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ORIGINAL ARTICLE

Plakophilin 1 but not plakophilin 3 regulates desmoglein clustering

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Abstract

Plakophilins (Pkp) are desmosomal plaque proteins crucial for desmosomal adhesion and participate in the regulation of desmosomal turnover and signaling. However, direct evidence that Pkps regulate clustering and molecular binding properties of desmosomal cadherins is missing. Here, keratinocytes lacking either Pkp1 or 3 in comparison to wild type (wt) keratinocytes were characterized with regard to their desmoglein (Dsg) 1- and 3-binding properties and their capability to induce Dsg3 clustering. As revealed by atomic force microscopy (AFM), both Pkp-deficient keratinocyte cell lines showed reduced membrane availability and binding frequency of Dsg1 and 3 at cell borders. Extracellular crosslinking and AFM cluster mapping demonstrated that Pkp1 but not Pkp3 is required for Dsg3 clustering. Accordingly, Dsg3 overexpression reconstituted cluster formation in Pkp3- but not Pkp1-deficient keratinocytes as shown by AFM and STED experiments. Taken together, these data demonstrate that both Pkp1 and 3 regulate Dsg membrane availability, whereas Pkp1 but not Pkp3 is required for Dsg3 clustering.

Keywords Desmosome · Cell adhesion · Desmosomal clustering · Atomic force microscopy · STED

Abbreviations

AFM	Atomic force microscopy
ARVC	Arrhythmogenic cardiomyopathy
DP	Desmoplakin
Dsc	Desmocollin
Dsg	Desmoglein
EC	Extracellular domains
FRAP	Fluorescence recovery after photobleaching
HaCaT	Cultured human keratinocytes
IF	Intermediate filament
MAPK	Mitogen-activated protein kinase
MKZ	Murine keratinocytes
PEG	Polyethylenglycol
Pg	Plakoglobin
Pkp	Plakophilins
PV	Pemphigus vulgaris
QI	Quantitative imaging
STED	Stimulated emission depletion
Sulfo-EGS	Ethylene glycolbis (sulfosuccinimidylsuccinate)
UFs	Unbinding forces
UP	Unbinding position
wt	Wild type

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Introduction

Desmosomes are cell–cell contacts crucial for proper intercellular adhesion. They are indispensable for the integrity of tissues which are exposed to repetitive mechanical stress, such as the heart or the epidermis [1]. Importance of desmosomal adhesion is reflected by severe diseases occurring

when cell cohesion is compromised, e.g., through autoantibodies in pemphigus vulgaris (PV) or genetic mutation in arrhythmogenic cardiomyopathy (ARVC) [2, 3] and skin fragility syndrome [4]. On the molecular level, desmosomes are composed of three major protein families: desmosomal cadherins, comprising three desmocollin (Dsc1-3) and four desmoglein (Dsg1-4) isoforms, which maintain intercellular adhesion via their extracellular domains in homo- and heterophilic Ca^{2+}-dependent manner [5–7]. Further, desmosomes consist of armadillo family proteins plakophilins (Pkp1-3) and plakoglobin (PG), and of desmoplakin (DP), the latter of which belongs to the family of plakin proteins. These proteins build up the desmosomal plaque, stabilize desmosomal cadherins and anchor them to the intermediate filament (IF) cytoskeleton [8–10].

Pkps, of which all three isoforms are expressed in a differentiation-dependent manner in keratinocytes, are important for desmosomal cadherin turnover [11, 12] and interact with various desmosomal proteins [13]. Different isoforms are involved in a broad range of signaling pathways and have in part opposing effects on the cellular processes [14]. For instance, loss of function of Pkp1 leads to ectodermal dysplasia-skin fragility syndrome [4, 15, 16]. In line with this, Pkp1 k.o. mice develop growth retardation, loss of desmosomal adhesion and impaired tight junction function [17], whereas Pkp3 k.o. mice reveal a mild phenotype with hair abnormalities and skin infections [18].

On a molecular level, studies using electron tomography and immune electron microscopy suggest that Pkps reveal a quasi-periodicity of approximately 7 nm in the desmosomal plaque neighboring intracellular domains of desmosomal cadherins [19], indicating that Pkps directly participate in the organization of the desmosomal plaque. In line with this, Pkps laterally cluster with desmoplakin to increase desmosome size [20–22]. Beside their structural function in desmosomes, Pkps regulate several signaling pathways [13, 14, 23, 24–26], which indicates specific roles for Pkps in the regulation of desmosomal adhesion. In line with this, Pkp3 is of importance for the formation of new desmosomes, whereas Pkp1 is involved in hyper-adhesion, a state in which desmosomes are insensitive to the reduction of extracellular Ca^{2+} levels [14, 26, 27].

Changes in desmosomal organization and desmoglein clustering are present in various desmosomal diseases such as pemphigus and skin fragility syndrome [28–33]. Thus, it is possible that Pkps also differentially cluster desmosomal cadherins and via organizing the desmosomal plaque, this could be a critical mechanism for maintenance of proper intercellular adhesion. However, this has not been directly tested so far. Further, it remains unknown whether Pkps also participate in the regulation of desmosomal adhesion at the level of single-molecule binding properties. Since binding properties of desmosomal cadherins are affected by intracellular proteins such as keratins and various signaling pathways [34], we here investigated the role of Pkp 1 and 3 for Dsg binding and clustering. We focused on Dsg1 and 3, since the autoimmune blistering skin disease pemphigus shows that targeting Dsg1 and 3 interferes with stable tissue cohesion [35, 36].

Materials and methods

Cell culture, transfection and reagents

Wild type (wt) and Pkp1- or 3- (Pkp k.o.) deficient murine keratinocytes (MKZ) were isolated and maintained as described before and in Supplement Materials and Methods [14, 17]. Cells were used at 48 h after confluency and switching to high Ca^{2+} conditions throughout all experiments. For some experiments, human keratinocyte cells (HaCaT) [37] were used (see Suppl. M&M). For Dsg3 overexpression, cells were grown to 70% confluency and subsequently transiently transfected with pEGFP-C1-Dsg3 (kindly provided by Dr. Yasushi Hanakawa, Ehime University School of Medicine, Japan) or pSNAPf-mDsg3-N (for cloning see Suppl. M&M) using Lipofectamine 3000 (Invitrogen, Carlsbad, USA). Change to high Ca^{2+}-containing media (1.2 mM) was carried out 24 h after transfection.

Purification of recombinant Dsg1 and 3-Fc construct

Purification of recombinant human Dsg1 and 3-Fc proteins was carried out as described before [38, 39] and in Suppl. M&M.

