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Dana Kirsten Tucker Date

Pemphigus Vulgaris: Pathomechanisms of a Desmosomal Disease and Protection by Plakophilin-1

By

Dana Kirsten Tucker
Doctor of Philosophy

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

_____
Andrew P. Kowalczyk, PhD
Advisor

_____
Victor Faundez, MD, PhD
Committee Member

_____
Michael Koval, PhD
Committee Member

_____
Lian Li, PhD
Committee Member

_____
Winfield S. Sale, PhD
Committee Member

Accepted:

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

_____
Date

Pemphigus Vulgaris: Pathomechanisms of a Desmosomal Disease and Protection by Plakophilin-1

By

Dana Kirsten Tucker
B.S., Missouri Southern State University, May 2002
M.S., Missouri State University, May 2005

Advisor: Andrew P. Kowalczyk, PhD

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Abstract

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By Dana Kirsten Tucker

Desmosomes are intercellular junctions that provide strong adhesion between epithelial cells by anchoring keratin intermediate filaments to cell-cell contact sites. Pemphigus vulgaris (PV) is a life-threatening epidermal blistering disease caused by the presence of autoantibodies against the desmosomal cadherin desmoglein 3. PV is characterized by loss of adhesion or acantholysis between the basal and suprabasal layers in the epidermis. Clinically, patients suffer from severe mucosal erosions and epidermal blistering. The mechanism by which PV IgG disrupts desmosomal adhesion is not fully understood. In my dissertation we hypothesize that PV IgG binding to desmoglein 3 promotes desmosome disassembly and perturbs desmosome assembly, thereby causing the loss of cell-cell adhesion. To test this hypothesis, primary human keratinocytes and patient IgG were used to define the morphological, biochemical and functional changes in desmosome adhesion triggered by PV IgG. Our results indicate that desmosome disassembly and the subsequent loss of cell-cell adhesion occurs sequentially in specific phases triggered by desmoglein 3 internalization and degradation. Furthermore, we found that increasing desmoglein 3 biosynthesis counteracts the increase in desmoglein 3 endocytosis and turnover caused by PV IgG. Based on these data we predicted that reinforcing desmosome adhesion by promoting assembly or slowing disassembly of desmosomes will counteract the effects of PV IgG and prevent the loss of adhesion.

We tested this prediction by exogenously expressing the desmosomal component, plakophilin-1, in basal keratinocytes to determine if this would result in desmosomes that are refractory to PV IgG. Plakophilin-1 is a differentially expressed armadillo family protein that stabilizes and increases keratinocyte adhesion by promoting desmosome formation in the suprabasal layers of the epidermis. We found that enhanced expression of plakophilin-1 blunted the effects of PV IgG and prevented the loss of cell-cell adhesion. Furthermore, plakophilin-1 expression transformed desmosome adhesion from a calcium-dependent to a calcium-independent and hyper-adhesive state. Collectively, these results demonstrate that PV IgG binding to Dsg3 triggers a series of keratinocyte responses that result in the loss of desmosomal cell-cell adhesion and that manipulating the adhesive states of desmosomes can block the pathogenic effects of PV IgG on keratinocyte adhesion.

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CHAPTER I

General Introduction

Dana K. Tucker

Section 1.2 was adapted from Saito M*, Tucker DK*, Kohlhorst D, Niessen CM, Kowalczyk AP (2012) Classical and desmosomal cadherins at a glance. Journal of Cell Science 125:2547-52. * These authors contributed equally

Significance and overview

The ability of cells to recognize and attach to one another, or rather to engage in cell-cell adhesion, is a fundamental step in the evolution of unicellular to multicellular organisms (Vleminckx, 2001). In multicellular organisms, cell-cell adhesion facilitates mechanical stability and communication between neighboring cells. This in turn allows for specialization and cooperation of cells to form diverse three-dimensional tissues (Gumbiner, 1996). The cell-cell adhesion structure most highly dedicated to mechanically coupling tissues is the desmosome. These highly ordered intercellular junctions provide strong tensile strength between cells by anchoring to the intermediate filament cytoskeleton. Desmosomes are especially abundant in tissues that experience a high level of mechanical stress, such as in the skin and cardiac muscle. Desmosomes are also present in epithelial cells of various tissues including the intestinal mucosa, gallbladder, uterus/oviducts, liver, pancreas, stomach, salivary and thyroid glands and the epithelial cells of the nephron (Brooke *et al.*, 2012; Getsios *et al.*, 2004). Weakening of desmosomes by mutation of individual desmosomal components, inhibitory autoantibodies or microbial proteases, cause arrhythmias in the heart and blisters within the epidermis (Holthöfer *et al.*, 2007). Beyond desmosome function as 'intercellular glue', these intercellular junctions play important roles in tissue morphogenesis, homeostasis and cell signaling (Getsios *et al.*, 2004). In my dissertation, I will focus on desmosomal cell-cell adhesion in the epidermis and on the pathomechanisms and possible treatment of Pemphigus Vulgaris (PV), a disease of the desmosome.

PV is a life-threatening, autoimmune epidermal blistering disease caused by autoantibodies (IgG) that compromise desmosome function causing the loss of cell-cell

adhesion. (Amagai *et al.*, 1991; Amagai and Stanley, 2012; Sharma *et al.*, 2007). In PV patients, this loss of adhesion, or acantholysis, manifests as severe mucosal erosions and epidermal blisters between the basal and suprabasal layers of the epidermis (Kottke *et al.*, 2006; Payne *et al.*, 2004). Evidence from our lab and others suggests that PV IgG binding to its desmosomal target, desmoglein 3, triggers a series of keratinocyte responses that result in desmosome disassembly and acantholysis. However, we do not fully understand the mechanisms by which PV autoantibodies disrupt keratinocyte adhesion. The overall goals of this dissertation are: 1) to understand the pathomechanisms of the disease pemphigus vulgaris, 2) to explore a novel approach to prevent the loss of cell-cell adhesion caused by PV autoantibodies, specifically by exogenously expressing the desmosomal component, Plakophilin-1, and 3) to further our understanding of the fundamental mechanisms that regulate desmosomal adhesion. In order to effectively present the findings in this dissertation and critically evaluate their implications I will first review 1) the epidermis 2) desmosome morphology and molecular architecture 3) mechanisms that regulate desmosomal adhesion 4) PV disease characteristics 5) treatments and therapeutic approaches and 6) pathomechanisms of PV.

Section 1.0 Function and organization of the epidermis

The skin is a protective barrier that guards the body from infection, dehydration, mechanical stress and UV irradiation. Consequently, development and maintenance of the two layers of the skin, the dermis and epidermis, is vital throughout the mammalian life cycle (Simpson et al., 2011). The dermis physically supports and provides nutrients to the epidermis, while the avascular epidermis forms a mechanically stable barrier (McLafferty *et al.*, 2012). The epidermis is a highly specialized stratified squamous

epithelium consisting of four distinct types of cells, keratinocytes, melanocytes, merkel cells and langerhan cells. Keratinocytes make up over 90% of the cells within the epidermis (Fuchs, 2007) and are the type of cells used for experimentation in this dissertation. While the other types of cells provide important functions within the epidermis, they are outside the scope of the work presented here and will not be discussed.

The epidermis is organized into four major keratinocyte layers or strata; the stratum basal, stratum spinosum, stratum granulosum and stratum corneum. These epidermal strata are also referred to as the basal, spinous, granular and corneal layers (Figure 1). The epidermis undergoes constant self-renewal and is generated from the 'stratum basal', which is a single layer of mitotically active, progenitor keratinocytes. While basal layer keratinocytes stay attached to the basement membrane, their daughter cells move up and thus comprise the various epidermal layers. During their transcendence to the stratum corneum, keratinoctyes mature into protective and terminally differentiated keratinocytes (Fuchs, 1990; Fuchs, 2007). Therefore the basal, spinous and granular layers are the living cell layers undergoing terminal differentiation, while the stratum corneum consists of terminally differentiated keratinocytes which are dead, flattened, and fully keratinized cells. Irrespective of keratinocyte position within the strata, all keratinocytes must form and properly regulate desmosomes in order to provide the epidermis with mechanical stability (Green and Simpson, 2007).

Section 1.1 Desmosome identification, morphology and molecular architecture

The adhesive intercellular junction, the desmosome, was named by Josef Schaffer in 1920 and the term is derived from the Greek word "desmos", meaning bond or

fastening, with "some" meaning body (Calkins and Setzer, 2007; Schaffer, 1920). This conspicuous structure was first observed over 150 years ago as technical advances were made in light microscopy. In 1864, Giulio Bizzozero accurately described desmosomes as points of cell-cell adhesion between adjacent cells and named them “nodes of Bizzozero” (Bizzozero, 1864). While this name did not stick, the name desmosome continues to the present day. More than a century later their morphological details were revealed by electron microscopy (Calkins and Setzer, 2007; Tamarin and Sreebny, 1963). Biochemical analysis of desmosomes began in the mid 1970’s and subsequently the identification and cloning of their molecular constituents followed in 1980’s and early 1990’s, respectively (Collins *et al.*, 1991; Garrod, 1993; Gorbsky and Steinberg, 1981; Hatzfeld *et al.*, 1994; Kapprell *et al.*, 1988; Parker *et al.*, 1991; Skerrow and Matoltsy, 1974; Takeichi, 1988).

Desmosomes appear as electron dense, bilaterally symmetrical disk-shaped structures with a diameter of 0.2-0.5 μm (Desai *et al.*, 2009). These highly regulated, complex macromolecular structures are comprised mainly of proteins from three major families: the desmosomal cadherins, desmocollins (Dsc1-3) and desmogleins (Dsg1-4); armadillo proteins, plakoglobin (PG) and the plakophilins (PKP 1-3); and the plakins, particularly desmoplakin (DP) (Green and Simpson, 2007). The transmembrane desmosomal cadherins form the intercellular adhesive interface, while the armadillo and plakin family members form the electron dense desmosomal plaques which tether the keratin intermediate filament cytoskeleton to cadherin intracellular domains (Waschke, 2008). Ultrastructurally, desmosomes are divided into three morphologically identifiable regions: the extracellular core region or desmoglea, the outer electron dense plaque

(ODP) and the inner electron dense plaque (IDP) (Figure 2). The extracellular core region spans 30-35 nm and consists of desmosomal cadherins which associate with each other in a calcium-dependent manner. The outer dense plaque is located closer to the plasma membrane and the inner dense plaque is further away from the membrane. The outer dense plaque spans 15-20 nm and consists of the cytoplasmic tails of the desmosomal cadherins and armadillo family members, plakoglobin and the plakophilins (1-3) (Al-Amoudi *et al.*, 2011; Al-Amoudi and Frangakis, 2008; Holthöfer *et al.*, 2007; North *et al.*, 1999). Plakoglobin and the plakophilins link the cadherin cytoplasmic tails via protein-protein interactions to the amino-terminus of the plakin family member, desmoplakin (Getsios *et al.*, 2004; Kowalczyk *et al.*, 1999). The inner dense plaque spans 15-20 nm and consists of the carboxyl -terminal ends of desmoplakin molecules which directly bind to keratin intermediate filaments thereby tethering the cytoskeletal network to the cadherin adhesion complex (Figure 2) (Kouklis *et al.*, 1994; Stokes, 2007).

Considering the electron dense nature of desmosomes, it is not surprising that upon Triton X-100 extraction of whole cells, desmosome components biochemically partition into two distinct Triton-soluble and insoluble protein fractions. Desmosomal components from the Triton-soluble pool reflect membrane-associated proteins not incorporated into desmosomes. The Triton-insoluble pool is called the desmosomal pool because it reflects proteins that are assembled into desmosomes and thus are associated with the intermediate filament cytoskeleton (Palka and Green, 1997). In cells with assembled desmosomes, roughly 60-80% of the total desmosomal protein pool partition to the insoluble fraction (Pasdar and Nelson, 1988). Investigation into the stability of the proteins within these two pools revealed that the establishment of desmosomal adhesion

within a cell significantly increases the metabolic stability of desmosomal proteins. In the absence of cell-cell contact, both the soluble and insoluble pools of desmoplakin are unstable and are degraded rapidly (t½ approximately 8 hours), while the desmosomal cadherins t½ are roughly ~4 hours. Upon cell-cell contact, desmosomes assemble and this acts to increase both the capacity and stability of the Triton-insoluble protein pool. Newly synthesized desmosomal proteins from the Triton-soluble pool titrate into the insoluble pool, creating approximately a three-fold increase in cytoskeletal-associated desmosomal proteins. Furthermore, the insoluble pool of desmoplakin becomes very stable (t½ greater than 72 hours), while the desmosomal cadherins t½ life increases to ~ 24 hours (Pasdar and Nelson, 1988, 1989; Penn *et al.*, 1987). Collectively, these data demonstrate that desmosomal adhesion acts to stabilize desmosomal proteins within the triton-insoluble, desmosomal pool.

The most recent and in-depth investigation into the arrangement or stoichiometry of desmosomal proteins has utilized cryoelectron tomography to examine human epidermal desmosomes both *in vivo* and in situ. The resolution of the 3D images collected did not allow for the absolute identification of desmosome stoichiometry, but rather provided several possibilities. The possible stochiometries are as follows, desmosomal cadherins to plakoglobin 1:1, 1:2, or 1:4, desmosomal cadherins to the plakophilins 1:1, 1:2, and finally the cadherins to desmoplakin 1:0, 1:1 or 1:2 (Al-Amoudi *et al.*, 2011). The insoluble nature of the desmosomal proteins has made elucidation of desmosome stoichiometry challenging (Green and Simpson, 2007). Further progress is needed to reveal the precise stoichiometry of individual proteins engaged in desmosomal adhesion (Stokes, 2007). Despite these limitations, structure-function,

biochemical, mutagenesis and yeast-two hybrid studies have provided a further understanding of how desmosomes establish and maintain cell-cell adhesive strength. The next sections will review these elements as well as the tissue and differentiation-specific expression of desmosomal proteins.

Section 1.2 Desmosomal cadherins – Desmogleins and Desmocollins

Section 1.2 was adapted from Saito M, Tucker DK, Kohlhorst D, Niessen CM, Kowalczyk AP (2012) Classical and desmosomal cadherins at a glance. Journal of Cell Science 125:2547-52.

The desmosomal cadherins are members of the cadherin superfamily of cell–cell adhesion molecules that are fundamental determinants of how and when cells interact, migrate and undergo morphogenetic conversions (Gumbiner, 2005; Halbleib and Nelson, 2006; Hulpiau and van Roy, 2011; Niessen *et al.*, 2011; Pokutta and Weis, 2007; Saburi and McNeill, 2005). The activity of cadherins was originally appreciated in the context of epithelial-cell compaction during development (Nose *et al.*, 1988). Since these early studies, the discovery of many additional cadherin superfamily members has resulted in a plethora of publications describing their structure and function in a wide range of molecular interactions and cellular activities. Many cadherins operate to mechanically couple adjacent cells by mediating cell-cell interactions within highly ordered junctional complexes. These complexes include the actin-associated adherens junctions, intermediate-filament-associated desmosomes, intercalated discs between cardiomyocytes, and a variety of other related junctions with tissue-specific functions (Delva *et al.*, 2009; Franke, 2009; Niessen and Gottardi, 2008; Niessen *et al.*, 2011). Cadherin-based adhesive intercellular junctions drive tissue morphogenesis during

development and are essential for the maintenance of adult tissue architecture in virtually all complex tissues (Gumbiner, 2005; Stepniak *et al.*, 2009).

Cadherins constitute a large superfamily with over 350 members (Hulpiau and van Roy, 2009; Hulpiau and van Roy, 2011). The most salient feature of this superfamily is the presence of a variable number of successive extracellular-cadherin (EC) repeat domains, each consisting of approximately 110 amino acids, that are rigidified by binding three calcium ions at linker regions between these domains (Figure 3a) (Boggon *et al.*, 2002; Ciatto *et al.*, 2010; Hulpiau and van Roy, 2009). Sequence homology categorizes cadherins into subfamilies that include classical, desmosomal, proto-cadherins, and a variety of other cadherin subfamily members that exhibit a wide range of activities and binding partners. In this section, I will review some of the features of the classical cadherins for the purpose of comparison with the desmosomal cadherins.

In humans, there are four desmoglein (Dsg1–4) and three desmocollin (Dsc1–3) genes (Figure 3a). All three desmocollin gene products are subjected to alternative splicing to generate the type ‘a’ form and the shorter type ‘b’ form. Both desmocollin isoforms localize to desmosomes, although the shorter ‘b’ form lacks the intracellular cadherin-like (ICS) domain, where plakoglobin binds, and thus may have less extensive cytoskeletal linkages (Dusek *et al.*, 2007; North *et al.*, 1999). The desmosomal cadherins are expressed in a tissue-specific and differentiation stage-specific manner. Desmoglein 2 and desmocollin 2, along with desmoplakin, plakoglobin and plakophilin-2, are ubiquitously expressed in all cells and tissues that form desmosomes (Waschke, 2008). Desmogleins 1, 3 and desmocollins 1, 3 are only expressed in stratified epithelia. Desmoglein 4 is expressed in the epidermis, as well as in salivary glands, testis and

prostate. All of the desmogleins and desmocollins are expressed in the epidermis, but have distinct, differentiation-dependent expression patterns (Figure 4) (Desai *et al.*, 2009). Desmoglein 3 and desmocollin 3 are primarily expressed in the basal and suprabasal layers, while desmoglein 1 and desmocollin 1 are most prominent in the highly differentiated upper spinous and granular layers of the skin (Garrod *et al.*, 2002). Desmoglein 2 expression is restricted to the basal layer, while desmoglein 4 expression is restricted to the granular layer (Figure 4) (Yin and Green, 2004).

The ectodomains of both classical and desmosomal cadherins comprises five highly conserved EC domains (EC1-EC5), with the most membrane-proximal (EC5) domain of the desmosomal cadherins being referred to as the extracellular anchor (EA) domain (Figure 3a) (Boggon *et al.*, 2002; Delva and Kowalczyk, 2009; Shapiro and Weis, 2009). In classical cadherins, the EC1 domains engage homophilically in *trans*-interactions through the exchange or swap of their amino-terminal β-strands (Harrison *et al.*, 2010). The formation of this strand-exchange dimer involves the insertion of a conserved Trp2 residue in EC1 of one cadherin into the hydrophobic pocket located in EC1 of a partner cadherin. The residues that flank Trp2 form additional interactions that stabilize the formation of this dimer. Additionally, other regions within EC1 participate in lateral *cis*-interactions with a region of the EC2 domain of a neighboring molecule. Cooperativity between the strong *trans*-dimers and weak *cis*-interactions is necessary for the production of stable and higher ordered junctional structures (Harrison *et al.*, 2011). Desmosomal cadherins have conserved the Trp2 and hydrophobic pocket required for the EC1 *trans* β-strand swap, but lack sequences similar to the *cis*-interface sequences of type I cadherins. However, visualization of the 3D organization of native desmosomes

revealed that desmosomal cadherins do participate in *cis*-interactions (Al-Amoudi *et al.*, 2007). Additionally, the desmosomal cadherins have been reported to interact both homophilically and heterophilically (Green and Simpson, 2007; Thomason *et al.*, 2010).

The intracellular domains of classical and desmosomal cadherins specify whether they are tethered to the actin or intermediate-filament cytoskeleton (Delva *et al.*, 2009; Shapiro and Weis, 2009). The cytoplasmic domain of desmosomal cadherins are coupled to intermediate filaments through associations with the α-catenin-related armadillo protein plakoglobin, and the plakophilins (Al-Amoudi *et al.*, 2011; Carnahan *et al.*, 2010; Hatzfeld, 2007). Desmoplakin links the desmosomal cadherin complex to the cytoskeleton by binding plakophilins and plakoglobin at its amino-terminus and intermediate filaments at its carboxyl-terminus (Desai *et al.*, 2009; Thomason *et al.*, 2010). The linkage between the intermediate filament cytoskeleton and desmosomal cadherin complexes is critical to tissues that experience substantial mechanical stress, such as the myocardium and stratified epithelia (Simpson *et al.*, 2011).

Desmosomal cadherins and their associated plaque proteins are critical for normal function of the skin and heart. In the epidermis, inactivation of desmosomal proteins can be caused by autoantibody inhibition, mutations in genes encoding these proteins, or by proteases released during staphylococcal bacterial infection (Stanley and Amagai, 2006). In many of these instances, the resulting clinical presentations include epidermal fragility and blistering. However, thickening of the epidermis (hyperkeratosis) and ectodermal dysplasia can also be observed, suggesting important roles for desmosomal components in epidermal differentiation (Getsios *et al.*, 2009; Simpson *et al.*, 2011). Similarly, in the heart, mutations in desmoglein 2, desmocollin 2 and several cadherin associated proteins,

including plakoglobin, desmoplakin and plakophilin-2, lead to cardiomyopathies (Lai-Cheong *et al.*, 2007; Thomason *et al.*, 2010). These cardiac disorders are characterized by both altered mechanical and signaling functions of desmosomes. The recent identification of desmoglein 2 as an adenoviral receptor further highlights the importance of cadherins in host pathogen-interactions (Wang *et al.*, 2011). Collectively, these examples highlight the ways in which cadherin function is impaired or hijacked during disease.

In the 30 years that have passed since cadherins were discovered it has been demonstrated that one of the primary functions of desmosomal cadherins is to establish and maintain cell adhesion on the cellular and tissue level. Although the engagement of classical cadherins in the regulation of the cytoskeleton and signaling pathways has been studied in great detail in cell culture models, it is largely unclear how desmosomal cadherins engage in signaling and cytoskeletal regulation. The further identification of cadherin dysfunction in disease states has provided important clues that expand our understanding of how desmosomal cadherins function at the molecular and tissue level. Advances made while studying the disease pemphigus vulgaris will be discussed in Chapter II.

Section 1.3 Armadillo Proteins

Armadillo proteins play important roles in a variety of cellular processes, including cell junction assembly, nuclear transport and transcriptional activation. Proteins in this family are characterized by multiple repeats of a 42 amino acid domain, called *arm*-repeat domains (Delva et al., 2009). The founding member of this family, the protein armadillo, was discovered as a regulator of segment polarity in *Drosophila melanogaster* (Riggleman *et al.*, 1989). In humans, the *Drosophila* armadillo protein is known as β-

catenin serves both as an adherens junction component and transcription factor (Ozawa *et al.*, 1989; Valenta *et al.*, 2012). An additional member closely related to β-catenin is the desmosomal component and transcription factor plakoglobin (Holthöfer *et al.*, 2007; Peifer and Wieschaus, 1990). Other family members include p120 catenin, p0071, δ-catenin/NPRAP, ARVCF and the more distantly related plakophilins (1-3). The armadillo protein family members that localize to desmosomes are plakoglobin and the plakophilins (Hatzfeld, 1999). In the desmosome, these proteins function to promote assembly by recruiting and linking desmoplakin to the junctional complex. In addition to facilitating desmosomal-cytoplasmic interactions, they also exhibit non-desmosomal roles in signaling and transcription (Garrod and Chidgey, 2008).

Section 1.3a Plakoglobin

Plakoglobin, also referred to as γ-catenin, is an obligatory desmosomal component that is expressed throughout the epidermis (Garrod and Chidgey, 2008) . Structurally, plakoglobin consists of 12 central *arm*-repeat domains flanked by non-*arm* amino- and carboxyl-terminal tail domains (Figure 3b) (Stokes, 2007). Plakoglobin localizes to both desmosomes and adherens junctions, however plakoglobin preferentially localizes to desmosomes in cells that harbor both types of intercellular junctions (Adams *et al.*, 1996; Nathke *et al.*, 1994). This observation is presumed to occur because plakoglobin has a greater affinity to the desmosomal cadherins than to the classical cadherins (Chitaev *et al.*, 1996). In the desmosome, plakoglobin targets desmosomal cadherins into cell-cell junctions and serves as a linker in the desmosomal plaque by directly binding to the desmosomal cadherins and desmoplakin (Getsios *et al.*, 2004; Troyanovsky *et al.*, 1996). Additionally, plakoglobin also binds to plakophilin-2 (Chen *et*

al., 2002), plakophilin-3 (Bonne *et al.*, 2003), p0071 (Hatzfeld *et al.*, 2003) and keratins (Smith and Fuchs, 1998). However, the specificity and implications of these interactions are not well understood (Holthöfer *et al.*, 2007). Deletion mutagenesis and co-immunoprecipitation studies revealed that several hydrophobic amino acids in the *arm*-repeats 1-3 of plakoglobin are required for binding to desmoglein 1 and desmocollin 2a (Chitaev *et al.*, 1998). Interestingly, the interaction of plakoglobin with desmocollin 1a requires the both the amino-terminal and carboxyl-terminal ends of the central *arm*-repeat region (Witcher *et al.*, 1996). Plakoglobin binds to desmoplakin via its central *arm*-repeat domain (Kowalczyk et al., 1997). Furthermore, the association of plakoglobin with desmoplakin can be influenced by Src-dependent tyrosine phosphorylation (Miravet et al., 2003). Outside of the *arm*-repeat region, plakoglobin's relatively short amino- and carboxyl-termini have been suggested to be important in regulating desmosome size and ligand specificities (Holthöfer *et al.*, 2007; Palka and Green, 1997). These data support a model where desmosomal cadherins are linked to desmoplakin and the intermediate filament cytoskeleton by plakoglobin in a simple linear chain. However, whether plakoglobin simultaneously interacts with desmosomal cadherins and desmoplakin has not been concretely demonstrated (Delva *et al.*, 2009). Nonetheless, the importance of plakoglobin in desmosome assembly has been revealed by human diseases and mouse genetic model systems.

A crucial role for plakoglobin in desmosome adhesion during development was first revealed by plakoglobin knockout studies in mice. Plakoglobin was found to be essential for proper desmosome formation of intercalated disks in the embryonic heart (Waschke, 2008). Absence of plakoglobin results in dramatic changes in the architecture

of intercalated disks and plakoglobin null mice die as early as embryonic day 10.5 and onward due to fragility and the loss of tissue integrity in the heart (Bierkamp et al., 1996). In a similar study, some mutant embryos survived and the mouse pups exhibited skin blistering and died shortly after birth due cardiac dysfunction (Ruiz et al., 1996). In humans, Naxos disease is caused by a recessively inherited two base-pair deletion in the plakoglobin gene which leads to the premature termination of the plakoglobin carboxyl-terminus. Patients with the Naxos disease suffer from arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) as well as cutaneous phenotypes, such as woolly hair and abnormal thickening of the palms and soles (palmoplantar keratoderma) (McKoy *et al.*, 2000). This mutation in plakoglobin was the first identified desmosomal component reported to cause ARVD/C (Judge and Johnson, 2008). Later, mutations of other desmosomal genes, desmoplakin, plakophilin-2, desmoglein 2 and desmocollin 2 genes have also been found to cause ARVD/C. Consistent with the mouse knockout studies, ARVD/C is a disorder of the cardiac desmosomes within intercalated discs in the heart and is characterized by life threatening right ventricular aneurysms, arrhythmias and sudden death (Basso *et al.*, 2006; Judge and Johnson, 2008; Thiene *et al.*, 2007).

Section 1.3b Plakophilins

The Plakophilins (1-3) represent an individual group with the p120ctn subfamily of armadillo-repeat proteins. X-ray crystallography structural analysis of the armadillo repeat domain of plakophilin-1 revealed the plakophilins contain 9 *arm*-repeat domains (Figure 3b). Distinct from their fellow armadillo family member plakoglobin, the plakophilins possess a long flexible insert present between the fifth and sixth *arm* repeats that produces a major bend in their overall structure (Choi and Weis, 2005b).

Additionally, the plakophilins have a rather large amino-terminal domain which thus far has been the only domain found to mediate protein-protein interactions, capable of binding an extensive array of desmosomal components (Hatzfeld, 2007). Unlike other armadillo proteins, no binding partners have been reported for the *arm*-repeats of the plakophilins (Stokes, 2007).

Initially, the plakophilins were considered accessory desmosomal plaque proteins, however they are now known be indispensable for desmosomal adhesion, playing crucial roles in the assembly and maintenance of desmosomes (Hatzfeld, 2007). The plakophilins interact with many different desmosomal components and are more than simple linkers within a linear chain. Rather they coordinate of a complex web of molecular interactions likely by associating simultaneously with multiple partners in the desmosomal plaque (Schmidt and Jäger, 2005). The plakophilins share similar structural and functional properties, but exhibit tissue and differentiation-specific expression patterns. Plakophilin-1 is predominantly expressed in stratified epithelia and in urothelium (Hatzfeld *et al.*, 1994; Heid *et al.*, 1994). Plakophilin-2 is expressed in all simple and stratified epithelia as well as in cardiomyocytes and purkinje fibers of the heart and in lymph node follicle cells (Brooke *et al.*, 2012; Mertens *et al.*, 1996). Plakophilin-3 is expressed in most simple and stratified epithelia (Schmidt *et al.*, 1999). In regards to the epidermis, plakophilin-1 is expressed as a gradient, with maximum expression in the uppermost spinous and granular layers, whereas plakophilin-2 has an inverse expression profile with maximum expression in the basal layer (Figure 4) (Desai *et al.*, 2009; Hatzfeld *et al.*, 1994; Heid *et al.*, 1994; Schmidt and Jäger, 2005). Finally, plakophilin-3 is uniformly expressed in all layers of the epidermis (Figure 4) (Bonne *et al.*, 2003; Schmidt *et al.*,

1999). The physiological implications of the plakophilins expression patterns are not fully understood, but may involve differential capacities in mediating desmosome assembly. These differences will be discussed in more detail below.

While there is only one gene product of plakophilin-3, plakophilins 1 and 2 are alternatively spliced, each existing as two isoforms (Figure 3b) (Hatzfeld, 2007). The shorter 'a' and the longer 'b' variants differ by an insertion of 21 and 44 amino acids in the third and fourth arm repeats, respectively (Figure 3b). Plakophilin 1a and b differ in their localization, with the smaller 1a isoform being nuclear and desmosomal, while 1b is exclusively localized to the nucleus (Klymkowsky, 1999; Schmidt *et al.*, 1997). Currently no experimental data exist comparing localization of plakophilin-2 isoforms a and b. All three plakophilins have known functions in desmosomes and in the nucleus (Hatzfeld, 2007; Hatzfeld, 2010), however only their roles in cell-cell will be discussed.

Section 1.3 Plakophilin-1

Plakophilin-1 was the first of the plakophilins to be identified and was originally known as "band-6 protein" from biochemical analysis of bovine desmosomal preparations (Kapprell *et al.*, 1988). The importance of plakophilin-1 in mediating desmosome stability and functional integrity of tissues is highlighted by the autosomal recessive disorder ectodermal dysplasia-skin fragility syndrome (EDSF). Loss of function mutations in plakophilin-1 cause EDSF and this clinically manifests as skin erosions, skin crusting, painful fissures, keratoderma, nail dystrophy and hypotrichosis (hair loss) (McGrath *et al.*, 1997). EDSF patient skin biopsies revealed poorly formed desmosomes with the loss of cell-cell adhesion or *acantholysis* (McMillan *et al.*, 2003). Along with these findings, subsequent studies have investigated the mechanisms of how plakophilin-

1 functions to regulate desmosomes size and stability. Plakophlin-1 binds to desmoplakin, desmoglein 1, desmocollin 1, actin and keratin (Hatzfeld *et al.*, 2000; Hofmann *et al.*, 2000; Kapprell *et al.*, 1988; Kowalczyk *et al.*, 1997; Smith and Fuchs, 1998). In one study, plakophilin-1 was clearly demonstrated to enhance recruitment of desmoplakin to the desmosomal plaque, which laterally extended the size of desmosomes and increased their interactions with the keratin cytoskeleton (Kowalczyk *et al.*, 1997).Furthermore, plakophilin-1 in coordination with plakoglobin was found to be required for the formation of clustered structures containing both desmoplakin and Desmoglein 1 (Bornslaeger *et al.*, 2001). These studies and others suggest that plakophilin-1 promotes desmosome formation by recruiting and clustering desmosomal proteins at the plasma membrane and within desmosomes (Hatzfeld, 2007; Hatzfeld *et al.*, 2000; South, 2004). Consistent with this model is the observation that desmosomes in suprabasal layers of the epidermis are larger than desmosomes from both simple epithelia and basal epidermal cells (Holthöfer *et al.*, 2007).

Interestingly, recent findings identified plakophilin-1 in stress granules suggesting an association with the translational machinery (Hofmann *et al.*, 2006). Moreover, plakophilin-1 was found to directly associate with and promote the activity of the eukaryotic translation initiation factor 4A1 (eIF4A1) (Wolf *et al.*, 2010). One possible nuclear role of plakophilin-1 could be to enhance transcriptional levels of desmosomal proteins to enhance adhesive strength. Importantly, a study examining the role of plakophilin-1 in stabilizing desmosomal adhesion found that while plakophilin-1 increased desmosomal protein content, it did not alter the expression levels of mRNA encoding desmosomal proteins (South *et al.*, 2003). Thus, these findings indicate that

plakophilin-1 has a direct role in stabilizing desmosomal adhesion, rather than by an indirect control of translation (Wolf *et al.*, 2010) Overall, the evidence demonstrates that plakophlin-1 is crucial in rendering the skin resistant to mechanical stress by mediating interactions with other desmosomal proteins to increase desmosome size and stability in the suprabasal layers of the epidermis (Wahl, 2005; Wolf and Hatzfeld, 2010).

