Supplementary information, Data S1

Materials and Methods

Fluorometric imaging plate reader (FLIPR)

Keratinocytes derived from C57BL/6 newborn mice were cultured in 6-well plates for 48 h. The grown keratinocytes were then washed, trypsinized (0.25% trypsin) and centrifuged (5 min, 1,000 rpm, room temperature (RT)) with a BSA column. The pellet was resuspended and plated in collagen IV-coated 384-well plates (6,000 cells/well). The cells were grown at 37 °C with 5% CO_2 for 2 days prior to FLIPR analysis. FLIPR experiments with adherent cells were performed essentially as described previously[1,2]. In brief, the cells were washed with 1× HBSS (1.3 mM Ca^{2+}) with 20 mM HEPES and 2.5 mM probenecid by an Embla plate-washer (Molecular Devices), loaded with the Ca^{2+} sensitive fluorophore Fluo-3 (Molecular Devices) for 1.5 h, and washed again. The cell plates were then transferred to a FLIPR (Molecular Devices) to monitor Fluo-3 fluorescence in response to temperature changes. The temperature change of the medium inside the well was controlled by a custom-designed temperature-control device as described previously[3].

Fura-2 single-cell Ca^{2+} imaging

Cell culture and transient transfection of HeLa, HEK293T, the Orai-KO-HEK cells (a general gift from Drs Donald Gill and Youjun Wang) and Fura-2 single cell Ca^{2+} imaging with the indicated extracellular Ca^{2+} were performed according to the

previous protocol [1]. The intracellular Ca^{2+} concentration is expressed as the 340/380 ratio of Fura-2 or converted to Ca^{2+} concentration (Fig. 1m) following the method described previously[1]. The temperature change of bath solution was controlled using a CL-100 temperature controller (Warner Instruments) and a SC-20 Solution In-Line Heater/Cooler (Harvard Apparatus), and recorded with a thermistor placed at the outlet of the perfusion. As reported previously[1], high temperatures can cause an increase of the Fura-2 ratio independent of the extracellular Ca^{2+} and intracellular Ca^{2+} store, due to the effect of temperature on the Fura-2 dye itself. In Fig. 2f (left panel), Fig. 3i (heat on) and Supplementary information, Fig. S1h (heat on), the STIM1-independent heat-induced change of the Fura-2 signal itself (measured from $STIM1^{Ker/Ker}$ (KO) keratinocytes that were subjected to a heating pulse in the absence of extracellular Ca^{2+} and ER Ca^{2+} store (depleted by CPA with 0 mM $[Ca^{2+}]_o$)) was subtracted from the heat-induced responses of cells in the presence of 10 mM $[Ca^{2+}]_o$.

Western blotting

For detecting STIM1 protein, keratinocytes derived from either wild type or STIM1-deficient littermates or STIM1-ΔK knock-in mice were grown in 6-well plates for 48 h prior to cell lysis using RIPA Lysis and Extraction Buffer (Sigma). Cell lysate derived from human STIM1-overexpressing HeLa cells was used for positive control as shown in Fig. 1a, d. 30 μg of whole cell lysate was subjected to SDS-PAGE electrophoresis and transferred to PVDF membrane at 100 V for 1 h. The membrane was blocked with 5% milk in TBS buffer with 0.1% Tween-20 (TBST buffer) at RT

for 1 h, and then incubated with the anti-STIM1 antibodies (1:1,000) at RT for 1 h, which were purchased from BD Biosciences (GOK/STIM1, clone 44, N-terminus) and Thermo (antibody ID: MA1-19451, C-terminus). After being washed with TBST buffer, the membrane was incubated with the anti-mouse IgG antibody (1:10,000) at RT for 1 h. Proteins were detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo).

Immunofluorescence staining

Dorsal skin specimens were collected from 7-week-old mice. Tissues were processed for immunostaining as previously described [4]. The primary antibodies include the anti-STIM1 antibody (Proteintech, 11561-1-AP), anti-KRT5 (Biorbyt, arb135811) and anti-Loricrin (Sigma-Aldrich, SAB2108557) for staining the basal and late differentiated granular layer of the skin, respectively. Fluorescent images were captured using a Nikon confocal microscope C2.

