Citing this paper
Please note that where the full-text provided on King’s Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher’s definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher’s website for any subsequent corrections.

General rights
Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain.
• You may freely distribute the URL identifying the publication in the Research Portal.

Take down policy
If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Pelota Regulates Epidermal differentiation by Modulating BMP and PI3K/AKT Signaling Pathways


PII: S0022-202X(16)31141-1
DOI: 10.1016/j.jid.2016.04.020
Reference: JID 315

To appear in: The Journal of Investigative Dermatology

Received Date: 10 August 2015
Revised Date: 4 April 2016
Accepted Date: 11 April 2016


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Pelota Regulates Epidermal differentiation by Modulating BMP and PI3K/AKT Signaling Pathways

Manar Elkenani1,2, Gunsmaa Nyamsuren1, Priyadharsini Raju1, Kifayathullah Liakath-Ali3,4, Aicha Hamdaoui1, Aleksandra Kata1, Ralf Dressel5, Thomas Klonisch6, Fiona M. Watt3, Wolfgang Engel1,*, James A. Thliveris6, D. V. Krishna Pantakani1,7, Ibrahim M. Adham1

1 Institute of Human Genetics, University of Göttingen, D-37073 Göttingen, Germany; 2 Faculty of Medicine, Mansoura University, Mansoura, Egypt; 3 Centre for Stem Cells and Regenerative Medicine, King’s College London, Guy’s Hospital Campus, London SE1 9RT, UK; 4 Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Old Addenbrooke's Site, Cambridge CB2 1GA; 5 Institute of Cellular and Molecular Immunology, University Medical Center Göttingen, D-37073 Göttingen, Germany; 6 Department of Human Anatomy and Cell Science, College of Medicine, Faculty of Health Sciences, University of Manitoba, Winnipeg, R3E 0J9, Manitoba, Canada; 7 Institute of Clinical Chemistry/UMG-Laboratories, University Medical Center Göttingen, D-37075, Göttingen, Germany. *Deceased.

Correspondence: Ibrahim Adham, Institute of Human Genetics, Heinrich-Düker-Weg 12, D-37073 Göttingen, Germany. Phone: +49-551-397522; Fax: +49-551-399303; Email: iadham@gwdg.de

Short title: PELO Regulates Epidermal Differentiation

ABBREVIATIONS: Pelo, Pelota; FLG, filaggrin; Tam, Tamoxifen; EPB, epidermal permeability barrier; BMP, Bone morphogenetic protein; PI3K, phosphoinositide 3-kinase; AKT, Protein kinase B; SC, stratum corneum; CE, cornified envelope.
ABSTRACT

The depletion of evolutionarily conserved Pelota protein (PELO) causes impaired differentiation of embryonic and spermatogonial stem cells. In this study, we show that temporal deletion of PELO prior to epidermal barrier acquisition leads to neonatal lethality due to perturbations in permeability barrier formation. Further analysis indicated that this phenotype is a result of failed processing of profilaggrin into filaggrin monomers, which promotes the formation of a protective epidermal layer. Molecular analyses revealed that PELO negatively regulates the activities of BMP and PI3K/AKT signaling pathways in the epidermis. To address whether elevated activities of BMP and PI3K/AKT signaling pathways were the cause for the perturbed epidermal barrier in Pelo-deficient pups, we made use of organotypic cultures of skin explants from control and mutant embryos at E15.5. Inhibition of PI3K/AKT signaling did not significantly affect the BMP activity. However, inhibition of BMP signaling caused a significant attenuation of PI3K/AKT activity in mutant skin and, more interestingly, the restoration of profilaggrin processing and normal epidermal barrier function. Therefore, increased activity of PI3K/AKT signaling pathway in Pelo-deficient skin might conflict with the dephosphorylation of profilaggrin and thereby affects its proper processing into filaggrin monomers and ultimately the epidermal differentiation.
INTRODUCTION

