Cellular/Molecular

Goofy Coordinates the Acuity of Olfactory Signaling

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The basic scheme of odor perception and signaling from olfactory cilia to the brain is well understood. However, factors that affect olfactory acuity of an animal, the threshold sensitivity to odorants, are less well studied. Using signal sequence trap screening of a mouse olfactory epithelium cDNA library, we identified a novel molecule, Goofy, that is essential for olfactory acuity in mice. *Goofy* encodes an integral membrane protein with specific expression in the olfactory and vomeronasal sensory neurons and predominant localization to the Golgi compartment. *Goofy*-deficient mice display aberrant olfactory phenotypes, including the impaired trafficking of adenylyl cyclase III, stunted olfactory cilia, and a higher threshold for physiological and behavioral responses to odorants. In addition, the expression of dominant-negative form of cAMP-dependent protein kinase results in shortening of olfactory cilia, implying a possible mechanistic link between cAMP and ciliogenesis in the olfactory sensory neurons. These results demonstrate that Goofy plays an important role in establishing the acuity of olfactory sensory signaling.

Introduction

The olfactory system integrates sophisticated molecular, cellular, and neural circuit mechanisms for detection and discrimination of a huge variety of odorants in the environment (Axel, 1995; Mori et al., 1999; Mori and Sakano, 2011). Odor molecules inhaled into an animal's nostrils are detected by the olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) (Buck, 2000). OSNs are bipolar, extending a single axon basally toward the olfactory bulb (OB) and a single dendrite apically toward the epithelial surface. Several long cilia emanate from the dendritic knob into the nasal mucus for efficient reception of odorants (McEwen et al., 2008).

Upon odorant binding to the olfactory cilia, action potentials are generated by the OSN's signal transduction machinery (Reed, 1992; Touhara and Vosshall, 2009). This signaling process occurs through a sequential activation of a cascade of functional molecules including odorant receptors (ORs), olfactory-specific GTP-binding protein (Golf), adenylyl cyclase III (ACIII), and cyclic nucleotide-gated cation channel, all of which are enriched in the olfactory cilia (Jones and Reed, 1989; Bakalyar and Reed, 1990; Dhallan et al., 1990; Buck and Axel, 1991). Genetic mutations in these signaling molecules lead to olfactory dysfunctions as exemplified by anosmia and hyposmia (McEwen et al., 2008).

Transmembrane and secreted proteins play important roles in a variety of developmental and functional aspects of OSNs. For example, the hierarchical and combinatorial expression of multiple guidance molecules directs convergent projection of OSN axons to target glomeruli in the OB (Mori and Sakano, 2011). In addition, there are other transmembrane and secreted proteins crucial for odor-sensing mechanisms in OE, which facilitate the OR trafficking to plasma membranes (Saito et al., 2004), carry odor molecules in nasal mucus to ORs (Tegoni et al., 2000), and convert odorant information into intracellular second messenger elevation (Gibson and Garbers, 2000). Most of these proteins are characterized by the presence of a signal sequence with a stretch of hydrophobic amino acids at the N terminus.

In this study, we performed a signal sequence trap screening of mouse OE cDNA library and identified a novel transmembrane protein that is specifically expressed in OSNs and vomeronasal sensory neurons (VSNs) and predominantly localized in the Golgi apparatus. Hence, we designated this gene product as Goofy (Golgi protein in olfactory neurons). Genetic ablation of the *Goofy* gene caused abnormal localization of ACIII, shortening of olfactory cilia, and reduced sensitivity to odorants, indicating a crucial role of Goofy in functioning of the olfactory system.

Materials and Methods

Signal sequence trap screening. Yeast signal sequence trap screening was performed essentially as described previously (Jacobs et al., 1997; Na-

A

poly(A+) RNA from olfactory epithelia of 40 mice at posnatal day 3

↓

double-stranded cDNA with *Eco*RI and *Xho*I adapters

↓ amplified by PCR

PCR products

↓ digested with *Eco*RI and *Xho*I

↓ ligated to <u>Yeast Signal Sequence Trap Vector</u> (pSUC2T7M13ORI)

cDNA library

↓ introduced into the invertase-deficient yeast strain (YTK12)

↓ selected on Trp⁻ plates

1.5 x 10⁶ transformants (TRP1⁺)

↓ selected on raffinose plates (1st screening)

686 colonies (capable of secreting invertase)

↓

yeast DNA

↓ PCR

inserted cDNA

↓ sequenced

258 sequence tags

↓ encoding signal peptide ?

215 sequence tags

49 individual genes

↓ homology search   database search (2nd screening)

37 known genes   12 novel genes

↓ domain search

↓ *in situ* hybrigization (3rd screening)

B

clone No.	Gene Name	Accession No.	Incidence
#104	mucin1	NM013605	4
#106	lactoferrin	D88510	3
#115	microfibril-associated glycoprotein	L23769	9
#119	m.neonate head cDNA/ NOVEL	AK017253	2
#123	NOVEL		2
#126	11days embryo cDNA / NOVEL	AK012310	6
#148	fetuin-like protein	AJ242927	1
#168	placenta cDNA/ NOVEL	AK005558	1
#176	FVB/N collagen	U08020	1
#192	neuropeptide FF	NM_018787	2
#201	clone rp21-657p21/ NOVEL	AC005743	1
#212	insulin-like growth factor 2	M14951	9
#214	chromogranin	M64278	1
#226	microfibril-associated glycoprotein 4	AK003537	1
#235	MGC-24: cell-surface sialomucin	AB028895	17
#251	epididymal secretory protein	AB021289	1
#252	fibromodulin	X94998	3
#275	cystatin C	U10098	1
#324	carbonic anhydrase	AF079835	1
#325	PLUNC	AF356785	7
#353	cystein-rich glycoprotein SPARC	X04017	1
#372	PDGF receptor	X04367	1
#378	follistatin-related protein	M91380	1
#391	NOVEL		1
#393	matrix carboxyglutamate	NM_008597	1
#406	NOVEL		1
#412	procollagen	AK018742	1
#422	DR5	AF176833	1
#430	NOVEL		1
#440	similar to rat RYF3	NM_021764	1
#444	similar to CG7146/ NOVEL	BC007479	2
#456	bone carboxyglutamate protein 1	NM007541	1
#484	lysozyme M	M21047	1
#491	calreticulin	X14926	1
#493	amelogenin: enamel-specific protein	D31768	34
#496	stromal cell derived factor receptor2	NM_009146	1
#522	NOVEL		1
#556	dentin matrix protein1	MMU65020	1
#570	rat Tmp21-I	AK003676	1
#575	rat odorant binding protein	J03093	8
#607	m.placenta cDNA/ NOVEL	AK005519	4
#623	rat CL3AC	AF081156	1
#702	rat odorant binding protein (RY2G12)	M76734	1
#733	signal sequence receptor	BC008172	1
#736	bone carboxyglutamate protein 2	NM_031358	1
#748	sarcoglycan	NM_009161	1
#781	NOVEL		1
#809	thymic dendritic cell-factor1	AF116911	1
#824	rat RYF3 :ligand binding protein	X60659	68

C

#119   **#123 (*Goofy*)**   **#126**
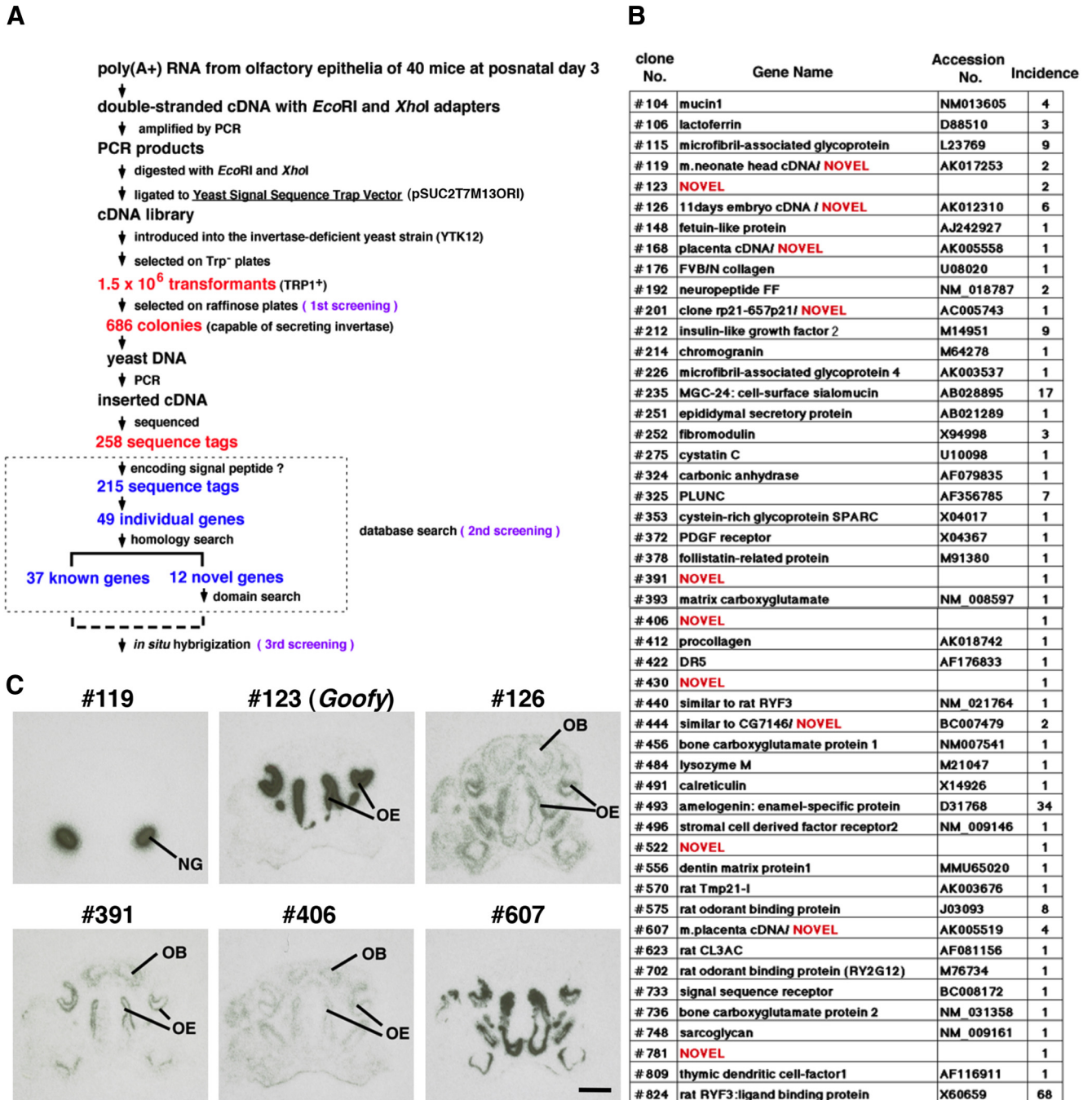
#391   **#406**   **#607**

