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The Requirement for Perp in Postnatal Viability and Epithelial Integrity Reflects an Intrinsic Role in Stratified Epithelia

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Abstract

Mice lacking the desmosome protein Perp exhibit blistering in their stratified epithelia and display postnatal lethality. However, it is unclear if these phenotypes are strictly related to Perp function in stratified epithelia, as *Perp* expression is not restricted to these tissues during embryogenesis, and certain desmosomal blistering diseases such as *pemphigus vulgaris* and *pemphigus foliaceus* have non-cell-intrinsic bases. Furthermore, we show here that Perp is expressed in the heart, raising the possibility that defects in heart function could account for lethality in the *Perp*-deficient mice. To determine conclusively if Perp function in stratified epithelia is crucial for postnatal survival and epithelial adhesion, we specifically ablated Perp in stratified epithelia by breeding conditional *Perp* knockout mice to keratin 5 (K5)-Cre transgenic mice. We found that the majority of mice lacking Perp in stratified epithelia die within 10 days after birth, accompanied by blistering and hyperproliferation in the epithelia, similar to the constitutive *Perp* null mice. Together, these findings indicate that Perp's requirement for both viability and epithelial integrity reflects a role in the stratified epithelial compartment.

INTRODUCTION

Desmosomes are cell–cell adhesion junctions important for the structural integrity of epithelia (Green and Gaudry, 2000). Desmosome complexes form when the desmosomal cadherins, desmoglein and desmocollin, participate in heterotypic interactions that bring the plasma membranes of adjacent cells in close apposition. The desmosomal cadherins nucleate cytoplasmic complexes of plakoglobin, plakophilin, and desmoplakin, in a structure known as the desmosomal plaque. Through desmoplakin, the plaque connects to the keratin cytoskeleton to form a supracellular network that confers strength and resiliency on an epithelium. In addition to their critical function in epithelial integrity, desmosomes are essential in other tissues subject to mechanical stress, including the heart.

The central role of desmosomes in tissue integrity has been appreciated through studies of human diseases attributed to compromised desmosomal function and knockout mice deficient for specific desmosomal proteins. In the absence of proper desmosome function, phenotypes can manifest themselves either in stratified epithelia or in other tissues such as the heart. For example, mice lacking desmoglein 3 or desmocollin 1 display blistering in stratified epithelia,

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CONFLICT OF INTEREST
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and mice lacking plakoglobin or plakophilin 2 die from heart defects (Bierkamp *et al*., 1996; Ruiz *et al*., 1996; Koch *et al*., 1997; Chidgey *et al*., 2001; Grossmann *et al*., 2004). In humans, mutations in plakophilin 1 are associated with epidermal fragility, whereas mutations in desmoglein 1 or desmoplakin are associated with striate palmoplantar keratoderma (McGrath *et al*., 1997; Norgett *et al*., 2000; Wan *et al*., 2004). In addition, mutations in plakophilin 2, desmoplakin, and plakoglobin are linked to ARVC (arrhythmic right ventricular cardiomyopathy), in which individuals succumb to sudden cardiac death (Gerull *et al*., 2004; Cheong *et al*., 2005). Additional human diseases that have been particularly informative for understanding desmosome function in tissue integrity include the autoimmune diseases pemphigus foliaceus and pemphigus vulgaris, in which antibodies are produced that inactivate the desmosomal cadherins desmoglein 1 or desmoglein 3, respectively, resulting in blistering (Payne *et al*., 2004). As these desmosomal diseases result from an immune reaction, corticosteroid treatment is typically employed to prevent the lethality originally observed with these disorders. Hence, inactivation of desmosomal function can occur through multiple mechanisms, ensuing from either direct dysfunction in epithelia or through non-cell-intrinsic mechanisms, and desmosomal defects can affect both stratified epithelia and other organs.