Atomic force microscopy (AFM)

Throughout all measurements, a NanoWizard® 3 AFM (JPK Instruments, Berlin, Germany) coupled with an inverted optical microscope (Carl Zeiss, Jena, Germany) was used, which enabled the selection of a scanning area by visualization of the cells through a 63 × objective. Recorded topographic images and adhesion measurements were performed according to the previously applied protocols [34, 40, 41]. Two different force spectroscopy-based imaging modes have been used, the quantitative imaging (QI) (setpoint: 0.5 nN, Z-length: 1500 nm, pulling speed: 50 µm/s) and the force mapping mode (setpoint: 0.5 nN, Z-length: 2000 nm, extend time: 0.2 ms, extend delay: 0.1 s). Recorded pixels contain the information of a single force–distance curve. For experiments, the D-Tip (Si_3N_4) of MLCT cantilevers (Bruker, Mannheim, Germany) with a nominal spring constant of 0.03 N/m and a tip radius of 20 nm was used. To detect specific single-molecule interactions, recombinant Dsg3- and Dsg1-Fc (concentration: 0.15 mg/ml) were utilized

for coating of the tips through a flexible heterobifunctional acetal-polyethylene glycol (PEG) linker (Gruber Lab, Institute of Biophysics, Linz, Austria) as described previously [42]. All experiments on MKZ cells were performed in full FAD medium supplemented with Ca^{2+} at 1.2 mM concentration. Distribution ratio and stability coefficient were calculated as described before and in Suppl. Materials and Methods.

Electrophoresis and Western blot analysis

Cells were lysed and Western blotting was conducted as shown previously and in Suppl. M&M [43]. For respective experiments, Triton X-100 fractionation or surface biotinylation was conducted.

Chemical crosslinking

For investigation of protein oligomerization of desmosomal cadherins, the membrane-impermeable cross-linker ethylene glycolbis (sulfosuccinimidylsuccinate) (Sulfo-EGS) (Pierce Biotechnology, Rockford, USA) was used. The experimental procedure has been described before [7]. Briefly, Sulfo-EGS was added to cells 48 h after Ca^{2+} switch at a concentration of 2 mM for 30 min at room temperature. TBS at a concentration of 50 mM was added to stop the reaction. Detection of crosslinked products was performed by Western blotting.

Keratinocyte dissociation assay

To determine the adhesive strength of keratinocytes, dispase-based keratinocyte dissociation assays were performed as described previously [44].

Immunostaining

For details regarding immunostaining please refer to Suppl. M&M.

Stimulated emission depletion microscopy (STED)

Dsg3-snap transfected cells were labeled with SNAP-tag-kit® according to the manufacturer's protocol (New England Biolabs, Massachusetts, USA). Fixation and incubation with further antibodies were performed as described in Suppl. M&M. Recordings were made with the STED-Expert line setup from Abberior (Abberior Instruments GmbH, Göttingen, Germany).

Data processing and statistics

Image processing was conducted with Photoline (Computerinsel, Bad Gögging, Germany) and Photoshop CS7 (Adobe, San José, USA). JPK data processing software (JPK Instruments) was used for AFM images and data analysis of force–distance curves. Further analysis of measured AFM data, such as, unbinding forces, peak fitting and step position was done with Origin Pro 2016, 93G (Northampton, MA, USA). Western blot bands were analyzed with ImageJ software (NIH, Bethesda, USA). Moreover, data were evaluated with Excel (Microsoft, Redmond, WA). The data of two groups were compared using two-tailed Student's *t* test. In case of more than two group comparisons, analysis of variance (one-way ANOVA) followed by Bonferroni post hoc test, was performed. The shown error bars represent standard error of the mean or the standard deviation as indicated. Statistical significance was assumed for p values < 0.05.

Results

Intercellular adhesion depends on Pkp1 and Pkp3

Human keratinocytes (HaCaT) were used to delineate the expression patterns of Pkp1, 2 and 3 and their co-localization with Dsg3. As described earlier, HaCaT cells express all three Pkp isoforms [45]. In *xy*-plane, Dsg3 is distributed linearly along cell borders (Fig. 1a). Further, we observed punctate Dsg3 immunostaining on the cell surface of keratinocytes in the *xz*-plane, similar to that described before [34] (Fig. 1a). Staining of Pkp1, 2 and 3 showed that all isoforms localize along the cell boundaries and co-localize with Dsg3 at the cell border areas. Similar localization patterns were detected on the cell surface (Fig. 1a). Thus, we assumed that Pkps might regulate Dsg-binding properties at both localizations. To dissect the role of Pkps for desmoglein distribution and binding properties, we used murine keratinocytes lacking either Pkp1 or Pkp3, respectively [14, 17] (Figure S1a) and compared them to wt keratinocytes. Dispase-based keratinocyte dissociation assay revealed an increased number of fragments in Pkp3- and more drastically in Pkp1-deficient cells under untreated conditions, indicating that both Pkp isoforms contribute to intercellular adhesion, but Pkp1 is more important for strong cellular cohesion than Pkp3 (Fig. 1b). To investigate the effect of Pkp deficiency on expression and localization of Dsg3, proteins at the cell membrane were detected by cell surface biotinylation assays. Here, we observed a decreased total Dsg3 expression and a corresponding decrease in the biotinylated protein fraction, indicating that Pkp deficiency leads to loss of Dsg3 from the cell membrane (Fig. 1c). We further investigated the amount of Dsg3 in the cytoskeleton-unanchored (Triton X-soluble) and the cytoskeleton-anchored (Triton X-non-soluble) fraction, where we observed a drastic decrease of Dsg3 levels in both fractions from Pkp1-deficient cells, whereas Dsg3 levels were not altered in both fractions in Pkp3-deficient

