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Regulation of intestinal epithelial homeostasis by the desmosomal cadherins

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Abstract Cover Page

Regulation of intestinal epithelial homeostasis by the desmosomal cadherins

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B.A., Johns Hopkins University, 2002

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An abstract of
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Abstract

Abstract

Regulation of intestinal epithelial homeostasis by the desmosomal cadherins
By Keli Nicole Kolegraff

Desmocollin-2 (Dsc2) and desmoglein-2 (Dsg2) are the transmembrane cell adhesion proteins of desmosomes. Down-regulation of Dsc2 has been reported in colorectal carcinomas, however no mechanistic studies have examined the contribution of Dsc2-deficiency to tumorigenesis. Using RNAi to down-regulate the expression of Dsc2, I have examined the effect of Dsc2 loss on the behavior of colonic epithelial cells. Down-regulation of Dsc2 in a model colonic epithelial cell line SK-CO15 increases cell proliferation and enhances β-catenin signaling, a well-established pro-proliferative pathway in intestinal epithelial cells. Furthermore, Dsc2-deficient cells exhibit activation of Akt, a serine/threonine kinase which has been reported to regulate the transcriptional activity of β-catenin. Inhibition of Akt with triciribine or siRNA-mediated Akt depletion prevents the activation of β-catenin-dependent transcription and cell proliferation following Dsc2 loss. These data suggest that decreased expression of Dsc2 promotes cell proliferation via activation of Akt/β-catenin signaling. Given that aberrant activation of β-catenin signaling plays an important role in the progression of colorectal carcinomas, I also examined the ability of Dsc2-deficient cells to grow as tumors in immunodeficient mice. Notably, the parental cell line SK-CO15 is non-tumorigenic and does not form tumors in mice, however, down-regulation of Dsc2 enables tumor formation *in vivo*. Furthermore, inhibition of Akt strongly attenuates the growth of Dsc2-deficient cells. Taken together, this work defines a novel link between the desmosomal cadherins, Akt, and β-catenin and provides mechanistic evidence that loss of Dsc2 may contribute to the progression of epithelial cancers.

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Table of Contents

Chapter 1: Introduction **1**
The Origin and Molecular Basis of Intercellular Adhesion **2**
1.1.1 How do cells adhere to one another? **2**
1.1.2 To each (tissue), his own (cadherin). **3**
1.1.3 Differential expression of cadherin family members mediates cell-type specific sorting. **4**
1.1.4 E-cadherin is required for epithelial cell polarity. **4**
1.1.5 Cadherin engagement promotes cell differentiation and altered expression of cadherins perturbs tissue homeostasis. **4**
1.2 *Intercellular Adhesive Junctions Confer Functional Properties to Tissues* **5**
1.2.1 Tight junctions regulate tissue permeability and barrier function. **5**
1.2.2 Desmosomes reinforce intercellular adhesive contacts and enable tissues to resist mechanical stress. **7**
1.3 *Intercellular Adhesive Junctions in the Context of Disease: Chronic Inflammation of the Intestine* **12**
1.4 *Hypothesis: Altered Expression of the Desmosomal Cadherins Affects Intestinal Epithelial Homeostasis* **14**
1.5 *References* **16**
1.6 *Figures and Legends* **21**
Chapter 2: Loss of desmocollin-2 confers a tumorigenic phenotype to colonic epithelial cells through activation of Akt/β-catenin signaling **24**
2.1 *Abstract* **26**
2.2 *Introduction* **27**
2.3 *Results* **30**
2.4 *Discussion* **38**
2.5 *Materials and Methods* **46**
2.6 *References* **52**
2.7 *Figure Legends* **58**
2.8 *Figures* **64**
2.9 *Supplementary Data* **70**
Chapter 3: Characterization of full-length and proteolytic cleavage fragments of desmoglein-2 in native human colon and colonic epithelial cell lines **77**
3.1 *Abstract* **78**
3.2 *Introduction* **79**
3.3 *Results* **81**
3.4 *Discussion* **85**
3.5 *Materials and Methods* **89**
3.6 *References* **92**
3.7 *Figure Legends* **94**
3.8 *Figures and Supplementary Data* **98**
Chapter 4: Conclusions and Future Directions **105**
4.1 The *Desmosomal Cadherins* **105**
4.2 *Loss of Desmocollin-2 Promotes Tumorigenesis* **106**
4.3 *Desmosomal Cadherins and the Regulation of Lipid Raft Signaling Pathways* **106**
4.4 *Evidence of Distinct Signaling Functions for the "Partner" Desmosomal Cadherins Desmocollin-2 and Desmoglein-2* **107**
4.5 *Controlling Desmoglein-2 Function by Proteolysis* **108**
4.6 *What is the Significance of Altered Desmoglein-2 Expression in Chronic Intestinal Inflammation?* **109**
4.7 *In Summary* **110**
4.8 *References* **112**
Appendix: Cellular Domains Chapter 19 – The Tight Junction and Desmosomes **114**

Table of Figures

Chapter 1: Introduction **1**
Figure 1: Molecular composition of the tight junction and desmosome **21**
Figure 2: Schematic depicting the intestinal epithelium and the intercellular junctions between individual epithelial cells **22**
Figure 3: Desmoglein-2 expression is increased in patients with inflammatory bowel disease (IBD) **23**
Chapter 2: Loss of desmocollin-2 confers a tumorigenic phenotype to colonic epithelial cells through activation of Akt/β-catenin signaling **24**
Figure 1. Loss of desmocollin-2 leads to activation of β-catenin/TCF-dependent transcription without inducing accumulation of total beta-catenin. **64**
Figure 2. Inhibition of PI3K/Akt signaling reduces β-catenin activation following desmocollin-2 knockdown. **65**
Figure 3. Loss of desmocollin-2 induces Akt/β-catenin signaling through activation of the Epidermal Growth Factor Receptor (EGFR). **66**
Figure 4. Desmocollin-2 loss enhances cell proliferation through an Akt- and β-catenin-dependent mechanism. **67**
Figure 5. Down-regulation of desmocollin-2 promotes cellular transformation and enhances growth in vitro and in vivo. **68**
Figure 6. Working model: Loss of desmocollin-2 promotes cellular transformation and proliferation through activation of the Akt/β-catenin signaling pathway. **69**
Supplementary Figure 1.Loss of desmocollin-2 in Caco-2 cells leads to activation of β-catenin/TCF-dependent transcription. **70**
Supplementary Figure 2. Desmocollin-2 and β-catenin do not form a complex in SK-CO15 cells. **71**
Supplementary Figure 3. Targeted depletion of Akt using specific siRNA recapitulates the effect of triciribine on β-catenin-dependent transcription following Dsc2 knockdown. **72**
Supplementary Figure 4. Stable shRNA-mediated knockdown of desmocollin-2 in SK-CO15 cells induces Akt/β-catenin signaling. **73**
Supplementary Figure 5. Down-regulation of desmocollin-2 induces nuclear accumulation of p-(Ser552)-β-catenin through an Akt-dependent mechanism. **74**
Supplementary Figure 6. Desmocollin-2 loss enhances cell proliferation through an EGFR and β-catenin-dependent mechanism. **75**
Supplementary Figure 7. Desmocollin-2 is down-regulated in moderately and poorly differentiated colonic carcinomas. **76**
Chapter 3: Characterization of full-length and proteolytic cleavage fragments of desmoglein-2 in native human colon and colonic epithelial cell lines **77**
Figure 1. Antibody AH12.2 recognizes the first extracellular domain of desmoglein-2. **98**
Figure 2. Desmoglein-2 specific antibodies detect full-length and lower molecular weight species in cell lysates from colonic epithelial cell lines and human colon. **99**
Figure 3. Antibodies against the extracellular domain of desmoglein-2 detect a shed fragment in colonic epithelial cell culture supernatants. **100**
Figure 4. RNAi down-regulation of desmoglein-2 expression demonstrates antibody specificity for full-length and lower molecular weight forms of desmoglein-2. **101**
Figure 5. Putative fragments of desmoglein-2 in colonic epithelial cells. **102**
Supplementary Figure 1. The specificity of antibody AH12.2 for desmoglein-2 does not depend on protein glycosylation. **103**
Supplementary Figure 2. A prominent ~90 kDa protein detected by antibody 6D8 in colonic epithelial cell lysates does not appear to be desmoglein-2 but may be β-catenin. **104**
Chapter 4: Conclusions and Future Directions **105**
Appendix: Cellular Domains Chapter 19 – The Tight Junction and Desmosomes **114**
Figure 1: Ultra-structure of cell-cell junctions in polarized intestinal epithelial cells. **145**
Figure 2: Molecular composition of the tight junction and desmosome. **146**

INTRODUCTION

As most individuals can attest by the end of their first decade of life, cells make tissues, tissues make organs, organs make organ systems, and organ systems make the organism. The fundamental basis for the multicellular, tissue- and organ-forming animal kingdom is the ability to form contacts between cells, that is, to generate intercellular adhesion, a function that is largely attributed to the adherens junction-forming proteins of the cadherin family. Furthermore, specialized intercellular contacts, namely tight junctions, desmosomes, and gap junctions contribute to cell-cell adhesion and form the structural basis for the regulation of additional tissue properties, including paracellular permeability (tight junctions), tissue integrity and strength (desmosomes), and intercellular communication via small molecules and ions (gap junctions). Disruption of any of these intercellular adhesion complexes interferes with the normal physiologic function of the affected tissue and often results in disease.

In the introductory paragraphs that follow, I will first highlight the general principles of intercellular adhesion and discuss the types and basic molecular components of cell-cell junctions. Then, I will address how these intercellular junctions are perturbed in disease states, with an emphasis on the effect of inflammatory insults on the cell-cell adhesive complexes of the human intestinal epithelium, and I will present a novel observation concerning the altered expression of a desmosomal protein in the setting of chronic colonic mucosal inflammation. Finally, I will outline my thesis work that has attempted to address the role of specific proteins of the desmosome (the desmosomal cadherins) in the regulation of intestinal epithelial cell biology.

THE ORIGIN AND MOLECULAR BASIS OF INTERCELLULAR ADHESION

The fundamental basis underlying the existence of multicellular (metazoan) life forms is the ability to create and stabilize contacts between cells. Interestingly, multiple mechanisms of intercellular adhesion have arisen independently over the course of evolution, thus generating the morphological diversity of animals, fungi, plants and other multicellular organisms [Abedin and King 2010].

How do cells adhere to one another? In animals, intercellular connections are mediated by cell-surface adhesion molecules. These adhesion molecules are typically transmembrane proteins that belong to one of four major families: the immunoglobulin domain-containing superfamily, the cadherins, the integrins, or the selectins [Aricescu and Jones, 2007; Halbleib and Nelson, 2006; McEver and Zhu 2010; Stepniak et al., 2009]. In general, these proteins interact with neighboring cells via an extracellular adhesive domain, which may bind to the exact same type of cell adhesion molecule on the apposing cell (homophilic binding) or may interact with another type of adhesion protein (heterophilic binding) to mediate attachment between cells. In addition to their role in intercellular adhesion, members of the integrin family also mediate interactions between cells and the extracellular matrix [Cabodi et al. 2010].

Notably, not all cell adhesion molecules are capable of forming strong, stable intercellular contacts that are a prerequisite for tissue strength and integrity. For instance, members of the integrin and selectin protein families are largely involved in transient cell-cell interactions, such as those that occur during immune cell migration out of the bloodstream and into tissues [McEver and Zhu 2010]. Likewise, many members of the immunoglobulin superfamily are also involved in transient cellular interactions. In contrast, members of the cadherin family

are capable of forming intercellular adhesive complexes known as adherens junctions which are strong, stable sites of contact between cells.

To each (tissue), his own (cadherin). As alluded to above, the basic intercellular junction that exists between cells of tissue is the adherens junction, which is formed by the transmembrane classical cadherin family members. Cadherins are widely-expressed in all animal tissues [Abedin and King, 2010; Angst et al., 2001; Halbleib and Nelson, 2006] and even the sponge, the most primitive multicellular animal ancestor, expresses a cadherin homolog [Abedin and King 2010]. In animals, these proteins mediate adhesive interactions via cadherin repeat domains in their extracellular domain and link to the cellular actin cytoskeleton via their intracellular cytoplasmic domains. Additional proteins, including members of the catenin/armadillo family, associate with the cadherin cytoplasmic tails and serve adhesive and signaling functions.

Cadherin proteins are responsible for stable interactions that are required between cells in a tissue, but a tissue is more than just a tightly-bonded multicellular aggregate. For instance, each of the major tissues types found in mammals (epithelia/endothelia, muscle, connective and nervous tissues) exhibit some degree of cell-type specific organization/patterning within the tissue, such as in the mammary gland, where basal myoepithelial cells surround the luminal epithelial cells of the mammary ducts. Many tissue types, most notably simple, single-layered epithelial tissues, also exhibit cell polarity/directionality. Furthermore, all tissues are formed from precursor progenitor cells that, given the right environmental cues, differentiate into the mature cell types of the tissue. In tissues such as the intestinal epithelium and epidermis, a carefully controlled homeostatic balance of progenitor/stem cell proliferation, differentiation, and apoptosis contribute to overall tissue maintenance and renewal. Remarkably, as described below, the cadherins play essential roles in each of these

aspects of tissue development and preservation and thus link the basic development of stable attachment sites between cells to the more complex process of tissue organization, morphogenesis, and maintenance.

Differential expression of cadherin family members mediates cell type-specific sorting. As homophilic adhesion molecules, the classical cadherins mediate contact between cells that express the same cadherin family member on their surface and many of the cadherins are expressed in a tissue-specific manner. Pioneering work with epithelial E-cadherin and neural N-cadherin, perhaps the most studied and best characterized of the classical cadherins, demonstrated that it is the first extracellular domain of these proteins that mediates homophilic adhesion thus allowing cell-type specific sorting of tissue progenitor cells during embryogenesis [Krauss et al., 2005; Sheikh et al., 2009; Takeichi and Abe, 2005; Zuppinger et al., 2000]. Other studies have shown that desmosomal cadherins also contribute to segregation of epithelial cell types in adult tissues, as adhesion blocking anti-desmosomal cadherin peptides interferes with the sorting of epithelial and myoepithelial cells in a cell culture model of mammary morphogenesis [Runswick et al., 2001].

E-cadherin is required for epithelial cell polarity. Adherens junctions are required for the establishment of apical-basal polarity in epithelial tissues such as the intestine. E-cadherin engagement has been proposed to contribute to the induction of cell polarity by recruiting polarity proteins such as Par3 to the lateral cell membrane thereby orchestrating the directional targeting of channels and secretory vesicles, which are essential for the absorptive and secretive properties of many epithelial tissues [Baum and Georgiou 2011].

Cadherin engagement promotes cell differentiation and altered expression of cadherins perturbs tissue homeostasis. In addition to promoting stable intercellular junction formation,

cadherin homotypic interactions have been shown to induce growth arrest and promote cell differentiation in a number of tissue types, including skeletal and cardiac muscle, nervous tissue, and multiple epithelial tissues [Armeanu et al., 1995; Burdsal et al., 1993; Larue et al., 1996; Wheelock and Jensen, 1992; Zeschnigk et al., 1995]. Cadherin engagement promotes cell differentiation through a number of mechanisms, including induction of cell cycle kinase inhibitors and activation of PI3K/Akt signaling [Calautti et al., 2005; Laprise et al., 2004; Levenberg et al., 1999; St Croix et al., 1998]. Importantly, studies in the epidermis and intestine have indicated that aberrant expression of cadherin family members not only affects intercellular adhesion, but also disrupts tissue homeostasis through effects on proliferation and differentiation [Chidgey et al., 2001; Elias et al., 2001; Eshkind et al., 2002; Hardman et al., 2005; Hermiston et al., 1996].

INTERCELLULAR ADHESIVE JUNCTIONS CONFER FUNCTIONAL PROPERTIES TO TISSUES

Adherens junctions form the basic, stable contacts between cells in a tissue and allow the cells to be organized/polarized in such a way that they can contribute to the overall physiologic function of the tissue. As detailed below, it is the formation of other types of intercellular junctions, such as tight junctions and desmosomes, that enables the tissue to operate as a collective unit rather than as individual, independently-functioning cells (Figure 1, page 21).

Tight junctions regulate tissue permeability and barrier function. The tight junction is a cell-cell adhesive junction that brings together adjacent cell membranes to create a tight seal in the paracellular space. By creating selective pores within the intercellular space, the tight junction regulates the movement of ions and small molecules between epithelial cells (gate

function). In addition, the junction maintains cell polarity by separating apical and basolateral plasma membrane domains within a given cell and restricting the mixing of membrane protein and lipids (fence function). The tight junction is composed of the transmembrane proteins, including the tetraspanin proteins claudins and occludin, and members of the Junctional Adhesion Molecule (JAM) family. Plaque proteins associate with the cytoplasmic domain of the transmembrane proteins and include MAGUK (membrane-associated guanylate kinase) family members like the zonula occludens (ZO) proteins. Numerous other cytoplasmic proteins localize to the tight junction, and these proteins include transcription factors such as ZONAB and cell polarity proteins including Par3/6 and Scribble. Functionally, the formation of tight junctions between neighboring epithelial cells allows the separation of distinct body compartments and is essential for the regulation of tissue permeability. Dysregulation of tight junction proteins has been implicated in numerous pathophysiological processes affecting epithelia, including inflammation and tumorigenesis.

Currently, the protein components of the tight junction can be broadly grouped as transmembrane or cytosolic/scaffolding proteins. Occludin, tricellulin, and the claudin family members are integral membrane proteins that localize in the tight junction strands [Furuse et al., 1996; Ikenouchi et al., 2005]. These proteins have four transmembrane domains and are believed to mediate cell-cell contact through their extracellular loops. The claudin family of proteins consists of at least 24 isoforms in humans and mice and studies in claudin-deficient fibroblasts have demonstrated the homotypic and/or heterotypic interactions of the different claudin isoforms [Asano et al., 2003; Daugherty et al., 2007; Tsukita and Furuse, 1999; Tsukita and Furuse, 2000a; Tsukita and Furuse, 2000b; Tsukita and Furuse, 2002]. In addition, claudin knockout mouse models have further supported the unique role of different claudin family members in tight junction-containing tissues, as the phenotype varies

greatly depending on the deleted isoform [Furuse and Tsukita, 2006; Kitajiri et al., 2004]. In addition to the tetraspan integral membrane proteins, single-pass transmembrane proteins of the immunoglobulin superfamily, JAM (Junctional Adhesion Molecule) and CAR (Coxsackie and Adenovirus Receptor), have also been shown to localize to the tight junction strands [Bergelson et al., 1997; Martin-Padura et al., 1998; Roelvink et al., 1998] and regulate barrier permeability [Liu et al., 2000; Mandell et al., 2004].

While expression of claudin family members is required for strand formation, it is the expression and localization of the ZO proteins that control where strand assembly occurs [Siliciano and Goodenough, 1988]. The ZO proteins are MAGUK (membrane-associated guanylate kinase homologues) family members that contain several PDZ domains, SH3 domains, and one guanylate kinase-like domain [Anderson et al., 1988; Gumbiner et al., 1991]. The ZO proteins interact with the cytoplasmic tails of claudins, occludin, and JAM proteins through PDZ-dependent and independent mechanisms [Itoh et al., 1999]. Importantly, the ZO proteins link the tight junction structure to the apical actin cytoskeleton, directly or indirectly through associations with additional proteins, including afadin/AF6 [Miyoshi and Takai, 2005]. Importantly, it has been shown that modulation of this tight junction-associated, circumferential actin-myosin "belt" can control epithelial permeability, by changing the paracellular pores formed by the tight junction strands.

Desmosomes reinforce intercellular adhesive contacts and enable tissues to resist mechanical stress. Desmosomes or "maculae adherentes" are cell-cell adhesive junctions that were originally identified in the epidermis using electron microscopy. While desmosomes are found in a number of tissue types, they are enriched in tissues that must withstand extensive mechanical stress, such as the skin or heart. The core protein components of the desmosome consist of the transmembrane cadherin family member

glycoproteins desmocollin and desmoglein, the cytoplasmic plaque proteins of the plakin family, such as desmoplakin, and armadillo family members including plakoglobin and the plakophilins. The extracellular domains of the desmosomal cadherins mediate adhesion between neighboring cells while their intracellular domains are linked to the intermediate filament cytoskeleton via the plaque proteins. The intimate association of the desmosomal plaque with the intermediate filament network reinforces the cell-cell contact sites and adds overall structural stability to the tissue. While the desmosome has always been described as a junction specialized for maintaining tissue integrity, recent evidence from animal models and human diseases demonstrate that desmosomal proteins likely have important signaling roles in proliferation, differentiation, apoptosis, and overall tissue morphogenesis, in addition to their structural role in forming desmosomes.

Desmosomal cadherins. Desmocollin and desmoglein are calcium-dependent cell-cell adhesion proteins that belong to the cadherin superfamily. There are four desmoglein isoforms and three desmocollin isoforms that are expressed in a tissue-specific and differentiation-dependent manner [Dusek et al., 2007]. The extracellular domain of these desmosomal cadherins consists of cadherin repeat domains (EC1-5) that are linked by flexible calcium binding regions. As has been described for other cadherin family members, the adhesive site has been proposed to be in the EC1 domain of the desmosomal cadherins and blocking peptides against this region inhibit homophilic and heterophilic interactions of desmocollin and desmoglein [Runswick et al., 2001]. While the adhesive function of the desmosomal cadherins usually depends on the presence of calcium, Garrod et al. have described calcium-independent “super-adhesive” desmosomes that no longer require the presence of calcium to be maintained [Garrod and Kimura, 2008]. The membrane proximal EC5 domain (or EA domain) is less conserved between the proteins and may allow

interactions between distinct cadherin family members [Getsios et al., 2004]. The membrane spanning TM domain is followed by a cytoplasmic juxtamembrane domain (IA, intracellular anchor), which has been proposed to mediate interaction with plakophilins [Bonne et al., 1999; Chen et al., 2002; Hatzfeld et al., 2000], and the intracellular cadherin sequence (ICS) which has been shown to bind plakoglobin [Andl and Stanley, 2001; Gaudry et al., 2001; Wahl et al., 2000]. In addition to the domains described above, the desmogleins have additional unique cytoplasmic segments, the repeating unit domains (RUD) and the desmoglein terminal domain (DTD). The function of these domains remains to be determined.

Numerous studies have examined the regulation of the assembly and disassembly of the desmosomal junction and have found that phosphorylation of the cadherin cytoplasmic tail is an important contributor to this process [Calautti et al., 1998; Garrod et al., 2002b]. Phosphorylation also regulates the interaction of the desmosomal cadherins with the plaque proteins, including plakoglobin [Gaudry et al., 2001]. Proteolytic cleavage of the desmosomal cadherins, especially desmogleins, can occur in the extracellular domain [Bech-Serra et al., 2006; Cirillo et al., 2007; Klessner et al., 2009] or intracellular domain [Cirillo et al., 2008; Dusek et al., 2006; Nava et al., 2007] and regulates processes such as junction disassembly and apoptosis. In agreement with these findings, at least one human disease, the staphylococcal scalded-skin syndrome (SSSS), is caused by cleavage of desmoglein 1, an isoform expressed in the upper layers of the skin. Cleavage of this protein by the bacterial toxin induces massive exfoliation of the upper layers of the epidermis and often is fatal if not treated appropriately [Amagai et al., 2000; Hanakawa and Stanley, 2004; Nishifuji et al., 2008].

Plakin family members. The desmoplakin isoforms I and II are the major plakin family members of the desmosome and immunolocalization of desmoplakin often serves as a reliable marker to identify the desmosomal junction. The desmoplakins are large (210-230 kDa) proteins that are currently thought to be the major plaque proteins that connects the cytoplasmic tail of the desmosomal cadherins to the intermediate filament network. Desmoplakins also interact with armadillo family members, thereby serving as a general scaffold for the association of a number of proteins with the desmosomal junction [Franke et al., 1982; Mueller and Franke, 1983]. Multiple binding domains allow numerous protein-protein interactions with desmoplakin, including a globular head domain formed by two spectrin repeats, a Src homology domain, and a central coiled coil domain that mediates desmoplakin dimerization. Other plakin family members that have been localized to the desmosome include envoplakin, periplakin, and spectrin [Garrod and Chidgey, 2008].

Armadillo family members. The 42 amino acid long "arm" repeat domains were first identified in the polarity gene armadillo of the fruit fly Drosophila melanogaster [Klymkowsky, 1999]. These domains were subsequently shown to be present in a number of signaling proteins, including beta-catenin and other catenin family members. The desmosome contains several members of the armadillo family, including plakoglobin (gamma-catenin) and plakophilins 1-3, and these proteins have been shown to interact with the desmosomal cadherins and/or desmoplakin [Garrod and Chidgey, 2008; Hatzfeld, 1999]. Interestingly, plakoglobin localizes to both the desmosome and the adherens junction, and may allow "cross-talk" between the different junction types. Also, pointing to non-desmosomal signaling roles, the plakophilins demonstrate junctional and nuclear pools in epithelial cells and may integrate signals from the cell membrane with changes in cell behavior and tissue morphogenesis [Bonne et al., 2003; Hatzfeld et al., 2000; Klymkowsky, 1999].

Intermediate filaments. All desmosomes interact with the intermediate filament network, although the cytoskeletal proteins that comprise this network differ depending on the tissue type [Jamora and Fuchs, 2002; Owens and Lane, 2003]. In epithelial cells, the intermediate filaments consist of cytokeratin isoforms, which are differentially expressed based on the type of epithelia (simple versus stratified) and the differentation of the cells. For instance, keratin 8 and keratin 18 (K8/K18) are the primary keratins expressed in simple epithelia, K5/K14 in the basal cells of stratified epithelia, and K1/K10 in the suprabasal layers of the epidermis. In contrast, in non-epithelial tissues, desmosomes interact with the intermediate filament protein desmin (cardiomyocytes) or vimentin (follicular dendritic cells, meninges).

Tissue expression of the desmosomal cadherin and desmoplakin isoforms. While numerous tissues are known to form desmosomes, the constituent protein isoforms of desmosomes are not always the same. Currently, it is thought that the obligatory proteins of the desmosome are desmocollin, desmoglein, plakoglobin, and desmoplakin although the isoforms expressed differ depending on the tissue type. In general desmoglein-2 and desmocollin-2 are the ubiquitously expressed desmosomal cadherins, and are present in all tissues that have desmosomes [Cowin et al., 1985; Franke et al., 1981a; Franke et al., 1981b]. In the skin, these proteins are expressed in the basal layer of the epidermis but are replaced in the suprabasal layer by desmoglein-3 and desmocollin-3 isoforms. In the uppermost layers of the epidermis, desmocollin-1 and desmoglein-1 replace desmoglein-3 and desmocollin-3, and the expression of these proteins is always restricted to the terminally differentiated upper layers of the epidermis. Desmoglein-4 expression is restricted to the upper layers of the skin and hair follicles [Bazzi et al., 2009; Bazzi et al., 2006; Mahoney et al., 2006]. Desmoplakins I and II have been identified in both simple and stratified epithelia, although some confusion

remains on whether variant II exists in all epithelia [Franke et al., 1982; Garrod and Kimura, 2008; Garrod et al., 2002a; Mueller and Franke, 1983].

Gap junctions form intercellular conduits that allow transmission of small molecules and ions from the cytoplasm of one cell to another. Gap junctions have been identified in all mammalian tissue types. These junctions are comprised of the transmembrane connexin family of proteins, which cluster to form a hexameric channel in the plasma membrane of one cell which then interacts with the same structure in a neighboring cell to form the intercellular junction. Most cells express multiple connexins, and these can form homotypic or heterotypic interactions to create gap junctions with distinct physiologic properties [Goodenough and Paul, 2009].

INTERCELLULAR ADHESIVE JUNCTIONS IN THE CONTEXT OF DISEASE: CHRONIC INFLAMMATION OF THE INTESTINE

As described above, intercellular adhesion is of the utmost importance, and defects in the protein components of cell-cell junctions lead to numerous developmental and pathologic conditions. In particular, inflammatory insults such as pro-inflammatory cytokines have been shown to perturb intestinal epithelial junctions and thereby induce barrier defects in gut-associated diseases such as inflammatory bowel disease (IBD). We would like to better understand the mechanisms by which these cytokines disrupt intestinal epithelial homeostasis and barrier in an effort to provide new strategies for mucosal barrier-targeted therapeutics in inflammation.

The intestinal epithelium is a single layer of epithelial cells that lines the intestinal lumen and is actively turned over in a process requiring proliferation of progenitor crypt stem cells, migration along the crypt-luminal axis, and regulated shedding via apoptosis/anoikis at the

lumen (Figure 2, page 22). The physiological role of the intestinal epithelium is to provide an adequate surface area for nutrient and fluid absorption while serving as an effective barrier to prevent entry of intestinal pathogens into the body. The epithelium also serves to reduce exposure to luminal antigens and commensual flora and may play an active part in the development of tolerance to these potential immunogens [Koch and Nusrat, 2009; Laukoetter et al., 2008]. Breakdown of the epithelial barrier and loss of epithelial homeostasis is thought to precipitate or contribute to a number of gut-associated diseases, including inflammatory bowel disease (IBD), celiac disease (gluten sensitivity), and food allergies [Koch and Nusrat, 2009; Laukoetter et al., 2008].

The barrier function of the intestinal epithelium is typically attributed to components of the tight and adherens junctions, collectively referred to as the apical junctional complex (AJC). Inflammation promotes the internalization of the transmembrane adhesion proteins of the AJC, leading to increased paracellular permeability (a "leaky" barrier) [Gassler et al., 2001; Gibson, 2004]. Furthermore, compromised AJC structure has been observed in inflammatory disorders such as IBD [Gassler et al., 2001; Kucharzik et al., 2001]. Notably, in animal models such as SAMP/Yit, increased epithelial permeability precedes the onset of mucosal inflammation and a link between altered AJC, epithelial barrier dysfunction, and the development of colitis has been proposed [Strober et al., 2001]. In vitro studies indicate that inflammatory cytokines such as Tissue Necrosis Factor (TNF)-α and Interferon-γ induce disruption of the AJC and increase permeability of the epithelium, providing additional evidence that inflammatory processes perturb the function of epithelial intercellular junctions [Bruewer et al., 2003; Ivanov et al., 2004].

