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Mark Yulis Date

Desmosomal Cadherin Cleavage in the Regulation of Apoptosis in the Context of Inflammation

By

Mark Yulis
Doctor of Philosophy

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

_____
Asma Nusrat, M.D.
Advisor

_____
Michael H. Koval, Ph.D.
Advisor

_____
Anita H. Corbett, Ph.D.
Committee Member

_____
Lawrence H. Boise, Ph.D.
Committee Member

_____
Paula M. Vertino, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Desmosomal Cadherin Cleavage in the Regulation of Apoptosis in the Context of Inflammation

By

Mark Yulis
B.S., Muhlenberg College, 2013

Advisor: Asma Nusrat, M.D.
Advisor: Michael H. Koval, Ph.D.

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Abstract

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The regulation of apoptosis sensitivity is critical to the maintenance of physiological homeostasis and dysregulation of apoptosis is known to be involved in the pathobiology or progression of many disease states. The single pass, transmembrane proteins of the cadherin family have been appreciated as important proteins that regulate intercellular adhesion. In addition to this critical function, cadherins contribute to important signaling events that control cellular homeostasis. Much of the work on cadherin mediated signaling focuses on classical cadherins or on specific disease states such as pemphigus vulgaris. Cadherin mediated signaling has been shown to play critical roles during development, in proliferation, apoptosis, disease pathobiology and beyond. We have previously reported that select pro-inflammatory cytokines induce ectodomain cleavage of the desmosomal cadherin desmoglein-2 (Dsg2) and shedding of the resulting extracellular fragment from intestinal epithelial cells (IECs). The Dsg2 extracellular cleaved fragment (Dsg2 ECF) functions to induce paracrine pro-proliferative signaling in epithelial cells. The goal of this study was to further understand the role of desmoglein cleavage in the regulation of enterocyte homeostasis. To address this, we focused on characterizing Dsg2 intracellular cleavage and the functional effects of the resultant intracellular cleaved fragment. In this study, we showed that exposure of IECs to pro-inflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) resulted in Dsg2 intracellular cleavage and generation of a ~55kDa fragment (Dsg2 ICF). Dsg2 intracellular cleavage is mediated by caspase-8 and occurs prior to Dsg2 extracellular cleavage and the execution of apoptosis. Expression of exogenous Dsg2 ICF in model IECs increased sensitivity to apoptotic stimuli and apoptosis execution. Additionally, expression of the Dsg2 ICF repressed the anti-apoptotic Bcl-2 family member proteins Bcl-XL and Mcl1. Taken together, our findings identify a novel mechanism by which pro-inflammatory mediators induce cleavage of Dsg2 to activate apoptosis and eliminate damaged cells, while also promoting release of Dsg2 ECF that enhances proliferation of neighboring cells and epithelial barrier recovery. These findings expand our understanding of the signaling capabilities of desmosomal cadherins and provide a new pathway through which apoptosis can be altered in the context of intestinal inflammation.

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Table of Contents:

Figures and Table:

Abbreviations:

ALPS	Autoimmune lymphoproliferative syndrome
CD	Crohn's Disease
Ch-IP	Chromatin-immunoprecipitation
Co-IP	Co-immunoprecipitation
CTF2	C-terminal fragment 2
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DD	Death Domain
DISC	Death Inducing Signaling Complex
Dsg2 ECF	Desmoglein-2 extracellular fragment
Dsg2 ICF	Desmoglein-2 intracellular fragment
DSS	Dextran sulfate sodium
EMT	Epithelial to mesenchymal transition
EoE	Eosinophilic esophagitis
GEF	Guanine nucleotide exchange factor
IBD	Inflammatory Bowel Disease
IEC	Intestinal epithelial cell
mAb	Monoclonal antibody
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
NTF	N-terminal fragment
PF	Pemphigus foliaceus
PV	Pemphigus vulgaris
qPCR	quantitative PCR
RNAseq	RNA sequencing
RUD	Repeated unit domain
siRNA	small interfering RNA
SPPK	Striate palmoplantar keratoderma
TNBS	2,4,6-trinitrobenzene sulfonic acid
UC	Ulcerative colitis
UV	Ultraviolet
UVB	Ultraviolet B
WT	Wild type

Chapter 1: Introduction

A portion of this chapter is adapted from the following published work:

Yulis, M., Kusters D.H.M., Nusrat, A. (2018) *J. Physiol.* "Cadherins: Cellular adhesive molecules acting as signaling mediators"

Mechanisms and pathways of apoptosis:

Regulated cell death is important to maintaining tissue homeostasis. There are many forms of regulated cell death, including the classic non-lytic apoptosis, the regulated cellular swelling and bursting of necroptosis, and the inflammasome mediated pyroptosis. Apoptosis, the most well understood form of programmed cell death is often thought of as the yin to proliferation's yang. In development, the timed and precise death of numerous cells of varying types in myriad locations throughout the developing embryo is necessary for embryonic viability in multicellular organisms (1). Apoptosis is vital in the establishment of many key bodily functions such as the development of the immune system (2). For example, patients with certain mutations or defects in caspase-10 and/or other mediators of extrinsic apoptosis such as the Fas Ligand (FasL)/Fas Receptor (FasR) ligand/receptor pair develop Autoimmune LymphoProliferative Syndrome (ALPS) due to the inability to clear excess proliferative lymphocytes or overactive immune cells following the initial proliferative bursts during development of the immune system (3-5). Physiological apoptosis ensures the balance between cell growth and cell death as well as prevents damaged or altered cells from posing harm to the organism. The importance of apoptosis is punctuated by the fact that dysregulation of apoptosis is important in the development of several human pathologies (3-9). Without the tight regulation of apoptosis, multicellular life would not be able to exist.

Key players in the execution and regulation of apoptosis are the caspase family of cysteine proteases that target aspartate residues, which is the origin of their now famous name Cysteine ASPartate proteASE. Arguably, the most famous celebrities of the family are the executioner caspases, caspases-3 and -7 whose activation is required for

essentially all apoptosis to occur (10). Their activation leads to the cleavage of many key targets, the direct consequences of which are the physical and biochemical characteristics of apoptosis execution (10). However, as is always true, the celebrities would not accomplish anything without the arguably more important work of their phenomenal supporting actors, the initiator caspases. Their work is multifaceted, complex, and tightly regulated. Not only do they need to distinguish between different types of apoptotic stimuli, they also have to do so in a way that is simultaneously able to be activated very quickly but also reversed if necessary. The initiator caspases are key players in the regulation of apoptosis sensitivity whereas, for the most part, the executioner caspases are not. This is largely due to the mechanistic nature of apoptosis itself and where each of these different caspases act within these mechanisms.

There are three primary pathways that together make up the core mechanistic framework of apoptosis. At the very top of the apoptotic cascade are the two activation or sensor pathways for apoptosis, the intrinsic and extrinsic pathways (**Figure 1.1**). The intrinsic pathway is activated, as its name implies, by apoptotic stimuli that originate from within the cell (**Figure 1.1A**) (10). There are many stimuli that can activate the intrinsic pathway. These include but are not limited to stimuli that result in some form of excessive DNA damage (i.e. radiation or free radicals), viral or bacterial infections that can make the infected cell pose a threat to the organism, deprivation of various factors that are required for proper cellular function (i.e. serum or hormones), or a great many situations that cause stress to the cell and make it difficult for the cell to function (i.e. excessive temperature shock, exposure to toxins, hypoxia) (11-14). These stimuli, through various mechanisms lead to the mitochondria undergoing a process known as

mitochondrial outer membrane permeabilization or MOMP (15). MOMP involves the formation of protein pores that allow for the release of mitochondrial components that are required for apoptosis activation. These include proteins like Smac/DIABLO, HtrA2/Omi, etc., which are inhibitors of proteins that inhibit apoptosis (16-18). The most well-known protein released during MOMP is cytochrome c, which is a critical component of a structure known as the apoptosome (19). The apoptosome is a multi-subunit complex of repeating units of apaf1 bound to cytochrome c (20). The main function of the apoptosome is to bind to multiple copies of the pro-caspase-9 zymogen bringing them into close proximity thereby promoting their self-cleavage and full activation (20). Once caspase-9 is active it is able to then cleave and activate caspase-3 and apoptosis execution can proceed (10).

As the intrinsic pathway responds to apoptotic stimuli that originate from within the cell, the extrinsic pathway responds to stimuli that originate from outside the cell in question (**Figure 1.1B**). There are three main stimuli/receptor pairs that account for essentially all of extrinsic apoptosis: TNF-α/TNFR1, TNF-α Related Apoptosis Inducing Ligand (TRAIL)/Death Receptors 4 and 5 (DR4 and 5), and FasL)/FasR (21). All death receptors contain a characteristic motif known as a death domain (DD) which allow them to form protein complexes that have an analogous function to the apoptosome in intrinsic apoptosis (21). For FasR and DR4/5 this complex is the Death Inducing Signaling Complex (DISC) and for TNFR1 it is complex II (reviewed in 22). Cell death activated by TNF-α binding to TNFR1 is more complex than activation through FasR or DR4/5. This is due to the fact that TNF-α's day job is to induce inflammatory responses with the ultimate goal of inflammatory resolution and survival (22). Cell death only occurs

through TNFR1 when the stimulus is too strong indicating that the cells need to be sacrificed for the greater good. A key inhibitor/modulator of caspase-8 activation, cellular FLICE Like Inhibitory Protein (cFLIP), is directly transcriptionally regulated by TNFR1 mediated NF-κB signaling (23). This protein competes with caspase-8 for binding to the DISC or complex II (23). This is a prime example of how complex and fine-tuned apoptotic signaling is. Most cell types (including epithelial cells) are classified as type II extrinsic apoptotic cells (**Figure 1.1C**) (24). Therefore, despite being a separate apoptotic pathway, activation of capsase-8 through extrinsic stimuli alone is not sufficient to cause apoptosis execution. MOMP is required for this, which is triggered by the caspase-8 mediated cleavage of the Bcl-2 family protein Bid into tBid (24). This will be discussed further below when I describe the Bcl-2 family in greater detail.

The execution pathway is of importance because it is the mechanistic endpoint for apoptosis. This pathway begins with the cleavage and activation of caspase-3 by initiator caspases (10). Active caspase-3 then cleaves many downstream target proteins resulting in the morphological, biochemical, etc. outcomes of apoptosis culminating in non-lytic cell death through the dead cell being efficiently phagocytosed by immune cells (10). Since the details of biochemical and cell physiological mechanisms that underpin the execution of apoptosis are not relevant to the regulation of apoptosis execution, these mechanisms will not be discussed in detail here and have been reviewed elsewhere (10,25).

Regulation of apoptosis sensitivity by the Bcl-2 family of proteins:

Since the activation of caspase-3 is essentially an irreversible "on" switch, the regulation of sensitivity to apoptotic stimuli is largely focused on managing whether or not MOMP occurs. Although MOMP is also somewhat irreversible, it has a tightly regulated threshold for execution. This regulation is accomplished primarily by the Bcl-2 family of proteins, which fall into three categories: pro-apoptotic (i.e. PUMA, NOXA, Bid, Bad, etc.), anti-apoptotic (i.e. Bcl-2, Bcl-X_L, Mcl1, etc.), and effectors (i.e. Bax, Bak, etc.) (26,27). MOMP is achieved when the effectors Bax or Bak self oligomerize at the mitochondrial outer membrane and form the protein pore (**Figure 1.2**) (26,27). This is facilitated by direct binding of the pro-apoptotic Bcl2 family members to these effectors (**Figure 1.2**). On the other hand, MOMP can be inhibited by anti-apoptotic Bcl-2 family members that either directly bind to Bax and/or Bak and prevent them from forming the pore or directly binding to the pro-apoptotic family members preventing them from enabling pore formation and the execution of MOMP (**Figure 1.2**) (26,27). Therefore, modulation of the activity or expression of Bcl-2 family members effectively alters the cell's sensitivity to the apoptotic stimulus by either changing the magnitude of the pro-MOMP response to a stimulus (pro-apoptotic or effector members) or altering the threshold of signal necessary to initiate MOMP (anti-apoptotic members) (26,27). Since MOMP is a requirement for essentially all apoptosis in type II extrinsic apoptotic cells, the Bcl-2 family of proteins can arguably be seen as "master regulators of apoptosis" in these cells. Since enterocytes are type II extrinsic apoptotic cells, the regulation of apoptosis in these cells is primarily through regulation of MOMP and Bcl-2 family members.

Desmosomal junctions:

The intercellular junctions of intestinal epithelial cells (IECs) are vital to their function and to the regulation of IEC homeostasis. Desmosomes have been visualized in ultrastructural studies as spot welds between IECs. These junctions are present below the tight junctions and adherens junctions within the lateral membrane (28). Desmosomes are comprised primarily of the transmembrane desmosomal cadherins (the desmogleins and desmocollins) as well as intracellular plaque proteins including members of the plakin, armadillo, and catenin protein families amongst others (**Figure 1.3**) (29,30). Desmosomal cadherins are essential for establishing and maintaining the adhesive properties of the desmosomes. Human IECs exclusively express the desmosomal cadherins desmoglein-2 (Dsg2) and desmocollin-2 (Dsc2) (ref. 29). The desmosomes are critical for the tensile strength of intercellular contacts and are present within non-epithelial cells that require very strong intercellular contacts such as cardiac myocytes (31). These tensile strength functions of the desmosomes are so critical that mice lacking the desmosomal catenin plakoglobin die within the first 12-16 days of embryogenesis due to defects in embryonic heart function (32). However, it has become very clear that the desmosomes and their protein components perform a wide variety of additional functions within the cell.

Overview of Cadherins as Signaling Mediators:

Cadherins serve as calcium dependent transmembrane adhesion proteins and are subdivided into classical-, proto-, desmosomal, and atypical cadherins (33). The classical

and desmosomal cadherins have an ectodomain composed of five extracellular cadherin repeats, a single transmembrane region, and a cytoplasmic domain that interacts with either β-catenin (classical cadherins) or plakoglobin (Pg) (desmosomal and classical cadherins) (34). In addition to the classical adhesive function of cadherins, they contribute to a number of other cellular processes (**Figure 1.4**). Dynamic changes in cell adhesion are needed to establish and re-establish cell-cell contacts in multiple contexts, including developmental cell movements, tissue renewal, and wound repair (33,35). These processes require cells to integrate and process a variety of signals from the external- as well as the internal milieu. In many cases, the precise mechanism by which the cadherins participate in a signaling pathway is not fully understood, even though their involvement in the pathway is clearly demonstrated. One of the most well characterized signaling functions of cadherins is by acting as scaffolds to either promote or inhibit certain signaling pathways, which most often depend on the presence of the cadherin in the junction or its adhesion state. Through these and other mechanisms, cadherins participate in regulation of homeostatic events including proliferation, differentiation and apoptosis (**Table 1.1**).

Cadherin mediated regulation of proliferation:

Cadherins have been implicated in the regulation of cell proliferation through interaction with a wide range of signaling mediators. One example, and arguably the most classic paradigm of cadherin mediated signaling, is by modulating β-catenin signaling (33,36). The classical cadherins E-cadherin, N-cadherin, and VE-cadherin can prevent nuclear localization of β-catenin by direct interaction, thereby inhibiting

TCF/LEF mediated stimulation of proliferation (37,38). In addition to sequestration through direct binding, E-cadherin can suppress β-catenin signaling through other mechanisms. For example, re-expression of E-cadherin in SW480 colon carcinoma cells leads to increased β-catenin phosphorylation and co-localization at the plasma membrane with APC, thus promoting β-catenin degradation (39). Furthermore, the authors show that neither phosphorylated β-catenin nor members of the destruction complex co-fractionate with E-cadherin. This suggests that regulation of β-catenin stability by E-cadherin is independent of direct binding (39). However, another study found that re-expression of E-cadherin in this same cell line results in repression of fibronectin and LEF1 gene expression through prevention of β-catenin nuclear localization via direct binding (38). Such differences highlight the intricacies and complexities of cadherin mediated signaling. Binding of E-cadherin to β-catenin also affects other signaling pathways in addition to TCF/LEF mediated transcriptional regulation. E-cadherin engagement directly regulates Hippo signaling pathway in a β-catenin dependent manner through prevention of YAP nuclear localization, thereby inhibiting cell proliferation (40). Treating model epithelial cells with Wnt3a leads to increased β-catenin signaling, however, E-cadherin knock-down completely abolished this effect (41). These data show that through modulation of β-catenin activity alone, E-cadherin can participate in a variety of signal transduction pathways. In addition to E-cadherin, other cadherins regulate cell proliferation through a variety of mechanisms. VE-cadherin inhibits cell growth by binding to a variety of growth factor receptors such as TGF-βR (42) and VEGF-RII (43). N-cadherin knock-down results in reduced β-catenin activity and Akt phosphorylation, as opposed to increasing β-catenin activity as is seen after knock-down

of E-cadherin and VE-cadherin (44). Some unconventional or atypical cadherins can also modulate proliferative signaling. For example, cadherin-17 knock-down has been shown to result in decreased proliferation through reduced Erk phosphorylation (45).

The role of desmosomal cadherins in proliferative signaling is complex and depends on the cell-type and/or cadherin involved. In intestinal epithelial cells, Dsc-2 knock-down results in increased proliferation through EGFR/β-catenin signaling (46), while Dsg-2 knock-down reduces EGFR phosphorylation and inhibits proliferation (47). In line with these results, expression of Dsg-2 in the suprabasal layer of the epidermis in mice leads to development of epidermal hyperplasia. In western blots comparing transgenic and wild-type mouse skin, changes in the phosphorylation of several signaling mediators known to stimulate proliferation were observed, including MAPK, Akt, PDK-1, and GSK-3β as well as increased PTEN, c-Myc, and NF-κB protein levels (48). Exposure of intestinal epithelial cells to either IL-1β or TNF-α results in extracellular cleavage of Dsg-2 by the matrix metalloproteases MMP9 and ADAM10. The resulting Dsg-2 cleaved fragment reduces intercellular adhesion and promotes proliferation through activation of Her2/3 signaling (**Figure 1.5A**) (49). Similar to the effects of Dsg-2 extracellular cleavage, ADAM15 cleaves E-cadherin extracellularly and the resulting extracellular fragment stimulates Her2/3 heterodimerization and activation in breast cancer cell lines (50). Since these extracellular fragments affect both proliferation and adhesion, I have categorized this signaling mechanism being adhesion dependent, despite the requirement for cadherin cleavage. Re-expression of Dsc-3 in Dsc-3-deficient lung cancer cell lines results in reduced proliferation and decreased basal levels of phosphorylated Erk1/2 (51).

Cadherin mediated modulation of apoptosis:

In addition to regulating proliferation, cadherins act as important mediators of apoptotic signaling. E-cadherin homophilic engagement enhances STAT3 phosphorylation and disrupting this binding reduces pSTAT3 levels through activation of Rac1 leading to increased apoptosis (**Figure 1.6A**) (52). Expression of E-cadherin in lung, colorectal and pancreatic cancer lines enhances apoptosis and caspase-8 activation through direct interaction with the TRAIL receptors, DR4/5 (Ref. 53). Apoptosis sensitization was enhanced by E-cadherin adhesion and promoted formation of the DISC in response to TRAIL treatment (**Figure 1.6B**) (53). This is an example of cadherins acting as scaffolds for transmembrane signaling proteins in addition to functioning as scaffolds for cytoplasmic/intracellular signaling proteins. E-cadherin adhesion deficient mutant proteins were used to show that E-cadherin mediated adhesion participates in the stimulation of notch signaling, resulting in increased expression of the anti-apoptotic protein Bcl-2 (Ref. 54). These data provide examples showing that the adhesion state of E-cadherin, in addition to ensuring cell-cell cohesion, acts as a signal that is used to modulate the activity of a variety of downstream signaling events and influence cell homeostasis. Using mouse models expressing different chimeric cadherins containing portions of N-cadherin and E-cadherin it was shown that the extracellular domain of E-cadherin interacts with the Igf-1 receptor, which promotes activity of this receptor and survival of trophectoderm cells during blastocyst formation (55).

As with proliferation, other classical cadherins in addition to E-cadherin participate in the regulation of apoptosis. In the absence of VE-cadherin, endothelial

cells express higher levels of N-cadherin and re-expression of VE-cadherin reverses this through repression of β-catenin signaling. Additionally, re-expression of VE-cadherin in these cells also decreases apoptosis, proliferation, and migration (56). However, co-expression of both VE- and N-cadherin has synergistic effect that further dampens the execution of apoptosis, proliferation, and β-catenin nuclear localization. Conversely, co-expression of N-cadherin with VE-cadherin modestly reverses the inhibition of migration seen with VE-cadherin expression alone (56). These data suggest that VE-cadherin and N-cadherin play similar but slightly divergent roles in the regulation of homeostasis and migration of endothelial cells. The role of desmosomal cadherin mediated adhesion or junctional residence in apoptotic signaling is less well understood, but a few such mechanisms have been described. For example, expression of Dsg-2 in suprabasal cells of the murine epidermis increases NF-κB and Bcl-X_L protein levels. Keratinocytes derived from these mice display greater survival compared to cells derived from WT mice. These effects are enhanced by EGF treatment and are abolished by inhibition of NF-κB (48).

