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RESEARCH ARTICLE

E-cadherin and plakoglobin recruit plakophilin3 to the cell border to initiate desmosome assembly

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Abstract A decrease in the levels of the desmosomal plaque protein, plakophilin3 (PKP3), leads to a decrease in desmosome size and cell-cell adhesion. To test the hypothesis that PKP3 is required for desmosome formation, the recruitment of desmosomal components to the cell surface was studied in the PKP3 knockdown clones. The PKP3 knockdown clones showed decreased cell border staining for multiple desmosomal proteins, when compared to vector controls, and did not form desmosomes in a calcium switch assay. Further analysis demonstrated that PKP3, plakoglobin (PG) and E-cadherin are present at the cell border at low concentrations of calcium. Loss of either PG or E-cadherin led to a decrease in the levels of PKP3 and other desmosomal proteins at the cell border. The results reported here are consistent with the model that PG and E-cadherin recruit PKP3 to the cell border to initiate desmosome formation.

Keywords Plakophilin3 · Desmosome · Plakoglobin

Abbreviations

ARM	Armadillo
WT	Wild type
PKP	Plakophilin
PG	Plakoglobin
DP	Desmoplakin
DSG	Desmoglein
DSC	Desmocollin

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Introduction

Desmosomes are specialized adhesion junctions that anchor intermediate filaments at membrane-associated plaques in adjoining cells. In addition to imparting adhesive strength to cells, desmosomes have been postulated to play a role in differentiation and signal transduction [1]. Three different protein families contribute to desmosome structure and function: the desmosomal cadherins, the armadillo (ARM) proteins, and the plakins (reviewed in [1, 2]). Desmosome composition varies with respect to tissue type and differentiation. This is because the cadherins and associated ARM family members show both tissue-specific and differentiation-specific expression, thus having an important impact on the assembly, morphology and function of desmosomes in different tissues and different cell types in the same tissue (reviewed in [3, 4]).

The ARM proteins participate in the regulation of desmosome assembly and cell-cell adhesion [5, 6]. Plakoglobin (PG) and the plakophilin (PKP) family members collaborate with the desmoplakin (DP) N-terminus to regulate the clustering of the desmosomal cadherins at the cell surface [7]. The PKP family members have been reported to be important for recruiting DP to the cell surface and have been postulated to serve as molecular scaffolds that are required for junctional integrity and cross-communication between different junctions (reviewed in [3]). Plakophilin1 (PKP1) enhances the recruitment of desmosomal proteins to the plasma membrane in cultured keratinocytes, and an increase

in the expression of PKP1 correlates with an increase in desmosome number and size [8, 9]. Plakophilin2 (PKP2) is also reported to function in desmosome organization and in the maintenance of tissue integrity [10].

Plakophilin3 (PKP3) is the most widely expressed of the PKP family members [11, 12]. PKP3 has been shown to form a complex with several proteins required for desmosome formation and has been postulated to play a crucial role in regulating desmosome function and maintaining desmosome structure [13]. Consistent with this hypothesis, we have previously demonstrated that PKP3 loss leads to a decrease in desmosome size and cell-cell adhesion [14]. The decrease in desmosome size was not due to a decrease in the levels of other desmosomal proteins [14], suggesting that PKP3 may be required to recruit other desmosomal components to the cell surface. The decrease in desmosome size was accompanied by a decrease in cell-cell adhesion, an increase in cell migration and increased tumor formation and metastasis, suggesting that PKP3 may function as a tumor suppressor [14].

In the present study, we show that PKP3 is required for the recruitment of other desmosomal proteins, including PKP2, to the cell border in human cell lines derived from both simple and stratified epithelia. A calcium switch assay demonstrated that PKP3 was required for formation of new desmosomes. PKP3, PG and E-cadherin were present at the cell border under low concentrations of calcium, and a decrease in PG or E-cadherin expression led to a decrease in the levels of PKP3 and other desmosomal proteins at the cell border. PKP3 mutants capable of forming a complex with PG localized to the cell border and were able to recruit DP to the cell border, suggesting that PG recruits PKP3 to the cell border, resulting in the initiation of desmosome assembly.

Materials and methods

Plasmids and constructs

To generate a DsRED (red fluorescent protein) tagged WT PKP3 construct, pDsRED N1 (Clontech) was digested with SmaI and SspI (Fermentas) and inserted into pEF6 WT PKP3 [13] digested with *Eco*RV (Fermentas). DsRED tagged WT PKP3 cDNA was excised using *Hin*dIII and *Dra*I (Fermentas) from the pEF6 vector backbone and cloned into pCDNA3 (Invitrogen) digested with *Hin*dIII and *Eco*RV (Fermentas) to generate a CMV promoter driven expression vector for DsRED tagged WT PKP3 (pCDNA DsRED WT PKP3). To generate a shRNA resistant PKP3 cDNA construct, a site directed mutagenesis was performed with the oligonucleotides listed in Supplementary Table 1 as per the manufacturer's instructions (Stratagene) and cloned into pCDNA3 DsRED WT PKP3 digested with *Hin*dIII and *Eco*RI (Fermentas). The PKP3 deletion mutants were amplified using the oligonucleotide primers shown in Supplementary table 1. The amplified fragments were cloned into a TA vector (Fermentas) and digested with *Kpn*I and *Bam*HI (Fermentas), the cloned into pEF6 WT PKP3 [13] and subsequently cloned into pCDNA3. To generate DsRed tagged shRNA resistant versions of the PKP3 deletion mutants, the deletion mutants were digested with *Hin*dIII and *Eco*RI (Fermentas) and cloned into pCDNA3 DsRED WT PKP3 digested with the same enzymes. All the mutants generated were confirmed by DNA sequencing.

A fragment containing an MCS was excised from pDesRedN1 with BshTI and *Eco*RI (Fermentas) (Clonetech) with and cloned into pLKO1 [15] digested with the same enzymes to generate the pS18 lentiviral vector. To generate shRNA constructs for E-cadherin the oligonucleotide pairs shown in Supplementary table 1 were cloned into pS18 puro digested with *Age*I and *Sal*I (Fermentas).

Lentivirus production and infection

The pS18 vector control or constructs expressing shRNA's targeting E-cadherin were transfected in 293T cells with Vira power packaging mix (Invitrogen) using lipofectamine 2000 (Invitrogen) to produce lentiviruses as per the manufacturer's protocol. Then 1.5 ml of viral supernatant containing 8 μg/ml polybrene (Sigma) was added to HCT116 cells in a 60-mm dish; 24 h post infection, the cells were washed 2× with PBS and fed with complete medium containing 0.5 μg/ml of puromycin (Sigma). Three days later the cells were fixed and processed for immunofluorescence microscopy as described below.

Cell lines and transfections

The HCT116 (ATCC), HEK293 cells, the HCT-derived stable cell lines, HaCaT and HaCaT-derived stable cell lines were cultured in Dulbecco's modified Eagles medium (DMEM) (GIBCO) supplemented with 10% Fetal bovine Serum (JRH), 100 U of penicillin (Nicholas Piramal), 100 μg/ml of streptomycin (Nicholas Piramal) and 2 μg/ml of amphotericin B (HiMedia). The PKP3 knockdown cells used in this were cultured as described previously [14]. Cells were transfected by calcium phosphate precipitation protocol as described [16] or by Lipofectamine reagent (Invitrogen) as per the manufacturer's protocol. To generate the PG knockdown clones in the HCT116 cell line, cells were transfected with 3 μg of the shRNA constructs. Sixty hours post transfection, the cells were transferred to medium containing 5 μg/ml of puromycin (Sigma) to

generate single-cell clones. The clones PKG-5 and PKG-6 were generated with two different shRNA constructs.

Antibodies, Western blotting and Immunoprecipitations

The primary antibodies for PKP3 (clone 23E3-4, Zymed, dilution 1:1,000), DP (mouse monoclonal Abexome, DP-200, dilution 1:1,000), β actin (mouse monoclonal, Sigma, dilution 1:5,000), PG (mouse monoclonal, Abcam, dilution 1:1,000) and E-cadherin (clone 36/E-Cadherin, mouse monoclonal, BD Transduction laboratories, dilution 1:1,000) were used for Western blot analysis. Respective secondary antibodies were used at a dilution of 1:1,000 (Invitrogen) or 1:5,000 (Pierce). Protein samples were resolved on a polyacrylamide gel by SDS PAGE and then transferred to a nitro cellulose membrane and processed for Western blot analysis as described [17]. Cells transfected with the PKP3 mutants were harvested in EBC buffer and immunoprecipitations performed as previously described [17].

Calcium switch experiments

To determine the role of PKP3 in de novo desmosome assembly, HCT116 and HaCaT derived vector control or PKP3 knockdown clones were grown on poly L-lysine coated glass coverslips. The cells were then incubated in low calcium medium for 16–20 h [18, 19]. Subsequently, cells were fed with normal calcium containing medium and at different time intervals after calcium replenishment, cells were fixed, and immunofluorescence staining was performed to determine the localization of the different desmosomal proteins.

