

A homozygous nonsense mutation in the gene for *Tmem79*, a component for the lamellar granule secretory system, produces spontaneous eczema in an experimental model of atopic dermatitis

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Background: Flaky tail (*ma/ma Flg^{fl/fl}*) mice have a frameshift mutation in the filaggrin (*Flg^{fl}*) gene and are widely used as a model of human atopic dermatitis associated with *FLG* mutations. These mice possess another recessive hair mutation, *matted (ma)*, and develop spontaneous dermatitis under specific pathogen-free conditions, whereas genetically engineered *Flg^{-/-}* mice do not.

Objective: We identified and characterized the gene responsible for the matted hair and dermatitis phenotype in flaky tail mice. **Methods:** We narrowed down the responsible region by backcrossing *ma/ma* mice with wild-type mice and identified the mutation using next-generation DNA sequencing. We attempted to rescue the matted phenotype by introducing the wild-type *matted* transgene. We characterized the responsible gene product by using whole-mount immunostaining of epidermal sheets.

Results: We demonstrated that *ma*, but not *Flg^{fl}*, was responsible for the dermatitis phenotype and corresponded to a *Tmem79* gene nonsense mutation (c.840C>G, p.Y280*), which encoded a 5-transmembrane protein. Exogenous *Tmem79* expression rescued the matted hair and dermatitis phenotype of

Tmem79^{ma/ma} mice. *Tmem79* was mainly expressed in the trans-Golgi network in stratum granulosum cells in the epidermis in both mice and humans. The *Tmem79^{ma/ma}* mutation impaired the lamellar granule secretory system, which resulted in altered stratum corneum formation and a subsequent spontaneous dermatitis phenotype.

Conclusions: The *Tmem79^{ma/ma}* mutation is responsible for the spontaneous dermatitis phenotype in *matted* mice, probably as a result of impaired lamellar granule secretory system and altered stratum corneum barrier function. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Atopic dermatitis, skin barrier, stratum corneum, trans-Golgi network, filaggrin

Loss-of-function mutations in the filaggrin (*FLG*) gene cause ichthyosis vulgaris, a common autosomal dominant keratinization disorder characterized by scaly skin, and are a major factor predisposing one to atopic disorders, including atopic dermatitis (AD), atopic asthma, hay fever, and food allergy.¹⁻⁶ Filaggrin is a major component of the stratum corneum (SC) and plays an important role in its structural integrity.⁷ Consequently, defects in the *FLG* mutation-associated SC barrier lead to increased percutaneous allergen exposure and a subsequent heightened immune reaction.^{8,9} *FLG* mutations exist in 15% to 55% of patients with AD of various ethnic groups.^{4,10} Other predisposing factors remain to be identified in the remaining 45% to 85% of patients with AD.

The flaky tail (*Flg^{fl}*) mouse phenotype first arose as a spontaneous recessive mutation in 1958 on the background of the existing recessive hair phenotype, *matted (ma)*. Since then, the mice have been maintained as a mixed strain because these loci are closely linked on mouse chromosome 3.^{11,12} The *ma/ma Flg^{fl/fl}* mice express a truncated profilaggrin (approximately 215 kDa) instead of the normal profilaggrin (>500 kDa) because of a 1-bp deletion mutation, c.5303delA, which leads to premature termination 154 codons downstream (p.Asn1768Thrfs*154) in the murine *Flg* gene.^{13,14} Therefore *ma/ma Flg^{fl/fl}* mice are widely used as a model of AD to investigate its pathophysiologic mechanisms.¹⁵⁻²⁰ The *ma/ma Flg^{fl/fl}* mice have enhanced percutaneous allergen priming and develop spontaneous dermatitis under specific pathogen-free (SPF) conditions.¹⁴ In contrast, genetically engineered *Flg^{-/-}* mice have altered SC barrier function and enhanced percutaneous allergen priming but do not develop spontaneous dermatitis under SPF conditions.⁸

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Abbreviations used

AD:	Atopic dermatitis
CDSN:	Corneodesmosin
Flg:	Filaggrin
LG:	Lamellar granule
<i>ma</i> :	Matted
SC:	Stratum corneum
SG:	Stratum granulosum
SNV:	Single nucleotide variation
SPF:	Specific pathogen free
TEWL:	Transepidermal water loss
TGN:	<i>Trans</i> -Golgi network
WT:	Wild type

To examine this conflicting finding, we segregated the *ma* and *Flg^{fl}* mutations by backcrossing with wild-type (WT) mice (C57BL/6) to generate congenic strains and demonstrated that *ma*, but not *Flg^{fl}*, is responsible for the dermatitis phenotype and corresponds to a nonsense mutation (c.840C>G, p.Y280*) in the *Tmem79* gene. We further characterized the epidermal expression pattern of *Tmem79* in addition to the effects on lamellar granule (LG) secretion and SC formation caused by the mutation.

METHODS**Mice**

Flaky tail mice (*ma/ma Flg^{fl/fl}*) were obtained from Jackson Laboratory (Bar Harbor, Me). Flaky tail mice were backcrossed with C57BL/6J mice (CLEA Japan, Tokyo, Japan) to generate *ma/ma* and *Flg^{fl/fl}* mice. We genotyped *Flg^{fl}*, as described previously.¹⁴

Biophysical skin measurements

Skin hydration was evaluated by analyzing the skin electrical impedance with a Corneometer ASA-M2 (Asahi Biomed, Yokohama, Japan). Transepidermal water loss (TEWL) was measured with a VAPOSCAN AS-VT100RS (Asahi Biomed). All data are the median of 3 recordings.

DNA sequencing and transmembrane topology analysis

To narrow the *ma* candidate region, we established 9 markers that distinguished between C57BL/6 and CBA mouse alleles based on single nucleotide polymorphisms (SNPs). Genomic DNAs from *Tmem79^{malma}*, CBA, and C57BL/6 mice were sequenced by means of targeted resequencing with the Next Generation DNA Sequencer. Briefly, the extracted genomic DNA was enriched with the SureSelect Target Enrichment System (Agilent Technologies, Tokyo, Japan) and sequenced with the Illumina Genome Analyzer II (Illumina, Tokyo, Japan). The DNA sequence reads were mapped to the mouse reference genome (mm19) by using bwa,²¹ and single nucleotide variations (SNVs)/indels were extracted with SAMtools²² (see the [Methods](#) section in this article's Online Repository at www.jacionline.org for details). SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) and Octopus (<http://octopus.cbr.su.se/>) were used to predict the transmembrane location and topology.^{23,24}

Construction of a *Tmem79* transgenic vector and generation of *Tmem79^{Tg}* mice

The 9223-bp genomic region, including the entire *Tmem79* gene on bacterial artificial chromosome DNA RP23-168E1, was subcloned into pUC118 vector by using the Red/ET recombination system (GeneBridges, Heidelberg, Germany; see [Fig E1](#) in this article's Online Repository at www.jacionline.org) to generate *Tmem79^{Tg}* mice. The linearized *Tmem79* transgenic vector

was injected into pronuclei of fertilized eggs and implanted into pseudopregnant females. The mouse transgene was screened by using PCR to amplify the boundary region between the pUC118 vector and the *Tmem79* genomic sequence.

