Cell. Mol. Life Sci. (2015) 72:4885–4897
DOI 10.1007/s00018-015-1977-0

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

E-cadherin and Src associate with extradesmosomal Dsg3 and modulate desmosome assembly and adhesion

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Received: 31 January 2015 / Revised: 11 June 2015 / Accepted: 22 June 2015 / Published online: 27 June 2015

Abstract Desmosomes provide strong intercellular cohesion essential for the integrity of cells and tissues exposed to continuous mechanical stress. For desmosome assembly, constitutively synthesized desmosomal cadherins translocate to the cell–cell border, cluster and mature in the presence of Ca^{2+} to stable cell contacts. As adherens junctions precede the formation of desmosomes, we investigated in this study the relationship between the classical cadherin E-cadherin and the desmosomal cadherin Desmoglein 3 (Dsg3), the latter of which is indispensable for cell–cell adhesion in keratinocytes. By using autoantibodies from patients with the blistering skin disease pemphigus vulgaris (PV), we showed in loss of function studies that E-cadherin compensates for effects of desmosomal disassembly. Overexpression of E-cadherin reduced the loss of cell cohesion induced by PV autoantibodies and attenuated activation of p38 MAPK. Silencing of E-cadherin abolished the localization of Dsg3 at the membrane and resulted in a shift of Dsg3 from the cytoskeletal to the non-cytoskeletal protein pool which conforms to the notion that E-cadherin regulates desmosome assembly. Mechanistically, we identified a complex consisting of extradesmosomal Dsg3, E-cadherin, β-catenin and Src and that the stability of this complex is regulated by Src. Moreover, Dsg3 and E-cadherin are phosphorylated on tyrosine residues in a Src-dependent manner and Src activity is required for recruiting Dsg3 to the cytoskeletal pool as well as for desmosome maturation towards a Ca^{2+}-insensitive state. Our data provide new insights into the role of E-cadherin and the contribution of Src signaling for formation and maintenance of desmosomal junctions.

Keywords Desmosome · Src signaling · Desmoglein 3 (Dsg3) · E-cadherin · Calcium switch assay · Pemphigus

Abbreviations

Dsg	Desmogleins
Dsc	Desmocollin
PV	Pemphigus vulgaris
PG	Plakoglobin
DP	Desmoplakin
E-cad	E-cadherin
Ab	Antibody
GFP	Green fluorescent protein
PKC	Protein kinase C
p38 MAPK	p38 mitogen-activated protein kinase
p-Tyr	Phospho-tyrosine
Src	Rous sarcoma (Src) kinase
Pkp	Plakophilin
AJ	Adherens junction
IgG	Immunoglobulin G
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
PBS	Phosphate buffered saline

Electronic supplementary material The online version of this article (doi:10.1007/s00018-015-1977-0) contains supplementary material, which is available to authorized users.

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Hbss	Hank's buffered saline solution
IP	Immunoprecipitation
siRNA	Small interfering RNA
n.t.	Non-targeting

Introduction

Desmosomes are highly organized adhesive contacts that provide strong cohesion of neighboring cells to preserve tissue integrity. The core of desmosomes is formed by desmosomal cadherins, i.e. desmogleins (Dsg 1–4) and desmocollins (Dsc 1–3). These transmembrane cadherins are linked to intermediate filaments via plaque proteins comprising plakoglobin (PG), plakophilins (Pkps) and desmoplakin (DP). Desmosomes are abundant in tissues exposed to continuous mechanical stress like the myocardium and epidermis [30, 64] and Dsg3 is known to exist either desmosome-associated or extradesmosomal in the membrane [5, 51, 62].

The importance of desmosomal adhesion is underlined by a number of human diseases, either inherited or of autoimmune origin, caused by desmosome impairment predominantly in the heart or skin [15, 19, 28, 63]. As an example, pemphigus vulgaris (PV) is a severe blistering skin disease in which pathogenic autoantibodies target the desmosomal cadherins Dsg1 and Dsg3. Since many years, pemphigus is a well-established disease model that allows the modulation of desmosomal adhesion in vitro by application of PV autoantibodies derived from patients [3, 51]. Autoantibody-binding results in loss of epithelial cell–cell adhesion, depletion of Dsg3 from desmosomes and activation of a growing number of signaling molecules such as PKC, p38 MAPK and Src [6–8, 12, 32, 48].

Besides desmosomes, adherens junctions (AJ) represent the second group of cell–cell contacts mediating adhesion of adjacent epidermal cells [18, 40]. Displaying large homologies to desmosome structures, AJs also consist of Ca^{2+}-dependent cadherins, i.e. the classical cadherins E-cadherin and P-cadherin. Unlike desmosomal cadherins, which are connected to intermediate filaments, classical cadherins are coupled with the actin cytoskeleton via an association with β-catenin or PG [52, 66]. Since more than two decades, it is accepted that AJs not only cooperate with desmosomes in providing tight intercellular adhesion, but also are obligatory for desmosome formation [2, 24, 33, 65]. Recent work demonstrated that Pkp3 is located to the cell border by E-cadherin and PG to initiate desmosome formation [23, 54]. Another example of a cross-talk of AJs and desmosomes is highlighted by experiments with DP-null mice keratinocytes displaying immature and reduced AJs [61] which were dependent on PG signaling [34]. Direct interaction of E-cadherin with Dsg3, one of the main pemphigus antigens, was initially detected after extracellular Ca^{2+} depletion [55].

Extradesmosomal Dsg3 was shown to associate with the cytoskeleton [56] and with E-cadherin, thereby facilitating the activation of Src [57, 58], the latter of which is involved in the turnover of AJs [10, 38, 43] and was implicated in the pathogenesis of PV [12, 13, 45, 64]. Nevertheless, the precise role of E-cadherin in the turnover of desmosomal cadherins and in the disease pemphigus is still not fully understood.

In this study, we provide new insights into the function of E-cadherin and Src for Dsg3-mediated adhesion and desmosomal assembly. We show that E-cadherin can compensate for the loss of Dsg3 function induced by pemphigus autoantibodies. Furthermore, E-cadherin mediates Dsg3 incorporation into the cytoskeletal fraction, which in addition is also dependent on the activity of Src. A complex containing non-desmosomal Dsg3, E-cadherin and Src may be involved in this regulation.

Materials and methods

Cell culture and transfection

The human immortalized cell line HaCaT was cultured in DMEM (Life Technologies, Carlsbad, CA) containing 1.8 mM Ca^{2+} supplemented with 10 % fetal bovine serum (Biochrom, Berlin, Germany), 50 U/ml penicillin and 50 µg/ml streptomycin (both AppliChem, Darmstadt, Germany) and cultivated in a humidified atmosphere with 5 % CO_2 at 37 °C. For transfection, HaCaT cells were seeded on 24-well plates, grown to 80 % confluence and transiently transfected the day after seeding using TurboFect™ (Fermentas, Waltham, MA, USA) according to the manufacturer's protocol. Small interfering RNA (human E-cadherin: L-003877-00-0005, human Dsg3: L-011646-00-0005, non-targeting control siRNA: D-001810-10-05) was purchased from Thermo Fisher Scientific/Dharmacon, Waltham, MA, USA. pEGFP-N1-E-cadherin was a generous gift by Dr. Yasushi Hanakawa (Ehime University School of Medicine, Japan). Depending on the best transfection effectivity, reaction was stopped after 48–72 h and cells were prepared for processing.

