Klf4 is a transcription factor required for establishing the barrier function of the skin

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Located at the interface between body and environment, the epidermis must protect the body against toxic agents and dehydration, and protect itself against physical and mechanical stresses[1–4]. Acquired just before birth and at the last stage of epidermal differentiation, the skin's proteinaceous/lipid barrier creates a surface seal essential for protecting animals against microbial infections and dehydration. We show here that Kruppel-like factor 4 (Klf4, encoded by the gene *Klf4*), highly expressed in the differentiating layers of epidermis, is both vital to and selective for barrier acquisition. *Klf4*$^{-/-}$ mice die shortly after birth due to loss of skin barrier function, as measured by penetration of external dyes and rapid loss of body fluids. The defect was not corrected by grafting of *Klf4*$^{-/-}$ skin onto nude mice. Loss of the barrier occurs without morphological and biochemical alterations to the well-known structural features of epidermis that are essential for mechanical integrity. Instead, late-stage differentiation structures are selectively perturbed, including the cornified envelope, a likely scaffold for lipid organization. Using suppressive subtractive hybridization, we identified three transcripts encoding cornified envelope proteins with altered expression in the absence of *Klf4*. *Sprr2a* is one, and is the only epidermal gene whose promoter is known to possess a functional Klf4 binding site. Our studies provide new insights into transcriptional governance of barrier function, and pave the way for unravelling the molecular events that orchestrate this essential process.

