Conditional targeting of plectin in prenatal and adult mouse stratified epithelia causes keratinocyte fragility and lesional epidermal barrier defects

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Summary
Plectin, a widespread intermediate filament-based cytolinker protein capable of interacting with a variety of cytoskeletal structures and plasma membrane-bound junctional complexes, serves essential functions in maintenance of cell and tissue cytoarchitecture. We have generated a mouse line bearing floxed plectin alleles and conditionally deleted plectin in stratified epithelia. This strategy enabled us to study the consequences of plectin deficiency in this particular type of tissues in the context of the whole organism without plectin loss affecting other tissues. Conditional knockout mice died early after birth, showing signs of starvation and growth retardation. Blistering was observed on their extremities and on the oral epithelium after initial nursing, impairing food uptake. Knockout epidermis was very fragile and showed focal epidermal barrier defects caused by the presence of small skin lesions. Stratification, proliferation and differentiation of knockout skin seemed unaffected by epidermis-restricted plectin deficiency. In an additionally generated mouse model, tamoxifen-induced Cre-ER1-mediated recombination led to mice with a mosaic plectin deletion pattern in adult epidermis, combined with micro blister formation and epidermal barrier defects. Our study explains the early lethality of plectin-deficient mice and provides a model to ablate plectin in adult animals which could be used for developing gene or pharmacological therapies.

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Key words: Conditional gene targeting, Epidermolysis bullosa, Hemidesmosomes, Keratinocytes, Plectin, Transepidermal water loss

Introduction
Plectin, a highly versatile cytolinker protein, is essential in maintaining the integrity of skin, muscle and heart cytoarchitecture. It is expressed in a wide variety of mammalian cells and tissues and plays an important role in mediating interactions between different cytoskeletal network systems and their anchorage at cell-cell and cell-matrix junctional complexes (Wiche, 1998). In skin, as well as in cultured keratinocytes, plectin is predominantly localized at hemidesmosomes (HDs) and cell-cell borders (Andrä et al., 2003). Association of plectin with desmosomes (Eger et al., 1997) has been shown in simple epithelial cells. With an N-terminal actin-binding domain (Andrä et al., 1998), which serves also as an integrin β4 (Intβ4)-binding site (Rezniczek et al., 1998), and a C-terminal intermediate filament (IF)-binding site, plectin is instrumental in the physical anchorage of keratin filaments at the hemidesmosomal complex (Rezniczek et al., 1998; Andrä et al., 1997).

In humans, mutations in the plectin gene (PLEC1) result in skin fragility, manifested as blister formation at the level of HDs (for a review, see Pfendner et al., 2005). Clinically, these blistering disorders belong to the spectrum of epidermolysis bullosa (EB) phenotypes. Most plectin mutations lead to EB-MD, a condition characterized by neonatal blistering and late onset muscular dystrophy. Recently, however, plectin mutations have also been found in EB patients with pyloric atresia (EB-PA) (Pfendner and Uitto, 2005), a combination that can lead to early postnatal demise of the affected individuals.

Plectin-deficient mice die 1-3 days after birth exhibiting skin blistering caused by disruption of basal keratinocytes, as well as myopathies in skeletal muscle and disintegration of intercalated disks in the heart (Andrä et al., 1997). Rupture of basal keratinocytes occurs in the cytoplasm just above the inner hemidesmosomal plaque structures (Andrä et al., 1997).

The early lethality of plectin-null mice precluded the analysis of possible plectin functions at later stages of postnatal development and maturation of mice. To circumvent this problem we have now generated mice with floxed plectin alleles, rendering the plectin locus susceptible to conditional elimination by Cre recombinase. We describe here the skin phenotype of a mouse line showing a complete knockout of plectin restricted to keratin 5 (KRT5; hereafter referred to as K5)-expressing epithelia. In addition, we have generated an inducible mouse model, where plectin expression can be
knocked out in small patches of epidermis at the adult stage of the animal. Our analysis of these mice demonstrates the importance of plectin in mediating mechanical stress resistance of newborn and adult epidermis and offers an explanation for the early death of plectin-deficient animals.