The skin epidermis serves as the first physical barrier that protects the body from fluid loss and entry of toxic and pathogenic agents. In the mouse, the development of the epidermal barrier is initiated at embryonic day 16.5 (E16.5) and must be accomplished before the end of gestation (Hardman et al., 1998). Disruption or delay in the acquisition of the epidermal barrier is known to result in neonatal lethality (Segre, 2006). The epidermal barrier is maintained by a balance between proliferation of self-renewing stem cells located in the basal layer and differentiation of their daughter cells (Blanpain et al., 2006). These cells initially differentiate from suprabasal, to granular cells and finally become flattened enucleated cells of the stratum corneum (SC). Besides the loss of the nucleus and other organelles, the aggregation of keratin intermediate filaments into macrofibrils and the formation of a cornified envelope (CE) are the major structural changes that occur during the differentiation of granular to cornified cells. The SC contains multiple layers of corneocytes embedded in a lipids matrix, which is responsible for the formation and maintenance of the skin barrier (Kubo et al., 2012). Filaggrin (FLG), a protein associated with late epidermal differentiation, promotes the assembly of keratin intermediate filaments to form macrofibrils in the cornified cells (Dale et al., 1985; Irvine et al., 2011). This process mediates the compaction and structural integrity of the cornified cells (Kubo et al., 2012). FLG is synthesized as a high molecular weight precursor protein called profilaggrin that consists of tandem filaggrin repeats and is stored in keratohyalin granules of granular cells as highly phosphorylated protein (Sandilands et al., 2009). Profilaggrin is dephosphorylated and proteolytically cleaved into filaggrin monomers that are released into the lower layer of the SC. Loss or defective processing of profilaggrin results in different forms of congenital ichthyosis that is essentially characterized by impaired epidermal barrier functions (Thyssen et al., 2013).
**Pelota (Pelo)** is an evolutionarily conserved gene that has been identified in diverse species from archaeabacteria to humans (Shamsadin et al., 2002). The function of PELO at the molecular level has been extensively investigated in yeast, where the PELO-orthologue Dom34 participates in an RNA quality control mechanism called No-Go decay, and in the recycling of ribosomes that are stalled at mRNAs lacking stop codons, truncated mRNAs, or the non-coding regions of many cellular mRNAs (Doma and Parker, 2006; Shoemaker and Green, 2011; Guydosh and Green, 2014).

In mice, Pelo is ubiquitously expressed in various tissues during embryonic development and in the adult stage (Shamsadin et al., 2002). We previously demonstrated the subcellular localization of PELO to the cytoskeleton and at the plasma membrane and identified the interaction of PELO with several cytoskeleton-associated proteins (Burnicka-Turek et al., 2010). Deletion of Pelo in mice results in embryonic lethality at an early post-implantation stage (Adham et al., 2003). To circumvent the early embryonic lethality and to study the role of PELO during embryonic and postnatal life, we generated conditional knockout mice and investigated the effect of Pelo deletion on pluripotency and differentiation of embryonic stem cells and spermatogonial stem cells (Nyamsuren et al., 2014; Raju et al., 2015).

We show here that temporal deletion of Pelo prior to epidermal barrier formation disrupts the acquisition of the proper epidermal permeability barrier (EPB) causing early neonatal lethality. The impaired function of the EPB has been identified as the result of a defect in profilaggrin processing. Further *in vitro* and *in vivo* experiments revealed that the elevated activities of BMP and PI3K/AKT signaling pathways are the cause of the perturbed function of the Pelo-deficient epidermis.
RESULTS

Deletion of Pelo prior to skin barrier formation causes neonatal lethality

Analysis of conditional Pelo knockout mice showed that Pelo deletion between E9.5 and E13.5 leads to neonatal lethality. To test whether the neonatal lethality is due to an impaired formation of the epidermal barrier, pregnant females from Pelo/F-/CreERT2+/− breeding were treated with Tamoxifen (Tam) at embryonic stages E13.5 and E14.5. Although Tam-treated Pelo/F-/CreERT2+/− females gave rise to living offspring, no living Pelo-Δ/Δ newborn pups were found, indicating that Pelo-null pups died immediately after birth. To determine the cause for neonatal lethality of Pelo-deficient mice, E18.5 embryos were delivered by caesarean section and incubated at 37°C. Genotyping and Western blot analyses revealed that Pelo was efficiently ablated in the skin of Pelo-Δ/Δ pups (Figure 1a and 1b). During the incubation time, we observed that Pelo-Δ/Δ skin was erythematous, glossy and sticky to the touch (Figure 1c). In contrast to control pups, the body weight of Pelo-deficient embryos decreased gradually (Figure 1d), and all E18.5 Pelo-Δ/Δ embryos died within 5-6 hours of caesarean delivery. These results suggest that the decrease in body weight of Pelo-Δ/Δ embryos was due to increased trans-epidermal water loss resulting from an impairment of their epidermal barrier.