Section 1.3d Plakophilin-2

Plakophilin-2 interacts directly with a broader repertoire of desmosomal components than plakophilin-1, including desmoplakin, plakoglobin, desmoglein 1 and 2, and desmocollin 1a and 2a (Hatzfeld, 2007). As with plakophilin-1, these interactions are mediated through its amino-terminal head domain (Chen *et al.*, 2002). Mutations in plakophilin-2, similar to plakoglobin but with a higher incidence, cause ARVD/C (Gerull *et al.*, 2004; Lai-Cheong *et al.*, 2007). Consistent with ARVD/C in humans, plakophilin-2 mouse knockouts exhibited embryonic lethality due to disruption of desmosomes in the intercalated discs in the heart (Grossmann *et al.*, 2004).

In the epidermis, plakophilin-2 is thought to have differential functions from plakophilin-1 in regulating desmosomal adhesion (Bass-Zubek *et al.*, 2009). Plakophilin-2 is found at high levels in desmosomes in cells of the basal layer of the epidermis (Figure 4) (Schmidt and Jäger, 2005). During nascent junction formation, plakophilin-2 colocalizes with cytoplasmic desmoplakin-containing precursors that form in response to cell–cell contact. Furthermore, plakophilin-2 acts as a scaffold for protein kinase Cα (PKCα), which in turn phosphorylates desmoplakin to facilitate incorporation of desmoplakin into the desmosomal plaque. The lack of plakophlin-2 in cells resulted the failure of desmoplakin to incorporate into desmosomes and thus desmoplakin did not

accumulate properly at cell-cell borders (Bass-Zubek *et al.*, 2008). Furthermore, overexpressed plakophilin-2 localized to desmosomes but was less efficient than overexpressed plakophilin-1 in recruiting desmoplakin and clustering other desmosomal proteins at the cell membrane (Chen *et al.*, 2002). Collectively, plakophilin-2 is not thought to increase desmoplakin content or desmosomal size as robustly as plakophilin-1, but plakophilin-2 is considered to be essential for transport of desmoplakin to the plasma membrane (Bass-Zubek *et al.*, 2009; Godsel *et al.*, 2005).

Section 1.3e Plakophilin-3

Plakophilin-3 is the most recently identified and least characterized plakophilin isoform. Plakophilin-3 is ubiquitously expressed in the epidermis (Figure 4) and binds to the broadest repertoire of desmosomal components including desmogleins 1-3, desmocollin 1-3, plakoglobin, desmoplakin and cytokeratin 18 (Bonne *et al.*, 2003). Plakophilin-3 knockout mice revealed a critical role for plakophilin-3 in the morphogenesis of hair follicles, hairs shafts and in regulating the inflammatory responses in the skin. In the basal layer of the epidermis, plakophilin-3 null mice were devoid of desmosomes in the outer root sheath of hair follicles as well as in matrix cells that contact the dermal papillae in the dermis (Sklyarova *et al.*, 2008). Additionally, fewer desmosomes were present in basal layer keratinocytes. Plakophilin-3 null mice did not exhibit severe epidermal integrity phenotypes, however this is presumed to be because the upregulation of plakophilin-2 and desmoplakin compensated for the loss of plakophilin-3 (Sklyarova *et al.*, 2008). Overall, the data suggest that plakophilin-3 contributes discrete functions in regulating cell-cell adhesion, but further studies are needed to elucidate the specific roles of plakophilin-3 in desmosomal adhesion.

In conclusion, all three plakophilins interact with desmosomal cadherins and other plaque proteins, but they appear to have distinct roles in regulating desmosomal cell-cell adhesion (Hatzfeld, 2007). Plakophilins 2 and/or 3 may provide the scaffold for the assembly of desmosomes in simple epithelia and in basal layer keratinocytes. Plakophilin-1 appears to enhance desmosome adhesion to a greater extent than plakophilins 2 and 3 and is critical for cell-cell adhesion in the epidermis which experiences a great deal of mechanical stress (Hatzfeld, 2007).

Section 1.4 Plakin family member - Desmoplakin

Desmoplakin is a member of plakin family of proteins which function to crosslink cytoskeletal filaments to plasma membrane-associated complexes. Plakin family members BPAG1 and plectin link keratin filaments to hemidesmosomes and thereby attach cells to the extracellular matrix. Plakin family members, periplakin and envoplakin are important in the attachment of intermediate filaments during development of the cornified envelope in the stratum corneum (Jefferson *et al.*, 2004). The focus of this discussion will be on desmoplakin and how its direct binding to the intermediate filament cytoskeleton is essential for desmosomes to mediate strong intercellular adhesion and tissue integrity (Yin and Green, 2004).

Desmoplakin was first identified from purified desmosomal fractions from stratified bovine epithelia (Skerrow and Matoltsy, 1974). The desmoplakin gene is alternatively spliced. The longer desmoplakin I isoform and the shorter isoform II differ by 599 amino acids in the region of the central rod domain (Figure 3c) (Green *et al.*, 1990; Virata *et al.*, 1992). Desmoplakin is dumbbell-shaped molecule that has three-distinct structural regions: a 1056-amino acid amino-terminal globular plakin domain, an

890-residue coiled-coil dimerization domain (called the central rod domain) and a 925-residue C-terminal tail that contains plakin repeats A-C (Figure 3c). Following the plakin repeats at the extreme carboxyl-terminus of desmoplakin is a glycine-serine-arginine (GSR) rich region (Choi and Weis, 2011; Jefferson *et al.*, 2004; O'Keefe *et al.*, 1989). The desmoplakin amino-terminal plakin domain binds to desmosomal plaque proteins plakophilins (1-3) and plakoglobin (Bornslaeger *et al.*, 2001; Kowalczyk *et al.*, 1997). The central rod domain contains repeats of an oligomerization motif and is responsible for the formation of parallel homodimers (Choi and Weis, 2005a; Green *et al.*, 1990; O'Keefe *et al.*, 1989; Sonnenberg and Liem, 2007). Desmoplakin II is missing roughly two-thirds of the rod domain and thus may be present as a monomer, although this remains unclear (O'Keefe *et al.*, 1989; Sonnenberg and Liem, 2007; Stokes, 2007). The carboxyl-terminal plakin repeats (A-C) contain multiple sites for intermediate filament binding, while the GSR rich domain regulates intermediate filament binding in a phosphorylation-dependent manner (Brooke *et al.*, 2012; Fontao *et al.*, 2003; Kowalczyk *et al.*, 1997; Stappenbeck *et al.*, 1994). Rotary-shadow electron microscopy demonstrates that the molecule can span up to 180 nm, with a 130 nm long central rod connecting two the amino- and carboxyl-terminal globular heads which function to link the desmosomal plaque to keratin intermediate filaments (O'Keefe *et al.*, 1989; Stokes, 2007).

Human disease and gene-knockout studies provide compelling evidence that desmoplakin is absolutely required for desmosomal adhesion (Jefferson *et al.*, 2004). In humans, the first mutation reported in the gene encoding desmoplakin was observed in patients with the dominantly inherited skin disorder, striate palmoplantar keratoderma (Leung *et al.*, 2002) . Affected individuals exhibit skin thickening on the fingers, palms

and soles caused by haploinsufficiency of desmoplakin. Light and electron microscopy demonstrated widened intercellular spaces and small desmosomes with fewer keratin linkages (Armstrong *et al.*, 1999). Following this initial finding, subsequent reports have identified desmoplakin recessive nonsense and missense gene mutations, as well as a single base deletion to cause palmoplantar keratoderma, cardiac disorders and woolly hair (Alcalai *et al.*, 2003; Norgett *et al.*, 2000). All of the mutations mapped to regions encoding the amino- or carboxyl-terminal globular domains, likely disrupting binding to desmosomal plaque proteins and intermediate filaments, respectively (Whittock *et al.*, 1999; Whittock *et al.*, 2002). In mice, desmoplakin null animals suffered early embryonic lethality at embryonic day 6.5 and displayed a dramatic reduction in desmosomes with loss of keratin filament attachment (Gallicano *et al.*, 1998). Epidermis-specific desmoplakin knockout mice were viable and although they exhibited same number of desmosomes in the skin as wild type mice, they lacked keratin filament attachments. Induction of mild mechanical stresses to desmoplakin-null mouse skin caused intercellular separations that led to large areas of peeled and denuded skin (Vasioukhin *et al.*, 2001). Collectively, these studies demonstrate that desmoplakin is required for the assembly of functional desmosomes and the generation of epidermal integrity.

Section 1.5 Regulation of desmosome adhesion

The previous discussion has focused on the crucial function of desmosomes in the epidermis and the structure-function characteristics of individual proteins that comprise the desmosome. The evidence overwhelmingly establishes desmosomes as stable providers of mechanical integrity in tissues that experience significant mechanical stress. Nonetheless, these molecular rivets are dynamic structures subject to rearrangements

and/or disassembly in order to modify desmosomal adhesive strength during various cell behaviors including cell division, migration, wound healing and tissue regeneration (Hofer and Schweighofer, 2007; Yin and Green, 2004). To some extent, the tissue and differentiation-specific expression patterns of desmosomal components tailors desmosome function within different cells and tissues. However, desmosome assembly and disassembly are regulated post-translationally by extracellular calcium levels, intracellular signaling, proteolytic cleavage, and cross talk with adherens junctions (Yin and Green, 2004). Most relevant to this dissertation are fluctuations in desmosomal adhesion by changes in extracellular calcium, binding of pemphigus vulgaris autoantibodies and activation of intracellular signaling pathways. This next section will review how changes in extracellular calcium levels affect desmosomal adhesion. Following this section, the next chapter will address autoantibody binding and intracellular signaling pathways that modify desmosome adhesion in the disease pemphigus vulgaris.

Section 1.5a Calcium-dependent alterations of desmosomes

Based on experimental data of desmosome behavior in cell culture, desmosomal adhesion is commonly referred to as calcium-dependent. Early cell culture experiments showed that desmosome assembly is a calcium-dependent process as extracellular calcium concentrations above 0.1 mM are required for nascent junction formation (Garrod, 2010). Upon the addition of calcium to concentrations over 0.1 mM (typically 0.6-1.2 mM), keratinocytes form desmosomes within minutes and continue nascent junction assembly for several hours (Hennings and Holbrook, 1983; Hennings *et al.*, 1980; Watt *et al.*, 1984). The availability and binding of calcium ions to the ectodomains of desmosomal cadherins induces a conformational change that allows for the

engagement in homophilic or heterophilic adhesive interactions with cadherins from adjacent cells (Nagar *et al.*, 1996; Oroz *et al.*, 2011; Yin and Green, 2004). In the epidermis, the precise concentration of extracellular calcium is unclear, however studies show that extracellular calcium content is lower in the basal and suprabasal layers, with a gradual increase in the higher layers of the stratum granulosum (Elias *et al.*, 2002; Menon *et al.*, 1985). Although a gradient of extracellular calcium exists in the epidermis, it is unlikely that keratinocytes *in vivo* experience extreme variations in calcium levels observed in cell culture. Rather every cadherin molecule in the epidermis that emerges onto the cell surface is most likely instantly exposed to calcium concentrations that favor desmosome assembly (Garrod 2010). Therefore, the *in vitro* 'calcium switch' model does not precisely mimic physiological conditions, however it has been a useful tool in revealing insights into the mechanisms that regulate desmosome adhesion.

A new concept in cell-cell adhesion is hyper-adhesion, which is a calcium-independent state of desmosomal adhesion. Recent studies have revealed that subsequent to calcium-dependent junction assembly, desmosomes exist in two alternative adhesive states coined, 'calcium-dependent' or 'calcium-independent' (Garrod and Kimura, 2008; Garrod *et al.*, 2005). Identification of these states is revealed by their differential sensitivity to changes in extracellular calcium levels. To determine whether desmosomes are calcium-dependent or independent a simple "calcium chelation assay' experiment is performed. This assay involves growing cells in high calcium medium and subsequently exposing them for a minimum of 90 minutes to calcium-free media supplemented with the calcium chelating agent EGTA (Garrod, 2012). After calcium chelation, the localization of desmoplakin is observed and used as a desmosomal marker. In response to

calcium chelation, calcium-dependent desmosomes lose desmoplakin localization at cell-cell contacts (Figure 5), indicating that desmosomes are downregulated and keratinocyte adhesion is lost. In contrast, calcium-independent desmosomes are resistant to calcium chelation and desmoplakin staining at cell-cell contacts is maintained (Garrod, 2010) . Using functional adhesion strength assays, calcium-independent desmosomes have been shown to exhibit stronger intercellular adhesion and thus are deemed 'hyper-adhesive' (Kimura *et al.,* 2007). In cell culture, the acquisition of hyper-adhesion is achieved gradually. Confluent epithelial cell monolayers become ~98% calcium-independent upon the sixth day of culture in high calcium media (Wallis *et al.,* 2000). Additionally, keratinocytes from sub-confluent cell cultures possess a constant state of calcium-dependency (Kimura *et al.,* 2007; Wallis *et al.,* 2000). Investigations into the mechanism that regulate desmosome adhesive states report that the acquisition of calcium-independence and the ability of desmosomes to transition between adhesive states occurs solely through intracellular PKCα activity. Moreover, the acquisition of hyper-adhesion has been reported to involve no change in the composition of desmosomes (Garrod, 2010). However, conflicting reports implicate desmoplakin and plakophilin-1 in the formation of calcium-independent desmosomes (Hobbs and Green, 2012; South *et al.,* 2003). The presence of calcium-dependent and independent-desmosome adhesion have been reported *in vivo* although the implications of these findings are not fully understood.

Calcium-independent, hyper-adhesive desmosomes may represent the normal physiological state of adhesion from intact, unperturbed epithelia. Furthermore, calcium-independent desmosomes quickly revert to a calcium-dependent state upon wounding to an intact epidermis or cultured cell sheet (Garrod *et al.*, 2005; Wallis *et al.*, 2000). Upon

injury to skin, migration and proliferation of keratinocytes into the wound matrix requires downregulation of desmosome adhesion (Santoro and Gaudino, 2005). Moreover, the calcium gradient *in vivo* is disrupted when the epidermis is damaged by mechanical and chemical injury as well as by cutaneous diseases (Cornelissen *et al.*, 2007; Elias *et al.*, 2002; Menon and Elias, 1991). Collectively, these studies suggest the calcium-dependent state of desmosome adhesion represents a weaker and more plastic state of cell-cell adhesion necessary for the downregulation of desmosomes during the process of wound healing (Garrod 2010).

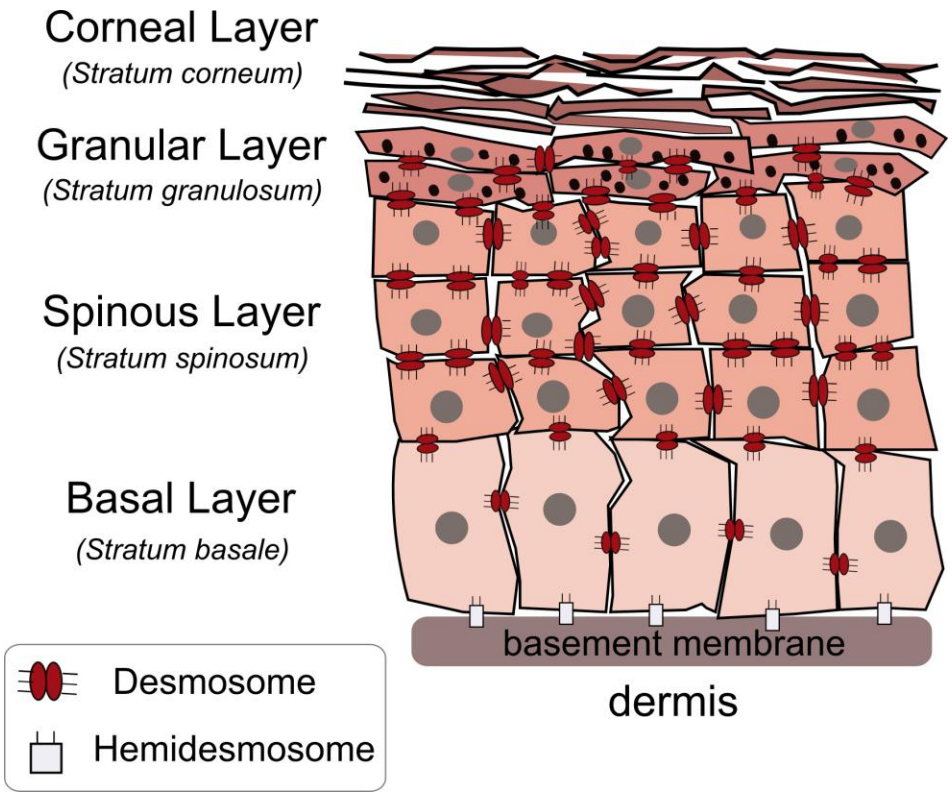

Figure 1. Organization of the epidermis. The human epidermis consists of four major cell layers or strata; the basal, spinous, granular and corneal layers. Each layer is primarily composed of keratinocytes and is constantly generated by proliferating basal cells that migrate up while undergoing terminal differentiation. Desmosomes are present throughout the cell layers and provide the epidermis with mechanical stability by anchoring the keratin intermediate filament cytoskeleton to sites of cell-cell contact.

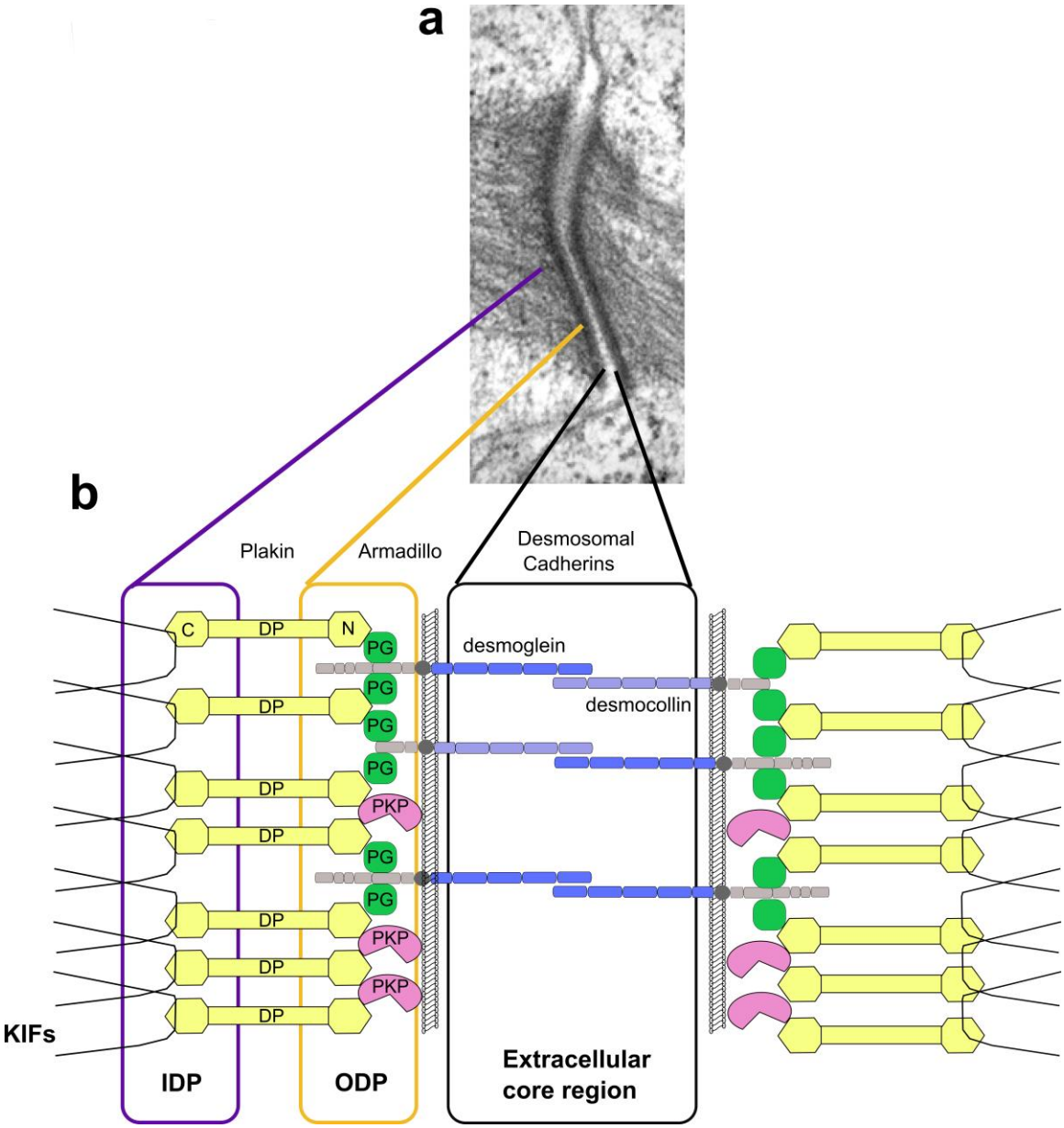

Figure 2. Desmosome ultrastructure and molecular architecture.

(a) Electron micrograph of a desmosome from primary human keratinocytes. **(b)** The proteins and interactions that comprise a desmosome. The extracellular domains of the desmosomal cadherins, desmogleins and desmocollins, mediate adhesive interactions within the extracellular core region. In the outer dense plaque (ODP) the cadherin cytoplasmic tails are linked to the desmoplakin (DP) amino-terminus (N) by the

armadillo proteins, plakoglobin (PG) and plakophilins (PKP). The inner dense plaque (IDP) consists of the desmoplakin carboxyl-terminus (C) that binds directly to keratin intermediate filaments (KIFs). The precise arrangement of proteins within the ODP is not known, however, desmoplakin binds directly to plakoglobin and the plakophilins, whereas plakoglobin binds directly to the cadherin tails.

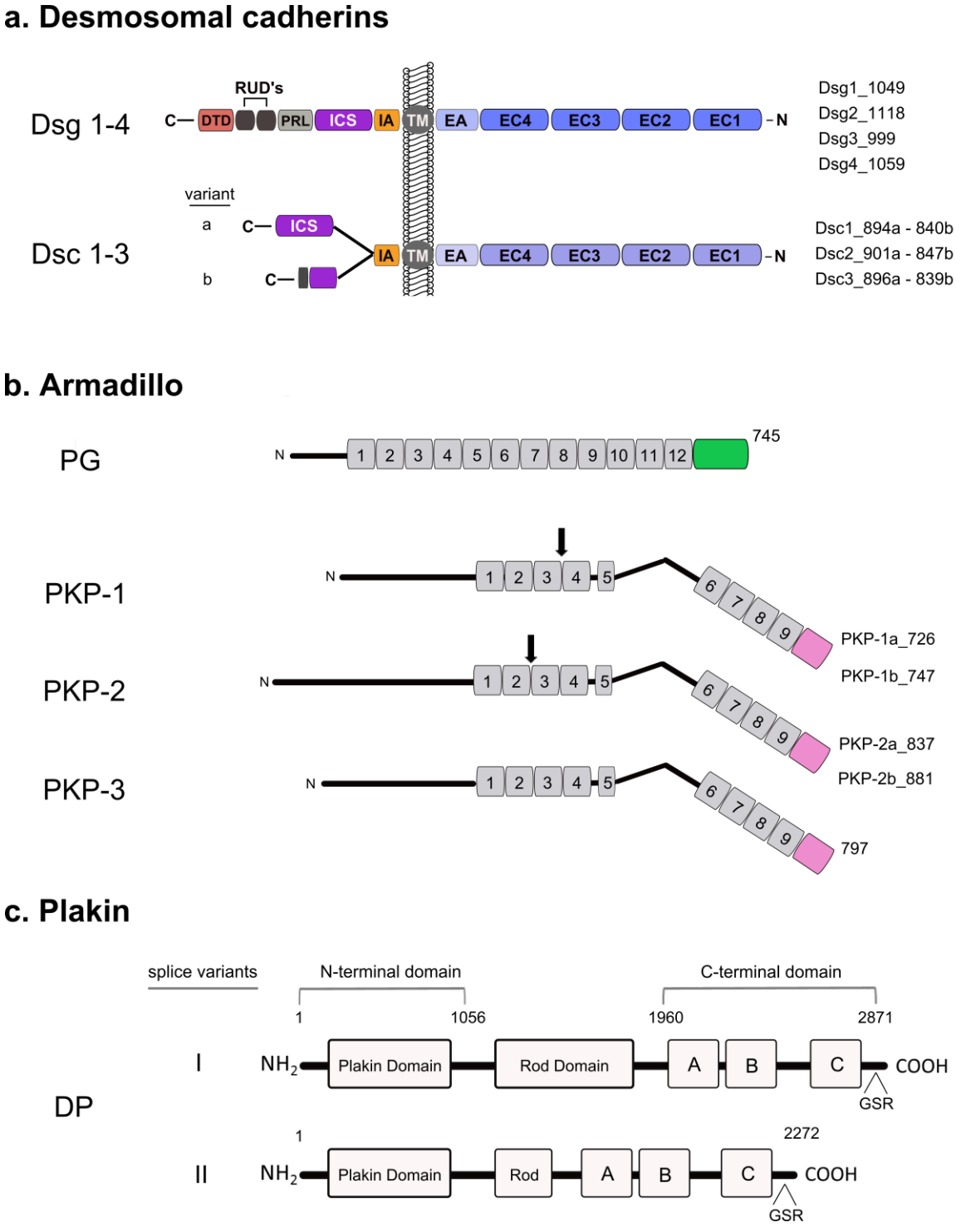

Figure 3. Protein domains of the major desmosomal components. (a) The desmosomal cadherins. The desmogleins (Dsg1-4) and desmocollins (Dsc1-3) contain four extracellular cadherin repeat domains (EC), with an extracellular anchor (EA)

followed by a transmembrane domain (TM) that is flanked by an intracellular anchor (IA) domain. All three desmocollin gene products are alternatively spliced generating the ‘a’ variant and the shorter ‘b’ variant. Desmocollin ‘a’ proteins and the desmogleins contain an intracellular cadherin-like domain (ICS) which binds plakoglobin. The desmogleins contain the additional intracellular proline-rich linker (IPL), a variable number of repeat unit domains (RUD, desmoglein 1 contains 5 RUDs; desmoglein 2, 6; desmoglein 3, 2 and desmoglein 4, 3) and the desmoglein terminal domain (DTD). The number of amino acid residues in each protein and splice variant are listed to the right. **(b) Armadillo protein family members’ plakoglobin and the plakophilins.** Plakoglobin (PG) consists of 12 central *arm*-repeat domains flanked by non-*arm* amino- and carboxyl-terminal tail domains. Plakophilins 1-3 (PKP 1-3) contain 9 central *arm*-repeats with an insert between repeats 5 and 6 that introduces a bend into their structure. Plakophilins 1 and 2 are alternatively spliced, each existing as two isoforms. The shorter ‘a’ and the longer ‘b’ variants differ by an insertion of 21 and 44 amino acids in the third and fourth arm repeats, respectively. **(c) The plakin family member desmoplakin.** Desmoplakin (DP) is alternatively spliced, the longer desmoplakin I isoform and the shorter isoform II differ by 599 amino acids. Desmoplakin consists of an amino-terminal globular plakin domain, a coiled-coil dimerization domain (the central rod domain) and a carboxyl-terminal tail that contains plakin repeats A-C and a glycine-serine-arginine (GSR) rich region.

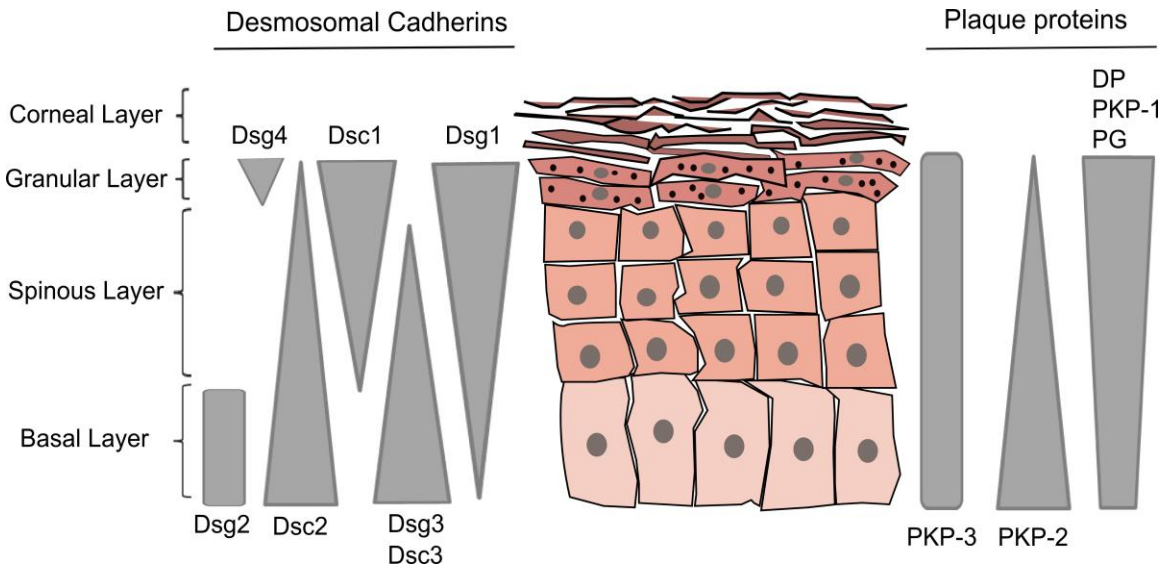

Figure 4. Differentiation-specific expression patterns of desmosomal proteins in the epidermis. The relative expression levels of the desmosomal cadherins (Desmoglein_Dsg1-4, desmocollin_Dsc 1-3) and the desmosomal plaque proteins (desmoplakin_DP, plakophilin_PKP-1-3, plakoglobin_PG) in the epidermal layers are depicted above (Desai *et al.*, 2009; Saito *et al.*, 2012b).

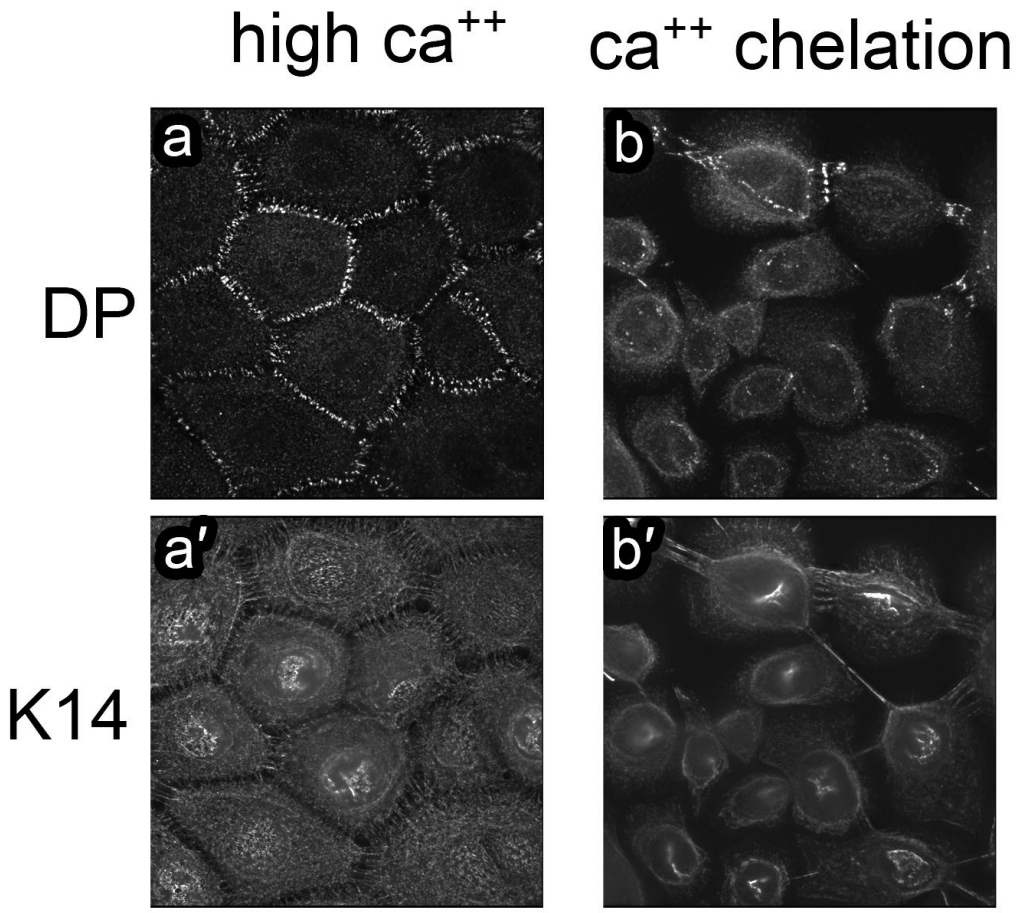

Figure 5. Sub-confluent keratinoctyes possess calcium-dependent desmosomes. Sub-confluent Keratinocytes in high calcium (ca^{++}) medium or after 4 hours of calcium chelation, fixed, permeabilized and immunostained for desmoplakin (DP) and keratin-14 (K14). Keratinocytes in high calcium media exhibit continuous desmoplakin staining and keratin filament extension at the cell-cell borders (a, a´). Following 4 hours of calcium chelation, keratinocytes exhibit loss of desmosomes marked by keratin retraction and decreased desmoplakin staining at cell-cell borders.

Chapter II

Introduction to Pemphigus Vulgaris

Dana K. Tucker

Figure 7 was adapted from Saito M, Tucker DK, Kohlhorst D, Niessen CM, Kowalczyk AP (2012) Classical and desmosomal cadherins at a glance. Journal of Cell Science 125:2547-52.

