**SUPPLEMENTARY INFORMATION**

**MATERIALS AND METHODS**

**Airway epithelia and cells**

Airway epithelial cells were obtained from trachea and bronchi of lungs removed for donation and prepared by the University of Iowa In Vitro Models and Cell Culture Core. Samples were collected with approval of the University of Iowa Institutional Review Board. Cells were grown on collagen-coated filters at the air-liquid interface using methods we previously described (Ref. *S1*). These epithelia differentiate within ~14 days of seeding and develop ciliated cells, goblet cells, basal cells, and non-ciliated columnar cells. For $[Ca^{2+}]_i$ imaging and ciliary beat experiments, cells were grown on transparent transwells. For some measurements of $[Ca^{2+}]_i$, cells were seeded on glass bottom dishes (MatTek Corporation, Ashland, MA) coated with human placental collagen and studied within 4 days.

**RT-PCR**

RNA was isolated from cultured, differentiated human airway epithelia following the protocol described in the RNeasy Plus Miniprep kit (Qiagen Inc, Valencia, CA) and converted to cDNA using the RT2 first strand kit (SABiosciences, Frederick, MD). RT-PCR was performed using PCR Supermix (Invitrogen, Carlsbad, CA). The primers used were: T2R4 (f-CAGACACTGCTCGAGTCCAA, r-GGGCATCACCAGGTTTCTTA); T2R16 (f-

CTTCACTGCCCTGAGGTCC, r-CCAACATCTCTTATCAAATAGAGTACC); T2R38 (f-GCAAGCTGGGTCTCCAGG, r-GAGCCTTGTATTGTTATTCATGAATAGC); T2R43 (f-GTAGTGGTTACATTTGTTATTGGAAATTT, r-CTCTGGAGACCGCCAGAGC); T2R46 (f-AAAATAGCAAAGGCCCCACT, r-ACCATTTCAGCAACTGGCTT); α-gustducin (f-CTCAGCAGCTTACTACCTTAATGATTTAG, r-CATCAAACATCCTGAAGTGCAA), PLC-β2 (f-GAAAAGTTCCCACTAAAACCAGG, r-CAGCCCACACTGTGCCC) and β-actin (f-CCTGGCACCCAGCACAAT, r-GCCGATCCACACGGAGTACT).

**Immunofluorescence analysis**

Human airway epithelia grown on permeable filter supports were fixed in 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA) for 15 min, followed by permeabilization with 0.1% Triton X-100 (Pierce, Rockford, IL) for 15 min. Following 3 washes with PBS, cells were treated with Superblock (Thermo Scientific, Rockford, IL) for 1 hr at room temperature. Primary antibody in Superblock was diluted and applied overnight at 4 °C. Then, after 3 washes with PBS, anti-acetylated α-tubulin primary antibody was applied for 1 hr at 37 °C. Cells were washed 4-5 times in PBS and the appropriate secondary antibody was applied (Alexa fluorophores, Invitrogen). Filters were cut from their supports and mounted in Vectashield (Vector Labs, Burlingame, CA) containing DAPI to label nuclei.

Samples were scanned for stacked XY-series with Z-steps of 0.5 μm, or for XZ-series with Z-steps of 0.35 μm. Image data sets were acquired and visualized on an Olympus FluoView1000 Confocal Microscope (Center Valley, PA.), and analysis was with Olympus FluoView software or NIH ImageJ software. The primary antibodies used are listed in Fig. S1. Confocal images were analyzed with Slidebook software (Leedsmicro, Minneapolis, MI) to obtain 3-D isosurface renderings. Isosurface rendering draws a 3-D surface within a volumetric data field, corresponding to points with a specific intensity value. For image analysis, the red channel opacity was set to 50% and all other channels were set to 100% opacity. The aspect ratio for the 3-D Surface rendered images was XY:Z of 1.0:0.8. Isosurface rendering emphasizes dominant staining, and hence distinct localization patterns.

In some experiments (Fig. S4-S6), we tested specificity of the antibodies using the immunogenic blocking peptides. Peptides were from T2R4 (Thermo Scientific, Waltham, MA), T2R43 (Sc-34850-p, Santa Cruz Biotechnology, Santa Cruz, CA), and T2R46 (Sc-34732-p, Santa Cruz Biotechnology). For each antibody, 2 μl primary antibody was added to 4 μl blocking peptide (10 mg/ml) and 34 μl Superblock. After incubating with gentle shaking at 37 °C for 1 hr, the antibody/peptide mix was used in place of the primary antibody in the immunostaining protocol outlined above.

**Calcium imaging**

Human airway epithelia were loaded with 1 μM Fura2-AM dye plus Pluoronic (Invitrogen) in saline containing 300 μM $CaCl_2$. Cells were loaded apically and basolaterally at 37 °C for 30 min followed by 2 washes. After 15 min at 37 °C in saline, $[Ca^{2+}]_i$ changes in epithelia were assessed using an inverted microscope (Nikon Eclipse TE200, Nikon Instruments Inc., Melville, NY), and the data were analyzed using NIS-Elements software (Nikon). Denatonium, thujone, salicin, quinine, and nicotine were applied apically at the indicated concentrations; PBS was applied as a control. To identify ciliated and nonciliated cells, we observed ciliary motion using phase contrast microscopy. To determine the latency of activation, we measured the time after ligand addition at which cells exhibited an increase in the 340nm/380nm emission ratio. Linear equations were fit to the data before and after ligand addition, and the X-intercept (time at which increase began) was determined to assess the latency between ligand addition and the 340nm/380nm emission increase. Analysis was done blinded to cell type.