Neonatal lethality in Pelo-null mice is due to skin barrier defects

To verify that the neonatal lethality of Pelo-null mice is a result of epidermal barrier dysfunction, control and mutant E18.5 embryos were subjected to toluidine blue dye penetration assay. Control embryos exhibited a well-developed epidermal barrier as judged by minimal epidermal penetration of the dye (Figure 1e). In contrast, there was extensive skin penetration of toluidine blue dye in Pelo-Δ/Δ embryos particularly in ventral areas, confirming the impaired skin barrier
formation in \(Pelo^{Δ/Δ}\) embryos (Figure 1e). To investigate the cause of the epidermal barrier defect observed in mutant embryos, we analyzed the histology, differentiation, and proliferation status of the skin of \(Pelo^{Δ/Δ}\) embryos from E15.5 until E18.5. Prior to epidermal barrier formation, the histology of \(Pelo\)-null skin at E15.5 and E16.5 did not reveal any obvious abnormalities compared to control skin (data not shown). However, after barrier acquisition the skin of mutant embryos at E17.5 and E18.5 displayed increased epidermal thickening in some regions and marked decrease of cornified layers (Supplementary Figure S1 online). The proliferation status in control and mutant E18.5 skin was assayed by BrdU incorporation. \(Pelo\)-deficient epidermis exhibited a significant elevation in the number of basal cells that incorporated BrdU as compared to controls (Figure 2a). Immunohistological analysis did not reveal any differences between control and mutant epidermis in the expression of the cell layer-specific marker proteins, basal-K14, subrabasal-K10 and granular cell marker loricrin (Lor) (Figure 2b). Expression of terminal differentiation marker FLG was first observed in control and mutant granular cell layers at E17.5 (Figure 2b). At E18.5, FLG accumulated in layers of the SG and in SC of control skin (Figure 2b). In contrast, in mutant skin FLG was present in all layers of the SG and at low levels in the SC (Figure 2b). The low levels of FLG immunostaining in the SC led us to assess whether there were any alterations in the cornified envelope (CE) of \(Pelo\)-deficient embryos. CEs of control skin were symmetrical and smooth, whereas \(Pelo^{Δ/Δ}\) CEs were rough, irregular and aggregated, indicating an immature state of CEs in the epidermis of mutant embryos (Figure 2c).

**Impaired epidermal barrier acquisition in \(Pelo\)-deficient pups is associated with altered profilaggrin processing**

We investigated whether the altered distribution of FLG in the epidermis of E18.5 \(Pelo^{Δ/Δ}\) pups was due to a defect in FLG processing. As expected, protein blot analysis of epidermal protein
lysates revealed that profilaggrin was processed to filaggrin monomers in the epidermis of E18.5 control embryos (Figure 2d). In contrast, almost no filaggrin monomers were detected in Pelo-null epidermis (Figure 2d). Collectively, these results demonstrated that the Pelo deletion during embryonic skin development results in altered profilaggrin processing, abnormal CE formation and skin barrier defects which could be the underlying cause of early neonatal lethality of PeloΔ/Δ pups.

**Failure of epidermal barrier formation in Pelo-null epidermis is associated with increased activity of BMP and PI3K/AKT signaling pathways**

Expression levels of PELO protein in the epidermis are high prior to barrier acquisition and significantly down-regulated after barrier formation (Figure 3a). These results were further confirmed by studying the expression pattern of Pelo transcripts (Figure 3b). These data indicated that the change in the levels of PELO during epidermal barrier formation is regulated at the transcription level.

