ishiyama et al., 2010). In contrast, VE-cadherin Y685 lies outside of the p120-binding region and mutation of this tyrosine residue only modestly affects VE-cadherin internalization (Figure 3.8 C and D). Furthermore, the Y685 endocytic signal apparently cannot mediate internalization in the absence of the DEE motif (Figure 3.4). These findings indicate that the DEE endocytic signal in VE-cadherin is the predominant endocytic motif in this cadherin and that p120 masking of the DEE signal is sufficient to regulate VE-cadherin endocytosis.

Although the endocytic function of the core p120-binding region is conserved in *Drosophila* DE-cadherin (Figure 3.8 A–B), the requirement for p120 binding to maintain cadherin stability is not, as p120-null flies are apparently normal (Myster et al., 2003). p120 also appears to be dispensable in *C. elegans* (Pettitt et al., 2003). The reason p120 is not

an essential gene in invertebrates remains unknown, but corresponding to the mammalian requirement for p120 stabilization of cadherins is a greatly expanded p120 sub-family of catenins, including p0071, δ-catenin/NPRAP, ARVCF, and the plakophilins, as well as an expanded repertoire of p120 splicing isoforms (Hatzfeld, 2005). Both the expanded role of p120 and the increased complexity of the p120 sub-family in mammals suggest that vertebrate tissue patterning requires additional pathways for fine-tuning cadherin trafficking not needed in simpler organisms.

But if p120 binding to the cadherin juxtamembrane domain is a key mechanism for controlling cadherin endocytosis, what causes p120 to dissociate from the cadherin? One possibility is that Src-mediated phosphorylation of E-cadherin Y753 and Y754 disrupts p120 binding and allows for ubiquitination by the ubiquitin ligase Hakai (Fujita et al., 2002). However, these tyrosine residues are not well conserved, so this mechanism may not be important for all classical cadherins. For example, VE-cadherin and N-cadherin lack the tyrosine residues that are critical for Hakai binding (Figure 3.1 C). Furthermore, there is evidence that Hakai-mediated ubiquitination of E-cadherin alters sorting of the cadherin after endocytosis, rather than mediating endocytosis directly (Palacios et al., 2005). In the case of VE-cadherin, VEGF may also induce cadherin endocytosis through Src activation, though the role of p120 in this pathway remains unclear (Gavard and Gutkind, 2006; Gavard et al., 2008; Hashimoto et al., 2011; Kidoya et al., 2010), and Src-mediated phosphorylation alone is apparently insufficient to disrupt p120 binding (Adam et al., 2010). Surprisingly, we were unable to measure any discernible impact of VEGF on VE-cadherin levels or endocytic rates in quiescent or wounded monolayers (Figures 3.13 and 3.15). Nonetheless, it is highly likely that p120 dissociation from the cadherin is a tightly controlled event that is modulated by a variety of humoral pathways and cellular adhesive interactions. Further study of the mechanisms driving p120 dissociation from the cadherin tail is needed to fully understand how cadherin cell surface levels are modulated in various developmental contexts and diseases states.

Although the mechanisms regulating p120 binding to the cadherin juxtamembrane domain have not been fully elucidated, the mutations we have identified within the core p120-binding region allow for the functions of cadherin in p120-bound and p120-unbound states

to be further explored. For example, the DEE mutation uncouples p120 binding to the cadherin cytoplasmic tail from control of cadherin endocytosis, resulting in a cadherin that is both unbound to p120 and yet also stable at the cell surface. Analysis of this cadherin mutant in a variety of cellular contexts, including p120-null backgrounds, will further our understanding of precisely how p120 binding modulates cadherin function. Furthermore, a number of recent studies have implicated cadherin endocytosis in the regulation of junction dynamics and in the modulation of cellular activities such as neuronal patterning (Kawauchi et al., 2010) and the establishment of planar polarity during development (Jarrett et al., 2002; Levayer et al., 2011). However, previous work in this area has relied on disruption of the endocytic pathway broadly, rather than selectively inhibiting cadherin endocytosis. For example, previous studies found that preventing endocytosis through dynamin inhibition either modestly increased (Canel et al., 2010) or significantly decreased (de Beco et al., 2009) the rate of E-cadherin FRAP. In contrast, selective inhibition of VE-cadherin endocytosis with the DEE 646–648 mutation does not affect the rate of fluorescence recovery, though it does increase the mobile fraction (Figure 3.9 and Table 3.1). Since specifically blocking cadherin endocytosis does not restrict cadherin mobility, endocytosis and recycling cannot explain cadherin FRAP. Rather, our FRAP results suggest that cadherins can diffuse rapidly in the membrane, but that their movement is limited by transient binding interactions, resulting in fluorescence recovery approximated by first-order kinetics (Sprague et al., 2004).

In contrast with our finding that inhibiting cadherin endocytosis does not inhibit FRAP, altering the ability of VE-cadherin to undergo endocytosis has a dramatic effect on endothelial cell migration (Figures 3.13 and 3.14). Mutation of the DEE sequence dramatically stalled endothelial cell migration both in response to VEGF and in cells cultured in the presence of serum. This inhibition was caused by changes in VE-cadherin trafficking, not p120 localization, since GGG-mutant VE-cadherin, which undergoes normal internalization but does not bind p120, did not slow migration. Furthermore, since exogenous expression of wild-type VE-cadherin did not slow migration, inhibition is not caused simply by increased cadherin levels. Rather, cadherin endocytosis is apparently essential for the dynamic regulation of cell contacts needed for directed cell migration. Furthermore, our results suggest

that VEGF does not stimulate migration by inducing VE-cadherin endocytosis (Figures 3.13 and 3.15). Rather, cell migration appears to require constitutive endocytic cycling of cadherins to impart plasticity to cell–cell contacts, thereby allowing a migratory signal to drive cell movement. Clearly, static models of cell–cell junctions are insufficient to explain important dynamic processes such as cell migration and tissue patterning. Studies of cadherin endocytic mutants in additional model systems will further reveal the biological importance of adherens junction regulation through endocytic mechanisms during a wider variety of developmental and disease processes.

3.4 Materials and Methods

Cell culture and reagents

Primary cultures of human dermal microvascular endothelial cells were isolated from neonatal foreskin and cultured in Endothelial Growth Medium 2 Microvascular (Lonza). Cells were grown for 24–48 hours to 80% confluence on plates or coverslips coated with 0.1% gelatin for most experiments. African green monkey kidney fibroblast-like cell line COS-7 (ATCC), human epidermoid carcinoma cell line A-431D (Lewis et al., 1997), human cervical adenocarcinoma cell line HeLa (ATCC), and, for adenovirus production, human embryonic kidney cell line QBI-293A (MP Biomedicals) were cultured in Dulbecco's modification of Eagle's medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Mediatech) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% antibiotic/antimycotic solution (Mediatech). Cells were transfected 24 hours prior to the start of experiments using Lipofectamine 2000 (Life Technologies). Dynamin was inhibited using 80μM Dynasore (Sigma-Aldrich) dissolved in culture medium.

cDNA constructs

Constructs encoding full-length human VE-cadherin in pBluescript (provided by E. Dejana, FIRC Institute of Molecular Oncology, Milan, Italy; Navarro et al., 1995), the extracellular and transmembrane domains of IL-2R in a CMV expression vector (provided by S. LaFlamme, Albany Medical College, Albany, NY; LaFlamme et al., 1994), and the IL-2R–VE-cadherin cytoplasmic tail chimera, constructed by PCR and ligation of amino acids

Table 3.2: *Primers used in this chapter.*

VE-cadherin Δ657 mutagenesis primers	
Forward	GC GAG ATG GAC ACC ACC AGC TAA TAA GAT GTG TCG G
Reverse	GCT GGT GGT GTC CAT CTC GCC GCC GCC CTC
VE-cadherin Δ644 mutagenesis primers	
Forward	TC CAC GAG CAG CTG GTC ACC TAA TAA GAC GAG GAG G
Reverse	GGT GAC CAG CTG CTC GTG GAT CTC CGG CAC
VE-cadherin DEE646–648AAA mutagenesis primers	
Forward	CTG GTC ACC TAC GCA GCA GCA GGC GGC GGC GAG ATG
Reverse	CAT CTC GCC GCC GCC TGC TGC TGC GTA GGT GAC CAG
VE-cadherin GGG649–651AAA mutagenesis primers	
Forward	TAC GAC GAG GAG GCA GCA GCA GAG ATG GAC ACC
Reverse	GGT GTC CAT CTC TGC TGC TGC CTC CTC GTC GTA
VE-cadherin EMD652–654AAA mutagenesis primers	
Forward	GAG GGC GGC GGC GCA GCA GCA ACC ACC AGC TAC
Reverse	GTA GCT GGT GGT TGC TGC TGC GCC GCC GCC CTC
VE-cadherin Y685A mutagenesis primers	
Forward	GCC CGG CCT TCC CTC GCG GCG CAG GTG CAG AAG CCA CC
Reverse	GG TGG CTT CTG CAC CTG CGC CGC GAG GGA AGG CCG GGC
E-cadherin DEE758–760AAA mutagenesis primers	
Forward	GTT TAT TAC TAT GCA GCA GCA GGA GGC GGA GAA GAG
Reverse	CTC TTC TCC GCC TCC TGC TGC TGC ATA GTA ATA AAC
E-cadherin EED764–766AAA mutagenesis primers	
Forward	GAA GGA GGC GGA GCA GCA GCA CAG GAC TTT GAC TTG
Reverse	CAA GTC AAA GTC CTG TGC TGC TGC TCC GCC TCC TTC
E-cadherin LL743–744AA mutagenesis primers	
Forward	GTG GTC AAA GAG CCC GCA GCA CCC CCA GAG GAT GAC
Reverse	GTC ATC CTC TGG GGG TGC TGC GGG CTC TTT GAC CAC

Underlined codons indicate sites of mutation.

621–784 of VE-cadherin with a C-terminal myc tag into pBluescript followed by subcloning in-frame into the IL-2R CMV expression vector, were described previously (Venkiteswaran et al., 2002; Xiao et al., 2003a), as were the wild-type and K444M mutant p120–RFP constructs in pcDNA3.1 expression vectors, which were constructed by PCR (Ishiyama et al., 2010). The IL-2R–E-cadherin cytoplasmic tail chimera construct, containing amino acids 728–878 of human E-cadherin ligated in-frame into the IL-2R construct using HindIII and XbaI, was provided by C. Niessen (Center for Molecular Medicine, University of Cologne, Germany). The IL-2R–VE-cadherin juxtamembrane domain chimera was constructed based on a report mapping the VE-cadherin catenin-binding domain (Navarro et al., 1995). As previously described, amino acids 621–702 of VE-cadherin were isolated by PCR and ligated in frame to the IL-2R construct using HindIII and XhoI restriction sites (Xiao et al., 2003a). The shorter IL-2R–VE-cadherin chimeras were constructed using site-directed mutagenesis to insert tandem stop codons after VE-cadherin residues 657 and 644. Full-length VE-cadherin, IL-2R–VE-cadherin chimera, and IL-2R–E-cadherin chimera point mutants were also constructed using site-directed mutagenesis (primers described in Table 3.2). Constructs encoding IL-2R–linker and IL-2R–linker–cadherin core p120-binding region chimeras were constructed by subcloning synthetic DNA plasmids (Table 3.3; Integrated DNA Technologies) coding for a poly-alanine linker with or without residues 644–664 of human VE-cadherin, residues 754–773 of human E-cadherin, residues 772–791 of human N-cadherin, or residues 1371–1390 of *Drosophila* DE-cadherin, into the existing IL-2R–VE-cadherin chimera construct using HindIII and XhoI.

Adenovirus production

The full-length VE-cadherin mutants were subcloned into Gateway TagRFP-AS-N (Evrogen), in-frame with a C-terminal TagRFP, a monomeric red fluorescent protein, then shuttled into pAd/CMV/V5-DEST using Gateway Clonase enzymes (Life Technologies). Plasmids were digested with PacI to expose the viral inverted terminal repeats, and transfected into QBI-293A cells for production of human adenovirus type 5 packaged with the desired gene. Virus was harvested by concentration and lysis of virus-producing QBI-293A cells. To induce gene expression, cells were infected 24 h prior to the start of experiments.

Table 3.3: *Synthetic genes used in this chapter.*

Synthetic gene	Sequence
Linker	AAGCTTCGGCG*GCGGGCTGCCGCAGCGGCTGCCGC-* *AGCGGCAGCTGTGGAGCAAAAGCTCATTTCTGAAG-* *AGGACTTG*TGACTCGAGGGATCCGATATC
VE-cadherin core p120-binding region	AAGCTTCGGCGGCGG*GCTGCCGCAGCGGCTGCCGC-* *AGCGGCAGCT***ACCTACGACGAGGAGGGCGGCGGCG-** **AGATGGACACCACCAGCTACGATGTGTCGGTGCTC-** **AAC***GTGGAGCAAAAGCTCATTTCTGAAGAGGACTT-* *G*TGACTCGAGGGATCCGATATC
E-cadherin core p120-binding region	AAGCTTCGGCGGCGG*GCTGCCGCAGCGGCTGCCGC-* *AGCGGCAGCT***TACTATGATGAAGAAGGAGGCGGAG-** **AAGAGGACCAGGACTTTGACTTGAGCCAGCTGCAC-** *GTGGAGCAAAAGCTCATTTCTGAAGAGGACTTG*TG- ACTCGAGGGATCCGATATC
N-cadherin core p120-binding region	AAGCTTCGGCGGCGG*GCTGCCGCAGCGGCTGCCGC-* *AGCGGCAGCT***AAATATGATGAAGAAGGTGGAGGAG-** **AAGAAGACCAGGACTATGACTTGAGCCAGCTGCAG-** *GTGGAGCAAAAGCTCATTTCTGAAGAGGACTTG*TG- ACTCGAGGGATCCGATATC
DE-cadherin core p120-binding region	AAGCTTCGGCGGCGG*GCTGCCGCAGCGGCTGCCGC-* *AGCGGCAGCT***AATTACGAGGACGAGGGTGGCGGCG-** **AGCGGGACACGGACTATGATCTGAATGTCCTGCGC-** *GTGGAGCAAAAGCTCATTTCTGAAGAGGACTTG*TG- ACTCGAGGGATCCGATATC

Boldface bases, core p120-binding regions; italicized bases, poly-alanine linker and myc tag.

Table 3.4: *Primary antibodies used in this chapter.*

Target	Antibody	Application
α-catenin	mouse IgG1 (BD Biosciences)	IF
β-catenin	rabbit polyclonal (NeoMarkers)	IF
	rabbit polyclonal (Sigma Aldrich)	WB
Clathrin heavy chain	mouse IgG1 (BD Biosciences)	IF
IL-2R	mouse IgG1 from hybridoma clone 7G7B6 (ATCC)	IF, WB
	mouse IgG2A, clone 24212 (R&D Systems)	IF, IP, WB
p120	rabbit polyclonal (S-19; Santa Cruz Biotech.)	IF, WB
p44/42 MAPK	rabbit polyclonal (Cell Signaling Tech.)	WB
phospho-p44/42	rabbit polyclonal (Cell Signaling Tech.)	WB
PECAM-1	goat polyclonal (M-20; Santa Cruz Biotech.)	IF
VE-cadherin	mouse IgG2A, clone BV6 (Corada et al., 2001)	IF
	mouse IgG1 (BD Biosciences)	IF
	rabbit polyclonal (Enzo Life Sciences)	WB

IF, immunofluorescence; WB, Western blot; IP, immunoprecipitation

Immunoprecipitation and Western blot analysis

For chimera immunoprecipitation experiments, cells were harvested in 0.5% Triton X-100 (Roche) with protease inhibitors (Roche Complete Mini tablets, EDTA free; Roche), 150 mM NaCl, 10 mM Hepes, 1 mM EGTA, and 0.1 mM $MgCl_2$, pH 7.4. After 30 min incubation at 4°C, cell lysates were centrifuged 16,100 × *g* for 10 min and the soluble fraction was diluted to a final protein concentration of 1 mg/mL. Cell lysate was then incubated with 2 µg antibody against IL-2R (Table 3.4) conjugated to ferromagnetic beads (Dynabeads; Life Technologies) for 1 h at 4 °C. The beads were then washed with 0.1% Triton X-100 and eluted into Laemmli sample buffer (Bio-Rad Laboratories). For other Western blot experiments, cells were harvested directly into Laemmli sample buffer. Samples were analyzed by SDS-PAGE and immunoblot. Primary antibodies are listed in Table 3.4. HRP-conjugated secondary antibodies (Bio-Rad Laboratories), a luminol-based detection system (ECL; GE Healthcare), and autoradiography film (Denville Scientific) were used for detection.

