Supplemental Information

Supplemental Data

Supplementary Figure 1. Generation of SASPase-deficient hairless mice.

(A) Anti-SASPase immunoblotting of the epidermal protein extracts of SASPase-deficient hairless mice. Epidermal protein extracts (10 µg) from SASP$^{+/+}$, SASP$^{+/-}$, and SASP$^{-/-}$ mice were immunoblotted with anti-SASP-C pAb (upper panel). CBB staining for each lane shows equivalent concentrations of loaded protein (lower panel). In the SASP$^{+/+}$ and SASP$^{+/-}$ epidermal extract, SASP32 (32 kDa) bands were detected, whereas in the homozygous one, this band was not detected.

(B) Loss of the SASPase protein in the epidermis of SASP$^{-/-}$ hairless mice examined by immunofluorescence microscopy. Frozen sections of back skin from SASP$^{+/+}$ and SASP$^{+/-}$ mice were stained with anti-SASP-C pAb (red). Nuclei were counterstained with bisbenzimide (blue). In the SASP$^{+/-}$ type of epidermis, the SASP32 protein was expressed in the granular layer of the epidermis, whereas in the SASP$^{-/-}$ epidermis, these signals became undetectable. Dashed lines represent the border between the epidermis and dermis. Scale bar: 10 µm.

Supplementary Figure 2. Normal expressions of epidermal differentiation markers in SASP$^{-/-}$ hairless mice. (A) Immunofluorescence staining of frozen sections of back skin of SASP$^{+/-}$ (A, C, E, F) and SASP$^{-/-}$ (B, D, F, H) hairless mice stained with anti-keratin 14 (A and B), keratin 1 (C and D), involucrin (E and F), and loricrin (G and H) pAbs (red). Nuclei were counterstained with bisbenzimide (blue). The SASP$^{+/-}$ epidermis showed a normal distribution and expression level of these markers. Dashed lines represent the border between the epidermis and dermis. Scale bar: 10 µm.
(B) Epidermal protein extracts from SASP<sup>−/−</sup> and SASP<sup>+/−</sup> mice were immunoblotted with anti-keratin 14, keratin 1, involucrin, and loricrin pAbs (0.3 µg of extracts for anti-keratin 14 and keratin 1, and 2 µg for involucrin and loricrin were subjected to immunoblotting, respectively.) Expression levels of these markers in the SASP<sup>−/−</sup> epidermis were indistinguishable from those in the SASP<sup>+/−</sup> epidermis.

**Supplementary Figure 3.** Physiological analysis of the SC of human epidermis with SASP(V243A) mutation. Trans-epidermal water loss (TEWL) (A) (number of areas tested =3) and SC hydration levels (B) (number of areas tested =5) of SASP(V243A)/+ and three SASP<sup>+/+</sup> individuals were measured using VAVO SCAN and ASA-M1. The horizontal lines indicate median values.

**Supplementary Table I.** Missense mutations of human SASPase in AD patients

Resulting from the mutation search of the human SASPase gene, four types of missense mutations (A54S, I186T, V187I, R311C) in AD patients and two types of missense mutations (D232Y, V243A) in the control subjects were identified. All mutations were heterozygous. A54S and R311C were found in the same patient and in the same allele. The V187I mutation was identified most frequently (three AD patients).

**Supplementary Table II.** Silent mutations of SASPase in AD patients

From the results of the mutation search on the human SASPase gene, three types of silent mutations (F101F, P206P, N276N) in the AD patients were identified. All mutations were heterozygous. P206P was found in the patient with N276N.
Supplemental materials and methods

Immunofluorescence

Mouse back skin was fixed in 2% paraformaldehyde / PBS for 1 h at room temperature. Next, samples were incubated with 10% sucrose in PBS for 3 h at 4 °C and then 20% sucrose in PBS overnight at 4 °C. Finally, samples were mounted in Tissue-Tek OCT compound (Sakura Fine-Technical, Tokyo, Japan) and frozen on dry ice. Frozen samples were cut into 5-µm thick sections on a cryostat, mounted on silane-coated glass slides and air-dried. The samples were then soaked in Block-Ace blocking solution (Dainippon Pharmacy, Osaka, Japan) for 1 h at room temperature and subsequently incubated in primary antibodies for 1 h at room temperature. Sections were washed three times with PBS and incubated with secondary antibodies for 30 min at room temperature. Cy3-labeled donkey anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA) and bisbenzimide (Sigma-Aldrich Japan) were used as secondary antibodies. Samples were washed three times with PBS and mounted in Mounting Reagent (Roche Diagnostics, Tokyo, Japan). Phase contrast and fluorescent images were obtained using a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan) using BZ Analyzer Software (Keyence).

Scanning electron microscopy

The specimens were fixed in 2.5% glutaraldehyde in 0.1 M PBS for 2 h. The specimens were washed overnight at 4°C in the same buffer and post-fixed with 1% OsO₄ buffered with 0.1 M PBS for 2 h. The specimens were dehydrated in a graded series of ethanol and dried in a critical point drying apparatus (HCP-2; Hitachi) with liquid CO₂. The specimens were sputter-coated with platinum and examined by SEM (S-4500; Hitachi,
Transmission electron microscopy

The specimens were fixed in 2.5% glutaraldehyde in 0.1 M PBS for 2 h. The specimens were washed overnight at 4°C in the same buffer and post-fixed with 1% OsO₄ buffered with 0.1 M PBS for 2 h. The specimens were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin (90 nm) sections were collected on copper grids, double-stained with uranyl acetate and lead citrate, and then examined by TEM (H-7100, Hitachi, Hitachinaka, Japan).

