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

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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 colon 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. 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, bolla and staphylococcal scalded skin syndrome (SSSS). Furthermore, altered expression of desmosomal proteins has been described in a number of disease states, including squamous cell carcinoma, colon adenocarcinoma and nasopharynx.

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. Isoforms of the desmosomal cadherins are expressed in a tissue-specific and differentiation-specific pattern, which may reflect differential adhesive capabilities of particular isoforms. For instance, in humans all isoforms (Dsg 1–4 and Dsc 1–3) 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 (Fig. 1A). Following the transmembrane (TM) domain, both Dsg and Dsc have a membrane proximal intracellular domain (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, 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. These findings suggest that proteolytic cleavage of Dsg may also affect other non-adhesive Dsg functions.

Assessing the role of Dsg cleavage fragments adds significant complexity to the study of Dsg function, because multiple Dsg
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. Immunoblot with anti-myc demonstrates that all proteins were properly expressed. The anti-myc antibody appears to cross-react with a CHO cellular antigen (~95 kDa), which may represent endogenous myc. Note that antibodies AH12.2 and 6D8 recognize distinct domains of Dsg2. (D) Schematic epitope diagram of the Dsg2-specific antibodies used in this study.
isoforms are expressed in the epidermis and in the keratinocyte cell lines that are commonly utilized to study desmosomes. Furthermore, the antibodies used to characterize Dsg expression often detect more than one Dsg isoform.

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.2,16,20 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.20 To gain insight into the binding site for AH12.2, we performed antibody binding experiments by expressing sequentially truncated forms of Dsg2 (Fig. 1A) in Chinese Hamster Ovary (CHO) cells, which do not express desmosomal cadherins. 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 DTO sequences (Dsg2RD). All expressed constructs were detected by the Dsg2 intracellular domain specific antibodies DG3.10 (Fig. 1B) and 4B2 (data not shown), which are known to bind an epitope within the RUD domain of Dsg2 (Fig. 1D). In contrast, AH12.2 and the known Dsg2 extracellular domain specific antibody 6D8 detected full-length Dsg2 but not its intracellular domain (Fig. 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,28 we found 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; Fig. 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 Supplemental 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 T84.20 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 part 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 (Fig. 2). In addition, a ~130 kDa protein band (indicated by *) and 65–100 kDa protein bands (solid bar) were detected by antibodies AH12.2 and 6D8 that recognize the extracellular domain of Dsg2. In contrast, using antibodies that detect the intracellular domain of Dsg2, DG3.10 (Fig. 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.26,29 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 this fragment in the media is 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 Dsg2,26,29 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 other protease inhibitors (presenilin/g-secretase inhibitor DAPT and cysteine protease inhibitor 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 downregulate the expression of Dsg2 in the colonic epithelial cell line SK-CO15. As shown in Figure 4, the ~150 kDa protein band detected by AH12.2, 6D8, DG3.10 (Fig. 4A) and 4B2 (data not shown) was decreased by Dsg2 siRNA treatment, thus confirming that this protein band
~95 kDa band detected by the mAb 6D8 (Hycult Biotech, The Netherlands) was not affected by downregulation of Dsg2 (Fig. 4C), 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 supernatant from the original source (Dr. James Wahl, University of Nebraska). Importantly, this antibody did not recognize the prominent non-Dsg2 protein.

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 mucosa. The extracellular domain antibodies AH12.2 and 6D8 detect a ~130 kDa band (indicated by *), 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 black bar) and ~110 kDa (indicated by <) protein forms. Actin is included as a loading control. (B) Lighter exposure of each of the blots displayed in (A).
band at 95 kDa (data not shown), suggesting that the commercially available antibody may cross-react with another cellular antigen that co-migrates with a Dsg2 cleavage fragment. Notably, we have observed that the commercial 6D8 antibody consistently co-immunoprecipitates β-catenin from human colonic epithelial cells (Sup. Fig. 2A), which also migrates as a 95 kDa band by SDS-PAGE. We confirmed cross-reactivity of the commercially available 6D8 with β-catenin by RNAi, which showed that β-catenin-specific siRNA decreases the appearance of the 6D8 reactive product at 95 kDa (Sup. Fig. 2B), suggesting that this band may in fact be β-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. 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 part of Dsg2-specific mAbs, all of which recognize distinct domains of Dsg2. RNAi mediated downregulation 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 (Fig. 5). 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.