We next investigated the expression levels of phosphorylated isoforms of SMAD 1/5 and AKT as indicators of the activity of BMP and PI3K/AKT, respectively, in control and mutant epidermis. Like the expression pattern of PELO, expression levels of pSMAD1/5 were down-regulated after completion of barrier development in control epidermis, but were unchanged in Pelo-deficient epidermis (Figure 3c). Consistent with results showing an essential role of PI3K/AKT signaling in keratinocyte differentiation (O'Shaughnessy et al., 2007), expression levels of pAKT were elevated at the onset of barrier formation at E16.5 and attenuated thereafter in control epidermis (Figure 3d). In Pelo-deficient epidermis, expression of pAKT remained at high levels even after barrier formation (Figure 3d), indicating that PELO deficiency leads to persistent activation of BMP and PI3K/AKT signaling pathways.
To address whether the elevated activities of BMP and PI3K/AKT signaling pathways were the cause of the perturbed epidermal barrier function in Pelo-deficient skin, we performed organotypic cultures with dorsal skin explants dissected from E15.5 embryos. H&E staining of skin explants after two days of culture showed the development of SC in control and mutant skin explants (Figure 4a). In order to determine whether epidermal barrier acquisition was disrupted in Pelo-null skin explants, we examined the diffusion of the fluorescent dye Lucifer yellow in cultured skin explants. The dye was retained in the SC (upper layer) of control explants (Figure 4b), whereas in mutant explants Lucifer yellow diffused through the SC down into the dermal layer (Figure 4b). These results supported our in vivo findings and also indicated that impaired EPB is a result of autonomous defects in Pelo-deficient skin and is not due to systemic defects of PELO deficiency.

To determine whether inhibition of PI3K/AKT signaling can restore epidermal development in mutant explants, E15.5 skin explants were cultured for 2 days in the presence or absence of PI3K inhibitor LY294002. Although LY294002 was able to significantly attenuate pAKT levels in mutant explants, these pAKT levels remained significantly higher than the basal pAKT levels found in control skin explants (Figure 4c). As shown in Figure 4c, LY294002 treatment reduced also the levels of pAKT in control epidermis. Proper skin development critically depends on tight control of AKT activity within the epidermis (O'Shaughnessy et al., 2009). Indeed, our histological analysis revealed a failure of SC development in both control and mutant explants in the presence of PI3K inhibitor (Figure 4a). We also analyzed the effect of LY294002 on BMP signaling in control and mutant skin and noticed a slight increase in the levels of pSMAD1/5 in LY294002-treated skin of both genotypes (Figure 4c).

Next, we addressed whether attenuation of BMP signaling would impact on PI3K/AKT activity and could restore the epidermal barrier function in Pelo-null skin. Skin explants were
cultured in the presence or absence of noggin, a BMP antagonist. The effectiveness of noggin in inhibiting BMP activity was confirmed by a decrease in pSMAD1/5 in control and mutant skin explants (Figure 4d). Interestingly, we observed a significant decrease in the levels of pSMAD1/5 and pAKT in mutant explants that corresponded to basal levels observed in control skin (Figure 4d). Importantly, we observed the development of SC in noggin-treated mutant explants (Figure 4a) and proper functioning of the skin barrier, as indicated by retention of Lucifer yellow in the upper layer of SC (Figure 4b). In contrast, inhibition of BMP signaling in control skin explants disrupted the development of SC (Figure 4a) and affected the EPB, as judged by the diffusion of dye through all epidermal layers and into the dermis of noggin-treated control explants (Figure 4b). In support of these results, levels of FLG monomers were markedly attenuated in noggin-treated control skin explants, whereas profilaggrin processing was restored and filaggrin monomers were readily detected in noggin-treated mutant skin explants (Figure 4e). These results support our conclusion that the restoration of epidermal barrier function by noggin treatment in Pelo-deficient pups is accompanied by proper processing of FLG. These results also establish a direct link between elevated levels of BMP and PI3K/AKT signaling with defective filaggrin processing in Pelo-deficient epidermis.

Epidermal barrier defects in Pelo-deficient mice result in hyperkeratosis and skin inflammation.