Results
Generation of mice bearing floxed plectin alleles
We have previously shown that the inactivation of the plectin gene in the mouse germline results in postnatal death, with pups showing severe skin blistering as well as muscular dystrophy (Andrä et al., 1997). To assess whether the blistering of the epidermis, or of other stratified epithelia, was caused by the animals’ death, we used a gene-targeting approach to create mice with a constitutive knockout of plectin in stratified epithelia, but not in other types of tissues. Our targeting vector contained single loxP sites in introns 25 and 31, so that upon conditional removal of exons 26-31, exon 25 would be spliced into exon 32, leading to a frameshift and a premature stop codon (see Fig. S1A in supplementary material). Selected ES cell clones were screened for correct recombination events by Southern blotting (Fig. S1B in supplementary material) and subsequently injected into blastocysts. After germline transmission of the targeted allele (Fig. S1C, lane 1 in supplementary material), the resulting mouse line carrying one Plecflox/̅alleles and one Plecalle were bred to Flp deleter mice to excise the FRT-flanked selection marker. Pups carrying the Plecflox allele were identified and then bred to homozygosity (Fig. S1C, lane 2 in supplementary material), the resulting mice homozygous for the floxed plectin allele (Plecflp/̅) appeared to be normal indicating that the genetic manipulation had not altered the function of plectin (data not shown).

Inactivation of the plectin gene in mouse skin leads to early postnatal death, caused by severe blistering of the oral epithelium
To inactivate plectin in the skin, we crossed Plecflox/̅ mice with transgenic mice expressing the Cre-recombinase under the control of the K5 promoter (Tarutani et al., 1997). Plec−/−:K5-Cre (Plec−/−:Krt5-Cre) mice thus generated would be referred to as K5-Cre KO. They were born at the expected Mendelian ratios and died 1-3 days after birth. They showed severe skin detachment, especially on the fore- and hindlimbs (Fig. 1A,B), and in some cases around the mouth and nasal cavities; rarely, aplasia cutis of the forelimbs was also noted (not shown). The extent of phenotypic alterations increased with age, reaching the size of up to 1 cm2 were found at the upper extremities, to the back skin of newborn K5-Cre KO and control pups, normally used to sequentially remove stratum corneum (Fig. 1S). To test whether such lesions could be induced by trauma we applied mild mechanical stress in the form of consecutive tape strippings with D-Squame disks (a procedure normally used to sequentially remove stratum corneum layers), to the back skin of newborn K5-Cre KO and control pups, followed by dye penetration assays. Indeed, we found small blue-stained spots in 10-times stripped K5-Cre KO (Fig. 1S) and control skin (data not shown). However, no differences in TEWL values were observed between intact skin of K5-Cre KO and control pups, demonstrating the absence of intrinsic barrier defects in K5-Cre KO mice. A histological examination of stained spots revealed microlesions at various stages of wound healing (Fig. 1O,P), Most lesions had a histological appearance similar to that shown in Fig. 1O, with a thick, acanthotic and weakly differentiated new epidermis having formed under a crust that was still covered with the old (dead) epidermis. In some lesions, a thin, multilayered sheet of keratinocytes could be seen under the crust, indicating ongoing re-epithelialization (Fig. 1P). To test whether such lesions could be induced by trauma we applied mild mechanical stress in the form of consecutive tape strippings with D-Squame disks (a procedure normally used to sequentially remove stratum corneum layers), to the back skin of newborn K5-Cre KO and control pups, followed by dye penetration assays. Indeed, we found small blue-stained spots in 10-times stripped K5-Cre KO (Fig. 1Q), but not in control skin (Fig. 1R), indicating localized loss of barrier function. Measurements of transepidermal water loss (TEWL), to detect increased fluid loss through lesions, revealed a ~3.5-fold increase in TEWL value compared to intact skin (data not shown). However, no differences in TEWL values were observed between intact skin of K5-Cre KO and control pups, demonstrating the absence of intrinsic barrier defects in K5-Cre KO mice. A histological examination of stained spots revealed microlesions at various stages of wound healing (Fig. 1O,P). Most lesions had a histological appearance similar to that shown in Fig. 1O, with a thick, acanthotic and weakly differentiated new epidermis having formed under a crust that was still covered with the old (dead) epidermis. In some lesions, a thin, multilayered sheet of keratinocytes could be seen under the crust, indicating ongoing re-epithelialization (Fig. 1P). To test whether such lesions could be induced by trauma we applied mild mechanical stress in the form of consecutive tape strippings with D-Squame disks (a procedure normally used to sequentially remove stratum corneum layers), to the back skin of newborn K5-Cre KO and control mice, followed by dye penetration assays. Indeed, we found small blue-stained spots in 10-times stripped K5-Cre KO (Fig. 1Q), but not in control skin (Fig. 1R). Histological examination of stripped K5-Cre KO skin areas showed that stripping had induced basal keratinocyte cytolysis (Fig. 1S). Moreover, a few tape strippings were often enough to induce the formation of a large blister (Fig. 1T). When stronger adhesive tape (Tesa 3M) was used, one stripping was sufficient to remove the entire epidermis of the stripped area (not shown). Together, these data demonstrated an extreme fragility of K5-Cre KO epidermis.
 Highly efficient reduction of plectin levels by K5-controlled Cre expression

