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Galectin-3 and Annexin A2 regulate the stability of cell surface proteins to control epithelial barrier function

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An abstract of a dissertation submitted to the Faculty of the
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Abstract

Galectin-3 and Annexin A2 regulate the stability of cell surface proteins to control epithelial barrier function

By Carl Robert Rankin

The gastrointestinal epithelium functions as an important barrier that separates luminal contents from underlying tissue compartments thereby maintaining mucosal homeostasis. Mucosal wounds in inflammatory disorders compromise the critical epithelial barrier. In response to injury, intestinal epithelial cells (IECs) remodel cell matrix adhesions and intercellular junctions as they proliferate and migrate to cover denuded surfaces. AnnexinA2 (AnxA2) and Desmoglein-2 (Dsg2) proteins have been reported to regulate wound closure and epithelial barrier function. In Chapter 2 I identify a mechanism by which AnxA2 controls IEC migration and wound closure. These studies revealed that AnxA2 controls IEC migration by regulating endocytosis of β1 integrin and turnover of cell-matrix adhesions. In Chapter 3 I identify the association of a glycan binding protein, Galectin-3, with an intercellular junction protein, Dsg2, in intestinal epithelial cells. Galectin-3 stabilizes Dsg2 at the cell surface thereby influencing intercellular adhesion and intestinal epithelial barrier function.

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Figures and Tables

CHAPTER 1

MOLECULAR MECHANISMS OF INTESTINAL EPITHELIAL BARRIER REGULATION AND REPAIR

1.1 Organization and function of the intestinal epithelium

The lining of the intestinal tract consists of a single layer of columnar epithelial cells that is organized in crypt-like structures **(Fig. 1)**. The epithelium functions as an important barrier that differentially regulates absorption of nutrients and fluids while excluding unwanted luminal antigens and bacteria. Intestinal epithelial cells (IECs) are actively renewed in less than five days depending on their location in the gut axis (Tsubouchi, 1981; Wiernik et al., 1962). Stem cells reside in the crypt base and generate daughter cells that migrate along the crypt-luminal axis to be eventually shed into the lumen. Thus, loss of this critical epithelial barrier is associated with exposure of underlying mucosal tissue to luminal antigens thus contributing to the pathogenesis of inflammatory diseases.

1.2 Intestinal epithelial wound closure

Epithelial injury resulting in mucosal wounds is observed in inflammatory disorders and following mechanical injury (Mizukami et al., 2011; Williams, 2001). The intestinal epithelium has a remarkable capacity to repair mucosal wounds (Buczacki et al., 2013; Lotz et al., 1997; Mammen and Matthews, 2003; Seno et al., 2009). Epithelial cells adjoining wounds proliferate and migrate as a sheet to rapidly cover denuded surfaces and repair the barrier defects **(Fig. 2A,B)** (Horwitz and Hunter, 1996; Lauffenburger and Horwitz, 1996). In chronic inflammatory disorders, such epithelial wound closure is perturbed resulting continued mucosal inflammation (Schmitz et al., 2000). In Chapter 2 of this thesis I will explore mechanism by which an epithelial

expressed protein referred to as Annexin A2 (AnxA2) that is up-regulated in the injured epithelium (Babbin et al., 2007), promotes IEC migration and wound closure.

1.3 Annexin A2

Annexins are a family of calcium and phospholipid binding proteins (Gerke et al., 2005; Gerke and Moss, 2002) that associate with membranes by forming a ternary complex consisting of Annexin, calcium and negatively charged membrane phospholipids (Marriott et al., 1990). The carboxy terminus of all Annexins contains phospholipid and calcium-binding sites. However, the sequence and structure of the amino terminus differs between annexins enabling differential binding of family members to distinct cytoplasmic proteins (Avila-Sakar et al., 1998; Geisow, 1986). Annexins have been reported to promote membrane aggregation and fusion in addition to participating in membrane repair (Emans et al., 1993; Mayran et al., 2003; Morel and Gruenberg, 2009; Zibouche et al., 2008). A unique amino terminal actin-binding domain of AnxA2 binds and caps F-Actin thereby providing a link between the actin cytoskeleton and plasma membrane (Hayes et al., 2004; Hayes et al., 2006; Hayes et al., 2009). In addition, AnxA2 has been reported to regulate a number of biological processes that include endocytosis and exocytosis (Futter and White, 2007; Senda et al., 1994; Uittenbogaard et al., 2002).

1.4 Intestinal epithelial cell migration

The epithelium adheres to matrix proteins in epithelial cell base that play an important role in regulating cell migration. Central to cell-matrix adhesion and migration are

transmembrane integrins that provide a link between the matrix and intracellular scaffold, signaling and cytoskeletal proteins. Thus, integrins play a central in providing the traction force required for forward cell movement (Huttenlocher et al., 1996). Cycles of polarized cell-matrix adhesion and de-adhesion, mediated by the assembly and disassembly of focal contacts is needed for forward cell movement (Larsen et al., 2006) **(Fig. 3)**. In addition to focal complexes the matrix is remodeled during cell migration and signals emanating from matrix-integrin adhesions control cell movement (Hocking et al., 2000). Thus, integrin based adhesion protein complexes serve as important mechanical sensors and signal transducers to orchestrate remodeling of the cytoskeleton and the extracellular matrix in migrating cells.

The structural core of transmembrane integrins in adhesions consist of α and β subunit heterodimers **(Fig. 4)**. IECs have been reported to express multiple α (1,2,3,5, and 6) and β (1,4,5, and 6) integrin subunits (Croyle et al., 1998; Lotz et al., 1997). In IECs β1 integrin controls cell-matrix adhesion and migration (Lotz et al., 1997). This subunit associates with different alpha integrins to mediate the biological responses in different cell types including epithelial cells (Margadant et al., 2011). Recent studies have demonstrated that integrins can reside with cholesterol glycosphingolipid-rich membrane microdomains also referred to as membrane rafts (Leitinger and Hogg, 2002; Vassilieva et al., 2008). Integrin attachment to the ECM is regulated by both extracellular and intracellular cues. For example, divalent cations and adaptor proteins such talin regulate integrin attachment to the ECM (Anthis et al., 2009; Tadokoro et al., 2003). Upon binding to the matrix, integrins undergo a conformational change that initiates signaling events including phosphorylation of kinases and adaptor proteins that in turn remodel

focal adhesions and the actin cytoskeletal required for cell movement (Burridge et al., 1992; Tremblay et al., 1996).

Integrins undergo internalization, endocytic trafficking and recycle back to the plasma membrane during remodeling of focal adhesions in migrating cells. (Caswell and Norman, 2006; Riggs et al., 2012; Roberts et al., 2004; White et al., 2007). In some cell types, endocytic recycling is rapid with internalization occurring within minutes to hours (Dukes et al., 2011; Rankin et al., 2013). Epithelial β1 integrin is internalized in membrane rafts (Shi and Sottile, 2008; Vassilieva et al., 2008). After internalization from the plasma membrane, integrins are targeted to distinct endocytic compartments. After internalization from the cell surface, β1 integrin containing vesicles first fuse with early endosomes containing Rab5 GTPase and Early Endosome Antigen 1 (EEA1). Subsequently, integrins traffic to Rab11 GTPase-containing recycling endosomes that are targeted back to new cell-matrix adhesions or to lysosomes for degradation **(Fig. 5)**.

1.5 Intestinal epithelial intercellular adhesion and desmosomes

A series of intercellular junctions mediate epithelial cell-cell adhesion required for barrier function. Intestinal epithelial intercellular junctions include an apical tight junction and subjacent adherens junction (AJ) and desmosomes (DM) (Chitaev and Troyanovsky, 1997; Rafei et al., 2011; Schlegel et al., 2010). AJ and DM proteins not only mediate intercellular adhesion but have also been reported to regulate epithelial proliferation and apoptosis (Kamekura et al., 2013; Kim et al., 2011; Lowy et al., 2002; Nava et al., 2007). Analogous to focal contacts, transmembrane proteins in intercellular

junctions associate with intracellular scaffold and cytoskeletal proteins, and are remodeled during epithelial migration and wound closure.

Key transmembrane proteins in epithelial DMs are cadherins referred to as desmoglein(s) and desmocollin(s). Human intestinal epithelial cells exclusively express Desmoglein 2 (Dsg2) and Desmocollin 2 (Dsc2) isoforms (Holthofer et al., 2007). Homophilic as well as heterophilic Dsg2 and Dsc2 interactions in the extracellular space mediate intercellular adhesion (Schlegel et al., 2010; Syed et al., 2002). Plakin and armadillo family of adaptor proteins provide a link between DM cadherins and intermediate filaments that further strengthens intercellular adhesion **(Fig. 6)** (Delva et al., 2009). In Chapter 3 of this thesis I demonstrate a role of a lectin, Galectin-3 (Gal-3) in controlling Dsg2 mediated IECs intercellular adhesion.

Cadherins consist of four to five eighty amino acid extracellular repeat (EC) domains. The distal most EC domain mediates cadherin association between cells. The cadherin association is mediated by a trypotphan residue in a cadherin EC1 domain that binds to a hydrophobic pocket in the opposing cadherin EC1 domain. (Sivasankar et al., 2009). Dsg2 and Dsc2 extracellular domains have cadherin repeat domains that only share 30% amino acid identity. Additionally40% amino acid identity has been reported in their intracellular domains In contrast to Dsc2, Dsg2 has a long cytoplasmic domain with six twenty-nine amino acid unique repeats. While both Dsg2 and Dsc2 have plakoglobin binding sites, Dsg2 has unique amino acid sequences that can be phosphorylated by Protein Kinase C (Garrod et al., 2005) and cleaved by Caspase-3 (Dusek et al., 2006). Cadherins are synthesized as precursor pro-peptides that are processed before being delivered to intercellular junction. Newly synthesized cadherins are delivered to the

plasma membrane in Rab GTPase containing endocytic vesicles emanating from the Golgi apparatus. Similar to β1 integrin, cadherins are internalized from the cell surface in endosomes and are either delivered back to the plasma membrane in recycling endosomes or targeted to lysosomes for degradation (Le et al., 1999). The glycosylation and phosphorylation of cadherins have been reported to influence their intracellular trafficking (Aoyama et al., 1999; Khare et al., 2013; Lommel et al., 2013; Pasdar et al., 1995). However, the biological impact of such DM cadherin post-translational modification on their intracellular trafficking and adhesion are not well understood (Gao et al., 2000).

The critical role of desmosomes in controlling epithelial intercellular adhesion is appreciated in diseases associated with loss of adhesion that is mediated by autoantibodies to Desmoglein 1 (Dsg1) and Desmoglein 3 (Dsg3) resulting in blistering skin diseases (Hashimoto et al., 1995; Ishii et al., 2005; Rafei et al., 2011). The bacterium *Staphylococcus aureus* expresses exfoliative toxins that cleave Dsg1, resulting in loss of keratinocyte adhesion and blistering (Hanakawa and Stanley, 2004). During inflammation, cytokines and growth factors such as epidermal growth factor (EGF) interferon gamma and transforming Growth Factor - b alter intercellular adhesion by influencing cadherin association, endocytosis and signaling (Bruewer et al., 2003; Bryant et al., 2007; Perry et al., 1999).

1.6 Galectin-3

Galectins are globular proteins that bind to β-galactosides containing glycoproteins (Krzeslak and Lipinska, 2004). Fifteen mammalian galectins have been reported to

influence diverse biological functions (Houzelstein et al., 2004; Perillo et al., 1998). Galectins can be categorized by three different quaternary structures based on their ability to bind to themselves and other galectins. While galectins have been reported to homodimerize and heterodimerize, Gal-3 is the only galectin known self-associate into complexes larger than two monomers (Yang et al., 2008). Upon binding to glycoproteins the self-association domain in Gal-3 participates in the generation of oligomers consisting of up to five Gal-3 molecules (Ahmad et al., 2004). Additionally, Gal-3 is secreted from cells by an unknown mechanism and has been showing to both stabilize and destabilize transmembrane proteins at the cell surface (Hughes, 1999; Menon et al., 2011).

Epithelial Gal-3 has been reported to control intercellular adhesion and overall homeostasis including proliferation and differentiation (Boscher and Nabi, 2013; Hikita et al., 2000; Partridge et al., 2004). At mucosal surfaces, Gal-3 participates in mucosal barrier function by interacting with high molecular weight apical mucin glycoproteins, MUC1 and MUC16 (Argueso et al., 2009). Gal-3 regulates epithelial matrix adhesion by associating with β-galactosides on β1 integrin thereby influencing cell surface integrin levels (Furtak et al., 2001; Saravanan et al., 2009). A role of Gal-3 in controlling epithelial proliferation by associating with Epidermal Growth Factor Receptor (EGFR) (Partridge et al., 2004), and differentiation by binding with Transforming Growth Factor Receptor - β (TβR) (Lau et al., 2007) has been reported. While these functional effects of Gal-3 in controlling epithelial homeostasis and barrier function have been described, the underlying mechanisms by which it controls intercellular adhesion are not understood.

Figure 1

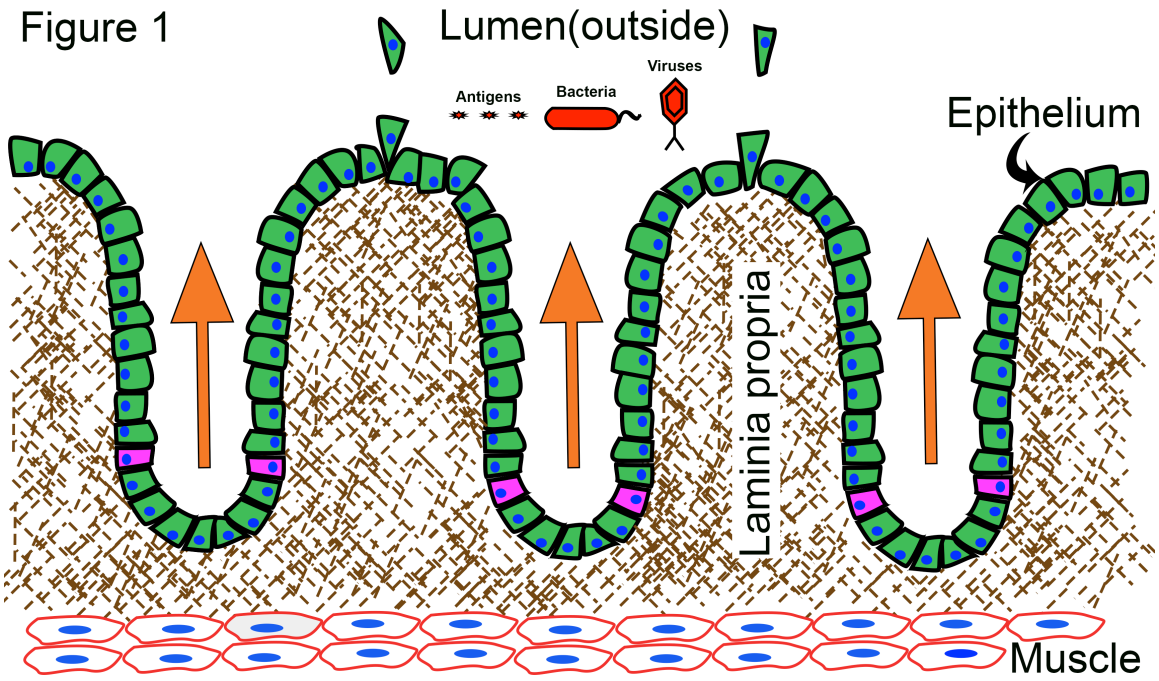
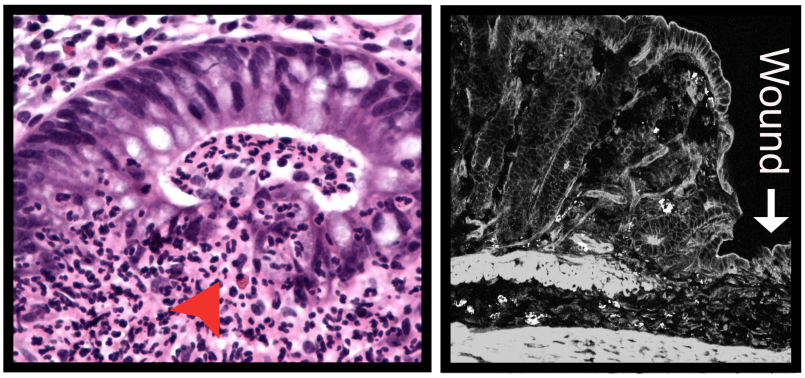

Lumen(outside)
Viruses
Antigens
Bacteria
Epithelium
Laminia propria
Muscle

Figure 1: Organization of the intestinal epithelium. The intestinal epithelial lining consists of a single layer of columnar epithelial cells (epithelium) that resides the underlying lamina propria. Long-lived cells, called stem cells (pink), reside at the base of the crypt and produce cells that renew the epithelium every 3-5 days. The progeny of stem cells migrate and proliferate up the crypt (orange arrows) until they are shed into the lumen.

Figure 2

A



B

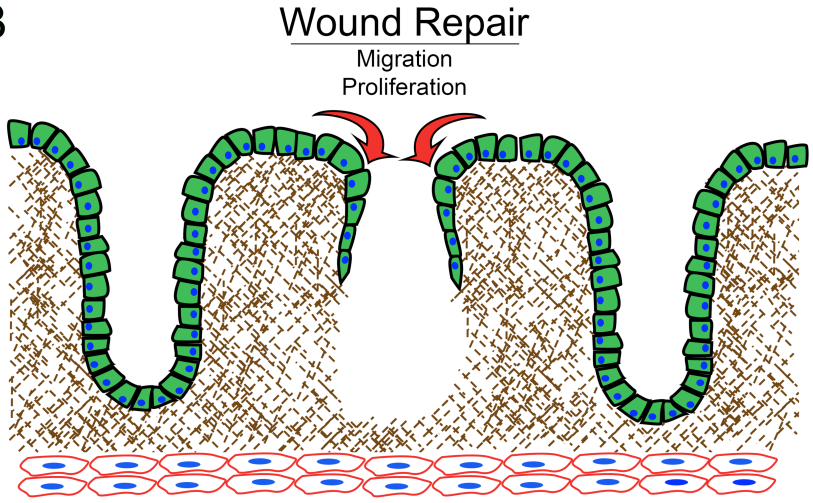


Figure 2: Intestinal epithelial wound closure. (A) An hematoxylin and eosin stained section of a mucosal biopsy from a patient with ulcerative colitis showing neutrophil infiltrate in the intestinal epithelium with an associated epithelial wound (red arrow, *left*). A resealing murine intestinal mucosal biopsy induced wound is shown in the right panel. Immunofluorescence labeling highlights F-actin (*right*). **(B)** A model showing epithelial cell migration and proliferation in resealing intestinal mucosal wounds.