The epidermis is a continuously regenerating organ composed

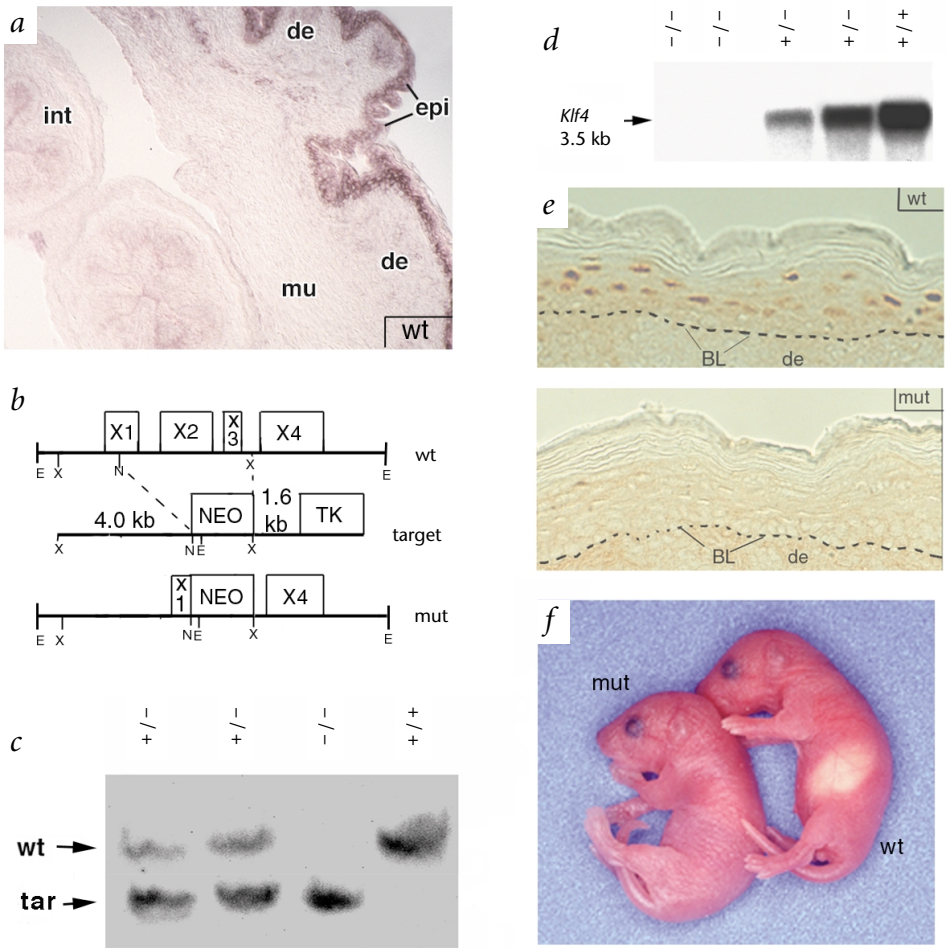

Fig. 1 *Klf4* expression and generation of mutant mice. ***a***, *In situ* hybridization of an E16.5 embryo section with a *Klf4* probe. Epi, epidermis; de, dermis; int, intestine; mu, muscle. ***b***, Schematic of the wild-type (wt) *Klf4* locus, targeting vector and predicted mutant (mut) targeted allele. *Klf4* was isolated from a 129/sv mouse genomic library and mapped by Southern-blot analysis[5,6]. The targeting vector was generated by inserting a phosphoglycerate kinase promoter-driven neomycin (*neo*) gene in place of a 2.8-kb fragment extending from 380 bp 5′ of the translation initiation codon (*Not*I site) to the first of three zinc fingers (*Xba*I site). Boxes denote *Klf4* exons and *neo* and herpes thymidine kinase (TK) genes used for positive and negative selection, respectively. Genotyping of G418/gancyclovir-selected ES clone DNAs was performed by Southern-blot screening of 5′ homologous recombination events using a 400-bp probe directly outside the 5′ arm; the 3′ event was assessed by PCR using a primer set from *neo* and exon 4. E, *Eco*RI; X, *Xba*I; N, *Not*I. ***c***, Southern-blot analysis of *Eco*RI-digested genomic DNAs from tails of four representative F2 intercross mice. The wild-type allele is 12 kb and the targeted (tar) allele is 5.7 kb. Genotypes are shown at the top. ***d***, Northern-blot analysis of epidermal mRNAs isolated from epidermis of five genotyped mice and probed with a *Klf4* cDNA probe. Note: the first '+/–' lane was underloaded relative to the others. ***e***, Anti-Klf4 immunohistochemistry of frozen sections of wild-type (wt) and *Klf4*$^{-/-}$ (mut) newborn epidermis. BL, basal layer. ***f***, Four-hour-old *Klf4*$^{-/-}$ and control mice.

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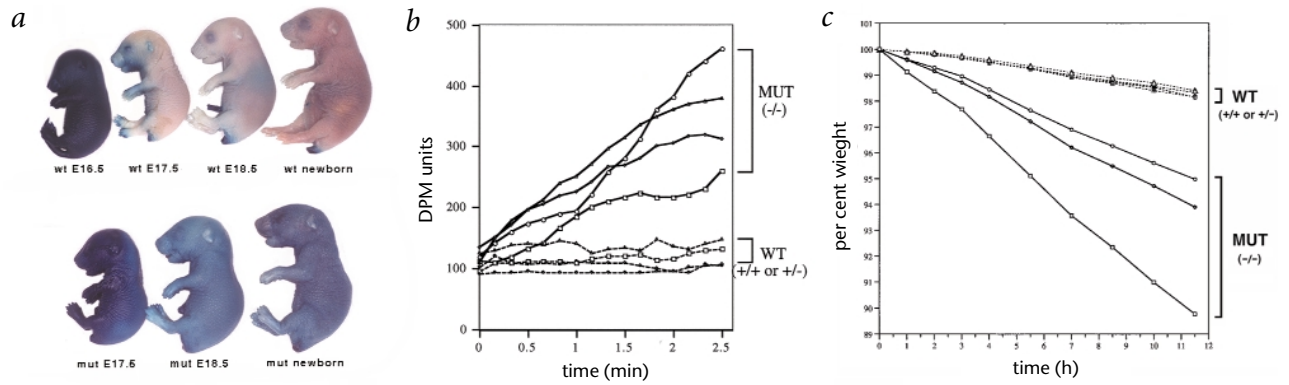

Fig. 2 Barrier defects in *Klf4*$^{-/-}$ mice. ***a***, Barrier-dependent dye exclusion assay on F2 *Klf4*$^{+/-}$ intercross litters taken at the ages indicated. ***b***, SEC of newborn mouse skin over time. ***c***, Dehydration assay over time. Data are presented as percentages of initial body weights (1.18–1.35 g); no differences in weight loss between wild-type and *Klf4*$^{+/-}$ mice were observed.