Test reagents and purification of PV-IgG fractions

The p38 MAPK inhibitor SB202190 (Calbiochem, Darmstadt, Germany) was used at 30 µM for 1 or 24 h, respectively. The c-Src inhibitor pp2 (Calbiochem) was used at a concentration of 10 µM for 0.5, 2 or 5 h. EGF (Sigma-Aldrich, Taufkirchen, Germany) was applied at

Table 1 Antibody profile of pemphigus patients' IgG

ELISA	Dsg1 (U/ml)	Dsg3 (U/ml)
PV1-IgG	60	1239
PV2-IgG	375	11,550

20 ng/ml for 1 h. Cells were incubated with 2.5 or 12.5 µg monoclonal Decma-1 antibody (without NaN_3, Santa Cruz Biotechnology, Dallas, TX, USA). AK23, a monoclonal pathogenic antibody derived from a pemphigus mouse model, was purchased from Biozol (Eching, Germany) and used at 75 µg/ml. PV-IgG was drawn from two patients with active PV suffering from both oral and skin lesions. Purification of IgG was performed as described previously [49]. PV-IgG fractions or IgG from healthy volunteers (control-IgG) were applied at 0.5 mg/ml. Patients' sera were tested by ELISA (Euroimmun, Luebeck, Germany) according to the manufacturer's protocols for reactivity against Dsg 1 and Dsg 3, respectively (see Table 1).

Immunofluorescence

Confluent HaCaTs grown on glass coverslips were fixed for 10 min with 2 % formaldehyde in PBS at room temperature and then permeabilized with 0.1 % Triton X-100 in PBS for 5 min. Afterwards cells were blocked for 40 min with 10 % normal goat serum and 1 % BSA in PBS and immunostained overnight at 4 °C with the following primary antibodies:

Dsg3 mAb (clone 5G11, Life Technologies), Dsg3 pAb (clone H-145, Santa Cruz Biotechnology), E-cadherin mAb (clone 36, BD Biosciences, San Jose, CA, USA), Src pAb, phospho-Tyr416 Src pAb (Cell Signaling, Danvers, MA, USA) and E-cadherin mAb (ECCD-2, Life Technologies). As secondary antibodies Cy3-, Cy2 or Cy5-labeled goat anti-mouse, goat anti-rabbit or goat anti-rat Abs were used (Dianova, Hamburg, Germany). Images were acquired using a Leica SP5 confocal microscope with a 63× NA 1.4 PL APO objective (both Leica, Mannheim, Germany).

Keratinocyte dissociation assay

Fully confluent HaCaT monolayers were washed with Hank's buffered saline solution (Hbss) and incubated with 150 µl of Dispase II (>2.4 U/ml in Hbss; both Sigma-Aldrich) for 20 min at 37 °C to detach monolayers from the well bottom. After replacement of Dispase II by 350 µl Hbss, cell monolayers were mechanically stressed by pipetting up and down seven times using a 1 ml electric pipette (Eppendorf, Hamburg, Germany). Resulting fragments were counted using a binocular microscope (Leica) at strictly the same magnification.

Calcium switch assay

To investigate desmosomal assembly/disassembly, HaCaT cells were grown to confluence (control) in normal DMEM medium containing 1.8 mM Ca^{2+}, incubated with DMEM supplemented with 5 mM EGTA for 1 h (Ca^{2+} depletion) and then treated with fresh DMEM containing 1.8 mM Ca^{2+} for additional 5 h (Ca^{2+} repletion).

Protein extraction and immunoprecipitation

After transfection or treatment with respective reagents, HaCaTs were washed with ice-cold phosphate-buffered saline (PBS). Total cell extraction was prepared by scraping cells in SDS-lysis buffer (containing 25 mM HEPES, 2 mM EDTA, 25 mM NaF and 1 % SDS) supplemented with protease-inhibitor cocktail (Roche, Mannheim, Germany). For protein fractionation, cells were incubated with Triton buffer (containing 0.5 % Triton X-100, 50 mM MES, 25 mM EGTA and 5 mM $MgCl_2$) for 10 min on ice under gentle shaking. Whole protein pool was centrifuged (5 min at 13,000 rpm) for separating in cytoskeletal- (Triton-insoluble, pellet) and a non-cytoskeletal (Triton-soluble, supernatant) fraction. Samples were sonificated and mixed with 3× Laemmli buffer prior to Western blot analysis.

For immunoprecipitation (IP) after Triton X-100 separation, HaCaTs grown to confluence in T75 flasks were washed 2× with PBS and first incubated with ice-cold Triton buffer (TX-100) for 30 min on ice under gentle shaking. After lysate preparation, non-cytoskeletal (supernatant, TX-100 S) fraction was separated from the cytoskeletal (pellet, TX-100 I) pool by centrifugation for 5 min at top spin. Resulting pellets were solved in RIPA buffer (0.05 M Tris–HCl, 0.15 M NaCl, 0.1 % SDS, 1 % Nonidet P-40, 0.1 mM EDTA) supplemented with protease-inhibitor cocktail. A protein amount of 600–1000 µg was pre-cleared with protein G beads (Calbiochem) or G/A beads (Santa Cruz Biotechnology) for 1 h before adding the specific antibody for 3 h at 4 °C. The antibody/lysate complex was applied to 40 µl of protein beads and incubated at 4 °C overnight with rotation. IP lysates were washed with RIPA buffer and, after mixing with 3× Laemmli buffer and boiling for 10 min, subjected to Western blot analysis. IgG-isotype controls from mouse or rabbit (both Santa Cruz Biotechnology) were used dependent on the origin of the capturing antibody.

For p-Tyr IPs, cells were incubated with lysis buffer (10 mM Na_2HPO_4, 150 mM NaCl 1 % Triton X-100, 0.25 % sodium deoxycholate) supplemented with protease- and phosphatase-inhibitor cocktail (Roche). Equal protein amounts were incubated with p-Tyr antibody (Tebu-Bio, Offenbach, Germany) for 1 h at 4 °C following incubation

with 30 µl G beads (Calbiochem) for 1 h at 4 °C. Before whole protein amount was subjected to gel electrophoresis, beads were washed several times and denatured for 10 min at 95 °C.

