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Desmosome dynamics: regulation via membrane rafts and dysregulation in pemphigus vulgaris

By

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By

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B.S., Clarkson University, 2008

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Abstract

Desmosome dynamics: regulation via membrane rafts and dysregulation in pemphigus vulgaris

By: Sara N. Stahley

Desmosomes provide adhesive strength to tissues such as the skin and heart by anchoring desmosomal cadherins of neighboring cells to the intermediate filament cytoskeleton. Compromised desmosome function results in human diseases, such as the autoimmune skin blistering disease pemphigus vulgaris (PV). In PV, autoantibodies (IgG) are directed against the desmosomal cadherin desmoglein 3 (Dsg3), resulting in the loss of desmosome-mediated cell-cell adhesion. We previously demonstrated that PV IgG induce membrane raft-mediated Dsg3 endocytosis. We hypothesized that raft microdomains play a broader role by regulating the dynamics of both desmosome assembly and disassembly. In human keratinocytes, Dsg3 was found to be raft associated biochemically and colocalized with raft markers at cell borders by structured illumination microscopy (SIM), a form of super-resolution. Raft disruption prevented desmosome assembly and adhesion, indicating a functional link between rafts and desmosomes. Furthermore, PV IgG-induced desmosome disassembly occurred by a redistribution of Dsg3 into raft-containing endocytic linear arrays, resulting in cholesterol-dependent loss of adhesion. Thus, rafts are critical for both desmosome assembly and disassembly.

Multiple PV pathomechanisms have been proposed based largely on *in vitro* studies. In order to better understand how PV IgG alters desmosome morphology and function *in vivo*, patient tissue was analyzed by SIM. Consistent with previous *in vitro* studies, we observed the following: aberrant clustering of desmosomal proteins, patient IgG colocalization with markers of rafts and endosomes, decreased Dsg3 levels and smaller desmosomes. Additionally, split desmosomes were detected in patient tissue, a finding not previously observed in cell culture models of PV. Desmosome splitting could be recapitulated in vitro by exposing cultured keratinocytes to both PV IgG and mechanical stress, demonstrating that desmosome splitting in patients is due to compromised desmosomal adhesion. These findings indicate that Dsg3 clustering and endocytosis are associated with reduced desmosome size and adhesion defects in PV patients. Defining the pathogenic alterations in PV patients provides a foundation for future *in vitro* studies investigating PV pathomechanisms and targeted therapeutics. Collectively, this dissertation demonstrates that raft membrane microdomains modulate the dynamics of desmosome assembly and disassembly, and that altered desmosome dynamics in PV result in the loss of desmosomal adhesion.

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Chapter 1

Dissertation Overview

Cell junctions play a fundamental role in cell biology, particularly in cell communication, cell movement, tissue organization and cell adhesion. The desmosome is an anchoring junction responsible for mediating strong adhesion between neighboring cells. Desmosomal adhesion provides tissues such as the skin and heart with the ability to resist mechanical stress. An introduction to desmosome structure and function is discussed in Chapter 2. The importance of desmosome-mediated adhesion is evidenced by the numerous human diseases of tissue fragility that result when desmosome function is compromised. Chapter 3 highlights diseases of compromised desmosomal adhesion, both inherited and acquired. Understanding the underlying cell biology of desmosomal diseases will contribute to the development of improved therapy options for desmosome-targeted diseases, and deepen our understanding of basic desmosome biology. One disease of particular relevance to this thesis is pemphigus vulgaris (PV), an autoimmune blistering disease in which patients produce autoantibodies (IgG) against the desmosomal cadherin desmoglein 3 (Dsg3), resulting in the loss of cell adhesion. The current understanding of pemphigus pathomechanisms is reviewed in Chapter 4. A host of studies, including the work presented in this thesis, indicate that pemphigus is ultimately a disease of altered desmosome dynamics.

In vitro, cultured keratinocytes can be treated with purified PV patient IgG to study the mechanisms by which PV IgG results in the loss of cell adhesion. Using this approach, the Kowalczyk lab previously identified distinct morphological changes and cellular responses to PV IgG, including Dsg3 clustering, endocytosis and endocytic linear array formation. PV IgG-induced Dsg3 endocytosis was found to occur through a lipid raft-mediated pathway, although the full implications of Dsg3 raft association in

desmosomal adhesion was unclear. We hypothesized that both desmosome assembly and PV IgG-induced disassembly were membrane-raft dependent. Dsg3 raft association was investigated using biochemical Triton X-100 insolubility and sucrose buoyancy criteria, super-resolution immunofluorescence microscopy and raft-perturbation. The results of this study indicating that desmosome assembly and disassembly are raft-dependent are presented in Chapter 5.

PV IgG has proven useful as a tool to disrupt desmosomes and thereby study desmosomal regulation. However, very little is known about the desmosomal alterations that occur in PV patients. Therefore, PV patient biopsies were analyzed using super-resolution immunofluorescence, and this analysis is presented in Chapter 6. Similar to previous *in vitro* studies, we observed desmosomal alterations including Dsg3 clustering, endocytosis, linear array formation and smaller desmosomes. Additionally, split desmosomes were also observed in patient tissue, a finding not previously reported *in vitro*. Desmosome splitting could be recapitulated in cultured keratinocytes upon exposing PV IgG-treated cells to mechanical stress, revealing that the application of mechanical force causes PV IgG-weakened desmosomes to split. These findings strongly indicate that Dsg3 clustering and endocytosis are associated with reduced desmosome size and adhesion defects in PV patient tissue.

A summary and discussion of future directions founded upon the results presented in this dissertation are detailed in Chapter 7. The overall objective of this dissertation was to investigate the significance of desmosomal raft association and define the desmosome alterations in PV patient tissue. Collectively, this work reveals that lipid raft membrane microdomains modulate the dynamics of desmosome assembly and disassembly, and that

these dynamics are altered in PV, resulting in the loss of desmosomal adhesion. Further, studies in this dissertation demonstrate that super-resolution light microscopy is capable of visualizing individual desmosomes as well as their morphology in patient tissue, which may prove useful for investigating other diseases in which adhesion structures are targeted. Super-resolution imaging will also be pivotal for future studies investigating desmosome- and raft-targeting mechanisms of desmosomal proteins, as well as, defining the structure and molecular composition of linear arrays. Importantly, the patient analysis presented here validates that PV pathomechanisms are accurately modeled *in vitro* through the use of cultured keratinoctyes and purified patient IgG. This finding has great import for future *in vitro* studies of PV pathomechanisms and for exploring better treatment options for patients of pemphigus and related bullous diseases. The work presented in this dissertation provides new insights into the fundamental cellular mechanisms that regulate desmosome dynamics and reveals how these processes are disrupted in the context of a devastating epidermal blistering disease.

Chapter 2

Introduction

2.1 The epidermis

Skin is essential for life and functions as our primary interface with the world. The skin is a protective barrier that protects us from infections, UV radiation and dehydration, but also plays important roles in thermoregulation, touch perception and wound healing. The skin is comprised of two major compartments, the dermis and the epidermis. The dermis provides support and nutrients to the epidermis, which is avascular and forms a barrier that is capable of resisting mechanical stress. The epidermis is a stratified squamous epithelium consisting of keratinocytes, melanocytes, Langerhans cells and Merkel cells. Keratinocytes are the most abundant cell type, making up 90-95% of the epidermis, and are organized into four major layers: the basal layer, the spinous layer, the granular layer and the stratum corneum (Figure 1). The basal layer contains a population of stem cells that allow the epidermis to be continually replenished with a turnover rate of about 28 days. Over a period of 14 days, keratinocytes differentiate as they transit through the spinous and granular layers. Another 14 days is spent within the stratum corneum after living keratinocytes undergo cornification into non-living corneocytes. This layer of corneocytes is most critical for providing the functional air-liquid barrier (Simpson *et al.*, 2011).

Cell-cell junctions are vital for a properly functioning epidermis, as evidenced by the many human disease states in which cell junctions are impaired (Ferone *et al.*, 2015; Ishida-Yamamoto and Igawa, 2014; Kobielak and Boddupally, 2014; Lai-Cheong *et al.*, 2007; Wei and Huang, 2013). Within the epidermis there are four types of intercellular junctions. Gap junctions are communicating junctions that allow for the passage of ions and small molecules between neighboring cells and are mostly located in the spinous

layer (Simpson *et al.*, 2011). Tight junctions are located in the upper regions of the granular layer and have recently been shown to contribute to the epidermal barrier by preventing trans-epidermal water loss through mechanisms that are not fully understood (Simpson *et al.*, 2011). Located in all three living layers of the epidermis are adherens junctions and desmosomes. Adherens junctions and desmosomes adhere neighboring cells through cadherin-based extracellular adhesive interactions and linkage to the actin and keratin intermediate filament cytoskeleton, respectively (Simpson *et al.*, 2011). Both desmosome size and number increase as cells differentiate through the epidermis. Desmosomes are particularly important for mediating strong intercellular adhesion and providing the epidermis with the ability to resist mechanical stress (Kowalczyk and Green, 2013). Desmosomes are the central focus of this dissertation, and their role in basic epidermal biology and disease will be discussed in detail in subsequent sections.

2.2 Desmosome ultrastructure and function

Since their first description as 'intercellular bridges' by Giulio Bizzozero in 1864 (Bizzozero, 1864; Calkins and Setzer, 2007; Mazzarello *et al.*, 2001), desmosomes have become recognized as sites of strong cell-cell adhesion (Berika and Garrod, 2014). Stemming from the Greek words *desmos,* meaning bond or to bind, and *soma* meaning body, the desmosome is a highly ordered and specialized 'spot-weld' of intercellular adhesion (Calkins and Setzer, 2007). Desmosomes tether adjacent cells through extracellular adhesive interactions and intracellular linkages to the intermediate filament cytoskeleton. When viewed by electron microscopy, desmosomes exhibit parallel, electron-dense plaques, one from each opposing cell, physically joined in the intracellular

space (Figure 2A) (Farquhar and Palade, 1963; Kelly, 1966; Odland, 1958; Overton, 1962). This electron dense appearance is due to extensive clustering and tight packing of the desmosomal components. Remarkably, desmosomes are also uniform in size, roughly 0.2-0.5 μm in diameter, depending on the tissue. Thus, desmosomes represent a unique membrane microdomain that is symmetrical and highly ordered.

The desmosome is comprised of proteins from three major families and is organized into three morphologically distinguishable regions (Figure 2B). The extracellular core region (ECR) consists of the extracellular space between adherent cells in which the desmosomal cadherins, desmoglein and desmocollin, engage in calcium-dependent interactions, physically adhering neighboring cells. The intracellular portion of the desmosome is commonly referred to as the 'desmosomal plaque' and is further divided into two regions, the plasma membrane proximal outer dense plaque (ODP) and the inner dense plaque (IDP). In the ODP, the cytoplasmic tails of the cadherins are linked to armadillo proteins plakoglobin and plakophilin, and the amino-terminus of the plakin family protein desmoplakin (Chen *et al.*, 2002; Chitaev *et al.*, 1996; Kami *et al.*, 2009). The precise organization and protein-protein interactions within the plaque, in particular the ODP, remain unknown. Within the IDP, the carboxyl-terminus of desmoplakin binds to keratin filaments, thereby tethering the intermediate filament cytoskeleton to the desmosomal adhesion complex (Choi *et al.*, 2002; Green *et al.*, 1992; Jones and Goldman, 1985; Stappenbeck *et al.*, 1993).

By mechanically anchoring neighboring cells to one another, the desmosome provides tissues with the ability to resist mechanical forces (Kowalczyk and Green, 2013). Desmosomes are prominent in epithelial and cardiac tissues, both of which

experience a high degree of mechanical stress (Berika and Garrod, 2014; Desai *et al.*, 2009). Though the desmosome must be rigid and sturdy enough to provide the strong adhesion necessary for tissue integrity, the desmosome is also thought to be a dynamic complex that undergoes remodeling during both normal homeostasis and cellular processes such as development, differentiation and wound healing, as well as disease (Kitajima, 2013, 2014; Nekrasova and Green, 2013). As discussed in Chapter 3, disruption of desmosome function causes numerous human diseases that are often associated with tissue fragility. The fact that desmosomal diseases often present with severe tissue fragility reinforces the notion that desmosomes provide strong adhesion and mechanical integrity to tissues.

2.3 Molecular components and organization of the desmosome

2.3.1 Desmosomal cadherins

The desmosomal cadherins, desmoglein and desmocollin, are type-1 transmembrane proteins that are members of the cadherin superfamily of cell adhesion molecules. Cadherins contain extracellular cadherin repeat domains that rigidify upon calcium binding in order to mediate cell-cell adhesive interactions. There are four desmogleins (Dsg 1-4) and three desmocollins (Dsc 1-3) (Figure 3A). Both desmosomal cadherins have four extracellular cadherin (EC1-4) repeats, with the EC1-2 domains thought to be primarily responsible for engaging in cis (on the same cell) and trans (on opposing cells) interactions to drive junction assembly and adhesion. The EC repeats are followed by an extracellular anchor (EA), a transmembrane (TM) domain and an intracellular anchor (IA) (Saito *et al.*, 2012b).

The desmogleins contain an intracellular cadherin-like sequence (ICS) domain which is responsible for binding plakoglobin, as well as, an extended tail comprised of an intracellular proline-rich linker (IPL), repeat unit domains (RUD) and a desmoglein terminal domain (DTD) (Saito *et al.*, 2012b). Evolutionarily and structurally, the desmocollins are more closely related to the classical cadherins, such as E-cadherin (Kljuic *et al.*, 2004). Alternative splicing results in a Dsc 'a' isoform that contains the ICS domain where plakoglobin binds and a shorter 'b' isoform which lacks the plakoglobin binding site (Cheng *et al.*, 2004; Collins *et al.*, 1991; Troyanovsky *et al.*, 1993; Witcher *et al.*, 1996). Unlike classical cadherins which engage in homophilic adhesive interactions, studies indicate that the desmosomal cadherins are capable of mediating both homo- and heterophilic interactions. Heterophilic interactions between Dsg and Dsc seem to be critical for desmosome formation and function (Green and Simpson, 2007; Thomason *et al.*, 2010). *In vitro*, the presence of both Dsg and Dsc is required to mediate cell-cell adhesion (Getsios *et al.*, 2004a; Kowalczyk *et al.*, 1996; Marcozzi *et al.*, 1998). Further, EC1-2 peptides of both Dsg1 and Dsc1 are required to prevent desmosomal adhesion in epithelial cells (Runswick *et al.*, 2001; Tselepis *et al.*, 1998). Binding partners of the cytoplasmic tail of the desmosomal cadherins make up the desmosomal plaque and will be discussed next.

2.3.2 Plakoglobin

Plakoglobin (PG) is a founding member of the armadillo family of proteins that are characterized by a central domain constructed of tandem 'armadillo' motifs. Armadillo was originally discovered in *Drosophila* and is the homolog of vertebrae

proteins β-catenin and PG (Peifer *et al.*, 1992). Armadillo domains tend to mediate protein-protein interactions. While PG is capable of binding to E-cadherin and localizes to the adherens junction, PG has a much greater affinity for binding to the desmosomal cadherins (Chitaev *et al.*, 1996). In the desmosome, PG serves as a bridge between the cytoplasmic tails of the cadherins and the intermediate filament binding protein desmoplakin (discussed in 2.3.4). PG contains 12 central arm repeat domains (similar to β-catenin) that are flanked by amino-terminal head and carboxyl-terminal tail domains (Figure 3B). Mutagenesis studies suggest that multiple arm repeats near the amino-terminus and the carboxyl-terminus are important for binding to the desmosomal cadherins (Chitaev *et al.*, 1996; Choi *et al.*, 2009; Wahl *et al.*, 1996; Witcher *et al.*, 1996). The central armadillo domain of PG has also been shown to bind to desmoplakin (Bornslaeger *et al.*, 2001; Kowalczyk *et al.*, 1997; Smith and Fuchs, 1998), thereby serving as bridge between the desmosomal cadherins and the keratin filament binding protein desmoplakin.

2.3.3 Plakophilins

The plakophilins (PKP1-3) are also members of the armadillo protein family, but are more closely related to the adherens junction protein p-120-catenin rather than PG (Hatzfeld, 2007). Structurally, the PKPs differ from PG in that they contain only nine arm repeat domains and harbor an insertion between repeats five and six which introduces a bend in the overall protein structure (Figure 3B) (Bass-Zubek *et al.*, 2009). Additionally, PKP1 and PKP2 are alternatively spliced resulting in a short ‘a’ isoform and a longer ‘b’ isoform (Bass-Zubek *et al.*, 2009). Interestingly, PKP1 and PKP2 localize to the nucleus

and the PKPs have been shown to bind to mRNA translational machinery (Bass-Zubek *et al.*, 2009; Fischer-Keso *et al.*, 2014; Klymkowsky, 1999; Wolf and Hatzfeld, 2010; Wolf *et al.*, 2010). While most armadillo proteins interact with others proteins via their arm repeat domains, all binding partners of the PKPs associate with the PKP amino-terminal head domain (Bass-Zubek *et al.*, 2009). The PKPs have been shown to bind to a wide variety of proteins including Dsg1, Dsg2, Dsg3, Dsc1a, Dsc2a, Dsc3, PG, desmoplakin, keratin and actin (Hatzfeld, 2007; Hatzfeld *et al.*, 2000). Though, binding of the PKP1 head domain to desmoplakin is particularly robust and is required to recruit desmoplakin to desmosomes (Kowalczyk *et al.*, 1999). Together with desmoplakin, the PKPs facilitate clustering and drive lateral interactions between the desmosomal cadherin complexes to reinforce the desmosomal plaque and strengthen desmosomal adhesion (Bornslaeger *et al.*, 2001; Kowalczyk *et al.*, 1999).

2.3.4 Desmoplakin

Desmoplakin (DP) is the most abundant desmosomal plaque protein and is an obligate component of the desmosome (Bornslaeger *et al.*, 1996; Delva *et al.*, 2009; Gallicano *et al.*, 1998; Kowalczyk and Green, 2013). DP is a member of the plakin family of proteins, large molecules that link the cytoskeleton to the plasma membrane and mediate associations between microtubules, actin and intermediate filament networks (Bouameur *et al.*, 2014). In keratinocytes, DP provides the crucial link between the keratin intermediate filament cytoskeleton and the desmosomal cadherins through interactions with PG and PKP (Delva *et al.*, 2009). Structurally, DP consists of an amino-terminal globular head domain, a coiled-coil rod domain and a carboxyl-terminal tail that

contains plakin repeats A-C, along with a glycine-serine-arginine (GSR) rich region (Figure 3C) (Choi *et al.*, 2002; Green *et al.*, 1999). DP is alternatively spliced within the rod domain resulting in a longer DPI isoform and a shorter DPII isoform (Green *et al.*, 1992). The plakin head domain is responsible for binding to PG and PKP, while the carboxyl-terminal tail domain binds to intermediate filaments (Kowalczyk and Green, 2013).

2.3.5 Other desmosomal proteins

While the previously discussed proteins are well characterized as major desmosomal components, other proteins are also known be associated with the desmosome. Perhaps the most notable is the recently discovered desmosomal protein Perp, a target of p53/63 transcriptional regulation and tetraspan membrane protein that localizes to desmosomes. Perp plays a role in desmosome assembly and is essential for epithelial integrity, as the loss of Perp leads to severe skin fragility in mice (Ihrie and Attardi, 2005; Ihrie *et al.*, 2005). Members of the plakin family, such as envoplakin and periplakin, contribute to the development of the cornified envelope as components of corneodesmosomes (Kalinin *et al.*, 2004; Ruhrberg *et al.*, 1997; Sevilla *et al.*, 2007). Another component of corneodesmosomes is corneodesmosoin, a glycoprotein primarily expressed in the upper epidermal layers and hair follicles, which contributes to keratinocyte adhesion in the stratum corneum (Jonca *et al.*, 2002; Jonca *et al.*, 2011). The periplakin-binding protein kazrin is an adaptor protein and RhoA regulator (Groot *et al.*, 2004; Sevilla *et al.*, 2008), while pinin is a known keratin-binding protein (Shi and

Sugrue, 2000). Additional desmosomal proteins include desmocalmin and kertocalmin (Fairley *et al.*, 1991; Tsukita and Tsukita, 1985).

2.4 Tissue distribution of desmosomal proteins

Desmosomal proteins have complex expression patterns that are tissue-specific and have been shown to be important for driving epithelial patterning and differentiation. The choreographed expression profile within the epidermis is a prime example (Figure 4). DP and PG, along with Dsg2 and Dsc2, are expressed in all desmosome-bearing tissues, including simple and stratified epithelia, as well as myocardium. All seven desmosomal cadherins are expressed in the epidermis, but are differentially expressed as keratinocytes undergo terminal differentiation while transiting to the granular layer (Holthofer *et al.*, 2007; Kottke *et al.*, 2006). Dsg2 and Dsg3 are expressed in the lower layers of the epidermis, while Dsg1 is predominantly expressed in the upper granular layers. Dsg4 is found within the granular layer but is primarily expressed in the hair follicle. Dsc2 and Dsc3 are expressed in the basal and spinous layers, while Dsc1 is restricted to the upper layers. These patterns of expression are critical for tissue organization and homeostasis as misexpression leads to a variety of proliferative, epidermal patterning and adhesion defects (Brennan *et al.*, 2007; Hardman *et al.*, 2005; Merritt *et al.*, 2002).

Similar to the desmosomal cadherins, the PKPs also display tissue and differentiation specific patterns of expression. PKP1 is expressed in the suprabasal layers of stratified epithelia, primarily the upper layers, and is important for tissue integrity of the upper epidermis, while PKP3 is expressed uniformly throughout the epidermis. PKP3

is also found in simple epithelia and has been shown to have important roles in the hair follicle (Bass-Zubek *et al.*, 2009). PKP2 is the most widely expressed plakophilin. PKP2 is found in the lower layers of stratified epithelia, as well as non-epithelial tissue such as myocardium and lymph nodes. PKP2 is required for normal cardiac function (Bass-Zubek *et al.*, 2009). The coordinated interaction among desmosomal proteins, as well as their appropriate expression patterns, is thought to be essential for desmosomal adhesion and epidermal function.

As mentioned above, the epidermis is a unique tissue with varying expression of desmosomal proteins, as well as differential expression of junctions themselves; with tight junctions located in the upper layers, etc. From the basal to granular layer, desmosomes increase in both number and size, along with an increasing gradient of extracellular calcium (Elias 2002, Menon 1985). Interestingly, cholesterol enriched microdomains known as lipid rafts, have been shown to be concentrated in the upper layers of the epidermis (Roelandt *et al.*, 2009). A significant portion of this dissertation investigates the significance of lipid rafts, or membrane rafts, in desmosomal adhesion, as well as their role in disease. A description of rafts, their functions and methods to study rafts will be introduced next, along with a discussion of how membrane rafts are integrated with the function of various cell junctions.

2.5 Membrane rafts

2.5.1 Membrane raft definition

Membrane rafts, also known as lipid rafts, membrane microdomains and detergent resistant membranes, are specialized regions or domains of the plasma membrane with

distinct properties from the surrounding lipid bilayer. Here, these microdomains will be simply referred to as ‘rafts’. Defined at the 2006 Keystone Symposium of Lipid Rafts and Cell Function, rafts are “small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” (Pike, 2006). The enrichment in cholesterol and sphingolipids results in a more ordered, packed and slightly thicker lipid bilayer relative to the surrounding plasma membrane (Figure 5A). Generally, there are two types of rafts, planar and caveolae. Planar rafts are not invaginated and are continuous with the plane of the membrane, and contain proteins such as flotillin. In contrast, caveolae are flask shaped invaginations in the plasma membrane containing the protein caveolin. Individual raft domains contain a small subset of select proteins and float freely within the membrane. However, rafts can cluster to form larger (> 450 nm), more stable domains that function as platforms to execute multiple cellular functions, such as signaling and endocytosis (Goswami *et al.*, 2008; Lajoie and Nabi, 2010; Simons and Sampaio, 2011).

2.5.2 Membrane raft function

Generally, rafts function as organizing hubs to facilitate a variety of cellular processes. By having an environment that is distinct from other areas of the plasma membrane, they are able to compartmentalize proteins and effector molecules for various processes, ranging from membrane protein trafficking to signal transduction to endocytosis (Lajoie and Nabi, 2010; Pike, 2006; Simons and Sampaio, 2011; Simons and Toomre, 2000). For example, rafts serve as spatial organizers to either promote interactions that favor signal transduction or prevent interactions by segregating

otherwise-interacting molecules (Simons and Toomre, 2000). The ability of rafts to cluster is critical for their function. For signaling to occur, many small individual rafts must cluster together to form a larger platform where proteins necessary for signaling are brought together and can interact regardless of what happens in the surrounding non-raft domains. Thus, rafts cluster to concentrate or bring together interacting molecules to execute cell functions. Raft clustering has been shown to be cholesterol-dependent and can occur through a variety of ways, driven from either the extracellular face or the cytosolic face of the membrane, as well as intracellularly (Simons and Ehehalt, 2002). Antibodies, ligands or raft-lipid-binding proteins can drive clustering on the extracellular side of the membrane while scaffolding proteins such flotillins or caveolins drive clustering from the cytoplasmic side of the membrane. Clustering can also occur through protein oligomerization within the membrane (Simons and Ehehalt, 2002).

A functional role for rafts in immune receptor signaling has emerged from studies of hematopoietic cells. The IgE, T-cell and B-cell receptors have been shown to translocate to rafts upon crosslinking, leading to kinase activation and signaling (Field *et al.*, 1995; Horejsi, 2003; Janes *et al.*, 2000; Sedwick and Altman, 2002). Rafts also play a role in disease, including a variety of bacterial and viral infections. For example, the HIV envelope is enriched in raft components and rafts play a functional role in both virus entry and exit (Aloia *et al.*, 1988; Aloia *et al.*, 1993; van Wilgenburg *et al.*, 2014). Rafts have been shown to regulate the protein trafficking and processing that lead to the accumulation of amyloid-β-peptide plaques, the hallmark of Alzheimer disease (Simons and Ehehalt, 2002).

A variety of proteins, including EGFR, TGFβ and GPI-linked proteins have been shown to undergo raft-mediated endocytosis, another important function of rafts (Hansen and Nichols, 2009; Lajoie and Nabi, 2010; Sharma *et al.*, 2002). Raft-mediated endocytosis falls within the category of clathrin-independent endocytosis and is characterized by a variety of criteria. Generally, raft endocytosis is categorized into caveolae-mediated, noncaveolar dynamin-dependent and noncaveolar dynamin-independent endocytosis (Lajoie and Nabi, 2010). Caveolae are plasma membrane invaginations of 60-80 nm in diameter enriched in the membrane-associated protein caveolin. Caveolae formation is dependent of the ability of caveolin to oligomerize and bind cholesterol (Lajoie and Nabi, 2010; Nichols, 2003). Fission of caveolae requires dynamin, a protein that is also important in clathrin-mediated endocytosis. Dynamin has been shown to regulate the formation of raft-derived endoctyic carries independently of caveolin, such as for Cholera toxin internalization, with some studies implicating the raft protein flotillin-1 in this process (Lajoie and Nabi, 2010). Additionally, raft endocytosis can occur independently of both caveolin or dynamin (El-Sayed and Harashima, 2013; Lajoie and Nabi, 2010). Regulators of this form of endocytosis may include Cdc42, GRAF1, Arf proteins and Rac1(El-Sayed and Harashima, 2013). Importantly, all raft-mediated endocytic pathways are sensitive to cholesterol depletion and are dependent on tyrosine kinase activity (Lajoie and Nabi, 2010). Multiple methods of cholesterol perturbation have been demonstrated to disrupt raft function, including endocytosis (Nichols, 2003; Nichols and Lippincott-Schwartz, 2001; Puri *et al.*, 2001; Sharma *et al.*, 2002).

