Supplemental Materials and Methods

Mouse keratinocytes- Epidermal keratinocytes were prepared and cultured as described (Lichti et al., 2008). To induce differentiation and cell-cell contacts, 0.15, 1.05 and 2 mM of CaCl\textsubscript{2}, respectively, was added to the medium.

Q-PCR- Equal amounts of total RNA samples were reverse transcribed by M-MLV reverse transcriptase, and qPCR was performed as previously described (Tu et al., 2011). Levels of mRNA were normalized to mitochondrial ribosomal protein L19.

Measurement of cytosolic Ca\textsuperscript{2+}- The cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in keratinocytes in response to elevated [Ca\textsuperscript{2+}]\textsubscript{o} and ionomycin was measured using a Dual-wavelength Fluorescence Imaging System (Intracellular Imaging Inc., Cincinnati, OH) as described (Tu et al., 2007). To measure the acute Ca\textsuperscript{2+} responses to elevated Ca\textsuperscript{2+}\textsubscript{o}, keratinocytes were maintained in 0.03 mM CaCl\textsubscript{2} and [Ca\textsuperscript{2+}]\textsubscript{i} was measured before and after exposure to 2 mM CaCl\textsubscript{2}. To assess the Ca\textsuperscript{2+} pool, [Ca\textsuperscript{2+}]\textsubscript{i} was measured before and after exposure to 2 \textmu M ionomycin in the absence of Ca\textsuperscript{2+}\textsubscript{o}. The data shown represent the average [Ca\textsuperscript{2+}] of 24-50 individual cells during recording.

Protein lysate preparation and immunoblotting- Total protein lysates were prepared by extracting epidermis of mouse neonates in lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Triton-100) containing 2% SDS. Conditions for immunoblotting were as described (Tu et al., 2008). 50 \textmu g protein samples were used in immunoblotting analyses.
**Immunohistochemical staining**- Mouse skins were fixed with 4% formaldehyde, embedded in paraffin, sectioned, and used for immunohistochemistry (Komuves et al., 2002). The sections were deparaffinized, rehydrated, and washed. Sections were incubated with primary antibodies to the proteins of interest and then with appropriate biotinylated secondary antibodies, followed by ABC-peroxidase (Vector, Burlingame, CA) and a DAB substrate. Secondary antibody alone was used as a control to establish the specificity of immunoreactivity.

**Electron microscopy**- Mouse skins were fixed with 2% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), then post-fixed with reduced osmium tetroxide, and processed for embedding in Spurr’s resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss 10CR transmission electron microscope.

**Assessment of epidermal Ca$^{2+}$ gradient**- Skin samples were processed for ion capture cytochemistry as previously described (Menon et al., 1985). Skin samples were fixed with an ice-cold fixative containing 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose (pH 7.4) in the dark. The samples were post-fixed in 1% osmium tetroxide containing 2% potassium pyroantimonate at 4°C in the dark for 2h, and then processed, embedded, sectioned and examined with a Zeiss 10CR transmission electron microscope.
Lanthanum perfusion- Skin samples were immersed in 4% lanthanum nitrate in 0.05 M Tris buffer (pH7.4) containing 2% glutaraldehyde and 1% paraformaldehyde for 1 h at room temperature. After lanthanum perfusion, the samples were washed and processed for electron microscopy as described above.

Skin permeability assay- The development of the skin barrier in Epid-CaR/- mouse embryos and their control littermates was assessed by toluidine blue penetration assay. Embryos of E15.5 and E16.5 were subjected to methanol dehydration and subsequent rehydration as described previously (Koch et al., 2000). They were then washed in PBS, stained in 0.0125% toluidine blue O (Sigma Chemical, St Louis, MO) in PBS for 1 min. After de-stained in PBS, embryos were photographed with a Leica M205C microscope.

Measurement of trans-epidermal water loss (TEWL)- Epid-CaR/- mice and their control littermates were maintained with a normal Ca²⁺ diet (1.3% Ca²⁺) or switched to a low Ca²⁺ (0.02%) diet after weaning. The backs of mice were shaved. Twenty-four hours later TEWL was measured using a Meeco electrolytic water analyzer to establish a basal rate. The permeability barrier was then disrupted acutely using sequential tape stripping until the rate exceeds 500ppm/cm²/hr, and TEWL was then measured at 0, 3 and 6 hours after tape stripping to determine the rate of barrier recovery.
Assessment of innate immune response - \( \text{EpidCaR}^-/- \) mice and their control littermates were maintained with a normal diet (1.3% Ca\(^{2+}\)) or switched to a low (0.02%) Ca\(^{2+}\) diet after weaning. The backs of mice were shaved. A 1-cm long full-depth skin incision was made on the back of the animals. Twenty-four hours later, total RNAs were collected from a 2 mm-wide area along wound edges and from skin of similar size in the uninjured area. Expressions of innate immune response genes were analyzed by qPCR.

Statistical analysis - Student’s t-Test (for comparing two sample groups) and one-way ANOVA (for comparing more than two groups) were performed to analyze the quantitated data of various assays described in this study. Statistical difference is determined significant if P value is lower than 0.05.

