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Lipid raft mediation of desmoglein function

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Abstract

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By Joshua D. Lewis

Desmosomes are robust cell-cell adhesion structures which enable tissues to withstand mechanical stress. Desmosomes are abundant in cardiac tissue, in the epidermis, and in various epithelial tissues which must withstand mechanical stress. Desmosomes are dense, protein-based structures which span the plasma membranes of two neighboring cells in order to anchor them together. Many of the basic mechanisms of desmosome assembly and regulation are currently unknown, although recent evidence indicates that specialized membrane microdomains, termed lipid rafts, are required for desmosome assembly. This dissertation explores the importance of lipid rafts for desmosome assembly and function.

Direct cell-cell adhesion is mediated by the desmosomal cadherins. Cadherins are transmembrane proteins which extend into the extracellular space between cells and directly bind to a cadherin from a neighboring cell. There are two families of desmosomal cadherins: desmogleins and desmocollins. The cytoplasmic domain of the cadherin is bound by desmosomal plaque proteins which serve as adaptor molecules to link the cadherin to the cell's keratin cytoskeleton. All of the major desmosomal proteins are associated with lipid rafts, and loss of lipid raft association impairs desmosome function. This dissertation explores a human disease caused by a mutation in a desmoglein, DSG1. The mutation causes a hydrophobic-to-hydrophilic substitution in the DSG1 transmembrane domain, abrogating the protein's lipid raft association. The non-raft mutant is defective in its trafficking through the secretory pathway, with a significant amount of protein retained in the Golgi apparatus. Mutant DSG1 which reaches the cell surface fails to incorporate into desmosomes.

This dissertation also investigates the desmoglein family more broadly, determining which features are and are not important for lipid raft targeting, and the functional consequences to loss of raft targeting. DSG3, which is targeted in the human autoimmune disease Pemphigus Vulgaris, is also investigated.

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Chapter 1

Dissertation Overview

Cell junctions are indispensable for the existence of multicellular organisms. They are critical for intercellular adhesion, organismal development, and intercellular communication. There are several types of cell junctions, and they are distinct in their structure and function. The desmosome is a robust adhesion junction which confers resilience upon tissues. Desmosomes are abundant in the heart, skin, and various epithelia which are subject to mechanical stress. Chapter 2 of this dissertation describes the desmosome, its constituent proteins, and its regulation. This chapter also details the role desmosomal proteins play in intracellular signaling, and diseases which result from disruption of desmosomal adhesion. Also included in this chapter is an introduction to severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome, a genetic disease which compromises the desmosome. A newly discovered instance of SAM syndrome is a major subject of this dissertation.

Desmosome assembly and function depends upon association with lipid rafts, sphingolipid and cholesterol rich microdomains found in cell membranes. Chapter 2 provides an overview of lipid rafts, their properties and lipid constituents, and the common mechanisms responsible for mediating protein targeting to lipid rafts. This chapter also describes the function of lipid rafts in protein trafficking through the secretory pathway and in facilitating segregation and concentration of raft constituent proteins in a variety of biological contexts. This chapter explores the role that lipid rafts play in human disease.

The association between desmosomes and lipid rafts is the final subject covered in chapter 2. The previously published evidence for this association is reviewed here, as are past findings which indicate that lipid raft perturbation, or loss of desmosomal protein association with lipid rafts, inhibits desmosome assembly, function, and disassembly.

Chapter 3 is adapted from Lewis et al, which reports a novel pathomechanism for SAM syndrome. A mutation in the desmosomal cadherin desmoglein 1 prevents its association with lipid rafts. The mutation occurs in the desmoglein 1 transmembrane domain,

rendering the protein defective in its trafficking through the secretory pathway and causing it to accumulate in the Golgi apparatus. The mutant protein is also defective in its ability to incorporate into desmosomes as assessed with super-resolution fluorescence microscopy both *in vitro* and in patient tissue. Finally, this chapter provides evidence that the lengthy desmoglein transmembrane domain is responsible for conferring lipid raft targeting on this family of proteins.

This dissertation is concluded in chapter 4, wherein the significance of the findings described above is discussed. Lipid rafts were previously known to play an important role in protein trafficking, desmosome assembly, immunological synapse formation, and other biological processes. This dissertation advances our understanding by illuminating how desmogleins are targeted to lipid rafts, and by issuing the first report of a human disease caused by a mutation which abrogates a protein's lipid raft targeting. This finding constitutes a novel pathomechanism for human disease. It also raises the possibility that other genetic diseases, particularly those caused by mutations in transmembrane domains and which result in trafficking defects, could also stem from loss of lipid raft association.

Chapter 2

Introduction

2.1 Introduction to desmosomes

Multicellularity is one of the hallmarks of eukaryotic life. Multi-cell organisms cannot exist without intercellular junctions which adhere neighboring cells to one another. One type of intercellular junction is the desmosome. Desmosomes are dense macromolecular structures which are visible using light microscopy. Their discovery is generally attributed to Giulio Bizzozero in 1864[1-3]. Since their discovery, desmosomes have come to be appreciated for their critical role in cell adhesion. Elucidating their regulation and disruption in human disease is an area of active research.

Desmosomes are robust adhesion structures. They are conserved throughout vertebrate animals[4-7]. They can be found in epithelial cells, which line the lumen of many organs, and they are particularly abundant in cardiac and epidermal tissue. The heart and skin are both subject to high levels of mechanical stress, and desmosomes confer upon these tissues the strength to withstand that stress[8]. When desmosome function is compromised, it typically manifests as diseases of the heart and/or skin[9].

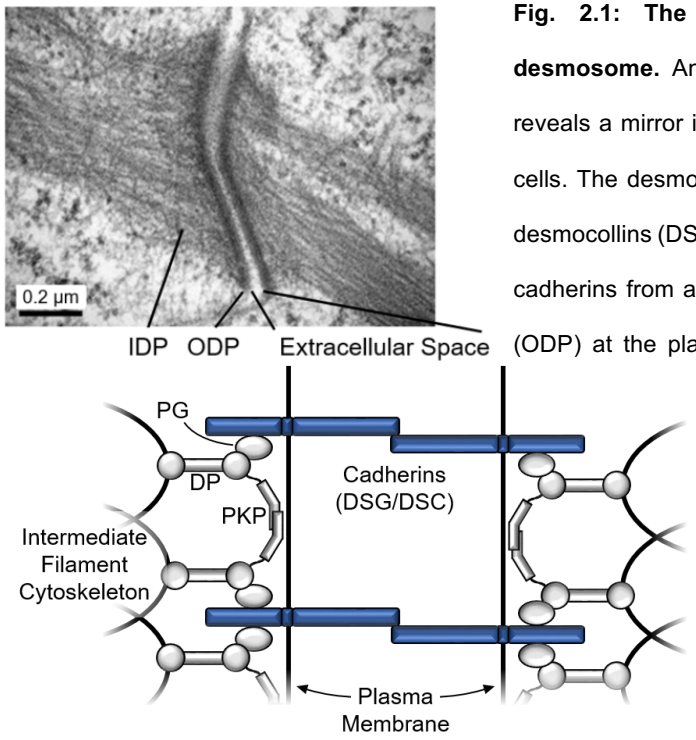

Fig. 2.1: The structure and components of the desmosome. An electron micrograph of the desmosome reveals a mirror image structure at the border between two cells. The desmosomal cadherins, desmogleins (DSG) and desmocollins (DSC), span the plasma membrane and bind to cadherins from a neighboring cell. The outer dense plaque (ODP) at the plasma membrane contains the cytoplasmic domains of DSG and DSC, as well as plakoglobin (PG), plakophilin (PKP), and the amino terminus of desmoplakin (DP). The inner dense plaque is comprised of the carboxy terminus of desmoplakin and of intermediate filaments.

2.2 Molecular components of the desmosome

The first ultrastructural insights into the desmosome stemmed from electron micrographs in 1963[10]. These micrographs revealed a symmetrical structure at the plasma membrane of two neighboring cells (the outer dense plaque) which extended into each cell's cytoplasm (the inner dense plaque) (see Fig. 2.1). Higher resolution structural information was obtained by mapping crystal structures of the classical, non-desmosomal C-cadherin[11] onto cryoelectron tomographs of the desmosome[12]. These data suggested the adhesion between desmosomal cadherins was mediated by insertion of a tryptophan residue from each cadherin into a hydrophobic pocket on its binding partner from the opposing cell, a mechanism shared with the classical cadherins of the adherens junction[12]. Technical improvements in sample preparation would yield further insights into the structure of the desmosome, suggesting that both the extracellular domains of the cadherins[13] and the intracellular plaque proteins[14] are arranged into a quasi-periodic lattice.

Desmosomal cadherins

Cadherins mediate cell-cell adhesion by directly binding to a cadherin from a neighboring cell. There are two families of desmosomal cadherins: desmogleins (DSG1-4) and desmocollins (DSC1-3). Desmosomal cadherins form heterophilic interactions; all DSGs are capable of forming adhesive dimers with all DSCs, but they do not form homophilic dimers[15,16]. Thus, expression of both a DSG and a DSC is required for desmosome formation and function[17,18]. Desmosomal cadherins are type I single-pass transmembrane proteins. Their extracellular domain is composed of four cadherin repeats (EC1-4) and a membrane-proximal extracellular anchor domain[19]. The most distal cadherin repeat, EC1, is directly engaged in trans binding (i.e. adhesion to a cadherin from an opposing cell)[12,13,15]. Like many proteins with an extracellular domain, the desmosomal cadherins are glycosylated[20,21]. A single transmembrane domain (TMD) spans the plasma

membrane. The desmoglein cytoplasmic domain is composed of an intracellular anchor, an intracellular cadherin-typical segment (ICS), a linker domain, a repeated unit domain (RUD), and a terminal domain. The ICS is directly bound by the plaque protein plakoglobin[22], facilitating cadherin binding to the desmosomal plaque. The desmocollins are subject to alternative splicing which can result in inclusion (isoform a) or exclusion (isoform b) of the ICS[23-25]. In desmogleins, evidence suggests that the domains carboxy-terminal of the ICS (i.e. the linker domain, the RUD, and the terminal domain) promote cadherin dimerization in order to inhibit endocytosis[26]. This would be consistent with the finding that the dimerization of the classical cadherins inhibits endocytosis[27].

Plakoglobin

While the desmosomal cadherins span the plasma membrane and directly mediate cell-cell adhesion, anchoring of desmosomes to the intermediate filament cytoskeleton is crucial for the ability of these junctions to bear load[28,29]. Desmoglein and desmocollin linkage to intermediate filaments is mediated by several adaptor proteins, beginning with plakoglobin[30,31]. Plakoglobin is a member of the armadillo family of proteins which are defined by the presence of repeated armadillo domains[32]. Plakoglobin contains 13 armadillo repeats, with both those near the amino- and carboxy-terminal interacting with the cadherin[30,33,34], although there appear to be multiple cadherin binding sites throughout plakoglobin[35]. Facilitating its role in linking the desmosomal cadherins to the intermediate filament cytoskeleton, plakoglobin also binds desmoplakin, which in turn directly binds intermediate filaments. Plakoglobin binding to desmoplakin occurs in the plakoglobin central armadillo domain[31].

In addition to its structural role within the desmosome, plakoglobin also localizes to other subcellular compartments. Plakoglobin is homologous to the adherens junction protein β-catenin and can bind E-cadherin and localize to adherens junctions[36,37]. However, plakoglobin binds the desmosomal cadherins with much greater affinity[35].

Plakoglobin is post-translationally modified by palmitoylation, the covalent attachment of the lipid palmitic acid, which is typically found on membrane bound proteins. This suggests that plakoglobin may directly contact the plasma membrane (and other cell membranes)[38,39]. However, plakoglobin is not constitutively membrane-bound. Rather, plakoglobin also localizes to the nucleus and modulates Wnt signaling and facilitates tumor suppression (see section 2.4)[40-42].

Desmoplakin

Desmoplakin completes the chain of desmosomal proteins anchoring the adhesive cadherins to the cytoskeleton by directly binding to intermediate filaments. Desmoplakin is a member of the plakin family of proteins, which are large adaptors that link desmosomes and hemidesmosomes to the cytoskeleton[43]. Desmoplakin is composed of a globular amino-terminal head domain, a coiled-coil rod domain, and a carboxyl-terminal tail domain. The desmoplakin head domain binds to plakoglobin[31], and its carboxyl-terminal domain binds to keratin filaments[44,45], mediating linkage between the desmosome and the cell's cytoskeleton. Phosphorylation of the desmoplakin carboxyl-terminal domain serves as a switch for binding and releasing from intermediate filaments, which is critical during desmosome assembly[46,47]. Alternative splicing yields two desmoplakin isoforms, I and II, with the latter having a shortened rod domain[48].

Plakophilin

Like plakoglobin, plakophilins are members of the armadillo family of proteins. Plakophilins possess 9 armadillo domains[49]. Three separate plakophilin paralogs are encoded in the human genome (PKP1-3), and PKP1 and 2 are alternatively spliced to produce a shorter a isoform and a longer b isoform[50,51]. Through their amino-terminal head domain, the plakophilins bind many desmosomal proteins, including DSG1-3, DSC1a/2a/3, plakoglobin, and desmoplakin, as well as keratin and actin (reviewed by Hatzfeld[52]). The binding of PKP to plakoglobin mediates clustering of desmosomal

proteins to facilitate desmosome assembly[31]. PKP1 and PKP2 also localize to the nucleus and interact with RNA polymerase III[50,51,53]. Unlike the signaling-dependent nuclear recruitment of the armadillo family members β-catenin and plakoglobin, PKP nuclear localization is constitutive, with the protein apparently serving as an integral component of the RNA polymerase III holoenzyme in interchromatin spaces of the nucleoplasm, suggesting the PKP-containing complex is not engaged in transcription, but instead represents an idle or pre-initiation form of the polymerase[53].

Other desmosomal proteins

In addition to the principal constituents of the desmosome described above, a number of other proteins are associated with this structure. These include perp, a four pass transmembrane protein expressed in stratified epithelia which localizes to the desmosome[54]. Perp is an effector of the transcription factor p53/p63, a master regulator of stratified epithelial development[54,55]. An armadillo family protein, p0071, is sometimes referred to as plakophilin 4 and may be present in desmosomes, although this has been the subject of debate[56,57]. While desmoplakin is thought to be the primary adaptor linking desmosomes to intermediate filaments, a number of other intermediate filament-binding proteins are present in desmosomes under various conditions. The keratin binding protein pinin localizes to mature, but not nascent desmosomes, and is thought to strengthen adhesion[58,59]. In polarized epithelia, the intermediate filament-binding protein plectin is localized to the cell periphery and binds desmoplakin[60]. Another such protein is IFAP 300, which binds vimentin intermediate filaments and localizes to desmosomes[61]. Desmocalmin and keratocalmin both localize to the desmosome and bind calmodulin, an effector of intracellular calcium signaling[62,63].

Several proteins are involved in the maturation of desmosomes into corneodesmosomes during terminal keratinocyte differentiation. Corneodesmosin is a glycoprotein secreted in the uppermost layers of the epidermis[64]. It is covalently bonded

to the cornified envelope of corneocytes and serves as an adhesion molecule[65]. Envoplakin and periplakin, members of the plakin family, are also involved in the formation of corneodesmosomes and are thought to reinforce linkage to the intermediate filament cytoskeleton[66-68].

2.3 Regulation of the desmosome

Little is known about the regulation of desmosome assembly, size, and disassembly. As mentioned above, the transcription factor p63 is a master regulator of stratified epithelial development[54,55]. One of its effectors, Perp, localizes to desmosomes, and genetic ablation of Perp in mice compromises cell-cell adhesion, causing blistering and postnatal lethality[54]. Desmosome assembly is also contingent upon the formation of adherens junctions. Mouse keratinocytes which are null for E-cadherin and P-cadherin lack adherens junctions and also fail to assemble desmosomes[69]. In fact, desmosome assembly may be nucleated by classical cadherins, which are then excluded as the desmosome matures (Shafraz & Sivasankar, personal communication).

Desmosome assembly requires the translocation of intracellular pools of desmosomal proteins to the plasma membrane. Cadherin-plakoglobin complexes are delivered to nascent desmosomes through microtubule-associated secretory vesicles[70,71]. Prior to the assembly of desmosomes, desmoplakin and plakophilin reside in keratin-filament-associated cytoplasmic granules[47]. Upon desmosome assembly, plakophilins recruit PKCα to cytoplasmic granules containing desmoplakin[72]. This recruitment allows PKCα to phosphorylate desmoplakin, releasing it from cytoplasmic keratin filaments and allowing it to translocate to the desmosome[72].

One facet of desmosome regulation which remains unexplained is how the size of the desmosome is governed. On a molecular level, the desmosome is a large array of repeated protein subunits[13,14]. There is no inherently obvious mechanism dictating the

number of subunits included in the desmosome. Yet desmosomes are consistent in size, varying between 0.2 – 0.5 μm in diameter[73-76]. The size of desmosomes varies some depending on the tissue or sub-compartment of a tissue in which they are found, with smaller desmosomes in the stratum basale and larger desmosomes in the stratum granulosum[76]. Human patients with mutations in plakophilin 1 have smaller than normal desmosomes in suprabasal keratinocytes[77], and conversely, exogenous expression of plakophilin 1 in cultured cells increases desmosome plaque size dramatically, indicating that the isoforms of desmosomal proteins present within a desmosome may regulate its size[78]. These data notwithstanding, the means by which uniformity of desmosome size is enforced remains unknown.