Figure 1. Discovery of *Goofy* (#123) by signal sequence trap screening. ***A***, Strategy of the signal sequence trap screening for identification of novel transmembrane and secreted molecules expressed in the OE. ***B***, A summary table for individual 49 cDNA clones obtained by the signal sequence trap screening. ***C***, mRNA expression patterns of six novel genes in P3 OE coronal sections assessed by *in situ* hybridization analysis. The clone #123 (*Goofy*) is abundantly and specifically expressed in the OE. NG, Nasal gland. Scale bar: ***C***, 500 μm.

kashiba et al., 2000). Poly(A)+ RNA was prepared from postnatal day (P) 3 mouse OE, reverse-transcribed, and PCR-amplified to generate double-stranded cDNA with EcoRI and XhoI adaptors. The double-stranded cDNA was digested with EcoRI and XhoI, size-fractionated by agarose gel electrophoresis (300–800 bp), and ligated unidirectionally into pSUC2T7M13ORI vector (Jacobs et al., 1997) to construct a cDNA library. For screening of cDNAs containing signal peptide sequences, the library was introduced into the invertase-deficient yeast strain YTK12, and selected on Trp⁻ plates followed by raffinose-containing plates on which only the transformants harboring cDNA-derived signal peptide-fused invertase gene can grow. Among 686 colonies grown on raffinose plates, cDNA inserts were amplified and sequenced for 258 recombi-

nants, and the presence of a putative signal peptide was confirmed for 215 clones that encoded 49 individual genes.

In situ *hybridization and Northern blot analysis. In situ* hybridization and Northern blot analysis were performed as described previously (Yoshihara et al., 1997). One to three mice at individual developmental stages were used for these analyses.

Antibody production. To generate anti-Goofy antibody, a peptide corresponding to the C-terminal 19 aa (RGPQRLEALSPAALSPNFF) of mouse Goofy protein was synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. This antibody labeled OSNs and VSNs, and its immunoreactivity was completely absent in *Goofy*-deficient mice, confirming its specificity to Goofy protein. Antibodies

against Olfr6/M50, Olfr17/P2, and rat OR-I7 were generated as described previously (Kaneko-Goto et al., 2008).

Immunohistochemistry. Section immunohistochemistry was performed as described previously (Kaneko-Goto et al., 2008). Immunolabeling of olfactory cilia in whole-mount OE preparations was performed as described previously (Strotmann et al., 2004). Primary antibodies used were as follows: rabbit anti-Goofy (1:1000); goat anti-OMP (1:10,000; Wako Pure Chemical Industries); rat anti-NCAM (1:500, Millipore Bioscience Research Reagents); rabbit anti-olfactory cell adhesion molecule (anti-OCAM; 1:1000) (Yoshihara et al., 1997); goat anti-NQO1 (1:500; Abcam); goat anti-neuropilin-1 (1:200; R&D Systems); goat anti-neuropilin-2 (1:200; R&D Systems); goat anti-contactin-4/BIG-2 (1:200; R&D Systems); rabbit anti-Golf (1:1000; Santa Cruz Biotechnology); rabbit anti-cyclic nucleotide-gated channel A2 subunit (anti-CNGA2; 1:200; Alomone Labs); rabbit anti-ACIII (1:1000; Santa Cruz Biotechnology); rabbit anti-Olfr6/M50 (1:2000); guinea pig anti-Olfr2/I7 (1:2000); guinea pig anti-Olfr17/P2 (1:1000); guinea pig anti-rat OR-I7 (1:5000); guinea pig anti-Olfr73/mOR-EG (1:1000); guinea pig anti-Olfr1507/MOR28 (1:1000) (Kaneko-Goto et al., 2008). To label intracellular organelles in OSNs, Organelle Sampler Kits (BD Biosciences) and Alexa488-conjugated wheat germ agglutinin (WGA; 1:100; Invitrogen) were used according to the manufacturers' instructions. Cy3-conjugated and Alexa488-conjugated secondary antibodies were purchased from Jackson ImmunoResearch and Invitrogen, respectively. Immunohistochemical analyses were performed on sections ($n = 3$–10 mice) and whole-mount OE ($n = 3$–4 mice) for each antibody.

Western blot analysis. Western blot analysis was performed on olfactory cilia fraction (3 μg of protein/lane), OE homogenate (10 μg of protein/lane), and OB homogenate (10 μg of protein/lane) from ≥3 mice of each genotype as described previously (Kaneko-Goto et al., 2008). Olfactory cilia fraction was prepared by a calcium shock method as described previously (Washburn et al., 2002). Primary antibodies used were as follows: rabbit anti-Goofy (1:1000); rabbit anti-ACIII (1:1000; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. Immunoreactive protein bands were visualized with a chemiluminescence reaction kit (ECL Plus Western Blotting Detection System, GE Healthcare) and an image analysis system (LAS-1000, Fujifilm).

Transgenic and mutant mice. Goofy-gapVenus transgenic mice were generated with a standard transgenic method. The 3.0 kb 5′-flanking region of *Goofy* gene was PCR-amplified from mouse genomic DNA and inserted into pBstN-*gapVenus* vector (Mitsui et al., 2011) to generate p*Goofy-gapVenus* plasmid. *Goofy-gapVenus* transgene was excised, gel-purified, and injected into the pronucleus of fertilized eggs that were obtained from crossing C57BL/6 and DBA/2J mice. The manipulated eggs were cultured to the two-cell stage and transferred into oviducts of pseudopregnant foster females (ICR strain). Integration of the transgene was screened by PCR of tail DNA.

Goofy-deficient mice were generated with a standard gene-targeting method. Two genomic DNA fragments of mouse *Goofy* gene (2.5 kb SpeI-KpnI short arm and 6.7 kb XbaI-EcoRI long arm) were subcloned into pSL301 vector (Invitrogen) together with a positive selection marker *pgk-neo* and a negative selection marker *DTA* to generate the *Goofy* targeting vector. Electroporation into 129/SVEv mouse ES cell line followed by G418 selection resulted in four positive homologous recombinants of 288 clones as determined by PCR screening and Southern blot analysis. Chimeric C57BL/6 males that transmitted the mutant allele were obtained, backcrossed with C57BL/6 mice more than nine times, and used for all the experiments.

Generation and characterization of *Olfr73(mOR-EG)-ires-gapEGFP* transgenic mice were described previously (Oka et al., 2006). *I7 (WT)-ires-ECFP* and *I7(WT)-ires-dnPKA-ires-EYFP* transgenic mice (RBRC02931 and RBRC02936) (Imai et al., 2006), generous gifts from Drs. Takeshi Imai and Hitoshi Sakano (The University of Tokyo), were provided by RIKEN Bio Resource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.
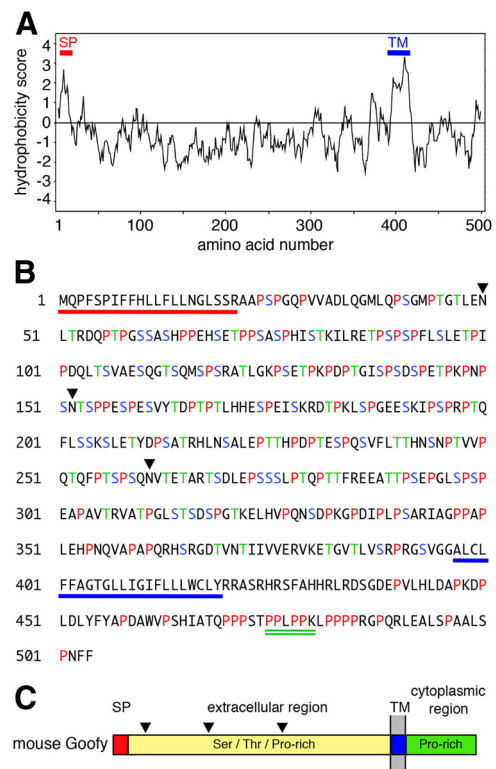

Figure 2. Structure of Goofy protein. ***A***, Hydrophobicity plot of mouse Goofy protein. Two highly hydrophobic regions are present: one at the N terminus and the other around amino acid number 400, which potentially serve as a signal peptide (SP) and a transmembrane segment (TM), respectively. ***B***, Amino acid sequence of mouse Goofy protein. Red underline, Signal peptide; blue underline, transmembrane segment; green double underline, canonical SH3-binding motif; black triangles, potential Asn-linked glycosylation sites. Ser, Thr, and Pro residues are highlighted in blue, green, and red letters, respectively. ***C***, A schematic diagram depicting the domain structure of Goofy protein. Goofy consists of an N-terminal signal peptide (SP, red), a Ser/Thr/Pro-rich extracellular region (yellow) with three potential glycosylation sites (black triangles), a transmembrane segment (TM, blue), and a Pro-rich cytoplasmic region (green).