Immunofluorescence

Cells cultured on glass coverslips were fixed either in methanol for 2 min or in 4% paraformaldehyde for 10 min followed by 0.1% Triton-X100 for 8 min, depending on the performance of the antibodies used. Primary antibodies are listed in Table 3.4. Secondary

antibodies conjugated to Alexa Fluor dyes (488 nm, 555 nm, or 647 nm; Life Technologies) were used to identify target molecules. Actin was labeled with Alexa Fluor 488-conjugated phalloidin (Life Technologies). Microscopy was performed using either a Leica DMR-XA2 wide-field fluorescence microscope equipped with 40×/0.65 NA, 63×/1.32 NA oil immersion, and 100×/1.40 NA oil immersion objectives, narrow bandpass filters, and an Orca digital camera (Hamamatsu) or an inverted Leica DMI-6000B microscope equipped with a 63×/1.40 NA oil-immersion objective, a VT Infinity 2D array scanner confocal module (VisiTech International), 561 nm and 491 nm solid-state lasers, a C9100-12 digital camera (Hamamatsu), and a temperature-regulated enclosure maintained at 37 °C. Images were captured using Simple PCI software (version 6.6; Hamamatsu). Super-resolution microscopy was performed using a Nikon N-SIM system in 3D structured-illumination microscopy mode on an Eclipse Ti-E microscope equipped with a 100×/1.49 NA oil immersion objective, 561 nm and 488 nm solid-state lasers, and a DU-897 EM-CCD camera (Andor). Images were captured and reconstructed using NIS-Elements software with the N-SIM module (version 3.22; Nikon).

Internalization assay

Assays to follow internalization of cadherin or IL-2R–cadherin chimeras were performed as previously described (Chiasson et al., 2009; Xiao et al., 2003a). Briefly, cells were incubated with an antibody against the extracellular domain of VE-cadherin or IL-2R dissolved in culture medium at 4 °C for 30 min. Unbound antibody was removed by washing with cold PBS. Cells were then incubated in culture medium at 37 °C for various time periods to allow internalization to occur. At the end of the internalization period, cells were returned to 4 °C, rinsed, and 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 rinsed and processed for immunofluorescence, with a second antibody against VE-cadherin or IL-2R, distinguishable based on isotype, or a fluorescent tag used to label the total cadherin or chimera pool. Internalization was quantified by taking the ratio of fluorescence signals corresponding to the internalized and total cadherin or chimera pools. Where indicated, clathrin-mediated endocytosis was inhibited by incubat-

ing cells in a potassium-depletion buffer (20 mM Hepes, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) instead of culture medium beginning 30 min prior to starting the internalization assay.

Fluorescence recovery after photobleaching

Primary human dermal microvascular endothelial cells expressing fluorescently-tagged VE-cadherin were grown in cover glass chambers (Thermo Fisher Scientific). Microscopy was performed using an A1-R laser-scanning confocal microscope (Nikon) equipped with a 60×/1.40 NA oil-immersion objective, a 561 nm laser, and a temperature-regulated enclosure maintained at 37 °C. Images were captured using NIS-Elements software (version 3.22; Nikon). Each FRAP sequence consisted of acquisition of two pre-bleach images, photobleaching of a section of a cell border (3.5–15.3 μm long × 5 μm wide; 33% laser power for 3.5 s), and acquisition of post-bleach images at 15 s intervals for 10 min. Images were acquired at 1.5% laser power. A Perfect Focus system (Nikon) was used to maintain focus during the FRAP sequence. Fluorescence recovery was calculated as the ratio of the average background-subtracted fluorescence intensity within the bleach area to the average background-subtracted fluorescence intensity of unbleached portions of cell borders to correct for acquisition-related photobleaching, normalized to the pre-bleach fluorescence intensity. Exponential curves ($f(t) = A(1 - e^{-kt})$, where $k = ln(2)/t_{\frac{1}{2}}$ and A is the mobile fraction) were fit using R (version 2.13; R Foundation for Statistical Computing).

Colocalization analysis

IL-2R–VE-cadherin chimera and clathrin colocalization experiments were designed as modified internalization assays. Chimera-expressing cells were incubated with an antibody against the extracellular domain of IL-2R dissolved in culture medium at 4 °C for 30 min, and unbound antibody was removed by washing with cold PBS. Cells were then incubated in culture medium at 4 °C for an additional 30 min to allow chimera clustering, but not endocytosis. After incubation, cells were processed for immunofluorescence. Colocalization was quantified by computing Pearson's product-moment correlation coefficient for chimera and clathrin pixel fluorescence intensities in individual cells using an algorithm built on the Commons Math library (version 2.0; Apache Software Foundation).

Migration assays

Migration of cells expressing wild-type or mutant cadherins was measured using scratch-wound assays. Cells were grown to confluent monolayers, scratched with a pipette tip, and imaged over time with a DMIL bright field microscope equipped with a 5×/0.12 NA objective and a DFC420 C camera (Leica). Images were acquired using FireCam software (version 3.4; Leica). For VEGF-induced migration, endothelial cells were serum-starved for 1 h prior to being scratched, and 100 ng/mL VEGF165 peptide (PeproTech) was added 12 h after wounding. Cell replication rates were measured by incorporation of a thymidine analogue, ethynyl-deoxyuridine (Click-iT EdU Imaging Kit; Life Technologies). For the single-cell migration assay, cells were imaged at 30-min intervals with an inverted Leica DMI-6000B microscope equipped with a 10×/0.30 NA objective, Retiga EXi camera (QImaging), and a temperature-regulated enclosure maintained at 37 °C. Phase-contrast images were acquired using SimplePCI software (version 6.6; Hamamatsu). Cell tracking data was extracted using the TrackMate plugin for ImageJ (version 1.2).

Image analysis and statistics

ImageJ software (versions 1.3–1.4; National Institutes of Health) was used for all image analysis and the application of lookup tables to produce display images. Custom ImageJ plugins were used to automate quantification. Statistics were computed using R (version 2.13; R Foundation for Statistical Computing). The Kruskal–Wallis rank sum test with Dunn's method for multiple comparisons was used to evaluate scaled data and the χ^2 test was used to evaluate count data.

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Chapter 4

p120-catenin guards cadherin stability against constitutive and inducible endocytic signals

Abstract

Vascular endothelial (VE)-cadherin undergoes constitutive internalization driven by a unique endocytic motif which also serves as a p120-catenin (p120) binding site. p120 binding masks the motif, stabilizing the cadherin at cell junctions. This mechanism allows constitutive VE-cadherin endocytosis and recycling to contribute to adherens junction dynamics without resulting in junction disassembly. Here, we identify an additional motif which drives VE-cadherin endocytosis and pathological junction disassembly associated with the endothelial-derived tumor Kaposi sarcoma. Human herpesvirus 8, which causes Kaposi sarcoma, expresses the MARCH family ubiquitin ligase K5. We report that K5 targets two membrane-proximal VE-cadherin lysine residues for ubiquitination, driving endocytosis and down-regulation of the cadherin. K5-induced VE-cadherin endocytosis does not require the constitutive endocytic motif. However, K5-induced VE-cadherin endocytosis is associated with displacement of p120 from the cadherin, and p120 protects VE-cadherin from K5. Thus, multiple context-dependent signals drive VE-cadherin endocytosis, but p120 binding to the cadherin juxtamembrane domain acts as a master regulator guarding cadherin stability.

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4.1 Introduction

Proper vascular function requires a balance between stability and plasticity of endothelial cell–cell contacts. Adhesion must be tight enough to resist vascular leak, yet also flexible enough to permit the cellular rearrangements necessary for new vessel formation during development and wound healing. Endothelial cell–cell adhesion is a dynamic and tightly regulated process, but the mechanisms controlling endothelial adhesion remain incompletely understood (Giannotta et al., 2013; Vincent et al., 2004). Because disruption of endothelial adhesion contributes to a wide variety of diseases, especially through excessive inflammation and facilitation of cancer metastasis, elucidating the basic cellular processes which determine the balance of endothelial adhesion is an important goal.

Endothelial cell–cell adhesion is mediated through the adherens junction complex. Vascular endothelial (VE)-cadherin, a member of the classical cadherin family and a principal adhesion molecule in the endothelium, joins adjacent cells through calcium-dependent homotypic trans interactions (Dejana and Orsenigo, 2013; Harris and Tepass, 2010; Ishiyama and Ikura, 2012). As with other classical cadherins, the cytoplasmic domain of VE-cadherin binds to armadillo family proteins called catenins, which perform important structural and regulatory functions. β-catenin binds to the C-terminal catenin-binding domain of VE-cadherin and, along with α-catenin and other proteins, links the cadherin to the actin cytoskeleton, mechanically coupling adjacent cells (Desai et al., 2013; Taguchi et al., 2011; Yamada et al., 2005). The juxtamembrane domain of VE-cadherin binds to p120-catenin (p120), which stabilizes cadherins at the adherens junction. In the absence of p120 binding, cadherins are rapidly endocytosed and degraded (Davis et al., 2003; Miyashita and Ozawa, 2007b; Xiao et al., 2003a,b). Thus, p120 binding to cadherins can function as a regulator of adherens junction stability (Nanes and Kowalczyk, 2012).

In the endothelium, p120 plays a particularly important role balancing the stability and flexibility of cell adhesion. The VE-cadherin juxtamembrane domain contains a dual-function motif which alternately serves as a p120 binding site or as an endocytic signal. p120 binding physically masks the endocytic signal, blocking its function and stabilizing the cadherin (Nanes et al., 2012). Endothelial-specific deletion of p120 in mice results in

hemorrhaging, vascular patterning defects, and embryonic lethality, underscoring the importance of p120 for maintenance of vessel stability (Oas et al., 2010). However, p120-modulation of VE-cadherin endocytosis allows a level of constitutive internalization of the cadherin. Constitutive VE-cadherin endocytosis confers plasticity to endothelial cell–cell junctions, and expression of a VE-cadherin mutant resistant to constitutive endocytosis inhibits collective migration of endothelial cells (Nanes et al., 2012). Because endothelial cell migration is an important component of angiogenesis, the junctional plasticity derived from constitutive VE-cadherin endocytosis likely has important physiologic roles.

If p120-modulated constitutive endocytosis of VE-cadherin is responsible for balancing stability and flexibility of endothelial adhesion, what causes the disruption of this mechanism in diseases associated with inappropriate loss of endothelial adhesion? One such disease is Kaposi sarcoma, an endothelial-derived tumor characterized by aberrant angiogenesis and leaky, slit-like vessels (Antman and Chang, 2000; Kaposi, 1872; Uldrick and Whitby, 2011). Kaposi sarcoma is caused by human herpesvirus 8 (HHV-8), which is always found within the lesions (Chang et al., 1994). Vascular permeability induced by HHV-8 likely occurs through multiple mechanisms, including expression of a viral G-protein–coupled receptor which activates Rac (Dwyer et al., 2011) and entry of the viral capsid into endothelial cells (Qian et al., 2008). In addition, HHV-8 encodes two membrane-associated RING-CH (MARCH)-family ubiquitin ligases, K3 and K5, which were originally identified to target host-cell mediators of immune function, such as MHC class I, for ubiquitination, endocytosis, and down-regulation (Coscoy and Ganem, 2000; Ishido et al., 2000). K5 also targets several other endothelial cell surface proteins for ubiquitination, including PECAM-1 and VE-cadherin (Mansouri et al., 2006, 2008). Interestingly, K5 expression increases permeability of endothelial monolayers (Mansouri et al., 2008), potentially linking ubiquitination of VE-cadherin to the aberrant angiogenesis and leaky vasculature seen in Kaposi sarcoma lesions.

The results presented here demonstrate that K5 disrupts endothelial cell–cell junctions by overriding the normal cellular regulation of VE-cadherin endocytosis. K5 targets VE-cadherin for ubiquitination, displaces p120 from the VE-cadherin juxtamembrane domain, and induces VE-cadherin endocytosis. Furthermore, we identify two membrane-proximal

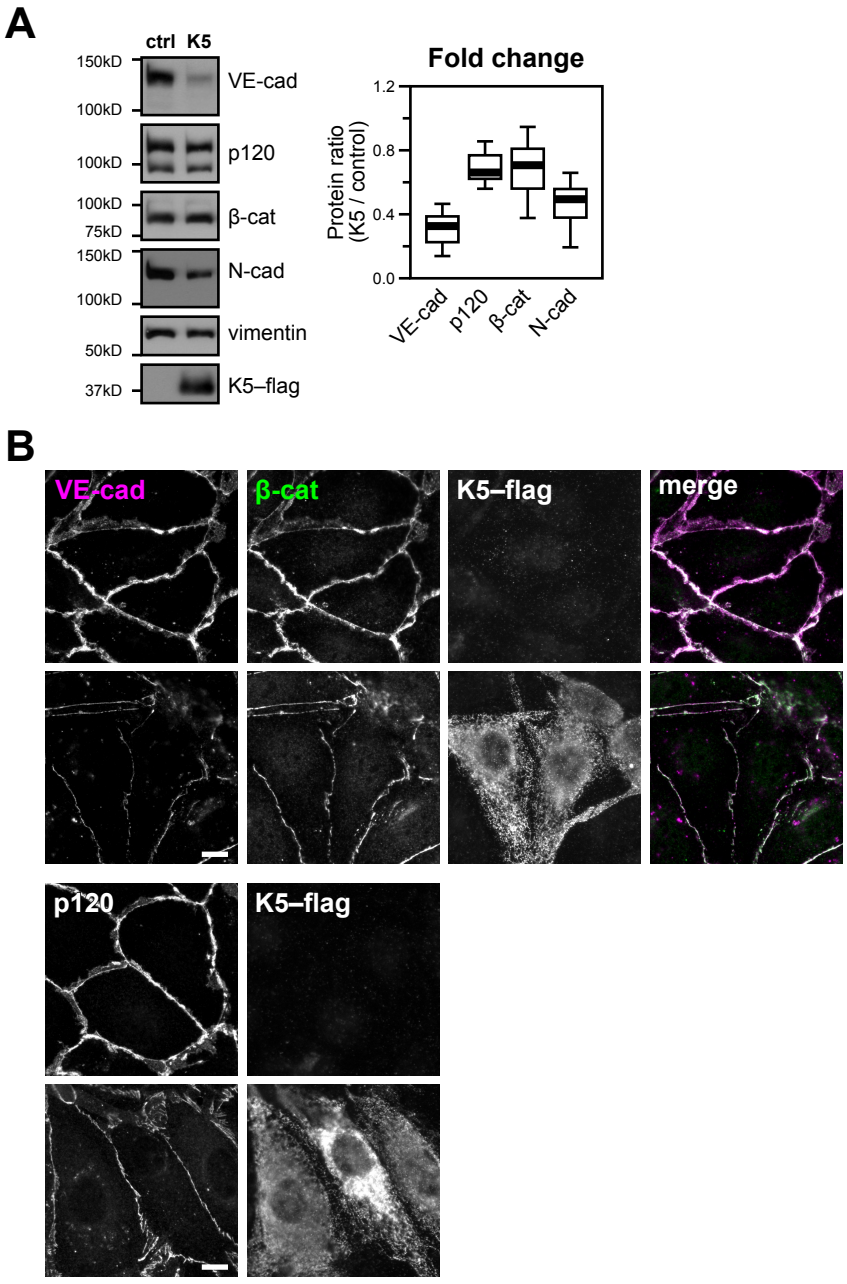

Figure 4.1: *K5 down-regulates VE-cadherin.* (A) K5–flag was expressed in cultures of the endothelial cell line HMEC-1 using adenoviral transduction. After 48 hours, cells were harvested and the lysates analyzed by Western blot. Thick line, median band intensity; boxes, interquartile range; whiskers, 90% range (n = 7 sample pairs per protein); $P < 0.01$, VE-cadherin compared to p120; $P < 0.05$, VE-cadherin compared to β-catenin. (B) K5 was expressed in primary cultures of dermal microvascular endothelial cells. After 48 hours, cells were fixed and processed for immunofluorescence. Bars: 10 µm.

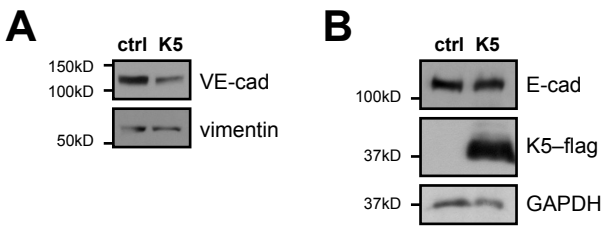

Figure 4.2: *K5-induced down-regulation of VE-cadherin is rapid and selective.* (A) K5–flag was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. After 24 hours, cells were harvested, and the lysates analyzed by Western blot. (B) K5–flag was expressed in primary human keratinocyte cultures by adenoviral transduction. After 48 hours, cells were harvested, and the lysates analyzed by Western blot.

lysines within the p120 binding site as the specific VE-cadherin residues targeted by K5, and demonstrate that p120 can protect VE-cadherin from K5-induced down-regulation. Interestingly, a VE-cadherin mutant resistant to constitutive endocytosis is still susceptible to down-regulation by K5, and a VE-cadherin mutant resistant to K5 still undergoes constitutive endocytosis. Thus, even though different endocytic signals drive constitutive- and K5-induced VE-cadherin internalization, p120 maintains a key role as the guardian of VE-cadherin stability through protection of the cadherin juxtamembrane domain.

