Lack of plakoglobin leads to lethal congenital epidermolysis bullosa: a novel clinico-genetic entity

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Epidermal integrity is essential for skin functions. It is maintained by adhesive structures between keratinocytes, mainly the desmosomes and adherens junctions, which provide resistance against mechanical stress and regulate the formation of the skin barrier. As a constituent of both types of intercellular junctions, plakoglobin has multiple interaction partners and mutations in its gene [junction plakoglobin (JUP)] have been associated with mild cutaneous disease, palmoplantar keratoderma and arrhythmogenic heart disease. Here we report a novel lethal phenotype caused by a homozygous nonsense JUP mutation, c.1615C>T, p.Q539X, which is very different from any human or murine JUP phenotype described so far. The patient suffered from severe congenital skin fragility with generalized epidermolysis and massive transcutaneous fluid loss, but apparently no cardiac dysfunction. In contrast to previously reported JUP mutations where truncated proteins were still present, in this case there was complete loss of plakoglobin in the patient’s skin, as demonstrated by immunofluorescence and immunoblot analysis. As a consequence, only very few abnormal desmosomes were formed and no adhesion structures between keratinocytes were recognizable. The expression and distribution of desmosomal components was severely affected, suggesting an essential role for plakoglobin in desmosomal assembly. Adherens junction proteins were localized to keratinocyte plasma membrane, but did not provide proper cell–cell adhesion. This lethal congenital epidermolysis bullosa highlights the fundamental role of plakoglobin in epidermal cohesion.

INTRODUCTION

The integrity of the epidermis, which is mainly dependent on the cohesion between keratinocytes, is achieved through specialized adhesion structures, such as desmosomes and adherens junctions (1). The function of these structures extends beyond that of mechanical cohesion, as they also participate in cell signalling which regulates homeostasis and responses to the environment. Desmosomes are robust intercellular attachment sites, which anchor the keratin intermediate filament cytoskeleton and are therefore important for the resistance of the tissue to high levels of mechanical stress (2). The formation of desmosomes depends on the adherens junctions, which precede them evolutionarily, during embryonic development as well as during assembly (3). Mutations in desmosomal components cause skin, hair and heart defects in humans, with a large spectrum of phenotypic variability ranging from severe lethal skin fragility to late onset cardiomyopathies (2). Because of the intimate functional interactions between different desmosomal constituents, mutations in their genes may cause similar phenotypes rendering the identification of the genetic cause of the disorders rather complicated. In contrast, only few mutations in adherens junction proteins have been reported so far; these are associated with relatively poorly defined phenotypes affecting skin, hair and other organs (4). Plakoglobin, a major cytoplasmic protein, is present in both adherens junctions and desmosomes, and presumably acts as a mediator of their interactions (2).

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Here, we report a novel severe phenotype associated with a homozygous nonsense mutation in the junction plakoglobin gene (JUP), which we refer to as ‘lethal congenital epidermolysis bullosa (LECEB)’. The complete loss of plakoglobin led to extreme skin fragility causing perinatal death, but was compatible with the development and function of the heart.

RESULTS
Clinical and morphological findings
After an uneventful pregnancy, the female index patient was born at 36 weeks of gestation by caesarean section because of pelvic presentation and anhydramnios. The parents are first cousins and have three other children. Except for total alopecia in the younger brother, no diseases are known in the family.

