Supporting Information

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SI Materials and Methods

Immunohistochemistry. Mice were anesthetized with sodium pentobarbital (40 mg/kg; Patterson Veterinary) and then transcardially perfused with a 0.025% heparin solution in 1% sodium nitrite, followed by a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.2). Following perfusion, the tongues were removed and placed into 4% paraformaldehyde (Electron Microscopy Services) for 2 h, followed by 4 °C overnight cryoprotection in 20% sucrose. On the following day, tongues were frozen in O.C.T. Compound (Sakura Finertek USA, Inc.) and 40-μm sections of the CV papillae were cut and washed in PBS (pH 7.4) three times for 10 min each time. Sections were incubated in blocking solution [0.3% Triton X-100, 1% normal goat serum, and 1% BSA in 0.1 M PBS (pH 7.4)] for 2 h at room temperature (RT) and then in primary antibodies for 2 h at RT before an overnight incubation at 4 °C. On the next day, sections were washed three times for 10 min each time and then incubated with an appropriate secondary antibody for 2 h in the dark. The sections were then washed in PBS three times for 10 min each time and mounted onto slides using Fluoromount-G (Southern Biotechnology Associates). Images were obtained with a Zeiss LSM 510 Meta NLO Confocal Microscope and were only adjusted for brightness and contrast.

The following primary antibodies were used: anti–SNAP-25 (1:250, catalog no.20-783-70323; GenWay Biotech, Inc.; RRID: AB-1024914), anti-TRPM4 (1:50, catalog no. OST00027W; Invitrogen; RRID: AB-1091055), anti-TRPM4 (1:50, catalog no. ab63080; Abcam; RRID: AB-956418), anti-PGP9.5 (1:100, catalog no. ab108986; Abcam; RRID: AB-10891773), anti-NTPDase2 [1:100 (1); RRID: AB-2314986], anti-PLCβ2 (1:250, catalog no. SC-206; Santa Cruz Biotechnology; RRID: AB-632197), anti-gustducin (1:100, catalog no. SC-395; Santa Cruz Biotechnology; RRID: AB-10177605), anti-IP_3R3 (1:50, catalog no. 610313; BD Biosciences; RRID: AB-397705), and anti-TRPM5 [1:200 (2)]. Secondary antibodies were purchased from Jackson ImmunoResearch and used at a dilution of 1:250. Anti-TRPM4 antibody was validated with the TRPM4-KO and TRPM4/5-DKO mice. Anti-TRPM5 antibody was validated with the TRPM5-KO and TRPM4/5-DKO mice.

ImageJ (NIH) was used to measure the corrected immunofluorescence intensity of TRPM4 and TRPM5, as shown in Fig. S2, and the corrected fluorescence intensity for TRPM5, gustducin, PLCβ2, IP_3R3, and TRPM4, as shown in Fig. S3. The area, mean gray value, and immunofluorescence intensity were measured in the area of interest and in five small areas selected as the background signal. The corrected integrated density was calculated as follows:

$$\text{Immunofluorescence intensity} = \text{Integrated Immunofluorescence intensity} - (\text{Area selected} \times \text{Mean value of background}).$$

The two-tailed Student's t test was done to determine the statistical difference between the immunofluorescence intensity of different mouse lines.

Live Cell Imaging.

Na^+ imaging. All measurements of intracellular Na^+ were performed in isolated taste receptor cells. Briefly, an enzyme solution containing 3 mg Dispase II, 1 mg trypsin inhibitor, and 0.7 mg Collagenase B (Roche Products) per milliliter of Tyrode's solution was injected between the lingual epithelium and the underlying muscles on the tongue. The tongue was incubated in oxygenated Tyrode's solution for 16 min. After this initial incubation, the lingual epithelium was separated and incubated in Ca^{2+}-free Tyrode's solution for 26 min. The taste receptors cells were isolated by manual aspiration and plated on coverslips coated with Celltak (Corning, Inc.). Cells were loaded for 20 min with the Na^+ indicator dye Asante NaTrium-2 (TEFLabs, Inc.) containing the nonionic dispersing agent Pluronic F-127 (Invitrogen, ThermoFisher Scientific) and then washed for 20 min with Tyrode's solution. Taste cells were identified as previously described in previous reports (3–5). Loaded taste cells were visualized using an Olympus IX73 microscope with a 40× oil immersion lens, and images were captured using a Hamamatsu ORCA-03G camera. Cells were excited at 488 nm, and images were captured at 540 nm. During experiments, the cells were kept under constant perfusion and images were collected every 1 s using Imaging Workbench (Indec Biosystems). Experiments were graphed and analyzed using Origin 2016 software. All Na^+ imaging data were collected as arbitrary fluorescence units. The taste-evoked Na^+ signals that reached a peak of 5 SD above a stable baseline and were reversible during the following washout period were considered to be responses. A taste cell had to meet two criteria to be used in our analyses. First, after dye loading, the cell had to have a fluorescence value of at least 200; most cells had a fluorescence value between 220 and 240 units. While these values are arbitrary, generally healthy cells take up comparable amounts of dye. A stable baseline was determined by collecting data in the absence of stimulation for at least 50 s. If there was no more than a 2- to 4-unit change in fluorescence during that time period, the cell was used for experiments. Data were normalized to baseline. Peak responses and response magnitudes were averaged and plotted with SEMs. Rise times to the peak responses were also averaged and plotted with SEMs. Data were analyzed for statistically significant differences using either a two-sided Student's t test or ANOVA with a Bonferroni's post hoc analysis as appropriate. Taste cell responsiveness was compared using a χ^2 analysis to determine if there were any significant differences between wild-type, TRPM4-KO, TRPM5-KO, and TRPM4/5-DKO mice. For all analyses, a significance level of $P < 0.05$ was used and SEMs were reported. In all figures, statistical significance is labeled as follows: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Sample numbers varied between experiments due to the variability in the responsiveness of the cells to particular stimuli. Before analysis, data were evaluated for normal distribution using the Shapiro–Wilk test and were determined to be normally distributed at $P < 0.05$.

Dual Ca^{2+} and Na^+ imaging. Cells were collected according to the procedures described above and were loaded simultaneously with Fura 2-AM (Invitrogen) and Asante NaTrium-2. The cells were excited at 340-, 380-, and 488-nm excitation wavelengths. The dual Ca^{2+} and Na^+ images were captured every 4 s using a multiedge dichroic beam-splitter that captures emission at both 510 and 540 nm and Imaging Workbench (Indec Biosystems). Experiments were graphed and analyzed using Origin 2016 software.

