Contribution of individual olfactory receptors to odor-induced attractive or aversive behavior in mice

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Odorants are recognized by multiple olfactory receptors (ORs) and induce innate behaviors like attraction or aversion via olfactory system in mice. However, a role of an individual OR is unclear. Muscone is recognized by a few ORs including MOR215-1 and MOR214-3, and attracts male mice. Odor preference tests using MOR215-1 knockout mice revealed that MOR215-1 and other OR(s), possibly including MOR214-3, are involved in the attraction. (Z)-5-tetradecen-1-ol (Z5-14:OH) activates ~3 ORs, including Olfr288, and evokes attraction at low levels but aversion at higher levels. Olfr288 knockout mice show no attraction but aversion, suggesting Olfr288 is involved in preference for Z5-14:OH, whereas activation of other low-affinity Z5-14:OH receptors evokes aversion. Each OR appears to send a signal to a neural circuit that possesses distinct valence, leading to a certain behavior. The final output behavior with multiple ORs stimulation is determined by summation (addition or competition) of valences coded by activated ORs.
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imals use up to a thousand odorant receptors (ORs), expressed in the olfactory epithelium, to detect a wide range of odorant molecules in the external world. In general, each odorant is recognized by dozens of ORs in a combinatorial fashion. For example, measurements of the odorant responses of dissociated olfactory sensory neurons revealed that octanal is recognized by more than 30 ORs in rat. 

Eugenol (EG) is recognized by about 45 ORs, as demonstrated by imaging and c-Fos mapping of the olfactory bulb. By contrast, odorants such as androstenone and muscone are recognized by a relatively small number of ORs. In any case, typical ORs are functionally redundant, although each OR has a distinct ligand spectrum, suggesting that this redundancy contributes to the discriminatory power of the olfactory system.

Because a defective mutation in one OR does not affect the ability of other ORs to detect an odorant, this functional redundancy helps to avoid complete anosmia to specific odorants. However, in humans, genetic variations in single ORs can affect sensitivity to, or the intensity of perception of, their cognate odorants. Similar observations have also been reported in mice. Genetic deletion of MOR215–1, the most sensitive receptor of muscone, results in reduced sensitivity to that compound. These results suggest that the effect on intensity or sensitivity is most obvious when the targeted OR has the highest affinity for a given odorant among the ORs that recognize it.

Odorants are recognized by multiple ORs in olfactory sensory neurons in the olfactory epithelium. Then the odor information is sent to the olfactory bulb (OB) and various higher brain areas, leading to behavioral outputs. In mice, urinary odorants such as (methyl-thio)methanethiol (MTMT) and (Z)-5-tetradecen-1-ol (Z5–14:OH) attract the opposite sex. By contrast, predator odorants such as trimethyl-thiazoline (TMT), secreted from the anal gland of foxes, induce aversive behavior. In addition to these semiochemicals utilized for intra- or inter-species communications, many general odorants cause attraction or avoidance behavior in mice, suggesting that odorants tend to possess positive or negative valence. Using various genetic tools, the brain areas that mediate attractive or aversive signals have gradually been revealed.

Olftr288 is the most sensitive OR for Z5–14:OH among ~3 ORs in mice. Z5–14:OH is secreted from the preputial gland in male mice, and activates ~3 ORs in female mice, including Olftr288, leading to attraction. To examine the involvement of Olftr288 in the attractive behavior, we generated Olftr288-KO mice. Olftr288-KO mice did not express Olftr288 in the main olfactory epithelium (Supplementary Fig. 1). The KO mice were stimulated with 1 mg and 0.1 ng Z5–14:OH, and the activated glomeruli in the olfactory bulb were counted. Consistent with a previous report, 1 mg Z5–14:OH activated four to seven glomeruli (corresponding to ~3 ORs, including Olftr288) and 0.1 ng Z5–14:OH activated one to three glomeruli (corresponding to ~1 ORs, likely including Olftr288) in WT mice (Fig. 2a, Supplementary Fig. 2). In Olftr288-KO mice, 1 mg Z5–14:OH activated three or four glomeruli (corresponding to ~2 ORs), and 0.1 ng Z5–14:OH activated almost zero glomeruli, which is reasonable because the KO mice lack one of the Z5–14:OH receptors, Olftr288 (Fig. 2a, Supplementary Fig. 2). As a control, no glomeruli activation was observed without odorant stimulation. The location of activated glomeruli were similar in different OBs/animals, suggesting that the same set of ORs are likely activated. (Supplementary Fig. 3).