of a basal layer of proliferating cells and suprabasal layers of terminally differentiating cells that transit toward and are sloughed from the skin surface. During their upward transit through the spinous and granular layers, keratinocytes remain transcriptionally active, synthesizing and assembling a durable cytoskeletal framework that provides inner mechanical strength. Cornified envelope (CE) proteins are deposited beneath the plasma membrane of the cell, and as transcription nears completion, specialized lipids are synthesized. As cells become permeable, CE proteins are irreversibly crosslinked by calcium-activated transglutaminases, creating a sac. As lipids are extruded, the CE seems to serve as a scaffold for lamellar organization and anchorage, sealing together CEs and creating a barrier at the skin surface[1–4].

Although many of the structural proteins and lipids involved in a barrier mechanical integrity and epidermal barrier have been identified, little is known about how barrier function is established or about the regulatory proteins that govern this late-stage process. A candidate for transcriptional orchestration of barrier function is Klf4, encoding a member of the 'kruppel' family of transcription factors[5,6]. The best characterized family members are Klf1 and Klf2, which are implicated in late-stage erythroid and T lymphocyte differentiation, respectively[7–9]. *Klf4* is more highly expressed in epidermis than in intestine, where it was first identified and initially referred to as gut Kruppel-like factor, or Gklf (Fig. 1*a*; ref. 6). Immunohistochemistry shows that Klf4 protein is restricted to the upper spinous and granular layers, where it is nuclear (Fig. 1*e*).

To assess whether the correlation between *Klf4* expression and epithelial differentiation is physiologically significant, we cloned and characterized the mouse gene and used homologous recombination in embryonic stem (ES) cells to produce two phenotypically identical lines of mice homozygous for a targeted mutation in the *Klf4* locus (Fig. 1*b*,*c*). Northern-blot analysis and immunohistochemistry of skin sections indicated that *Klf4* mRNA and protein were abolished (Fig. 1*d*,*e*, respectively).

Klf4$^{+/-}$ mice were phenotypically and histologically normal. *Klf4*$^{-/-}$ mice were born at the expected mendelian ratio and were initially indistinguishable from their littermates. *Klf4*$^{-/-}$ mice were unsuccessful at feeding, however, and soon lacked the white 'milk spot' of normal mice (Fig. 1*f*). They were fed by pipette, although defects in the tongue may have prevented the mutant mice from forming the suction necessary to withdraw milk. In contrast to control littermates that survived more than 24 hours when unfed, *Klf4*$^{-/-}$ animals died within 15 hours after birth.