Cadherin mediated control of cellular differentiation:

In addition to being important regulators of proliferation and apoptosis, cadherins also regulate cell differentiation in various contexts. Loss of E-cadherin in embryonic stem cells decreases Klf4 and Nanog expression as well as pSTAT3 levels and interaction of STAT3 with the Nanog promoter (57). In another study using embryonic stem cells, treatment with E-cadherin adhesion blocking antibodies reduced expression of several transcriptional regulators as well as Tet1 and Esrrb (58). Overexpression of cadherin-11

in *Xenopus* embryos, inhibits cranial neural crest migration and is phenocopied by ADAM13 inhibition. The authors found that ADAM13 cleaves cadherin-11 generating an extracellular fragment and treatment with this cadherin-11 extracellular fragment alleviates the effects of cadherin-11 overexpression and ADAM13 inhibition on cranial neural crest migration through binding of cadherin-11 (Ref. 59). Much of the research describing roles of cadherins in the regulation of differentiation are centered on the intricately regulated expression patterns of desmosomal cadherins, which play an essential role in ensuring proper epidermal differentiation. Exogenously expressed Dsg-1 co-immunoprecipitates (Co-IPs) Erbin in normal human epidermal keratinocytes (NHEKs) (60). Induction of differentiation in NHEKs leads to increased expression of many differentiation specific genes including loicrin and involucrin. However, after induction of differentiation in Erbin knock-down cells, these changes are all diminished. In differentiated wild-type NHEKs pErk is predominantly confined to the basal layer, whereas using the Erbin knock-down cells, pErk was also found within the first couple of suprabasal layers. Whereas expression of WT Dsg-1 reduces pErk1/2 levels by two thirds, expression of Dsg-1 lacking the Erbin binding site decreases pErk1/2 levels by half. These data suggest that while the ability of Dsg-1 to bind Erbin plays an important role in its regulation of Erk1/2 phosphorylation, this signaling function is not exclusively mediated by Erbin binding (60). Dsg-1 expression enables the Ras-Raf scaffold protein Shoc2 to Co-IP Erbin and diminishes Erbin's ability to Co-IP K-Ras (60). These data suggest that desmogleins can modulate binding properties of other important signaling scaffolds, in addition to acting as a scaffold themselves. In differentiated epidermal raft cultures generated using Dsg-1 knock-down NHEKs there is abnormal differentiation of

the suprabasal layers. Furthermore, inducing differentiation in Dsg-1 knock-down keratinocytes increases pEGFR and pErk1/2 compared to control cells. Moreover, Erk1/2 inhibition was found to reverse the effects of Dsg-1 knock-down on suprabasal epidermal differentiation (61). In NHEKs cultured in organotypic raft cultures, knock-down of the GEF Bcr or blockade of MAL/SRF-induced transcriptional regulation leads to reduced expression of multiple epidermal differentiation associated proteins. Re-expression of Dsg-1 in these cells rescues the loss of these differentiation markers (62). Exposure of NHEKs to UVB prior to the induction of differentiation causes a dose dependent decrease in Dsg-1, Dsc-1, and multiple keratins as well as an increase in pErk. Overexpression of Dsg-1 helps prevent these effects, but at higher doses of UVB exposure Dsg-1 overexpression loses this protective effect. Additionally Dsg-1 knock-down intensifies the effects of UVB exposure prior to NHEK differentiation (63).

Other signaling mediated by cadherins:

The role of cadherins in signaling extends beyond the homeostatic mechanisms of life, death, and differentiation. In fact, recent research into cadherin mediated signaling has begun to focus more on the roles that cadherins play in non-homeostatic signaling pathways. Since research into these aspects of cadherin signaling is in its infancy, this section will primarily focus on some isolated examples from various systems ranging from regulation of Schwann cell mediated myelination (64) to insulin production by pancreatic β-cells (65). The rate and extent of myelination in the peripheral nervous system of E-cadherin knock-out mice is significantly reduced compared to wild-type animals (64). It was found that E-cadherin together with neuregulin 1 increase

phosphorylation of HER2, Akt, and Erk to facilitate myelination (**Figure 1.6C**) (64). Pernaud et al. observed that primary human pancreatic β-cells subjected to high glucose, display a noticeable increase in insulin production when plated on E-cadherin Fc coated glass compared to controls. This effect is blocked by treatment with the E-cadherin adhesion blocking antibody DECMA-1 (Ref. 65). These data strongly suggest that engagement of E-cadherin is important to ensure maximal insulin production in β-cells. In addition to modulating insulin production, Park et al have recently found that E-cadherin can regulate aspects of glucose metabolism. Re-expression of E-cadherin in gastric cancer epithelial cells enhances oxidative phosphorylation, glycosylation, expression of glucose transporters, and increases proliferation. These effects are all reduced following NF-κB knock-down, suggesting that NF-κB participates in this process (66). Using MCF10A human mammary epithelial cells, Bays et al. showed that application of extracellular force to endogenous E-cadherin stimulates AMPK activation as well as leads to increased phosphorylation of MLC, vinculin, and CrkL. Additionally, this application of force to E-cadherin increases glucose uptake and cellular ATP levels. These effects are inhibited by knock-down of E-cadherin, AMPK, and Lkb1 as well as E-cadherin adhesion blocking antibody treatment (67). Knock-down of E-cadherin in A549 NSCLC cells increases invasiveness as well as enhances expression of multiple MMPs, transcriptional regulators, and various other target proteins. These effects are reversed by MEK inhibition (68). These data suggest that loss of E-cadherin in cancer contributes to the epithelial to mesenchymal transition (EMT) through activation of MEK/Erk signaling. Recently, other classical cadherins have been shown to mediate non-homeostatic signaling as well. For example, N-cadherin can be cleaved

extracellularly by both ADAM10 and MMP9 and treatment of cultured primary microglial cells with the resulting protein fragment causes increased nuclear NF-κB staining as well as increased release of TNF-α, MMP9, and MCP-1 (Ref. 69).

Tsang et al. performed a detailed examination of the effects of Dsg-3 on junctional and cytoskeletal components finding that knock-down of Dsg-3 in HaCaT cells results in decreased expression of many adherens junction, desmosome, and actin cytoskeleton associated proteins. In addition, the Dsg-3 knock-down cells displayed decreased β-catenin tyrosine phosphorylation as well as several other distinct effects including diminished re-establishment of junctional E-cadherin and β-catenin staining following calcium switch (70,71). On the other hand, the authors found that overexpression of Dsg-3 increases the number of actin protrusions and the rate of actin turnover as well as increasing activation of several Rho family small GTPases. Additionally, and when combined with Rac1 inhibition, Dsg-3 overexpression increases migration (70). In HaCaT cells, the authors observed interaction of Triton soluble Dsg-3 with E-cadherin in a calcium, p120, and plakoglobin dependent manner (71). Such observations suggest that Dsg-3 (and potentially other desmogleins) play a much larger role in overall junctional and cytoskeletal regulation and function than previously appreciated.

Cadherin mediated signaling in the context of disease:

Cadherin mediated signaling mechanisms have been shown to play an important role in multiple disease states and pathologies. One of the most well studied examples is in the context of cancer. A number of previous reports have addressed cadherin

dependent regulation of cancer development and progression as well as their regulation of proliferation, apoptosis, invasiveness, metabolism, and metastasis in the context of this disease (38,39,53,66,68,72). Although E-cadherin is a classic example of cadherin mediated signaling in cancer, N-cadherin, VE-cadherin, desmosomal cadherins, and other cadherins have also been shown to participate in oncogenic signaling.

The role of cadherin signaling in the autoimmune skin diseases pemphigus vulgaris (PV) and pemphigus foliaceus (PF) will be primarily discussed because these conditions have been extensively studied. Pemphigus vulgaris and pemphigus foliaceus are known to be at least partially caused by autoantibodies that target Dsg-3 and Dsg-1 respectively (73,74). Several signaling mechanisms have been implicated in the pathobiology of pemphigus. Multiple studies have shown that the use of monoclonal antibodies targeting desmogleins can recapitulate pemphigus pathology (73,75). However, the contribution of desmogleins to these signaling events remains incompletely understood (76). Key examples of signaling caused by pemphigus autoantibodies will be highlighted because this field of study is vast and has been reviewed more comprehensively previously (77-79). Following 48 hours of treatment with PV autoantibodies, mouse keratinocytes display a strong reduction in nuclear localization of plakoglobin. After prolonged PV autoantibody treatment, keratinocytes display noticeably increased proliferation, c-Myc expression, and c-Myc nuclear localization, which was shown to be reversed after plakoglobin knock-out. Similar increased proliferation is also seen in human PV patient samples (80). NHEKs treated with PV autoantibodies display internalization and depletion of Dsg-3, which are prevented by p38MAPK inhibition (81). It was later shown that in response to patient derived PV

autoantibody treatment p38MAPK phosphorylation occurs prior to the activation of EGFR. The PV autoantibody treatment causes an increase in pErk1/2, which is enhanced by inhibition of either p38MAPK or EGFR phosphorylation. PV autoantibody or AK23 (a monoclonal antibody against mouse Dsg-3 that causes PV phenotypes) treatment causes endocytosis of EGFR, which is prevented by p38MAPK inhibition. Treatment of NHEKs with PV autoantibodies leads to internalization of Dsg-3 and an overall loss of the protein and these effects are reduced by pre-treatment with inhibitors of either p38MAPK or EGFR or knock-down of EGFR. The functional outcome of EGFR inhibition in mouse models of PV is decreased blister formation (75). These data suggest that EGFR activation in response to PV autoantibody treatment contributes to the blister formation seen in PV patients and that these effects are dependent on p38MAPK activation.

Dysregulation of apoptosis is also known to be important in PV pathobiology. A mouse model often used to study these diseases is referred to as a passive transfer mouse, in which IgG or serum derived from PV or PF patients is injected into the tail vain after which the mice develop symptoms and clinical manifestations of the parent disease. PV passive transfer mice show higher Bax and lower Bcl-2 protein levels, as well as increased caspase-3 and -9 activity. In addition, the PV transfer treated mice have enhanced phosphorylation of m-TOR and Src as well as increased blister formation and apoptosis, all of which are abolished or reduced by FAK inhibition (82). Increased apoptosis was observed in the epidermis of PF passive transfer mice which led to the development of experimental PF. These effects were reversed using pan-caspase or caspase-3 selective inhibitors (83). In PF passive transfer mice, Lee et al. observed an

increase in phospho-p38MAPK in two peaks following treatment. The authors also described apoptosis activation beginning 30 hours post treatment and increasing in severity over time, which is prevented by p38MAPK inhibition. They found that only inhibition of the first p38MAPK peak but not the second prevented blister formation in these mice (84). These data suggest that this first burst of p38MAPK activation is the most critical in blister formation in PF. In the basal cells of PV passive transfer mice, Pretel et al. describe increased phosphorylated HER family growth factor receptors and downstream signaling molecules including Src and m-TOR, which were all inhibited by EGFR inhibitor treatment. The authors also found increased in betacellulin, TGF-α, and EGF in these cells. Pre-treatment with an EGFR inhibitor, a Src kinase family inhibitor, rapamycin, or pan-caspase inhibitors all prevent the apoptosis and other clinical manifestations normally seen in PV passive transfer mice (85). These data suggest that the increased apoptosis seen in response to pemphigus autoantibody treatment is necessary for the development of clinical symptoms. Using passive transfer of AK23 (monoclonal mouse anti-Dsg-3 antibody), Schulze et al. observed increased activation or expression of several signaling mediators that promote proliferation as well as decreased the expression of multiple desmogleins, total EGFR, total p38MAPK, and phosphorylated p38MAPK (73). Treatment of *ex vivo* human skin tissue with PV or PF autoantibodies results in blister formation, but pre-treatment with RhoA or Rho family activators inhibits this process. In HaCat cells, inhibition of p38MAPK or activation of Rho family members prevents the disruption of several desmosomal or desmosome associated proteins and E-cadherin seen in response to PV autoantibody treatment. Treatment of HaCaT cells with PV or PF autoantibodies also results in reduced active

RhoA, which is prevented by p38MAPK inhibition (86). These data suggest that inhibition of RhoA is an important step downstream of p38MAPK activation in pemphigus pathobiology. Using a panel of PV monoclonal antibodies, only those that cause a loss of intercellular adhesion activate p38MAPK. The authors also found that inhibition of p38MAPK prevents Dsg-3 internalization and depletion in response to the PV mAbs (74).

Cadherin mediated signaling has been shown to participate in other disease states besides cancer and pemphigus, but these are far fewer. For example, Dsg-1 mRNA and protein expression is reduced in eosinophilic esophagitis (EoE), that has been associated with reduced barrier integrity and esophageal epithelial cell adhesion (87). These effects are recapitulated by Dsg-1 knock-down of cultured esophageal epithelial cells. Dsg-1 knock-down promotes pro-inflammatory transcriptional responses by RNAseq. Both Dsg-1 knock-down cultured esophageal epithelial cells and human EoE patient samples display noticeably increased expression of the integrin ligand protein periostin compared to controls (87). Samples from human patients with striate palmoplantar keratoderma (SPPK) arising from mutations in Dsg-1 display increased Erk activity and reduced differentiation marker expression. These samples also display increased interaction of Shoc2 with K-ras and reduced interaction of this protein with Erbin (60).

Cadherin intracellular fragment mediated signaling:

The findings I have described thus far highlight the diversity and importance of cadherin mediated signaling. However, all of these mechanisms are either dependent on or in some way influenced by changes in cadherin adhesion. In the next section I will

highlight an emerging class of cadherin mediated signaling mechanisms that do not appear to be influenced by changes in cadherin adhesion. Most of these mechanisms are based on intracellular cadherin cleavage and the generation of a functional protein fragment. For example, E-cadherin can be intracellularly cleaved by gamma secretase resulting in generation of the E-cadherin C-Terminal Fragment 2 (CTF2) (Ref. 88). Expression of CTF2 results in its nuclear localization and increased proliferation, which are both enhanced by co-expression with p120. E-cadherin CTF2 promotes Kaiso mediated transcriptional regulation and is able to Co-IP both with Kaiso and p120 (**Figure 1.4B**) (89). In line with the E-Cadherin CTF2, expression of N-cadherin c-terminal fragment 2 (CTF2) leads to N-Cadherin CTF2 nuclear localization and increased expression of β-catenin and cyclin D1 as well as increased emigration of neural crest cells from the developing neural tube in quail embryos (90). In response to UV induced apoptosis in keratinocytes, desmoglein-1 is cleaved intracellularly by caspases-3 and -7 and knock-down of desmoglein-1 protects the cells from UV induced apoptosis (91).

Cadherin signaling in cells lacking intercellular adhesion:

There are also a few examples of cadherin mediated signaling that remain intact in cells lacking intercellular adhesion. In immortalized untransformed human mammary epithelial cells E-cadherin knock-down results in increased N-cadherin and vimentin expression as well as reduced expression of cytokeratin 18, indicative of an epithelial to mesenchymal transition. However, this was not seen after expression of adhesion blocking dominant negative E-cadherin, indicating that the signaling mechanisms preventing cells from undergoing EMT in this system do not require cells to be

participating in intercellular adhesion (72). In addition, E-cadherin knock-down results in increased motility, invasiveness, and reduced sensitivity to apoptosis, all of which are rescued by expression of the adhesion blocking E-cadherin dominant negative protein (72). These data support a role of E-cadherin in preventing metastatic phenotypes through signaling mechanisms that remain intact in the absence of intercellular adhesion. Similarly, in *Xenopus* embryos it was found that knock-down of cadherin-11 inhibits cranial neural crest migration and this is rescued by expression of cadherin-11 lacking the extracellular domain. However, expression of cadherin-11 lacking the β-catenin binding domain or the transmembrane domain does not rescue the effects of cadherin-11 knock-down. These effects are also reversed by supplementation with recombinant Rho family small GTPases or the Rho GEF Trio (92).

The importance of epithelial apoptosis in Inflammatory Bowel Disease (IBD) pathogenesis:

Chronic inflammatory disorders of the intestine, such as Ulcerative Colitis (UC) and Crohn's Disease (CD) (collectively referred to as Inflammatory Bowel Disease [IBD]), are characterized by recurring bouts of inflammation and compromise of the intestinal epithelial barrier. The barrier is comprised of IECs and their intercellular junctions. These junctions include tight junctions, adherens junctions, and desmosomes (28). This barrier serves many crucial functions including preventing enteric pathogens and antigens from accessing the underlying mucosal tissue (**Figure 1.7**). Loss of the epithelial barrier plays a critical role in the pathology of IBD by exacerbating the inflammatory and other symptoms (93,94,95). These diseases have complex and

multifactorial etiologies and the mechanistic basis of their pathobiology is incompletely understood (96).

There is evidence to support aberrant and/or excessive apoptosis as a factor that can contribute to the development of UC and CD as well as to their pathologies (97-107). There are multiple apoptotic regulators or pathways the loss of which have been have been shown to result in the development of spontaneous colitis in mice or exacerbate the colitis (98,102-104,107). For example, IEC specific knock-out of NF-κB Essential Modulator (NEMO) or X-box Binding Protein 1 (XBP1) in mice leads to noticeably increased epithelial apoptosis in the intestine, especially in Paneth cells, as well as the development of spontaneous colitis (102,103). Increased steady state mRNA and protein levels of the pro-apoptotic Bcl-2 family member p53 Upregulated Modulator of Apoptosis (PUMA) have been found in UC patient derived samples as well as in mice following Dextran Sodium Sulfate (DSS) treatment for 24 h (98). The authors found that the severity of DSS or TNBS (2,4,6-trinitrobenzene sulfonic acid) induced colitis was noticeably reduced in PUMA knock-out mice compared to wild type controls which was correlated with reduced apoptosis in the colonic epithelium (98). Junctional Adhesion Molecule-A (JAM-A) knock-out mice also displayed a noticeable increase in severity of DSS induced colitis compared to wild type controls or endothelial cell specific JAM-A knock-out mice. The JAM-$A^{-/-}$ mice also displayed noticeably increased rates of enterocyte apoptosis following DSS treatment compared to controls (104). These studies in mouse models suggest that dysregulation of apoptosis contributes to colitis development and severity.

The relationship between increased IEC apoptosis and IBD has not only been observed in laboratory models. Data from patient derived clinical samples show that increased enterocyte apoptosis is associated with UC and CD especially in more severe cases as well as in regions of the intestine involved in active inflammation (98-101,106). Therefore, gaining a better understanding of mechanisms by which the various apoptotic pathways are engaged in IBD pathobiology is of great importance. Identifying links between apoptosis and IBD is also attractive because of our knowledge of mechanisms by which apoptosis is regulated. Additionally, the development or use of therapeutic strategies that modulate apoptosis sensitivity in the treatment of other disease states (especially cancer) is already a major focus of clinical and translational research (27,108).

Scope and significance of dissertation:

It is becoming clear that cadherin mediated signaling plays a vital role in the regulation of many important cellular outcomes including homeostatic functions such as apoptosis. An emerging class of cadherin mediated signaling mechanism are those involving intracellular cadherin protein fragments generated through specific and regulated cleavage. Despite clear evidence for their existence and importance, very little is known about these types of mechanisms. The role of cadherin mediated signaling in the development or progression of IBD is incompletely understood. To investigate the role of cadherin mediated signaling in intestinal inflammation, I characterized the cleavage of the desmosomal cadherin Dsg2 in the context of inflammatory stimulation and determined the functional effects of the resulting intracellular protein fragment (Dsg2 ICF).

In chapter 2, we used *in vitro* model IEC culture and TNF-α and IFN-γ as inflammatory stimuli to characterize the cleavage of Dsg2 and generation of the Dsg2 ICF. We describe an important cadherin cleavage mechanism in which intracellular cleavage occurs before extracellular cleavage, which is in direct contrast to many cadherin cleavage mechanisms described thus far (88,109-112). Caspase-8 was identified as the protease responsible for Dsg2 ICF generation through the use of selective caspase inhibitors and Dsg2 mutants lacking putative caspase-8 cleavage sites. The fact that the Dsg2 ICF is generated by an initiator caspase and that its generation is stimulated by extrinsic apoptotic stimuli (TRAIL) led us to hypothesize that the Dsg2 ICF may play a role in the regulation of apoptosis. Exogenous expression of the Dsg2 ICF revealed distinct functional effects of this fragment on apoptosis sensitivity through repression of the anti-apoptotic Bcl-2 family members Bcl-X_L and Mcl1. We measured the increase in MOMP in cells expressing the Dsg2 ICF through assessment of cytoplasmic cytochrome-c, thereby confirming that the presence of the Dsg2 ICF results in increased execution of apoptosis. The ultimate goal of my dissertation work was to characterize the role of Dsg2 mediated signaling in the severity of enterocyte apoptosis in the context of intestinal inflammation. This work contributed to the understanding of the scope of cadherin mediated signaling as well as provided a new mechanism by which inflammatory stimuli can increase the severity of clinically relevant cellular outcomes in the intestinal epithelium.