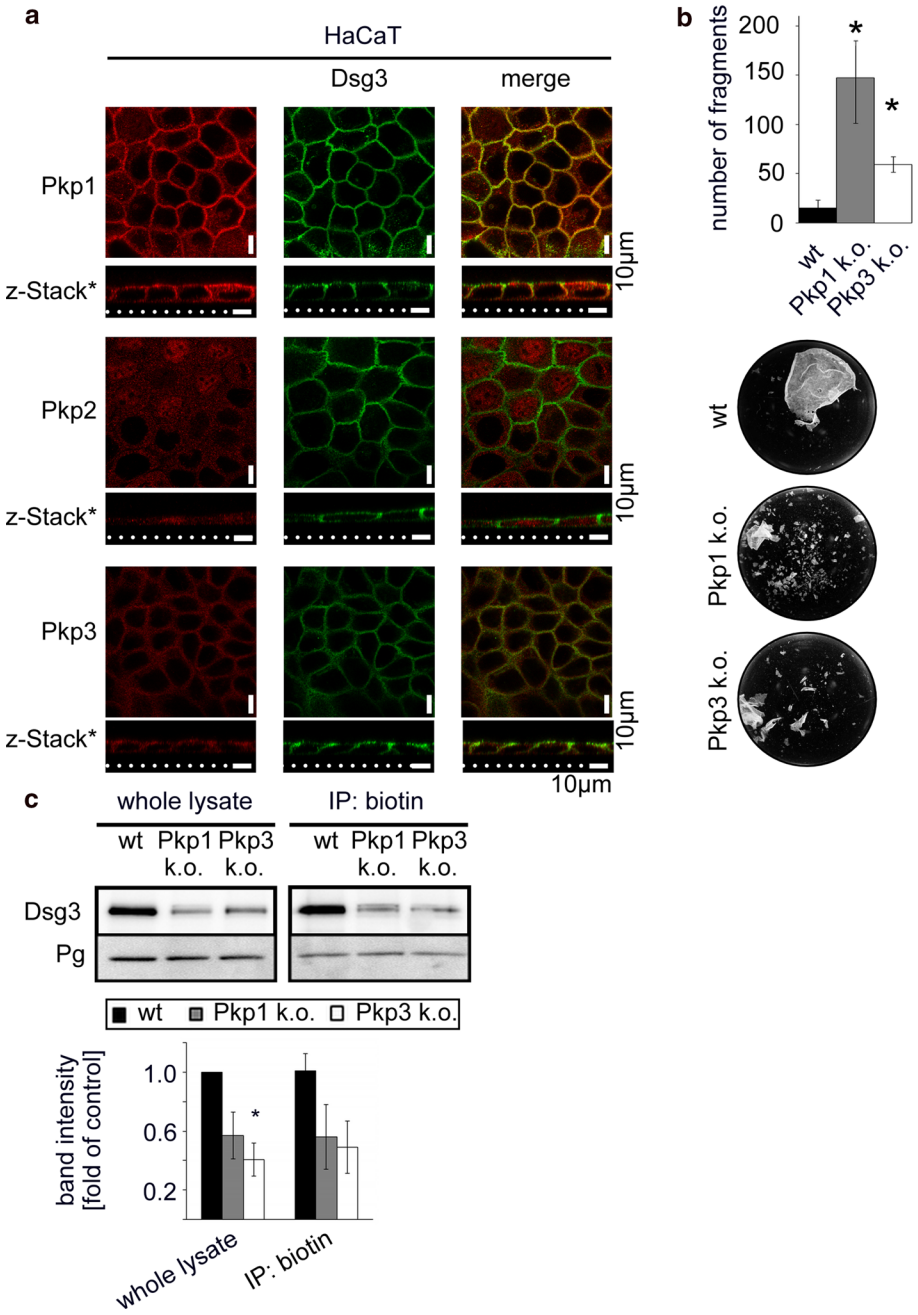

Fig. 1 Intercellular adhesion is Pkp1- and 3-dependent. **a** HaCaT cells were stained with Dsg3 and Pkp1, 2 or 3-antibodies, respectively. Dsg3 colocalizes with Pkps both at the cell borders and on the cell surface. **b** Dissociation assay in murine keratinocytes show reduced intercellular adhesion in cells lacking Pkp1 or 3, indicated by increased fragmentation. $^*p < 0.05$ vs. wt control. Error bars show mean ± SEM ($n = 4$). **c** Biotinylation assay and quantification of MKZ cells reveal reduced Dsg3 expression and membrane levels in Pkp1- and Pkp3-deficient cells. $^*p < 0.05$ vs to wt. Error bars indicate mean ± SEM ($n = 4$)

keratinocytes (Figure S1b, c). Interestingly, Dsc1, 2 and 3 expression was not altered in Pkp1- and 3-deficient keratinocytes (Figure S1d, e) indicating that Pkps primarily regulate desmogleins. These data show that both Pkp1 and 3 control Dsg3 membrane availability, whereas Pkp1 is only required for cytoskeletal anchorage.

Pkp loss reduces Dsg3 and Dsg1 interactions at cell borders

Although the impact of Pkps for intercellular adhesion has been described before [13, 14, 26], the underlying molecular mechanism is not yet known. Thus, we investigated the impact of Pkps for Dsg3-binding properties using atomic force microscopy (AFM). To do so, AFM tips were functionalized with recombinant Dsg3 extracellular domains (EC) and respective cell lines were mapped to detect specific Dsg3 single-molecule interactions. In previous studies, we have shown that Dsg3 undergoes homophilic and heterophilic interactions both under cell-free conditions as well as on the surface of living keratinocytes [34, 38, 40, 41]. Specificity of Dsg3 interactions on murine keratinocytes was confirmed in previous studies using inhibitory aDsg3 antibodies [34]. Scanning electron microscopy and

AFM topography showed elevated cell borders with similar morphology in all cell lines investigated (Figure S2a, b). Small areas from AFM topography images were selected to record adhesion maps (Figure S2b, green rectangles) at cell borders (10 µm^2) and on the cell surface (10 µm^2 or 4 µm^2) above the nucleus. In adhesion maps, each pixel represents an approach and retrace cycle of the AFM cantilever, gray values represent the topography at the respective position and every blue dot depicts a Dsg3-binding event (Fig. 2a). At cell borders, reduction of Dsg3-binding frequency ν (ν = #binding events/total#pixels) to 52% and 29% of wt levels was significant in both Pkp1- and Pkp3-deficient cells, respectively. In Pkp3-deficient cells, Dsg3-binding frequency was also reduced to about 28% on the cell surface (Fig. 2a, b). These data were in line with the results from the biotinylation assay (Fig. 1c) and suggest that Pkps are crucial for proper membrane availability of Dsg3-binding partners. Next, we evaluated more closely the distribution ratio between Dsg3-binding events on cell junctions (as indicated within the red dotted lines) and the junctional area in close proximity (Fig. 2a, c). No changes were observed in Pkp1- and Pkp3-deficient cells compared to wt cells indicating that junctional and peri-junctional Dsg3 availability was reduced to a similar extent as membrane availability in the peri-junctional compartment.

Furthermore, we investigated the strength of the single-molecule interactions, the so-called unbinding forces (UFs), of the remaining Dsg3-binding events (Fig. 2d and Figure S2c). For both areas, a significant reduction of UFs in cells lacking Pkp1 (42.8 pN at cell borders and 45.3 pN on the cell

