Skin Fragility and Impaired Desmosomal Adhesion in Mice Lacking All Keratins

Janina Bär[1], Vinod Kumar[1], Wera Roth[2], Nicole Schwarz[3], Miriam Richter[1], Rudolf E. Leube[3] and Thomas M. Magin[1]

Keratins perform major structural and regulatory functions in epithelia. Owing to redundancy, their respective contribution to epidermal integrity, adhesion, and cell junction formation has not been addressed in full. Unexpectedly, the constitutive deletion of type II keratins in mice was embryonic lethal ~E9.5 without extensive tissue damage. This prompted us to analyze keratin functions in skin where keratins are best characterized. Here, we compare the mosaic and complete deletion of all type II keratins in mouse skin, with distinct consequences on epidermal integrity, adhesion, and organismal survival. Mosaic knockout (KO) mice survived ~12 days while global KO mice died perinatally because of extensive epidermal damage. Coinciding with absence of keratins, epidermal fragility, inflammation, increased epidermal thickness, and increased proliferation were noted in both strains of mice, accompanied by significantly smaller desmosomes. Decreased desmosome size was due to accumulation of desmosomal proteins in the cytoplasm, causing intercellular adhesion defects resulting in intercellular splits. Mixing different ratios of wild-type and KO keratinocytes revealed that ~60% of keratin-expressing cells were sufficient to maintain epithelial sheets under stress. Our data reveal a major contribution of keratins to the maintenance of desmosomal adhesion and epidermal integrity with relevance for the treatment of epidermolysis bullosa simplex and other keratinopathies.

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INTRODUCTION

The mammalian epidermis is a stratified epithelium that protects the body against mechanical injury, dehydration, and infections. The family of keratin proteins that forms the major cytoskeleton of all epithelia is believed to contribute largely to these specialized keratinocyte functions by forming protein interactions in a context-dependent manner, in particular to desmosomal, hemidesmosomal, and cornified envelope proteins (Jones and Green, 1991; Kouklis *et al.*, 1994; Candi *et al.*, 1998) Keratin genes are located in two clusters on mouse chromosome 11 (type I) and 15 (type II) and are coordinately transcribed to allow formation of keratin intermediate filaments (KIFs) from heterodimers of a type I and a type II protein (Hesse *et al.*, 2004; Rogers *et al.*, 2004; Schweizer *et al.*, 2006; Kurokawa *et al.*, 2011). Keratin expression is tightly linked to specialized epidermal functions; whereas proliferating basal cells express K5, K14, and K15 (Nelson and Sun, 1983; Lloyd *et al.*, 1995; Porter *et al.*, 2000), the switch to terminal differentiation is accompanied by K1, K2e, and K10 expression in suprabasal cells, whereas disruption of epidermal homeostasis and tissue repair result in transient expression of K6, K16 and K17 (Fuchs and Green, 1980; Byrne *et al.*, 1994; Freedberg *et al.*, 2001). The notion that KIFs confer mechanical stability to epithelia upon physical trauma (Coulombe *et al.*, 1991) is substantiated by several keratin knockout (KO) mouse models and human keratinopathies including epidermolysis bullosa simplex (EBS) and epidermolytic hyperkeratosis resulting from missense mutations in keratin genes *KRT5, KRT14, KRT1,* and *KRT10,* respectively (Bonifas *et al.*, 1991; Coulombe *et al.*, 1991; Fuchs *et al.*, 1992; Lane *et al.*, 1992; Rothnagel *et al.*, 1992; Lloyd *et al.*, 1995; Peters *et al.*, 2001; Reichelt and Magin, 2002; Jack Fu *et al.*, 2013). EBS is a hereditable skin blistering disorder in which ruptures occur in the subnuclear cytoplasm of basal cells (Haneke and Anton-Lamprecht, 1982) causing fragility of the basal cell compartment upon mechanical trauma. Most cases of EBS are due to dominantly acting mutations in genes encoding K5 or K14 (Bonifas *et al.*, 1991; Coulombe *et al.*, 1991; Lane *et al.*, 1992) and differ in the severity of the phenotype, depending on the site of mutation. Homozygous recessive cases of EBS have also been reported where a premature termination codon mutation in KRT14

[1]*Translational Center for Regenerative Medicine and Institute of Biology, University of Leipzig, Leipzig, Germany;* [2]*German Center for Neurodegenerative Diseases, Bonn, Germany and* [3]*Institute of Molecular and Cellular Anatomy, RWTH Aachen University, Aachen, Germany*

Correspondence: Thomas M. Magin, Translational Centre for Regenerative Medicine (TRM) and Institute of Biology, Cell and Developmental Biology, University of Leipzig, Philipp-Rosenthal-Straße 55, Leipzig D-04103, Germany. E-mail: thomas.magin@trm.uni-leipzig.de

Abbreviations: DP, desmoplakin; EBS, epidermolysis bullosa simplex; HD, hemidesmosome; KIF, keratin intermediate filament; KO, knockout; KtyII, keratin type II gene cluster; PM, plasma membrane; WT, wild-type

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gene resulted in the lack of K14 (Rugg *et al.*, 1994). In the latter case, the extent of blistering was comparable to the severity of dominant-negative mutations (McLean and Moore, 2011) suggesting that the degree of cytoskeletal damage positively correlated to the extent of skin integrity. Similarly, mutations in the suprabasal keratins K1 and K10 cause widespread blistering and erosions due to continuous lysis of suprabasal keratinocytes accompanied by hyperkeratosis in humans (Rothnagel *et al.*, 1992) and in corresponding mouse models (Arin and Roop, 2001). In contrast, constitutive deletion of K1 or K10 had only moderate effect on skin integrity, because of compensatory suprabasal K5/K14 expression in the latter model (Reichelt and Magin, 2002; Roth *et al.*, 2012).

Altogether these findings raised the question of how much keratin IF are needed to maintain epithelial stability. Previously, we reported that the deletion of all keratins in cultured keratinocytes led to fragility of epithelial sheets when exposed to mechanical stress but re-expression of K5/K14 reaching ~13% of wild-type (WT) levels rescued sheet integrity (Kroger *et al.*, 2013). We had shown that improved intercellular adhesion was mediated by a keratin-dependent stabilization of desmosomes. However, owing to keratin redundancy, the respective contribution of keratins to desmosome formation and maintenance was difficult to analyze in single keratin KO mice (Hesse *et al.*, 2000; Roth *et al.*, 2012). Combined deletion of K1 and K10 in mice was accompanied by formation of smaller desmosomes (Wallace *et al.*, 2012). To further dissect the role of keratins in intercellular adhesion formation, skin integrity and stress resistance, we here present a comparative analysis of two strains of mice in which the entire keratin protein family was deleted either completely or in a mosaic pattern, using Cre-mediated genome engineering. We show that complete absence of keratins permits epidermal morphogenesis and stratification but strongly impairs desmosomal adhesion and causes cytolysis in basal and suprabasal layers combining major phenotypes of EBS and epidermolytic hyperkeratosis in one mouse model. This results in perinatal lethality. In contrast, mice with mosaic patches of keratin-free and keratin-expressing epidermis survive ~12 days, defining the respective contribution of keratins to skin integrity.