To investigate whether impaired barrier function results in increased susceptibility to skin inflammation in adult Pelo−/− mice as showed in other mouse models with a defect in skin barrier acquisition (Boguniewicz and Leung, 2011), we injected 8-week-old PeloF/F, PeloF/+CreERT2 and PeloF/F CreERT2 mice with Tam for 5 subsequent days. By 2-4 months after this treatment, control PeloF/F and Pelo−/+ mice presented a normal skin appearance (Figure 5a), whereas the tail
and ear skin of *Pelo*ΔΔ mice was scaly. In addition, facial and neck areas of *Pelo*ΔΔ mice exhibited alopecia, erythema and ulcerations, which were aggravated over time by increased scratching behavior (Figure 5b). For these reasons, *Pelo*-deficient animals had to be sacrificed once these symptoms appeared to minimize suffering. Histological analysis revealed marked epidermal hyperplasia and a hyperkeratotic stratum corneum in *Pelo*ΔΔ mice (Figure 5c and d). The hair follicles were hyperplastic, consistent with the alopecic phenotype in *Pelo*ΔΔ mice (Figure 5d). Transmission electron microscopic analysis revealed an enlarged cornified epidermal layer with thick, over-compacted corneal sheaths of different electron density exclusively in skin sections of adult *Pelo*ΔΔ (Supplementary Figure S2 online). These corneal sheaths were separated by narrow ragged spaces and corneocytes contained microvesicular inclusions (white arrowheads in b, d) In addition, a massive and diffused inflammatory infiltrate was observed in the dermis of *Pelo*ΔΔ skin as detected by a significant increase in the number of mast cells in these skin sections as determined by TEM (not shown) and staining with Toluidine blue (Figure 5e-g).

To assess whether the inflammatory skin phenotype is a result of impaired keratinocyte differentiation we determined the expression profiles of specific markers of different epidermal layers. The expression of K14 was restricted to basal cells of control epidermis, whereas K14 expression extended into the supra-basal cell layers of mutant epidermis and overlapped with expression of the early differentiation marker K10 (Supplementary Figure S3a-d online). Expression of the late differentiation markers loricrin and FLG revealed increased layers of SG and SC in mutant epidermis (Supplementary Figure S3e-h online), indicating perturbed keratinocyte differentiation to be the cause for the disruption of the epidermal barrier function in *Pelo*ΔΔ mutants.

Next, we addressed the origin of the inflammatory phenotype and investigated whether it correlated with epidermal hyperplasia and scratching behavior in *Pelo*ΔΔ mice. Expression levels
for epidermal *IL-4* and *Tslp*, which are implicated in the pathogenesis of atopic dermatitis (Chan et al., 2001; Demehri et al., 2009), were dramatically increased in *Pelo*<sup>ΔΔ</sup> epidermis compared to that of *Pelo*<sup>F/F</sup> mice (Supplementary Figure S3i and j online). In addition, mutant mice also showed increased serum IgE levels when compared to control mice (Supplementary Figure S3k online).
DISCUSSION

In the present study, we showed that the loss of PELO prior to epidermal barrier acquisition results in neonatal lethality. Disruption of barrier formation in Pelo-deficient pups is due to defective processing of profilaggrin into filaggrin monomers that occurs during the terminal differentiation of corneocytes (Sandilands et al., 2009). *In vivo* and *in vitro* studies demonstrated elevated activities of BMP and PI3K/AKT signaling pathways in Pelo-deficient epidermis suggesting that PELO regulates epidermal barrier acquisition through the orchestration of both signaling pathways. Further studies revealed that the disruption of epidermal barrier formation as a consequence of PELO depletion in adult life leads to dermal infiltration by immune cells and epidermal hyperplasia.

A general decrease in phosphorylation of profilaggrin is thought to be required before its proteolytic processing into filaggrin monomers (Resing et al., 1993). PI3K/AKT signaling mediates the phosphorylation of many proteins including proteins that are involved in epidermal barrier acquisition (O'Shaughness et al., 2007; O'Shaughnessy et al., 2009). Our observations led us to suggest that the significant elevation of pAKT in PELO-depleted epidermis might counteract the dephosphorylation of profilaggrin and thereby negatively affect its processing. Expression analysis of PELO as well as activity levels of BMP and PI3K/AKT signaling pathways during epidermal barrier formation of control embryos showed similar kinetics. Further results showed a significant elevation in the activity of both signaling pathways in the absence of PELO, leading us to the conclusion that PELO negatively regulates BMP and PI3K/AKT signaling pathways during epidermal barrier development. Inhibition of BMP signaling caused a significant attenuation of PI3K/AKT activity in mutant skin highlighting that PELO, either directly or indirectly, initiates the modulation of BMP activity which subsequently regulates
PI3K/AKT signaling. Several lines of evidences revealed that BMP signaling regulates the PI3K/AKT activity in the development of different tissues (Ghosh-Choudhury et al., 2002; He et al., 2004; Sui et al., 2009). Regulation of PI3K/AKT signaling by PELO has been demonstrated by results showing that PELO antagonizes the binding of the p85 regulatory subunit of PI3K to tyrosin kinase receptors, hence, attenuating PI3K/AKT signaling in tumor cell lines (Pedersen et al., 2013).

