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Cadherin endocytosis: mechanisms of regulation and implications for endothelial cell migration

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Abstract

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Dynamic regulation of endothelial cell adhesion is central to vascular development and maintenance. Furthermore, altered endothelial adhesion is implicated in numerous diseases. Thus, normal vascular patterning and maintenance require tight regulation of endothelial cell adhesion dynamics. VE-cadherin is an adhesive protein found in adherens junctions of endothelial cells. VE-cadherin mediates adhesion through trans interactions formed by its extracellular domain. Trans binding is followed by cis interactions that laterally cluster the cadherin in junctions. Many proteins interact with the cadherin to regulate expression at the plasma membrane, including catenins. p120-catenin binds to the cytoplasmic tail of the cadherin and stabilizes it at the plasma membrane. In addition, VE-cadherin is linked to the actin cytoskeleton through cytoplasmic interactions with β- and α-catenin, which increases the adhesive strength of the junction. However, the relationship between cadherin endocytosis and cadherin adhesive interactions is still not fully understood. In addition, the role of VE-cadherin endocytosis during developmental processes, such as collective cell migration, is not known.

Here, we provide insight into the dynamic relationship between adhesion and endocytosis. We find that cis dimerization of VE-cadherin inhibits endocytosis independent of both p120 binding and trans interactions. Importantly, we find that ankyrin-G, a cytoskeletal adaptor protein, associates with and inhibits the endocytosis of VE-cadherin cis dimers independent of p120 binding. Ankyrin-G binding is important for junctional organization. Depletion of ankyrin-G results in disrupted localization of junctional proteins, including VE-cadherin, p120, and β-catenin.

In addition, we define a role for VE-cadherin endocytosis during directed, collective cell migration. Previously, our lab found that mutation of specific amino acids in the VE-cadherin cytoplasmic tail prevents endocytosis of the cadherin and inhibits collective cell migration. Here we report that the VE-cadherin endocytic mutant inhibits collective cell migration through a mechanism that involves adhesion and cytoskeletal linkage. Furthermore, we have found that VE-cadherin endocytosis is required for leading edge accumulation of the cadherin and for Golgi orientation at the wound edge, processes which polarize cells and promote directed cell migration. Understanding the mechanisms that regulate cadherin adhesion and endocytosis provides insight into processes that are important for development and disease pathology.

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List of VE-cadherin mutants

VE-cadherin	Mutation	Reported functional outcome
DEE	DEE 646-648->AAA	inhibited endocytosis, disrupted p120 binding
GGG	GGG 649-651->AAA	disrupted p120 binding, increased endocytosis
W2/Trp2	W->A	disrupted adhesion
ΔCBD	deletion of catenin-binding domain	disrupted β-catenin binding, disrupted cytoskeletal linkage
EE-GGG	E637, E640 ->AA & GGG 649-651->AAA	decreased association with ankyrin-G, disrupted p120 binding, increased endocytosis
W2-DEE	W->A & DEE 649-651->AAA	inhibited endocytosis, disrupted adhesion
DEEΔCBD	DEE 649-651->AAA & deletion of catenin-binding domain	inhibited endocytosis, disrupted β-catenin binding, disrupted cytoskeletal linkage

Chapter 1

Dissertation Overview and Significance

1.0 Overview and significance

The vascular system is made up of a complex network of vessels comprised of arteries, veins and capillaries. For centuries, the vascular endothelium was viewed as a semipermeable inert cellophane-like membrane that lined the circulatory system (Cines *et al*., 1998). After the cell theory was proposed in the nineteenth century, it was recognized that the endothelium is a thin layer of cells that line the interior of blood vessels (Cines *et al*., 1998). However, it was thought that the primary function of endothelial cells was to simply act as a physical barrier to separate blood from tissue (Cines *et al*., 1998). Yet, George Palade and others argued that the function of endothelial cells went beyond a static barrier (Cines *et al*., 1998). In 1953, Palade reported his findings of electron microscopy of endothelial cells; he found that in addition to the organelles found in most cell types (nucleus, mitochondria, and endoplasmic reticulum), endothelial cells had "a number of vesicles concentrated directly underneath the plasma membrane" (Palade, 1953). He proposed that these vesicles represent a system for transporting fluids across the capillary wall, suggesting an active role of endothelial cells in vessel wall biology (Palade, 1953). The ability to culture endothelial cells contributed to the explosive growth in the field of vascular biology in the 1970s and 80s (Nachman *et al*., 2004). By the mid 1980s enough evidence to support the hypothesis that tumor growth is dependent neovascularization emerged, leading to an intense interest in angiogenesis (Folkman J., 2003).

Angiogenesis is defined as the sprouting of new vessels from existing vessels (Adair *et al*., 2010). Angiogenesis is important during development and throughout the lifetime of an organism for tissue homeostasis. During vascular development, an initial

primitive vascular plexus is established through vasculogenesis (Risau *et al*., 1995). This is followed by angiogenesis, which involves extensive vessel expansion and remodeling, resulting in a mature network of vessels (Carmeliet *et al*., 2000). Dynamic regulation of endothelial cell adhesion is essential for normal vascular development. Strong adhesion between endothelial cells is required to withstand the force of blood circulation and maintain barrier function. Yet, adhesion between endothelial cells must also be dynamic for cellular rearrangements during development and wound healing. Thus, modulation of adhesion is central to the functions of endothelial cells. However, despite the importance to endothelial cell function, the mechanisms that regulate the molecular components of adhesive cell junctions, such as the adherens junction, are not fully understood.

The adherens junction provides strong mechanical attachments between cells through calcium dependent homophilic associations between cadherins, a family of adhesive proteins (Niessen *et al*., 2008). The endothelial cell adhesion molecule Vascular Endothelial (VE) cadherin plays a key role in regulating vascular growth and morphogenesis (Vincent *et al*., 2004). Endothelial cells regulate adhesion through the modulation of VE-cadherin localized at the plasma membrane. The adhesive strength of an adherens junction is dependent upon the amount of cadherin at the plasma membrane. Therefore, elucidating the mechanisms that regulate VE-cadherin stability at the plasma membrane is essential to understanding how cell adhesion is modulated. Endocytosis of the cadherin is one way that the level of cadherin at the cell surface is regulated (Niessen *et al*., 2011). VE-cadherin undergoes clathrin-mediated endocytosis, and can either be recycled back to the plasma membrane or directed to the lysosome to be degraded (Delva *et al*., 2009). Cadherin recycling allows for quick modulation of adhesion in response to

environmental cues. A number of mechanisms to regulate cadherin endocytosis have been described. These mechanisms include regulation by a family of "linker" proteins, known as catenins, which bind to the cadherin and link it to the cytoskeleton. Other regulators of cadherin endocytosis include adaptor proteins, ubiquitin ligases, and growth factors (Delva *et al*., 2009). Because regulation of cadherin endocytosis is fundamental to the modulation of cell adhesion, it is likely that there are numerous regulators yet to be discovered. Interestingly, there are reports that support a role for adhesion in the regulation of cadherin endocytosis. Studies using classical cadherins have determined that a loss of adhesion is observed when cells are switched from media containing high calcium to low calcium (Kartenbeck *et al*., 1991). Additionally, cadherin internalization is reported with calcium depletion (Mattey *et al*., 1986). One interpretation of these observations is that endocytosis of the cadherin leads to a loss of adhesion. However, an alternative interpretation is that the loss of adhesion leads to endocytosis. Indeed, based on the literature there are two potential models that explain the relationship between adhesion and cadherin endocytosis. One possibility is that endocytosis is a mechanism to dissemble cadherin adhesive dimers (Troyanovsky *et al*., 2006), while another possibility is that disruption of adhesion induces endocytosis (Ivanov *et al*., 2004). Thus, the relationship between cadherin adhesion and endocytosis is complex and not fully understood. Moreover, it is not known how VE-cadherin adhesion and endocytosis are coordinated to regulate endothelial functions in physiologically relevant processes, such as angiogenesis. During angiogenesis, endothelial cells migrate in a directed and collective manner (Lamalice *et al*., 2007). Endothelial cells must coordinate movement as a cohesive group. Importantly, they must maintain contact while migrating to establish

new vessels. Because endothelial cells must quickly modulate adhesion in response to environmental cues during angiogenesis, cadherin endocytosis is likely to play a fundamental role. However, a specific role for VE-cadherin endocytosis during angiogenic processes, such as collective cell migration has not been defined.

The overall goals of this dissertation are to define the relationship between adhesion and VE-cadherin endocytosis and to understand how cadherin endocytosis regulates endothelial cell functions during angiogenic processes. These goals are addressed through two specific questions: 1) How do VE-cadherin homophilic interactions regulate cadherin endocytosis? 2) How do adhesion and endocytosis regulate collective cell migration, a key angiogenic process? To effectively present the findings of this dissertation, in chapter 2 I will review the molecular mechanisms of cadherin-based junctions, including a description of cadherin adhesive interactions, mechanisms that regulate cadherin endocytosis, and the role of cadherins in cell migration. This chapter is a broad overview of the mechanisms that regulate adherens junctions of many tissues, including but not specific to endothelial adherens junctions. Therefore, I provide a review of the adherens junctions of the vascular endothelium, including an overview of vascular development and endothelial cell migration during angiogenesis, followed by the role of VE-cadherin in these processes, and concluding with a discussion of endothelial cell junctions in vascular disease (chapter 3). Together, these review chapters provide a current understanding of the molecular mechanisms that regulate cadherins of many tissues, including the vascular endothelium, and provide insight into their function in important physiological processes, such as collective endothelial cell migration. In addition, these chapters provide information critical for understanding of the implications

of the findings presented in chapters 4 and 5 of dissertation.

The work in this dissertation has lead to the following discoveries regarding mechanisms that regulate VE-cadherin endocytosis and the relationship between VE-cadherin endocytosis and adhesion in endothelial cell function:

1. Lateral (cis) dimerization of VE-cadherin downstream of adhesion inhibits endocytosis of the cadherin (chapter 4).
2. Ankyrin-G, a cytoskeletal adaptor protein, associates with and inhibits endocytosis of VE-cadherin cis dimers, independent of adhesion (chapter 4).
3. An ankyrin-G isoform that associates with VE-cadherin is required for proper adherens junction organization in primary endothelial cells (chapter 4).
4. VE-cadherin endocytosis regulates Golgi orientation and leading edge accumulation of the cadherin, two processes that establish cell polarity during collective cell migration (chapter 5).
5. VE-cadherin endocytosis regulates collective cell migration through a mechanism that involves adhesion and linkage to the cytoskeleton (chapter 5).

Together, these findings contribute to the understanding of the relationship between cadherin endocytosis and adhesion and provide insight into processes important for development and disease pathology.

Chapter 2

Molecular mechanisms of cadherin-based junctions

2.0 Introduction to cadherin based junctions

Intercellular junctions are dynamic structures that enable cells to adhere to and directly communicate with their environment and each other. To facilitate these interactions, cells form different types of junctions, including tight junctions, gap junctions, and adherens junctions. Tight junctions form a barrier that is restrictive to fluid and are important for the regulation of cell polarity (Hartsock *et al*., 2008). Gap junctions allow the free passage of small molecules and ions between adjacent cells, allowing cells to communicate with each other (Mese *et al*., 2007). Adherens junctions and desmosomes are cadherin-based junctions that provide strong mechanical attachments between cells (Saito *et al*., 2012). The adherens junction encircles a cell forming a circumferential belt that is attached to the cytoskeleton, while desmosomes are spot like plaques and link to intermediate filaments (Green *et al*., 2007; Niessen *et al*., 2008). Both the adherens junctions and desmosomes are essential for the development of and maintenance of cohesive tissues.

2.1 Molecular components of Adherens junctions

The main components of adherens junctions are cell adhesion molecules, such as cadherins, and catenins, which are a family of proteins that link the cadherin to the actin cytoskeleton (Figure 1). However, there are many proteins that localize to adherens junctions that function in stabilizing the cadherins at the plasma membrane, linking the cadherin to the cytoskeleton, and signaling. Example of these proteins will be highlighted in the following sections.

2.1.1 Cadherins

Cadherins are a family of proteins named for their calcium-dependent adhesive interactions. Kartenbeck and colleagues observed that depletion of calcium resulted in the disruption of adherens junctions (Kartenbeck *et al*., 1982). Other reports subsequently demonstrated that cadherins are removed from the junction through endocytosis after calcium depletion (Kartenbeck *et al*., 1991; Mattey *et al*., 1986). Cadherins are single-pass transmembrane proteins. They consist of an extracellular domain made up of five cadherin repeats, termed EC1-5 (Figure 2). The adhesive interactions(Shapiro *et al*., 2009) of the cadherins are mediated through the EC domains. The cadherin cytoplasmic tail is involved in many protein-protein interactions that are essential for cadherin function. Both cadherin adhesive interactions and protein interactions of the cytoplasmic tail will be discussed in greater detail in later sections. The desmosomal cadherins, desmoglein and desmocollin, are closely related to classic cadherins in their ectodomains and adhesion mechanism (Shapiro *et al*., 2009). However, in order to facilitate interactions with the intermediate filament rather than the actin cytoskeleton, they differ from classic cadherins in their cytoplasmic domain. For the purposes of this dissertation, classical cadherins will be referred to simply as cadherins.

A. Cadherin ectodomain

The classic cadherin ectodomain is made up of five extracellular repeat domains (EC1-5) with three calcium-binding sites between each repeat (Figure 2). Calcium binding rigidifies the protein resulting in a conformation that activates adhesive potential (Shapiro *et al*., 2009). The EC1 domain mediates *trans* interactions, which are responsible for cell-cell adhesion and to play a role in cadherin specificity (Brasch *et al*., 2012). Based on

sequence homology, classic cadherins are divided into subcategories, type I and type II. Differences between type I and type II cadherins are mostly found in the EC1 domain (Shapiro *et al*., 2009). Type I cadherins mediate strong cell adhesion and express a histadine-alanine-valine (HAV) sequence, which is not conserved in type II cadherins (Halbleib *et al*., 2006; Nollet *et al*., 2000). Additionally, type I cadherins have a single conserved tryptophan at the extracellular N-terminal region, while type II cadherins have two tryptophans at the N-terminal region (Halbleib *et al*., 2006). Type I cadherins, including E-, N-, P-, and C, typically have a broad distribution in tissues. Type II cadherins such as VE-cadherin exhibit a more restricted expression. For example, N-cadherin is expressed throughout mesodermal and neural tissue, while VE-cadherin is restricted to the cells of the mesodermal endothelium (Halbleib *et al*., 2006).

B. Cadherin cytoplasmic tail

The most highly conserved region of the cadherin is the cytoplasmic tail, which consists of about 150 amino acids (Shapiro *et al*., 2009). The cytoplasmic tail is divided into regions referred to as the juxtamembrane domain (JMD) and the catenin-binding domain (CBD) (Figure 2). Each of these regions is known to bind to specific members of the catenin family, proteins defined by their function as "linkers" to the cytoskeleton (Figure 1). The CBD is C-terminal to the juxtamembrane domain and binds to β-catenin and plakoglobin (Figures 1 and 2). Through direct associations with α-catenin, β-catenin links the cadherin to the cytoskeleton (Figure 1). The membrane proximal JMD binds to p120-catenin (p120), an interaction that stabilizes the cadherin at the plasma membrane (Nanes *et al*., 2012).

2.1.2 Catenins: structure and function

Members of the catenin family were first identified in association with the cytoplasmic domains of members of the cadherin family, and have since been defined as proteins that link cadherins to the underlying cytoskeleton (McCrea *et al*., 2010). Most catenins are similar in structure and are characterized by a central armadillo domain. One notable exception is α-catenin; it lacks an armadillo domain and is homologous with vinculin, a focal adhesion protein (McCrea *et al*., 2010; Pokutta *et al*., 2008). The catenin central armadillo domain contains between 9-12 repeats that fold into a super-helix containing a groove that forms the binding interface with the cadherin tail (McCrea *et al*., 2010). Vertebrate catenins are divided into subfamilies based on sequence homology. The β-catenin family includes β-catenin and γ-catenin, also known as plakoglobin. The p120 family consists of p120, ARVCF, δ-catenin, and p0071. The plakophilin family includes plakophilins 1-3. Since their identification, catenins have been shown to have multiple roles. Along with serving as linkers of cadherins to the cytoskeleton, some catenins function to modulate cadherin endocytosis and regulate the activity of small GTPases, while others act in the nucleus. The contributions of catenins are important for development and tissue homeostasis through their roles in cadherin dependent adhesion, motility, polarity, and gene regulation.

A. p120-catenin

p120 is an important regulator of cadherin stability at the plasma membrane and acts as a "rheostat" to modulate cadherin expression (Xiao, Allison, Buckley*, et al*., 2003). p120 was first identified as an Src substrate (Reynolds *et al*., 1989), but has since been classified as a member of the catenin family after sequence analysis revealed an

armadillo repeat domain that interacts with the cadherin tail (Reynolds *et al*., 1992). As an armadillo family protein, p120 is related to other catenins in structure and in the ability to interact with cadherins at adherens junctions. p120 contains a central armadillo domain consisting of 9 armadillo repeats, an N-terminal regulatory region and a C-terminal tail. The N-terminal region is involved in the regulation of Rho GTPase activity. Phosphorylation of the N-terminal region is thought to be an important regulatory mechanism for p120 function. However, the central p120 function is attributed to the central armadillo domains. Armadillo repeats 1-7 mediate interaction with the highly conserved juxtamembrane domain of members of the cadherin family (Shapiro *et al*., 2009), and have been shown to play a role in clustering and increasing the adhesive activity of the cadherin (Yap *et al*., 1998). Further evidence for the role of p120 in cadherin adhesion comes from Ireton et al. They reported a decrease in E-cadherin levels and a decrease in adhesion in a colon carcinoma cell line lacking p120, which could be rescued by exogenous expression of p120. Additionally, they found that p120 expression stabilized E-cadherin through a posttranscriptional mechanism; E-cadherin mRNA levels were unchanged (Ireton *et al*., 2002). This work established p120 as an upstream regulator of E-cadherin surface expression. However, the mechanism by which the regulation occurred was unknown. Cadherin regulation by p120 was subsequently determined to be through the modulation of cadherin internalization (M. A. Davis *et al*., 2003).

In addition to its function in stabilizing the cadherin at the plasma membrane, p120 is involved with cytoskeletal reorganization. p120 regulates Rho family GTPases, though the exact mechanism is not fully understood. p120 inhibits RhoA and activates

Cdc42 and Rac1. p120-regulation of Rho GTPase activity is dependent upon cadherin expression at the plasma membrane. p120 binds to the cadherin at the junction leading to a reduction of the cytoplasmic pool of the catenin. Disruption of the cadherin-p120 interaction at the junction increases the cytoplasmic pool of p120 and increases Rac1 and Cdc42 activity, promoting cell motility (Noren *et al*., 2000). Overexpression of p120 in epithelial cells and fibroblasts results in changes in cell shape and motility, including increased filopodia and lamellipodia activity, and a concomitant increase in Rac1 and Cdc42 activity (Grosheva *et al*., 2001). In addition to regulating cytoskeletal reorganization through Rho GTPases, p120 associates with microtubules (MTs) in a cadherin-independent manner that may be inversely related to Rho GTPase regulation (Franz *et al*., 2004). In addition, when bound to cadherins, p120 associates with a protein that recruits the MT minus end-binding protein Nezha, leading to anchorage of the MT at adherens junctions (Meng *et al*., 2008). Furthermore, Shahbazi and colleagues report that CLASP2, a protein involved in MT-dependent cytoskeletal remodeling during cell migration, associates with p120 at the adherens junctions of mouse keratinocytes. Reduced levels of either protein results in decreased localization of the other at junctions and altered junction stability (Shahbazi *et al*., 2013).

B. β-catenin

β-catenin is a highly conserved protein and, unlike the many catenins that have tissue specific variants, only a single isoform is found in vertebrates and insects (Shapiro *et al*., 2009). β-catenin is composed of an amino terminal region, a central domain consisting of 12 armadillo repeats, and a carboxy-terminal region (Shapiro *et al*., 2009). The β-catenin armadillo domain binds to the cadherin tail. The interaction is dynamic and it is regulated

by phosphorylation. α-catenin binds upstream, N-terminal to the cadherin-binding site (Choi *et al*., 2006). Cytosolic β-catenin is rapidly degraded. However, in the presence of Wnt signaling, cytosolic β-catenin is stabilized and can translocate to the nucleus where it functions as a transcriptional activator through its C-terminal tail (Brembeck *et al*., 2006).

In adherens junctions, β-catenin is a key regulator of cadherin-mediated adhesion. β-catenin binds to the CBD of the cadherin cytoplasmic tail and to α-catenin, thereby linking the cadherin to the actin cytoskeleton (Shapiro *et al*., 2009). A study using a chimera containing the cytoplasmic VE-cadherin tail fused to the transmembrane domain of the IL-2 receptor, found that β-catenin binding was essential for strengthening adhesion (Oas *et al*., 2013). The strength of cell adhesion under hydrodynamic shear force was assessed using chimeras harboring mutations that specifically disrupt either β-catenin or p120 binding to the cadherin tail. These assays used micropatterned coverslips to limit cell-substrate contact area, providing insight into the differential roles of p120 and β-catenin binding to the cadherin with respect to cell adhesion. Interestingly, while p120 binding to the cadherin tail was necessary for cell spreading, it did not alter adhesion strength. Instead, the interaction between β-catenin and the cadherin cytoplasmic tail was essential for strong adhesion (Oas *et al*., 2013).

In addition to its role in junctions, β-catenin binds to newly synthesized cadherin in the endoplasmic reticulum and traffics to the plasma membrane with the cadherin. This interaction is thought to protect the cadherin from proteolytic degradation and ensure its delivery to the plasma membrane (Pokutta *et al*., 2007).

C. α-catenin

Despite its name, α-catenin lacks an armadillo domain and more closely resembles the actin-binding protein vinculin, a focal adhesion protein. α-catenin forms a homodimer that binds to and bundles F-actin and inhibits Arp2/3 and cofilin activities(Shapiro *et al*., 2009). The homodimer dissociates upon binding to β-catenin to form a 1:1 heterodimer. Homodimerization and β-catenin binding are mutually exclusive as the binding site for both is mediated by the N-terminus of α-catenin (Shapiro *et al*., 2009). Other important regions of α-catenin are the M-domain, which contains a binding site for afadin, an actin-binding protein, and the actin-binding domain (Shapiro *et al*., 2009).

At adherens junctions, α-catenin is a key component of the cadherin-catenin complex. This complex is formed though cadherin binding to β-catenin, which recruits α-catenin. α-catenin binds to F-actin, linking the cadherin to the cytoskeleton (Shapiro *et al*., 2009). The simple model that α-cadherin directly links the cadherin-catenin complex to the cytoskeleton became controversial when in vitro studies were unable to reconstitute linkage to F-actin by the cadherin-catenin complex in solution using purified proteins (Yamada *et al*., 2005). Further studies found that α-catenin could not bind to both β-catenin and actin simaltaneouslty (Yamada *et al*., 2005). One potential explanation suggested that additional proteins are involved in linking the catenin to the cytoskeleton. Indeed, α-catenin interacts with other actin binding proteins, such as vinculin (Yao *et al*., 2014). Nonetheless, this controversy has recently been reconciled by the observation that force is needed to form a stable bond between the cadherin-catenin complex and F-actin (Buckley *et al*., 2014). Using a cadherin-catenin complex with F-actin reconstituted in an optical trap based assay, Buckley et al. found that α-catenin was required for the complex to bind to F-actin. Still, investigation of other actin binding proteins, including vinculin,

at the cadherin-catenin complex may provide additional insight into cadherin-catenin linkage to the cytoskeleton and the role of tension in recruiting these proteins.

2.2 Mechanisms of cadherin based adhesion

Cadherin-based adhesion was classically described as a calcium-dependent zipper-like structure. However, recent advances in defining the molecular mechanisms governing cadherin adhesive interactions have revealed that adhesion is much more complicated than originally thought. It is now recognized that adhesion involves trans interactions, first formed through an encounter complex, most likely a complex referred to as the X-dimer, and followed by the strand swap dimer (Harrison *et al*., 2010; Sivasankar *et al*., 2009). These interactions are reinforced by cis interactions, which, in addition to interactions mediated by the cadherin cytoplasmic tail, help to cluster the cadherin and increase overall strength of the junction (Zhang *et al*., 2009). A current model of cadherin-based adhesion and junction assembly is presented in the following sections.

2.2.1 Trans interaction

All classic cadherins share a common binding mechanism. Cadherin ectodomains extend out from opposing cell surfaces and form adhesive homodimers through *trans* interactions (Figure 3A). The *trans*-binding interface occurs at the EC1 domain, where the N-terminal region of the β-A-strand, the A* strand, is swapped between EC1 domains of two cadherins. The A* strand contains conserved tryptophans, at position 2 for type I cadherins and at 2 and 4 for type II cadherins, that form the trans dimer by insertion into a hydrophobic pocket on the EC1 domain of the partner cadherin (Brasch *et al*., 2012). While all vertebrate cadherins use a similar strand swapping mechanism to form adhesive

dimers, there are differences in the crystal structure between type I and type II cadherins (Brasch *et al*., 2012). As mentioned above, type I cadherins have a single conserved tryptophan at position 2 (Trp2, W2) involved in strand swapping (Figure 3B), while type II cadherin have two tryptophans at positions 2 and 4 (Trp2 and Trp4) that are involved in strand swapping. However, mutation of the Trp2 residue disrupts adhesion for both type I and type II cadherins. In addition to the second tryptophan involved in type II strand swapping, hydrophobic interactions occur between conserved residues Phe8, Ile10, and Tyr13, which results in an extended binding interface along the entire face of the EC1 domain (Brasch *et al*., 2012). The binding interface of type I cadherin is restricted to the most N-terminal region of the EC1 domain. Interestingly, VE-cadherin is an exception. Though VE-cadherin utilizes both Trp2 and Trp4 in forming strand swap dimers, it lacks the hydrophobic interactions along the rest of the EC1 domain (Brasch *et al*., 2011). Therefore, this divergent type II cadherin has an overall dimer arrangement more similar to type I cadherins.

2.2.2 X-dimer

The formation of strand swap dimers requires a transition from the "closed" monomer form, where the Trp2 is docked in its own hydrophobic pocket of the cadherin, to the "opened" form, where it can engage in an adhesive interaction. This conversion takes a substantial amount of time for other proteins that engage in strand swapping (Hong *et al*., 2011). However, cadherin binding is fast (Bayas *et al*., 2006; Katsamba *et al*., 2009; Vunnam *et al*., 2011). To explain this discrepancy, a model in which an X-dimer is formed has gained increasing acceptance. The X dimer is a non-strand swap intermediate (Figure 3C). Evidence of the X-dimer comes from studies using strand swap

impaired E-cadherin (type I) and cadherin-6 (type II) mutants, where the critical Trp2 was mutated to alanine (Harrison *et al*., 2010). Crystallography has revealed that the X-dimer, named for its resemblance to an X, occurs through the EC1-EC2 linker region, and the most N-terminal region of EC2 (Figure 3C). This dimer can occur without transition to an "opened" formation, therefore the kinetics of binding are fast. Additionally, this dimer positions the A-strand of each cadherin in a favorable conformation to form a strand swap dimer (Harrison *et al*., 2010). Experiments using epithelial cells expressing X-dimer mutants result in very stable cell junctions, suggesting that this dimer is an intermediate for dissociation of the strand swap dimer (Hong *et al*., 2011). It is not entirely clear if the X-dimer is an intermediate for assembly of the strand swap dimer (Hong *et al*., 2011; Sivasankar *et al*., 2009). However, a Trp2-independent initial encounter complex that may correspond to the X-dimer has been reported through the use of in vitro assays with cadherin Trp2 mutants (Sivasankar *et al*., 2009). Because the X-dimer has been identified for both a type I and II cadherins, it is likely that this mechanism is common amongst the members of the cadherin subfamilies.

2.2.3 Cis interaction

Trans interactions form the basis for cadherin adhesion. However, other interactions such as lateral cis interactions are important for adherens junction assembly and stability. Currently, it is thought that adhesion, through trans interactions, occurs first, followed by lateral clustering through cis interactions (Brasch *et al*., 2012; Harrison *et al*., 2011; Zhang *et al*., 2009). Structural studies have revealed an interface for cis interactions that is potentially conserved amongst type I cadherins. The interface has been observed in crystal structures of full-length E-, N-, and C- cadherins (Brasch *et al*., 2012).

The cis interaction occurs between the EC1 domain of one cadherin and the EC2 domain of a cadherin on the same cell (Figure 3). The region of the EC1 domain that mediates cis interactions is opposite the domain involved in strand swapping, enabling cis and trans interactions to occur simultaneously (Figure 3). Both cis and trans interactions contribute to forming the ordered lattice of cadherins observed at adherens junctions. Evidence to support this comes from experiments using mutations that disrupt the cis interface of E-cadherin. Harrison et al. found that cis-dimer mutants result in reduced adhesion of liposomes. Additionally, the expression of cis-dimer mutants disrupts junctions when incorporated into junctions containing endogenous cadherin (Harrison *et al*., 2011). Finally, cis-dimer mutants localize to cell contacts but fail to cluster into junction-like structures in cells lacking endogenous cadherin (Harrison *et al*., 2011). Interestingly, the cis interface is not present in the chicken VE-cadherin crystal lattice (Brasch *et al*., 2011). However, because VE-cadherin is localized to and clustered in adherens junctions, it is likely that cis interactions occur through an interface that has not yet been defined.

2.2.4 Cadherin clusters

Cis interactions occur through the EC domain. However, the EC domain is not sufficient to form higher order lateral clusters of the cadherin (Niessen *et al*., 2011). Instead, the cadherin tail is important for increasing adhesive strength and it has been implicated in cadherin clustering (Katz *et al*., 1998; Yap *et al*., 1998). Early experiments with tailless E- and C-cadherin mutants resulted in decreased adhesion (Brieher *et al*., 1996). These data suggest that the weak binding of the cadherin ectodomain must be strengthened to mediate cell-cell adhesion. Indeed, the stability of the ectodomain-based junctions is increased upon linkage to the actin cytoskeleton (Hong *et al*., 2013).