Electrophoresis and western blot analysis

Western blot lysates were subjected to gel electrophoresis and blotted according to the standard protocol after determining the protein amount using the BCA protein assay kit (Pierce/Thermo Scientific, Waltham, MA, USA). Blots were probed with Desmoplakin pAb, Dsg3 pAb (clone H-145), GAPDH mAb (Santa Cruz Biotechnology), α-tubulin mAb (Abcam, Cambridge, UK), p38MAPK pAb, phospho-Thr180/182 p38MAPK pAb, Src pAb, phospho-Tyr416 Src pAb, (Cell Signaling, Danvers, MA, USA), E-cadherin mAb, β-catenin mAb (clone14) (BD Transduction, Heidelberg, Germany), plakoglobin mAb (Progen, Heidelberg, Germany), phospho-Tyr mAb (Tebu-Bio), phospho-Y845 EGFR pAb (Abcam, Cambridge, USA) and EGFR mAb (Calbiochem) as primary antibodies. For protein detection polyclonal HRP-conjugated goat anti-rabbit (Cell Signaling) or goat anti-mouse (Dianova) IgG Abs and an ECL reaction system (self-made solutions) were used.

Image processing and statistics

Images were processed with Photoshop CS5 (Adobe Systems). ImageJ was used for quantification of band intensities of Western blot experiments. Band intensities were referred to the respective loading controls (e.g. α-tubulin, p38 MAPK, DP or GAPDH) and expressed as folds of controls. Error bars represent standard error of mean. Data for two groups were compared using two-tailed Student's t test. For multiple group comparisons, analysis of variance (ANOVA) was performed followed by Bonferroni post hoc test. Statistical significance was assumed when $p < 0.05$.

Results

E-cadherin compensates for effects of desmosomal perturbation

In the present study, we used autoantibodies from pemphigus patients (PV-IgG) as a tool to modulate desmosomal adhesion in human keratinocytes. First, we investigated whether overexpression of E-cadherin reduced PV-IgG-induced loss of cell adhesion and p38 MAPK activation. Controls or cells expressing E-cadherin-GFP were incubated for 24 h with PV-IgG and subjected to dispase-based dissociation assays (Fig. 1a, b). Overexpression of E-cadherin-GFP significantly reduced loss of cell cohesion induced by PV-IgG (297 ± 29 vs. 140 ± 37 fragments per well). A hallmark of pemphigus is the activation of p38 MAPK. Compared to controls, overexpression of E-cadherin-GFP blocked the dose-dependent activation of p38 MAPK induced by AK23 (monoclonal anti-Dsg3 antibody from a pemphigus mouse model [60]) or PV-IgG treatment for 30 min. Levels of p38 MAPK phosphorylation are indicated above each Western blot band (Fig. 1c).

Vice versa, we used Decma-1, a monoclonal Ab that blocks binding of E-cadherin by maintaining it in a monomeric, inactive state [44]. Dissociation assays showed that 24 h incubation with Decma-1 at high (12.5 µg/ml) as well as low (2.5 µg/ml) doses resulted in loss of cell cohesion (number of fragments per well: 16 ± 2 and 7 ± 1) compared to control cells (1 ± 1). Similar to the situation of impaired Dsg3 binding as previously published [7, 25], co-incubation with the p38 MAPK inhibitor SB202190 significantly reduced the number of fragments (12.5 µg/ml Decma-1: 10 ± 2 and 2.5 µg/ml Decma-1: 4 ± 1) (Fig. 1d). Surprisingly, in contrast to PV-IgG treatment, incubation of Decma-1 for 30 min did not enhance p38 MAPK phosphorylation levels (Fig. 1e). Although the applied PV-IgG fraction contains both Dsg3 and Dsg1 autoantibodies, we focused on Dsg3 because under the culture conditions used for this study HaCaTs express Dsg3 but not Dsg1 [14].

To further dissect the roles of E-cadherin and Dsg3 for cell–cell adhesion and signaling, we specifically silenced Dsg3 or E-cadherin. SiRNA-mediated depletion of either E-cadherin or Dsg3 did not alter the levels of the respective other protein (Fig. 2a). In accordance with our previous findings [25], depletion of Dsg3 led to 1.5-fold p38 MAPK activation compared to non-targeting (n.t.) siRNA controls. Activation of p38 MAPK by Dsg3 silencing correlated with increased number of fragments compared to controls (23 ± 1 vs. 8 ± 1 fragments per well), and SB202190-mediated p38 MAPK inhibition blunted loss of cell cohesion in Dsg3-depleted cells (16 ± 1 fragments per well) (Fig. 2b). In line with the experiments using Decma-1 Ab, E-cadherin-depleted cells displayed no alterations in p38 MAPK activity. Nevertheless, the increased fragment numbers caused by E-cadherin silencing were also significantly reduced after 24 h SB202190 incubation (22 ± 1 vs. 14 ± 0.4). Together, these experiments demonstrate that E-cadherin overexpression significantly ameliorates two hallmarks of PV, namely loss of cell–cell cohesion and activation of p38 MAPK. Furthermore, p38 MAPK inhibition reduces monolayer fragmentation as a result of either Dsg3 or E-cadherin interference.

