

SUPPORTING INFORMATION

METHODS

Generation of mEar2-deficient mice

All mice were housed in a specific pathogen-free animal facility with a regular 12-h light/dark cycle at an appropriate temperature and humidity. All experimental procedures used in this study were approved by the Animal Care and Use Committee of the Kansai Medical University (No:22-048). Female C57BL/6J mice (6-8 weeks old) were purchased from Shimizu Experimental Materials (Kyoto, Japan).

The mEar2-targeting vector was designed to replace exon 2 with a Cre-Enhanced Green Fluorescent Protein (Cre-EGFP) and PGK promoter-driven neomycin resistance cassette (Fig. S1A). The 4.3 kb long and 2.1 kb short arm genomic DNA fragments were obtained from the C57BL/6 mouse Bac genomic clone (ID: RP23-262F9 and RP23-344C13, Roswell Park Cancer Institute, USA). To produce the long arm fragment and Cre-EGFP, the 1.4 kb 3' side of the long arm fragment and Cre-EGFP fragment amplified from the pCAG-Cre:GFP plasmid (Addgene, #13776) were inserted in pBlueScriptII SK+ (Invitrogene, USA). The 5' side of the long arm fragment was amplified via PCR, digested using PspOMI and XmaI, and inserted in pBS-NEO-DTA by Unitech Co. (Kashiwa, Japan). The short-arm fragment was amplified using PCR, digested with XmaI and PspOMI, and inserted into the pBS-loxP-FRT-Neo-FRT vector (Unitech, Japan). The targeting vector was constructed by subcloning the 5' long arm and Cre-EGFP digested using XmaI and MluI and the short arm fragment digested using MluI and SacII into the pBS-DTA vector with the 5' side of the long arm fragment digested using Xma and SacII. The sequences of the targeted vectors were confirmed via DNA sequencing. The targeting vector was linearized via SacII digestion and electroporated into C57BL/6-derived embryonic stem cells. Southern blotting and PCR were performed to verify homologous recombination in G418-gancyclovir-resistant clones. To generate chimeric mice, targeted embryonic stem clones were injected into ICR blastocysts. Heterozygous F1 offspring (derived from ES clone #53 and 65) were produced by mating chimeric males with C57BL/6 females. Heterozygous offspring were crossed to generate homozygous embryos. mEAR2 deficient mice are available from RIKEN BioResource Research Center. Genotyping via PCR using DNA samples from tail biopsies was performed to classify mice into wild type (mEAR2^{+/+}), heterozygous (mEAR2^{+/-}), and homozygous (mEAR2^{-/-}) animals. For the list of all primers, see the supplementary table S1.

AAI model

AAI was induced in mEAR2^{-/-} and mEAR2^{+/-} mice as our previous report.¹ Briefly, mice were sensitized through 2 intraperitoneal (i.p.) injections of 10 µg unpurified Der f 1. (*Dermatophagoides farinae*) (Institute of Tokyo Environmental Allergy, Japan), referred to HDM antigen, mixed with 2 mg of aluminum hydroxide (Thermo Fisher Scientific, USA) at days 0 and 14. Then, mice were intranasally (i.n) challenged with 20 µL PBS or 5 µg HDM in 20 µL PBS from day 21 to day 25. AHR was evaluated by measuring the airway resistance upon nebulization of increasing methacholine concentrations using invasive plethysmography (FinePointe RC; Buxco Research Systems, Winchester, UK) 48 hours after the last challenge. Lung inflammation was assessed using histology and bronchoalveolar lavage fluid (BALF) analysis. The mRNA and/or protein levels of cytokines, mEAR2 in the lung tissue and/or BALF, and the total immunoglobulin E (IgE) in the serum were measured by qPCR and ELISA on days 0, 14, 20, and 27.

Lung sample preparation

The left and right lung lobes were collected for histological analysis and RNA extraction, respectively. The left lung was fixed with 4% paraformaldehyde for 24 h and then freeze-dried in super cry embedding medium (Leica Microsystems, Land Hessen, Germany) in isopentane (Nacalai Tesque, Kyoto, Japan) cooled in liquid nitrogen. Freshly frozen samples

51 were sectioned following the Kawamoto method³ and stained with hematoxylin and eosin for
52 morphological analysis.

