Letter to the Editor

Selective role of neurokinin B in IL-31–induced itch response in mice

To the Editor:

Atopic dermatitis (AD) is a chronic inflammatory skin disease affecting 15% to 20% of the general population in developed countries.[1] It is characterized by recurrent eczematous legions and intense itch. Because the itch sensation induces scratching behavior, which exacerbates the skin inflammation and disturbs the quality of life of affected individuals, chronic itch is a challenging clinical problem in the treatment of AD.

Itch can be induced by various chemical mediators. Among them, much attention has been paid to IL-31 as an AD-associated itch mediator since the discovery of the pruritogenic action of IL-31 in mice.[2] IL-31 is mainly produced by $CD4^+$ T cells and transmits the signals via a heterodimeric receptor composed of IL-31 receptor A and oncostatin M receptor (OSMR), both of which are expressed in various cell types including dorsal root ganglion (DRG) neurons.[3] A recent clinical study has demonstrated that blockade of IL-31 signals by a specific antibody for IL-31 receptor A alleviates pruritus in patients with AD.[4] However, the neuronal mechanism underlying IL-31–induced itch sensation is poorly understood.

Mutations of *DOCK8* in humans cause a combined immunodeficiency characterized by AD. We have previously reported that DOCK8-deficient ($Dock8^{-/-}$), but not $Dock8^{+/-}$, mice spontaneously develop AD-like skin disease when crossed with transgenic mice expressing AND T-cell receptor (designated AND Tg mice). In this model, the disease manifestations were completely lost when either OSMR or IL-31 was deleted (see Fig E1 in this article's Online Repository at www.jacionline.org). To identify candidate molecules that mediate IL-31–induced itch sensation, we performed microarray analysis of the DRG neurons and found that 698 genes were expressed at higher levels in $Dock8^{-/-}$ AND Tg mice than in $Dock8^{+/-}$ AND Tg mice (see Table E1 in this article's Online Repository at www.jacionline.org), the second highest of which was *Tac2* encoding neurokinin B. To confirm the microarray data, we performed real-time PCR analyses. Although the expression of *Il31ra* was comparable between $Dock8^{-/-}$ AND Tg and $Dock8^{+/-}$ AND Tg littermates, the expression of *Tac2* in the DRG neurons increased 23-fold in $Dock8^{-/-}$ AND Tg mice, which was also canceled by deleting OSMR or IL-31 (Fig 1, *A*; see Fig E2 in this article's Online Repository at www.jacionline.org). Immunohistochemical analyses of $Dock8^{-/-}$ AND Tg mice revealed that neurokinin B was expressed by the IL-31 receptor A^+ DRG neurons (Fig 1, *B*). Neurotransmitters are stored in synaptic vesicles and released on stimulation. Indeed, neurokinin B was released *in vitro* from primary DRG neurons of wild-type (WT; C57BL/6) mice in response to IL-31 (Fig 1, *C*). However, the release of neurokinin B was completely abolished when primary DRG neurons lack OSMR expression (Fig 1, *C*). Thus, neurokinin B is induced in and released from DRG neurons in response to IL-31.

To examine the physiological significance of neurokinin B in IL-31–induced itch sensation, we developed 2 lines of *Tac2*-deficient (Δ4 and Δ15) mice by using the CRISPR/Cas9 nuclease system (see Fig E3 in this article's Online Repository at www.jacionline.org). Intradermal injection of histamine, chloroquine, and protease-activated receptor 2 agonist (SLIGRL-NH2) comparably induced scratching in both $Tac2^{-/-}$ (Δ4) and $Tac2^{+/-}$ mice (Fig 1, *D*), as was previously reported.[5] However, although IL-31 administration induced scratching in $Tac2^{+/-}$ mice, $Tac2^{-/-}$ (Δ4) mice were significantly less sensitive to IL-31 stimulation (Fig 1, *D*). Similar results were obtained when $Tac2^{-/-}$ mice (Δ15) were analyzed (see Fig E4 in this article's Online Repository at www.jacionline.org). Interestingly, *Tac2* deficiency markedly improved the skin inflammation and scratching behavior in $Dock8^{-/-}$ AND Tg mice without affecting serum IL-31 levels (Fig 1, *E-G*). Thus, neurokinin B is selectively required for transmission of IL-31–induced itch sensation.

Many pruritogens require natriuretic polypeptide b (Nppb) and gastrin-releasing peptide (GRP) to transmit itch sensation in the spinal cord.[6] To examine whether IL-31 uses GRP or Nppb to transmit itch sensation in the spinal cord, we specifically ablated neurons expressing GRP receptor or Nppb receptor by intrathecally injecting toxin (saporin)-conjugated GRP or Nppb, respectively. Although Nppb has been implicated in IL-31–mediated skin inflammation in the periphery,[7] IL-31–induced scratching was unaffected by intrathecal injection of Nppb-saporin (Fig 2, *A*). In contrast, treatment with GRP-saporin reduced IL-31–induced scratching (Fig 2, *A*). The *Grp* expression in the DRG neurons increased 4.5-fold in $Dock8^{-/-}$ AND Tg mice as compared with that in $Dock8^{+/-}$ AND littermates (see Fig E2). Although GRP was released *in vitro* from WT DRG neurons in response to IL-31, GRP release was hardly detected when primary DRG neurons from $Tac2^{-/-}$ (Δ4) mice were similarly stimulated (Fig 2, *B*). In addition, itch response induced by intrathecal injection of neurokinin B was lost when neurons expressing GRP receptor were ablated beforehand by toxin treatment (Fig 2, *C*). Neurokinin B transmits the signal through neurokinin 3 receptor (NK3R), a G protein–coupled tachykinin receptor. Immunohistochemical analyses of $Dock8^{-/-}$ AND Tg mice revealed that GRP was expressed by the $NK3R^+$ DRG neurons and neurokinin B^+ DRG neurons (Fig 2, *D*). These results indicate that neurokinin B acts upstream of GRP to transmit IL-31–induced itch sensation (Fig 2, *E*).