Evaluation of dermatitis severity

Mouse behavior was videotaped, and the number of scratches within a 5-minute period was recorded 3 times; the median value was analyzed. We scored the total clinical skin severity of the mice from 0 to 3 for 5 skin-associated phenotypes (pruritus, erythema, edema, excoriation, and scaling), as previously described.²⁵ The total serum IgE level was measured with an ELISA kit (Bethyl Laboratories, Montgomery, Tex).

Whole-mount immunostaining of epidermal sheets

Epidermal sheets from mouse ear and human shoulder skin were immunostained for *Tmem79* and other marker proteins. The skin specimens were treated with 3.8% ammonium thiocyanate, and the dermis and epidermis were separated. Whole-mount staining of epidermal sheets was performed, as described previously.²⁶ *Tmem79* and other organelle marker proteins were observed by using z-stack scanning with a Leica TCS SP5 confocal microscope (Leica Microsystems, Tokyo, Japan).

Antibodies

Rabbit antibodies were raised against synthetic peptides containing *Tmem79* N-terminal residues 99 to 115 (Ab^{matN}) and C-terminal residues 376 to 391 (Ab^{matC}). Other antibodies used in this study are shown in the [Methods](#) section in this article's Online Repository.

Immunoelectron microscopy

Postembedding immunoelectron microscopy was performed, as described previously, with slight modification.²⁷ Anti-TMM79 rabbit antibody (Abgent, San Diego, Calif) and anti-human *trans*-Golgi network (TGN) 46 sheep antibody (AbD Serotec, Oxford, United Kingdom) were used as primary antibodies, and 10-nm gold-labeled donkey anti-sheep IgG (BBInternational, Cardiff, United Kingdom) and 5-nm gold-labeled goat anti-rabbit IgG (BBInternational) were used as secondary antibodies.

Tape stripping

The dorsal skin of P5 neonatal mice was repeatedly tape stripped at the same sites with Cryofilm Type 2C (Leica Microsystems) to assess SC layer detachment under mechanical stress. The remaining SC layer was evaluated by using safranin staining, and the number of focal abnormal desquamated regions in which all layers peeled off were counted.

Statistical analysis

All experiments were analyzed by using 2-tailed Student *t* tests with GraphPad Prism 5.0 (GraphPad Software, San Diego, Calif). All results are presented as means and SEMs. A *P* value of less than .05 was considered to indicate statistical significance.

RESULTS**Matted mutation, but not filaggrin mutation, is responsible for the matted hair and spontaneous dermatitis phenotype**

To generate each congenic mouse strain (*ma/ma* and *Flg^{fl/fl}*), we segregated the *ma* and *Flg^{fl}* mutations by backcrossing with WT mice (C57BL/6). As a result, *ma/ma* mutant mice, which displayed the *matted* hair phenotype, developed spontaneous

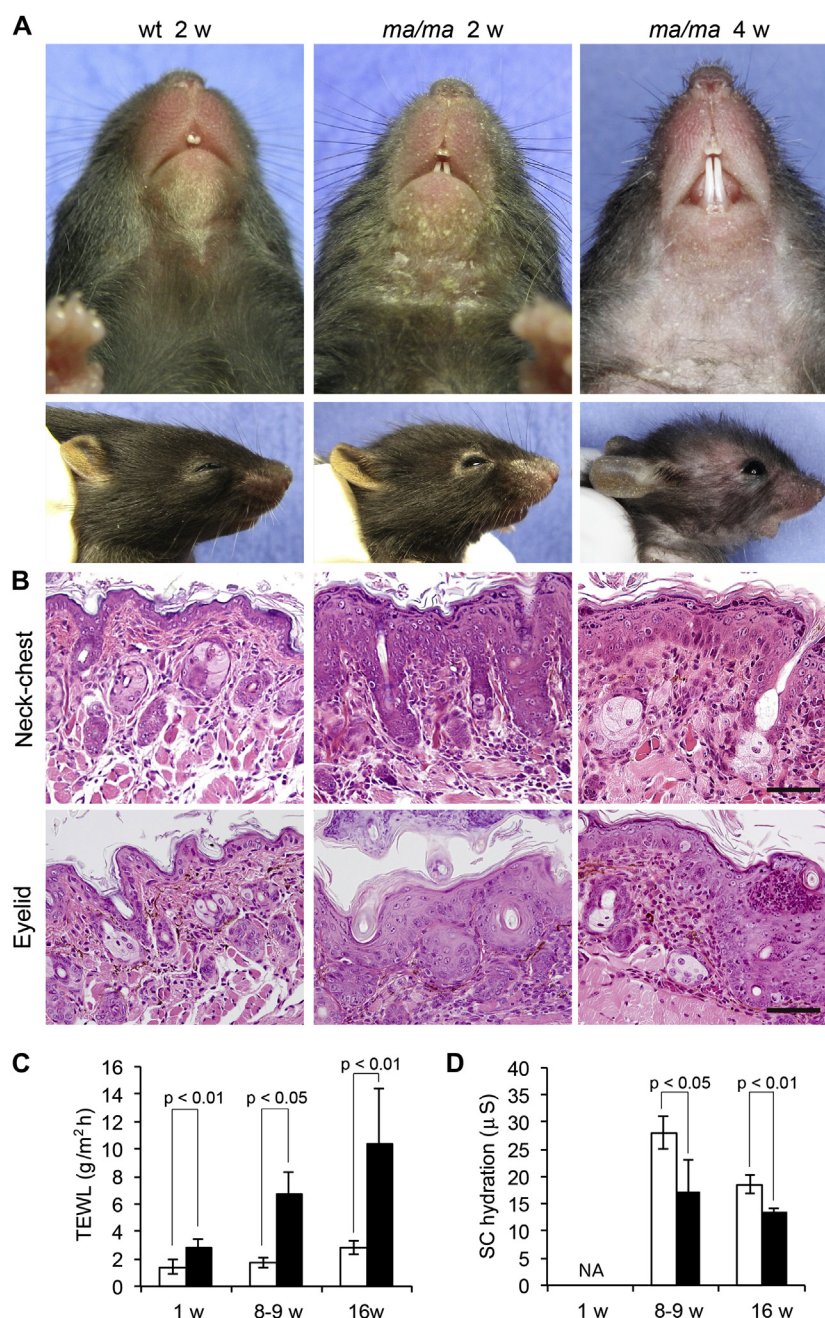


FIG 1. Development of spontaneous dermatitis in *ma/ma* mice. **A**, Gross phenotype of 2-week-old WT (C57BL/6), 2-week-old *ma/ma*, and 4-week-old *ma/ma* mice. **B**, Histology of the skin on the neck-chest area and eyelid. Bars = 100 μm. **C** and **D**, TEWL and SC hydration of the skin on the neck-chest area of 1-, 8-, and 16-week-old WT (C57BL/6: white bars, n = 5) and *ma/ma* (black bars, n = 5) mice. NA, Not analyzed.

dermatitis with scratching behavior, whereas *Flg^{fl/fl}* mice did not have dermatitis under SPF conditions (Fig 1, A and B, and see Table E1 in this article's Online Repository at www.jacionline.org). TEWL was higher and SC hydration was lower in *ma/ma* mice compared with WT mice (Fig 1, C and D). The enhanced TEWL and histologic changes, such as acanthosis with leukocyte infiltration, were observed as early as 1 week, when no apparent scratching behavior was observed, indicating that there is a primary defect in the skin leading to the development of dermatitis in *ma/ma* mice. Therefore we concluded that *ma*,

but not *Flg^{fl}* or *Flg^{-/-}*, is responsible for the development of spontaneous dermatitis in *ma/ma Flg^{fl/fl}* mice.

