Supporting Information

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SI Materials and Methods

Lineage Tracing. *Lgr6-EGFP-ires-CreERT2* mice were crossed with *Rosa26-tdTomato* mice to generate $Lgr6^{+/-}$; $Rosa\text{-}tdTomato^{+/-}$ progeny, which were confirmed by genotyping using primer sets recommended by Jackson Lab. Mice 8–16 wk old received a single i.p. injection of tamoxifen (0.22 mg/g) dissolved in sunflower seed oil (Sigma, S5007). Mice were killed 1 d, 2 wk, and 1 mo after induction with tamoxifen. Taste buds with $tdTomato^+$ cells in fungiform papillae from 10-μM sections were identified, and then the number of $tdTomato^+$ cells in each taste bud was counted (a single taste bud spans two to four consecutive sections, and we chose the section with the most labeled cells for a single taste bud for statistical analysis. The boundary of taste buds was determined by differential interference contrast microscopy). Data represent mean ± SE and total number of taste buds examined. $tdTomato^+$ cells in taste buds in circumvallate papilla were counted the same way. Statistical analyses were performed with unpaired two-tailed Student *t* test.

Cell Sorting. To obtain taste $Lgr5^+$ or $Lgr6^+$ cells, tongues from *Lgr5-EGFP-ires-CreERT2* or *Lgr6-EGFP-ires-CreERT2* mice were injected with ~0.5 mL of an enzyme mixture containing elastase (0.1 U/mL, Sigma), protease (2 mg/mL, Sigma), and DNase I (10 mg/mL, Invitrogen) or dispase (2 mg/mL, Roche) and collagenase (1 mg/mL, Roche) in Tyrode's solution (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM $NaHCO_3$, 10 mM pyruvate, 10 mM glucose) for 15 min at 37 °C. Tongue epithelium was peeled gently from the connective tissue underneath, and the regions surrounding the circumvallate and foliate papillae were dissected out and collected. Tissues were minced using scissors, further treated with trypsin (0.25%) for 15–30 min, and mechanically dissociated into single cells using a fire-polished glass pipette. Single-cell suspensions were filtered using 70-μm nylon mesh (BD Falcon #352350) to remove large aggregates, followed by further filtering with 35-μm nylon mesh (BD Falcon #352235). Cells were sorted using a FACS machine (Flow Cytometry and Cell Sorting Resource Laboratory, University of Pennsylvania), according to the green fluorescent EGFP signal (excitation, 488 nm; emission, 530 nm). For clonal analysis, we sorted $Lgr5\text{-}EGFP^+$ cells from six $Lgr5\text{-}EGFP\text{-}ires\text{-}CreERT2^{+/-}$; $Rosa\text{-}tdTomato^{+/-}$ mice; three of them received a single tamoxifen injection 1 d before sorting. Cells were sorted into individual wells in 200 μL taste culture medium.

3D Organoid Cultures. Sorted $Lgr5^+$ or $Lgr6^+$ cells from taste tissue were cultured with minor modifications according to Clevers' protocol (1) for culturing intestinal stem cells. Taste culture medium is based on DMEM/F12 medium (Life Technologies) supplemented with R-spondin-1 (200 ng/mL, R&D), Noggin (100 ng/mL, PeproTech), Jagged-1 (1 μM, Anaspec), Y27632 (10 μM), *N*-acetylcysteine (1 mM, Sigma), epidermal growth factor (50 ng/mL, Life Technologies), N2 (1%, Life Technologies), and B27 [2% (vol/vol), Life Technologies]. Sorted cells in taste culture medium were mixed with chilled Matrigel (BD Biosciences) at a volume ratio of 1:1 and then seeded on prewarmed 24-well plates at a density of 100–200 cells per well or 96-well plates at a density of 1–10 cells per well. In some cases, sonic hedgehog (shh) was added into the medium; however, we did not observe any significant effect of shh on our organoid cultures. Medium was changed every 2–3 d. For passage studies, organoids were digested into smaller pieces using trypsin (0.25%) for 10 min after 10 d in culture and reseeded into fresh Matrigel. To determine the area of an organoid, we used SPOT 5.1 Advanced software. Organoids with a typical round shape were counted for the experiments performed in Fig. S1. Plating efficiency was estimated by the number of round organoids (typically around day 7) over the number of sorted cells plated (~300 organoids grown out from ~3,000 sorted cells from two to three mice for a typical experiment). Organoids with intrinsic GFP were visualized under an inverted microscope. About 100 organoids from $Lgr5^+$ cells were found to have strong intrinsic GFP fluorescence from more than ~1,500 organoids (day 7) counted from two independent preparations (from two preparations we found 43 and 61 organoids with GFP fluorescence, out of 374 and 1201 organoids, respectively; and only two of 12 preparations of $Lgr5^+$ cells had organoids with intrinsic GFP fluorescence); ~130 organoids from $Lgr6^+$ cells were found to have strong intrinsic GFP fluorescence from more than ~1,500 organoids (day 7) counted from two independent preparations (from two preparations we found three and 129 organoids with GFP fluorescence out of 106 and 1447 organoids, respectively; and only two of eight preparations of $Lgr6^+$ cells had organoids with intrinsic GFP fluorescence).