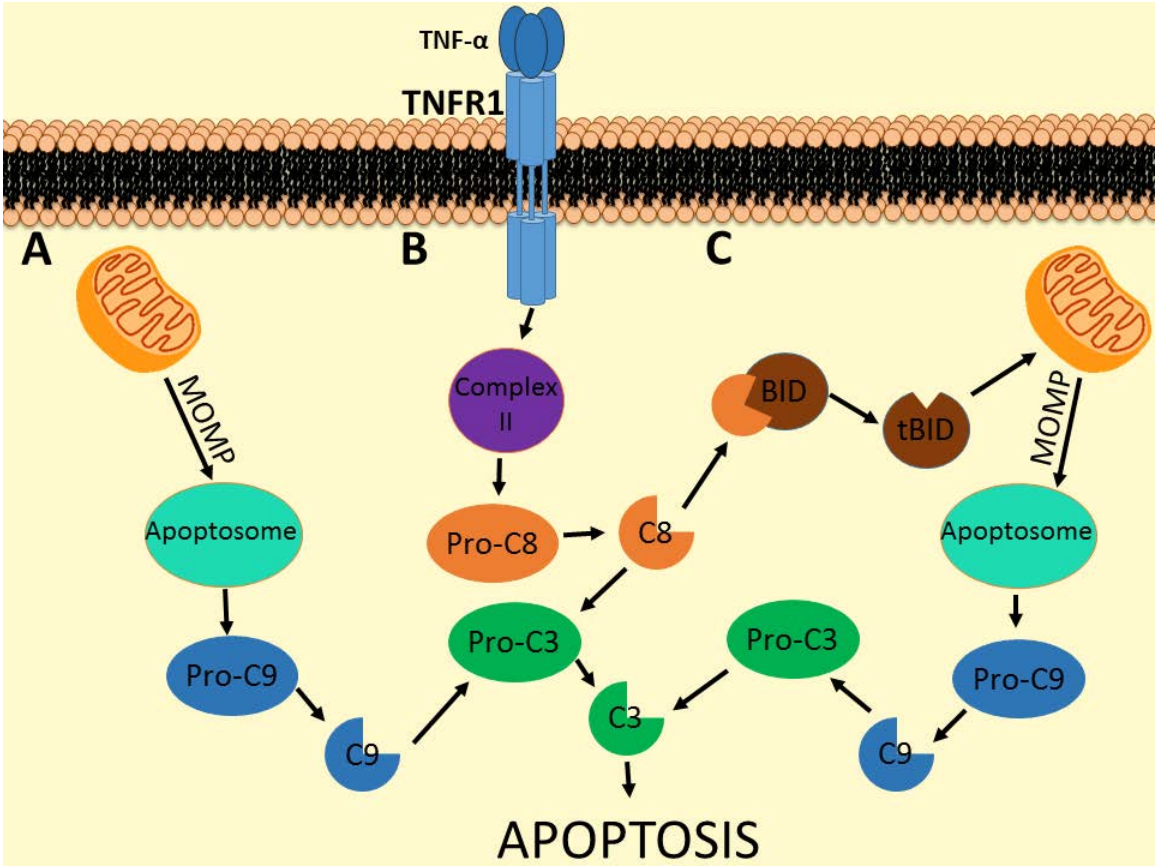

Figure 1.1: Basic model of apoptosis initiator pathways. A) The intrinsic initiator pathway is activated through numerous mechanisms that all converge on the sensitization to or direct stimulation of MOMP. MOMP leads to the formation of a multiprotein complex known as the apoptosome. The apoptosome consists of multiples of the following protein in repeating multimer units: Apaf-1, Cytochrome-c, and pro-caspase-9. The exact nature of the formation of and structure of the apoptosome has been reviewed more comprehensively elsewhere (113,114). The formation of the apoptosome leads to the proximity induced autoactivation of caspase-9 which then cleaves multiple downstream targets including caspase-3 resulting in the full execution of apoptosis. **B)** A classic example of the extrinsic apoptotic initiator pathway is the response to excessive TNF-α signaling through TNFR1. In the presence of sufficient TNF-α signal, another multiprotein complex referred to as Complex II consisting of pro-caspase-8, FADD, and TRADD (Complex IIA) or pro-caspase-8, FADD, and cleaved RIP1 (Complex IIB) is formed. The molecular mechanisms of extrinsic apoptosis have been reviewed more thoroughly elsewhere (22). Complex II facilitates the proximity mediated auto-cleavage of pro-caspase-8 leading to its full activation. Caspase-8 then cleaves multiple substrates including pro-caspase-3 leading to apoptosis execution. **C)** In type II extrinsic apoptotic cell such as enterocytes, the activation of the intrinsic apoptosis initiator pathway is required for efficient apoptosis activation. This is accomplished through cleavage of the pro-apoptotic Bcl-2 family member Bid generating tBid, which is a potent inducer of mitochondrial outer membrane permeabilization (MOMP) leading to activation of the intrinsic initiator pathway.

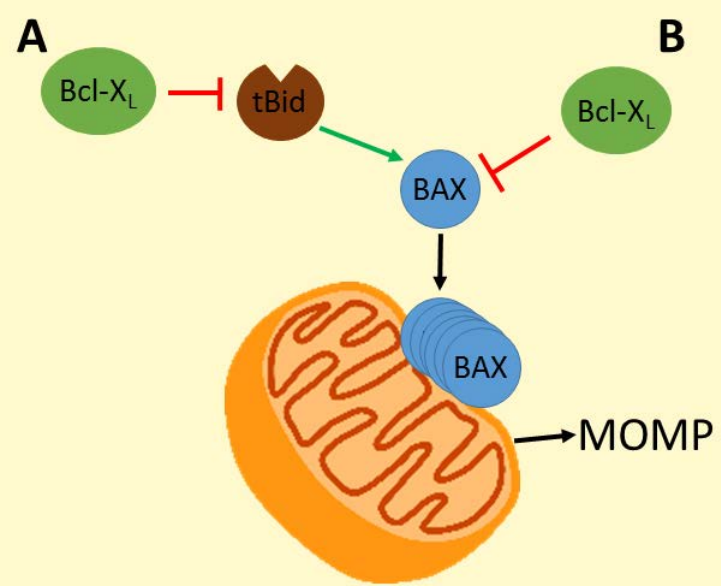

Figure 1.2: Model of Bcl-2 family regulation of MOMP. The effector Bcl-2 family members (e.g. BAX) directly execute MOMP through homo-oligomerization on the mitochondrial outer membrane forming the protein pore that causes MOMP. This is facilitated by direct binding to these effector family members by pro-apoptotic Bcl-2 proteins (e.g. tBid). MOMP is inhibited through the action of anti-apoptotic Bcl-2 family members (e.g. Bcl-XL). This is accomplished through two primary methods: **A)** Direct competitive binding to pro-apoptotic Bcl-2 family members thereby preventing them from facilitating pore formation and/or **B)** direct competitive binding to the effector Bcl-2 family proteins and preventing pore formation in that way. The precise molecular and biochemical nature of Bcl-2 family mediated regulation of MOMP and apoptosis has been more comprehensively reviewed elsewhere (27).

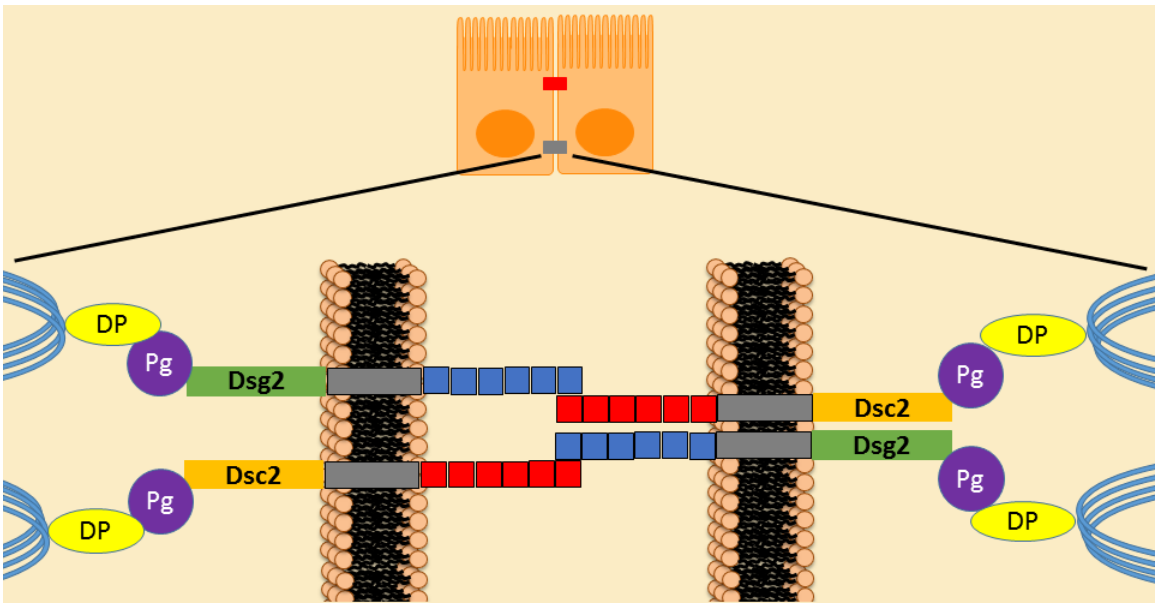

Figure 1.3: Epithelial desmosomes. Desmosomes are multiprotein structures comprised primarily of three groups of proteins: the desmosomal cadherins (the desmogleins and the desmocollins), catenin proteins (which in desmosomes is predominantly the armadillo family member plakoglobin [Pg]), and other intracellular plaque proteins including plakins such as Desmoplakin (DP). The desmosomal cadherins are transmembrane proteins that establish the intercellular contacts of the desmosomes through extracellular binding. The intracellular catenin proteins bind to the C-terminal tails of the cadherins and help mediate interaction between these tails and other intracellular plaque proteins. The remaining intracellular plaque proteins perform a wide variety of roles including mediating the interaction between the desmosomes and the intermediate filaments cytoskeleton. The structure of the desmosomes and the many functions of its components have been reviewed more comprehensively elsewhere (29,115).

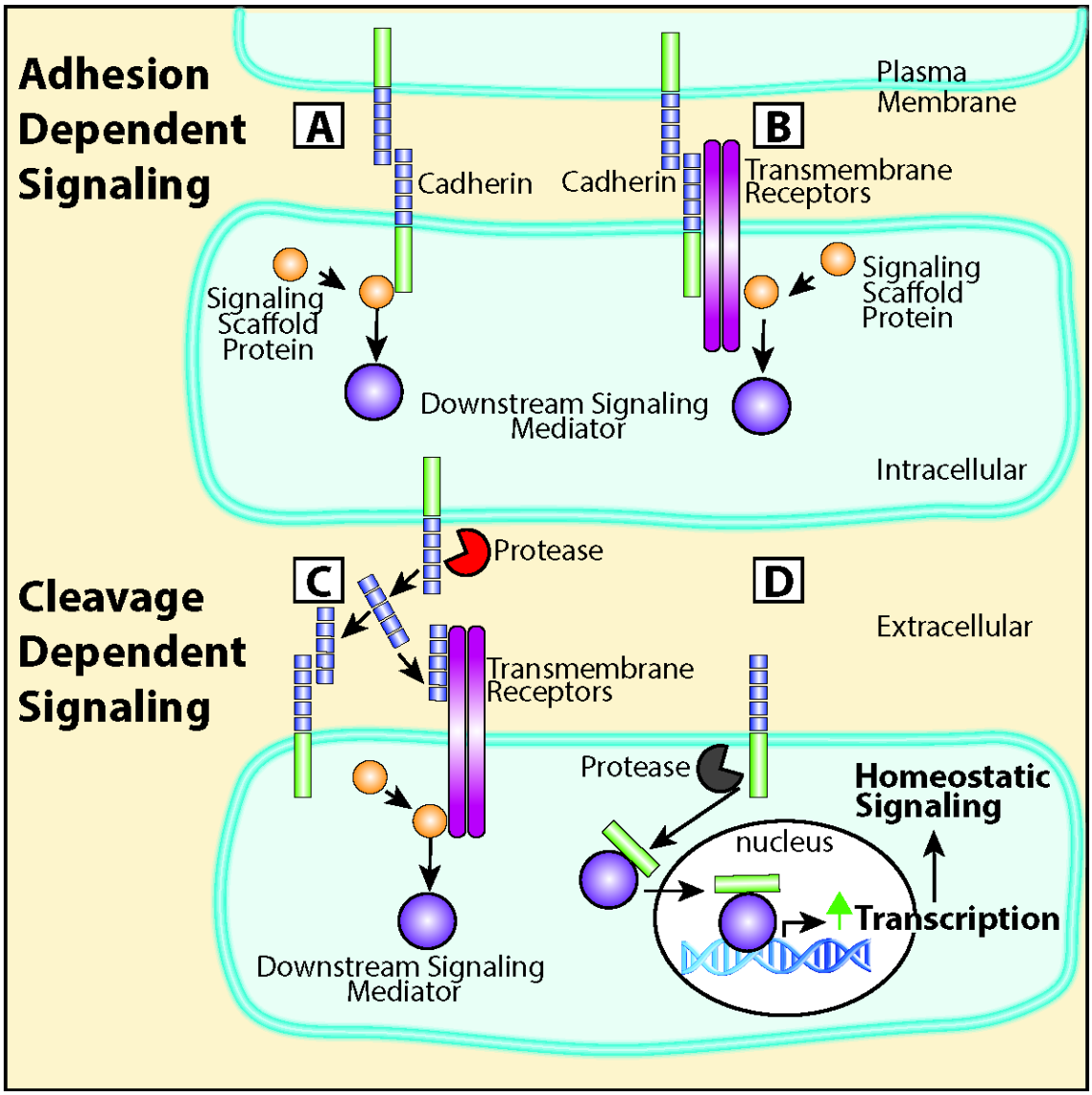

Figure 1.4: Modalities of cadherin mediated signaling. Cadherins have been shown to participate in many diverse signaling pathways and mechanisms. Not only is the range of functional outcomes that cadherins regulate wide, the number of different types of mechanisms that cadherins participate in to mediate their signaling is also impressive. **A)** Many times, cadherins themselves act as scaffolds for important signaling events and the ability of the cadherin to participate in these signaling mechanisms is often dependent upon their participation in cadherin mediated adhesion. **B)** Other mechanisms requiring cadherin mediated adhesion include cadherin protein association with other transmembrane signaling proteins and receptors. **C+D)** There are several reports of cadherin mediated signaling that requires the formation of stable cadherin protein fragments via regulated proteolytic cleavage. Most of these mechanisms require cadherin extracellular fragment generation and often times both stimulate receptor signaling pathways and interfere with cadherin mediated adhesion **(C)**. There are, however, multiple studies describing cadherin intracellular protein fragment generation (**D)**. These

mechanisms often entail binding of the intracellular fragment to a cytoplasmic signaling partner, nuclear re-localization, and regulation of gene transcription.

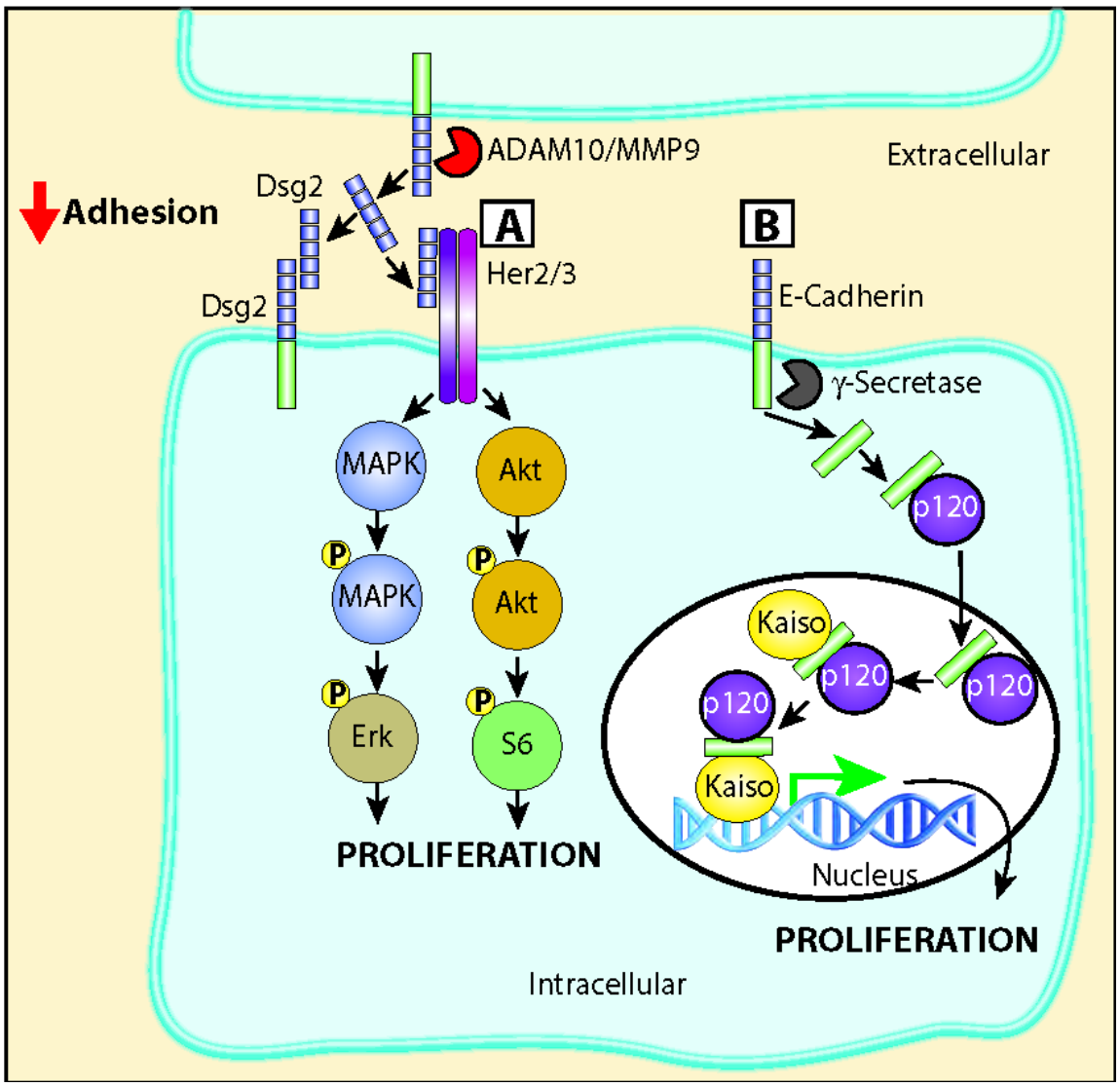

Figure 1.5: Examples of cleavage dependent cadherin mediated signaling. A growing number of reports have shown that cadherin protein fragments generated through regulated cleavage play important signaling roles. **A)** Many of these mechanisms are dependent upon cadherin extracellular fragment generation. These usually influence signaling of transmembrane receptors and/or cadherin mediated adhesion in a paracrine and/or autocrine manner. The extracellular fragment of Dsg2 has been shown to participate in both of these activities interfering with Dsg2 extracellular adhesive binding and increasing proliferation through stimulation of the Her2 and Her3 receptors (116). **B)** Cadherin intracellular fragments also mediate important signaling events. An example of this is the enhancement of E-cadherin C-Terminal Fragment 2 (CTF2) nuclear localization through binding to p120 and increased Kaiso mediated nuclear transcription (89).