Identification of the gene responsible for the spontaneous dermatitis phenotype in *ma/ma* mice

Next we sought to identify the genetic mechanism underlying the *ma* mutant and study the detailed function of the responsible gene product. The *ma* locus is located between *spa* (*Glrh*) and *ft* (*Flg*) in an 80.7- to 93.1-Mb region of mouse chromosome 3.¹²

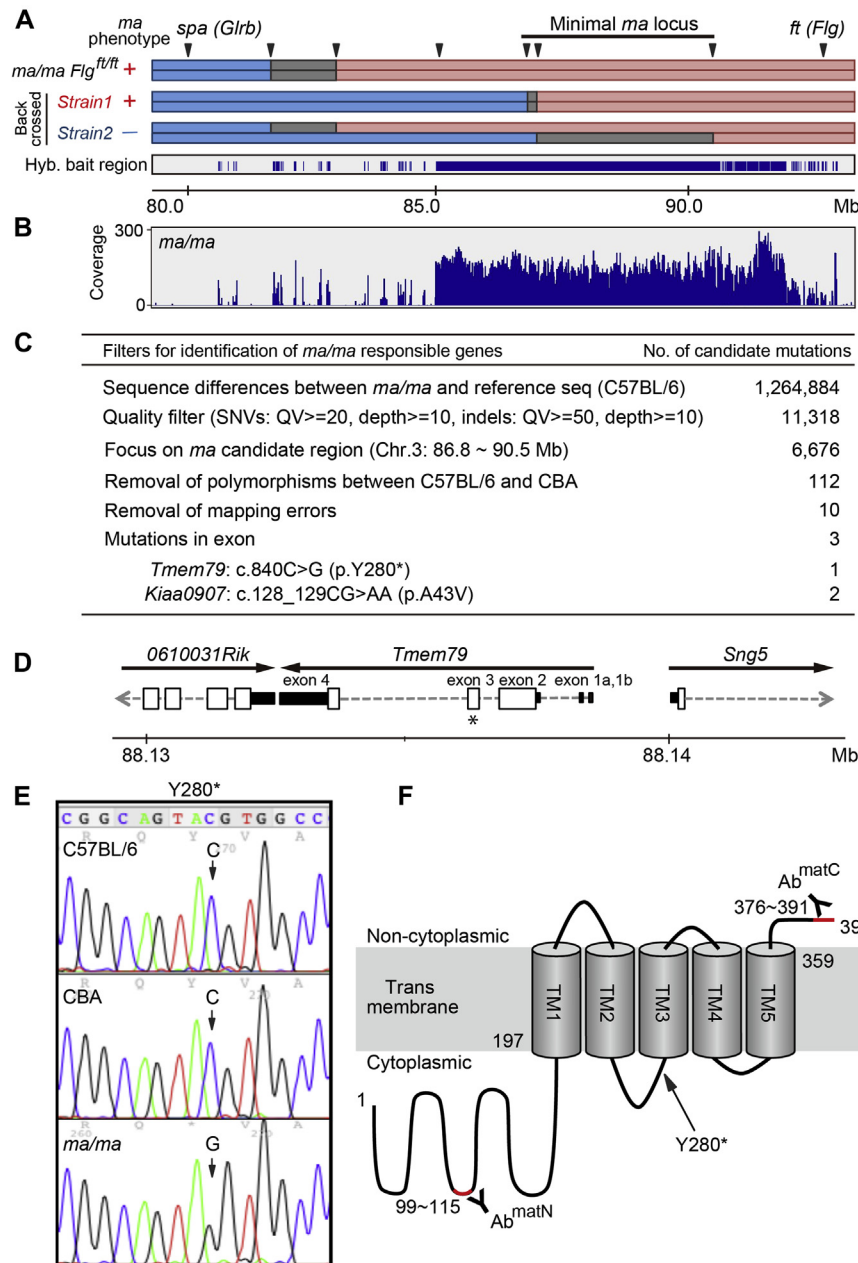


FIG 2. Identification of the *Tmem79* mutation as the gene responsible for *ma*. **A**, The minimal *ma* locus was defined by backcrossing *ma/ma* mice with C57BL/6 mice. The 2 rectangles indicate the mouse alleles. *ma/ma Flg*^{ft/ft}, Original flaky tail mouse; *ma* (+/-), matted positive or negative mice; blue, C57BL/6 allele; pink, CBA allele; gray, indistinguishable region. Triangles indicate the positions of polymorphic markers in C57BL/6 and CBA. Hyb. bait regions are the hybridization bait regions for targeted resequencing. **B**, Sequence coverage of the *ma* candidate region with targeted resequencing. **C**, Identification of the *ma* gene and a number of additional candidates carrying variations identified by means of targeted resequencing. **D**, Genomic structure of *Tmem79*. The asterisk in exon 3 indicates the c.840C>G mutation. White and black boxes indicate coding and noncoding regions, respectively. **E**, Confirmation of the *Tmem79* mutation in *ma/ma* mice by means of Sanger sequencing. **F**, Predicted protein structure of *Tmem79*. The location of transmembrane domains and topology were predicted by using the programs SOSUI and Octopus. Two rabbit anti-*Tmem79* antibodies (Ab^{matN} and Ab^{matC}) were raised against synthetic peptides derived from amino acids 99 to 115 and 376 to 391, respectively. p.Y280* is located between the second and third transmembrane domains of the *Tmem79* protein.

