Supporting Online Material.

Materials and methods

Mouse strains

Details of production of the Grhl3+/– mice have been previously reported (1). Grhl3 heterozygous mice were interbred to produce homozygous embryos for analyses. All experiments were approved by the Melbourne Health Animal Ethics Committee.

Barrier function assays

The skin permeability and transepidermal water loss (TEWL) assays were performed as described previously (2). For the TEWL assay, the surface area of skin was determined using high resolution imaging (Zeiss Axiocam, Carl Zeiss, Thornwood NY), and the TEWL was calculated as milligrams water loss per square millimetre of epidermis per hour (mg/mm²/hr).

Embryo wound assay

Gestational day 12.5 and 16.5 mice were dissected from the uterus in sterile Tyrode's saline as described previously (3). The left hind limb was amputated with the aid of fine dissecting tools (#5 watchmaker forceps and ophthalmic iris dissecting scissors) and an SMZ-U dissecting microscope (Nikon) and retained for genotyping. Embryos were then submerged in 4 mls of a 1:3 mix of rat serum: Tyrode's saline and cultured for 6-24 hours in an atmosphere of 95% oxygen, in which they were rolled at 30 rpm at 37°C. Following culture, embryos were fixed overnight in 2.5% glutaraldehyde (Pro SciTech) at 4°C, rinsed in 0.1 M sodium cacodylate buffer (3 x 15 minute changes) and post fixed with 2.5% osmium tetroxide (Pro SciTech) for 1 hour. Following extensive rinsing in distilled water (3 x 15 minute changes) samples were dehydrated through increasing concentrations of acetone (5 minute changes in each of: 30%, 50%, 70%, 80%, 95% and 100%). Samples were then critical point dried (Polaron critical point dryer), mounted on stubs with carbon dag (ProSciTech) and sputter coated with
gold in an Edwards Sputter Coater. Gold-coated samples were observed using a Phillips 515 SEM at 20 kV.

CASTing and EMSA

CASTing was performed as described previously (4) with cellular extract derived from the human cell line A431 transfected with the pcDNA3.1 expression vector (Invitrogen) containing HA-tagged Grhl3. Expression of HA-Grhl3 was confirmed by Western blot (not shown). EMSAs were performed as described previously (5) with the following oligonucleotide probes (sense strand only given):

**Grhl3 consensus** - 5' - AACTAGATAAAACCGGTTTTACTAGTT - 3'
**Grhl3 mutant** - 5' - AACTAGATAAAaCGTTTTTACTAGTT - 3'
**TGase1-A** - 5' - CACTGACTCAAACCTGGCTGGGTGGGGAG - 3'
**TGase1-B** - 5' - GATGGCAGGAACCGGCCCAGCCATGT - 3'

Histology and Electron Microscopy

For histologic analysis, skin samples were immersion fixed in 4% paraformaldehyde in PBS (pH 7.3), embedded in paraffin and stained with haematoxylin and eosin. For EM analysis, skin samples were taken from E18.5 embryos at autopsy, and fixed overnight in Karnovsky’s fixative, washed two times with 0.1 M cacodylate buffer, and post-fixed in either 1% aqueous osmium tetroxide (OsO₄) in 0.1 M cacodylate buffer, pH 7.3, or in ruthenium tetroxide (RuO₄), both containing 1.5% potassium ferrocyanide, as described previously (6). After fixation, samples were dehydrated in graded ethanol solutions and embedded in an Epon/epoxy mixture. Ultrathin sections were examined both with and without further contrasting with lead citrate in an electron microscope (Zeiss 1A, Carl Zeiss) operated at 60 kV.

Morphometric measurement of CE

In order to quantify CE integrity, CE thickness was assessed in electron micrographs of E18.5 epidermis. Ten or more pictures were taken by an independent observer from three separate tissue
samples in each experiment; i.e., a total of at least 30 micrographs. In each case, a total magnification of 148,050 was achieved, and the combined CE-corneocyte lipid envelope (CE-CLE) thickness was measured at intervals of 10 mm, as determined with a planimeter (7), within the inter-desmosomal regions. Combined CE-CLE thickness was determined for the first (apical aspect) and second (basolateral aspect) layers of the lower SC only. A two-tailed Mann-Whitney test was used to determine statistical significance.
Figure S1. Specific expression of Grhl3 in the surface ectoderm at E12.5 in a representative region of a developing forelimb as analysed by in situ hybridisation. fb, forelimb bud; se, surface ectoderm.
Figure S2. Abnormalities in differentiation and CE formation in Grhl3-null epidermis. Skin from wild type (+/+) and mutant (–/–) embryos taken at E15.5 and E18.5 as indicated. Epithelial ridges (arrows) in wild type skin at E15.5 (top left panel) are absent in the E15.5 mutant skin (top right panel). Basal disorganisation and suprabasal hyperproliferation and a compacted SC are evident in the E18.5 mutant skin (bottom right) compared to the wild type (bottom left). d, dermis; sb, stratum basale; ss, stratum spinosum; sg, stratum granulosum; sc, stratum corneum.
Fig S3

A

B

Cornified Envelope Thickness
grhl-3-Deficient Mice (EGA 18.5 d)

+/-

-/-

- 11.5%

17.4

15.4

Cornified Envelope Thickness (nm)

0

5

10

15

20

+/-

-/-

* p < 0.002

C

D

E

+/-
Figure S3. Grlh3-null epidermis displays abnormal lamellar body contents, and decreased stratum corneum membrane structures. (A) Ultrastructural analysis by electron microscopy of skin sections from E18.5 embryos. In the wild type skin (left panel, +/+), the stratum corneum (SC) has normal multiple layers of corneocytes (C) with normal EM artefact-induced, well-spaced intercellular layers (asterisk). In comparison, although the number of corneocyte layers is similar in mutant skin (right panel, –/–), the intercellular layers are essentially non-existent (asterisk). Keratohyaline granules (arrows) seem somewhat larger in the mutant skin. Scale bar = 2 µm (B) Morphometric analysis of cornified envelope thickness from multiple E18.5 wild type (+/+) and mutant (–/–) epidermis sections shows a significant decrease (11.5%) in the overall CE thickness in the mutant epidermis. (C) Focusing on the lipid structures shows that the E18.5 wild type (+/+ stratum corneum displays a full complement of extracellular lamellar membrane structures (arrows) and that the lamellar bodies show multiple layers of lipid containing lamellae (insert of panel E, arrows). Whereas, the extracellular spaces of the (D) mutant (–/–) stratum corneum displays a paucity of lamellar bilayers (open arrows), although occasional fragments of replete lamellar arrays are seen (solid arrow). (E) This is as a result of almost all the lamellar organelles lacking internal contents (arrowheads) in the mutant epidermis, even though there are a normal complement of lamellar bodies. Panels (A & E), osmium tetroxide post-fixation; Panels (C & D), ruthenium tetroxide post-fixation.
**Fig S4A.** Sequence alignment of 49 clones obtained by simultaneous CASTing with cellular extract from A431 cells transduced with pcDNA3-HA-Grhl3 and anti-HA antisera.
Fig S4B. Grhl3 DNA binding consensus sequence derived from the clones outlined in Fig S4A.
Fig S4C. Complete image of the EMSA showing DNA binding of Grhl3 to the TGase1 promoter.
Supporting Online Material.

References