Section 2.0 Introduction to Pemphigus

The name “pemphigus” is derived from the Greek word “pemphix” meaning bubble or blister (Venugopal and Murrell, 2012). In 1791 the word “pemphigus” was given its present meaning, that of chronic skin blistering diseases (Lever, 1953). Pemphigus represents a class of autoimmune skin blistering diseases characterized by the loss of cell-cell adhesion, or *acantholysis,* of keratinocytes in the skin and mucous membranes due to binding of autoantibodies to the desmosomal cadherins, Desmoglein 3 and/or desmoglein 1 (Amagai *et al.*, 1991; Payne *et al.*, 2004). In pemphigus, the passive transfer of purified pemphigus autoantibodies to normal human skin and mice induces blistering. Furthermore, pemphigus autoantibodies primarily belong to the IgG4 subclass (Ayatollahi *et al.*, 2004; Ding *et al.*, 1999) and do not require the complement system or leukocytes to cause disease (Anhalt *et al.*, 1982; Anhalt *et al.*, 1986; Schiltz and Michel, 1976). Pemphigus is divided into two major subtypes, pemphigus vulgaris (PV) and pemphigus foliaceus (PF). PV is further categorized as either mucosal dominant or mucocutanous. Each PV subtype is based on a specific desmoglein autoantibody profile and clinical presentation (Amagai *et al.*, 1999b). Patients with PV exhibit the most severe clinical presentations and account for roughly 70% of pemphigus cases (Venugopal and Murrell, 2012).

Section 2.1 Autoantibody profile and the location of blisters in pemphigus

Pemphigus foliaceus (PF) is characterized by the generation of only anti-desmoglein 1 IgG autoantibodies and patients develop blisters in the more superficial granular layer of the epidermis (Amagai *et al.*, 1999b; Shirakata *et al.*, 1998). In pemphigus vulgaris (PV), patients present with painful non-healing erosions of mucous

membranes and blisters in the skin between the basal and suprabasal layers of the epidermis (Waschke, 2008). Furthermore, PV patients generate either solely anti-desmoglein 3 autoantibodies or both anti-desmoglein 3 and anti-desmoglein 1 autoantibodies. These two distinct autoantibody profiles represent the two subtypes of PV, mucosal dominant or mucocutanous PV (Figure 6). Mucosal dominant PV is characterized by the generation of anti-desmoglein 3 autoantibodies and areas affected are limited to mucous membranes such as in the mouth, nostrils, lips, eyelids and genital areas (Amagai *et al.*, 1991; Amagai *et al.*, 1999b; Shimizu *et al.*, 1995; Venugopal and Murrell, 2012). Mucocutaneous PV accounts for roughly 50% of PV cases and patients generate both anti-desmoglein 3 and anti-desmoglein 1 autoantibodies. Those afflicted with mucocutanous PV exhibit blisters and erosions deep in the epidermis, as well as erosions of mucous membranes (Amagai *et al.*, 1999b; Kottke *et al.*, 2006).

The desmoglein compensation hypothesis has been proposed to explain the different locations of blisters within the epidermis observed in PV and PF patients (Figure 6) (Hanakawa *et al.*, 2002). This hypothesis suggest that the blister site is dependent upon: 1) the differential expression of desmoglein 3 and desmoglein 1 and 2) the specific isoform of desmoglein that is targeted by the autoantibodies in PV or PF (Waschke, 2008). Desmoglein 1 and desmoglein 3 possess inverse expression patterns in the epidermis. Desmoglein 1 expression is prominent in the superficial epidermis and less so deep in the epidermis, whereas desmoglein 3 is primarily expressed in the basal and lower spinous layers (Figure 3, 6) (Shimizu *et al.*, 1995). In mucous membranes the expression profiles of desmoglein 1 and desmoglein 3 differ from the skin in that desmoglein 3 is expressed throughout the cell layers of mucus membrane epithelia

(Figure 6). According to the desmoglein compensation hypothesis, PV autoantibodies against desmoglein 3 cause blistering where desmoglein 3 is most highly expressed deep within the epidermis, where desmoglein 1 is lacking. Furthermore, high expression of desmoglein 1 in the more superficial layers of the epidermis is thought to compensate for PV-induced loss of desmoglein 3 function within the upper epidermis. Concordantly, PF desmoglein 1-specifc autoantibodies cause blistering that is restricted to the superficial epidermis because expression of desmoglein 3 in the lower epidermis compensates for the lack of functional desmoglein 1. However, the desmoglein compensation hypothesis does not explain why superficial blisters do not form in mucocutaneous PV patients considering that both anti desmoglein 1 and desmoglein 3 autoantibodies are generated (Figure 6) (Payne *et al.*, 2004; Waschke, 2008). Nevertheless, the desmoglein compensation hypothesis does in part explain the clinical manifestations of PV and PF. The following sections of this chapter will highlight the characteristics of PV and will focus only on the effects of anti-desmoglein 3 autoantibodies on desmosomal cell-cell adhesion.

Section 2.2 Epidemiology

PV is a rare disease with a yearly incidence approximately 1 in 100,000 people (Venugopal and Murrell, 2012). The incidence of PV increases to roughly 2-3 per 100,000 people in the Ashkenazi Jewish population and in people of Mediterranean decent (Zakka *et al.*, 2012). Although PV is not a genetic disease, people with certain human leukocyte antigen (HLA) alleles of the major histocompatibility complex (MHC) are more likely to develop the disease. Additionally, various environmental and pharmacologic etiological factors have been reported to trigger PV (Venugopal and

Murrell, 2012). The mean onset is 50-60 years old, although cases of PV have been reported in children and the elderly. PV is distributed equally between men and women (Yeh *et al.*, 2005). Beyond humans, PV afflicts horses, dogs and cats (Waschke, 2008).

Section 2.3 Diagnosis and Treatment

Diagnosis of PV is based on a combination of clinical, histopathological, and immunofluorescence analysis (McCuin *et al.*, 2006). Hematoxylin and eosin (H&E) analysis of biopsies from early, intact blisters are used to determine the location of acantholysis (intraepidermal or subepidermal). PV is characterized by suprabasilar acantholysis resulting in a split just above the stratum basale, thus leaving a single layer of basal keratinocytes attached to the basement membrane (Figure 7) (Venugopal and Murrell, 2012). Immunofluorescence (direct and indirect) reveals the presence of IgG (IgA and IgM in some cases) as well as the specific autoantibody target, either desmoglein 3 and/or desmoglein 1 (Mutasim, 1999; Venugopal and Murrell, 2012). Finally, the most recent advance in diagnosing pemphigus is the availability and use of standardized enzyme-linked immunosorbent assays (ELISA) to detect PV autoantibodies (Amagai *et al.*, 1999a; Bhol *et al.*, 1998).

Prior to the 1950's, the mortality rate of PV was between 70-100 % (Jeffes and Ahmed, 1983; Yeh *et al.*, 2005). Following the 1950's, the introduction of oral corticosteroids to suppress the immune system resulted in a significant drop in mortality, but the rate is still relatively high at around 12% (Grando, 2012). Currently, prolonged, high-dose systemic corticosteroid treatment remains the standard treatment for PV patients. Unfortunately, severe and sometimes life threatening side effects are associated with prolonged immune suppression including hypertension, osteoporosis,

atherosclerosis, peptic ulcer disease, aseptic necrosis, diabetes mellitus/glucose intolerance, susceptibility to infections and septicemia (Yeh *et al.*, 2005). Therefore, alternative treatment approaches are being sought in an attempt to reduce or eliminate the need of long-term corticosteroids (Jeffes and Ahmed, 1983; Yeh *et al.*, 2005).

Adjuvant therapies, such as anti-inflammatory and other immunosuppressant agents, are now commonly used in combination with corticosteroids to reduce the negative effects of prolonged corticosteroid treatment. However, some patients have been resistant to these treatments and/or develop additional adverse effects, thus resulting in their discontinuation (Yeh *et al.*, 2005). In addition to adjuvant therapies, a number of other treatments have been developed including intravenous immunoglobulin replacement therapy, plasmapheresis and immunoadsorption. On a smaller scale, several case reports have documented the use of extracorporeal photochemotherapy, tumor necrosis factor-alpha (TNF-α) antagonists (infliximab and etanercept) and cholinergic agonists to treat PV (Prajapati and Mydlarski, 2008). A new promising and more widely used treatment for PV is rituximab, which is a chimeric monoclonal antibody that targets the B-cell CD20 antigen to result in their lysis (Feldman and Ahmed, 2011). The rationale for the use of rituximab in PV is based on its ability to deplete CD20+ B cells so to result in a decrease in the production of pathogenic PV IgG. Preliminary studies have had promising results; however whether these positive results can be maintained long term, life long, or only of for limited duration is not known (Zakka *et al.*, 2012).

Section 2.4 Pathomechanisms of PV

Although an extensive set of literature exists on the mechanisms by which PV autoantibodies (IgG) disrupt desmosome adhesion and cause acantholysis, this area of

research remains a major focus because the pathomechanisms of PV are not fully understood. Three main models of PV pathogenesis have been proposed including direct inhibition (steric hindrance) of desmoglein 3 adhesive interactions, PV IgG-induced intracellular signaling that downregulates desmosomal adhesion and that destabilization of desmosomal components disrupts desmosome dynamics (assembly and disassembly). These models will be discussed in the following sections.

Section 2.4a Steric hindrance model

Since the discovery that autoantibodies in PV are directed against cell surface desmosomal proteins, it was proposed that these autoantibodies directly interfere with desmoglein adhesive *cis* and/or *trans* binding interactions, a mechanism called "steric hindrance" (Amagai *et al.*, 1991; Jones *et al.*, 1986; Waschke, 2008). Several lines of evidence support this model, starting with the identification of PV IgG epitopes that map to the desmoglein 3 amino-terminal EC1 domain (Amagai *et al.*, 1992; Sekiguchi *et al.*, 2001; Tsunoda *et al.*, 2003). Crystal structures of classical cadherins indicate that this region forms the *trans*-adhesive interface between cells (Boggon *et al.*, 2002; Nagar *et al.*, 1996; Shapiro *et al.*, 1995). To determine the pathogenicity of antibody binding to either the amino-terminal EC1-2 domains or the more carboxyl EC3-5 domains of desmoglein 3, PV IgG was affinity purified on columns containing glutathione fusion proteins encoding these specific regions. The purified antibodies were then injected into mice. Subsequent to injection, PV IgG affinity-purified from the EC1-2 fusion protein column caused suprabasilar acantholysis and blistering, whereas PV IgG from the EC3-5 column did not (Amagai *et al.*, 1992). These results demonstrate that the pathogenic activity or rather, the formation of blisters occurs upon the binding of PV IgG to the

region of desmoglein 3 thought to be responsible for cadherin adhesive interactions. Further evidence in support of the steric hindrance model was revealed by the presence of pathogenic activity by the mouse monoclonal antibody (mAb) AK23, which targets the amino-terminal adhesive interface of desmoglein 3. Mouse mAbs that were found to not be pathogenic, bind to the carboxyl-terminal EC3-5 domains of desmoglein 3 and are the regions where no direct adhesive *trans*-interactions are thought to occur (Tsunoda *et al.*, 2003). Finally, more recent findings utilizing atomic force microscopy have demonstrated that PV IgG directly block desmoglein 3 *trans*-interactions (Heupel *et al.*, 2008).

Beyond the use of mice and atomic force microscopy, passive transfer of anti-desmoglein 3 antibodies (PV IgG or mouse mAbs) to primary cultures of keratinoctyes has been developed as an effective model to investigate the pathomechanisms of PV. In vitro keratinocyte cell-dissociation assays are utilized to measure cell-cell adhesion strength and assess the pathogenicity of anti-desmoglein 3 antibodies (Ishii *et al.*, 2005). In these functional *in vitro* assays, keratinocyte monolayers are incubated with anti-desmoglein 3 antibodies and then treated with the enzyme dispase, which specifically disrupts cell to matrix interactions. Following release from their substrate, monolayers are subjected to mechanical stress. Depending on the strength of adhesion between cells this stress may or may not cause the cell sheet to fall apart (Ishii *et al.*, 2005). Utilizing cell-dissociation assays, evidence from our laboratory suggest that steric hindrance alone is not sufficient to cause acantholysis. Primary human keratinocytes treated with PV IgG at 4°C do not lose cell-cell adhesion strength, rather the disruption of keratinocyte adhesion only occurred in cells several hours after being incubated with PV IgG at 37 °C (Calkins *et al.*, 2006). Additional findings from this study, which will be discussed later,

support the model by which PV IgG binding disrupts desmosome dynamics acting to destabilize desmosomal components. The next section discusses the role of cell signaling in the pathogenesis of PV.

Section 2.4b Intracellular signaling model

A multitude of studies have implicated the involvement of signal transduction pathways in mediating PV IgG-induced acantholysis. Indeed, PV IgG binding to desmoglein 3 causes the activation of several signaling pathways including protein kinase C (PKC), p38 mitogen activated protein kinase (MAPK) and epidermal growth factor receptor (EGFR) and amongst others (Sharma *et al.*, 2007). Furthermore, modulations of the aforementioned pathways have partially or completely prevented PV IgG-induced keratinocyte acantholysis. These findings are reviewed below.

PKC:

Several studies have shown that PV IgG induces a rapid, transient phospholipase C (PLC)-dependent increase of inositol 1,4,5 triphosphate (IP3) and intracellular calcium leading to activation of PKC (Kitajima *et al.*, 1999; Memar *et al.*, 1996; Osada *et al.*, 1997; Seishima *et al.*, 1995; Seishima *et al.*, 1999). Furthermore, inhibitors of phospholipase C and PKC prevented PV IgG-induced acantholysis in mice (Sánchez-Carpintero *et al.*, 2004). A more recent study reported that the acquisition of hyper-adhesive, calcium-independent desmosomes by mechanisms that involve PKCα attenuates PV IgG-induced keratinocyte acantholysis. However, in this study, pharmacological induction of the hyper-adhesive desmosome state with the PKC inhibitor Go6976 only partially reduced PV IgG-induced acantholysis (Cirillo *et al.*, 2010).

p38MAPK:

The three main subfamilies of the MAPKs are p38MAPK, extracellular signal-regulated kinases (ERK) and c-jun amino-terminal kinases (JNK) (Johnson and Lapadat, 2002). Three of the four p38MAPK family members, α, β and δ isoforms are expressed in human keratinocytes and can be activated by environmental stresses such as heat or osmotic shock (Sharma et al., 2007). Additionally, *in vivo* both in mice and in humans, PV IgG has been demonstrated to activate p38MAPK and one of its downstream targets heat shock protein (HSP) 27 (Berkowitz *et al.*, 2008; Berkowitz *et al.*, 2006). In mice, inhibition of p38MAPK abrogated blister formation (Berkowitz *et al.*, 2006). Whether or not p38MAPK inhibition prevents blister formation in pemphigus patients has not been determined. However, clinical trials are underway to determine the safety and efficacy of an oral p38MAPK inhibitor for treatment of PV (Berkowitz *et al.*, 2008; Prajapati and Mydlarski, 2008). The mechanisms by which p38MAPK inhibition abrogates keratinocyte dissociation are not entirely clear but may involve the preventing disorganization of the keratinocyte cytoskeleton caused by PV IgG (Berkowitz *et al.*, 2005; Waschke, 2008).

EGFR:

Increases in the activation of EGFR following binding of PV IgG has been reported after 30 minutes and peaked after 60 minutes in cultured keratinocytes (Frusic-Zlotkin *et al.*, 2006). Furthermore, PV IgG-induced activation of EGFR resulted in phosphorylation of its downstream substrates, the MAP kinase ERK and transcription factor c-Jun, and internalization of EGFR. Specific inhibitors of EGFR blocked PV IgG-induced acantholysis along with phosphorylation of EGFR and its downstream substrates

(Frusic-Zlotkin *et al.*, 2006). However the significance of these findings are unclear considering the very high concentrations of PV IgG were used (5mg/mL vs. the typical 150 µg/mL) and that 60 hours incubation of PV IgG was required to induce acantholysis (Frusic-Zlotkin *et al.*, 2006; Waschke, 2008).

Section 2.4c Pemphigus is a disease of desmosome instability

Prior to the identification of desmosomal cadherins as the targets of PV IgG, early studies noted that on murine epidermal cell monolayers the binding of PV IgG caused cell surface clustering and internalization of PV IgG and its cell surface antigen within 30 minutes. Furthermore, these PV IgG complexes fused with lysosomes suggesting that PV IgG marks its target for an endocytic-degradation pathway (Patel *et al.*, 1984). Following this initial observation, multiple studies have further investigated PV IgG-induced desmoglein 3 internalization and have proposed that the internalization of desmoglein 3 plays a key role in the pathogenesis of PV. In a report by Sato *et al*., cell surface clustering of non-junctional desmoglein 3 molecules was observed just prior to their incorporation into desmosomes. Upon treatment with PV IgG, these non-desmosomal desmoglein 3 proteins were internalized and desmosome assembly was prevented (Sato *et al.*, 2000). Another study demonstrated that PV serum decreases the half-life of non-desmosomal desmoglein 3 proteins from 24 hours to 18 hours and suggest that this destabilization of desmoglein 3 contributes to the loss of cell-cell adhesion (Cirillo *et al.*, 2006). Studies from our lab demonstrate that the PV IgG-internalized non-desmosomal desmoglein 3 proteins are targeted for degradation thru an endo-lysosomal pathway (Calkins *et al.*, 2006). Additionally, our lab found that keratinocytes do not exhibit less

adhesion strength immediately upon PV IgG binding, rather that the loss of adhesion occurs over time after the cells have mounted some sort of response.

A time course examining both desmoglein 3 and DP localization after PV IgG treatment revealed that mislocalization of desmoplakin occurs several hours after desmoglein 3 internalization and degradation. Moreover, this disruption of desmoplakin temporally correlates with the loss of cell-cell adhesion strength, whereas the disruption of desmoglein 3 occurs prior to the loss of cell adhesion (Calkins *et al.*, 2006). These and the other findings suggest that desmoglein 3 endocytosis and degradation play a key role in the pathogenesis of PV by destabilizing desmosomes and triggering their disassembly.

Section 2.5 Dissertation hypotheses

My central hypothesis is that PV IgG binding to desmoglein 3 promotes desmosome disassembly and perturbs desmosome assembly thereby causing the loss of cell-cell adhesion. To further our understanding of the pathomechanisms of PV, our goal described in Chapter III was to reveal more precisely how PV IgG-induced desmosome disassembly occurs by defining the temporal and spatial relationships between desmosomal components during this process. The results presented in Chapter III suggest that desmosome disassembly and the subsequent loss of cell-cell adhesion occurs sequentially in specific phases triggered by desmoglein 3 internalization and degradation. *A prediction based on the central hypothesis is that reinforcing desmosome adhesion by promoting assembly or slowing disassembly of desmosomes will counteract the effects of PV IgG and prevent the loss of adhesion.* Indeed, we demonstrate in Chapter III that increasing desmoglein 3 biosynthesis counteracts the increase in desmoglein 3 endocytosis and turnover caused by PV IgG. Furthermore, using this approach we were

able to prevent the downregulation of desmoglein 3, disruption of desmoplakin organization and loss of adhesion in PV IgG-treated cells. These findings suggest that induction of endocytosis and turnover of desmoglein 3 is plays a key role in the pathogenicity of PV.

In Chapter IV, we explore a novel approach to confer desmosome resistance to autoimmune attack in pemphigus. We found that that altering the expression of a single desmosomal plaque protein, Plakophilin-1 (PKP-1), reinforces desmosomal adhesion and prevents the pathogenic effects of PV IgG. Furthermore, we found that PKP-1 interacts with desmoglein 3, promotes desmosome formation and transforms desmosomes into a calcium-independent and hyper-adhesive state. These results reveal new insights into the fundamental regulation of desmosomal adhesion.

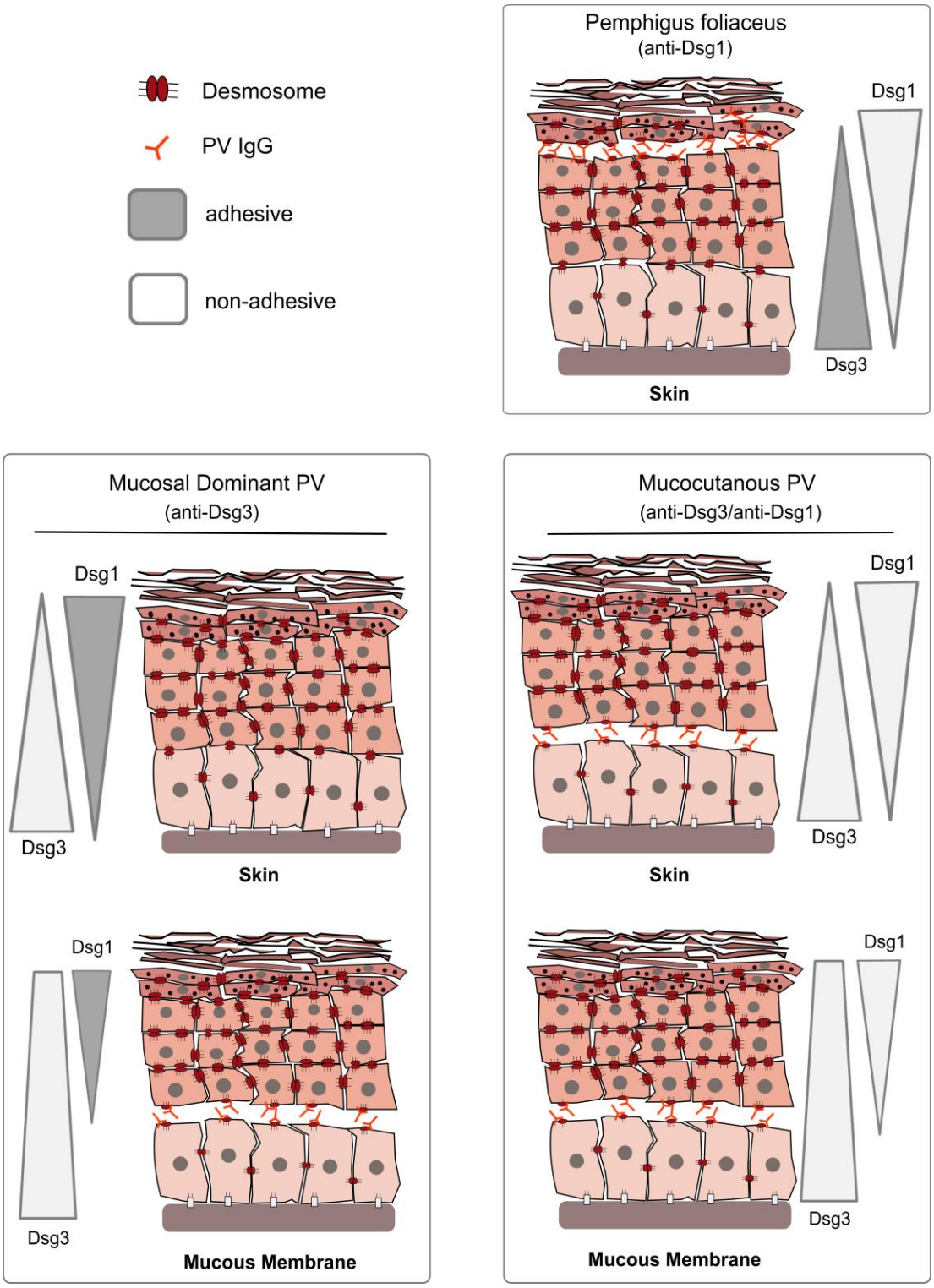

Figure 6. Autoantibody-induced loss of desmoglein 1 and/or 3 (Dsg1, 3) adhesion and sites of blister formation in pemphigus foliaceus, mucosal pemphigus vulgaris (PV) and mucocutaneous pemphigus vulgaris.

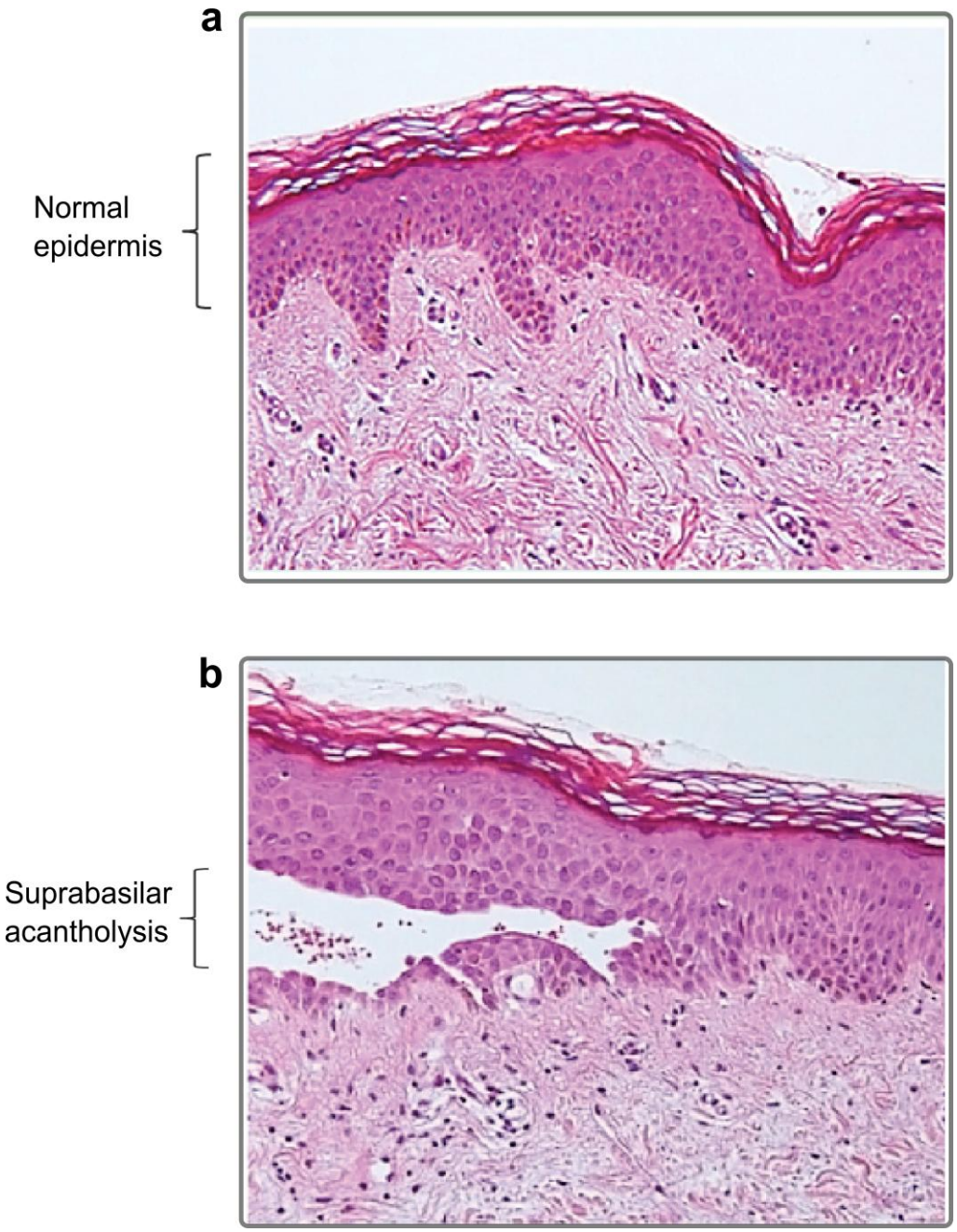

Figure 7. Histologic biopsies stained with hematoxylin and eosin (a). Normal epidermis (b) Pemphigus vulgaris (PV) patient. The loss of desmosomal adhesion in response to PV IgG causes suprabasilar acantholysis in the epidermis.

Chapter III

Desmosome Disassembly in Response to Pemphigus Vulgaris IgG Occurs in Distinct Phases and can be Reversed by Expression of Exogenous Dsg3

Adapted from Jennings JM *, Tucker DK*, Kottke MD, Saito M, Delva D, Hanakawa Y, Amagai M, and Kowalczyk AP The Journal of Investigative Dermatology 2011 March; 131 (3):706-18. * These authors contributed equally

Section 3.0 Abstract

Pemphigus vulgaris (PV) is an epidermal blistering disorder caused by antibodies directed against the desmosomal cadherin desmoglein-3 (Dsg3). The mechanism by which PV IgG disrupt adhesion is not fully understood. To address this issue, primary human keratinocytes and patient IgG were utilized to define the morphological, biochemical and functional changes triggered by PV IgG. Three phases of desmosome disassembly were distinguished. Analysis of fixed and living keratinocytes demonstrated that PV IgG cause rapid desmoglein 3 internalization which likely originates from a non-junctional pool of desmoglein 3. Subsequently, desmoglein 3 and other desmosomal components rearrange into linear arrays that run perpendicular to cell contacts. desmoglein 3 complexes localized at the cell surface are transported in a retrograde fashion along these arrays before being released into cytoplasmic vesicular compartments. These changes in desmoglein 3 distribution are followed by depletion of detergent insoluble desmoglein 3 pools and by the loss of cell adhesion strength. Importantly, this process of disassembly can be prevented by expressing exogenous desmoglein 3, thereby driving desmoglein 3 biosynthesis and desmosome assembly. These data support a model in which PV IgG cause the loss of cell adhesion by altering the dynamics of desmoglein 3 assembly into desmosomes and the turnover of cell surface pools of desmoglein 3 through endocytic pathways.

Section 3.1 Introduction

Desmosomes are adhesive intercellular junctions that mediate attachment of intermediate filament networks to sites of cell to cell contact (Desai *et al.*, 2009; Green and Simpson, 2007; Holthöfer *et al.*, 2007). Desmosomes are prominent in tissues that

experience substantial mechanical stress, including the heart and the epidermis (Bazzi and Christiano, 2007). The desmogleins and desmocollins are the major desmosomal adhesion molecules and are members of the cadherin gene superfamily (Garrod and Chidgey, 2008; Wheelock and Johnson, 2003). Adhesive interactions mediated by the desmogleins and desmocollins are coupled to the intermediate filament cytoskeleton by an elaborate arrangement of cytoplasmic proteins. These desmosomal plaque molecules include the armadillo family proteins plakoglobin and the plakophilins (Hatzfeld, 2007), as well as members of the plakin cytolinker family, such as desmoplakin (Green and Simpson, 2007; Sonnenberg and Liem, 2007).

Desmosome function is compromised in a number of inherited (Awad *et al.*, 2008; Bazzi and Christiano, 2007; Lai-Cheong *et al.*, 2007) and autoimmune disorders (Stanley and Amagai, 2006). Pemphigus vulgaris (PV) is an acquired disease in which the desmosomal cadherin desmoglein 3 is targeted by autoantibodies (Kottke *et al.*, 2006; Waschke, 2008). Patients with this disorder exhibit severe oral erosions as well as epidermal blistering if the disease progresses to include targeting of desmoglein 1. Importantly, IgG can be isolated from PV patient serum and used in mouse and tissue culture models to investigate the mechanism of disease pathogenesis (Amagai, 2008; Kottke *et al.*, 2006). For example, injection of IgG from pemphigus patients into newborn mice causes epidermal blistering that is indistinguishable from that observed in patients (Stanley and Amagai, 2006). Similarly, PV IgG disrupts desmosomes and compromises adhesion strength when incubated in the media of cultured keratinocytes (Payne *et al.*, 2004). This ability to passively transfer the disease from the patient to various model systems has resulted in significant advances in our understanding of PV pathobiology.

Nonetheless, the precise mechanism by which PV IgG causes loss of keratinocyte adhesion is not fully understood.

Evidence from mouse models as well as atomic force microscopy suggests that PV IgG may directly disrupt desmoglein 3 *trans* (adhesive) interactions (Heupel *et al.*, 2008; Shimizu *et al.*, 2004). Similarly, mapping of pathogenic epitopes from both patient IgG and experimentally generated monoclonal antibodies indicate that pathogenic antibodies target the amino-terminal region of desmoglein 3, a cadherin domain critical for adhesive interactions (Tsunoda *et al.*, 2003). These findings offer compelling evidence that disruption of desmoglein 3 adhesive interactions causes the loss of adhesion and blistering that characterizes the disease in patients. However, other work suggests a more complicated effect of PV IgG on keratinocytes (Muller *et al.*, 2008; Sharma *et al.*, 2007; Waschke, 2008). For example, a number of studies have demonstrated that pharmacological approaches targeting various signaling pathways can prevent loss of adhesion both *in vitro* and *in vivo* (Berkowitz *et al.*, 2005; Berkowitz *et al.*, 2006; Williamson *et al.*, 2006). These studies suggest that PV IgG trigger keratinocyte responses and signal transduction cascades that are required for loss of adhesion. Similarly, previous work from our laboratory demonstrated that keratinocyte exposure to PV IgG at 4°C is insufficient to cause loss of adhesion, even though PV IgG bind desmoglein 3 and decorate cell-cell borders (Calkins *et al.*, 2006). In fact, incubation in PV IgG for several hours is required before detectable changes in cell-cell adhesive strength are measurable. These and other data suggest that PV IgG binding to desmoglein 3 is necessary but not sufficient to disrupt keratinocyte adhesion (Kitajima, 2002; Mao *et al.*, 2009; Sato *et al.*, 2000; Yamamoto *et al.*, 2007).