Unlike the transmembrane proteins of the tight junction/AJC which are down-regulated and/or internalized in the setting of inflammation, we have observed increased expression of

the transmembrane desmosomal cadherin desmoglein-2 in the colonic epithelium of patients with inflammatory bowel disease (Figure 3, page 23). However, the functional relevance of this finding is unclear and little is known about the basic biology of the desmosomal cadherins in the intestinal epithelium.

In stratified epithelia and cardiac muscle, altered expression of the desmosomal cadherins has been shown to perturb numerous physiologic processes, such as cell differentiation, proliferation, and apoptosis [Chidgey et al., 2001; Elias et al., 2001; Eshkind et al., 2002; Hardman et al., 2005]. Furthermore, studies in our group have demonstrated that intracellular cleavage fragments of desmoglein-2 induce apoptosis in the colonic epithelium, suggesting that changes in desmosomal cadherin expression also affect intestinal epithelial homeostasis [Nava et al., 2007].

HYPOTHESIS: ALTERED EXPRESSION OF THE DESMOSOMAL CADHERINS AFFECTS INTESTINAL EPITHELIAL HOMEOSTASIS

Based on these findings, I hypothesized that altered expression of the desmosomal cadherins regulates intestinal epithelial homeostasis by modulating cell proliferation, differentiation, and/or apoptosis. To test this hypothesis, I utilized a loss of function approach and employed RNAi techniques to down-regulate the expression of either desmocollin-2 (Dsc2) or desmoglein-2 (Dsg2) in a model intestinal epithelial cell line (SK-CO15) and assessed the effect of decreased protein expression on cell proliferation, differentiation, and apoptosis. The results from the studies with Dsc2 are presented in Chapter 2 of this dissertation. On-going loss of function studies with Dsg2 will be discussed in the Conclusions/Future Directions section. In addition to these functional studies, I also characterized the Dsg2-specific mouse monoclonal antibody AH12.2 previously generated by our group and

demonstrated that this antibody recognizes the first extracellular domain of Dsg2 (Chapter 3 of this dissertation). Together, the data from my thesis work demonstrate that alterations in desmosomal cadherin expression influence processes within the intestinal epithelium, such as proliferation. Furthermore, the results presented herein raise additional questions regarding the function of these cadherin family members in epithelia and other tissue types.

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FIGURES

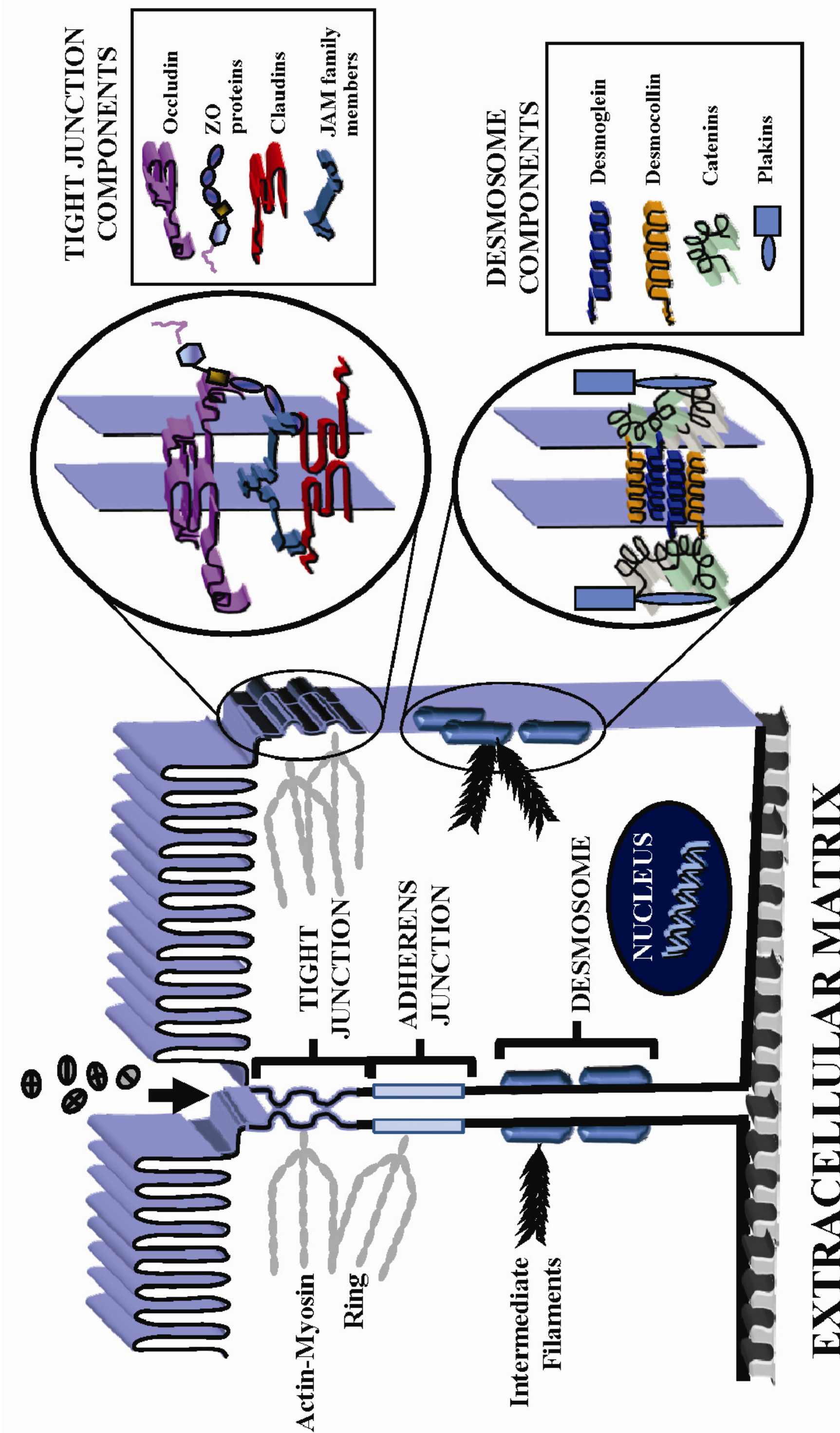


Figure 1. Molecular composition of the tight junction and desmosome. Schematic diagram depicting the transmembrane, plaque, and cytoskeletal proteins of each junction.

Figure 2. Schematic depicting the intestinal epithelium and the intercellular junctions between individual epithelial cells. Tight junctions (yellow), adherens junctions (black), desmosomes (green), nuclei (blue). The integrity of the intestinal epithelial barrier is maintained through a balance of cell proliferation within the crypts and apoptosis/cell shedding at the luminal surface.

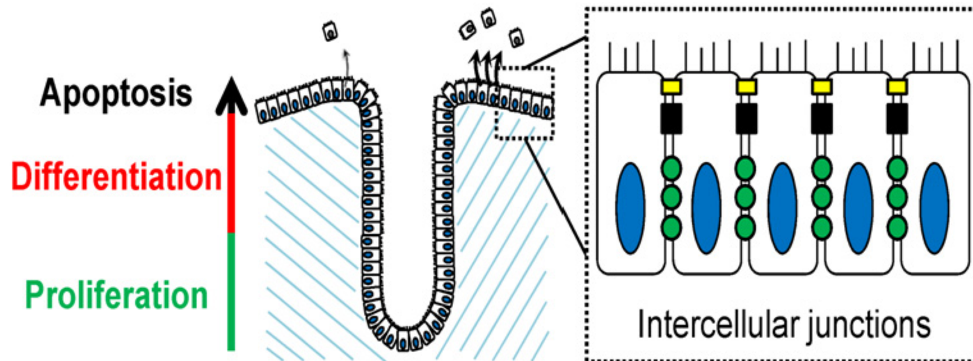
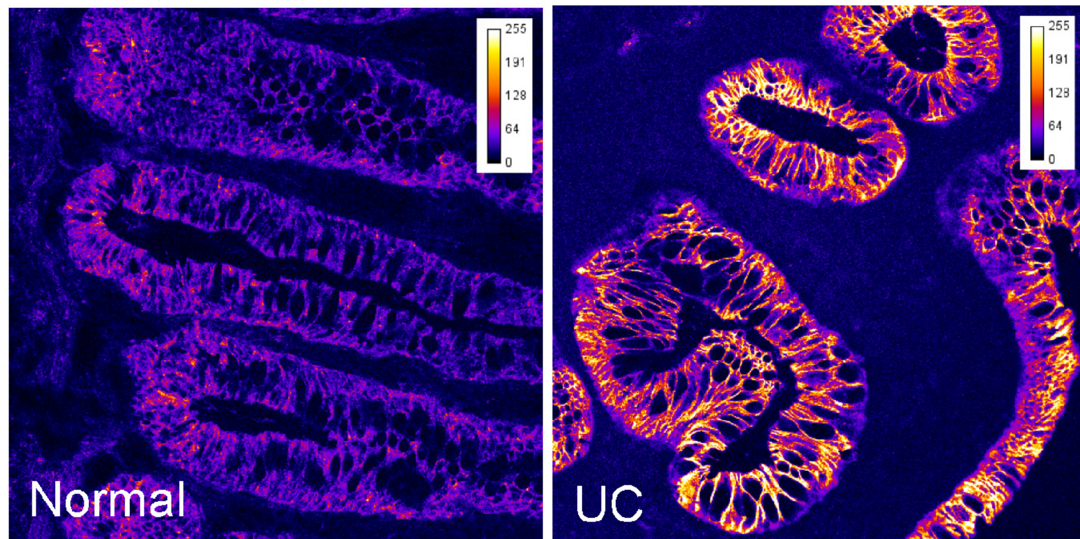


Figure 3. Desmoglein-2 (Dsg2) expression is increased in the intestine of patients with inflammatory bowel disease (IBD). Heat map representation of Dsg2 expression levels in Normal and inflamed colonic mucosa. Dsg2 is increased in the intestinal epithelium of patients with ulcerative colitis (UC) and Crohn's Disease (not shown).



CHAPTER 2:

Loss of desmocollin-2 confers a tumorigenic phenotype to colonic epithelial cells through activation of Akt/β-catenin signaling

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Loss of desmocollin-2 confers a tumorigenic phenotype to colonic epithelial cells through activation of Akt/β-catenin signaling

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Running title: Desmocollin-2 regulates Akt/β-catenin signaling

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ABSTRACT

Desmocollin-2 (Dsc2) and desmoglein-2 (Dsg2) are transmembrane cell adhesion proteins of desmosomes. Reduced expression of Dsc2 has been reported in colorectal carcinomas, suggesting that Dsc2 may play a role in the development and/or progression of colorectal cancer. However, no studies have examined the mechanistic contribution of Dsc2-deficiency to tumorigenesis. Here, we report that loss of Dsc2 promotes cell proliferation and enables tumor growth *in vivo* through the activation of Akt/β-catenin signaling. Inhibition of Akt prevented the increase in β-catenin-dependent transcription and proliferation following Dsc2 knockdown and attenuated the *in vivo* growth of Dsc2-deficient cells. Taken together, our results provide evidence that loss of Dsc2 contributes to the growth of colorectal cancer cells and highlight a novel mechanism by which the desmosomal cadherins regulate β-catenin signaling.

Keywords: *desmocollin, β-catenin, Akt, intestinal epithelial cells, proliferation, tumorigenesis*

INTRODUCTION

Colorectal cancer is a leading cause of cancer morbidity and mortality worldwide (Jemal *et al.*, 2008). In the United States alone, 160,000 people are diagnosed with colorectal cancer every year and close to 60,000 of these patients will die from their disease (Markowitz and Bertagnolli, 2009). Colorectal cancer begins as a benign adenoma and progresses through the adenoma-carcinoma sequence to eventually acquire features of an invasive cancer with metastatic potential (Humphries and Wright, 2008). Numerous genetic changes occur throughout the evolution of the tumor; however, hyper-activation of the Wnt/β-catenin signaling pathway is largely regarded as the initiating event underlying colorectal cancer development and sustained activation of β-catenin signaling is required for tumor progression (Gavert and Ben-Ze'ev, 2007; Markowitz and Bertagnolli, 2009; Oving and Clevers, 2002).

In the normal adult intestinal epithelium, Wnt/β-catenin signaling preserves the crypt stem cell population and drives epithelial cell proliferation, thereby contributing to epithelial cell renewal and the maintenance of tissue homeostasis (Pinto and Clevers, 2005). Central to the regulation of Wnt/β-catenin signaling is the control of the protein stability of β-catenin, the core effector molecule of this pathway. In the absence of the appropriate Wnt signals, β-catenin is targeted for degradation by a destruction protein complex, containing Axin, GSK3β (glycogen synthase kinase 3β), and APC (adenomatosis polyposis coli), which binds to and phosphorylates the N-terminus of β-catenin, leading to its ubiquitination and degradation by the proteasome (Jin *et al.*, 2008). Inhibition of the destruction complex allows the cytosolic accumulation and nuclear localization of β-catenin, where it cooperates with TCF/LEF (T cell factor/Lymphoid enhancer factor) family members to mediate the transcription of pro-proliferative genes (Clevers, 2006). The most common mutations in colorectal cancer

inactivate the gene that encodes APC, leading to constitutive activation of Wnt/β-catenin signaling and hyper-proliferation of cells. Other mutations include those in the N-terminal sequence of β-catenin, which interfere with the ability to phosphorylate and degrade the protein (Markowitz and Bertagnolli, 2009). In addition to Wnt signaling and regulation via the destruction complex, Wnt-independent mechanisms also modulate β-catenin-dependent transcription. Notably, regulation of β-catenin by the cadherin family of cell adhesion molecules has received significant attention in the field of tumor biology and, more recently, the PI3K/Akt signaling axis has been shown to promote β-catenin signaling and cell proliferation in mouse models of intestinal carcinogenesis and inflammation (He *et al.*, 2007; He *et al.*, 2004; Lee *et al.*, 2010; Nava *et al. 2010*).

Classical cadherins such as E-cadherin and N-cadherin, directly bind β-catenin and this interaction is essential for their function as cell adhesion proteins (Nelson and Nusse, 2004). In addition, by associating with β-catenin, these proteins sequester β-catenin at the cell membrane and antagonize its transcriptional activity in colon cancer cells (Gottardi *et al.*, 2001; Sadot *et al.*, 1998; Stockinger *et al.*, 2001). Down-regulation of E-cadherin has been shown to promote β-catenin/TCF transcription in a number of cancer cell types (Eger *et al.*, 2000; Kuphal and Behrens, 2006), and this is one general mechanism by which loss of cell adhesion proteins is thought to contribute to tumor progression.

Desmosomal cadherins, consisting of desmocollin (Dsc 1-3) and desmoglein (Dsg 1-4) family members in humans, do not interact with β-catenin (Kowalczyk *et al.*, 1994b; Wahl *et al.*, 2000) but have been proposed to regulate β-catenin signaling through various mechanisms (Chen *et al.*, 2002; Hardman *et al.*, 2005; Merritt *et al.*, 2002). However, in contrast to the extensively studied classical cadherins E- and N-cadherin, very little is known

about the regulation of β-catenin signaling by the desmosomal cadherins and whether these proteins have a role in tumorigenesis (Chidgey and Dawson, 2007). In the human intestinal epithelium, only desmocollin-2 (Dsc2) and desmoglein-2 (Dsg2) are expressed (Holthofer *et al.*, 2007). Recently, it has been reported that Dsc2 is down-regulated in colon carcinomas (Funakoshi *et al.*, 2008; Khan *et al.*, 2006), suggesting that loss of Dsc2 may contribute to colorectal cancer development and/or progression. However, complementary mechanistic studies are lacking.

Here, we investigated the effect of Dsc2 down-regulation on the behavior of non-tumorigenic, transformed colonic epithelial cells. For our studies, we used the colonic adenocarcinoma-derived SK-CO15 cell line (Anzano *et al.*, 1989; Lisanti *et al.*, 1989), which has constitutive activation of β-catenin/TCF signaling due to an APC mutation (Rowan *et al.*, 2000) but retains features of a differentiated intestinal epithelium (growth as a monolayer, apical-basal polarity, and formation of intercellular adhesive junctions, including tight junctions, adherens junctions, and desmosomes) and have the added advantage of being amenable to transfection (Ivanov *et al.*, 2007; Nava *et al.*, 2007). Our results demonstrate that down-regulation of Dsc2 directly contributes to tumor progression and enables tumor cell growth in immunodeficient mice, by promoting cell proliferation and transformation through the activation of Akt/β-catenin signaling. Collectively, these results highlight a previously unrecognized contribution of Dsc2 loss to the malignant transformation of intestinal epithelial cells and will help to reevaluate the role of the desmosomal cadherins in tumorigenesis and epithelial homeostasis.

RESULTS

Loss of desmocollin-2 enhances β-catenin-dependent transcription in transformed colonic epithelial cell lines. Studies in stratified epithelia and cardiac muscle have implicated desmosomal proteins in the regulation of the transcriptional activity of β-catenin (Garcia-Gras *et al.*, 2006; Hardman *et al.*, 2005; Wang *et al.*, 2007). To investigate whether Dsc2 influences β-catenin signaling in colonic epithelial cells, we down-regulated Dsc2 expression in SK-CO15 cells using RNA interference. Dsc2 down-regulation was confirmed by immunoblot analysis and immunofluorescence labeling/confocal microscopy (Figure 1A). Dsc2-specific siRNA did not decrease the total protein levels of other cadherin superfamily members desmoglein-2 (Dsg2) or E-cadherin (E-cad), thus demonstrating the specificity of the siRNA for Dsc2 (Figure 1A). To assess β-catenin following Dsc2 knockdown, we first examined total protein levels of β-catenin by immunoblotting. Dsc2 down-regulation did not significantly alter total levels of β-catenin (Figure 1B). However, because total protein levels of β-catenin do not necessarily correlate with its transcriptional activity, we also used a luciferase-based transcriptional reporter assay (TOP Flash) to measure β-catenin-dependent transcription in cells with decreased Dsc2 expression (Korinek *et al.*, 1997). Cells were co-transfected with control or Dsc2-specific siRNA and the TOP reporter plasmid containing the β-catenin/TCF binding motifs. A parallel set of co-transfections was carried out using the FOP plasmid with mutated binding sites as a negative control. Seventy-two hours after transfection, luciferase activity was assessed in control and Dsc2-deficient cells. As shown in Figure 1C, β-catenin/TCF signaling was significantly increased ~5-fold in cells with decreased Dsc2 expression. Importantly, down-regulation of Dsc2 enhanced β-catenin transcriptional activity in other epithelial cell types, including the colonic epithelial cell line, Caco-2 (Supplementary Figure 1) and in renal epithelial MDCK (Madin-Darby Canine

Kidney) cells (data not shown), demonstrating that this effect is not specific to the SK-CO15 cell line. To further verify increased β-catenin/TCF transcriptional activity, we analyzed the protein levels of β-catenin/TCF target genes, CD44 and cyclin D1 (Rimerman *et al.*, 2000; Wielenga *et al.*, 1999). In keeping with the reporter assay results, we observed up-regulation of CD44 (Figure 1D) and cyclin D1 (data not shown). To confirm that the effects we observed in these cells were due to β-catenin, we utilized siRNA to down-regulate the expression of Dsc2, β-catenin, or both proteins (Figure 1E). Indeed, TOP Flash activity following Dsc2 down-regulation was completely inhibited by β-catenin knockdown (Figure 1F). These findings demonstrate that the increased β-catenin activity induced by Dsc2 knockdown was mediated by β-catenin and was not due to TOP Flash reporter activation by another catenin family member.

Since Dsc2 down-regulation enhanced the transcriptional activity of β-catenin, we investigated whether β-catenin could be regulated through association with Dsc2 in a protein complex, as has been reported for the classical cadherins. Studies in keratinocytes and co-immunoprecipitation experiments with Dsc and Dsg have shown that β-catenin does not directly associate with desmosomal cadherins, although conflicting results have been reported (Bierkamp *et al.*, 1999; Wahl *et al.*, 2000). To our knowledge, no studies to date have examined the interaction of Dsc2 and β-catenin in intestinal epithelial cells, and to address this possibility, we first determined if Dsc2 could co-immunoprecipitate with β-catenin in SK-CO15 cells. As shown in Supplementary Figure 2B and C, co-immunoprecipitation experiments failed to reveal association of these proteins. To further verify this result, we expressed full-length Dsc2 protein in Chinese Hamster Ovary (CHO) cells, which express β-catenin but lack cadherin family members (Nava *et al.*, 2007; Niessen and Gumbiner, 2002). Consistent with our results in SK-CO15 cells, immunoprecipitation

experiments failed to reveal an association between Dsc2 and β-catenin (data not shown). In contrast, exogenous expression of E-cadherin, which is known to interact directly with β-catenin, co-immunoprecipitated with β-catenin and induced β-catenin membrane localization in CHO cells (data not shown). Thus, these data are in agreement with published reports demonstrating that desmocollin does not associate with β-catenin in a protein complex (Kowalczyk *et al.*, 1996; Kowalczyk *et al.*, 1994a).

Down-regulation of desmocollin-2 induces β-catenin signaling through activation of Akt. Since we did not observe an interaction between Dsc2 and β-catenin, we explored other mechanisms by which Dsc2 loss could activate β-catenin signaling. It has been shown that the serine/threonine kinase Akt, also known as Protein Kinase B (PKB), can regulate β-catenin-dependent transcription either by inhibiting GSK3β or by directly phosphorylating and activating β-catenin (Fang *et al.*, 2007; He *et al.*, 2007; He *et al.*, 2004; Sharma *et al.*, 2002; Tian *et al.*, 2004). We therefore investigated if Akt could mediate the effects of Dsc2 down-regulation on β-catenin signaling. Indeed, in cells with down-regulated Dsc2 expression we observed increased phosphorylation of Akt at Ser473 and Thr308, which is consistent with Akt activation (Figure 2A). Because the phosphorylation and activation of Akt depends on the generation of phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) in the inner cell membrane, we also examined the levels of PIP_3 in control and siDsc2 knockdown cells. As shown in Figure 2B, PIP_3 levels are consistently increased in Dsc2-deficient cells, in agreement with the observed increase in Akt activity. To further determine the contribution of Akt activation to increased β-catenin signaling, we utilized a specific Akt inhibitor, triciribine (API-2/Akt Inhibitor V), and assessed its effect on β-catenin-dependent transcription (Koon *et al.*, 2007; Yang *et al.*, 2004). As shown in Figure 2C, treatment of cells with triciribine effectively reduced the activation of β-catenin/TCF signaling following

Dsc2 down-regulation, as determined by the TOP Flash reporter assay (3-fold decrease compared to DMSO alone). Since active PI3-Kinase (PI3K) catalyzes the formation of PIP_3 and thereby promotes the activation of Akt, we next investigated the influence of PI3K inhibition on β-catenin dependent transcription. Analogous to the results obtained with Akt inhibition, we observed that treatment of Dsc2 knockdown cells with a PI3K inhibitor LY294002 (Vlahos *et al.*, 1994), also reduced β-catenin transcriptional activity (Figure 2D; 4.7-fold decrease compared to DMSO alone). In order to confirm that Akt mediates the activation of β-catenin signaling in Dsc2-deficient cells, we performed knockdown experiments using specific siRNAs against Dsc2, Akt, or both proteins and examined the effects on β-catenin-dependent transcription (Supplementary Figure 3). Consistent with the results obtained using the Akt and PI3K inhibitors, targeted-depletion of Akt also reduces the activation of β-catenin signaling following Dsc2 down-regulation. These results suggest that PI3K/Akt signaling contributes to increased β-catenin-dependent transcription in Dsc2-deficient cells.

Sustained down-regulation of desmocollin-2 activates Akt/β-catenin signaling. Transient down-regulation of Dsc2 allowed the characterization of signaling events and changes in cell behavior at short time periods following Dsc2 loss. To examine the long term effects of Dsc2 down-regulation, we developed a stable cell line expressing small hairpin RNA (shRNA) against Dsc2 (Supplementary Figure 4). Consistent with results obtained with transient Dsc2 knockdown, we observed a 3.5-fold increase in the level of β-catenin-dependent transcription, increased CD44 expression, and increased Akt activation (Supplementary Figure 4C and D) in the stable Dsc2 knockdown cells (shDsc2). In addition, immunofluorescence analysis revealed a prominent nuclear localization of β-catenin in cells with decreased expression of Dsc2, consistent with enhanced activation of β-catenin

signaling (Supplementary Figure 5A, arrows). To confirm that the increase in β-catenin signaling was dependent on Akt, we treated control and shDsc2 cells with the Akt inhibitor triciribine. As shown in Figure 2E, treatment with triciribine diminished the activation of Akt, as determined by immunoblot analysis of phosphorylated Akt (Ser473 and Thr308), and reduced the expression of the β-catenin/TCF target CD44 (Figure 2E). In addition, Akt inhibition resulted in decreased nuclear localization of β-catenin (Supplementary Figure 5A) and decreased β-catenin/TCF-dependent transcription in shDsc2 cells (Figure 2F). Furthermore, we observed that Akt inhibition enhanced the membrane staining of β-catenin in control cells, an effect that was not seen in shDsc2 knockdown cells (Supplementary Figure 5A). Taken together, these data support the conclusion that PI3K/Akt regulates β-catenin signaling in Dsc2-deficient cells.

Loss of desmocollin-2 promotes the nuclear localization of p(Ser552)-β-catenin. Next we examined the mechanism by which Akt enhances the activity of β-catenin in Dsc2-deficient cells. As mentioned above, previous work has shown that Akt promotes β-catenin-dependent transcription by phosphorylating and inhibiting GSK3β (Ser9) and/or by activating β-catenin through direct phosphorylation at Serine 552. Therefore, we assessed the phosphorylation status of these Akt target residues using phosphorylation site-specific antibodies. As shown in Supplementary Figure 5B, transient down-regulation of Dsc2 promoted the cytosolic and nuclear accumulation of p(Ser552)-β-catenin. In addition, p(Ser552)-β-catenin accumulated in the nucleus of stable Dsc2 knockdown cells and this nuclear localization was inhibited by triciribine (Supplementary Figure 5C). Furthermore, we observed that loss of Dsc2 led to an increase in inactivated GSK3β (pGSK3β; Supplementary Figure 4D and 5D), which was reduced by treatment with triciribine. Interestingly, despite the Akt-mediated inhibition of GSK3β, total levels of β-catenin were decreased in the stable Dsc2 knockdown cells

compared to controls (Supplementary Figure 5D). Taken together, these results suggest that Akt enhances β-catenin signaling in Dsc2-deficient cells by promoting the nuclear localization of active β-catenin without inducing accumulation in the total protein level of β-catenin.

Enhanced activation of the epidermal growth factor receptor promotes Akt/β-catenin signaling in desmocollin-2 deficient cells. Given the observed effects of Dsc2 loss on the activation of PI3K/Akt signaling, we next sought to elucidate the mechanism by which Dsc2 regulates the activity of Akt. Previous studies have linked the desmosomal cadherins to the regulation of epidermal growth factor receptor (EGFR) signaling (Getsios *et al.*, 2009; Lorch *et al.*, 2004), a well-recognized activator of PI3K/Akt that has been shown to enhance the transcriptional activity of β-catenin (Hu and Li, 2010; Lu *et al.*, 2003). Therefore, we hypothesized that activation of EGFR may promote Akt/β-catenin signaling in Dsc2-deficient cells. To first examine whether EGFR activation could induce Akt/β-catenin signaling in SK-CO15 cells, we treated cells with epidermal growth factor (EGF) and assessed the effect on β-catenin-dependent transcription. Importantly, β-catenin signaling was increased in EGF-treated cells, an effect that was blocked by inhibition of EGFR using a specific inhibitor (GW2974) (Figure 3A). Importantly, similar results were observed following inhibition of PI3K (data not shown) or Akt (Figure 3A and B). Next, we assessed the total levels of EGFR in Dsc2-deficient cells by immunoblotting. As shown in Figure 3C, down-regulation of Dsc2 consistently increased the total level of EGFR. Furthermore, inhibition of EGFR diminished the activation of β-catenin following Dsc2 knockdown (Figure 3D), recapitulating the effects of PI3K or Akt inhibition. Taken together, these data suggest that enhanced activation of EGFR promotes Akt/β-catenin signaling in Dsc2-deficient cells.

Desmocollin-2 loss enhances cell proliferation through an EGFR/Akt/β-catenin-dependent mechanism. Since enhanced β-catenin activity is known to mediate epithelial proliferation (Hermiston *et al.*, 1996; Wong *et al.*, 1996), we next examined the effect of Dsc2 knockdown on cell proliferation. To assess proliferation, cells were transfected with control or Dsc2-specific siRNA and, 72 hours after transfection, pulse-labeled for 1 hour with a thymidine analog EdU. As shown in Figure 4A, down-regulation of Dsc2 led to increased incorporation of EdU. This effect was also seen in cells with stable Dsc2 knockdown (data not shown). Furthermore, simultaneous knockdown of β-catenin and Dsc2 prevented the increase in cell proliferation observed with Dsc2 down-regulation in stable (Figure 4B) and transient knockdowns (Supplementary Figure 6A). These findings further support a role of β-catenin in promoting cell proliferation following Dsc2 down-regulation.

Since inhibition of either EGFR or Akt signaling reduced the transcriptional activity of β-catenin, we next determined if the same treatments could also prevent the increase in cell proliferation following Dsc2 knockdown. Control and Dsc2 knockdown cells were cultured for two days in the presence of triciribine, GW2974, or vehicle alone (DMSO) and EdU incorporation was used to measure the number of proliferating cells in each condition. Inhibition of either EGFR or Akt prevented the increase in cell proliferation following transient (data not shown) and stable Dsc2 down-regulation (Akt inhibition, Figure 4C; EGFR inhibition, Supplementary Figure 6B). In addition, when cells were cultured for longer time periods (4-5 days) in the presence of triciribine, we observed that cells with reduced Dsc2 were more sensitive to Akt inhibition than their control counterparts. As shown in Figure 4D, shDsc2 knockdown cells no longer grew as adherent monolayers and large gaps were observed between cell colonies, suggesting that triciribine treatment induced

cell detachment and/or death, an effect that was not observed in control cells. Taken together, these results suggest that Dsc2 regulates an EGFR/Akt/β-catenin signaling axis and that, in the absence of Dsc2, cell proliferation and/or survival is dependent on Akt/β-catenin signaling.