2.5.3 Studying membrane rafts

A variety of biochemical, imaging and drug-based techniques have been employed to analyze membrane rafts (Connell and Smith, 2006; Damjanovich *et al.*, 2002; de Almeida *et al.*, 2005; de Almeida *et al.*, 2009; Shogomori and Brown, 2003; Xu *et al.*, 2001; Zidovetzki and Levitan, 2007), and those applicable to this dissertation will be briefly discussed. Utilized most heavily is the biochemical raft fractionation technique to assay for raft association. We used the standard method in the field for preparing detergent resistant membranes (DRMs, a biochemical definition of lipid rafts) as described by Lingwood and Simons (Lingwood and Simons, 2007). Following detergent solubilization, cell lysates are subjected to sucrose gradient fractionation. Rafts and raft-associated proteins are Triton X-100 insoluble at 4°C and are relatively buoyant compared to non-raft membranes on a sucrose gradient following ultracentrifugation. Protein composition at different buoyant densities within the sucrose gradient fractions can be analyzed via western blot to identify proteins that co-fractionate together (Figure 5B). Flotillin-1 and caveolin-1 are commonly used as markers of raft fractions, while calnexin, an endoplasmic reticulum resident protein, is commonly used as a non-raft marker. It is important to note that this assay on its own is simply reflective of a protein's biochemical ability to fractionate with raft DRMs, and thus it is not necessarily direct evidence that a protein is raft associated in intact cells (Lingwood and Simons, 2007; Sharma *et al.*, 2002). Nonetheless, the buoyant properties of a protein on a sucrose gradient provides a powerful biochemical criterion for raft association under different biological circumstances, such as a change in cell-cell adhesive or migratory status.

Visualizing rafts by microscopy is technically challenging. Due to conventional light microscopy resolution limits and the variable and small size of rafts, sometimes 10-100 nm, it is often difficult or near impossible to visualize individual raft domains in living cells, though lipid ordered phases are readily observable in model membranes (Owen and Gaus, 2013; Simons and Toomre, 2000). Colocalization with different raft markers such as the GPI-anchored protein CD59 and caveolin-1 can be used to suggest raft association, although this approach does not provide definitive evidence that a particular protein is 'in a raft'. However, colocalization with a variety of markers for proteins that have an affinity for different raft sub-types can help identify the type of raft a protein of interest likely associates with. This type of information is not obtainable by the fractionation technique discussed above as it potentially isolates a heterogeneous population of rafts that exhibit the same buoyant density (Duggan *et al.*, 2008). Thus, proteins that fractionate together are not necessarily in the same raft domain in the living cell. For this reason, we utilized a combination of biochemical and high resolution optical approaches, such as structured-illumination microscopy, in the work reported in this thesis.

In addition to biochemical isolation and imaging techniques, rafts can be functionally perturbed by a variety of reagents (George and Wu, 2012; Zidovetzki and Levitan, 2007). Methyl-beta-cyclodextrin (mβCD) is widely used to disrupt raft function by depleting plasma membrane cholesterol (Simons and Toomre, 2000). Other raft altering reagents include those that bind cholesterol such as filipin and nystatin, as well as, those that inhibit cholesterol or sphingolipid synthesis such as the statin class of drugs (lovastatin, simvastatin, fluvastatin) and myriocin respectively (Hanada *et al.*, 2000;

Hothersall *et al.*, 2006; Miyake *et al.*, 1995; Simons and Toomre, 2000; Zeki *et al.*, 2011). Both filipin and nystatin have been used in previous Kowalczyk lab studies (Delva *et al.*, 2008). More recently, we have utilized mβCD to perturb raft function, as reported here in Chapter 5. Tyrosine kinase signaling has also been shown to be important for raft-mediated functions such as endocytosis, and for this reason tyrosine kinase inhibitors have been implemented in a variety of studies for preventing endocytosis via raft domains (Damm *et al.*, 2005; Minshall *et al.*, 2000; Sharma *et al.*, 2004).

2.5.4 Rafts and cell junctions

In addition to the functions described above, rafts have been shown to play important roles in cell junction regulation. The neuromuscular junction (NMJ) is a specialized junction that connects the nervous and muscular systems with synaptic contact points and is critical for muscle contraction. The NMJ *in vivo* is enriched in GM1, a key component of rafts, and rafts have been demonstrated to be required for acetylcholine receptor clustering, which is necessary for NMJ formation and function (Cartaud *et al.*, 2011; Zhu *et al.*, 2006). Studies also suggest that raft microdomains organize cell-cell contacts by clustering and stabilizing N-cadherin in myoblasts for functional adhesion. N-cadherin was raft associated biochemically and raft-disruption perturbed junction assembly and dynamics (Causeret *et al.*, 2005). Localization of the N-cadherin-p120 catenin complex in rafts was required for N-cadherin-dependent activation of RhoA during the induction of myogenesis (Taulet *et al.*, 2009). Additionally, the tight junction (TJ) has been classified as a membrane microdomain. Major TJ proteins occludin and zonula occludin-1 were found to be raft associated. Importantly, TJ

disassembly resulted in a displacement of TJ proteins from rafts. Furthermore, immunogold electron microscopy revealed occludin and caveolin-1 in close proximity (Nusrat *et al.*, 2000). Thus, emerging evidence suggests a raft requirement for junction organization and function, ranging from neuronal to muscle to epithelial junctions.

Growing evidence is revealing a role for rafts in desmosome regulation and function. Mature desmosomes are remarkably detergent insoluble structures, and this property has been useful in monitoring desmosome assembly status. For example, desmosome components partition into a Triton-soluble pool and a Triton-insoluble pool when whole cells are extracted with Triton X-100. The insoluble pool reflects proteins that are incorporated into the desmosomal complex associated with the intermediate filament cytoskeleton, and thus is referred to as the desmosomal pool, while the soluble pool reflects the non-desmosomal pool (Palka and Green, 1997). It is interesting that the desmosome has been studied classically using a similar solubility criteria as rafts, with the exception of 'floatation'. Intriguingly, a connection between desmosomes and rafts actually goes back to the 1970's, before the term 'lipid raft' was coined, and even before the concept of a membrane microdomain was appreciated. Initial studies isolating and biochemically characterizing desmosomes revealed that the lipid fraction of desmosomal membrane was enriched in cholesterol and sphingolipids (Drochmans *et al.*, 1978; Skerrow and Matoltsy, 1974).

More recently, desmosomal proteins Dsg2, Dsc2, PG and DP were confirmed to be raft associated biochemically by the standard raft fractionation protocol discussed above in multiple epithelial cell types. Furthermore, raft disruption was found to weaken cell adhesion (Brennan *et al.*, 2012; Nava *et al.*, 2007; Resnik *et al.*, 2011). As rafts can

cluster to form more stable platforms for cellular functions, we reasoned that desmosomes might represent a sub-type of these raft-containing stable membrane-ordered assemblies. However, previous work in the Kowalczyk lab has demonstrated that Dsg3 is internalized via a raft-mediated pathway upon triggering desmosome disassembly and that this internalization can be prevented by inhibiting various signaling pathways (Delva *et al.*, 2008; Saito *et al.*, 2012a). Overall, the literature and our previous studies support a broader hypothesis in which rafts play a functional role in desmosome regulation, signaling and adhesion. Yet, the mechanisms by which desmosomal proteins associate with rafts and the significance of this association remain largely unknown. The functional significance of desmosomal raft association is investigated in Chapter 5 and future studies on the mechanisms regulating raft association will be discussed in Chapter 7.

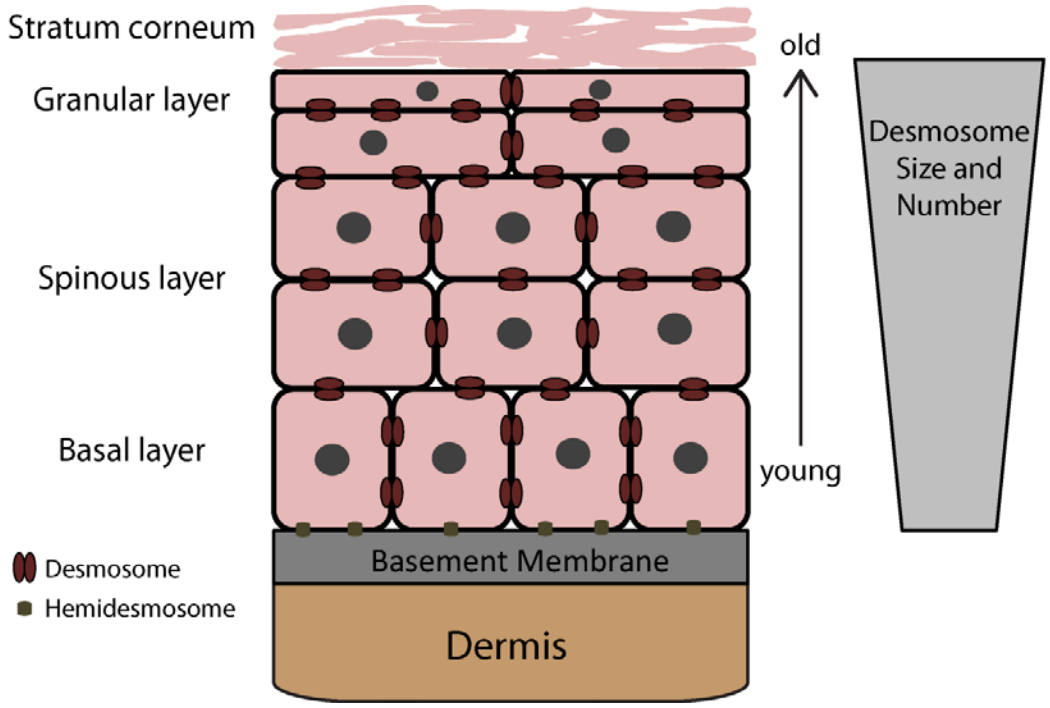

Figure 1. Structure of the epidermis. The skin is divided into two major regions, the epidermis and the underlying dermis, which are separated by a basement membrane. The epidermis is mainly comprised of keratinocytes that are organized into four distinct layers: the basal layer, the spinous layer, the granular layer and the stratum corneum. The epidermis is replenished by basal cells that proliferate and migrate toward the outer layers while undergoing terminal differentiation. Desmosomes (red oval pairs) provide strong adhesion to the epidermis by mechanically anchoring neighboring cells to one another. Both desmosome size and number increase as the cells age and transit through the granular layer.

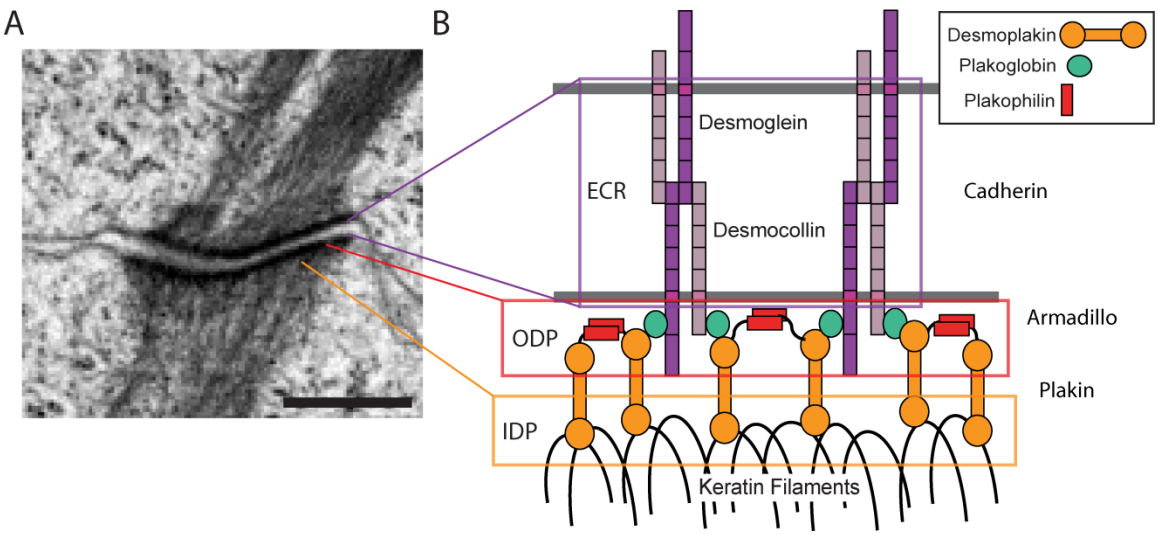

Figure 2. Desmosome ultrastructure and organization. A) Electron micrograph of a desmosome in cultured human keratinocytes. Scale bar, 0.5 µm. Image by Dana K. Tucker. **B)** Desmosome schematic. The extracellular core region (ECR) comprises the extracellular space between two cells that are engaged in cell adhesion mediated by the extracellular domains of the desmosomal cadherins, desmoglein and desmocollin. The cytoplasmic tails of the cadherins are linked to plaque proteins plakoglobin and plakophilin of the armadillo protein family, along with the amino-terminus of plakin family member desmoplakin within the outer dense plaque (ODP). The carboxyl-terminus of desmoplakin binds to keratin filaments within the inner dense plaque (IDP).

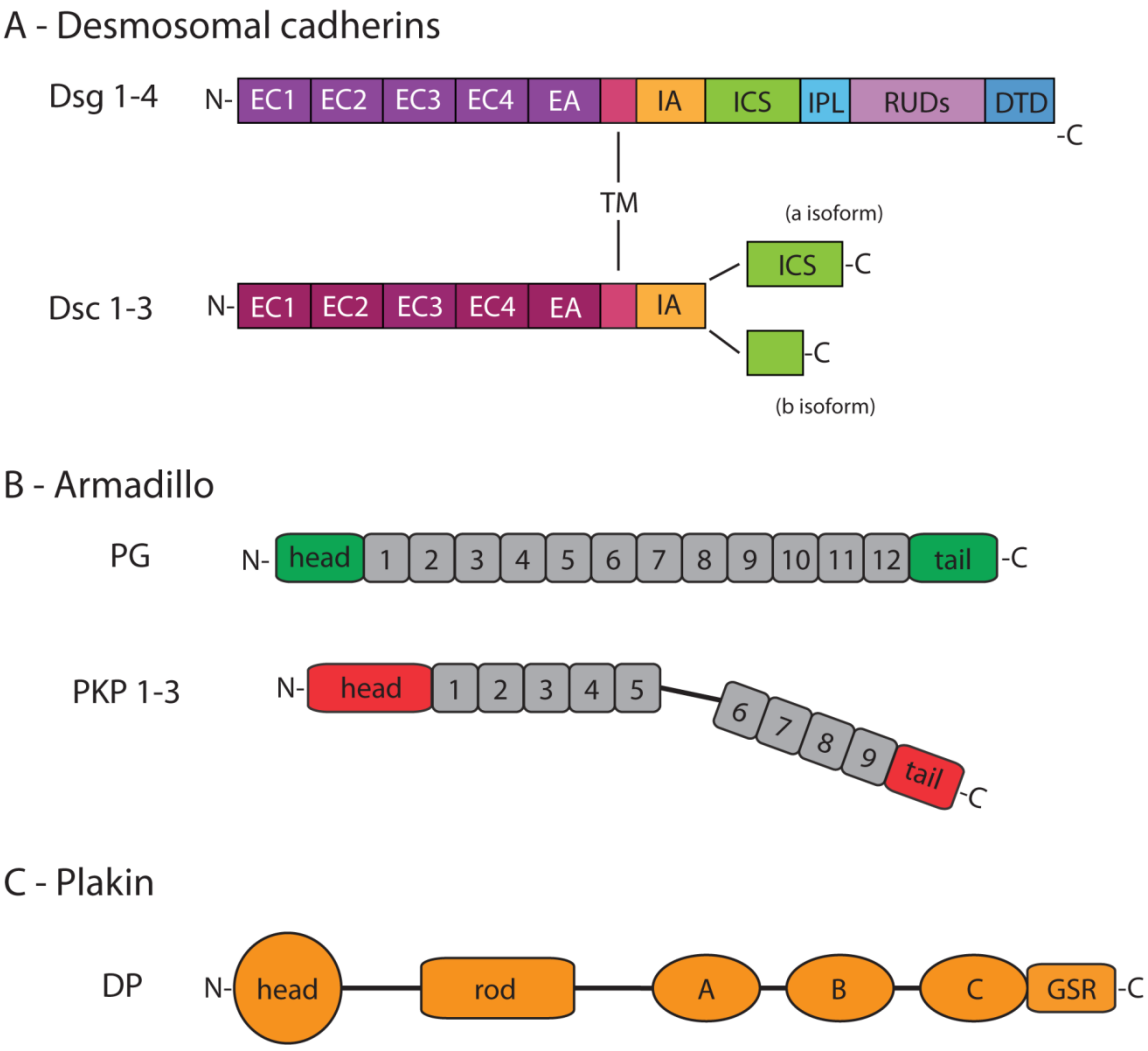

Figure 3. Domain organization of desmosomal proteins. A) Desmosomal cadherin domain structure. The desmogleins (1-4) and desmocollins (1-3) contain four extracellular cadherin (EC) repeat domains followed by extracellular anchor (EA), transmembrane (TM) and intracellular anchor (IA) domains. Following the IA, the desmogleins contain an intracellular cadherin-like sequence (ICS) domain which binds plakoglobin, as well as, an extended tail comprised of an intracellular proline-rich linker (IPL), variable repeat unit domains (RUD) and a desmoglein terminal domain (DTD). Desmocollins are alternatively spliced into an 'a' isoform containing an ICS domain and

a shorter ‘b’ isoform. **B)** Domain structure of armadillo family proteins. Plakoglobin (PG) contains 12 central *arm*-repeat domains (similar to β-catenin), while the plakophilins (PKP) (1-3) contain nine *arm*-repeats with an insert between repeats 5 and 6, introducing a bend in the overall structure. Both PG and PKP contain amino-terminal head and carboxyl-terminal tail domains that flank the central repeat domains. **C)** Desmoplakin domain structure. Desmoplakin (DP) consists of an amino-terminal globular plakin domain (head), a coiled-coil dimerization domain (rod) and a carboxyl-terminal tail that contains plakin repeats A-C and a glycine-serine-arginine (GSR) rich region. DP is alternatively spiced into a longer DPI isoform and a shorter DPII isoform.

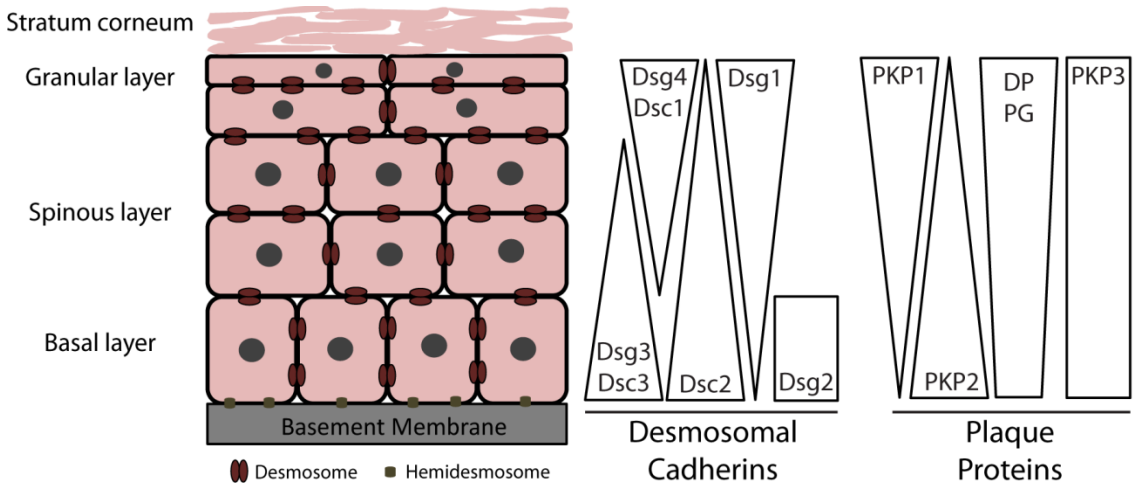

Figure 4. Expression profile of desmosomal proteins in the epidermis. Dsg3, Dsg2, Dsc3 and Dsc2 are primarily expressed in the lower epidermal layers, while Dsg4, Dsc1 and Dsg1 are highly expressed in the upper layers of the epidermis. PKP1 is primarily expressed in the upper layers, PKP2 is found primarily in the lower layers and PKP3 is expressed throughout the epidermis. DP and PG are expressed in all epidermal layers. Dsg, desmoglein; Dsc, desmocollin; PKP, plakophilin, DP, desmoplakin; PG, plakoglobin.

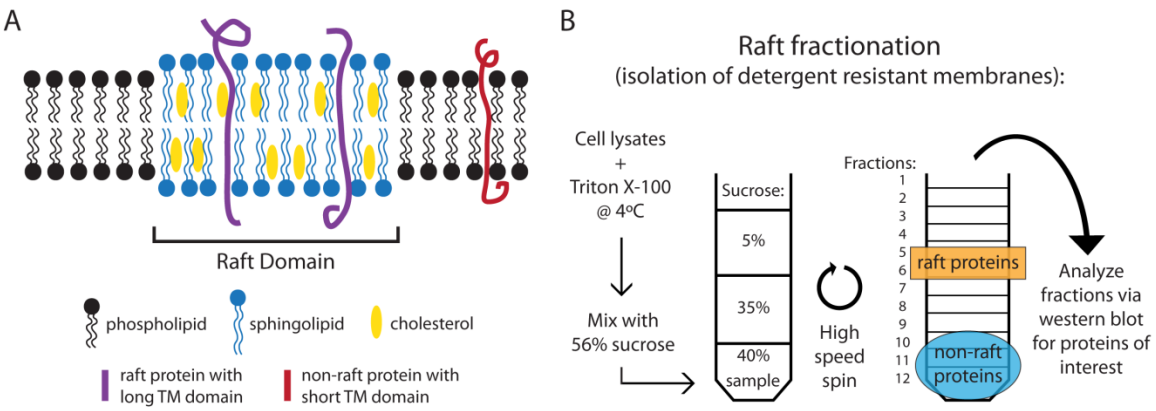

Figure 5. Membrane rafts and their biochemical isolation. A) Depiction of a membrane raft microdomain. The raft domain is enriched in sphingolipids and cholesterol, resulting in a more ordered and slightly thicker lipid bilayer relative to the surrounding plasma membrane. Proteins that associate with rafts typically have a longer transmembrane (TM) domain than non-raft proteins and/or are lipid modified to facilitate raft targeting (not shown). **B)** Schematic for isolating detergent resistant membranes (raft fractionation). Cell lysates are extracted in Triton X-100 at 4°C and mixed with 56% sucrose for a final concentration of 40%. Volumes of 35% and 5% sucrose are then layered on top of the sample. The sample is spun at high speed until equilibrium. During centrifugation, rafts 'float' along with any associated proteins. Fractions are sequentially removed from the gradient and protein composition is assayed via western blot.

Chapter 3

Compromised desmosomal adhesion in disease

Section 3.2 & 3.3 adapted from:

Stahley S.N. and Kowalczyk A.P. “Desmosomes in acquired disease”. Cell and Tissue Research. 2015. DOI: 10.1007/s00441-015-2155-2. *In press.*

The importance of desmosome function is evidenced by the numerous human diseases that result when desmosomal adhesion is compromised. Affecting nearly every tissue where desmosomal proteins are expressed, these disorders include inherited, auto-immune and infectious diseases (Table 1), as well as cancer (Table 3). The focus of this chapter will be on acquired desmosomal disorders, as the acquired disease pemphigus vulgaris is central to this dissertation.

3.1 Inherited disorders of the desmosome

Mutations in multiple genes encoding desmosomal proteins result in a variety of skin and heart disorders that typically present with either skin fragility and/or cardiomyopathy (Table 1). The first human desmosomal mutations were discovered in the gene encoding for the armadillo protein plakophilin 1 (PKP1) and result in the autosomal recessive disorder ectodermal dysplasia-skin fragility syndrome (McGrath *et al.*, 1997). Mutations within the amino-terminus and multiple arm domains of PKP1 have since been found to cause the disorder which is characterized by a combination of skin fragility (erosions and blistering, keratoderma) and abnormalities in ectodermal development (nail dystrophy, growth delay) (McGrath and Mellerio, 2010). Another disorder that presents with epidermal thickening of the palms and soles, striate palmoplantar keratoderma, results from mutations in DSG1, DSC2 or DP (Amagai and Stanley, 2012). Mutations in DSC2 and DP also can result in woolly hair, a syndrome characterized by frizzy and wiry hair on the scalp, giving it a wool-like appearance. Hypotrichosis, a condition of abnormal hair patterns (sparse, fragile, hair-follicle defects) is caused by mutations in DSG4 or DSC3 (Kljuic *et al.*, 2003). Frameshift mutations in

DP lead to lethal acantholytic epidermolysis bullosa, a devastating and fatal condition that presents with rapidly progressive and generalized epidermal blistering upon birth (McGrath *et al.*, 2010). Naxos disease and the Ecuadorian variant Carvajal syndrome are caused by carboxyl-terminal mutations in either DP or PG and are characterized by a combination of woolly hair, palmoplantar keratoderma and arrhythmogenic right ventricular cardiomyopathy (ARVC) (Carvajal-Huerta, 1998; Protonotarios and Tsatsopoulou, 2004). In ARVC, arrhythmias originate in the right ventricle and the muscle wall thins, leading to sudden cardiac arrest. Mutations in desmosomal genes accounts for 50-70% of ARVC cases, with PKP2 being the most commonly mutated desmosomal gene in ARVC (Brooke *et al.*, 2012). Mutations in DSG2, DSC2, PG and amino- or carboxy-terminal mutations in DP have also been reported in ARVC (Brooke *et al.*, 2012). Another form of cardiomyopathy, dilated cardiomyopathy (DC), can be caused by mutations in DSG2, DSC2 and DP and is characterized by an enlargement and weakening of the left ventricle, leading to impaired cardiac function (Towbin, 2014).

3.2 Desmosomes in acquired disease

Acquired desmosomal diseases fall into two main categories, epidermal autoimmune disorders and infections (Table 1). The roles of desmosomal genes in tumor biology will also be discussed in this section.

3.2.1 Pemphigus

Pemphigus, stemming from the Greek word *pemphix*, meaning blister, is a family of diseases characterized by circulating autoantibodies that target desmosomal proteins

and compromise cell-cell adhesion (Amagai, 2009, 2010; Kneisel and Hertl, 2011a). These relatively rare autoimmune diseases (1-16 new cases per million people per year) (Kneisel and Hertl, 2011a, b; Ruocco *et al.*, 2013) present clinically with mucosal erosions and/or epidermal blisters. The mean age of onset is 50-60 years old, although a younger age of onset is observed for endemic variants of pemphigus (Joly and Litrowski, 2011; Rocha-Alvarez *et al.*, 2007). Unlike other bullous diseases that require complement fixation and inflammatory cascades (Gammon *et al.*, 1980), pemphigus autoantibodies directed against the extracellular domains of desmosomal cadherins are necessary and sufficient to cause the loss of keratinocyte adhesion, a process termed acantholysis (Amagai, 2010). This assertion has been demonstrated definitively by three decades of research, in particular through an elegant series of passive transfer experiments using antibodies (IgG) purified from patients (Table 2). As discussed below, several subtypes of pemphigus are defined by the desmosomal proteins targeted and by the tissues affected. We refer readers to a pair of reviews by Kneisel and Hertl that provide excellent clinical descriptions and key diagnostic indicators for this family of diseases (Kneisel and Hertl, 2011a, b).

Pemphigus vulgaris. Pemphigus vulgaris (PV) is the most common form of pemphigus, accounting for about 70% of cases. Most cases are sporadic, with a few rare familial cases, as well as a higher incidence rate in Ashkenazi Jewish and Japanese populations (Joly and Litrowski, 2011; Rocha-Alvarez *et al.*, 2007). PV affects both men and women, and age of onset is typically between 40-60 years, although a few cases have been observed in children (Ariyawardana *et al.*, 2005; Kneisel and Hertl, 2011a). Treatment

with immunosuppressive agents decreases the otherwise high mortality rate of PV patients to 5-10%. PV is characterized clinically by painful mucosal lesions and histologically by an intraepidermal cleavage between the basal and spinous layers of the epidermis (Amagai, 2010). In the mucosal dominant form of PV, autoantibodies directed against Dsg3 result in blistering restricted to the oral mucosal, where Dsg3 is the predominant Dsg expressed. In these patients, the skin is uninvolved because Dsg1 is expressed sufficiently in the lower layers of the epidermis to compensate for the loss of Dsg3 function. When both Dsg1 and Dsg3 are targeted by autoantibodies, Dsg1 can no longer compensate in the basal layers of the epidermis and the patients develop skin blisters in addition to oral erosions (mucocutaneous form) (Amagai, 2010; Mahoney *et al.*, 1999a; Mahoney *et al.*, 1999b; Sharma *et al.*, 2007). Dscs can be the primary targets in PV (Dmochowski *et al.*, 1993; Hashimoto *et al.*, 1995b; Mao *et al.*, 2010; Rafei *et al.*, 2011), although these cases are less common. Importantly, targeting of the extracellular domain of the desmosomal cadherins compromises adhesion and causes tissue fragility. The pathomechanisms by which these autoantibodies cause loss of adhesion are discussed in detail in Chapter 4.