Recently, the Perp tetraspan membrane protein was shown to be essential for desmosomal adhesion in stratified epithelia (Ihrie *et al*., 2005). *Perp* null mice die neonatally with blisters resembling those seen in pemphigus-related diseases. Perp localizes to desmosomes, and desmosome structure and assembly are perturbed in the skin from *Perp*-deficient mice. However, although Perp is robustly expressed in stratified epithelia, its expression is not restricted to these epithelia, as suggested by *in situ* hybridization of late-gestation embryos showing RNA expression in cartilage of the developing skeleton and in the heart (Ihrie *et al*., 2005). This localization pattern suggested a potential role of Perp in these tissues, and raised the question of whether the blistering and lethality observed in mice lacking Perp ubiquitously are exclusively attributable to a role in stratified epithelia. Furthermore, the fact that mice lacking other central desmosomal components, including desmoglein 3 and desmocollin 1, display blistering but not lethality suggests the possibility that there could be an additional factor contributing to the lethality in $Perp^{-/-}$ mice (Koch *et al*., 1997; Chidgey *et al*., 2001). Therefore, to systematically establish whether the lethality and the associated phenotypes observed upon Perp deficiency are due to Perp loss in stratified epithelia, we have examined the consequence of inactivation of Perp specifically in these tissues. We find that this tissue-specific Perp ablation recapitulates the phenotypes observed in the germline *Perp* knockout mice, indicating that Perp's primary role is in stratified epithelia.

RESULTS

Perp is expressed in the heart

Perp germline null mice die postnatally with blisters of the oral mucosa and skin (Ihrie *et al*., 2005). However, as epithelial blistering is not necessarily sufficient to cause lethality, it is possible that some other function of Perp is critical for viability. Our previous *in situ* hybridization analysis indicated that during embryogenesis, *Perp* message is present in the heart, a vital organ that depends on desmosome function. To determine if the crucial role for Perp in postnatal viability could relate to a function in the heart, we examined Perp protein expression in the heart of newborn mice. Analysis of Perp protein levels in newborn mice demonstrated that it is indeed expressed in the heart, but not in tissues containing simple epithelia, such as the lung, consistent with our previous findings that Perp is absent from simple epithelia in adult mice (Figure 1a). Through immunohistochemical analysis, we found that Perp localizes to the intercalated discs of cardiac muscle, a site of known function for desmosomes (Figure 1b and c). This expression profile for Perp suggests that Perp could play

a role in the heart and that the observed lethality in its absence could reflect a defect in heart function.

Mice lacking Perp specifically in stratified epithelia display postnatal lethality

To examine whether the postnatal requirement for Perp reflects a direct role in stratified epithelia of the skin and oral mucosa, we sought to delete Perp specifically in these tissues through the use of keratin 5 (K5)-Cre transgenic mice (Ramirez *et al*., 2004). A recent skin carcinogenesis study we performed using adult mice lacking Perp in stratified epithelia suggested that fewer K5-Cre; $Perp^{fl/fl}$ (*fl*, floxed) adults survived than the number expected by Mendelian ratios (Marques *et al*., 2005). To examine this more closely and to determine if deletion of *Perp* in stratified epithelia is sufficient to recapitulate the postnatal lethality and associated phenotypes seen in constitutive *Perp* knockout mice, we crossed K5-Cre transgenic; $Perp^{fl/+}$ males with $Perp^{fl/+}$ females and carefully tracked the viability of progeny after birth. As our crosses have suggested that the K5-Cre transgene is on the same chromosome as the $Perp^{fl}$ mutant allele, we compared the number of K5-Cre; $Perp^{fl/+}$ and K5-Cre; $Perp^{fl/fl}$ survivors, rather than examining K5-Cre; $Perp^{+/+}$ mice, which would only occur as a product of chromosomal recombination. We observed that while K5-Cre; $Perp^{fl/+}$ mice survived, the majority of the K5-Cre; $Perp^{fl/fl}$ mice die before postnatal day 10 (Figure 2a). The kinetics of lethality are similar to those seen with *Perp* constitutive knockout mice, suggesting that the requirement for Perp in viability is directly related to its expression in stratified epithelia. This idea is supported by the observation that Perp expression in the heart is retained in the K5-Cre; $Perp^{fl/fl}$ mice (Figure 2b). To verify the efficiency of Cre-mediated deletion of Perp in the stratified epithelia of K5-Cre; $Perp^{fl/fl}$ mice, we performed immunohistochemistry using antibodies directed against Perp. We noted a complete absence of Perp expression in all layers of the epidermis (Figure 2c and d). In the tongue and the palate, however, we detected incomplete ablation of Perp expression (Figure 2e–h). We hypothesized previously that the lethality in $Perp^{-/-}$ newborns relates to the loss of Perp function in the oral cavity, which leads to impaired feeding. Therefore, this variable deletion in the tongue of conditional knockout mice could explain the finding that only 62% of the K5-Cre; $Perp^{fl/fl}$ mice die rather than the >95% lethality seen in the constitutive *Perp* null mice.

