PAR2 absence completely rescues inflammation and ichthyosis caused by altered CAP1/Prss8 expression in mouse skin

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Altered serine protease activity is associated with skin disorders in humans and in mice. The serine protease channel-activating protease-1 (CAP1; also termed protease serine S1 family member 8 (Prss8)) is important for epidermal homeostasis and is thus indispensable for postnatal survival in mice, but its roles and effectors in skin pathology are poorly defined. In this paper, we report that transgenic expression in mouse skin of either CAP1/Prss8 (K14-CAP1/Prss8) or protease-activated receptor-2 (PAR2; Grhl3PAR2+/−), one candidate downstream target, causes epidermal hyperplasia, ichthyosis and itching. K14-CAP1/Prss8 ectopic expression impairs epidermal barrier function and causes skin inflammation characterized by an increase in thymic stromal lymphopoietin levels and immune cell infiltrations. Strikingly, both gross and functional K14-CAP1/Prss8-induced phenotypes are completely negated when superimposed on a PAR2-null background, establishing PAR2 as a pivotal mediator of pathogenesis. Our data provide genetic evidence for PAR2 as a downstream effector of CAP1/Prss8 in a signalling cascade that may provide novel therapeutic targets for ichthyoses, pruritus and inflammatory skin diseases.
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uman skin disorders such as atopic dermatitis, psoriasis and ichthyoses range in severity from mild to life threatening, and share characteristics of inflammation, defects in epidermal barrier function, skin desquamation and pruritus in affected patients (reviewed in refs 1 and 2). Our understanding of their molecular and genetic origins is improving3–5, with serine proteases emerging among possible disease-causing culprits. Gene-targeting experiments in mice revealed a critical role for membrane-bound serine proteases, particularly those of the S1 or trypsin-like family6 in postnatal epidermal barrier function7–9. It also emerged that inadequate regulation of serine protease activity in the skin of both mice6,10 and humans11 could disrupt skin homeostasis. However, which serine proteases need to be tightly regulated to prevent disease and how they mediate skin pathology when deregulated are still incompletely defined.

Channel-activating protease-1 (CAP1; also termed protease serum S1 family member-8 (Prss8)), the mouse homologue of human prostaticastatin, is a glycosylphosphatidylinositol-anchored serine protease12. CAP1/Prss8 is expressed at high levels in the prostate gland13, and is also found in a variety of other organs such as skin, colon, lung and kidney14. We have previously shown that CAP1/Prss8 is predominantly expressed in granular and spinous layers of the epidermis where it is crucial for epidermal barrier function and thereby indispensable for postnatal survival15. Membrane-bound serine proteases exhibit pleiotropic functions not only by clearing growth factors, zymogen proteases and extracellular matrix proteins16 but also by inducing cell signalling through members of the proteinase-activated receptor (PAR) family of G-protein-coupled receptors17. It was recently demonstrated that CAP1/Prss8 can function upstream in a proteolytic cascade that culminates in PAR2 activation18–20; yet, genetic evidence that CAP1/Prss8 can evoke PAR2 signalling in vivo is lacking. PAR2 is widely expressed in skin, including keratinocytes, endothelial cells and sensory nerves19–21, in which it has already been implicated in the regulation of keratinocyte proliferation and differentiation22, maintenance of the epidermal barrier23, inflammation24,25 and pruritus26–28. Both CAP1/Prss8 and PAR2 are expressed in the suprabasal granular layers of the epidermis28–31, supporting a possible interaction between the two.

In this study, to directly address the potential contribution of deregulated CAP1/Prss8 and PAR2 expression to skin pathology, we manipulated epidermal CAP1/Prss8 and PAR2 expression in transgenic mouse lines (K14-CAP1/Prss8, Ghrh23CAP22–24). The two models yielded modestly increased as well as ectopic expression of CAP1/Prss8 or PAR2, and exhibited similar phenotypes characterized by scaly skin, hyperplastic epidermis and scratching behaviour. Having established the ability of protease and signalling receptor to independently cause skin pathology, we addressed whether they also cooperated to drive pathogenesis as might be suggested by signalling studies16. We indeed observed that the K14-CAP1/Prss8 transgene completely lost its capacity to induce skin pathology in the absence of PAR2, placing PAR2 as a pivotal mediator downstream of CAP1/Prss8 in this model. We thus demonstrate that altered CAP1/Prss8 expression triggers PAR2-dependent inflammation, ichthyosis and itching, supporting the requirement for tight control of protease activity in skin homeostasis, and implicating PAR2 as a potential mediator of pathologies linked to loss of serine protease regulation in skin.

Results

Generation and characterization of K14-CAP1/Prss8 mice.