In the current study, a combination of biochemical and imaging approaches was used to define the dynamics of the PV-IgG-desmoglein 3 complex during desmosome disassembly. The results reveal that desmosome disassembly occurs in discrete phases that proceed in a temporally predictable sequence. These phases of disassembly include endocytosis of non-desmosomal desmoglein 3 as previously demonstrated by Sato and colleagues using immunogold electron microscopy (Sato *et al.*, 2000). Internalization of non-junctional desmoglein 3 is followed by the rearrangement of desmosomal components into linear structures that appear to function as sites for endocytosis of desmosomal pools of desmoglein 3. This second phase is followed by depletion of junctional desmoglein 3 and the loss of cell adhesion. Remarkably, this process can be prevented by expressing exogenous desmoglein 3 to counteract this disassembly pathway with enhanced desmoglein 3 biosynthesis. These findings have important implications for understanding the basic cellular mechanisms of desmosome disassembly, and for designing therapeutic strategies to treat PV and related disorders.

Section 3.2 Results

Results section 3.2a Time Course of Desmosome Disassembly in Response to PV IgG

In the current study, a series of biochemical and imaging approaches were used to define the spatial and temporal relationships between desmosomal components during desmosome disassembly in response to PV IgG. As shown in Figure 8, PV IgG binds to keratinocyte intercellular junctions when cells are incubated at 4°C and colocalizes with desmoplakin along cell borders (Figure 8A-C). After 1 hour at 37°C, PV IgG-desmoglein 3 complexes clustered into punctuate structures that were distal to cell contacts and lacked desmoplakin (Figure 8D-F). This pool of PV IgG-desmoglein 3 is likely to

represent internalization of desmoglein 3 from non-desmosomal compartments as previously described by Sato and colleagues (Sato *et al.*, 2000). This change in desmoglein 3 distribution preceded disruption of desmoplakin organization (Figure 8E). However, by 3-6 hours, desmoplakin was markedly disrupted and junctional desmoglein 3 localization decreased (Figure 8G-L). By 24 hours, desmoglein 3 was largely depleted, desmoplakin localization was highly disorganized (Figure 8M-O) and cell-cell adhesion strength was significantly compromised (Figure 15H and (Calkins *et al.*, 2006; Delva *et al.*, 2008)). Control experiments using a monoclonal antibody against desmoglein 3 confirmed that PV IgG colocalized with desmoglein 3 over the 24 hour time course (Supplemental Figure 1). To determine if PV IgG-induced desmosome rearrangement leads to depletion of desmosomal components, keratinocytes were incubated with NH or PV IgG for 24 hours. Sequential Triton X-100 detergent extraction and western blot analysis was carried out to determine if membrane associated (Triton soluble) or cytoskeleton associated (Triton insoluble) pools of desmosomal proteins were depleted after 24 hours exposure to PV IgG (Supplemental Figure 2). The Triton soluble pools of desmoplakin, desmocollin-2 (Dsc2) and plakoglobin in PV IgG treated cells was similar to the NH control, whereas the soluble pool of desmoglein 3 was dramatically reduced. The Triton insoluble pools of desmoplakin and plakoglobin were not altered by PV IgG. The insoluble pool of desmocollin 2 exhibited a slight decrease in response to PV IgG, whereas desmoglein 3 levels were considerably down regulated (see also Supplemental Figure 4 and (Calkins *et al.*, 2006)). These data demonstrate that morphological changes in desmoglein 3 are followed by selective depletion of desmoglein 3 protein levels.

To further assess changes in the distribution of junction components and overall cell shape, keratinocytes were treated with either NH IgG (Figure 9A-C) or PV IgG (Figure 9D-F) and the localization of the adherens junction protein β-catenin was used to outline cell-cell contacts. After 6 hours in PV IgG, alterations in desmoplakin localization were observed, but only minimal changes in β-catenin localization were detected (Figure 9D, E). Furthermore, differential interference contrast microscopy demonstrated that PV IgG treatment did not cause a dramatic alteration in cell shape (Figure 9F). These data are consistent with previous findings (De Bruin *et al.*, 2007) and indicate that changes in desmosomal protein distribution precede alterations in adherens junctions and changes in cell shape.

Results section 3.2b Non junctional desmoglein 3 is internalized and degraded before desmosomes are disrupted by PV IgG

The results shown in Figure 8 suggest that the distribution of desmosomal components is altered in a sequential manner after exposure of keratinocytes to PV IgG. To monitor sequential changes in desmoglein 3 localization after addition of PV IgG, time lapse fluorescent imaging experiments were conducted using fluorescently tagged desmoglein 3 monoclonal antibodies (AK15 or AK23) (Tsunoda *et al.*, 2003) to label cell surface pools of desmoglein 3. To verify that these tracer antibodies did not cause significant alterations in cell surface desmoglein 3 distribution, AK23 was allowed to bind to keratinocytes at 4°C. Excess fluorescently tagged AK23 was then removed from the medium and keratinocytes treated with either NH IgG or PV IgG for 6 hours at 37°C (Supplemental Figure 3). Note that very little change in AK23 distribution was observed after 6 hour exposure to NH IgG, whereas the distribution of the AK23 complex was

dramatically altered by addition of PV IgG. Similar results were obtained using AK15 (not shown). Therefore, a series of experiments were conducted using fluorescently tagged AK15 or AK23 monoclonal antibodies to monitor desmoglein 3 distribution after exposure to PV IgG using four dimensional time lapse fluorescence microscopy of living keratinocytes. Over a time course of 4 hours, time lapse imaging demonstrated that desmoglein 3 is first clustered by PV IgG into puncta, followed by rearrangement of junctional pools of desmoglein 3 (Supplemental Movie 1). Additional time lapse imaging focusing on earlier time points revealed that desmoglein 3 formed clusters on the dorsal surface of keratinocytes (Figure 10A-D and Supplemental Movie 2). This clustering occurred rapidly and preceded any obvious changes in desmoglein 3 at cell-cell borders. To determine the fate and composition of these desmoglein 3 complexes, longer time lapse imaging experiments were conducted. In addition, the cells were fixed at the end of the time lapse series and processed for retrospective immunofluorescence microscopy using antibodies directed against early endosome antigen-1 (EEA-1). This approach demonstrated that clusters of desmoglein 3 formed within the first two hours of PV IgG incubation at 37°C (Figure 10E-G). Furthermore, retrospective analysis revealed that many of the desmoglein 3 particles observed colocalize with EEA-1 (Figure 10H-I), demonstrating that desmoglein 3 is internalized. Immunoblot analysis of Triton X-100 extracts of keratinocytes indicated that the soluble pool of desmoglein 3 is only slightly diminished after incubation with PV IgG at these early time points, and no consistent changes were observed in the insoluble pool of desmoglein 3 (Figure 10J). Taken together, these results suggest that the internalization of non-junctional pools of

desmoglein 3 occurs rapidly upon exposure of keratinocytes to pathogenic PV IgG and that these changes precede alterations in the junctional pools of desmoglein 3.

Results section 3.2c PV IgG cause rearrangement of cell surface desmoglein 3 into linear arrays and subsequent internalization of desmoglein 3 from cell-cell junctions

The data shown above indicate that internalization of non-junctional desmoglein 3 precedes alterations in junctional pools of desmoglein 3 and desmoplakin. Therefore, additional time lapse microscopy was carried out using fluorescently tagged AK23 at later time points after addition of PV IgG. As shown in Figure 11, over a time course of 2 hours, desmoglein 3 reorganizes from the typical desmosomal pattern into linear arrays that extend perpendicularly from cell borders (Figure 11A-C and Supplemental Movie 3). Interestingly, the AK23-desmoglein 3 complex appears to be released from the tips of these linear arrays into vesicular structures that are transported in a retrograde fashion away from cell-cell junctions. Retrospective immunolocalization was used to define the composition of these linear arrays and vesicular structures. AK23-desmoglein 3 complexes in linear arrays colocalized with desmoplakin, whereas vesicular desmoglein 3 that is released from the linear arrays did not (Figure 11D, see also Figure 12). These findings suggest that the desmoglein 3-desmoplakin complex is disrupted upon desmoglein 3 endocytosis from the cell surface. To test this possibility, cell surface desmoglein 3 was labeled with AK23 and keratinocytes were treated with PV IgG for 3 hours. The cells were then transferred to 4°C and either fixed immediately (Figure 11E) or washed in a low pH buffer before fixation to remove AK23 bound to the cell surface (Figure 11F) (Delva *et al.*, 2008). Importantly, the low pH wash removed AK23 that localized to linear arrays, but the punctate AK23-desmoglein 3 particles remained after

the acid wash, indicating that this pool of desmoglein 3 had been internalized. At this time point, soluble levels of desmoglein 3 are notably reduced, whereas the Triton-X 100 insoluble pool of desmoglein 3 exhibits little or no change (Figure 11G). These data suggest that the desmoglein 3 localized to linear arrays is on the cell surface, whereas the vesicular structures released from the linear arrays represent desmoglein 3 that has entered an endocytic compartment.

To define the molecular composition of the desmoglein 3 linear arrays, immunofluorescence microscopy was conducted on keratinocytes treated with PV IgG for 6 hours. Interestingly, the PV IgG-desmoglein 3 complex in the linear arrays co-localized with all desmosomal components tested, including desmoplakin (Figure 12A-D), desmocollin 2 (Figure 12 E-H), plakophilin-2 (Figure 12I-L), and plakoglobin (Figure 12M-P). However, PV IgG in these linear arrays did not colocalize with the adherens junction component E-cadherin (Figure 12Q-T). The composition of these structures was analyzed further to determine if cytoskeletal elements were present in the linear arrays. In keratinocytes exposed to NH IgG, actin bundles were prominent at cell-cell contacts (Figure 13A). As reported previously (Berkowitz *et al.*, 2005), exposure to PV IgG resulted in retraction of actin bundles away from cell borders (Figure 13D). In addition, actin filaments colocalized with the PV IgG-desmoglein 3 complex that had reorganized into linear arrays (Figure 13D-G). Keratin filaments also aligned with PV IgG in the linear arrays (Figure 13K-N). Together, these data indicate that PV IgG causes reorganization of desmosomal components into linear arrays that contain all of the major desmosomal components, as well as actin and keratin filaments.

A number of studies have implicated actin and actin associated proteins in the regulation of desmosome assembly (Godsel *et al.*, 2005; Hatzfeld, 2007). The reorganization of actin in response to pathogenic PV antibodies suggests that the actin network may also be playing a role in desmoglein 3 endocytosis and desmosome disassembly in PV IgG treated cells (Berkowitz *et al.*, 2005). To test this possibility, keratinocytes were treated with latrunculin A to disrupt actin polymerization. Interestingly, latrunculin A treatment resulted in dramatically increased endocytosis of desmoglein 3 in PV IgG treated cells (Figure 14). Furthermore, time lapse imaging revealed that latrunculin A treatment eliminated the formation of the typical linear arrays that form in PV IgG treated cells (compare Figure 14J-M, O-R, and Supplemental Movies 4-5). Instead, the PV IgG-desmoglein 3 complex was internalized in large aggregates directly from cell-cell contacts in latrunculin A treated cells. To investigate the role of actin in PV IgG-induced desmoglein 3 down regulation, western blots were performed on keratinocytes incubated for 24 hours with NH or PV IgG, with or without latrunculin A to inhibit actin polymerization (Supplemental Figure 4). No changes in desmosomal protein levels were observed in cells treated with NH IgG and latrunculin A for 24 hours. However, both the soluble and insoluble pools of desmoglein 3 were reduced by PV IgG in the absence and presence of latrunculin A. Interestingly, in cells treated with both PV IgG and latrunculin A the Triton-X soluble pools of desmocollin 2 and plakoglobin were modestly decreased. Furthermore, the insoluble pools of desmoplakin, desmocollin 2 and plakoglobin were reduced similarly to desmoglein 3. Together these data indicate that PV IgG and actin depolymerization act synergistically to decrease steady state desmosomal protein levels.

Results section 3.2d Expression of exogenous desmoglein 3 prevents desmosome disassembly and loss of adhesion

The rearrangement of desmoglein 3 into linear arrays followed by release of desmoglein 3 into vesicular structures precedes the dramatic loss of desmoglein 3 and desmoplakin from cell-cell borders that are observed by 24 hours (Figure 8M-O). By 24 hours, both the Triton soluble and insoluble pools of desmoglein 3 are depleted (Figure 15A, Supplemental Figure 2 and 4). Recently, we reported that desmoglein 3 is internalized through a clathrin and dynamin independent mechanism in PV IgG treated keratinocytes (Delva *et al.*, 2008). Therefore, we hypothesized that the disruption of desmosomes and the loss of adhesion in response to PV IgG is caused by the internalization of both non-junctional and junctional pools of desmoglein 3, leading to depletion of cell surface desmoglein 3 and the loss of adhesion. Therefore, we reasoned that the loss of adhesion in PV IgG treated cells might be prevented by enhancing desmoglein 3 biosynthesis rates to counter the increase in desmoglein 3 endocytosis and turnover. To test this possibility, a GFP tagged desmoglein 3 fusion protein (Dsg3.GFP) was expressed exogenously using an adenoviral delivery system. As shown in Figure 15A, PV IgG treatment for 24 hours results in decreased desmoglein 3 levels in both the Triton soluble and insoluble pools. Importantly, expression of exogenous Dsg3.GFP prevented the down-regulation of desmoglein 3 levels in PV IgG treated keratinocytes. To determine if expression of exogenous Dsg3.GFP could also prevent alterations in desmoplakin localization in PV IgG treated cells, Dsg3.GFP was expressed in NH IgG (Figure 15B-D) and PV IgG (Figure 15E-G) treated keratinocytes. Interestingly, expression of Dsg3.GFP prevented the disruption of desmoplakin organization in PV IgG

treated keratinocytes (Figure 15E-G). Note that cells expressing Dsg3.GFP exhibit well organized desmoplakin staining at cell borders, whereas desmoplakin staining is dramatically reduced in adjacent cells lacking exogenous desmoglein 3 expression. These data suggested that exogenous expression of desmoglein 3 may prevent the loss of adhesion triggered by PV IgG. Remarkably, expression of exogenous Dsg3.GFP reduced the number of fragments generated in PV IgG treated keratinocytes exposed to mechanical stress (Figure 15H). Together, these findings indicate that exogenous expression of desmoglein 3 prevents the down-regulation of total cellular desmoglein 3 protein levels, the disruption of desmoplakin localization, and the loss of adhesion strength in response to PV IgG.

Section 3.3 Discussion

The results of this study indicate that desmosome disassembly in response to PV IgG occurs in three sequential but temporally overlapping phases. Time lapse fluorescence microscopy indicates that the first phase of PV IgG-induced disassembly (Phase 1) is characterized by endocytosis of the non-junctional pool of desmoglein 3 (Figure 8 and 10). These findings are consistent with previous studies using immunogold electron microscopy and time lapse labeling (Sato *et al.*, 2000). The internalized desmoglein 3 is then delivered to early endosomes (Figure 10), where it is most likely sorted for degradation in lysosomes (Calkins *et al.*, 2006). This model is supported by the observation that the internalization of desmoglein 3 is accompanied by a decrease in the Triton soluble pool of desmoglein 3 as assessed biochemically (Figure 10J). In contrast, junctional pools of desmoglein 3 are largely unaffected over the course of the first 1-2

hours after PV IgG binding to the keratinocyte cell surface (Figure 8D-F). Furthermore, there is no significant loss of adhesive strength at these early time points (Calkins *et al.*, 2006), consistent with the observation that the desmosomal pool of desmoglein 3 remains intact even while the non-desmosomal pool decreases. Thus, the non-desmosomal pool of desmoglein 3 is most susceptible to the effects of pathogenic antibody binding, and internalization of this pool from the cell surface occurs during the first phase of desmosome disassembly in response to PV IgG.

A prominent feature of keratinocytes treated for 2-6 hours with PV IgG (Phase 2) is the rearrangement of desmosomal components into linear arrays which align with keratin filaments and extend away from cell-cell borders (Figure 12 and Figure 13). The linear arrays colocalize with actin and contain not only the PV IgG-desmoglein 3 complex, but also all other desmosomal components examined (Figure 12 and Figure 13). Importantly, the desmoglein 3 complex appears to emerge from the tips of these arrays into vesicular structures that are internalized (Figure 11). At these intermediate time points, adherens junction components are well organized and cells remained closely apposed (Figure 9). These data indicate that alterations in desmosomal organization precede changes in adherens junctions and alterations in cell shape. Finally, Phase 3 of disassembly (6-24 hours) is characterized by disruption of desmosomal components and loss of adhesion strength. At these later time points, the depletion of desmoglein 3 from both the soluble and insoluble pools was dramatic (Figure 8M-O, Figure 15 and Supplemental Figure 2 and 4).

Previous studies using time lapse imaging of desmosome assembly revealed that desmosome formation also takes place in discrete phases (Bass-Zubek *et al.*, 2008;

Godsel *et al.*, 2005). In these studies by Green and colleagues, desmoplakin and the desmosomal cadherins appear to form distinct complexes during assembly and are transported separately to sites of junction formation. This process appears analogous to that observed during disassembly. For example, desmoglein 3 colocalizes extensively with plakoglobin upon internalization. However, none of the other desmosomal components examined, including plakophilin-2 and desmoplakin, colocalize with the internalized pool of desmoglein 3 (Calkins *et al.*, 2006). Cytoskeletal elements are also involved in desmosomal assembly and disassembly. Cytochalasin D disrupts actin filaments and impairs desmosome assembly, implicating the actin cytoskeletal network in the process of desmosome formation (Godsel *et al.*, 2005). Similarly, we observed colocalization between the actin cytoskeleton and the linear arrays that characterize phase 2 of disassembly in response to PV IgG (Figure 13). In addition, disruption of actin polymerization caused a dramatic increase in desmoglein 3 endocytosis from both the cell surface and from intercellular junctions (Figure 14), consistent with a recent report suggesting that the actin cytoskeleton regulates desmosome disassembly in response to PV IgG (Gliem *et al.*, 2010). These data suggest that actin plays roles in both assembly and disassembly of desmosomes, and that desmosomal protein association with the actin cytoskeleton may be most prominent and functionally relevant when desmosomal components are undergoing dynamic regulation.

A key issue in pemphigus pathophysiology is the mechanism by which PV IgG binding to desmoglein 3 causes loss of adhesion. The data presented here, along with a number of other studies, suggest that PV IgG binding to desmoglein 3 is insufficient to cause loss of keratinocyte adhesion (Muller *et al.*, 2008; Waschke, 2008). Rather, it

appears that PV IgG binding disrupts the kinetics of assembly and disassembly by favoring endocytosis of desmoglein 3 and alterations in desmosome organization that lead to disassembly. A prediction based on this hypothesis is that increasing desmoglein 3 biosynthesis should counteract the increase in desmoglein 3 endocytosis and turnover caused by PV IgG. A shown in Figure 15, expression of exogenous desmoglein 3 using an adenoviral delivery system prevented the down-regulation of desmoglein 3, disruption of desmoplakin organization, and loss of adhesion in PV IgG treated cells. These findings suggest that induction of endocytosis and turnover of desmoglein 3 causes loss of adhesion, and that increased desmoglein 3 biosynthesis is sufficient to restore desmosome assembly and adhesion. The precise mechanism by which expression of exogenous desmoglein 3 prevents loss of adhesion is not fully understood, and may involve upregulation of other adhesion molecules or desmosomal plaque proteins. Interestingly, we find that keratinocytes become more resistant to the effects of PV IgG on adhesion with increasing time in high calcium medium (Saito and Kowalczyk, unpublished). This increased resistance to PV IgG is most likely due to increased desmoglein 3 cell surface levels and decreased desmoglein 3 endocytosis when cells are cultured in high calcium (Delva and Kowalczyk, 2009). These findings suggest that treatment of pemphigus patients with agents that enhance desmoglein 3 biosynthesis or stabilize desmosomes may be beneficial. Consistent with this notion, corticosteroids have been found to increase desmoglein 3 transcription (Nguyen *et al.*, 2004). Along with the results present here, these findings suggest that corticosteroids may be beneficial in pemphigus treatment due to increase expression of adhesion molecules in addition to immune suppression.

Section 3.4 Materials and Methods

Cell Culture

Primary human keratinocytes were isolated from neonatal foreskin and cultured in keratinocyte growth medium (Cascade Medium 154 or Gibco Defined Keratinocyte-SFM, Invitrogen, Carlsblad, CA) as described previously (Calkins *et al.*, 2006; Delva *et al.*, 2008). Keratinocytes, no later than passage 3, were then switched into media containing 0.55 mM or 1.2 mM calcium for 16-18 hours before experimental manipulations.

Immunofluorescence

Keratinocytes were seeded onto glass coverslips and allowed to proliferate to desired confluence. Cells were switched to 0.55 mM calcium containing media for 16-18 hours prior to treatment with NH IgG (Invitrogen, Carlsblad, CA) or PV IgG for varying amounts of time. Sera from pemphigus vulgaris patients were acquired without identifiers and IgG was affinity purified as previously described (Calkins *et al.*, 2006; Delva *et al.*, 2008). Data shown are representative of at least two different PV patient IgG and/or pathogenic monoclonal desmoglein 3 antibodies (Tsunoda *et al.*, 2003). NH IgG or PV IgG was added to cell culture media at 100-150 µg/ml and allowed to bind to keratinocyte cell surfaces at 4°C for 30 minutes prior to incubation at 37ºC. Upon completion of the incubation at 37°C, cells were washed three times with PBS+, and then fixed on ice using either -20ºC methanol for 2-4 minutes or 4.0% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes followed by extraction with 0.1% Triton X-100 (Roche Diagnostics, Indianapolis, IN) for 7 minutes. The localization of desmosomal components was assessed using the following antibodies: rabbit anti-

desmoplakin (GeneTex, Inc., San Antonio, TX), rabbit anti- desmoplakin (NW6 a gift from Dr. Kathleen Green, Northwestern University), mouse anti-desmoplakin (Fitzgerald Industries International, Inc., Concord, MA.), mouse anti-plakoglobin (BD Transduction Laboratories, Rockville, MD), mouse anti-desmocollin-2 (7G6, a gift from Dr. James Wahl), mouse anti- plakophillin-2 (Meridian Life Sciences, Inc. Saco, ME), rabbit anti-β-catenin (Lab Vision Products, Thermo Fisher Scientific, Fremont, CA.), mouse anti-E-cadherin (BD Transduction Laboratories, Rockville, MD), and mouse anti-cytokeratin (Immunotech, Marseille, France). Secondary antibodies to appropriate species conjugated to various Alexa Fluors (Invitrogen, Carlsbad, CA.) were used for dual-label staining. Localization of actin was monitored using Alexa Fluor 555 phalloidin (Invitrogen, Carlsblad, CA). Endocytosis of desmoglein 3 was quantified by measuring fluorescence intensity of intracellular AK23/desmoglein 3 using a low pH wash to remove cell surface antibody as previously described (Delva *et al.*, 2008). Latrunculin A (Invitrogen) was used at 250 nM.

Time-Lapse Microscopy

Immunofluorescence analysis and time lapse microscopy were carried out using an inverted Leica DMI-6000B microscope equipped for wide field and confocal microscopy. Confocal microscopy was carried out using two solid state lasers (491nm and 561nm) and a VT Infinity 2D array scanner (VisiTech International, Sunderland, UK). Images were captured using a Hamamatsu electron multiplier deep cooled CCD camera (C9100-12). An automated stage and image acquisition were driven by Simple PCI software (Hamamatsu Corporation, Sewickley, PA). For live cell analysis, temperature control was achieved using an environmental control chamber (Pecon

Incubator ML) and heated stage insert (Pecon Heating Insert P). For time lapse experiments, keratinocytes were seeded into chamber slides (Lab-Tek/Nunc, Rochester, NY) and then switched to medium containing 0.55mM calcium for 16-18 hours prior to the launch of the experiment. Keratinocytes were placed on ice and labeled with Alexa Flour 555-tagged AK23 or AK15 monoclonal antibodies directed against desmoglein 3 (Tsunoda *et al.,* 2003) for 20-30 minutes. Unbound AK23 or AK15 antibody was removed using a PBS+ wash and fresh media containing PV IgG was added and then to the cells were warmed to 37º C. Time lapse images and movies represent projection images of the entire cell z-plane of the cell. Retrospective analysis was achieved by removing the cells from the microscope chamber, immediately fixing in 4.0 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes followed by 0.1% Triton X-100 (Roche Diagnostics, Indianapolis, IN) extraction and staining with appropriate primary antibody. Upon completion of staining, fixed keratinocytes were placed back on the microscope in the exact location where time lapse imaging occurred.

Sequential Detergent Extraction and Western Blot Analysis

For western blot analysis, keratinocytes were grown in 35mm or 4 well tissue culture plates and extracted sequentially in Triton buffer (1% Triton X-100, 10mM Tris, pH 7.5, 140 mM NaCl, 5mM EDTA, 2 mM EGTA, with protease inhibitor cocktail tablets from Roche Diagnostics, Indianapolis, IN) followed by extraction with Urea-SDS buffer (1% SDS, 8 M Urea, 10mM Tris-HCL, pH 7.5, 5 mM EDTA, 2 mM EGTA) as described previously (Calkins *et al.*, 2006). Rabbit anti-Glyceraldehyde 3-phosphate dehydrogenaseA (GAPDH, Santa Cruz), mouse anti-cytokeratin and anti-plakoglobin (mentioned above) were used as loading controls for the Triton soluble and insoluble

fractions. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA) were detected using enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Dispase Cell Dissociation Assay

Keratinocytes were grown to 100 % confluence in 35 mm tissue culture plates. Cells were switched into 1.2 mM calcium containing media 16-18 hours prior to the addition of NH or PV IgG at approximately 100 µg/ml. After 24 hours, keratinocytes were washed with PBS+ and incubated with 1 unit/ml dispase (Roche Diagnostics, Indianapolis, IN) for 30 minutes to an hour. Following release from the culture plate, the cell sheets were transferred to 15-ml conical tubes containing 11 ml final volume of PBS+ and subjected to mechanical stress by 50 inversion cycles on a rocker panel. Fragments were counted using a dissecting microscope. Adenovirus expressing Dsg3.GFP or empty vector adenovirus (GFP alone) was added to cells 24 hours prior to the addition of PV IgG or NH IgG.

Adenovirus Expression

A full length GFP tagged desmoglein 3 adenoviral expression constructed was generated as follows. The cDNA encoding mouse desmoglein 3 was subcloned into pEGFP-N1 (Clontech, Mountain View, CA). GFP tagged desmoglein 3 (Dsg3.GFP) was further subcloned into pENTR11 vector (Invitrogen, Carlsblad, CA). A pAd-CMV-Dsg3.GFP expression vector was then generated with pENTR11- Dsg3.GFP using the Gateway system. *PacI* digested pAd-CMV-Dsg3.GFP was transfected into 293 cells and recombinant Dsg3.GFP adenovirus was generated and purified as described previously (Delva *et al.*, 2008).

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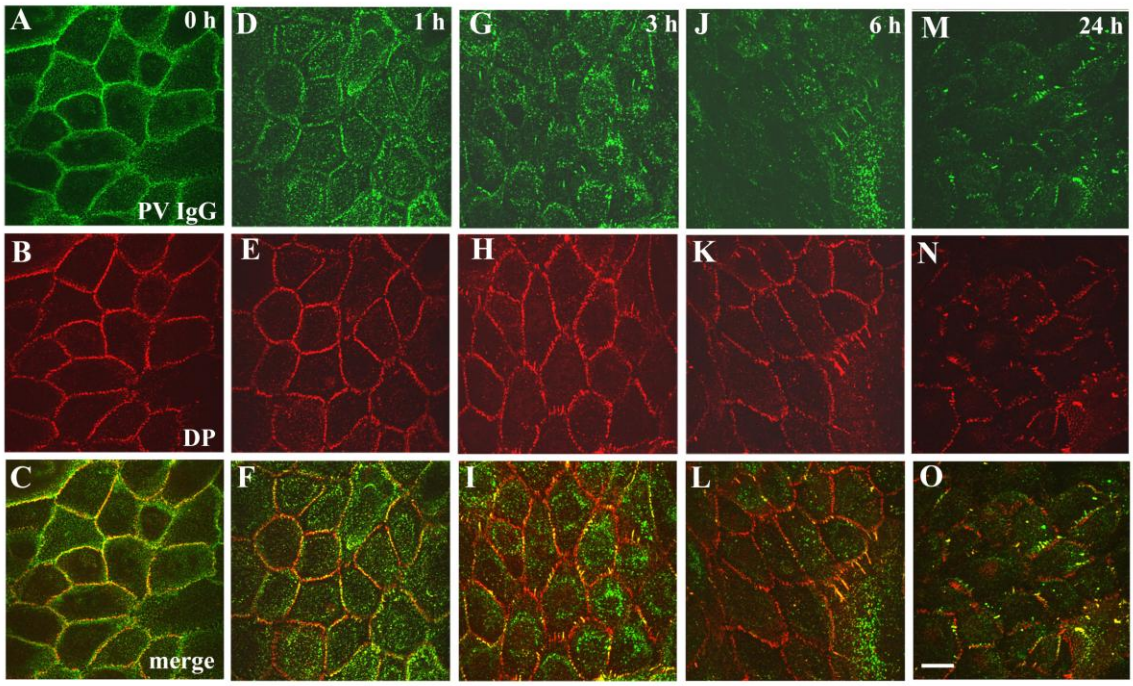


Figure 8. Time course of desmosome disassembly in response to PV IgG.

Keratinocytes were exposed to PV IgG at 4ºC for 20 minutes and subsequently shifted to 37ºC for 1, 3, 6, and 24 hours. The localization of human IgG and desmoplakin (DP) was monitored by immunofluorescence microscopy. In cells incubated at 4ºC (A-C), PV IgG labels cell borders and desmoplakin staining is predominantly in punctate linear patterns at cell-cell junctions. After 1 hour treatment with PV IgG (D-F), the PV-IgG-desmoglein 3 molecules accumulate in puncta that are distal to cell-cell borders, while desmoplakin (E) staining is unchanged. Keratinocytes treated with PV IgG for 3 and 6 hours exhibit a rearrangement of desmoplakin into linear arrays emanating from cell borders which contain both desmoglein 3 and desmoplakin. Following treatment with PV IgG for 24 hours (M-O) both desmoglein 3 and desmoplakin are noticeably mislocalized and/or absent from cell-cell junctions. *Bar*, 10µm.

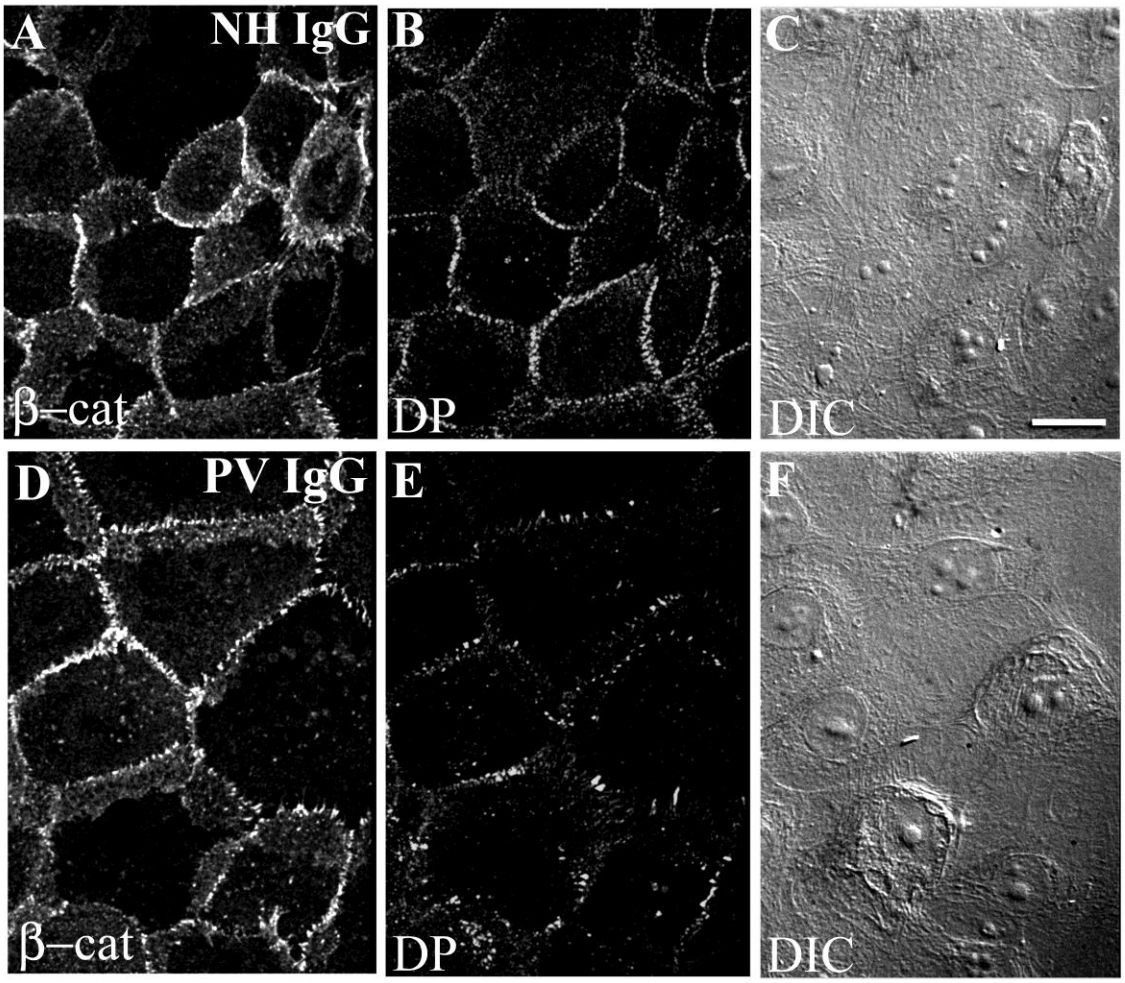


Figure 9. Desmosomes are disrupted by PV IgG but β-catenin is minimally affected and cells remain in close apposition. Keratinocytes were treated with NH IgG (A-C) or PV IgG (D-F) for 6 hours and subsequently processed for immunofluorescence. Note robust β-catenin and desmoplakin (DP) staining (A, B) in cells treated with NH IgG. The PV IgG treated cells exhibit markedly disrupted desmoplakin staining at borders (E), whereas β-catenin remains largely unaltered (D). Differential interference contrast imaging shows that cells do not undergo major changes in cell shape (F). *Bar*, 10μm.