Impaired development of SC in control and mutant skin explants as a result of inhibition of PI3K/AKT signaling is consistent with a previous report which showed that the down-regulation of PI3K/AKT activity in double null mice for Akt1 and Akt2 disrupts the development of the SC and causes neonatal lethality (Peng et al., 2003). Although the levels of pAKT in mutant skin were attenuated by LY294002 treatment, the development of epidermis was not restored. These results suggest that loss of PELO may affect other signaling pathway(s) necessary for normal skin development.

In contrast to the effect of AKT inhibition, cultures of mutant skin explants with the BMP antagonist noggin resulted in the restoration of epidermal permeability barrier as judged by the retention of the Lucifer yellow fluorescent dye in the upper layer of the SC. At the molecular level, treatment of mutant skin explants with noggin led to a significant decrease in the levels of pSMAD and pAKT, to levels seen in untreated control epidermis. Collectively, these results suggest that an adequate threshold level of BMP activity is required for proper acquisition of epidermal barrier functions. Certainly, it remains to address how PELO modulates the activities of both signaling pathways during epidermal barrier development.

Mutations in either the profilaggrin gene or genes encoding for proteins regulating the proteolytic processes of FLG monomers have been identified as the cause of ichthyosis vulgaris (IV) and are the major predisposing factors for atopic dermatitis (AD) (Sandilands et al., 2009;
Agrawal and Woodfolk, 2014). Defective processing of profilaggrin in the epidermis of Pelo-null mice and similarities of epidermal defects in skin of Pelo-deficient mice with those in IV and AD patients led to speculation that mutations in PELO may represent another predisposing factor for IV and AD. The screening of patients with AD is required to address this.

The proper development and maintenance of the epidermal permeability barrier requires equilibrium between the proliferation of keratinocytes in the basal layer and the coordinated differentiation of corneocytes (Hsu et al., 2014). We showed that the defective barrier function in Pelo-deficient mice is associated with a significant increase in cell proliferation in the basal layer of epidermis and defective terminal differentiation of keratinocytes. Both phenotypes are likely to arise from increased activities of PI3K/AKT and BMP signaling pathways and might be responsible for the perturbation of epidermal barrier acquisition.

In conclusion, we identified PELO as a modulator of the BMP-AKT signaling axis which promotes, either directly or indirectly, epidermal barrier formation.
MATERIAL AND METHODS

Mice

The generation of conditional Pelo knockout (Pelo\textsuperscript{\textDelta/\textDelta}) mice was described elsewhere (Nyamsuren et al., 2014). To inactivate Pelo before the development of the skin barrier, pregnant Pelo\textsuperscript{F/F} Cre\textsubscript{ERT2} and control Pelo\textsuperscript{F/F} mice were injected intra-peritonally with tamoxifen (Tam) at embryonic stages E13.5 and E14.5 as described previously (Raju et al., 2015). To delete Pelo in 8-week-old Pelo\textsuperscript{F/F}, Pelo\textsuperscript{F+/+} Cre\textsubscript{ERT2} and Pelo\textsuperscript{F/F} Cre\textsubscript{ERT2} mice, animals were injected with 1 mg Tam for 5 consecutive days. Skin samples were prepared from Tam-treated mutant Pelo\textsuperscript{\textDelta/\textDelta} and control Pelo\textsuperscript{F/F} embryos at E15.5, E16.5, E17.5 and E18.5 and from adult animals after 2-3 months of Tam injection. To detect Cre-mediated Pelo recombination, a piece of tail was genotyped by polymerase chain reaction (PCR) as previously described (Nyamsuren et al., 2014). All experiments performed in this study comply with EU regulation and were approved by the Institutional Animal Care and Use Committee of Medical University of Göttingen.