So far, several selective antagonists for NK3R such as osanetant and fezolinetant have been developed.[8,9] When osanetant was intraperitoneally injected into WT mice, IL-31–induced scratching was significantly attenuated (Fig 2, *F*). Similar results were obtained when fezolinetant was administered orally (see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast, treatment with NK3R antagonists failed to suppress itch response induced by histamine, chloroquine, and SLIGRL-NH2 (Fig 2, *F*; see Fig E5). Thus, pharmacological inhibition of NK3R selectively attenuates IL-31–induced itch sensation. Although NK3R antagonists suppress sex hormones by modulating gonadotropin secretion, its effect is transient and reversible, with no major side effects reported as yet.[8,9] Therefore, NK3R antagonists may be another option for treating AD-associated itch particularly in adults.

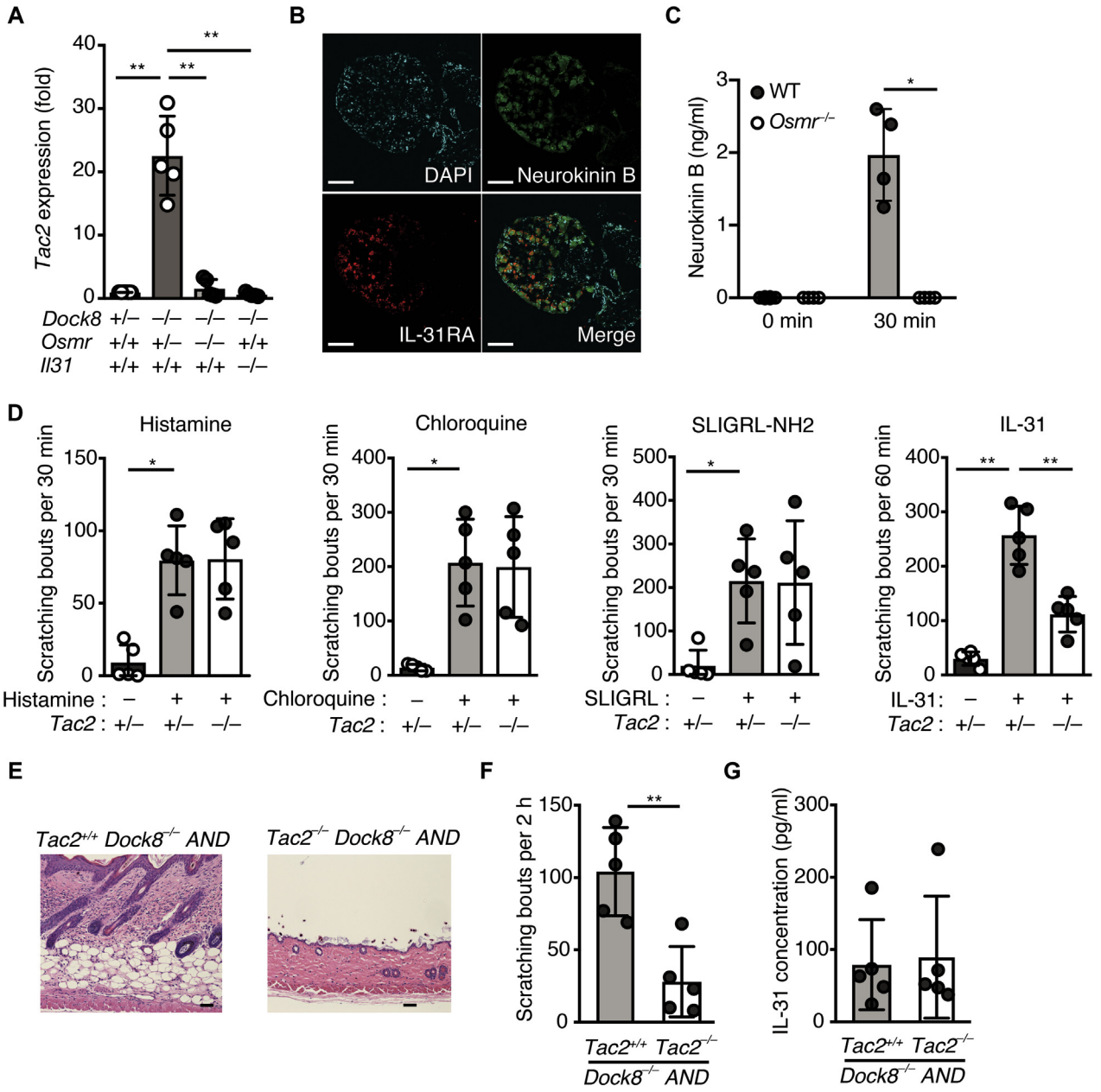