The disassembly of desmosomes is also poorly understood. Turnover of desmosomal proteins from junctions is quite slow; the half-lives of detergent-insoluble desmoglein 1 and desmoplakin are greater than 24 and 72 hours, respectively[71]. Internalization of the desmosomal cadherins is accelerated via proteolytic cleavage by matrix metalloproteases. This process appears to be EGFR-mediated, as inhibition of EGFR blocks DSG2 ectodomain cleavage and intracellular accumulation[78,79]. In certain disease contexts, the binding of auto-antibodies triggers rapid cadherin internalization[80]. In the context of wound healing, desmosomal adhesion is downregulated in response to recruitment and activation of PKCα to the wound edge[81]. Other reports have disputed whether desmosomes are ever disassembled; in some cases, half desmosomes or entire desmosomes have been observed inside of cells by electron microscopy, suggesting they were internalized without being decomposed into smaller subunits[82-84]. In summary, the regulation of desmosomes is largely unexplored frontier, with fundamental concepts such as the mechanisms governing desmosome assembly, disassembly, and size poorly understood. This dissertation addresses this gap by identifying factors which regulate the

secretory trafficking and desmosome incorporation of the desmoglein family of desmosomal cadherins (see chapter 3).

2.4 Desmosome involvement in intracellular signaling

While the desmosome is principally a cell-cell adhesion structure, its protein components are also involved in intracellular signaling events. As described above, plakoglobin and plakophilin can enter the nucleus and interact with transcriptional machinery. Like β-catenin, plakoglobin binds to the Wnt signaling pathway transcription factor TCF/LEF[42,85]. Wnt signaling is a critical developmental pathway conserved throughout Animalia[86], and loss of β-catenin causes embryonic lethality in mice[87]. However, the role plakoglobin plays in this pathway may only be to elevate endogenous levels of β-catenin[88], and it cannot rescue β-catenin-null mice during embryonic development[87]. Plakoglobin also acts as a tumor suppressor, binding to promotors which are targeted by the tumor suppressor p53. Plakoglobin/p53 binding increases expression of the tumor suppressor 14-3-3σ[89] and decreases expression of the tumor promoter SATB1[90]. Plakophilin involvement in intracellular signaling has been less thoroughly explored. It can bind single-stranded DNA[91], and as mentioned above, interacts with RNA polymerase III[50,51,53]. In the cytoplasm, plakophilins interact with RNA and RNA-processing machinery and is present in stress granules[92].

Within the epidermis, keratinocytes terminally differentiate as they progress from the stratum basale to the stratum corneum. Differentiation is programmed by an isoform switch from expression of DSG2/3 to DSG1[93]. DSG1 expression suppresses signaling from epidermal growth factor receptor (EGFR), a key step in differentiation[94]. Loss of DSG1 expression in a tissue culture model of the epidermis prevents the morphological and protein expression changes associated with normal differentiation, such as expression of filaggrin, loricrin, and the isoform switch from keratin 14 to keratin 10[94].

DSG1 expression also promotes differentiation by suppressing MAPK/ERK signaling[95]. By binding to the ERK-regulatory protein Erbin, DSG1 promotes Erbin interaction with SHOC2, a Ras/Raf scaffolding protein in the ERK pathway. DSG1-Erbin binding inhibits Ras-SHOC2 interaction, resulting in decreased MAPK/ERK signaling. Neither of these signaling functions require the adhesive ectodomain of DSG1[94,95]. The central role played by DSG1 in the terminal differentiation program of keratinocytes underscores the importance of the desmosome in vertebrate animals.

2.5 Diseases of the desmosome

Genetic diseases of the desmosome

Mutations in desmosomal proteins typically manifest as diseases of the skin and/or heart, organs in which desmosome adhesion is crucial to withstand mechanical stress[8]. The organ or organs affected in these diseases reflects the tissues in which the mutated protein is expressed. For example, cardiomyopathies are diseases in which the heart becomes enlarged or rigid. They can be caused by mutations in DSG2, DSC2, PKP2, and desmoplakin, all of which are expressed in cardiac tissue[9]. A variety of skin diseases are caused by mutations in desmosomal proteins. Striate palmoplantar keratoderma presents as aberrant thickening of the skin covering the palms and the soles of feet. It is caused by mutations in DSG1, desmoplakin, or keratin 1[9]. Epidermolysis bullosa is skin fragility disease in which patients are prone to blistering. It is caused by mutations in plakoglobin or desmoplakin. Mutations in desmosomal proteins can also manifest as hair defects, including wooly hair caused by mutations in DSC2 or desmoplakin and hair loss (hypotrichosis) caused by mutations in DSG4[96].

Sometimes mutations in desmosomal proteins cause syndromes with more complicated clinical presentations. In Naxos disease, homozygous plakoglobin mutations cause diffuse palmoplantar keratoderma, cardiomyopathy, and woolly hair[9]. The array of phenotypes which manifest in Naxos reflect the variety of tissues in which desmosomes

play a critical role in development and tissue strength; skin, heart, and hair are all organs in which desmosomes are abundant and indispensable.

Another recently discovered disease is SAM syndrome, or severe dermatitis, multiple allergies, and metabolic wasting. SAM syndrome patients have compromised barrier function in their skin, leading to multiple allergies and rendering them vulnerable to repeated infection[97]. This causes metabolic wasting, and most affected individuals die in early childhood. SAM syndrome is caused by mutations in DSG1[97-99] or desmoplakin[100]. A novel case of SAM syndrome has afforded new insight into desmosome biology and is the focus of chapter 3 of this dissertation.

Treatments for patients afflicted with genetic diseases compromising the desmosome are generally lacking, whereas autoimmune diseases are somewhat more tractable, with efficacious clinical interventions available[101].

Autoimmune diseases of the desmosome

In contrast to genetic mutations in proteins of the desmosome which give rise to congenital diseases, autoimmune diseases develop when a patient's immune system targets one or more desmosomal proteins. The pemphigus family of skin blistering diseases were named by Boissier de Sauvages[102]. Translating from Latin as "pustule," pemphigus is caused by circulating IgG autoantibodies which bind to desmosomal cadherins, disrupting desmosome adhesion. Different target proteins lead to varying degrees of severity. Pemphigus vulgaris is primarily caused by antibodies targeting DSG3, which is the predominant desmoglein isoform in the basal layer of the epidermis[103] This causes severe blistering and is lethal if it is not treated with immune suppression. In pemphigus foliaceus, antibodies target DSG1, the expression of which is limited to the upper layers of the epidermis[104]. This gives rise to a milder blistering phenotype than pemphigus vulgaris. Paraneoplastic pemphigus is the most severe disease in this family, and it is typically associated with lymphoproliferative disorders such as non-Hodgkin's

lymphoma[105,106]. Patient autoantibodies target multiple desmosomal proteins, including DSGs, DSCs, plakophilins, and desmoplakin. It is rarer and more severe than pemphigus vulgaris or foliaceus, with painful oral lesions and inflammation of the mouth and lips. Even with treatment, paraneoplastic pemphigus is almost always fatal due to sepsis, gastrointestinal bleeding, multiorgan failure, and respiratory failure[107]. Finally, IgA pemphigus is a rare and relatively mild form of pemphigus targeting DSC1 or DSG1 and DSG3[108,109]. We recently reported that some of the consequences of pemphigus vulgaris can be blocked by disrupting lipid rafts[110,111], an observation which laid the foundation for the research described in chapter 3.

Immune suppression is a highly effective clinical intervention for patients suffering from pemphigus. Corticosteroids are the most widely used treatment for pemphigus, often in combination with other immunosuppressive agents[112]. Retuximab, a monoclonal antibody targeting $CD20^+$ B-cells, is also highly efficacious[113] and may overtake corticosteroids as the predominant treatment for pemphigus[114]. Retuximab treatment depletes patient B-cells, halting the production of pathogenic autoantibodies[113]. A drawback of this approach is that non-pathogenic B-cells are also destroyed, partially compromising the patient's immune system. An emerging alternative to indiscriminate B-cell depletion uses gene therapy to target only pathogenic B-cells. By introducing chimeric antigen receptors into patient T-cells, a patient's immune system can be directed against only those B-cells which are secreting pathogenic autoantibodies[115]. In contrast to genetic diseases of the desmosome, the treatments available for autoimmune diseases of the desmosome are generally efficacious. Treatment of infectious diseases which compromise the desmosome is still more straightforward.

Infectious diseases of the desmosome

Several pathogens target desmosomal proteins. DSG1 is targeted for cleavage by *Staphylococcus aureus* via secretion of exfoliative toxin A (ETA). This causes the

blistering disease bullous impetigo, in which large, flaccid bullae develop on the skin at the site of the infection[116]. In infants and immune-compromised adults, *S. aureus* infection can be extensive, leading to systemic release of toxin. This causes a more severe clinical presentation known as staphylococcal scalded skin syndrome[117], in which large areas of the skin are involved and symptoms also include fever and malaise[118]. For both bullous impetigo and staphylococcal scalded skin syndrome, treatment of the causative infection with antibiotics is effective[119], however the mortality rate is substantially greater for immune-compromised adults[118].

The desmosomal cadherin DSG2 is a receptor for certain adenoviruses that infect respiratory and urinary epithelia[120]. Adenovirion binding to DSG2 in epithelial cells triggers protein expression changes suggestive of epithelial-to-mesenchymal transition, opening intercellular junctions[120]. Clinically, adenoviruses typically cause mild infections of the respiratory or gastrointestinal tracts, or conjunctiva[121]. While the vast majority of individuals recover from these infections, they can be deadly for immune-compromised patients, and there is currently no Federal Drug Administration-approved treatment for adenoviral infection[121]. Because of their recognition of DSG2 and other epithelial-specific proteins, adenoviruses are also potential vectors in gene therapy treatments for cancers of epithelial origin[120,122].

The array of human diseases which arise from the loss of desmosome function reveals the indispensability of these adhesion structures. While genetic, autoimmune, and infectious diseases affecting the desmosome vary in terms of severity and tractability, elucidating their pathogenesis often yields insights into the fundamental biology of the desmosome. The central focus of this dissertation is an unusual disease-causing mutation which highlights an emerging concept in desmosome biology: the dependence of desmosomes on lipid rafts.

2.6 Introduction to lipid rafts

As mentioned in section 2.5, the body of work presented in this dissertation grew from the observation that disruption of lipid rafts is protective against desmosome disruption in pemphigus vulgaris. The association between desmosomes and lipid rafts is a central theme of this document. Conceptually, lipid rafts can be thought of as a means of achieving compartmentalization in the cell. Compartmentalization is a hallmark of eukaryotic cells, where cellular functions are often carried out in specialized organelles. For example, many proteins are synthesized in the endoplasmic reticulum, ATP is mainly produced in mitochondria, and protein degradation is performed in lysosomes. Compartmentalization increases the efficiency of these processes by bringing together all the necessary machinery into one location. Lipid rafts are another means by which cells achieve spatial segregation, in this case within the two-dimensional plane of a membrane.

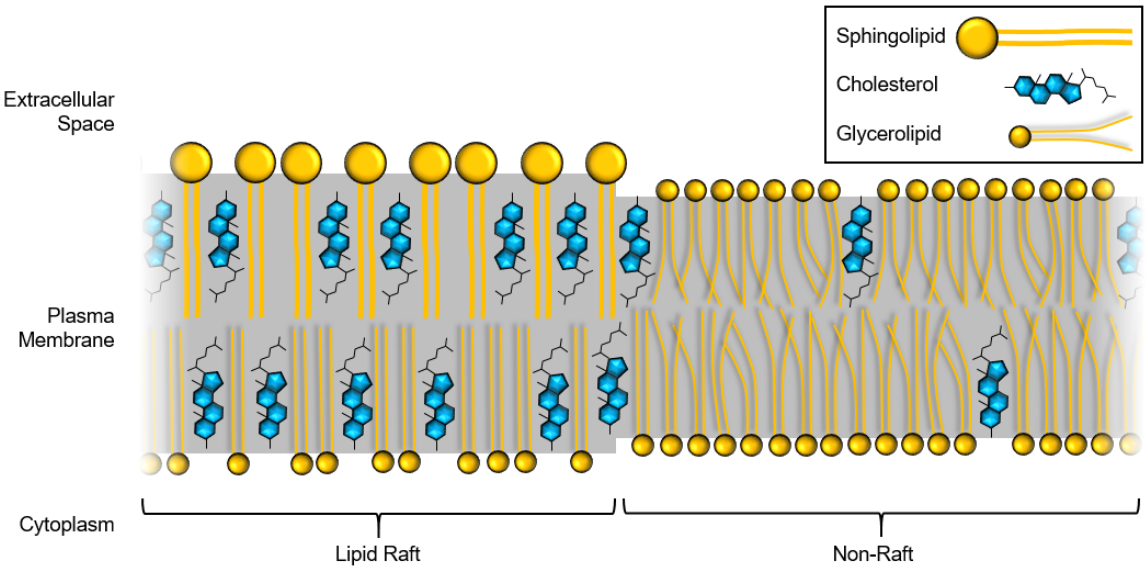

Fig. 2.2: Lipid rafts are sphingolipid- and cholesterol rich membrane microdomains. The membrane is asymmetric, with sphingolipid predominantly in the outer leaflet. Lipid rafts are dominated by lipids with long, saturated hydrophobic chains, and these domains tend to be more orderly in their packing than non-raft membrane. Lipid rafts are also substantially thicker (up to 5.6 nm) than non-raft membranes (3.5 nm). Lipid rafts facilitate tight packing of membrane proteins in a variety of biological contexts.

Cell membranes are composed of a great variety of lipids. These lipids tend to spontaneously self-segregate based on their physical properties, forming lipid raft microdomains which are enriched for long, saturated side chains, and non-raft domains in which lipids with unsaturated side chains are abundant. Lipid rafts play important roles in a variety of cellular processes, including intracellular signaling[123], protein trafficking through the secretory pathway[124,125], and protein clustering at the plasma membrane[110,126-128].

2.7 Lipid diversity in cell membranes

Lipid diversity is important for cell membranes because changing the lipid composition of a membrane can alter its properties. Membrane fluidity and permeability are heavily influenced by lipid composition[129]. Cell membranes contain a substantial diversity of lipids, with thousands of species present. Cells facing the intestinal lumen, for example, have greater than average concentrations of sphingolipids, which are less fluid and less permeable, helping these cells to withstand their harsh environment[130]. Changing lipid composition can alter local membrane architecture, promoting deformations (evidence reviewed by McMahon & Gallop 16319878). For example, lysophosphatidic acid promotes positive membrane curvature, while phosphatidic acid promotes negative curvature[131]. Lipids themselves can serve as signaling molecules. A classic example of this is the cleavage of phosphatidylinositol 4,5-bisphosphate into the secondary messengers inositol trisphosphate and diacylglycerol (initial observations made by [132-134], reviewed by [135]). Sphingosine and ceramide, lipids found in raft microdomains, are also signaling molecules[136,137]. As described above, lipid diversity also facilitates compartmentalization within the two-dimensional plane of a membrane by segregating into raft and non-raft domains.

Most broadly, lipids are categorized as glycerolipids, sphingolipids, and sterols, with the latter two being the principle components of lipid rafts (see Fig. 2.2). Glycerolipids are

composed of a head group, a glycerol, and two fatty acids. Glycerolipid head groups include phosphorylcholine, phosphorylethanolamine, phosphorylinositol, and others. Glycerolipids have two fatty acid side chains which come in different lengths and contain varying numbers of carbon-carbon double bonds. Further adding to glycerolipid diversity, fatty acid side chains can be covalently attached to glycerol using several different kinds of linkages (ester, alkyl ether, or alkenyl ether).

Sphingolipids are also diverse. Unlike glycerolipids, they are ceramide-based. Sphingolipids possess a sphingosine backbone with a fatty acid amide-bonded to the sphingosine. As with glycerolipids, there are many head groups, including phosphorylcholine and numerous glycans[138].

Sterols are four-ring, isoprenoid-based hydrocarbons. The primary animal sterol is cholesterol (major discoveries reviewed by Olson[139]). The hydrocarbon rings of cholesterol are hydrophobic, while its single hydroxyl group is hydrophilic. Cholesterol promotes orderly packing of fatty acid side chains, making membranes thicker and more impermeable. Cholesterol preferentially associates with saturated hydrocarbon tails and is a major constituent of lipid raft domains. Cholesterol is synthesized in the endoplasmic reticulum, but its concentration in this organelle is quite low[140,141]. Its concentration increases within the secretory pathway, peaking at the plasma membrane. In mammalian cells, approximately 30% of plasma membrane lipids are cholesterol[142-146].

2.8 Characteristics of lipid rafts

In a cell-free context, lipids in a membrane will spontaneously segregate into large raft and non-raft domains[147]. Raft domains are enriched for sphingolipids and cholesterol, and the fatty acid side chains in rafts tend to be saturated. These lipids preferentially interact with one another, excluding the kinked fatty acid chains present in unsaturated lipids. Their orderly, fully extended fatty acid tails also make lipid rafts substantially thicker than non-

raft membranes, and membrane spanning proteins can effect even greater membrane thickness[148]. Measurements of membrane thickness have been generated from supported monolayers and bilayers of purified lipids using a variety of methodologies (see examples in Table 1, below). A consensus estimate is that non-raft domains are 3.5 nm (35Å) thick, whereas lipid rafts may achieve up to 5.6 nm (56Å) thickness depending on the length of transmembrane peptides present[148-151].

Ref.	Methodology	Membrane thickness reported
[148]	Circular dichroism and x-ray diffraction on oriented multilayers composed of various lipids	30.8Å – 35.3Å (Hydrophobic region is 20.8Å – 25.3Å) TMD can thicken bilayer by 1.3Å
[149]	Nuclear magnetic resonance on supported bilayers composed of phosphatidylcholine with or without cholesterol	35.8Å without cholesterol 39.9Å with 30% cholesterol (Hydrophobic region is 25.8Å - 29.9Å thick without & with cholesterol, respectively)
[150]	Atomic force microscopy and near field scanning optical microscopy on monolayers composed of dipalmitoylphosphatidylcholine and cholesterol	Up to 14Å difference between domain thicknesses (0.7 nm difference between liquid crystal and liquid expanded domains of a monolayer)
[151]	Atomic force microscopy on supported bilayers of sphingomyelin, phosphatidyl-choline, and cholesterol	10Å – 13Å difference between domain thicknesses

Table 2.1: Published estimates of membrane thickness

Rafts in living cells are smaller than those observed in cell-free membranes, ranging from 10-200 nm in diameter[152]. One conceptual explanation for this discrepancy lies in the picket fence model, wherein cytoskeleton-anchored transmembrane proteins obstruct the diffusion of lipids and constrain the size of membrane domains in living cells[153]. Under certain conditions, however, rafts can be aggregated into far larger domains, as occurs in the formation of the immunological synapse[126], and in response to antibody- or toxin-mediated clustering of raft-residing proteins[154].