Behavioral Experiments.

Two-bottle preference test. Mice from C57BL/6 (wild-type), TRPM5-only, TRPM4-only, and TRPM4/5-DKO lines were subjected to two-bottle preference tests in blind conditions in which each mouse tested was not identified by genotype during the analysis. Each test concentration was presented simultaneously with water for a total of 48 h. Test solutions were switched with water every 24 h to control for side preferences. Preference ratios were calculated as

volume of taste solution intake/total volume intake (taste solution + water). Five male mice from each background were used for a total of 20 mice. Two taste stimuli for each taste quality were tested: sweet [sucrose (10, 50, 100, 300, and 500 mM) and SC45647 (0.001, 0.01, 0.05, and 0.1 mM)], bitter [Den (0.1, 0.5, 1, 5, 10, and 20 mM) and quinine hydrochloride (0.001, 0.01, 0.1, 1, and 5 mM)], and umami [MPG (1, 10, 50, 100, and 300 mM) and inosine monophosphate (0.01, 0.1, 1, 10, and 50 mM)]. Preference ratios between different groups of mice were compared using a repeated-measures two-way ANOVA with a Bonferroni's post hoc analysis and two-sided Student's t test to determine significant differences for each concentration. Average values were plotted with the SD for each stimulus concentration. Significance level was set at $P < 0.05$.

Analysis of licking behavior. We recorded the unconditioned licking responses to varying concentrations of taste stimuli in a test chamber designed to measure brief-access licking (Davis MS80 Rig; Dilog Instruments and Systems). This apparatus consisted of a Plexiglas cage with a wire mesh floor. An opening at the front of the cage allowed access to one of 16 spill-proof glass drinking tubes that reside on a sliding platform. A mechanical shutter opened and closed to allow the mouse access to one of the tubes for a user-specified length of time. A computer controlled the movement of the platform, order of tube presentation, opening and closing of the shutter, duration of tube access, and interval between tube presentations. Each individual lick was detected by a contact lickometer and recorded on a computer via DavisPro collection software (Dilog Instruments and Systems).

Mice were adapted to the test chamber and trained to drink from the sipper tubes for five consecutive days. During training, mice were water-deprived for 20 h. On the first day of training, the mouse was presented with a single stationary bottle of water for 30 min. On the second day, a tube containing water was presented, but the mouse was given 180 s to initiate licking this time, and once licking was recorded, the mouse was given access to the tube for 30 s. At the conclusion of either the 30-s access or the 180-s limit, the shutter was closed again for 10 s. Each of the eight tubes, all containing water, was presented three times. The entire training program took an average of 15 min. During the remaining 3 d of training, the mouse was given 30 min to initiate licking to one of eight tubes of water. Once the mouse began licking, it was given 10 s to lick before the shutter closed for 10 s, after which a new tube was presented.

During testing, animals were allowed to take as many trials as possible in 30 min. Mice were tested on varying concentrations of SC45647 (0, 0.001, 0.01, 0.05, 0.1, and 1 mM), quinine hydrochloride (0, 0.001, 0.01, 0.1, 1, and 5 mM), and MSG (0, 50, 100, 200, 400, and 800 mM) in 10 μM amiloride, in that order. Each stimulus was presented in randomized blocks on Monday, Wednesday, and Friday in a single week. Animals were water-deprived for 22 h for all testing except SC45647, when water-replete animals were tested. Once an animal began licking the tube, it was allowed 10 s of access before the shutter closed.

For stimuli tested in the water-deprived condition (MSG and quinine), lick scores were calculated by dividing the average number of licks at each concentration by the average number of licks to water. For stimuli tested while the mice were water-replete (SC45647), lick scores were calculated by subtracting the average number of licks at each concentration by the average number of licks to water. These corrections are used to standardize for individual differences in lick rate and are based on water need (6). Lick scores and licks relative to water were compared by repeated-measures ANOVA with genotype as a between-factor variable and concentration as a repeated-measures within-factor variable. Significant interaction terms were followed by Tukey's honestly significant difference tests. Statistical analyses were performed in Statistica.

Solutions. Normal Tyrode's solution consists of the following: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose, and 1 mM pyruvic acid. Ca^{2+}-free Tyrode's solution contained the following: 140 mM NaCl, 5 mM KCl, 10 mM Hepes, 2 mM EGTA, 2 mM 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 10 mM glucose, and 1 mM pyruvic acid. The low-sodium Tyrode's solution used as the bath solution during dual-imaging experiments with NaCl and citric acid was composed of the following: 30 mM NaCl, 110 mM *N*-methyl-D-glucamine (NMDG), 5 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose, and 1 mM pyruvic acid. The 50 mM KCl and 50 mM citric acid stimuli were dissolved in low-sodium Tyrode's solution with an equimolar substitution of KCl or citric acid for NMDG. NaCl solution was made by completely replacing the NaCl normally found in Tyrode's solution with the indicated concentration of NaCl. The pH of all solutions was adjusted to 7.4 using NaOH or HCl, except for 50 mM citric acid solution, which was adjusted to pH 4.

Normal Tyrode's solution with 1 μM U73122 (Tocris Bioscience) and 3 μM thapsigargin (Tocris Bioscience) was used to block either PLC or Ca^{2+} reuptake via the SERCA pump into Ca^{2+} stores. Normal Tyrode's solution with 1 μM U73443 (Tocris Bioscience), which is the inactive analog of the PLC blocker U73122, was used to show the specificity of the taste-evoked Na^+ response. The inhibitor 9-phenanthrol (50 μM) was used to block TRPM4 channels, and TPPO (50 μM) was used to block TRPM5 channels. All blockers and tastants were dissolved in normal Tyrode's solution. The concentrations of the tastants used were as follows: Den (10 mM, catalog no. D5765; Sigma–Aldrich), sucralose (20 mM, catalog no. 69293; Sigma–Aldrich), saccharin (5 mM, catalog no. 109185; Sigma–Aldrich), and MPG (20 mM, catalog no. 49601; Sigma–Aldrich). For the concentration gradient experiments, the following concentrations were used: bitter [Den (0.5, 1, 2.5, 5, 7.5, and 10 mM)], sweet [sucralose (0.1, 0.5, 1, 10, 15, and 20 mM)], and umami [MPG (1, 5, 10, 15, and 20 mM)]. All chemicals were purchased from Sigma–Aldrich unless otherwise noted.

