The Role of Desmoglein 1 in Gap Junction Turnover Revealed through the Study of SAM Syndrome


An effective epidermal barrier requires structural and functional integration of adherens junctions, tight junctions, gap junctions (GJ), and desmosomes. Desmosomes govern epidermal integrity while GJs facilitate small molecule transfer across cell membranes. Some patients with severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome, caused by biallelic desmoglein 1 (DSG1) mutations, exhibit skin lesions reminiscent of erythrokeratodermia variabilis, caused by mutations in connexin (Cx) genes. We, therefore, examined whether SAM syndrome-causing DSG1 mutations interfere with Cx expression and GJ function. Lesional skin biopsies from SAM syndrome patients (n = 7) revealed decreased Dsg1 and Cx43 plasma membrane localization compared with control and nonlesional skin. Cultured keratinocytes and organotypic skin equivalents depleted of Dsg1 exhibited reduced Cx43 expression, rescued upon re-introduction of wild-type Dsg1, but not Dsg1 constructs modeling SAM syndrome-causing mutations. Ectopic Dsg1 expression increased cell-cell dye transfer, which Cx43 silencing inhibited, suggesting that Dsg1 promotes GJ function through Cx43. As GJA1 gene expression was not decreased upon Dsg1 loss, we hypothesized that Cx43 reduction was due to enhanced protein degradation. Supporting this, PKC-dependent Cx43 S368 phosphorylation, which signals Cx43 turnover, increased after Dsg1 depletion, while lysosomal inhibition restored Cx43 levels. These data reveal a role for Dsg1 in regulating epidermal Cx43 turnover.


INTRODUCTION

Intercellular junctions, including adherens junctions, tight junctions, gap junctions (GJ), and desmosomes are required for proper epidermal barrier formation and function (Meše et al., 2007). GJs are formed by proteins from the connexin (Cx) family encoded by genes subdivided into five subfamilies (GJA1–GJ6; Martin et al., 2014; Beyer et al., 2018). Cx proteins form homomeric or heteromeric hexamers (termed connexons) on two adjacent cells to produce conducted hemichannels for direct cell-cell molecular exchange (Boyden et al., 2016; Laird, 2008). Cx43, one of the Csx expressed in the epidermis, has a short half-life of one to three hours. Its dynamics are regulated by phosphorylation of several C-terminal amino acids, which affects connexin internalization, degradation, and assembly into gap junctions (Solan and Lampe, 2018).

GJs play diverse roles in normal physiology, and skin diseases have been attributed to mutations in genes encoding five of the Cxs- Cx26, Cx30, Cx30.3, Cx31 and Cx43 (LaiCheong et al., 2007; Scott et al., 2011; Boyden et al., 2015; Shuja et al., 2016). Erythrokeratodermia variabilis (EKV) is a group of diseases mainly caused by mutations in Cx-encoding genes. Most frequently mutated are GJB3 and GJB4, encoding Cx30.3 and 31, respectively (Richard et al., 1998; Macari et al., 2000). Although less frequent, mutations in GJA1, encoding Cx43, also cause EKV (Boyden et al., 2015), and EKV-causing mutations in the Cx31-encoding gene can exert trans-dominant functional inhibition on Cx43 (Schnichels et al., 2007; Scott et al., 2011).

Severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome is caused by bi-allelic mutations in DSG1, encoding the desmosomal cadherin desmoglein 1 (Dsg1) (Samuelov et al., 2013). Curiously, some SAM syndrome patients present with chronic circumscribed, well-demarcated, hyperkeratotic, erythematous to brownish
plaques mimicking EKV (Has et al., 2015; Cheng et al., 2016; Schlief et al., 2016; Taiber et al., 2018). Desmosomal components such as desmoglein 2, desmoplakin (DP), plakoglobin, and plakophilin 2 can regulate Cx expression, trafficking, localization, and ubiquitination, especially in cardiac tissue (Oxford et al., 2007; Asimaki et al., 2009; Asimaki et al., 2014; Gehmlich et al., 2010; Patel et al., 2014; Kam et al., 2018). Therefore, we hypothesized that Dsg1 plays a role in GJ dynamics in the skin. Here, clinical and histological analysis of biopsies from patients representing families with three different SAM syndrome mutations, along with in vitro analysis of keratinocytes deficient for Dsg1 or expressing Dsg1 constructs representing SAM syndrome mutations, revealed a role for Dsg1 in regulating Cx43 stability and function.

RESULTS

EKV-like clinical features in SAM syndrome
Seven patients from three families were investigated, six females and one male. Ages ranged from 4–32 years. Clinical follow-up ranged from 4–28 years. Figure 1a presents a schematic of the DSG1 mutations associated with these patients.

The homozygous c.2659C>T mutation introduces a premature termination codon (p.R887X; Has et al., 2015; Taiber et al., 2018). The homozygous 1861delG mutation is expected to cause a frameshift resulting in a premature stop codon (p.A621Gfs*3; Samuelov et al., 2013). The homozygous c.49-1G>A mutation was previously shown to cause an in-frame skipping of DSG1 exon 2. The resulting 12-amino-acid deletion in the Dsg1 signal peptide leads to Dsg1 mislocalization and accumulation in the ER and Golgi (Samuelov et al., 2013; Figure 1a).

All patients included in the study presented with severe palmoplantar keratoderma and food allergies. In five of the patients, the predominant cutaneous clinical feature was hyperkeratotic, scaly, erythematous to brown plaques, reminiscent of EKV (Dsg1 mutations c.2659C>T and c.49–1G>A). Two patients suffered from longstanding, nonremitting erythroderma (Dsg1 mutation c.1861delG). Representative clinical images are shown in Figure 1b and Supplementary Figure S1.

Dsg1 expression varies within the same individual between lesional and nonlesional skin
To determine how DSG1 mutations impact Dsg1 protein expression and localization in patient skin, we conducted immunofluorescence staining of skin biopsies from each of the patients using two different antibodies recognizing Dsg1. One antibody recognizes the Dsg1 ectodomain (EC). The second antibody recognizes the C-terminal domain (amino acids, 1000–1049).