For some studies (Fig. S8), we used cells grown on glass. Cells were loaded with Oregon Green 488 BAPTA-1, AM (Invitrogen) for 30 min, followed by a 15-min wash. Changes in $[Ca^{2+}]_i$ were assayed using relative fluorescence. Imaging was performed using a Zeiss LSM 510 META NLO confocal microscope (Carl Zeiss Micro Imaging Inc., Thornwood, NY). Ciliated and nonciliated cells were

identified by observing ciliary motion. Analysis was done blinded to cell type.

**Ciliary beat frequency**

Human airway epithelia were transferred to a 37 °C temperature-controlled stage and imaged on a Zeiss LSM 510 META NLO. Ciliated cells were randomly located by eye and imaged using transmitted light. These images served as a guide for selecting regions for subsequent line scans which were collected at 2 ms intervals (2000 lines collected over 4 sec). The data were then analyzed and the beat frequency quantified using NIH ImageJ software (Ref. *S2*).

**Supplementary References**

S1. P. H. Karp *et al.*, in *Epithelial Cell Culture Protocols,* C. Wise, Ed. (Humana Press, Inc., Totowa, NJ, 2002), vol. 188, pp. 115-137.

S2. A. S. Shah *et al.*, *Proc. Natl. Acad. Sci. U. S .A.* **105**, 3380 (2008).

**Supplemental Figure Legends**

**Figure S1**. Antibodies used for immunostaining assays listed by protein, vendor, product number, and type. We also indicate if the antibody immunostained human airway epithelia, the localization of the immunostaining, and the corresponding figure number.

**Figure S2**. Immunostaining of human airway epithelia. All epithelia were labeled with DAPI to identify nuclei. (**A**) Epithelia immunostained with anti-T2R38

antibody (green). (**B**) Non-immune rabbit serum was applied instead of the primary antibody. (**C**) Non-immune goat serum was applied instead of the primary antibody. (**D**) No primary antibodies were applied and both donkey anti-rabbit and donkey anti-goat secondary antibodies were applied. These controls indicate no non-specific binding of the sera or secondary antibodies.

**Figure S3**. Immunolocalization of T2Rs, PLC-β2, and TRPM5 in human airway epithelia. Anti-acetylated α-tubulin is in red and DAPI is blue. For identification of antibodies, see Fig. S1. Immunostaining with multiple antibodies to the same protein in this Figure and in Fig. 2 and 3 in the manuscript gave similar patterns of staining.

**Figure S4**. T2R4 blocking peptide prevented immunostaining with an anti-T2R4 antibody. Human airway epithelia were immunostained with monoclonal anti-acetylated α-tubulin antibody and rabbit polyclonal anti-T2R4 antibody. Epithelia were pretreated with T2R4, T2R43, or T2R46 blocking peptides. The T2R4 peptide, but not the T2R43 or T2R46 peptides prevented immunostaining with the anti-T2R4 antibody, indicating specificity.

**Figure S5**. T2R43 blocking peptide prevented immunostaining with an anti-T2R43 antibody. Human airway epithelia were immunostained with monoclonal anti-acetylated α-tubulin antibody and goat polyclonal anti-T2R43 antibody.

Epithelia were pretreated with T2R4, T2R43, or T2R46 blocking peptides. The T2R43 peptide, but not the T2R4 or T2R46 peptides prevented immunostaining with the anti-T2R43 antibody, indicating specificity.

**Figure S6**. T2R46 blocking peptide prevented immunostaining with an anti-T2R46 antibody. Human airway epithelia were immunostained with monoclonal anti-acetylated α-tubulin antibody and goat polyclonal anti-T2R46 antibody. Epithelia were pretreated with T2R4, T2R43, or T2R46 blocking peptides. The T2R46 peptide, but not the T2R4 or T2R43 peptides prevented immunostaining with the anti-T2R46 antibody, indicating specificity.

**Figure S7**. T2Rs appear to differentially localize along the cilium. Images are 3-D isosurface renderings (see supplemental methods) of images from Fig. 2. In all panels the nuclei are stained with DAPI (blue). (**A**) Anti-T2R4 is in green and anti-acetylated α-tubulin is in red. T2R4 appears to localize to the distal portion of the cilium. (**B**) Anti-T2R43 is in green and anti-acetylated α-tubulin is in red. T2R43 appears to localize to the proximal portion of the cilium. (**C**) Anti-T2R4 is in red and anti-T2R43 is in green. T2R4 appears to localize to the distal portion and T2R43 to the proximal portion of cilia. (**D**) Anti-T2R38 is in red and anti-T2R46 is in green. T2R38 appears to localize to the distal portion and T2R46 to the proximal portion of cilia. In all panels, the background grids are at 0.5 μm.

**Figure S8.** Denatonium increased $[Ca^{2+}]_i$ and ciliary beat frequency in individual ciliated epithelial cells. Human airway epithelial cells were grown on a collagen-coated glass-bottom dish for 4 days. Ciliated and nonciliated cells were identified by observing the motion of the cilia. (**A**) Changes in Oregon Green 488 fluorescence in response to 1 mM denatonium. Data for ciliated cells are in circles and non-ciliated cells are in squares. (**B**) Ciliary beat frequency was measured in individual ciliated cells before and after the measuring $[Ca^{2+}]_i$-dependent changes in fluorescence. Asterisk indicates $P<0.01$.