Cadherin cis mutants are unable to form clusters via their extracellular domain (Hong *et al*., 2013). However, they gain the ability to cluster when they interact with actin through a covalently linked actin-binding domain (Hong *et al*., 2013). Recently, Wu et al. used super resolution microscopy techniques to study E-cadherin organization at junctions of A431D cells on a nanoscale level (Wu *et al*., 2015). They found that cadherin mutants lacking the cytoplasmic tail or cadherin mutants lacking the EC domain formed clusters and that the cadherin clusters were encircled by a "fence" of F-actin (Wu *et al*., 2015). However, cis and trans mutations of a tailless E-cadherin resulted in the inability to form clusters. This result suggests that clusters can form in the absence of cis and trans interactions, through the cytoplasmic tail. Importantly, actin restricted clusters, upstream of adhesion, would provide a mechanism for cadherin observed at the free edge of cells or clusters of cadherin at the lateral junctions of A431D cells that are not engaged in adhesive interactions (Iino *et al*., 2001; Wu *et al*., 2015).

2.2.5 Junction assembly

Adherens junction assembly is thought to occur through an initial contact of cell protrusions, such as lamellipodia (Ehrlich *et al*., 2002; Hoelzle *et al*., 2012). One potential model for junctional assembly is that initially, actin corrals cadherin clusters independent of adhesion. These clusters may increase the probability that a cadherin will interact with a cadherin on a neighboring cell if they are in close proximity. Then, trans interactions occur through the formation of the X-dimer encounter complex between two cadherins. This interaction switches to the strand swap configuration, and is followed by cis interactions. Finally, adhesion and clustering is further reinforced through p120 inhibition of cadherin internalization and through β-catenin and α-catenin mediated

linkage to the actin cytoskeleton.

2.3 Cadherin trafficking

Cadherin-mediated cell adhesion is dynamic which allows the junctional reorganization required for proper development and growth. A key mechanism for the modulation of adhesion is through regulation of levels of cadherin at the plasma membrane. The level of cadherin at the cell surface is balanced through endocytosis and degradation, which decrease surface levels, and through synthesis of new protein and recycling, which increase levels at the plasma membrane (Figure 4). Together, these mechanisms modulate adhesion to obtain junction plasticity.

2.3.1 Trafficking pathways

Cadherins are internalized through several distinct mechanisms that fall into one of two categories, clathrin dependent or clathrin independent endocytosis. Of these mechanisms, clathrin-mediated endocytosis is the best characterized. Membrane proteins, including E-, N-, and VE-cadherin, are targeted for clathrin-mediated endocytosis through the binding of adaptor proteins. Adaptor proteins recruit components of the endocytic machinery and cluster the targeted protein in clathrin-coated pits. Vesicles then bud off from the plasma membrane after dynamin-mediated scission. Targeted proteins can be recycled back to the plasma membrane or directed to the lysosome for degradation.

In addition to clathrin-mediated internalization, cadherins have been shown to undergo endocytosis independent of clathrin, though these processes are less well studied. Clathrin independent endocytosis can occur through caveolin-mediated internalization, macropinocytosis, and in the case of the desmosomal protein,

desmoglein-3 (Dsg3), lipid raft mediated internalization. E-cadherin has been reported to undergo caveolin-mediated endocytosis in response to EGF (Lu *et al*., 2003). In contrast, Bryant et al. report that in response to EGF in a breast cancer carcinoma cell line, E-cadherin is internalized with p120 and β-catenin through macropinocytosis (Bryant *et al*., 2007). However, it is unclear the reason for these contrasting modes of endocytosis in response to EGF. Further evidence for macropinocytosis of cadherin comes from Paterson and colleagues, who describe E-cadherin endocytosis that is both clathrin- and caveolin- independent. Interestingly, this endocytic pathway affects E-cadherin that is not engaged in trans interactions at the cell junction (Paterson *et al*., 2003). The various endocytic pathways utilized by cadherins suggest a fine-tuned system for the modulation of adhesion that is context specific.

2.3.2 Regulation of cadherin fate

Under normal physiological conditions, cadherins are constitutively recycled and degraded. While most of the cadherin is located at cell junctions, a small pool of cadherin is internalized and found in vesicular compartments (Figure 4). Evidence to suggest that recycling is important for cadherin trafficking comes from the finding that newly synthesized E-cadherin does not travel directly from the Golgi complex to the plasma membrane. Instead, the cadherin is transited through Rab11 positive endosomes (Lock *et al*., 2005). Additionally, in Drosophila, Rab11 mediated recycling of cadherin is responsible for rearrangement of cell contacts in the hexagonal packing of wing disk cells (Classen *et al*., 2005).

Once internalized, additional sorting mediators regulate the fate of the cadherin. In some cases, such as in MDCK cells, E-cadherin is rapidly recycled back to the plasma

membrane (Le *et al*., 1999). In other cases, the cadherin is fated for degradation by the lysosome (Xiao, Allison, Buckley, *et al*., 2003; Xiao, Allison, Kottke, *et al*., 2003). Little is known about the molecular mechanisms the govern cadherin recycling after internalization, though studies have begun to elucidate important components. For example, members of the exocyst complex, sec5, sec6, and sec15 are required for DE-cadherin recycling in Drosophila (Langevin *et al*., 2005). In addition, the adaptor protein AP-1B mediates recycling of E-cadherin through association with phosphatidylinositol-4-phosphate 5 kinase type Iγ (PIPKIγ), which binds to the cadherin tail (Ling *et al*., 2007). In addition to mediators of cadherin recycling, there are signals that trigger lysosomal degradation, including the loss of p120-catenin binding to the cadherin and ubiquitination of the cadherin by E3-ligases, such as Hakai. The role of both p120 and ubiquitination in cadherin endocytosis will be discussed in greater detail in sections to follow. While there are a few mediators that are known, there is still much to learn about the components that determine whether the cadherin is recycled or degraded.

2.4 Regulation of cadherin endocytosis

Cadherin turnover is regulated through numerous mechanisms that exert their influence at different regions of the cadherin. For example, interactions of the extracellular domain can influence cadherin internalization. In addition, many of the mechanisms involve the cadherin juxtamembrane domain. Multiple proteins interact with this region of the cadherin tail to influence cadherin stability, including catenins, ubiquitin ligases, and adaptor proteins. Important regulators of cadherin internalization are highlighted below.

2.4.1 Cadherin homophilic interactions

The interplay between adhesion and cadherin endocytosis is not fully understood. There is some evidence to suggest that homophilic interactions play a role in regulating cadherin endocytosis. First, an increase in E-cadherin endocytosis was observed in sub-confluent MDCK cells lacking stable cell contacts (Le *et al*., 1999). Additionally, disruption of cell adhesion by calcium chelation increased E-cadherin endocytosis (Ivanov *et al*., 2004; Le *et al*., 1999). Further evidence comes from a study using soluble recombinant cadherin ectodomains. In a cell free system, Izumi and colleagues found that the presence of recombinant cadherin ectodomains inhibited E-cadherin internalization (Izumi *et al*., 2004). Alternatively, other studies have suggested that endocytosis is the driving force for disassembly of cadherin adhesive dimers. Disassembly of cadherin-based adhesion is an active process that can be blocked by depletion of ATP or inhibitors of endocytosis (de Beco *et al*., 2009; Hong *et al*., 2010; Troyanovsky *et al*., 2006). Accordingly, E-cadherin dimers were stabilized in the presence of endocytic inhibitors in MDCK cells (de Beco *et al*., 2009; Troyanovsky *et al*., 2006). However, further examination of the process of dimer disassembly revealed a more complicated story. In MCF cells, endocytosis inhibitors did not block cadherin exchange in the adherens junction (de Beco *et al*., 2009). This result suggested that alternative mechanisms function in cadherin dimer disassembly. Furthermore, mutations in the cadherin cytoplasmic tail that specifically block E-cadherin endocytosis did not affect the ATP dependent turnover of the cadherin (Hong *et al*., 2010). Therefore, it is likely that clathrin-mediated endocytosis is not the only mechanism responsible for junctional cadherin turnover.

Recent work has provided insight into the complex mechanism of the disassembly of cadherin dimers. Using a combination of live cell imaging and biochemistry, Hong and colleagues found that the cadherin exits the junction through the destabilization of the strand swap dimer to the intermediate X-dimer configuration (Hong *et al*., 2011). In chapter 3, I will present data that demonstrates that cadherin cis interactions strongly inhibit VE-cadherin internalization through a mechanism involving a cytoskeletal adaptor protein, ankyrin-G.

2.4.2 Catenins

The cadherin cytoplasmic tail associates with members of the catenin family of proteins, which link the cadherin to the actin cytoskeleton and stabilize junctions. Members of this family include p120-catenin (p120), β-catenin, and α-catenin. The contributions of each of these catenins to the regulation of cadherin stability will be discussed in the following sections.

A. p120

The cadherin juxtamembrane domain acts a site for the integration of multiple mechanisms that regulate cadherin endocytosis. Much of this regulation involves p120 both directly and indirectly. Multiple mechanisms for how p120 regulates cadherin stability at the cell surface have been suggested. However, much of the data supports a model in which p120 binds the juxtamembrane domain and blocks an endocytic motif, inhibiting adaptor proteins from recruiting the cadherin into clathrin coated pits. NMR studies of the E-cadherin JMD in a complex with p120, supports the idea that p120 binding masks dileucine and tyrosine residues important for clathrin mediated

endocytosis and association of the ubiquitin ligase, Hakai (discussed in greater detail in the ubiquitin section), respectively (Ishiyama *et al*., 2010). Further support for this model comes from the finding that p120 inhibits the entry of VE-cadherin into clathrin and AP-2 enriched membranes, independent of RhoA activity (Chiasson *et al*., 2009). In addition, our lab has demonstrated that a well-conserved cluster of acidic residues in the core p120-binding domain of the VE-cadherin cytoplasmic tail (DEE 646-648) is both necessary and sufficient to mediate VE-cadherin internalization. When p120 is bound to the cadherin this endocytic motif is masked.

Mutation of the DEE sequence inhibits p120 binding and inhibits endocytosis of the cadherin. However, mutation of the GGG (GGG 649-651) sequence in the core p120-binding region also disrupts p120 binding, but the cadherin is still internalized. Interestingly, endothelial cells expressing the VE-cadherin-DEE mutant exhibit a defect in collective cell migration, while the GGG mutation does not result in an obvious migration defect in vitro (Nanes *et al*., 2012). Thus, the migration defect caused by the DEE mutation highlights the importance of cadherin endocytosis for junctional plasticity.

B. β-catenin

In the adherens junction, the major role of β-catenin is to link the cadherin to the cytoskeleton through interactions with both the cadherin and α-catenin. However, there are a few studies that suggest that β-catenin may play a role in cadherin trafficking to the plasma membrane and endocytosis of the cadherin. Dupre-Crochet et al., found that inhibition of casein kinase I (CK1) resulted stable cadherin based cell contacts and overexpression of CK1 resulted in disrupted contacts. They found that CK1

phosphorylates E-cadherin on a serine in the catenin-binding domain. Phosphorylation of the site weakens the interaction between β-cadherin and the cadherin and results in increased cadherin endocytosis (Dupre-Crochet *et al*., 2007). Further data supporting a role for β-catenin in regulating cadherin endocytosis comes from a study that found that N-cadherin endocytosis in decreased upon activation of the NMDA receptor in hippocampal neurons. After stimulation of the receptor, β-catenin binding to N-cadherin is increased. Overexpression of a β-catenin phosphorylation mutant that exhibits increased association with N-cadherin inhibits internalization of the cadherin independent of receptor stimulation (Tai *et al*., 2007). Studies from Sharma and colleagues present a somewhat contradictory finding that β-catenin mediates macropinocytosis of N-cadherin in NIH 3T3 fibroblasts (Sharma *et al*., 2007). Nonetheless, whether it inhibits or mediates cadherin endocytosis, it appears that junctional β-catenin is involved in more than linking the cadherin to the cytoskeleton. Because β-catenin binds to both the cadherin and α-catenin, it is possible that α-catenin is plays a role in regulating cadherin endocytosis. However, this seems unlikely because endocytosis of a cadherin/α-catenin fusion protein was internalized at a rate similar to a cadherin control (Schulte *et al*., 2011). Still, a role for α-catenin in cadherin trafficking is conceivable.

2.4.3 Regulation of cadherin endocytosis through adaptor proteins

Because clathrin-mediated endocytosis requires adaptor proteins, one mechanism used in the regulation of cadherin trafficking is sorting signals within the cargo protein. These signals are motifs that mediate interactions with specific adaptor proteins. The adaptor protein AP-2 can bind cargo molecules, clathrin, and phospholipids at the plasma membrane, making it a key regulator of clathrin mediated endocytosis and a predicted

adaptor protein for cadherins. AP-2 recognizes cargo proteins with tyrosine or dileucine based motifs (Traub, 2003). E-cadherin harbors a dileucine motif in the juxtamembrane domain of its cytoplasmic tail that is required for clathrin-mediated internalization, which likely occurs through AP-2 binding (Miyashita *et al*., 2007). The dileucine motif is conserved in many other classical cadherins, but it is not present in VE-cadherin or Drosophila DE-cadherin. Interestingly, VE-cadherin undergoes clathrin, dynamin, and AP-2 dependent internalization (Chiasson *et al*., 2009). Additionally, during the establishment of planar cell polarity in Drosophila germ band extension, DE-cadherin is internalized through a clathrin and AP-2 dependent mechanism (Levayer *et al*., 2011). Therefore, other motifs in the cadherin tail must be responsible for AP-2 associated endocytosis.

AP-2 is not the only adaptor protein involved in regulation of cadherin internalization. Other adaptor molecules, including AP-1B, Dab2, and Numb are implicated in cadherin regulation to establish and maintain cell polarity. The adaptor protein AP-1B mediates recycling of E-cadherin to the basolateral membrane of polarized MDCK cells through association with a lipid kinase that binds to the cadherin tail (Ling *et al*., 2007). Down-regulation of Disabled-2 (Dab2), another an adaptor protein associated with clathrin mediated endocytosis, results in the loss of apicobasal polarity in murine embryonic endoderm and an accumulation of cell surface E-cadherin (Yang *et al*., 2007). Though it was not directly assessed, it is likely that the increased surface E-cadherin is due to decreased internalization caused by Dab2 down-regulation. Additionally, the adaptor protein Numb interacts with the E-cadherin-p120 complex to regulate cadherin endocytosis and maintain apicobasal polarity in epithelial cells (Sato *et*

al., 2011). Interestingly, Sato and colleagues found that Numb interacts directly with p120 to induce E-cadherin endocytosis. This is in contrast to AP-2 mediated internalization of E-cadherin which requires p120 dissociation (Miyashita *et al*., 2007). These two distinct mechanisms may enable cells to fine-tune E-cadherin surface expression in response to different stimuli.

In addition to endocytic adaptor proteins, the spectrin-actin cytoskeletal adaptor protein, ankyrin-G, regulates cadherin endocytosis and is involved in maintaining cell polarity of epithelial cells (P. Jenkins, Meng, H., Bennett, V., 2015; P. M. Jenkins *et al*., 2013). Ankyrin-G binds to E-cadherin and retains it at the lateral membrane of polarized MDCK cells and in cooperation with clathrin establishes apical-lateral polarity (P. M. Jenkins *et al*., 2013). Moreover, a recent study from Jenkins et al. reports micron-sized domains consisting of an underlying membrane skeleton comprised of ankyrin-G and its partner β-spectrin that inhibit endocytosis of E-cadherin through the exclusion of clathrin and clathrin dependent cargo in epithelial lateral membranes (P. Jenkins, Meng, H., Bennett, V., 2015). Additionally, in chapter 3, I will present data to support a role for ankyrin-G in inhibiting internalization of VE-cadherin cis-dimers.

2.4.4 Ubiquitin

Ubiquitin is another regulator of cadherin endocytosis. Proteins are targeted for ubiquitination through the interaction with an E3 ubiquitin ligase. Single ubiquitin molecules or a poly-ubiquitin chain are attached to the target protein, usually on lysine residues. Typically, a poly-ubiquitin chain targets a protein for degradation by the proteasome, while a single ubiquitin molecule signals for endocytosis and degradation by the lysosome.

The first report of an ubiquitin ligase regulating cadherin endocytosis was Hakai. Hakai is a c-Cbl-like protein that binds to and mono-ubiquitinates the E-cadherin, inducing internalization of the cadherin (Fujita *et al*., 2002). The Hakai binding site is in the JMD of the E-cadherin tail. The phosphorylation of two tyrosine residues in the JMD is required for Hakai to bind to the cadherin. p120 binding to the cadherin could block the phosphorylation of the tyrosine residues, which may explain how p120 inhibits the internalization of E-cadherin. However, the tyrosine residues are not conserved in all classic cadherins. Therefore, it is unlikely that Hakai regulates the endocytosis of all cadherins.

In addition to Hakai, other ubiquitin ligases have been reported to regulate cadherin endocytosis. VE-cadherin is targeted by K5, an ubiquitin ligase expressed by human herpesvirus-8 (HHV-8) (Mansouri *et al*., 2008). HHV-8 causes Kaposi's sarcoma, an angioproliferative neoplasm, which is associated with increased vascular permeability. Work from our lab suggests that increased vascular permeability is the result of K5-mediated down-regulation of VE-cadherin (Nanes et al., submitted) K5 directly targets VE-cadherin for ubiquitination and down-regulation through endocytosis (Nanes et al., submitted). Interestingly, K5 does not require the constitutive endocytic signal, DEE646-648 (see p120 regulation of VE-cad). Instead, two membrane proximal lysine residues are required for K5-induced ubiquitination and internalization. This indicates that distinct motifs in the juxtamembrane domain regulate constitutive and K5-mediated internalization of VE-cadherin. Because K5 is a viral protein, it will be interesting to determine if cellular ubiquitin ligases are also involved in the regulation of VE-cadherin stability. Of particular interest are the cellular K5 homologs, membrane-associated

RING-CH (MARCH) family of ubiquitin ligases. Nonetheless, ubiquitin-mediated cadherin endocytosis is an important mechanism for the regulation of cadherin cell surface expression.

2.4.5 Growth Factors

A number of growth factor signaling pathways are associated with regulation of cadherin endocytosis. A few examples of these growth factors include hepatocyte growth factor (HGF), fibroblast growth factor (FBF), epithelial growth factor (EGF), and vascular endothelial growth factor (VEGF). Often the relationship between the growth factor and cadherin regulation is bidirectional. This two-way regulation shows that adherens junctions are not only acted upon, but also participate in signaling in response to intercellular cues.

HGF is important for angiogenesis, tumorigenesis, and wound healing. HGF was shown to induce the co-internalization of both the HGF receptor (c-met) and E-cadherin through the activation of the GTPase Arf6 in MDCK cells (Kamei *et al*., 1999; Palacios *et al*., 2001). In addition, HGF signaling causes the endocytic adaptor protein Numb to dissociate from E-cadherin and instead associate with members of the Par polarity complex, disrupting cell polarization (Wang *et al*., 2009).

The FGF family of growth factors are involved in embryonic patterning and the coordination of cellular morphogenic movements during development as well as angiogenesis and tissue homeostasis during adulthood (Dorey *et al*., 2010). In MCF-7 cells, a breast cancer cell line, the activation of FGF receptor (FGFR) by FGF results in the co-internalization of FGFR and E-cadherin and subsequent translocation of FGFR to

the nucleus (Bryant *et al*., 2005). In addition, overexpression of E-cadherin or p120 blocked internalization of the cadherin and receptor, nuclear translocation of FGFR and FGF-induced signaling (Bryant *et al*., 2005). Here, FGF signaling results in down regulation of the cadherin and internalization of the cadherin provides a mechanism to inhibit FGF signaling. Interestingly, Suyama and colleagues found that N-cadherin also inhibits FGFR-1 internalization in MCF-7 cells, which results in prolonged expression of the receptor on the cell surface. This sustained surface expression lead to persistent MAPK signaling and promoted tumor cell invasion (Suyama *et al*., 2002).

A third example of a growth factor that is involved in regulating cadherin endocytosis is VEGF. VEGF is a proangiogenic factor that stimulates the growth of new blood vessels. It is also associated with increasing vascular permeability by disrupting endothelial cell junctions. Gavard and Gutkind demonstrated that the mechanism by which VEGF disrupts endothelial junctions is through inducing internalization of VE-cadherin, by Src-mediated phosphorylation of serine 665 (S665) in the juxtamembrane domain of the cadherin tail. Phosphorylation of S665 recruits β-arrestin and promotes clathrin-mediated endocytosis of VE-cadherin (Gavard *et al*., 2006). VEGF-mediated internalization of VE-cadherin is disrupted by angiopoietin-1, a proangiogenic factor that is associated with stabilizing blood vessels, by inhibiting Src activation by VEGF (Gavard *et al*., 2008). Interestingly, FGF also plays a role in stabilizing endothelial adherens junctions and counter-acting VEGF. However, FGF regulates cadherin stability by disrupting the expression of a VE-cadherin phosphatase, SHP2, resulting in the dissociation of p120 from the cadherin (Hatanaka *et al*., 2012). The loss of p120 binding leads to a decrease in VE-cadherin at the cells surface and concomitant increase in the

cytosolic pool (Murakami *et al*., 2008).

Similar to FGF and E-cadherin, regulation of VE-cadherin and VEGF is bidirectional. Association of VE-cadherin with the VEGF receptor (VEGFR) retains the receptor at the cell surface and inhibits downstream MAPK signaling of the receptor (Grazia Lampugnani *et al*., 2003). When VEGFR is retained at the plasma membrane by association with VE-cadherin, it results in dephosphorylation of the receptor through a mechanism that requires β-catenin and the density-enhanced phosphatase-1 (DEP-1) (Lampugnani *et al*., 2006). The interaction between VEGFR and VE-cadherin is discussed in detail in the following chapter.

2.5 Adherens junctions in cell migration

Classic cadherins have numerous roles during development, including mediating cell sorting and morphogenic movements. Beyond development, cadherins are involved in tissue maintenance, such as their role in wound healing. These processes require the dynamic regulation of adhesion. When adhesion is not tightly controlled it can lead to disease, such as cancer. The specific role of cadherins during developmental processes has been extensively reviewed (Gumbiner, 2005; Niessen *et al*., 2011; Takeichi, 1995). The role of VE-cadherin during vascular development is discussed in detail in the following chapter. Therefore, the next section will focus on the role of cadherins in cell migration.

2.5.1 Directed, collective cell migration

Cell migration is central to development, tissue homeostasis in adulthood, and cancer progression. As cell adhesion molecules, force sensors, and signaling proteins,

cadherins have active roles in coordinating cell migration. Cells can migrate in a variety of ways, including as single and as collective groups. These modes of migration can be further classified into directed or random for both single and collective cell migration (Rorth, 2009). Additionally, collective cell migration can occur as a sheet of cells or as a chain. For each cell type, the mode of migration depends on their adhesive potential and cytoskeletal organization. Many cell types utilize multiple modes of migration depending upon the stimulus. Environmental factors including the composition of the extracellular matrix, substrate stiffness, and external cues also contribute to the mode of migration.

In response to environmental cues, cells migrate in a specific direction. Directional migration is critical for development, wound healing, and immune function, and it is defined by the velocity and persistence of migration (Petrie *et al*., 2009). Both autonomous and collective cell migration can be directed. However, collective migration requires establishing and maintaining polarity of the cohesive group (Haeger *et al*., 2015; Petrie *et al*., 2009). The free edge contributes to establishing polarity (See polarity section below). Cells at the leading edge, "leader" cells, exhibit more protrusive activity and are highly motile (Petrie *et al*., 2009). Cells behind the leader cell, "follower" cells, are also motile and contribute to collective movement. However, leader cells confer directionality.

Collective cell migration occurs during development of the vasculature, neural crest, and many epithelial tissues, and during processes such as wound healing. In addition, many tumor cells invade tissue through collective migration (Rorth, 2009). Generally, collective cell migration is characterized by the preserved integrity of cell-cell junctions during motility, though some consider it collective movement if cells are

loosely attached or detach and reattach. There are different types of collective cell migration. These types include migration as a sheet, which is common to epithelial cells, sprouting or branching, such as in the vasculature, and streaming of the neural crest cells (Rorth, 2009). Collective migration requires the coordination of cell-cell contacts. Thus, cadherins play important roles in collective cell migration.

VE-cadherin is required during angiogenic sprouting, when cells migrate collectively as leader (tip) cells or follower (stalk) cells. In primary endothelial cells, down regulation of VE-cadherin resulted in the inability of stalk cells to follow tip cells, leading to a disruption in collective cell migration (Vitorino *et al*., 2008). Another example where down regulation of a cadherin can disrupt collective cell migration is during epithelial-mesenchymal transition (EMT). EMT is important during development and beyond for numerous processes, such as neural tube formation, wound healing, and tumor metastasis. Epithelial cells express high levels of E-cadherin, which mediates cell-cell adhesion during collective cell migration. However, during EMT the down regulation of E-cadherin promotes single cell migration and invasiveness (Hazan *et al*., 2004). In addition to the invasive phenotype, the loss of E-cadherin contributes to the loss of cell polarity, a hallmark of cancer cells (Hazan *et al*., 2004). The role of cadherins in cell polarity during migration is discussed in the next section.

2.5.2 Migratory polarization

Cell polarity is central to nearly every aspect of development and homeostasis. It is important for proliferation, morphogenesis, wound healing, and immune function. In addition to their established role in apical-basal polarity, cadherins are emerging as a key determinant of cell polarity during migration. During migratory polarization, many cell

structures organize towards the leading edge, including lamellipodia and filopodia, the centrosome, and the Golgi apparatus (Figure 5). Other cell structures localize towards the rear of the cell. These structures include the nucleus, stress fibers, and focal adhesions (Figure 5). Asymmetrical localization of cell structures is essential for effective directed migration.

Filopodia and lamellipodia are membrane protrusions that facilitate cell motility. Filopodia are thin, finger-like projections, which extend from lamellipodia, characterized by their thin sheet-like morphology (Figure 5). Both filopodia and lamellipodia are located at the leading edge of migrating cells, which establishes front-rear polarity. Cadherins have been reported to localize to the leading edge and to both types of protrusions (Almagro *et al*., 2010; Ehrlich *et al*., 2002; Hoelzle *et al*., 2012; McNeill *et al*., 1993; Peglion *et al*., 2014; Vasioukhin *et al*., 2000; Vasioukhin *et al*., 2001). This non-junctional pool of cadherin is thought to be important for the initiation of new stabile contacts between cells (Lenard *et al*., 2013). In a zebra-fish model in which the vessels do not express functional VE-cadherin, the tip cells continuously extend additional filopodia after making contact with opposing cells. These cells make multiple contacts with gaps in between and the vessels are unable to make proper connections. This is in contrast to wild type cells that, after initial contact, stop extending filopodia and form a single contact that enlarges, sealing vessels (Lenard *et al*., 2013).

Cadherins that accumulate at the front edge of migrating cells can become incorporated into continuously forming new adherens junction of lateral contacts between adjacent cells during cadherin treadmilling (Figure 6). Kametani and Takeichi were the first to describe this as cadherin treadmilling. Using live cell imaging of A431D cells

expressing fluorescently tagged cadherins, including VE-cadherin, N-cadherin, and P-cadherin, they observed basal-to-apical flow of cadherin at junctions in non-migrating cells (Kametani *et al*., 2007). Cadherin treadmilling was disrupted when actin treadmilling was halted. In addition, cadherin treadmilling required the C-terminal catenin-binding region of the cadherin tail, suggesting that cadherin flow is based on actin treadmilling (Kametani *et al*., 2007). Interestingly, VE-cadherin expressed in MDCK cells did not treadmill until induced by a scratch wound and the flow only occurred only on protrusions facing the direction of migration, indicating that cadherin treadmilling only occurs when these cells are moving in a fixed direction (Kametani *et al*., 2007). A recent study provides additional evidence to support actin-dependent cadherin treadmilling, as well as provides a potential mechanism for replenishing cadherin at the leading edge. Astrocytes expressing N-cadherin with a photoconvertable tag were found to undergo treadmilling after a scratch wound. Peglion et al. tracked the cadherin from the rear of the cell to the leading edge. They found that pharmacological inhibition of endocytosis disrupted N-cadherin treadmilling (Peglion *et al*., 2014). This led to a model in which migrating astrocytes undergo cadherin treadmilling sustained by polarized cadherin recycling. In both of the mentioned reports, treadmilling cadherin remained associated with other adherens junction proteins including p120 and β-catenin. p120 and β-catenin were both visible at the leading edge and colocalized with VE-cadherin (Peglion *et al*., 2014). p120 depletion prevented the accumulation of N-cadherin at the leading edge and disrupted the formation of new lateral junctions, which was rescued by expression of siRNA resistant p120 (Peglion *et al*., 2014).