Pemphigus foliaceus. Pemphigus foliaceus (PF) is characterized by superficial epidermal blisters without mucosal involvement. Intraepidermal blistering in PF results from autoantibodies directed against Dsg1, and thus blistering is restricted to the outer (granular) layer of the epidermis where Dsg1 is expressed. Most cases of PF are sporadic and account for 20-30% of pemphigus cases. However, endemic forms of PF (also known as fogo selvagem) occur in regions of Brazil, Columbia and Tunisa (Aoki *et al.*, 2011;

Joly and Litrowski, 2011). Brazilian endemic pemphigus foliaceus patients in particular also generate autoantibodies to Dsc1 and Dsc2 (Dmochowski *et al.*, 1993). Relative to PV, those diagnosed with PF typically have a better prognosis as the lesions are more superficial and do not involve mucous membranes. Importantly, autoantibodies directed against the extracellular domain of Dsg1 cause PF (Amagai *et al.*, 1995). Thus, PV and PF represent well characterized autoimmune disorders in which autoantibodies disrupt target antigen function (adhesion) and thereby cause disease.

Paraneoplastic pemphigus. First described in 1990 by Anhalt and colleagues (Anhalt *et al.*, 1990), paraneoplastic pemphigus (PNP) is a rare variant of pemphigus that typically presents in patients diagnosed with lymphoproliferative disorders, primarily malignancies such as non-Hodgkin's lymphoma and chronic lymphocytic leukemia (Allen and Camisa, 2000; Yong and Tey, 2013). Age of onset is 45-70 years old, but children and adolescents can be afflicted. While less common than PV or PF, it is the most severe disease within the pemphigus family. PNP is characterized by refractory inflammation of the mouth and lips (Amagai, 2010) and extremely painful oral lesions, followed by the development of cutaneous lesions that can affect any body area. This includes the palms and/or soles, a feature that is unusual in other forms of pemphigus (Allen and Camisa, 2000). Conjunctival involvement is more common in PNP than with the other pemphigus variants. Also differing from the other forms of pemphigus, simple squamous epithelia can be involved, with 30-40% of patients developing pulmonary symptoms (Amagai, 2010). A recent study utilizing the PNP model mouse suggests that the fatal pulmonary symptoms in patients are most likely due to ectopic expression of Dsg3 and other

epidermal antigens (Hata *et al.*, 2013). Autoantibodies in PNP are directed against multiple desmosomal proteins, including Dsgs, Dscs, desmoplakin and plakophilins (Futei *et al.*, 2003; Gallo *et al.*, 2014; Hashimoto *et al.*, 1995a; Lambert *et al.*, 2010; Seishima *et al.*, 2004). Similar to other forms of pemphigus, blistering in this disease can be attributed to autoantibodies directed against Dsgs (Amagai *et al.*, 1998; Nishifuji *et al.*, 2007; Saleh *et al.*, 2012), particularly the Dsg3 EC2-3 domains (Saleh *et al.*, 2012). However, autoantibodies directed against the plakin family including envoplakin and periplakin are useful diagnostic markers that help to distinguish PNP from other forms of pemphigus (Amagai, 1999; Amagai *et al.*, 1998; Mahoney *et al.*, 1998; Poot *et al.*, 2013; Probst *et al.*, 2009). Importantly, histopathological studies have revealed keratinocyte necrosis with the presence of inflammatory cells into the epidermis, supporting the notion that cell-mediated immunity is an important component of PNP (Yong and Tey, 2013). PNP patient prognosis is poor as these individuals often develop sepsis and organ failure, contributing factors in mortality rates approaching 90% (Yong and Tey, 2013).

IgA pemphigus. IgA pemphigus is characterized by circulating IgA autoantibodies that target both desmosomal and non-desmosomal components of the keratinocyte cell surface. Histologically, acantholysis and extensive neutrophilic infiltration are observed within the upper layers or all layers of the epidermis (Tsuruta *et al.*, 2011). Two subtypes of IgA pemphigus have been characterized. In subcorneal pustular dermatosis, Dsc1 is the target antigen. In contrast, Dsgs 1 and 3 are targets in the intraepidermal neutrophilic dermatosis subtypes (Kneisel and Hertl, 2011b). Only about 60 cases have been reported

and the disease is considered to be less life-threatening than the other variants of pemphigus.

3.2.2 Bullous impetigo / Staphylococcal scalded skin syndrome

Bullous impetigo is an infectious skin disease caused by the release of exfoliative toxin (ET) by *Staphylococcus aureus*. ET is a serine protease that cleaves the extracellular domain of Dsg1 with high specificity. The protease does not cleave Dsg3 or related cadherin family members such as E cadherin, and does not appear to have any other substrates (Amagai *et al.*, 2000). There are three types of ETs produced (ETA, ETB, and ETD), all specifically targeting Dsg1, with ETA being the most common. The result of Dsg1 cleavage by these ETs is an intraepidermal cleavage in the uppermost layers of the epidermis, strikingly similar to the granular layer blister caused by IgG targeting Dsg1 in PF. Primarily affecting children, bullous impetigo is very common and highly contagious. Another infectious skin disease, Staphylococcal scalded skin syndrome (SSSS), also known as Ritter disease, was first described in 1878 by Baron Gottfried Ritter von Rittershain and was soon acknowledged to be a more generalized and severe form of bullous impetigo (Mockenhaupt *et al.*, 2005). SSSS commonly affects children under age 6, particularly neonates (Amagai, 2010). Adults with chronic renal insufficiency or immunosuppression are occasionally affected. In contrast to bullous impetigo, in which blisters form locally at the site of infection, SSSS presents with blisters at sites distant from the infection due to systemic circulation of the ET. Typically, both bullous impetigo and SSSS are effectively treated by topical or oral antibiotics to eliminate the underlying infection (Bernard, 2008; Hartman-Adams *et al.*, 2014).

Additional treatment of pain as well as anti-septic measures are undertaken when treating SSSS. While mortality in neonates and children remains low, management of SSSS in adults often presents a challenge with a mortality rate over 60% (Patel, 2004).

3.2.3 Cancer

Recent work in cancer biology has revealed striking alterations in desmosomal protein expression patterns in various epithelial derived tumors. The majority of cancer cases are acquired, with only 5-10 percent of cancer cases being inherited (Anand *et al.*, 2008). In order to become migratory and metastatic, cells must loosen, rearrange and dissociate their junctions, a process known as epithelial-mesenchymal transition (EMT) (Birchmeier *et al.*, 1996). Anchoring junctions are often down-regulated during tumorigenesis (Birchmeier *et al.*, 1996; Birchmeier *et al.*, 1995; Defamie *et al.*, 2014). In fact, nearly all desmosomal components have been shown to be mis-regulated in various forms of cancer (Table 3). Nearly 90% of acantholytic squamous cell carcinomas display a reduction in at least one desmosomal protein, as assessed by immunofluorescence of tumor sections (O'Shea *et al.*, 2014). Dsg2 expression is reduced in familial and gastric cancer (Biedermann *et al.*, 2005; Yashiro *et al.*, 2006), while Dsc2 (Hamidov *et al.*, 2011) and Dsc3 (Oshiro *et al.*, 2005) are reduced in pancreatic and breast cancers. The loss of Dsc3 is also a contributing factor in skin tumor development and progression in a mouse model (Chen *et al.*, 2013). Oropharyngeal tumors have been shown to exhibit reduced expression of desmoplakin (Papagerakis *et al.*, 2009), while plakophilin 1 is down-regulated in prostatic adenocarcinoma (Breuninger *et al.*, 2010) and squamous cell carcinoma (Sobolik-Delmaire *et al.*, 2007); and plakophilins 2 and 3 are reduced in

gastric cancer (Demirag *et al.*, 2011). Finally, loss of the recently identified desmosomal protein Perp, a p53/p63 target gene, promotes carcinogenesis in both mouse and cell culture model systems (Beaudry *et al.*, 2010; Dusek *et al.*, 2012; Kong *et al.*, 2013). There are examples in which up-regulation occurs as well. Dsg2 overexpression has been documented in squamous cell carcinoma (Brennan and Mahoney, 2009; Kurzen *et al.*, 2003), melanoma (Schmitt *et al.*, 2007) and prostate cancer (Trojan *et al.*, 2005), while Dsg3 overexpression occurs in head and neck cancer (Chen *et al.*, 2007). Further, plakophilin 3 has been shown to be up regulated in prostatic adenocarcinoma (Breuninger *et al.*, 2010) and lung (Furukawa *et al.*, 2005) and breast cancer (Demirag *et al.*, 2012).

Mechanistic details are beginning to emerge regarding how desmosomal protein levels are altered in cancer. For example, the transcription factor Slug has been associated with reduced expression of Dsg3, Dsc2 and plakophilin 1 in oral squamous cell carcinomas (Katafiasz *et al.*, 2011). Slug has also been implicated in FGF-1-induced alterations in border localization of Dsg1/2 and desmoplakin in NBT-II bladder carcinoma cells (Savagner *et al.*, 1997; Thiery and Chopin, 1999). DNA methylation also plays a role in the reduced expression of Dsc1, Dsc2, Dsc3 and desmoplakin in lung cancer (Cui *et al.*, 2012a; Cui *et al.*, 2012b). Furthermore, the protease kallikrein has been linked to the reduced expression of Dsg1 and Dsg2 in pancreatic adenocarcinoma (Ramani *et al.*, 2008). Finally, EGFR signaling in cooperation with metalloprotease-mediated ectodomain shedding, regulates Dsg2 endocytosis in the highly invasive SCC68 cell line (Klessner *et al.*, 2009). Overall, the majority of data suggest that desmosomes play tumor-suppressive roles similar to E-cadherin. However, these studies have only begun to uncover the influence of the desmosome in cancer and future studies are needed

in order to understand how desmosomal components might be used as either prognostic markers or as targets for therapy.

Interestingly, some of the therapies derived from studying and treating pemphigus may be translatable to other disorders of desmosome disruption, including cancer. An intriguing example of this possibility is genistein, a soy isoflavone and tyrosine kinase inhibitor that prevents loss of desmosome adhesion in various models of pemphigus vulgaris (Delva *et al.*, 2008; Marquina *et al.*, 2008; Saito *et al.*, 2012a). Genistein has also shown promise in cancer prevention and treatment, as it reverses promoter hypermethylation of tumor suppressor genes (Mahmoud *et al.*, 2014). Interestingly, several desmosomal genes have recently been linked to tumor suppressive activity (Chun and Hanahan, 2010; Dusek and Attardi, 2011), with the onset of cancer progression through methylation of desmosomal genes (Cui *et al.*, 2012a; Cui *et al.*, 2012b). Genistein has been shown to have anti-cancer effects through the inhibition of tyrosine kinases and the modulation of related cellular pathways (Mahmoud *et al.*, 2014; Nagaraju *et al.*, 2013). It is interesting to speculate that perhaps one of the many ways in which genistein acts to impede cancer progression is by affecting desmosomal adhesion through pathways similar to its protective effects in pemphigus models.

3.2.4 Other desmosomal diseases

In addition to Dsg1 being a target in bullous impetigo, desmosomal components are now known to be targeted in a variety of infectious conditions. Dsg2 is a receptor for adenovirus serotypes (Ad) 3, 7, 11 and 14 that cause respiratory and urinary tract infections (Amagai and Stanley, 2012). Interaction between Dsg2 and Ad3 triggers EMT-

like events that result in the opening of intercellular junctions (Wang *et al.*, 2011). Dsc 2/3 is targeted by *Giardia duodenalis*, a parasitic protozoan that disrupts the arrangement of tight, adherens and desmosomal junctions of intestinal cells (Maia-Brigagao *et al.*, 2012). Impaired desmosome formation and a reduction in the epithelial barrier is the result of desmoplakin and plakoglobin down-regulation by *Bacillus anthracis* lethal toxin in alveolar epithelial cells (Langer *et al.*, 2012). Furthermore, infection by the enterovirus coxsackievirus B3 (CVB3) leads to cardiac failure by inducing miR-21 expression, which targets the deubiquitinating enzyme YOD1. This change leads to enhanced K48-linked ubiquitination and degradation of desmin, a critical intermediate filament that provides anchorage to the intercalated disc, which ultimately results in desmosome disruption (Ye *et al.*, 2014). Lastly, ultrastructural analysis has revealed smaller desmosomes in *Candida* infections of the oral mucosa, although the specific targets remain unknown (Nagai *et al.*, 1992).

A variety of other acquired conditions in which desmosomal components are targeted have also been described. Dsgs 1 and 3, the primary targets in pemphigus vulgaris, are also targets in pemphigus erythematosus, a condition with pathology that overlaps clinically and serologically with pemphigus foliaceus and lupus erythematosus (Oktarina *et al.*, 2012; Perez-Perez *et al.*, 2012). In this autoimmune disease, IgG deposition is mainly found in the granular layer of the epidermis, suggesting that Dsg1 is the primary pathogenic autoantigen (Amerian and Ahmed, 1985). Thus, the condition has recently been classified as a variant of pemphigus foliaceus (Steffen and Thomas, 2003). Additionally, antinuclear antibodies are also present in most patients with pemphigus erythematosus (Kneisel and Hertl, 2011a; Perez-Perez *et al.*, 2012; Steffen and Thomas,

2003). In patients with Grover's disease, an itchy acantholytic dermatosis, staining for Dsg1 and/or Dsg3 was shown to be decreased.. Though patients are positive for IgA or IgG autoantibodies, it remains unclear whether the autoantibodies cause the disease (Phillips *et al.*, 2013). Autoantibodies against Dsc3 have been implicated in acquired palmoplantar keratoderma (Bolling *et al.*, 2007), and autoantibodies against desmosomal components have also been reported in erythema multiforme (Foedinger *et al.*, 1995; Foedinger *et al.*, 1998; Foedinger *et al.*, 1996), a hypersensitivity reaction that is associated with infections or medications. The precise pathomechanism of this disease, and the related disorder Stevens-Johnson syndrome, are not known, but the presence of autoantibodies against desmoplakin is considered characteristic of these diseases. Lastly, reduced numbers of desmosomes have been reported in papular acantholytic dyskeratosis (de Almeida Junior *et al.*, 2001). Thus, a rather extensive number of epidermal disorders involve alterations in desmosome morphology and function, although additional research in this area is needed to clarify precisely how these changes might relate to clinical presentation of these disorders.

3.3 Future directions

The study of inherited and acquired diseases has revealed significant insight into both the critical role desmosomes play in epithelial biology and the fundamental cellular mechanisms that regulate desmosomal adhesion. Still, many pieces of the puzzle are missing. Little is known about what controls the tissue-specific and differentiation-dependent expression of the desmosomal genes. Furthermore, identification of the downstream targets of desmosomal cadherin signaling is needed to shed light on the

precise roles these cadherins play in tissue patterning and development. Though poorly understood, it is clear there is cross-talk between desmosomes and other junctions, including adherens and gap junctions. A better understanding of junctional communication during both normal tissue homeostasis and disease conditions will reveal how the dynamics of one junction affect the other. These insights will yield a deeper understanding of the basic cell biology of cell-cell contact and reveal new targets for disease treatments.

Desmosomes exhibit a high level of structural organization, as well as remarkable spatial and temporal control during assembly and disassembly. A combination of structural analyses including X-ray crystallography, NMR spectroscopy and cyro-electron tomography have all provided significant insight into the structure of desmosome components and their protein interactions (Al-Amoudi and Frangakis, 2008; Al-Jassar *et al.*, 2013; He *et al.*, 2003; Owen *et al.*, 2008; Stokes, 2007). While these techniques are generating an appreciation for the network of protein interactions within the desmosome, the entire macromolecular structure has yet to be visualized at high resolution. Even at low resolution, the entire desmosome has yet to be comprehensively analyzed in diseased states. Future studies using proteomics, advanced optical imaging, and atomic level structural approaches are needed to understand how desmosomes are organized, and how assembly and disassembly pathways regulate this critically important cell adhesion structure.

Table 1: Desmosomal targets in disease

Protein	Tissue Distribution	Disease Type		
		Autoimmune	**Infection**	**Inherited**
Dsg1	stratified epithelia	pemphigus foliaceus mc pemphigus vulgaris paraneoplastic pemphigus pemphigus erythematosus	SSSS bullous impetigo	striate PPK
Dsg2	simple and stratified epithelia, myocardium	none known	ad 3,7,11 & 14 respiratory uninary tract	ARVC DC
Dsg3	stratified epithelia	md pemphigus vulgaris mc pemphigus vulgaris paraneoplastic pemphigus pemphigus erythematosus IgA pemphigus	none known	none known
Dsg4	Outermost epithelia, hair follicle epithelia	mc pemphigus vulgaris	none known	hypotrichosis
Dsc1	stratified epithelia, hair follicle epithelia	IgA pemphigus pemphigus vulgaris	none known	none known
Dsc2	simple and stratified epithelia, myocardium	pemphigus vulgaris paraneoplastic pemphigus	giardiasis	striate PPK wolly hair ARVC
Dsc3	stratified epithelia	pemphigus vulgaris paraneoplastic pemphigus	giardiasis	hypotrichosis
Desmoplakin	all desmosome-bearing epithelia	paraneoplastic pemphigus erythema multiform Stevens-Johnson syndrome	BA-LT	striate PPk woolly hair LAEB Carvajal syndrome Naxos disease ARVC DC
Plakoglobin	all desmosome-bearing epithelia	mc pemphigus vulgaris paraneoplastic pemphigus	BA-LT	Naxos disease ARVC
Plakophilins 1-3	stratified epithelia	paraneoplastic pemphigus (PKP3)	none known	EDSF (PKP1) ARVC (PKP2)
Envoplakin	stratified epithelia, myocardium	paraneoplastic pemphigus	none known	none known
Periplakin	stratified epithelia, myocardium	paraneoplastic pemphigus	none known	none known

Abbreviations: mc, mucocutaneous; md, mucosal dominant; SSSS, staphylococcal scalded skin syndrome; ad, adenovirus serotypes; BA-LT, Bacillus anthracis lethal toxin; PPK, palmoplantar keratoderma; ARVC, arrhythmogenic right ventricular cardiomyopathy; DC, dilated cardiomyopathy; LAEB, lethal acantholytic epidermolysis bullosa; EDSF, ectodermal dysplasia-skin fragility

Table 2: Anti-Dsg autoantibodies cause acantholysis in pemphigus

Finding	**Reference**
Patient autoantibody titer correlates with disease activity	(Sams and Jordon, 1971)
Purified patient autoantibodies cause blisters in human skin organ culture	(Schiltz and Michel, 1976)
Passive transfer of patient IgG induces blister formation in neonatal mice	(Anhalt *et al.*, 1982)
IgG4 subclass of autoantibodies are pathogenic	(Rock *et al.*, 1989)
PV IgG purified with Dsg EC domains causes suprabasal acantholysis in mice	(Amagai *et al.*, 1992)
PV sera that is immunoabsorbed with EC domain of Dsg3 is no longer pathogenic	(Amagai *et al.*, 1994)
PF sera that is immunoabsorbed with EC domain of Dsg1 is no longer pathogenic	(Amagai *et al.*, 1995)

Table 3: Desmosomal targets in cancer

Protein	Cancer Type	↑↓ Regulated	Mechanism	References
Dsg1	SCC	down		(Harada *et al.*, 1996; Myklebust *et al.*, 2012; Ramani *et al.*, 2008; Wong *et al.*, 2008)
	pancreatic AC	down	protease kallikrein	(Ramani *et al.*, 2008)
Dsg2	familial & gastric	down		(Biedermann *et al.*, 2005; Yashiro *et al.*, 2006)
	pancreatic AC	down	protease kallikrein	(Ramani *et al.*, 2008)
	melanoma, prostate, SCC	up		(Brennan and Mahoney, 2009; Kurzen *et al.*, 2003; Schmitt *et al.*, 2007; Trojan *et al.*, 2005)
Dsg3	SCC	down		(Harada *et al.*, 1996)
	oral SCC	down	Slug expression	(Katafiasz *et al.*, 2011)
	head & neck	up		(Chen *et al.*, 2007; Chen *et al.*, 2013)
Dsg4	none know	N/A	N/A	N/A
Dsc1	anal SCC	down		(Myklebust *et al.*, 2012)
	lung	down	DNA methylation	(Cui *et al.*, 2012b)
Dsc2	pancreatic ductal AC	down		(Hamidov *et al.*, 2011)
	oral SCC	down	Slug expression	(Katafiasz *et al.*, 2011)
	lung	down	DNA methylation	(Cui *et al.*, 2012b)
Dsc3	lung	down	DNA methylation	(Cui *et al.*, 2012a; Cui *et al.*, 2012b)
Desmoplakin	lung	down	DNA methylation	(Yang *et al.*, 2012)
	SCC	abnormal		(Harada *et al.*, 1996)
Plakoglobin	oral SCC, prostate	down		(Franzen *et al.*, 2012; Katafiasz *et al.*, 2011)
	SCC	abnormal		(Harada *et al.*, 1996)
Plakophilin 1	prostate AC	down		(Breuninger *et al.*, 2010)
	oral SCC	down	Slug expression	(Katafiasz *et al.*, 2011)
Plakophilin 2	gastric	down		(Demirag *et al.*, 2011)
Plakophilin 3	colorectal carcinoma	down		(Khapare *et al.*, 2012)
	lung, prostate AC, breast	up		(Breuninger *et al.*, 2010; Demirag *et al.*, 2012; Furukawa *et al.*, 2005)
Perp	SCC, mammary	down		(Beaudry *et al.*, 2010; Dusek *et al.*, 2012; Kong *et al.*, 2013)
Periplakin	esophageal SCC	down	DNA methylation	(Otsubo *et al.*, 2015)

Abbreviations: SCC - squamous cell carcinoma, AC - adenocarcinoma

Chapter 4

Pathomechanisms of pemphigus: altered desmosome dynamics

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While it is well-established that blistering is caused by autoantibodies directed against Dsgs (Section 3.2.1; Table 2), the precise pathomechanisms of the disease are not fully understood. Two general hypotheses for explaining pemphigus pathomechanisms have been considered (Getsios *et al.*, 2010; Stanley and Amagai, 2006). First, the direct inhibition of Dsg adhesive interactions may result from autoantibodies sterically interfering with adhesion. Second, cellular responses such as Dsg3 endocytosis, decreased desmosome assembly and/or increased disassembly, activation of various cell signaling pathways, and keratinocyte apoptosis have been suggested to be pathogenic. It should be noted that these mechanisms are not necessarily mutually exclusive. This chapter discusses pemphigus pathomechanisms in detail and highlights how the study of pemphigus vulgaris in particular is revealing fundamental aspects of desmosome biology and desmosome dynamics. It is likely that some of the pathways targeted in pemphigus contribute to desmosome homeostasis mechanisms that are altered in other diseases. Thus, therapies developed to treat PV may also prove useful in other diseases characterized by altered desmosome function.

4.1 Steric hindrance

Dsgs, like other cadherins, are thought to engage in extracellular domain interactions that bridge two cells and mediate cell-cell adhesion. It has been hypothesized that pemphigus autoantibodies cause a loss of adhesion by physically interfering with Dsg adhesive function. In fact, once a cadherin-type molecule was discovered as the target, it was suggested that patient IgG directly disrupt adhesive interactions (Amagai *et al.*, 1991). There is now substantial evidence that pemphigus autoantibodies disrupt

desmosomal adhesion by directly interfering with Dsg cis (same cell)- and trans (opposing cells)-interactions. Many of the blister-inducing autoantibodies found in patient sera recognize the amino-terminal Dsg domains that are thought to be responsible for engaging in these adhesive interactions. For example, PV patient IgG that was affinity purified using the first two cadherin repeats (EC1,EC2) of the Dsg3 extracellular domain induced intraepidermal blisters when injected into mice (Amagai *et al.*, 1992) or human skin cultures (Bhol *et al.*, 1995). Competition ELISA experiments revealed that nearly 70% of PF sera and nearly 80% of PV sera bound to the N-terminal 161 residues of Dsg1 and Dsg3, respectively. The authors further mapped the binding site of pathogenic IgG, defined as autoantibodies that were sufficient to induce acantholysis, to the putative adhesive interface based on structural models of classical cadherins (Sekiguchi *et al.*, 2001). Additional epitope mapping studies have confirmed that regions within the EC1 and EC2 domains of Dsgs contain the dominant epitopes recognized by pathogenic autoantibodies from both PF (Chan *et al.*, 2010) and PV (Ohyama *et al.*, 2012). Recently, the putative cis binding interface of Dsg3 was found to represent a dominant epitope recognized by pathogenic autoantibodies in patients (Di Zenzo *et al.*, 2012). Thus, pemphigus autoantibodies often target the Dsg3 domains that are believed to be most critical for extracellular adhesion, supporting the notion that steric hindrance mechanisms are a significant factor in the disruption of desmosomal adhesion in pemphigus patients.

Additional evidence supporting the idea that direct inhibition of adhesion contributes to PV pathogenesis comes from the isolation of single chain (scFv) monoclonal IgG cloned from pemphigus patients, and from monoclonal IgG isolated from an active pemphigus vulgaris mouse model (eg. AK19, AK23). In both instances,

these highly characterized monoclonal reagents were found to target the Dsg3 EC1 domain and to be pathogenic in mouse models of disease (Payne *et al.*, 2005; Tsunoda *et al.*, 2003). These studies strongly suggest that pemphigus autoantibodies directly inhibit Dsg adhesive interactions. Indeed, both PV IgG and the mouse monoclonal AK23 IgG directly disrupted Dsg3-Dsg3 trans-interactions as assessed using atomic force microscopy (Heupel *et al.*, 2008), suggesting that at least some portion of IgG in patients acts by steric hindrance. It remains unknown if antibodies interfere with heterophilic adhesive interactions between Dsgs and Dscs. Further studies with atomic level resolution are needed to define the precise mechanism of desmosomal cadherin adhesion, and how PV IgG might disrupt adhesive interactions at the molecular level.

4.2 Beyond steric hindrance

As discussed above, steric hindrance of Dsg adhesion is an important factor in pemphigus pathophysiology. Additional mechanisms that likely contribute to pemphigus pathogenesis are discussed below.

4.2.1 IgG targeting the Dsg EC3-5 domains play a role in acantholysis

As discussed above, PV patient IgG directed against the N-terminal domains of Dsg3 are sufficient to cause loss of adhesion in various models of disease. However, adsorption of PV IgG to remove anti-EC1 domain IgG only partially abolished blister formation, indicating that autoantibodies against membrane proximal Dsg3 domains may also contribute to pathogenesis (Amagai *et al.*, 1992). Subsequent studies demonstrated that adsorption of patient sera against the entire EC domain of Dsg3 (PV sera) or Dsg1

(PF sera) was required to completely abolish pemphigus IgG activity (Amagai *et al.*, 1995; Amagai *et al.*, 1994; Langenhan *et al.*, 2014). Additionally, nearly 20 percent of patient auto-antibodies bind to regions of the Dsg3 extracellular domain other than the adhesive EC1 and EC2 domains (Sekiguchi *et al.*, 2001). Finally, a rare subset of pemphigus patients with active disease lack autoantibodies that target either EC1 and/or EC2 domains (Saito *et al.*, 2012a; Sekiguchi *et al.*, 2001). Collectively, these data indicate that PV IgG directed against the putative adhesive interfaces of the Dsgs are not required for disruption of desmosome function and blistering in patients. Thus, other considerations are required to understand how these antibodies compromise adhesion.

4.2.2 PV IgG cause alterations in the desmosomal plaque

A recent study using post-embedding immunogold-EM directly compared the Dsg3 -/- mouse to the active PV mouse model (Saito *et al.*, 2009). The PV clinical hallmark of suprabasal acantholysis was also observed in the Dsg3 -/- mouse, along with split desmosomes containing an intact desmosomal plaque (Koch *et al.*, 1997). These observations confirm the importance of Dsg3 in epidermal adhesion and are consistent with the idea that PV autoantibodies disrupt adhesion by directly interfering with adhesive function of Dsg3 (Koch *et al.*, 1997). However, in the PV mouse model, a distinct and measurable shift in the positioning of desmoplakin within the desmosomal plaque was observed. This is an interesting observation because simple disruption of Dsg3 trans-interactions would be predicted to cause desmosome splitting at the adhesive interface, but not necessarily alterations in desmosome organization. The authors speculated that the large shift in desmoplakin localization might be the result of a

signaling event or be mechanistically related to the keratin retraction that has been observed in cell culture models of PV (Bektas *et al.*, 2013; Calkins *et al.*, 2006; Tucker *et al.*, 2014). This study highlights that IgG-induced blistering is morphologically, and thus mechanistically, distinct from a simple Dsg3 loss of function.

