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Supplemental Information

RNF43/ZNRF3 negatively regulates taste tissue homeostasis and positively regulates dorsal lingual epithelial tissue homeostasis

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Supplemental Figures

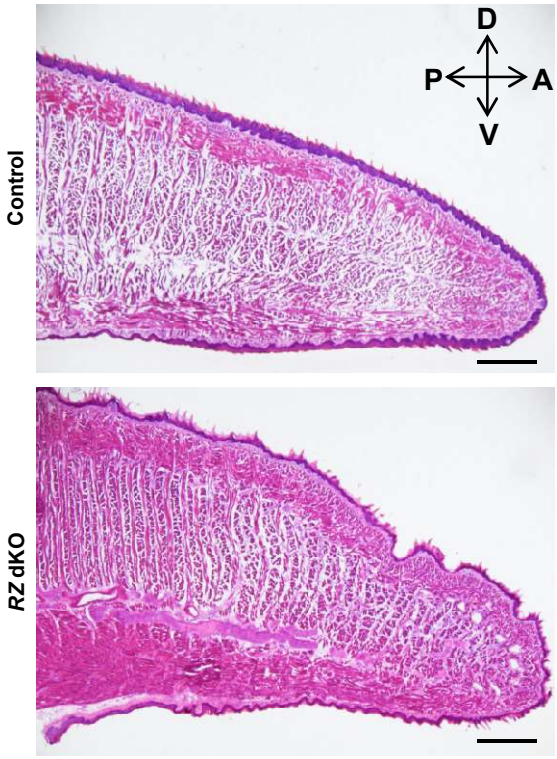

Figure S1 (related to Figure 2). Representative images of hematoxylin and eosin staining of anterior tongue sections from control and *RZ* dKO mice (large view).
Note the thinner dorsal lingual epithelium (i.e., filiform papillae) in *RZ* dKO mice. n=3 for each group. Scale bars, 500 μm.

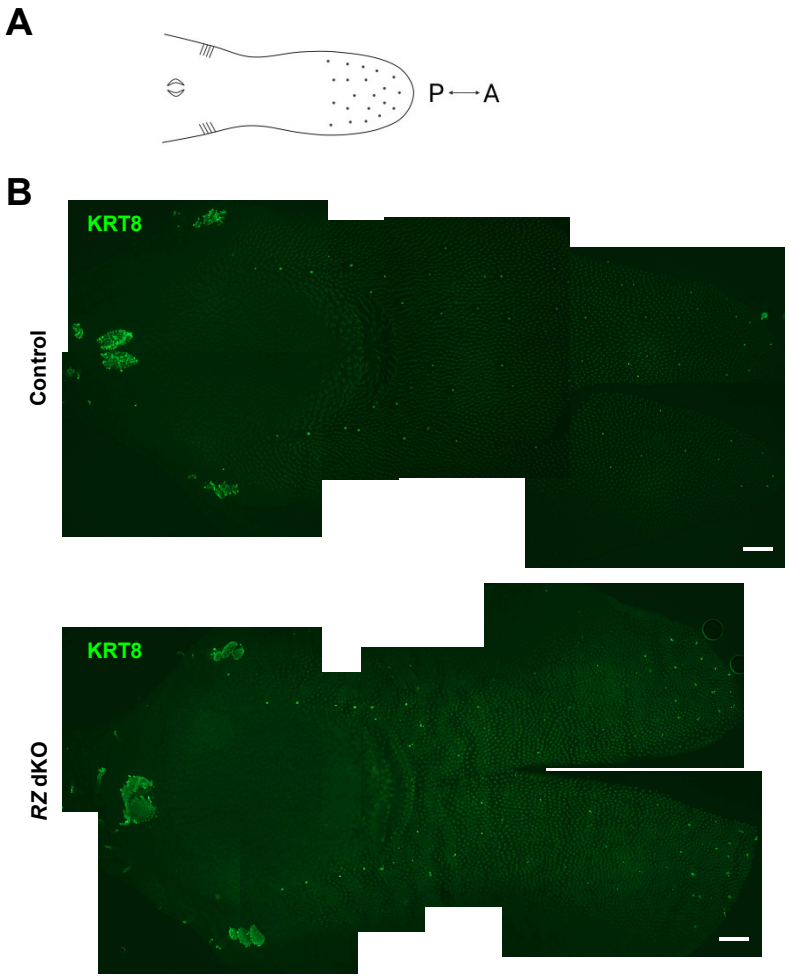

Figure S2 (related to Figure 3B). Representative images of KRT8 immunostaining of whole-mount tongue epithelium.