At birth, the patient presented with extensive areas of superficially eroded skin, with epidermal sheets detached on fingers and toes. Therefore, the patient was hospitalized in a neonatal intensive care unit immediately after birth. The condition rapidly progressed to a generalized erythema and epidermolysis with fluid loss through the epidermis, which apparently lacked any barrier function (Fig. 1A). Massive fluid substitution was necessary to maintain the fluid balance and the cardiac function. Under these conditions, ultrasound revealed normal morphology and function of the heart and no internal abnormalities. Further examination revealed complete absence of the scalp hair and onycholysis. Systemic antimycotic and antibiotic therapy as well as topical antiseptic treatment was initiated to prevent infection. The skin became eroded all over the body, and even if small areas seemed to re-epithelialize, the upper epidermal layers detached extremely easily, and appeared to be loose. The blood count was normal, as were T and B lymphocyte numbers. Six days after birth, the skin apparently became more stable and dry. The following day, greyish deposits appeared, which rapidly extended to large skin areas. Skin and mucosal swabs demonstrated the presence of Aspergillus fumigatus. In spite of intensive antibiotic and antimycotic therapy, a sepsis developed. Therapy with granulocyte colony-stimulating factor was initiated, and erythrocyte and thrombocyte concentrates were administrated because of progressive anaemia and thrombocytopenia. At postnatal day 11, the patient developed purpura fulminans with incipient necrosis of the fingers and toes. After administration of protein C, progression of cutaneous necrosis was visibly stopped. The patient died 12 days after birth because of respiratory failure due to compression of the thorax by massive coprostasis and by the infected and stiffered skin. An autopsy was declined by the family.

Histopathology of the skin showed pronounced acantholysis, and cleavage within the epidermis, with the loss of upper spinous, granular and horny layers. Basal keratinocytes were attached to the basement membrane but had only little or no contact to neighbouring and to suprabasal cells (Fig. 1B).

Transmission electron microscopy (TEM) revealed morphologically intact basement membrane and hemidesmosomes at the dermal–epidermal junction (Fig. 2A and B). Apart from single remnants of desmosomal structures between basal cells and within the hair follicle cortex, desmosomes were absent on the lateral aspects of basal cells and around all spinous cells (Fig. 2C). Epidermal keratinocytes formed numerous interdigitating protrusions, but no adhesion structures were recognizable (Fig. 2E and F).

Indirect immunofluorescence (IIF) staining of the patient’s skin was performed with antibodies to laminin 332, collagen XVII, collagen IV and collagen VII and showed no abnormalities of the dermal–epidermal basement membrane. The staining of keratin 5 and 14 was positive, but revealed loss of cell–cell contacts in the basal layer. Immunoreactivity to desmoplakin and desmoglein-3 (DSP3) was lost (Fig. 3A and B). Plakophilin-1 was diffusely distributed in the cytoplasm of the patient’s keratinocytes when compared with the intercellular staining in the control skin (Fig. 3C). The adherens junction proteins β-catenin and E-cadherin stained positively at the periphery of the cells, but intercellular adhesion was obviously disturbed (Fig. 3D and E).

Disease causing mutation and its biological consequences
Initially, we suspected lethal acantholytic epidermolysis bullosa (LAEB, MIM #609638) due to mutations in the desmoplakin gene (DSP). However, no pathogenic mutations were disclosed. Based on a candidate gene approach and IIF findings, other desmosomal or adherens junction components were screened for mutations. Pathogenic sequence variants were excluded from the genes for desmoglein-1 and -3 (DSG1, DSG3), plakophilin-1 and -3 (PKP1, PKP3), desmocollin-1 (DSC1) as well as α-catenin (CTNNA1).
Considering the TEM and IIF findings, we concluded that an essential component of adhesive structures, interacting with multiple binding partners, is defective in our patient, leading to devastating effects on the desmosomes and desmosomal proteins. Therefore, focus was put on plakoglobin, which is an essential component of both desmosomes and adherens junctions and has a plethora of interaction partners, including desmoplakin, desmogleins and plakophilin (2). In the DNA of the patient, we disclosed the homozygous nonsense mutation c.1615C>T, p.Q539X in exon 9 of the JUP gene (transcript NM_002230.2 and NG_009090) (Fig. 4A), which is predicted to lead to premature termination of the translation in the tenth Armadillo repeat of plakoglobin (Fig. 4B). The parents were heterozygous carriers of the mutation.