Therefore we defined a minimal *ma* locus by backcrossing *ma/ma* mice with WT mice and applied target enrichment, followed by next-generation DNA sequencing (Fig 2, A-C). After filtering sequence differences and making a detailed comparison of *ma/ma*

and reference genome sequences, we found 3 nucleotide substitutions in the exon of *ma/ma* mice: c.840C>G in *Tmem79* (nonsense mutation p.Y280*; Fig 2, C and E) and a 2-base substitution in *Kiaa0907*, c.128_129CG>TT (missense mutation p.A43V;

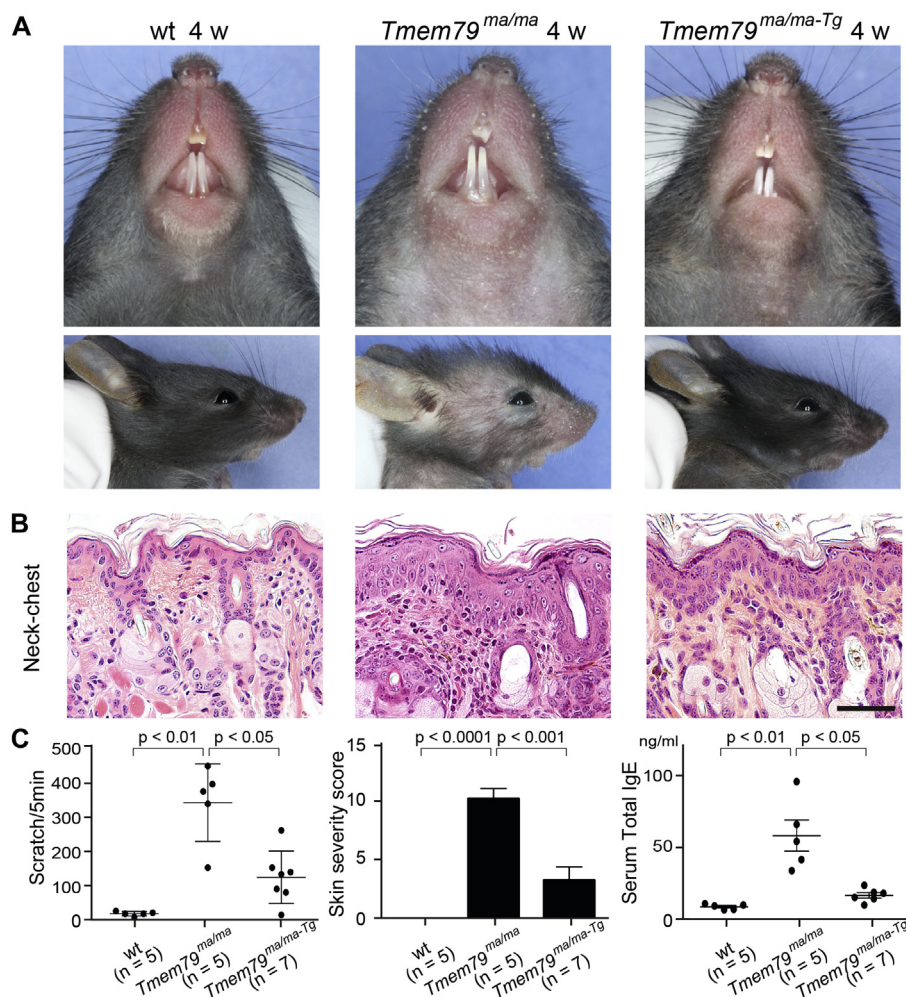


FIG 3. Rescue of the *ma* phenotype in *Tmem79*^{ma/ma} mice by means of transgenic reintroduction of WT *Tmem79*. **A**, Gross dermatitis phenotype of 4-week-old WT (C57BL/6), *Tmem79*^{ma/ma}, and *Tmem79*^{ma/ma-Tg} mice. **B**, Histology of the skin on the neck-chest. Bars = 100 μ m. **C**, Scratching frequency, skin severity score, and serum total IgE level were compared among 4-week-old WT (C57BL/6, n = 5), *Tmem79*^{ma/ma} (n = 5), and *Tmem79*^{ma/ma-Tg} (n = 7) mice.

Fig 2, C). Because *Tmem79* carried a nonsense mutation and was predominantly expressed in the skin of WT mice (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org), we focused on further characterization of *Tmem79*. The *Tmem79* gene was composed of 5 exons that encode a 43.4-kDa protein with 5 transmembrane domains (exon 1a and exon 1b for spliced variant). The *Tmem79* protein N-terminus is located in the cytoplasm, as predicted by transmembrane prediction programs. The c.840C>G mutation is located in exon 3, and p.Y280* is located between the second and third transmembrane domains (Fig 2, F).

Transgenic rescue of the matted hair and dermatitis phenotype of *Tmem79*^{ma/ma} mice

To confirm that *Tmem79* was responsible for the *matted* hair phenotype and spontaneous dermatitis in *Tmem79*^{ma/ma} mice, we attempted to rescue the phenotype by introducing a WT *Tmem79* transgene under control of the *Tmem79* promoter. We subcloned a fragment containing the entire *Tmem79* genomic locus, including the *Tmem79* promoter region from bacterial

artificial chromosome clone RP23-168E1, into pUC118 and used this for pronuclear injection of the *Tmem79* transgene (see Fig E1). The resultant *Tmem79*^{Tg} mice were subsequently mated with *Tmem79*^{ma/ma} mice. These *Tmem79*^{ma/ma-Tg} mice no longer developed matted hair or spontaneous dermatitis at 4 weeks, when the phenotype became apparent in *Tmem79*^{ma/ma} mice (Fig 3, A and B). *Tmem79*^{ma/ma-Tg} mice also displayed significantly less frequent scratching behavior than *Tmem79*^{ma/ma} mice (Fig 3, C). The skin severity scores and serum total IgE levels of *Tmem79*^{ma/ma-Tg} mice were significantly lower than those of *Tmem79*^{ma/ma} mice. *Tmem79*^{ma/ma-Tg} mice remained free from any apparent dermatitis phenotype for as long as 1 year. These findings confirm that *Tmem79* was responsible for the matted hair and dermatitis phenotype observed in *Tmem79*^{ma/ma} mice.

Tmem79 is primarily expressed in the outermost cells of the stratum granulosum

After examining various organs, *Tmem79* mRNA was expressed most strongly in the epidermis (see Fig E2). Rabbit antibodies raised against the synthesized peptides containing N-terminal