4.2.3 IgG-induced Dsg3 clustering

Altered organization of Dsg3 is emerging as a hallmark feature of pemphigus pathogenesis (Ko and McNiff, 2014) and has been observed in pemphigus epidermis for many years (Iwatsuki *et al.*, 1999). An analysis of pemphigus patient biopsies revealed that Dsg1, Dsg3 and plakoglobin are misorganized into a clustered pattern in PV patient epidermis compared to control biopsies (Oktarina *et al.*, 2011). The authors further demonstrate that bivalent IgG was able to induce clustering, but that Fab fragments were not. Wier et al. also reported a clustered IgG and Dsg1 and/or 3 in PF and PV skin. Furthermore, desmosome size was reduced in PV patients with skin involvement and with autoantibodies against both Dsg1 and Dsg3 (van der Wier *et al.*, 2014). The authors concluded that Dsg clustering was a marker for the cadherin that was being targeted, but that clustering was unlikely to be part of a pathogenic mechanism. This conclusion was based on the observation that Dsg3 clustering was observed in clinically unaffected epidermis, where desmosome size was also normal. However, desmosomes would not be functionally compromised in uninvolved skin, such as in a mucosal dominant PV patient lacking autoantibodies against Dsg1. While mucosal dominant PV patients have IgG to Dsg3, and therefore Dsg3 is clustered, Dsg1 is likely compensating for the clustering and

loss of function of Dsg3. Thus, additional analysis of Dsg3 clustering in PV patients and in PV model systems is needed to further clarify the pathogenic role of Dsg3 clustering.

In vitro studies of Dsg3 distribution in cultured keratinocytes exposed to PV IgG represent a model system to understand how alterations in cell surface organization of desmosomal proteins might compromise desmosome function. Following PV IgG treatment, lateral aggregation of Dsg3 is followed by internalization, suggesting that clustering impairs desmosome dynamics and the stability of cell surface desmosomal cadherins (Sato *et al.*, 2000). Work by Saito et al. directly compared the effects of the pathogenic monoclonal IgG AK23 to that of polyclonal IgG isolated from PV patient sera (Saito *et al.*, 2012a). Upon exposure to AK23, Dsg3 and desmoplakin staining at cell borders was unchanged. In contrast, exposure to PV patient IgG dramatically altered desmosomal protein organization. Similarly, at the ultrastructural level, desmosomes were morphologically intact when exposed to AK23, but highly disorganized and smaller when exposed to PV IgG. However, both AK23 and PV IgG similarly compromised keratinocyte adhesion, suggesting that the monoclonal IgG and PV patient IgG were disrupting desmosomal adhesion by different mechanisms. Consistent with this hypothesis, loss of adhesion in response to PV IgG occurred in a p38MAPK-dependent manner, whereas AK23-induced loss of adhesion was independent of p38MAPK signaling.

The observation that patient IgG causes Dsg3 clustering and down-regulation does not exclude the possibility that some portion of PV patient IgG, which represents a polyclonal mixture in each patient, is disrupting adhesion by steric hindrance. In fact, this possibility is quite likely as several pathogenic monoclonal antibodies have been isolated

from PV patients (Di Zenzo *et al.*, 2012; Heupel *et al.*, 2008; Payne *et al.*, 2005; Sharma *et al.*, 2007). Collectively, these studies suggest that pathogenic monoclonal IgG against Dsg3 disrupt adhesion primarily by steric hindrance, whereas polyclonal patient IgG disrupts adhesion by both steric hindrance and clustering, as well as other mechanisms downstream of IgG ligation of the cadherin. Additional mechanisms that may contribute to PV pathogenesis are discussed in subsequent sections.

4.2.4 Dsg endocytosis and down-regulation

A number of studies provide compelling evidence that Dsg endocytosis and down-regulation of surface pools of the cadherins occur in response to PV IgG binding (Calkins *et al.*, 2006; Cirillo *et al.*, 2007; Iwatsuki *et al.*, 1999; Iwatsuki *et al.*, 1989; Jolly *et al.*, 2010; Kitajima, 2014; Mao *et al.*, 2014; Mao *et al.*, 2011; Patel *et al.*, 1984; Sato *et al.*, 2000; Schulze *et al.*, 2012). After PV IgG treatment in cultured keratinocytes, PV IgG and Dsg3 are rapidly internalized (Aoyama and Kitajima, 1999; Calkins *et al.*, 2006; Delva *et al.*, 2008; Jennings *et al.*, 2011; Milner *et al.*, 1989; Sato *et al.*, 2000). Interestingly, plakoglobin, but not desomplakin or the plakophilins, appear to be associated with the majority of these internalized IgG/Dsg complexes (Calkins *et al.*, 2006; Jennings *et al.*, 2011). Subsequent to internalization, the PV IgG-Dsg3 complex was found to traffic to endosomal and lysosomal compartments (Calkins *et al.*, 2006; Jennings *et al.*, 2011), leading to a decrease in both cell surface and steady-state Dsg3 levels (Calkins *et al.*, 2006; Jennings *et al.*, 2011; Saito *et al.*, 2012a; Yamamoto *et al.*, 2007). The Triton-soluble or non-desmosomal pool of Dsg3 appears to be depleted as early as 30 minutes after exposure to PV IgG, while the triton-insoluble desmosomal pool

begins to decrease by 6 hours (Aoyama and Kitajima, 1999; Jennings *et al.*, 2011; Sato *et al.*, 2000; Yamamoto *et al.*, 2007). Work by Delva et al. revealed that PV IgG-induced endocytosis occurs through a clathrin-independent, lipid raft-mediated pathway (Delva *et al.*, 2008). Furthermore, disruption of lipid rafts with cholesterol perturbing drugs prevented both the loss of Dsg3 cell surface levels and the loss of keratinocyte adhesion (Delva *et al.*, 2008; Stahley *et al.*, 2014). Ongoing studies in the Kowalczyk lab indicate that PV IgG causes Dsg3 clustering and colocalizes with the early endosomal marker EEA1 in PV patient biopsies (Stahley, *unpublished,* Figure 17). Collectively, these studies suggest that clustering and destabilization of cell surface pools of Dsg3 are characteristics of keratinocytes exposed to PV IgG *in vitro* and in patients, and that these alterations in Dsg3 localization may contribute to PV pathogenesis.

4.2.5 Cell signaling

A growing body of evidence has implicated other cellular responses in pemphigus pathogenesis, including several cell signaling pathways. EGFR, PKC, Src, p38MAPK, RhoA, c-myc, HSP27, AChR, TNFα and tyrosine kinase pathways have all been implicated in contributing to PV IgG-induced loss of adhesion (Berkowitz *et al.*, 2005; Berkowitz *et al.*, 2006; Chernyavsky *et al.*, 2007; Delva *et al.*, 2008; Getsios *et al.*, 2004b; Jolly *et al.*, 2010; Koga *et al.*, 2013; Nguyen *et al.*, 2000; Saito *et al.*, 2012a; Sanchez-Carpintero *et al.*, 2004; Sayar *et al.*, 2014; Seishima *et al.*, 1999; Spindler and Waschke, 2011; Waschke *et al.*, 2006; Williamson *et al.*, 2006). Specifically, inhibition of cell signaling pathways was found to prevent the loss of adhesion induced by PV IgG in several cell culture and mouse models of the disease (Berkowitz *et al.*, 2005;

Berkowitz *et al.*, 2006; Delva *et al.*, 2008; Saito *et al.*, 2012a; Sanchez-Carpintero *et al.*, 2004; Waschke *et al.*, 2006; Williamson *et al.*, 2006). Although various pathways have been implicated in PV pathogenesis, several labs have used both cell culture and mouse models to establish roles for EGFR and p38MAPK signaling in Dsg3 endocytosis and the loss of keratinocyte adhesion in response to PV IgG (Bektas *et al.*, 2013; Berkowitz *et al.*, 2005; Berkowitz *et al.*, 2006; Jolly *et al.*, 2010; Saito *et al.*, 2012a; Sayar *et al.*, 2014). EGFR is activated in response to the pathogenic IgG AK23, followed by an increase in c-myc levels (Schulze *et al.*, 2012). Additionally, both heat shock protein 27 (HSP27) and p38MAPK were rapidly phosphorylated in response to PV IgG. p38MAPK inhibition prevented HSP27 phosphorylation and cytoskeletal reorganization in cultured keratinocytes (Berkowitz *et al.*, 2005) and the loss of adhesion in a mouse model of PV (Berkowitz *et al.*, 2006). Furthermore, increased phosphorylation of both p38MAPK and HSP27 were detected in PV patient skin (Berkowitz *et al.*, 2008). Experiments in cultured keratinocytes revealed that EGFR activation is downstream of p38MAPK and that inhibition of EGFR prevented PV IgG-induced Dsg3 endoctyosis, keratin retraction and the loss of cell adhesion (Bektas *et al.*, 2013). Though p38MAPK has been shown to be upstream of EGFR activation, EGFR inhibitors have also been shown to suppress p38MAPK activity (Li *et al.*, 2014), suggesting interplay between EGFR and p38MAPK signaling in the context of PV pathogenesis.

A role for the actin cytoskeleton in regulating desmosome adhesion in the context of PV pathogenesis has also emerged. Actin disruption by latrunculin exacerbated the effects of PV IgG (Jennings *et al.*, 2011), whereas stabilization of the actin cytoskeleton by jasplakinolide blunted the effects of PV IgG (Gliem *et al.*, 2010). Consistent with

actin regulatory mechanisms modulating desmosomal responsiveness to PV IgG, several studies have implicated the GTPase RhoA, a key regulator of actin organization, in pemphigus. PV IgG-induced RhoA inactivation was found to be p38MAPK-dependent, and experimental activation of RhoA prevented PV IgG-induced loss of adhesion in both cultured cells and human skin. RhoA activation also prevented the loss of Dsg3 from the cytoskeleton-bound fraction typically observed upon PV IgG treatment (Waschke *et al.*, 2006). Recently, adducin was shown to be rapidly phosphorylated at serine 726 in a PKC-dependent and p38MAPK-independent manner in response to PV IgG. Adducin is an actin-organizing protein that is a substrate for PKC and a target for RhoA. The protective effect of RhoA was dependent on the presence of adducin, as well as its phosphorylation (Rotzer *et al.*, 2014). PKC signaling has also been implicated in pemphigus and is activated by PV IgG binding. Activation of PKC causes desmosomes to become calcium-dependent and less adhesive (Osada *et al.*, 1997; Wallis *et al.*, 2000). Recently, DP has been demonstrated to be phosphorylated in a PKC-dependent manner shortly after PV IgG treatment in cultured keratinocytes (Dehner *et al.*, 2014). Collectively, these studies suggest that mechanisms involving RhoA, the actin cytoskeleton, and PKC may be involved in keratinocyte responses to PV IgG ligation of Dsg3. It is interesting to speculate that various drugs (eg. RhoA activation) that protect against PV IgG-induced loss of adhesion may do so by strengthening other junctional complexes rather than acting directly on desmosomes. There is substantial evidence that the functional status of adherens junctions, which are actin associated, can influence desmosome assembly and disassembly (Getsios *et al.*, 2004b; Gumbiner *et al.*, 1988; Kitajima, 2013; Vasioukhin *et al.*, 2000). It will be important to further understand this

crosstalk in the search for new therapeutics that may regulate overall cell-cell contact in the context of pemphigus and other disorders that are characterized by altered cell adhesion, such as cancer (see below).

4.2.6 Desmosome non-assembly vs. disassembly

A common theme that has arisen from studies of pemphigus pathomechanisms is that PV IgG destabilize the homeostasis of desmosome assembly and disassembly kinetics, presumably by disrupting cadherin dynamics (Kitajima, 2013, 2014). One hypothesis is that autoantibodies bind to and down-regulate non-desmosomal Dsg3, thereby upsetting the balance of Dsg3 exchange into and out of the desmosome (Sato *et al.*, 2000). The effect of PV IgG on depletion of non-desmosomal pools of Dsg3 would result in the depletion of desmosomal Dsg3 due to constitutive exchange of Dsg3 out of desmosomes, thereby leading to desmosomes with weakened adhesive interactions. Support for this hypothesis is manifest in the observation that PV IgG depletes desmosomes of Dsg3 by approximately 80%, leading to a 40% reduction in adhesive strength in a dissociation assay (Yamamoto *et al.*, 2007). Furthermore, immunoelectron microscopy time-course experiments of desmosome assembly suggest that PV IgG-Dsg3 complexes that form outside of the desmosome are excluded from entering the mature desmosome core (Aoyama and Kitajima, 1999; Sato *et al.*, 2000). Overall, these studies support a model in which PV IgG bind to the assembly pool of Dsg3 and prevent these molecules from entering desmosomes, thereby leading to Dsg3 depleted desmosomes which are vulnerable to mechanical stress (Kitajima, 2013).

An alternative and complementary hypothesis is that PV IgG drive disassembly by increasing the exchange of Dsg3 out of mature desmosomes. During normal desmosome assembly, the desmosomal cadherin-plakoglobin complex is trafficked to the plasma membrane separately from the desmoplakin-plakophilin complex (Green *et al.*, 2010; Nekrasova and Green, 2013). Once at the surface, the cadherin and plaque complexes are thought to associate to drive desmosomal cadherin clustering and tight packing of desmosomal constituents. It is reasonable to speculate that the reverse process occurs during desmosome disassembly and turnover. Post-embedding immunoelectron microscopy revealed that the majority of IgG in the PV mouse model localized to desmosomes and was uniformly distributed along the entire length of desmosomes (Shimizu *et al.*, 2004). Additionally, IgG on the surface of keratinocytes was found to have a strong affinity for desmosomal structures *in vivo* (Iwatsuki *et al.*, 1989). Furthermore, endocytosis of Dsg3-plakoglobin complexes occurs independently from desmoplakin dislocation from the desmosomal plaque in cell culture models of PV. These observations are consistent with the hypothesis that PV IgG target desmosomal pools of cadherin and trigger disassembly of Dsg complexes. Further support of a desmosome disassembly hypothesis comes from a study using AK23 isotypes. An experimentally constructed IgM isotype of AK23 was found at the edges of desmosomes and between desmosomes, presumably bound to non-desmosomal Dsg3, but was excluded from the desmosomal core where AK23 IgG is frequently located. Importantly, and unlike the IgG isotype, AK23 IgM was unable to induce blistering in mice, indicating that binding to the desmosomal core is necessary to confer pathogenicity (Tsunoda *et al.*, 2011). These findings suggest that anti-Dsg3 IgG, and thus PV IgG, are only pathogenic when they

bind within the extracellular region of intact desmosomes, consistent with the hypothesis that autoantibodies are triggering disassembly of intact desmosomes. Additional experiments using advanced optical imaging approaches and time lapse are needed to sort out precisely how assembly and disassembly kinetics are impacted by PV IgG binding to Dsg3.

4.3 Model for PV pathomechanisms and therapeutic implications

As outlined above, pemphigus research over the past 20 years supports a multifactorial model for pathophysiological mechanisms that result in epidermal blistering, including steric hindrance, endocytosis and cell signaling (Figure 6). First, it is clear that anti-Dsg autoantibodies are both necessary and sufficient to disrupt adhesion and cause disease (Table 2). PV IgG bind to surface pools of Dsg3, causing clustering and disorganization of both desmosomal and non-desmosomal pools of the protein. A subset of the autoantibodies act directly to block Dsg3 adhesive interactions, perhaps by sterically hindering protein interactions, thus weakening cell adhesion. Clustering of surface Dsg3, along with unbinding of adhesive interactions, increases the rate of raft-mediated internalization and degradation of desmosomal protein complexes. This depletion of surface Dsg3 further weakens cell-cell adhesion. The physical effects of the antibodies are integrated with IgG-induced alteration of several signaling pathways, including p38MAPK and other pathways outlined above. Disrupted signaling activity serves to feed forward the pathogenic response to the autoantibodies by further weakening adhesive interactions, enhancing endocytosis, and/or uncoupling cytoskeletal linkages.

The value of resolving the precise pathogenic mechanisms of pemphigus IgG is the promise of developing more targeted and safer therapies to effectively treat this class of disorders. Corticosteroids and immunosuppressive agents are broad-spectrum treatments that have a wide range of potential side-effects while also predisposing the patient to infections. In addition to more selective immunological suppression, agents that stabilize and/or enhance desmosome dynamics in favor of strong adhesion may represent an important addition to current therapeutic approaches. Tucker et al. recently demonstrated that increased expression of the plaque protein plakophilin 1 protected cultured keratinocytes from PV IgG-induced desmosome disruption and loss of adhesion (Tucker *et al.,* 2014). This work supports the idea that cytoplasmic molecular interactions within the plaque can be modulated to bolster desmosomal strength to prevent disease. Further, the authors provided evidence that plakophilin 1 expression prompted the transition of calcium-dependent desmosomes into calcium-independent, hyperadhesive desmosomes, a state typical of fully mature desmosomes *in vivo*. PKC inhibition has been shown to induce desmosome hyperadhesion (Wallis *et al.,* 2000). Importantly, hyperadhesive desmosomes are resistant to the effects of PV serum, likely through mechanisms involving PKC inhibition (Cirillo *et al.,* 2010). Similarly, expression of a desmoplakin point mutant (S2849G) that exhibits enhanced association with keratin filaments and causes desmosomes to become hyperadhesive (Hobbs and Green, 2012), was shown to ameliorate PV IgG-induced loss of cell adhesion (Dehner *et al.,* 2014). Collectively, these studies suggest that reinforcing desmosomal adhesion through altered expression of desmosomal genes and/or exploiting the ability of the desmosome to

achieve hyperadhesion may be an effective strategy to prevent acantholysis in pemphigus.

An additional promising target for therapies is lipid rafts, for which a critical role in regulating desmosomal adhesion is emerging. Membrane rafts, microdomains enriched in sphingolipids and cholesterol, can cluster to form larger, ordered domains that function as platforms for various cellular processes including signaling, endocytosis and adhesion (Pike, 2006; Simons and Ehehalt, 2002; Simons and Sampaio, 2011). Recent studies have supported a role for raft association of desmosomal components in regulating adhesion. All desmosomal proteins have been shown to be raft-associated under normal cell culture conditions(Brennan *et al.*, 2012; Nava *et al.*, 2007; Resnik *et al.*, 2011; Roberts *et al.*, 2014; Stahley *et al.*, 2014). Functional rafts are also required for desmosome assembly and adhesion (Resnik *et al.*, 2011; Stahley *et al.*, 2014). Interestingly, PV IgG-induced Dsg3 endocytosis occurs through a lipid-raft mediated pathway (Delva *et al.*, 2008). Further, raft perturbation through cholesterol depletion was shown to prevent entry of Dsg3 into PV IgG-induced endocytic structures termed “linear arrays” (Jennings *et al.*, 2011) and the loss of keratinocyte adhesion, in addition to preventing proper assembly during a calcium switch assay (Stahley *et al.*, 2014). Collectively, these data indicate that raft disruption alters the ability of Dsg3 to traffic normally into and out of the desmosome during both assembly and disassembly, suggesting that raft-targeting drugs may be therapeutic in modulating Dsg3 dynamics to favor the formation of stable desmosomes. Rafts may also function as signaling hubs for desmosomal regulatory proteins such as EGFR, p38MAPK and Src, all of which have been found to associate with lipid rafts (Simons and Toomre, 2000). Whether these proteins signal from lipid rafts during PV

pathogenesis remains to be elucidated. Nonetheless, raft-perturbing agents have potential to be effective in modulating desmosome responsiveness to pemphigus IgG.

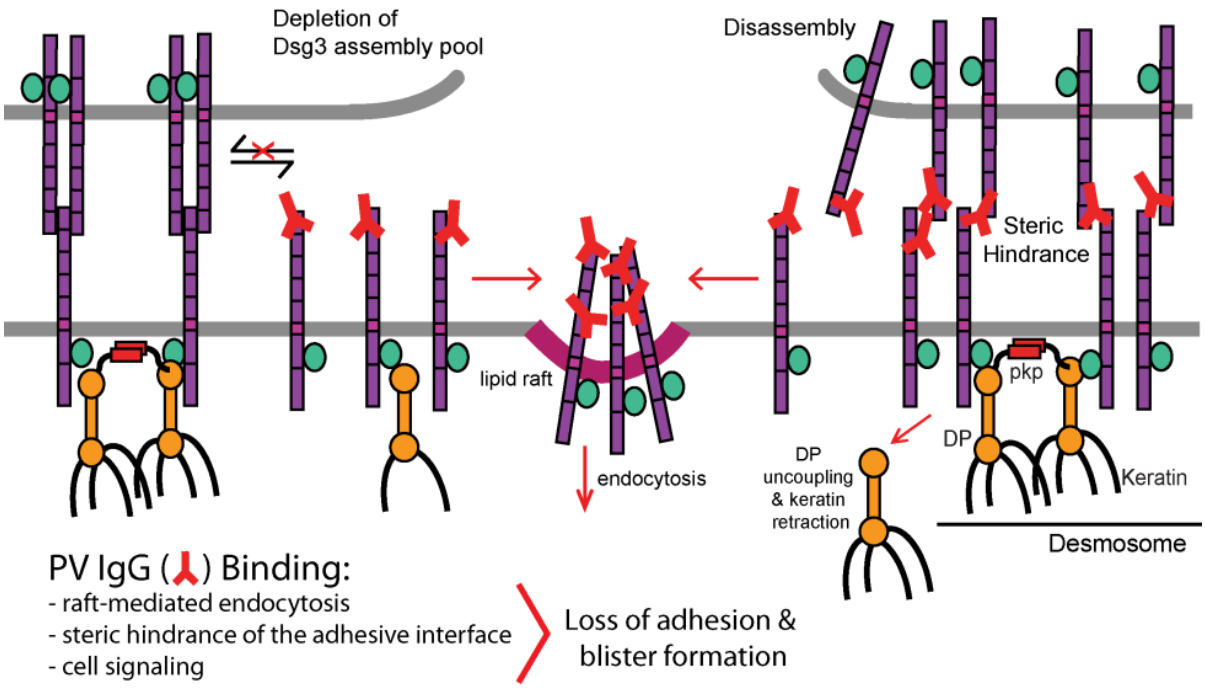

Figure 6. Model for PV pathomechanisms. Data support a multifactorial model for pathophysiological mechanisms resulting in epidermal blistering. PV IgG binding results in raft-mediated endocytosis of Dsg3, steric hindrance of the adhesive interface, as well as the involvement of cell signaling pathways which collectively contribute to the loss of adhesion and subsequent blister formation.

Chapter 5

Desmosome assembly and disassembly are membrane raft-dependent

This chapter is adapted from:

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5.1 Introduction

The desmosome is an intercellular junction that mediates strong adhesion and anchors the intermediate filament cytoskeleton to the plasma membrane at sites of cell-cell contact (Delva *et al.*, 2009; Getsios *et al.*, 2004b; Saito *et al.*, 2012b). Desmosomes are prominent in tissues that experience substantial mechanical stress, such as the skin and heart (Desai *et al.*, 2009; Green and Simpson, 2007). Adhesive interactions in the desmosome are mediated by desmogleins and desmocollins, members of the cadherin superfamily of adhesion molecules (Saito *et al.*, 2012b). Desmosomal plaque proteins, including plakoglobin and desmoplakin, tether the cytoplasmic tails of the desmosomal cadherins to the intermediate filament cytoskeleton. Plakophilins, a subgroup of the armadillo family, further cluster desmosomal cadherin complexes. This architectural arrangement integrates intercellular adhesive interactions and cytoskeletal elements, thereby mechanically coupling adjacent cells (Delva *et al.*, 2009; Getsios *et al.*, 2004b; Holthofer *et al.*, 2007; Yin and Green, 2004). Importantly, the function of both the desmosomal cadherins and the plaque proteins is essential for establishing and maintaining strong cell-cell adhesion, as evidenced by the numerous genetic, auto-immune, and infectious diseases that result when desmosomal protein function is compromised (Amagai and Stanley, 2012; Kottke *et al.*, 2006; Payne *et al.*, 2004; Thomason *et al.*, 2010; Waschke, 2008).

Although desmosomes mediate strong cell-cell adhesion, these structures are dynamic and exhibit tissue and differentiation specific changes in size and composition. The dynamics of desmosome assembly and disassembly must be precisely controlled to yield a junction both rigid enough to provide mechanical integrity to tissues, yet plastic

enough to allow for remodeling during wound healing and development (Green *et al.*, 2010). Alterations in desmosome assembly and disassembly are thought to compromise desmosome function in diseases such as the autoimmune blistering disease pemphigus vulgaris (PV) (Getsios *et al.*, 2010; Green *et al.*, 2010; Kitajima, 2013). In PV, IgG auto-antibodies target the extracellular domain of the desmosomal cadherin desmoglein 3 (Dsg3), or both Dsg3 and Dsg1 (Amagai, 2010; Amagai *et al.*, 1991; Anhalt and Diaz, 2004; Getsios *et al.*, 2010; Payne *et al.*, 2004; Waschke, 2008). Histologically, the pemphigus family of diseases is characterized by the loss of adhesion, or acantholysis, between adjacent keratinocytes. Clinically, PV manifests as severe mucosal erosions as well as epidermal blisters (Kottke *et al.*, 2006; Waschke, 2008).

Recently, we and others have demonstrated that PV IgG aberrantly clusters cell surface Dsg3 (Oktarina *et al.*, 2011; Saito *et al.*, 2012a), leading to increased Dsg3 endocytosis and decreased steady state levels of Dsg3 at the plasma membrane (Calkins *et al.*, 2006; Yamamoto *et al.*, 2007), resulting in desmosome disassembly. PV IgG-induced internalization occurs via a membrane raft-mediated pathway (Delva *et al.*, 2008), indicating that Dsg3 raft association provides a means for desmosome regulation. Also known as lipid rafts or detergent resistant membranes (DRMs), membrane rafts (here, simply referred to as rafts) are highly ordered microdomains within the plasma membrane enriched in cholesterol and sphingolipids (Pike, 2006; Simons and Ehehalt, 2002). Individual raft domains contain a small subset of select proteins and float freely within the membrane, but can cluster to form larger, ordered domains that function as platforms for a variety of cellular processes, such as signaling, endocytosis and membrane organization (Simons and Ehehalt, 2002; Simons and Sampaio, 2011).

Therefore, we speculated that rafts regulate the dynamics of desmosome assembly and disassembly, and thereby modulate normal keratinocyte adhesion, as well as keratinocyte responses to PV IgG. Indeed, several recent studies have demonstrated that desmosomal proteins, including Dsg2, Dsc2, plakoglobin and desmoplakin are raft associated (Brennan *et al.*, 2012; Nava *et al.*, 2007; Resnik *et al.*, 2011). Furthermore, classical preparations of desmosomes isolated from bovine snout are enriched in cholesterol and sphingolipids, providing further evidence of a tight association of desmosomes with membrane raft components (Drochmans *et al.*, 1978; Skerrow and Matoltsy, 1974).

In the current study, we sought to determine if the PV antigen Dsg3 is also raft associated and if rafts play a functional role in regulating desmosomal adhesion. Using primary human keratinocytes, we demonstrate that Dsg3 is raft associated biochemically and colocalizes with raft markers as assessed by super resolution immunofluorescence microscopy. Disruption of membrane rafts via cholesterol depletion prevents desmosome assembly in response to increased extracellular calcium, thus establishing a role for rafts as critical regulators of desmosome formation. Interestingly, Dsg3 did not partition to rafts in cells lacking desmosomal proteins. Furthermore, in response to PV IgG, cell surface Dsg3 reorganizes into linear arrays, membrane projections that extend perpendicular from cell-cell borders. Super resolution immunofluorescence microscopy revealed that these linear arrays, which we have previously found to be active sites for Dsg3 endocytosis (Jennings *et al.*, 2011), are highly enriched in raft markers. Importantly, raft disruption prevents linear array formation, desmosome disassembly and the loss of cell adhesion in PV IgG treated cells. These results support a model in which

membrane raft microdomains serve as a critical platform for the regulation of both desmosome assembly and disassembly.