FIG 1. Neurokinin B mediates IL-31–induced itch response. **A,** *Tac2* expression in the DRG from mice of the indicated genotypes (n = 5-10). **B,** Immunofluorescence staining of the DRG from *Dock8*$^{-/-}$AND Tg mice. *Scale bar,* 100 μm. **C,** Release of neurokinin B from the DRG neurons in response to IL-31 (n = 4). **D,** Scratching behavior of *Tac2*$^{+/-}$ and *Tac2*$^{-/-}$ mice in response to various pruritogens (n = 5). **E-G,** Hematoxylin and eosin staining of the skin, scratching behavior, and serum concentration of IL-31 of *Dock8*$^{-/-}$ AND Tg mice with or without *Tac2* expression (n = 5). *Scale bar*, 100 μm. *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride. **P* < .05, ***P* < .01 (2-tailed unpaired Student *t* test for Fig 1, *A*, *F*, and *G*; Mann-Whitney test for Fig 1, *C* and *D*).

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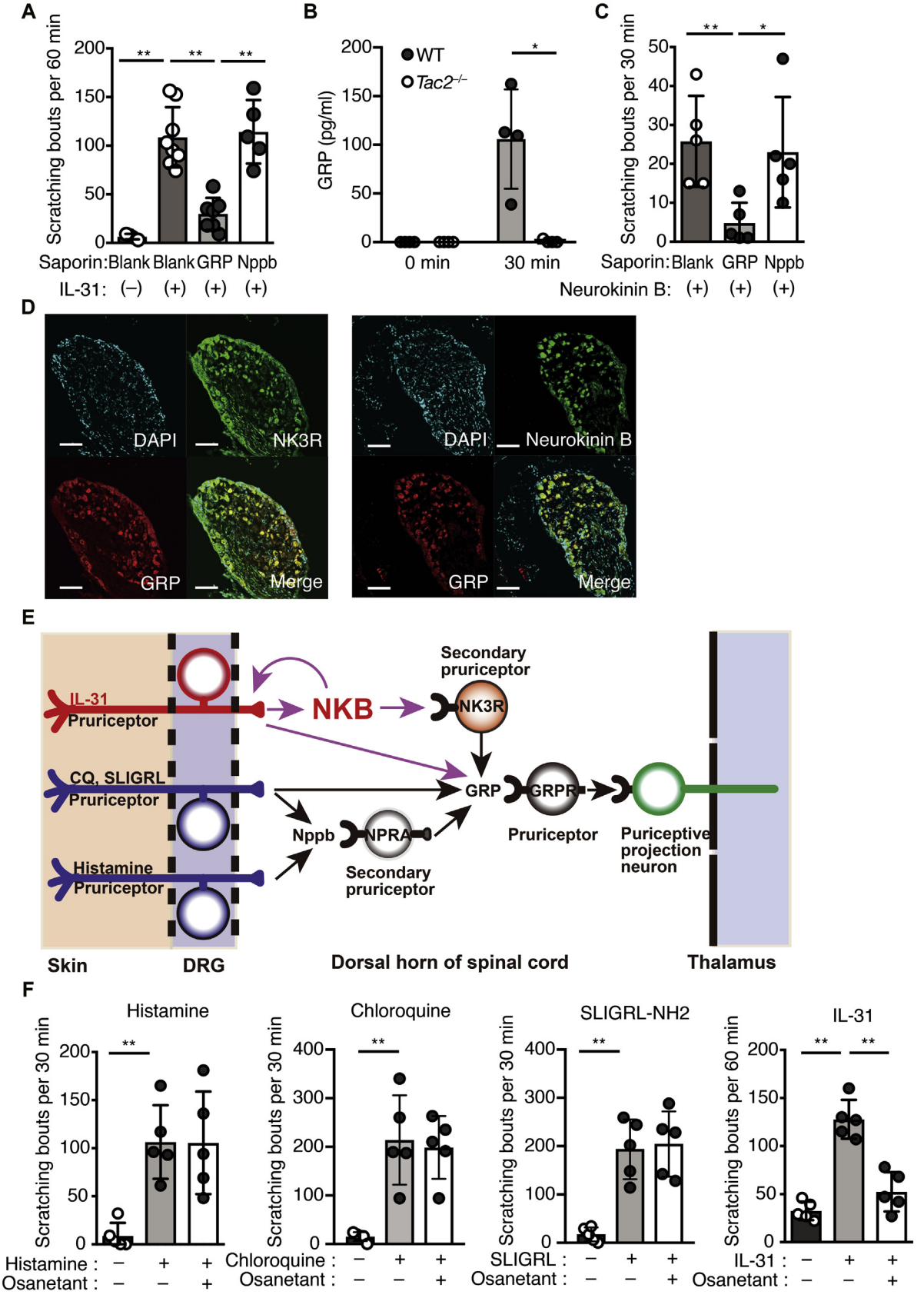