To study the biological consequence of deregulated CAP1/Prss8 expression in skin, we generated transgenic mice expressing the full-length mouse CAP1/Prss8 coding sequence32 under the control of the human keratin-14 promoter33 (Fig. 1a). This promoter targets gene expression to keratinocytes of the basal layer of the epidermis and the outer root sheath of hair follicles34. Pronuclear injections of the K14-CAP1/Prss8 transgene construct yielded five founders, two of which were fertile and transmitted the transgene to produce two independent stable transgenic lines (termed lines 1 and 2; Fig. 1b,c). The transgene was transmitted according to Mendelian inheritance in both lines (line 1: 54% tg, n = 95; line 2: 54%tg, n = 72), suggesting that the level of CAP1/Prss8 expression obtained did not interfere with embryonic development. For line 1, only male mice (all of them) were transgenic, indicating integration of the transgene into the Y chromosome. Both transgenic lines expressed the transgene in skin as determined by immunohistochemistry (Fig. 1d; Supplementary Fig. S1a). Analysis of total (transgene-driven plus endogenous) expression of CAP1/Prss8 demonstrated a 1.1- and 4.7-fold increase in transcript levels and a 1.1- and 2.9-fold increase in protein levels in lines 1 and 2, respectively (Fig. 1e,f), indicating higher CAP1/Prss8 expression levels in line 2.

Epidermal defects and death in K14-CAP1/Prss8 mice.

K14-CAP1/Prss8 transgenic mice were easily discernible from their control littermates just a few days after birth by their scaly skin (ichthyosis), which progressed with age and was evident most prominently in the ventral-abdominal region and the tail (line 2, Fig. 2a, line 1; Supplementary Fig. S1b). Both transgenic lines manifested ichthyosis with 100% penetrance, as well as abnormal hair growth (hypotrichosis) relative to littermate controls (Fig. 2b). No histopathological abnormalities were observed in other organs, such as thymus, tongue, oesophagus, heart, liver, lung and spleen (Supplementary Fig. S2). Histological analysis revealed epidermal hyperplasia (acanthosis) in transgenic mice from as early as 2 days of age (Fig. 2c; Supplementary Fig. S1c) associated with enhanced proliferation of keratinocytes, as evidenced by a threefold increase in the number of Ki67-positive cells in the stratum basale (Fig. 2d), without an accompanying change in the number of apoptotic cells in the epidermis (Fig. 2e). In addition to epidermal hyperplasia, the dermis of K14-CAP1/Prss8 transgenic mice was more cellular with a notable increase in the number of haematoxylin-stained nuclei (Fig. 2c).

Despite differences in CAP1/Prss8 expression levels, mice from both transgenic lines also manifested a significant reduction in body weight (Fig. 2f; Supplementary Fig. S1d) accompanied by an up to twofold increase in transepidermal water loss (TEWL) evident in 2-week-old animals (Fig. 2g; Supplementary Fig. S1e) and maintained in adults (TEWL: 3.31 g m−2 h−1 in wild type (wt) versus 6.90 g m−2 h−1 in tg, P < 0.05, n = 6 and 7, respectively, in line 1. 2.87 g m−2 h−1 in wt versus 6.02 g m−2 h−1 in tg, P < 0.05, n = 6 and 4, respectively, in line 2; Body weight: 37.49 g in wt versus 32.62 g in tg, n = 6 and 7, respectively, in line 1 and 37.64 g in wt versus 26.06 g in tg, P < 0.001, n = 6 and 4, respectively, in line 2) indicative of a defect in the skin barrier function. Many transgenic mice from line 2 died within the first 2 weeks (~55 versus 10% in line 1, Fig. 2h; Supplementary Fig. S1f), but survival was not further impaired thereafter. Although we did not address it directly, we speculate that death of these animals may be a consequence of severe dehydration.

Altered lipid and protein content in K14-CAP1/Prss8 mice.

To explore the basis for skin barrier defects observed in K14-CAP1/Prss8 transgenic mice, we first looked for alterations in expression of keratinocyte differentiation markers by immunohistochemistry and western blot. Expression of keratin-1, loricrin, filaggrin and involucrin appeared to be more widespread within the epidermis of K14-CAP1/Prss8 mice, but there was no obvious change in distribution within the differentiated layers (Fig. 3a; Supplementary Fig. S1g), and expression levels were normal with the exception of filaggrin. Levels of all proteolytically cleaved intermediates of filaggrin were increased (Fig. 3b,c; Supplementary Fig. S1h,i). Moreover, the differentiation marker keratin-14, normally expressed...
exclusively in the stratum basale and hair follicles, was now detected in all nucleated epidermal cell layers. The expression of keratin-6, normally present only in hair follicles, was also detectable in interfollicular keratinocytes (Fig. 3a; Supplementary Fig. S1g), consistent with epidermal hyperplasia.

To test the integrity of intercellular tight junctions in the granular layer of the epidermis, we injected NHS-LC-biotin (600 Da) into the dermis and subsequently visualized its diffusion on sections stained with fluorescently conjugated streptavidin. NHS-LC biotin diffused freely between cells up to the second-last nucleated layer of the epidermis with no discernable difference between transgenic mice and littermate controls (Fig. 3a). Thus, the epithelial barrier within the granular layer appeared fully functional at least for molecules ≥600 Da.

