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Role of the cytoplasmic tail in regulating cadherin trafficking

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Biochemistry, Cell, and Developmental Biology

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Wenji Su
B.S., Hong Kong University of Science and Technology, 2010

Advisor: Andrew P. Kowalczyk, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies
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Abstract

Role of the cytoplasmic tail in regulating cadherin trafficking

By Wenji Su

Cell adhesion is essential in many biological processes including embryonic development and disease progression. Dynamic regulation of cadherin-based adhesion is important in modulating cadherin function and adhesion strength. The cytoplasmic tail of classical cadherins acts as a regulatory domain for cadherin activities such as attachment to the cytoskeleton and cadherin turnover, through interactions with a variety of binding partners.

In this dissertation, I focused on two different mechanisms that regulate cadherin trafficking. First, I provided insights into a novel proteolytic event that alters the post-endocytic trafficking itinerary of VE-cadherin. By using a combination of biochemical and fluorescence imaging approaches, I demonstrated that the VE-cadherin tail is cleaved by calpain upon entrance into clathrin-enriched membrane domains before reaching the early endosome, and this cleavage occurs between the juxtamembrane domain and the catenin-binding domain of VE-cadherin. I also found that the cleaved fragment exhibits a higher turnover rate when compared to the full-length cadherin, likely due to a decreased recycling rate implied by reduced colocalization with the recycling markers, as well as increased degradation rate implied by increased colocalization with lysosomal markers.

In addition, I also demonstrated that mutation of a dileucine motif to alanine, which inhibits E-cadherin endocytosis, reduces collective cell migration when measured by a 2-D migration assay, in both normal epithelial cells and lung cancer cells. By disrupting cadherin endocytosis with the dileucine mutation, cancer cell invasion was increased in a 3-D spheroid invasion assay. These results together suggest a context-dependent function of cadherin endocytosis in different biological systems.

In summary, these findings contribute to a better understanding of the role of the cytoplasmic tail in cadherin trafficking and how cadherin trafficking affects cell mobility.

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Chapter 1

Dissertation Overview and Significance

The human body consists of trillions of cells, which require constant interaction with one another to maintain proper function of the organism. One way cell-cell interaction is established is through physical contact (Figure 1-1). Cell-cell adhesion plays a major role in establishing and maintaining physical cell-cell interactions, and is essential in development and diseases.

Cell-cell adhesion is universal in almost all cells types. For example, in skin epithelial cells, strong cell-cell adhesion is important in resisting external forces and maintaining the protective barrier for the organism (Nekrasova and Green 2013). Pemphigus vulgaris, an autoimmune disease that targets adhesion proteins in the skin epithelial keratinocytes, results in loss of cohesion between keratinocytes in epidermis, leading to symptoms including blistering and can ultimately be fatal (Stahley and Kowalczyk 2015, Cholera and Chainani-Wu 2016). In endothelial cells that lines the inside of blood vessels, cell-cell adhesion is required for maintaining proper barrier function to restrict trans-endothelial movements of molecules and cells (Gavard 2013, Schnittler 2016). Cell adhesion is also important during angiogenesis, which refers to the formation of new blood vessels (Dorland and Huveneers 2016). In general, cell-cell adhesion allows adjacent cells to stick together and is essential in establishing three-dimensional multicellular organization, which allows the cells to form functional units that are the foundation of a biologically active organism.

Despite providing adhesive strength, the plasticity of adhesive junctions is also important, especially during development. For example, vascular endothelial adherens junctions need to remodel during vasculogenesis and angiogenesis to allow cell movement (Dorland and

Huveneers 2016). One way to achieve plasticity is through vesicle trafficking, a process during which adhesion molecules are removed from and reinserted into the plasma membrane (Cadwell, Su et al. 2016, Dorland and Huveneers 2016). Adhesion molecules can be removed from the plasma membrane via endocytosis (Cadwell, Su et al. 2016). Endocytosed molecules then enter endocytic compartments followed by various sorting mechanisms. A subset of those molecules are transported to recycling compartments to return to the plasma membrane. Typically, the remainder of the endocytosed molecules enter late endosomes and are eventually degraded (Cadwell, Su et al. 2016). The endocytosis-recycling/degradation pathway is effective in regulating the surface dynamics of adhesion molecules and is therefore a very important topic in the cell adhesion field.

Cell-cell adhesion is mainly mediated by adhesive cell junctions. There are two types of adhesive cell junctions, adherens junction and desmosome (Cadwell, Su et al. 2016). Adherens junction mainly consists of classical cadherins. On the other hand, desmosomes contain desmosomal cadherins. Other than cadherins, various types of catenins are also major components of adherens junctions and desmosomes. Both the adherens junction and desmosome are connected to the cytoskeleton, which provides mechanical strength to the junction (Cadwell, Su et al. 2016). Cadherins engage in homophilic interactions at cell-cell border to mediate adhesion. In general, the strength of adhesion is relative to the amount of surface cadherins. Through dynamic trafficking, the surface levels of cadherins can be modulated to allow releasing and reattachment of a single cell. In vascular endothelial cells, this process is essential during the growth and development of blood vessels (Dorland and Huveneers 2016). Local rearrangement of vascular endothelial cadherins (VE-cadherin) is also important in allowing trans-endothelial migration during inflammation (Gavard 2013).

Despite mediating adhesion, cadherins are also known to be involved in signal transduction pathways. For instance, VEGF promotes VE-cadherin endocytosis through activation of tyrosine kinase Src (Sarelius and Glading 2015). It is also believed that the establishment of stable adherens junctions triggers signaling events that result in contact inhibition (Roycroft and Mayor 2016). Another way to regulate cadherin activities is through proteolytic processing. There are a variety of known proteases that interact with cadherins. For example, metalloproteases have been shown to cleave the ectodomain of cadherins and lead to loss of adhesion (Saftig and Lichtenthaler 2015). Calpain has also been implicated in cleaving the cytoplasmic domain of cadherins and also results in disruption of adhesion (Miyazaki, Taketomi et al. 2011, Konze, van Diepen et al. 2014, Kudo-Sakamoto, Akazawa et al. 2014).

The overall goal of this dissertation is to expand our knowledge of cadherin regulation by focusing on two different regulatory mechanisms in the cadherin cytoplasmic tail: one involves a proteolytic processing event that alters post-endocytic trafficking of VE-cadherin, and the other is to further investigate the effect of a dileucine endocytic motif on cell mobility in different model systems. This goal is addressed through three specific questions:

1. How does VE-cadherin undergo proteolytic processing during endocytosis?

2. How does VE-cadherin proteolysis affect cadherin trafficking?

3. How does cadherin endocytosis affect cell motility and function?

To better present the findings of the dissertation, I will first review the overall structure and molecular mechanism of the adherens junction in chapter 2, including detailed reviews of the adherens junction components, cadherin turnover and post-translational processing of cadherins. This chapter provides an overview of adherens junctions and insights into how cadherins are regulated, thereby providing a foundation for the findings presented in chapter 3 and 4 of this dissertation.

The work in this dissertation leads to the following discoveries regarding the cadherin tail and its role in cadherin trafficking:

1. VE-cadherin cytoplasmic tail is cleaved during endocytosis.
2. Cleavage of VE-cadherin occurs upon entry of the cadherin into the clathrin-enriched domain and is completed before reaching the early endosome.
3. Cleavage of the VE-cadherin tail leads to altered post-endocytic trafficking resulting in reduced recycling and increased degradation.
4. VE-cadherin tail is cleaved by the protease calpain to remove the catenin-biding domain.
5. A dileucine motif in E-cadherin cytoplasmic tail is required for E-cadherin endocytosis.
6. E-cadherin harboring the endocytic mutation leads to different cell migration manners in different *in vitro* migration models.

Together, these findings contribute to our understating of the cadherin cytoplasmic tail and its role in cadherin trafficking, and thereby provide insights into processes important in development and disease.

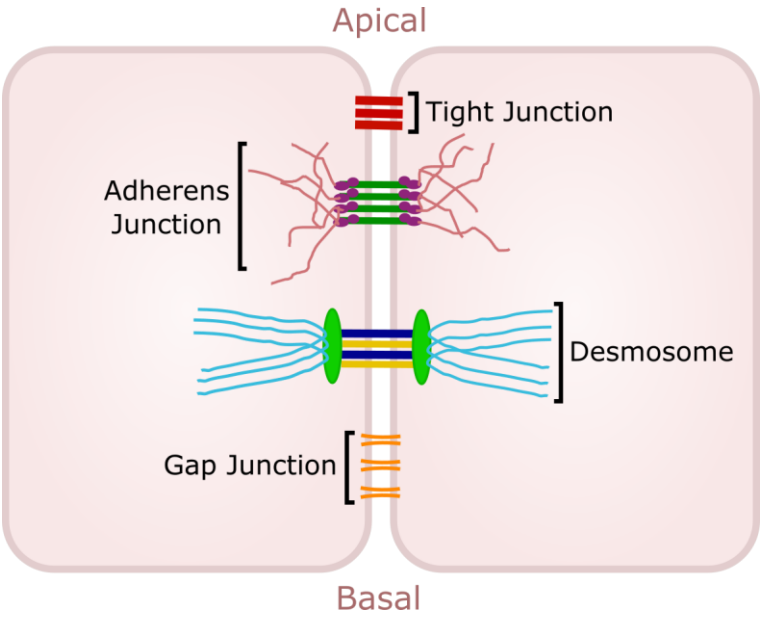


Figure 1-1. Schematic illustration of cell junctions in polarized cells. Tight junctions define the apical-lateral boundary, followed by adherens junctions. Desmosomes and gap junctions locate to the basal-lateral surface of the cell. Tight junctions restrict molecular movement between the apical and the basal-lateral regions. Adherens junctions mediate cell adhesion and regulate cell migration. Desmosomes link to intermediate filaments to withstand force and maintain cellular integrity. Gap junctions mediate transport of molecules between connected cells.

Chapter 2

Overview of adherens junctions

2.1 Introduction to the adherens junction

Thanks to the advancement of electron microscopy techniques in the 1950s, scientists were able to closely examine fine structures at cell-cell contacts (Palade 1952, Farquhar and Palade 1963), leading to the identification of the adherens junction (Farquhar and Palade 1963). Adherens junctions were first named *zonula adherens* (intermediary junction) in the 1960s, characterized by intermediary space (~200Å) occupied by low density homogeneous materials (Farquhar and Palade 1963). It was initially considered to act as a physical bridge to connect two adjacent cells. Further investigation revealed the importance of adherens junction in various biological processes including cell segregation, signal transduction, cell polarity and mobility (Harris and Tepass 2010). Research in this field have progressed over the ensuing decades to dramatically improve our understanding of adherens junctions, not only on the cellular level, but also on the molecular level. In this chapter, I will give a comprehensive overview of adherens junctions by focusing on the following aspects: molecular components, cadherin turnover, and post-translational processing. The purpose of this chapter is to set a foundation to help understand the implications of the findings presented in the dissertation.

2.2 Molecular components of adherens junctions

2.2.1 Cadherins

Cadherins are the major components of adherens junctions. The name "cadherin" came from the observation that this protein is calcium-dependent and carries the function of adhesion (Yoshida-Noro, Suzuki et al. 1984). By loose definition in the field, proteins that contain extracelluar cadherin (EC) repeats are considered members of cadherin superfamily, which includes structurally diverse proteins (Oda and Takeichi 2011). There are four major subfamilies in the cadherin superfamily: classical cadherins, desmosomal cadherins, protocadherins and unconventional cadherins. The cadherin superfamily includes single-pass transmembrane cell adhesion proteins, G-protein coupled receptors and others (Oda and Takeichi 2011). The diversity of the cadherin family is accomplished by having multiple cadherin encoding genes and alternative splicing (Oda and Takeichi 2011).

Classical cadherins, which are the major focus of this dissertation, are structurally similar to each other (Ivanov, Philippova et al. 2001). There are two subfamilies in classical cadherin, Type I and Type II, both have similar structures with a distinction in the ectodomain (Ivanov, Philippova et al. 2001) (Figure 2-1). Type I classical cadherin, including E-, N-, P-, and C-cadherin, are more widely expressed in multiple cell types, whereas type II classical cadherin such as VE-cadherin, often have a more restricted expression pattern (Ivanov, Philippova et al. 2001). Classical cadherins are the best studied amongst the cadherin superfamily. A classical cadherin is composed of four domains: an ectodomain that contains five tandem extracellular cadherin repeats, a transmembrane

domain, a juxtamembrane domain, and a catenin-binding domain. Both the juxtamembrane domain and the catenin-binding domain are part of the cytoplasmic tail and are involved in binding to other cytoplasmic proteins such as catenins (Figure 2-1).

2.2.1a Cadherin ectodomain

The ectodomain of classical cadherins is composed of five EC repeats, each has approximately 110 amino acids (Shapiro, Fannon et al. 1995, Ivanov, Philippova et al. 2001). Binding of calcium ions to cadherin ectodomain occurs through three calcium-binding sites between two adjacent EC repeats, one at the base of the one EC domain, one at the top of the other EC domain, one in the linker between the two (Ozawa, Engel et al. 1990, Shapiro, Fannon et al. 1995, Shapiro and Weis 2009). Without calcium binding, the ectodomain remains flexible. Upon binding of calcium, the cadherin ectodomain gains structural rigidity, adopting a curved shape, which allows the extracellular domain to engage in homophilic adhesive interactions between opposing cells that is essential for carrying out biological functions (Shapiro, Fannon et al. 1995, Shapiro and Weis 2009) (Figure 2-1).

Homophilic interactions between the cadherin mainly involve the cadherin repeat EC1 (Nose, Tsuji et al. 1990, Boggon, Murray et al. 2002, Klingelhofer, Laur et al. 2002, Troyanovsky, Sokolov et al. 2003, Shapiro and Weis 2009). However, in the absence of the other extracelluar domains, the N-terminal EC1 domain alone cannot maintain functional binding or adhesive activity (Yap, Brieher et al. 1997, Ivanov, Philippova et al. 2001, Shapiro and Weis 2009). This homophilic interaction also mediates cell sorting: cells that bear identical cadherins on their surfaces first aggregate (Takeichi, Atsumi et al. 1981,

Ogou, Okada et al. 1982, Takeichi 1990, Shapiro and Weis 2009). This binding specificity is the potential mechanism behind complex tissue patterning and segregation of cell types during development.

X-ray crystallography analysis revealed that cadherins exist in the form of lateral dimers (Shapiro, Fannon et al. 1995, Ivanov, Philippova et al. 2001). Despite the initial model that suggests cadherin interact in a zipper-like fashion, recent studies have recognized multiple forms of adhesive interactions between cadherin, including *trans* and *cis* interactions (Figure 2-2). When two opposing cells approach each other, *trans* interactions are first formed through an intermediate encounter complex, most likely a complex referred to as the X-dimer (Sivasankar, Zhang et al. 2009, Harrison, Bahna et al. 2010). During the maturation of adhesive interactions, strand swap dimers are formed, which occur between two EC1 from two opposing cells with the insertion of a conserved Trp2 side chain into a hydrophobic binding pocket on its binding partner in type I classical cadherins (Harrison, Bahna et al. 2010) (Figure 2-2). These interactions are reinforced by *cis* interactions, which, in addition to interactions mediated by the cadherin cytoplasmic tail, help to cluster the cadherins and increase overall strength of the junction (Zhang, Sivasankar et al. 2009). Recent studies have shown that *cis* interactions can occur between EC1 and EC2 on two adjacent cadherins from the same cell (Shapiro and Weis 2009, Zhang, Sivasankar et al. 2009). The region of EC1 involved in *cis* interaction is the opposite domain of the one involved in strand swapping (Shapiro and Weis 2009). This enables both *trans* strand swap interactions and *cis* interactions to occur simultaneously (Figure 2-2).

2.2.1b Cadherin cytoplasmic tail

The cytoplasmic tail of classical cadherins, which is the most highly conserved region in these proteins, mainly consists of two domains: the juxtamembrane domain and the catenin-binding domain, both of which are involved in catenin binding (Figure 2-1). The first evidence of cadherin association with the cytoskeleton was based on studies that show cadherins cannot be extracted with non-ionic detergents which can effectively solubilized other membrane proteins (Nagafuchi and Takeichi 1988, Ozawa, Baribault et al. 1989, McCrea and Gumbiner 1991, Ivanov, Philippova et al. 2001). It was shown later that the cadherin C-terminal catenin-binding domain binds to β-catenin, which then associates with α-catenin to mediate the interaction between the cadherins and actin cytoskeleton (Cadwell, Su et al. 2016).

In 2001, Huber and colleagues crystalized the E-cadherin cytoplasmic tail in complex with β-catenin, and utilized X-ray crystallography to determine its structure (Huber and Weis 2001). Another report of cadherin cytoplasmic domain structure came from Ishiyama and colleagues, in which they used X-ray crystallography and nuclear magnetic resonance (NMR) study to analyze the binding interface between E-cadherin juxtamembrane domain and p120 catenin (Ishiyama, Lee et al. 2010). Together with previous reports of β-catenin-E-cadherin complex (Huber and Weis 2001) and β-catenin-α-catenin complex structures (Pokutta and Weis 2000, Yang, Dokurno et al. 2001), they were able to put together the entire cadherin cytoplasmic domain structure in complex with catenins (Ishiyama, Lee et al. 2010). As the structure reveals, the cadherin cytoplasmic domain is intrinsically unstructured (Huber, Stewart et al. 2001, Huber and Weis 2001, Ishiyama, Lee et al. 2010). This intrinsically unstructured nature of the cadherin tail and the dynamic binding manner

of catenins leave room for potential regulation through post-translational modifications (Dyson and Wright 2005, Tompa, Szasz et al. 2005). Binding of catenins to the cadherin cytoplasmic tail not only stabilizes it structurally, but also protects the tail from being modified (Huber, Stewart et al. 2001, Huber and Weis 2001, Ishiyama, Lee et al. 2010). In fact, it was shown that newly synthesized E-cadherin is associated with β-catenin in the endoplasmic reticulum (Hinck, Nathke et al. 1994, Shapiro and Weis 2009). By disrupting this interaction, E-cadherin becomes vulnerable to proteosomal degradation (Chen, Stewart et al. 1999, Shapiro and Weis 2009). On the other hand, the juxtamembrane domain, which associates with p120 catenin, was shown to be essential in mediating cadherin stability through regulating cadherin endocytosis (Ohkubo and Ozawa 1999, Thoreson, Anastasiadis et al. 2000, Davis, Ireton et al. 2003, Xiao, Allison et al. 2003, Nanes, Chiasson-MacKenzie et al. 2012). Studies have shown that the juxtamembrane domain in VE-cadherin, a type II classical cadherin, harbors an endocytic motif which is masked by p120 catenin upon binding (Nanes, Chiasson-MacKenzie et al. 2012). By disrupting this interaction, VE-cadherin exhibits increased endocytosis resulting in reduced steady-state level (Xiao, Allison et al. 2003, Nanes, Chiasson-MacKenzie et al. 2012). In summary, the cytoplasmic tail of classical cadherin is a major regulatory domain for cadherin cytoskeletal linkages and endocytic trafficking.

2.2.2 Other components of the adherens junction

Over 400 different proteins were found to interact directly or indirectly with epithelial cadherins at the adherens junction by recent proximity biotinylation proteomics studies (Guo, Neilson et al. 2014, Van Itallie, Tietgens et al. 2014, Padmanabhan, Rao et al. 2015). These proteins can be divided into more than 20 functional groups including cytoskeleton

regulators, scaffolding proteins, signaling kinases and phosphatases, transcription elements, endocytic machinery, and proteolytic enzymes - enabling adherens junctions to perform a variety of biological functions (Padmanabhan, Rao et al. 2015). Catenins were the first identified and best studied amongst them. Catenins include a family of cytoplasmic proteins characterized by signature central armadillo domain (Ozawa, Baribault et al. 1989, Ozawa, Ringwald et al. 1990, Ozawa and Kemler 1992, Shapiro and Weis 2009). Classical cadherins are known to associate directly with both p120-catenin and β-catenin. As stated above, p120-catenin binds to cadherin through cytoplasmic juxtamembrane domain, while β-catenin binds to the C-terminal catenin-binding domain. α-catenin, a non-armadillo family protein related to vinculin, does not bind to cadherin directly. Instead, α-catenin binds to β-catenin and thus physically connects the cadherin to F-actin (Shapiro and Weis 2009, Buckley, Tan et al. 2014) (Figure 2-1).

Although p120 and β-catenin are structurally similar to each other, they carry very different biological functions. p120-catenin, through its binding to the juxtamembrane domain, acts as a master regulator of cadherin endocytosis by masking a conserved endocytic motif (Ishiyama, Lee et al. 2010, Nanes, Chiasson-MacKenzie et al. 2012). On the other hand, β-catenin not only serves as a bridge to connect cadherin to F-actin, but also helps protect newly synthesized cadherin from proteosomal degradation (Lickert, Bauer et al. 2000, Shapiro and Weis 2009).

2.2.2a p120-catenin

p120-catenin is an armadillo family protein, named after its 120kDa molecular size (Anastasiadis and Reynolds 2000). Initial discovery of p120 identified it as a substrate of

the tyrosine kinase Src (Reynolds, Roesel et al. 1989). It was first found to be in a complex with E-cadherin by Reynolds and colleagues in 1994 and was characterized to be a member of the armadillo family (Reynolds, Daniel et al. 1994). Subsequent studies demonstrated that p120 associates with cadherin juxtamembrane domain (Finnemann, Mitrik et al. 1997, Lampugnani, Corada et al. 1997, Ozawa and Kemler 1998, Yap, Niessen et al. 1998, Aono, Nakagawa et al. 1999, Ohkubo and Ozawa 1999, Thoreson, Anastasiadis et al. 2000). Binding of p120 to the cadherin juxtamembrane domain is involved in regulation of cadherin endocytosis. Most data support a model that p120 binding blocks an endocytic motif, inhibiting adaptor proteins from recruiting the cadherin into clathrin-coated pits. Structural studies of E-cadherin in complex with p120 suggest that p120 binding masks dileucine residues that are important for cadherin endocytosis and lysine residues that can be modified by ubiquitination (Ishiyama, Lee et al. 2010) (Figure 2-5). Further evidence comes from the finding that p120 inhibits the entry of VE-cadherin into clathrin and AP-2 enriched membranes (Chiasson, Wittich et al. 2009). Yeast two-hybrid data and mutational analyses identified a region of about 10 residues that makes up the core p120-binding sequence (Ohkubo and Ozawa 1999, Thoreson, Anastasiadis et al. 2000). A well-conserved cluster of acidic residues in the core p120-binding domain of the VE-cadherin cytoplasmic tail (DEE 646-648) was characterized recently to be both necessary and sufficient to mediate VE-cadherin internalization (Nanes, Chiasson-MacKenzie et al. 2012) (Figure 2-5). p120 binding masks this endocytic motif and therefore prevents VE-cadherin from being internalized (Nanes, Chiasson-MacKenzie et al. 2012). Mutation of this DEE sequence not only blocks p120 binding and VE-cadherin endocytosis, but also inhibits collective cell migration (Nanes, Chiasson-MacKenzie et al. 2012). This data suggests the

importance of cadherin endocytosis cell mobility, which is critical during development and disease.

2.2.2b β-catenin

β-catenin was one of the first catenins identified in the adherens junction via immunoprecipitation of detergent-solubilized cell extracts using anti-cadherin antibodies (Ozawa, Baribault et al. 1989). It was later found to bind to the cadherin cytoplasmic domain and α-catenin, which associates with actin cytoskeleton (Shapiro and Weis 2009, Buckley, Tan et al. 2014). Other than the stabilizing the adherens junction by linking cadherin to actin cytoskeleton, several studies have suggested that β-catenin may also play a role in cadherin trafficking to the plasma membrane and endocytosis of the cadherin. In 2009, Lickert et al. discovered that several serine residues in the catenin-binding domain of newly synthesized E-cadherin is phosphorylated by casein kinase II, which increases the binding affinity between cadherin and β-catenin and therefore protects E-cadherin from degradation (Lickert, Bauer et al. 2000). In contrast, Dupre-Crochet et al. found that casein kinase I overexpression leads to disruption of adherens junction by phosphorylating serine846 residue in the catenin-binding domain in E-cadherin (Dupre-Crochet, Figueroa et al. 2007). Interestingly, this phosphorylation reduces the binding affinity of β-catenin and thus lead to increased cadherin endocytosis (Dupre-Crochet, Figueroa et al. 2007). The opposite effect of casein kinase I and II on cadherin stability provides an example of how phosphorylation of a similar region at different stages of the cadherin life cycle can lead to dramatically different outcomes. Another example of how β-catenin is involved in cadherin trafficking came from a study that found decreased N-cadherin endocytosis upon activation of the NMDA receptor in hippocampal neurons (Tai, Mysore et al. 2007). β-catenin binding

with N-cadherin was found to be increased in response to NMDA receptor activation, leading to adherens junction stabilization (Tai, Mysore et al. 2007). However, studies from Sharma and colleagues suggested that β-catenin mediates macropinocytosis of N-cadherin in NIH 3T3 fibroblasts (Sharma and Henderson 2007). Whether β-catenin inhibits or mediates cadherin endocytosis, it appears that β-catenin is involved in more than simply linking the cadherin to the cytoskeleton. In addition, studies from Chen et al. showed that β-catenin binding with E-cadherin is important in successful targeting of the cadherin to the basal lateral membrane in MDCK cells (Chen, Stewart et al. 1999). This study suggested an important role of β-catenin in cadherin trafficking and sorting. The work presented in this dissertation also show data indicating the role of β-catenin during cadherin sorting and fate after internalization (Chapter 3).