5.2 Results

5.2.1 Dsg3 and other desmosomal proteins are membrane raft associated

To assess desmosomal protein association with membrane rafts, detergent resistant membranes (DRMs) were isolated from primary human keratinocytes. Following extraction in cold Triton X-100 and ultracentrifugation, buoyant DRMs and associated proteins partition to a characteristic density (~25%) on a sucrose gradient (Lingwood and Simons, 2007). Western blot analysis confirmed Dsg3 raft association with DRMs (Figure 7, fractions 6 and 7) as identified by raft markers flotillin-1 and caveolin-1, and non-raft marker calnexin. Additionally, desmosomal proteins plakoglobin (PG) and plakophilin 2 (pkp-2) were found to be raft associated. Desmocollin 3 and desmoplakin also displayed partitioning to raft fractions (not shown). E-cadherin, a classical cadherin found in adherens junctions, failed to partition to rafts, demonstrating specificity for an enrichment of desmosomal components in rafts. Super resolution structured illumination microscopy (SIM) was used to determine if Dsg3 colocalized with raft markers at cell-cell borders. CD59, a GPI-anchored protein, and caveolin-1 (Cav-1) are proteins known to localize to membrane rafts and are commonly used as raft markers (Brennan *et al.*, 2012; Hogue *et al.*, 2011). Dsg3 was found to colocalize with both CD59 and caveolin-1, although to a much greater extent with CD59 (Figure 8), suggesting a potential specificity for Dsg3 in CD59 containing raft domains. In contrast, Dsg3 did not

colocalize with the non-raft marker clathrin. Together, these results suggest that raft association of desmosomal proteins is an integral aspect of desmosome regulation.

5.2.2 Desmosome assembly and adhesion are cholesterol dependent

To test if desmosome assembly was raft dependent, human keratinocytes were treated with methyl β-cyclodextrin (mβCD) during a low to high calcium switch to induce desmosome formation. Cholesterol depletion with mβCD is widely used as a method to disrupt membrane rafts (Lingwood and Simons, 2007; Simons and Toomre, 2000; Zidovetzki and Levitan, 2007). Many cell types, including keratinocytes (Bang *et al.*, 2005; Zidovetzki and Levitan, 2007), remain viable when treated with mβCD doses as high as 20 mM. However, at these doses we observed extensive cell rounding and shape changes (not shown) suggesting non-specific effects not directly attributable to cholesterol depletion. Additionally, high doses (≥ 10 mM) of mβCD exposure lead to cholesterol depletion from both raft and non-raft regions of the membrane, whereas lower doses have been shown to preferentially remove cholesterol from raft membrane domains (Zidovetzki and Levitan, 2007). Therefore, we treated cells with 1 mM mβCD (Figure 9), which did not cause the cell shape changes observed with higher mβCD concentrations. Accumulation of both Dsg3 and DP at cell borders was reduced in cells treated with mβCD (Figure 9A), suggesting that mβCD treatment prevented desmosome assembly. In contrast, border localization of adherens junction protein p120 was largely unchanged with mβCD treatment. A monolayer fragmentation assay was performed to confirm that mβCD treatment weakened adhesive strength (Ishii and Amagai, 2013). Briefly, confluent keratinocyte monolayers were lifted off the culture dish with the enzyme

dispase and then subjected to mechanical stress via pipetting. In this assay, increased monolayer fragmentation is indicative of weakened adhesion. Relative to control, cells switched to high calcium in the presence of mβCD showed a significant increase in fragmentation (Figure 9B). Collectively, these findings indicate that raft disruption prevents desmosome assembly and weakens keratinocyte cell-cell adhesion strength.

To further test the effect of cholesterol depletion on the process of desmosome assembly, human keratinocytes were treated with varying concentrations of mβCD during a low to high calcium switch and then processed by sequential detergent extraction and western blot to distinguish between the non-desmosomal (Triton soluble) and desmosomal (Triton insoluble) pools of DP and Dsg3. The amounts of desmoplakin and Dsg3 in the insoluble or desmosomal pool are an indication of the relative assembly state of desmosomes. Treatment with mβCD caused a dose dependent shift of both desmoplakin and Dsg3 from the Triton insoluble to soluble pool (Figure 9C, D). These results further indicate that desmosome assembly and raft association are intimately coupled.

5.2.3 Dsg3 raft partioning in associated with desmosome assembly

A prediction derived from the results above is that only desmosomal Dsg3 is raft associated. To test this idea, raft association of Dsg3 was first compared between human keratinocytes cultured in low or high calcium medium. Keratinocytes cultured in low calcium medium do not form desmosomes as indicated by the cytoplasmic staining of both Dsg3 and desmoplakin (Figure 10A). However, once exposed to high calcium, keratinocytes readily form desmosomes as indicated by the concentrated border staining

of Dsg3 and desmoplakin (Figure 10A). Interestingly, Dsg3 raft association increased significantly upon shifting cells from low to high calcium conditions (Figure 10B, C). Dsg3 raft association was further analyzed in cell types with varying abilities to form desmosomes. For these experiments, wild type Dsg3.GFP was expressed in normal human keratinocytes and A431 cells, an epidermoid carcinoma cell line that forms desmosomes, or in CHO (Chinese hamster ovary) and HMEC-1 (immortalized human microvascular endothelial cells) cells, both of which lack various desmosomal components and therefore do not form desmosomes. As expected, Dsg3.GFP partitioned to rafts similarly to endogenous Dsg3 in primary HKs and showed substantial partitioning to rafts in A431s (Figure 11A). However, Dsg3.GFP exhibited little or no raft association in CHOs and HMEC-1s (Figure 11B), indicating that Dsg3 preferentially targets to raft fractions in a cell-type specific manner.

5.2.4 PV IgG-induced desmosome disassembly is raft-dependent

Previous work from our laboratory has shown that Dsg3 endocytosis in response to PV IgG occurs via a raft-mediated pathway (Delva *et al.*, 2008). Recently, we have shown that PV IgG causes clustering and rearrangement of cell surface Dsg3 into endocytic linear structures that we have termed linear arrays. These structures extend perpendicularly from cell contacts and are sites for internalization of desmosomal components (Jennings *et al.*, 2011). Given that endoyctic vesicles bud from these arrays and that internalization is raft-mediated, we predicted that raft components were enriched in these structures. Following PV IgG treatment, SIM revealed that raft markers CD59 and caveolin-1 were enriched in linear arrays, and colocalized significantly with Dsg3

relative to the non-raft marker clathrin in cultured human keratinocytes (Figure 12A, C). Fluorescence intensity measurements of lines drawn perpendicularly through the linear arrays confirmed alignment of Dsg3 and raft markers (Figure 12B). To determine if raft-enriched linear arrays also occur in human epidermis, excised human skin was injected with PV IgG and then analyzed by SIM (Figure 12D). Dsg3, along with the raft marker CD59, was found in linear array structures remarkably similar to those observed *in vitro* (Figure 12A). These results confirm enrichment of raft components in linear arrays, suggesting that linear array formation and subsequent desmosome disassembly are raft-dependent.

To test the possibility that PV IgG-induced linear array formation and loss of adhesion are raft-dependent, human keratinocytes were cultured in high calcium media to first allow for desmosome assembly, and subsequently treated with NH or PV IgG either in the absence or presence of mβCD. PV IgG disrupted Dsg3 staining as indicated by extensive cell surface clustering and linear array formation (Figure 13A). These morphological changes were prevented by mβCD treatment, suggesting that PV IgG-induced desmosome disassembly was abrogated by cholesterol depletion. A fragmentation assay confirmed that desmosomes were functionally protected against PV IgG by mβCD (Figure 13B). These results indicate that desmosome disassembly and loss of cell-cell adhesion in response to PV IgG require functional raft domains.

5.3 Discussion

Significant advances have been made in characterizing desmosomes morphologically and biochemically (Kowalczyk and Rubenstein, 2007). A wide variety

of human diseases have revealed critical functions of these adhesive junctions in the skin and heart (Kottke *et al.*, 2006; Lai-Cheong *et al.*, 2007; Thomason *et al.*, 2010). However, we lack a comprehensive understanding of how desmosome assembly and function are regulated. The results presented here provide insight into how cells spatially control the dynamics of desmosome assembly and how these dynamics are altered to facilitate desmosome disassembly in diseases such as PV. Here, we demonstrate that desmosomal proteins associate with membrane rafts biochemically and that Dsg3 colocalizes with raft markers at cell borders. Dsg3 raft association increased during calcium-mediated desmosome assembly, and raft disruption by cholesterol depletion prevented both desmosome assembly and PV IgG-induced desmosome disassembly. These results support a model in which membrane raft microdomains serve as a crucial platform for desmosome regulation.

Several junctional proteins have been shown to be raft associated, including the tight junction proteins occludin, ZO-1 and JAM-A (Dodelet-Devillers *et al.*, 2009; Nusrat *et al.*, 2000) and the adherens junction protein N-cadherin (Causeret *et al.*, 2005; Nakai and Kamiguchi, 2002). More recently, desmosomal proteins Dsg2, Dsc2, plakoglobin and desmoplakin also have been shown to be associated with membrane rafts (Brennan *et al.*, 2012; Morel *et al.*, 2008; Nava *et al.*, 2007; Resnik *et al.*, 2011). Here, we demonstrate for the first time that Dsg3 and other desmosomal proteins are raft associated in primary human keratinocytes (Figure 7). The association of desmosomal proteins with rafts in cell types ranging from colonic and kidney epithelia to keratinocytes and carcinoma cell lines suggests that lipid raft modulation of desmosome function is a fundamental feature of desmosome regulation. Super-resolution imaging revealed that Dsg3 colocalized with raft

markers CD59 and caveolin-1 at sites of cell-cell borders (Figure 8), suggesting that rafts play a role in mediating desmosome assembly at regions of cell contact.

Current models for desmosome assembly suggest that desmosomal cadherins are stabilized in response to cell-cell contact and cluster with plaque proteins to yield a highly ordered and compact structure (Desai *et al.*, 2009). Our results showing that desmosome assembly is raft-dependent (Figure 9) supports a model in which the raft milieu allows for and facilitates the extensive clustering that yields a mature and tightly packed desmosome. Even with a mβCD dose 10-fold lower than previously reported to weaken desmosomal adhesion (Resnik *et al.*, 2011), we found desmosome assembly and adhesion to be cholesterol dependent (Figure 9). Importantly, Dsg3 was unable to associate with rafts in both CHO and HMEC-1 cells (Figure 11), suggesting that desmosomal or other proteins absent in these cells are responsible for Dsg3 raft targeting. Although Dsg3 colocalizes extensively with CD59 (Figure 8), this protein does not appear sufficient for raft targeting of Dsg3 since HMEC-1 cells express this raft associating protein (Supplemental Figure 1). Collectively, these observations suggest that association of desmosomal proteins with raft domains plays an important role in desmosomal protein clustering and desmosome assembly. Raft dependent protein clustering has been demonstrated for both the immunological synapse and the neuromuscular junction (Balamuth *et al.*, 2004; Bi and Altman, 2001; Pato *et al.*, 2008; Vogt *et al.*, 2002). Evidence suggests that nanoscale rafts coalesce into larger and more stable membrane-ordered assemblies (Lingwood and Simons, 2010; Simons and Sampaio, 2011). Desmosomes may represent a sub-type of these large raft-containing, stable membrane-ordered assemblies which result from the clustering of precursor pools

associated with smaller nanoscale raft subcomplexes. Consistent with this possibility, early studies revealed that the lipid content of desmosome cores is enriched in cholesterol and sphingolipids (Drochmans *et al.*, 1978; Skerrow and Matoltsy, 1974).

Our results investigating compromised adhesion in response to PV IgG also support a model in which raft-dependent clustering enables cadherin endocytosis and desmosome disassembly. We recently demonstrated that PV IgG cause extensive clustering of cell surface Dsg3, an effect that is attributed to the polyclonal nature of anti-Dsg3 IgG present in PV patients (Saito *et al.*, 2012a). This combination of both loss of adhesion and clustering appears to drive Dsg3 endocytosis through a membrane raft pathway. Interestingly, polyclonal antibody-induced clustering of raft-localized proteins, such as placental alkaline phosphatase (PLAP), is cholesterol dependent (Harder *et al.*, 1998). The results in Figure 13 showing PV IgG-induced desmosome disassembly and loss of adhesion are cholesterol dependent, along with our work showing PV IgG cause clustering of cell surface Dsg3, suggest this antibody-induced clustering is raft-mediated. In addition to clustering of Dsg3, we previously demonstrated that PV IgG cause rearrangement of desmosomal proteins into linear array structures that function as sites for endocytosis (Jennings *et al.*, 2011). Consistent with our study showing that PV IgG-induced internalization occurs via a raft-mediated pathway (Delva *et al.*, 2008), super-resolution immunofluorescence imaging confirms that these linear array structures are enriched in raft markers (Figure 12). Furthermore, raft-enriched linear arrays were also present in excised normal human skin treated with PV IgG (Figure 12C). These findings suggest that drugs that alter raft dynamics may have therapeutic potential in treating PV.

The results presented here support a model (Figure 14) in which desmosomal protein association with ordered membrane raft domains is essential for clustering driven by cadherin ectodomain and plaque protein interactions during desmosome assembly, as well as for desmosomal cadherin clustering and endocytosis during disassembly in response to PV IgG. This model accounts for the somewhat counterintuitive notion that disruption of raft microdomains impacts both desmosome formation on the one hand, and desmosome disassembly in the context of PV on the other. In addition to functioning as platforms for desmosomal protein clustering during desmosome formation and disassembly, rafts may also function as signaling hubs for the recruitment of regulatory proteins that modulate desmosome formation and turnover. For example, desmosome disassembly in disease states such as PV has been linked to EGFR, p38MAPK and Src signaling (Chernyavsky *et al.*, 2007; Getsios *et al.*, 2010; Waschke, 2008), all of which have been found to be raft associated (Simons and Toomre, 2000). The ability of rafts to compartmentalize proteins at the membrane is a fundamental mechanism by which domains form platforms of specific composition for various cellular processes (Kabouridis, 2006; Lingwood and Simons, 2010; Simons and Sampaio, 2011). Thus, raft association is likely an efficient mechanism for compartmentalization of desmosomal components and their effectors (kinases, caspases, etc.) to precisely control desmosome assembly and disassembly dynamics. These findings further suggest that manipulation of raft dynamics may be a promising therapy to treat desmosomal diseases, such as pemphigus vulgaris and other disorders where desmosome function or signaling is compromised.

5.4 Materials and Methods

Ethics statement. Use of human IgG and skin was approved by the Institutional Review Boards at Emory University and the University of Pennsylvania. This study used existing or otherwise discarded, de-identified human samples obtained for clinical purposes during medical evaluation or skin surgery repair procedures. These studies were approved for human subjects research exemption (informed consent not required) by the Institutional Review Board at the University of Pennsylvania and the Institutional Review Board at Emory University according to the United States Health and Human Services Code of Federal Regulations 46.101(b)(4).

Cells and culture conditions. Human epidermal keratinocytes were isolated from neonatal foreskin as previously described (Calkins *et al.*, 2006) and cultured in KBM-Gold basal medium (Lonza, Walkersville, MD) with growth supplements (KGM-Gold Single-Quot Kit, Lonza). Keratinocytes, no later than passage 4, were switched to 550 μM calcium 16-18 hours prior to experimental manipulation, unless other noted. For the experiments in Figure 9B and Figure 13B, keratinocytes were grown in 50 μM calcium to prevent desmosome assembly for 16-18 hours and then switched to 550 μM calcium for the indicated times. Chinese hamster ovary (CHO) cells were cultured in F12 media (ATCC, Manassas, VA). HMEC-1s were cultured in 0.1% gelatin-coated flasks in EBM-2 media (Lonza) with growth supplements (EGM-2 MV SingleQuots, Lonza). Where indicated, cells were infected 24-48 hours prior to experimentation with adenovirus for expression of Dsg3.GFP, as previously described (Jennings *et al.*, 2011).

Antibodies and reagents. Antibodies used were as follows: mouse anti-Dsg3 antibodies AK15 and AK23 (Tsunoda *et al.,* 2003) were kind gifts from Dr. Masayuki Amagai (Keio University, Tokyo); mouse anti-Dsg3 antibody 5G11 (Invitrogen, Carlsbad, CA); mouse anti-plakophilin 2 antibody (Biodesign, Saco, Maine); rabbit anti-calnexin antibody (Enzo Life Sciences, Farmingdale, NY); mouse anti-CD59-FITC conjugated antibody (Millipore, Billerica, MA; Invitrogen); rabbit anti-desmoplakin antibody NW6 was a kind gift from Dr. Kathleen Green (Northwestern University); rabbit anti-gamma catenin antibody (plakoglobin, H-80, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-E-cadherin antibody, mouse anti-flotillin-1, rabbit anti-caveolin-1, and mouse anti-clathrin antibodies (BD Biosciences, San Jose, CA). Secondary antibodies conjugated to Alexa Fluors were purchased from Invitrogen while horseradish peroxidase-conjugated secondary antibodies were purchased from BioRad (Hercules, CA). Normal human (NH) IgG was purchased from Bethyl Labs (Montgomery, TX). PV sera were kind gifts from Dr. M. Amagai, Dr. John Stanly (University of Pennsylvania, Philadelphia, PA) and Dr. Robert Swerlick (Emory University, Atlanta, GA). IgG was purified from PV sera using Melon Gel IgG Purification Resins and Kits (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. methyl-β-cyclodextrin (mβCD) was purchased from Sigma (St. Louis, MO). 10 mM mβCD working stock was prepared by dissolving 15mg in 1mL pre-warmed keratinocyte culture medium. The solution was rotated for 30min and subsequently sterile filtered (0.22μm) prior to use.

Isolation of detergent resistant membranes. Detergent resistant membranes were isolated as described previously (Lingwood and Simons, 2007). Briefly, cells were cultured in 25

cm^2 flasks (two per gradient) and washed with PBS^+. Cells were collected by scraping in TNE buffer supplemented with protease inhibitors (Roche Diagnostic GmbH) followed by centrifugation at 0.4 rcf at 4°C for 5 min (5415R, Eppendorf). Cells were re-suspended in TNE buffer and homogenized using a 25-guage needle. TNE buffer with detergent was added to lysate for a 1% Triton-X 100 final concentration followed by incubation on ice for 30 min. 400 μL was mixed with 800 μL 56% sucrose and placed at the bottom of a centrifuge tube. 1.9 mL volumes of 35% and 5% sucrose were layered on top of the sample. Following an 18 hour centrifugation at 4°C (44,000 rpm, SW55 rotor, Beckman Optima LE-80K Ultracentrifuge), 420 μL fractions (1-11, remaining volume combined to make up fraction 12) were removed from top to bottom of the gradient and stored at -20°C until processed for western blot analysis. Flotillin-1 and calnexin were used as raft and non-raft markers respectively. Sucrose concentrations across gradients were measured using a AR200 Digital Refractometer (Leica).

Differential detergent extraction. Keratinocytes were cultured until confluent in 4-well tissue culture plates. Cells were extracted sequentially in Triton buffer (1% Triton X-100, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, with protease inhibitor) followed by extraction with urea-SDS buffer (1% SDS, 8 M Urea, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM EGTA) as described previously (Calkins *et al.*, 2006) and then processed for western blot analysis.

Western blot analysis. Samples were mixed 1:1 with Laemmli sample buffer containing β-mercaptoethanol and heated to 95°C for 5 min. Proteins were resolved by 7.5% SDS-

PAGE and transferred to a nitrocellulose membrane according to standard protocols. Dsg3 was detected using an anti-Dsg3 antibody mixture (AK15 and 5G11). HRP-conjugated secondary antibodies were detected using enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunofluorescence. Human keratinocytes were cultured to 70% confluence on glass coverslips and then fixed on ice in either methanol for 2 min or 4% paraformaldehyde for 10 min followed by 0.2% Triton X-100 for 7 min. Primary antibodies described above were detected with Alexa Fluor-conjugated secondary antibodies. Wide field fluorescence microscopy was performed using a DMRXA2 microscope (Leica, Wetzler, Germany) equipped with a 63x/1.32 NA oil immersion objective and narrow band pass filters. Images were acquired with an ORCA digital camera (Hamamatsu Photonics, Bridgewater, NJ) and processed using Simple PCI software (Hamamatsu Corporation, Sewickley, PA). Super-resolution microscopy was performed using a Nikon N-SIM system on an Eclipse Ti-E microscope system equipped with a 100x/1.49 NA oil immersion objective, 488- and 561-nm solid-state lasers in 3D structured illumination microscopy mode. Images were captured using an EM charge-coupled device camera (DU-897, Andor Technology) and reconstructed using NIS-Elements software with the N-SIM module (version 3.22, Nikon). Colocalization analysis was performed by obtaining Mander's coefficient using ImageJ plugin JACoP (Bolte and Cordelieres, 2006).

Dispase-based fragmentation assay. Keratinocytes were cultured until confluent in 4-well tissue culture plates and processed as previously described (Saito *et al.*, 2012a). Keratinocytes were switched to 50 μM calcium, to ensure no desmosome assembly, 16-18 hours prior to a second switch 550 μM calcium for 3 hours to assemble desmosomes. Where indicated, PV IgG (100 μg/mL) was added for another 3 hours at 37°C. Cells were then incubated with 1 U/mL dispase (Roche) for 30-45 min or until keratinocyte sheets were lifted from the culture dish. Cell sheets were rinsed with PBS^{+} and subjected to mechanical stress via pipetting. Cells were then fixed in paraformaldehyde, stained with methylene blue, and fragments counted using a dissecting microscope.

Human skin explant injections. Normal human skin explants were cultured as previously described (Saito *et al.*, 2012a). PV IgG (160-400 μg) was injected intradermally in the presence of 0.8 μg ETA for 16 hours. Skin sections were processed for structured illumination immunofluorescence using a mouse anti-human CD59-FITC antibody and goat anti-human Alexa Fluor 555 secondary antibody.

Statistics. Statistical analysis of fluorescence colocalization measurements in Figure 8 was performed using Kruskal-Wallis one-way analysis of variance on ranks with multiple comparisons performed by Dunn's method with a significance level of 0.05. Statistical analysis of fluorescence colocalization measurements in Figure 12 and the dispase-based fragmentation assay in Figure 13 were performed using Shapiro-Wilk one-way analysis of variance on ranks with multiple comparisons performed by Holm-Sidak method with a significance level of 0.05. Statistical analysis of the dispase-based fragmentation assay in

Figure 9 and raft association in Figure 10 were performed using a *t*-test assuming unequal variances with a significance level of 0.05.

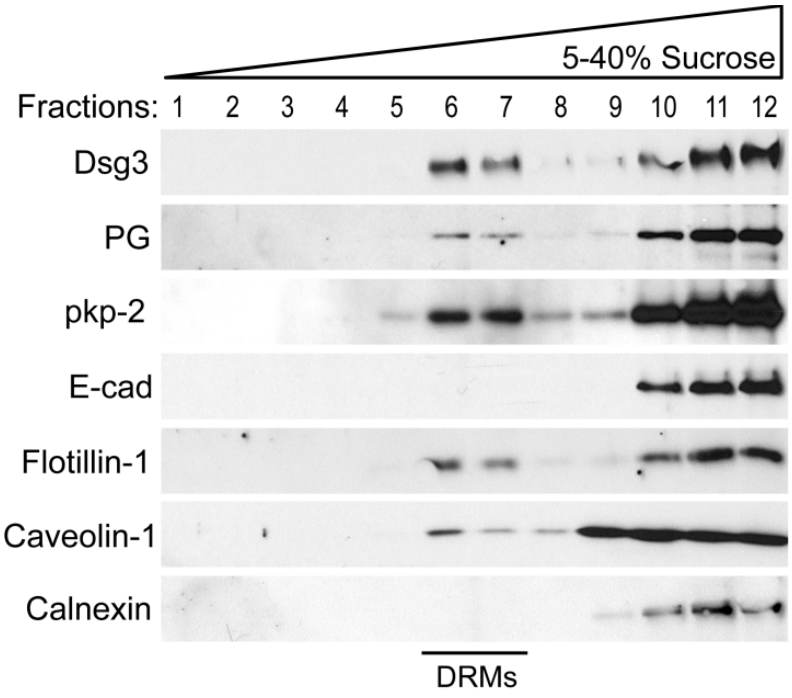


Figure 7. Dsg3 and other desmosomal proteins are membrane raft associated. Primary human keratinocytes were grown to confluence and switched to high calcium media for 16-18 hrs. Following detergent extraction (1% Triton X-100) and ultra-centrifugation on a 5-40% sucrose gradient, 12 fractions were sequentially removed from the gradient and processed via western blot. Dsg3 partitions to the buoyant raft fractions (DRMs, detergent resistant membranes) as indicated by the positive controls flotillin-1 and caveolin-1, and negative control calnexin. Desmosomal components plakoglobin (PG) and plakophilin 2 (pkp-2) were also found to be raft associated. E-cadherin, a classical cadherin of adherens junctions, is not enriched in membrane rafts.

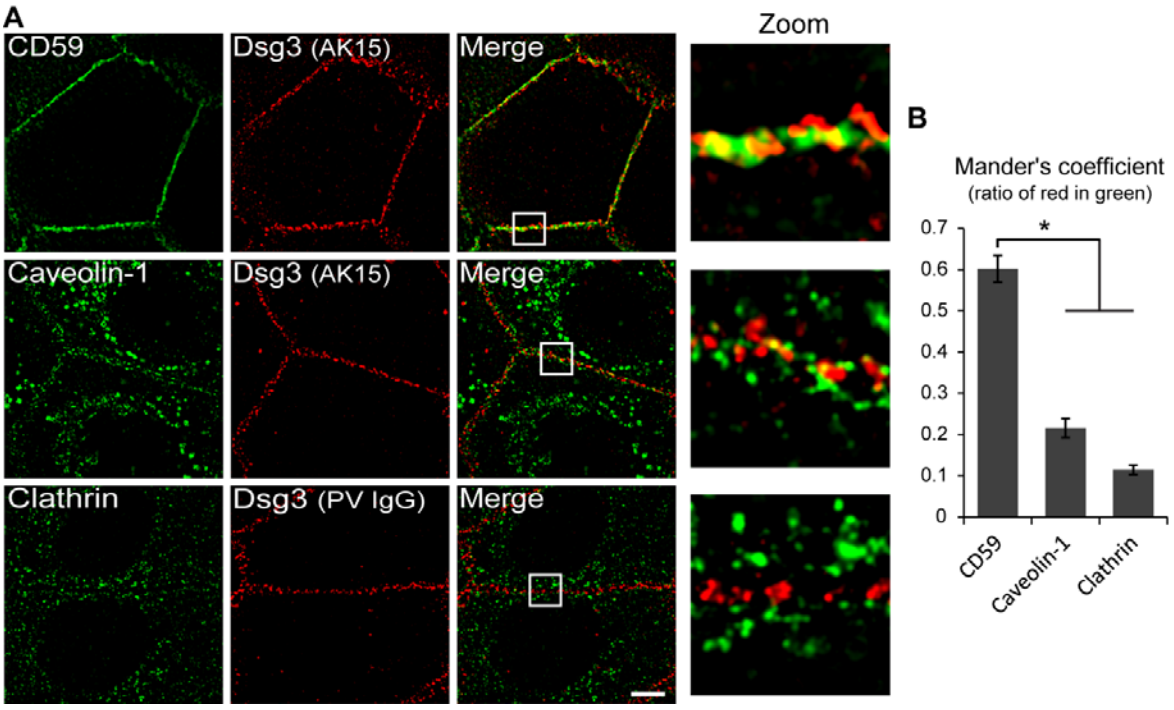


Figure 8. Dsg3 colocalizes with raft markers at cell-cell borders. A) After switching human keratinocytes to high calcium media for 16-18 hrs, surface Dsg3 was labeled live for 10 min with Alexa Fluor 555-conjugated AK15 mAb (top 2 rows) or PV IgG. Dsg3 colocalization with raft markers CD59 (a GPI-anchored protein) and caveolin-1 were compared to colocalization with clathrin, a non-raft membrane component using SIM. Dsg3 colocalized substantially with CD59, moderately with caveolin-1 and very weakly with clathrin. **B)** Quantification of Dsg3 colocalization. Mander's coefficient was used to define the ratio of red fluorescence (Dsg3) found within green (CD59, caveolin-1 or clathrin). Means ± SEM (n = 20-37 border regions); *$p < 0.05$. Scale bar in A, 5 μm.