Compared with the plasma membrane localization of Dsg1 in control skin, plasma membrane Dsg1 was partially lost in all of the patient biopsies using the EC antibody (Figure 2a, b, and Supplementary Figure S2). Increased cytoplasmic signal with patchy plasma membrane staining was observed in patients harboring the c.1861delG and c.49–1G>A mutations with the EC antibodies, particularly in the lower spinous layers. However, Dsg1 staining was not observed in biopsies from patients carrying either the c.2659C>T or c.1861delG mutation when stained with the C-terminal antibody, consistent with the predicted premature termination of protein translation. The mixed junctional and cytoplasmic staining pattern for the c.49–1G>A mutation was similar when using either the EC or C-terminal antibody.

The c.2659C>T and c.1861delG mutations were both previously reported to result in negligible amounts of both protein and RNA, the latter reportedly due to mRNA decay (Samuelov et al., 2013; Has et al., 2015). Because we observed Dsg1 protein in the skin biopsies from these patients, we hypothesized that mutant Dsg1 expression and localization might vary between diseased and healthy skin. Using the EC antibody, we compared Dsg1 expression in biopsies from lesional and nonlesional trunk skin from the same patient (Figure 2c). Significantly reduced membraneto-cytoplasmic Dsg1 ratios were observed in all lesional areas compared with the nonlesional skin from the same patient, independent of the mutation type (Figure 2d). Patients with c.1861delG and c.49–1G>A mutations also showed prominent cytoplasmic Dsg1 staining.

Considering that most patients with SAM syndrome do not manifest blistering disease, we hypothesized that the expression of other junctional proteins is sufficient to prevent loss of adhesion. To test this, we first stained lesional skin samples for DP, β-catenin and desmocollin 1 (Supplementary Figure S3), which showed that membrane to cytoplasm ratios of DP and β-catenin were not reduced compared with control, while the desmocollin 1 ratio was even increased in the c.1861delG and c.49–1G>A biopsies. Furthermore, the expression and localization of the junctional proteins DP, plakoglobin, E-cadherin and β-catenin were not altered in submerged normal human epidermal keratinocyte cultures (NHEKs) or epidermal raft cultures upon Dsg1 depletion compared with controls (Supplementary Figure S4a, b, c, d).

Finally, the dispase based dissociation assay was used to test the adhesive properties of Dsg1-silenced NHEKs compared with control and DP-silenced NHEKs (Supplementary Figure S4e, f, g). Control and Dsg1-silenced cell sheets remained intact compared with DP-silenced cell sheets, which showed significant fragmentation (Supplementary Figure S4f). These results suggest that loss of Dsg1 is insufficient to weaken keratinocyte adhesion when other desmosomal proteins are still expressed and functioning to maintain adhesion at the plasma membrane.

Plasma membrane-localized Cx43 is reduced in patients with SAM syndrome and correlates with Dsg1 expression
Several of the SAM syndrome patients analyzed in this report presented with clinical features similar to EKV. In this work, we focused on Cx43, as it is one of the GJ proteins linked to EKV (Boyden et al., 2015) and its expression and localization are influenced by desmosomes in skin and heart. (Oxford et al., 2007; Noorman et al., 2013; Asimaki et al., 2014; Patel et al., 2014; Kam et al., 2018). We hypothesized that loss of Dsg1 might lead to abnormal GJ protein expression and impaired function.

To test this idea, we first carried out immunofluorescence staining for Dsg1 and Cx43 in lesional and nonlesional patient skin (Figure 3a–c). Then, using E-cadherin as a plasma membrane marker, we quantified plasma membrane-associated
**Cx43** (see methods for detailed explanation). This analysis showed that mean plasma membrane-localized Cx43 intensity was higher in control and nonlesional epidermis than in lesional skin from patients with the c.2659 C>T mutation and c.49-1 G>A mutation (Figure 3d, e). Particularly compelling is a side-by-side comparison of adjacent regions within a biopsy taken at the boundary between lesional and nonlesional skin in a patient with the c.1861delG mutation. In this example, relatively normal Dsg1 expression is observed next to areas lacking Dsg1 staining (Figure 3c). The analysis revealed higher Cx43 levels in Dsg1 positive cells compared with Dsg1 negative cells (Figure 3f).

**Dsg1 loss reduces Cx43 expression, which is restored by ectopically expressed wild-type Dsg1, but not SAM syndrome mutants**

To test the hypothesis that Dsg1 loss affects Cx43 expression, we compared Dsg1 and Cx43 expression in NHEK controls and cells in which Dsg1 was depleted by shRNA. Cx43 expression was significantly reduced when Dsg1 was depleted compared with control cells (Figure 4a, b).