In addition to regulating cadherin trafficking, β-catenin, known to be a key member of Wnt pathway, was also found to functionally link adherens junction and Wnt signaling (Fagotto 2013). Wnt signaling is an important pathway that regulates cell proliferation and differentiation by leading to expression of effector genes (Nusse 2005). β-catenin participates in the Wnt pathway as a T-cell factor (TCF) transcription factor (Wheelock and Johnson 2003). One key element of Wnt pathway regulation is the distribution of β-catenin in the cell. As binding of β-catenin to TCF and cadherin is mutually exclusive, the binding of β-catenin to cadherin at the junction sequesters β-catenin at the junction (Wheelock and Johnson 2003). Studies from Simcha and colleagues identified a small sequence within the E-cadherin cytoplasmic region that can successfully sequester β-catenin and inhibit transcriptional activities (Simcha, Kirkpatrick et al. 2001). Studies by Stockinger and colleagues supported the model that cadherin binding with β-catenin can

regulate cell growth by modulating proliferation-dependent β-catenin transcriptional activity (Stockinger, Eger et al. 2001). However, van de Wetering et al. used an E-cadherin mutant that does not bind to β-catenin to show that without cadherin binding, TCF-mediated transcriptional activity was not elevated in breast cancer cells (van de Wetering, Barker et al. 2001). The studies from Gottardi et al. showed a different result that overexpression of E-cadherin can effectively sequester β-catenin from the cytosol and lead to decreased Wnt signaling in colon cancer cells (Gottardi, Wong et al. 2001). These studies suggested different effects of cadherins on the Wnt pathway through β-catenin, which may be dependent on the specific biological context such as cell types and the external environment.

In summary, increasing amounts of evidence have supported the idea of β-catenin being more than just a scaffold to connect cadherin and actin-cytoskeleton. More studies in the future are required to further clarify the mechanisms of how β-catenin participates in cadherin trafficking and signaling.

2.2.2c Plakoglobin

Despite being a major member of desmosomes, plakoglobin, or γ-catenin, has also been found to bind to classical cadherins in a mutually exclusive manner to β-catenin binding (Shapiro and Weis 2009). Plakoglobin is highly homologues to β-catenin and can also directly bind to α-catenin and therefore link to the actin cytoskeleton (Ben-Ze'ev and Geiger 1998). In endothelial cells, VE-cadherin was found to exist in two distinct pools, one binds to β-catenin and the other binds to plakoglobin (Dejana, Orsenigo et al. 2008). Blood vessels formed by β-catenin null endothelial cells have abnormal lumen and are

frequently hemorrhagic, in which most endothelial adherens junctions have plakoglobin as substitution of β-catenin (Cattelino, Liebner et al. 2003). This results in a different and weaker organization of adherens junctions (Cattelino, Liebner et al. 2003). In addition, Nottebaum and colleagues showed that dissociation of a vascular endothelial protein phosphatase from VE-cadherin results in weakened endothelial cell contacts, via a mechanism that requires only plakoglobin not β-catenin (Nottebaum, Cagna et al. 2008). To date, the role of plakoglobin in classical cadherin-based adherens junction is not fully understood and further research is required to advance our understanding of the function of cadherin/plakoglobin complex.

2.2.2d Ankyrin

Ankyrin is a family of scaffolding proteins that mediates the attachment integral membrane proteins to spectrin-actin based membrane skeleton (Bennett and Baines 2001). The membrane skeleton is a filamentous network that is closely opposed to the cytoplasmic face of the plasma membrane, and is an important element that defines cell shape and gives mechanical support to the membrane (Pumplin and Bloch 1993). Ankyrin was first discovered to associate with cadherin through studies by Nelson et al., in which they identified a complex containing ankyrin in complex with E-cadherin by sucrose gradient fractionation followed by nondenaturing gel electrophoresis from MDCK cell extracts (Nelson, Shore et al. 1990). The assembly of this ankyrin-cadherin complex was later found to contribute to the establishment of epithelial cell surface polarity (Nelson, Hammerton et al. 1990). Kizhatil et al. identified Ankryin-G as a direct binding partner of E-cadherin at the cytoplasmic domain. After binding with E-cadherin, ankyrin-G then recruits β-spectrin to the cadherin-ankyrin complex. Knocking down of either ankyrin-G or β-spectrin results

in failure of E-cadherin localization at cell-cell contacts indicating a regulatory role of ankryin-spectrin complex in E-cadherin mediated cell adhesion and membrane assembly (Kizhatil, Davis et al. 2007, Kizhatil, Yoon et al. 2007). Ankyrin-G was found to bind to E-cadherin in the juxtamembrane domain. Mutation of a conserved polarity motif in the E-cadherin juxtamembrane, which leads to loss of ankyrin binding, results in a highly mobile fraction of cadherin. This observation indicated a role of ankyrin binding to restrict cadherin lateral movement (Jenkins, Vasavda et al. 2013). Recent studies from Cadwell et al. demonstrated that ankyrin-G binds to VE-cadherin juxtamembrane domain independently of p120 binding and inhibits VE-cadherin endocytosis (Cadwell, Jenkins et al. 2016). These studies have suggested a role of ankyrin-G in modulating cadherin trafficking and cell adhesion.

2.2.2f α-catenin and actin

The very basic function of cadherin is to physically connect the cytoskeletal networks between neighboring cells. Studies on the organization of adherens junction revealed that F-actin is associated with cadherin through α-catenin (Buckley, Tan et al. 2014). Despite its name, α-catenin lacks an armadillo domain and more closely resembles the actin-binding protein vinculin, a focal adhesion protein (Shapiro and Weis 2009). At adherens junctions, α-catenin is a key component of the cadherin-catenin complex. This complex is formed though cadherin binding to β-catenin, which recruits α- catenin. α-catenin binds to F-actin, linking the cadherin to the cytoskeleton (Shapiro and Weis 2009). Regions of α-catenin that are important in binding to actin includes the M-domain, which contains a binding site for afadin, an actin-binding protein, and the actin-binding domain (Shapiro and Weis 2009). Mature adherens junctions often associate with stress fibers

formed by large bundles of F-actin and myosin (Schnittler, Taha et al. 2014). One important role of actin cytoskeleton attachment by cadherins is to withstand and transmit force (Padmanabhan, Rao et al. 2015). With the presence of contractile force generated by the cytoskeleton, external stretching or shear stress, the cadherin/catenin/actin complex is often under tension (Padmanabhan, Rao et al. 2015). Interestingly, recent studies show that the cadherin/α-/β-catenin complex can effectively bind to F-actin only when the interaction is under tension (Buckley, Tan et al. 2014), and this force has been shown to reinforce adherens junctions. Lack of tension results in downsizing of adherens junction organization (le Duc, Shi et al. 2010, Liu, Tan et al. 2010, Yonemura, Wada et al. 2010, Thomas, Boscher et al. 2013, Barry, Tabdili et al. 2014, Engl, Arasi et al. 2014). Therefore, association of cadherin with F-actin through α-catenin is essential in maintaining strong adhesion strength.

2.3 Cadherin turnover

The total amount of cadherins present at cell-cell junction is a major factor that affects the adhesive strength. There are several ways that the junctional level of cadherin is modulated. Figure 2-3 depicts the potential mechanisms by which cadherin levels can be modified: biosynthesis, transportation towards the plasma membrane, endocytosis, recycling and degradation. Among these mechanisms, biosynthesis, transportation towards cell surface and recycling regulate the "gain" of cadherins in the junction, whereas endocytosis and degradation affect the "loss" of cadherins from the junction. Together, these mechanisms contribute to the dynamic regulation of adhesion in the cell and are therefore important subjects to study.

2.3.1 Biosynthesis and transportation to the plasma membrane

Biosynthesis of cadherins, as proteins in general, starts from transcription. At the level of transcriptional regulation, a classic example is epithelial-mesenchymal transition during cancer metastasis, in which E-cadherin expression is downregulated while N-cadherin expression is upregulated. This transition is achieved by action from a variety of transcription factors and through epigenetic regulations (Serrano-Gomez, Maziveyi et al. 2016).

Classical cadherins are first synthesized as a larger precursor polypeptide (Shore and Nelson 1991, Ivanov, Philippova et al. 2001). Sequence analysis of E-cadherin cDNA revealed that the mRNA encodes a polypeptide with an additional coding region at the N-terminus, which is located to the extracellular domain of the protein (Gallin, Sorkin et al. 1987, Ozawa, Baribault et al. 1989). Proteolytic cleavage by Furin, a member of the

proprotein convertase family, to remove this extra sequence is required for proper adhesive activity of E-cadherin (Ozawa and Kemler 1990, Posthaus, Dubois et al. 1998) (Figure 2-6). During the transportation of cadherin towards the plasma membrane, the 135kDa precursor polypeptide is rapidly converted to mature E-cadherin of 120kDa before reaching the plasma membrane (Shore and Nelson 1991, Posthaus, Dubois et al. 1998). Cadherins follow a classic secretory pathway with synthesis in the endoplasmic reticulum, followed by processing in the Golgi apparatus, then entering post-Golgi carriers and transporting via microtubule-dependent motors (Bryant and Stow 2004). Studies have shown that newly synthesized E-cadherin, instead of getting directly into the plasma membrane, transports towards basolateral membrane domains via vesicles associated with Rab11, a small GTPase that plays critical roles in post-Golgi trafficking and recycling (Lock and Stow 2005, Welz, Wellbourne-Wood et al. 2014).

Other than the cleavage of precursor polypeptides into mature cadherins, a number of post-translational modifications are required for proper transportation of newly synthesized cadherins and assembly of adherens junctions. Geng and colleagues showed that O-linked β-N-acetylglucosamine modification of the cytoplasmic domain retains E-cadherin in the endoplasmic reticulum during E-cadherin transportation (Zhu, Leber et al. 2001, Geng, Zhu et al. 2012). In addition, E-cadherin can also be modified by N-glycosylation at multiple sites in the ectodomain, which has been shown to affect the stability of adherens junctions by influencing both the organization of E-cadherin based protein complexes and their association with the actin cytoskeleton (Liwosz, Lei et al. 2006). It was also shown that inhibition of N-glycosylation does not prevent E-cadherin assembly into adherens junction, indicating N-glycan itself does not function as an adhesive structure (Shirayoshi,

Nose et al. 1986). Lastly, the cytoplasmic domain of E-cadherin can also be modified by phosphorylation. As stated in section 2.2.2b, phosphorylation of newly synthesized E-cadherin protects cadherin from proteosomal degradation (Lickert, Bauer et al. 2000, McEwen, Maher et al. 2014). McEwen and colleagues also showed that phosphorylation during biosynthesis on several serine residues in the E-cadherin catenin-binding domain is important for cadherin-catenin complex formation and adherens junction stability (McEwen, Maher et al. 2014). Thus ultimately, phosphorylation on the catenin-binding domain of E-cadherin facilitates transport of newly synthesized cadherin towards the plasma membrane and stabilizes the adherens junction.

To summarize, biosynthesis and transportation of E-cadherin to the plasma membrane involves multiple critical steps. After the precursor protein being synthesized in endoplasmic reticulum, proprotein convertase Furin located in Golgi cleaves at the ectodomain to remove the excess N-terminal sequence from the precursor cadherin, which allows for maturation of the ectodomain structure and is required for proper adhesive function. Before reaching the plasma membrane, E-cadherin is also subjected to both N- and O-glycosylation and phosphorylation. These post-translational modifications provide multiple layers for regulating E-cadherin transportation and the assembly of adherens junctions.

2.3.2 Endocytosis

Endocytosis is a form of active transport in which cell surface molecules are engulfed into the cell in an energy-consuming process. Endocytosis can be divided into two broad categories: clathrin-dependent and clathrin-independent pathways (Figure 2-4). Between

the two, clathrin-dependent endocytosis is the best characterized (Bonifacino and Traub 2003). Cadherin was first recognized to undergo clathrin-mediated endocytosis by Le and colleagues, who observed constitutive clathrin-mediated endocytosis and recycling of E-cadherin in MDCK cells (Le, Yap et al. 1999). VE-cadherin also has been shown to undergo constitutive endocytosis through a clathrin-mediated pathway (Xiao, Allison et al. 2003, Cadwell, Su et al. 2016). Proteins are targeted for clathrin-mediated endocytosis by the binding of adaptor protein complexes. Once bound, other components of the endocytic machinery are recruited and cluster into clathrin-coated pits (Doherty and Lundmark 2009, Traub and Bonifacino 2013). Vesicles then bud off from the cell surface through a scission event mediated by the GTPase dynamin (Doherty and Lundmark 2009). Internalized proteins can be recycled back to the plasma membrane or sorted to the lysosome for degradation (Lock and Stow 2005, Palacios, Tushir et al. 2005, Kowalczyk and Nanes 2012).

In addition to clathrin-dependent endocytosis, studies have also identified a number of clathrin-independent pathways via which cadherins can get internalized. Though less studied, cadherins were found to endocytose through caveolin-mediated endocytosis, micropinocytosis-like pathways and specifically for desmosomal cadherins, lipid-raft mediated endocytosis (Delva, Jennings et al. 2008, Doherty and McMahon 2009, Kowalczyk and Nanes 2012, Stahley, Saito et al. 2014) (Figure 2-4). A study by Lu and colleagues demonstrated that EGF signaling could disrupt cell-cell adhesion by triggering the caveolin-mediated internalization of E-cadherin, a mechanism which may be relevant to epithelial-to-mesenchymal transition in cancers (Lu, Ghosh et al. 2003). In contrast, Bryant and colleagues reported that E-cadherin is co-internalized with p120 and β-catenin

through macropinocytosis in response to EGF in a breast carcinoma cell line (Bryant, Kerr et al. 2007). Further evidence for macropinocytosis comes from Paterson and colleagues, who have observed E-cadherin endocytosis that is both clathrin- and caveolin-independent, but dynamin-dependent, which they identified as a macropinocytosis-like pathway (Paterson, Parton et al. 2003). Lastly, desmosomal cadherins were shown to internalize through a lipid raft-mediated pathway (Delva, Jennings et al. 2008, Stahley, Saito et al. 2014), which has not been demonstrated for classical cadherins. Together, these studies suggested multiple pathways available for cadherin endocytosis in different biological context. Though future studies are required to clarify details for these endocytic process, it is important to take them into consideration when studying cadherin turnover under different physiological and pathological conditions.

2.3.2a Endocytic motifs in the cadherin tail

Several studies done in mammalian systems suggest that p120 physically occupies the cadherin juxtamembrane domain, which harbors multiple types of endocytic signals (Figure 2-5) (Kowalczyk and Nanes 2012). Binding of p120 to cadherin controls access of endocytic adaptors to these motifs, thereby regulating cadherin endocytosis (Ishiyama, Lee et al. 2010, Nanes, Chiasson-MacKenzie et al. 2012). In fact, studies using a chimera polypeptide that consists of the interleukin-2 receptor extracellular and transmembrane domain fused with VE-cadherin juxtamembrane domain suggest that the juxtamembrane alone is sufficient to drive cadherin endocytosis (Xiao, Allison et al. 2003). In addition, p120 binding to these chimera blocks endocytosis of the polypeptide (Xiao, Allison et al. 2003). Although p120 is known to inhibit Rho A signaling (Anastasiadis, Moon et al. 2000, Anastasiadis and Reynolds 2001), this GTPase regulatory ability of p120 does not affect

its role in regulating VE-cadherin endocytosis (Chiasson, Wittich et al. 2009). Studies from Chiasson et al. showed that the mutant of p120 that does not inhibit Rho A activity but still binds to cadherin were still able to block VE-cadherin endocytosis (Chiasson, Wittich et al. 2009). Together, these studies provide evidence supporting the model that p120 binding to cadherin physically masks endocytic signals that reside in the juxtamembrane domain to inhibit endocytosis.

Further support for this model came from structural studies and mutagenesis analysis of the juxtamembrane domain (Ishiyama, Lee et al. 2010). X-ray crystallography and NMR studies of E-cadherin juxtamembrane domain in complex with p120 demonstrated that p12- binds to cadherin in a dynamic-static manner (Ishiyama, Lee et al. 2010) (Figure 2-5). Due to the highly-conserved nature of the juxtamembrane domain in the classical cadherin family, this p120 binding manner is likely conserved in other classical cadherins such as VE-cadherin and N-cadherin. In the case of VE-cadherin, gain and loss of function studies demonstrated a three-amino acid acidic motif (DEE) in the core static p120 binding region of the juxtamembrane domain (Nanes, Chiasson-MacKenzie et al. 2012) (Figure 2-5). When these three amino acids were mutated to alanine, p120 binding with VE-cadherin is disrupted (Nanes, Chiasson-MacKenzie et al. 2012). Interestingly, without p120 binding, this mutant VE-cadherin remains stable at the cell surface and does not get endocytosed, indicating the DEE sequence is not only required for p120 binding, but also is critical to endocytosis of VE-cadherin (Nanes, Chiasson-MacKenzie et al. 2012). In contrast, mutation of a GGG sequence immediately downstream of DEE, which also leads to abolishment of p120 binding, slightly increases the endocytosis rate of VE-cadherin,

suggesting that GGG is only required for p120 binding but not for mediating endocytosis (Nanes, Chiasson-MacKenzie et al. 2012).

Sequence analysis shows that the DEE sequence is highly conserved in classical cadherins (Figure 2-5). However, mutation of the DEE motif in E-cadherin only modestly decreases cadherin endocytosis (Nanes, Chiasson-MacKenzie et al. 2012). Instead, E-cadherin harbors a dileucine sequence that is upstream of the DEE sequence, and by NMR studies, identified to locate in the dynamic binding region of p120 (Figure 2-5) (Ishiyama, Lee et al. 2010). Mutational studies of the dileucine motif in E-cadherin suggest that it is the major endocytic motif in E-cadherin (Miyashita and Ozawa 2007). Since the dileucine motif is in the dynamic binding region of p120, mutation of the dileucine motif is unlikely to completely disrupt p120 binding, but the presence of p120 is still likely to hinder the access of adaptor protein to the dileucine sequence. Indeed, disruption of p120 binding was shown to lead to increased E-cadherin endocytosis (Miyashita and Ozawa 2007). Data presented in this dissertation confirms that the dileucine motif is required for E-cadherin endocytosis, and the disruption of E-cadherin endocytosis by mutating the dileucine motif can have somewhat contradictory effects in different *in vitro* models of cell migration, which will be discussed in more detail in chapter 4.

The dileucine motif is also conserved in other cadherins, including cadherin-6B (Figure 2-5). However, studies suggest that the dileucine motif is not required for cadherin-6B endocytosis, suggesting another dominant endocytic motif existing in cadherin-6B (Padmanabhan and Taneyhill 2015). Nonetheless, mutations that disrupt p120 binding to cadherin-6B still increases endocytosis, suggesting the mechanism of p120 regulating

cadherin endocytosis is conserved in cadherin-6B (Padmanabhan and Taneyhill 2015). Sequence analysis shows that cadherin-6B harbors an NDE sequence in the equivalent location as DEE in VE-cadherin (Figure 2-5). It is possible that these acidic residues in the core p120-binding site of cadherin-6B function as an endocytic signal, but this possibility has yet to be tested.

In addition to DEE and dileucine motif, a recent study has identified a new sequence that mediates endocytosis of cadherin-11 and is potentially subjected to regulation by p120 (Satcher, Pan et al. 2015). Cadherin-11, a mesenchymal cadherin, interacts with clathrin directly through a membrane proximal sequence VFEEE in the juxtamembrane domain (Satcher, Pan et al. 2015) (Figure 2-5). By using mutational analysis and endocytosis assay, Scatcher et al. demonstrated that the VFEEE sequence mediates clathrin-dependent endocytosis of cadherin-11 and removal of this sequence results in cadherin-11-mediated cell migration in C4-2B and PC3-mm2 prostate cancer cells (Satcher, Pan et al. 2015). The VFEEE is similar to the LxEx(E/D) sequence, which binds to clathrin, found in c-terminal tail of arrestin3 (Krupnick, Santini et al. 1997). Sequence alignment shows that the LxEx(E/D) sequence also exists in N- and P-cadherin (Figure 2-5), though it's unclear if this motif mediates endocytosis in other cadherins. Importantly, the LxEx(E/D) motif is present in the dynamic binding region of p120, suggesting that it can potentially be regulated by p120 binding. Cadherin-11 also harbors an acidic sequence DDE, similar to the DEE motif in VE-cadherin, that can potentially also drive endocytosis of cadherin-11 (Figure 2-5). It will be interesting to know if the VFEEE motif in cadherin-11 can regulate endocytosis independently of the DDE sequence, which resides in the core p120 binding region of the cadherin.

In summary, the juxtamembrane domain of cadherin harbors multiple endocytic motifs and through binding with p120, the access of endocytic adaptors to these motifs is regulated. There can potentially be more motifs in the juxtamembrane domain that have not been identified, which requires further investigation.

2.3.3 Recycling and degradation

After proteins are successfully internalized from the cell surface, additional sorting steps are required to determine their fate. A portion of internalized proteins can be recycled back to the plasma membrane. Recycling pathways are particularly important for cadherins, and the dynamics between degradation and recycling can help fine-tune the amount of cadherin present at adherens junctions and the strength of cell-cell adhesion (Figure 2-4). The first evidence of the importance of a recycling pathway to cadherin trafficking came from the discovery that E-cadherin does not travel directly from the Golgi complex to the cell surface, but transits first through Rab11-positive recycling endosomes (Lock and Stow 2005). Rab11 is a small GTPase that plays critical roles in post-Golgi trafficking and the recycling of a variety of receptors, including adhesion molecules (Welz, Wellbourne-Wood et al. 2014). In addition to mediating transportation of newly synthesized E-cadherin towards the plasma membrane, Rab11-positive recycling endosomes can also sort internalized cadherin for recycling back to the cell -surface. In fact, Classen and colleagues found that in Drosophila, Rab11-mediated recycling of cadherin is responsible for rearrangement of cell contacts in the hexagonal packing of wing disk cells (Classen, Anderson et al. 2005). In addition, a recent study found that VE-cadherin recycling and endothelial barrier function is regulated by Rab11a (Yan, Wang et al. 2016), further

highlighting the importance of this GTPase in modulating classical cadherin availability at cell-cell junctions in a variety of cell types.

Although the exact mechanism that determines the fate of internalized cadherins is still not clear, many studies have emerged to identify potential mechanisms and components of this pathway. It was shown Sec5, sec6, and sec15 are all required for DE-cadherin trafficking from recycling compartments to the plasma membrane (Langevin, Morgan et al. 2005). Depletion of the scaffolding protein PALS1 also disrupts recycling of E-cadherin (Wang, Chen et al. 2007). Adaptor protein complex AP-1B, which usually mediates recycling of basolaterally targeted proteins, was also found to be a potential regulator of cadherin recycling through interaction with phosphatidylinositol-4-phosphate-5 kinase type Iγ (PIPKIγ) (Ling, Bairstow et al. 2007).

In addition to mediators of cadherin recycling, there are signals that trigger lysosomal degradation, such as ubiquitination of the cadherin by E3-ligases, such as Hakai and K5 (Fujita, Krause et al. 2002, Mansouri, Rose et al. 2008, Nanes, Grimsley-Myers et al. 2016). The work presented in this dissertation (Chapter 3) demonstrated a novel mechanism that cadherin fate after endocytosis could be determined by proteolytic processing. This work identifies a proteolytic event mediated by calpain that appears to dictate the trafficking itinerary after VE-cadherin is endocytosed.

Regulation of cadherin post-endocytic trafficking, though only partially understood, is an area that is crucial to our understanding of cadherin turnover. So far, increasing numbers of mechanisms and mediators have been identified, and the list is likely to grow in the future.

2.4 Proteolytic processing of cadherin

2.4.1 Overview of cadherins proteolysis

In addition to endocytosis, cadherin cell surface levels can also be regulated by proteolysis. Proteolysis of cadherins can occur at different stages during the cadherin life cycle (Figure 2-6). As stated above in section 2.3.1, E-cadherin is first synthesized in a precursor form, which needs to be cleaved by a proprotein convertase Furin to remove the extra N-terminal sequence. This proteolytic processing allows for maturation of E-cadherin to achieve an adhesive state, and disruption of this process will lead to abolishment of E-cadherin processing in fibroblasts (Ozawa and Kemler 1990, Posthaus, Dubois et al. 1998).

Cadherin ectodomain can also be a target for proteases after being delivered to the cell surface. Initial observations of cadherin ectodomain cleavage showed that an 80kDa soluble E-cadherin fragment in the conditioned media of a breast cancer cell line could disrupt adhesion functions (Damsky, Richa et al. 1983, Wheelock, Buck et al. 1987). ADAM10, a member of metalloproteinase family, has been implicated in generating soluble E-cadherin in normal keratinocytes as well as melanoma cell lines (Billion, Ibrahim et al. 2006, Maretzky, Scholz et al. 2008). Recent studies also reported that ADAM10 cleaves the VE-cadherin extracellular domain, leading to reduced cell-cell adhesion strength (Dreymueller, Pruessmeyer et al. 2012, Flemming, Burkard et al. 2015). In addition, both γ-secretase and caspase-3 cleave cadherins to promote disassembly of adherens junctions and reduce adhesion (Hunter, McGregor et al. 2001, Steinhusen, Weiske et al. 2001, Marambaud, Shioi et al. 2002). Lastly, calpain, a calcium-dependent cysteine protease, has emerged more recently as a novel protease that cleaves several

classical cadherins and thereby down-regulates cell-cell adhesion (Jang, Jung et al. 2009, Miyazaki, Taketomi et al. 2011, Ye, Tian et al. 2013, Kudo-Sakamoto, Akazawa et al. 2014, Trillsch, Kuerti et al. 2016). In summary, proteolytic processing of cadherins can serve multiple functions in regulation of cadherin turnover and adhesion strength and thus is an important consideration in our comprehensive understanding of adherens junctions.

