Methods

Subjects  Forty-six adult AD patients (17 female and 29 male subjects; mean, 34.8 years old ± 10.4 SD) and 46 adult healthy controls (19 female and 27 male subjects; mean, 31.8 years old ± 6.2 SD) between 21 to 63 years of age were invited from Kyushu University Hospital to participate in the study during 2014–2016. All study subjects were Japanese living in Japan (ethnicity, Asian). The characteristics for all participants are shown in Table S1. No age or gender disparities were observed between the two groups (P = 0.095 and P = 0.669, respectively). The two groups were recruited using the following criteria: the AD group, (i) being diagnosed with AD at the time of the survey and (ii) above 20 years old; the control group, (i) no previous history of AD and (ii) above 20 years old. AD was diagnosed by dermatologists according to current criteria provided by the Japanese Dermatological Association.1 This study was conducted according to STROBE statement2 and approved by the Ethics committee of Kyushu University Hospital. Written informed consent was obtained from all participants before their participation according to the Helsinki Declaration as revised in 2013. The assessment of the pruritus severity was performed using the Investigator Global Assessment (IGA) and the Eczema Area and Severity Index (EASI). The IGA is a 5-point scale ranging from 0 to 4 (0, clear; 1, almost clear; 2, mild; 3, moderate; 4, severe).

Genotyping  Venous whole blood was drawn from the participants, and genomic DNA was extracted using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). PCR reactions were performed in a 96-well plate (volume of 50 μL) containing genomic DNA, KOD plus DNA polymerase, 2 mM dNTPs, 25 mM MgSO4, 10X PCR buffer (all from TOYOBO, Osaka, Japan), and DOCK8 exon-specific primers (listed in Table S2). Then, PCR fragments were prepared using Big Dye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and DTR Gel Filtration Cartridges (EdgeBio, San Jose, CA, USA) for the following DNA sequencing. Sequencing was performed by Applied Biosystems 3130xl Genetic Analyzers (Thermo Fisher Scientific), and the sample genotypes were determined using ApE software (by Wayne Davis from the University of Utah).

Preparation of HCT116 cell lines and transfectants  Wild-type HCT116 cells (human colon cancer cell line) were purchased from ATCC. HCT116 cells were maintained in D-MEM medium (Wako, Osaka, Japan) containing 10% (vol/vol) heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 50 μM 2-mercaptoethanol (Nacalai Tesque Inc., Japan), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1x MEM non-essential amino acids (all from Thermo Fisher Scientific). Human DOCK8 (+1790C)-HA and DOCK8 (+1790T)-HA expressing cells were prepared by the transfection of the pBJ vectors encoding neomycin and human DOCK8 (+1790C or +1790T) with an HA-tag at its C-terminus [pBJ-neo-DOCK8 (+1790C)-HA and pBJ-neo-DOCK8 (+1790T)-HA, respectively]. HCT116 cells transfected with pBJ-neo backbone vector were prepared as negative control (empty-vector transfected HCT116...
cells). Plasmids were transfected with polyethylenimine (PEI; Sigma-Aldrich, St Louis, MO, USA) and stable transfectants were isolated by limiting dilution and selection with 2 mg/mL G418 (Wako).

**Immunoblotting** Total cell lysates were prepared and separated by SDS-PAGE as previously described. Blots were probed with goat anti-β-Actin antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP)-conjugated mouse anti-goat IgG (1:2,000, Santa Cruz Biotechnology), and rabbit anti-DOCK8 antibody (1:1,000, custom-made) and rabbit anti-EPAS1 antibody (#100-122; 1:1,000, Novus Biologicals, Centennial, CO, USA) followed by HRP-conjugated mouse anti-rabbit IgG (1:2,000, Santa Cruz Biotechnology).