The extracellular lipid barrier in the stratum corneum consists mainly of ceramides, free fatty acids and cholesterol present in a unique and stoichiometric composition. The ceramides are preferentially composed of probarrier lipids glucosylceramide, sphingomyelin and cholesterol sulphates. Ceramide levels were significantly higher in transgenic mice (Fig. 3d; Supplementary Fig. S1j), and more ceramide forms with longer fatty acids were present as seen by ceramide fractionation and densitometric quantification (Cer(C-6)-NS, Cer(C26-AS), Cer(C26-NH) and Cer(EOS); Supplementary Table S1), as well as more ceramide precursors glucosylceramide and sphingomyelin. Cholesterol sulphate levels were nearly 1.8-fold higher than that of controls (Fig. 3e; Supplementary Fig. S1k). Covalently bound lipids, which are also important for skin barrier function, were then separated into ω-hydroxylated fatty acid and Cer(OS), showing that Cer(OS) levels were only 6.3% of that of control mice in line 2 (Fig. 3f) and 14% of that of control mice in line 1 (Supplementary Fig. S1l).

The hydrophobic water-protective barrier of the skin consisting of lipids originates from lamellar bodies secreted by keratinocytes of the granular layer. Electron microscopy analyses revealed that secretion of lamellar bodies is diminished in the transgenic epidermis and that numerous lamellar body-like inclusions are present in transgenic corneocytes (Fig. 3g). The stratum granulosum-stratum corneum interface appeared to be widened in transgenic mice, containing less vesicular lamellar bodies and more electrondense amorphous material. The process of corneodesmosome degradation and stratum corneum desquamation was delayed in the transgenic epidermis relative to control epidermis (Fig. 3g). Thus, defective epidermal barrier function in K14-CAP1/Prss8 transgenic mice is likely to originate from altered epidermal protein-lipid composition.

**Itching and inflamed skin in K14-CAP1/Prss8 mice.** Transgenic animals surviving till adulthood frequently exhibited skin lesions (Fig. 4a; Supplementary Fig. S1m) possibly caused or aggravated by increased scratching behaviour (Fig. 4b; Supplementary Fig. S1n), which was evident after weaning and persistent throughout life. Lesions were sometimes accompanied by inflammation and swelling of regional lymph nodes, which were found to be enriched in plasma cells (Fig. 4a; Supplementary Fig. S1m).

Epidermal thickening and increased dermal cellularity (Fig. 2c) are often observed in mice with skin inflammation. To address the characteristics and the origin of the inflammatory phenotype and how it correlated with epidermal hyperplasia, barrier dysfunction
Ichthyosis and itching in Grhl3<sup>−/−</sup> mice. To probe the contribution of PAR2 signalling to skin disease, we addressed whether increased expression of PAR2 in mouse skin might induce a pathological transformation similar to what had been observed with altered CAP1/Prss8 activity. We inserted a cassette consisting of the mouse PAR2 coding sequence followed by an internal ribosomal entry site and the lacZ reporter gene at the start codon of the grainyhead-like-3 (Grhl3) gene (Grhl3<sup>−/−</sup>) by homologous recombination (Fig. 5a,b). The Grhl3 locus drives the expression of PAR2 throughout the epidermal ectoderm from mid-gestation onwards<sup>16,34,35</sup>. Consistent with simultaneous interruption of the Grhl3 gene, mice with PAR2 inserted in both alleles (Grhl3<sup>−/−</sup>/<sup>−</sup>) died perinatally with spina bifida<sup>34</sup>. Therefore, Grhl3<sup>−/−</sup>/<sup>−</sup> mice were used throughout this study for transgenic PAR2 overexpression. Importantly, mice carrying one functional allele of the Grhl3 gene (Grhl3<sup>+/-</sup>) do not exhibit phenotypic differences compared to wt controls<sup>4</sup>, and knock-in of the Cre recombinate into the Grhl3 gene locus also affects Grhl3 gene expression, but does not trigger skin pathology<sup>16</sup>.

The expression of the Grhl3<sup>−/−</sup> allele, as monitored by lacZ staining, was found predominantly in the suprabasal layers of the epidermis (Fig. 5c). Although Grhl3<sup>−/−</sup>/<sup>−</sup> mice appeared normal at birth, they started to exhibit a scaly skin, mostly evident on the tail, and epidermal hyperplasia from 2 weeks onwards (Fig. 5d). Similar to K14-CAP1/Prss8 transgenic mice, Grhl3<sup>−/−</sup> mice displayed increased scratching behaviour in adulthood (Fig. 5e) and severe skin lesions associated with occasional lymph node swelling (Fig. 5f). Notably, scratching behaviour was not observed in young mice or in adult mice without lesions, and did not appear to precede lesion formation. The common features in the phenotype of Grhl3<sup>−/−</sup> mice and K14-CAP1/Prss8 transgenic mice support a possible role for PAR2 in CAP1/Prss8-induced skin pathology.