**Figure S9**. Denatonium increases cilia beat frequency in human airway epithelia. (**A**) Images are 1 sec of line scans of ciliary motion. Intervals between arrows indicate one beat. Addition of denatonium increased cilia beat frequency. (**B**) Scanning electron micrograph of apical surface of cultured human airway epithelia showing cilia. (**C**) Transmission electron micrograph showing motile cilia.

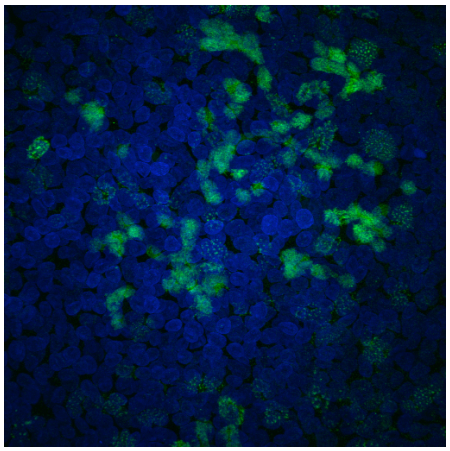
| Protein | Vendor | Product Number | Type | Immuno-staining | Localization | Figure |
|---|---|---|---|---|---|---|
| T2R4 | Abcam | Ab65489 | Rabbit polyclonal | Y | Distal Cilium | 2A |
| T2R4 | Novus Biologicals | NB110-74890 | Rabbit Polyclonal | Y | Distal Cilium | S3B |
| T2R4 | Thermo Scientific | OSR00153W | Rabbit Polyclonal | Y | Distal Cilium | S3A, S4 |
| T2R38 | Abcam | Ab65509 | Rabbit polyclonal | Y | Distal Cilium | S3E, S2A |
| T2R38 | Santa Cruz Biotechnology | Sc-67108 | Rabbit Polyclonal | Y | Distal Cilium | 2C |
| T2R43 | Thermo Scientific | OSR00171W | Rabbit Polyclonal | N | - | - |
| T2R43 | Thermo Scientific | OSR00167W | Rabbit Polyclonal | Y | Proximal Cilium | S3C |
| T2R43 | Abcam | Ab65518 | Rabbit Polyclonal | N | - | - |
| T2R43 | Santa Cruz Biotechnology | Sc-34850 | Goat Polyclonal | Y | Proximal Cilium | S3D, S5 |
| T2R43 | Santa Cruz Biotechnology | Sc-34851 | Goat Polyclonal | Y | Proximal Cilium | 2B |
| T2R44 | Santa Cruz Biotechnology | Sc-34729 | Rabbit Polyclonal | N | - | - |
| T2R46 | Thermo Scientific | OSR00173W | Rabbit Polyclonal | Y | Proximal Cilium | S3F |
| T2R46 | Abcam | Ab65520 | Rabbit Polyclonal | Y | Proximal Cilium | S3G |
| T2R46 | Santa Cruz Biotechnology | Sc-34732 | Goat Polyclonal | Y | Proximal Cilium | 2C, S6 |
| T2R46 | Santa Cruz Biotechnology | Sc-34734 | Goat Polyclonal | N | - | - |
| T2R49 | Santa Cruz Biotechnology | Sc-34531 | Goat Polyclonal | N | - | - |
| Alpha Gustducin | Santa Cruz Biotechnology | Sc-395 | Rabbit Polyclonal | Y | Cilium | 3A |
| PLC-β2 | Santa Cruz Biotechnology | Sc-9018 | Rabbit Polyclonal | Y | Apical Portion of Cell | 3B |
| PLC-β2 | Santa Cruz Biotechnology | Sc-31757 | Goat Polyclonal | Y | Apical Portion of Cell | S3H |
| TRPM5 | Santa Cruz Biotechnology | Sc-27366 | Goat Polyclonal | Y | Cilium | 3C |
| TRPM5 | Santa Cruz Biotechnology | Sc-27367 | Goat Polyclonal | Y | Cilium | S3I |
| Acetylated Tubulin | Sigma | T6793 | Mouse Monoclonal | Y | Cilium | |