Immunofluorescence and confocal microscopy

To determine the intracellular localization of the desmosomal proteins in the PKP3 knockdown clones, immunofluorescence analyses were performed. Transfected cells were fixed and permeabilized 36–48 h post transfection. To detect desmogleins and desmocollins, cells were fixed in 4% paraformaldehyde (Sigma) for 20 min at room temperature. To detect DP, PG and PKP3, cells were fixed in chilled methanol for 10 min at −20°C. The cells were permeabilized and washed as described [17]. Primary antibodies to DSG2 (mouse monoclonal, Abcam, dilution 1:25), DSC 2 and 3 (mouse monoclonal, Zymed, dilution 1:25), DP (rabbit polyclonal, ABD Serotec, dilution 1:400), DP (mouse monoclonal Abexome, clone # 1B8 DP-200, dilution 1:100), PG (mouse monoclonal, Abcam, dilution 1:25), PKP3 (clone 23E3-4, Zymed, dilution 1:25), PKP3 (rabbit polyclonal, Santacruz, clone H-170, dilution 1:50), PKP2 (mouse monoclonal, BD clontech, dilution 1:25), ZO-1 (rabbit monoclonal, Zymed, dilution1:25), β-catenin (rabbit monoclonal, Abcam-32572, dilution 1:25), α-catenin (goat polyclonal, Santacruz Biotechnology, dilution 1:25) and E-cadherin (clone 36/E-Cadherin, mouse monoclonal, BD Transduction laboratories, dilution 1:100) were incubated with the cells for 1 h at room temperature. The secondary antibodies, Alexa 568 conjugated anti-mouse IgG (Molecular Probes), Alexa 488 conjugated anti-mouse IgG (Molecular Probes), Alexa 555 conjugated anti-rabbit IgG (Molecular probes) and FITC conjugate anti-mouse IgG (Sigma) were used at a dilution of 1:100 and incubated for half an hour at room temperature. Confocal images were obtained by using a LSM 510 Meta Carl Zeiss Confocal system with an Argon 488-nm and helium/neon 543-nm lasers. All images were obtained using an Axio Observer Z.1 microscope [numerical aperture (NA) 1.4] at a magnification of $\times$63 with 2$\times$ optical zoom. The surface intensity of staining for the different proteins was measured using the Axiovision software.

Hanging drop assay to determine cell-cell adhesion

Hanging drop assays were performed to estimate the cell-cell adhesive properties of the PKP3 or PG knockdown cells compared to the vector control cell line [20]; 2×10^4 cells were suspended in 35-µl drops of complete medium from the lid of a 24-well plate for 16 h. The corresponding wells contained PBS to maintain humidity. After the incubation, the drops were pipetted five times with a 200-µl standard tip, fixed with 3% glutaraldehyde, and aliquots were spread on coverslips. Images of five random fields from three independent suspensions were taken with a plan-Neofluar lens (NA 0.3) at 10$\times$ on an upright Axio-Imager.Z1 microscope (Carl Zeiss, Germany) for each sample. The area of cell clusters was determined using the Axiovision rel 4.5 software (Zeiss).

Fluorescence resonance energy transfer (FRET)

HEK293 cells were transfected with Dsred (acceptor) tagged PKP3 WT or deletion constructs using lipofactamin 2000 (Invitrogen). Transfected cells were fixed and permeabilized 36–48 h post transfection. Cells were stained with primary antibodies specific to PG followed by staining with a flurophore labeled secondary antibody, FITC (donor).

HaCaT-derived vector control HpTU6 and PKP3 knockdown clone HB8 were cultured in calcium-depleted medium for 16–20 h followed by immunostaining with primary antibodies specific to endogenous proteins, PKP3 (rabbit polyclonal IgG) and E-Cadherin (mouse monoclonal IgG) and respective secondary antibodies, Alexa 555 (acceptor) and Alexa 488 (donor). FRET measurements were done

using the sensitized emission method in fixed cells using the following three samples: Donor only, Acceptor only and FRET sample. The following images were acquired for FRET corrections and efficiency calculations.

1. Acceptor only using Acceptor filter set
2. Acceptor only using FRET filter set
3. Donor only using Donor filter set
4. Donor only using FRET filter set
5. FRET Specimen only using FRET filter set

All the images were captured at ×630 magnification in 12-bit format using Zeiss LSM 510 Meta confocal laser scanning microscope. The images were acquired using the following lasers: donor excitation using a 488-nm argon laser line and acceptor excitation using a 543-nm helium-neon laser line. Images acquired were further processed using LSM 510 image examiner software. The nomenclature and equations for FRET calculations were as previously described [21, 22], and the FRET protocol was obtained from the Centre for Optical Instrumentation Laboratory, Wellcome Trust Center for Cell Biology, University of Edinburgh (http://coil.bio.ed.ac.uk/Protocols/Sensitized.htm).

FRET corrections:

Acceptor in FRET channel (co-efficient A) = Average intensity of Acceptor only using FRET set/Average intensity of Acceptor only using acceptor set.

Donor in FRET channel (co-efficient B) = Average intensity of Donor only using FRET filter set/Average intensity of Donor only using Donor filter set.

Average FRET efficiency = FRET Specimen − (A × FRET Specimen using Acceptor filter set) − (B × FRET Specimen using Donor filter set).

All the images were acquired in 12-bit format with maximum grey value of 4,096.

Average FRET efficiency % = (FRET efficiency/4,096) × 100

Statistical analysis

Error bars represent standard deviation. Statistical analysis was performed using the two-tailed Student's *t* test.

Results

A decrease in PKP3 levels leads to a decrease in desmosome size in HCT116 [14] and HaCaT cells (supplementary figure 1A and B). This decrease is not due to a decrease in the protein levels of DP, desmocollins2/3 (DSC2/3), desmoglein 2 (DSG2) PKP2 or PG (Supplementary figure 1C–1F and [14]). To determine why PKP3 loss leads to a decrease in desmosome size, the localization of desmosomal proteins was determined in the vector control and PKP3 knockdown clones by immunofluorescence analysis followed by confocal microscopy. DP, DSG2 and PKP2 showed a typical border staining in the HaCaT-derived vector control cells (HpTU6). In contrast, a diffused cytoplasmic localization was observed for these proteins in the HaCaT-derived PKP3 knockdown clones (HA1, HA5 and HB8) with greatly diminished staining at the cell border. The decrease in cell border staining was statistically significant (Fig. 1a). The levels of PG at the cell border were not appreciably altered in the PKP3 knockdown clones as compared to the vector controls (Fig. 1a), presumably because PG localizes to both desmosomes and adherens junctions [23, 24]. Similar results were observed for DSC2/3, DP, DSG2 and PG in the HCT116-derived vector control (pTU6) and PKP3 knockdown clones (S9 and S10) (Fig. 1b). These results suggest that PKP3 is required to recruit other desmosomal proteins to the cell border.

To demonstrate that the phenotypes observed above are not due to off-target effects of the shRNA, a DsRed-tagged PKP3 cDNA resistant to the shRNA construct pkp3.7 (DsRed pkp3.7R) was generated. As shown in Fig. 2a, DsRed pkp3.7R was expressed in cells transfected with either the vector control or the pkp3.7 shRNA construct, but not in cells transfected with the pkp3.5 or pkp3.6 shRNA constructs. Further, DsRed pkp3.7R was expressed in either the vector control (pTU6) or a PKP3 knockdown clone generated with the pkp3.7 construct (S9), but not in a clone generated with the pkp3.5 construct (S10) [14] (Fig. 2a). To test whether expression of DsRed pkp3.7R could restore the cell border localization of desmosomal proteins, DsRed pkp3.7R was transfected into the S9 clone. At 48 h post transfection, cells were fixed and stained either with a monoclonal antibody to DP or a monoclonal antibody that recognizes both DSC2 and DSC3. The DsRed pkp3.7R protein showed a punctuate staining at the cell border similar to that observed for endogenous PKP3 and to that reported for a GFP-tagged PKP3 protein [13]. It was observed that both DP and DSC2/3 re-localized to the cell border in cells expressing DsRed pkp3.7R, but not in untransfected cells in the same field (Fig. 2b). These results suggest that the depletion of desmosomal proteins from the cell surface is due to a decrease in PKP3 levels and not due to off target effects of the shRNA.

