Supplementary Information

Skn-1a/Pou2f3 specifies taste receptor cell lineage

Ichiro Matsumoto, Makoto Ohmoto, Masataka Narukawa, Yoshihiro Yoshihara & Keiko Abe

METHODS

Differential screening of gene expression data. Candidate genes encoding transcription factors and showing taste bud–specific expression in the tongue epithelium were extracted from gene expression data obtained for isolated taste buds and circumvallate epithelium after taste bud dissection, and non–gustatory tongue epithelium, as described previously [1].

Histological study. *In situ* hybridization, immunohistochemistry, and their combinatorial analyses were carried out as described previously [1-3]. For immunohistochemistry, goat anti–GFP antibody (Abcam, 1:500), rat anti–GFP antibody (Nacalai, 1:500), rabbit anti–Skn-1a/i antibody (Santa Cruz, 1:1,000), rabbit anti–T1R3 antibody (1:300) [3], rabbit anti–Ggust antibody (Santa Cruz, 1:500), and rabbit anti–KCNQ1 antibody (Chemicon, 1:3,000) were used.

***skn1a–FinG* transgenic mice.** For the transgenic construct, approximately 5 kb of the 5' upstream region of the ATG start codon of *skn-1a* was obtained from BAC clone RP23–400E15 and used as the *skn-1a* regulatory sequence. The plasmid pires–nGFP, which encodes enhanced GFP fused to the C-terminal nuclear localization signal of the SV40 large T antigen, was generated by substituting the *Bsr*G I–*Afl* II fragment of pIRES2–EGFP (Clonetech) for the *Bsr*G I–*Afl* II fragment of pECFP–Nuc (Clonetech).

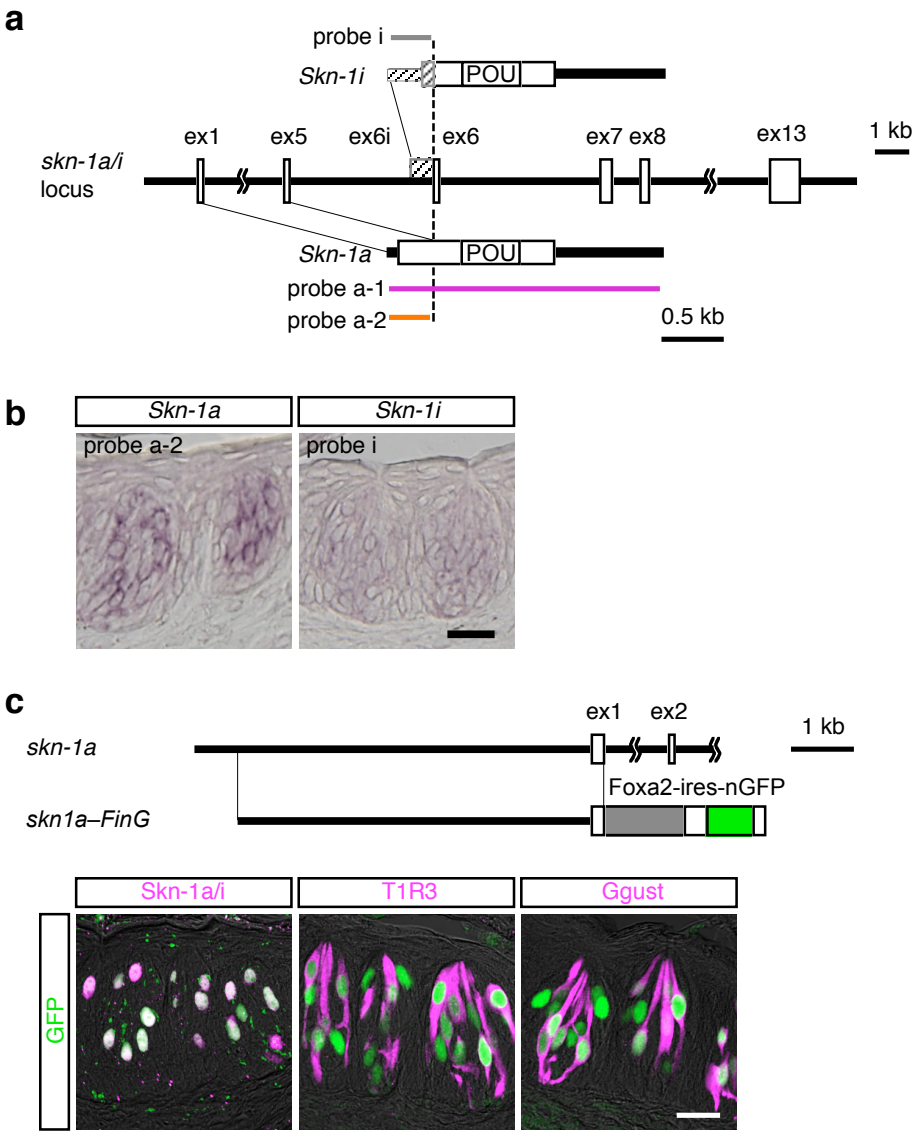
Foxa2 cDNA was fused upstream of ires–nGFP. Transgenic mice were generated by pronuclear injection from BDF1 (C57BL/6 x DBA/2 hybrids) embryos by pronuclear injection, as described previously [3]. Detailed phenotypic analyses of the *skn1a–FinG* mice will be published elsewhere.