2.4.2 Proteolytic processing of the cadherin tail

Several proteolytic processing events have been found to occur on the cadherin cytoplasmic tail (Figure 2-6). In contrast to the cleavage in the ectodomain, the discovery of cadherin cytoplasmic tail cleavage came much later. The first report of cleavage in cadherin tail was in 1995 when Sato and colleagues found N-cadherin is cleaved in response to calcium ion increase (Sato, Fujio et al. 1995). They demonstrated that this cleavage was conducted by calpain, a cytoplasmic cysteine protease, and can be inhibited by leupeptin and calpeptin (Sato, Fujio et al. 1995). Later, several studies further confirmed calpain cleavage of cadherins in the cytoplasmic tail (Rios-Doria, Day et al. 2003, Rios-Doria and Day 2005, Jang, Jung et al. 2009, Miyazaki, Taketomi et al. 2011, Ye, Gao et al. 2012, Konze, van Diepen et al. 2014, Kudo-Sakamoto, Akazawa et al. 2014, Rodriguez-Fernandez, Ferrer-Vicens et al. 2016, Trillsch, Kuerti et al. 2016, Xu, Sobue et al. 2016). Details of calpain cleavage of cadherins will be discussed in the section to follow. Apart from calpain, presenilin-1 and caspase-3 have also been indicated to cleave cadherin in the cytoplasmic domain. Presenilin-1 is a transmembrane protease that was found to cleave cadherins at the juxtamembrane domain after ectodomain cleavage to generate a C-terminal fragment (Marambaud, Shioi et al. 2002, Marambaud, Wen et al. 2003, Uemura, Kihara et al. 2006, Ferber, Kajita et al. 2008, McCusker and Alfandari 2009). In the case of caspase-

3, Hunter and colleagues demonstrated that caspase-3 can cleave N-cadherin in osteoblasts undergoing apoptosis (Hunter, McGregor et al. 2001). They showed that when osteoblasts were treated with antibodies that disrupt N-cadherin mediated adhesion, apoptosis gets activated and so does caspase-3, which in turn cleaves N-cadherin and β-catenin, leading to further disruption of cell adhesion (Hunter, McGregor et al. 2001). Later in 2003, Keller and Nigam found cleavage of E-cadherin by caspase-3 in MDCK cells under stress, in response to apoptosis (Keller and Nigam 2003). Also, E-cadherin was found to be cleaved by an unknown ATP-independent intracellular protease to generate an 80kDa fragment in whole ischemic kidneys and ATP-depleted Madin-Darby canine kidney (MDCK) cells (Bush, Tsukamoto et al. 2000).

Cleavage of the cadherin cytoplasmic tail, in most cases, results in disassembly of adherens junction and disruption of cell adhesion. However, in some cases, the cleavage can play a role in other signaling pathways that is related to cadherin. Calcium influx was shown to trigger E-cadherin cleavage in both the ectodomain and cytoplasmic tail, and the cleavage leads to disassembly of adherens junctions and translocation of junctional β-catenin, potentially affecting the Wnt pathway (Ito, Okamoto et al. 1999). E-cadherin fragment generated by cytoplasmic tail cleavage was shown to potentiate cell death in prostate epithelial cells, indicating a role of cadherin cleavage in epithelial cell survival (Rios-Doria and Day 2005). The C-terminal fragment generated by presenilin-1 cleavage has been suggested to be involved in multiple pathways, including CREB-mediated transcription in cell culture that is important in neuronal growth and survival, and Wnt pathway that is involved in cell proliferation and differentiation (Marambaud, Shioi et al. 2002, Marambaud, Wen et al. 2003, Uemura, Kihara et al. 2006, Ferber, Kajita et al. 2008,

McCusker and Alfandari 2009). A recent study by Schiffmacher and colleagues showed that a C-terminal fragment generated from γ-secretase cleavage of cadherin-6B modulates β-catenin–responsive EMT effector genes (Schiffmacher, Xie et al. 2016). To date, the fate of cadherin fragment generated from cytoplasmic proteolysis is still not fully understood. More research is required to gain insight into this subject, which is of potential importance in further understanding of the adherens junction.

2.4.3 Calpain and cadherins

Calpain, as its name suggests, is a calcium-dependent cysteine protease. The best characterized member of the calpain family are calpain 1 or μ-calpain and calpain 2 or m-calpain, named after the amount of calcium required to activate them *in vitro (Goll, Thompson et al. 2003)*. Calpain consists of two subunits, one large at about 80kDa and one at about 28kDa (Figure 2-7). Studies have shown that the large subunit of calpain has the complete function of calpain in terms of protease activity. The small subunit, instead, is believed to be a regulatory subunit that is required for modulation of calpain activity (Goll, Thompson et al. 2003). Also, the 28Kda subunit acts as a chaperon to ensure correct conformation of the 80kDa subunit (Suzuki, Hata et al. 2004). Another member of the calpain system is calpastatin, which is an endogenous inhibitor protein that is specific for calpain (Goll, Thompson et al. 2003). The mechanism of calpain activation involves binding of calcium ion to both large and small subunits of calpain, which leads to dissociation of the small subunit from the complex, and conformational changes to form and expose the catalytic domain (Khorchid and Ikura 2002, Goll, Thompson et al. 2003, Suzuki, Hata et al. 2004) (Figure 2-7). The substrate specificity of calpain is not based on amino acid sequences, but rather is dependent on protein tertiary structure with a preference

to unstructured open regions between defined domains (Goll, Thompson et al. 2003). Calpain has been found to have a large variety of target substrates, including ankryin, cadherins, catenins, tubulin and many others. This allows calpain to be involved in many biological processes such as cell mobility, signal transduction, cell cycle control, gene expression, apoptosis, and long-term potentiation.

Recently, several studies have reported an interaction between calpain and classical cadherins. The initial report came from Covault and coworkers in 1991, in which they demonstrated proteolysis of N-cadherin occurs in response to increased intracellular calcium levels (Covault, Liu et al. 1991). Based on the micromolar level threshold requirement of calcium for this proteolysis, they hypothesized the protease to be calpain and found that the cleavage disrupts the connection between the cadherin and cytoskeleton (Covault, Liu et al. 1991). Since then, a few studies have reported cleavage of cadherins by calpain, although studies of cadherin cleavage by calpains are still very sparse and the mechanisms behind this proteolytic event are not fully understood. In 1995, another report showed that N-cadherin is cleaved in heart, induced by an elevated level of intracellular calcium, and this cleavage can be inhibited by calpeptin, a known inhibitor for calpain (Sato, Fujio et al. 1995). In 2003, researchers found that E-cadherin is cleaved by calpain in prostate and mammary epithelial cells to generate a 100kDa fragment, which does not bind to β- and p120-catenin, suggesting a disruption of adhesion (Rios-Doria, Day et al. 2003). Then later in 2005, the same group demonstrated that overexpression of the 100kDa E-cadherin fragment results in downregulation of endogenous E-cadherin at the membrane and, in conjunction with PKC activation, leads to potential cell death (Rios-Doria and Day 2005). In 2009, another report came out from Jang and coworkers suggesting that N-

cadherin cleavage by calpain, which generates a 110kDa fragment, significantly affects cell adhesion, AKT signaling, the N-cadherin/β-catenin interaction and Wnt target gene expression through the accumulation of nuclear β-catenin (Jang, Jung et al. 2009). The first evidence of calpain cleavage of VE-cadherin came from Miyazaki and colleagues, who found that VE-cadherin is cleaved specifically by m-calpain in response to signals induced by atherosclerotic lesions in aorta (Miyazaki, Taketomi et al. 2011). They also narrowed down the cleavage site to be five amino acids between the juxtamembrane domain and the catenin-binding domain (Miyazaki, Taketomi et al. 2011). They found that this cleavage event, induced by LPC *in vitro*, can lead to disorganization of the adherens junction (Miyazaki, Taketomi et al. 2011). Recently more studies have demonstrated calpain cleavage on E- and N-cadherin, adding to the evidence to support the observation of cadherin cleavage on classical cadherins (Konze, van Diepen et al. 2014, Kudo-Sakamoto, Akazawa et al. 2014, Rodriguez-Fernandez, Ferrer-Vicens et al. 2016, Trillsch, Kuerti et al. 2016, Xu, Sobue et al. 2016). To date, not much research has been done on this subject but the consensus so far is that calpain cleavage of cadherins seems to be involved in disruption of cell adhesion. However, the mechanism of how calpain cleavage leads to disruption of adhesion is still not clear. The work in this dissertation provides a possible explanation to why calpain cleavage results in adhesion disruption (Chapter 3).

2.5 Other post-translational modifications of the cadherin tail

2.5.1 Phosphorylation

Multiple sites in the cadherin cytoplasmic tail are potential targets for phosphorylation. When E-cadherin is synthesized in the endoplasmic reticulum, the C-terminal end of its cytoplasmic tail is phosphorylated at several serine residues, this allows for increased binding affinity to β-catenin and protect the cadherin from being degraded (Lickert, Bauer et al. 2000). Also, VE-cadherin serine phosphorylation on the S665 residue has been shown to modulate AJ assembly (Gavard and Gutkind 2006, Spring, Chabot et al. 2012). Tyrosine residues are also common targets for phosphorylation. In VE-cadherin, there are nine putative phospho-tyrosine sites, among which Y645, Y658, Y685, Y731, and Y733 have been implicated in barrier integrity (Potter, Barbero et al. 2005, Wallez, Cand et al. 2007, Turowski, Martinelli et al. 2008, Orsenigo, Giampietro et al. 2012). Both Src and focal adhesion kinase (FAK) have been shown to drive phosphorylation of VE-cadherin and lead to junction disassembly (Gavard 2013). In addition to VE-cadherin, E- and N-cadherin have also been found to be phosphorylated on tyrosine residues in the cytoplasmic tail (Behrens, Vakaet et al. 1993, Hamaguchi, Matsuyoshi et al. 1993, Adam 2015). In fact, recent structural studies have confirmed and advanced our understanding of phosphorylation in E-cadherin. Structural studies of E-cadherin cytoplasmic domain in complex with β-catenin show that the catenin-binding domain is heavily phosphorylated on serine residues, leading to strengthened β-catenin binding through formation of hydrogen bonds and salt bridges (Huber and Weis 2001). NMR study of E-cadherin juxtamembrane domain in complex with p120-catenin revealed tyrosine phosphorylation sites within the static p120 binding region, indicating that these tyrosine residues, when

phosphorylated, can potentially lead to dissociation of p120 from cadherin and junction disassembly (Ishiyama, Lee et al. 2010). Indeed, in the case of VE-cadherin, when increasing the tyrosine 658 phosphorylation, VE-cadherin-p120 interaction is destabilized (Hatanaka, Lanahan et al. 2012).

To summarize, post-translational phosphorylation of cadherin cytoplasmic tail has been shown to play an important role in cadherin trafficking, junction assembly and adhesion strength. Regulation of cadherin cytoplasmic tail phosphorylation therefore is essential in understanding the molecular mechanism and function of cadherin-based adherens junction.

2.5.2 Ubiquitination

Ubiquitin is a small regulatory protein that was first discovered in 1975 by Goldstein and colleagues (Goldstein, Wolinsky et al. 1975). It was named after it's ubiquitous distribution in wide variety of cells. Since then, numerous studies have been done to characterize this protein and its function (Wilkinson 2005). Ubiquitin modifies its substrate by attaching to the protein via covalent bonds, usually to a lysine residue (Pickart 2001). This process is called ubiquitination. To date, ubiquitination has been shown to be involved in many major cellular processes. For example, ubiquitination can be a signal for protein degradation via the proteasome (Glickman and Ciechanover 2002). In addition, ubiquitination can alter protein trafficking, distribution and activity (Schnell and Hicke 2003, Mukhopadhyay and Riezman 2007). Recent studies have suggested that cadherin can also undergo ubiquitination. In 1995, Beesley and colleagues discovered enrichment of N-cadherin in post-synaptic density (PSD) and the PSD proteins are covalently modified by ubiquitination (Beesley, Mummery et al. 1995). This hinted potential ubiquitin

modification of classical cadherins at the junction. Later, in 2002, E-cadherin was found to be ubiquitinated by a ubiquitin ligase Hakai in response to activation of tyrosine kinase Src (Fujita, Krause et al. 2002). This was the first report that confirmed ubiquitination on a classical cadherin. Since then, increasing numbers of studies have demonstrated ubiquitin modification of classical cadherins. Another report also showed Src activation resulting in ubiquitination and lysosomal degradation of E-cadherin (Shen, Hirsch et al. 2008). In the same year, Bonazzi et al. also showed E-cadherin ubiquitination occurs after Src activation, induced by interaction between E-cadherin and listeria monocytogenes surface proteins internalin A (Bonazzi, Veiga et al. 2008). In 2012, Andrea Hartsock and William J. Nelson reported a competitive relationship between p120 binding and E-cadherin ubiquitination by Hakai (Hartsock and Nelson 2012). In this study, they found that two lysine residues in the juxtamembrane domain of E-cadherin, K5 and K83, are ubiquitinated by Hakai (Hartsock and Nelson 2012). Ubiquitination of the E-cadherin juxtamembrane domain was indicated to prevent p120 binding (Hartsock and Nelson 2012). Recently, this competitive relationship between p120 binding and cadherin ubiquitination was also reported in VE-cadherin, where two membrane-proximal lysine residues can be ubiquitinated by K5, a MARCH family ubiquitin ligase expressed by human herpesvirus 8 (Nanes, Grimsley-Myers et al. 2016) (Figure 2-5). This ubiquitination, which is blocked by p120 binding, drives VE-cadherin endocytosis and degradation, leading to disassembly of endothelial adherens junction (Nanes, Grimsley-Myers et al. 2016). Until now, in most cases, ubiquitination of classical cadherins has been found to drive cadherin endocytosis and degradation, which then results in disruption of cell adhesion. Therefore, ubiquitin

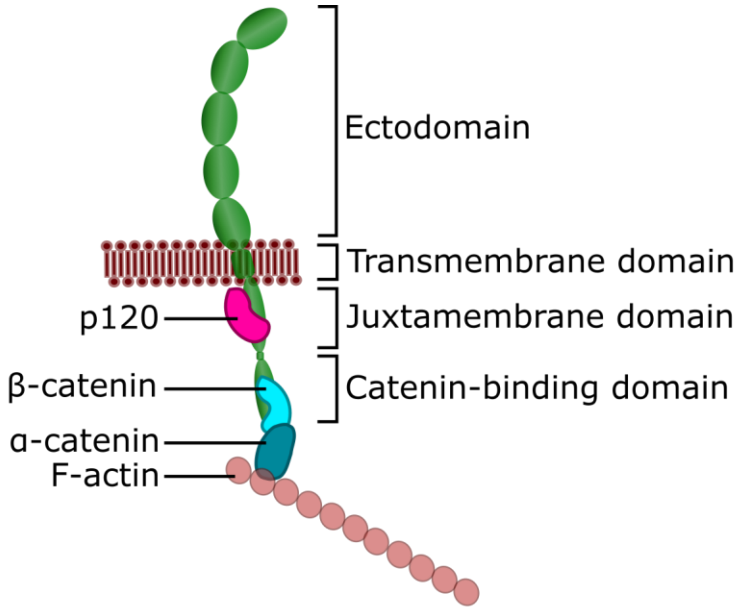
modification of cadherin tail can be an important regulatory mechanism for cadherin turnover and cell adhesion.

2.6 Concluding remarks

The importance of cell adhesion in biological systems is widely appreciated. Despite extensive studies that have been done to advance our understanding of the regulation of cell adhesion, the mechanisms of how cells regulate adhesive strength is still not fully understood. Studies suggest that cadherin endocytosis is a particularly important mechanism for the disassembly of cadherin-based adhesive contacts (Troyanovsky, Sokolov et al. 2006). To date, the significance of cadherin endocytosis to the dynamic regulation of cell adhesion has been well established. In recent years, numerous studies have been conducted to understand the molecular mechanisms involved in cadherin endocytosis. Yet a universal model to elucidate the molecular mechanism of cadherin endocytosis has not been established. In addition, the role of cadherin endocytosis in development and disease is not fully understood. Most studies took approaches such as cadherin knock down or disrupting endocytosis globally rather than selectively preventing endocytosis of the cadherin. In VE-cadherin, a DEE sequence within the juxtamembrane domain was shown to be a motif sufficient to drive cadherin endocytosis (Nanes, Chiasson-MacKenzie et al. 2012). In E-cadherin, however, there is another motif containing two leucine residues upstream of the DEE sequence, which was shown to be critical to E-cadherin endocytosis (Miyashita and Ozawa 2007). Mutation of the dileucine motif allows us to address the role of cadherin endocytosis specifically under developmental or pathological conditions. Results of these data will be discussed in chapter 4 of this dissertation.

A relatively new mechanism to modulate cell adhesion involves proteolytic activities. Cadherins are subjected to cleavage by several different kinds of proteases. Well known

examples include cleavage by metalloproteinases in the ectodomain, caspase-3 during apoptosis, and calpain in the cytoplasmic domain. Interestingly, these proteolytic events all seem to be involved in disorganization of adherens junctions and disruption of cell adhesion. So far, the molecular mechanism of how the cleavage of cadherins by calpain disrupts cell adhesion is unclear. The work in this dissertation provides a potential explanation of how calpain cleavage could be involved in reduced adhesion (Chapter 3). This would not only improve our understanding of cadherin cleavage by calpain, but also represents a new model for understanding how cadherin turnover could be regulated.



VE-cadherin (*Homo sapiens*)

```
  1 mqrlmmllat sgaclgllav aavaaaganp aqrdthsllp thrrqkrdwi wnqmhideek
 61 ntslphhvgk ikssvsrkna kyllkgeyvg kvfrvdaetg dvfaierldr eniseyhlta
121 vivdkdtgen letpssftik vhdvndnwpv fthrlfnasv pessavgtsv isvtavdadd
181 ptvgdhasvm yqilkgkeyf aidnsgriit itksldrekq aryeivvear daqglrgdsg
241 tatvlvtlqd indnfpfftq tkytfvvped trvgtsvgsl fvedpdepqn rmtkysilrg
301 dyqdaftiet npahnegiik pmkpldyeyi qqysfiveat dptidlryms ppagnraqvi
361 initdvdepp ifqqpfyhfq lkenqkkpli gtvlamdpda arhsigysir rtsdkgqffr
421 vtkkgdiyne keldrevypw ynltveakel dstgtptgke sivqvhievl dendnapefa
481 kpyqpkvcen avhgqlvlqi saidkditpr nvkfkfilnt ennftltdnh dntanitvky
541 gqfdrehtkv hflpvvisdn gmpsrtgtst ltvavckcne qgeftfcedm aaqvgvsiqa
601 vvaillcilt itvitllifl rrrlrkqara hgksvpeihe qlvtydeegg gemdttsydv
661 svlnsvrrgg akpprpalda rpslyaqvqk pprhapgahg gpgemaamie vkkdeadhdg
721 dgppydtlhi ygyegsesia eslsslgtds sdsdvdydfl ndwgprfkml aelygsdpre
781 elly
```

Cadherin Repeat
Transmembrane Domain
Juxtamembrane Domain
Catenin-binding Domain

Figure 2-1. Structure and binding partners of a classical cadherin. (Top) Classical cadherins are highly conserved in structure, consisting of an ectodomain, a transmembrane domain, and a cytoplasmic tail. The cytoplasmic tail can be further divided into a juxtamembrane domain which binds to p120, and a catenin-binding domain which binds to β-catenin. α-catenin binds to both β-catenin and F-actin to allow association of cadherin to actin cytoskeleton. (Bottom) Amino acid sequence of VE-cadherin with cadherin repeats,

transmembrane domain, juxtamembrane domain and catenin-binding domain labeled respectively.

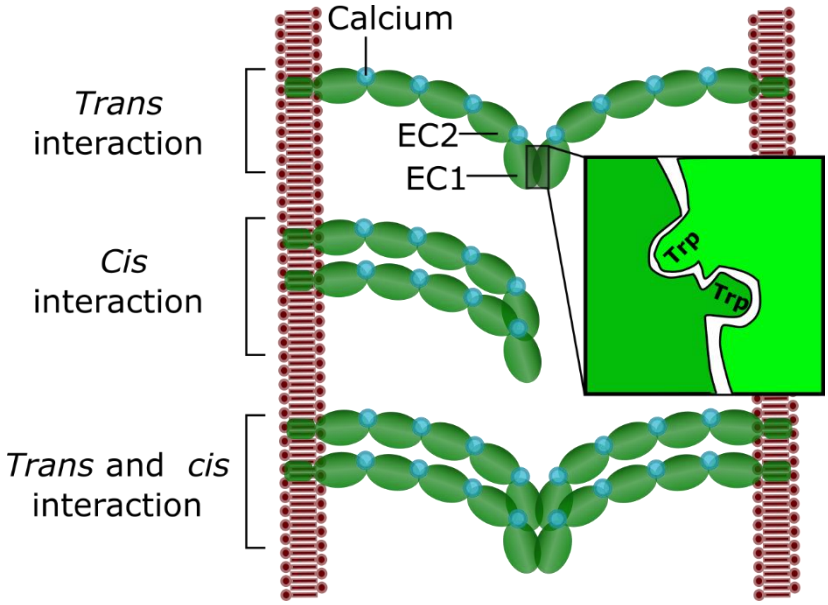


Figure 2-2. Cadherin ectodomain structure and interactions. Ectodomain of classical cadherin has five extracellular cadherin (EC) repeats. With calcium binding, cadherin ectodomain gains structural rigidity with a curved organization. Two cadherin ectodomains can engage in both *trans* and *cis* interaction. Zoomed panel shows a strand swap interaction at the *tran* binding interface, in which the Trp residue from one cadherin is inserted into the hydrophobic binding pocket of the opposing cadherin. *Cis* dimer can form involving both EC1 and EC2 from two adjacent cadherin from the same cell. The *cis* interaction requires sequences that are not engaged in *trans* interaction, allowing for simultaneous formation of both *trans* and *cis* dimers.

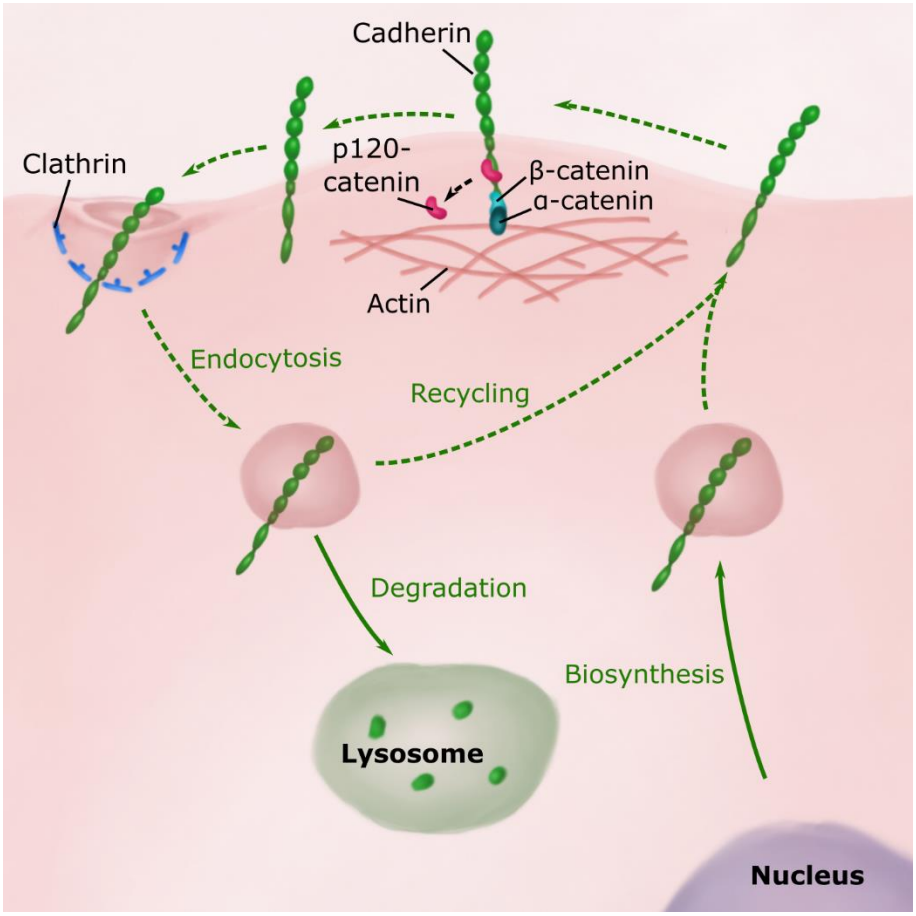


Figure 2-3. Overview of cadherin turnover. Newly synthesized cadherins are transported to the plasma membrane. When bound to catenins, cadherins are stabilized at the cell junction. Upon release of catenins, cadherin can undergo endocytosis, typically through a clathrin-mediated pathway. Endocytosed cadherins can either be degraded in the lysosome or recycled back to the cell surface.