To examine to what extent plectin expression was reduced in the epidermis of conditional knockout compared to control animals, immunoblotting of tissue homogenates was performed. Using an antiserum recognizing all isoforms of plectin (anti-pan-plectin), only a very weak, in fact barely detectable, signal was obtained in K5-Cre KO epidermis compared with the strong signal in control tissue (Fig. 2A). This remaining signal was likely to originate from non- keratinocyte cells present in the epidermis, such as dendritic (Langerhans) cells and pigment cells (melanocytes), which are known to express a set of plectin isoforms different from that of keratinocytes (Abrahamsberg et al., 2005) (our unpublished data). In fact, when an antiserum specific to plectin 1a, the major plectin isoform found in keratinocytes (Andrä et al., 2003), was used instead of the anti-pan-plectin antiserum, not even a weak signal was detectable in epidermal tissue extracts (data not shown). As expected, in tissue extracts from the dermis of K5-Cre KO animals, reduction of plectin expression was much less dramatic (Fig. 2A). Since neither the anti-plectin 1a nor the anti-pan-plectin antiserum (both recognizing epitopes in plectin domains preceding the rod), detected proteins in K5-Cre KO tissue homogenates, that were of lower molecular mass than full-length plectin, the expression of rodless-plectin in K5-Cre KO epidermis could be ruled out.

To examine the plectin gene locus of K5-Cre KO animals in Cre-affected (epidermal) tissue compared to putatively unaffected (dermal) tissue, DNAs isolated from epidermal and dermal K5-Cre KO tissues were subjected to Southern blot analysis, along with corresponding samples from control (Pleclox/lox) animals. In K5-Cre KO epidermis, the only signal detected (9 kb) corresponded to the Pleclox/lox allele, indicating a recombination efficiency of 100% (Fig. 2B). In K5-Cre KO dermis, however, the 15 kb wild-type signal was predominant, with an additional (lesser) signal of 9 kb (Fig. 2B). Considering that enzymatic detachment of the epidermis leaves some hair follicles embedded in the dermis (Vasioukhin et al., 1999) (our unpublished data), the presence of the wild-type signal in K5-Cre KO dermis is not surprising.
unpublished observation), it is likely that the recombination signal observed in K5-Cre KO dermis stemmed from a contamination with keratinocytes. The absence of the 9 kb mutated gene signal in both controls (epidermis and dermis) indicated that aberrant (non-Cre-mediated) recombination between the \textit{loxP} sites did not occur.