RESULTS

Mosaic and total deletion of type II keratin genes in skin cause distinct epidermal fragility

We have previously shown that ubiquitous deletion of the keratin type II gene cluster (KtyII) (Supplementary Figure S1a online) in mice resulted in loss of the entire keratin protein family. Resulting embryos died before onset of epidermal development ~E9.5 due to severe growth defects and, surprisingly, embryonic epithelia remained intact without keratins (Vijayaraj *et al.*, 2009; Kroger *et al.*, 2011). To investigate keratin function during epidermal morphogenesis, we devised two different KO strategies allowing formation of KIFs in simple epithelia but not in skin (Figure 1a, Supplementary Figure S1a and b online). In the first mouse model, epidermal-specific deletion of type II keratin genes was performed, using a K14-Cre variant that deletes in a mosaic pattern (Huelsken *et al.*, 2001), resulting in patches of normal and keratin-deficient epidermis. Thus, WT and keratin-depleted skin become comparable in the same individual. The second mouse model aimed to analyze the consequences of total keratin deficiency in skin. To overcome embryo mortality, a genetic rescue experiment was performed mating KtyII$^{-/-}$ to transgenic mice expressing murine K8 controlled by its own promoter. The skin of K8 transgenic mice appeared normal without overt phenotype. Mating these mice to KtyII$^{-/-}$ mice enabled KIF formation between transgenic K8 and endogenous simple epithelial keratins 19 and 20 encoded by the KtyI gene locus (Zhou *et al.*, 2003; Hesse *et al.*, 2004; Moll *et al.*, 2008). As K8 expression is confined to simple and glandular epithelia (Wu *et al.*, 1982; Byrne *et al.*, 1994; Moll *et al.*, 2008), these KIFs maintain morphogenesis of internal epithelia and rescue embryo development until birth (Kumar *et al.*, in preparation). In contrast, K8 is not expressed in epidermal keratinocytes that allows analysis of epidermal development in a keratin-free background. K14-Cre KO mice are referred to as KtyII$_m^{-/-}$ (mosaic KO) and were born alive at approximate Mendelian ratio, showing no overt phenotypic abnormalities at birth (Supplementary Figure S2a online). From postnatal day 5 onward, they developed a hyperkeratotic, scaly, unelastic skin (Figure 1b) with skin lesions restricted to limb and neck folds, regions exposed to natural mechanical strain. The skin phenotype, accompanied by weight loss (Figure 1c) worsened and the mice died at ~8–12 days of age (Supplementary Figure S2b online). Next, histology and immunofluorescence of dorsal skin of 8-day-old mutant and control mice were performed. The former revealed acanthotic and hyperkeratotic epidermis patches (Figure 1f) along with neighboring unaffected areas (Figure 1g) that were similar to WT skin (Figure 1e). Affected skin patches showed enlarged intercellular spaces, cytolysis at the dermo-epidermal interface, and throughout all epidermal layers (Figure 1f and h). Unlike K1/K10 double-deficient mice where nuclei were lost prematurely (Wallace *et al.*, 2012), loss of all keratins caused accumulation of parakeratotic keratinocytes in the stratum corneum of hyperkeratotic skin patches (Figure 1f). The basis for the mosaic distribution of skin lesions became evident upon antibody staining for several keratins at postnatal day 8, demonstrating patches of keratin-free and keratin-expressing epidermis (Figure 2 a–e). In those epidermal patches, K5 and K14 remained expressed in basal and suprabasal epidermis, whereas neighboring patches were keratin-free following cre-mediated deletion. Western blot analysis of whole skin lysates of KtyII$_m^{-/-}$ mice showed insignificantly altered amounts of K5, whereas K14 was increased (Supplementary Figure S2c and d online). In contrast to K5, K1 was reduced by 60% (Figure 2b, Supplementary Figure S2c and d online), whereas the amount of K10 was increased although it could not be detected by immunofluorescence in spinous and granular keratinocytes (Figure 2c; Supplementary Figure S2c and d online). In unaffected, histologically normal KtyII$_m^{-/-}$ skin patches, the deletion of keratins had occurred only in a minor number of keratinocytes (Figure 2e) whereas affected skin patches contained a large number keratin-free keratinocytes (Figure 2a–d).