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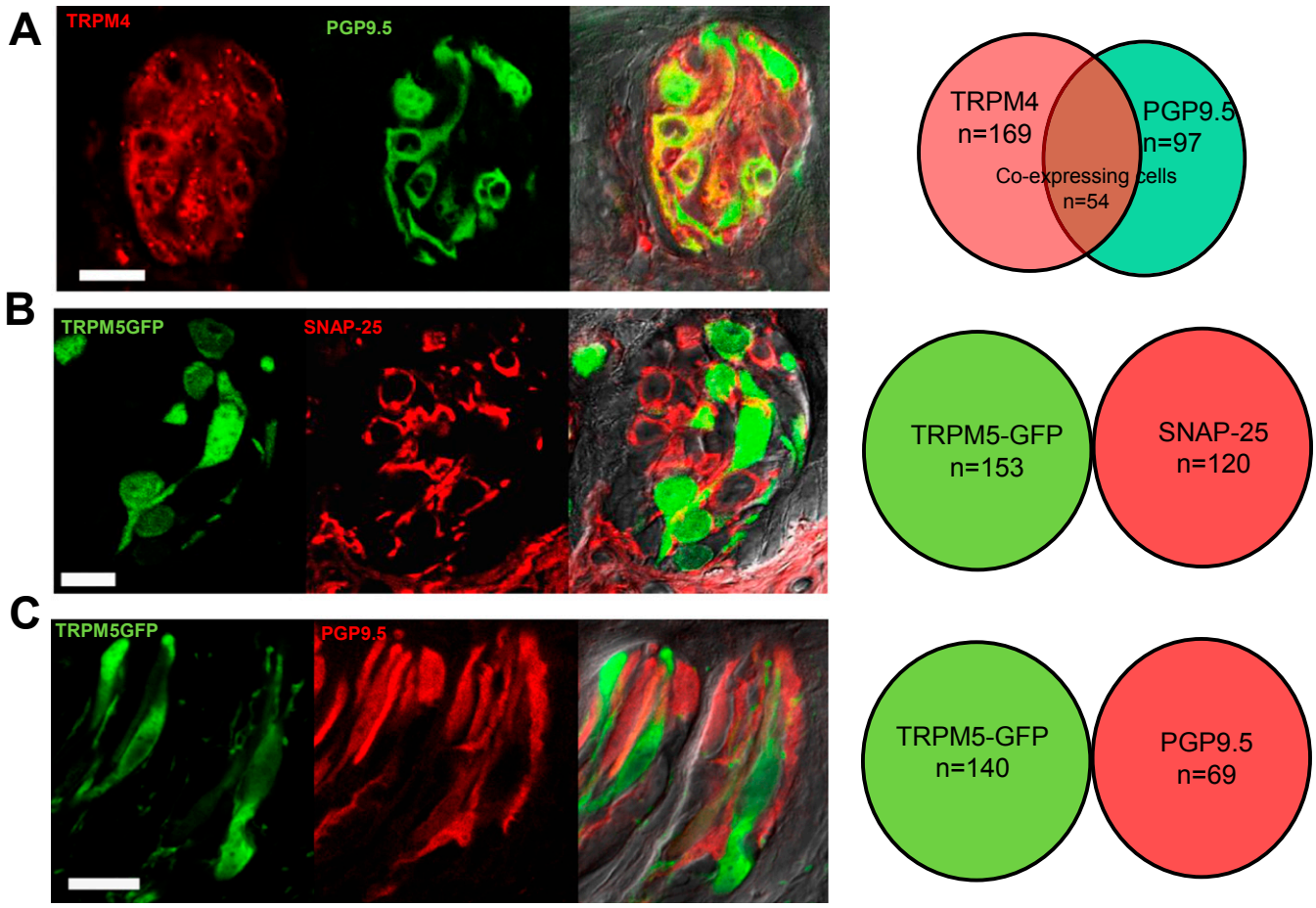

Fig. S1. TRPM4 is expressed in type III taste cells, but TRPM5-GFP is not expressed in type III taste cells. (*A*) Expression of TRPM4 (*Left*) expression of PGP9.5 (*Center*), and an overlay with a DIC image (*Right*) are shown. (*Far Right*) Corresponding Venn diagram represents the cell count data. Of the 169 TRPM4^{+} cells in CV papillae, 54 cells also expressed PGP9.5, indicating an overlap of 32% between TRPM4 and PGP9.5. (*B*) Expression of TRPM5-GFP (*Left*), expression of SNAP-25 (*Center*), and an overlay with a DIC image (*Right*) are shown (n = 153 TRPM5-GFP cells and 120 SNAP-25^{+} cells). (*Far Right*) Corresponding Venn diagram represents the cell count data. (*C*) Expression of TRPM5-GFP (*Left*), expression of PGP9.5 (*Center*), and an overlay with a DIC image (*Right*) are shown (n = 140 TRPM5-GFP cells and 69 PGP9.5^{+} cells). (*Far Right*) Corresponding Venn diagram represents the cell count data (n = 3 mice for each). (Scale bar: 20 μm.)