Keratinocytes cultured on coverslips were fixed with 4% paraformaldehyde (PFA) followed by cell permeabilization using 0.02% Triton X-100, and then blocked with 3% bovine serum albumin (BSA) in 1× PBS buffer. The cells were then incubated with the mouse KRT-5 antibody (1:500, Sigma, SAB1402245) at RT for 1 h. After being washed with the TBST buffer, cells were incubated with the Alexa Fluor 488 donkey anti-mouse IgG secondary antibody (Invitrogen, 1:500) at RT for 1 h followed by washing. The cells were incubated with DAPI for nuclear staining before the coverslips were mounted onto the glass slide for confocal imaging. All the imaging

procedures were performed on the Nikon A1 confocal microscope (Nikon Instruments) with 20× or 60× objectives. The images were analyzed using the Nikon NIS-Elements AR software.

shRNA-mediated knockdown in primary keratinocytes using lentivirus infection

A third generation of lentivirus packaging system was used to insert oligonucleotides encoding shRNAs. shRNA sequences targeting mouse STIM1, Orai1 or Orai3 sequences were selected by web-based RNAi designer (Supplementary information, Table S1). Annealed shRNA oligonucleotides were ligated into HpaI-XhoI sites of the PLL3.7-mCherry vector after U6 promotor. Lentivirus was produced by co-transfecting PLL3.7-shRNA and helper vectors (pMDLg/pRRE, pRSV-Rev and pCMV-VSV-G) into HEK293T cells. Viral supernatants were collected after 72 h and concentrated by 300-fold using polyethylene glycol precipitation (P5413, Sigma). Virus was aliquoted, flash-frozen in liquid nitrogen and stored at -80 °C. For keratinocytes infection, new medium containing 5 μL lentivirus and 8 μg/mL Hexadimethrine bromide (H9268, Sigma) was added into one well of 24-well plate (1/2 total cells from 1 pup plated in a 24-well plate) for 12 h. Keratinocytes were used for Ca^{2+} imaging or other assays 72 h after lentivirus infection.

Quantitative real-time PCR (qRT-PCR)

T cells isolated by kit (STEMCELL, 19851A), spleen from the adult mouse, HEK293T and RNA from wild-type keratinocytes or lentivirus-infected keratinocytes

after 4 days of culture was isolated using the RNA miniprep kit (Axygen, 04113KD1). The concentration and purity of the isolated RNA was measured by NanoDrop (Thermo Scientific). Total RNA (2 μg) was reverse transcribed in a reaction volume of 20 μL using the RevertAid first-strand cDNA synthesis Kit (Thermo Scientific, K1622). Primers for all qPCR experiments were used as the previously described [5]:

STIM1-Forward: 5'-GGCCAGAGTCTCAGCCATAG-3';

STIM1-Reverse: 5'-TCCACATCCACATCACCATT -3';

Orai1-Forward: 5'-CCTGTGGCCTGGTTTTTATC -3;

Orai1-Reverse: 5'-GTGCCCGGTGTTAGAGAATG -3';

Orai3-Forward: 5'-GGGTAAACCAGCTCCTGTTG -3';

Orai3-Reverse: 5'-GCCTGGTCCATGAGCACTAT -3'.

Real-time PCR reactions were conducted using the SYBR® Premix Ex Taq™ (Takara, RR420) in the ViiA™ 7 Real-Time PCR System. Expression of STIM1, Orai1 or Orai3 was normalized to β-actin. Cycling conditions were as follows: Hold on 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Melting curve analysis was performed at 95 °C for 15 s and 65 °C for 15 s. The results were analyzed by the ViiA™ 7 Real-Time PCR software, and the gene expression was determined using the comparative Ct method [6]. The mRNA levels were expressed as normalized mRNA levels (%).