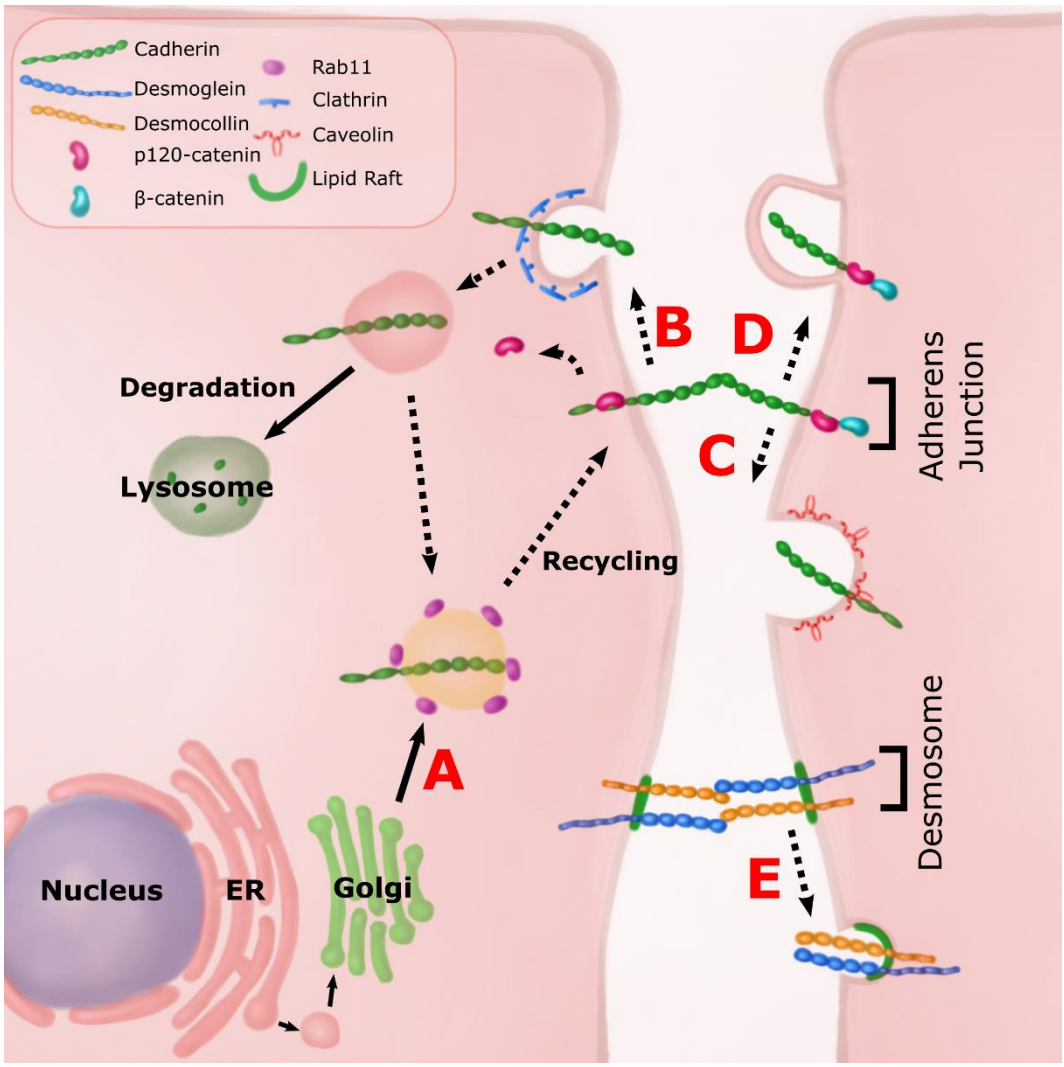


Figure 2-4. Endocytosis pathways of cadherins (Adapted from Cadwell, Su et al. 2016). (A) Newly synthesized cadherins are transported to the plasma membrane either directly or through Rab11 positive endosomes. (B) Classical cadherins undergoes clathrin-mediated endocytosis. Classical cadherins can also internalize through (C) caveolin-mediated endocytosis, or (D) macropinocytosis. Internalized cadherins are either degraded in the lysosome or recycled back to the cell surface through Rab11 positive endosomes. (E) Desmosomal cadherins undergoes lipid raft mediated endocytosis.

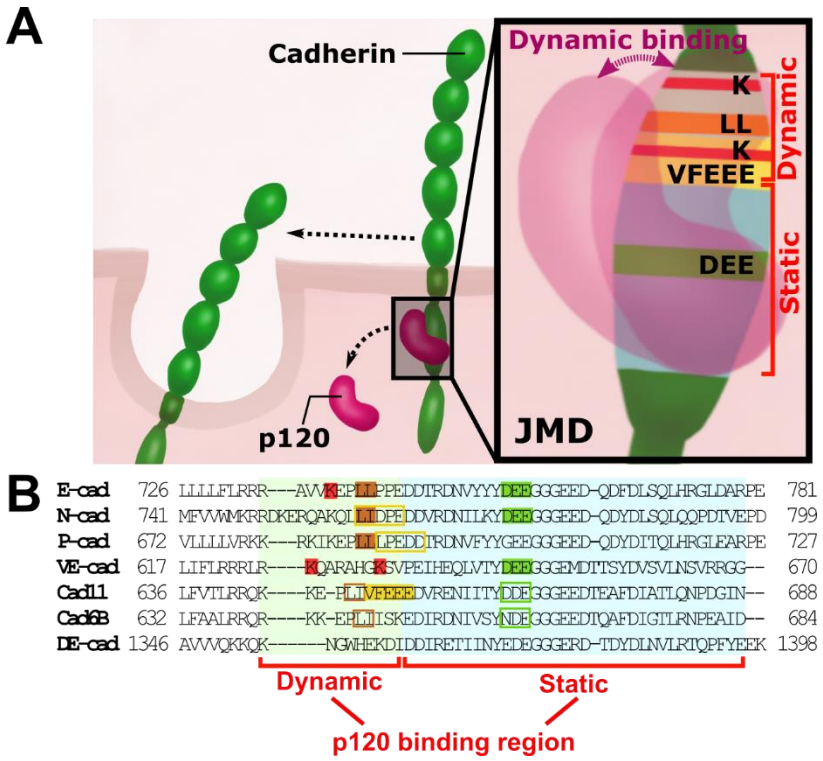


Figure 2-5. Dynamic-static binding of p120 on the juxtamembrane domain guards cadherin from endocytosis or ubiquitination (Adapted from Cadwell, Su et al. 2016). (A) p120 binds to the juxtamembrane domain (JMD) of cadherin in a dynamic-static manner. The binding of p120 masks multiple endocytic motifs from access of adaptor proteins or post-translational modifications that drive endocytosis. Releasing of p120 exposes these motifs, resulting in cadherin internalization. Detailed view of the juxtamembrane domain on the right illustrates endocytic motifs and important residues in the dynamic or static binding region of p120. (B) Sequence alignment of p120 biding regions in classical cadherins. Red: lysine residues that can be modified by ubiquitination. Orange: dileucine motif that drives endocytosis in E-cadherin. Yellow: VFEEE sequence that directly binds to clathrin. Green: conserved DEE sequence that drives endocytosis in VE-cadherin. Open boxes: potential endocytic motifs that need

experimental testing. Pale green: dynamic p120 binding region. Pale blue: static p120 binding region.

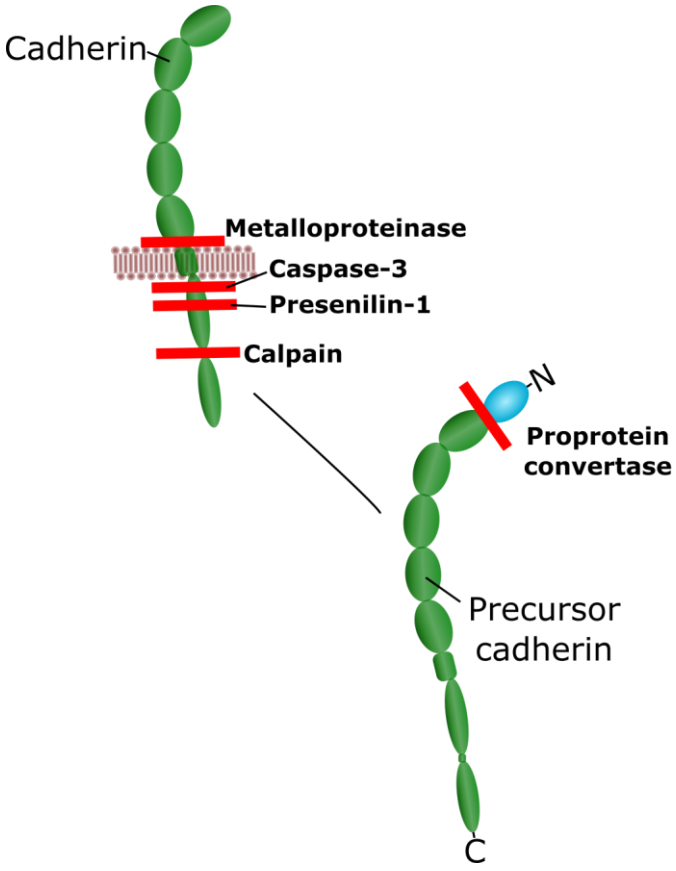


Figure 2-6. Notable proteolytic processing sites in classical cadherins. Precursor cadherin needs to be cleaved by proprotein convertase (Furin) to remove excess N-terminal sequences before reaching the plasma membrane. Metalloproteinases have been found to cleave cadherin ectodomains, resulting in cadherin shedding. Caspase-3 has been shown to cleave the membrane proximal region of the cadherin cytoplasmic tail. Presenilin-1 cleaves cadherin in the juxtamembrane domain. Calpain was shown to cleave cadherin between the juxtamembrane domain and the catenin-binding domain.

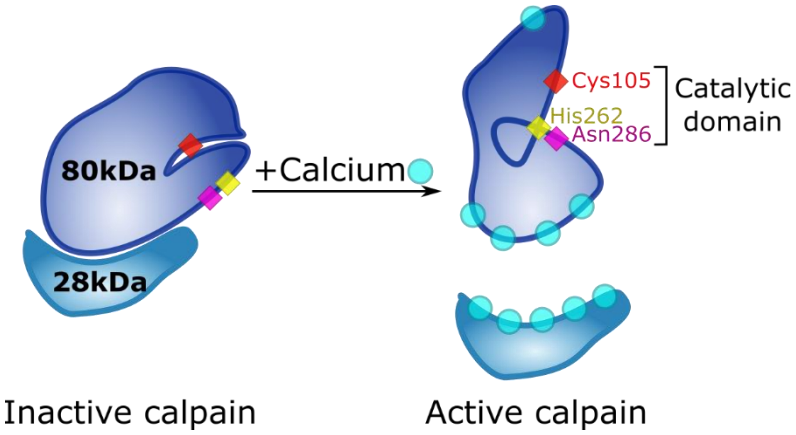


Figure 2-7. Illustration of calpain structure and activation. Calpain consists two subunits, one large subunit at about 80kDa and one small 28kDa subunit. The catalytic site, which requires Cys105, His262 and Asn286 to be in close proximity, are located only in the large subunit. Without calcium binding, large and small subunits of calpain form a complex and the catalytic domain is not formed. Therefore, calpain remains inactive when calcium is not present. Upon binding of calcium ions to both subunits, the large subunit undergoes conformational changes to form and expose the catalytic domain. Meanwhile, the small subunit is dissociated from the calpain complex to allow activating the protease activity in the large subunit. In this way, calpain becomes active when calcium is bound.

Chapter 3

Proteolytic processing of VE-cadherin tail regulates cadherin trafficking

*This chapter is adapted from W. Su, A. P. Kowalczyk. The VE-cadherin cytoplasmic domain undergoes proteolytic processing during endocytosis. MBoC. PMID: 27798242. doi: 10.1091/mbc.E16-09-0658. 2016.

3.1 Introduction

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

VE-cadherin dynamics at the plasma membrane are thought to be essential in modulating endothelial adhesion strength and adherens junction plasticity (Cadwell, Su et al. 2016).

The level of cell surface VE-cadherin is regulated in part by endocytosis. Previous work has found that VE-cadherin undergoes clathrin-mediated endocytosis (Xiao, Garner et al. 2005, Chiasson, Wittich et al. 2009, Kowalczyk and Nanes 2012, Semina, Rubina et al. 2014, Zhang, Zhang et al. 2014, West and Harris 2016). This process is inhibited by p120-catenin, which binds to the cadherin tail and masks an endocytic motif in the juxtamembrane domain (Chiasson, Wittich et al. 2009, Nanes, Chiasson-MacKenzie et al. 2012). This regulatory mechanism is thought to be a major control point for cadherin expression levels on the plasma membrane in a variety of mammalian cell types and tissues. In addition to endocytosis, cadherin cell surface levels can also be regulated by proteolysis. For example, metalloproteinases cleave the VE-cadherin extracellular domain, leading to reduced cell-cell adhesion strength (Dreymueller, Pruessmeyer et al. 2012, Flemming, Burkard et al. 2015). Both γ-secretase and caspase-3 cleave cadherins to promote disassembly of adherens junctions and reduce adhesion (Hunter, McGregor et al. 2001, Steinhusen, Weiske et al. 2001, Marambaud, Shioi et al. 2002). Calpain, a calcium-dependent cysteine protease, also has been shown to cleave several classical cadherins and thereby down-regulate cell-cell adhesion (Jang, Jung et al. 2009, Miyazaki, Taketomi et al. 2011, Ye, Tian et al. 2013, Kudo-Sakamoto, Akazawa et al. 2014, Trillsch, Kuerti et al. 2016).

Despite the growing evidence of a role for calpain in modulating the activity of cadherins and other adhesion receptors, the subcellular localization of cadherin cleavage by calpain and the relationship of this processing to VE-cadherin endocytosis have not been established. Here, we provide evidence that VE-cadherin is proteolytically processed upon entry into clathrin-enriched membrane domains during the process of endocytosis. This

cleavage event, which removes the catenin-binding domain of the cadherin tail, is mediated by calpain and fates the cadherin for a degradative rather than recycling pathway. These findings reveal a novel mechanism for how proteolytic processing could modulate cadherin surface levels by altering the itinerary of the cadherin during endocytosis.

3.2 Result

3.2.1 VE-cadherin is cleaved during endocytosis

Our previous studies demonstrated that a fragment of VE-cadherin missing the β-catenin binding domain accumulates in endothelial cells treated with chloroquine, which inhibits lysosomal degradation (Xiao, Allison et al. 2003). Data shown in Figure 1A confirms that chloroquine treatment results in the accumulation of an approximately 95kDa fragment of VE-cadherin. This processed form of VE-cadherin can be detected using antibodies to the VE-cadherin extracellular domain but not with antibodies directed against the catenin-binding domain of the cadherin tail (Xiao, Allison et al. 2003). To determine if this fragment was generated during cadherin internalization, we labeled the cell surface pool of VE-cadherin using an antibody directed against the extracellular domain (BV6) and followed the fate of the cadherin during endocytosis over a 3-hour period in the presence of chloroquine. To distinguish cell surface from internalized pools of cadherin, BV6 bound to the surface pool of VE-cadherin was removed using a low pH wash. The presence of the cytoplasmic catenin-binding domain was monitored using C19, an antibody directed against the VE-cadherin carboxyl-terminal domain (Figure 3-1B). Co-localization of the antibodies directed against the VE-cadherin extracellular (BV6) and carboxyl-terminal tail (C19) was measured at cell-cell borders and in endosomal compartments. As expected, BV6 and C19 exhibited extensive co-localization at cell-cell borders (Figure 1C top panels, and Figure 3-1D). In contrast, the internalized pool of VE-cadherin remained labeled with BV6, but the majority of this internalized cadherin failed to label with C19 (Figure 1C bottom panels and Figure 3-1D). Similar results were obtained in COS7 cells exogenously expressing VE-cadherin labeled with a carboxyl-terminal RFP tag (Figure 3-10). These

data were further confirmed by the absence of β-catenin co-localization with internalized pools of VE-cadherin. Whereas β-catenin exhibited high levels of co-localization with the VE-cadherin extracellular domain at cell-cell borders, very little co-localization was observed at endosomes (Figure 3-2). Collectively, these data indicate that the carboxyl-terminal tail, including the β-catenin-binding domain of VE-cadherin is removed during endocytosis or subsequent trafficking of the cadherin through the endosomal system.

3.2.2 Endocytosis is required for VE-cadherin cleavage.

To determine if endocytosis is required for VE-cadherin processing, we utilized several approaches to inhibit VE-cadherin internalization from the plasma membrane. First, we used a VE-cadherin endocytic mutant in which the "DEE" amino acid residues within the juxtamembrane domain were mutated to alanines. Mutation of these DEE residues results in a dramatic inhibition of VE-cadherin endocytosis and a failure to enter clathrin-enriched membrane domains (Nanes, Chiasson-MacKenzie et al. 2012). Cells expressing wild-type or the VE-cad DEE endocytic mutant were treated with chloroquine and analyzed by western blot. In comparison to the wild-type VE-cadherin, the VE-cad DEE mutant exhibited dramatically reduced fragmentation (Figure 3-3A, 3-3B). These findings suggest that VE-cadherin endocytosis is required for cleavage. Previously we found that over-expression of p120-catenin reduces VE-cadherin endocytosis, whereas loss of p120-catenin accelerates VE-cadherin internalization (Xiao, Allison et al. 2003, Nanes, Chiasson-MacKenzie et al. 2012). Therefore, we manipulated p120-catenin expression levels as an alternative approach to alter VE-cadherin endocytosis rates. Similar to the results obtained with the VE-cad DEE mutation, over-expression of p120-GFP dramatically inhibited VE-cadherin fragment formation (Figure 3-3C, 3-3D). This over-

expression system was complemented by a loss of function approach in which VE-cadherin fragmentation was monitored in a p120 null background where cadherin internalization rates are increased (Oas, Xiao et al. 2010). p120 null endothelial cells (Oas, Xiao et al. 2010) were treated with chloroquine overnight and VE-cadherin fragmentation was monitored by western blot analysis. Increased VE-cadherin fragmentation was observed in p120 null cells compared to controls (Figure 3-3E, 3-3F). Collectively, these observations indicate that endocytosis is required for VE-cadherin cleavage and removal of the catenin-binding domain.

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

To determine the subcellular location and endocytic step in which the cadherin cytoplasmic tail is processed, we performed three-channel co-localization analysis for the VE-cadherin extracellular domain, the VE-cadherin carboxyl-terminal domain, and various endocytic markers. For these experiments, we expressed VE-cadherin with a C-terminal RFP tag and utilized the BV6 antibody against the cadherin extracellular domain as illustrated in Figure 4-4A. Cell surface cadherin was labelled with BV6 at 4^0C followed by incubation at 37^0C for various amounts of time depending on the marker (5 min for clathrin or 30 min for EEA1). As expected, BV6 and the carboxyl-terminal RFP tag exhibited extensive co-localization at cell-cell junctions, indicating the presence of intact, full length cadherin. However, co-localization is reduced as the cadherin enters clathrin-enriched membrane domains (Figure 4-4B), and is further reduced by the time the cadherin enters early endosomes (Figure 4-4C). These findings, together with the data demonstrating that endocytosis is required for cleavage (Figure 3-3), suggest that VE-cadherin is

proteolytically processed in clathrin-enriched domains and the cleavage is completed before VE-cadherin reaches the early endosome.

3.2.4 The catenin-binding domain regulates VE-cadherin turnover rates.

Previous studies have suggested that the catenin-binding domain of classical cadherins regulates cadherin transport to the plasma membrane (Chen, Stewart et al. 1999). Therefore, we hypothesized that VE-cadherin cleavage, which has been shown above (Figure 1, 2) and previously to remove the catenin-binding domain (Xiao, Allison et al. 2003), alters the trafficking dynamics of VE-cadherin and leads to cadherin degradation instead of recycling. To determine how loss of the catenin-binding domain alters the trafficking dynamics of cell surface cadherin, we examined the turnover rate of VE-cadherin and a VE-cadherin truncation mutant lacking the catenin-binding domain (VE-cadherin ΔCBD) (Figure 3-5A). The VE-cadherin ΔCBD co-migrates with the VE-cadherin cleavage fragment observed in chloroquine treated cells (Xiao, Allison et al. 2003) and therefore was used to mimic the N-terminal cleaved fragment of VE-cadherin. Cell surface biotinylation and pulse chase analysis was used to monitor VE-cadherin turnover rates. Interestingly, deletion of the catenin-binding reduced the half-life of cell surface VE-cadherin from around 7.5hrs to approximately 3.8hrs (Figure 3-5B, 3-5C). The rate constant k of VE-cadherin turnover increased from -0.09 (wild-type) to -0.18 (ΔCBD).

To determine if the increased rates of cadherin turnover were due to increased endocytosis or alterations in post-endocytic trafficking of the cadherin, we measured endocytosis rates and the subcellular localization of internalized full length VE-cadherin and the ΔCBD mutant cadherin. To avoid misinterpretation due to the cleavage of full length VE-cadherin,

we used another VE-cadherin extracellular domain antibody (Cad-5, BD TransLab, 610252) instead of C-terminal RFP to visualize the total pool of VE-cad. We were unable to detect any significant difference in endocytosis rates when full length VE-cadherin was compared to VE-cadherin ΔCBD (Figure 3-6A, 3-6B). However, using both transferrin (Figure 3-6C, 3-6D) and Rab11 (Figure 3-6E, 3-6F) as markers for recycling compartments (Lock and Stow 2005, Mayle, Le et al. 2012, Yan, Wang et al. 2016), we observed significantly less co-localization of internalized ΔCBD VE-cadherin with recycling markers than we did for the full-length cadherin. On the other hand, internalized VE-cadherin ΔCBD exhibited more co-localization with the lysosomal marker CD63 than the full-length VE-cadherin (Figure 3-6G, 3-6I). Together, our data suggest that loss of the catenin-binding domain alters the VE-cadherin post-endocytic trafficking itinerary, resulting in a higher turnover rate due to less recycling and more degradation.

3.2.5 VE-cadherin is cleaved by calpain.

Several previous studies have shown that calpain cleaves the cytoplasmic domain of cadherins (Jang, Jung et al. 2009, Miyazaki, Taketomi et al. 2011, Ye, Tian et al. 2013, Kudo-Sakamoto, Akazawa et al. 2014, Trillsch, Kuerti et al. 2016). To determine if calpain activity is required for VE-cadherin processing and the removal of the catenin-binding domain, we pre-treated cells with the calpain inhibitor calpeptin and then analyzed co-localization between the cadherin extracellular domain and carboxyl-terminal tail after a 30-minute internalization period. Immunofluorescence data show that calpeptin treatment reduced the loss of the cadherin tail during endocytosis (Figure 3-7A and 7B). Finally, we directly tested if VE-cadherin can be cleaved by calpain *in vitro*. Cell lysates from adenovirus infected COS7 cells expressing VE-cadherin-RFP were treated with purified

active calpain 1 large subunit in the presence of $CaCl_2$ with or without calpain inhibitors (Calpeptin and ALLM). Calpain treatment resulted in the formation of a 95kDa fragment identical to that observed in cells treated with chloroquine (Figure 3-7C, 3-7D). Together with the observation that calpeptin inhibits cleavage of the cadherin tail in living cells, these findings suggest that calpain cleaves VE-cadherin during endocytosis.

3.3 Discussion

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

A number of studies have demonstrated that cadherins are targeted by extracellular and cytoplasmic proteases, including metalloproteinases, elastase and cathepsin G (Dejana, Orsenigo et al. 2008, Dreymueller, Pruessmeyer et al. 2012). One previous study suggested that the VE-cadherin cytoplasmic tail is cleaved by m-Calpain in response to inflammatory mediators during atherosclerosis (Miyazaki, Taketomi et al. 2011). In the current study, we found that VE-cadherin is cleaved by calpain during endocytosis. This interpretation is based on the fact that inhibiting endocytosis prevents cleavage (Figure 3-2), and by imaging data suggesting that the cadherin tail is removed as cell surface cadherin is recruited into clathrin-enriched membrane domains (Figure 4-4). This is also supported by our data showing that VE-cadherin DEE mutant, which fails to enter clathrin-enriched domains (Nanes, Chiasson-MacKenzie et al. 2012), does not get cleaved (Figure 3-3). Interestingly, binding to phospholipids at the plasma membrane is known to facilitate calpain activation (Kuboki, Ishii et al. 1992, Tompa, Emori et al. 2001, Shao, Chou et al. 2006) and calpain is a component of clathrin-coated vesicles (Sato, Saito et al. 1995). These

findings suggest that calpain may cleave the cadherin upon recruitment into clathrin-coated pits and/or early in endocytic trafficking and before the clathrin coat dissociates from endocytic vesicles. Additional studies will be needed to define with higher spatial and temporal resolution precisely where and when the cadherin tail is cleaved by calpain.

