Supplementary Materials and Methods

Generation of RNF186-deficient mice

A 5.8-Kb fragment encoding the exon region of Rnf186 gene was replaced with a neomycin-resistant gene cassette, and the HSV thymidine kinase gene was inserted into the genomic fragment for negative selection. V6.5 embryonic stem cells were transfected with the targeting vector, and colonies resistant to both G418 and ganciclovir were further analyzed for homologous recombination by PCR and Southern blot methods. For microinjection of homologous recombinants, blastocysts of C57BL/6 females were used, and heterozygous F1 mice were intercrossed to generate Rnf186-deficient mice. Successful gene targeting of Rnf186 was confirmed by Southern blot analysis. Rnf186-deficient and wild-type littermates from heterozygote intercresses were used for experiments. All animal experiments were conducted with the approval from the Animal Research Committee of the Graduate School of Medicine at Osaka University.

Generation of Rnf186A117T/A117T mice by the CRISPR/Cas9 system

RNF186-gAS11-F (5’-CACCTAGCAGAAGCTTCAGGCATA-3’) and RNF186-gAS11-R (5’-AAACTATGCTGAAGCTTCTGCTA-3’) oligonucleotides (Life Technologies) were annealed and inserted into the BbsI restriction site in the px330 vector (Addgene plasmid 42230) by Ligation high Ver. 2 (Toyobo). This plasmid was designated as px330-RNF186-gAS11. For the EGxxFP system\(^1\), part of exon 1 in the Rnf186 gene was amplified with KOD FX (Toyobo) using the following primers:

RNF186-EGxxFP-F:
5’-AAGCTAGCCATGCAAGTCCTGGCAGCTCCGGTGGATGT-3’;
RNF186-EGxxFP-R:
5’-AAGAATTCACAGTGTCCTGTTCTTCTCTCTCTGTCAG-3’. The PCR product was purified with an illustra GFX PCR DNA and Gel Band Purification Kit (GE
Healthcare) and inserted into pCAG-EGxxFP using Ligation high Ver. 2. This plasmid was designated as pCAG-EGxxFP-RNF186. The two vectors, px330-RNF186-gAS11 and pCAG-EGxxFP-RNF186, were purified with a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The vectors were transfected into HEK293T cells by Lipofectamine 2000. EGFP fluorescence in HEK293T cells was observed by fluorescence microscopy (FV1000-D). Then, the px330-RNF186-gAS11 DNA vector and single-stranded DNA (RNF186-Ala117Thr:

5′-GAGCCCTACAACGTGCTCGGTCCCCCAAGCTGCTTAGCTGTCAGCACATTCTGTACAGTATGCTGAAACTTCTGCTATATGTGCAGGAAGACACCTGGTCCATCCCCTGTCCG-3′) were injected into the pronuclei of one-cell-stage embryos from B6D2F1 mice as described previously\(^1\). The eggs were cultivated in kSOM overnight and then transferred into the oviducts of pseudopregnant ICR females. Screening of founder mice was performed by PCR and direct sequencing using DNA obtained from the tail. PCR was performed with rTaq DNA Polymerase (Toyoobo) and the following primers: RNF186-EGxxFP-F and RNF186-EGxxFP-R. The PCR products were purified with a GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), and sequences were analyzed using an Applied Biosystems 3130 Genetic Analyzer (Life Technologies) with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and the RNF186-EGxxFP-F primer. \(Rnf186^{A117T/A117T}\) mice and their \(Rnf186^{wt/wt}\) littermates from intercrosses of \(Rnf186^{A117T/wt}\) mice were used for experiments.

**Isolation of lamina propria lymphocytes**

To isolate colonic lamina propria lymphocytes, large intestines were opened, washed to remove fecal content with sterile PBS, shaken in HBSS containing 5 mM EDTA for 20 min at 37°C to remove epithelial cells and fat tissue, cut into small pieces, and incubated with RPMI 1640 containing 4% heat-inactivated fetal bovine serum (FBS), 1
mg/ml collagenase D (Roche), 0.5 mg/ml dispase (Invitrogen), and 40 µg/ml DNase I (Roche) for 40 min at 37°C in a shaking water bath. After filtration through nylon mesh, the fraction was washed with RPMI 1640 containing 4% FBS, resuspended in 2.5 ml 40% Percoll (GE Healthcare), and overlaid on 2.5 ml 80% Percoll in a 15-ml Falcon tube. Percoll-gradient separation was performed by centrifugation at 780 × g for 20 min at 25°C. The lamina propria lymphocytes were collected at the interface of the Percoll gradient and washed with RPMI 1640 containing 10% FBS.