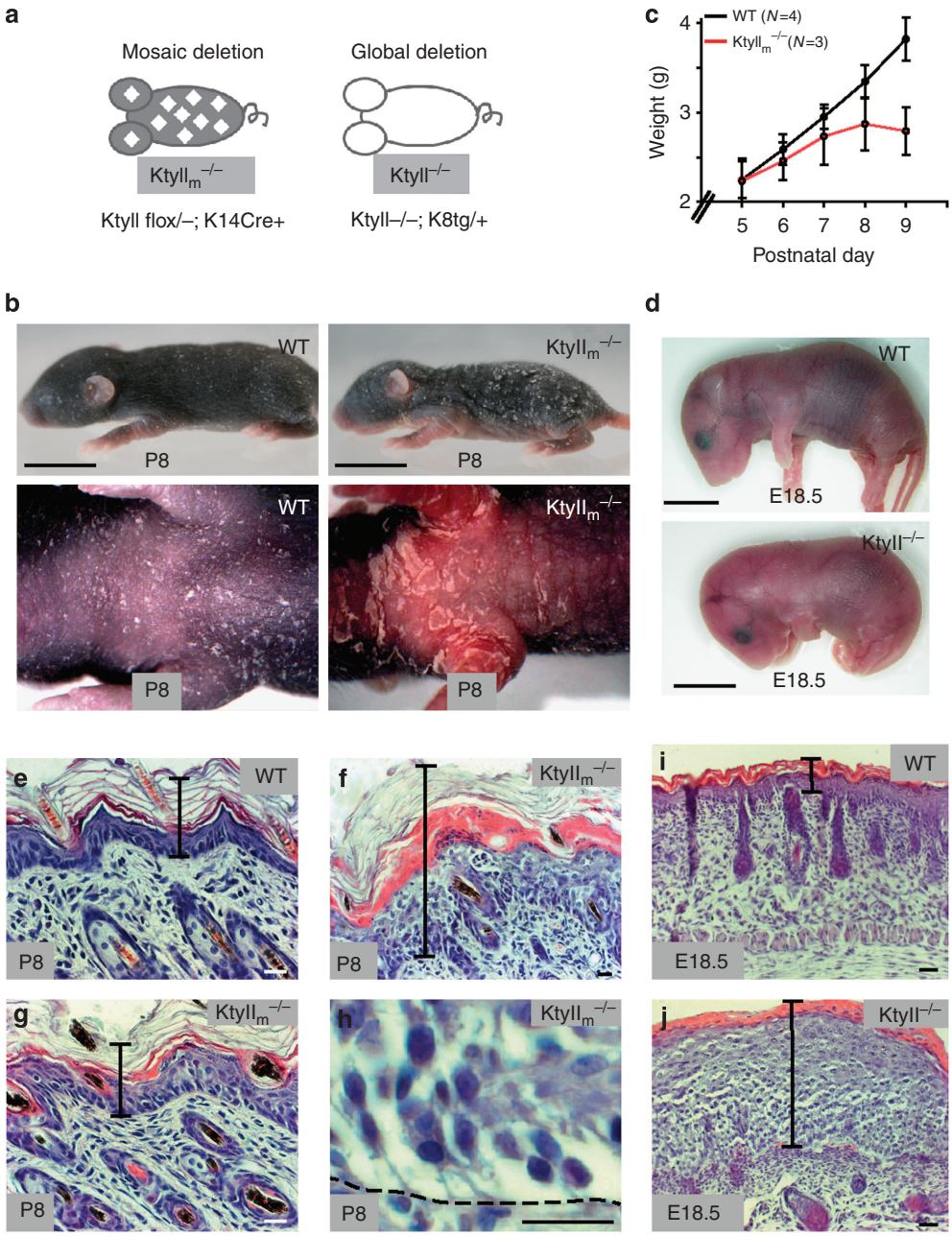

Figure 1. Comparison of mosaic and complete deletion of type II keratins. (**a**) Scheme of the two KO strategies. (**b**) KtyII$_m^{-/-}$ mice acquired a hyperkeratotic skin at postnatal day 8 (P8) with scaly squames covering the body. (**c**) Death of KtyII$_m^{-/-}$ is preceded by weight loss (mean ± SD). (**d**) KtyII$^{-/-}$ mice had a translucent, fragile skin, and shorter extremities. Histology of keratin-deficient patches of KtyII$_m^{-/-}$ skin at P8 revealed acanthotic, hyperkeratotic epidermis, larger intercellular gaps, and cytolysis at the dermo-epidermal junction (**f**, **h**), whereas neighboring keratin-expressing patches were unaffected (**g**), like controls (**e**). The epidermis of E18.5 KtyII$^{-/-}$ mice was 6-fold thicker with large intercellular gaps and prominent cytolysis throughout the epidermis (**j**). Black dotted line = basement membrane; vertical black bars = epidermis thickness; bars: **b** = 1 cm; **d** = 5 mm; **e–h** = 10 μm; **i–j** = 20 μm. KO, knockout; KtyII, keratin type II gene cluster; WT, wild-type.

Next, we analyzed mice with a complete deletion of type II keratin genes, referred to as KtyII$^{-/-}$. Owing to extensive detachment of the epidermis from the dermis and pronounced defects in intercellular adhesion, these mice died immediately after birth and all analyses were performed on E18.5 embryos upon Cesarean section. Most KtyII$^{-/-}$ embryos were smaller than WT embryos and had a shiny, extremely delicate skin (Figure 1d). Keratin immunofluorescence and western blotting demonstrated the complete loss of K5, K1, K10, and the presence of K14 aggregates throughout the epidermis (Figure 2a–d, Supplementary Figure S2e online). Global loss of keratins resulted in a ~6-fold thicker epidermis compared with WT mice (Figure 1i and j, Supplementary Figure S2i online). The overall proliferation rate was ~5-fold higher with