Stratified epithelia in K5-Cre; $Perp^{fl/fl}$ mice exhibit adhesion defects and enhanced proliferation

At the time of lethality, constitutive $Perp^{-/-}$ newborn mice show numerous defects in the tongue, palate, and skin, including adhesion defects and hyperproliferation. While we had observed signs of blistering in K5-Cre; $Perp^{fl/fl}$ skin (Marques *et al*., 2005), we had not yet examined the oral epithelial compartment. To establish whether defects in the oral mucosa might explain the lethality in newborn K5-Cre; $Perp^{fl/fl}$ mice, we examined these tissues for the presence of lesions. We observed blisters in both the tongue and palate of K5-Cre; $Perp^{fl/fl}$ newborn mice similar to the *Perp* germline null mice (Figure 3b, data not shown). These blisters were detected between the basal and suprabasal layers of the epithelia as well as in the more superficial layers of the epithelia. We also observed separation between individual cells in the skin and oral epithelia, perhaps reflecting incipient acantholysis (Figure 3d, data not shown). Together, these findings are consistent with the idea that compromised integrity of the oral mucosa contributes to postnatal lethality.

A common response to disrupted adhesion is enhanced proliferation. Consistent with this notion, the stratified epithelia in K5-Cre; $Perp^{fl/fl}$ newborn mice appeared thicker than those in K5-Cre; $Perp^{+/+}$ controls (Figure 3a–d). To examine proliferation more directly, we performed immunofluorescence for Ki67, a marker of cycling cells. We detected significantly increased Ki67 positivity in newborn mice lacking Perp in stratified epithelia relative to K5-Cre; $Perp^{fl/+}$ newborns, with a proliferation index similar to that observed in the constitutive

Perp null mice (Figure 3e–g). Moreover, we observed that the interfollicular epidermis in K5-Cre; $Perp^{fl/fl}$ mice expressed keratin 6, a marker typically induced in response to hyperproliferative signals (Figure 3h and i). In contrast, analysis of markers for the basal, spinous, and granular layers of the epidermis demonstrated that terminal differentiation occurred normally in K5-Cre; $Perp^{fl/fl}$ newborn mice, as in constitutive $Perp^{-/-}$ newborn mice (data not shown). Therefore, in all respects examined, the K5-Cre; $Perp^{fl/fl}$ mice replicate the properties of the $Perp^{-/-}$ newborns (Ihrie *et al*., 2005). Together, these data indicate that the changes observed in the skin and oral mucosa upon uniform Perp deficiency ensue as a direct consequence of loss of Perp in stratified epithelia. Moreover, the lack of epithelial integrity evidenced by the presence of blisters supports the idea that an intrinsic adhesion defect, likely in the oral cavity, is the cause of lethality in the absence of Perp.

DISCUSSION

Here, by crossing *Perp* conditional knockout mice with K5-Cre transgenic mice, we examine the consequences of ablating Perp specifically in stratified epithelia. Sixty-two percent of neonatal mice lacking Perp in these epithelia die within 10 days after birth, with similar kinetics to the *Perp* germline null mice, indicating that Perp function in stratified epithelia is important for viability. Like $Perp^{-/-}$ mice, the lethal phenotype is incompletely penetrant, indicating a stochastic element. In the case of $Perp^{-/-}$ mice, however, a greater proportion (>95%) of the mice show lethality. We hypothesize that this difference in the extent of lethality is due to variegated Cre expression, as staining for Perp indicates that K5-Cre-driven deletion of the *Perp* locus in the oral cavity is variable. If lethality is accounted for by impaired feeding ensuing from lesions in the oral mucosa, it may be that some of these K5-Cre; $Perp^{fl/fl}$ mice retain sufficient Perp to survive. Alternatively, variations in genetic background could explain the differences in the extent of lethality observed in constitutive and conditional *Perp* knockout mice.