**Immunofluorescence** HCT116 cells (5 x 10^4) were cultured on the poly-L-lysine coated glass-bottom dishes (Matsunami, Osaka, Japan) in 1% O₂ (hypoxia) or 20% O₂ (normoxia) for 30 h. Then, cells were fixed with 4% paraformaldehyde (Wako) for 20 min and permeabilized with 0.2% Triton X-100 (Wako) in PBS for 30 min. After being blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature, cells were incubated overnight at 4°C with rabbit anti-EPAS1 antibody (#100-122; 10 µg/mL, Novus Biologicals) and biotinylated rat anti-hemagglutinin tag (HA, 3F10; 1 µg/mL, Roche, Basel, Switzerland). The staining was detected with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (H+L; 4 µg/mL, Thermo Fisher Scientific) and Alexa Fluor 546-conjugated streptavidin (2 µg/mL, Thermo Fisher Scientific). DAPI (1:5,000, DOJINDO, Kumamoto, Japan) was used for nucleus staining. All images were obtained with a laser scanning confocal microscope (FV3000; Olympus, Tokyo, Japan). The fluorescence intensity of EPAS1 in each nucleus was measured with ImageJ software (NIH, Bethesda, USA). Relative fluorescence intensity of intranuclear EPAS1 is expressed as the proportion in comparison to empty-vector transfected HCT116 cells under the hypoxic condition.

**Statistics** Statistical analyses were conducted with Prism 8 (GraphPad Software). The difference in allele and genotype frequencies was compared between case and control samples and evaluated using the chi-square test. A logistic regression model was used to calculate the odds ratio (OR) and 95% confidence intervals. The confidence interval of the OR was calculated by using Baptista-Pike method. Multiple logistic regression models were built to analyze genetic data: Dominant (major allele homozygotes vs. heterozygotes and minor allele homozygotes), Co-dominant 1 (heterozygotes vs major allele homozygotes), Co-dominant 2 (minor allele homozygotes vs. major allele homozygotes), Recessive (minor allele homozygotes vs. major allele homozygotes and heterozygotes), Over-dominant (major and minor allele homozygotes vs. heterozygotes). In Figure 2B, the data were analyzed with a Kruskal-Wallis test with Dunn’s multiple comparison test (vs. Empty vector). P-values less than 0.05 were considered significant. The post-hoc power analysis about Table 1 was conducted with G*power 3.1 (α error probability = 0.05, sample size = 46), and the values of power in each comparison were as
follows: CC vs. (CT+TT), 0.622; CT vs. CC, 0.256; TT vs. CC, 0.982; TT vs. (CC+CT), 0.745; (CC+TT) vs. CT, 0.744.

**Supplemental References**

1. Katoh N, Ohya Y, Ikeda M, et al. Japanese guidelines for atopic dermatitis 2020. *Allergol Int.* 2020;69:356–69.

2. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol.* 2008;61:344–9.

3. Kunimura K, Sakata D, Tun X, et al. S100A4 Protein Is Essential for the Development of Mature Microfold Cells in Peyer’s Patches. *Cell Rep.* 2019;29:2823-2834.e7.

4. Yamamura K, Uruno T, Shiraishi A, et al. The transcription factor EPAS1 links DOCK8 deficiency to atopic skin inflammation via IL-31 induction. *Nat Commun.* 2017;8:13946.

**Supplemental Figures**

**FIGURE S1**

![Bar graph showing % of the Investigator Global Assessment (IGA) scores in each genotype of the AD group.](image1)

**FIGURE S2**

![Bar graph showing the Eczema Area and Severity Index (EASI) scores in CT and TT genotypes of moderate-to-severe AD patients with IGA scores of ≥3. The data are shown as means ± SDs and analyzed with two-tailed unpaired Student's t-test.](image2)

**FIGURE S3**

![Immunoblots showing DOCK8, EPAS1, and β-Actin expression in each HCT116 cell line. Data are representative of three independent experiments.](image3)

**FIGURE S1**: Bar graphs showing % of the Investigator Global Assessment (IGA) scores in each genotype of the AD group.

**FIGURE S2**: Bar graphs showing the Eczema Area and Severity Index (EASI) scores in CT and TT genotypes of moderate-to-severe AD patients with IGA scores of ≥3. The data are shown as means ± SDs and analyzed with two-tailed unpaired Student's t-test.