To demonstrate a cause-and-effect relationship between Dsg1 and Cx43, we infected control or Dsg1-silenced NHEKs with retroviruses expressing full-length-Dsg1-FLAG (Dsg1-FL, FL) or constructs simulating the SAM syndrome-causing mutations. The Dsg1-ΔExon2-FLAG (ΔExon2, ΔEx2) construct simulates the c.49-1 G>A mutation; whereas, the Dsg1-Δ909-FLAG (Δ909) has a premature termination codon close to that created by the c.2659 C>T mutation (p.887X). A schematic of these constructs, their expression in NHEKs, and quantification of membrane-to-cyttoplasmic fluorescence intensities are provided in Supplementary Figure S5. FL-Dsg1 expression restored Cx43 staining intensity in Dsg1 knockdown NHEKs, but neither Dsg1-ΔExon2-FLAG nor Dsg1-Δ909-FLAG was able to do so (Figure 4c, d, S5d). Reduced expression of Cx43 at the plasma membrane was also observed in Dsg1-silenced 3D organotypic cultures when compared with controls (Supplementary Figure S6a, b).
Figure 2. Dsg1 expression varies within the same individual between lesional and nonlesional skin. (a) Immunofluorescence staining of biopsies from lesional skin obtained from a patient from each family in the study compared with control skin, using an anti-Dsg1 EC antibody and an anti-Dsg1 CT antibody (scale bar = 20 μm). (b) The ratio of plasma membrane to cytoplasmic Dsg1 staining using the ectodomain antibody (E-cadherin was used as a membrane marker). n = 20 borders per sample; error bars, mean difference ± SD. ****P < 0.0001, one-way analysis of variance with Dunnet post hoc test. (c) Immunofluorescence staining of lesional and nonlesional skin sections with the ectodomain Dsg1 antibody (scale bar = 20 μm). Red boxed regions of the suprabasal layers of the patient epidermis are shown magnified to the right of the lower magnification images (scale bar = 20 μm). (d) Plasma membrane-to-cytoplasmic Dsg1 ratio (E-cadherin was used as a membrane marker). n = 20 borders per sample; error bars, mean difference ± SD. ****P < 0.0001, two-tailed Student t test. L, lesional; CT, C-terminal; EC, ectodomain; N.L., nonlesional.
Figure 3. Plasma membrane-localized Cx43 intensity is reduced in patients with SAM syndrome, correlating with Dsg1 expression. (a, b) Immunofluorescence staining of Dsg1 (EC antibody) and Cx43 in lesional and nonlesional skin sections from patients with c.2659C>T and c.49-1G>A Dsg1 mutations (scale bar = 20μm). Insets are magnified regions of plasma membranes demonstrating the level of Dsg1 and Cx43 localization in lesional and nonlesional areas of the biopsies. (c) Immunofluorescence staining at the edge of lesional skin (c.1861delG-I) showing areas of low and normal Dsg1 expression and corresponding Cx43 levels (scale bar = 20 μm). (d–f) Quantification of plasma membrane-associated Cx43 staining intensity from panels a–c. n = 30 borders per sample; error bars, mean difference ± SD. *P = 0.023; **P = 0.0045; ***P = 0.0003; ****P < 0.0001, one-way analysis of variance followed by Bonferroni post hoc test. EC, ectodomian; L, lesional, N.L., nonlesional.
Dsg1 promotes GJ function through Cx43
We next used a dye transfer assay to test the functional impact of Dsg1 on GJ intercellular communication (GJIC), evaluating the cell-to-cell transfer of the GJ permeable lucifer yellow dye as an indicator of GJIC. NHEKs infected with green fluorescent protein (GFP) or FLAG-tagged Dsg1-FL were grown to confluence and cultured in 1.2 mM calcium-containing media for 16 hours, which is enough time to ensure that Dsg1-FL is localized to the membrane, but not enough time for endogenous Dsg1 to be expressed (Figure 4e). Cells expressing Dsg1-FL had significantly higher cell-to-cell lucifer yellow dye transfer than controls (115.36 ± 4.95 vs. 68.2 ± 4.26) (Figure 4f), indicating a functional role for Dsg1 in GJIC.

Plasma membrane-localized Cx43 was significantly higher in the Dsg1-FL infected cells compared with GFP infected controls (7.08 ± 1.32 vs. 1.36 ± 1.09), consistent with the idea that Dsg1 promotes GJIC through Cx43 (Figure 4g, h). To directly test the role of Cx43 in Dsg1-mediated GJIC, we silenced Cx43 using siRNA against Cx43 in NHEKs infected with Dsg1-FL, effectively reducing lucifer yellow dye transfer compared with the transfer in control Dsg1-FL expressing NHEKs treated with nontargeting siRNA (52.2 ± 12.9 vs. 111.9 ± 15.2) (Figure 4i–k).

Dsg1 loss is associated with enhanced Cx43 degradation involving PKC-mediated phosphorylation of Cx43 Serine 368
We next determined whether Dsg1 regulates the expression of Cx43 at the mRNA or protein level. GJA1 mRNA expression was not reduced in shDsg1- compared with shControl-infected NHEKs (Figure 5a), suggesting that post-transcriptional regulation is involved. Cx43 and other GJ proteins have short half-lives, indicating high rates of remodeling and turnover (Laird et al., 1991; Lampe and Lau, 2004; Solan and Lampe, 2014). Cx43 turnover is tightly regulated, and misregulation contributes to pathogenicity (Fernandes et al., 2004; Kam et al., 2018). The lysosome is the main site of Cx43 degradation (Cone et al., 2014; Su and Lau, 2014). To test whether loss of Dsg1 results in Cx43 protein degradation, we treated shControl- and shDsg1-infected NHEKs with the lysosomal inhibitor chloroquine. Chloroquine increased Cx43 protein to comparable levels in control and shDsg1-expressing NHEKs, consistent with a role for Dsg1 in preventing Cx43 degradation (Figure 5b, c). Similar results were observed using leupeptin, another lysosome inhibitor (Supplementary Figure S6c).

Phosphorylation of multiple serine residues at the C-terminus of Cx43 regulates GJ transport, assembly, and internalization; Cx43 phosphorylation is regulated by several signaling pathways (ERK, AKT, PKC; Solan and Lampe, 2014). Phosphorylation of S325, S328, S330, S364/365, and S373 enhance GJIC; whereas, phosphorylation of S255, S262, S279/282, and S368 downregulate Cx43 (Alström et al., 2015; Bao et al., 2004). We previously demonstrated that DP loss results in Cx43 degradation through Erk-dependent phosphorylation of S279/282 (Kam et al., 2018); therefore, we evaluated the level of phosphorylation at Cx43 residues S279/282 in shDsg1-infected NHEKs compared with shControl-infected NHEKs. There was no change in the level of pCx43 S279/282 phosphorylation (Supplementary Figure S6d, e). PKC regulates the internalization and degradation of Cx43 by phosphorylating S368 (Rivedal and Opsahl, 2001; Cone et al., 2014; Su and Lau, 2014) and desmosomes have also been linked to PKC regulation (Johnson et al., 2014a). Thus we next evaluated the PKC-dependent Cx43 phosphorylation site, S368. The ratio of pCx43 S368/total Cx43 was significantly higher in shDsg1-infected cells compared with shControl-infected NHEKs, suggesting Cx43 phosphorylation at that site leads to increased degradation secondarily to Dsg1 loss (Figure 5d, e).

We then stained skin biopsies obtained from SAM syndrome patients for pCx43 S368 to determine whether SAM syndrome-associated Dsg1 mutations correlated with increased pCx43 S368 phosphorylation in vivo (Figure 5f). A significantly higher ratio of pCx43 S368/Cx43 was observed in lesional patient skin compared with nonlesional and control skin (Figure 5g).

Finally, we tested whether PKC inhibition with bisindolylmaleimide would reduce pCx43 S368 and upregulate total Cx43 expression in NHEKs infected with shControl- or shDsg1-expressing retroviruses. PKC inhibition significantly decreased the ratio of pCx43 S368/total Cx43 in the context of Dsg1 depletion (Figure 5h, i). In addition, we observed an increase in total Cx43 in bisindolylmaleimide-treated control and recovery of total Cx43 in bisindolylmaleimide-treated shDsg1 cells. Together, these data suggest that PKC-mediated phosphorylation of pCx43 S368 is responsible for Cx43 turnover downstream of Dsg1 loss.