**PAR2 deficiency rescues K14-CAP1/Prss8-induced skin defects.** To address whether CAP1/Prss8 can trigger PAR2 activation in vivo and to experimentally test whether the similarities between Grhl3<sup>−/−</sup> and K14-CAP1/Prss8 transgenic animals were due to excessive PAR2 activation in both models, we crossed K14-CAP1/Prss8 transgenic mice from line 2 with mice constitutively and globally deficient in PAR2 (PAR2<sup>−/−</sup>) that do not exhibit obvious skin abnormalities<sup>36</sup>. Mice carrying the K14-CAP1/Prss8 transgene that were either wt (K14-CAP1/Prss8) or heterozygous mutant for PAR2 (K14-CAP1/Prss8;PAR2<sup>−/−</sup>) displayed scaly skin with epidermal

and itching, we explored the expression of cytokines and procytokines in the skin of K14-CAP1/Prss8 transgenic mice. Interleukin-1α (IL-1α), interleukin 1β (IL-1β), thymic stromal lymphopoietin (TSLP) and the extracellular matrix remodelling enzyme matrix metalloproteinase-9 (MMP9) were upregulated relative to littermate controls (<i>P</i> < 0.05). Consistent with the increase in antigen-presenting cells, CD3-positive T cells undergoing apoptosis in the epidermis of 2-week-old animals. (<i>P</i> < 0.01). (h) Survival curves of K14-CAP1/Prss8 versus wild-type littermates. All data are presented as mean±s.e.m.

Figure 2 | Phenotype of K14-CAP1/Prss8 transgenic mice. (a) Macroscopic appearance of transgenic mice and littermate controls at 1 week (1 wk), 2 weeks (2 wk) and 5 months (5 mo) of age. (b) Quantification of the number (Nb.) of hair follicles in transgenic versus control skin. n=3 mice per genotype, *<i>P</i> < 0.05. (c) Haematoxylin and eosin analyses of 2-day (2 d)-, 2-week (14 d)- and 5-month (5 mo)-old K14-CAP1/Prss8 mice compared with wild-type littermates. White bars indicate the thickness of the epidermis. Scale bars indicate 20 μm. n=3 mice per genotype. (d) Quantification of the number of Ki67-positive cells in the epidermis of 2-week-old animals. (e) Quantification of the number (Nb.) of cells undergoing apoptosis in the epidermis of K14-CAP1/Prss8 versus wild-type littermates (n=3 mice per genotype, NS: not significant). (f) Body weight measurements of 2-week-old male (M) and female (F) transgenic mice and littermate controls, *<i>P</i> < 0.05. (g) Transepidermal water loss (TEWL) measurements of male and female transgenic mice. n=4 animals per group. **<i>P</i> < 0.01. (h) 5 mo. Two-day-old transgenic pups also

suggests an inflammatory phenotype in the skin of transgenic mice with very early onset, and a potential initiating role for dendritic cells in this process.

As dendritic cell-mediated skin inflammation has been linked to TSLP production by keratinocytes<sup>33</sup>, we further investigated TSLP expression in the skin by real-time PCR. Although no difference was seen at embryonic day 17.5 (E17.5) or postnatal day 1 (P1), a dramatic increase in TSLP expression was observed in transgenic skin at P2, P7 and P14 (Fig. 4i). No difference in TEWL or body weight was observed at P1, P2 and P5 (Fig. 4j,k), indicating that the defective barrier function and decrease in body weight might be secondary to epidermal hyperplasia and skin inflammation. Primary keratinocytes derived from transgenic and control littermates at P2 showed increased TSLP expression (Fig. 4l) and proliferation rates (Fig. 4m), suggesting that hyperplasia and inflammation might both originate in the transgenic keratinocytes. Transcripts for proinflammatory mediators IL-1α, IL-1β and MMP9 were barely or not at all detectable in the same cells (Fig. 4l). Thus, chronic skin inflammation, characterized by immune cell infiltration and expression of procytokines and cytokines, occurs early in parallel to epidermal hyperplasia in K14-CAP1/Prss8 transgenic mice. Although apparently not causative, barrier dysfunction and itching may later aggravate these phenotypes.

Ichthyosis and itching in Grhl3<sup>−/−</sup> mice. To probe the contribution of PAR2 signalling to skin disease, we addressed whether increased expression of PAR2 in mouse skin might induce a pathological transformation similar to what had been observed with altered CAP1/Prss8 activity. We inserted a cassette consisting of the mouse PAR2 coding sequence followed by an internal ribosomal entry site and the lacZ reporter gene at the start codon of the grainyhead-like-3 (Grhl3) gene (Grhl3<sup>−/−</sup>) by homologous recombination (Fig. 5a,b). The Grhl3 locus drives the expression of PAR2 throughout the epidermal ectoderm from mid-gestation onwards<sup>16,34,35</sup>. Consistent with simultaneous interruption of the Grhl3 gene, mice with PAR2 inserted in both alleles (Grhl3<sup>−/−</sup>/<sup>−</sup>) died perinatally with spina bifida<sup>34</sup>. Therefore, Grhl3<sup>−/−</sup>/<sup>−</sup> mice were used throughout this study for transgenic PAR2 overexpression. Importantly, mice carrying one functional allele of the Grhl3 gene (Grhl3<sup>+/-</sup>) do not exhibit phenotypic differences compared to wt controls<sup>4</sup>, and knock-in of the Cre recombinate into the Grhl3 gene locus also affects Grhl3 gene expression, but does not trigger skin pathology<sup>16</sup>.