Figure 3

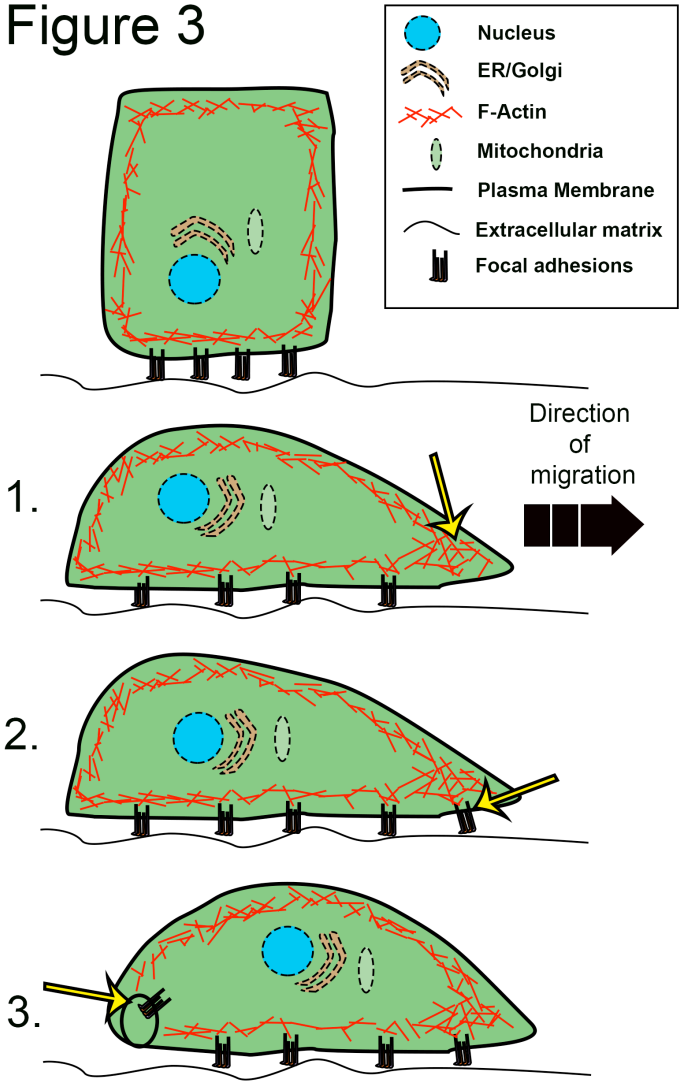

Nucleus
ER/Golgi
F-Actin
Mitochondria
Plasma Membrane
Extracellular matrix
Focal adhesions
Direction of migration
1.
2.
3.

Figure 3: Mechanism of epithelial cell migration. Migration of an epithelial sheet is mediated by: 1) polarized extrusion of the plasma membrane at the leading edge that is mediated by actin cytoskeletal (short red lines) restructuring, 2) assembly of new cell-matrix adhesions, and 3) disassembly of cell-matrix adhesions at the rear of the cell. Arrows refer to the major event during each step.

Figure 4

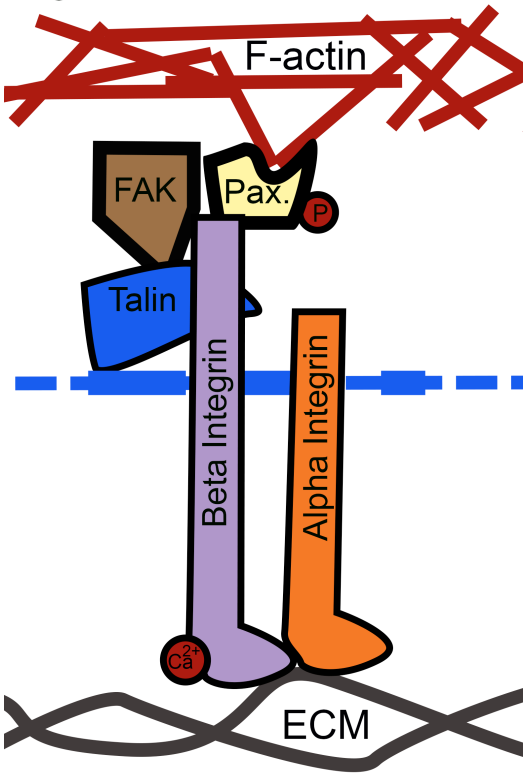


Figure 4: Cell-matrix adhesions. Cell-matrix adhesions contain transmembrane integrins that span the plasma membrane to mediate association of the extracellular matrix (ECM) with the actin cytoskeleton. The intracellular cytoskeletal associations of integrins are mediated by scaffold and signaling proteins that include paxillin, talin and focal adhesion kinase (FAK) (Calderwood et al., 1999; Goult et al., 2010). (Burridge et al., 1992). Thus, integrins mediate bidirectional cross talk between the extracellular matrix and intracellular cytoskeleton to coordinate cell movement (Bellis et al., 1995; Critchley et al., 1999).

Figure 5

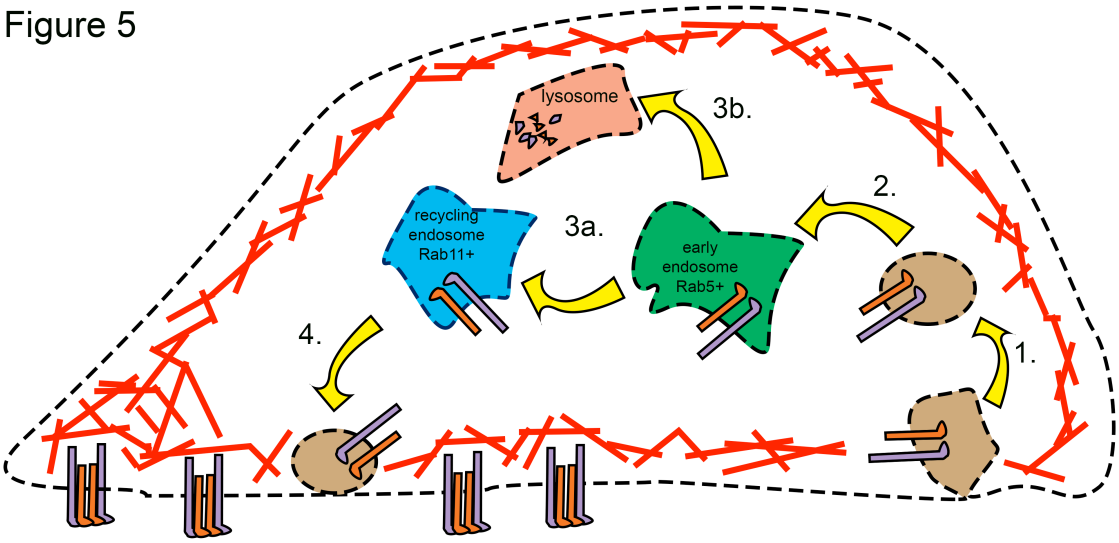

lysosome
3b.
recycling
endosome
Rab11+
3a.
early
endosome
Rab5+
2.
4.
1.

Figure 5: Endocytic recycling of integrins. The model depicts mechanisms by which integrins in adhesions at the trailing edge translocate to new adhesions at the leading edge of migrating cells (Caswell and Norman, 2006; Caswell et al., 2009; Jones et al., 2006). Integrins in cell matrix adhesions at the trailing edge of migrating cells are endocytosed **(1)** and delivered to Rab5 GTPase-containing early endosomes **(2).** Subsequently, integrins are delivered either to Rab11 recycling endosomes **(3a)** and back to the plasma membrane **(4)** or to lysosomes for degradation **(3b)**.

Figure 6

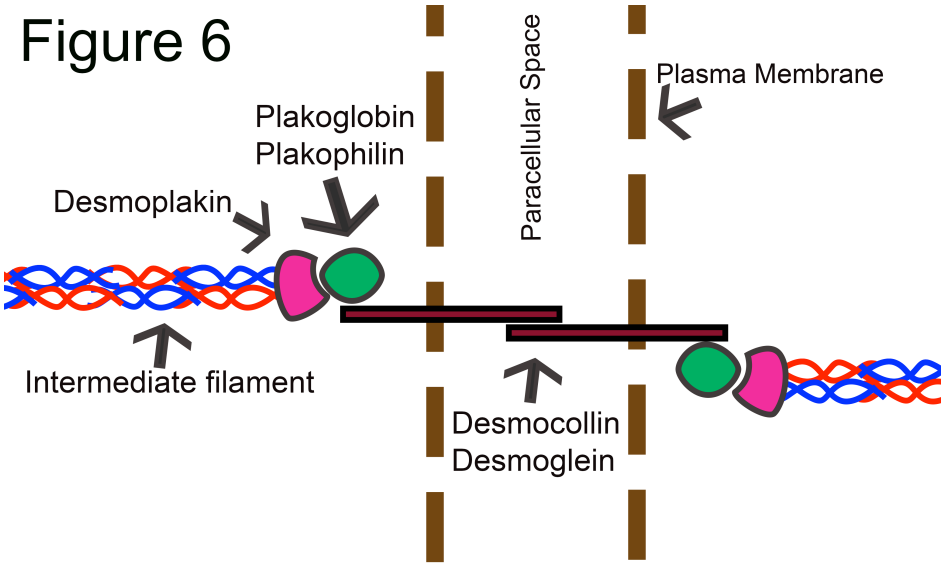

Paracellular Space
Plasma Membrane
Plakoglobin
Plakophilin
Desmoplakin
Intermediate filament
Desmocollin
Desmoglein

Figure 6: Molecular composition of an epithelial desmosome. Transmembrane cadherins, Desmoglein and Desmocollin associate in the paracellular space to mediate cell-cell adhesion. These cadherins are linked to intermediate filaments by cytoplasmic plaque proteins that include plakophilin and plakoglobin (Bornslaeger et al., 2001; Schmidt et al., 1994).

CHAPTER 2

ANNEXIN A2 REGULATES β1 INTEGRIN INTERNALIZATION AND INTESTINAL EPITHELIAL CELL MIGRATION

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2.1 Introduction

The intestinal epithelium functions as a selectively permeable barrier that allows for the absorption of nutrients and fluids while excluding bacteria and toxins from underlying tissue compartments. The epithelium is highly dynamic as it is actively turned over by proliferation of crypt epithelial cells, migration of cells along the crypt-luminal axis, and apoptosis at the luminal surface. Epithelial injury such as that observed in inflammatory diseases leads to fluid and electrolyte loss as well as exposure to luminal antigens that further exacerbate the inflammatory response. It is therefore critical that epithelial wounds efficiently reseal so as to re-establish the intestinal epithelial barrier and restore the physiologic state. The intestinal epithelium has a remarkable capacity to efficiently reseal wounds. An important mechanism of intestinal epithelial wound closure involves migration of the epithelial cell sheet that rapidly covers denuded surfaces. (Blikslager et al., 2007; Crosnier et al., 2006; Rutten and Ito, 1983)

Cell migration requires regulated cycles of polarized cell-matrix attachment at the leading edge of the cell with corresponding detachment at its trailing edge. These critical migratory events are mediated by dynamic turnover of integrin-mediated adhesions. Indeed, disassembly of integrin-based cell-matrix adhesions is important for mediating forward cell movement. Past studies have shown that integrin based adhesions form at the leading edge, mature, and then disassemble at the trailing edge. Focal adhesion disassembly requires endocytosis of integrins that are then recycled back to the plasma membrane at the leading edge of migrating cells or destroyed by lysosomal degradation (Bretscher, 1989; Caswell et al., 2009; Margadant et al., 2011; Raub and Kuentzel, 1989). A central component of such biological events is the coordinated intracellular movement

of proteins allowing for reuse of non-damaged proteins. Studies from others and our group have demonstrated that β1 integrin plays an important role in IEC cell-matrix adhesion and migration (Chen et al., 2009a; Lotz et al., 1997; Mandell et al., 2005). Additionally, endocytosis and trafficking of β1 integrin is lipid raft-mediated (Vassilieva et al., 2008; Wang et al., 2010).

We have previously reported that the calcium dependent phospholipid binding protein, Annexin A2 (AnxA2), facilitates IEC migration (Babbin et al., 2007). AnxA2 is a highly conserved protein in invertebrates and plants, and is expressed in most cell types. AnxA2 contains four 70-80 amino acid Annexin core repeats that allow for Ca^{2+} dependent attachment to phosphatidylinositol 4,5-bisphosphate (PIP_2) rich membranes, a component of lipid rafts (Babiychuk and Draeger, 2000; Glenney, 1986; Harder et al., 1997; Harrist et al., 2009; Jacob et al., 2004; Rescher et al., 2004; Uittenbogaard et al., 2002). Additionally, AnxA2 can cap filamentous Actin while associated with lipid rafts (Ali and Burgoyne, 1990; Gerke and Weber, 1984; Hayes et al., 2006). Since AnxA2 regulates membrane-membrane and membrane-cytoskeletal interactions, it has been implicated in functioning as a regulatory protein for a wide range of membrane trafficking events that include endocytosis, endosome fusion, and exocytosis (Blackwood and Ernst, 1990; Emans et al., 1993; Law et al., 2009; Morel et al., 2009; Rescher and Gerke, 2004; Sarafian et al., 1991).

While the structure function relationship between AnxA2 and migration has been investigated, the mechanisms by which AnxA2 controls IEC migration remain poorly understood. Using an AnxA2 shRNA gene silencing approach, we identify a role for

AnxA2 in regulating the internalization of cell surface β1 integrin that is needed for the turn-over of focal adhesions, forward cell movement and ultimately wound closure.

2.2 Materials and methods

Cell Culture and Constructs - Lentiviruses were generated in HEK293T TLA cells by PEI transfection (Open Biosystems). Titered lentiviruses were then used to transduce SK-CO15 and Caco2 human colonic cancer epithelial cells. Clonal lines were grown as previously described except for the addition of 2 μg/mL puromycin (Severson et al., 2009). Cycloheximide (MP biomedical) was used at the concentration of 50 μg/mL for the given amount of time at 37^{o}C before harvesting cells. Bafilomycin A1 (Acros) was used at the concentration of 30 nM for two hours at 37^{o}C before fixation. For transient rescue experiments, a pShuttle eGFP plasmid containing a shRNA resistant AnxA2 (Addgene) was electroporated into cells in solution L, program 15 (Lonza). For scratch wound rescue experiments a shRNA resistant AnxA2-eGFP was subcloned from pShuttle into pLEX to generate a shRNA resistant AnxA2-eGFP lentivirus. Cells were transduced with the lentivirus and sorted for eGFP expression by flow cytometry. Supplemental table 1 contains the shRNA sequences and primers used for the creation of shRNA resistant constructs.

Immunoblots - Cells were lysed in RIPA buffer (20 mM Tris pH7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% Sodium Deoxycholate, 1% Triton X-100, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Lysates were cleared by centrifugation before boiling in a reducing SDS sample buffer. After SDS-PAGE, resulting gels were transferred to PVDF-fl membranes (Millipore) overnight. Immunoblots were incubated in casein for an hour to inhibit non-specific binding

followed by primary and secondary antibody treatment and scanned (LI-COR). The following primary antibodies were used: β1 integrin clone EP1041Y (Novus), AnxA2 clone ZO14 (Invitrogen), Paxillin clone 165 (BD), pPaxillin Y118 (2541, Cell Signaling), Actin (A2668, Sigma), Cyclin D1 clone DCS-6 (Santa Cruz), E-cadherin clone HECD (in house). Caveolin-1 clone N-20 (Santa Cruz), Transferrin receptor clone C-20 (Santa Cruz). Secondary antibodies were from KPL labs.

Immunofluorescence Microscopy - Cells were fixed in 3.7% formalin for 20 minutes and permeabilized in 0.5% Triton X-100 for 3 minutes. Fixed cells were then incubated with 5% bovine serum albumin for an hour before primary antibody incubation overnight. After secondary antibody incubation, cells were mounted in ProLong® gold antifade agent (Invitrogen) before confocal microscopy (Zeiss LSM 510). The following primary antibodies were used: pPaxillin Y118 (Cell Signaling), β1 integrin clone Mab13 (BD), AnxA2 clone 3E8-B6 (Sigma). Alexa conjugated secondary antibodies were purchased from Invitrogen. Proliferation was monitored by a 1 hour pulse with EdU (Invitrogen). Cells were fixed and permeabilized as described above before counterstaining with To-Pro®3-iodide (Invitrogen) to highlight nuclei. For Rab5a co-localization experiments, cells were transduced in suspension with Celllight® early endosomes RFP, Bacmam 2.0 (Invitrogen) before culturing. Focal adhesions in individual cells were quantified using To-Pro®3-iodide to highlight nuclei and ImageJ software (NIH).

Scratch Wound Assay - Scratch wounds were generated using suction via a micropipette. Time-lapse imaging of wound closure was then performed. Images of the leading edge were captured every 2 minutes for 4 hours at 37^{o}C with an EMCCD camera (Photometrics). For the 24-hour time-point the bottom of the culture plate was scored by

a marker perpendicular to the wound and images were taken at this intersection immediately after wounding and 24 hours later. ImageJ (NIH) was used to quantify wound areas.

ECM Adhesion Assay - Single cell suspensions were generated using a non-enzymatic cell dissociation buffer (Gibco). Equal concentrations of cells (10000 cells in 0.1 mL) were resuspended in optimem with 0.1% BSA and then incubated with BCECF-AM (Invitrogen) according to the manufacturer's protocol. Antibodies against β1 integrin, clone Mab13 (BD) or clone TS2/16 (eBioscience), were dialyzed in PBS overnight and used at a concentration of 5 μg/mL. Cells were incubated with these antibodies for 2 hours prior to the adhesion assay. Antibody treated cells were then allowed to adhere to a matrix coated/BSA blocked 96 well plate for 30 min at 37^{o}C. Plates were coated overnight with 10 μg/mL of Matrigel™ (356234, BD) or fibronectin (354008, BD).