Electro-olfactogram recording. Electro-olfactogram (EOG) recording was performed as described previously (Kimoto et al., 2005; Fukuda et al., 2008).

Behavioral analysis. Behavioral responses to 2,4,5-trimethylthiazoline (TMT; Phero Tech) were analyzed as described previously (Kobayakawa et al., 2007) with the following modifications. Group-housed adult male mice (4–6 month old; wild type, $n = 36$; *Goofy*-deficient, $n = 35$) were used. For habituation to the experimental environment, mice were placed individually in a cage that was identical to the test cage (14 × 30 × 20 cm). After 30 min, mice were transferred to a new cage. This habituation process was repeated four times for each animal. Then, the mice were transferred to the test cage, and a filter paper (2 × 2 cm) scented with various amounts (0, 0.4, 4, 40 μl) of TMT was introduced. The test cage was subdivided into two equally sized areas. Time spent in the area without a filter paper was evaluated as avoidance. Freezing time and stay time in each area during the 5 min test period were measured. The mouse behavior was recorded with a digital video camera (Canon).

Results

cDNA cloning and structure of *Goofy*

To search for membrane-bound or secreted proteins involved in development and/or function of OSNs, we adopted a yeast signal sequence trap method, which enables us to selectively isolate cDNAs encoding proteins with an N-terminal hydrophobic signal peptide. Poly(A)+ RNA was prepared from the OE of P3 mice and a cDNA library was constructed in a signal sequence trap
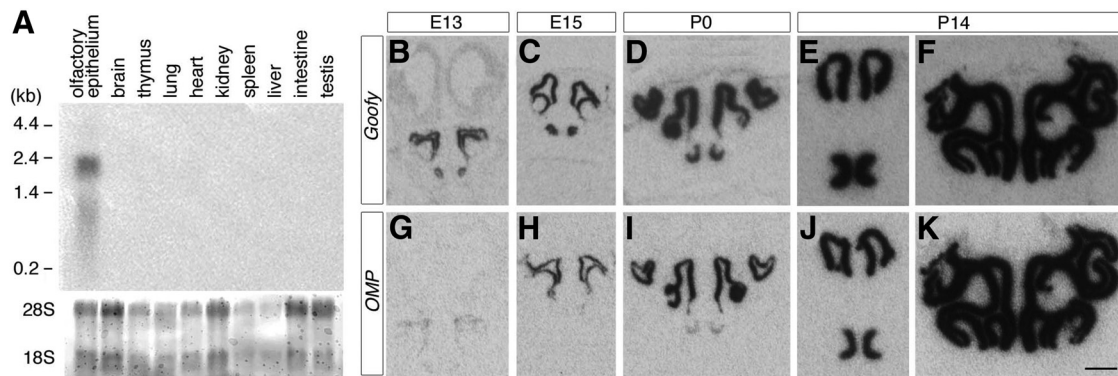
Figure 3. Expression of *Goofy* mRNA. ***A***, Tissue distribution of *Goofy* mRNA in adult mouse assessed by Northern blot analysis. Top, Autoradiogram of a blot probed with ^{32}P-labeled *Goofy* cDNA. Bottom, Same blot stained with methylene blue. *Goofy* mRNA is specifically expressed in the olfactory epithelium. ***B–K***, Ontogenic expression of *Goofy* and *OMP* mRNAs assessed by *in situ* hybridization analysis. Coronal sections containing the OE and VNO from E13 (***B, G***), E15 (***C, H***), P0 (***D, I***), and P14 (***E, F, J, K***) mice were hybridized with ^{35}S-labeled *Goofy* (***B–F***) and *OMP* (***G–K***) cRNA probes. In both OE and VNO, the expression of *Goofy* mRNA precedes that of *OMP* mRNA. Scale bar: ***B–K***, 500 μm.

plasmid (pSUC2T7M13ORI), which harbors a truncated invertase gene without a signal sequence (Jacobs et al., 1997). When the cDNA library was introduced into the invertase-deficient yeast strain YTK12 (total: 1.5×10^6 transformants), only 686 transformants capable of secreting invertase could grow on the glucose-free, raffinose-containing plates, due to in-frame fusion of the truncated invertase with a cDNA-derived signal peptide (Jacobs et al., 1997; Nakashiba et al., 2000). Of the 258 clones sequenced, canonical signal peptides with a stretch of hydrophobic amino acids were present in 215 clones (83.3%), among which 12 novel genes were included (Fig. 1*A,B*). Subsequently, we performed *in situ* hybridization analysis for these genes on mouse head sections (Fig. 1*C*) and identified the clone #123 (designated as *Goofy*) with specific and robust expression in the OE.

The mouse *Goofy* gene encodes a type I integral membrane protein consisting of an N-terminal signal peptide (21 aa), a long extracellular region (375 aa), a single membrane-spanning domain (23 aa), and a short cytoplasmic region (85 aa) (Fig. 2). The extracellular region of Goofy protein is rich in Ser (15%), Thr (12%), and Pro (18%) residues, contains three potential Asn-linked glycosylation sites, and shows no significant sequence homology to any other proteins. The cytoplasmic region of Goofy is abundant in Pro (21%) and contains a canonical SH3-binding motif (PxxPx+) (Fig. 2*B*). The *Goofy* gene appears to be present only in mammalian species, because its orthologs were identified in genomic DNA databases of various mammals, including human, chimpanzee, monkey, dog, cattle, rat, and mouse, but not of nonmammalian organisms, such as chicken, *Xenopus*, zebrafish, *Drosophila*, and *Caenorhabditis elegans* (data not shown).

Expression of *Goofy* mRNA

Northern blot analysis was performed to survey the tissue distribution of *Goofy* mRNA in adult mouse. *Goofy* mRNA was detected specifically in the OE, but not in other tissues, such as the
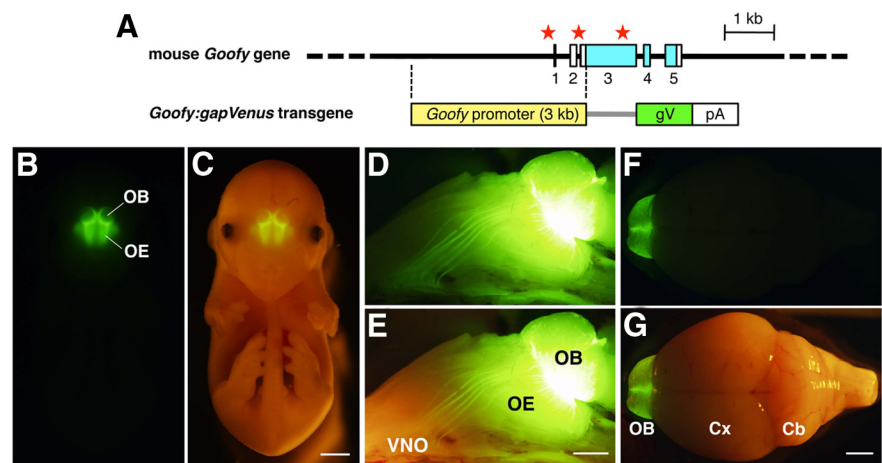

Figure 4. Promoter analysis of *Goofy* gene. ***A***, Organization of mouse *Goofy* gene (top). Exons are indicated by boxes with numbers: light blue boxes indicate the open reading frame, and white boxes represent 5′-nontranslated and 3′-nontranslated regions. Red asterisks denote canonical Olf-1-binding sites. Transgene construct (bottom). The *Goofy* gene's 5′-flanking region (3 kb) was fused to rabbit β-globin intron (gray bar), membrane-targeted Venus cDNA [gapVenus (gV)], and polyadenylation (pA) signals. ***B, C***, Whole-mount fluorescence views of *Goofy-gV* transgenic mouse at E15.5. Intense gapVenus fluorescence is specifically observed in the olfactory axons projecting from OE to OB even from the outside of embryo. A merged view of fluorescence and visible light is shown in ***C***. ***D–G***, Whole-mount fluorescence views of OE, VNO, OB (***D, E***), and brain (***F, G***) from adult *Goofy-gV* transgenic mouse. Specific and intense fluorescence of gapVenus is detected in the olfactory and vomeronasal axons in all the transgenic mice examined (*n* = 5). Merged views of fluorescence and visible light are shown in ***E*** and ***G***. Cx, Cerebral neocortex; Cb, cerebellum. Scale bars: ***B, C***, 1 mm; ***D–G***, 2 mm.

brain, thymus, lung, heart, kidney, spleen, liver, small intestine, and testis (Fig. 3*A*).

Ontogenic expression of *Goofy* mRNA was investigated by *in situ* hybridization analysis in comparison with *OMP* (olfactory marker protein) mRNA, a well characterized molecule expressed in all mature OSNs and VSNs (Margolis et al., 1991; Halpern et al., 1998). *Goofy* mRNA was first observed at embryonic day (E) 11.5 in the OE and at E13.5 in the vomeronasal organ (VNO), steadily increased thereafter, reached maximal level at P14, and maintained robust expression level into adulthood (Figs. 1*C*, 3*B–F*; data not shown). In contrast, *OMP* mRNA was first detectable at E15.5 in the OE and postnatally in the VNO (Fig. 3*G–K*).