4.2 Results

4.2.1 K5 targets VE-cadherin for ubiquitination and down-regulation

Previous studies have shown that expression of K5 in endothelial cells leads to VE-cadherin down-regulation (Mansouri et al., 2008). We sought to determine whether K5 targets VE-cadherin directly, or if down-regulation of VE-cadherin is secondary to K5-induced down-regulation of other adherens junction components. In agreement with previous reports, expression of K5 caused a sharp reduction in VE-cadherin protein levels (Figure 4.1 A). This reduction occurred rapidly, and was observed within 24 hours after adenoviral transduction of endothelial cells with K5 (Figure 4.2 A). In contrast, total protein levels of p120 and β-catenin were either slightly decreased or unchanged (Figure 4.1 A). N-cadherin, which does not typically assemble into endothelial cell–cell junctions, was also decreased, but not as sharply as levels of VE-cadherin, indicating the K5 has some specificity for VE-cadherin

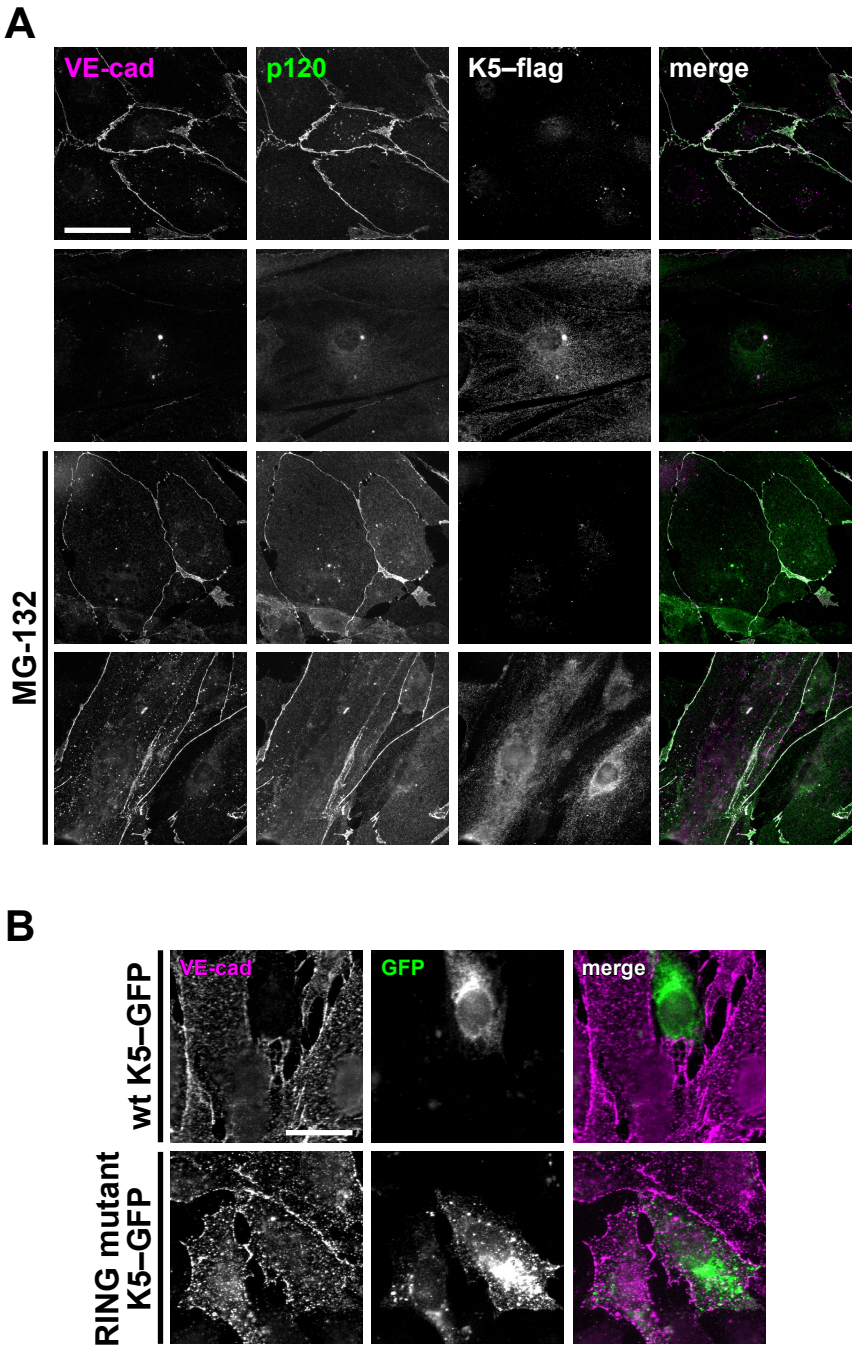

Figure 4.3: *K5-induced down-regulation of VE-cadherin requires ubiquitin ligase activity.* (A) K5–flag was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. After 6 hours, cells were treated with 50 μM MG-132 to disrupt the ubiquitin–proteasome system, or with vehicle as a control. After 24 hours, cells were fixed and processed for immunofluorescence. (B) CHO cell lines stably expressing VE-cadherin were transfected with wild-type K5–GFP or a K5 mutant lacking ligase activity (RING mutant; Means et al., 2007). After 24 hours, cells were fixed and processed for immunofluorescence. Bars: 20 μm.

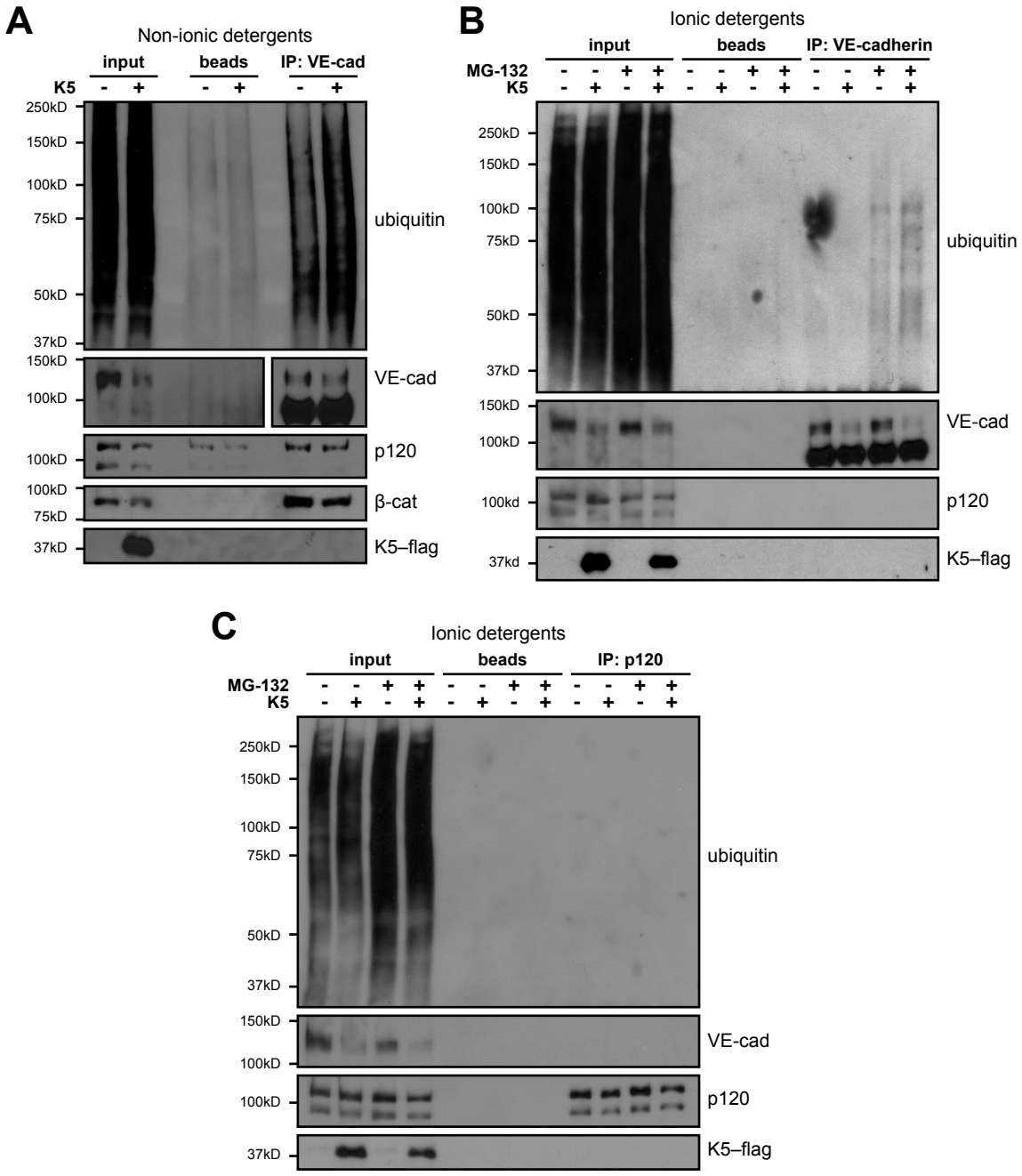

Figure 4.4: *K5 targets VE-cadherin for ubiquitination.* K5–flag was expressed in HMEC-1 cultures using adenoviral transduction. After 24 hours, cells were pre-treated with 10 μM MG-132 for 2 hours to preserve protein ubiquitination, then lysed, either in non-ionic detergents to preserve protein–protein interactions (A) or with 0.1% SDS to disrupt non-covalent interactions (B and C). VE-cadherin (A and B) or p120 (C) were isolated by immunoprecipitation, and the products analyzed by Western blot.

in endothelial cells (Figure 4.1 A). Expression of K5 in keratinocytes failed to decrease levels of E-cadherin (Figure 4.2 B), also indicating that K5-mediated down-regulation of VE-cadherin is a specific process, rather than the result of non-specific targeting of cell surface proteins. K5-mediated down-regulation of VE-cadherin in endothelial cells was also evident by immunofluorescence (Figure 4.1 B). Furthermore, even though K5 did not cause a significant reduction in total protein levels of β-catenin or p120, K5 expression did displace both catenins from cell–cell junctions (Figure 4.1 B).

Additionally, we found that K5-mediated down-regulation of VE-cadherin is associated with ubiquitination of the cadherin. Prolonged treatment of endothelial cells with MG-132 to broadly disrupt the ubiquitin–proteasome system blocked the ability of K5 to remove VE-cadherin and p120 from cell–cell junctions (Figure 4.3 A). Furthermore, a K5 mutant lacking ubiquitin ligase activity (Means et al., 2007) failed to down-regulate VE-cadherin stably expressed in a CHO cell line (Figure 4.3 B). We also used immunoprecipitation and Western blot to detect VE-cadherin ubiquitination directly. Expression of K5 in endothelial cells significantly increased the amount of ubiquitination detected in VE-cadherin complexes captured by immunoprecipitation (Figure 4.4 A). However, standard immunoprecipitation conditions with non-ionic detergents isolate cadherin-binding proteins along with the cadherin. Therefore, this result has two possible explanations. Either K5 targets VE-cadherin directly, or K5-mediated ubiquitination of another adherens junction component, such as p120, leads to the subsequent down-regulation of VE-cadherin. In order to determine if K5 targets VE-cadherin for ubiquitination, we added ionic detergents to disrupt non-covalent interactions. Increased ubiquitination of VE-cadherin was

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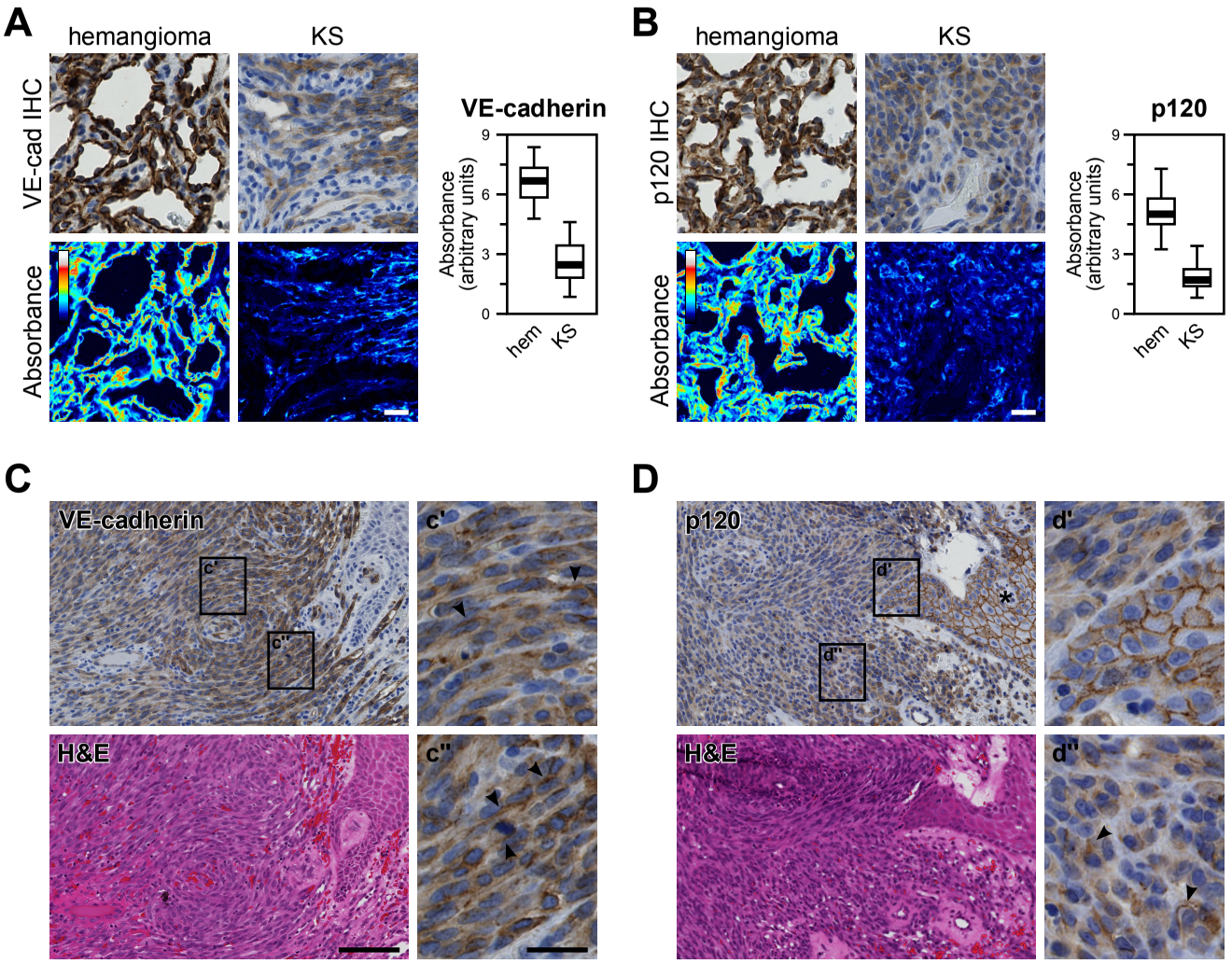
Figure 4.5 ***(facing page)*****:** *Adherens junction proteins are disrupted in Kaposi sarcoma.* Formalin-fixed paraffin-embedded tissue sections from Kaposi sarcoma lesions and hemangiomas were stained for VE-cadherin or p120 by immunohistochemistry. (A and B) A linear unmixing algorithm was used to estimate diaminobenzidine absorbance, which was then quantified in the endothelial cells lining vascular spaces in each section. Thick line, median; box, interquartile range; whiskers, 90% range (*n* = 116 vessels from 4 Kaposi sarcoma lesions and 89 vessels from 2 hemangiomas). (C and D) Kaposi sarcoma spindle cells stained diffusely positive for both VE-cadherin and p120, with only occasional junctional localization (arrowheads). In epidermal keratinocytes overlying the lesion (D, asterisk), p120 maintained junctional localization. Bars: A, 20 μm; B main panels, 100 μm; insets, 400 μm.


A
hemangioma
KS
VE-cad IHC
Absorbance
VE-cadherin
Absorbance (arbitrary units)
9
6
3
0
hem
ks
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hemangioma
KS
p120 IHC
Absorbance
p120
Absorbance (arbitrary units)
9
6
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hem
ks
C
VE-cadherin
c'
c''
H&E
D
p120
d'
d''
*
H&E

still detected with the addition of ionic detergents (Figure 4.4 B), and no K5-induced ubiquitination was detected in p120 captured by immunoprecipitation (Figure 4.4 C), indicating that ubiquitin is ligated directly to VE-cadherin. Thus, K5 targets VE-cadherin for ubiquitination and down-regulation, leading to disassembly of the endothelial adherens junction.