DISCUSSION
In the present work, recognition of shared clinical phenotypes in patients with SAM syndrome and EKV led to the elucidation of a role for Dsg1 in Cx43 stabilization and GJ function in the epidermis. Supporting a role for Dsg1 in stabilizing Cx43, we observed a positive association between plasma membrane-localized Dsg1 and robust Cx43 intensity in nonlesional skin, while areas of Dsg1 loss were associated with decreased Cx43 intensity in lesional skin of SAM syndrome patients. In vitro, Dsg1 loss decreased Cx43 expression, which was rescued by ectopic Dsg1-FL expression, but not by constructs modeling SAM syndrome mutations. Ectopic expression of Dsg1-FL enhanced dye transfer between cells, which was prevented by Cx43 silencing. This observation supports a role for Dsg1 in promoting GJIC through regulation of Cx43 expression and localization in the connexon.

As desmosomal components can regulate and scaffold PKC (Bass-Zubek et al., 2008; Johnson et al., 2014a) and desmosome instability can activate PKC (Osada et al., 1997), we hypothesized that PKC-mediated phosphorylation of Cx43 S368 regulates Cx43 turnover (Cone et al., 2014) downstream of Dsg1 loss. Indeed, Dsg1 loss was associated with an increased pCx43 S368/total Cx43 ratio both in genetically manipulated cells and in the lesional skin of SAM syndrome patients. PKC inhibition blocked S368 phosphorylation, significantly reversing the pCx43 S368/total Cx43 ratio in Dsg1-depleted cells. Thus, disturbance of Dsg1 expression alters PKC signaling to impact GJs.

Desmosomal proteins have previously been reported to play roles in Cx43 dynamics in both the heart and skin.
Figure 4. Dsg1 loss reduces Cx43 expression while Dsg1 expression promotes GJ function through Cx43. (a) Immunoblot of Dsg1 and Cx43 in normal human epidermal keratinocytes (NHEKs) infected with shControl or shDsg1 72 hours after calcium switch (tubulin = loading control, n = 6). (b) Quantification of fold changes in Cx43 protein expression presented in panel a. Error bars, mean ± SD. ****P < 0.0001, two-tailed Student t test. (c) Immunofluorescence of Dsg1, FLAG, and Cx43 in NHEKs infected with shDsg1, or shDsg1 in combination with Dsg1-ΔExon2-FLAG (ΔExon2), Dsg1-Δ909-FLAG (Δ909), or Dsg1-FL-FLAG (FL) 72 hours after switching cells to high calcium-containing medium. Red arrowheads highlight examples of Cx43 localization at cell borders, coincident with Dsg1-FL construct expression (scale bar = 10 μm). Refer to Supplementary Figure S5d for Dsg1 and Cx43 staining of shControl-infected suprabasal cells. (d) Quantification of plasma membrane-localized Cx43 from panel c (plakoglobin intensities were comparable among samples and was used as a membrane marker. Average of three independent experiments with at least 20 borders/condition/experiment; error bars, mean ± SD. *P = 0.012, by one-way analysis of
Cx43 is the main GJ protein affected by modulation of desmosomes (Oxford et al., 2007; Noorman et al., 2013; Asimaki et al., 2014; Patel et al., 2014; Kam et al., 2018; Boyden et al., 2016). For instance, we previously showed that DP’s N-terminus interacts with EB1 to promote microtubule stabilization at the cell surface, associated with an increase in plasma membrane-localized Cx43 in both cardiomyocytes and keratinocytes. Furthermore, N-terminal DP mutations associated with arrhythmogenic cardiomyopathy and skin fragility-wooly hair syndrome interfere with the DP-EB1 interaction, resulting in Cx43 mislocalization. This mislocalization is thought to be due to a failure of Cx43 to undergo plus-ended microtubule-mediated delivery of Cx43 to the membrane (Patel et al., 2014). We also previously demonstrated that loss of DP triggers ERK1/2/MAPK dependent phosphorylation of Cx43 at residues S279/S282, signaling the internalization and lysosomal degradation of the connexin (Kam et al., 2018). Other desmosomal proteins have also been shown to regulate Cx43 localization. For example, arrhythmogenic cardiomyopathy resulting from desmoglein 2 mutations was associated with Cx43 mislocalization (Gehmlich et al., 2010; Schlipp et al., 2014; Kant et al., 2015). Mis-localized Cx43 was observed in a zebrafish model of arrhythmogenic cardiomyopathy caused by a JUP (i.e., junctional plakoglobin encoding gene) 2057del2 mutation (Asimaki et al., 2014). Thus, diverse mechanisms have evolved to mediate Cx43 positioning and function via desmosome molecules.

In light of these observations, we tested the extent to which Cx43 loss in SAM syndrome is caused directly by impaired Dsg1 or by a more general adhesion defect. Toward this end, we determined the expression and distribution of other junctional proteins, including DP, desmocollin 1, E-cadherin, and β-catenin. All of these proteins were preserved in the skin of the SAM syndrome patients analyzed in this study (Supplementary Figure S3), as well as in cell culture and epidermal raft models of Dsg1 loss (Supplementary Figure S4). We further found no significant change in the adhesive properties of Dsg1-deficient NHEKs compared with controls; whereas, DP-silencing increased monolayer fragility, as has been previously reported (Carbal et al., 2012). Consistent with these results, SAM syndrome patients do not typically present with blistering of the sort observed in toxin-autoantibody-mediated Dsg1 diseases like staphylococcal scalded skin syndrome and pemphigus foliaceus (Amagai, 2010). Although we do not rule out that other junctional proteins contribute to GJ function, Dsg1 plays a critical role in controlling Cx43 under the in vitro and in vivo conditions studied here (Figure 4). Together with our previous observations, Dsg1 and DP, through distinct mechanisms, both inhibit plasma membrane turnover of Cx43.