Down-regulation of desmocollin-2 promotes cell invasion and xenograft tumor formation in mice. In addition to enhancing cell proliferation, aberrant activation of β-catenin signaling is also thought to contribute to the transformation of colonic epithelial cells, enabling cells to eventually acquire an invasive phenotype (Behrens *et al.*, 1993; Birchmeier *et al.*, 1993; Shimizu *et al.*, 1997). Consistent with the notion that Dsc2 loss enhances cell transformation, SK-CO15 cells stably expressing Dsc2 shRNA lacked the classical epithelial phenotype and were less cohesive than their Dsc2-expressing epithelial counterparts (Figure 5A). To analyze the invasive growth potential of cells lacking Dsc2, cells were cultured in a three-dimensional extracellular matrix. As shown in Figure 5B, shDsc2 knockdown cells grew as large cellular aggregates when compared to control cells, suggesting that loss of Dsc2 may confer invasive capabilities to non-tumorigenic colonic epithelial cells.

Given our *in vitro* findings that cells lacking Dsc2 exhibited features of invasive cells, we examined the *in vivo* tumorigenicity of control and shDsc2 knockdown cells using immunodeficient Rag1$^{-/-}$ mice. Mice were injected subcutaneously with 1 x 10^6 control or shDsc2 cells and monitored for tumor formation. Three weeks post-injection the mice were euthanized and tumor burden was assessed. As expected, mice injected with control cells did not develop tumors (Figure 5C, upper image). In contrast, large tumors grew in mice injected with cells lacking Dsc2 (Figure 5C, lower image). Upon histologic examination, the shDsc2-derived growths showed evidence of tumor formation with characteristic features of

a poorly differentiated carcinoma (Figure 5D). Thus, these data support the hypothesis that loss of Dsc2 promotes tumor growth.

Our studies *in vitro* demonstrated that cells lacking Dsc2 are sensitive to Akt inhibition. To determine whether the *in vivo* growth of these cells was also dependent on Akt signaling, we assessed the effect of Akt inhibition on tumor growth in $Rag1^{-/-}$ mice. Mice were injected with control or shDsc2 cells (1×10^6) and monitored for tumor formation. At 16 days post-injection, palpable tumors were detected in all mice injected with shDsc2 cells and no tumors were evident in mice injected with control SK-CO15 cells, supporting the findings highlighted in Figure 5C and D. Tumor-bearing mice were then treated with the Akt inhibitor triciribine (1mg/kg) or DMSO. After five days of treatment, mice were sacrificed and tumor burden and histology analyzed. To assess the effect of Akt inhibition on tumor size, *in vivo* images documenting tumor size were obtained immediately prior to treatment (Figure 5E, Day 16) and compared to images obtained at the end of treatment (Figure 5E, Day 20). Fold change in tumor size was then estimated by calculating the total tumor area using Image J analysis software. As seen in Figure 5E, Akt inhibition markedly reduced the *in vivo* growth of tumors derived from shDsc2 SK-CO15 cells. Taken together with the *in vitro* studies, our findings suggest that the increased proliferation and tumor growth following Dsc2 down-regulation is mediated by activation of Akt/β-catenin signaling.

DISCUSSION

In this study, we report that loss of the desmosomal cadherin Dsc2 confers a tumorigenic phenotype to transformed colonic epithelial cells. Our data demonstrate that decreased expression of Dsc2 enhances β-catenin signaling and promotes proliferation of colonic

epithelial cells. Furthermore, loss of Dsc2 enables cells to grow as tumors in immunodeficient mice, a phenomenon that is not observed in parental cell lines. Importantly, our results also identify activated Akt as a key component driving β-catenin-dependent transcription in Dsc2-deficient cells. Taken together, these data provide the first mechanistic evidence that loss of Dsc2 may contribute to the malignant transformation of intestinal epithelial cells and demonstrate a novel mechanism to explain the regulation of β-catenin signaling by desmosomal cadherin family members.

Dsc2 is the only desmocollin expressed by simple epithelial tissues such as the colon. Dsc2 loss or down-regulation has been observed in sporadic and inflammation-associated colonic adenocarcinomas (Khan *et al.*, 2006) and in highly tumorigenic colonic adenocarcinoma cell lines (Funakoshi *et al.*, 2008). In support of these findings, we also have observed decreased Dsc2 protein expression and/or diffuse membrane localization in moderately and poorly differentiated colonic adenocarcinomas (Supplementary Figure 7). Dsc2 and Dsc3 proteins, which are both expressed in stratified epithelial tissues, are down-regulated or mislocalized in breast, skin, esophageal, and hepatocellular carcinomas (Cao *et al.*, 2007; Fang *et al., 2010*; Kurzen *et al.*, 2003; Oshiro *et al.*, 2005). Decreased expression of Dsc3 in oral squamous cell carcinomas correlated with poor histological grade, lymph node metastasis, and altered localization of β-catenin (Wang *et al.*, 2007) and Fang et al. recently reported similar prognostic correlations for Dsc2 loss in esophageal carcinomas (Fang *et al. 2010*). These data suggest that loss of Dsc proteins may contribute to tumor formation and/or progression; however, no study to date has examined whether Dsc has a direct role in tumorigenesis.

Here, we report that down-regulation of Dsc2 in a colonic epithelial cell line increased the activation of β-catenin/TCF signaling, enhanced cell proliferation, and conferred tumorigenic capacity to SK-CO15 cells. These findings are in agreement with a large body of evidence

that strongly implicates aberrant activation of β-catenin signaling in the development and progression of colorectal cancers (Korinek *et al.*, 1997; Morin *et al.*, 1997; Munemitsu *et al.*, 1995). Importantly, increased activity of β-catenin/TCF transcription has been shown to drive cell proliferation by directly enhancing the expression of pro-proliferative target genes (Rimerman *et al.*, 2000; Tetsu and McCormick, 1999; Wong *et al.*, 1996; Wong *et al.*, 1998) and activation of this pathway also contributes to cellular transformation and the acquisition of invasive properties (Behrens *et al.*, 1993; Birchmeier *et al.*, 1993; Shimizu *et al.*, 1997). Interestingly, Dsc2-deficient colonic epithelial cells appear to share similar properties to those described for colon cancer stem cells (hyper-proliferation, activation of Akt/β-catenin, expression of putative stem cell markers such as CD44, and tumorigenic capability). It is tempting to speculate that loss or low levels of Dsc2 may play an important role in the maintenance of colon cancer stem cells (Vermeulen *et al.*, 2010; Vermeulen *et al.*, 2008).

Germline or acquired somatic mutations in proteins of the Wnt/β-catenin signaling pathway occur in the majority of colorectal cancers and interfere with β-catenin degradation (Fodde and Brabletz, 2007; Markowitz and Bertagnolli, 2009). However, Wnt-independent mechanisms are also known to regulate the activity of the β-catenin/TCF transcriptional complex, including regulation by cell adhesion proteins, such as E-cadherin (Hermiston *et al.*, 1996; Jeanes *et al.*, 2008), as well as regulation by the serine/threonine kinase Akt (Fang *et al.*, 2007; He *et al.*, 2007; He *et al.*, 2004) which has recently emerged as an important regulator of β-catenin transcriptional activity in the intestine (Brown *et al.*, 2010; Lee *et al.*, 2010; Nava *et al.*, 2010; Vermeulen *et al.*, 2008).

As opposed to the extensively studied classical cadherin E-cadherin, the mechanism(s) by which the desmosomal cadherins regulate β-catenin signaling remain unclear. Unlike E-cadherin, our findings reveal that Dsc2 does not associate with β-catenin in colonic epithelial

cells, indicating that activation of β-catenin is not due to "release" of this protein from a Dsc/β-catenin complex, as has been proposed for E-cadherin. Furthermore, the amount of β-catenin bound to E-cadherin does not appear to be altered in Dsc2-deficient cells (unpublished observations) suggesting that the effects on β-catenin signaling are not due to changes in the interaction between E-cadherin and β-catenin. In addition, we have not detected a change in E-cadherin membrane localization by immunofluorescence/confocal imaging following Dsc2 down-regulation (data not shown). The total levels of E-cadherin, as assessed by immunoblotting, are unchanged in transient knockdown cells (Figure 1A) and are modestly decreased (by ~20%) in stable shDsc2-expressing cells (data not shown), which mirrors the effects we observed for total β-catenin levels. At the present time, it is not clear why there is a difference between transient and stable Dsc2 knockdown cells; however, these results suggest that decreased expression of E-cadherin in SK-CO15 cells may be a longer term consequence of loss of Dsc2.

Studies in the epidermis have suggested that Dsc proteins may regulate β-catenin signaling indirectly through effects on plakoglobin, a catenin family member that interacts with Dscs and may "compete" with and displace β-catenin from E-cadherin, when plakoglobin is in excess (Hardman *et al.*, 2005; Miravet *et al.*, 2003; Zhurinsky *et al.*, 2000). However, simultaneous knockdown experiments in which Dsc2 and β-catenin or plakoglobin were down-regulated in SK-CO15 cells demonstrated that β-catenin but not plakoglobin knockdown completely abolished the activation of the β-catenin transcriptional reporter and reversed the proliferation phenotype of Dsc2-deficient cells (Figure 1F, 4B; data not shown).

Instead we observed that loss of Dsc2 induced the activation of serine/threonine kinase Akt, which has been shown to positively regulate β-catenin signaling in a number of cell types, including intestinal epithelial cells. Akt has been proposed to enhance β-catenin-dependent

transcription through direct phosphorylation and activation of β-catenin (Ser 552) or through the inhibition of GSK3β (Ser 9), a signaling component that is shared with the Wnt/β-catenin pathway. In our study, activation of Akt in Dsc2-deficient cells was associated with an increase in the number of cells staining positively for phospho-β-catenin (Ser552), an Akt phosphorylation site on β-catenin which is associated with enhanced β-catenin transcription (Fang *et al.*, 2007; He *et al.*, 2007). Furthermore, we observed an enrichment of phospho-β-catenin (552) in the nucleus of mitotic cells (data not shown), a phenomenon that has been reported previously for β-catenin (Kaplan *et al.*, 2004; Zhang *et al., 2010*). We also observed an increase in phosphorylation of the inhibitory Ser9 residue of GSK3β, which has been proposed to enhance β-catenin signaling by promoting the accumulation of β-catenin protein levels, in a manner analogous to Wnt-mediated GSK3β inactivation (Desbois-Mouthon *et al.*, 2001; Mulholland *et al.*, 2006; Naito *et al.*, 2005; Sharma *et al.*, 2002). Interestingly, despite the inhibition of GSK3β, we observed a decrease in the total levels of β-catenin in stable Dsc2 knockdown cells, suggesting that Akt-mediated GSK3β inhibition may promote the nuclear redistribution of β-catenin and enhance β-catenin-dependent transcription without inducing stabilization of total β-catenin protein levels. These findings are in agreement with a number of reports suggesting that it is the nuclear localization of β-catenin, rather than the total protein levels that indicates enhanced transcriptional activity of β-catenin (Gottardi and Gumbiner, 2004; Maher *et al.*, 2010; Miller and Moon, 1997) and indicate that in addition to GSK3β, other cellular components (cell-cell adhesion complexes, etc.) likely also influence the total levels of β-catenin in cells. Furthermore, our results also suggest that inhibition of GSK3β by Akt may enhance β-catenin signaling in a distinct manner than Wnt-mediated GSK3β inactivation, even though this component is shared by both Wnt/β-catenin and Akt/β-catenin pathways (See Figure 6). Of note, while numerous reports have demonstrated a clear role for Akt activation in the regulation of β-catenin-dependent transcription, PI3K/Akt does

not enhance the transcriptional activity of β-catenin in all cell types, suggesting that Akt/β-catenin signaling may occur in a tissue and/or context-dependent manner (Ng *et al.*, 2009).

Our data suggest that loss of Dsc2 may promote Akt/β-catenin signaling through the EGFR, which has been previously shown to regulate β-catenin dependent transcription (Hu and Li, 2010). We find that EGFR protein levels are increased following Dsc2 down-regulation (Figure 3C) and that inhibition of EGFR diminishes the activation of β-catenin-dependent transcription and cell proliferation following Dsc2 knockdown (Figure 3D; Supplementary Figure 6B). Furthermore, treatment of SK-CO15 cells with EGF enhances β-catenin signaling, an effect that is diminished by treatment with EGFR, PI3K, and Akt inhibitors or by targeted depletion of Akt using siRNA (Figure 3A and B). Thus, EGFR activation by EGF recapitulates the effect of Dsc2 down-regulation on β-catenin-dependent transcription and strongly supports a role for EGFR in the activation of Akt/β-catenin signaling in Dsc2-deficient cells. These findings complement a recent report linking increased desmosomal cadherin expression to the suppression of EGFR signaling in a model of epidermal differentiation (Getsios *et al.*, 2009).

Interestingly, activation of EGFR/Akt/β-catenin signaling appears to be specific to loss of Dsc2 rather than a general response to the disruption of desmosomal adhesion, as down-regulation of the related desmosomal cadherin Dsg2 does not appear to activate the same signaling cascade in SK-CO15 cells (our unpublished observations).

Finally, the mechanisms by which Dsc2 may be down-regulated in colorectal cancers are not well defined. Funakoshi et al. reported that loss of the intestine-specific homeobox transcription factor CDX2 correlated with loss of Dsc2 expression in colon cancer cell lines, and that re-expression of CDX2 restored Dsc2 expression, suggesting that transcriptional

regulation may lead to its down-regulation (Funakoshi *et al.*, 2008). In contrast, Khan et al. noted that loss of Dsc2 protein in colonic adenocarcinomas occurred without changes in mRNA levels, indicating changes in protein stability rather than transcriptional down-regulation (Khan *et al.*, 2006). Furthermore, these authors found evidence of "Dsc switching" in colonic adenocarcinoma, as loss of Dsc2 was associated with increased expression of Dsc3. In our study, we found no evidence of Dsc3 protein or mRNA induction following down-regulation of Dsc2, suggesting that loss of Dsc2 is not sufficient to induce Dsc3 expression. Lastly, others have proposed that enhanced proteolytic cleavage of the extracellular domain of Dsc2 may contribute to its loss in transformed cells (Mathias *et al.*, 2009). While these studies provide important insight, additional work is required to better characterize the mechanisms regulating changes in Dsc expression during tumorigenesis.

Based on the data from the current study, a hypothetical model to explain how loss of Dsc2 contributes to tumor formation and growth can be proposed (Figure 6A). In this model, down-regulation of Dsc2 induces the activation of the EGFR, which stimulates PI3K activity and enhances PIP_3 levels. Akt is recruited to the membrane via interactions with PIP_3 and is activated by phosphorylation. Activated Akt phosphorylates and inhibits GSK3β and phosphorylates β-catenin directly, increasing the nuclear localization and transcriptional activity of β-catenin. Interestingly, our data suggest that loss of Dsc2 may lead to activation of Akt/β-catenin signaling through the presence of "solitary" (Schmitt *et al.*, 2007) or excess Dsg2, which has been shown to activate Akt ((Brennan *et al.*, 2007); our unpublished observations). Although not addressed in the work presented here, in addition to promoting β-catenin signaling and cell proliferation, active Akt also phosphorylates other downstream targets, which are known to promote the survival of tumor cells (dashed arrows). These pro-

survival effects of Akt may also contribute to the enhanced tumorigenicity of Dsc2-deficient SK-CO15 cells by allowing the cells to "seed" a tumor *in vivo*.

In summary, this work defines a mechanistic role for Dsc2 in the progression of colorectal cancer and identifies Akt as a novel link between the desmosomal cadherins and β-catenin signaling.

MATERIALS AND METHODS

Cell culture and antibodies.

The transformed human intestinal epithelial cell lines SK-CO15 and Caco-2 were grown in high glucose (4.5 g/L) DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 15 mM HEPES (pH 7.4), 2 mM L-glutamine, and 1% nonessential amino acids as previously described (Ivanov *et al.*, 2007). The following primary polyclonal antibodies (pAb) and monoclonal antibodies (mAb) were used to detect proteins by immunoflurescence labeling and/or Western blotting: anti-desmocollin 2/3 mAb, clone 7G6 (kind gift from K. Green, Northwestern University, USA); anti-actin, tubulin, and GAPDH pAbs (Sigma-Aldrich, St. Louis, MO); anti-desmoglein 2 mAb, clone AH12.2 (generated in-house); anti-E-cadherin mAb, clone HECD-1 (hybridoma supernatant; a generous gift from A. S. Yap, University of Queensland, Australia); anti-β-catenin mAb and anti-JAM-A pAb (Zymed Laboratories, South San Francisco, CA); anti-CD44 mAb, clone BBA10 (R&D Systems); pAkt (Ser473), pAkt (Thr308), pGSK3β (Ser9), and p-β-catenin (Ser552) pAbs (Cell Signaling, MA); anti-PML pAb (Abbiotec, San Diego, CA); anti-EGFR pAb (a.a. 961-972, ECM Biosciences, KY); Alexa 488-conjugated phalloidin (Invitrogen). Fluorophore-conjugated secondary antibodies were obtained from Invitrogen (Alexa Dye Series). Peroxidase-conjugated secondary antibodies were obtained from Jackson Laboratories.

RNAi reagents, transient transfection and generation of stable cell lines.

siGENOME SMARTpool siRNA for Human Dsc2, β-catenin, E-cadherin and non-targeting control were purchased from Dharmacon RNA Technologies (Lafayette, CO). Akt1 and Akt2-specific siRNAs were purchased from Sigma and shRNA against Dsc2 or empty vector was obtained from Open Biosystems. SK-CO15 cells cultured to 60–70% confluence were

transfected with the reagents at a final concentration of 20 nM by using Lipofectamine 2000 (Invitrogen). The cells were incubated for an additional 2-3 d after transfection to allow for sufficient knockdown of the target proteins. To generate stable cell lines, SK-CO15 cells were transfected with control (empty vector with puromycin resistance cassette) or a Dsc2-specific shRNA plasmid (also puromycin resistant) and grown in the presence of puromycin to select for transfected cells. Monoclonal cell populations were isolated by limiting dilution and clones were chosen for further analysis based on the efficiency of Dsc2 down-regulation by immunoblot analysis. β-catenin + Dsc2 knockdown was achieved by co-transfecting siRNA targets for β-catenin and Dsc2 together or β-catenin siRNA in the shRNA Dsc2 stable cell line. Akt knockdown studies were performed using a combination of siRNAs targeting both Akt1 and Akt2 isoforms, which are the two major Akt isoforms expressed in colonic epithelial cells, including the SK-CO15 cell line (unpublished observations). Confluent epithelial cultures were analyzed. Each experiment was performed at least three times.

Akt, PI3-kinase, and EGFR inhibitors.

AKT inhibition was achieved using the AKT inhibitor (final 10 μM), Triciribine (Inhibitor V, 35943-35-2; Calbiochem, Germany; (Cheng *et al.*, 2005; Yang *et al.*, 2004)) or the PI3K inhibitor LY294002 (final 20 μM) (L9908, Sigma Aldrich, MO). EGFR inhibition was achieved using the specific inhibitor GW2974 (final 2.5 μM) (Sigma Aldrich, MO). Briefly, after Dsc2 knockdown, medium was replaced with DMEM containing 1% fetal calf serum with vehicle alone (DMSO) or the inhibitor and cells maintained for an additional 12-48 hours before processing and analysis.

Immunoblotting.

Confluent monolayers were washed two times with $HBSS^+$ on ice and collected in lysis buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4), containing a proteinase inhibitor cocktail (1:100, Sigma). Lysates were then cleared by centrifugation (30 min at 14, 0000 x *g*) and immediately boiled in SDS sample buffer. Gel electrophoresis and immunoblotting were conducted by standard methods with 10–20 µg protein per lane.

Immunofluorescence microscopy.

Confluent monolayers were fixed/permeabilized in 3.7% paraformaldehyde (10 min at room temperature [RT]) followed by 0.5% Triton X-100 in $HBSS^+$ (30 min at RT), or in ice-cold absolute ethanol (20 min at –20°C). Frozen sections (6 µm) of normal human colonic mucosa were fixed in absolute ethanol. Nonspecific protein binding was blocked in 3% bovine serum albumin in Hanks balanced salt solution (HBSS)+ (1 h at RT) and incubated with primary antibodies (1 h at RT), washed in HBSS+, and subsequently labeled with secondary antibodies (1 h at RT). Nuclei were stained using To-Pro3-iodide (Invitrogen, Carlsbad, CA) in HBSS+ (10 min at RT). Monolayers were mounted in 1:1:0.01 (vol/vol/vol) phosphate-buffered saline (PBS):glycerol:*p*-phenylenediamine, and they were visualized on a Zeiss LSM 510 Meta Confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

β-catenin/TCF transcriptional reporter assay (TOP Flash).

In vitro activation of β-catenin was assessed using the optimized reporter kit for β-catenin/TCF (Upstate Biotechnology, Charlottesville, VA). Briefly, SK-CO15 or Caco-2 cells were trypsinized and plated (60,000 cells/well) in 48 well plates. 12h after plating cells were transfected with the reporter plasmid pTOPGlow (TOP) or the negative control pFOPGlow (FOP) along with TK *Renilla* (50:1 ratio) as an internal control. After 12h, media

was replaced with DMEM complemented with 1% fetal calf serum (time 0). TOP/FOP activities were measured with the Dual Luciferase Reporter Assay System (Promega, Madison, WI) at 48h after transfection in the GloMax® 96 Luminometer (Cat.# E6511, E6521) and normalized to *Renilla* to control for transfection efficiency. For EGF studies, cells were treated with 100ng/ml EGF (BD Biosciences, MA) and luciferase activity was assessed 4-6hrs post-treatment.

PIP_3 isolation and detection.

PIP_3 levels were determined using the commercially available PIP_3 Mass Strip Kit from Echelon Biosciences Inc.

Nuclear and cytosolic cellular fractionation.

Cytosolic and nuclear fractions of SK-CO15 cells were isolated using the commercially available NE-PER® Nuclear and Cytoplasmic Extraction Reagents from ThermoScientific, following the manufacturer's protocol.

EdU incorporation assay.

Cells were grown to confluency on glass coverslips and pulse-labeled with EdU (10 nM) for 1h. Processing was carried out as described by the manufacturer (Invitrogen, Click-It EdU). Nuclei were counterstained with TOPRO, mounted in standard mounting media and imaged by fluorescence microscopy.

3-Dimensional growth in Matrigel/Invasion assay.

Cells were plated at equal density (10,000 per well) in matrix solution (Matrigel; BD Biosciences, Franklin Lakes, NJ) and incubated at 37°C. Seventy-two hours after plating,

Matrigel plugs were washed, fixed with 3.7% formaldehyde, and permeabilized with 5% Triton-X100. Plugs were then processed for imaging by immunofluorescent labeling/confocal microscopy.

Tumor formation in Rag1-/- mice.

Rag1 homozygous knockout mice were used to assay tumorigenicity *in vivo*. Control or shDsc2 expressing cells were grown to logarithmic growth phase, harvested, washed and resuspended in PBS for injection. Each cell line (1×10^6 cells in 150 μl PBS) was injected subcutaneously into the dorsum of Rag1-/- mice. Tumor formation was monitored daily. All mice were sacrificed at 3 weeks and tumors were immediately isolated and analyzed. For Akt inhibition studies, the Akt inhibitor triciribine (1mg/kg) or DMSO was administered daily via intraperitoneal injection for the indicated time. After treatment, all mice were sacrificed and tumors were isolated and processed for histology. All animal experiments were performed in accordance with protocols approved by the Emory University School of Medicine Institutional Review Board (IRB).

***In vivo* imaging.**

Images documenting tumor growth were obtained using the KODAK In-Vivo Multispectral System FX.

Statistics.

Statistical analysis was carried out using GraphPad PRISM software.

CONFLICT OF INTEREST

The authors have no competing financial interests in relation to the work described herein.

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FIGURE LEGENDS

Figure 1. **Loss of desmocollin-2 leads to activation of β-catenin/TCF-dependent transcription without inducing accumulation of total beta-catenin.** (A) SK-CO15 cells were transfected with Dsc2-specific siRNA (siDsc2) in parallel with a non-targeting siRNA control. Knockdown of Dsc2 expression was confirmed by immunoblot (left panel) and immunofluorescence labeling/confocal microscopy (right panel; green, Dsc2; scale bar is 50 μm). Protein levels of other cadherin family members E-cadherin (E-cad) and desmoglein-2 (Dsg2) are not down-regulated following treatment with Dsc2 siRNA, demonstrating the specificity of the siRNA for Dsc2. (B) Protein extracts from control siRNA and Dsc2-specific siRNA transfected SK-CO15 cells were immunoblotted and probed with antibodies against β-catenin and actin and the expression levels quantified by densitometry. Graph represents mean ± SD from three independent experiments. (C) Luciferase-based reporter assay for β-catenin/TCF-dependent transcription (TOP Flash). SK-CO15 cells were co-transfected with control or Dsc2-specific siRNA and the β-catenin luciferase reporter plasmid

(TOP) or a negative control plasmid (FOP). At 72 hours post-transfection, luciferase activity was assessed. The results are representative of at least five independent experiments. (D) Immunoblot analysis of known β-catenin/TCF target, CD44, using an antibody that detects all variant forms; expression levels were quantified by densitometry. Graph represents mean ± SD from three independent experiments. (E) SK-CO15 cells were transfected with control, Dsc2 (siDsc2), β-catenin (siβ-catenin), or Dsc2 and β-catenin-specific siRNAs. Knockdown was confirmed by immunoblot using Dsc2 and β-catenin-specific antibodies. Actin is included as a loading control. (F) SK-CO15 cells transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids, as described in C.

Figure 2. **Inhibition of PI3K/Akt signaling reduces β-catenin activation following desmocollin-2 knockdown.** (A) Western blot analysis of Akt activation status following Dsc2-specific siRNA treatment. Phospho-specific antibodies against phosphorylated Akt (Serine 473 or Threonine 308) and total Akt were used and the expression levels quantified using densitometry. Graphs represent the mean ± SEM from at least three independent experiments. (B) PIP_3 content of control versus siDsc2 knockdown cells. Total cellular PIP_3 was isolated as described in the Methods section. Graph represents the densitometric quantification of two independent experiments; mean ± SD. (C) SK-CO15 cells were transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids. Twelve hours after transfection, the cells were treated with the Akt inhibitor triciribine or vehicle alone (DMSO). At 72 hours post-transfection, luciferase activity was assessed. (D) SK-CO15 cells were transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids. 12 hrs after transfection, the cells were treated with the PI3K inhibitor LY294002 or vehicle alone (DMSO). At 72 hours post-transfection, luciferase activity was assessed. (E) Cells stably expressing Dsc2-specific shRNA (shDsc2)

or vector alone control plasmid (control) were grown in the presence of triciribine or vehicle only (DMSO). Protein extracts from each condition were assayed for expression of the indicated proteins by immunoblot. Actin is included as a loading control. (F) Luciferase-based reporter assay for β-catenin/TCF-dependent transcription (TOP Flash) in control and shDsc2 cells. Cells were co-transfected with the β-catenin luciferase reporter plasmid (TOP) or a negative control plasmid (FOP). Twelve hours after transfection, the cells were treated with the Akt inhibitor triciribine, PI3K inhibitor LY294002, or vehicle alone (DMSO). At 48 hours post-transfection, luciferase activity was assessed.

Figure 3. **Loss of desmocollin-2 induces Akt/β-catenin signaling through activation of the Epidermal Growth Factor Receptor (EGFR).** (A) SK-CO15 cells were transfected with the TOP or FOP luciferase reporter plasmids. Twelve hours after transfection, cells were pre-treated with the Akt inhibitor Triciribine, the EGFR inhibitor GW2974, or vehicle alone (DMSO) for 8 hours prior to the addition of epidermal growth factor (EGF). Luciferase activity was assessed 4 hours post-EGF treatment. Graph is representative of 3 independent experiments.

(B) SK-CO15 cells were transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids. Twelve hours after transfection, cells were treated with EGF and luciferase activity was assessed 4 hours post-treatment. Graph is representative of 2 independent experiments. (C) Protein extracts from control siRNA and Dsc2-specific siRNA transfected SK-CO15 cells were immunoblotted and probed with antibodies against Dsc2, EGFR, and GAPDH and the expression levels quantified by densitometry. Graph represents mean ± SEM from three independent experiments. (D) SK-CO15 cells were transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids. 12 hrs after

transfection, the cells were treated with the EGFR inhibitor GW2974 or vehicle alone (DMSO). At 48 hours post-transfection, luciferase activity was assessed.

Figure 4. **Desmocollin-2 loss enhances cell proliferation through an Akt- and β-catenin-dependent mechanism.** (A) SK-CO15 cells were transfected with Dsc2-specific siRNA (siDsc2) in parallel with a non-targeting siRNA control. At 72 hours post-transfection, cells were pulse-labeled with EdU for 1 hour and processed to detect incorporated EdU. Images were obtained using confocal microscopy and the number of EdU positive nuclei was scored. The histogram shows the mean ± SEM of EdU positive nuclei from at least 10 different fields. (EdU, red; nuclei, blue). (***p< 0.0002, Student's T-test) (B) Stable shDsc2 knockdown and control cells were transfected with non-targeting or β-catenin-specific siRNA (siβ-catenin) and EdU incorporation assessed 48 hours after transfection. Images were obtained using confocal microscopy and the number of EdU positive nuclei was scored. The histogram shows the mean ± SEM of the number of EdU positive nuclei from at least 10 different fields. (***p< 0.0001, ANOVA) (C) EdU incorporation assay for cells stably expressing Dsc2-specific shRNA (shDsc2) or vector alone control plasmid (control) and grown in the presence of triciribine or vehicle only (DMSO). The histogram shows the mean ± SEM of EdU positive nuclei from at least 10 different fields. (***p< 0.0001, ANOVA) (D) Images obtained by light microscopy of control and shDsc2 knockdown cells treated for 96 hours with triciribine or DMSO. The results are representative of at least three independent experiments.