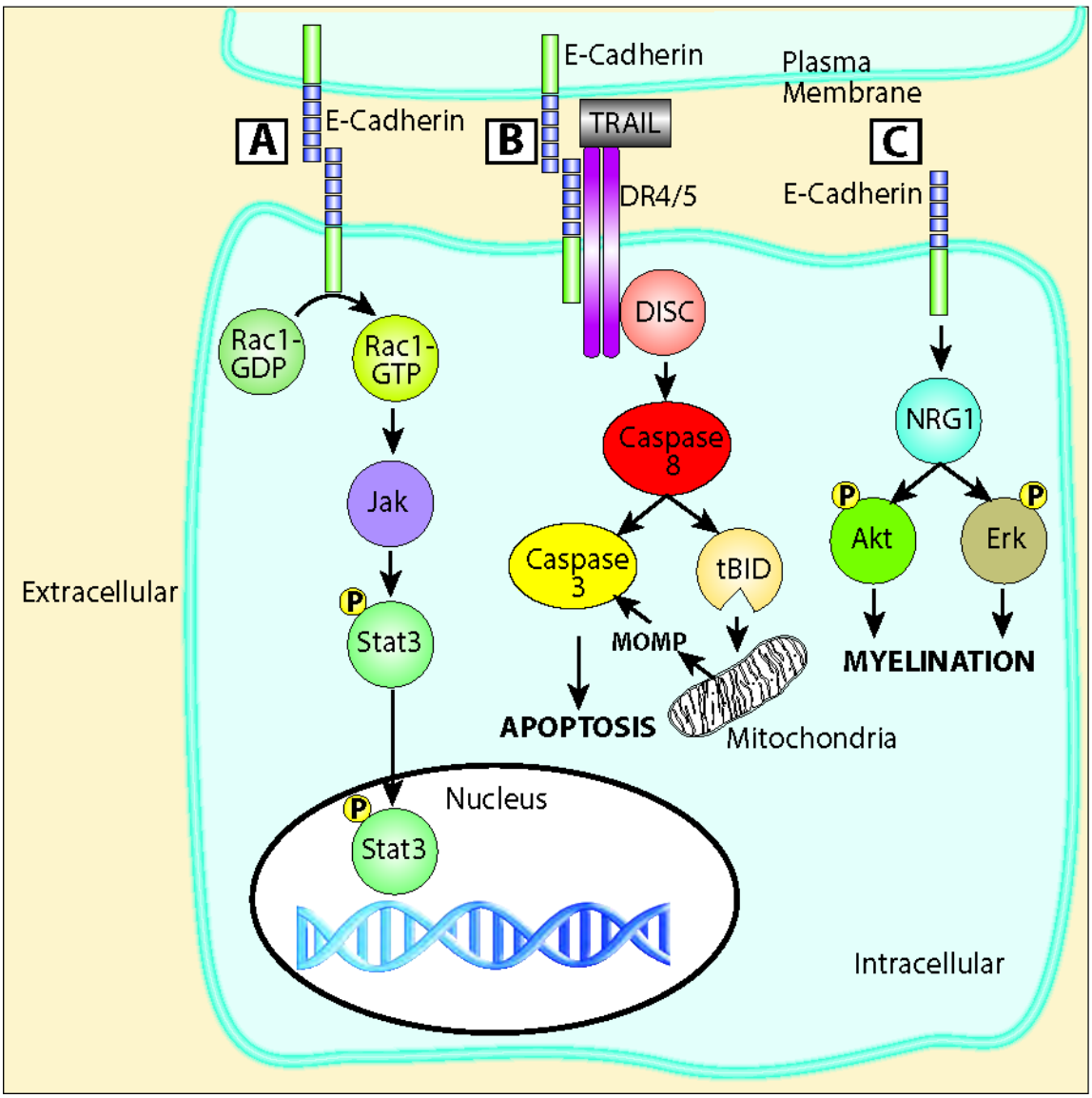

Figure 1.6: Examples of cadherin mediated signaling dependent on junctional residence and adhesive state. Cadherin cytoplasmic tails can act as scaffolds for intracellular signaling molecules thereby promoting distinct signaling pathways under various conditions. These scaffolding functions are often dependent upon the following: **A+B)** The adhesive state of the cadherin or **C)** the presence of the cadherin within a stable junction. Examples of these types of mechanisms include activation of Rac1 and Stat3 signaling through E-cadherin adhesive binding leading to repression of apoptosis (52) or reduced peripheral nervous system myelination in the absence of E-cadherin (64). There have even been a few reports of cadherin adhesive binding leading to the cadherin acting as a scaffold for and enhancing the activity of transmembrane signaling receptors such as the receptors for TNF-α Related Apoptosis Inducing Ligand (TRAIL), Death Receptors 4 and 5 (DR4 and 5) leading to enhanced Death Inducing Signaling Complex (DISC) formation and apoptosis sensitivity (53).

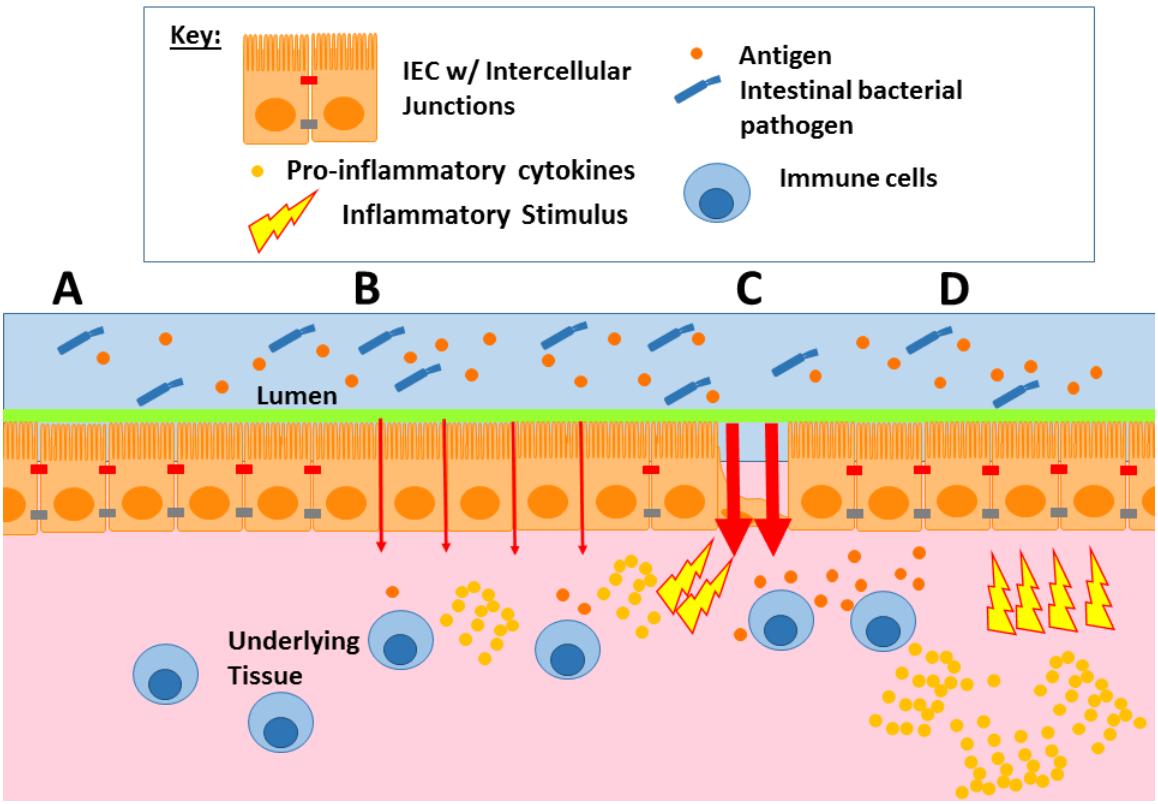

Figure 1.7: Intestinal epithelial barrier and barrier compromise in mucosal inflammation.
The intestinal epithelial barrier consists of the following:

1. Mucus that interfaces the apical surface of epithelial cells and the intestinal lumen (light green).
2. A monolayer of intestinal epithelial cells (IECs) that lines the intestine (light orange) and a series of intercellular junctions (dark grey and red boxes) through which epithelial barrier function is achieved.

A) The epithelial barrier protects mucosal tissue from luminal pathogens and antigens. **B)** Compromise of this critical barrier during mucosal inflammation results in antigen access to tissues resulting in inflammatory and other immune responses. **C)** Immune mediators can further decrease epithelial barrier function thereby contributing to disease pathogenesis and chronic inflammation **D)**.

Table 1.1: Overview of cadherin mediated signaling.

Functional Output	Cadherin (/fragment)	Associated Proteins	Effectors	Reference
Proliferation	E-Cadherin	β-catenin	TCF/LEF; Fibronectin	38
			TCF/LEF; β-catenin	39
		β-catenin	Hippo; YAP	117
		β-catenin*	TCF/LEF[†]; Wnt3a[‡]Wnt3A	41
	(NTF[#])	HER2/3	ErBb	50
	(CTF2[#])	P120; Kaiso	Kaiso[†]	89
		EGFR	EGF[‡]; Stat5; ERK	118
	N-Cadherin	β-catenin	Akt	44
		β-catenin*	TCF/LEF[†]; Wnt3a[‡]; TBX6	44
	(CTF2[#])		Cyclin D1; β-catenin	90
	VE-Cadherin	TGF-βR; ALK5; ALK1	PAI-1, Id1; TGF-β[‡]; Smad1/5, 2, 3	42
		VEGF-RII; β-catenin	p44/42 MAPK VEGF[‡]; EGF[‡]	43
[Multiple processes affected]	VE-Cadherin / N-Cadherin	β-catenin	PI3K; FoxO1; Akt; SOD[‡]; β-catenin; Caspase-3; FGF-2; MMP3; PDGFRA; ANGPT2; FRS-2α	56
	Cadherin-17		Ras; Raf; MEK; Erk; Integrin β1, β4, β5; p21; p53;	45
	Dsg-2		EGFR; Dsc-2; Histone H3; Src; Erk1/2	47
[Multiple processes affected]			MAPK; Akt; PDK-1; GSK-3β; PTEN; c-Myc; NFκB; MEK1/2; p90RSK; Raf; Stat3; Bcl-X_L	48
	(ECF[#])	HER2/3	S6; mTOR; MAPK; Akt; Erk1/2	49
	Dsc-2		EGFR; CD44; Akt; β-catenin[†]	46

	Dsc-3		Erk1/2	51
Apoptosis	E-Cadherin	DR4/5	DISC *[complex formation]*	53
			Stat3; Rac1; Cdc42	52
			Notch; Bcl-2	54
		Igf-1r	Igf-1‡	55
[Multiple processes affected]			Twist; β-catenin; Cytokeratin-8; N-cadherin; vimentin; fibronectin	72
[Multiple processes affected]	VE-Cadherin / N-Cadherin	β-catenin	PI3K; FoxO1; Akt; SOD‡; β-catenin; Caspase-3; FGF-2; MMP3; PDGFRA; ANGPT2; FRS-2α	56
	Dsg-1		Caspase-3	91
[Multiple processes affected]			MAPK; Akt; PDK-1; GSK-3β; PTEN; c-Myc; NFκB; MEK1/2; p90RSK; Raf; Stat3; Bcl-X_L	48
Differentiation	E-Cadherin		Klf4; Nanog; Stat3; Erk1/2; Akt; N-cadherin	57
			Stat3; Tet1; Esrrb; Tbx3; Nanog; Klf4; Nr0B1; Nr5a2; Erk1/2	58
	Dsg-1	Erbin; Shoc2	Erk1/2; K-Ras; loricrin; keratin-10	60
			EGFR; Erk1/2; Lamin A/C; keratin-10; desmocollin-1; fillagrin; loricrin; c-Raf; Mek1/2; EphA2; ErbB2	61
		Bcr◊	desmocollin-1; MAL; SRF; keratin-10; keratin-1; loricrin	62
	Dsg-1/Dsc-1		keratin1; keratin 10; p63;	63

			desmocollin-1	
	Cadherin-11 [extracellular fragment[#] *{fragment is unnamed}*]	ADAM13; ADAM9	?	59
Non-homeostatic signaling / disease	E-Cadherin	Neurgulin1[◊]; HER2; ErbB2	Akt; Erk; P0	64
		ROCK[▪]; Rac1[▪]; FAK[▪]		65
[Multiple processes affected]		β-catenin	NFκB; Wnt; c-Myc; IκBα; GLUT1; GLUT3; GLUT4	66
		Lkb1; AMPK[*]	MLC; Vinculin; CrkL; Abl; RhoA	67
			EGFR; Erk; ZEB1; N-cadherin; CDH1; Twist; Slug; ZEB1; MMP2; p53; GSK3β	68
[Multiple processes affected]			Twist; β-catenin; Cytokeratin-8; N-cadherin; vimentin; fibronectin	72
	N-cadherin [extracellular fragment[#] *{fragment is unnamed}*]	ADAM10; MMP7	NFκB; Iba-1; TNF-α; MMP9; MCP-1	69
	Dsg-1		periostin	119
	Dsg-3	E-Cadherin; Actin; Rac1; RhoA; Cad42	Src; p120; β-catenin; MLC	120
		β-catenin; E-cadherin; P-cadherin; desmoglein-2; plakoglobin[*]; p120[*]	desmolgien-1/2; desmocollin-2; plakoglobin; PKP2; desmoplakin; PKP1; Src;	121

	Cadherin-11	β-catenin	GEF-Trio; Rho; Rac; Cdc42	92

*Interaction is inferred but not directly demonstrated

†Inferred through effects on promoter based reporter activity assay

‡The cadherin influences the outcome of this mediator's signaling, but is not activating or repressing this mediator directly

#Cadherin cleavage fragment shown to mediate this signaling

◊Although no interaction is shown or inferred, the cadherin directly modulates the signaling of this protein

▪Inhibition of this protein enhances effects of cadherin mediated signaling, although no direct binding was detected or inferred

Chapter 2: Desmosomal Cadherin Cleavage and Modulation of Enterocyte Apoptosis in the Context of Inflammation

A portion of this chapter is adapted from the following published work:

Yulis, M., Quiros, M., Hilgarth, R., Parkos, C.A., Nusrat, A. (2018) *Cell Death Dis.* "Intracellular Desmoglein-2 cleavage sensitizes epithelial cells to apoptosis in response to pro-inflammatory cytokines"

INTRODUCTION:

Intestinal epithelial cells are a critical component of the intestinal mucosal barrier. This barrier serves as an interface between distinct luminal and mucosal environments and is essential to maintaining tissue homeostasis (122). The intestinal epithelium is highly dynamic and is actively turned over in less than a week. Yet, throughout this process, the epithelial barrier properties are maintained. Intestinal epithelial barrier compromise has been reported to contribute to the pathogenesis of mucosal inflammatory disorders such as inflammatory bowel disease (123). Epithelial barrier function is achieved by a series of intercellular junctions that include the tight junctions, adherens junctions, and desmosomes (28,124). Intercellular junctional proteins not only serve to control epithelial adhesion and barrier function but also play an active role in regulating epithelial homeostasis encompassing cell proliferation, migration, and differentiation (49,80,125,126).

Ultrastructural studies have visualized desmosomes as spot welds between intestinal epithelial cells (IECs). These junctions are located within the lateral membrane below the tight junctions and adherens junctions (28). The basic structural components of desmosomes are the transmembrane cadherin proteins (the desmogleins and desmocollins) and intracellular plaque proteins including members of the plakin, armadillo, and catenin families amongst others that serve a diverse range of critical functions (29,30). Desmosomal cadherins are essential for establishing and maintaining the adhesive properties of the desmosomes. IECs exclusively express the desmosomal cadherins desmoglein-2 (Dsg2) and desmocollin-2 (Dsc2) (ref. 29). Previous studies have identified pro-inflammatory mediators that initiate proteolytic cadherin cleavage

during mucosal inflammation (49,126). Cadherin cleavage products have been shown to have biological properties that influence epithelial homeostatic functions and intercellular adhesion (49,91,111,126-130).

We have previously shown that an intracellular fragment (ICF) of Dsg2 was generated in response to camptothecin, an intrinsic apoptotic stimulus. Dsg2 ICF generation was associated with increased IEC apoptosis (129). Apoptosis can occur through two main pathways, intrinsic and extrinsic (131). The intrinsic pathway is activated in response to apoptotic stimuli originating within the cell (i.e. excessive DNA damage) and is characterized by release of pro-apoptotic proteins from within the mitochondria through mitochondrial outer membrane permeabilization (MOMP). The extrinsic pathway is activated in response to stimuli originating from outside the cell (i.e. TNF-α). However, IECs are type 2 extrinsic apoptotic cells, which require MOMP for full execution of apoptosis even in response to extrinsic stimuli (132). Therefore, regulation of mitochondrial engagement in these cells is important for the execution of apoptosis.

The Bcl-2 protein family are key regulators of mitochondrial engagement in apoptosis (26,132). This family consists of three major groups, anti-apoptotic members (e.g. Bcl-X_L, Bcl-2, Mcl1, etc.), pro-apoptotic members (e.g. BID, BAD, NOXA, PUMA, etc.), and effectors (BAX, BAK, and BOK) (26,132). The anti-apoptotic members prevent MOMP either through direct interaction with the effectors or by directly interacting with pro-apoptotic family members (26,132). Modulation of Bcl-2 protein function is primarily achieved through two types of mechanisms, 1) changing Bcl-2 protein stability/expression and/or 2) interfering with their binding (26).

In this study, we report that the pro-inflammatory cytokines TNF-α and IFN-γ induce generation of the Dsg2 ICF. We also demonstrate that this occurs prior to Dsg2 extracellular cleavage and the execution of apoptosis. Our data show that Dsg2 intracellular cleavage is mediated by caspase-8 and also occurs in response to another extrinsic apoptotic mediator, TNF-α Related Apoptosis Inducing Ligand (TRAIL). Using adenoviral expression vectors encoding myc-tagged Dsg2 ICF, we show that the Dsg2 ICF promotes apoptosis sensitization that is associated with downregulation of the anti-apoptotic Bcl-2 family proteins Bcl-X_L and Mcl1. These data indicate that pro-inflammatory cytokines promote Dsg2 intracellular cleavage which contributes to the signaling pathways leading to epithelial apoptosis.

RESULTS:

TNF-α and IFN-γ promote Dsg2 Intracellular Cleavage:

Pro-inflammatory cytokines released into the epithelial milieu during inflammation influence cellular homeostasis and barrier function (122). We have previously reported that select pro-inflammatory cytokines induce Dsg2 ectodomain cleavage and shedding from intestinal epithelial cells (49). Given these observations, we next determined if TNF-α and IFN-γ promote intracellular cleavage of Dsg2, influencing epithelial cell fate. TNF-α and IFN-γ dose response studies were performed in the barrier forming model intestinal epithelial cell line T-84 **(Figure 2.1)**. Using an epitope mapped monoclonal antibody (4B2) that binds to a Dsg2 intracellular epitope **(Figure 2.2A)** we observed that TNF-α and IFN-γ exposure for 24hrs promoted Dsg2 intracellular cleavage

with generation of a ~55kDa Dsg2 intracellular fragment (Dsg2 ICF) (133) **(Figure 2.2B)**. Immunofluorescence labeling and confocal microscopy using the Dsg2 C-terminal specific antibody 4B2 revealed that TNF-α and IFN-γ exposure for 24hrs led to redistribution of Dsg2 cytoplasmic tail from the plasma membrane (**Figure 2.3**). As we have previously reported, TNF-α and IFN-γ treatment also promoted Dsg2 ectodomain shedding into the cell culture supernatant **(Figure 2.2C)** as detected by immunoblotting using the Dsg2 ectodomain specific epitope mapped monoclonal antibody, AH12.2 (ref. 49).

We next asked if there is a direct mechanistic link between Dsg2 ectodomain shedding and generation of the Dsg2 ICF. T-84 model IECs were treated with TNF-α and IFN-γ in the presence or absence of a specific ADAM 10 inhibitor (GI454023X) which was previously demonstrated to prevent Dsg2 extracellular cleavage (49). While ADAM 10 inhibition influenced Dsg2 ectodomain shedding, this treatment did not impact Dsg2 ICF generation in response to TNF-α and IFN-γ treatment **(Figure 2.2D and E)**. To characterize the kinetics of Dsg2 cleavage, time course experiments of TNF-α and IFN-γ treatment were performed. Dsg2 ICF was generated within 6 hours of TNF-α and IFN-γ treatment, which is prior to Dsg2 ECF cleavage and release into the cell culture supernatant **(Figure 2.2F and G)**. Since Dsg2 ICF generation was previously associated with increased apoptosis (129), we assayed the kinetics of PARP cleavage, which was observed 12 hours after cytokine exposure **(Figures 2.2F)**. These data demonstrate that TNF-α and IFN-γ induce Dsg2 ICF cleavage, which occurs prior to Dsg2 ectodomain shedding and apoptosis execution.

Calpain Inhibition Leads to Accumulation of Active Caspase-8 and Promotes Dsg2 ICF Generation:

Pro-inflammatory cytokine signaling has been shown to activate proteases that influence cellular outcomes (49,126,134). To examine the molecular mechanisms by which TNF-α and IFN-γ promote intracellular cleavage of Dsg2, we next explored the contribution of candidate intracellular proteases that mediate this cleavage event. Since γ-secretase is responsible for generating the intracellular C-terminal fragment-2 (CTF2) fragments of both E- and N-cadherin, we assessed the role of this protease in mediating Dsg2 cleavage (88,109). However, inhibition of γ-secretase with DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) did not affect Dsg2 intracellular cleavage in response to TNF-α and IFN-γ **(Figure 2.4A)**.

The calpain family of intracellular cysteine proteases have been previously implicated in cadherin cleavage (135). To test the potential role of calpains in the generation of the Dsg2 ICF, we treated IECs with TNF-α and IFN-γ in the presence and absence of the calpain inhibitor, calpeptin. In the presence of these cytokines, calpeptin treatment resulted in increased generation of the Dsg2 ICF as well as Dsg2 ectodomain shedding **(Figure 2.4B and C)**. Another family of intracellular proteases shown to have a role in cadherin cleavage are the caspases (88,91). Calpains have been shown to inhibit the activity of caspases-8 and -9 through cleavage of the active form of these caspases (136). Caspase activity was modulated by inhibition of calpains following TNF-α and IFN-γ treatment as evidenced by an increased abundance of active caspase-8 **(Figure 2.4D)**. These data suggest that calpain inhibition enhances the generation of both the

Dsg2 ICF and ECF and this effect may be mediated by alleviation of calpain induced cleavage of caspases.