The observation that VE-cadherin is cleaved into only two fragments is consistent with the fact that calpain only partially digests its substrates, and is therefore considered to be a regulatory protease (Goll, Thompson et al. 2003, Ono and Sorimachi 2012). The VE-cad ΔCBD polypeptide, which mimics the N-terminal fragment of VE-cadherin after cleavage (Xiao, Allison et al. 2003), exhibits decreased co-localization with recycling markers and increased co-localization with lysosomal markers when compared to full length VE-cadherin (Figure 3-6). These findings suggest that the cleavage of VE-cadherin in the cytoplasmic domain results in an altered trafficking and sorting itinerary of VE-cadherin. Thus, calpain cleavage appears to be a fate-determining step during VE-cadherin endocytic processing. Previous studies showed that β-catenin facilitates E-cad transport to the plasma membrane (Chen, Stewart et al. 1999). Therefore, it is likely that deceased VE-cadherin recycling after cleavage (Figure 3-6) is due to the removal of the β-catenin binding domain of VE-cadherin. These altered trafficking dynamics are likely to impact VE-cadherin cell surface levels. Thus, calpain activity could be a potential regulatory target for modulating the VE-cadherin surface levels during a variety of pathophysiological circumstances.

Calpain has been shown to cleave a variety of classical cadherins (Jang, Jung et al. 2009, Ye, Tian et al. 2013, Kudo-Sakamoto, Akazawa et al. 2014, Trillsch, Kuerti et al. 2016), suggesting that the mechanism of cadherin regulation reported here may apply to other

cadherins in a variety of cell types. For example, it has been shown that E-cadherin cleavage by calpain is involved in tumor progression (Ye, Tian et al. 2013, Trillsch, Kuerti et al. 2016) and that calpain cleavage of N-cadherin reduces cell-cell adhesion (Jang, Jung et al. 2009, Kudo-Sakamoto, Akazawa et al. 2014). VE-cadherin cleavage by calpain has also been shown to result in disorganization of adherens junctions and hyperpermeability of vascular endothelial cells (Miyazaki, Taketomi et al. 2011). It is possible that the alteration in cadherin adhesive function observed in these other model systems and cell types could reflect an underlying mechanism of calpain-mediated regulation of adherens junctions through altered cadherin trafficking. Calpain is a calcium-dependent protease that is activated by intracellular calcium (Campbell and Davies 2012, Ono and Sorimachi 2012). Thus, increases in intracellular calcium downstream of inflammatory or angiogenic signaling could result in increased calpain activity and altered endothelial cell-cell adhesion. In fact, increases in the intracellular calcium levels have been shown to disrupt endothelial adherens junction (Sandoval, Malik et al. 2001, Komarova, Huang et al. 2012). Although the exact mechanism of how the adherens junction is disrupted in response to increased intracellular calcium levels is still not clear, our findings provide evidence that supports calpain activation as an intermediate step during the disassembly of the adherens junction in response to increased intracellular calcium.

The work presented here and in our previous studies indicates that VE-cadherin is cleaved between the juxtamembrane and the catenin-binding domain (Xiao, Allison et al. 2003). It has also been shown that calpain cleaves E-cadherin and N-cadherin in a similar region and generates similar sized fragments (around 100kDa) (Jang, Jung et al. 2009, Ye, Tian

et al. 2013, Kudo-Sakamoto, Akazawa et al. 2014, Trillsch, Kuerti et al. 2016). Calpain is known to act based on protein tertiary structures instead of specific amino acid sequences and exhibits a preference to inter-domain unstructured regions (Stabach, Cianci et al. 1997, Goll, Thompson et al. 2003, Ono and Sorimachi 2012). Based on structural studies of the E-cadherin cytoplasmic domain (Ishiyama, Lee et al. 2010), there is a flexible linker region between the juxtamembrane and the catenin-binding domain which is a likely target for calpain cleavage (Figure 3-8A). A previous study indicated that five amino acids within this linker region in VE-cadherin are required for m-calpain cleavage (Miyazaki, Taketomi et al. 2011). To determine if this region was also involved in VE-cadherin processing during endocytosis, we generated a similar VE-cadherin construct lacking these five amino acids (692 to 696) between the juxtamembrane domain and catenin-binding domain (VE-cadherin 691Δ5-RFP). Indeed, when tested in immunofluorescence based assays we observed reduced cleavage of the cadherin tail during endocytosis (Figure 3-11). However, biochemical assays indicated that calpain-1 could indeed cleave this VE-cadherin polypeptide *in vitro* (Data not shown). Although further studies will be needed to map the precise site of calpain cleavage, these studies suggest that the linker sequence between the juxtamembrane and the catenin-binding domain is a potentially important regulatory site that is subject to cleavage by calpain during endocytosis. Lastly, additional studies will be needed to determine the fate of both β-catenin and the carboxyl-terminal VE-cadherin fragment after cleavage since these polypeptides may exhibit biological activity upon release from the cadherin during endocytosis.

3.4 Material and methods

Cell Culture

Human dermal microvascular endothelial cells were cultured in endothelial growth medium 2 (Clonetics, CC-3159) supplemented with EGM-2 MV SingleQuots (Clonetics, CC-4147) on gelatin-coated plates. The African green monkey kidney fibroblast-like (COS7, ATCC) and HEK QBI-293A cell lines (MP Biomedicals) were cultured in Dulbecco's modification of Eagle's medium (Corning, 10-013-CV) supplemented with 10% FBS (HyClone, GE Healthcare, SH30071) and 1% antibiotic antimycotic solution (Corning, 30-004-CI). Primary mouse endothelial cells were cultured as described previously (Oas, Xiao et al. 2010).

Virus Production

To generate adenovirus for protein expression in mammalian cells, the gene of interest was cloned into the gateway pAd/CMV/V5-DEST vector (Invitrogen, V49320). The vector was linearized using PacI and transfected into HEK QBI-293A cells using TransFectin (Bio-Rad, 1703350) to produce virus. After a second round of infection, cells were lysed and virus was harvested.

Internalization Assay and Image Analysis

Cells were infected with adenovirus or transfected with plasmid expressing the protein of interest 48 hours prior to the experiment. Transfection was conducted using Lipofectamine 2000 (ThermoFisher, 11668-019) or 3000 (ThermoFisher, L3000008) according to the protocol provided by the manufacturer. Internalization assays were performed as described previously (Xiao, Allison et al. 2003, Chiasson, Wittich et al. 2009, Cadwell, Jenkins et al.

2016). Briefly, cultured cells on glass coverslips were incubated with an antibody against the VE-cadherin extracellular domain (BV6, Millipore, MABT134) in cell culture medium for 30 minutes at 4 °C. Cells were washed three times with cold PBS containing calcium and magnesium to remove unbound antibody. To allow internalization, cells were incubated in prewarmed medium at 37 °C. Cells were then returned to cold medium. A low-pH buffer (100mM glycine, 20mM magnesium acetate, and 50mM potassium chloride (pH 2.2)) was used to remove any remaining antibody from the cell surface. Cells were then fixed and permeabilized by incubation in 4% paraformaldehyde for 10 minutes, followed by 0.1% Triton X-100 for 10 minutes at room temperature. Goat anti-VE-cadherin antibody (C19, Santa Cruz, sc-6458) or C-terminal RFP tag was used to visualize the C-terminal domain of VE-cadherin. Mouse anti-VE-cadherin (Cad-5, BD TransLab, 610252), an alternative antibody recognizes the extracellular domain of VE-cadherin, was used to determine the total amount of VE-cadherin. Additional primary antibodies used including Mouse anti-clathrin heavy chain (BD TransLab, 610500), mouse anti-EEA1 (BD TransLab, 610457), mouse anti-p120 (BD TransLab, 610134), rabbit anti-β-catenin (Sigma, C2206), rabbit anti-Rab11 (Invitrogen 71-3500), mouse anti-CD63 (H5C6, IowaLabs). Secondary antibodies conjugated to fluorescent dyes (Alexa Fluor 488, 555, or 647 nm; Life Technologies) were used to visualize antibody binding.

To quantify cleavage, co-localization between the signals from endocytosed VE-cadherin (labeled at the extracellular domain) and C-terminal end of VE-cadherin (labeled at the C-terminal domain) were quantified using Manders' Correlation Coefficient:

$M1 = \frac{\sum_i Ri,colocal}{\sum_i Ri}$ And $M2 = \frac{\sum_i Gi,colocal}{\sum_i Gi}$ where R_i: red signal, $R_{i,colocal}$: red signal that co-localizes with green signal, G_i: green signal, $G_{i,colocal}$: green signal that co-localizes with red signal. Coloc 2 program from ImageJ/Fiji was used for quantification.

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

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

Western Blot Analysis

Samples were prepared by either scraping cells into Laemmli Sample Buffer (Bio-Rad, #1610737) with 5% β-mercaptoethanol (β-ME) or adding the Laemmli Sample buffer with 5% β-ME directly into the reaction mixture. Samples were then heated at 95^0C for 5 minutes before SDS-PAGE, and analyzed by immunoblotting on nitrocellulose membranes (Whatman, Maidstone, United Kingdom). Quantification was done using gel analysis program in ImageJ/Fiji and statistical analysis was performed in RStudio. Primary antibodies used are as follows: mouse anti-VE-cadherin (Cad-5, BD TransLab, 610252), mouse anti-VE-cadherin (BV6, Millipore, MABT134), rat anti-mouse VE-cadherin (eBioscience, E028599), rat anti-mouse VE-cadherin (BD Pharmingen, 550548), rabbit

anti-p120 (Santa Cruz, sc-1101), Secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) and ECL western blot detection reagents (GE Healthcare, RPN2106) were used to visualize proteins by western blot.

Surface biotinylation pulse-chase assay and VE-cadherin half-life determination

COS7 cells were transfected with plasmid of interest 48 hours prior to the experiment. Cells were pulse-labeled with biotin (ThermoFisher, 21331) on ice for 30 minutes. Excess biotin was quenched by 50mM Ammonium Chloride. Cells were then incubated at 37^0C for various amounts of time, returned to ice and then lysed with 1% Triton X-100 in PBS with calcium and magnesium for 30 minutes. Cell lysates were centrifuged at 13,200 rpm for 30 minutes at 4^0C. The supernatants were added to streptavidin beads (ThermoFisher, 20349) and incubated for 1 hour at 4^0C. Beads were washed 4 times using ice cold PBS with 0.1% Tween-20. Biotinylated protein was eluted using Laemmli Sample Buffer with 5% β-ME at 95^0C for 5 minutes, followed by western blot analysis.

To calculate VE-cadherin half-life, the following equation was used: $t_{1/2} = \frac{t}{\log_{1/2}(\frac{N_t}{N_0})}$ and the rate constant $k = \frac{\ln(\frac{N_t}{N_0})}{t}$ where $t_{1/2}$: half-life, t: given time, N_t: the amount of protein at given time, N_0: the amount of protein at time 0.

***In vitro* calpain digestion**

COS7 cells were infected with adenovirus expressing VE-cadherin RFP 48 hours prior to the experiment. Cells were lysed in M-PER lysis buffer (ThermoFisher, 78501) supplemented with 1% protease inhibitors (Sigma, P8849) for 10 minutes on ice. 1mM

CaCl2, 1 unit of active calpain 1 large subunit (Sigma, C6108), 100μM Calpeptin (Millipore, 03-3-0051) and 250μM ALLM (Santa Cruz, CAS 136632-32-1) were added separately to the cell lysates. The reaction mixture was incubated at room temperature for 30 minutes. The reaction was then terminated by adding Laemmli Sample Buffer with 5% β-ME to the mixture, followed by western blot analysis.

Figures

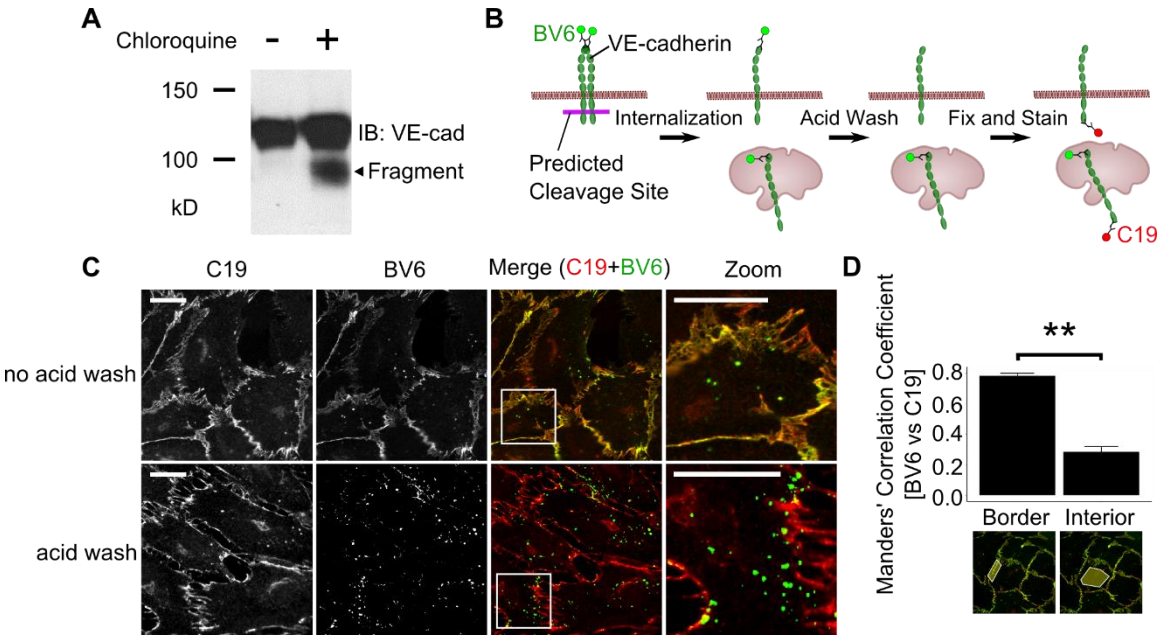


Figure 3-1. VE-cadherin is cleaved during endocytosis. (A) Western blot showing that overnight chloroquine treatment leads to the accumulation of a VE-cadherin cleavage fragment, detected using antibodies against the VE-cadherin extracellular domain. (B) Illustration of the experimental procedure for an internalization assay to monitor VE-cadherin extracellular domain (BV6) and carboxyl-terminal domain (C19). (C) Immunofluorescence following the protocol in B suggesting VE-cadherin is cleaved during endocytosis, resulting in the removal of the VE-cadherin carboxyl-terminal tail. Cell surface VE-cadherin was labeled in MECs and cells were incubated for 3 hours with 100µM Chloroquine treatment at 37^0C. Scale bar = 20µm. (D) Quantification of the results in C. Co-localization between endocytosed VE-cadherin (BV6, acid wash panel) and c-terminal end of VE-cadherin (C19) was quantified. Border regions of MECs without acid wash were used for comparison. Data are presented as the means ± SEM. N (number of

cells) = 21 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. **$p<0.01$, *two tailed t*-test

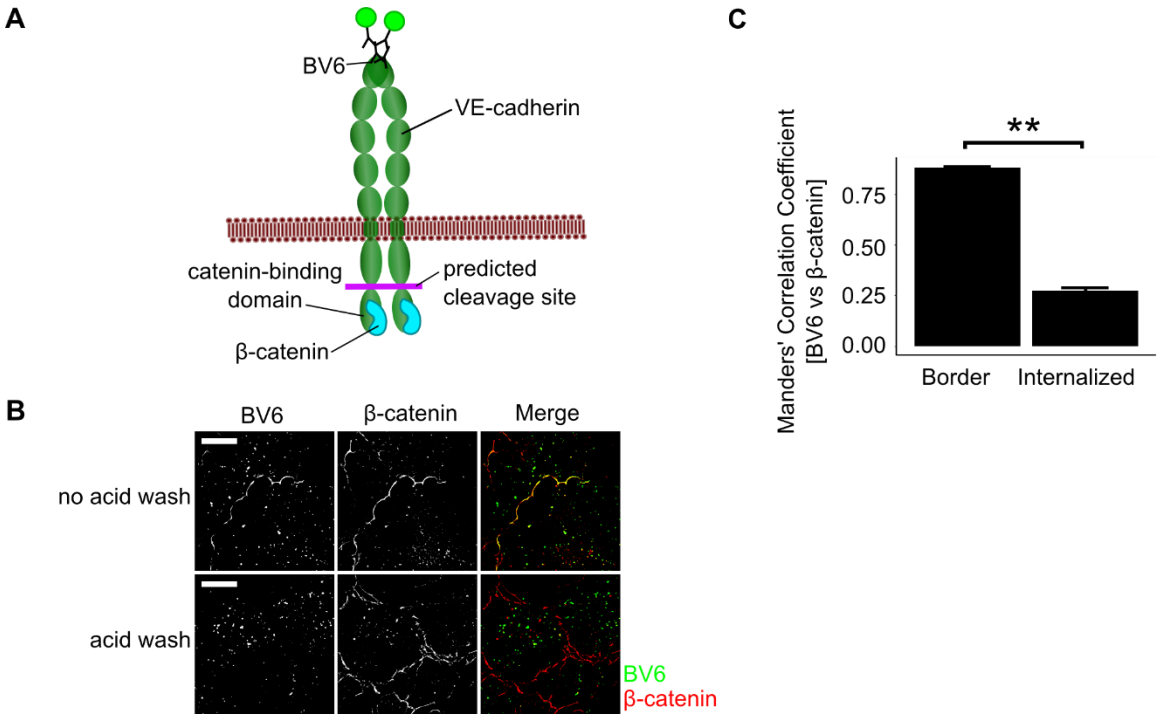


Figure 3-2. β-catenin co-localizes with junctional but not internalized VE-cadherin. (A) Schematic illustration of the labeling setup of the experiment in B. (B) COS7 cells were infected with adenovirus expressing VE-cadherin. Immunofluorescence imaging reveals β-catenin does not co-localize with the VE-cadherin extracellular domain (BV6) after endocytosis (acid wash panel). Scale bar = 20μm. (C) Quantification of co-localization between β-catenin and the VE-cadherin extracellular domain at cell-cell borders and in acid wash samples (Internalized). Data are presented as the means ± SEM. N ≥ 69 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. $^{**}p<0.01$, *two tailed t*-test.

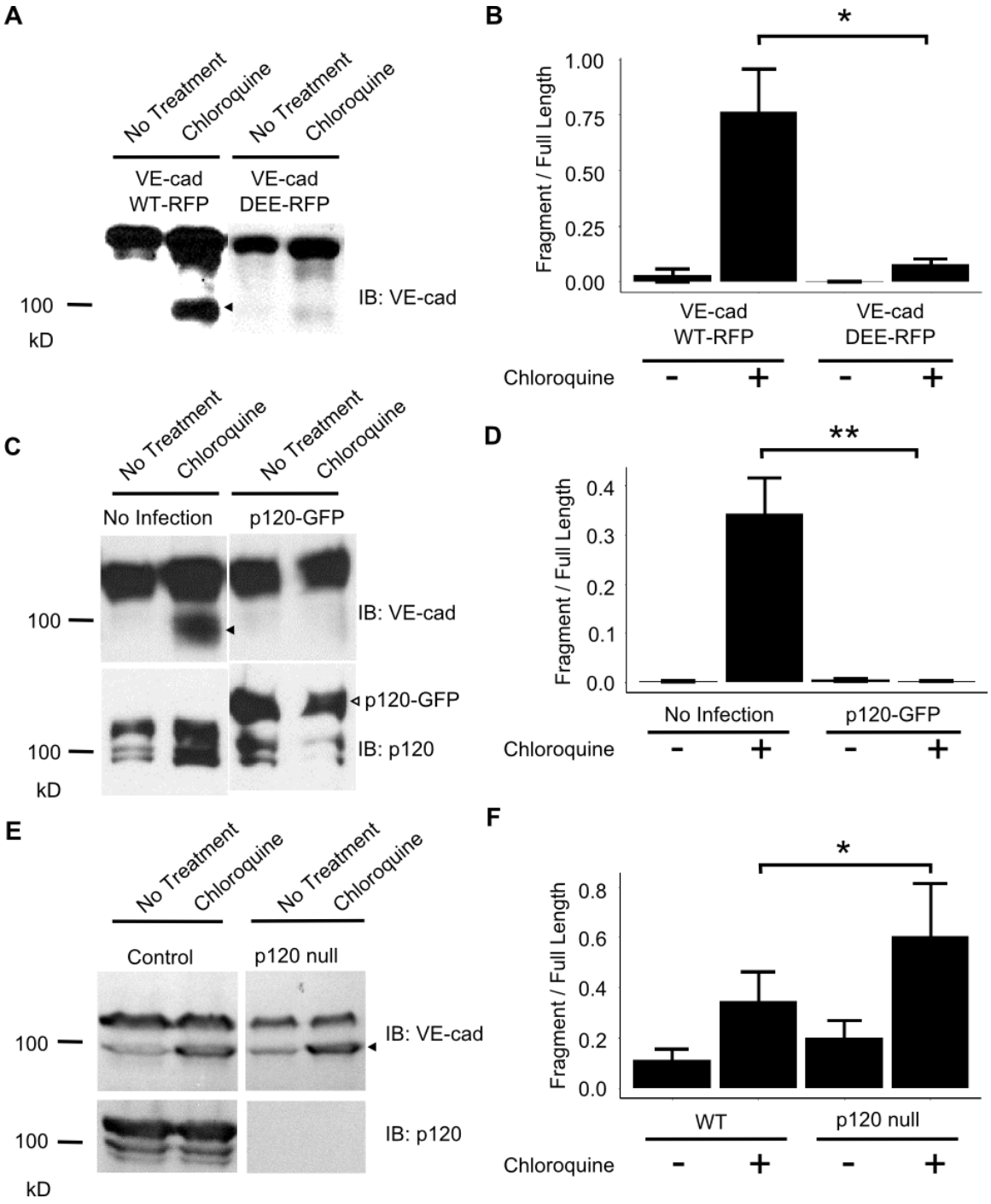


Figure 3-3. VE-cadherin endocytosis is required for cleavage. (A) COS7 cells were transfected with indicated constructs and treated with chloroquine for 6 hours prior to lysis. Western blot analysis reveals significantly less cleavage of the VE-cadherin DEE endocytic mutant compared to wild type VE-cadherin. (B) Quantification of the results in A. Data are

presented as the means ± SEM. Each condition was conducted in triplicate and represents data from three independent experiments. *p<0.05, *two tailed t*-test. (C) MECs were infected with adenovirus expressing p120-GFP 48 hours prior to the experiment. Cells were treated with chloroquine overnight. Western blot analysis reveals significantly reduced fragment accumulation of VE-cadherin with p120 overexpression compared to the control. (D) Quantification of the result in C using the same method as described in B. **p<0.01, *two tailed t*-test. (E) p120 knock out results in increased VE-cadherin cleavage. Mouse skin endothelial cells with or without p120 were treated with chloroquine overnight. Western blot shows increased fragmentation in p120 null cells compared to the control. (F) Quantification of the result in E using the same method as described in B. *p<0.05, *two tailed paired t*-test.

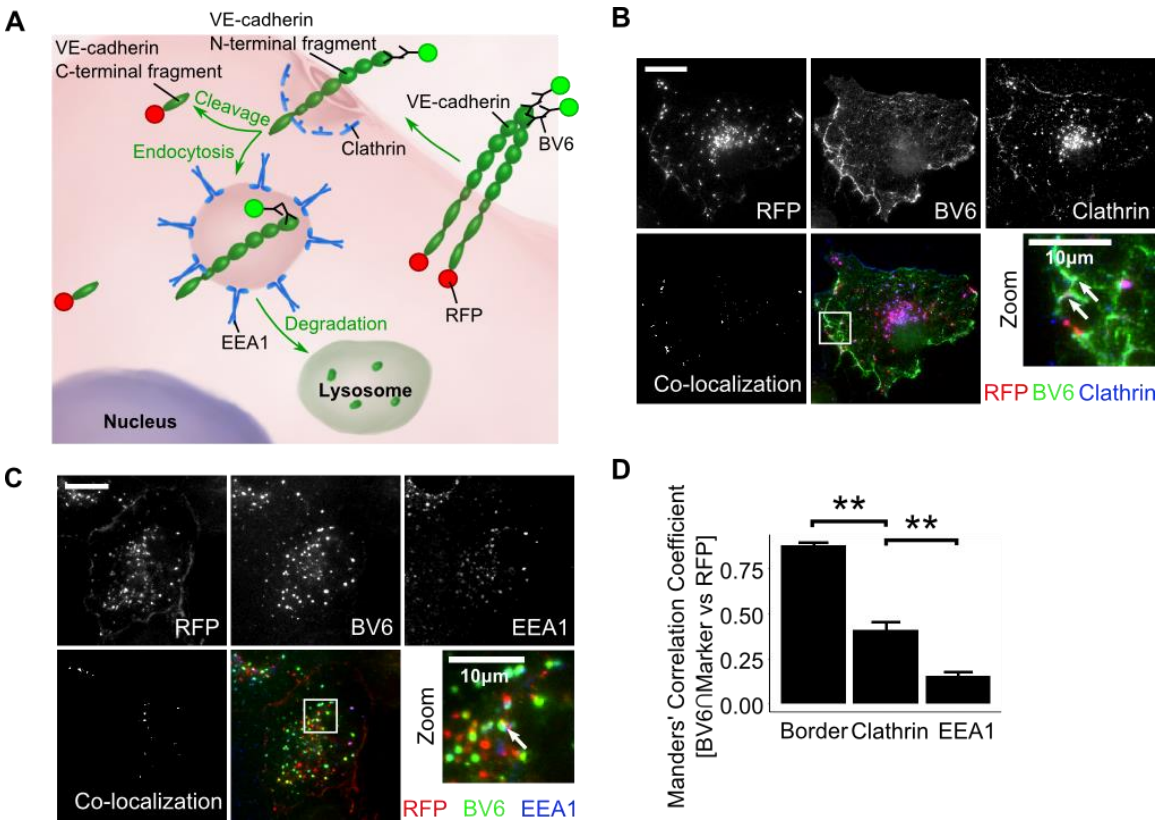


Figure 3-4. VE-cadherin cleavage occurs after VE-cadherin enters clathrin-enriched membrane domains but before it reaches the early endosome. (A) Illustration showing where VE-cadherin cleavage may occur during endocytosis and the labeling approach used for experiments in B and C. (B) Adenovirus infected COS7 cells expressing VE-cadherin-RFP were labeled with BV6 and then warmed to 37^{0}C for 5 minutes to visualize surface pools and very early endocytic events. Three channel co-localization reveals that cleavage occurs while VE-cadherin enters clathrin-enriched domains. Scale bar = 20µm. (C) COS7 cells were warmed to 37^{0}C for 30 minutes to induce endocytosis, followed by acid wash to remove cell surface antibodies. Three channel co-localization reveals cleavage occurs before VE-cadherin reaches the early endosome. Scale bar = 20µm. (D) Quantification of the results in B and C. Border regions of COS7 cells expressing VE-cadherin-RFP without

acid wash were used as positive controls. Co-localization analysis between BV6 and Clathrin or BV6 and EEA1 was used to identify cadherin pools at different steps of endocytosis. Co-localization analysis between RFP and VE-cadherin in clathrin-enriched domains or early endosomes was then performed to reveal whether the VE-cadherin carboxyl-terminal domain was present at these endocytic steps. Data are presented as the means ± SEM. N ≥ 16 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. $^{**}p<0.01$, *two tailed t*-test.