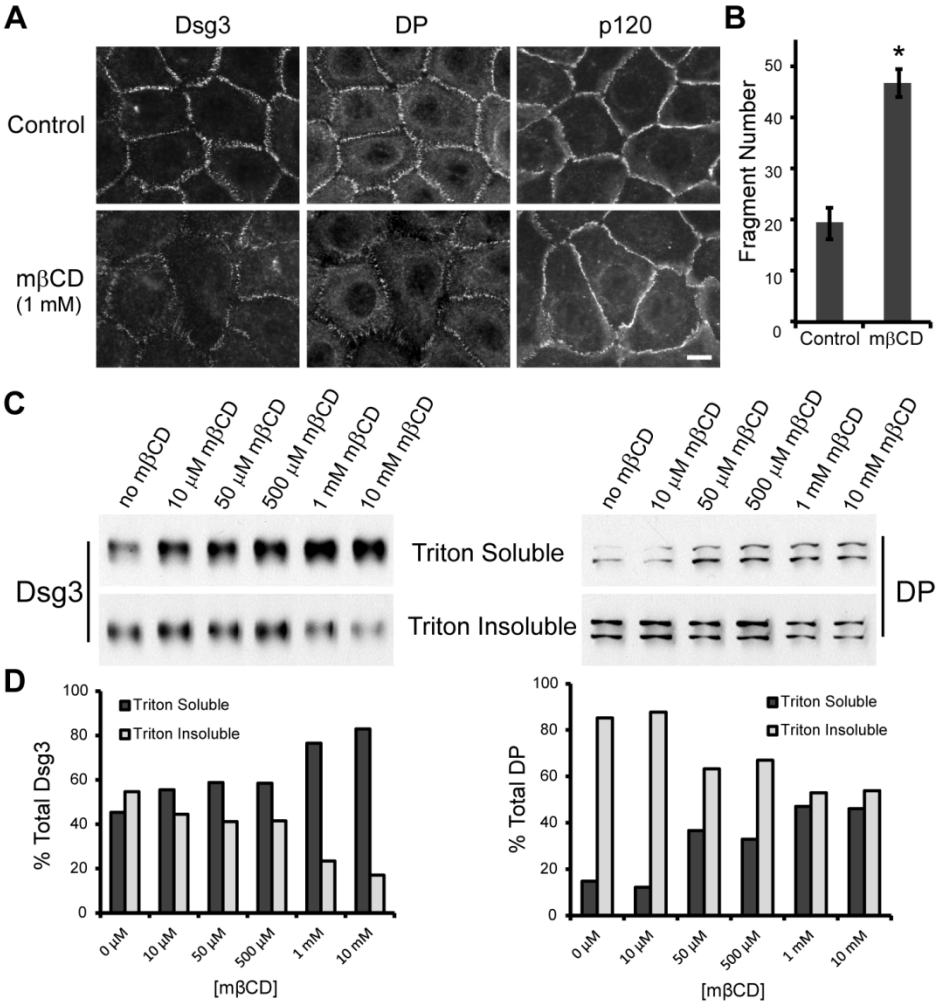

A
Dsg3
DP
p120
Control
mβCD
(1 mM)
B
Fragment Number
50
40
30
20
10
0
*
Control
mβCD
C
no mβCD
10 μM mβCD
50 μM mβCD
500 μM mβCD
1 mM mβCD
10 mM mβCD
Dsg3
Triton Soluble
Triton Insoluble
DP
D
% Total Dsg3
Triton Soluble
Triton Insoluble
100
80
60
40
20
0
0 μM
10 μM
50 μM
500 μM
1 mM
10 mM
[mβCD]
% Total DP
Triton Soluble
Triton Insoluble
[mβCD]

Figure 9. Desmosome assembly and adhesion are cholesterol dependent. A) Human keratinocytes were treated with 1 mM mβCD (methyl-β-cyclodextrin) during a 3 hr switch from 50 μM to 550 μM calcium. Dsg3 was detected by labeling cells live with AK15 mAb during the last 15 min of the calcium switch. Under control conditions (no mβCD), Dsg3 and DP are recruited to cell borders. Border staining of both Dsg3 and DP is dramatically reduced in cells treated with mβCD, while border staining of adherens junction protein p120 remained similar to control. **B)** Dispase-based fragmentation assay after keratinocytes were switched from a 50 μM to 550 μM calcium either in the absence or presence of 1mM mβCD. Cells switched in the presence of mβCD showed a significant increase in the amount of fragmentation relative to control (no mβCD). Means ± SEM (n = 8 monolayers per group); *$p < 0.05$. **C)** Keratinocytes treated with varying concentrations of mβCD during a 6hr switch from 50 μM to 550 μM were processed by sequential detergent extraction with 1% Triton X-100 and western blot to distinguish between the non-desmosomal and desmosomal pools of Dsg3 and DP. mβCD treatment caused a dose dependent shift of both Dsg3 and DP from Triton insoluble (desmosomal) to soluble (non-desmosomal) pool. **D)** Quantification of Dsg3 and DP solubility changes in response to increasing mβCD concentrations. Scale bar in A, 10 μm.

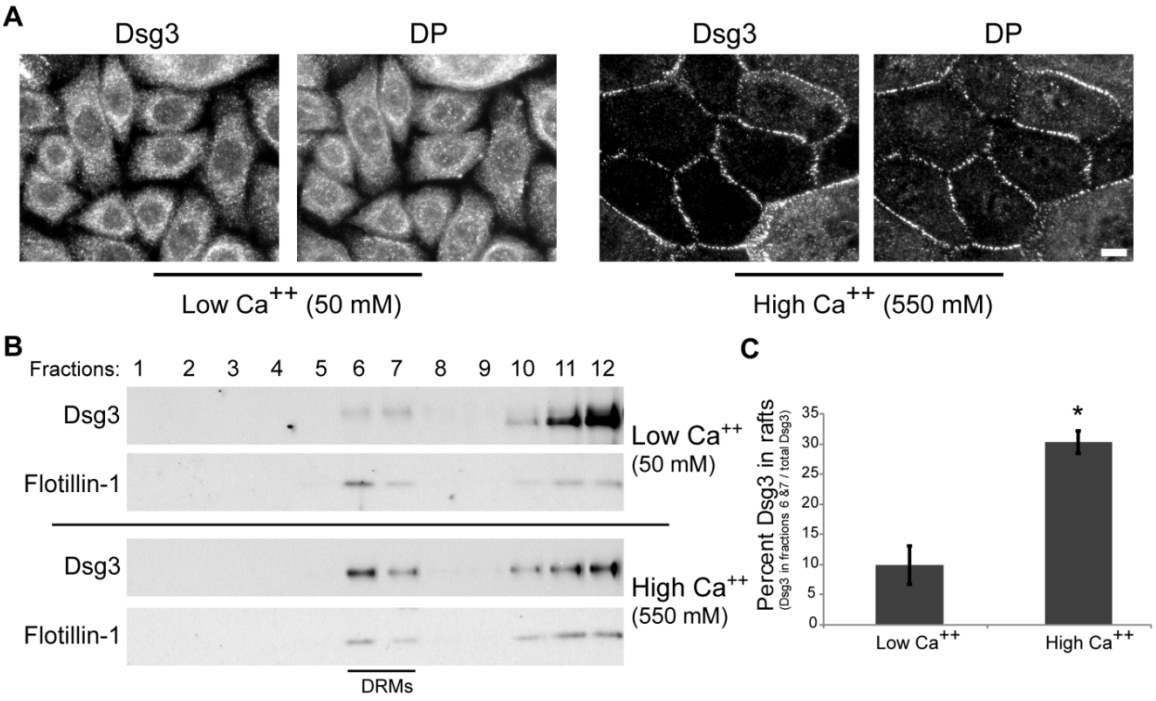


Figure 10. Dsg3 raft association increases upon calcium addition. A) Dsg3 and DP (desmoplakin) remain cytoplasmic when human keratinocytes are cultured in low (50 μM) calcium media. Protein staining increases at regions of cell contact when keratinocytes are cultured in high (550 μM) calcium media and desmosomes assemble. Dsg3 was detected with AK23 mAb post fixation. **B)** Confluent keratinocytes cultured in 50 μM or 550 μM calcium media 16-18 hrs prior to solubilization with 1% Triton X-100 and membrane raft fractionation. Western blots were probed for Dsg3 and the raft marker flotillin-1. Dsg3 raft partitioning increases significantly upon shifting cells from low to high calcium conditions. **C)** Quantification of relative Dsg3 levels normalized to total Dsg3 across all fractions. Means ± SEM ($n = 3$); *$p < 0.05$. Scale bar in A, 10 μm.

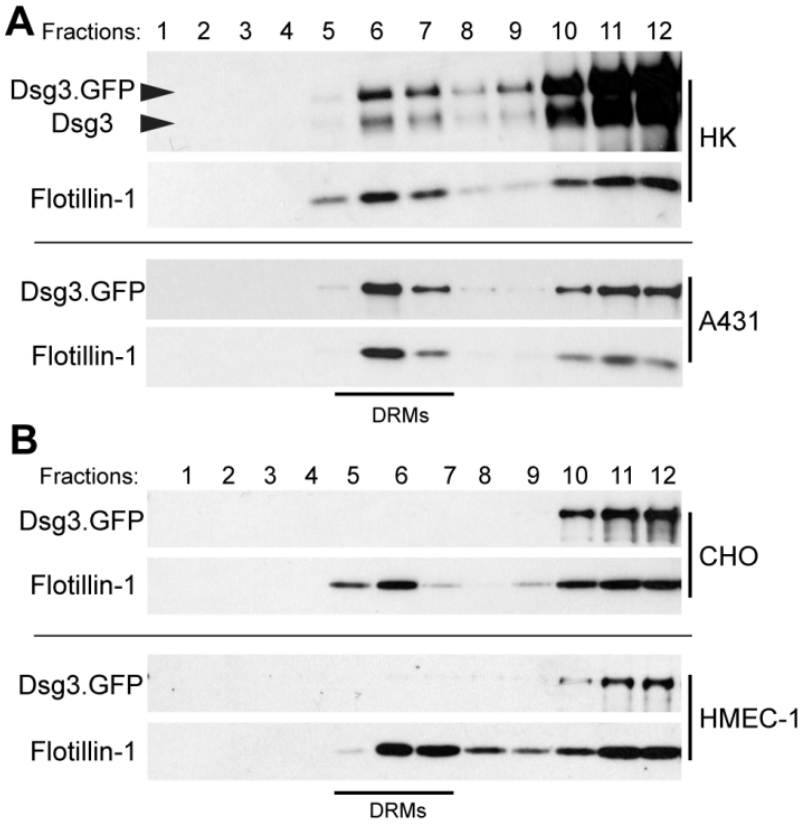


Figure 11. Dsg3 does not partition to rafts in cells lacking desmosomal proteins. A) In human keratinocytes (HKs) cultured in high calcium media for 16-18 hrs and A431 cells, GFP-tagged Dsg3 (top arrow) partitions to rafts similar to endogenous Dsg3. **B)** Dsg3.GFP was expressed in CHO (Chinese hamster ovary) cells and HMEC-1s (immortalized human microvasular endothelial cells), cell types that do not form desmosomes. Dsg3.GFP did not partition to the raft containing fractions in either CHOs or HMEC-1s.

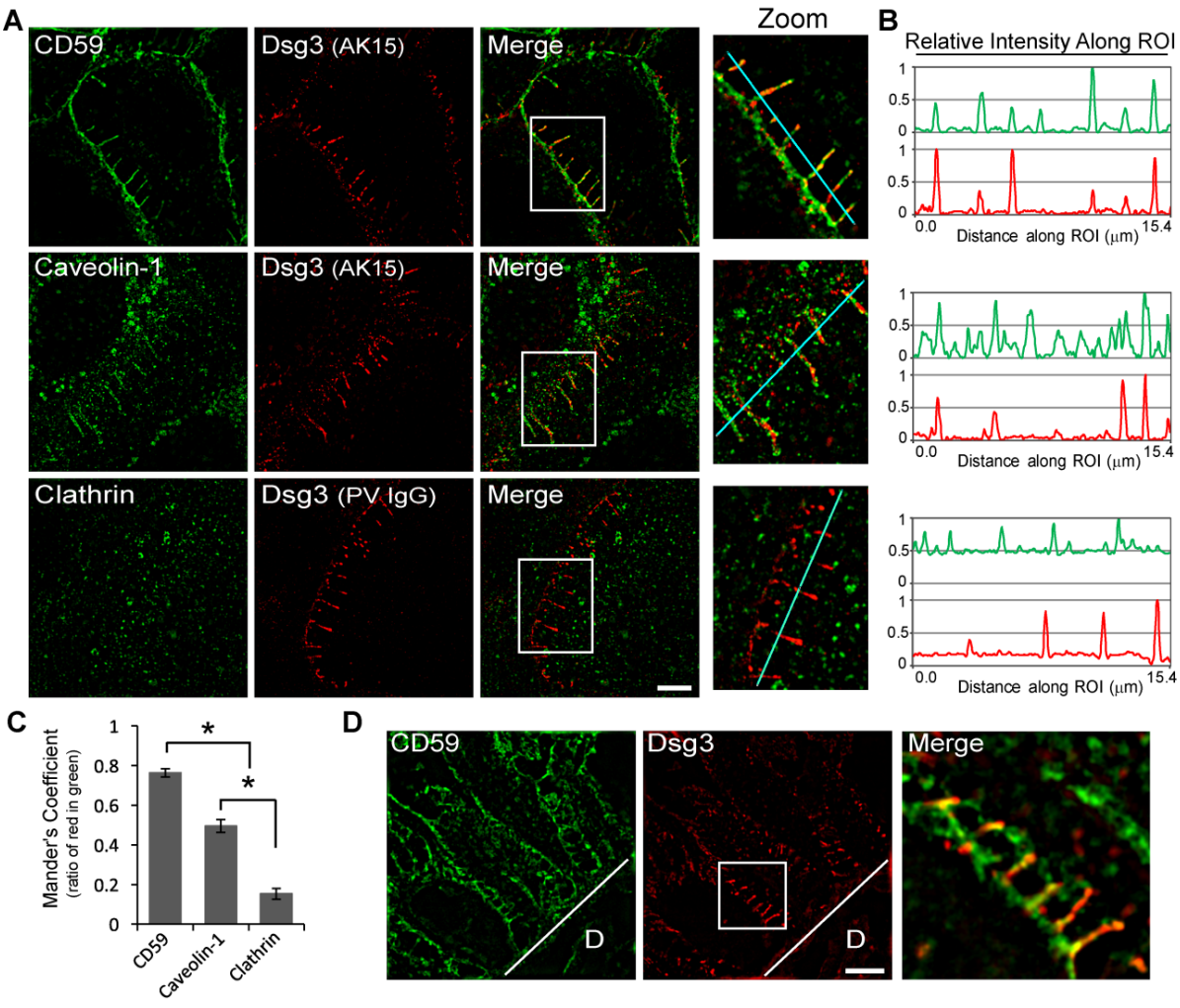

A
CD59
Dsg3 (AK15)
Merge
Zoom
Caveolin-1
Dsg3 (AK15)
Merge
Clathrin
Dsg3 (PV IgG)
Merge
B
Relative Intensity Along ROI
1
0.5
0
0.0
Distance along ROI (μm)
15.4
C
Mander's Coefficient
(ratio of red in green)
1
0.8
0.6
0.4
0.2
0
*
*
CD59
Caveolin-1
Clathrin
D
CD59
Dsg3
Merge
D
D

Figure 12. PV IgG causes redistribution of Dsg3 into raft-containing linear arrays. **A-C)** Dsg3 colocalization with various membrane markers was analyzed using structured illumination microscopy (SIM) in human keratinocytes cultured in high calcium for 16-18 hrs and then treated with PV IgG for 3 hrs. CD59 was detected with FITC conjugated antibody by live labeling for 10 min prior to fixing. Dsg3 was detected with Alexa Fluor 555 conjugated AK15 mAb by live labeling for 10 min prior to fixing for the top two rows. For clathrin colocalization Dsg3 was monitored using PV IgG and secondary antibody detection of human IgG. In response to PV IgG, Dsg3 enters endocytic linear structures (previously termed 'linear arrays') that emanate perpendicular from cell-cell borders and extend toward the cell center (Jennings *et al.*, 2011). **A)** Raft markers CD59 and caveolin-1 were enriched in linear arrays and colocalizaed significantly with Dsg3 relative to the non-raft marker clathrin. **B)** Fluorescence intensity measurements of lines drawn perpendicularly through linear arrays show alignment of Dsg3 (bottom line) and raft marker (top line) fluorescence. **C)** Quantification of Dsg3 colocalization in linear arrays as indicated by Mander's coefficient (ratio of red in green). Means ± SEM (n = 27-36 arrays per group); *$p < 0.05$. **D)** SIM was also used to view Dsg3 colocalization with CD59 in linear arrays in excised normal human epidermis injected with PV IgG. Basal keratinocytes are shown. D, dermis. Scale bar in A and D, 5 μm.

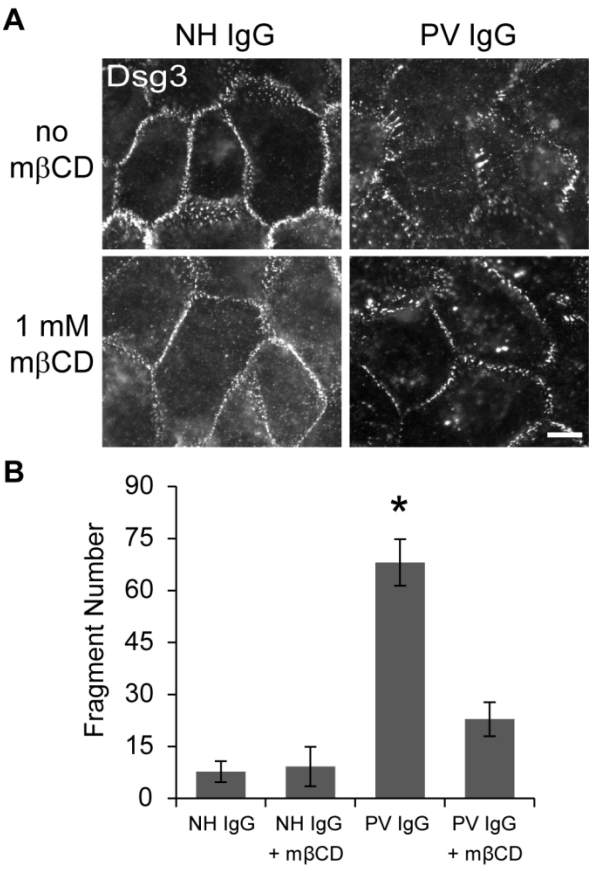


Figure 13. Cholesterol depletion prevents PV IgG-induced Dsg3 redistribution and weakened adhesion. A) Human keratinocytes with assembled desmosomes were treated with NH or PV IgG for 3 hrs either in the absence or presence of 1 mM mβCD. Dsg3 was detected by live labeling with AK15 for 30 min on ice. Keratinocytes treated with PV IgG exhibit disrupted Dsg3 staining (surface clustering and linear array formation). mβCD treatment prevented both Dsg3 clustering and linear array formation in response to PV IgG. **B)** mβCD treatment protected desmosomes against PV IgG-induced fragmentation. Means ± SEM (*n* = 4-8 monolayers per group); *p < 0.05. Scale bar in A, 10 μm.

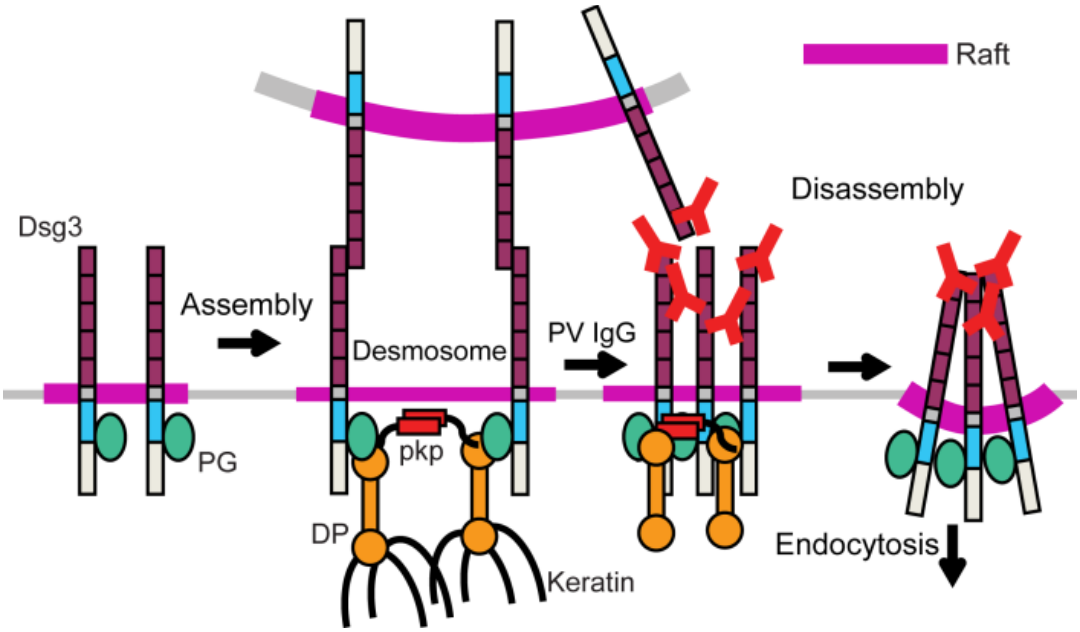
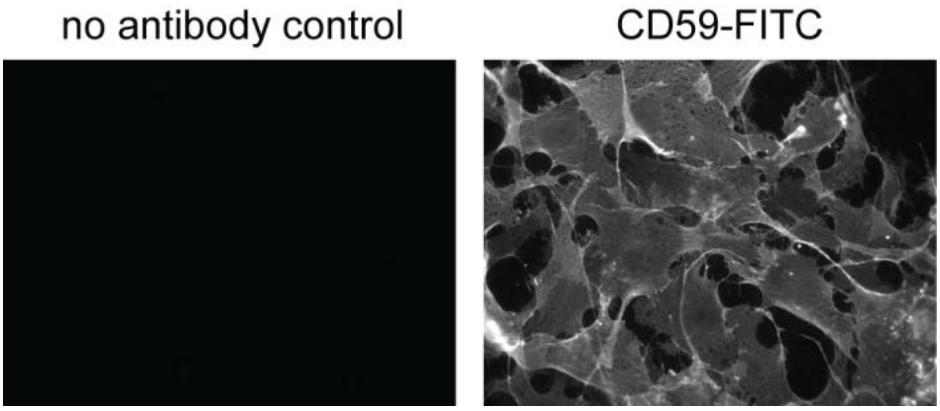


Figure 14. Model for membrane rafts as platforms for desmosome regulation. Desmosomal protein targeting to membrane rafts is required for the extensive clustering driven by cadherin ectodomain and plaque protein interactions during assembly that yields a mature and tightly packed desmosome. When adhesion is compromised (i.e. in response to PV IgG), clustering in a raft facilitates desmosomal cadherin endocytosis. PG, plakoglobin; pkp, plakophilin; DP, desmoplakin.



Supplemental Figure 1. CD59 is expressed on the surface of HMEC-1 cells. HMEC-1s were unlabeled (no antibody control) or labeled live with FITC-conjugated CD59 for 10 min at 37°C. The cells were then fixed in methanol and imaged. Labeling demonstrated that HMEC-1 cells express CD59. Negative control showed a lack of background fluorescence.

Chapter 6

Super-resolution microscopy reveals altered desmosomal protein organization in pemphigus vulgaris patients

This chapter is adapted from:

Stahley SN, Warren MF, Feldman RJ, Swerlick RA, Mattheyses AL and Kowalczyk AP. 2015. Journal of Investigative Dermatology. *Submitted.*

6.1 Introduction

The desmosome is a robust anchoring junction responsible for strong adhesion between neighboring cells (Berika and Garrod, 2014; Kowalczyk and Green, 2013). Desmosomes are present in all epithelia, but are especially prominent in the skin and the heart (Berika and Garrod, 2014; Desai *et al.*, 2009). Because these tissues experience a high degree of mechanical stress, desmosome dysfunction leads to skin and heart defects that are often characterized by tissue fragility (Al-Jassar *et al.*, 2013; Cirillo, 2014; Kottke *et al.*, 2006; Thomason *et al.*, 2010). The desmosome is comprised of desmosomal cadherins, which engage in extracellular adhesive interactions, and cytoplasmic plaque proteins, including plakoglobin, plakophilin and desmoplakin (Kowalczyk and Green, 2013). Desmogelin (Dsg) and desmocollin are transmembrane proteins of the cadherin superfamily that mediate calcium-dependent adhesive interactions between cells. The armadillo family protein plakoglobin links the cytoplasmic tail of the cadherins to desmoplakin, an intermediate filament binding protein and member of the plakin family of cytolinkers. Plakophilin, also an armadillo protein, facilitates lateral interactions between desmosomal cadherin complexes and further strengthens the plaque (Desai *et al.*, 2009; Kowalczyk and Green, 2013). This architectural arrangement of desmosomal proteins couples extracellular adhesive interactions mediated by the desmosomal cadherins to the intermediate filament cytoskeleton, thereby building an integrated adhesive and cytoskeletal network that extends throughout a tissue (Kowalczyk and Green, 2013).

Desmosome assembly and disassembly dynamics must be precisely controlled to allow for both strong adhesion and tissue integrity, but also for plasticity during processes

such as wound healing and development (Nekrasova and Green, 2013). Likewise, alterations in desmosome dynamics are thought to contribute to desmosome disruption and loss of adhesion in disease states, including the autoimmune blistering disease pemphigus vulgaris (PV) (Kitajima, 2013, 2014). Pemphigus is a family of potentially fatal bullous diseases caused by autoantibodies (IgG) directed against the extracellular domain of desmosomal cadherins (Amagai, 2009, 2010). Patients with PV produce autoantibodies that target Dsg3, or both Dsg3 and Dsg1 (Amagai, 2009). PV IgG binding to the desmosomal cadherins results in the loss of keratinocyte adhesion, or acantholysis, between the basal and spinous layers of the epidermis (Amagai, 2010). Clinically, the disease presents as painful mucosal erosions and epidermal blisters (Kneisel and Hertl, 2011a). Although it has been established that anti-Dsg3 antibodies are sufficient to cause disease, the precise pathomechanisms by which PV autoantibodies cause loss of adhesion and blister formation are not fully understood (Stahley and Kowalczyk, 2015).

Much of what we know about PV pathomechanisms comes from *in vitro* work in which cultured keratinocytes are exposed to PV IgG. Previous work by our group and others using cell culture models has revealed that PV IgG cause cell surface Dsg3 to become aberrantly clustered, leading to increased endocytosis and decreased steady state levels of Dsg3 (Calkins *et al.*, 2006; Iwatsuki *et al.*, 1999; Iwatsuki *et al.*, 1989; Jolly *et al.*, 2010; Mao *et al.*, 2014; Mao *et al.*, 2011; Patel *et al.*, 1984; Saito *et al.*, 2012a; Sato *et al.*, 2000). In response to PV IgG, Dsg3 and other desmosomal components arrange into streaks, or linear arrays, which extend perpendicularly from cell-cell borders (Jennings *et al.*, 2011). These structures appear to be associated with lipid raft mediated endocytosis of the Dsg3-PV IgG complex (Delva *et al.*, 2008; Stahley *et al.*, 2014).

Furthermore, multiple signaling pathways, including tyrosine kinases and p38MAPK-dependent pathways, have been implicated in PV pathogenesis (Berkowitz *et al.*, 2005; Berkowitz *et al.*, 2006; Chernyavsky *et al.*, 2007; Delva *et al.*, 2008; Getsios *et al.*, 2004b; Jolly *et al.*, 2010; Koga *et al.*, 2013; Nguyen *et al.*, 2000; Saito *et al.*, 2012a; Sanchez-Carpintero *et al.*, 2004; Sayar *et al.*, 2014; Seishima *et al.*, 1999; Spindler and Waschke, 2011; Waschke *et al.*, 2006; Williamson *et al.*, 2006). Finally, there is substantial evidence suggesting that steric hindrance of Dsg3 mediated adhesion causes keratinocyte acantholysis. The majority of autoantibodies isolated from PV patients are directed against the amino terminal domain of Dsg3, a region of cadherins known to mediate adhesion (Amagai *et al.*, 1992; Di Zenzo *et al.*, 2012; Sekiguchi *et al.*, 2001). Further, monoclonal Dsg3 antibodies that target the adhesive interface cause loss of adhesion in cultured keratinocytes and blistering in mouse models of disease (Payne *et al.*, 2005; Tsunoda *et al.*, 2003). Thus, a large number of studies suggest that multiple mechanisms, perhaps acting synergistically, contribute to PV pathogenesis. However, previous studies have not systematically and directly compared the effects of PV IgG on desmosomal protein organization and function in cell culture models to alterations that occur in PV patient skin.

