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Cellular Mechanisms for the Regulation of VE-cadherin Endocytosis

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Abstract

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Cadherins maintain adult tissue architecture and direct morphogenic events through homotypic cellular interactions and via signaling pathways that direct cell growth and migration. In the vascular endothelium, the dynamic regulation of cadherin-based cell adhesion is crucial for vascular development. Vascular endothelial (VE)-cadherin is the central adhesion molecule in endothelial adherens junctions and its regulation is crucial for vascular function. p120-catenin (p120) binds to cadherins and regulates cadherin levels by preventing cadherin endocytosis and degradation. In vivo studies have demonstrated that p120 is required for vascular development, and mice lacking p120 in endothelial cells exhibit severe defects in vascular patterning, barrier function, and proliferation. These phenotypes are at least partially dependent on the loss of VE-cadherin observed in vessels lacking p120.

The primary goal of the research described in this dissertation is to understand the mechanism by which p120 regulates VE-cadherin endocytosis. Studies shown here have demonstrated that p120 prevents VE-cadherin from entering a clathrin-mediated endocytic pathway through a mechanism that requires binding of p120 to the cadherin cytoplasmic tail. We demonstrate that p120 prevents VE-cadherin internalization independently of its role in regulating RhoGTPase activity. Rather, we present evidence that p120 stabilizes VE-cadherin at the plasma membrane by competing with the clathrin adaptor AP-2 for interactions with the VE-cadherin juxtamembrane domain.

We have identified a key role for the clathrin adaptor complex AP-2 in regulating VE-cadherin endocytosis. AP-2 interacts with the VE-cadherin cytoplasmic tail, and is required for VE-cadherin endocytosis. Mutation of a tyrosine motif in the VE-cadherin juxtamembrane domain compromises VE-cadherin endocytosis and reduces the efficiency of AP-2 binding to the VE-cadherin tail. These findings support a model in which p120 binding to the VE-cadherin tail inhibits the entry of VE-cadherin into a clathrin-mediated endocytic pathway by preventing interactions with the clathrin adaptor AP-2.

These studies further elucidate the mechanism by which p120 functions as a setpoint for VE-cadherin expression and establish a line of inquiry to investigate the role of VE-cadherin membrane trafficking during vascular development, when it is likely to play an important role in regulating the blood vessel growth and reorganization that occurs during angiogenesis.

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Chapter 1

Cadherin-mediated Adhesion in the Vascular Endothelium

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1.1 Cadherin-mediated cell adhesion

Cadherins make up a superfamily of adhesion molecules that engage in calcium dependent, homophilic cell adhesion. Cadherins are organized within the cell into highly structured junctional complexes, which are anchored to cytoskeletal networks. Cadherin-based cell-cell adhesion forms the basis for the highly ordered, multicellular structures that comprise all the solid tissues of the body. Two intrinsic features of cadherins, namely their adhesiveness and the specificity achieved by homophilic binding, underlie cadherin function in cell-cell recognition and the morphological changes required for tissue development and the maintenance of tissue architecture in adult organisms (Takeichi, 1990). The wide range of morphological processes that are controlled by cadherins, including the separation of tissue layers (Larue et al., 1994; Riethmacher et al., 1995), epithelial to mesenchymal transitions (Cano et al., 2000), and long range cell migration (Matsunaga *et al.*, 1988; Hermiston *et al.*, 1996; Geisbrecht and Montell, 2002), demonstrates that cadherins must engage in strong adhesion, yet remain primed for dynamic modulation according to the changing physiological needs of the organism and the local cellular signaling environment (Tepass *et al.*, 2002; Gumbiner, 2005).

Cells have evolved several mechanisms for regulating the molecular composition of the plasma membrane, including transcriptional and post-transcriptional modifications. One major means for rapidly changing the adhesive state of cells is through membrane trafficking of junctional molecules. Endocytic and exocytic transport provides a finely tuned mechanism for the spatial and temporal regulation of membrane receptors at the cell surface (Bryant and Stow, 2004). Over the past decade, an ever-expanding body of research has revealed a fundamental role for membrane trafficking in the regulation of

cadherin function during development, normal homeostasis, and the disease state. A myriad of studies indicate that cadherins can be transported to and from the cell surface through a variety of pathways that include; rapid retrieval to the plasma membrane, temporary sequestration in cytoplasmic vesicles, or long term down regulation through lysosomal degradation (Bryant and Stow, 2004). These multiple pathways enable cells to tightly regulate their adhesive capacity and morphology.

This dissertation will focus of the regulation of cadherin endocytosis in the vascular endothelium, where cell adhesion must be dynamically regulated both during development of the vascular system and in the adult organism to maintain normal vessel homeostasis. The primary emphasis will be on the dynamic regulation of two types of molecular interactions that occur during cadherin trafficking; alterations to the cadherin-catenin complex itself and interactions with the membrane trafficking machinery. First, however, a discussion of the vascular endothelium and its dynamic regulation by cadherin-mediated adhesion is necessary.

1.2 The vascular endothelium

The vascular endothelium provides an excellent model for studying the dynamic regulation of cell adhesion. A single layer of endothelial cells lines blood vessels throughout the vascular tree, providing a network of tubes for the transport of blood and nutrients and acting as a barrier between the lumen of blood vessels and the surrounding tissue (Dejana, 2004; Gavard and Gutkind, 2008; Vestweber, 2008a). The regulation of the endothelium as a semi-permeable barrier is crucial for many important functions, including tissue fluid homeostasis, angiogenesis, and immune responses (Mehta and

Malik, 2006). Endothelial cells function as gatekeepers to regulate the exchange of nutrients, fluids, and cells between the blood compartment and the underlying tissue (Dejana, 2004). The dynamic regulation of barrier function requires tight control of intercellular endothelial junctions. Junctions between endothelial cells must selectively reorganize in response to normal physiologic cues during development and immune responses. Additionally, the deregulation of junctions can be either a cause or an effect of pathologic disorders, such as chronic inflammation, edema, or arteriosclerosis (Aghajanian et al., 2008; Vandenbroucke et al., 2008).

The passage of macromolecules and blood cells across the vascular barrier can occur through either a transcellular pathway, involving the passage of solutes through endothelial cells in vesiculo-vacuolar organelles (Dvorak *et al.*, 1995; Carman, 2009), or a paracellular pathway, which requires the opening and closing of endothelial junctions to allow passage of molecules and cells between cells (Orrington-Myers *et al.*, 2006; Vandenbroucke *et al.*, 2008; Vestweber, 2008a). Both pathways function as active, rather than passive means of regulating vessel permeability, and therefore require tight regulation of cellular processes in response to stimuli. The dynamic regulation of endothelial junctions is particularly important for the paracellular pathway, as evidenced by the fact that the intercellular junctions are the primary target of permeability inducing agents (Mehta and Malik, 2006), including thrombin (Rabiet *et al.*, 1996; Konstantoulaki *et al.*, 2003), histamine (Andriopoulou *et al.*, 1999; Winter *et al.*, 1999; Alexander *et al.*, 2000) and vascular endothelial growth factor (VEGF) (Esser et al., 1998; Weis and Cheresh, 2005).

Endothelial cell barrier function is also subjected to dynamic modulation during angiogenesis, both during development and in tumors. In the developing embryo, angiogenesis is required for the expansion and remodeling of a primitive vascular plexus into a more mature vascular network composed of large vessels that branch into smaller capillaries (Carmeliet, 2005). Early steps in the angiogenic process require the dilation of existing blood vessels and increases in endothelial cell permeability (Eliceiri et al., 1999; Kimura et al., 2000). These processes occur through a VEGF mediated mechanism that involves redistribution of endothelial adhesion molecules, including VE-cadherin (Conway et al., 2001). Following the migration of the active and proliferating endothelial cells, the reestablishment of endothelial intercellular junctions is important for the assembly of new vascular cords, which ultimately mature into a complex vascular network lined by differentiated endothelial cells that have been optimized to function according to the needs of the local tissue environment (Thurston et al., 2000). Similar processes occur during tumor angiogenesis, when malignant cells co-opt the growth of new blood vessels to provide a source of nutrients for the tumor to grow and metastasize. The importance of angiogenesis for tumor growth and metastasis has made it an attractive target of therapeutics aimed at treating cancers (Folkman, 2006). However, due to the highly unregulated tumor signaling environment, blood vessels that infiltrate tumors are both structurally and functionally abnormal (Hashizume *et al.*, 2000; Carmeliet *et al.*, 2009; Mazzone *et al.*, 2009). In combination with the complexities of developing a normal, stabilized vascular network, this has limited the effectiveness of current anti-angiogenic strategies, and underscores the necessity of understanding the molecular mechanisms that regulate the dynamics of the vascular network (Carmeliet, 2005).

1.3 Structure and function of endothelial adherens junctions

Adhesion between cells in the vascular endothelium is mediated through a series of junctional complexes, primarily adherens junctions and tight junctions. Junctions in endothelial cells are comparable to those in epithelial cells; however, in epithelial cells, the junctions display a highly organized spatial distribution along the apico-basal surface of the cell, while in endothelial cells, the junctions are less well defined and adherens junctions and tight junctions tend to overlap along the intercellular cleft (Wallez and Huber, 2008). Additionally, junctional architecture varies throughout the vascular system, depending on the local organ-specific requirements (Bazzoni and Dejana, 2004; Aghajanian *et al.*, 2008; Dejana *et al.*, 2009). For example, junctions at the blood-brain barrier, where permeability is relatively low and must be strictly regulated, are enriched in well-developed tight junctions (Sandoval and Witt, 2008). At the other extreme, junctions in post capillary venules are loose and disorganized, indicative of the role of these vessels in mediating leukocyte extravasation (Aird, 2007). These examples speak to the dynamic nature of junctional complexes, both during the establishment and maturation of cell contacts and in stable junctions. The following sections will discuss the organization and function of endothelial adherens junctions, the most prominent junctions in endothelial cells and the focus of this dissertation.

The adherens junction forms a circumferential belt around the cell that is associated to the actin cytoskeleton (Figure 1) (Hartsock and Nelson, 2008). Cadherins form the molecular backbone of adherens junctions. Adherens junctions in endothelial cells contain VE-cadherin, a type II classical cadherin expressed exclusively in vascular endothelial cells (Dejana, 2004). The cadherin ectodomain contains a series of five

cadherin repeat domains (EC) with a calcium binding site between each domain (Figure 2) (Vincent et al., 2004). The binding of calcium at these sites rigidifies the protein structure, activates the adhesion potential, and prevents degradation by proteases. The N-terminal EC1 domain is important for *trans*- interactions of cadherins and also plays a role in determining cadherin selectivity (Pokutta and Weis, 2007). The cytoplasmic face of the adherens junction interacts with the catenins, which are members of the armadillo family of proteins (Figure 1). β-catenin binds to the distal region of the cadherin cytoplasmic tail, to a core region of 30 amino acids termed the catenin binding domain. β-catenin recruits the actin binding protein α-catenin to the cadherin complex, an interaction that serves to tether the cadherin-catenin complex to the actin cytoskeleton, though the mechanism regulating this association has proven to be more complex than originally thought (Shapiro and Weis, 2009). p120-catenin (p120) interacts with the membrane proximal region of the cadherin cytoplasmic domain. p120 was originally identified as a substrate for Src phosphorylation, but was later found to contain a central armadillo repeat domain that mediates association with the cadherin tail (Daniel and Reynolds, 1995). p120 plays an important role in the modulation of cadherin expression levels by regulating cadherin membrane trafficking (Xiao et al., 2007). The role of p120 in cadherin trafficking will be discussed extensively within the context of this dissertation.

The organization of cadherins into highly organized macromolecular cell junctions facilitates cadherin function in cell adhesion and tissue organization. Cellular junctions form a hierarchy of interactions that transforms the relatively weak homophilic binding ability of the cadherin extracellular domain into stable cell adhesion (Figure 1).

The assembly of cadherins into junctional complexes promotes the association of cadherins to cytoskeletal networks, which provides tensile strength and allows the cell to resist mechanical stress (Pokutta and Weis, 2007). However, in addition to their roles in cell adhesion, cadherin-based junctional complexes are important for cellular organization and movement, and also function as scaffolds to transmit signals from the extracellular environment to the interior of the cell (Dejana, 2004; Gumbiner, 2005). These functions of cadherins, which help to mediate a wide range of cellular processes, are dependent on interactions with the catenins and other signaling molecules that associate with the cytoplasmic face of junctional complexes. The diverse and dynamic roles of cadherin-based junctions in modulating morphogenesis, cell communication, and tissue homeostasis require that the entire complex be tightly regulated in a spatial and temporal manner. The following sections will describe the structure and function of the individual components of endothelial adherens junctions in order to understand their important contributions to regulating cell adhesion.

1.4 VE-cadherin: A unique member of the cadherin family

VE-cadherin was originally identified and cloned using an RT-PCR approach to identify new cadherin family members (Suzuki et al., 1991). In this study, the cDNA sequence of clone 5 was identified as an endothelial cadherin with a divergent cytoplasmic tail. Its identity and localization in vascular endothelial cells was confirmed by the development of a monoclonal antibody (7B4) that labeled intercellular borders in endothelial cells and regulated cellular permeability (Lampugnani et al., 1992). The localization of VE-cadherin is highly specific to endothelial cells, such that it can be used

as a marker for the endothelium, and is turned on in development when cells become committed to an endothelial lineage (Breier *et al.*, 1996; Bazzoni and Dejana, 2004). Although endothelial cells contain several other adhesion molecules, VE-cadherin appears to be of primary importance for maintaining the stability of endothelial cell junctions, as antibodies directed against other adhesion molecules fail to dramatically disrupt adhesion (Vestweber et al., 2009). VE-cadherin, however, is crucial to the establishment and maintenance of a mature vascular network during early embryonic development. Mice deficient in VE-cadherin are able to form a primitive vascular plexus, indicating that initial vessel formation does not require VE-cadherin. However, VE-cadherin null embryos die at mid-gestation due to severe impairments in the expansion and remodeling of the vascular network in both embryonic and extraembryonic tissues, indicating that VE-cadherin is essential for sprouting angiogenesis and blood vessel stability (Carmeliet *et al.*, 1999; Gory-Faure *et al.*, 1999). Importantly, the phenotypes observed in the mice lacking VE-cadherin were found to be at least partially related to defects in VE-cadherin mediated signaling through a pathway that promotes cell survival via VEGF, β-catenin, PI3K, and Akt (Carmeliet *et al.*, 1999).

Sequence analysis revealed that like other members of the cadherin family, VE-cadherin consists of five extracellular cadherin repeat domains, a single transmembrane domain, and a cytoplasmic tail (Figure 2) (Lampugnani et al., 1992). Cadherins can be categorized into four major groups according to domain structure, genomic structure, and phylogenetic analysis (Nollet et al., 2000). These groups include the classical/type I cadherins, the atypical/type II cadherins, the desmogleins and desmocollins, and the protocadherins. Although VE-cadherin can be characterized as an atypical/Type II

classical cadherin according to its genomic organization, sequence and phylogenetic analysis have revealed that it is unique among other members of the cadherin family (Vincent et al., 2004).

Like other cadherins, VE-cadherin mediates calcium-dependent, homophilic interactions between adjacent cells. The extracellular domain of VE-cadherin contains five cadherin repeat domains (EC1-EC5) that are linked by calcium binding sites that serve to rigidify the structure when bound by calcium ions (Shapiro and Weis, 2009). Evidence from several crystal structures demonstrates that cadherins engage in homophilic interactions through intimate associations between two EC1 domains through a strand swapping mechanism (Shapiro *et al.*, 1995; Boggon *et al.*, 2002; Miloushev *et al.*, 2008). VE-cadherin, along with other type II cadherins, contains two conserved tryptophans (W2 and W4) in the N-terminal EC domain. The EC1 domain also contains a region of conserved nonpolar contacts that forms a hydrophobic binding pocket that can accommodate both tryptophan residues of a partner cadherin molecule. This strand swapping mechanism has been hypothesized to mediate homophilic *trans* interactions between cadherins on neighboring cells (Patel *et al.*, 2006a). Mutagenesis of these tryptophan residues has been shown to disrupt cadherin interactions, indicating that this mode of dimer formation is important for adhesion (Tamura *et al.*, 1998; May *et al.*, 2005; Pokutta and Weis, 2007). In addition to engaging in *trans* dimerization events, cadherins are thought to form *cis* interactions. Lateral, or *cis* interactions are those that occur between cadherins on the same cell. Although the mechanism for how *cis* dimerization of cadherins occurs is not fully understood, evidence suggests that these

lateral interactions are crucial for mediating cadherin-based cell adhesion (Shapiro and Weis, 2009).

Although the precise mechanisms by which VE-cadherin mediates homophilic cell adhesion are not yet fully understood, it is clear that the VE-cadherin extracellular domain plays a crucial role in modulating endothelial cell function. Antibodies against the EC1 domain of VE-cadherin have been shown to disrupt endothelial barrier function and prevent angiogenesis both *in vitro* and in blood vessels *in vivo* (Corada et al., 2001). Antibodies against the VE-cadherin EC4 domain, however, were able to disrupt angiogenesis independently of changes to barrier function, suggesting that distinct functions of VE-cadherin are separable by domain, and underscoring the role that cadherin structure plays in modulating cellular function (Corada et al., 2001). While the cadherin extracellular domain directly engages in homophilic interactions in order to mediate cell adhesion, an important contribution to the adhesive potential of the adherens junction is made by the cytoplasmic tail of the cadherin, specifically through its interactions with members of the armadillo family of proteins, the catenins. The next section will discuss the importance of the catenins to regulating VE-cadherin function.

1.5. The catenins: cytoskeletal linkers and signaling scaffolds

1.5.1. p120-catenin

p120-catenin (p120) was originally identified as a Src substrate (Reynolds et al., 1989), but sequence analysis later identified it as a member of the catenin family by the presence of an armadillo repeat domain that mediates interactions with the cadherin cytoplasmic tail (Reynolds *et al.*, 1992; Daniel and Reynolds, 1995). p120 is only

distantly related to β-catenin, but is the prototypical member of a subfamily of armadillo domain containing proteins that includes ARVCF, p0071, delta-catenin, and the plakophillins (Hatzfeld, 2005). p120 is expressed as several different isoforms as a result of alternative splicing of a single gene. Isoforms 1-4 result from the use of alternative ATG start codons, while exons A, B, and C are internal alternatively spliced sequences, giving rise to the potential for 64 different isoforms (Keirsebilck et al., 1998). Different cell types usually express characteristic ratios of multiple isoforms(Reynolds and Roczniak-Ferguson, 2004). For instance, endothelial cells primarily express p120 isoforms 1A and 3A (Ferreri and Vincent, 2008).

The structure of p120 consists of a central armadillo domain made up of 9 tandem armadillo repeats flanked by a 325 amino acid N-terminal regulatory region and a carboxy terminal tail (Figure 3) (Choi and Weis, 2005). The N-terminal region of p120 contains several tyrosine and serine/threonine phosphorylation sites and phosphorylation has been predicted to be an important regulatory mechanism for p120 function. However, despite the fact that p120 was originally identified as a substrate for phosphorylation by Src kinases, surprisingly little is understood about p120 regulation by phosphorylation (Reynolds, 2007). This region has also been found to play a role in the regulation of RhoGTPase activity, a key function of p120 that will be discussed in greater detail below. The function of the C-terminal tail is also poorly understood. The core functions of p120 in the cell have been primarily liked to the central armadillo domains. Arm domains 1-7 of p120 mediate interactions with the juxtamembrane domain (JMD) of most type I and type II cadherins (Ireton et al., 2002; Reynolds and Roczniak-Ferguson, 2004). This region of the cadherin tail is highly conserved, and a core region of eight amino acids has

been identified as the core binding site for p120 within the cadherin JMD (Thoreson et al., 2000). Although the interaction between p120 and the cadherin tail occurs at relatively low affinity, the association has found to be of critical importance for the regulation of cadherin function. Mutations within this octapeptide sequence disrupt p120 binding to the cadherin tail and result in defects in cell adhesion (Thoreson et al., 2000). Yap et al first identified an important role for the cadherin JMD in adhesion using laminar flow and cell aggregation assays to measure the adhesive strength of cadherin truncation mutants. These early experiments suggested indirectly that p120 might positively regulate cell adhesion, potentially through a mechanism involving lateral cadherin clustering (Yap et al., 1997; Yap et al., 1998).

The first direct evidence for a role for p120 in cadherin-based adhesion came from the identification of a tumor cell line with cell adhesion deficiencies due to mutations in p120. The absence of p120 in these cells results in a corresponding decrease in E-cadherin stability, even though mRNA levels are not affected (Ireton et al., 2002). Cell adhesion and E-cadherin expression can be rescued by exogenous expression of either p120 or E-cadherin, suggesting that p120 functions in regulating E-cadherin turnover (Ireton et al., 2002). Further studies have helped to elucidate the mechanism by which p120 acts to regulate E-cadherin expression and function. siRNA knockdown and overexpression of p120 in mammalian cell culture revealed that p120 functions as a set point, or rheostat, of cadherin expression levels in multiple cell types and for multiple cadherins, including E-cadherin, N-cadherin and VE-cadherin (Davis et al., 2003; Xiao et al., 2003a). Loss of p120 by siRNA leads to a concomitant reduction in cadherin levels, in a dose dependent manner, as well as decreases in cell adhesion. Conversely, the

expression of exogenous p120 causes increased cadherin expression. In cells depleted of p120 by siRNA, unbound cadherin is properly delivered to the cell surface (Davis et al., 2003), but cannot be retained and therefore is immediately targeted for endocytosis and degradation in the lysosome (Xiao et al., 2003a; Xiao et al., 2003b). This dissertation primarily focuses on understanding the mechanism by which p120 regulates the endocytosis and turnover of VE-cadherin in endothelial cells.

These findings also provided important insight governing the mechanism of action of the dominant negative cadherin chimeras, which, as described above, lead to the endocytosis and degradation of endogenous cadherins when expressed in cells at high levels. Several studies have demonstrated this phenomenon upon expression of cadherin molecules consisting of a cadherin cytoplasmic domain tethered to a non-adhesive extracellular domain. These cadherin mutants act in a dominant fashion, inducing the downregulation of endogenous cadherin (Norvell and Green, 1998; Nieman *et al.*, 1999; Troxell *et al.*, 1999). In endothelial cells, the expression of an IL2R-VE-cadherin chimera results in the internalization of endogenous VE-cadherin that is processed and degraded through an endo-lysosomal pathway (Xiao et al., 2003a; Xiao et al., 2003b). The VE-cadherin JMD was found to be sufficient to cause downregulation of the endogenous cadherin (Xiao *et al.*, 2003a; Iyer *et al.*, 2004). Additionally Xiao et al. found that an IL2R-VE-cadherin chimera with a mutation in the p120 binding site does not cause cadherin downregulation. Furthermore, VE-cadherin levels could be restored upon exogenous expression of p120, suggesting that the dominant negative cadherin mutants function by competing with endogenous cadherins for interactions with p120 (Xiao et al., 2003a). Thus, p120 acts as a limiting factor in stabilizing cadherins at the cell surface. In

cells expressing dominant negative cadherins, p120 is sequestered by the mutant cadherin and therefore unable to bind to endogenous cadherin, resulting in its endocytosis and degradation. The downregulation of endogenous cadherins by the expression of dominant negative chimeric cadherins is reminiscent of the cadherin switching that occurs during EMT (Cowin et al., 2005). During EMT, E-cadherin expression is transcriptionally repressed while non-epithelial cadherins such as N-cadherin, R-cadherin or cadherin 11 are upregulated, a change that is required for the increased motility associated with EMT, especially in metastasis of breast cancer cells (Pishvaian *et al.*, 1999; Hazan *et al.*, 2000; Maeda *et al.*, 2005). When R-cadherin is expressed in A431 epithelial cells, which normally express E- and P-cadherin, the endogenous cadherin is shifted from a recycling pathway to a lysosomal degradation pathway. Interestingly, this phenomenon was found to result from competition for the availability of p120 (Maeda et al., 2006). The ability of non-epithelial cadherins to downregulate E-cadherin expression is cell type specific, as evidenced by the fact that N-cadherin expression in oral squamous epithelial cells reduces levels of E-cadherin but has no effect on E-cadherin in some breast epithelial cells (Islam et al., 1996; Nieman et al., 1999). These findings may be explained by the relative abundance of p120 in the cell, based on p120's function as a setpoint for overall cadherin levels.

In addition to its functions in modulating cadherin expression at the plasma membrane, p120 also regulates cytoskeletal organization and function though its ability to regulate the activity of the RhoGTPases RhoA, Rac1, and Cdc42. p120's role in regulating RhoGTPase activity was first appreciated as a result of the observation that overexpression of p120 in fibroblasts causes a dramatic branching phenotype that could

be attributed to inhibition of RhoA (Anastasiadis et al., 2000) and activation of Rac1 (Noren et al., 2000). While the precise mechanism of how p120 regulates RhoGTPases is not well understood, it appears to depend largely on the cell type and the local signaling microenvironment (Yanagisawa and Anastasiadis, 2006; Soto *et al.*, 2008; Yanagisawa *et al.*, 2008). In some cell types, cytoplasmic p120 directly inhibits RhoA by sequestering it in a GDP-bound manner, acting like a Guanine Nucleotide Dissociation Inhibitor (GDI) (Anastasiadis et al., 2000). However, in other systems, p120 has been shown to inhibit RhoA downstream of Rac1, through a mechanism that involves the p120-dependent recruitment of p190RhoGAP to adherens junctions (Wildenberg et al., 2006). Activation of Rac1 and Cdc42 by p120 appears to occur indirectly through interactions of p120 with guanine nucleotide exchange factors such as Vav-2 (Noren et al., 2000). The downstream effects of p120-mediated regulation of RhoGTPase activity also seem to be dependent on the cellular context, particularly on the type of cadherin expressed. For example, in cells expressing E-cadherin, local activation of Rac1 and Cdc42 by p120 leads to enhanced cell adhesion, while in cells expressing mesenchymal cadherins, including N-cadherin, R-cadherin, and cadherin-11, changes in RhoGTPase activity lead to increased cell motility (Yanagisawa and Anastasiadis, 2006). Interestingly, in endothelial cells, VE-cadherin mediated adhesion appears to be refractory to regulation by Rho or Rac activity; however, when expressed in CHO cells, inhibition of Rho or Rac caused disruption of VE-cadherin from junctions (Braga et al., 1999). Together, these studies help to illustrate the incredible complexity of p120 functions in adhesion and signaling and suggest that they must be tightly regulated during normal development and tissue morphogenesis.

The importance of understanding the cellular mechanisms underlying p120's function are underscored by *in vivo* studies in mice using p120 conditional knock out models in a variety of tissues. The loss of p120 in several epithelial tissues, including the salivary gland, results in decreased E-cadherin expression, ultimately leading to hyperproliferation or inflammation (Davis and Reynolds, 2006; Perez-Moreno et al., 2006). Additionally, mice with a conditional knock out of p120 in endothelial cells die embryonically and display severe defects in endothelial barrier function, blood vessel patterning, and endothelial cell proliferation (Oas et al., 2010). These defects are at least partially associated with a decrease in VE-cadherin levels in endothelial cells in vivo, highlighting the importance of p120-mediated regulation of cadherin turnover to the development and maintenance of proper vascular function.

1.5.2. β–catenin and Plakoglobin

β-catenin is a 92 kD protein that was originally identified in *Drosophila* as the segment polarity protein armadillo (Peifer et al., 1992). β -catenin and related proteins contain a series of armadillo domains – a 42 amino acid repeat motif that forms a helical structure. β-catenin contains 12 armadillo domains that are tightly packed into a superhelix of helices that form a positively charged groove for protein binding to several interacting partners, including cadherins (Figure 3) (Huber and Weis, 2001). β -catenin binds to a highly conserved region in the distal portion of the cadherin cytoplasmic tail called the catenin-binding domain (CBD). Studies have demonstrated that β -catenin plays a fundamental role in regulating cadherin-mediated adhesion, owing to its roles in preventing proteolytic degradation of the cadherin tail, directing delivery of the cadherin

from the endoplasmic reticulum to the plasma membrane, and linking the cadherin complex to the actin cytoskeleton (Daugherty and Gottardi, 2007). Mutational analysis of VE-cadherin has revealed that deletion of the β -catenin binding domain dramatically compromises the ability of the cadherin to mediate tight cell adhesion (Navarro et al., 1995). In a mouse model, the same mutation leads to lethality due to defects in vascular remodeling, comparable to the phenotype seen by inactivation of the entire VE-cadherin gene (Carmeliet *et al.*, 1999). Furthermore, studies using a conditional knockout of β-catenin in endothelial cells demonstrated that β-catenin is essential for vascular patterning and integrity during later stages of embryonic development. Embryos lacking β-catenin in endothelial cells display abnormal vessel branching, blind ending vessels, localized hemorrhaging, and changes in vascular organization (Cattelino et al., 2003). These phenotypes appear to be largely attributable to defects in adherens junction integrity in the absence of β-catenin.