Because of the striking difference between our case and the previously described patients who harboured plakoglobin mutations, we explored the consequences of p.Q539X on

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**Figure 2.** Transmission electron microscopic analysis of the patient’s skin. (A and B) Electron microscopic examination of the patient’s skin showed structurally normal basement membrane and hemidesmosomes but loss of cell–cell contacts. (C) Desmosomes were almost completely absent in the patient’s skin; only very few desmosomal remnants could be observed (black arrow). (D) In control skin morphologically intact desmosomes between keratinocytes were present (open arrow). (E and F) Despite abundant interdigitating protrusions, no adhesive structures were detectable between keratinocytes in the patient’s skin. Magnification: ×1500.

**Figure 3.** Immunofluorescence staining revealed abnormal expression and distribution of desmosomal and adherens junction proteins in the skin of the patient. IIF staining of control (left panels) and the patient’s skin (right panels) were performed with antibodies to desmoplakin, desmoglein-3, plakophilin-1, E-cadherin and β-catenin. (A) Desmoplakin staining in control skin showed a normal distribution in the intercellular spaces. (B) Desmoglein-3 staining revealed a normal distribution in the control skin, whereas in the patient’s skin, desmoglein-3 staining was absent. (C) Plakophilin-1 was diffusely distributed in the cytoplasm of the patient’s keratinocytes, whereas in control skin, plakophilin-1 staining was restricted to the localization at intercellular contacts. (D) E-cadherin staining at the keratinocyte membrane was comparable in the patient’s skin and in controls. Magnification: ×1500.
RNA and protein level. This demonstrated a strong reduction in the JUP transcript in the patient's skin by \( \approx 90\% \), presumably as a consequence of nonsense-mediated mRNA decay (Fig. 5A). In agreement with the IIF results, the expression levels of DSP and DSG1 were also significantly reduced by 70 and 80\%, respectively, whereas PKP1 and CTNNB1 (the gene encoding β-catenin) were only moderately affected.

Immunostaining with antibodies to both N-terminal and C-terminal epitopes of plakoglobin (Fig. 4B) yielded negative signals in the patient's skin (Fig. 5B). The prediction that the mutation leads to loss of plakoglobin was confirmed by immunoblotting of skin extracts using the antibody specific to the N-terminal domain of plakoglobin (Fig. 5C). There was no evidence of the presence of a truncated plakoglobin in the patient's skin.

**DISCUSSION**

The phenotype described here is by far the most severe ever associated with mutations in the JUP gene. Lack of plakoglobin led to extreme skin fragility and did not allow skin barrier...
formation, thus exposing the patient to environmental pathogens and permanent fluid loss. The phenotype observed in our patient differs from those previously described in patients with JUP mutations or in plakoglobin knockout mice. Plakoglobin is mainly expressed in the heart and the skin, and consequently, mutations in its gene give rise to a broad spectrum of phenotypes affecting these two organs.

In humans, mutations in the JUP gene were associated with either arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease, MIM#601214) (5), arrhythmogenic right ventricular dysplasia type 12 (ARVD12, MIM#611528) (6), or mild cutaneous disease (7). Interestingly, all mutations described so far presumably allowed expression of altered plakoglobin molecules. The mutation described in Naxos disease, c.2157del2, led to expression of a C-terminally truncated plakoglobin (8), whereas the mutations p.S24X and c.468G>A, which caused mild skin fragility, diffuse palmoplantar keratoderma, and woolly hair, but no cardiomyopathy, resulted in reduced amounts of truncated plakoglobin (7). In autosomal dominant ARVD12, the mutation p.S39_K40insS was associated with expression of an abnormal plakoglobin, which is subjected to enhanced ubiquitylation. Despite strongly reduced plakoglobin immunoreactivity in the myocard, no skin symptoms were observed in those patients (6).

Two different plakoglobin knockout mouse models exhibited severe structural and functional abnormalities of the heart leading to early embryonic death (9,10). In contrast to the murine phenotypes, the heart function of our patient did not seem to be impaired and was not the primary cause of death. However, we hypothesize that cardiac dysfunction could have developed later in life similar to patients with Naxos disease and ARVD12. The normal function of the heart during first days of life, despite lack of plakoglobin, might be explained by differences in the cardiac anatomy and physiology between humans and mice, resulting in an altered response to mechanical stress (11). It should also be kept in mind that in our patient, haemodynamic changes due to massive fluid loss may have simulated normal heart function. Because autopsy was denied, we cannot definitely exclude morphologic abnormalities of the heart.