These results indicate that the expression of *Goofy* mRNA is confined to the OE and VNO throughout development and the onset of *Goofy* expression significantly precedes OMP.
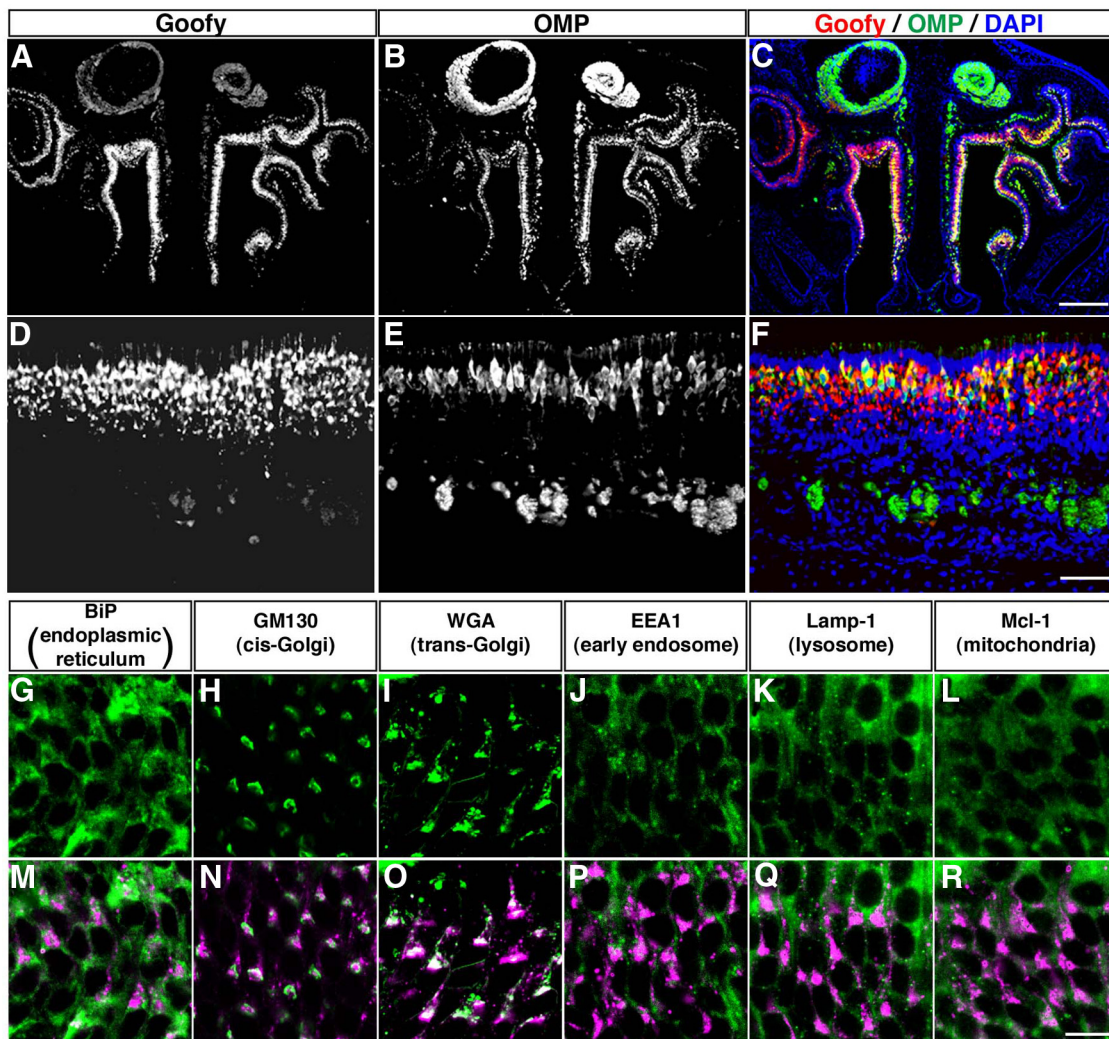
Figure 5. Localization of Goofy protein. *A–F*, Double immunofluorescence labeling of a coronal section of P3 mouse head with anti-Goofy (*A, C, D, F*) and anti-OMP (*B, C, E, F*) antibodies. *D–F*, Higher magnification of OE. *C, F*, Merged images showing Goofy (red), OMP (green), and DAPI (blue). *G–R*, Double fluorescence labeling of adult OE sections with various organelle markers (green) and anti-Goofy antibody (*M–R*, magenta). The organelle markers include anti-BiP (*G, M*, endoplasmic reticulum;), anti-GM130 (*H, N*, *cis*-Golgi), WGA (*I, O*, *trans*-Golgi), anti-EEA1 (*J, P*, early endosome), anti-Lamp-1 (*K, Q*, lysosome), and anti-Mcl-1 (*L, R*, mitochondria). Goofy is predominantly localized to *cis*-Golgi and *trans*-Golgi apparatus (*N, O*). Reproducibility of the result was confirmed for individual markers in at least three experiments. Scale bars: *A–C*, 500 μm; *D–F*, 100 μm; *G–R*, 20 μm.

Promoter analysis of *Goofy* gene

The unique expression of *Goofy* mRNA prompted us to investigate its transcriptional regulatory mechanism. The mouse *Goofy* gene consists of five exons and four introns that are compactly arranged within 2.5 kb on the chromosome 7 (Fig. 4A). Intriguingly, the 5′-upstream region of mouse *Goofy* gene is highly homologous to the previously reported rat DNA sequence *50.06*, which was discovered as one of the potential targets of the olfactory transcription factor Olf-1 (Wang and Reed, 1993; Wang et al., 1993). The mouse *Goofy* gene contains three canonical Olf-1-binding motifs (YTC-CCYRGGGAR; Fig. 4A, red asterisks) that are commonly present in olfactory neuron-specific genes, such as *ORs*, *Golf*, *ACIII*, *CNGA2*, and *OMP* (Wang and Reed, 1993; Wang et al., 1993; Rothman et al., 2005). We constructed a transgene (*Goofy-gV*) that harbors a 3.0 kb sequence upstream of the transcriptional start site of *Goofy* gene, rabbit β-globin intron, membrane-targeted yellow fluorescence protein (gapVenus) cDNA, and SV40 polyadenylation signal (Fig. 4A).

Eight independent lines of *Goofy-gV* transgenic mice were established, among which *gapVenus* transgene was detected specifically in the OE and VNO of six lines (75%) at different expression levels.

Line #3 mice showed the strongest fluorescence in olfactory axons projecting from the OE to the OB, which could be observed in intact E15.5 embryos by epifluorescent stereomicroscopy (Fig. 4B,C). In adults from this line, intense gapVenus fluorescence was detected in the OE, VNO, and OB, but not in other brain regions or in other tissues (Fig. 4D–G). In addition, we observed gapVenus expression in two other olfactory subsystems: the septal organ and Grueneberg ganglion (data not shown).

These results reinforce the OE-specific and VNO-specific expression of *Goofy* gene. In addition, the 3.0 kb upstream region of mouse *Goofy* gene can be used as an efficient promoter to drive specific and robust expression of transgenes in all types of sensory neurons in the olfactory system.