The cadherin-catenin complex is tightly coupled to the actin cytoskeleton. Attachment of the cadherin to the actin cytoskeleton limits lateral mobility of the cadherin, thus promoting lateral clustering and increased strength of adhesion (Adams et al., 1996; Adams et al., 1998; Chu et al., 2004). A key role of β-catenin in stabilizing adherens junctions and strengthening cell adhesion is thought to originate from β-catenin's ability to link the cadherin cytoplasmic tail to the actin cytoskeleton. The function of β-catenin as a cytoskeletal linker stems from its role in recruiting α-catenin to the cadherin-catenin complex (Ozawa et al., 1990). Despite its name, α-catenin lacks an armadillo domain and is more closely related to vinculin and other actin binding proteins than it is to β-catenin (Nagafuchi et al., 1991). According to the textbook model of

adherens junction structure, β-catenin anchors the cadherin complex to the actin cytoskeleton through interactions with α-catenin. However, recent attempts to reconstitute the adherens junction complex *in vitro* revealed that while a 1:1:1 stochiometric complex containing the cadherin cytoplasmic tail, β-catenin, and α-catenin could be isolated, this complex did not bind to actin (Drees et al., 2005; Yamada et al., 2005). Further studies demonstrated that α-catenin cannot simultaneously bind to β-catenin and actin. Furthermore, α-catenin was found to exist as monomers that bind to β-catenin at the cadherin complex, or as homodimers that interact with actin and regulate actin dynamics (Drees et al., 2005; Yamada et al., 2005). These findings necessitate the development of a new model to explain the linkage of the cadherin-catenin complex to the actin cytoskeleton. One possibility is that β-catenin recruits α-catenin to adherens junctions, where it then dissociates from the β-catenin and locally regulates actin organization without maintaining a connection to the cadherin complex. Another possible scenario is that additional molecules are required to bridge the cadherin complex to the actin cytoskeletal network. A newly identified candidate for this role is eplin, an actin binding protein that was recently found to interact with α-catenin in a manner that is crucial for maintaining stable junctions (Abe and Takeichi, 2008).

In addition to its role in mediating cadherin-based adhesion, β-catenin is also a key component of a Wnt signaling axis that regulates expression of genes involved in growth control and tissue patterning (Cadigan and Peifer, 2009). The balance of β-catenin activity between adhesion and signaling is largely dictated by its binding partners, as β-catenin does not possess intrinsic enzymatic activity of its own. Interactions between β-catenin and its binding partners are largely regulated by phosphorylation (Daugherty and

Gottardi, 2007). Serine/threonine phosphorylation of E-cadherin (Lickert et al., 2000) or β-catenin (Bek and Kemler, 2002) has been shown to increase the affinity of the interaction between β-catenin and the cadherin complex, while tyrosine phosphorylation of β-catenin (Roura *et al.*, 1999; Piedra *et al.*, 2003) or VE-cadherin (Potter et al., 2005) disrupts the association of β-catenin with the cadherin tail. The incorporation of β-catenin into cadherin complexes acts to stabilize the protein, while the cytoplasmic pool of β-catenin is normally targeted for proteasomal degradation by the axin/APC/GSK3β complex in a phosphorylation dependent manner (Clevers, 2006). However, in the presence of Wnt signaling through its receptor frizzled, the axin/APC/GSK3β complex is inhibited, preventing β-catenin degradation and allowing for its translocation to the nucleus, where it interacts with LEF/TCF transcription factors to modulate expression of genes involved in cell growth (Arce et al., 2006). Recent studies have found evidence that Wnt signaling through β-catenin plays an important role in vascular development. During chick development, a proper gradient of Wnt signaling is crucial for the formation of the heart (Marvin *et al.*, 2001; Armstrong and Bischoff, 2004). Additionally, studies in zebra fish demonstrated that cardiac cushion formation, an important step in the development of cardiac valve formation, requires Wnt/β-catenin signaling (Hurlstone et al., 2003).

Like β-catenin, plakoglobin is closely related to the *Drosophila* protein armadillo (Peifer and Wieschaus, 1990). Plakoglobin is highly enriched in desmosomal junctions, but can also bind to the CBD of classical cadherins and assemble into adherens junctions (Zhurinsky et al., 2000). In adherens junctions, plakoglobin's function overlaps with that of β-catenin, as plakoglobin is able to bind to α-catenin and form linkages to actin. While plakoglobin is not a required component of adherens junctions, it is thought to play an

important signaling role in regulating cross-talk between adherens junctions and desmosomes. In fact, its association with classical cadherins is required for the formation of desmosomes in epithelial cells (Lewis et al., 1997). Interestingly, although endothelial cells do not contain desmosomal cadherins, plakoglobin is able to link VE-cadherin to the vimentin intermediate filament cytoskeleton by recruiting the intermediate filament linker protein desmoplakin, to form a unique endothelial junction, termed the "complexus adhaerentes" (Kowalczyk et al., 1998). These junctions also contain the p120-catenin related protein p0071, which also mediates interactions with desmoplakin (Calkins et al., 2003). This novel junctional complex may help to mediate tight adhesion in endothelial cells subjected to high levels of mechanical stress. In cultured endothelial cells that are highly confluent, the amount of plakoglobin bound to VE-cadherin is higher (Lampugnani et al., 1995). Furthermore, expression of plakoglobin in endothelial cells enhances endothelial barrier function and increases endothelial cell growth rates (Venkiteswaran et al., 2002).

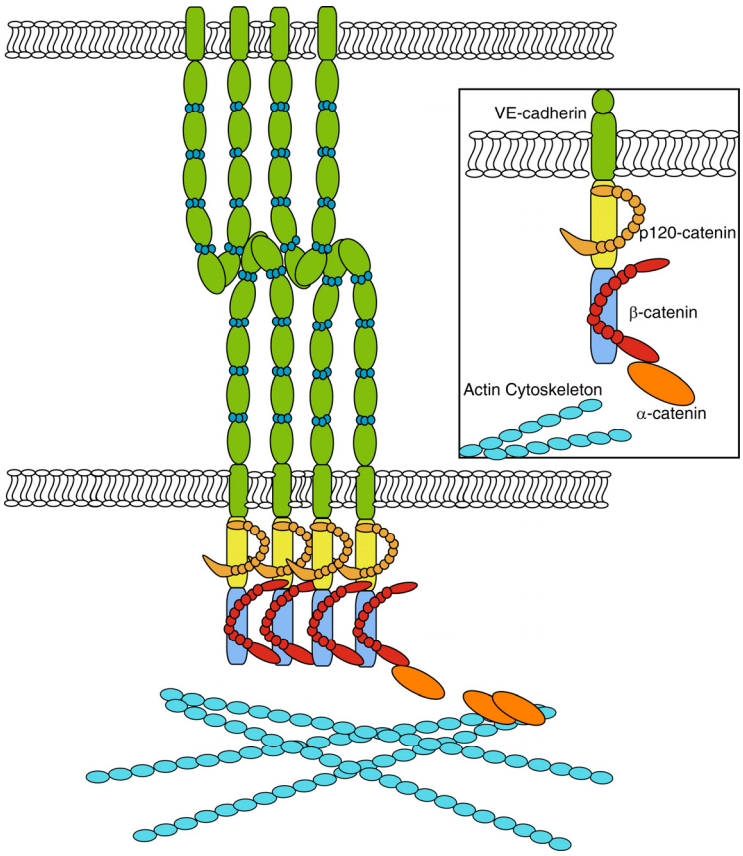

Figure 1. Organization of the adherens junction and the cadherin-catenin complex.

Cadherins are organized into zipperlike structures along borders between neighboring cells. Cadherins engage in both *trans* and *cis* homophilic interactions in a Ca^{2+} dependent manner. The core cytoplasmic components of adherens junctions include p120-catenin and β-catenin. p120 interacts with the juxtamembrane domain of VE-cadherin, and β-catenin interacts with the distal region of the cadherin, called the catenin binding domain. β-catenin interacts with α-catenin, an actin binding protein that serves as a link between the cadherin and the actin cytoskeleton, though the mechanism is not well understood. Only the core components of the adherens junction are depicted, but cadherins and catenins have been found to interact with many other proteins, including a wide range of signaling molecules.

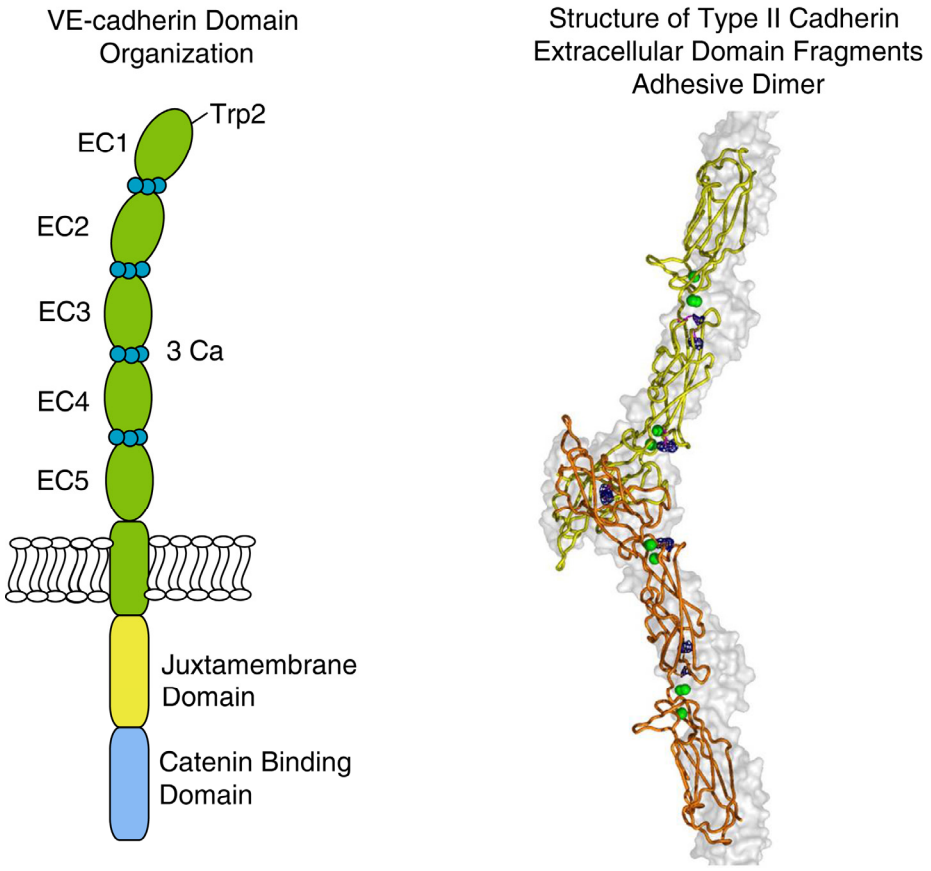

Figure 2. Structure and domain organization of VE-cadherin.

The VE-cadherin extracellular domain consists of five cadherin type repeats (EC domains – extracellular cadherin domains). The binding of three Ca^{2+} ions between each EC domain serves to rigidify the otherwise unstructured domain. Like other type II cadherins, VE-cadherin contains two conserved tryptophan residues (Trp2, Trp4) that are required for cadherin adhesive interactions through a proposed strand swapping mechanism. The VE-cadherin cytoplasmic tail is made up of the juxtamembrane domain, which interacts with p120-catenin, and the catenin binding domain, which contains the β-catenin binding domain. The crystal structure of a type II cadherin adhesive dimer is shown to the right and is modified from (Patel *et al.*, 2006a).

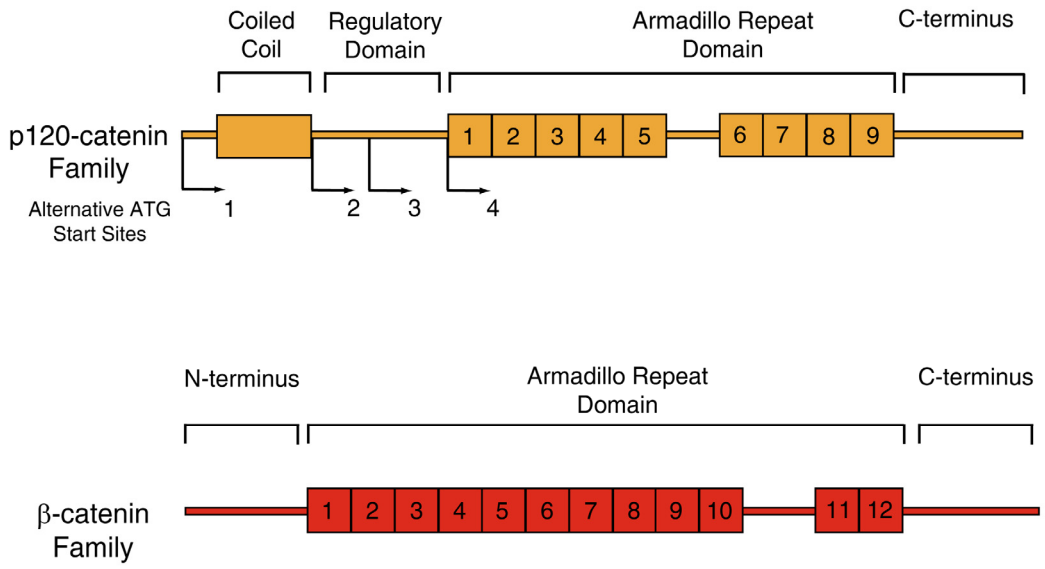

Figure 3. Domain organization of catenin family proteins.

Two major families of armadillo proteins, the p120 family and the β-catenin family, assemble into adherens junctions in endothelial cells. The p120 family also includes ARVCF, p0071, delta-catenin, and the plakophillins. The structure of p120 includes an N-terminal domain, a central armadillo domain, and carboxy terminal tail. The N-terminal region includes a coiled-coil domain and a regulatory domain that contains several tyrosine phosphorylation sites. The use of alternative ATG start codons results in several isoforms that lack varying regions of the N-terminal domain. The p120 armadillo domain contains 9 tandem armadillo repeats. Arm domains 1-7 mediate interactions with the cadherin juxtamembrane domain. β-catenin and plakoglobin make up the other family of armadillo proteins. The structure of β-catenin is similar to that of p120, but β-catenin contains 12 armadillo repeats that are tightly packed into a superhelical structure. The entire armadillo domain mediates interactions with the cadherin catenin binding domain. β-catenin binds to α-catenin through sequences in the N-terminus. The C-terminal region functions as a transcriptional activator in Wnt signaling.

Chapter 2

Regulation of Cadherin Trafficking

This chapter is adapted from the peer reviewed version of the following article:

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2.1. Endocytosis of adherens junction proteins

Over the past decade, research has increasingly indicated that the expression and function of cadherins at adherens junctions is largely influenced by a balance between exocytic and endocytic transport mechanisms (Figure 1). The regulated uptake of cadherins from the cell surface has been documented during both development and post-natal life when large scale cellular rearrangements are required. For example, in gastrulating sea urchin embryos, cells undergoing epithelial-mesenchymal transition (EMT) exhibit adherens junction disassembly, which correlates with changes in cadherin localization from junctions to intracellular organelles (Miller and McClay, 1997). Dynamic changes in cadherin expression have also been observed during EMT in invasive tumors, angiogenesis, and wound healing (Alexander *et al.*, 1998; Detmar, 2000; Tonnesen *et al.*, 2000; Thiery, 2002).

Cadherin function at cellular junctions is not static, but rather requires the dynamic modulation of adhesive strength in response to changing developmental or environmental cues (Emery and Knoblich, 2006). Adherens junctions are highly dynamic and undergo continual remodeling, as evidenced by live cell imaging of GFP-tagged E-cadherin (Adams et al., 1998). The molecular mechanisms underlying the dynamic nature of cadherin based cell adhesion are not fully understood and have been a matter of great controversy within the field. The traditional model of the dynamic nature of cadherin mediated adhesion is based on low-affinity initial cadherin-cadherin interactions that are subsequently strengthened by lateral cadherin clustering and cytoskeletal anchoring (Figure 1) (Adams et al., 1998; Kusumi et al., 1999). However, more recent studies point to an alternative model in which cadherin adhesive dimers are formed by strong, but

dynamic interactions that are rapidly assembled and disassembled (Klingelhofer et al., 2002; Troyanovsky, 2005).

Cadherin recycling and degradation occur both constitutively and under conditions in which cell adhesion is compromised. Membrane trafficking of cadherins to and from the cell surface serves as a crucial determinant of cellular adhesive strength and acts as a way to dynamically modulate cadherin expression levels within cells (Delva and Kowalczyk, 2008). The degree of endocytosis and the fate of the cadherin are dependent on the degree of cell-cell contact and the cellular signaling environment. Under normal physiological conditions, the majority of cadherin is located on the cell surface at intercellular junctions; however, a certain pool is endocytosed into intracellular vesicular compartments. In the case of E-cadherin in MDCK cells, the internalized pool of cadherin is rapidly recycled to the cell surface (Le et al., 1999). However, in endothelial cells, VE-cadherin undergoes a certain level of constitutive lysosomal degradation (Xiao et al., 2003a; Xiao et al., 2003b). Basal levels of constitutive cadherin endocytosis could involve a small pool (predicted to represent 10-15% of total cadherin) of predominantly non-cytoskeletal associated cadherins that are able to flow within the plane of the plasma membrane and enter endocytic routes (Le *et al.*, 1999; Izumi *et al.*, 2004; Miyashita and Ozawa, 2007; de Beco *et al.*, 2009). Alternatively, recent reports suggest that endocytosis functions as the driving mechanism for disassembly of adhesive cadherin dimers. Using ATP depletion or hypertonic sucrose treatment to inhibit endocytosis, Troyanovsky et al. demonstrated that E-cadherin adhesive dimers are stabilized in the absence of endocytosis. This blockage of dimer dissociation resulted in a dramatic increase in the amount of adhesive dimers, along with a parallel decrease in the pool of cadherin

monomers (Troyanovsky et al., 2006). This model is supported by further studies that utilized 2 photon fluorescence recovery after photobleaching (FRAP) to measure E-cadherin membrane dynamics in mature adherens junctions (de Beco et al., 2009; Wirtz-Peitz and Zallen, 2009). In this study de Beco et al. found that less than 5% of E-cadherin in confluent MDCK or MCF7 cultures was subject to membrane diffusion. Rather, E-cadherin undergoes rapid recycling through a pathway that requires dynamin-dependent endocytosis. Together, these studies support the idea that the endocytic machinery can access and remove cadherin molecules that are actively engaged in adhesive interactions. Recent work in *Drosophila* lends further support for this model of cadherin-based adhesion. Several studies have demonstrated that membrane trafficking of cadherin is necessary for the maintenance of stable cell adhesion (Harris and Tepass, 2008; Leibfried et al., 2008; Wirtz-Peitz and Zallen, 2009). Inhibition of E-cadherin endocytosis leads to disruption of adherens junctions and eventually causes decreases in epithelial cell adhesion. These findings may indicate the importance of cadherin trafficking in dynamically modulating adherens junctions both in nonmoving cells and during cell rearrangements and suggest a tight coupling between cadherin endocytosis and cell adhesion. In both of these models, cadherin endocytosis plays a fundamental role in regulating the dynamics and plasticity of adherens junctions.

In addition to continual recycling of cadherins at stable cell junctions, levels of endocytosis are greatly increased in cells lacking stable cell-cell contacts, such as in preconfluent monolayers, or following disruption of cell junctions by Ca^{2+} depletion (Kartenbeck *et al.*, 1991; Ivanov *et al.*, 2004). Upon replenishment of extracellular Ca^{2+}, recycling of surface E-cadherin is sufficient, and necessary, to restore the epithelial

monolayer in the absence of protein synthesis (Le et al., 1999). Under conditions in which cadherin based adhesion is disrupted, cadherin expression is often downregulated. For instance, EMT correlates with loss of E-cadherin, which is often attributed to transcriptional repression or genetic mutations (Cavallaro and Christofori, 2001). However, in some model systems, cadherin expression is greatly reduced despite normal gene expression levels (Thiery, 2002; D'Souza-Schorey, 2005; Janda *et al.*, 2006). During EMT induced by v-Src activation, E-cadherin is degraded in the lysosome following endocytosis. Expression of v-Src leads to the phosphorylation dependent ubiquitination of E-cadherin. Ubiquitinated E-cadherin is trafficked to the lysosome for degradation through a pathway that requires hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and the GTPases Rab5 and Rab7 (Palacios et al., 2005). Another study identified Hakai as a c-Cbl-like E3 ubiquitin ligase that promotes the monoubiquitination and endocytosis of E-cadherin upon phosphorylation of the E-cadherin JMD by Src (Fujita et al., 2002). It is not fully understood whether Hakai functions at the plasma membrane or at endocytic compartments. Interestingly, recent studies have suggested that VE-cadherin undergoes ubiquitination and degradation in response to infection by the Kaposi's Sarcoma-associated herpesvirus (KSHV), a virus that causes the most common AIDs related malignancy. In this system, VE-cadherin ubiquitination and downregulation is mediated by the KSHV protein K5, an E3 ubiquitin ligase with homology to human membrane-associated RING-CH proteins (Mansouri et al., 2008; Qian et al., 2008).

2.2 Routes of cadherin endocytic trafficking

Endocytosis can occur through several pathways, which can be generally classified as clathrin dependent or clathrin independent (Figure 2). Clathrin-dependent endocytosis is the most well characterized mechanism of transmembrane receptor internalization. In clathrin-dependent endocytosis, adaptor proteins recruit cargo receptors into clathrin coated invaginations in the plasma membrane by recognizing distinct sorting motifs in the cytosolic domain of the receptor. While not as well understood, clathrin-independent endocytosis has become increasingly recognized as an important pathway for the regulation of receptor trafficking. Clathrin-independent mechanisms for endocytosis include caveolae dependent pathways, lipid raft mediated pathways, and macropinocytosis.

Numerous studies have indicated that cadherin endocytosis can be mediated by both clathrin dependent and clathrin independent pathways and that the cellular machinery used by cadherins is likely dependent on the cellular context. Recycling of E-cadherin in MDCK cells, both constitutive and in the absence of extracellular Ca^{2+}, occurs in a clathrin dependent manner (Le et al., 1999). In T84 cells, Ivanov et al observed coordinated internalization of the entire apical junctional complex into an intracellular compartment enriched in syntaxin-4 upon Ca^{2+} depletion (Ivanov et al., 2004). While adherens junction and tight junction proteins appeared to be segregated into distinct populations within the syntaxin-4 vesicles, endocytosis of both types of junctions was blocked by inhibitors of clathrin dependent endocytosis, but not by disruption of caveolae/lipid rafts or macropinocytosis (Ivanov et al., 2004). In endothelial cells, VE-cadherin is also internalized through a clathrin dependent manner, and is trafficked

through early and late endosomes to the lysosome, where it undergoes degradation (Figure 3) (Xiao et al., 2003b; Xiao et al., 2005).

Clathrin independent pathways have also been implicated in cadherin endocytosis. In response to EGF treatment, E-cadherin in A431 cells is internalized through a pathway that requires caveolin-1 (Lu et al., 2003). A similar mechanism may be involved in E-cadherin endocytosis in keratinocytes upon Rac activation, as cadherin containing intracellular vesicles exhibited colocalization with caveolin (Akhtar and Hotchin, 2001). The desmosomal cadherin Desmoglein-3 (Dsg3) also undergoes clathrin independent endocytosis. Dsg3 is also the target of the autoimmune disease Pemphigus Vulgaris (PV). PV is an epidermal blistering disease characterized by the loss of epidermal cell adhesion in response to autoantibodies generated against Dsg3. In a cell culture model, treatment of keratinocytes with PV IgG induces the loss of cell adhesion associated with the endocytosis and degradation of Dsg3 in the lysosome (Calkins et al., 2006). Further characterization of the endocytic machinery involved in Dsg3 endocytosis indicates that Dsg3 is internalized through a cholesterol dependent, but clathrin and dynamin independent pathway (Delva et al., 2008). The multitude of endocytic pathways utilized by cadherins for the modulation of cellular adhesive contacts points towards a fine tuned system for the regulation of cadherin levels that is highly dependent on the cellular context.

2. 3 Regulation of cadherin trafficking by the endocytic machinery

The selective internalization of transmembrane receptors from the cell surface requires exquisite regulation of the process of protein sorting. Sorting is largely regulated

by heterotetrameric protein complexes called adaptor protein complexes, or adaptors. Adaptors recruit cargo receptors into clathrin coated vesicles by binding to sorting signals in the cytoplasmic domain of the cargo. AP-2 is the most prominent non-clathrin protein in clathrin coated vesicles, and is considered the core sorting adaptor at the plasma membrane (Traub, 2009). AP-2 is composed of four distinct subunits, namely, the large subunits α and β2, the medium subunit μ2, and the small subunit σ2. Together, these subunits assemble to form a structure consisting of a trunk domain and an ear domain that are linked together by a flexible hinge region (Figure 4). This unique structure enables AP-2 to serve as a protein interaction hub, a function that is central to its role in clustering numerous proteins at sites of clathrin coated vesicle formation (Collins et al., 2002). Through its ability to bind to cargo molecules and clathrin, as well as other proteins and phospholipids at the plasma membrane, AP-2 is a key regulator of clathrin coated vesicle formation (Benmerah and Lamaze, 2007; Rappoport, 2008). Interactions of AP-2 with short, linear sorting motifs in the cytoplasmic domains of transmembrane protein cargoes help to concentrate the cargo in membrane domains from which clathrin coated vesicles will bud. AP-2 recognizes tyrosine or dileucine based motifs. Tyrosine-based sorting signals consist of a tyrosine residue followed by any two amino acids, followed by a bulky, hydrophobic residue (YXXØ (in which X is any amino acid and Ø is a hydrophobic residue) (Canfield et al., 1991; Jadot et al., 1992). Dileucine motifs contain an acidic residue, usually aspartate or glutamate, followed by any three amino acids, followed by a leucine and either another leucine or isoleucine ([DE]XXXL[LI]) (Letourneur and Klausner, 1992). These different types of cargo are sorted by direct interactions of the sorting motif with the μ2 subunit or the α/σ2 subunits of AP-2,

respectively (Ohno *et al.*, 1995; Kelly *et al.*, 2008). Studies have demonstrated that sorting motifs are both necessary and sufficient for the endocytosis of cargo molecules (Letourneur and Klausner, 1992; Bonifacino and Traub, 2003). In addition to targeting proteins for removal from the plasma membrane, sorting signals also function in directing trafficking of molecules within the cell, for example in making decisions between delivery to the lysosome and recycling back to the cell surface.

Despite the central role for AP-2 in clathrin-dependent endocytosis, studies have demonstrated that certain ligands, including the low density lipoprotein (LDL), can be incorporated into clathrin coated vesicles in the absence of AP-2 (Motley et al., 2003). These data indicate that alternative clathrin adaptors must exist. Indeed, upwards of 20 additional adaptor molecules, termed clathrin-associated sorting proteins (CLASPS) have been characterized in recent years (Traub, 2009). Most of these proteins are able to simultaneously bind to clathrin and AP-2, suggesting a highly complex web of interactions that occurs during the process of vesicle formation. Due to the interconnectivity between these CLASPs, it is likely that in some cases they are able to initiate vesicle formation and cargo recruitment in the absence of AP-2 (Sorkin, 2004). The function of adaptor molecules, including AP-2, in cargo recognition and recruitment to clathrin coated vesicles suggests an important role for components of the clathrin endocytic machinery in regulating the endocytic turnover of cell surface receptors, including cadherins. However, little is known about the role of adaptors in regulating cadherin endocytosis.