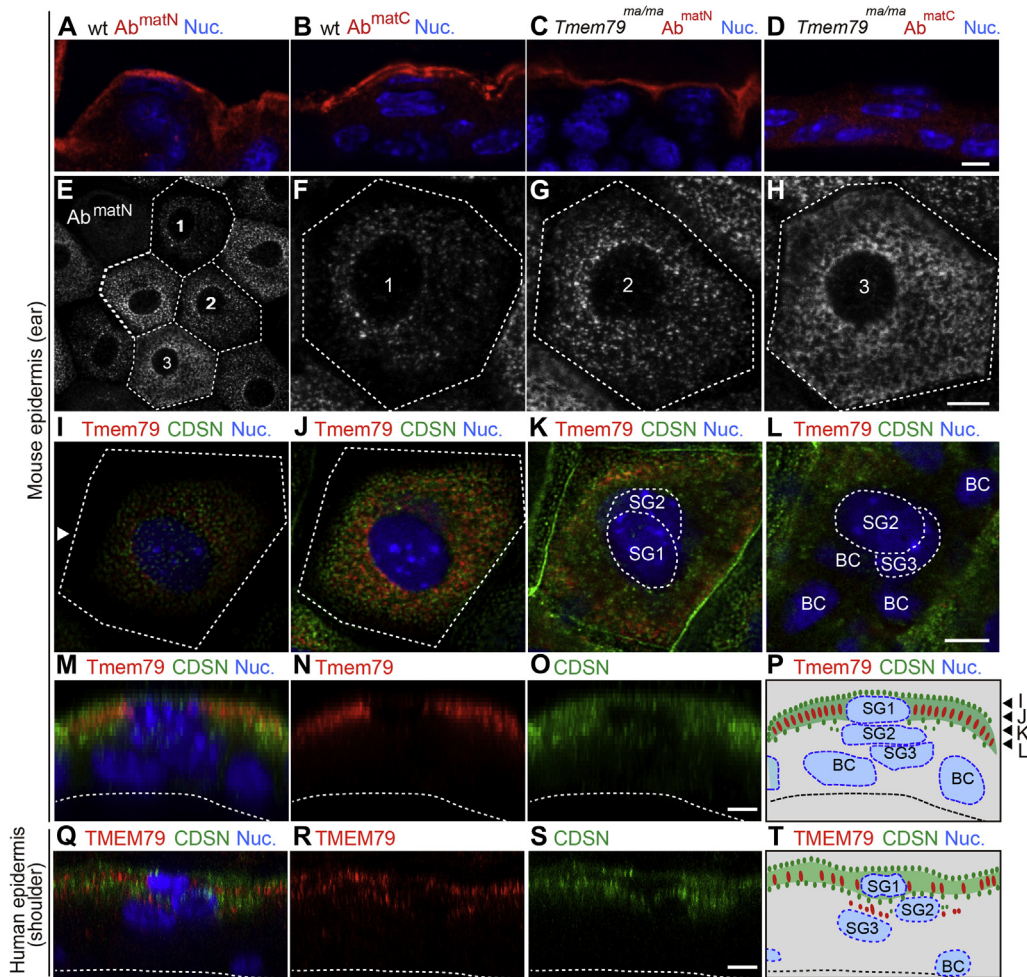


FIG 4. Localization of Tmem79 in the epidermis. **A-D**, Vertical skin sections were immunostained with Ab^{matN} and Ab^{matC} for WT (C57BL/6) and *Tmem79*^{ma/ma} mice. Red, Tmem79; blue, nucleus. **E-H**, Horizontal confocal images of Tmem79 stained with Ab^{matN} in SG1 cells of the mouse epidermal sheet. Dotted lines indicate the boundary of the SG1 cells, and the numbers (1-3) in the centers of the cells indicate the cell location in Fig 4. **E, I-L**, Z-axis scanning confocal images of Tmem79 (Ab^{matN}) and CDSN in the layers of the mouse epidermal sheet. Red, Tmem79; green, CDSN; blue, nucleus. Dotted lines indicate the boundary of the SG1 cells (Fig 4, I and J) and nucleus (Fig 4, K and L). BC, Basal cell. The triangle in Fig 4, I, indicates the position of the reconstituted vertical image in Fig 4, M. See also Video E1. **M-O**, Reconstituted vertical images from the z-axis scanning confocal images from Fig 4, I to L. The dotted line indicates the position of the basement membrane zone. See also Video E2. **P**, The scheme for the reconstituted vertical images of the mouse epidermal sheet. The positions of the triangles indicate the levels of each confocal horizontal image (Fig 4, I-L). **Q-S**, The reconstituted vertical images of Tmem79 (Ab^{matN}) and CDSN from z-axis scanning confocal images of the human epidermal sheet. Red, Tmem79; green, CDSN; blue, nucleus. **T**, Scheme of the reconstituted vertical images of the human epidermal sheet. Bars = 5 μm.

residues 99 to 115 (Ab^{matN}) and C-terminal residues 376 to 391 (Ab^{matC}) of Tmem79 stained the outermost layer of the stratum granulosum (SG) cells (SG1 cells) of WT mice, whereas Ab^{matN} staining, but not Ab^{matC} staining, was observed in vertical epidermal sections from *Tmem79*^{ma/ma} mice (Fig 4, A-D), indicating that the truncated Tmem79 is expressed in *Tmem79*^{ma/ma} mice. Because SG cells, which differentiate to form the SC, are largely flattened and piled up at different stages of differentiation, whole-mount staining of epidermal sheets was used to examine the expression pattern in SG1 cells.²⁶ When the epidermal sheet *en face* views were examined by using confocal microscopy, the Tmem79 staining showed a dot-like, tubular, or reticular pattern depending on the maturation stage of SG1 cell differentiation during SC biogenesis (Fig 4, E-H). Costaining with corneodesmosin (CDSN), which

labeled SG1 cell membranes, revealed that Tmem79 was exclusively expressed in the cytoplasm of SG1 cells in mice, as seen in vertically reconstituted images (Fig 4, I-P; see Videos E1 and E2 in this article's Online Repository at www.jacionline.org). Similarly, human TMEM79 was primarily detected in the cytoplasm of SG1 cells and was detected in SG2 cells to a lesser degree (Fig 4, Q-T), suggesting that the function of Tmem79 is conserved between mice and humans.

Colocalization of Tmem79 and TGN marker in SG1 cells

Tmem79 was costained with antibodies for various organelle markers to further characterize the intracellular localization of

Tmem79 in SG1 cells (Fig 5, A-D). Tmem79 most closely colocalized with TGN46, a marker of the TGN.²⁸ LAMP1, a marker for lysosomes, tended to costain at the apical tips of Tmem79-positive organelles.²⁹ Tmem79 did not colocalize with GM130 (*cis*-Golgi network), EEA1 (early endosome), SNAP29 (vesicle transport), or CDSN (CDSN and LG).³⁰⁻³³ Immunoelectron microscopy confirmed that Tmem79 and TGN46 were colocalized in human epidermal SG1 and SG2 cells (Fig 5, E).³⁰⁻³³

Reduced expression of LG-secreted proteins in *Tmem79^{ma/ma}* mice

The TGN plays a central role in protein sorting among the organelles of the secretory pathway.^{34,35} The LG secretory system specializes in the polarized secretion of various proteases, protease inhibitors, and lipids in the SG layers to form the SC.³⁶ Therefore we examined LG-secreted proteins in *Tmem79^{ma/ma}* mice to examine whether Tmem79 is involved in the LG secretory system (Fig 6). Although there were no obvious changes in the vertical section staining for FLG and LOR or whole-mount staining for TGN46 and Tmem79 N-terminal, both vertical sections and whole-mount staining for LG-secreted proteins (KLK5 and LEKTI) was significantly reduced in *Tmem79^{ma/ma}* mice compared with WT mice (Fig 6, A-C). In contrast, this reduced staining was remarkably rescued in *Tmem79^{ma/ma}-Tg* mice (Fig 6, D). These results suggest that *Tmem79* is involved in the LG secretory system and that *Tmem79* deficiency directly or indirectly causes dysfunction of this system.