Real Time PCR - RNA was isolated using Trizol® (Invitrogen) and treated with DNase I (Qiagen prior to cDNA synthesis using an oligo dT primer and Superscript® III (Invitrogen). SYBR® Green master mix (ABI) was used to perform real time PCR on a BioRad iQ5 cycler. Cycle threshold values (Ct) were used to calculate the fold change in mRNA according to the 2^-ΔΔCt method (Livak and Schmittgen, 2001). GAPDH was used as a reference gene. Supplemental table 1 includes PCR primer sequences.

Lipid Raft Isolation - Cells were harvested in HBSS^{+} supplemented with a protease inhibitor cocktail (Sigma) before nitrogen cavitation at 200 psi for 15 minutes (Macdonald and Pike, 2005). The post-nuclear supernatant was placed in the bottom of a 5-35% continuous sucrose gradient. Gradients were then centrifuged at 39000 RPM for

19 hours in a SW41 rotor (Beckman) to isolated light density lipid rafts (~21% sucrose). Sucrose fractions were harvested and immunoblotted.

Cell surface biotinylation and endocytosis - Pulse chase biotinylation experiments were performed as previously described (Dukes et al., 2011; Roberts et al., 2001). Briefly cell surface proteins were biotinylated (0.5 mg/ml) on ice using NHS-SS-biotin (Pierce). Internalization of cell surface proteins was induced by placing the cells at 37^{o}C for 2 hours. Biotin was stripped from non-internalized proteins by treating cells twice with 20 mM MESNA (Genscript) for 5 minutes. Cells were then treated with 20 mM iodoacetamide (Sigma) for 15 minutes to quench any remaining MESNA. Biotinylated proteins from soluble lysates (200 μg) were purified using monomeric avidin agarose (Pierce), eluted in a reducing SDS sample buffer, and subjected to immunoblot analysis.

2.3 Results

AnxA2 promotes epithelial cell migration and wound closure. We have previously reported that Annexin A2 (AnxA2) is up-regulated in migrating intestinal epithelial cells (IECs) and controls cell motility and wound closure (Babbin et al., 2007). To further identify the mechanisms by which AnxA2 regulates epithelial cell motility, we generated cell lines with stable down-regulation of AnxA2 using two different shRNA sequences (shAnxA2) and a control cell line with a non-silencing shRNA target (shCtrl). Additionally, to verify our results, AnxA2 was stably down-regulated in two model IECs (SK-CO15, Caco2). Immunoblot analysis revealed a significant down-regulation of AnxA2 (>80% reduction) in shAnxA2 cells as compared to shCtrl cells **(Fig. 7A)**. Furthermore, to determine if any effects of the AnxA2 shRNA were specific to loss of AnxA2 protein, we transduced shAnxA2 cells with a shRNA resistant eGFP tagged

AnxA2. As shown in figure 7A, the AnxA2 eGFP fusion protein was identified at 70 kDa using an AnxA2 antibody. Actin and tubulin were used as loading controls. We next tested the functional effects of stable AnxA2 down-regulation and rescue on IEC wound closure. Using an *in vitro* scratch wound resealing assay and time-lapse imaging, we observed that loss of AnxA2 resulted in a four-fold delay in wound closure over a four hour time period and re-expression of AnxA2 was able to significantly rescue the delay in wound closure ($p<0.001$) (**Fig. 7B, Online Supp. Videos 1-3)**. Similarly, after twenty-four hours of wound closure, shAnxA2 cells displayed a two-fold decrease in wound closure that was abrogated in shAnxA2 cells re-expressing AnxA2 **(Supp. Fig. 1A)**. These experiments support a role of AnxA2 in controlling intestinal epithelial wound closure. Since wound closure can be mediated by cell proliferation in addition to cell migration, epithelial proliferation was evaluated by determining EdU incorporation in cells during wound closure. In spite of decreased wound closure in shAnxA2 cells as compared to control cells, we observed increased proliferation in cells with down-regulated AnxA2 **(Supp. Fig 1B)**. These findings suggest that the decreased wound closure following AnxA2 down-regulation is mediated by an influence of this protein on epithelial cell migration.

Down-regulation of AnxA2 increases cell-matrix adhesion. Since AnxA2 facilitates IEC migration that in turn requires dynamic turnover of cell-matrix associations, we next investigated the adhesion of epithelial cells to a complex extracellular matrix. Down-regulation of AnxA2 increased cell-matrix adhesion (40% increase) **(Fig. 8A, *left panel*)**. In an analogous manner, increased adhesion of shAnxA2 cells to fibronectin was also observed **(Fig. 8A, right panel)**. Since SK-CO15 cells were

used for these assays, we verified adhesive properties of another model intestinal epithelial cell line, Caco2. Analogous to SK-CO15 cells, increased cell-matrix adhesion was seen in Caco2 cells with down-regulated AnxA2 (data not shown). To biochemically link functional cell-matrix adhesion to the activity of proteins that mediate cell-matrix adherence, we evaluated the phosphorylation/activation status of the β1 integrin scaffold protein, paxillin. Phosphorylated paxillin at tyrosine 118 (pPaxY118) has been previously reported in engaged cell-matrix adhesions (Bellis et al., 1997; Burridge et al., 1992). In keeping with the increased adhesive properties of shAnxA2 cells, immunoblot analysis revealed pPaxY118 and total Paxillin levels were increased two-fold in shAnxA2 cells compared to shCtrl IECs **(Fig. 8B)**. Immunofluorescence labeling and confocal microscopy of spreading cells corroborated the biochemical results. In shCtrl cells, pPaxY118 is visualized in short punctate basal structures representing focal adhesions. In shAnxA2 cells, the pPaxY118 containing cell-matrix adhesions were more prominent in size and staining intensity. Quantification of pPaxY118-positive adhesions per spreading cell revealed a two-fold increase in the number adhesions in cells with down-regulated AnxA2 **(Fig. 8D)**. These findings support a role of AnxA2 in regulating cell-matrix adhesions in IECs migrating to close wounds.

Cell surface β1 integrin stability is increased in cells with down-regulated AnxA2. Cell surface β1 integrin mediates IEC-matrix adhesion and plays an important role in cell movement. Since we had observed that AnxA2 down-regulation resulted in increased cell-matrix adhesion with a concomitant increase in pPaxY118 containing focal adhesions, we next determined if the increase in cell-matrix adhesion in shAnxA2 cells was dependent upon β1 integrin function. Inhibitory (Mab13) but not non-inhibitory

(TS2/16) β1 integrin antibodies abrogated the increase in cell-matrix adhesion in shCtrl and shAnxA2 cells **(Fig. 8A, *right panel*)**. Additionally, the inhibition of β1 integrin abrogated the difference in pPax.Y118 in shCtrl and shAnxA2 cells **(Fig. 8C)**. To better understand how AnxA2 regulates β1 integrin, we first examined the steady state levels of β1 integrin mRNA and protein. IEC immunoblots revealed a two-fold increase in β1 integrin protein in shAnxA2 cells as compared to shCtrl cells **(Fig. 9A)**. The increased β1 integrin protein in shAnxA2 cells was not associated with a corresponding increase in β1 integrin mRNA **(Fig. 9B)** suggesting an influence of AnxA2 on β1 integrin protein dynamics/stability. We next determined if AnxA2 regulates the post-translational stability of β1 integrin. As shown in figure 3C, the stability of β1 integrin protein was increased four hours after cycloheximide treatment in shAnxA2 cells as compared to shCtrl cells. Additionally, β1 integrin stability was significantly increased after eight hours of cycloheximide treatment in shAnxA2 cells as compared to shCtrl cells ($p<0.05$).

Given that down-regulation of AnxA2 resulted in increased stability of β1 integrin protein, we next determined β1 integrin subcellular localization by immunofluorescence labeling and confocal microscopy. In control cells β1 integrin is visualized in intracellular punctate, "vesicle-like" structures that were prominent at the leading edge of migrating cells in addition to a small pool in the basolateral membrane. However, in migrating shAnxA2 cells, β1 integrin was localized predominantly in the basolateral membrane of migrating cells **(Fig. 9D)**. These findings suggest that loss of AnxA2 results in increased β1 integrin at the cell surface. To further support these results, we biochemically analyzed cell surface β1 integrin levels. Cell surface biotinylation, avidin pull-down, and β1 integrin immunoblotting revealed a six-fold increase in cell surface β1 integrin

relative to total β1 integrin in shAnxA2 cells as compared to control cells **(Fig. 9E)**. The AnxA2 effects observed were specific for β1 integrin as we did not observe a change in total and cell surface levels of another basolateral plasma membrane associated protein, E-cadherin. The influence of AnxA2 loss on total and cell surface β1 integrin levels was also confirmed in another model intestinal epithelial cell line, Caco2 **(Fig. 9F)**.

AnxA2 promotes β1 integrin localization in endosomes. Cell surface proteins undergo trafficking into early endosomes. Thus, to further characterize β1 integrin trafficking in cells with down-regulated AnxA2, we co-localized β1 integrin with Rab5a, a marker of early endosomes. To highlight early endosomes a Celllight® early endosome RFP, Bacmam 2.0 (RFP-Rab5a) construct was expressed in migrating IECs. In shCtrl cells, a large pool of β1 integrin co-localized with early endosomes. However, in shAnxA2 cells, β1 integrin was distributed in the basolateral plasma membrane with minimal localization in RFP-Rab5a containing early endosomes **(Fig. 10A)**. In keeping with the imaging data, subcellular fractionation of migrating epithelial cells by isopycnic sucrose density gradient sedimentation revealed co-sedimentation of AnxA2 with β1 integrin and an early endosome protein, EEA1 (Early Endosome Antigen 1). Down-regulation of AnxA2 decreased co-sedimentation of β1 integrin with EEA1 (data not shown). To ensure the specificity of the AnxA2 shRNA, we performed a rescue experiment to determine if the expression of a shRNA resistant eGFP tagged AnxA2 in shAnxA2 cells would promote the redistribution of β1 integrin to endosomes. Immunofluorescence labeling and confocal microscopy revealed that in cells lacking eGFP-AnxA2, β1 integrin resided mainly in the basolateral membrane. In cells re-

expressing AnxA2, as marked by GFP expression, β1 integrin redistributed to endosomal-like vesicle structures **(Fig. 10B)**.

β1 integrin internalization is delayed in IECs with down-regulated AnxA2. Since loss of AnxA2 increases the cell surface level of β1 integrin, we investigated if AnxA2 functions to either increase the rate of β1 integrin internalization or decrease the rate of β1 integrin trafficking back to the cell surface. To distinguish between these two possibilities we first tested the effect of BafilomycinA1 (BafA1) on β1 integrin localization in shCtrl and shAnxA2 cells. BafA1 inhibits the vacuolar-type H^+ ATPases, retarding protein trafficking between endosomes needed for recycling (Presley et al., 1997). In shCtrl cells, BafA1 treatment increased the intracellular accumulation of β1 integrin. However, in BafA1 treated shAnxA2 cells, β1 integrin primarily localized in the plasma membrane, suggesting that loss of AnxA2 delays β1 integrin internalization **(Fig. 11A)**. To determine the rate of β1 integrin internalization in shCtrl and shAnxA2 cells, biotin pulse chase experiments were performed. After biotin labeling of cell surface proteins at 4^{o}C, cells were transferred to 37^{o}C to induce protein internalization. Cells were then placed back at 4^{o}C and biotin was chemically removed (stripped) from non-internalized cell surface proteins, leaving only internalized proteins biotinylated. Biotinylated proteins from resultant lysates were pulled-down using avidin agarose and immunoblotted for β1 integrin. To ensure complete biotin stripping from non-internalized proteins, one group of cells was left at 4^{o}C after labeling, and then stripped and lysed (labeled Strip). Additionally, to account for internalized and degraded biotinylated protein, cells were lysed directly after the internalization period (Non-Deg.). In shCtrl IECs, all cell surface β1 integrin was internalized after two hours at 37^{o}C. In contrast to

shCtrl cells, the rate of β1 integrin internalization was significantly decreased in shAnxA2 cells (two-fold) **(Fig. 11B)**. To better define how AnxA2 regulates the internalization of β1 integrin, we determined if these proteins co-fractionate with the lipid raft associated protein Caveolin-1. Lipid rafts from control and AnxA2 down-regulated cells were isolated by floatation in sucrose gradients. Indeed, immunoblot analysis identified AnxA2 and β1 integrin with Caveolin-1 in lipid raft containing sucrose fractions **(Fig. 11C)**. To verify the specificity of this finding, we immunoblotted fractions for the transferrin receptor as it has been previously reported that this receptor does not associate with lipid rafts (Macdonald and Pike, 2005). In conclusion, these results suggest that AnxA2 and β1 integrin associate with IEC Caveolin-1 containing lipid rafts that have been previously shown to mediate β1 integrin internalization from the cell surface (Vassilieva et al., 2008; Wang et al., 2010).

2.4 Discussion

Directed migration of a cohesive sheet of epithelial cells mediates mucosal wound closure. In this report, we identify a novel role of AnxA2 in controlling traffic of β1 integrin need for the turnover of focal adhesions in the migrating epithelial sheet. In a previous report, AnxA2 has been implicated in mediating cell-matrix adhesion by controlling RhoA GTPase activity (Rescher et al., 2008). In our current study, we show that AnxA2 modulates β1 integrin endocytosis from the cell surface thereby promoting cell migration. In the absence of AnxA2, β1 integrin internalization into early endosomes is inhibited thereby resulting in increased cell surface β1 integrin that is available for remodeling of cell matrix adhesions in the migrating epithelial sheet. This finding is analogous to a report indicating that down-regulation of Sorting Nexin 27 results in

disruption of Multidrug Resistance Protein 4 receptor (MRP4) internalization, causing increased steady state levels of this receptor at the cell surface (Hayashi et al., 2012). In a previous study, we reported that AnxA2 controls intestinal epithelial wound closure by modulating the small GTPase, RhoA (Babbin et al., 2007). Our current findings are complementary to this report since Rho GTPases control F-Actin organization that in turn influences dynamics of membrane raft associated proteins (Lamaze et al., 2001). Interestingly, in our previous report, we also observed that transient and incomplete down-regulation of AnxA2 did not significantly alter β1 integrin protein levels. In contrast, our current findings were derived from stable cell lines having greater than 90% down-regulation of AnxA2 which clearly show significantly increased β1 integrin protein levels. These differences can be explained either by limited ability to detect changes under conditions of low transfection efficiency or that there is a threshold for AnxA2 down-regulation, beyond which significant AnxA2 loss inhibits traffic of β1 integrin and epithelial cell migration. We have proposed that AnxA2 associates with β1 integrin in lipid rafts and regulates β1 integrin endocytosis from the plasma membrane needed for turnover of cell matrix contacts **(Fig. 12A)**. Following the loss of AnxA2, focal adhesions containing β1 integrin and paxillin accumulate at the plasma membrane resulting in increased cell-matrix adhesion and decreased cell migration **(Fig. 12B)**. Interestingly, we observed that steady state levels of paxillin mimic β1 integrin. A functionally inhibitory β1 integrin antibody (Mab13) decreased paxillin protein levels. The association of paxillin with β1 integrin decreases the rate of paxillin degradation. Paxillin tyrosine 118 phosphorylation was also increased in shAnxA2 cells. Since β1 integrin association with the extracellular matrix promotes paxillin phosphorylation at this residue (Burridge et al.,

1992) our results suggest that β1 integrin can still signal after loss of AnxA2.

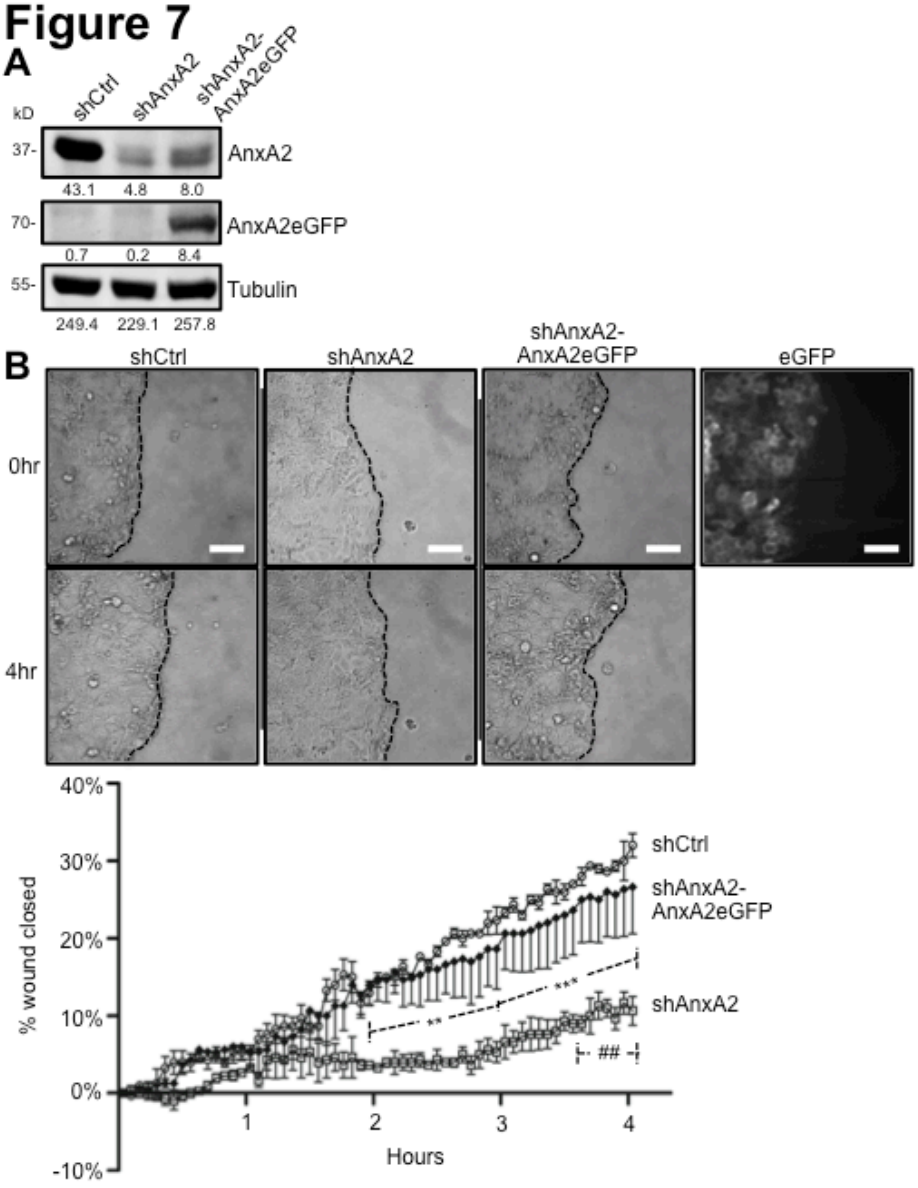
AnxA2 has been implicated in regulating traffic of intracellular proteins by promoting fusion and exocytosis of endosomes. AnxA2 has been reported to promote aggregation and fusion of vesicles (Emans et al., 1993; Mayran et al., 2003; Zibouche et al., 2008). Interestingly, previous studies in cells with down-regulated AnxA2 revealed that epidermal growth factor and transferrin accumulate in late endosomes (Mayran et al., 2003; Zobiack et al., 2003). Since we have observed that down-regulation of AnxA2 inhibits β1 integrin endocytosis from the cell surface it is likely AnxA2 has diverse roles that are dependent on the cargo in the vesicular compartments. Furthermore, our loss-of-function study exclusively analyzes the influence of AnxA2 on an early event in β1 integrin endocytic recycling during epithelial cell migration. We therefore do not exclude a role of AnxA2 in controlling β1 integrin trafficking between other endosomal compartments such as β1 integrin recycling back to the plasma membrane in migrating epithelial cells.