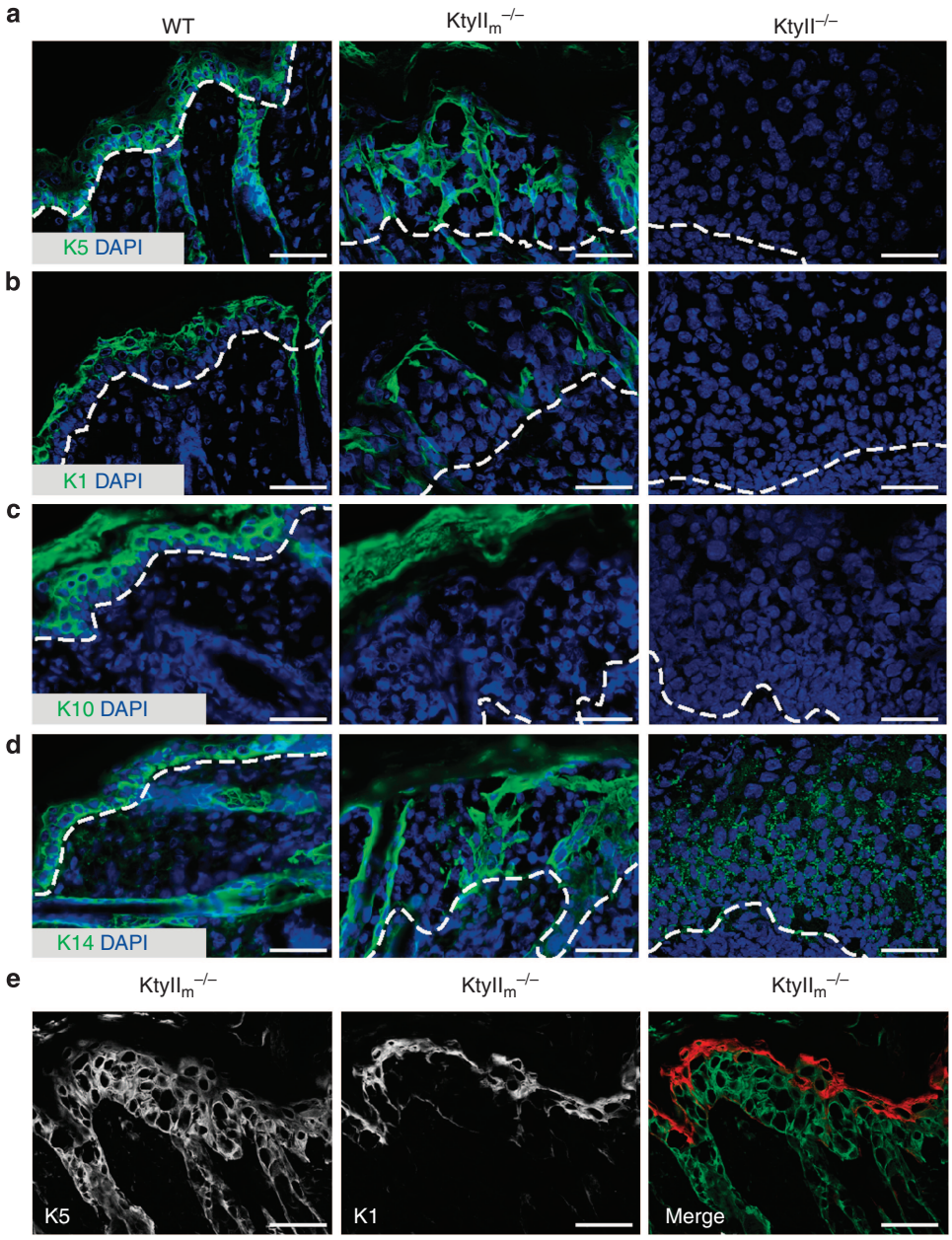

Figure 2. Extent of residual keratin expression determines skin fragility. (**a**–**d**) Immunofluorescence of K5, K1, K10, and K14 revealed partial deletion of type II keratins in KtyII$_m^{-/-}$ skin at P8 versus normal keratin expression in WT skin. K5, K1, and K10 were absent in the skin of KtyII$^{-/-}$ mice at E18.5 but aggregates of the type I keratin K14 were present throughout the epidermis (**d**). (**e**) Co-immunofluorescence of K1 and K5 shows a WT-like patch of KtyII$_m^{-/-}$ epidermis with a high number of keratin-expressing cells. White dotted line = basement membrane. Bars = 20 μm. DAPI-4′,6-diamidino-2-phenylindole; KtyII, keratin type II gene cluster; WT, wild-type.

50% increase in basal cell proliferation and a high number of proliferating suprabasal cells as indicated by Ki67 staining (Supplementary Figure S2f and g online). Unlike in other keratin KO mice reported so far, a strong increase in suprabasal apoptotic keratinocytes was noted (Supplementary Figure S2f and h online). In comparison with the mosaic KO, cytolysis occurred throughout the epidermis and intercellular spaces were extremely large (Figures 1i, j and 4e). Like in dorsal skin, keratin deficiency caused increased epidermal thickness and cytolysis in tail skin, whereas in tongue epithelium cytolysis but no hyperkeratosis was observed and the stratum corneum of ventral tongue epithelium appeared loser (Supplementary Figure S1c online). In both strains of mice, we observed an increase in inflammatory cells. Although the number of mast cells was similar between WT and KtyII$_m^{-/-}$ skin, the number of T cells (CD3$^+$) and leukocytes (CD45$^+$) was strongly increased (Supplementary Figure S2j and k online) in the dermis, independent of the extent of keratin deletion in the corresponding skin patch. This indicated a systemic inflammation, possibly resulting from a

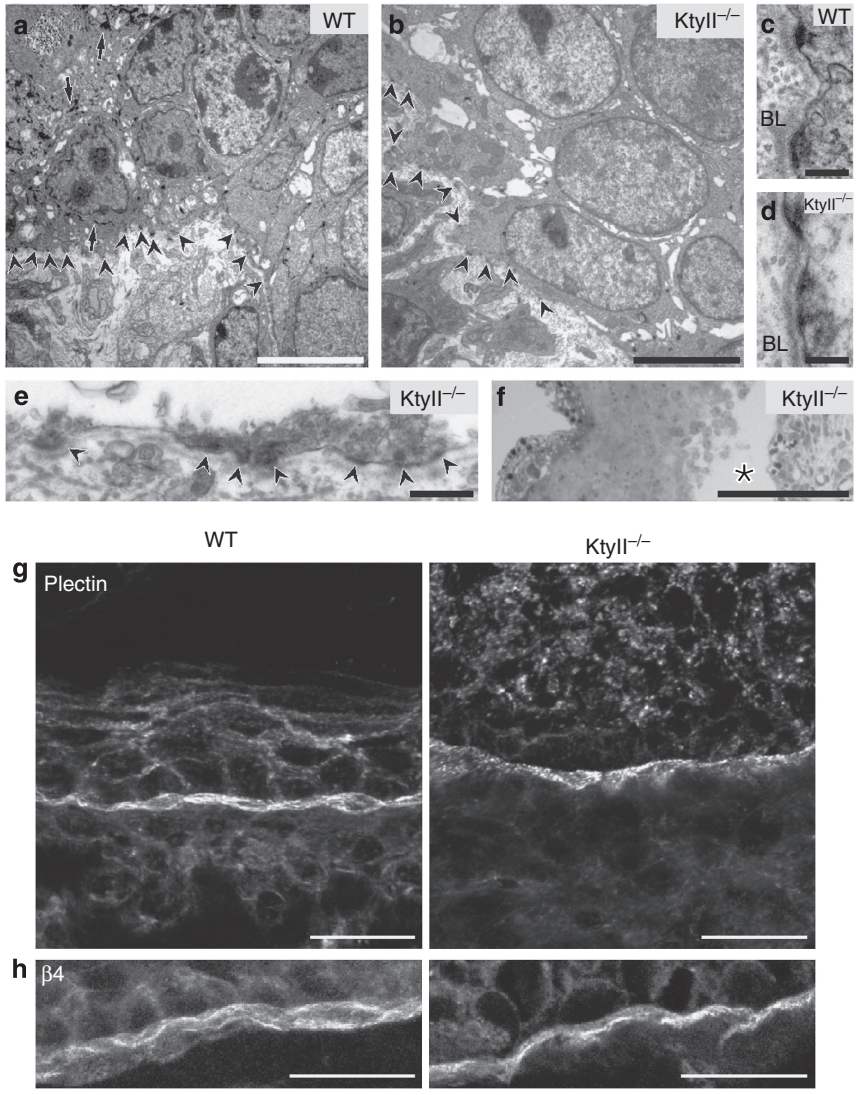

Figure 3. Hemidesmosome (HD) morphology appears normal upon keratin loss. (**a**–**e**) Ultrastructure of HDs. HDs of KtyII$^{-/-}$ appeared indistinguishable from those of WT mice. Black arrows = keratin filaments, arrowheads = HDs. (**e**) Tissue splits occurred within basal keratinocytes. Note basal cell remnants attached to the basement membrane whereas HDs appear intact. (**f**) Semithin section of KtyII$^{-/-}$ skin revealed skin fracturing at the dermo-epidermal interface (black asterisk). (**g, h**) Immunofluorescence analysis of the hemidesmosomal protein plectin revealed a more punctuate pattern along the basement membrane and was shifted away from the plasma membrane to the cytoplasm upon keratin loss whereas β4-integrin showed wild-type-like localization. Bars: **a**, **b** = 5 μm; **c**–**e** = 200 nm; **f** = 100 μm; **g**, **h** = 20 μm. BL, basal lamina; KtyII, keratin type II gene cluster.

barrier defect. The notion that CD3$^+$ cells were increased even in prenatal KtyII$^{-/-}$ mice *in utero*, in the absence of infection, further supports an involvement of keratinocytes in modulating immune responses, in agreement with recent reports (Depianto *et al.*, 2010; Roth *et al.*, 2012).