A significant gap in our understanding of PV pathomechanisms in patients is due to the difficulty in assessing the organization and localization of the various desmosomal proteins in multiple patients at high levels of spatial resolution. While ultrastructural approaches such as electron microscopy provide high resolution, this approach is limited by difficult sample preparation, small sample size, and the challenges associated with quantitatively assessing the colocalization of various desmosomal proteins with other

cellular antigens. In contrast, optical microscopy approaches such as wide-field and confocal immunofluorescence allow for high throughput of samples, but suffer from a lack of spatial resolution sufficient to discern how desmosome structure is altered in disease states such as PV.

Here, we take advantage of recent advances in optical imaging approaches that bridge the resolution gap between electron microscopy and standard immunofluorescence imaging. Using structured illumination microscopy (SIM), a form of super-resolution optical imaging (Galbraith and Galbraith, 2011), we assessed how desmosomes are altered in the skin of PV patients. Further, we directly compared the changes in desmosomes that we observed in patient skin with the alterations in desmosomal protein organization that PV IgG cause in vitro. Our results indicate that desmosomal protein clustering and Dsg endocytosis occur in patient skin, and that these changes are associated with reduced desmosome size and with splitting of the desmosome at the Dsg adhesive interface. We further demonstrate that desmosome splitting can be replicated in cultured keratinocytes by exposing PV IgG treated cells to mechanical stress. These findings reveal new insights into the changes in desmosomal protein organization, trafficking and function that occur in PV patient tissue, and serve to validate the use of purified PV IgG and cultured keratinocytes as an invaluable model system to understand PV pathomechanisms.

6.2 Results and discussion

6.2.1 PV IgG causes desmosomal protein clustering in patient tissue and in cultured keratinocytes

Desmosomal protein organization in the basal layer of the epidermis of normal human (NH) and PV patient tissue was analyzed using structured-illumination microscopy (SIM). PV patients with severe clinical blistering and high Dsg3 ELISA scores (>100) were selected for analysis. Both mucosal dominant and muccocutaneous PV variants were analyzed, as well as lip and skin biopsy sites (Supplemental Table 1).

Biopsies from normal human epidermis were negative for hIgG deposition (Figure 15a). SIM imaging revealed uniform organization of Dsg3, desmoplakin and E-cadherin along cell borders (Figure 15a, top panel). In contrast, PV patient skin exhibited hIgG deposition and disrupted Dsg3 and desmoplakin organization. Importantly, E-cadherin staining remained largely unaltered (Figure 15a, bottom panel). Alterations in junctional protein organization were quantified using a clustering index derived from measurements of the distance between peak fluorescence intensity along cell borders ((Saito *et al.*, 2012a)and Methods). Average Dsg3 and desmoplakin clustering was found to be increased significantly in PV patients (Figure 15b). Clustering scores from individual patients are displayed in Supplemental Figure 2. Altered organization of desmosomal proteins was also observed in lip biopsies from PV patients (Supplemental Figure 3). In addition to Dsg3 and desmoplakin, the desmosomal plaque protein plakoglobin also displayed altered organization in PV patient lip mucosa and epidermis (Supplemental Figure 4). Lastly, we verified that the IgG from each patient analyzed in Figure 15 caused similar alterations in Dsg3 organization in vitro. Samples of IgG from each patient were added to cultured keratinocytes and Dsg3 clustering was assessed. Similar to patient tissue, Dsg3 was also clustered by the IgG from all six PV patients

tested (Figure 15c). Collectively, these results demonstrate that clustering of desmosomal proteins is a hallmark feature of both PV IgG treated cells and patient tissue.

6.2.2 PV IgG associates with lipid raft enriched linear array structures in vivo and is trafficked to endosomes

Dsg3 and other desmosomal proteins have been found to enter linearly organized membranes, or linear arrays, extending perpendicularly from cell-cell borders in both cultured cells and in human epidermal explants exposed to PV IgG (Jennings *et al.*, 2011; Stahley *et al.*, 2014). These linear arrays in cell culture models are enriched in lipid raft markers (Stahley *et al.*, 2014) and are thought to be sites for desmosome disassembly and endocytosis (Jennings *et al.*, 2011). Using SIM analysis of PV patient tissue, we observed that desmoplakin, plakoglobin and the lipid raft marker CD59 colocalized with hIgG in linear array-like structures in PV patient tissue (Figure 16). Similar to cultured cells, adherens junction markers such as E-cadherin (Figure 16) were not detected in linear arrays. To examine further if the Dsg-PV IgG complex colocalizes with lipid raft markers *in vivo*, PV patient issue was immunostained for hIgG and multiple lipid raft markers. Patient IgG (hIgG) colocalized with raft markers CD59 and caveolin-1 both at cell borders and in vesicular-like puncta (Figure 17a). A number of studies using cell culture models of PV have found that the PV IgG-Dsg complex is internalized from the plasma membrane, resulting in degradation of Dsg3 and reduced plasma membrane levels of the adhesive protein (Calkins *et al.*, 2006; Cirillo *et al.*, 2007; Delva *et al.*, 2008; Jolly *et al.*, 2010; Kitajima, 2014; Mao *et al.*, 2014; Mao *et al.*, 2011; Saito *et al.*, 2012a; Sato *et al.*, 2000; Schulze *et al.*, 2012). To determine if human PV IgG is internalized to endocytic

compartments in patient tissue, biopsy sections were stained for hIgG and the early endosomal marker EEA-1. Nearly every cell, particularly near sites of blister formation, displayed multiple puncta at or near the cell periphery containing hIgG and EEA1 (Figure 17b). Interestingly, steady state Dsg3 levels were decreased in PV patient tissue compared to normal human control tissue (Figure 17c, Supplemental Figure 5), further suggesting that PV IgG induces Dsg3 endocytosis and degradation in patient tissue.

6.2.3 Desmosomes are smaller and split in PV patients

Ultrastructural studies of desmosome morphology in PV patient skin and mouse models of disease have suggested that desmosomes either split at the adhesive interface or are reduced in size in tissues exposed to pathogenic IgG (Shimizu *et al.*, 2004; van der Wier *et al.*, 2014). Here, we utilized SIM imaging to assess desmosome morphology in patient skin, and to directly compare these changes to cultured keratinocytes exposed to patient IgG. We took advantage of previous immune-gold EM studies defining the position of various desmosomal proteins (North *et al.*, 1999) and the sub-diffraction limit resolution of SIM in order to identify bona fide desmosomes in tissues and cells using immunofluorescence localization. Using this approach, antibodies directed against the carboxyl terminal domain of desmoplakin result in a “rail-road track” pattern of fluorescence that defines the localization of the desmosomal plaque, whereas antibodies against the Dsg extracellular domain identify the adhesive core (Supplemental Figure 6).

This pattern of desmoplakin rail-road track staining was used to identify and measure desmosome size in human tissue. Desmosomes in NH samples averaged 0.43 microns in size, while desmosomes in PV patients were significantly smaller, averaging

0.35 microns (Figure 18a,c). This observation is consistent with recent electron microscopy studies of PV patients (van der Wier *et al.*, 2014) and analysis of desmosomes in cultured keratinocytes treated with PV IgG (Saito *et al.*, 2012a; Tucker *et al.*, 2014). Interestingly, SIM microscopy and dual label immunofluorescence for both desmoplakin and hIgG revealed two types of fluorescence staining patterns (Figure 18b). When desmoplakin railroad track staining was intact, we often observed hIgG deposited in the desmosome core (red) and bracketed by desmoplakin staining on opposing keratinocyte membranes (green). A few instances of desmoplakin railroad track staining devoid of hIgG deposition in the desmosomal core were also observed, possibly indicating Dsg-depleted desmosomes (Supplemental figure 7). In addition, we observed numerous split desmosomes in which hIgG (red) was adjacent to only one "rail" of desmoplakin staining (green) (Figure 18b, asterisks), indicating desmosome splitting. The presence of split desmosomes in patients and mouse models of PV has been attributed to pathogenic antibodies physically interfering with, or sterically hindering, desmosomal cadherin adhesive interactions. If steric hindrance were the sole mechanism for loss of adhesion, we would predict that desmosomes would be split but remain otherwise unchanged morphologically. Interestingly, the majority of split desmosomes in PV patient tissue were also found to be smaller than intact desmosomes in NH tissue (Figure 18c). The observation that desmosomes are smaller and split supports a pathomechanism model for PV that includes altered desmosome assembly or disassembly dynamics, in addition to steric hindrance.

6.2.4 Mechanical stress causes desmosome splitting

Our analysis of patient tissue using SIM revealed striking similarities in desmosomal protein organization and trafficking in cultured keratinocyte models and PV patient skin. However, split desmosomes have not been reported in cell culture models of disease. We reasoned that desmosomes do not split in culture because the cells, unlike patient tissue, are not exposed to mechanical stress. To test the hypothesis that mechanical stress causes desmosome splitting, cultured keratinocyte monolayers were subjected to a dispase cell fragmentation assay (Ishii *et al.*, 2005) and then processed for immunofluorescence analysis by SIM. Similar to normal human tissue, the keratinocyte cell sheet exposed to NH IgG remained adhesive and numerous intact desmosomes were observed (Figure 19). As expected, keratinocytes exposed to PV IgG dissociated upon exposure to mechanical stress. SIM analysis of the free edge of the fragmented cell sheet exposed to PV IgG revealed extensive desmosome splitting (Figure 19) that was strikingly similar to that observed at the blister edge in PV patient biopsies (Figure 18b). These results indicate that the application of mechanical force causes desmosomes that are weakened by PV IgG to split at the adhesive interface.

6.2.5 Conclusions and implications

In this study, sub-diffraction limit optical microscopy and immunofluorescence imaging revealed changes in desmosomal protein distribution and trafficking that occur in PV patient epidermis. We compared these changes in patient tissue to those that occur in cultured keratinocytes exposed to PV IgG. In both cases, desmosomal proteins become clustered, enter lipid raft-enriched linear arrays, and are internalized to endosomes. These changes are accompanied by a reduction in steady state Dsg3 levels, as well as a

reduction in desmosome size. High resolution imaging also revealed examples of individual desmosomes in patient tissue that were depleted of desmogleins. It is likely that these changes, along with the ability of PV IgG to sterically interfere with desmosomal cadherin adhesion, compromise desmosome function, resulting in mechanical failure upon exposure to mechanical stress. Indeed, desmosome splitting could be recapitulated *in vitro* by exposing PV IgG treated keratinocytes to physical forces. These results support a multifactorial model in which PV IgG weaken cell adhesion by altering desmosomal protein distribution, by perturbing the dynamics of desmosome assembly and/or disassembly, and by sterically interfering with adhesion (Kitajima, 2013, 2014; Stahley and Kowalczyk, 2015). Lastly, this study provides a foundation for using advanced optical imaging techniques to investigate alterations in epidermal adhesion structures in a variety of epidermal diseases, and for the development of new optical imaging-based diagnostic metrics for pemphigus and related disorders.

6.3 Materials and methods

Antibodies. The following antibodies were used in this study: mouse anti-Dsg3 antibody AK15 (Tsunoda 2003) was a kind gift from Dr. Masayuki Amagai (Keio University, Tokyo); rabbit anti-desmoplakin antibody NW6 was a kind gift from Dr. Kathleen Green (Northwestern University); mouse anti-desmoplakin I/II antibody (Fitzgerald, Acton, MA); rabbit anti-γ-catenin (plakoglobin, H-80) and rabbit anti-p120 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-E-cadherin and rabbit anti-caveolin-1 antibodies (BD Biosciences, San Jose, CA); mouse anti-CD59-FITC conjugated antibody (Millipore, Billerica, MA; Invitrogen); rabbit anti-early endosomal antigen-1 antibody

(EEA1) (Thermo Scientific, Waltham, MA). Secondary antibodies conjugated to Alexa Fluors were purchased from Invitrogen. PV sera used in Figure 19 was a generous gift from Dr. M. Amagai. PV patient sera used in all other Figures was obtained from patients seen at Emory University, Department of Dermatology. IgG was purified from PV sera according to the manufacturer's protocol using Melon Gel IgG Purification Resins and Kits (Thermo Fisher Scientific, Rockford, IL).

Human Skin Biopsy Processing. Perilesional biopsies from patients seen at the Emory Clinic Dermatology Department were collected and stored at -80°C. 5 μm sections from the biopsies were mounted onto glass slides and processed for immunostaining as described below.

Cells and Culture Conditions. Primary human keratinocytes (HKs, passage 2 or 4) were isolated as previously described (Calkins *et al.*, 2006)and cultured in KBM-Gold basal medium (100 μM calcium) supplemented with KGM-Gold Single-Quot Kit (Lonza, Walkersville, MD). For Figure 15c, HKs were cultured to 70% confluence on glass coverslips and switched to 550 μM calcium 16-18 hrs to induce junction assembly. HKs were exposed to NH IgG or IgG from PV patients for 6 hrs at 37°C, processed for wide-field immunofluorescence and then analyzed for clustering as described below. For dispase assay in Figure 19, HKs were cultured to 100% confluence in 4-well tissue culture plates and switched to 50 μM calcium to prevent any junction assembly for 16-18 hrs prior to switching to 550 μM calcium for 3 hrs to allow for junction assembly. HKs

were exposed to NH or PV IgG (100 μg/mL) for 3hrs at 37°C and processed for a dispase fragmentation assay followed by N-SIM, as described below.

Immunofluorescence. Patient tissue slices were allowed to come to room temperature and immunostained with primary and secondary antibodies for 1 hr each at room temperature with triple PBS^{+} washes between antibody incubations. HKs in Figure 15 were fixed in methanol and processed for immunfluorescence. Primary antibodies described above were detected with Alexa Fluor-conjugated secondary antibodies. Widefield fluorescence microscopy was performed as previously described (Stahley *et al.*, 2014). Super-resolution microscopy was performed using a Nikon N-SIM system on an Eclipse Ti-E microscope system. The N-SIM system was equipped with a 100x/1.49 NA oil immersion objective and 488- and 561-nm solid-state lasers in 3D structured illumination microscopy mode. Images were captured with an EM charge-coupled device camera (DU-897, Andor Technology) and reconstructed using NIS-Elements software with the N-SIM module (version 3.22, Nikon).

Clustering analysis. Protein clustering was measured as previously described (Saito *et al.*, 2012a). Briefly, lines were drawn along cell borders to measure fluorescence intensity using ImageJ Fiji software (NIH, Bethesda, MD). MATLAB was used to count peaks which were defined by a slope of zero. Increased distance between peaks indicates increased clustering. The clustering index was calculated by dividing the ratio of peaks per given distance by one.

Dispase-based fragmentation assay with immunofluorescenc. Following PV IgG treatment, cells were subjected to a dispase fragmentation assay as previously described (Saito *et al.*, 2012a). In parallel, cell sheets/fragments were fixed in paraformaldehyde, permeablized in Triton X-100 and incubated with primary and secondary antibodies in the tissue culture wells. Gentle washes with PBS^{+} were carried out between Triton and antibody incubations. Cell sheets/fragments were then mounted in ProLong Gold (Molecular Probes, Eugene, OR) and then imaged by N-SIM.

Statistics. Statistical analysis was performed using a *t*-test assuming unequal variances with a significance level of 0.05 and comparing the PV group to NH.

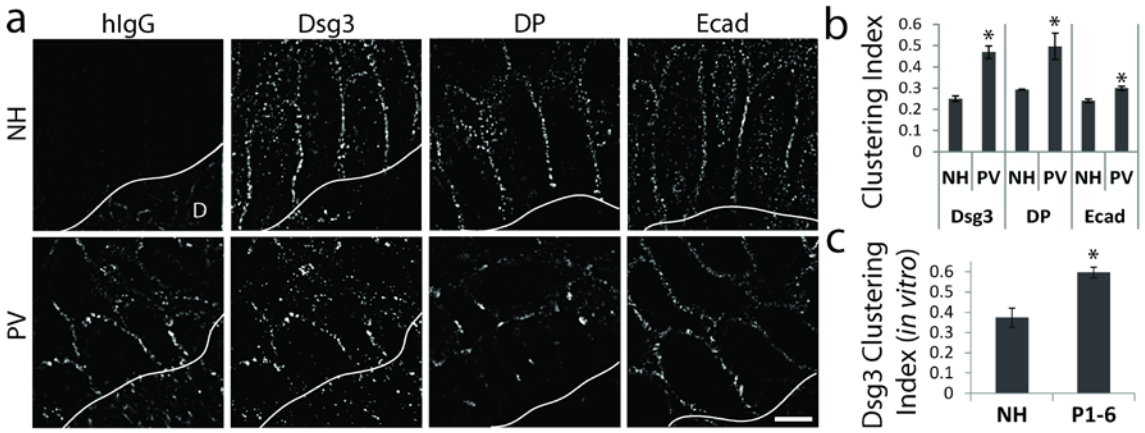


Figure 15. PV IgG causes desmosomal protein clustering in patient tissue and in cultured keratinocytes. a) Top panel: Normal human (NH) epidermal tissue is negative for IgG (hIgG) deposition and junctional proteins desmoglein 3 (Dsg3), desmoplakin (DP) and E-cadherin (Ecad) are uniformly distributed along cell borders. Bottom panel: PV patient epidermis is positive for hIgG deposition and border localization of Dsg3 and DP is highly disorganized and clustered. Ecad staining is largely unaltered. D, dermis. Solid line, epidermis-dermis interface. Images oriented dermis down. Scale bar, 5 μm. **b)** Quantification of protein clustering. Means ± SEM; *$p < 0.05$ (Dsg3 – 0.0003, DP – 0.0227, Ecad – 0.0046). **c)** Quantification of average Dsg3 clustering using patient sera (P1-6) added to cultured keratinocytes. Means ±SEM, *$p<0.05$.

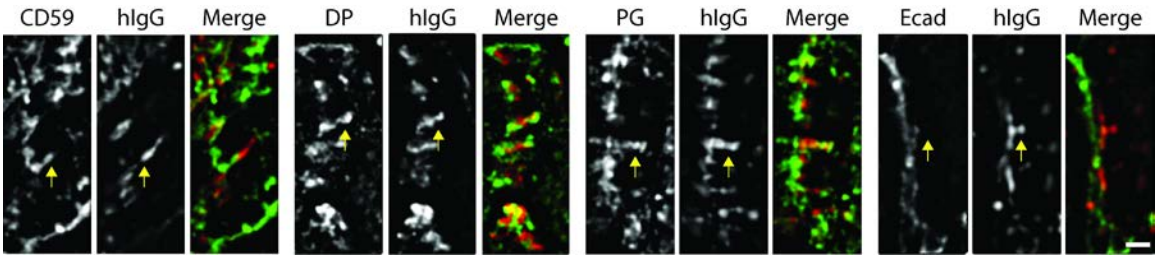


Figure 16. PV IgG associates with linear arrays. PV patient IgG (hIgG) is found in linear array structures (yellow arrows) and colocalizes with raft marker CD59, desmoplakin (DP) and plakoglobin (PG) in biopsies from PV patient tissue. E-cadherin (Ecad) does not colocalize with hIgG in linear arrays. Images oriented with cell-cell borders vertical. Scale bar, 1 μm.

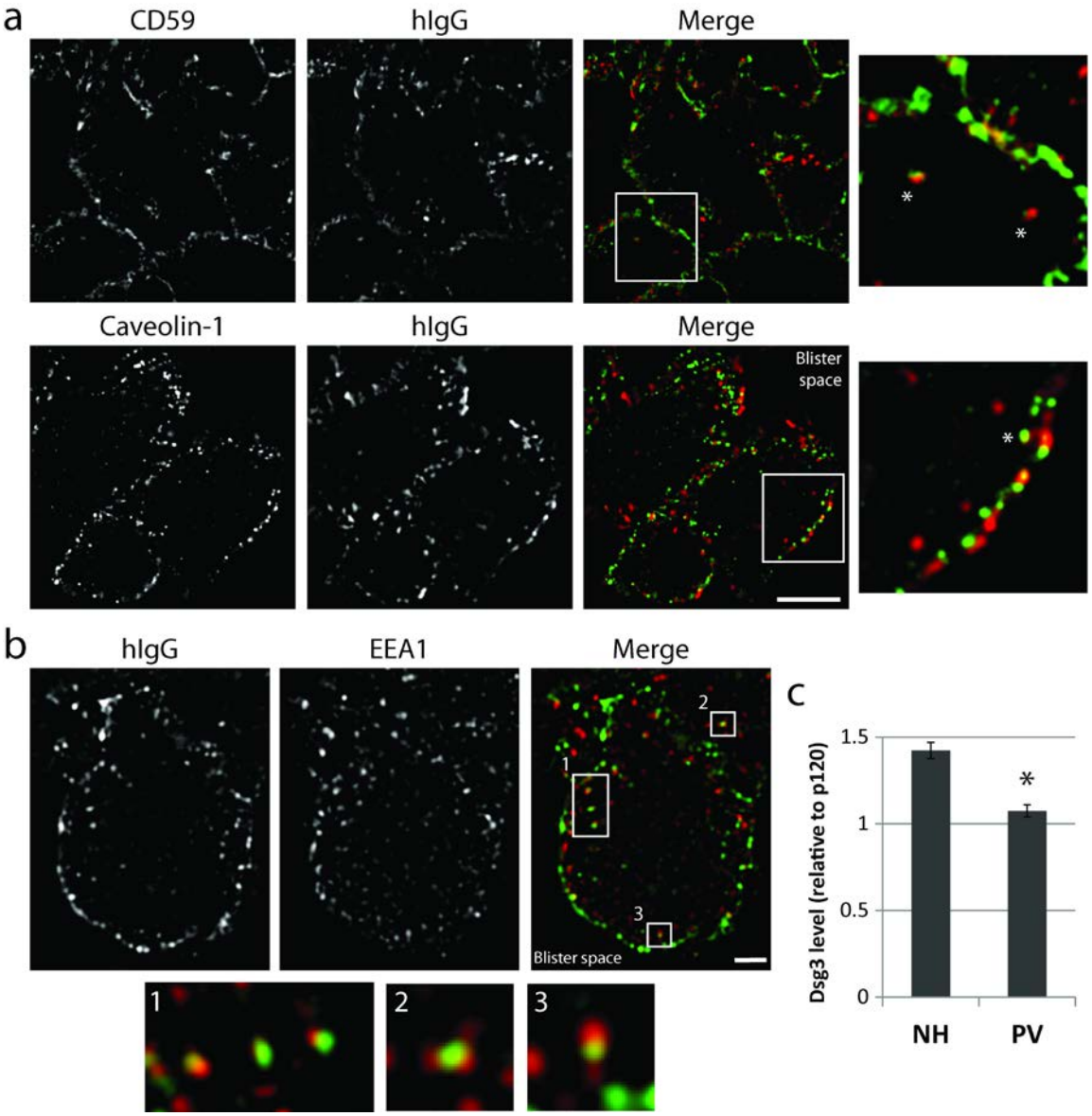


Figure 17. PV patient IgG colocalizes with lipid raft and endosomal markers. a) Colocalization of PV patient IgG (hIgG) with lipid raft markers CD59 (top panel) and caveolin-1 (bottom panel). Colocalization is observed both at cell borders and in vesicular-like structures (asterisks). Scale bar, 5 µm. **b)** PV IgG (hIgG) colocalizes with early endosomal marker EEA1. Zooms highlight vesicular colocalization. Scale bar, 2 µm. **c)** Quantification of Dsg3 protein levels relative to p120 in normal and PV patient tissue. Means ± SEM; *$p < 0.05$.

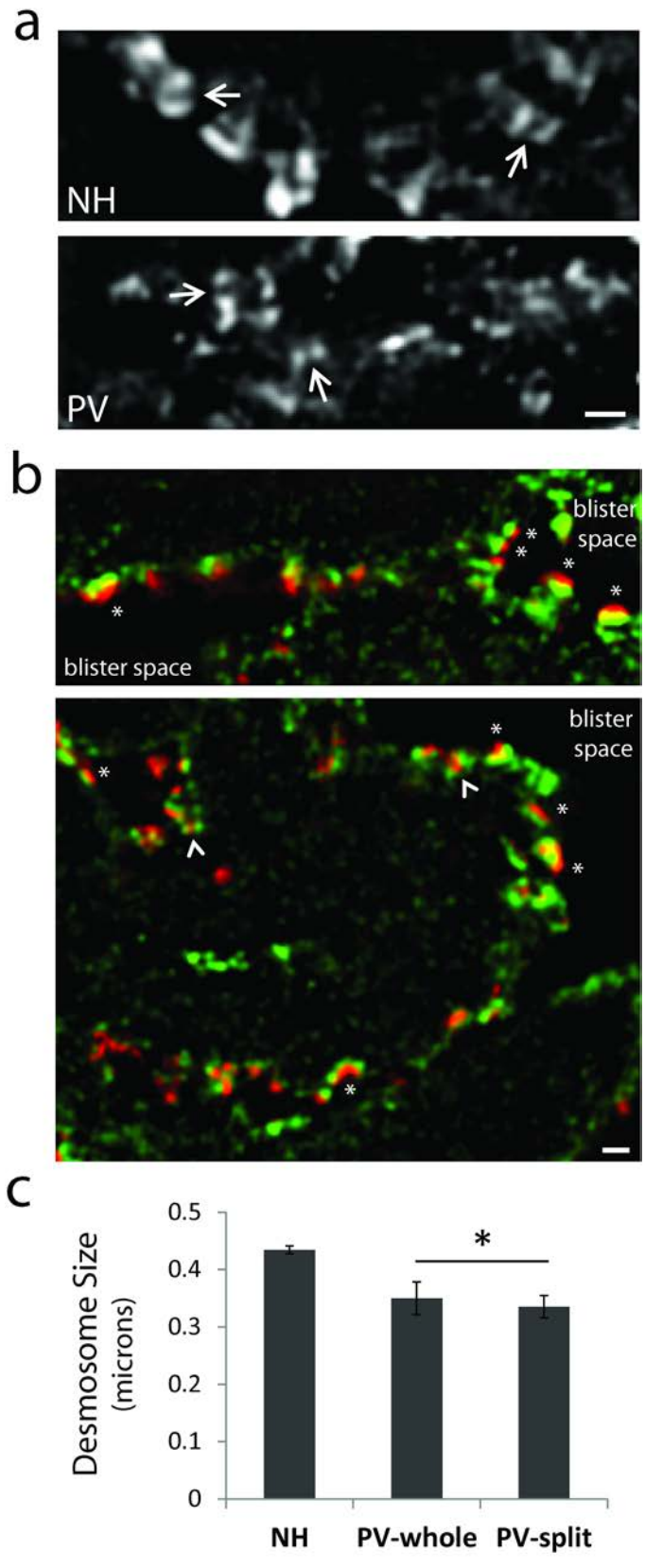

a
NH
PV
b
blister space
blister space
blister space
c
Desmosome Size (microns)
0.5
0.4
0.3
0.2
0.1
0
*
NH
PV-whole
PV-split

Figure 18. Desmosomes in PV patients are smaller and split. Normal human (NH) and PV patient biopsies stained for desmoplakin (green) and hIgG (red) were imaged by SIM. Supplemental Figure 6 details the staining scheme. Desmosomes appear as regions of parallel desmoplakin staining along cell borders, referred to as 'rail-road' tracks, which were used to identify and measure desmosome size. **a)** Desmoplakin staining. Arrows (oriented toward intercellular space) highlight smaller desmosomes. Scale bar, 0.5 μm. **b)** Many split (or half) desmosomes with IgG staining (asterisks) are observed adjacent to the blister space. Arrowheads, small but intact desmosomes. Scale bar, 0.5 μm. **c)** Quantification of desmosome size. Means ± SEM; * $p < 0.05$ comparing PV-whole or PV-split to NH.