Current knowledge of how AnxA2 functions in the endocytosis of lipid raft associated proteins is very limited. A single study to date suggested that AnxA2 promotes the internalization of a cell surface receptor (Biener et al., 1996). To build a more comprehensive model on the role of AnxA2 in endocytosis, studies have analyzed key AnxA2 binding partners. These reports have shown that AnxA2 can simultaneously interact with Actin and lipid rafts (Babiychuk and Draeger, 2000; Glenney, 1986; Harder et al., 1997; Harrist et al., 2009; Jacob et al., 2004; Rescher et al., 2004; Uittenbogaard et al., 2002). The role of Actin in endocytosis and the involvement of lipid rafts in endocytosis have been previously reported (Huckaba et al., 2004; Kaksonen et al., 2005).

Actin nucleating proteins attach to the plasma membrane thereby promoting polymerization of Actin at these sites. Vesicles are attached to filamentous Actin (F-Actin) that facilitates inward budding (Liu et al., 2009). Since AnxA2 can cap filamentous Actin while being associated with lipid rafts, we propose that AnxA2 provides a link between F-Actin and internalization of β1 integrin from cell surface rafts to early endosomes (see model in Figure 12A). It is likely that Protein Kinase C (PKC) mediated phosphorylation of AnxA2 plays a regulatory role in the endocytosis of lipid rafts and cell-matrix detachment since PKC has been reported to phosphorylate AnxA2 and also regulate the endocytosis of β1 integrin (Ivaska et al., 2002; Oudinet et al., 1993; Rescher et al., 2008; Upla et al., 2004).

In summary, our findings implicate an important role of AnxA2 in controlling β1 integrin dynamics, epithelial migration and wound closure

Figure 7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shCtrl
shAnxA2
shAnxA2-AnxA2eGFP
kD
37-
AnxA2
43.1 4.8 8.0
70-
AnxA2eGFP
0.7 0.2 8.4
55-
Tubulin
249.4 229.1 257.8
B
shCtrl
shAnxA2
shAnxA2-AnxA2eGFP
eGFP
0hr
4hr
40%
30%
20%
10%
0%
-10%
% wound closed
1
2
3
4
Hours
shCtrl
shAnxA2-AnxA2eGFP
shAnxA2
**
***
##

Figure 7. AnxA2 down-regulation inhibits epithelial cell migration and wound closure. (**A**) SK-CO15 epithelial cells lines expressing control non-silencing shRNA (shCtrl) and Annexin A2 shRNA (shAnxA2) were generated by lentivirus transduction of the respective shRNA. To re-express AnxA2 in shAnxA2 cells, a shRNA resistant eGFP fusion with AnxA2 was transduced into shAnxA2 cells. Lysates from representative clones and eGFP expressing cells were immunoblotted for AnxA2 and Tubulin. The numbers below the lanes represent fluorescence intensity quantifications. (**B**) Scratch wounds were made in epithelial monolayers and images were taken at the same position every four minutes for four hours. Mean±SEM. ** $p<0.005$ and *** $p<0.0001$, shCtrl vs. shAnxA2. ## $p<0.001$, shAnxA2 vs. shAnxA2-AnxA2eGFP. Scale bar = 250 μm.

Figure 8

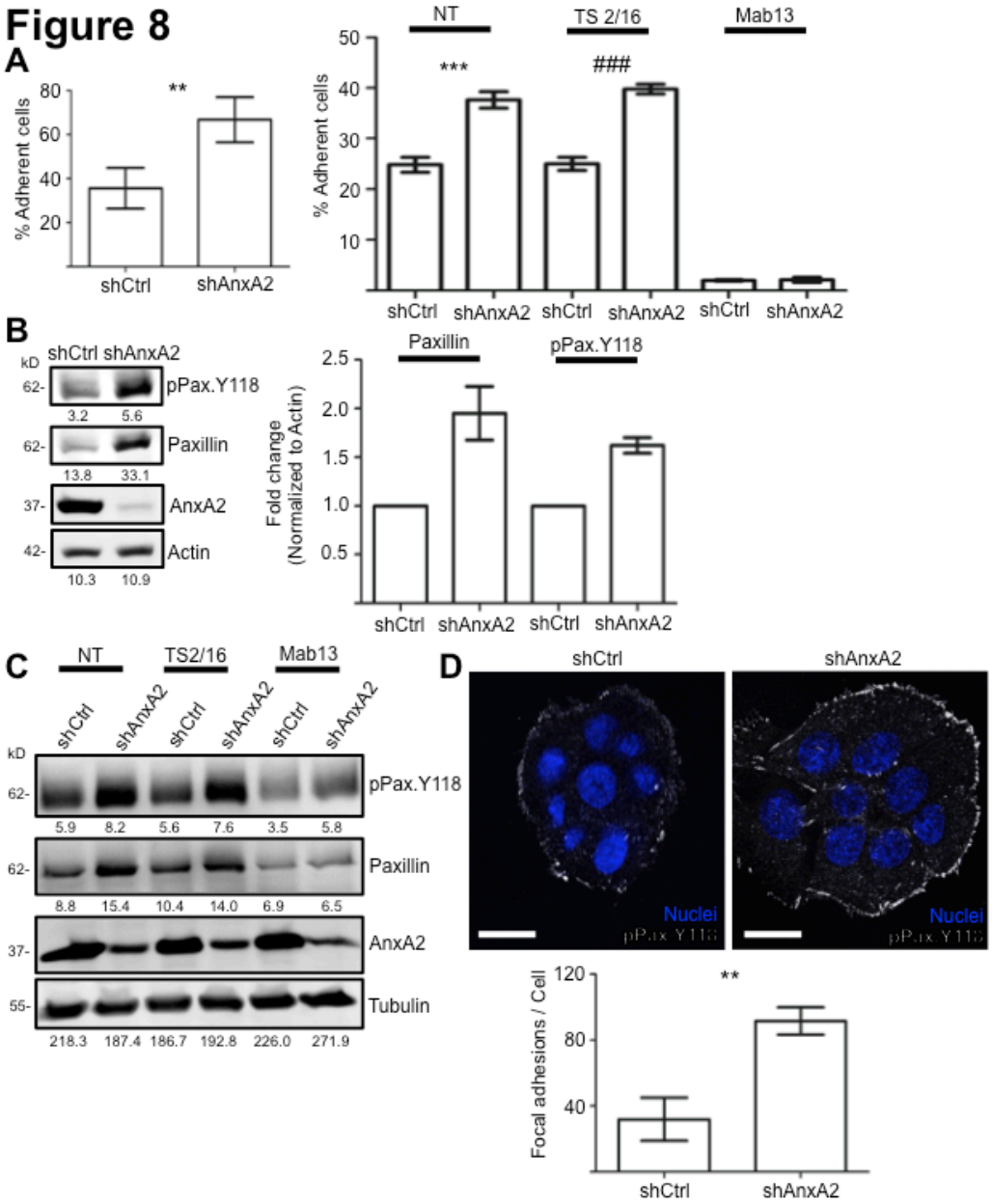

A
% Adherent cells
80
60
40
20
**
shCtrl
shAnxA2
NT
TS 2/16
Mab13
50
40
30
20
10
***
###
shCtrl
shAnxA2
shCtrl
shAnxA2
shCtrl
shAnxA2
B
kD
shCtrl shAnxA2
62-
pPax.Y118
3.2
5.6
62-
Paxillin
13.8
33.1
37-
AnxA2
42-
Actin
10.3
10.9
Paxillin
pPax.Y118
Fold change
(Normalized to Actin)
2.5
2.0
1.5
1.0
0.5
shCtrl
shAnxA2
shCtrl
shAnxA2
C
NT
TS2/16
Mab13
shCtrl
shAnxA2
shCtrl
shAnxA2
shCtrl
shAnxA2
kD
62-
pPax.Y118
5.9
8.2
5.6
7.6
3.5
5.8
62-
Paxillin
8.8
15.4
10.4
14.0
6.9
6.5
37-
AnxA2
55-
Tubulin
218.3
187.4
186.7
192.8
226.0
271.9
D
shCtrl
shAnxA2
Nuclei
Nuclei
Focal adhesions / Cell
120
80
40
**
shCtrl
shAnxA2

Figure 8. Down-regulation of AnxA2 increases cell-matrix adhesion. (**A**) Single suspensions of control shRNA (shCtrl) and Annexin A2 shRNA (shAnxA2) cells were labeled with a fluorescent dye prior to adhesion to either matrigel (left panel) or fibronectin (right panel) coated plates. The pre-wash fluorescent signal was compared to the signal of adherent cells after washing the cells with a buffer. Incubation with functionally non-inhibitory (TS2/16) and inhibitory (Mab13) β1 integrin antibodies was used to determine the contribution of β1 integrin to cell-matrix adhesion. Results represent the Mean±SD of three independent experiments. ** $p<0.001$. shCtrl vs. shAnxA2, NT, *** $p<0.0001$. shCtrl vs. shAnxA2, TS2/16, ### $p<0.0001$. (**B**) Cell lysates were immunoblotted with antibodies to phosphorylated Paxillin tyrosine 118 (pPax.Y118), total Paxillin (Paxillin), AnxA2, and Actin. The bar graph shows fluorescence intensity quantifications that are presented as Mean±SEM. **(C)** Cells were incubated with β1 integrin antibodies for two hours and then subjected to immunoblotting with antibodies to phosphorylated Paxillin tyrosine 118 (pPax.Y118), total Paxillin (Paxillin), AnxA2, and Tubulin. Cyclin D1 was used as a positive control. (**D**) Immunofluorescence labeling and confocal microscopy of pPax.Y118 (white) in shCtrl and shAnxA2 colonies. To quantify the number of adhesions per field, pPax.(Y118) puncta were divided by the total number of nuclei (blue). The data is representative of 3 independent experiments and expressed as Mean±SD. ** $p<0.001$. Scale bar = 20 μm.

Figure 9

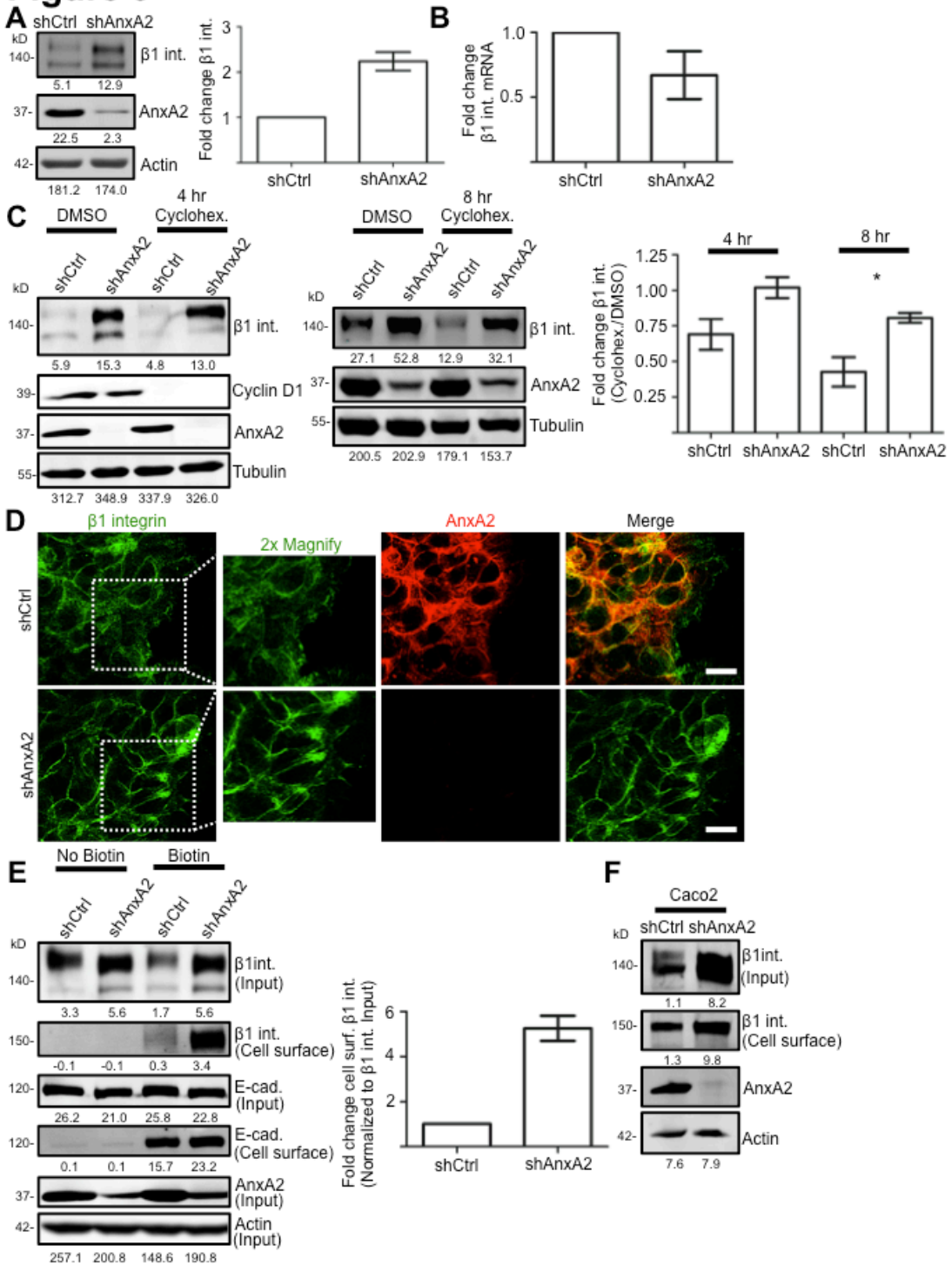

A
shCtrl shAnxA2
kD
140-
β1 int.
5.1 12.9
37-
AnxA2
22.5 2.3
42-
Actin
181.2 174.0
Fold change β1 int.
3
2
1
shCtrl
shAnxA2
B
Fold change β1 int. mRNA
1.0
0.5
shCtrl
shAnxA2
C
DMSO
4 hr Cyclohex.
shCtrl shAnxA2 shCtrl shAnxA2
kD
140-
β1 int.
5.9 15.3 4.8 13.0
39-
Cyclin D1
37-
AnxA2
55-
Tubulin
312.7 348.9 337.9 326.0
DMSO
8 hr Cyclohex.
shCtrl shAnxA2 shCtrl shAnxA2
kD
140-
β1 int.
27.1 52.8 12.9 32.1
37-
AnxA2
55-
Tubulin
200.5 202.9 179.1 153.7
Fold change β1 int. (Cyclohex./DMSO)
4 hr
8 hr
1.25
1.00
0.75
0.50
0.25
*
shCtrl shAnxA2 shCtrl shAnxA2
D
β1 integrin
2x Magnify
AnxA2
Merge
shCtrl
shAnxA2
E
No Biotin
Biotin
shCtrl shAnxA2 shCtrl shAnxA2
kD
140-
β1int. (Input)
3.3 5.6 1.7 5.6
150-
β1 int. (Cell surface)
-0.1 -0.1 0.3 3.4
120-
E-cad. (Input)
26.2 21.0 25.8 22.8
120-
E-cad. (Cell surface)
0.1 0.1 15.7 23.2
37-
AnxA2 (Input)
42-
Actin (Input)
257.1 200.8 148.6 190.8
Fold change cell surf. β1 int. (Normalized to β1 int. Input)
6
4
2
shCtrl
shAnxA2
F
Caco2
shCtrl shAnxA2
kD
140-
β1int. (Input)
1.1 8.2
150-
β1 int. (Cell surface)
1.3 9.8
37-
AnxA2
42-
Actin
7.6 7.9

Figure 9. Cell surface β1 integrin stability is increased following loss of AnxA2. (**A**) Control non-silencing shRNA (shCtrl) or Annexin A2 shRNA (shAnxA2) migrating epithelial cells were isolated and immunoblotted for β1 integrin, AnxA2, and Actin. The bar graph show densitometric quantification with Mean±SEM. (**B**) After purification of total RNA and cDNA synthesis, real time PCR was performed to determined the relative levels of β1 integrin mRNA in shCtrl and shAnxA2 cells. Relative β1 integrin mRNA levels in shCtrl vs. shAnx2 cells was calculated using the 2^-ΔΔCt method. GAPDH was included as a reference gene. Results are presented as Mean ± SEM. **(C)** shCtrl and shAnxA2 cells were treated with either DMSO (1:500) or cycloheximide for the stated amount of time and cell lysates were immunoblotted for β1 integrin (β1 int.), AnxA2, and Tubulin. Mean±SEM. * $p<0.05$. (**D**) Immunofluorescence labeling and confocal microscopy of AnxA2 and β1 integrin in shCtrl and shAnxA2 migrating epithelial cells (24 hrs). Representative confocal images of leading edge cells adjoin the wound are shown. Scale bar = 20 μm. (**E**) Cell surface proteins from shCtrl and shAnxA2 SK-CO15 cells were biotinylated on ice, captured with streptavidin, and immunoblotted to detect β1 integrin (β1 int.), E-Cadherin (E-Cad.), AnxA2, and Actin. A non-biotinylated (No Bio.) sample was included as negative control. **(F)** Cell surface proteins from shCtrl and shAnxA2 Caco2 cells were biotinylated on ice, captured with streptavidin, and immunoblotted to detect β1 integrin (β1 int.), AnxA2, and Actin.