Alongside with the loss of the plakoglobin protein, strongly reduced levels of desmoplakin and desmogleins were found in the skin of the patient, whereas adherens junction components were not significantly affected. Similar findings were reported in murine plakoglobin null keratinocytes (12), and might be explained by the crucial role of plakoglobin for the assembly of the desmosomes (13). In addition, quantitative real-time PCR (qPCR) results indicate that in our case reduced expression of direct binding partners of plakoglobin occurs at transcriptional level. Therefore, we hypothesize that binding of plakoglobin with E-cadherin regulates gene expression of desmosomal cadherins and desmoplakin (13). Interestingly, plakophilin-1, the only plakophilin not directly linked to plakoglobin (14–16), was expressed at a similar level as in control samples, but remained diffusely distributed in the cytoplasm, since it could not be incorporated into the desmosomes. However, even though both transcriptional and
post-transcriptional levels of E-cadherin and $\beta$-catenin remained unaffected and both proteins were targeted to the keratinocyte cell membrane, no proper cell–cell adhesion was established. In plakoglobin knockout mice, $\beta$-catenin, which is highly homologous to plakoglobin, partially replaced the absent plakoglobin in adherens junctions and modified desmosomes (17). However, desmosomal proteins did not assemble properly and the stability of the new, modified adhesion structures was not sufficient to resist mechanical stress (17). Therefore, $\beta$-catenin cannot fully compensate for the loss of plakoglobin in the desmosomes (12). One can hypothesize that a similar situation may have occurred in our patient. As desmosomal proteins were either strongly reduced, or remained in the cytoplasm, we predict that if $\beta$-catenin had been present in fetal skin, they did not resist the postnatal environment.

The molecular pathology of desmosomes exhibits significant complexity. Mutations in binding partners may cause similar phenotypes, whereby genotype–phenotype correlations are difficult to establish (18). Particularly, $DSP$ and $JUP$ mutations seem to cause comparable phenotypes (Table 1). LAEB has been reported as a consequence of C-terminal truncation (19,20) or near-complete loss of desmoplakin (18). Histologically, both LAEB and LECB are characterized by suprabasal acantholysis. Interestingly, this is also a feature in Hailey–Hailey disease (MIM#169600), a genodermatosis caused by mutations in the $ATP2C1$ gene [encoding the secretory pathway Ca$^{2+}$/Mn$^{2+}$ ATPase pump type I (SPCA1)]. In contrast to LAEB and LECB, Hailey–Hailey disease is relatively benign with typical onset in the adulthood. The integrity of the skin is largely maintained, and acantholysis is accompanied by orthokeratotic hyperkeratosis, parakeratosis and presence of dyskeratotic cells (21). Although the functions of SPCA1 are not known, it may influence Ca$^{2+}$ signalling, posttranslational modification of desmosomal proteins and thereby intercellular adhesive strength (22,23).

Our results show that complete lack of plakoglobin abolishes stable desmosomes, whereas loss of the desmoplakin tail was compatible with the assembly and maintenance of desmosomes. Therefore, both well-organized desmosomal structures and functional interactions with the intermediate filament network are essential for sealing epithelial sheets of the skin (19). As multimolecular adhesion complexes, desmosomes seem relatively stable in vivo, and small amounts of plakoglobin may be sufficient for their assembly and partial function in the skin and the myocard, as observed in Naxos disease and ARVD12. Plakoglobin truncations may cause variable clinical phenotypes, including skin and/or cardiac symptoms. In the present case, the first fully plakoglobin-deficient human dramatically demonstrates the essential function of this Armadillo protein in the assembly of desmosomes and the maintenance of skin integrity.