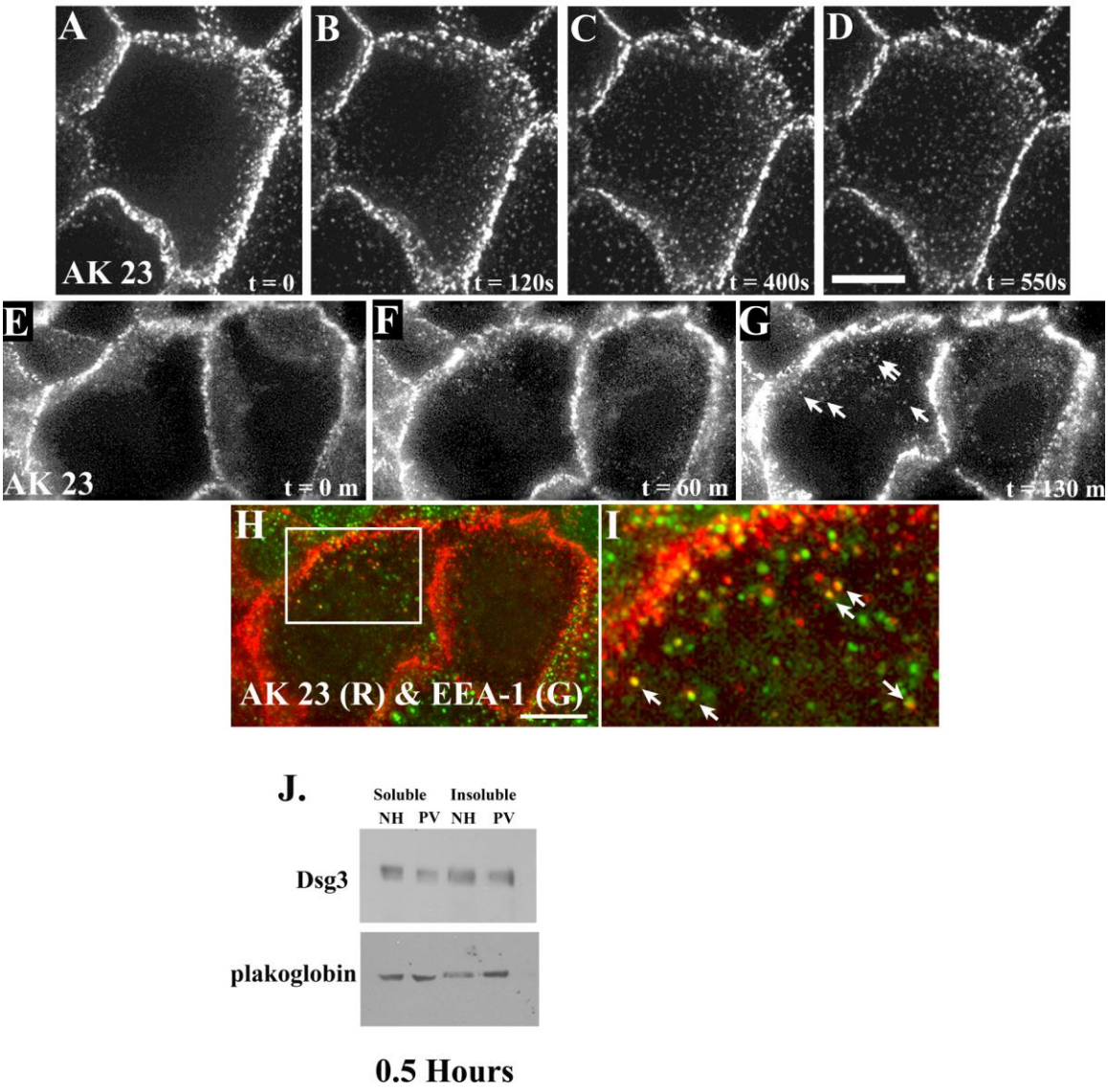


Figure 10. Non-junctional pools of desmoglein 3 are rapidly internalized after exposure to PV IgG. The desmoglein 3 antibody AK23 was fluorescently tagged with Alexa Flour 555 and incubated with human keratinocytes for 30 minutes at 4ºC. Unbound antibody was removed and media containing PV IgG was added. Living cells were imaged using time lapse fluorescence microscopy (A-D and E-G) to follow the dynamics of the desmoglein 3 complex in PV IgG treated cells. Note the appearance of AK23-desmoglein 3 clusters and vesicular structures that form rapidly after exposure to PV IgG,

while junctional desmoglein 3 remains largely unaltered (Supplemental movie 2). To determine the composition of the AK23-desmoglein 3 puncta, cells shown in panel G were rapidly fixed and processed for indirect immunofluorescence using antibodies directed against EEA-1. Note co-localization between the AK23-desmoglein 3 complex and EEA-1 (H, I), indicating that the AK23-desmoglein 3 complex undergoes internalization to endosomes. Each panel in the series represents a single projection image of 7 (A-D) or 8 (E-G) z-axis images collected at each time point. Sequential detergent extraction and western blot analysis was carried out to determine if membrane associated (Triton X-100 soluble) or cytoskeleton associated (Triton X-100 insoluble) pools of desmoglein 3 were depleted. A decrease in the soluble pool of desmoglein 3 was detected within 30 minutes of incubation with PV IgG (J). Results are representative of at least three independently conducted experiments. *Bar*, 10μm.

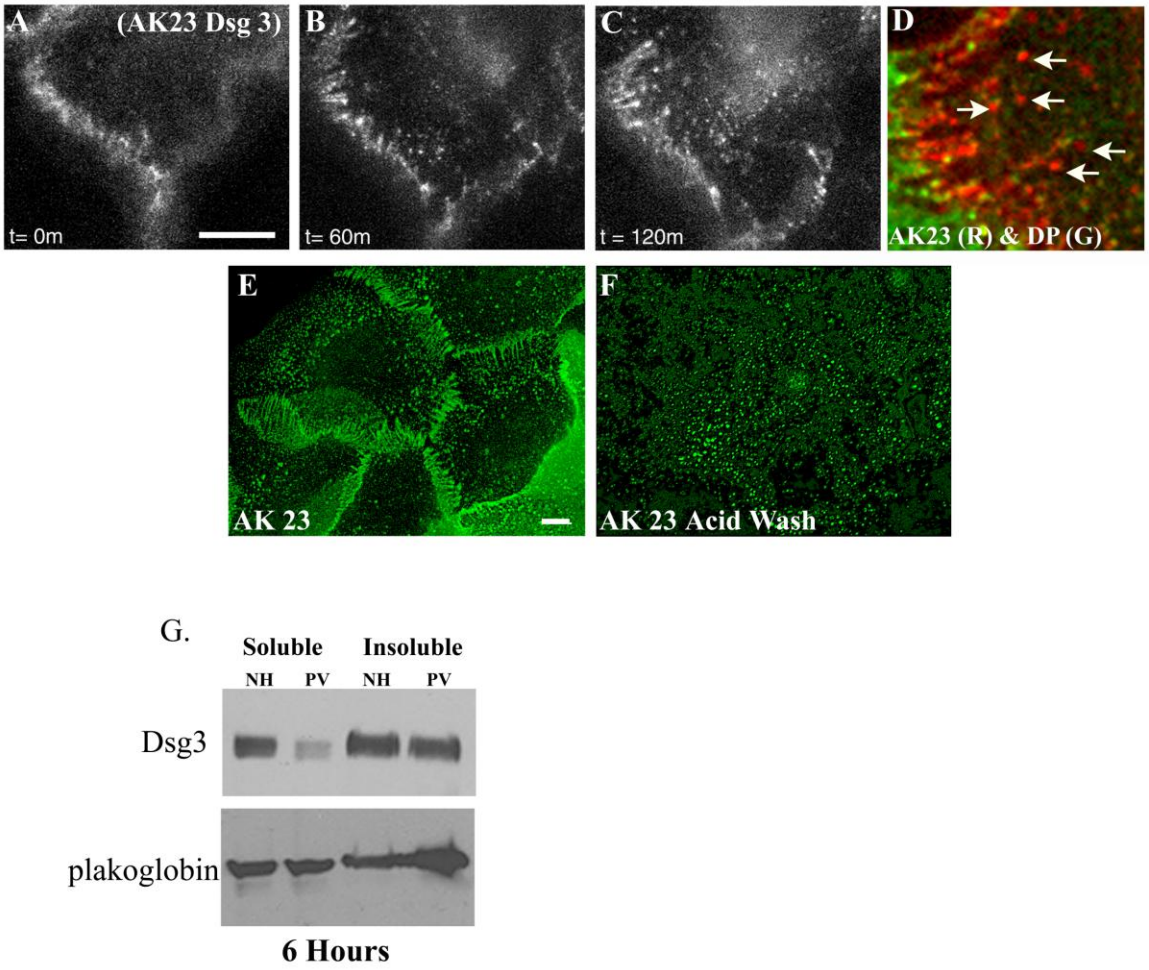


Figure 11. The PV IgG-desmoglein 3 complex reorganizes into linear arrays that exhibit retrograde movement before entering vesicular compartments. Keratinocytes were incubated at 4ºC with Alexa Flour labeled AK23 monoclonal antibody for 30 minutes. Unbound antibody was removed followed by the addition of keratinocyte media containing PV IgG. Cells were then imaged every 5 minutes for two hours at 37ºC. Notice the formation of linear arrays of AK23-desmoglein 3, followed by apparent budding of vesicular structures from the tips of the arrays (B, C). (Supplemental movie 3). Retrospective immunofluorescence analysis (D) indicates that desmoglein 3 that has entered this vesicular compartment is no longer associated with desmoplakin (arrows, panel D). To determine if desmoglein 3 in the linear arrays was present on the cell

surface, living cells were incubated with the pathogenic monoclonal AK23 for 3 hours in the presence of PV IgG. Cells were then incubated on ice and either fixed immediately (E) or incubated in a low pH acid wash before fixation (F) to remove cell surface bound AK23 IgG. Note that the linear arrays are not observed in cells exposed to the low pH wash, demonstrating that desmoglein 3 in these structures is present at the cell surface and that the punctate vesicular desmoglein 3 observed in panel F is intracellular. Western blot analysis of keratinocyte lysates treated for 6 hours in PV IgG reveals a decrease in the non-junctional Triton X-100 soluble pool of desmoglein 3, whereas the Triton insoluble pool of desmoglein 3 exhibited little or no change. No changes in plakoglobin levels were observed (G). Results are representative of at least three independently conducted experiments. *Bar,* 10μm.

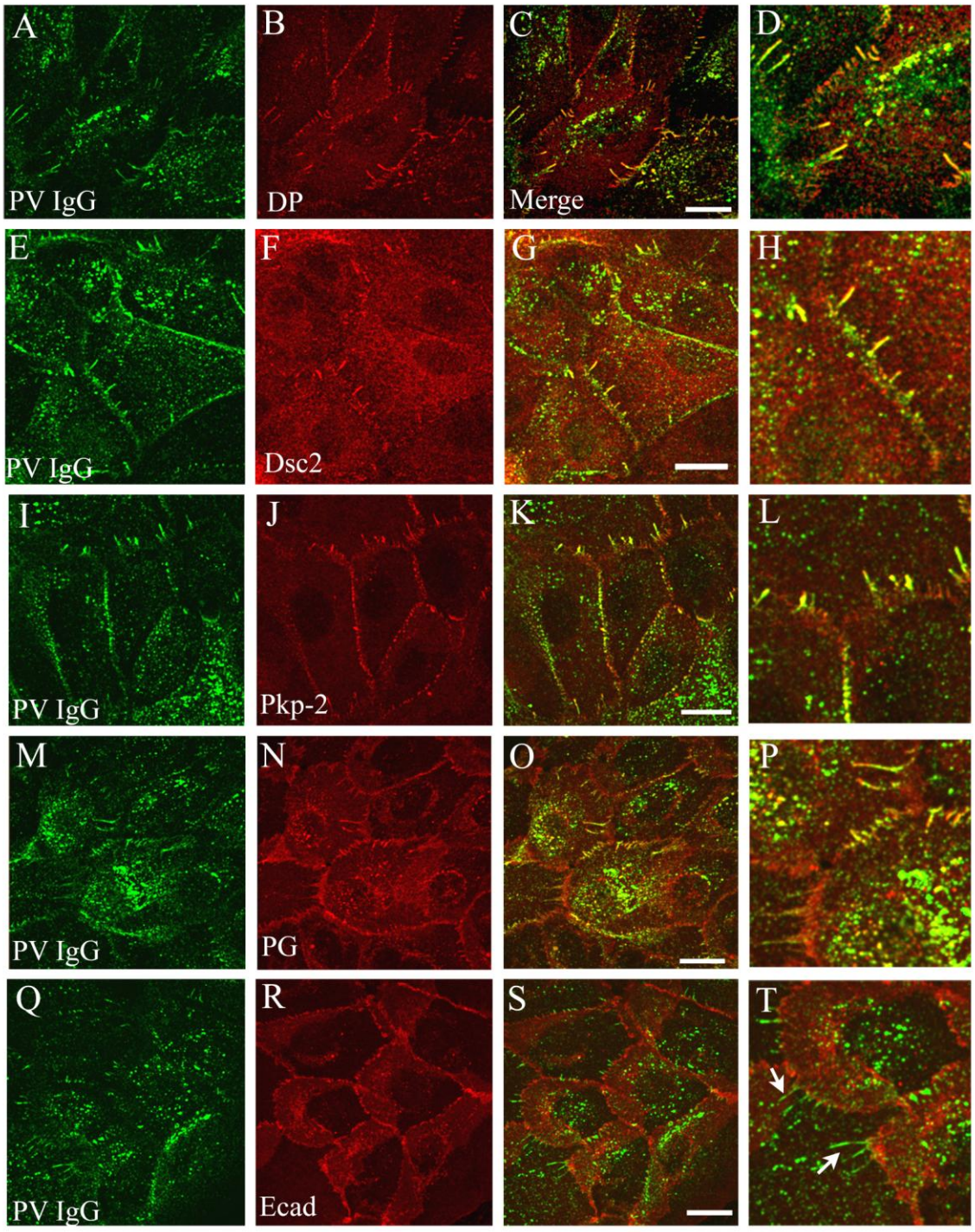


Figure 12. Linear arrays contain desmosomal but not adherens junction components. Primary human keratinocytes were incubated with PV IgG for 6 hours and then processed for confocal immunofluorescence microscopy for PV IgG and either

desmoplakin (DP, A-D), desmocollin-2 (Dsc2, E-H), plakophilin-2 (Pkp-2, I-L), plakoglobin (M-P), or E-cadherin (Ecad, Q-T). Note that the PV IgG complex colocalizes with each of the desmosomal components in linear arrays, but does not colocalize with the adherens junction marker E-cadherin (arrows panel T). *Bar*, 10μm.

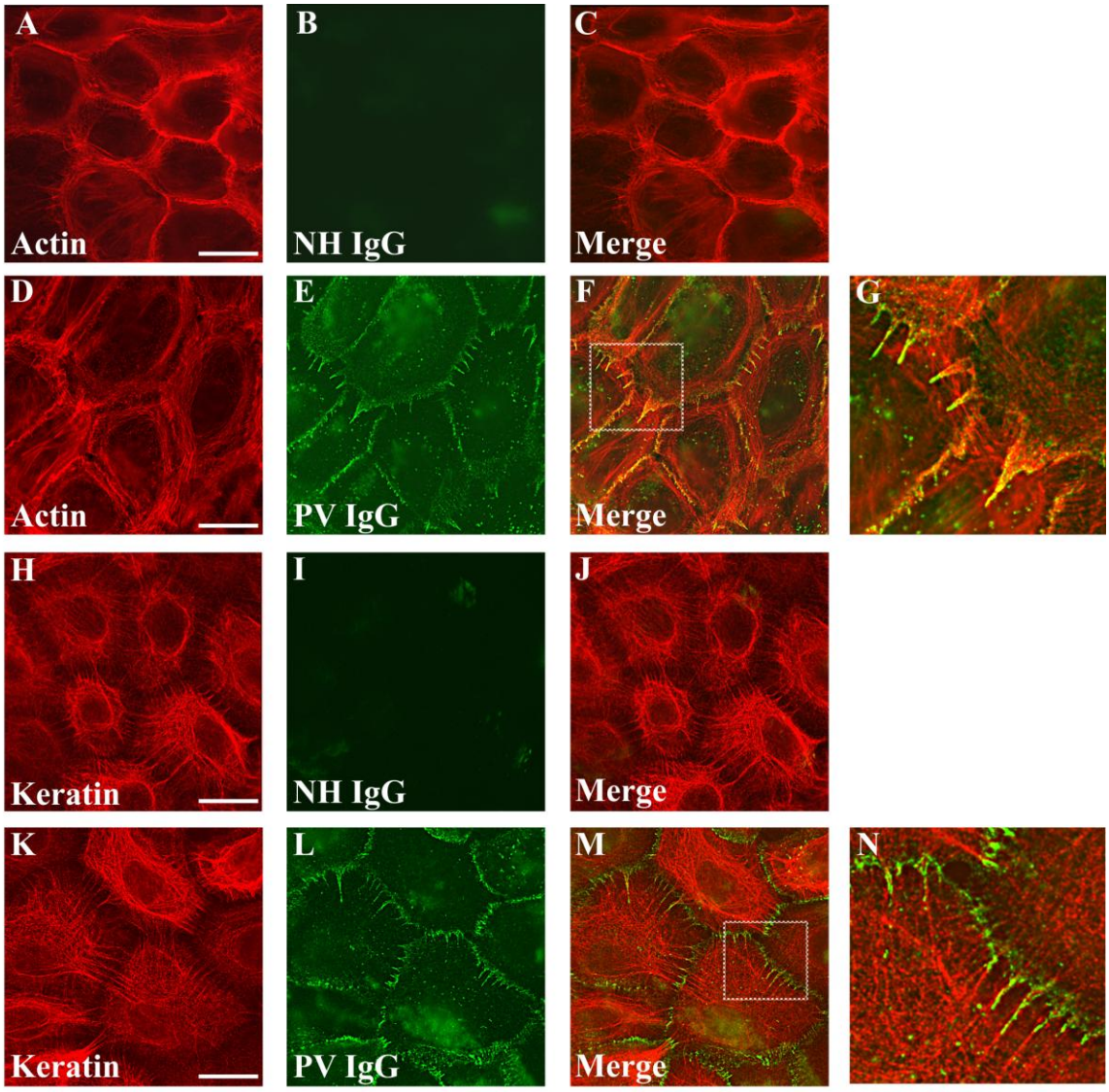


Figure 13. PV IgG-desmoglein 3 in linear arrays colocalize with actin and align with keratin filaments. Keratinocytes were treated with either NH IgG or PV IgG for 6 hours and processed for immunofluorescence localization of NH and PV IgG with actin (A-G) or NH and PV IgG and cytokeratin (H-N). Note colocalization of PV IgG in linear arrays with actin, which emanate away from cell-cell borders in alignment with keratin filaments. Images shown are single z-plane representations that were captured using wide field microscopy followed by deconvolution. *Bar*, 20µm.

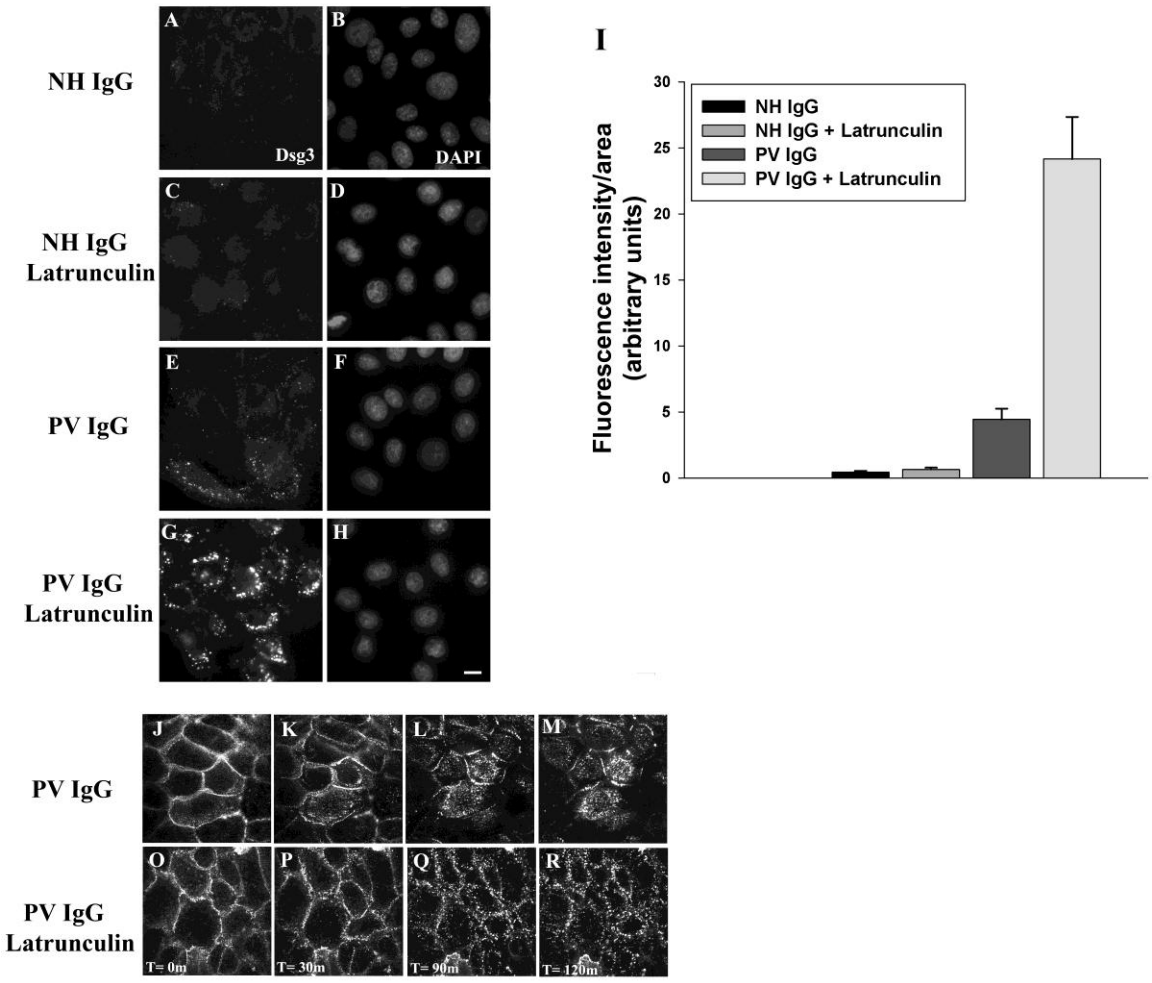
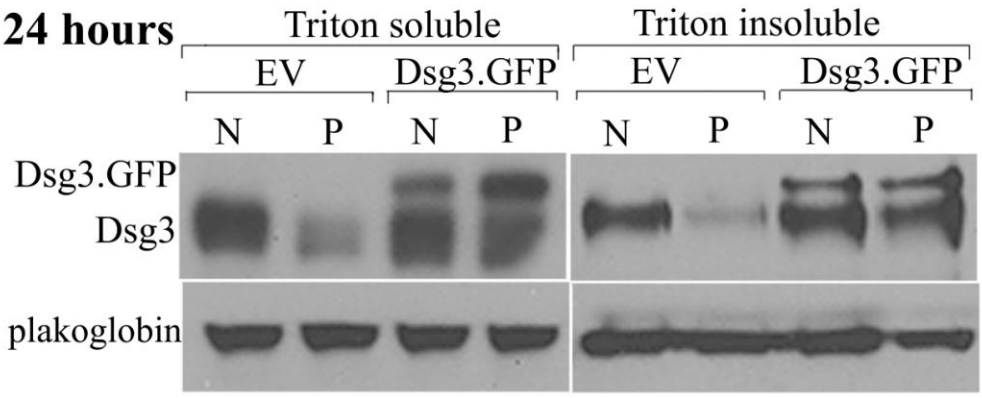


Figure 14. Actin depolymerization increases PV IgG-induced desmoglein 3 internalization. Keratinocytes were untreated (A-B and E-F) or treated with latrunculin A (250 nM) (C-D and G-H) at 37ºC for 1 hour. Cells were then incubated at 4ºC for 30 min with AK23 IgG to label cell surface pools of desmoglein 3. Excess AK23 antibody was removed from the medium, NH IgG or PV IgG were added, and the cells were shifted to 37ºC for 3 hours. To visualize only the intracellular pool of desmoglein 3, cells were washed in a low pH buffer at 4ºC to remove surface bound antibody prior to processing for immunofluorescence. *Bar*, 5μm. The amount of internalized AK23-desmoglein 3 was then quantified (I). Error bars represent the standard error of the mean,

where n = 15 fields of view. In parallel experiments, time lapse microscopy was used to visualize desmoglein 3 dynamics in PV IgG (J-M) or PV IgG + Latrunculin A (O-R) treated keratinocytes (Supplemental movies 4 and 5).

A.


24 hours
Triton soluble
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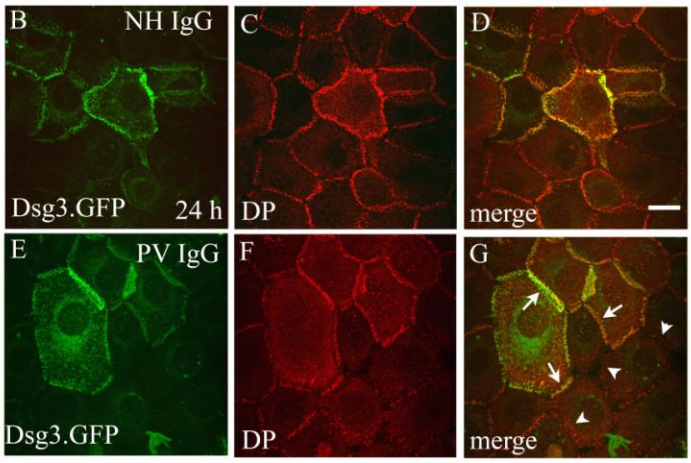
B
NH IgG
Dsg3.GFP
24 h
C
DP
D
merge
E
PV IgG
Dsg3.GFP
F
DP
G
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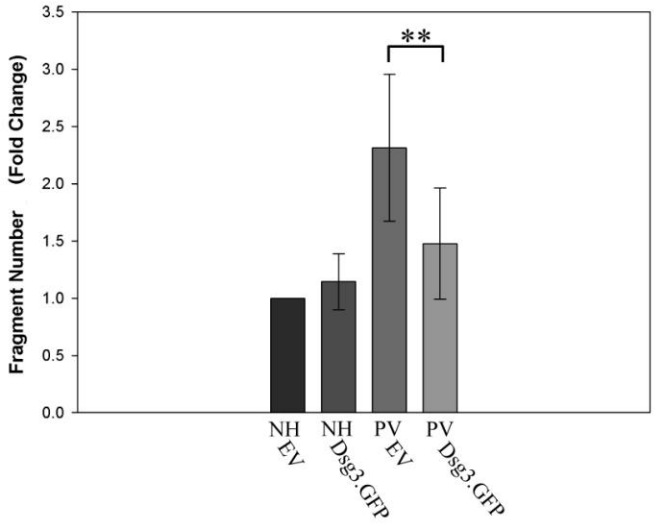
H.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NH EV
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PV EV
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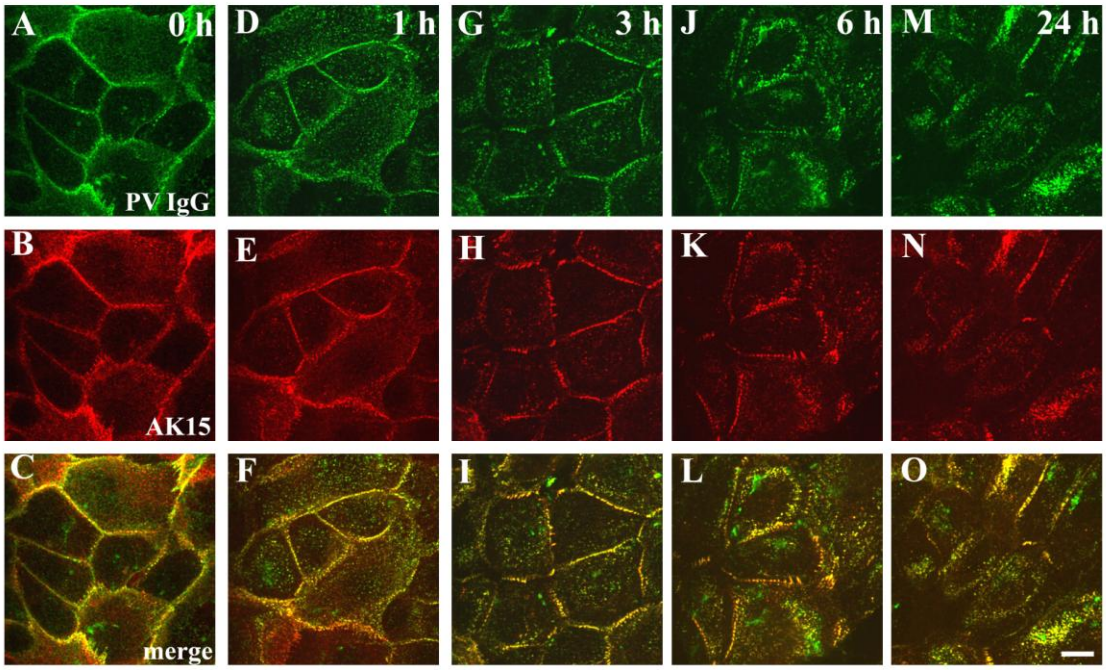
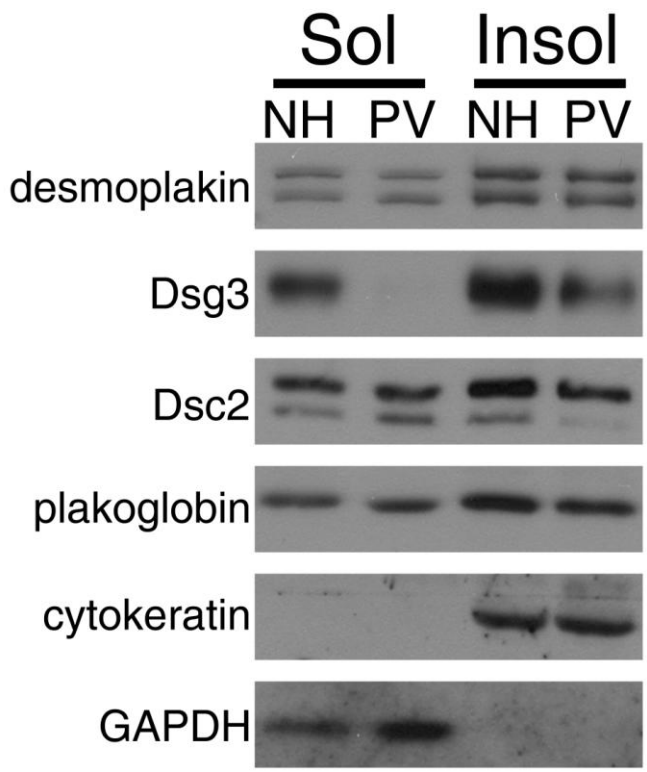
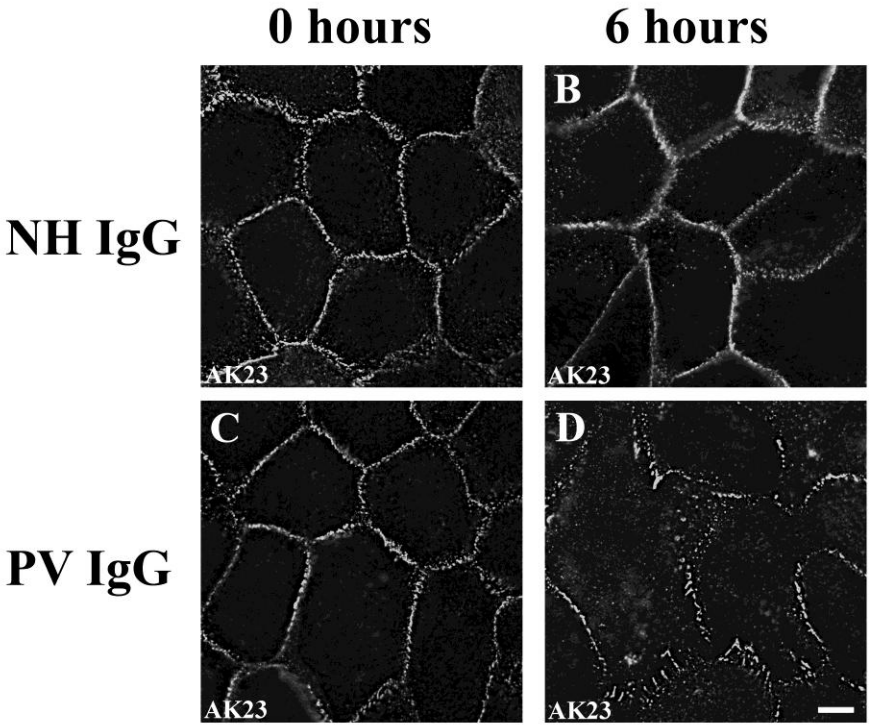
Figure 15. Expression of exogenous Dsg3.GFP prevents desmoplakin mislocalization and loss of cell adhesion in PV IgG treated keratinocytes. Dsg3.GFP was expressed in keratinocytes using an adenoviral delivery system 24 hours prior to treatment with NH or PV IgG. After an additional 24 hours, cells were processed for sequential detergent extraction and western blot (Panel A), immunofluorescence (Panels B-G), or subjected to mechanical stress to measure cell adhesion strength (Panel H). Note that expression of exogenous Dsg3.GFP prevented the down-regulation of desmoglein 3 in both the Triton X-100 soluble and insoluble pools in PV IgG (P) treated cells compared to cells treated with NH IgG (N) (Panel A). Note that Dsg3.GFP colocalized with desmoplakin in NH IgG treated cells (B-D) and prevented mislocalization of desmoplakin in PV IgG treated cells (E-G). Note also the robust desmoplakin staining in cells that are expressing Dsg3.GFP (arrows, G) in sharp contrast to adjacent cells lacking Dsg3.GFP (arrowheads, G). To determine if Dsg3.GFP expression could prevent loss of cell adhesion strength in PV IgG treated cells, NH IgG or PV IgG treated keratinocytes were released from the substrate using the enzyme dispase and subjected to mechanical stress. The number of resulting monolayer fragments was quantified. Note that exogenous Dsg3.GFP rendered PV IgG treated keratinocytes resistant to mechanical stress. The graph represents an average of three separate experiments quantified by a blinded observer. PV IgG treated keratinocytes expressing Dsg3.GFP exhibited significantly less fragments than those expressing empty adenovirus as determined using ANOVA with post hoc least significant difference analysis (**$p < 0.01$). Error bars represent the standard deviation. *Bar*, 10μm.