Figure 10

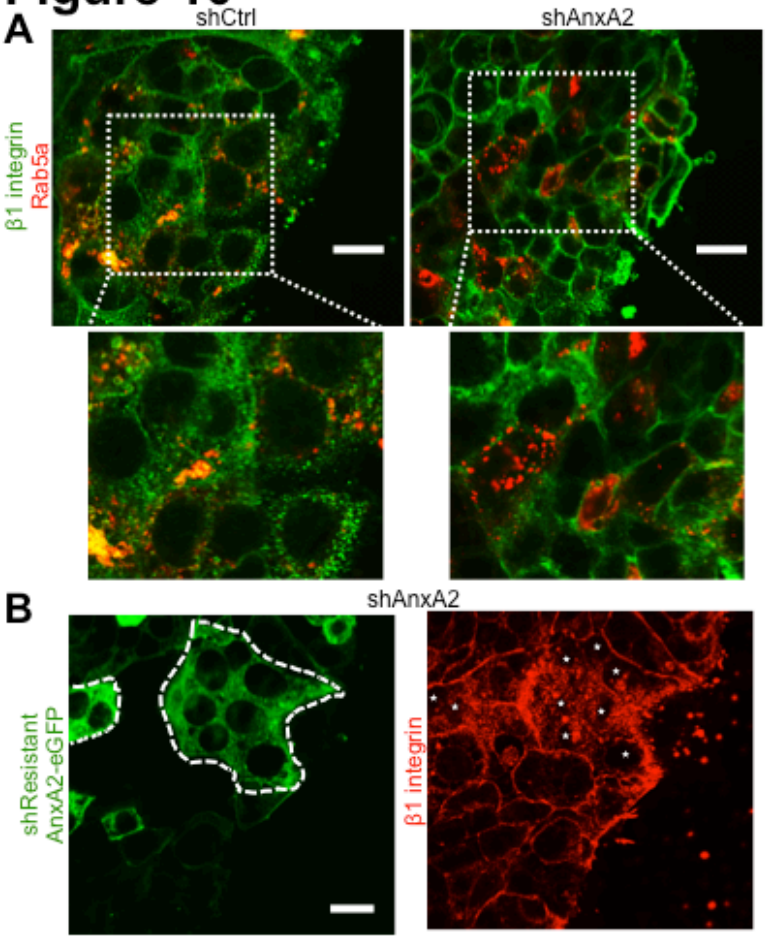

A
shCtrl
shAnxA2
β1 integrin
Rab5a
B
shAnxA2
shResistant
AnxA2-eGFP
β1 integrin

Figure 10. AnxA2 promotes β1 integrin localization in early endosomes. (**A**) Control shRNA (shCtrl) and Annexin A2 shRNA (shAnxA2) cells were transduced with a Rab5a-RFP Bacmam 2 baculovirus (red) and then wounded. Immunofluorescence labeling of β1 integrin is shown in green. Cells were analyzed by confocal microscopy. Scale bar = 10 μm. (**B**) shAnxA2 cells were transduced with shRNA resistant eGFP tagged AnxA2 (green). Epithelial cells were allowed to migrate for 24 hours. Confocal images were taken at the leading edge of cells stained for β1 integrin (red). Cells expressing the eGFP tagged Annexin A2 are highlighted with dashed lines. * shows AnxA2 expressing cells with internalized β1 integrin. Scale bar = 20 μm.

Figure 11

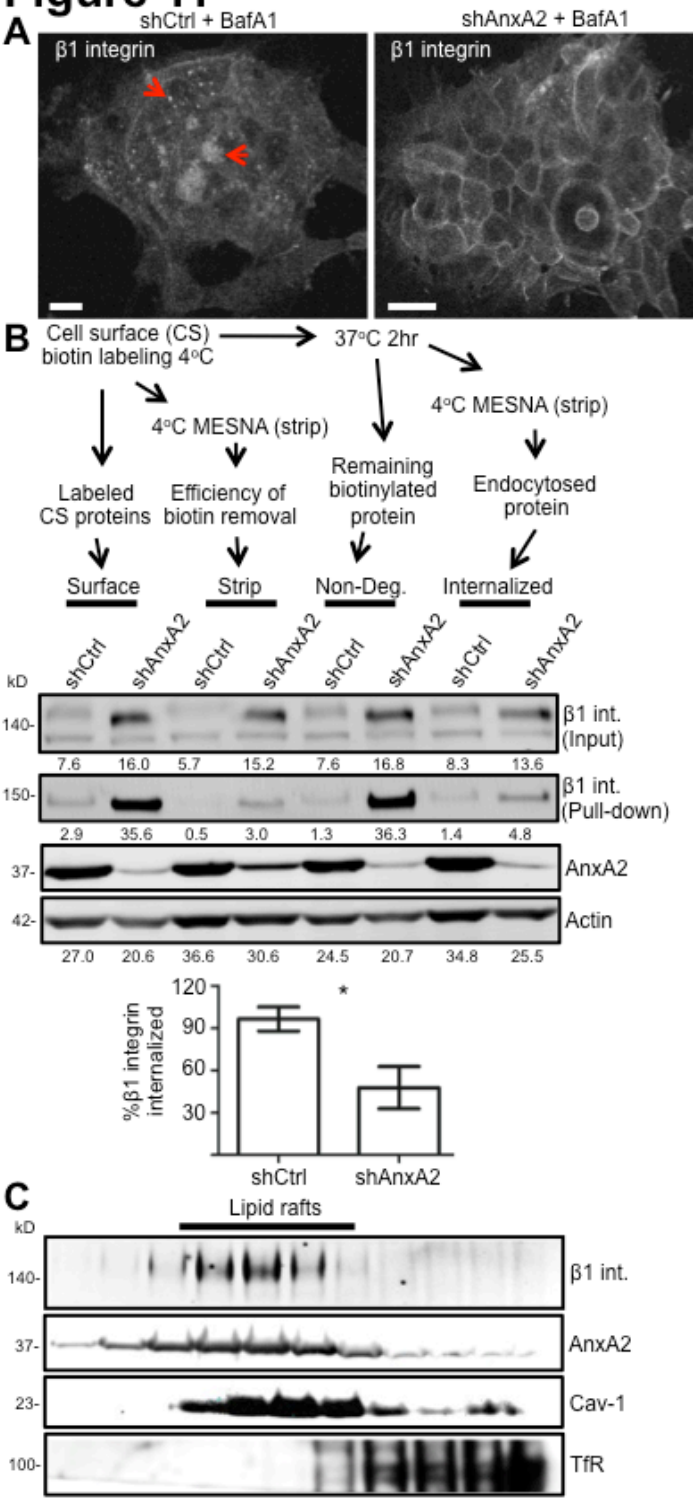

A
shCtrl + BafA1
shAnxA2 + BafA1
β1 integrin
β1 integrin
B
Cell surface (CS) biotin labeling 4°C
37°C 2hr
4°C MESNA (strip)
4°C MESNA (strip)
Labeled CS proteins
Efficiency of biotin removal
Remaining biotinylated protein
Endocytosed protein
Surface
Strip
Non-Deg.
Internalized
shCtrl
shAnxA2
kD
140-
β1 int. (Input)
7.6 16.0 5.7 15.2 7.6 16.8 8.3 13.6
150-
β1 int. (Pull-down)
2.9 35.6 0.5 3.0 1.3 36.3 1.4 4.8
37-
AnxA2
42-
Actin
27.0 20.6 36.6 30.6 24.5 20.7 34.8 25.5
%β1 integrin internalized
120
90
60
30
*
shCtrl
shAnxA2
C
Lipid rafts
kD
140-
β1 int.
37-
AnxA2
23-
Cav-1
100-
TfR

Figure 11. Down-regulation of AnxA2 delays β1 integrin internalization. (**A**) After treating control shRNA (shCtrl) and Annexin A2 shRNA (shAnxA2) cells with Bafilomycin A1 (BafA1), immunofluorescence labeling of β1 integrin (white) and confocal microscopy were performed. Red arrows highlight intracellular accumulation of β1 integrin. Scale bar = 20 μm. (**B**) A pulse chase biotinylation assay was performed in shCtrl and shAnxA2 cells to determine β1 integrin internalization rates. Cell surface proteins were biotinylated on ice (Cell surface) and protein internalization was allowed to occur at 37^{o}C for 2 hours. Biotin from non-internalized proteins was chemically removed and internalized biotinylated proteins were pulled-down with avidin agarose and subjected to immunoblotting (Internalized). A stripping control (Strip) was performed to ensure the removal of biotin from non-internalized proteins. To control for protein degradation, a non-degraded control (Non-Deg.) was included after the internalization period. Percent internalization was quantified by adding the degraded fraction to the internalized fraction divided by the surface fraction. A non-biotinylated (No Bio.) sample was included as negative control. The graph is represents three independent experiments. Mean±SEM. * $p<0.05$. (**C**) shCtrl cells were lysed by nitrogen cavitation and membrane raft fractions were isolated by floatation in continuous sucrose gradient (5-30%). The fractions were subjected to immunoblot analysis of β1 integrin (β1 int.), AnxA2, Caveolin-1 (Cav-1), and Transferrin receptor (TfR).

Figure 12

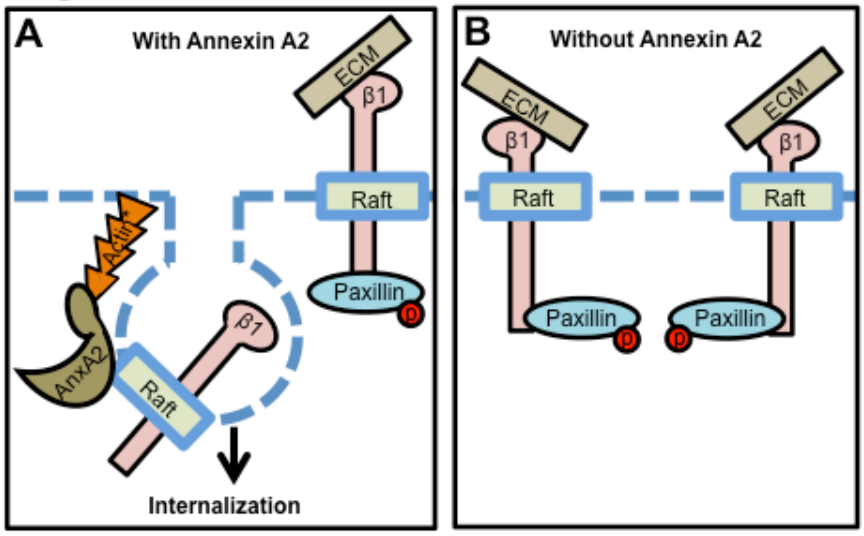
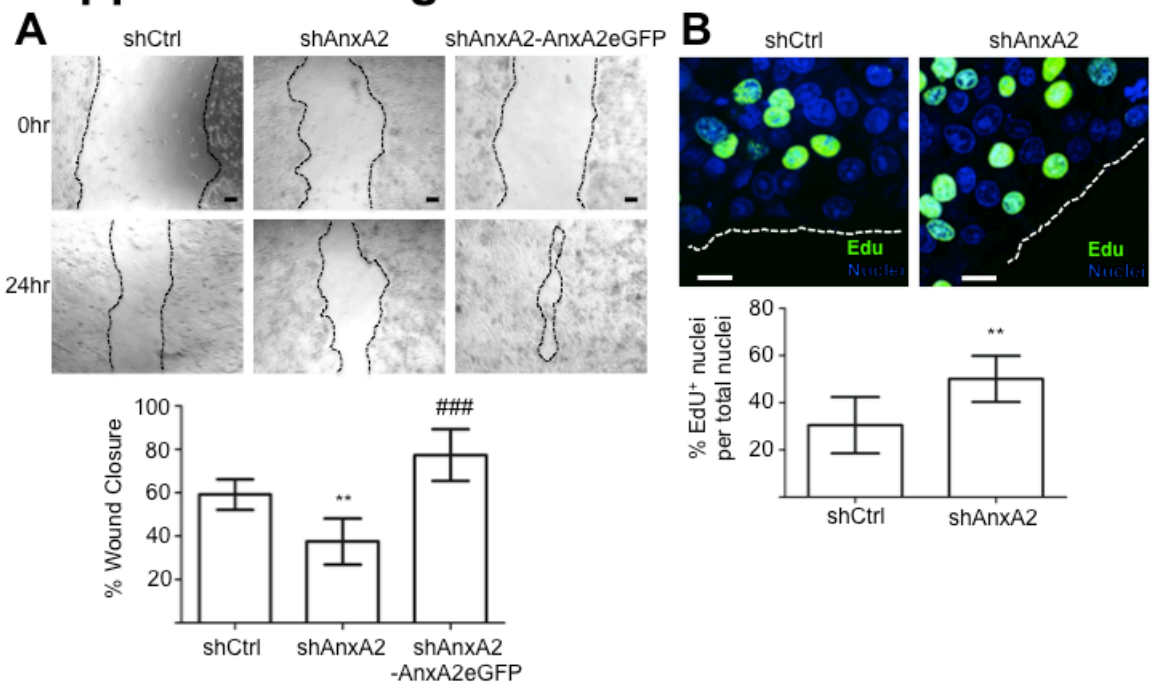

A
With Annexin A2
ECM
β1
Raft
Paxillin
P
Actin
AnxA2
β1
Raft
Internalization
B
Without Annexin A2
ECM
β1
Raft
Paxillin
P
ECM
β1
Raft
Paxillin
P

Figure 12. Proposed model for Annexin A2 mediated endocytosis of β1 integrin. (**A**) In intestinal epithelial cells, Annexin A2 (AnxA2) associates with lipid rafts (Rafts) that contain β1 integrin (β1). * To promote internalization of rafts the amino terminus of AnxA2 caps plasma membrane associated polymerizing F-Actin (Huckaba et al., 2004; Kaksonen et al., 2005; Liu et al., 2009). Internalized β1 integrin can then be re-used at the new leading edge of cells, allowing for efficient cell migration. **(B)** In IECs lacking AnxA2, lipid raft (Raft) associated β1 integrin (β1) builds up at the plasma membrane. Paxillin then becomes phosphorylated and stabilized as a consequence of the increased adhesion. Without being able to detach from the matrix, IECs lacking AnxA2 do not efficiently migrate to close wounds.

Supplemental Figure 1



Supplemental Figure 1. (A) Scratch wounds were made in epithelial monolayers and the wound size was analyzed immediately after wounding and twenty-four hours later. The graph is representative of three independent experiments. Mean±SD. Statistically significant decreased wound closure was observed in shCtrl vs. shAnxA2 cells, ** $p<0.005$; and statistically significant increased wound closure was observed in shAnxA2 vs. shAnxA2-AnxA2eGFP, $^{###}$ $p<0.001$. Scale bar = 100 μm. **(B)** shCtrl and shAnxA2 monolayers were scratch wounded and allowed to migrate for three hours. During the last hour of migration, cells were pulsed with EdU (green) to monitor proliferation. Cells were counterstained with To-Pro®3-iodide to highlight nuclei (blue). The data is representative of three independent experiments and expressed as Mean±SD.

Table 1

A

Non-silencing control
CCUAAGGUUAAGUCGCCCUCG
AnxA2 Target 1
CGGGAUGCUUUGAACAUUGAA
AnxA2 Target 2
GCAGGAAAUUAACAGAGUCUA

B

β1 integrin
FP: CCTGTCTTACTCAATGAAAG
RP: GGAAAACACCAGCAGCCGTG
GAPDH
FP: GGGTGTGAACCACGAGAAAT
RP: CCTTCCACAATGCAAAAGTT

C

AnxA2 site directed mutagenesis
FP: GATGCTGAGAGGGACGCATTAAACATTGAAACAGCCATC
RP: GTTTCAATGTTTAATGCGTCCCTCTC

D

AnxA2-eGFP subcloning
FP: TTTCCCTTGGCGCGAGCCACCATGTCTACTGTTCACGAAATCCTG
RP: TCGGTGGTGGCGCGACTAGTCATCTCCACCACACAGGTACAG

Table 1. (A) shRNA sequences used to down-regulate the corresponding gene. **(B)** Primer sequences used for real time PCR. **(C)** Primer sequences used to create a shRNA resistant AnxA2-eGFP. **(D)** Primer sequences used for sub-cloning AnxA2-eGFP from pShuttle into pLEX.

CHAPTER 3

GALECTIN-3 REGULATES DESMOGLEIN-2 AND INTESTINAL EPITHELIAL INTERCELLULAR ADHESION

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3.1 Introduction

The epithelial lining of the gastrointestinal tract serves as an important barrier that separates luminal contents from underlying tissue compartments, thereby playing an important role in host defense and mucosal homeostasis. Intestinal epithelial (IEC) intercellular junctions include adherens junction (AJ) and desmosomes (DM) that mediate cell-cell adhesion thereby providing mechanical strength to the epithelium. Intercellular junctions are actively remodeled during epithelial proliferation, migration and differentiation. Intercellular junction proteins regulate signaling events to control these biological processes (Chen et al., 2012; Eshkind et al., 2002; Kamekura et al., 2013; Nava et al., 2007; Schlegel et al., 2010; Wang et al., 2013). Thus, altered function of intercellular junction proteins contributes not only to the compromised epithelial barrier but also to changes in epithelial homeostasis observed in disease states associated with mucosal inflammation and neoplasia (Brooke et al., 2012; Dusek and Attardi, 2011; Fukushima et al., 2003; Kamekura et al., 2013; Kolegraff et al., 2011a; Yashiro et al., 2006).

Transmembrane cadherin glycoproteins in desmosomes include desmoglein (Dsg) and desmocollin (Dsc) that mediate calcium dependent intercellular adhesion in epithelial cells (Klessner et al., 2009; Marcozzi et al., 1998; Schlegel et al., 2010). N-terminal DM cadherin ectodomains bind in *trans* between neighboring cells to mediate intercellular adhesion, while their C-terminal tails associate with plaque proteins and ultimately with intermediate filaments to stabilize the protein complexes and provide mechanical strength to the epithelium (Vasioukhin et al., 2001).

Four human Dsg genes are expressed in a tissue and differentiation specific manner (Cheng and Koch, 2004; Garrod et al., 2002; Green and Simpson, 2007; Mahoney et al., 2006) The human simple columnar epithelium only expresses Dsg2 isoform (Holthofer et al., 2007). Dsg2 has highly conserved extracellular repeat domains that contain N-linked glycans (Chen et al., 2009b; Wollscheid et al., 2009). The Dsg2 distal cadherin repeat domain self-associates and also interacts with Dsc2 to mediate intercellular adhesion (Schlegel et al., 2010; Syed et al., 2002).