We previously observed that *Perp* message is expressed in compartments other than stratified epithelia during embryogenesis, including the heart. We hypothesized that ablating Perp in the heart might result in lethality from impaired cardiac function, as in the case of deficiencies in other desmosome proteins in both mice and humans (Bierkamp *et al*., 1996; Ruiz *et al*., 1996; Norgett *et al*., 2000; Grossmann *et al*., 2004). Our data here indicate, however, the crucial function of Perp in the stratified epithelial compartment. Specifically, our results rule out both heart-related lethality and an autoimmune-based mechanism for lethality, as in the pemphigus diseases. An essential role for Perp in stratified epithelia, however, does not exclude the possibility that it may also be important in other contexts, including the heart, and that Perp loss in the heart may have more subtle consequences that affect long-term survival. In fact, individuals with certain mutations in desmoplakin display a host of symptoms, including striate palmoplantar keratoderma in the skin, as well as arrhythmic right ventricular cardiomyopathy, a disease of the heart in which individuals undergo sudden cardiac arrest (McKoy *et al*., 2000; Norgett *et al*., 2000). Similarly, mutations in plakoglobin or plakophilin 2 are associated with this heart condition (Gerull *et al*., 2004; Cheong *et al*., 2005). Thus, mice deficient for Perp in the heart may succumb to heart failure upon aging, a possibility that can be addressed in the future by inactivating Perp in the heart using a heart-specific Cre transgenic strain.

A number of mice lacking specific desmosomal components, or associated intermediate filament constituents, have been generated (Bierkamp *et al*., 1996; Ruiz *et al*., 1996; Koch *et al*., 1997; Gallicano *et al*., 1998; Chidgey *et al*., 2001; Peters *et al*., 2001; Wong and Coulombe, 2003; Grossmann *et al*., 2004). While desmoglein 2, plakoglobin, desmoplakin, and plakophilin 2 are essential for embryonic development, the requirement for other components is less stringent. For example, desmoglein 3 knockout mice display oral blistering and are runted from an apparent difficulty in feeding, but do not typically show lethality (Koch *et al*.,

1997). Similarly, mice lacking another desmosomal cadherin, desmocollin 1, manifest flaky skin and acantholysis in the granular layer of the skin, without oral blistering, but also do not show lethality (Chidgey *et al*., 2001). In contrast, disruption of both keratin 6 isoforms, α and β, leads to severe blistering in both the tongue and the palate of knockout mice, which causes death within a week after birth, likely due to feeding difficulties (Wong *et al*., 2000). The differences in the penetrance of lethality in these various mouse mutant strains may reflect differences in the genetic background of the mice analyzed. Alternatively, the absolute requirement for different desmosome components or associated keratins may vary, with some proteins performing such a central function that their loss results in lethality, and in other cases, playing a less essential part. This could relate to compensation by family members: specifically, for each desmoglein and desmocollin, there are at least three different related gene products (Green and Gaudry, 2000). Our results highlight the crucial role for Perp in stratified epithelia, which likely relates to the fact that Perp is highly expressed throughout the stratified epithelium and that there is no clear related protein that could substitute in its absence (Ihrie *et al*., 2005).

Establishing a clear intrinsic role for Perp in stratified epithelia provides important insight for the future identification of human diseases related to *Perp* inactivation. Like the desmogleins, Perp is a membrane protein and theoretically might be susceptible to attack by autoantibodies in human pemphigus syndromes. Blocking Perp function in stratified epithelia may be expected to be lethal, given its central role in the skin and oral mucosa of the mouse, and would therefore necessitate systemic immunosuppressant treatment for patients. Alternatively, *Perp* may be the target of mutation in blistering disorders of unknown cause. However, such diseases would be unlikely to result from recessive, loss-of-function mutations, as the complete absence of Perp would also likely cause a lethal phenotype in humans. Rather, blistering diseases could be due to partial loss-of-function or dominant, gain-of-function mutations in *Perp*. Future analyses will reveal the exact role of Perp in human skin integrity and disease.

MATERIALS AND METHODS

Mouse work

Perp conditional knockout and K5-Cre transgenic mice have been described (Ramirez *et al*., 2004; Marques *et al*., 2005). Mice used in these experiments were on a mixed 129/Sv/C57BL/6 genetic background. All animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

Histology and immunohistochemistry

Paraffin sections were prepared for immunohistochemical or hematoxylin and eosin staining by standard methods. Immunohistochemistry was performed as described (Ihrie *et al*., 2005).

TUNEL and Ki67 assays

TUNEL and Ki67 assays were performed as described (Ihrie *et al*., 2005). Proliferation was quantified by counting the number of labeled cells as a percentage of total basal cells in each 40× field.