Cadherins contribute to cell polarity beyond leading edge localization. In many

cell types, the microtubule-organizing center (MTOC) is polarized during collective cell migration (Dupin *et al*., 2009; Gotlieb *et al*., 1981; Gotlieb *et al*., 1983; Magdalena *et al*., 2003; Nemere *et al*., 1985; Schaar *et al*., 2005). The Golgi complex colocalizes with and reorients with the MTOC (Kupfer *et al*., 1983). Orientation of these two complexes towards the leading edge involves the concomitant repositioning of the nucleus towards the rear of the cell (Figure 5). It is thought that the reorientation of the MTOC and Golgi contributes the polarized delivery of membrane proteins. In response to sheer flow, endothelial cells elongate and align stress fibers and microtubules in the direction of flow (Tzima *et al*., 2003). In addition, the MTOC is oriented toward the heart *in vivo*, and in migrating endothelial cell sheets, the MTOC and Golgi are localized toward the leading edge in front of the nucleus *in vitro* (Palazzo *et al*., 2001). Tzima et al., found that localized activation of Cdc42 and Par6 and PKCζ direct the orientation of the MTOC in response to sheer flow (Tzima *et al*., 2003). The positioning of the Golgi and MTOC in front of the nucleus involves rearward movement of the nucleus coupled with retrograde actin flow and is regulated by a pathway involving Cdc42 in a fibroblast cell line (Gomes *et al*., 2005). During nuclear rearward movement the Golgi and MTOC remain stationary, a process that requires dynein, Par6, and PKCζ (Gomes *et al*., 2005).

Polarization of the MTOC and Golgi is important for polarized cell migration for many cell types (Dupin *et al*., 2009; Gotlieb *et al*., 1981; Gotlieb *et al*., 1983; Magdalena *et al*., 2003; Nemere *et al*., 1985; Schaar *et al*., 2005). Interestingly, nuclear positioning, and MTOC and Golgi orientation seems to involve cadherin-mediated cell contacts. Studies using micropatterned substrates to impose asymmetries in cell contacts have provided insight into the role of cadherins in mediated polarity. Desai and colleagues

found that cell contact induced displacement of the nucleus, centrosomal orientation, and lamellipodial ruffling in kidney epithelial cells. Disruption of E-cadherin-mediated contact inhibited scrape-wound-induced cell orientation, including nuclear position and polarized lamellipodia ruffling, but did not disrupt migration rate (R. A. Desai *et al*., 2009). Another study using a micropatterned substrate found that nuclear positioning and centrosome orientation is controlled by N-cadherin in astrocytes (Dupin *et al*., 2009). In a recent study combining micropatterning technology with live cell imaging, it was reported that N-cadherin-mediated adherens junctions promote activation of PI3K and Rac at the free edge through p120, and promoted the accumulation of actin filaments near the cell junction through β-catenin (Ouyang *et al*., 2013). Thus, classical cadherins contribute to the establishment and maintenance of cell polarity through multiple mechanisms.

2.6 Concluding Remarks

Membrane trafficking plays a crucial role in regulating cell-cell adhesion. Recent work has advanced our understanding of cadherin trafficking and has provided a more detailed description of cadherin homophilic interactions. Yet, the relationship between cadherin endocytosis and cadherin adhesive interactions is still not fully understood at the molecular level. For example, does endocytosis disrupt cadherin adhesive bonds or do cadherin adhesive bonds inhibit endocytosis? Early work has shown that disruption of adhesion through calcium depletion results in increase endocytosis (Ivanov *et al*., 2004). This suggests that adhesion inhibits internalization of the cadherin. However, other studies have shown that endocytosis is a driving force to disrupt cadherin adhesion (Troyanovsky *et al*., 2006). Moreover, cadherin adhesive interactions have proved to be

more complicated than originally thought. For instance, cadherin adhesion is not simply due to the formation calcium-dependent trans interactions. Instead, there is an intermediate, the X-dimer, which is crucial for cadherin disassociation (Hong *et al*., 2011). Though, whether or not the X-dimer is as an intermediate to forming trans interactions is still an open question. In addition, cis interactions occur downstream of trans interactions and strengthen adhesion through lateral clustering of the cadherin. Notably, the cis interface described for type I cadherins, is not conserved for type II cadherins. Because type II cadherins cluster in junctions, cis interactions must occur through an interface that has yet to be described. More work is needed to elucidate the mechanisms of cadherin homophilic interactions and to understand how the interactions are coordinated to provide strong adhesion than can be quickly modified in response to environmental cues.

Cadherin binding partners integrate trafficking and adhesion through their role in regulating cadherin endocytosis. Indeed, many of these partners stabilize the cadherin at the surface or contribute to overall adhesive strength of the junction. However, the mechanisms that regulate the binding of these proteins to the cadherin are not fully understood. An example of this is the interaction between the cadherin and p120. p120-binding masks an endocytic motif (Nanes *et al*., 2012). When p120 is not bound the cadherin undergoes rapid internalization. However, numerous questions remain unanswered. For instance, what regulates p120 binding to the cadherin? What role do adapter proteins play in the regulation of p120 binding? Thus, further investigation is needed to understand how endocytic signals and cadherin binding partners are integrated at the cadherin tail.

In addition, the role of cadherin endocytosis in development and disease is not fully understood. Many studies have focused on cadherin knock down or methods that disrupt endocytosis globally, rather than selectively preventing endocytosis of the cadherin. The finding that a mutation in the cadherin tail that disrupts cadherin endocytosis results in migration defect is particularly interesting (Nanes *et al*., 2012). While recent studies have advanced our understanding of the role of cadherin in cell migration, many questions remain. Peglion and colleagues reported that polarized recycling of N-cadherin is required to replenish lateral junctions(Peglion *et al*., 2014). Further, Oubaha and colleagues report the localization of VE-cadherin and members of the Par polarity complex at the leading edge (Oubaha *et al*., 2012). It would be interesting to determine what directs polarized endocytosis and exocytosis of the cadherin. One intriguing possibility is that polarity proteins may play a role in determining cadherin fate during trafficking or that cadherin endocytosis is required for establishing cell polarity in cooperation with polarity proteins. Selective inhibition of cadherin endocytosis through the use of cadherin mutants is indeed an important approach for future studies. Moreover, studies directed at understanding the mechanisms involved in the integration of cadherin adhesion and endocytosis will provide important insight into development and disease pathology.

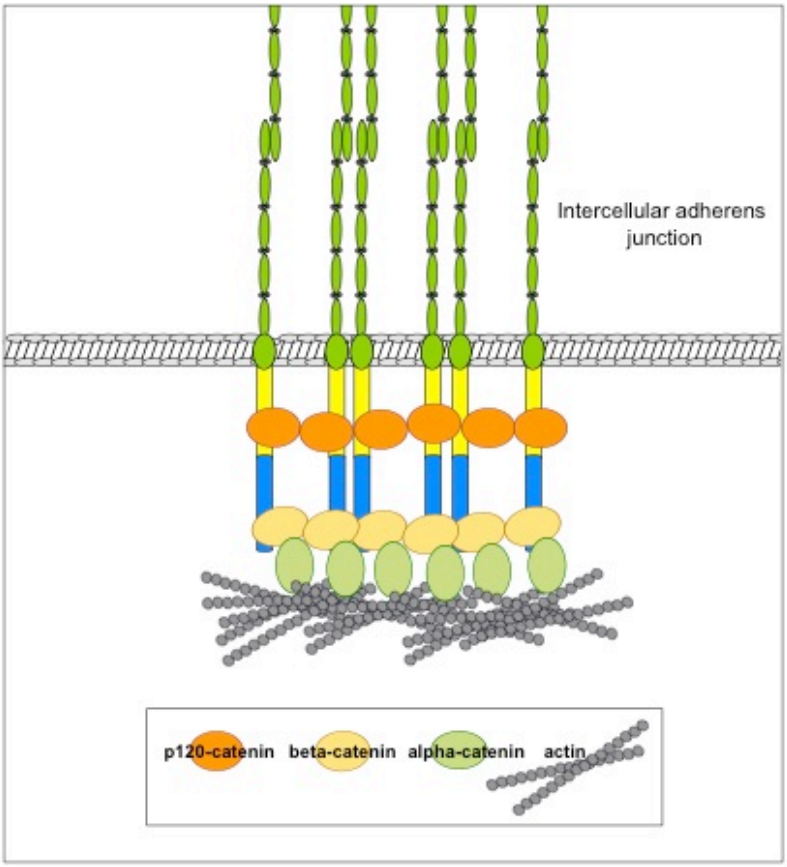


Figure 1. Molecular components of the adherens junction

Cadherins are organized in to junctions where they engage in homophilic interactions with cadherins on both the same cell and neighboring cells. Catenins bind to the cadherin tail. p120-catenin binds to the juxtamembrane domain of the cadherin tail and stabilizes it at the cell surface. β-catenin binds to the catenin-binding domain of the cadherin tail and to α-catenin. α-catenin binds to actin, which links the complex to the cytoskeleton and increases the adhesive strength of the junction. While there are numerous proteins that localize and function in adherens junctions, this figure depicts the core junctional complex.

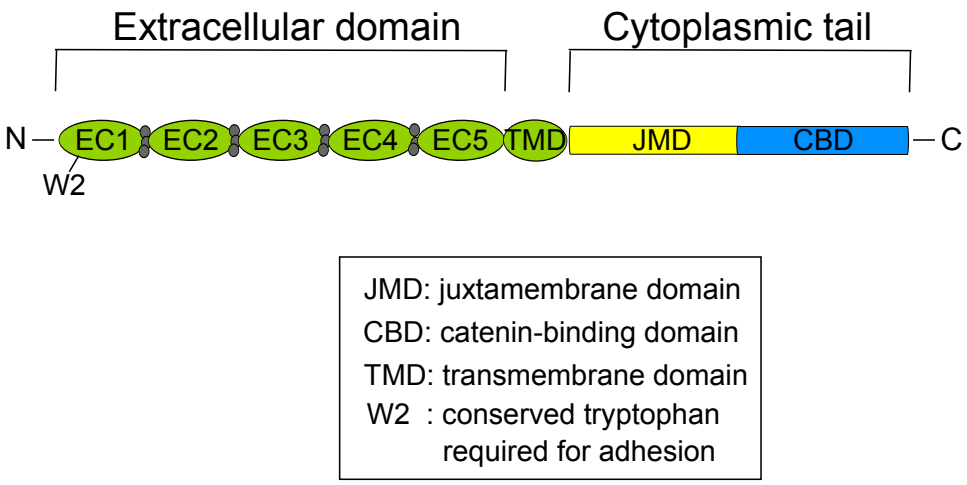


Figure 2. Domains of classical cadherins

Classic cadherins are single pass transmembrane proteins that have an extracellular (EC) domain made up of five cadherin repeats, a transmembrane domain, and a cytoplasmic tail. The EC domains are referred to as EC1-5. Between each EC domain there are three calcium-binding sites. Calcium binding rigidifies the cadherin and it is required for adhesion. EC1 harbors a conserved tryptophan (W2, Trp2) that is required for adhesion. The EC2 domain is important for lateral cadherin interactions. The juxtamembrane domain of the cadherin tail interacts with numerous proteins, including p120 catenin, and it is an important site for the integration of signals that regulate cadherin expression at the cell surface. β-catenin binds to the catenin-binding domain of the cadherin tail and to α-catenin.

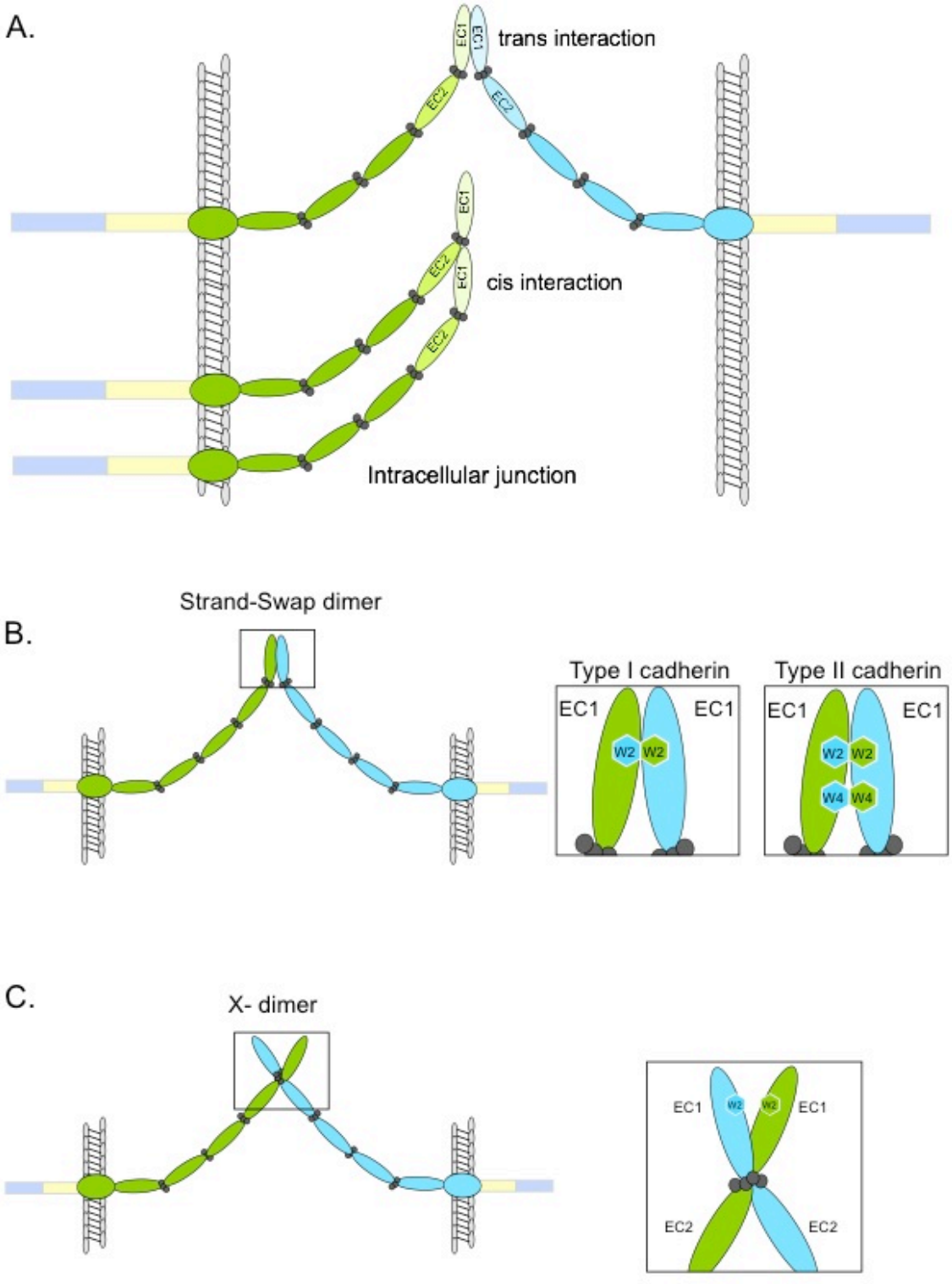

A.
EC1
EC1
trans interaction
EC2
EC2
EC1
EC2
EC1
cis interaction
EC2
Intracellular junction
B.
Strand-Swap dimer
Type I cadherin
EC1
EC1
W2
W2
Type II cadherin
EC1
EC1
W2
W2
W4
W4
C.
X- dimer
EC1
W2
W2
EC1
EC2
EC2

Figure 3. Homophilic cadherin interactions

Cadherins engage in homophilic interactions that are involved in adhesion and clustering the cadherin in the junction. (A) Trans interactions are the adhesive interactions between two cadherins on neighboring cells. Cis interactions occur between two cadherins on the surface of the same cell. For type I cadherins, the interaction occurs between the EC1 domain of one cadherin and the EC2 domain of the adjacent cadherin. Both cis and trans interactions cluster the cadherin into the junction. (B) Strand swap dimers are trans interactions that occur between the EC1 domains of cadherins on neighboring cells. This interaction occurs though a reciprocal process in which a conserved tryptophan from one cadherin is inserted into the hydrophobic pocket of the neighboring cadherin. Type I cadherins have one conserved tryptophan (Trp2, W2) involved in the strand swap dimer. Type II cadherins have two tryptophans (Trp4, W4) involved in strand swap dimer. (C) The X-dimer is an intermediate for the dissociation (and possibly the association) of the strand swap dimer. It occurs through the EC1-EC2 linker region, and the most N-terminal region of EC2 of the extracellular domain of the cadherin.

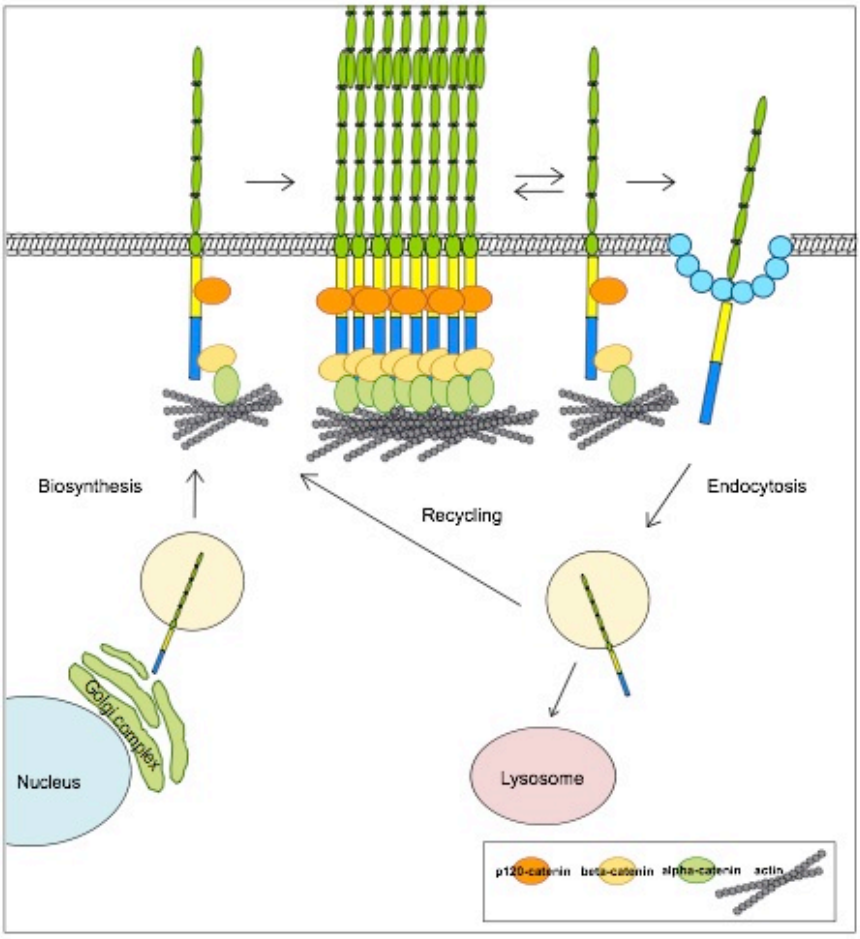


Figure 4. Membrane trafficking pathways of cadherins

Cadherins are synthesized and localized to the plasma membrane, where they undergo constitutive internalization. Cadherins undergo both clathrin dependent and independent endocytosis. Clathrin dependent internalization is well understood and is depicted in this figure. The cadherin tail harbors a motif that when exposed signals for its internalization. Once internalized the cadherin can either be recycled back to the plasma membrane or degraded by the lysosome.

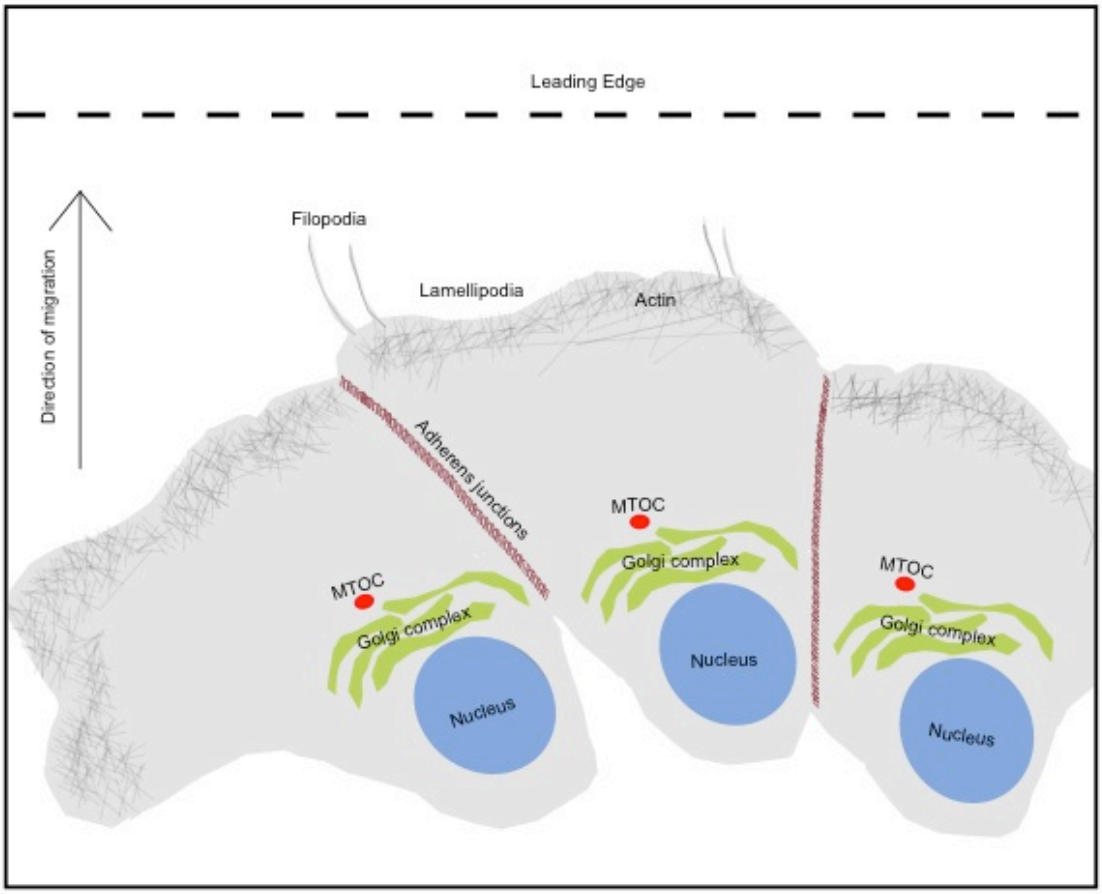


Figure 5. Polarized migratory cells

This figure depicts directed, collective cell migration. In response to a scratch wound, cells polarize by forming protrusions, such as lamellipodia and filopodia at the free edge. In addition, many organelles orient towards the leading edge of the cell, including the Golgi complex and the microtubule-organizing center (MTOC). Other organelles such as the nucleus move to the rear of the cell. Adherens junctions are modulated to maintain adhesion while cells migrate.

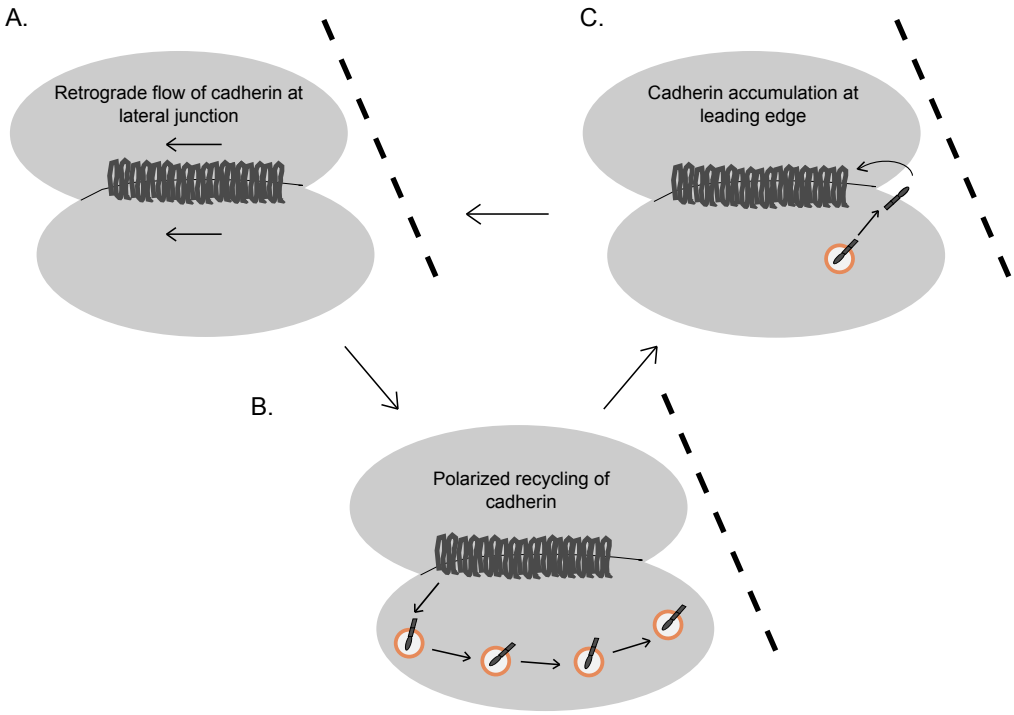


Figure 6. Cadherin treadmilling

Cadherins undergo basal-to-apical flow. A) retrograde flow of cadherin and associated catenins. B) cadherin at the rear of the cell is internalized and recycled to the leading edge. C) leading edge cadherin is incorporated into lateral junctions, replenishing the junction, enable the cycle to continue.

Chapter 3

Adherens junctions of the vascular endothelium

3.0 Adherens junctions of the vascular endothelium

The heart, major veins and arteries, and networks of small capillaries form the cardiovascular system. The vascular system provides oxygen and nutrients through the circulation of blood to tissues throughout the body during development and throughout the lifetime of the organism. The vascular endothelium is a thin layer of endothelial cells that form a monolayer lining the lumen of blood vessels.

The adherens junctions of the vascular endothelium need to be dynamically regulated. They must be strong enough to withstand mechanical force as blood is pumped through the vascular system. Yet, they must also be plastic to enable new vessel formation during development and wound healing during adulthood. Much of this junctional plasticity comes from the regulation of adhesion molecules, such as VE-cadherin, the major adhesive molecule found in the adherens junction of endothelial cells.

3.1 Vascular development

Vasculogenesis refers to the initial *de novo* formation of blood vessels. The formation of new blood vessels *in situ* begins through the formation of blood islands in the yolk sac. Blood islands consist of mesodermal-derived hemangioblasts. The inner mass of the blood island is formed by hematopoietic precursors, which give rise to blood cells, while the outer layer is formed by angioblasts, endothelial cell precursors. As endothelial cells migrate and proliferate, they connect the blood islands to form a primitive vascular plexus.

Once the primitive vascular plexus is established, the vascular network matures through a process of vessel growth and regression. Most of the vascular network is

formed through angiogenesis, which is defined as the formation of new vessels from existing vessels (Adair *et al*., 2010). Sprouting was the first recognized mode of angiogenesis (Adair *et al*., 2010). As its name implies, it occurs through sprouting of existing vessels to form new vessels. In response to angiogenic growth factors, endothelial cells migrate and proliferate to form sprouts that eventually connect to neighboring vessels. While most angiogenic growth occurs through sprouting, vessels in a few organs, such as the lung and skeletal muscle, form by intussusception, or splitting angiogenesis (Udan *et al*., 2013). This type of angiogenesis involves an ingrowth from surrounding connective tissue that divides and splits an existing vessel to form two vessels.

Most vessels in the primitive vascular plexus undergo regression (Korn *et al*., 2015). Through vascular pruning and regression, vessels formed by sprouting angiogenesis undergo extensive remodeling to establish a mature network. Regression occurs by migration of endothelial cells and apoptosis (Korn *et al*., 2015). Both vessel expansion and regression are necessary for proper vascular development. As such, both processes are tightly regulated by angiogenic signals.

One of the first receptors expressed by angioblasts is the growth factor vascular endothelial growth factor receptor 2 (VEGFR2) (Flk-1 in mice) (Risau *et al*., 1995). VEGF is the principle regulator of new vessel formation and it is essential for vascular development. VEGF is unique in that it acts to both form new vessels and to destabilize existing vessels. It induces endothelial cells to proliferate, migrate, and assemble in to tubes. VEGF can act as a survival signal for endothelial cells by stimulating VEGFR2 and subsequent PI3-kinase signaling (Gerber *et al*., 1998). However, VEGF can also

increase vessel permeability.

Expression of VEGF must be tightly regulated; both under-expression and over-expression result in vascular abnormalities or disease. In mice, the deletion of a single VEGF allele results in abnormal vascular development and lethality (Carmeliet *et al*., 1996). Mice homozygous for VEGF-deficiency have more pronounced vascular defects (Carmeliet *et al*., 1996; Ferrara *et al*., 1996). Conversely, VEGF over-expression can result in pathological neovascularization, contributing to tumor growth and metastasis (Drake *et al*., 2000). In addition, VEGF-induced vessel destabilization can lead to pathological effects including vascular leakage and inflammation. However, other growth factors such as angiopoietin-1 (Ang-1) act to stabilize vessels and can counter the effects of VEGF.

The Tie-2 receptor binds to the angiopoietins 1 and 2 (Ang-1 and Ang-2). Ang-1 activates Tie-2 and Ang-2 antagonizes Ang-1 binding to Tie-2, though Ang-2 can also activate Tie-2 in various contexts (Herbert *et al*., 2011). While VEGF is the primary growth factor in early in vessel development, Ang-1 is involved in vessel remodeling and stabilization later in development (Herbert *et al*., 2011). Initial expression of the Tie-2 receptor occurs slightly after VEGFR expression in mice and it is also essential for vascular development. Knockout of either Tie2 or Ang-1 results in embryonic lethality at E12.5 due to vascular defects (Suri *et al*., 1996). Ang-1 stabilizes vessels by promoting interactions between endothelial cells and mural cells (vascular smooth muscle cells and pericytes), and by influencing junctional molecules, such as integrins (Cleaver *et al*., 2003). However, as a proangiogenic factor, Ang-1 stimulates endothelial cell migration (S. Davis *et al*., 1996). Interestingly, it was recently reported that activation of endothelial

cells with VEGF lead to enhanced single cell migration, while Ang-1 resulted in collective cell migration (Oubaha *et al*., 2012), suggesting that VEGF and Ang-1 function together, but in different ways, to fine tune endothelial cell migration during blood vessel formation and maturation. Indeed, Shin et al., report a 3D system for studying endothelial cell sprouting angiogenesis into a collagen extracellular matrix in which a diffusion-based gradient of growth factors can influence migration. They found that together, VEGF and Ang-1generated stable and connected 3D capillary structures. In addition, Ang-1 stabilizes connections between tip cells and stalk cells, thus regulating stalk cell migration (Shin *et al*., 2011).