53 ***Eosinophil differentiation from bone marrow and functional assays***

54 Eosinophils were differentiated from the bone marrow (BM) of mEAR2^{-/-} and mEAR2^{+/+}
55 mice as previously described.² BM cells were cultured at 5×10^6 /mL in medium containing
56 RPMI 1640 (Invitrogen, USA) with 20% FBS (Gibco, USA), 100 IU/mL penicillin, 10 µg/mL
57 streptomycin (Gibco, USA), 2 mM glutamine (Invitrogen), 25 mM HEPES, 1x non-essential
58 amino acids, 1 mM sodium pyruvate (Gibco, USA), 50 µM β-mercaptoethanol (Sigma,
59 Germany), 100 ng/mL stem cell factor (SCF; PeproTech, USA), and 100 ng/mL FLT3-ligand
60 (FLT3-L; PeproTech, USA) from day 0 to day 4. On day 4, the medium containing 10 ng/mL
61 recombinant interleukin-5 (rmIL-5; R&D Systems, USA) was replaced with a medium
62 without SCF and FLT3-L. On day 8, the cells were seeded into new dishes, and half of the
63 medium was replaced with fresh medium containing rmIL-5 every other day. Morphological
64 and functional analyses of BM-derived eosinophils (BMDE) from mEAR2^{-/-} and mEAR2^{+/+}
65 mice were performed via microscopic counting following diff-quick staining, chemotaxis
66 assay, and cytokine profiling using qPCR on day 14.

67 ***Chemotaxis assay***

68 For the chemotaxis assay, an eosinophil suspension (0.5×10^6 cells/mL) was applied to the
69 upper chamber of a 24-well-type Chemotaxis cell chamber (5 µm pore, polycarbonate
70 membrane) in RPMI-1640 medium. Chemoattractant solution (10 µg eotaxin-1, PeproTech,
71 USA) was injected into the lower chamber, and the plate was incubated at 37 °C for 3 h. After
72 gentle washing, both the upper and lower cells were incubated with APC-SiglecF (BD
73 Bioscience, USA) and analyzed using the FACS Calibur flow cytometer acquire the events
74 within 30 s. At the end of the assays, we calculated the chemotactic index by dividing the
75 number of eosinophils that migrated over the membrane towards the chemoattractant by the
76 number of eosinophils remaining at baseline.⁴

77 ***Co-culture of sensitized splenocytes and BMDE***

78 Co-culture experiments with splenocytes and BMDE from mEAR2^{+/+} or mEAR2^{-/-} mice were
79 performed as previous described with modification.⁵ Briefly, spleens were collected from
80 mEAR2^{+/+} and mEAR2^{-/-} 8-weeks-old mice after sensitization using 10 µg HDM with 2 mg
81 aluminum hydroxide i.p. at day 0 and 7. Splenocytes were resuspended in RPMI with 10%
82 FBS, followed by removal of red blood cells treated with lysis buffer (BD Bioscience, USA).
83 3×10^6 splenocytes /mL and 3×10^6 BMDE cells/mL were individually cultured or co-
84 cultured in the RPMI medium with or without 40 µg/mL HDM and incubated for 96 h at
85 37 °C with 5% CO₂. Then, the cells and supernatant were harvested, and type 2 cytokines (IL-
86 4, -5, and -13) analysis was performed using qPCR and ELISA.

87 ***Statistical analysis***

88 Data are presented as the means ± standard error of the mean (SEM). Statistical significance
89 was determined using the Student's t-test or Mann–Whitney U test. The threshold of
90 significance for all tests was considered $p < 0.05$ for all tests.