Figure 5. **Down-regulation of desmocollin-2 promotes cellular transformation and enhances growth *in vitro* and *in vivo*.** (A) Images obtained by light microscopy of control and shDsc2 knockdown cells plated at sub-confluent densities. Note the altered cell

morphology in Dsc2-deficient cells. (B) Control and Dsc2 stable knockdown clones were plated at equal density (10,000 cells per well) in a Matrigel matrix to assess 3D growth. Seventy-two hours later, the Matrigel plugs were processed for immunofluorescence microscopy. Images were collected from at least 10 fields per condition using a confocal microscope and the size of each cell cluster was measured. (Actin, green; nuclei, blue) (†$p < 0.05$, Student's T-test) The results are representative of at least three independent experiments. (C) *In vivo* tumor growth for control versus shDsc2 knockdown cells in Rag -/- mice. Mice were injected subcutaneously with 1 x 10^6 control or shDsc2 cells. Animals were euthanized on day 21 and the extent of tumor development was assessed macroscopically. (D) Microscopic analysis of shDsc2-derived tumors by H & E analysis. (E) The effect of Akt inhibition on tumor growth *in vivo*. Using *in vivo* imaging, tumor size was recorded immediately prior to treatment with triciribine or vehicle alone and compared with final tumor size. The fold change in tumor size was then calculated. The graph represents the mean ± SD. Note the absence of tumors in mice injected with control, Dsc2-expressing cells. Images are representative of baseline and final tumor sizes in each treatment group. White arrows denote tumor location.

Figure 6. **Working model: Loss of desmocollin-2 promotes cellular transformation and proliferation through activation of the Akt/β-catenin signaling pathway.** (A) [1] Down-regulation of Dsc2 leads to activation of the epidermal growth factor receptor [2] and PI3K/Akt-dependent activation of β-catenin/TCF transcription in colonic epithelial cells. Activation of this pathway leads to enhanced cell proliferation and tumor growth *in vitro* and *in vivo*. The contribution of Wnt/β-catenin signaling (C), "solitary" Dsg2, or the additional pro-survival targets of Akt that may enhance tumorigenicity of Dsc2-deficient cells have not been directly investigated in this study. (B) Akt/β-catenin signaling. Akt is recruited to

phosphatidylinositol 3,4,5 triphosphate (PIP_3)-rich regions of the plasma membrane via its pleckstrin homology domain where it is activated by phosphorylation at residues Ser473 and Thr308. Active Akt has been shown to enhance β-catenin signaling either by phosphorylation of GSK3β at Ser9 or by directly phosphorylating β-catenin at Ser552. Note that GSK3 is also part of the β-catenin destruction complex of the Wnt pathway. (C) Wnt/β-catenin signaling. In the absence of a Wnt signal, β-catenin is bound by the destruction complex and targeted for degradation (not depicted). In the presence of Wnt ligands, Wnt bind to the Frizzled (Fz) and LRP co-receptors, leading to the dissociation of the GSK3-containing destruction complex from β-catenin thereby promoting accumulation of β-catenin. Abbreviations: Frizzled (Fz), Dishevelled (Dvl), low-density lipoprotein receptor-related protein (LRP), Casein Kinase (CK), Glycogen Synthase Kinase-3 (GSK3), Epidermal Growth Factor Receptor (EGFR).

FIGURES

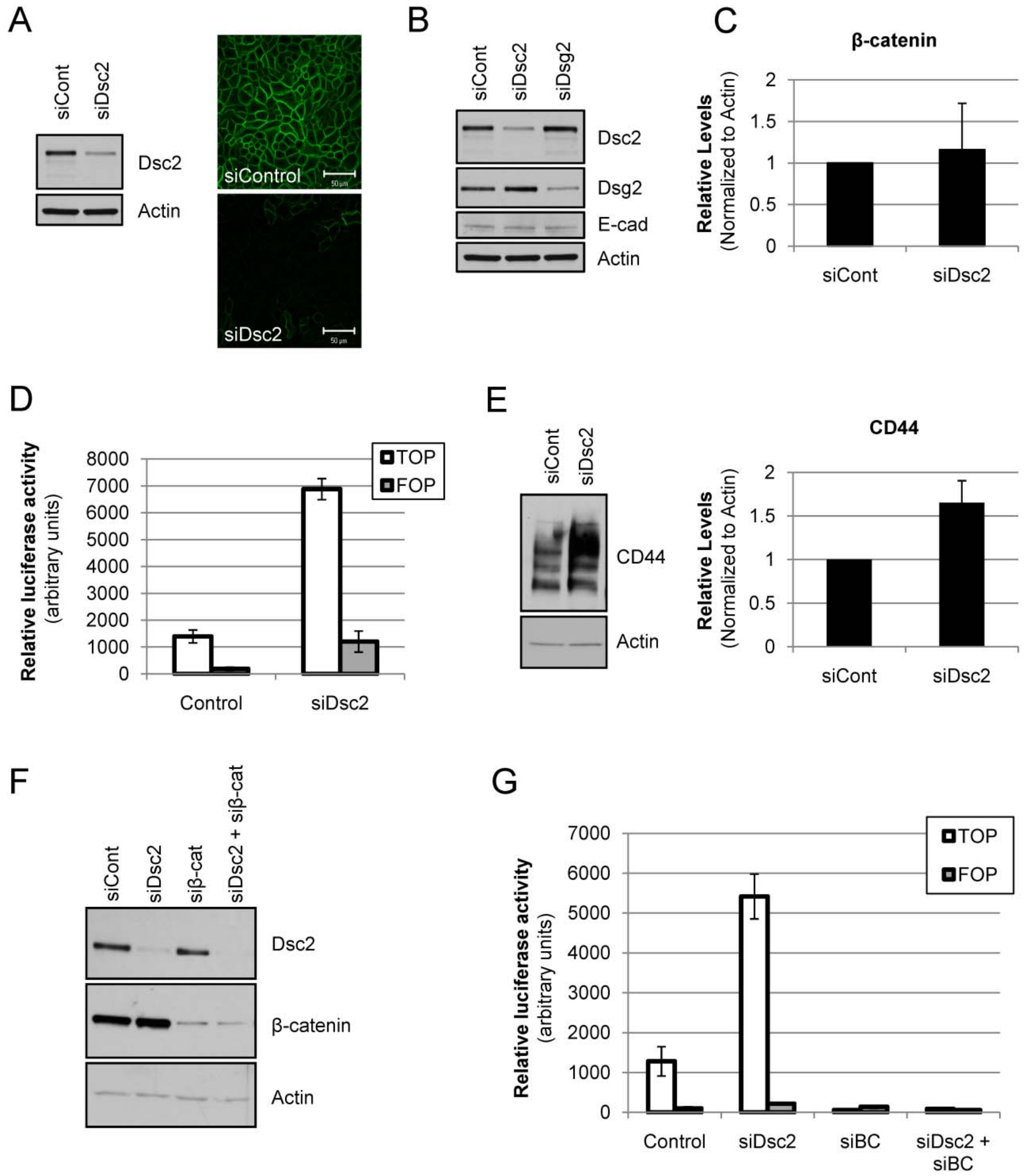


FIGURE 1

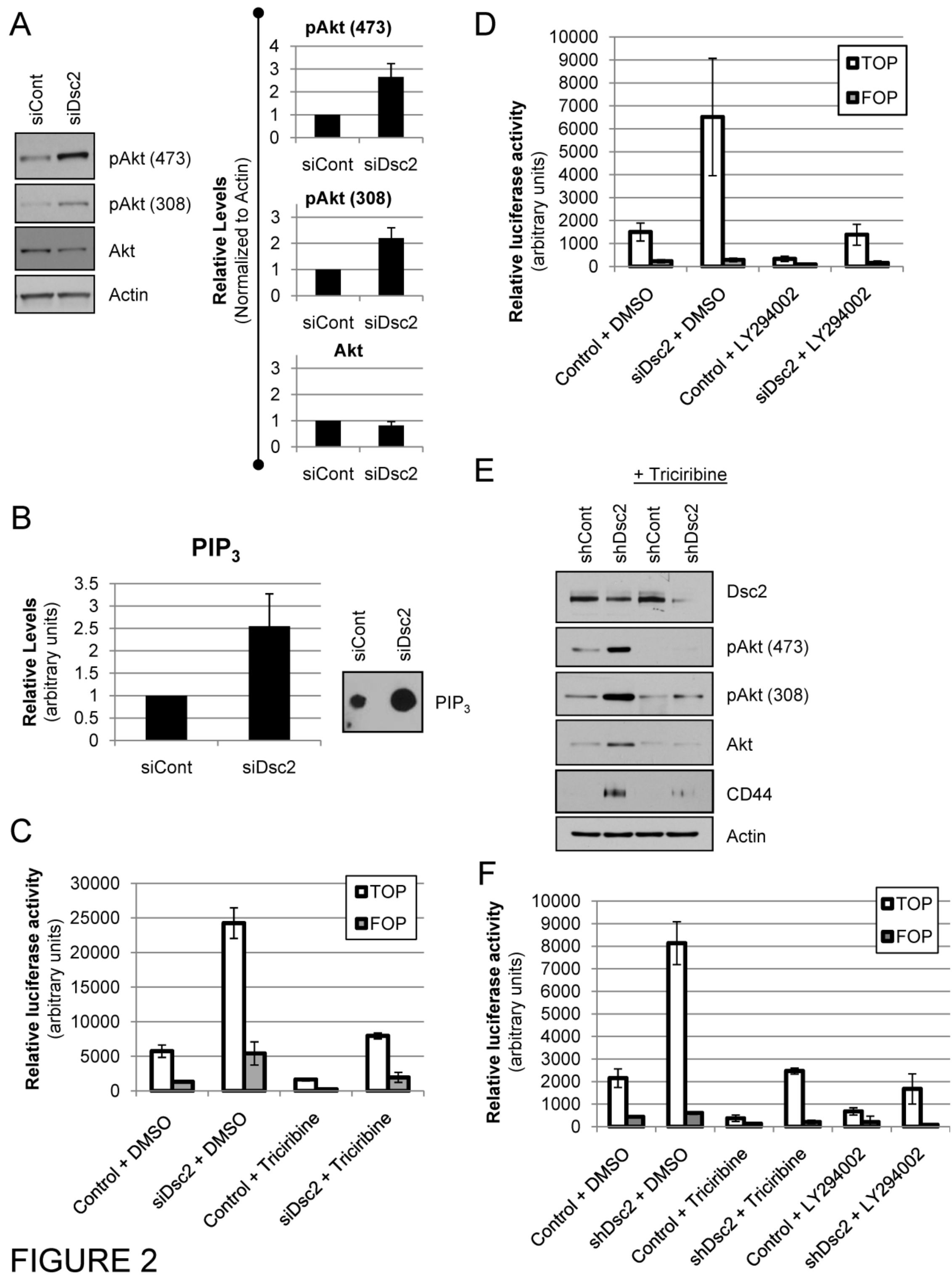


FIGURE 2

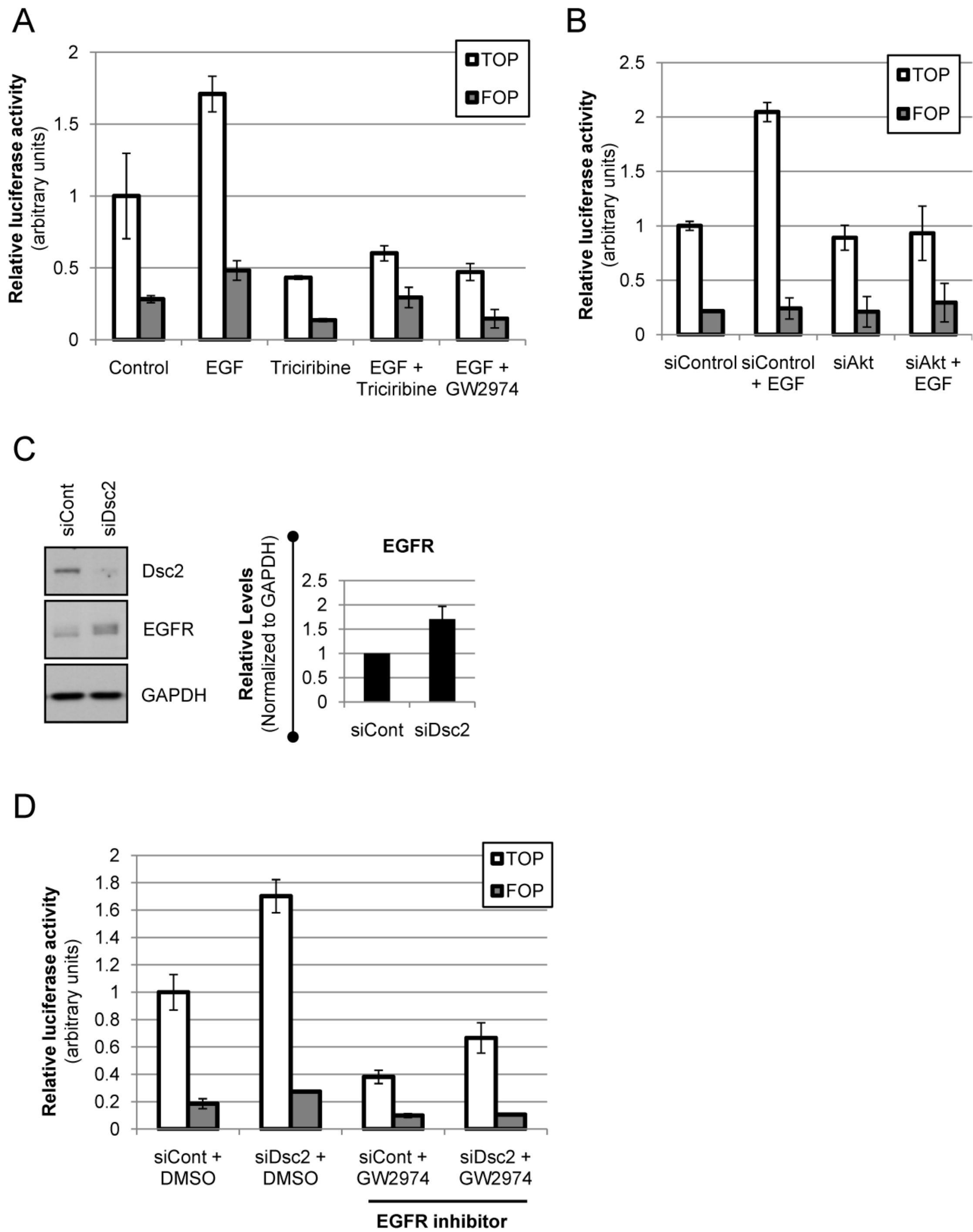


FIGURE 3

A

Control

siDsc2

Number EdU positive nuclei per field

800
600
400
200
0

***

Control
siDsc2

B

Control
shDsc2
Control + siβ-catenin
shDsc2 + siβ-catenin

Number EdU positive nuclei per field

450
400
350
300
250
200
150
100
50
0

***

Control
shDsc2
Control
shDsc2
+ siβ-catenin

C

DMSO
Triciribine

Control
shDsc2
Control
shDsc2

Number EdU positive nuclei per field

600
500
400
300
200
100
0

***

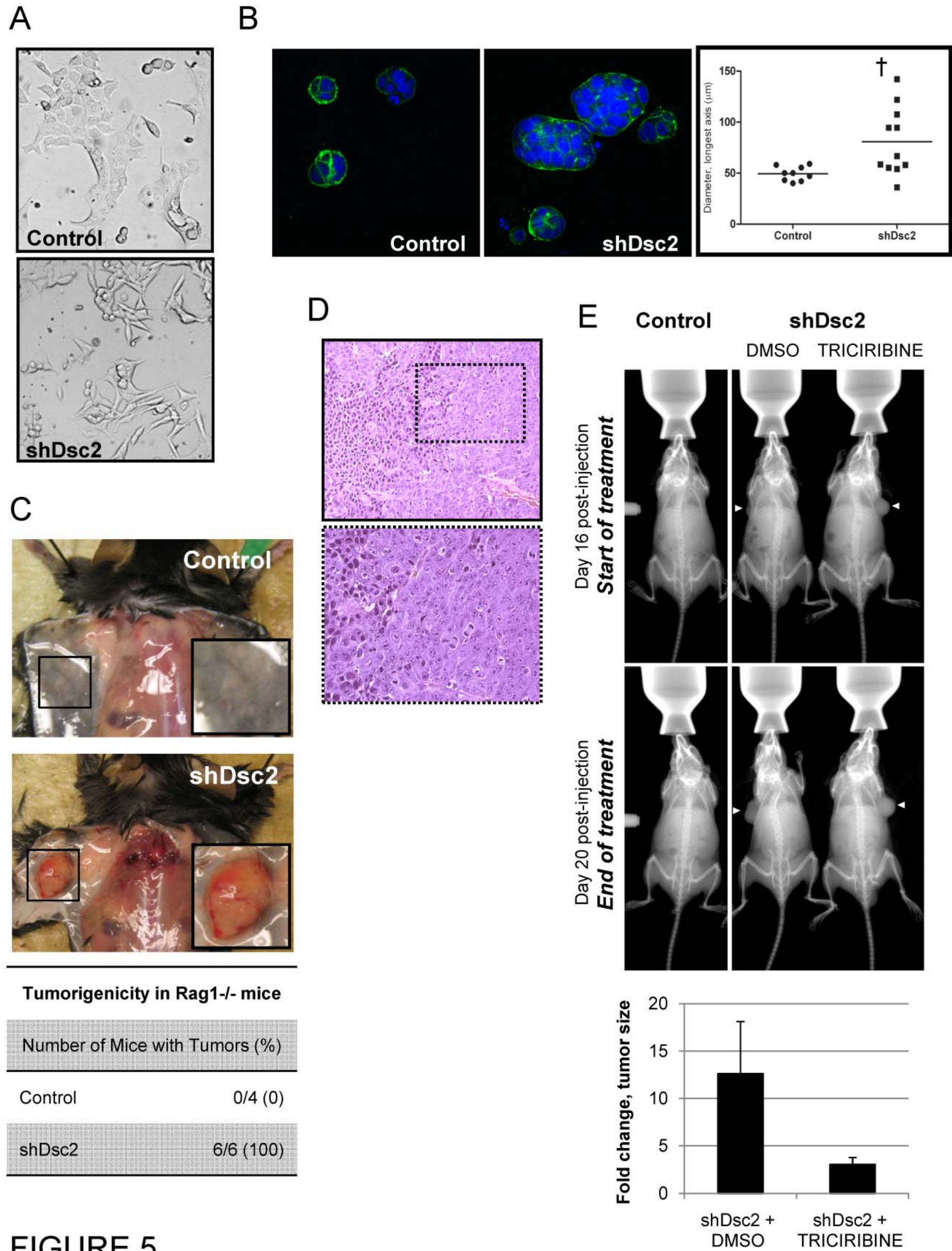
Control + DMSO
shDsc2 + DMSO
Control + Tricribine
shDsc2 + Triciribine

D

DMSO
Triciribine

Control
shDsc2
Control
shDsc2

FIGURE 4



Tumorigenicity in Rag1-/- mice	
Number of Mice with Tumors (%)	
Control	0/4 (0)
shDsc2	6/6 (100)

FIGURE 5

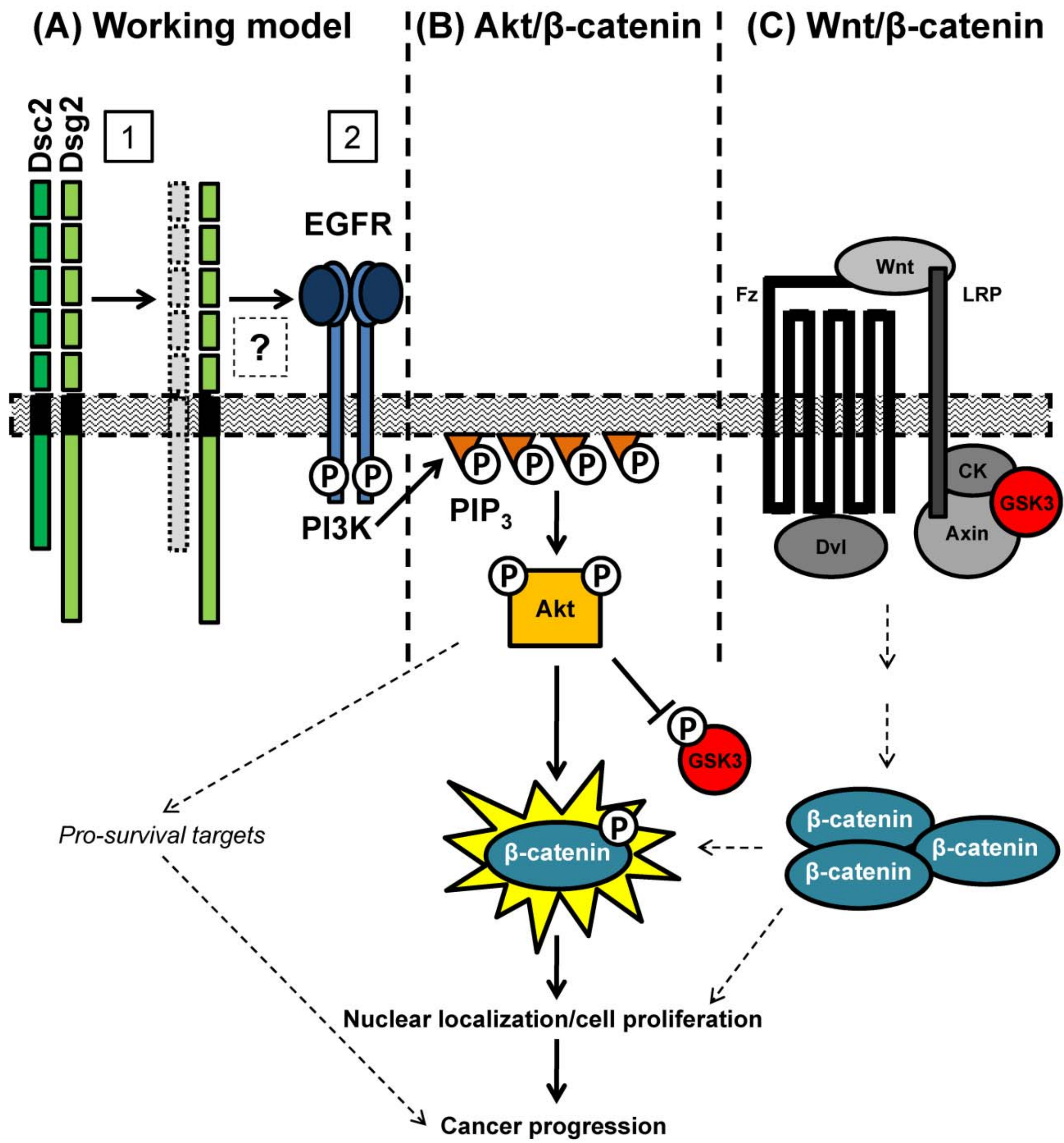
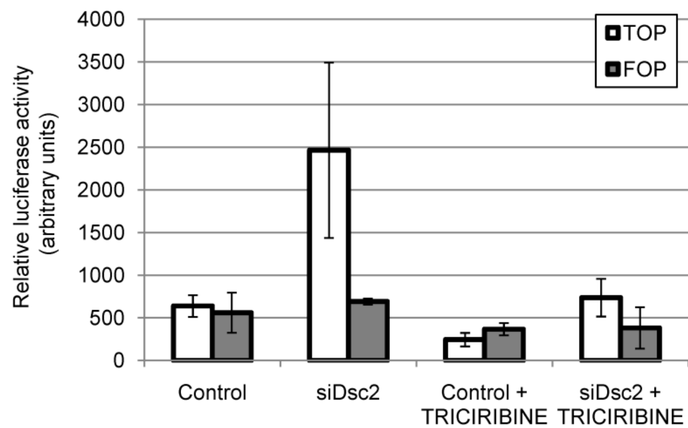
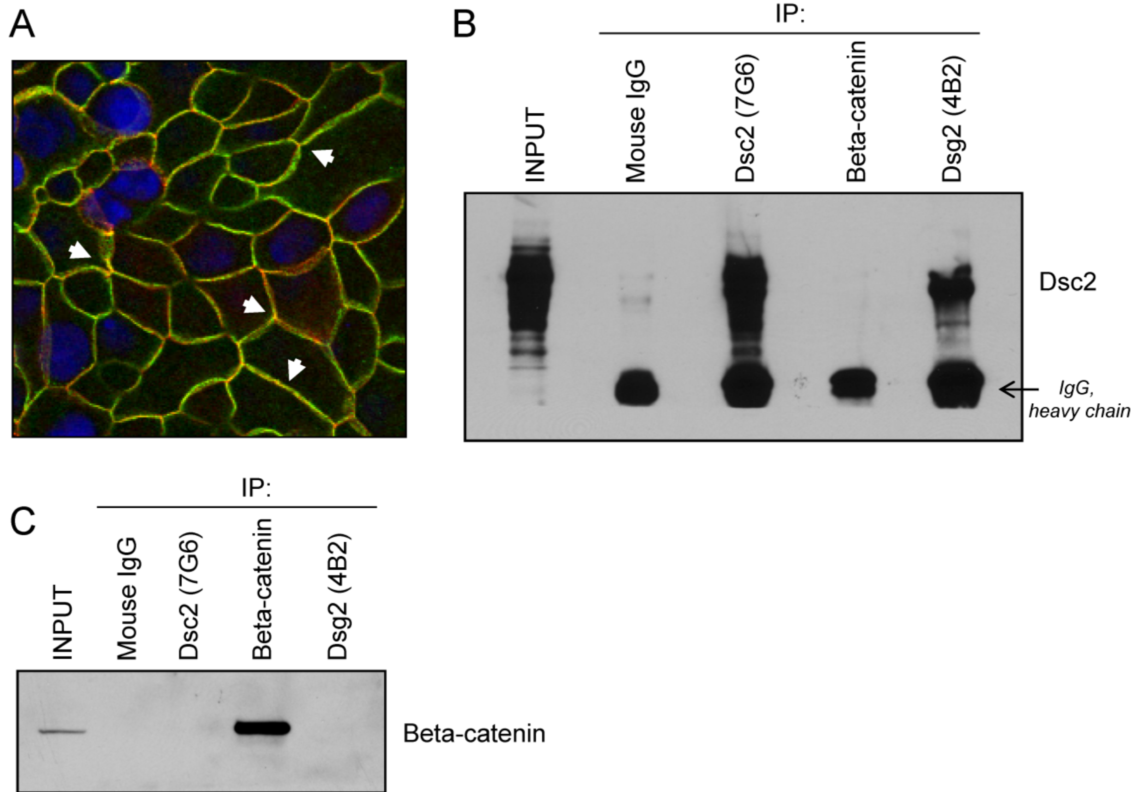


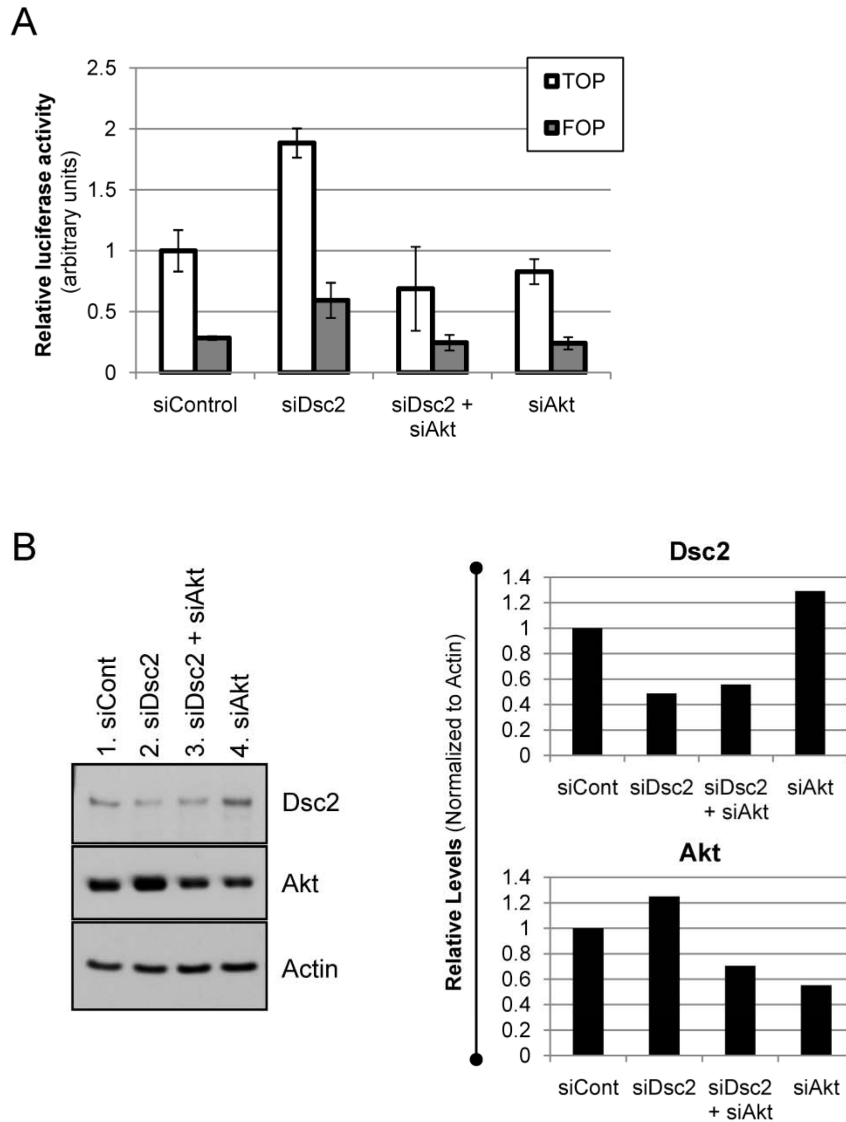
FIGURE 6



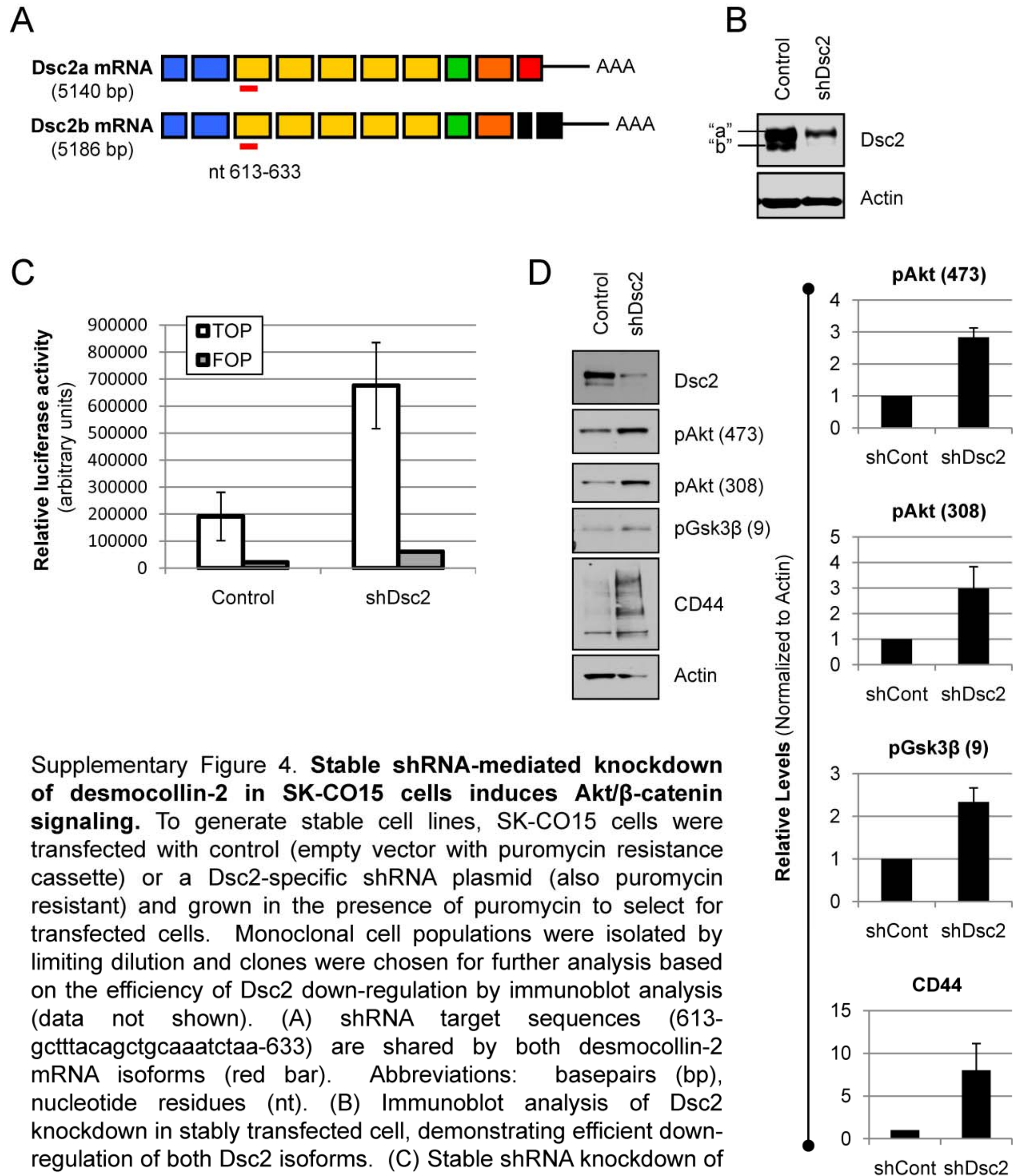
Supplementary Figure 1. **Loss of desmocollin-2 in Caco-2 cells leads to activation of β-catenin/TCF-dependent transcription.** Luciferase-based reporter assay for β-catenin/TCF-dependent transcription (TOP Flash). Caco-2 cells were transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids. Twelve hours after transfection, the cells were treated with the Akt inhibitor triciribine or vehicle alone (DMSO). At 72 hours post-transfection, luciferase activity was assessed.