**FIGURE S3**: Immunoblots showing DOCK8, EPAS1, and β-Actin expression in each HCT116 cell line. Data are representative of three independent experiments.

**Supplemental Tables**

**TABLE S1**: Demographic characteristics of participants recruited into the study (N = 92)
| AD patients (N = 46) | Healthy controls (N = 46) | P value |
|---------------------|--------------------------|--------|
| Age (years)         | 34.86 ± 10.41            | 31.84 ± 6.26 | 0.095 |
| Gender, no.         |                          |        |
| Female              | 17                       | 19     | 0.669 |
| Male                | 29                       | 27     |       |
| IGA score           | 2.39 ± 1.02              | NA     |       |

Data are presented as mean ± SD. P values were tested by two-tailed Student’s t-test for continuous variables and the chi-square test for categorical variables. NA, not applicable.

**TABLE S2:** List of primers for the direct DNA sequencing of the *DOCK8* exons

| Exon No. | Forward Sequence (5´- 3´) | Reverse Sequence (5´- 3´) |
|----------|----------------------------|---------------------------|
| 1        | CCGACTTGCCCTACATTCC        | GGCCACCTGTAAGAACCT        |
| 2        | GTCTCCTCTTCTGTCATCATC      | GATGTCCAGACACAGTCAACA     |
| 3        | TGCCCTTTGTCGTCTTAACAG      | GGCATCTCCCTACCCAC         |
| 4        | TTGGAAATGATGCGGGAAGACACAG | TGTGTGATATGGAGTGAGGGG     |
| 5        | TCTAATGGCTTTGGCGCTTCTCG    | CCGTGCAAAGACAGCGCC        |
| 6        | CCAATTTGGAGACAGTGATTCTTG   | AATAATGAACACACCAAATACCTCCAC |
| 7        | TTAACCTTGGACCGGTGAGGGG     | GAAGATGACCATCAAGAGTGG     |
| 8        | TCAAATATTCTCGGGAACAGTGC    | TCAAACTGATAGTTCTTGCGTGC   |
| 9        | TTTAAACCATTGAAATGCAACAGAG | TGTGCTGGGAACACACACAGC     |
| 10       | AGCTTCCTATGTGCAATATTGTC    | GGGACACATTTGGAGCTAAAGCC   |
| 11       | AGGCAGTTGATCTTGGTGCTG      | AGTAAGCTTGAGACACCTGC      |
| 12       | TGGCCATATTCAGTTGCTTCTG     | ATCTCCTCTCTGTTGGCTC       |
| 13       | TCCAGAAAATCTGTGTGGCTCC     | TGTGTCAACGATAAGCAGTTTG    |
| 14       | GCAATGCAGTTGGAAAGCTAC     | CAGAGTCTTGCTTGCTCGTCC     |
| 15       | AAGCACCACATGGAATGGAGAAG    | TCCCGACTTTGTTGAAGTGG      |
| 16       | ACATCCTGGCTGGCTGATG        | AAAACAGGAGATGATGAAACC     |
| 17       | GGCTGTGGCTGTTTATAACATC     | CTGCAAGGACCTCATAGTC       |
| 18       | TTGCATTTGATTATGGCGAGC      | CCACATGGGAATGGGAAAGT      |
| 19       | GGGCTCCTGACTCCAGAAACAG     | AGAGGTGCTGTTGCTGCC        |
| 20       | CTCTCTGGTTGAAAGCCTG        | GCACACATCATCAGTGGAAGAG    |
| 21       | AAACCTCCACCTGAGGGCTCC      | ACTGCACTTGAGGAGGGGAAG     |