Caspase-8 is Responsible for Generating the Dsg2 ICF:

To further determine the role of caspases in mediating Dsg2 cleavage, T-84 model IECs were incubated with TNF-α and IFN-γ in the presence and absence of the pan-caspase inhibitor, Z-VAD-fmk. Pan-caspase inhibition resulted in complete loss of the Dsg2 ICF **(Figure 2.5A)**. This treatment also inhibited shedding of the Dsg2 ectodomain into the cell culture supernatant **(Figure 2.5B)**. To identify the specific caspase(s) responsible for Dsg2 ICF generation, IECs were incubated with TNF-α and IFN-γ in the presence of increasing concentrations of selective caspase inhibitors targeting caspases-3/-7 (Z-DEVD-fmk), caspase-8 (Z-IETD-fmk), and caspase-9 (Z-LEHD-fmk), or vehicle. The caspase-8 selective inhibitor, Z-IETD-fmk, had the maximal effect in reducing Dsg2 ICF generation **(Figure 2.5C)**. To confirm that caspase-8 is the primary protease responsible for generation of Dsg2 ICF, myc-tag Dsg2 constructs with mutation of two putative intracellular caspase-8 cleavage consensus sites were generated (D715A and D675A). Myc tagged Dsg2 wild type (Dsg2 WT) and mutant proteins were expressed in Cos7 cells. Cos7 cells were chosen because they are readily transfected to express exogenous proteins and robustly cleave endogenous and exogenous Dsg2 to generate the Dsg2 ICF. As shown in figure 2.5D, D715A inhibited generation of the Dsg2 ICF while D675A had minimal effects on Dsg2 ICF cleavage in response to TNF-α and IFN-γ treatment. These data indicate that caspase-8 is the primary protease responsible for Dsg2 ICF cleavage following TNF-α and IFN-γ exposure.

TRAIL promotes Dsg2 cleavage analogous to TNF-α and IFN-γ:

To assess if Dsg2 cleavage can be mediated another mechanism which activates the extrinsic apoptotic pathway, a time-course experiment was performed using TRAIL. TRAIL activates the TNF receptor family members Death Receptor 4 and Death Receptor 5 (DR4 and DR5), which initiate the extrinsic apoptosis pathway and caspase-8 activation (137). As with TNF-α and IFN-γ, Dsg2 ICF generation was induced within the first 6 hours of TRAIL treatment **(Figure 2.6A)**. Furthermore, TRAIL also induced Dsg2 ectodomain shedding into the cell culture supernatant after initial generation of the Dsg2 ICF (12-24 hrs) **(Figure 2.6B)**. Analogous to TNF-α and IFN-γ treatment, these effects were seen prior to maximal PARP cleavage 12-24 hrs after TRAIL exposure **(Figure 2.6A)**. These data suggest that Dsg2 ICF and ECF generation are a response to stimulation of extrinsic apoptosis.

Expression of the Dsg2 ICF sensitizes epithelial cells to Apoptosis:

To determine if Dsg2 ICF generation sensitizes IECs to apoptosis, we exogenously expressed the Dsg2 ICF in SKCO15 model IECs by transduction with an adenovirus expressing myc-tagged Dsg2 cytoplasmic tail (amino acids 634-1117) (Dsg2 ICF) (**Figures 2.7A-C**). SKCO15 model IECs were used for these experiments since they have well developed intercellular junctions and can be readily transfected to express proteins. Additionally, to evaluate if plasma membrane tethering influences Dsg2 ICF mediated signaling and apoptosis, cells were transduced with adenoviruses expressing a fusion construct encoding myc-Dsg2 ICF and the extracellular and transmembrane

regions of the IL2R-α receptor (**Figures 2.7A and B**). The IL2R-α plasma membrane tether tagged with myc was expressed as a control (**Figure 2.7A and B**). These adenoviral constructs all encode the EGFP gene expressed through an Internal Ribosomal Entry Site (IRES) to mark transduced cells. As shown in Figure 2.7B, Dsg2 ICF expression was detected by western blotting using the intracellular specific epitope mapped monoclonal Dsg2 antibody 4B2. Analysis of the functional outcome revealed maximal PARP cleavage after expression of the non-tethered Dsg2 ICF-Myc supporting its role in increasing sensitivity to apoptotic stimuli (**Figure 2.7C**). Cells expressing the non-tethered Dsg2 ICF also displayed a decrease in the protein levels of the anti-apoptotic Bcl-2 family members Bcl-X_L and Mcl1 (~49% and ~57% of control respectively) (**Figure 2.7C**). To assay the effects of Dsg2 ICF expression on mitochondrial outer membrane permeabilization and cytochrome c release into the cytoplasm, we harvested cytoplasmic and mitochondria containing fractions of model IECs transduced with the above adenovirus constructs. Fractionation controls are shown in Figure 2.11. The cytoplasmic cytochrome c levels were increased in cells expressing the non-tethered Dsg2 ICF compared to controls (~218% of control) (**Figure 2.7D**). To further confirm increased apoptosis in cells expressing the Dsg2 ICF, Annexin V labeling was performed in live cells. Cells expressing the Dsg2 ICF displayed increased Annexin V labeling and cell loss compared to controls (**Figure 2.8)**. These findings suggest that cleavage and release of Dsg2 ICF from the plasma membrane is associated with sensitization to apoptotic signaling and that downregulation of anti-apoptotic Bcl-2 family members contributes to this process.

DISCUSSION:

Mechanisms of epithelial barrier compromise in response to inflammation remain incompletely understood. Mucosal inflammation perturbs IEC homeostatic signaling that is associated with compromised barrier function, which results in translocation of luminal antigen that further perpetuate the inflammatory response. Failure to control inflammation and epithelial barrier dysfunction has been observed in chronic diseases such as inflammatory bowel disease (IBD) (123,138). Thus, understanding mechanisms of epithelial barrier compromise is important in developing effective therapeutic strategies. Homeostatic mechanisms that are perturbed during mucosal inflammation include proteolytic cleavage of transmembrane cadherin proteins resulting in the generation of functional protein fragments (49,88,109-112,126,127,129,139-141). Few studies describe cadherin cleavage specifically in response to inflammatory stimuli. These primarily focus on the generation of extracellular cadherin fragments, which usually interfere with cadherin based adhesion or the maintenance of endothelial barrier tightness (49,140,141). Regardless of the stimulus responsible, in most scenarios the extracellular cadherin cleavage precedes intracellular cleavage. These sequence of events have been described for E-cadherin, N-cadherin, and VE-cadherin as well as Dsg2 and Dsg1 in keratinocytes (88,109-112). In contrast to these reports, we observed that Dsg2 undergoes intracellular cleavage prior to extracellular cleavage in response to pro-inflammatory cytokine signaling in intestinal epithelial cells. These differences are likely related to the cell type and stimulus that induces such responses. Little is known about

the relationship between intracellular and extracellular cadherin cleavage in response to inflammatory stimuli. However, one report showed that upon exposure of HUVECs to TNF-α, VE-cadherin undergoes cleavage in its extracellular and intracellular domains, and inhibiting extracellular cleavage suppresses intracellular cleavage (112). This suggests that, in response to TNF-α, VE-cadherin extracellular cleavage occurs prior to its intracellular cleavage.

Knowledge of the protease(s) responsible for generation of protein fragments provides crucial context to the functional effects of that fragment within the cell and in the overall pathology. Cadherin extracellular cleavage is most often mediated by matrix metalloproteinases. ADAM 10 promotes extracellular cleavage of E-, N-, and VE-cadherin resulting in the generation of N-terminal fragments (NTFs). In a recent report, we observed that ADAM 10 and MMP9 were responsible for extracellular cleavage of Dsg2 in intestinal epithelial cells (49,110,127,139). In keratinocytes, ADAM 17 has been implicated in mediating extracellular cleavage of Dsg2 (111).

Cadherin intracellular cleavage however, can be somewhat complicated and several protease families have been implicated in mediating this event. For example, the most abundant E-cadherin C-terminal fragment (CTF2) is generated by γ-secretase, but other reports implicate cysteine proteases in mediating E-cadherin intracellular cleavage (88,135,142). Surprisingly, despite both the mechanistic similarities to notch signaling (extracellular cleavage by ADAM 10 and generation of a functional intracellular fragment) as well as the similarities of the Dsg2 ICF to the CTF2s of both E-cadherin and N-cadherin, we found that γ-secretase inhibition had no effect on Dsg2 ICF generation

(88,109,143,144). We therefore investigated the influence of other protease families in mediating Dsg2 intracellular cleavage.

The calpain family of intracellular cysteine proteases have been shown to mediate E-cadherin intracellular processing (135). Such E-cadherin intracellular cleavage was shown to leave behind a membrane bound ~100 kDa N-terminal fragment (135). Thus, analogous to the processing of Dsg2 in IECs, calpain mediated E-cadherin cleavage appears to occur before extracellular cleavage. However, we observed that inhibition of calpains caused an increase in Dsg2 ICF generation indicating that the activity of the protease responsible for generating this fragment was enhanced by calpain inhibition. We also observed that calpain inhibition resulted in increased abundance of active caspase-8. Not only have members of the caspase family of cysteine proteases been implicated in cadherin cleavage, their activity is known to be regulated by the calpains (88,91,136). While it is generally thought that calpains cleave and activate caspase-3, a previous report has shown that calpains cleave and inactivate the initiator caspases, caspase-8 and caspase-9 (Refs. 136,145-147). In keratinocytes, intracellular Dsg1 cleavage is mediated by caspases-3/-7. The cleavage site was identified to reside within the third Dsg1 repeated unit domain (RUD) thereby generating a ~17 kDa fragment (91). Cirillo et al. previously attributed generation of the Dsg2 ICF to caspase-3 based upon inhibitor studies using the caspase-3 selective inhibitor Z-DEVD-fmk (148). In our studies, Dsg2 ICF generation by TNF-α and IFN-γ was minimally influenced by this inhibitor at a concentration of 30 μM. However, using 100 μM Z-DEVD-fmk inhibited Dsg2 intracellular cleavage. It is to be cautioned that at high doses such inhibitors often have off target effects (149). Our study suggests a more definitive role of caspase-8 in

mediating Dsg2 intracellular cleavage. These results were supported by performing dose response studies using selective caspase inhibitors as well as by expression of two Dsg2 mutant constructs lacking putative caspase-8 cleavage sites (D715A and D675A). These sites were chosen based upon their ability to generate a ~55 kDa ICF and their similarities to known caspase-8 cleavage consensus sites. The identification of caspases as mediators of cadherin intracellular cleavage suggests that these mechanisms may play a larger role in apoptosis regulation.

The two primary pathways that lead to the execution of apoptosis are the extrinsic and intrinsic pathways. Caspase-8 is one of the initiator caspases for the extrinsic pathway. This pathway is closely related to TNF-α pro-inflammatory signaling (131). This is because TNFR1 is one of the founding members of the "death receptor" family of transmembrane receptors whose activation is the first step in the extrinsic pathway (150). TNF-α Related Apoptosis Inducing Ligand (TRAIL) is another canonical extrinsic apoptotic stimulus that activates Death Receptors 4 and 5 (DR4/5) (ref. 22). In this study, we found that the Dsg2 ICF is generated in response to TRAIL treatment with similar kinetics to TNF-α and IFN-γ treatment. These observations indicate that generation of the Dsg2 ICF is a response to stimulation of the extrinsic apoptotic pathway. Thus, Dsg2 ICF generation may play a role in the response to extrinsic apoptosis activation through inflammatory mediators and in other scenarios such as use of chemotherapy agents that target DR4/5 or CD95 (Refs. 151,152). Following stimulation of death receptors, the extrinsic apoptotic cascade proceeds through activation of caspases-8 and/or -10. Once these initiator caspases have been activated, they cleave target proteins including pro-caspase-3 leading to its activation and the execution of apoptosis (131). Due to the

irreversible nature of activation through proteolysis, caspase-3 activation and apoptosis execution are tightly regulated through numerous upstream sensitization pathways. Therefore, the generation of the Dsg2 ICF by caspase-8 in response to both TNF-α and IFN-γ as well as TRAIL suggests that Dsg2 cleavage can represent a potential mechanism of sensitization in the apoptotic cascade. Since the sensitivity of IECs to apoptosis is a key factor in whether or not mucosal inflammation progresses towards effective resolution or development of chronic disease, this mechanism may play a role in the pathobiology of epithelial barrier dysfunction in inflammatory diseases.

There is a precedent for cadherin intracellular cleavage playing a role in apoptotic mechanisms. Most reports describing cadherin intracellular cleavage events used apoptotic stimuli. Although most of these have been intrinsic stimuli such as UV exposure, staurosporine, or camptothecin (88,91,142). However, the generation of the VE-cadherin intracellular fragment in response to TNF-α has been described (110). The same report that identified Dsg1 intracellular cleavage by caspases-3/-7 also showed that keratinocytes displayed increased resistance to UV induced apoptosis in response to shRNA knockdown of Dsg1 (Ref. 91). We have obtained similar results previously using siRNA knockdown of Dsg2 in IECs treated with camptothecin (129). In the current study, we observed that expression of Myc-tagged Dsg2 ICF in model IECs leads to increased sensitivity to apoptosis. IECs expressing the Dsg2 ICF also displayed reduced levels of the anti-apoptotic Bcl-2 family proteins, Bcl-X_L and Mcl1, at the protein level. The Bcl-2 family of proteins are key regulators of mitochondrial engagement in apoptosis, which is essential for intrinsic apoptosis as well as extrinsic apoptosis in type II extrinsic apoptotic cells such as IECs (132). These data indicate that the Dsg2 ICF

does, in fact, participate in sensitization to apoptotic stimuli and suggest that downregulation of Bcl-X_L and Mcl1 plays a role in this process.

We have shown that TNF-α and IFN-γ exposure induces cleavage of Dsg2 mediated by caspase-8 leading to the generation of the Dsg2 ICF. Presence of this Dsg2 ICF causes sensitization to apoptosis through downregulation of the anti-apoptotic Bcl-2 family members Bcl-X_L and Mcl1. This establishes Dsg2 intracellular cleavage as a mechanism that mediates apoptosis of cells that are exposed to an inflammatory insult (**Figure 2.9A**). Of interest, the dying cells subsequently release the Dsg2 ECF, which can signal to surrounding cells to promote proliferation and repair through activation of HER2 and HER3 signaling (**Figure 2.9B**) (49). Considering that the Dsg2 ICF is generated before the Dsg2 ECF, we propose that during mucosal inflammation these events mediate death and loss of damaged IECs through Dsg2 ICF signaling, while subsequent generation of the Dsg2 ECF can have paracrine effects on surrounding epithelial cells to promote proliferation and repair.

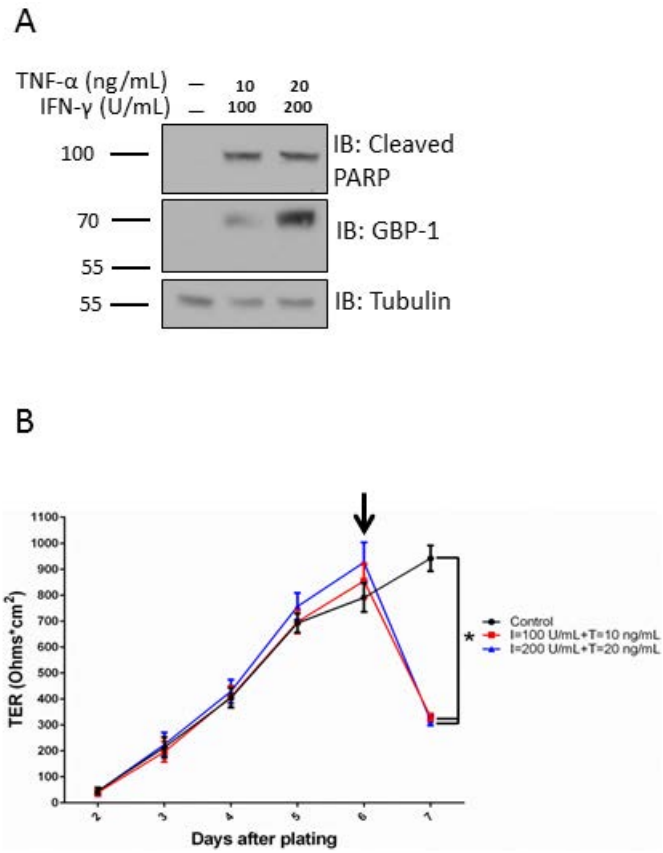

Figure 2.1: **Characterization of T-84 response to TNF-α and IFN-γ treatment. A)** Western blot for GBP-1 and PARP of cell lysates of T-84s treated with TNF-α and IFN-γ for 24 h. All blots are representative of at least three independent experiments. **B)** TEER of cells used in Figure 2.1A and in Figure 2.2. Treatment was applied on day 6 post plating as indicated by the black arrow. TEER measurements were collected as described in Materials and Methods. *, p=0.0001 n=11 per group.

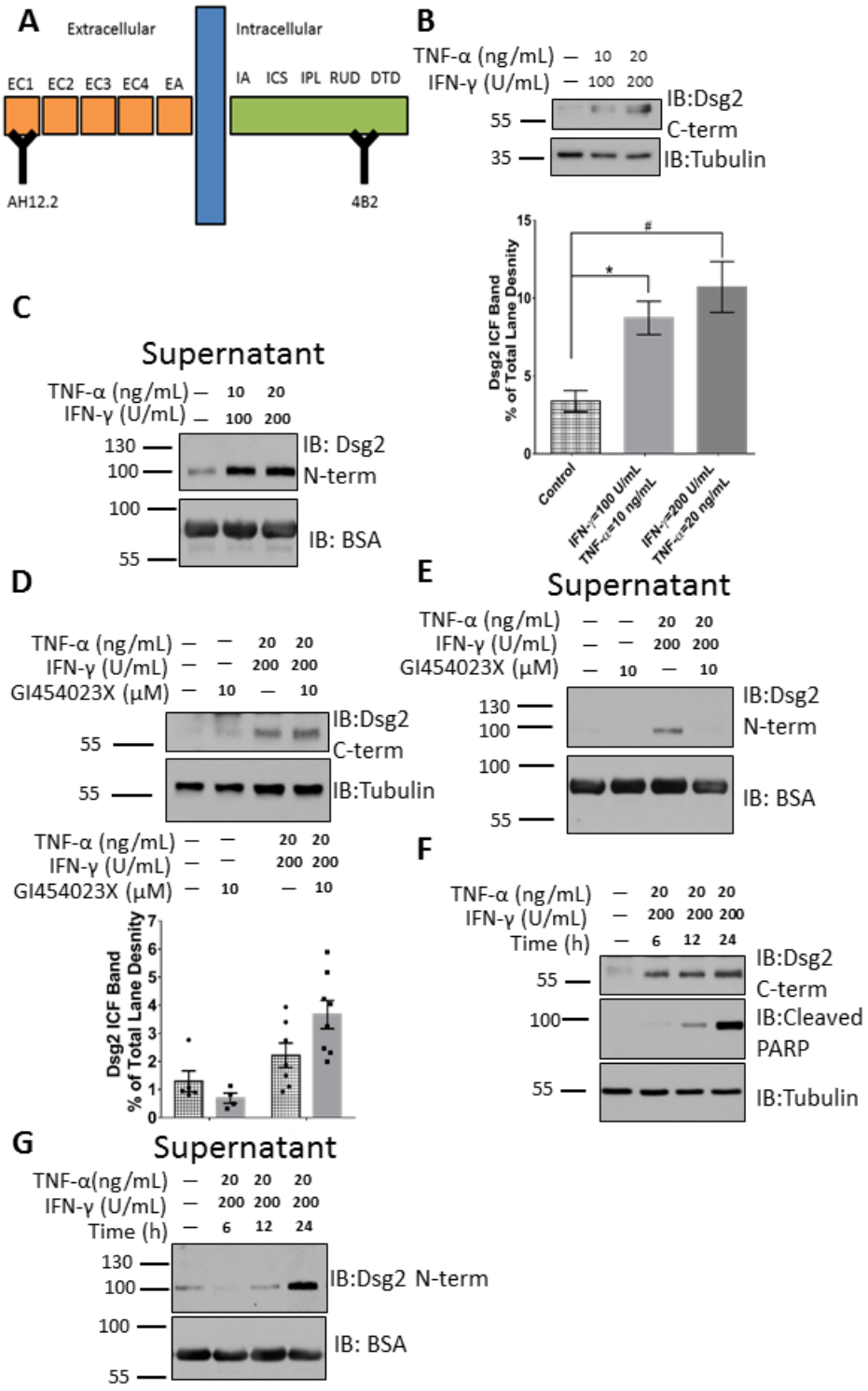

Figure 2.2: TNF-α and IFN-γ promote Dsg2 intracellular and extracellular cleavage. **A)** Model of Dsg2 with mapped epitopes for AH12.2 (extracellular [N-term] specific) and 4B2 (intracellular [C-term] specific) monoclonal antibodies. EC=extracellular cadherin domain; EA=extracellular anchor; TM=transmembrane domain; IA=intracellular anchor; ICS=intracellular cadherin-typical sequence; IPL=intracellular

proline-rich linker domain; RUD=repeated-unit domain; DTD=desmoglein-specific terminal domain. **B)** Top. Western blot using an antibody against Dsg2 C-term of cell lysates of T-84s treated with TNF-α and IFN-γ for 24 h. Bottom. Densitometry of blots. Density for all bands in each lane were collected. The ratio of the density of the ~55 kDa Dsg2 ICF band to the density of all bands for a given lane was then calculated. The data in the graph is the average of these ratios within each experimental group. *, p=0.0067; #, p=0.0003 n=11 per group. For full lanes of Dsg2 C-term blot please see **Figure 2.10A**. **C)** Western blot using an antibody against Dsg2 N-term of cell culture supernatants of T-84s treated as in Figure 2.2B. For full lanes of Dsg2 N-term blot please see **Figure 2.10B**. **D)** Top. Western blot using an antibody against Dsg2 C-term of cell lysates of T-84s treated with TNF-α and IFN-γ in the presence or absence of GI254023X for 24 h. Bottom. Densitometry of blots. Densitometry collected and analyzed as for Figure 2.2B. n≥4 per group. **E)** Western blot using an antibody against Dsg2 N-term of cell culture supernatants of T-84s treated as in Figure 2.2D. **F)** Western blot using an antibody against Dsg2 C-term of cell lysates of T-84s treated with TNF-α and IFN-γ for 6 h, 12 h, or 24 h. **G)** Western blot using an antibody against Dsg2 N-term of T-84 cell culture supernatants of cells treated as in Figure 2.2F. All blots are representative of at least three independent experiments.