(**A**) Schematic of the tongue epithelium (P, posterior; A, anterior).

(**B**) Images stitched manually to show the whole field of tongue surface (left to right corresponds to posterior to anterior tongue). Note the presence of the circumvallate papilla (middle) and foliate papillae (lateral) in the posterior tongue. n=3 for each group. Scale bars, 500 μm.

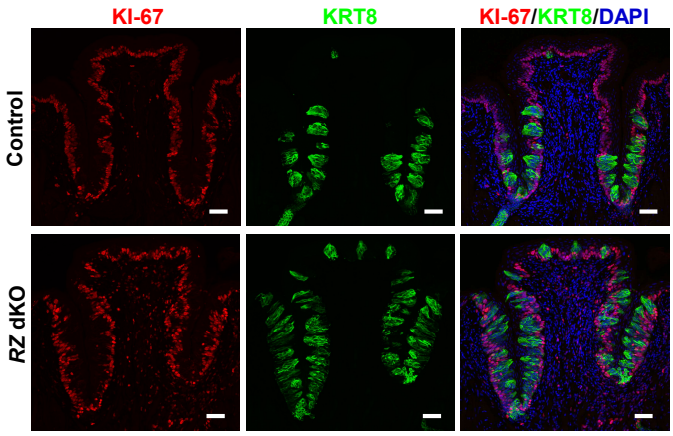

Figure S3. KI-67 staining in the circumvallate papilla in control and *RZ* dKO mice.
Representative images of KI-67 and KRT8 immunostaining of circumvallate papilla sections. No apparent changes are apparent in the distribution pattern of KI-67^{+} proliferating basal cells in *RZ* dKO mice compared to control mice. Cell nuclei were counterstained with DAPI (blue). n=3 for each group. Scale bars, 50 μm.

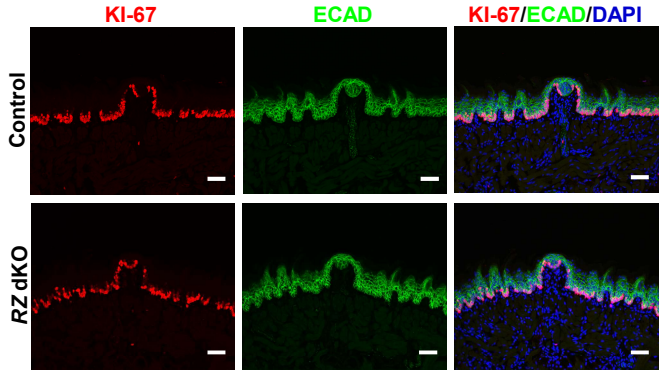

Figure S4. KI-67 staining of the anterior tongue epithelium section (including fungiform papilla and filliform papillae) in control (n=2) and *RZ* dKO mice (n=4) at day 2 after tamoxifen induction.
Representative images of KI-67 and ECAD immunostaining of anterior dorsal epithelium sections. No apparent changes are detected in the distribution pattern of KI-67^{+} proliferating basal cells in *RZ* dKO mice compared with control mice. Cell nuclei were counterstained with DAPI (blue). Scale bars, 50 μm.