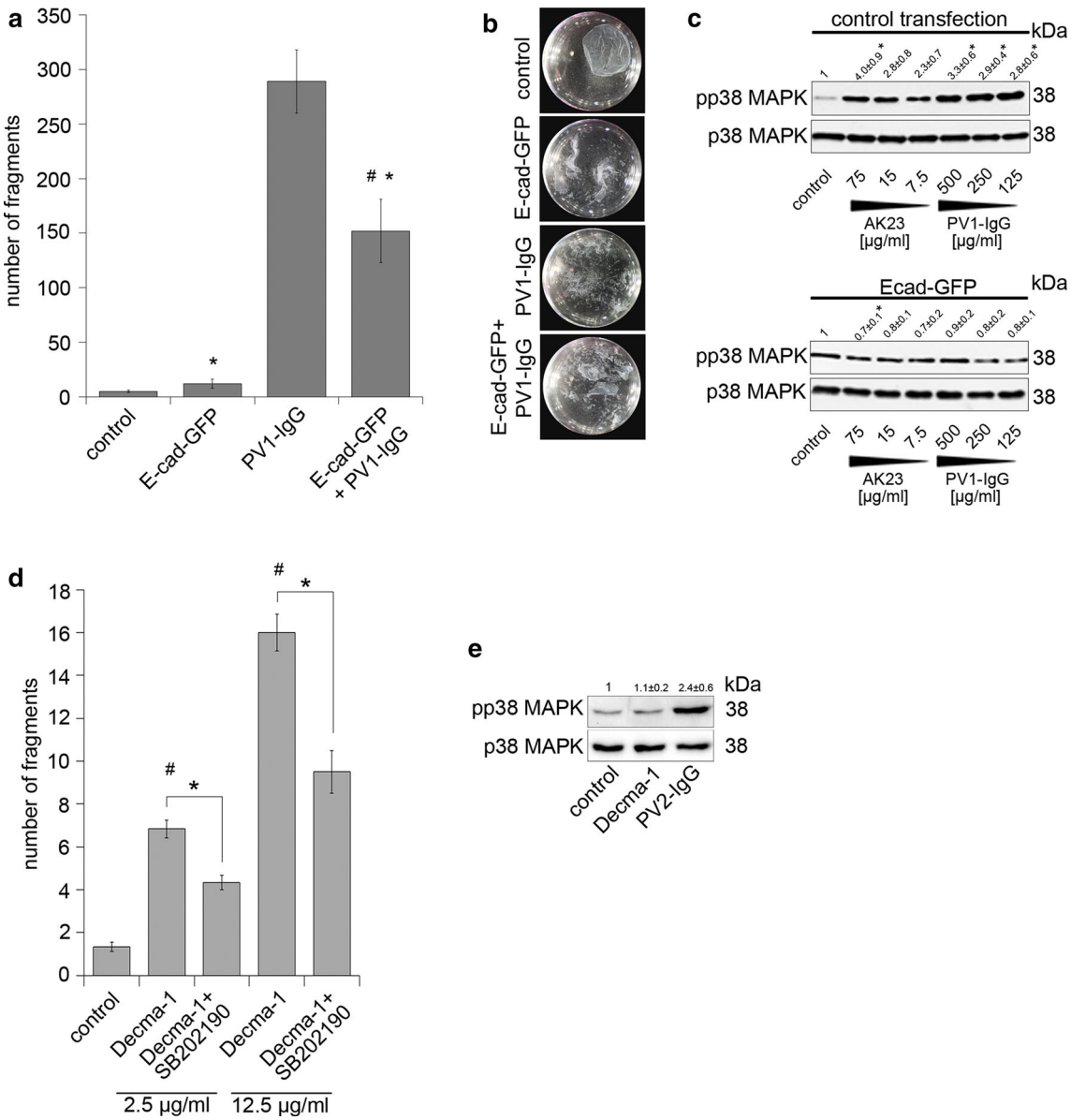

Fig. 1 E-cadherin blocks PV-IgG-mediated loss of cell cohesion and p38 MAPK activation. **a** Expression of GFP-tagged E-cadherin reduced fragment numbers induced by pemphigus autoantibodies (PV-IgG), but had no effect on cell cohesion under control transfection ($n = 6$; $^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. PV-IgG). **b** Representative pictures of HaCaT monolayers subjected to dispase-based dissociation assays. **c** In control cells, both 30 min AK23 or PV-IgG incubation induced dose-dependently p38 MAPK phosphorylation that was blocked by E-cadherin overexpression. Band intensity of pp38 MAPK was measured by densitometry, referred to p38 MAPK intensities and expressed as fold of control ($n = 6$, $^{*}p < 0.05$ vs. control). **d** Inhibition of E-cadherin binding by Decma-1 antibody at high (12.5 µg/ml) as well as low dose (2.5 µg/ml) for 24 h promoted loss of cell cohesion that was significantly reduced by parallel p38 MAPK inhibition (SB202190) ($n = 6$; $^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. Decma-1 control). **e** In Western blot analysis, incubation with PV-IgG but not with Decma-1 antibody for 30 min altered the phosphorylation levels of p38 MAPK. Band intensity of pp38 MAPK was referred to p38 MAPK and indicated as fold of control ($n = 3$)

E-cadherin interacts with Dsg3 and regulates Dsg3 subcellular localization

Since numerous studies demonstrated that E-cadherin is required for desmosome assembly [33, 36, 67], we next examined the spatial distribution of Dsg3 following E-cadherin silencing. As shown in Fig. 3a, a partial co-localization of E-cadherin and Dsg3 at the cell cortex was detectable under control conditions (n.t. siRNA, left panels). Interestingly, cells with prominent E-cadherin knockdown also showed a depletion of Dsg3 at the membrane. Because E-cadherin silencing was not associated with reduced Dsg3 levels (see Fig. 2a), we next performed Triton extraction experiments to separately investigate the

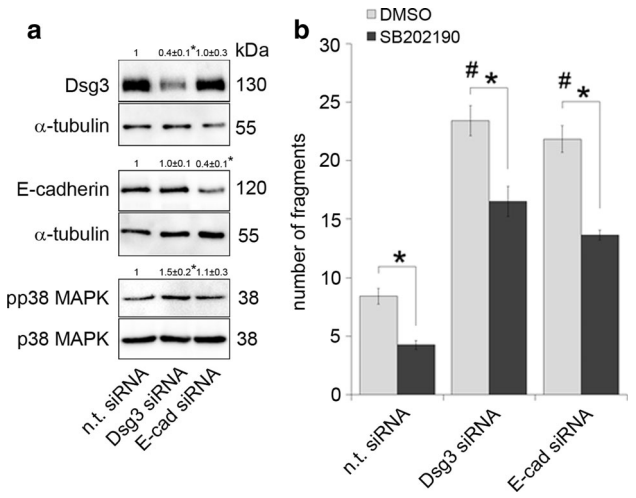

Fig. 2 Dsg3 but not E-cadherin silencing enhances p38 MAPK activity. **a** In Western blot, siRNA-mediated silencing of Dsg3 but not of E-cadherin activated p38 MAPK. Relative values of pp38 MAPK, Dsg3 and E-cadherin are indicated above each Western blot band as fold of n.t. siRNA controls. Protein amount of pp38 MAPK was referred to p38 MAPK, Dsg3 and E-cadherin to the loading control α-tubulin ($n = 4$, $^{*}p < 0.05$ vs. n.t. siRNA). **b** In contrast, increased loss of cell cohesion after both Dsg3 and E-cadherin depletion was blocked by p38 MAPK inhibition for 24 h ($n \geq 8$; $^{\#}p < 0.05$ vs. control,$^{*}p < 0.05$ vs. SB202190)

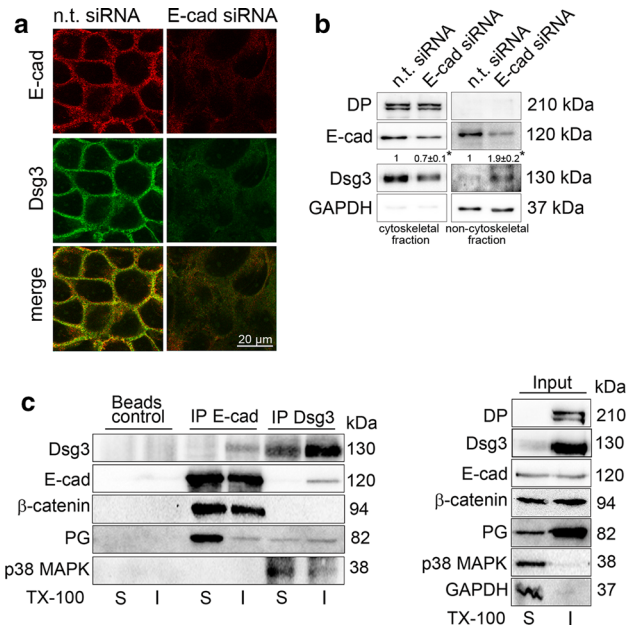

Fig. 3 E-cadherin interacts with Dsg3 and promotes its cytoskeletal anchorage. **a** Under non-targeting (n.t.) siRNA conditions, Dsg3 partially co-localized with E-cadherin at the cell membrane whereas in cells transfected with E-cadherin siRNA, Dsg3 staining was depleted ($n = 4$; *scale bar* 20 µm, *insets* represents 5× magnification of indicated areas). **b** E-cadherin depletion caused a shift of Dsg3 from the cytoskeletal (Triton-insoluble) to the non-cytoskeletal (Triton-soluble) protein pool. Band intensity was quantified by densitometry, referred to either DP or GAPDH and indicated as fold of the n.t. siRNA control ($n = 4$, $^{*}p < 0.05$ compared to n.t. siRNA control). **c** Pull down after Triton-mediated separation of either E-cadherin or Dsg3 revealed complex formation with the respective other protein within the cytoskeletal fraction. In both Triton-soluble (TX-100 S) and -insoluble (TX-100 I) pools E-cadherin co-immunoprecipitated with β-catenin and PG whereas Dsg3 associated with p38 MAPK and PG ($n = 3$). GAPDH and DP were used to identify either the Triton X-100-soluble or insoluble fraction