Additionally, we used immunofluorescence microscopy to assess the absence of plectin from the epidermis of knockout mice (Fig. 2C). A pronounced staining of the dermo-epidermal borderline, along with a weaker staining of the cytoplasm and membranes of all epidermal keratinocytes, was detected in normal mouse skin (Fig. 2Ca) but in the corresponding K5-Cre KO tissue, staining above background was observed in only a few insular cells (arrows in Fig. 2Cb). These plectin-positive cells most likely are melanocytes and Langerhans cells (arrows in b). Also note strongly reduced plectin-specific label in K5-Cre KO esophagus (f), but in K5-Cre KO heart, plectin expression was not reduced (h). p, palate; t, tongue. Dashed lines (in b and d) indicate dermo-epidermal borders. Bars, 20 μm.

Unaltered epidermal stratification and differentiation in K5-Cre KO mice
To investigate whether plectin deficiency had any influence on skin development and differentiation we undertook a detailed immunohistological analysis of skin sections from 1-day-old K5-Cre KO and control mice. First, we assessed the expression of two major hemidesmosomal proteins, Intβ4, which binds directly to plectin (Rezniczek et al., 1998), and BPAG1 (also known as dystonin; Dst). As expected, the Intβ4 signal was found confined to the basal membrane of basal keratinocytes and the outer root sheath of hair follicles (Fig. 3A,B). Similar
To investigate whether plectin deficiency affects epidermal proliferation, sections of leg skin from 1-day-old wild-type and knockout mice were immunostained using antibodies to Ki-67 to evaluate proliferation. Sections of leg skin from 1-day-old wild-type and knockout mice were also stained with antibodies to involucrin and K10 to examine differentiation patterns. In addition, electron microscopy was performed on macroscopically intact K5-Cre KO epidermis to further analyze cell morphology and desmosomal structures.

Conditional targeting of plectin does not affect proliferation and survival of keratinocytes. This suggests that plectin is not essential for keratinocyte proliferation under normal conditions. However, the role of plectin in the differentiation of epidermal keratinocytes is still under investigation.
epidermis showed that similar numbers of stained cells were present per linear millimeter of basal membrane, namely 58±13.1 and 61±14.3 for wild-type and knockout, respectively (Fig. S6C in supplementary material). This result indicated that plectin deficiency did not affect proliferation of keratinocytes in vivo. We also examined the effects of plectin deficiency on apoptosis by TUNEL analysis of the skin of newborn mice. Neither K5-Cre KO basal keratinocytes, nor control cells showed signs of apoptotic cell death (Fig. S6D,E in supplementary material). Occasionally, singular TUNEL-positive cells lining the exposed epidermal surface of blister roofs were found in K5-Cre KO mice (data not shown), indicating the occurrence of some apoptotic events after epidermal detachment.

Inducible knockout of plectin in adult skin leads to increased epidermal fragility and lesional barrier defects

Since K5-Cre KO mice, similar to plectin-null mice, died within a few days after birth, they were not suitable as an animal model for studying consequences of plectin loss at later stages in life. To overcome these limitations, we employed conditional gene targeting, using the Cre-loxP recombination system with an inducible chimeric Cre-ER<sup>T</sup> recombinase that can be activated by 4-hydroxy-tamoxifen (OHT), but not by endogenous estradiol (Feil et al., 1996; Indra et al., 1999). To achieve temporally controlled somatic plectin ablation in the epidermis, we crossed mice with one floxed and one null allele (Plec<sup>flox/–</sup>) with transgenic mice expressing the chimeric Cre-ER<sup>T</sup> genotype were injected intraperitoneally with 1 mg OHT per day for five consecutive days and were subjected to phenotypic analyses 2 weeks after the last injections. Southern blotting analysis showed that ~35% of the floxed plectin allele was converted to the <sup>H9004</sup> allele under these conditions (Fig. 4A).

Histological analysis of sections from tail and shaved back skin revealed normal organization of the epidermis and the absence of microblisters in unstressed skin (Fig. 4G). Transepidermal water loss (TEWL; H) and quantification of stratum corneum protein removal (I) after serial tape strippings of skins from Plecflox/–:K5-Cre-ERT mice, that had been either untreated (–OHT) or treated (+OHT). Basal TEWL was measured before the first stripping. Values are mean ± s.e.m. from 5 OHT-treated and 5 untreated (control) Plecflox/–:K5-Cre-ERT littermates. Measurements were taken after two subsequent tape strippings. For statistical analyses the Student’s t-test was used (*P<0.1; **P<0.05). Note that for quantification of stratum corneum protein removal, disks from two subsequent strippings were pooled. Bars, 20 μm.
of microblisters (Fig. 4E). However, small blisters could be induced by repeated tape stripplings using D-Square disks (Fig. 4F,G), indicating that localized (mosaic) plectin deficiency made the epidermis of adult mice more susceptible to mechanical stress, although the plectin-expressing cells were sufficient to prevent epidermal detachment.