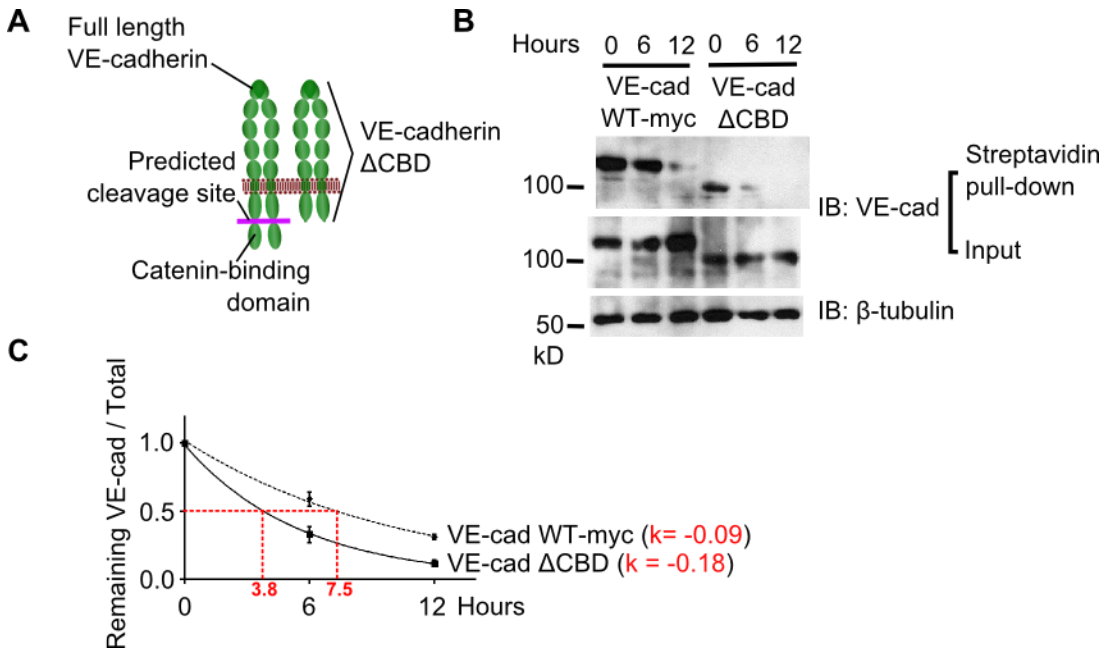


Figure 3-5. Deletion of the VE-cadherin catenin-binding domain increases VE-cadherin turnover rates. (A) Illustration showing that VE-cadherin ΔCBD mimics the cleaved fragment of VE-cad. (B) Surface biotinylation reveals faster turnover of VE-cadherin ΔCBD compared to full length VE-cadherin. COS7 cells were transfected with indicated constructs. (C) Quantification of the results in B. Data are presented as means ± SEM. The half-life of each cadherin is labeled with red dotted lines. Rate constant k was labeled on the right in red. Each condition was conducted in triplicate and represents data from three independent experiments.

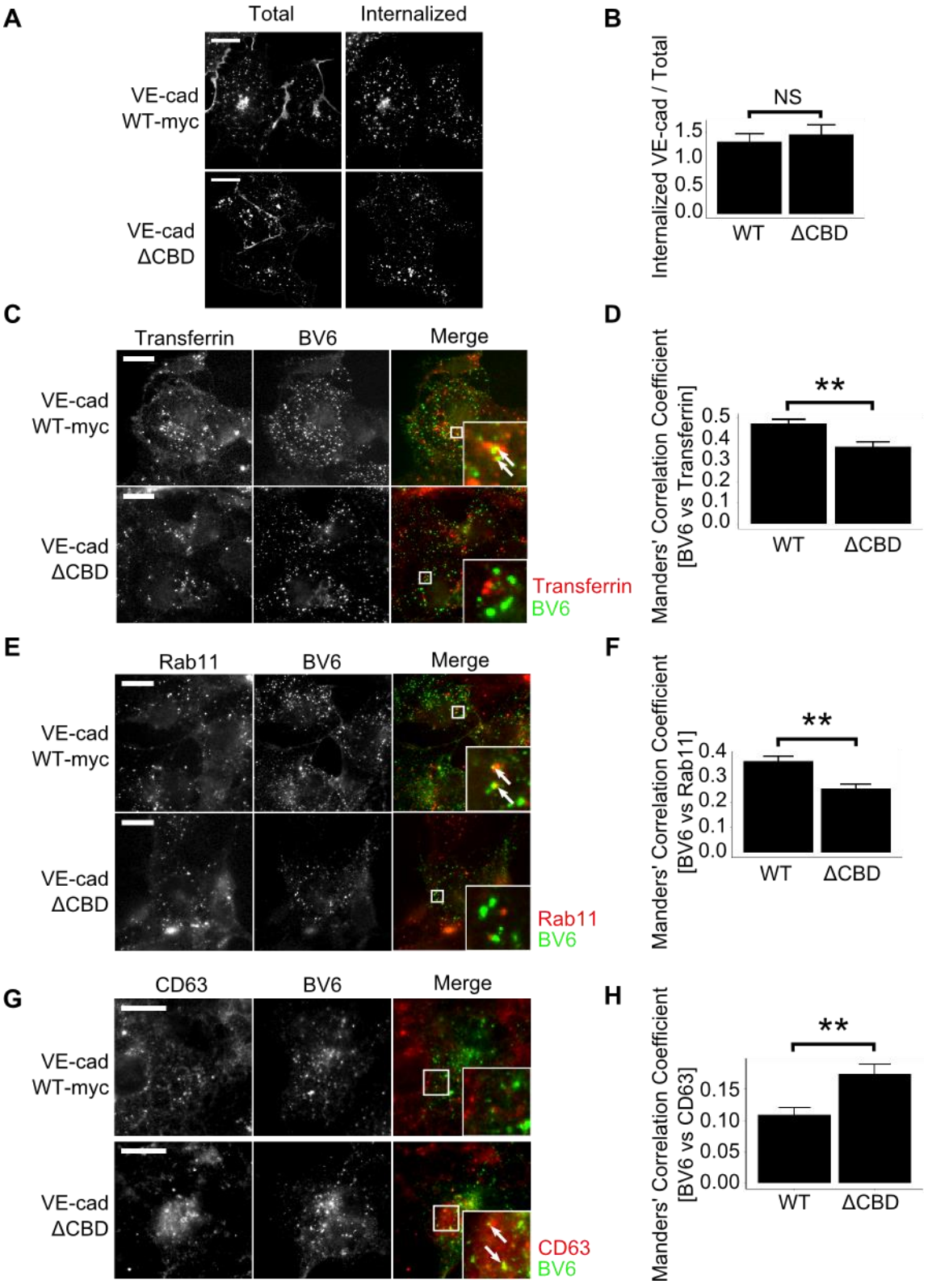

A
Total
Internalized
VE-cad WT-myc
VE-cad ΔCBD
B
Internalized VE-cad / Total
NS
1.5
1.0
0.5
0.0
WT
ΔCBD
C
Transferrin
BV6
Merge
VE-cad WT-myc
VE-cad ΔCBD
Transferrin
BV6
D
Manders' Correlation Coefficient [BV6 vs Transferrin]
**
0.5
0.4
0.3
0.2
0.1
0.0
WT
ΔCBD
E
Rab11
BV6
Merge
VE-cad WT-myc
VE-cad ΔCBD
Rab11
BV6
F
Manders' Correlation Coefficient [BV6 vs Rab11]
**
0.4
0.3
0.2
0.1
0.0
WT
ΔCBD
G
CD63
BV6
Merge
VE-cad WT-myc
VE-cad ΔCBD
CD63
BV6
H
Manders' Correlation Coefficient [BV6 vs CD63]
**
0.15
0.10
0.05
0.00
WT
ΔCBD

Figure 3-6. The VE-cadherin catenin-binding domain regulates cadherin fate after endocytosis. (A) VE-cadherin ΔCBD has a similar endocytosis rate as wild type cadherin. An internalization assay was performed using BV6 antibody to label internalized VE-cadherin. Total VE-cadherin was detected using Cad-5 antibody. Scale bar = 20μm. (B) Quantification of the results in panel A. Data are presented as the means ± SEM. N ≥ 17 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. NS: non-significant, *two tailed t*-test. (C and E) VE-cadherin ΔCBD exhibits reduced co-localization with recycling markers compared to full length VE-cadherin. Internalization assays were performed using BV6 to monitor the internalized VE-cadherin. Cells were warmed to 37^0C for 30 minutes in the presence of tetramethylrhodamine-conjucated transferrin at 50μg/mL in C or for 60 minutes and followed by Rab11 staining in E. Scale bar = 20μm. (D) Quantification of the results in C. Data are presented as the means ± SEM. N ≥ 43 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. $^{**}p<0.01$, *two tailed t*-test. (F) Quantification of the results in E. Data are presented as the means ± SEM. N ≥ 31 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. $^{**}p<0.01$, *two tailed t*-test. (G) VE-cadherin ΔCBD exhibits more co-localization with the lysosomal marker CD63 than observed for wild-type cadherin. Cells were warmed to 37^0C for 60 minutes and were then fixed and stained for CD63. Scale bar= 20μm. (H) Quantification of the results in G. Data are presented as the means ± SEM. N ≥ 33 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. $^{**}p<0.01$, *two tailed t*-test.

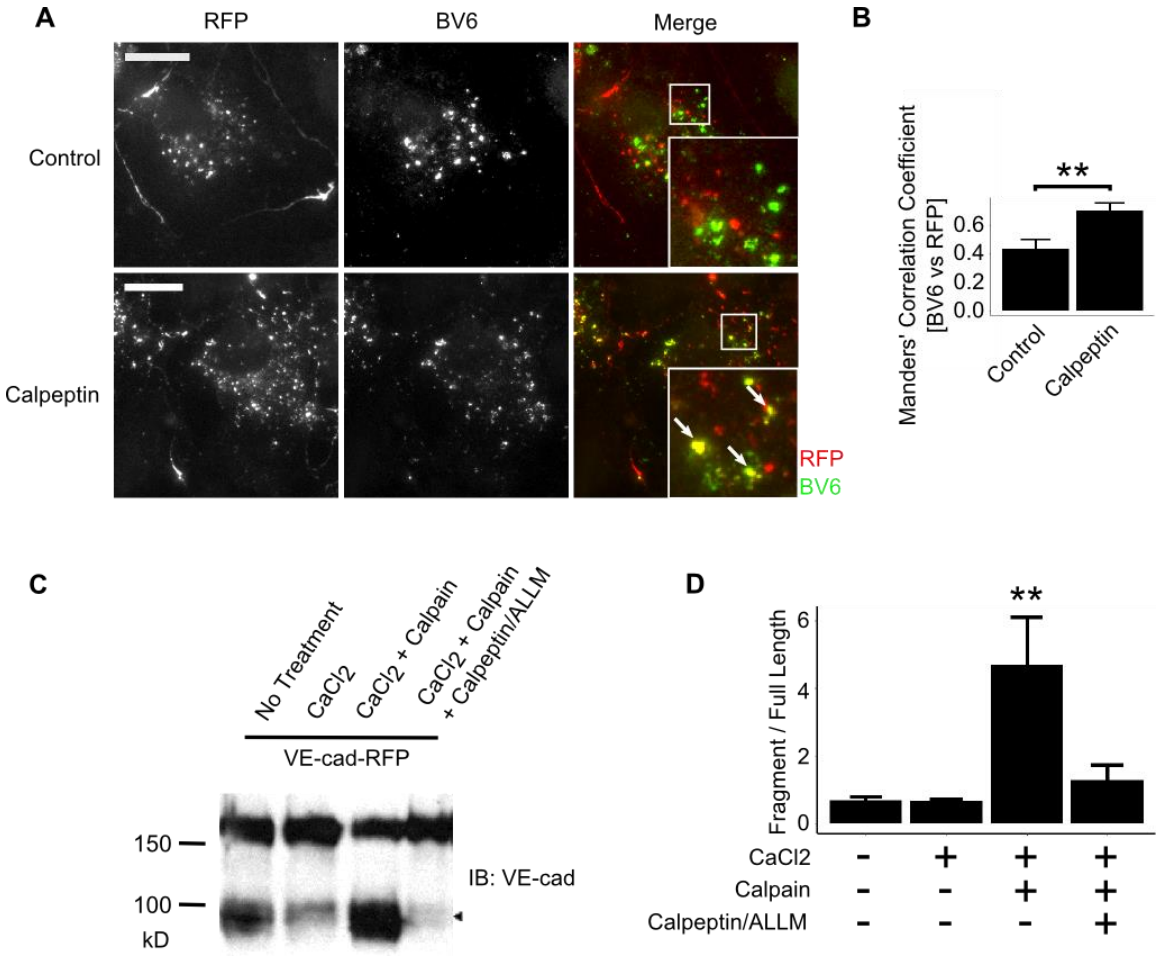


Figure 3-7. VE-cadherin is cleaved by calpain. (A) Immunofluorescence staining indicates that inhibiting calpain activity reduces VE-cadherin cleavage. COS7 cells were pre-treated with 10μM calpeptin for 30 minutes followed by a 30-minute internalization assay. Scale bar = 20μm. (B) Quantification of the results in A. Data are presented as the means ± SEM. N ≥ 14 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. ***p*<0.05, *two tailed t-test*. (C) Western blot showing VE-cadherin is digested by calpain *in vitro*. COS7 cells infected with adenovirus expressing VE-cadherin-RFP were lysed in M-PER lysis buffer. Active calpain large subunit was added to the lysate in the presence of $CaCl_2$ with or without calpain inhibitors (Calpeptin

and ALLM). (D): Quantification of the results in C. Data are presented as the means ± SEM. Each condition was conducted in triplicate and represents data from three independent experiments. **$p<0.01$, *one -way ANOVA*.

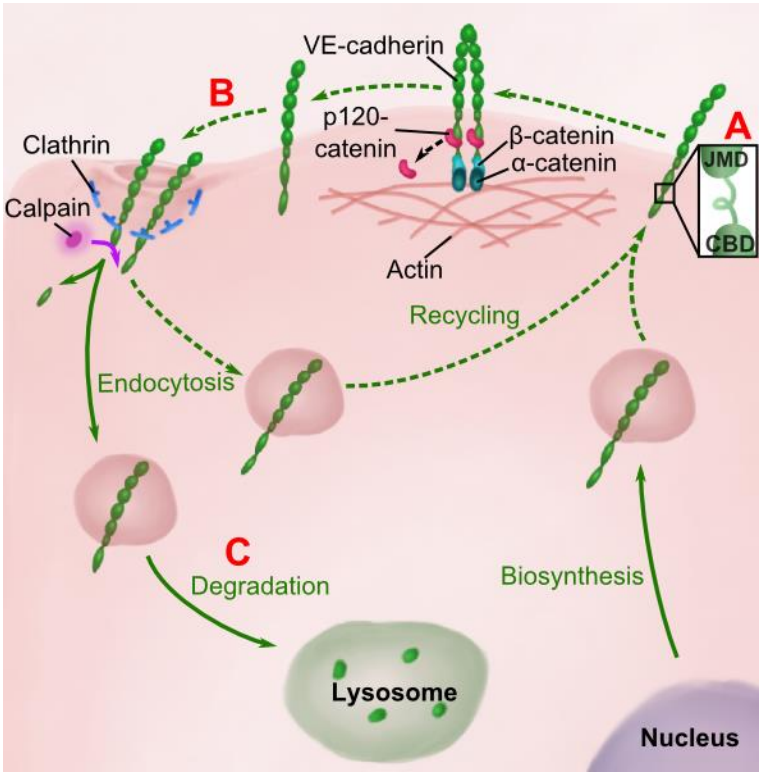


Figure 3-8. Model. (A) Open structure region between the juxtamembrane domain (JMD) and the catenin-binding domain (CBD) is likely the cleavage site for calpain. (B) Upon entry into clathrin-enriched domains, a portion of the VE-cadherin is cleaved by active calpain. (C) Cleaved VE-cadherin is more likely to be degraded rather than recycled back to the cell surface.

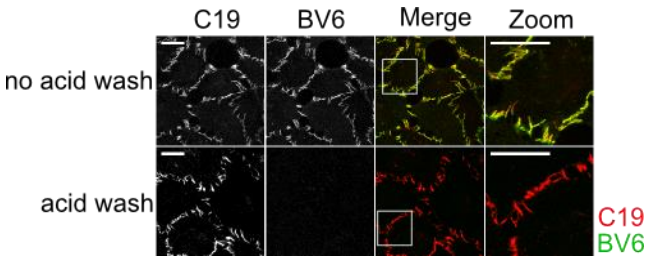


Figure 3-9. 4^0C control for internalization assays. MECs were kept at 4^0C after surface labeling rather than returned to 37^0C. 4^0C temperature blocks VE-cadherin endocytosis and the acid wash effectively removes remaining surface antibodies, indicating that fluorescent signal present after acid wash reflects internalized cadherin. Scale bar = 20μm.

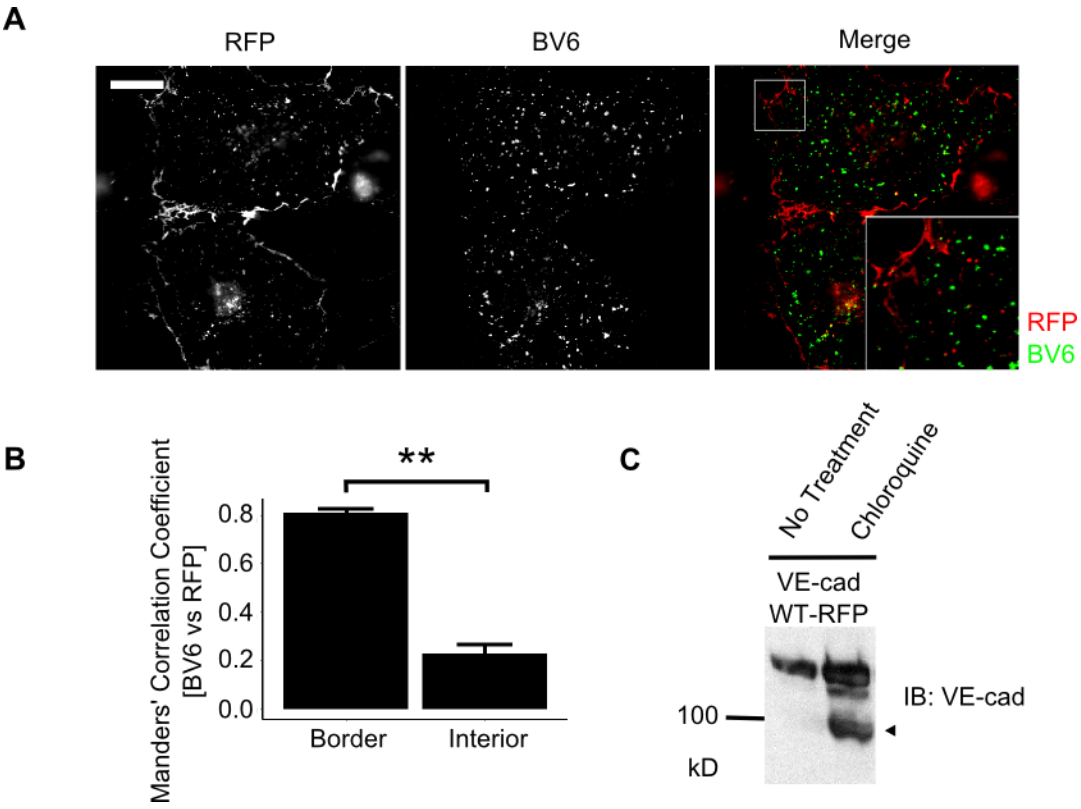


Figure 3-10. Exogenous VE-cadherin is cleaved during endocytosis. (A) COS7 cells infected with adenovirus expressing VE-cadherin-RFP were warmed to 37^{0}C to allow for endocytosis. Immunofluorescence staining indicates that exogenous VE-cadherin is cleaved during endocytosis. Scale bar = 20μm. (B) Quantification of the results in A. Border regions without acid wash were used as positive control. Data are presented as the means ± SEM. N ≥ 29 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. $**p<0.01$, *two tailed t*-test. (C) Western blot showing that chloroquine treatment leads to the accumulation of VE-cadherin cleaved fragment. COS7 cells were treated with chloroquine for 6 hours.

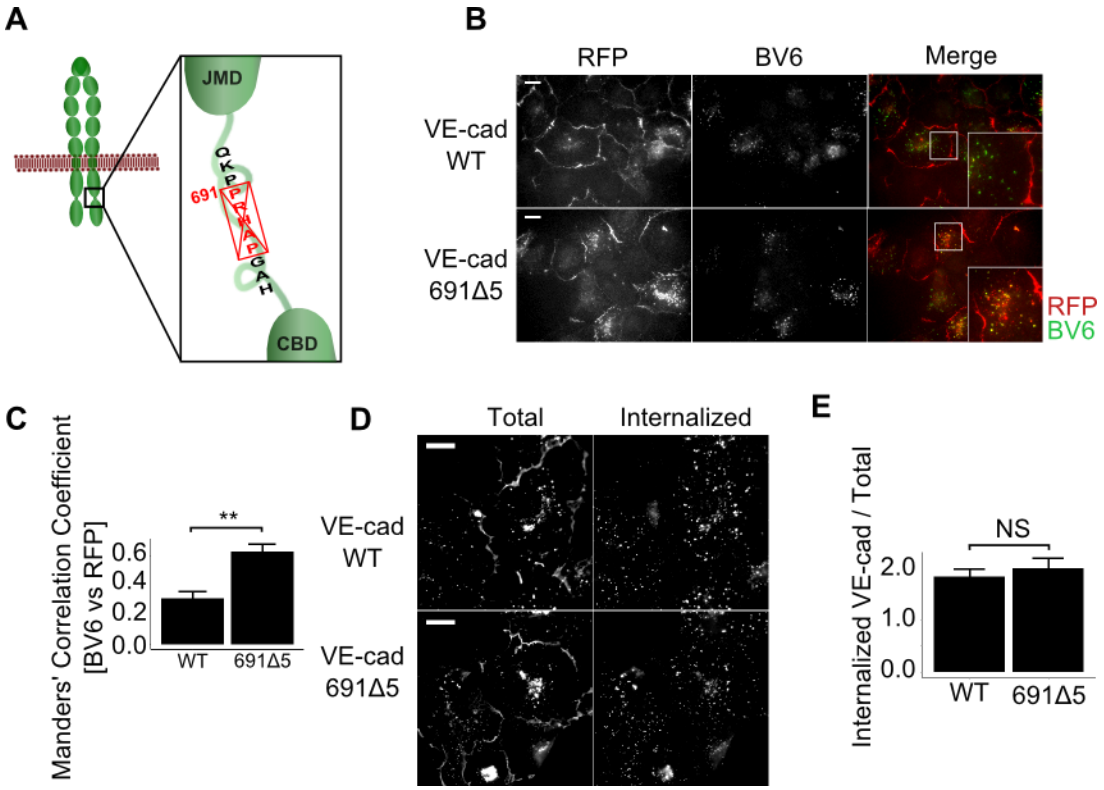


Figure 3-11. VE-cadherin 691Δ5 is resistant to calpain cleavage. (A**)** Illustration showing VE-cadherin 691Δ5 construct with the deletion of five amino acids in the linker region between the juxtamembrane domain (JMD) and the catenin-binding domain (CBD). (B) Immunofluorescence staining indicates reduced cleavage of the VE-cadherin 691Δ5 compared to wild type VE-cadherin. COS7 cells were infected with adenovirus 48 hours prior to the experiment, followed by a 30-minute internalization assay. Scale bar = 20µm. (C) Quantification of the results in B comparing the co-localization of BV6 to RFP fluorescence signals. Data are presented as the means ± SEM. N ≥ 11 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. ***p*<0.01, *two tailed t*-test. (D) Internalization assay suggests VE-cadherin 691Δ5 is endocytosed at similar rates compared to the wild-type VE-cadherin. BV6 was used to label the internalized pool of VE-cadherin while Cad-5 was used to label the total

VE-cadherin. Scale bar = 20μm. (E) Quantification of the result in D. Data are presented as the means ± SEM. N ≥ 17 in each group. Each condition was conducted in triplicate and represents data from three independent experiments. NS: non-significant, *two tailed t*-test.