Immunostaining. For coimaging taste cell markers and intrinsic tdTomato fluorescence, we used mice that were killed 1 mo after induction with tamoxifen. Tissues were fixed in 4% (wt/vol) paraformaldehyde for 2 h, cryoprotected with 30% sucrose overnight, and sectioned at 10 μm. To distinguish distinct types of taste cells, we used specific primary antibodies against taste cell markers and species-specific secondary antibodies: type 2 taste cells, guinea pig anti-Trpm5 (transient receptor potential cation channel subfamily M member 5; gift from Emily Liman; 1:500) and rabbit anti-gustducin (Santa Cruz; 1:500, Research Resource Identifier [RRID] #AB_673678); type 3 taste cells, goat anti-5-HT (serotonin; Immunostar; 1:100, RRID #AB_572262). For serotonin detection, mice were injected with 5-hydroxytryptophan (Sigma) 1 h before being killed to allow detection of 5-HT in type 3 cells. Species-specific Alexa-Fluo 647-conjugated secondary antibodies were used to visualize specific taste cell markers.

For cultured organoids, individual colonies were picked from culture plates using a 1-mL pipette and reseeded briefly in a slide chamber to allow attachment to the glass slide coated with polylysine for immunostaining. Organoids were then fixed for 15 min with 4% paraformaldehyde in 1× PBS supplemented with $MgCl_2$ (5 mM), EGTA (10 mM), and sucrose [4% (wt/vol)] (2). Organoids were examined for the intrinsic EGFP fluorescence before immunostaining to determine whether the secondary antibodies with 488 fluorophore can be used for triple-color staining. Organoids were washed 3× with PBS, followed by incubation with a blocking buffer [SuperBlock (Thermo Scientific PI-37525) + 2% (vol/vol) donkey serum + 0.3% Triton X-100] for 1 h. Primary antibody incubation was performed overnight at 4 °C. After washing three times with PBS, appropriate secondary antibodies were used to visualize staining as a whole-mount preparation. The primary antibodies were used as follows: rabbit anti-K14 (Covance; 1:500, RRID #AB_10063486), rabbit anti-K5 (Abcam, 1:500, RRID #AB_24647), rat anti-K8 (Developmental Studies Hybridoma Bank, 1:10, RRID #AB_531826), rabbit anti-gustducin (Santa Cruz, 1:500, RRID #AB_673678), goat anti-CA4 (Abcam, 1:20, RRID #AF2414), rabbit anti-Plcβ2 (Santa Cruz, 1:100, RRID #AB_632197), rabbit anti-NTPDase2 (Centre de Recherche du CHUL, Quebec, Canada, 1:500, RRID #AB_2314986), and goat anti-T1R3 (Santa Cruz, 1:200,

RRID #AB_2200953). As negative controls, secondary antibodies showed no staining. All images were acquired by either a Nikon Eclipse E800 microscope or a Leica Sp2 confocal microscope. Confocal images were compressed z-stacks (~3 μm); single optical sections gave rise to the same results as the z-stacks.

BrdU Labeling. BrdU (Invitrogen, 00–0103) tracing was performed by following the manufacture's protocol. Briefly, BrdU was added into medium in which organoids were cultured at 1:100 dilution (3 μg/mL) overnight. Organoids were then fixed as described earlier. BrdU immunostaining was performed according to a standard acid treatment protocol, using a mouse anti-BrdU antibody (R&D, 1:200, RRID #AB_10972641).