Constitutive STIM1 KO mice (STIM1$^{-/-}$)

We obtained a gene trap embryonic stem (ES) cell line 335A11, derived from R1 ES cells, containing an insertional disruption in the *STIM1* gene (http://www.genetrap.org/cgi-bin/annotation.py?cellline=CMHD-GT_335A11-3) from Centre for Modeling Human Disease (CMHD), Toronto. In this ES cell line, a poly-A trap vector (UPA)[7] is inserted in intron 6 of the *STIM1* gene. The ES cell line 335A11 was injected into mouse blastocysts to generate chimeric mice. Male chimeras from this ES cell line were bred to C57BL/6J females to generate $STIM1^{+/-}$ mice, which were intercrossed to generate $STIM1^{-/-}$ mice. The exact insertion site of the gene-trap vector was determined by PCR using primers in the gene-trap cassette and extending out into intron 6 up to neighbouring exons 6 and 7. Based on the insertion site, a genotyping PCR strategy was designed using PCR with the primers: 335A11 geno R: gcctatctctctacctgccaagtcc; 335A11 geno F: ccaggtggttgtgtgctcttgaatg; UPA geno 5' end rev: cagtggtccaggctctagttttgac. We detected PCR products with the predicted sizes (385 bp for the wild-type allele and 766 bp for the allele with the integration of the UPA trap vector). Integration of the UPA trap vector into intron 6 of the STIM1 gene would result in the generation of two transcripts. RT-PCR confirmed that there is a disruption of the STIM1 transcript in STIM1-deficient keratinocytes by using primers located in exon 5 and exon 7 (data not shown). Furthermore, we did not detect STIM1 protein from cultured keratinocytes derived from newborn (P0-P3) STIM1-deficient mice (Fig. 1d). Importantly, SOCE was dramatically reduced in STIM1-deficient keratinocytes (Fig. 1e), suggesting that there is a lack of functional STIM1 in STIM1-deficient mice. Consistent with the other STIM1-deficient mouse

lines reported previously[8-11], STIM1-deficient mice display early postnatal lethality (19 live STIM1-deficient mice out of 142 pups during the period from P0 to P2) and growth retardation for the survived mice.

Generation of STIM1-ΔK knock-in mice

An ES cell targeting construct was generated that would allow for knocking in a new stop codon (replacing the sequence of AGGAAG with TGATCA) into the exon 12 of the mouse *STIM1* gene (Fig. 2a). The pBSACN vector contains a testes-specific promoter (tACE-promoter)-driven Cre-encoding region, followed with a neomycin-encoding region driven by a constitutive promoter[12]. The Cre and neomycin (Neo) cassette was flanked by two LoxP sites. A 6.0 kb 5' homology arm and a 3.0 kb 3' homology arm were amplified from mouse embryonic stem cell DNA and inserted upstream (SalI-XbaI) and downstream (XmaI-SacII) of the loxP-Cre-Neo-loxP cassette, respectively. The HSV-TK negative selection gene was inserted after the 3' homology arm. The entire targeting construct was sequence verified and linearized by SalI in preparation for embryonic stem cell electroporation. C57BL/6-derived embryonic stem cell clones that survived neomycin selection were screened by PCR. For the PCR positive clones, the PCR product containing the 3′ arm (PCR-F2: GCTGACCGCTTCCTCGTGCTTTA; PCR-R: CTCTGACCATCCAAACCCTCGTG) was gel excised and sequence verified for the integration of the TGATCA sequence. Five sequence verified positive clones were then subjected to southern blotting using probes located upstream of the 5′ arm

(Probe1, digestion with XbaI, 22.6 kb of wild type allele and 17.9 kb of recombinant allele) and in the Neo region (Probe2, digestion with HindIII, 7.2 kb of recombinant allele), respectively, to confirm the integration of both arms of homology. All five clones were confirmed positive (data not shown). The positive clones were subjected to blastocyst injections. The resulting male chimeras were mated to wild-type C57BL/6 female mice to obtain $STIM1^{WT/\Delta K}$ heterozygous mice. The loxP-Cre-Neo-loxP cassette was self-excised by the tACE-driven Cre during spermatogenesis [12]. The $STIM1^{WT/\Delta K}$ heterozygous mice were intercrossed to generate $STIM1^{\Delta K/\Delta K}$ homozygous mice (STIM1-ΔK mice). Genotypes were determined by PCR using the primers: STIM1-ΔK-F, 5'-GCCCACCTATAAATTCTGTGAGAGGG-3' and STIM1-ΔK-R, 5'-TGGGCCTGATCCCTGCTGAA-3'.