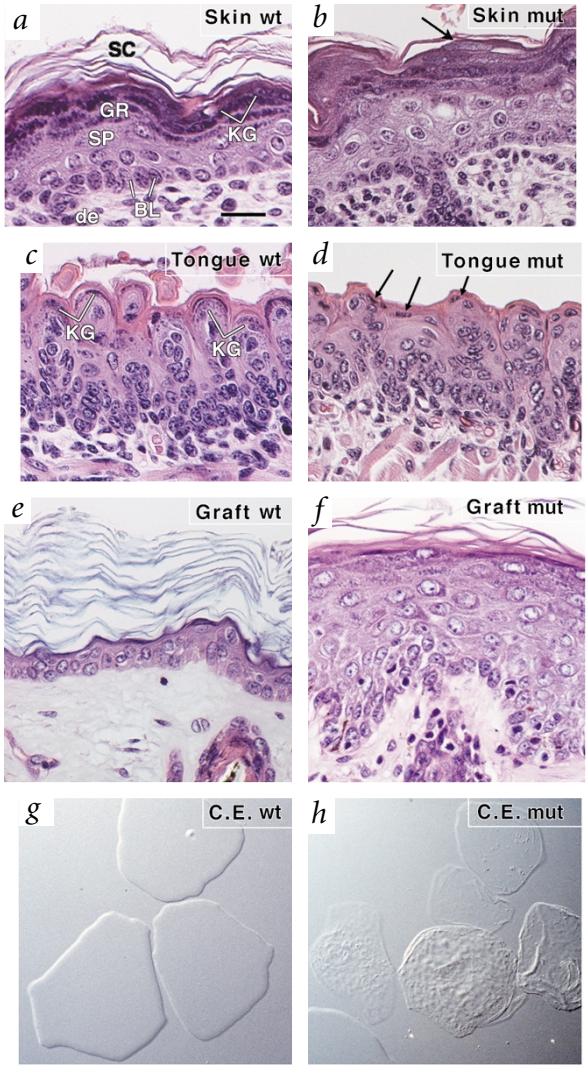

Fig. 3 Aberrations in differentiation and CE formation in *Klf4*$^{-/-}$ stratified epithelia. Skin (***a***,***b***) and tongue (***c***,***d***) from wild-type (*a*,*c*) and mutant (*b*,*d*) newborn mice were fixed in Bouin's, paraffin embedded, sectioned (5 μm) and stained with haematoxylin and eosin. Arrow in (*b*) denotes intracellular material in upper SC layers. Arrows in (*d*) denote nuclei in SC. SC, stratum corneum; GR, granular layer; KG, keratohyalin granules; SP, suprabasal layer; BL, basal layer; de, dermis; and hf, hair follicle. ***e***,***f***, Skin from the backs of newborn wild-type and *Klf4*$^{-/-}$ mice was grafted to the backs of nude mice, and skins were processed for histology after one month. Representative examples from two sets of animals (e, wild type; *f*, mutant) are shown. ***g***,***h***, Cornified envelopes prepared as described from newborn back skin[19]. Bar, 150 μm for all frames.