Evidence has been found for sorting motifs in regulating the trafficking of cadherins. In MDCK cells a highly conserved dileucine motif in the E-cadherin

cytoplasmic tail has been shown to be required for endocytosis of E-cadherin. Mutation of the dileucine motif at amino acids 587-588 results in the accumulation of E-cadherin at the basolateral surface (Miyashita and Ozawa, 2007). This dileucine motif has also been implicated in targeting of E-cadherin to the basolateral membrane. The use of chimeric proteins containing the cytoplasmic tail of E-cadherin fused to the extracellular domain of the interleukin 2 receptor α subunit (TAC) revealed that the cytoplasmic tail of E-cadherin is sufficient for proper sorting to the basolateral membrane. Mutagenesis of this site causes missorting of E-cadherin to the apical surface, resulting in the disruption of cell polarity and morphology (Miranda et al., 2001). These results raise the possibility that the same sorting motif may regulate multiple aspects of cadherin trafficking within the cell.

An adaptor interaction with the E-cadherin dileucine motif has not been identified, but it has been suggested that this motif may interact with the β subunit of AP1B, an adaptor that regulates delivery of cargo from endosomes to the plasma membrane (Rapoport et al., 1998). A recent study identified an indirect association of E-cadherin with the μ subunit of the AP1B adaptor complex through type 1γ phosphatidylinositol phosphate kinase (PIPK1γ) (Ling et al., 2007). The interaction of PIPK1γ with AP1B is functionally important for E-cadherin trafficking. In MDCK cells expressing the isoform of PIPK1γ lacking the AP1B binding site, E-cadherin is sequestered in a cytosolic compartment and its internalization and recycling are inhibited. Interestingly, a naturally occurring germline mutation in E-cadherin, found in hereditary diffuse gastric cancer, contains a mutation (V832M) in the proposed PIPK1γ binding site and has impaired PIPK1γ binding and accumulates in a cytosolic compartment,

highlighting the importance of efficient E-cadherin trafficking. The authors propose a model in which PIPK1γ serves a dual function as both scaffold and signaling molecule. PIPK1γ acts as a cargo adaptor to enable the recruitment of AP1B to E-cadherin and also regulates the localized generation of $PI4,5P_2$, which drives the function of the trafficking machinery (Ling et al., 2007). As AP1B is preferentially localized to the recycling endosome rather than the TGN, it is possible that PIPK1γ functions as an additional sorting step to the dileucine motif. Additionally, the dileucine motif may also interact with AP1B, perhaps with the β subunit, to stabilize the interaction (Rapoport et al., 1998).

Importantly, while this dileucine motif is conserved in several classical cadherins, it is absent in VE-cadherin, indicating that alternative motifs mediate VE-cadherin internalization. Gain of function experiments with the IL2R-VE-cadherin chimera demonstrate that the VE-cadherin cytoplasmic domain harbors information that positively mediates endocytosis (Xiao et al., 2005). Sequence analysis of the VE-cadherin cytoplasmic domain reveals the presence of several putative tyrosine and dileucine motifs, suggesting a potential mechanism for the recruitment of VE-cadherin into a clathrin-mediated endocytic pathway. A central focus of this dissertation is defining sequences in the VE-cadherin tail that mediate endocytosis and identifying the adaptor molecules that regulate this process.

One potential role for adaptor mediated sorting of VE-cadherin has been identified. Gavard and Gutkind recently demonstrated that VEGF signaling through VEGFR-2 can also promote VE-cadherin endocytosis via a novel signaling pathway that appears to regulate endothelial monolayer permeability (Gavard and Gutkind, 2006). They identified a serine-threonine cluster (Ser 665) unique to VE-cadherin, but highly

conserved among vertebrate species, downstream of the p120 binding site. Phosphorylation of serine 665 is required for VEGF mediated endocytosis of VE-cadherin, due to its ability to interact with β-arrestin-2, an adaptor that is best known for its role in GPCR ligand dependent endocytosis. Furthermore, knockdown of β-arrestin-2 by shRNA prevents VEGF mediated internalization of VE-cadherin in addition to loss of endothelial barrier function associated with VEGF signaling (Gavard and Gutkind, 2006). Further studies will be needed to determine if this pathway is also used as a general mechanism of VE-cadherin endocytosis, or is specific to VEGF signaling.

2.4 Rho GTPase regulation of cadherin endocytosis

Rho GTPases, including RhoA and Rac, have been implicated in cadherin endocytosis, either through regulation of the cadherin/catenin complex, or through general regulation of intracellular trafficking steps. Activation of Rac1 has been reported to have both stimulatory (Akhtar and Hotchin, 2001; Gavard and Gutkind, 2006) and inhibitory (Kamei *et al.*, 1999; Palacios *et al.*, 2002; Izumi *et al.*, 2004) effects on cadherin endocytosis, likely depending on the adhesive status and cell type. Braga et al demonstrated that the requirement for Rho and Rac activity in cadherin dependent adhesion varies greatly among different cadherin receptors and cell types, and is also dependent on cell confluency. For example, in endothelial cells, VE-cadherin is refractory to regulation by Rho or Rac activity; however, when expressed in CHO cells, inhibition of Rho or Rac caused disruption of VE-cadherin from junctions (Braga et al., 1999). On the other hand, E-cadherin and P-cadherin, which are both expressed in keratinocytes and form independent complexes, are both lost from cell junctions and

degraded upon inhibition of Rho or Rac activity (Braga et al., 1999). Interestingly, when E-cadherin is expressed in fibroblast L cells, its function is regulated by Rho but not Rac, again suggesting that Rho GTPase activity and its role in regulating cadherin based adhesion and trafficking is highly sensitive to cellular context (Braga et al., 1999).

A recent study has identified a potential mechanism by which p120 may regulate cadherin function by integrating diverse signaling systems through an interaction with p190RhoGAP. Using PDGF activated actin remodeling as a model system, Wildenberg et al. observed a dramatic increase in actin stress fibers and the loss of actin remodeling, accompanied by cell transformation and serum-free proliferation in NIH3T3 cells depleted of p120 by siRNA. These changes in actin cytoskeleton dynamics and cell growth result from a dramatic increase in Rho GTPase activity upon loss of p120 (Wildenberg et al., 2006). By dissecting the molecular pathway of PDGF mediated actin remodeling, it was found that activation of Rac by PDGF causes transient translocation of p190RhoGAP to cadherin complexes through an interaction with p120. Through this interaction, p120 mediates antagonism between Rac and Rho signaling by locally inhibiting Rho at cadherin complexes. In the absence of p190RhoGAP, p120 and N-cadherin are mislocalized, suggesting a role for p190RhoGAP in adherens junction stability (Wildenberg et al., 2006). Together, these findings support a model whereby p120 regulates the stability of cadherin complexes by acting as a nexus to integrate extracellular signals into a coordinated cellular response to environmental cues. However, the relationship between p120's regulation of cadherin endocytosis and its role in regulating RhoGTPase activity is not clear. One issue addressed directly in this

dissertation is whether p120-mediated inhibition of RhoA activity is important for p120's function in preventing VE-cadherin endocytosis.

2.5 Regulation of cadherin endocytosis by receptor tyrosine kinases

The multitude of endocytic pathways utilized by cadherins for the modulation of cellular adhesive contacts points towards a fine tuned system for the regulation of cadherin levels by differences in the local signaling environment. Evidence suggests that even the same signaling molecules can stimulate cadherin internalization through different pathways depending on the cellular context. For instance, short term treatment of tumor cells overexpressing EGFR with EGF leads to cell contact disruption and EMT as a result of E-cadherin endocytosis through a cholesterol dependent pathway that may involve caveolae (Lu et al., 2003). In another system, Rac1 activation downstream of EGF signaling induces macropinocytosis of E-cadherin, along with its associated catenins, p120 and β-catenin, into a recycling endosome (Bryant et al., 2007). Macropinocytosis occurs from regions of the cell lacking cell contacts but is recycled back to cell contacts through a pathway requiring sorting nexin 1 (SNX1). This pathway could therefore be useful in remodeling of existing cell contacts without the loss of adhesion (Conacci-Sorrell *et al.*, 2002; Qian *et al.*, 2004; Bryant *et al.*, 2007). Taken together, these results suggest that endocytosis of junctional proteins is tightly regulated and cells utilize several different membrane trafficking pathways to regulate cadherin function depending on signaling cues in the cellular environment.

The functional interplay between cell adhesion molecules and receptor tyrosine kinases (RTKs) has recently been identified as a novel mechanism for the co-regulation

of adhesion and signaling activity (Conacci-Sorrell et al., 2002; Qian et al., 2004). Several examples of bi-directional regulation of cadherin-RTK function by endocytic trafficking machinery have recently been reported in the literature, suggesting a synergistic mechanism for the regulation of cell growth, proliferation, and migration. In MDCK cells, hepatocyte growth factor (HGF) activation results in adherens junction disruption and endocytosis of E-cadherin. The HGF/SF receptor c-met colocalizes with E-cadherin at cell junctions and is endocytosed into a perinuclear vesicular compartment along with E-cadherin upon HGF treatment or Ca^{2+} depletion. HGF induced endocytosis of E-cadherin and c-met is specifically dependent on GTPase activity. Expression of DA Rho, DA Rac1, or DN Rab5 mutants prevented E-cadherin and c-met endocytosis upon HGF treatment, but had no effect on internalization following Ca^{2+} depletion (Kamei et al., 1999). This suggests an indirect mechanism for GTPase regulation of cadherin and receptor trafficking in this system, and again underscores the importance of cellular context in the regulation of cadherin function. Activation of the small GTPase ARF6, which lies downstream of the HGF receptor (c-met), has also been identified as a positive regulator of E-cadherin endocytosis in response to HGF. ARF6 promotes clathrin and dynamin dependent endocytosis of E-cadherin (Palacios et al., 2001; Palacios et al., 2002). Paterson et al. also found ARF6 activity to be required for endocytosis of unbound pools of E-cadherin in MCF-7 cells lacking cell-cell contacts. Interestingly, however, in these cells endocytosis occurred through a dynamin-dependent, but clathrin-independent pathway resembling macropinocytosis (Paterson et al., 2003).

Fibroblast growth factor (FGF) signaling is involved in embryonic patterning during development and epithelial cell morphogenesis through its role in EMT.

Activation of the fibroblast growth factor receptor (FGFR) by FGF stimulates the co-endocytosis of FGFR1 and E-cadherin and the eventual nuclear translocation of FGFR1 (Bryant et al., 2005). The retention of E-cadherin at cell-cell contacts, either by overexpression of E-cadherin or p120, directly impacts FGFR function by preventing its endocytosis, signaling to the MAPK pathway, and nuclear translocation (Bryant et al., 2005). The parallel regulation of E-cadherin and FGF function through the modulation of intracellular trafficking suggests an elegant mechanism for maintaining the balance between epithelial and mesenchymal phenotypes during changes in the cellular environment. Interestingly, the association of N-cadherin with FGFR1 at the cell surface also prevents FGFR internalization, but inhibition of FGFR endocytosis by N-cadherin leads to the prolonged activation of MAPK signaling downstream of FGF activation, thereby promoting invasiveness of tumor cells (Suyama et al., 2002).

In endothelial cells, VE-cadherin mediates contact inhibition of endothelial cell growth through a mechanism involving attenuation of VEGF induced signaling. In confluent cell monolayers, VE-cadherin forms a complex with VEGF receptor (VEGFR)-2 upon VEGF stimulation. The association of VE-cadherin with VEGFR-2 results in the downregulation of VEGFR-2 phosphorylation and downstream MAPK signaling through a β-catenin dependent mechanism involving the phosphotyrosine phosphatase density enhanced phosphatase-1 (DEP-1) (Grazia Lampugnani et al., 2003). In endothelial cells, the phenomenon of cadherin mediated attenuation of RTK signaling also requires endocytosis of growth factor receptors. Analogous to the situation in epithelial cells, association of VE-cadherin with VEGFR-2 in endothelial cells prevents internalization of VEGFR-2 through a clathrin-dependent pathway. In VE-cadherin null cells, VEGFR-2 is

rapidly internalized into early endosomes, where it retains its signaling abilities (Lampugnani et al., 2006). In contrast to the co-endocytosis of FGFR and E-cadherin, VE-cadherin does not colocalize with internalized VEGFR-2, suggesting that the proteins dissociate prior to internalization. This mechanism of VE-cadherin mediated regulation of VEGF induced signaling helps to explain the persistence of proliferative signaling in the absence of VE-cadherin. This suggests a model whereby VE-cadherin modulates cell growth through the inhibition of VEGFR-2 signaling by retaining it at the cell surface, where it is maintained in an inactive state by DEP phosphatase activity (Lampugnani et al., 2006).

2.6 Working model and central hypothesis:

The overall goal of this research was to define the mechanistic basis of VE-cadherin endocytosis and the role of p120 in regulating this process. Previous work from our lab has described a core function for p120 in regulating VE-cadherin turnover by preventing its entry into a clathrin dependent endocytic pathway. In this work, a chimeric molecule consisting of the cytoplasmic tail of VE-cadherin fused to the extracellular domain of the IL-2 Receptor (IL2R-VE-cad$_{cyto}$) was utilized to demonstrate that the cytoplasmic tail of VE-cadherin harbors information that mediates internalization of the receptor, as this molecule is rapidly internalized from the cell surface (Xiao et al., 2005). Expression of p120 potently inhibits internalization of this molecule, through a mechanism that requires the interaction of p120 with the VE-cadherin cytoplasmic tail. Furthermore, while p120 colocalizes extensively with junctional VE-cadherin, it is not

present in vesicular pools of VE-cadherin internalized from the cell surface (Xiao et al 2005).

From this data, a model emerges in which the VE-cadherin cytoplasmic tail engages in interactions with components of the clathrin endocytic machinery, and that p120 prevents these associations (Figure 5). Upon dissociation of p120 from the cadherin tail, amino acid sequences in the cytoplasmic domain of VE-cadherin are exposed, enabling endocytic adaptor molecules to recruit the cadherin receptor into clathrin coated pits. In other words, p120 functions as a "cap" on the VE-cadherin tail to prevent it from being recruited into a clathrin-dependent endocytic pathway. This model suggests that the mechanism by which p120 stabilizes VE-cadherin at the plasma membrane is direct, and based on the physical ability of p120 to bind to the cadherin JMD, rather than on an enzymatic activity. An alternative possibility is that p120's function in preventing VE-cadherin internalization is based on its role in regulating Rho GTPase activity (Figure 6).

We hypothesized that the VE-cadherin cytoplasmic tail contains sorting motifs that bind to adaptor complexes, such as AP-2, to recruit the cadherin into clathrin coated pits, and that by binding to the VE-cadherin JMD, p120 prevents adaptors from interacting with the cadherin tail. Several predictions can be derived from this hypothesis. (1) VE-cadherin undergoes endocytosis through a pathway that is dependent on components of the clathrin endocytic machinery; (2) The VE-cadherin JMD contains endocytic sorting motifs that interact with clathrin adaptor molecules, such as AP-2; (3) expression of p120 disrupts these associations in a manner dependent on the binding of p120 to the cadherin cytoplasmic tail, and (4) p120-mediated inhibition of VE-cadherin

endocytosis occurs independently of p120's ability to regulate RhoA GTPase activity. These predictions are systematically tested in the following chapters.

2.7 Scope and conceptual contribution of this dissertation:

An emerging concept in the field of cell adhesion is that cells utilize endocytic trafficking pathways to modulate the number of adhesion molecules available at the cell surface to engage in adhesive interactions. In the case of cadherins, it has become clear in recent years that p120 plays a key role in regulating the expression and stability of cadherins by preventing cadherin endocytosis and degradation. A central focus of this dissertation has been to understand the molecular mechanism by which p120 carries out this core function. In order to understand how p120 promotes VE-cadherin stability, it is necessary to first define the molecular machinery that regulates the entry of VE-cadherin into an endocytic pathway.

In Chapter III of this dissertation, we utilized a series of pharmacological, biochemical, and genetic approaches to identify components of the endocytic machinery required for internalization of VE-cadherin. We found that VE-cadherin is primarily internalized through a pathway that is dependent on clathrin, AP-2, and dynamin. Furthermore, the VE-cadherin tail was found to specifically associate with clathrin and AP-2. Knowing that VE-cadherin endocytosis occurs through a clathrin-dependent pathway that requires the AP-2 adaptor complex enabled us to test the hypothesis that p120 interferes with this pathway by preventing associations between VE-cadherin and AP-2. We found that expression of exogenous p120 was able to prevent VE-cadherin from entering into membrane domains enriched for clathrin and AP-2. Importantly, this

function of p120 was found to be dependent on its ability to bind to the VE-cadherin JMD, but independent of its role as an inhibitor of RhoA GTPase activity. Together, these data provide support for our model that the binding of p120 to the cadherin cytoplasmic tail prevents adaptor molecules from recruiting the cadherin into a clathrin-dependent endocytic pathway. Furthermore, these data demonstrate that p120's function in stabilizing VE-cadherin at the plasma membrane can be uncoupled from its role in inhibiting RhoA GTPase activity.

In chapter IV, we further examine our central hypothesis that p120 competes with clathrin adaptors for interactions with the VE-cadherin tail by defining sequences in the VE-cadherin tail that mediate endocytosis. As mentioned above, previous studies using a gain of function approach demonstrated that the VE-cadherin tail harbors information that promotes internalization. Importantly, the amino acid sequence of the VE-cadherin cytoplasmic tail is unique amongst cadherin family members. For instance, sequences that have found to be important for membrane trafficking events in E-cadherin are not conserved in the VE-cadherin tail, indicating that VE-cadherin must contain unique sequences that mediate endocytic sorting. Mutational analysis of the IL2R-VE-cad_{cyto} chimera demonstrated that the JMD of VE-cadherin is sufficient for endocytosis. Further sequence analysis of this region revealed the presence of a putative tyrosine-based motif in the VE-cadherin JMD. Using site directed mutagenesis, we found that mutating the tyrosine in this motif prevents efficient internalization of the IL2R-VE-cad_{cyto} chimera, and disrupts the interaction of AP-2 with the cadherin tail, indicating that this site plays an important role in the sorting of VE-cadherin into an endocytic pathway.

The work described in this dissertation serves to further our understanding of the mechanisms regulating VE-cadherin endocytosis, a process that is thought to be important for the dynamic morphological events that occur in the endothelium during vascular development and disease responses. First, this research identified a novel interaction between VE-cadherin and the clathrin adaptor AP-2, and demonstrated a requirement for AP-2 in VE-cadherin endocytosis. Additionally, we identified a tyrosine-based sorting motif in the VE-cadherin cytoplasmic tail that at least partially mediates the interaction with AP-2. Second, this work has served to further elucidate the mechanism by which p120 functions as a set point to regulate cadherin expression levels and also uncouples this activity of p120 from its role in inhibiting RhoA. Our results support a model in which by binding to the VE-cadherin JMD, p120 prevents the clathrin adaptor AP-2 from associating with the cadherin and recruiting it into clathrin coated pits. Together with previous work from our lab, these findings establish a novel mechanism whereby a cytoplasmic binding partner functions as a plasma membrane retention signal for a transmembrane receptor. The importance of p120 in VE-cadherin stability and function is underscored by phenotypes observed in a conditional knockout of p120 in vascular endothelial cells. Cells and tissues from embryos lacking p120 have reduced levels of VE-cadherin due to rapid internalization and degradation of the cadherin. Loss of p120 in blood vessels leads to severe defects in barrier function, vascular organization, and proliferation, phenotypes that can be at least partially explained by the loss of VE-cadherin expression. Understanding the relationship between VE-cadherin membrane trafficking and endothelial cell adhesion will allow for the development of therapies

aimed at modulating VE-cadherin based functions such as barrier function and angiogenesis.

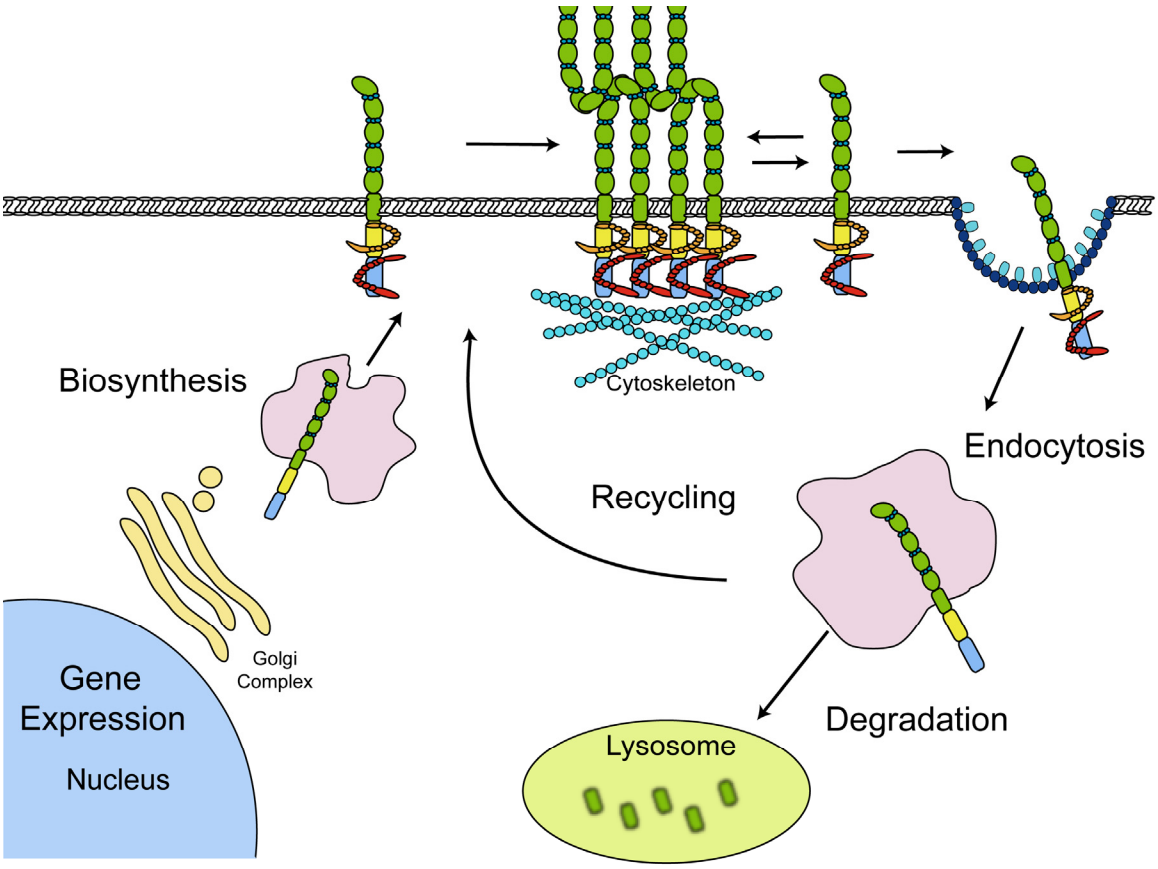

Figure 1. Cadherin dynamics are regulated by membrane trafficking pathways. Over the past decade, membrane trafficking has emerged as a central mechanism for the regulation of cadherin adhesive function at cellular junctions. The adhesive potential of cells is tightly regulated by maintaining a balance between exocytic and endocytic transport mechanisms. Alterations in cadherin turnover occur both during development and in post-natal life when large scale cell rearrangements are required. Some level of constitutive turnover is also necessary for the maintenance and renewal of stable intercellular junctions. Newly synthesized cadherin is actively sorted from the trans-golgi network through exocytic pathways that mediate delivery to the plasma membrane, where the cadherin is associated into junctional complexes. Endocytosis of cadherins from the

cell surface could occur from soluble pools that are non-junctional, or cadherin molecules could be extracted directly from the junction. Once internalized, cadherins can be recycled back to the cell surface, or targeted to the lysosome for degradation.

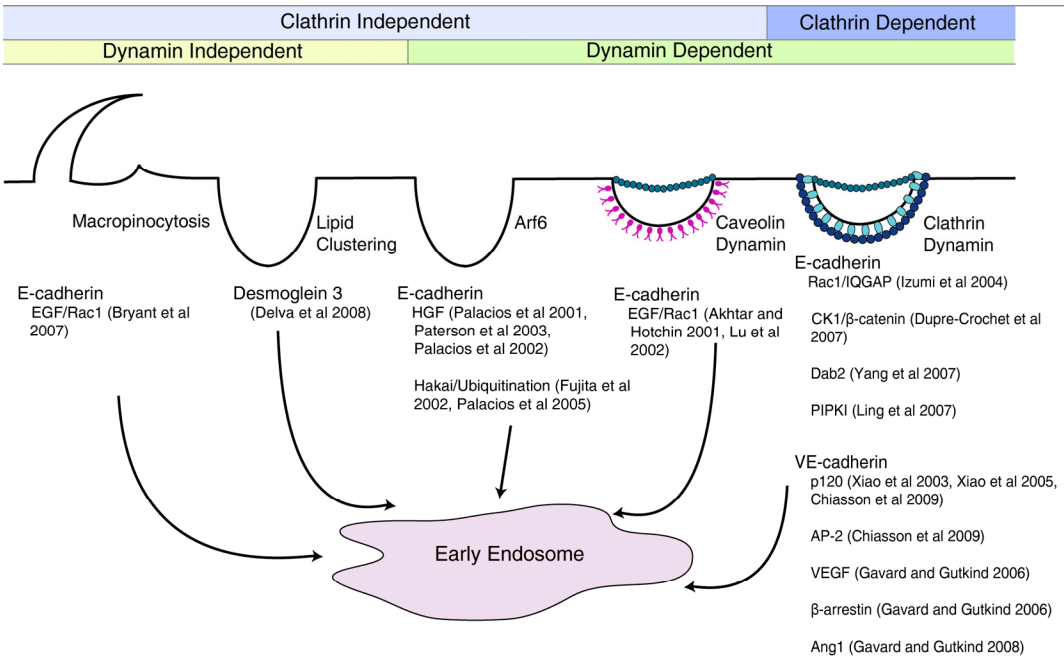

Figure 2. Cadherin endocytosis occurs through many endocytic pathways and is regulated by different adaptors, binding partners, and signaling molecules. Studies have demonstrated that cadherins can be internalized through both clathrin-dependent and clathrin-independent pathways. The choice of pathway used is likely to be dependent on the cellular context and the local signaling environment. Classical cadherins, including E-cadherin and VE-cadherin seem to primarily undergo clathrin-mediated endocytosis. However, under different signaling conditions E-cadherin has also shown to be internalized by several different pathways, including, caveolae-dependent, Arf6-dependent, or macropinocytosis. The desmosomal cadherin Desmoglein 3 is internalized through a unique pathway that is independent of clathrin and dynamin, but may occur through lipid rafts. Many different mechanisms have been identified for the regulation of cadherin endocytosis, suggesting a fine tuned system for the modulation of cadherin levels within cells. Figure is modified from (Wieffer *et al.*, 2009).

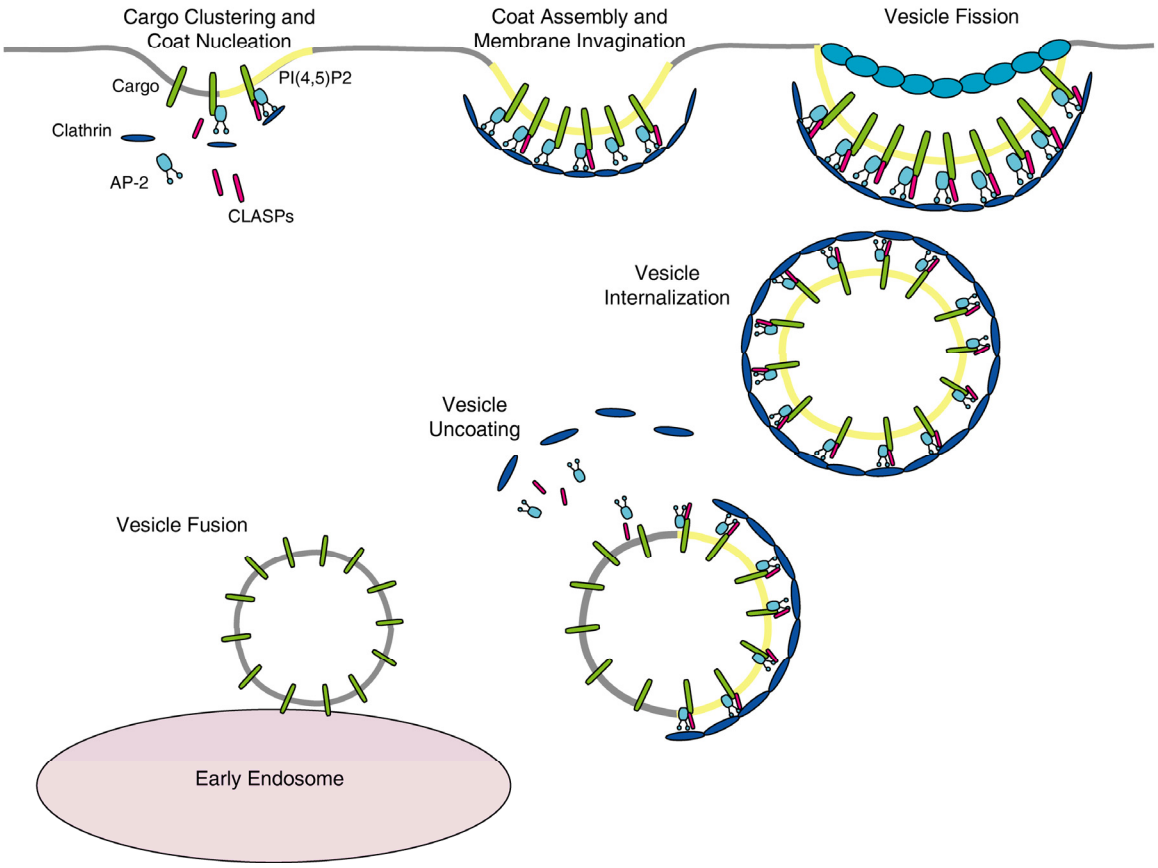

Figure 3. Mechanisms of clathrin-mediated endocytosis.