Fig. S1

A

Primary antibody:
rabbit anti-T2R38 antibody

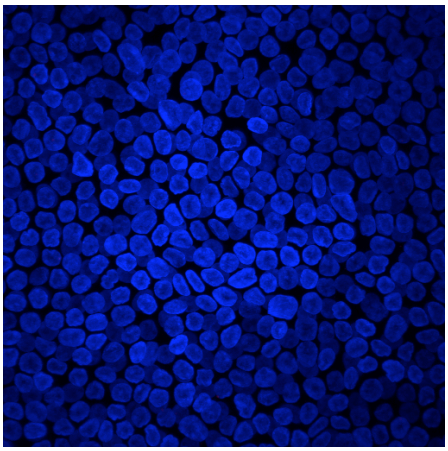
Secondary antibody:
goat anti-rabbit



B

Primary antibody:
non-immunized rabbit serum

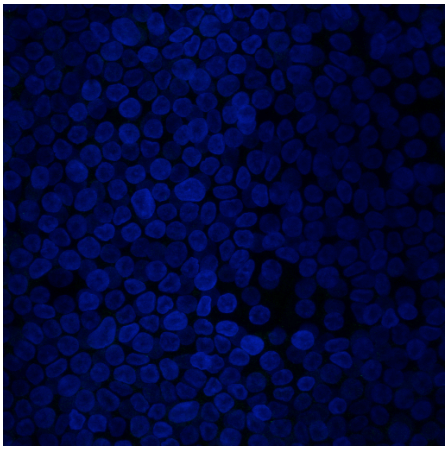
Secondary antibody:
goat anti-rabbit



C

Primary antibody:
non-immunized goat serum

Secondary antibody:
donkey anti-goat



D

Primary antibody:
no primary antibody

Secondary antibody:
donkey anti-rabbit +
donkey anti-goat

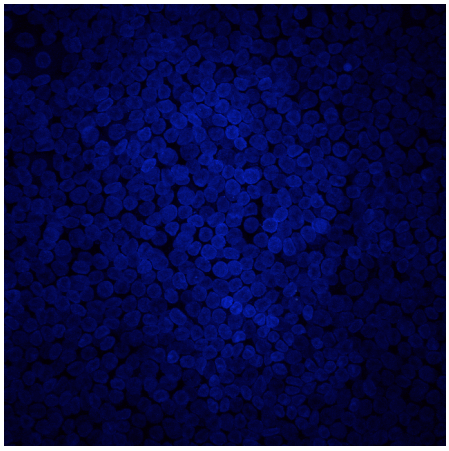


Fig. S2

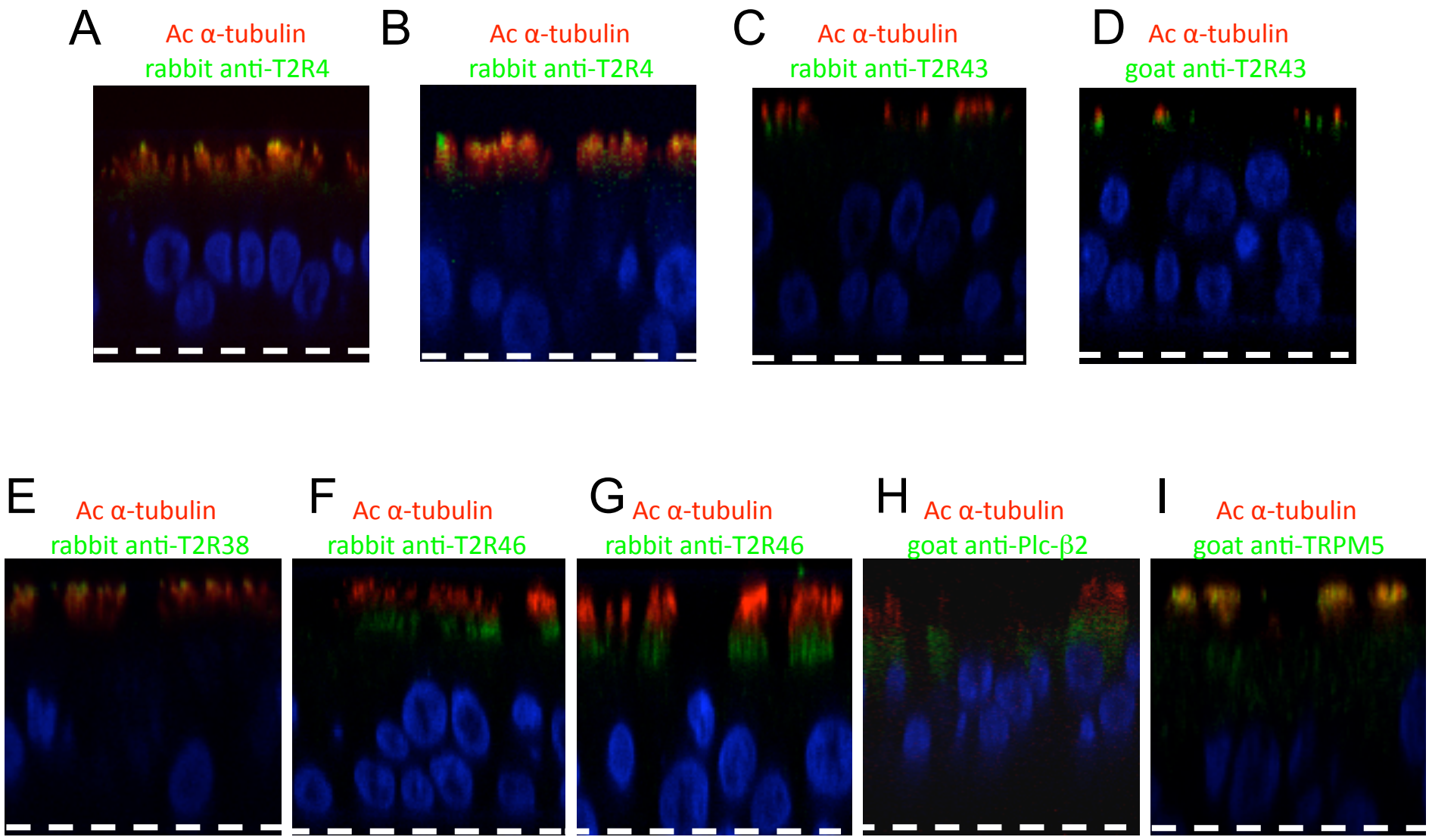

A
Ac α-tubulin
rabbit anti-T2R4
B
Ac α-tubulin
rabbit anti-T2R4
C
Ac α-tubulin
rabbit anti-T2R43
D
Ac α-tubulin
goat anti-T2R43
E
Ac α-tubulin
rabbit anti-T2R38
F
Ac α-tubulin
rabbit anti-T2R46
G
Ac α-tubulin
rabbit anti-T2R46
H
Ac α-tubulin
goat anti-Plc-β2
I
Ac α-tubulin
goat anti-TRPM5