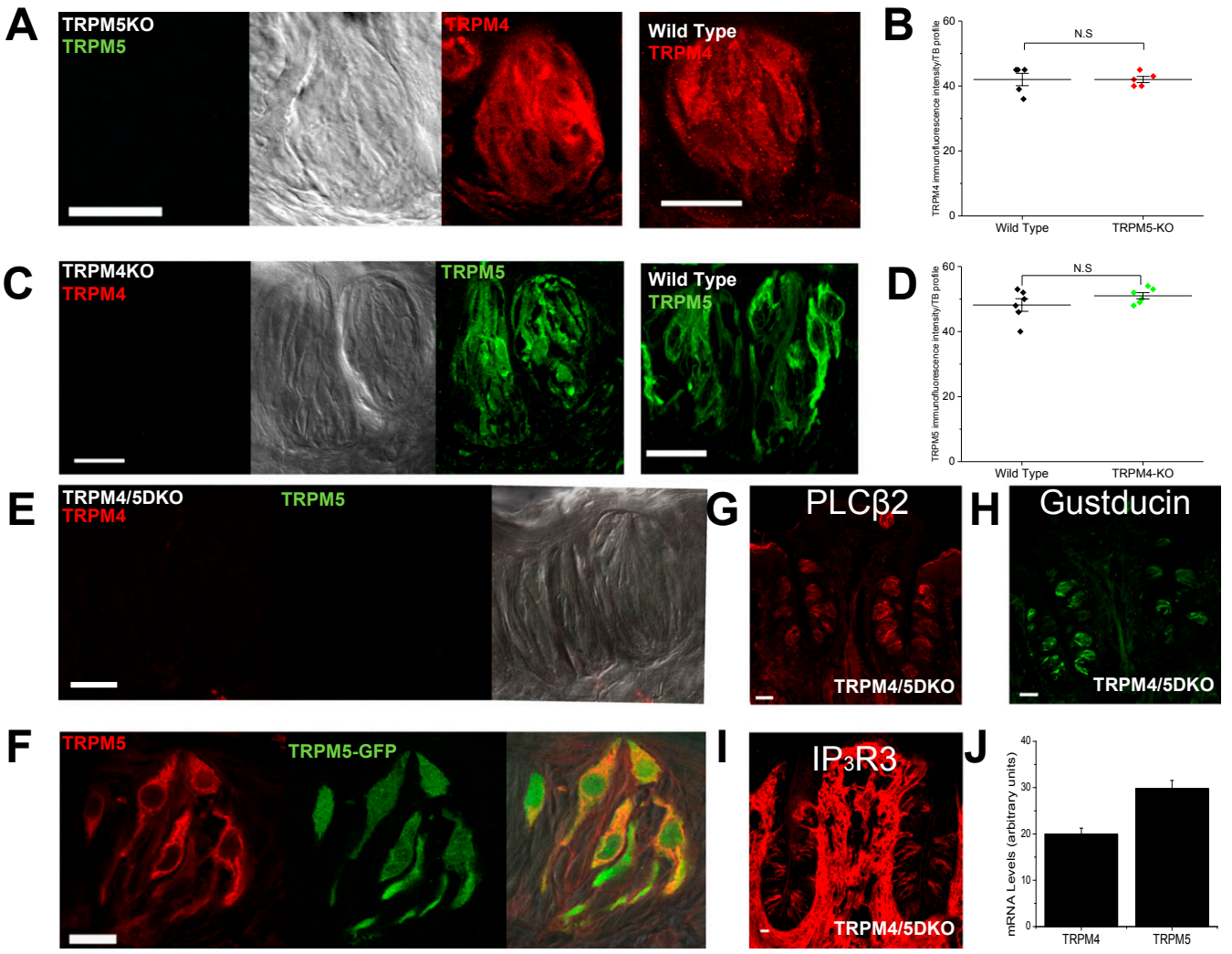

Fig. S2. Validation of transgenic mouse lines. (*A*) TRPM5-KO mice lack TRPM5 expression but retain TRPM4 labeling: TRPM5 (*Left*), overlay with a DIC image (*Center*), and TRPM4 labeling (*Right*). There is no change in TRPM4 labeling compared with wild-type mice. (*B*) Quantification of fluorescence intensity for TRPM4 immunoreactivity in wild-type and TRPM5-KO mice found no significant differences. N.S., not significant. (*C*) TRPM4-KO mice lack TRPM4 expression but retain TRPM5 labeling: TRPM4 (*Left*), overlay with a DIC image (*Center*), and TRPM5 labeling (*Right*). (*D*) Comparison of TRPM5 immunofluorescence intensity between wild-type and TRPM4-KO mice revealed no significant differences ($n = 5$ mice for each). (*E*) TRPM4/5-DKO mice lack both TRPM4 and TRPM5 proteins: TRPM4 (*Right*), TRPM5 (*Center*), and an overlay with a DIC image (*Right*). (*F*) Anti-TRPM5 labeling colocalizes completely with the TRPM5-GFP cells: anti-TRPM5 (*Left*), TRPM5-GFP (*Center*), and an overlay with a DIC image (*Right*). Expression of PLCβ2 (*G*), gustducin (*H*), and IP_3R3 (*I*) in TRPM4/5-DKO mice confirms that the upstream signaling components of the PLCβ2 signaling pathway are intact in the TRPM4/5-DKO mice ($n = 3$ mice for each). (Scale bars: 20 μm.) (*J*) RNA-sequencing analysis identified the presence of mRNA levels for both TRPM4 and TRPM5 in isolated CV and foliate taste receptor cells (data from ref. 30).