Impaired SC formation in *Tmem79^{ma/ma}* mice

Next we examined SC formation in *Tmem79^{ma/ma}* mice. Although there were no apparent structural changes to the SC layers on safranin staining, physical stress caused by tape stripping resulted in quite different peeling patterns of the SC layers in WT and *Tmem79^{ma/ma}* mice (Fig 7, A and B). Although the SC layers of WT mice peeled off evenly and the amount of desquamated SC decreased with the number of strippings, the SC layers of *Tmem79^{ma/ma}* mice peeled off unevenly as blocks, and the entire SC layer was focally removed after 10 tape-stripping applications. The number of regions in which tape stripping removed the entire SC layer was significantly greater in *Tmem79^{ma/ma}* mice than in WT mice (Fig 7, C). These findings indicate that *Tmem79^{ma/ma}* mice have impaired SC formation with less resistance to physical external stress.

DISCUSSION

Original double-mutant flaky tail (*ma/ma Flg^{fl/fl}*) mice express truncated profilaggrin and significantly reduced mature filaggrin, show enhanced TEWL and decreased SC hydration, and develop spontaneous dermatitis with increased IgE levels.^{13,14} Thus *Tmem79^{ma/ma} Flg^{fl/fl}* mice have been widely used as a mouse model of AD with filaggrin deficiency. However, genetically engineered *Flg^{-/-}* mice had no apparent change in TEWL or SC hydration compared with WT mice and did not develop dermatitis under SPF conditions, although filaggrin was completely absent.⁸ The onset of dermatitis in *Flg^{-/-}* mice under conventional conditions remains to be determined. This conflicting observation between flaky tail mice and *Flg^{-/-}* mice led us to investigate the gene responsible for the *ma* phenotype. We identified a nonsense mutation (c.840C>G, p.Y280*) in the *Tmem79*

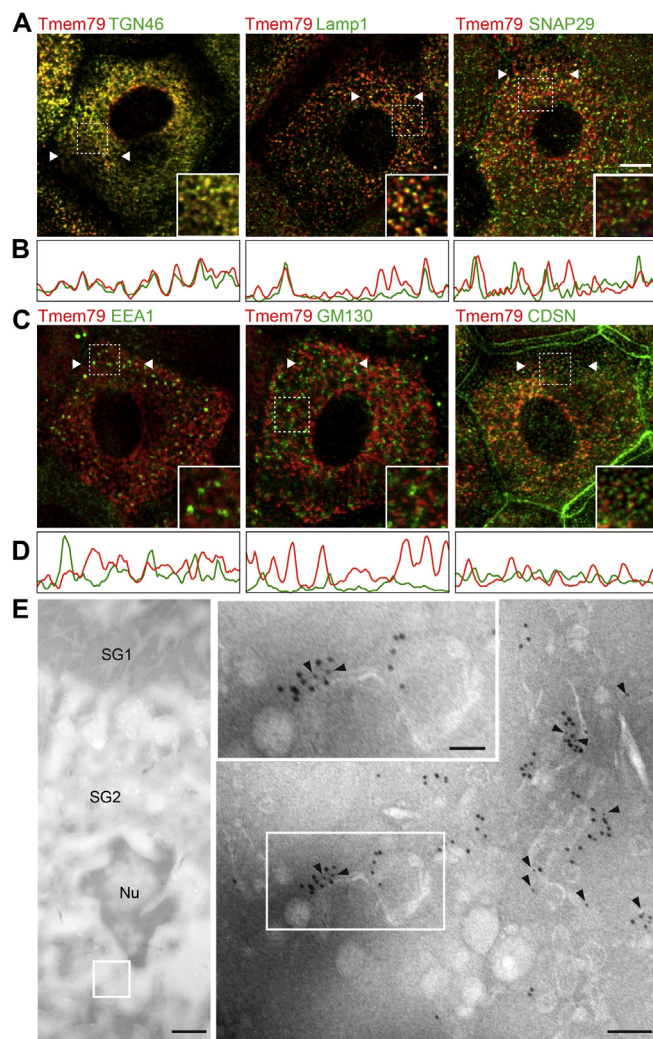


FIG 5. Costaining of Tmem79 with intracellular organelle markers in SG1 cells. **A and C,** Horizontal confocal images of coimmunostaining with Tmem79 (Ab^{matN}; red) and various intracellular organelle markers (green), including TGN46 (*trans*-Golgi network), LAMP1 (lysosomes), SNAP29 (vesicle transport), EEA1 (early endosomes), GM130 (*cis*-Golgi), and CDSN (corneodesmosome and LG). **Insets** are magnified images of the indicated areas (dotted squares). Bars = 5 μm. **B and D,** Colocalization analysis of multicolor confocal immunostaining. Each color curve indicates the relative intensity of the immunostaining for each protein (red, Tmem79; green, each organelle marker) between the 2 triangles in each image. **E,** Immunoelectron microscopic image of colocalized Tmem79 and TGN46 in human skin. The square in the left column is enlarged in the right column. The rectangle in the right column is enlarged in the upper left corner. Tmem79, arrowheads, 5 nm gold; TGN46, 10 nm gold. Nu, Nucleus. Bars = 1 μm (left), 100 nm (right), and 50 nm (center).

gene. *Tmem79^{ma/ma}* mice without the filaggrin mutation have enhanced TEWL and decreased SC hydration and develop spontaneous dermatitis with increased IgE levels. Mutations of both the *Flg* and *Tmem79* genes result in altered SC formation and impaired SC barrier, although in different pathways. Therefore all of the previous work with the double-mutant flaky tail mice in the context of filaggrin deficiency needs to be carefully re-evaluated.^{15-19,37} The contribution of the *Tmem79* mutation has to be reconsidered in those studies.

Several skin barrier-related genes responsible for SC formation have already been isolated, and *Tmem79* is a new member in that category. Defects in these skin barrier-related genes often