To examine the sensitivity of KO mice to Z5–14:OH, we performed the odor-finding test as described above for the MOR215–1 KO mice. Individual mice were exposed for 5 min to the tip of a glass capillary with various amount of Z5–14:OH during the dark period. In WT female mice, the threshold amount of Z5–14:OH was ~3 pg under this experimental condition: no mouse could detect 1 pg, but more than a half of the tested mice detected 3 pg. In contrast, none of the KO mice could detect 3 pg Z5–14:OH, indicating that KO mice are anosmic to Z5–14:OH.
could find 3 pg (Fig. 2b). Almost all WT female mice could detect 10 pg Z5–14:OH, whereas almost none of the Olfr288-KO female mice could do so (Fig. 2b). By contrast, the detection threshold for eugenol, a neutral odorant, was similar between WT and Olfr288-KO female mice (Fig. 2c). These results indicate that the threshold amount for Olfr288 is around 3 pg, and the other ORs detect Z5–14:OH in amounts >30 pg. Therefore, it is reasonable to conclude that Olfr288 is the most sensitive OR for Z5–14:OH.

Preference for Z5–14:OH is mediated by Olfr288. Next, we examined the involvement of Olfr288 in attractive behavior by performing the two-choice odor-preference test in WT and Olfr288-KO female mice. First, we performed a series of control experiments. Female mice spent similar time nose-poking two vents when the same urine from intact male urine was placed in both rooms (Fig. 3a). No difference in investigation time was observed between rooms when a neutral odorant, eugenol, was placed (Fig. 3a). In contrast, female mice were more attracted to urine from intact male mice (hereafter, intact urine) than urine from castrated males (hereafter, castrated urine) or water controls (Fig. 3a). In this experimental paradigm, aversion could not be measured because no difference was observed for TMT, a fox odorant, whereas almost none of the Olfr288-KO female mice (Fig. 2b), these results suggest that Olfr288 is involved in OlfrY(s), (one of the least sensitive OR among ~3 Z5–14:OH) activates OlfrX(s), in addition to Olfr288; (2) 3 ng Z5–14:OH activates OlfrY(s), (one of the least sensitive OR among ~3 Z5–14:OH ORs); and (3) the activation of OlfrY(s) suppresses the Olfr288-mediated attractive behavior. In this experiment, however, we could not determine whether mice were simply not interested in 3 ng Z5–14:OH, or actively avoided it, because this nose-poking preference test cannot evaluate aversive behavior (Fig. 3a, TMT experiment). Number of poking noses into two vents was similar between water and 3 ng Z5–14:OH, or between castrated urine and 3 ng Z5–14:OH-spiked castrated urine than to castrated urine alone (Fig. 3d), as reported previously, whereas this preference was not observed in Olfr288-KO mice (Fig. 3e). Because 10 pg Z5–14:OH activates only Olfr288 (Fig. 2b), these results suggest that Olfr288 is involved in Z5–14:OH-mediated attraction in female mice.

In order to correct for any intrinsic special biases of individual mice, the “preference index” was calculated (Supplementary Fig. 5). Preference index means the ratio of investigation time of poking a nose into a hole with a targeted sample to that of the total investigation time into both holes. WT mice significantly preferred Z5–14:OH (10 pg or 1 ng)-spiked castrated urine in comparison to Olfr288-KO mice (P = 0.0049 for 10 pg, P = 0.0031 for 1 ng; Mann–Whitney test). There was no significant difference but a tendency for WT mice to prefer 10 pg and 1 ng Z5–14:OH in comparison to Olfr288-KO mice.