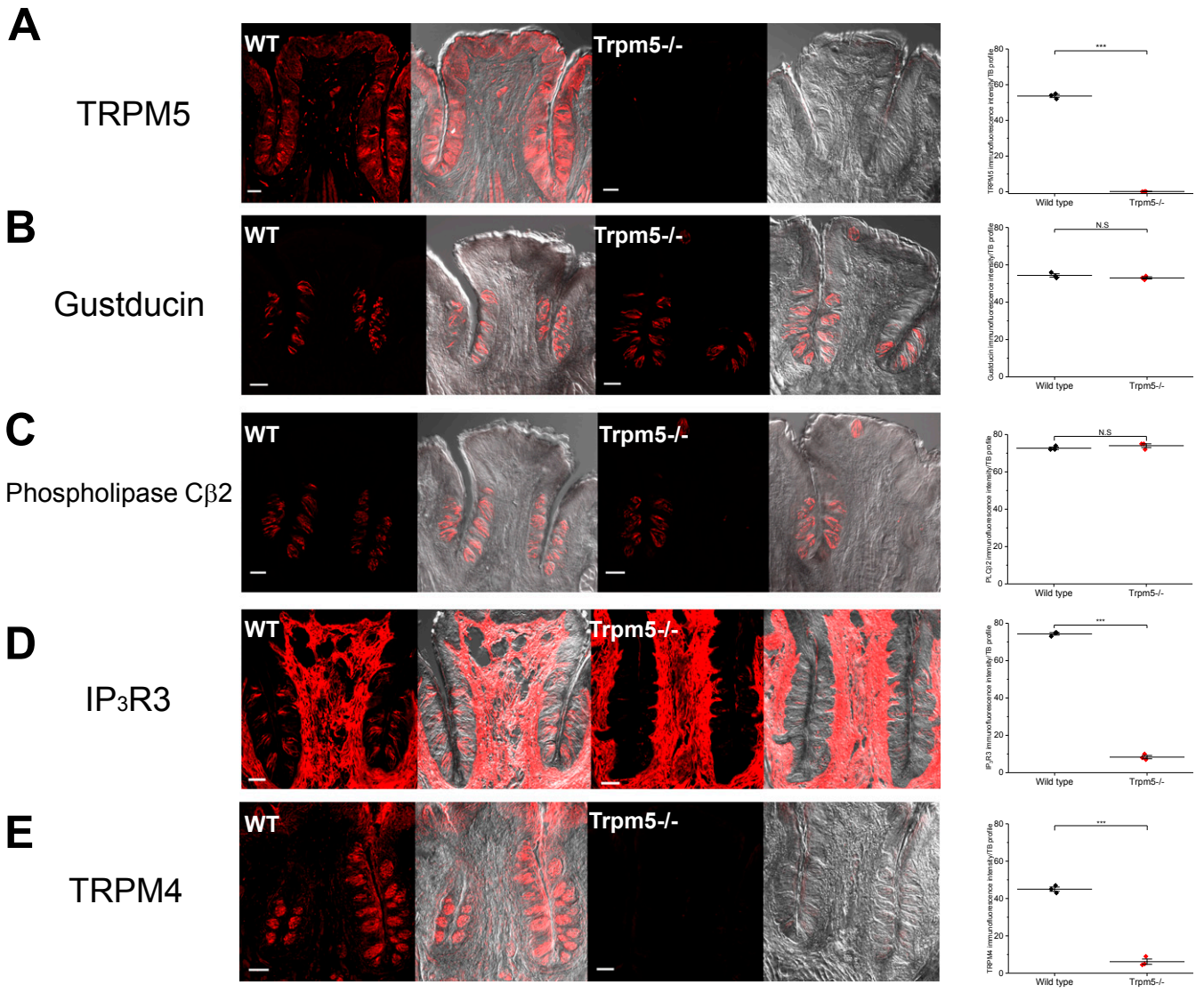

Fig. S3. Evaluation of the Trpm5$^{-/-}$ mouse line used by Zhang et al. (6). Immunohistochemical analyses of the Trpm5$^{-/-}$ mouse line evaluated the expression of signaling components normally expressed in type II taste cells from the CV papillae compared with C57BL/6 wild-type mice (n = 3 mice for all experiments). (*A*) TRPM5 expression in wild-type (WT) mice (*Left*; anti-TRPM5 labeling and overlay with DIC) and Trpm5$^{-/-}$ mice (*Right*; anti-TRPM5 labeling and overlay with DIC). (*Far Right*) ImageJ analysis of fluorescence intensity found no TRPM5 labeling in the Trpm5$^{-/-}$ mouse compared with WT (***$P < 0.001$). (*B*) Gustducin expression in WT mice (*Left*; anti-gustducin labeling and overlay with DIC) and Trpm5$^{-/-}$ mice (*Right*; anti-gustducin labeling and overlay with DIC). (*Far Right*) ImageJ analysis of fluorescence intensity found no difference in gustducin labeling between the WT and Trpm5$^{-/-}$ mice. N.S., not significant. (*C*) PLCβ2 expression in WT mice (*Left*; anti-PLCβ2 labeling and overlay with DIC) and Trpm5$^{-/-}$ mice (*Right*; anti-PLCβ2 labeling and overlay with DIC). (*Far Right*) ImageJ analysis of fluorescence intensity found no difference in PLCβ2 labeling between the WT and Trpm5$^{-/-}$ mice. (*D*) IP$_3$R3 expression in WT mice (*Left*; anti-IP$_3$R3 labeling and overlay with DIC) and Trpm5$^{-/-}$ mice (*Right*; anti-IP$_3$R3 labeling and overlay with DIC). (*Far Right*) ImageJ analysis of fluorescence intensity found that IP$_3$R3 labeling in the Trpm5$^{-/-}$ CV papillae was only 11% of WT labeling (***$P < 0.001$). (*E*) TRPM4 expression in wild-type mice (*Left*; anti-TRPM4 labeling and overlay with DIC) and Trpm5$^{-/-}$ mice (*Right*; anti-TRPM4 labeling and overlay with DIC). (*Far Right*) ImageJ analysis of fluorescence intensity found that TRPM4 labeling in the Trpm5$^{-/-}$ CV papillae was only 13% of WT labeling (***$P < 0.001$). (Scale bars: 20 μm.)