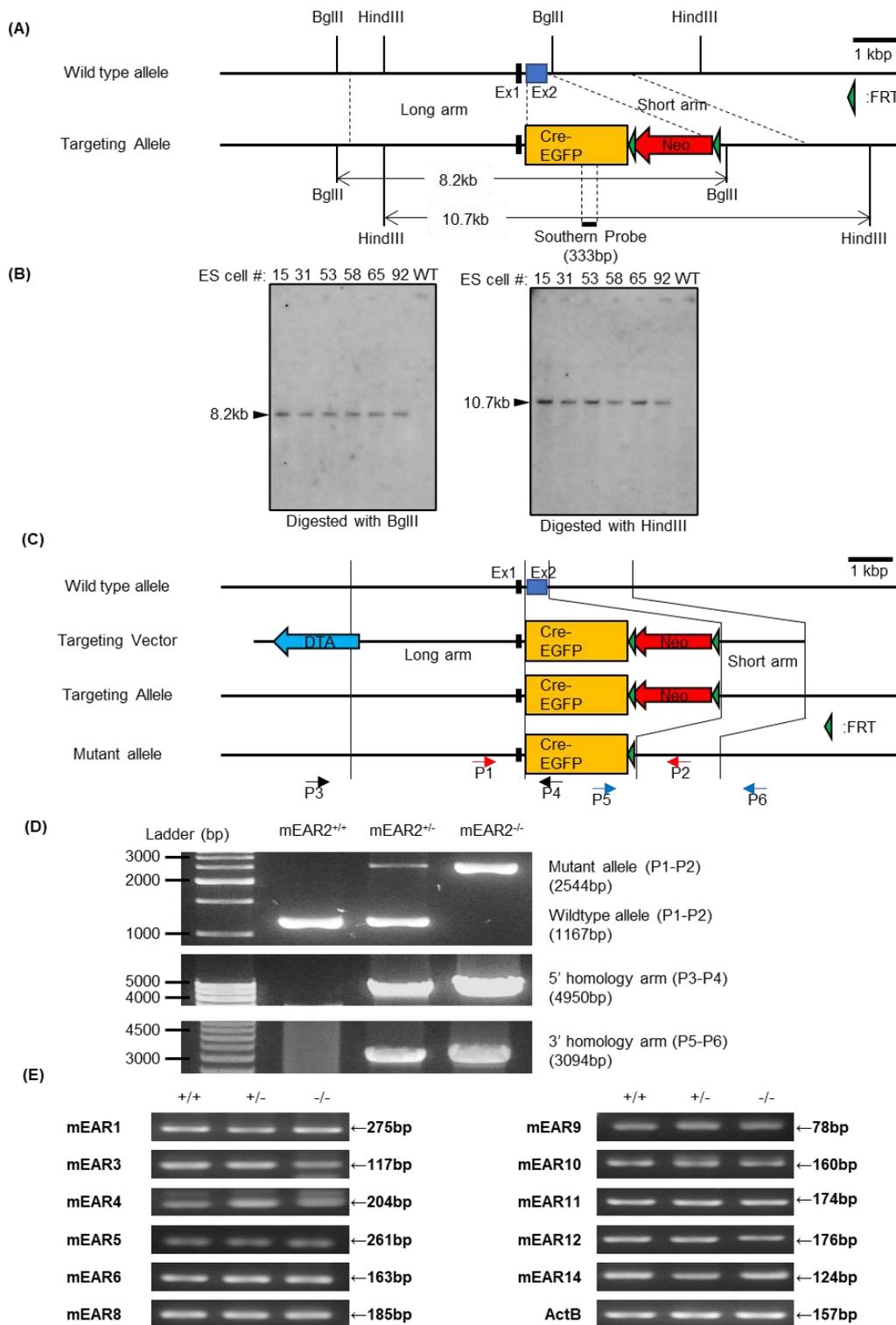
91 **REFERENCES**

- 92 1. Kanda A, Kondo K, Hosaka N, Kobayashi Y, Bui DV, Yun Y, et al. Eosinophilic
93 upper airway inflammation in a murine model using an adoptive transfer system induces
94 hyposmia and epithelial layer injury with convex lesions. *Med Sci (Basel)* 2019;7(2):22. doi:
95 10.3390/medsci7020022
- 96 2. Lu TX, Rothenberg ME. Bone Marrow Derived Eosinophil Cultures. *Bio Protoc.*
97 2014;4(12). doi: [10.21769/bioprotoc.1161](https://doi.org/10.21769/bioprotoc.1161)
- 98 3. Kawamoto T, Kawamoto K. Preparation of Thin Frozen Sections from Nonfixed and
99 Undecalcified Hard Tissues Using Kawamoto's Film Method (2020). In: Hilton MJ, editor.
100 *Skeletal Development and Repair: Methods and Protocols*. New York, NY: Springer US;

- 101 2021. p. 259-81. doi: [10.1007/978-1-0716-1028-2_15](https://doi.org/10.1007/978-1-0716-1028-2_15)
- 102 4. Wen T, Besse JA, Mingler MK, Fulkerson PC, Rothenberg ME. Eosinophil adoptive
 103 transfer system to directly evaluate pulmonary eosinophil trafficking in vivo.
 104 2013;110(15):6067-72. doi: [10.1073/pnas.1220572110](https://doi.org/10.1073/pnas.1220572110)
- 105 5. Peng B, Sun L, Zhang M, Yan H, Shi G, Xia Z, Dai R and Tang W (2022). Role of IL-
 106 25 on Eosinophils in the Initiation of Th2 Responses in Allergic Asthma. Front. Immunol.
 107 13:842500. doi: [10.3389/fimmu.2022.842500](https://doi.org/10.3389/fimmu.2022.842500)