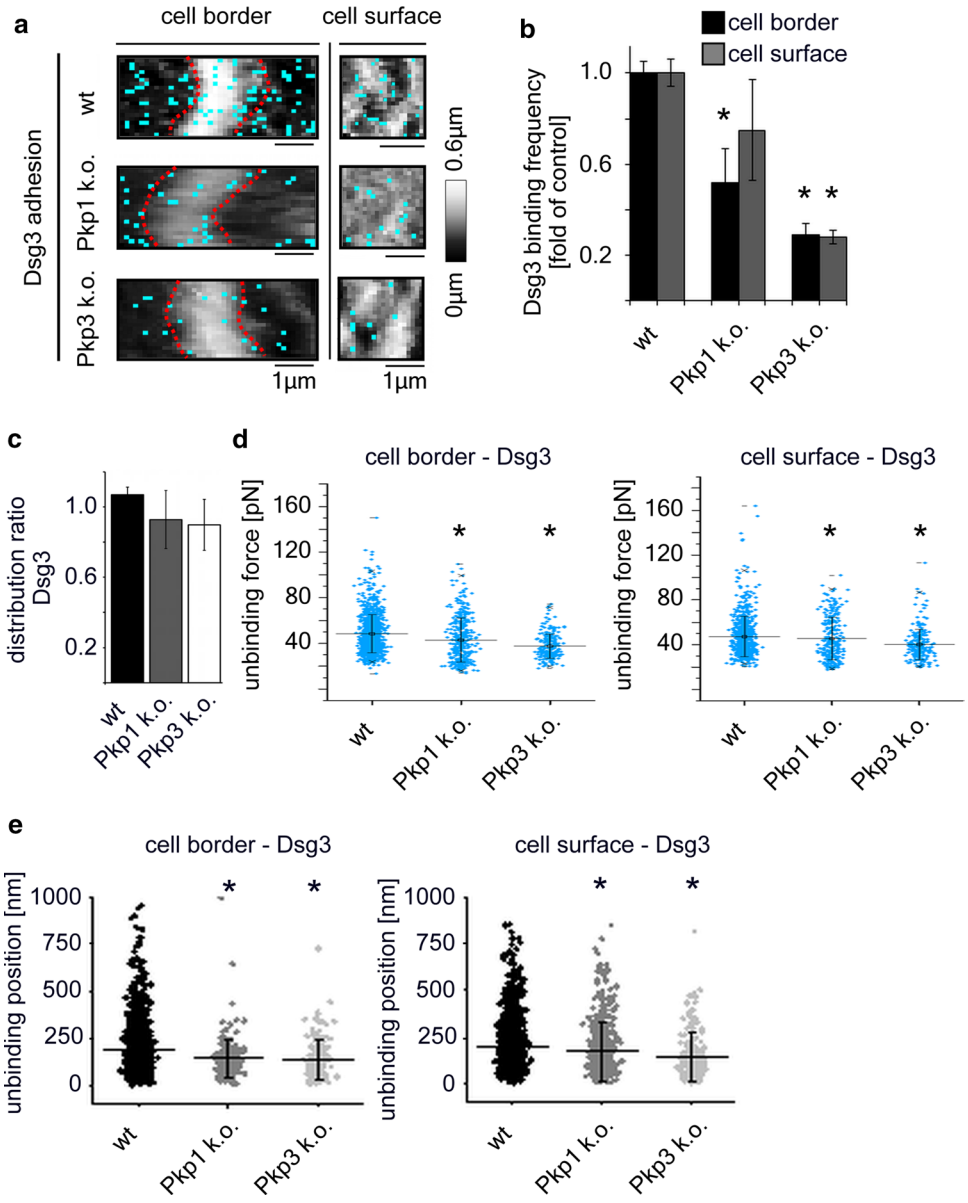

Fig. 2 Pkp1- and 3-deficiency reduces Dsg3 single-molecule interactions at cell borders. **a** AFM adhesion maps, with each pixel displaying a force–distance curve, gray background colors show topography of cell borders and blue dots indicate Dsg3-binding events. Cell borders are marked by red dotted lines. **b** Dsg3-binding frequency is significantly reduced at cell borders and on cell surface of Pkp3-deficient cells as well as at cell borders in Pkp1-deficient keratinocytes. **c** Dsg3 distribution ratio between junctional and peri-junctional compartments shows no changes in Pkp-deficient keratinocytes. *$p < 0.05$ vs. wt. Error bars show mean ± SEM. $n \geq 6$ cell borders with 1000 force–distance curves each. **d** Analysis of unbinding forces (UF) of remaining Dsg3 molecules shows reduced values for cells lacking either Pkp1 or 3. **e** Unbinding positions (UP) of remaining Dsg3 molecules are reduced in Pkp1- and Pkp3-deficient cells. **d/e** *$p < 0.05$ vs. wt. Error bars indicate mean ± SD

surface) or Pkp3 (37.5 pN at cell borders and 40.1 pN on the cell surface) was detected compared to wt cells (48.5 pN at cell borders and 47.2 pN on the cell surface) (Suppl. Table 1, Fig. 2d). We further analyzed the unbinding position (UP) of the remaining Dsg3 interactions (Figure S2c). This measure describes the distance from the contact point at which the bond ruptures. Higher UPs could be a possible indication of the so-called tethers [46] and the UP was supposed to be an indirect measure for cytoskeletal anchorage [47, 48]. UP values were significantly reduced in cells deficient for Pkp1 by about 25.6% at cell borders and 13.6% on the cell surface and by 28.3% at the cell borders and 27.9% on the cell surface in Pkp3-deficient keratinocytes when compared to controls (Fig. 2e and Suppl. Table 1). Taken together, these data indicate that Pkp1 and 3 are required to maintain the number of Dsg3-binding events at cell junctions. In contrast, the subpopulation of remaining Dsg3 molecules is small and suggests that altered binding properties of this subpopulation are less important for intercellular adhesion compared to the drastic reduction in binding frequency along cell borders.

As Dsg1 represents the other main antigen for autoantibodies in pemphigus [49] and Dsg1 expression pattern in human skin is different from the expression pattern of Dsg3 [1, 50], we further investigated the binding properties of Dsg1. For these experiments, AFM tips were coated with Dsg1-EC recombinant proteins. Specificity of Dsg1 interactions on murine keratinocytes was shown in previous studies using an inhibitory aDsg1 antibody [51]. Similar to the data obtained for Dsg3, in both Pkp1- and 3-deficient cell lines, a significant reduction in Dsg1-binding frequency was observed mainly at cell borders (Fig. 3a, b). Moreover, the distribution ratio of Dsg1-binding events between the