FIG 2. NKB acts upstream of GRP to transmit IL-31–induced itch sensation. **A,** IL-31–induced scratching in WT mice after intrathecal injection of saporin-conjugated GRP or Nppb (n = 5-8). **B,** Release of GRP from the DRG neurons in response to IL-31 (n = 4). **C,** Effect of GRP-saporin or Nppb-saporin on scratching in WT mice induced by intrathecal injection of NKB (n = 5). **D,** Immunofluorescence staining of the DRG from *Dock8*$^{-/-}$AND Tg mice. *Scale bar*, 100 μm. **E,** A model for IL-31–induced itch sensation. **F,** Effect of osanetant on scratching behavior of WT mice in response to various pruritogens (n = 5). *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride; *GRPR*, GRP receptor; *NKB*, neurokinin B; *NPRA*, Nppb receptor. *$P < .05$, **$P < .01$ (2-tailed unpaired Student *t* test for Fig 2, *A*, *C*, and *F*; Mann-Whitney test for Fig 2, *B*).

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Disclosure of potential conflict of interest: Kyushu University and University of Toyama have a pending patent application related to the work reported herein, in which D. Sakata, T. Urono, T. Andoh, and Y. Fukui are listed as coinventors. The rest of the authors declare that they have no relevant conflicts of interest.

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METHODS

Animals

Tac2$^{-/-}$ mice were developed by using the CRISPR/Cas9 genome editing system. A targeting site within the exon 4 of mouse *Tac2* was selected using the CHOPCHOP web design tool (https://chopchop.rc.fas.harvard.edu/). Two complementary oligonucleotides (5′-CACCGAGTGCTGAGCAAGGCTAGCG-3′ and 5′-AAACCGCTAGCCTTGCTCAGCACTC-3′) containing the guide sequence (underlined) and *Bbs* I ligation adaptors were synthesized, annealed, and ligated into the *Bbs* I–digested px330 vector for coexpression of single guide RNA and Cas9 protein. The pX330 vector (5 ng/μL in Dulbecco's PBS) was injected into the pronuclei of *in vitro* fertilized eggs of C57BL/6 mice in M2 medium (Sigma-Aldrich, St Louis, Mo). The injected zygotes were cultured in CZB medium at 37°C, 5% CO_2 until 2-cell stage embryos develop. Then, 24 to 36 embryos were transferred into the oviducts of pseudo-pregnant ICR female mice. Genotype of offspring mice was identified by genomic PCR using primers (5′-CTCTCCCCTACAAGGACTCTGA-3′ and 5′-CCAATCTAATCTTCAGAACGCC-3′) followed by TA cloning of the products and sequencing. The offspring mice carrying desired mutation (Δ4 and Δ15) were crossed with C57BL/6 mice or *Dock8*$^{-/-}$ AND Tg mice.[E1] *Dock8*$^{+/-}$ AND Tg and *Dock8*$^{-/-}$ AND Tg mice with or without OSMR expression have been described previously.[E1] For generation of *Il31*$^{-/-}$ mice, a targeting vector was designed on the basis of pNT1.1 vector to insert the gene encoding–enhanced green fluorescent protein and a flippase recognition target–flanked neomycin-resistant cassette (*neo*) immediately after the initiation codon and was introduced into embryonic stem cells by electroporation. Correctly targeted embryonic stem clones were microinjected into C57BL/6 blastocysts, and the male chimeras obtained were crossed with C57BL/6 female mice. Heterozygous mutant mice were crossed with CAG-FLPe transgenic mice (RBRC01843) to remove *neo*, and *neo*-deleted mutant mice were backcrossed with C57BL/6 mice for more than 5 generations before crossing with *Dock8*$^{+/-}$ AND Tg and *Dock8*$^{-/-}$ AND Tg mice. All mice were maintained under specific-pathogen-free conditions in the animal facility of Kyushu University, and age- and sex-matched littermate mice were used as controls. *Dock8*$^{+/-}$ AND Tg and *Dock8*$^{-/-}$ AND Tg mice with or without *Osmr*, *Il31*, and *Tac2* were analyzed at age 12 to 18 weeks. *Tac2*$^{-/-}$ mice, *Tac2*$^{+/-}$ mice, and WT mice were analyzed at age 7 to 10 weeks. All animal experiments were conducted according to relevant national and international guideline contained in the "Act on Welfare and Management of Animals" (Ministry of Environment of Japan) and the "Regulation of Laboratory Animals" (Kyushu University). The protocol of animal experiments was approved by the Committee of Ethics on Animal Experiments of Kyushu University.

Ablation of specific neurons by toxin treatment

Ablation of spinal cord neurons expressing GRP receptor or Nppb receptor was performed by intrathecal (segment L3/4) injection of GRP-saporin or Nppb-saporin, respectively (2 μg in 5 μL each, Advanced Targeting Systems, San Diego, Calif). As a control, nontargeted saporin (designated Blank) was also injected. Mice were used for experiments 2 weeks after toxin injection.