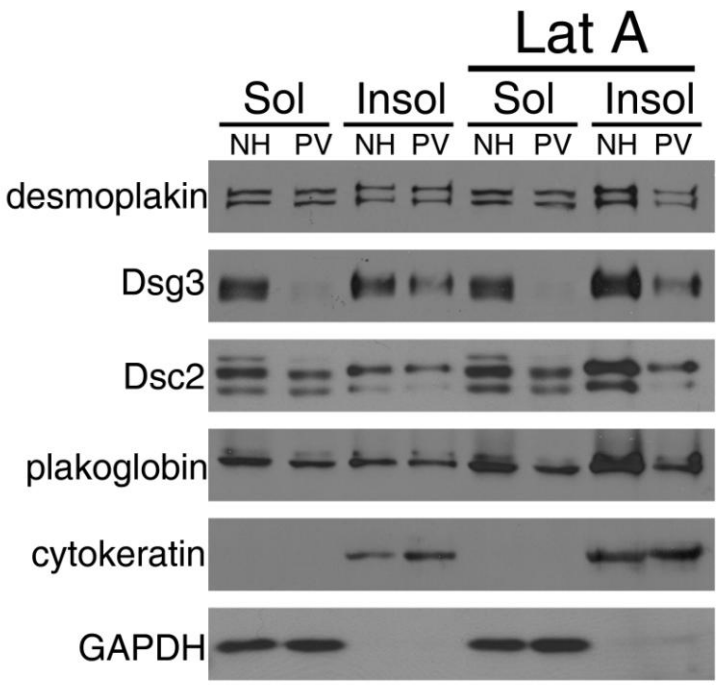
Supplemental Figure 1. **PV IgG colocalize with desmoglein 3.** Keratinocytes were exposed to PV IgG and the desmoglein 3 monoclonal antibody AK15 at 4ºC for 20 minutes and subsequently shifted to 37ºC for 1, 3, 6, and 24 hours. The localization of both human IgG and AK15 were monitored by immunofluorescence microscopy. Throughout the time course human IgG and the desmoglein 3 monoclonal antibody AK15 colocalize. In cells incubated at 4ºC (A-C), PV IgG and AK15 both label cell borders. After 1 hour, the PV IgG-desmoglein 3 and AK15-desmoglein 3 complexes localize in areas away from cell-cell borders. Keratinocytes treated with PV IgG for 3 and 6 hours (G-L) exhibit a rearrangement of desmoglein 3 into linear arrays emanating from cell borders. Following treatment with PV IgG for 24 hours (M-O) both human IgG and AK15-desmoglein 3 are depleted and co-localize in areas away from cell-cell borders and in large clusters at cell contacts. *Bar*, 10µm.



Supplemental Figure 2. **PV IgG decreases steady state desmoglein 3 protein levels.** Sequential detergent extraction and western blot analysis was carried out to determine if membrane associated (Triton X-100 soluble) and cytoskeleton associated (Triton X-100 insoluble) pools of various desmosomal components were depleted in response to 24 hour PV IgG treatment. Neither desmoplakin nor plakoglobin exhibited decreases in either the soluble or insoluble pools by PV IgG. The insoluble pool of Desmocollin-2 (Dsc-2) slightly decreased in cells incubated with PV IgG. Importantly, both the soluble and insoluble pools of desmoglein 3 are noticeably reduced by PV IgG. GAPDH and cytokeratin were used as loading controls for the Triton soluble and insoluble fractions, respectively.



Supplemental Figure 3. **Tracer amounts of the desmoglein 3 monoclonal antibody AK23 do not cause alterations in desmoglein 3 localization.** Cell surface desmoglein 3 was labeled with AK23 at 4°C for 30 minutes as a tracer to monitor desmoglein 3 localization (A-D). Unbound AK23 IgG was removed and the cells were incubated with NH IgG (A-B) or PV IgG (C-D). Cells were then either fixed (A, C) or shifted to 37ºC for 6 hours. Note that surface labeling with AK23 in the presence of NH IgG does not cause significant changes in desmoglein 3 distribution, whereas addition of PV IgG dramatically alters the distribution of cell surface AK23-desmoglein 3. *Bar*, 2.5μm



Supplemental Figure 4. **PV IgG and actin depolymerization act synergistically to decrease steady state desmosomal protein levels.** Sequential detergent extraction and western blot analysis was carried out to determine if membrane associated (Triton X-100 soluble) and cytoskeleton associated (Triton X-100 insoluble) pools of various desmosomal components were depleted in response to 24 hour treatment with PV IgG and latrunculin A. No changes in desmosomal protein levels were observed in cells treated with NH IgG and latrunculin A for 24 hours. Both the soluble and insoluble pools of desmoglein 3 were reduced by PV IgG in the absence and presence of latrunculin A. Additionally, PV IgG and latrunculin A treatment caused a modest decrease in the soluble pools of desmocollin-2 (Dsc2) and plakoglobin. The insoluble pools of desmoplakin, Dsc2 and plakoglobin were reduced similarly to desmoglein 3 in PV IgG and latrunculin A treated cells. GAPDH and cytokeratin were used as loading controls for the Triton soluble and insoluble fractions, respectively.

Supplemental Movie 1: The desmoglein 3 monoclonal antibody AK15 was fluorescently labeled with Alexa Fluor-555 and incubated with living keratinocytes at 4ºC. Excess antibody was removed, PV IgG was added to the medium, and cells were shifted to 37ºC and imaged every three minutes for 3 hours and 51 minutes. The AK23-desmoglein 3 complex is first clustered by PV IgG into puncta, followed by disruption of desmoglein 3 at cell-cell borders. Each time point represents a single projection of 10 images taken through the z-axis at 0.8µm intervals. Projection images are shown at a rate of 10 frames/second.

Supplemental Movie 2: Figure 11 Panels A-D: Keratinocytes were labeled with AK23 tagged with Alexa-Fluor 555 at 4ºC, shifted to 37ºC in medium containing PV IgG and imaged every 10 seconds for 9 minutes. In response to PV IgG, AK23-desmoglein 3 molecules rapidly form punctate clusters. Each panel represents a single projection of 9 z-axis images at 0.5µm images. Projection images are shown at a rate of 10 frames/second.

Supplemental Movie 3: Figure 12 Panels A-D: Alexa-Fluor 555 tagged AK 23 was incubated with living keratinocytes at 4ºC to label desmoglein 3. Excess tagged AK23 was removed and PV IgG was added to the medium. After shifting cells to 37ºC, time lapse imaging was initiated every two minutes for 2 hours and 20 minutes. AK23-desmoglein 3 molecules reorganize from cell contacts into linear arrays that extend perpendicularly from cell borders (Figure 12A-C and Supplemental Movie 3). Furthermore, AK23-desmoglein 3 complexes are released from the tips of these linear

arrays. Single projection images at each time point were compiled using 6 z-axis slices at 0.5μm intervals. Projection images are shown at a rate of 10 frames/second.

Supplemental Movie 4: Figure 15 Panels N-Q: Living keratinocytes were labeled at 4ºC with Alexa-Fluor tagged AK15. The fluorescent antibody was removed, PV IgG was added to the medium, and cells were shifted to 37ºC. Time lapse imaging occurred every two minutes for two hours. In response to PV IgG, AK23-desmoglein 3 molecules form punctate clusters. Linear arrays form and extend perpendicularly from cell borders. Each panel represents a projection image of 6 slices through the z-axis at a 1μm interval, and these images are shown at a rate of 15 frames/second.

Supplemental Movie 5: Figure 15 Panels R-U: Keratinocytes were pretreated with 250 nM latrunculin A for 1 hour to inhibit actin polymerization and then labeled at 4ºC with Alexa-Fluor tagged desmoglein 3 antibody, AK15. Excess AK15 was removed and keratinocyte medium was replaced containing PV IgG and latrunculin A. Keratinocytes were shifted to 37°C and imaged every two minutes for two hours. In response to PV IgG and latrunculin A, AK23-desmoglein 3 molecules form large aggregates that appear to be rapidly internalized. Six 1μm slices were taken at each time point through the z-axis. Projection images of the six slices were combined and shown at a frame rate of 15 frames/second.

Section 3.6 Current Perspectives

Findings presented in Chapter III demonstrate that the binding of PV IgG promote desmosome disassembly by triggering desmoglein 3 internalization and degradation. Furthermore, PV IgG cause pathogenic rearrangements of desmosomes into linear arrays. Subsequently, desmoglein 3 is lost from desmosomal protein pools, desmosomes disassemble and keratinoctyes fail to exhibit strong intercellular adhesion. Following the work presented in Chapter III, our lab sought to examine and directly compare the pathogenic mechanisms between polyclonal PV IgG from patients and the monoclonal desmoglein 3 antibody AK23. Polyclonal PV IgG bind to an array of epitopes on the extracellular domain of desmoglein 3 including those that are thought to be non-adhesive interacting regions, as well as regions responsible for *cis* and *trans*-adhesive interactions. The sterically hindering monoclonal antibody AK23 specifically binds to the region of EC1 that is thought to be responsible for *trans*-adhesive interactions (Tsunoda *et al.*, 2003). Masataka Sato, a postdoc in our lab, found that polyclonal PV IgG-induced loss of adhesion was accompanied by extensive clustering and endocytosis of desmoglein 3, as well as significant structural defects in desmosomes (Saito *et al.*, 2012a). Interestingly, AK23 caused the loss of adhesion without altering desmoglein 3 trafficking and desmosome morphology. Furthermore, we found that cell signaling pathways triggered by polyclonal PV IgG were not part of the pathomechanisms caused by the monoclonal antibody AK23. Inhibition of tyrosine kinase or p38 MAPK prevented desmoglein 3 endocytosis and the loss of keratinocyte adhesion in response to polyclonal PV IgG. In contrast, disruption of adhesion by AK23 was not prevented by these inhibitors. These results reveal that the some of pathogenic activity of polyclonal PV IgG can be attributed

to clustering and p38 MAPK-dependent endocytosis of desmoglein 3, whereas pathogenic monoclonal desmoglein 3 antibodies function independently of this pathway (Saito *et al.*, 2012a). Collectively, these data suggest that signaling pathways play a key role in the pathogenicity of PV and contributes to desmosome instability by mediating desmoglein 3 internalization that occurs in phase 1 of desmosome disassembly.

As reviewed in the introduction, several lines of evidence suggest that steric hindrance and intracellular signaling pathways play a key role in the pathogenicity of PV. A very recent report by Di Zenzo *et al.* found that the primary target of PV autoantibodies is to regions of desmoglein 3 that are expected to be involved in *cis*-adhesive interactions. Furthermore, the Di Zenzo *et al.* suggest that pathogenic antibodies interfere with desmoglein 3-desmoglein 3 *cis*-interactions in the non-desmosomal pool as well as in the desmosomal compartment (Di Zenzo *et al.*, 2012). In support of the findings by Di Zenzo *et al*., several reports suggest that lateral or *cis* cadherin interactions are essential in mediating cadherin cell-cell adhesion (Brieher *et al.*, 1996; Yap *et al.*, 1997). Based on these and other studies, we suggest that subsequent desmosome disassembly which occurs in three distinct phases, that steric hindrance of *cis* and *trans*-desmoglein 3 interactions prevents de novo desmosome assembly by inhibiting the incorporation of new desmoglein 3 molecules into desmosomes.

Altogether, the findings presented in Chapter III and from other studies support *my central hypothesis that PV IgG binding to desmoglein 3 promotes desmosome disassembly and perturbs desmosome assembly thereby causing the loss of cell-cell adhesion.* In line with this hypothesis, we propose a model that incorporates the phases of desmosome disassembly with other PV pathomechanisms that contribute to the loss of

cell-cell adhesion (Figure 16). These pathomechanisms collectively act to destabilize desmosomes and include: 1) signaling pathways are required to mediate desmoglein 3 internalization and degradation characterized by phase 1 of desmosome disassembly 2) pathogenic rearrangement of desmosomes into linear arrays facilitate the loss of junctional desmoglein 3 and 3) steric hindrance of newly synthesized desmoglein 3 proteins inhibits their incorporation into desmosomes and thus new desmosome assembly is prevented (Figure 16). *Based on our central hypothesis we predict that reinforcing desmosome adhesion by promoting assembly or slowing disassembly of desmosomes will counteract the effects of PV IgG and prevent the loss of adhesion.* This prediction led us to focus on the protein, Plakophilin-1, which is thought to regulate the processes of desmosome assembly and disassembly. In Chapter IV, we present findings that test our prediction and ultimately provide further evidence in support of the central hypothesis.

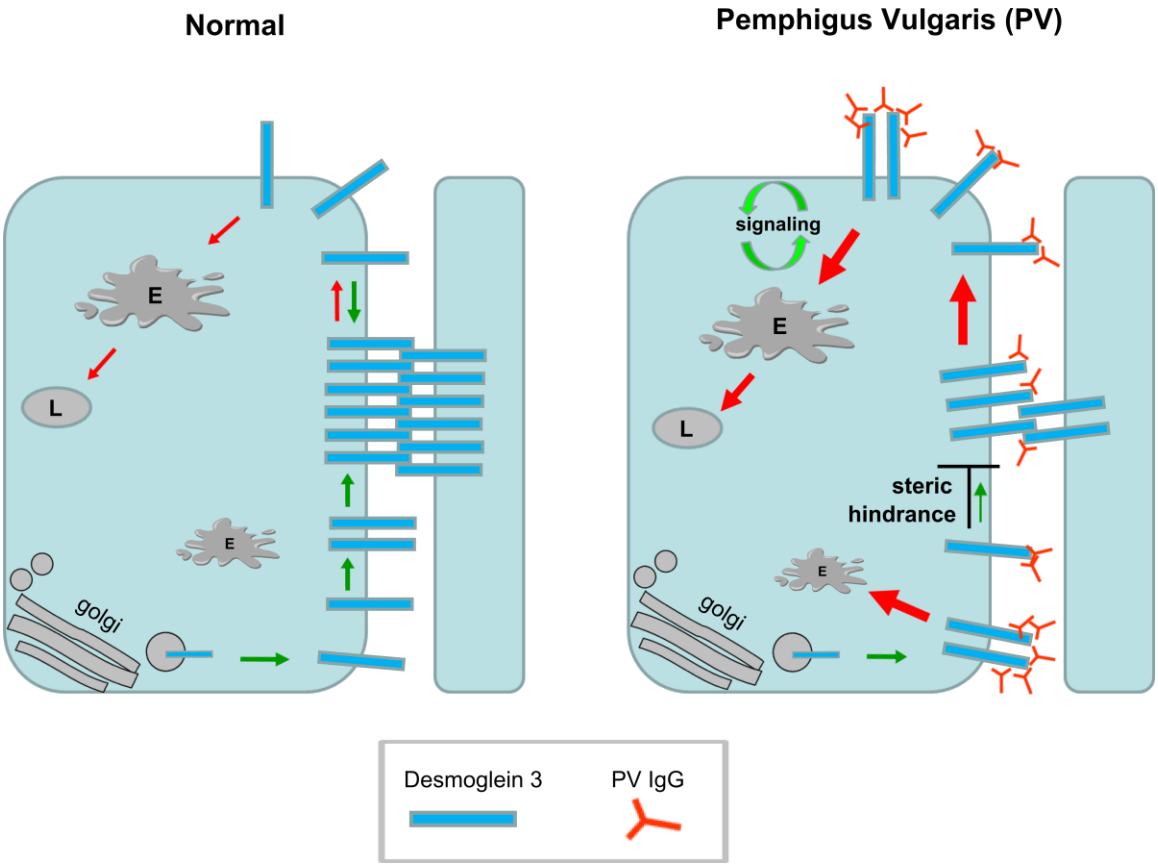


Figure 16. Model: Pemphigus vulgaris is a disease of desmosome instability. In our model, we propose that PV IgG induced-desmosomal disassembly occurs in three distinct but overlapping phases where both intracellular signaling and steric hindrance contribute to the loss of desmosomal adhesion caused by PV IgG. In Phase 1, PV IgG binding triggers endocytosis of non-desmosomal desmoglein 3 to endosomes (E) in a manner dependent upon the activation of signaling pathways. Subsequently, this pool of desmoglein 3 subjected to lysosomal degradation (L). Phase 2 involves the rearrangement of desmosomes into linear arrays at the cell surface that facilitate the loss of desmosomal desmoglein 3 in vesicles that bud off from the ends of these arrays. At later time points, Phase 3 occurs, in which we observe depletion of desmoglein 3 from cell-cell contacts,

desmoplakin disruption and overall loss of cell-cell adhesion. Finally, steric hindrance of newly synthesized desmoglein 3 proteins prevents de novo desmosome assembly.

Chapter IV

Plakophilin-1 protects keratinocytes from pemphigus vulgaris IgG by forming calcium-independent desmosomes

Dana K. Tucker

Section 4.0 Abstract

Plakophilin-1 (PKP-1) is an armadillo family protein critical for desmosomal adhesion and epidermal integrity. In the autoimmune skin blistering disease pemphigus vulgaris (PV), autoantibodies (IgG) target the desmosomal cadherin desmoglein 3 (Dsg3) and compromise keratinocyte cell-cell adhesion. Here, we report that enhanced expression of PKP-1 protects keratinocytes from PV IgG-induced loss of cell-cell adhesion. PKP-1 prevents loss of desmoglein 3 and other desmosomal proteins from cell-cell borders and prevents alterations in desmosome ultrastructure in keratinocytes treated with PV IgG. Using a series of desmoglein 3 chimeras and deletion constructs, we find that PKP-1 clusters desmoglein 3 with the desmosomal plaque protein desmoplakin in a manner dependent upon the plakoglobin binding domain of the desmoglein 3 tail. Furthermore, PKP-1 expression transforms desmosome adhesion from a calcium-dependent to a calcium-independent and hyper-adhesive state. These results demonstrate for the first time that manipulating the expression of a single desmosomal plaque protein can block the pathogenic effects of PV IgG on keratinocyte adhesion.

Section 4.1 Introduction

Desmosomes are intercellular junctions that provide strong adhesion between keratinocytes by anchoring keratin intermediate filaments to cell-cell contact sites. Desmosomal adhesion is critical in tissues that experience mechanical stress, such as in the skin and heart (Getsios *et al.*, 2004). These highly regulated, complex macromolecular junctions are comprised mainly of proteins from three major families: the desmosomal cadherins, desmocollins (Dsc 1-3) and desmogleins (Dsg1-4); armadillo

proteins, plakoglobin (PG) and the plakophilins (PKP 1-3); and the plakin family protein, desmoplakin (DP) (Green and Simpson, 2007). The transmembrane desmosomal cadherins mediate calcium-sensitive adhesive interactions between adjacent cells (Saito *et al.*, 2012b). The armadillo and plakin family members form the desmosomal plaque that tethers the keratin intermediate filament cytoskeleton to cadherin intracellular domains (Delva *et al.*, 2009). The importance of desmosomes in regulating tissue integrity, differentiation and morphogenesis is evidenced by numerous inherited and autoimmune disorders in which desmosome function is compromised (Brooke *et al.*, 2012; Simpson *et al.*, 2011; Thomason *et al.*, 2010).

Pemphigus vulgaris (PV) is a life-threatening epidermal blistering disease caused by autoantibodies (IgG) directed against the desmosomal cadherin desmoglein 3 (Dsg3) (Amagai *et al.*, 1991; Amagai and Stanley, 2012). PV IgG binding to desmoglein 3 results in desmosome disruption and the loss of cell-cell adhesion (Sharma *et al.*, 2007). In PV patients, this loss of adhesion, or acantholysis, manifests as severe mucosal erosions and epidermal blisters between the basal and suprabasal layers of the epidermis where desmoglein 3 is highly expressed (Kottke *et al.*, 2006). Studies reveal distinct, but likely synergistic pemphigus pathomechanisms which include interference of extracellular desmoglein 3 *cis* or *trans* interactions (steric hindrance), endocytosis and depletion of cell surface desmoglein 3, and activation of cellular signaling pathways (Di Zenzo *et al.*, 2012; Getsios *et al.*, 2010; Kitajima and Aoyama, 2007). Inhibition of various PV pathomechanisms can reinforce desmosome adhesion and prevent acantholysis in both cell culture and animal model systems (Waschke, 2008). These findings collectively suggest that reinforcing desmosomal adhesion, rather than

suppressing the immune response is a promising approach in the search for better pemphigus therapies. Apart from the discovery of potentially superior disease treatments, these studies contribute to our understanding of the complex mechanisms that regulate desmosome adhesion.

A fundamental feature of desmosomes not fully understood is the tissue and differentiation specific expression of desmosomal components (Dusek *et al.*, 2007). Changes in the molecular composition of desmosomes correlate with distinct structural and functional differences in desmosomal adhesion (Holthöfer *et al.*, 2007). The differentially expressed armadillo family protein plakophilin-1(PKP-1) stabilizes and increases keratinocyte adhesion by promoting desmosome formation in the suprabasal layers of the epidermis (Hatzfeld, 2007). PKP-1 promotes desmosome formation by recruiting and clustering desmosomal proteins at the plasma membrane and within desmosomes (Bornslaeger *et al.*, 2001; Kowalczyk *et al.*, 1999; Wahl, 2005). PKP-1 binds to DP, desmoglein 1, Dsc1, actin and keratin (Hatzfeld *et al.*, 2000; Hofmann *et al.*, 2000; Kapprell *et al.*, 1988; Smith and Fuchs, 1998). Mutations in PKP-1 cause the autosomal recessive disorder ectodermal dysplasia-skin fragility syndrome (EDSF) (McGrath *et al.*, 1997). EDSF patient skin biopsies revealed a significant reduction in the number and size of desmosomes in the spinous and granular layers (McMillan *et al.*, 2003). Similar to PV, these patients suffer from mechanical stress-induced skin blistering (McGrath and Mellerio, 2010). Finally, PKP-1 has been associated with the formation of calcium-independent desmosomes (South *et al.*, 2003), which exhibit increased intercellular adhesion strength and decreased sensitivity to the depletion of extracellular calcium (Garrod and Kimura, 2008).

The observation that PKP-1 stabilizes and enhances desmosome adhesion raised the possibility that keratinocytes expressing PKP-1 might assemble desmosomes that are refractory to PV IgG. To test this idea, exogenous PKP-1 was expressed in primary keratinocyte cultures. We found that PKP-1 enhanced desmosome formation and clustered desmoglein 3 with desmoplakin through a mechanism that requires the plakoglobin binding domain on the desmoglein 3 tail. Furthermore, we found that PKP-1 prevented PV IgG induced desmosome disruption by inducing the formation of calcium-independent and hyper-adhesive desmosomes. These findings demonstrate for the first time that the increased expression of a desmosomal plaque protein can modulate keratinocyte sensitivity to the pathogenic effects of PV IgG, and provide new insights into the mechanism by which keratinocytes acquire calcium-independent adhesion.

Section 4.2 Results

Results section 4.2a PKP-1 promotes desmosome formation

To determine the role of PKP-1 in modulating keratinocyte adhesion and responsiveness to PV IgG, we used an adenoviral delivery system to manipulate PKP-1 expression in primary human epidermal keratinocytes. We first examined the effect of increased PKP-1 expression on desmoglein 3 and desmoplakin localization in cells cultured in low or high calcium media (Figure 17a-d). To detect desmoglein 3, cells were labeled just prior to fixation with AK15, a non-pathogenic anti-desmoglein 3 monoclonal antibody (mAb). Total desmoplakin and PKP-1.myc were detected after methanol fixation. In empty vector (EV) control keratinocytes, minimal desmoglein 3 and desmoplakin localization was observed at cell borders in low calcium medium (Figure

17a, a′). Upon the addition of calcium, both proteins localized in punctate patterns at cell-cell contacts (Figure 17c, c′). Interestingly, in low calcium culture conditions, PKP-1.myc expression substantially increased desmoglein 3 and DP localization at cell-cell contacts (Figure 17e, compare b, b′ to a, a′). Under high calcium conditions, desmoglein 3 fluorescence intensity was similar between control and cells with increased PKP-1 expression, whereas desmoplakin localization at cell-cell contacts increased significantly with increased PKP-1 expression (Figure 17f, compare c, c′ to d, d′). Sequential detergent extractions from keratinocyte cell lysates followed by western blot analysis was used to compare partitioning of desmosomal proteins between the membrane-associated pool (Triton X-100 soluble) and the desmosomal, cytoskeleton-associated pool (Triton X-100 insoluble). In cells with increased PKP-1 expression, the Triton-insoluble pools of desmoglein 3, desmoglein 2 and desmoplakin increased, suggesting enhanced desmosome formation (Figure 17g). Ultrastructural analysis confirmed that PKP-1.myc expression enhanced desmosome formation. Desmosomes in keratinocytes expressing PKP-1.myc more than doubled in length compared to control, increasing from ~0.4 to ~1.0 μm (Figure 17h, i). Together, these results demonstrate that PKP-1 increases desmosome formation in primary basal keratinocytes.

Results section 4.2b PKP-1 prevents PV IgG-induced desmosome disruption and loss of cell-cell adhesion

To determine if desmosomes in cells with enhanced PKP-1 expression are responsive to PV IgG-mediated disruption, we examined desmosomal protein distribution and keratinocyte adhesion strength in cells expressing EV or PKP-1.myc, treated with NH IgG or PV IgG. To monitor cell surface desmoglein 3 after 24 hours of PV IgG

treatment, cells were labeled with AK15 just prior to fixation. Similar to previous reports, compared to NH IgG treated cells, PV IgG disrupted desmoglein 3 localization and significantly reduced the amount of desmoglein 3 at cell-cell contacts (Figure 18a-b, e) (Calkins *et al.*, 2006; Jennings *et al.*, 2011). Interestingly, expression of PKP-1.myc reduced the loss of desmoglein 3 border localization in PV IgG treated cells (Figure 18c-e). Furthermore, PV IgG treatment of control cells disrupted desmoplakin localization and caused keratin filament retraction from cell-cell borders (Figure 18f-g). In contrast, cells expressing PKP-1.myc and treated with PV IgG displayed prominent desmoplakin localization at cell-cell contacts with extensive keratin associations at desmosomes (Figure 18i). Control experiments verified that PV IgG, as well as two well characterized desmoglein 3 mAb directed against the EC1 (AK23) and EC2-3 (AK15) domains of desmoglein 3 (Tsunoda *et al.*, 2003), bound to keratinocyte cell surfaces in both control and PKP-1.myc expressing cells (Figure 19). These findings suggest that PKP-1 expression results in desmosomes that are refractory to disruption when desmoglein 3 is occupied by pathogenic antibodies.

At the ultrastructural level, desmosomes exhibited a variety of severe abnormalities in keratinocytes treated with PV IgG, including desmosomes with disordered electron dense plaques and few keratin attachments, as well as desmosomes separated at the midline (Figure 20a). Additionally, PV IgG treatment of control cells caused substantial decreases in desmosome size, from ~0.4 to ~0.1µm (Figure 20b). In contrast, PV IgG treated keratinocytes expressing PKP-1.myc exhibited elongated desmosomes with well-organized and electron dense plaques with extensive keratin filament attachments (Figure 20a). Furthermore, desmosome lengths in keratinocytes

expressing PKP-1.myc did not change with PV IgG treatment and averaged ten times longer than the length of desmosomes in PV IgG treated control cells (Figure 20b). Lastly, keratinocyte adhesion strength was assessed by performing cell-dissociation assays (Ishii *et al.*, 2005) on keratinocytes treated with PV IgG or the pathogenic mAb AK23. PV IgG and AK23 both caused substantial monolayer fragmentation, indicating the loss of intercellular adhesion strength (Figure 20c, d). Interestingly, increased expression of plakoglobin, which has been implicated in mediating PV IgG effects (Caldelari *et al.*, 2001), exacerbated the effects of PV IgG and AK23 (Figure 20c-d). In contrast, PKP-1 expression maintained strong intercellular adhesion and significantly reduced fragmentation of keratinocytes exposed to pathogenic antibodies (Figure 20c-d). Collectively, these results demonstrate that increasing PKP-1 expression in basal keratinocytes protects desmosomes from PV IgG-induced disruption and loss of keratinocyte adhesion strength.

Results section 4.2c PKP-1 clusters desmoglein 3 with desmoplakin

To investigate the cytoplasmic interactions between PKP-1 and desmoglein 3 independently of desmoglein 3 adhesive interactions, we fused either the full length desmoglein 3 cytoplasmic domain ($Dsg3_{cyto}$), or truncated versions of the desmoglein 3 cytoplasmic tail ($Dsg3_{cyto\Delta866}$, $Dsg3_{cyto\Delta715}$), to the non-adhesive extracellular and transmembrane domains of the interleukin-2 receptor alpha-chain (IL-2R) (Figure 21a). The IL-2R-$Dsg3_{cyto\Delta866}$ chimera lacks the unique desmoglein sequences including the proline-rich linker (PRL), repeating unit domains (RUD) and the desmoglein terminal domain (DTD). The IL-2R-$Dsg3_{cyto\Delta715}$ chimera lacks these domains and the intracellular cadherin-specific domain (ICS), which binds plakoglobin. These proteins were expressed

in keratinocytes, with or without PKP-1.myc, and their localization at the cell surface was monitored by live cell IL-2R antibody labeling prior to fixation. Without PKP-1.myc co-expression, all four IL-2R proteins localized to the cell surface and at cell-cell borders (Figure 21b-e). However, only the IL-2R-Dsg3$_{cyto}$ and IL-2R-Dsg3$_{cyto\Delta866}$ chimeras co-localized with desmoplakin as revealed by detergent pre-extraction (Figure 22a-d insets). Moreover, the addition of desmoglein 3 cytoplasmic sequences to the IL-2R conferred resistance to detergent pre-extraction in both control and PKP-1.myc keratinocytes (Figure 22 compare a′ to b′-d′ and e′, to f′-h′). Interestingly, co-expression of PKP-1.myc induced extensive co-clustering of cell surface IL-2R-Dsg3$_{cyto}$ and IL-2R-Dsg3$_{cyto\Delta866}$ chimeras with desmoplakin, but not the IL-2R or the IL-2R-Dsg3$_{cyto\Delta715}$ chimera (Figure 21f-i, Figure 22e-h). These results demonstrate that PKP-1 mediates lateral co-clustering of desmoglein 3 and desmoplakin and this activity requires the plakoglobin binding domain on the desmoglein 3 tail.