Protein preparation and immunoblotting

For protein extracts, tissues were resuspended in 1% Triton X-100 solubilization buffer plus 9 M urea, as described previously (Ihrie *et al*., 2005). Western blotting was performed according to standard methods, with 40 μg of protein per lane.

Antibodies

Perp antibodies have been described (Ihrie *et al*., 2005). Antibodies against Ki67 (Pharmingen, San Diego, CA), keratin 6 (Covance, Berkeley, CA), and α-tubulin (Sigma, St Louis, MO) were used for immunofluorescence and immunoblotting.

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Abbreviations

fl	floxed
K5	keratin 5

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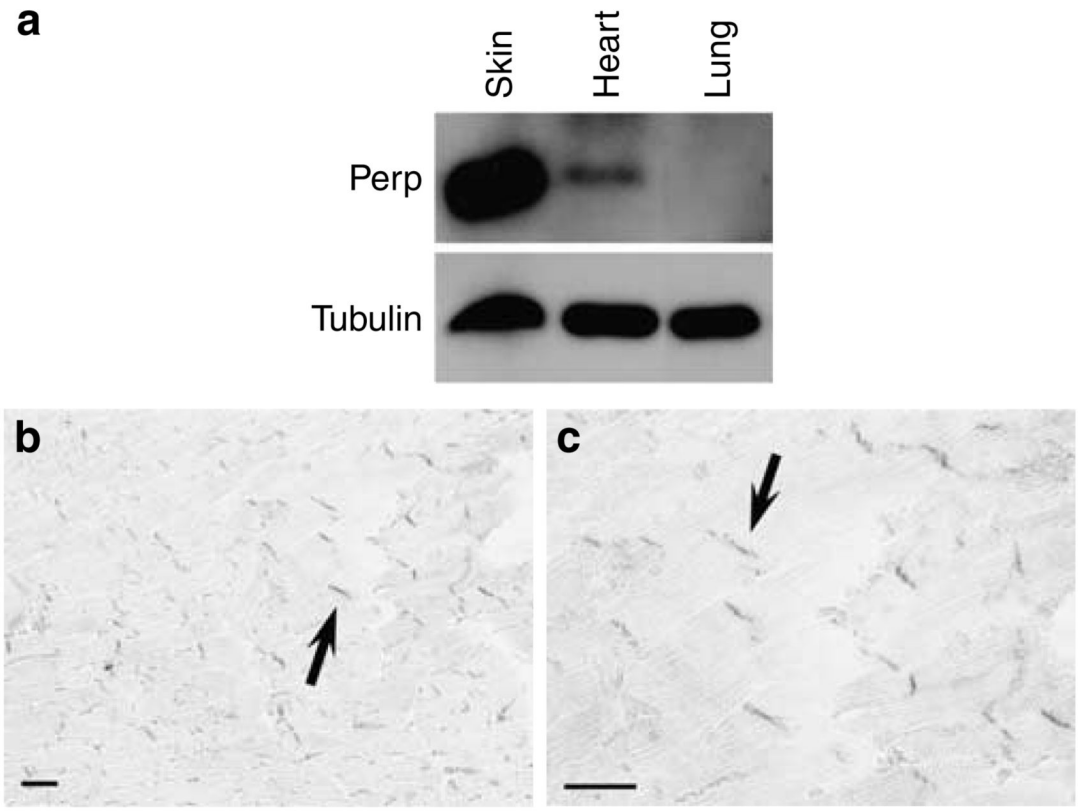

Figure 1. Perp is expressed in the heart

(a) Western blot analysis examining Perp protein levels in newborn tissues (skin, heart, lung). α-Tubulin serves as a loading control. (**b** and **c**) Low- and high-power magnification images, respectively, showing Perp expression (dark precipitate) in the intercalated discs of the heart of adult mice by immunohistochemistry (arrows). Scale bars correspond to 25 μm.