Intraepidermal blistering and smaller desmosomes upon keratin loss

Owing to severe skin fragility and adhesion defects in both mice, we performed ultrastructural analysis of hemidesmosomes (HDs) and desmosomes in KtyII$^{-/-}$ and WT skin. HDs appeared morphologically indistinguishable from WT mice (Figure 3 a–d). Cytolysis occurred in the subnuclear cytoplasm of basal cells similar to EBS lesions and resulted in the splitting of epidermis and dermis (Figure 3 e–f). Intracellular fractures were observed frequently throughout the epidermis (Figure 4e) showing the fragility of cells in the absence of keratins. Plectin localized to the plasma membrane (PM) and along the basement membrane in WT epidermis. In KtyII$^{-/-}$ mice, plectin localized to the basement membrane in a more punctate pattern when compared with the WT and was shifted away from the PM to the cytoplasm in suprabasal layers (Figure 3g; Seltmann *et al.*, 2013b). The distribution of β4-integrin was similar to that in WT skin (Figure 3h). Ultrastructural analysis of the stratum spinosum of KtyII$^{-/-}$ epidermis revealed readily detectable desmosomal plaque structures lacking attached intermediate filaments (Figure 4 b, c and f, g). However, the number of desmosomes was reduced in comparison with the WT (Supplementary Figure S3f online; 0.18 vs. 0.74 desmosomes/μm PM; $n = 3$ for each genotype; $P < 0.01$).

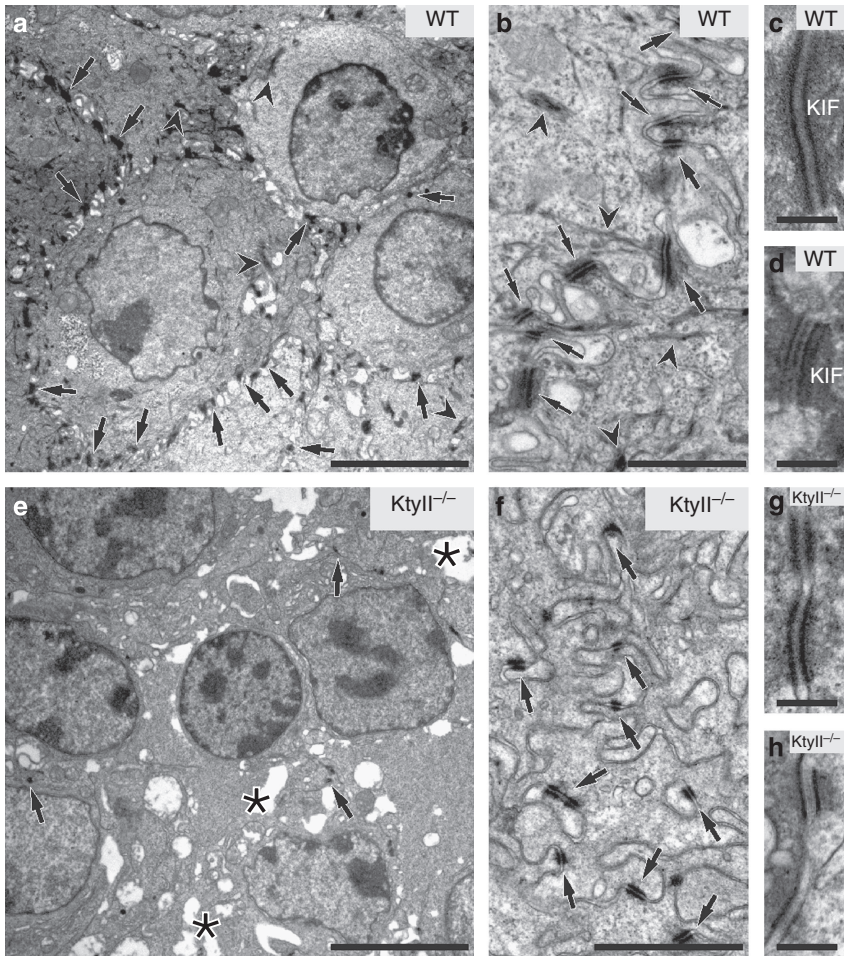

Figure 4. Keratin-dependent reorganization of desmosomes contributes to skin fragility. (**a**–**h**) Ultrastructure of desmosomes. Desmosomes in basal and spinous layer of KtyII$^{-/-}$ epidermis revealed fewer and smaller desmosomes when compared with WT skin (**a, b** and **e, f**). Arrows = desmosomes, arrowheads = keratin filaments, black asterisks = cytolysis. Higher magnification of desmosomes (**c**, **g**) and corneodesmosomes (**d**, **h**) in KtyII$^{-/-}$ versus WT epidermis. Bars: **a**, **e** = 5 μm; **b**, **f** = 1 μm; **c**, **d**, **g**, **h** = 200 nm. KtyII, keratin type II gene cluster; WT, wild-type.

Furthermore, desmosomes were significantly smaller (Supplementary Figure S3e online). Corneodesmosomes were present in KtyII$^{-/-}$ epidermis (Figure 4d and h) although they also appeared to be reduced in numbers compared with the WT. In contrast, desmosomes in desmoplakin (DP)-deficient skin were not significantly altered in size and number although desmosomes completely lacked the attachment to the keratin cytoskeleton (Vasioukhin *et al.*, 2001).

Cytoplasmic accumulation of desmosomal proteins in keratin-depleted epidermis

In keratin-free patches of KtyII$_m^{-/-}$ epidermis, a subset of DP molecules remained localized in a punctate pattern along the PM indicating persistence of desmosomes, whereas a large fraction was scattered throughout the cytoplasm of keratin-deficient cells in all epidermal layers (Supplementary Figure S3a and b online). In contrast, keratin-expressing patches of KtyII$_m^{-/-}$ epidermis showed WT-like distribution of DP along the PM. Remarkably, at the interface of keratin-expressing and keratin-deficient keratinocytes, DP remained localized at the PM (Supplementary Figure S3b online) and only few DP molecules were found in the cytoplasm close to the PM. This suggests that there might be a trans-stabilization effect of keratins on desmosomes in neighboring keratin-free cells. In KtyII$^{-/-}$ epidermis, DP was found mostly in the cytoplasm (Figure 5a). The highly similar distribution of the desmosomal cadherins desmoglein 1 + 2 and of the armadillo protein plakophilin 1 (Figure 5b and c) suggested that desmosome particles became internalized in the absence of keratins. In contrast, the armadillo protein plakoglobin, a constituent protein of desmosomes and adherens junctions (Cowin *et al.*, 1986), remained at the PM along with E-cadherin, the major transmembrane protein of adherens junctions, but displayed a broader distribution along the PM (Supplementary Figure S3c and d online).