Localization of Goofy protein

To address the localization of Goofy protein in the OE, we generated polyclonal antibodies directed against the C-terminal 20 aa peptide of Goofy protein and performed immunohistochemical analysis in comparison with OMP protein, a marker of mature OSNs. On coronal sections of P7 mouse head, both Goofy
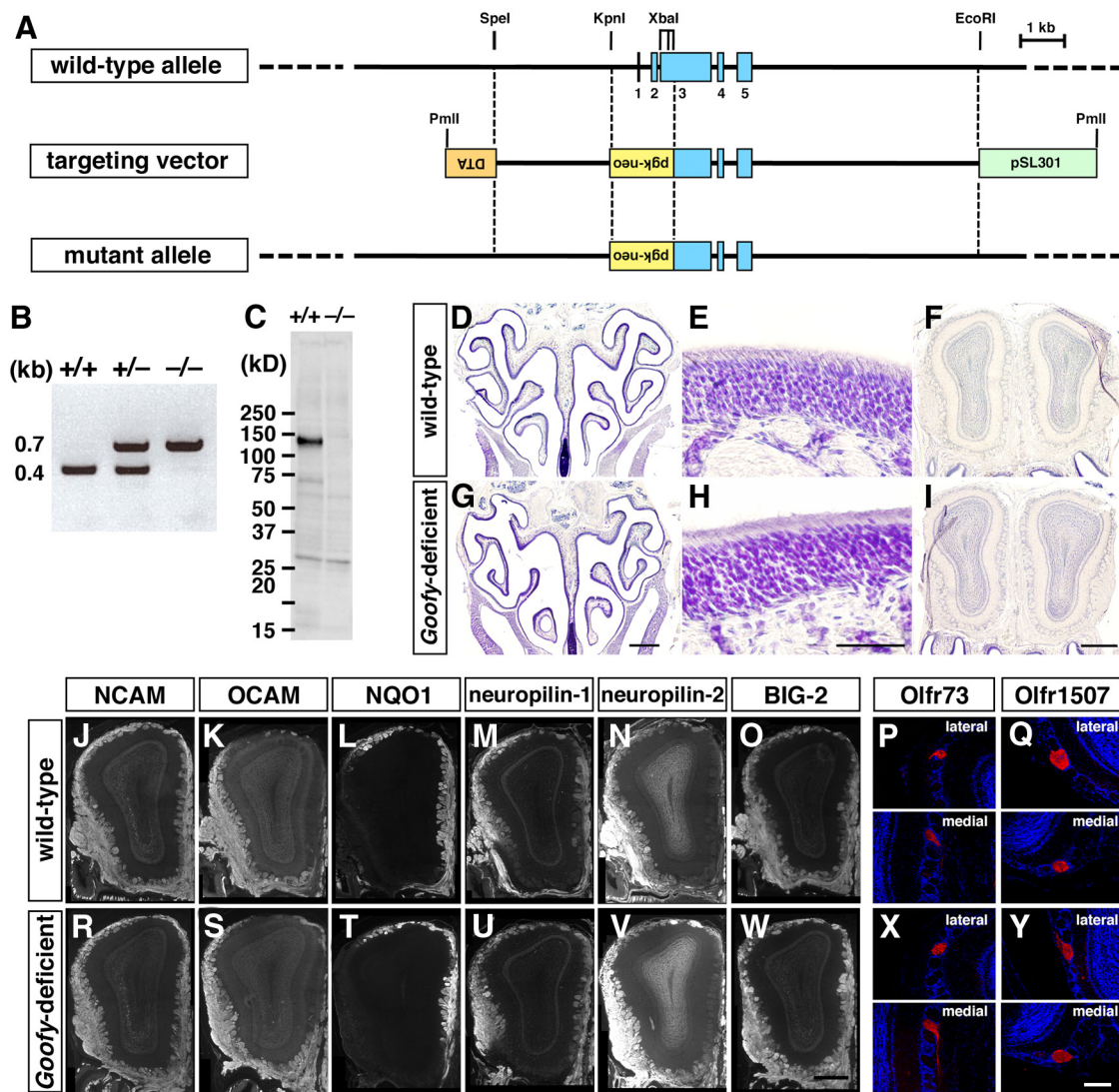
Figure 6. Generation of *Goofy*-deficient mice. *A*, Gene targeting strategy to generate *Goofy*-deficient mice. A *pgk-neo* selection marker was inserted into the *Goofy* gene. *DTA*, *Diphtheria toxin A subunit*. *B*, PCR genotyping of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. *C*, Immunoblot analysis of Goofy protein. Goofy protein is absent in OE homogenates from mutant mice. *D–I*, Nissl-stained sections of OE (*D, E, G, H*) and OB (*F, I*) from wild-type (*D–F*) and *Goofy*-deficient (*G–I*) mice. No obvious difference in gross anatomy is observed at both low (*D, F, G, I*) and high (*E, H*) magnifications. *J–Y*, Immunofluorescence labeling of OB sections from wild-type (*J–Q*) and *Goofy*-deficient (*R–Y*) mice with antibodies against NCAM (*J, R*), OCAM (*K, S*), NQO1 (*L, T*), neuropilin-1 (*M, U*), neuropilin-2 (*N, V*), BIG-2 (*O, W*), Olfr73 (*P, X*), and Olfr1507 (*Q, Y*). No difference is observed in the expression of cell adhesion molecules (*J–O, R–W*) and the olfactory axon convergence to both lateral and medial target glomeruli (*P, Q, X, Y*). Scale bars: *D, G, J–O, R–W*, 500 μm; *E, H, P, Q, X, Y*, 200 μm.

and OMP proteins were present throughout the OE with no zonal preference (Fig. 5A–C). Higher-magnification views revealed that Goofy protein was expressed in both immature and mature OSNs and most abundantly localized to the cell bodies, while OMP protein was present only in mature OSNs and showed cytoplasmic localization with an almost equal level of immunoreactivity in cell bodies, dendrites, and axons (Fig. 5D–F). Goofy protein was not detected in the globose and horizontal basal cells that are located in the most basal region of OE.

The intracellular localization of Goofy protein in OSNs was examined by double fluorescence labeling with various organelle markers. Goofy protein was colocalized almost perfectly with GM130 (*cis*-Golgi marker) and WGA (*trans*-Golgi marker) and partially with BiP (endoplasmic reticulum marker), but not with EEA1 (early endosome marker), Lamp-1 (lysosome marker), and Mcl-1 (mitochondria marker; Fig. 5G–R).

These results indicate that Goofy protein is predominantly localized to the Golgi apparatus in both immature and mature

OSNs. A similar Golgi apparatus-specific localization of Goofy protein was also observed in the sensory neurons of the VNO (both apical and basal zones), septal organ, and Grueneberg ganglion, as well as in *Goofy cDNA*-transfected N2a cells (data not shown).

Abnormal localization of ACIII in *Goofy*-deficient mice

To elucidate the physiological function of Goofy, we generated *Goofy*-deficient mice by disrupting the noncoding exons 1 and 2 and a part of exon 3, which encodes the signal peptide of Goofy protein (Fig. 6A, B). The absence of Goofy protein in the mutant mice was validated by Western blot and immunohistochemical analyses (Figs. 6C, 7A, F). Gross anatomy and layer organization of OE and OB in *Goofy*-deficient mice were not different from those of wild-type mice, as assessed by Nissl staining (Fig. 6D–I). The olfactory axon wiring from the OE to the OB appeared normal in *Goofy*-deficient mice, as assessed by both the differential expression patterns of various axon guidance molecules (e.g.,
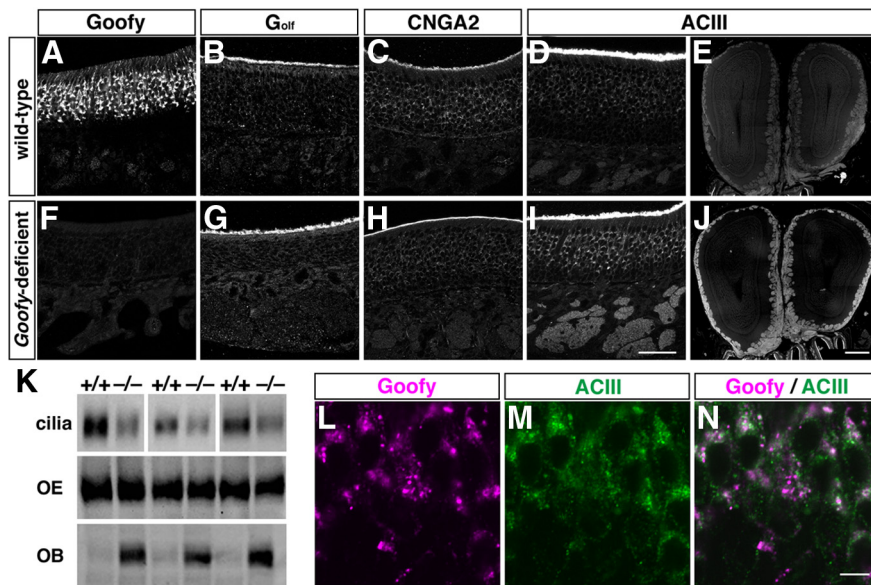
Figure 7. Abnormal localization of ACIII in *Goofy*-deficient mice. *A–J*, Immunofluorescence labeling of OE (*A–D*, *F–I*) and OB (*E*, *J*) sections with antibodies against Goofy (*A*, *F*), Golf (*B*, *G*), CNGA2 (*C*, *H*), and ACIII (*D*, *E*, *I*, *J*). In wild-type mice, Golf, CNGA2, and ACIII are highly localized to olfactory cilia, while strong ACIII immunoreactivity is observed also in olfactory axons and glomeruli of mutant mice. *K*, Immunoblot analysis of ACIII protein in olfactory cilia, whole OE, and OB from three individual mice of each genotype. In *Goofy*-deficient mice, ACIII is significantly decreased in the cilia (60 ± 7%), slightly decreased in the OE (94 ± 2%), and dramatically increased in the OB (496 ± 41%), compared with wild-type mice (*n* = 3). *L–N*, Double immunofluorescence labeling of wild-type OE section with anti-Goofy (*L*, *N*) and anti-ACIII (*M*, *N*) antibodies. *N*, A merged image showing colocalization of Goofy (magenta) and ACIII (green) at Golgi apparatus in OSNs. Scale bars: *A–D*, *F–I*, 200 μm; *E*, *J*, 500 μm; *L–N*, 20 μm.

NCAM, OCAM, NQO-1, neuropilin-1, neuropilin-2, BIG-2, kirrel 2) and the convergence of particular OR-expressing OSNs to their target glomeruli (e.g., Olfr73 [mOR-EG], Olfr1507 [MOR28], and Olfr2 [I7]) (Fig. 6*J*–*Y*; data not shown). Furthermore, no obvious change was detected in expression patterns of various functional molecules in OSNs, such as Golf, CNGA2, NKCC1, and OMP (Fig. 7*B*,*C*, *G*, *H*; data not shown).

However, we noticed abnormal localization of ACIII in OSNs of *Goofy*-deficient mice. In wild-type mice, ACIII was predominantly localized to the olfactory cilia where the odorant-induced signal transduction occurs through sequential activation of ORs, Golf, ACIII, and a cyclic nucleotide-gated cation channel. In *Goofy*-deficient mice, however, a high level of ACIII immunoreactivity was observed not only in the olfactory cilia and cell bodies, but also in axons innervating OB glomeruli (Fig. 7*D*,*E*,*I*,*J*). Western blot analysis also revealed the abnormal localization of ACIII in *Goofy*-deficient mice with a substantial decrease in the olfactory cilia (60 ± 7% of wild-type mice) and a drastic increase in the OB (496 ± 41% of wild-type mice) (Fig. 7*K*). A high-magnification image of wild-type OE revealed that Goofy and ACIII proteins are colocalized in Golgi apparatus of OSNs (Fig. 7*L*–*N*). These results suggest that the intracellular trafficking of ACIII from Golgi apparatus to olfactory cilia is impaired in *Goofy*-deficient mice.