While a number of studies have described mechanisms by which Dsg(s) are stabilized by proteins associations with the cadherin cytoplasmic domains, our understanding of proteins that bind to their extracellular domains to mediate adhesion is not well understood. Thus, to identify proteins that regulate Dsg2 mediated intercellular adhesion, we performed mass spectrometry of proteins that co-immunoprecipitated with Dsg2 in intestinal epithelial cells. This study identified a lectin referred to as galectin-3 (Gal3) in a complex with Dsg2. Galectins are β-galactoside-binding proteins that are not only localized in the nucleus and cytoplasm, but are also secreted and bind cell surface glycans (Chabot et al., 2002; Cho and Cummings, 1995; Danielsen and van Deurs, 1997; Moutsatsos et al., 1986; Thomsen et al., 2009). Gal3 has a C-terminus carbohydrate recognition domain (Cherayil et al., 1990). A unique feature of Gal3 is the N-terminus self-association domain that enables Gal3 oligomerizaton after glycoprotein binding, thereby facilitating lattice formation at the cell surface (Ahmad et al., 2004; Lepur et al., 2012; Morris et al., 2004). This ability of Gal3 to oligomerize at cells surface provides a unique property by which it can influence stability of proteins at the cell surface. Gal3 has been reported to regulate cell-cell adhesion and other key homeostatic properties such

as cell-matrix adhesion, proliferation, and differentiation by associating with N-cadherin, β1 integrin, Epithelial Growth Factor Receptor, and Transforming Growth Factor β Receptor (Boscher and Nabi, 2013; Boscher et al., 2012; Friedrichs et al., 2008; Furtak et al., 2001; Inohara and Raz, 1995; Lajoie et al., 2007; Lau et al., 2007; Partridge et al., 2004).

We observed that Gal3 association with Dsg2 is mediated by N-linked glycans on Dsg2 and this lactose-sensitive interaction promotes Dsg2 stability at the cell surface and epithelial intercellular adhesion.

3.2 Materials and Methods

Cell Culture and reagents - SKCO-15 and T84 human model intestinal epithelial cell lines were cultured and maintained as previously described (Kolegraff et al., 2011a). MG262 (Enzo) or Chloroquine (MP Biomedical) were used at concentrations of 10 ng/ml and 200 μg/ml respectively. The Gal3 amino terminal antibody M3/38.1.2.8 HL.2 was harvested from a hybridoma TIB166 (American Type Culture Collection) and used at a concentration of 20 μg/mL. Lipofectamine 2000 was used for siRNA transfections according to the manufacturers protocol (Invitrogen). The following siRNA (Sigma Aldrich) sequences were used: siCtrl (CCUAAGGUUAAGUCGCCCUCG), siGal3_1 (GAGUCAUUGUUUGCAAUAC), siGal3_2 (CAGAAUUGCUUUAGAUUUC). Sucrose (Sigma Aldrich) and α-lactose (Sigma Aldrich) were both used at a concentration of 20 mM and incubated for eighteen hours unless stated otherwise. Two days before antibody, lactose, or siRNA treatment, cells were cultured in 1% FBS containing media and then treated/incubated overnight in serum free DMEM (Cellgro).

Immunoblots - Lysates were prepared as previously described (Rankin et al., 2013). After SDS-PAGE, resulting gels were transferred to PVDF membranes (Millipore) overnight. For human intestinal epithelial cell lines the following primary antibodies were used: Dsg2 clone AH12.2 (Kolegraff et al., 2011b; Nava et al., 2007), E-cadherin clone HECD-1 (Shimoyama et al., 1989), GAPDH (Sigma Aldrich, G9545), Gal3 clone EPR2774 (Novus Biologicals), Flotillin-1 clone 18 (Becton Dickinson). HRP labeled secondary antibodies were from Jackson ImmunoResearch Laboratories and infrared dye labeled secondary antibodies were from Kerry Perry Laboratories. For mouse intestinal epithelial cells, the primary antibody against Dsg2 was used: clone EPR6767 (Novus). Additionally, immunoblots from mouse lysates were imaged using an odyssey scanner (Licor).

Co-Immunoprecipitations - SK-CO15 monolayers ($\sim 10^6$ cells) were lysed in 100 mM KCl, 2 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 10 mM HEPES, 1% triton x-100, with protease inhibitor cocktails (Sigma Aldrich). Post-nuclear fractions were pre-cleared for two hours with sepharose beads conjugated with FLAG antibody clone M2 (Sigma Aldrich). Pre-cleared lysates were then used to immunoprecipitate Dsg2 or Gal3. The following antibodies were used for immunoprecipitation: Dsg2 clone AH12.2, Gal3 clone M3/38.1.2.8 HL.2 (American Type Culture Collection).

Mass Spectrometry - T84 monolayers ($\sim 10^9$ cells) were washed in HBSS+ and harvested in PBS with 0.05% triton x-100. Post-nuclear fractions were pre-cleared for two hours with sepharose beads conjugated with an isotype matched control mouse IgG (Sigma, I5381) and Dsg2 was immunoprecipitated using AH12.2 antibody. Immunoprecipitates were then electrophoresed in 7.5% polyacrylamide gels and Silver stained. The protein

bands were excised and mass spectrometry was performed by the Emory Microchemical Core Facility by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF)-mass spectrometry (MS) and nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Immunofluorescence labeling and confocal microscopy - For immunofluorescence labeling cells were fixed in 100% ethanol at -20^{o}C for 20 minutes. 10 μm sections were made from intestinal tissue frozen in OCT (Tissuetek). The following primary antibodies were used for immunofluorescence labeling of SKCO15 cells: Dsg2 clone AH12.2 and Gal3 clone M3/38. To immunostain murine frozen sections Dsg2 clone EPR6767 and Gal3 (clone AF1197; R&D Systems) were used. Alexa conjugated secondary antibodies were purchased from Invitrogen and images of labeled proteins were captured and analyzed using a Zeiss LSM 510 confocal microscope.

Recombinant Proteins - The extracellular domain Dsg2 was cloned into pcDNA3.0 with a His tag and transfected into CHO or HEKT29T cells with 25 μg/mL of polyethylenimine. Supernates were collected and Dsg2 was purified using nickel beads (Pierce). Recombinant human Gal3 was purchased from Abcam (#ab89487). The extracellular domain of Dsg2 was deglycosylated with PNGase F according to the manufacturer instructions.

ELISA - Immulon 2HB plates (Thermo Scientific) were coated with 5 μg of Dsg2 ectodomain (ecDsg2), incubated with Casein (Roche), 0.5 μg/mL of Gal3 in PBS for 1 hour. 20 mM sucrose or lactose (Sigma Aldrich) were added to the wells during Gal3 incubation. Gal3 binding was detected using 0.5 μg/mL M3/38.1.2.8 HL.2 (American

Type Culture Collection) and a goat anti rat HRP conjugated antibody (Jackson ImmunoResearch Laboratories).

Dispase Assay - Monolayers were treated with 2 mg/mL dispase (Roche) in HBSS+ for 30 minutes to degrade the extracellular matrix. Monolayers were then subjected to orbital shaking and pictures were taken to document the extent of monolayer fragmentation.

In Vivo Loop Model - Mice were anesthetized and the small intestine was exteriorized. After securing the ends of a 2 cm section with surgical thread, antibodies or vehicle (PBS) were injected into the lumen of the loop (Monteiro et al., 2013; Sumagin et al., 2013). After two hours of treatment, mice were euthanized and small intestinal loops were resected. Intestinal epithelial crypts were isolated from the mucosa by incubating with a cell recovery solution (Becton Dickinson) for 15 min. Harvested crypt epithelial cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma Aldrich). For antibody treatment 0.5 μg/μL of a Gal3 antibody (clone M3/38.1.2.8 HL.2; American Type Culture Collection) or Gal-1 antibody (clone 201066; R&D Systems) were used. All animal experiments were performed in accordance with protocols approved by the Emory University IACUC.

Lipid Raft Isolation - Cells were harvested in HBSS+ with 1.5% Triton X-100 supplemented containing a protease inhibitor cocktail (Sigma) and Dounced (Wheaton). A 5-30% (w/w) sucrose gradient was constructed over the lysate. The gradients were then centrifuged for 19hrs (4^{o}C, 39,000 rpm) in a Beckman SW41 rotor. Fractions (0.5ml) were collected and analyzed.

Statistics - Immunoblots were quantified using ImageJ (NIH) or ImageStudioLite (Licor). A *z*-test was performed for control-normalized experiments. An unpaired students *t*-test

was performed for all other experiments. Results were considered significant when $p<0.05$.

3.3 Results

Gal3 associates with Dsg2 in a glycosylation dependent manner. Dsg2 is key structural component of desmosomes in intestinal epithelial cells (IECs) (Chen et al., 2012; Schlegel et al., 2010). To identify proteins that regulate Dsg2 mediated intercellular adhesion, we performed Mass spectrometry of proteins that co-immunoprecipitated with Dsg2 using model intestinal epithelial cell line (T84). These studies identified Galectin-3 (Gal3) in a complex with Dsg2. The presence of Gal3 in a Dsg2 protein complex was further confirmed by immunoblotting for Gal3 after Dsg2 immunoprecipitation **(Fig. 13A)**. To determine if Gal3 association with Dsg2 is mediated through glycan binding, IECs were incubated with lactose that competitively inhibits galectin-glycan interaction prior to Dsg2 immunoprecipitation (Hirabayashi et al., 2002). The disaccharide lactose that is composed of glucose-galactose has been used as a competitor of galectin binding. In contrast, the disaccharide sucrose comprised of glucose-fructose does not inhibit galectin recognition of glycans and can therefore be used as a negative control for lactose mediated inhibition of galectin glycan binding. Indeed, Gal3 co-immunoprecipitation with Dsg2 was inhibited by lactose, but not sucrose. These findings support a galactoside-mediated association of Dsg2 with Gal3. Immunofluorescence labeling and confocal microscopy demonstrated localization of Dsg2 and Gal3 in the lateral membrane of cell-cell junctions **(Fig. 13B)**. Since Dsg2 is enriched in lipid rafts (Nava et al., 2007), experiments were performed to determine if Gal3 co-sediments with Dsg2 in lipid rafts. As shown in figure 13C, Dsg2 was enriched

in IEC lipid raft fractions that also contained Gal3. Additionally, ELISA demonstrated direct binding of recombinant Dsg2 ectodomain (ecDsg2) and Gal3 **(Fig. 13D)**. The binding of ecDsg2 and Gal3 was inhibited by lactose but not sucrose further confirming galactoside-mediated mediated interaction of these proteins **(Fig. 13D)**. Dsg2 contains multiple extracellular N-linked gylcans (Chen et al., 2009b; Wollscheid et al., 2009). Thus, we next determined if these N-linked glycans mediate Gal3 association with Dsg2. Incubation of recombinant ecDsg2 with the N-Glycosidase PNGase F resulted in a molecular weight shift from 100kDa to 70kDa supporting presence of N-linked glycans on the ectodomain of Dsg2. Lastly, PNGase F inhibited binding of ecDsg2 and Gal3 **(Fig. 13E).** Taken together, these findings support Gal3 association with N-linked glycans in ecDsg2.

Intercellular adhesion is controlled by Gal3. The contribution of Gal3 in controlling epithelial cell-cell adhesion was explored since it associates with Dsg2 that regulates intercellular adhesion. Gal3 was down-regulated with siRNA and intercellular adhesion was measured using a previously published dispase assay (Chen et al., 2012; Schlegel et al., 2010). The immunoblot in figure 14A confirms siRNA mediated down-regulation of Gal3. A non-silencing siRNA was used as control. Additionally, a Gal3 antibody M3/38 that binds to its N-terminus self-association domain (Ho and Springer, 1982), lactose, and sucrose were used independently to analyze the contributions of Gal3 to epithelial intercellular adhesion. As shown in figure 14B, down-regulation of Gal3 (>10 fold, $p<0.05$), M3/38 antibody (>10 fold, $p<0.05$) or lactose (>10 fold, $p<0.001$) but neither sucrose nor control siRNA increased monolayer fragmentation consistent with

decreased intercellular adhesion. These findings support a role of Gal3 in regulating IEC intercellular adhesion.

Dsg2 protein stability is influenced by Gal3. The above findings independently suggested that Gal3 associates with Dsg2 and regulates cell-cell adhesion. Previous studies have shown that DM cadherins are recruited to the plasma membrane after which they are incorporated into the junction and stabilized by associating with underlying plaque and cytoskeletal proteins (Kowalczyk et al., 1999). Additionally, remodeling of desmosomes in response to environmental stimuli is associated with destabilization of junction associated cadherins and their internalization from the cell surface. Internalized cadherins can be degraded or recycled back to the plasma membrane. Additionally, the Gal3 cell surface lattice has been reported to inhibit internalization and degradation of cell surface receptors such as Epidermal Growth Factor Receptor (Lau et al., 2007; Partridge et al., 2004). Thus, to further explore the relationship between Gal3 and Dsg2, we determined if Gal3 down-regulation influences Dsg2 protein levels. Indeed, as shown in the western blot in figure 15A down-regulation of Gal3 resulted in a 2-fold decrease in Dsg2 protein. Consistent with these results immunofluorescence labeling and confocal microscopy revealed decreased Dsg2 staining in cells treated with Gal3 siRNA **(Fig. 15B)**. Analogous to Gal3 siRNA treatment, incubation of IECs with lactose or Gal3 mAb (M3/38) also decreased Dsg2 steady state protein levels compared to cells that were treated with media alone, control siRNA, or sucrose **(Fig. 15C)**. We next determined if Gal3 antibody (M3/38) or lactose treatment resulted in proteasomal or lysosomal degradation of Dsg2. While treatment with lactose and Gal3 antibody (M3/38) decreased

Dsg2 protein, co-incubation with MG262 to inhibit proteasomal degradation but not chloroquine an inhibitor of lysosomal degradation, restored Dsg2 protein **(Fig. 15C)**.

Inhibition of Gal3 in vivo decreases intestinal epithelial Dsg2 protein. The above *in vitro* results using model intestinal epithelial cell lines support a role for Gal3 in regulating Dsg2 protein stability and intercellular adhesion. We next verified the influence of Gal3 on Dsg2 protein *in vivo* using a murine intestinal loop model **(Fig. 16A)** (Monteiro et al., 2013; Sumagin et al., 2013). The lumen of isolated small intestinal loops from anesthetized mice were perfused with either Gal3 mAb (M3/38), Gal-1 mAb (201066) or vehicle (PBS) for two hours prior to harvesting intestinal epithelial cells for immunoblotting. Analogous to the *in vitro* results, a two-fold decrease in Dsg2 protein was observed after infusion of the intestinal lumen with Gal3 antibody (M3/38), but not Gal-1 antibody (201066) or PBS **(Fig. 16B)**. Additionally, immunofluorescence labeling and confocal microscopy of the intestinal mucosa revealed Dsg2 distribution in the lateral membrane of epithelial cell-cell contacts. However, perfusion with the Gal3 antibody (M3/38) induced redistribution of Dsg2 into intracellular compartments **(Fig. 16C)**. Similarly, Gal3 was re-localized from the lateral membrane of intestinal epithelial cells into intracellular vesicle-like structures after perfusion with the Gal3 antibody (M3/38) **(Fig. 16C)**.

3.4 Discussion

DMs in intestinal epithelial cells are visualized as multiple spot-welds in the lateral membrane that reside below the AJ. Cadherins in these junctions mediate strong intercellular adhesion in addition to controlling epithelial homeostasis (Kolegraff et al.,

2011a; Nava et al., 2007; Schlegel et al., 2010). While mass spectroscopy has shown that Dsg2 contains N-linked glycans (Chen et al., 2009b; Wollscheid et al., 2009), their functional significance has not been established. However, N-glycans on N-Cadherin have been shown to influence cadherin mediated intercellular binding (Langer et al., 2012).This study identified Gal3 association with N-linked glycans in Dsg2 ectodomains that stabilizes Dsg2 at the cell surface and inhibits its proteasomal degradation. Furthermore, Gal3 regulates intercellular adhesion in IECs. Previous reports have shown that lactose, peptides specific to Gal3 or the Gal3 mAb (M3/38) weaken intercellular association (Inohara et al., 1996; Inohara and Raz, 1995; Zou et al., 2005). Interestingly lactose did not decrease Gal3 levels in whole cell lysates. Additionally, the antibody that binds epitopes in the self-association domain of Gal-3 (M3/38) resulted in the redistribution of Gal3 into intracellular compartments. Importantly these findings suggest that changes in localization and/or association of Gal3 with its target glycoprotein are important for its function rather than changes in its steady state levels. We therefore believe disruption of the Gal3 lattice with M3/38 influences Dsg2 stability and intercellular adhesion.

Our study demonstrates a role of Gal3 in regulating the steady state level of a desmosomal cadherin protein. Gal3 has been reported to stabilize cell surface receptors including Epithelial Growth Factor Receptor and Transforming Growth Factor β Receptor (Lau et al., 2007; Partridge et al., 2004). However, not all Gal3-associated receptors are stabilized by Gal3. Gal3 increases the mobility another cadherin, N-cadherin (Boscher et al., 2012). Additionally, Gal3 promotes internalization of β1 integrin from the cell surface (Friedrichs et al., 2008; Furtak et al., 2001).

Gal3 has been reported to self-associate into oligomers of up to five Gal3 molecules. The extracellular Gal3 lattice can be viewed as cell surface microdomains that recruit glycoproteins based on the N-glycan number and branching (Lau et. al. 2007). Gal3 oligomers therefore represent higher order structures that retain cell surface Dsg2 at points of intercellular adhesion in a way that is reminiscent of Gal3 lattice stabilization of Epidermal Growth Factor Receptor and Transforming Growth Factor β Receptor at the cell surface. Desmosomes are dynamic structures that undergo remodeling in response to a number of physiologic and pathologic stimuli (Kowalczyk et al., 1999). The Gal3 higher order structure presumably keeps Dsg2 localized within cell-cell contacts thereby inhibiting its internalization and subsequent proteasomal degradation **(Fig. 17)**. However, the precise mechanism by which Gal3 stabilizes Dsg2 remains to be identified. Dsg proteins have an extended C-terminal unique region (DUR) that can mediate Dsg2 self-interaction to influence stabilization of Dsg2 at the cell surface by inhibiting its internalization (Chen et al., 2012). Similarly, in the extracellular space Gal3 could influences Dsg2 clustering and associations at the cell surface that could serve as a complementary mechanism to inhibit its internalization from the plasma membrane. Additionally, since E-cadherin undergoes proteasomal degradation (Hartsock and Nelson, 2012) in a ubiquitin-dependent manner, Gal3-dependent clustering of Dsg2 within the plasma membrane might inhibit its ubiquitination and proteasomal degradation. Since Dsg2 and Gal3 co-fractionate in lipid rafts, the Gal3 lattice could regulate partitioning of Dsg2 in membrane rafts (Brennan et al., 2012; Resnik et al., 2011). Thus, a multimeric Gal3/Dsg2 lattice might hinder the ability of Dsg2 to undergo internalization from the plasma membrane and subsequent degradation. In future studies, it will be important to

elucidate the relationship between Gal3 mediated stabilization of plasma membrane-associated Dsg2, cytoplasmic domain phosphorylation, and signaling events that serve to strengthen cell-cell adhesion in desmosomes.