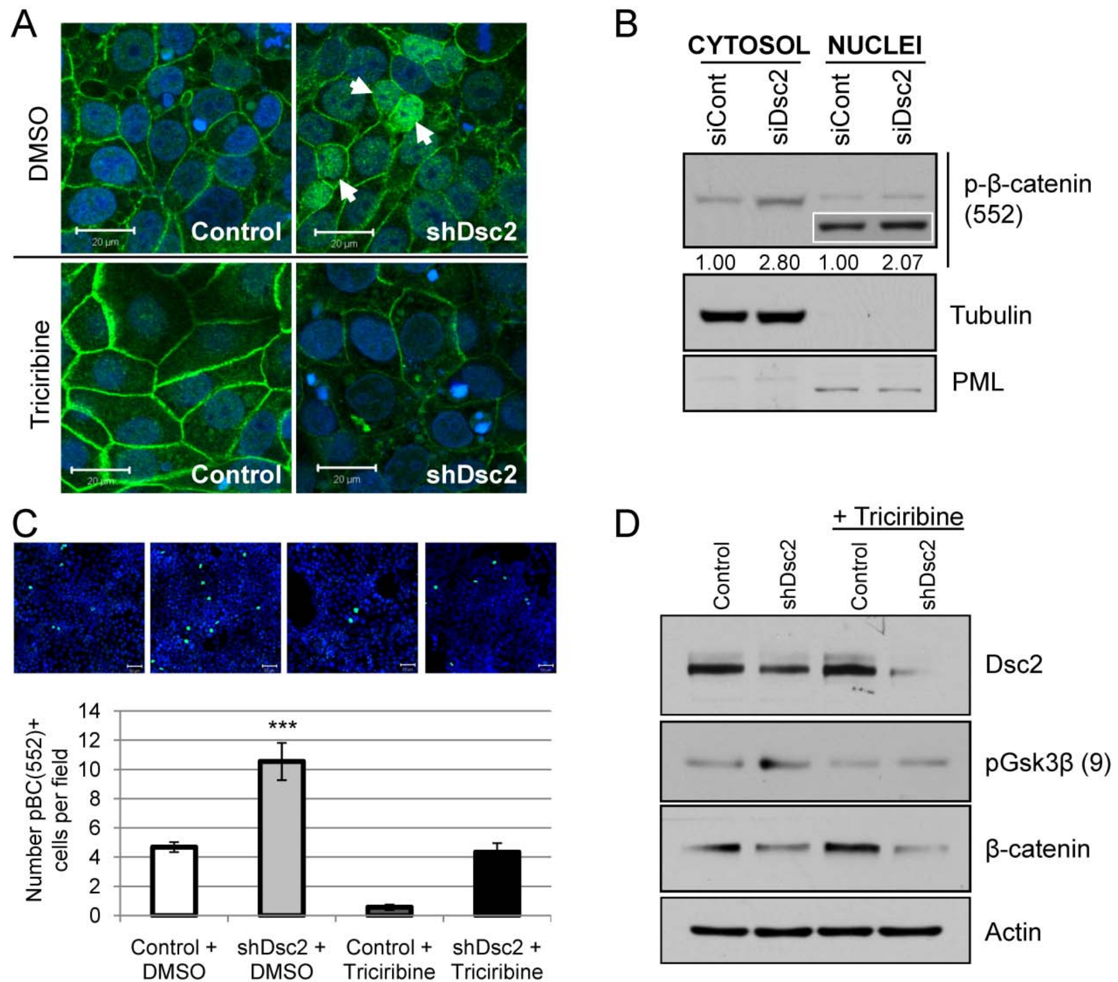
Supplementary Figure 2. **Desmocollin-2 and β-catenin do not form a complex in SK-CO15 cells.** (A) SK-CO15 cells stained for Dsc2 (green) and β-catenin (red). These proteins co-localize at some areas along the lateral membrane of SK-CO15 cells (arrows). (B) and (C) Co-immunoprecipitation (IP) experiments using SK-CO15 cell lysates. Antibody used for IP is indicated across the top of the blot; membranes were probed with Dsc2 (B) or B-catenin (C). There is no evidence of complex formation between Dsc2 and β-catenin. As expected, an association between Dsg2 and Dsc2 was detected (positive control).



Supplementary Figure 3. **Targeted depletion of Akt using specific siRNA recapitulates the effect of triciribine on β-catenin-dependent transcription following Dsc2 knockdown.** (A) SK-CO15 cells were transfected with the indicated siRNAs along with the TOP or FOP luciferase reporter plasmids. At 48 hours post-transfection, luciferase activity was assessed. The graph is representative of at least three independent experiments. (B) Immunoblot analysis of Dsc2 and Akt protein levels following treatment with siRNA specific for Dsc2, Akt, or both (left panel). Densitometric quantification of the western blot data, normalized to total actin levels.

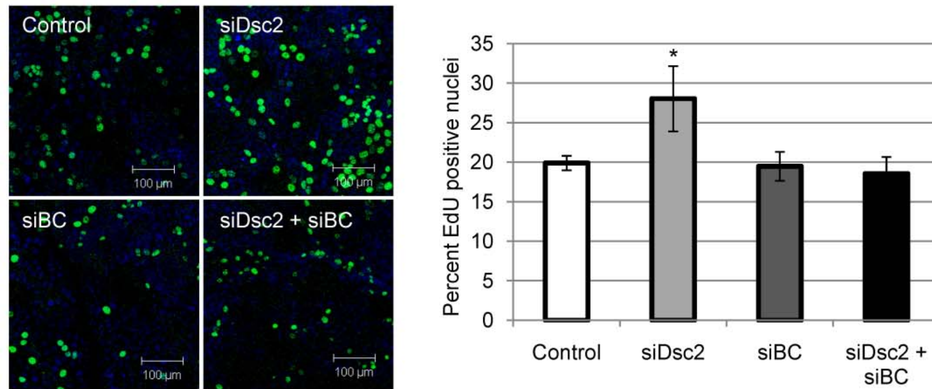


Supplementary Figure 4. **Stable shRNA-mediated knockdown of desmocollin-2 in SK-CO15 cells induces Akt/β-catenin signaling.** To generate stable cell lines, SK-CO15 cells were transfected with control (empty vector with puromycin resistance cassette) or a Dsc2-specific shRNA plasmid (also puromycin resistant) and grown in the presence of puromycin to select for transfected cells. Monoclonal cell populations were isolated by limiting dilution and clones were chosen for further analysis based on the efficiency of Dsc2 down-regulation by immunoblot analysis (data not shown). (A) shRNA target sequences (613-gctttacagctgcaaatctaa-633) are shared by both desmocollin-2 mRNA isoforms (red bar). Abbreviations: basepairs (bp), nucleotide residues (nt). (B) Immunoblot analysis of Dsc2 knockdown in stably transfected cell, demonstrating efficient down-regulation of both Dsc2 isoforms. (C) Stable shRNA knockdown of Dsc2 increases β-catenin/TCF-dependent transcription. (D) Western blot analysis of phosphorylated Akt (Thr308 and Ser473, active), phospho-Gsk3β (Ser9, inactive), and CD44 in control and shDsc2 knockdown cells. Actin is included as a loading control. Densitometric analysis of at least three experiments. Graph represents the mean ± SEM for each protein analyzed.

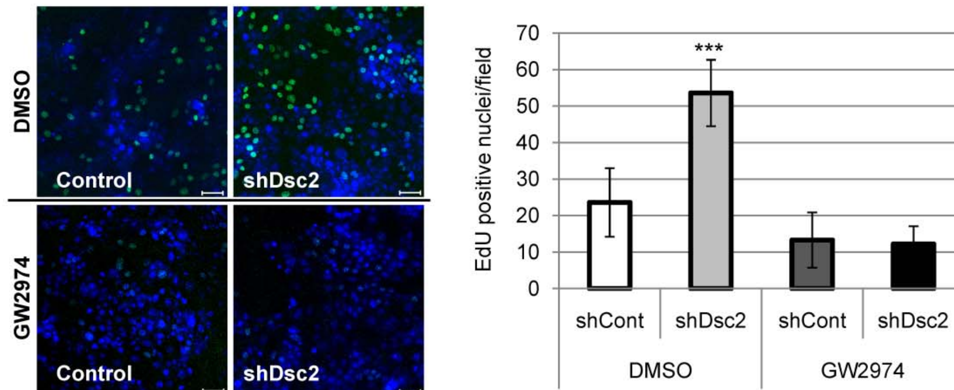


Supplementary Figure 5. **Down-regulation of desmocollin-2 induces nuclear accumulation of p-(Ser552)-β-catenin through an Akt-dependent mechanism.** (A) Immunofluorescence labeling/confocal microscopy of β-catenin in control and shDsc2 cells after Akt inhibition, nuclear accumulation is noted by the arrows (green, β-catenin; blue, nuclei; scale bar is 20 µm). (B) Nuclear and cytosolic fractionation of siControl and siDsc2 transfected cells, at 24 hours post-transfection. Tubulin and PML mark the cytosolic and nuclear compartments, respectively. Signal intensities were determined using densitometry and the relative difference for each fraction in indicated. Inset is a darker exposure of nuclear immunoblot. Results are representative of three independent experiments. (C) Immunofluorescence labeling/confocal microscopy of phospho-β-catenin (Serine 552, active; pBC(552)) in control and shDsc2 cells after Akt inhibition (green, pBC(552); blue, nuclei; scale bar is 10 µm). The histogram shows the mean ± SEM of pBC(552) positive nuclei per field from at least 10 different fields. (***$p < 0.0001$, ANOVA). The results are representative of at least three independent experiments. (D) Cells stably expressing Dsc2-specific shRNA (shDsc2) or vector alone control plasmid (control) were grown in the presence of triciribine or vehicle only (DMSO). Protein extracts from each condition were assayed for expression of phosphorylated Gsk3β (Serine 9, inactive) and total β-catenin by immunoblot. Actin is included as a loading control.

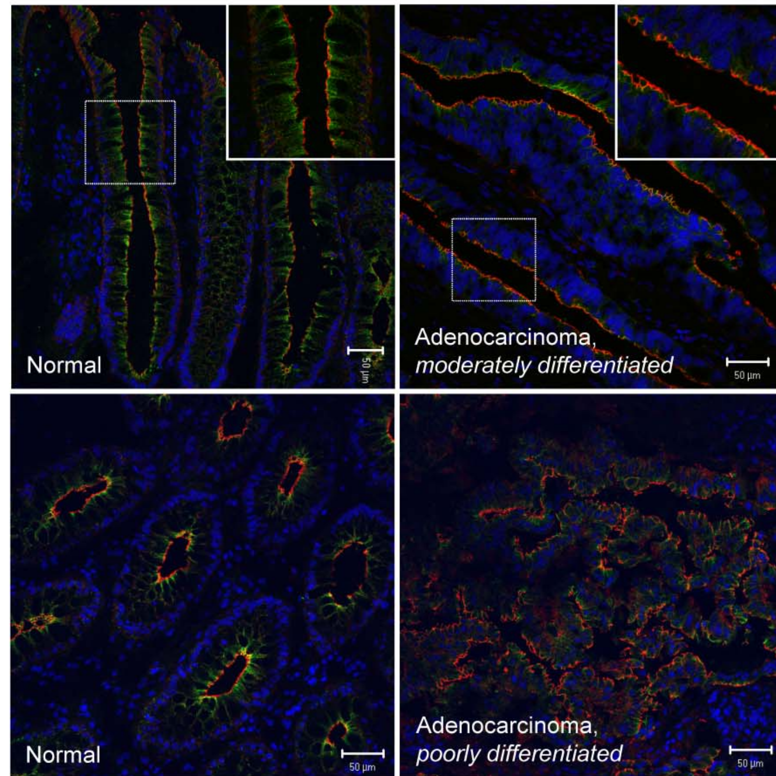
A



B



Supplementary Figure 6. **Desmocollin-2 loss enhances cell proliferation through an EGFR and β-catenin-dependent mechanism.** (A) SK-CO15 cells transfected with control, Dsc2, β-catenin (BC), or Dsc2 and BC-specific siRNAs. At 72 hours post-transfection, cells were pulse-labeled with EdU for 1 hour and processed to detect incorporated EdU. Images were obtained using confocal microscopy and the percent of EdU positive nuclei was scored. The histogram shows the mean ± SEM of the percent of EdU positive nuclei from at least 10 different fields. The results are representative of three independent experiments. (EdU, green; nuclei, blue). (*$p < 0.05$, ANOVA) (B) EdU incorporation assay for cells stably expressing Dsc2-specific shRNA (shDsc2) or control shRNA plasmid (control) and grown in the presence of the EGFR inhibitor GW2974 or vehicle only (DMSO). The histogram shows the mean ± SD of EdU positive nuclei from at least 10 different fields. (***$p < 0.0001$, ANOVA).



Supplementary Figure 7. **Desmocollin-2 is down-regulated in moderately and poorly differentiated colonic carcinomas.** Dsc2 distribution in human colonic epithelium (Normal, left panels) and adenocarcinoma of the colon (right panels) using confocal microscopy. Co-staining with anti-JAM-A delineates the apical/lumenal domain of the epithelium. (Dsc2, green; JAM-A, red; nuclei, blue).

CHAPTER 3:

Characterization of full-length and proteolytic cleavage fragments of desmoglein-2 in native human colon and colonic epithelial cell lines

This chapter consists of a manuscript in preparation, March 2011.

Characterization of full-length and proteolytic cleavage fragments of desmoglein-2 in native human colon and colonic epithelial cell lines

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ABSTRACT

The desmosomal cadherin desmoglein-2 (Dsg2) is a transmembrane cell adhesion protein that is widely expressed in epithelial and non-epithelial tissues, such as the intestine, epidermis, testis, and heart. Dsg2 has been shown to regulate numerous cellular processes, including proliferation and apoptosis, and we have previously reported that intracellular fragments of Dsg2 promote apoptosis in colonic epithelial cells. While several studies have shown that both the extracellular and intracellular domains of Dsg2 can be targeted by proteases, identification of these putative Dsg2 fragments in colonic epithelial cells has not been performed. Here, we report that the mouse monoclonal antibody (mAb) AH12.2 binds to the first extracellular domain of Dsg2. Using this antibody along with previously described mAb against the extracellular (6D8) and intracellular (DG3.10) domains of Dsg2, we characterize the expression and identify the cleavage fragments of Dsg2 in colonic epithelial cells. This study provides a detailed description of the extracellular and intracellular Dsg2 cleavage fragments that are generated in the simple epithelium of the intestine and will guide future studies examining the relationship of these fragments to cellular fate and disease states.

INTRODUCTION

Desmosomes are specialized intercellular junctions that have been identified in all epithelial tissues, myocardium, and lymph nodes, where they reinforce cell-cell adhesion and strengthen tissue integrity [Delva et al., 2009; Green and Simpson, 2007; Holthöfer et al., 2007]. In particular, desmosomes are highly enriched in tissues that experience extensive mechanical stress, such as cardiac muscle and epidermis. Abnormal desmosomal function results in weakened intercellular adhesion and disease, as exemplified by the human pathologies arrhythmogenic right ventricular cardiomyopathy (ARVC), pemphigus, bullous impetigo, and staphylococcal scalded skin syndrome (SSSS) [Amagai, ; Delmar and McKenna, ; Waschke, 2008]. Furthermore, altered expression of desmosomal proteins has been described in a number of disease states, including squamous cell carcinoma, colonic adenocarcinoma, and nasal polyposis [Brennan and Mahoney, 2009; Chen et al., 2007; Khan et al., 2006; Wang et al., 2007; Wong et al., 2008; Zuckerman et al., 2008].

The extracellular adhesive interface of the desmosome is formed by the desmosomal cadherins desmoglein (Dsg) and desmocollin (Dsc), which are single-pass transmembrane glycoproteins of the cadherin superfamily. The cytoplasmic domains of Dsg and Dsc mediate interactions with linker/adaptor plaque proteins such as plakoglobin, plakophilin, and desmoplakin and thereby connect the desmosome to the intermediate filament network of the cell [Dusek et al., 2007]. Isoforms of the desmosomal cadherins are expressed in a tissue-specific and differentiation-specific pattern, which may reflect differential adhesive capabilities of particular isoforms [Dusek et al., 2007; Mahoney et al., 2006]. For instance, all isoforms (Dsg 1-4 and Dsc 1-3 in humans) are expressed in the epidermis, albeit in a differentiation-dependent manner, whereas only Dsg2 and Dsc2 are expressed in cardiac myocytes and in the intestinal epithelium.

Desmosomal cadherins share common features, including an amino-terminal extracellular domain that consists of four cadherin repeats (EC1-4) and the membrane proximal extracellular anchor (EA) sequence (Figure 1A) [Green and Simpson, 2007; Holthöfer et al., 2007]. Following the transmembrane (TM) domain, both Dsg and Dsc have a membrane proximal intracellular anchor (IA) domain and an intracellular catenin binding site (ICS) which associates with plakoglobin. Unique to the Dsg isoforms are additional cytoplasmic domains of unknown function, consisting of the intracellular proline-rich linker (IPL), repeated unit domain (RUD), and the glycine-rich Dsg-terminal domain (DTD).

Interestingly, the extracellular and intracellular domains of the Dsgs have been shown to be targeted by matrix metalloproteinases and cysteine proteases, respectively [Amagai et al., 2000; Bech-Serra et al., 2006; Borgono et al., 2007; Cirillo et al., 2008; Dusek et al., 2006; Jiang et al., ; Nava et al., 2007; Ramani et al., 2008], and proteolysis may be a physiologic and/or pathologic mechanism by which desmosomal adhesion is regulated. Furthermore, studies from our laboratory and others have demonstrated that Dsg cleavage fragments, as opposed to the full-length protein, actively regulate cellular processes, including apoptosis and differentiation [Getsios et al., 2009; Nava et al., 2007]. These findings suggest that proteolytic cleavage of Dsg may also affect other non-adhesive Dsg functions.

Given that multiple Dsg isoforms are expressed in the epidermis and in the keratinocyte cell lines which are commonly utilized to study desmosomes and that the antibodies used to characterize Dsg expression often detect more than one Dsg isoform, assessing the role of Dsg cleavage fragments adds significant complexity to the study of Dsg function.

Given the recent evidence of specific functional effects induced by Dsg cleavage fragments, we sought to characterize the major Dsg2 fragments that are generated in human colonic

epithelial cells, which only express the Dsg2 isoform of Dsg [Holthöfer et al., 2007; Nava et al., 2007]. Here, we demonstrate that the mAb AH12.2 recognizes the first extracellular domain of Dsg2 and show that multiple extracellular and intracellular Dsg2 cleavage fragments can be detected in colonic epithelial cell lines and native colonic mucosa. The generation of these fragments may be related to intestinal epithelial cell fate and therefore may influence tissue homeostasis.

RESULTS

Antibody AH12.2 detects the first extracellular domain of desmoglein-2. We previously identified desmoglein-2 (Dsg2) as the antigen recognized by mAb AH12.2 and showed that this antibody does not detect other human Dsg isoforms [Nava et al., 2007]. To gain insight into the binding site for AH12.2, we performed antibody binding experiments by expressing sequentially truncated forms of Dsg2 (Figure 1A) in Chinese Hamster Ovary (CHO) cells. To assess whether AH12.2 detected an intracellular epitope of Dsg2, we expressed full-length Dsg2 (Dsg2FL), the Dsg2 intracellular domain (Dsg2CT), or a minimal fragment of the intracellular domain, containing only the RUD and DTD sequences (Dsg2RD). All expressed constructs were detected by the Dsg2 intracellular domain specific antibodies DG3.10 (Figure 1B) and 4B2 (data not shown), which are known to bind an epitope within the RUD domain of Dsg2 (Figure 1D). In contrast, AH12.2 and the known Dsg2 extracellular domain specific antibody 6D8 detected full-length Dsg2 but not its intracellular domain (Figure 1B), suggesting that AH12.2 recognizes an extracellular epitope of Dsg2. To confirm that AH12.2 detects an extracellular region of Dsg2 and to further map the epitope, we expressed the entire Dsg2 extracellular domain (Dsg2EC1-EA) or overlapping

extracellular domain segments (Dsg2EC1+2, Dsg2EC2+3, etc.) in CHO cells and examined the ability of AH12.2 to detect these expressed proteins by immunoblotting. As shown in Figure 1C, AH12.2 detects the full extracellular domain of Dsg2 and the EC1+EC2 but not EC2+EC3 domains. These results demonstrate that AH12.2 binds to the first extracellular domain of Dsg2 or the "joint" between EC1 and EC2. Consistent with previous reports [Wang et al.], we show that antibody 6D8 specifically binds EC3/EC4. Importantly, none of the Dsg2-specific antibodies tested in this study detected the related cadherins desmocollin-2 (Dsc2; Figure 1B and C) or E-cadherin (data not shown) by immunoblot analysis, thereby demonstrating specificity of these antibodies for desmoglein. The schematic in Figure 1D summarizes the results of the antibody mapping experiments. Since some Dsg-specific antibodies have been shown to recognize glycoepitopes of Dsg, we next assessed whether non-glycosylated Dsg2 could be detected by AH12.2 by expressing a GST-tagged Dsg2 extracellular domain in bacteria. As shown in Supplementary Figure 1, bacterially-expressed Dsg2 is detected by AH12.2, indicating that this antibody likely detects the protein backbone of Dsg2 rather than a glycoepitope.

Desmoglein-2 protein expression in colonic epithelial cells. We previously described the generation of lower molecular weight Dsg2 cleavage fragments in the model colonic epithelial cell lines SK-CO15 and T84 [Nava et al., 2007]. To determine whether these Dsg2 cleavage products could be detected in additional intestinal epithelial cell lines (Caco-2 and HT-29) as well as in native human colonic mucosa, we prepared a panel of cell lysates from each of these sources and analyzed Dsg2 expression by immunoblotting. Full-length Dsg2 (~150 kDa) was detected in each of the cell lines examined, as well as in the native human colonic mucosa (Figure 2). In addition, a ~130 kDa protein band (indicated by *) and 65-100 kDa protein bands (solid bar) were detected by AH12.2 and 6D8 that recognize the

extracellular domain of Dsg2. In contrast, using antibodies that detect the intracellular domain of Dsg2, DG3.10 (Figure 2) and 4B2 (data not shown), we identified ~110 kDa (indicated by <) and 50-65 kDa protein bands (dashed bar) in addition to the full-length ~150 kDa Dsg2. These data suggest that the generation of cell-associated, lower molecular weight fragments of Dsg2 may have functional significance in the intestinal epithelium.

In addition to cell-associated fragments, shedding of the Dsg2 extracellular domain has been reported in epithelial model systems but has not been described for the intestine [Bech-Serra et al., 2006; Lorch et al., 2004]. Therefore, we assessed whether a shed Dsg2 extracellular domain could be detected in the culture medium of colonic epithelial cell lines. As shown in Figure 3A, both Dsg2 extracellular domain-specific antibodies AH12.2 and 6D8 recognized a ~90 kDa protein band in the supernatants collected from each of the cell lines; however, the intracellular domain antibodies DG3.10 or 4B2 (data not shown) did not detect these bands, suggesting that the presence of these bands were due to ectodomain shedding, rather than non-specific cell lysis. Furthermore, the ability of AH12.2 and 6D8 to detect this band was not due to cross-reactivity of the antibodies with media proteins, as these antibodies only detected the ~90 kDa protein band in conditioned cell culture media but not media alone (data not shown). Since metalloproteases have been shown to mediate the ectodomain shedding of Dsg2, we assessed the ability of GM6001, an inhibitor of metalloproteases, to reduce the release of this fragment into the culture medium. As shown in Figure 3B, treatment with GM6001, but not the presenilin inhibitor DAPT or the inhibitor of cysteine proteases E64, reduced the shedding of the Dsg2 extracellular domain into the culture supernatant.

RNAi knockdown confirms the specificity of AH12.2 for the full-length and lower molecular weight fragments of desmoglein-2. To confirm that each of the species detected

by the Dsg2 specific antibodies were indeed derived from Dsg2, we utilized siRNA to down-regulate the expression of Dsg2 in the colonic epithelial cell line SK-CO15. Indeed, the ~150 kDa protein band detected by AH12.2, 6D8, DG3.10 (Figure 4A) and 4B2 (data not shown) was decreased by Dsg2 siRNA treatment, thus confirming that this protein band represents full-length Dsg2. Likewise, the shed fragment detected in the cell culture medium by AH12.2 was also diminished following down-regulation of Dsg2 (Figure 4B) as were the 110 kDa and 50-65 kDa bands recognized by the intracellular domain antibodies DG3.10 (Figure 4C, left panel) and 4B2 (data not shown). Lastly, Dsg2 knockdown reduced the intensity of the 95-100 kDa bands detected by AH12.2 and a ~100 kDa band detected by 6D8 (Figure 4C). Unexpectedly, the prominent ~95 kDa band detected by the mAb 6D8 (Hycult Biotech, The Netherlands) was not affected by down-regulation of Dsg2 (Figure 4C; indicated by #), suggesting that this protein band does not represent a Dsg2 cleavage fragment. Since our results suggested cross-reactivity of 6D8 (Hycult Biotech, The Netherlands) with a non-Dsg2 protein, we obtained 6D8 hybridoma supernant from the original source (Dr. James Wahl, University of Nebraska). Importantly, this antibody did not recognize the prominent non-Dsg2 protein band at 95 kDa (data not shown), suggesting that the commercially available antibody cross-reacts with another cellular antigen that co-migrates with a Dsg2 cleavage fragment. Notably, we have observed that the commercial 6D8 antibody consistently co-immunoprecipitates beta-catenin from human colonic epithelial cells (Supplementary Figure 2A), which also migrates as a 95 kDa band by SDS-PAGE. We confirmed cross-reactivity of the commercially available 6D8 with beta-catenin by RNAi which showed that beta-catenin-specific siRNA decreases the appearance of the 6D8 cross-reactive product at 95 kDa (Supplementary Figure 2B), suggesting that this band may in fact be beta-catenin.

DISCUSSION

Recent evidence suggests that proteolytic fragments of Dsgs can influence intracellular signaling cascades and thereby control cellular processes such as apoptosis and differentiation [Getsios et al., 2009; Nava et al., 2007]. To characterize the Dsg2 fragments generated in human colonic epithelial cell lines (SK-CO15, T84, Caco-2, and HT-29) and in native human colonic mucosa, we used a panel of Dsg2-specific mAbs, all of which recognize distinct domains of Dsg2. RNAi mediated down-regulation of Dsg2 expression was employed to confirm that each of the protein bands detected were indeed Dsg2.

The results from this study demonstrate that multiple cleavage fragments of Dsg2 are generated in colonic epithelial cells. Major proteolytic products identified here represent shed extracellular domain "shed Dsg2", a cell-associated extracellular domain-containing cleavage product "truncated Dsg2", and fragments containing the cytoplasmic/c-terminal RUD/DTD domains of Dsg2 "c-terminal Dsg2". Furthermore, we provide evidence that a c-terminally truncated Dsg2 "RUD/DTD-lacking Dsg2" may be expressed in colonic mucosa however more work is needed to determine whether this protein band represents a Dsg2 cleavage fragment or an alternatively spliced form of Dsg2. These data extend earlier studies from our laboratory and others demonstrating that the Dsg2 extracellular and intracellular domains are cleaved by proteolytic enzymes and that these cleavage events can be regulated by pro-apoptotic and other stimuli [Amagai et al., 2000; Bech-Serra et al., 2006; Borgono et al., 2007; Cirillo et al., 2008; Dusek et al., 2006; Jiang et al., 2010; Nava et al., 2007; Ramani et al., 2008; Zuckerman et al., 2008].