Another noteworthy property of lipid rafts is the asymmetry of the lipid bilayer. Sphingolipids are heavily concentrated in the exoplasmic leaflet (the surface of the membrane facing away from the cell)[155]. Bilayer asymmetry is generated by flippases and floppases, which transport specific lipids to the cytoplasmic and exoplasmic leaflets, respectively[156-159]. The scrambling of bilayer asymmetry is an important step in myoblast fusion[160-164], oocyte fertilization[165-167], extracellular vesicle budding[168-172], and other biological processes (reviewed by Whitlock and Hartzell[173]). Finally, lipid rafts in cells are not monolithic. Most broadly, they are divided into caveolar and non-caveolar rafts, with the former being defined by the presence of caveolin, a membrane-deforming protein which mediates endocytosis[174,175]. More nuanced distinctions can also be drawn among raft compartments, as various protein markers of lipid rafts do not perfectly colocalize in cells, revealing further heterogeneity[176].

The properties of lipid rafts lend themselves to detection with a number of assays. One widely used technique is the biochemical isolation of detergent resistant membranes[177]. The properties of lipid rafts make them more resistant to detergent extraction than non-raft membranes. Detergent extraction, particularly extraction with Triton X-100 at 4°C, solubilizes most cell membranes while preserving sphingolipid- and cholesterol-enriched membranes. Surviving membranes can then be isolated via sucrose gradient fractionation[177]. This approach is typically coupled with western blotting to determine

whether a protein of interest was present in detergent resistant membranes, which is indicative of lipid raft association. One pitfall of this method is that different detergent extraction methods yield contradictory answers about whether a given protein is raft-associated[178]. More recently, other techniques have been used as an alternative to this approach (reviewed by Simons & Gerl[179]). Although the size of most lipid rafts is below the diffraction limit of conventional fluorescence microscopy, super-resolution fluorescence microscopy can be used to investigate colocalization between a protein of interest and a marker of lipid rafts. For example, cholera toxin B binds to GM1 glycosphingolipid and is widely used to label raft domains[126,180]. Fluorescently labeled lipid dyes can also be used to specifically label either raft or non-raft domains, depending on the lipid[181]. One of the shortcomings of this technique is that a given raft marker, particularly a lipid raft associated protein, will not be present in all rafts.

Another approach to investigating lipid rafts involves the isolation of giant plasma membrane vesicles (GPMV) from cultured cells[182-185]. GPMVs lack cytoskeletal support which would ordinarily limit lipid diffusion, allowing whole-sale phase separation of lipids into vast raft and non-raft domains. The partitioning of a fluorescently-tagged protein of interest can be measured using fluorescent dyes which preferentially label raft or non-raft domains[186]. Unfortunately, none of these approaches can be used to visualize native lipid rafts in living cells. Given their small size and temporal transience, this goal remains out of reach with current technology.

2.9 Membrane proteins and lipid rafts

According to computational predictions, 15% - 39% of the human proteome consists of integral membrane proteins[187], with many more proteins spending part of their lives on a membrane. There are several mechanisms which drive protein association with membranes. Transmembrane proteins possess one or more domains containing

hydrophobic residues which span the lipid bilayer. During synthesis, these proteins typically insert into the endoplasmic reticulum membrane, and the hydrophobicity of their transmembrane domain(s) (TMD) keeps them anchored in cell membranes as they are trafficked throughout the cell. Some proteins, notably members of the Ras superfamily of small GTPases, are post-translationally modified with lipids to promote membrane association (reviewed by Magee & Newman[188]). Some lipid modifications are insufficient to confer membrane localization and must be coupled with other mechanisms. For example, positively charged residues on a protein's surface can promote membrane association by interacting with the negatively charged head groups of phospholipids[189].

Like lipids, integral membrane proteins will preferentially partition into raft or non-raft domains depending on their properties. In a forthcoming publication, Lorent & Levental describe a tripartite system for predicting the raft affinity of transmembrane proteins. The three properties which they identify as important for conferring lipid raft association are TMD length, TMD surface area, and palmitoylation (Levental, personal communications). TMD length is likely important in mediating lipid raft association because lipid rafts are thicker than non-raft membranes, and spanning these thicker microdomains would require a longer series of hydrophobic residues[186]. This dissertation provides additional examples of this phenomenon (see Chapter 3). Experimental and computational data indicate that surface area of the amino acid side chains found in TMDs are inversely related to lipid raft affinity (Levental, personal communications). This phenomenon is probably due to the fact that amino acids with large steric size have greater surface tension in raft domains than in a non-raft environment. Palmitoylation, a post-translational modification in which the lipid palmitate is covalently bonded to a protein, has been previously identified as a lipid raft targeting mechanism[190,191]. Although not all palmitoylated proteins partition to lipid rafts, this modification is necessary for the raft targeting of the majority of integral raft proteins[192], including several junctional proteins[39,193,194]. Glycosylphosphatidylinositol (GPI) anchored

proteins reliably partition to lipid rafts[195]. This lipid modification is always added the C-terminus of the target protein, with the GPI anchor inserted into the exoplasmic leaflet of the plasma membrane.

2.10 Lipid raft involvement in secretory pathway trafficking

Conceptually, lipid rafts were conceived to explain why polarized epithelial cells have glycolipid-rich apical membranes[146,155]. Later, it became clear that proteins bearing a glycosylphophatidylinositol (GPI) anchor were preferentially associated with these domains[195-197]. This association occurs after transport to the Golgi apparatus but prior to delivery to the plasma membrane, supporting the model of lipid raft-mediated transport of proteins[198]. Bretscher & Munro proposed a model in which proteins were either retained early in the secretory pathway or trafficked to the plasma membrane as a function of their ability to associate with sphingolipid- and cholesterol-rich membranes[124]. They noted that Golgi-resident proteins had shorter TMDs than those residing on the plasma membrane (averaging 15 and 20 residues, respectively). This trend correlates with the concentrations of sphingolipids and cholesterol in these membranes, which determine membrane thickness. Bretscher & Munro reasoned that proteins with shorter TMDs would be unable to span the thicker membranes found in later compartments and would therefore be retained in the Golgi.

Subsequent research supported this model. Vesicles departing the Golgi apparatus which are destined to the plasma membrane can be isolated by targeting vesicles carrying a plasma membrane resident protein[199]. When the lipid content of these vesicles was analyzed, they were found to be enriched for sphingolipids and cholesterol[199]. The concentration of lipid raft constituents in the plasma membrane is, therefore, partially explained by their preferential inclusion in vesicles departing the Golgi apparatus en route to the plasma membrane. When a Golgi-resident protein is mutated to lengthen its TMD,

the protein mislocalizes to the plasma membrane[125]. This finding provides further evidence that TMD length regulates inclusion into the sphingolipid- and cholesterol-enriched secretory vesicles destined for the plasma membrane. Finally, the lipid raft partitioning of Linker for Activation of T-cells (LAT) is also necessary and sufficient for trafficking to the plasma membrane[186], which is also consistent with this model. This model is further supported by and provides a conceptual framework for understanding the data presented in Chapter 3, wherein TMD shortening of the desmosomal cadherins results in loss of lipid raft partitioning and a protein trafficking defect characterized by retention in the Golgi apparatus.

2.11 Platform for protein segregation and concentration

Several fields have converged on a model in which lipid raft association is necessary for the clustering of membrane proteins to facilitate a physiological process[200]. The saturated hydrocarbon chains which dominate lipid rafts tend to pack tightly and orderly, ostensibly permitting tighter packing of transmembrane domains. One example of this is the formation of the immunological synapse. When a T-cell receptor (TCR) binds to the major histocompatibility complex (MHC) of an antigen presenting cell, a signaling cascade results in T-cell activation[201] (activation reviewed by Smith-Garvin et al.[202]). Numerous copies of these proteins are recruited to the contact site between the cells, forming the immunological synapse. This process is facilitated by costimulation of coreceptors, including CD28 and CD3[126]. These coreceptors are lipid raft associated[126], as are many TCR effectors, including Fyn[203], Lck[203], LAT[186,204], Ras[205], and GTP-binding proteins[206]. Stimulation of CD28 and CD3 by antibody coated beads causes the coalescence of large lipid rafts around the beads. Conversely, artificial clustering of lipid raft domains promotes TCR signaling, but only when CD28 and CD3 are recruited to these rafts using antibodies[126]. These data demonstrate that lipid rafts promote TCR signaling response,

apparently by serving as a platform for the concentrating the protein components of the TCR signaling pathway.

Similar to T-cell activation, immunoglobulin E (IgE) receptor signal transduction may be facilitated by the coalescence of lipid raft microdomains (evidence reviewed by Sheets et al.[127]). In its basal state, the IgE receptor molecule FcεRI has a weak association with lipid rafts[207,208]. Antigen binding causes FcεRI cross-linking and the coalescence of large membrane domains containing markers of lipid rafts. Loss of lipid raft association prevents FcεRI phosphorylation, an important step in signal transduction[209]. Thus IgE signal transduction constitutes another example in which protein clustering and lipid raft microdomain consolidation are correlated, and loss of raft association inhibits protein function.

Lipid rafts have also been implicated in viral replication and virion budding (reviewed by Veit & Thaa[128]). Both the influenza glycoproteins, hemagglutinin and neuraminidase, are associated with lipid rafts[190,210,211]. Hemagglutinin partitioning to rafts is mediated by palmitoylation three of its cysteine residues[190]. Interestingly, loss of lipid raft partitioning impairs hemagglutinin trafficking through the secretory pathway[212]. Influenza virion budding from the plasma membrane requires the concentration of viral proteins and ribonucleoprotein particles (RNPs) at a nascent bud site. Hemagglutinin and neuraminidase recruit matrix protein M1 to lipid rafts[213], and other viral proteins may also accumulate in lipid rafts[214,215]. Disruption of lipid rafts via cholesterol depletion prevents accumulation of viral RNPs at the apical membrane, where viral budding occurs[215]. These data are consistent with a mechanism of virion budding in which lipid rafts serve as a platform for the clustering of viral proteins and RNPs to facilitate viral bud formation.

In each of these examples, lipid rafts play a role in the clustering of membrane proteins, often via the coalescence of small raft domains into large ones. As discussed in

Chapters 3 and 4, we hypothesize that an analogous process occurs in the assembly of the desmosome.

2.12 Lipid rafts and human disease

Lipid rafts have a well-established role in signaling, and there are many cases in which disease-associated signaling pathways have components in lipid rafts. These have been recently reviewed[216,217]. Links between cardiovascular disease and lipid rafts form one area of active research. In vascular smooth muscle cells, the binding of angiotensin II to its eponymous receptor causes the receptor to partition to lipid rafts[218]. This pathway causes vasoconstriction and has been linked to hypertension. Likewise, several potassium channels whose disruption causes hypertension, ischemia, and heart failure are also associated with lipid rafts[219-221]. Caveolin-3, a muscle-specific caveolin isoform, is upregulated in a cell culture model of cardiac hypertrophy[222]. Interestingly, mutations in caveolin-3 also cause autosomal dominant limb-girdle muscular dystrophy[223]. Macrophage clearance of oxidized LDL-cholesterol is important in preventing atherosclerosis. CD36, a receptor for oxidized LDL-cholesterol, is associated with lipid rafts[224].

Lipid rafts and their constituents are also involved in tumorigenesis pathways. Several studies indicate that caveolin-1 is a breast cancer suppressor[225-228], although its expression is up-regulated in colon cancer[229,230]. Several proteins in apoptotic signaling pathways are associated with lipid rafts, including ROCK[231,232] and caspase-3[233]. Sphingomylein synthesis promotes apoptosis through Fas signaling[234]. Though these examples illustrate that perturbation in raft components is correlated with tumorigenesis, they fall short of demonstrating a causal relationship.

Lipid rafts are also implicated in neurological disorders and diseases of the immune system. As described above, IgE-mediated allergic response occurs when FcεRI binds

antigen, resulting in receptor cross linking and increased lipid raft partitioning. Uncontrolled signaling causes Quinke edema and allergic shock[217]. High blood levels of cholesterol and LDL-cholesterol are a risk factor for Alzheimer disease[235], whereas cholesterol depletion inhibits pathogenic Aβ plaque formation[236]. Prion protein (PrP), the protein which misfolds and causes human prion disease, is GPI-anchored and resides in lipid rafts[237,238]. Lipid raft disruption via cholesterol depletion inhibits generation of the pathogenic version of the protein, demonstrating the importance of raft domains in prion protein function and pathology.

To date, the most direct link between lipid rafts and a human disease can perhaps be found in a Fc receptor polymorphism associated with systemic lupus erythematosus. The mutation, an isoleucine-to-threonine substitution, prevents FcγRIIb from associating with lipid rafts and is thought to cause unopposed pro-inflammatory signaling[216,239,240]. However, this polymorphism is present in many individuals who are not afflicted with the disease. Overall, previous attempts to link lipid rafts with human disease have been indirect. Chapter 3 of this dissertation describes the first reported case in which a genetic disease has been caused by a mutation which abrogates the lipid raft partitioning of a protein.

2.13 Introduction to the role of lipid rafts in desmosome assembly, function, and disassembly

Since the earliest biochemical isolation of desmosomes, evidence has been accumulating that they are associated with lipid rafts. Skerrow & Matolsky found that their preparations of desmosomes were enriched for sphingolipids and cholesterol[241]. They made this observation well before the lipid raft model was proposed. More recent work has confirmed this early finding, and research is ongoing to determine how and why the desmosome and its constituent proteins are associated with lipid rafts[39,110,242-244]. In light of the role lipid rafts play in other biological contexts (see section 2.11), it is likely that lipid rafts help to cluster desmosomal proteins during desmosome assembly (see Fig. 2.3). Raft partitioning may also be the mechanism by which desmosomal proteins are spatially segregated away from proteins of the adherens junction, allowing these two adhesive junctions to remain discrete in cells. Disruption of lipid rafts, and even the failure of a desmosomal protein to partition into rafts, can inhibit desmosome assembly and function and, as described in chapter 3, cause fatal disease.

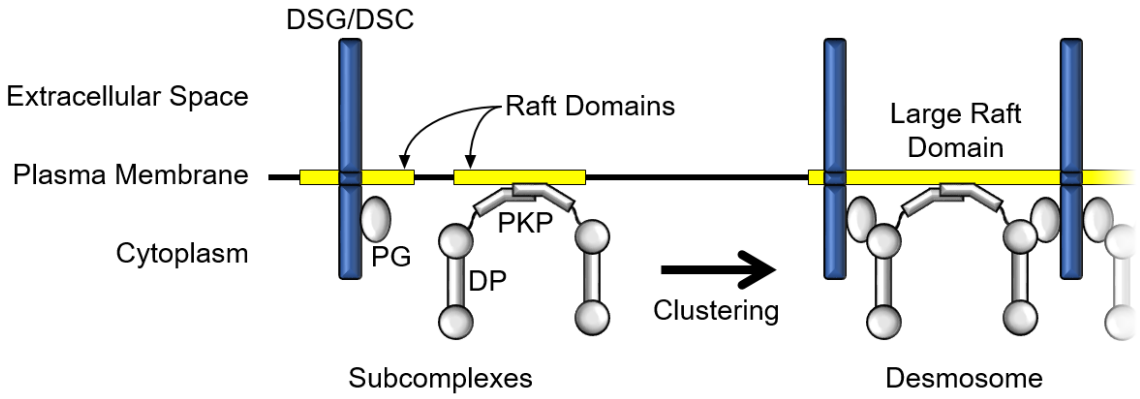

Fig. 2.3: Model for desmosome assembly in lipid rafts. Subcomplexes of desmosomal proteins are composed of a desmosomal cadherin (desmoglein (DSG) or desmocollin (DSC)) and plakoglobin (PG), or of plakophilin (PKP) and desmoplakin (DP). These subcomplexes independently associate with lipid rafts. During assembly, these subcomplexes cluster, facilitating formation of the desmosome.

2.14 Evidence for desmosome association with lipid rafts

Skerrow & Matolsky used low pH acetic acid buffers and velocity sedimentation on a sucrose gradient to biochemically isolate desmosomes[245]. When examined by electron microscopy, the desmosomes isolated by this technique are free of cell debris and organelles, although some plasma membrane remains associated. Thin layer chromatography indicated that cholesterol, a major constituent of lipid rafts, was heavily enriched in isolated desmosomes[241]. More recently, the converse of this finding has also been demonstrated: biochemical isolations of lipid rafts are enriched for the components of desmosomes[39,110,242-244].

As described earlier, detergent extraction of cell lysate is a commonly used method of biochemically isolating lipid rafts[177]. The discovery[246] that cell membranes could be separated into detergent soluble and detergent resistant fractions was a foundational observation in lipid raft research[247]; however, even pioneers in this field caution that detergent extraction alters membrane organization and does not isolate pre-existing membrane domains, making the isolation of detergent resistant membranes an imperfect proxy for the isolation of lipid rafts[248]. Isolation of detergent resistant membranes is accomplished by extracting cell lysate with Triton X-100 at 4°C and then subjecting the extract to equilibrium centrifugation under a sucrose gradient[177]. When lipid rafts are isolated from epithelial cells, every major desmosomal protein is present[39,110,242-244]. The partitioning of desmosomal proteins to lipid rafts is disrupted when cholesterol is depleted from cell membranes using methyl-β cyclodextrin (mβCD)[243]. mβCD is commonly used as a reagent for disrupting lipid rafts in living cells[249]. The association between desmosomal proteins and lipid rafts is also evinced by colocalization between lipid raft markers and desmosomal proteins[110,243]. The reciprocal findings of desmosomal components in lipid rafts and vice versa convincingly demonstrate an association between the two.