Behavioral assays

The temperature gradient assay and two-temperature choice assay shown in Fig. 4 were performed as described previously using the custom generated thermal gradient or two-temperature choice apparatus[13]. Briefly, mice were individually tracked for 2 h in the apparatus in ambient light using a video camera controlled by the software provided by the manufacturer. The time spent in each zone in a 30 min of interval for temperature gradient assay or 15 min of interval for two-temperature choice assay was determined.

For the two-temperature choice assay shown in Figs. 5-7 and Supplementary information, Figs. S4, 6, 7, the experiment was performed in the two-temperature choice apparatus purchased from Bioseb as described previously[14]. In brief, each of the two plates was pre-set to the desired temperatures. Mice were then placed at the border of the two plates and tracked on a second-basis for 1 or 2 h using a video camera controlled by the software provided by the Bioseb. The time spent on each plate in either 10 or 20 min of interval was analyzed for determining the thermal preference behaviors. As a control, when both plates were set to 33 °C , WT mice preferred equally to both sides (not shown), excluding any side preference of the apparatus.

For tryptase-induced itch behaviors, the experiments were carried out as described previously[15]. All other behaviors, including cold or hot plate assays, Hargreaves for radiation heat and Von Frey assay, were conducted according to previously described methods[14,16].

TSLP detection

Primary mouse keratinocytes were seeded into three 24-well plates with 1 mL medium in each well, and cultured at 33 °C, 35 °C and 37 °C for 60 h, respectively. Each genotype had three replicates in each plate. The cultured supernatant medium was collected and centrifuged for 20 min at 12,000 rpm at 4 °C to get rid of debris. The level of TSLP was measured by using the TSLP ELISA kit (eBioscience, 88-7490). For total protein levels, keratinocytes were lysed with RIPA buffer (Sigma,

R0278) and analyzed using Pierce™ BCA Protein Assay Kit. The measured amounts of TSLP were normalized to the protein levels. The experiments were repeated at least three times.

ATP detection

Primary mouse keratinocytes were seeded into 12-well plates with 1 mL medium in each well, and cultured at 33 °C for 2 days. Each genotype had three replicates in each plate, two plates at least once. After discarding the supernatant medium, 600 μL of medium with the ATPase inhibitor ARL 67156 (Sigma, A265) was added. Then the plates were kept at 33 °C, 35 °C, 37 °C and 39 °C incubators for 50 min, respectively, and then 100 μL of supernatant medium was collected for ATP detection using the CellTiterGlo® Luminescent Cell Viability Assay (Promega, G7570). For total protein levels, keratinocytes were lysed with RIPA buffer (Sigma, R0278) and analyzed using Pierce™ BCA Protein Assay Kit. The measured amounts of ATP were normalized to the protein levels. The experiments were repeated at least three times except for 35 °C (two times).

Endothelin-1 detection

Primary mouse keratinocytes were seeded into 6-well plates with 2 mL medium in each well, and cultured at 33 °C and 37 °C for 60 h, respectively. Each genotype had three replicates in each plate. Then the cells were washed with DPBS once after removal of the supernatant medium. RNA was extracted from each well using the

RNA miniprep kit (Axygen, 04113KD1). The concentration and purity of the isolated RNA were monitored by NanoDrop (Thermo Scientific). Total RNA (2 μg) was reverse transcribed in a reaction volume of 20 μL using the RevertAid firststrand cDNA synthesis Kit (Thermo Scientific, K1622). Primers for RT-PCR experiments include Endothelin-1-Forward (5'-ACTCCATTCTCAGCTCCGGT-3') and Endothelin-1-Reverse (5'- CTCTTCTGACCCCTTTGCAG-3'). RT-PCR reactions were conducted using the SYBR® Premix Ex Taq™ (Takara, RR420) in the ViiA™ 7 Real-Time PCR System. Expression of Endothelin-1 was normalized to GAPDH. The results were analyzed using the ViiA™ 7 Real-Time PCR software, and the gene expression was determined using the comparative Ct method. The mRNA levels were expressed as normalized mRNA levels.