Histological and immunohistochemical analysis

Histology and immunohistochemical analyses are described in Supplementary Materials online.

Transmission electron microscopy

Control and mutant 8-week-old mice were i.p. injected with TAM. After 2 months of Tam administration, mice were sacrificed and skin samples were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) for 3 hours followed by an overnight rinse in 0.1 M phosphate buffer containing 5% sucrose (pH 7.3). Tissues were then post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3) for two hours, dehydrated in ascending concentrations of alcohol and embedded in Epon 812 (Luft, 1961). Transmission electron microscopy (TEM) was performed on ultra-thin sections (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate. Images were taken with a Philips CM10 instrument at 80 kV.
Skin permeability staining

An *in situ* dye permeability assay with toluidine blue was performed using *Pelo*\(^{+/+}\) and *Pelo*\(^{N/d}\) embryos at E18.5 as previously described (Hardman et al., 1998).

**Proliferation assay**

Cell proliferation was measured by intra-peritoneal injection of pregnant mice at gestational day 18.5 with 50 mg/kg BrdU and the embryos were sacrificed 2 h later. Skin sections prepared as described in Supplementary Materials online were incubated with rat anti-BrdU antibody (1:500; Abd seroTec, Puchheim, Germany). The number of BrdU-positive nuclei was counted at x200 magnification in 5-7 microscopic fields with skin samples from 4 different embryos per genotype.

**Immunoblotting**

Skin samples were harvested for protein isolation and Western blots were performed as described in Supplementary Materials online.

**Cornified envelope preparation**

Isolated epidermis from E18.5 embryos was cut into 5 mm\(^2\) and CE was prepared as described in Supplementary Materials online.

**Embryonic epidermal explant culture**

Organotypic cultures of embryonic skin were prepared as previously described (O'Shaughnessy et al., 2009) and details are listed in Supplementary Materials online.

**RNA preparation and real time PCR**

Methods of RNA isolation and real time PCR were described in Supplementary Materials online.

**Enzyme-linked immunosorbent assay (ELISA) for IgE**

A sandwich ELISA was used for the determination of IgE in serum as described in Supplementary Materials online.
Statistical analyses

Data were expressed as mean ± SEM. Differences among groups were tested by the Student’s *t* test or two-way ANOVA test when appropriate. A *P* value < 0.05 was considered to be significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary materials are linked to the online version of the paper.
REFERENCES


Figure Legends

Figure 1: Temporal deletion of Pelo leads to perturbation of the EPB and early neonatal lethality. (a) Genotyping PCR analysis of E18.5 embryos. The presence of a 455-bp fragment of $Pelo^\Lambda$ allele ($\Lambda$) and loss of a 376-bp of $Pelo^F$ allele (F) in mutant embryos demonstrates successful Cre-mediated recombination. (b) Western blot analysis of epidermis lysates from E18.5 $Pelo^{F/F}$ and mutant $Pelo^{\Lambda\Lambda}$ pups shows efficient loss of PELO in $Pelo^{\Lambda\Lambda}$. For loading control, the blot was stripped and reprobed with anti $\alpha$-tubulin antibody (TUB). (c) Gross morphology of E18.5 $Pelo^{F/F}$ and $Pelo^{\Lambda\Lambda}$ pups is shown. (d) Graph showing the weight loss of $Pelo^{F/F}$ ($n = 4$) and $Pelo^{\Lambda\Lambda}$ embryos ($n = 6$) over the indicated time period. Body weight over the time is presented relative to that of 0 h. Each value represents mean ± SEM. *$P < 0.05$ compared with control. (e) Skin permeability assay on E18.5 $Pelo^{F/F}$ and $Pelo^{\Lambda\Lambda}$ embryos using toluidine blue.