After blood circulation begins, platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ) recruit mural cells to the nascent vessels, which contributes to the stabilization of vessel walls. Finally, the deposition of the basement membrane and strengthening of cell-cell contacts suppresses sprouting resulting in quiescent endothelial cells.

3.2 Endothelial cell migration during angiogenesis

Sprouting angiogenesis forms new vessels from existing vessels through the migration and proliferation of specialized endothelial cells. A specialized endothelial cell known as a "tip cell" recognizes signals and converts this information into directional migration. "Stalk cells", another type of specialized endothelial cell, follow behind the tip cell and elongate the stalk of the sprout. "Phalanx cells" are quiescent endothelial cells that line the vessel after new branches have been formed.

Tip and stalk endothelial cells migrate in a directed and collective manner. The

directionality is regulated by chemotactic, haptotatic, and mechanotactic signals (Lamalice *et al*., 2007). The tip cells sense these signals, determine directionality, and coordinate with stalk cells. The chemotactic signals are primarily VEGF, FGF, and Ang-1 (Lamalice *et al*., 2007). However, other growth factors are known to be involved. Haptotatic factors, generally involving the extracellular matrix, and mechanotactic signals, such as sheer flow, also contribute to the environmental cues that endothelial cells must integrate for effective migration. Below, I will discuss the regulation of endothelial cell migration by growth factors, and in particular, VEGF.

A. Tip cells

Tip cells are located at the front of vessel branches. They are highly polarized and form numerous filopodia that probe the environment during migration. Endothelial cells are induced to become tip cells through VEGF signaling. Tip cells have a specific molecular fingerprint which is characterized by the expression of different receptors, including VEGFR2 and importantly, Delta-like ligand-4 (Dll-4) (De Smet *et al*., 2009). Tip cells are exposed to the highest levels of VEGF, which induces the expression of Dll-4. This expression is important for inhibiting neighboring endothelial cells from becoming tip cells. Lateral inhibition occurs when the Dll-4 on the tip cell binds to Notch on an adjacent endothelial cell and down regulates VEGFR2 signaling (De Smet *et al*., 2009). This interaction suppresses the tip cell phenotype and signals to the cell to become a stalk cell.

The formation of filopodia and lamellipodia polarize the tip cell and allow it to sense environmental cues that aid in directional migration. In response to VEGF, Cdc42

induces the formation of filopodia on the leading edge of tip cells, while the back of the tip cell maintains contact with stalk cells (De Smet *et al*., 2009). Tip cells also form polarized lamellipodia through VEGFR-mediated activation of Rac and simultaneous activation of WAVE2 (Lamalice *et al*., 2007). In addition, Ang-1 can induce the formation of lamellipodia on the leading edge of tip cells (Cascone *et al*., 2003).

B. Stalk cells

Stalk cells trail behind tip cells. Notch signaling reduces the migratory response to VEGF and inhibits filopodia formation in stalk cells. To elongate the stalk of a sprouting vessel, stalk cells must proliferate, migrate, form a lumen and maintain contact with the tip cell. Stalk cells maintain contact with tip cells through VE-cadherin. In human umbilical vein endothelial cells (HUVECs), down regulation of VE-cadherin using siRNA resulted in the inability of stalk cells to follow tip cells, leading to a disruption in collective cell migration (Vitorino *et al*., 2008). After elongation of the stalk, a lumen is formed and blood flow is initiated. Finally, the phalanx cells line the vessel and help to form a tight barrier. Low levels of VEGF are required to maintain quiescence and survival of these cells (Lee *et al*., 2007). Additionally, VE-cadherin plays a role in the shift from migration and proliferation to quiescence and survival through its ability to sequester VEGFR at the plasma membrane, disrupting the downstream signaling pathway (De Smet *et al*., 2009).

3.3 VE-cadherin in vascular development

VE-cadherin is the major adhesion molecule found strictly in the adherens junction of the endothelium. The role of VE-cadherin during development has been

extensively studied both *in vitro* and *in vivo*. In mouse embryos, VE-cadherin transcripts can be detected as early as E7.5 in the mesodermal-derived hemangioblasts (Breier *et al*., 1996; Vittet *et al*., 1997). The early expression of VE-cadherin in endothelial precursors, suggests that it contributes to differentiation. However, multiple groups have determined that VE-cadherin is not required for the differentiation of angioblasts to endothelial cells (Carmeliet *et al*., 1999; Crosby *et al*., 2005). In fact, VE-cadherin appears to be dispensable for the initial stages of vasculogenesis. VE-cadherin knockdown or truncation of the catenin-binding domain in mice results in embryonic lethality due to vascular defects at E9.5, after the formation of the primitive vascular plexus (Carmeliet *et al*., 1999; Crosby *et al*., 2005). Instead, VE-cadherin is essential for angiogenic processes, such as vessel expansion, branching, and remodeling, by preventing vascular regression through signaling for endothelial cell survival (Carmeliet *et al*., 1999). Endothelial cell survival is impaired in knockout mice, and apoptosis increases in cultured VE-cadherin knockout cells (Carmeliet *et al*., 1999). VE-cadherin knockout or truncation resulted in cells that were refractory to VEGF-mediated survival signals (Carmeliet *et al*., 1999). Therefore, the increased apoptosis is likely due to the inability of VE-cadherin null cells to respond to VEGF. Vascular defects become increasingly severe in VE-cadherin knockout mice at the start of angiogenesis in the mouse (around E8.75-9.0). Endothelial cells become disconnected from each other and the basement membrane (Carmeliet *et al*., 1999). Sprouting and remodeling were impaired resulting in gaps and disconnected or dilated vessels (Carmeliet *et al*., 1999). Taken together these data indicate that VE-cadherin is not required for initial vascular formation, but is essential for angiogenesis through its contributions to endothelial cell survival.

Interestingly, conditional knockout of endothelial p120 in mice results in a reduction in VE-cadherin (Oas *et al*., 2010). p120 deficiency results embryonic lethality starting at E11.5 due to hemorrhages and disorganized vascular networks in the endothelial embryonic tissues. It is possible that the reduction of surface VE-cadherin levels caused by the loss of p120 stabilization of the cadherin might be responsible for the defects in remodeling observed in the p120 mutant mice. VE-cadherin also contributes to angiogenesis through other mechanisms such as the establishment of cell polarity for lumen formation and tubulogenesis (Lampugnani *et al*., 2010; Strilic *et al*., 2009).

Anastomosis, the process of joining two branching vessels, results in quiescent endothelial cells. Through contact inhibition, endothelial cells inhibit their growth and motility and form stable cell-cell contacts. The process of contact inhibition has been reported to occur through multiple VE-cadherin-mediated methods. The most common method of contact inhibition is thought to be through β-catenin-dependent VE-cadherin regulation of the VEGF receptor. A suggested model is that VEGF stimulation causes VE-cadherin and VEGFR to associate. This interaction requires β-catenin binding to the cadherin and leads to the dephosphorylation of VEGFR which inhibits it internalization, preventing downstream signaling. Evidence to support this mechanism comes from multiple groups. First, VEGF-induced phosphorylation of VEGFR2 is reduced when cells are plated at a high cell density and increased when the cells are pretreated with adhesion blocking antibodies against VE-cadherin (Rahimi *et al*., 1999). VEGF triggers clathrin-mediated endocytosis of VEGFR, which is increased when VE-cadherin is absent or in sparsely plated cells (Grazia Lampugnani *et al*., 2003). VEGFR remains phosphorylated when internalized and blocking endocytosis restores contact inhibition of growth

(Lampugnani *et al*., 2006).

In quiescent endothelial cells, the interaction between VE-cadherin and VEGFR2 is long lasting, maintaining the dephosphorylation of the receptor (Hayashi *et al*., 2013). However, during angiogenesis, VEFG induces the sprouting of new vessels by activating VEGFR2 in tip cells. Hayashi and colleagues found that VEGFR2 activation is decreased in stalk cells through dephosphorylation of the receptor by vascular endothelial-phospho 9 phosphatase (VE-PTP) indirectly through the Tie2 receptor. Inactivation of VE-PTP in mouse embryoid bodies, leads to increased VEGFR2 signaling, increased tyrosine phosphorylation of VE-cadherin (which is a generally accepted mechanism to increase permeability), and loss of cell polarity and subsequently, lumen formation (Hayashi *et al*., 2013).

3.4 The role of VE-cadherin in cell polarity

To form a lumen in vessels, apical-basal polarity of cells must be established. VE-cadherin contributes to endothelial cell polarity. In both mice and zebrafish, the absence of VE-cadherin results in improper vascular lumen formation (Lampugnani *et al*., 2010). VE-cadherin is required for the localization of specific proteins that define the lumen (Strilic *et al*., 2009). The partitioning defect (PAR) polarity complex is a key regulator of cell polarity (McCaffrey *et al*., 2012). PAR3, PAR6, and atypical protein kinase C (aPKC) form a physical complex, referred to as the PAR complex. The interactions between members of the PAR complex are not constitutive and are regulated by protein kinases, small GTPases, notably Cdc42, and other binding proteins. VE-cadherin is known to interact directly with members of the PAR complex, including Par3 and Par6 (Iden *et al*., 2006). Specifically, both of these PAR proteins bind to non-overlapping

regions of the VE-cadherin tail at endothelial cell junctions (Iden *et al*., 2006). However, aPKC is not found in this junctional complex (Iden *et al*., 2006). Of note, Par6 binds amino acids 621-689 in the juxtamembrane domain of the cadherin tail, spanning the core p120-binding domain. Interestingly, VE-cadherin is required for localization and activation of Par3, Par6, and PKCζ complex in HUVECs to determine apical-basal polarity (Lampugnani *et al*., 2010). In addition, Koh and colleagues report the requirement of Par3 and Par6 in endothelial lumen and tube formation to establish polarity through association with Cdc42 and aPKC in an *in vitro* 3D matrix assay using HUVECs (Koh *et al*., 2008).

Endothelial cells can establish polarity through the localization of VE-cadherin at the leading edge during migration. Cadherin at the free edge of the cell has been reported for a number of cadherins, including E-cadherin and N-cadherin, both during migration and at gaps between cells in a confluent monolayer (Iino *et al*., 2001; Peglion *et al*., 2014; Wu *et al*., 2015). Oubaha et al., report that, in response to a scratch wound and upon stimulation with Ang-1, VE-cadherin localizes to the leading edge of the wound. Interestingly, at the leading edge, VE-cadherin colocalizes with β-catenin and PKCζ, and PKCζ colocalizes with Par3 and Par6. However, they did not directly show colocalization of VE-cadherin and Par3 or Par6 (Oubaha *et al*., 2012). Whether or not VE-cadherin is required for the localization and activation of the Par proteins at the leading edge is not known.

3.5 VE-cadherin endocytosis and migration

Endocytosis of VE-cadherin is important for a number of key mechanisms during angiogenesis. For example, during angiogenic sprouting, stalk cells must maintain

contact with tips cells and this is facilitated through homophilic VE-cadherin interactions. While endothelial cell migration occurs as a chain of cells overall, inside the chain the cells often change position. They slide past one another, while maintaining contact as they follow the tip cell. For cells to change positions within a group while maintaining contact, adhesion and migration must be coordinated. One way that endothelial cell adhesion is modulated is through endocytosis of VE-cadherin. Our lab reported a mutation in the cytoplasmic tail of the cadherin, the DEE sequence in the core p120-binding domain, results in inhibited endocytosis of the cadherin (Nanes *et al*., 2012). This mutation has allowed us to explore the role of cadherin endocytosis in endothelial cell function specifically, without disrupting endocytosis globally. One question addressed was the role of VE-cadherin endocytosis in endothelial cell migration. Cells expressing the VE-cadherin-DEE mutant (VE-cadherin-DEE) exhibited a migration defect and were unable to close the gap created by a scratch in the monolayer (Nanes *et al*., 2012). This migration defect was not due to disrupted p120 binding as a control VE-cadherin mutant, VE-cadherin-GGG, that does not bind to p120 but undergoes endocytosis, migrated similar to cell expressing wild type VE-cadherin (Nanes *et al*., 2012). The migration defect was not due to decreased mobility of the mutant cadherin with in the plasma membrane. FRAP analysis revealed a larger mobile fraction in cells expressing VE-cadherin-DEE (Nanes *et al*., 2012). Therefore, VE-cadherin endocytosis is a requirement for collective endothelial cell migration. However, VE-cadherin endocytosis is not required for single cell migration. Sparsely seeded endothelial cells expressing VE-cadherin-DEE migrated similar to wild type cells plated at the same density (Nanes *et al*., 2012). Together these data suggest that junctional plasticity, achieved through cadherin

endocytosis, is required for collective cell migration.

Because much of angiogenesis and vessel remodeling depends on endothelial migration, disruption of VE-cadherin endocytosis would most likely result in severe vascular malformation and possibly lethality. It will be interesting to know how mutations that inhibit VE-cadherin endocytosis or disrupt p120 binding affect angiogenic processes *in vivo*.

3.6 Endothelial junctions in vascular disease

The loss of proper endothelial cell function is associated with a variety of diseases. The hallmarks of these diseases include vascular leakage, inflammation, and inappropriate neovascularization. A balance between junctional plasticity and the maintenance of vascular integrity must be tightly coordinated. Alterations in cell junctions and maintenance are involved in many vascular disorders.

A common feature of many vascular disorders is defective barrier function, which can occur through the disruption of VE-cadherin's adhesive and signaling functions. One example of this is the autosomal dominant hereditary condition known as cerebral cavernous malformation (CCM). In CCM, adherens junctions in vessels are disassembled leading to the loss of endothelial apical-basal cell polarity and the formation of hemorrhagic cavernomas, which are a cluster of abnormal blood vessels mainly found in the brain and spinal cord. Lampugnani et al. report that the CCM1 protein stabilizes VE-cadherin at junctions where it activates the Par polarity complex to establish and maintain correct endothelial cell polarity and vascular lumen (Lampugnani *et al*., 2010).

In addition to heritable conditions, disrupted vascular integrity also can occur

through VEGF signaling and inflammation. VE-cadherin has emerged as a key molecule targeted by multiple pro-permeability and pro-inflammatory mediators to perturb endothelial barrier integrity. During inflammation, endothelial cell adhesion is disrupted, by temporary loss of VE-cadherin at the junction, to allow leukocytes to pass through the vessel wall into the surrounding tissue (Allport *et al*., 2000). After leukocyte transmigration, the junction is reestablished. Pro-inflammatory mediators, such as histamine, thrombin, and fibrinogen, have been reported to influence VE-cadherin function at junctions (Le Guelte *et al*., 2012). Both histamine and fibrinogen destabilize VE-cadherin through dissociation of β-catenin from the cadherin (Le Guelte *et al*., 2012).

VEGF increases permeability through tyrosine phosphorylation of VE-cadherin resulting in the destabilization of the adherens junction. In response to hypoxia, VEGF signaling can result in hyperpermeability following stroke or myocardial infarction (Weis *et al*., 2005). VEGF signaling is also implicated in aberrant angiogenesis in both ocular disorders, such as diabetic retinopathy and in cancers (Weis *et al*., 2005).

VE-cadherin-mediated permeability aids in tumorigenesis through its role in tumor-induced angiogenesis and inflammation. However, VE-cadherin can be linked to metastasis through both intra- and extra- vasation of tumor cells. In one example, breast cancer tumor cells were reported to adhere to the endothelium and disrupt adherens junctions, likely by internalization of VE-cadherin, with the additional observation of disrupted β-catenin binding (Cai *et al*., 1999). In addition, EMT during breast cancer has been reported to occur with increased expression of VE-cadherin. The increased VE-cadherin expression promotes proliferation of the tumor cells through TGF-β signaling pathway (Labelle *et al*., 2008).

VE-cadherin levels are decreased in vascular tumors such as angiosarcomas (Martin-Padura *et al*., 1995). In addition, Zanetta et al. induced vascular tumors in mice and found that the loss of VE-cadherin correlated with invasiveness and hemorrhaging (Zanetta *et al*., 2005). Kaposi Sarcoma is an endothelial-derived tumor caused by human herpesvirus 8 (HHV8) and characterized by abnormal angiogenesis and leaky vessels (Mansouri *et al*., 2008; Qian *et al*., 2008). HHV8 encodes two membrane-associated RING-CH (MARCH)-family ubiquitin ligases, K3 and K5. K5 directly targets VE-cadherin for ubiquitination and down-regulation through endocytosis (Nanes et al., submitted). HHV8-induced vascular permeability is likely to occur through multiple mechanisms, including K5-mediated down-regulation of VE-cadherin (Nanes et al., submitted).

3.7 Summary

Vascular development can be separated in to two phases, vasculogenesis and angiogenesis. While VE-cadherin seems to be dispensable during vasculogenesis, it is essential to angiogenesis through its role in signaling for endothelial cell survival and in collective angiogenic migration. VE-cadherin expression must be tightly regulated during angiogenesis as the misregulation is implicated in numerous diseases.

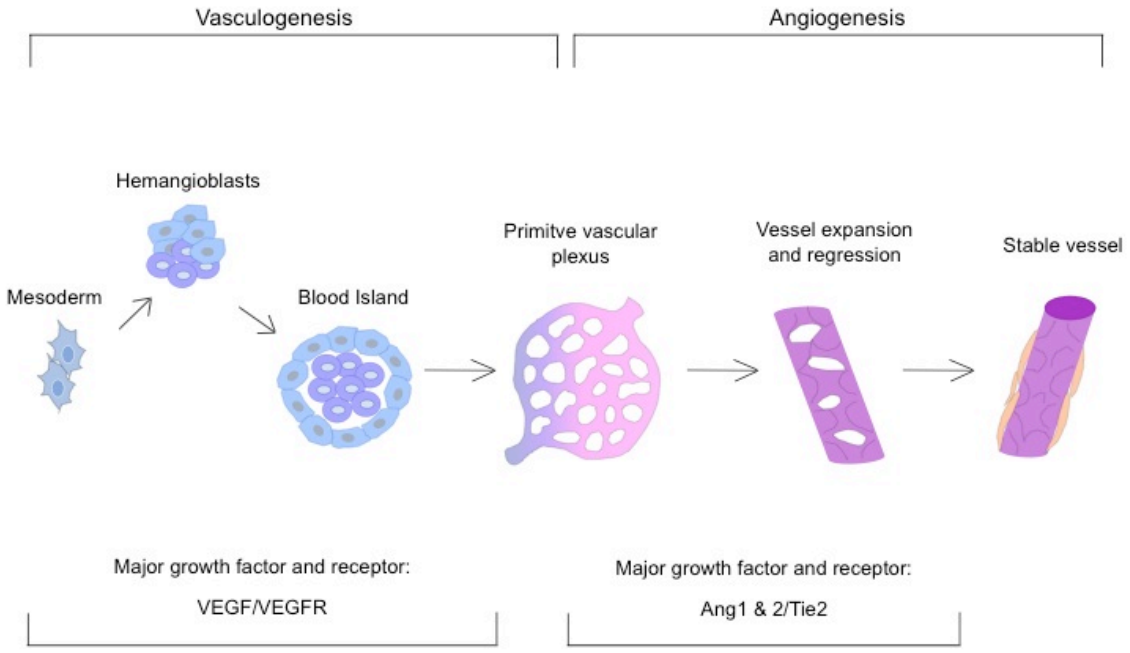


Figure 7. Vascular Development

Vascular development can be loosely separated into two phases: vasculogenesis and angiogenesis. Vasculogenesis is defined as *de novo* vessel formation, while angiogenesis occurs through the sprouting of existing vessels to form a complex network of vessels. During vasculogenesis mesodermal derived hemangioblasts form blood islands in the yolk sac of a developing embryo. Next, the blood islands connect to form the primitive vascular network. Vascular endothelial growth factor (VEGF) is the major growth factor responsible for vessel development during vasculogenesis. During developmental angiogenesis, the primitive vascular network is remodeled through vessel expansion and regression to establish stable vessels and the mature network. Angiopoietin (Ang) 1 and 2 are the major growth factors involved in angiogenic remodeling.

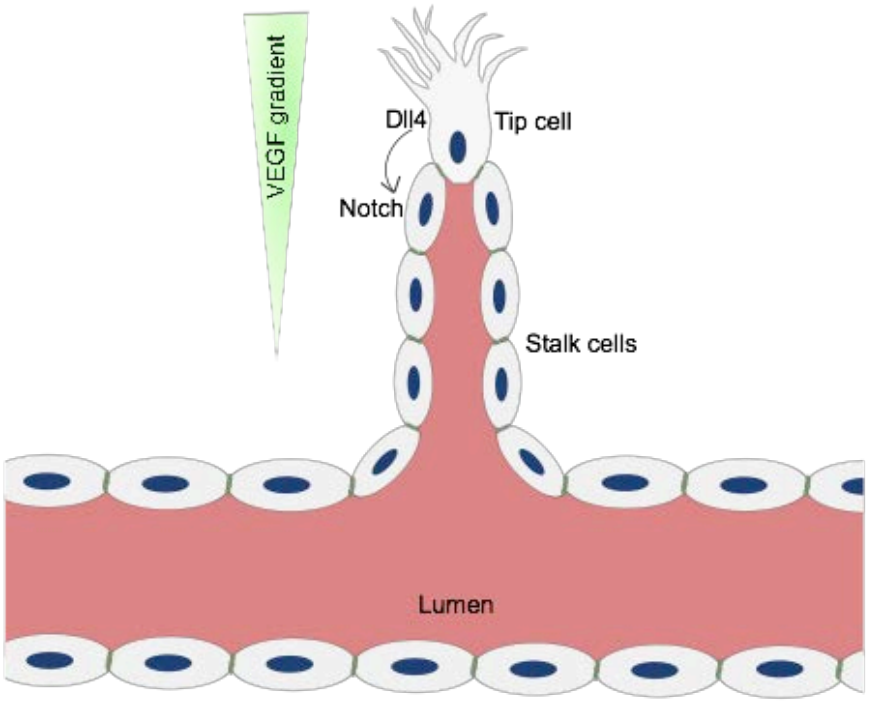


Figure 8. Spouting angiogenesis

Sprouting angiogenesis forms the majority of blood vessels in a mature network. It occurs through the migration and proliferation of endothelial cells in response to growth factors. "Tip" cells are highly polarized cells at the tip of the stock. They receive the highest VEGF signal, which induces Delta-Notch mediated lateral inhibition. This signaling pathway is important for inhibiting stalk cells from becoming tip cells. "Stalk" cells follow tips cells, migrating and proliferating to develop new vessels.

Chapter 4

Ankyrin-G inhibits endocytosis of cadherin dimers

This chapter is adapted from:

Cadwell, C. M., Jenkins, P. M., Bennett, V., & Kowalczyk, A. P. (2015). Ankyrin-G inhibits endocytosis of cadherin dimers. *J Biol Chem*.

4.0 Introduction

Dynamic regulation of endothelial cell adhesion is central to normal vascular development and maintenance. Furthermore, altered endothelial adhesion is associated with aberrant angiogenesis and contributes to numerous diseases through increased inflammation (Carmeliet, 2003; Dejana *et al*., 2008; Vincent *et al*., 2004). Thus, normal vascular patterning and maintenance requires tight regulation of endothelial cell adhesion dynamics. Yet, the mechanisms that control junctional plasticity are not fully understood.

Adherens junctions (AJs) are cadherin-based intercellular structures that mediate adhesion and mechanically link adjacent cells (Saito *et al*., 2012). Vascular endothelial cadherin (VE-cadherin) is the major adhesive protein in the AJs of the endothelium. Modulation of VE-cadherin levels at the plasma membrane contributes to the dynamic regulation of adhesion (Harris *et al*.). Like other classical cadherins, VE-cadherin binds to members of the armadillo-family proteins called catenins through its cytoplasmic tail. p120-catenin (p120) stabilizes VE-cadherin at the cell surface through binding to the juxtamembrane (JMD) domain and masking an endocytic motif (Chiasson *et al*., 2009; Nanes *et al*., 2012). When p120 is not bound, the cadherin undergoes rapid clathrin-dependent endocytosis and degradation (M. A. Davis *et al*., 2003; Xiao, Allison, Buckley*, et al*., 2003; Xiao, Allison, Kottke*, et al*., 2003). β-catenin binds to the catenin-binding domain of the cadherin cytoplasmic tail. Through interactions between β and α-catenin the cadherin is linked to the actin cytoskeleton, which increases adhesive strength of the junction (R. Desai *et al*., 2013; Oas *et al*., 2013; Pokutta *et al*., 2008; Taguchi *et al*., 2011; Yamada *et al*., 2005).

VE-cadherin mediates adhesion through its extracellular domain by the formation

of calcium dependent homophilic trans interactions. Trans interactions occur between two cadherins on neighboring cells and are believed to be the initial recognition event in the formation of adherens junctions (Brasch *et al*., 2012; Zhang *et al*., 2009). Adhesion occurs through a reciprocal process in which a conserved tryptophan (W2) is inserted into a hydrophobic pocket of a cadherin on a neighboring cell (Brasch *et al*., 2011; Shapiro *et al*., 1995). Cis interactions, interactions between two cadherins on the surface of the same cell, laterally cluster VE-cadherin (Brasch *et al*., 2012; Harrison *et al*.). Together, cis and trans interactions coalesce the cadherin into cell junctions.

Neighboring endothelial cells are mechanically coupled through linkage of VE-cadherin to the cytoskeleton. The actin cytoskeleton of endothelial cells is composed of three separate but inter-related structures, the membrane skeleton, the cortical actin ring, and actomyosin-based stress fibers (Prasain *et al*., 2009). The membrane skeleton, often referred to as the spectrin-actin cytoskeleton, is immediately adjacent to the plasma membrane and it is responsible for membrane architecture (Prasain *et al*., 2009). It primarily consists of spectrin and spectrin binding partners, including ankyrin-G. Ankyrin-G binds to membrane proteins and through associations with spectrin links them to the cytoskeleton. Ankyrin-G binding partners include cell adhesion molecules, such as L1 CAMs, E- and N- cadherin (Bennett *et al*., 2009).

Amongst most classical cadherins, including E-cadherin, the ankyrin-G-binding site is highly conserved and spans a region of the cytoplasmic tail that includes the JMD (Kizhatil *et al*., 2007). In polarized epithelial cells, ankyrin-G binds to E-cadherin and retains it at the lateral wall (P. M. Jenkins *et al*., 2013). In MDCK cells, E-cadherin at the apical membrane undergoes clathrin-mediated endocytosis. In contrast, E-cadherin at the

lateral membrane is bound by ankyrin-G and stabilized at the surface. In this way ankyrin-G, in cooperation with clathrin, contributes to the polarized epithelial phenotype.

To better understand the mechanisms that regulate endothelial cell adhesion, we studied the relationship between homophilic VE-cadherin interactions involved in adherens junction formation and cadherin endocytosis. Our data demonstrate that cis-dimerization inhibits VE-cadherin endocytosis independent of trans interactions. Inhibition of endocytosis through cis dimerization is not dependent on p120 binding to the cadherin. However, we find that ankyrin-G associates with cadherin cis-dimers and inhibits endocytosis of VE-cadherin. Our findings support a novel mechanism for regulation of VE-cadherin endocytosis through ankyrin-G association with cadherin engaged in lateral interactions.

4.1 Results

4.1.1 Induced dimerization of VE-cadherin inhibits endocytosis independent of adhesion

To determine how adhesion affects VE-cadherin internalization, we introduced a point mutation to a conserved tryptophan (W2) in the extracellular domain of VE-cadherin (VE-cadherin-W2). The W2 mutation has previously been shown to disrupt VE-cadherin homophilic adhesion (May et al., 2005). Using a fluorescence-based internalization assay, which allows us to specifically observe the internalized pool of the cadherin, we found that VE-cadherin-W2 endocytosis was significantly increased over wild type VE-cadherin (Fig. 1A and B). Additionally, instead of junctional clustering, we observed that the W2 mutation resulted in a diffuse localization of the cadherin on the plasma membrane (Fig. 1A). Expression levels between wild type VE-cadherin and VE-cadherin-W2 were relatively similar by western blot (Fig. 1C).

Trans interactions are thought to occur before cis interactions (Brasch et al., 2012; Zhang et al., 2009). Therefore, the increase in VE-cadherin internalization may be the result of either the loss of adhesive trans interactions or lateral cis-dimerization. To distinguish between these two possibilities, we used the FKB506 binding protein homodimerizing system to model cadherin cis clustering. AP20187 is a cell -permeant molecule that can induce the dimerization of proteins containing FKB506 protein repeats. We fused a FK506 binding protein (FKBP) repeat to the intracellular C-terminus of the VE-cadherin tail (VE-cadherin-FKBP) (Fig. 2A). FK binding proteins have previously been used to study endocytosis of other membrane proteins and are not known to disrupt cadherin localization (Broermann et al., 2011; Chen et al., 2012; Hofman et al., 2010; Song et al., 2005; Yap et al., 1997). We verified expression by western blot and junctional localization of VE-cadherin-FKBP using immunofluorescence (IF) microscopy via a HA-tag fused to the C-terminus of the protein (Fig. 2B and C, lower left panels). We then introduced the W2 mutation to VE-cadherin-FKBP to generate a VE-cadherin mutant that is not adhesive but that can be induced to form a dimer (VE-cadherin-W2-FKBP). Using an internalization assay, we observed that induced dimerization of the both VE-cadherin-FKBP and VE-cadherin-W2-FKBP cadherin fusion proteins resulted in decreased internalization (Fig. 2C and D). Therefore, cis interactions independent of trans interactions strongly inhibit VE-cadherin endocytosis.