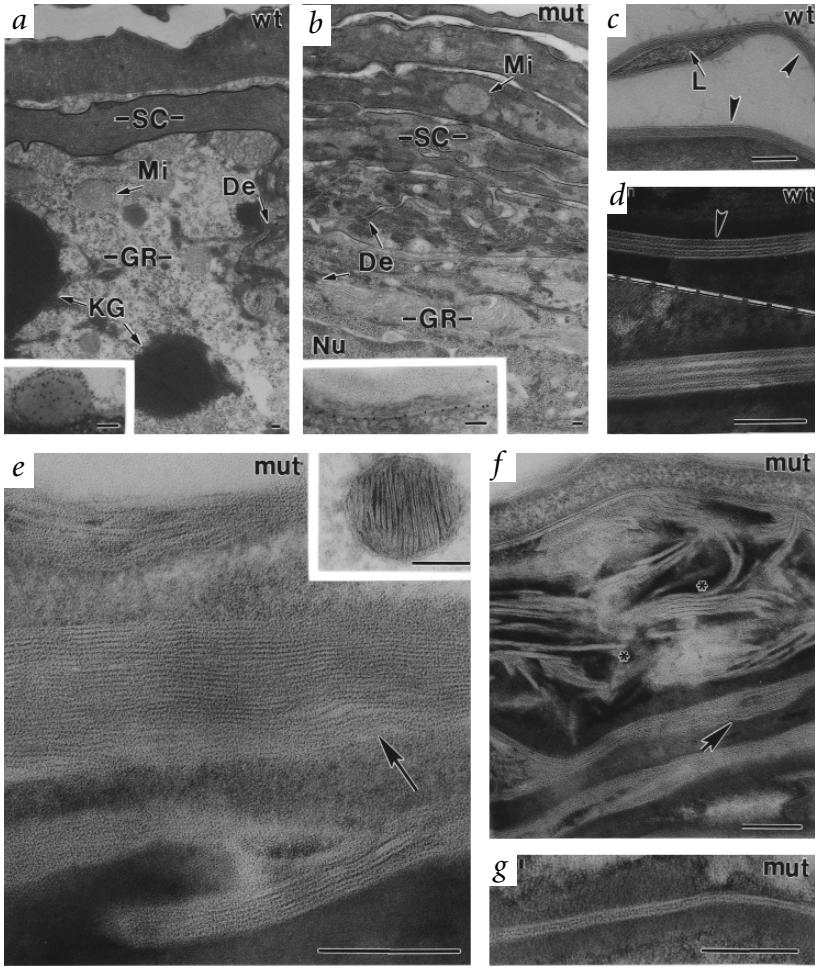

Fig. 4 Ultrastructural abnormalities in *Klf4*$^{-/-}$ epidermis. ***a***,***b***, Conventional and immunoelectron microscopy of ultrathin sections of wild-type (*a*) and mutant (*b*) arm skin. Inset in (*a*) shows lightly stained loricrin-labelled keratohyalin granule attached to larger densely stained filaggrin-containing keratohyalin granule; inset in (*b*) shows loricrin-labelled inner surface of cornified envelope in mutant squame. SC, stratum corneum; GR, granular layer; KG, filaggrin-containing keratohyalin granules; Mi, mitochondria; De, desmosomes; Nu, nuclei. Ruthenium tetroxide-stained samples of the SC from wild-type (***c***,***d***) and mutant (***e***,***f***,***g***) newborn back skins are shown. Arrowheads in (*c*) and (*d*) denote highly ordered alternating extracellular lipid tracks of electron-lucent and electron-dense bands. Occasionally, small amounts of desmosomal material appear trapped (L, lacuna; arrow in *c*). Note: dotted white line in (*d*) denotes where SC cell interior was cropped to show that the number of lipid tracks varies typically from 2–4 in wild-type sections (shown are 3 tracks upper and 4 tracks lower). In contrast, variation is much greater in *Klf4*$^{-/-}$ sections (compare *e* and *g*). Arrows, wavy, irregular lipid tracks; asterisks, large intracellular aggregates of lamellae. Inset to (*e*) shows a morphologically normal intracellular lamellar granule. Bar, 100 nm.

The strong expression of *Klf4* in skin, coupled with the rapid post-natal lethality, suggested that *Klf4*$^{-/-}$ mice might possess a defective epidermal barrier. To test this directly, we first examined the ability of the animals to prevent penetration of external solutions[10]. In agreement with prior studies[10], dye exclusion in control embryos was established as a wave, with a marked transition between embryonic day (E) 16.5 and E17.5 (Fig. 2*a*, top). In contrast, *Klf4*$^{-/-}$ embryos and newborn mice all turned an intense blue colour when immersed in solution (Fig. 2*a*, bottom).

The ability of the skin to prevent fluids from escaping into the environment was tested by surface electrical capacitance (SEC), which measures hydration of the skin surface[11]. Newborn control and mutant skins exhibited similar initial capacitances, reflecting humidity at the air-skin interface. Over a period of 2.5 minutes, however, the SEC increased exclusively in all mutant mice, indicating elevated hydration of their stratum corneum (SC; Fig. 2*b*). *Klf4*$^{-/-}$ skin remained defective when grafted onto foster mice, as judged by a high SEC compared with control skin (900 units versus 120 units). These data ruled out a developmental delay in barrier function acquisition by *Klf4*-null mice, and indicated that the defect was inherent to the skin and was not a secondary event.

To assess whether the observed water loss was sufficient to cause the perinatal lethality observed, we removed *Klf4*$^{+/-}$ F2 intercross newborns before feeding, monitored their weights hourly, and determined their identity by genotyping after 12 hours. In a representative experiment, three mice suffered steady weight loss, whereas four maintained weight (Fig. 2*c*). A perfect correlation was subsequently found between weight loss and *Klf4*$^{-/-}$ genotype. Although the percentage of weight loss varied, *Klf4*$^{-/-}$ animals always lost 5–10% of their birth weight. As none of the animals fed or urinated during the evaluation period and all were similarly active, we attribute the weight loss to fluid evaporation due to a compromised skin barrier function. In humans, such a rapid rate of dehydration would result in hypovolaemic shock leading to death, as observed in *Klf4*$^{-/-}$ mice. A complete necropsy revealed no obvious defects in any organ other than the skin.