To determine if PKP3 was required for de novo desmosome formation, calcium switch assays were performed in the vector or PKP3 knockdown clones. As shown in Fig. 3a, DP, DSC 2/3 and PKP2 were not localized to the cell surface in the HaCaT-derived vector control and PKP3 knockdown clones in the absence of calcium (0 min). Upon addition of calcium to the medium, these molecules localized to the cell border in the vector control within

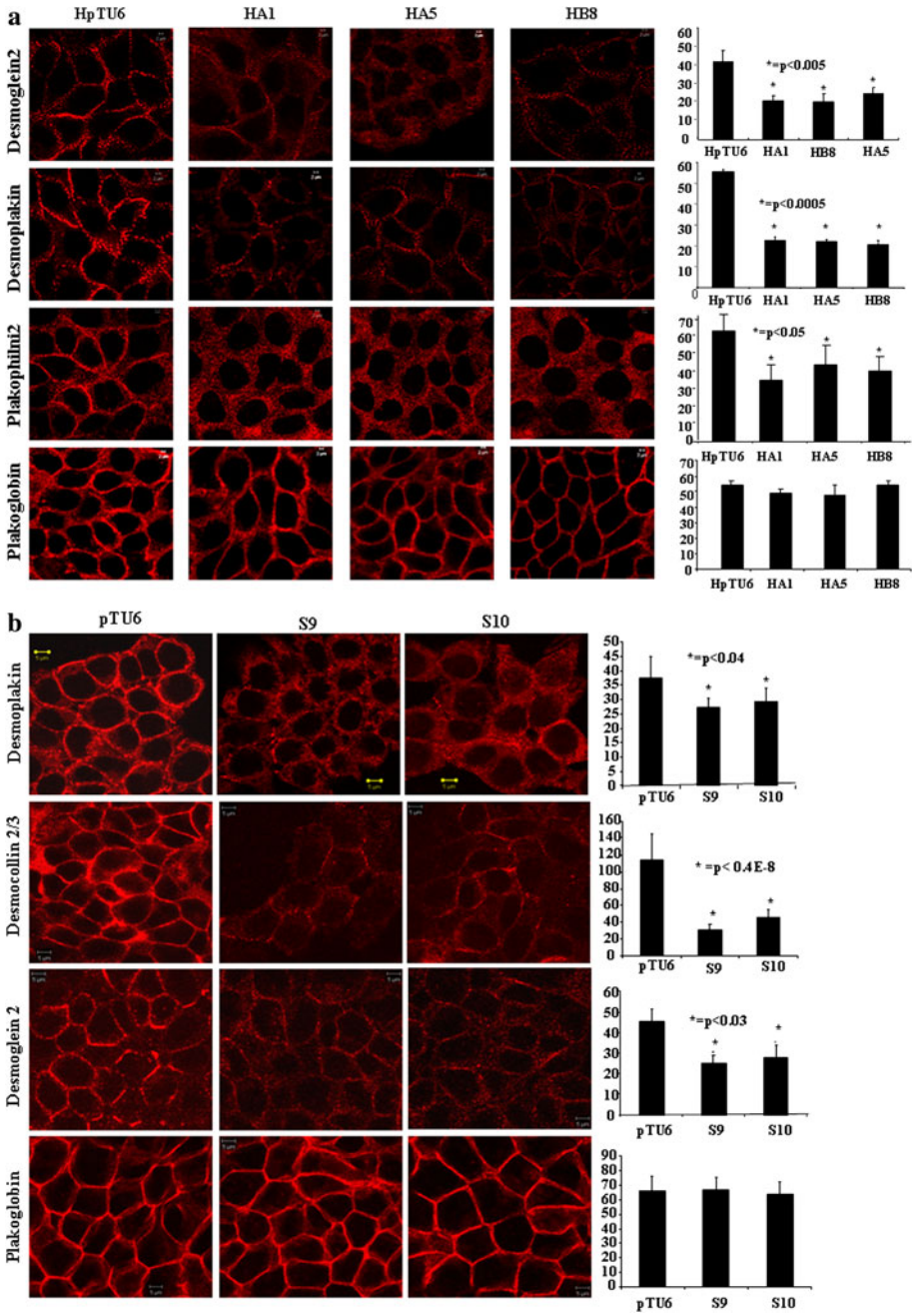


Fig. 1 Cell border localization of desmosomal components is significantly reduced in epithelial cells upon PKP3 downregulation. **a** HaCaT-derived pTU6 vector control (HpTU6) or the PKP3 knockdown clones (HA1, HA5 and HB8) were fixed and immunostained with the indicated antibodies. **b**. HCT116-derived pTU6 vector control cells (pTU6) or the PKP3 knockdown cells (S9 and S10) were fixed and immunostained the indicated antibodies. The mean fluorescence intensities at the cell borders were measured for 24 cells in three independent experiments for each immunofluorescence analysis. *p* values indicated were obtained using a Student's *t* test. (Original magnification ×630 with 2× optical zoom)

30 min. This re-localization to the cell border upon calcium addition was not observed in the PKP3 knockdown clone (HB8). In contrast, PKP3 was present at the cell border in the absence of calcium in the vector control (Fig. 3a) and PG localized to the cell border in the absence of calcium in both the vector control and the PKP3 knockdown cell lines (Fig. 3b). Similar results were observed in the HCT116-derived vector control (pTU6) and PKP3 knockdown clone (S9) (Supplementary figure 2A and data not shown). The levels of E-cadherin and PG were not altered at the cell border in the HaCaT-derived vector control and PKP3 knockdown clones in the presence or absence of calcium (Fig. 3b). Addition of calcium resulted in a further increase in PG and E-cadherin levels at the cell border in both the control and vector knockdown clones (Figs. 3c, d).

To determine whether PKP3 loss leads to a decrease in the levels of other junctional components from the cell border, the localization of the adherens junction components β-catenin and α-catenin and the tight junction protein ZO-1 was determined in the vector control and PKP3 knockdown cells in the calcium switch assay. As shown in Figs. 3e and g, β-catenin and α-catenin were present at the cell border in the absence of calcium in both the vector

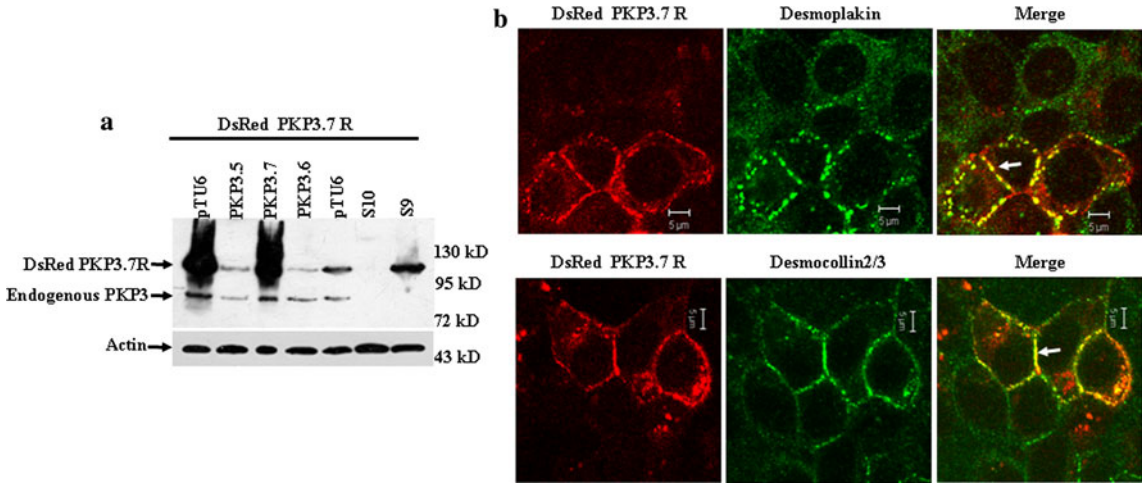


Fig. 2 PKP3 expression restores the cell border localization of DP and DSC 2/3 in the PKP3 knockdown clones. **a** HCT116 cells were cotransfected with DsRed pkp3.7R and the indicated PKP3 shRNA constructs (pkp3.5, pkp3.6 and pkp3.7) or the vector control (pTU6). pTU6 vector control cells or PKP3 knockdown cells S9 and S10 were transfected with the DsRed PKP3.7R construct. Extracts were prepared and resolved on SDS PAGE gels and Western blots performed with antibodies to PKP3. Western blots for β-actin served as a loading control. **b** PKP3 knockdown S9 cells were transfected with DsRed pkp3.7R and stained with antibodies to DSC2/3 or DP (*green*) followed by confocal microscopy. The individual images and the merged images are shown. The *white arrows* indicate regions where PKP3 and DSC2/3 or DP co-localize in the S9 cells. (Original magnification ×630 with 2× optical zoom)

control and PKP3 knockdown clones when cells were grown in low calcium medium. The levels of both proteins increased at the cell border upon calcium addition (Figs. 3f, h), a phenotype consistent with the increase in levels of E-cadherin at the cell border (Figs. 3b, c). Similarly, ZO-1 was present at the cell border in both the vector control and PKP3 knockdown cells in the absence of calcium (Fig. 3g), and its levels increased upon calcium addition (Fig. 3i) as previously described [25]. Thus, PKP3 loss leads to a selective depletion of just desmosomal proteins from the cell border.