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expression in murine skin 

hyperplasia (Fig. 6a). In contrast, no scaling, epidermal hyperplasia or premature lethality was observed in K14-CAP1/Prss8 transgenic mice that were homozygous mutant for PAR2 (K14-CAP1/Prss8:PAR2−/−, Fig. 6a and Table 1). Despite the absence of PAR2, these mice still expressed the K14-CAP1/Prss8 transgene (Fig. 6a,b). The rescue was also evident functionally, as K14-CAP1/Prss8:PAR2−/− mice exhibited TEWL in the same range as that of wt controls but significantly different from that of K14-CAP1/Prss8:PAR2−/− littermates (Fig. 6c). Moreover, whereas K14-CAP1/Prss8 mice displayed a significantly lower body weight compared with controls, the K14-CAP1/Prss8:PAR2−/− mice were indistinguishable from littermate controls in this regard (Fig. 6d). Surprisingly, heterozygosity for PAR2 was sufficient to reverse the increased water loss and reduced body weight observed in K14-CAP1/Prss8 transgenic mice, despite persistence of ichthyotic and hyperplastic phenotypes (Fig. 6e,d). Moreover, exposure levels of inflammatory markers IL-1α, IL-1β, TSLP and MMP9, as well as of macrophage and dendritic cell marks anti-F4/80 and anti-S100, were indistinguishable from control levels in K14-CAP1/Prss8:PAR2−/− animals (Figs 4c and 6e,f). K14-CAP1/Prss8:PAR2−/− mice manifested an intermediate phenotype, suggesting a dose-dependent effect of PAR2. Thus, absence of PAR2 completely rescued hyperplasia, barrier dysfunction, inflammation and ichthyosis caused by altered CAP1/Prss8 expression in the skin, establishing PAR2 as a pivotal mediator of K14-CAP1/Prss8-driven skin pathology in this model.

Expression of CAP1/Prss8 and PAR2 in human skin. CAP1/Prss8 immunodetection on skin cross-sections from four healthy controls and from five acute, two atopic and three chronic dermatitis patients showed defined localization of CAP1/Prss8 in the granular layer of the human epidermis (Fig. 7) similar to CAP1/Prss8 expression in murine skin4. In healthy controls and acute dermatitis patients, CAP1/Prss8 was localized both intracellularly and at the plasma membrane. In patients with chronic and atopic dermatitis,
CAP1/Prss8 appeared to be predominantly expressed at the plasma membrane. PAR2 expression was seen in suprabasal keratinocytes in the same samples and appeared to be more extensive in chronic and atopic dermatitis patients, following hyperplasia. It is thus conceivable that differential expression of CAP1/Prss8 and/or PAR2 in diseased versus healthy skin may contribute to the pathogenesis of these disorders.

**Discussion**

Altered activity of serine proteases and serine protease inhibitors can severely compromise skin homeostasis in mice and humans. In this study we used mouse models to demonstrate that increased expression of either the membrane-tethered serine protease CAP1/Prss8, or PAR2, a candidate downstream effector, is sufficient to induce skin disease in mice and that CAP1/Prss8-elicited pathogenesis is fully dependent on PAR2.

Transgenic K14-CAP1/Prss8 mice exhibited severe ichthyosis, hyperplasia, epidermal barrier defects and inflammation. Intriguingly, lack of one PAR2 allele associated with CAP1/Prss8 overexpression was sufficient to completely reverse the increased TEWL and body weight loss, but not ichthyosis and hyperplasia, suggesting that barrier dysfunction was not secondary to epidermal hyperplasia. Increased proliferation and TSLP production in primary keratinocytes isolated from K14-CAP1/Prss8 transgenic mice suggested that hyperplasia and inflammation were both, at least in part, directly triggered by a protease signalling cascade in keratinocytes, supporting a cell-autonomous phenotype. TSLP production by epithelial cells has been shown to trigger dendritic cell-mediated effects.