Mice with null mutations in several other genes have been found to display defective epidermal barrier function, but all exhibit overt abnormalities in their appearance, indicative of defects at earlier stages of differentiation[12–18]. In contrast, the normal appearance of *Klf4*$^{-/-}$ mice represented the first possible primary lesion to be identified in barrier function. To explore this possibility further, we examined the histology and biochemistry of control versus *Klf4*$^{-/-}$ skin. Normal newborn epidermis consists of a single basal layer of dividing cells, 2–3 layers each of transcriptionally active spinous and granular cells, and 4–6 layers of metabolically inert, flattened enucleated SC cells (Fig. 3*a*). Indeed, the inner portion of newborn *Klf4*$^{-/-}$ epidermis, including basal and spinous layers, appeared morphologically normal (Fig. 3*b*). Immunohistochemistry with antibodies against the proliferation markers Ki67 and PCNA displayed typical basal staining patterns, and TUNEL assays showed no signs of apoptosis (data not shown). In contrast, the overall balance between growth and differentiation seemed unaffected, whereas the upper layers displayed subtle morphological abnormalities. Most notably, the granular layer exhibited alterations in its characteristic, darkly stained keratohyalin granules, and the granular and SC cells failed to flatten and discard their cytoplasmic organelles (Fig. 3*b*). These defects were

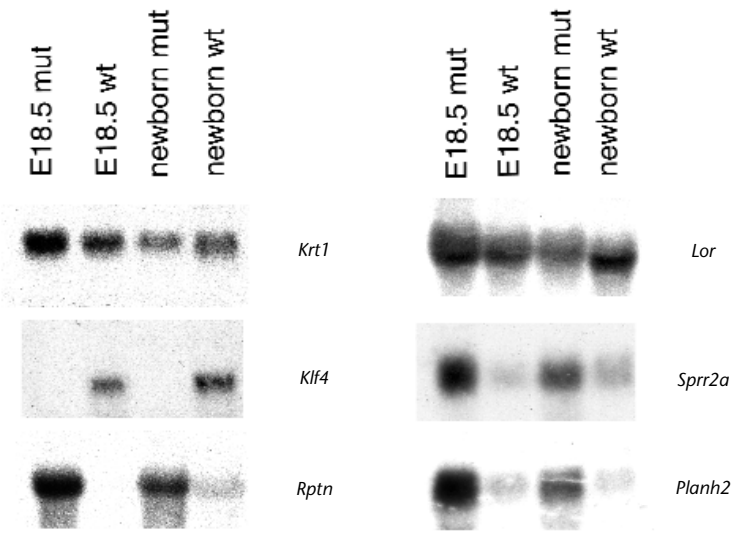

Fig. 5 Differentially expressed genes in *Klf4*$^{-/-}$ mice. Northern-blot analysis of approximately equal amounts of skin mRNAs isolated from mice of the phenotype and age indicated on the diagram. RNA levels were quantitated spectrophotometrically and by ethidium bromide staining of rRNA bands. Blots were probed with cDNAs specific to *Krt1* (2.5 kb), *Lor* (1.6 kb), *Klf4* (3.5 kb), *Sprr2a* (1.0 kb), *Rptn* (4.5 kb) and *Planh2* (2.2 kb).

also prominent in *Klf4*$^{-/-}$ tongue unlike wild-type tongue, in which keratohyalin granules normally crest the dorsal taste buds and nuclei are not typically seen in the upper SC layers (Fig. 3*c*,*d*).

Defects in epidermal morphology persisted when *Klf4*$^{-/-}$ skin was grafted onto nude mice. In contrast to control epidermis, which thinned normally when grafted and adopted the appearance of adult skin (Fig. 3*e*), mature *Klf4*$^{-/-}$ skin continued to exhibit the same defects in the granular and SC layers (Fig. 3*f*). The mutant graft epidermis was unusually thick, unlike newborn *Klf4*$^{-/-}$ skin. Because hyperproliferation is a typical compensatory response of adult epidermis that is severely compromised in barrier function, this difference is likely to be secondary.