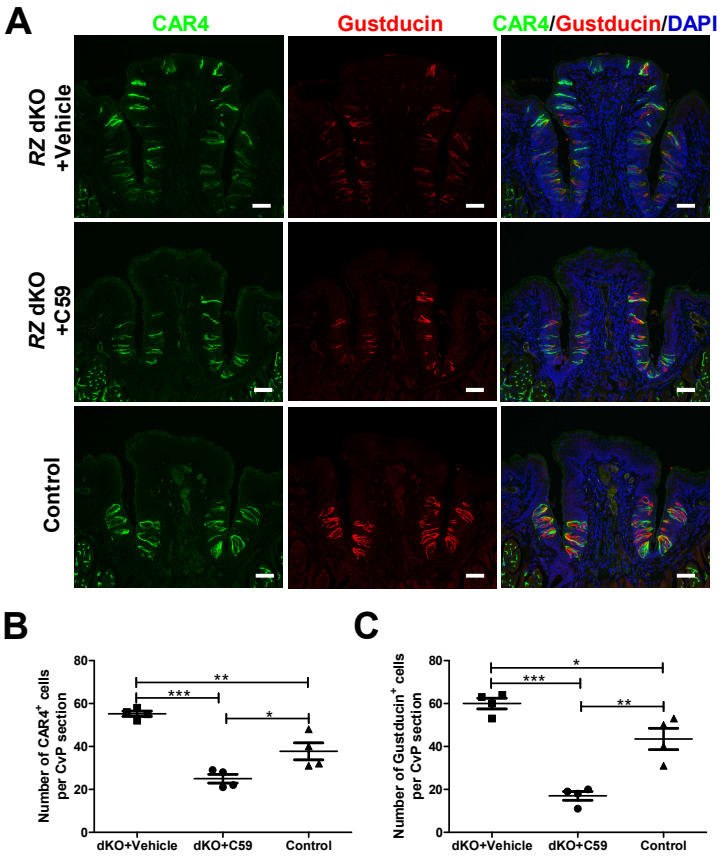

Figure S5 (related to Figure 7). WNT signaling blockade prevents the increase of CAR4⁺ type III and α-Gustducin⁺ type II taste cells in the circumvallate papilla in *RZ* dKO mice.
(**A**) **Immunofluorescence** staining for CAR4 (green) and α-Gustducin (red) of circumvallate papilla sections from *RZ* dKO mice receiving vehicle or C59 or from control mice. Taste bud cells are frequently present in the upper cleft and dorsum of the circumvallate papilla in *RZ* dKO mice receiving vehicle but not in *RZ* dKO mice receiving C59 or in control mice. Cell nuclei were counterstained with DAPI (blue). Scale bars, 50 µm.
(**B, C**) Numbers of CAR4⁺ taste cells (**B**) and α-Gustducin⁺ taste cells (**C**) in circumvallate papilla (CvP) in *RZ* dKO mice receiving vehicle or C59 or in control mice. Data are presented as mean ± SEM. * p<0.05. ** p<0.01. *** p<0.001. n=4 for each group. Each point represents a single mouse.

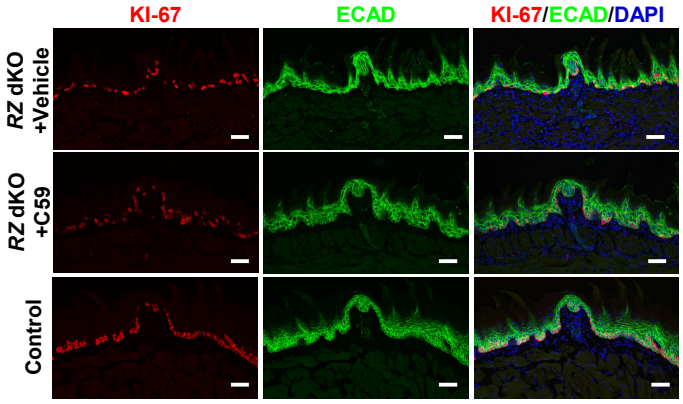

Figure S6 (related to Figure 7). Blocking WNT signaling partly rescues decreased proliferation in lingual epithelium in *RZ* dKO mice.
Immunofluorescence staining of KI-67 (red) and E-cadherin (ECAD, green) of anterior tongue sections from *RZ* dKO mice receiving vehicle or C59 or from control mice. Cell nuclei were counterstained with DAPI (blue). n=4 for each group. Scale bars, 50 μm.