Shortened olfactory cilia in *Goofy*-deficient mice
Next, we compared the OR protein localization in OSNs between wild-type and *Goofy*-deficient mice. Specific antibodies recognizing four well characterized OR proteins were used for this analysis: anti-Olfr2 (I7), anti-Olfr6 (M50), anti-Olfr17 (P2), and anti-Olfr73 (mOR-EG) (Kaneko-Goto et al., 2008). By whole-mount immunofluorescence labeling of wild-type OE, Olfr2 and Olfr6 proteins were clearly detectable on the olfactory cilia ema-

nating from dendritic knobs of OSNs (Fig. 8*A*). In contrast, the length of Olfr2-positive and Olfr6-positive cilia appeared markedly shorter in *Goofy*-deficient OE (Fig. 8*B*). To verify whether the use of OR antibodies is sufficient to assess the cilia length, we used *Olfr73-ires-gapEGFP* transgenic mice in which membrane-tethered GFP is expressed in Olfr73-expressing OSNs (Oka et al., 2006). Double immunofluorescence labeling of OE sections of *Olfr73-ires-gapEGFP* mice on *Goofy*-deficient background revealed that Olfr73 signals in olfactory cilia almost completely overlapped with GFP signals (Fig. 8*C–E*). Thus, OR proteins are properly trafficked even to the most distal part of olfactory cilia, validating the appropriateness of using OR antibodies for assessment of the cilia length. When the OE sections of wild-type and *Goofy*-deficient mice were immunostained with anti-Olfr2, anti-Olfr17, and anti-Olfr73 antibodies, a clear difference was observed in the extent of cilial coverage of individual OSNs on OE surface (Fig. 8*F–K*). Quantification of cilia length revealed that the olfactory cilia in *Goofy*-deficient mice were significantly shorter than those in wild-type mice for all three OR-expressing OSNs examined (Fig. 8*L–N*).

In various cell types, the length of primary cilia is regulated through the cAMP signaling pathway. Elevation of intracellular cAMP levels results in the elongation of primary cilia, whereas decreased cAMP leads to cilium shortening (Besschetnova et al., 2010; Abdul-Majeed et al., 2012). Because the olfactory cilia are classified as a specialized form of the primary cilia (Singla and Reiter, 2006; Berbari et al., 2009), it is conceivable that the cAMP signaling may also control cilia length in the OSNs. To address this possibility, we analyzed two lines of transgenic mice, *I7(WT)-ires-ECFP* and *I7(WT)-ires-dnPKA-ires-EYFP*), in which rat OR-I7 is expressed in a small population of OSNs with or without the dominant-negative form of cAMP-dependent protein kinase (dnPKA), respectively (Imai et al., 2006). Immunofluorescent labeling of OE sections with rat OR-I7-specific antibody revealed that the length of olfactory cilia was greatly shortened in the dnPKA-expressing OSNs, compared with the control OSNs (Fig. 8*O–Q*). These results suggest the role of cAMP as a regulator of ciliogenesis in OSNs, implying a possible mechanistic link between the mislocalized ACIII and the shortened olfactory cilia in *Goofy*-deficient mice.

Reduced olfactory sensitivity in *Goofy*-deficient mice
The above observation of shortened olfactory cilia led us to examine whether the *Goofy* deficiency results in impairment of odor-induced physiological and behavioral responses. To evaluate the physiological responses of OSNs, local field potentials (EOG) were recorded from the epithelial surface using intact OE preparations of wild-type and *Goofy*-deficient mice. The EOG responses to various odorants (eugenol, 2-heptanone, 1-heptanol, safrole, and lilial) were smaller in *Goofy*-deficient mice than in wild-type mice (Fig. 9*A*,*B*; data not shown). A concentration–response curve for eugenol demonstrates a clear difference of EOG amplitudes between the two genotypes

(Fig. 9C). Thus, *Goofy*-deficient OSNs displayed reduced sensitivity to odorants.

Finally, olfactory behaviors were compared between wild-type and *Goofy*-deficient mice. We used TMT (a component of fox feces odor) as a stimulus odorant, which evokes innate fear responses in rodents (Varnet-Maury et al., 1984; Fendt et al., 2005). TMT exposure induced avoidance and freezing responses in both wild-type and *Goofy*-deficient mice in a dose-dependent manner, but their sensitivities to TMT were clearly different (Fig. 9D,E). Wild-type mice showed significant responses even at the lowest amount (0.4 µl) of TMT. In contrast, the response threshold to TMT was higher in *Goofy*-deficient mice, with smaller values of avoidance index and shorter durations of freezing time to the lowest and middle amounts. At the highest amount of TMT, however, there was no difference in the responses between the two genotypes.

Thus, the electrophysiological and behavioral analyses revealed that the olfactory sensitivity of *Goofy*-deficient mice is significantly lower than that of wild-type mice, suggesting that mice become "dull-nosed" without Goofy function.

Discussion

We discovered a novel membrane protein Goofy that is specifically expressed in OSNs and VSNs and predominantly localized to the Golgi apparatus. The *Goofy*-deficient mice displayed various olfactory phenotypes, such as abnormal localization of ACIII, shortened olfactory cilia, and reduced electrophysiological and behavioral sensitivities to odorants. These findings demonstrate that Goofy is a crucial molecule for proper function of the olfactory system.

Northern blot, *in situ* hybridization, and immunohistochemical analyses revealed that *Goofy* mRNA and its translation product are expressed by sensory neurons of the olfactory system in a highly restricted manner. This rigorous regulation of *Goofy* gene transcription was verified by generating transgenic mice in which a fluorescent reporter protein was strongly and specifically expressed in the OE, VNO, septal organ, and Grueneberg ganglion under the control of *Goofy* gene promoter encompassing the 3 kb upstream sequence. The mouse *Goofy* gene contains three Olf-1-binding sites with a consensus nucleotide sequence of YTCCCYRGGGAR. Olf-1, also known as early B-cell factor-1, was first identified in rats as an olfactory neuron-specific transcription factor that binds to the conserved *cis*-acting regulatory elements commonly present upstream of *ORs, OMP, Golf, ACIII,* and *CNGA2* as well as two functionally uncharacterized genes *50.06* and *50.11* (Wang and Reed, 1993;
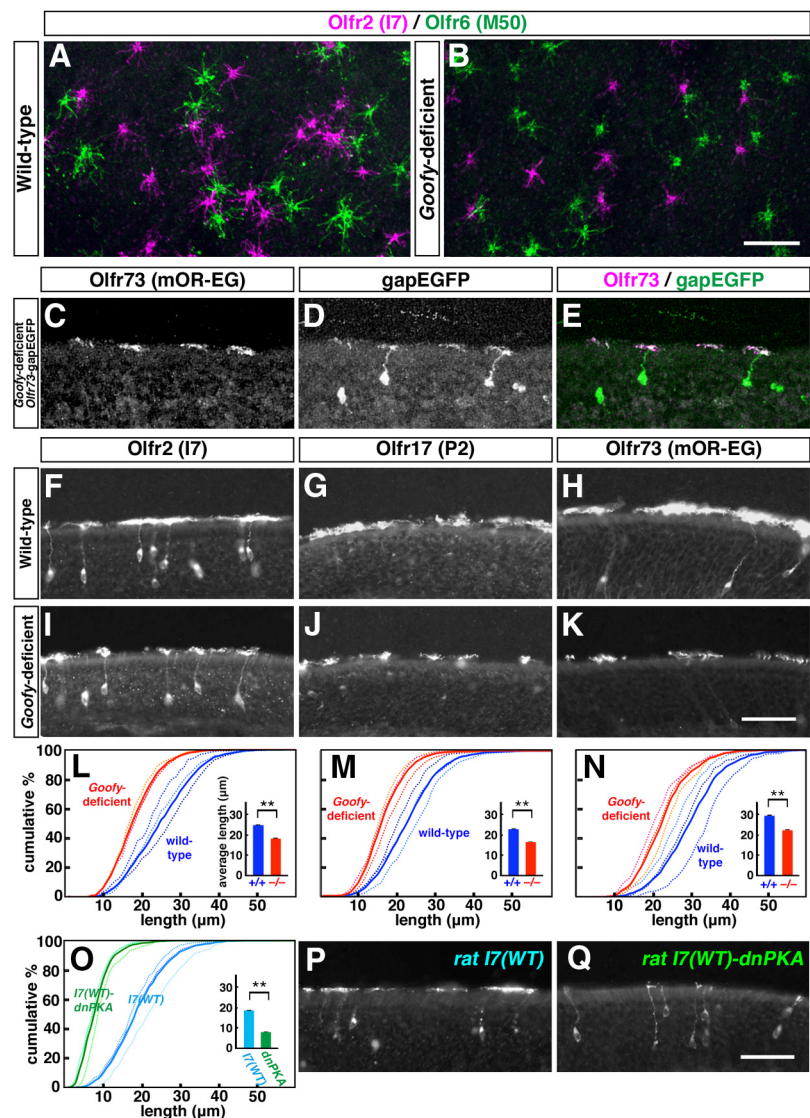