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 (Fig. 1). Interestingly, previous studies with this
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; 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. Given recent findings indicating that loss of Dsc2 leads to hyperproliferation and confers a tumorigenic phenotype to colonic epithelial cells, it will be interesting to examine the role of Dsg2 in this context, and whether antibodies against Dsg2 have any effect of these protumorigenic pathways in colonic epithelial cells.

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. 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 proproliferative 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. In addition, pathogenic autoantibodies or the functional antibodies used in a variety of experimental systems to study Dsg may exert their functional effects by altering the generation of Dsg cleavage fragments, as the ability of antibodies to interfere with or promote proteolysis of transmembrane cell-cell adhesion proteins has already been reported.

**Figure 4.** RNAi downregulation 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 black bar) and ~110 kDa (indicated by * ) protein forms detected by DG3.10 were all reduced by Dsg2 knockdown as were the ~95–100 kDa bands recognized by AH12.2 (solid black bar). In contrast, only the higher ~100 kDa band detected by 6D8 was decreased in siDsg2 treated monolayers.

antibody suggested recognition of an intracellular epitope of Dsg2, as permeabilization was required for antigen detection by immunofluorescence labeling/confocal microscopy. 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...
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) Dulbecco’s Modified Eagle Medium (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 in reference 36. The human colonic epithelial cell line T84 was cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium supplemented with 10 mM HEPES, 14 mM NaHCO3, 40 μg/ml penicillin, 90 μg/ml streptomycin, 5% newborn calf serum, also previously described in references 37 and 38. The following mouse monoclonal antibodies were used to detect proteins by protein gel blotting: anti-desmocollin 2/3, clone 7G6 and anti-desmoglein-2, clone 4B2 (kind gifts from K. Green, Northwestern University);
anti-desmoglein 2, clone AH12.2 (generated in-house); anti-desmoglein 2, clone 6D8 (Hycult Biotech, The Netherlands and generous gift from J. Wahl, University of Nebraska); anti-desmoglein 2, clone DG3.10 (Progen, Germany); and anti-β-catenin (Cat.#13-8400, Invitrogen, Carlsbad, CA). Rabbit polyclonal anti-actin, anti-β-catenin, anti-GST (all from Sigma-Aldrich, St. Louis, MO) and anti-myc tag (Abcam, Cambridge, MA) antibodies were also used in immunoblot studies. Peroxidase-conjugated secondary antibodies were obtained from Jackson Laboratories. Dylight Infrared dye-conjugated secondary antibodies were obtained from ThermoScientific. Mouse IgG control antibody used in co-immunoprecipitation studies was obtained from Sigma-Aldrich (Catalog #I5381).

**Preparation of samples for immunoblot analysis.** For in vitro cell cultures, cell lysates were prepared as follows: confluent monolayers were washed two times with HBSS on ice and collected in RIPA lysis buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS, pH 7.4), containing protease and phosphatase inhibitor cocktails (1:100, Sigma). Lysates were cleared by centrifugation (15 min at 14,000g) and protein concentration determined by a BCA protein assay and samples were boiled in reducing SDS sample buffer. For studies with conditioned media, cells were grown to confluency and switched to serum-free media 24 h prior to sample collection. After collection, supernatants were concentrated 10-fold using Amicon Ultra-15 (5,000 MW) columns (Millipore, Billerica, MA) and boiled in SDS sample buffer. Equal sample volumes were then analyzed by SDS-PAGE/immunoblotting as described below. For analysis of protein expression in native human colon, normal colonic mucosa specimens were obtained from the Human Tissue Procurement Service at Emory University. Tissue specimens were washed with cold HBSS+, re-suspended in RIPA lysis buffer containing protease and phosphatase inhibitors (1:100, Sigma), sonicated and cleared by centrifugation. Protein concentration was determined using a BCA protein assay and samples were boiled in reducing SDS sample buffer. All procedures on discarded human tissue were carried out with approval from the Institutional Review Board at Emory University.