Gene targeting of *skn-1*. ES cells of 129SvEv origin were used for gene targeting, and the targeting vector was derived from BAC clone RP22–526M22. Approximately 4 kb of the *skn-1* gene, including exons 7 to 9, was replaced with a *tk–neo* cassette. In addition, 2 kb and 6 kb, respectively, of the homologous sequences flanking the 4–kb *skn-1* sequence at the 5' and 3' ends were included, and the 5'-homologous sequence was flanked by a DT–A cassette for negative selection. Skn-1 knockout mice were generated by homologous recombination following standard procedures [4,5]. The mice used for gene targeting were of a mixed 129 x C57BL/6J background.

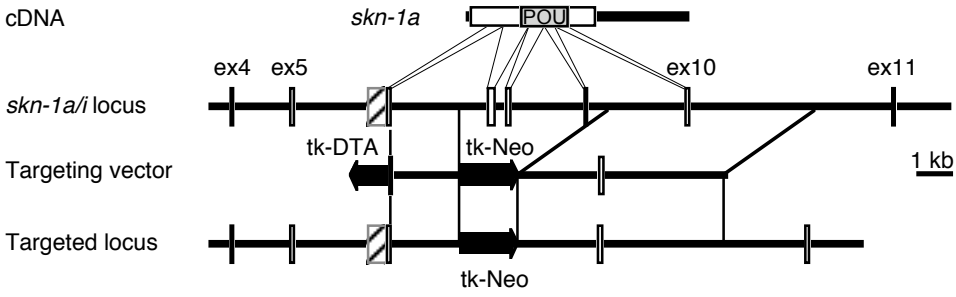
Behavioral assays. Wild–type (B6) and *skn-1*$^{-/-}$ mice (male, 2 to 10 months-old, n = 10) were individually caged and given free 48-h access to two bottles: one contained deionized water and the other a tastant solution. The taste solutions used were 300 mM sucrose, 3 mM saccharin, 30 mM monosodium glutamate with 0.5 mM inosine monophosphate (MSG (+IMP)), 1 mM denatonium benzoate, 0.1 mM quinine hydrochloride (QHCl), 30 mM citric acid, and 100 mM sodium chloride. After 24 h, the bottle positions were switched to control for positional effects. The ratio of tastant volume to total liquid consumed was recorded, and the intake of each tastant solution was expressed as the mean ± s.e.m. The differences in tastant preferences between wild–type and *skn-1*$^{-/-}$ mice were statistically evaluated using Student's t–test.