Figure 4 | K14-CAP1/Prss8 mice exhibit itching and severe skin inflammation. (a) Skin injuries with open wounds and lymph node inflammation (haematoxylin and eosin staining, bar represents 20 μm) in a representative 7-month-old transgenic mouse. (b) Dot plot indicating the duration of the scratching behaviour expressed in percentage. Each dot represents a single event per animal. Bars indicate the average of the time spent scratching, P < 0.01. (c) Semiquantitative RT-PCR analysis of TSLP, MMP9, IL-1β and IL-1α (30 cycles) in transgenic versus wild-type skin. The reaction is controlled by detection of β-actin (25 cycles). Water was used as negative control. Data are representative of n = 3 mice per genotype. (d) Giemsa, periodic acid-Schiff staining and s100, F4/80 and CD3 labelling in skin of 2-week-old wild-type and K14-CAP1/Prss8 transgenic mice. Pictures are representative of n = 3 animals per genotype. (e) Quantification of the number of CD3-positive cells in transgenic versus wild-type skin. P < 0.001. (f) FACs analysis of dendritic cells (CD11c + ), macrophages (F4/80 + ) and T cells (CD3 + ) in skin of 6-day-old pups (n = 3 mice per genotype). (g) F4/80 and s100-positive cells in skin of 2-day-old transgenic and littermate controls. Bar represents 20 μm; pictures are representative of n = 3 mice per genotype. (h) Quantification of the number of s100-positive cells in 2-day-old transgenic and littermate animals. n = 3 mice per genotype, *P < 0.05. (i) TSLP quantitative RT-PCR analyses of wild-type and transgenic skin in 2-day-old pups (n =3 mice per group, *P < 0.05, **P < 0.01). (j) TEWL and body weight (k) measurements at P1, P2 and P5 (n=4 mice per group). (l) Quantitative RT-PCR examination of TSLP, IL-1α, IL-1β and MMP9 in transgenic primary keratinocytes, *P < 0.001. (m) Quantification of cell growth assessed in primary keratinocytes derived from 2-day-old transgenic and control littersmates. (i, m) The data are representative of three independent experiments. All data are presented as mean ± s.e.m.
inflammation\(^3\), and it is well established that dendritic cells migrate from inflamed peripheral tissues to the closest draining lymph node to orchestrate adaptive immune responses\(^2\). PAR2 signalling was recently shown to promote dendritic cell trafficking to lymph nodes and subsequent T-cell activation\(^4\). In our K14-CAP1/Prss8 model, CD11c- and S100-positive dendritic cells were found to be more abundant in the skin, supporting a potential role for TSLP expression in immune cell recruitment and activation. Consistent with these findings, CAP1/Prss8 transgenic skin exhibited an increase in CD3-positive T cells, as demonstrated by both immunohistochemistry and FACS quantification. Taken together, these data suggest a possible CAP1/Prss8-PAR2-TSLP cascade in which TSLP, ...
mobilized by CAP1/Prss8-elicited PAR2 activation on keratinocytes, stimulates dendritic cells that then recruit T cells to start an adaptive inflammatory response.

Increased scratching behaviour was documented in both Grhl3<sup>+/−</sup> knock-in and K14-CAP1/Prss8 transgenic mice. Itching sensation can be induced by dry, damaged and inflamed skin, and numerous epithelial and immune mediators can activate and sensitize sensory nerve endings and even modulate their growth (reviewed in ref. 41). However, there is also a body of evidence to suggest that PAR2 can function as a direct mediator of pruritus in humans and mice. Functional PAR2 is present on primary spinal afferents, and proteases might activate PAR2 on sensory nerves in the skin. Importantly, however, we aimed to drive PAR2 expression in epithelial cells, not in sensory nerves, and, as predicted from the expression pattern of Grhl3 (ref. 35), dorsal root ganglia or peripheral nerves are not targeted. Thus, although itching might increase

Table 1 | PAR2 deficiency prevents the K14-CAP1/Prss8-induced phenotype in double-transgenic K14-CAP1/Prss8:PAR2<sup>−/−</sup> mice (line 2).

| Genotype Breeding pairs | K14-CAP1/Prss8 PAR<sup>−/−</sup> | PAR2<sup>−/−</sup> | K14-CAP1/Prss8 PAR2<sup>−/−</sup> | PAR2<sup>−/−</sup> | K14-CAP1/Prss8 PAR2<sup>−/−</sup> | PAR2<sup>−/−</sup> |
|-------------------------|-------------------------------|----------------|-------------------------------|----------------|-------------------------------|----------------|
| K14-CAP1/Prss8 PAR2<sup>−/−</sup>×PAR2<sup>−/−</sup>, n=135 | 18 | 20 | 34 | 31 | 16 | 16 |
| Observed, %            | 13.3 | 14.8 | 25.2 | 23.0 | 11.9 | 11.9 |
| Expected, %           | 12.5 | 12.5 | 25 | 25 | 12.5 | 12.5 |
| K14-CAP1/Prss8 PAR2<sup>−/−</sup>×PAR2<sup>−/−</sup>, n=71 | 12 | 16 | 26 | 17 | – | – |
| Observed, %            | 17 | 23 | 37 | 24 | – | – |
| Expected, %           | 25 | 25 | 25 | 25 | – | – |
| CAP1/Prss8 scaly skin phenotype | – | – | + | – | + | – |
| Animals found dead | 0 | 0 | 0 | 6 | 0 | 0 |

*Pups were analysed during the first 3 weeks after birth. Number of mice for the recorded genotypes is indicated.
we never observed spontaneous tumour formation in CAP1/Prss8 transgenic animals, even when histopathologically followed up until their death. Finally, CAP1/Prss8 has been reported to have biological activity independent of its catalytic triad41,42; hence, we cannot exclude that CAP1/Prss8 elicits a protease cascade leading to PAR2 signalling by a cleavage-independent mechanism.