The first step in clathrin-mediated endocytosis is the clustering of cargo and nucleation of the clathrin coat. This process is mediated by the clathrin adaptor AP-2 and plasma membrane lipids, particularly phosphatidylinositol(4,5)-bisphosphate, PI(4,5)P_2. As the coat continues to assemble other clathrin accessory proteins function to facilitate membrane invagination to further form the coated pit. Once the pit is fully formed, the GTPase dynamin assembles at the neck of the vesicle and mediates scission from the plasma membrane with the help of other clathrin accessory proteins. Following removal from the plasma membrane clathrin coated vesicles are rapidly uncoated during transport to early endosomes. Figure is modified from (Wieffer *et al.*, 2009).

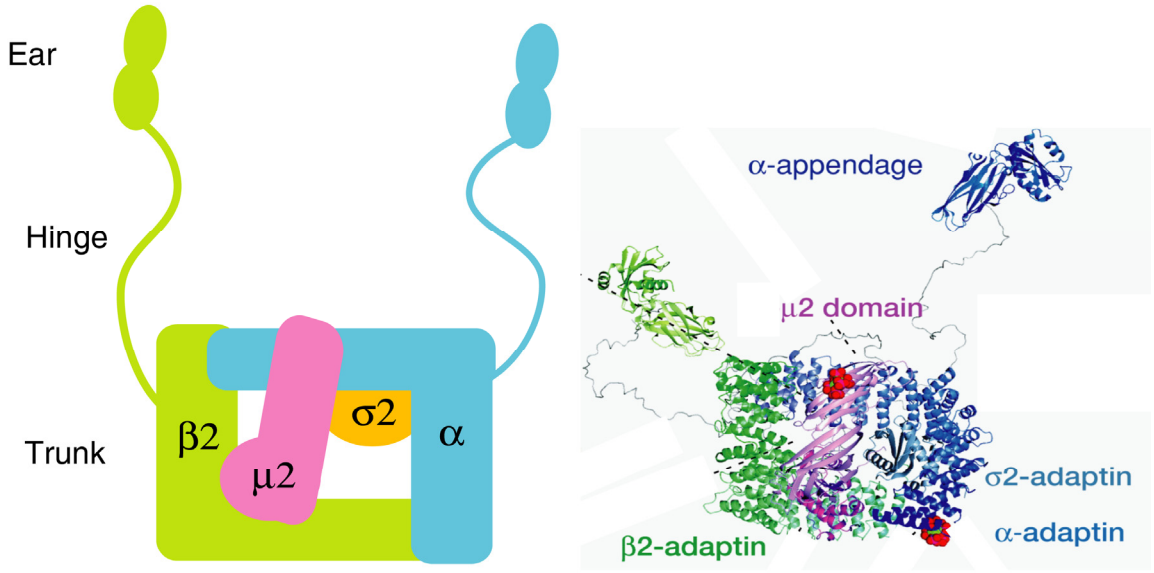

Figure 4. Structure and organization of the heterotetrameric adaptor complex AP-2. AP-2 is a heterotetrameric adaptor molecule that mediates cargo recruitment and clathrin coated vesicle formation. AP-2 is composed of four distinct subunits: the large subunits α and β2, the medium subunit μ2, and the small subunit σ2. AP-2's function as a protein interaction hub follows from its unique structure, which consists of trunk domains and ear domains that are linked by a flexible hinge region. This structure enables the complex to mediate simultaneous interactions with clathrin, transmembrane cargo receptors, other clathrin accessory proteins, and lipids in the plasma membrane. A crystal structure of AP-2 is shown on the right and is modified from (Owen *et al.*, 2004).

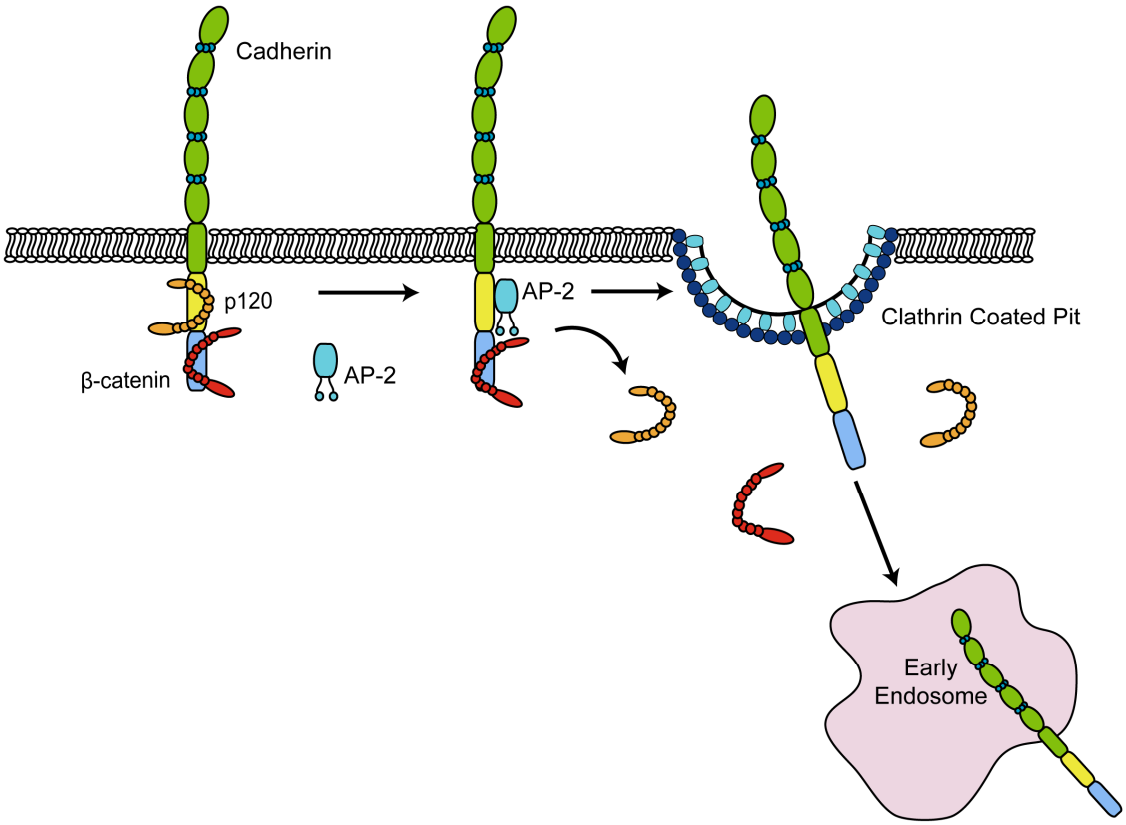

Figure 5. Model for the regulation of VE-cadherin endocytosis by p120 preventing adaptor interactions with the cadherin tail.

p120 may function as a "cap" on the cadherin cytoplasmic tail. In this model, the binding of p120 to the cadherin juxtamembrane domain prevents clathrin adaptor proteins, such as AP-2 from interacting with sorting motifs in the VE-cadherin tail. Upon dissociation of p120 from the cadherin juxtamembrane domain, the cadherin would be recruited into clathrin-coated vesicles and internalized from the plasma membrane.

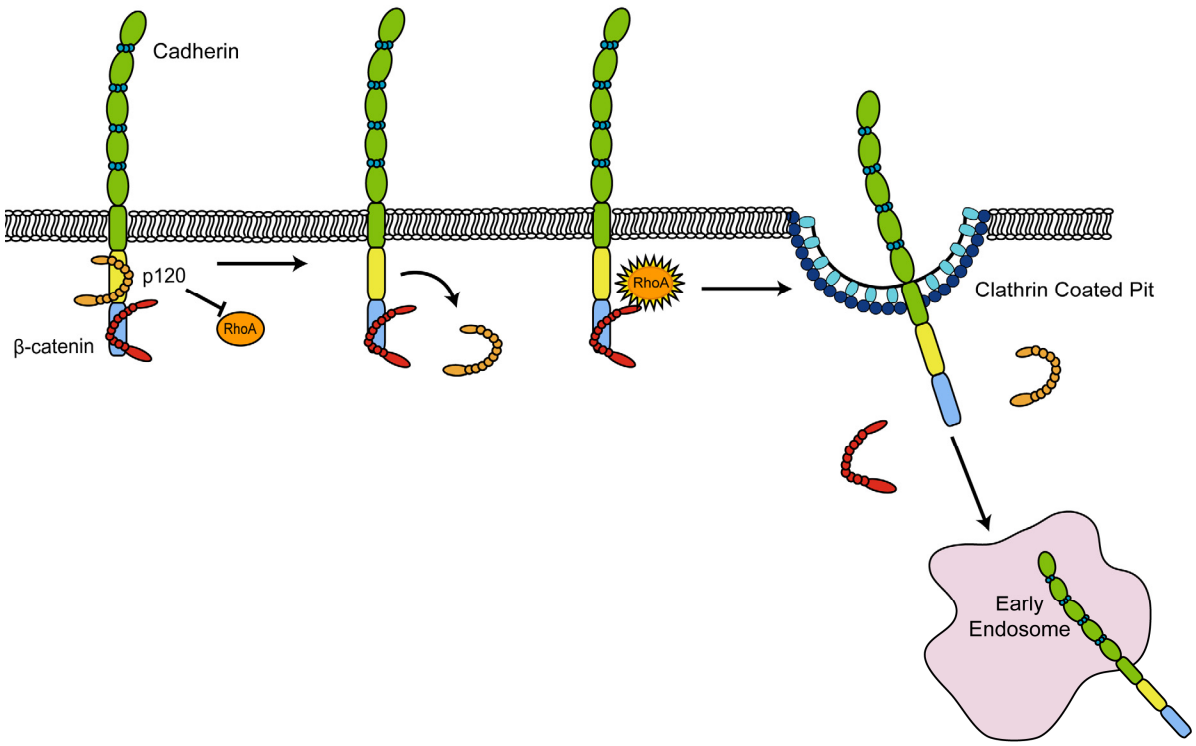

Figure 6. Model for the regulation of VE-cadherin endocytosis by p120-mediated inhibition of RhoA activity.

p120's regulation of RhoGTPase activity may also be involved in the regulation of cadherin endocytosis. In this model, p120 locally inhibits RhoA activity at adherens junctions when bound to the cadherin juxtamembrane domain. The inhibition of RhoA activity would prevent the cadherin from being internalized. Upon p120 dissociation from the cadherin juxtamembrane domain, the inhibition of RhoA would be relieved, leading to increased RhoA activity and cadherin endocytosis.

Chapter 3

p120-Catenin Inhibits VE-cadherin Internalization Through a Rho-Independent Mechanism

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INTRODUCTION

VE-cadherin is a central component of endothelial adherens junctions and mediates homophilic interactions between neighboring endothelial cells. Adhesion and signaling events mediated by VE-cadherin play key roles in vascular barrier function and angiogenesis (Vestweber, 2008a; Wallez and Huber, 2008). Likewise, several endothelial signaling pathways are known to regulate endothelial cell-cell interactions through regulation of VE-cadherin or other adherens junction molecules (Gavard and Gutkind, 2006; Lampugnani et al., 2006). The cytoplasmic domain of VE-cadherin interacts with members of the armadillo family of proteins, including β-catenin and p120-catenin (p120). These interactions regulate association of cadherins with the actin cytoskeleton and are important for strong cell-cell adhesion (Pokutta and Weis, 2007; Hartsock and Nelson, 2008). Furthermore, gene ablation studies have revealed key roles for VE-cadherin, β-catenin and p120 in vascular development, underscoring the importance of adherens junction components in vascular biology and vertebrate development (Carmeliet *et al.*, 1999; Gory-Faure *et al.*, 1999; Cattelino *et al.*, 2003) (Xiao, Oas, and Kowalczyk unpublished observation).

An important aspect of the ability of cells to dynamically modulate their adhesive state is the regulation of cadherin availability at the cell surface. One cellular mechanism that is commonly used to rapidly alter the expression of cell surface receptors is membrane trafficking. Cells utilize several endocytic pathways to retrieve receptors from the cell surface. The most common of these is clathrin dependent, in which transmembrane cargos are sorted into clathrin coated pits at the plasma membrane.

Endocytic adaptor proteins, including the tetrameric adaptor complex AP-2, play a fundamental role in the recruitment and formation of clathrin coated vesicles at the plasma membrane through the ability to bind to lipids, cargo, clathrin, and clathrin accessory proteins (Bonifacino and Traub, 2003; Maldonado-Báez and Wendland, 2006). Clathrin independent pathways for receptor endocytosis are increasingly recognized as important portals of internalization (Mayor and Pagano, 2007). Cadherins can be internalized through both clathrin dependent and independent pathways, depending on the cadherin and the cellular context (Chiasson and Kowalczyk, 2008; Delva and Kowalczyk, 2008). Evidence suggests that E-cadherin and VE-cadherin are primarily internalized through clathrin dependent pathways. However, a recent study demonstrated that the desmosomal cadherin desmoglein 3 is internalized through a clathrin and dynamin independent pathway (Delva *et al.*, 2008). It remains unclear how the decision for cadherins to enter different endocytic compartments is regulated according to the needs of the cell.

Several years ago, a series of studies revealed a core function for p120 in regulating cadherin mediated adhesion. p120 acts as a setpoint for cadherin expression by controlling the amount of cadherin available at cell junctions for adhesion (Davis *et al.*, 2003; Xiao *et al.*, 2003a; Xiao *et al.*, 2007; Chiasson and Kowalczyk, 2008). In the absence of p120, cadherin-based junctions are destabilized and the cadherin is targeted for degradation through an endosomal-lysosomal pathway. In addition to regulating cadherin endocytosis, p120 also functions in a signaling capacity as a potent regulator of Rho-GTPase activity within the cell (Anastasiadis, 2007). Rho-GTPases make up a large family of proteins that regulate cytoskeletal organization. The first evidence for the

involvement of p120 in regulating Rho-GTPase signaling was the observation of a dramatic branching phenotype in cells expressing high levels of exogenous p120 characterized by the formation of long dendritic processes extending from the cell body (Reynolds *et al.*, 1996; Anastasiadis *et al.*, 2000; Noren *et al.*, 2000). These and other studies have demonstrated that p120 acts as an inhibitor of RhoA, and through this activity p120 appears to regulate cell migration, proliferation, and inflammatory responses.

Although p120's functions in stabilizing cadherins and regulating RhoA signaling are well established, the relationship between these roles is unclear. Early studies suggested that p120-mediated inhibition of RhoA activity is spatially restricted to the cytoplasm, and therefore mutually exclusive with the role of p120 at adherens junctions (Anastasiadis et al., 2000; Fox and Peifer, 2007; Yanagisawa et al., 2008). However, a recent study has proposed a potential mechanism linking these functions of p120, at least in certain cellular contexts. Following PDGF signaling in fibroblasts, p120 was found to be required for the localized inhibition of RhoA activity through a mechanism that requires the recruitment of p190RhoGAP to cadherin based junctions. Interestingly, p190RhoGAP not only inhibits RhoA, but also promotes adhesion (Wildenberg et al., 2006). In the absence of p190RhoGAP, N-cadherin is mislocalized from cell junctions. These data provide the first evidence that localized inhibition of Rho activity by p120 at cell junctions may regulate cadherin localization and stability. These data raise the possibility that p120 mediated inhibition of RhoA may in turn regulate entry of cadherins into an endocytic pathway.

In the present manuscript, we directly tested whether p120 inhibition of endocytosis and regulation of RhoA are mechanistically interdependent. Using a series of approaches, we demonstrate that p120 potently inhibits entry of the cadherin into clathrin and AP-2 enriched membrane domains. VE-cadherin is internalized through a pathway that is dependent upon clathrin, dynamin, and the clathrin adaptor complex AP-2. Lastly, we found that the ability of p120 to inhibit cadherin endocytosis can be experimentally uncoupled from p120 inhibition of RhoA, indicating that binding to the cadherin tail, but not the ability of p120 to inhibit Rho, is the crucial function of p120 in preventing endocytosis. These findings clarify the mechanism by which p120 regulates cadherin endocytosis and distinguish this activity from p120 regulation of RhoA.

MATERIALS AND METHODS

Cell Culture. Primary cultures of dermal microvascular endothelial cells (MECs) from human neonatal foreskin were isolated and cultured in MCDB 131 medium as described previously (Xiao et al., 2005). The medium was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), L-anyl-glutamine (Mediatech, Herndon, VA), camp (Sigma-Aldrich), hydrocortisone (Sigma-Aldrich), epidermal growth factor (Intergen, Purchase, NY), and antibiotic/antimyotic (Invitrogen, Carlsbad, CA). HMEC-1 cells were cultured as previously described (Venkiteswaran et al 2002). Cells were generally cultured overnight on 0.1% gelatin-coated plates and grown to ~80% confluency for experiments. For adenovirus production human embryonic kidney cell line QBI-293A (Qbiogene, Carlsbad, CA) were routinely cultured in DMEM supplemented with 10% FBS and antibiotic/antimycotic. Chloroquine was purchased

from Sigma-Aldrich and used at 100 μM. Cell permeable C3 Transferase was purchased from Cytoskeleton, Inc.(Denver, CO) and used at 1.0 μg/ml. Y27632 was purchased from Calbiochem (San Diego, CA) and used at 10 μM.

Adenovirus Production. Adenoviral reagents for the IL-2R-VE-cad$_{cyto}$ chimera, IL-2R-Dsg3$_{cyto}$ chimera and p120ctn 1A were generated as described previously (Xiao *et al.*, 2005). To generate the p120-4A K622,628A adenovirus, PCR was performed using a p120-1A K622,628A construct (provided by A. Reynolds, Vanderbilt University, Nashville, TN) as a template and the forward primers consisting of the start site of p120-4A isoform with a Not1 restriction site and Kozak (5'-GCGGCCGCGCCACCATGATTGGTGAGGAGGTGCCA-3') along with the reverse primer containing a Swa1 site and the C-terminus of p120 (5'-ATTTAAATGAATCTTCTGCATCAAGGGTGC-3'). The recombinant fragment was placed in pShuttle in front of EGFP. Adenovirus was then produced using the AdEasy packaging system described previously (Xiao et al., 2005). The dominant negative DynII (K44A) adenovirus (Altschuler et al., 1998) was provided by S. Schmid (The Scripps Institute, La Jolla, CA) and the dominant negative RhoA (N19) adenovirus (Kalman et al., 1999) was provided by D. Kalman (Emory University, Atlanta, GA).

Immunofluorescence: Immunofluorescence was carried out as described previously (Xiao et al., 2005). Antibodies used were as follows: rabbit anti VE-cadherin (Alexis Biochemicals, San Diego, CA), anti-IL-2R IgG from 7G7B6 mouse hybridoma (American Type Culture Collection, Manassas, VI), chicken anti myc epitope tag (Bethyl

Laboratories, Montgomery, TX), a mouse monoclonal antibody (mAb) (BD Biosciences Pharmigen) or a polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) against p120, mouse mAB against dynamin (BD Biosciences Pharmigen), mouse mAb against RhoA (Santa Cruz Biochemicals), mouse mAbs against clathrin (BD Transduction Labs) or AP-2 (Santa Cruz Biochemicals), a rabbit antibody against caveolin-1 (BD Biosciences Pharmigen). The localization of actin was determined with an Alexa fluor 488 conjugated phalloidin (Molecular Probes, Eugene, OR). Secondary antibodies conjugated to Alexa Fluors (Molecular Probes) were used for dual and triple-label experiments. Microscopy was performed using either a wide field fluorescence microscope (model DMR-E; Leica, Wetzlar, Germany) equipped with narrow band pass filters and a digital camera (model Orca; Hamamatsu, Bridgewater, NJ) or an inverted Leica DMI-6000B microscope equipped with an Infinity II confocal scanning module, 561nm and 491nm lasers, and a Hamamatsu CCD camera (C9100-12). Images were captured, pseudo colored, and processed using Simple PCI software (Compix, Cranberry Township, PA) or Metamorph Software (Universal Imaging, West Chester, PA). For colocalization experiments, a nearest neighbors deconvolution algorithm was performed on successive .20 μm-focal planes.

Internalization Assay: Assays to follow internalization of transferrin and the IL-2R-VE-cadherin polypeptides were carried out as previously described (Xiao et al., 2005). For colocalization experiments with clathrin and AP-2, cells were labeled at 4ºC with IL-2R antibodies and then incubated for 5 minutes at 37ºC to allow for internalization. Cells were then fixed and processed for immunofluorescence without being acid washed.

Potassium depletion was performed as described previously (Xiao et al., 2005), and cells were treated with 5 μg/ml chlorpromazine for 30 min. at 37ºC . To measure internalization of endogenous VE-cadherin, cells were treated with 100 μM chloroquine for 3 hours. Cells were then rinsed, fixed, and processed for immunofluorescence as described above. Internalization was quantified using Simple PCI software to measure either total intracellular fluorescence or object number. Statistical analysis was performed using two-tailed *t* test. Error bars represent standard error of the mean.

Cross-linking and Immunoprecipitation: To analyze low affinity interactions between VE-cadherin and AP-2, we performed cross-linking in MEC with DSP [dithiobis(succinimidylpropionate)] as described (Craige *et al.*, 2008; Salazar *et al.*, 2008). Briefly, MEC were grown to confluency in 10 cm dishes and infected with Il-2R-VE-cadherin constructs. On the day of the experiment, cells were placed on ice, rinsed twice with PBS, and incubated with 1 mM DSP (Pierce, Rockford, IL) or vehicle for 2 hours on ice. The reaction was quenched by adding 25 mM Tris. The cells were then lysed in buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM $MgCl_2$, pH 7.4) + 0.5% TX-100, scraped from the dish, and incubated on ice for 30 min. Cell homogenates were centrifuged at 16,100 x *g* for 10 min. and supernatants were diluted to 1 mg/ml in 0.5 ml of Buffer A + 0.5% TX-100. The supernatants were incubated overnight at 4ºC with Dynal magnetic beads (Invitrogen) conjugated to mAbs against AP-2 (α subunit, BD Transduction Labs) or clathrin (Calbiochem, San Diego, CA). The beads were then washed with buffer A + 0.1% TX-100 and eluted with SDS-PAGE

sample buffer at 75ºC for 5 min. Immunoblotting under reducing conditions reverses the DSP cross-linking and allows for the detection of immunoprecipitated material.

siRNA Knockdown of AP-2: siRNA oligonucleotides to the AP-2 μ subunit and control, non-targeting siRNA were purchased from Dharmacon (Lafayette, CO) and have been previously described (Urs et al. 2008, Motley et al. 2003). HMEC-1 cells were seeded in 60 mm dishes at 50% confluency. Cells were transfected on consecutive days with 200 pM siRNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Twenty-four hours after the second transfection, cells were trypsinized and plated onto coverslips or 35 mm dishes to assay internalization or efficiency of knockdown. Cells were infected with adenovirus expressing IL-2R-VE-cad$_{cyto}$ at the time of plating. Internalization assays were performed as described above.

RESULTS

VE-cadherin is internalized via a clathrin, AP-2, and dynamin dependent pathway

The cytoplasmic domain of VE-cadherin mediates internalization of the cadherin from the cell surface, and p120 is a potent inhibitor of this process (Xiao et al., 2005). However, the molecular machinery utilized for VE-cadherin internalization and the mechanism of p120 inhibition are not fully understood. In previous studies, we demonstrated that a chimeric molecule consisting of the cytoplasmic domain of VE-cadherin fused to the extracellular domain of the interleukin (IL)-2 receptor (IL-2R-VE-cad$_{cyto}$) is rapidly internalized from the cell surface (Xiao et al., 2005). To further elucidate the endocytic machinery used by VE-cadherin, the IL-2R-VE-cad$_{cyto}$ chimera

was expressed in primary cultures of human MECs and a series of pharmacological and genetic approaches were utilized to inhibit endocytosis. Cells were subjected to K+ depletion (Salazar and Gonzalez, 2002; Xiao *et al.*, 2005) or treatment with chlorpromazine (Wang *et al.*, 1993)to inhibit clathrin dependent endocytosis. To measure internalization of IL-2R-VE-cad$_{cyto}$ chimera, MECs were incubated at 4ºC with antibodies directed against IL-2R to label the cell surface. Following incubation at 37ºC for 5 minutes to allow for endocytosis, cells were washed with a low pH acid wash buffer to remove antibody remaining at the cell surface, making it possible to distinguish internalized IL-2R-VE-cad$_{cyto}$ from that at the cell surface. A myc epitope at the carboxy-terminal tail of the VE-cadherin cytoplasmic domain was used to identify cells expressing the IL-2R-VE-cad$_{cyto}$. As shown in Figure 1, untreated cells expressing the IL-2R-VE-cad$_{cyto}$ chimera were found to display high levels of internalized IL-2R-VE-cad$_{cyto}$ following 5 minutes of internalization at 37ºC (Figure 1, A, a'-b', B). As expected from previous data (Xiao et al. 2005) K+ depletion dramatically reduced the extent of internalization of the IL-2R-VE-cad$_{cyto}$ chimera (Figure 1, A, c'-d', B). Treatment with chlorpromazine, which disrupts localization of the clathrin adaptor complex AP-2 to membranes and prevents clathrin assembly at the plasma membrane (Wang et al., 1993), also strongly inhibited internalization of the IL-2R-VE-cad$_{cyto}$ chimera (Figure 1, A, e'-f', B).

To determine if dynamin, which is required for scission of clathrin-coated vesicles from the plasma membrane (Ungewickell and Hinrichsen, 2007), is involved in VE-cadherin endocytosis, a dominant negative dynamin II (DynII K44A) mutant was utilized (Altschuler et al., 1998). Transferrin internalization was entirely blocked by expression of

DN DynII (Figure 1, C and D). Intracellular accumulation of endogenous VE-cadherin following treatment with chloroquine to inhibit lysosomal degradation was used as a measure of VE-cadherin endocytosis (Xiao *et al.*, 2003a). Chloroquine treated cells expressing DN DynII (Figure 1, E, e'-f', F) had limited vesicular staining of VE-cadherin compared to cells expressing empty vector (Figure 1, E c'-d', F), suggesting a role for dynamin in VE-cadherin endocytosis.

Inhibition of VE-cadherin endocytosis by inhibitors of clathrin mediated endocytosis suggests a role for adaptor molecules involved in the formation and transport of clathrin coated vesicles. To determine if the adaptor complex AP-2 is required for VE-cadherin endocytosis, we used siRNA to deplete expression of AP-2. For these experiments, HMEC-1 cells, an immortalized line of dermal microvascular endothelial cells, were used to overcome transfection inefficiencies in primary cells. Treatment of cells with siAP-2, but not siControl, reduced expression of AP-2 by 75% as measured by western blot (Figure 1, G). Treatment with siAP-2 also inhibited internalization of transferrin, a well characterized AP-2 cargo, by 80% (Figure 1, H-I). Internalization of IL-2R-VE-cad$_{cyto}$ was also reduced by 80% in siAP-2 treated cells compared to siControl treated cells (Figure 1, J-K). A dominant negative AP-2 mutant also reduced internalization of endogenous VE-cadherin in chloroquine treated cells (data not shown). Together, these results demonstrate that VE-cadherin endocytosis is dependent on components of the clathrin endocytic machinery, including AP-2 and dynamin.