Nerve recordings. Whole–nerve responses to the lingual application of tastants were recorded from the chorda tympani (CT) and glossopharyngeal (GL) nerves. Male mice (n = 5 for *skn-1*$^{-/-}$, n = 5–6 for wild–type) were anaesthetised by intraperitoneal

injection of sodium pentobarbital (50 mg/kg) and urethane (500 mg/kg). A tracheal cannula was implanted in each animal, and each animal was then secured with a headholder. The CT nerve was exposed at its exit from the lingual nerve by removal of the internal pterygoid muscle, dissected free from surrounding tissues, and cut at the point of its entry into the bulla. The GL nerve was exposed by removal of the digastricus muscle and posterior horn of the hyoid bone, dissected free from underlying tissues, and cut near its entrance into the jugular foramen. The entire nerve was placed on a platinum wire electrode. An indifferent electrode was positioned nearby in the wound. Whole–nerve activities were amplified, displayed on an oscilloscope, and monitored with an amplifier (DAM50, World Precision Instruments, Inc., Sarasota, Florida, USA). The amplified signal was passed through an integrator with a time constant of 1 sec. The magnitude of the whole–nerve response was measured as the height of the integrated response from baseline (before stimulation) approximately 5 sec after the onset of stimulation to avoid the tactile effects of stimuli. The taste solution was applied for 30 s, followed by a >30 s rinse with deionized water. Application of the taste solution was repeated at least three times for each solution, and the mean response was calculated. The taste solutions used were 300 mM sucrose, 30 mM monosodium glutamate with 0.5 mM inosine monophosphate (MSG (+IMP)), 1 mM denatonium benzoate, 30 mM citric acid, and 100 mM sodium chloride. The relative response magnitude for each tastant was calculated by comparison with that of 100 mM ammonium chloride as a control. The differences in nerve responses between wild–type and *skn-1*$^{-/-}$ mice were statistically evaluated using Student's *t*–test.



Supplementary Figure 1 Skn-1 variant expressed in the taste buds. (**a**) Schematic structures of the gene, transcripts, and probe regions for *Skn-1*. *Skn-1a* mRNA consists of 13 exons (open boxes). The first exon of *Skn-1i* (exon 6i) contains approximately 300 bases from the 3' end of intron 5 (dashed box) and the entire following exon 6. *Skn-1a* and *-1i* mRNA coding regions are depicted by an open box. Non–coding regions of *Skn-1a* and *-1i* common to *Skn-1a* are depicted by a thick black line, and those specific to *Skn-1i* are depicted by a thick dashed line. (**b**) *In situ* hybridization using antisense probes specific to *Skn-1a* (probe a–2) and *-1i* (probe i). (**c**) Expression of GFP in *Skn-1a/i*–expressing cells driven by the 5' upstream region of *skn-1a*. The transgene construct *skn1a–FinG* consists of approximately 5 kb of the 5' upstream region of *skn-1a* (thick line), the 5' non–coding sequence of *skn-1a* (left open box), the Foxa2 coding sequence (shaded box), the internal ribosome entry site (IRES; middle open box), the GFP coding sequence (green box), and the SV40 polyA site (right open box). Nuclear–localized GFP (green) in sweet/umami (magenta in T1R3 panel) and bitter (magenta in Ggust panel) TRCs of circumvallate papillae was observed by double–labeling fluorescent immunohistochemistry. Scale bars measure 20 μm.



Supplementary Figure 2 Gene–targeting strategy used for the *skn-1a/i* locus. Exons 7–9 encoding POU homeodomain and a part of the POU–specific domain were deleted in the targeted allele.