Fig. S3

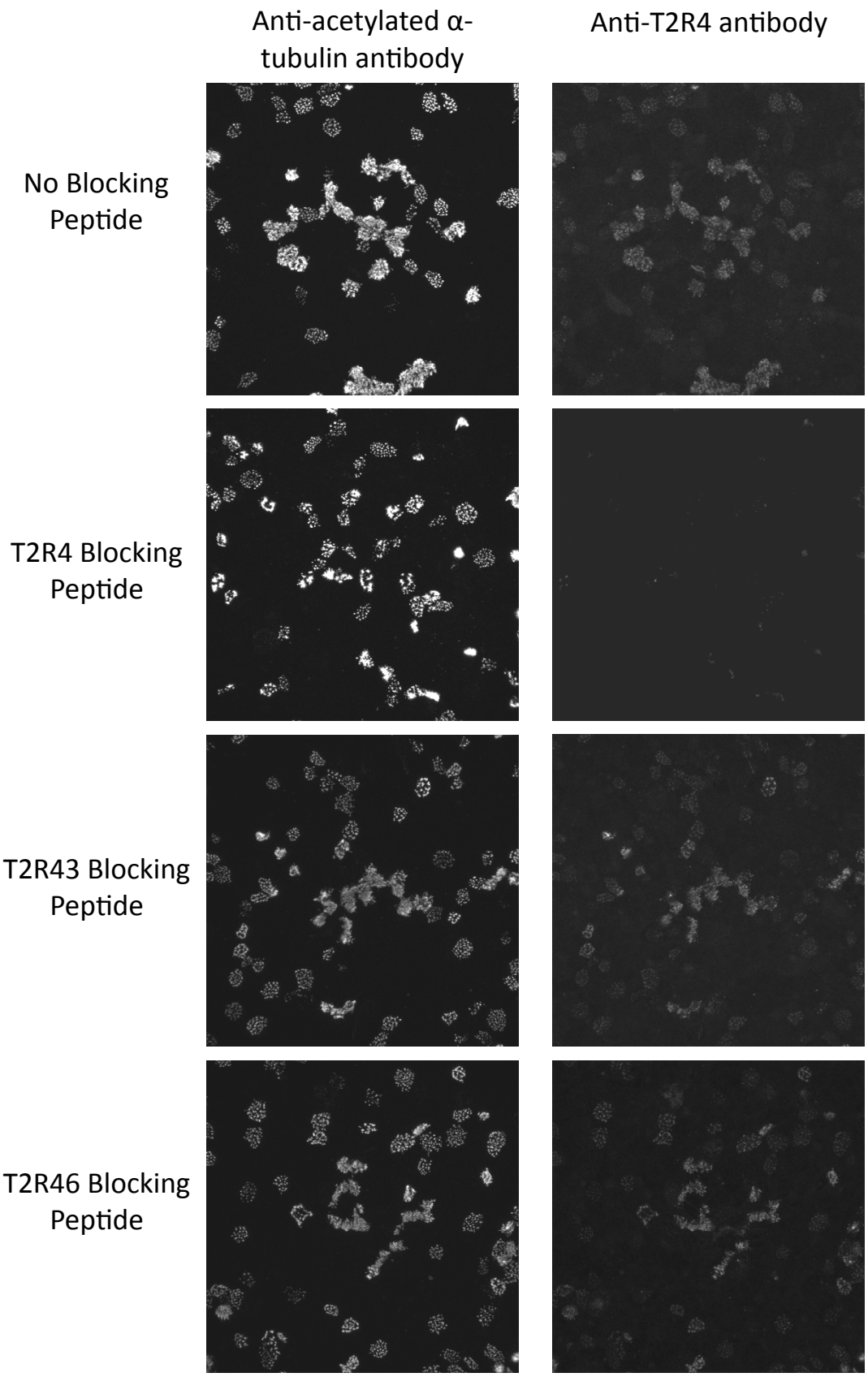

Anti-acetylated α-tubulin antibody
Anti-T2R4 antibody
No Blocking Peptide
T2R4 Blocking Peptide
T2R43 Blocking Peptide
T2R46 Blocking Peptide