Measurement of scratching behavior

Before experiments, mice were put into an acrylic cage (11 × 14 × 20 cm) for at least 1 hour for acclimation. Then, the itch-inducing substances dissolved in sterile saline were injected intradermally into the shoulder of the mice at a volume of 50 μL, and their behaviors were video-recorded using HDR-CX390 (Sony, Tokyo, Japan). Playback of the video was used for determination of the total number of scratching bouts per the specified times. When mice scratch, they stretch their hind paw toward the itchy spot, lean the head toward the hind paw, rapidly move the paw several times, and then lower it back to the floor. A series of these movements was counted as 1 bout of scratching. The following itch-inducing substances were used for intradermal injection in this study: IL-31 (1 μg in 50 μL; Peprotech, Rocky Hill, NJ), SLIGRL-NH2 (100 μg in 50 μL; BACHEM, Bubendorf, Switzerland), chloroquine (100 μg in 50 μL; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and histamine (100 μg in 50 μL; FUJIFILM Wako Pure Chemical Corporation). In some experiments, the NK3R antagonist osanetant (Axon Medchem, Groningen, The Netherlands) or fezolinetant (Haoyuan ChemExpress, Shanghi, China) was administered intraperitoneally or orally at concentration of 5 mg/kg or 10 mg/kg, respectively, 45 minutes before intradermal injection of itch-inducing substances.

Histology and immunohistochemistry

Skin tissues were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin blocks. Sections (3 μm thick) were stained with hematoxylin and eosin, and examined by light microscopy. For immunofluorescence analyses of the DRG, mice were euthanized with isoflurane and subjected to perfusion with 4% paraformaldehyde in PBS. The DRG tissues were collected, postfixed overnight at 4°C, and cryoprotected overnight in 30% sucrose-PBS. Then, tissue samples were embedded in OCT compound (Sakura Finetech, Tokyo, Japan) and frozen on dry ice. After cryostat sections (10 μm thick) were prepared, they were blocked with G-Block (GenoStaff, Tokyo, Japan; GB-01) for 30 minutes at room temperature and incubated overnight at 4°C with primary antibodies. The staining was detected and visualized with fluorophore-conjugated secondary antibodies (Thermo Fisher, Waltham, Mass). 4′-6-Diamidino-2-phenylindole, dihydrochloride (DOJINDO, 1:5000) was used for nucleus staining. All images were obtained with a laser scanning confocal microscope (FV3000, Olympus, Tokyo, Japan). The following primary antibodies were used in this study: rabbit antibody for neurokinin B (Novus Biologicals, Centennial, Colo; NB300-201, 1:500), goat antibody for IL-31 receptor A (R&D Systems, Minneapolis, Minn; AF2107, 1:100), rabbit antibody for NK3R (Novus Biologicals; NB300-102, 1:50), and goat antibody for GRP (Santa Cruz Biotechnology, Santa Cruz, Calif; sc-7788, 1:100).

Microarray analysis

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan), and cRNA was amplified and labeled using a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, Calif). The cRNA was then hybridized to a 44 K 60-mer oligomicroarray (Whole Mouse Genome oligo DNA Microarray Kit version 2.0; Agilent Technologies). The hybridized microarray slides were scanned using an Agilent scanner. The relative hybridization intensities and background hybridization values were calculated using Feature Extraction Software version 9.5.1.1 (Agilent Technologies). Raw signal intensities and flags for each probe were calculated from the hybridization intensities and spot information, according to the procedures recommended by Agilent Technologies. To identify up- or downregulated genes in experimental samples, we calculated *Z* scores and ratios from the normalized signal intensities of each probe (upregulated genes, *Z* score > 2.0 and ratio > 1.5-fold; downregulated genes, *Z* score < −2.0 and ratio < 0.66-fold). The microarray data that support the finding of this study are available in Gene Omnibus with the primary accession code GSE118986.

Real-time PCR

Total RNA from each tissue was isolated using ISOGEN (Nippon Gene). After treatment with RNase-free DNase I (Life Technologies), RNA samples were reverse-transcribed with oligo (dT) primers (Life Technologies) and SuperScript III reverse transcriptase (Life Technologies) for amplification by PCR. The following primers were used for real-time PCR: for *Tac2*, 5′-TCGTGAAAGTGCTGAGCAAG-3′ and 5′-GTGTCTGGTTGGCTGTTCCT-3′; for *Grp*, 5′-CACGGTCCTGGCTAAGATGT-3′ and 5′-ATACAGGGACGGGGATTCAT-3′; for *Il31ra*, 5′-TCCTGAGGATCCCAGATGTC-3′ and 5′-GGAGCCACTCCACTATCCAA-3′; and for *Hprt*, 5′-CTGGTGAAAAGGACCTCTCG-3′ and 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′. Real-time PCR was performed on CFX Connect Thermal Cycler (BIO-RAD, Hercules, Calif) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif). The expressions of mouse target genes were normalized to expression of *Hprt* gene. CFX Manager software (version 3.1) supplied with the instrument was used for analyses. Data are expressed as relative values to that of a sample from *Dock8*$^{+/-}$*Osmr*$^{+/+}$*Il31*$^{+/+}$ AND Tg mice (Fig 1, *A*) or a sample from *Dock8*$^{+/-}$ AND Tg mice (Fig E2).