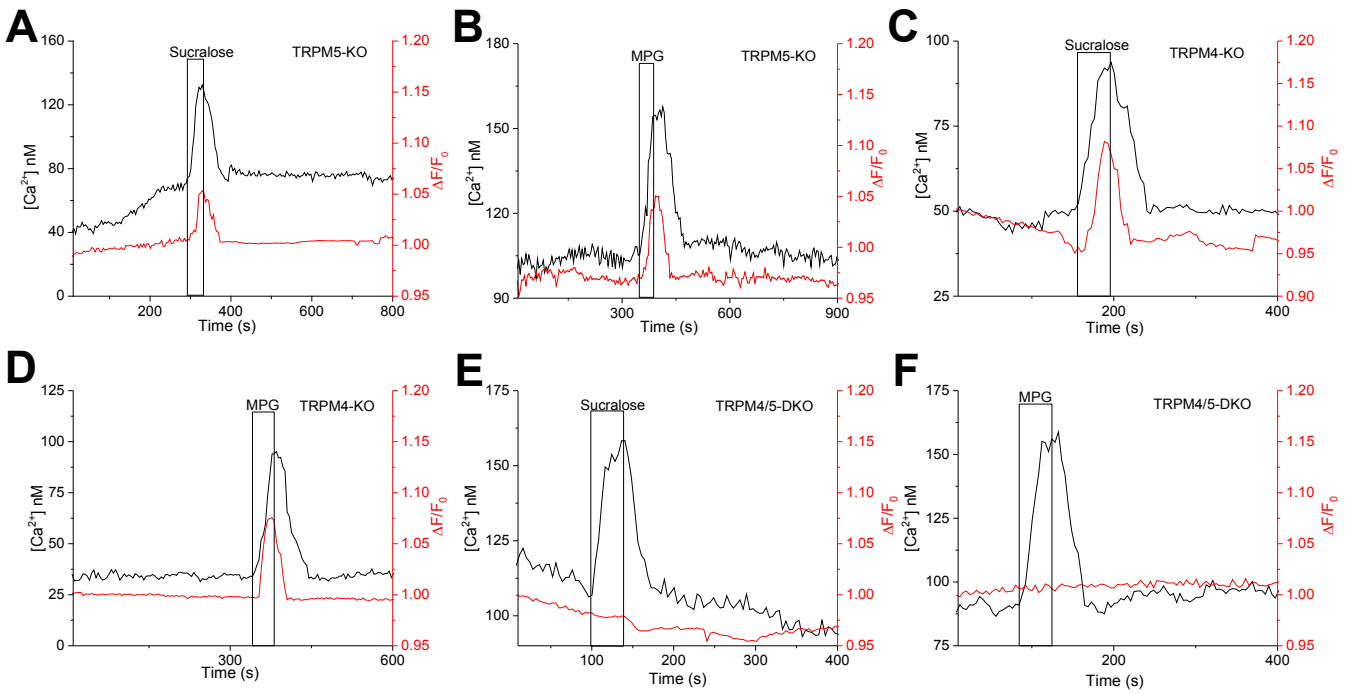

Fig. S4. Taste-evoked Na^+ signals in isolated taste cells depend on the Ca^{2+}-activated cation channels TRPM4 and TRPM5. Representative dual Ca^{2+} (black trace) and Na^+ (red trace) imaging traces from TRPM4-only (TRPM5-KO) mice show cytosolic Ca^{2+} and Na^+ responses to different taste stimuli: sweet (*A*; 20 mM sucralose) and umami (*B*; 20 mM MPG) in TRPM4-only cells. Representative dual Ca^{2+} and Na^+ imaging traces from a TRPM5-only (TRPM4-KO) cell show an evoked response to sweet (*C*; 20 mM sucralose) and umami (*D*; 20 mM MPG). TRPM4/5-DKO cells never responded to any taste stimuli presented with a Na^+ response; however, the upstream Ca^{2+} signaling pathway is still functional for sweet (*E*; 20 mM sucralose) and umami (*F*; 20 mM MPG).