4.2.2 K5 induces VE-cadherin endocytosis

Because K5 expression caused adherens junction disassembly in cultured endothelial cells, we also asked whether biopsies of Kaposi sarcoma lesions showed evidence of junctional alterations. Kaposi sarcoma lesions are characterized by fascicles of endothelial-derived spindle cells, abnormal slit-like vascular spaces, and extravasated erythrocytes (Radu and Pantanowitz, 2013). We used immunohistochemistry to stain biopsies of Kaposi sarcoma lesions and assess the organization of endothelial cell–cell junctions. Consistent with previous reports (Dwyer et al., 2011), we found lower levels of VE-cadherin staining in endothelial cells lining the vascular spaces in Kaposi sarcoma lesions compared to similarly located cells in hemangiomas (Figure 4.5 A). Interestingly, we also found substantially decreased p120 staining (Figure 3B). Because K5 expression in endothelial cells did not induce a substantial decrease in p120 protein levels (Figure 4.1 A), decreased p120 staining in Kaposi sarcoma lesions may reflect an HHV-8 pathomechanism unrelated to K5. Given their thin profile, we were unable to determine the subcellular localization of VE-cadherin or p120 in cells lining the vascular spaces. However, the endothelial-derived spindle cells expressed both VE-cadherin and p120, and were large enough to distinguish junctional from cytoplasmic staining patterns (Figure 4.5 C and D). Although limited cell border localization was observed in some spindle cells (Figure 4.5 C and D, arrowheads), both VE-cadherin and p120 stains were predominantly diffuse and cytoplasmic. The cytoplasmic staining pattern of p120 in spindle cells was particularly striking when compared to the junctional localization of p120 in keratinocytes adjacent to the lesions (Figure 4.5 D, inset d'). Because VE-cadherin undergoes rapid endocytosis and degradation in the absence of p120 binding (Xiao et al., 2003a), disruption of p120 in Kaposi sarcoma lesions suggests that down-regulation of VE-cadherin in the lesions may result from increased internalization of the cadherin. In fact, we found that expression of K5 in endothelial cells significantly in-

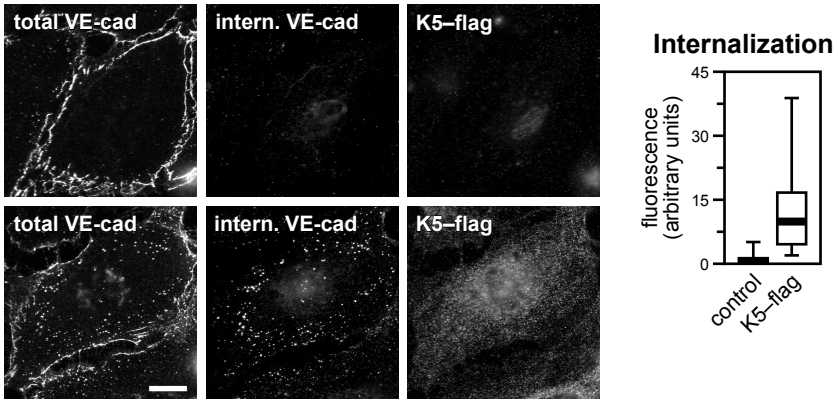


Figure 4.6: *K5 induces VE-cadherin endocytosis.* K5–flag was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. Cells were treated with 1 µg/mL doxyxycline to suppress K5 expression until 6 hours prior to beginning the assay. 24 hours after transduction, VE-cadherin endocytosis was measured using a fluorescence-based internalization assay. Internalized VE-cadherin (center column) was identified by antibody-labeling cell-surface VE-cadherin, incubating cells for 10 minutes to allow endocytosis, than washing cells with a low-pH buffer to remove any antibody remaining on the cell surface. A second antibody was used to label the total VE-cadherin pool for comparison (left column). Thick line, median; box, interquartile range; whiskers, 90% range (n = 55–58 cells per group); $P < 0.001$. Bar: 20 µm.

creased VE-cadherin endocytosis (Figure 4.6). Although we cannot rule out the possibility that K5 might affect VE-cadherin synthesis or trafficking in other ways, increased endocytosis of the cadherin is consistent with the disruption of endothelial cell junction proteins seen both in cell culture and in Kaposi sarcoma lesions.

4.2.3 Distinct endocytic motifs drive constitutive- and K5-induced VE-cadherin endocytosis

VE-cadherin undergoes constitutive endocytosis and recycling driven by an internalization signal in the cadherin juxtamembrane domain. This constitutive endocytic motif is anchored by three acidic amino acids within the core p120 binding region (DEE646–648; Figure 4.7 A), and mutation of these residues results in a cadherin variant that is resistant to constitutive endocytosis. We therefore asked whether K5-induced VE-cadherin down-regulation requires the constitutive endocytic signal. Surprisingly, the constitutive edocytosis–defective VE-cadherin mutant was not resistant to down-regulation by K5 (Figure 4.7 B), indicating that K5-mediated ubiquitination of the cadherin is sufficient to drive

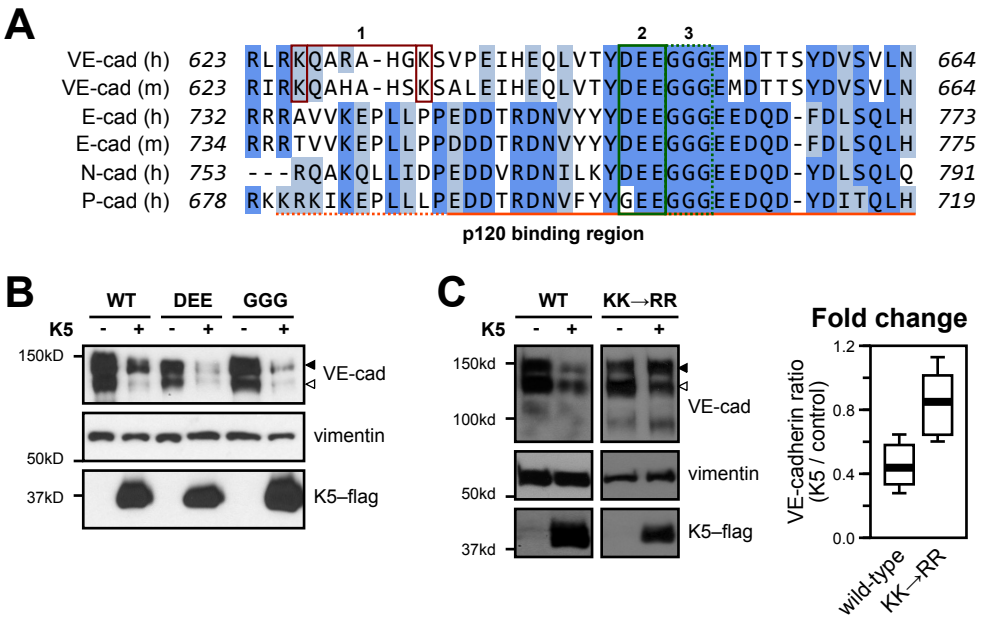


Figure 4.7: *K5 induces VE-cadherin down-regulation through an alternate endocytic motif.* (A) Multiple sequence alignment of the juxtamembrane domains of classical cadherins. 1, K626 and K633 mutated in (C); 2, DEE646–648 mutated in (B); 3, GGG649–651 mutated in (B). The p120 binding region is marked with an orange line below the alignment: solid line, static binding region; dotted line, dynamic binding region. (B) Wild-type or mutant VE-cadherin–RFP and K5–flag were expressed in primary dermal microvascular endothelial cells by adenoviral transduction. VE-cadherin with a DEE646–648AAA mutation (DEE) does not bind p120, but is resistant to constitutive endocytosis (Nanes et al., 2012). VE-cadherin with a GGG649–651AAA mutation (GGG) does not bind p120 and undergoes constitutive endocytosis normally. 48 hours after transduction, cells were harvested and the lysates analyzed by Western blot. Empty arrowhead, endogenous VE-cadherin; filled arrowhead, VE-cadherin–RFP. (C) Wild-type (WT) or mutant (K626R, K633R; KK→RR) VE-cadherin–RFP was stably expressed in HMEC-1 cells using lentiviral transduction. K5–flag was expressed by adenoviral transduction 48 hours before cells were harvested and the lysates analyzed by Western blot. Empty arrowhead, endogenous VE-cadherin; filled arrowhead, VE-cadherin–RFP. Thick line, median; box, interquartile range; whiskers, 90% range (n = 6–7 sample pairs per group); $P < 0.05$.

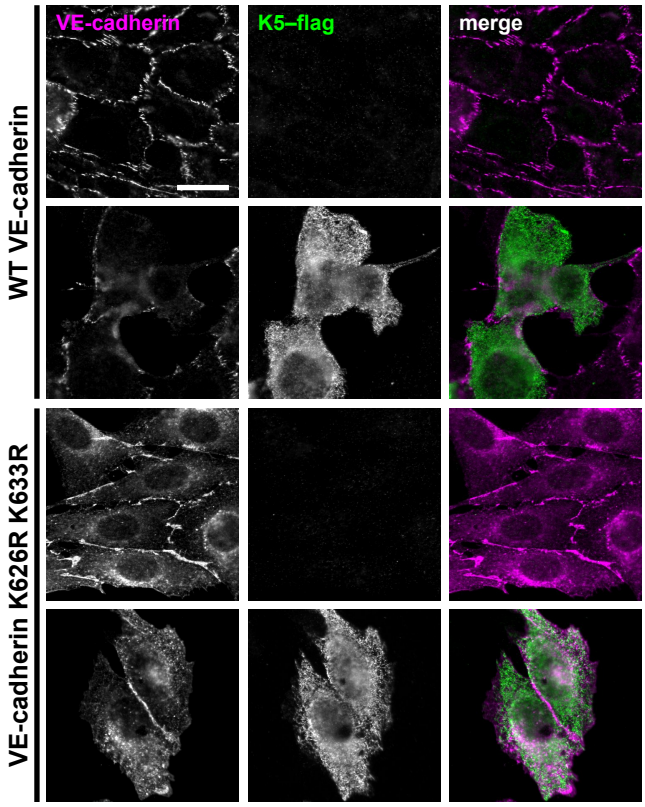


Figure 4.8: *K5 targets two membrane-proximal lysine residues on VE-cadherin.* K5–flag was expressed in CHO cell lines stable expressing wild-type or mutant (K626R K633R) VE-cadherin by adenoviral transduction. After 30 hours, cells were fixed and processed for immunofluorescence.

cadherin endocytosis, even in the absence of the constitutive endocytic signal. A control VE-cadherin mutant which does not bind p120 but undergoes constitutive endocytosis normally (GGG649–651; Nanes et al., 2012) was also susceptible to K5-induced down-regulation (Figure 4.7 B). Since the constitutive endocytic signal is not required for K5-mediated down-regulation of VE-cadherin, we reasoned that other motifs in the cadherin cytoplasmic tail might be important. In particular, two membrane-proximal lysines, K626 and K633, are potential target residues for K5-mediated ubiquitination (Figure 4.7 A). To test whether K5 targets these particular residues, we mutated them to arginines, which are not suitable targets for ubiquitination. Unlike the constitutive endocytosis–resistant VE-cadherin mutant, the K626R, K633R mutant was resistant to down-regulation by K5 (Figures 4.7 C and 4.8). Thus, distinct motifs in the cadherin juxtamembrane domain drive constitutive- and K5-induced VE-cadherin internalization.

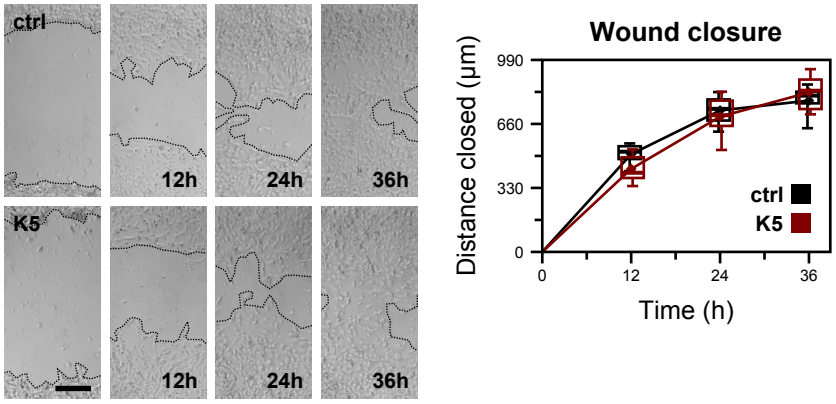
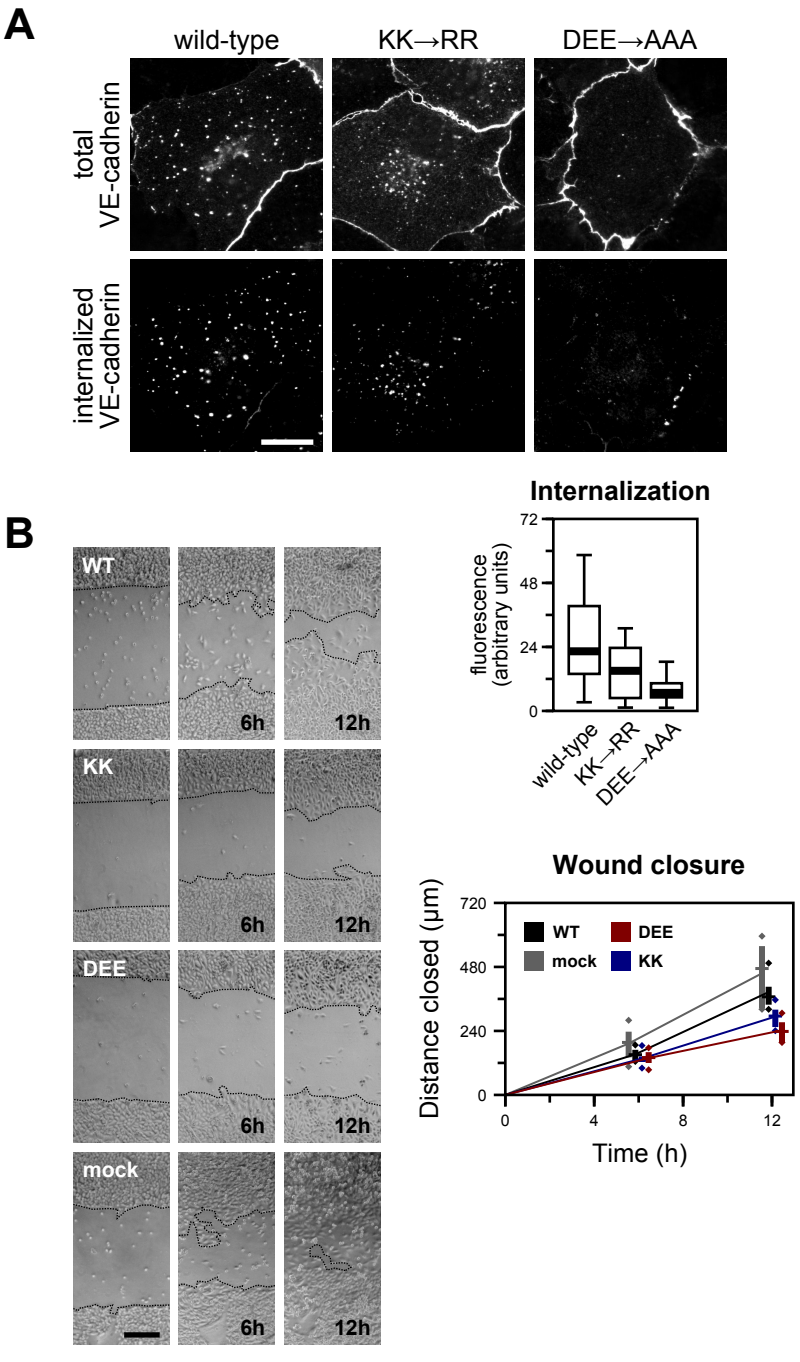


Figure 4.9: *K5-induced VE-cadherin endocytosis does not affect collective migration of endothelial cells.* K5–flag was expressed in HMEC-1 cultures by adenoviral transduction. Transduction with an empty adenovirus was used as a control. 24 hours after transduction, cell monolayers were scratched with a pipette tip, and migration into the wound area was tracked over time. Diamonds and connecting lines, mean; thick lines, median; boxes, interquartile range; whiskers, 90% range (n = 8 per group). Bar: 200 µm.