cytoskeleton-bound (Triton-insoluble) and the cytosolic/membrane (Triton-soluble) protein pools (Fig. 3b). E-cadherin silencing resulted in reduced levels of Dsg3 in the cytoskeletal (protein level 0.7 ± 0.1), desmosome-containing pool but increased amounts of Dsg3 in the cytosolic/membrane protein pool (protein level 1.9 ± 0.2) compared to control siRNA. Thus, our data demonstrate that the anchorage of Dsg3 to the cytoskeleton is dependent on the presence of E-cadherin and the loss of the latter molecule causes a redistribution of Dsg3 from the cytoskeletal to the non-cytoskeletal pool.

Given the partial co-localization of E-cadherin and Dsg3, which has also been observed by others [58], we next immunoprecipitated either Dsg3 or E-cadherin separately from the Triton-soluble and the Triton-insoluble pool (Fig. 3c). Correct separation of the two pools was indicated by DP (Triton-insoluble pool, TX-100 I) and GAPDH (Triton-soluble pool, TX-100 S) detection. Pull down of either Dsg3 or E-cadherin co-precipitated PG in both protein pools whereas β-catenin associated with E-cadherin, but not with Dsg3. The slightly reduced PG amount in the soluble compared to the insoluble fraction may result from less efficient capturing of Dsg3 in the soluble fraction. In contrast, an association of Dsg3 with E-cadherin and vice versa was detectable within the cytoskeleton-bound pool (TX-100 I) only. Complex formation of p38 MAPK and Dsg3 was detectable in both pools, as it has been observed previously [25], whereas E-cadherin was not associated with p38 MAPK.

The specific association of E-cadherin and Dsg3 within the cytoskeleton-bound pool suggested localization within a junctional complex. Thus, we performed additional immunoprecipitation experiments (IPs) specifically within the Triton-insoluble protein fraction (Fig. 4a) and pulled down either β-catenin, a member of AJs [1, 29] or DP, the marker component of desmosomes [37]. IP of β-catenin displayed association with E-cadherin, Dsg3 and the tyrosine kinase Src but not with DP or p38 MAPK. In contrast, Co-IP analysis of DP showed complex formation with Dsg3 and p38MAPK but not with β-catenin, E-cadherin or Src. In line with these experiments, we detected partial co-localization of Dsg3 both with Src and with p38 MAPK at the cell cortex by immunostaining. In contrast to Src, co-localization between Dsg3 and p38 MAPK was also detectable in the cell cytoplasm

(Fig. 4b). This is in agreement with the two different complexes containing either Dsg3 and Src (β-catenin pull down) or Dsg3 and p38 MAPK (DP pull down). For further experiments, we focused on the complex containing Dsg3, E-cadherin and Src. Pre-incubation with the specific Src inhibitor pp2 (10 μM) for 1 h reduced EGF-mediated cell dissociation [27] and EGFR phosphorylation in response to EGF stimulation after 1 h (Fig. S1a and S1b), demonstrating the efficacy of pp2 under the conditions used. Interestingly, application of pp2 for 2 h reduced the association of Dsg3 and Src with β-catenin within the cytoskeleton-bound pool (Fig. 4c). This indicates that Src activity is required for retention of Dsg3 within the complex of Dsg3, E-cadherin and β-catenin. Since Src is a tyrosine kinase [46, 53], we next investigated whether Dsg3 is phosphorylated dependent on Src activity by pull down of tyrosine phosphorylated proteins from the Triton-insoluble fraction. As shown in Fig. 4d, specific Src inhibition by pp2 treatment reduced tyrosine phosphorylation of Dsg3 but also of E-cadherin and PG. Since DP phosphorylation was not altered, these data indicate that Dsg3, E-cadherin and PG are specifically phosphorylated by Src. Detection of pEGFR (Y845) served as positive control.

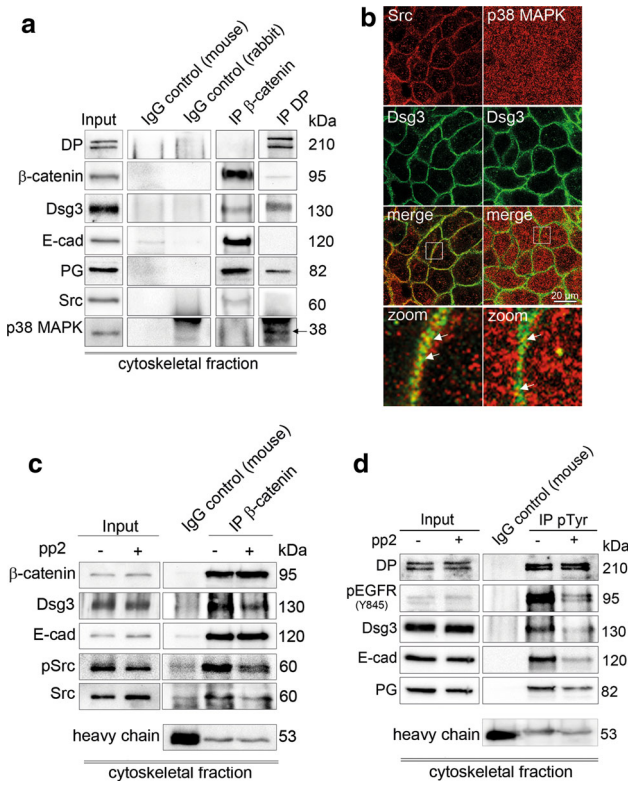

Fig. 4 Dsg3 forms a Src-dependent complex with E-cadherin in the cytoskeletal protein fraction. **a** Co-IP analysis after pull down of β-catenin within the Triton-insoluble fraction revealed a complex consisting of Dsg3 and E-cadherin together with Src and PG. These associations are absent in desmosomes, as shown by pull down of desmoplakin (DP) ($n = 4$). **b** Immunofluorescence analysis displayed a partial co-localization of Dsg3 with Src and p38 MAPK (*arrows*, $n = 4$; *scale bar* 20 μm; *insets* are 6× magnification of indicated areas). **c** Application of pp2, a potent Src inhibitor, for 2 h reduced the association of Dsg3 with E-cadherin and Src bound to β-catenin within the cytoskeletal pool ($n = 4$). **d** IP analysis within the cytoskeleton-bound fraction showed a reduction of tyrosine phosphorylation of Dsg3, E-cadherin and PG but not of DP after 2 h treatment with pp2. Detection of pEGFR (Y845) served as a positive control ($n = 3$)