PG localizes to both desmosomes and adherens junctions, and is required for the initiation of desmosome formation by adherens junctions [23, 26, 27]. To test the hypothesis that PG may recruit PKP3 to the cell surface, thus initiating desmosome formation, the expression of PG was inhibited in HCT116 cells using vector-driven RNA interference. Two PG knockdown cell lines (PG-5 and PG-6) showed decreased PG levels as compared to the vector control (Vec) by Western blot analysis, whereas the levels of PKP3 and DP were not appreciably altered in the PG knockdown clones (Fig. 4a). The clones were generated using two different shRNA constructs, limiting the possibility that the phenotypes observed are due to off-target effects of the shRNAs. To determine if the knockdown of PG lead to a decrease in cell-cell adhesion, a hanging drop assay was performed. As shown in Fig. 4b and Table 1, the vector control cells formed larger clumps and more clumps than the PG knockdown clones, similar to results reported for the PKP3 knockdown clones [14]. These results are similar to results reported in PG −/− cell lines and tissues [23, 24, 28]. The localization of PG and E-cadherin in these cell lines was determined using immunofluorescence followed by confocal microscopy. PG localized to the cell border in the vector control cells (Vec), but showed decreased cell border staining in the PG knockdown clones (PG-5 and PG-6). Some residual staining for PG can be observed in the knockdown clones, though the levels are considerably reduced compared to the vector control. E-cadherin localized to the cell border in both the vector control and PG knockdown clones (Fig. 4c). To determine if the loss of PG led to a decrease in the levels of PKP3 and DP at the cell border, an immunofluorescence assay was performed. The levels of PKP3 and DP were reduced at the cell border in the PG knockdown clones as compared to the vector controls, and this decrease was statistically significant (Fig. 4d, e).

To test the hypothesis that association with PG was required for the localization of PKP3 to the cell surface, myc epitope tagged deletion mutants of PKP3 (Fig. 5a) were tested for their ability to form a complex with PG. The myc epitope tagged PKP3 constructs were transfected into HEK293 cells and immunoprecipitations performed with antibodies to the myc epitope. WT PKP3 and the mutant 101–797 formed a complex with endogenous PG, while the mutants 51–797 and 151–797 were unable to form a complex with PG (Fig. 5b). Ds-Red tagged shRNA-resistant versions of WT PKP3 or the PKP3 deletion mutants were transfected into the PKP3 knockdown clone S9, and the cells were stained with antibodies to DP. Consistent with the hypothesis that PG is required to recruit PKP3 to the cell surface, Ds-Red tagged versions

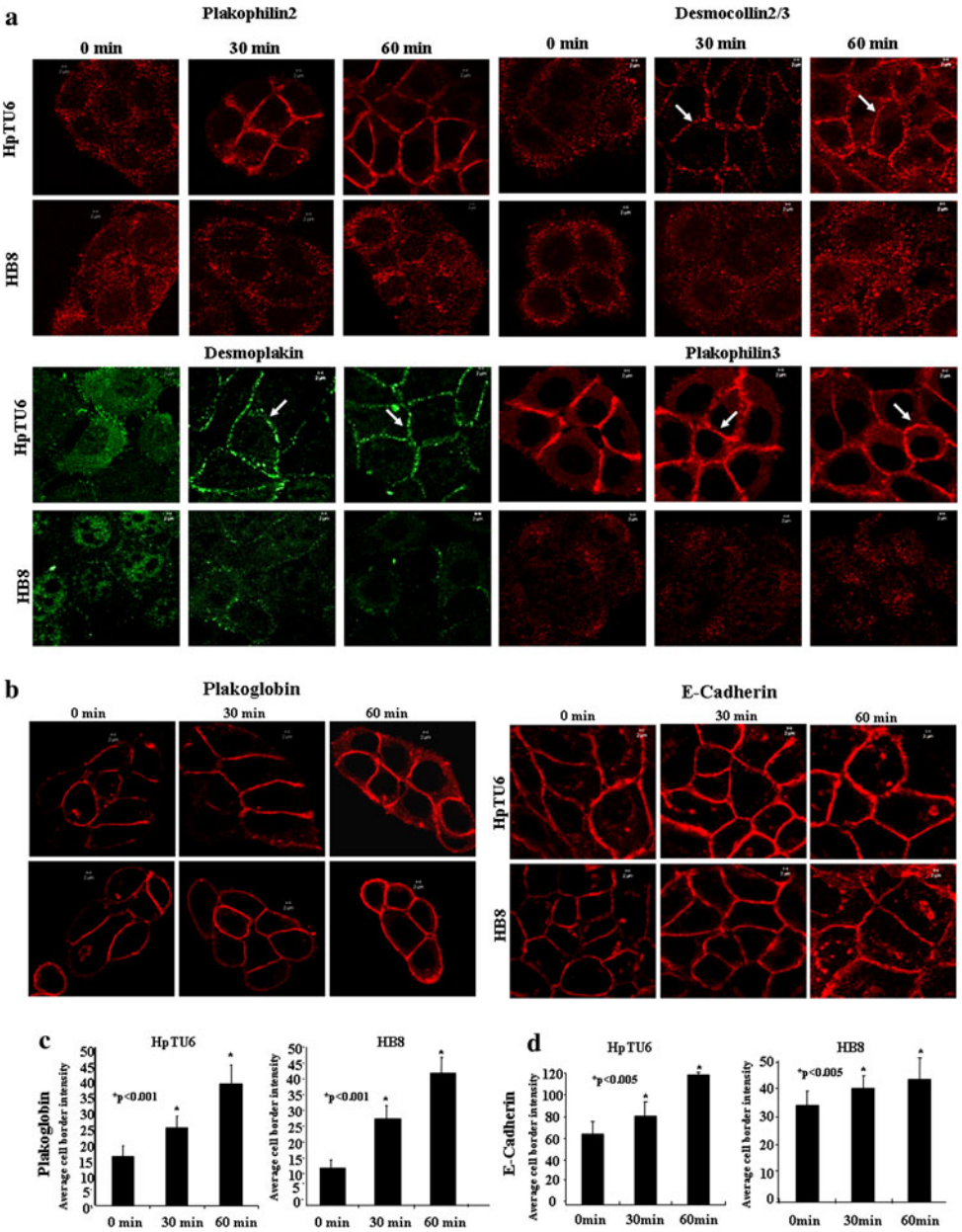
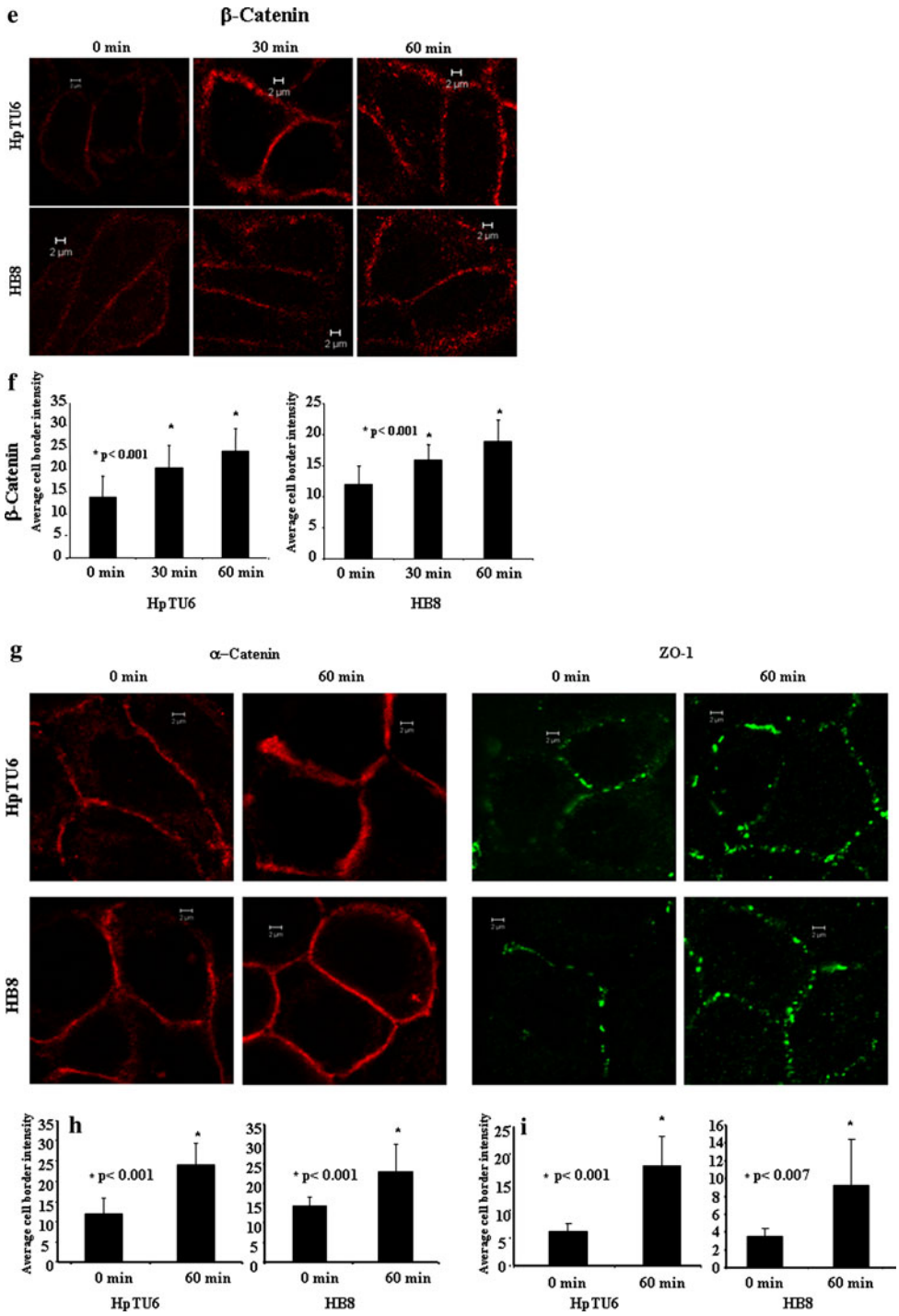