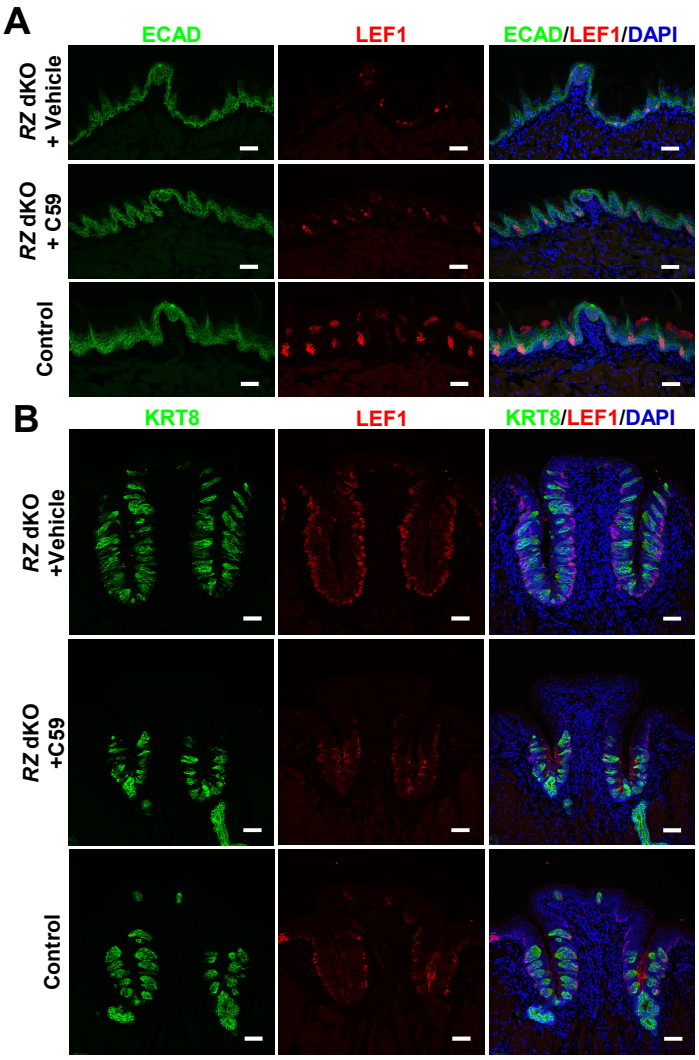

Figure S7 (related to Figure 7). LEF1 expression in lingual epithelium and circumvallate papilla.

Immunofluorescence staining of E-cadherin (ECAD, green) and LEF1 (red) of anterior tongue sections (**A**) and KRT8 (green) and LEF1 (red) of circumvallate papilla sections (**B**) from *RZ* dKO mice receiving vehicle or C59 or from control mice. Cell nuclei were counterstained with DAPI (blue). n=4 for each group. Scale bars, 50 μm.

Supplemental Experimental Procedures

Glossopharyngeal nerve transection

An incision was made along the ventral neck midline; the digastric muscle was retracted to expose the glossopharyngeal nerve, identified as coursing anterior and lateral to the internal carotid artery; and bilateral transection was performed.

In situ hybridization

Tongues were harvested from C57BL/6 mice and embedded in the O.C.T. compound (Sakura, no. 4583). Fresh-frozen sections 8 μm thick were prepared using a Leica CM1900 cryostat (Leica Biosystems). In brief, digoxigenin-labeled antisense RNAs were used as probes after fragmentation of linearized complete coding sequence of *Rnf43* or *Znrf3* under alkaline conditions. Fresh-frozen sections were fixed with 4% paraformaldehyde, treated with diethylpyrocarbonate, prehybridized with salmon sperm DNA for 2 h at 58°C, and hybridized with fragmented antisense riboprobes (~150 bases) overnight at 58°C after alkaline fragmentation. After hybridization, the sections were washed in 0.2× SSC at 58°C and blocked with 0.5% blocking reagent (Roche Diagnostics) in Tris-buffered saline. The sections were then incubated with alkaline phosphatase–conjugated anti-digoxigenin primary antibody (1:500, Roche Diagnostics) for 1 h, followed by overnight incubation with chromogenic substrates 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. Images were acquired with a Nikon Microphot microscope.