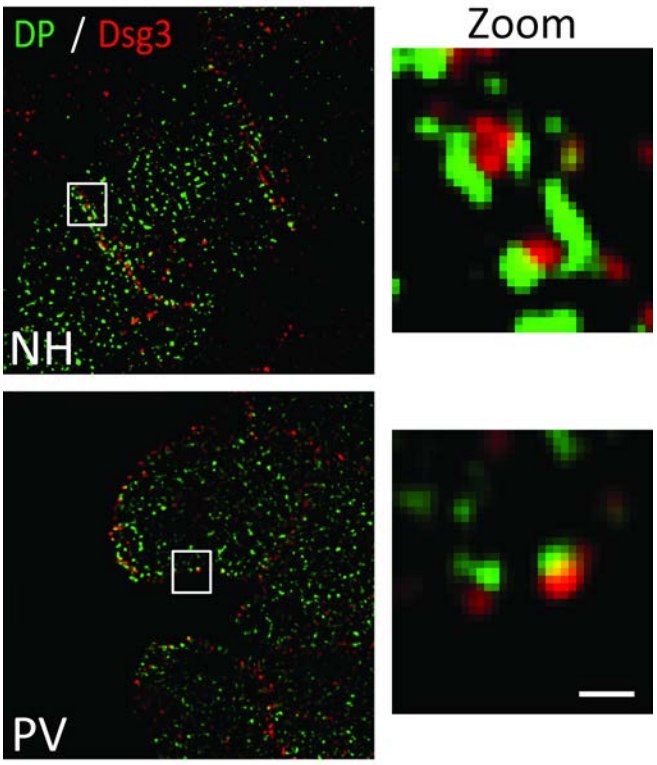
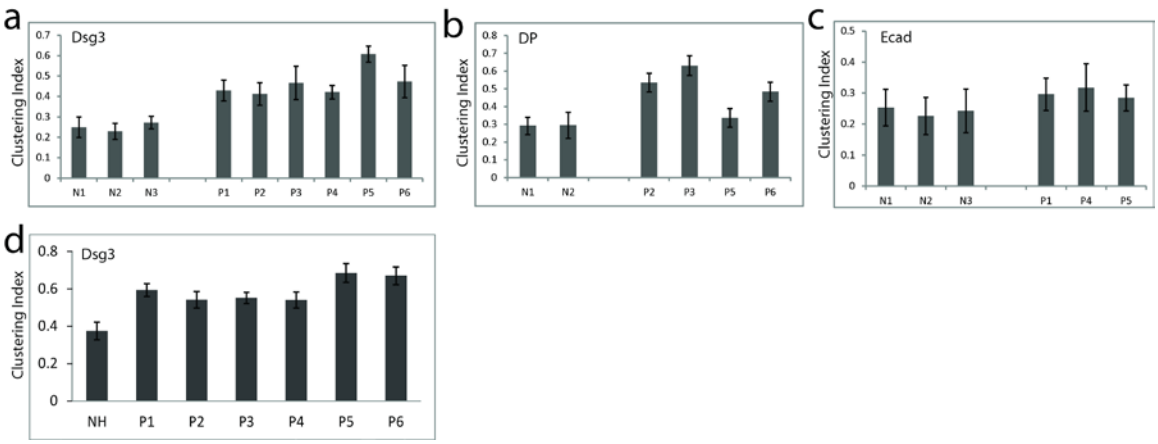


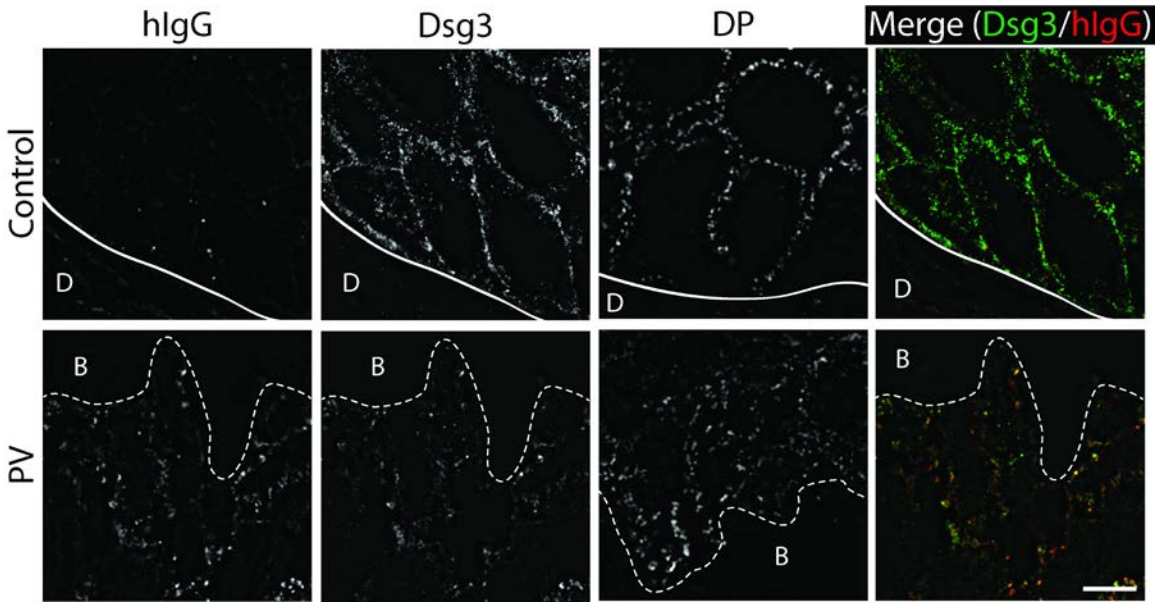
Figure 19. Mechanical stress causes desmosome splitting in cultured keratinocytes exposed to PV IgG. Keratinocyte monolayers exposed to either normal human (NH) or PV IgG were subjected to mechanical stress by repeated pipetting and then processed for structured illumination microscopy. The keratinocyte sheet exposed to NH IgG display intact desmosomes at cell borders indicated by the green-red-green 'rail-road' track staining pattern (zoom, top panel). The free edge of the fragmented sheet exposed to PV IgG exhibits multiple split desmosomes (zoom, bottom panel) indicated by only a green-red staining. Scale bar, 0.5 µm.

Biopsy Site	Patient ID#	ELISA Score	
		α-Dsg1	α-Dsg3
skin	P1	128	178
	P2	217	146
	P3	18	166
lip	P4	176	196
	P5	36	164
	P6	0	114

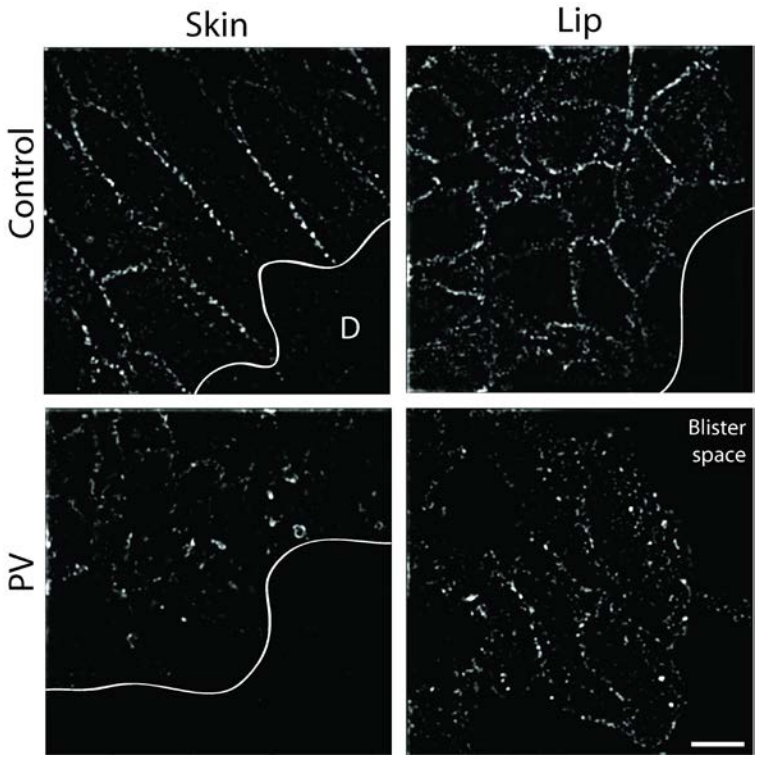
Supplemental Table 1. PV patient ELISA scores.



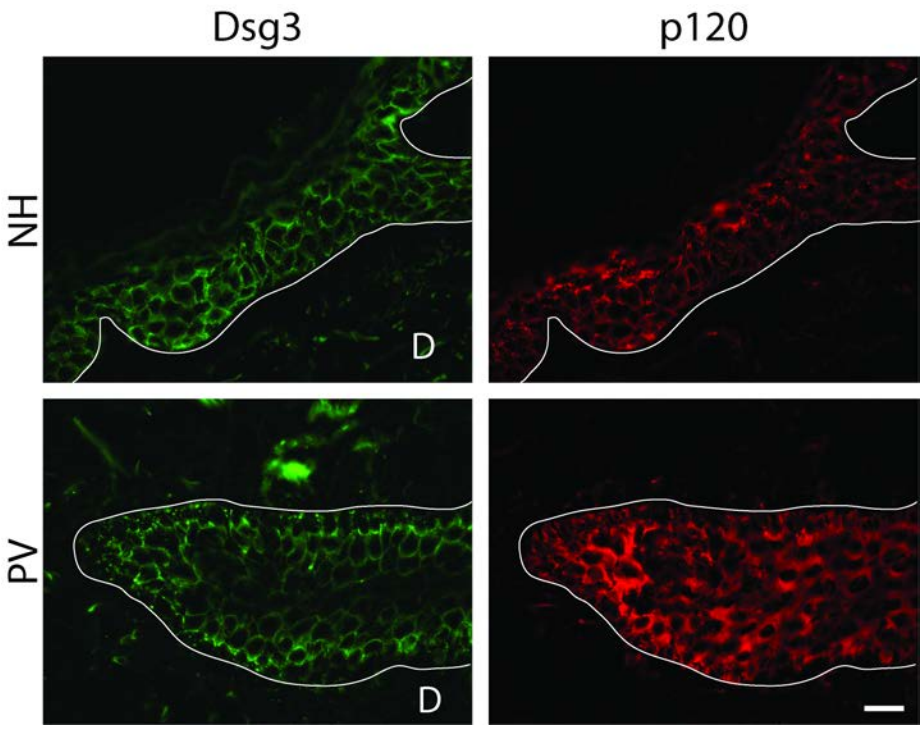
Supplemental Figure 2. Clustering analysis of individual samples used in figure 15. **a-c)** Quantification of Dsg3 **(a)**, desmoplakin (DP) **(b)** and E-cadherin (Ecad) **(c)** clustering *in vivo*. **d)** Quantification of Dsg3 clustering in cultured keratinocytes exposed to either NH IgG or IgG from PV patients (P) 1-6.



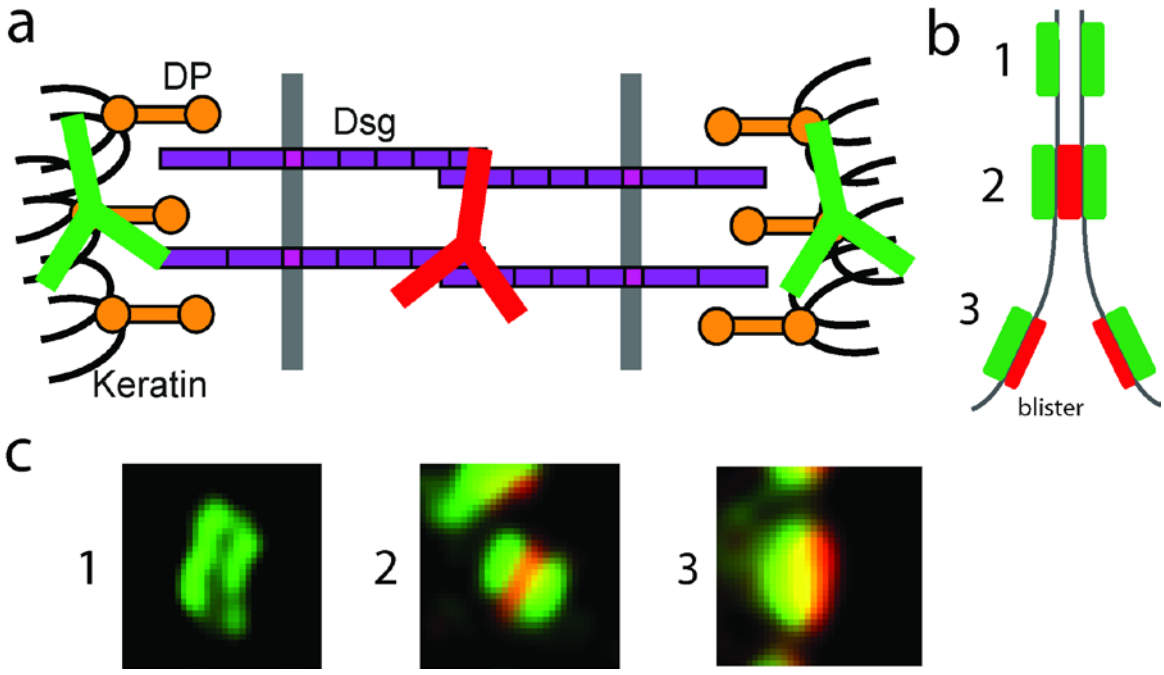
Supplemental Figure 3. Altered desmosomal protein organization in PV patient lip tissue. Top panel: Control (non-PV) mucosal tissue is negative for IgG (hIgG) deposition and junctional proteins desmoglein 3 (Dsg3) and desmoplakin (DP) uniformly distributed along cell borders. Bottom panel: PV patient mucosa is positive for hIgG deposition and border localization of Dsg3 and DP is highly disorganized and clustered. D, dermis. Solid line, dermis interface. B, blister space. Dashed line, blister floor or roof. Images oriented dermis down. Scale bar, 5 μm.



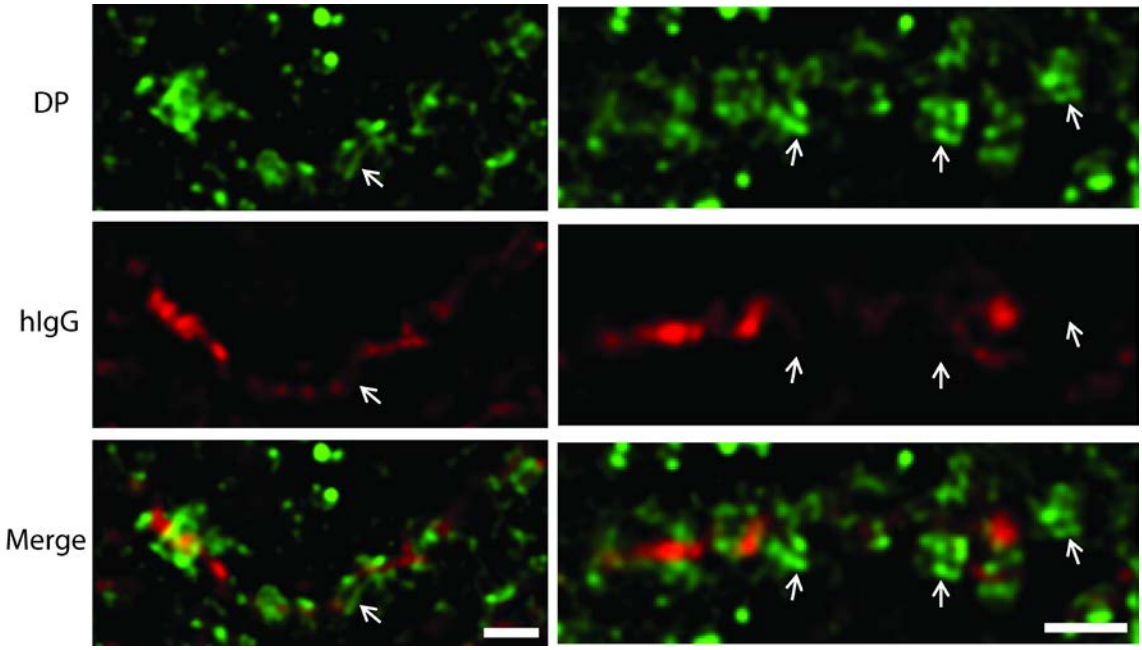
Supplemental Figure 4. Altered plakoglobin organization in PV patient tissue. Top panel: Control (NH, skin and non-PV, lip) tissue displays uniform border localization of plakoglobin. Bottom panel: Disrupted and clustered plakoglobin staining in PV patient tissue. D, dermis. Solid line, dermis interface. B, blister space. Images are oriented dermis down. Scale bar, 5 μm.



Supplemental Figure 5. Dsg3 levels are decreased in PV patient tissue. Dsg3 levels were analyzed via widefield microscopy and normalized to adherens junction protein p120 (Figure 17c). Total Dsg3 levels were slightly decreased in PV patient tissue, particularly in basal cell keratinocytes. D, dermis. Solid line, epidermis-dermis interface. Scale bar, 20 μm.



Supplemental Figure 6. Viewing desmosome 'rail-road' tracks via SIM. a) Simplified desmosome schematic depicting staining with N-terminal Dsg3 antibody (hIgG if in patient tissue) and a C-terminal desmoplakin (DP) antibody. **b)** SIM is able to resolve the distance from plaque to plaque and thus desmosomes appear as regions of parallel desmoplakin staining along a cell border (1) or a sandwich of DP-Dsg3-DP staining (2). Split desmosomes appear as regions of green-red staining (3). **c)** *In vivo* examples of desmosome staining depicted in (B).



Supplemental Figure 7. Dsg-depleted desmosomes in PV patient tissue. Patient tissue stained for desmoplakin (DP) and hIgG (red). Arrows highlight desmosome rail-road tracks lacking hIgG staining. Scale bar, 1 μm.

Chapter 7

Summary and future directions

The overall goal of this dissertation was to investigate the significance of desmosomal raft association and to define the desmosomal alterations in patients with the skin blistering disease pemphigus vulgaris (PV). Previously, PV IgG-induced Dsg3 endocytosis was found to occur via a raft-mediated pathway, yet PV pathomechanisms *in vivo* remained largely unknown. Specifically, the work presented in this dissertation sought to achieve the following objectives: 1) to determine if Dsg3 and other desmosomal proteins are raft associated biochemically, 2) to analyze the role of raft microdomains in regulating desmosome assembly and PV IgG-induced disassembly and 3) to define the desmosomal alterations in PV patients. Using biochemical and super-resolution imaging approaches, the dynamics of desmosome assembly and disassembly were determined to be raft-dependent. These dynamics were found to be disrupted in PV patients, leading to the loss of adhesion through altered desmosomal protein organization. The studies presented in this dissertation have advanced our understanding of desmosome dynamics in adhesion and disease, but have also raised additional questions for future investigation that will be discussed next.

What regulates Dsg3 raft association?

The study in chapter 5 demonstrated that Dsg3, along with other desmosomal proteins, targets to raft microdomains biochemically (Figure 7) and that Dsg3 colocalized with raft markers CD59 and caveolin-1 by super-resolution microscopy (Figure 8). Further, desmosome assembly and disassembly required functional raft domains (Figures 9 and 13). It remains to be elucidated how Dsg3 targets to rafts. Dsg3 raft targeting could be regulated by at least three mechanisms: post-translational lipid modifications, the

transmembrane domain or by protein-protein interactions with other raftophilic desmosomal proteins, or even a combination of these mechanisms.

Palmitoylation is considered to be the primary mechanism by which transmembrane proteins target to rafts and as the only reversible post-translational lipid modification, it has been suggested to regulate dynamic raft targeting (Aicart-Ramos *et al.*, 2011; Levental *et al.*, 2010b; Simons and Sampaio, 2011). Palmitoylation typically occurs on one or more membrane-proximal cysteine residues (Levental *et al.*, 2010a). Interestingly, Dsg3 along with all the other desmosomal cadherins, contains highly conserved cysteine residues immediately adjacent to the transmembrane domain (not shown). These residues have recently been confirmed to be palmitolayted (Lewis, Wahl and Kowalczyk, *unpublished*). Surprisingly, current studies in the lab indicate that Dsg3 lacking these residues required for palmitoylation still partitioned to rafts (not shown). These results are consistent with other studies indicating that palmitoylation is not always sufficient for raft-targeting (Simons and Toomre, 2000). Ongoing studies in the lab are focused on the possible role of the Dsg3 transmembrane domain in raft targeting. The desmosomal cadherins contain an extended transmembrane domain, a typical feature of raft transmembrane domains (McIntosh *et al.*, 2003; Vidal and McIntosh, 2005). Preliminary experiments do suggest the Dsg3 transmembrane domain is critical for raft affinity. Yet, this does not preclude a role for Dsg3 palymitoylation. It is likely that both the transmembrane domain and palmitoylation contribute to raft association. Future studies will investigate the contribution of palmitoylation in regulating Dsg3 mobility and/or stability. Based on the work presented in Chapter 5 demonstrating that Dsg3 raft association directly correlated with desmosomal assembly and functional adhesion, Dsg3

defective in either raft targeting or altered raft dynamics would be predicted to negatively impact desmosomal adhesion.

Dsg3 failed to partition to rafts in CHO cells or HMEC-1s (Figure 11), suggesting that other proteins also play a role in Dsg3 raft targeting. Neither of these cell types assemble desmosomes and lack expression of multiple desmosomal proteins. This experiment is consistent with the observation that there is a strong correlation between desmosome assembly and Dsg3 raft association (Figures 9-11). Epithelial cells lacking E-cadherin (A431Ds) do not form adherens junctions and also do not form desmosomes (Kim *et al.*, 2000; Lewis *et al.*, 1997). Surprisingly, preliminary data indicates that Dsg3 still partitions to rafts in A431Ds, even though desmosomes do not assemble (not shown). These cells express all the desmosomal proteins suggesting that other desmosomal proteins aid in Dsg3 raft targeting. Dsg3 mutants that lack desmosomal protein-interacting domains will prove useful in identifying Dsg3 binding partners that mediate Dsg3 raft association. Both loss of function experiments using A431D cells, as well as gain of function experiments using CHO cells or HMEC-1s will help determine the desmosomal proteins responsible for Dsg3 raft association.

Are there distinct raft-mediated pathways that mediate Dsg3 endocytosis?

Studies have shown that within the umbrella of raft endocytosis, proteins can be targeted to different endocytic pathways (El-Sayed and Harashima, 2013; Hansen and Nichols, 2009; Lajoie and Nabi, 2010). For example, the epidermal growth factor receptor (EGFR) has been shown to undergo both dynamin-dependent and –independent raft endocytosis under different cellular conditions (Lajoie and Nabi, 2010). There is

evidence to suggest that Dsg3 raft endocytosis might occur via different mechanisms, as well. Biochemically, desmosomal proteins have been classified to either partition to a 'desmosomal' or 'non-desmosomal' pool (Palka and Green, 1997). Are non-desmosomal and desmosomal Dsg3-interalization regulated by distinct mechanisms? Previous work from the lab convincingly demonstrated that PV IgG-induced Dsg3 endocytosis was caveolin- and dynamin-independent (Delva *et al.*, 2008). However, these studies were performed with relatively short time periods of PV IgG treatment, ranging from 1-2 hrs. During this period of time, the desmosomal pool of Dsg3 is largely unaffected and only the non-desmosomal pool of Dsg3 is internalized (Jennings *et al.*, 2011). Overexpression of the plaque protein PKP1 results in extremely robust desmosomes (larger and hyperadhesive) that are resistant to PV IgG-induced loss of adhesion (Tucker *et al.*, 2014). PKP1 expression however, does not prevent Dsg3 endocytosis of the non-desmosomal pool (Tucker and Kowalczyk, *unpublished*) suggesting that these pools of Dsg3 are regulated by different mechanisms, yet share raft commonality.

Over longer periods of time, the desmosomal pool of protein is affected and eventually down-regulated. A time course study analyzing keratinocyte responses following PV IgG treatment revealed distinguishable phases of PV IgG-induced desmosomal alterations. A prominent feature in the 2-6 hr PV IgG treatment window is that desmosomal components rearrange into linear arrays, dynamic structures that form at cell borders and extend perpendicularly toward the cell center. Additionally, time lapse microscopy revealed that endocytic vesicles bud off linear array tips. Linear arrays contain all the desmosomal components, including obligate desmosomal protein desmoplakin, suggesting that these structures likely represent turnover of the desmosomal

pool of Dsg3 (Jennings *et al.*, 2011). One prediction would be that linear arrays should not form in cells in which Dsg3 targets to rafts but does not form desmosomes (such as A431Ds, not shown) following PV IgG treatment, though Dsg3 should still undergo raft endocytosis.

Internalization of desmosomal Dsg3 via linear arrays might require different regulatory mechanisms than the rapid non-desmosomal internalization does. Work presented in Chapter 5 indicated that linear arrays are enriched in raft components by super-resolution SIM (Figure 12) and that their formation was raft-dependent (Figure 13). It remains unknown whether desmosomal Dsg3 endoctyosis via PV IgG-induced linear arrays would also be perturbed by inhibiting dynamin and caveolin function. By SIM, caveolin-1 was heavily enriched in these structures and caveolin-1 puncta were observed at the tip of nearly every array (Figure 12, additional data not shown), possibly indicating a role for caveolae in endocytic budding from array tips. Interestingly, p38MAPK inhibition protects against PV (Berkowitz *et al.*, 2006; Saito *et al.*, 2012a) but linear arrays still form to some extent (not shown). Vesicle budding at array tips appeared to be perturbed, while broader signaling inhibition with a pan tyrosine kinase inhibitor prevented all morphological Dsg3 changes (Dsg3 clustering and linear array formation) (not shown). These results suggest that Dsg3 raft endocytosis of the non-desmosomal pool that is caveolin- and dynamin-independent, but that internalization of desmosomal pools of Dsg3 via linear arrays is caveolin- and p38MAPK-dependent. Future studies are needed to test this possibility.

What morphological features of PV correlate with disease severity?

The work presented in Chapter 6, for the first time, directly compared the morphological observations in PV patients to the desmosomal alterations that have been characterized *in vitro*. This study has validated use of the *in vitro* cell culture model system and will prove invaluable to future PV studies. Precise disease pathomechanisms that have been confirmed to occur in patients can now be investigated in detail using the *in vitro* model system. An important future direction will be determining if the morphological criteria used to evaluate patient tissue can also be used to generate a clinical readout that quantifies disease severity, such as the clustering index used to measure desmosomal protein clustering in Figure 15. Clinically, patients are characterized by their pemphigus disease area index (PDAI) score (Daniel *et al.*, 2012; Rosenbach *et al.*, 2009), a standardized and quantitative measure of disease activity, and their Dsg1/3 antibody titer or ELISA scores. Generally, PDAI scores are positively correlated with ELISA scores (not shown), i.e. patients with high antibody titers present with more severe blistering. However, there are exceptions to this generality (Patsatsi *et al.*, 2014), and ELISA scores do not provide a functional readout for IgG pathogenicity, which is likely to vary between patients. In contrast, desmosomal protein clustering in patient tissue presumably reflects pathogenic activity of patient IgG.

The clustering index needs to be validated by comparing clustering both in a patient's biopsy and *in vitro*, to their clinical PDAI and ELISA scores. We predict that patients with high PDAI and high ELISA scores will correspondingly display a high degree of Dsg3 clustering. Clustering of obligate desmosomal protein desmoplakin (DP) should also be evaluated. If clustering of either Dsg3 or DP proves to be an accurate index of disease severity, it could potentially be used by physicians to determine how

aggressively a particular patient should be treated. Might linear arrays also be indicative of disease severity? The fact that linear arrays have now been observed in cultured keratinocytes (Figures 12, 13, (Jennings *et al.*, 2011), in human skin explants (Figure 12) and in PV patient biopsies (Figure 16) strongly supports a pathogenic role for linear arrays in PV, and thus they might also be indicative of disease severity. As discussed above for Dsg3 endocytosis, the degree of clustering and the degree of linear array formation (turnover of the desmosomal pool) could be different and thus indicate differences in the pathogenicity of a patient's IgG. Standardized clustering and linear array indices could prove valuable for treatment as well as therapy development for PV.

Concluding remarks

The research in this dissertation has made important contributions to the field of desmosome biology by revealing that raft microdomains modulate the dynamics of desmosome assembly and disassembly, and that these dynamics are altered in pemphigus vulgaris. Studies aimed at defining the raft- and desmosome-targeting mechanisms of the desmosomal proteins, as well as, their spatial organization will uncover key regulatory mechanisms of desmosome biology. Further, the super-resolution analysis of PV patient tissue in combination with cell culture experiments has identified hallmark features of the disease that occur both in patients and in cultured keratinocytes. Future studies utilizing the cell culture model system will help to define the precise cellular mechanisms that regulate the desmosomal alterations observed *in vivo*. These types of studies will allow for the development of optimal therapies for pemphigus and related disorders of the desmosome, further advancing the field of desmosome biology.

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