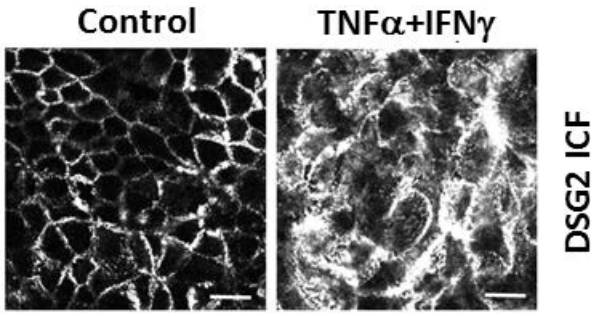

Figure 2.3: **Re-localization of Dsg2 C-terminal tail in response to TNF-α and IFN-γ**. Immunofluorescence staining and confocal microscopy using an antibody against Dsg2 C-term in T-84 cells treated with TNF-α and IFN-γ for 24 h. Scalebars=20 μm. Images are representative of at least three independent experiments.

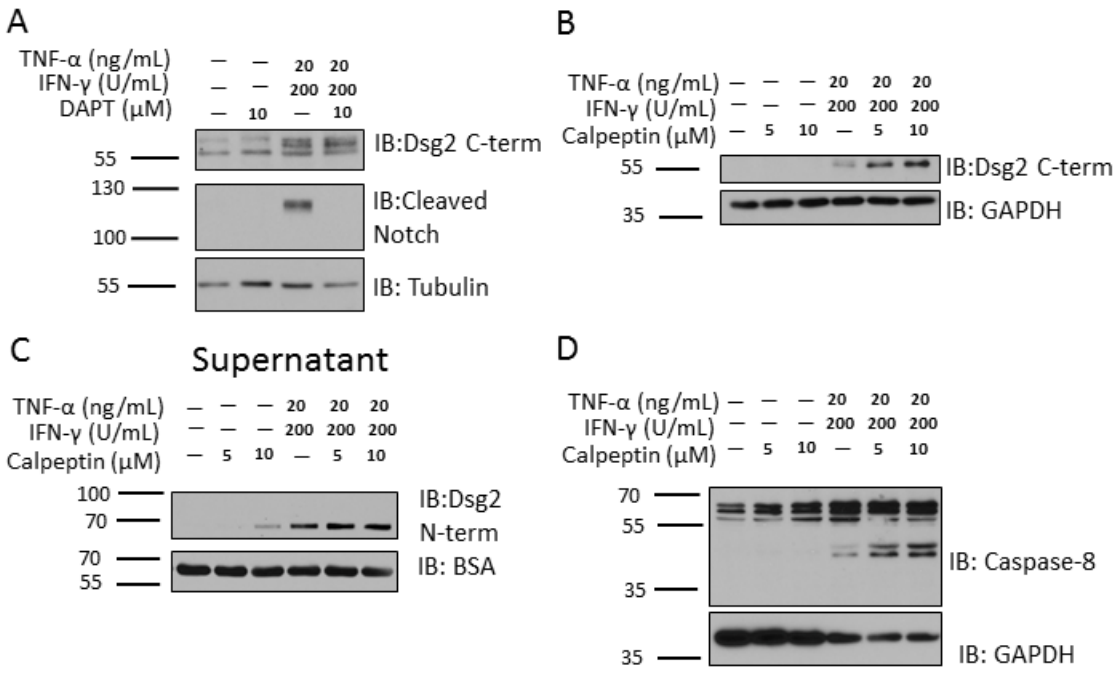

Figure 2.4: Effects of inhibition of candidate proteases on Dsg2 cleavage. A) Western blot using an antibody against Dsg2 C-term and also for cleaved Notch of cell lysates of T-84s treated with TNF-α and IFN-γ for 24 h in the presence or absence of the γ-secretase inhibitor DAPT. **B)** Western blot using an antibody against Dsg2 C-term of cell lysates of T-84s treated with TNF-α and IFN-γ in the presence or absence of increasing concentrations of calpeptin for 24 h. **C)** Western blot using an antibody against Dsg2 N-term of cell culture supernatants of T-84s treated as in Figure 2.4B. **D)** Western blot for caspase-8 cell lysates of T-84s treated as in Figure 2.4B. All blots are representative of at least three independent experiments.

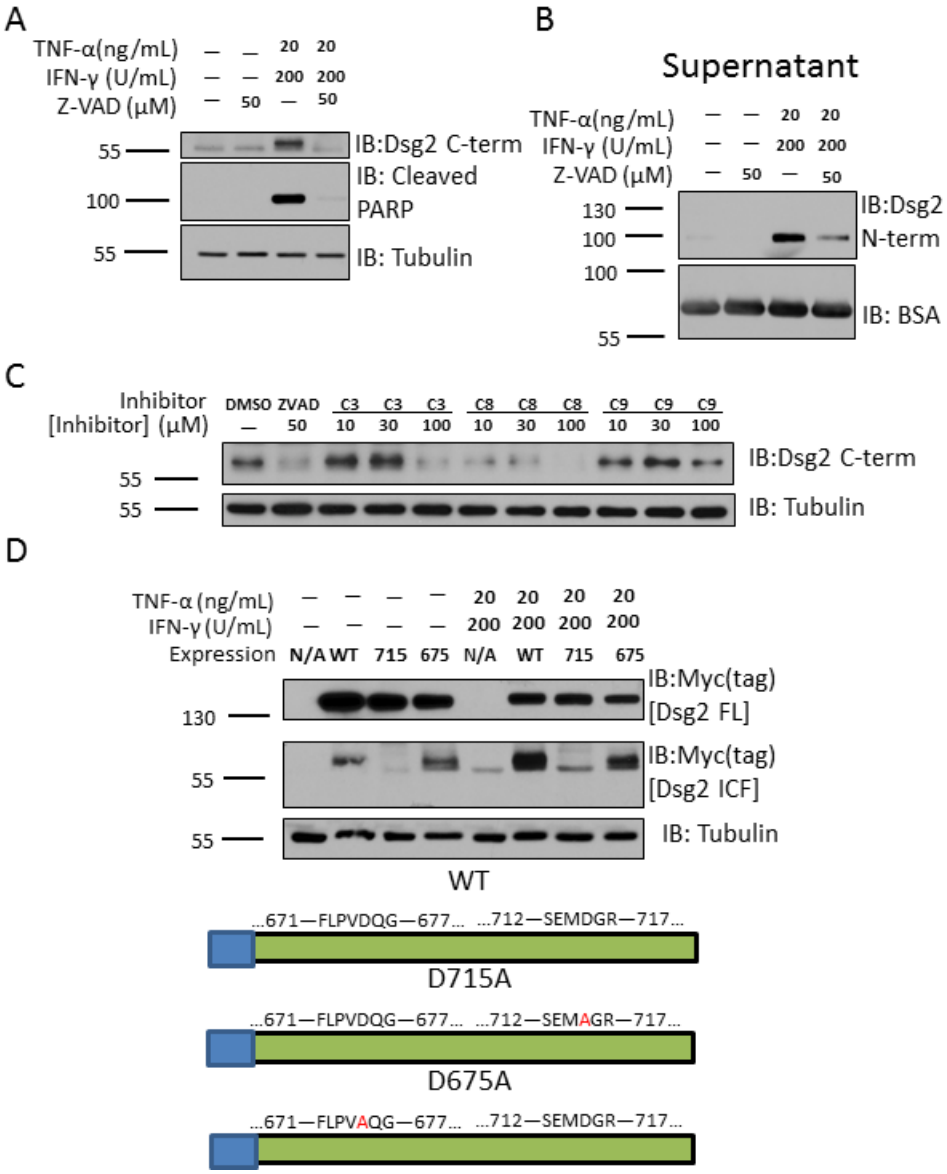

Figure 2.5: Caspase-8 is responsible for Dsg2 ICF generation. A) Western blot using an antibody against Dsg2 C-term of cell lysates of T-84s treated with TNF-α and IFN-γ in the presence or absence of Z-VAD-fmk for 24 h. **B)** Western blot using an antibody against Dsg2 N-term of cell culture supernatants of T-84s treated as in Figure 2.5A. **C)** Western blot using an antibody against Dsg2 C-term of cell lysates of T-84s treated with TNF-α and IFN-γ in the presence or absence of increasing concentrations of either Z-DEVD-fmk, Z-IETD-fmk, or Z-LEHD-fmk for 24 h. **D)** Cos7 cells were transfected with expression plasmids encoding either WT Dsg2 [WT], or Dsg2 containing one of two putative caspase-8 cleavage consensus site mutations (D715A [715] or D675A [675]). Cells treated with Lipofectamine 3000 reagents alone with no DNA were used as controls [N/A]. *Top.* Western blot against the Myc tag (Top=Dsg2 full length band; Middle=Dsg2 ICF band; Bottom=Tubulin). *Bottom.* Model of WT Dsg2, D715A, and D675A depicting region of Dsg2 containing sequences mutated in each highlighting the mutated residues in red. All blots are representative of at least three independent experiments.

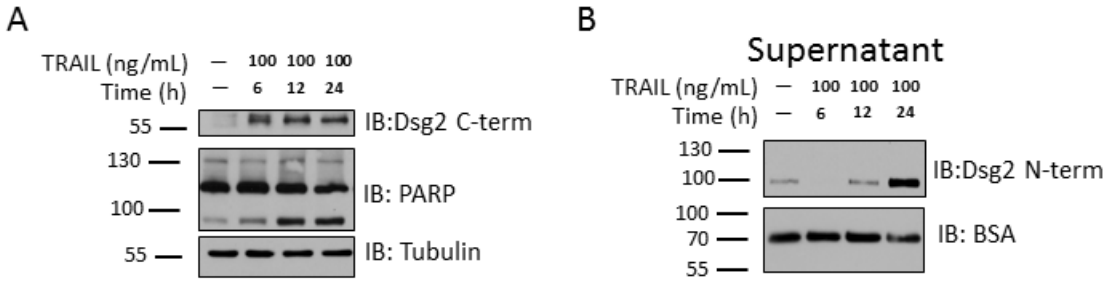

Figure 2.6: TRAIL treatment induces Dsg2 ECF and ICF generation similarly to TNF-α and IFN-γ. A) Western blot using an antibody against Dsg2 C-term and against PARP of cell lysates of T-84s treated with TRAIL for 6 h, 12 h, or 24 h. **B)** Western blot using an antibody against Dsg2 N-term of cell culture supernatants of T-84s treated as in Figure 2.6A. All blots are representative of at least three independent experiments.

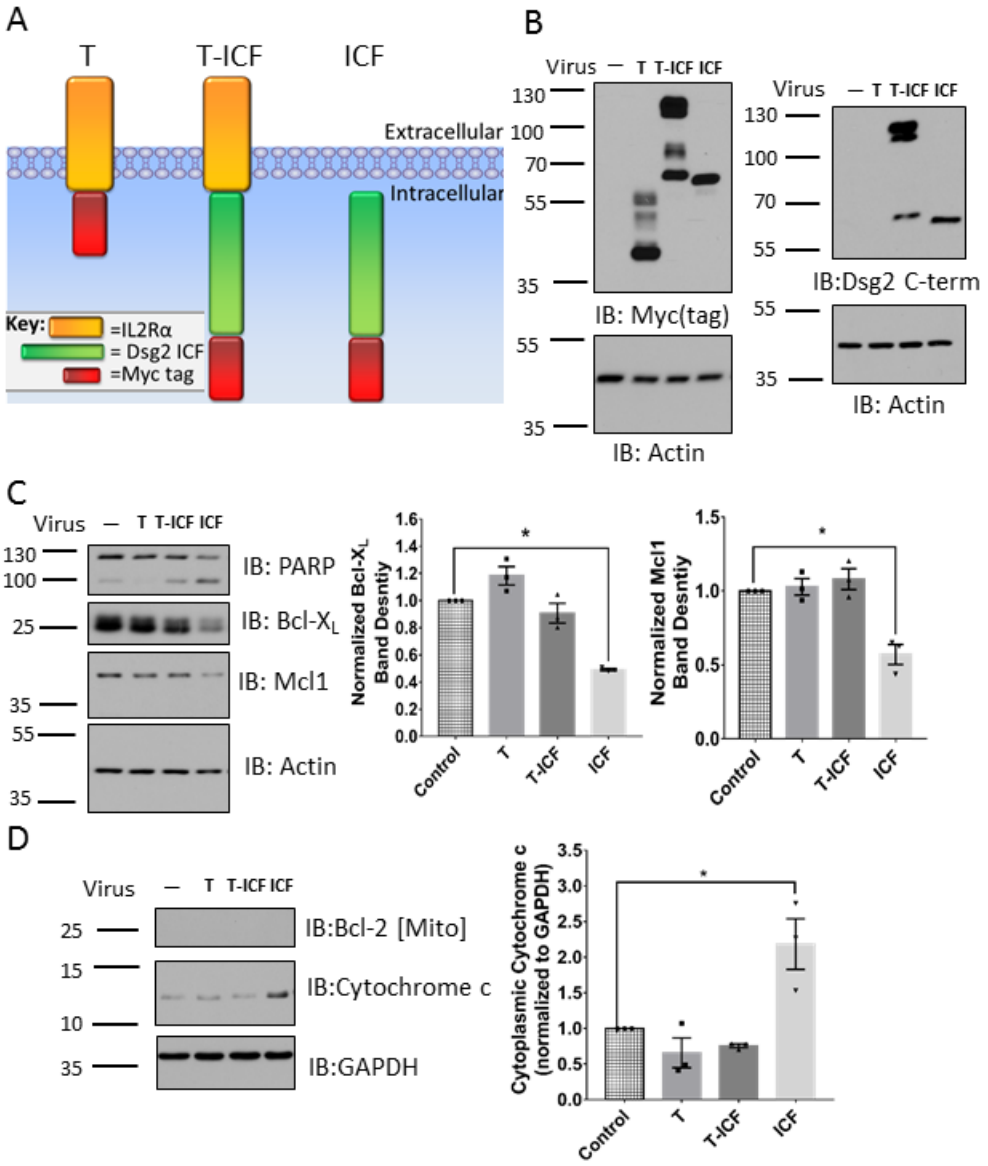

Figure 2.7: Exogenous expression of Myc-tagged Dsg2 ICF sensitizes cells to apoptosis through downregulation of anti-apoptotic Bcl-2 family members. A) Model of the protein products of the adenoviral expression constructs used in this paper. **B)** Western blot of cell lysates from SKCO15s expressing the indicated adenoviral expression constructs. *Left.* Western blot for the Myc tag. *Right.* Western blot using an antibody against Dsg2 C-term. **C)** *Left.* Western blot for PARP, Bcl-X_L, and Mcl1 of cell lysates from SKCO15s treated as in Figure 2.7B. *Right.* Densitometry of blots for Bcl-X_L and Mcl1. *, $p \leq 0.05$ n=3 per group. For Bcl-X_L p=0.0003 and for Mcl1 p=0.0017. **D)** *Left.* Western blot for cytochrome c, GAPDH, and Bcl-2 of cytoplasmic fractions from SKCO15s treated as in Figure 2.7B. *Right.* Densitometry of blots for cytochrome c normalized to GAPDH control. [For similar analysis of mitochondria containing fractions see **(Figure 2.11)**.] *, p=0.0093 n=3 per group**.** All blots are representative of at least three independent experiments.
Abbreviations: Mito, mitochondria; Cyto-c, cytochrome c.

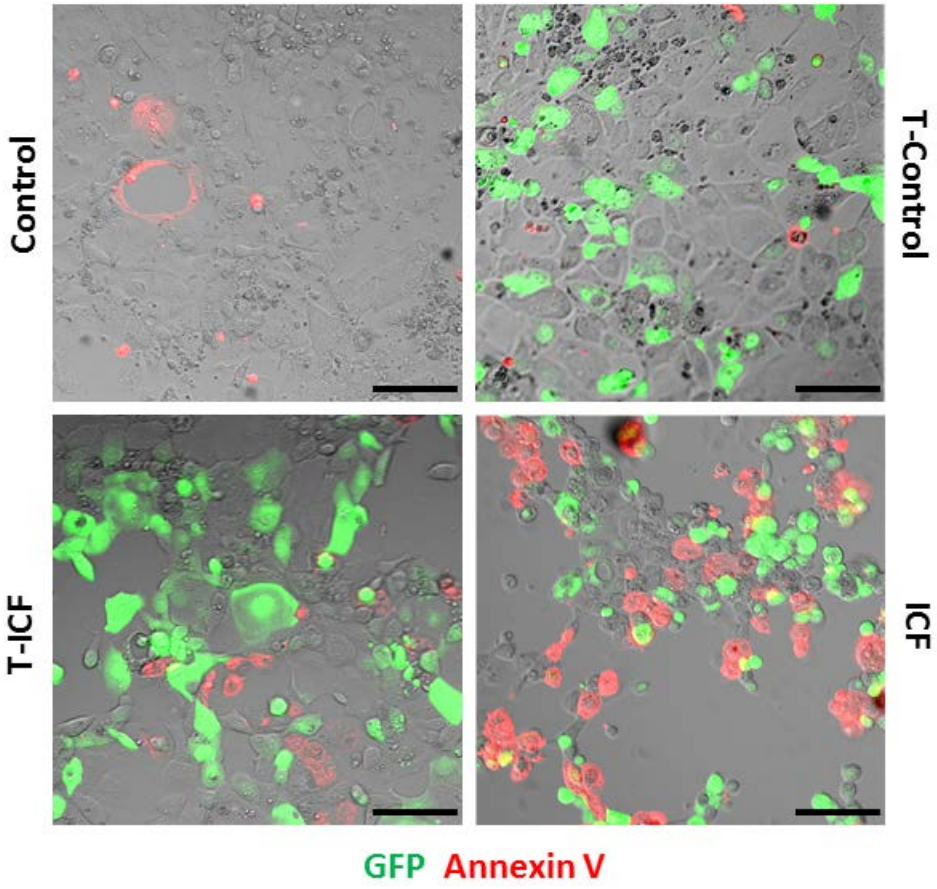

Figure 2.8: Annexin V staining also indicates increased apoptosis sensitivity in cells expressing Dsg2 ICF. Fluorescence and light microscopy merged images of SKCO15 cells treated as in Figure 2.7B and labeled with Alexa fluor 568 conjugated annexin V [red]. GFP is in green. All images are representative of at least three independent experiments. Scalebars=100 μm. Images are representative of at least three independent experiments.