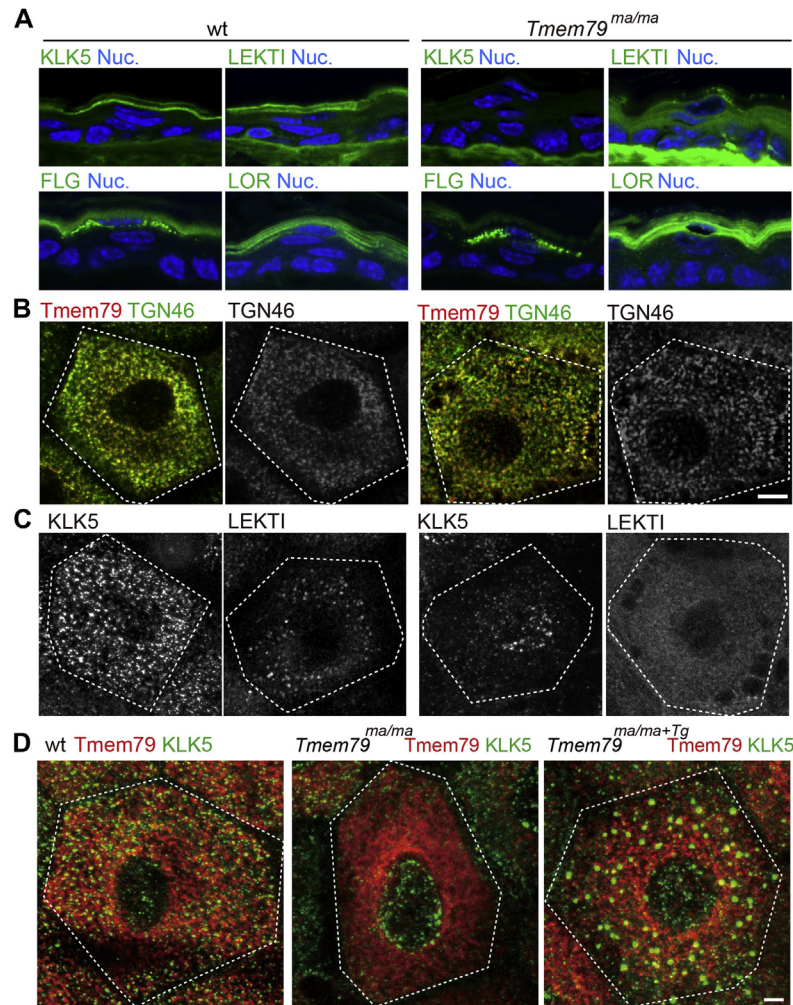


FIG 6. LG secretory system dysfunction in *Tmem79*^{ma/ma} mice. **A**, Vertical skin sections were immunostained with KLK5, LEKTI, FLG, and LOR in WT (C57BL/6) and *Tmem79*^{ma/ma} mice. Green, each protein; blue, nucleus. **B**, Horizontal confocal images of *Tmem79* and TGN46 in the SG1 cells of the mouse epidermal sheet in WT (C57BL/6) and *Tmem79*^{ma/ma} mice. Red, *Tmem79* (Ab^{matN}); green, TGN46. Dotted lines indicate the boundary of the SG1 cells. Bars = 5 μ m. **C**, Horizontal confocal images of the LG secretory proteins (KLK5 and LEKTI) in SG1 cells of the mouse epidermal sheet in WT (C57BL/6) and *Tmem79*^{ma/ma} mice. Dotted lines indicate the boundary of the SG1 cells. Bars = 5 μ m. **D**, Horizontal confocal images of *Tmem79* (Ab^{matN}) and KLK5 proteins in the SG1 cells of the mouse epidermal sheets from 8-week-old (C57BL/6), *Tmem79*^{ma/ma}, and *Tmem79*^{ma/ma+Tg} mice. Red, *Tmem79*; green, KLK5. Bars = 5 μ m.

result in skin disorders in humans. *CDSN* encodes a component of CDSN that plays the role of an adhesion molecule for corneocytes, and *CDSN* defects cause peeling skin syndrome, in which patients have ichthyosiform erythroderma with superficial exfoliation.³⁸ *ST14* encodes the protease matriptase, and *ST14* defects cause ichthyosis with hypotrichosis syndrome.³⁹ *SPINK5* encodes the protease inhibitor LEKTI, and *SPINK5* defects cause Netherton syndrome, which is characterized by congenital ichthyosiform erythroderma with bamboo hair formation.⁴⁰ *ABCA12* encodes a lipid transporter, and *ABCA12* defects cause both autosomal recessive congenital ichthyosis 4A (ARCI4A) and Harlequin ichthyosis.^{41,42} SNAP29 encodes a SNARE protein involved in intracellular trafficking, and *SNAP29* mutations cause cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma (CEDNIK) syndrome. Among those skin disorder-related genes, several are linked to the LG secretory system, which plays an essential role in intact SC formation and maturation. CDSN

and LEKTI are secreted through the LG, *ABCA12* is localized in the LG and functions as a lipid transporter, and SNAP29 is member of the t-SNARE proteins that are associated with granule fusion to the cell membrane in the LG secretory system.⁴³ *Tmem79* is a novel skin barrier-related gene involved in the LG secretory system, and *Tmem79* deficiency causes abnormal secretion of several secreted proteins, which results in impaired SC barrier formation and spontaneous dermatitis in mice. The involvement of *Tmem79* in any human skin disorder remains to be determined.

Tmem79^{ma/ma} *Flg*^{fl/fl} and *Tmem79*^{ma/ma} mice also display the matted hair phenotype, which was originally described as (1) erection of hair, (2) matted clumps of hair (hair with a tendency to stick together), (3) a tendency to baldness, and (4) change in hair color.¹¹ The hair phenotype was rescued by introducing with WT *Tmem79* transgene, therefore, *Tmem79*^{ma/ma} was also responsible for the hair phenotype. Because *Tmem79*^{ma/ma} mice

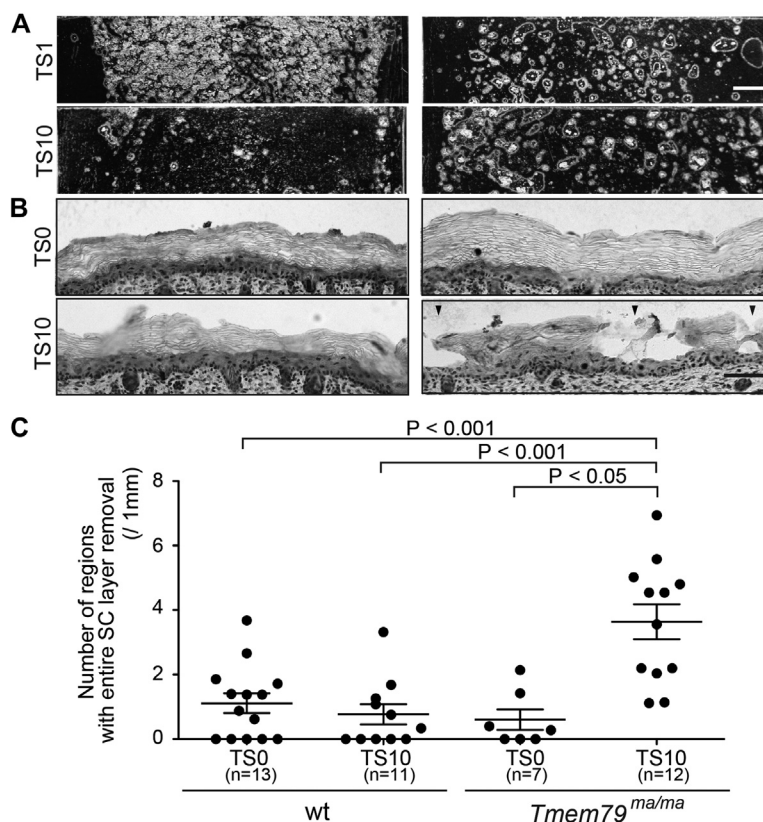


FIG 7. Impaired SC formation in *Tmem79*^{ma/ma} mice. **A**, The SC layers that came off with the tape after tape stripping are shown for 5-day-old WT (C57BL/6) and *Tmem79*^{ma/ma} mice. TS1, First tape stripping; TS10, 10th tape stripping. Bars = 2 mm. **B**, The SC layers were visualized by means of safranin staining before (TS0) and after (TS10) tape stripping of 5-day-old WT and *Tmem79*^{ma/ma} mice. Triangles indicate regions with entire SC layer removal. Bars = 100 μ m. **C**, The number of regions with removal of the entire SC layer per 1 mm was counted and compared between WT and *Tmem79*^{ma/ma} mice before (TS0; WT mice, n = 13, *Tmem79*^{ma/ma} mice, n = 7) and after (TS10; WT mice, n = 11; *Tmem79*^{ma/ma} mice, n = 12) tape stripping.

have moist and oily hair, we suspected that matted clump formation might be due to differences in lipid composition of the surface of the hair. This hypothesis was indeed true; lipid composition analysis revealed more cholesterol ester members in the hair of *Tmem79*^{ma/ma} *Flg*^{fl/fl} mice than in WT mice (data not shown). This difference is likely due to an impaired LG secretory system in the hair follicles. Further investigation is necessary to understand how *Tmem79*^{ma/ma} induces the matted hair phenotype.