Chapter 4

Role of the dileucine motif in E-cadherin endocytosis and cell mobility

4.1 Introduction

Epithelia, one of the four basic types of animal tissues, is widely distributed throughout the organism (Vogelmann, Amieva et al. 2004, Gonzalez-Mariscal, Garay et al. 2009). Notable examples of epithelia the most outer layer of skin, and cell layers that lines the inside of organs such as lung and intestines. One of the most basic functions of epithelium is to separate compartments in an organism to protect tissues from external elements. In order to achieve effective protection, epithelial cells need to be held tightly together and be able to withstand external forces (Citi, Guerrera et al. 2014). Apical junctional complexes are one of the key organelles that allow strong association between neighboring epithelial cells. In skin epithelial cells, there are two types of cadherin-based adhesive junctions: adherens junctions and desmosomes (Citi, Guerrera et al. 2014). Adherens junctions contain classical cadherins that are connected to the actin cytoskeleton via catenins (Cadwell, Su et al. 2016). Desmosomes, on the other hand, are associated with intermediate filaments through interactions between desmosomal cadherins and adaptor proteins (Kowalczyk, Bornslaeger et al. 1999, Kowalczyk and Green 2013). In contrast to desmosomes, which mainly provide structural support to withstand mechanical forces, adherens junctions mainly mediate intercellular adhesion to maintain the cell-cell contact between adjacent epithelial cells and to allow for initial attachment of two cells (Citi, Guerrera et al. 2014).

Dynamic regulation of cell adhesion is required for release and reattachment of cells, which is an essential process during development and disease. During epidermal differentiation, the adherens junction needs to be remodeled to allow movement of new layers of differentiating epithelial cells towards the surface (Nelson 2009, Abhishek and Palamadai Krishnan 2016). Precise modulation of cell adhesion is critical to achieve proper

differentiation. Another example where dynamic regulation of cell adhesion is essential is during epithelial-mesenchymal transition (EMT). EMT describes a process in which polarized epithelial cells lose their epithelial properties and acquire mesenchymal properties, which is a key step that leads to the invasive ability of metastatic cancer cells (Diepenbruck and Christofori 2016, Sun and Fang 2016). One of the important characteristics of cells undergoing EMT is loss of cell adhesion and polarity (Diepenbruck and Christofori 2016). Current hypothesis is that weakened cell adhesion allows for easier movement of cells into their surroundings, which eventually leads to cancer metastasis.

The most prominent cadherin expressed by epithelial tissues is E-cadherin, which is a type I classical cadherin (Shapiro and Weis 2009, Saito, Tucker et al. 2012, Cadwell, Su et al. 2016). Cell adhesion mediated by E-cadherin can be regulated in multiple ways, one of which is endocytosis. Endocytosis allows for removal of E-cadherin from the intercellular contacts, resulting in a decreased amount of cadherin present at adherens junctions therefore reducing adhesion strength. Numerous studies have been conducted on the regulation of E-cadherin endocytosis. A major regulator of E-cadherin endocytosis is p120, which binds to E-cadherin at the juxtamembrane domain (Ireton, Davis et al. 2002, Davis, Ireton et al. 2003, Ishiyama, Lee et al. 2010). It was demonstrated that the juxtamembrane domain of E-cadherin per se drives cadherin endocytosis (Nanes, Chiasson-MacKenzie et al. 2012). Binding of p120 was shown to prevent the assess of the endocytic machinery and thereby prevents E-cadherin endocytosis (Ishiyama, Lee et al. 2010). Structural studies suggest that p120 also masks multiple endocytic motifs (Ishiyama, Lee et al. 2010). One of the putative endocytic motifs on the juxtamembrane domain is a dileucine motif that is in the dynamic binding region of the p120 binding site (Figure 2-5). Miyashita and Ozawa

found that when mutating these leucine residues to alanine, E-cadherin endocytosis was effectively inhibited (Miyashita and Ozawa 2007). Together, these studies suggest that the dileucine motif is the putative endocytic motif that is required for efficient E-cadherin internalization.

Although extensive research has been done examining E-cadherin endocytosis, the biological consequences of selectively inhibiting E-cadherin endocytosis has not been fully explored. In the case of VE-cadherin, decreased endocytosis was shown to inhibit collective cell migration (Nanes, Chiasson-MacKenzie et al. 2012). Due to the high structural and functional similarity between E- and VE-cadherin, it is likely that inhibiting E-cadherin endocytosis also inhibits cell migration. By utilizing the dileucine mutant E-cadherin as a tool to inhibit E-cadherin endocytosis, we were able to address the effect of cadherin endocytosis in cell migration. Here, we present data supporting the idea that inhibiting E-cadherin endocytosis leads to reduced collective cell migration. One of the biggest challenges in studying collective cell migration, which currently are largely based on 2-D migration of cell sheets, is to relate *in vitro* findings to various *in vivo* models of collective cell migration and tissue morphogenesis that have very different architecture (Friedl, Locker et al. 2012). In an effort to address this challenge, we collaborated with the Marcus lab to test the effect of E-cadherin endocytic mutant in an *in vitro* 3-D cancer metastasis model. The results indicated that, rather than inhibiting migration, inhibiting E-cadherin endocytosis leads to increased invasive ability of cancer cells. Our data suggest that the biological consequences of inhibiting cadherin endocytosis are context dependent and thus require careful interpretation.

4.2 Results

4.2.1 Dileucine motif is required for E-cadherin endocytosis

In order to verify if the dileucine (LL) motif is required for E-cadherin endocytosis, we generated a RFP-tagged E-cadherin construct in which the LL residues were mutated to AA. Using a lentivirus approach, we were able to express the mutant E-cadherin in various mammalian cell lines to determine endocytosis rates of the mutant cadherin compared to wild type. To examine E-cadherin endocytosis, we used HECD-1 antibody to label surface E-cadherin at 4ºC followed by a 30-minute internalization at 37ºC. With removal of remaining surface antibodies by low pH wash, signals from the internalized pool of E-cadherin were divided by signals from the total pool of E-cadherin, which was visualized by the C-terminal RFP tag from the E-cadherin construct. Our results showed that in COS7 cells, the LL mutant E-cadherin exhibits a lower rate of endocytosis when compared to wild type E-cadherin (Figure 4-1 A, B). We also verified that in the human non-small cell lung carcinoma cell line H1299, the LL E-cadherin again exhibits a reduced rate of endocytosis rate compared to the wild type cadherin (Figure 4-1 C, D). Together, our data indicate that the LL motif regulates E-cadherin endocytosis.

4.2.2 Mutation of the dileucine motif results in reduced collective cell migration rate

A VE-cadherin mutant defective in endocytosis was shown to inhibit collective cell migration when expressed in endothelial and other cell types (Nanes, Chiasson-MacKenzie et al. 2012). To determine if the E-cadherin LL mutant, which shows reduced endocytosis, can also inhibit cell migration, we measured the collective migration rate in various cell types expressing either the wild type or LL mutant E-cadherin by performing the scratch

wound assay. We first measured the migration rate in A341D cells, an epithelial cell deficient in endogenous classical cadherins. The results showed that the LL mutant can indeed reduce cell migration (Figure 4-2 A). This result was then verified in primary human keratinocytes (Figure 4-2 B), which have endogenous E-cadherin, suggesting that the LL mutant acts dominantly over endogenous E-cadherin to inhibit collective cell migration. Lastly, to determine if the ability of the LL mutant to inhibit migration also occurs in lung cancer cells, we conducted scratch wound assay in H1299 cells to measure their migration rate. As expected, the collective migration was inhibited with LL mutant E-cadherin expression (Figure 4-3). Together, our data suggest that comparable to VE-cadherin, E-cadherin that harbors endocytosis inhibiting mutations can also inhibit collective cell migration in two dimensional cell migration assays.

4.2.3 Mutation of the dileucine motif results in increased invasive ability of lung cancer cells

During cancer metastasis, increased cell migration is often observed to allow easier invasion of cancer cells into neighboring tissues. Therefore, we speculated that with inhibited cell migration, the LL mutant E-cadherin would inhibit cancer cell invasion. In order to test this hypothesis, we collaborated with Dr. Adam Marcus at Emory whose lab is experienced with lung cancer models and *in vitro* cancer invasion assays. Using H1299 expressing either the wild type or LL mutant E-cadherin, Jessica Konen from the Marcus lab performed a spheroid invasion assay, which is an *in vitro* assay that models cancer metastasis. To our surprise, the results were opposite to what we predicted. Instead of inhibiting cell invasion, cells expressing the LL mutant E-cadherin showed an increased ability to invade into surrounding collagen matrix when compared to wild type cadherins

(Figure 4-4). Wild type E-cadherin, on the other hand, showed decreased invasive behavior even when compared to parental cells (Data not shown). These studies are preliminary and additional experiments such as flow cytometry are required to verify expression levels of various cadherin mutants are equal. In addition, cell proliferation controls such as BrdU incorporation assay would be required to eliminate potential difference in cell proliferation rate. Nonetheless, our results thus far point to a key role for cadherin endocytosis in cell migration and potentially in tumor cell metastasis.

4.3 Discussion

The results of this study demonstrate a context-dependent effect of cadherin endocytosis in different biological systems. Our data suggested that by mutating the dileucine motif into alanine in E-cadherin, which inhibits cadherin endocytosis, can effectively inhibit collective migration of various types of cells in two dimensional cell culture models. However, in *in vitro* spheroid invasion assay, which models cancer metastasis, the dileucine mutant of E-cadherin promoted lung cancer cell invasion. These findings suggested that the effect of inhibiting cadherin endocytosis is dependent on the biological system.

In VE-cadherin, an acidic motif (DEE) was found to mediate endocytosis and mutation of this motif inhibited collective cell migration (Nanes, Chiasson-MacKenzie et al. 2012). DEE residues are conserved in E-cadherin but only has modest effect in endocytosis (Nanes, Chiasson-MacKenzie et al. 2012). Rather, a dileucine motif, which was not present in VE-cadherin, was found to be the dominant endocytic motif for E-cadherin (Miyashita and Ozawa 2007). However, the role of E-cadherin endocytosis in epithelial cell migration has not been elucidated. Epithelial morphogenesis typically involves migration and branching of tube-like structures (Hirata, Park et al. 2014). During *Drosophila* embryo development, disruption of endocytosis globally leads to cell intercalation defects, which results from impaired epithelial morphogenesis (Levayer, Pelissier-Monier et al. 2011), suggesting that dynamic regulation of cadherin endocytosis is important for proper cell migration. In this study, we utilized a more specific approach to inhibit E-cadherin endocytosis by mutating the dileucine motif to examine its effect in cell migration. Indeed, our data suggest that inhibiting E-cadherin directly through dileucine mutation can effectively reduce migration

in various cell types including skin epithelial cells (A431D and HK) and lung cancer cells (H1299) (Figure 4-2 and 4-3).

As mentioned before, one of the biggest challenges in studying cell migration is to relate the 2-D *in vitro* models to *in vivo* conditions which have diverse multicellular composition and organization (Friedl, Locker et al. 2012). Having identified the key endocytic motifs in both VE-cadherin and E-cadherin, we can now address this question by specifically disrupting E-cadherin endocytosis in various *in vitro* or *in vivo* models that can better relate to actual *in vivo* conditions. The spheroid invasion assay is a well-established *in vitro* model that utilizes 3-D Matrigel to better mimic cancer metastasis *in vivo* (Yang, Garcia et al. 2013, Konen, Wilkinson et al. 2016). Based on the observations that E-cadherin dileucine mutants inhibit 2-D cell migration, we speculated that this mutant can also inhibit spheroid invasion in a 3-D environment. However, to our surprise, the result showed the opposite effect. The E-cadherin dileucine mutant promoted spheroid invasion rather than inhibiting invasion (Figure 4-4). Though unexpected, this result is an example of how 2-D monoculture migration studies can differ dramatically from three-dimensional culture systems. Spheroid invasion typically involves the formation of multiple protrusions created by “leader” cells, which drag a group of “follower” cells to initiate the invasion process (Konen, Wilkinson et al. 2016). Therefore, it is possible that with E-cadherin dileucine mutants, the adhesion between leader cells and follower cells is strengthened due to lack of endocytosis. This stronger adhesion between invasive leader cells and follower cells leads to increased invasion ability of the cells as a collective. It is likely that the mechanisms involved in 2-D migration and 3-D spheroid invasion are different. One possibility is that the movement of invasive leader cells in 3-D matrix is mainly mediated

by cell-matrix adhesion rather than intercellular adhesion. In this case, strong intercellular adhesion is beneficial to forming an effective leader-follower cell invasion. On the other hand, 2-D collective migration requires dynamic modulation of intercellular adhesion.

In addition to cancer metastasis, another *in vivo* cell migration model involving dynamic regulation of cell adhesion is keratinocyte differentiation. Keratinocytes are skin epithelial cells that form a layered 3-D structure (Houben, De Paepe et al. 2007). During terminal differentiation, the epidermal keratinocytes undergo a series of programmed cellular events which allows them to become part of the cornified layer of keratinized "inactive" corneocytes from the basal layer of "active" keratinocytes (Abhishek and Palamadai Krishnan 2016). This process involves not only differentiation of keratinocytes in terms of protein expression profile, but also movement of "younger" keratinocytes towards the top of the epidermis. To date, very little is known about what roles cadherin endocytosis may play in keratinocyte differentiation. Our prediction is that by inhibiting E-cadherin endocytosis, regulation of cell adhesion will be disrupted, which will result in failure of keratinocytes to move upwards and eventually incomplete keratinocyte differentiation. This has yet to be tested experimentally. With the E-cadherin dileucine mutant, which specifically disrupts cadherin endocytosis, future studies can be conducted by knocking in this mutant E-cadherin into either *in vitro* or *in vivo* keratinocyte differentiation models. This approach will allow for a direct way to test the role of cadherin endocytosis in keratinocyte differentiation.

In summary, our findings suggest a context-dependent effect of E-cadherin endocytosis in cell motility and migration. Future studies utilizing the dileucine mutant of E-cadherin

developed in this study will be required to further understand the role of cadherin endocytosis in important biological processes including cancer metastasis, as well as normal epithelial cell morphogenesis and differentiation.

4.4 Material and methods

Cell Culture

The African green monkey kidney fibroblast-like (COS7, ATCC), HEK293T cell lines (MP Biomedicals) and human squamous carcinoma cell line (A431D) were cultured in Dulbecco's modification of Eagle's medium (Corning, 10-013-CV) supplemented with 10% FBS (HyClone, GE Healthcare, SH30071) and 1% antibiotic antimycotic solution (Corning, 30-004-CI). Human non-small cell lung carcinoma cell line (H1299) were cultured in Roswell Park Memorial Institute medium (RPMI, Corning, 10-040) supplemented with 10% FBS (HyClone, GE Healthcare, SH30071) and 1% antibiotic antimycotic solution (Corning, 30-004-CI). Primary human keratinocytes are cultured in keratinocyte growth medium-2 (KGM-2, Lonza, CC-3103) supplemented with KGM-2 SingleQuot Kit Suppl. & Growth Factors (Lonza, CC-4152).

To generate stable cell lines, A431D and H1299 cells were infected with the lentivirus expressing protein of interest then selected in the presence of blasticidin (ThermoFisher, R21001) for 7 days to ensure high percentage of protein expression.

cDNA constructs

To generate the dileucine mutation, site-directed mutagenesis was performed using E-cadherin construct in pcDNA3 vector. Primers used were as follows: (Forward primer) 5'-GTG GTC AAA GAG CCC GCA GCA CCC CCA GAG GAT GAC-3', (Reverse primer) 5'-GTC ATC CTC TGG GGG TGC TGC GGG CTC TTT GAC CAC-3'. PCR products were subjected to digestion by DpnI to remove the methylated template plasmids, followed by transformation into competent E. Coli strain DH5α (ThermoFisher, 18265017).

Virus Production

To generate lentivirus for protein expression in mammalian cells, the gene of interest was cloned into the gateway pLenti6/V5-DEST vector (ThermoFisher, V49610). This vector, together with two other plasmids (pSPAX2 and pMD2.G) were transfected into HEK293T cells using $CaCl_2$ to produce virus. After 72 hours, supernatants were collected and concentrated by ultracentrifuge at 20,000g. Virus were then resuspended in PBS with 1% BSA.

Internalization assay and Image Analysis

H1299 expressing protein of interest were seeded onto glass coverslips. In the case of COS7 cells, cells were transiently infected with lentivirus expressing protein of interest for 72 hours prior to the experiment. Internalization assay was performed as described previously (Chapter 4.4). Briefly, cultured cells on glass coverslips were incubated with an antibody against the E-cadherin extracellular domain (HECD-1, Abcam, ab1416) in cell culture medium for 30 minutes at 4 °C. To allow internalization, cells were incubated in prewarmed medium at 37 °C for 30 minutes then returned to cold medium followed by a low-pH buffer (100mM glycine, 20mM magnesium acetate, and 50mM potassium chloride (pH 2.2)) wash to remove any remaining antibody from the cell surface. Cells were then fixed and permeabilized by incubation in 4% paraformaldehyde for 10 minutes, followed by 0.1% Triton X-100 for 10 minutes at room temperature. C-terminal RFP tag was used to visualize the total pool of E-cadherin. To quantify internalization, signals from internalized E-cadherin were divided by signals from the total pool of E-cadherin, which was performed in ImageJ/Fiji.

Scratch Wound Migration Assay

Cells expressing protein of interest were seeded onto 4-well cell culture plates. Scratch wound assay was performed as described before (Nanes, Chiasson-MacKenzie et al. 2012). Briefly, cells were scratched manually with a p200 pipette tip, followed by incubation at 37^0C for varying amounts of time. For quantification, the wound was outlined manually then the area was measured using ImageJ/Fiji and normalized to the size of the image. Then the difference of the wound size between each time point was calculated.

Spheroid Invasion Assay

Spheroid invasion assay was performed by Jessica Konen from the Marcus lab as described before (Konen, Wilkinson et al. 2016). Briefly, H1299 cell spheroids expressing protein of interest were resuspended in collagen 3-D matrix with added culture media. Cells were imaged live after 21hours.

Figures

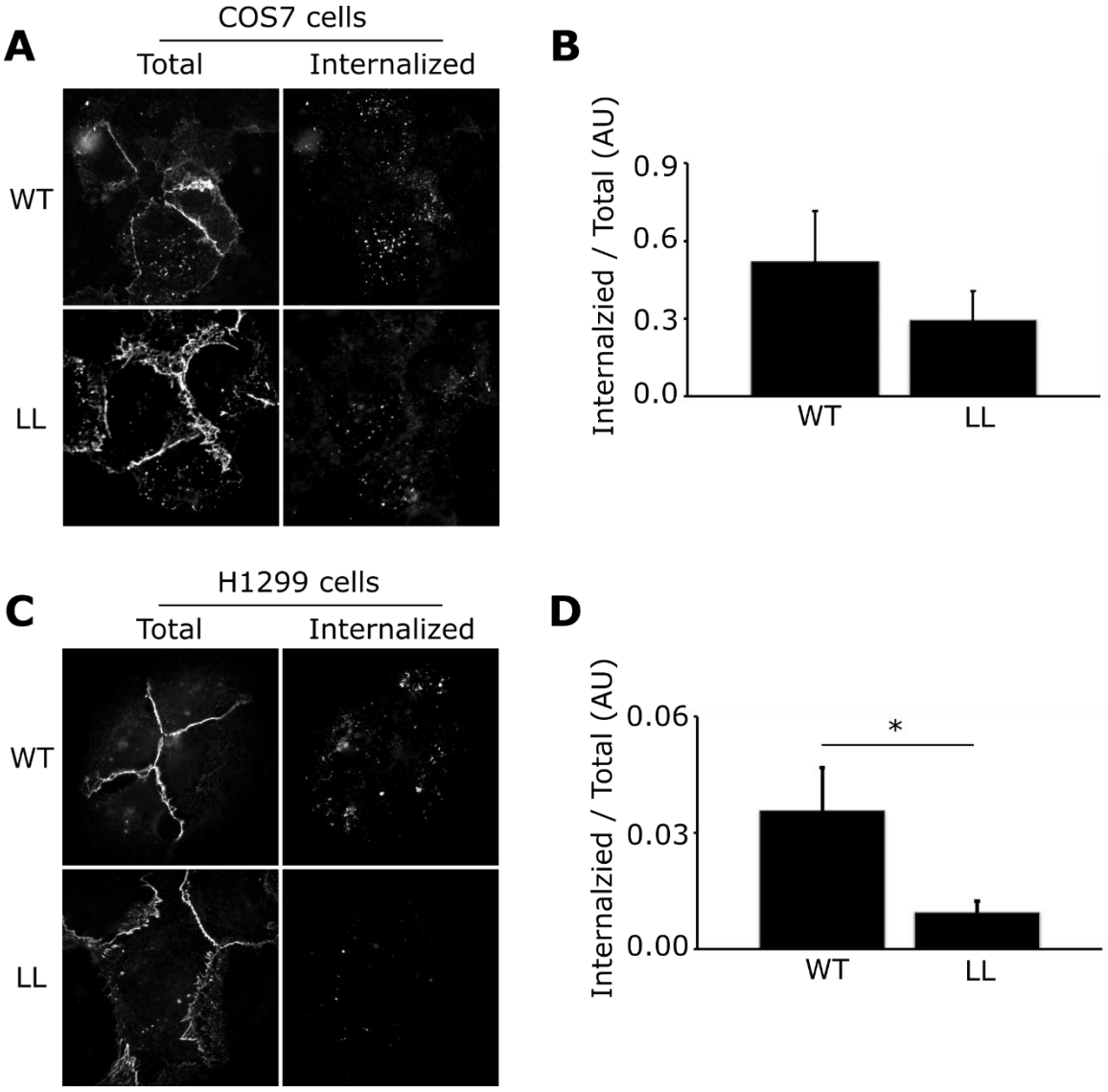


Figure 4-1. Dileucine motif (LL) is required for E-cadherin endocytosis. (A) E-cadherin dileucine mutant exhibits reduced endocytosis when compared to wild type E-cadherin in COS7 cells. An internalization assay was performed using HECD-1 antibody to follow E-cadherin during endocytosis. Total E-cadherin was detected using C-terminal RFP tag. (B) Quantification of the results in panel A. Data are presented as the means ± SEM. N ≥ 7 in each group. (C) E-cadherin dileucine mutant shows a significant reduction

in endocytosis rate compared to wild type E-cadherin in H1299 cells. An internalization assay was performed as described in A. (B) Quantification of the results in panel C. Data are presented as the means ± SEM. $N \geq 5$ in each group. *$p \leq 0.05$, *one tailed t*-test.