ELISAs

DRG neurons were prepared from the specified mouse lines and stimulated *in vitro* with IL-31 (10 μg/mL; Peprotech). The culture supernatants were recovered 30 minutes after stimulation, and the concentrations of neurokinin B and GRP were measured with ELISA kits (MyBiosource, San Diego, Calif; MBS744693 for neurokinin B and RayBiotech, Norcross, Ga; EIAM-GRP for GRP), according to the manufacturers' instructions.

Statistical analyses

Statistical analyses were performed using GraphPad Prism7 (GraphPad Software, La Jolla, Calif). The data were initially tested with a Kolmogorov-Smirnov test for normal distribution. Parametric data were analyzed using a 2-tailed unpaired Student *t* test when 2 groups were compared. Nonparametric data were analyzed with a Mann-Whitney test when 2 groups were compared. Data are expressed as mean ± SD, and *P* values of less than .05 were considered significant.

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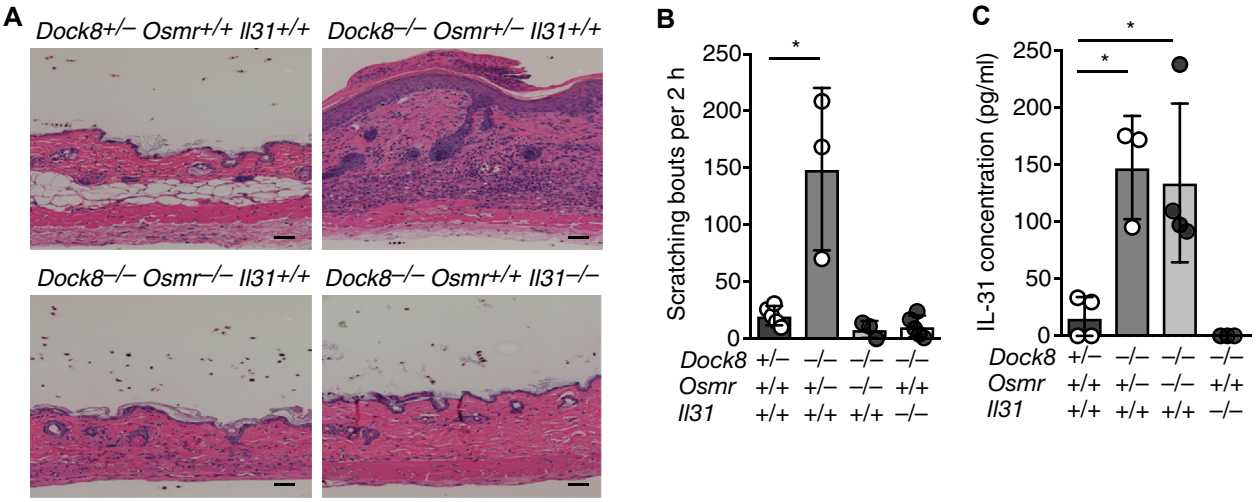

FIG E1. Comparison of *Dock8*$^{+/-}$ AND Tg mice and *Dock8*$^{-/-}$ AND Tg mice with or without the expression of *Osmr* and *Il31*. **A,** Hematoxylin and eosin staining of the skin from 15-week-old mice of the indicated genotypes. Data are representative of 3 independent experiments. *Scale bar,* 100 μm. **B,** Scratching behavior of 12- to 18-week-old mice of the indicated genotypes (n = 3-5). **P* < .05 (Mann-Whitney test). **C,** Serum concentration of IL-31 in 12- to 18-week-old mice of the indicated genotypes (n = 3-4). **P* < .05 (Mann-Whitney test).

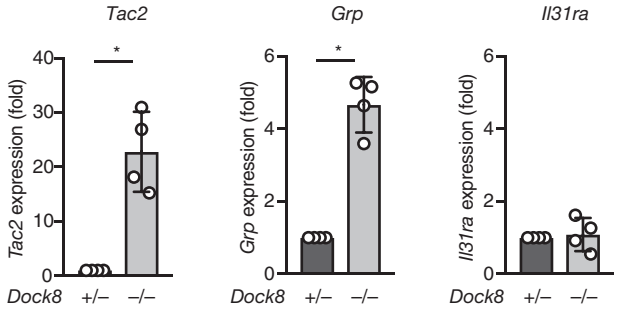

FIG E2. The expressions of *Tac2, Grp,* and *Il31ra* in the DRG are compared between 12- to 18-week-old *Dock8*$^{+/-}$ AND Tg and *Dock8*$^{-/-}$ AND Tg littermate mice (n = 4). **P* < .05 (Mann-Whitney test).