4.2.4 Constitutive- and K5-induced VE-cadherin endocytosis are functionally separable

We previously reported that collective migration of endothelial cells in a scratch-wound assay required constitutive endocytosis of VE-cadherin (Nanes et al., 2012). Therefore, we asked whether K5-induced endocytosis of VE-cadherin could increase the rate of endothelial cell migration. We found, however, that K5-expressing endothelial cells migrated comparably to endothelial cells which did not express K5 (Figure 4.9). Apparently, en-

Figure 4.10 ***(facing page)*****:** *Collective migration of endothelial cells correlates with constitutive VE-cadherin endocytosis.* (A) Wild-type or mutant VE-cadherin–RFP was expressed in COS-7 cells by lentiviral transduction. 72 hours after transduction, constitutive VE-cadherin endocytosis over a 20-minute period was measured using a fluorescence-based internalization assay. KK, VE-cadherin K626R K633R, the K5-resistant mutant; DEE, DEE646–648AAA, the constitutive endocytosis–resistant mutant. Thick lines, median; boxes, interquartile range; whiskers, 90% range (n = 30–33 cells per group). DEE compared to WT, $P < 0.001$; DEE compared to KK, $P < 0.05$. (B) CHO cells were transfected to express either wild-type or mutant VE-cadherin. Treatment with transfection reagent by no DNA was used as a control (mock). After 24 hours, cell monolayers were scratched with a pipette tip, and migration into the wound area was tracked over time. Vertical lines, interquartile range; diamonds, 90% range; cross lines, median; connecting lines, mean (n = 8 per group). At 12 h: DEE compared to WT, $P < 0.01$; DEE compared to mock, $P < 0.001$; KK compared to mock, $P < 0.05$. Bars: (A) 20 µm; (B) 200 µm.


A
wild-type
KK→RR
DEE→AAA
total VE-cadherin
internalized VE-cadherin
B
WT
KK
DEE
mock
6h
12h
Internalization
fluorescence (arbitrary units)
72
48
24
0
wild-type
KK→RR
DEE→AAA
Wound closure
Distance closed (µm)
720
480
240
0
WT
DEE
mock
KK
0
4
8
12
Time (h)

dothelial cell migration requires only a basal level of constitutive VE-cadherin endocytosis to confer plasticity to cell–cell junctions, and inducing VE-cadherin endocytosis through K5 does not increase migration speed. In contrast, even modest suppression of constitutive cadherin endocytosis can slow collective cell migration. The K5-resistant VE-cadherin mutant does undergo constitutive endocytosis, although at a lower rate than wild-type VE-cadherin (Figure 4.10 A). Correlating with that finding, cells expressing the K5-resistant VE-cadherin mutant migrated more slowly in a scratch-wound assay than cells expressing wild-type VE-cadherin, and faster than cells expressing a VE-cadherin mutant which is almost completely resistant to constitutive endocytosis (Figure 4.10 B). These results indicate that constitutive- and K5-induced VE-cadherin endocytosis are both driven by different signals and have different functional effects.

4.2.5 K5 displaces p120 from VE-cadherin

Binding of p120 to the VE-cadherin juxtamembrane domain modulates cadherin endocytosis by masking the constitutive endocytic signal. Therefore, we asked whether p120 might similarly regulate K5-induced VE-cadherin endocytosis. In particular, we tested whether K5-induced VE-cadherin endocytosis was associated with separation of p120 from the cadherin cytoplasmic tail and, conversely, whether p120 could protect VE-cadherin from down-regulation by K5. Expressing K5 in endothelial cells did not measurably decrease the amount of p120 immunoprecipitated with VE-cadherin (Figure 4.4 A). However, VE-cadherin that is not bound to p120 is rapidly internalized and degraded, so the size of any p120-unbound cadherin pool is likely quite small. To overcome this challenge, we treated endothelial cells with chloroquine, an inhibitor of lysosomal acidification, which causes proteins targeted for degradation in the lysosome to instead accumulate in intracellular vesicles. Chloroquine treatment of K5-expressing endothelial cells resulted in the loss of VE-cadherin from cell–cell junctions and accumulation of the cadherin intracellularly, consistent with K5-induced VE-cadherin endocytosis (Figure 4.11 A and B). Interestingly, most of the VE-cadherin–containing vesicles did not contain p120, indicating that in the process of K5-induced VE-cadherin endocytosis, p120 is displaced from the cadherin (Figure 4.11 C). Since K5-induced VE-cadherin endocytosis is associated with displacement

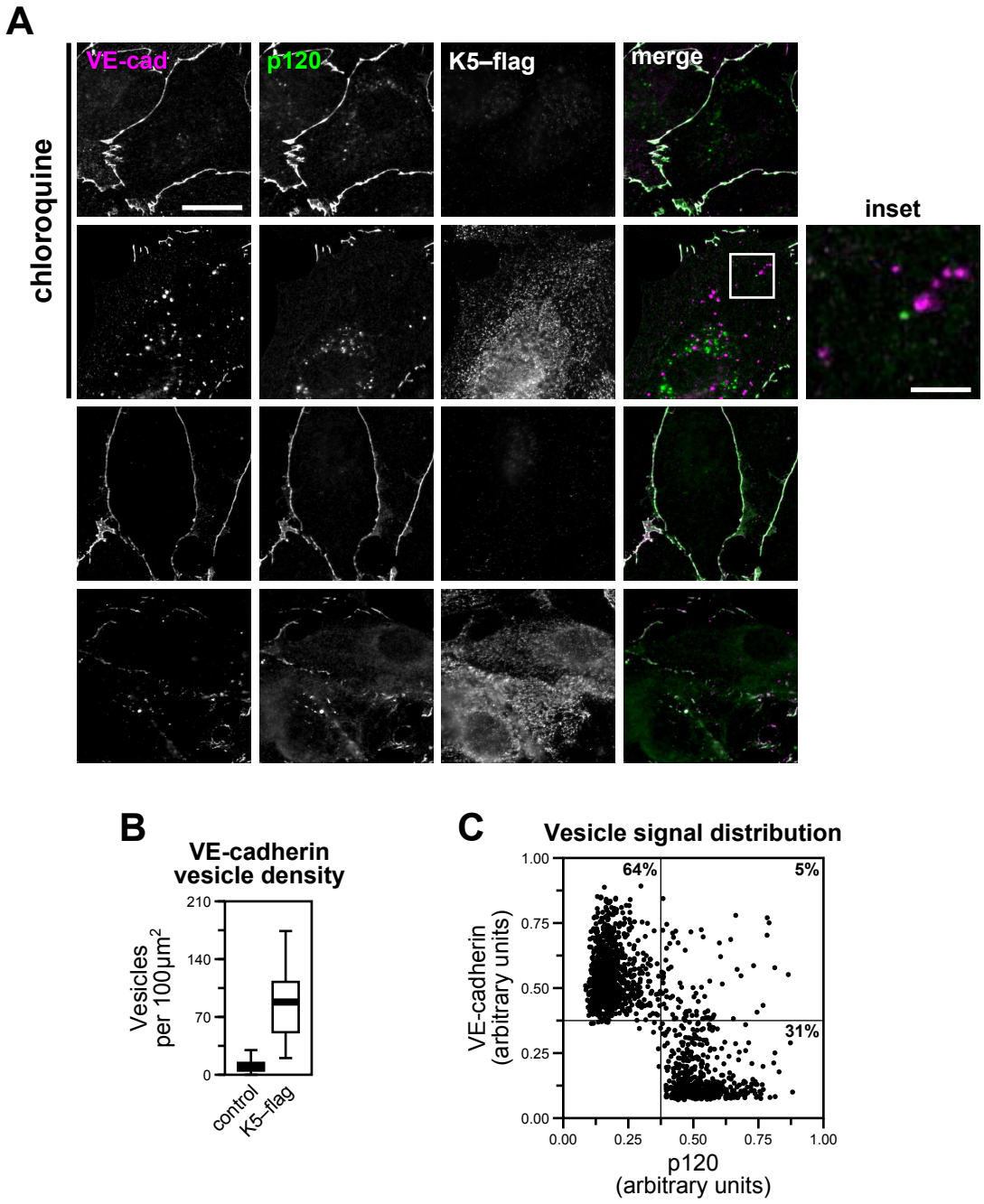


Figure 4.11: *K5 displaces p120 from VE-cadherin.* K5–flag was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. Cells were treated with vehicle or 100 μM chloroquine. (A) After 24 hours, cells were fixed and processed for immunofluorescence. (B) The density of VE-cadherin–containing vesicles in individual cells was quantified. Thick line, median; box, interquartile range; whiskers, 90% range (n = 20–22 cells per group); $P < 0.001$. (C) Individual vesicles were selected from K5-expressing chloroquine-treated cells, and the amount of VE-cadherin and p120 fluorescence signals were quantified. Most vesicles contain high levels of VE-cadherin fluorescence or high levels of p120 fluorescence, but not both. Bars: main panel, 20 μm; inset, 80 μm.

of p120 from the cadherin, we also tested whether p120 could protect VE-cadherin from down-regulation by K5. Indeed, increased expression of p120 in endothelial cells limited the reduction of VE-cadherin protein levels induced by K5 expression (Figure 4.12). These results indicate that even though constitutive- and K5-induced VE-cadherin endocytosis are driven by distinct internalization signals, p120 functions as a common modulator of both mechanisms.

4.3 Discussion

The aberrant angiogenesis and leaky vasculature observed in Kaposi sarcoma lesions indicate that normal cellular control of VE-cadherin stability has been overridden. We have identified one mechanism potentially responsible. The HHV-8 ubiquitin ligase K5 targets two membrane-proximal lysine residues on VE-cadherin (Figure 4.7 C), displaces p120 from the cadherin (Figure 4.11), and induces removal of VE-cadherin from the membrane by endocytosis (Figure 4.6). Interestingly, K5-induced internalization of VE-cadherin does not depend on the same motif which drives constitutive endocytosis of VE-cadherin required for collective migration of endothelial cells (Figure 4.7 B; Nanes et al., 2012). However, p120 maintains a key role modulating VE-cadherin endocytosis by protecting against both the constitutive- and K5-mediated endocytic signals (Figure 4.12). The p120 binding site in the cadherin juxtamembrane domain can be divided into a region mediating tight binding and a region mediating more dynamic interactions (Ishiyama et al., 2010). Since the VE-cadherin lysines targeted by K5 lie within the dynamic p120 binding region, our results support a model where p120 and K5 compete for access to the VE-cadherin juxtamembrane domain. Thus, p120 protects VE-cadherin from down-regulation by K5, and K5-induced ubiquitination of VE-cadherin displaces p120 (Figure 4.13). These findings indicate that a variety of signals may trigger VE-cadherin endocytosis both in the context of normal physiology and in disease states, but p120 binding to the cadherin juxtamembrane domain serves as a common control point guarding cadherin stability.

Because p120 binding to classical cadherins both in the endothelium and in other tissues is an important regulator of adherens junction dynamics, understanding other pro-

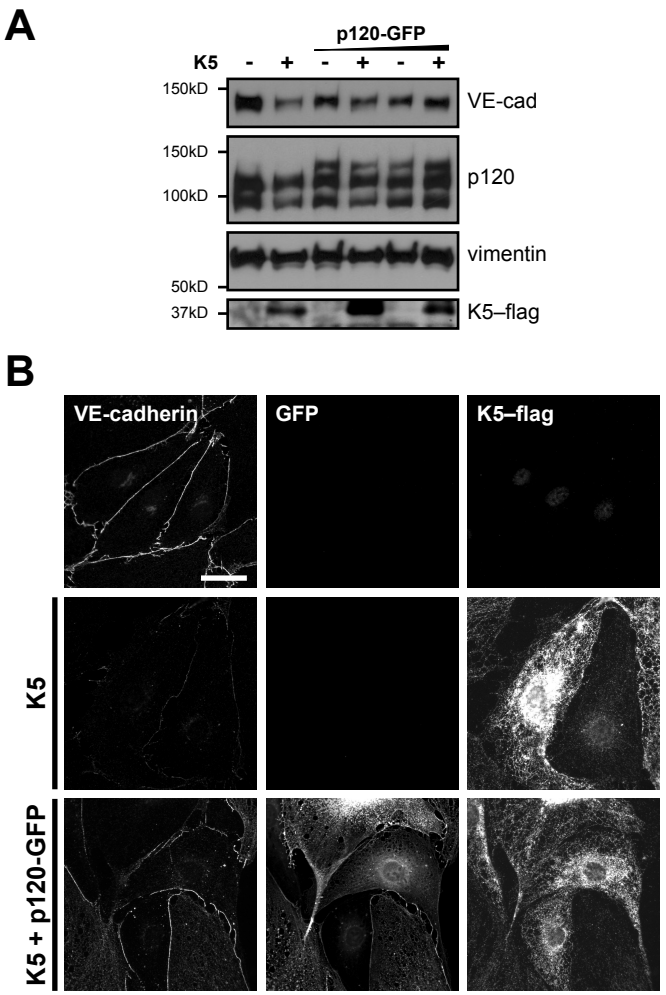


Figure 4.12: *p120 protects VE-cadherin from down-regulation by K5.* (A) K5–flag was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. After 24 hours, cells were additionally transduced to express varying levels of p120–GFP, or transduced with an empty adenovirus as a control. After another 24 hours, cells were lysed and analyzed by Western blot. (B) K5–flag and p120–GFP were expressed in primary dermal microvascular endothelial cells by adenoviral transduction. After 24 hours, cells were fixed and processed for immunofluorescence. Bar: 20 μm.

cesses which may displace p120 from cadherins remains an important area for research. Many studies of VE-cadherin have focused on the potential of inflammatory mediators such as histamine and vascular endothelial growth factor (VEGF) to induce phosphorylation of the cadherin (Andriopoulou et al., 1999; Esser et al., 1998). However, there is considerable disagreement over which sites may be phosphorylated under different conditions and whether p120 binding is disrupted (Hatanaka et al., 2011; Wallez et al., 2007). Furthermore, it remains unclear whether phosphorylation alone is sufficient to drive cadherin down-regulation (Adam et al., 2010). Nonetheless, VEGF signaling has been linked to phosphorylation and β-arrestin–dependent endocytosis of VE-cadherin (Gavard and Gutkind, 2006; Hebda et al., 2013). Even if the precise mechanisms activated in different physiological contexts remain unclear, VE-cadherin phosphorylation, disruption of p120 binding, and cadherin endocytosis are emerging hallmarks of the endothelial response to inflammatory signals.

Most studies of cadherin ubiquitination have focused on epithelial (E)-cadherin rather than VE-cadherin. The c-Cbl–like ligase Hakai targets E-cadherin for ubiquitination in a manner dependent on Src-mediated phosphorylation of two juxtamembrane domain tyrosine residues which are not conserved in VE-cadherin (Fujita et al., 2002). Hakai-mediated ubiquitination of E-cadherin is associated with increased endocytosis and down-regulation of the cadherin. While the specific residues ubiquitinated by Hakai remain unknown, the phospho-tyrosines required for targeting of E-cadherin are within the p120 binding region. This raises the possibility that p120 binding may compete with phosphorylation and Hakai-mediated ubiquitination of the cadherin. Although this hypothesis has not been tested, it would, if correct, represent another instance of p120 modulation of a cadherin endocytic mechanism. One study supporting this possibility used mitochondrial targeting assays to demonstrate that E-cadherin ubiquitination and p120 recruitment were mutually exclusive (Hartsock and Nelson, 2008). However, there is also evidence that Hakai does not trigger E-cadherin endocytosis directly. Instead, Hakai-mediated ubiquitination may target E-cadherin that has already entered the endocytic pathway for lysosomal degradation rather than recycling back to the cell surface, a mechanism dependent on Hrs and Src (Palacios et al., 2005). Interestingly, depletion of Hrs causes up-regulation of E-cadherin, sug-

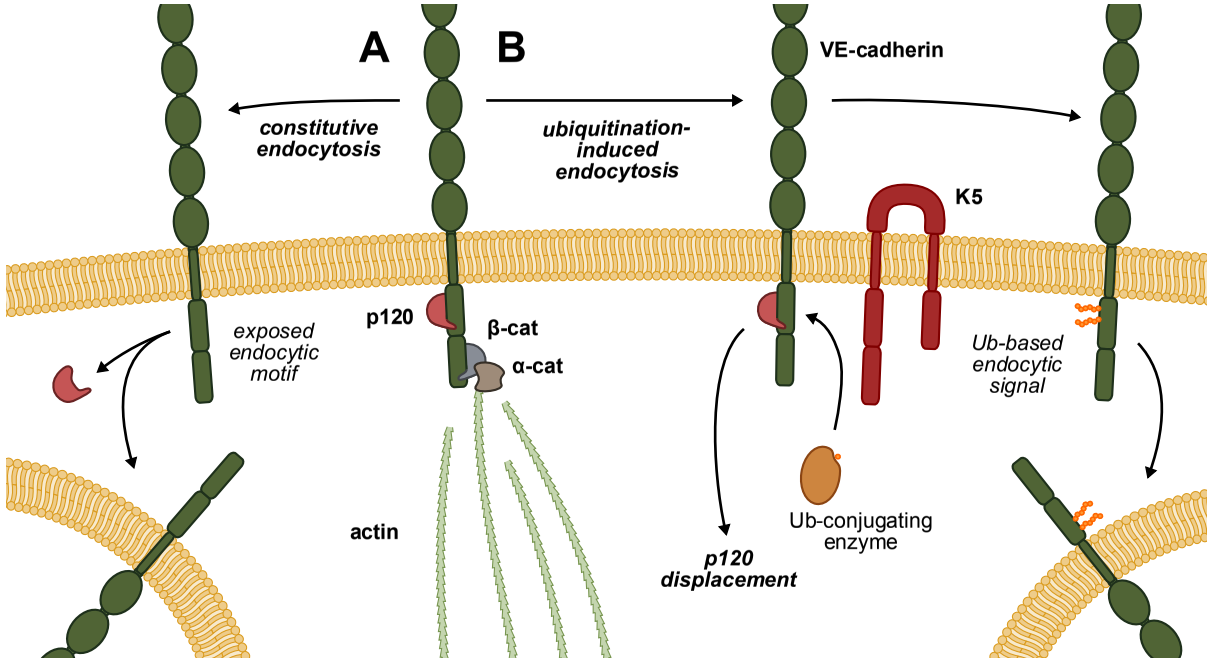


Figure 4.13: *p120 guards against multiple internalization signals.* (A) Displacement of p120 from VE-cadherin by an unknown mechanism unmasks an endocytic motif in the cadherin juxtamembrane domain. This motif drives constitutive VE-cadherin endocytosis which confers plasticity to the endothelial adherens junction. (B) K5 targets VE-cadherin for ubiquitination, displaces p120 from the cadherin, and induces VE-cadherin endocytosis and down-regulation. K5-induced VE-cadherin endocytosis does not require the constitutive endocytic motif.

gesting this mechanism may play a role in balancing cellular cadherin levels (Toyoshima et al., 2007). The role of p120 in this pathway is unclear. A second ubiquitin ligase, MDM2, has also been reported to target E-cadherin, and in human breast carcinomas, high levels of MDM2 expression correlated with decreased E-cadherin protein levels (Yang et al., 2006). However, as with cadherin down-regulation by Hakai, the relevance of p120 to E-cadherin down-regulation by MDM2 remains unknown.