4.1.2 p120-catenin binding is not required for inhibited endocytosis of VE-cadherin dimers

Because p120-catenin is a known regulator of cadherin stability at the cell surface, we hypothesized that p120 binding stabilizes VE-cadherin dimers. To determine the role of

p120 in VE-cadherin dimer endocytosis, we first compared the amount of p120 bound to dimerized cadherin to non-dimerized cadherin by immunoprecipitation. However, we found that there was no observable difference in the amount of p120 that co-immunoprecipitated with VE-cadherin in cells treated with AP20187 compared to VE-cadherin in cells treated with vehicle control (Fig. 3A).

To further investigate the role of p120 binding in endocytosis of VE-cadherin dimers, we mutated three glycine residues to alanines in the p120 core-binding domain in the VE-cadherin-FKBP fusion protein (VE-cadherin-GGG-FKBP). Mutation of these three glycine residues (649-651) has previously been described to disrupt p120 binding and to increase endocytosis of the cadherin compared to wild type (Nanes et al., 2012). As expected, dimerization of VE-cadherin-GGG-FKBP did not result in p120 binding (Fig. 3B). However, using an internalization assay, we found that inducing dimerization of VE-cadherin-GGG-FKBP inhibited endocytosis (Fig. 3C and D). Additionally, we found that dimerization of a VE-cadherin mutant lacking the catenin-binding domain (VE-cadherin-ΔCBD-FKBP) also inhibited endocytosis (Fig. 3E and F). Together, these results indicate that VE-cadherin dimers are resistant to endocytosis in the absence of either p120 or β-catenin binding.

4.1.3 VE-cadherin colocalizes with and co-immunoprecipitates with Ankyrin-G

Because p120 binding is not required to inhibit endocytosis of dimerized VE-cadherin, we reasoned that another protein might be involved in stabilizing the cadherin at the plasma membrane. One such candidate protein is ankyrin-G, which binds to E-cadherin at conserved sites in the cadherin tail (Fig. 4A) and retains the cadherin at the lateral membrane in polarized epithelial cells (P. M. Jenkins et al., 2013). The canonical

190/210kD ankyrin-G isoforms are expressed in endothelial cells including HUVECs, although expression of both isoforms was not detected in all endothelial cells examined (Fig. 4B). Interestingly, the ankyrin-G-binding motif identified in the cytoplasmic tail of E-cadherin is not fully conserved in VE-cadherin (Fig. 4A)(P. M. Jenkins et al., 2013). However, by IF microscopy, we observed that in HUVECs endogenous ankyrin-G localized to various regions of the cells, including cell-cell junctions, where the protein colocalized with VE-cadherin (Fig. 4C). Additionally, ankyrin-G colocalized with wild type VE-cadherin when co-expressed in COS-7 cells (Fig. 4D and E). Interestingly, we observed a decrease in colocalization between ankyrin-G and VE-cadherin-W2 (Fig. 4D and E).

To further investigate the association between ankyrin-G and VE-cadherin we performed co-immunoprecipitation assays. We exogenously expressed the 190kD isoform of HA-tagged ankyrin-G and VE-cadherin in COS-7 cells, which do not express endogenous VE-cadherin. Wild type VE-cadherin co-immunoprecipitated with ankyrin-G (Fig. 4F and G). Consistent with the colocalization results, we were unable to detect ankyrin-G association with VE-cadherin-W2 (Fig. 4F and G).

4.1.4 Ankyrin-G inhibits internalization of VE-cadherin independent of p120-catenin binding

Because ankyrin-G inhibits the endocytosis of E-cadherin localized to the lateral membrane of polarized epithelial cells, we reasoned that it might inhibit VE-cadherin endocytosis. To address this question, we performed internalization assays in cells exogenously expressing both VE-cadherin and ankyrin-G. Expression of ankyrin-G inhibited internalization of VE-cadherin (Fig. 5A and B). Additionally, expression of

ankyrin-G inhibited internalization of VE-cadherin-GGG (Fig. 5C and D) and VE-cadherin-ΔCBD (Fig. 5E and F), which are unable to bind to p120 or β-catenin, respectively. However, Ankyrin-G expression did not inhibit internalization of transferrin receptor (Fig. 5G and H). Therefore, ankyrin-G does not inhibit endocytosis globally, but instead it selectively inhibits the endocytosis of VE-cadherin. These data suggest that ankyrin-G specifically inhibits endocytosis of VE-cadherin independent of catenin binding.

4.1.5 *Ankyrin-G selectively associates with dimerized VE-cadherin and does not inhibit endocytosis of W2 mutant*

Our co-immunoprecipitation and colocalization results suggest that ankyrin-G binds to dimerized VE-cadherin. Because cis-dimers may not form in the absence of trans interactions, we wanted to determine which type of VE-cadherin dimer, cis or trans, associates with ankyrin-G. To address this question, we induced cis-dimerization of the VE-cadherin-W2-FKBP mutant in cells exogenously expressing ankyrin-G. We observed that induced dimerization of the W2 mutant resulted in ankyrin-G colocalization similar to wild type VE-cadherin (Fig. 6A and B). Therefore, we conclude that ankyrin-G selectively associates with cis-dimerized VE-cadherin.

Our colocalization results indicate that ankyrin-G associates with VE-cadherin upon cis dimerization of the cadherin. Therefore, we predicted that ankyrin-G would not inhibit endocytosis of the W2 mutant. Consistent with this prediction, we found that VE-cadherin-W2 internalization was not inhibited by ankyrin-G expression (Fig. 6C and D). These data indicate that ankyrin-G mediated inhibition of VE-cadherin internalization requires cadherin cis dimerization.

4.1.6 Ankyrin-G association is required to inhibit VE-cadherin dimer internalization

Our data suggest that ankyrin-G associates with and inhibits the internalization of cis-dimerized VE-cadherin. To directly test this idea, we mutated two glutamic acid residues, E637 and E640, to alanines. These amino acids represent acidic residues that are conserved in the ankyrin-G binding site in E-cadherin (Fig. 4A). To avoid complications from p120 binding to the dimers, we made the mutations in the VE-cadherin-GGG-FKBP mutant to create VE-cadherin-EE-GGG-FKBP. We observed a decrease in ankyrin-G colocalization with VE-cadherin-EE-GGG compared to VE-cadherin-GGG (Fig.7A and B). Additionally, we observed a significant increase in internalization of VE-cadherin-EE-GGG compared to wild type VE-cadherin. Internalization of VE-cadherin-EEGGG was also increased compared to VE-cadherin-GGG, though the increase was not statistically significant (Fig. 7C and D). Similar to the W2 mutant, ankyrin-G expression did not inhibit internalization of the VE-cadherin-EE-GGG mutant (Fig. 7E and F). Moreover, unlike the other VE-cadherin-FKBP fusion proteins, we found that inducing the dimerization of VE-cadherin-EE-GGG-FKBP did not result in significant inhibition of internalization (Fig. 7G and H). These data indicate that ankyrin-G association is required to inhibit VE-cadherin dimer internalization.

4.1.7 Ankyrin-G regulates adherens junction organization in endothelial cells

Our data demonstrate that ankyrin-G inhibits endocytosis of VE-cadherin dimers in COS-7 cells and localizes with VE-cadherin at endothelial cell junctions. Therefore, we reasoned that ankyrin-G may play a role in stabilizing VE-cadherin at the cell surface of endothelial cells. To test this hypothesis, we transfected HUVECs with shRNA against ankyrin-G. We observed a significant decrease in ankyrin-G signal in HUVECs

transfected with ankyrin-G shRNA compared to cells transfected with control shRNA by immunofluorescence microscopy (Fig. 8A). Additionally, a western blot of cell lysate from HUVECs transfected with control shRNA against luciferase or shRNA against ankyrin-G, confirmed a decrease in the 190kD isoform of ankyrin-G (Fig.8B). We observed a significant decrease in adherens junction proteins including VE-cadherin, p120-catenin and ß-catenin at junctions after ankyrin-G knockdown compared to control knockdown (Fig. 8A). Importantly, co-transfection of ankyrin shRNA with a HA-tagged 190kD ankyrin-G that is refractory to the shRNA (Fig. 8B, right panels) resulted in levels of junctional VE-cadherin similar to control (Fig. 8C). Moreover, we found that knockdown of ankyrin-G increased the internalization of wild type VE-cadherin, though it did not reach statistical significance. This outcome is likely due to p120 binding to the cadherin and masking effects of ankyrin knockdown (Fig. 8D) Therefore, to assess the affect of ankyrin-G knockdown in the absence of p120 binding, we performed an internalization assay of VE-cadherin-GGG in cells transfected with ankyrin-G shRNA and found a significant increase in internalization compared to cells transfected with control shRNA (Fig. 8E). Together, these data suggest that ankyrin-G inhibits endocytosis of VE-cadherin in endothelial cells and regulates the organization of endothelial adherens junctions.

4.2 Discussion

Membrane trafficking has emerged as a major mechanism that regulates cadherin adhesion. However, the mechanisms that control VE-cadherin endocytosis are not fully understood. The results presented here demonstrate that cis-dimerization of VE-cadherin inhibits endocytosis. Furthermore, inhibition of endocytosis occurs in a manner

independent of adhesive interactions (trans dimerization) (Fig. 2C and D) and independent of p120 binding (Fig. 3). The mechanism by which cis dimerization inhibits endocytosis was also investigated. Our data reveal that ankyrin-G stabilizes the cadherin at the cell surface (Fig. 5A and B). This process requires cadherin cis-dimerization, but not p120 binding nor cadherin linkage to the actin cytoskeleton through association with β-catenin. Collectively, our results support a model in which ankyrin-G associates with and inhibits the internalization of VE-cadherin cis-dimers (Fig. 9).

The role of adhesion in regulation of cadherin endocytosis is not fully understood. Interestingly, dimerization of other cell surface receptors, including receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs), results in increased endocytosis from the plasma membrane (Hofman et al., 2010; Song et al., 2005). The results presented here suggest a distinct mechanism of regulation for cadherin endocytosis. We find that mutating a tryptophan residue critical for adhesion results in increased cadherin endocytosis. However, increased endocytosis appears to result from a loss of cadherin cis dimers, rather than from a loss of adhesion. This conclusion is based on the finding that forcing cis dimerization of a cadherin mutant unable to engage in adhesion virtually abolished VE-cadherin endocytosis. Additionally, p120 binding to the cadherin tail is not required for cis dimerization to inhibit endocytosis (Fig. 3B, C, and D). This result was surprising, as p120 is widely known to be a key regulator of cadherin stability at the plasma membrane.

Here, we report that ankyrin-G associates with VE-cadherin and inhibits its endocytosis (Figs. 4 and 5). Ankyrin-G binding partners are known to include other cell adhesion molecules, including L1 CAMs, E- and N- cadherin. The ankyrin-G binding

sites in these proteins are stretches of 10-20 amino acids that do not contain a single defining motif (Bennett et al., 2009). Instead, the ankyrin-G binding site is different between families of proteins. However, it is typically conserved within a family of proteins. For example, the ankyrin-G-binding site in E- and N- cadherin consists of a stretch of 21 amino acids with 7 conserved amino acids that are critical for binding. This ankyrin-G-binding motif is not fully conserved in VE-cadherin (Fig. 4A). In spite of these differences in this region of the VE-cadherin tail, our data demonstrate that ankyrin-G associates with the cytoplasmic tail of VE-cadherin. First, we observe colocalization of ankyrin-G and VE-cadherin in primary endothelial cells and when exogenously expressed in other cell types (Fig. 4C, D, and E). In addition, we found that wild type VE-cadherin co-immunoprecipitates with ankyrin-G. Importantly, the VE-cadherin-W2 mutant, which is unable to engage in adhesion, does not co-immunoprecipitate with ankyrin-G, indicating ankyrin-G association is specific and requires dimerization (either cis or trans) of the cadherin.

The data presented here, as well as previously published work by Jenkins et al. establish ankyrin-G as a novel regulator of cadherin endocytosis. Furthermore, ankyrin-G and p120-catenin inhibit cadherin internalization in mechanistically distinct ways. For example, p120 associates with and potently inhibits endocytosis of cadherins that are unable to engage in adhesion, including IL2-VE-cadherin chimeras which lack the entire cadherin extracellular domain (Xiao, Allison, Buckley, et al., 2003). In contrast, ankyrin-G selectively associates with dimerized cadherin (Fig. 4), and does not associate with or prevent endocytosis of VE-cadherin mutants that are unable to form dimers. This finding is consistent with reports that ankyrin-G binds to specific receptors, such as neurofascin,

only when dimerized (Jefford et al., 2000). The precise mechanisms by which ankyrin-G regulates internalization is not fully understood, but it does not appear to require linkage to the actin cytoskeleton through β-catenin because ankyrin-G inhibited the internalization of a VE-cadherin mutant lacking the catenin-binding domain. Because VE-cadherin shares only a portion of the conserved ankyrin-G-binding motif, it is possible that dimerization provides a platform capable of stabilizing the VE-cadherin-ankyrin-G interaction.

The VE-cadherin juxtamembrane domain in the cytoplasmic tail contains a dual function motif that serves as either a p120-binding site or as an endocytic motif (Nanes et al., 2012). When p120 is bound to this motif, clathrin-dependent endocytosis of the cadherin is potently inhibited. Because ankyrin-G binds to the juxtamembrane domain of classic cadherins (Kizhatil et al., 2007), it is possible that ankyrin-G inhibits VE-cadherin endocytosis in a similar manner. However, we cannot rule out that ankyrin-G may regulate VE-cadherin endocytosis indirectly through association with other proteins reported to modulate cadherin endocytosis, such as Numb. However, this seems unlikely because Numb associates with p120-catenin to regulate endocytosis of E-cadherin (Sato et al., 2011), and we find that ankyrin-G inhibits endocytosis of the VE-cadherin-GGG mutant, which does not bind to p120 (Fig. 3B). Therefore, we favor a model in which ankyrin-G interacts with VE-cadherin directly to modulate endocytosis (Fig. 9).

It remains to be determined if the binding of p120 and ankyrin-G to the cadherin is mutually exclusive. However, given the mass of both p120 and ankyrin-G, and the location of critical ankyrin-G residues in the core p120-binding region, it is unlikely that both proteins bind to the cadherin simultaneously. Thus, it is highly likely that different

subcellular pools of cadherin are stabilized by either p120 or ankyrin-G, and that these binding partners differentially regulate cadherin stability at the plasma membrane. For example, it is possible that ankyrin-G and p120 inhibit the endocytosis of different pools of VE-cadherin at different membrane domains, such as at junctional or non-junctional regions of the plasma membrane. Consistent with this possibility, a recent study found that ankyrin-G is concentrated in microdomains in the lateral membrane of MDCK cells. Furthermore, the giant ankyrin-G isoform stabilizes GABAergic synapses by opposing endocytosis in microdomains in the somatodendidritic plasma membrane of hippocampal neurons (He et al., 2014; Tseng et al., 2015). Moreover, a recent report from Jenkins et al. reports micron-sized domains consisting of an underlying membrane skeleton comprised of ankyrin-G and its partner β-spectrin that inhibit endocytosis through the exclusion of clathrin and clathrin dependent cargo in epithelial lateral membranes. It is possible that ankyrin-G, in conjunction with β-spectrin, inhibits VE-cadherin endocytosis through clathrin exclusion at microdomains in endothelial cells (P. Jenkins, Meng, H., Bennett, V., 2015).

Our data suggest that ankyrin-G regulates adherens junction organization as knockdown of the protein results in significantly less cadherin, p120-catenin and ß-catenin at junctions (Fig. 8A). These observations suggest a key role for ankyrin-G in the regulation of endothelial functions that rely on adherens junctions, such as signaling and barrier function (Dejana et al., 2008; Giannotta et al., 2013; Hordijk et al., 1999). The identification of ankyrin, in addition to p120, as a modulator of cadherin trafficking suggests that the cadherin juxtamembrane domain may interact with a number of different proteins in different cellular contexts to regulate cadherin levels at the plasma

membrane. It is likely that additional binding partners for this cadherin domain will emerge as regulators of cadherin endocytosis. Further studies are needed to gain insight into the specific role of p120 and ankyrin-G in regulating cadherin internalization to modulate cell adhesion in various developmental and disease contexts.

4.3 Methods and Materials

Cell Culture

African green monkey kidney fibroblast-like (COS-7) (American Type Culture Collection, ATCC) and human embryonic kidney (HEK) QBI-293A cell lines (MP Biomedicals) were cultured as previously described (Nanes *et al*., 2012). Primary mouse endothelial cells were cultured as previously described (Oas *et al*.). Human dermal microvascular endothelial cells were cultured in Endothelial Growth Medium 2 Microvascular (Lonza). Human umbilical vein endothelial cells were cultured in M199 (Mediatech, Inc.) supplemented with 20% fetal bovine serum (FBS), and 1% pen/strep on gelatin-coated plates.

Virus production

To generate an adenoviral expression system for protein expression in mammalian cells, the gene of interest was cloned into the Gateway pAd/CMV/V5-DEST vector (Invitrogen). The vector was linearized using PacI and transfected into HEK QBI293 cells to produce virus. After several rounds of infection, cells were lysed and virus was harvested.

Generation of VE-cadherin cDNA constructs

FKBP fusion proteins were generated using the ARGENT Regulated Homodimerization Kit (ARAID Pharmaceuticals Inc., Cambridge, MA), by subcloning a single FKBP domain followed by a HA tag in frame with N-terminus of the cadherin. VE-cadherin-ΔCBD-FKBP was generated by the addition of a single FKBP domain with HA-tag to the end of the juxtamembrane domain of the VE-cadherin tail. The W2 mutation was introduced using site directed mutagenesis with the following primers: 5'-CGCCAAAAGAGAGATGCAATTTGGAACCAGATG-3', 5'-CATCTGGTTCCAAATTGCATCTCTCTTTTGGCG-3'. The GGG -> AAA mutation was introduced using previously described primers (Nanes *et al*., 2012).

Dimerization of VE-cadherin-FKBP fusion proteins

To induce dimerization, cells were incubated with 1uL of 100uM AP20187 (ARGENT, ARAID Pharmaceuticals, Inc., Cambridge, MA) or 1uL of Ethanol (vehicle control) in 1mL DMEM + 10% FBS for two hours at 37°C. Cells were washed three times with DMEM before use for downstream applications.

shRNA transfection in HUVECs

HUVECs cultured on gelatin coverslips were transfected using Targefect-HUVEC (targetingsystem, El Cajon, CA) according to the protocol provided by the supplier with a pLentilox3.7 plasmid encoding shRNA against ankyrin-G (5'-GGATTAAGCAGGAAAGCAACC-3') or luciferase (5'-CGTTACCGCGGAATACTTCGA-3') under U6 promoter and mCherry under ß-actin promoter. For rescue experiments, endothelial cells were co-transfected with ankyrin-G shRNA and HA-tagged 190kD ankyrin-G. HA-tagged ankyrin-G was generated using the

coding sequence of the previously described 190kDa isoform of ankyrin-G (Kizhatil *et al*., 2004) was inserted into the EcoRI and PmeI sites of the pEGFP-N3 vector in which the eGFP had been replaced by a 3x hemagglutinin (HA) tag using standard molecular biology techniques. After 48 hours, immunofluorescence or western blot was performed. For immunofluorescence, mCherry was used to detect transfected cells.

Immunoprecipitation and western blot analysis

For immunoprecipitation experiments, COS-7 cells were pretreated with 1mM Dithiobis[succinimidyl propionate] (DSP) (Thermo Scientific, Pierce) for two hours at 4°C to crosslink proteins. To quench the crosslinking reaction, cells were incubated with 25mM Tris pH 7.5 at 4°C for 15 minutes. Cells were harvested in 0.5% Triton X-100 (Roche) containing protease inhibitor cocktails (Complete Mini tablets, EDTA free; Roche), 150 mM sodium chloride, 10 mM Hepes, 1 mM EGTA, and 0.1 mM magnesium chloride. After a 30-minute incubation at 4°C, cell lysates were centrifuged at 16,100 x g for 10 minutes, then incubated with 2 μg anti-HA (Bethyl Laboratories, Inc.) conjugated to ferromagnetic beads (Dynabeads, Life Technologies) for 2 hours at 4°C with full rotation. 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 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). Primary antibodies: rabbit anti-ankyrin-G C-terminal domain, anti-p120 (Rabbit polyclonal S-19; Santa Cruz Biotechnology, Inc.), Cadherin-5 (BD Transduction Laboratories), monoclonal anti-

vimentin clone V9 (Sigma-Aldrich), rabbit anti-β-tubulin (Santa Cruz).

For protein detection, horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) and a luminol-based detection system (ECL, GE Healthcare) were used, followed by exposure to autoradiography film (Denville Scientific).

Internalization and localization assays

Cells were infected with VE-cadherin mutants using adenoviral expression system, or cells were transfected using Lipofectamine 2000 (Invitrogen) according to the protocol provided by the supplier.

Internalization assays were performed as previously described (Chiasson *et al*., 2009; Xiao, Allison, Buckley, *et al*., 2003). Briefly, cells cultured on glass coverslips were incubated with an antibody against the VE-cadherin extracellular domain in media for 30 minutes at 4°C. Cells were washed 3 times with cold PBS to remove unbound antibody. To allow internalization, cells were incubated in prewarmed media for 30 minutes for VE-cadherin, or 5 minutes for transferrin receptor, at 37°C. Cells were returned to cold media. A low pH buffer (PBS with 100 mM glycine, 20 mM magnesium acetate, and 50 mM potassium chloride, pH 2.2) was used to remove any remaining antibody from the cell surface. Cells were then fixed and permeabilized by incubating in 4% paraformaldehyde for 10 minutes followed by 0.1% Triton X-100 for 8 minutes at room temperature. Rabbit anti-HA antibody (Bethyl Laboratories, Inc.) was used to determine the total cadherin pool. Secondary antibodies conjugated to fluorescent dyes (Alexa Fluor 488, 555, or 647 nm; Life Technologies) were used to visualize antibody binding. For each cell, internalization was quantified as the ratio of fluorescence signals corresponding

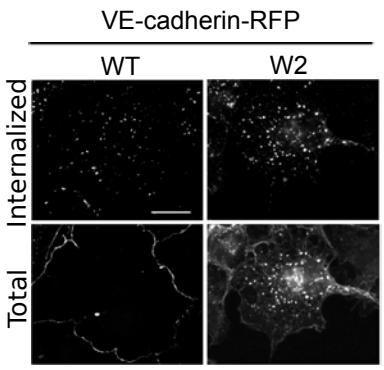
to the internalized and total cadherin pools. For localization assays, endothelial cells or cells cotransfected with VE-cadherin and GFP-tagged or HA-tagged 190kD ankyrin-G were fixed and processed for IF. Antibodies used include Chicken c-myc antibody (Bethyl Laboratories, Inc.), Transferrin from human serum, Alexa Fluor 555 conjugate (Molecular Probes), anti-VE-cadherin antibody, clone BV6 (Millipore), and rabbit anti-ankyrin-G, anti-p120-catenin (Rabbit polyclonal S-19; Santa Cruz Biotechnology, Inc.) and anti-ß-catenin (Rabbit, Sigma-Aldrich). Colocalization was quantified in individual cells using Pearson's correlation coefficient for VE-cadherin and ankyrin-G pixel intensities.

Microscopy was performed using an epifluorescence microscope (DMRXA2, Leica) equipped with 63X and 100X 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).

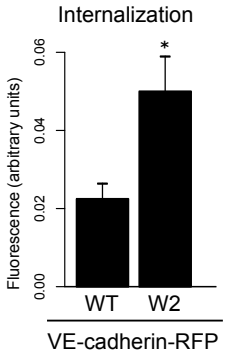
Image analysis and statistics

ImageJ software was used for all image analysis (Schneider *et al*., 2012). Custom ImageJ plugins were used to automate data quantification. R was used to compute statistics.

A.


VE-cadherin-RFP
WT
W2
Internalized
Total

B.


Internalization
Fluorescence (arbitrary units)
0.00
0.02
0.04
0.06
*
WT
W2
VE-cadherin-RFP

C.

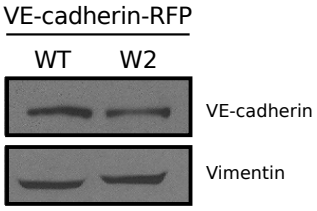

VE-cadherin-RFP
WT
W2
VE-cadherin
Vimentin

Figure 9. Mutation of conserved tryptophan (W2) increases VE-cadherin internalization

(A) Fluorescence based internalization assay of wild type (left) or non-adhesive (W2) VE-cadherin (right) proteins expressed in COS-7 cells. VE-cadherin internalized pool (upper panels) identified using anti-VE-cadherin antibody, after a 30 minute internalization period, a low pH wash was used to remove remaining surface antibody. Total pool (lower panels) was determined by fluorescence of RFP-tag fused to the N-terminus of the cadherin. Bar, 20 μm. (B) Quantification of internalization determined by normalizing internalized pool to total pool. Error bars represent SEM. N>20 cells/group. *, $P<0.05$. (C) western blot for expression levels of cadherin (upper panel) or vimentin as a loading control (lower panel) in COS-7 cells.

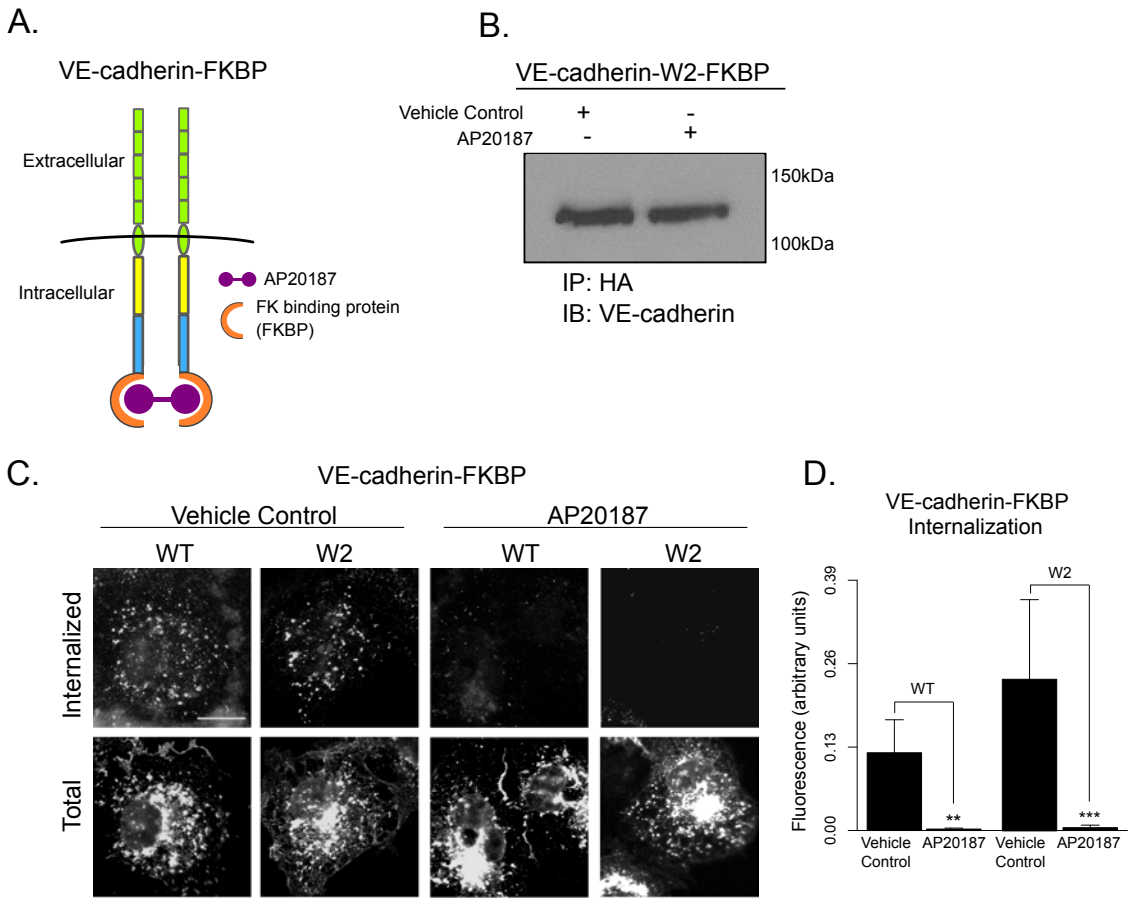


Figure 10. Induced dimerization of VE-cadherin inhibits its endocytosis independent of adhesion

(A) Schematic of VE-cadherin-FKBP fusion protein. FK binding protein (FKBP) is dimerized by the cell permeant bivalent molecule AP20187. (B) Isolation of HA-tagged VE-cadherin-W2-FKBP by immunoprecipitation of lysate from COS-7 cells expressing the protein treated with vehicle control or AP20187, followed by western blot for VE-cadherin. (C) Internalization assay of cells expressing either wild type VE-cadherin or VE-cadherin-W2 after treatment with vehicle control or AP20187. Bar, 20 μm. (D)

Quantification of internalization assay. Error bars represent SEM. N= 10-25 cells/group. **, $P<0.01$ compared to vehicle control treated WT. ***, $P<0.001$ compared to vehicle control treated W2.