The number of keratin-expressing cells determines tissue integrity *in vivo* and *in vitro*

We hypothesized that spatio-temporal Cre activation was responsible for the progressive and context-dependent epidermal deterioration in KtyII$_m^{-/-}$ mice. To address this, keratin

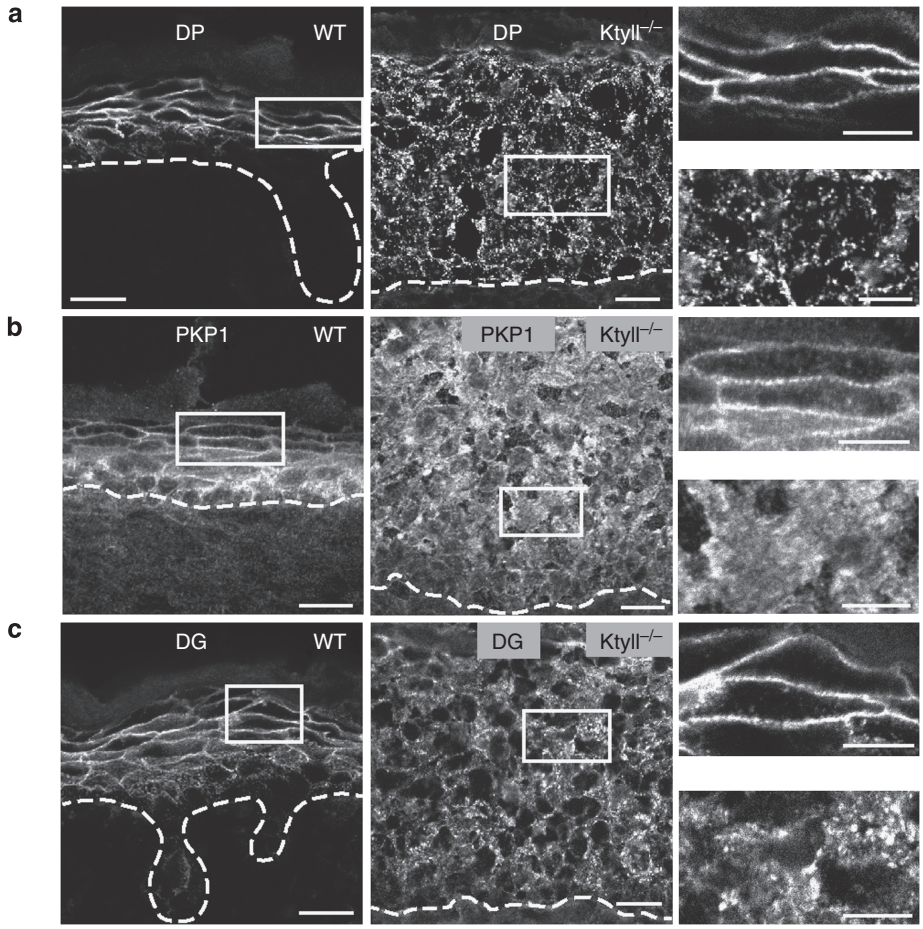

Figure 5. Mislocalization of desmosomal proteins upon keratin loss. (**a–c**) Immunofluorescence of desmoplakin (DP), plakophilin 1 (PKP1), and desmoglein 1/2 (DG) on E18.5 KtyII$^{-/-}$ skin. DP, DG, and PKP1 localized at the plasma membrane in WT skin but accumulated in the cytoplasm upon keratin loss. (**d**) Model depicting the organization of adherens junctions (AJ), desmosomes (DM), and hemidesmosomes (HDs) between WT cells, between KO cells, and at the WT/KO interface. Fractures occur in the subnuclear cytoplasm of basal cells (arrow). (**e**) Table comparing the major phenotypes of keratin type II KO mice with single and double keratin KO mice (Lloyd *et al.*, 1995; Peters *et al.*, 2001; Reichelt *et al.*, 2001; Reichelt and Magin, 2002; Roth *et al.*, 2012; Wallace *et al.*, 2012). White box = magnified area, white dotted line = basement membrane (BM), bars: **a–c** = 20 μm, insets = 10 μm. Acan., acanthosis; Ep., epidermal; Hyperker., hyperkeratosis; KIF, keratin intermediate filament; KO, knockout; KtyII, keratin type II gene cluster; n, not analyzed; WT, wild-type; +, yes; –, no; +/–, mild.

expression in KtyII$_m^{-/-}$ dorsal skin at different time points during development was studied (Figure 6 a and c). At E18.5, hardly any keratin-depleted cell was found in the epidermis and in neonatal mice only ~5% of basal cells were negative for K5. At P3, already 20% of basal cells were devoid of keratins and their descendants expanded into suprabasal layers, in line with the occurrence of epidermal proliferative units (Allen and Potten, 1974). At P8, large epidermal patches lacked keratin staining. Thus, progressive Cre activity and resulting gradual keratin loss underlie the mosaic skin phenotype, which permitted survival of mice for up to 12 days. To substantiate this further, primary keratinocytes were isolated from 2-day-old KtyII$_m^{-/-}$ mice to monitor keratin expression over time, revealing a similar increase in keratin-depleted cells *ex vivo* (Supplementary Figure S2l and m online). Of note, the number of keratin-deficient cells