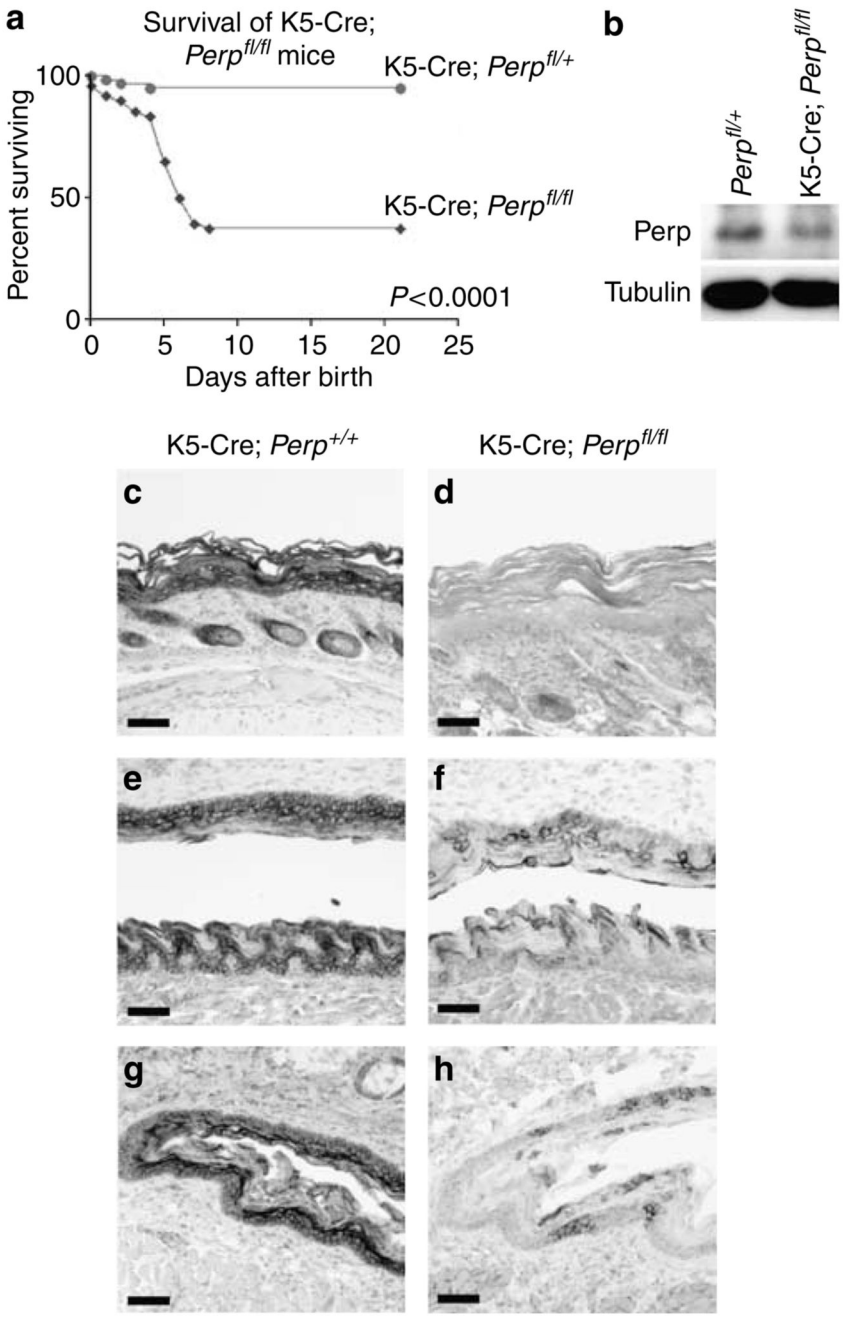

Figure 2. Mice lacking Perp in stratified epithelia display postnatal lethality

(**a**) Kaplan–Meier analysis shows that while K5-Cre; *Perp*$^{fl/+}$ mice survive up to weaning age, many K5-Cre; *Perp*$^{fl/fl}$ mice die within the first 10 days of life. *P* is <0.0001 by the log-rank test. (**b**) Western blot analysis examining Perp protein levels in the hearts of K5-Cre; *Perp*$^{fl/fl}$ and control newborn mice. (**c**-**h**) Perp immunohistochemistry examining the extent of Perp deletion in stratified epithelial tissues of K5-Cre; *Perp*$^{fl/fl}$ newborn mice compared to K5-Cre; *Perp*$^{+/+}$ newborn mice. Scale bars correspond to 25 μm. (**c** and **d**) Perp staining of the dorsal epidermis. (**e** and **f**) Perp staining of a sagittal section of the tongue and palate. (**g** and **h**) Perp staining of a parasagittal section of the posterior pharynx.