Antibody AH12.2 binds to the first extracellular domain of desmoglein-2. We report that mAb AH12.2 recognizes a protein epitope contained within the first extracellular domain of

human Dsg2 (Figure 1). Interestingly, previous immunofluorescence labeling/confocal microscopy studies with this antibody suggested recognition of an intracellular epitope of Dsg2 [Nava et al., 2007]. However, detailed analysis of domain binding using Dsg2 expression constructs revealed that the antibody in fact recognizes the EC1 domain of Dsg2. Based on these findings, it is likely that the lack of AH12.2 binding to non-permeabilized colonic epithelial cell monolayers reflects the inaccessibility of the EC1 epitope, either due to adhesive interactions that have been proposed to occur through the EC1 domain of Dsg or due to the inability of the antibody to access desmosomal proteins through intact tight junctions.

Other Dsg2-specific extracellular domain antibodies have been shown to affect intercellular adhesion [Schlegel et al., 2010] however our preliminary studies suggest that AH12.2 does not have the same adhesion-disrupting effect on colonic epithelial cells (unpublished observations). Interestingly, a recent report from Wang et al. demonstrated Dsg2 antibodies inhibited adenovirus entry into epithelial cells and could also induce the activation of signaling pathways involved in epithelial-to-mesenchymal transition [Wang et al., 2010]. Given recent findings indicating that loss of Dsc2 leads to hyperproliferation and confers a tumorigenic phenotype to colonic epithelial cells [Kolegraff et al., 2011], it will be interesting to examine the role of Dsg2 in this context, and whether antibodies against Dsg2 have any effect of these pro-tumorigenic pathways in our model system.

Proteolysis as a potential mechanism to regulate adhesive and non-adhesive functions of desmoglein-2. In addition to supporting cell-cell adhesion, Dsgs have also been shown to regulate numerous cellular processes, including proliferation, differentiation, tissue morphogenesis/sorting, and apoptosis [Brennan et al., 2007; Dusek et al., 2007; Getsios et al., 2009; Runswick et al., 2001]. Furthermore, Dsg family members are the antigenic target of

at least two autoimmune diseases, pemphigus foliaceus (Dsg1) and pemphigus vulgaris (Dsg3/Dsg1), in which anti-Dsg antibodies induce blistering of the skin and mucosal membranes, due to antibody interference with Dsg function. These data along with that of others suggests that proteolytic "uncoupling" of the Dsg extracellular and intracellular domains may contribute to the regulation of Dsg function in epithelia and other cell types as fragmentation of Dsg is altered in a number of disease states. For instance, enhanced generation of pro-proliferative signaling fragments may explain how a putative cell-cell adhesion protein promotes, rather than inhibits, epithelial cell proliferation as has been demonstrated for Dsg family members [Brennan et al., 2007; Merritt et al., 2002]. In addition, pathogenic autoantibodies or the functional antibodies used in a variety of experimental systems to study Dsg may in fact alter the generation of Dsg cleavage fragments and thereby exert their functional effects as the ability of antibodies to interfere with or promote proteolysis of transmembrane cell-cell adhesion proteins has already been reported [Brazil et al., 2010]. We have preliminary evidence that both antibodies AH12.2 and 6D8 may influence the generation of "truncated Dsg2" in colonic epithelial cell cultures without affecting Dsg2 shedding (unpublished observations), however more work is needed to examine the ability of anti-Dsg2 antibodies to influence Dsg2 proteolysis.

Finally, the data presented here also show the need to re-examine earlier studies using antibody 6D8, as we report that a commercially-available Dsg2 antibody (6D8, Hycult, The Netherlands) cross-reacts with a cellular antigen that co-migrates with the truncated Dsg2 cleavage fragment but is not Dsg2 (Figure 4; Supplementary Figure 2). Further characterization of this antigen suggests that it may be beta-catenin, given the results of immunoprecipitation and RNAi silencing experiments. Importantly, we tested 6D8 hybridoma supernatant from the original source (J. Wahl, University of Nebraska) and this

antibody preparation did not detect the prominent 95 kDa band recognized by the commercially available antibody (data not shown). These results suggest that the commercially-available antibody may contain an additional cross-reactive antibody that complicates interpretation of expression/localization studies.

In summary, the data presented here show that the Dsg2-specific antibody AH12.2 binds to the Dsg2 EC1 domain. In addition, our results show that multiple Dsg2 cleavage fragments are detected in colonic epithelial cells. We speculate that the generation of these fragments may be related to intestinal epithelial cell fate and therefore may influence tissue homeostasis.

MATERIALS AND METHODS

Cell culture and antibodies. The transformed human intestinal epithelial cell lines HT-29, SK-CO15 and Caco-2 and Chinese Hamster Ovary (CHO) cells were grown in high glucose (4.5 g/L) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES (pH 7.4), 2 mM L-glutamine, and 1% nonessential amino acids as previously described [Ivanov et al., 2007]. The following monoclonal antibodies (mAb) were used to detect proteins by Western blotting: anti-desmocollin 2/3 mAb, clone 7G6 (kind gift from K. Green, Northwestern University, USA); anti-actin, tubulin, and GAPDH pAbs (Sigma-Aldrich, St. Louis, MO); anti-desmoglein 2 mAb, clone AH12.2 (generated in-house); anti-desmoglein 2 mAb, clone 6D8 (Hycult Biotech, The Netherlands); anti-desmoglein 2 mAb, clone DG3.10 (Progen, Germany). Peroxidase-conjugated secondary antibodies were obtained from Jackson Laboratories. Dylight Infrared dye-conjugated secondary antibodies were obtained from ThermoScientific.

Immunoblotting. Confluent monolayers were washed two times with HBSS+ on ice and collected in lysis buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4), containing a proteinase inhibitor cocktail (1:100, Sigma). Lysates were then cleared by centrifugation (30 min at 14, 0000 x g) and immediately boiled in SDS sample buffer. Gel electrophoresis and immunoblotting were conducted by standard methods with 10–20 μg protein per lane. Two-color infrared immunoblots were imaged using the Licor Odyssey Detection System.

Generation of fusion proteins. The intracellular domain fragments were generated as previously described [Nava et al, 2007]. The DNA fragments encoding the Dsg2 extracellular fragments were generated by polymerase chain reaction (PCR) as follows. GST-

tagged extracellular domain of Dsg2 (gene nucleotides 143 to 1735) was cloned in pGEX-4T1 (Pharmacia) primers forward 5-gcgtggatccgcctggatcaccgcccccgtggc-3; reverse 5 aagcactcgagctaactaaaaccctgattgtctgaaa-3. For the generation of the extracellular fragments of Dsg2, the DNA fragments encoding the Dsg2 extracellular were cloned in pcDNA3.1 (Invitrogen) using the following primers. Dsg2 EC1-EC2 (gene nucleotides 1 to 813) forward 5-acggggtaccgccaccatggcgcggagcccgggacgcgc-3; reverse 5-gatgggatccgtgatggtgatggtgatggtgatggtgatgtactacaggtatattgtcat-3; Dsg2 EC2-EC3 (gene nucleotides 473 to 1161) forward 5-tcatttagtgcggcaaaagcgcacacaggatgtctttgttgggt -3; reverse 5-gatgggatccgtgatggtgatggtgatggtgatggtgatgaaaatgaatgccttctttca-3; Dsg2 EC3-EC4 (gene nucleotides 813 to 1507) forward 5- tcatttagtgcggcaaaagcgcgaaaataaagtgcttgaaggga-3; reverse 5-gatggg atccgtgatggtgatggtgatggtgatggtgatgcacaggctctatcagtgtgg-3; Dsg2 EC4-EA (gene nucleotides 1161 to 1735) forward 5-tcatttagtgcggcaaaagcgcaaaagcagcgtcatctcaattt-3; reverse 5-gatgggatccgtgatggtgatggtgatggtgatggtgatgtggtttgtcaatgacggaga-3. The cloning was verified by sequencing. Expression and purification of GST fusion proteins was performed following previously described standard procedures (Frangioni and Neel, 1993 Anal. Biochem. 210, 179-187). The constructs encoding the c-myc epitope tagged Dsg2 extracellular fragments were transfected into Chinese Hamster Ovary (CHO) cells (70% confluence) using Lipofectamine 2000 (Invitrogen,) in Opti-MEM medium (Invitrogen). The media was replaced after 6 h with fresh media (Opti-MEM) and cells were cultured for other 24 h before media was collected and concentrated using Amicon Ultra-15 (5000 MW) columns (Millipore). For Western blot analysis, fusion proteins were resuspended in phosphate-buffered saline (PBS) (pH 7.4) to a concentration of 1µg/µl and subject to SDS–PAGE analysis.

Protease inhibitors. For inhibitor studies, the cells were preincubated for 8-12 h with different inhibitors as follow: presenilin inhibitor DAPT (Tocris, Cat. No. 2634; 200 nM), metalloprotease inhibitor GM6001 (Calbiochem, Cat. No.364206; 10 μM), cysteine protease inhibitor E64 (Sigma, Cat. No. E3132; 10μM), or DMSO control before media was collected concentrated a previously describe and subject to Western blot analysis.

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FIGURE LEGENDS

Figure 1. Antibody AH12.2 recognizes the first extracellular domain of desmoglein-2. **(A)** Desmoglein-2 (Dsg2) constructs used to characterize the epitope of AH12.2. Top schematic depicts the protein domains of Dsg2. The domains represented by each construct are indicated below the diagram, along with the amino acids (aa). PRO, prosequence; EC, extracellular domain; EA, extracellular anchor; TM, transmembrane domain; IA, intracellular anchor; ICS, intracellular cadherin segment; IPL, intracellular proline-rich linker; RUD, repeating unit domain; DTD, Dsg terminal domain; FL, full-length; RD, RUD + DTD domains; CT, c-terminus; open triangle, Myc tag; open rectangle, Flag tag. **(B)** AH12.2 does not recognize an intracellular domain of Dsg2. Full-length and c-terminal constructs of Dsg2 were expressed in Chinese Hamster Ovary (CHO) cells and the samples were processed for immunoblot analysis with the indicated antibodies. **(C)** AH12.2 recognizes the first extracellular domain of Dsg2. CHO cells were transfected with constructs encoding segments of the extracellular portion of Dsg2 and samples were then analyzed by immunoblot with the indicated antibodies. Antibodies AH12.2 and 6D8 recognize distinct domains of Dsg2. **(D)** Schematic epitope diagram of the Dsg2-specific antibodies used in this study.

Figure 2. Desmoglein-2 specific antibodies detect full-length and lower molecular weight species in cell lysates from colonic epithelial cell lines and human colon. **(A)** Total cell lysates were prepared from colonic epithelial cell lines (SK-CO15, T84, Caco-2 passage 26, Caco-2 passage 41, HT-29) and two human colonic mucosa specimens (Colon 1 and 2). As expected, extracellular and intracellular domain antibodies detect the full-length, ~150 kDa form of Dsg2 in the colonic epithelial cell lines examined as well as in human colonic biopsy specimens. The extracellular domain antibodies AH12.2 and 6D8 detect a ~130 kDa band

(*), which is predominately found in the human colon samples and is not detected by intracellular domain antibody DG3.10, as well as ~65-95 kDa species (solid black bar) in human colonic epithelial cell lines and colon. In contrast, the intracellular domain antibody DG3.10 recognizes 50-65 kDa (dashed bar) and ~110 kDa (<) protein forms. Actin is included as a loading control. **(B)** Lighter exposure of each of the blots displayed in (A).

Figure 3. Antibodies against the extracellular domain of desmoglein-2 detect a shed fragment in colonic epithelial cell culture supernatants. **(A)** Antibodies AH12.2 and 6D8 recognize a ~90 kDa band in conditioned media from colonic epithelial cell line cultures. This band is not detected by the intracellular domain antibody DG3.10 (bottom panel). **(B)** Inhibition of metalloproteases reduces the shedding of Dsg2. SK-CO15 monolayers were treated with the presenilin inhibitor DAPT, metalloprotease inhibitor GM6001, cysteine protease inhibitor E64, or DMSO control. Following treatment, culture medium was collected and assayed for the presence of shed Dsg2 using antibody AH12.2. Non-treated total cell lysate is included in the panel, demonstrating the distinct sizes of the shed (~90 kDa) and cell-associated (~95-100 kDa) fragments detected by AH12.2.

Figure 4. RNAi down-regulation of desmoglein-2 expression demonstrates antibody specificity for full-length and lower molecular weight forms of desmoglein-2. **(A)** Cell lysates from non-silencing control (siCont) and Dsg2 siRNA (siDsg2) treated SK-CO15 cells were probed with antibodies against the extracellular (AH12.2 and 6D8) and intracellular (DG3.10) domains of Dsg2. The 150 kDa band detected by all antibodies is reduced by siDsg2 treatment. Actin is included as a loading control. **(B)** Cell culture supernatants from non-silencing control (siCont), Dsc2 siRNA (siDsc2), and Dsg2 (siDsg2) siRNA treated SK-CO15 cells were processed and analyzed by immunoblot with antibody AH12.2. The ~90 kDa shed fragment detected by AH12.2 is reduced by siDsg2 treatment. **(C)** The effect of

siDsg2 treatment on the lower molecular species detected by antibodies AH12.2, 6D8 and DG3.10 was also determined. The 50-65 kDa (dashed bar) and ~110 kDa (<) protein forms detected by DG3.10 were all reduced by Dsg2 knockdown as were the ~95-100 kDa bands recognized by AH12.2. In contrast, only the higher ~100 kDa band detected by 6D8 (white #) was decreased in siDsg2 treated monolayers.

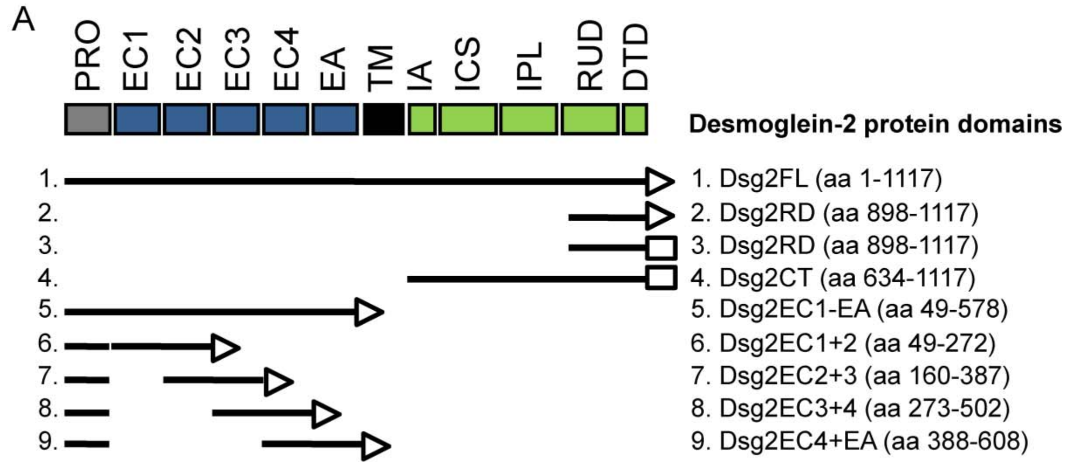
Figure 5. Putative fragments of desmoglein-2 in colonic epithelial cells. Based on the data presented herein, the various smaller molecular weight bands detected by Dsg2-specific antibodies likely represent a shed form of Dsg2 (~90 kDa; sDsg2), a cell-associated, truncated Dsg2 that lacks the intracellular domain (~95-100 kDa; tDsg2), and a c-terminal only form of Dsg2, which lacks the extracellular domain (~50-65 kDa; ctDsg2). In addition, a prominent ~130 kDa form containing the extracellular domain of Dsg2 but lacking the RUD domain was detected in human colon biopsy specimens, which may represent an additional form of Dsg2 in colonic epithelial cells.

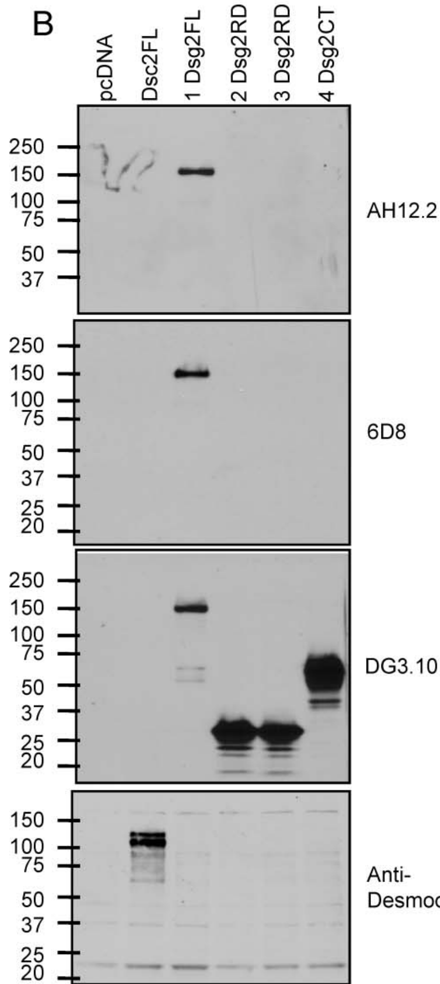
Supplementary Figure 1. The specificity of antibody AH12.2 for desmoglein-2 does not depend on protein glycosylation. The GST-tagged extracellular domain of Dsg2 "Dsg2EC1-EA" was expressed in E. coli along with a control GST-tagged protein UNC-95. The bacterially-expressed non-glycosylated extracellular domain of Dsg2 is detected by both antibodies AH12.2 and 6D8. Immunoblot against GST confirms the expression of both proteins.

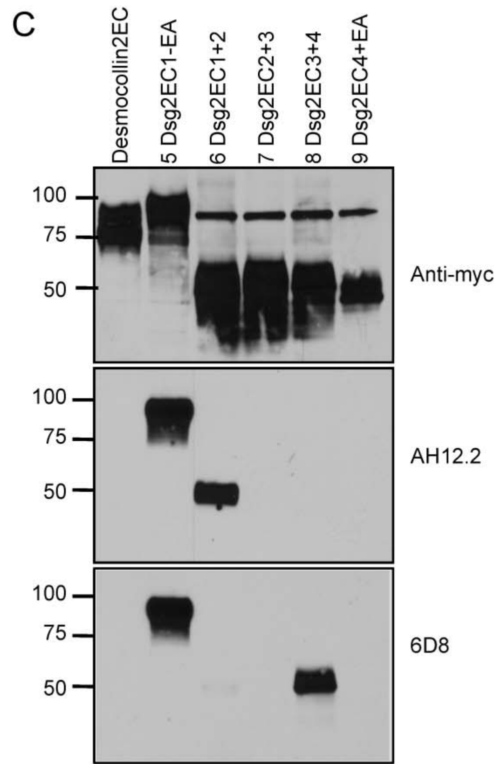
Supplementary Figure 2. A prominent ~90 kDa protein detected by antibody 6D8 in colonic epithelial cell lysates does not appear to be desmoglein-2 but may be β-catenin. **(A)** Immunoprecipitation (IP) from SK-CO15 cell lysates was carried out using antibodies AH12.2, 6D8, and the β-catenin-specific antibody (Zymed; mouse monoclonal). Samples

were analyzed by 2-color immunoblot by probing with antibodies 6D8 (left panel, red), AH12.2 (right panel, red), and β-catenin-specific antibody (β-cat., both panels, green). As expected, both AH12.2 and 6D8 recognize a 150 kDa band in the 6D8 and AH12.2 IP lanes, corresponding to full-length Dsg2. Antibody 6D8 also recognizes a ~90 kDa band in the β-catenin IP lane (white arrowhead), which co-migrates with β-catenin (see merged 6D8/ β-cat. Image) and is not detected by AH12.2. In addition, in contrast to antibody AH12.2, 6D8 immunoprecipitates β-catenin (white stars). **(B)** RNAi knockdown of β-catenin but not Dsg2 reduces the expression of the prominent ~90 kDa 6D8 reactive band. Cell lysates from non-silencing control (siCont), Dsg2 siRNA (siDsg2), and β-catenin siRNA-treated SK-CO15 cells were probed with antibodies AH12.2, 6D8, and anti-β-catenin. The ~150 kDa band detected by both AH12.2 (see lighter exposure inset) and 6D8 is reduced by siDsg2 treatment. However, the ~90 kDa band detected by 6D8 is reduced by β-catenin but not Dsg2 knockdown. Actin is included as a loading control.

FIGURES







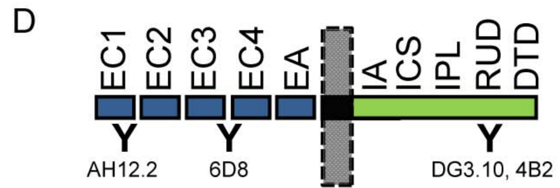
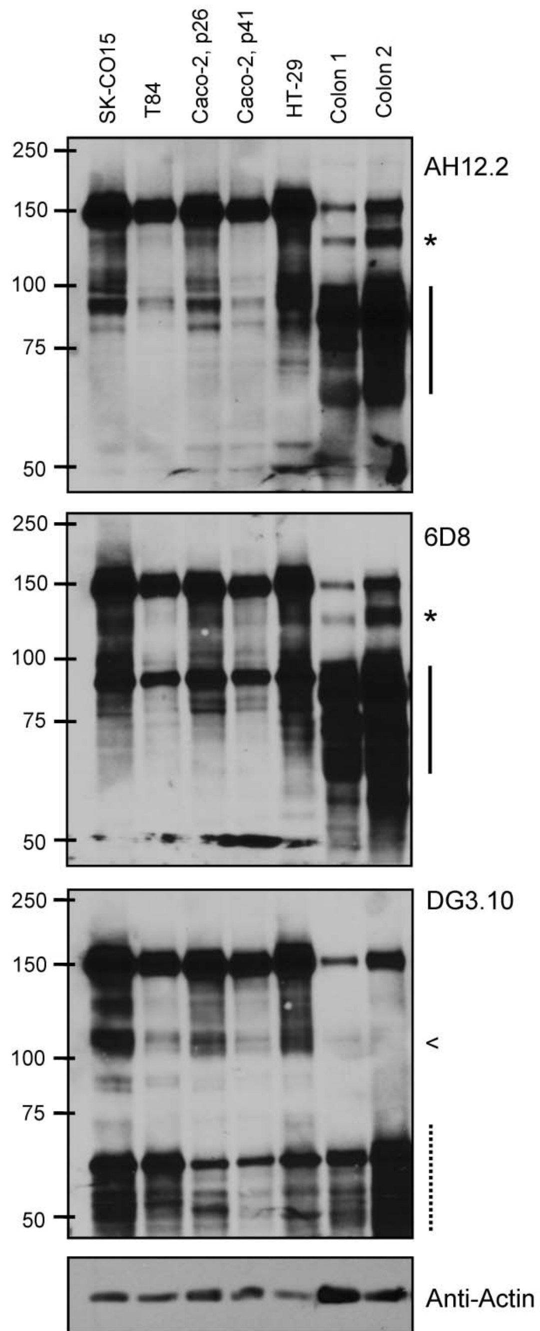


Figure 1



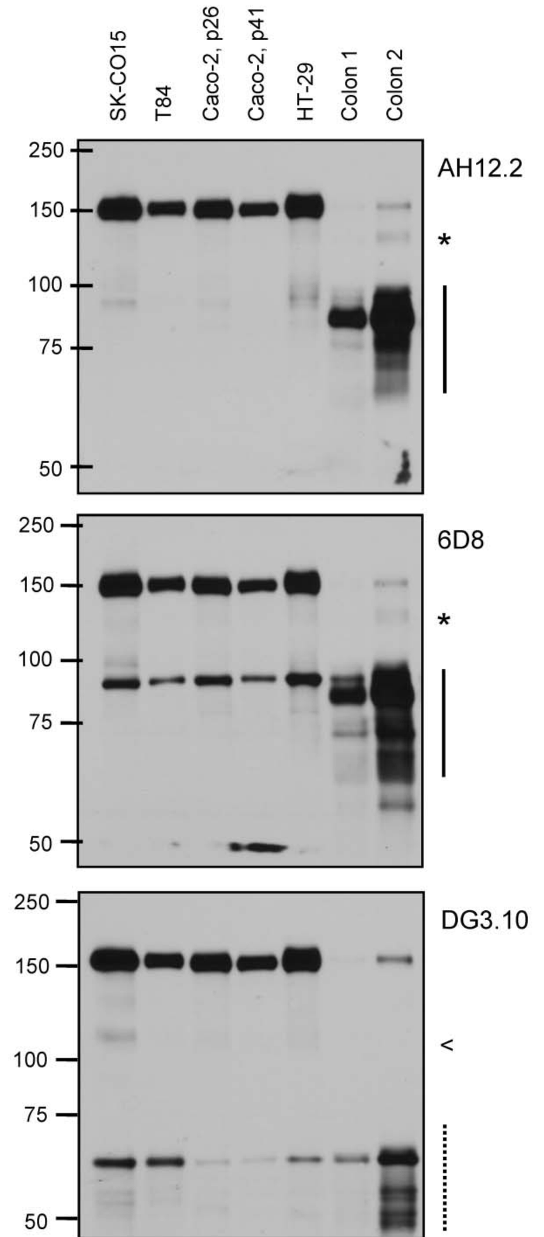
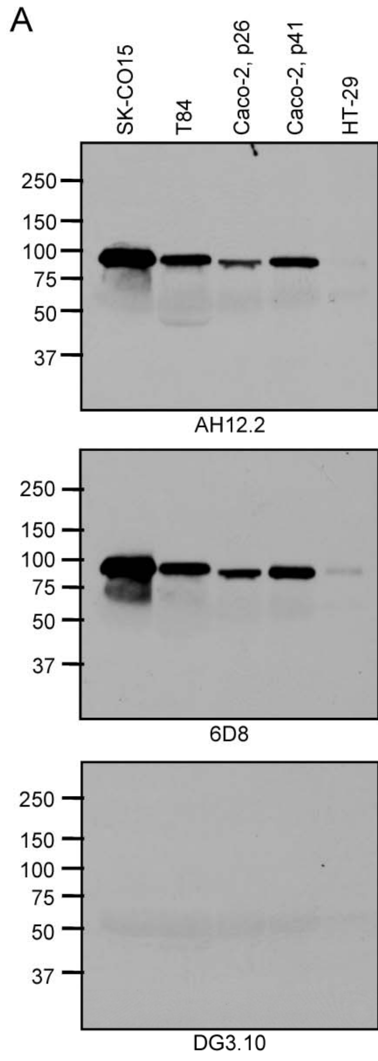