So far, SAM syndrome has been reported in 13 patients from nine families (Samuelov et al., 2013; Has et al., 2015; Cheng et al., 2016; Schlifé et al., 2016; Dănescu et al., 2017; Lee et al., 2018; Taiber et al., 2018). The patients share features of dermatitis, palmpoplantar keratoderma (PPK), and allergy, yet present with phenotypic variability, even between patients harboring the same mutation (Cheng et al., 2016). In this study, we showed differences in Dsg1 expression at the same anatomic location and within the same patient when comparing samples obtained from lesional and nonlesional areas. We show that at least some SAM syndrome-associated Dsg1 mutants are expressed, and are capable of localization at the cell membrane.

It is known that physiologic expression of Dsg1 is impaired by external stimuli such as UV irradiation (Johnson et al., 2014b), S. aureus-stimulated serine proteases (Williams et al., 2017) and inflammatory cytokines (Davis et al., 2016), the latter which are expressed by keratinocytes isolated from SAM syndrome patients (Samuelov et al., 2013). Mutant Dsg1 proteins may be more susceptible to degradation or turnover compared with wild-type Dsg1. Future delineation of the detailed mechanisms underlying Dsg1 turnover could provide new therapeutic targets for SAM syndrome patients.

Shared clinical features between SAM syndrome and KEV patients led us to uncover molecular and mechanistic connections between Dsg1 and Cx43. However, even though Cx43 is reduced in areas of Dsg1 loss in lesional biopsies from patients with c.1861delG Dsg1 mutations, these patients present with sustained erythoderma, rather than KEV-like symptoms. It may be the case that additional epidermal barrier components were compromised in these patients, resulting in the observed differences in phenotype.

The role of Cx43 in epidermal differentiation is still obscure. Cx43 KD caused impaired differentiation in 3D epidermal rafts (Langlois et al., 2007). A truncated Cx43 variant caused lethal permeable epidermis in a knock-in mouse model (Maass et al., 2004), despite the preserved ability of the truncated protein to form functional GJs, which implies that the role of Cx43 in epidermal barrier formation may go beyond GJIC. Cx43 interacts with other important components of epidermal differentiation, such as the tight junction protein zonula occludens 1, and β-Catenin (Thévenin et al., 2017; Leithé et al., 2018). Hence, Dsg1-governed stabilization of Cx43 might contribute directly to
Figure 5. Dsg1 loss associates with enhanced Cx43 degradation involving PKC-mediated phosphorylation of Cx43 Serine 368. (a) GJA1 gene expression in shControl- or shDsg1-infected NHEKs (n = 3; error bars, mean ± SD, two-tailed Student t test). (b) Immunoblot of Dsg1, Cx43 in shControl or shDsg1 infected NHEKs treated with PBS or 100 μM Cq for 16 hours (tubulin used as loading control; representative of n = 3). (c) Fold changes of total Cx43 from panel b (error bars, mean ± SD. *P = 0.03, two-way analysis of variance with Sidak post hoc test). (d) Immunoblot of Cx43, pCx43 S368 in NHEKs infected with shControl or shDsg1 harvested 72 hours after the switch to high calcium medium (representative of n = 5). (e) Fold changes in the ratio of pCx43 S368 to total Cx43 from panel d (error bars, mean ± SD. ***P = 0.0005, two-tailed Student t test). (f) Lesional and nonlesional skin from two patients with different Dsg1 mutations stained for total Cx43 and pCx43 S368 (scale bar = 30 μm). (g) Quantification of plasma membrane pCx43 S368/Cx43 ratios from panel f (n = 30 borders from each sample; error bars, mean ± SD. ****P < 0.0001 by one-way analysis of variance with Dunnet post hoc test). (h) Immunoblot of total

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**GJA1 Gene Expression Fold**

**ShCo**

**ShDsg1**

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**pCx43 S368/Cx43 Ratio**

**shControl**

**shDsg1**

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**DMSO**

**BIM**

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**Figure 5. Dsg1 loss associates with enhanced Cx43 degradation involving PKC-mediated phosphorylation of Cx43 Serine 368. (a) GJA1 gene expression in shControl- or shDsg1-infected NHEKs (n = 3; error bars, mean ± SD, two-tailed Student t test). (b) Immunoblot of Dsg1, Cx43 in shControl or shDsg1 infected NHEKs treated with PBS or 100 μM Cq for 16 hours (tubulin used as loading control; representative of n = 3). (c) Fold changes of total Cx43 from panel b (error bars, mean ± SD. *P = 0.03, two-way analysis of variance with Sidak post hoc test). (d) Immunoblot of Cx43, pCx43 S368 in NHEKs infected with shControl or shDsg1 harvested 72 hours after the switch to high calcium medium (representative of n = 5). (e) Fold changes in the ratio of pCx43 S368 to total Cx43 from panel d (error bars, mean ± SD. ***P = 0.0005, two-tailed Student t test). (f) Lesional and nonlesional skin from two patients with different Dsg1 mutations stained for total Cx43 and pCx43 S368 (scale bar = 30 μm). (g) Quantification of plasma membrane pCx43 S368/Cx43 ratios from panel f (n = 30 borders from each sample; error bars, mean ± SD. ****P < 0.0001 by one-way analysis of variance with Dunnet post hoc test). (h) Immunoblot of total

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the establishment of an efficient epidermal barrier, and its loss may result in the perturbed barrier as occurs in SAM syndrome.

In summary, here we demonstrate a role for Dsg1 in stabilizing the GJ protein Cx43, strengthening the evidence that desmosomes and GJs work together to govern proper epidermal architecture and barrier formation.

MATERIALS AND METHODS

Patients

Patients were recruited from the Department of Dermatology of Emek Medical Center, Afula, Israel during the years 2016–2018. Some of the patients were reported previously (Samuelov et al., 2013; Taiber et al., 2018). Patients or their legal guardians signed written informed consent before their inclusion in the study, according to a protocol approved by the Emek Medical Center (IRB Protocol #0086–15). Patients or their legal guardians signed written informed consent for publication of clinical photos. De-identified control skin biopsies were obtained from volunteers that signed written informed consents and the biopsies were provided by the Skin Biology and Diseases Resource-Based Center of Northwestern University (IRB Protocol#STU00009443).