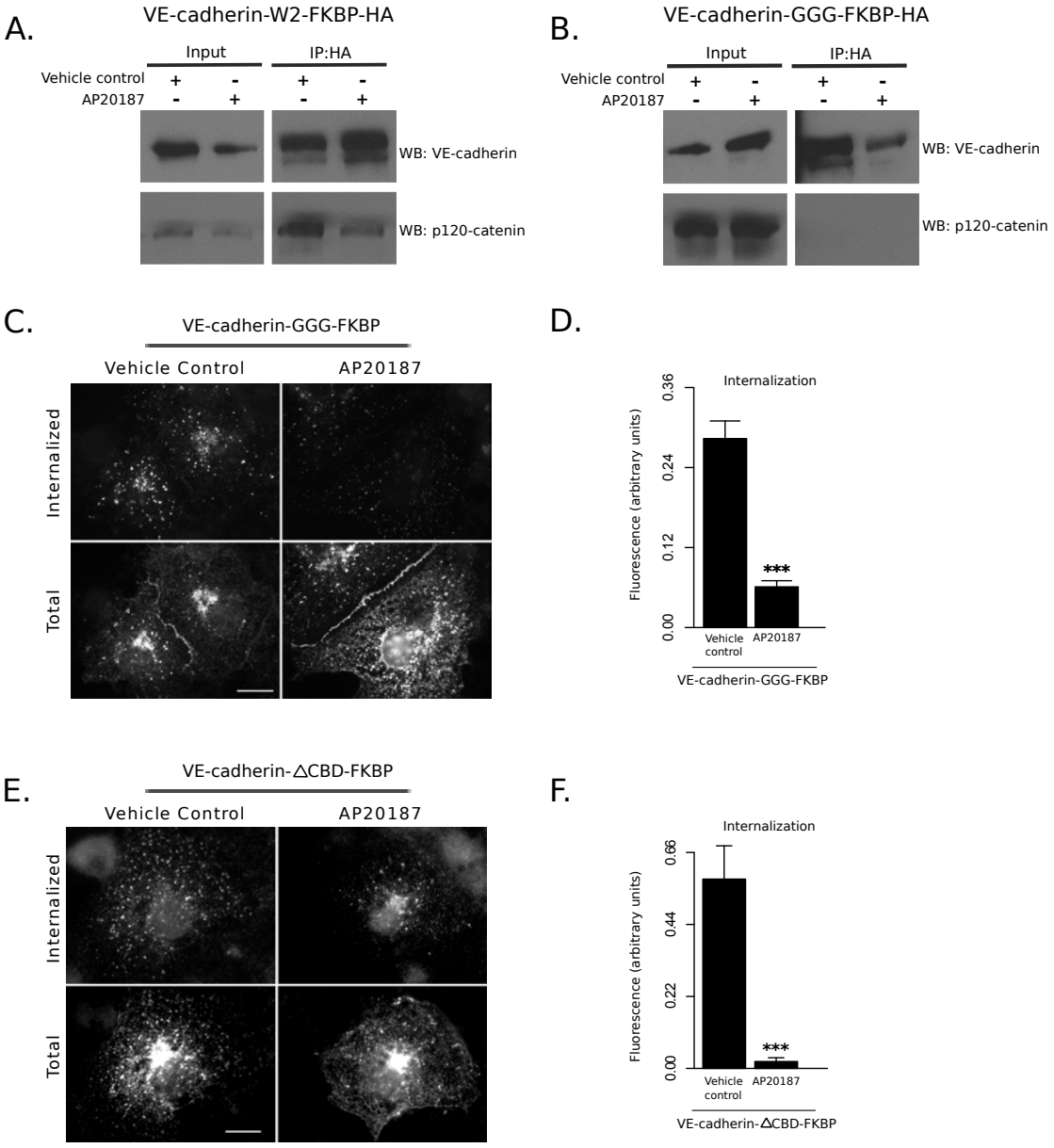
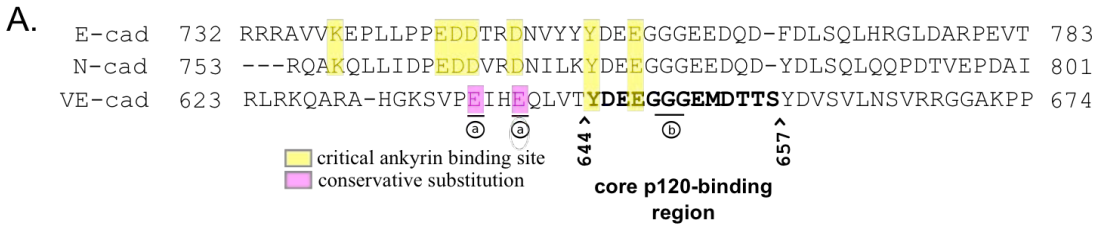
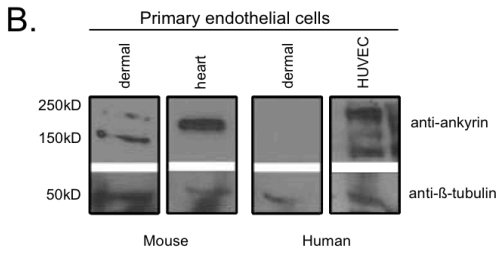


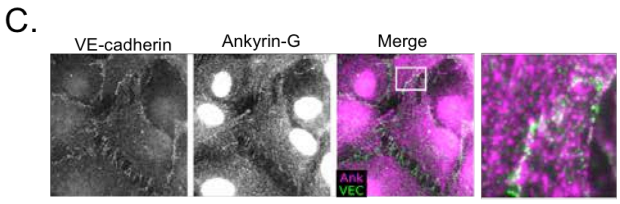
Figure 11. p120-catenin binding is not required for inhibited dimer internalization

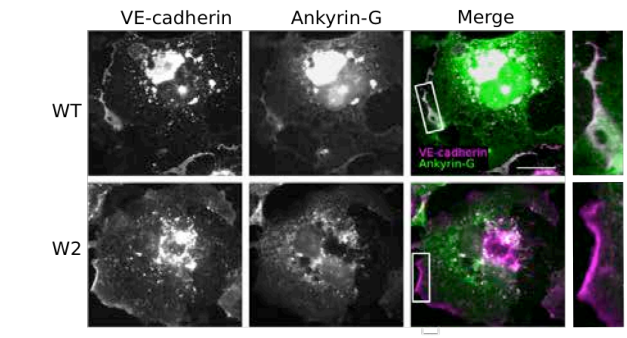
(A and B) Isolation of VE-cadherin-W2-FKBP (A) or VE-cadherin-GGG-FKBP (B) by immunoprecipitation using an antibody against the C-terminus HA-tag. Western blot for VE-cadherin (upper panel) and p120 (lower panel). (C and E) Fluorescence-based internalization assay of VE-cadherin-GGG-FKBP (C) or VE-cadherin-ΔCBD-FKBP (E)

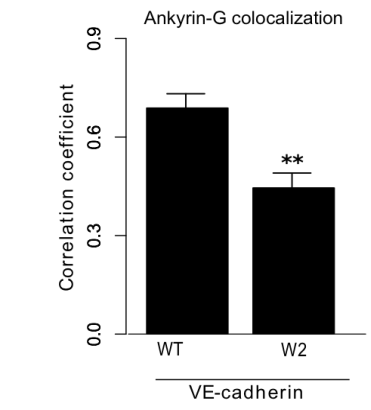
in COS-7 cells after treatment with vehicle control (left panels) or AP20187 (right panels). Bar, 20 μm. (D and F) Quantification of internalization. Error bars represent SEM. N>20 cell/group. ***, P<0.001.

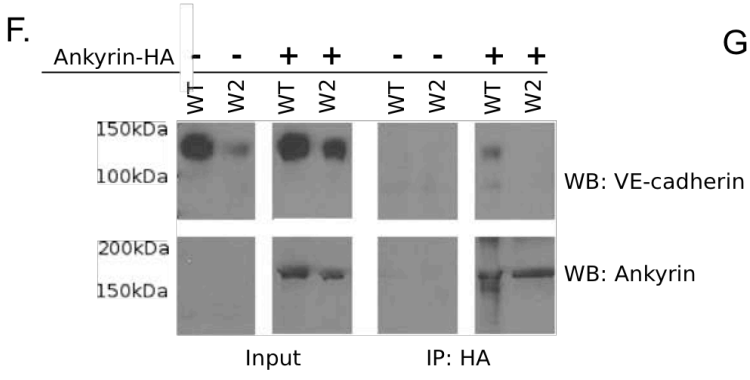

A.
E-cad 732 RRRAVVKEPLLPPEDDTRDNVYYYDEEGGGEEDQD-FDLSQLHRGLDARPEVT 783
N-cad 753 ---RQAKQLLIDPEDDVRDNILKYDEEGGGEEDQD-YDLSQLQQPDTVEPDAI 801
VE-cad 623 RLRKQARA-HGKSVPEIHEQLVTYDEEGGGEMDTTSYDVSVLNSVRRGGAKPP 674
critical ankyrin binding site
conservative substitution
644 >
657 >
core p120-binding region


B.
Primary endothelial cells
dermal
heart
dermal
HUVEC
250kD
150kD
50kD
anti-ankyrin
anti-ß-tubulin
Mouse
Human


C.
VE-cadherin
Ankyrin-G
Merge
Ank
VEC


D.
VE-cadherin
Ankyrin-G
Merge
WT
W2
VE-cadherin
Ankyrin-G


E.
Ankyrin-G colocalization
Correlation coefficient
0.0
0.3
0.6
0.9
**
WT
W2
VE-cadherin


F.
Ankyrin-HA
- - + + - - + +
WT W2 WT W2 WT W2 WT W2
150kDa
100kDa
200kDa
150kDa
WB: VE-cadherin
WB: Ankyrin
Input
IP: HA

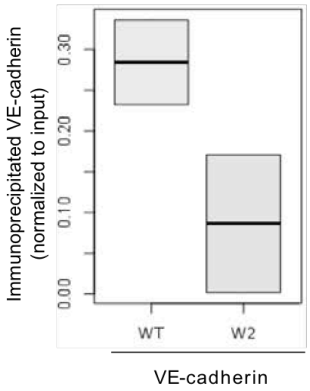

G.
Immunoprecipitated VE-cadherin
(normalized to input)
0.00
0.10
0.20
0.30
WT
W2
VE-cadherin

Figure 12. Ankyrin-G associates with the juxtamembrane domain of VE-cadherin

(A) Sequence alignment of juxtamembrane of classical cadherins. Critical ankyrin-G binding sites highlighted in yellow. Conservative substitutions highlighted in pink. Core p120-binding region identified by bold print. (a)VE-cadherin mutations used in Fig. 7. (b) VE-cadherin mutations used in Figs. 3, 5, and 7. (B) Western blot for ankyrin-G (upper panel) and β-tubulin (lower panel) as a loading control. Samples from left to right: primary mouse dermal endothelial cells or heart endothelial cells and primary human dermal microvascular endothelial cells or Human umbilical vein endothelial cells (HUVECs). Note that only the 210kD ankyrin-G isoform is detectable in mouse heart endothelial cells. (C) Immunofluorescence of VE-cadherin and ankyrin-G in HUVECs. Area inside white box is enlarged in last panel. (D) Colocalization of ankyrin-G with wild type (upper panels) or W2 (lower panel) cadherin mutant. Area inside white rectangle is enlarged in far right panel. Intense perinuclear signal was excluded from analysis. Bar, 20 μm. (E) Quantification of colocalization in B. y-axis: Pearson's correlation coefficient. N>20 cells/group **, P<0.01 (F) Co-immunoprecipitation of cell lysates exogenously expressing either VE-cadherin or both ankyrin-G-HA and VE-cadherin. An anti-HA antibody was used to isolate ankyrin-G-HA and western blot for VE-cadherin (upper panel) or ankyrin-G (lower panel) was performed. Cadherin proteins: wild type (WT) or non-adhesive (W2). (G) Densitometric quantification of western blot in D. N=2.

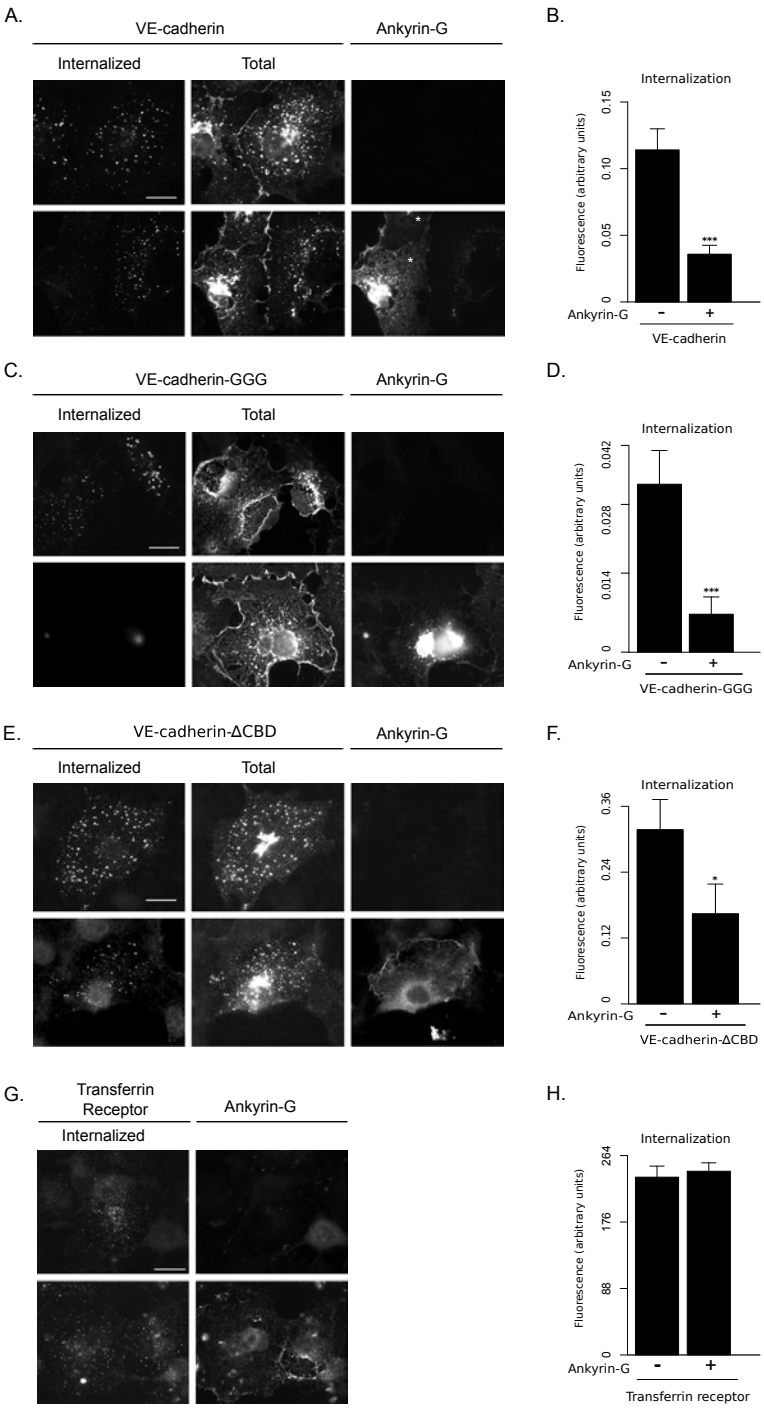


Figure 13. Ankyrin-G inhibits internalization of VE-cadherin

Endocytosis of wild type VE-cadherin (A and B), VE-cadherin-GGG (C and D), VE-

cadherin-ΔCBD (E and F), or transferrin receptor (G and H) without (upper panels) or with (lower panels) exogenous ankyrin-G expression measured with fluorescence-based internalization assay. (A) Asterisks mark cells in view expressing both VE-cadherin and ankyrin-G. (B and D) Error bars represent SEM. N=20 cells/group. ***, $P<0.001$. (F) Error bars represent SEM. N=10 cells/group. *, $P<0.05$ (H) Error bars represent SEM. N>20 cells/group. Bars, 20 μm.

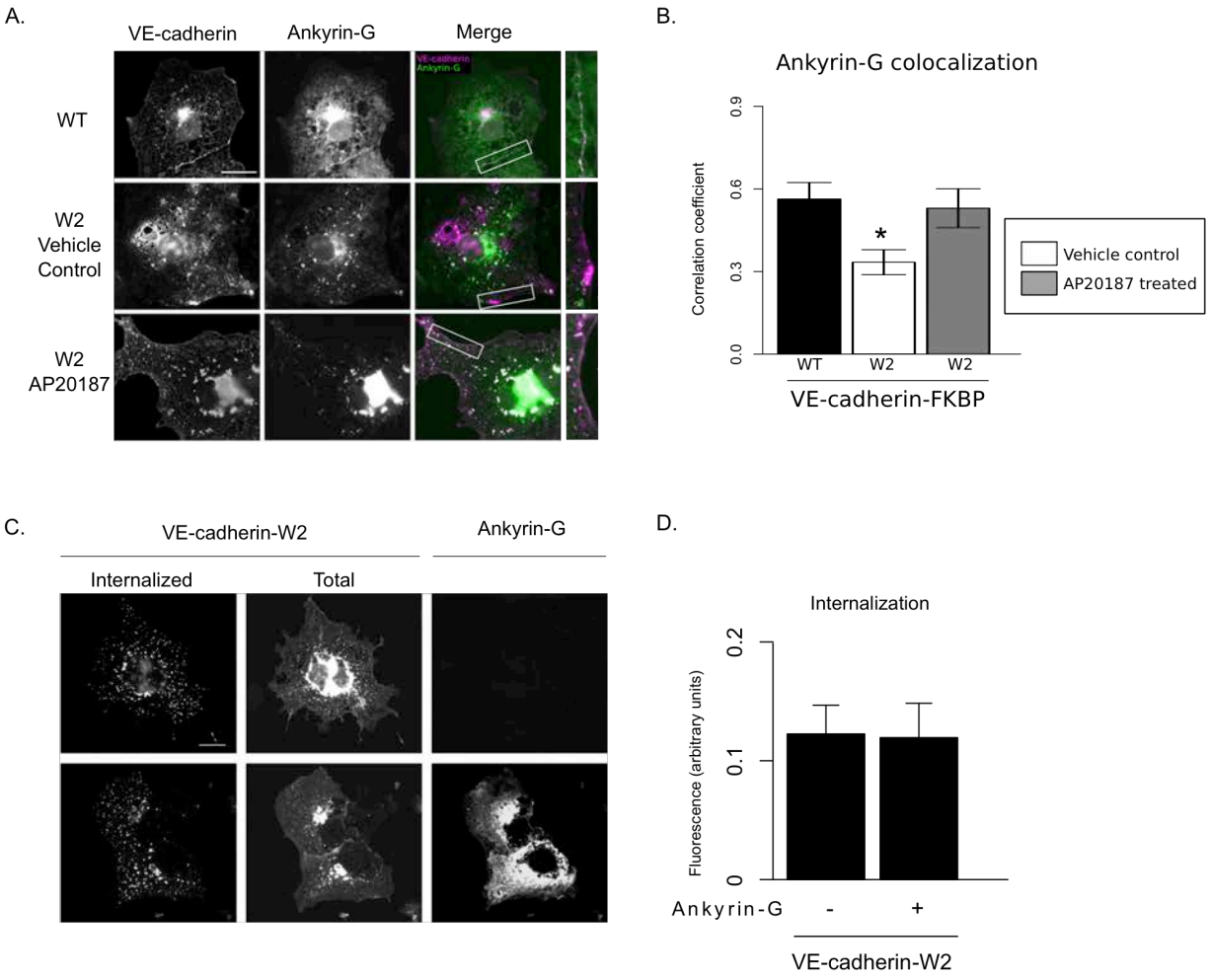


Figure 14. Ankyrin-G associates with VE-cadherin dimers and does not inhibit endocytosis of W2 mutant

(A) Colocalization of ankyrin-G with wild type VE-cadherin (upper panel), vehicle control treated W2 mutant (middle panel), or AP20187 treated W2 mutant (lower panel). Area inside white rectangle is enlarged in far right panel. Intense perinuclear signal was excluded from analysis. (B) Quantification of colocalization. y-axis: Pearson's correlation coefficient. Error bars represent SEM. N=8 cells/group. *, $P<0.05$ compared to WT. (C) Internalization assay of VE-cadherin-W2 without (upper panel) or with

(lower panel) exogenous ankyrin-G expression. (D) Quantification of internalization. Error bars represent SEM. N>15 cells/group. Bars, 20 μm.

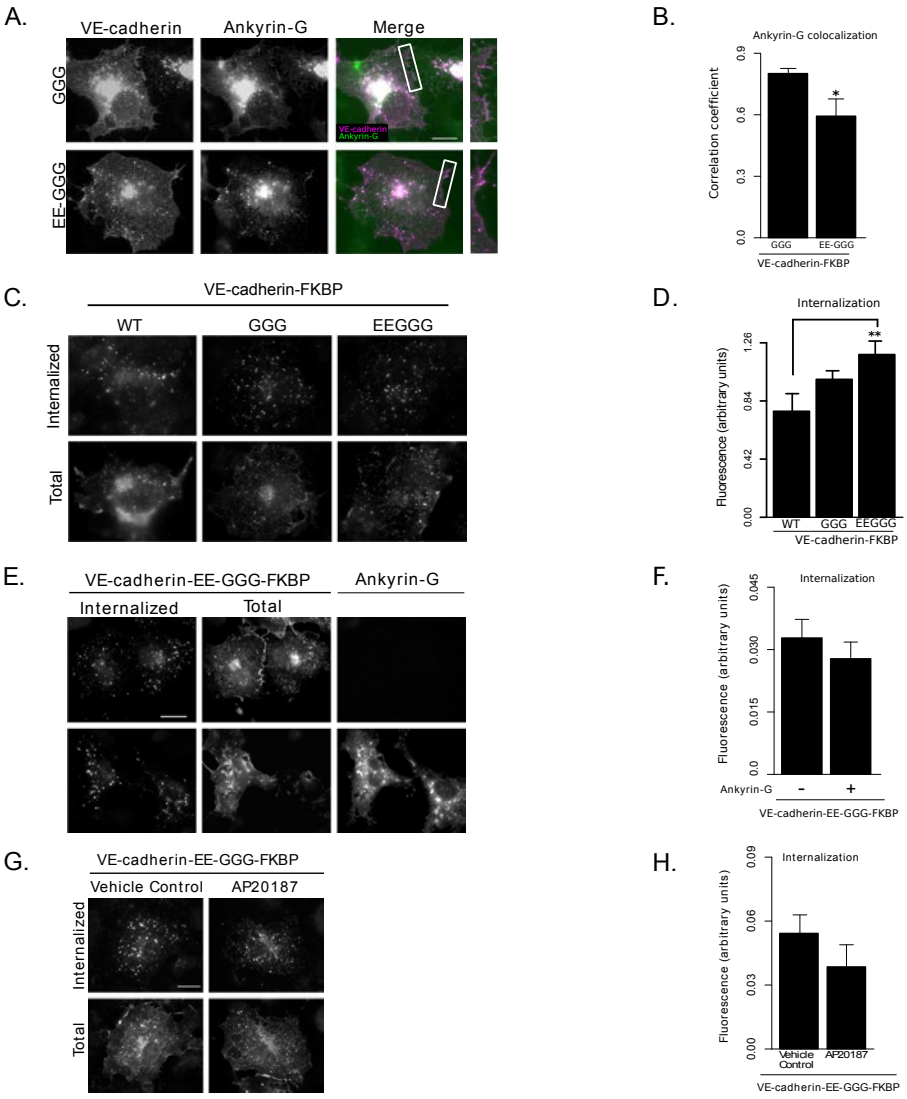


Figure 15. Ankyrin-G association is required to inhibit VE-cadherin dimer internalization

(A) Colocalization of ankyrin-G with VE-cadherin-GGG mutant (upper panel) or VE-cadherin-EE-GGG mutant (lower panel). Area inside white rectangle is enlarged in far right panel. Intense perinuclear signal was excluded from analysis. Bar, 20 μm. (B) Quantification of colocalization. y-axis: Pearson's correlation coefficient. Error bars represent SEM. N>20 cells/group. *, P<0.05 compared to VE-cadherin-GGG. (C) Internalization assay of VE-cadherin-EE-GGG-FKBP compared to VE-cadherin-WT and

VE-cadherin-GGG in COS-7 cells (D) Quantification of internalization shown in C. Error bars represent SEM. N>15 cells. **, P<0.01 compared to VE-cadherin-WT. (E) Endocytosis of VE-cadherin-EE-GGG without (upper panel) or with (lower panel) exogenous ankyrin-G expression measured with fluorescence-based internalization assay. (F) Quantification of internalization. Error bars represent SEM. N>20 cells/group. (G) Fluorescence-based internalization assay of VE-cadherin-EE-GGG-FKBP after treatment with vehicle control (left panels) or AP20187 (right panels). Bar, 20 μm. (H) Quantification of internalization. Error bars represent SEM. N>25 cell/group.

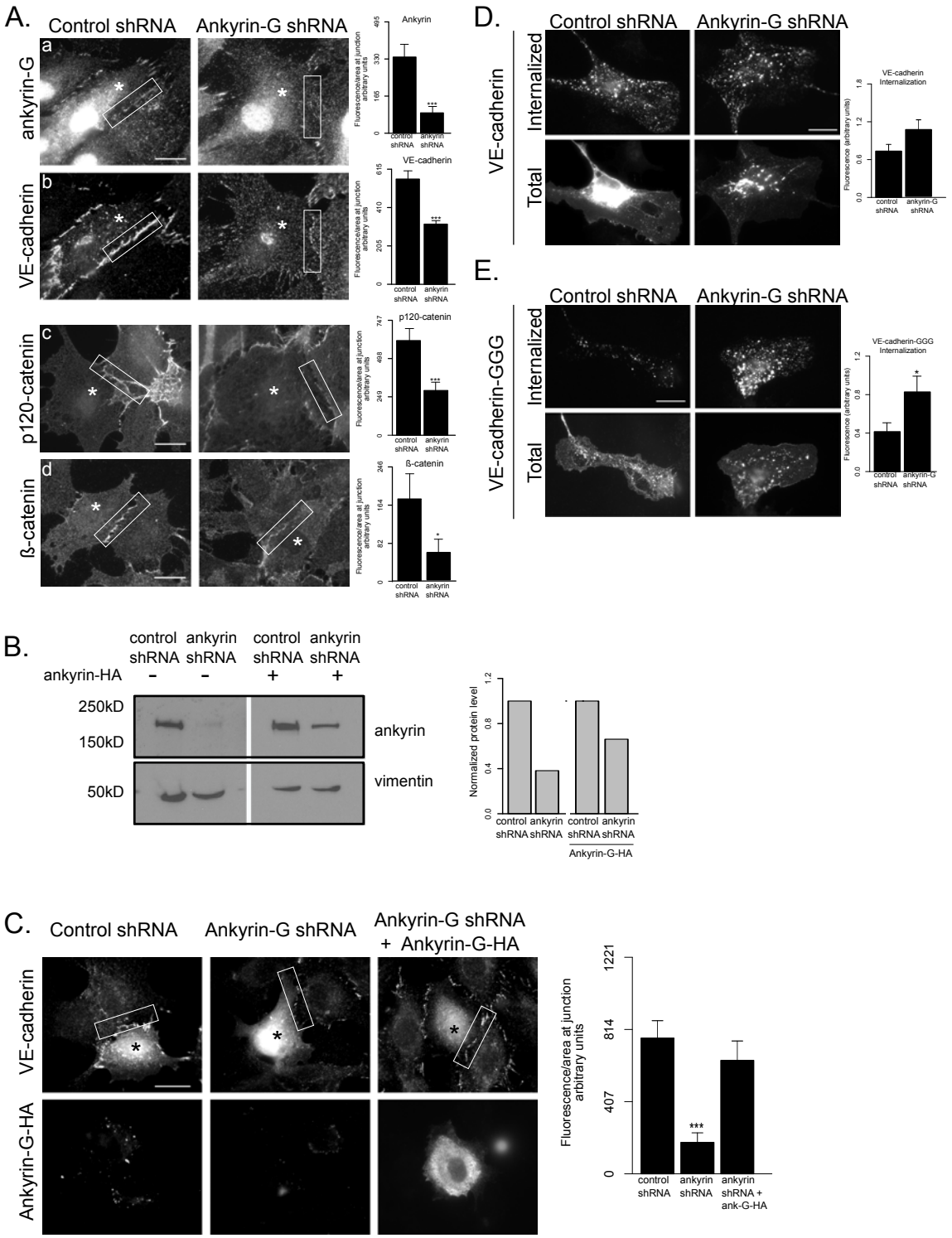


Figure 16. Ankyrin-G regulates adherens junction organization in endothelial cells

(A) Immunofluorescence of HUVECs after transfection with luciferase shRNA (control)

or ankyrin-G shRNA. Immunofluorescence of ankyrin-G (a), VE-cadherin (b), p120-catenin (c) and ß-catenin (d) after transfection with luciferase shRNA or ankyrin-G shRNA, quantification to the right. Asterisks mark transfected cells determined by mCherry expression. Rectangles highlight junctions. Bar, 20 μm. Error bars represent SEM. N>15 junctions/groups. ***, P<0.001, *, P<0.05. (B) Western blot of whole cell lysates from HUVECs after transfection with luciferase shRNA (control) or ankyrin-G shRNA. Upper panel: ankyrin-G, lower panel: vimentin loading control. Left: shRNA only. Right shRNA plus HA-tagged 190kD ankyrin-G. Quantification of protein levels normalized to luciferase shRNA control or luciferase shRNA plus HA-tagged 190kD ankyrin-G on the right. (C) Immunofluorescence of HUVECs expressing control shRNA, ankyrin-G shRNA, or ankyrin-G shRNA plus HA-tagged 190kD ankyrin-G. VE-cadherin, upper panels. Ankyrin-G-HA, lower panels. Rectangles highlight cell junctions. Asterisks mark transfected cells determined by mCherry expression. Quantification of VE-cadherin at junctions to the right. Bar, 20 μm. Error bars represent SEM. N>25 junctions/groups. ***, P<0.001. (D) Internalization of exogenously expressed VE-cadherin (wild type) in HUVECs in cells transfected with luciferase shRNA or ankyrin-G shRNA. Bar, 20 μm. Error bars represent SEM. N>25 cell/group.. (E) Internalization of exogenously expressed VE-cadherin-GGG in HUVECs in cells transfected with luciferase shRNA or ankyrin-G shRNA. Bar, 20 μm. Error bars represent SEM. N>30 cells/group. *, P<0.05.