The mechanisms responsible for driving desmosomal protein partitioning to lipid rafts are the subject of ongoing research. Recently published findings demonstrated that palmitoylation is responsible for conferring lipid raft targeting to plakophilin[39]. With the exception of desmoplakin, every major desmosomal protein is palmitoylated, raising the possibility that palmitoylation drives lipid raft partitioning for all of these proteins[39]. However, a non-palmitoylated desmoglein mutant still partitioned to lipid rafts[250]. One report suggested that desmoglein 2 is recruited to lipid rafts through direct interaction with caveolin 1, however the authors did not investigate whether mutating DSG2 to prevent this association would abrogate lipid raft targeting[244]. As discussed in chapter 3, we find that the length of the desmoglein transmembrane domain is responsible for conferring lipid raft partitioning on this family of proteins.

The means by which plakoglobin, desmoplakin, and desmocollin are recruited to desmosomes is currently unknown. Plakoglobin is not an integral membrane protein; it can dissociate from the membrane and mediate signaling in the nucleus. Plakoglobin is subject to palmitoylation, so it is possible that this lipid modification is directly responsible for conferring both membrane association and lipid raft partitioning on plakoglobin. An alternative explanation is that protein-protein association between plakoglobin and other proteins, particularly the desmosomal cadherins, is responsible recruiting plakoglobin to lipid rafts. Furthermore, plakoglobin binds the lipid raft resident proteins flotillin-1 and flotillin-2, providing additional means by which it could be recruited to lipid rafts[251]. Protein-protein interaction is almost certainly responsible for the presence of desmoplakin in lipid rafts, since this protein does not directly interact with cell membranes[14].

Finally, there are several possible lipid raft targeting mechanisms for desmocollin. Its raft partitioning could be driven by its transmembrane domain, as is the case with the desmogleins. However, desmocollin isoforms have shorter transmembrane domains than do desmogleins, with predicted lengths of 18 – 21 residues. This is substantially shorter

than the 24 – 25 residues predicted for desmogleins. Desmocollin 2 is palmitoylated, and all desmocollin isoforms possess one or more conserved cysteine residues at the interface between their transmembrane and cytoplasmic domain, a common target for palmitoylation and lipid raft targeting[39]. Given the shorter desmocollin transmembrane domain, palmitoylation could be more important in determining lipid raft partitioning than for desmogleins.

2.15 Lipid raft perturbation inhibits desmosome assembly, function, and disassembly

A growing body of evidence demonstrates that lipid rafts are indispensable for desmosome assembly. The disruption of lipid rafts in cultured epithelial cells via treatment with mβCD inhibits assembly of new desmosomes[110,243]. In these studies, assembly of desmosomes was assessed using immunofluorescence to monitor recruitment of desmoplakin and other desmosomal proteins to the sites of cell-cell contact. mβCD prevents accumulation of desmosomal proteins at cell borders[110,243]. Desmosomal desmoplakin forms a characteristic staining pattern of two parallel bands, one contributed by each neighboring cell, but this staining pattern is lost when desmosome assembly is triggered in the presence of mβCD, indicating desmosome assembly is inhibited[110]. Treatment with mβCD also severely inhibits cell-cell adhesion, providing further evidence of the necessity of lipid rafts for the formation of functional desmosomes[110,243].

Like all drug treatment experiments, mβCD-mediated disruption of lipid rafts may cause off-target effects. An alternative approach would be to prevent the lipid raft partitioning of one or more individual desmosomal proteins and then determine whether desmosome assembly and function have been compromised. This approach was recently used by Roberts et al, who mutated plakophilin 2 (PKP2) to prevent its association with lipid rafts[39]. Expression of this non-raft mutant PKP2 inhibited desmosome assembly and cell-cell adhesion, providing compelling evidence that association with lipid rafts is crucial

for desmosome assembly and function. Further evidence of this relationship is provided using a non-raft desmoglein 1 mutant (see chapter 3).

mβCD can also prevent loss of cell-cell adhesion in an *in vitro* model of Pemphigus vulgaris. As described above, Pemphigus vulgaris patient autoantibodies (PV IgG) bind to DSG3, causing its internalization from the plasma membrane[80]. This internalization occurs in a lipid-raft dependent manner[111], and lipid raft disruption via mβCD is protective against DSG3 internalization and loss of adhesion[110].

One conceptual framework for explaining these data is that the lipid environment of the desmosome, particularly at the sites of assembly and disassembly, must be composed of raft lipids. The location(s) in which the desmosome is dynamic, wherein copies of DSG3 and other proteins are added to or removed from the junction, would be reliant on the orderly lipid environment of rafts to either integrate or disintegrate proteins from nascent or extant desmosomes. Disruption of lipid rafts would then be expected to prevent both assembly and disassembly of desmosomes, as has been observed.

As this chapter has described, evidence for the association between desmosomes and lipid rafts has been accumulating for over forty years, but only recently have *in vitro* experiments revealed the importance of these membrane microdomains for desmosome function and dynamics. In chapter 3, we offer the first report of a human disease caused by a mutation in a desmosomal protein which abrogates lipid raft targeting. This finding offers the most robust evidence to date of the indispensability of lipid rafts for desmosomes.

Chapter 3

A mutation in the desmoglein 1 transmembrane domain abrogates lipid raft targeting and causes severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome

This chapter is adapted from:

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We describe a novel mutation which causes severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome. Whole exome sequencing of two related patients revealed a point mutation in the desmosomal cadherin desmoglein 1 (DSG1). This ominantly acting mutation substitutes a hydrophilic arginine residue into the DSG1 transmembrane domain (G562R). DSG1 levels in patient epidermis were reduced, and DSG1 was mislocalized and aberrantly clustered at cell-cell borders. Expression of the mutant in a cell culture model revealed that the mutant protein is delivered to the cell surface, but its trafficking through the secretory pathway is partially impaired. Furthermore, once at the cell surface, the DSG1 mutant is deficient in desmosome incorporation. Biochemical and imaging approaches revealed that the DSG1 mutant is defective in targeting to lipid raft membrane microdomains. These findings demonstrate that lipid raft association is essential for normal desmoglein function and suggest that defects in lipid raft targeting may be an under-appreciated pathomechanism in human disease.

3.1 Introduction

Desmosomes are robust cell-cell junctions that are abundant in epithelial and cardiac tissues and confer resilience to mechanical stress. Loss of desmosome function results in skin and heart diseases characterized by tissue fragility [9,101]. Desmosomes are comprised of desmosomal cadherins, which span the plasma membrane to directly bind desmosomal cadherins from neighboring cells, and adaptor proteins which mediate linkage to the intermediate filament cytoskeleton. The mechanisms of assembly of desmosomal cadherins into these densely packed and uniformly sized adhesion structures are poorly understood. Recent studies indicate that desmosomal proteins are recruited into lipid rafts, and that recruitment to these specialized membrane microdomains may be essential for normal desmosome assembly and function [39,110,242-

[244]. However, the mechanisms and physiological importance of desmosomal cadherin recruitment to lipid raft membrane domains is not known.

In the skin, loss of desmosomal adhesion manifests clinically as epidermal blisters and erosions, and in some disorders, aberrant thickening of the epidermis [96]. One recently discovered example of such a disease is severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome. Most cases of SAM syndrome are caused by homozygous functional null mutations in the desmosomal cadherin desmoglein 1 (*DSG1*) [97-99]. DSG1 is the primary desmoglein expressed in the upper layers of the epidermis, and most individuals afflicted with SAM syndrome succumb to chronic infection in early childhood. Here, we report the first dominantly inherited SAM-causing mutation in *DSG1* in which patients harbor a missense mutation in the DSG1 transmembrane domain. This mutation abrogates DSG1 targeting to lipid raft membrane microdomains, slows DSG1 trafficking to the plasma membrane, and impairs DSG1 incorporation into desmosomes. Previous investigations into lipid raft involvement in human disease have found only indirect links and no instances in which a mutation abrogates raft association and causes disease [216,217]. Thus, we report a novel pathomechanism for a human disease and demonstrate the importance of desmosomal protein association with lipid rafts in epidermal homeostasis.

3.2 Results and Discussion

Loss of DSG1 function is associated with a number of autoimmune, infectious, and genetic diseases [9,101]. We have identified a family harboring a novel, dominantly inherited DSG1 missense mutation (Fig. 3.1). The probands presented with ichthyosiform erythrokeratoderma, diffuse palmoplantar keratosis and multiple allergies (Fig. 3.1A). The proband III-2 suffered metabolic wasting and died of status asthmaticus and recurrent infections. Hemotoxylin and eosin staining of skin biopsied from this proband revealed

compact hyperkeratosis with parakeratosis, frequent detachment of the entire stratum corneum and dissociation of individual corneocytes (Fig. 3.1B), suggesting an adhesion defect. Altered keratin organization in patient desmosomes was detected in the granular layer of the epidermis by electron microscopy (Fig. 3.1C). These clinical and genetic observations led us to diagnose the patients with SAM syndrome. Unlike previously reported instances of *DSG1* mutations in SAM syndrome (Cheng et al., 2016; Danescu et al., 2017; Has et al., 2015; Samuelov et al., 2013), this novel missense mutation introduces a hydrophilic arginine residue (p.G562R) into the otherwise hydrophobic transmembrane domain of DSG1 (Fig. 3.1F).

To determine how the p.G562R mutation impacted DSG1 organization in patient skin, biopsies from the proband were processed for immunofluorescence microscopy. DSG1 levels were markedly reduced (~40%) in the spinous and granular layers of patient epidermis (Fig. 3.2A,B), and DSG1 localized in cytoplasmic puncta and aberrant clusters at cell-cell borders. Interestingly, DSG1 staining in patient stratum corneum was markedly increased, perhaps reflecting increased antibody penetration. Desmoplakin levels were slightly reduced in patient epidermis, whereas DSG3 levels were markedly increased (Fig. 3.2C-E). To further investigate alterations in DSG1 distribution, we employed super-resolution structured illumination microscopy (SIM). Using this approach, bone fide desmosomes can be identified as two parallel bands of desmoplakin fluorescence intensity at cell-cell borders [252,253]. We determined that DSG1 fluorescence intensity within patient and control desmosomes was comparable in basal keratinocytes, where DSG1 expression is low and other DSG isoforms (DSG2, DSG3) are expressed. However, in the spinous and granular layers where DSG1 is prominently expressed, DSG1 fluorescence intensity in patient desmosomes was significantly reduced (Fig. 3.2F,G). Previously reported DSG1 mutations causing SAM syndrome are characterized by recessive inheritance patterns and total loss of DSG1 expression and/or localization at the plasma

membrane [97-100]. Furthermore, haploinsufficiency of DSG1 results in a non-syndromic palmoplantar keratoderma[254,255]. Thus, the SAM-causing DSG1(p.G562R) mutation presented here is unique in that it is transmitted in a dominant fashion and is characterized by a defect in DSG1 incorporation into desmosomes in the upper layers of the epidermis.

To investigate the mechanism by which the DSG1(p.G562R) mutation causes SAM syndrome, GFP-tagged murine wild type Dsg1α (WT Dsg1) and a mutant harboring the equivalent G-to-R substitution (Dsg1(G578R)) were expressed in A431 epithelial cells. Fluorescence activated cell sorting and drug selection were utilized to achieve stable cell lines expressing similar levels of WT Dsg1 and Dsg1(G578R). Widefield fluorescence imaging revealed that both WT Dsg1 and Dsg1(G578R) were present at cell-to-cell borders (Fig. 3.3A). Interestingly, the Dsg1(G578R) mutant exhibited a prominent perinuclear staining pattern. There were no obvious differences in desmoplakin localization in the two cell lines (Fig. 3.3A), and plakoglobin generally co-localized with both cell-cell border and perinuclear pools of WT Dsg1 and Dsg1(G578R) (Fig. 3.3B). To determine if the Dsg1(G578R) mutant was defective in desmosome targeting, SIM was performed and Dsg1 fluorescence intensity was measured at cell borders both within and outside of individual desmosomes to control for variations in Dsg1 levels at different cell-cell contact sites. The enrichment of Dsg1(G578R) fluorescence intensity within desmosomes was significantly reduced compared to WT Dsg1 (Fig. 3.3C,D). Furthermore, parallel bands of Dsg1.GFP fluorescence overlapping with DP staining were commonly observed for WT Dsg1 but not for Dsg1(G578R) (Fig. 3.3C,E). In addition to SIM, we also assessed desmosome incorporation of wild type and mutant DSG1 biochemically (Fig. 3.3F,G). WT Dsg1 efficiently entered a detergent resistant pool, consistent with incorporation into insoluble desmosomal complexes, whereas mutant Dsg1 remained predominantly soluble. Together, these findings indicate that in cultured A431 cells, the

G-to-R TMD mutation reduces Dsg1 incorporation into desmosomes, similar to what we observed in patient tissue.

In addition to a desmosome targeting defect, we observed that DSG1 was present in cytoplasmic puncta in patient epidermis (Fig. 3.2A) and that Dsg1(G578R) was concentrated in perinuclear compartments in A431 cell lines (Fig. 3.3A). TMD length has been identified as a mechanism driving subcellular protein localization, with lengthy TMDs facilitating trafficking to the thicker, more lipid raft-enriched plasma membrane [124,125]. Recently, raft association was shown to be a determinant of receptor recycling back to the plasma membrane after endocytosis [186]. These observations suggested that the DSG1 G-to-R mutation may alter DSG1 membrane trafficking in addition to desmosome incorporation. To address this possibility, cell surface proteins were cleaved using trypsin and the rate of Dsg1 recovery at the cell surface was monitored (Fig. 3.4A). At steady state, cell surface expression of WT and mutant Dsg1 was comparable. Following cleavage, however, the surface pool of WT Dsg1 recovered within 3-6 hours, while Dsg1(G578R) exhibited a severe reduction in delivery to the plasma membrane. No difference was observed in the rate of Dsg1(G578R) turnover from the plasma membrane as assessed by cell surface biotinylation and pulse-chase experiments (Fig. 3.4B). To determine if Dsg1(G578R) was being retained in biosynthetic compartments, we grew A431 cells in low calcium medium, wherein there is minimal Dsg1 on the cell surface. When cells were switched to high calcium medium to allow Dsg1 to traffic out to cell-cell borders, Dsg1(G578R) was aberrantly retained in GM130-labeled compartments (Fig. 3.4C,D). These findings indicate that the G-to-R mutation causes retention of Dsg1 in the Golgi apparatus, delaying its trafficking through the secretory pathway.

As discussed above, desmosomal proteins, including desmogleins, associate with lipid raft membrane microdomains. Transmembrane domain length is a critical determinant for integral membrane protein targeting to lipid rafts [186,256,257], (Levental,

submitted). Sequence alignments (Fig. 3.8A) reveal that the TMDs of the raft-targeting desmogleins are longer than the corresponding TMDs of classical cadherins, which exhibit minimal raft association [110]. Furthermore, arginine residues play an important role in terminating TMDs and establishing TMD orientation within the lipid bilayer [258,259]. Together, these observations led us to hypothesize that the G-to-R mutation would prevent DSG1 from partitioning to lipid rafts and potentially alter desmosome targeting. To test this hypothesis, we biochemically isolated detergent resistant membranes (DRM) from our A431 cell lines using Triton X-100 extraction and sucrose gradient fractionation [177]. A substantial portion of WT Dsg1 was present in the DRM fractions (Fig. 3.5A,B), as we have reported previously for Dsg3. However, Dsg1(G578R) was absent from DRM. Interestingly, plakoglobin association with DRM displayed a detectable although not statistically significant reduction in cell lines expressing Dsg1(G578R), providing further evidence that the mutant altered plakoglobin subcellular distribution as observed in Fig. 5.3B. To further test the ability of WT Dsg1 and Dsg1(G578R) to associate with lipid rafts, cDNAs encoding these proteins were transiently expressed in rat basophilic leukemia cells and giant plasma membrane vesicles were chemically isolated. Non-raft membrane domains were marked with F-DiO, a dialkylcarbocyanine dye. WT Dsg1 efficiently partitioned into areas of the vesicle lacking F-DiO, indicating partitioning to the liquid ordered, raft domain (Fig. 3.5C,D). In contrast, Dsg1(G578R) almost entirely co-segregated with F-DiO, indicating minimal association with lipid raft domains.

In addition to Dsg1, We have previously shown that Dsg3 also associates with lipid rafts [110]. To determine if the TMD is the principle motif conferring lipid raft affinity on the desmoglein family of proteins, we generated a chimeric cadherin in which the Dsg3 TMD was replaced with the E-cadherin TMD (Dsg3(ETMD)). Subsituting the Dsg3 TMD with that of E-cadherin abrogated lipid raft targeting, similar to our findings with the SAM-causing DSG1 mutant (Fig. 3.6A,B). Furthermore, Dsg3(ETMD) was more soluble in

Triton X-100 than WT, both in a biochemical context (Fig. 3.6D-F) and when cells were pre-extracted immediately prior to fixation and immunofluorescence (Fig. 3.6C). To determine whether the Dsg3 TMD was sufficient to confer lipid raft targeting, we used an interleukin 2 receptor (IL2R) α chain-Dsg3 chimeric protein (Fig. 3.7). In these chimeras, raft partitioning was conferred by the Dsg3 TMD. In addition to TMD length and content, raft targeting of membrane spanning proteins can also be regulated by palmitoylation. We confirmed recent findings [39,250] that Dsg3 is palmitoylated and that mutation of conserved, membrane proximal cysteine residues abrogates palmitoylation (Fig. 3.8B). However, the palmitoylation null Dsg3(cc) mutant displayed no discernable defect in lipid raft association (Fig. 3.8C) nor any change in Triton-X100 solubility (Fig. 3.8D-F). Collectively, these results suggest that the TMD rather than palmitoylation is the dominant lipid raft targeting mechanism for the desmoglein protein family.