Figure 2



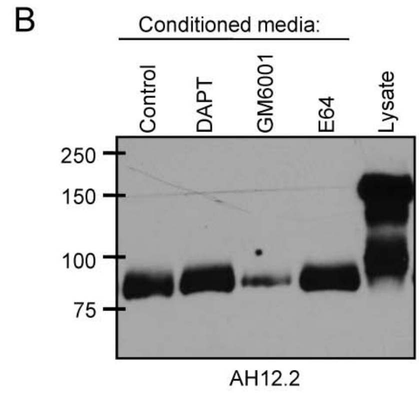


Figure 3

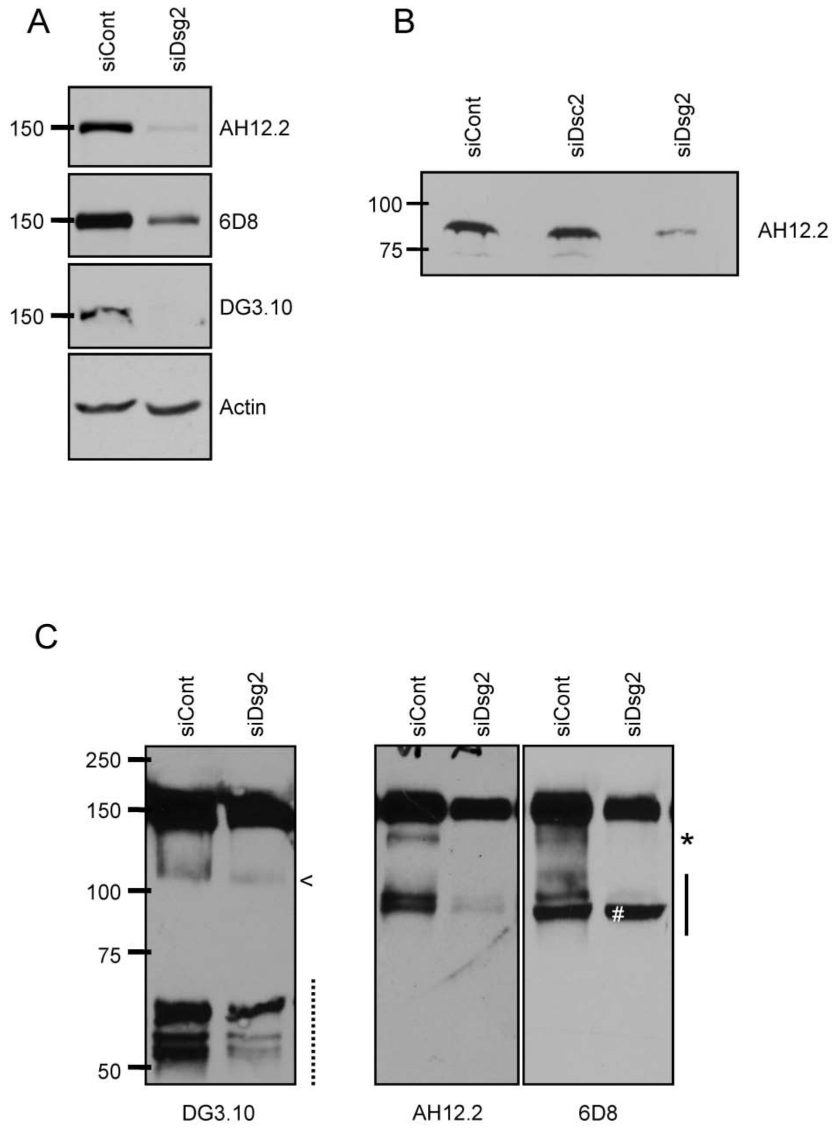


Figure 4

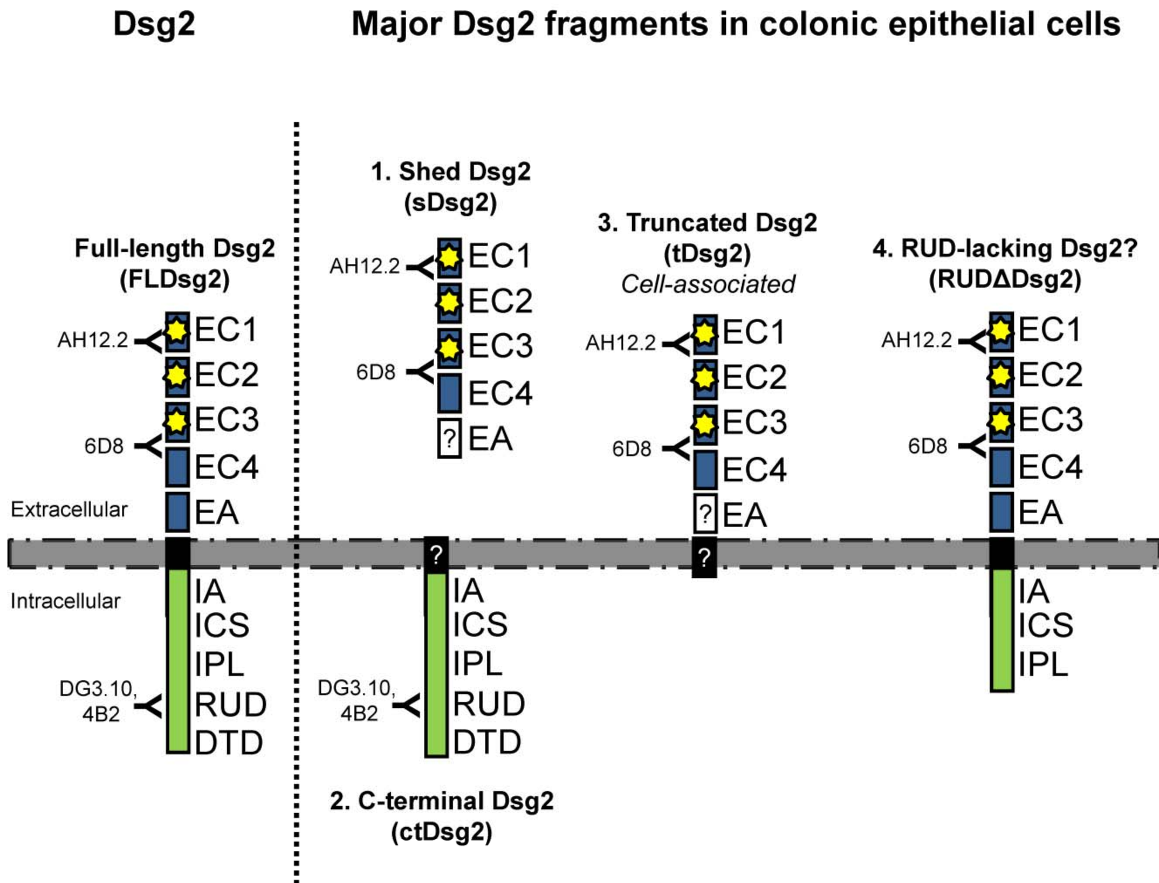
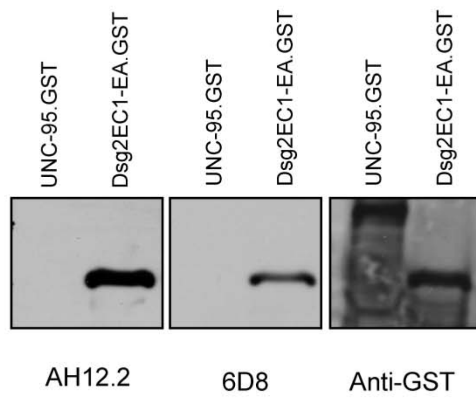
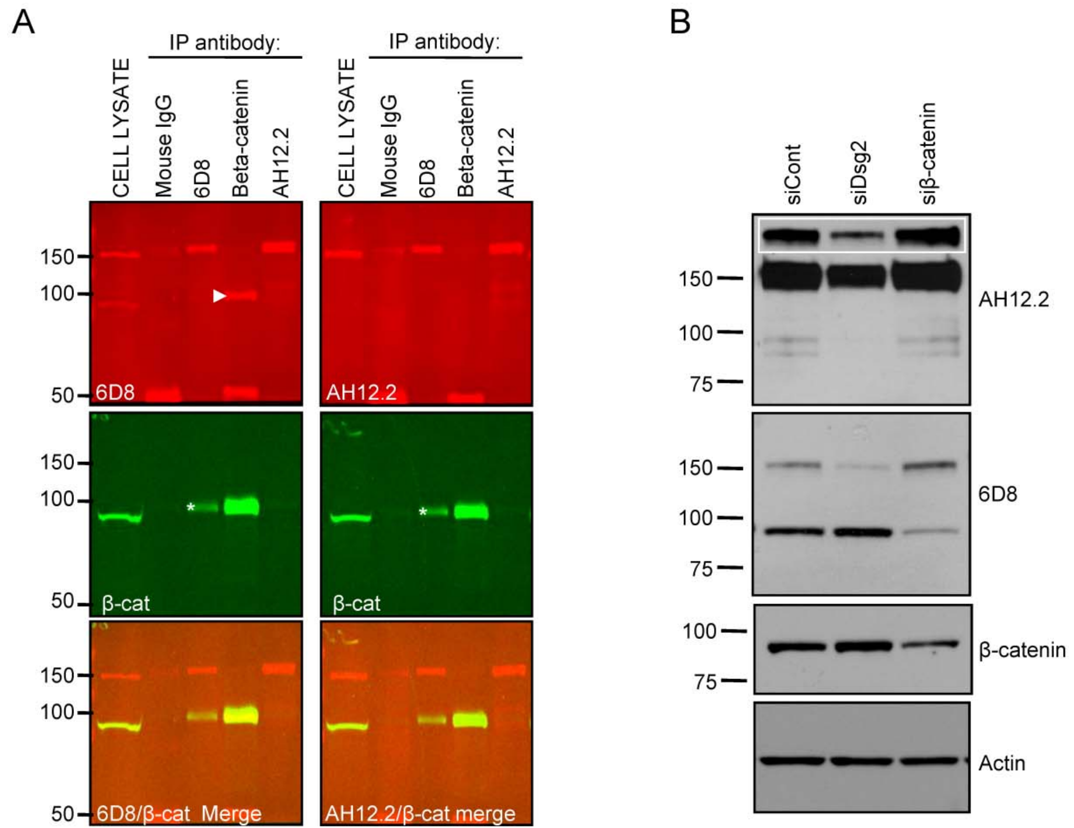


Figure 5



Supplementary Figure 1



Supplementary Figure 2

CONCLUSIONS AND FUTURE DIRECTIONS

THE DESMOSOMAL CADHERINS

The desmosomal cadherins desmoglein and desmocollin are essential transmembrane glycoproteins that form the extracellular adhesive face of the desmosome. Through intracellular interactions with scaffolding proteins such as desmoplakin and plakoglobin, the desmosomal cadherins link the desmosome to the intermediate filament network within the cell and thereby reinforce the junction.

Several human diseases have been attributed to defective desmosomal adhesion, such as pemphigus and staphylococcal scalded skin syndrome, which are caused by autoantibodies and proteolytic bacterial toxins, respectively, that target desmoglein in the skin and/or oral mucosa [Amagai, 2010; Delmar and McKenna 2010]. Arrhythmogenic right ventricular cardiomyopathy has been linked to mutations in a number of desmosomal component proteins in the heart, including desmoglein-2, desmocollin-2, plakophilin, desmoplakin, and plakoglobin [Herren et al., 2009]. Altered expression of both desmocollins and desmogleins has been reported in a number of carcinomas [Brennan and Mahoney, 2009; Chen et al., 2007; Fang et al., 2010; Khan et al., 2006; Kurzen et al., 2003; Wang et al., 2007; Wong et al., 2008], suggesting that changes in expression of the desmosomal cadherins may contribute to the development and/or progression of tumors. In addition, we have observed that desmoglein-2 expression is consistently increased in the colonic mucosa of patients with inflammatory bowel disease; however, the significance of this finding is unknown.

Within the dermatology community, an intense research effort has been devoted to the desmogleins in an attempt to understand the pathologic mechanisms underlying the potentially-fatal blistering diseases pemphigus and staphylococcal scalded skin syndrome. In

contrast, the desmocollins have received less attention from the research community, despite the fact that these proteins are often lost or mislocalized in cancers.

LOSS OF DESMOCOLLIN-2 PROMOTES TUMORIGENESIS

Importantly, the data from my thesis work advances our understanding of how down-regulation of desmocollins may contribute to tumor progression and provides several important links between the desmocollins and pro-oncogenic signaling pathways in colonic epithelial cells.

Loss of desmocollin-2 has been described in several tumor types, including colonic adenocarcinoma. Thus, we were able to use our intestinal epithelial model system to assess the effect of decreased expression of desmocollin protein on the behavior of colonic epithelial cells. As discussed in Chapter 2 of this dissertation, our results demonstrated that down-regulation of desmocollin-2 enhances cell proliferation and enables tumor formation *in vivo*. Furthermore, we identified the serine/threonine kinase Akt as a novel link between the desmosomal cadherins and the regulation of the transcriptional activity of β-catenin, a major player in the regulation of intestinal epithelial homeostasis and a known pro-oncogenic transcription factor. As discussed below, this study also raises important questions regarding desmosomal cadherin biology and the signaling roles of these proteins in epithelia and other tissue types.

DESMOSOMAL CADHERINS AND THE REGULATION OF LIPID RAFT SIGNALING PATHWAYS

Up until recently, most studies examining the function of the desmosomal cadherins in tissues have focused on their effects on intercellular adhesion. However it is becoming apparent that these proteins may also regulate intracellular signaling cascades that influence

homeostatic processes, such as the balance between proliferation and apoptosis that is required for renewal of epithelial tissues. Importantly, my thesis work demonstrates that loss of desmocollin-2 promotes activation of Epidermal Growth Factor Receptor/PI3-Kinase/Akt signaling (Chapter 2, Figure 6), a pro-survival and pro-proliferative pathway that is often hyperactivated in cancers. Interestingly, the components of this pathway are associated with cholesterol-enriched membrane microdomains called "lipid rafts" which are important regulators of cellular signal transduction. We previously reported that desmoglein-2 is highly enriched in these lipid raft domains of intestinal epithelial cells [Nava et al., 2007] and a similar distribution was observed for desmocollin-2 (unpublished observation; [Resnik et al.]). Thus, the lipid raft localization of the desmosomal cadherins may explain how these adhesion proteins influence PI3K/Akt signaling. Numerous studies have demonstrated that movement into or out of lipid rafts influences the function of cell surface receptors, including receptor tyrosine kinases such as the EGFR. Notably, Curto et al. demonstrated that adherens junction formation triggers the suppression of EGFR signaling by sequestering the receptor into a non-signaling microdomain of the cell membrane [Curto et al., 2007]. In addition, a recent report has shown that lipid raft membrane microdomains are required for desmosome assembly in a model renal epithelial cell line [Resnik et al., 2010]. It will be interesting to further explore the relationship between lipid rafts and the function of the desmosomal cadherins in intestinal epithelial cells and how the lipid raft localization of these proteins influences intracellular signaling cascades.

EVIDENCE OF DISTINCT SIGNALING FUNCTIONS FOR THE "PARTNER" DESMOSOMAL CADHERINS DESMOCOLLIN-2 AND DESMOGLEIN-2

As mentioned in the discussion of Chapter 2, down-regulation of desmoglein-2 does not appear to activate EGFR/Akt/β-catenin signaling in model colonic epithelial cell lines, even

though both of these proteins are required for intercellular desmosomal adhesion (unpublished observation). This observation strongly suggests that these partner desmosomal cadherins have distinct signaling function in intestinal epithelial cells. In support of this notion, we have observed that over-expression of desmoglein-2 but not desmocollin-2 enhances β-catenin transcriptional activity in the SK-CO15 colonic epithelial cell line. While more work is needed to elucidate the mechanism(s) of these differential effects, these observations are in agreement with seemingly 'contradictory' reports that desmocollins are down-regulated in carcinomas, whereas desmogleins are often upregulated. Furthermore, we have noted that pro-inflammatory cytokines significantly up-regulate desmoglein-2 expression while desmocollin-2 expression appears to be only modestly increased (unpublished observation; *in vitro* cultures) in colonic epithelial cells. It is tempting to speculate that the unique cytoplasmic domains of the desmogleins may provide additional signaling functions to these cadherin family members.

CONTROLLING DESMOGLEIN-2 FUNCTION BY PROTEOLYSIS

In addition to studies examining the effect of desmocollin-2 down-regulation on the behavior of colonic epithelial cells, my thesis work also involved the characterization of the mouse monoclonal anti-desmoglein-2 antibody AH12.2 and examining the cleavage fragments of desmoglein-2 that are generated in colonic epithelial cell lines and native colonic mucosa (Chapter 3). While the functional significance of these fragments remains to be determined, studies from our lab and others suggests that proteolytic fragments of the desmogleins can play active signaling roles within cells [Getsios et al., 2009; Nava et al., 2007]. Importantly, we have observed that intracellular cleavage fragments of desmoglein-2 (ctDsg2) induce apoptosis when expressed in colonic epithelial cell lines. Furthermore, Interferon-γ or TNF-α treatment of intestinal epithelial cell monolayers increases the generation of these fragments,

suggesting desmoglein-2 cleavage may enhance the pro-apoptotic effect of pro-inflammatory cytokines on the intestinal epithelium. These findings may also relate to the observation that desmoglein-2 is up-regulated during chronic inflammation (see below).

The generation of extracellular-domain containing fragments of desmoglein-2 may also have important implications that extend beyond the understanding of desmosomal cadherin biology. Notably, desmoglein fragments have been detected in the sera of patients with pemphigus [Lanza et al., 2006] and may relate to the pathology of the disease. It would be interesting to examine if shed desmoglein can be detected in the sera of patients with other clinical entities, such as IBD or carcinoma. In addition, because the pathogenic pemphigus autoantibodies bind to the extracellular domain of the desmogleins, it is possible that these antibodies exert their function via interactions with a cell-associated truncated form of desmoglein, rather than the full-length molecule found in desmosomes. Alternatively, these antibodies may bind to full-length desmoglein and alter its proteolytic cleavage because antibodies have been shown to interfere with the shedding of other transmembrane receptors [Brazil et al., 2010].

WHAT IS THE SIGNIFICANCE OF ALTERED DESMOGLEIN-2 EXPRESSION IN CHRONIC INTESTINAL INFLAMMATION?

As discussed above, we have found that intracellular fragments of desmoglein-2 promote intestinal epithelial apoptosis and that the generation of these fragments is increased by exposure to pro-inflammatory cytokines. In addition, we have observed that desmoglein-2 is increased in the colonic mucosa of patients with IBD. While the relevance of this finding to the pathology of chronic inflammation remains to be determined, there are several possibilities that warrant further investigation. One possibility is that desmoglein-2 up-

regulation contributes to the pro-apoptotic effects of inflammatory cytokines and therefore is a detrimental change that occurs in the epithelium. A second possibility is that increased desmoglein-2 is protective and is a compensatory response to long-standing inflammation in the gut. Lastly, it is possible that desmoglein-2 only appears to be up-regulated because of an inflammation-induced change in the solubility/extractability of this protein during the preparation of cell lysates or a change in the accessibility of the protein to antibodies used in immunostaining, as both techniques have been used to document increased 'expression' of desmoglein-2. Either of these scenarios would still support the conclusion that the overall state of desmosomes is altered during chronic inflammation. However, it is likely that the increased expression reflects a transcriptional change within the inflamed tissue, as we have seem a similar increase in desmoglein-2 transcript levels in a pilot *in vitro* experiment. Importantly, it is not known if the ultra-structure of desmosomes is altered in patients with IBD which could help to understand how changes in desmoglein-2 expression are affecting desmosomal adhesion. Future studies in the laboratory will continue to characterize the changes in desmosomal biology that occurs during acute and chronic inflammation, using both cell culture and mouse model systems. Interestingly, a recent report demonstrated that desmoglein-2 is the receptor for the adenovirus subtypes Ad3, Ad7, Ad11 and Ad14 which cause respiratory and urinary tract infections [Wang et al., 2010]. While not directly addressed by this study, our results suggest that in this context, an inflammatory response to the virus infection may actually increase desmoglein-2 expression and aid further spread of virus within the host.

IN SUMMARY

The fundamental hypothesis guiding my thesis work was that altered expression of the desmosomal cadherins affects intestinal epithelial homeostasis. Using a loss of function

approach, my studies demonstrated that desmocollin-2 regulates intestinal cell proliferation by controlling the activation of EGFR/Akt/β-catenin signaling and that activation of this pathway confers tumorigenicity to the SK-CO15 cell line [Kolegraff et al., 2011]. These data therefore provide evidence that desmocollin-2 may also contribute to the regulation of intestinal epithelial homeostasis. Interestingly, my data also suggest that desmocollin-2 and desmoglein-2 may contribute to the regulation of intestinal epithelial homeostasis through distinct mechanisms even though these proteins share a common function as desmosomal adhesion molecules. Future studies will attempt to address the function of desmocollin-2 and desmoglein-2 in the intestine *in vivo* and will be guided by my *in vitro* work using model colonic epithelial cell lines.

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APPENDIX: Cellular Domains Chapter 19 – The Tight Junction and Desmosomes

This appendix consists of a book chapter that will be published in *Cellular Domains*, Edited by Ivan R. Nabi, PhD [In Press, July 2011].

SPECIALIZED INTERCELLULAR JUNCTIONS IN EPITHELIAL CELLS: THE TIGHT JUNCTION AND DESMOSOME

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ABSTRACT

Epithelial cells line the body surfaces that are in contact with the outside world and thereby create an important barrier that defines distinct body compartments. Epithelial tissues include the multilayer (stratified) epithelium of the epidermis, the single layer (simple) epithelium of the intestine and kidney, and the transitional epithelium of the bladder. As with other tissue types, intercellular junctions between adjacent cells allow proper organization of tissue structures and confer specialized physiologic functions to the tissue. In this chapter, we will discuss two types of cell-cell junctions in epithelial cells: the tight junction and the desmosome. Other classes of intercellular junctions, such as the adherens junction and gap junction, are discussed in other chapters of this book.

TIGHT JUNCTIONS

Definition of the tight junction. The tight junction is a cell-cell adhesive junction that brings together adjacent cell membranes to create a tight seal in the paracellular space. By creating selective pores within the intercellular space, the tight junction regulates the movement of ions and small molecules between epithelial cells (gate function). In addition, the junction maintains cell polarity by separating apical and basolateral plasma membrane domains within a given cell and restricting the mixing of membrane protein and lipids (fence function). The tight junction is composed of the transmembrane proteins, including the tetraspanin proteins claudins and occludin, and members of the Junctional Adhesion Molecule (JAM) family. Plaque proteins associate with the cytoplasmic domain of the transmembrane proteins and include MAGUK (membrane-associated guanylate kinase) family members like the zonula occludens (ZO) proteins. Numerous other cytoplasmic proteins localize to the tight junction, and these proteins include transcription factors such as ZONAB and cell polarity proteins including Par3/6 and Scribble. Functionally, the formation of tight junctions between neighboring epithelial cells allows the separation of distinct body compartments and is essential for the regulation of tissue permeability. Dysregulation of tight junction proteins has been implicated in numerous pathophysiological processes affecting epithelia, including inflammation and tumorigenesis.

Kisses in the dark: the original description of the zonula occludens. In the early 1960's, electrophysiological studies established that epithelial tissues could function as an electrical barrier and separate charge across the cell layers thereby creating and defining distinct body compartments [Diamond, 1962a; Diamond, 1962b; Diamond, 1964; Diamond and Tormey,

1966a; Diamond and Tormey, 1966b; Tormey and Diamond, 1967; Wright and Diamond, 1968]. This observation led to the notion that sites of intercellular contact must exist to impede the flow of ions and fluid across the epithelium. Examination of cells using light microscopy hinted at the presence of such intracellular bridges [Bizzozero, 1864; Bizzozero, 1870], but it was not until the 1960's that this intercellular "seal" was characterized [Farquhar and Palade, 1963]. Using electron microscopy to analyze polarized epithelial tissues, Farquhar and Palade described a point of contact between adjacent epithelial cells that appeared to result from a fusion of the outer leaflet of the plasma membrane from each cell (see Figure 1A). This close apposition of cell membranes occurred at the apical or luminal domain of the lateral cell membrane and tracer studies demonstrated that macromolecules and dyes could not penetrate this paracellular region [Kaye and Pappas, 1962; Kaye et al., 1962; Miller, 1960]. Thus, this restrictive or occluding zone of the plasma membrane was referred to as the "zonula occludens" or tight junction. Other electron microscopists took a more romantic approach in their description and referred to these characteristic zones as "kisses" between neighboring epithelial cells [Diamond, 1974].

Initial studies supported the role of the tight junction as a paracellular "gate" which restricted the passage of ions and macromolecules between individual epithelial cells. Interestingly, it was shown that not all tight junction-containing tissues exhibit characteristics of a "tight" seal. Using a glass micropipette as a sensitive probe to monitor regions of electrical current, it was found that the paracellular space is a region of high conductance and different epithelial tissues have characteristic rates of conductance [Fromter, 1972]. Thus, epithelial tissues could be classified as "tight" or "leaky" based on these conductance parameters. Early electron microscopic studies could not distinguish the basis for this difference in ion selectivity and tissue permeability and it was not clear what possible tight junction structure-

function correlation existed in these different tissue types [Misfeldt et al., 1976; Wright and Diamond, 1968].

Nature of the gate: are tight-junction strands composed of lipid, protein, or both? During the 1970's and 1980's, technological advances in electron microscopy led to the development of the freeze fracture technique, in which frozen tissues are broken apart to allow the visualization of structural detail in the fracture plane by electron microscopy. Using this technique, the tight junction appears as parallel fibrils or strands running along the circumference of the epithelial cell with the strands interconnected by shorter branches of fibrils [Chalcroft and Bullivant, 1970]; see Figure 1B). These strands are embedded in the inner leaflet of the plasma membrane, also referred to as the protoplasmic (P) face. Complementary furrows or grooves are evident in the corresponding exoplasmic leaflet (E face). The first hint at a possible tight junction structure-function correlation came from the work of Claude and Goodenough in the 1970's, who demonstrated that "tight" epithelial tissues with reduced conductance had more parallel strands than their "leaky" counterparts [Claude and Goodenough, 1973]. Further work demonstrated that fragmentation or disruption of these strands correlated with changes in tissue permeability, as was observed during the absorption of glucose in the small intestine or in pathological settings such as inflammation or exposure to toxic stimuli [Fujita et al., 2000; Laukoetter et al., 2008; Sonoda et al., 1999]. Therefore, the ability of a tissue to regulate permeability seemed to lie in the formation of these strands. However, it was not clear how these strands were formed and how they were able to act as an intercellular seal. Perhaps more pressing at the time was the looming question in the tight junction field: what is the molecular composition of these anastomosing fibrils?

Early studies examining the composition of the tight junction fibrils pointed toward a lipid-based composition of the strands and several tight junction models proposed that the exoplasmic leaflets of neighboring cells were fused into a single membrane at the zonula occludens, forming a cylindrical pore that controlled the passage of ions and macromolecules [Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982]. However, these models fell out of favor as the tight junction strands were shown to be sensitive to protein-fixatives such as gluteraldehdye [Staehelin, 1973] and were not extractable by detergents [Meller and El-Gammal, 1983]. Furthermore, strand formation was shown to be disrupted by protein synthesis inhibitors, supporting a protein-based composition of the strands [Sang et al., 1980]. In 1986 Stevenson and Goodenough identified a large cytoplasmic protein that localized to the strands of the zonula occludens and appropriately named it "ZO-1" [Stevenson et al., 1986]. However, it was not until the 1990's that the first integral membrane proteins of the tight junction were described. Using modern biological techniques, Furuse and Tsukita identified two tetraspan proteins that localize to the tight junction strands, naming them occludin, from the Latin "occludure" or "to occlude" and claudin, from the Latin "claudure" or "to close". Further studies from the same group demonstrated that expression of the claudin proteins in fibroblasts allowed the formation of tight junction-like strands and that claudin is the obligatory integral membrane of tight junctions [Furuse et al., 1998a; Furuse et al., 1993; Furuse et al., 1998b; Furuse et al., 1999]; see Figure 2A). Tsukita's group and others went on to show that charged extracellular loops of the claudin protein allow homotypic and heterotypic interactions among claudin family members [Colegio et al., 2003; Tsukita and Furuse, 2002]. These claudin interactions create a charged aqueous pore in the extracellular space (see Figure 2B), whose electrophysiological characteristics depend on the claudin composition, as 24 claudin isoforms have been described to date. Our studies have revealed that the tight junction proteins localize in raft-

like compartments at the cell membrane [Nusrat et al., 2000]. These micro-structures play an important role in the spatial organization of the tight junction and contribute to the regulation of paracellular permeability in epithelial cells.

A second function for the zonula occludens: the tight junction as a membrane fence between apical and basolateral "pastures". An important characteristic of epithelial cells is the asymmetric localization of membrane components into at least two specialized regions: the apical surface, which faces the organ lumen, and the basolateral surface, which is in contact with the underlying connective tissue. The first evidence of such "polarized" organization of the epithelium came from electron microscopic studies of the small intestine, which demonstrated the presence of small finger-like projections (micro-villi) that were present only on the apical surface of the cells [Berridge and Oschman, 1969; Bizzozero, 1870]. During the 1980's, a great deal of effort in the field of cell biology was focused on understanding the fundamental process of epithelial cell polarity and how proteins are targeted to and retained by specific plasma membrane domains. Because epithelial cells were known to segregate proteins and lipids to apical and basolateral surfaces, it was proposed that cells maintain this polarity through the actions of a fence-like structure within the plasma membrane [Pisam and Ripoche, 1976; Sang et al., 1980]. The work of Dragsten et al. using fluorescent membrane probes demonstrated that the tight junction restricts the lateral diffusion and mixing of apical or basolateral membrane components [Dragsten et al., 1981; Dragsten et al., 1982a; Dragsten et al., 1982b; Rigos et al., 1983]. Interestingly, their work showed that the tight junction only restricts the movement of lipids and proteins in the outer leaflet of the plasma membrane bilayer, as labeled probes added to the inner leaflet were not restricted in their diffusion. Thus the evidence suggested that while the tight junction does

not establish epithelial cell polarity *per se*, this structure was important for the maintenance of the distinct plasma membrane domains.

Molecular composition of the tight junction. After the discovery of ZO-1 in 1986, the number of tight-junction associated proteins has grown substantially (see Figure 2). Currently, the protein components of the tight junction can be broadly grouped as transmembrane or cytosolic/scaffolding proteins. The major components are discussed here, for more comprehensive reviews see [Gonzalez-Mariscal et al., 2003; Gonzalez-Mariscal and Nava, 2005].

Tetraspan proteins of the tight junction. Occludin, tricellulin, and the claudin family members are integral membrane proteins that localize in the tight junction strands [Furuse et al., 1999; Ikenouchi et al., 2005]. These proteins have four transmembrane domains and are believed to mediate cell-cell contact through their extracellular loops. The claudin family of proteins consists of at least 24 isoforms in humans and mice and studies in claudin-deficient fibroblasts have demonstrated the homotypic and/or heterotypic interactions of the different claudin isoforms [Asano et al., 2003; Daugherty et al., 2007; Tsukita and Furuse, 1999; Tsukita and Furuse, 2000a; Tsukita and Furuse, 2000b; Tsukita and Furuse, 2002]. In addition, claudin knockout mouse models have further supported the unique role of different claudin family members in tight junction-containing tissues, as the phenotype varies greatly depending on the deleted isoform [Furuse and Tsukita, 2006; Kitajiri et al., 2004]. In addition to the tetraspan integral membrane proteins, single-pass transmembrane proteins of the immunoglobulin superfamily, JAM (Junctional Adhesion Molecule) and CAR (Coxsackie and Adenovirus Receptor), have also been shown to localize to the tight junction strands

[Bergelson et al., 1997; Martin-Padura et al., 1998; Roelvink et al., 1998] and regulate barrier permeability [Liu et al., 2000; Mandell et al., 2004].

Cytoplasmic/scaffolding proteins of the tight junction. While expression of claudin family members is required for strand formation, it is the expression and localization of the ZO proteins that control where strand assembly occurs [Siliciano and Goodenough, 1988]. The ZO proteins are MAGUK (membrane-associated guanylate kinase homologues) family members that contain several PDZ domains, SH3 domains, and one guanylate kinase-like domain [Anderson et al., 1988; Gumbiner et al., 1991]. The ZO proteins interact with the cytoplasmic tails of claudins, occludin, and JAM proteins through PDZ-dependent and independent mechanisms [Itoh et al., 1999]. Importantly, the ZO proteins link the tight junction structure to the apical actin cytoskeleton, directly or indirectly through associations with additional proteins, including afadin/AF6 [Miyoshi and Takai, 2005]. Importantly, it has been shown that modulation of this tight junction-associated, circumferential actin-myosin "belt" can control epithelial permeability, by changing the paracellular pores formed by the tight junction strands.

Pathophysiology of the tight junction. Since their initial description, tight junctions have been intimately linked with pathological conditions and diseases, and an alteration in their structure corresponds with changes in tissue permeability in both physiologic and pathologic settings. To date, numerous studies have examined the role of tight junction defects in disease, and human genetic disorders and mouse models have further elucidated the roles of individual tight junction proteins in normal tissue function and homeostasis.