To quantify any focal defects in epidermal barrier function, we measured TEWL before and during acute barrier disruption by repeated tape stripplings, as described above for constitutive conditional plectin knockout mice. In addition, the amount of proteins removed by serial tape stripplings was measured to analyze stratum corneum cohesion. Under basal (non-stripped) conditions, animals with induced mosaic plectin deletion did not show a difference in TEWL, compared to control animals (Fig. 4H). However, after four subsequent stripplings, TEWL began to increase sharply in mice with induced plectin deletion, but only modestly in control mice (Fig. 4H). No differences in TEWL could be observed between non-induced \textit{Plec\textsuperscript{floox}/K5-Cre-ER\textsuperscript{T}} and non-induced \textit{Plec\textsuperscript{floox}/K5-Cre-ER\textsuperscript{T}} mice either under basal conditions or after tape stripping (data not shown).

Estimates of the amount of proteins removed after each stripping demonstrated that during the last stripplings proportionally more cells had been removed from the skin of mice with induced mosaic plectin deletion compared to control mice, suggesting increased fragility of the cell layers adjacent to the induced lesions (Fig. 4I). Overall, the data obtained with the inducible mouse model demonstrated that even localized plectin deficiency in the epidermis renders keratinocytes more prone to mechanical stress-induced damage.

**Discussion**

Similar to plectin-null mice, K5-Cre KO mice with a specific deletion of plectin in stratified epithelia died within 1-3 days postnatally exhibiting severe skin fragility, focal skin barrier defects and growth retardation. Focal skin barrier defects appeared to arise as a consequence of localized keratinocyte cytolsis and thus did not imply a direct role of plectin in barrier formation. Similar skin lesions with increased TEWL have previously been reported for desmocollin 1-null mice (Chidgey et al., 2001) where they were related to localized acantholysis. Small Toluindine-Blue-stained lesions were also found in the skin of K5 knockout mice, where they were attributed to cytolsis of basal keratinocytes (Peters et al., 2001). The early onset of blister formation and the severity of tissue fragility clearly showed that plectin is more important than BPAG1 in linking keratin filaments to hemidesmosomes, as BPAG1 knockout mice grow to adulthood and display only mild blistering (Guo et al., 1995). Apart from the formation of blisters and lesions due to epidermal detachment, the epidermis of K5-Cre KO displayed a normal stratified organization and correct expression patterns of differentiation markers, and proliferation and survival of keratinocytes were unaffected. The occasional presence of apoptotic cells in the lumen-proximal keratinocyte layer of blister roofs indicated that, although cytolysis due to keratinocyte disruption above the HDs predominated, some still intact keratinocytes underwent programmed cell death. However, massive apoptosis of basal keratinocytes, as it was observed upon epidermal detachment in Intrb4-deficient mice (Dowing et al., 1996; DiPersio et al., 2003), was not evident.

The skin phenotype of K5-Cre KO mice resembled that of children suffering from EB-PA (EB-PA; OMIM accession no. 226730), who are born with an extremely fragile skin (Charlesworth et al., 2003; Pfendner and Uitto, 2005). As most of these patients harbored premature termination codon or nonsense mutations in exons encoding N-terminal parts of the protein, a rapid decay of the prematurely terminated short RNA transcripts formed is very likely, leading to a complete absence of the protein (Charlesworth et al., 2003). EB-PA mutations being located upstream of exon 31 [the exon encoding plectin’s rod domain (Liu et al., 1996)] could therefore not be compensated for by rodless plectin isoforms, explaining the more severe phenotype of patients with plectin-associated EB-PA, compared with patients suffering from EB-MD. Thus, mice with a specific deletion of plectin in stratified epithelia, such as our K5-Cre KO mice, can be considered as an ideal model for the stratified epithelia phenotypes of patients with severe forms of plectin-associated EB-PA.