In summary, a point mutation in the transmembrane domain of DSG1 causes SAM syndrome. This mutation abrogates lipid raft association of DSG1 and causes at least two defects in DSG1 function. Firstly, the G-to-R substitution slows DSG1 anterograde trafficking to the plasma membrane due to retention in GM130-positive Golgi compartments. Secondly, once at the plasma membrane, the mutation impairs incorporation of DSG1 into desmosomes as assessed both biochemically and using super-resolution imaging of patient epidermis and cultured epidermoid cell lines. Emerging evidence indicates that TMD amino-acid number and content are both determinants of raft association (Levental, personal communication). Furthermore, a variety of TMDs have been shown to contribute to protein function, including members of the cadherin family[260-262]. Further studies will be needed to determine the precise structural and functional features of the desmoglein TMD, and to determine how this domain and raft association contribute to desmosome morphology and function. Importantly, our findings reveal that failure of a protein to associate with lipid raft microdomains is a novel pathomechanism of

a desmosomal disease, and raise the possibility that other human disorders may result from alterations in lipid raft association or raft homeostasis. Indeed, loss of lipid raft targeting may be an under-appreciated pathomechanism in human diseases which were previously conceived as generalized protein trafficking defects.

3.3 Acknowledgements

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The authors declare no competing financial interests.

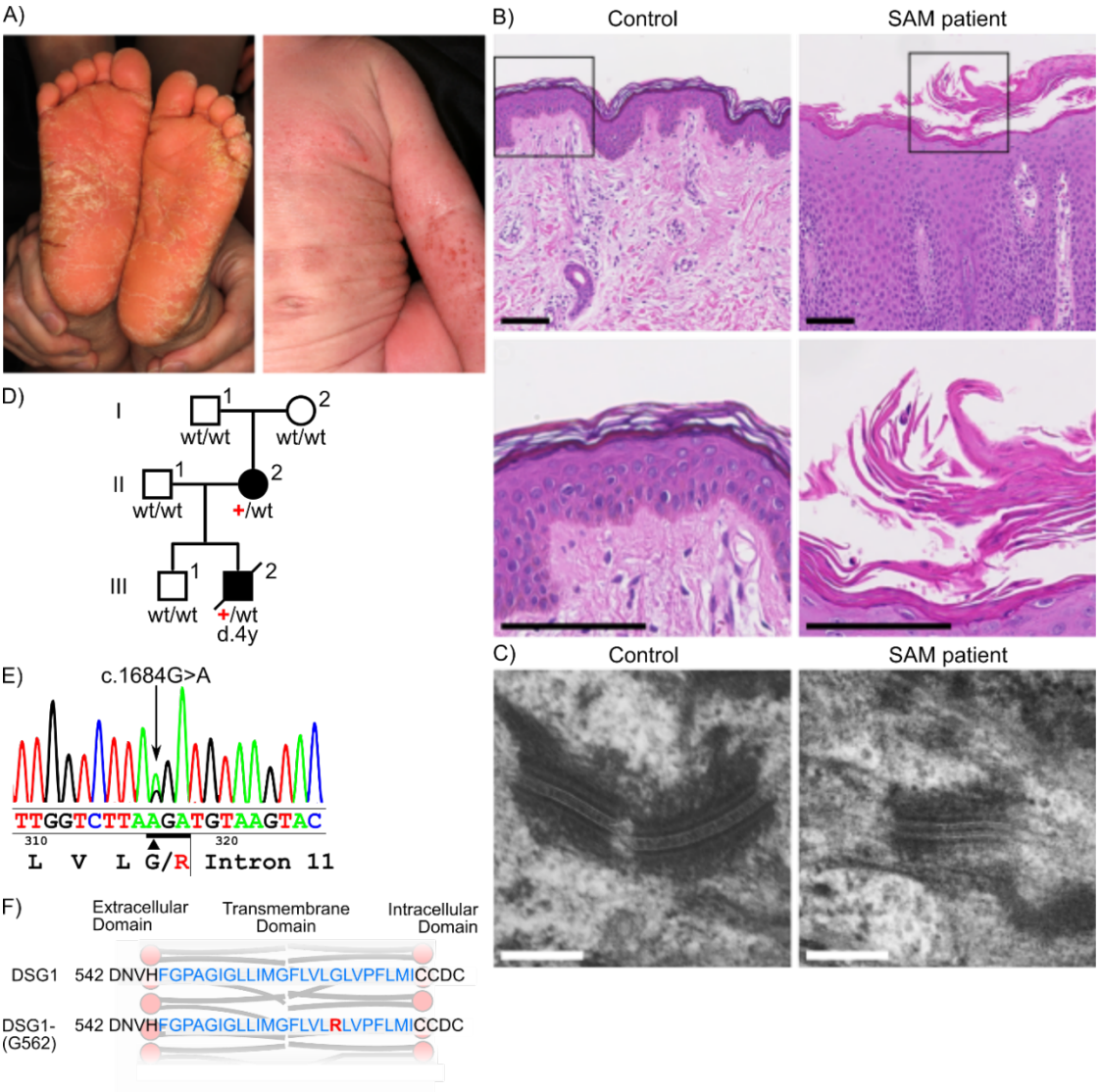

Fig. 3.1: Desmoglein 1 (DSG1) transmembrane domain mutation causes severe dermatitis, multiple allergies, and metabolic wasting (SAM) Syndrome. A) Individual III-2 displays feet covered with hyperkeratotic yellowish papules and plaques, and ichthyosiform erythroderma with severe itch occur over much of his body. B) Hemotoxylin and eosin staining of III-2's skin biopsy reveals acantholytic lesions in the upper layers of the epidermis. Scale bar = 100 μm. C) Electron micrographs of epidermal sections from the proband indicate keratin filaments are poorly developed in keratinocytes of the stratum granulosum. Scale bar = 200 nm. D) Pedigree of affected individuals and near relatives. Inheritance determined by genomic DNA sequencing. +, c.1684G>A in *DSG1*. E) Genomic DNA sequencing of white blood cells reveals these SAM patients have a heterozygous point mutation, c.1684G>A (black arrow) in DSG1. The adjacent splice site is unaffected. F) Schematic showing the location of the SAM-causing G-to-R substitution (red) within the transmembrane domain (blue).

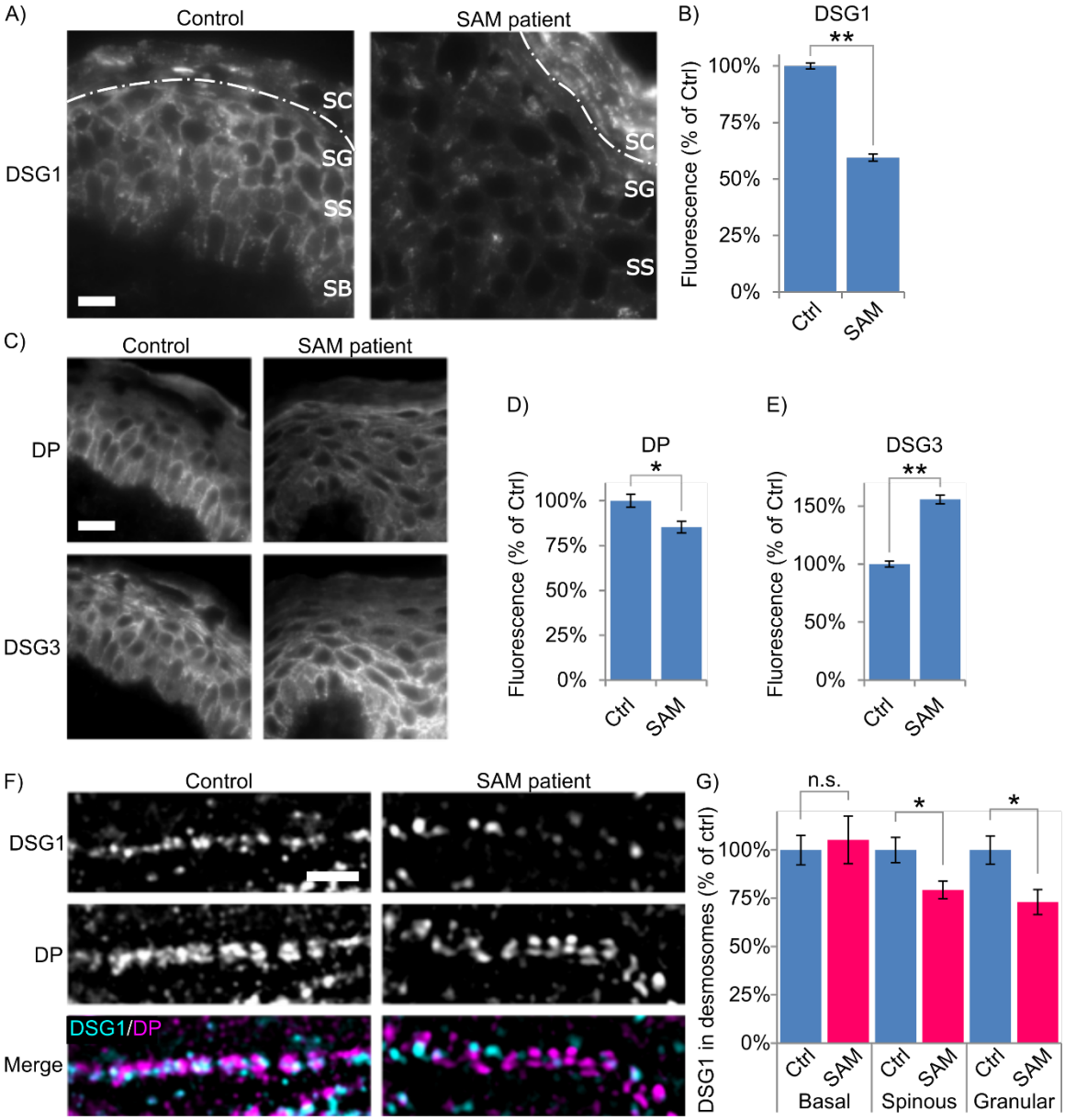

Fig. 3.2: Imaging of SAM patient tissue confirms desmosome disruption and DSG1 junction targeting defect. A,B) Widefield microscopy of DSG1 immunofluorescence in human skin biopsies reveals both DSG1 downregulation and inappropriate clustering at cell borders in SAM patient epidermis. SC = stratum corneum, SG = stratum granulosum, SS = stratum spinosum, SB = stratum basale. SC/SG boundary demarcated by dashed line. Downregulation of DSG1 is severe in the SG and SS. C-E) Desmoplakin (DP) is slightly downregulated in patient skin, and DSG3 is upregulated. Quantification in B, D, and E was performed in the SG and SS, scale bar = 20 μm. F,G) Structured Illumination Microscopy (SIM) images indicate that less DSG1 is present in desmosomes in SAM patient tissue in the stratum granulosum. Scale bar = 5 μm. *p<0.01, **p<0.001

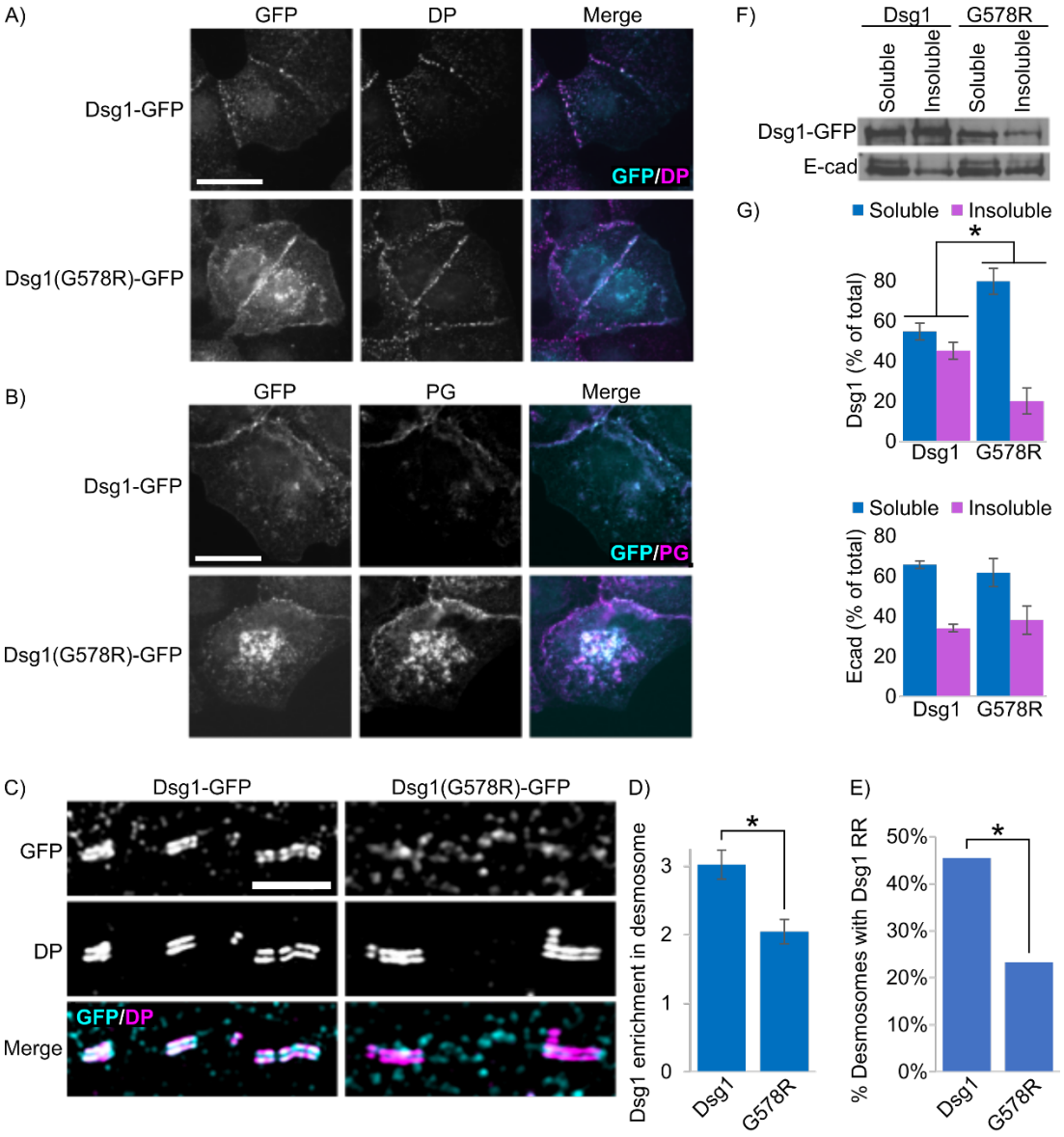

Fig. 3.3: SAM-causing DSG1 mutation causes defects in junction targeting. A) Widefield micrographs of A431 cell lines stably expressing murine Dsg1α-GFP (wild type or bearing the SAM-causing glycine-to-arginine mutation, G578R) reveal broadly similar distribution of desmoplakin (DP) and B) colocalization between DSG1 and plakoglobin (PG). Scale bar = 20 μm. C) Super-resolution micrographs of A431 stable cell lines acquired using structured illumination microscopy (SIM) reveal defects in Dsg1(G578R) desmosome targeting. Scale bar = 5 μm. D) Quantification of Dsg1-GFP enrichment in desmosomes compared to non-desmosomal cell border areas. E) Quantification of Dsg1-GFP railroad track (RR) patterning at desmosomes (trend representative of two independent experiments, Chi-square test to determine significance). F,G) The G578R mutation also increases solubility of Dsg1 in Triton X-100, suggesting decreased cytoskeletal association. E-cadherin solubility was similar in both cell lines. *p<0.05

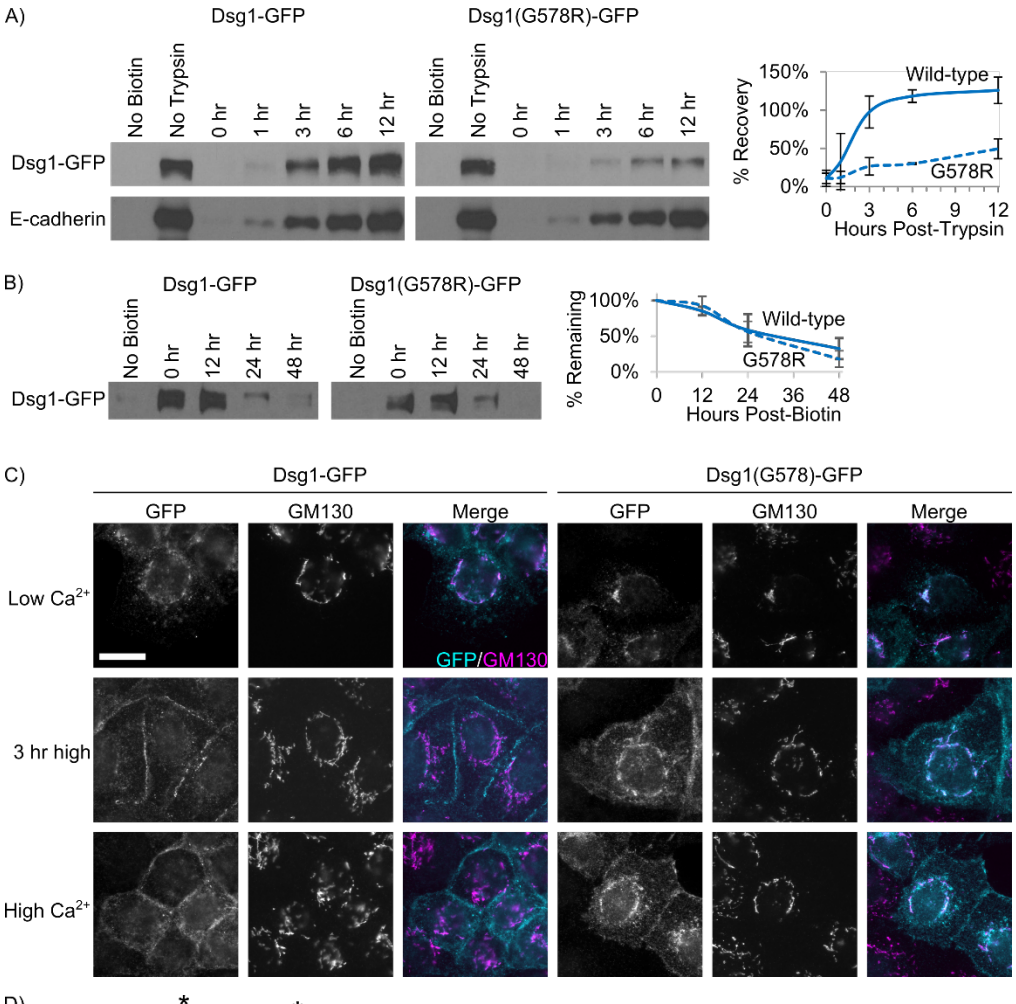

Fig. 3.4: SAM-causing DSG1 mutation delays trafficking to the plasma membrane. A) Dsg1(G578R) is trafficked to the plasma membrane substantially more slowly than wild-type. Dsg1 present on the cell surface was cleaved using trypsin at t=0. Cells were incubated for the indicated time and the amount of newly delivered surface Dsg1 was assayed via biotin labeling. B) The rate of turnover of Dsg1 from the plasma membrane was assessed in A431 cells exogenously expressing murine Dsg1α-GFP. Cells were biotinylated at t=0 and lysate was collected after the indicated time. No difference in the rate of turnover can be detected between the wild type and mutant protein. C,D) Dsg1(G578R) displays increased colocalization with the Golgi Apparatus protein GM130, indicating a trafficking defect. Cells were grown in low calcium medium and switched to high calcium for the indicated duration to permit cell surface accumulation of desmoglein. Cells were then processed for immunofluorescence. Scale bar = 20μm. *p<0.001