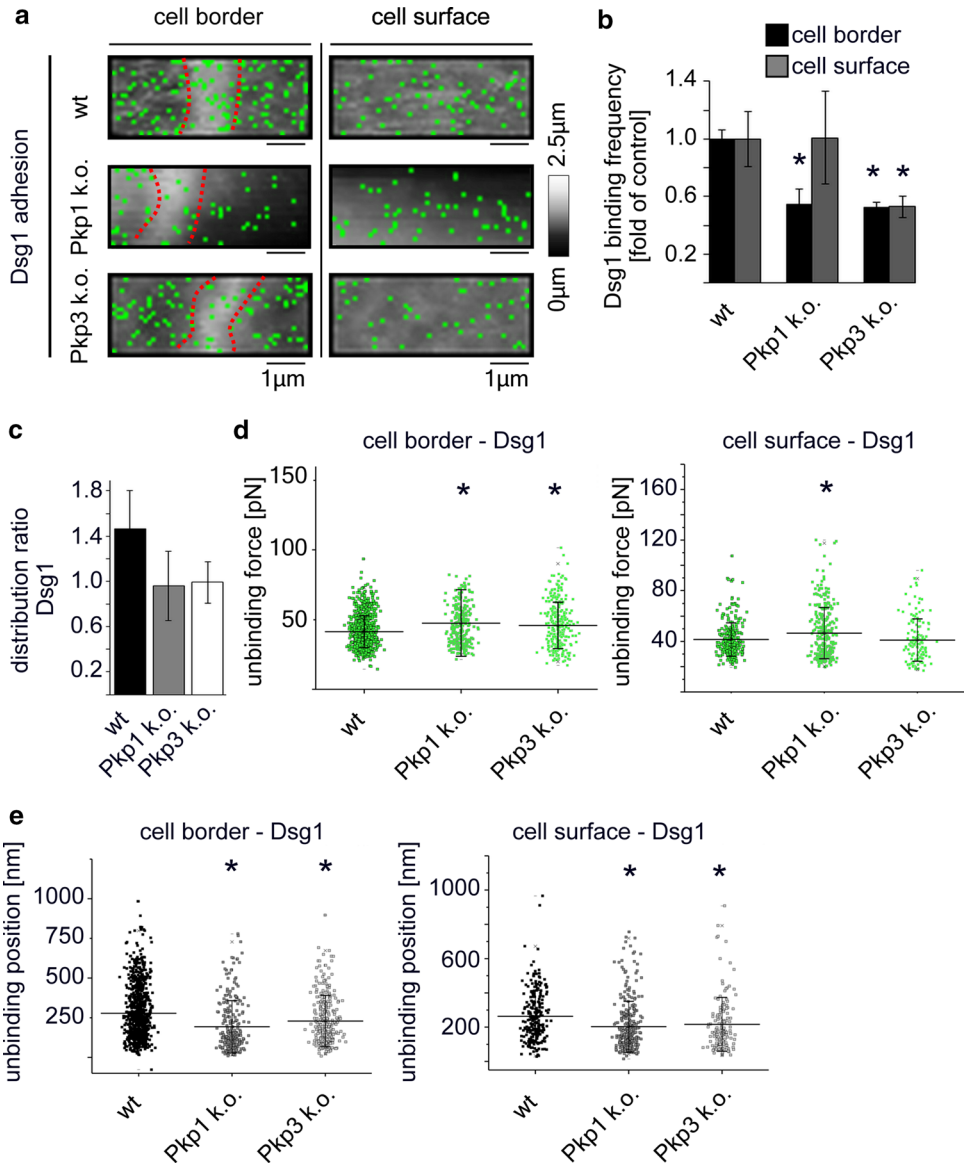

Fig. 3 Pkp1- and 3-deficiency also reduces Dsg1 interactions at cell borders. **a** AFM adhesion maps for Dsg1 as described above. 2. **b** Dsg1-binding frequency is significantly reduced in Pkp-deficient cells at cell borders. **c** Distribution ratio for Dsg1 between junctional and peri-junctional compartment is not significantly altered in Pkp-deficient keratinocytes. $^*p < 0.05$ vs. wt at respective localization, error bars indicate mean ± SEM. $n = 6$ cell borders with 1000 force–distance curves each. **d** Analysis of UF showed increased forces in binding strength for Pkp1 and 3 k.o. cells. $N = 6$ for independent measurements with 1000 force–distance curves each. $^*p < 0.05$ vs. wt. **e** For Pkp1 and 3 k.o. cells the UP are shorter for both compared to wt. $^*p < 0.05$ vs. wt

junctional and peri-junctional compartment was not significantly altered neither in Pkp1- and nor in Pkp3-deficient cells (Fig. 3c). In Pkp1- and Pkp3-deficient keratinocytes, the remaining Dsg1-binding events displayed slightly higher unbinding forces at cell borders and for Pkp1-deficient keratinocytes also on the surface when compared to wt cells (Fig. 3d and Suppl. Table 1), whereas UPs were reduced for Dsg1 in Pkp-deficient cell lines similar to Dsg3 as outlined above (Fig. 3e and Suppl. Table 1). These data indicate that both Pkp 1 and 3 are critical for the maintenance of Dsg1 and 3 availability at cell borders.

Pkp deficiency does not alter p38MAPK signaling

So far, AFM experiments delineate that Dsg1 and 3 junctional availability is dependent on Pkps, therefore we next investigated the mechanisms underlying this phenomenon. As signaling pathways such as p38MAPK are central for the regulation of desmosomal adhesion and also modulate Dsg-binding properties [34, 51, 52], we further tested whether modulation of p38MAPK affects intercellular adhesion in the respective cell lines. Dispase-based dissociation assay using anisomycin for 1 h to activate p38MAPK showed compromised intercellular adhesion in wt- and Pkp-deficient keratinocytes. The effect was significant for wt- and Pkp3-deficient cells but not for Pkp1-deficient cells, most likely because in the latter adhesion was severely compromised under resting conditions as shown above (Fig. 4a, compare to Fig. 1b). Accordingly, Western blot analysis showed unaltered p38MAPK activity in all cell lines. This was not only true under resting conditions but also after anisomycin treatment (Fig. 4b). Hence, this signaling pathway seems not to be relevant for Pkp-dependent loss of cell cohesion.

Pkp1 but not Pkp3 regulates Dsg3 clustering

Membrane-impermeable crosslinking was used to investigate Dsg3 oligomerization as an indirect indicator for Dsg3 clustering. Interestingly, Dsg3 oligomers were significantly reduced in Pkp1-deficient cells and also diminished in Pkp3-deficient cells (Fig. 4c, d). To investigate whether this is an effect specific for desmosomal cadherins, we checked for oligomers of the classical cadherin E-Cad. However, no significant difference between the cell lines was observed (Fig. 4c, d), underlining that Pkp-dependent oligomerization is specific for desmosomal cadherins. These results give a first hint that Pkp-dependent clustering may account for the observed differences in overall cell cohesion between Pkp1- and Pkp3-deficient cells as revealed by dispase-based assay.

To confirm the above results and correlate them with AFM experiments, we performed repetitive AFM scans of the same region to detect Dsg3 clusters at cell borders of the respective keratinocytes. To do so, the size of connected bond positions in these AFM measurements was determined (Fig. 4e, f, see Suppl. Materials and Methods). Interestingly, cluster size was reduced in both Pkp1- and Pkp3-deficient keratinocytes when compared to wt (Fig. 4e, f) which would be in line with impaired oligomerization and thus this provides a further approach to investigate Dsg clustering in living keratinocytes. Next, we tested whether there is a link between the amount of clustered proteins and their mobility. As molecule mobility seems to negatively correlate with strong intercellular adhesion [34], this could serve as a further explanation for impaired intercellular adhesion in Pkp-deficient keratinocytes. Therefore, we analyzed repetitive AFM experiments with respect to stable molecule clusters as described before [34]. AFM mobility measurements show higher mobility of the Dsg3 molecules as indicated by a significantly lower stability coefficient in Pkp1-deficient and a slight but not significantly reduced stability coefficient in Pkp3-deficient keratinocytes (Fig. 4g, h), indicating that Pkp1 is primarily required for Dsg3 clustering and immobilization. This reflects differential roles of Pkp1 and Pkp3 in promoting proper intercellular adhesion.