Besides functioning upstream of PAR2, as demonstrated here, CAP1/Prss8 is known to be involved in the activation of the highly amiloride-sensitive epithelial sodium channel ENaC43. Complete ENaC knockout mice die postnatally because of impaired lung fluid clearance44 and exhibit dehydration similar to skin-specific CAP1/Prss8-deficient animals45,46. Other epidermal effectors of CAP1/Prss8 are filaggrin and occludin, both of which are processed normally in ENaC-deficient skin47,48. We therefore consider a contribution of ENaC to the K14-CAP1/Prss8-mediated skin phenotype as unlikely.

PAR2 has been implicated in inflammatory dermatosis49,50 and two natural mutations of CAP1/Prss8 have been found as causative of skin defects in rodents51. In human epidermis we found that CAP1/Prss8 expression, mainly in the granular layer, appeared more concentrated to the plasma membrane in affected skin from patients with atopic and chronic dermatitis relative to skin from healthy controls, and was accompanied by enhanced PAR2 expression in the epidermis of the same affected subjects. Observations in our transgenic models should prompt further investigation into whether CAP1/Prss8, its inhibitors, PAR2 and its other activators are deregulated in diseased human skin. K14-CAP1/Prss8 and Grhl3<sup>−/−</sup> mice may also serve as models to test the efficacy of protease inhibitors and/or PAR2 antagonists against ichthyosis, pruritus and inflammatory skin disorders.

Methods

Mice and primary cell culture. K14-CAP1/Prss8 transgenic mice were generated by cloning mouse CAP1/Prss8 cDNA (Genbank g.i. 19111159) into the pBHR2(Smal) vector<sup>25</sup> (See Supplementary Methods). The linearized vector was microinjected into B6D2F1 hybrid zygotes. Transgenic lines were maintained in the hemizygous state. Transgenic mice were genotyped by PCR and Southern blot analysis of genomic DNA extracted from tail biopsies<sup>25</sup>. Knock-in mice were generated by targeting 129X1/SvJ-derived E14 ES cells using standard methods, essentially as described for Grhl3<sup>−/−</sup> mice<sup>52</sup> except that Cre DNA was replaced by mouse PAR2 cDNA. Further details of cloning are provided in Supplementary Information. The Grhl3<sup>−/−</sup> phenotype was observed in both C57Bl/6j / 129X1/SvJ mixed and FVB/n strain backgrounds. Mice from both strains were used for the experiments. Experimental procedures and animal maintenance followed federal guidelines and were approved by local authorities.

Mouse epidermal keratinocytes were isolated from pups and cultured in vitro<sup>53</sup>. Cell proliferation was quantified using the Quick Cell Proliferation Testing Solution (GenScript) according to instructions provided by the manufacturer.

Measurement of scratching behaviour. For K14-CAP1/Prss8 mice and littermate controls, simultaneous experiments on three mice in three individual cages were conducted in the absence of investigators. Mice could not see each other during the experiment. The mice were videotaped for 15–20 min for later quantification of scratching behaviour. One scratch was considered as lifting of a limb towards the body with subsequent replacement of the limb on the bedding. For Grhl3<sup>−/−</sup> mice and littermate controls, mouse behaviour was recorded in the absence of investigators and the time spent grooming and/or scratching quantified in a 5 min time period and expressed as percentage of total time.

Immunofluorescence and histology. For paraffin-embedded sections: Slides were incubated with primary antibodies for 1 h at room temperature following rinsing with PBS. Antibodies against keratin-14, keratin-1, keratin-6, involucrin, loricrin and filaggrin were purchased from Covance and diluted 1:1,000 or 1:4,000 (keratin-14). Affinity-purified CAP1/Prss8 rabbit anti-mouse antiseraum<sup>54</sup> was diluted 1:200. Rabbit polyclonal antibody PAR2 (H-99; sc-5997) was provided by Santa Cruz Biotechnology and diluted 1:1,000. Slides were rinsed three times for 10 min in PBS containing 0.2% gelatin and 0.05% saponin at room temperature and the secondary antibody (Alexa Fluor 488, diluted 1:5,000) was diluted in 0.1% BSA, 0.3% Triton X-100 in PBS. Nuclei were counterstained with 4,6-diamidino-2-
phenylindole (DAPI) 0.2 μg/ml−1 in mounting media (Dako Schweiz AG). Staining was visualized using an LSM confocal microscope (LSM 510 Meta, Carl Zeiss MicroImaging Inc.). For cryosections: Tissue-Tek OCT-C. Embedded skin was fixed in 2% paraformaldehyde (PFA) in PBS and permeabilized with 0.1% SDS in PBS. After blocking with 2% BSA and 3% normal goat serum (NGS), sections were incubated with the keratinocyte-specific isoforms (Dako) for 3 h, followed by a 45 min incubation with rabbit anti-mouse CD3 antibody (552724, BD Pharmingen, dilution 1:100) for 1 h. LaC2 staining: LaC2 expression was detected by incubating the tissue at 30 °C overnight in 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM magnesium chloride, 0.002% NF-1, 0.01% sodium deoxycholate, PBS, pH 7.8 (ref. 38). Giemsa: Parafin sections were dehydrated and stained for 7–10 min at 77 °C. Giemsa stain solution and subsequently rinsed in distilled water. The sections were differentiated with 0.5% aqueous acetic acid and subsequently rinsed thereafter. For periodic acid Schiff staining: Paraffin sections were dehydrated, stained with periodic acid 0.5% for 10 min, washed and stained again with Schiff reagent for 5 min. After dehydration and xylene clearing, slides were mounted and visualized.