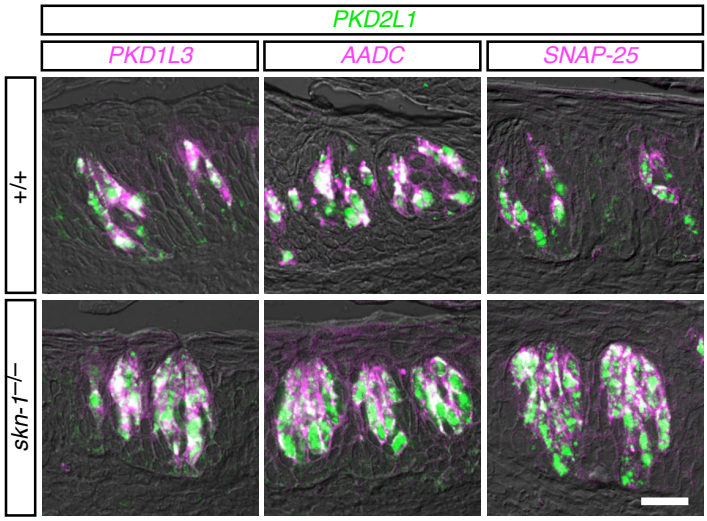
a

Taste substances	Concentration	Preference ratio (to water)		*p*-value
		+/+	*skn-1*$^{-/-}$	
Sucrose	300 mM	0.95±0.01	0.50±0.05	1.58×10^{-6}
Saccharin	3 mM	0.90±0.01	0.48±0.02	4.20×10^{-12}
MSG (+IMP)	30 mM	0.68±0.06	0.49±0.02	3.28×10^{-3}
Denatonium	1 mM	0.09±0.00	0.47±0.05	1.58×10^{-6}
QHCl	0.1 mM	0.09±0.00	0.52±0.02	5.86×10^{-15}
Citric acid	30 mM	0.11±0.01	0.15±0.02	0.0751
NaCl	100 mM	0.38±0.06	0.50±0.04	0.0936

b

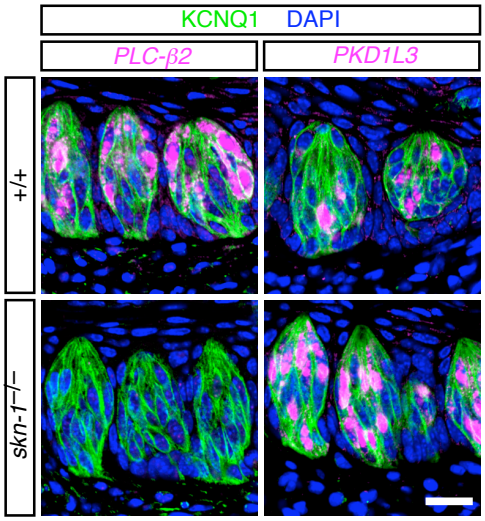
Taste substances	Concentration	Nerve	Ratio to response to NH_4Cl		*p*-value
			+/+	*skn-1*$^{-/-}$	
Sucrose	300 mM	CT	0.63±0.26	0.06±0.03	0.0591
		GL	0.35±0.03	0.06±0.02	3.02×10^{-5}
MSG (+IMP)	30 mM	CT	1.03±0.15	0.26±0.07	5.68×10^{-3}
		GL	0.19±0.05	0.13±0.03	0.326
Denatonium	1 mM	CT	0.17±0.05	0.03±0.01	0.0283
		GL	1.32±0.15	0.02±0.01	2.41×10^{-5}
Citric acid	30 mM	CT	0.93±0.26	1.41±0.41	0.353
		GL	1.56±0.16	1.38±0.17	0.451
NaCl	100 mM	CT	1.10±0.13	0.77±0.12	0.106
		GL	1.02±0.08	0.79±0.13	0.157

Supplementary Figure 3 Altered behavior and nerve responses of Skn-1a–deficient mice. (**a**) Loss of behavioral responses to sweet, umami, and bitter taste solutions. Preference for each taste solution was indicated as a ratio to that for the control water intake. (**b**) Loss of electrophysiological responses of chorda tympani (CT) and glossopharyngeal (GL) nerves to sweet, umami, and bitter taste solutions. Nerve response to each taste solution was indicated as a ratio to that of the response to NH_4Cl. Alterations in these ratios were evaluated using Student's *t*–test. Data are means ± s.e.m.