To test the hypothesis that the VE-cadherin cytoplasmic tail forms a complex with clathrin, we immunoprecipitated clathrin from MEC lysates expressing either IL-2R or IL-2R-VE-cad$_{cyto}$ chimera. Reducing SDS-PAGE and immunoblotting with IL-2R

antibodies revealed that the IL-2R-VE-cad$_{cyto}$ chimera co-precipitated with clathrin but the IL-2R lacking the VE-cadherin tail did not (Figure 2, compare lanes 13 and 14), demonstrating that clathrin specifically associates with the VE-cadherin cytoplasmic domain. To analyze interactions between VE-cadherin and adaptor complexes, we used limited whole cell crosslinking followed by immunoprecipitation. DSP, a homobifunctional cell permeable crosslinker, was used to stabilize cargo-adaptor interactions (Craige et al., 2008). In cells treated with DSP, IL-2R-VE-cad$_{cyto}$, but not IL-2R, was consistently immunoprecipitated with AP-2 (Figure 2, lanes 11-12). IL2-R and IL-2R-VE-cad$_{cyto}$ did not coprecipitate with beads without antibody (Figure 2, lanes 5-8), demonstrating that clathrin and AP-2 specifically interact with the VE-cadherin cytoplasmic tail.

To determine if the association between clathrin, AP-2 and the VE-cadherin tail could be observed in cells, the localization of these proteins was examined by deconvolution immunofluorescence microscopy. Transferrin was used as a positive control for colocalization with clathrin. A chimeric molecule consisting of the extracellular domain of the IL-2 receptor fused to the cytoplasmic tail of the desmosomal cadherin desmoglein 3 (IL-2R-Dsg3$_{cyto}$), which has been shown to be internalized through a clathrin-independent pathway (Delva et al. 2008), was used as a negative control. To measure colocalization, cells expressing IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ were labeled with IL-2R antibodies at 4ºC and transferred to 37ºC for 5 minutes to allow for internalization. For transferrin, uninfected cells were labeled with fluorescently conjugated transferrin at 4ºC and transferred to 37ºC for 5 minutes. In contrast to internalization assays, cells were not acid washed, allowing for the

visualization of clusters of the chimeric molecules at early endocytic steps. As expected, extensive colocalization was observed between transferrin and clathrin (Figure 3, A, a'-d', B). We also observed significant levels of colocalization between IL-2R-VE-cad$_{cyto}$ and clathrin (Figure 3, A, e'-h', B). This colocalization was dependent on the VE-cadherin cytoplasmic tail, since only minimal colocalization was observed between IL-2R or IL-2R-Dsg3$_{cyto}$ and clathrin (Figure 3, A, i'-p', B). Furthermore, the IL-2R-VE-cad$_{cyto}$ did not colocalize with caveolin-1 (Figure 3, A, q'-t', B), providing additional evidence that VE-cadherin is internalized through a clathrin dependent pathway. Significant colocalization was also observed between IL-2R-VE-cad$_{cyto}$ and AP-2 (Figure 3, C, e'-h', D), but not between IL-2R and AP-2 (Figure 3, C, a'-d', D) or IL-2R-Dsg3$_{cyto}$ and AP-2 (data not shown). These data indicate that VE-cadherin associates with clathrin and AP-2 and localizes to membrane domains enriched in both proteins during internalization.

p120 prevents VE-cadherin from entering a clathrin and AP-2 enriched membrane domain

To test the hypothesis that p120 inhibits the entry of VE-cadherin into membrane domains enriched in clathrin and AP-2, we monitored the localization of the IL-2R-VE-cad$_{cyto}$ chimera in cells expressing exogenous p120. Interestingly, in cells expressing exogenous p120 the IL-2R-VE-cad$_{cyto}$ chimera did not form punctate clusters as seen in control cells (Figure 4, A and C, a'-j'). Additionally, colocalization between the IL-2R-VE-cad$_{cyto}$ chimera and clathrin or AP-2 was dramatically reduced in these cells (Figure 4, B and D). To test whether this function of p120 requires its interaction with the

juxtamembrane domain (JMD) of VE-cadherin, we expressed a mutant IL-2R-VE-cad$_{cyto}$ construct which contains a triple alanine substitution in the p120 binding site of VE-cadherin (IL-2R-VE-cad$_{JMD-AAA}$) (Xiao et al. 2005). This mutation completely abrogates the binding of p120 to VE-cadherin (Calkins et al 2003). The IL-2R-VE-cad$_{JMD-AAA}$ displays a punctate localization and colocalizes with clathrin following incubation at 37ºC in cells containing endogenous levels of p120 (Figure 4, A, k'-o'). However, unlike the wild type IL-2R-VE-cad$_{cyto}$ chimera, the distribution and colocalization of the IL-2R-VE-cad$_{JMD-AAA}$ chimera was not affected by exogenous expression of p120 (Figure 4, A, p'-t', B). These results indicate that p120 prevents VE-cadherin from localizing to clathrin and AP-2 enriched membrane domains in a manner that requires its binding to the VE-cadherin JMD.

p120 inhibits VE-cadherin endocytosis independently of RhoA

In addition to its central function in regulating cadherin expression at adherens junctions, an important role has also been established for p120 in regulating Rho GTPases, particularly as an inhibitor of RhoA activity. To determine if this function of p120 is integral to its ability to stabilize VE-cadherin at the plasma membrane by preventing VE-cadherin endocytosis, we utilized several methods to inhibit Rho activity in endothelial cells and measured the effect on internalization of endogenous VE-cadherin. As expected, expression of exogenous p120 inhibited the accumulation of intracellular VE-cadherin following chloroquine treatment (Figure 5, A, e'-f'). Expression of a dominant negative RhoA mutant (N19) (Figure 5, A, g'- i'), treatment with the selective Rho inhibitor C3 toxin (Figure 5, A, j'-k'), or treatment with Y27632

(Figure 5, A, l'- m'), an inhibitor of ROCK, a downstream Rho effector, were effective in reducing RhoA activity within endothelial cells, as indicated by the dramatic loss of actin stress fibers. However, none of these treatments had any discernable impact on internalization of VE-cadherin in chloroquine treated cells (Figure 5, B and C), suggesting that inhibition of RhoA does not phenocopy the activity of p120.

To look specifically at the role of local p120-dependent regulation of RhoA on VE-cadherin stability, we tested the effects of a p120 mutant that is unable to inhibit RhoA activity. The p120 mutant lacks the N-terminal regulatory domain and also contains mutations within the Rho binding domain (p120 4A K622,628A). Deletion of these domains eliminates p120s ability to inhibit RhoA activity (Castano et al., 2007; Yanagisawa et al., 2008). In MECs expressing high levels of wild type p120, cells exhibit a dramatic branching phenotype characterized by dendritic outgrowths, which have been shown to result from loss of RhoA activity (Figure 6, A, a'). No cells with this branching phenotype could be found in cells expressing the p120 Rho uncoupled mutant, even at very high levels (Figure 6, A, b'). Next, internalization of endogenous VE-cadherin in cells expressing either wild type p120 (Figure 6, B, c'-d') or the Rho uncoupled p120 mutant (Figure 6, B, e'-f') was assessed. Both wild type p120 and the Rho uncoupled p120 mutant significantly reduced the amount of internalized VE-cadherin (Figure 6, C). To determine if p120 Rho uncoupled mutant can block the association of VE-cadherin with clathrin and the AP-2 adaptor, we measured colocalization between the IL-2R-VE-cad_{cyto} and clathrin (Figure 7, A) or AP-2 (Figure 7, C) in cells expressing either wild type (Figure 7, A and C, f'- j') or mutant p120 (Figure 7, A and C, k'- o'). The p120 Rho uncoupled mutant was able to prevent colocalization between the IL-2R-VE-cad_{cyto} and

clathrin (Figure 7, B) and AP-2 (Figure 7, D) similarly to wild type p120. Together, these findings suggest that p120 stabilizes VE-cadherin at the plasma membrane by preventing the cadherin from entering a clathrin and AP-2 dependent endocytic pathway. The mechanism by which p120 functions in this manner requires its interaction with the VE-cadherin cytoplasmic tail, but occurs independently of RhoA activity.

DISCUSSION

The results presented in this study demonstrate that p120 stabilizes VE-cadherin at the plasma membrane by preventing its entry into a clathrin, AP-2, and dynamin dependent endocytic pathway through a mechanism that does not involve RhoA. These studies identify the endocytic machinery involved in VE-cadherin endocytosis and help to clarify the mechanism by which p120 functions as a set point to regulate cadherin expression levels. Additionally, they establish a novel role for p120 as part of a plasma membrane retention mechanism which modulates the availability of a cargo protein for recruitment into membrane trafficking pathways.

In previous studies, we demonstrated that the cytoplasmic domain of VE-cadherin mediates rapid endocytosis of the protein and that p120 prevents VE-cadherin from being internalized (Xiao et al., 2005). However, these previous studies did not fully explain how p120 functions to stabilize the cadherin at the plasma membrane. In the present study, we found that internalization of endogenous VE-cadherin or an IL-2R-VE-cad$_{cyto}$ chimera was reduced by inhibition of clathrin, AP-2, or dynamin dependent endocytosis (Figure 1). Following a short period of internalization, a significant fraction of the IL-2R-VE-cad$_{cyto}$ chimera colocalizes with clathrin and AP-2 (Figure 3), providing further

evidence that VE-cadherin is internalized through a clathrin mediated pathway. We were also able to specifically immunoprecipitate the IL-2R-VE-cad$_{cyto}$ with antibodies against clathrin and the clathrin adaptor AP-2 (Figure 2). Together, these results provide the first evidence for a role for the AP-2 adaptor complex in the trafficking of VE-cadherin through a clathrin mediated pathway.

Expression of exogenous p120 dramatically inhibits internalization of VE-cadherin during the earliest stages of endocytosis. The IL-2R-VE-cad$_{cyto}$ colocalizes extensively with clathrin and AP-2. However, in cells expressing exogenous p120, the IL-2R-VE-cad$_{cyto}$ fails to colocalize with clathrin or AP-2. Importantly, the interaction of p120 with the VE-cadherin JMD is crucial for p120's ability to prevent VE-cadherin from clustering with components of the clathrin machinery (Figure 4). These data support previous findings that p120 prevents VE-cadherin endocytosis and indicate that p120 acts to stabilize VE-cadherin at the plasma membrane at an early step in endocytosis, most likely by preventing adaptor binding and entry into a clathrin enriched membrane domain.

In addition to functioning as a critical regulator of adherens junction stability, p120 also plays a key signaling role in regulating activity of Rho-GTPases (Anastasiadis, 2007). The relationship between the function of p120 in regulating RhoA activity and its role in stabilizing cadherins at cell junctions is not understood. The data presented in this study indicate that p120 regulates cadherin stability at the plasma membrane independently of Rho activity. Internalization of VE-cadherin was not impacted by inhibition of Rho activity by either genetic or pharmacological manipulation (Figure 5). Similarly, a p120 mutant that is unable to inhibit Rho activity retains its ability to prevent

VE-cadherin endocytosis (Figure 6) and also prevents the IL-2R-VE-cad$_{cyto}$ from colocalizing with clathrin and AP-2 (Figure 7). Importantly, these data indicate that VE-cadherin endocytosis occurs independently of Rho activity and that p120 stabilizes VE-cadherin at the cell surface through a mechanism that does not require its Rho inhibitory function.

Based on our findings that the function of p120 in preventing VE-cadherin endocytosis can be uncoupled from the ability of p120 to inhibit RhoA, we hypothesize that p120 prevents the VE-cadherin cytoplasmic tail from interacting with components of the endocytic machinery that selectively recruit transmembrane receptors into a clathrin-mediated pathway. The identification of a complex containing VE-cadherin, clathrin, and AP-2, together with the inhibitory effect of silencing AP-2 expression on the internalization of VE-cadherin, demonstrates an important role for the AP-2 adaptor complex in the regulation of VE-cadherin endocytosis. In general, clathrin interacts with transmembrane cargo through multiple adaptor and accessory proteins (Bonifacino and Traub, 2003; Robinson, 2004; Sorkin, 2004). Recently, VEGF-induced internalization of VE-cadherin was found to involve β-arrestin (Gavard and Gutkind, 2006), a clathrin adaptor that often works cooperatively with AP-2 to regulate clathrin-dependent endocytosis. Studies are currently underway to further characterize the interaction between VE-cadherin and AP-2, to identify additional adaptor proteins that may be required for VE-cadherin endocytosis, and to determine if p120 prevents them from interacting with the VE-cadherin cytoplasmic tail.

The ability of p120 to prevent VE-cadherin from entering domains enriched in clathrin and AP-2 in a manner that requires p120 binding to the VE-cadherin

juxtamembrane domain further supports a model in which p120 functions as a cap on the VE-cadherin cytoplasmic tail to prevent adaptor interactions. An alternative model is that p120 stabilizes cadherins at the plasma membrane by inhibiting the activity of another component of the endocytic machinery. Based on this hypothesis, one would predict that p120 may function as a general inhibitor of clathrin-dependent endocytosis. Thus far, we have found no evidence that this is the case. p120 has no effect on internalization of transferrin receptor or epidermal growth factor receptor (EGFR) (Xiao et al. 2005, unpublished observation). Additionally, the requirement for p120 binding to the cadherin tail for the inhibitory effect suggests that p120's function is cadherin specific. Therefore, we currently favor a model in which the association of p120 with the VE-cadherin JMD prevents the clathrin adaptor AP-2 from recruiting VE-cadherin into clathrin coated pits.

Endocytic adaptor proteins play a fundamental role in the recruitment and formation of clathrin coated vesicles at the plasma membrane. The recruitment of transmembrane cargo receptors into clathrin-coated vesicles by AP-2 and the formation of a stable network of interactions between cargo, adaptors, and clathrin is a key step in vesicle formation (Maldonado-Báez and Wendland, 2006). For these reasons, the regulation of the interaction between adaptor proteins and cargo molecules is crucial to the process of clathrin coated vesicle formation and trafficking. The ability of p120 to prevent cadherin recruitment into clathrin and AP-2 enriched membrane domains reveals a previously unappreciated mechanism by which clathrin mediated endocytosis is regulated. It remains to be determined whether this mechanism for endocytic regulation is unique to the cadherin tail-p120 interaction, or if other receptors and clathrin cargo are similarly regulated by cytoplasmic binding partners.

Figure 1

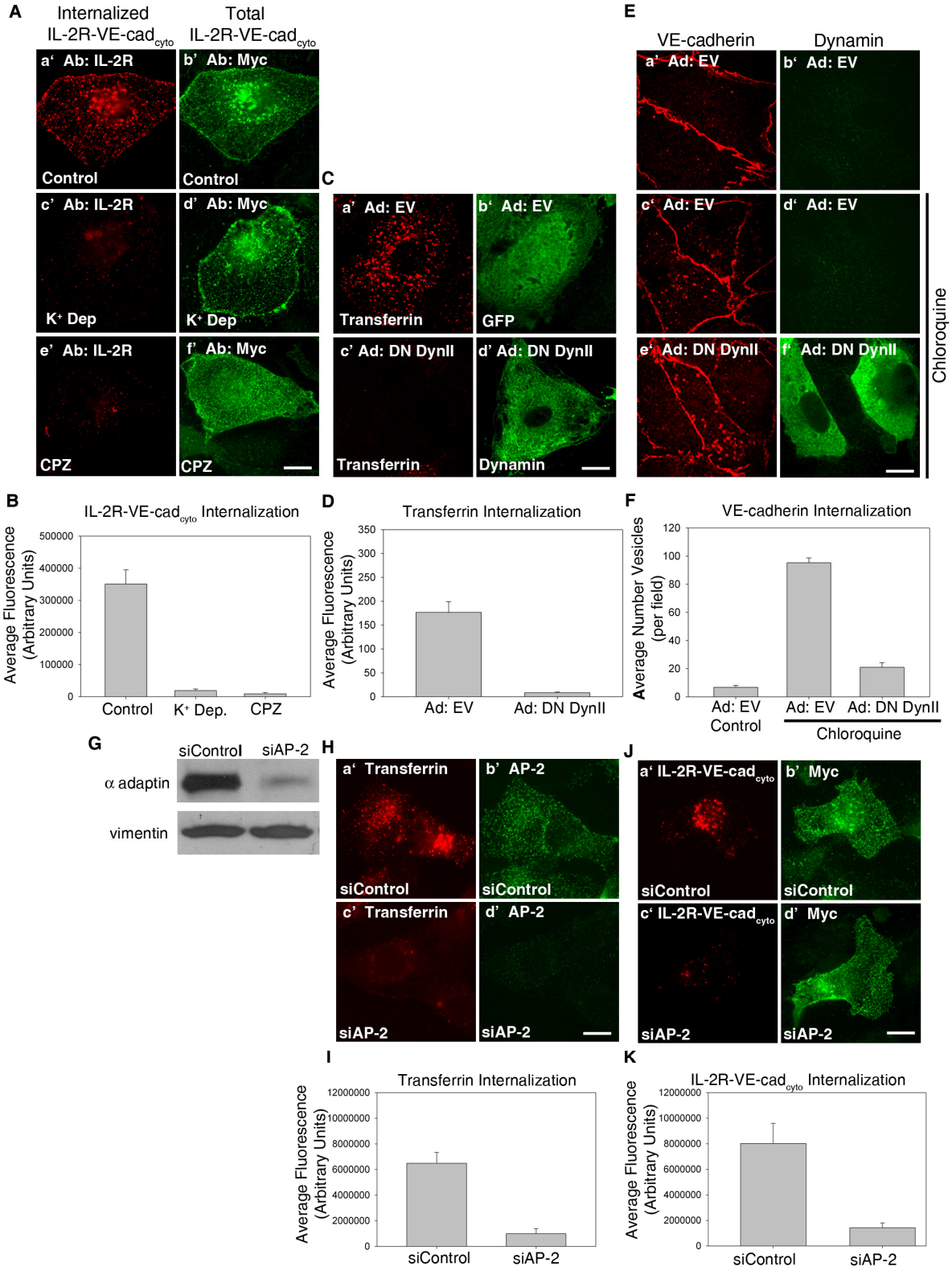
A
Internalized IL-2R-VE-cad$_{cyto}$
Total IL-2R-VE-cad$_{cyto}$
a' Ab: IL-2R
b' Ab: Myc
Control
Control
c' Ab: IL-2R
d' Ab: Myc
K+ Dep
K+ Dep
e' Ab: IL-2R
f' Ab: Myc
CPZ
CPZ
C
a' Ad: EV
b' Ad: EV
Transferrin
GFP
c' Ad: DN DynII
d' Ad: DN DynII
Transferrin
Dynamin
E
VE-cadherin
Dynamin
a' Ad: EV
b' Ad: EV
c' Ad: EV
d' Ad: EV
e' Ad: DN DynII
f' Ad: DN DynII
Chloroquine
B
IL-2R-VE-cad$_{cyto}$ Internalization
Average Fluorescence (Arbitrary Units)
500000
400000
300000
200000
100000
0
Control
K+ Dep.
CPZ
D
Transferrin Internalization
Average Fluorescence (Arbitrary Units)
350
300
250
200
150
100
50
0
Ad: EV
Ad: DN DynII
F
VE-cadherin Internalization
Average Number Vesicles (per field)
120
100
80
60
40
20
0
Ad: EV Control
Ad: EV
Ad: DN DynII
Chloroquine
G
siControl
siAP-2
α adaptin
vimentin
H
a' Transferrin
b' AP-2
siControl
siControl
c' Transferrin
d' AP-2
siAP-2
siAP-2
J
a' IL-2R-VE-cad$_{cyto}$
b' Myc
siControl
siControl
c' IL-2R-VE-cad$_{cyto}$
d' Myc
siAP-2
siAP-2
I
Transferrin Internalization
Average Fluorescence (Arbitrary Units)
12000000
10000000
8000000
6000000
4000000
2000000
0
siControl
siAP-2
K
IL-2R-VE-cad$_{cyto}$ Internalization
Average Fluorescence (Arbitrary Units)
12000000
10000000
8000000
6000000
4000000
2000000
0
siControl
siAP-2

Figure 1.

VE-cadherin endocytosis is mediated through a clathrin, AP-2, and dynamin dependent pathway. (A-B). Cell surface IL-2R-VE-cad$_{cyto}$ was labeled with IL-2R antibodies at 4ºC. Cells were incubated at 37ºC for 5 min, washed in a low pH buffer at 4ºC to remove surface bound antibody, and then processed for immunofluorescence to visualize intracellular IL-2R-VE-cad$_{cyto}$ (A, a',c',e'). Antibodies against the myc epitope tag at the carboxyl terminal domain of the IL-2R-VE-cad$_{cyto}$ were used to verify expression of the polypeptide (A, b',d',f'). Note that untreated cells exhibited high levels of internalized IL-2R-VE-cad$_{cyto}$, whereas treatment with K+ depletion or chlorpromazine completely inhibited internalization. (C-F) MECs were infected with adenoviruses expressing either GFP (C, a'-b') or dominant negative DynII (C, c'-d'). The DN DynII completely blocked internalization of Alexa Fluor 555 conjugated transferrin (C-D). To monitor internalization of VE-cadherin, MECs were either untreated (E, a'-b') or treated with 100 μM chloroquine for 3 hours (E, c'-f'). Note that intracellular vesicular accumulation of VE-cadherin is reduced in cells expressing DN DynII (E, e'- f', F). (G-K) HMEC-1 cells were transfected with an siRNA oligo targeted to the AP-2 μ subunit or a non-targeting control siRNA. Lysates prepared from cells treated with control siRNA or AP-2 siRNA were separated by SDS-PAGE and immunoblotted for AP-2 to confirm AP-2 knockdown (G). Western blot and immunofluorescence reveals about 75% reduction in AP-2 levels in siRNA treated cells compared to control, as well as an 85% decrease in transferrin internalization (G-I). IL-2R-VE-cad$_{cyto}$ internalization assays were performed and AP-2 siRNA treated cells (J, c'-d') exhibited an 80% decrease in internalization of IL-2R-VE-cad$_{cyto}$ compared to control (J, a'-b'), indicating a requirement for AP-2 in

VE-cadherin internalization (K). Error bars represent standard error of the mean where n>20 cells. Scale bar, 20 μm.

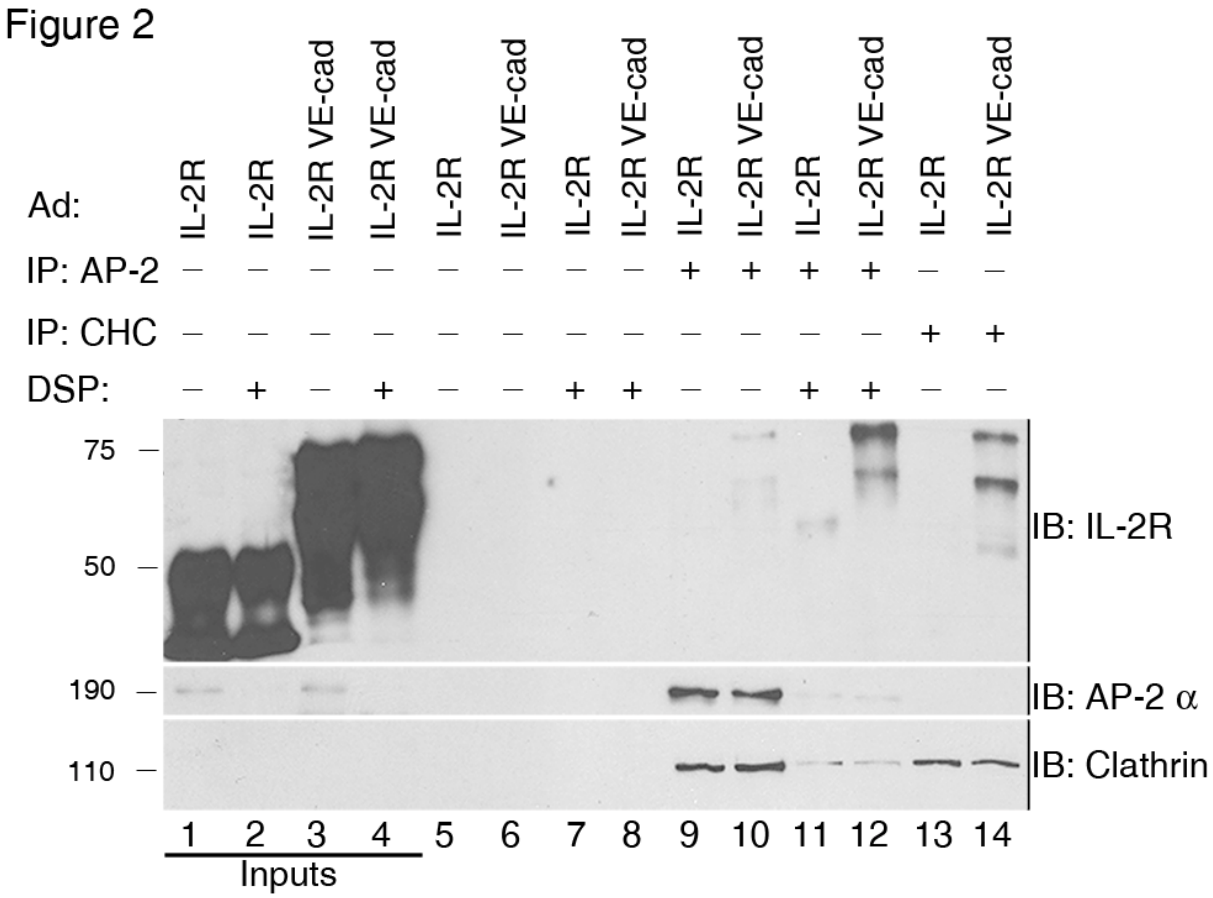

Figure 2.

The VE-cadherin cytoplasmic tail specifically associates with clathrin and AP-2.

MECs expressing IL-2R or IL-2R-VE-cad$_{cyto}$ were treated with DSP (lanes 2, 4, 7-8, 11-12) or DMSO vehicle (lanes 1, 3, 5-6, 9-10, 13-14), extracted in detergent, and proteins immunoprecipitated with beads alone (lanes 5-8), beads coated with AP-2 α antibodies (lanes 8-12), or beads coated with clathrin antibodies (CHC) (lanes 13-14). Western blot analysis using antibodies against IL-2R to detect IL-2R or IL-2R-VE-cad$_{cyto}$ demonstrates that the IL2R-VE-cad$_{cyto}$ specifically interacts with AP-2 (lane 12) and clathrin (lane 14). Inputs represent 5% of sample.