Calcium Imaging. Cultured organoids (days 15–32) were reseeded onto coverslips coated with polylysine and laminin (Sigma, L2020) and allowed to attach and grow into 2D culture for up to 2 wk in the same taste culture medium as described for 3D cultures. Before calcium imaging, cells were loaded with calcium indicator dye fura-2 in a modified Tyrode's buffer (30 mM NaCl, 110 mM *N*-methyl-D-glucamine, 5 mM KCl, 4 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, 1 mM Na-pyruvate at pH 7.4) for 30 min and mounted onto a perfusion chamber with continuous perfusion of the modified Tyrode's buffer. Cells were imaged under an Olympus fluorescence microscopy. Fluorescence images were obtained at excitation wavelengths of 340 and 380 nm with an emission wavelength of 460–600 nm using MetaFluro 7.5 imaging software (Olympus). Cells were then assayed for their responses to tastants: denatonium (5 mM, 10 mM, and 20 mM), acesulfame-K (5 mM, 20 mM), sucralose (15 mM, 30 mM), NaCl (200 mM, 250 mM), KCl (50 mM), citric acid (50 mM at pH 4), and monosodium glutamate (20 mM, 50 mM). In some experiments, U73122 (10 μM, Sigma) was added to cultured cells for 10 min to determine whether responses were mediated by the Plcβ2 pathway.

Reverse Transcription PCR. cDNAs from sorted cells, cultured organoids, or attached cells growing out from organoids were made using Clontech smart cDNA technology [SMARTer Pico PCR cDNA Synthesis Kit (cat. #634928) for sorted cells and cultured organoids and SMARTer PCR cDNA Synthesis Kit (cat. #634925) for 2D attached cells growing out from organoids]. Lgr and taste signaling element primers are intron-spanning primers with these sequences: 5′-taaagacgacggcaacagtg-3′ (Lgr5 forward), 5′-gattcggatcagccagctac-3′ (Lgr5 reverse), 5′-agataacagcccccaagacc-3′ (Lgr4 forward), 5′-gcaggcagtgatgaacaaga-3′ (Lgr4 reverse), 5′-gatggggacagaggactcaa-3′ (Lgr6 forward), 5′-gcaaagactgtcagcagcac-3′ (Lgr6 reverse), 5′-cacgggaagaacaatcaggt-3′ (T1R1 forward), 5′-gcagcagcaatagcgtgtta-3′ (T1R1 reverse), 5′-ggtccccatatccatgtgtt-3′ (T1R2 forward), 5′-agaatggccagcgtactgat-3′ (T1R2 reverse), 5′-aaatgtactggccaggcaac-3′ (T1R3 forward), 5′-caggaggagtgacagcacaa-3′ (T1R3 reverse), 5′-tgcctatgacatggtgcttg-3′ (gustducin forward), 5′-ggtgcaccttagccactttc-3′ (gustducin reverse), 5′-gcgctgtagccatgttcata-3′ (NTPDase2 forward), 5′-aagagcagcaggagagcaac-3′ (NTPDase2 reverse), 5′-ggcattgctctcaatgacaa-3′ (GAPDH forward), and 5′-tgtgagggagatgctcagtg-3′ (GAPDH reverse). The sizes of the amplified fragments for Lgr5, Lgr4, Lgr6, T1R1, T1R2, T1R3, gustducin, NTPDase2, and GAPDH were 199, 261, 171, 283, 285, 269, 164, 217, and 200 bp, respectively. The amplified fragments were confirmed by direct sequencing. *GAPDH* was used as a positive control.

1. Sato T, Clevers H (2013) Primary mouse small intestinal epithelial cell cultures. *Methods Mol Biol* 945:319–328.
2. Marchenko S, Flanagan L (2007) Immunocytochemistry: Human neural stem cells. *J Vis Exp* (7):267.

Fig. S1. R-spondin-1 promotes the growth of organoids derived from single Lgr5$^+$ cells. (*A*) Representative bright-field images of cultured organoids derived from individual Lgr5-EGFP$^+$ cells in the presence and absence of R-spondin-1. (Scale bars: 250 μm.) (*B*) Statistical analysis of the average size (mean ± SD) of organoids in the presence ($n = 35$) and absence ($n = 28$) of R-spondin-1 at indicated points. *t* test $P < 0.001$ for all points except day 1. Data were collected from two independent preparations.

Fig. S2. Organoids with intrinsic EGFP signal contain K5$^+$ cells. Representative confocal images of an organoid with the EGFP signal (green; labels Lgr5$^+$ cells) immunostained with the anti-K5 antibody (basal cell marker, red). (Scale bar: 50 μm.)

Fig. S3. Lgr homologs and taste signaling elements are expressed in organoid cells. (*A*) RT-PCR analysis showed the presence of transcripts of Lgr4, Lgr5, and Lgr6 in cultured organoids derived from Lgr5$^+$ cells. (*B*) Representative confocal images of an organoid derived from an Lgr5$^+$ cell immunostained with anti-NTPDase2 antibody (type 1 cell marker, red) and anti-K8 antibody (red), counterstained with DAPI. (Scale bar: 50 μm.) (*C*) Transcripts for T1R1, T1R2, T1R3, gustducin, and NTPDase2 were all detected in cultured organoids derived from Lgr5$^+$ cells. Experiments were performed in triplicate.