Fig. S4

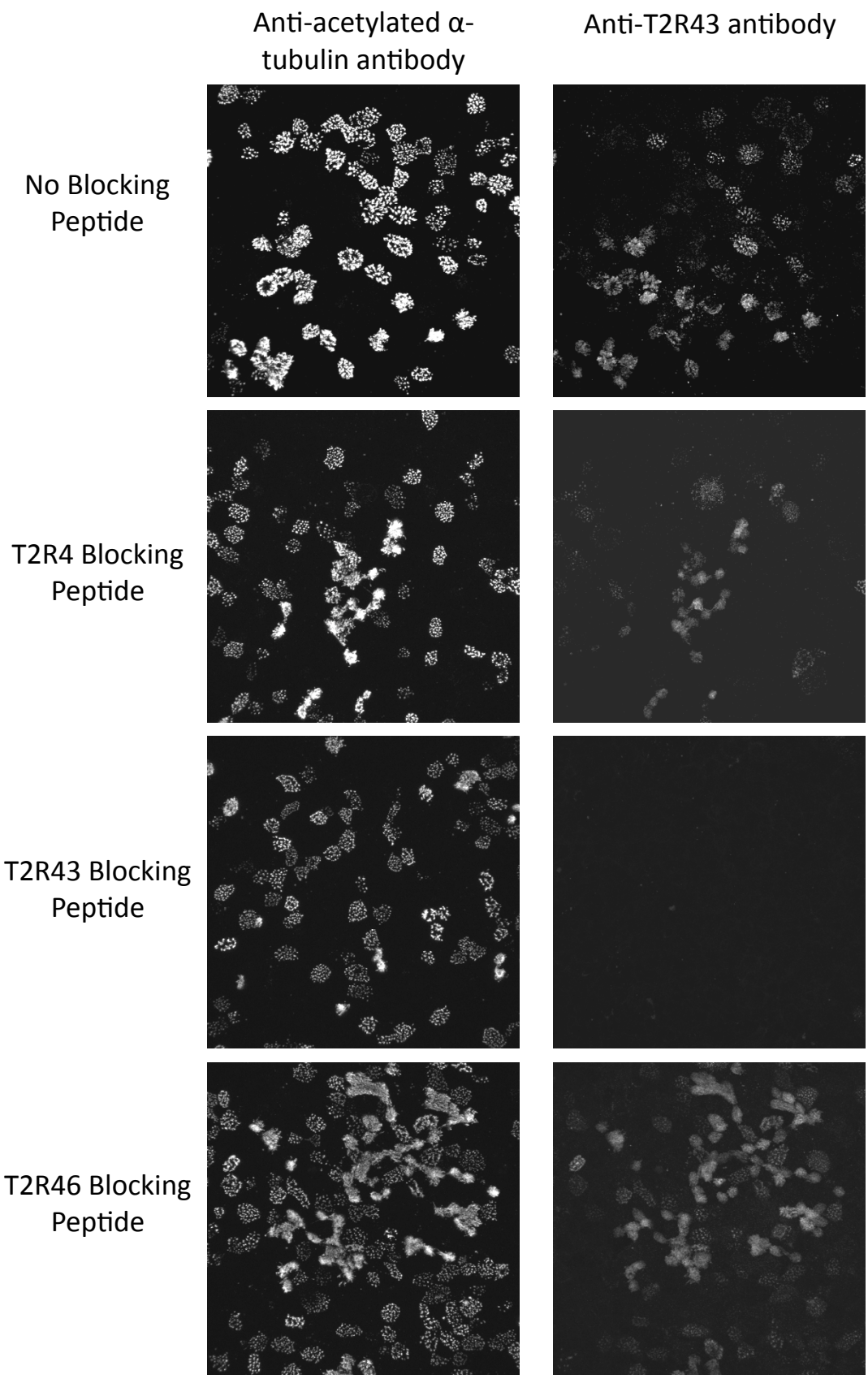

Anti-acetylated α-tubulin antibody
Anti-T2R43 antibody
No Blocking Peptide
T2R4 Blocking Peptide
T2R43 Blocking Peptide
T2R46 Blocking Peptide

