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Supplemental Information

Desmosomal Hyperadhesion Is Accompanied with Enhanced Binding Strength of Desmoglein 3 Molecules

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Results

Cells lacking Pkp1 or 3 have reduced levels of Dsg3 after EGTA treatment

We investigated the expression and distribution of Dsg3 and actin using immunostainings. Interestingly, in wt murine keratinocytes Dsg3 and actin staining showed gaps (indicated by white arrows) when cells were treated with EGTA after 24 h in high Ca^{2+}-medium. After 72 h in high Ca^{2+} we found that Dsg3 and actin are properly located at the cell membrane and observed no gaps even after EGTA treatment in wt. However, Dsg3 staining was slightly reduced (Figure S1A). In contrast, Pkp1-deficient cells revealed a drastic phenotype with gaps after EGTA treatment at 24 h and 72 h of differentiation with reduced levels of Dsg3 at the cell membranes (Figure S1B) resembling the results from dissociation assays. Pkp3-deficient cells showed no reduced intensity of Dsg3 at the cell membrane after EGTA treatment at both time points (Figure S1C) but a more blurred distribution along the cell membrane. This is in line with their diminished ability to acquire hyper-adhesion in dissociation assays. The results suggest that Pkp1 and 3 both are important for hyper-adhesion in murine keratinocytes by contributing to a proper localization of Dsg3.

Materials and Methods

Tissue culture and human *ex-vivo* hyper-adhesion model

A small piece of skin from the shoulder area, < 24 h post mortem, was taken from each body donor and cut into equal pieces (1 cm × 1 cm). The age of the body donors ranged from 74 to 95 years. The samples were incubated in Dulbecco modified Eagle medium (DMEM) with 5 mM of ethylene glycol tetraacetic acid (EGTA) for 1.5 h or 24 h respectively at 37°C and 5% CO_2. Skin samples were afterwards embedded in TissueTec (Leica Biosystems, Nussloch,

Germany) and cut into 7 µm thick slices using a cryostat microtome (CryoStar™ NX70, Thermo Scientific™, Waltham, Massachusetts, USA).

Cell culture

Wild type (wt) and Pkp1- or 3- (Pkp k.o.) deficient murine keratinocytes (MKZ) were isolated and immortalized as described before (kindly provided by Prof. Dr. Hatzfeld, University of Halle (Saale)) [1, 2]. A stable Dsg3 k.o. cell line of mouse epidermal keratinocytes (MKZ) was generated by spontaneous immortalization from Dsg3$^{-/-}$ and $^{+/+}$ C57BL/6J mice. Epidermis was gained from neonatal mice by overnight incubation of the skin with dispase II (Sigma-Aldrich, Munich, Germany). Epidermal cells were physically washed out after treatment for 1 h with accutase (Sigma-Aldrich, Munich, Germany) and seeded in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies; Carlsbad; CA; USA) supplemented with 10 % Chelex-treated fetal calf serum (Biochrom, Berlin, Germany), 50 units/ml penicillin (AppliChem, Darmstadt, Germany), 50 µg/ml streptomycin (AppliChem), 1 mM sodium pyruvate, 0.18 mM adenine, 0.5 µg/ml hydrocortison, 5 µg/ml insulin, 100 pM cholera toxin (all 5 from Sigma-Aldrich, Munich, Germany), 10 ng/ml epidermal growth factor (Life Technologies; Carlsbad; CA; USA) and 2 mM GlutaMAX (Life Technologies; Carlsbad; CA; USA) on collagen I (rat tail; BD Bioscience, New Jersey, US) coated T25 culture flasks and maintained at 35°C and 5 % CO_2. After passaging for 10-15 times keratinocytes immortalized spontaneously while other epidermal cells died. For all experiments with MKZ, cells were switch to high Ca^{2+} (1.2 mM) containing medium after reaching confluency and grown for 24 h or 72 h, respectively, before experiments were conducted.