Interestingly, no preference was observed between water and 3 ng Z5–14:OH, nor between castrated urine and 3 ng Z5–14:OH-spiked castrated urine, in either WT or Olfr288-KO female mice (Fig. 3b–d). Because Olfr288-KO mice can still detect Z5–14:OH at amounts greater than 1 ng via the other Z5–14:OH ORs (Fig. 2b), we propose the following models; (1) 1 ng Z5–14:OH activates OlfrX(s), in addition to Olfr288; (2) 3 ng Z5–14:OH activates OlfrY(s), (one of the least sensitive OR among ~3 Z5–14:OH ORs); and (3) the activation of OlfrY(s) suppresses the Olfr288-mediated attractive behavior. In this experiment, however, we could not determine whether mice were simply not interested in 3 ng Z5–14:OH, or actively avoided it, because this nose-poking preference test cannot evaluate aversive behavior (Fig. 3a, TMT experiment). Number of poking noses into two vents was similar between water and 3 ng Z5–14:OH, or between castrated urine and 3 ng Z5–14:OH-spiked castrated urine, in either WT or Olfr288-KO female mice (Supplementary Fig. 4).
Aversion to Z5–14:OH is mediated via the least sensitive Z5–14:OH OR. To evaluate attraction and aversion in the same experiment, we performed an odor investigation assay in which a filter paper scented with an odorant was placed in the home cage, and the investigation time for the filter paper was measured during a 3-min test period (Fig. 4a, b). The investigation time for eugenol (EG), a neutral odor, was similar to that for water (Fig. 4c). Consistent with a previous report, in WT female mice the investigation time for TMT, an aversive odor, was significantly shorter (Fig. 4c). In addition, the investigation time for intact or castrated urine was significantly longer than for water control (Fig. 4c). These data demonstrate that both attractive and aversive behaviors can be evaluated in this investigation test.

We then investigated whether Z5–14:OH elicited attraction or aversion in this assay. The investigation time for 10 pg or 1 ng Z5–14:OH was significantly longer than for water in WT female mice (Fig. 4b, c), consistent with the results of the nose-poking preference test (Fig. 3). By contrast, the investigation time was significantly shorter for 3 ng Z5–14:OH than for water, suggesting that mice were aversive to 3 ng Z5–14:OH (Fig. 4c).

The degree of aversion was similar to that of TMT. These results led us to propose that activation of the least sensitive Z5–14:OH OR evokes aversive behavior that overrides the attraction mediated by Olfr288.

In Olfr288-KO female mice, behavior was similar for intact urine, castrated urine, EG, and TMT, whereas the attraction to 10 pg or 1 ng Z5–14:OH was completely diminished (Fig. 4c), consistent with the results obtained in Fig. 3. Further, the aversion to 3 ng Z5–14:OH was still observed in Olfr288-KO mice (Fig. 4c). All these results support our model that the attraction to Z5–14:OH is mediated via Olfr288, while aversion to a larger amount of Z5–14:OH is mediated by the least sensitive of ~3 Z5–14:OH ORs.
Discussion

Many odorants are neutral for mice (general odorants), whereas others possess specific valences such as attraction or aversion. The response is sometimes concentration-dependent, such that as the concentration increases, the output behavior shifts from attraction to aversion. An outstanding question is how odor valence is coded at the level of receptors. We can propose several models: the valence could be coded in the pattern of activated ORs, in individual OR(s), or by a combination of the two. In addition, valence may not be solely dependent on receptors, but also on the anatomy of activated regions in the olfactory bulb. In this study, to determine how behavioral output is regulated by ORs, we took advantage of odorants that are recognized by only a few ORs, generated mice in which the most sensitive OR for a given odorant was deleted, and then performed behavioral analysis. Our results support the combination model in which activation of a single OR elicits preference or aversive behavior, and output behavior is determined by summation (addition or competition) of valences encoded by activated ORs.

We presented two cases: muscone and its two ORs, and Z5–14:OH and its ~3 ORs. In the case of muscone, as described in the bottom panels in Fig. 1c and d, MOR215–1 is likely to be involved in the attractive behavior, and MOR214–3 is an additional candidate OR that possesses the positive valence in MOR215–1 KO mice. In the case of Z5–14:OH, Fig. 5 shows a model that explains the mechanism underlying the concentration-dependent switch from attraction to aversion at the receptor level, and how individual Z5–14:OH ORs are involved in this behavior. There appear...
needed, as reported previously for TMT24. Neurons expressing
induce a specific or aversive odorants21. It remains to be determined how
amygdala are capable of eliciting innate responses to either pre-
innate behaviors. Distinct cell populations within the cortical
from the olfactory bulb to cortical amygdala, resulting in various
paradigms must be developed for assessment of positive valences.