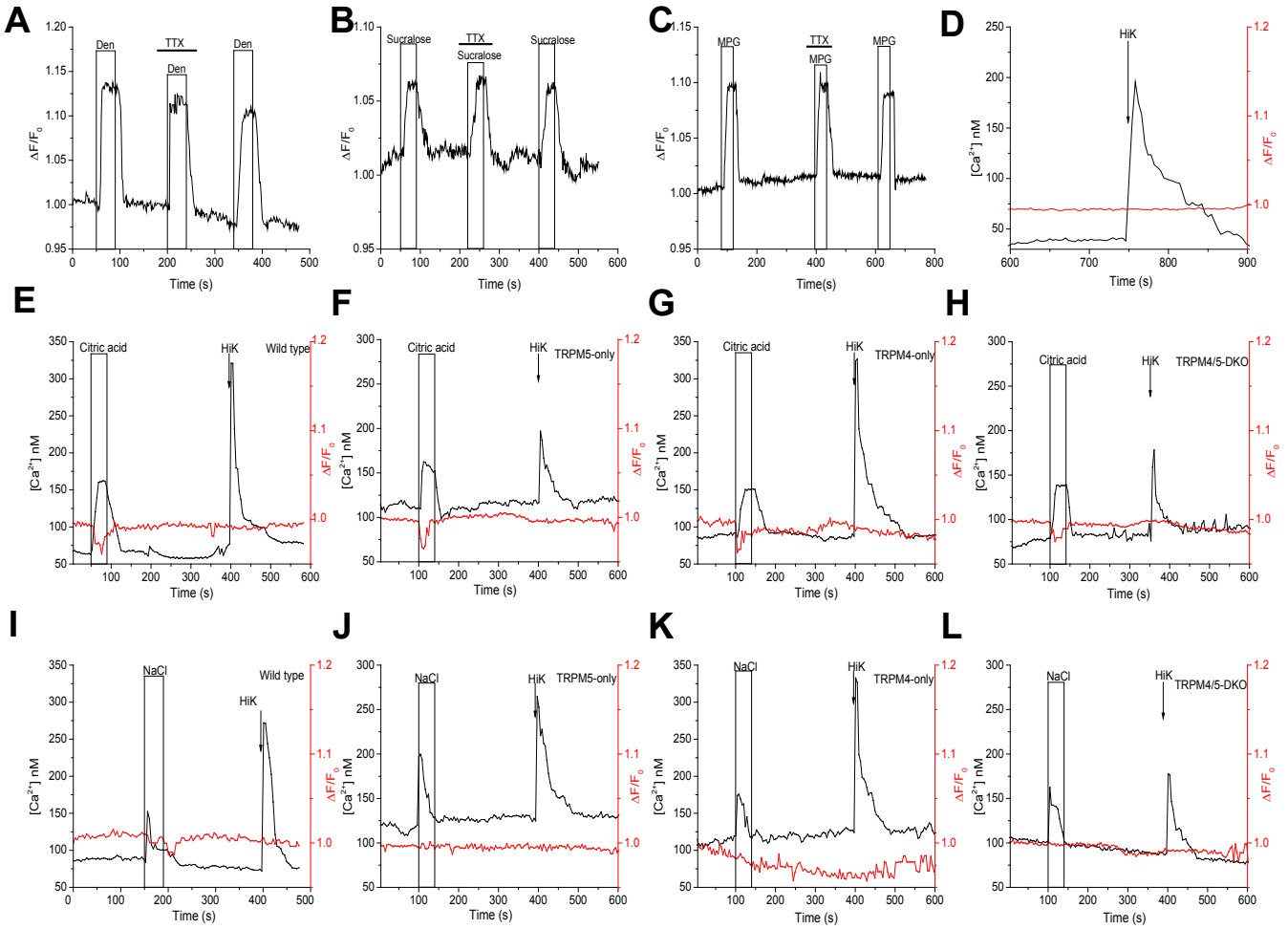

Fig. S5. Voltage-gated Na^+ channels and Ca^{2+} influx are not influencing the taste-evoked Na^+ responses in taste cells, while salty and sour responses are not affected by loss of TRPM4 or TRPM5. Application of the specific voltage-gated Na^+ channel blocker TTX did not block taste-evoked Na^+ responses to Den (*A*; bitter), sucralose (*B*; sweet), or MPG (*C*; umami). (*D*) High K^+ (HiK)-induced Ca^{2+} influx through VGCCs did not affect the intracellular Na^+ levels in taste cells. The sour stimulus [50 mM citric acid (pH 4)] did not generate a Na^+ signal but did evoke a Ca^{2+} signal in wild-type taste cells (*E*) that was not affected by loss of TRPM4 (*F*; TRPM5-only), TRPM5 (*G*; TRPM4-only), or both TRPM4/TRPM5 (*H*). The salt stimulus (250 mM NaCl) did not generate a Na^+ signal but caused a Ca^{2+} signal in wild-type taste cells (*I*) that was not affected by loss of TRPM4 (*J*; TRPM5-only), TRPM5 (*K*; TRPM4-only), or both TRPM4/TRPM5 (*L*). The acidification of the taste cells caused a transient bleaching of the sodium dye that recovered upon washout. Data shown are representative of at least $n = 3$ cells.

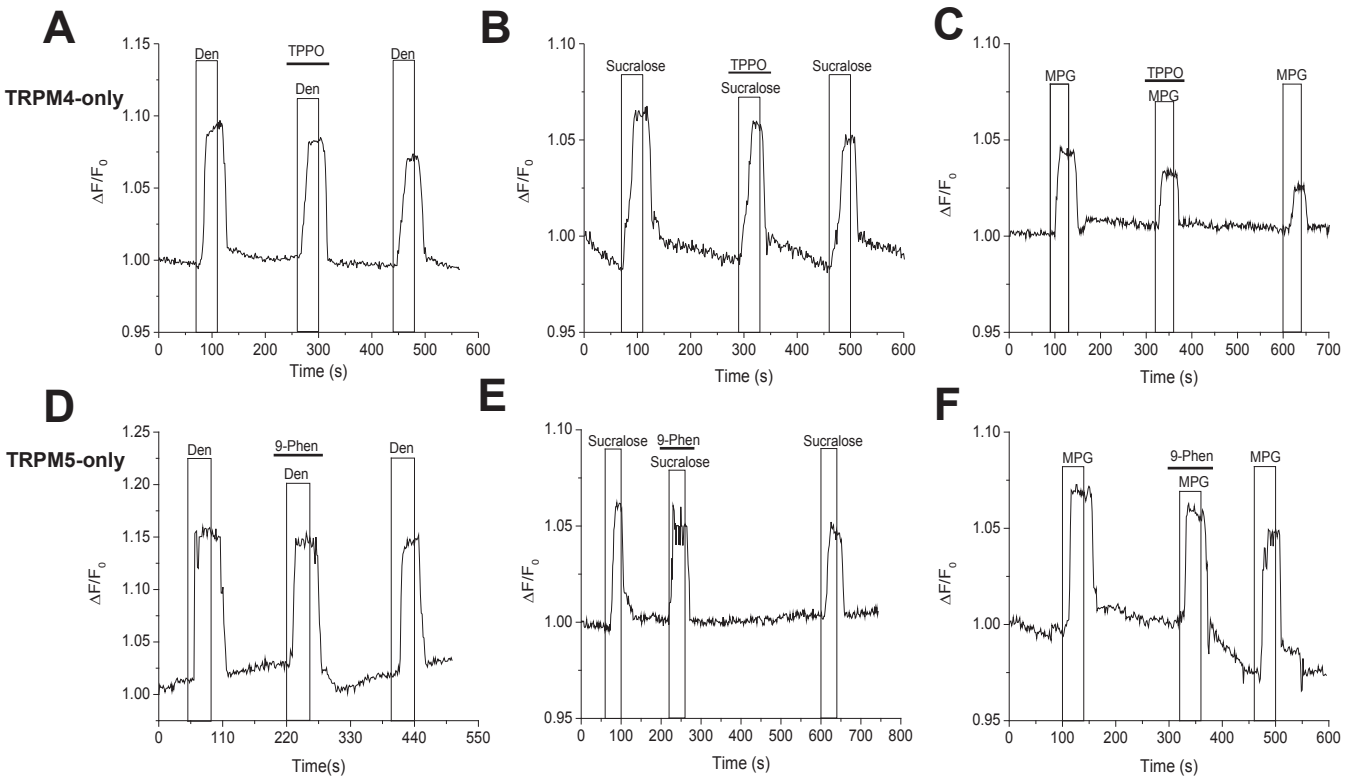

Fig. S6. TRPM4 blocker and TRPM5 blocker are specific. Application of the specific TRPM5 blocker TPPO on TRPM4-only (TRPM5-KO) cells did not affect taste-evoked Na^+ responses to Den (*A*), sucralose (*B*), or MPG (*C*). Application of the specific TRPM4 blocker 9-phenanthrol (9-Phen) on TRPM5-only (TRPM4-KO) cells did not affect taste-evoked Na^+ responses to Den (*D*), sucralose (*E*), or MPG (*F*).