Immunostaining

Immunofluorescence of skin sections was done as described elsewhere [3]. Briefly summarized, skin sections were fixed with 4% formaldehyde, permeabilized with 1% Triton

X-100 and incubated with primary antibodies. For human skin samples two different primary antibodies were used (Table S1).. As secondary antibodies Cy-labelled goat-anti-mouse and –rabbit secondary antibodies (Dianova, Hamburg, Germany) were used, respectively. Further DAPI (Roche, Mannheim, Germany) was used to visualize the cells nuclei. Quantification was done with ImageJ Software using the selection brush tool. The raw integrated density was measured along all cell membranes and then divided by the measured area. Murine keratinocytes were fixed, 24 h or 72 h after Ca^{2+} switch respectively, in pure ethanol for 30 min on ice followed by three min aceton at room temperature. Primary antibodies were Desmoglein 3 pAb (Biozol Diagnostica, Eching, Germany), Alexa 488-phalloidin (Dianova, Hamburg, Germany) and DAPI. Images were taken with a Leica SP5 confocal microscope using a 63x NA 1.4 PL APO objective controlled by LAS AF software (Leica, Mannheim, Germany).

Antibody against:	In Figure 4, S3 and S4	Figure S4
Dsg1	Desmoglein 1-P124 mAb (Progen Biotechnik GmbH, Heidelberg, Germany)	A9812 pAb (Abclonal Technology, USA)
Dsg3	Desmoglein 3 pAb, E-AB-62720 (Biomol GmbH, Hamburg, Germany)	5G11 mAb (Invitrogen, USA)
Dsc1	Desmocollin 1 pAb (1), abx176152 (Abbexa, Cambridge, United Kingdom)	L15, sc-18115, pAb (2), (Santa Cruz, Dallas, TX, USA)
Dsc3	Desmocollin 3 mAb (Progen Biotechnik GmbH, Heidelberg, Germany)	Abx334157 pAb, (Abbexa, Cambridge, United Kingdom)

Table S1: Used primary antibodies for human skin samples.

Crosslinking, Electrophoresis and Western blot analysis

After washing with PBS cells were lysed with SDS-lysis buffer (25 mmol/l HEPES, 25 mmol/l NaF and 1% SDS, pH 7.4) followed by sonication on ice. The amount of protein was determined with the Pierce^{TM} BCA Protein Assay Kit (Thermo Fisher, USA). Western blotting was implemented following established protocols [4].

The membrane-impermeable cross-linker ethylene glycolbis (sulfosuccinimidylsuccinate) (Sulfo-EGS) (Pierce Biotechnology, Rockford, USA) was used for detection of oligomerization of desmosomal cadherins. The experimental approach followed a well-established protocol [5, 6]. In brief, cells were subjected to respective experimental conditions, washed three times with cold PBS and Sulfo-EGS was added to the cells at a concentration of 2 mM for 30 min at room temperature. In order to stop the reaction, TBS was added at a concentration of 50 mM and incubated for 15 min. Western blotting to detect crosslinked proteins was performed following a standard protocol. For quantification, the raw integrated density of the oligomer band was first divided by the band of oligomer plus monomer. Afterwards the EGTA-treated column was divided by the non-EGTA treated column to obtain the oligomerization ratio.

Data processing and Statistics

For image processing Photoline software (Computerinsel, Bad Gögging, Germany) was applied. AFM images and data analysis of measured force-distance curves were processed with JPK data processing software (Bruker Nano GmbH, Berlin, Germany). Further AFM parameters were determined with Origin Pro 2016, 93G (Northampton, MA, USA). For densitometric measurements ImageJ software (NIH, Bethesda, USA) was used. Other data shown in this study were evaluated and depicted with Excel (Microsoft, Redmond, WA, USA).

For statistical significance in case of two groups we applied two-tailed Student´s t test. For multiple groups analysis of variance (one-way ANOVA) followed by Bonferroni post hoc test was done. Error bars are standard error of the mean or standard deviation as indicated. Significance was assumed at a p-value < 0.05.