Results section 4.2d PKP-1 expression induces the formation of calcium-independent and hyper-adhesive desmosomes

Previous studies indicate that desmosomes can become resistant to calcium depletion (Garrod, 2012) and that calcium-independent desmosomes are resistant to PV IgG (Cirillo *et al.*, 2010). Therefore, we performed ‘calcium chelation assays’ to determine if expression of exogenous PKP-1 leads to an increase in the formation of calcium-independent desmosomes. Sub-confluent basal keratinocytes pre-incubated in high calcium medium (14-18 hours) were subjected to calcium chelation for 4 hours (calcium-free medium with 3 mM EGTA). After calcium chelation, the localization of PKP-1, desmoplakin and keratin-14 was examined. Cells were scored as calcium-

independent if cell-cell contact sites were positive for desmoplakin staining. Similar to previous findings (Kimura *et al.*, 2007), the majority of sub-confluent keratinocytes were calcium-dependent, with only ~34% being calcium-independent (Figure 23e). Interestingly, within this subset of calcium-independent keratinocytes, over 90% were positive for endogenous PKP-1 localization at cell-cell contact sites (Figure 23f, b, b′ insets,). Although one-third of control keratinocytes were calcium-independent, the entire population exhibited a rounded morphology, extensive keratin retraction and reduced cell border desmoplakin localization after calcium chelation (Figure 23 compare a-a″ to b-b″). Conversely, keratinocytes expressing exogenous PKP-1.myc exhibited a flattened, cobblestone morphology with prominent desmoplakin localization and keratin attachments at cell-cell contacts (Figure 23 compare d-d″ to b-b″). Remarkably, we observed that 100% of sub-confluent keratinocytes with increased PKP-1.myc expression possessed calcium-independent desmosomes (Figure 23d, e). To determine if these junctions maintain adhesive strength after calcium chelation we performed cell-dissociation assays. Fragmentation of keratinocyte monolayers expressing PKP-1.myc after calcium chelation was reduced significantly compared to control monolayers (Figure 23g). These data indicate that PKP-1 induces the formation of calcium-independent and hyper-adhesive desmosomes that are resistant to PV IgG.

Section 4.3 Discussion

We report here that the armadillo family protein PKP-1 increases desmosome formation and prevents PV IgG-induced desmosome disruption in primary human keratinocytes. Keratinocytes with increased PKP-1 expression maintained strong

intercellular adhesion in the presence of PV IgG or the pathogenic desmoglein 3 antibody AK23. Furthermore, PKP-1 co-clusters desmoglein 3 with desmoplakin, resulting in the formation of calcium-independent and hyper-adhesive desmosomes. These findings raise the interesting possibility that modulating desmosomal plaque protein expression represents a novel approach to reduce blister formation in PV patients.

Pemphigus and related autoimmune disorders are typically treated using immune suppressive therapies (Kasperkiewicz *et al.*, 2012). Although effective and often life-saving, these non-selective approaches cause undesirable side effects (Yeh *et al.*, 2005). Currently, efforts in the field are aimed at developing more directed immune suppressive approaches and to discover ways to render the skin resistant to an ongoing autoimmune response. The latter approach has focused on uncovering signaling pathways that regulate desmosome responses to PV IgG, including p38 MAPK, c-myc, EGFR, among others (Sharma *et al.*, 2007). Here, we report that enhanced expression of a single desmosomal plaque protein, PKP-1, protects keratinocytes from PV IgG-induced loss of adhesion by inducing the formation of calcium-independent, hyper-adhesive desmosomes. These findings indicate that manipulating desmosomal plaque protein gene expression should be considered as an additional approach to facilitate desmosome resistance to autoimmune attack in pemphigus.

Recent studies demonstrate that desmosomes in confluent cell cultures undergo a transition from a calcium-dependent to calcium-independent state over time (Garrod, 2010). Our immunofluorescence analysis revealed that the small percentage of calcium-independent desmosomes that exist in sub-confluent keratinocytes stain positive for PKP-1. Furthermore, in sub-confluent cultures, exogenous PKP-1 expression resulted in a

complete transition of desmosomes to a calcium-independent state. These data and others suggest that the composition of the desmosomal plaque (Hobbs and Green, 2012; South *et al.*, 2003) along with intracellular signaling pathways (Garrod, 2010), modulate the transition of desmosomes from a calcium-dependent to a calcium-independent state. The mechanism of this transition is unclear, but is likely to involve alterations or stabilization of desmosomal cadherin extracellular domain interactions (Garrod *et al.*, 2005).

The mechanism by which PKP-1 stabilizes and increases desmosome formation is not fully understood. Previous studies demonstrate that PKP-1 enhances desmoplakin recruitment to desmosomes (Hatzfeld, 2007). Here, we show that PKP-1 co-clusters desmoplakin with desmoglein 3 through interactions dependent upon the plakoglobin binding domain in the cadherin tail. Based on previous data of known cadherin and desmosomal plaque protein binding partners, we have drawn a schematic of the interactions thought to occur in the PKP-1-IL-2R-Dsg3 chimera clusters (Figure 24) (Kowalczyk *et al.*, 1997; Troyanovsky *et al.*, 1996). These results suggest that PKP-1 may stabilize lateral interactions between desmosomal cadherins and desmosomal plaque proteins. Cadherin lateral interactions are also mediated by *cis*-binding interactions in the cadherin extracellular domain (Al-Amoudi *et al.*, 2007; Brasch *et al.*, 2012). An intriguing possibility is that PKP-1 clustering of the desmoglein 3 tail helps to stabilize desmoglein 3 extracellular domain *cis*-interactions. Interestingly, a recent study demonstrated that PV IgG bind to putative desmoglein 3 *cis*-interacting sequences in the cadherin extracellular domain (Di Zenzo *et al.*, 2012). Together, these findings suggest that PKP-1 may protect desmosomes from PV IgG mediated disruption by stabilizing desmoglein 3 *cis*-interactions. Further studies using structural and biophysical approaches

will be needed to test these possibilities, and to fully understand the basis for the transition of desmosomes to a calcium-independent and PV IgG resistant state. Such studies are likely to yield insights into the fundamental mechanisms that govern desmosome assembly as well as new therapeutic approaches to treat PV and related disorders.

Section 4.4 Materials and Methods

Cell culture

Normal human epidermal keratinocytes (NHEKs) isolated from neonatal foreskin were propagated in low calcium (0.1mM) keratinocyte growth media (KBM-Gold 00192151 with KGM-GoldSingleQuot 00192152, Lonza, Walkersville, MD) as previously described (Calkins *et al.*, 2006). Sub-confluent (50-80%) NHEKs, passages 2-4, were switched into KGM containing 0.6mM CaCl 14-18 hours prior to experimentation.

Antibodies

PV patient sera were kind gifts from M. Amagai (Keio University, Tokyo), J. Stanley (University of Pennsylvania, Philadelphia, PA), and R. Swerlick (Emory University, Atlanta, GA). PV sera recognized desmoglein 3, but not desmoglein 1, determined by ELISA. PV IgG was purified using a Melon Gel IgG Purification Kit (45212, ThermoFisher Scientific, Rockford, IL). Mouse mAb AK15 and AK23 were generated as previously described (Tsunoda *et al.*, 2003). NH and PV IgG were used at 100-150 µg/ml, while AK23, AK15 and IL-2R were added at 10-15µg/ml. Other antibodies: NH IgG (Invitrogen), rabbit anti-DP (NW6; gift KJ Green, Northwestern

University), rabbit anti-gamma catenin (plakoglobin, H-80) and rabbit anti-GAPDH (25778) (Santa Cruz Biotechnology, Santa Cruz, CA), chicken (5560) and rabbit anti-keratin 14 (gift J. Segre, NIH, Bethesda, MD), polyclonal chicken anti-c-myc (A190-103A; Bethyl Laboratories, Montgomery, TX), mouse anti-desmoglein 2 (AH12.2; gift A. Nusrat, Emory University (Nava *et al.*, 2007)), mouse anti-IL-2R IgG2a alpha (7G7B6; American Type Culture Collection, Manasssas, VA), mouse anti-E-cadherin (61081; BD Transduction Laboratories), mouse anti-PKP-1 (14B11; gift J. Wahl III, University of Nebraska (Sobolik-Delmaire *et al.*, 2006)), mouse anti-cytokeratin (Immunotech, Marseille, France). Secondary antibodies: AlexaFluor 647, 555, 488 and HRP-conjugated goat IgGs (Invitrogen).

Adenoviruses

Myc-tagged full length PKP-1 was subcloned into pAd-Track and adenoviruses made from pAdEeasy adenovirus-packaging system that co-expresses GFP with the cDNA of interest (Delva *et al.*, 2008). The full-length "a" form of PKP-1 originally cloned into pCMV-Script was described previously (Hatzfeld *et al.*, 1994; Kowalczyk *et al.*, 1999). The IL-2R and IL-2R-Dsg3 chimeras were generated as described previously (Saito *et al.*, 2012a). NHEKs were infected with adenoviruses 24-36 hours prior to experimentation for adenoviral protein expression. All constructs and their expression were verified by DNA sequencing, western blotting, and immunohistochemistry. High titers were generated as described previously (Setzer *et al.*, 2004; Xiao *et al.*, 2003). Infection rates (70-90%) were determined visually by GFP and immunofluorescence of the protein of interest.

Immunofluorescence and cell-cell border quantification

NHEKs on glass coverslips were washed 3x with phosphate-buffered saline (PBS+), fixed on ice using either -20°C methanol (2 minutes) or were pre-extracted with 0.2% TritonX-100 + 3mM sucrose (45 seconds) and fixed with 4% paraformaldehyde (10 minutes) followed by extraction with 0.2% TritonX-100 (5 minutes). Live cell labeling with mAb AK15, AK23 or IL-2R was performed for 30 minutes prior to fixation at 37°C. To remove surface bound antibody (supplemental Figure 1), cells were incubated on ice with a low pH wash (PBS+ with 100 mM glycine, 20 mM magnesium acetate, and 50 mM potassium chloride, pH 2.2) for 30 minutes prior to fixation. Image acquisition, deconvolution and quantification was conducted with Simple PCI software (version 6.6, Hamamatsu Corporation, Sewickley, PA) on a wide-field fluorescence microscope (DMRXA2; Leica) equipped with 63×/1.32 NA, and 100×/1.40 NA oil immersion objectives, and a CCD camera (ORCA; Hamamatsu Photonics). Images shown are deconvolved z-stack projections. For quantification, exposure times and fluorescence intensity thresholds were consistent between experimental data sets. Images were thresholded and the average intensity above threshold was calculated within 4.5µm x 4.5µm region of interests (ROIs) selecting cell borders from individual images.

Calcium chelation assays

NHEKs on glass coverslips, infected with the appropriate adenoviruses in 0.1mM calcium KGM, were switched to high calcium media (0.6 mM) for 14-18 hours prior to three, one-minute washes in calcium and magnesium-free PBS supplemented with 3mM Methylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA). Cells were incubated in calcium free KBM with 3mM EGTA for 4 hours and processed for immunofluorescence. Images were captured at random (63x oil objective), the total

number of cells counted and scored as calcium-independent if cell-cell contact sites were positive for DP staining (8 images per group, per experiment (~ 40 cells per image)). This number, a proportion of the total cell count, is displayed as the percentage of cells that are calcium-independent.

Cell-Dissociation assays

Dispase based cell-dissociation assays were performed as described previously (Saito *et al.*, 2012a). Briefly, 100% confluent NHEKs in 1.9cm² culture dishes were switched to high calcium medium ~16 hours before addition of the appropriate antibodies. Recombinant exfoliative toxin A (ETA, 0.25µg/ml) cleaves desmoglein 1 and was added 2 hours prior to the assay. After overnight incubation with antibodies, monolayers were released (1U/ml dispase, Roche) and subjected to mechanical stress (pipetting 25x with a 1ml micropipette tip). Monolayers were fixed, stained and fragments counted using a dissecting microscope. In assays with calcium chelation, monolayers were released from the culture dish prior to calcium chelation.

Electron microscopy

NHEKs on coverslips, infected with the appropriate adenovirus, were treated with NH or PV IgG as described above. Cells were fixed (2.5% glutaraldehyde in 0.1M cacodylate) and processed for conventional electron microscopy (Robert P. Apkarian Integrated Electron Microscopy Core, Emory University).

Sequential detergent extraction and western blot analysis

NHEKs were extracted sequentially in Triton buffer (1% TritonX-100, 10mM Tris-pH 7.5, 140mM NaCl, 5mM EDTA, 2mM EGTA, (cOmplete, Mini, EDTA-free Protease

Inhibitor Tablets)) followed by extraction with urea–SDS buffer (1% SDS, 8M urea, 10mM Tris-HCL-pH 7.5, 5mM EDTA, 2mM EGTA, 70 mM NaCl) as described previously (Calkins *et al.*, 2006). The triton-soluble samples were 2x the insoluble samples for visualization.

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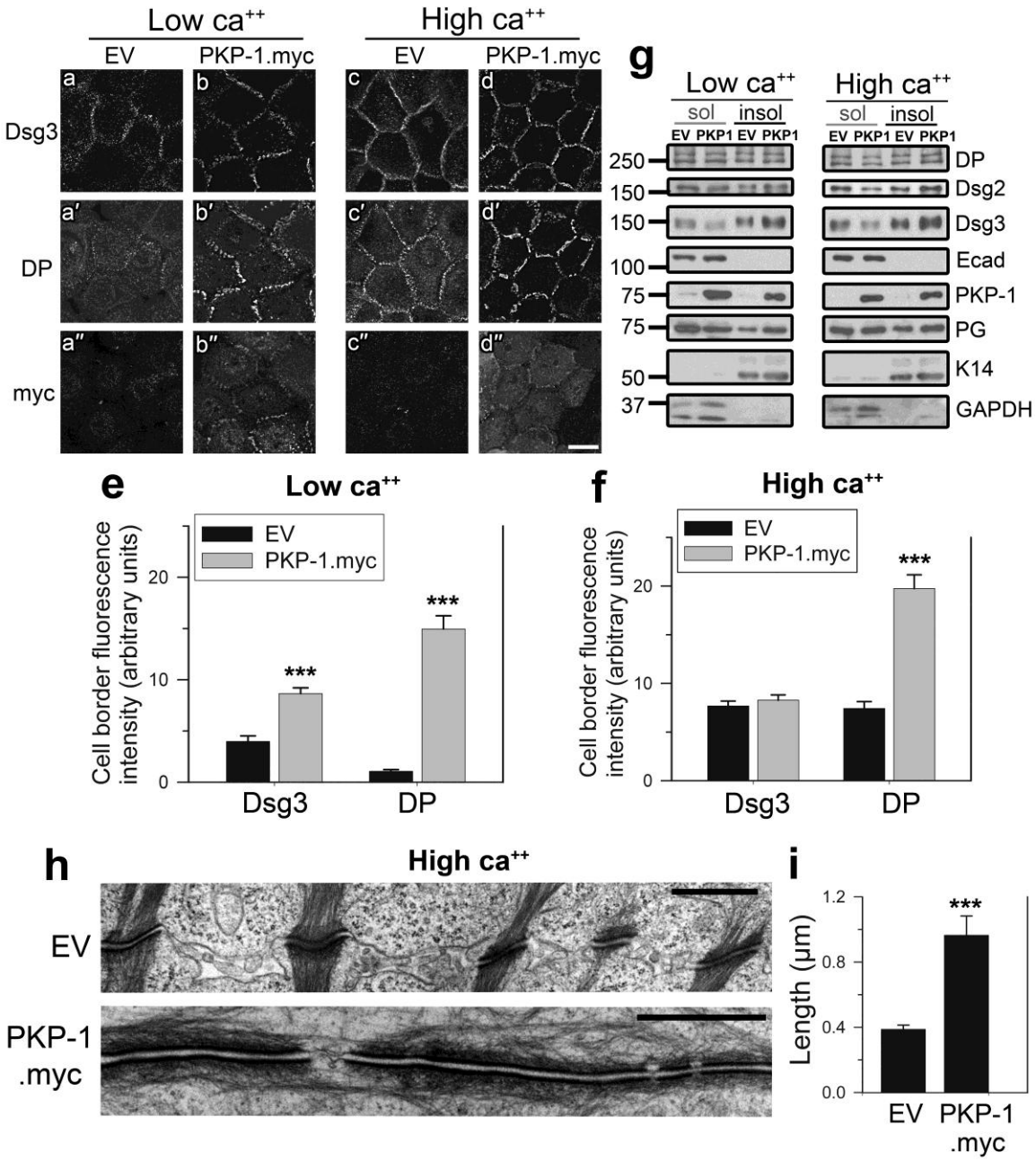

Figure 17. PKP-1 promotes desmosome formation. (**a-d**) Immunofluorescence images and (**e, f**) quantification of cell-cell border fluorescence intensity from keratinocytes expressing empty vector (EV) or PKP-1.myc in low (0.1mM) or high calcium (ca^{++}) media (0.6mM) for 24 hours and immunostained for surface desmoglein 3, total desmoplakin (DP) and myc (PKP-1.myc). Scale bar: 20µm. (**e, f**) Mean ± SEM (n= 50 borders per group); *** $p < 0.001$ compared with EV (Mann Whitney). (**g**) Sequential

detergent extraction and western blot analysis of Triton X-100 soluble (sol) and insoluble (insol) proteins. Blot represents four independent experiments. (**h, i**) Electron micrographs and quantification of desmosome lengths. (**i**) Mean ± SEM (n= 25 desmosomes per group); *** $p < 0.001$ compared with EV (Mann Whitney). Scale bars: 1µm.

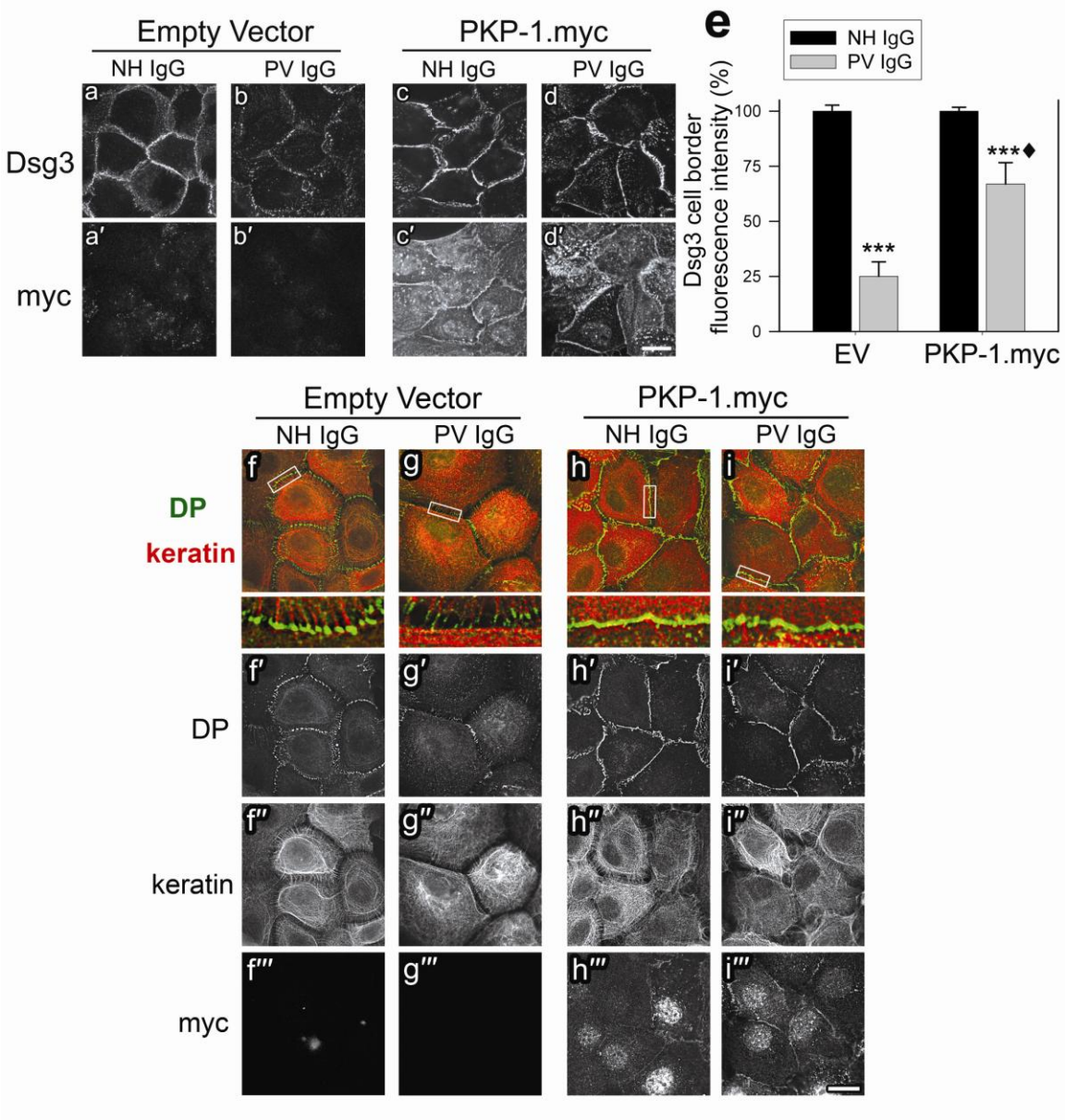

Figure 18. PKP-1 protects desmosomal components from disruption by PV IgG.

Keratinocytes expressing empty vector (EV) or PKP-1.myc were treated with NH or PV IgG for 24 hours. (**a-d**) The mAb AK15 was used live to detect cell surface desmoglein 3 and total myc was detected after methanol fixation (a′-d′). (**e**) Quantification of desmoglein 3 fluorescence intensity at cell-cell borders. Data are mean percentages normalized to EV and PKP-1 NH IgG controls. Mean ± SEM (n= 50 borders per group);

*** $p < 0.001$ compared with EV-NH IgG, ♦ compared with EV-PV IgG and PKP-1.myc-NH IgG (Two-way ANOVA, Holm-Sidak method). (**f-i)** Cells were briefly pre-extracted prior to fixation and immunostained for desmoplakin (DP) (green, f′-i′), cytokeratin (keratin, red, f″-i″) and myc (f‴-i‴). Data represent four independent experiments.

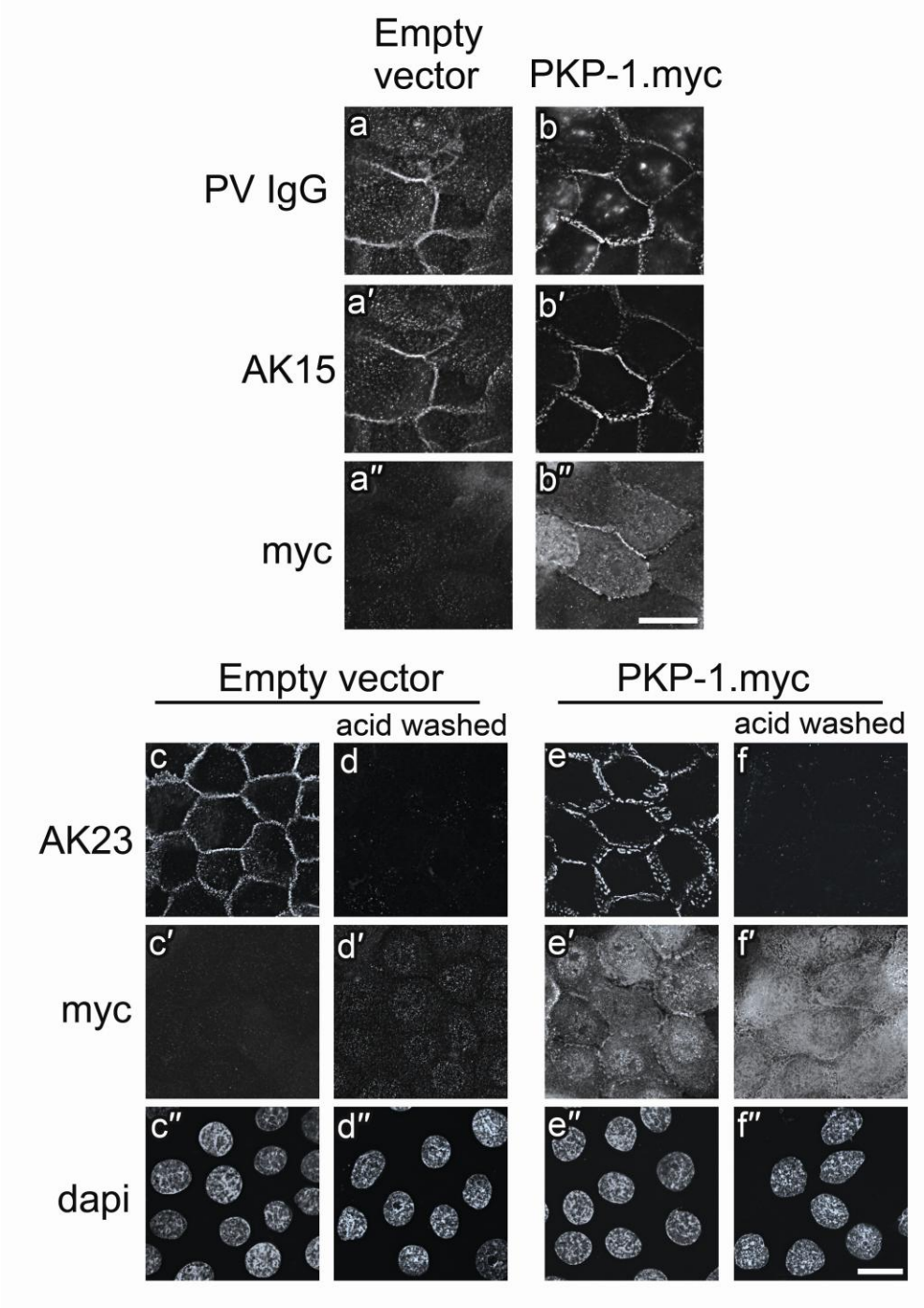

Figure 19. PKP-1 does not prevent anti-desmoglein 3 antibodies from binding to the cell surface. (**a-b**) Keratinocytes expressing empty vector (EV) or PKP-1.myc were co-incubated with PV IgG and the non-pathogenic desmoglein 3 mAb AK15 for 30 minutes at 37°C. The localization of human IgG, AK15 and myc were visualized. (**c-f**)

Keratinocytes expressing EV or PKP-1.myc were incubated with the pathogenic desmoglein 3 mAb AK23 for 30 minutes at 37°C. The cells were then either processed for immunofluorescence (c, e) or subjected to a low pH acid to remove surface-bound antibody prior to immunofluorescence processing (d, f). The localization of surface AK23, total myc and DNA (DAPI) were visualized. Scale bars, 20μm.

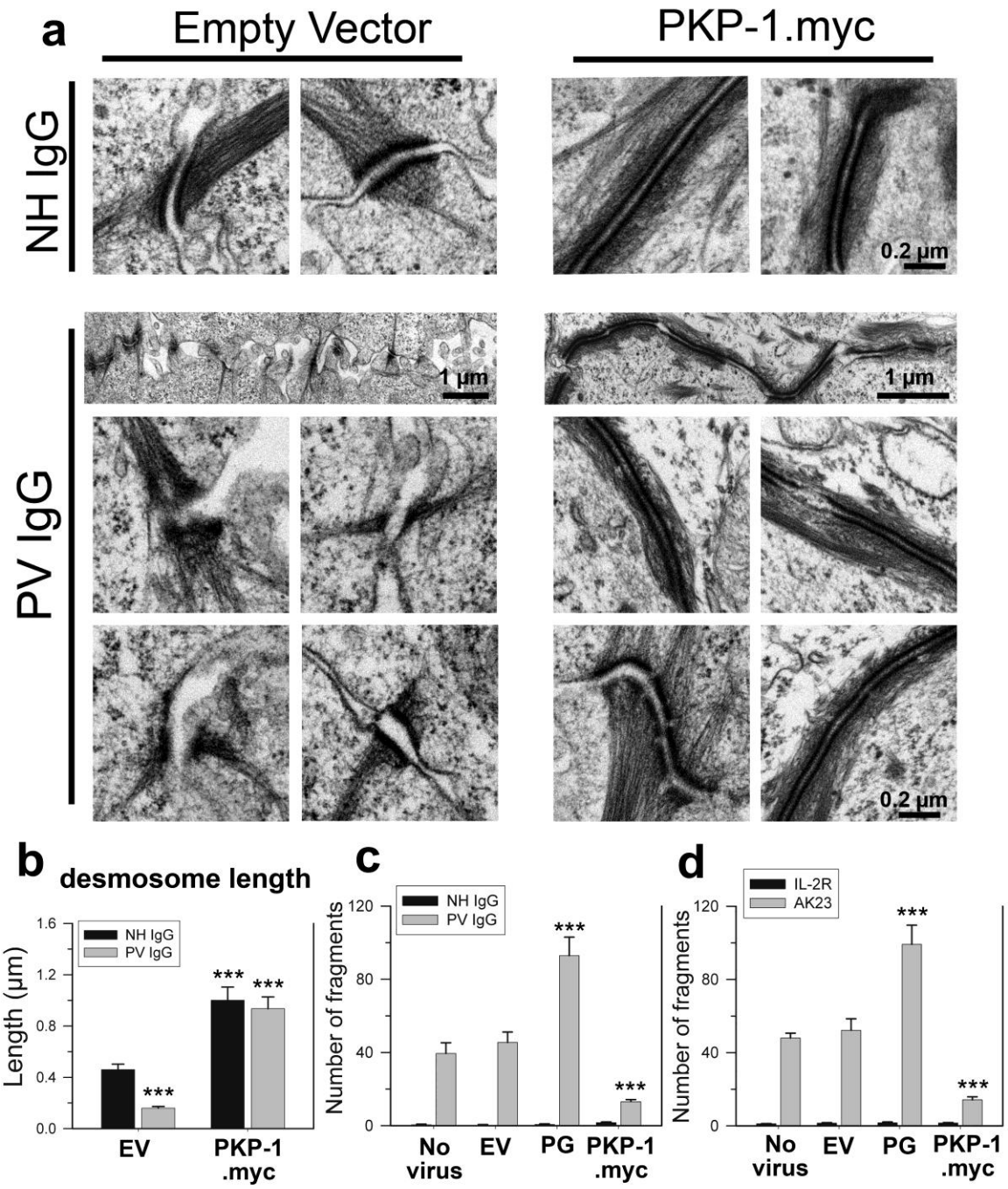

Figure 20. PKP-1 protects desmosome ultrastructure and keratinocyte adhesion strength from disruption by PV IgG. (**a**) Electron micrographs and (**b**) quantification of average desmosome lengths from keratinocytes expressing empty vector (EV) or PKP-1.myc treated with NH or PV IgG for 24 hours. (**b**) Means ± SEM (n= 25-50

desmosomes per group); *** $p < 0.001$ compared with EV-NH IgG (Mann Whitney). (**c, d**) Quantification of monolayer fragmentation after cell dissociation assays using control keratinocytes (no virus, EV, and plakoglobin (PG)) or keratinocytes expressing PKP-1.myc exposed for 24 hours to (**c**) NH or PV IgG or (**d**) mAb IL-2R or AK23, a pathogenic desmoglein 3 mAb. Mean number of fragments ± SEM;*** $p < 0.001$ compared with no virus and EV (Two-way ANOVA, Holm-Sidak method).

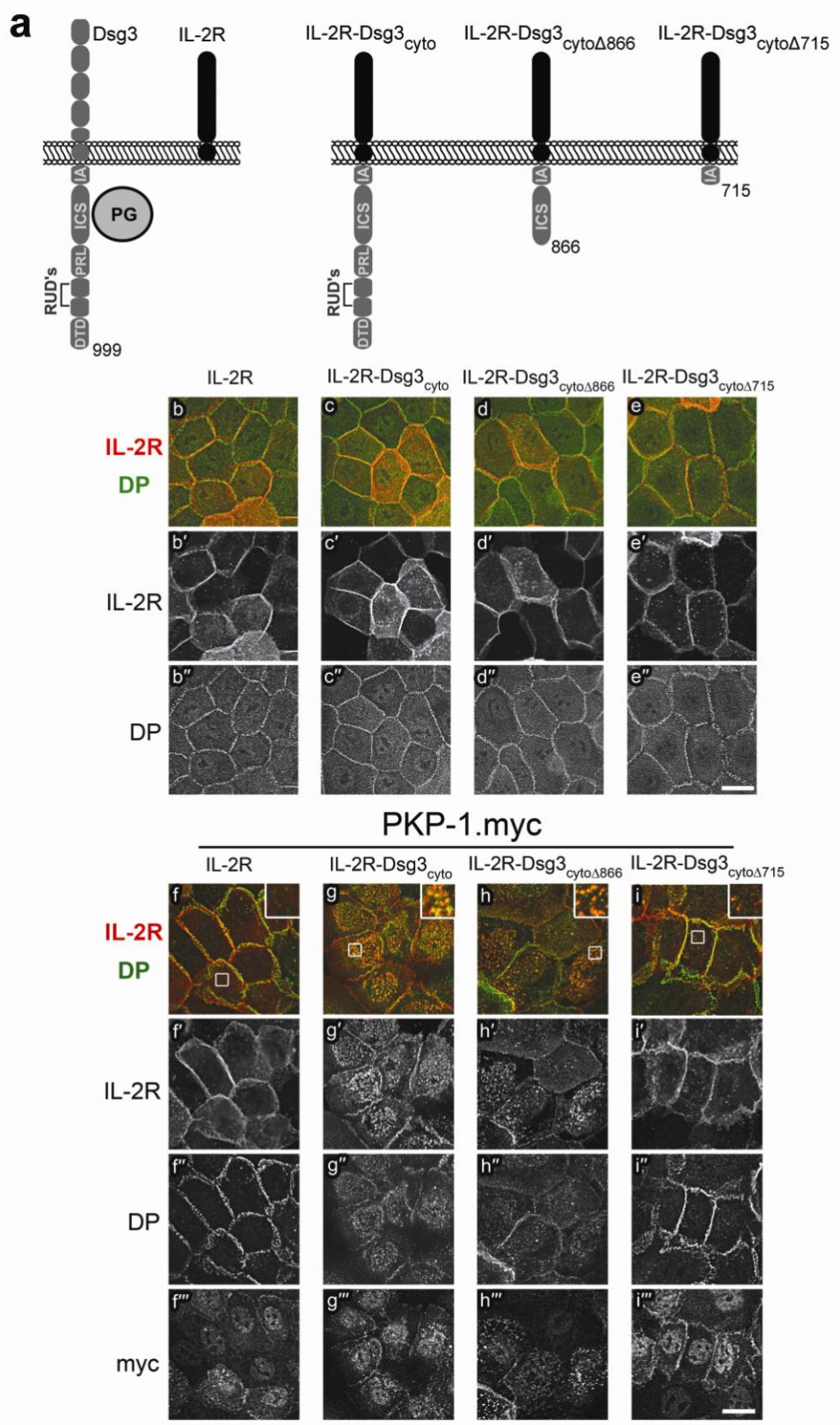
a
Dsg3
IL-2R
IL-2R-Dsg3cyto
IL-2R-Dsg3cytoΔ866
IL-2R-Dsg3cytoΔ715
IA
ICS
PRL
DTD
PG
RUD's
999
866
715
IL-2R
DP
PKP-1.myc
myc

Figure 21. PKP-1 clusters the cytoplasmic tail of desmoglein 3 with desmoplakin (DP). (**a**) Portions of the desmoglein 3 cytoplasmic tail (grey) were fused to the extracellular and transmembrane domains of the interleukin-2 receptor alpha chain (IL-2R, black) to create chimeric IL-2R-Dsg3 proteins. IA, intracellular anchor; ICS, intracellular cadherin-specific; IPL, proline-rich linker; RUD, repeating unit domain; DTD; desmoglein terminal domain; cyto, entire desmoglein 3 cytoplasmic tail; cytoΔ866 and cytoΔ715, desmoglein 3 cytoplasmic tail truncated at residues 866 and 715. (**b-e**) IL-2R and IL-2R-Dsg3 chimeras expressed in keratinocytes, methanol fixed and immunostained for cell-surface IL-2R (red, b′-e′) and total desmoplakin (DP) (green, b″-e″). (**f-i**) IL-2R and IL-2R-Dsg3 chimeras were co-expressed with PKP-1.myc and immunostained for cell surface IL-2R (red, f′-i′), total DP (green, f″-i″) and myc (f‴-i‴). Scale bars 20μm.