Horse-radish peroxidase staining and TUNEL analysis

For horse-radish peroxidase staining. Endogenous peroxidase activity was inhibited by incubating sections for 15 min in 1% H₂O₂/methanol. Sections were then boiled in 10 mM citrate (pH 6) for 15 min for antigen retrieval, rinsed in Tris-HCl pH 7.6, and blocked with normal goat serum (1:10) for 5 min. Primary antibodies (α-smooth muscle actin clone A45-B7, Dako M72499, rat anti-mouse F4/80, Invitrogen MF48000 and S100 (Dako Z0311) antibodies were diluted 1:50, 1:20 and 1:1000, respectively, in Tris-HCl, pH 7.6, 0.5% BSA and applied to sections for 1 h at room temperature. Following rinsing, specific binding was revealed by incubating with horse-radish peroxidase (HRP)–conjugated goat anti-rat polyclonal antibody (1:200) for 30 min and subsequent incubation with 50-fold diluted HRP substrate (DAB; DAKO) for 7 min. Nuclei were counterstained using Harris haematoxylin. For TUNEL analysis: Following deparaffinization and dehydration, sections were pretreated with proteinase K (20 μg/ml−1 in PBS, 30 min) at room temperature. PBS-rinsed sections were preincubated in TdT reaction buffer (25 mM Tris-HCl, 200 mM sodium cacodylate, 0.25 mg/ml−1 BSA, 1 mM cobalt chloride) for 10 min and incubated for 1–2 h at 37 °C in a humidified chamber. The reaction was stopped by incubation with standard 300 mM NaCl and 30 mM sodium citrate for 10 min, and detection was performed with streptavidin–HRP in PBS for 30 min. Nuclei were counterstained with Gill’s haematoxylin. Slides were dehydrated and covered with mounting medium. Positively stained cells were counted from three animals independently analysed per group. A minimum of three random pictures per section were taken using an Axio HRC (Carl Zeiss MicroImaging Inc.)

FACS analysis. Single cell suspensions were prepared from postnatal day-6 ventral skin22. After washing in PBS/0.5% FCS, the cells were stained for flow cytometry using standard procedures. The following monoclonal antibody conjugates were used to define cell subsets: CD45–Alexa 700 (30.2H12), CD11c–APC (N418), CD3–PE (17A2), F4/80–APC-eFluoro 780 and TCRVγ3–FITC (536). All were purchased from eBiosis, except TCR Vγ3–FITC, which was purchased from BD Pharmingen. DAPI (Invitrogen) was used for dead cell discrimination. Samples were analysed on an LSR II Flow Cytometer (Becton Dickinson) equipped with 488, 407 and 640 lasers and data analysed with FlowJo software (TreeStar).

Electron microscopy. Skin biopsy samples were minced to < 0.5 mm−1, fixed in modified Karnovsky’s fixative overnight and postfixed in either 0.2% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide. After fixation, samples were dehydrated in a graded ethanol series, and embedded in an Epon–epoxy mix. After selection of sites with perpendicular cross-sectioning in 1 mm toluidine blue-stained, epoxy-embedded sections, ultrathin sections were examined, with or without further contrasting with lead citrate, using a Zeiss 10A electron microscope (Carl Zeiss), operated at 60 kV.

Measurement of TEWL. The rate of TEWL from the ventral skin of preshaved, 2-week-old, anaesthetized transgenic and littermate controls was measured using a Tewameter TM210 (Courage and Khazaka). Measurement of TEWL from newborns was removed at autopsy, frozen and stored at −20 °C until further treatment. Stratum corneum lipids were homogenized, lyophilized and weighted. Epidermal lipids were extracted for 24 h at 37 °C in solvent mixtures (chloroform/methanol/water)9. Statistical analysis. All data were expressed as mean ± s.e.m. Individual groups were compared using the Wilcoxon Mann–Whitney test, except for lipid analyses for which the Welch Two Sample Student’s t-test was applied. A level of P<0.05 was considered statistically significant for all comparisons; *P<0.05, **P<0.01 and ***P<0.001.

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Author contributions
S.F. conducted most of the experiments. E.C. and S.R. designed and performed Grhl3’ generation and characterization. G.C., S.R., M.M., R.-P.C., F.B., J.-C.S., B.B., K.S., A.W., M.H. and S.M. conducted and assisted in experiments. S.F., E.C. and E.H. wrote the manuscript. E.H. designed and supervised the study.

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