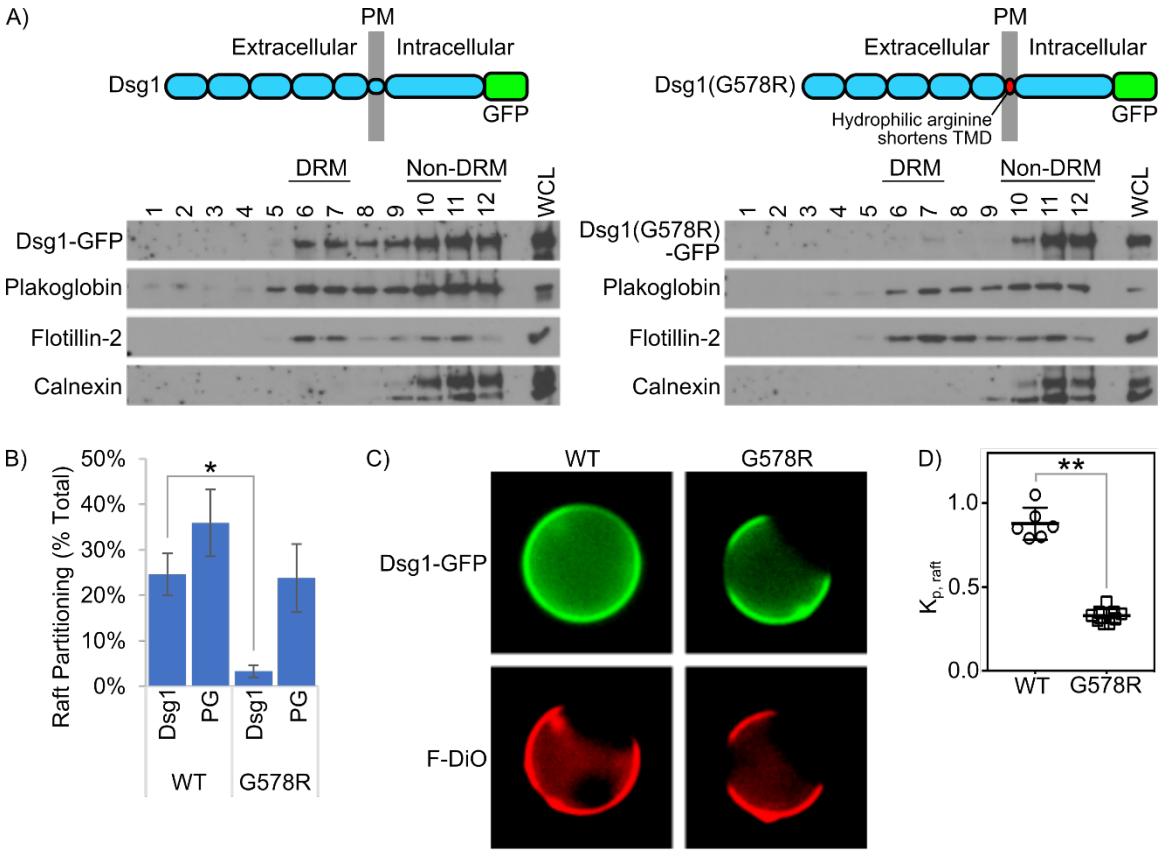

Fig. 3.5: SAM-causing DSG1 mutation abolishes lipid raft targeting. A) Sucrose gradient fractionation of A431 cell lines stably expressing WT and mutant Dsg1. DRM = detergent resistant membranes. The lipid raft-associating protein flotillin was used as a positive control for DRM partitioning, while the non-raft protein calnexin served as a negative control. B) Quantification of A indicates SAM-causing mutation abolishes Dsg1 partitioning to DRM. C) Representative images of giant plasma membrane vesicles isolated from rat basophilic leukemia cells expressing GFP-tagged transmembrane polypeptide from WT Dsg1 and Dsg1(G578R). Unsaturated marker FAST-DiO (F-DiO) to visualize the nonraft phase. D) Normalized line scans of Dsg1 fluorescence intensity were measured through peaks corresponding to Dsg1 intensity in raft and nonraft membrane, respectively. Background-subtracted ratios of these two intensities yield raft partition coefficients, $K_{p,raft}$. Compared to WT Dsg1, the G578R mutation abrogates lipid raft partitioning. Data are shown as mean ± SEM from three independent experiments. *p<0.05, **p<0.001

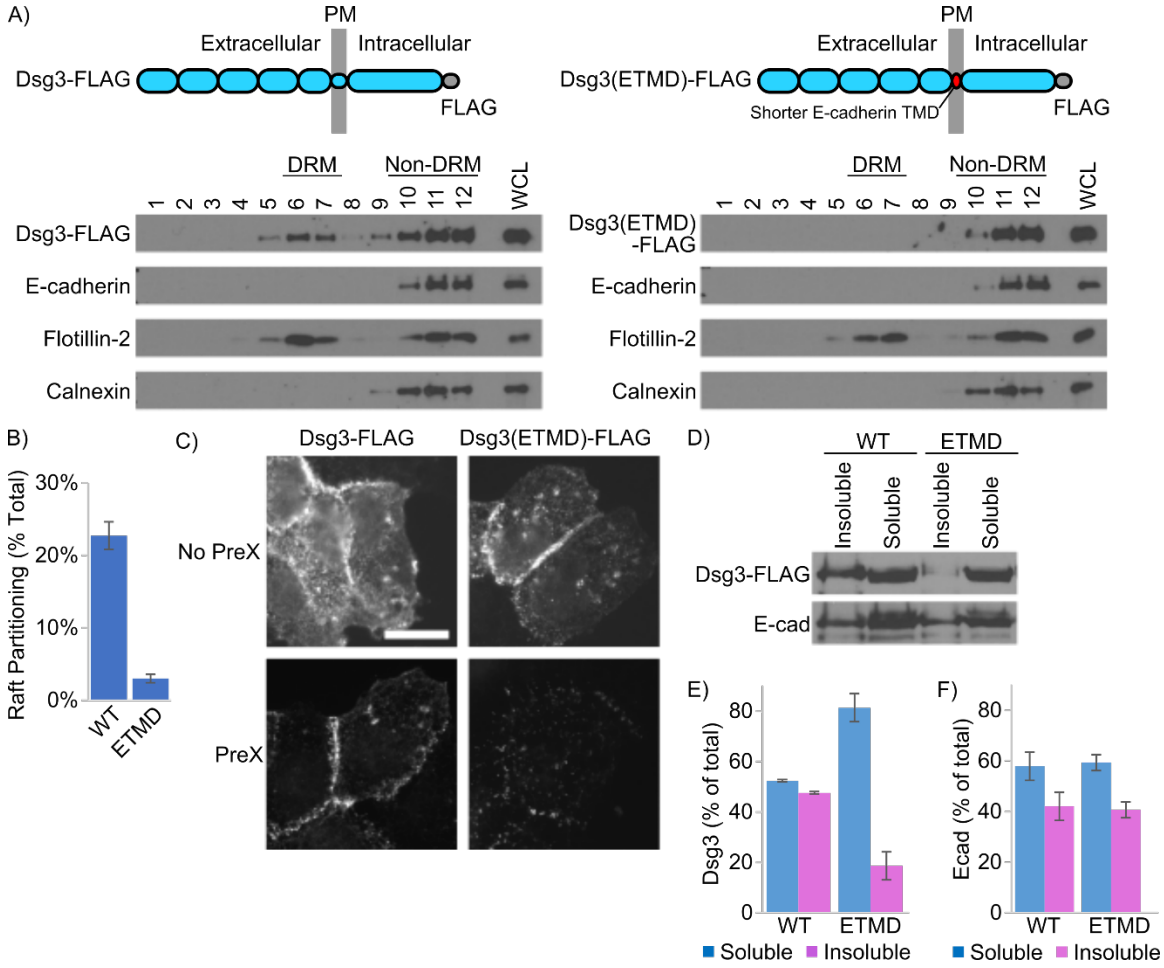

Fig. 3.6: The Dsg3 TMD is necessary for lipid raft targeting. A,B) Fractionation of A431 cells stably expressing murine Dsg3 (wild-type or ETMD mutant). Replacing the Dsg3 TMD with the shorter E-cadherin TMD abolishes lipid raft targeting. C) Dsg3(TMD) is more susceptible to pre-extraction in Triton X-100. Scale bar = 20 μm. D-F) Dsg3(ETMD) is more soluble in Triton X-100.

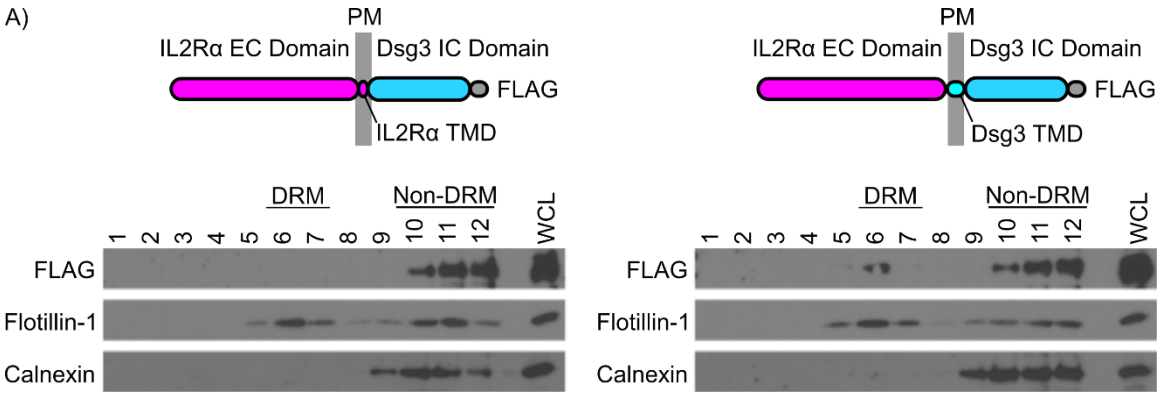

Fig. 3.7: Dsg3 TMD confers lipid raft targeting on an interleukin 2 receptor-DSG3 chimera. A) Lipid raft fractionation of HeLa cells expressing FLAG-tagged IL2R-Dsg3 chimeras from adenoviruses. Inclusion of the lengthy Dsg3 TMD in the chimera confers lipid raft targeting.

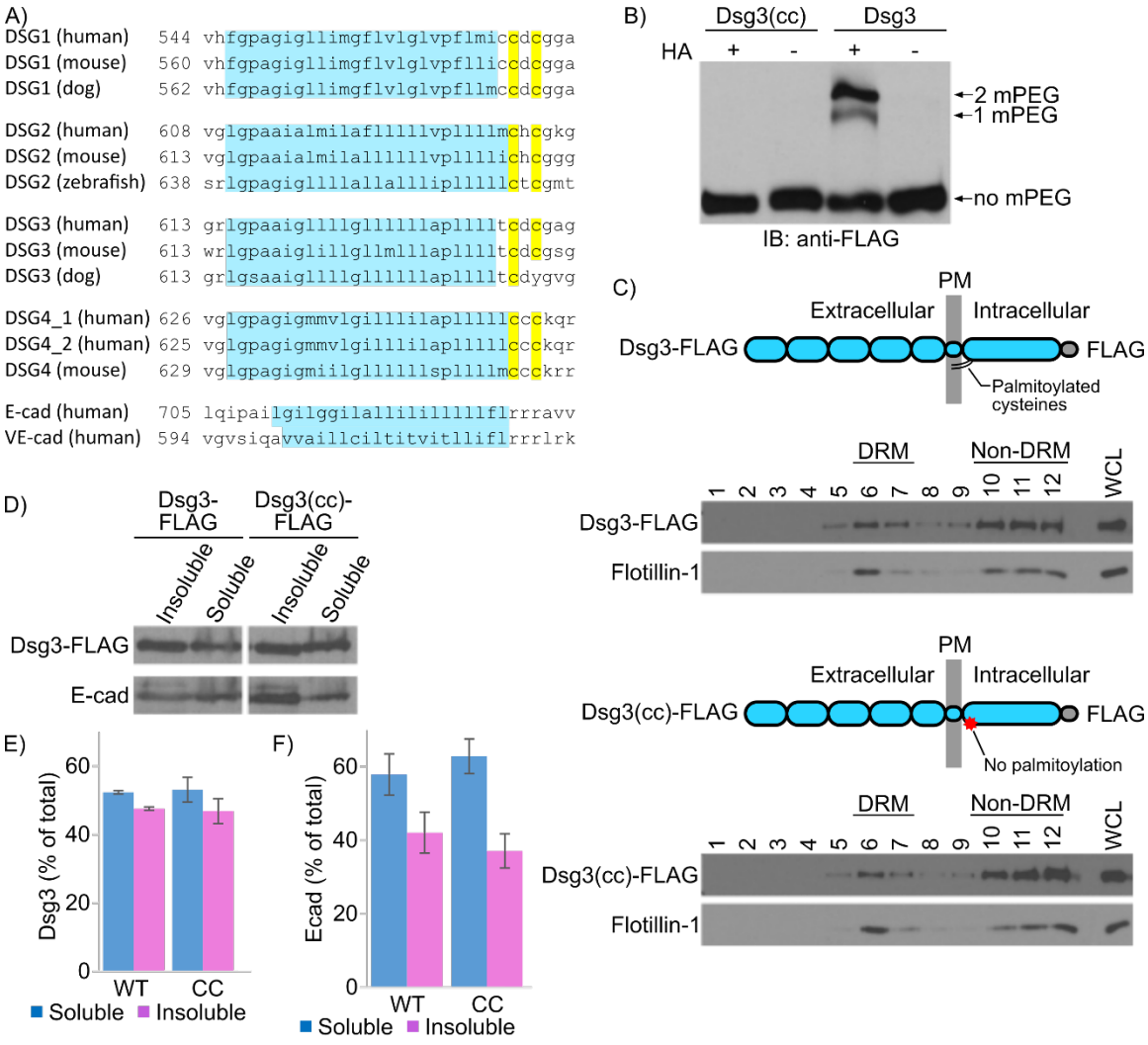

Fig. 3.8: Palmitoylation is not required for Dsg3 lipid raft-targeting or junction targeting. A) Alignment of the desmogleins reveals a pair of highly conserved cysteine residues (yellow highlight) at the interface between these proteins' sole transmembrane domain (light blue) and their cytoplasmic domain. B) Mass-tag labeling replaces palmitate on palmitoylated cysteine residues with mPEG, causing a size shift. Dsg3 is doubly palmitoylated, and mutation of the membrane-proximal cysteine residues to alanine (Dsg3(cc)) negates palmitoylation. C) Lipid raft fractionation of HeLa cells expressing Dsg3-FLAG from adenoviruses reveals no defect in lipid raft targeting of the palmitoylation-null mutant. D-F) Loss of palmitoylation has no detectable effect on the solubility of Dsg3 in Triton X-100, a classic measure of desmosome association.

Materials and methods

Subjects. All affected and healthy family members or their legal guardians provided written and informed consent in accordance with the guidelines of the Institutional Review Board of Keio University School of Medicine in adherence to the Helsinki guidelines. The investigators were not blinded to the allocation during experiments and outcome assessment.

Mutation analysis. Whole-exome sequencing was performed using genomic DNA isolated from the probands (II-2 and III-2) and their parents (I-1, I-2 and II-1). Whole exome sequencing libraries were constructed using SureSelect Human All Exon V5 (Agilent) and sequenced by HiSeq2500 (Illumina). Sequencing reads were mapped to a human reference genome sequence (hs37d5) by BWA software (0.7.12-r1039). The mapped reads were realigned and variation sites were detected by GATK-3.30 software. The detected variation sites were annotated by SnpEff/SnpSift 4.1d software. Since the phenotype appeared in the proband II-2 (delivered from healthy parents) and transmitted to the proband III-2 (Fig. 3.1D), we searched for genetic variations that de novo mutated in the proband II-2 and transmitted to the proband III-2. Only one variation was identified to fulfill the criteria, which was c.1684G>A (p.G562R) of *DSG1*, coding the desmosomal cadherin desmoglein 1. Sanger sequencing confirmed the mutation was identified in the probands but not from other healthy family members (Fig. 3.1D,E). The mutation had not been identified in cohort studies [263-266]. The whole exome sequencing of the probands II-2 and III-2 revealed no other variations in the exons and exon-intron boundaries of *DSG1*.