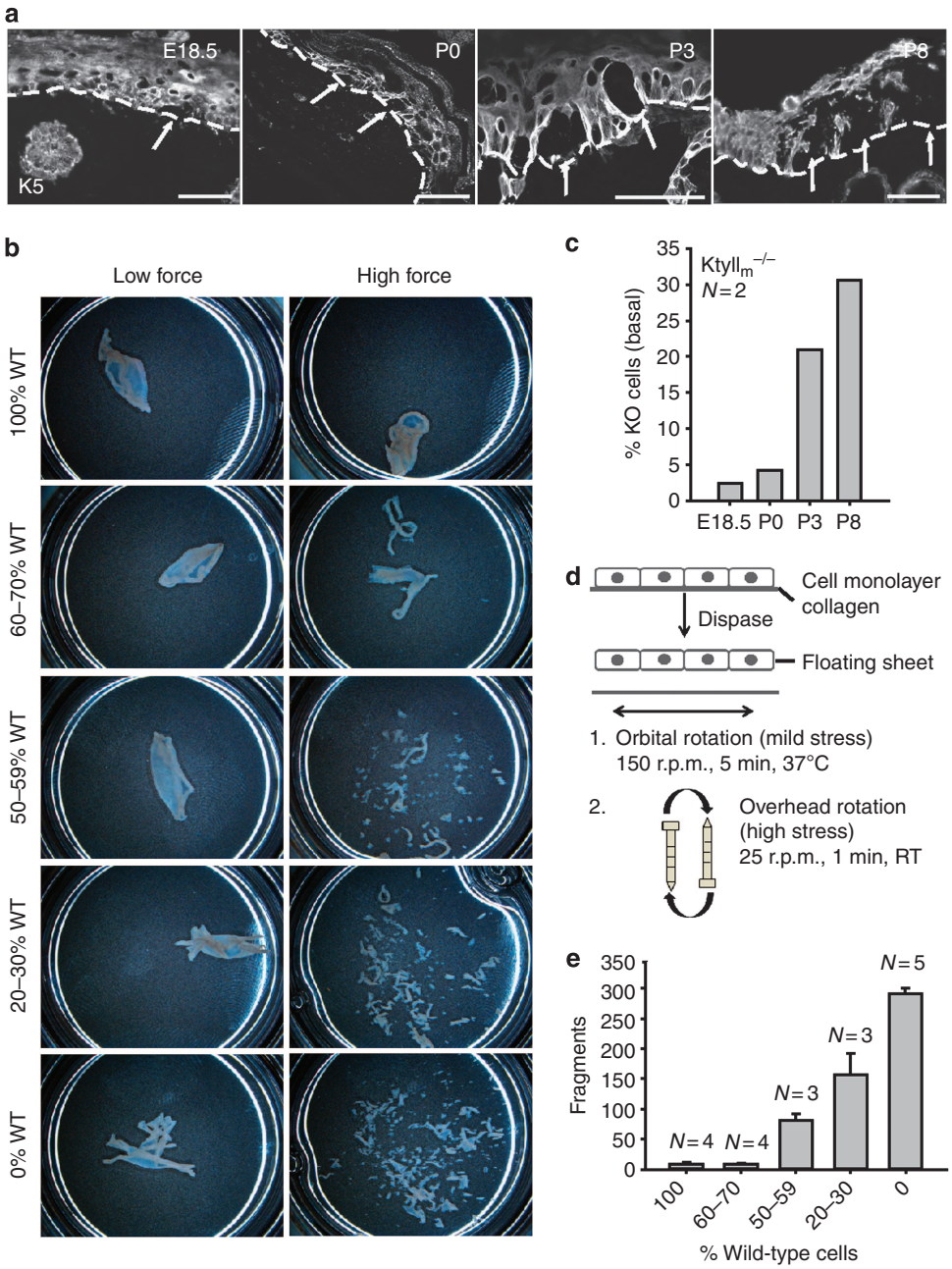

Figure 6. The number of keratin-expressing cells determines tissue integrity *in vivo* and *in vitro*. (**a, c**) Immunofluorescence of K5 on tail sections of KtyII$_m^{-/-}$ mice at different stages of postnatal skin differentiation revealed increased keratin deletion over time. (**b**) Epithelial sheets composed of different ratios of WT and KO keratinocytes were subjected to low and subsequent high mechanical force. Upon high force strong fragmentation was observed in sheets with <60% WT cells, whereas sheets with >60% WT cells remained largely intact. Schematic representation (**d**) and quantitation of the shear assay (**e**). Error bars = mean ± SEM. White dotted line = basement membrane, white arrows = keratin depletion. Bars = 50 μm. KO, knockout; KtyII, keratin type II gene cluster; RT, room temperature; WT, wild-type.

remained stable at ~65% KO between 13 and 90 days in culture, indicating that lack of keratins represents no growth advantage. Considering that keratin-free cells were already seen at E18.5 and onset of Cre-activity was observed ~E15 (Huelsken *et al.*, 2001), our data are compatible with a t1/2 of epidermal keratins of 48–72 hours *in vivo*.

The direct comparison of KtyII$_m^{-/-}$ and KtyII$^{-/-}$ mice demonstrated that the presence of keratin-expressing keratinocytes promoted skin integrity and survival of mice for up to 12 days. To address how many keratin-expressing cells are required to restore epithelial integrity in diseased conditions, KtyII$^{-/-}$ and WT keratinocytes were mixed at different ratios and cultured under conditions allowing epithelial sheet formation, followed by dispase-mediated release of epithelial sheets from the culture dish and shear force application (Kroger *et al.*, 2013; Figure 6b, d and e). After

low force exposure, all sheets remained intact except those containing 0% WT cells that became fragmented and the sheets containing 20–30% WT cells in which small ruptures were observed (Figure 6b). Application of high mechanical force resulted in the fragmentation of sheets containing <60% WT cells, whereas sheets with higher number of keratin-expressing cells remained largely intact. We conclude that the presence of at least 60% keratin-expressing cells is needed to rescue skin blistering and fragility under *in vitro* conditions.

DISCUSSION

The unique setting of mosaic keratin deletions with corresponding regions of disrupted and intact epidermis in KtyII$_m^{-/-}$ provides an excellent model to examine keratin contribution to the formation of desmosomes, HDs, to epidermal adhesion and integrity in the same animal and to relate it to WT and KtyII$^{-/-}$ settings. This study strengthens the importance of keratins in maintaining mechanical stability and resistance of epithelia *in vivo* and *in vitro*, although epidermal fragility is less severe than one might expect from combining the effects of single keratin gene KOs (Lloyd *et al.*, 1995; Peters *et al.*, 2001; McGowan *et al.*, 2002; Lessard and Coulombe, 2012). Although the complete absence of keratins results in shedding of the skin immediately upon trauma, KtyII$_m^{-/-}$ survive ~2 weeks because of a proportion of keratin-expressing cells that is sufficient to prevent skin blistering. The cause of death in KtyII$_m^{-/-}$ mice remains to be determined and may be partially due to a barrier defect (to be discussed elsewhere) and inflammation (Roth *et al.*, 2012). The observed weight loss in KtyII$_m^{-/-}$ mice was rather because of increased trans-epidermal water loss than to impaired feeding, which had been reported to result from oral and tongue lesions in K6a/K6b double-deficient mice (Wojcik *et al.*, 2001) because milk was present in the stomachs of KtyII$_m^{-/-}$ mice. In agreement, tongue and esophageal epithelia of KtyII$_m^{-/-}$ mice appeared histologically indistinguishable from WT mice (Supplementary Figure S1c online). This was due to persistence of K5/14 KIF in most keratinocytes, indicating that deletion of the KtyII locus had not occurred in these epithelia to a significant degree at this point of time (Supplementary Figure S1d–f online). Intraepidermal cytolysis and fractures in KtyII$_m^{-/-}$ skin, together with a failure of keratin-deficient skin to terminally differentiate might altogether contribute to the disruption of the skin barrier and cause water loss.