Supporting References

1. Keil, R., K. Rietscher, and M. Hatzfeld, *Antagonistic Regulation of Intercellular Cohesion by Plakophilins 1 and 3.* Journal of Investigative Dermatology, 2016. **136**: p. 8.
2. Rietscher, K., A. Wolf, G. Hause, A. Rother, R. Keil, T.M. Magin, M. Glass, C.M. Niessen, and M. Hatzfeld, *Growth Retardation, Loss of Desmosomal Adhesion, and Impaired Tight Junction Function Identify a Unique Role of Plakophilin 1 In Vivo.* Journal of Investigative Dermatology, 2016. **136**(7): p. 8.
3. Egu, D.T., E. Walter, V. Spindler, and J. Waschke, *Inhibition of p38MAPK signalling prevents epidermal blistering and alterations of desmosome structure induced by pemphigus autoantibodies in human epidermis.* British Journal of Dermatology, 2017. **177**(6).
4. Hartlieb, E., B. Kempf, M. Partilla, B. Vigh, V. Spindler, and J. Waschke, *Desmoglein 2 Is Less Important than Desmoglein 3 for Keratinocyte Cohesion.* PLoS ONE, 2013. **8**(1): p. 12.
5. Nie, Z., A. Merritt, M. Rouhi-Parkouhi, L. Tabernero, and D. Garrod, *Membrane-impermeable Cross-linking Provides Evidence for Homophilic, Isoform-specific Binding of Desmosomal Cadherins in Epithelial Cells.* JOURNAL OF BIOLOGICAL CHEMISTRY, 2011. **286**(3): p. 11.
6. Fuchs, M., M. Foresti, M.Y. Radeva, D. Kugelmann, R. Keil, M. Hatzfeld, V. Spindler, J. Waschke, and F. Vielmuth, *Plakophilin 1 but not plakophilin 3 regulates desmoglein clustering.* Cellular and Molecular Life Sciences, 2019.

Figures:

Figure S1

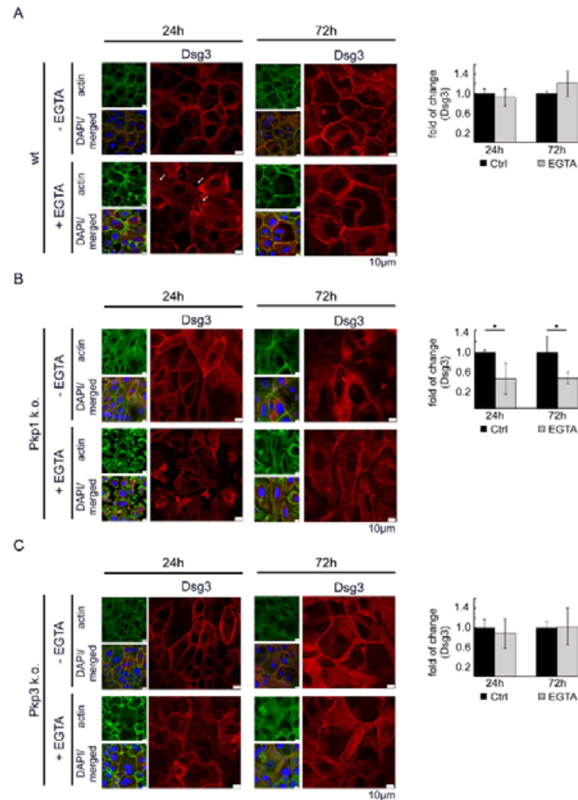

Figure S1: Reduced levels of Dsg3 after EGTA treatment for Pkp1 or 3 lacking cells.

A/B/C: Murine keratinocytes were maintained for 24 h or 72 h in high Ca^{2+} medium and were subsequently subjected to Ca^{2+} chelation with EGTA for 90 min. Immunostaining for Dsg3 and actin revealed disturbed membrane localization after 24 h in high Ca^{2+} medium as well as gap formation between the cells (white arrows). In contrast, membrane staining was preserved after Ca^{2+} chelation in wt and Pkp3-deficient but not in Pkp1-deficient keratinocytes after 72h in high Ca^{2+} medium. DAPI was used to stain cell nuclei. Pictures show representatives of $n \geq 3$. Scale bar = 10 µm. $^*p<0.05$ vs. corresponding control, error bars represent standard deviation.