Figure 3

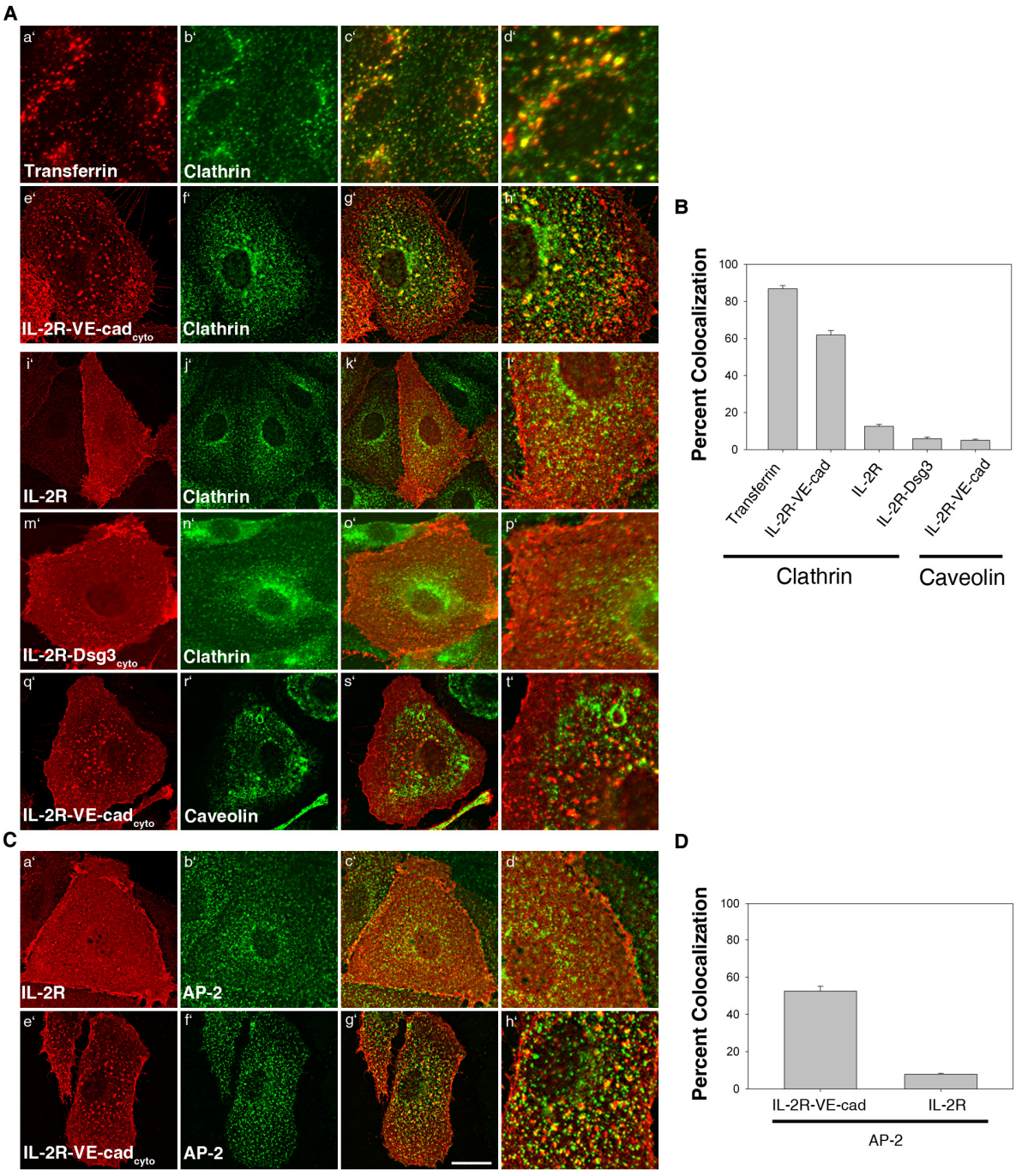
A
Transferrin
Clathrin
IL-2R-VE-cad cyto
Clathrin
IL-2R
Clathrin
IL-2R-Dsg3 cyto
Clathrin
IL-2R-VE-cad cyto
Caveolin
B
Percent Colocalization
100
80
60
40
20
0
Transferrin
IL-2R-VE-cad
IL-2R
IL-2R-Dsg3
IL-2R-VE-cad
Clathrin
Caveolin
C
IL-2R
AP-2
IL-2R-VE-cad cyto
AP-2
D
Percent Colocalization
100
80
60
40
20
0
IL-2R-VE-cad
IL-2R
AP-2

Figure 3.

The VE-cadherin cytoplasmic tail specifically colocalizes with clathrin and AP-2. MECs expressing IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ were labeled for 30 minutes at 4ºC with IL-2R antibodies, transferred to 37ºC for 5 minutes, and then processed for immunofluorescence microscopy. As a positive control, internalization of transferrin receptor was monitored by labeling MECs with fluorescently conjugated transferrin at 4ºC, transferring cells to 37ºC for 5 minutes, and then processing for immunofluorescence microscopy. (A-B) Colocalization of transferrin, IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ with clathrin or caveolin was monitored. Colocalization was quantified as the percentage of transferrin, IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ that colocalize with clathrin or caveolin, using Metamorph software. (C-D) Colocalization of IL-2R and IL-2R-VE-cad$_{cyto}$ with AP-2 was also measured. Colocalization was quantified as the percentage of transferrin, IL-2R or IL-2R-VE-cad$_{cyto}$ that colocalize with AP-2. Error bars represent standard error of the mean where n=25 cells. Scale bar, 20 μm.

Figure 4

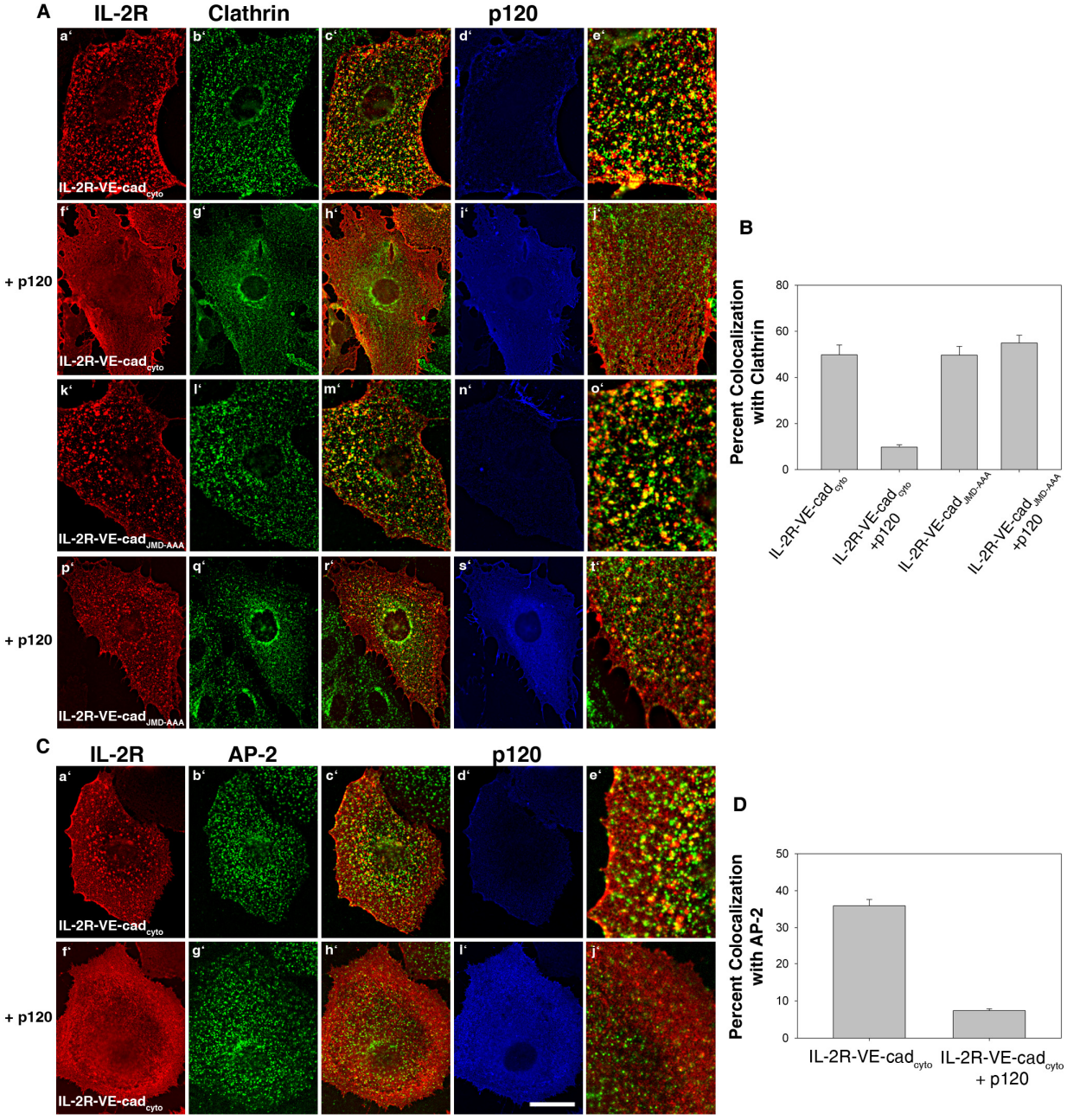
A
IL-2R
Clathrin
p120
IL-2R-VE-cad$_{cyto}$
+ p120
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{JMD-AAA}$
+ p120
IL-2R-VE-cad$_{JMD-AAA}$
B
Percent Colocalization with Clathrin
0
20
40
60
80
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{cyto}$ +p120
IL-2R-VE-cad$_{JMD-AAA}$
IL-2R-VE-cad$_{JMD-AAA}$ +p120
C
IL-2R
AP-2
p120
IL-2R-VE-cad$_{cyto}$
+ p120
IL-2R-VE-cad$_{cyto}$
D
Percent Colocalization with AP-2
0
10
20
30
40
50
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{cyto}$ + p120

Figure 4.

Exogenous expression of p120 prevents IL-2R-VE-cad$_{cyto}$ from colocalizing with clathrin and AP-2 in a manner dependent on the interaction of p120 with the VE-cadherin JMD. (A) p120 was coexpressed in MECs with either IL-2R-VE-cad$_{cyto}$ or IL-2R-VE-cad$_{JMD-AAA}$, which is unable to bind to p120, and internalization assays were conducted as described in Figure 3. Cells were fixed and processed for triple-label immunofluorescence to monitor the IL-2R-VE-cadherin polypeptides, p120, and clathrin. (B) Colocalization was quantified as the percentage of IL-2R-VE-cad$_{cyto}$ or IL-2R-VE-cad$_{JMD-AAA}$ that colocalize with clathrin. In the presence of p120, colocalization between IL-2R-VE-cad$_{cyto}$ and clathrin is greatly reduced. High levels of colocalization between IL-2R-VE-cad$_{JMD-AAA}$ and clathrin in cells expressing exogenous p120 demonstrate that p120 must bind to the VE-cadherin JMD to prevent VE-cadherin recruitment into clathrin enriched membrane domains. (C) Colocalization between IL-2R-VE-cad$_{cyto}$ and AP-2 was also monitored. In the presence of p120, IL-2R-VE-cad$_{cyto}$ colocalization with AP-2 is dramatically reduced (D). Error bars represent standard error of the mean where n=25 cells. Scale bar, 20 μm.

Figure 5

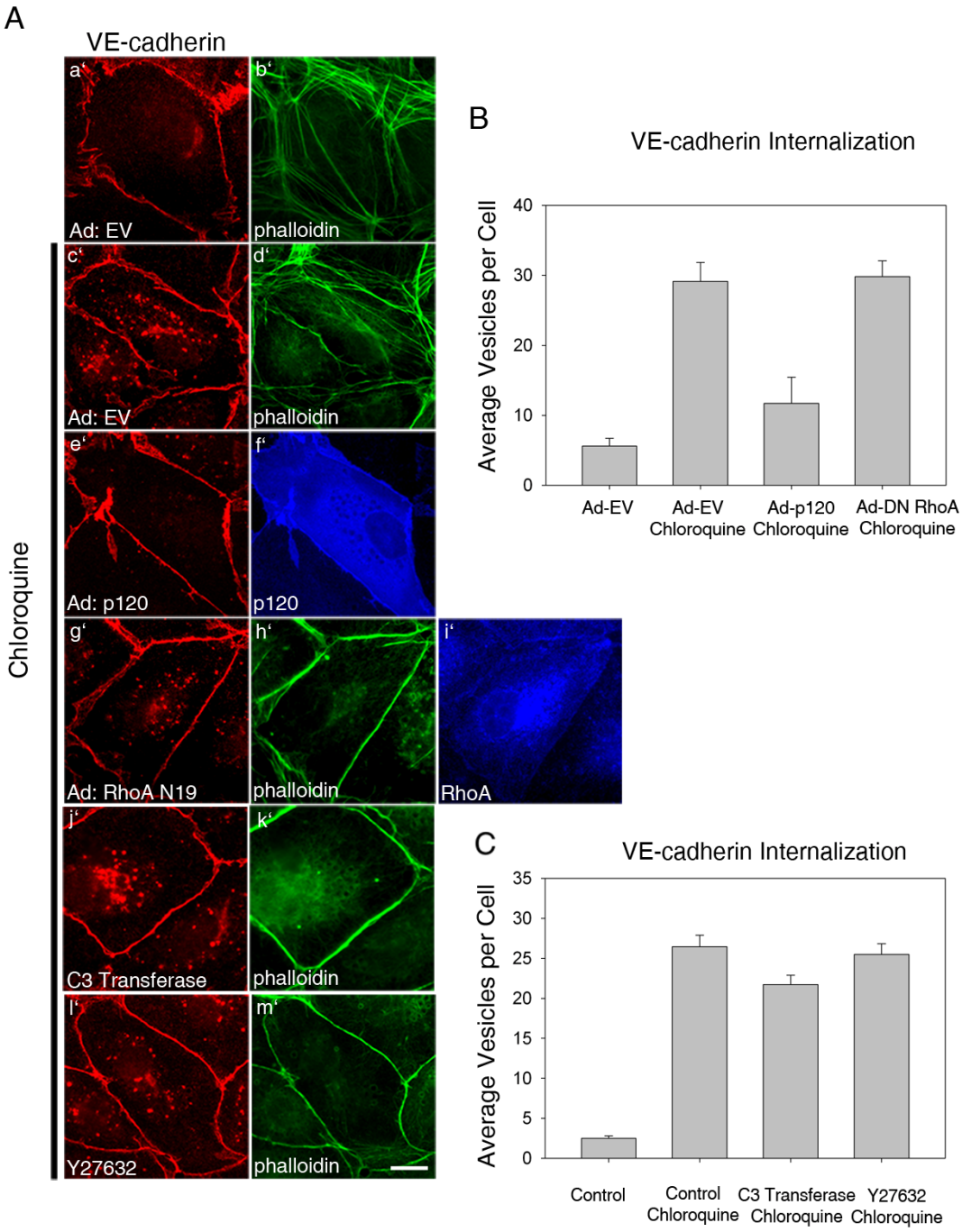
A
VE-cadherin
a'
b'
Ad: EV
phalloidin
c'
d'
Ad: EV
phalloidin
e'
f'
Ad: p120
p120
Chloroquine
g'
h'
i'
Ad: RhoA N19
phalloidin
RhoA
j'
k'
C3 Transferase
phalloidin
l'
m'
Y27632
phalloidin
B
VE-cadherin Internalization
Average Vesicles per Cell
0
10
20
30
40
Ad-EV
Ad-EV Chloroquine
Ad-p120 Chloroquine
Ad-DN RhoA Chloroquine
C
VE-cadherin Internalization
Average Vesicles per Cell
0
5
10
15
20
25
30
35
Control
Control Chloroquine
C3 Transferase Chloroquine
Y27632 Chloroquine

Figure 5.

Inhibition of RhoA activity does not inhibit VE-cadherin endocytosis. (A) MECs were infected with empty adenovirus (a'-d'), virus expressing p120 1A (e'-f'), or a RhoA dominant negative mutant (N19) (g'-i'). RhoA activity was also inhibited by treating cells with a cell permeable C3 transferase (j'-k') or Y27632 (l'-m'), an inhibitor of Rho Kinase. Accumulation of vesicular VE-cadherin was measured in untreated or chloroquine treated cells as described in Figure 1 and inhibition of Rho activity was verified by loss of stress fibers as detected by Alexa Fluor 488-conjugated phalloidin (b', d', h', k', m'). The number of VE-cadherin containing vesicles were quantified as shown in panels B and C. Error bars represent standard error of the mean where n=50 cells.

Figure 6

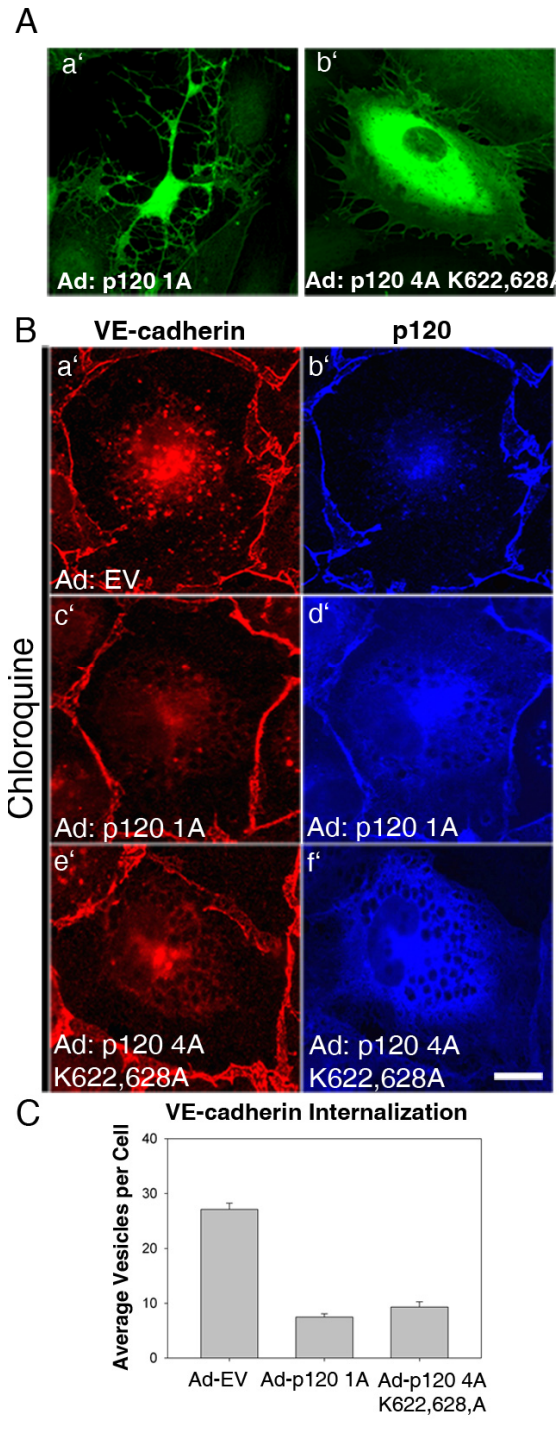
A
a'
b'
Ad: p120 1A
Ad: p120 4A K622,628A
B
VE-cadherin
p120
Chloroquine
a'
b'
Ad: EV
c'
d'
Ad: p120 1A
Ad: p120 1A
e'
f'
Ad: p120 4A K622,628A
Ad: p120 4A K622,628A
C
VE-cadherin Internalization
Average Vesicles per Cell
40
30
20
10
0
Ad-EV
Ad-p120 1A
Ad-p120 4A K622,628,A

Figure 6.

A p120 Rho uncoupled mutant is effective at preventing VE-cadherin endocytosis. (A) Wild type p120 1A or a RhoA uncoupled mutant (p120 4A K622,628A) were expressed in MECs. Note dendritic appearance of cells expressing wild type but not mutant p120. (B) Internalization of VE-cadherin was measured in MECs expressing EV (a'-b'), p120 1A (c'-d'), or the RhoA uncoupled p120 4A K622,628A mutant (e'-f') by treating cells with chloroquine for 3 hours. Total intracellular levels of VE-cadherin were quantified as shown in panel C. VE-cadherin internalization was dramatically reduced in cells expressing either exogenous wild type p120 or the RhoA uncoupled p120 mutant. Error bars represent standard error of the mean where n=100 cells. Scale bar = 20 μm.

Figure 7

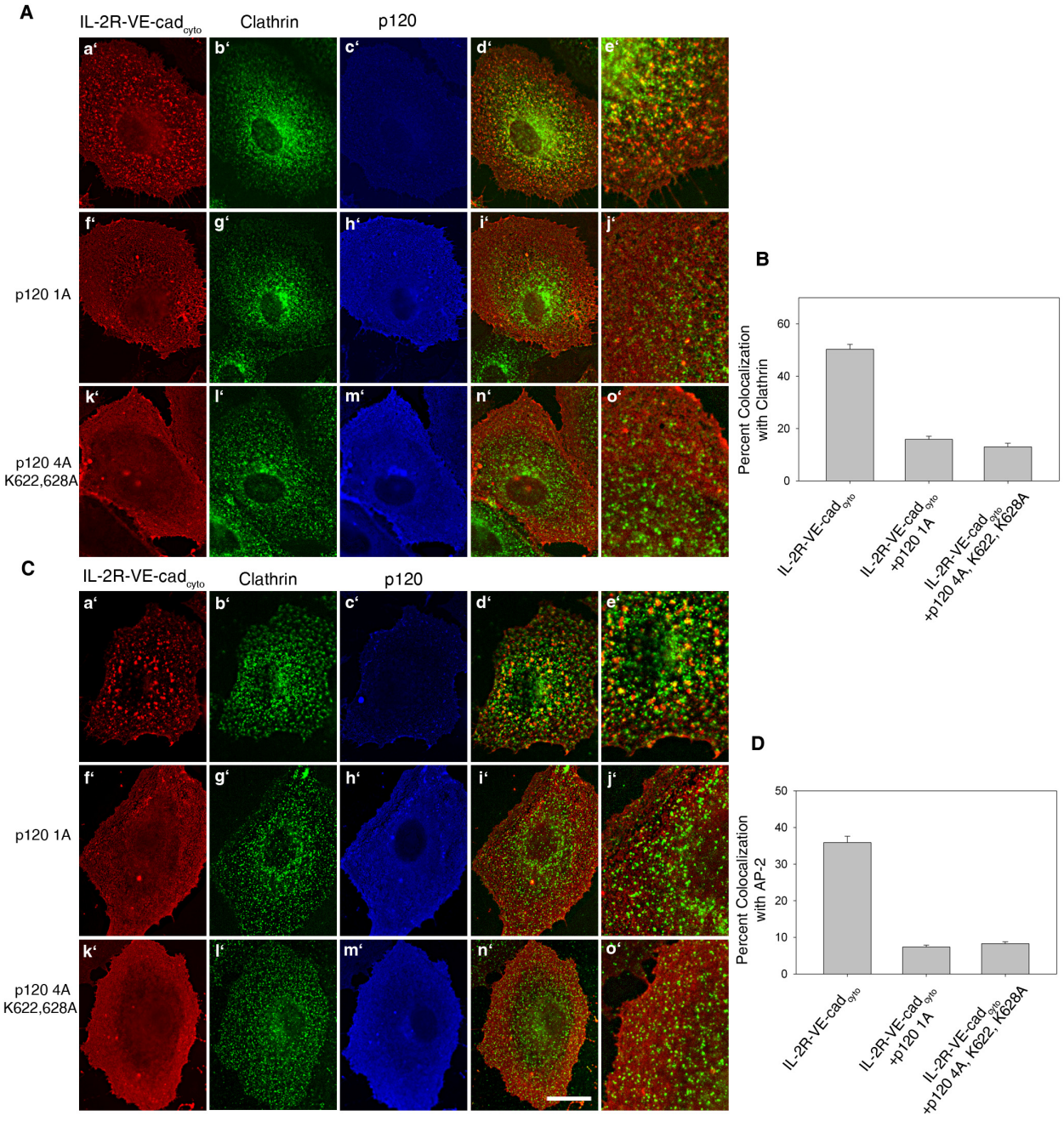
A
IL-2R-VE-cad$_{cyto}$
Clathrin
p120
p120 1A
p120 4A
K622,628A
B
Percent Colocalization with Clathrin
60
40
20
0
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{cyto}$ +p120 1A
IL-2R-VE-cad$_{cyto}$ +p120 4A, K622, K628A
C
IL-2R-VE-cad$_{cyto}$
Clathrin
p120
p120 1A
p120 4A
K622,628A
D
Percent Colocalization with AP-2
50
40
30
20
10
0
IL-2R-VE-cad$_{cyto}$
IL-2R-VE-cad$_{cyto}$ +p120 1A
IL-2R-VE-cad$_{cyto}$ +p120 4A, K622, K628A

Figure 7.

A p120 Rho uncoupled mutant prevents entry of VE-cadherin into membrane compartments containing clathrin and AP-2. (A, C) MECs were infected with adenovirus carrying IL-2R-VE-cad$_{cyto}$ and either p120 1A or p120 4A K622,628A. Cells were then processed for immunofluorescence to determine the extent of colocalization between IL-2R-VE-cad$_{cyto}$ and clathrin (A) or AP-2 (C) in cells expressing either wild type or Rho uncoupled p120 (p120 4A K622,628A). Colocalization was quantified as the percentage of IL-2R-VE-cad$_{cyto}$ that colocalize with clathrin or AP-2. The RhoA uncoupled p120 mutant was as effective at preventing colocalization of IL-2R-VE-cad$_{cyto}$ as wild type p120 (B, D). Error bars represent standard error of the mean where n=25 cells. Scale bar, 20 μm.

Chapter 4

Identification of a tyrosine sorting motif that regulates VE-cadherin endocytosis and AP-2 binding

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INTRODUCTION

The vascular endothelial cells that line blood vessels form a dynamic barrier between the plasma compartment of blood vessels and the tissue extracellular space (Lampugnani and Dejana, 1997; Braverman, 2000). In addition to maintaining normal tissue homeostasis, endothelial cells are actively involved in inflammatory responses, wound healing, and angiogenesis (Dvorak *et al.*, 1995; Cavallaro *et al.*, 2006; Dejana *et al.*, 2008). The modulation of endothelial intercellular junctions represents an important mechanism for regulating cell permeability, migration, and proliferation in the endothelium. In particular, the adhesion molecule VE-cadherin has been identified as playing a critical role in the regulation of vascular barrier function, angiogenesis, and endothelial growth control (Bazzoni and Dejana, 2004; Vestweber, 2008a; Wallez and Huber, 2008). Disruption of VE-cadherin from adherens junctions in endothelial cells is associated with increased vascular permeability during inflammatory responses and ischemic injury (Conacci-Sorrell *et al.*, 2002; Thiery, 2002; Yuan, 2002). Furthermore, the loss of VE-cadherin due to gene disruption in mice causes embryonic lethality due to severe defects in angiogenesis and vascular remodeling (Carmeliet *et al.*, 1999a).

The regulation of cell surface expression of cadherins represents an important mechanism for modulating the adhesive potential of cells. An emerging concept in the field of cell adhesion is that membrane trafficking pathways play a fundamental role in the regulation of adhesion molecule expression and function (Chiasson and Kowalczyk, 2008). Previous work from our lab has demonstrated that VE-cadherin undergoes constitutive endocytosis and degradation through an endo-lysosomal pathway under steady state conditions (Xiao *et al.*, 2005). Additionally, we have identified a core

function for the VE-cadherin binding partner, p120-catenin (p120), in stabilizing VE-cadherin at the plasma membrane by preventing its entry into a clathrin-dependent endocytic pathway (Xiao *et al.*, 2003a; Xiao *et al.*, 2005). Other studies have implicated a role for VE-cadherin internalization in conditions that lead to junctional disruption, including VEGF stimulation (Gavard and Gutkind, 2006) and treatment with hydrogen peroxide (Kevil *et al.*, 1998; Kevil *et al.*, 2001a; Kevil *et al.*, 2001b). These observations suggest that VE-cadherin is a constitutive process that may be dynamically regulated to maintain junctional plasticity in response to changing physiological cues. However, there is little direct evidence linking VE-cadherin endocytosis to endothelial cell function. Therefore, an important long-term goal of this study is to understand how VE-cadherin endocytosis contributes to endothelial cell functions that are crucial to vascular biology, including cell permeability, migration, and proliferation.

Transmembrane receptors can be internalized through several different endocytic pathways, which can be characterized as either clathrin-dependent, or clathrin-independent (Bonifacino and Traub, 2003). While cadherins have been found to undergo both clathrin-dependent and –independent endocytosis, VE-cadherin appears to be internalized primarily through a clathrin-dependent pathway (Xiao *et al.*, 2005; Gavard and Gutkind, 2006; Chiasson *et al.*, 2009). The formation of clathrin coated vesicles at the plasma membrane and the recruitment of cargo transmembrane receptors to these sites is mediated by a complex network of protein-protein and protein-lipid interactions (Traub, 2009). AP-2 is a heterotetrameric complex that is considered the core adaptor molecule in clathrin-mediated endocytosis. Through its ability to interact with clathrin, plasma membrane lipids, other adaptor proteins, and cargo molecules, AP-2 serves as a

key regulator of clathrin coated vesicle formation (Maldonado-Báez and Wendland, 2006). AP-2 also carries out a critical function in the recruitment of cargo molecules into clathrin coated vesicles by binding to short, linear sorting motifs in the cytoplasmic tail of transmembrane cargo receptors. AP-2 recognizes either tyrosine-based (YXXØ (in which X is any amino acid and Ø is a hydrophobic residue) or dileucine-based ([DE]XXXL[LI]) motifs (Bonifacino and Traub, 2003). Studies have shown that sorting motifs are both necessary and sufficient for the endocytosis of cargo receptors.