Reference

Lichti U, Anders J, Yuspa SH (2008) Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. *Nat Protoc* 3:799-810.
Figure S1. Reduced Ca^{2+}-induced expression of differentiation markers in EpidCaR-/- keratinocytes. Epidermal keratinocytes were isolated from the back skin of 5-day-old EpidCaR-/- and control mice and cultured in medium containing 0.05, 0.15 or 1.05 mM CaCl_{2} for 48 hours. The levels of differentiation markers keratin 1, involucrin, transglutaminase (TG) 1, loricrin and filaggrin and the basal cell marker keratin 14 in cell lysates were assessed by immunoblotting. Ca^{2+}-induced expression of involucrin, TG1, loricrin and filaggrin in the EpidCaR-/- keratinocytes was reduced as compared to control cells, while the level of basal cell marker keratin 14 was not affected.
Figure S2. Increased proliferation in epidermis of EpidCaR-/- mice. (a, c) Proliferation in epidermis of 7-day-old EpidCaR-/- (CaR-KO) and control mice was assessed by PCNA staining. (a) PCNA (+) cells in epidermis of each genotype in representative fields (200x magnification) were quantified using a computer-assisted program (BIOQUANT, Nashville, TN). Data were presented as mean ± SE, n=30. *P<0.01. (b) Epidermal keratinocytes were isolated from the back skin of 3-day-old EpidCaR-/- and control mice and cultured in medium containing 0.05 mM CaCl₂ for 24 hours. Cell proliferation was determined by measuring ³H-incorporation into cellular DNA and normalized to controls. Each data point shows mean ± SE, n=6. #P<0.05, EpidCaR-/- vs. controls. (c) Immunohistochemical staining of skin for PCNA. Positive staining sites are visualized as brown. Bar = 50 μm. (d) H & E staining of epidermis of 3-day-old EpidCaR-/- and control mice. Bar = 50 μm.
Figure S3. Inefficient formation of adherens junctions in Ca\textsuperscript{2+}-treated \textit{EpidCaR-/-} keratinocytes. Epidermal keratinocytes were isolated from newborn \textit{EpidCaR-/-} mice and their control littermates and then exposed to 2 mM Ca\textsuperscript{2+} for 20 minutes to induce formation of cell-cell junctions. Fluorescent immunostaining was performed using E-cadherin polyclonal antibody (green), and monoclonal antibodies (red) against β-catenin, Rho A and Fyn followed by the appropriate FITC- or Texas red-conjugated secondary antibody. Overlapped staining sites are visualized as yellow. Bar = 20 μm. Ca\textsuperscript{2+}-induced recruitment of E-cadherin, β-catenin, Rho A and Fyn to the cell-cell junctions was suppressed in \textit{EpidCaR-/-} keratinocytes.
**Figure S4.** EpidCaR-/− mice exhibited normal barrier recovery when maintained on a normal (1.3%) calcium diet. Four-month-old EpidCaR-/− mice and their control littermates were maintained on a regular diet containing 1.3% Ca^{2+}. Sequential cellophane tape stripping was used to disrupt the permeability barrier and increase trans-epidermal water loss (TEWL) levels until rates exceeded 500 ppm/cm^2/hr. TEWL was measured before (a) and 0, 3, and 6 hours after (b) barrier disruption. The degree of barrier recovery was expressed as % decrease of TEWL at 3 and 6 verses 0 hour after barrier disruption. Data were presented as mean ± SE, n=11. No significant difference in basal TEWL and barrier recovery between EpidCaR-/− and control mice was found.
Figure S5. Expression of differentiation markers and mediators of epidermal sphingolipid synthesis, transport and processing in EpidCaR/- mice and control littermates maintained on either normal (1.3%) or a low (0.02%) calcium diet for 4 months. (a) QPCR analyses of full skin RNA for differentiation markers keratin 1, involucrin (INV), transglutaminase (TGM1), loricrin (LOR) and filaggrin (FLG). (b) QPCR analyses of full skin RNA for lipid transporter ABCA12, glucosylceramide synthase (UGCG), sphingomyelinase (Smase), β-glucocerebrosidase (GCB) and fatty acid elongase (ELOVL4). The message levels in EpidCaR/- (KO) epidermis were normalized to that in controls and presented as mean ± SE, n=7-11. * P<0.01, # P<0.05.
Figure S6. *EpidCaR-/-* mice displayed normal innate immune response and 1,25-dihydroxyvitamin D3/VDR-mediated signaling when raised on a normal (1.3%) calcium diet. Four-month old *EpidCaR-/-* (CaR-KO) mice and control littermates were maintained on a regular diet containing 1.3% Ca$^{2+}$. (a) Full-depth skin incisions were made on the back of the animals. Twenty-four hours later, total RNAs were collected from wounded and uninjured areas. Expressions of TLR2, CD14, cathelicidin (camp), IL-1β and IL-6 were analyzed by qPCR. The message levels in the wound were normalized to that in the uninjured area. (b) The message levels of 25-hydroxyvitamin D3 1-α-hydroxylase (CYP27B1) and vitamin D receptor (VDR) in *EpidCaR-/-* (KO) skin were normalized to that in controls. Data were presented as mean ± SE, n=7-10. No statistically significant difference between *EpidCaR-/-* (CaR-KO) and control mice was observed.
Figure S7. EpidCaR-/- mice and control littermates have similar levels of serum calcium and phosphate when maintained on either normal (1.3%) or a low (0.02%) calcium diet for 4 months. Data were presented as mean ± SD, n=9-19. # P<0.05, normal vs. low calcium diet in control mice. Values of average serum calcium and phosphate levels (mg/dL) of each group are shown above the bars. No statistically significant difference between EpidCaR-/- (CaR-KO) and control mice was observed regardless of diets.
Figure S8. *EpldCaR-/-* mice and control littermates have equivalent body size when maintained on either normal (1.3%) or a low (0.02%) calcium diet for 6 months. Data were presented as mean ± SD, n=7-13. # P<0.05, normal vs. low calcium diet in *EpldCaR-/-* mice. No statistically significant difference between *EpldCaR-/-* (CaR-KO) and control mice was observed regardless of diets.