This study highlights an important role of Gal3 and glycosylated Dsg2 in intercellular adhesion, contributing to epithelial barrier integrity. Interestingly, loss of Gal3 protein (Brazowski et al., 2009; Jensen-Jarolim et al., 2002) and Gal3 autoantibodies (Jensen-Jarolim et al., 2001) have been observed in patients with ulcerative colitis a pathologic state associated with chronic active mucosal inflammation (Bodger et al., 2006; Campbell et al., 2001). Furthermore, changes in glycosylation of proteins have been reported in patients with chronic inflammatory disorders such as ulcerative colitis and Crohn's disease (Bodger et al., 2006; Shinzaki et al., 2013). Such altered glycosylation states have been linked to compromised mucin production and function that in turn results in barrier dysfunction with subsequent increase in mucosal inflammation (Campbell et al., 2001). In summary, these studies highlight an important role of Gal3 in controlling Dsg2 protein stability and intercellular adhesion that is intimately linked to barrier function of the intestinal epithelium.

Figure 13

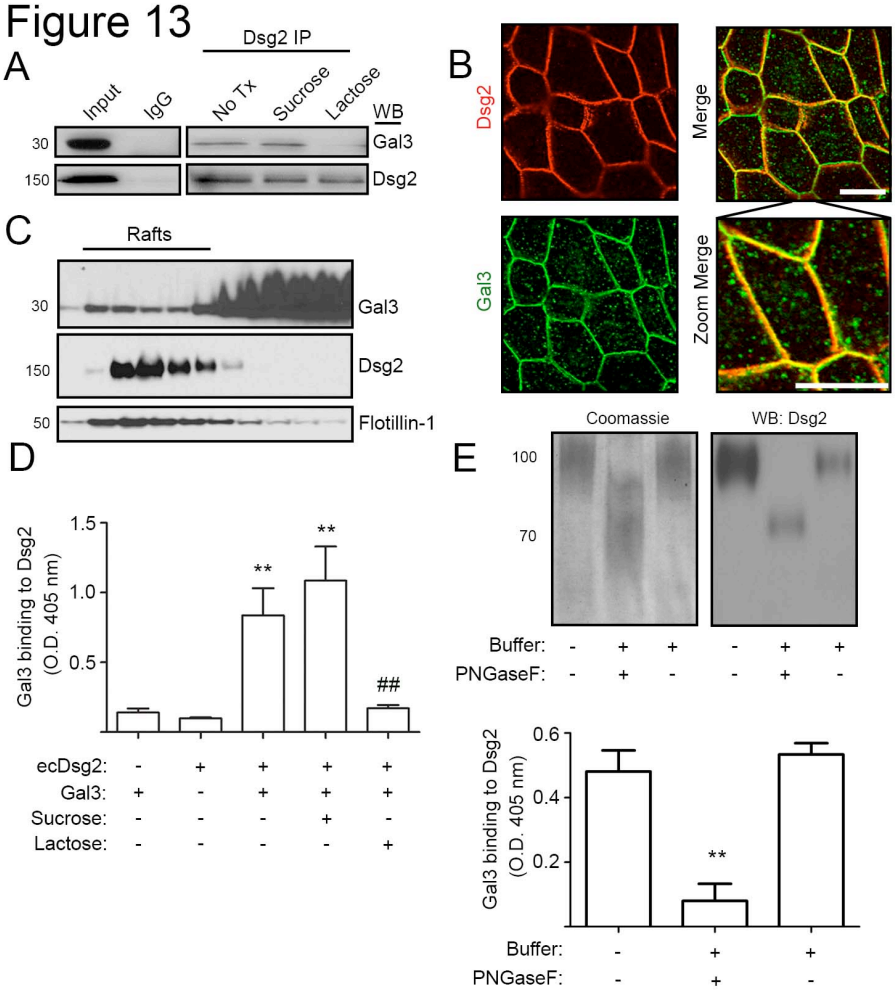
A
Dsg2 IP
Input
IgG
No Tx
Sucrose
Lactose
WB
30
150
Gal3
Dsg2
B
Dsg2
Merge
Gal3
Zoom Merge
C
Rafts
30
150
50
Gal3
Dsg2
Flotillin-1
D
Gal3 binding to Dsg2 (O.D. 405 nm)
1.5
1.0
0.5
**
**
##
ecDsg2:
Gal3:
Sucrose:
Lactose:
E
Coomassie
WB: Dsg2
100
70
Buffer:
PNGaseF:
Gal3 binding to Dsg2 (O.D. 405 nm)
0.6
0.4
0.2
**
Buffer:
PNGaseF:

Figure 13. Gal3 associates with Dsg2 in a glycosylation dependent manner. (A) Dsg2 was immunoprecipitated of from SKCO15 cells in the absence and presence of 20 mM sucrose or lactose. Representative immunoblots show Gal3 and Dsg2 in the Dsg2 in the protein complex. Immunoblots are representative of three independent experiments. **(B)** Immunofluorescence labeling and confocal microscopy to localize Dsg2 and Gal3 in SKCO-15 cells. Scale Bar = 20 μm. **(C)** Isolation of membrane rafts from SKCO15 cells by floatation in continuous sucrose gradients (5-30%). Gradient fractions were immunoblotted for Gal3, Dsg2, and Flotillin-1 protein. **(D)** ELISA to measure binding of recombinant Gal3 to immobilized Dsg2 ectodomain in the presence and absence of 20 mM sucrose and lactose. The results represent the Mean±SD from three independent experiments. Gal3 and Dsg2 or Gal3 and Dsg2 with sucrose vs. Gal3 or Dsg2 only, ** $p<0.01$. Lactose vs. Sucrose treatment, ## $p<0.01$. **(E)** The ectodomain of Dsg2 was treated with either a deglycosylation buffer alone (buffer) or buffer with PNGaseF to remove N-linked glycans. Samples subjected to SDS-PAGE were either stained with coomassie or immunoblotted to detect Dsg2 protein (upper panel). ELISA was performed to determine binding of Dsg2 ectodomain with Gal3 in the presence and absence of PNGase F (lower panel). The results represent the Mean±SD from three independent experiments. ** $p<0.01$.

Figure 14

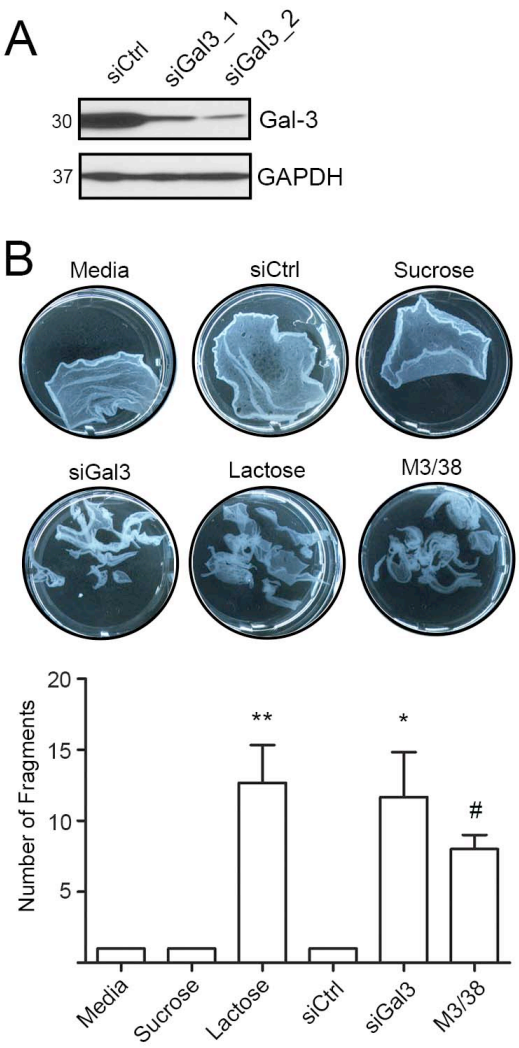
A
siCtrl
siGal3_1
siGal3_2
30
Gal-3
37
GAPDH
B
Media
siCtrl
Sucrose
siGal3
Lactose
M3/38
Number of Fragments
20
15
10
5
**
*
#
Media
Sucrose
Lactose
siCtrl
siGal3
M3/38

Figure 14. Intercellular adhesion is controlled by Gal3. (A) Immunoblots to verify down-regulation of Gal3 using two different siRNA sequences (siGal3_1,siGal3_2 in SKCO15 cells. A non-silencing siRNA control was included (siCtrl). GAPDH was used as a loading control. **(B)** Dispase assay was performed to determine the strength of intercellular adhesion. Gal3 was down-regulated with siRNA (siGal3) or its functional association was inhibited with either lactose or a Gal3 mAb that recognizes its homodimerization domain (M3/38). Monolayers were subjected to the dispase assay and epithelial fragments were quantified as a measure of cell-cell adhesion. Mean±SEM. Lactose vs. Sucrose or Media, ** $p<0.001$. siCtrl vs. siGal3, * $p<0.05$. M3/38 vs. Media, # $p<0.05$.

Figure 15

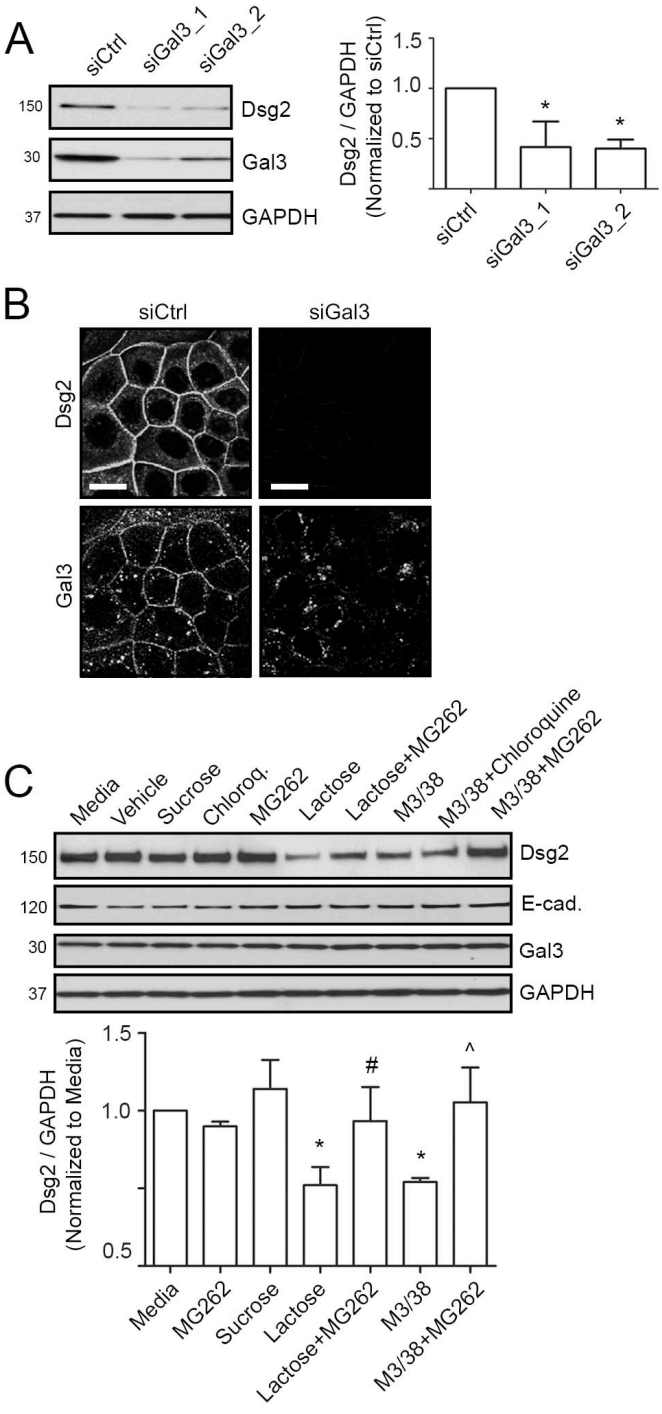
A
siCtrl
siGal3_1
siGal3_2
150
Dsg2
30
Gal3
37
GAPDH
Dsg2 / GAPDH
(Normalized to siCtrl)
1.5
1.0
0.5
*
*
siCtrl
siGal3_1
siGal3_2
B
siCtrl
siGal3
Dsg2
Gal3
C
Media
Vehicle
Sucrose
Chloroq.
MG262
Lactose
Lactose+MG262
M3/38
M3/38+Chloroquine
M3/38+MG262
150
Dsg2
120
E-cad.
30
Gal3
37
GAPDH
Dsg2 / GAPDH
(Normalized to Media)
1.5
1.0
0.5
*
#
*
^
Media
MG262
Sucrose
Lactose
Lactose+MG262
M3/38
M3/38+MG262

Figure 15. Dsg2 protein stability is influenced by Gal3. (A) Gal3 protein was down-regulated using two different siRNAs in SKCO15 cells (siGal3_1, siGal3_2). Immunoblots to show decreased Dsg2 protein in cells with down-regulated Gal3. Non-silencing siRNA was used as a control (siCtrl) and GAPDH to demonstrate equal loading per lane. Mean±SEM. siCtrl vs. siGal3_1, * $p<0.05$. siCtrl vs. siGal3_2, * $p<0.05$. **(B)** Immunofluorescence labeling and confocal microscopy to assess Dsg2 and Gal3 localization in SKCO15 cells treated with non-silencing control (siCtrl) and or Gal3 siRNA (siGal3) in SKCO15 cells. Scale Bar = 25 μm **(C)** Immunoblots to determine the influence Gal3 mAb (M3/38), lactose, or sucrose on Dsg2 steady state protein levels. MG262 or chloroquine was used to evaluate the influence of Gal3 on proteasomal vs. lysosomal degradation of Dsg2. Mean±SEM. Treatment vs. Media only, * $p<0.05$. Lactose vs. Lactose+MG262, # $p<0.05$. M3/38 vs. M3/38+MG262, ^ $p<0.05$.

Figure 16

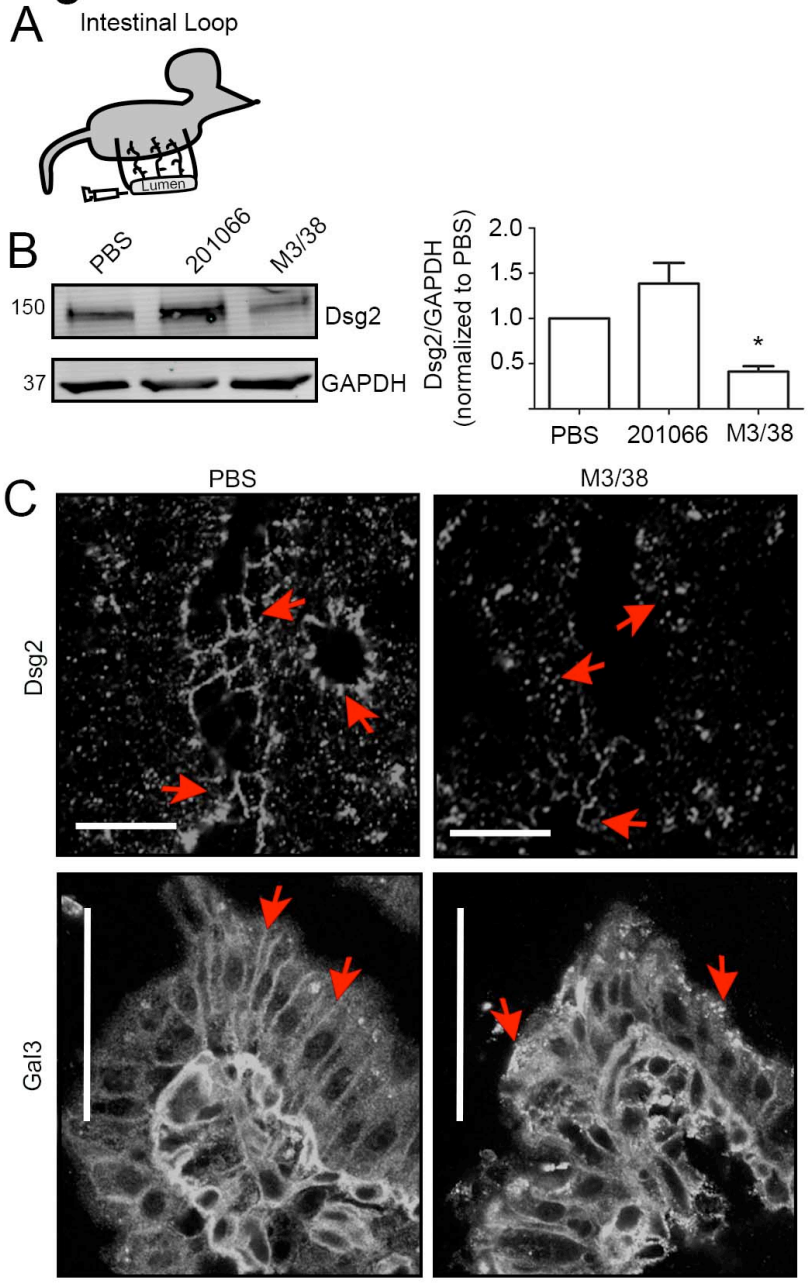
A
Intestinal Loop
Lumen
B
PBS
201066
M3/38
150
Dsg2
37
GAPDH
Dsg2/GAPDH
(normalized to PBS)
2.0
1.5
1.0
0.5
*
PBS
201066
M3/38
C
PBS
M3/38
Dsg2
Gal3

Figure 16. Inhibition of Gal3 in vivo decreases intestinal epithelial Dsg2 protein. (A) A cartoon representation of the intestinal loop method**. (B)** Vehicle alone (PBS), Gal-1 mAb (201066, rat anti mouse), or a Gal3 mAb (M3/38, rat anti mouse) was introduced into the lumen of murine small intestinal loops as described in the methods. Two hours after treatment intestinal epithelial cells were isolated and immunoblotted for Dsg2 and GAPDH protein. Mean±SEM. PBS vs. Gal3, * $p<0.05$. **(C)** Immunofluorescence labeling and confocal microscopy to detect distribution of Dsg2 or Gal3 in frozen sections of the small intestinal loop treated with PBS or M3/38 antibody. Scale bar = 50 μm.