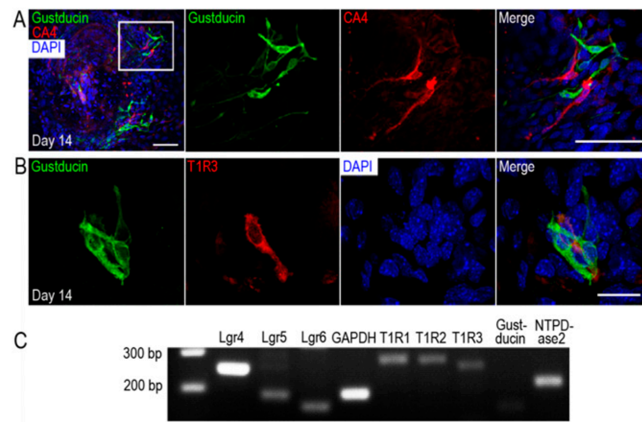

Fig. S4. Cells grown out from organoids derived from Lgr5^{+} cells on 2D surfaces express taste-cell markers. (*A*) Representative confocal images show the expression of gustducin (green) and CA4 (red) in cells that attached to the laminin-coated coverslip after growing out from organoids. (*B*) Representative image showing the presence of gustducin (green) and T1R3 (red) immunoreactive cells in cells that attached to the laminin-coated coverslip. Experiments were performed in triplicate. (*C*) RT-PCR results show the presence of transcripts for Lgr4, Lgr5, Lgr6, T1R1, T1R2, T1R3, gustducin, and NTPDase2 in attached cells that grew out from organoids. Experiments were performed in triplicate. (Scale bars: *A*, 50 μm; *B*, 20 μm.)

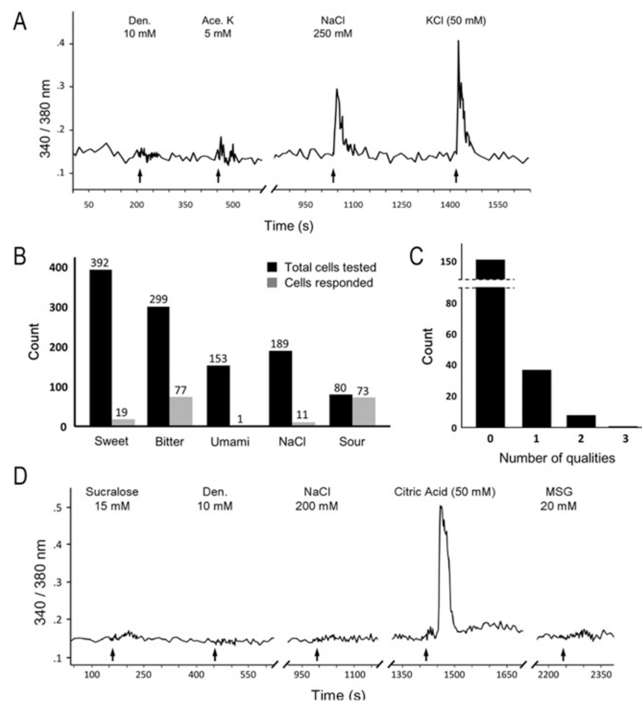

Fig. S5. Mature taste-like cells derived from single Lgr5^{+} cells are functional. (*A*) Representative trace of an organoid-derived salt-responsive taste cell responding to NaCl (250 mM) and KCl (50 mM). (*B*) Total number of cells tested with and number of cells that gave calcium responses to tastants representing different basic taste qualities [sweet, 5 mM or 20 mM acesulfame-K and 15 mM or 30 mM sucralose; bitter, 10 mM denatonium; umami, 20 or 50 mM monosodium glutamate (MSG); salty, 200 or 250 mM NaCl; sour, 50 mM citric acid at pH 4]. This graph includes data from 19 different imaging fields across 15 different organoid-derived 2D cultures. (*C*) Breadth of tuning in recorded cells. Data are from cells recorded at eight different imaging fields across seven different organoid-derived 2D cultures. Only imaging fields in which cells were tested for responses to at least three taste qualities (sweet, bitter, umami, and/or salty), not including sour, are included in this analysis. Sour responses are not included in the analysis because acid stimulation apparently evoked calcium mobilization through a nonspecific mechanism. (*D*) Representative trace of a cell that responded to 50 mM citric acid but not to any of the other stimuli tested.