Fig. S5

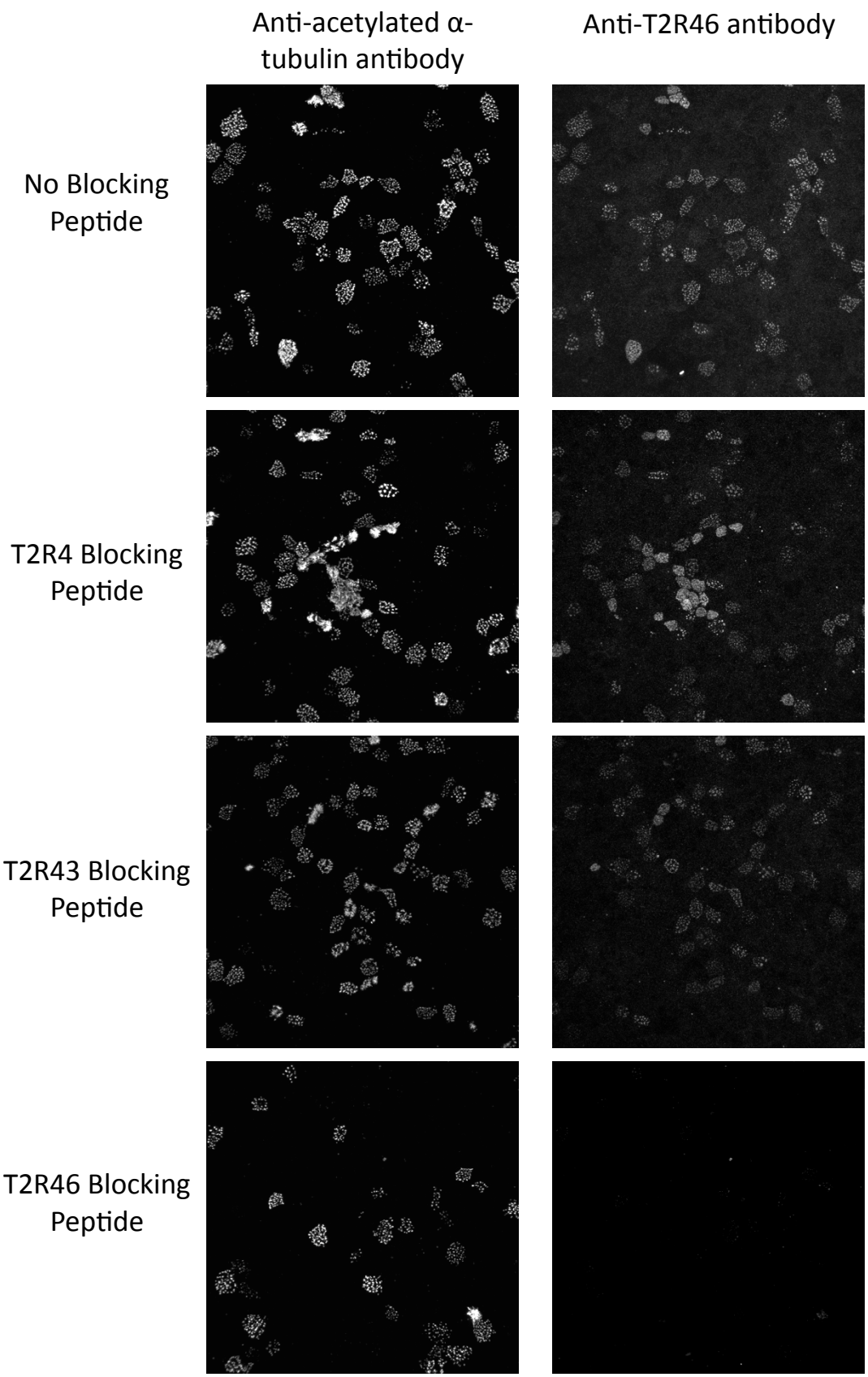

Anti-acetylated α-tubulin antibody
Anti-T2R46 antibody
No Blocking Peptide
T2R4 Blocking Peptide
T2R43 Blocking Peptide
T2R46 Blocking Peptide

Fig. S6

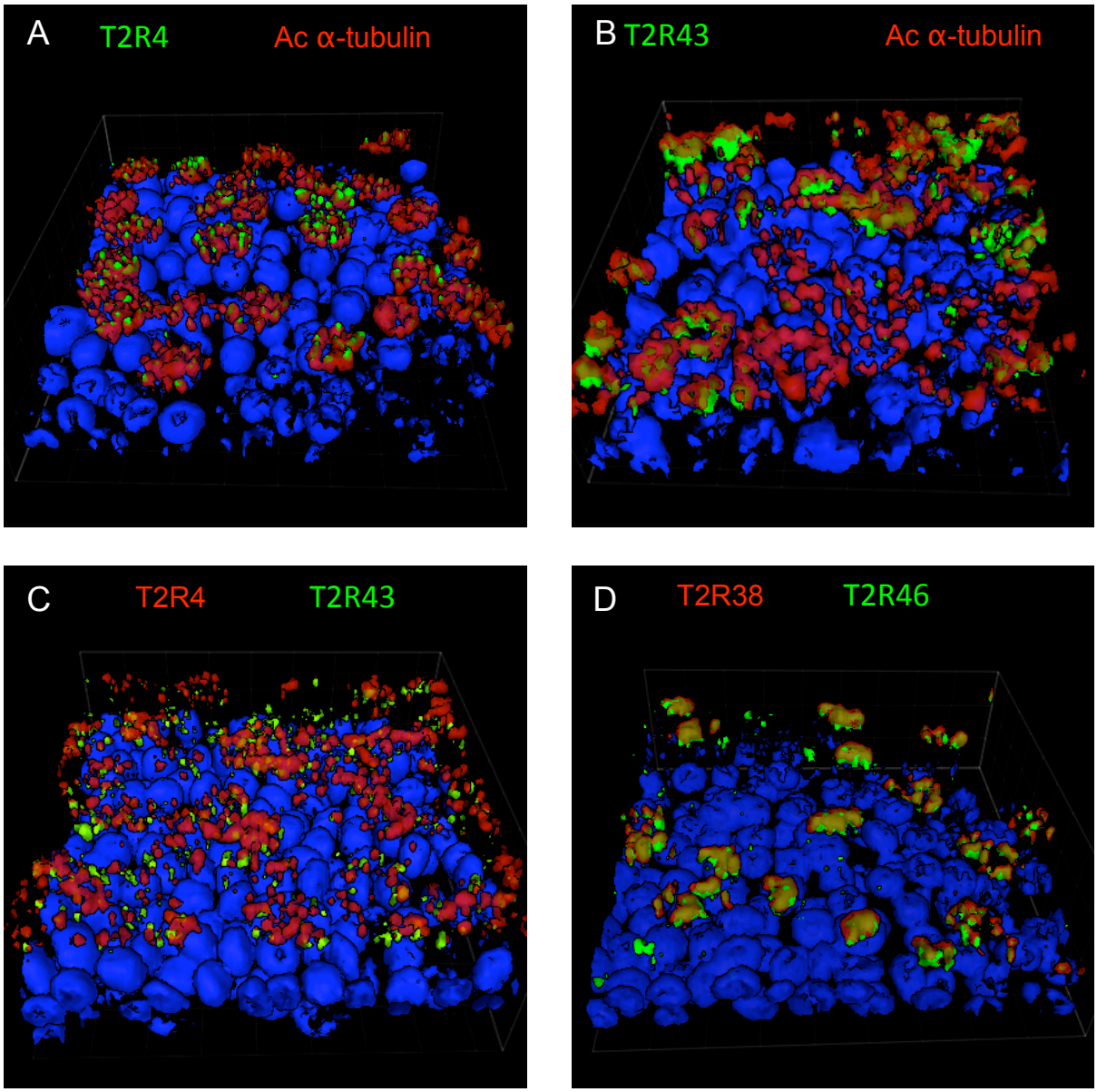
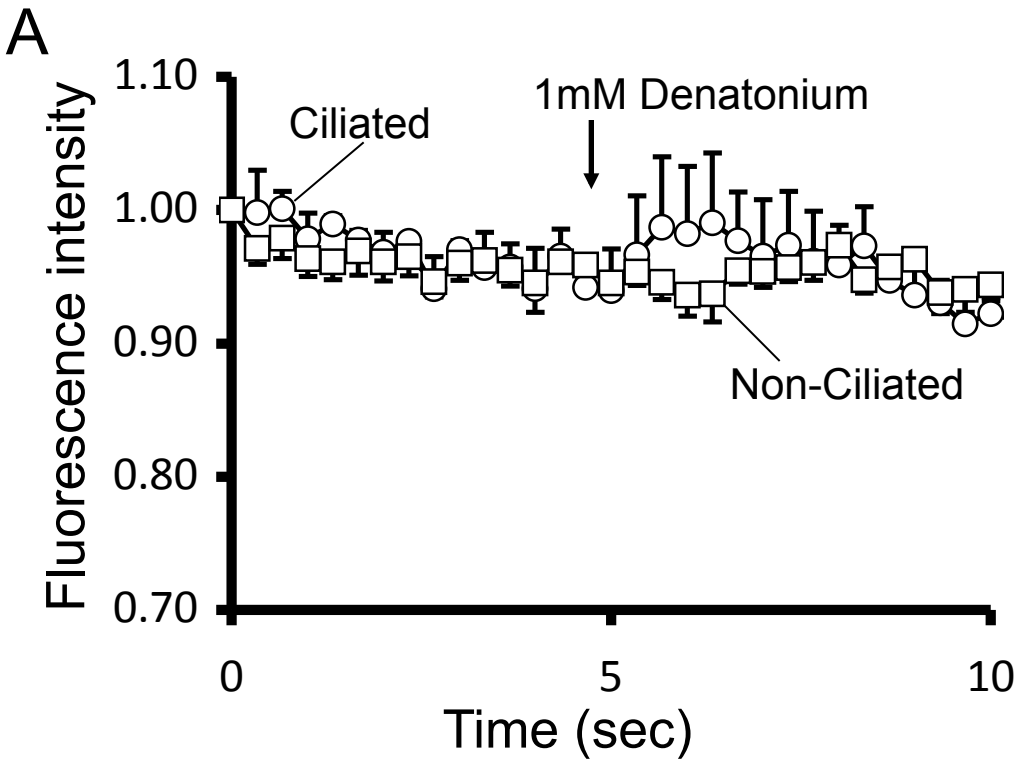