Preparation of mice SC urea-extracts

The dorsal and back skin of SASP⁺/⁺, SASP⁺/- and SASP⁻/- hairless mice were washed with 10 ml per mouse of an urea-buffer (5 M urea/2M thiourea, PBS, 1 mM EDTA supplemented with protease inhibitor cocktail (Nacalai Tesque, Tokyo, Japan)). Thereafter, the washed area was scraped with the edge of a microscope slide and continuously rinsed with the 8 M urea solution. The buffer containing corneocytes was collected in a container placed below the mice. Corneocytes were removed by centrifugation at 3,000 × g for 30 min at 4 °C. The supernatant was concentrated using an Amicon-ultra (Millipore, Billerica, MA).

cDNA cloning and recombinant protein expression

Human poly (A)+ RNA was purchased from Invitrogen. First strand cDNA was prepared by a PrimeScript® 1st strand cDNA Synthesis Kit (TAKARA, Shiga, Japan). The DNA fragment encoding open reading frame of the human SASPase 28 kDa form
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(hSASP28) was amplified by Prime STAR MAX (TAKARA) from a human skin cDNA library by PCR using the 5’-SalI-hSASP28 primer (5’-AATT-GTCGAC-ATG-gccggagcggagcagggt-3’) and 3’-NotI-hSASP28 primer (5’-ATAT-GCGGCCGC-TCA-gtggatatagctctgccg-3’), respectively. PCR products were subcloned into a pMD20 vector using a Mighty TA cloning kit (TAKARA). After digestion with SalI and NotI, the cDNAs were subcloned into the SalI-NotI sites of pGEX4T-3 (GE Healthcare, Japan) to yield pGEX-hSASP28. Various mutants of pGEX-hSASP28(I186T), (V187T), (R311C), (D232Y) and (V243T) were generated by PCR from each patient’s genome using the 5’-SalI-hSASP28 primer and 3’-NotI-hSASP28 primer. Recombinant proteins including, GST-hSASP28(I186T), (V187T), (R311C), (D232Y) and (V243T) were produced and purified according to the manufacturer’s instructions (GE Healthcare Japan, Tokyo, Japan) and dialyzed against 50 mM phosphate buffer (pH 6.0) and 0.7 M NaCl. The DNA fragment encoding the open reading frame of the human monomeric filaggrin with the linker peptide was amplified as described above using the 5’-SalI-hFil primer (5’-ATAT-GTCGAC-tctggacgctcaggttctctaccag -3’) and 3’-NotI-Fil primer (5’-AATT-GCGGCCGC-CTA-gctttccctgactgcaagcgtgaccactc -3’), respectively. PCR products were subcloned and the cDNAs were subcloned into the SalI-NotI sites of pMal-c2 (New England Biolab, Beverly, MA, USA) to yield pMal-linker-hFil. MBP-hFilaggrin was purified according to the manufacturer’s instruction and dialyzed against 50 mM phosphate buffer (pH 6.0) and 0.7 M NaCl.

Clinical materials
Blood samples were obtained from 196 Japanese AD patients and 28 control subjects. The diagnostic criteria of AD patients are described (Sasaki et al., 2008), and the controls had never been given a diagnosis of AD. This study was approved by the committee for medical ethics at Keio University, Japan, and written consent was always obtained in the formal style. Basal SC hydration was measured with a moisture meter (ASA-M1; Asahi Biomed, Tokyo, Japan) on a forearm area (5 x 5 cm) of three male individuals without SASPase and filaggrin mutation and a male individual with V243A mutation at a room temperature of 24°C and a relative humidity of 40%.

Mutation analysis on human SASPase

All DNA samples were amplified by PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA) and the gene specific primers. PCR amplification conditions were as follows: 1 cycle of 95 °C for 9 min, 55 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 3 min, and 1 cycle of 72 °C for 5 min. PCR products were treated with ExoSAP-IT (USB, Cleveland, OH). Sequencing reactions were performed with the ABI BigDye Terminator Sequencing Kit and the ABI3100 capillary sequencer (Applied Biosystems). The primers for PCR and sequencing included:

PCR primer forward 5'- ATGTGGTAGGAGCTCAGTACATGTAAAC -3'
PCR primer Reverse 5'- AGAAGAGCAAGAGTTGATAAGCAGACTG -3'
Sequencing primer F1 5'- TTCTTTCACTGGGCTGATGAC -3'
Sequencing primer F2 5'- TTGCTGCTGAGGTTCCAGAG -3'
Sequencing primer F3 5'- TCACTGATGGCGATCTGGAC -3'
Sequencing primer R1 5'- AGAAGAGCAAGAGTTGATAAGC -3'
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Sequencing primer R2 5’- CCCAGGATCTTCATTTTACGC -3'
Sequencing primer R3 5’- GATGACTTCAAGCTGTGCAG -3'

Supplementary References


Matsui et al., Supplementary Figure 1
Matsui et al., Supplementary Figure 2
Matsui et al., Supplementary Figure 3
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Total 5 2

†These mutations were found in the same patient.
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| Total  | 9                 | 0                 |

†These mutations were found in the same patient.