The defects in epidermal morphology were supportive of a late-stage lesion in the differentiation process. To explore this in greater detail, we prepared CEs from *Klf4*$^{-/-}$ and littermate control skins. After boiling epidermis for 20 minutes in the presence of ionic detergent and a reducing agent, these crosslinked cellular ghosts remain[19]. Control CEs were uniformly rigid, polygonal, plump and smooth, and are thought to reflect the presence of covalently attached lipids that organize at the CE outer surface[20] (Fig. 3*g*). In contrast, most *Klf4*$^{-/-}$ CEs were rough, irregular and crumpled (Fig. 3*h*). Despite these defects, normal immunostaining patterns were obtained with antibodies against major CE proteins (involucrin and loricrin), and *in situ* transglutaminase activity assays revealed no gross differences in γ-glutamyl ε-lysine crosslinkages among CE proteins (data not shown).

The first signs of ultrastructural defects were also in the late stages of terminal differentiation (Fig. 4). In the granular layers, the two types of keratohyalin granules[21–22] (KGs) were still present in *Klf4*$^{-/-}$ skin, but they were often fewer and smaller (Fig. 4*a*,*b*). L-type KGs normally label with antibodies against loricrin (Fig. 4*a*, inset), and when barrier function is established embryologically, loricrin redistributes and is crosslinked into the cytoplasmic surface of the CE (ref. 10). This redistribution process did not appear defective, as judged by anti-loricrin labelling found at the inner surface of mutant squames (Fig. 4*b*, inset). Similarly, despite a reduction in electron-dense KGs, immunoblot analyses revealed that its major constituent, profilaggrin, is processed normally (data not shown). Finally, immunoblot analysis revealed no major differences in loricrin or filaggrin levels (data not shown), suggesting that the paucity of these granules reflects an organizational defect of these proteins.

The abnormal appearance of CEs suggested that lipid synthesis, secretion or deposition might be aberrant in *Klf4*$^{-/-}$ epidermis. Quantitative analysis of polar and nonpolar lipids and phospholipids showed no statistical difference between the lipid profiles of *Klf4*$^{-/-}$ and control mice (P. Wertz and J.A.S., unpublished data), suggesting that lipid synthesis proceeded normally. In contrast, ultrastructural differences emerged when skin was fixed with ruthenium tetroxide to preserve lipid structure[23]. In control skin, this process revealed highly ordered tracks of alternating electron-dense and electron-lucent lipid lamellae interconnecting SC cells (Fig. 4*c*,*d*). In *Klf4*$^{-/-}$ SC, intercellular lamellae were often discontinuous and highly variable in thickness (Fig. 4*e*,*f*,*g*). The lamellae often exhibited a wavy, disorganized pattern (Fig. 4*e*,*f*, arrows), which in some regions was abnormally thick (Fig. 4*e*) and in others atypically thin (Fig. 4*g*). Although intracellular lamellar granules appeared normal (Fig. 4*e*, inset), large internal aggregates of disorganized lamellae (Fig. 4*f*, asterisks) were evident. The abnormal CE morphology (Fig. 3*g*,*h*) and ultrastructural data suggest that the absence of Klf4 results in a downstream alteration in CEs and either concomitant or subsequent gross defects in the secretion and deposition of lipids onto the CE scaffold.

The lack of major biochemical changes in the skin prompted us to wonder what target genes *Klf4* might regulate. The only epidermal gene thus far shown to have a Klf4 regulatory element encodes Sprr2a, a CE protein thought to be important in the organization of loricrin, the major CE constituent[24]. Northern-blot analysis revealed that *Sprr2a* RNA expression was upregulated in *Klf4*$^{-/-}$ skin (Fig. 5), in contrast to the unaffected RNAs encoding two major structural proteins, keratin 1 (*Krt1*) and loricrin (*Lor*). This difference was also found in mRNAs isolated from embryonic day 18.5 skins, and thus was not attributed to a secondary consequence of the loss of barrier function in a terrestrial environment. The upregulation is consistent with *in vitro* data suggesting that full-length *Klf4* may in some cases act as a repressor, in contrast to *Klf1*, but similar to *Klf3* (refs 25,26). Further studies will be needed to ascertain the mode(s) of action of *Klf4* on specific epidermal promoters *in vivo*.