In addition, a stereotyped neural circuit transmits information

to be at least three ORs that recognize Z5–14:OH. The model
most consistent with our results is that the most sensitive OR,
Olfr288, encodes information about attraction, whereas the least
sensitive (and still-unknown) ORs, a few OlfrX(s), sends the
signal for aversion. The shift from attraction to aversion occurs
when all ORs, including Olfr288, OlfrX(s), and OlfrY(s), for
Z5–14:OH are activated. Taken together, our results suggest that
each OR sends a signal to a neural circuitry leading to a specific
behavior, and therefore encodes an odor-associate valence; the
summation of valences coded by activated ORs determines the
final output behavior. To conclude that activation of a single OR is sufficient to
induce a specific behavior, gain-of-function experiments will be
needed, as reported previously for TMT24. Neurons expressing
muscone receptors send axons to the dorsomedial part of the
olfactory bulb. Glomeruli innervated with Z5–14:OH ORs are all
located in the ventral region. Thus, accessing these bulbar regions
are challenging upon applying optogenetic or pharmacological
tools to activate individual muscone ORs or yet-identified OlfrXs
or Ys. In addition, another challenge relates to the behavioral paradigm. Aversion can be assessed relatively easily by optoge-
nic or pharmacological stimulation24, whereas preference behavior is more difficult to evaluate. A more refined behavioral
paradigm must be developed for assessment of positive valences.
In addition, a stereotyped neural circuit transmits information
from the olfactory bulb to cortical amygdala, resulting in various
innate behaviors. Distinct cell populations within the cortical
amygdala are capable of eliciting innate responses to either pre-
fERENCE or aversive odorants21. It remains to be determined how
the behavioral outputs of muscone and Z5–14:OH are correlated
with activation patterns in the higher brain areas, especially the
cortical amygdala.

Based on our findings, we propose that some ORs code a
valence such as information about preference or aversive beha-
vor, thus linking individual ORs are with specific behavioral
outputs. The final output behavior is determined by addition/competition of valences coded by activated ORs, suggesting that
convergence and cross talk among stimuli from multiple ORs
occur within the brain. From the standpoint of applications, the
concept of OR-associated negative or positive valence could be
exploited to regulate animal behavior, e.g., controlling a pest or a
harmful animal by development of agonists or antagonists for the
target OR. In human society, this concept could be applied to
design of improved fragrances or flavors by targeting an essential
OR involved in detecting a specific odorant quality.

Methods
Odorants. Muscone and mineral oil were purchased from Wako. Propylene glycol (PG) was purchased from ADEKA. (Z)–5-tetradecen-1-ol (Z5–14:OH) (>90%;
including 7.1% of the E-isomer) was synthesized by Chegnis Co. via a synthetic
pathway described previously26. Eugenol (EG) and TMT were purchased from
Tokyo Chemical Industry Co. or Phero Tech.

Animals. All experiments were carried out in accordance with the guidelines of
the Animal Care Committee at The University of Tokyo. The animal room was
maintained under a 12-h light/dark cycle (light from 7:00 to 19:00) at a constant
temperature (23 ± 1 °C). C57BL/6 male and female mice (>8 weeks old; CLEA
Japan) were used for all experiments. MOR215–1 deletion mice were obtained
from Dr. Sakano27. The coding sequence of MOR215–1 was changed to that of
MOR103–1 using recombinant PCR in the MOR103–1->MOR215–1 IRES tau-
**Fig. 5** A receptor-based model for concentration-dependent switching from attraction to aversion to Z5-14:OH. Behavioral data of wild-type mice (a) and Olfr288 knockout mice (b) in response to various amounts of Z5-14:OH (10 pg, 1 ng, 3 ng) are consistent with a model (a, b right) in which activation of Olfr288 (red) and OlfrYs (blue) are involved in attraction and aversion to Z5-14:OH, respectively; activation of OlfrXs (green) are neutral.

ECFP mouse (Acc. no. CDB0545K; http://www.cdb.riken.jp/arg/mutant%20mice%20list.html). Olfr288 deletion mice were generated as follows. A genomic fragment of mouse Olfr288 was isolated from the B6N Mouse BAC clone B6Ng01-336F02 (RIKEN BioResource Center). A 2.9-kb fragment spanning the 5′ region of the third exon was amplified by PCR with KOD FX (Toyobo) and inserted into the pBlueScript II SK(−). A self- excision Neo<sup>R</sup> cassette derived from pAC2<sup>RP</sup> and a 1.1-kb fragment of Olfr288 genomic sequence spanning from upstream of the second exon to downstream of first exon was then inserted into the 3′ region of the Neo<sup>R</sup> cassette. To allow negative selection, a diphertheria toxin A fragment gene cassette derived from pMCIDTPα<sub>RP</sub> was inserted. The targeting vector was linearized with NotI and electroporated into C57BL/6 mouse ES cells. Cells that underwent recombination were selected with G418, and colonies were screened by PCR. Male chimaeras were crossed to wild-type C57BL/6 females (Japan SLC) to establish a C57BL/6 inbred background. The mutant mice were analyzed by PCR using two primer pairs (5′–gtagttcg/acgagggtaag-3′ and 5′–gacattgacagc-tccttg-3′ for the wild-type allele, and 5′–gacattgactagtcgcttg-3′ and 5′–gacattgactacgcttgcttg-3′ for the mutated allele).