Studies of E-cadherin, the prototypical cadherin family member, have identified sequences within the cytoplasmic tail that regulate trafficking of the receptor (Miranda *et al.*, 2001; Fujita *et al.*, 2002; Ling *et al.*, 2007; Miyashita and Ozawa, 2007). Interestingly, however, these sites are not conserved in VE-cadherin, indicating that unique sequences must mediate internalization and turnover of VE-cadherin. In the previous chapter of this dissertation, we identified the cellular machinery involved in VE-cadherin endocytosis. Our results demonstrated that internalization of VE-cadherin requires clathrin, dynamin, and the clathrin adaptor complex AP-2. We were also able to identify a novel interaction between the VE-cadherin cytoplasmic tail and AP-2 and showed that VE-cadherin forms a complex with clathrin and AP-2. Furthermore, expression of p120 was able to prevent the recruitment of VE-cadherin into membrane domains enriched for clathrin and AP-2. Together, this data support a model in which, by binding to the cadherin cytoplasmic tail, p120 functions as a cap to prevent AP-2 from binding to sorting signals in the VE-cadherin tail and recruiting it into clathrin coated vesicles. A further prediction of this model is that the VE-cadherin cytoplasmic tail

harbors sorting information that forms the basis for the interaction with AP-2 and mediates recruitment into clathrin coated vesicles.

Previously, our lab used a gain of function approach to demonstrate that the VE-cadherin cytoplasmic tail mediates internalization (Xiao *et al.*, 2005). In the present study, we utilized site directed mutagenesis to identify sequences in the VE-cadherin cytoplasmic tail that are required for internalization of VE-cadherin and binding to AP-2. We found that the VE-cadherin JMD, which contains the p120 binding site, is sufficient for efficient turnover of VE-cadherin. Within this region we identified a tyrosine-based sorting motif at tyrosine 685 that conforms to the consensus sequence of YXXØ. Mutagenesis of this site reduced the efficiency of VE-cadherin internalization and binding to AP-2 by approximately 50%. Together, these data indicate that tyrosine 685 is part of an AP-2 sorting motif and plays an important role in VE-cadherin endocytosis. These findings support our model that binding of p120 to VE-cadherin prevents AP-2 from interacting with the cadherin tail and recruiting it into clathrin coated vesicles. The identification of sequences in the VE-cadherin tail that are required for endocytosis provide us with important tools to directly examine the role of VE-cadherin endocytosis in endothelial cell functions, such as migration and barrier function.

MATERIALS AND METHODS

Cell Culture: Primary cultures of dermal microvascular endothelial cells (MECs) from human neonatal foreskin were isolated and cultured in Microvascular Endothelial Cell Growth Media-2 (EGM-2MV) (Lonza, Basel, Switzerland), supplemented with cAMP (Sigma-Aldrich, St. Louis, MO). Cells were generally cultured overnight on 0.1%

gelatin-coated plates and grown to ~80% confluency for experiments. HeLa ATTC cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin/amphotericin. For adenovirus production human embryonic kidney cell line QBI-293A (Qbiogene, Carlsbad, CA) were routinely cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin/amphotericin.

cDNA constructs and Transfections: The IL-2R-VE-cad$_{cyto}$ and IL-2R-VE-cad$_{JMD}$ chimeric constructs, consisting of the IL-2R extracellular domain, either the entire VE-cadherin cytoplasmic domain or a deletion mutant of the VE-cadherin cytoplasmic tail lacking the catenin binding domain of VE-cadherin, and a carboxyl-terminal c-myc epitope tag, were generated as previously described (Xiao et al 2003). Site directed mutagenesis was carried out using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). HeLa cells transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

Adenovirus Production: The IL-2R, IL-2R-VE-cad$_{cyto}$, and IL-2R-VE-cad$_{JMD}$ chimera cDNAs were subcloned into the pAd-Track vector which coexpresses green fluorescent protein (GFP) (He *et al*., 1998). Adenoviruses were produced using the pAdeasy adenovirus-packaging system as described previously (Xiao *et al.*, 2005). For most experiments, infection rates of 50-60% were used as monitored by GFP expression.

Immunofluorescence: Immunofluorescence was carried out as described previously (Xiao et al., 2005). The IL-2R and IL-2R-VE-cadherin chimeras were followed using an

affinity purified anti-IL-2R IgG produced from 7G7B6 mouse hybridoma (American Type Culture Collection, Manassas, VI). The VE-cadherin constructs were also detected by using a mouse monoclonal antibody (mAB) against IL-2R (R&D Systems, Minneapolis, MN) or by using a chicken antibody against the c-terminal myc epitope tag (Bethyl Laboratories, Montgomery, TX). Secondary antibodies conjugated to Alexa Fluors (Molecular Probes) were used for dual label experiments. Microscopy was performed using either a wide field fluorescence microscope (model DMR-E; Leica, Wetzlar, Germany) equipped with narrow band pass filters and a digital camera (model Orca; Hamamatsu, Bridgewater, NJ) or an inverted Leica DMI-6000B microscope equipped with an Infinity II confocal scanning module, 561nm and 491nm lasers, and a Hamamatsu CCD camera (C9100-12). Images were captured, pseudo colored, and processed using Simple PCI software (Compix, Cranberry Township, PA).

Internalization Assay: Assays to follow internalization of the IL-2R-VE-cadherin polypeptides were carried out as previously described (Chiasson et al 2009). Cells were plated on glass coverslips for experimentation. MECs were infected with adenovirus or HeLa cells were transfected overnight to allow time for expression of the polypeptides. The cells were surface labeled using IL-2R antibody at 4°C and transferred to 37°C for various amounts of time. To remove cell surface bound antibody while retaining internalized antibody, cells were acid washed for 30 min in phosphate-buffered saline (PBS), pH 2.7, containing 25 mM glycine and 3% bovine serum albumin (BSA). The cells were rinsed, fixed, and processed for immunofluorescence as described above. To measure loss of surface expression of the VE-cadherin chimeras, MECs expressing the

appropriate polypeptides were labeled with IL-2R antibodies as described above. Following a time course of internalization for various amounts of time, cells were fixed with paraformaldehyde, but not permeabilized. Processing with secondary antibodies enabled detection of IL-2R antibodies remaining at the cell surface. Internalization was quantified using Simple PCI software to measure either total fluorescence or object number. Error bars represent standard error of the mean.

Immunoprecipitation: Briefly, HeLa cells were grown to confluency in 60 mm dishes and transfected with IL-2R-VE-cadherin constructs. On the day of the experiment, cells were placed on ice, rinsed twice with PBS, then lysed in buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM $MgCl_2$, pH 7.4) + 0.5% TX-100, scraped from the dish, and incubated on ice for 30 min. Cell homogenates were centrifuged at 16,100 x *g* for 10 min. and supernatants were diluted to 1 mg/ml in 0.5 ml of Buffer A + 0.5% TX-100. The supernatants were incubated overnight at 4ºC with Dynal magnetic beads (Invitrogen) conjugated to mAbs against AP-2 (α subunit, BD Transduction Labs). The beads were then washed with buffer A + 0.1% TX-100 and eluted with SDS-PAGE sample buffer at 75ºC for 5 min. Immunoblotting under reducing conditions allows for the detection of immunoprecipitated material. Polyclonal antibodies against IL-2R (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect precipitated proteins.

RESULTS

The JMD of VE-cadherin mediates internalization and turnover

In previous studies, we used a gain of function approach to elucidate the role of the VE-cadherin cytoplasmic domain in mediating endocytosis and turnover of the receptor. We showed that the addition of the VE-cadherin cytoplasmic tail to the extracellular domain of the IL-2 Receptor (IL2R-VE-cad$_{cyto}$) dramatically increases internalization compared to the IL-2R alone (Xiao *et al.*, 2005). This data indicates that the cytoplasmic domain of VE-cadherin harbors information that directs its localization and trafficking.

Sequence analysis of the cytoplasmic domain of VE-cadherin revealed the presence of putative tyrosine and di-leucine based sorting motifs that could be important for VE-cadherin endocytosis. Specifically, the JMD contains one tyrosine based motif, and the catenin binding domain (CBD) contains two tyrosine motifs and one di-leucine motif (Figure 1). Based on our model of p120 mediated regulation of VE-cadherin endocytosis, we predicted that the sorting motif responsible for VE-cadherin endocytosis would be located near the p120 binding site, within the JMD of VE-cadherin. Therefore, we first asked whether the VE-cadherin JMD was sufficient to mediate loss of VE-cadherin from the cell surface. As described in previous studies, we used chimeric polypeptides consisting of the extracellular domain of IL2R fused to either the full cytoplasmic tail of VE-cadherin (IL2R-VE-cad$_{cyto}$) or the VE-cadherin JMD (IL2R-VE-cad$_{JMD}$) to determine the contribution of the cytoplasmic tail to VE-cadherin internalization. Fluorescence based pulse-chase assays were used to measure loss of surface expression of IL2R and determine the region of VE-cadherin that mediates

endocytosis. Briefly, IL2R, IL2R-VE-cad$_{cyto}$, or IL2R-VE-cad$_{JMD}$ were expressed in primary dermal microvascular endothelial cells (MEC) using an adenoviral expression system. Cell surface pools of IL2R, IL2R-VE-cad$_{cyto}$ or IL2R-VE-cad$_{JMD}$ were labeled with a monoclonal antibody against the IL2R extracellular domain at 4°C. Cells were then switched to 37°C to allow for endocytosis. Cells were fixed in paraformaldehyde without permeabilization and stained with secondary antibodies to IL2R to measure surface expression of the chimeras following internalization. As shown in Figure 2 (A-C), IL2R, IL2R-VE-cad$_{cyto}$, and IL2R-VE-cad$_{JMD}$ are localized at the cell surface at 4°C. However, following internalization for three hours, surface levels of IL2R-VE-cad$_{cyto}$, and IL2R-VE-cad$_{JMD}$ are significantly lower, approximately 10% of levels at 4°C, compared to IL2R alone, which retains 55% of surface expression after three hours of internalization (Figure 2, D-F). The graphical representation of these data clearly show that both IL2R-VE-cad$_{cyto}$ and IL2R-VE-cad$_{JMD}$ are rapidly internalized from the cell surface within the first 30 minutes of internalization, while IL2R alone is not (Figure 2, G).

To confirm more directly that the JMD is sufficient to mediate VE-cadherin endocytosis, we compared the internalization of the IL2R-VE-cad$_{cyto}$ to that of the IL2R-VE-cad$_{JMD}$. To measure internalization of IL-2R-VE-cad chimeras, MECs were incubated at 4ºC with antibodies directed against IL-2R to label the cell surface. Following incubation at 37ºC for 5 minutes to allow for endocytosis, cells were washed with a low pH acid wash buffer to remove antibody remaining at the cell surface, making it possible to distinguish internalized IL-2R-VE-cad$_{cyto}$ from that at the cell surface. A myc epitope at the carboxy-terminal tail of the VE-cadherin cytoplasmic domain was used to identify cells expressing the IL-2R-VE-cad$_{cyto}$. Similarly to the results shown in

figure 2, we found that the IL2R-VE-cad$_{JMD}$ undergoes internalization from the cell surface at a similar rate as the IL2R-VE-cad$_{cyto}$ (Figure 3.). These results indicate that sequences in the juxtamembrane domain mediate VE-cadherin endocytosis.

A tyrosine motif in the VE-cadherin JMD mediates the association between AP-2 and VE-cadherin.

Based on our findings that the VE-cadherin JMD is sufficient for VE-cadherin endocytosis, we hypothesized that this region of the cadherin should mediate the interaction with AP-2. We used *in silico* analysis to identify a putative tyrosine motif, $_{685}$YAQV$_{688}$, that conforms to the consensus tyrosine sorting signal, YXXØ. This motif is highly conserved in VE-cadherin amongst vertebrate species (Figure 1). Interestingly, this motif is unique to VE-cadherin, as it is not conserved in the sequence of other cadherin family members. We hypothesized that this motif may function as an AP-2 binding site.

To test this hypothesis, we mutated the critical tyrosine residue in this motif to alanine in the context of the IL2-VE-cad$_{cyto}$ chimera and expressed wild type (WT) or tyrosine mutant (Y685A) IL2-VE-cad$_{cyto}$ in HeLa cells. AP-2 antibodies were used to perform immunoprecipitations from lysates from cells expressing either WT or Y685A IL2-VE-cad$_{cyto}$. Reducing SDS-PAGE and immunoblotting with IL-2R antibodies revealed that mutagenesis of Y685 reduced the interaction of AP-2 with the VE-cadherin cytoplasmic tail by approximately 50% compared to the WT IL2-VE-cad$_{cyto}$ (Figure 4). Control immunoprecipitations using beads without antibody were unable to co-precipitate IL2-VE-cad$_{cyto}$, confirming that the interaction is specific. These data suggest that the

YAQV tyrosine motif plays a role in mediating interactions between AP-2 and the VE-cadherin cytoplasmic tail, but indicate that additional sequences in the VE-cadherin tail may also contribute to AP-2 binding.

Mutagenesis of the tyrosine 685 prevents efficient internalization of VE-cadherin

We next asked if mutagenesis of the YAQV tyrosine motif would prevent internalization of the IL2-VE-cad$_{cyto}$. To answer this question, we expressed IL-2R, WT IL2-VE-cad$_{cyto}$ or Y685A IL2-VE-cad$_{cyto}$ in HeLa cells. Internalization was measured as described above. Following fixation, a second IL-2R antibody was used to detect total IL-2R expression as a means of normalizing expression levels between constructs. The Y685A IL2-VE-cad$_{cyto}$ was expressed at the cell surface at similar levels to IL-2R and WT IL2-VE-cad$_{cyto}$, suggesting that the mutation does not affect delivery of the VE-cadherin chimera to the plasma membrane (Figure 5 A-C). Mutagenesis of Y685 resulted in a significant decrease in internalization of IL2-VE-cad$_{cyto}$, to levels that are approximately half those observed in WT IL2-VE-cad$_{cyto}$ (Figure 5 E and F). Similar results were observed with a truncation mutant lacking the entire tyrosine motif (data not shown). As seen previously, IL-2R is internalized at rates that are about 80% less than WT IL2-VE-cad$_{cyto}$. Together, these findings demonstrate that mutation of tyrosine 685 reduces AP-2 binding and IL2R-VE-cad$_{cyto}$ endocytosis by 50%, indicating an important role for this residue in VE-cadherin internalization. The decrease in internalization of IL2-VE-cad$_{cyto}$ caused by mutation of the tyrosine motif is similar to the decrease in AP-2 binding, suggesting that these events are related.

DISCUSSION

A key finding from our previous studies is that the clathrin adaptor AP-2 binds to the VE-cadherin cytoplasmic tail and is required for VE-cadherin endocytosis (Chiasson *et al.*, 2009). While several earlier studies have defined pathways for cadherin endocytosis based on pharmacological inhibition, relatively little was known about the molecular machinery involved in VE-cadherin endocytosis. Here, we have extended our studies of the role of AP-2 in VE-cadherin internalization by identifying sequences in the VE-cadherin cytoplasmic tail that are important for endocytosis. The results presented here demonstrate that a tyrosine motif located in the VE-cadherin JMD mediates interactions with AP-2 and is required for efficient VE-cadherin endocytosis. Together with data presented in the previous chapter, these findings provide further support for our proposed model of p120 mediated regulation of VE-cadherin endocytosis. Previous work from our lab has identified p120 as a potent inhibitor of VE-cadherin endocytosis. Our data suggests that p120 functions as a "cap" on the VE-cadherin cytoplasmic tail, and that by binding to the VE-cadherin JMD, p120 prevents interactions between AP-2 and the tyrosine sorting signal in the JMD.

Previously, we used a gain of function approach to demonstrate that when fused to the extracellular domain of the IL-2 Receptor, the VE-cadherin cytoplasmic tail mediates rapid internalization from the plasma membrane (Xiao et al., 2005). Here, we have taken advantage of these chimeric molecules that undergo rapid endocytosis to identify specific sequences within the VE-cadherin tail that are required for internalization. First, we were able to show that the VE-cadherin JMD is sufficient for internalization and turnover of the cadherin. A deletion mutant lacking the c-terminal

region of VE-cadherin, which includes the catenin binding domain (CBD), was internalized from the cell surface and degraded at similar rates as the full length VE-cadherin tail, suggesting that the JMD harbors sorting signals that mediate internalization of the receptor. A recent study identified a dileucine motif in the E-cadherin JMD that is important for endocytosis (Miyashita and Ozawa, 2007). Sequence analysis revealed that VE-cadherin does not contain this dileucine motif, indicating that VE-cadherin may contain unique endocytic sorting signals. Further analysis identified a putative tyrosine motif ($_{685}$YAQV$_{688}$) approximately twenty amino acids downstream of the p120 binding site. This motif conforms to the consensus sequence for tyrosine motifs (YXXØ). YXXØ signals mediate tyrosine dependent interactions with the μ2 subunit of AP-2. The requirement for a bulky hydrophobic residue (Ø) is best fulfilled by leucine, but isoleucine, phenylalanine, methionine, and to a lesser extent valine are also tolerated (Traub, 2009). Although valine is not an ideal hydrophobic residue, there are several examples of receptors that contain sorting signals that include valine, including cation dependent mannose 6-phosphate receptor (CDM6-PR), PAR1, and acid phosphatase (Bonifacino and Traub, 2003). Deletion of this motif or replacement of the tyrosine residue with an alanine resulted in impaired binding of VE-cadherin to AP-2 and reduced levels of VE-cadherin endocytosis, indicating that the YAQV motif is a bona fide sorting signal.

Interestingly, while mutation of the YAQV motif significantly reduces both AP-2 binding and internalization, VE-cadherin maintains approximately 50% of its AP-2 binding and internalization capacity in the absence of the tyrosine residue. Together with our previous finding that depletion of AP-2 resulted in almost complete inhibition of VE-

cadherin internalization, the fact that AP-2 binding and internalization are similarly affected by mutation of the tyrosine implies that AP-2 is the primary adaptor for VE-cadherin endocytosis. These results suggest that although the YAQV motif functions to mediate interactions with AP-2, the VE-cadherin tail must contain other sequences that mediate AP-2 binding. There are in fact two other tyrosine motifs and a dileucine motif in the VE-cadherin CBD. However, since the JMD is sufficient for internalization, and mutation of the $_{685}$YAQV$_{688}$ motif in the context of a deletion mutant lacking the CBD results in similar levels of internalization to the mutant with the full cytoplasmic tail, the sequences in the CBD do not appear to be required for VE-cadherin internalization. This result would suggest that other sequences in the VE-cadherin JMD play an important role in AP-2 recognition and sorting. While the JMD does not contain additional sequences that conform to known AP-2 binding sites, it is possible that AP-2 may interact with the cadherin tail indirectly, by cooperating with other adaptor molecules to recruit VE-cadherin into clathrin coated vesicles.

In recent years, mounting evidence has revealed that adaptors other than AP-2 participate in clathrin-dependent endocytosis, and that in some cases, including low-density lipoprotein (LDL), these alternative adaptors can function to mediate endocytosis in the absence of AP-2 (Motley *et al.*, 2003). Proteins in this family of monomeric adaptors, known as clathrin-associated sorting proteins (CLASPs), interact with AP-2 and clathrin through short peptide interaction motifs, creating a complex network of protein-protein interactions that form the basis for clathrin coated vesicle formation (Traub, 2009). Our previous data showing that depletion of AP-2 by siRNA almost completely eliminated internalization of VE-cadherin indicates that alternative adaptors cannot

compensate for AP-2 function in recruiting VE-cadherin into clathrin coated vesicles. However, in light of our new findings, the possibility remains that AP-2 may cooperate with CLASPs in binding to the VE-cadherin JMD.

One possibility is that ubiquitination plays a role in VE-cadherin endocytosis. Post-translational modification with ubiquitin, which enables recognition by ubiquitin selective CLASPs, including epsin 1 and EPS15, is a well established mechanism for sorting of cargo into clathrin coated vesicles (Traub and Lukacs, 2007; Traub, 2009). In fact, evidence suggests that ubiquitination may play a role in E-cadherin trafficking. E-cadherin is ubiquitinated by Hakai, an E3 ubiquitin ligase, through mechanism that requires tyrosine phosphorylation downstream of Src activation. Overexpression of Hakai leads to endocytosis of E-cadherin and disruption of cell junctions (Fujita *et al.*, 2002). The precise mechanisms by which Hakai mediates endocytosis or whether adaptors or CLASPs are involved, is not understood. Interestingly, however, the Hakai binding sites are not conserved in VE-cadherin, indicating that other sites may mediate ubiquitination of VE-cadherin.

Another possible role for CLASPs in VE-cadherin endocytosis has been suggested by a recent study of VE-cadherin internalization in response to VEGF signaling. Gavard and Gutkind defined a signaling pathway involving Rac-mediated phosphorylation of VE-cadherin, which is activated in endothelial cells following treatment with VEGF. Phosphorylation of a conserved serine cluster in the VE-cadherin JMD (S665) leads to β-arrestin binding, which subsequently mediates VE-cadherin endocytosis and results in increased endothelial cell permeability (Gavard and Gutkind, 2006). The requirement for β-arrestin in VEGF induced internalization of VE-cadherin is

interesting, as β-arrestin is a CLASP molecule that is generally associated with clathrin dependent internalization of GPCRs. β-arrestin has been shown to mediate recruitment of cargo into clathrin coated vesicles occurs in cooperation with AP-2 (Bonifacino and Traub, 2003). However, it is not clear whether the endocytic pathway utilized by VE-cadherin in response to VEGF stimulation is the same as the one used for constitutive turnover. One possible explanation for the residual AP-2 binding and internalization we observed in the Y685A mutant is that the serine cluster in the VE-cadherin JMD provides an additional AP-2 binding site, perhaps indirectly through β-arrestin. Therefore, it would be interesting to determine if the mutagenesis of the serine cluster, or depletion of β-arrestin would inhibit internalization of the VE-cadherin chimera.

The identification of sequences that mediate internalization of VE-cadherin represents an important step in understanding the mechanisms that dynamically modulate the adhesive potential of cadherins by regulating their availability at the plasma membrane. The results reported here extend our previous findings by demonstrating that the $_{685}$YAQV$_{688}$ motif in the VE-cadherin JMD is a bona fide sorting motif that mediates interactions with AP-2, and is thus required for efficient VE-cadherin internalization. The location of this sorting motif in the JMD, near the p120 binding site, lends further support for our model, in which p120 stabilizes VE-cadherin at the plasma membrane by preventing AP-2 from interacting with sorting motifs and recruiting the cadherin into clathrin coated vesicles. The importance of p120 to maintaining proper VE-cadherin function in the endothelium is underscored by recent *in vivo* studies from our lab, in which we conditionally knocked out expression of p120 in vascular endothelial cells. Mice lacking endothelial p120 die embryonically due to severe defects in endothelial

barrier function, blood vessel patterning and endothelial cell proliferation (Oas *et al.*, 2010). These defects can be at least partially explained by decreased expression of VE-cadherin associated with the loss of p120. However, p120 has additional functions in endothelial cells outside of regulating VE-cadherin endocytosis, including regulating the stability of N-cadherin and regulating the activity of Rho GTPases, making it difficult to directly attribute the defects observed in the p120 null mice to misregulation of VE-cadherin endocytosis. Our identification of a tyrosine motif that mediates VE-cadherin internalization and AP-2 binding provide us with important tools for understanding how VE-cadherin endocytosis contributes to endothelial cell function. An important future direction of this project involves studying mutants of VE-cadherin that are incapable of undergoing efficient endocytosis to elucidate the role of cadherin endocytosis in endothelial cell functions, including migration, barrier function, and proliferation.

Figure 1

A.

```
Human E-Cad          (1)  EEDQDFDLSQLHRGLDARPEVTR-----NDVAPTLMSVP
Human N-cad          (1)  EEDQDYDLSQLQQPDTVEPDAIKPVGIRRMDERPIHAEP

Human VE-cad         (1)  EMDTTSYDVSVLNSVRRGGAKPPRPALDARPSLYAQVQK
Bovine VE-cad        (1)  EMDTTSYDVSVLNSARHGGAKPPRPALDARPSLYAQVQK
Porcine VE-cad       (1)  EMDTTSYDVSVLNSVRHGGAKPPRPALDARPSLYAQVQK
Chicken VE-cad       (1)  EMDTTSYDVSVLNSVRKNGIKP-----EVVPSAYAQVQK
Rat VE-cad           (1)  EMDTTSYDVSVLNSVRTGSNKPLRSTMDARPAVYTQVQK
Mouse VE-cad         (1)  EMDTTSYDVSVLNSVRGGSTKPLRSTMDARPAVYTQVQK
Zebra Fish VE-cad    (1)  EMDTNGYDVSILSSACHDSSFRP----SVGPALYAMVKK
```

B.

JMD

AAA Y685A

RRRLRKQARAHGKSVPEIHEIHEQLVTYDEEGGGEMDTTSYDVSVLNSVRRGGAKPPRPALDARPSLYAQV
QKPPRHAPGAHGGPGEM

CBD

AAMIEVKKDEADHDGDGPPYDTLHIYGYEGSESIAESLSSLGTDSSDSDVDYDFLNDWGPRFKMLAELYGS
DPREELLY

C.

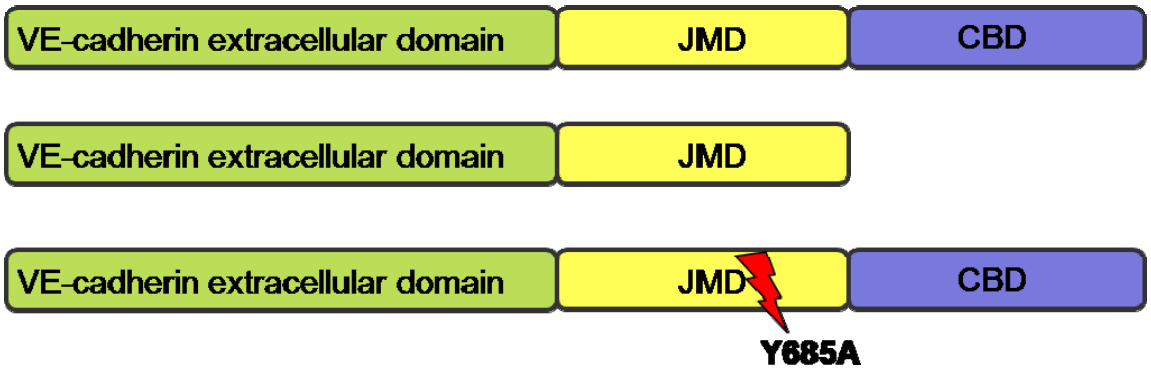

Figure 1. VE-cadherin sequences.

A. Sequence analysis of the VE-cadherin cytoplasmic tail revealed the presence of a highly conserved tyrosine motif in the JMD at $_{685}$YAQV$_{688}$, which conforms to the consensus sequence for AP-2 binding. Y685 and V688 are conserved in VE-cadherin from zebra fish to human, but not in E-cadherin or N-cadherin. B. This region of the VE-cadherin tail also contains the p120 binding site. The catenin binding domain contains two additional putative tyrosine motifs and a dileucine motif. C. In this study, we constructed mutants of the IL-2R-VE-cad$_{cyto}$ construct, including a truncation mutant lacking the CBD (IL-2R-VE-cad$_{JMD}$) and a mutation in which Y685 was substituted with an alanine (IL-2R-VE-cad$_{Y685A}$).