**Immunoblotting.** For analysis of cell and tissue lysates, equal amounts of protein (10–20 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h with 5% wt/vol dry milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS), incubated with primary antibodies in blocking buffer overnight at 4°C and washed with TTBS. For standard immunoblots, membranes were incubated in HRP-conjugated secondary antibodies for 1 h prior to development with HyGlo Chemiluminescent HRP Detection Reagent (Denville Scientific, Metuchen, NJ). For two-color infrared immunoblots, membranes were incubated with Dylight 680 or 800 dye-conjugated secondary antibodies for 1 h prior to imaging with the LI-COR Odyssey Detection System.

**Generation of Dsg2 fusion proteins.** The intracellular domain fragments were generated as previously described. The myc-tagged extracellular domains of Dsg2 were cloned into the pcDNA3.1 vector (Invitrogen) using the following primers: Dsg2 EC1-EC2 (gene nucleotides 1 to 813) forward 5-ACG GGG TAC CGC CAC CAT GGC GCG GAG CCC GGG ACG CGC-3; reverse 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GTA GTC CAG GAA AAT GAA TGC CCT TCT TGT GGG TCT-3; reverse 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGA AAT GAA TGC CCT TCT TGT GTA TAT TGT GAT-3; Dsg2 EC2-EC3 (gene nucleotides 473 to 1,161) forward 5-TCA TTC AGT GCG GCA AAA GCG CAC ACA GGA TGT CTT TGT TGG GTG-3; reverse 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGA AAT GAA TGC CCT TCT TGT GTA TAT TGT GAT-3; Dsg2 EC3-EC4 (gene nucleotides 813 to 1,507) forward 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GTA CTA GGA AGG GA-3; reverse 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGA AAT GAA TGC CCT TCT TGT GTA TAT TGT GAT-3; Dsg2 EC4-EC5 (gene nucleotides 1,161 to 1,735) forward 5-TCA TTG TGG ATC CGG CATCCC ACG CAG GTA TAT TGT CAT CTC AAT TT-3; reverse 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GTG GTT TGT GTA CAA TGA CGG AGA-3. The GST-tagged extracellular domain of Dsg2 (gene nucleotides 143 to 1,735) was cloned into the pGEX-4T1 vector (Pharmacia) using the forward 5-GCC TGG ATC CGC CTG GCA AAA GCG CAA AAG CAG CGT CAT CTC AAT TT-3; reverse 5-GAT GGG ATC CGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GGT GAT GTG GTT TGT GTA CAA TGA CGG AGA-3. The GST-tagged extracellular domain fragments were transfected into Chinese Hamster Ovary (CHO) cells (70% confluency) using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium (Invitrogen). The media was replaced after 6 h with fresh Opti-MEM and cells were cultured for other 24 h before media was collected and prepared for immunoblot analysis as described above. Expression and purification of Dsg2 EC-GST fusion protein was performed following previously described standard procedures. The control GST fusion protein UNC95-GST was obtained from Dr. Hiroshi Qadota, Emory University.

**Protease inhibitors.** For inhibitor studies, confluent SK-CO15 monolayers were switched to serum-free media and pre-incubated with the following protease inhibitors for 8–12 h before media was collected: 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; 10 μM) or DMSO control.

**RNAi reagents and transient transfection.** siGENOME SMARTpool siRNA for human Dsg2, β-catenin, Dsc2 and non-targeting control were purchased from Dharmacon RNA Technologies (Lafayette, CO). SK-CO15 cells cultured to 60–70% confluence were transfected with the siRNA reagents at a final concentration of 20 nM 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.

**Co-immunoprecipitation studies.** Confluent SK-CO15 cell monolayers were washed in HBSS, scraped into RIPA lysis buffer and cleared by centrifugation as described above. Equal volumes of cell lysate were then incubated overnight (4°C) with 5 μg of AH12, 6D8, anti-β-catenin or control mouse IgG antibody. Pull-down was achieved by rotation with Protein G-coupled
Sepharose beads (GE Healthcare, UK) for 3 h at 4°C. Beads were washed, pelleted, boiled in reducing SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis.

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