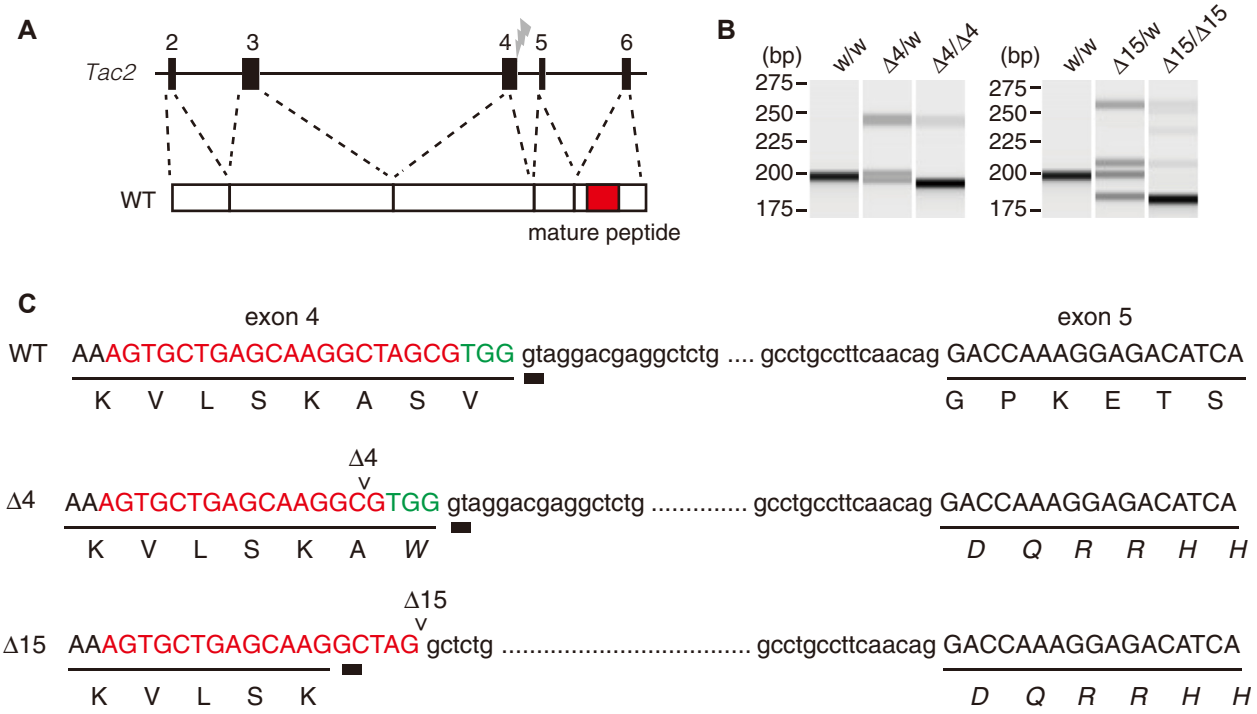

FIG E3. Generation of *Tac2*$^{-/-}$ mice. **A,** Schematic of the structure of mouse *Tac2* gene and encoded protein. Mature, active form of *Tac2* is encoded by exon 6 and generated from the precursor peptide through processing. The guide RNA for genome editing by CRISPR/Cas9 system was designed within exon 4. **B,** PCR genotyping of 2 *Tac2*-deficient lines using primers (s: CTCTCCCCTACAAGGACTCTGA, as: CCAATCTAATCTTCAGAACGCC). The PCR samples were subjected to electrophoresis on the MultiNA microchip electrophoresis system (MCE-202, Shimadzu, Kyoto, Japan). **C,** Genomic sequences of 2 mutant alleles and the deduced amino acid sequences based on the transcripts. Guide sequence (*red*) and PAM (*green*). Positions of nucleotide deletion (Δ4 and Δ15) were indicated. For transcript analysis, total RNA was isolated from the DRG neurons of each mouse, and reverse-transcribed to obtain cDNA. The cDNA encoding *Tac2* was amplified by PCR using primers (s2: AGGGAGGGAGGCTCAGTAAGGAC, as1: CCAACAGGAGGACCTTACAGGCAGG), and the products were analyzed by TA cloning followed by DNA sequencing. Splice donor sites for each transcript of each allele are indicated by solid line. Both deletions led to frame-shift in the last amino acid sequence encoded by exon4, and thereby no longer encode mature peptide sequence.

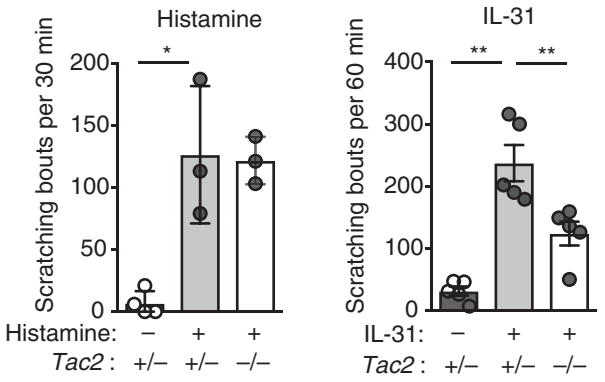

FIG E4. *Tac2*$^{-/-}$ mice (Δ15) also exhibit selective impairment of IL-31–induced itch sensation. Scratching behaviors of 7- to 10-week-old *Tac2*$^{+/-}$ and *Tac2*$^{-/-}$ (Δ15) mice in response to intradermal injection of histamine (*left*; n = 3-4) or IL-31 (*right*; n = 5) were analyzed. **P* < .05, ***P* < .01 (Mann-Whitney test).