Figure 8. Shortened olfactory cilia in *Goofy*-deficient mice and dnPKA-expressing transgenic mice. ***A, B***, Whole-mount OE preparations from wild-type (***A***) and *Goofy*-deficient (***B***) mice immunolabeled with anti-Olfr2 (I7, magenta) and anti-Olfr6 (M50, green) antibodies. The length of both Olfr2-expressing and Olfr6-expressing olfactory cilia in *Goofy*-deficient mice is markedly shorter than that in wild-type mice. ***C–E***, Double immunofluorescence labeling of an OE section from *Olfr73-ires-gapEGFP* transgenic mouse on *Goofy*-deficient background with anti-Olfr73 (***C, E***) and anti-GFP (***D, E***) antibodies. ***E***, A merged image showing perfect overlapping of Olfr73 (magenta) with GFP (green) in the olfactory cilia. ***F–K***, Immunofluorescence labeling of OE sections from wild-type (***F–H***) and *Goofy*-deficient (***I–K***) mice with antibodies against Olfr2 (I7; ***F, I***), Olfr17 (P2; ***G, J***), and Olfr73 (mOR-EG; ***H, K***). ***L–N***, Cumulative frequency plot for length of olfactory cilia expressing Olfr2 (***L***), Olfr17 (***M***), and Olfr73 (***N***) in wild-type (blue) and *Goofy*-deficient (red) mice. Thin broken lines represent the results from individual mice (n = 3 mice for each genotype; n = 259–805 OSNs for each mouse), while bold lines represent pooled data (n = 1196–1815 OSNs). Insets, Averages of cilia length. For all the three ORs, the length of olfactory cilia in *Goofy*-deficient mice is significantly shorter than that in wild-type mice. ***O***, Cumulative frequency plot for length of rat I7-expressing olfactory cilia in *I7-ires-ECFP* (light blue) and *I7-ires-dnPKA-ires-EYFP* (green) transgenic mice. Thin broken lines represent the results from individual mice (n = 3 mice for each genotype; n = 292–693 OSNs for each mouse), while bold lines represent pooled data (n = 1538 for *I7-ires-ECFP*; n = 1229 for *I7-ires-dnPKA-ires-EYFP*). Insets, Averages of cilia length. ***P, Q***, Immunofluorescence labeling of coronal sections of OE from *I7-ires-ECFP* (***P***) and *I7-ires-dnPKA-ires-EYFP* (***Q***) transgenic mice with anti-rat I7-specific antibody. **p < 0.01 (2-tailed *t* test). Scale bars: ***A, B***, 20 µm; ***C–K, P, Q***, 50 µm.

Wang et al., 1993). Although the following studies reported that the *50.11* gene encodes an olfactory signal-modulating protein, SLP3/SRO, belonging to the stomatin family (Kobayakawa et al., 2002; Goldstein et al., 2003), the functional role of *50.06* gene product remained to be elucidated. Interestingly, the reported nucleotide sequence (1.0 kb) in the 5′-upstream region of rat *50.06* gene (Wang et al., 1993) is highly homologous to that of mouse *Goofy* gene with 86% identity. This significant sequence
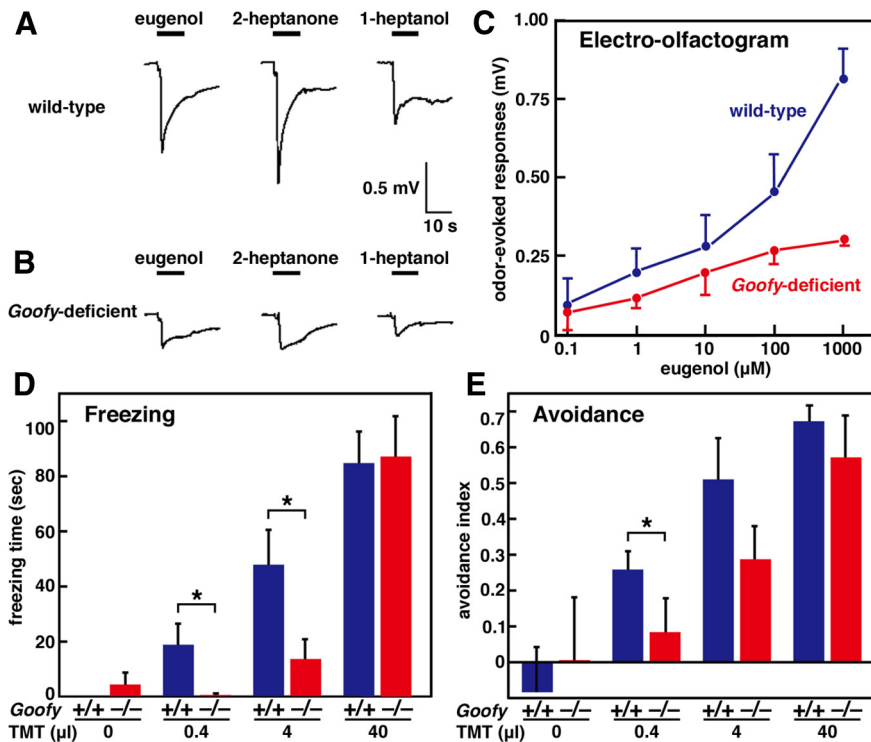
Figure 9. Reduced olfactory sensitivity in *Goofy*-deficient mice. *A–C*, Electrophysiological properties of OSNs assessed by measurement of odor-evoked EOG responses (*A*, *B*). Representative EOG traces to eugenol, 2-heptanone, and 1-heptanol in wild-type (*A*) and *Goofy*-deficient (*B*) mice. *C*, Relationship between applied eugenol concentrations and EOG responses in wild-type (blue) and *Goofy*-deficient (red) mice (*n* = 12 for each genotype). *$p < 0.05$ (2-tailed *t* test). *D*, *E*, Behavioral responses of wild-type (blue) and *Goofy*-deficient (red) mice to an aversive odor TMT stimulation (*n* = 8–10 for each genotype and each TMT concentration). Freezing time (*D*) and avoidance index (*E*) were measured for the two genotypes to different amounts of TMT. *$p < 0.05$ (Mann–Whitney *U* test).

homology indicates that the *50.06* gene encodes a rat ortholog of *Goofy* and that Olf-1 plays a crucial role in regulating the restricted expression of *Goofy/50.06* in rodent olfactory neurons.

Another remarkable feature of *Goofy* expression is its early appearance in both developmental and differentiation aspects in sharp contrast to the later onset of *OMP* expression. Ontogenic analysis revealed that *Goofy* mRNA is detectable as early as at E11.5 in the OE and at E13.5 in the VNO, much earlier than the appearance of *OMP*. In the neonates and adults, Goofy protein is present in both the immature and mature OSNs residing in the deep and superficial layers of OE, respectively, but not in the globose or horizontal basal cells, the progenitors of OSNs and sustentacular cells (Schwob, 2002). Thus, the Goofy expression starts at a postmitotic, immature stage during the differentiation process of OSNs.

For the OSN-specific transgene expression, the transcriptional regulatory region of *OMP* gene has been widely used so far (Mombaerts et al., 1996; Walters et al., 1996; Yoshihara et al., 1999; Bozza et al., 2004; Nguyen et al., 2007). Although the *OMP* gene promoter/enhancer has proved powerful in many cases, it can drive transgene expression only in the mature OSNs. In contrast, the present study demonstrates that the 3 kb fragment upstream of mouse *Goofy* gene can efficiently induce transgene expression in both the immature and mature OSNs from developmental stages earlier than the expression onset of *OMP* and other olfactory signaling molecules. Thus, the *Goofy* gene promoter/enhancer will become a useful and convenient tool for various studies on differentiation, maturation, and function of OSNs.

Immunohistochemical analysis with various organelle markers revealed that Goofy protein is predominantly localized to

Golgi apparatus of OSNs. The Golgi apparatus is an intracellular central organelle with a well established role as a processing and sorting station in the transport of secretory and transmembrane cargo proteins to their final destination (Wilson et al., 2011). By surveying the expression patterns of various functional molecules in OSNs, we found the abnormal localization of ACIII, an enzyme playing a pivotal dual role in odor-induced signal transduction cascade (Bakalyar and Reed, 1990) and olfactory axon targeting to OB (Imai et al., 2006). ACIII is a transmembrane protein normally highly enriched in olfactory cilia (Bakalyar and Reed, 1990). In *Goofy*-deficient mice, however, ACIII was also abundantly detected in olfactory axons and glomeruli. Thus, it is likely that Goofy associates with ACIII in Golgi apparatus of OSNs, regulating either its post-translational modification or intracellular trafficking. Further biochemical and proteomics analyses are now in progress to gain mechanistic insight into the interaction between Goofy and ACIII as well as to search for other functional molecules that associate with Goofy.

In addition to this molecular phenotype of ACIII mislocalization, *Goofy*-deficient mice displayed morphological abnormality of OSNs: the shortening of olfactory cilia. How does Goofy protein in the Golgi apparatus affect the morphology of olfactory cilia? We observed that the length of olfactory cilia is drastically shortened by the transgenic expression of dnPKA, suggesting a crucial role of cAMP signaling in ciliogenesis of OSNs. A similar regulatory mechanism of olfactory cilia architecture by cyclic nucleotide was reported in *C. elegans*, where cGMP signaling plays an important role (Mukhopadhyay et al., 2008). Taking into consideration all of these findings, we speculate that the abnormal localization of ACIII in *Goofy*-deficient mice might cause insufficiency of cAMP production in dendrites, resulting in the impaired elongation of olfactory cilia. Consequently, *Goofy*-deficient mice may display reduced sensitivity to odorants in both physiological and behavioral analyses, rendering themselves goofy-nosed.

The cAMP second messenger system appears to play three important roles in OSN functioning: odor-induced neuronal excitation (Bakalyar and Reed, 1991; Schild and Restrepo, 1998), olfactory axon guidance (Imai et al., 2006; Col et al., 2007), and ciliogenesis (this study). In *Goofy*-deficient mice, the odor-induced responses were smaller and the olfactory cilia were shorter than in wild-type mice, whereas the olfactory axon projection to target glomeruli appeared normal. It is likely that the degree of odor-induced activation and the length of olfactory cilia are attributable to the absolute cAMP level in individual OSNs in a cell-autonomous manner. In contrast, the olfactory axon innervation may be determined by the relative difference in cAMP-regulated expression levels of axon guidance molecules among distinct OR-expressing OSNs (Mori and Sakano, 2011). Therefore, it is conceivable that the olfactory axons can precisely innervate target glomeruli in appropriate OB regions in *Goofy*-

deficient mice where cAMP signaling cascade is partially toned down in all OSNs evenly.