Figure 2

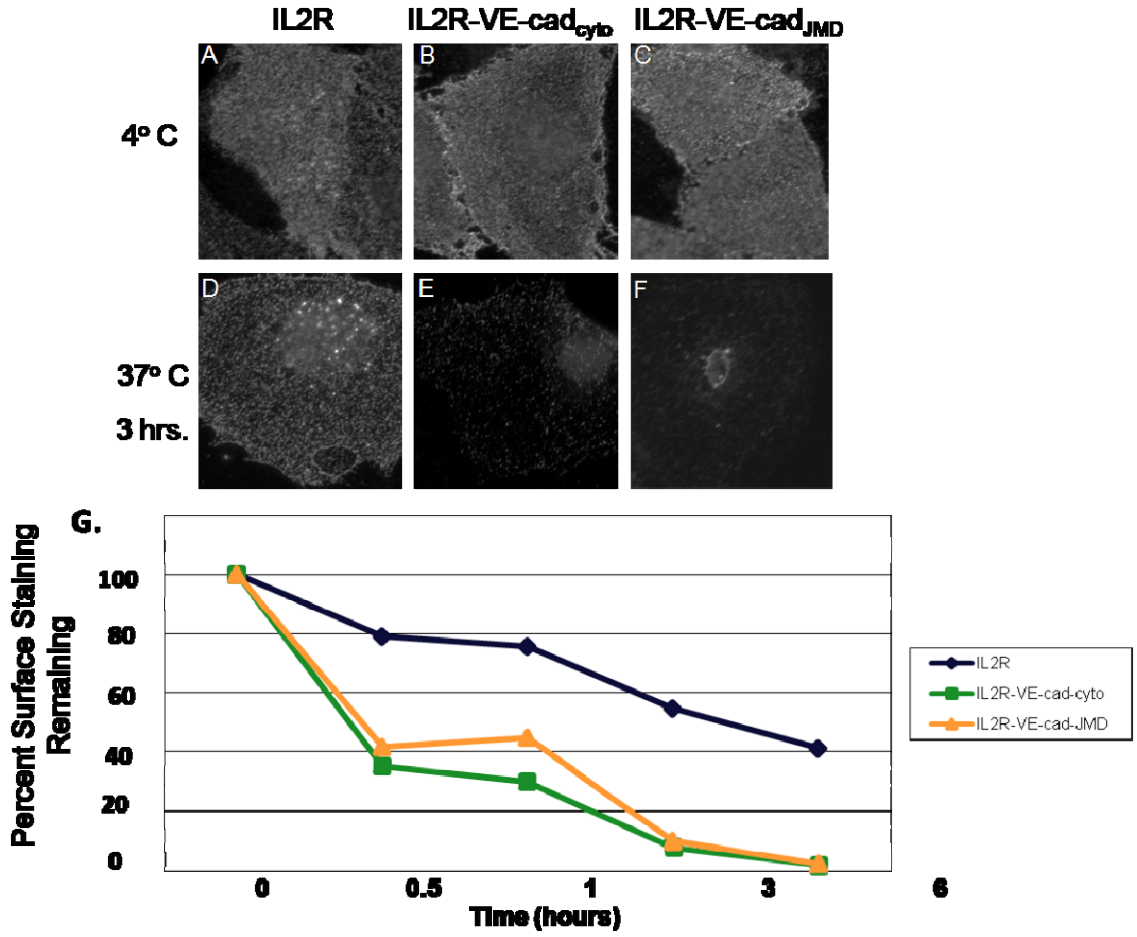

Figure 2.

The VE-cadherin JMD mediates turnover of VE-cadherin. To determine if the JMD of VE-cadherin is sufficient to mediate cadherin turnover, an IL2R-VE-cadherin chimera comprising the VE-cadherin JMD but lacking the CBD was generated. To examine the rate of turnover of the cell surface pool of the various receptors, cells infected with IL2R, IL2R-VE-cad$_{cyto}$, or IL2R-VE-cad$_{JMD}$ were labeled at 4°C and allowed to undergo internalization for 30 min, 1 hr., 3 hrs., or 6 hrs. at 37ºC. Following internalization, cells were fixed without permeabilization and processed for immunofluorescence microscopy to visualize surface levels of the IL2R-VE-cadherin chimeras. After 3 hours of internalization, cells infected with IL2R alone exhibit surface staining of IL2R similar to

cells incubated at 4ºC (A, D). Cells infected with IL2R-VE-cad$_{cyto}$ or IL2R-VE-cad$_{JMD}$ exhibit similar levels of surface staining to IL2R alone at 4ºC (B, E) and almost complete loss of surface staining following 3 hrs of internalization (C, F). Data is quantified as percentage of surface staining remaining at each time point, as compared to 4ºC (G).

Figure 3

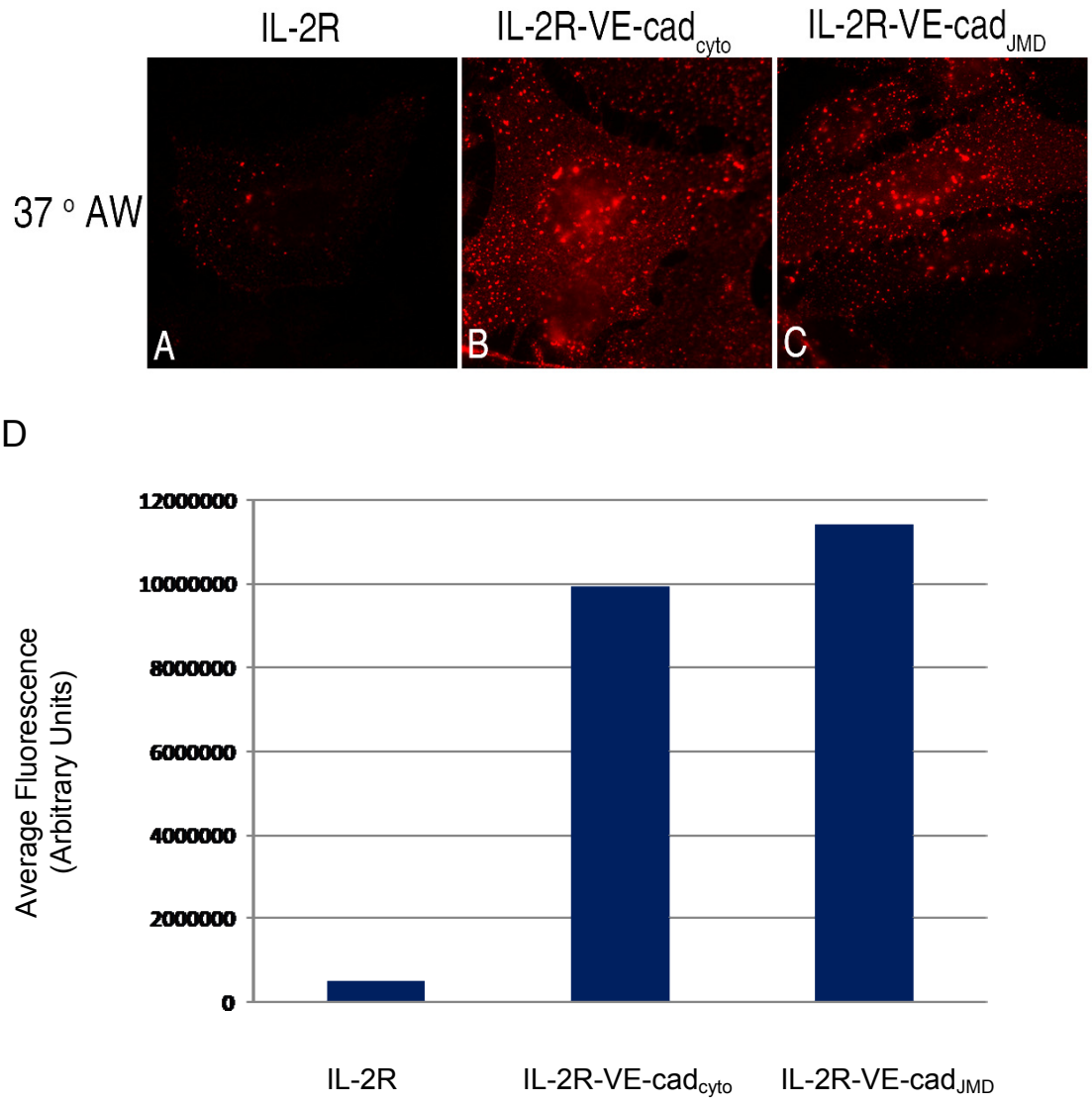

Figure 3.

The VE-cadherin JMD is sufficient for VE-cadherin internalization. To determine if the JMD of VE-cadherin is sufficient to mediate cadherin internalization, MECs expressing either IL-2R, IL2R-VE-cad$_{cyto}$, or IL2R-VE-cad$_{JMD}$ were labeled at 4°C with an antibody directed against IL-2R and allowed to undergo internalization for 5 minutes at 37ºC. Following internalization, cells were acid washed to remove antibodies

remaining at the cell surface. Cells were then fixed and processed for immunofluorescence microscopy. Following 5 minutes at 37ºC, both the IL2R-VE-cad_{cyto} (B) and the IL2R-VE-cad_{JMD} (C) exhibited similar levels of internalization, while very little IL-2R (A) was internalized. Data is quantified as average fluorescence intensity and is normalized to total IL2-R expression (D).

Figure 4

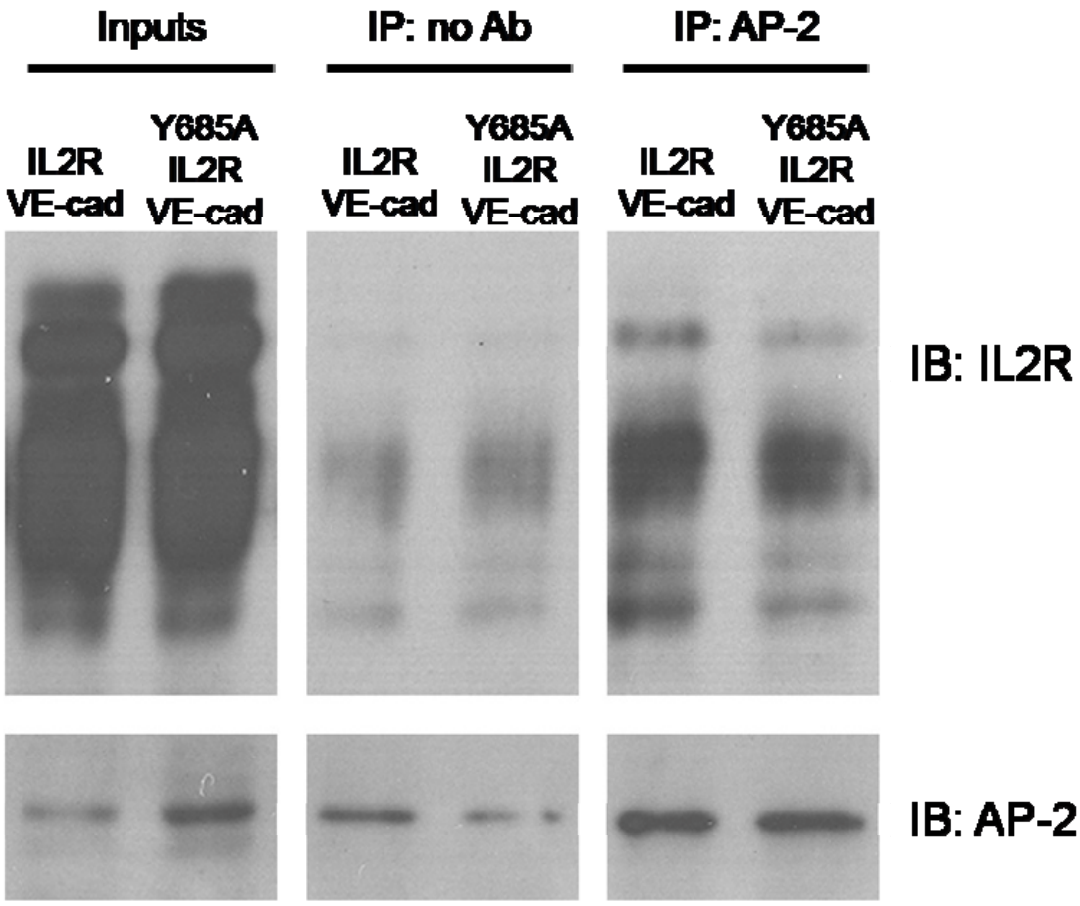

Figure 4.

Mutation of Y685 disrupts the interaction between VE-cadherin and AP-2. MECs expressing IL-2R-VE-cad$_{cyto}$ or IL-2R-VE-cad$_{Y685A}$ were immunoprecipitated with beads alone (lanes 3-4) or beads coated with AP-2 α antibodies (lanes 5-6). Western blot analysis using antibodies against IL-2R was used to detect the interaction between the IL-2R-VE-cad chimeras and AP-2. Immunoprecipitation of AP-2 coprecipitates IL-2R-VE-cad$_{Y685A}$ less efficiently than the WT IL-2R-VE-cad$_{cyto}$ (compare lane 6 to lane 5). Inputs represent 5% of sample.

Figure 5

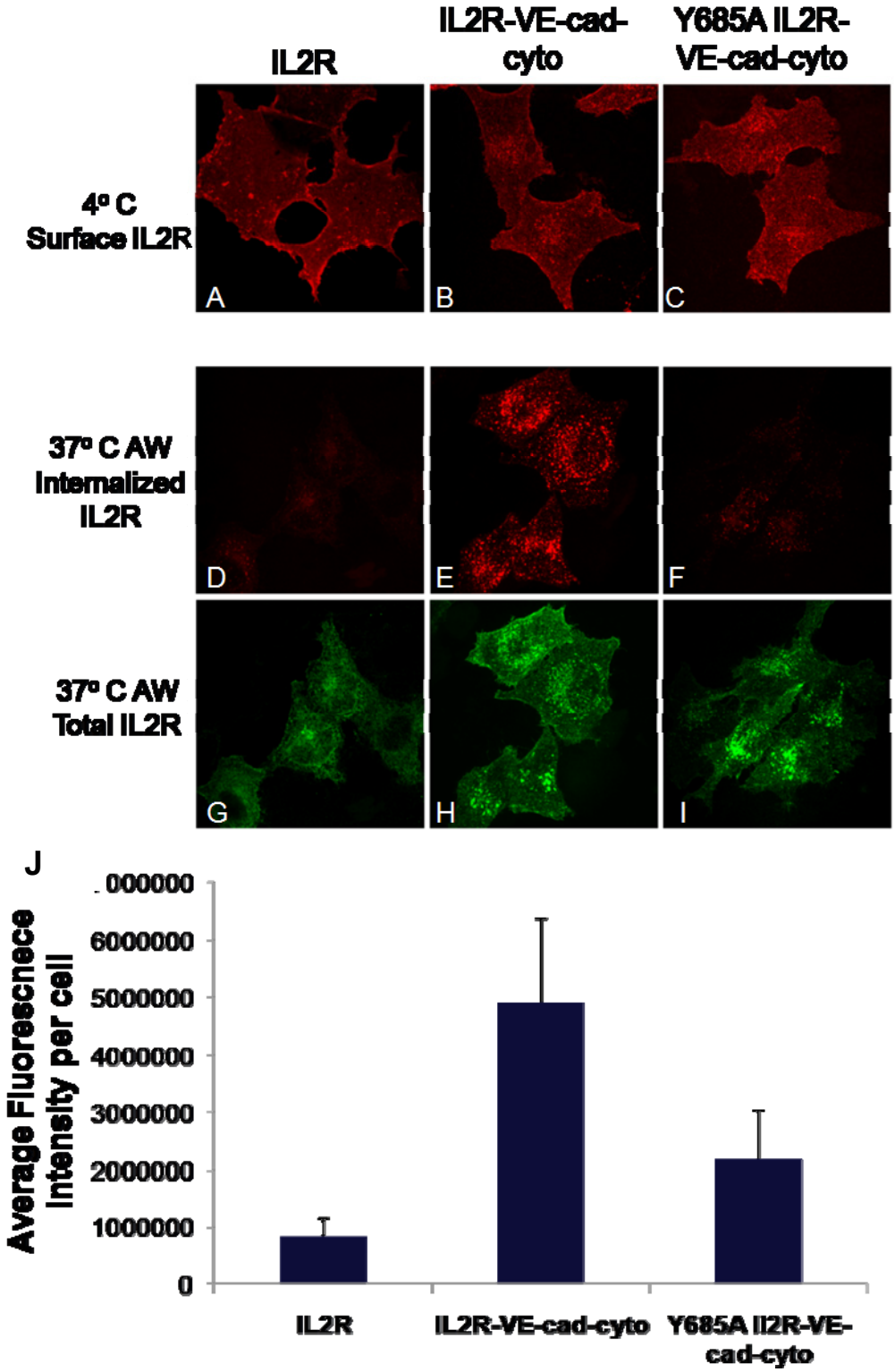
IL2R
IL2R-VE-cad-cyto
Y685A IL2R-VE-cad-cyto
4° C Surface IL2R
37° C AW Internalized IL2R
37° C AW Total IL2R
A
B
C
D
E
F
G
H
I
J
Average Fluorescnece Intensity per cell
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
IL2R
IL2R-VE-cad-cyto
Y685A II2R-VE-cad-cyto

Figure 5:

Tyrosine 685 is required for efficient VE-cadherin endocytosis. Site directed mutagenesis was used to determine if the putative tyrosine based sorting motif ($_{685}$YAQV$_{688}$) is important for internalization of the IL2R-VE-cad$_{cyto}$. HeLa cells expressing IL2R, IL2R-VE-cad$_{cyto}$, or a IL2R-VE-cad$_{cyto}$ Y685A point mutant, were labeled at 4^{o}C with the IL2R antibody and then incubated at 37^{o}C for 15 min. to allow for internalization. Cells were then acid washed to remove surface antibody and processed for immunofluorescence. A second IL2R antibody was used after fixation to detect total levels of expression of the chimeras. Similarly to the IL2R alone (A), internalization in cells expressing the IL2R-VE-cad$_{cyto}$Y685A substitution (C) was significantly reduced as compared to the IL2R-VE-cad$_{cyto}$ (B).

Chapter 5

Summary and Future Directions

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Overview of the Dissertation

The primary goal of the work described in this dissertation was to understand the mechanisms underlying how p120 levels function as a setpoint for cadherin expression. A thorough understanding of the cellular mechanisms regulating the dynamics of cadherin function is essential because these pathways are often misregulated during disease. Previous work determined that p120 stabilizes cadherin expression at the plasma membrane by preventing cadherin degradation through an endo-lysosomal pathway. As a necessary prerequisite for elucidating the mechanistic basis of p120's function, the results presented here achieve three additional objectives: 1) to define the (adaptor) endocytic machinery involved in VE-cadherin internalization, 2) to identify sequences in the VE-cadherin cytoplasmic tail that mediate internalization and 3) to mechanistically uncouple the function of p120 in preventing cadherin endocytosis from its role in the regulation of RhoGTPase activity.

Prior studies suggested that VE-cadherin may be internalized through a clathrin dependent endocytic pathway, but little was known about the molecular machinery involved. Here, we used a series of pharmacological, biochemical, and genetic approaches to confirm that VE-cadherin endocytosis occurs through a clathrin and dynamin dependent pathway. A key result from these studies was the finding that depletion of the clathrin adaptor AP-2 by siRNA prevents internalization of VE-cadherin. We went on to identify a novel interaction between AP-2 and the VE-cadherin cytoplasmic tail. These data provide the first evidence for a role for AP-2 in cadherin endocytosis. The finding that AP-2 interacts with VE-cadherin and recruits it into clathrin coated vesicles led us to look for AP-2 binding sites in the cadherin tail. Analysis of the

VE-cadherin cytoplasmic tail revealed the presence of a putative tyrosine sorting motif ($_{685}$YAQV$_{688}$) within the VE-cadherin JMD and mutagenesis studies confirmed that this motif functions as a bona fide sorting signal that is required for efficient AP-2 binding and VE-cadherin endocytosis. Together, our findings lend support to a model in which the binding of p120 to the VE-cadherin cytoplasmic tail stabilizes VE-cadherin at the plasma membrane by preventing the clathrin adaptor AP-2 from interacting with a tyrosine sorting motif in the VE-cadherin tail and recruiting the cadherin into clathrin coated vesicles. The ability of p120 to prevent cadherin recruitment into clathrin and AP-2 enriched membrane domains reveals a previously unappreciated mechanism by which clathrin-mediated endocytosis is regulated. While these studies have provided key answers to questions regarding p120's function, they have also raised important new questions, which we will discuss next.

Future Directions

What mechanisms regulate the dissociation of p120 from the VE-cadherin tail?

In our model, p120 functions as a cap on the VE-cadherin tail that prevents other interactions from occurring through steric hindrance. An important prediction that arises from this model is that p120 must dissociate from the cadherin tail prior to internalization. Consistent with this idea, previous data suggests that p120 does not colocalize with the intracellular pool of VE-cadherin (Xiao *et al.*, 2005). The mechanism regulating the association of p120 with the VE-cadherin tail is thus of central importance to our model. The loss of p120 from the cadherin JMD may be explained by changes in the affinity of p120 for the cadherin tail or by competition from other proteins for

cadherin binding. To date, however, very little is known about regulatory factors and signaling pathways that modulate this association. Interestingly, the affinity of the p120-cadherin association is relatively low compared to that of β-catenin, suggesting that the weak nature of the interaction may be important for regulating the plasticity of adherens junctions (Xiao *et al.*, 2007). One hypothesis is that similarly to β-catenin, phosphorylation of p120 may be important in regulating the affinity of the interaction between p120 and the cadherin tail. p120 contains numerous phosphorylation sites and functions as a substrate for both tyrosine and serine/threonine kinases (Alema and Salvatore, 2007). In epithelial cells, Src family kinases (SFKs), the nonreceptor protein tyrosine kinase Fer, and receptor tyrosine kinases have all been shown to regulate adherens junction integrity, and p120 has been implicated as a potential target of regulation (Alema and Salvatore, 2007). However, the precise mechanisms regulating these processes remain unclear. It is likely that different kinases regulate p120 phosphorylation in different ways, allowing for subtle changes in function depending on the local signaling microenvironment. Interestingly, the kinases regulating p120 phosphorylation in endothelial cells have yet to be elucidated. A related possibility is that phosphorylation of the cadherin tail regulates the p120 association. Again, while the cadherin tail is a known substrate for both tyrosine and serine/threonine phosphorylation, the evidence for a role for cadherin phosphorylation in regulating the affinity of p120 binding is unclear (Potter *et al.*, 2005; Wallez and Huber, 2008; Adam *et al.*, 2010). One study reported that phosphorylation of VE-cadherin at Y658 disrupts p120 binding and leads to increased cell permeability. However, these experiments were performed in CHO cells, and subsequent studies in endothelial cells have produced contradictory results

(Monaghan-Benson and Burridge, 2009; Adam *et al.*, 2010). Further studies will be required to more definitively examine how VE-cadherin phosphorylation contributes to the interaction with p120 and how this process is modulated by upstream signaling molecules.

Alternatively, the dissociation of p120 from the cadherin tail may be reflective of competition from other cadherin binding partners. Presenilin-1 and Hakai are two molecules that have been proposed to play such a role in regulating cadherin stability. It has been hypothesized that by binding to the cadherin tail, these or other molecules may cause p120 dissociation, resulting in endocytosis. Presenilin-1 causes proteolytic cleavage of the cadherin tail, resulting in cadherin degradation. Studies have demonstrated that presenilin-1 and p120 bind to the same region of the cadherin JMD and that this binding is mutually exclusive, suggesting that presenilin-1 may compete with p120 for interactions with the cadherin tail (Baki *et al.*, 2001; Rubio *et al.*, 2005). Another molecule that has been found to function through a similar mechanism is the E3 ubiquitin ligase Hakai, which interacts with a phosphorylated tyrosine residue in the E-cadherin JMD. Upon binding to the cadherin tail, Hakai causes ubiquitination and degradation of E-cadherin (Fujita *et al.*, 2002). While it has not been directly tested, it is possible that p120 may block cadherin phosphorylation and/or Hakai binding. Interestingly, VE-cadherin does not contain the residue required for Hakai binding, indicating that alternative molecules may carry out similar functions in endothelial cells.

An intriguing example of such a molecule has recently been illustrated by studies of Kaposi's sarcoma, an angiogenic vascular tumor that causes aberrant endothelial cell proliferation, angiogenesis, and inflammation (Mansouri *et al.*, 2008; Qian *et al.*, 2008).

Kaposi's sarcoma is the most common AIDS associated malignancy and is caused by infection with Kaposi sarcoma-associated herpesvirus (KSHV). Recent studies have revealed that VE-cadherin is a target of K5, an E3 ubiquitin ligase that is expressed by the KSHV. K5, along with K3, another protein expressed by KSHV, functions as a membrane-associated viral E3 ubiquitin ligase, and is homologous to the human membrane-associated RING-CH (MARCH) proteins. Upon K5-mediated ubiquitination, VE-cadherin is degraded and adherens junctions are disrupted, leading to increased cellular permeability (Mansouri *et al.*, 2008; Qian *et al.*, 2008). Interestingly, K3 has been shown to mediate the addition of lysine-63 linked polyubiquitin chains to MHC class I molecules. Following polyubiquitination by K3, MHC class I undergoes clathrin-dependent endocytosis that is mediated by the epsin endocytic adaptor (Duncan *et al.*, 2006). The precise mechanism by which K5 promotes VE-cadherin downregulation is not yet clear, but the identification of a disease that directly modulates VE-cadherin turnover could serve as an invaluable tool for studying VE-cadherin endocytosis. The VE-cadherin JMD contains several lysine residues that represent potential targets for ubiquitination by K5. We have begun to analyze these sites to determine if ubiquitination may also contribute to the constitutive internalization of VE-cadherin, potentially in cooperation with endogenous MARCH proteins.

How does VE-cadherin endocytosis contribute to vascular development and disease?

VE-cadherin is the central component of endothelial adherens junctions, and its adhesive and signaling functions are crucial for maintaining the integrity of the vascular endothelium, both during development and in the adult organism. While the disruption of

VE-cadherin from adherens junctions has long been correlated with decreased barrier function and changes to cellular morphology, surprisingly little is known about the direct contribution of VE-cadherin endocytosis during vascular development and disease responses. Our identification of a tyrosine motif ($_{685}$YAQV$_{688}$) that mediates VE-cadherin internalization and AP-2 binding provides us with important tools for understanding how VE-cadherin endocytosis contributes to endothelial cell function. An important future direction of this project involves studying mutants of VE-cadherin that are incapable of undergoing efficient endocytosis to elucidate the role of cadherin endocytosis in endothelial cell functions, including migration, barrier function, and proliferation.

An immediate goal is to confirm the requirement for Y685 for internalization of full length VE-cadherin. These experiments will be carried out in the context of p120-null endothelial cells, which exhibit reduced levels of endogenous VE-cadherin and should promote rapid turnover of exogenously expressed cadherins. This system will enable us to determine if the Y685A mutant is stabilized at the plasma membrane compared to the WT VE-cadherin. Interestingly, the tyrosine mutant retains about 50% of activity compared to WT VE-cadherin, indicating the VE-cadherin tail must contain other sequences that mediate AP-2 binding. These sequences are likely to be located within the VE-cadherin JMD, as this domain is sufficient for internalization. In recent years, numerous “alternative adaptors”, or CLASPs have been identified and shown to cooperate with AP-2 and the clathrin machinery in clathrin coated vesicle formation and cargo recruitment (Traub, 2009). One possibility is that one or more CLASPs may function cooperatively with AP-2 to mediate VE-cadherin recruitment into clathrin coated vesicles. A future goal is to identify additional sequences within the JMD that are

important for VE-cadherin internalization and characterize the protein interactions that mediate endocytosis. As discussed in Chapter IV, previous studies of cadherin endocytosis provide us with hints of potential candidate adaptors, including roles for ubiquitination dependent adaptors and the GPCR adaptor β-arrestin. Therefore, one focus of our future studies will be to determine the contribution of these adaptors to constitutive internalization of VE-cadherin.

As described above, these experiments will be conducted using mutants in the context of full length VE-cadherin that we will express in a cadherin null background. We believe that these studies will elucidate important roles for VE-cadherin endocytosis in functions of the vascular endothelium that are dynamically regulated during development and disease. In addition to the Y685A mutant, the identification of other sites in the VE-cadherin tail that are required for endocytosis will hopefully provide us with mutants that are stably expressed at the plasma membrane. Ultimately, the generation of a transgenic mouse containing a VE-cadherin mutant that is unable to undergo endocytosis could provide valuable information about the contribution of membrane trafficking to vascular development.

The research presented in this dissertation represents an important contribution to the collective body of evidence that supports a pivotal role for the regulation of cadherin based cell adhesion by membrane trafficking pathways. The importance of membrane trafficking in modulating cellular adhesive potential is underscored by the widespread misregulation of cadherin trafficking in human pathologies, including cancer, inflammation, and viral pathogenesis. Ultimately, novel aspects of cadherin trafficking may be targeted by therapies aimed at modulating cadherin function. Overall, however,

there is a need to better understand the functional interplay between membrane trafficking pathways and cadherin function in the context of development and human disease.

Chapter 6

References

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