In addition to the disease-associated K5-mediated ubiquitination and down-regulation of VE-cadherin reported here, VE-cadherin ubiquitination may also play a role in normal cellular processes. Proteasome inhibitors can block constitutive VE-cadherin endocytosis (Xiao et al., 2003b) as well as K5-induced VE-cadherin down-regulation (Figure 4.3 A). Furthermore, treatment with the inflammatory signal bradykinin induces VE-cadherin ubiquitination in vitro and in vivo, and drives VE-cadherin endocytosis in vitro (Orsenigo et al., 2012). These findings suggest that cellular ubiquitin ligases may participate in the regulation of cadherin stability. One intriguing possibility is that cellular homologs of K5, MARCH-family ubiquitin ligases (Bartee et al., 2004; Nicholas et al., 1997), might serve such a function. As with many viral pathomechanisms, K5-induced down-regulation of VE-cadherin may result from the hijacking of existing cellular machinery.

Somewhat surprisingly, the junctional remodeling induced by K5 in cultured endothelial cells was not accompanied by profound changes in cell morphology or the retraction of cells to form large gaps in the monolayer (Figure 4.1 B). Such changes sometimes accompany endothelial cell–cell junction disassembly in response to inflammatory mediators (for example, Chen et al., 2012; Gong et al., 2014). This raises the possibility that inflammatory mediators may induce changes in endothelial cells beyond the removal of VE-cadherin from junctions, such as activation of myosin (Vandenbroucke et al., 2008; Wallez and Huber, 2008). It is also likely that HHV-8 expresses factors in addition to K5 which disrupt endothelial cell–cell adhesion, potentially with more inflammatory-like effects. Additional HHV-8 virulence factors could also explain why expression of K5 in cultured endothelial cells did not decrease p120 protein levels (Figure 4.1 A), while p120 staining in Kaposi sarcoma lesion biopsies was substantially decreased (Figure 4.5 B). Since disruption of cell–cell junctions can change cell morphology, and changing cell morphology, such as

through cytoskeletal remodeling, can disrupt cell–cell junctions, disentangling the causal mechanisms in cases where both phenotypes occur may be difficult. Interestingly, p120 can influence both cell–cell junctions and cell morphology. In addition to regulating cadherin stability, p120 also affects cytoskeletal dynamics through regulation of Rho GTPases (Anastasiadis, 2007; Beckers et al., 2010). This function is separable from p120 modulation of cadherin endocytosis (Chiasson et al., 2009), but recruitment of p120 to cell borders can influence the spreading of individual cells on an adhesive substrate (Oas et al., 2013). Further work will be needed to fully understand the connections between adherens junction disassembly and cytoskeletal remodeling in inflammatory conditions, and the roles of p120 in both processes.

Our results indicate that p120 binding to the cadherin juxtamembrane domain modulates VE-cadherin endocytosis driven by two very different signals. p120 regulates both the constitutive endocytosis and recycling of VE-cadherin which establishes junctional plasticity necessary for collective migration of endothelial cells and the K5-induced ubiquitination, endocytosis, and down-regulation of VE-cadherin associated with the endothelial-derived tumor Kaposi sarcoma. Constitutive- and K5-induced VE-cadherin endocytosis are driven by different internalization signals, but both signals are located within the p120 binding region of the cadherin. Thus, the cadherin juxtamembrane domain serves as the integration site for multiple mechanisms regulating cadherin stability. A variety of different endocytic signals drive cadherin internalization and promote junctional plasticity in different contexts, while p120 functions as the master regulator stabilizing the cadherin and guarding junctional stability.

4.4 Materials and Methods

Cell culture

Primary human dermal microvascular endothelial cells and keratinocytes were isolated from neonatal foreskin. Primary endothelial cells and HMEC-1 immortalized endothelial cells (Ades et al., 1992) were cultured in Endothelial Growth Medium 2 Microvascular (Lonza) and grown on tissue culture-treated plastic or glass coverslips coated with

0.1% gelatin. Keratinocytes were cultured in Keratinocyte Growth Medium Gold (Lonza). African green monkey kidney fibroblast-like cell line COS-7 (American Type Culture Collection, ATCC), human embryonic kidney cell line HEK-293T (ATCC), and, for adenovirus production, human embryonic kidney cell line QBI-293A (MP Biomedicals) were cultured in Dulbecco's modification of Eagle's medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% antibiotic/antimycotic solution (Corning). Chinese hamster ovary epithelial cell line CHO (ATCC) was cultured in Kaighn's Modification of Ham's F-12 Medium (ATCC) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% antibiotic/antimycotic solution (Corning). Cells were transfected using Lipofectamine 2000 (Life Technologies). CHO-derived cell lines were created by transfecting CHO cells with the construct of interest and a plasmid conferring resistance to neomycin/G-418. Transfected cells were selected in 5–10 mg/mL G-418 (Mediatech), then clonal populations were isolated and expanded. After selection, CHO lines were maintained in 0.5–1 mg/mL G-418. Stable lentiviral transduction of HMEC-1 cells was achieved through selection in 20 μg/mL blasticidin (Valeant Pharmaceuticals), followed by further enrichment for cells expressing fluorescently-tagged constructs by fluorescence activated cell sorting (BD Biosciences FACS Aria II). After selection, cells were maintained in 1–5 μg/mL blasticidin.

Table 4.1: *Primers used in this chapter.*

VE-cadherin K626R, K633R mutagenesis primers	
Forward	5'-G CGG CTC CGG AGG CAG GCC CGC GCG CAC GGC AGG AGC GTG CCG G-3'
Reverse	5'-C CGG CAC GCT CCT GCC GTG CGC GCG GGC CTG CCT CCG GAG CCG C-3'
VE-cadherin DEE646–648AAA mutagenesis primers	
Forward	5'-CTG GTC ACC TAC GCA GCA GCA GGC GGC GGC GAG ATG-3'
Reverse	5'-CAT CTC GCC GCC GCC TGC TGC TGC GTA GGT GAC CAG-3'
VE-cadherin GGG649–651AAA mutagenesis primers	
Forward	5'-TAC GAC GAG GAG GCA GCA GCA GAG ATG GAC ACC-3'
Reverse	5'-GGT GTC CAT CTC TGC TGC TGC CTC CTC GTC GTA-3'

Underlined codons indicate sites of mutation.

cDNA constructs

The K5–GFP constructs, containing HHV-8 K5 ligated between EcoRI and BamHI in pEGFP-N1 (Clontech), in-frame with GFP, were provided by R. Means (Yale University, New Haven, CT) and J. Jung (University of Southern California, Los Angele, CA). The ligase-dead K5 mutant (C30A, C32A, H40A, C43A), described previously, was created by site-directed mutagenesis (Means et al., 2007). The construct encoding human VE-cadherin, ligated between EcoRI sites in pECE, was provided by E. Dejana (Italian Foundation for Cancer Research Institute of Molecular Oncology, Milan, Italy). The K5-resistant mutant (K626R, K633R), constitutive endocytosis–resistant mutant (DEE646–648AAA; Nanes et al., 2012), and p120 binding control mutant (GGG649–651AAA) were created by site-directed mutagenesis (primers described in Table 4.1). For virus production, VE-cadherin constructs were subcloned between BamHI and AgeI restriction sites in Gateway TagRFP-AS-N (Evrogen), in-frame with monomeric C-terminal TagRFP, then shuttled into pAd/Cmv/V5-DEST for adenovirus production or pLenti6/V5-DEST for lentivirus production using LR Clonase recombination (Life Technologies). The pSV2-neo plasmid was used to confer neomycin/G-418 resistance (Southern and Berg, 1982).

Virus production

To create replication-deficient human adenovirus type 5 packaged with a gene of interest, the gene was cloned into pAd/CMV/V5-DEST, then digested with PacI to expose the viral inverted terminal repeats. Linearized DNA was transfected into virus-producing QBI-293A cells, which were harvested, concentrated, and lysed after 48–72 hours to recover adenovirus. To create replication-deficient second-generation lentivirus packaged with a gene of interest, the gene was cloned into pLenti6/V5-DEST and transfected into HEK-293T cells using a kit combining transfection reagent with the necessary lentiviral regulatory genes (LENTI-Smart, InvivoGen). Lentivirus was collected from culture supernatants 48–72 hours after transfection.

Immunoprecipitation and Western blot analysis

For immunoprecipitation experiments, cells were harvested either in 0.5% Triton X-100 (Roche) to preserve non-covalent interactions or 1% Triton X-100 with 0.1% sodium dode-

Table 4.2: *Primary antibodies used in this chapter.*

Target	Antibody	Application
β-catenin	rabbit polyclonal (NeoMarkers, cat. #RB-090-P0)	IF
	rabbit polyclonal (Sigma Aldrich, cat. #C-2206)	WB
E-cadherin	mouse IgG2a (BD Biosciences, cat. #610182)	WB
flag	chicken polyclonal (Bethyl Laboratories, cat. #A190-100A)	IF, WB
GAPDH	rabbit polyclonal (Santa Cruz Biotech., cat. #sc-25778)	WB
N-cadherin	mouse IgG1 (BD Biosciences, cat. #610920)	WB
p120	rabbit polyclonal (Santa Cruz Biotech., cat. #sc-1101)	IF, IP, WB
	rabbit IgG (Abcam, cat. #ab92514)	IHC
ubiquitin	mouse IgG1 (Santa Cruz Biotech., cat. #sc-8017)	WB
VE-cadherin	mouse IgG2A, clone BV6 (Corada et al., 2001)	IF
	mouse IgG1 (BD Biosciences, cat. #610252)	IF
	rabbit polyclonal (Novus Biologicals, cat. #18940002)	IP, WB
	goat polyclonal (Santa Cruz Biotech., cat. #sc-6458)	IHC
vimentin	mouse IgG (Sigma Aldrich, cat. #V6630)	WB

IF, immunofluorescence; WB, Western blot; IP, immunoprecipitation; IHC, immunohistochemistry

cyl sulfate (Fisher). Both buffers also contained protease inhibitor cocktails (Complete Mini tablets, EDTA free; Roche), 5 mg/mL N-ethylmaleimide to inhibit deubiquitinase enzymes, 10 μM MG-132 to inhibit the proteasome, 150 mM sodium chloride, 10 mM Hepes, 1 mM EGTA, and 0.1 mM magnesium chloride. In addition, pretreatment of cells with 10 μM MG-132 for 2 h prior to harvesting was used to increase the amount of ubiquitinated protein recovered. After 30-minute incubation at 4 °C, cell lysates were centrifuged at 16 100 g for 10 minutes, and the soluble fraction was diluted to a final protein concentration of 1 mg/mL. Cell lysates were then incubated with 2 μg antibody against VE-cadherin or p120 (Table 4.2) conjugated to ferromagnetic beads (Dynabeads, Life Technologies) for 1 hour at 4 °C. The beads were then washed with 0.1% Triton X-100 and eluted into Laemmli sample buffer (Bio-Rad Laboratories) with 5% β-mercaptoethanol. For other Western blot experiments, cells were harvested directly into sample buffer. Samples were heated at 95 °C for 5 minutes, then separated by SDS-PAGE and analyzed by immunoblotting on nitrocellulose membranes (Whatman). To increase detection of ubiquitin, membranes were covered with deionized water and autoclaved for 30 minutes. Primary antibodies are listed in Table 4.2. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories), a luminol-based detection system (ECL, GE Healthcare), and autoradiography film (Denville Scientific) were used for detection.

Immunofluorescence

Cells cultured on glass coverslips were fixed either in methanol for 2 minutes at 4 °C or in 4% paraformaldehyde for 10 minutes followed by 0.1% Triton X-100 for 8 minutes at room temperature, depending on the performance of the primary antibodies used (Table 4.2). Secondary antibodies conjugated to fluorescent dies (Alexa Fluor 488, 555, or 647 nm; Life Technologies) were used to identify target molecules. Microscopy was performed using an epifluorescence microscope (DMRXA2, Leica) equipped with 63×/1.32 NA and 100×/1.40 NA oil immersion objectives with apochromatic aberration and flat field corrections, narrow band pass filters, and a digital camera (ORCA-ER C4742-80, Hamamatsu Photonics). Images were captured using Simple PCI software (Hamamatsu Photonics).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were cut to 5 μm sections, affixed to glass slides, deparaffinized in Xylene, and processed for heat-induced antigen retrieval in either 10 mM sodium citrate, pH 6.0, for VE-cadherin staining, or Tris/EDTA, pH 9.0, for p120 staining. Primary antibodies are described in Table 4.2. Horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine substrate were used to detect antibody labeling. Hematoxylin was used as a counterstain. Digital images were captured using whole-slide scanning (Nanozoomer 2.0HT, Hamamatsu Photonics). For quantification, each image channel was log-transformed, then a linear unmixing algorithm was used to separate the resulting red, green, and blue absorbances into diaminobenzidine and hematoxylin absorbance components. Vascular spaces were outlined, and for each vessel, average diaminobenzidine absorbance was calculated within 1.1 μm of the border.

Internalization assay, vesicle analysis, and migration assay

In order to measure K5-induced or constitutive internalization of VE-cadherin, cells were incubated in antibody against the VE-cadherin extracellular domain dissolved in culture medium for 30 minutes at 4 °C. Unbound antibody was removed by washing with cold PBS. Cells were then incubated in culture medium for various time periods at 37 °C to allow internalization to occur. At the end of the internalization period, cells were returned to 4 °C and washed with PBS. Any antibody remaining at the cell surface was removed by washing cells with a low-pH buffer (PBS with 100 mM glycine, 20 mM magnesium acetate, and 50 mM potassium chloride, pH 2.2). Cells were then fixed and processed for immunofluorescence, with a second antibody against VE-cadherin, distinguished based on isotype, used to label the total cadherin pool. Internalization was quantified as the ratio of fluorescence signals corresponding to the internalized and total cadherin pools. For vesicle analysis experiments, cells were treated with 100 μM chloroquine (Sigma Aldrich) dissolved in culture medium for 24 hours, refreshed after 12 hours, then fixed and processed for immunofluorescence. Vesicles were identified by automated selection of 4-connected regions of pixels above background thresholds with areas of 2.56×10^{-2} to 16.0×10^{-2} μm^2. Collective cell migration was measured using a scratch wound assay. Cells were grown to confluent

monolayers, scratched with a pipette tip, and imaged over time using a bright-field microscope (DM IL, Leica) equipped with a 5×/0.12 NA objective with apochromatic aberration correction and a digital camera (DFC420 C, Leica). Images were acquired using FireCam software (version 3.4; Leica).

Image analysis and statistics

The Fiji distribution of ImageJ (Schindelin et al., 2012) with custom plugins was used for all image analysis and automated quantification. The JAMA linear algebra library (version 1.0.3; National Institute of Standards and Technology) was used to implement the linear unmixing algorithm. Statistical analyses were implemented in R (version 2.15; R Foundation for Statistical Computing). The Kruskal-Wallis rank sum test with Dunn's method for multiple comparisons was used to evaluate non-parametric scaled data (Dunn, 1964).