108 **Supplementary Table S1. Primers List**

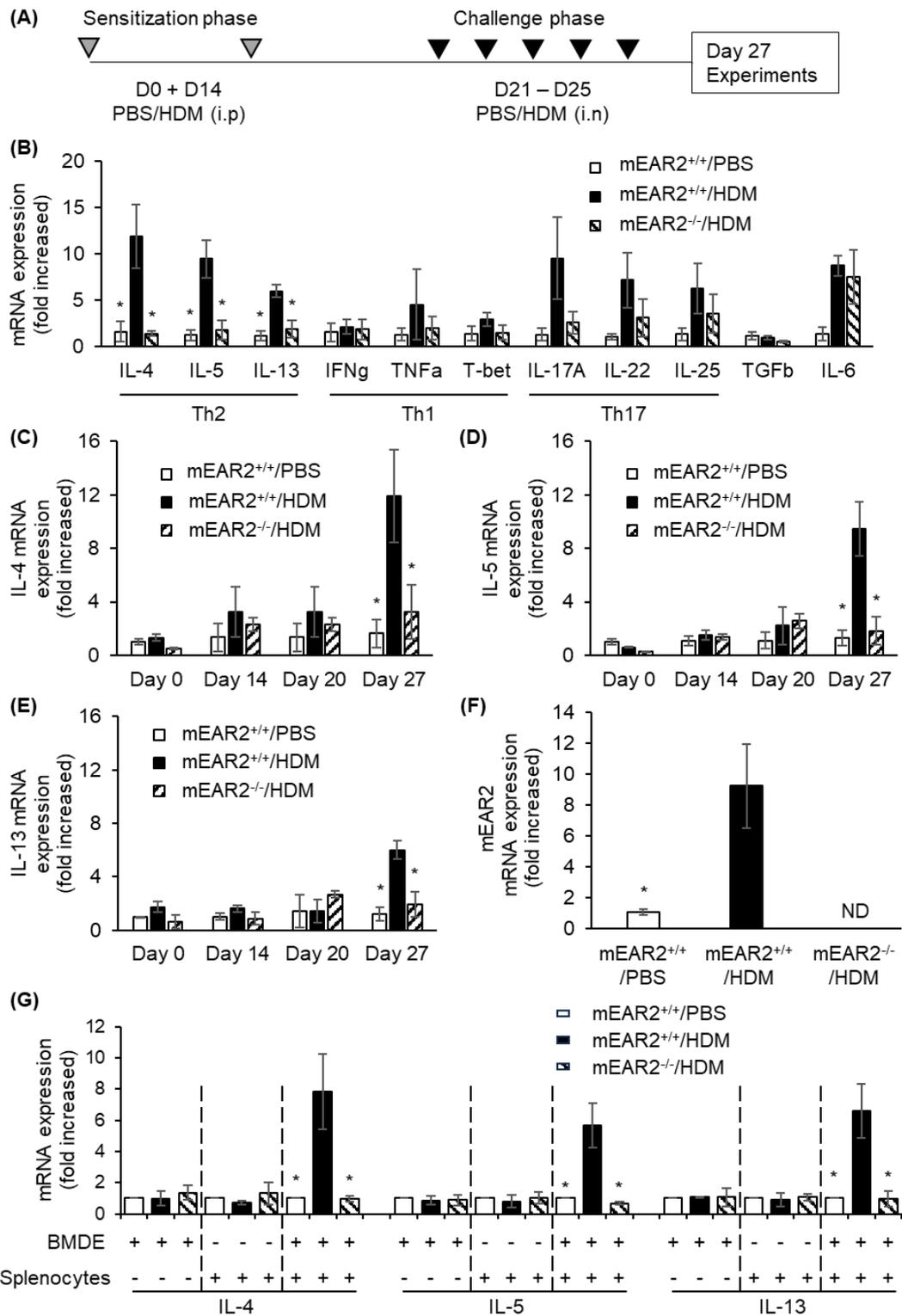
Gene	Forward	Reverse
P1-P2	ACTGAGGCAAGGTGGGAATAGT	GCATTTTCATTCTAGTCCATTAG
P3-P4	AGCAAGAGAGCTCACGTACACC	TGCACGTTACCGGCATCAAC
P5-P6	TGAAGTTCATCTGCACCACCGG	TTCTGACTCAGGAGAGGCGAC
Southern probe	AGCTGACCCTGAAGTTCATCTG	TATAGACGTTGTGGCTGTTGTAGTT
ActB	TCGAGTTCGCGTCCACC	GGGAGCATCGTCGCCC
IL-4	ACACCACAGAGAGTGAGCTCG	TGCAGCTCCATGAGAACAACACTAG
IL-5	AGGATGCTTCTGCACTTGAG	AGCTGTGTCAAGGTCTCTTTC
IL-13	AAAAGTGCAGCAAGACCGTG	CCAGAGCCCCTGCTTCAAT
IFN γ	ACTCAAGTGGCATAGATGTGG	AAGACTTCAAAGAGTCTGAGGTAG
TNF α	TGTCTACTCCCAGGTTCTCTTC	GCAGAGAGGAGGTTGACTTTC
T-bet	CCCACAAGCCATTACAGGATGT	TGCCTTCTGCCTTCCACAC
IL-17A	TACAGTGAAGGCAGCAGCGATC	ACATTCTGGAGGAAGTCCTTGG
IL-22	ATCGTCAACCGCACCTTTAT	GACTCCTCGGAACAGTTTCTC
IL-25	ACAGGGACTTGAATCGGGTC	TGGTAAAGTGGGACGGAGTTG
TGF- β	ACCATCCATGACATGAACCGG	AGCAGTTCTTCTCTGTGGAGC
IL-6	CCACGGCCTTCCCTACTTCA	CCACGATTTCCAGAGAACATG
mEAR1	GGTCAAACCCCTTCCAGAA	CATTGGGTATAATTTGTTGC
mEAR2 – Exon1	CAGGACAACCAGCCCTA	CTGTCTGCTTTGTGGCT
mEAR2 – Exon2	TGCTTGAGTCTCGACTTTGT	TTGGGTATAAGGTGTTCTCC
mEAR3	CTGCTGCTAAGACTTGTC	CAGCATTTCAACATTACATCGG
mEAR4	CGATGTGATGCTGTAATGAGG	TCTTGTCGGCATAGTGAGG
mEAR5	GTCTGAAGCTGCTTGAATCC	CGTCTTTGCAAGTCTTGGG
mEAR6	CCAATGCAGATACCAAACAAGG	GCAGTAGCAGATGATCGAAGG
mEAR8	AACAGGTTTAGAAGAACATGTAAGG	GTATAATTTGTTGCCCGACTGG
mEAR9	GCCATCCAGTATATCAATAATAATGCC	TGTTCTTCTAGACCTGTTAATACGC
mEAR10	ATGGGTCCAAAGCTGCTTGAG	TACGCTGCATTCAACATTAC
mEAR11	CTCCGGCCAGTCATTATTCC	CGATGAATGAAGGTACAAAGTGC
mEAR12	AACAGGTTTAGAAGAACATGTAAGG	CTTCTCCGACTGGTGTATGC
mEAR14	TGTTGGGACTTGTCTAATGC	CTGTTAACAACCCTCATTACAGC