Cell culture

Primary NHEKs were obtained through the Skin Biology and Diseases Resource-Based Center of Northwestern University, where mycoplasma testing is routinely performed. The NHEKs were isolated from human foreskin as previously described (Halbert et al., 1992) and grown in M154 media (Life Technologies, Thermo Fisher Scientific) supplemented with 0.07 mM CaCl₂, human keratinocyte growth supplement, and gentamicin/amphotericin B solution (Life Technologies, Thermo Fisher Scientific). Keratinocytes were infected as described in the Supplementary Materials with supernatants from Phoenix cells that produce retroviruses (provided by G. Nolan, Stanford University, Stanford CA) as previously described (Getsios et al., 2004). Keratinocytes were then grown to confluency in M154 media (0.07 mM CaCl₂) and switched to M154 media supplemented with 1.2 mM CaCl₂ for 72 hours to induce differentiation. To test inhibition of lysosomal degradation NHEKs were differentiated for 72 hours and then treated overnight with 100 μM chloroquine (lysosomal inhibitor; MP Biomedicals, Solon, OH) or 200 μM Leupeptin (MilliporeSigma, St. Louis, MO) vs. phosphate-buffered saline (PBS) as control. To inhibit PKC activity, NHEKs differentiated for 72 hours were treated for 1 hour with 12.5 μM bisindolylmaleimide (PKC inhibitor; MilliporeSigma) or DMSO as a control.

DNA constructs

LZRS-Non Target-GFP (i.e., shControl), LZRS-GFP, LZRS-Dsg1-FLAG, and LZRS-shDsg1-GFP were generated as described (Getsios et al., 2004). LZRS-Dsg1-DΔ909-FLAG was generated as described (Nekrasova et al., 2018). LZRS-Dsg1-DΔExon2-FLAG was generated to correspond with the human Dsg1 mutation c.49-1 G > A reported by our group (Samuelov et al., 2013). This mutation was shown to cause in-frame skipping of exon 2 in two patients with SAM syndrome. The construct was made using the PCR product of full-length Dsg1 NM_001942.2; nucleotides 213–3359 with deletion of exon 2, corresponding to nucleotides 261–296 and ligating this product to a C-terminal flag epitope followed by a stop codon. The resulting ΔEx2-Dsg1-FLAG was then cloned into the pLZRS vector (provided by M. Denning, Loyola University, Chicago, IL). Briefly, NHEKs were infected with LZRS-shControl-GFP, LZRS-shDsg1-GFP, or with dual infection of LZRS-shDsg1-GFP to deplete endogenous expression of wild-type Dsg1 plus the following constructs, LZRS-Dsg1-FL-FLAG, LZRS-Dsg1-DΔ909-FLAG, or LZRS-Dsg1-DΔExon2-FLAG. Further details about infection procedures are provided in the Supplementary Materials and Methods.

Antibodies

Antibodies utilized in this study are listed in Table S1.

Western blotting

For analysis of protein expression levels, cells were washed twice with PBS and lysed in urea sample buffer (10 M deionized urea, 1% sodium dodecyl sulfate, 10% glycerol, 60 mM Tris, pH 6.8, and 5% β-mercaptoethanol). Total protein concentrations were equalized, and samples were run on 7.5–10% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes to be probed with primary and secondary antibodies against proteins of interest (Table S1). Chemiluminescent imaging was performed using an Odyssey Fc imaging system, and densitometry was performed using Fc image studio version 5.x (LI-COR, Lincoln, NE). Densitometry quantification was normalized using GAPDH or tubulin as loading controls. Full blots are presented in Supplementary Figures S7 and S8.

Immunofluorescence of cells and paraffin-embedded skin sections

Cells grown on glass coverslips were washed in PBS, fixed using 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, at room temperature. Primary antibodies diluted in 0.5% bovine serum albumin in PBS were added to coverslips and incubated at 37 °C in a humidified chamber for 1h followed by multiple washes in PBS. Alexa Fluor—conjugated secondary antibodies diluted 1:300 in 0.5% bovine serum albumin in PBS were added and incubated at 37 °C for 30 min followed by PBS washes and mounting of coverslips in ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Formalin-fixed tissues were processed by the Northwestern University Skin Biology and Disease Resource-Based Center. Paraffin-embedded sections were baked, deparaffinized in sequential xylene and ethanol washes, followed by permeabilization as described above. Antigen retrieval was performed by heating samples to 95 °C in 0.01 M citrate buffer. Sections were blocked in 0.5% BSA in PBS and incubated with primary antibodies for 16 hours at 4 °C, followed by PBS washes. Alexa Fluor—conjugated secondary antibodies were added and incubated at 37 °C for 30 minutes. After mounting in ProLong Gold Antifade Mountant (Thermo Fisher Scientific), sections were visualized as described in the Supplementary Materials and Methods.

Real time quantitative PCR

The real time quantitative PCR procedures utilized to quantify Cx43 mRNA were according to the manufacturer’s instructions and are described in the Supplementary Materials and Methods.
Statistics
Statistics were analyzed using Prism 8 (GraphPad Software, San Diego, CA). Biological replicates were quantified separately and compared as repeated measures. All quantifications are presented as mean ± SD. One-way analysis of variance followed by Dunnett post hoc analysis was used for multiple comparisons. For data comparing two conditions, statistical differences were analyzed with a two-tailed Student t test. For data comparing two conditions at baseline and after treatment, we used two-way analysis of variance followed by Sidak post hoc tests. \( P < 0.05 \) was considered significant.

For the analysis presented as fold change (bar graphs), the mean of data from control samples was assigned a value of 1, and all other samples were calculated relative to the control.

Data availability statement
No datasets were generated or analyzed during the current study.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This work was supported by the National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases R01 AR041836 and National Institutes of Health R37 AR43380 to KJG, and by a fullbright postdoctoral fellowship grant to ECB. MH was supported by the National Institutes of Health T32 Training Grant (T32 CA009560). Additional support was provided by the JL Mayberry endowment to KJG. We acknowledge support and materials from the Northwestern University Skin Biology and Diseases Resource-Based Center supported by 5P30AR057216. We thank Chen Gafni and Efrat Mamluk from “Emek” Medical Center for their assistance.

AUTHOR CONTRIBUTIONS
Conceptualization: ECB, KJG, ES; Data Curation: ECB, LMG, JLJ; Formal Analysis: ECB, MH; Funding Acquisition: ECB, MH, KJG; Investigation: ECB; Methodology: ECB, LMG, KJG; Project Administration: KJG; Resources: JLK, DKG, HH, NFD, MK, MR, JK, RH, RB, AP, MH, OS, MZ, SAS, ES; Software: ECB; Supervision: LMG, KJG; Validation: LMG, KJG; Visualization: LMG, KJG, JLJ; Writing - Original Draft Preparation: ECB, JLJ; Writing - Review and Editing: JLJ, LMG, KJG.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.08.433.