Fig. 3 PKP3 is required to initiate desmosome formation. **a**, **b**, **e**, and **g** HaCaT-derived HpTU6 vector control cells and the PKP3 knockdown HB8 cells were incubated in low calcium medium for 16–18 h. Cells were washed and fed with normal medium with optimum calcium concentration, fixed and immunostained at different time intervals of 0, 30 and 60 min. Immunostaining was performed with the indicated antibodies. *Scale bars* are indicated on the figures. *Arrows* indicate cell border localization. (Original magnification ×630 with 2× optical zoom). **c**, **d**, **f**, **h** and **i** The mean fluorescence intensities at the cell borders were measured for 24 cells in three independent experiments for each immunofluorescence analysis. *p* values indicated were obtained using a Student's *t* test

of WT PKP3 and the 101–797 mutant localized to the cell border in S9 cells and were able to recruit DP to the cell border (data not shown). In contrast, a Ds-Red tagged version of the PG binding defective mutant 151–797 showed a cytoplasmic localization (Fig. 5c) similar to that reported for a deletion mutant that contains only the head domain of PKP3 [13]. The other PG binding defective mutant, 51–797, showed a cytoplasmic localization and could not recruit DP to the cell border (data not shown). Similar localization phenotypes were observed in HEK293 cells (Fig. 5d). To confirm the results of the immunoprecipitation experiments, HEK293 cells were transfected with the DsRed-tagged PKP3 mutants and stained with antibodies to PG. A FRET analysis was performed to determine whether PG and WT or mutant PKP3 proteins were in close physical proximity. As shown in Fig. 5e, a high percentage FRET efficiency was observed between endogenous PG and WT PKP3 or the 101–797 mutant, a result consistent with the results of the immunoprecipitation analysis. In contrast, the percentage FRET efficiency between PG and the 51–797 and 151–797 mutants were significantly lower as compared to WT PKP3 or the 101–797 mutant. The values for individual cells in each transfection are shown in Supplementary figure 3A. These results when considered with the immunoprecipitation experiments described above suggest that only PKP3 mutants that form a complex with PG can localize to the cell border.

Fig. 3 continued



It has been previously reported that E-cadherin forms a complex with both β-catenin and PG and that these proteins are not present in a tripartite complex [29]. One possible hypothesis is that an E-cadherin PG complex recruits PKP3 to the cell border to initiate desmosome formation. To test this hypothesis, E-cadherin expression was inhibited using vector-driven RNA interference. Lentiviruses expressing shRNAs targeted against E-cadherin were used to infect HCT116 cells. As shown in Fig. 6a, two of the shRNAs (E2 and E3) were able to inhibit the expression of E-cadherin, in contrast to the vector control and the E1 shRNA. The E3 shRNA is more efficient at inducing a knockdown than the E2 shRNA (Fig. 6f); however, both shRNAs show a knockdown of greater than 70% as compared to the vector control (Supplementary figure 3C). The use of two different shRNAs limits the possibility that any phenotype observed is due to off-target effects of the shRNA. Loss of E-cadherin from the cell

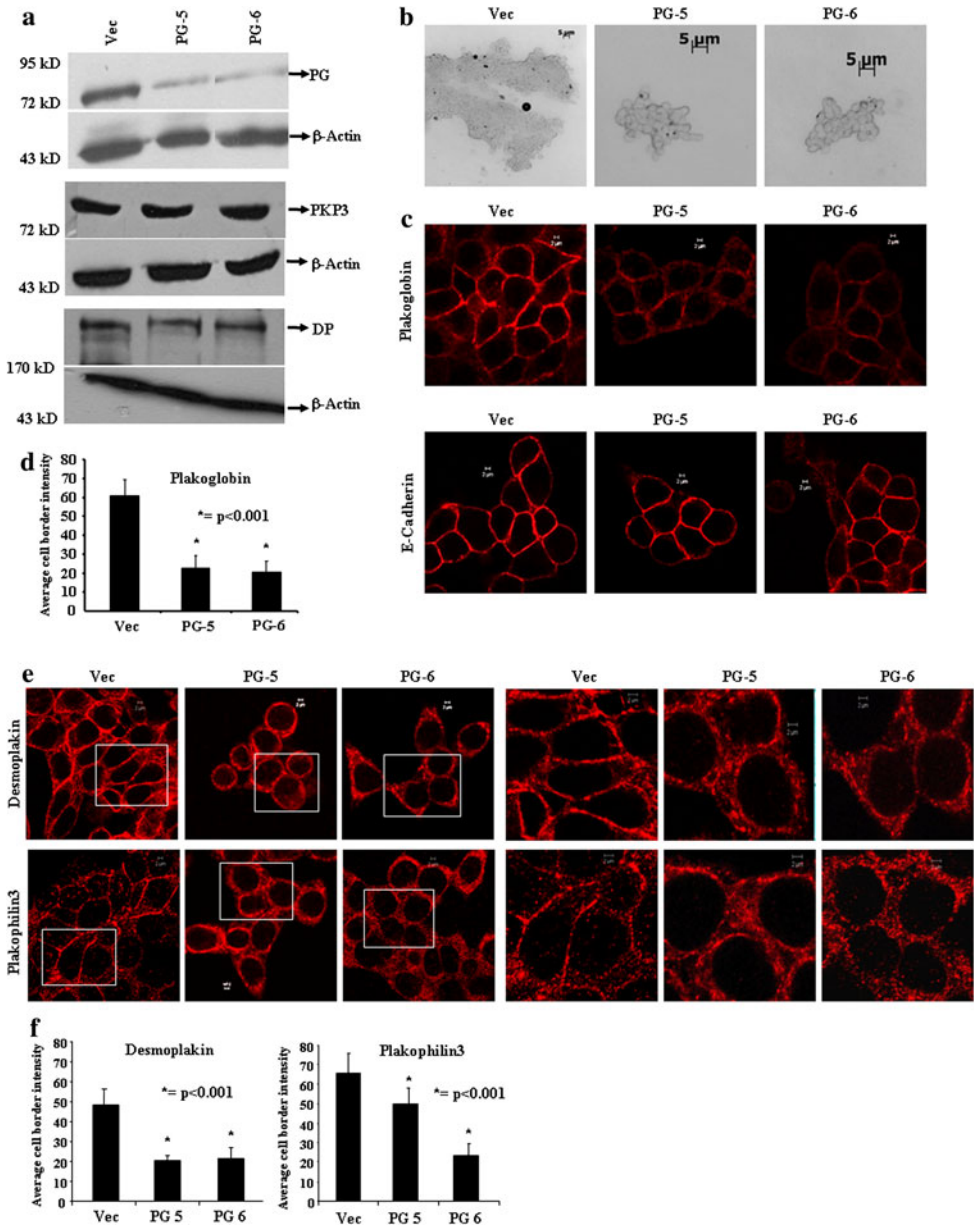


Fig. 4 PG is required for recruitment of PKP3 to the cell border. **a** Protein extracts from either vector control cells (Vec) or the PG knockdown clones (PG-5 and PG-6) were resolved on SDS-PAGE gels and Western blots performed with antibodies to PG, PKP3 and DP as indicated. PG levels were decreased in the knockdown clones as compared to the vector control cells, whereas PKP3 and DP levels were not altered in the knockdown clones as compared to the vector control. Western blots to β-actin were performed to serve as a loading control. **b** 2×10^4 of the vector control (Vec) cells or PG knockdown clones (PG-5 and PG-6) were suspended in 35 μl of complete medium for 16 h. The cells were then fixed and visualized for formation of aggregates. Representative images for each clone are shown. **c** The HCT116-derived PG knockdown clones (PG-5 and PG-6) or the vector control (Vec) were stained with antibodies to either PG (*upper panel*) or E-cadherin (*lower panel*) followed by confocal microscopy. **d** The mean fluorescence intensities at the cell borders were measured for 24 cells in three independent experiments for each immunofluorescence analysis. **e** The HCT116-derived PG knockdown clones (PG-5 and PG-6) or the vector control (Vec) were stained with antibodies to either DP (*upper panel*) or PKP3 (*lower panel*) followed by confocal microscopy. Higher resolution inserts of the *boxed regions* are are shown on the *right* (Original magnification ×630 with 2× optical zoom). **f** The mean fluorescence intensities at the cell borders were measured for 24 cells each in three independent experiments. $p < 0.001$ by Student's t test

border resulted in a depletion of PKP3 from the cell border (Fig. 6a), and this was not seen in cells infected with viruses expressing either a non-targeting construct (E1) or the vector control. Similarly, E-cadherin loss also leads to a loss of both DP and PKP2 from the cell border (Fig. 6b, d), which is consistent with the observation that PKP3 loss leads to a depletion of DP and PKP2 from the cell border (Fig. 1). The levels of PKP3 and DP are not altered upon

Table 1 PG knockdown clones show a defect in cell adhesion. Cell-cell adhesion was measured by the hanging drop assay as described. Then 2×10^4 cells of the indicated knockdown clones were resuspended in 35 µl of complete medium on the lid of a 24-well dish; 16 h later the cells were fixed and the number and area of aggregates in 15 fields was measured. The numbers of aggregates of different sizes are shown

Cell lines	Number of aggregates		
	$<2 \times 10^4 - 1 \times 10^4$ µm^2	$<10^4 - 3.5 \times 10^3$ µm^2	$<3.5 \times 10^3$ µm^2
Vec-puro	6	10	0
PKG 5	0	0	15
PKG 6	0	0	16

knockdown of E-cadherin as determined by Western blot analysis (Fig. 6f). Western blots for actin were performed as loading controls. Similarly, β-catenin was also depleted from the cell border under conditions of E-cadherin knockdown as previously reported [25, 30–33]. Surprisingly, PG was not depleted from the cell border upon E-cadherin knockdown (Fig. 6c). This maybe due to the fact that PG has been reported to form a complex with both P-cadherin and E-cadherin [29], and it is likely that in the absence of E-cadherin PG can be recruited to the border by P-cadherin. HCT116 cells have previously been shown to express P-cadherin [34].