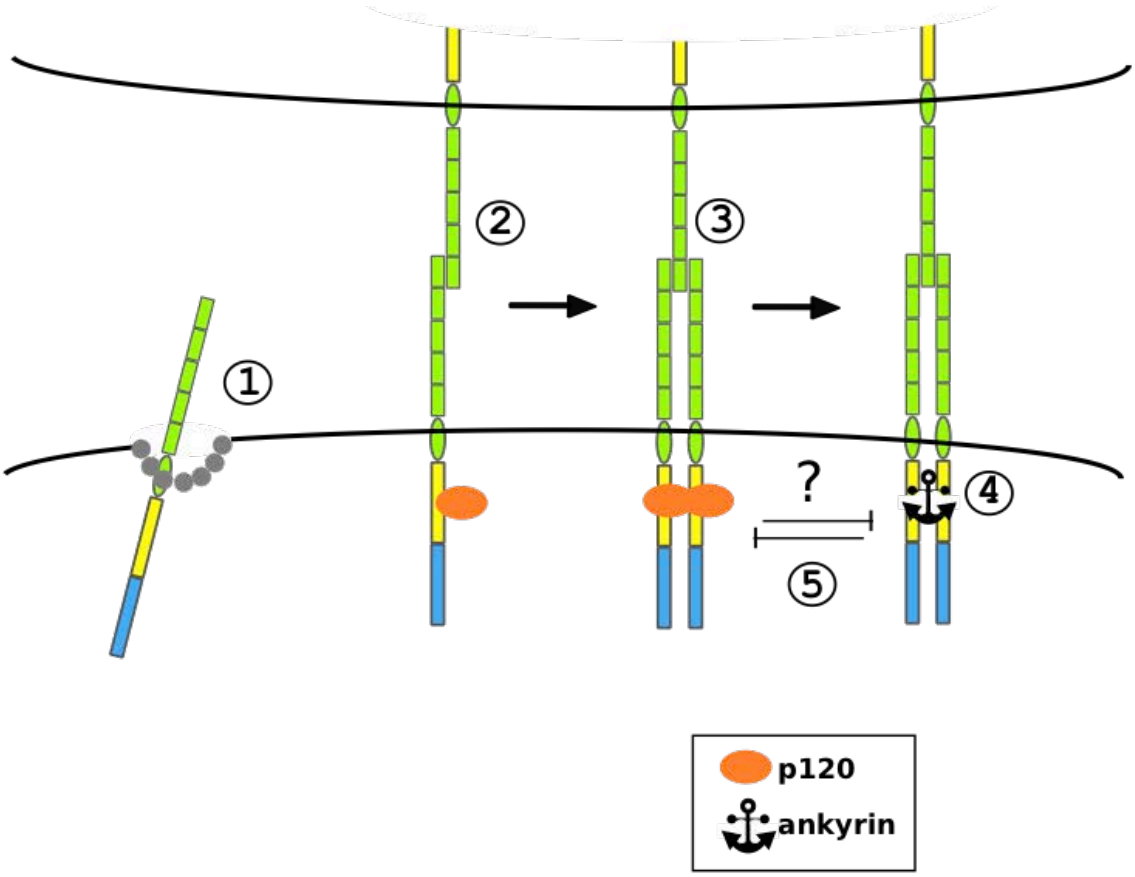


Figure 17. Model of ankyrin-G mediated inhibition of the internalization of VE-cadherin dimers

VE-cadherin monomers are internalized if not bound by p120 (1). p120 stabilizes cadherin at plasma membrane and *trans* interactions form between two VE-cadherin proteins on neighboring cells (2). After the formation of *cis* interactions p120 (2) or ankyrin-G (3) bind to and stabilize VE-cadherin. Whether p120 and ankyrin-G bind to VE-cadherin simultaneously in not known (4).

Chapter 5

Cadherin endocytosis regulates cell polarity during directed collective cell migration

5.0 Introduction

During angiogenesis, endothelial cells must coordinate adhesion and migration. One way that endothelial cell adhesion is modulated is through endocytosis of VE-cadherin. Previously, our lab reported a mutation in a cluster of acidic amino acids in the cytoplasmic tail of the cadherin (DEE646-648) in the core p120-binding domain that results in disrupted p120-binding and in inhibited endocytosis of the cadherin (Nanes *et al*., 2012). This mutation has allowed us to specifically assess the role of cadherin endocytosis in endothelial cell function without disrupting endocytosis globally. One question addressed was the role of VE-cadherin endocytosis in endothelial cell migration. Cells expressing the VE-cadherin-DEE mutant (VE-cadherin-DEE) exhibited a migration defect and were unable to close the gap created by a scratch wound in the monolayer.

An important mechanism for directed cell migration is establishing polarity. One hallmark of cell polarization during directional migration is Golgi orientation. The Golgi orients to face the direction of the wound, in front of the nucleus. Previous reports have demonstrated the requirement for E-cadherin in Golgi orientation in epithelial cells (R. A. Desai *et al*., 2009). However, the mechanism by which E-cadherin regulates Golgi orientation is not known. In addition, it is not known if VE-cadherin regulates Golgi orientation similar to E-cadherin or if cadherin endocytosis is required.

In addition to Golgi orientation, leading edge cells form filopodia and lamellipodia establishing front-rear polarity. Cadherins have been reported to localize to both of these types of protrusions (Almagro *et al*., 2010; Ehrlich *et al*., 2002; Hoelzle *et*

al., 2012; McNeill *et al*., 1993; Peglion *et al*., 2014; Vasioukhin *et al*., 2000; Vasioukhin *et al*., 2001). This non-junctional pool of cadherin is thought to be important for the initiation of new contacts between cells (Hoelzle *et al*., 2012). A recent report found that shortly after a wounding event in response to angiopoietin-1 (Ang-1), VE-cadherin was localized to the leading edge in cultured primary endothelial cells (Oubaha *et al*., 2012). However, the mechanism for leading edge accumulation of VE-cadherin is not fully understood. One possibility is cadherin treadmilling, a process that has been reported for multiple cadherin family members, including VE-cadherin. Recently it was shown for N-cadherin that during cadherin treadmilling, the lateral junctions are replenished through polarized recycling of the cadherin (Peglion *et al*., 2014). Pharmacologically inhibiting endocytosis disrupted N-cadherin treadmilling in migrating astrocytes, indicating that cadherin internalization is required for this process. We hypothesized that VE-cadherin endocytosis is required for leading edge accumulation of the cadherin.

Here, we report that VE-cadherin endocytosis modulates directional cell migration through a mechanism that involves adhesion and actin cytoskeletal linkages In addition, we found that VE-cadherin endocytosis is required for proper Golgi orientation and for leading edge accumulation of the adherens junction proteins at the migratory front. These findings reveal a novel mechanism by which cadherin endocytosis regulates cell polarity and directed cell migration.

5.1 Results

5.1.1 Cadherin endocytosis, adhesion and cytoskeletal linkages cooperate to regulate cell migration

Our lab previously determined that mutation of an acid cluster of amino acids (DEE) in the cytoplasmic tail of VE-cadherin inhibits endocytosis of the cadherin (Nanes *et al*., 2012). Furthermore, when expressed in endothelial cells and other cell types, this VE-cadherin DEE mutant inhibits cell migration. To test the hypothesis that adhesion is required for the endocytic mutant to suppress migration, we generated a non-adhesive endocytic VE-cadherin mutant, VE-cadherin-W2-DEE. This mutant combines a mutation of a conserved tryptophan in the EC1 domain that is required for adhesion with the DEE mutation. We then performed a scratch wound assay and found that cells expressing VE-cadherin-W2-DEE migrated similar to VE-cadherin-WT. This is in contrast to cells expressing VE-cadherin-DEE, which exhibited inhibited migration (Fig. 1A and B). Therefore, we conclude that adhesion is required for the VE-cadherin endocytic mutant to suppress migration.

Because linkage to the cytoskeleton is important for adhesive strength, we reasoned that disruption of cadherin linkage to the cytoskeleton might also relieve the migration suppression observed in cells expressing VE-cadherin-DEE. To test this hypothesis, we mutated the DEE sequence in the cytoplasmic tail of a VE-cadherin truncation mutant lacking the catenin-binding domain (CBD). This truncation mutant has previously been described and does not associate with β-catenin, and is not linked to the actin cytoskeleton (Navarro *et al*., 1995). To determine if the DEE mutation would inhibit the internalization of the cadherin in the absence of β-catenin binding, we performed an

internalization assay. We did not observe a difference in the internalization of VE-cadherin-CBD compared to wild type. However, we found that introduction of the DEE mutation to the truncation mutant inhibited internalization of the cadherin (Fig. 2A and B). Next, we performed a scratch wound assay and found that cells expressing VE-cadherin-CBD migrated similar to cell expressing wild type VE-cadherin (Fig. 2C and D). Importantly, we observed that cells expressing VE-cadherin-DEE-CBD also migrated similar to wild type (Fig. 2C and D). We conclude that linkage to the cytoskeleton is required for the endocytic mutant to inhibit migration.

5.1.2 VE-cadherin endocytosis is required for Golgi orientation toward the wound edge in endothelial cells

Classical cadherins have been reported to play a role in cell polarization, such as centrosome orientation, which is tightly coupled with Golgi orientation and nuclear position (R. A. Desai *et al*., 2009; Dupin *et al*., 2009). We predicted that VE-cadherin endocytosis would be required for Golgi orientation. To test this prediction, we analyzed the orientation of the Golgi in primary dermal microvascular endothelial cells (MECs) using a cis Golgi marker. Because the DEE mutation disrupts p120 binding to the cadherin in addition to inhibited endocytosis, we compared Golgi orientation of the endocytic mutant to wild type cadherin and a control cadherin with a mutation in the cytoplasmic tail that disrupts p120 binding (VE-cadherin-GGG), but that does not inhibit endocytosis of the cadherin (Nanes *et al*., 2012). Golgi was considered oriented if it was within a 90° angle (45° in either direction) from the wound edge and through the center of the nucleus (Fig. 4A). As predicted, Golgi orientation toward the wound edge was established in cell expressing wild type and VE-cadherin-GGG (Fig. 4A and B).

However, fewer cells expressing VE-cadherin-DEE had oriented their Golgi to face the wound edge (Fig. 4A and B). These data indicate that cell polarization during the initiation of migration requires cadherin endocytosis.

5.1.3 VE-cadherin endocytosis is required for leading edge localization of adherens junction proteins

N-cadherin and VE-cadherin have been shown to accumulate at the leading edge of cells during directed migration. Pharmacological inhibition of endocytosis was found to prevent N-cadherin from localizing at the leading edge of migrating cells, suggesting that cadherin endocytosis is required for this localization (Peglion *et al*., 2014). Furthermore, VE-cadherin localizes to the leading edge in response to angiopoietin I (Ang-1), though the mechanism modulating this localization has not been determined. Based on these results, it is likely that VE-cadherin endocytosis is required for leading edge localization in migrating endothelial cells. Therefore, we predicted that the leading edge accumulation would be disrupted in cells expressing VE-cadherin-DEE. To test this prediction, we treated MECs with Ang-1 and analyzed the cadherin localized to the leading edge one hour after a wounding event. We observed wild type VE-cadherin, as well as p120 and β-catenin at the wound edge (Fig. 3A and B). However, leading edge localization was disrupted in cells expressing VE-cadherin-DEE (Fig. 3A and B). We found that localization of VE-cadherin-GGG at the leading edge was similar to wild type cadherin (Fig. 3A and B). Therefore, we conclude that VE-cadherin endocytosis is required for localization of the cadherin and its catenin partners at the leading edge of cells induced to migrate in response to Ang-1.

5.2 Discussion

Our lab previously demonstrated that a mutation in the DEE sequence in the cytoplasmic tail of VE-cadherin resulted in inhibited migration when expressed in endothelial cells. This migration defect was not due to disrupted p120 binding, as cells expressing a control VE-cadherin mutant, VE-cadherin-GGG, that does not bind to p120 but undergoes endocytosis, migrated similar to cell expressing wild type VE-cadherin. The migration defect was not due to decreased mobility of the mutant cadherin with in the plasma membrane. This interpretation is based on our previous findings using FRAP analysis which revealed that the DEE mutation increased the fraction of cadherin that was mobile in the plasma membrane (Nanes *et al*., 2012). Here, we examined the interplay between adhesion or linkage of the cadherin to the cytoskeleton and cadherin endocytosis. Because collective cell migration requires the reorganization of junctions, we reasoned that disrupting adhesion or linkage to the actin cytoskeleton would restore the junctional plasticity and relieve the migration suppression caused by the endocytic VE-cadherin mutant. We found that cells expressing a VE-cadherin endocytic mutant that is unable to engage in adhesive interactions (VE-cadherin-W2-DEE) migrated similar to cells expressing wild type cadherin. In addition, cells expressing an endocytic VE-cadherin mutant that is not linked to the actin cytoskeleton through interactions with β-catenin (VE-cadherin-DEEΔCBD) migrated similar to wild type. Taken together, these data support the hypothesis that VE-cadherin endocytosis is required to facilitate plasticity of junctional pools of VE-cadherin during collective cell migration.

Interestingly, VE-cadherin endocytosis is not required for undirected single cell migration. Sparsely seeded endothelial cells expressing VE-cadherin-DEE migrated at a

rate similar to wild type cells plated at the same density (Nanes *et al*., 2012). However, whether VE-cadherin endocytosis is required for directed migration of single cell migration was not determined. Cell polarization is required for directed migration of a wide range of cell types, including cells of mesenchymal, epithelial and neuronal origin. An important mechanism of polarization for directed migration is the orientation of the microtubule-organizing center (MTOC) and the Golgi apparatus (Gomes *et al*., 2005). The MTOC and Golgi orient together, and disrupted orientation of one results in disrupted orientation of the other (Gotlieb *et al*., 1983; Kupfer *et al*., 1982; Palazzo *et al*., 2001). During this process, the nucleus moves to the rear of the cells, while the Golgi is held in place and oriented towards the free edge (Gomes *et al*., 2005). Nuclear movement is coupled to retrograde actin flow and MTOC orientation requires local activation of Cdc42, Par3, and PKCζ, members of the Par polarity complex. Both E- and N-cadherin were also reported to be critical for both of these processes (R. A. Desai *et al*., 2009; Dupin *et al*., 2009). Interestingly, Dupin et al. reported calcium dependent E- and N-cadherin contacts promote cell orientation. They found that the nucleus and centrosome have a random orientation in isolated astrocytes and JEG3 epithelial cells. In addition, a similar intracellular organization was observed after calcium depletion for both cell types (Dupin *et al*., 2009). However, the addition of calcium induced the movement of nucleus and centrosome and orientation of these organelles towards the free edge (Dupin *et al*., 2009). Because calcium depletion results in cadherin endocytosis (Ivanov *et al*., 2004), we hypothesized that VE-cadherin endocytosis would be important for Golgi orientation. We tested this hypothesis in primary endothelial cells and found that cell expressing the VE-cadherin endocytic mutant failed to orient their Golgi. This is in contrast to cells

expressing wild type VE-cadherin or VE-cadherin-GGG. Because cells expressing VE-cadherin-GGG oriented their Golgi similar to wild type, we conclude that cadherin endocytosis, and not p120 binding to the cadherin, is required for proper orientation.

A wounding event initiates the establishment front-rear polarity including the formation of protrusions, such as lamellipodia, which occurs before Golgi orientation. Cadherins are reported to accumulate at the leading edge of cells, and therefore may play a role in establishing front-rear polarity (Almagro *et al*., 2010; Ehrlich *et al*., 2002; Hoelzle *et al*., 2012; McNeill *et al*., 1993; Peglion *et al*., 2014; Vasioukhin *et al*., 2000; Vasioukhin *et al*., 2001). Using pharmological approaches, it was reported that accumulation of N-cadherin at the leading edge was prevented in cells treated with endocytosis inhibitors, supporting a model in which cadherin is internalized at the rear of the cell and recycled to the leading edge (Peglion *et al*., 2014). Here, using a mutation in the cadherin tail that selectively inhibits cadherin endocytosis, we show that endocytosis is required for leading edge accumulation of VE-cadherin and associated adherens junction proteins. Endothelial cells expressing wild type or VE-cadherin-GGG localized VE-cadherin, p120, and β-catenin to the leading edge, while cells expressing the endocytic VE-cadherin mutant had very little accumulation of these proteins. We propose a model in which leading edge accumulation of VE-cadherin is required to establish front-rear polarity, which in turn directs the orientation of cell. Ongoing studies are directed at testing the role of cadherin endocytosis and junctional plasticity in the context of vascular development and disease.

5.4 Materials and Methods

Cell Culture

Human dermal microvascular endothelial cells were cultured in Endothelial Growth Medium 2 Microvascular (Lonza). Human umbilical vein endothelial cells were cultured in M199 (Mediatech, Inc.) supplemented with 20% fetal bovine serum (FBS), and 1% pen/strep on gelatin-coated plates. African green monkey kidney fibroblast-like (COS-7) (American Type Culture Collection, ATCC) and human embryonic kidney (HEK) QBI-293A cell lines (MP Biomedicals) were cultured as previously described (Nanes *et al*., 2012).

Generation of VE-cadherin cDNA constructs

Site directed mutagenesis of VE-cadherin was used to generate the VE-cadherin-W2-DEE using previously described primers ((Nanes *et al*., 2012). The W2 mutation was introduced by site directed mutagenesis into a VE-cadherin truncation mutant, a generous gift from E. Dejana, (Navarro *et al*., 1995) lacking the C-terminal catenin-binding domain.

Virus production

To generate an adenoviral expression system for protein expression in mammalian cells, the gene of interest was cloned into the Gateway pAd/CMV/V5-DEST vector (Invitrogen). The vector was linearized using PacI and transfected into HEK QBI293 cells to produce virus. After several rounds of infection, cells were lysed and virus was harvested.

Migration assay

Migration of MECs or COS-7 cells expressing wild type or VE-cadherin mutants was

measured using a scratch wound assay. Briefly, cells were plated on gelatin-coated coverslips at confluency, and then scratched with a pipette tip. Migration distance was measured over time using a bright-field microscope (DM IL; Leica) equipped with a 5x/0.12 NA objective and a camera (DFC420 C; Leica). Images were acquired using FireCam software (version 3.4; Leica).

Internalization assay

Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the protocol provided by the supplier. Internalization assays were performed as previously described (Chiasson *et al*., 2009; Xiao, Allison, Buckley*, et al*., 2003). Briefly, cells cultured on glass coverslips were incubated with an antibody against the VE-cadherin extracellular domain in media for 30 minutes at 4°C. Cells were washed 3 times with cold PBS to remove unbound antibody. To allow internalization, cells were incubated in prewarmed media for 30 minutes for VE-cadherin, or 5 minutes for transferrin receptor, at 37°C. Cells were returned to cold media. A low pH buffer (PBS with 100 mM glycine, 20 mM magnesium acetate, and 50 mM potassium chloride, pH 2.2) was used to remove any remaining antibody from the cell surface. Cells were then fixed and permeabilized by incubating in 4% paraformaldehyde for 10 minutes followed by 0.1% Triton X-100 for 8 minutes at room temperature. Chicken anti-myc antibody (Bethyl labs) was used to determine the total cadherin pool. Secondary antibodies conjugated to fluorescent dyes (Alexa Fluor 488, 555, or 647 nm; Life Technologies) were used to visualize antibody binding. For each cell, internalization was quantified as the ratio of fluorescence signals corresponding to the internalized and total cadherin pools.

Leading edge localization and Golgi orientation assay

MECs expressing RFP-tagged wild type or VE-cadherin mutants were plated on gelatin-coated coverslips and grown to confluency. Cells were serum starved overnight. Cells were scratched with a pipette tip and media was changed.

For leading edge localization, media was supplemented with 100ng/ml VEGF peptide (PeproTech) or 100ng/ml recombinant Angiopoietin-1 (R&D Systems) with 10% FBS. After one hour, the cells were then fixed and permeabilized by incubating in 4% paraformaldehyde for 10 minutes followed by 0.1% Triton X-100 for 8 minutes at room temperature. Rabbit Anti-RFP (Molecular Probes) was used to detect VE-cadherin. Hoescht dye was used to visualize nucleus.

For Golgi orientation assay, six hours after scratch, cells were fixed and permeabilized as described above. GM190 (TransLabs), Hoescht, and Rabbit Anti-RFP were used to detect Golgi, nucleus, and VE-cadherin, respectively. Golgi orientation was quantified by measuring the angle from the nucleus to the wound edge that contained the Golgi. The Golgi was considered oriented towards the wound edge if it was within a 90° angle (45° in either direction) from the wound edge and through the center of the nucleus.

Microscopy

Microscopy was performed using an epifluorescence microscope (DMRXA2, Leica) equipped with 40X, 63X, and 100X 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).

Image analysis and statistics

ImageJ software (version 1.5) was used for all image analysis (Schneider *et al*., 2012). Custom ImageJ plugins were used to automate data quantification. R was used to compute statistics. Kruskal-Wallis rank sum test followed by Dunn's method for multiple comparisons was used for scaled data. One-way ANOVA with Tukey's Post Hoc test was used to evaluate scratch wound assays.

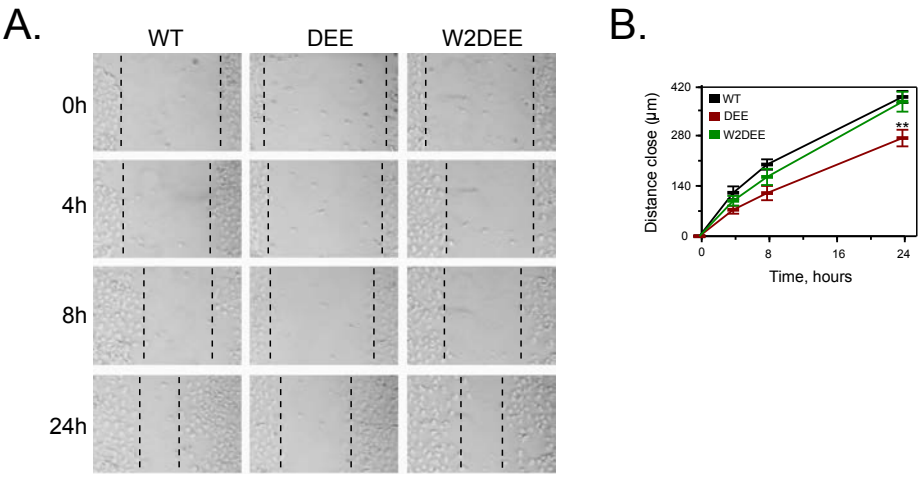


Figure 18. VE-cadherin endocytic mutant requires adhesion to suppress migration

A) A confluent monolayer of Cos-7 cells expressing either wild type VE-cadherin (WT), VE-cadherin endocytic mutant (DEE), or VE-cadherin endocytic and adhesive mutant (W2DEE) was scratched with a pipette tip. Migration into the wound was measured over 24 hours. B) Quantification of scratch wound assay shown in A. Error bars represent SEM. N = 8 wounds/groups. **, P< 0.01 compared to WT.

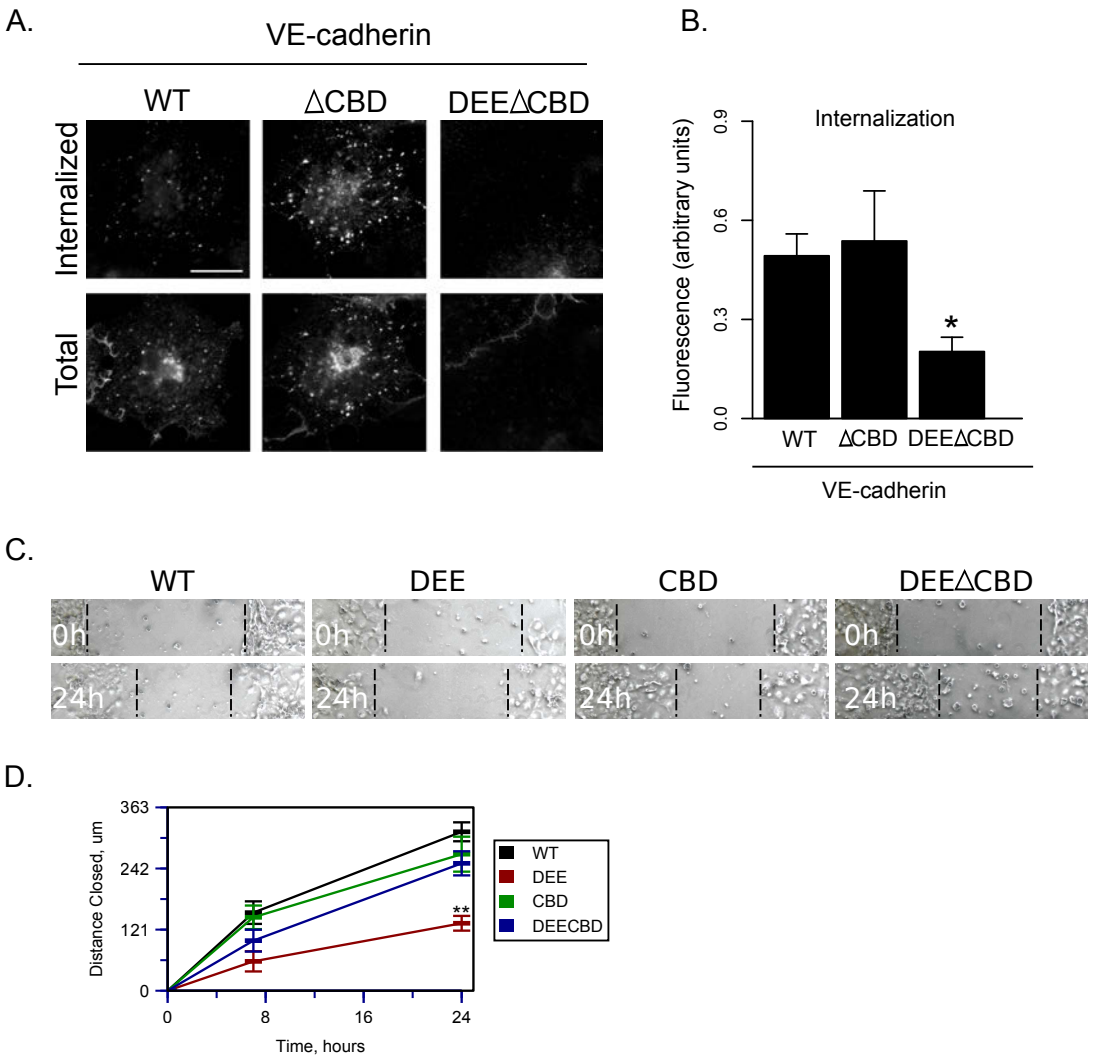


Figure. 19 VE-cadherin endocytic mutant requires catenin-binding domain to suppress migration

A) Internalization of wild type VE-cadherin (WT), VE-cadherin truncation mutant lacking the catenin-binding domain (ΔCBD), or VE-cadherin endocytic mutant lacking the catenin binding domain (DEEΔCBD). Top panel, internalized VE-cadherin. Lower panel, total VE-cadherin. Bar, 20μM. B) Quantification of internalization assay in A. Internalized VE-cadherin was normalized to total. Error bars represent SEM. N>20 cells/group. *, P<0.05. C) Scratch wound assay of Cos-7 cells expressing either WT,

DEE, ΔCBD, or DEEΔCBD VE-cadherin. Migration into the wound was measured over 24 hours. D) Quantification of scratch wound in C. Error bars represent SEM. N = 8 wounds/group. **, P<0.01 compared to WT.

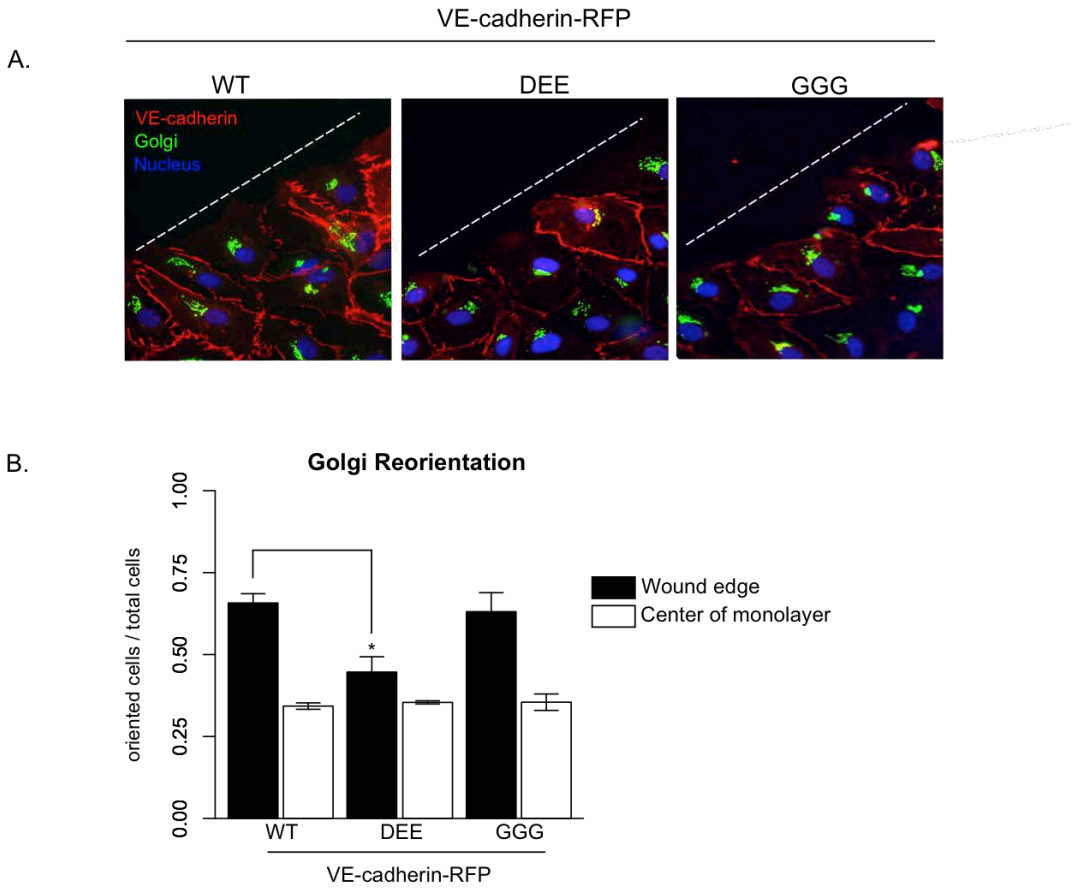


Figure 20. VE-cadherin endocytosis is required for Golgi orientation

A) Immunofluorescence of endothelial cells expressing wild type VE-cadherin-RFP (WT), VE-cadherin-DEE-RFP, or VE-cadherin-GGG-RFP were scratched with a pipette tip. Six hours after scratch, oriented Golgi were quantified. B) Quantification of oriented Golgi at wound edge and center of monolayer. Golgi was considered oriented if it was within a 90° angle (45° in either direction) from the wound edge and through the center of the nucleus. White dashed line represents leading edge. Graph represents average of three independent replicates. Error bar represents SEM. N> 45 cells/group/replicate. *, $P<0.05$ compared to cells expressing VE-cadherin-RFP at the leading edge.