Dsg3 clustering can be rescued by overexpression of Dsg3-GFP in Pkp3- but not in Pkp1-deficient keratinocytes

Pkp-deficient cells show reduced levels of Dsg3 (see Fig. 1c, Suppl. Figure 1b and c). Therefore, we asked whether impaired clustering and higher mobility may be due to reduced junctional availability of Dsg3 molecules or whether they are a direct result of Pkp deficiency. To address this, we overexpressed Dsg3 in all cell lines and performed AFM adhesion measurement according to Fig. 2 (Suppl. Figure 3a, b). Dsg3-binding frequency at cell borders was slightly higher after Dsg3 overexpression in wt cells but not in Pkp1- or Pkp3-deficient cells (Fig. 5a), suggesting that in the absence of Pkps, no additional Dsg3 molecules were incorporated in areas accessible for the AFM. In accordance, we observed no significant changes in the unbinding forces after Dsg3 overexpression in Pkp1- and Pkp3-deficient cell lines, i.e., unbinding forces of th remaining Dsg3 molecules remained slightly but significantly lower when compared to wt cells (Fig. 5b). Interestingly, the unbinding position dropped significantly in wt keratinocytes suggesting that overexpression of Dsg3 molecules enhanced its anchorage to the cytoskeleton [53]. This was accompanied by enhanced Dsg3 clustering indicating that in wt, molecule availability at the cell membrane correlates with molecule clustering (Fig. 5c). We further checked for the clustering size by performing repetitive Dsg3 AFM experiments at defined cell border areas. After Dsg3 overexpression the cluster size increased in wt- and Pkp3-deficient keratinocytes but not in Pkp1-deficient keratinocytes (Fig. 5d, e compared to

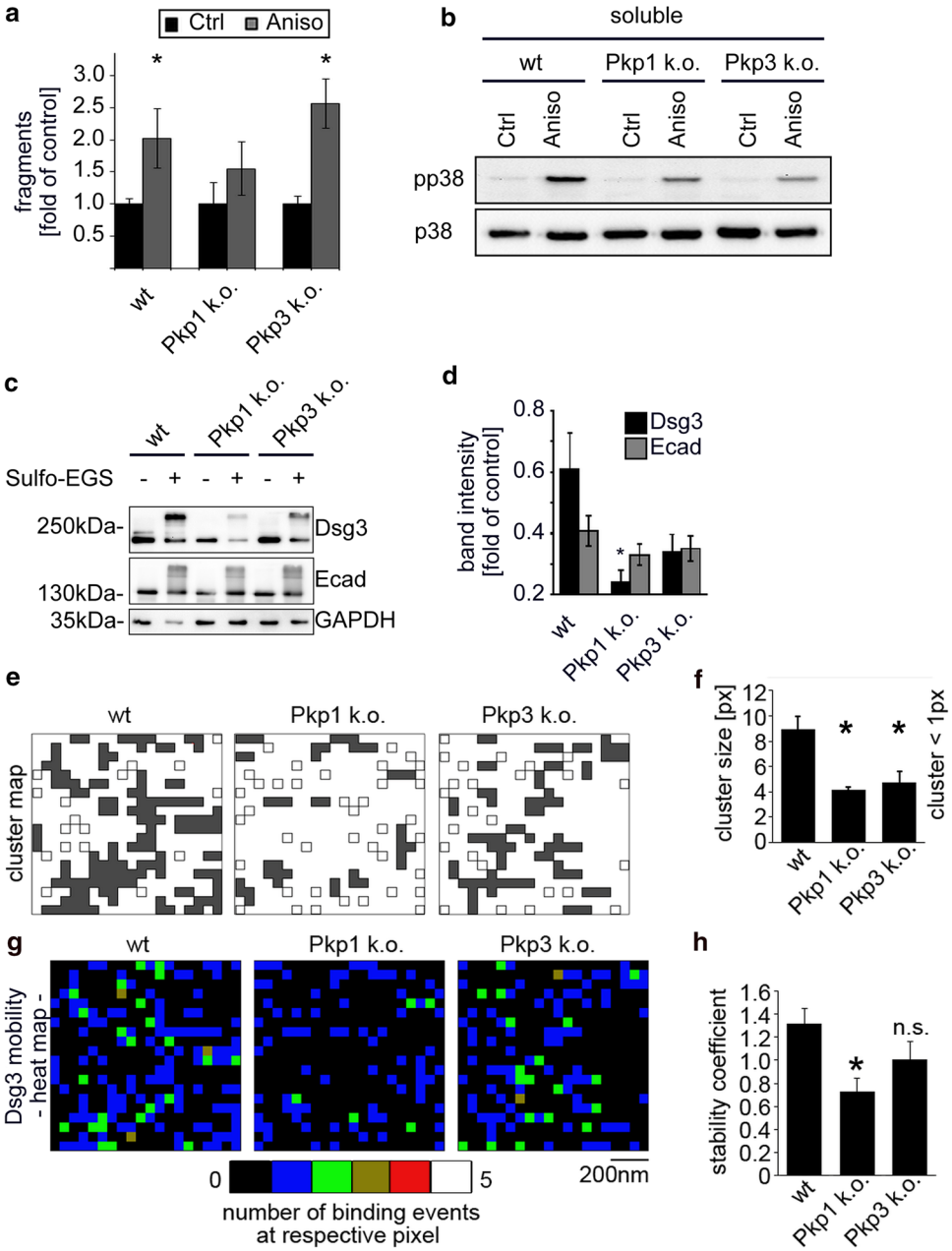

Fig. 4 Dsg3 clustering is altered in Pkp-deficient keratinocytes. **a** p38MAPK activation using anisomycin (Aniso) reduced intercellular adhesion in all cell lines. $*p < 0.05$ vs. control (Ctrl). Error bars indicate mean ± SEM ($n \geq 4$). **b** Basal activity and anisomycin-induced activation of p38MAPK were similar in wt and Pkp-deficient cells. $n = 5$. **c** Membrane-impermeable crosslinking with sulfo-EGS significantly showed reduced numbers of Dsg3 oligomers in Pkp1-deficient cells whereas effect was minor in cells lacking Pkp3. **d** Densitometric quantification of Dsg3 and Ecad bands shows the ratio of crosslinked proteins to total protein. $N = 5$ and $*p < 0.05$ vs. wt. **e**, **f** Dsg3 clusters from AFM measurements show impaired cluster size in Pkp1- and Pkp3-deficient cells. **g** Mobility heat map shows color-coded maps of five successively scanned AFM adhesion maps (area = 1 µm², 20 × 20 pixels). **h** Quantification of g, by determining the stability coefficient which was reduced in Pkp1-deficient cells only (see Suppl. M&M). $N = 8$, $*p < 0.05$, error bars show ± SEM