PGE_2 and mKC measurement

Primary mouse keratinocytes were cultured from neonatal mice. Cells from each pup were plated in each well of 6-well plate and cultured for 3 days at 37 °C, 5% CO_2 before experimental test. Cells were washed with 1× HBSS buffer with 2 mM Ca^{2+} (pH = 7.4) twice and acclimated at RT for 30 min in 1 mL buffer with 2 mM Ca^{2+} (3 wells for each condition). After 30 min acclimation at RT, 1 mL of supernatant was collected as the baseline sample. 1 mL of preheated buffer (45 °C) was immediately added into the cells. The cell plates were then maintained in a 45 °C water bath for 30 min before 1 mL of supernatant was collected as the heat on sample. 1 mL of room temperature buffer was immediately added into the cells. The cell plates were then

maintained at RT for 30 min before 1 mL of supernatant was collected as the heat off sample. Finally, 1 mL of buffer with 10 μM ionomycin was added and the plate was maintained at RT for 30 min before the supernatant was collected as the ionomycin sample. Keratinocytes were lysed with 50 μL RIPA buffer (Sigma, R0278) and the resulting protein level was detected by PierceTM BCA Protein Assay kit (Thermo Scientific, Prod# 23227). The collected supernatant was spun at 4 °C at 10,000 rpm for 1 min to remove cells and debris. The samples were subjected to ELISA analysis for detecting mKC using the mouse CXCL1/KC Quantikine ELISA Kit (R&D, MKC00B) and LC-MS/MS analysis for detecting PGE_2 (MW, 352.47), respectively. PGE_2 measurement was conducted by following the previously described protocol[17]. 10 μL of d4-PGE_2 solution (1,000 ng/mL, Cayman Chemical, CAS 34210-10-1) was spiked into all samples before processing to track endogenous PGE_2 recovery. C18 column (Sep Pak Vac C18 1cc) was conditioned with 0.5 mL MeOH (HPLC level), and 0.5 mL 75/25 H_2O/MeOH. Samples were loaded onto column, respectively, and then the column was washed with 1 mL water. PGE_2 was eluted with 1.5 mL 65/35 MeOH/H_2O at 4 °C. The elutant was dried by SpeedVac and then reconstituted with 100 μL 30/70 ACN/H_2O before analyzed on AB SCLEX Q TRAP 4500 MS with the following conditions: linear gradient from 70% isopropanol with 30% MeOH with water with 0.1% formic acid at 200 μL/min, C8 HPLC column (Unison UK-C8), electrospray negative ion multiple reaction mode (351.20---315.1; 355.2---319.2). Standard curves were generated by pure PGE_2 stock solution (Cayman Chemical, CAS 363-24-6) at the following concentrations (ng/mL): 0.25, 0.25, 0.5, 1, 5, 25, 125,

250 and 500. Additionally, 10 μL of 1,000 ng/μL d4-PGE2 solution was spiked into the above solution (90 μL) before detection. Finally, the amount of PGE_2 was corrected for recovery and protein content in each culture well. The PGE_2 levels were expressed as normalized PGE_2 fold changes.

NO measurement

Keratinocytes from neonatal mice were grown on 8 mm coverslips in 24-well plate for three days before experiment. The coverslips were loaded with DAF-FM deacetate (Life Technologies, D-23844) for 20 min in 2 mM Ca^{2+} (pH = 7.2) buffer solution. Heat stimulation was conducted using bath solution controlled by a CL-100 temperature controller (Warner Instruments) and a SC-20 Solution In Line Heater/Cooler (Harvard Apparatus). Images of the loaded cells were recorded with a LCD camera using MetaFluor with the excitation/emission wavelengths at ~488/515 nm. The fluorescence intensity of individual cells in each experiment was normalized to the baseline (F/F_0).

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