Before dying, K5-Cre KO mice became progressively weaker, ceased to grow and apparently became unable to suckle, as their stomachs remained empty. Inspection of the oral cavities of newborn K5-Cre KO mice after at least one nursing showed the presence of multiple blisters on their palates and in some cases also on their tongues. Therefore, we reason that the mechanical stress applied to the oral epithelia by sucking and swallowing was the cause of the oral blister formation. As a consequence, the pups’ desire for food was probably reduced due to pain and possibly obstruction of the oral cavity by especially large blisters or cellular debris. A similar running due to prevention of feeding by erosive mucosal blistering was described for mice lacking desmoglein 3 (Koch et al., 1997). In these mice, lesions were initially caused by sucking and became even worse when growing mice began eating solid food. The finding that K5-Cre KO mice did not show epithelial detachment in the esophagus was not surprising, as the liquid diet of neonatal mice exerts only low mechanical stress on such organs.

Mucosal involvement is common in various forms of EB and cases of mucosal blistering associated with EB-MD (OMIM accession no. 226670) have been described (Kunz et al., 2000; Schara et al., 2004). The mouth and throat problems of patients suffering from EB have been reported to often cause a serious disruption of nutrient intake, similar to what we observed with K5-Cre KO mice. Because of oral blistering these children barely grow, as there is a higher need for nutrients because the body loses proteins along with the wound exudate and also has to sustain normal metabolism (information available at the DEBRA website: www.debra-international.org). Pyloric atresia manifests with gastric abnormalities, primarily pyloric and duodenal atresia. However, in K5-Cre KO mice, the movement of coagulated milk from the pylorus into the intestine was not obstructed and consequently, the stomachs were not distended, ruling out that conditional knockout animals were suffering from pyloric atresia. Moreover, the absence of gastric pathologies in K5-Cre KO mice proved the targeting of the plectin gene to be exclusive for stratified epithelia, without affecting simple epithelia.

Even though the epidermis of OHT-treated \textit{Plec\textsuperscript{floox}/K5-Cre-ER\textsuperscript{T}} mice showed only a mosaic pattern of plectin deletion, its susceptibility towards mechanical stress-induced damage was found to be clearly increased. Evidently, the patchwise deletion of plectin rendered the epidermis more prone to trauma, as
revealed by increased TEWL and lesion formation. The observed more readily removable cells of the deeper epidermal cell layers by tape stripping may also be related to the lesion formation, although reduced cohesion of keratinocytes in the outer epidermal layers due to weakened desmosomal connections cannot be ruled out at the present stage.

Although we used quite high concentrations of OHT to activate Cre recombinase, plectin deletion was achieved only in patches of adult mouse epidermis. Inducible deletion of floxed genes in adult mice via Cre-ERT recombinase is not always 100% effective and appears to be especially difficult to achieve when cytoskeleton-associated proteins with a long half-life are targeted (López-Rovira et al., 2005). The very low turn-over rate of plectin in polarized epithelia (Eger et al., 1997) might explain the relatively weak deletion of plectin expression 14 days after Cre activation, since Cre is activated within 24 hours of the final OHT treatment (Indra et al., 1999). It might be advantageous for future studies to use K14-Cre-ERT2 mice, in which induction efficiency is more potent than in K5-Cre-ERT mice (Indra et al., 1999), and to combine this approach with topical application of OHT, which is less toxic than intraperitoneal injection (Vasioukhin et al., 1999) and can be done over longer periods (Benitah et al., 2005). Once more efficient deletion of plectin in adult stratified epithelia has been achieved, it will be interesting to re-evaluate skin barrier function, and moreover, investigate other yet unaddressed skin-related topics, such as wound healing and hair growth.