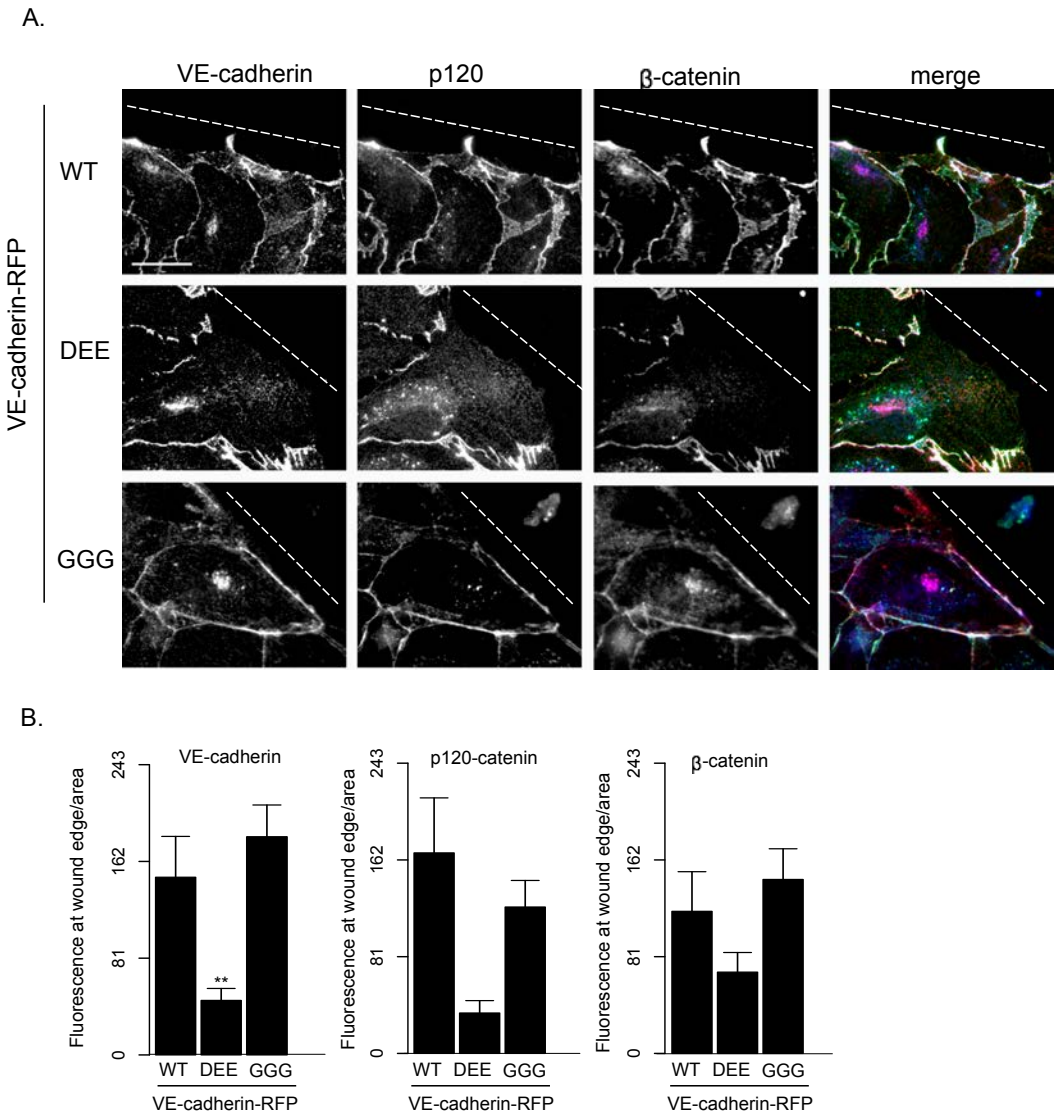


Figure 21. Endocytosis is required for leading edge accumulation of VE-cadherin

A) Immunofluorescence of endothelial cells expressing wild type VE-cadherin, VE-cadherin-DEE, or VE-cadherin-GGG were serum starved over night. The monolayer was scratched with a pipette tip and media was changed to media supplemented with angiopoietin-1 (Ang-1). Cells were imaged one hour after scratch. White dashed line represents wound edge. Bar, 20μM. B) Quantification of Ang-1 induced leading edge accumulation of VE-cadherin, p120, and β-catenin. Fluorescence at wound edge was

normalized to area. Error bars represent SEM. VE-cadherin, N>30 cells. p120 and β-catenin, N>10 cells. **, P<0.01 compared to WT.

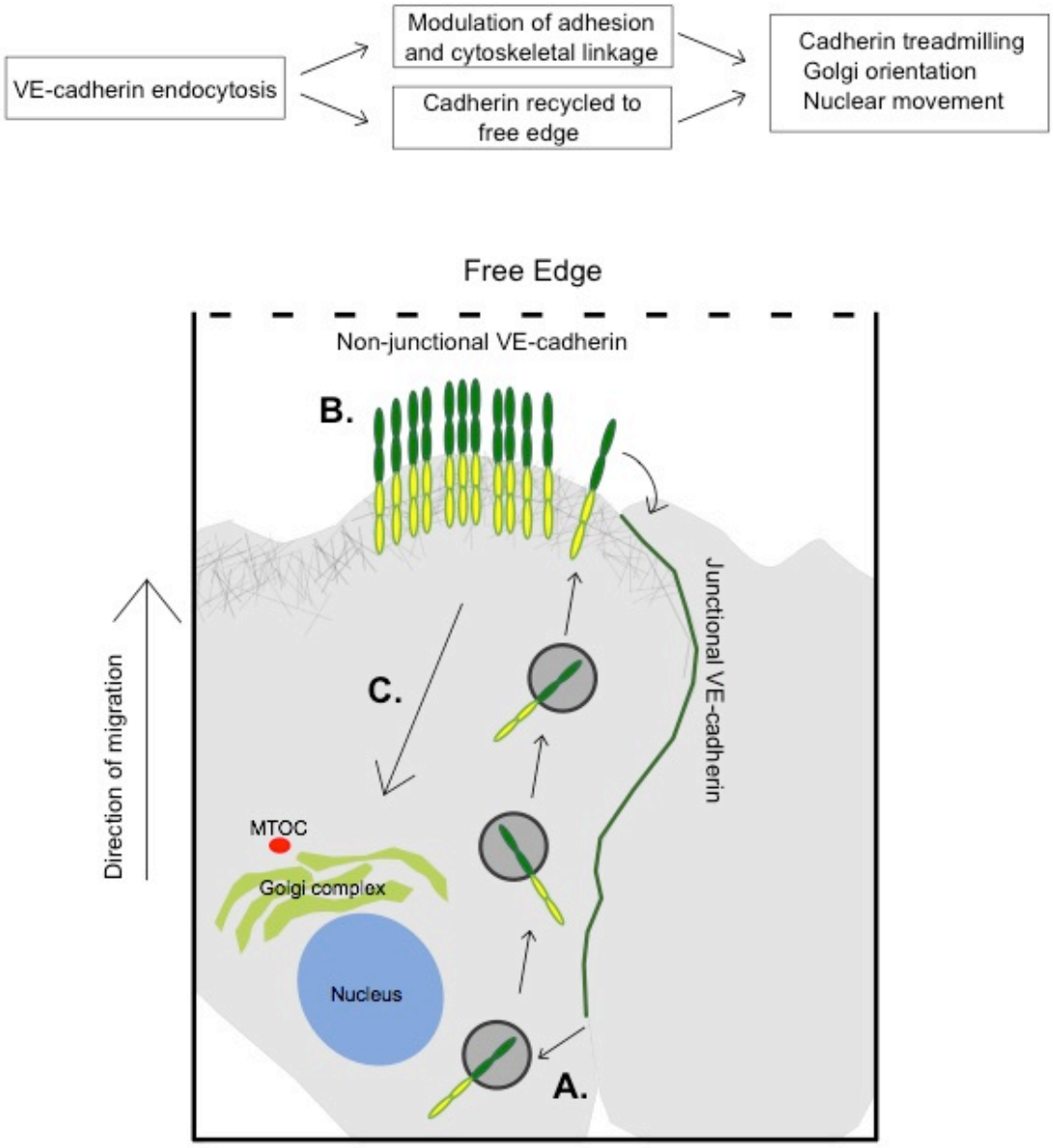


Figure 22. Model of VE-cadherin endocytosis during directed, collective cell migration

A) VE-cadherin internalization enables the modulation of adhesion and cytoskeletal linkage, and replenishes lateral junctions through polarized recycling of the cadherin to the free edge. B) Non-junctional, leading edge VE-cadherin can be incorporated into lateral junctions to facilitate cadherin treadmilling. C) Non-junctional cadherin marks the

leading edge and induces Golgi orientation towards the free surface.

Chapter 6

Dissertation summary and future directions

6.0 Dissertation Summary

The balance between junctional plasticity and maintenance of vascular integrity must be tightly coordinated for normal endothelial cell functions during developmental angiogenesis and for adult tissue homeostasis. The loss of endothelial cell function is associated with many vascular disorders. Hallmarks of these disorders include vascular leakage, inflammation, and inappropriate neovascularization. The overall objective of this dissertation was to define mechanisms that regulate cadherin endocytosis and to understand how cadherin endocytosis regulates endothelial cell function during angiogenesis. Two specific questions guided the approach to the overall objective: 1) How do VE-cadherin homophilic adhesive interactions regulate cadherin endocytosis? 2) How do adhesion and endocytosis regulate collective cell migration? These questions were addressed in chapters 4 and 5. In chapter 4, I presented data demonstrating that cadherin cis dimerization downstream of adhesion inhibits endocytosis through association with the cytoskeletal adapter protein, ankyrin-G. Moreover, in chapter 5 I presented findings that show that VE-cadherin endocytosis regulates Golgi orientation and leading edge accumulation of the cadherin and that VE-cadherin endocytosis regulates collective cell migration through a mechanism that involves adhesion and linkage to the cytoskeleton. Thus, through the work in this dissertation, a model of the relationship between VE-cadherin endocytosis and adhesive interactions and the role of VE-cadherin endocytosis during collective endothelial cell migration emerges (Figure 23). However, these insights have led to additional questions that will be important for a deeper understanding of the role of VE-cadherin endocytosis in endothelial cell functions

during vascular development and angiogenesis. I will discuss these questions in detail below.

6.1 Future Directions

What are the cellular pathways that modulate ankyrin regulation of VE-cadherin endocytosis?

Based on the assumption that ankyrin-G directly binds to VE-cadherin and that binding of ankyrin-G is mutually exclusive to p120 binding, many questions emerge regarding the cellular pathways that regulate ankyrin-G binding to the cadherin. Although, it remains to be determined if ankyrin binds directly to VE-cadherin, it has been reported that ankyrin-G binds directly to E-cadherin *(P. M. Jenkins et al., 2013)*. Furthermore, mutation of residues predicted to be important for ankyrin-G binding based on sequence homology to E-cadherin resulted in decreased association between ankyrin-G and VE-cadherin (Figure 12). Therefore, it seems likely that ankyrin-G directly binds to VE-cadherin. A cell free in vitro binding assay, using purified GST-tagged VE-cadherin and purified 210/190kD isoforms of ankyrin-G, followed by a GST-pull down assay and western blot for ankyrin-G could be used to determine if there is a direct interaction. In addition, it is not known if the binding of p120 and ankyrin-G to the cadherin is mutually exclusive. However, given the mass of both proteins and the shared binding region in the core p120-binding domain, it is unlikely that both proteins bind to the cadherin simultaneously. This could be determined using the cell free in vitro binding assay previously described to perform a competitive binding assay between purified p120 and purified ankyrin-G (Kizhatil *et al.*, 2007). I would predict that as the amount of one of the cadherin-binding partners was increased, there would be a concomitant decrease in

amount of the other bound to the cadherin.

Determining the molecular mechanisms that regulate ankyrin binding to VE-cadherin is an important pursuit. In addition to providing insights into endocytic regulation of the cadherin, the findings may contribute to another important question in the field: what regulates p120 binding to the cadherin? In chapter 4, I present data that suggests that ankyrin binds to cadherin dimers. First, induced cis dimerization potently inhibits internalization of the cadherin (Figure 10). Next, ankyrin association with VE-cadherin is increased upon cis dimerization of the cadherin (Figure 14). Ankyrin-G does not associate with or inhibit endocytosis of a mutant cadherin that does not engage in adhesive interactions, and therefore most likely does not engage in cis interactions (Figure 14). Finally, selectively disrupting ankyrin association with cis-dimerized cadherin results in endocytosis of the dimers (Figure 15). Taken together, these data suggest that ankyrin-G associates with dimerized cadherin, but does not dimerize the cadherin itself. That ankyrin-G selectively associates cadherin dimers, while p120 can bind to both cadherin monomers or dimers may provide a clue to the function of ankyrin-G binding. Given the specific role for ankyrin-G in epithelial cells, it is likely that ankyrin has a similar function in endothelial cells. Ankyrin binds to E-cadherin and in cooperation with clathrin, retains the cadherin at the lateral membrane of polarized epithelial cells (P. M. Jenkins *et al*., 2013). Similar to epithelial cells, endothelial cells are also polarized in that their apical domain faces the vessel lumen while their basal domain is attached to the basement membrane (Kissa *et al*., 2010). Additionally, endothelial cells elongate and orient their MTOC in response to sheer flow, establishing a type of planar polarity (Tzima *et al*., 2003). It is possible that different pools of cadherin are stabilized

by either p120 or ankyrin-G to establish or maintain polarity. For example, ankyrin-G and p120 may inhibit the endocytosis of different pools of VE-cadherin at different membrane domains, such as at apical or basal domains or at junctional or non-junctional regions of the plasma membrane. Though different pools of cadherin bound to either p120 or ankyrin-G were not detected in this work, it is possible that difference pools could be detected upon the establishment of apical-basal polarity in response to sheer flow or by using high-resolution microscopy, such as Structure Illumination Microscopy (SIM). The high-resolution capability of SIM may allow the detection of different pools of cadherin bound to p120 and ankyrin-G within close proximity to each other. The localization of these pools may provide a clue to the function of this additional mechanism for the regulation of VE-cadherin endocytosis.

Functional studies may also provide insight into the cellular pathways that result in ankyrin-G regulation of VE-cadherin endocytosis. In chapter 4, I presented data demonstrating that ankyrin-G is required for proper organization of junctional proteins, including VE-cadherin (Figure 16). Given that result, I would speculate that ankyrin-G is important for the regulation of endothelial adherens junction proteins to maintain barrier function. I would predict increased permeability of a monolayer of cells after ankyrin-G depletion. This prediction could be tested using a transwell permeability assay of a cell line expressing an inducible shRNA against the 210/190kD isoforms of ankyrin-G. However, if permeability of the monolayer is not increased after ankyrin-G knockdown, it may suggest that ankyrin-G is less important for maintaining junctions and instead important for the formation of new junctions. This conclusion is based on the observation that while ankyrin-G knock down in endothelial cells resulted in loss of junctional VE-

cadherin, the cells were not a confluent monolayer after transfection of the shRNA (not shown). Consequently, the loss of junctional organization could be due to the disruption of existing junctions or the inability of the cells to form new junction (or both). Therefore, either result from the permeability assay would be interesting and could provide insight into the functional outcome of ankyrin-G regulation of VE-cadherin endocytosis.

While many questions remain, the identification of ankyrin in addition to p120 as a modulator of cadherin trafficking suggests that the cadherin juxtamembrane domain may interact with a number of different proteins in different cellular contexts to regulate cadherin levels at the plasma membrane. It is likely that additional binding partners for this cadherin domain will emerge as regulators of cadherin trafficking, perhaps both to and from the plasma membrane.

What are the cellular pathways downstream of VE-cadherin endocytosis that result in proper cell polarity during directed collective cell migration?

In chapter 5, I reported that inhibited VE-cadherin endocytosis results in disrupted leading edge accumulation of the cadherin (Figure 21) and Golgi orientation toward the wound edge (Figure 20). A scratch wound induced leading edge accumulation of VE-cadherin temporally upstream (within an hour) of Golgi orientation, which occurred by six hours post wounding. However, it is not known if Golgi orientation is dependent upon leading edge accumulation of cadherin-catenin complexes. Furthermore, it is not known if the migration defect conferred by the VE-cadherin endocytic mutant is the result of loss of leading edge polarity and Golgi orientation. One hypothesis is that the leading edge

accumulation of VE-cadherin through polarized endocytosis and recycling contributes to establishing leading edge polarity, which in turn establishes proper Golgi orientation. If this hypothesis is correct, then the next question is: what molecules direct the polarized endocytosis and recycling of VE-cadherin? One intriguing possibility is members of the Par polarity proteins, which have been shown to associate with VE-cadherin at junctions (Iden *et al*., 2006). In addition, the Par proteins localize to the leading edge and colocalize with adherens junction proteins, including β-catenin (Oubaha *et al*., 2012). Par6 binds to the juxtamembrane of the cadherin tail, most likely in the absence of p120, which could potentially signal for cadherin endocytosis. One hypothesis is that VE-cadherin and Par proteins traffic to the leading edge together. Performing a localization assay for the Par proteins in cells expressing either wild type VE-cadherin or VE-cadherin-DEE could determine if cadherin endocytosis is required for localization of Par proteins to the leading edge. In addition, depletion of the Par proteins may disrupt cadherin trafficking to the leading edge. Therefore, it would be worthwhile to knock down Par6 with siRNA, which has been reported to be effective in endothelial cells (Koh *et al*., 2008), with the prediction that if Par proteins are required, the cadherin would not traffic to the leading edge. However, the depletion of Par6 will likely disrupt other Par polarity protein functions. Therefore, if disrupted cadherin trafficking to the leading edge were observed, selectively disrupting the interaction between Par6 and VE-cadherin would be an important experiment. Since Par6 binds to VE-cadherin in the juxtamembrane domain, mutations to the cadherin may disrupt p120. Consequently, a better choice would be to mutate residues in the cadherin-binding region of Par6 to uncouple the interaction.

Because the disruption of Golgi orientation does not affect the rate of migration in other cells types, such as kidney epithelial cells, it is unlikely that disruption of polarity (Golgi orientation and leading edge accumulation of the cadherin) through inhibited VE-cadherin endocytosis is alone responsible for the migration defect observed. Indeed, in chapter 4, I report that disrupting adhesion or cytoskeletal linkage enable cells expressing the endocytic mutant to migrate. It is possible that disrupting these interactions allows the cells to migrate autonomously, but without directionality due to the loss polarity. In this case, the migration rate would not be affected; cells would migrate in random directions, but still fill in the wound. Support for this idea comes from a report by Desai and colleagues that found disruption of E-cadherin-mediated contact inhibited scrape-wound-induced cell orientation, including nuclear position and polarized lamellipodia ruffling, but did not disrupt migration rate (R. A. Desai *et al*., 2009). Though these cells had lost orientation, they migrated to fill in the wound similar to control cells. Further evidence to support this idea is the observation that cells expressing VE-cadherin-DEE migrate at a similar rate as cells expressing wild type cadherin plated at the same density during undirected, single cell migration (Nanes *et al*., 2012). One way to test if disrupting adhesion relieves the migration defect caused by the endocytic mutant by allowing cells to migrate autonomously is by live tracking of the cells expressing either wild type VE-cadherin-RFP, VE-cadherin-DEE-RFP, or VE-cadherin-W2-DEE-RFP after a wounding event and comparing the number of single migrating cells for each group. My prediction is that there would be a significant increase in single cell migration into the wound in cells expressing VE-cadherin-W2-DEE versus VE-cadherin-DEE. In addition, it would be interesting to know if VE-cadherin-DEE acts as a dominant negative to disrupt cell

migration of cells expressing wild-type VE-cadherin. Live imaging of a scratch wound assay of a mixed population of cells expressing wild-type VE-cadherin with one fluorescent tag and cells expressing VE-cadherin-DEE with a different fluorescent tag would address this question.

Finally, it would be interesting to determine the role of cadherin endocytosis on migration independent of Golgi orientation or leading edge accumulation of VE-cadherin. One way to separate leading edge cadherin accumulation or Golgi orientation from migration is to inhibit VE-cadherin endocytosis after the polarizing events. VE-cadherin-FKBP endocytosis can be inhibited using the dimerizing molecule AP20187. Endocytosis could be inhibited one hour after a scratch wound to allow leading edge accumulation to occur. Alternatively, endocytosis could be inhibited six hours after a scratch wound to allow both leading edge accumulation and Golgi orientation to occur. Then, the distance closed and directional persistence over time could be measured and compared to control cells that did not undergo the polarizing steps. This kind of approach will allow us to inhibit cadherin endocytosis with high temporal precision to determine which steps in endothelial cell polarization and migration are dependent on cadherin endocytosis. Current studies in the lab are focused on addressing these important questions to provide insight into the role of VE-cadherin endocytosis in directed, collective endothelial cell migration.

What is the role of VE-cadherin endocytosis in vascular development?

Gene ablation experiments in mouse models indicate that VE-cadherin is dispensable for initial vascularization (Carmeliet *et al*., 1999). However, it is essential for

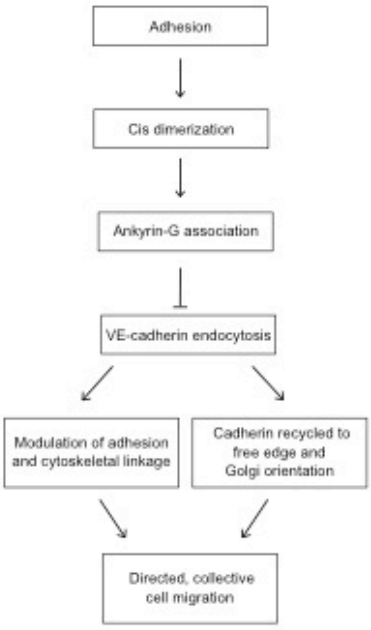
angiogenic processes after the formation of the primary vascular plexus (Carmeliet *et al*., 1999). VE-cadherin plays an important role in cell survival, migration, and regulating VEGF signaling (Carmeliet *et al*., 1999). As discussed above and shown in this dissertation, junctional plasticity is required for effective collective migration. The VE-cadherin endocytic mutant most likely disrupts the ability of cells to modulate their junctions. In addition, inhibited VE-cadherin endocytosis is likely to affect the internalization and subsequent downstream signaling of VEGFR in response to VEGF. One prediction is that knock-in mice expressing in which the endogenous VE-cadherin gene is replaced with the VE-cadherin-DEE mutation will exhibit defects in angiogenesis, with the prediction that the phenotype will result in embryonic lethality due to insufficient angiogenesis. However, it is possible that VE-cadherin-DEE knock-in mice will survive. In this case, it is possible that a phenotype will present itself upon injury or exposure to a pathogen. VEGF regulates VE-cadherin endocytosis to induce vessel permeability during wound healing. If the DEE mutant VE-cadherin is not internalized in response to VEGF, wound healing may be affected. In addition, inhibited VE-cadherin endocytosis may disrupt leukocyte transmigration resulting an ineffective immune response.

Another interesting question is how does VE-cadherin that does not bind to p120 and is therefore internalized more rapidly affect vascular development? The mutation of three glycine residues (GGG) in the core p120-binding region of the cadherin tail disrupts p120 binding and increases VE-cadherin internalization. However, cells expressing this mutant polarize and migrate similar to wild type. Therefore it appears that the VE-cadherin-GGG mutant does not disrupt junctional plasticity. However, it is possible that

because it is internalized rapidly, VE-cadherin-GGG may disrupt VEGF signaling. If the cadherin is unable to stabilize the receptor at the surface, which is required in quiescent endothelial cells, it may result in continuous downstream signaling. This could impact cell survival, proliferation, and migration *in vivo*, resulting in aberrant angiogenesis. Our lab is currently generating mice harboring either the DEE mutation or the GGG mutation in the cytoplasmic tail of VE-cadherin. These knock-in mice will provide valuable insight into the role of VE-cadherin during vascular development and angiogenesis.

The work in this dissertation has made important contributions to understanding the role of VE-cadherin endocytosis during angiogenic processes, such as cell migration, and to determining regulators of cadherin stability at the cell surface. This work includes evidence of a novel interaction between a cytoskeletal adaptor protein and dimerized cadherin that may have implications for establishing and maintaining endothelial cell polarity, which is important for vessel function. Further, this work provides a new understanding of the dynamic relationship between adhesion and VE-cadherin endocytosis during directed, collective cell migration, processes that are essential for development and homeostasis. Understanding the mechanisms that regulate VE-cadherin endocytosis is key to understanding vascular development and elucidating the pathways that lead to vascular disease.

A.


Adhesion
Cis dimerization
Ankyrin-G association
VE-cadherin endocytosis
Modulation of adhesion and cytoskeletal linkage
Cadherin recycled to free edge and Golgi orientation
Directed, collective cell migration

B.

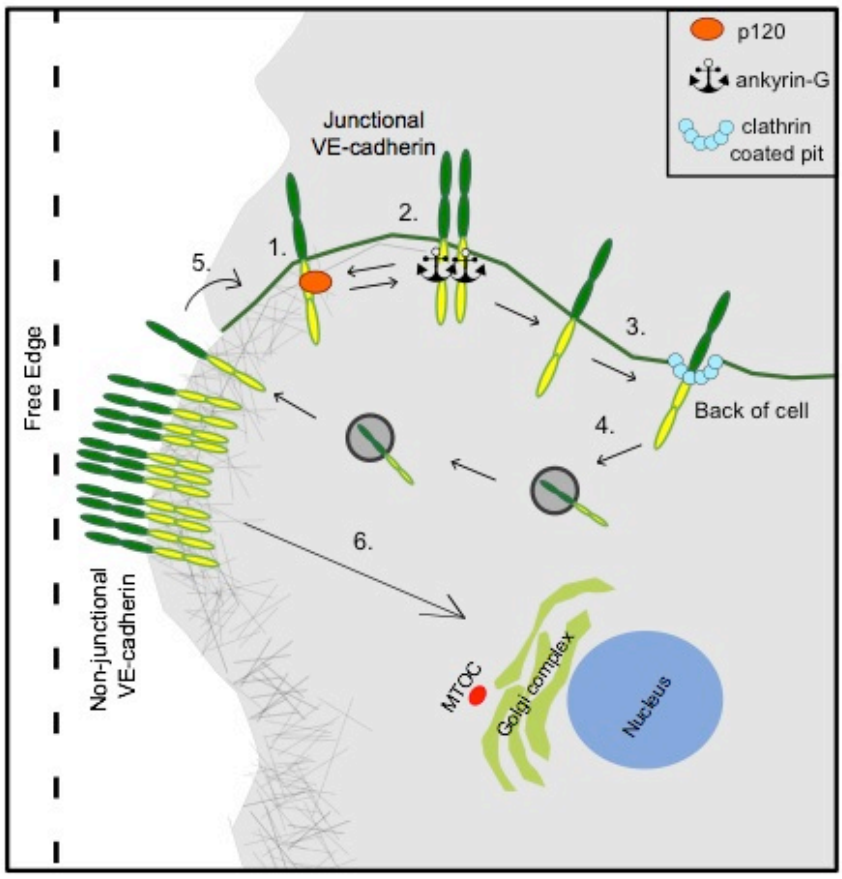

p120
ankyrin-G
clathrin coated pit
Junctional VE-cadherin
1.
2.
3.
4.
5.
6.
Free Edge
Back of cell
Non-junctional VE-cadherin
MTOC
Golgi complex
Nucleus

Figure 23. Summary and model

(A) VE-cadherin adhesion occurs upstream of cis dimerization. Ankyrin-G binds to VE-cadherin cis dimers. Ankryin-G binding inhibits VE-cadherin endocytosis. VE-cadherin endocytosis is required for modulation of adhesion and cytoskeletal linkage, Golgi orientation and cadherin accumulation at the free edge, and for directed collective cell migration. (B) (1). p120 stabilizes cadherin at plasma membrane and *trans* interactions form between two VE-cadherin proteins on neighboring cells (not depicted) (2). After the formation of *cis* interactions p120 (2) or ankyrin-G (3) bind to and stabilize VE-cadherin. (3) VE-cadherin monomers are internalized if not bound by p120. (4) Cadherin from the back of the cell is recycled to the free edge. (5) Non-junctional cadherin accumulates at the free edge and can be incorporated into the front of the lateral junction. (6) Golgi is oriented to face the free edge.

Chapter 7

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