To further explore downstream targets, we performed suppressive subtractive hybridization (SSH) with RNAs isolated from newborn *Klf4*$^{-/-}$ and control littermate skin. *Sprr2a* was

identified in this screen along with two additional cornified envelope proteins: repetin (encoded by *Rptn*) and plasminogen activating inhibitor 2 (encoded by *Planh2*; refs 27,28). Expression of these mRNAs was also upregulated in *Klf4*$^{-/-}$ skin (Fig. 5). These data lead us to posit that upregulation of transcription of some, but not all, CE genes may lead to an imbalance in CE assembly or composition, thereby altering the structural scaffold on which the lipid lamellae are organized.

Our data are the first to reveal an essential role for Klfs beyond the haematopoietic system. Like the haematopoietic system, the epidermis undergoes a process of self-renewal, and like *Klf1* and *Klf2*, *Klf4* is not expressed in stem cells, but rather appears in the late stages of terminal differentiation[7–9]. All three genes identified as targets for *Klf4* are organized in chromosomal clusters of related genes, reminiscent of *Klf1* regulation of globin genes[27,29,30]. This raises the possibility that Kruppel family members may function generally to control gene switching within activated regions of chromosomes.

Klf4 represents the first essential transcription factor that is critical to and selective for establishing the skin's barrier function. Although targeting of some other skin genes has abrogated barrier function by perturbing epidermal differentiation, *Klf4* is the first mutant in which barrier function is selectively perturbed without gross alterations to the epidermal differentiation program that defines mechanical integrity. Our ability to use these mice to identify target genes whose expression is affected by loss of *Klf4* underscore the power of discovering a transcription factor directly involved in this key epidermal process. The *Klf4*$^{-/-}$ mice will provide a valuable resource for further dissecting the molecular events governing epidermal barrier function. Understanding this process and generating mouse models are important steps in developing new and improved methods of accelerating skin barrier function acquisition and reducing medical risks in premature infants.

Methods

***In situ* hybridization, immunohistochemistry, Northern-blot analysis and SSH.** We performed *in situ* hybridization using a digoxygenin-UTP cRNA specific to the *Klf4* cDNA probe (bp 537–1,471) on cryosections (15 μm) of E16.5 embryos. We performed immunohistochemistry on cryosections (8 μm) incubated with an affinity-purified rabbit polyclonal antibody raised against aa 300–320 of Klf4 and processed with the Elite Vectastain antibody kit (Vector Laboratories). We prepared RNAs from Trizol (Gibco BRL) extractions of E18.5 or newborn tissues. We hybridized northern blots with RT-PCR generated, labelled fragments corresponding to *Klf4* cDNA (bp 537–1,471), *Sprr2a* cDNA (bp 2–779), *Planh2* cDNA (bp 323–1,700) or *Rptn* cDNA (bp 6,468–7,650). *Krt1* probe was a gift of S. Yuspa. *Lor* probe was a gift of A. Joyner. We carried out SSH according to the manufacturer's instructions with poly(A)$^+$ skin mRNA (2 μg) from *Klf4*$^{-/-}$ and control littermates (Clontech).

Barrier function assays. We performed dye penetration assays as described[10]. Briefly, we rinsed the samples in PBS and immersed them in 5-bromo-4-chloro-e-indolyl-b, D-galactopyranoside dye solution, pH 4.5, at 37 °C for 8 h. We genotyped and photographed the animals following the assay. We obtained SEC measurements with an impedance-based NOVA Dermal Phase Meter 9003 and a capacitance probe (model 9105; NOVA Technology Corporation).

Electron microscopy and immunoelectron microscopy. We enzymatically removed the epidermis and fixed it overnight at 4 °C in 2% glutaraldehyde, 2% paraformaldehyde, $CaCl_2$ (2 mM) in sodium cacodylate (0.1 M, pH 7.3). We postfixed the samples for 30 min with either 0.5% osmium tetroxide at 4 °C followed by 2% aqueous uranyl acetate, or with 0.2% ruthenium tetroxide at RT. We then dehydrated the samples in a graded series of ethanol and embedded them in LX-112 resin (Ladd Research Industries). For immunoelectron microscopy, we processed the epidermis using Lowicryl K4M, a rabbit polyclonal anti-loricrin antibody (Babco), and a 10 nm gold particle-conjugated secondary antibody.

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