Supplementary Figure 4 Coexpression of *PKD1L3*, *AADC*, and *SNAP-25* with *PKD2L1* in the taste buds. Scale bar measures 20 μm.

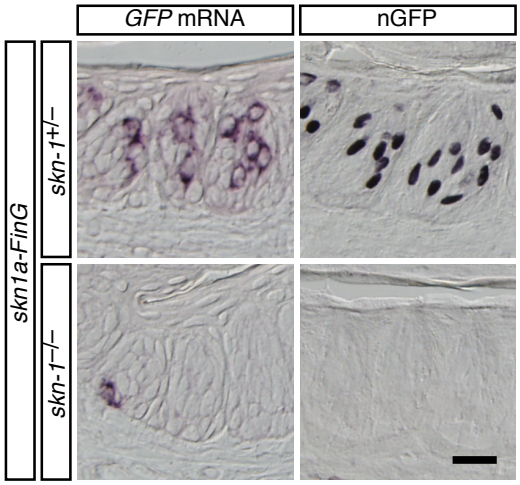
a



b

Cell type	Marker	Ratio to KCNQ1$^+$ cells (%)		*p*-value
		+/+	*skn-1*$^{-/-}$	
Sweet/ Umami/ Bitter	*PLC-β2*	29.8±0.7	0	1.86×10^{-6}
Sour	*PKD1L3*	16.0±0.5	44.6±1.4	4.81×10^{-5}
Others		54.1±1.1	55.4±1.4	0.505

Supplementary Figure 5 Quantitative estimation of the altered composition of TRCs in the taste buds of Skn-1a–deficient mice. (**a**) The mRNA signals for *PLC-β2* and *PKD1L3* in conjunction with KCNQ1 immunoreactivity. Sections were counterstained with DAPI. Scale bar measures 20 μm. (**b**) Numbers indicating *PLC-β2* and *PKD1L3* mRNA signals and KCNQ1 immunoreactivity were counted in the taste buds of wild–type (n = 3) and Skn-1a–deficient mice (n = 3). Alteration of the ratios of *PLC-β2* and *PKD1L3* mRNA–positive cell populations to KCNQ1–positive cells were evaluated using Student's *t*–test. Data are means ± s.e.m.



Supplementary Figure 6 Loss of sweet, umami, and bitter TRCs in Skn-1a–deficient mice. Robust expression of *GFP* mRNA and GFP localized in the nuclei were observed in the taste buds of *skn1a–FinG* transgenic mice in the *skn-1*$^{+/-}$ heterozygous background (*top panels*) and in the wild–type background (Supplementary Fig. S1c). Substantial *GFP* mRNA expression was observed in the basal cells of the taste buds in *skn1a–FinG* transgenic mice in the *skn-1*$^{-/-}$ homozygous background (*left, bottom*), but no GFP was observed in the taste buds (*right, bottom*). Scale bar measures 20 μm.

References

1. Ohmoto, M., Matsumoto, I., Misaka, T. & Abe, K. *Chem. Senses* **31**, 739-746 (2006).

2. Matsumoto, I., Emori, Y., Ninomiya, Y. & Abe, K. *Brain Res. Mol. Brain Res.* **93**, 105-112 (2001).

3. Ohmoto, M., Matsumoto, I., Yasuoka, A., Yoshihara, Y. & Abe, K. *Mol. Cell. Neurosci.* **38**, 505-517 (2008).

4. Joyner, A. L. (ed.) *Gene Targeting* (Oxford University Press Inc., New York, 2000).

5. Kaneko-Goto, T., Yoshihara, S.-i., Miyazaki, H. & Yoshihara, Y. *Neuron* **57**, 834-846 (2008).