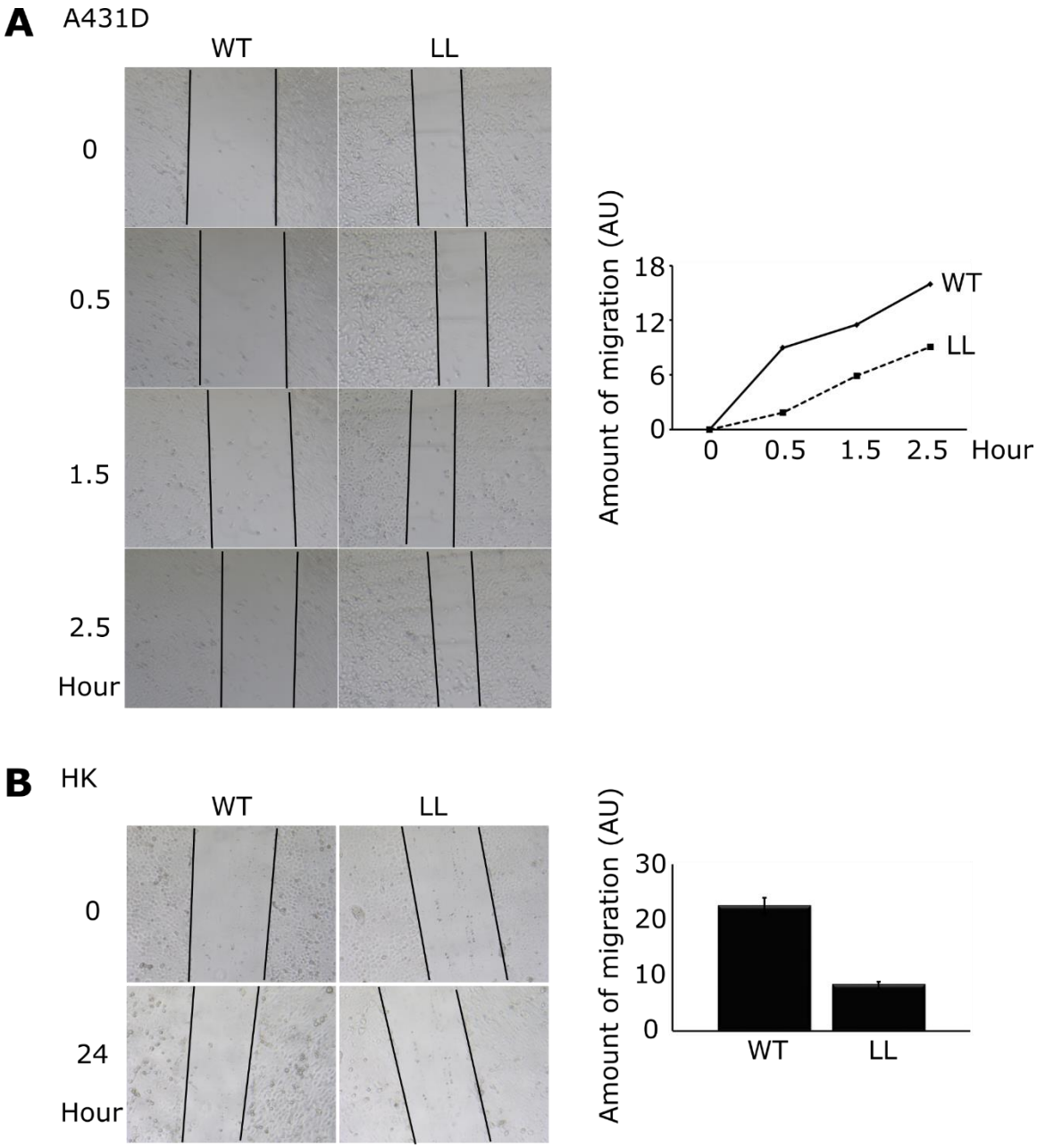


Figure 4-2. Dileucine mutant of E-cadherin inhibits collective cell migration in both A431D and primary epithelial cells. (A) A431D cells expressing wild type or LL mutant E-cadherin were scratched and imaged at different time points. Quantification of migration is shown on the right. Data are presented as mean at different time points. $N \geq 4$ in each group. (B) primary human keratinocytes (HK) were infected with lentivirus expressing

wild type or LL mutant E-cadherin. A scrape wound assay was perfumed and samples were imaged at different time points. Quantification of migration is shown on the right. Data are presented as mean ± SEM. $N \geq 8$ in each group.

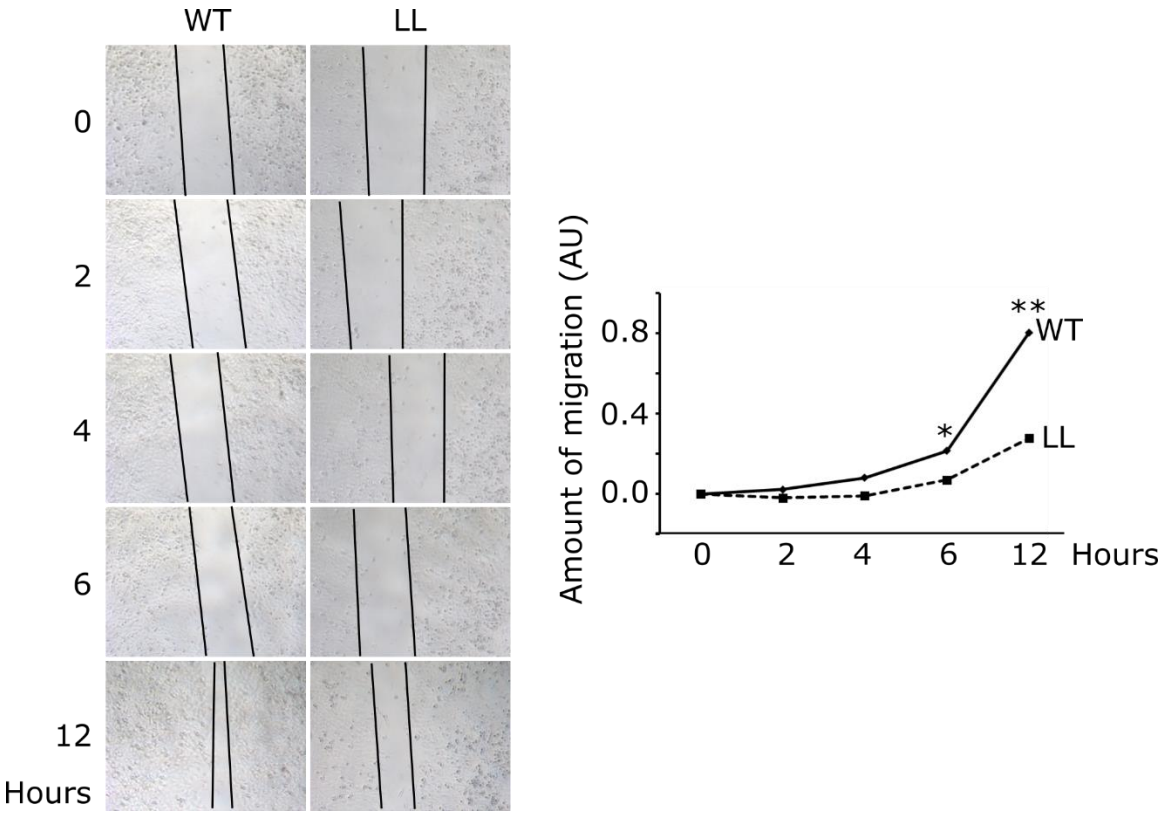


Figure 4-3. Dileucine mutant of E-cadherin inhibits collective cell migration in lung cancer cells. H1299 cells expressing wild type or LL mutant E-cadherin were scratched and imaged at different time points. Quantification of migration is shown on the right. Data are presented as mean at different time points. N ≥ 18 in each group. *$p \leq 0.05$, **$p \leq 0.01$ *two tailed t*-test.

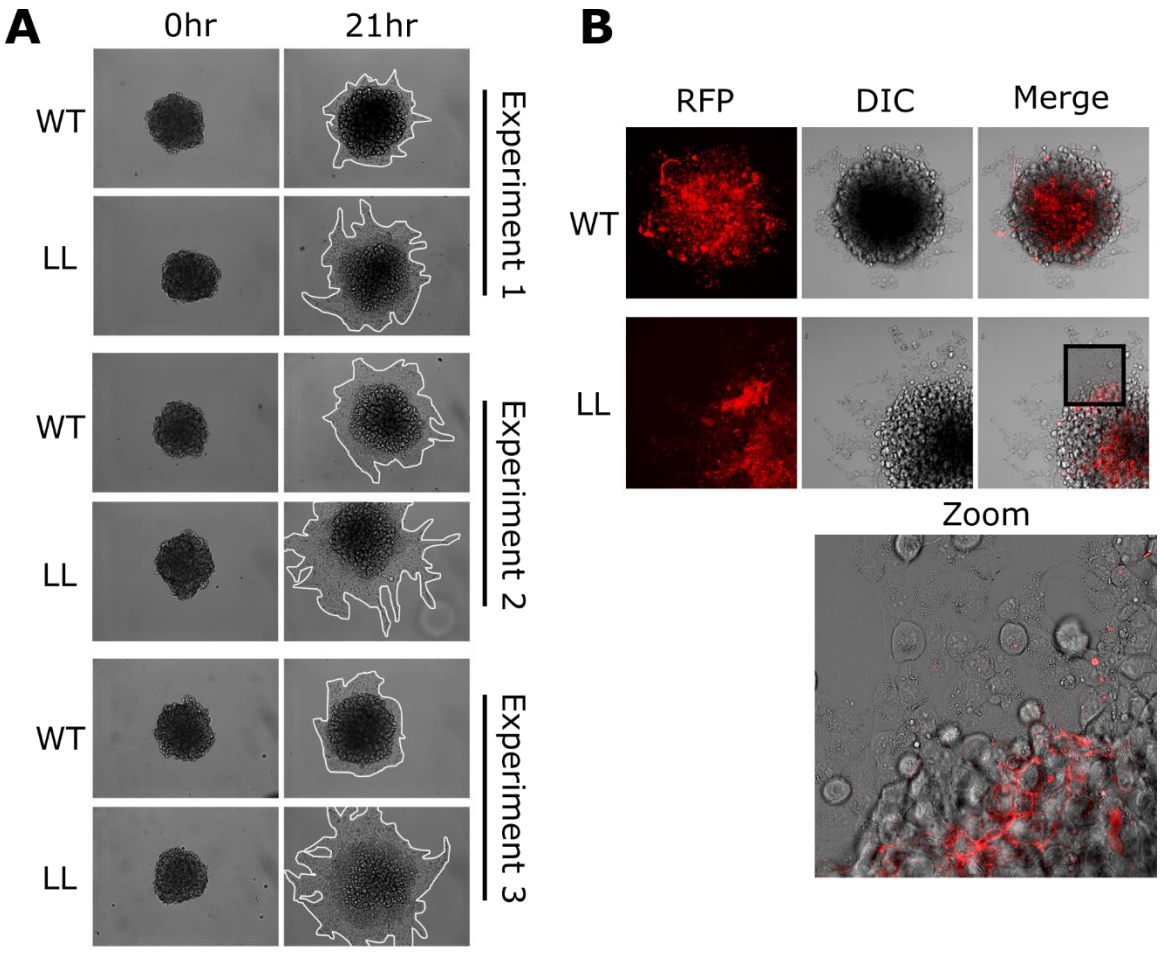


Figure 4-4. Dileucine mutant of E-cadherin promotes spheroids invasion when compared to the wild type cadherin (In collaboration with Jessica Konen from Marcus lab). H1299 cells expressing RFP-tagged WT or LL mutant E-cadherin were seeded onto a 3-D Matrigel. Spheroid invasion assay was performed. (A) Cells expressing LL E-cadherin showed increased invasive ability when compared to the wild type cadherin. Experiments were repeated three types with representative images shown from each repeat. Edges of each spheroid are outlined in white. (B) Merged fluorescence and DIC imaging show relatively less cadherin expression at the leading spikes. Detailed look of the invading cells from LL mutant group is shown at the bottom.

Chapter 5

Dissertation summary and future direction

5.1 Summary of findings

Modulation of cell adhesion is essential during development and disease. Dynamic regulation of cadherin trafficking is thought to be one of the major mechanisms to modulate cell adhesion (Cadwell, Su et al. 2016). The importance of the cytoplasmic tail in regulating cadherin trafficking has been extensively studied and is well established. The unstructured organization of the cadherin tail and its large interacting network of binding partners leads to versatility in biological function that can be modulated by post-translational modifications. To date, the possible mechanisms that modify the cadherin tail, which can potentially modulate cadherin function, have not yet been fully explored. The overall goal of this dissertation is to advance our understanding of how the cadherin tail plays roles in regulating cadherin trafficking and the biological consequences of the altered cadherin trafficking. This overall objective was approached through two specific questions: (1) How does a proteolytic event that cleaves in the VE-cadherin cytoplasmic domain regulate cadherin trafficking? (2) What effect does inhibiting cadherin endocytosis have in different model systems? These questions were addressed in chapter 3 and 4. The findings presented in chapter 3 are summarized as follows:

1. By using a combination of protein electrophoresis and fluorescence imaging analysis, I demonstrated that the VE-cadherin tail is cleaved upon entrance into clathrin-enriched membrane domains before reaching the early endosome, and this cleavage occurs between the juxtamembrane domain and the catenin-binding domain of VE-cadherin.
2. Drug treatment and *in vitro* enzyme digestion assays suggested that VE-cadherin is cleaved by calpain, a calcium-dependent cysteine protease.

3. By utilizing a construct that resembles the N-terminal fragment of VE-cadherin after cleavage, I found that the cleaved fragment exhibits a higher turnover rate when compared to the full length cadherin. Interestingly, the cleaved fragment is endocytosed at a similar rate as the full length cadherin, but has a decreased recycling rate, implied by reduced colocalization with the recycling markers. In addition, this fragment exhibits increased colocalization with lysosomal markers, suggesting that its subjected to faster degradation than the full length cadherin.

Together, these findings suggest that cleavage of VE-cadherin tail by calpain alters the post-endocytic trafficking itinerary of VE-cadherin.

Other than calpain cleavage, the cadherin tail also was found to harbor multiple motifs that mediate cadherin endocytosis. In chapter 4, I focused on a well-characterized endocytic motif, the dileucine residues, in the E-cadherin cytoplasmic domain to examine how cadherin endocytosis affects cell mobility in different model systems. The findings in this chapter demonstrate:

1. Mutation of the dileucine motif to alanine, which indeed inhibits E-cadherin endocytosis, reduces collective cell migration when measured by a 2-D migration assay, in both normal epithelial cells and lung cancer cells.
2. Disrupting cadherin endocytosis by the dileucine mutation, instead of inhibiting cell migration as suggested by 2-D migration study, promotes cancer cell invasion in a 3-D spheroid invasion assay. This result suggests a context-dependent function of cadherin endocytosis in different biological systems.

To conclude, my studies in this dissertation not only demonstrated a novel mechanism by which cytoplasmic tail modifications can regulate cadherin trafficking, but also suggested cadherin endocytosis could have different roles in different biological systems. These findings contribute to a better understanding of the role of the cytoplasmic tail in cadherin trafficking and how cadherin trafficking affects cell mobility. However, these insights lead to more questions that will be important for a deeper understanding of cadherin and cell adhesion in general, which will be discussed in the section to follow.

5.2 Remaining questions and future directions

What upstream signaling events activate calpain to cleave VE-cadherin?

Calpain is a calcium-dependent cysteine protease. Under normal conditions, calpain remains inactive by forming a complex containing the large and small subunits of calpain, and, in most cases, calpastatin. In the past decades, extensive studies have been done on calpain activation mechanisms. To date, the mechanisms for calpain activation *in vivo* are believed to involve the following: (1) An increase of intracellular calcium levels beyond the activating threshold of calpain. (2) Binding of phospholipids that facilitates calpain activation, which under *in vivo* conditions requires calpain to localize to the plasma membrane. (3) Phosphorylation of calpain and calpastatin disrupts calpain-calpastatin binding and thereby increases calpain activity. Among these requirements, plasma membrane localization can be achieved if the substrate protein interacts with other proteins in proximity of the plasma membrane. Findings presented in chapter 3 suggest that VE-cadherin is cleaved upon entrance into the clathrin-enriched membrane domain. In this case, active calpain that associates with plasma membrane can indeed cleave VE-cadherin at the cell surface.

Past studies have identified a number of potential upstream signaling events that activate calpain, including calcium influx and phosphorylation of calpain (Sandoval, Malik et al. 2001, Letavernier, Perez et al. 2008, Scalia, Gong et al. 2011, Smolock, Mishra et al. 2011, Komarova, Huang et al. 2012, Sheng, Leshchyns'ka et al. 2013). However, the molecular mechanisms of how these signals lead to calpain activation are not fully understood. Upstream signals that regulate calpain activity remain an open question that requires more

investigation in the future. In order to identify new upstream calpain activation signals, pathways that link to regulation of intracellular calcium levels and protein phosphorylation in particular, are main areas in need of study. For example, vascular endothelial growth factors and signaling proteins involved in apoptosis could be considered.

What amino-acid sequences in the VE-cadherin tail are required for calpain cleavage?

Rather than recognizing a specific amino acid sequence, calpain cleavage specificity is dependent upon protein tertiary structure with a preference to inter-domain regions that are open and flexible (Goll, Thompson et al. 2003). In the case of cadherin, several reports have suggested that the cleavage site lays between the juxtamembrane domain and the catenin-binding domain. Studies from Jang et al. suggested four potential calpain cleavage sites in N-cadherin cytoplasmic tail, mostly in between the juxtamembrane and catenin-binding domain (Jang, Jung et al. 2009). Another study on E-cadherin showed that calpain cleavage of E-cadherin produces a fragment containing the juxtamembrane but lacking the catenin-binding domain (Rios-Doria, Day et al. 2003). Interestingly, this fragment, though containing the juxtamembrane region, does not bind to p120 (Rios-Doria, Day et al. 2003). They also found that by removing six amino acids at amino acid location 782 (VTRNDV), which is between the juxtamembrane and catenin-binding domain, calpain cleavage of E-cadherin is inhibited (Rios-Doria, Day et al. 2003). A similar observation was made in VE-cadherin that removal of five amino acids between the juxtamembrane and catenin-binding domain (^{691}PRHAP) inhibits calpain cleavage (Miyazaki, Taketomi et al. 2011). However, in my attempt to map the VE-cadherin cleavage site, removal of ^{691}PRHAP only showed

partial inhibition of cadherin cleavage by fluorescence imaging (Chapter 3, Figure 3-11) but not by *in vitro* calpain digestion assay (Data not shown).

So far, the exact site for calpain cleavage in various cadherin has yet been conclusively identified. However, studies on this subject all reach a consensus that the cleavage site is between juxtamembrane and catenin-binding domain. Structural studies of E-cadherin showed that there is a linker sequence between the juxtamembrane and catenin-binding domain (Ishiyama, Lee et al. 2010), which is likely the target of calpain cleavage. Further studies will be required to map out the cleavage site, keeping in mind that the tertiary structure of this linker sequence between juxtamembrane and catenin-binding domain likely plays a crucial role. The most direct method is to mutate the sequence, by either removing or switching amino acids in the linker region. One possibility is that the degree of flexibility or accessibility of the linker region is important to the efficiency of calpain cleavage. If this is the case, we predict that by reducing the length of the linker sequence, which brings the juxtamembrane domain and the catenin-binding domain closer together, calpain cleavage can be inhibited. On the other hand, adding more amino acids to this linker region can potentially increase the efficiency of calpain cleavage. Potential calpain cleavage cadherin mutants can then be verified by *in vitro* or *in vivo* calpain digestion assay. Cadherin mutants with reduced or increased calpain cleavage efficiency will become a very useful tool for future studies on cadherin function and cell adhesion in general as these tools would be helpful in testing the biological function of calpain cleavage in different model systems such as angiogenesis.

What biological function could calpain cleavage of cadherin have other than altering cadherin trafficking?

Cadherin not only mediates cell adhesion, but also is involved in various signaling pathways through its interacting partners. One notable example is β-catenin, which is a key member of the Wnt signaling pathway. Binding of β-catenin to cadherin was shown to sequester β-catenin at the junction thus inhibiting the Wnt pathway (Fagotto 2013). Cleavage of VE-cadherin was shown to remove the catnin-binding domain in cadherin (Chapter 3) (Xiao, Allison et al. 2003), which could potentially affect the distribution of β-catenin in the cell.

Two approaches can be taken to identify the biological function of calpain cleavage. One is to use a cadherin truncation mutant that mimics the cleaved form of cadherin. This method was used in this dissertation to reveal that calpain cleavage alters VE-cadherin trafficking (Chapter 3). Likewise, expression of the C-terminal fragment of cadherin could also reveal novel functions for calpain cleavage of cadherins. Studies in this dissertation (Chapter 3) and from the past (Rios-Doria, Day et al. 2003, Jang, Jung et al. 2009, Miyazaki, Taketomi et al. 2011) have suggested that the C-terminal fragment from calpain cleavage contains the catenin-binding domain. It would be interesting to know if this fragment continues to bind to β-catenin once released from full-length cadherin. Binding of the C-terminal fragment to β-catenin might either facilitate or prevent translocation of β-catenin into the nucleus, a key step of Wnt signaling. There are multiple approaches that could be taken to determine how calpain cleavage and the release of the catenin binding domain might alter cell signaling. By over-expressing the C-terminal fragment, immunoprecipitation could be used to test if β-catenin binds to the free cytosolic C-terminal

cadherin fragment. Also, over-expression of the C-terminal fragment could be used to visualize the distribution of this fragment. If this fragment co-localizes with β-catenin and accumulates inside the nucleus, it's likely that the fragment facilitates the translocation of β-catenin into nucleus. In addition, Wnt pathway reporter assays could be used to examine if the Wnt pathway is indeed activated.

Another way to test how calpain cleavage affects cadherin function could be achieved by using a calpain-resistant cadherin mutant. This requires prior knowledge of the calpain cleavage site in cadherin, which was discussed in the section above. With this mutant, various *in vitro* and *in vivo* assays could be used to examine the function of calpain cleavage, potentially over longer time frames. For example, stable cell lines that express cleavage resistant VE-cadherin could be generated and used with the electrical cell-substrate impedance sensor (ECIS) method to determine the effect on endothelial permeability. Murine models expressing the mutant cadherin could also be generated and assay for physiological effects of the cleavage resistant cadherin such as vasculogenesis and angiogenesis, and potential embryonic or growth defects.

To date, not much is known about the function of cadherin cleavage by calpain except that it leads to junction disassembly. It is likely that the cleavage has additional biological consequences, given the importance of cadherin and catenin in development and disease. Further investigation will be required to advance our understanding of this subject.

What role does E-cadherin endocytosis play in epidermal differentiation?

We predicted that E-cadherin endocytosis, which is important in the dynamic regulation of epithelial adherens junction, is required for proper epidermal differentiation. To test this hypothesis, we generated a mutant E-cadherin that exhibits reduced endocytosis (E-cadherin LL). Epidermal differentiation is a long-term process that requires weeks of development. To help achieve this, a lentivirus was made to express the E-cadherin LL mutant which allows us to infect primary keratinocytes that can be used for epidermal differentiation assay (Arnette, Koetsier et al. 2016). One way to examine the function of E-cadherin endocytosis in epidermal differentiation is to use 3D organotypic culture, which is one of the best characterized *in vitro* model for studying epidermal differentiation (Arnette, Koetsier et al. 2016). Based on findings supporting the model that VE-cadherin and E-cadherin endocytosis are required for collective cell migration (chapter 4) (Nanes, Chiasson-MacKenzie et al. 2012), we predicted that keratinocytes expressing E-cadherin dileucine mutant would exhibit slow or abolished epidermal differentiation. In an effort to test this prediction, we collaborated with Dr. Spiro Getsios at Northwestern University, whose lab has expertise in studying epithelial biology using the 3D organotypic culture (Getsios, Simpson et al. 2009, Arnette, Koetsier et al. 2016). We provided the Gatsios Lab with lentivirus to infect keratinocytes to express the E-cadherin dileucine mutant. So far, this study is still ongoing. At a larger time scale, a knock in mouse model with E-cadherin dileucine mutant can be an ultimate experiment to test its effect in epidermal differentiation.

In addition to modulating adhesion, remodeling of E-cadherin based adherens junctions is important in several other biological processes including when epithelia change their overall phenotypes to either squamous or columnar (Grammont 2007, Kolahi, White et al.

2009, St Johnston and Sanson 2011, Coopman and Djiane 2016). E-cadherin endocytosis could play an essential role in reestablishing apical-basal polarity during these processes and the E-cadherin LL mutant could be a useful tool to address these questions. Nonetheless, the study of E-cadherin endocytosis in tissue morphogenesis is just at the beginning and further investigation is required.

How does E-cadherin endocytosis affect cancer metastasis?

There are many misregulated pathways in metastasizing cancer cells, and loss of adhesion is often one of them. Studies from our lab and many others have suggested that cadherin endocytosis is critical in modulating cell adhesion. Therefore, it is natural to hypothesize that cadherin endocytosis could play an important role during cancer metastasis. In collaboration with Dr. Adam Marcus, our data (Chapter 4) indicated a model that the cadherin endocytic mutant provides a strong adhesion between cancer cells. This strong adhesion facilitates the pulling of follower cells by leader cells to start the invasion process. We found that increased cell adhesion promotes cancer cell invasion, which opens more questions that need to be tested experimentally. One way is to use other methods to modulate cadherin adhesive strength such as using the Trp2 mutant cadherin, which lacks trans-dimerization (Cadwell, Jenkins et al. 2016), to test the effect of adhesion on cancer cell invasion. Our prediction is that with reduced adhesion, cancer cell invasion will be inhibited. In addition, overexpression of p120, which leads to a more stable adherens junction (Chiasson, Wittich et al. 2009), can also be utilized to study cancer invasion. The prediction would be that with p120 overexpression, cancer cell invasion will be enhanced.

It is also possible that cell adhesion plays different role at different stages of cancer metastasis. Our data indicated that strong adhesion can promote initial invasion by cancer cells, but it is likely that when transiting into a later stage where cancer cells migrate individually in the organism, the ability of cancer cells to detach from each other becomes important, which requires loss of adhesion. This hypothesis could be tested by orthotopic inoculation of tumor cells into murine models followed by examining the progression of cancer (Koya, Kajiyama et al. 2016). These tumor cells could be genetically manipulated to express either relatively stable adherens junction such as the E-cadherin dileucine mutant, or less adhesive adherens junctions such as the Trp2 mutant cadherin. The prediction is that different adhesive strength of cadherin could result in different metastatic patterns. For example, cell expressing dileucine mutant E-cadherin could lead to a highly protrusive tumor, but fewer individually invasive cancer cells. On the other hand, cells expressing the Trp2 mutant cadherin could lead to small and restricted tumor growth but highly invasive pool of individual cancer cells.

In the end, there are still a lot of questions remain unanswered in the role of cadherin-based cell adhesion in important biological processes, including tissue morphogenesis and cancer progression. Moreover, the mechanisms of cadherin regulation still require further clarification. The work in this dissertation contributes to our understanding of cadherin regulation and function, and future investigation is required to continue exploring this subject.

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