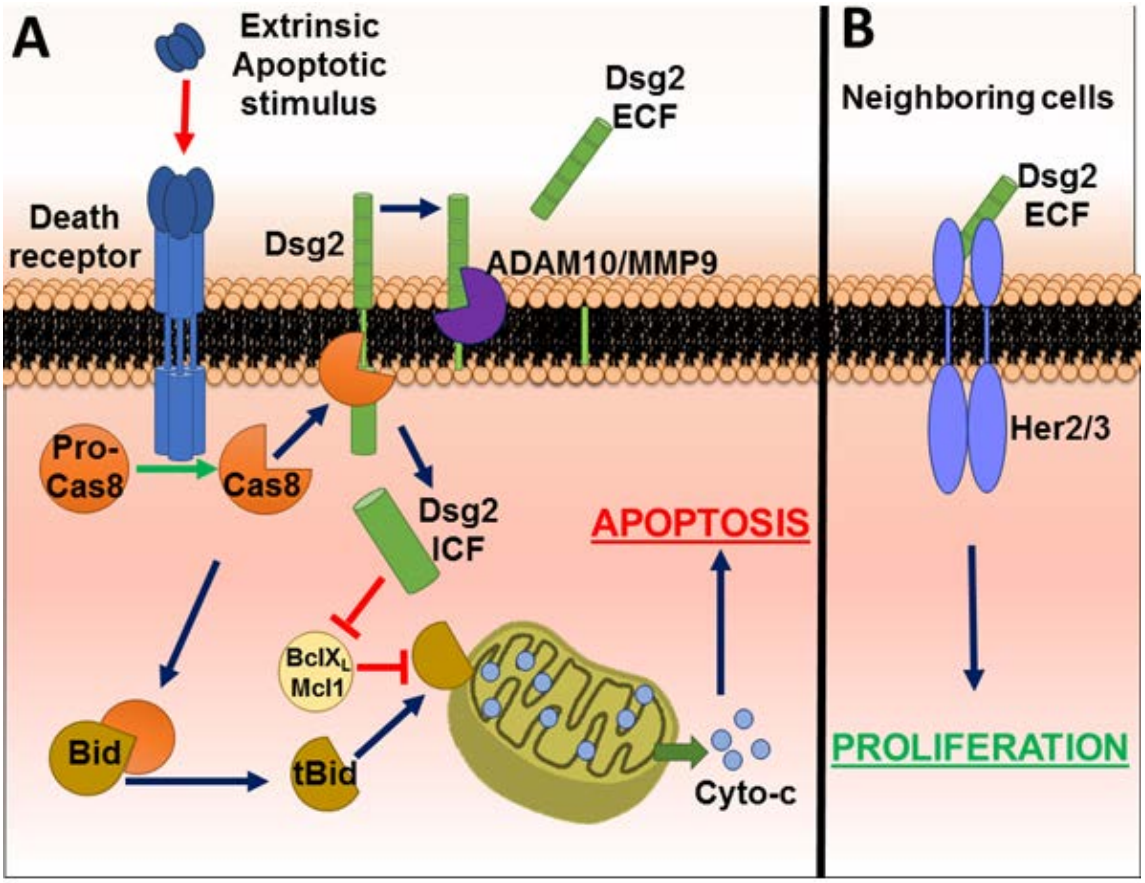

Figure 2.9: Dsg2 ICF generation sensitizes IECs to apoptosis. A) Stimulation of death receptors (such as TNFR1 or DR4/5) results in activation of caspase-8, which cleaves Dsg2 intracellularly generating the Dsg2 ICF as well as other substrates including the Bcl-2 family member Bid generating tBid. The presence of the Dsg2 ICF reduces Bcl-X_L and Mcl1 protein levels, thereby alleviating their inhibition of tBid and allowing for MOMP and the execution of apoptosis to proceed. Following Dsg2 ICF generation, MMP9 and ADAM10 cleave Dsg2 in the extracellular domain generating the Dsg2 ECF. **B)** We have previously shown that this Dsg2 ECF promotes wound closure by stimulating IEC proliferation through activation of Her2/3 signaling. We propose that the Dsg2 ICF and ECF work in concert to remove damaged IECs and promote wound closure to help ensure effective resolution of inflammation.
Abbreviations: Pro-Cas8, Pro-caspase-8; Cas8, caspase-8; Cyto-c, cytochrome-c.

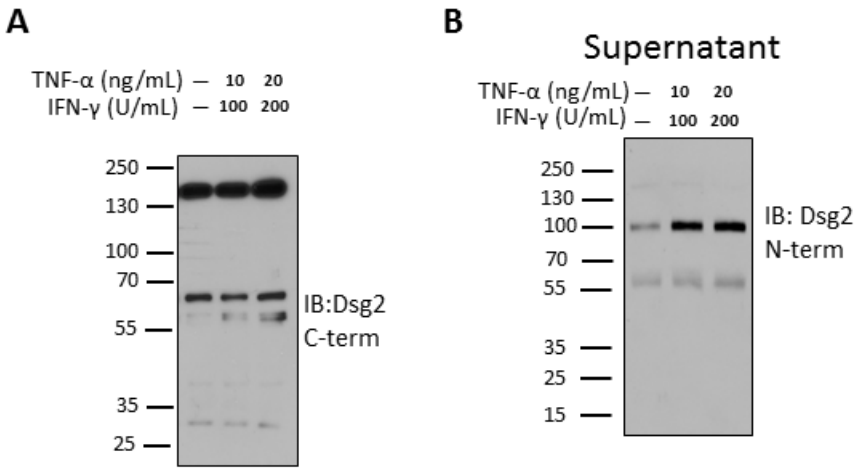

Figure 2.10: Full lanes of blots for C-terminal and N-terminal Dsg2. A) Full lanes of western blot using an antibody against Dsg2 C-term shown in Figure 2.2B. **B)** Full lanes of western blot using an antibody against Dsg2 N-term shown in Figure 2.2C. All blots are representative of at least three independent experiments.

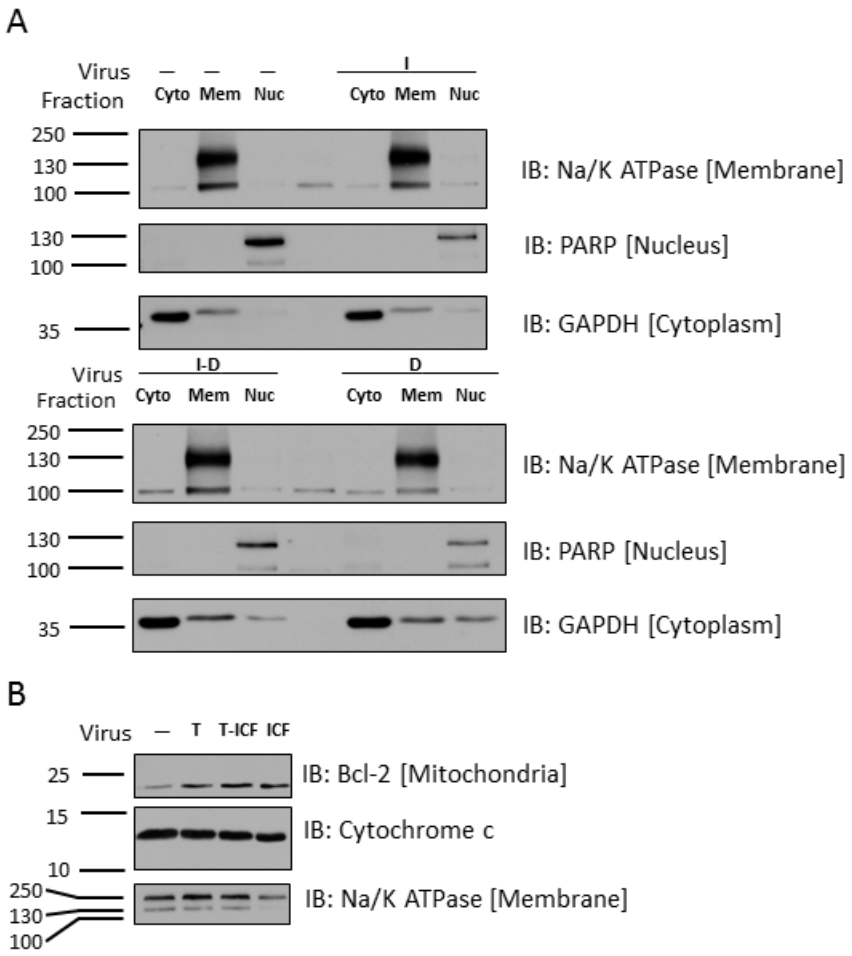

Figure 2.11: Fractionation controls for Figure 2.7D. A) *Top.* Western blot for Na/K ATPase, PARP, and GAPDH from indicated fractions of SKCO15 cells treated as in Figure 2.7B [Control and T virus transduced samples]. *Bottom.* Western blot for Na/K ATPase, PARP, and GAPDH from indicated fractions of SKCO15 cells treated as in Figure 2.7B [T-ICF and ICF virus transduced samples]. All blots are representative of at least three independent experiments. We have found that the mitochondria are enriched in the membrane fraction and are absent from the cytoplasmic fraction when using this fractionation kit (Milipore; Tamecula, CA, USA). Abbreviations: Cyto, cytoplasmic; Mem, membrane (mitochondria containing); Nuc, nuclear. **B)** Western blot for cytochrome c, Na/K ATPase, and Bcl-2 of mitochondria containing fractions from SKCO15s treated as in Figure 2.7B.

EXPERIMENTAL PROCEDURES:

Antibodies and Reagents:

The following primary monoclonal and polyclonal antibodies were used to detect proteins by immunoblot analysis: mouse anti-Dsg2 {extracellular} (clone AH12.2, generated in-house; (129,153) [1:1000], mouse anti-Dsg2 {intracellular} [1:750] (clone 4B2; a kind gift from Dr. K.J. Green, Northwestern University, Evanston, IL), mouse anti-beta-actin (clone AC-74) [1:5000-1:10000], rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [1:20000], mouse-anti-tubulin (clone DM1A [1:10000]; Sigma-Aldrich; St. Louis, MO, USA), rabbit anti-cleaved PARP (clone D64E10 XP(r)) [1:1000], rabbit anti-PARP [1:1000], rabbit anti-Bcl-X_L (clone 54H6) [1:2000], rabbit anti-cleaved Notch (clone D3B8) [1:1000], rabbit anti-cytochrome c (clone D18C7) [1:1000], mouse anti-caspase-8 (clone 1C12), mouse anti-Myc tag (clone 9B11; Cell Signaling Technology; Danvers, CT, USA) [1:5000], rabbit anti-Na/K ATPase [1:100000] (clone EP1845Y; Abcam; Cambridge, MA, USA), rabbit anti-Mcl1 [1:3000] (Enzo Life Sciences; Farmingdale, NY, USA), Armenian hamster anti-Bcl-2 [1:125] (BD Biosceicnes; San Jose, CA, USA), rabbit anti-bovine serum albumin (BSA) (clone B-140) [1:10000], rat anti-guanylate binding protein-1 (GBP-1 (clone 1B1) [1:750]; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Goat anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and used at a concentration of 1:10000. Mouse anti-armenian/Syrian hamster HRP conjugated secondary antibody cocktail (clones G70-204/G94-56) was purchased from BD Biosciences and used at a concentration of 1:1000. Recombinant human TNF-α, IFN-γ, and TRAIL were purchased from PeproTech (Rocky

Hill, NJ, USA). Z-VAD-fmk was purchased from Enzo Life Sciences, Calpeptin was purchased from EMD Millipore (Billerica, MA, USA), GI454023X was purchased from Sigma-Aldrich, Z-DEVD-fmk, Z-LEHD-fmk, and Z-IETD-fmk were all purchased from R&D Systems (Minneapolis, MN, USA).

Cell Culture:

T-84 and SKCO15 human model IEC were cultured both on Transwell™ permeable supports and on tissue culture treated plastic as previously described (154,155). Cos7 monkey model kidney fibroblast cells were cultured as described previously (156). For TNF-α+IFN-γ dose dependency experiments, cells were treated with media alone (control), 100 Units/mL of IFN-γ+10 ng/mL TNF-α, or 200 Units/mL of IFN-γ+20 ng/mL TNF-α. For time-course experiments, T-84 model IECs were treated with either 200 Units/mL of IFN-γ+20 ng/mL TNF-α or 100 ng/mL of TRAIL for 0 h, 6 h, 12 h, and 24 h. For transient plasmid transfection, Lipofectamine 3000 (Invitrogen; Carlsbad, CA, USA) was used according to manufacturer's instructions. Cell culture media was replaced 24 h after transfection with either media alone or media containing 200 Units/mL of IFN-γ+20 ng/mL TNF-α. Samples were collected for immunoblotting 24 h after application of cytokine or control treatment. For cells cultured on Transwell permeable supports with 3 μm pore size filters (Corning), transepilthelial electrical resistance was measured using an EVOM voltmeter with an ENDOHM-12 (World Precision Instruments; Sarasota, FL, USA). Electrical resistance was expressed as $\Omega x cm^2$. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

Inhibitor Treatment:

Confluent T-84 cells on Transwell permeable supports were treated with the following inhibitors 1 h before treatment with 200 Units/mL of IFN-γ+20 ng/mL TNF-α: 10 μM GI254023X, 50 μM Z-VAD-fmk, 20 μM/50 μM calpeptin, 10 μM DAPT,10 μM/30 μM/100 μM Z-DEVD-fmk, 10 μM/30 μM/100 μM Z-IETD-fmk, 10 μM/30 μM/100 μM Z-LEHD-fmk.

Immunoblotting:

For *in vitro* cell cultures, confluent monolayers were collected in RIPA (20 mM Tris-Base, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Sodium deoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.4) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cells were incubated at 4°C for 20 mins and centrifuged (8000xg at 4°C for 10 min). For cell culture supernatants, supernatants were cleared by centrifugation (5000xg 4°C for 5 min). Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; Marietta, OH, USA). NuPAGE LDS Sample Buffer (Life Technologies; Eugene, OR, USA) with a final concentration of 100 mM DTT (Sigma-Aldrich) was used. Samples were boiled for 5 mins at 100°C and loaded onto polyacrylamide gels (TNFα+IFN-γ or TRAIL lysate samples=30 μg protein/lane; adenoviral transduction lysates=10 μg protein/lane; cell culture supernatants=30 μL of sample/lane; cell fraction samples=22 μg protein/lane). After electrophoresis, the samples were transferred to a nitrocellulose membrane (Bio-Rad; Hercules, CA, USA) and probed with primary antibodies and HRP conjugated

secondary antibodies (Jackson ImmunoResearch Labs). Immunoblots were quantified using ImageJ (National Institutes of Health; Bethesda, MD, USA) or an Amersham 600 Blot Imaging System (GE Healthcare; Piscataway, NJ, USA). To calculate the amount of the Dsg2 ICF generated under each condition in Figures 1B and 1F, the density of bands representing Dsg2 cleavage products as well as the full length protein were collected and the ratio of the density of the Dsg2 ICF band to the density of all bands for a given lane was then calculated. For all densitometric analysis, each individual data point was derived from measurement of a distinct sample.

Adenovirus Transduction:

Fifteen minutes prior to adenoviral transduction, SKCO15 model IECs were calcium switched using calcium switch reagent (10 mM HEPES, 25 mM EGTA, pH 7.4). Following this, the Dsg2 ICF-Myc and IL2R-Dsg2 ICF-Myc viruses were added at an MOI of 1 and the IL2R-Myc virus was added at an MOI of 10 to ensure equal exogenous protein expression. Cells were centrifuged for 1 h at 1200xg at 37°C and then placed into a humidified incubator with 5% CO_2 at 37°C. Samples were used 48h following initial virus addition unless otherwise specified.

For western blot analysis, sample preparation was as described above.

Cell Fractionation:

To generate the cytoplasmic and mitochondria containing cellular fractions, we used the Compartmental Protein Extraction Kit (Millipore; Temecula, CA, USA) according to manufacturer's instructions.

Immunofluorescence labeling:

Confluent T-84 monolayers grown on Transwell permeable supports were fixed using 4% paraformaldehyde in PBS+ for 15 minutes. Samples were washed three times using PBS+ and then permebialized using 0.5% Triton X-100 in PBS+ for 10 minutes. Following washing, samples were blocked in 3% BSA in PBS+ for 30 minutes and labeled with 4B2 [1:100] in 3% BSA in PBS+ for 2 hours. The samples were then washed and incubated with goat anti-mouse secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen; [1:1000]) in 3% BSA in PBS+ for 1 hour, washed and mounted onto charged glass slides using Prolong gold (Life Technologies; Eugene, OR, USA). Images were collected on a Leica SP5 confocal microscope and processed using ImageJ.

Annexin V Fluorescent Imaging:

Alexa Fluor 568 conjugated Annexin V (Life Technologies) was used for fluorescent live-cell imaging according to manufacturer's instructions with minor modifications. Briefly, model SKCO15 cells were transduced with the Dsg2 ICF-Myc, IL2R-Dsg2 ICF-Myc, and IL2R-Myc adenoviral constructs as described above. Cells were washed in ice cold PBS+ three times followed by incubation with 10 μL of labeled Annexin V per 100 μL of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) for 15 minutes. Cells were washed three times using annexin-binding buffer and imaged using a Zeiss Axiovert 200M microscope. Images were exported as TIFFs and processed using ImageJ.

Statistics:

Statistical significance was calculated using either One-Way ANOVA followed by Dunnet's test for multiple comparisons or Two-Way ANOVA followed by Tukey's test for multiple comparisons dependent upon the experimental design. Results displayed as means ± SEM; $p<0.05$ was considered significant.

Chapter 3: Discussion

Here we described how the intracellular cleavage of the desmosomal cadherin desmoglein 2 (Dsg2) constitutes a mechanism by which extrinsic apoptotic stimuli can sensitize intestinal epithelial cells (IECs) to apoptosis. I characterized the extracellular and intracellular cleavage of Dsg2 in response to TNF-α and IFN-γ as well as TNF-α Related Apoptosis Inducing Ligand (TRAIL) stimulation. I showed that the generation of the Dsg2 intracellular fragment (ICF) is the first step in this mechanism, preceding both the execution of apoptosis itself and the generation of the Dsg2 extracellular fragment (ECF). I identified the intracellular cysteine aspartate protease caspase-8 as the protease responsible for generating the Dsg2 ICF. Using adenoviral expression vectors encoding a Myc-tagged Dsg2 ICF as well as other control constructs, I demonstrated that the presence of the Dsg2 ICF causes a noticeable repression of the steady state protein levels of the anti-apoptotic Bcl-2 family members Bcl-X_L and Mcl1. Preliminary data suggested that the Dsg2 ICF represses Mcl1 through modulation of its stability whereas it represses the mRNA and protein expression of Bcl-X_L through yet to be fully defined mechanisms (Yulis and Nusrat, unpublished data). Furthermore, I showed that the presence of the Dsg2 ICF results in increased apoptosis sensitivity and execution as well as directly measure increased MOMP and cytochrome c release in cells expressing free-floating Dsg2 ICF compared to controls. These studies add to a small but growing body of literature showing that distinct cadherin protein fragments can operate as important signaling mediators.

To date, only a few examples of cadherin protein fragment mediated signaling are described within the literature (50,89,90,116). Despite our incomplete understanding of these mechanisms, cadherin protein fragments have already been shown to regulate key

cellular functions including apoptosis (Chapter 2), proliferation (50,116), and neural crest cell delamination during development (90). Our studies reveal an important role for Dsg2 cleavage in the effective execution and resolution of inflammation.

A model for Dsg2 cleavage as a beneficial signaling mechanism during inflammation:

Our findings that the Dsg2 ICF is generated by the physiologically relevant inflammatory stimuli TNF-α and IFN-γ and sensitizes IECs to apoptosis suggest that the generation of the Dsg2 ICF may play a role in ensuring effective clearance of damaged cells following inflammatory insult. My mentor's laboratory has previously published that in response to TNF-α or IL-1β stimulation, Dsg2 undergoes extracellular cleavage that is mediated by ADAM 10 and MMP9 resulting in the release of a stable extracellular fragment of Dsg2, the Dsg2 ECF that has distinct paracrine signaling properties (116). Most notably, it enhances the activity of the Her2 and Her3 receptor signaling pathways to promote cell proliferation (116). In Chapter 2, we show that in response to both TNF-α and IFN-γ as well as TRAIL the Dsg2 ICF is generated well before the Dsg2 ECF, which accumulates following 24 hours of treatment. These studies lead to a model where these two Dsg2 cleavage events work together to enhance the clearance of damaged cells following an inflammatory insult and resolution of the inflammatory response (**Figure 2.9**). In this model, following exposure to pro-inflammatory cytokines including TNF-α and IFN-γ, IECs activate caspase-8 and cleave Dsg2 resulting in generation of the Dsg2 ICF. Through yet to be fully characterized mechanisms, this Dsg2 ICF represses the anti-apoptotic Bcl-2 family members Bcl-X_L and Mcl1 thereby promoting mitochondrial

outer membrane permeabilization (MOMP) and apoptosis. It is likely that Mcl1 is lost first since repression due to changes in protein stability often occur fairly quickly. However, if cells endure prolonged exposure to high levels of inflammatory stimuli and there is sustained generation of the Dsg2 ICF, the loss of Bcl-X_L mRNA and protein expression due to the presence of the Dsg2 ICF will synergize with the loss of Mcl1 to further stimulate apoptosis in these cells. This will help ensure effective elimination of cells that have been too damaged by the inflammatory response. This is likely to target certain cells that are more directly localized in the inflamed areas of the intestine, which would be closer to the threshold of apoptotic stimulation necessary to undergo MOMP. Following this, Dsg2 will then be extracellularly cleaved by ADAM 10 and MMP9 resulting in the release of the Dsg2 ECF into the extracellular milieu. The extracellular fragment stimulates proliferation of nearby healthy cells by binding to the Her2 and Her3 receptors and activating their downstream signaling including through the Akt, mTOR, and p38MAPK pathways (116). These events serve to promote healing of epithelial injury that is associated with excessive cell death. Thus, inhibition of Dsg2 cleavage in inflammatory states can have detrimental effects on elimination of dead cells and repair of the epithelial barrier.