Fig. 4e, f). Here, Pkp3-deficient cells reached cluster sizes similar to untransfected wt, whereas cluster sizes in Pkp1 k.o. cells remained comparable to its non-transfected control (Fig. 5d, e compared to Fig. 4e, f), indicating that Dsg3 overexpression restored Dsg3 clustering in Pkp3-, but not in Pkp1-deficient keratinocytes. Similarly, in FRAP experiments using Dsg3-GFP, Dsg3 mobility was increased in Pkp1-deficient cells but not in Pkp3-deficient keratinocytes (Suppl. Figure 3c, d). To further check the morphology of Dsg3 clusters, we performed STED microscopy imaging. To do so, cells were transfected with Dsg3-snap and labeled using SNAP®-Cell 647-SiR. Both wt- and Pkp3-deficient keratinocytes displayed dense clusters along cell borders and rounded clusters on the cell surface (Fig. 5f), which in size were comparable to Dsg3 clusters as measured by AFM. In contrast, molecule clusters were reduced in number and seemed irregular in shape in Pkp1-deficient cells, thus confirming disturbed Dsg3 clustering in Pkp1-deficient cells. Moreover, at cell borders, Dsg3 staining was stronger and broader in wt cells compared to both Pkp-deficient cell lines which is in line with the observation that Dsg3 overexpression did not rescue Dsg3-binding frequency at cell junctions in AFM (Fig. 5f, compared to Fig. 5a).

Taken together, these data demonstrate that restoration of Dsg3 levels is sufficient to rescue Dsg3 clustering in

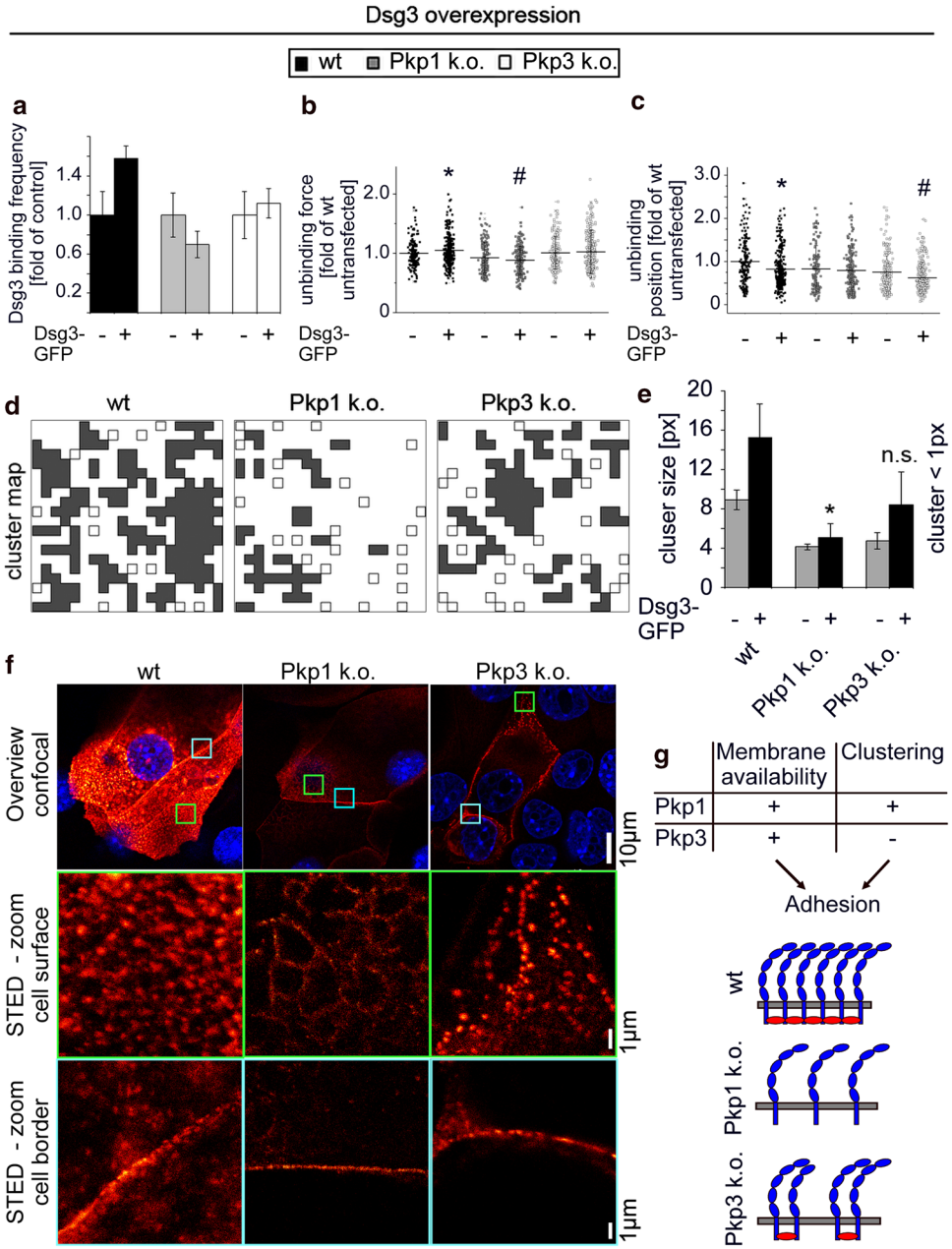

Fig. 5 Dsg3 clustering can be rescued by Dsg3 overexpression in Pkp3- but not in Pkp1-deficient cells. **a** Dsg3-binding frequency was slightly increased in wt but not in Pkp1- and Pkp3-deficient keratinocytes when Dsg3-GFP was overexpressed. **b** Unbinding forces (UF) of remaining Dsg3 molecules were not significantly altered by Dsg3 overexpression in Pkp1- and Pkp3 k.o. cells. **c** Unbinding positions (UP) were significantly reduced in wt keratinocytes following Dsg3 overexpression. **a**–**c** $N=6$ cell borders with 1000 force–distance curves each, $*p<0.05$ vs. not Dsg3-overexpressed cells, $\#<0.05$ vs. wt Dsg3-overexpressed cells. **d**, **e** Dsg3 cluster sizes as evaluated by AFM after Dsg3 overexpression cells shows significantly reduced cluster size in Pkp1- but not in Pkp3-deficient cells. $N=6$, $*p<0.05$, error bars show ± SEM. **f** STED microscopy images to visualize Dsg3 clusters stained with SNAP®-Cell 647-SiR and DAPI. **g** A schematic summary of the mechanisms by which Pkp1 and Pkp3 control adhesion on the level of Dsg3 binding (Dsg3 in blue; Pkp1 in red)

Pkp3-deficient but not in Pkp1-deficient cells, indicating that Pkp1 in contrast to Pkp3 drives Dsg clustering.