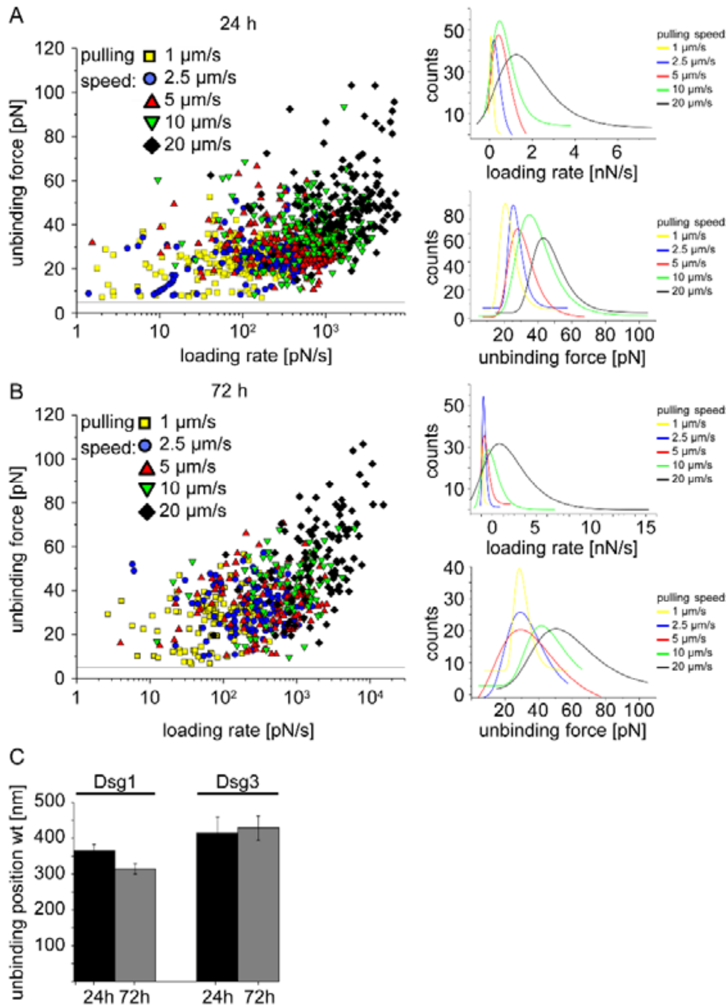

Figure S2: A-B: UF plotted against logarithmic loading rate with respect to their pulling speed after 24 h and 72h in high Ca2+ medium, shows an increase of UF and loading rate for higher pulling forces. Grey line indicates 5 pN threshold level, values below that line were excluded from analysis. On the right side the distribution of the loading rates and unbinding forces of the increasing pulling speeds is shown. n=8 with 2 cell borders/experiment. **C:** Unbinding position of Dsg1 and 3 coated tips comparing 24 h and 72 h in high Ca2+ medium shows no significant alterations. For Dsg1 n=4 and for Dsg3 n=6 were used for analysis, error bars represent error of the mean.