REFERENCES
Kant S, Holthöfer B, Magin TM, Krusche CA, Leube RE. Desmoglein 2-dependent arrhythmogenic cardiomyopathy is caused by a loss of adhesion.


SUPPLEMENTARY MATERIALS AND METHODS

Retroviral infection of keratinocytes
Briefly, 70% confluent Phoenix cells that had been transfected with retroviral CDNA constructs and selected in puromycin were incubated for 12–24 h at 32 °C and then the supernatant was collected. Supernatants were concentrated using Centricon Plus-20 columns (MilliporeSigma, St. Louis, MO). Keratinocytes at 20% cell confluency were incubated at 32 °C for 1 hour in M154 media containing 4 μg/ml polybrene hexadimethrine bromide (MilliporeSigma) and retrovirus supernatants followed by washing the culture with phosphate-buffered saline and feeding the cells with fresh M154 media.

Organotypic epidermal rafts
For stratified organotypic epidermal raft cultures, normal human epidermal keratinocytes (NHEKs) were infected with shControl or shDsg1 retroviruses, as described above. The cells were then expanded and grown at an air-medium interface according to published protocols for six days (Arnette et al., 2016), followed by processing and imaging as described in materials and methods.

Visualization of cells and tissues by microscopy
Cells and sections were visualized with epifluorescence microscopes (DMR; Leica, Buffalo Grove, IL or AxioVision Z1; Carl Zeiss, Peabody, MA). The DMR microscope images were generated using a ×40 objective (PL Fluorat, NA 1.0; Leica, Germany), a digital camera (Orca 100, model C4742–95; Hamamatsu, Skokie, IL), and MetaMorph 7.6 software (Molecular Devices, Sunnyvale, CA). The AxioVision Z1 microscope images were generated using a ×40 (Plan-Neofluar, NA 0.5; Carl Zeiss) objective, a digital camera (Axio-Cam MRm; Carl Zeiss), an Apotome slide module (Carl Zeiss), and Axio-Vision software (Carl Zeiss). The images were obtained with identical conditions and analytically processed uniformly by ImageJ software (National Institutes of Health, Bethesda, MD).

Real time quantitative PCR of Cx43
Total RNA was isolated from cells using the RNEasy Mini kit (ZymoResearch, Irvine, CA) according to the manufacturer’s instructions. Samples were then equalized for total RNA concentration, and cDNA was synthesized using the Superscript III First-Strand kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Primers for GJA1 (5’-GCC AAA GAC TGT GGG TCT CA-3’, 5’-TTA GAG GAAUAUCAUUGAACUCUUC, siNT (No Target #D-001206-14-20, Dharmacon, Lafayette, CO) included four sequences- GAACCUCAUAUCAGAUAA, UGAAGCA-GAUUGAGAUAAA, GCUCAGUGUUCCUAGUGA, and GAUAUAUCAUGACUCUCU, siNT (No Target #D-001206-14-20, Dharmacon Lafayette, CO) and DP (#10620318, Invitrogen/Thermo Fisher Scientific) CAGACCCGUGGCCAAGGAUAGAUAA.

Scrape loading dye transfer assay
Dsg1 FL- and GFP- infected NHEKs were grown to confluence on glass coverslips. M154 media (Thermo Fisher Scientific) supplemented with 1.2 mM CaCl2 was added for 16 hours, and a surgical blade was used to scrape lines in the confluent monolayer. The medium was aspirated and replaced with 300 μl lucifer yellow at 12.5 mg/ml (Thermo Fisher Scientific). Cells were incubated at 37 °C for 15 minutes, followed by PBS washes. Subsequently, samples were fixed in 4% paraformaldehyde for 15 minutes at room temperature, and images were collected as described above. In the experiments presented in Figure 4 e GFP retrovirus was utilized as the control for NHEKs infection; therefore, lucifer yellow dye was visualized using the 568 nm channel. In the experiments presented in Figure 4 i, NHEKs were infected with FL-Dsg1-Flag and were grown for 48 hours, followed by transfection with siRNA against GJA1 and compared with siNT (Non Targeting) oligo transfected NHEKs. Monolayers were scrape wounded as described above and then incubated for 15 min with 2 mg/ml lucifer yellow and 2 mg/ml of the dextran Alexa Fluor 647 (Thermo Fisher Scientific). In this experiment, lucifer yellow was visualized using the 488 nm channel and the dextran Alexa Fluor 647 at the 647 nm channel.

Dispase based dissociation assay
NHEKs were infected as described above, with shControl, shDsg1 retrovirus or transfected with siDP as described above. The cells were grown to confluence in triplicates on six-well plates for at least 48 hours. After reaching 100% confluence, M154 media supplemented with 1.2 mM CaCl2 was added for 48 hours. Then, keratinocyte cultures were washed twice with PBS, incubated in 2 ml of dispase II (2.4 units/ml, MilliporeSigma) at 37 °C for 30 minutes and quantification. For tissue sections (Figures 2, 3, 5, and Supplementary Figures S3, S4, S6), five images from each biopsy were analyzed. Each image was split into individual channels (e.g. Dsg1, Cx43, pCx43 S368, and E-cadherin) and regions of interest were chosen along the plasma membrane (E-cadherin was used as a plasma membrane marker, using the Synchronized function in Image J), and pixel intensities were measured. For the quantification of Cx43 membrane staining in cell cultures on coverslips, images were analyzed using plakoglobin as a plasma membrane marker. The averages of three independent experiments were compared.

siRNA mediated knockdown
NHEKs were electroporated with siRNA oligonucleotides at a final concentration of 20 μM via AMAXA nucleoporation (Lonza, Walkersville, MD) using the Ingenio Electroporation Solution (Mirus, Madison, WI) and the program X-001. Pool siRNA directed toward GJA1 (#M-011042-01-0005, Dharmacon, Lafayette, CO) included four sequences- GAACCUCAUAUCAGAUAA, UGAAGCA-GAUUGAGAUAAA, GCUCAGUGUUCCUAGUGA, and GAUAUAUCAUGACUCUCU, siNT (No Target #D-001206-14-20, Dharmacon Lafayette, CO) and DP (#10620318, Invitrogen/Thermo Fisher Scientific) CAGACCCGUGGCCAAGGAUAGAUAA.
detached from the plate as epidermal sheets. Cell sheets were first rotated, in parallel, on an orbital shaker for 20 minutes (150 rpm) followed by trituration of the monolayers by gently pipetting monolayers eight times each with 1000 μl pipette tips. Fragments were visualized using a dissecting microscope (Leica MZ6, Buffalo Grove, IL) and images obtained using a digital camera (Leica DFC295, Buffalo Grove, IL) followed by processing using Adobe Photoshop CS3. Fragments were counted using the cell counter function in Image J (National Institutes of Health, Bethesda, MD).