Specific mouse genetic models will be required to best test this hypothesis. The most ideal experiment to address this would one in which CRISPR/Cas9 technology is used to create knock in mice replacing wild type Dsg2 with mutant Dsg2 incapable of generating the Dsg2 ICF, ECF, or both that would then be subjected to multiple colitis models. The severity of disease activity, proliferation of cells surrounding ulcers, degree of apoptosis or epithelial erosion, etc. would all be characterized in these models to

understand the roles that the Dsg2 ICF and ECF play throughout the course of colitis. Since we are unsure if Dsg2 ECF generation requires generation of the ICF first, it is possible that the mice expressing the Dsg2 ICF deficient mutant protein and the double mutant protein will have the same phenotype.

Dependency of Dsg2 ECF generation on Dsg2 ICF generation:

The mechanisms by which most cadherins are cleaved following apoptotic stimulation entail generation of an extracellular fragment which then facilitates the generation of an intracellular fragment (91,110-112,157,158). In several of these mechanisms, inhibition of the extracellular fragment inhibits generation of the intracellular fragment (110,112). We however, found that the Dsg2 ICF is generated prior to the generation of the Dsg2 ECF. While we showed that inhibition of the Dsg2 ICF using the pan-caspase inhibitor Z-VAD-fmk diminishes the presence of the Dsg2 ECF, whether or not the generation of the Dsg2 ECF is completely dependent upon Dsg2 ICF generation is unknown. This could be addressed by first generating knock-in cells replacing wild type Dsg2 with the caspase-8 cleavage site null mutant. These cells would then be treated with TNF-α and IFN-γ followed by analysis of Dsg2 ECF shedding into the cell culture media will address this question.

Mechanistic basis for Dsg2 ICF signaling:

In Chapter 2, we showed that cells expressing the non-tethered Dsg2 ICF display an increase in PARP cleavage indicating increased caspase-3 activation and apoptosis execution. The presence of the Dsg2 ICF also increases cytoplasmic cytochrome C,

indicating that the Dsg2 ICF participates in a mechanism that results in increased MOMP. We show that the presence of the Dsg2 ICF suppresses the steady state protein levels of the anti-apoptotic Bcl-2 family members Bcl-X_L and Mcl1, suggesting that this is the mechanism through which the Dsg2 ICF mediates increased MOMP. However, the molecular mechanisms by which the Dsg2 ICF itself suppresses Bcl-X_L and Mcl1 steady state protein levels have not been fully described.

The E-cadherin CTF2, which is similar in nature to the Dsg2 ICF, has been shown to participate in nuclear transcriptional regulation (89). Cytoplasmic binding of the E-cadherin CTF2 to p120 catenin facilitates its nuclear localization. Once in the nucleus, these proteins complex with the transcription factor Kaiso to enhance its transcriptional signaling program (89). In support of the Dsg2 ICF potentially participating in a similar mechanism, we have seen by qPCR that expression of the Dsg2 ICF results in a ~50% reduction in relative steady state mRNA levels for Bcl-X_L. These findings suggest that the presence of the Dsg2 ICF transcriptionally represses Bcl-X_L expression (Yulis and Nusrat, unpublished data).

In light of the data presented in Chapter 2 two major questions regarding how the Dsg2 ICF influences Bcl-X_L transcription remain: 1) Does the Dsg2 ICF localize to the nucleus, 2) what are the Dsg2 ICF binding partner(s) and how do they influence its signaling? The immunofluorescence labeling and confocal microscopy presented in Chapter 2 were inconclusive regarding the exact sub-cellular localization of the Dsg2 ICF. Additionally, previous attempts to perform immunofluorescence labeling in cells expressing the Myc-tagged Dsg2 ICF were inconclusive. Therefore, alternative approaches are needed to determine if the Dsg2 ICF localizes to the nucleus.

Biochemical cellular fractionation of cells expressing myc-tagged Dsg2 ICF would allow for determination of the cellular compartment(s) in which the fragment resides. Other potential methods include the proximity ligation assay, however this would only work if the Dsg2 ICF comes into close enough proximity to whichever nuclear protein is used as the second target in the PLA and would not show if the fragment localizes to other compartments. In order to address the question of the Dsg2 ICF's binding partner(s), a co-immunoprecipitation experiment followed by mass spectrometry proteomic analysis would allow for identification of proteins that form a stable complex with the Dsg2 ICF. Highly enriched proteins that are known to influence transcriptional regulation (i.e. transcription factors, p120, plakoglobin, β-catenin, etc.) would be validated. Following validation, specific siRNA mediated knock down of the putative binding partner in the context of Dsg2 ICF expression can be used to determine if it rescues the functional effects of the Dsg2 ICF. This could also be achieved through use of a similar strategy but instead utilizing BioID technology to have any protein that comes within ~10 nm of Dsg2 ICF tagged with the enzyme BirA to be covalently tagged with biotin. This would then be followed by an affinity precipitation using streptavidin coated beads and the samples would then be processed as described above for the proposed Co-IP mass spec experiment. A chromatin immunoprecipitation (Ch-IP) assay using an antibody against the Myc tag from cells expressing the non-tethered Dsg2 ICF followed by sequencing could determine if the Dsg2 ICF is part of a regulatory complex that directly interfaces with the Bcl2L1 gene. This is because, if the Dsg2 ICF were part of a stable protein complex that regulates Bcl2L1 gene expression through direct binding, this complex should co-immunoprecipitate and will remain bound to any DNA sequences it was bound

to (following crosslinking of the DNA to all bound proteins using formaldehyde or UV light). These bound DNA sequences can then be used to determine the DNA targets of any protein complexes containing the Dsg2 ICF. This method could also potentially identify other sequences that the Dsg2 ICF may bind to. This, of course, is assuming that the mechanism through which the Dsg2 ICF influences Bcl-X_L's transcription is similar in nature to the mechanism described for the E-cadherin CTF2.

There are other mechanisms through which the Dsg2 ICF could influence transcription including regulation of transcription factor expression and modulation of epigenetic mechanisms. Since in our experiments the functional effects of the Dsg2 ICF are assayed 48 hours post adenovirus transduction and Dsg2 ICF overexpression, it is not unreasonable to propose an epigenetic component to the mechanism of Dsg2 ICF signaling. A qPCR based array plate containing primers specific to many important transcription factors could be utilized to determine whether or not the Dsg2 ICF regulates transcription factor expression. As with the mass spectrometry experiment described above, the initial identification of such factors would be validated. Following validation, candidate proteins would be either knocked down or overexpressed in the context of Dsg2 ICF expression to assess their potential role in the functional effects of the Dsg2 ICF. Transcriptional regulation is often mediated by altering the patterns of various histone modifications (i.e. acetylation and/or histone mono-, di-, or tri-methylation, etc.) that can dramatically affect gene expression. Ch-IP Seq based assays utilizing antibodies against specific histone modifications could be used to determine if the Dsg2 ICF influences these modifications genome wide. Additionally, whole genome bisulfite sequencing using cells expressing the Dsg2 ICF would determine if the Dsg2 ICF

influences the placement or removal of repressive DNA methylation marks. Not only would these data potentially tell us a great deal about the mechanistic basis of Dsg2 ICF signaling, it could potentially identify additional signaling targets of the fragment to study further.

While our qPCR results suggested that the Dsg2 ICF regulates Bcl-X_L through transcriptional repression, they also showed that expression of the Dsg2 ICF results in an increase in the steady state levels of Mcl1 mRNA despite the reduction in protein levels shown in chapter 2 (Yulis and Nusrat, unpublished data). This indicates that the Dsg2 ICF regulates Mcl1 repression through a mechanistically distinct mechanism from the one it uses to regulate Bcl-X_L. Mcl1 is a notoriously labile protein that is rapidly degraded by the proteasome, suggesting that the Dsg2 ICF influences Mcl1 protein stability likely through regulation of its proteasomal degradation (159). This could be tested through the use of proteasome inhibitors. However, the use of proteasome inhibitors dramatically increased baseline Mcl1 levels, and made the analysis of the resulting data complicated (Yulis and Nusrat, unpublished data). Use of densitometric analysis only comparing samples that have received the same proteasome inhibitor treatment may be able to address the question of whether or not the Dsg2 ICF enhances the proteasomal degradation of Mcl1. Additionally, immunoprecipitating Mcl1 out of Dsg2 ICF expressing cells and performing a subsequent western blot for the levels of ubiquitination present on the precipitated protein could begin to address the role of the Dsg2 ICF in regulating the proteasomal degradation of MCl1.

Previously published evidence show that the Dsg2 binding partner plakoglobin (Pg) performs signaling functions similar to those displayed by the Dsg2 ICF (80,160).

However, when we assayed the apoptotic functional outputs of Dsg2 ICF expression with and without siRNA mediated Pg knock down, we observed that the loss of Pg increased the magnitude of PARP cleavage and Bcl-X_L repression instead of reversing them (Yulis and Nusrat, unpublished data). This suggests that plakoglobin (Pg) may suppress these pro-apoptotic effects of the Dsg2 ICF. This could involve direct binding of Pg to the Dsg2 ICF preventing it from participating in the mechanism it uses to suppress Bcl-X_L. Co-IP experiments in which antibodies against Pg were used to pull the Dsg2 ICF and vice versa would show whether or not there is appreciable binding between them. If Pg does stably bind to the Dsg2 ICF, whether or not this affects the Dsg2 ICF's functional effects could be addressed by mutagenesis studies. This could be achieved by expressing the Dsg2 ICF in cells that have a knock-in of a Pg mutant that lacks the residues required for Dsg2 binding. Alternatively, a mutant form of the Dsg2 ICF in which the residues required for binding to Pg were mutated could be expressed in WT cells and compared to cells expressing the WT Dsg2 ICF for their ability to elicit the functional effects described in Chapter 2.

The implications of cadherin intracellular fragments as independent signaling mediators:

We showed that the Dsg2 ICF, which is generated by caspase-8 following extrinsic apoptotic stimulation, acts as a signaling mediator independently of other stimuli (Chapter 2). The only two other cadherin intracellular fragments known to behave in a similar way are the E-cadherin and N-cadherin CTF2s (89,90,157,158). However, this does not mean that other cadherin intracellular fragments are not also

acting as important signaling mediators in other contexts. It has been previously described that caspases-3 and -7 can cleave Dsg1 generating a ~17 kDa intracellular fragment and shRNA knock down of Dsg1 desensitizes cells to apoptosis (91). Exogenous expression of the Dsg1 intracellular fragment in keratinocytes would more specifically address whether or not the caspase generated fragment itself is responsible for the apoptosis sensitization that is lost upon Dsg1 knock down. Similarly to Dsg1, Dsg3 has been shown to be cleaved intracellularly by caspase-3 resulting in an intracellular fragment (161). However, whether or not this fragment can mediate signaling on its own has not been directly addressed. The use of exogenous expression based studies similar to those proposed above would be able to answer this question. Previous studies have shown that VE-cadherin is cleaved intracellularly generating an intracellular fragment during the process of its degradation following internalization (162). The same study also showed that the accumulation of this fragment only occurred following lysosomal inhibition using chloroquine (162). This suggests that while VE-cadherin may be processed somewhat similarly to E-cadherin, N-cadherin, and Dsg2, the resulting fragment likely has little to no relevant signaling capabilities. Even though it is possible that the desmocollins may have signaling competent intracellular fragments, these studies await demonstration of stable intracellular fragment generation in response to stimulation of a physiologically relevant mechanism.

Additional functions of Dsg2 ICF signaling:

The Dsg2 ICF likely influences other aspects of homeostasis and epithelial cell function. In fact, preliminary data suggests that even in the absence of caspase activity

the expression of the Dsg2 ICF suppresses proliferation. We have shown that in the absence of caspase activity through treatment with Z-VAD-fmk, cells expressing the Dsg2 ICF display noticeably lower levels of Ki67 positivity, which is a marker for proliferation (Yulis and Nusrat, unpublished data). The transcription factor array and Co-IP mass spec proteomic analyses that were proposed earlier to study the general mechanisms by which the Dsg2 ICF mediates its signaling as well as an array to assay expression profiles of proteins that classically modulate cell proliferation will likely shed light onto the mechanisms at play here. Even though much of our work on the Dsg2 ICF has been focused on its modulation of intracellular signaling mechanisms, there is still a high likelihood that its presence influences Dsg2's adhesive functions as well. This leads to a model in which the presence of the Dsg2 ICF competes with junction associated Dsg2 for binding to key intracellular plaque proteins (i.e. plakoglobin) that are required for full desmosomal adhesive strength and thereby compromises cell-cell adhesion. The question of whether or not the Dsg2 ICF influences IEC intercellular adhesion can be addressed by performing either a dispase assay or a hanging drop assay. However, since the Dsg2 ICF's expression promotes cell death and loss of epithelial monolayer integrity, the hanging drop assay is a more viable method (Chapter 2). Another likely mechanism for the loss of desmosomal adhesion in the presence of the Dsg2 ICF would be internalization of Dsg2. To address this, a surface biotinylation assay followed by streptavidin affinity precipitation using cells expressing the Dsg2 ICF could be used. The samples would then be run on an immunoblot using the extracellular specific epitope mapped Dsg2 monoclonal antibody AH12.2 to compare the abundance of biotinylated

Dsg2 in Dsg2 ICF expressing samples to controls. This would determine whether or not the Dsg2 ICF's presence influences Dsg2 internalization.

The role of inflammation in Dsg2 cleavage:

Inflammation is a multifaceted process that involves complex signaling and activates multiple mechanisms. Many of these mechanisms can influence and synergize with one another. Since the extrinsic apoptotic stimuli used in Chapter 2 to generate the Dsg2 ICF (TNF-α and TRAIL) are both in some way involved in activating non-apoptotic inflammatory signaling pathways, it is arguably unknown whether or not non-apoptotic inflammatory signaling mechanisms may also be playing a role in generation of the Dsg2 ICF. This could be done by combining one of the extrinsic apoptotic stimuli used in Chapter 2 with inhibition of canonical inflammatory signal transduction. Since TRAIL is arguably less directly involved in the stimulation of inflammatory signaling processes than TNF-α, it is a more suitable choice for the stimulus. However, several reports have provided evidence that TRAIL treatment can result in the expression of multiple inflammatory cytokines and chemokines as well as activation of JNK, p38MAPK, and NF-κB (163-166). This suggests that TRAIL could potentially influence other canonical inflammatory signaling pathways. Additionally, these reports show that the TRAIL stimulated expression of these inflammatory cytokines and chemokines is inhibited or reversed by the inhibiting of the activation of p38MAPK, NF-κB, and/or JNK (163-165). Therefore, pre-treatment of model IECs with inhibitors to these three inflammatory signaling mediators followed by a time-course of TRAIL treatment should at least begin to address the potential role of canonical inflammatory signaling in the

generation of the Dsg2 ICF. It is of note that the activation of caspase-8 was found to be required for effective activation of JNK, p38MAPK, and NF-κB following TRAIL treatment (166). Since mutation of the putative caspase-8 cleavage site centered on aspartate 715 in human Dsg2 eliminates intracellular fragment generation (Chapter 2), this suggests that the Dsg2 ICF may also be generated before the activation of JNK, p38MAPK, and NF-κB by TRAIL.

Dsg2 cleavage during intrinsic apoptosis:

We have previously published that the Dsg2 ICF is also generated in response to intrinsic apoptotic stimulation using the topoisomerase II inhibitor camptothecin (129). Since caspase-8 is not involved in the execution of intrinsic apoptosis, the identity of the caspase responsible for generating the Dsg2 ICF under these conditions requires further investigation. A dose response study using selective caspase inhibitors similarly to the one presented in Chapter 2 would more definitively address this question. However, for this experiment the cells would be stimulated using camptothecin or another intrinsic apoptotic stimulus. Additionally, to avoid any off target effects of the selective caspase inhibitors, targeted knock down experiments could be performed to address this question. Cells transfected with siRNA targeting caspases-3, -8, and -9 could then be treated with an intrinsic apoptotic stimulus followed by a western blot for Dsg2 ICF generation will determine which caspase is responsible for generating the Dsg2 ICF under these conditions. Since the repression of Bcl-X_L and Mcl1 following MOMP should not have great physiological relevance, this experiment would primarily resolve the lingering

question of the identity of the protease that generates the Dsg2 ICF following intrinsic apoptotic stimulation.

Placing the Dsg2 ICF and its signaling functions into the broader context of cadherin mediated signaling:

We have used *in vitro* human model intestinal epithelial cell culture as a system to characterize a cadherin cleavage mechanism and determine the functional effects of the resulting intracellular fragment. *In vitro* cell culture allowed for interrogating the nature of the generation of the Dsg2 ICF, its signaling potential, and the outcomes of that signaling with relative ease and efficiency. This system permitted the use of various transfection or transduction techniques as well as use of inhibitors or stimuli to more directly probe the roles that each potential member of this mechanism played. Since these are cancer cells with different dynamics regarding pro- and anti-apoptotic Bcl-2 family member expression, the degree of apoptosis stimulation seen here may differ from that seen in primary cells. However, this does not at all diminish the fact that in either cancer or primary cells, repression of anti-apoptotic Bcl-2 family proteins by the Dsg2 ICF will sensitize these cells to apoptosis. The work presented here lays the foundation for follow-up studies to more fully characterize the breadth of signaling that the Dsg2 ICF participates in as well as provides valuable precedent for studies into similar signaling mechanisms involving other cadherins.

Our current understanding of cadherin mediated signaling is focused on specific groupings or generally accepted fields of inquiry (i.e. the classical cadherins E-cadherin and VE-cadherin in the regulation of apoptosis and/or proliferation; the desmosomal

cadherins in the proper differentiation of the skin, etc.). For example, there is a large body of work that addresses signaling functions of desmoglein-3 (Dsg3) and desmoglein-1 (Dsg1) specifically in the context of the autoimmune diseases pemphigus vulgaris and pemphigus foliaceus respectively (77-79). Even so, whether or not these effects are even specifically mediated by Dsg3 or Dsg1 is still not well established (73,75,76). However, a number of studies have provided support for the participation of different cadherins in different functional contexts (64,65,116,129). This suggests that we have only begun to explore the breadth of cadherin mediated signaling.

Additionally, the majority of studies that have analyzed cadherin mediated signaling focus mostly on knock-down or knock-out of the cadherin in question or interference with its adhesive functions resulting in perturbation of the signaling mechanism being studied. More often than not, these studies either do not definitively define the nature of the cadherin's role in the mechanism or describe mechanisms in which the cadherin acts as an important scaffold at the membrane and in their respective junction. Several examples exist of other mechanisms for cadherin mediated signaling, predominantly through the generation of stable protein fragments through regulated cleavage. However, most of these involve extracellular cadherin protein fragments (50,59,69,116). The few instances of cadherin intracellular fragments directly modulating cellular signaling pathways have been described in complex experimental systems and involved the classical cadherins N-cadherin and E-cadherin (89,90).

Here we characterized the generation of a Dsg2 intracellular fragment in the context of intestinal inflammation and demonstrate that the presence of this fragment alone is sufficient to directly influence apoptotic regulatory pathways (**Figure 2.9**). This

mechanism is fairly unique within the literature in terms of the identity of the cadherin involved, the organismal context of the system interrogated, the nature of the signaling mechanism itself, and the type of cellular function being regulated. We thereby lay the groundwork for a wider range of possibilities for future research into cadherin mediated signaling.

Final conclusions:

These studies provide a new mechanism by which intestinal epithelial homeostasis may be altered in the context of inflammatory pathobiology as well as highlight the diversity of cadherin mediated signaling. The disruption of enterocyte homeostasis, especially apoptosis, is a clear contributor to the overall pathobiology of chronic inflammatory disorders of the intestine such as inflammatory bowel disease. Despite this, mechanisms by which intestinal epithelial homeostasis is altered under inflammatory conditions remains incompletely understood. Our studies show that the Dsg2 intracellular fragment acts as a signaling mediator to sensitize cells to apoptosis and is generated in response to pro-inflammatory cytokines. In light of the previously described pro-proliferative roles of the Dsg2 extracellular fragment, these two mechanisms likely cooperate in the inflammatory response and in repair after injury. Thus, the generation of Dsg2 protein fragments in response to inflammatory signaling may play a critical role in the inflammatory response.

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