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Chapter 5

From static linkers to effectors of tissue dynamics

Studies elucidating the structure of adherens junctions, their components, and the interactions between them have greatly contributed to our understanding of cell–cell adhesion and its role in tissue patterning and morphogenesis. The molecular organization of adherens junctions is intimately related to their function linking individual cells into tissues—as the saying goes, form follows function.[1] However, both the forms and the functions of tissues in multicellular organisms undergo constant change. This is particularly true in the vasculature, where vessels are remodeled during development and wound healing, and endothelial cells must balance between static and dynamic cell–cell adhesion. Thus, understanding endothelial adherens junctions only as structural components linking adjacent cells is clearly insufficient. We must also understand how endothelial adherens junctions contribute to vessel dynamics. Toward that end, this dissertation identifies two signals driving vascular endothelial (VE)-cadherin internalization in different contexts. A constitutive endocytic motif establishes endothelial cell–cell junction plasticity, while a tumor-associated viral ubiquitin ligase targets VE-cadherin for ubiquitination, endocytosis, and down-regulation, leading to disassembly of the adherens junction.

[1] In fact, Sullivan used the language of biology, albeit not at the molecular level, to present his guiding principle for modernist architecture (1896):

> "Whether it be the sweeping eagle in his flight, or the open apple-blossom, the toiling work-horse, the blithe swan, the branching oak, the winding stream at its base, the drifting clouds, over all the coursing sun, *form ever follows function*, and this is the law. Where function does not change, form does not change."

5.1 Context-dependent signals drive VE-cadherin endocytosis

Chapter 3 identifies a motif in the VE-cadherin juxtamembrane domain responsible for driving constitutive endocytosis of the cadherin. This motif is anchored by three acidic residues, and mutation of these residues creates a cadherin variant that is resistant to constitutive endocytosis (Figures 3.1, 3.4, and 3.6). Conversely, attachment of the VE-cadherin endocytic signal to an unrelated transmembrane protein can drive internalization (Figure 3.2). When exposed, this signal triggers rapid removal of the cadherin from the membrane. However, p120-catenin (p120) binding to the cadherin juxtamembane domain physically masks the signal, stabilizing the cadherin (Figure 3.7). This mechanism allows a level of constitutive endocytosis and recycling of the cadherin, which confers plasticity to the junction. Expression of the endocytosis-resistant VE-cadherin mutant in endothelial cells inhibits collective migration in a scratch wound assay (Figures 3.13 and 5.1), and because endothelial migration is an important component of angiogenesis, this signal likely has important roles in endothelial function.

In contrast, Chapter 4 identifies a signal responsible for induced VE-cadherin endocytosis. This signal is associated with a disease characterized by inappropriate loss of VE-cadherin, the endothelial-derived tumor Kaposi sarcoma. Kaposi sarcoma lesions contain fascicles of spindle cells, believed to be of endothelial origin, and abnormal, leaky vascular proliferations. The endothelial cells lining the vascular spaces in Kaposi sarcoma lesions express lower levels of VE-cadherin and p120 than ordinary endothelial cells, indicating that endothelial adherens junctions are disrupted (Figure 4.5). Human herpesvirus 8 (HHV-8), which causes Kaposi sarcoma, may disrupt endothelial cell–cell junctions through multiple mechanisms, at least one of which involves inducing VE-cadherin endocytosis. The HHV-8 ubiquitin ligase K5 targets VE-cadherin for ubiquitination on two membrane-proximal lysine residues (Figure 4.7), displacing p120 from the cadherin (Figure 4.11), and driving cadherin endocytosis (Figure 4.6). Interestingly, the constitutive endocytosis-resistant VE-cadherin mutant is not resistant to down-regulation by K5, and a K5-resistant VE-cadherin mutant still undergoes constitutive endocytosis (Figures 4.7 and 4.10).

Thus, distinct signals drive VE-cadherin endocytosis in different contexts and with very

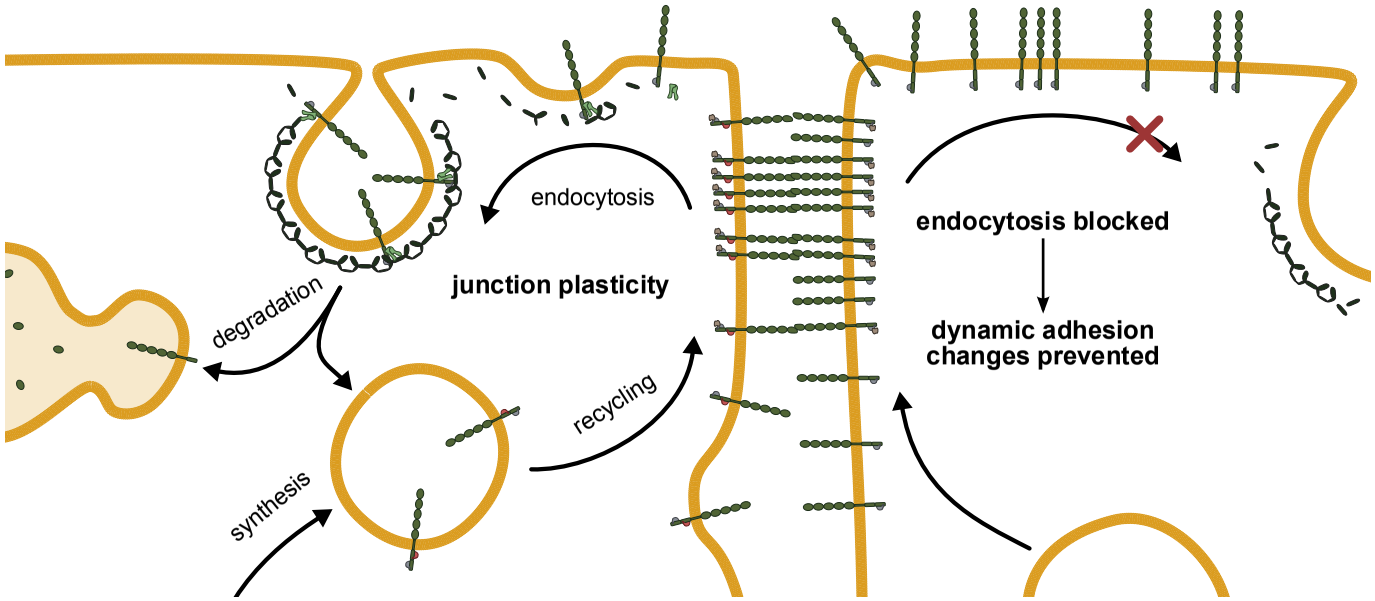

Figure 5.1: *Cadherin endocytosis and recycling establish junction plasticity.* Left: Constitutive endocytosis and recycling of the cadherin confers plasticity to the adherens junction, facilitating the cellular rearrangements necessary for collective migration. Right: In contrast, cells expressing endocytosis-defective cadherin cannot rearrange cell–cell contacts, preventing dynamic adhesion changes and disrupting collective migration.

different results. Constitutive VE-cadherin endocytosis is important for endothelial function, while K5-induced VE-cadherin endocytosis disassembles endothelial adherens junctions. Despite these differences, both the constitutive and K5-induced endocytic signals share two important features. First, both signals involve sites in the VE-cadherin juxtamembrane domain, the same region of the cadherin cytoplasmic tail bound by p120. Second, p120 functions as a brake on both signals, modulating constitutive endocytosis and protecting VE-cadherin from K5. This indicates that the cadherin juxtamembrane domain serves as a common platform integrating multiple signals controlling cadherin trafficking, with p120 acting as a master regulator, guarding cadherin stability. However, while these results advance our understanding of the dynamic regulation of endothelial cell–cell adhesion, and of the adherens junction more generally, several important questions remain.

5.2 p120 dissociation: *Quis custodiet ipsos custodes?*

First, the central role of p120 in ensuring cadherin stability raises an issue which recurs throughout this dissertation. What controls p120 binding to cadherins, and what triggers its dissociation? The fact that VE-cadherin undergoes constitutive endocytosis and recycling indicates that p120 dissociation must be a routine event. Is this the result of an active process, such as a kinase targeting VE-cadherin with some low basal rate of phosphorylation, or simply passive competition for access to the cadherin juxtamembrane domain between p120 and the endocytic machinery? Structural and biochemical data suggest that p120 binds to cadherins quite tightly (Ishiyama et al., 2010), but very little is known about the dynamics of the cadherin–p120 interaction *in vivo*. Additionally, the endocytic adapter responsible for recognizing the VE-cadherin constitutive endocytic signal has not yet been identified, further hindering a detailed understanding of the mechanism of constitutive VE-cadherin endocytosis.

In contrast, a number of mechanisms have been proposed for induced VE-cadherin endocytosis in response to various inflammatory and permeability signals. As discussed in Section 1.5.2, many studies have focused on VE-cadherin phosphorylation as a trigger for endothelial adherens junction disassembly. While some of the VE-cadherin phosphoryla-

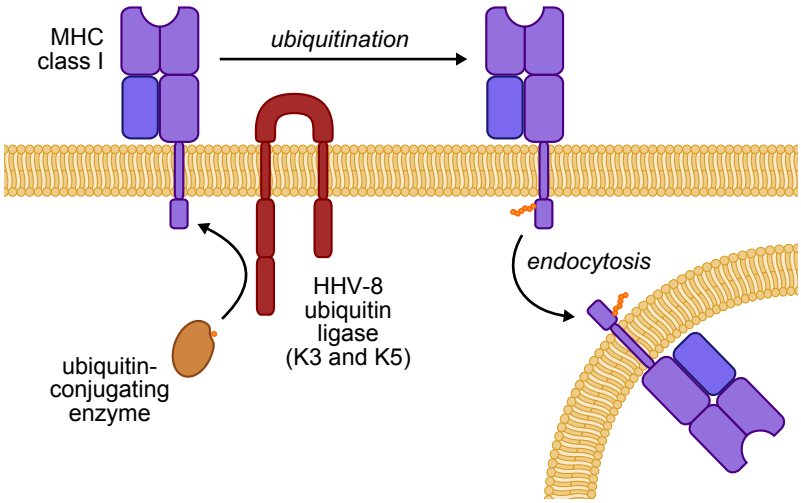

Figure 5.2: *Viral ubiquitin ligases facilitate evasion of the host immune response.* Human herpesvirus 8 (HHV-8) ubiquitin ligases K3 and K5 target cellular components of the immune system, such as major histocompatibility complex (MHC) class I, for ubiquitination, endocytosis, and down-regulation.

tion sites identified do lie within the p120 binding region, the actual effect of VE-cadherin phosphorylation at those sites on p120 binding remains unclear.

This dissertation identifies VE-cadherin ubiquitination as a mechanism for displacing p120 (Figure 4.11). While K5 is a viral ubiquitin ligase originally identified as part of an immune evasion mechanism (Figure 5.2), it does have cellular homologs, the membrane-associated RING-CH (MARCH) family proteins (Bartee et al., 2004; Nicholas et al., 1997). Several MARCH family ligases are expressed in endothelial cells (Figure 5.3), raising the possibility that they might regulate VE-cadherin stability under physiologic conditions. Indeed, one report has identified VE-cadherin ubiquitination in response to an inflammatory signal *in vivo* (Orsenigo et al., 2012), further suggesting a broader role for ubiquitin ligases in the regulation of cadherin stability. Just as VE-cadherin phosphorylation can be tuned by phosphatases (Bäumer et al., 2006), cadherin ubiquitination may also be tuned by deubiquitinase enzymes, enabling precise control of ubiquitin-dependent signaling. Recent studies have found important roles for deubiquitinases in clathrin-mediated endocytosis in yeast (Weinberg and Drubin, 2014), as well as in vessel branching and angiogenesis in mouse development (Rivkin et al., 2013). While the latter effect is thought to involve modulation of Wnt signaling rather than VE-cadherin trafficking, the connection of

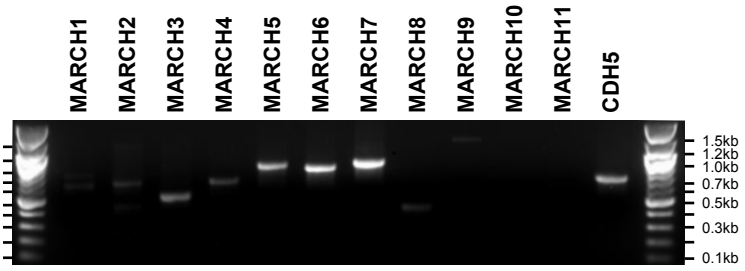

Figure 5.3: *MARCH-family ubiquitin ligases are expressed in endothelial cells.* Cultures of primary dermal microvascular endothelial cells were lysed and processed for reverse transcription polymerase chain reaction (RT-PCR) using primers recognizing transcripts for each of the 11 cellular MARCH genes or VE-cadherin (CDH5). RT-PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide and ultraviolet light.

deubiquitinases to both endocytic pathways and angiogenesis does present a potential role for deubiquitinases in endothelial adherens junction dynamics.

In addition to binding competition and post-translational modification of the cadherin, post-translational modification of p120 itself may regulate cadherin–p120 binding. One study found that phosphorylation of p120 at a serine residue in the C-terminal regulatory region was associated with decreased interaction with VE-cadherin and disassembly of endothelial cell–cell junctions (Vandenbroucke St Amant et al., 2012). However, this residue does not directly participate in the cadherin–p120 interaction (Ishiyama et al., 2010). Other potential targets for post-translational modification of p120 are lysine residues in the third and fifth armadillo repeat domains. These lysines interact through hydrogen bonds with the three acidic residues that form the core of the constitutive endocytic signal (Figures 3.1 and 3.7), and ubiquitination at either lysine would very likely prevent p120 from binding. While we were not able to detect K5-induced ubiquitination of p120 (Figure 4.4 C), the possibility that post-translational modification of p120 might regulate cadherin stability warrants further investigation.

5.3 Understanding catenin role switching

Second, while this dissertation focuses primarily on the roles of catenins in the adherens junction, catenins also function in signaling pathways influencing transcription, and the relationships between these different roles are not well understood. β-catenin in particular

is perhaps better known as an effector of Wnt signaling than as a link between cadherins and the actin cytoskeleton (Clevers and Nusse, 2012; Valenta et al., 2012). When free in the cytoplasm, β-catenin is typically unstable and rapidly degraded. However, Wnt signaling through the Frizzled receptor prevents β-catenin degradation. β-catenin then translocates to the nucleus, where it interacts with TCF/LEF-family transcription factors to activate gene expression. In addition to this canonical Wnt–β-catenin signaling pathway, other non-canonical Wnt signaling pathways can influence planar cell polarity and affect the cytoskeleton. While adherens junctions could, in theory, provide a reservoir of β-catenin available for signaling functions, almost no data are available to connect the two catenin pools. Ever since β-catenin was independently identified based on its junctional and signaling functions (Ozawa et al., 1989; Wieschaus et al., 1984), the connection between the two roles has remained unclear.

Like β-catenin, transcriptional functions have also been identified for p120, although they are somewhat less well understood. p120 interacts with the transcriptional repressor Kaiso (Daniel and Reynolds, 1999; Prokhortchouk et al., 2001). However, unlike β-catenin, which translocates to the nucleus to activate TCF/LEF, p120 sequesters Kaiso in the cytoplasm, relieving its transcriptional repression. Interestingly, Kaiso suppresses Wnt signaling (Kim et al., 2004) and the Wnt pathway effector Dishevelled stabilizes p120 (Park et al., 2006), potentially connecting the p120 and β-catenin signaling cascades. Additionally, Kaiso may suppress transcription of β-catenin itself, possibly establishing a positive-feedback mechanism (Liu et al., 2014). While p120–Kaiso signaling does not involve p120 translocation to the nucleus, nuclear localization of p120 has been detected (Hosking et al., 2007). Furthermore, nuclear localization has also been reported for the p120-family protein armadillo repeat gene deleted in velocardiofacial syndrome (ARVCF), where it influences mRNA splicing (Rappe et al., 2014). However, as with β-catenin, p120's signaling functions have not been connected to its adherens junction role.

Complicating matters even further, in addition to preventing cadherin endocytosis and influencing transcription, p120 also modulates Rho-family GTPases (Section 2.4.1; Oldenburg and de Rooij, 2014). This function does seem to be connected to p120's adherens junction role. Although p120-mediated stabilization of cadherins is Rho-independent (Chi-

asson et al., 2009), p120 recruitment to cell borders promotes cell spreading on an adhesive substrate (Oas et al., 2010), presumably due to local cytoskeletal effects. Still, the functional relationship between p120 promotion of cadherin stability and modulation of Rho GTPases *in vivo* remains murky. Much more work is needed to truly understand the connections between the remarkably different roles shared by the catenins.