Discussion

In the present study, we demonstrate for the first time that Pkps regulate clustering of desmosomal cadherins in keratinocytes in an isoform-specific manner. Both Pkp1 and Pkp3 are required for junctional membrane availability of desmosomal cadherins Dsg1 and 3 (Fig. 5g). In contrast, Dsg3 clustering, as shown here by AFM and STED imaging, is a specific function of Pkp1 which correlates with the more pronounced adhesion defect in keratinocytes lacking Pkp1 compared to cells deficient for Pkp3 (Fig. 5g).

Plakophilins regulate junctional membrane availability of desmogleins

It is known that Pkps participate in desmosome formation, where they act as scaffolds and are important for cellular signaling [9, 54]. Although Pkps share some functions, they display distinct roles in regulating intercellular adhesion. For instance, loss of Pkp1 has dramatic effects on the size and number of desmosomes [17] and mutations in Pkp1 lead to skin fragility syndrome in which the epidermal cohesion is

severely impaired [4, 15, 16]. In contrast, Pkp3 loss reveals a much milder phenotype [13, 18] but is associated with tumor formation and metastasis [55]. In accordance, although cells lacking either Pkp1 or Pkp3 display impaired cellular cohesion, Pkp1 is much more important for stable cohesion than Pkp3 [14].

Binding properties of adhesion molecules can be investigated on a single-molecule level using AFM [5, 56, 57]. Using this technique, we have previously shown that, Dsg1- and 3-binding properties are not uniformly distributed in keratinocytes and intracellular molecules such as keratins are involved in regulating the binding properties of desmogleins [34, 51]. The data presented show that frequencies of Dsg1- and Dsg3-binding events were drastically reduced to about 30% of wt levels in Pkp1- and Pkp3-deficient keratinocytes which was accompanied by less Dsg3 available at the cell membrane. As membrane availability of desmosomal cadherins was shown to correlate with firm intercellular adhesion [31, 58], these data indicate that Pkp1 and Pkp3 stabilize desmosomal adhesion by maintaining junctional membrane availability of desmogleins (Fig. 5g). This is in line with former studies providing evidence that desmosomal cadherin levels depend on Pkp1 [14, 15, 59]. Interestingly, binding properties of remaining binding events in Pkp1- and 3-deficient keratinocytes show weaker single-molecule interactions and a reduced unbinding position. Thus, it could be speculated that Pkps regulate especially the membrane availability of a desmoglein pool serving as desmosomal precursors, which is in line with the former studies showing that Pkp1 and 3 are crucial for desmosome assembly [11, 12, 14, 60, 61].

Reduced binding frequency and impaired intercellular adhesion were also observed in keratin-deficient keratinocytes for Dsg1 [51]. In contrast, Dsg3 was upregulated in keratin-deficient keratinocytes, which appears to be an insufficient rescue mechanism depending on PKC signaling [34]. Interestingly, this mechanism seems to be missing in Pkp1- and 3-deficient keratinocytes underlining their importance for PKC-dependent signaling [14, 62].

Since keratins also regulate p38MAPK activity, which is important for loss of cell adhesion in pemphigus, and regulates desmosome number and size [34, 35, 63], we studied whether altered p38MAPK signaling may account for impaired binding in keratinocytes deficient for Pkp1 and 3. Interestingly, activation of p38MAPK was not altered in Pkp-deficient keratinocytes and direct activation of p38MAPK was effective in further reducing cell cohesion. This indicates that loss of cell cohesion induced by loss of Pkp1 and Pkp3 is not p38MAPK dependent. Moreover, the data show that p38MAPK is not generally and unspecifically activated whenever desmosomal adhesion is impaired. This is in agreement with a previous study where loss of cell cohesion caused by siRNA-mediated depletion of plakoglobin but not of desmoplakin was paralleled by p38MAPK activation [64].

Dsg clustering is regulated by Pkp1 and maybe crucial for proper desmosomal adhesion

It is well established that adhesion molecules are densely packed at cell contact areas. This is important for proper intercellular adhesion, because the force exerted on the cell is portioned to multiple binding sites [65]. Further, as lifetimes of desmogleins are short [5], it is conceivable that strong intercellular adhesion is dependent on proper cluster formation [65]. These clusters are well characterized for classical cadherins in adherens junctions but were also suggested to be present for desmosomal cadherin [66, 67]. As already described, Pkp1 plays a role in the clustering of Dsg3 with desmoplakin [26]. Here, we observed by AFM, STED and crosslinking that clustering of Dsg3 was strictly dependent on Pkp1. In contrast, in cells lacking Pkp3, these effects were less pronounced and significant only for a reduced cluster size.

This clustering function of desmosomal cadherins may be important for proper intercellular cohesion. This is indicated by a previous study which demonstrated homophilic interaction of desmogleins in living keratinocytes and oligomerization to correlate with strong intercellular adhesion [7]. Further, NMR, high-resolution fluorescence microscopy and electron microscopy studies provide models for the organization of the desmosomal plaque, revealing a localization of Pkps at the membrane-facing side of the outer dense desmosomal plaque neighboring desmogleins and thereby suggest interdependence of the order of intra- and extracellular parts of the desmosome [19, 28, 68, 69]. Thus, it is conceivable that Pkp isoforms contribute to cell adhesion by both maintaining junctional availability of desmogleins as well as by regulating clustering of desmogleins, the latter of which is a function specific for Pkp1 but not Pkp3 (Fig. 5g).

To exclude that changes in clustering are a result of impaired membrane availability in Pkp-deficient cell lines, we overexpressed Dsg3, which increased cluster sizes in AFM experiments and restored cluster formation and size in Pkp3-deficient keratinocytes, whereas no effect was observed in Pkp1-deficient cells. In line with this, FRAP experiments revealed that Dsg3 mobility in the membrane depends on Pkp1 but not on Pkp3, which is consistent with previous studies showing that molecule mobility is inversely proportional to intercellular adhesion [34]. These data demonstrate that Pkp1 is critical for Dsg3 clustering whereas Pkp3 is not, at least as long as junctional Dsg3 availability is sufficient (Fig. 5g). The data can explain the profound difference in overall cell cohesion for cell lines lacking Pkp1 and Pkp3 as detected by dissociation assays.

Taken together, the data provided show a new isoform-specific function of Pkp1 and Pkp3 in the regulation of desmosomal adhesion.

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Compliance with ethical standards

Conflict of interest The authors state that there was no conflict of interest.

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