In summary, the generation of conditional stratified epithelia-specific plectin knockout mice has improved our understanding of the phenotypic manifestations of plectin deficiency in multilayered epithelia. Most importantly, the generation of mice carrying floxed plectin alleles makes it now possible to analyze the function of plectin in various other tissues known to be affected by EB-MD, such as skeletal muscle and brain. In addition, by refining the inducible knockout of plectin in skin, deeper insights into plectin functions in the adult organism might be gained, and a test system for the development of therapeutic approaches for skin blistering in EB-MD/EB-PA might be obtained.

Materials and Methods
All experiments involving animals were performed in accordance with Austrian Federal Government laws and regulations.

Engineering of plectin conditional knockout mice
To prepare the targeting construct, an FRT-PGKneo–FRT sequence plus a loxP site was inserted into intron 26. The targeting construct (linearized with Sall) was electroporated into E14.1 embryonic stem cells. Colonies resistant to geneticin (G418) were screened for the desired homologous recombination by Southern blotting. To generate chimeric mice, two recombinant ES cell clones (10-26, respectively. Heterozygous mice were bred to FLP deleter mice and offspring (26, respectively. Heterozygous mice were bred to FLP deleter mice and offspring were transgenic for the K5-Cre recombinase and carried the deletion alleles of the plectin gene. The K5-Cre transgene was detected by PCR amplification with the primers pleIn25-26U and pleEx32/L7714 (5'-GCCAGCGCTGGTGGTTT-CCTCCGCCGCA-3').

Preparation of 4-hydroxy-tamoxifen solutions
A 10 mg/ml 4-hydroxy-tamoxifen (OHT; Sigma) solution was prepared as described previously (Metzger and Chambon, 2001). Mice were injected with 1 mg OHT per day for 5 consecutive days.

Antibodies
For a complete list of primary antibodies refer to Table S1 in supplementary material. All secondary antibodies were purchased from Jackson Immuno-Research Laboratories (West Grove, PA).

Immunofluorescence and phase contrast microscopy of tissue sections, apoptosis assay, and dye penetration assay
Tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin, or shock frozen in isopentane (cooled with liquid N2), sectioned (2 µm) on a cryostat microtome, and fixed in acetone at −20°C (Spazierer et al., 2006). Samples were examined using a Zeiss fluorescence laser-scanning microscope (LSM 510) or a Zeiss Axiohot microscope equipped with an AxioCam Hrc camera (Zeiss). Digital images were processed using Adobe Photoshop and Adobe Illustrator software.

Apoptosis was analyzed using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI). Dye penetration assays were performed as described previously (Spazierer et al., 2006).

Preparation of cell and tissue extracts, and immunoblotting
Epidermis and dermis from newborn mice were separated by treatment with dispase (30 units/ml, 37°C), frozen in liquid nitrogen and pulverized. Tissue homogenates were prepared in Triton X-100 high-salt buffer (Eger et al., 1997) by mechanical disruption using a Polytron PT-3000 homogenizer (Kinematica, Littau-Luzern, Switzerland). Cell fractions were obtained from ground frozen tissue following the protocol of Vasioukhin et al. (Vasioukhin et al., 2001). Proteins were subjected to SDS-PAGE and immunoblotting as previously described (Spazierer et al., 2006).

Tape stripping, protein removal assay and TEWL
Tape stripping and measurement of transepidermal water loss (TEWL) were performed as described previously (Spazierer et al., 2006). TEWL of 1-day-old mice was measured with a Tewameter TM-300 equipped with a small-diameter (3 mm) probe, and measurements were digitally recorded via a MPAS multiprobe adapter (Courage and Khazaka, Cologne, Germany). The amount of protein removed per D-Squame disk (CuDerm corporation, Dallas, TX) was measured by a modification of the method of Dreher et al. (Dreher et al., 1998). Disks from two subsequent stripings were combined and the NaOH-soluble protein content after neutralization with HCl was determined using the Bio-Rad Protein Assay Kit. Absorption was measured with a spectrophotometer at 595 nm. An empty D-Squame disk and distilled water incubated with the Bio-Rad dye, served as negative controls. The amount of protein measured was then normalized to skin surface area (µg/cm2).

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