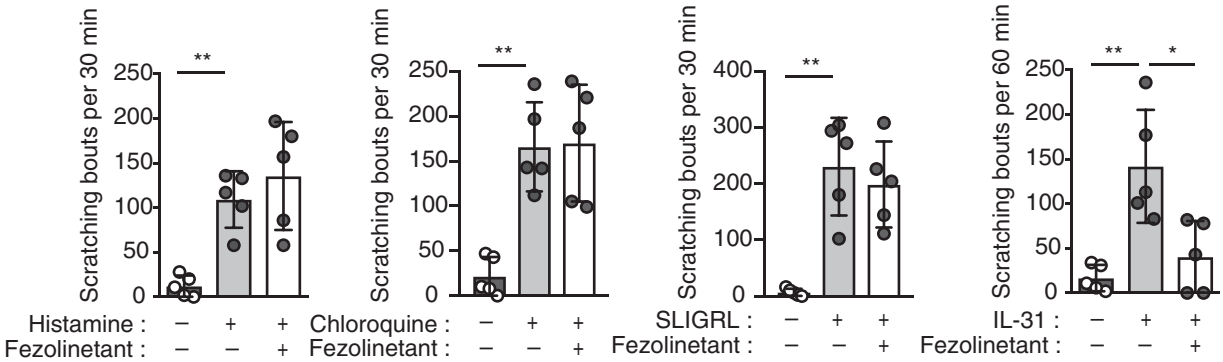

FIG E5. NK3R antagonist fezolinetant also selectively inhibits IL-31–induced itch response. Effect of fezolinetant on scratching behaviors of WT mice in response to various pruritogens. Forty-five minutes before assays, mice (n = 5) were treated with or without fezolinetant. *$P < .05$, **$P < .01$ (2-tailed unpaired Student *t* test).

TABLE E1. Top 50 genes that are upregulated in the DRG neuron from *Dock8*$^{-/-}$ AND Tg mice, compared with *Dock8*$^{+/-}$ AND Tg control

List no.	Gene symbol	Signal value for Dock8$^{-/-}$ AND sample	Signal value for Dock8$^{+/-}$ AND sample	*Z* score
1	*Polg*	25059.91309	131.60595	11.59500
2	***Tac2***	**1061.98115**	**16.79421**	**7.02463**
3	*Trp53rkb*	26796.715	2622.0525	6.08060
4	*Plin4*	4265.5005	219.17575	6.03909
5	*Syn3*	2634.8025	151.647705	5.80919
6	*Phyhipl*	35585.94	3869.65	5.80499
7	*Tbx22*	9567.6395	703.0649	5.77195
8	*Shc4*	37813.335	4228.3795	5.73202
9	*Dcaf12l2*	29924.9756	3363.7015	5.71846
10	*Cd300lf*	12531.68	949.6657	5.70400
11	*Dsg1b*	249.57585	3.8240735	5.67923
12	*Lzts3*	41246.425	4790.573	5.63296
13	*St6galnac1*	12747.9037	1109.5959	5.39827
14	*Cabp5*	40772.645	5208.665	5.38427
15	*Rabgap1*	6006.822	550.03765	5.28636
16	*Tdgf1*	8140.282	819.101	5.07827
17	*Mrgprg*	2062.2515	173.52145	5.03959
18	*Ddc*	1657.524	140.62649	5.02281
19	*Cdcp1*	2394.0065	203.1315	5.02260
20	*Opcml*	5325.982776	473.0818	4.92981
21	*Klra1*	4445.9955	409.6638	4.85551
22	*Anxa7*	2333.107	217.63445	4.83062
23	*Nudt5*	7748.6555	880.51705	4.80989
24	*Pou4f1*	14542.092	1671.67	4.78441
25	*Gfap*	7228.1925	831.614	4.78254
26	*Pinx1*	5107.1435	612.0913	4.69236
27	*Phactr3*	503.5882	16.509495	4.64595
28	*Pole2*	427.78415	28.81568	4.57212
29	*Rs1*	40260.475	7019.798489	4.57195
30	*Herc2*	13638.32	1826.8075	4.44695
31	*Rmst*	658.85205	47.932365	4.44182
32	*Lsamp*	6295.404	846.24615	4.43912
33	*Camkv*	1281.75195	95.369055	4.40378
34	*Gm11974*	3275.5705	377.4753	4.40220
35	*Phactr3*	4831.169	720.0565	4.21125
36	*Slc6a1*	1522.78495	194.20005	4.19683
37	*Agt*	6413.059	987.2563	4.13985
38	*Rnu3b1*	12692.165	1958.6015	4.13454
39	*Gcm1*	4401.5455	699.5915	4.06934
40	*Pip5k1a*	17049.945	3686.792	4.00982
41	*Mobp*	2778.7755	400.82595	3.94721
42	*Mt2*	75372.04	18862.395	3.93820
43	*Slco2a1*	929.748	92.21826	3.91727
44	*Sptbn1*	2888.314	431.60695	3.87562
45	*Ermn*	30312.04	6931.194	3.86389
46	*Slc9a3*	597.00745	61.973665	3.84021
47	*Gm20753*	53705.875	14057.213	3.81242
48	*Skor1*	167.65605	10.185202	3.80799
49	*B020031M17Rik*	4581.977	828.54815	3.78464
50	*Zbtb16*	1930.8435	302.44065	3.78020

The expression data on *Tac2* gene encoding neurokinin B are indicated in boldface.