The tight junction was once thought of as a static, rigid structure that merely formed a selective seal between adjacent cells, but it is now known that this specialized zone is a dynamic, complex structure that can quickly be modulated in the context of physiology or disease. As early as the 1970's it was reported that tight junction structure was altered during inflammation [Sosula, 1975] and infection [Barker, 1975]. Soon it became evident that many pathogens and pathogen products could actually target the tight junction structure to cause increased tissue permeability and enhance their invasion through the body's epithelial barriers [Laukoetter et al., 2007; Schulzke et al., 2009]. Recently, tight junction proteins have been identified as receptors for a number of viruses, including coxsackie virus and adenovirus (CAR) [Bergelson et al., 1997; Roelvink et al., 1998], reovirus (JAM) [Barton et al., 2001], and most recently, Hepatitis C virus [Beard and Warner, 2007; Evans et al., 2007] and HIV (claudins) [Andras et al., 2003; Zheng et al., 2005]. Furthermore, bacterial toxins such as clostridium perfringes enterotoxin have been shown to bind specifically to and degrade claudins to disrupt the intestinal epithelial barrier [Fujita et al., 2000; Sonoda et al., 1999]. Interestingly, perturbation of tight junction function is also a hallmark of several inflammatory diseases, such as Crohn's disease and ulcerative colitis [Laukoetter et al., 2008]. In these disorders inflammatory cytokines induce the down-regulation and internalization of tight junction proteins or target components of the actin-myosin machinery, leading to a compromised epithelial barrier, enhanced exposure to luminal antigens and further inflammation.

A number of congenital syndromes have been described for patients with mutations in claudin family members. Interestingly, mutations in different claudin isoforms can have drastically different phenotypes. For instance, mutations in claudins 11 and 14 have been shown to cause some forms of hearing loss [Kitajiri et al., 2004; Wilcox et al., 2001] due to

sensory epithelial defects whereas claudin-16 (paracellin) mutations have been detected in some patients with hypomagnesemia and hypercalciuria caused by kidney malfunction [Simon et al., 1999]. Deletion of claudin-5 underlies the phenotype of the Velo-Cardio-Facial Syndrome, which affects multiple organ systems including the heart and kidneys [Morita et al., 1999].

The tight junction has also been shown to be altered in cancers and several tight junction proteins have been shown to contribute to tumorigenesis [Darido et al., 2008; Dhawan et al., 2005; Resnick et al., 2005]. In 1977 it was reported that, in contrast to their normal cell counterparts, prostatic cancer cells possess a loosely organized network of fibrils and many individual, short strands that are disorganized and likely dysfunctional [Sinha et al., 1977]. Given that a characteristic property of epithelial cancers is the loss of cell polarity, a breakdown in tight junction structure contributes to this defect in normal cellular asymmetry. Interestingly, the tumorigenic potential of several viral oncoproteins has been shown to correlate with their ability to sequester and/or inactivate tight junction proteins [Gonzalez-Mariscal et al., 2009; Latorre et al., 2005].

DESMOSOMES

Definition of a desmosome. Desmosomes or "maculae adherentes" are cell-cell adhesive junctions that were originally identified in the epidermis using electron microscopy. While desmosomes are found in a number of tissue types, they are enriched in tissues that must withstand extensive mechanical stress, such as the skin or heart. The core protein components of the desmosome consist of the transmembrane cadherin family member

glycoproteins desmocollin and desmoglein, the cytoplasmic plaque proteins of the plakin family, such as desmoplakin, and armadillo family members including plakoglobin and the plakophilins. The extracellular domains of the desmosomal cadherins mediate adhesion between neighboring cells while their intracellular domains are linked to the intermediate filament cytoskeleton via the plaque proteins. The intimate association of the desmosomal plaque with the intermediate filament network reinforces the cell-cell contact sites and adds overall structural stability to the tissue. While the desmosome has always been described as a junction specialized for maintaining tissue integrity, recent evidence from animal models and human diseases demonstrate that desmosomal proteins likely have important signaling roles in proliferation, differentiation, apoptosis, and overall tissue morphogenesis, in addition to their structural role in forming desmosomes.

Discovery of the desmosome. The existence of a link or "node" between neighboring epithelial cells was first described in 1864 by the Italian medical doctor Bizzozero, who examined sections of epidermis using a light microscope [Bizzozero, 1864; Bizzozero, 1870; Calkins and Setzer, 2007]. The original descriptions declared these novel structures "desmosomes", which derives from the Greek words "desmo", meaning link or bond and "soma", or body [Schaffer, 1920]. With the advent of electron microscopy, it soon became evident that there were specialized structures linking adjacent cells in stratified epithelial tissues. Several descriptions reported the existence of symmetrical electron dense plaques that were present in membranes of adjacent epithelial cells [Kelly, 1966; Porter et al., 1945]; see Figure 1A). These plaques appeared to also have a less defined substructure in the intercellular space. In addition, it was evident that densely packed, cytoplasmic tonofilaments converged on these plaques [Odland, 1958; Porter, 1956]. The plaques were circular or oval in shape and multiple plaques could be detected along the membranes of

adjacent cells, leading to their description as "spot welds" in epithelial tissues. Interestingly, it was noted that these structures were almost always shared between neighboring cells, that is, the plaque was formed between cells in contact, and each cell membrane had an identical, aligned electron dense plaque region with associated tonofilaments [Arnn and Staehelin, 1981; Overton, 1962]. Occasionally, a "half" plaque would be detected, but these were usually cytoplasmic bodies suggesting that they were traveling to, or returning from, the cell membrane. Additionally, structures along the basal surface of epithelial cells were described as "hemi-desmosomes" [Kelly, 1966], even though they were formed between the cell and the underlying extracellular matrix and therefore only had one half of a circular desmosomal-like plaque.

Through careful examination, electron microscopists identified three major regions of the desmosomal plaque: the midline, an electron dense plaque consisting of an outer dense region and an inner, less dense region, and the associated tonofilaments that contacted the plaque at the inner region [Farquhar and Palade, 1963; Odland, 1958; Porter, 1956]. Around the same time, the cytoskeletal filaments were being carefully studied using the same techniques. Three major classes of filaments were known to exist in the cells: microfilaments with the smallest diameters (5-6 nm), intermediate filaments (7-10 nm), and microtubules with the largest diameters (20-25 nm). Based on these morphological criteria, it was clear that the desmosomal tonofilaments belonged to the intermediate filament class [Drochmans et al., 1978].

The desmosome was first described as a structure enriched in the stratified epithelial tissues of the epidermis and the work of many investigators demonstrated that desmosomes are also formed in other epithelial tissues, namely the simple or single-layered epithelial tissues of the

intestine and kidney. In their careful characterization of junctional complexes in epithelial cells, Farquhar and Palade noted that desmosomes or "maculae adherentes" (adherent spots) were one of three cytoskeletal-associated plaques in polarized, simple epithelial tissues such as the mucosal linings of the intestine and kidney, the other junctions being the zonulae occludens (tight junctions) and the zonulae adherens (adherens junctions) [Farquhar and Palade, 1963]. The desmosome was distinguished from the other cell-cell junctions based on its association with the intermediate filament network in contrast to the microfilament network, which associates with the tight and adherens junctions, and because it is the most basally located of the three junction types.

Around the time of the seminal Farquhar and Palade publication in 1963, structures with desmosomal morphology were also described in many other non-epithelial tissue types, including in meninges, between follicular dendritic cells of lymph nodes, and in the intercalating disks of cardiomyocytes [Fawcett and Selby, 1958; Gusek, 1962; Swartzendruber, 1965]. Importantly, it was also noted that desmosomal structures were highly enriched, and often larger, in tissues that routinely experience high mechanical stress, such as the heart and skin. While it seemed plausible that these junctions were specialized for resistance to mechanical stresses and in reinforcing tissue integrity, there was no direct evidence demonstrating this function of desmosomes.

The desmosome as intercellular adhesive cement. The desmosome was a junction defined based on morphological criteria as observed using an electron microscope. Little was known about the exact role of this structure in the tissue and whether or not this junction could "mal-function" had yet to be determined. However, in the medical field, the existence of blistering skin diseases had already been described, in which patients suffer from a fragile epidermis

that would separate easily from the underlying dermis [Waschke, 2008]. This class of diseases was referred to as "pemphigus" and histologic examination of skin biopsies demonstrated splitting of the epidermis just above the basal cell layer and detachment of individual epithelial cells from their neighbors (acantholysis). In 1964, a possible disease mechanism was proposed, as Beutner et al. described the presence of anti-epithelial antibodies coating the surface of keratinocytes isolated from patients with pemphigus [Beutner and Jordon, 1964]. These antibodies were not found in healthy skin samples, strongly suggesting that the anti-keratinocyte antibodies were at least partly responsible for the disease. A series of studies using electron microscopy later demonstrated that the acantholytic keratinocytes have altered desmosomal junctions, detachment of tonofilaments, or both. Notably, Hashimoto and Lever described the appearance of abnormal granules on the surface of acantholytic keratinocytes and also observed a disruption of the amorphous intercellular substance that seemed to bridge adjacent cells [Hashimoto and Lever, 1967a; Hashimoto and Lever, 1967b]. They hypothesized that the granules were responsible for transporting the intercellular adhesive "cement" to the cell surface and that somehow this substance was abnormal in patients with pemphigus or that a lytic material was acting on the intercellular substance to break it down. Other EM studies of a different form of pemphigus, pemphigus foliaceus, also demonstrated breakdown of the desmosome, especially loss of the cementing substance, as well as loss of the tonofilament-plaque association at the membrane [Arnn and Staehelin, 1981]. Together with the findings that keratinocytes are coated with antibodies, it was proposed that the blistering disease pemphigus was caused by an "anti-intercellular cement" antibody that disrupted the function of desmosomes and made the skin fragile. Thus, pemphigus was the first group of diseases that were linked to desmosomal dysfunction. Later, through advances in molecular cloning and genetic studies, mutations in

multiple desmosomal proteins were shown to underlie the pathogenesis of diseases affecting not only the skin, but hair and heart as well.

The molecular structure of the desmosome. Advances in biomedical technology of the 1980's and 1990's allowed the identification of the protein components of the desmosome (see Figure 2A). Scientists exploited the highly insoluble nature of the desmosome to enrich and isolate relatively pure desmosomal fractions. Initial biochemical characterization of these fractions identified a mixture of glycosylated and non-glycosylated proteins that comprise the desmosomal plaque [Drochmans et al., 1978; Franke et al., 1981a; Franke et al., 1981b]. We now know that these proteins include the transmembrane glycoproteins desmocollin and desmoglein, as well as the non-glycosylated cytoplasmic plaque proteins desmoplakin, plakoglobin, and the plakophilins [Garrod and Chidgey, 2008; Getsios et al., 2004; Holthöfer et al., 2007]. Additional proteins have been described as being localized to desmosomes or interacting with known desmosomal proteins, but have received less attention: desmocalmin, desmoyokin, erbin, pinin, p120 catenin, p0071 [Garrod and Chidgey, 2008].

The desmosomal cadherins. Desmocollin and desmoglein are calcium-dependent cell-cell adhesion proteins that belong to the cadherin superfamily. There are four desmoglein isoforms and three desmocollin isoforms that are expressed in a tissue-specific and differentiation-dependent manner [Dusek et al., 2007]. The extracellular domain of these desmosomal cadherins consists of cadherin repeat domains (EC1-5) that are linked by flexible calcium binding regions. As has been described for other cadherin family members, the adhesive site has been proposed to be in the EC1 domain of the desmosomal cadherins and blocking peptides against this region inhibit homophilic and heterophilic interactions of

desmocollin and desmoglein [Runswick et al., 2001]. While the adhesive function of the desmosomal cadherins usually depends on the presence of calcium, Garrod et al. have described calcium-independent "super-adhesive" desmosomes that no longer require the presence of calcium to be maintained [Garrod and Kimura, 2008]. The membrane proximal EC5 domain (or EA domain) is less conserved between the proteins and may allow interactions between distinct cadherin family members [Getsios et al., 2004]. The membrane spanning TM domain is followed by a cytoplasmic juxtamembrane domain (IA, intracellular anchor), which has been proposed to mediate interaction with plakophilins [Bonne et al., 1999; Chen et al., 2002; Hatzfeld et al., 2000], and the intracellular cadherin sequence (ICS) which has been shown to bind plakoglobin [Andl and Stanley, 2001; Gaudry et al., 2001; Wahl et al., 2000]. In addition to the domains described above, the desmogleins have additional unique cytoplasmic segments, the repeating unit domains (RUD) and the desmoglein terminal domain (DTD). The function of these domains remains to be determined.

Numerous studies have examined the regulation of the assembly and disassembly of the desmosomal junction and have found that phosphorylation of the cadherin cytoplasmic tail is an important contributor to this process [Calautti et al., 1998; Garrod et al., 2002]. Phosphorylation also regulates the interaction of the desmosomal cadherins with the plaque proteins, including plakoglobin [Gaudry et al., 2001]. Proteolytic cleavage of the desmosomal cadherins, especially desmogleins, can occur in the extracellular domain [Bech-Serra et al., 2006; Cirillo et al., 2007a; Klessner et al., 2009] or intracellular domain [Cirillo et al., 2007b; Dusek et al., 2006; Nava et al., 2007] and regulates processes such as junction disassembly and apoptosis. In agreement with these findings, at least one human disease, the staphylococcal scalded-skin syndrome (SSSS), is caused by cleavage of desmoglein 1, an

isoform expressed in the upper layers of the skin. Cleavage of this protein by the bacterial toxin induces massive exfoliation of the upper layers of the epidermis and often is fatal if not treated appropriately [Amagai et al., 2000; Hanakawa and Stanley, 2004; Nishifuji et al., 2008].

Plakin family members. The desmoplakin isoforms I and II are the major plakin family members of the desmosome and immunolocalization of desmoplakin often serves as a reliable marker to identify the desmosomal junction. The desmoplakins are large (210-230 kDa) proteins that are currently thought to be the major plaque proteins that connects the cytoplasmic tail of the desmosomal cadherins to the intermediate filament network. Desmoplakins also interact with armadillo family members, thereby serving as a general scaffold for the association of a number of proteins with the desmosomal junction [Franke et al., 1982; Mueller and Franke, 1983]. Multiple binding domains allow numerous protein-protein interactions with desmoplakin, including a globular head domain formed by two spectrin repeats, a Src homology domain, and a central coiled coil domain that mediates desmoplakin dimerization. Other plakin family members that have been localized to the desmosome include envoplakin, periplakin, and spectrin [Garrod and Chidgey, 2008].

Armadillo family members. The 42 amino acid long “arm” repeat domains were first identified in the polarity gene *armadillo* of the fruit fly *Drosophila melanogaster* [Klymkowsky, 1999]. These domains were subsequently shown to be present in a number of signaling proteins, including beta-catenin and other catenin family members. The desmosome contains several members of the armadillo family, including plakoglobin (gamma-catenin) and plakophilins 1-3, and these proteins have been shown to interact with the desmosomal cadherins and/or desmoplakin (Hatzfeld 1999; Garrod and Chidgey 2008). Interestingly,

plakoglobin localizes to both the desmosome and the adherens junction, and may allow "cross-talk" between the different junction types. Also, pointing to non-desmosomal signaling roles, the plakophilins demonstrate junctional and nuclear pools in epithelial cells and may integrate signals from the cell membrane with changes in cell behavior and tissue morphogenesis [Bonne et al., 2003; Hatzfeld et al., 2000; Klymkowsky, 1999].

Intermediate filaments. All desmosomes interact with the intermediate filament network, although the cytoskeletal proteins that comprise this network differ depending on the tissue type [Jamora and Fuchs, 2002; Owens and Lane, 2003]. In epithelial cells, the intermediate filaments consist of cytokeratin isoforms, which are differentially expressed based on the type of epithelia (simple versus stratified) and the differentation of the cells. For instance, keratin 8 and keratin 18 (K8/K18) are the primary keratins expressed in simple epithelia, K5/K14 in the basal cells of stratified epithelia, and K1/K10 in the suprabasal layers of the epidermis. In contrast, in non-epithelial tissues, desmosomes interact with the intermediate filament protein desmin (cardiomyocytes) or vimentin (follicular dendritic cells, meninges).

Tissue expression of the desmosomal cadherin and desmoplakin isoforms. While numerous tissues are known to form desmosomes, the constituent protein isoforms of desmosomes are not always the same. Currently, it is thought that the obligatory proteins of the desmosome are desmocollin, desmoglein, plakoglobin, and desmoplakin although the isoforms expressed differ depending on the tissue type. In general desmoglein-2 and desmocollin-2 are the ubiquitously expressed desmosomal cadherins, and are present in all tissues that have desmosomes [Cowin et al., 1985; Franke et al., 1981a]. In the skin, these proteins are expressed in the basal layer of the epidermis but are replaced in the suprabasal layer by desmoglein-3 and desmocollin-3 isoforms. In the uppermost layers of the epidermis,

desmocollin-1 and desmoglein-1 replace desmoglein-3 and desmocollin-3, and the expression of these proteins is always restricted to the terminally differentiated upper layers of the epidermis and other stratified epithelia. Desmoglein-4 expression is restricted to the upper layers of the skin and hair follicles [Bazzi et al., 2009; Bazzi et al., 2006; Mahoney et al., 2006]. Desmoplakins I and II have been identified in both simple and stratified epithelia, although some confusion remains on whether variant II exists in all epithelia [Franke et al., 1982; Garrod and Kimura, 2008; Garrod et al., 2002; Mueller and Franke, 1983].

Alterations in desmosome function: lessons from human diseases and knockout mouse studies. Since the description of the defective "intercellular cement" between acantholytic keratinocytes in patients with pemphigus, alterations in desmosomes and desmosomal proteins have been shown to contribute to disease pathogenesis of a number of conditions affecting the skin, hair, and heart.

Inflammatory/infectious etiologies. In addition to the autoimmune blistering skin diseases pemphigus vulgaris (anti-Dsg3 autoantibodies) and pemphigus foliaceus (anti-Dsg1 autoantibodies), another type of skin disease called the Staphylocccal scalded skin syndrome, leads to disease through the ability of the *Staphylococcus aureus* exfoliative toxins A and B to specifically cleave desmoglein-1, inducing blister formation and massive skin exfoliation [Amagai et al., 2000; Hanakawa and Stanley, 2004; Nishifuji et al., 2008].

Inherited desmosomal diseases. Given their widespread distribution, it is not surprising that inherited mutations in desmosomal proteins have been shown to affect mainly the heart, skin, and hair. For instance, numerous mutations in desmosomal proteins have been linked to arrhythmogenic right ventricular cardiomyopathy (ARVC), a heart disease in which patients

experience fibrofatty replacement of cardiac muscle tissue, resulting in abnormal electrical conductance and contraction of the muscle. Mutations in desmoglein-2, desmocollin-2, desmoplakin, and plakophilin-2 have all been linked to ARVC. Interestingly, heart disease was the only manifestation of the particular mutations described in these patients, likely due to the serious and often fatal presentation of this disease. Other desmosomal protein mutations have shown a mix of skin, hair, and heart involvement, including a deletion mutation in plakoglobin, which results in Naxos disease: ARVC-like cardiomyopathy, woolly hair, and palmoplantar keratoderma. Mutations in a given gene do not always lead to the same phenotype; for instance, a second type of mutation in desmoplakin leads to striate palmoplantar keratoderma without cardiac involvement [Bazzi and Christiano, 2007].

Desmosomes and cancer. A number of studies have observed changes in the protein and/or mRNA of desmosomal proteins in tumor samples; however, it is not currently understood what contribution these changes may have on the development, progression, or metastasis of the tumor [Funakoshi et al., 2008; Khan et al., 2006; Kurzen et al., 2003; Schmitt et al., 2007].

Knockout mouse models. While the important role of desmosomes in the skin and heart are further supported by studies using knockout mice, many of the observed phenotypes also suggest that desmosomal proteins have essential functions beyond cell-cell adhesion [Bazzi and Christiano, 2007; Green and Simpson, 2007]. Notably, embryos lacking the ubiquitously expressed desmoplakin or desmoglein-2 exhibit embryonic lethal phenotypes. Surprisingly, desmocollin-3 knockout embryos are also not viable and die before the appearance of desmosomes in the developing embryo [Den et al., 2006]. These findings suggest that desmosomal proteins function early in embryonic development perhaps playing a role in

embryonic adhesion or in proliferation of the embryonic stem cells [Eshkind et al., 2002].

FUTURE PERSPECTIVES

Epithelial tissues provide an important barrier between the external environment and the body's internal compartments. Proper function and integrity of these tissues is essential and breakdown in epithelial function underlies the pathogenesis of a number of human diseases. Specialized adhesive contacts between epithelial cells allow the regulation of epithelial permeability (tight junctions) and reinforce cell-cell contacts to increase tissue strength (desmosomes). Furthermore, proteins of these junctions also contribute to regulation of the homeostatic processes of the epithelium, including cell proliferation, differentiation, and apoptosis; however, the mechanisms by which this occurs are not well understood. Additional studies are needed to address several important questions, which apply to both tight junctions and desmosomes: How are these junctions assembled and disassembled? Can assembly or disassembly be modulated as a therapeutic strategy, for instance to minimize and/or reverse the effects of inflammation, infection or autoantibodies or to improve drug delivery through epithelial linings such as the intestine and skin? In addition, and even less understood, is the role of junction proteins in the process of tumorigenesis and whether or not these proteins are able to protect against the development and/or progression of cancers. Future studies should further explore the contribution of these proteins to the regulation of basic cellular processes.

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FIGURES

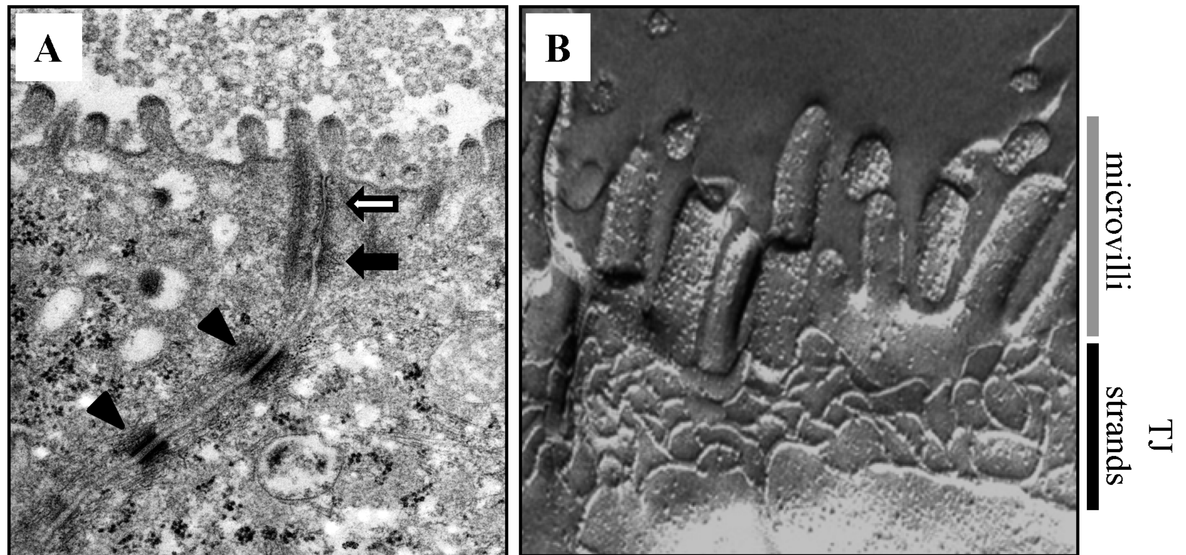
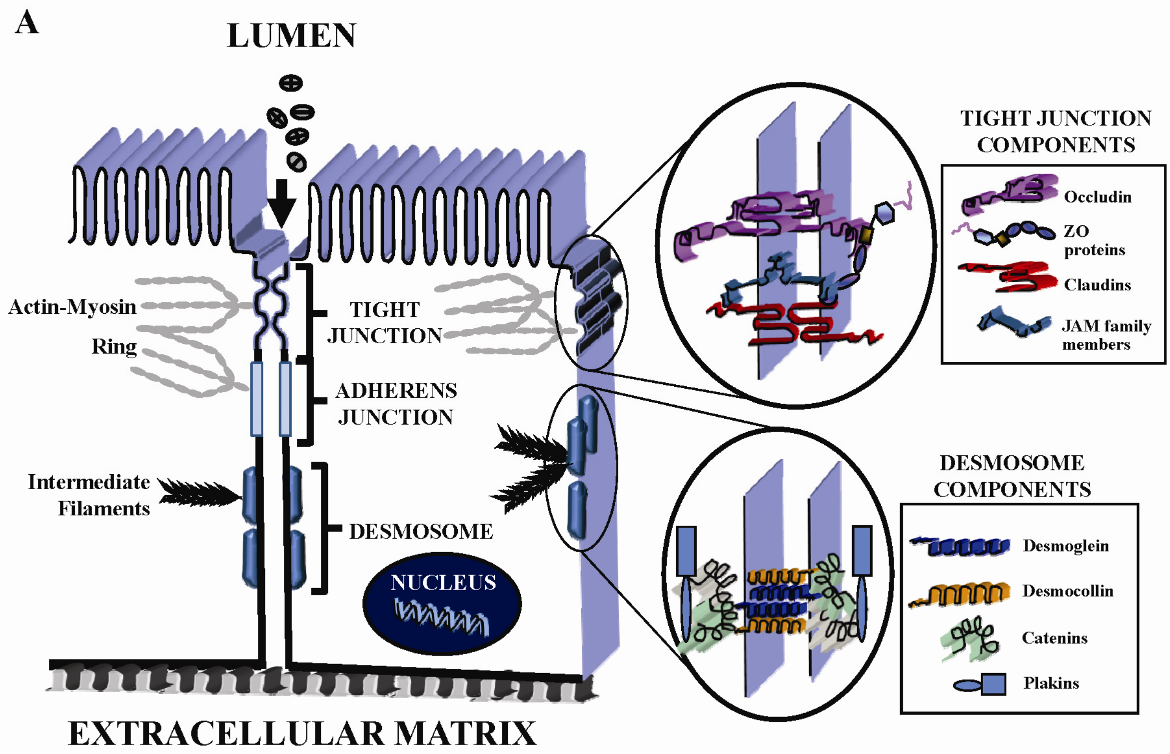


Figure 1: Ultra-structure of cell-cell junctions in polarized intestinal epithelial cells. A. Transmission electron microscopy of two adjacent intestinal epithelial cells (T84). At the level of the tight junction (white arrow), the intercellular space is obliterated by the close apposition of the cell membranes. The adherens junction (black arrow) is located below the tight junction. Numerous desmosomes (black arrowheads) can be observed along the lateral border of the cell membranes. (Image courtesy of Dr. James L. Madara) B. Freeze fracture replica of the mouse colon, demonstrating the anastomosing tight junction strands that run along the circumference of the epithelial cells. PF, P face; EF, E face; MV, microvilli, TJ, tight junction. (Image courtesy of Dr. Hartwig Wolburg, Institute of Pathology, University Hospital Tübingen, Germany)


A
LUMEN
Actin-Myosin
Ring
TIGHT
JUNCTION
ADHERENS
JUNCTION
Intermediate
Filaments
DESMOSOME
NUCLEUS
EXTRACELLULAR MATRIX
TIGHT JUNCTION
COMPONENTS
Occludin
ZO
proteins
Claudins
JAM family
members
DESMOSOME
COMPONENTS
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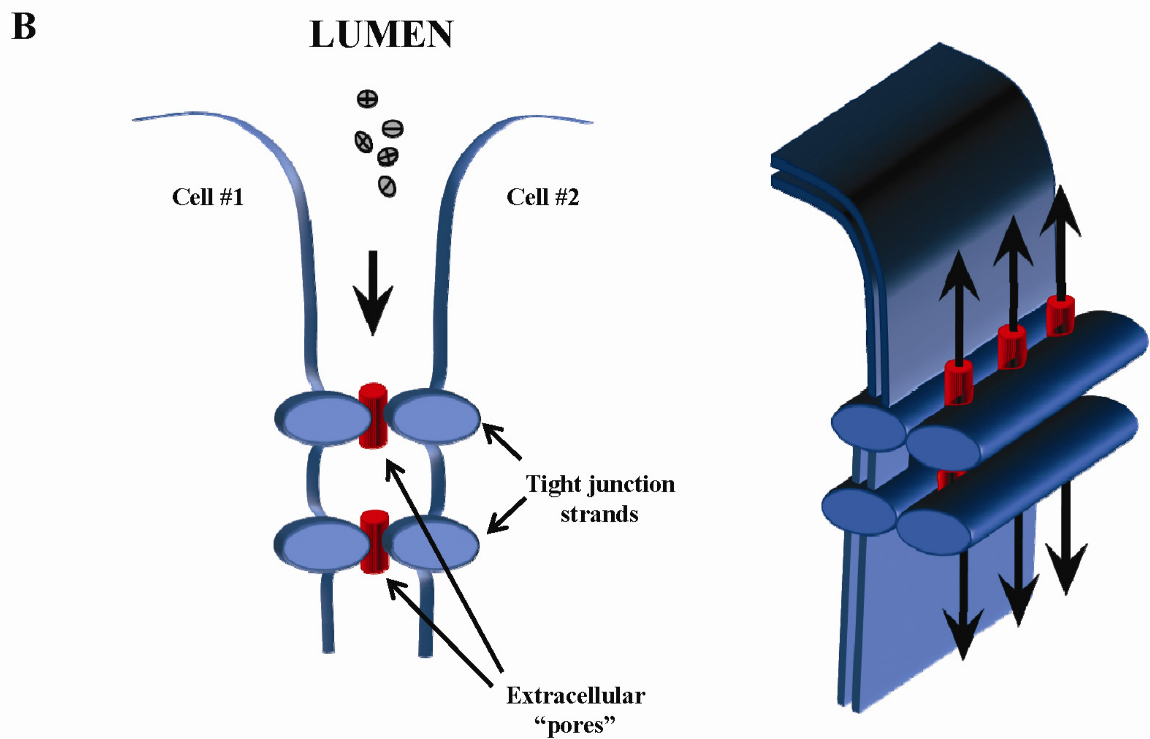
B
LUMEN
Cell #1
Cell #2
Tight junction
strands
Extracellular
"pores"

Figure 2: Molecular composition of the tight junction and desmosome. A. Schematic diagram depicting the transmembrane, plaque, and cytoskeletal proteins of each junction. B. Current model of how the tight junction functions as a paracellular gate. The extracellular loops of tight junction proteins from adjacent cells interact to create aqueous pores in the paracellular space (red cyclinders). The electrophysiological characteristics of these pores vary depending on the claudin composition.