**Two-choice odor-preference test.** Two-choice odor-preference tests were performed in a custom-made acrylic box (300 mm × 210 mm × 100 mm) consisting of three compartments: one mouse compartment (155 mm × 210 mm) with clean bedding, and two odorant compartments (143 mm × 104 mm), as described previously<sup>5,15</sup> in the same cage. A 2.5-kb fragment spanning the 5′ region upstream of the second exon was amplified by PCR with KOD FX (Toyobo) and inserted into the pBlueScript II SK(−). A self- excision Neo<sup>R</sup> cassette derived from pAC2<sup>RP</sup> and a 1.1-kb fragment of Olfr288 genomic sequence spanning from upstream of the second exon to downstream of first exon was then inserted into the 3′ region of the Neo<sup>R</sup> cassette. To allow negative selection, a diphertheria toxin A fragment gene cassette derived from pMCIDTPα<sub>RP</sub> was inserted. The targeting vector was linearized with NotI and electroporated into C57BL/6 mouse ES cells. Cells that underwent recombination were selected with G418, and colonies were screened by PCR. Male chimaeras were crossed to wild-type C57BL/6 females (Japan SLC) to establish a C57BL/6 inbred background. The mutant mice were analyzed by PCR using two primer pairs (5′–gtagttcg/acgagggtaag-3′ and 5′–gacattgacagc-tccttg-3′ for the wild-type allele, and 5′–gacattgactagtcgcttg-3′ and 5′–gacattgactacgcttgcttg-3′ for the mutated allele).

**Odor finding test.** Each mouse was housed individually in its home cage with clean bedding. After 2 days of isolation, the odor-finding test was performed as described previously<sup>5,15</sup> in the same cage. The mouse was exposed to the tip of a glass capillary with an odorant. The tip of each capillary was set carefully so that air could pass or the mouse could poke its nose. A stream of air was drawn through a charcoal filter from each odorant compartment into the mouse compartment passing along a Y-shaped tube using a vacuum pump. Each odorant was counterbalanced among tests. The partition between the mouse compartment and each odorant compartment contained a round vent through which air could pass or the mouse could poke its nose. A stream of air was drawn through a charcoal filter from each odorant compartment into the mouse compartment passing along a Y-shaped tube using a vacuum pump. Mice were recorded with a digital video camera. Each test was performed for 5 min, and the amount of time a mouse spent poking the tip of its nose through the vent, designated as the investigation time, was measured by using a stopwatch in a double-blind manner. The investigation time was compared statistically between odorant compartments by paired Student’s <i>t</i>-test. To avoid confounding of the data due to learning, each mouse was used only once.

**c-Fos immunostaining.** Mice were housed individually with clean bedding. After 2 days of isolation, the tip of a glass capillary with each odorant was set carefully in the home cage. The glass capillary was kept in the home cage for 1 min after freely behaving mice found and began sniffing the odorant. Then the glass capillary with odorant was removed from the home cage carefully. As a control, mice were stimulated through a glass capillary with no odorant for 1 min. After 70 min, mice were anesthetized with sodium pentobarbital and perfused with PBS and 4% paraformaldehyde in PBS at 4 °C. The skull was removed so that the olfactory bulb (OB) was exposed, and the OB was post-fixed for 3 h and stored overnight in cryopreservation solution (30% sucrose in PBS). Thirty-micron coronal cryosections of the OB were collected, placed onto MAS-coated glass slides (Matsunami Glass), and incubated with anti-c-Fos rabbit polyclonal antibody (1:500, sc-52, Santa Cruz Biotechnology). The slides were then incubated with biotinylated goat anti-rabbit secondary antibody (1:200, PK-6101, Vector Laboratories), subjected to ABC amplification (Vector Laboratories), and stained with 3,3′ diaminobenzidine (Sigma). Number of activated glomeruli that expressed c-Fos in juxtaglomerular cells were counted from one side of the OB from each mouse. No c-Fos induction was observed in the OB of mice with no odor as a control.