Figure 17

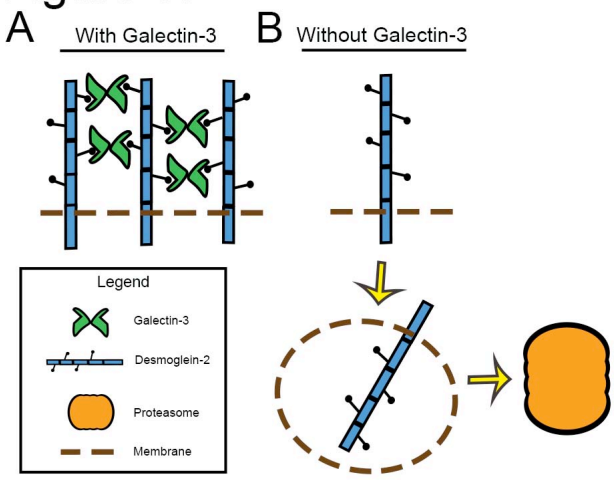
A
With Galectin-3
B
Without Galectin-3
Legend
Galectin-3
Desmoglein-2
Proteasome
Membrane

Figure 17. A proposed model for Gal3 lattice regulation of Dsg2 protein. (A) Gal3 is secreted into the extracellular space oligomerizes and binds N-linked glycans in Dsg2 ectodomain to stabilize Dsg2 in the plasma membrane. **(B)** In the absence of Gal3, Dsg2 is internalized from the plasma membrane and undergoes proteasomal degradation.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

4.1 Summary - AnxA2 regulation of endocytosis

Chapter 2 demonstrated that AnxA2 regulates cell-matrix adhesion by controlling endocytosis of β1 integrin (Rankin et al., 2013). In the proposed model AnxA2 associates with the inner leaflet of the plasma membrane and regulates internalization of β1 integrin into endosomes without directly associating with β1 integrin. It has been proposed that endocytosis from the cell surface requires proteins that associate with the inner leaflet of the plasma membrane at sites of budding vesicle and cap polymerizing F-Actin (Liu et al., 2009). AnxA2 has both these properties as it caps F-Actin and associates with membranes thereby linking the force of actin polymerization to endocytosis of cell surface b1integrin. Thus, our model supports a role for AnxA2 as a key component of the endocytic machinery **(Fig. 18A)**. This endocytic machinery also contains caveolin and dynamin and likely regulates endocytosis of other cell surface transmembrane proteins. In Chapter 2, the steady state and cell surface levels of E-cadherin did not change after down-regulation of AnxA2. In unpublished data presented herein, the steady state and cell surface level of a tight junction transmembrane protein, Junctional Adhesion Molecule-A (JAM-A) are not regulated by AnxA2 **(Fig. 18A)**. However the stability of another tight junction protein, Occludin, is controlled by AnxA2. This suggests that AnxA2 differentially controls endocytosis of intercellular junction transmembrane proteins but the underlying mechanism remain **(Fig. 18B,C)**.

My model furthers our understanding of the relationship between intracellular calcium control of cell migration. Localized calcium bursts have been observed in disassembling focal adhesions (Tsai and Meyer, 2012; Wei et al., 2012). Since AnxA2 association with membranes is calcium dependent, it is likely that localized calcium

release enables AnxA2 binding to the plasma membrane thereby promoting internalization of b1 integrin in focal cell matrix adhesions of migrating epithelial cells. In addition to calcium mediated plasma membrane binding, the phosphorylation of AnxA2 has been suggested to influence its biological function (de Graauw et al., 2008; Hubaishy et al., 1995). For example, phosphorylation of AnxA2 at tyrosine 23 residue coincides with insulin mediated cell-matrix detachment (Biener et al., 1996; Rescher et al., 2008). Thus the phosphorylation of AnxA2 and depletion of local intracellular calcium levels are likely inhibit AnxA2 mediated endocytosis of β1 integrin and cell migration.

This is the first study that has demonstrated a role of AnxA2 in the internalization of a cell surface receptor or a transmembrane protein involved in cell-matrix adhesion. Three prior studies have observed that AnxA2 participates in budding of vesicles from the cell surface or AnxA2 expression is correlated with receptor internalization. Expression of AnxA2 has been correlated with internalization of the insulin receptor (Biener et al., 1996), phagocytosis (Law et al., 2009) and the internalization of viral particles (Gonzalez-Reyes et al., 2009; Law et al., 2009). Lastly AnxA2 has only been previously correlated to regulating cell-matrix adhesion (Rescher et al., 2008).

4.2 Future directions - AnxA2 regulation of endocytosis

Identification of AnxA2 domains that regulate endocytosis of cell surface proteins.

The contribution of AnxA2 calcium binding and F-Actin capping domains in controlling endocytosis of cell surface transmembrane proteins remains to be defined. AnxA2 threonine 282 and aspartate 321 residues bind single calcium ions to mediate

membrane attachment (Shao et al., 2006). Additionally, the F Actin capping domain resides in nine amino acids in its carboxy terminus (Filipenko and Waisman, 2001). Thus, generation of epithelial cell lines expressing AnxA2 proteins with mutation in these residues will be a helpful tool in identifying the contribution specific AnxA2 domains in controlling endocytosis.

Does AnxA2 regulate basolateral and/or FA endocytosis?

Although the proposed model suggests that AnxA2 regulates β1 integrin in focal adhesions, this thesis did not specifically address the precise location where AnxA2 regulates β1 integrin internalization from the plasma membrane. β1 integrin localizes in focal adhesions and in the lateral membrane of epithelial cells, and therefore AnxA2 could potentially regulate endocytosis of β1 integrin from either of these sites. Additional studies utilizing tagged AnxA2 and β1 integrin will help to more clearly understand the spatial relationship of these proteins in the lateral vs. basal plasma membrane. Additionally, complementary immunogold labeling and ultrastructural studies will also help in mapping the distribution of AnxA2 and integrins in vesicles budding from the plasma membrane of migrating IECs.

AnxA2 - direct or indirect regulation of endocytosis

While our model suggests that AnxA2 is a component of the endocytic machinery, AnxA2 could also indirectly contribute to the endocytosis of cell surface transmembrane proteins. Previous studies have demonstrated that AnxA2 influences activity of the small GTPase, RhoA (Babbin et al., 2007; Garrido-Gomez et al., 2012; Rescher et al., 2008) that in turn modulates membrane lipid raft dynamics (Lamaze et al., 1996). However, β1 integrin has also been reported to negatively regulate RhoA GTPase

activity (Arthur et al., 2000; Zhou and Kramer, 2005). Given these scenarios, AnxA2 could control cell-matrix adhesion by multiple mechanisms that involve control of integrin, membrane lipid raft and GTPase dynamics. Further defining the relationship between AnxA2, β1 integrin, and RhoA will help in understanding mechanisms of focal adhesion assembly and disassembly during cell movement and wound closure.

AnxA2 – a component of the endocytic machinery?

My data suggests that AnxA2 is a component of the endocytic machinery. Thus, future directions should include identification of a role of AnxA2 in regulating endocytosis of other membrane associated proteins. An initial approach would include a proteomic screen that compares transmembrane proteins in IECs expressing AnxA2 vs. cell lacking this protein (Ong et al., 2002).

Does AnxA2 regulate endocytosis in vivo?

The *in vitro* studies in this thesis utilized model intestinal epithelial cell lines. It is important to validate some of our observations in *in vivo* studies using AnxA2$^{-/-}$ mice (Law et al., 2009) and in human tissue. A previous study demonstrated that AnxA2 protein is increased in the intestinal epithelium of patients with inflammatory bowel disease (Shkoda et al., 2007). Therefore an extensive characterization of AnxA2 using human tissue from IBD patients is important in understanding the role of AnxA2 in the pathogenesis of colitis.

4.3 Summary – Gal-3 regulation of Dsg2

The data presented in Chapter 3 reveals that Gal-3 associates with and stabilizes Dsg2 to strengthen intercellular adhesion in intestinal epithelial cells. Since Gal-3 is

secreted outside the cell and forms oligomers (Ahmad et al., 2004; Yang et al., 1998), it has been suggested that Gal-3 oligomerizaton allows Gal-3 to cluster receptors, thereby stabilizing them at the cell surface. I have therefore proposed that Gal-3 forms multivalent structures either before or after association with Dsg2 to mediate clustering of Dsg2. These higher order structures stabilize Dsg2 in desmosomes by inhibiting their internalization and proteasomal degradation through an undefined mechanism **(Fig. 15B)**. Studies in other cell types have proposed that Gal-3 peptides mimicking the self-association domain or carbohydrate-binding domain inhibit cell-cell adhesion (Zou et al., 2005). Our study uses a genetic approach to demonstrate for the first time that Gal-3 regulates the stability of a cadherin and controls cell-cell adhesion.

4.4 Future directions - Gal-3 mediated stabilization of Dsg2

Contribution of a Gal-3 lattice in stabilization of Dsg2at the cell surface

Further experiments are needed to understand the contribution of the Gal-3 lattice in stabilizing Dsg2 at the cell surface. The results obtained with the Gal-3 antibody (M3/38) used in Chapter 3 suggest that the N-terminal domain of Gal-3 is necessary for Gal-3 mediated stabilization Dsg2. While the model suggests that Gal-3 self-association and therefore lattice formation regulates Dsg2, Gal-3 could simply stabilize Dsg2 by binding to it as a monomer. For instance, Gal-3 could stabilize Dsg2 by enhancing Dsg2 *trans* associations. Biochemical techniques such as size exclusion chromatography, native gel electrophoresis, and analytical centrifugation will be useful in identifying the stoichiometry of Gal-3/Dsg2 complexes. Furthermore, the contribution of Gal-3 carboxy terminus in stabilizing Dsg2 should be explored.

Is the cytoplasmic domain of Dsg2 modified in the absence of Gal-3?

While our studies suggest that Gal-3 stabilizes the extracellular domain of Dsg2, its role in regulating the Dsg2 cytoplasmic domains needs to be evaluated. The association of the extracellular Gal-3 lattice with Dsg2 could allosterically influence the conformation of a Dsg2 cytoplasmic internalization motif. Dsg2 cytoplasmic domain has ten rapid endocytosis motifs (TXXΦ) and two dileucine internalization motifs. Additionally, loss of Gal-3 could result in differentially phosphorylation or ubiquitination of Dsg2 leading to its degradation. Thus, analyzing the impact of expressing Dsg2 cytoplasmic domain mutant proteins will be helpful in elucidating the mechanism of Gal-3 mediated stabilization of Dsg2.

Biological function of Gal-3 - Dsg2 association in vivo

The *in vivo* relevance of Gal-3 mediated stabilization of Dsg2 on intercellular adhesion and intestinal epithelial barrier function should be further explored. Dsg2 protein expression is altered in intestinal mucosal inflammation that is associated with epithelial barrier defect (Gassler et al., 2001). Thus, future studies directed at understanding the mechanisms by which Gal-3 and Dsg2 glycosylation contribute barrier compromise in inflammation are important. Furthermore, a screen for therapeutic agents that promote Gal-3 mediated Dsg2 stabilization will be useful in treating barrier defect observed in mucosal inflammatory disorders such as inflammatory bowel disease.

Figure 18

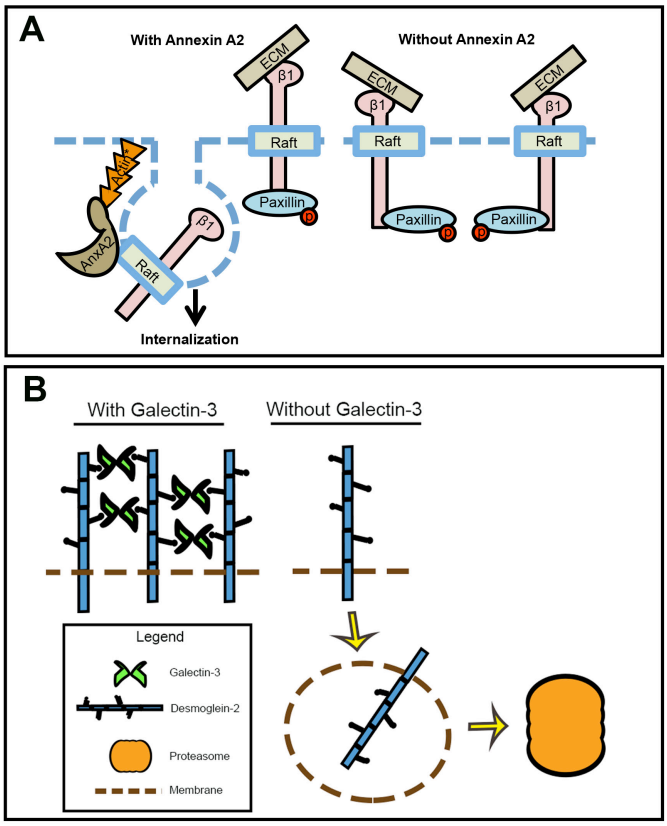
A
With Annexin A2
Without Annexin A2
ECM
β1
Raft
Paxillin
AnxA2
Internalization
B
With Galectin-3
Without Galectin-3
Legend
Galectin-3
Desmoglein-2
Proteasome
Membrane

Figure 18. Comparison of the models generated in Chapters 2 and 3. (A) Chapter 2 identified that in IECs without AnxA2: β1 integrin internalization is defective, cell-matrix adhesion is enhanced, and wound closure is delayed. As AnxA2 resides in the cytosol, caps F-Actin, and associates with membranes in a calcium dependent manner, we proposed a model whereby AnxA2 is part of the endocytic machinery linking the force of F-Actin polymerization to vesicle budding **(B)** Chapter 3 both identified that Gal-3 associated with Dsg2 and regulated its stability. In contract to AnxA2 mediated internalization of β1 integrin Gal-3 directly bound to glycans Dsg2 thereby negatively regulating the internalization of Dsg2. As a Mab against the homodimerization domain destabilized Dsg2 and cell-matrix adhesion, the model presented in Chapter 3 suggests Gal-3 clustering of Dsg2 inhibits the endocytosis of Dsg2. Lastly, as a proteasome inhibitor rescued the stability of Dsg2 in the absence of Gal-3, we suggest Dsg2 degraded at the proteasome in the absence of Gal-3.

Figure 19

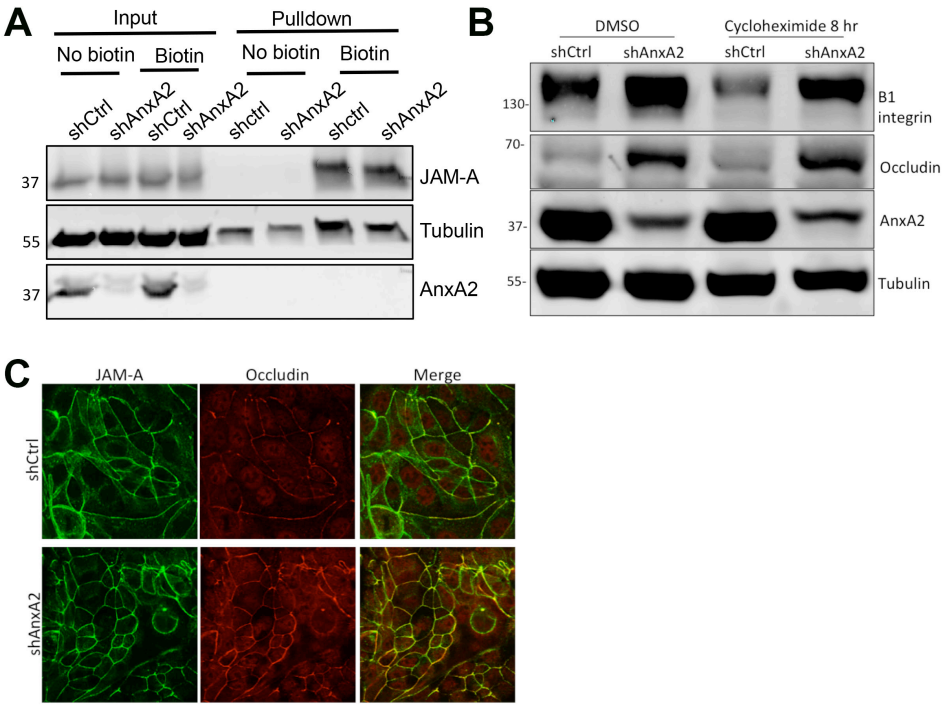
A
Input
Pulldown
No biotin
Biotin
No biotin
Biotin
shCtrl
shAnxA2
shCtrl
shAnxA2
shctrl
shAnxA2
shctrl
shAnxA2
37
JAM-A
55
Tubulin
37
AnxA2
B
DMSO
Cycloheximide 8 hr
shCtrl
shAnxA2
shCtrl
shAnxA2
130-
B1 integrin
70-
Occludin
37-
AnxA2
55-
Tubulin
C
JAM-A
Occludin
Merge
shCtrl
shAnxA2

Figure 19. AnxA2 regulates occludin but not JAM-A. (A) Cell surface biotinylation was performed in a model intestinal epithelial cell line, SK-CO15, that had been transduced with control shRNA (shCtrl) or AnxA2 shRNA (shAnxa2) containing lentivirus. Biotinylated proteins were captured with avidin Sepharose beads and subjected to immunoblotting. Loss of AnxA2 does not influence the steady state and cell surface levels of JAM-A. **(B)** Occludin steady state levels were increased in SK-CO15 cells lacking AnxA2 and are stable over the period of eight hours after inhibition of protein translation with cycloheximide. **(C)** Immunofluorescence labeling and confocal microscopy to detect occludin and JAM-A in shCtrl and shAnxA2 cells. While increased occludin labeling was observed in shAnxA2 cells, JAM-A labeling remained unchanged.

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