Tongue epithelium peeling

Tongues from $Rnf43^{fl/fl};Znrf3^{fl/fl};Krt5^{CreERT2/+}$ and $Rnf43^{fl/fl};Znrf3^{fl/fl}$ mice were injected with ~0.5 mL of an enzyme mixture containing dispase II (4 mg/mL; Roche, no. 0494207800) and collagenase A (2 mg/mL; Roche, no. 10103578001) 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.

Immunostaining and imaging

Slides or peeled epithelium were washed three times in PBS and blocked in SuperBlock™ Blocking Buffer (Thermo Scientific, no. 37515) containing 2% donkey serum and 0.3% Triton X-100 for 1 h at room temperature. Primary antibodies were incubated overnight at 4˚C, and secondary antibodies were applied for 1.5 h at room temperature. Primary antibodies were rat anti-KRT8 (Developmental Studies Hybridoma Bank, no. TROMA-I; 1:10), rabbit anti-P2X3 (Alomone Labs, no. APR-016; 1:1000), goat anti-CAR4 (R&D, no. AF2414; 1:100), rabbit anti-α-Gustducin (Santa Cruz, no. sc-395; 1:100), goat anti-E-cadherin (R&D Systems, no. AF648; 1:500), rabbit anti-LEF1 (Cell Signaling Technology, no. 2230S; 1:200), and rabbit anti-KI-67 antibody (Novus, no. NB600-1252; 1:100). For immunostaining with anti-KI-67 antibody, the sections were treated in a preheated target retrieval solution (pH 9) (Dako, no. S2367) at 80˚C for 20 min for antigen retrieval before blocking. Secondary antibodies (1:500) included donkey anti-rat Alexa Fluor 488 (Molecular Probes, no. A-21208), donkey anti-rabbit Alexa Fluor 555 (Abcam, no. ab150074), and donkey anti-goat Alexa Fluor 488 (Abcam, no. ab150129). Images were captured by a Leica TCS SP8 confocal microscope at the Cell and Developmental Biology Core at the University of Pennsylvania or by a Nikon ECLIPSE 80i and Olympus SZ61 microscope at Monell Chemical Senses Center. Confocal images were compressed z-stacks of the entire section (~10 μm).

Cell counting

The numbers of taste buds and type II/III taste cells were counted manually, including both lateral trench walls and the dorsum of the circumvallate papilla. Typically, a total of 30-40 sections that spanned the entire circumvallate papilla were collected on 10 glass slides, as follows: slide 1 contained sections 1, 11, 21 (and 31 if available); slide 2 contained sections 2, 12, 22, (32), and so forth. This way, each slide provides an accurate sampling of an entire circumvallate papilla. For each slide, the section in the middle of the circumvallate papilla with slightly more taste buds or taste cells was used for cell counting, measurement of the depth of the trench, and statistical analysis, to alleviate potential sampling bias

associated with sections at the most anterior or posterior portions of the circumvallate papilla, for every mouse, regardless of genotype. KI-67^{+} cells in the dorsal lingual epithelium were counted using serial sagittal sections of anterior tongues. Sections that did not have fungiform papillae were used for counting (one section counted for each mouse).

RNA isolation and quantitative PCR

cDNA was synthesized using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen, no. 11766500), and qPCR was performed using Fast SYBR™ Green Master Mix Kit (Applied Biosystems, no. 4385612). *Gapdh* was used as control to normalize the expression levels of analyzed gene transcripts. The relative gene expression was calculated as $2^{-(CTTarget-CTGapdh)}$. The primers used were intron spanning, with the following sequences: 5'-accatagcagaccggatcctc-3' (*Rnf43* forward), 5'-ctcgtggaggcacgaaatga-3' (*Rnf43* reverse), 5'-acattgacggagaggagctt-3' (*Znrf3* forward), 5'-cacacggcctgggtaatgta-3' (*Znrf3* reverse), 5'-TGGCCTTCCGTGTTCCTAC-3' (*Gapdh* forward), 5'-GAGTTGCTGTTGAAGTCGCA-3' (*Gapdh* reverse).