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**Table 1. Major clinical and morphological findings in lethal acantholytic and lethal congenital epidermolysis bullosa (EB)**

<table>
<thead>
<tr>
<th>Disease-causing gene</th>
<th>Lethal acantholytic EB</th>
<th>Lethal congenital EB</th>
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<tbody>
<tr>
<td>DSP</td>
<td>Desmoplakin</td>
<td>Plakoglobin</td>
</tr>
<tr>
<td>JUP</td>
<td>Four (18–20)</td>
<td>One (this study)</td>
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</table>

**Clinical phenotype**

<table>
<thead>
<tr>
<th>Skin</th>
<th>Generalized epidermolysis, milia</th>
</tr>
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<tbody>
<tr>
<td>Mucous membranes</td>
<td>Oral, conjunctival and genital erosions</td>
</tr>
<tr>
<td>Hair</td>
<td>Universal alopecia</td>
</tr>
<tr>
<td>Nail abnormalities</td>
<td>Nail loss, anonychia</td>
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<tr>
<td>Neonatal teeth</td>
<td>May be present</td>
</tr>
<tr>
<td>Ears</td>
<td>Malformed</td>
</tr>
<tr>
<td>Hands and feet</td>
<td>Malformed (e.g. syndactyly, clinodactyly)</td>
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<tr>
<td>Heart involvement</td>
<td>Cardiomyopathy in one case</td>
</tr>
<tr>
<td>Proneness to infection</td>
<td>No</td>
</tr>
</tbody>
</table>

**Purpura fulminans**

No

**Skin morphology**

<table>
<thead>
<tr>
<th>Level of cleavage</th>
<th>TEM</th>
<th>Keratinocytes</th>
<th>Desmosomes</th>
<th>Basement membrane and hemidesmosomes</th>
<th>Immunofluorescence mapping of major desmosomal markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suprabasal</td>
<td></td>
<td>Incomplete separation of keratinocytes, increase in mitochondrial size</td>
<td>Variable morphology—partially hypoplastic and reduced in number, inner dense plaque may be lost, keratin filaments are disconnected</td>
<td>Normal, but hemidesmosome size and tonofilament insertion can be reduced</td>
<td>Punctate intercellular stainingbNegative staining</td>
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<td>Plakoglobin</td>
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<td>Desmoplakin</td>
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<td>Desmoglein-3</td>
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<td></td>
<td></td>
<td></td>
<td>Plakophilin-1</td>
</tr>
</tbody>
</table>

**Proneness to infection**

No

**Heart involvement**

Cardiomyopathy in one case

**Proneness to infection**

No

**Mucous membranes**

Generalized epidermolysis

**Hair**

Universal alopecia

**Nail abnormalities**

Nail loss, anonychia

**Neonatal teeth**

May be present

**Ears**

Malformed

**Hands and feet**

Malformed (e.g. syndactyly, clinodactyly)

**Heart involvement**

Cardiomyopathy in one case

**Proneness to infection**

No

**Skin morphology**

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<td>Plakophilin-1</td>
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*aNo suprabasal epidermis was present in the skin sample, b as reported in ref. (19).*
MATERIALS AND METHODS

Human tissues
After informed consent, skin samples and ethylenediaminetetra-acid (EDTA) blood were obtained from the patient, and EDTA-blood from the parents. The project was approved by the Ethics Committee of the University of Freiburg.

Morphological analyses
For histopathological examination using light microscopy, skin biopsy specimens were embedded in paraffin, and the sections were stained with haematoxylin and eosin by standard procedures.

For TEM, formalin-fixed skin sections were transferred to a 3% glutaraldehyde solution in 0.1 M cacodylate buffer pH 7.4 after 1 day, cut into pieces of 1 mm³, washed, post-fixed for 1 h at 4 °C in 1% osmium tetroxide, rinsed in water, dehydrated through graded ethanol solutions, transferred into propylene oxide and embedded in epoxy resin (glycidether 100). Semithin and ultrathin sections were cut with an ultramicrotome (Reichert Ultracut E). Semithin 1 μm sections were stained with methylene blue. Ultrathin sections (70–80 nm) were treated with uranyl acetate and lead citrate, and examined with an electron microscope (Philips EM 400 and Zeiss EM900).