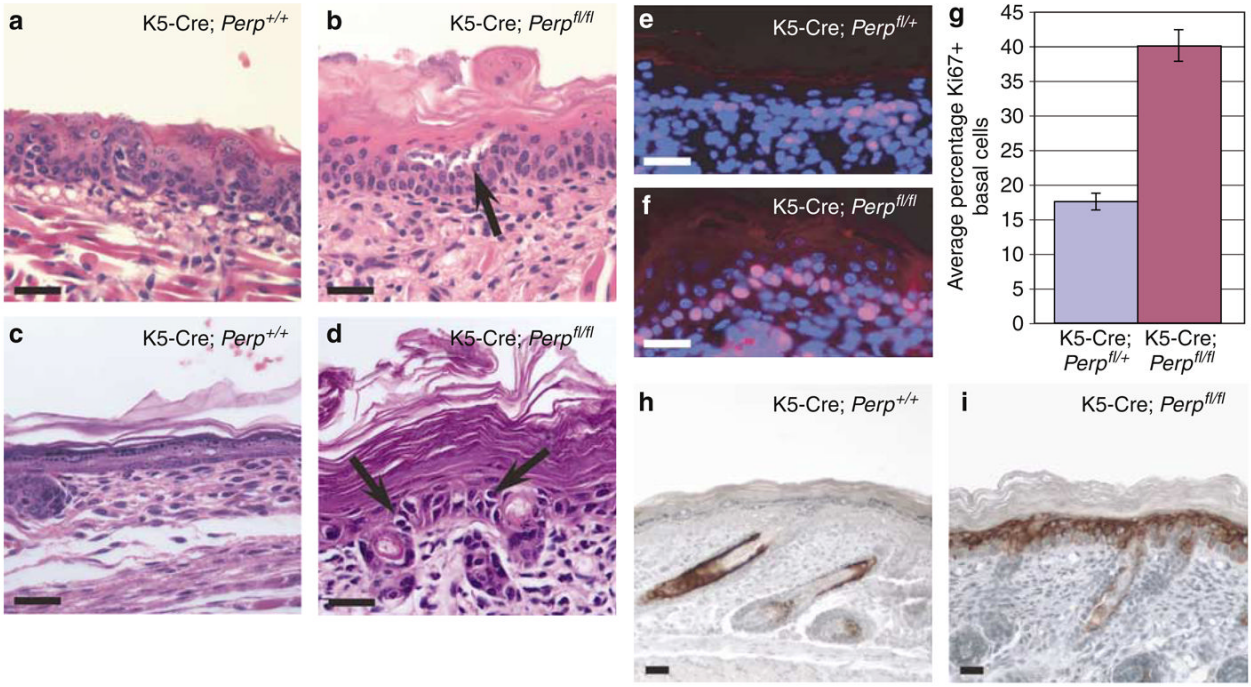

Figure 3. K5-Cre; *Perp*$^{fl/fl}$ mice exhibit blistering and hyperproliferation in stratified epithelia

(**a–f**) Hematoxylin and eosin staining of stratified epithelia from K5-Cre; *Perp*$^{+/+}$ and K5-Cre; *Perp*$^{fl/fl}$ newborn mice. Scale bars correspond to 25 μm. (**a**) Normal newborn tongue. (**b**) Blister in the tongue of a K5-Cre; *Perp*$^{fl/fl}$ newborn (arrow). (**c**) Normal newborn dorsal epidermis. (**d**) Separation of cells in the dorsal epidermis of a K5-Cre; *Perp*$^{fl/fl}$ newborn (arrows). (**e** and **f**) Immunofluorescence against Ki67 examining the proliferation index of basal cells of the epidermis in K5-Cre; *Perp*$^{fl/+}$ and K5-Cre; *Perp*$^{fl/fl}$ newborn mice. Nuclei are marked by DAPI staining. (**g**) Graph displaying the average percentage of Ki67-positive basal cells, ±standard error of mean, in the interfollicular epidermis. (**h**) Immunohistochemistry for K6 in K5-Cre; *Perp*$^{+/+}$ mice shows normal hair follicle staining but no interfollicular epidermis staining. (**i**) Immunohistochemistry for K6 in K5-Cre; *Perp*$^{fl/fl}$ newborn mice shows K6 expression in the interfollicular epidermis and in the hair follicles.