Immunohistochemistry and electron microscopy of patient samples. Biopsies were embedded in optimum cutting temperature (OCT) solution and stored at -80°C. Prior to

immunostaining, 5 μm cryosections were prepared on glass microscope slides. Primary and secondary antibodies are described below. Sections were sealed using mounting medium (ProLong Gold by ThermoFisher Scientific) and a coverslip. For electron microscopic studies, the biopsied sample was fixed in an ice-cold 2% glutaraldehyde/60 mM Hepes (pH 7.4) buffer followed by fixation with 1% osmium tetroxide, staining with 1% uranyl acetate, and embedding in Epon812. Ultrathin sections were stained with 1.5% uranyl acetate and Reynolds lead citrate and examined with an electron microscope (JEM-1010, JEOL) at the accelerating voltage of 80 kV.

Construction of mutants. Constructs were cloned using PCR and mutagenesis by the Cloning Division within Emory Integrated Genomics Core.

Cell line generation, culture, and reagents. A431 cells were cultured in DMEM (Corning 10-013-CV) with 10% fetal bovine serum (Hyclone SH30071.03) and 1% penicillin/streptamycin (Corning 30-004-CI). Cells were stably infected with lentiviruses expressing the various murine desmoglein constructs. 5 μg/mL blasticidin was used to select for infected cells. No clonal isolation was performed. Cell lines expressing wild type and mutant DSG1-GFP were subjected to fluorescence activated cell sorting in order to obtain populations with roughly equal DSG1-GFP expression levels. For experiments utilizing a calcium switch, low calcium medium was prepared as described previously [267]: no calcium DMEM (Gibco/Molecular Probes 21068028), 10% fetal bovine serum, calcium chelating BT Chelex 100 resin (Biorad 143-2832), and 1% penicillin/streptamycin.

Immunofluorescence. A431 cells were cultured to ~70% confluence on glass coverslips. In experiments in which pre-extraction is explicitly used, cells were treated with PBS+ containing 0.2% Triton X-100 and 300 mM sucrose on ice for 1 min prior to fixation. Cells

were fixed in 4% paraformaldehyde in PBS+ on ice for 10 min. Cells were permeabilized in PBS+ containing 0.1% Triton X-100 and 0.3% bovine serum albumin for 10 min. Non-specific antibody binding was prevented with a blocking step in PBS+ containing 0.3% bovine serum albumin and 0.05% Triton X-100. Primary and secondary antibodies (listed below) were diluted into blocking solution. For rinse buffer, we used PBS+ containing 0.0002% bovine serum albumin and 0.05% Triton X-100. Cells were mounted to glass microscope slides using mounting medium (described above).

Antibodies. Mouse anti-DSG3 AK15 was described previously [268]. Rabbit anti-calnexin (Enzo Life Sciences ADI-SPA-860). Mouse anti-desmoplakin1/2 (Fitzgerald 10R-D108AX). Rabbit anti-desmoplakin NW6 was a kind gift from Dr. Kathleen Green (Northwestern University). Mouse anti-plakoglobin (gamma catenin) (BD TransLabs 610253). Mouse anti-E-cadherin (BD Biosciences 610252). Mouse anti-flotillin 1 (BD 610820). Mouse anti-flotillin 2 (BD 610383). Rabbit anti-Green Fluorescent Protein Life A11122). Rabbit anti-FLAG (Bethyl A190-102A). Secondary antibodies conjugated to Alexa Fluors were purchased from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies were purchased from Biorad.

Image acquisition and processing. Widefield fluorescence microscopy was performed using a DMRXA2 microscope (Leica, Wetzler, Germany) equipped with a 100X/1.40 NA oil immersion objective and narrow band pass filters. Images were acquired with an ORCA digital camera (Hamamatsu Photonics, Bridgewater, NJ) and processed using Fiji ImageJ. Super-resolution microscopy was performed using a Nikon N-SIM system on an Eclipse Ti-E microscope system equipped with a 100X/1.49 NA oil immersion objective, 488- and 561-nm solid-state lasers in 3D structured illumination microscopy mode. Images were captured using an EM charge-coupled device camera (DU-897, Andor Technology) and

reconstructed using NIS-Elements software with the N-SIM module (version 3.22, Nikon). Imaging results are representative for at least two independent experiments containing at least 10 cells each.

Desmosome targeting analysis using SIM. To quantify desmosome targeting in cultured cells, Dsg1.GFP fluorescence was measured within regions of interest (ROI) drawn around desmoplakin rail road track staining at cell-cell borders. This Dsg1.GFP fluorescence intensity was compared to adjacent ROI at regions of cell borders lacking desmosomes. For both wild type and mutant Dsg1, targeting to desmosomes was measured as a fold-enrichment of Dsg1.GFP fluorescence in desmosomes compared to non-desmosomal regions. For SAM patient and control tissue, desmosomal ROIs were defined using desmoplakin railroad tracks and DSG1 fluorescence was measured therein.

Triton solubility/insolubility. A431 cells were cultured until confluent in 6 well tissue culture plates. Cells were washed twice with phosphate buffered saline. The triton soluble pool was isolated by incubating cells with triton buffer (1% Triton X-100, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, with protease inhibitor) for 10 min on ice. Lysate was then centrifuged at 16,000 x *g* for 10 min at 4°C to pellet triton insoluble fraction. Triton-soluble supernatant was collected. The triton-insoluble pellet was resuspended in 2X laemmli sample buffer (Biorad 161-0737) sample buffer containing 5% β-mercaptoethanol. All samples were heated to 95°C for 5 minutes prior to being run on a gel for western blotting.

Isolation of detergent resistant membranes. Detergent resistant membranes were isolated as described previously [177]. Briefly, cells were cultured in 25 cm^2 flasks (two per gradient) and washed with PBS+. Cells were collected by scraping in TNE buffer

supplemented with protease inhibitors (Roche) and pelleted by centrifugation at 0.4 x *g* at 4°C for 5 min (5415R, Eppendorf). Cells were re-suspended in TNE buffer and homogenized using a 25-guage needle. TNE buffer containing Triton X-100 was added (final concentration of 1%) and cells were incubated on ice for 30 min. 400 µL of detergent extract was mixed with 800 µL of 56% sucrose in TNE and placed at the bottom of a centrifuge tube. 1.9 mL volumes of 35% and 5% sucrose were layered on top of the sample. Following an 18 hour centrifugation at 4°C (44,000 rpm, SW55 rotor, Beckman Optima LE-80 K Ultracentrifuge), 420 µL fractions (1–11, remaining volume combined to make up fraction 12) were removed from top to bottom of the gradient and stored at −20°C until processed for western blot analysis. Flotillin-1 and calnexin were used as raft and non-raft markers respectively. Unless otherwise stated, all films shown are representative for at least three independent experiments.

Giant plasma membrane vesicle (GPMV) isolation and partitioning measurements. GPMVs were isolated and imaged as described [183,185]. Before GPMV isolation, cell membranes were stained with 5 µg/mL of FAST-DiO (Invitrogen), a fluorescent lipidic dye that strongly partitions to disordered phases because of double bonds in its fatty anchors [184].

Biotin labeling in pulse-chase experiments. For Dsg1 cleavage and recovery experiments, cells were grown to confluence in a 35 mm cell culture plates (Corning 430165). Cells were trypsinized using TrypLE (Gibco 12605-010) for ~8 min and suspended. After the indicated refractory period, surface proteins were biotinylated. For experiments monitoring protein turnover from the plasma membrane, surface proteins were biotinylated before the indicated period. Biotinylation was achieved using PBS+ containing 0.5 mg/ml EX-Link sulfo NHS SS Biotin (Thermo Scientific 21331) for 30 min

at 37°C (or on ice for surface turnover experiments). Unbound biotin was quenched in PBS+ containing 50 mM NH_4Cl for 1 min. Cells were lysed in RIPA (PBS+ containing 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 10 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and protease inhibitor cocktail (Roche 11836170001)), scraped to transfer from culture plate to an Eppendorf tube, and incubated for 10 min on ice. Lysate was cleared via centrifugation at 16,000 x *g* at 4°C for 10 min. Biotinylated protein was captured on streptavidin-coated beads (manufacturer) during overnight incubation at 4°C. Beads were collected via centrifugation at 2,500 x *g* at 4°C for 1 min. Protein was released from beads using Laemmli buffer containing 5% β-mercaptoethanol.

Mass-tagging of palmitoylated proteins. For mass-tag labeling, we followed the procedure described by [269]. Lysates from A431 cells expressing the indicated constructs were prepared in TEA buffer (50 mM triethanolamine; pH7.3, 150 mM NaCl, and 5 mM EDTA) containing 4% SDS. 200 µg of total cellular protein was treated with a final concentration of 10 mM neutralized TCEP for 30 min with end over end rotation. NEM was added to a final concentration of 25 mM and rocking continued for 2 hours. NEM was removed by 3 rounds of chloroform/methanol/H_2O precipitation. The final pellet was resuspended in TEA buffer containing 0.2% Triton X-100. Samples are treated with 0.75 M NH_2OH (+HA) or without hydroxylamine (-HA) and incubated at room temperature for 1 hour. Excess hydroxylamine was removed with one round of chloroform/methanol/H_2O precipitation and the pellet was resuspended in TEA buffer containing 0.2% Triton X-100 supplemented with 1 mM mPEG-Mal (10 kDa; Sigma). Samples were incubated with rocking for 2 hours and reactions were terminated by 1 round of chloroform/methanol/H_2O precipitation. The final pellet was suspended in 1x Laemmli sample buffer and resolved by SDS-PAGE.

Statistics. Error bars represent standard error of the mean. Significance was determined using a student's t-test (two tailed, heteroscedastic) and p-values have been indicated. Unless otherwise indicated, statistical analysis of immunofluorescence results was conducted on at least two independent experiments with ten images per condition per replicate. Statistical analysis of western blotting was conducted on results from three independent experiments.

Chapter 4

Loss of Desmoglein Partitioning to Detergent Resistant Membranes is not Sufficient to Cause Trafficking Defect or Mislocalize an Endogenous Desmoglein

In addition to the data presented in chapter 5, we have accumulated other data relevant to the relationship between desmosomes, lipid rafts, and the novel SAM pathomechanism. In light of the trafficking defect observed for the SAM-causing DSG1 mutation, we investigated whether a similar defect would be seen in the non-raft partitioning Dsg3(ETMD) mutant. In contrast with our findings in the Dsg1(G578R) mutant (Fig. 3.4), we observed no difference in the rate of trafficking of wild type Dsg3 and non-raft Dsg3(ETMD) (Fig. 4.1). Given that this construct is a chimera bearing the E-cadherin transmembrane domain, and E-cadherin is efficiently trafficked to the plasma membrane, it is perhaps unsurprising that Dsg3(ETMD) also efficiently localizes to the plasma membrane. Still, this raises a question. Heretofore, we have focused on the loss of DSG1 partitioning to lipid rafts as the probable mechanism driving defective trafficking and exclusion from desmosomes. Yet the trafficking defect was not replicated by a non-raft Dsg3 mutant. This calls into question whether the SAM mutant's loss of raft targeting is responsible for its trafficking defect. It is possible that the glycine-to-arginine mutation in DSG1 is disrupting trafficking through some altogether different mechanism. For example, perhaps protein-protein interactions mediated by the DSG TMD are required for trafficking, and these are disrupted by the SAM-causing G-to-R mutation, but not by replacement with the E-cadherin TMD. We plan to test this hypothesis by replacing the DSG1 TMD with that of a completely unrelated protein, the linker for activation or T-cells (LAT). LAT partitions to lipid rafts[186] and its TMD bears little sequence similarity to that of DSG1 (data not shown). If DSG1(LAT.TMD) traffics efficiently to the plasma membrane, this would suggest that the trafficking defect in the SAM mutant was not caused by disrupting TMD-mediated protein-protein binding. By contrast, failure of DSG1(LAT.TMD) to traffic efficiently would be consistent with a TMD-mediated protein-protein interaction facilitating DSG1 trafficking.

An alternative explanation is that the more severe shortening of the TMD caused by the SAM mutation (from 24 amino acids to 16 amino acids) results in more complete exclusion from sphingolipid- and cholesterol-enriched domains than does the milder truncation in Dsg3(ETMD) (24 amino acids to 21 amino acids). This interpretation is consistent with other data, including the general agreement between sphingolipid/cholesterol content of organelles and the TMD length of their resident proteins[124]. This question is explored in greater detail in chapter 5.

We also sought to determine how the SAM mutant acts dominantly in our patients. Heterozygous loss of function mutations cause a milder disease, striate palmoplantar keratoderma[254,255]. The DSG1(G562R) mutant must have acquired a novel function which is more deleterious than simple loss of function. One possibility is that the mutant is interacting with and mislocalizing wild type desmogleins, either DSG1 or other endogenous desmogleins. To investigate this hypothesis, we queried the subcellular localization of endogenous DSG2 in the presence of the SAM-causing mutant (Fig. 4.2). In A431 cells, which stably express either wild type or SAM mutant DSG1, we see no recruitment of DSG2 to the perinuclear compartments in which Dsg1(G578R) is mislocalized. We do note some diminution of border staining (not shown), but this finding has not been fully investigated. We also assessed the lipid raft partitioning of endogenous DSG2 in our A431 cell lines which stably express either wild type or SAM mutant DSG1 (Fig. 4.3). We find no change in the lipid raft partitioning of endogenous DSG2 in these cell lines, however the low percentage of cells exogenously expressing Dsg1-GFP could be masking such a change if it existed. These findings and future directions are further explored in the following chapter.

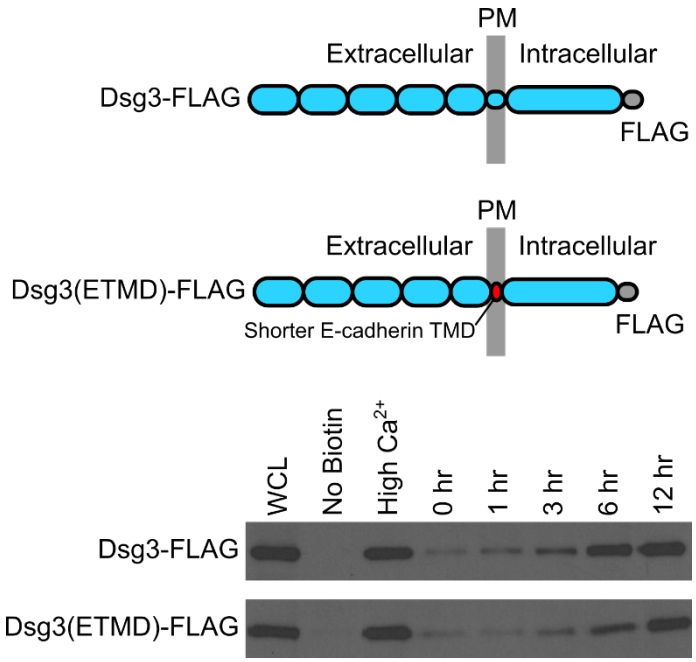

Fig. 4.1: Loss of lipid raft targeting in the Dsg3(ETMD) mutant does not affect protein trafficking to the plasma membrane. A431 cells were grown in low calcium medium prior to the experiment. At t=0, cells were switched to high calcium medium, allowing Dsg3-FLAG to accumulate on the cell surface. After the indicated time, Dsg3 present on the cell surface was assessed via biotin pull-down and subsequent western blotting.

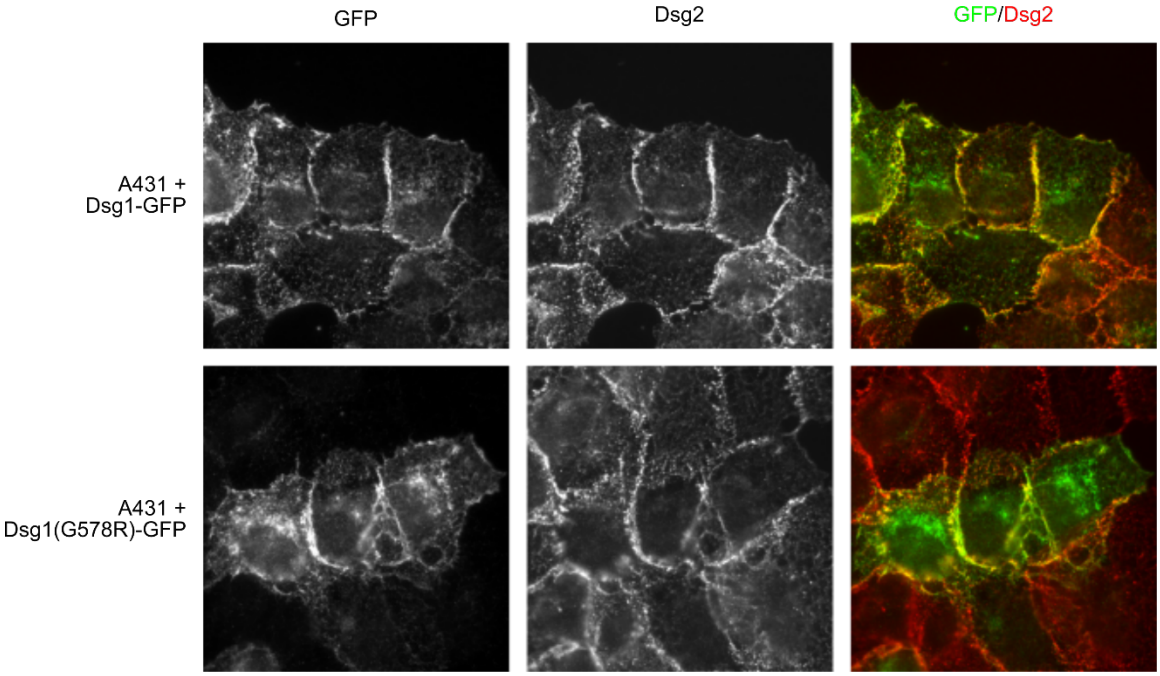

Fig. 4.2: Expression of SAM-causing Dsg1 mutant does not result in mislocalization of endogenous DSG2. A431 cells were grown in low calcium medium prior to the experiment. Three hours before fixation, cells were switched to high calcium. Immunofluorescence was performed to assess subcellular localization. Although Dsg1(G578R) accumulates in a perinuclear compartment, there is no apparent recruitment of endogenous DSG2 into this compartment.