Src together with E-cadherin regulates cytoskeletal anchorage of Dsg3 during desmosome assembly

Because we detected a complex containing Dsg3, E-cadherin and Src and that loss of E-cadherin reduced the amount of Dsg3 in the cytoskeletal pool, we tested the role of Src in the regulation of desmosomal assembly. This can be tested by raising extracellular Ca^{2+} levels [26, 41]. HaCaT keratinocytes were first depleted of Ca^{2+} for 1 h to induce desmosome disassembly followed by a 5 h period of Ca^{2+} repletion to re-establish desmosomes. Cells that were constantly exposed to 1.8 mM Ca^{2+} served as controls and were designated control condition. Cell contact disassembly following 1 h Ca^{2+} depletion and reassembly after Ca^{2+} supply were monitored by staining of Dsg3 as well as by ECCD-2, a monoclonal antibody that detects E-cadherin when bound homophilically (Fig. S2), as reported previously [11]. In Ca^{2+} switch assays, we detected increased staining of activated Src at the cell membrane after 30 and 120 min of Ca^{2+} repletion compared to controls and after 1 h Ca^{2+} depletion (upper panels, Fig. 5a). In contrast, levels of total Src were not altered (lower panels, Fig. 5a).

Further experiments with HaCaTs in Ca^{2+} switch assays showed that under physiological Ca^{2+} (control) conditions, incubation of pp2 for 2 h had no effects on the localization of Dsg3 at the cell border (Fig. 5b, left panels). However, compared to cells repleted with 1.8 mM Ca^{2+} for 5 h, Dsg3 staining at cell borders was perturbed and appeared fragmented after 5 h Ca^{2+} repletion under simultaneous pp2-mediated Src inhibition compared to respective controls treated with DMSO (Fig. 5b, right panels, arrows). Under conditions of Ca^{2+} depletion Dsg3 staining disappeared completely (Fig. 5b, middle panels). In line with this, analyses of the Dsg3 distribution after separation into cytoskeletal-anchored and cytosolic/membrane protein pools revealed that Src inhibition reduced the shift of Dsg3 into the cytoskeletal, desmosome-containing fraction. Compared to repletion without pp2, treatment with the Src inhibitor led to reduced Dsg3 levels in the cytoskeletal protein pool and to a significant increase in the cytosolic/membrane fraction (Fig. 5c). Taken together, or data indicate that Src activity is required to assemble Dsg3 in the desmosomal pool.