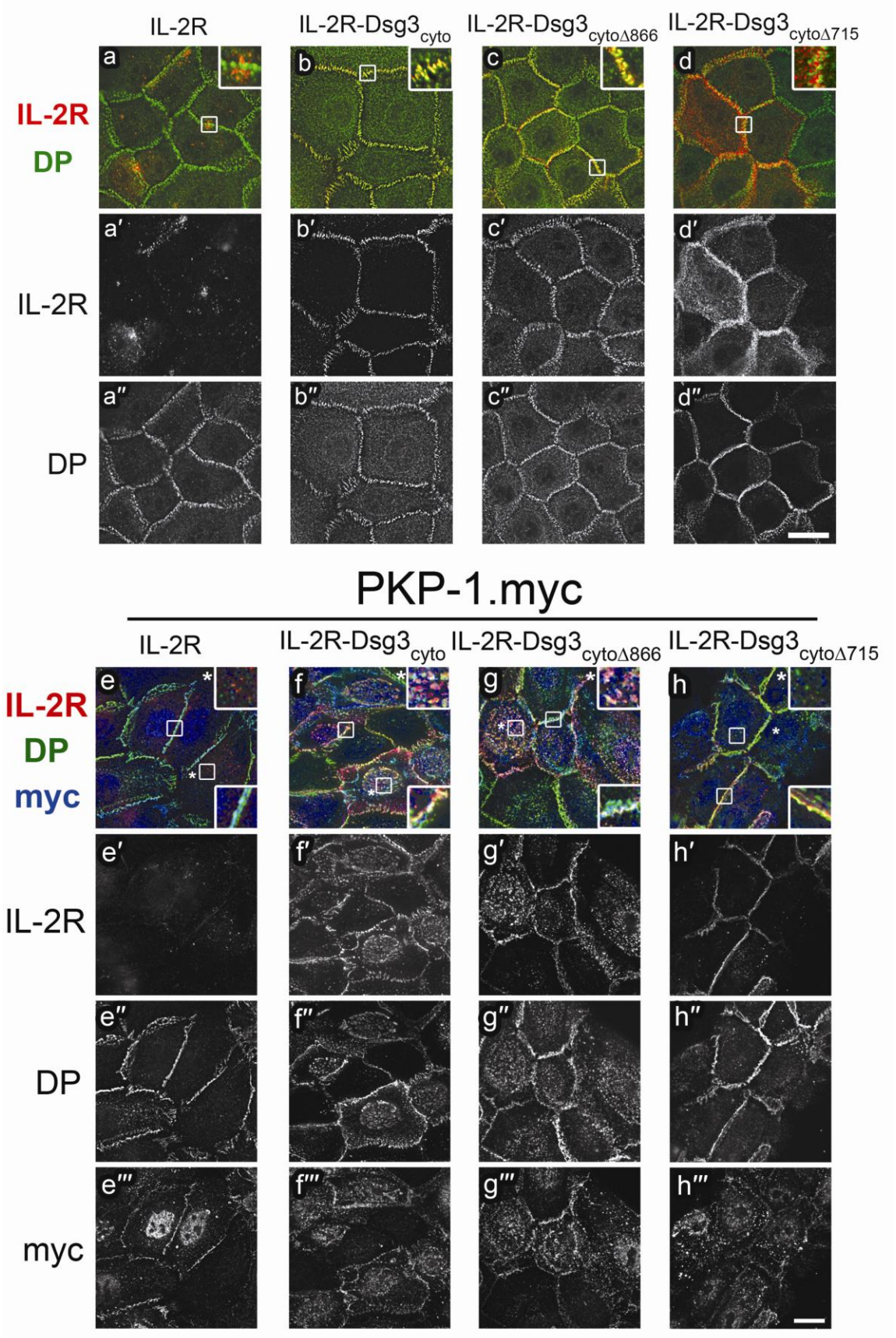
IL-2R
IL-2R-Dsg3cyto
IL-2R-Dsg3cytoΔ866
IL-2R-Dsg3cytoΔ715
IL-2R
DP
a
b
c
d
a′
b′
c′
d′
IL-2R
a″
b″
c″
d″
DP
PKP-1.myc
IL-2R
IL-2R-Dsg3cyto
IL-2R-Dsg3cytoΔ866
IL-2R-Dsg3cytoΔ715
IL-2R
DP
myc
e
f
g
h
e′
f′
g′
h′
IL-2R
e″
f″
g″
h″
DP
e‴
f‴
g‴
h‴
myc

Figure 22. Desmoglein 3 cytoplasmic sequences mediate co-localization with desmoplakin (DP) and differential sensitivity to detergent pre-extraction. (**a-h**) Images of IL-2R and IL-2R-Dsg3 chimeras expressed in keratinocytes, with and without PKP-1.myc co-expression. Cells were labeled live with a mAb IL-2R (red and a′-h′) for 30 minutes and then immediately subjected to a 45 second detergent pre-extraction with 0.2% Triton-X100. Following fixation, the cells were immunostained for total desmoplakin (DP) (green and a″- h″) and myc (blue and e‴- h‴). Scale bars, 20μm.

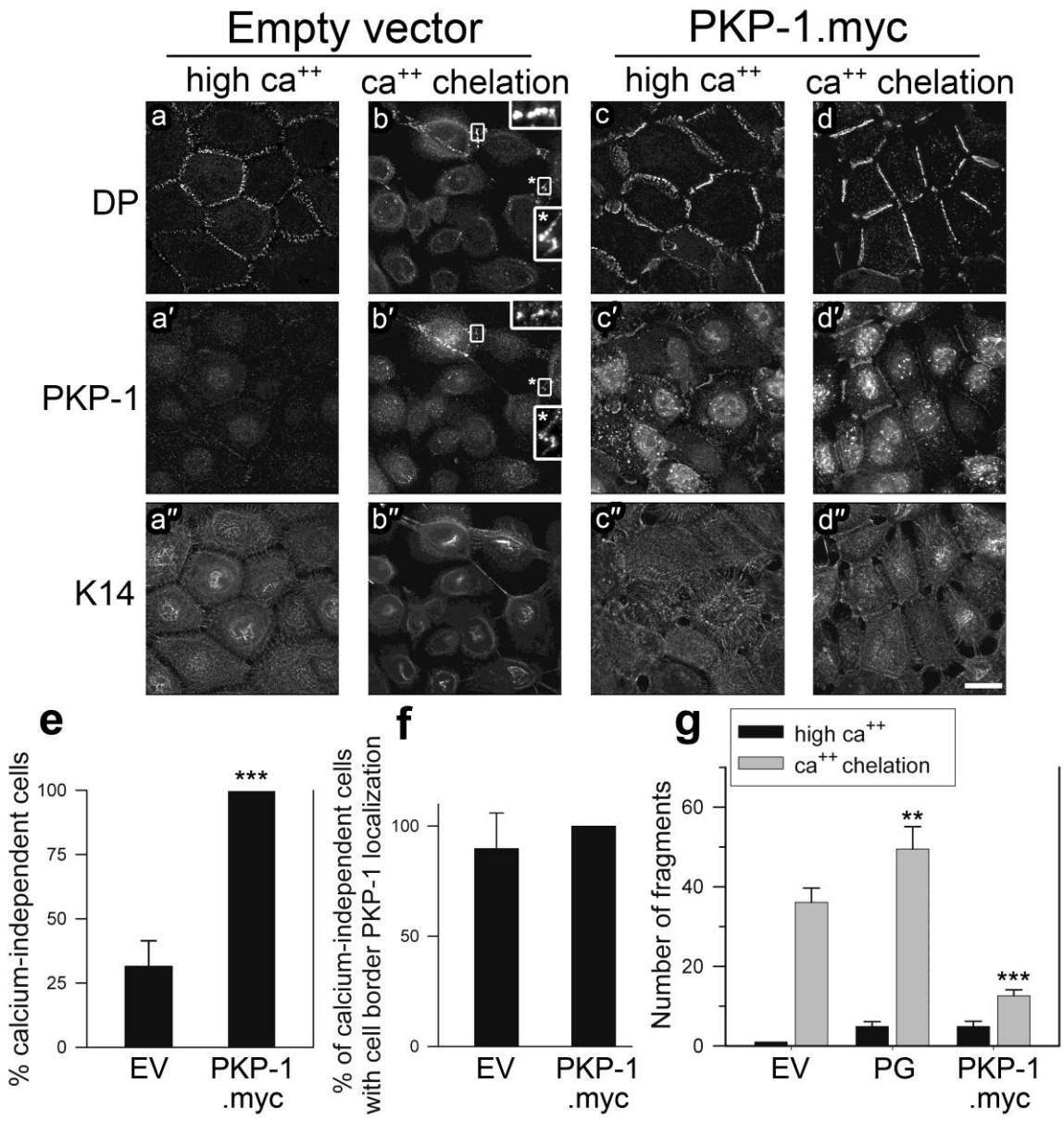

Figure 23. PKP-1 induces the formation of calcium-independent, hyper-adhesive desmosomes. (**a-d**) Keratinocytes expressing empty vector (EV) or PKP-1.myc in high calcium (ca^{++}) medium or after 4 hours calcium chelation, immunostained for desmoplakin (DP), PKP-1 and keratin-14 (K14), bar 20µm. (**e**) Percent calcium-independent cells per microscope field. Cells were scored as calcium-independent if cell-cell contact sites were positive for desmoplakin (DP). Means ± SEM (n=24 fields);*** p

< 0.001 compared with EV (Mann Whitney). (f) Percent cells with PKP-1 localization at cell-cell contacts among calcium-independent cells per microscope field. Means ± SEM (n=24 fields). (**g**) Quantification of cell dissociation assays using monolayers subjected to 4 hours calcium chelation. Means ± SEM;*** p < 0.001 compared with EV (Two-way ANOVA, Holm-Sidak).

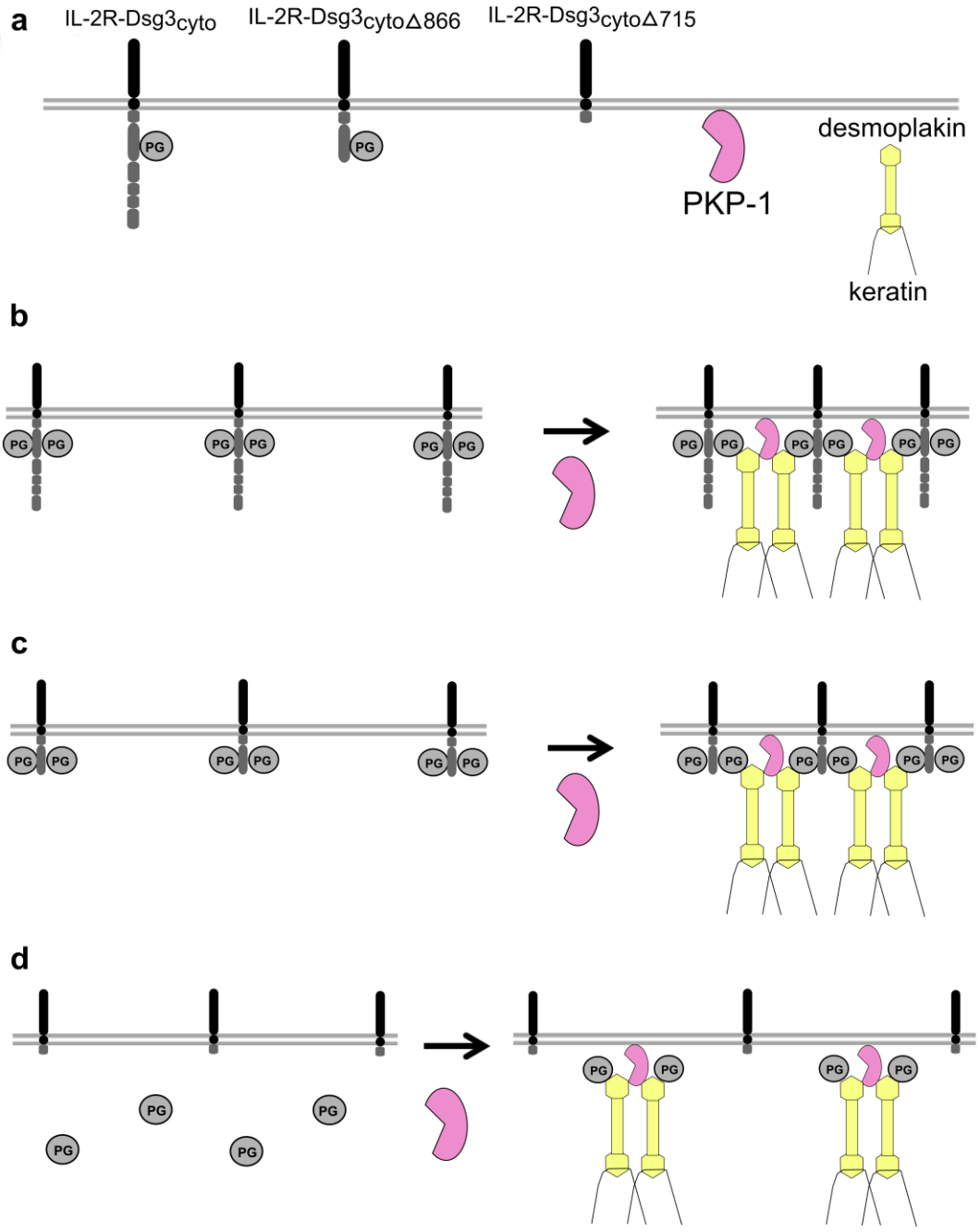

Figure 24. Model of interactions proposed to occur between PKP-1 and the IL-2R-Dsg3 chimeras. (a) key **(b)** IL-2R-Dsg3$_{cyto}$ **(c)** IL-2R-Dsg3$_{cyto\Delta 866}$ **(d)** IL-2R-Dsg3$_{cyto\Delta 715}$.

Chapter V

Future Directions and Concluding Remarks

Dana K. Tucker

Section 5.0 Future Directions

The findings presented in Chapter IV demonstrate that reinforcing desmosome adhesion can prevent the loss of adhesion caused by PV IgG. Furthermore, we have revealed new insights into the regulation of desmosome adhesion states by identifying a role for PKP-1 in the formation of calcium-independent, hyper-adhesive desmosomes. Several questions have arisen from these studies. What phase of PV IgG-induced desmosome disassembly does PKP-1 prevent? How does PKP-1 transform desmosomes to a calcium-independent adhesive state? Does the transformation of desmosome adhesive states mediated by PKP-1 reveal differences between plakophilin isoforms that relate to their differentiation specific expression in the epidermis?

What phase of PV IgG-induced desmosome disassembly does PKP-1 prevent?

As reviewed in Chapter III, we found that the loss of adhesion caused by PV IgG can be distinguished in three phases. Phase 1 is characterized by rapid desmoglein 3 internalization and degradation of non-junctional desmoglein 3 molecules. In phase 2, desmosomes rearrange into linear arrays and desmoglein 3 complexes are released from the ends of these arrays into cytoplasmic vesicular compartments. Phase 3 is characterized by depletion of desmoglein 3 from cell contacts, desmoplakin and keratin localization is disrupted and keratinocytes exhibit significant decreases in cell-cell adhesion strength. In chapter IV we demonstrate that PKP-1 prevents desmosome disruption and the loss of adhesion caused by PV IgG. One hypothesis could be that PKP-1 interferes with phase 1 by preventing PV IgG-induced desmoglein 3 endocytosis thereby stabilizing desmoglein 3 cell surface levels. However, internalization assays of

PV IgG treated cells, with and without increased PKP-1 expression, demonstrated that PKP-1 does not prevent desmoglein 3 internalization (data not shown). Furthermore, western blot analysis indicated that the soluble pool of desmoglein 3 is reduced in keratinocytes with increased PKP-1 expression that were treated with PV IgG (data not shown). These preliminary results suggest that PKP-1 does not prevent phase 1 of desmosome disassembly. Nonetheless, results in Chapter IV (Figure 18) demonstrate that desmoglein 3 is being maintained at cell-cell contacts. Thus, it may be that PKP-1 prevents the loss of desmosomal desmoglein 3, whereas the non-desmosomal pool of desmoglein 3 is affected by PV IgG. An observation that supports this idea is that PKP-1 only partially rescued desmoglein 3 levels at cell-cell contacts, thus the reduction in desmoglein 3 immunofluorescence intensity we observed may be due to the loss of non-desmosomal desmoglein 3 (Figure 18). One possible mechanism by which PKP-1 circumvents the loss of soluble desmoglein 3 proteins could be promotion of new desmosome assembly. Thus one prediction we can make is that PKP-1 mediates new desmosome assembly even in the presence of PV IgG. This may be possible if prior to PV IgG binding, PKP-1 directly incorporates desmoglein 3 into desmosomes by clustering newly synthesized desmoglein 3 proteins with desmoplakin. To test the idea that PKP-1 can assemble desmosomes in the presence of PV IgG, cells with and without increased PKP-1 expression, would be treated with PV IgG in low calcium media. Subsequent to PV IgG treatment, the cells would be switched to high calcium media to allow for desmosome assembly. If PKP-1 is able to promote desmosome assembly in the presence of PV IgG, the cells would exhibit strong cell-cell adhesion strength when examined via the cell dissociation assay. Additionally, immunofluorescence microscopy

would be used to determine if desmosomal components localize normally at cell-cell contacts.

In Chapter IV (Figure 20), we found that PKP-1 rescues desmoplakin and keratin localization in cells treated with PV IgG for 24 hours. Based on these data we predict that PKP-1 prevents the formation of linear arrays observed in phase 2 (Chapter III, Figure 12). To test this idea we would treat cells, with and without increased PKP-1 expression, for 6 hours with PV IgG and examine various desmosomal components to determine if linear array formation is prevented.

Evidence from Chapter IV demonstrates that PKP-1 increases desmosome formation and prevents PV IgG-induced desmosome disassembly. Furthermore, our preliminary data suggest that PKP-1 does not prevent phase 1 of PV-IgG induced desmosome disassembly (data not shown). Thus based on these data we propose a model by which PKP-1 mediates desmosome resistance to the pathogenic effects of PV IgG (Figure 25). In this model, PKP-1 does not prevent PV IgG-induced internalization and degradation of the non-desmosomal pool of desmoglein 3. However, we propose that PKP-1 counteracts this process by forming calcium-independent desmosomes which stabilize the junctional pool of desmoglein 3. Secondly, we speculate that PKP-1 mediates the incorporation of a subset of newly synthesized desmoglein 3 into desmosomes and thus allows for desmosome assembly to occur even in the presence of PV IgG (Figure 25).

How does PKP-1 transform desmosomes into a calcium-independent adhesive state?

Recent work indicates that PKCα modulates desmosome adhesive states (Garrod, 2010). Evidence suggests that wounding of confluent monolayers triggers PKCα activation which then transforms desmosomes from a calcium-independent to a calcium-dependent state (Kimura *et al.*, 2007). Furthermore, in sub-confluent keratinocytes, desmosomes are thought to be maintained in a constant state of calcium-dependent adhesion due to PKCα signaling (Garrod, 2010). The molecular mechanism by which PKCα mediates transitions between desmosome adhesion states is unknown. Our immunofluorescence analysis in Chapter IV (Figure 23) revealed that the small percentage of calcium-independent desmosomes that exist in sub-confluent keratinocytes stain positive for endogenous PKP-1. Furthermore, in sub-confluent cultures, exogenous PKP-1 expression resulted in a complete transition of desmosomes to a calcium-independent state. The relationship between PKCα signaling and PKP-1 mediated calcium-independent desmosome adhesion is unclear. However, our results suggest that PKP-1 renders desmosomes refractory to transitions between adhesive states mediated by PKCα. Future work is needed to reveal the mechanisms by which PKCα and PKP-1 regulate calcium-dependent and calcium-independent desmosome adhesion.

One mechanism proposed to explain the two adhesion states suggests that calcium-independent desmosomes exhibit a more highly ordered arrangement of extracellular cadherin domains to yield binding interactions so tight they become resistant to calcium chelation. Furthermore, this ordered arrangement may be reflected by and dependent upon the molecular architecture of the desmosomal plaque (Garrod and Kimura, 2008). We sought to examine the relationship between PKP-1 and desmoglein 3 in the desmosomal plaque. Our electron microscopy (EM) ultrastructural analysis of

desmosomes from cells with increased PKP-1 expression demonstrates that PKP-1 greatly increases the size of desmosomes (Figure 17). Measurement of immunofluorescence intensities indicated that while PKP-1 increases desmoplakin recruitment, desmoglein 3 recruitment to cell-cell borders was no different from control cells (Figure 17). This may suggest that the elongation of desmosomes we see by EM is simply due to increased recruitment of more desmoplakin to the desmosomal plaque. However, examination of desmosomal proteins biochemically told a different story. Western blot analysis of non-desmosomal (Triton-soluble) and desmosomal (insoluble) protein pools revealed that in cells with increased PKP-1 expression the desmosomal pool of desmoglein 3, desmoglein 2 and desmoplakin increased (Figure 17). This suggested to us that PKP-1 increases desmoglein 3 incorporation into junctions and interacts with desmoglein 3 within the desmosomal plaque. We next set out to investigate the relationship between PKP-1 and desmoglein 3. However, this is not a trivial pursuit because the highly insoluble nature of desmosomal proteins makes it difficult to study their protein-protein interactions. Thus to circumvent this issue, we utilized the IL-2R-desmoglein 3 chimera's and found that PKP-1 mediates cytoplasmic lateral interactions between desmoglein 3 and desmoplakin (Figure 21, 22, 24). This experiment demonstrates that PKP-1 is interacting with desmoglein 3 and suggests that PKP-1 is recruiting desmoglein 3 to desmosomes by mediating an association with desmoplakin in the desmosomal plaque. Considering that we now know that PKP-1 interacts with desmoglein 3, one possibility is that in the desmosomal plaque PKP-1 clustering of the cadherin cytoplasmic domains facilitates an ordered arrangement of cadherin extracellular domains to form calcium-independent adhesive interactions. Thus, one

prediction would be that desmosomes formed by PKP-1 adopt a more ordered structure than desmosomes formed by plakophilin-2, which are known to become calcium-dependent. Currently, elucidation of the precise molecular architecture of the desmosomal plaque is beyond our experimental and technical capabilities. Therefore, any possible difference in the arrangements of proteins in the plaque that may be mediated by different plakophilin isoforms is entirely unknown. More advanced structural studies are needed to fully understand the mechanisms of calcium-independent desmosome adhesion and to determine if plakophilin isoforms mediate distinct differences in the organization of the desmosomal plaque. Despite these limitations, functional studies suggest that the plakophilins play distinct roles in regulating desmosomal cell-cell adhesion (Hatzfeld, 2007). Overexpression experiments indicate that PKP-1 enhances desmosome adhesion to a greater extent than plakophilin-2 and plakophilin-3. Thus, we are interested in determining whether or not plakophilin-2 and -3 cluster the desmoglein 3 tail in a similar manner as PKP-1.

Does the transformation of desmosome adhesive states mediated by PKP-1 reveal differences between plakophilin isoforms that relate to their differentiation specific expression in the epidermis?

A fundamental feature of desmosome adhesion not fully understood is the differentiation-specific expression of the plakophilin isoforms in the epidermis (Dusek *et al.*, 2007). In the epidermis, PKP-1 is expressed as a gradient, with maximum expression in the uppermost spinous and granular layers, whereas plakophilin-2 has an inverse expression profile with maximum expression in the basal layer (Desai *et al.*, 2009; Hatzfeld *et al.*, 1994; Heid *et al.*, 1994; Schmidt and Jäger, 2005). The differentiation-

specific expression of plakophilin isoforms may be partially explained by their differential abilities in modulating desmosome adhesion. The physiological relevance of the calcium-dependent state is thought to represent a more plastic state of desmosomal adhesion that is necessary for the downregulation of desmosomes during wound healing (Garrod, 2010). Indeed, during the process of wound healing, migration and proliferation of keratinocytes into the wound matrix requires downregulation of desmosome adhesion (Santoro and Gaudino, 2005). The role for calcium-dependent adhesion in wound healing is supported by the observation that *in vivo* the normal state of adhesion from intact and unperturbed epithelium is calcium-independent. Furthermore, it is only upon wounding that keratinoctyes in the epidermis become calcium-dependent (Garrod, 2010). Interestingly, wound healing primarily involves the basal and a small subset of suprabasal layer keratinocytes. Whereas, keratinocytes from the upper spinous and granular layers, do not participate in wound healing (Santoro and Gaudino, 2005). Findings presented in Chapter IV (Figure) demonstrate that in sub-confluent control keratinocytes, which have high levels of plakophilin-2 expression (data not shown), the majority of desmosomes were calcium-dependent. Furthermore, we found that PKP-1 induces the formation of calcium-independent, hyper-adhesive desmosomes. We suspect that our observations involving PKP-1 and desmosome adhesion states may represent a functional difference between plakophilin isoforms within basal and differentiated keratinocytes during the process of wound healing. Thus because PKP-1 induces the formation of calcium-independent desmosomes in sub-confluent basal keratinoctyes, we suspect that desmosomes enriched with PKP-1 inherently exhibit less plasticity. If this is the case then PKP-1 would impede cell migration. Interestingly, the effect of PKP-1 expression on cell

motility in oral squamous cell carcinoma cells was recently examined. Decreased expression of PKP-1 resulted in an increase in cell motility, whereas overexpression of PKP-1 reduced cell migration (Sobolik-Delmaire *et al.*, 2007). This strongly supports the role of PKP-1 in forming desmosomes that are less capable of undergoing rearrangements during cell migration. Whether or not cell migration is hindered in primary human keratinocytes with increased PKP-1 expression has yet to be examined. One experiment to determine if PKP-1 affects keratinocyte migration could be to perform scratch wound assays in basal keratinocytes with and without increased PKP-1 expression.

Section 5.2 Concluding Remarks

The overall goals of this dissertation were to 1) to understand the pathomechanisms of the pemphigus vulgaris 2) to explore a novel approach to prevent the loss of cell-cell adhesion caused by PV autoantibodies by exogenously expressing the desmosomal component, PKP-1 and 3) to further our understanding of the fundamental mechanisms that regulate desmosomal adhesion. In Chapter III we furthered our understanding of the pathomechanisms of PV by revealing more precisely how PV IgG-induced desmosome disassembly occurs by defining the temporal and spatial relationships between desmosomal components during this process. Our findings demonstrate that desmosome disassembly and the subsequent loss of cell-cell adhesion occurs sequentially in specific phases that are triggered by desmoglein 3 internalization and degradation. Importantly, we proposed a PV disease model that incorporates the phases of desmosome disassembly with the PV pathomechanisms of steric hindrance and intracellular signaling which have both been shown to play a key role in the loss of cell-cell adhesion (Figure 16, 25).

In Chapter IV we explored a novel approach to render the skin resistant to an ongoing autoimmune response. We found that enhanced expression of PKP-1 protects keratinocytes from PV IgG-induced loss of cell-cell adhesion by inducing the formation of calcium-independent, hyper-adhesive desmosomes. Additionally, we demonstrated that PKP-1 mediates lateral clustering of desmoglein 3 with desmoplakin. Our results demonstrate for the first time that manipulating the expression of a single desmosomal plaque protein can block the pathogenic effects of PV IgG on keratinocyte adhesion. Furthermore, these findings suggest the reinforcement of desmosome adhesion is a viable approach to facilitate desmosome resistance to autoimmune attack in pemphigus. Finally, the identification of PKP-1 as a regulator of desmosome adhesive states may have broad implications for functional differences in desmosome adhesion in wound healing and between basal and upper layer keratinoctyes during this process.

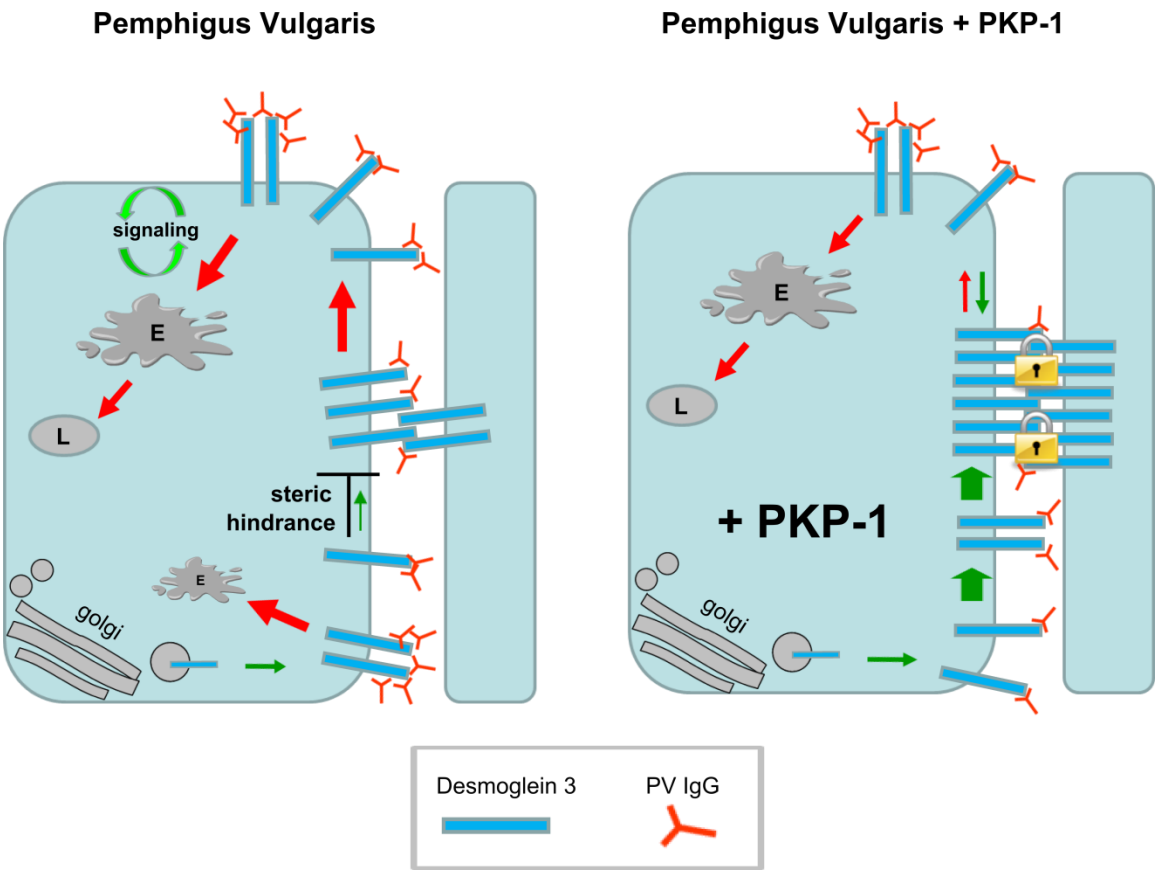

Figure 25. Pemphigus vulgaris (PV) disease model and protection by Plakophilin-1 (PKP-1). In our model, we propose that PV IgG induced-desmosomal disassembly occurs in three distinct but overlapping phases where both intracellular signaling and steric hindrance contribute to the loss of desmosomal adhesion caused by PV IgG. Increased expression of PKP-1 prevents desmosome disassembly and prevents the loss of adhesion caused by PV IgG. PKP-1 counteracts internalization and degradation of non-desmosomal desmoglein 3 by forming calcium-independent desmosomes and by promoting desmosome assembly in keratinoctyes treated with PV IgG.

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