References

Abdul-Majeed S, Moloney BC, Nauli SM (2012) Mechanisms regulating cilia growth and cilia function in endothelial cells. Cell Mol Life Sci 69: 165–173. CrossRef Medline

Axel R (1995) The molecular logic of smell. Sci Am 273:154–159. CrossRef Medline

Bakalyar HA, Reed RR (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science 250:1403–1406. CrossRef Medline

Bakalyar HA, Reed RR (1991) The second messenger cascade in olfactory receptor neurons. Curr Opin Neurobiol 1:204–208. CrossRef Medline

Berbari NF, O'Connor AK, Haycraft CJ, Yoder BK (2009) The primary cilium as a complex signaling center. Curr Biol 19:R526–R535. CrossRef Medline

Besschetnova TY, Kolpakova-Hart E, Guan Y, Zhou J, Olsen BR, Shah JV (2010) Identification of signaling pathways regulating primary cilium length and flow-mediated adaptation. Curr Biol 20:182–187. CrossRef Medline

Bozza T, McGann JP, Mombaerts P, Wachowiak M (2004) In vivo imaging of neuronal activity by targeted expression of a genetically encoded probe in the mouse. Neuron 42:9–21. CrossRef Medline

Buck L, Axel R (1991) A novel multigene family may encode odorant receptors: a molecular basis for odorant recognition. Cell 65:175–187. CrossRef Medline

Buck LB (2000) The molecular architecture of odor and pheromone sensing in mammals. Cell 100:611–618. CrossRef Medline

Col JA, Matsuo T, Storm DR, Rodriguez I (2007) Adenylyl cyclase-dependent axonal targeting in the olfactory system. Development 134: 2481–2489. CrossRef Medline

Dhallan RS, Yau KW, Schrader KA, Reed RR (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature 347:184–187. CrossRef Medline

Fendt M, Endres T, Lowry CA, Apfelbach R, McGregor IS (2005) TMT-induced autonomic and behavioral changes and the neural basis of its processing. Neurosci Biobehav Rev 29:1145–1156. CrossRef Medline

Fukuda N, Shirasu M, Sato K, Ebisui E, Touhara K, Mikoshiba K (2008) Decreased olfactory mucus secretion and nasal abnormality in mice lacking type 2 and type 3 IP3 receptors. Eur J Neurosci 27:2665–2675. CrossRef Medline

Gibson AD, Garbers DL (2000) Guanylyl cyclases as a family of putative odorant receptors. Annu Rev Neurosci 23:417–439. CrossRef Medline

Goldstein BJ, Kulaga HM, Reed RR (2003) Cloning and characterization of SLP3: a novel member of the stomatin family expressed by olfactory receptor neurons. J Assoc Res Otolaryngol 4:74–82. Medline

Halpern M, Jia C, Shapiro LS (1998) Segregated pathways in the vomeronasal system. Microsc Res Tech 41:519–529. CrossRef Medline

Imai T, Suzuki M, Sakano H (2006) Odorant receptor-derived cAMP signals direct axonal targeting. Science 314:657–661. CrossRef Medline

Jacobs KA, Collins-Racie LA, Colbert M, Duckett M, Golden-Fleet M, Kelleher K, Kriz R, LaVallie ER, Merberg D, Spaulding V, Stover J, Williamson MJ, McCoy JM (1997) A genetic selection for isolating cDNAs encoding secreted proteins. Gene 198:289–296. CrossRef Medline

Jones DT, Reed RR (1989) Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. Science 244:790–795. CrossRef Medline

Kaneko-Goto T, Yoshihara S, Miyazaki H, Yoshihara Y (2008) BIG-2 mediates olfactory axon convergence to target glomeruli. Neuron 57:834–846. CrossRef Medline

Kimoto H, Haga S, Sato K, Touhara K (2005) Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature 437:898–901. CrossRef Medline

Kobayakawa K, Hayashi R, Morita K, Miyamichi K, Oka Y, Tsuboi A, Sakano H (2002) Stomatin-related olfactory protein, SRO, specifically expressed in the murine olfactory sensory neurons. J Neurosci 22: 5931–5937. Medline

Kobayakawa K, Kobayakawa R, Matsumoto H, Oka Y, Imai T, Ikawa M, Okabe M, Ikeda T, Itohara S, Kikusui T, Mori K, Sakano H (2007) Innate versus learned odour processing in the mouse olfactory bulb. Nature 450:503–508. CrossRef Medline

Margolis FL, Verhaagen J, Biffo S, Huang FL, Grillo M (1991) Regulation of gene expression in the olfactory neuroepithelium: a neurogenetic matrix. Prog Brain Res 89:97–122. CrossRef Medline

McEwen DP, Jenkins PM, Martens JR (2008) Olfactory cilia: our direct neuronal connection to the external world. Curr Top Dev Biol 85:333–370. CrossRef Medline

Mitsui S, Igarashi KM, Mori K, Yoshihara Y (2011) Genetic visualization of the secondary olfactory pathway in Tbx21 transgenic mice. Neural Syst Circuits 1:5. CrossRef Medline

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R (1996) Visualizing an olfactory sensory map. Cell 87:675–686. CrossRef Medline

Mori K, Sakano H (2011) How is the olfactory map formed and interpreted in the mammalian brain? Ann Rev Neurosci 34:467–499. CrossRef Medline

Mori K, Nagao H, Yoshihara Y (1999) The olfactory bulb: coding and processing of odor molecule information. Science 286:711–715. CrossRef Medline

Mukhopadhyay S, Lu Y, Shaham S, Sengupta P (2008) Sensory signaling-dependent remodeling of olfactory cilia architecture in C. elegans. Dev Cell 14:762–774. CrossRef Medline

Nakashiba T, Ikeda T, Nishimura S, Tashiro K, Honjo T, Culotti JG, Itohara S (2000) Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. J Neurosci 20:6540–6550. Medline

Nguyen MQ, Zhou Z, Marks CA, Ryba NJ, Belluscio L (2007) Prominent roles for odorant receptor coding sequences in allelic exclusion. Cell 131: 1009–1017. CrossRef Medline

Oka Y, Katada S, Omura M, Suwa M, Yoshihara Y, Touhara K (2006) Odorant receptor map in the mouse olfactory bulb: in vivo sensitivity and specificity of receptor-defined glomeruli. Neuron 52:857–869. CrossRef Medline

Reed RR (1992) Signaling pathways in odorant detection. Neuron 8:205–209. CrossRef Medline

Rothman A, Feinstein P, Hirota J, Mombaerts P (2005) The promoter of the mouse odorant receptor gene M71. Mol Cell Neurosci 28:535–546. CrossRef Medline

Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H (2004) RTP family members induce functional expression of mammalian odorant receptors. Cell 119:679–691. CrossRef Medline

Schild D, Restrepo D (1998) Transduction mechanisms in vertebrate olfactory receptor cells. Physiol Rev 78:429–466. Medline

Schwob JE (2002) Neural regeneration and the peripheral olfactory system. Anat Rec 269:33–49. CrossRef Medline

Singla V, Reiter JF (2006) The primary cilium as the cell's antenna: signaling at a sensory organelle. Science 313:629–633. CrossRef Medline

Strotmann J, Levai O, Fleischer J, Schwarzenbacher K, Breer H (2004) Olfactory receptor proteins in axonal processes of chemosensory neurons. J Neurosci 24:7754–7761. CrossRef Medline

Tegoni M, Pelosi P, Vincent F, Spinelli S, Campanacci V, Grolli S, Ramoni R, Cambillau C (2000) Mammalian odorant binding proteins. Biochim Biophys Acta 1482:229–240. CrossRef Medline

Touhara K, Vosshall LB (2009) Sensing odorants and pheromones with chemosensory receptors. Annu Rev Physiol 71:307–332. CrossRef Medline

Varnet-Maury E, Polak EH, Demael A (1984) Structure-activity relationship of stress-inducing odorants in the rat. J Chem Ecol 10:1007–1018. CrossRef

Walters E, Grillo M, Oestreicher AB, Margolis FL (1996) LacZ and OMP are co-expressed during ontogeny and regeneration in olfactory receptor neurons of OMP promoter-lacZ transgenic mice. Int J Dev Neurosci 14: 813–822. CrossRef Medline

Wang MM, Reed RR (1993) Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature 364:121–126. CrossRef Medline

Wang MM, Tsai RY, Schrader KA, Reed RR (1993) Genes encoding components of the olfactory signal transduction cascade contain a DNA binding site that may direct neuronal expression. Mol Cell Biol 13:5805–5813. Medline

Washburn KB, Turner TJ, Talamo BR (2002) Comparison of mechanical agitation and calcium shock methods for preparation of membrane frac-

tion enriched in olfactory cilia. Chem Senses 27:635–642. CrossRef Medline

Wilson C, Venditti R, Rega LR, Colanzi A, D'Angelo G, De Matteis MA (2011) The Golgi apparatus: an organelle with multiple complex functions. Biochem J 433:1–9. CrossRef Medline

Yoshihara Y, Kawasaki M, Tamada A, Fujita H, Hayashi H, Kagamiyama H, Mori K (1997) OCAM: a new member of the neural cell adhesion mol-ecule family related to zone-to-zone projection of olfactory and vomero-nasal axons. J Neurosci 17:5830–5842. Medline

Yoshihara Y, Mizuno T, Nakahira M, Kawasaki M, Watanabe Y, Kagamiyama H, Jishage K, Ueda O, Suzuki H, Tabuchi K, Sawamoto K, Okano H, Noda T, Mori K (1999) A genetic approach to visualization of multisynaptic neural pathways using plant lectin transgene. Neuron 22:33–41. CrossRef Medline