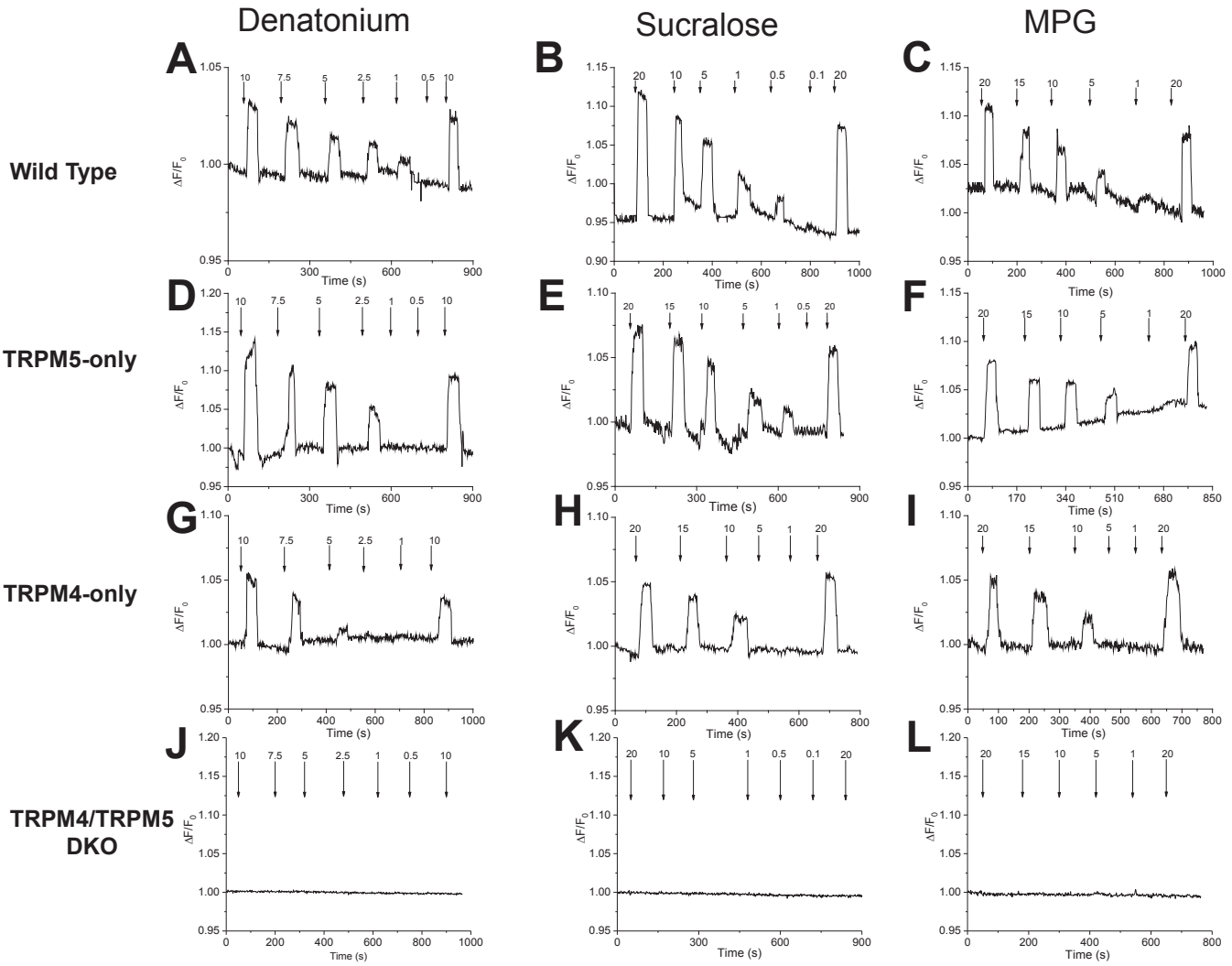

Fig. S7. Amplitudes of taste-evoked Na^+ responses are concentration-dependent, and TRPM4 and TRPM5 have different concentration sensitivities. Representative Na^+ imaging traces show the amplitudes of taste-evoked Na^+ responses to the bitter stimulus (Den) at different concentrations for wild-type (*A*), TRPM5-only (*D*), TRPM4-only (*G*), and TRPM4/TRPM5-DKO (*J*) cells. Representative Na^+ imaging traces show the amplitudes of taste-evoked Na^+ responses to the sweet stimulus (sucralose) at different concentrations for wild-type (*B*), TRPM5-only (*E*), TRPM4-only (*H*), and TRPM4/TRPM5-DKO (*K*) cells. Representative Na^+ imaging traces showing the amplitudes of taste-evoked Na^+ responses to the umami stimulus (MPG) at different concentrations for wild-type (*C*), TRPM5-only (*F*), TRPM4-only (*I*), and TRPM4/TRPM5-DKO (*L*) cells.

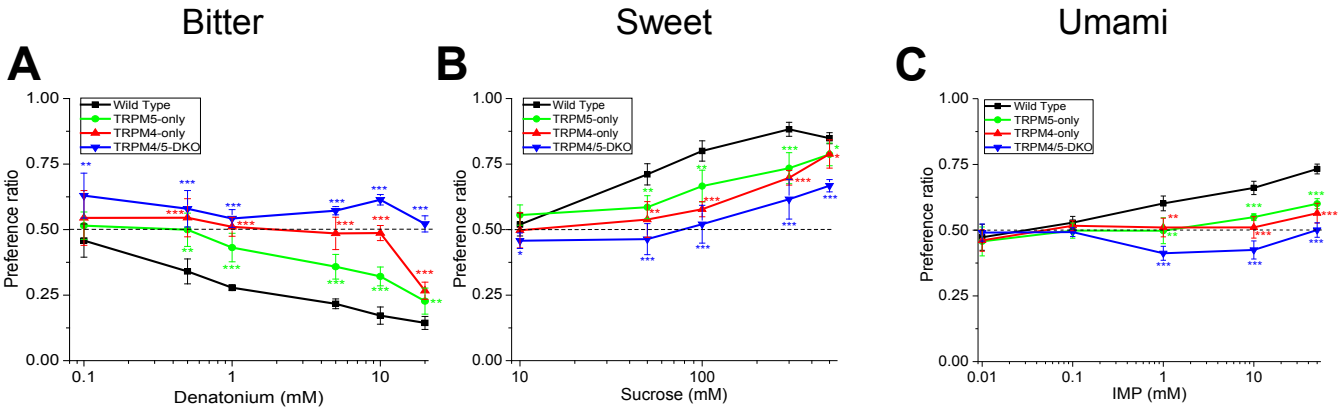

Fig. S8. Preference ratios for bitter, sweet, and umami taste stimuli are affected in TRPM5-only, TRPM4-only, and TRPM4/5-DKO mice. Mean preference ratios (±SD) from two-bottle preference tests compare the responses of TRPM5-only (green line), TRPM4-only (red line), and TRPM4/5-DKO (blue line) mice with those of wild-type mice (C57BL/6, black line). Preferences for the bitter stimulus Den (*A*; 0.1, 0.5, 1, 5, 10, and 20 mM), sweet stimulus sucrose (*B*; 10, 50, 100, 300, and 500 mM), and umami stimulus inosine monophosphate (IMP) (*C*; 0.01, 0.1, 1, 10, and 50 mM) were significantly altered in TRPM5-only, TRPM4-only, and TRPM4/5-DKO mice compared with the wild-type mice. Five mice of each genotype were used for all experiments (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).