Fig. S6. Lgr6 marks taste progenitor cells in circumvallate papilla. (*A*) Representative confocal images of $Lgr6^+$ cells in circumvallate papilla. Lgr6-EGFP transgene was detected by intrinsic fluorescence (green). Lgr6-$EGFP^+$ cells were found at the base of taste buds. (*B*) Tamoxifen-induced Lgr6-Cre generates tdTomato activity to mark cells from the $Lgr6^+$ lineage. More cells were labeled in taste buds in circumvallate papillae of Lgr6-Cre mice at 2 wk (4.80 ± 0.47 cells per labeled taste bud in a 10-μM section; $n = 36$) and 1 mo (5.23 ± 0.50; $n = 30$) than at 1 d (0.71 ± 0.13; $n = 38$) after a single tamoxifen induction ($P < 0.0001$). No significant difference was found between the numbers of labeled taste bud cells at 1 mo and 2 wk after tamoxifen induction ($P = 0.54$). (*C*) $Lgr6^+$ stem/progenitor cells generate multiple subtypes of taste bud cells in circumvallate papillae. Mature taste bud cells marked by Lgr6-Cre-generated tdTomato 1 mo after tamoxifen induction were stained with markers for type 2 (Trpm5, green) and type 3 (5-HT, serotonin, green) in circumvallate papilla. Arrows denote $tdTomato^+$ cells that are immunopositive for a specific taste cell marker. Three mice were used for each point of the lineage tracing experiments. (Scale bars: 100 μm.)

Fig. S7. Lgr transcripts are detected in sorted $Lgr5^+$ or $Lgr6^+$ cells. RT-PCR analysis showed the presence of transcripts of Lgr4, Lgr5, and Lgr6 in sorted $Lgr5^+$ cells from posterior tongue and in sorted $Lgr6^+$ cells from either anterior or posterior tongue. No or barely detectable transcripts for Lgr5 and Lgr6 were amplified in sorted cells negative for Lgr5-EGFP.

Fig. S8. Expansion of isolated Lgr6$^+$ cells from taste tissue in 3D culture and generation of mature taste-like cells in cultured organoids derived from Lgr6$^+$ cells. (*A*) Representative bright-field images of cultured organoids derived from isolated Lgr6-EGFP$^+$ cells at indicated points. At day 1, the lower panel is a representative fluorescence image of a cultured organoid derived from Lgr6-EGFP cells showing detectable EGFP signal (green). Data from at least three independent preparations. (*B*) Representative bright-field and fluorescence images of organoids with or without the intrinsic GFP signal. Data from two independent preparations. (*C*) Whole-mounts of day 14 organoids immunostained with anti-gustducin (red) and anti-T1R3 (blue) antibodies and displaying intrinsic EGFP fluorescence (green). (*D*) Whole-mount of day 14 organoids immunostained with anti-gustducin (red) and anti-CA4 (blue) antibodies and displaying intrinsic EGFP fluorescence (green). (*E*) Whole-mount of day 14 organoids without intrinsic EGFP fluorescence immunostained with anti-gustducin (green) and anti-T1R3 (red) antibodies. (*F*) Whole-mount of day 14 organoids without intrinsic EGFP signal immunostained with anti-gustducin (green) and anti-CA4 (red) antibodies. Experiments were performed in multiple organoids. (Scale bars: *A* and *B*, 100 μm; *C*–*F*, 100 μm.)

Table S1. Summary of organoids staining positive for taste cell markers over total organoids examined (n/N)

	Organoid source			
	Lgr5$^+$ cells		Lgr6$^+$ cells	
Taste receptor cell marker	Without intrinsic GFP fluorescence	With intrinsic GFP fluorescence	Without intrinsic GFP fluorescence	With intrinsic GFP fluorescence
Single-marker staining				
K8	23/43 (53%)	ND	3/4 (75%)	ND
NTPDase2	37/53 (70%)	ND	6/23 (26%)	7/13 (54%)
Gustducin	48/71 (68%)	17/17 (100%)	29/58 (50%)	26/28 (93%)
CA4	88/134 (66%)	10/10 (100%)	22/31 (71%)	10/10 (100%)
T1r3	77/105 (73%)	7/7 (100%)	8/33 (24%)	11/13 (85%)
Dual-marker staining				
Gustducin + CA4	18/32 (56%)	10/10 (100%)	13/29 (45%)	8/10 (80%)
Gustducin + T1r3	10/12 (83%)	7/7 (100%)	5/27 (19%)	8/9 (89%)

Scoring an organoid as positive for a marker required staining above-background (compared with control with secondary antibody alone) of at least one cell within the organoid that displayed a taste cell-like morphology. ND, not determined.