The extensive epidermal thickness most likely resulted from a combination of reduced intercellular adhesion and increased proliferation. Hyperplasia as a consequence of increased proliferation is generally presumed to result from a response to injury or a requirement for cell renewal (Weinstein *et al.*, 1984; Proksch *et al.*, 1991). Intraepidermal ruptures observed upon keratin loss stimulate wound-healing processes and the requirement for cell replacement. In addition, disturbances in epidermal barrier function are closely linked to increased DNA synthesis (Proksch *et al.*, 1991). Based on this, increased proliferation upon type II keratin cluster ablation might indicate a compensatory response to impaired barrier function and inflammation (Proksch *et al.*, 2008). The linkage between barrier dysfunction, inflammation, and epidermal hyperproliferation is well known from inflammatory skin disorders like psoriasis (Weinstein and McCullough, 1973; Ziboh, 1988; Roberson and Bowcock, 2010). In support of this hypothesis, we noted an increased production of selected cytokines, chemokines, and antimicrobial peptides in KtyII$^{-/-}$ mice. IL1β expression was strongly upregulated in KtyII$^{-/-}$ skin. IL1β on the one hand has proinflammatory function in skin and contributes to immune cell attraction but on the other hand signals to the dermis and stimulates epidermal growth factor production such as keratinocyte growth factor and epidermal growth factor in adjacent fibroblasts (Cohen, 1965; Maas-Szabowski *et al.*, 1999; Gibbs *et al.*, 2000). Thus, alterations in the cross-talk between keratinocytes and dermal fibroblast very likely contribute to the observed changes in proliferation. In the context of immune cell accumulation, cytokines produced by keratinocytes and dermal fibroblasts might trigger the activation of dermal dendritic cells, which then promote expansion of dermal T cells. This might explain enrichment of CD3-positive (general T-cell marker) and CD45-positive (general leukocyte marker) cells in the dermis of keratin KO mice.

Apoptosis could either be increased to balance increased cell proliferation and thereby maintain epidermal homeostasis (Budtz and Spies, 1989) but it was also reported to be increased in acantholytic skin disorders upon disruption of cell–cell contacts (Gniadecki *et al.*, 1998). We propose a model in which loss of keratins causes cytoplasmic accumulation of desmosomal proteins (Figure 5d; see also Kroger *et al.*, 2013). Consequently, intercellular adhesion is reduced and larger intercellular gaps appear. We assume that the minor relocalization of E-cadherin and plakoglobin might be due to a shift of traction forces away from the keratin–desmosome complex to adherens junctions (Seltmann *et al.*, 2013a). However, epidermal fractures appear in the subnuclear cytoplasm of basal cells and in suprabasal layers upon mechanical strain. Thus, keratins are required to maintain desmosome integrity and stability, to an extent that intercellular adhesion is insufficiently maintained by adherens junctions.

The reduced desmosome size in KtyII$^{-/-}$ mice seems to result from the high number of desmosomal proteins that is shifted to the cytoplasm upon keratin loss. Knockout of DP showed that the attachment of keratin filaments to the desmosomal plaque alone is not required for desmosome maintenance and stability (Vasioukhin *et al.*, 2001). This suggests that keratins are important for the stabilization of desmosomes independent of their attachment to the desmosome. This is in line with previous studies showing that desmosomes assemble but are endocytosed at accelerated rates in cells without keratins because of increased protein kinase Cα-mediated phosphorylation of DP (Kroger *et al.*, 2013). HDs instead were intact but plectin localization was altered. Plectin has binding sites for actin, intermediate filaments, and microtubules and constitutes the link between the integrins α6β4 to the keratin cytoskeleton. The loss of direct keratin interaction in KtyII$^{-/-}$ skin alone (Seltmann

et al., 2013a, b) or altered actin organization upon keratin loss (unpublished data) might affect plectin localization.

Our findings strongly support a major requirement of keratins in the maintenance of desmosomal adhesion and keratinocyte integrity, suggesting more complex pathomechanisms underlying EBS and related disorders. The restoration of epithelial sheets able to withstand mechanical force upon careful titration of WT and keratin-deficient keratinocytes provide a rationale for therapy approaches of keratinopathies although the shear force assay only considers the mechanical properties of keratin filaments but does not address or measure other aspects or functions in which keratins might be involved like barrier integrity, tight junction integrity, inflammation, and signaling pathways. Applied to therapy this would mean that restoration of keratin expression in a subset of cells into EBS patient skin might stabilize the lesions and prevent blistering. This could possibly be achieved by transplanting corrected cell suspensions on meshgrafts. The deletion of the entire type II keratin cluster uncovered a number of major phenotypes of which individual traits have already been described in single and double keratin-KO mice. However, most defects appeared much more severe in the absence of all keratins, suggesting considerable compensation by other keratins in single- or double-deficient mice. In strong support, the accumulation of desmosomal protein complexes is unique in the cytoplasm of KtyII-deficient mice and highlights a major role of keratins in desmosome maintenance (Kroger *et al.*, 2013; Figure 5e). Given that the single keratin pair K8/K19 was sufficient to maintain development of simple epithelia in complete KtyII$^{-/-}$ mice, our mouse model invites the opportunity to examine isotype-specific keratin functions *in vivo*.

MATERIALS AND METHODS

Tissue preparation and histochemistry

Animals were killed and dorsal skin and organs were either embedded in Tissue-Tek (Sakura, Alphen aan den Rijn, The Netherlands) freezing medium and snap-frozen in isopentane precooled at −80 °C for cryosections or fixed overnight in freshly prepared 4% formaldehyde in phosphate-buffered saline and processed for routine paraffin embedding. Sections were cut at 7–14 μm depending on the type of tissue and method. Hematoxylin/eosin staining for routine histology was performed according to Roth *et al.* (2012). For visualization of mast cells, cryosections were stained with May–Grünwald–Giemsa.

Immunofluorescence analysis

Frozen sections were cut with CM3050 S cryotome (Leica, Wetzlar, Germany) and were processed for immunofluorescence as described (Vijayaraj *et al.*, 2009). Antibody staining on cells was performed as described (Kroger *et al.*, 2013). Nuclei were counterstained using 1:1,000 diluted 4,6-diamidino-2-phenylindole (Roth, Karlsruhe, Germany). Antibodies are listed in Supplementary Table S1 online.

Protein lysate preparation, SDS-PAGE, and western blotting

Dorsal skin of 8-day-old KtyII$_m^{-/-}$ and head skin of E18.5 old pups was kept in SDS-sample buffer, cut into small pieces, homogenized with T10 basic ULTRA-TURRAX (IKA, Staufen, Germany), and treated with repeated cycles of heating (95 °C) and sonication to extract total proteins. SDS-PAGE, western blotting, and subsequent immunostaining was performed as described (Vijayaraj *et al.*, 2009). Total protein was loaded on 10% polyacrylamide gels. Equal loading was assessed by Coomassie staining of SDS-polyacrylamide gels run in parallel. Tubulin was used as housekeeping control and run in parallel. Primary and secondary antibodies are listed in Supplementary Table S1 online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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