To determine if PKP3 forms a complex with E-cadherin in low calcium medium, the HaCaT-derived vector control and PKP3 knockdown clones were grown in low calcium medium and stained with antibodies against E-cadherin and PKP3. As shown in Fig. 6g, the polyclonal PKP3 antibody (Santa Cruz) showed a punctuate staining at the cell border in the vector control but not the PKP3 knockdown clone. A diffused cytoplasmic signal was also observed for this antibody in both cell lines, which may be due to a non-specific reaction. E-cadherin was localized to the border in both cell types. The merged image shows co-localization between E-cadherin and PKP3 as shown by the yellow signal in the bottom panel. The co-localization is only observed in the vector control, but not in the PKP3 knockdown clones suggesting that it is specific. To determine whether the co-localization reflected an association between the two proteins, we performed a FRET analysis as described in the "Materials and methods." As shown in Fig. 6h, both proteins localized to the cell border when viewed with the appropriate filter sets (top two panels). When FRET filter sets where the excitation wavelength was for the E-cadherin fluorophore and the emission wavelength for the PKP3 fluorophore, a signal for PKP3 was detected in the vector control, but not in the PKP3 knockdown clone. The percentage of FRET efficiencies are shown in Fig. 6i. The values for individual cells in each transfection are shown in Supplementary figure 3B. These results suggest that E-cadherin and PKP3 are in close physical proximity at the cell border.

Discussion

The results described herein show that PKP3 is present at the cell border prior to desmosome formation and is required to recruit multiple desmosomal proteins to the cell border to initiate desmosome formation. The effects of PKP3 loss on the localization of other desmosomal components can be reversed by expression of a shRNA-resistant PKP3, suggesting that the effects observed are due to the decrease in PKP3 levels and not due to off-target effects of the shRNAs. The recruitment of PKP3 to the cell border is dependent on the expression of PG and E-cadherin and PG binding defective PKP3 mutants fail to localize to the cell border. These results suggest that the presence of PKP3 at the cell border is essential for the formation of desmosomes in epithelial cells in culture.

PKP3 is the most widely expressed of the PKP family members [11, 12] and has been shown to form a complex with several proteins required for desmosome formation [13]. These data suggest that PKP3 may play a central role in organization of the desmosome as the other PKP family members do not exhibit this wide array of interactions with other desmosomal proteins. While multiple experiments have demonstrated that overexpression of PKP family members can recruit DP and other desmosomal plaque proteins to the cell surface [13, 35–39], this is the first report that suggests that a PKP family member is required to recruit the desmosomal cadherins (DSC2/3 and DSG2), DP and other PKP family members (PKP2) to the cell surface at *physiological levels of expression*. Further, the data reported here suggest that PKP3 does not affect the localization of adherens junction components (β-catenin and α-catenin) or tight junction components (ZO-1) to the cell border, suggesting that the effects observed upon PKP3 loss are specific to desmosome organization and function. These results were observed in cell lines derived from both complex (HaCaT) and simple (HCT116) epithelia, suggesting that PKP3 functions are conserved in most tissues, which is consistent with the broad tissue distribution of PKP3 [11, 12], and are consistent with data that the skin of PKP3 −/− mice show loose sealing of cells of the basal

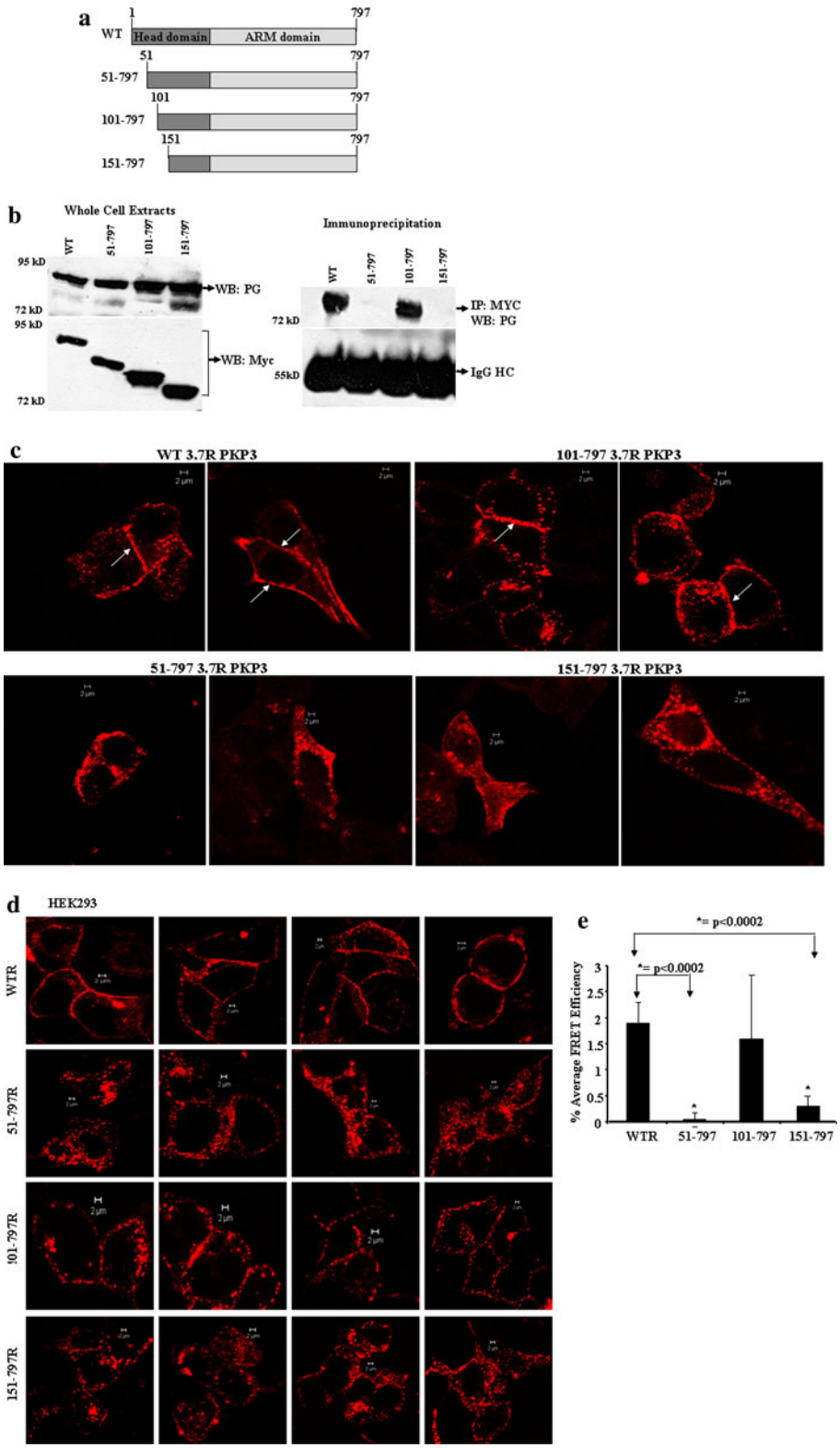


Fig. 5 PKP3 mutants that form a complex with PG localize to the cell border. **a** Schematic of PKP3 deletion constructs. Wild-type or the different deletion mutant constructs with the respective amino acids in each have been shown. **b** Myc tagged WT or the indicated deletion mutant constructs (51–797, 101–797 and 151–797) of PKP3 were transfected into HEK293 cells and immunoprecipitations were performed with an antibody against the Myc epitope. Whole cell extracts or immunoprecipitated complexes were resolved on a 10% SDS PAGE gel, and Western blot analysis was performed using antibodies against the Myc epitope or against PG as indicated. **c** The PKP3 knockdown clone, S9, was transfected with Ds-Red tagged WT or mutant constructs of PKP3. At 40 h post transfection, cells were fixed and the cells visualized by confocal microscopy. The *white arrows* indicate regions where WT PKP3 or the 101–797 mutant localize at the cell border in S9 cells. (Original magnification ×630 with 2× optical zoom). **d** HEK293 cells were transfected with Ds-Red tagged WT or mutant constructs of PKP3. At 40 h post transfection, cells were fixed and the cells visualized by confocal microscopy. (Original magnification ×630 with 2× optical zoom). **e** HEK293 cells were transfected with Ds-Red tagged WT or mutant constructs of PKP3. At 40 h post transfection, cells were fixed and stained with antibodies to PG and a FRET analysis performed. The *graph* shows the mean and standard deviation for percentage FRET efficiency from ten different cells. *p* values indicated were obtained using a Student's *t* test

layers and a huge reduction of lateral desmosomes in the basal and the supra basal layers of the epidermis [40]. The absence of a stronger phenotype in other tissues in the PKP3 −/− mice may be due to the observation that an increase in the levels of the PKP family members, PKP1 and PKP2, was observed in the tissue derived from PKP3 −/− mice as opposed to wild-type mice [40]. A similar increase in the levels of either PKP2 or PG was not