**Odor investigation assay.** Mice were housed individually in their home cages with clean bedding. After 1 day of isolation, the odor investigation assay was performed as described previously<sup>5,15</sup> with minor modifications<sup>36</sup>, in the same cage. A filter paper (15 mm diameter) scented with an odorant (5 μl) was introduced to the home cage, and the time spent investigating the filter paper during a 3-min test period was measured. Mouse behavior was recorded with a digital video camera for analysis. To avoid confounding of the data due to learning, each mouse was used only once. Tests were conducted during the late part of the light periods, and were performed in a double-blind manner. The latency of finding the odorant was compared statistically between WT and KO mice by the Scheirer–Ray–Hare test, a nonparametric version of repeated two-way ANOVA<sup>30,31</sup>.

**RT-PCR.** Olfactory sensory neurons were isolated from olfactory epithelium and RNAs were purified by RNeasy Plus Mini Kit (Qiagen). 1000 ng RNAs were each placed into lysis mix (0.5 μl Oligo dT (invitrogen), 2.5 mM dNTPs (Takara)) and RNase free water was added till 14 μl. PCR tubes each containing lysed cells were heated to 65 °C for 5 min and then cooled at 4 °C. 6 μl of RT-positive mix (1 μl Superscript III (Invitrogen), 1 μl RNase inhibitor (Takara), 4 μl 5 × ES buffer
In situ hybridization. To make the Olfr288 gene probes, DNA fragments of 371 bp containing the 3′ untranslated region (UTR) sequences were amplified by PCR from the C57BL/6 mouse genomic DNA. Primer sequences for Olfr288 were AGAAGGGGCTGCTGGAGAAG (forward) and AATTTACCTCCTACAAGGGTCGCTAATACGTG (reverse, with T3-sequence at 5′ end). To make the c-Fos gene probes, DNA fragments of 888 and 905 bp containing the coding sequence were amplified by PCR from the C57BL/6 mouse cDNA from the brain. Primer sequences for c-Fos were CCAAGCTCTGCCTGCAATGCC and CGGTCTTCCTATCAGCACG (forward) and AATTTACCTCCTACAAGGGTCGCTAATACGTG and AATTTACCTCCTACAAGGGTCGCTAATACGTG (reverse, with T3-sequence at 5′ end). Antisense cRNA probes were synthesized using T3 RNA polymerase (NEB) and digoxigenin (DIG) labeling mix (Roche) from PCR templates. Thirty-week-old C57BL/6 and Olfr288-KO mice were anesthetized with sodium pentobarbital (2.5 mg/animal) in PBS for 5 min at 37 °C. After fixing in 4% paraformaldehyde in PBS, tissues were decalcified using 4.5 M EDTA at 4 °C, placed in 30% sucrose. Fifteen μm coronal sections of olfactory epithelium were prepared. After drying, the samples were fixed for 10 min in 4% paraformaldehyde in PBS at room temperature. The sections were rinsed with PBS and incubated with 1 μg/ml Proteinase K in PBS for 5 min at 37 °C. After fixing again with 4% paraformaldehyde in PBS for 10 min and rinsing with PBS, the sections were incubated with 0.1 μM trithramolamine with 2.5 μM/ml acetic anhydride, pH 8.0, washed with PBS, and the slide was incubated with the hybridization solution (50% formamide, 5 × SSC, 1 × Denhardt’s, 250 μg/ml yeast rRNA) for 10 min. Probes were diluted (1:200) with the hybridization solution, and 300 μl of each sample was applied to a slide. After 12 h of incubation at 68 °C, the sections were washed, first with 5 × SSC for 5 min, then with 0.2 × SSC times for 30 min at 68 °C. After blocking with the blocking reagent (PerkinElmer), slides were incubated with anti-DIG-POD (Roche, at 1/100 dilution in blocking reagent) for 3 h at room temperature. Slides were washed three times with TBST (100 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 10 min and treated with TSA-plus Cyanine 3 (PerkinElmer, NEL744001KT, 1:100 in 1× plus amplification diluent) for 20 min. Sections were washed three times with TBST for 10 min and mounted with cover glass using PermaFluor (Lab Vision Corporation), and imaged by Keyence microscope (x4 or x10 objective). Images were processed in Photoshop CC (Adobe).

Data availability. The data that support the findings in this study are available from the corresponding author on request.

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Author contributions
N.H. and K.T. conceived the project, designed the experiments, and wrote the manuscript. N.H. performed all experiments except that K.M. performed expression analysis of
knockout mice. K.Y. performed an initial experiment related to Fig. 3. Y.Y. helped generation of knockout mice.

**Additional information**

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