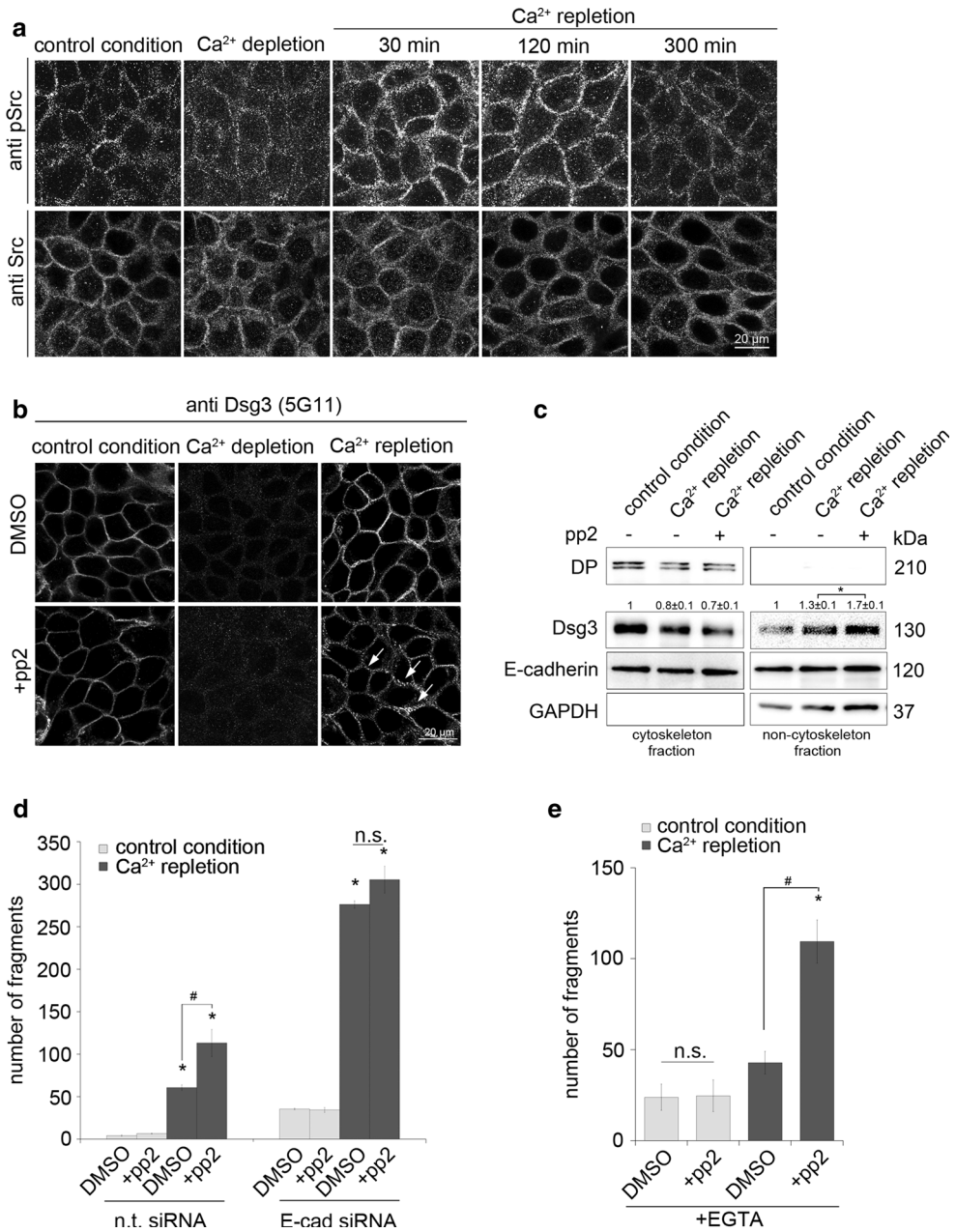

Fig. 5 Src activity is necessary for Dsg3 cytoskeletal anchorage. **a** HaCaTs subjected to Ca^{2+} switch assay displayed enhanced pSrc staining at the membrane after 30 and 120 min of Ca^{2+} repletion compared to cells of control and Ca^{2+} depletion conditions (*upper panel*). In contrast, no changes of total Src levels at the membrane were detectable during Ca^{2+} switch assay (*lower panel*, $n = 4$). **b** Dsg3 staining at the cell cortex was not modulated by pp2 treatment for 2 h under physiological Ca^{2+} supply, but appeared disrupted after parallel pp2 incubation (Ca^{2+} repletion, *right panel*, *arrows*) compared to the DMSO control. After Ca^{2+} depletion, staining of membranous Dsg3 was abolished (*middle panel*, $n = 3$). **c** After Ca^{2+} replacement for 5 h, Dsg3 levels were increased in the non-cytoskeletal pool and slightly decreased in the cytoskeletal fraction by parallel pp2 incubation. Dsg3 band intensity was quantified by densitometry, referred to either DP or GAPDH and indicated as fold of the control condition (DMSO) ($n = 4$, $^{*}p < 0.05$ compared to Ca^{2+} repletion condition of either cytoskeletal or non-cytoskeletal fraction). **d** Under condition of desmosome re-assembly (Ca^{2+} repletion), pp2 incubation for 5 h significantly increased fragment numbers in n.t. siRNA control cells, whereas cohesion of E-cadherin-deficient cells was not further impaired by Src inhibition ($n = 12$, $^{\#}p < 0.05$ vs. pp2 condition, $^{*}p < 0.05$ vs. respective DMSO control condition). **e** Dissociation assays showed that pp2 treatment during Ca^{2+} repletion significantly blocked transition of HaCaT cells to become Ca^{2+}-insensitive compared to cells treated with DMSO only ($n = 12$, $^{*}p < 0.05$ compared to the respective control)

To examine a potential role of E-cadherin in Src-dependent Dsg3 assembly, we combined siRNA-mediated silencing of E-cadherin with Ca^{2+} switch experiments and analyzed intercellular cohesion under these conditions by dispase-based assays (Fig. 5d). Interestingly, fragment numbers were still increased following Ca^{2+} repletion although immunostaining of Dsg3 (Fig. 5b) and E-cadherin ligation detected by ECCD-2 (Fig. S2) were restored. Fragment numbers of cells transfected with n.t. siRNA and grown in medium with physiological Ca^{2+} content were not elevated by simultaneous pp2 treatment. The combination of Ca^{2+} repletion and Src inhibition resulted in nearly doubled fragment numbers compared to repletion alone (113 ± 16 vs. 61 ± 3 fragments per well), which is in line with impaired assembly of Dsg3 into desmosomes we observed. We compared this to monolayers depleted for E-cadherin as shown in Fig. 2a. E-cadherin silencing resulted in higher fragment numbers which were not affected by pp2 incubation compared to DMSO controls (number of fragments per well: 34 ± 3 vs. 36 ± 1). However, during Ca^{2+} repletion, the inhibitory effect of pp2 on the restoration of cell cohesion was blunted, as fragment numbers were not significantly different compared to the respective controls (276 ± 4 vs. 306 ± 16 fragments per well). These results demonstrate that Src-regulated desmosome assembly is dependent on the presence of E-cadherin. During cell contact maturation, desmosomes undergo a process which finally leads to a state termed hyper-adhesion, in which cells resist Ca^{2+} depletion and are still adhesive [20]. To investigate the role of Src in this context, we addressed hyper-adhesion of HaCaT cells before Ca^{2+} withdrawal and after Ca^{2+} repletion in combination with inhibition of Src. Cell monolayers were detached from the well-bottom by dispase incubation and then treated with 5 mM EGTA in DMEM containing 1.8 mM Ca^{2+} for additional 2 h. In control conditions (constant Ca^{2+}-supply), pp2-induced Src inhibition did not alter the intercellular adhesion strength compared to cells treated with DMSO as control agent (24 ± 7 vs. 25 ± 9 fragments per well). In contrast, after Ca^{2+}-repletion for 5 h pp2-treated HaCaTs were significantly less resistant against mechanical stress compared to the respective DMSO-treated control cells (109 ± 12 vs. 43 ± 6 fragments per well) (Fig. 5e). Thus, Src inhibition interferes with maturation of cell–cell contacts towards Ca^{2+} insensitivity.

In summary, based on data from this study together with findings from a previous work [25], three Dsg3-containing signaling complexes can be assumed (Fig. 6). Complex I consists of non-cytoskeletal (Triton-soluble) Dsg3, PG and p38 MAPK whereas cytoskeleton-bound (Triton-insoluble) Dsg3 binds either to E-cadherin, β-catenin, PG and Src outside of desmosomes (complex II) or to DP, PG and p38 MAPK (complex III) within desmosomes. Specifically the

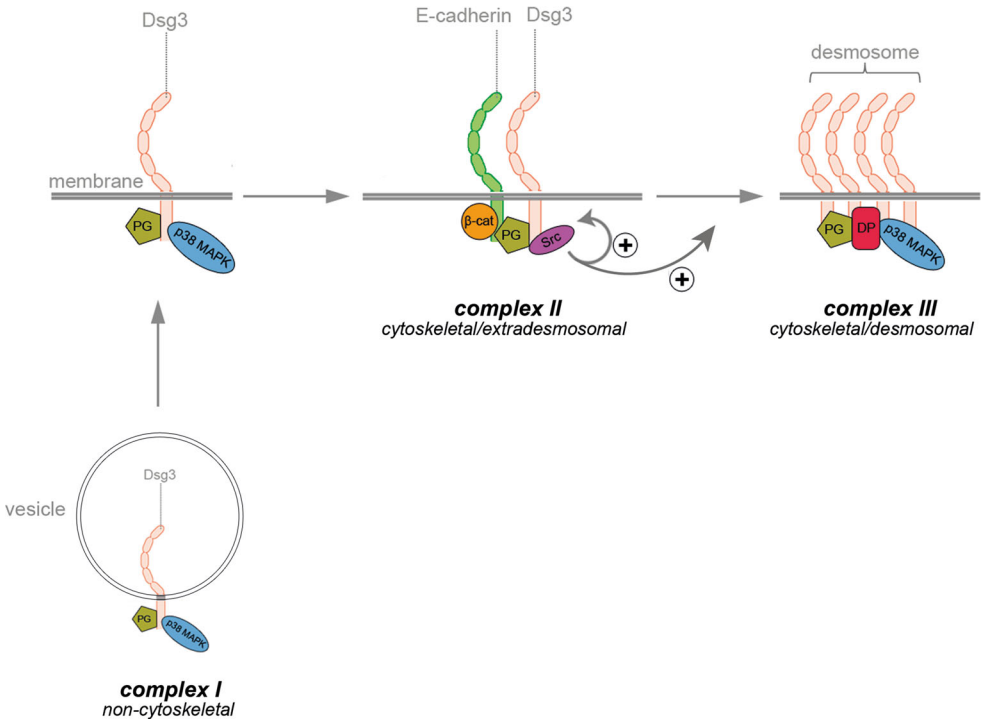

Fig. 6 Src regulates Dsg3 cytoskeletal anchorage. Schematic of the three distinct Dsg3-containing complexes that may be relevant for the formation and maintenance of desmosomes

interaction of E-cadherin with Src and Dsg3 may be required for establishing of proper desmosomal adhesion.