Figure 2: Aberrant processing of profilaggrin in Pelo-deficient epidermis. (a) Epidermal proliferation was assayed in skin sections of E18.5 $Pelo^{F/F}$ and $Pelo^{\Lambda\Lambda}$ embryos by immunostaining using antibodies to BrdU and K14, a basal cell-specific marker. Histogram showing the percentage of BrdU-positive nuclei in basal layer relative to total number of K14-immunopositive cells (right panel). More than 400 K14-positive cells were examined from 3 pups of each genotype. Nuclei (blue) were stained with DAPI. Values are expressed as mean ± SEM. *$P < 0.05$. (b) Immunofluorescent staining for expression of epidermal K10, K14, Lor and FLG in skin sections from E17.5 and E18.5 embryos. (c) Morphological appearance of purified cornified envelopes. (d) Western blot analysis for profilaggrin processing in E18.5 $Pelo^{F/F}$ and $Pelo^{\Lambda\Lambda}$ epidermal extracts. The positions of profilaggrin intermediates (2x and 3x FLG) and filaggrin monomers (1x FLG) and molecular weight markers (kDa) are indicated. Bars = 40 µm (a), 100 µm (b) and 200 µm (c).
Figure 3. Loss of PELO elevates the activity of BMP and PI3K/AKT signaling pathways. (a) Expression of PELO in epidermal extracts of control embryos during skin barrier acquisition. Epidermal protein extracts from mutant embryos were used to verify the specificity of the anti-PELO antibody. On the right panel, PELO levels normalized to α-tubulin (TUB) are presented as mean ± SEM (n = 3). A.U. indicates arbitrary units. Protein levels in the epidermis of E15.5 were expressed as 1.0. *P < 0.05 compared with E15.5. (b) Expression of Pelo mRNA at different stages was determined by qRT-PCR. Values of Pelo expression levels normalized to Hprt are presented as mean ± SEM of three experiments. Transcript levels in the epidermis of adult mice are expressed as 1.00. *P < 0.05 compared with adult. (c, d) Western blot analysis showing the expression levels of pSMAD1/5 (c) and pAKT (d) at different stages (left panels). On the right panels, expression levels of pAKT and pSMAD1/5 normalized to that of total AKT and α-tubulin, respectively, are presented as mean ± SEM (n = 3 per stage). *P < 0.05.

Figure 4. Noggin treatment restores the functional EPB in mutant skin organotypic culture. (a) Histological analysis of E15.5 Pelo<sup>F/F</sup> and Pelo<sup>∆/∆</sup> skin explants before (d0) and after culture for 2 days (d2) in the presence (+) and absence (-) of LY294002 or Noggin. (b) Micrographic images showing Lucifer Yellow dye (green) applied on skin explants. Sections were counterstained with DAPI (blue). (c, d) Western blot showing pAKT and pSMAD1/5 expression in skin explants cultured either with (+) or without (-) PI3K inhibitors LY294002 (c) or recombinant Noggin (d). Expression levels of pAKT and pSmad1/5 were normalized to that of total AKT (AKT) and α-tubulin (TUB), respectively, and presented as mean ± SEM (n = 6). Protein levels in untreated skin explants from control embryos were expressed as 1.0. *P < 0.05, **P < 0.001, ***P < 0.0001. (e) Protein blot analysis for profilaggrin processing in skin explants cultured in either presence (+) or absence of Noggin (-). Filaggrin monomers are labeled by arrowhead. Bars = 20 µm (a) and 40 µm (b).
Figure 5. Depletion of PELO during mouse adult life displays atopic dermatitis-like phenotypes. (a, b) Gross morphology of 5-month-old control (a) and mutant (b) mouse after 3 months of Tam administration. Alopecia is noticed in the neck region of mutant mice (insert in b). (c, d) Hematoxylin/eosin-stained skin sections from the neck region of control (c) and mutant mice (d). (e, f) Toluidine blue-stained sections of Pelo^{F/F} (e) and Pelo^{∆/∆} (f) skin. Arrowheads indicate mast cells with intense blue color in the dermis. (g) The number of mast cells in the skin sections of Pelo^{F/F} and Pelo^{∆/∆} animals is represented as mean ± SEM. *P > 0.05. Data represent the number of mast cells in 20 microscopic fields from 3 animals per genotype. Bars = 100 µm (c, d, e) and 50 µm (f).