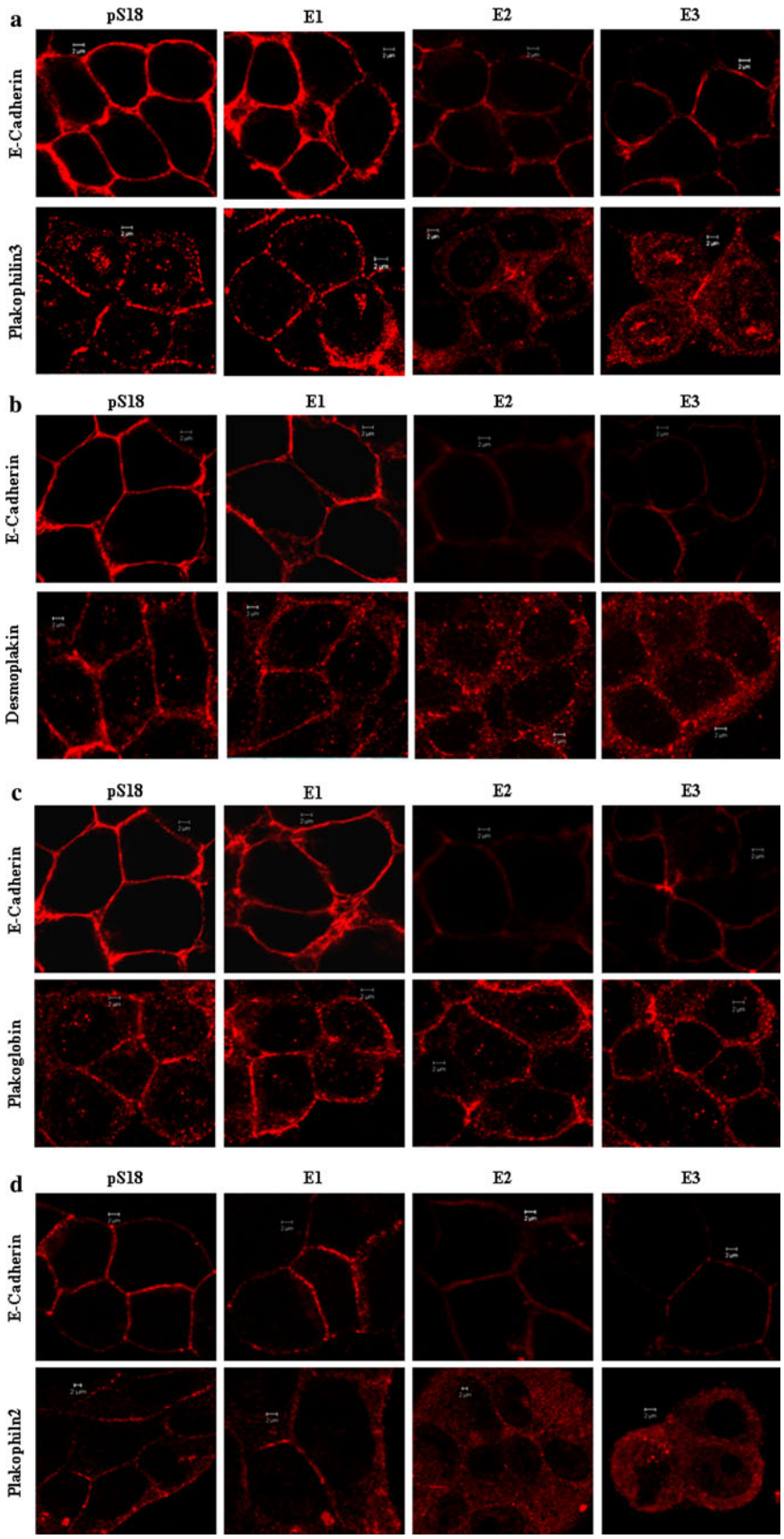
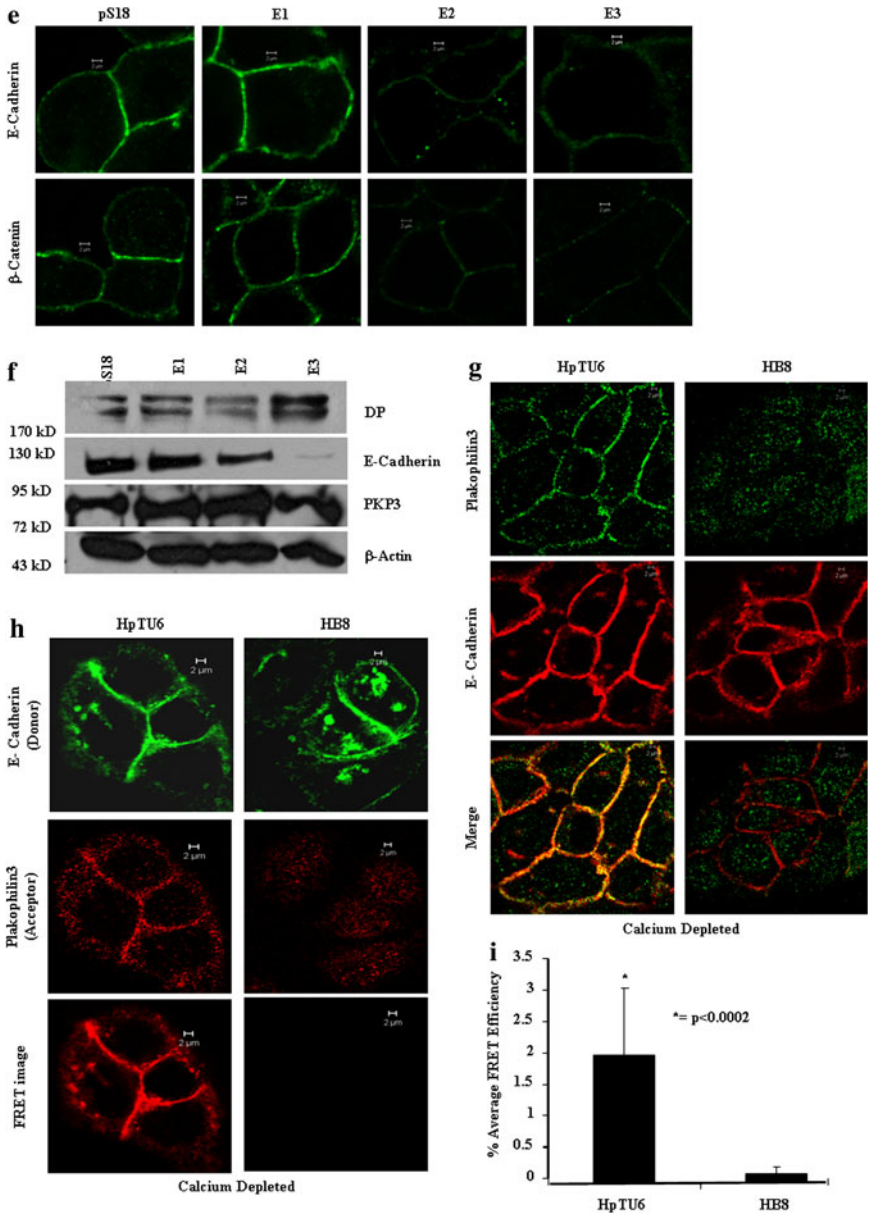