110
111
112
113
114
115
116
117
118
119

Fig. S1. Construction of mEAR2 genetically modified mice

(A) Locus of restriction enzyme recognition sites and probe for Southern blot analysis. (B) Southern blot to identify correctly targeted embryonic stem cell clones. Southern probe detected targeted band following digestion with BglIII or HindIII. (C) Gene maps of the targeting construct and mEAR2 locus before and after homologous recombination with primer sets used for genotyping (D) Genotyping results of mEAR2-deficient mice indicated as wild-type (mEAR2^{+/+}), heterogenous (mEAR2^{+/-}), and homogenous (mEAR2^{-/-}). (E) RT-PCR of mEARs in the lung tissues from wild-type (mEAR2^{+/+}), heterogenous (mEAR2^{+/-}), and homogenous (mEAR2^{-/-}) mice.



120
121
122
123
124
125
126
127

Fig. S2. mEAR2 contributes to the development of Allergic Airway Inflammation

(A) HDM-induced allergic airway inflammation model (AAI). (B) qPCR of Th1, Th2, Th17 cytokines, TGF- β , and IL-6 in the lung tissues. (C-E) qPCR of IL-4, -5, and -13 in the lung tissues on days 0, 14, 20, and 27. (F) qPCR of mEAR2 in the lung tissues upon HDM-induced AAI. (G) qPCR of IL-4, -5, and -13 for culture experiments. Data are expressed as means \pm SEM (n = 3 – 4 for each group). * Significantly different from mEAR2^{+/+}/HDM group (p < 0.05). ND: not detected. BMDE: bone marrow-derived eosinophils.