Supplementary Figure S2. Representative images of SAM syndrome patient biopsies analyzed in the manuscript presented at 20X magnification. Dsg1 immunofluorescence staining in the lesional and nonlesional areas of skin biopsies from representative patients harboring each of the SAM syndrome mutations using the Dsg1 EC antibody (scale bar = 20 μM). Roman numerals indicate which patient biopsy is included, refer to Figure 1b for clinical features (c.2659C>T-II, c.49-1G>A-III and c.1861delG-I; note that the area of the c.1861delG-I biopsy contains both lesional and nonlesional areas). EC, ectodomain; L, lesional, N.L., nonlesional; SAM, severe dermatitis, multiple allergies, and metabolic wasting syndrome.

Supplementary Figure S1. Additional images presenting the clinical features of patients with SAM syndrome. c.1861delG-I presents with non-remitting diffuse dermatitis. c.49-1G>A-III presents with well-defined hyperkeratotic plaques similar to features of erythrodermatodermia variabilis. Note the tip of the gastrostomy tube.

Supplementary Figure S2. Representative images of SAM syndrome patient biopsies analyzed in the manuscript presented at 20X magnification. Dsg1 immunofluorescence staining in the lesional and nonlesional areas of skin biopsies from representative patients harboring each of the SAM syndrome mutations using the Dsg1 EC antibody (scale bar = 20 μM). Roman numerals indicate which patient biopsy is included, refer to Figure 1b for clinical features (c.2659C>T-II, c.49-1G>A-III and c.1861delG-I; note that the area of the c.1861delG-I biopsy contains both lesional and nonlesional areas). EC, ectodomain; L, lesional, N.L., nonlesional; SAM, severe dermatitis, multiple allergies, and metabolic wasting syndrome.
Supplementary Figure S3. Plasma membrane-to-cytoplasmic ratios of Desmoplakin, β-catenin, and Desmocollin1 are preserved in skin sections of patients with SAM syndrome. (a) Immunofluorescence staining of DP, β-catenin, and Dsc1 from sections of control and lesional skin of patients with SAM syndrome (scale bar = 20 μm). (b) Quantification of the plasma membrane to cytoplasm ratios of patients shown in panel a (n = 20 borders per sample; error bars, mean difference ± SD. *P = 0.0112, ****P < 0.0001, one-way analysis of variance with Dunnet post hoc test). DP, Desmoplakin; Dsc1, desmocollin1; SAM, severe dermatitis, multiple allergies, and metabolic wasting syndrome.
Supplementary Figure S4. Junctional protein is preserved in Dsg1-silenced models. (a) Immunoblot of DP, PG, β-catenin, and E-cadherin in shControl- and shDsg1-infected NHEKs differentiated for 72 hours in high calcium-containing medium. Tubulin used as a loading control. (b) Fold changes of DP, PG, β-catenin and E-cadherin of shDsg1 and shControl-infected NHEKs (n = 3; error bars, mean ± SD, two-tailed Student t test). (c) Immunofluorescence of Dsg1, DP, PG, and β-catenin in shControl and shDsg1 organotypic epidermal equivalents, six days after being lifted to the air/liquid interface (scale bar = 20 μM). (d) Membrane-to-cytoplasmic ratios of Dsg1, PG, β-catenin, and DP from panel c (n = 20 borders per sample; error bars, mean difference ± SD. ****P < 0.0001, two-tailed Student t test). (e) NHEK monolayers infected with shControl and shDsg1 or transfected with siDP were lifted from the substrate using dispase and...
subjected to mechanical stress. An increase in monolayer fragments is indicative of decreased cell-cell adhesive function. (f) The number of cell sheet fragments was counted and graphed as a measure of cell adhesive strength (n = 3; error bars, mean ± SD. *P = 0.03, one-way analysis of variance with Dunnet post hoc test).

(g) Immunoblot of DP, Dsg1, and tubulin demonstrating DP and Dsg1 knockdown. DP, Desmoplakin; N.S., not significant; PG, plakoglobin.
Supplementary Figure S6. (a) Immunofluorescence staining for Dsg1 and Cx43 in 6-day epidermal organotypic equivalents silenced by shRNA against Dsg1 compared with shControl-infected cultures (scale bar = 20 μM). (b) Quantification of plasma membrane pixel intensities of Dsg1 and Cx43 in the organotypic cultures shown in panel a (E-cadherin was used as a membrane marker; n = 25 borders per sample; error bars represent mean difference ± SD. ****P < 0.0001, two-tailed Student t test). (c) Immunoblot of Dsg1, Cx43 in shControl or shDsg1 infected NHEKs treated with PBS or 200 μM leupeptin for 16 hours (representative of n = 3; densitometry, normalized by tubulin, are presented below each lane). (d) Immunoblot of DP, pCx43 S279/282 and total Cx43 in shControl- and shDsg1-infected NHEKs, differentiated for 72 hours in high calcium-containing media (n = 3). Tubulin used as a loading control. (e) Fold changes of pCx43 S279/282 of shDsg1 and shControl-infected NHEKs analyzed using the two-tailed Student t test, N.S., not significant.
Supplementary Figure S7. Full membranes of immunoblots contained in Figures 4a, 4k, 5b, 5d, and 5h.
Supplementary Figure S8. Full membranes of immunoblots contained in Supplementary Figures S4a, S4g, S5b, S6c, and S6d.
### Supplementary Table S1. Antibodies utilized in this study

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Abbreviation: FFPE, formalin-fixed paraffin-embedded.