5.4 Cadherin diversity

Lastly, how does the diversity of cadherins, both among the different cadherin family members expressed in different cell types and among the multiple pools of cadherin within each cell, affect junction dynamics? At any particular moment, not every cadherin molecule within the cell is part of an adherens junction complex. Some cadherins may be at the cell surface, but not incorporated into junctions, or contained within endocytic and recycling vesicles. The fluorescence recovery after photobleaching experiments presented in this dissertation suggest a complex relationship between these different cadherin pools, since VE-cadherin mobility cannot be attributed solely to diffusion within the membrane or endocytosis and recycling (Figures 3.9 and 3.10). But how are these cadherin subpopulations related? Is cadherin removed directly from the adherens junction by endocytosis, or does it separate from the junctional complex before internalization? Does incorporation into a junction stabilize the cadherin, and, if so, is this the result of p120 binding to the cadherin juxtamembrane domain, or other processes as well? Understanding the establishment and regulation of cell–cell adhesion dynamics will require answers to these questions.

The different classical cadherins have most often been compared based on the strength and specificity of their *trans* interactions (Nose et al., 1988). However, while the interactions between different cadherins and the catenins are broadly similar, the cadherin cytoplasmic domains do contain substantial differences which may affect their dynamics in important ways. For example, while the acidic amino acid–based endocytic motif described in Chapter 3 is conserved in many classical cadherins, it serves as the primary internalization signal only in VE-cadherin (Figure 3.8). Similarly, K5 targets VE-cadherin, but not E-cadherin, for ubiquitination and endocytosis (Figure 4.2), and the dileucine-based endo-

cytic motif contained in E-cadherin is not conserved in VE-cadherin (Miranda et al., 2001; Miyashita and Ozawa, 2007b). The existence of different trafficking motifs in different cadherins is not entirely surprising, since different cell types and tissues have different requirements for adhesion dynamics. Clearly, multiple classical cadherins do not exist solely for the purpose of adhesion-based cell sorting. Differences in intracellular regulatory mechanisms are also important, although they are considerably less well understood.

5.5 Outlook

The results presented in this dissertation begin to establish the importance of cadherin endocytosis for adherens junction plasticity and dynamic cell–cell adhesion. However, as discussed above, many questions remain, particularly regarding the mechanistic details of how the complex adhesive properties of different tissues emerge from cadherin trafficking and catenin regulation. But more broadly, why use the relatively complicated process of removing cadherin molecules from the membrane and recycling them back to junctions to modulate adhesion, when switching adhesion molecules between adhesive and non-adhesive states could provide a simpler solution? Perhaps the complexity of the system is exactly the point, since it provides more opportunities for regulation and fine-tuning of adhesion in different biological contexts. Precise regulation of cell–cell adhesion is particularly important in the endothelium, which must constantly balance the need to resist vascular leak with the flexibility required for vascular patterning, remodeling, and angiogenesis.

Going forward, it will be important to connect the cellular mechanisms regulating VE-cadherin trafficking in endothelial cells to dynamic changes in the vasculature *in vivo*. Transgenic animals with the cadherin trafficking mutants identified in this dissertation are likely to be particularly informative, as they will facilitate investigation of VE-cadherin endocytosis in development and disease pathogenesis. As discussed in Section 1.6, disrupted regulation of endothelial cell–cell adhesion contributes to a variety of diseases, especially through excessive inflammation. Of note, all the available classes of anti-inflammatory drugs, including cyclooxygenase inhibitors, tumor necrosis factor inhibitors, and steroids, affect signaling through inflammatory cytokines or transcriptional regulation. Therapies

targeting the downstream effectors of inflammation, especially disassembly of endothelial adherens junctions, are likely to be more focused and effective. Developing such therapies will require a detailed understanding of the basic cellular mechanisms regulating endothelial cell–cell adhesion, including VE-cadherin trafficking. This dissertation contributes to that foundation.

Appendix A

Derivation of the fluorescence recovery after photobleaching models

This appendix presents derivations of the diffusion-limited and first-order–limited recovery models used to analyze the VE-cadherin fluorescence recovery after photobleaching (FRAP) studies in Chapter 3 (Figures 3.9 and 3.10; Table 3.1), and further explains the selection of the first-order–limited model as the more appropriate of the two.

A.1 Diffusion-limited recovery

Diffusion-limited models assume free and undirected movement of the protein of interest within the cell, resulting in net movement from regions of high concentration to those of low concentration. The model does not preclude the possibility of protein–protein or other interactions, but those interactions are assumed to be fast compared to diffusion so that they are always in equilibrium. Because VE-cadherin localization is generally restricted to cell borders, this model allows diffusion in one dimension. Although the cell membrane is a two-dimensional surface, both photobleaching and imaging integrate over the depth of the relatively thin cells used for the FRAP experiments, reducing the effective region of interest from a plane to a line. Similarly, FRAP analyses of protein diffusion in the three-dimensional cytoplasmic space use a two-dimensional model (Sprague et al., 2004). Since the one-dimensional equivalent has not been previously published, it is presented here.

The diffusion-limited model derives from Fick's law, describing concentration changes

over time in an uneven concentration field:

$$\frac{\partial f}{\partial t} = k\nabla^2 f \quad . \tag{A.1}$$

In one dimension, this simplifies to

$$\frac{\partial f}{\partial t} = k\frac{\partial^2 f}{\partial x^2} \quad , \tag{A.2}$$

where f is concentration and k is the diffusion coefficient, a parameter describing the rate of diffusion relative to the concentration gradient. This model assumes that the diffusion coefficient of VE-cadherin within the cell membrane does not vary with the concentration of the cadherin, at least over the range of concentrations observed in the experiment.

Fick's law takes the same form as the heat equation, which describes the diffusion of heat through a uniform solid, and has been analyzed extensively in that context. In particular, a solution is available for initial conditions appropriate to the experiment in Section 3.2.6, with a rectangular bleach region (Carslaw and Jaeger, 1959, p 54):

$$f(x,t) = \frac{A}{2}\left(\operatorname{erfc}\left(\frac{r-x}{2\sqrt{kt}}\right) + \operatorname{erfc}\left(\frac{r+x}{2\sqrt{kt}}\right)\right), \qquad \begin{matrix} -\infty < x < \infty \\ t \geq 0 \end{matrix}$$

$$f(x,0) = \begin{cases} 0\,, & -r < x < r \\ A\,, & |x| \geq r \end{cases} \quad . \tag{A.3}$$

Here, r is the radius of the bleach, so $|x| < r$ defines the bleach region, A is the baseline fluorescence or height of the bleach well, and

$$\operatorname{erfc}(z) = \frac{2}{\sqrt{\pi}}\int_z^\infty e^{-t^2}\mathrm{d}t \tag{A.4}$$

is the complementary error function. Note that these conditions make the further assumption that the length of the membrane is large relative to the length of the bleach region.

The parameters of interest for this model are average fluorescence within the bleach region, designated $\overline{\text{frap}}(t)$, and total fluorescence within the bleach region, designated

$\text{frap}(t) = 2r\,\overline{\text{frap}}(t)$. These can be found by integrating Equation A.3 over $|x| < r$:

$$\text{frap}(t) = \int_{-r}^{r} \frac{A}{2}\left(\text{erfc}\left(\frac{r-x}{2\sqrt{kt}}\right) + \text{erfc}\left(\frac{r+x}{2\sqrt{kt}}\right) \right) \mathrm{d}x \quad . \tag{A.5}$$

With transformation of variables:

$$\text{frap}(t) = \frac{A}{2}\left(\int_{x=-r}^{x=r} -2\sqrt{kt}\,\text{erfc}(u)\,\mathrm{d}u + \int_{x=-r}^{x=r} 2\sqrt{kt}\,\text{erfc}(v)\,\mathrm{d}v \right) ,$$
$$u = \frac{r-x}{2\sqrt{kt}} \qquad v = \frac{r+x}{2\sqrt{kt}} \quad . \tag{A.6}$$

Using

$$\int \text{erfc}(\xi)\,\mathrm{d}\xi = \xi\,\text{erfc}(\xi) - \frac{e^{-\xi^2}}{\sqrt{\pi}} + C \quad , \tag{A.7}$$

the integral evaluates to

$$\text{frap}(t) = A\sqrt{kt}\Bigg(-\left(\frac{r-x}{2\sqrt{kt}}\right)\text{erfc}\left(\frac{r-x}{2\sqrt{kt}}\right) + \frac{e^{-\left(\frac{r-x}{2\sqrt{kt}}\right)^2}}{\sqrt{\pi}}$$
$$+ \left(\frac{r+x}{2\sqrt{kt}}\right)\text{erfc}\left(\frac{r+x}{2\sqrt{kt}}\right) - \frac{e^{-\left(\frac{r+x}{2\sqrt{kt}}\right)^2}}{\sqrt{\pi}} \Bigg)\Bigg|_{-r}^{r} \quad . \tag{A.8}$$

Finally, simplifying gives the one-dimensional diffusion-limited recovery model:

$$\overline{\text{frap}}(t) = \frac{A}{r}\left(\frac{\sqrt{kt}}{\sqrt{\pi}}\left(1 - e^{-\left(\frac{r^2}{kt}\right)}\right) + r\,\text{erfc}\left(\frac{r}{\sqrt{kt}}\right) \right) \quad . \tag{A.9}$$

This function has $\lim_{t\to 0} \overline{\text{frap}}(t) = 0$ and $\lim_{t\to\infty} \overline{\text{frap}}(t) = A$, which is consistent with complete bleaching of the fluorescence signal and eventual recovery to the initial fluorescence level. It depends on three parameters: r, the radius of the bleach region; k, the diffusion coefficient; and A, the initial fluorescence level. Note that if some fraction of VE-cadherin is immobile on the timescale of the experiment, fluorescence within the bleach region will not recover to the initial fluorescence level. In this case, if A_0 is the initial fluorescence level, A/A_0 represents the mobile fraction of VE-cadherin.

A.2 First-order–limited recovery

An alternative model of fluorescence recovery assumes movement limited by some process with first-order kinetics. There are probably several biologically plausible processes which could result in a first-order–limited recovery model, including endocytosis and recycling of the cadherin as well as binding and unbinding of the cadherin to other proteins which restrict cadherin mobility. Regardless of the underlying biology, the model assumes two pools of VE-cadherin, one pool that is positionally fixed along the cell border and a second pool that is in constant equilibrium. Interpreted as the result of binding–unbinding, the fixed pool represents VE-cadherin bound to some scaffold, and the equilibrium pool represents VE-cadherin rapidly diffusing through the membrane so that it is always evenly distributed along the entire cell border. Interpreted as the result of endocytosis–recycling, the equilibrium pool instead represents VE-cadherin in intracellular vesicles rapidly diffusing through the cytoplasm, and no diffusion occurs along cell borders. The model assumes exchange between the fixed and equilibrium pools with first-order kinetics. Furthermore, the size of the equilibrium pool is assumed to be constant, which is reasonable if exchange between the two pools occurs across the entire cell border, and if the cell border is large compared to the bleach area. This assumption implies that only the fixed pool contributes to fluorescence changes, and that if bleaching is complete, the equilibrium pool must either be outside of the bleach area—intracellular vesicles in the endocytosis–recycling interpretation—or small enough not to contribute measurably to the fluorescence signal.

Therefore, the change in fluorescence signal within the bleach region is equal to movement from the equilibrium pool to the fixed pool less movement from the fixed pool to the equilibrium pool. For a one-dimensional model with a $2r$ long bleach area,

$$\frac{\mathrm{d}\,\mathrm{frap}(t)}{\mathrm{d}t} = 2r\frac{\mathrm{d}[\mathrm{F}]}{\mathrm{d}t} = 2r\left(\zeta - k[\mathrm{F}]\right) , \qquad \lim_{t\to 0}\mathrm{frap}(t) = 0 \quad , \tag{A.10}$$

where $[\mathrm{F}]$ is the concentration of VE-cadherin in the fixed pool within the bleach region, k is the fixed pool to equilibrium pool transition rate constant, and $\zeta = k'[\mathrm{E}]$ is the equilibrium pool to fixed pool transition rate, assumed to be constant.

This differential equation is separable and easily solved:

$$\int \frac{\mathrm{d}[\mathrm{F}]}{\zeta - k[\mathrm{F}]} = \int \mathrm{d}t \tag{A.11}$$

$$\frac{\log\left(\zeta - k[\mathrm{F}]\right)}{-k} = t + C \tag{A.12}$$

$$[\mathrm{F}] = \frac{\zeta}{k} - e^{-kC}e^{-kt} \quad . \tag{A.13}$$

Given the initial condition $\lim_{t\to 0} \mathrm{frap}(t) = 0$ and the assumption of constant [E], $[\mathrm{E}] \approx 0$ within the bleach region, and $\mathrm{frap}(t) = 2r[\mathrm{F}]$. Therefore,

$$\overline{\mathrm{frap}}(t) = \frac{\zeta}{k}\left(1 - e^{-kt}\right) \quad . \tag{A.14}$$

Note that $\lim_{t\to\infty} \overline{\mathrm{frap}}(t) = \zeta/k$. This is the equivalent of parameter A from the diffusion-limited recovery model, and, divided by the initial fluorescence level, gives the mobile fraction of VE-cadherin.

A.3 Model evaluation

As shown in Section 3.2.6, the first-order–limited recovery model fits well to experimental data. However, both the first-order–limited model and the diffusion-limited model have broadly similar behavior, increasing rapidly at first, than slowly approaching a final fluorescence value. In fact, the diffusion-limited model also fits the experimental data reasonably well (Figure A.1 and Table A.1). Distinguishing between the two models requires examining their respective behaviors as the bleach area radius changes.

In the first-order–limited recovery model, none of the parameters depend on the bleach

Table A.1: *VE-cadherin FRAP diffusion-limited model parameters*

Protein	A/A_0	$k \times 10^3$ (μm^2/s)
WT VE-cadherin-RFP	0.859 [0.799 – 1]	0.497 [0.364 – 0.643]
VE-cadherin-RFP (DEE→AAA)	1.00 [0.955 – 1]	0.679 [0.630 – 0.779]
VE-cadherin-RFP (GGG→AAA)	0.877 [0.755 – 1]	0.351 [0.259 – 0.506]

Square brackets indicate 95% confidence interval.

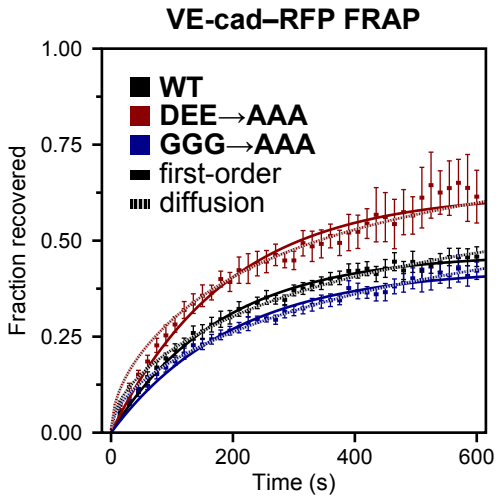

Figure A.1: *Fluorescence recovery after photobleaching model comparison.* RFP-tagged wild-type (WT), DEE-mutant, or GGG-mutant VE-cadherin was expressed in primary dermal microvascular endothelial cells and used in fluorescence recovery after photobleaching (FRAP) experiments. Average fluorescence within the bleach area, corrected for image acquisition–related photobleaching, was quantified over time and used to fit first-order– and diffusion-limited recovery models. Points, mean ± SEM (*n* = 16–18 sequences per group); solid lines, first-order–limited recovery models; dashed lines, diffusion-limited recovery models. See also Section 3.2.6.

radius, r. In contrast, r does affect the diffusion-limited model. In particular, k and r are closely related. To see this relationship, let r be scaled by a factor of γ, giving the altered recovery model

$$\overline{\text{frap}}'(t) = \frac{A}{\gamma r}\left(\frac{\sqrt{kt}}{\sqrt{\pi}}\left(1 - e^{-\left(\frac{\gamma^2 r^2}{kt}\right)}\right) + \gamma r \operatorname{erfc}\left(\frac{\gamma r}{\sqrt{kt}}\right)\right) \quad . \tag{A.15}$$

Now, if k is also scaled by a factor of γ^2,

$$\overline{\text{frap}}''(t) = \frac{A}{\gamma r}\left(\frac{\gamma\sqrt{kt}}{\sqrt{\pi}}\left(1 - e^{-\left(\frac{\gamma^2 r^2}{\gamma^2 kt}\right)}\right) + \gamma r \operatorname{erfc}\left(\frac{\gamma r}{\gamma\sqrt{kt}}\right)\right) \quad , \tag{A.16}$$

which equals $\overline{\text{frap}}(t)$ (Equation A.9). Thus, scaling r by γ is equivalent to scaling k by γ^{-2}. As a result, fluorescence recovery should be faster for smaller bleach sizes and slower for larger bleach sizes. However, this is not the case with the experimental data (Figure 3.10), so the diffusion-limited recovery model is rejected in favor of the first-order–limited model.

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