| 22       | GTCTTTTCTCAACCTACTATCC     | GTGGGCAAGCAGATTGGTTG      |
| 23       | TCCAGATGTCTGGCTTACCTCTTG   | CACAGTGATGAGGGCATAGGC     |
| 24       | GAACATCTAAGCACAAGTCTTCCAGG | CAGCAGGCGAACACTAGTG       |
| 25       | GCCACCCACGACAGAATAAAG      | CCACATCAGGCTGCCAAGC       |
| 26       | CCCACCAGTGCACTGGGAAG       | CGAACACATCAATGAGAAGAAGAAC |
| 27       | GCCAACCTCAACTCCACTTACC     | GCTAGGTGGAATCTGCCAGGAG    |
| 28       | TGGCCATCGCTATTTTACATTCC    | TTTACCAAGTGTGAGAGCTAGACTG |
| 29       | TGCTTGGTTCCTACAGTCACC      | CAGCAGGTGAAAGAATGACAG     |
| 30       | GTAGGGGACATGGGGAATG        | GCTGGAGCATGAAGAACC        |
|   | GATCTCCAGCCTAGCAGTGATG | CTCCATGGCCCCAAACAAAG |
|---|------------------------|----------------------|
| 31| TTAGCTGGCATCACCTGGGAG  | GGACATTCCCTCCCAAAAC  |
| 32| GAACCTTCTGTTATCTTGGAGGG | TCAGTAATACAAGCAAGCTGGG |
| 33| CCATCATGGGAACCTGGC     | TGGGATCACATTATGCTTTTAC |
| 34| CACTGGACATGGGACATCAGC  | CTGTGACTTTTGGCTCCACCTG |
| 35| ATTTCAACGGTCCAGAAAGTG  | TTTTCGAGCTGATTTCCTTAC |
| 36| TGCCATGCTGCTTTTCCAG    | CATACACAAATGTTGGCAGATCCC |
| 37| GTGGCCTCTCTGACCTGGGAC   | AGTGACAAATCCTTGACCCC |
| 38| AAAAGGTCACACAAAGTAGAAAGAACAG | GACAAAATCGCCCAAGTGG |
| 39| ATTCGCGGTTCTGTCAGTC     | CCAGCACCAAAGTCCAG |
| 40| GGACAATGACCTCTGGGGC     | ATCTGTAGGACAGGCTCAGCC |
| 41| CACAATGAGAGACCCCTGCC    | TGGTGATGACCCACTCTAACTTG |
| 42| TCACCCTCAAGAGCAGGAGTGG  | TTCTTGGGATAGAAGCAAGGG |
| 43| TCATTGCCTGCAAGGGATG     | GTTTTGAGGTCTTCCTTGG |
| 44| TGCCCTCTCTGATAGCTCC     | AAGGAAGAAGGGGTGCAGACG |
| 45| TCCTGAGATGTCCAAAGACCTTC | CCACACCTCCAGATTTCAA |
| 46| AGGTGATCCCGATATGCCCA    | TGACTGTGGGACCCTTCTCC |
| 47| (First half) TGATCTTTTTCTCCTGTCAGC | ACTCCAATAAATTCAATATGGGC |
| 48| (Second half) TCCACAATGTACAAAGAGTTG | TCCATTTAAGTGAAAGCAGATCTGTG |

**TABLE S3**: Association between genotypes of rs17673268 and IGA scores in the AD group (N = 46)

| IGA score | Genotype | CC | CT | TT |
|-----------|----------|----|----|----|
|           |          | N  | Frequency | N  | Frequency | N  | Frequency |
| ≤3        | CC       | 13 | 1.00 | 20 | 1.00 | 13 | 1.00 |
|           | CT       |    |       |    |       |    |       |
|           | TT       |    |       |    |       |    |       |
| 4         | CC       | 13 | 1.00 | 20 | 1.00 | 13 | 1.00 |
|           | CT       |    |       |    |       |    |       |
|           | TT       |    |       |    |       |    |       |

| Model     | x2 value | P value† |
|-----------|----------|----------|
| CC vs. CT | 2.959    | 0.065    |
| CC vs. TT | 4.727    | 0.029*   |
| CT vs. TT | 0.497    | 0.48     |

†P values were tested by the chi-square test. Statistical significance, *P < 0.05