Discussion

This study provides novel evidence that the cooperation of adherens junctions and desmosomes is crucial for intercellular adhesion in keratinocytes. First, forced overexpression of E-cadherin can partially rescue cell dissociation and p38 MAPK activation as a result of autoantibody-mediated impairment of desmosomes. Second, we identified E-cadherin and Dsg3 being phosphorylated at a tyrosine residue dependent on Src activity. In this context, we also detected that E-cadherin and Src are required to assemble Dsg3 into desmosomes. Third, we propose that a complex consisting of extradesmosomal Dsg3, E-cadherin and Src, which has been reported previously [57, 58], is a potential candidate to elicit this function (Fig. 6).

Src and E-cadherin regulate Dsg3 assembly

The requirement of E-cadherin for correct desmosome formation is well established [24, 34, 36]. In line with this, we show that E-cadherin modulates the sorting of Dsg3 from the Triton-soluble to the Triton-insoluble pool which is required to achieve proper cell cohesion. Interestingly, silencing of either E-cadherin or Dsg3 with a similar efficacy reduces cell cohesion to roughly the same extent. Given the role of E-cadherin for Dsg3 turnover, the question arises whether the loss of cell cohesion observed following E-cadherin knockdown is not alone an effect resulting from the missing adhesive function of E-cadherin. Loss of intercellular cohesion induced by E-cadherin depletion may rather result in reduced cytoskeletal association of Dsg3 and consequently in impaired desmosome formation. Moreover, inhibition of Src during junction assembly has similar effects on Dsg3 distribution as E-cadherin-silencing: (1) reduction of Dsg3 at the membrane and (2) a shift from the Triton-insoluble to the Triton-soluble fraction. Therefore, it is possible that both E-cadherin and Src share similar mechanisms to modulate Dsg3 turnover. Indeed, we were able to detect a complex containing E-cadherin, Src and Dsg3. A similar association of Dsg3 and E-cadherin has been observed before, albeit in the non-cytoskeletal fraction [57], using a different fractionation regime. It is possible that the complex detectable under our experimental conditions is located in adherens junctions because it is localized in the cytoskeletal pool and both β-catenin and PG are associated, whereas DP is not. It is unclear whether the desmosomal molecules are stable within this complex and participate in cell cohesion or rather represent a temporary association on the way to or from desmosomes (Fig. 6). In addition, Src, which is well known to localize to adherens junctions [17, 59], is required for the stability of this complex, as demonstrated by the exclusion of Dsg3 but not of E-cadherin following Src inhibition. Because Src also regulates the shift from the Triton-soluble to the Triton-insoluble fraction, it might either regulate Dsg3 incorporation into desmosomes and/or into the β-catenin-associated complex observed in our study.

Src-mediated phospho-tyrosine signaling is controversially discussed regarding its regulatory function for cell cohesion, which might be dependent on the cellular context and the activity levels of Src [35]. Nevertheless, the activity of Src family kinases and tyrosine phosphorylation of junctional components is important to regulate keratinocyte cell cohesion in vitro and in vivo [9]. Importantly, by means of Src inhibition, we identify E-cadherin, Dsg3 and PG within the cytoskeletal protein pool as direct or indirect target of Src. Together with the finding that active Src is predominantly localized at the cell cortex at early time points of Ca^{2+} repletion and the maintenance of E-cadherin in this complex following Src inhibition, it is conceivable that E-cadherin binding results in Src-dependent phosphorylation of desmosomal molecules to foster desmosome assembly. Interestingly, vice versa, overexpression of Dsg3 was also shown to increase phosphorylation of adherens junction molecules in a Src-dependent manner [58]. This further highlights the interdependence of adherens junctions and desmosomes.

It has recently been shown that the translocation of DP to the desmosome is mediated via Pkp3 and the cAMP target Epac1 [54]. This indicates that different pathways exist for assembly of the desmosomal components. DP, probably by already attached keratin filaments, is translocated to the membrane by a mechanism involving initially Pkp3 and Epac1, followed by the concerted action of Pkp2, PKC and RhoA [4, 22, 54] In contrast, desmosomal cadherins are transported to the membrane by a microtubule-driven transport [39] and then laterally incorporated into desmosomes [47]. According to our data, at least Dsg3 is guided to the junctions by a mechanism that also requires E-cadherin and Src activity.

E-cadherin can compensate for loss of Dsg3 function

Using PV-IgGs as tools to impair desmosomal adhesion, we demonstrated that overexpression of E-cadherin is sufficient to at least partially inhibit two hallmarks of PV: complete loss of cell–cell adhesion and activation of p38 MAPK. Although E-cadherin antibodies can be present in pemphigus patients [16, 42], E-cadherin is assumed to be of minor importance for loss of cell cohesion [31, 64].

Similarly, we did not observe major alterations of E-cadherin distribution in recent studies [21, 50]. Nevertheless, obviously E-cadherin can partially compensate for loss of desmosome function. The most obvious explanation is that enhanced E-cadherin levels directly result in increased adhesion due to enhanced binding events with respective counterparts of the adjacent cell. However, the impact of E-cadherin on overall cell cohesion can be more indirect. For instance, E-cadherin likely contributes to epithelial integrity by regulating desmosome assembly which in part involves the activation of Src.

In this context, it is intriguing to speculate that E-cadherin and Dsg3 regulate desmosome assembly and disassembly via different kinases. Interestingly, Src was detected as part of the intermediate complex consisting of non-junctional/cytoskeleton-bound Dsg3 and E-cadherin but not in the desmosomal protein pool (Fig. 6) indicating that E-cadherin but not Dsg3 may regulate Src function which is required for desmosome formation. Contrary, p38 MAPK, which is known to regulate desmosome turnover by controlling Dsg3 depletion and cytoskeletal anchorage [51] was present in both the non-cytoskeletal and the desmosomal fraction of Dsg3. Although loss of cell cohesion and p38 MAPK activation in response to PV-IgG treatment were efficiently blocked by E-cadherin overexpression, the facts that E-cadherin silencing or antibody-mediated inhibition of E-cadherin binding resulted in loss of cell cohesion without affecting p38 MAPK activity indicate that p38 MAPK is more indirectly regulated by E-cadherin than by Dsg3. In line with this, we did not observe an interaction of p38 MAPK with E-cadherin (Fig. 6) but instead with Dsg3, which supports our previous studies [25, 50]. Thus, the control of Dsg3 turnover by E-cadherin, as discussed above, may cause the lack of p38MAPK activation observed in cells overexpressing E-cadherin.

These observations indicate that Dsg3 and E-cadherin contribute to cell cohesion by different mechanisms. More refined studies are required to dissect these differences in more detail.

Acknowledgments We thank Dr. Yasushi Hanakawa for providing the pEGFP-N1-Ecad construct. Furthermore, we thank Veronika Heimbach, Martina Hitzenbichler, Claudia Meyerhofer, Linda Jakobi and Andrea Wehmeyer for their skillful technical assistance. This work was supported by Deutsche Forschungsgemeinschaft Grant DFG SP1300/1-1 and 1-3 (to V.S. and J.W.)

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