IIF staining of the patient’s and control skin was performed on 5 μm cryosections, which were air dried and incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: laminin 332 (a3, clone BM165, provided by Dr Eble, Muenster, Germany; β3, clone 6F12, Santa Cruz, Heidelberg, Germany; γ2, clone GB3, Sera-lab, Cambridge, UK), collagen XVII (24,25), collagen IV (clone CIV-22, Abcam, Cambridge, UK), collagen VII (clone LH7.2, Chemicon, Nürnberg, Germany), keratin 5 (clone CK5, Sigma, Munich, Germany) and 14 (clone LL001, Abcam), desmoplakin (clone 2Q40, Abcam), desmoglein-3 (clone AK23, MBL, Nagoya, Japan), pakophilin-1 (clone 10B2, Santa Cruz), β-catenin (Abcam), E-cadherin (clone 36, Biosciences, Heidelberg, Germany), plakoglobin N-terminus (clone 15F11, Abcam) and plakoglobin C-terminus (clone PG5.1, Progen, Heidelberg, Germany). The secondary antibodies were Alexa-488 anti-mouse IgG, Alexa-594 anti-rabbit IgG (both Invitrogen, Darmstadt, Germany) or Cy3-conjugated Anti-Guinea Pig IgG (Jackson Immunoresearch, Suffolk, UK). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Millipore, Temecula, CA, USA). Stained sections were observed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

Mutation detection
Genomic DNA was extracted from EDTA-blood using QIAMP® DNA mini kit (QIAGEN, Hilden, Germany). Exon/intron boundaries of the genes for desmoglein-1 and -3, pakophilin-1 and -3 as well as α-catenin were amplified by PCR using primers designed with Primer3 Software (version 0.4.0). The genes for desmoplakin, desmocollin-1 and plakoglobin were analysed as described previously (26–28), with the modification of the forward primer for exon 2 (5’-TGGGCCAGGTGACTTC-3’) for the JUP gene. All PCR products were submitted to automated nucleotide sequencing in an ABI 3130XL genetic analyzer using Big Dye Terminator Chemistry (Applied Biosystems, Darmstadt, Germany). DNA sequences were compared with the reference sequence from NCBI Entrez Nucleotide database (desmoglein-1 NC_000018.9; desmoglein-3, NC_000018.9; plakophilin-1, NC_000011.10; plakoglobin-3, NC_000011.9; α-catenin, NC_000005.9; desmoplakin, NC_000006.11; desmocollin-1, NC_000018.9 and plakoglobin, NG_009090) using Mutation Surveyor™ DNA variant analysis software (version 2.61 Softgenetics, State College, PA, USA).

RNA extraction and qPCR
Total RNA was isolated from the skin of the patient and of a normal control using RNAeasy® FFPE kit (QIAGEN), transcribed into cDNA (Fermentas, St Leon-Rot, Germany) and subjected to qPCR using iQ™ SYBR® Green Supermix and Biorad CFX96 Real-Time PCR Detection System (both BioRad, Munich, Germany) (Supplementary Material, Table). The data were analysed using the BioRad CFX Manager Software (version 1.5).

Protein extraction and immunoblot analysis
Five-μm-thick cryosections were prepared from the skin of the patient and of a normal control. For protein extraction, 20 cryosections mounted on microscope slides were put on dry ice and the tissue was scraped with a needle from the slides. The specimens were directly transferred to lysis buffer (25 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 1% NP-40, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride block, protease inhibitor cocktail set III (Calbiochem, Merck, Darmstadt, Germany), incubated with agitation for 1 h at 4°C, and clarified by centrifugation at 2000g for 20 min. Immunoblot analysis was performed as described elsewhere (29) using the primary antibody to plakoglobin N-terminus (clone 15F11, Abcam) and to keratin 5/6 (clone D5/16 B4, Dako, Glostrup, Denmark) to control loading.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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