**Intracellular cytokine staining and flow cytometry**

Intracellular expression of IFN-γ and IL-17 in CD4+ T cells was analyzed using a Cytofix/Cytoperm Kit Plus (with GolgiStop; BD Biosciences), according to the manufacturer’s instructions. Lymphocytes obtained from the colonic lamina propria were incubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 5 µM calcium ionophore A23187 (Sigma-Aldrich), and GolgiStop at 37°C for 4 h. Surface staining was performed with anti–CD4–PerCP/Cy5.5 (cat# 100434) (BioLegend) for 20 min at 4°C after incubation with Fc block (cat# 553141) (BD Biosciences) for 15 min at 4°C. The cells were permeabilized with Cytofix/Cytoperm solution for 20 min at 4°C, and intracellular cytokine staining was performed with anti–IFN-γ–FITC (cat# 505806) (BioLegend) and anti–IL-17A–APC (cat# 506916) (BioLegend) for 30 min. Data were acquired using a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Antibodies**

Primary antibodies used in this study were: mouse anti-Flag antibody (cat# F3165) (Sigma-Aldrich); mouse anti-Myc antibody (cat# 2276), rabbit anti-sXBP1 antibody (cat# 12782), and rabbit anti-cleaved caspase-3 antibody (cat# 2276) (Cell Signaling); mouse anti-HA antibody (cat# sc-7392), mouse anti-actin antibody (cat# sc-8432),
mouse anti-ubiquitin antibody (cat# sc-8017), goat anti-GRP78 antibody (cat# sc-1050),
mouse anti-CHOP antibody (cat# sc-7351), and rabbit anti-MUC2 antibody (cat# sc-15334) (Santa Cruz Biotechnology); rabbit anti-K48 ubiquitin antibody (cat# 05-1307) and rabbit anti-K63 ubiquitin antibody (cat# 05-1308) (Millipore); rabbit anti-lysozyme antibody (cat# ab108508) (Abcam); rabbit anti-SRPRB antibody (cat# 14636-1-AP) and rabbit anti-GNB2 antibody (cat# 16090-1-AP) (Proteintech); mouse anti-KDEL antibody (cat# ADI-SPA-827) (Enzo); Antibodies against occludin has been described previously. Secondary antibodies used were: donkey anti-rat IgG (H+L), Alexa Fluor® 488 conjugate (cat# A-21088), goat anti-rabbit IgG (H+L), Alexa Fluor® 568 conjugate (cat# A-11011), and goat anti-mouse IgG (H+L), Alexa Fluor® 647 conjugate (cat# A-21235) (Thermo Fisher Scientific Inc.); horseradish peroxidase (HRP)-conjugated anti-rat IgG (cat# NA9350V), HRP-conjugated anti-mouse IgG (cat# NA931V), HRP-conjugated anti-rabbit IgG (cat# NA934V) (Amersham Biosciences); donkey anti-goat IgG HRP (cat# sc-2056: Santa Cruz Biotechnology); biotin-conjugated goat anti-rabbit IgG (cat# 71-00-30: Kirkegaard & Perry Laboratories).