Fig. 6 E-cadherin is required for recruitment of PKP3 to the cell border. **a–e** Lentiviruses expressing shRNAs targeting E-cadherin (E1, E2 and E3) or the vector control were used to transduce HCT116 (**a–c**, **e**, **f**) or HaCaT (**c**) cells. After infection, the cells were stained with antibodies to E-cadherin (*top panel*, **a–e**) or PKP3 (*bottom panel*, **a**), DP (*bottom panel*, **b**), PG (*bottom panel*, **c**), PKP2 (*bottom panel*, **d**) and *β*-catenin (*bottom panel*, **e**). Note that the shRNAs that inhibit E-cadherin expression (E2 and E3) also induce a depletion of PKP3, DP, PKP2 and *β*-catenin from the cell border. (Original magnification ×630 with 2× optical zoom). **f** Lentiviruses expressing shRNAs targeting E-cadherin (E1, E2 and E3) or the vector control were used to transduce HCT116 cells. After infection, protein extracts prepared from the cells were resolved on SDS PAGE gels and Western blots performed with the indicated antibodies. Western blots for actin were performed as a loading control. **g** The HaCaT-derived vector control (HpTU6) and the PKP3 knockdown clone (HB8) were cultured in low calcium medium and stained with antibodies to PKP3 (*green*) and E-cadherin (*red*). Note that PKP3 and E-cadherin co-localize in the vector control but not in the knockdown clones. **h** The HaCaT-derived vector control (HpTU6) and the PKP3 knockdown clone (HB8) were cultured in low calcium medium and stained with antibodies to E-cadherin (*green*) and PKP3 (*red*) followed by a FRET analysis. The *top two panels* show the staining of the individual proteins and the *bottom panel* shows a FRET image. **i** A FRET analysis was performed in the vector control (HpTU6) and PKP3 knockdown (HB8) clones. The *graph* shows the mean and standard deviation for percentage FRET efficiency from ten different cells. *p* values indicated were obtained using a Student's *t* test

Fig. 6 continued



observed in the PKP3 knockdown cells used in these studies ([14] and Supplementary figure 1), which may account for the observation that PKP3 seems to be required for the initiation of desmosome formation in these human cell lines. Further, it has been previously demonstrated that both PKP2 and PG are required for desmosome formation in cardiac muscle in the mouse [28, 41]. It is possible that in cardiac muscle, PKP2 or PKP1 performs functions normally performed by PKP3 in epithelial cells resulting in the formation of intact desmosomes in cardiac myocytes. It has been reported previously that desmosomal components show differential levels of expression in the layers of the stratified epithelium (reviewed in [1]). Thus, it is possible that there are tissue- and cell-type-specific differences in the formation of desmosomes.

Multiple reports suggest that all three PKP family members can cooperate with PG and DP to induce desmosome formation in cell lines that do not have desmosomes [23, 36, 38, 42, 43]. Our results suggest that at physiological levels of expression of the PKP family members, PKP3 is required to initiate desmosome formation, even in cells expressing PKP2, and that it is required to recruit PKP2 to the cell surface. Previous reports have shown that PKP3 is expressed in all living layers in both simple and stratified epithelium [13], unlike PKP1 and 2, which show a much more limited pattern of expression [44, 45]. We would like to postulate that at physiological levels of expression, PKP3 localized at the cell border might initiate desmosome formation in epithelial cells and that PKP1 and PKP2 are subsequently recruited to the cell

border to further stabilize the desmosome. This is consistent with the observation that loss of PKP2 does not prevent the initial accumulation of DP at the cell border, but is required to stabilize DP at the cell border [35]. Further, PKP3 is present at the cell border in the absence of calcium, unlike PKP1 [42] or PKP2 ([35] and Fig. 4a), and PKP3 expression is required for the recruitment of PKP2 to the cell surface (Fig. 1a). These data suggest that it is not levels of the individual PKP family proteins that matter, but the sequence in which they localize to the cell border in the absence and presence of calcium that is essential for the initiation of desmosome formation.

Godsel et al. [46] have reported that PKP2 and a GFP-tagged DP showed a greater level of colocalization 15 min after a calcium switch than PKP3 and a GFP-tagged DP in SCC9 cells. These data when considered with the data in this report would suggest that the presence of PKP3 at the cell border might result in the recruitment of other desmosomal proteins, including DP and PKP2, to the cell border, following which the mechanics of desmosome assembly will determine the association of individual proteins at the cell border. The data reported by Bass-Zubek et al. [35] that PKP2 is required to induce the phosphorylation of DP by PKC-α are also consistent with the model that PKP3 is required for the initial recruitment of desmosomal proteins to the cell border and that other PKP family members then further stabilize and increase desmosome size.

PG is required for the recruitment of PKP3 to the cell border and for desmosome formation as measured by DP staining at the cell surface and in cell-cell adhesion assays (Fig. 4). Further data that support this hypothesis come from the observation that PKP3 deletion mutants that bind to PG localize to the cell border and can recruit DP to the cell border, while a PKP3 mutant (151–797) that does not form a complex with PG fails to recruit DP to the cell border (Fig. 5 and data not shown). PG has been shown to induce desmosome formation in cooperation with DP and PKP1 in A431DE cells [36] and with DP and PKP2 in HT-1080 SL1 cells [38]. Our results are consistent with the hypothesis that a complex between PG and one of the PKP family members is required for desmosome formation. As discussed above, our results also suggest that a complex between PKP3 and PG might be required for the initiation of desmosome formation when none of the PKP family members are over expressed. The inhibition of desmosome formation observed in the PG knockdown clones is similar to data from PG knockout mice, which show severe defects in desmosome formation [28].

The formation and assembly of desmosomes has been shown to be dependent upon the presence of pre-formed adherens junctions in the same cell, as treating cells with antibodies to the adherens junction cadherins can inhibit the assembly of desmosomes [31, 32, 47, 48]. It has also been reported that expression of classical cadherins in combination with PG could initiate desmosome formation [27]. Our results demonstrate that PG and E-cadherin levels are not altered at the cell border in the PKP3 knockdown cells (Fig. 3), suggesting that the presence of PG and E-cadherin at the cell border will initiate desmosome formation only in the presence of PKP3. Further, a decrease in E-cadherin expression leads to a decrease in PKP3, PKP2 and DP at the cell border, but not PG (Fig. 6). It has been previously demonstrated that E-cadherin can exist in complex with either β-catenin or PG and that these two complexes are distinct in epithelial cells [27, 29]. Our data also suggest that PKP3 forms a complex with E-cadherin in the absence of calcium at the cell border (Fig. 6). It is possible that this complex formation is essential for PKP3 border localization. Further, the result that a decrease in E-cadherin levels does not result in an alteration in PG levels at the border suggests that both PG and E-cadherin are required for the localization of PKP3 to the border in the absence of calcium. PG has been reported to form a complex with both P-cadherin and E-cadherin [29], and it is likely that in the absence of E-cadherin PG can be recruited to the border by P-cadherin. These are also consistent with reports from Michels et al. that suggest that after a calcium switch PG was present at the cell border 48 h post-calcium addition in mouse keratinocytes null for E-cadherin [31]. These results suggest that in the presence of calcium, E-cadherin loss is not sufficient to deplete PG from the cell border. They also suggest the possibility that loss of both E- and P-cadherin is required for the complete loss of both PG and PKP3 from the cell border, which presumably would result in a complete disruption of desmosome function as reported in experiments done in tissues and primary keratinocytes derived from mice that do not express both cadherins [31, 32].

Based on the data presented in this paper, we would like to put forward the following model for desmosome formation (Fig. 7). PG and E-cadherin are independently required for recruitment of PKP3 to the cell border, and the two proteins might serve as a molecular bridge to connect adherens junction formation to the formation of desmosomes. Once calcium is added to the medium, leading to the appropriate folding of the desmosomal cadherins, they and other desmosomal proteins are recruited to the cell surface by PKP3 resulting in the formation of an intact desmosome. Further, it has been previously reported that while PKP2 is required for recruitment of DP to the cell border, loss of PKP2 does not prevent the initial accumulation of DP at the cell border, but is required to stabilize DP at the cell border [35]. Our results would suggest that PKP3 is required for the initial accumulation of PKP2, DP and other desmosomal components at the cell border, following which PKP2 may mediate the phosphorylation of

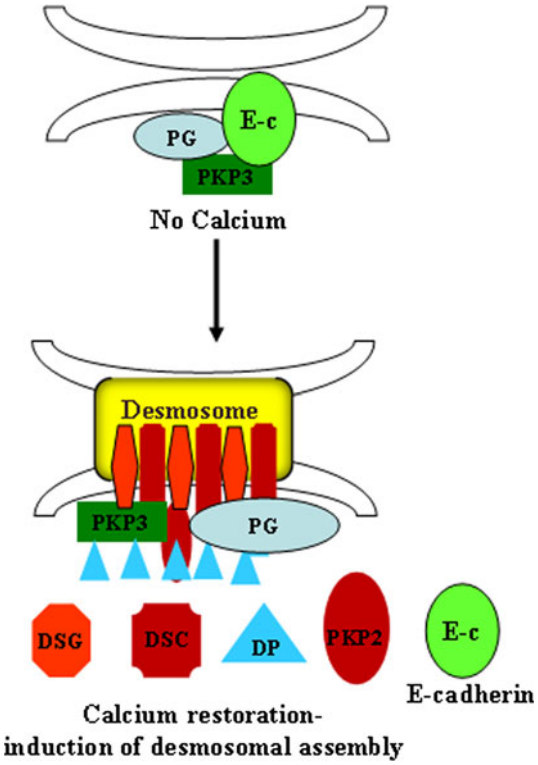


Fig. 7 Model for desmosome formation

DP by PKC-α resulting in the formation of additional lateral contacts that stabilize the desmosome. Thus, PKP3 is crucial for the initiation of desmosome assembly in human cell lines derived from both simple and stratified epithelia.

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