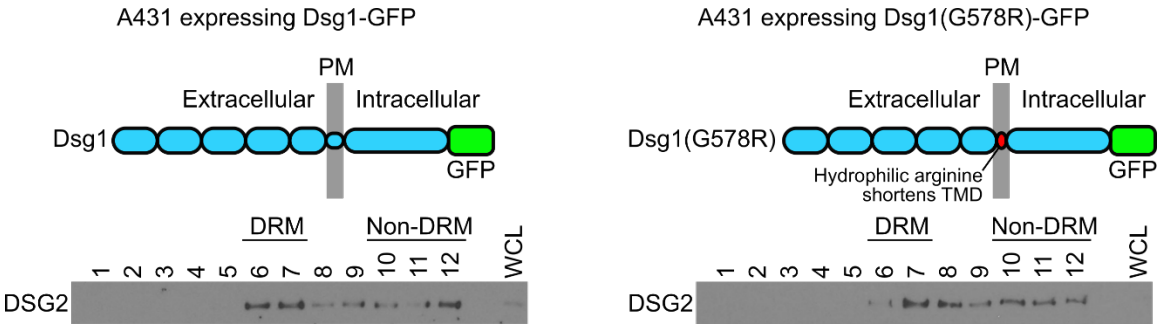

Figure 4.3: Expression of SAM-causing Dsg1 mutant does not diminish DSG2 targeting to lipid rafts. A431 cells stably expressing wild type or SAM mutant DSG1 were subjected to detergent extraction and fractionation. Partitioning of endogenous DSG2 to lipid rafts was not diminished in the presence of Dsg1(G578R).

Chapter 5

Dissertation Summary and Future Directions

This dissertation investigates the association between desmosomes and lipid rafts. Previous work has clearly demonstrated such an association and provided evidence that desmosome assembly, function, and disassembly depend on lipid raft association[39,110,243] (see chapter 3). We have identified the mechanism and demonstrated the importance of lipid raft association for the desmoglein family of desmosomal cadherins. We have also reported an instance of SAM syndrome caused by a mutation in DSG1 which abrogates lipid raft targeting (chapter 3). We believe this finding constitutes the first report of a mutation which prevents raft association and causes a human disease.

In chapter 2, we provide background on the desmosome, lipid rafts, and the association between the two. In chapter 3, we describe two related patients who were diagnosed with SAM syndrome. Unlike previous cases of SAM, this instance is caused by a mutation in the DSG1 transmembrane domain. The resulting non-raft partitioning mutant is defective in its trafficking through the secretory pathway and its incorporation into desmosomes. In this chapter, we also provide evidence that the broader desmoglein family of proteins is targeted to lipid rafts via their lengthy transmembrane domain. While these proteins are subject to palmitoylation, a post-translational lipid modification often responsible for lipid raft targeting, loss of DSG palmitoylation does not prevent partitioning into detergent resistant membranes. Thus we have elucidated the lipid raft targeting mechanism for the DSG family of proteins.

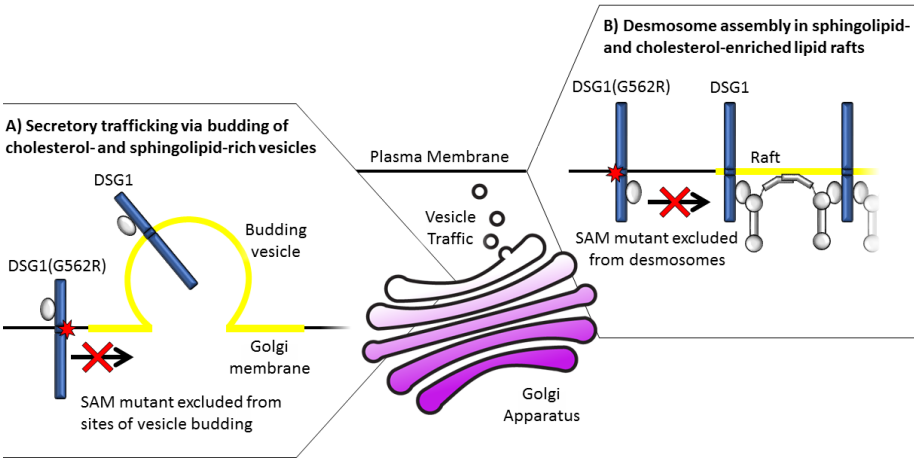

Fig. 5.1: Model: Association with lipid rafts facilitates desmosome assembly. Desmosomal proteins are synthesized and delivered to cell-cell borders during junction assembly. A) With its severe TMD truncation, the DSG1(G562R) SAM mutant is excluded from the cholesterol- and sphingolipid-rich sites of secretory vesicle budding, rendering it partially defective in its trafficking out of the Golgi apparatus. B) Upon arrival at the plasma membrane, association with lipid rafts promotes protein clustering during desmosome assembly. DSG1(G562R) which reaches the plasma membrane is unable to efficiently incorporate into desmosomes, again due to exclusion from the cholesterol- and sphingolipid-rich environment of the desmosome.

A number of questions merit consideration and future investigation. Using a variety of Dsg constructs, cell lines, and experimental regimes, we have demonstrated that the Dsg family of proteins is targeted to lipid rafts via their lengthy transmembrane domain (Fig. 3.5, 3.6, 3.7). However, data from our lab have indicated that wild type Dsg3 fails to partition to lipid rafts in both Chinese Hamster Ovary (CHO) and Human Microvascular Endothelial Cell-1 (HMEC) cells[110]. These cells do not express all desmosomal proteins, nor do they assemble desmosomes. This finding is unexpected; if lipid raft partitioning is an intrinsic property of DSGs, conferred by the biophysical properties of its transmembrane domain, Dsg3 would be expected to partition to lipid rafts in any cell type. In A431D cells, which do not assemble desmosomes, Dsg3 still partitions to lipid rafts. This indicates lipid raft association is not contingent on desmosome assembly. Together, these results suggest that there remain unidentified factors which impact DSG partitioning

to lipid rafts. One explanation is that in A431D cells, Dsg3 could be forming protein complexes, particularly with plakoglobin and plakophilin, which may serve to promote raft association. These complexes would be smaller in scale than a desmosome, but could still promote raft association in a manner similar to FcεRI recruitment to lipid rafts upon clustering of the IgE signal transduction pathway components[207-209] (see section 2.11). This would explain Dsg3 association with lipid rafts in A431D cells, wherein plakoglobin, plakophilin, and desmoplakin are expressed[270,271], and its lack of raft partitioning in CHO cells, where plakophilin and desmoplakin are absent. The hypothesis that desmoglein raft association is driven by protein clustering could be tested by co-expressing Dsg3 with other desmosomal proteins in CHO cells. Pinpointing each factor which is necessary for and contributes to desmoglein association with lipid rafts is particularly important given the human patient described in chapter 3, for whom loss of DSG1 raft partitioning causes disease.

The conceptualization of desmosome assembly as a clustering of lipid raft-associated protein subcomplexes is consistent with other existing data. Prior to desmosome assembly, desmosomal cadherins and plakoglobins form a separate complex from plakophilins and desmoplakin. Classical cadherins bind β-catenin immediately upon synthesis[272], and the same is likely true of the desmosomal cadherins and plakoglobin. Furthermore, protein levels of plakoglobin are precisely regulated to saturate cadherin binding partners[273], suggesting that the desmosomal cadherins are bound to plakoglobin from synthesis to degradation. In the absence of desmosomes, desmoplakin and plakophilin exist in cytoplasmic granules anchored to the keratin cytoskeleton, constituting a separate subcomplex[47,70,72]. During assembly, these subcomplexes are transported to cell borders and cluster to form a desmosome (model and further evidence reviewed by Nekrasova & Green[274]). Mutations in either plakophilin[39] or DSG1 (see chapter 3) which abrogate lipid raft portioning compromise desmosomes, suggesting that these proteins

may be the primary raft targeting component of their respective subcomplexes, and that loss of raft targeting interferes with junction assembly.

Another unresolved question is how this SAM-causing mutation acts dominantly. As described in chapter 3, SAM syndrome is typically caused by homozygous recessive loss-of-function mutations in DSG1. Exceptions include a report of compound heterozygosity[275] and a dominantly acting desmoplakin mutation[100,275]. However, every case investigated to date has shown DSG1 is not expressed in patient epidermis or has been mislocalized away from the plasma membrane[97-100]. The SAM case we describe does not conform to this paradigm. The causative mutation occurs in the DSG1 transmembrane domain (Fig. 3.1F), acts dominantly (Fig. 3.1D), and does not result in total loss of expression on the plasma membrane (Fig. 3.2-3.4). DSG1 expression is diminished in patient epidermis (~60% of control) (Fig. 3.2), raising the possibility that DSG1 haploinsufficiency is pathogenic. However, DSG1 haploinsufficiency manifests as palmoplantar keratoderma and is not sufficient to cause SAM syndrome[254,255]. This strongly suggests there is a mechanism by which DSG1 is acting to inhibit desmosome function. One possibility is that the mutant copy of DSG1 is causing mislocalization of the wild type DSG1 (or other desmogleins or desmocollins), sequestering it away from the sites of cell-cell contact. Classical cadherins form both homo-[27,276,277] and heterodimers[260], and desmosomal cadherin dimerization has also been reported[26]. This hypothesis is consistent with the immunofluorescence from patient skin biopsies (Fig. 3.2), wherein patient DSG1 fluorescence is overwhelmingly punctate. If the patient's wild type copy of DSG1 was normally localized, one would expect a combination of punctate staining contributed by the mutant and normal border staining contributed by wild type DSG1. This hypothesis could be tested by co-expressing wild type and mutant DSG1 with distinguishable epitope or fluorescent tags. Mislocalization of the wild type copy in the presence of the mutant would confirm this hypothesis. Alternatively, the ability of the SAM-causing mutant DSG1

to mislocalize other desmogleins or desmocollins could be tested by staining endogenously expressed desmosomal cadherins in the A431 cell lines stably expressing Dsg1(G578R). Our preliminary results found no evidence of recruitment of DSG2 into perinuclear, Dsg1(G578R)-positive compartments (Fig. 4.2), nor loss of DSG2 partitioning to lipid rafts (Fig. 4.3). However, the cell lines in which these experiments were conducted were not monolithic in terms of Dsg1 expression; only 10-30% of cells appear Dsg1-GFP-positive by immunofluorescence, which would have the effect of understating biochemical readouts like DSG2 partitioning to lipid rafts.

An alternative explanation for the dominant negative activity of the SAM-causing DSG1 mutant is that the mutant is sequestering its binding partner plakoglobin and depleting it from desmosomes. Some support for this hypothesis can be found in the altered localization of plakoglobin in cells expressing mutant DSG1 (Fig. 3.3). Plakoglobin partitioning to lipid rafts also trends downward in cells expressing mutant DSG1, although this finding does not rise to statistical significance (Fig 3.5). As with raft targeting of DSG2, the loss of plakoglobin partitioning to rafts is likely understated because of the low percentage of cells expressing Dsg1. This explanation is further undermined by the fact that plakoglobin expression is tuned to saturate the available cadherin binding partners in mammalian cells[273], rendering it unlikely that a shortage of plakoglobin is limiting the formation of functional desmosomes.

Another means by which the mutant could be acting dominantly is through perturbation of EGFR signaling. As described in section 2.4, DSG1 downregulation of EGFR signaling is a crucial step driving the programmed terminal differentiation of keratinocytes in skin[94]. In SAM patients, thickening of the epidermis, particularly hypergranulosis, has been observed[97,278]. This strongly suggests a differentiation defect in which mutant DSG1 is preventing the EGFR downregulation required for proper keratinocyte differentiation. This is likely the pathomechanism of striate palmoplantar keratoderma, wherein DSG1

haploinsufficiency causes epidermal expansion[254,255]. However, it is possible that the SAM-causing DSG1(G562R) has acquired a novel, dominantly acting activity, resulting in more severe disruption of EGFR signaling on par with the complete loss of DSG1 expression seen in other SAM cases. This hypothesis could be tested by exogenously expressing the SAM-equivalent murine Dsg1(G578R) mutant in a tissue culture model system[279] to see whether it suppresses differentiation. The contribution of EGFR signaling to SAM pathogenesis is under-investigated and could shed further light on the role of DSG1 in epidermal differentiation.

Also unresolved is the exact nature of the contribution of raft partitioning to intracellular trafficking. Our data suggest the SAM-causing mutation causes loss of Dsg1 raft targeting (Fig. 3.5) and Golgi retention (Fig. 3.4), while the non-raft mutant Dsg3(ETMD) traffics normally (Fig. 4.1). There are other examples of mutations which decrease raft affinity and cause trafficking defects[186] (reviewed[200,280]), but certainly many plasma membrane-resident proteins are absent from detergent resistant membranes and are thought to be non-raft associating. The explanation probably lies in a more nuanced understanding of the nature of lipid raft association. As discussed in section 3.5, cholesterol and sphingolipid content increases through the secretory pathway, peaking at the plasma membrane. Vesicles departing the Golgi apparatus en route to the plasma membrane are enriched for sphingolipids and cholesterol[199]. Proteins with minimal affinity for lipid rafts, i.e. those with 15 amino acid TMDs and no palmitoyl groups, are likely excluded from these budding vesicles. Proteins like E-cadherin, with a 21 amino acid TMD, have sufficient affinity for these orderly domains to permit entry and trafficking, but they fail to partition into detergent resistant membranes (DRMs). DRMs probably represent only the most robust, long-lived membrane microdomains, perhaps those stabilized by protein-protein interactions. In this conceptual framework, a protein's affinity for lipid rafts exists on a spectrum, wherein moderate raft affinity is required for anterograde trafficking through

the secretory pathway, and robust raft affinity is required for detection in detergent resistant membranes. This regime is consistent with findings reported by Diaz-Rhorer et al., wherein progressive shortening of the linker for activation of T-cells TMD by 3, 6, or 9 residues resulting in gradual diminution of partitioning to the liquid-ordered phase of giant plasma membrane vesicles[186].

When Roberts et al. mutated plakophilin 2 to prevent its palmitoylation and therefore its lipid raft targeting, they found that expression of a non-raft mutant inhibited desmosome assembly[39]. As mentioned previously, prior to desmosome assembly, plakophilin resides in non-membrane-bound cytoplasmic granules with desmoplakin[47]. While there is no experimental evidence to support this, it seems unlikely that plakophilin is palmitoylated while it resides in cytoplasmic granules. Rather, it seems most likely that plakophilin is palmitoylated by a protein acyl-transferase (PAT) upon arrival at the plasma membrane. DHHC5 and DHHC21 are the PATs most likely to be responsible for palmitoylating PKP, given their plasma membrane localization and widespread expression in various tissues[281]. It also seems likely that palmitoylation not required for recruitment of plakophilin from cytoplasmic granules to the plasma membrane, but instead for its retention after its arrival.

Intriguingly, expression of non-raft desmoglein mutants did not produce the inhibitory effect on desmosome assembly observed for non-raft plakophilin. Neither expression of non-raft Dsg1 nor Dsg3 mutants inhibited desmosome assembly in A431 and other cells (Fig. 3.3, data not shown). This finding is somewhat unexpected; the non-raft DSG1 mutant acts dominantly to cause SAM syndrome. Furthermore, cadherin recruitment to nascent desmosomes precedes plakophilin recruitment. We would have predicted expression of a non-raft DSG mutant would have a more substantial impact on desmosome assembly. This finding could be an artifact stemming from the ratios of mutant and endogenous proteins present in the cell. One way to address this would be to express

non-raft desmoglein in a desmoglein-null background which still expresses the remaining complement of desmosomal proteins. Such a cell line would not assemble desmosomes, but exogenous desmoglein expression should rescue desmosome assembly. However, we would predict that expression of non-raft DSG would fail to rescue desmosome assembly. To date, no such cell line exists.

The mechanism and importance of the lipid raft partitioning of other desmosomal proteins remains undiscovered. As described in chapter 2, all major desmosomal proteins are associated with lipid rafts, but plakophilin and desmoglein are the only proteins whose raft-targeting mechanism has been identified[39] (see chapter 3). Desmocollins could be targeted to lipid rafts by their transmembrane domain, similar to desmogleins. Their shorter transmembrane domain suggests that this might not be the case, and that palmitoylation is more important in conferring lipid raft partitioning on desmocollins. Desmoplakin is not palmitoylated[39], nor is it believed to directly interact with cell membranes[14]. Rather, its recruitment to lipid rafts is likely mediated by protein-protein interactions with other raft-targeting desmosomal proteins such as plakophilin, plakoglobin, and desmosomal cadherins. Investigating the means and functional consequences of raft targeting for these proteins will further elucidate the role lipid rafts play in facilitating desmosome assembly and function.

Concluding remarks

This dissertation provides the first evidence for a human disease caused by the loss of a protein's lipid raft association. Lipid rafts have been recognized as playing important roles in a variety of biological processes, but previous attempts to link lipid rafts to human disease have been indirect. This dissertation describes the first case in which a mutation abrogates a protein's lipid raft partitioning and gives rise to a human disease, SAM syndrome. The mutated protein, desmoglein 1, is rendered defective in its trafficking through the secretory pathway and in its incorporation into desmosomes. This finding constitutes a novel pathomechanism for desmosomal disease and for human disease writ large.

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