**Plasmid construction**

Flag-tagged human RNF186 (Flag-RNF186WT) was obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the EcoRI and SacII sites of the pcDNA-Flag expression vector (Invitrogen). Flag-tagged RNF186 mutants (Flag-RNF186H60W and Flag-RNF186A64T) were generated using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Myc-Occludin was obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the HindIII and Sall sites of the pcDNA-Myc expression vector (Invitrogen). HA-Ub was obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the BamHI and EcoRI sites of the pcDNA-HA expression vector (Invitrogen). HA-tagged mutant ubiquitin (HA-UbK48R) was generated as described previously. mCherry-RNF186 was
obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the EcoRI and SalI sites of the pmCherry vector (Takara Bio). GFP-occludin was obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the HindIII and SalI sites of the pEGFP vector (Takara Bio). Myc-SRPRB was obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the BamHI and NotI sites of the pcDNA-Myc expression vector. Myc-GRP78, Myc-CDK5RAP3, and Myc-STX17 were obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the SacII and NotI sites of the pcDNA-Myc expression vector. Myc-GNB2 and Myc-ACSL1 were obtained by PCR using genomic DNA from Caco-2 cells as a template and ligated into the BamHI and EcoRI sites of the pcDNA-Myc expression vector.

Cell culture and transfection
HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Nacalai Tesque) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Caco-2 cells were grown in minimum essential medium (Eagle) (MEM; Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum, MEM non-essential amino acids (0.1 mM, Nacalai Tesque), sodium pyruvate (0.5 mM, Nacalai Tesque), penicillin (100 U/ml), and streptomycin (100 mg/ml). All cells were cultured at 37°C with 5% CO₂. The cells were transfected with the indicated plasmids using Lipofectamine 2000 (Life Technologies) or Lipofectamine LTX and PLUS Reagent (Life Technologies) according to the manufacturer’s protocols.

Immunofluorescence analysis
Caco-2 cells were transfected with mCherry-RNF186 and GFP-occludin using Lipofectamine 2000 and cultured for 24h at 37°C with 5% CO₂. The cells were washed with cold sterile PBS and were fixed with 100% methanol at -20°C for 5 min. After
washing with PBS, the samples were blocked with 1% bovine serum albumin in PBS for 1 h at room temperature and were stained with anti-KDEL for 1 h at room temperature. The samples were washed and incubated with Alexa 647-conjugated anti-mouse IgG for 30 min at room temperature. The samples were washed with PBS, embedded in mounting medium, and were observed under the confocal microscope.

**Human genomic DNA sequence**

All genomic DNA from UC patients and healthy individuals was collected at Tokyo Medical and Dental University, and analyzed at Osaka University. The Tokyo Medical and Dental University, and Osaka University ethics committee approved this study and informed consent was obtained from all study subjects.

**Analysis of public microarray data**

The microarray datasets (GSE36807 and GSE38713), which represent whole-genome transcriptional analysis of colonic biopsies from UC patients and non-inflammatory controls, were obtained from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The GEO2R web application (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used for analyzing expression values of *RNF186* in the selected datasets.

**Thin section electron microscopy**

Under anesthesia, 8-week-old *Rnf186<sup>−/−</sup>* mice and their *Rnf186<sup>+/+</sup>* littermates were fixed by vascular perfusion of 2% PFA and 2.5% glutaraldehyde in 0.1 M Hepes buffered saline (HBS; pH 7.2). Then, the colons were removed, cut into small pieces, and fixation was continued overnight at 4°C. After being washed thoroughly with 0.1 M HBS, specimens were postfixed in 1% OsO<sub>4</sub> in the same buffer for 2 hours on ice. The segments were washed with distilled water and stained en bloc with EM stainer (Nisshin
EM, Tokyo, Japan) for 2 hours. They were dehydrated in ethanol and embedded in Polybed 812 resin (Polysciences, Warrington, PA). Thin sections were cut and then examined under an electron microscope (Model JEM-1400Plus; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

**Induction of oxazolone-induced colitis**

Eight-week-old *Rnf186* /− mice and their *Rnf186* +/+ littermates were used for oxazolone (cat# E0757: Sigma-Aldrich)-induced colitis experiments as described previously. Mice were carefully shaved a 1.5 cm x 1.5 cm field of the abdominal skin of the mouse using an electric razor and sensitized with 150 µl of 3% oxazolone in 100% ethanol. Five days after the sensitization, anaesthetized mice were intracolonically administrated with 1% oxazolone in 50% ethanol using a thin round-tip needle. The tip of the needle was inserted 4 cm proximal to the anal verge, and mice were held in a vertical position for 30s after the injection. Mice were analyzed for changes in body weight and colonic histology.

**References**