Although we identified *Tmem79*^{ma/ma} as the gene responsible for spontaneous dermatitis in double-mutant flaky tail mice and matted mice, the exact mechanisms involved in the onset of dermatitis remain to be elucidated. What is the exact molecular role of *Tmem79* in the LG secretory system? How does the impaired LG secretory system affect SC formation? Why do impaired SC barriers result in spontaneous dermatitis? Are any commensal bacteria involved in this process? Although many questions remain to be answered to fully understand the pathophysiologic mechanism of dermatitis caused by *Tmem79*^{ma/ma} deficiency, the identification of *Tmem79* as a novel skin barrier-related gene will open a new path to dissect the fundamental mechanisms of SC barrier formation and provide a new target for the development of therapeutic strategies for atopic diseases.

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Clinical implications: *Tmem79* is a novel skin barrier-related gene involved in the pathogenesis of dermatitis, and matted mice or *Tmem79*-deficient mice provide a valuable tool for dissecting pathophysiologic mechanisms of skin barrier disruption and AD.

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METHODS

DNA sequencing with the Next Generation DNA Sequencer

Genomic DNA from *Tmem79^{ma/ma}*, CBA, and C57BL/6 mice was obtained from the liver. The extracted genomic DNAs were sequenced by using the SureSelect Target Enrichment System (Agilent Technologies Japan), which targeted an 85.0- to 92.0-Mb region excluding repetitive sequences and the 80.0- to 85.0-Mb and 92.0- to 93.0-Mb exonic regions on mouse chromosome 3. The enriched DNA fragments were sequenced for 75-bp single-end reads with an Illumina Genome Analyzer II (Illumina), according to the manufacturer's instructions. The raw image files were processed with Illumina SCS2.8 software using the default parameters. The extracted DNA sequence reads were mapped to the mouse reference genome (mm19) by using bwa. SAMtools was used to call SNVs/indels for each mouse based on the following filter conditions: base quality of 20 or greater and sequence depth of 10 or greater for SNVs and base quality of 50 or greater and sequence depth of 10 or greater for indels.

Then we removed the SNVs/indels found in both CBA and *Tmem79^{ma/ma}* as SNVs/indels between C57BL/6 and CBA. Finally, all candidate mutations were evaluated manually, and the exonic mutations were identified with the IGV viewer.

Antibodies

Anti-CDSN mouse mAb (Abcam, Tokyo, Japan), anti-EEA1 mouse mAb (MBL, Nagoya, Japan), anti-GM130 mouse mAb (Abcam), anti-FLG rabbit antibody (Covance, Princeton, NJ), anti-KLK5 mouse mAb (R&D Systems, Minneapolis, Minn), anti-LEKTI mouse mAb (Santa Cruz Biotechnology, Dallas, Tex), anti-Lamp1 mouse mAb (Santa Cruz Biotechnology), anti-LOR rabbit mAb (Gene Tex, Taiwan), anti-SNAP29 mouse mAb (Sigma, Fukushima, Japan), and anti-TGN46 sheep polyclonal antibody (Abcam) were used as primary antibodies for immunostaining. Alexa Fluor 488 Goat Anti-mouse IgG, Alexa Fluor 488 Donkey Anti-sheep IgG, and Alexa Fluor 568 Goat Anti-rabbit IgG (Life Technologies, Tokyo, Japan) were used as secondary antibodies.

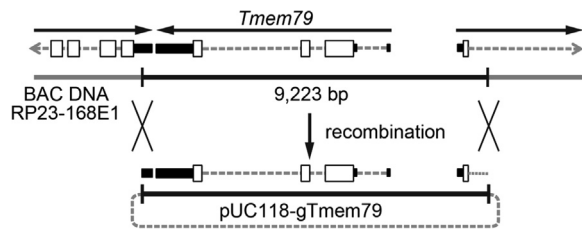


FIG E1. Transgene construct for *Tmem79* transgenic mice. Whole genomic regions, including *Tmem79*, and the 3' untranslated region and exon 1 of adjacent genes were subcloned into pUC118 by using a Red/ET DNA recombination system and used to generate *Tmem79* transgenic mice.

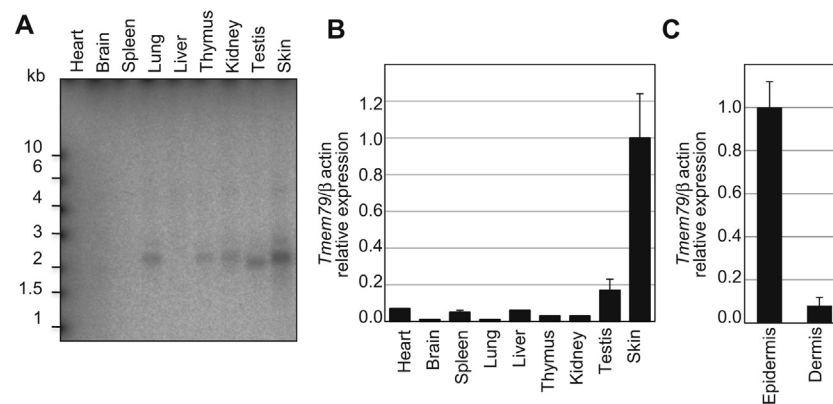


FIG E2. Transcription levels of *Tmem79* in various tissues. **A**, Northern blot analysis for *Tmem79* in various tissues in 8-week-old C57BL/6 mice. **B**, Quantitative PCR analysis for *Tmem79* in 8-week-old C57BL/6 mice. **C**, Quantitative PCR analysis for *Tmem79* in dermis and epidermis in 5-day-old C57BL/6 mice. These results indicate that *Tmem79* is mainly expressed in the epidermis of the skin.

TABLE E1. Difference of phenotype in flaky tail (*maFlg^{ft/ft}*), *Flg^{-/-}*, *Flg^{ft/ft}*, and *ma/ma* mice

	Flaky tail (<i>maFlg^{ft/ft}</i>)	<i>Flg^{-/-}</i>	<i>Flg^{ft/ft}</i>	<i>ma/ma</i>
Spontaneous dermatitis	+	—	—	+
Scratching behavior	+	—	—	+
Keratohyalin granules	—	—	—	+
TEWL	↑	→	→	↑
SC hydration	↓	→	→	↓