Figure S3

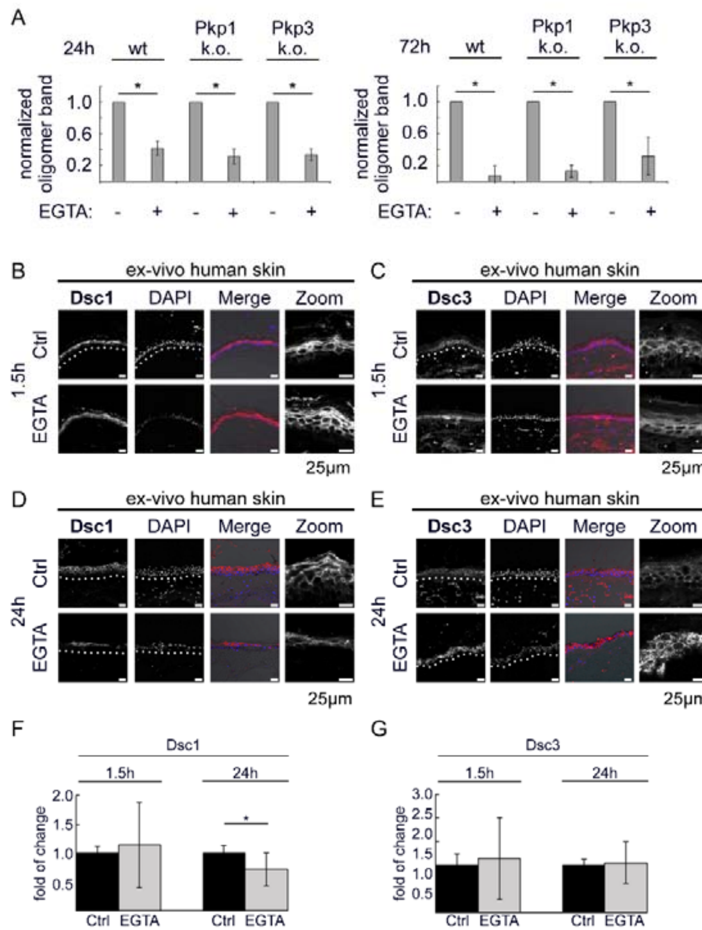

Figure S3: A: Quantification of oligomer band density of ECad shows significant decrease after EGTA treatment for all cell lines after 24h and 72h in high Ca^{2+} medium. n≥4, *p<0.05 vs. corresponding control, error bars represent standard deviation. B**-G:** Immunostaining for Dsc1 and 3 of human epidermis after 1.5h or 24h of EGTA incubation reveals reduced and fragmentated levels of Dsc1 but not Dsc3 staining. n≥3, *p<0.05 vs respective control, error bars represent standard deviation.

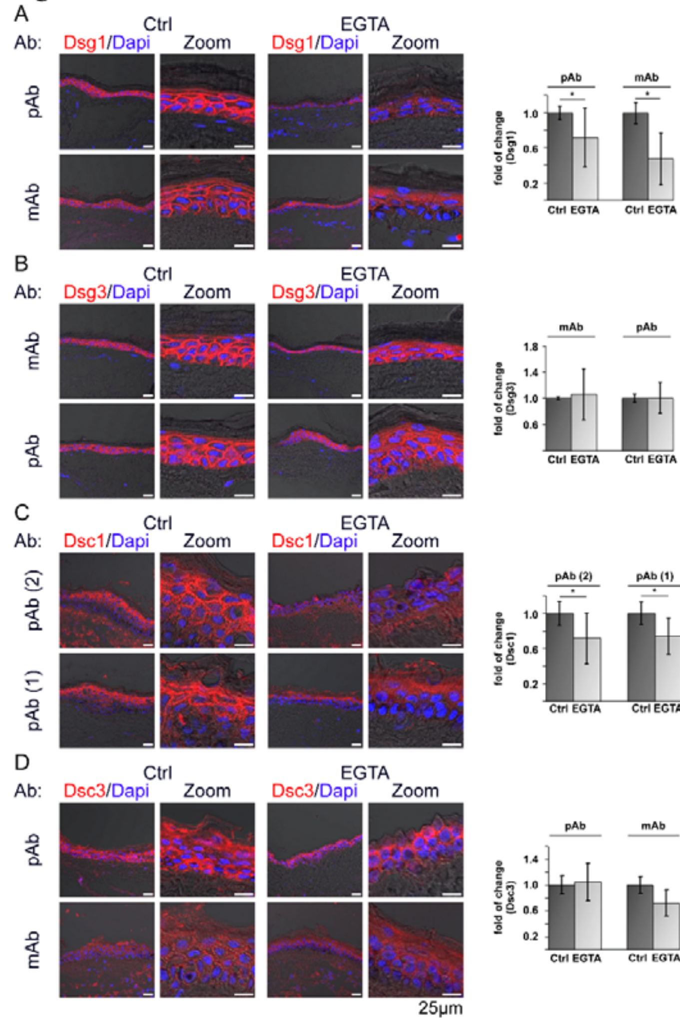

Figure S4: Immunostaining of respective desmosomal cadherins in human epidermis after 24h of EGTA treatment using two sets of primary antibodies. **A:** For Dsg1 both antibodies show a significant reduced membrane staining and protein amount after 24h EGTA treatment. **B:** Dsg3 immunostaining with two different primary antibodies show little alterations after 24h EGTA incubation. **C:** Significantly reduced membrane staining of Dsc1 after EGTA treatment is shown for both primary antibodies. **D:** For Dsc3 staining no difference can be found for the used primary antibodies. **A-D:** n=4, *p<0.05 vs corresponding control, error bars represent standard deviation.