A
T2R4
Ac α-tubulin
B T2R43
Ac α-tubulin
C
T2R4
T2R43
D
T2R38
T2R46

Fig. S7


A
1.10
1.00
0.90
0.80
0.70
Fluorescence intensity
Ciliated
1mM Denatonium
Non-Ciliated
0
5
10
Time (sec)

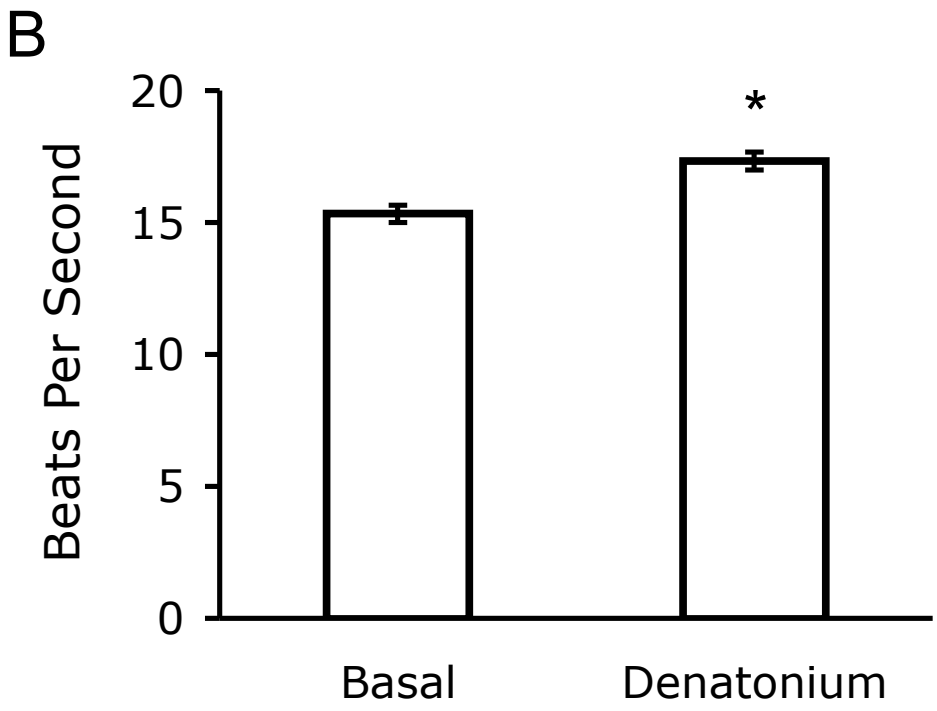
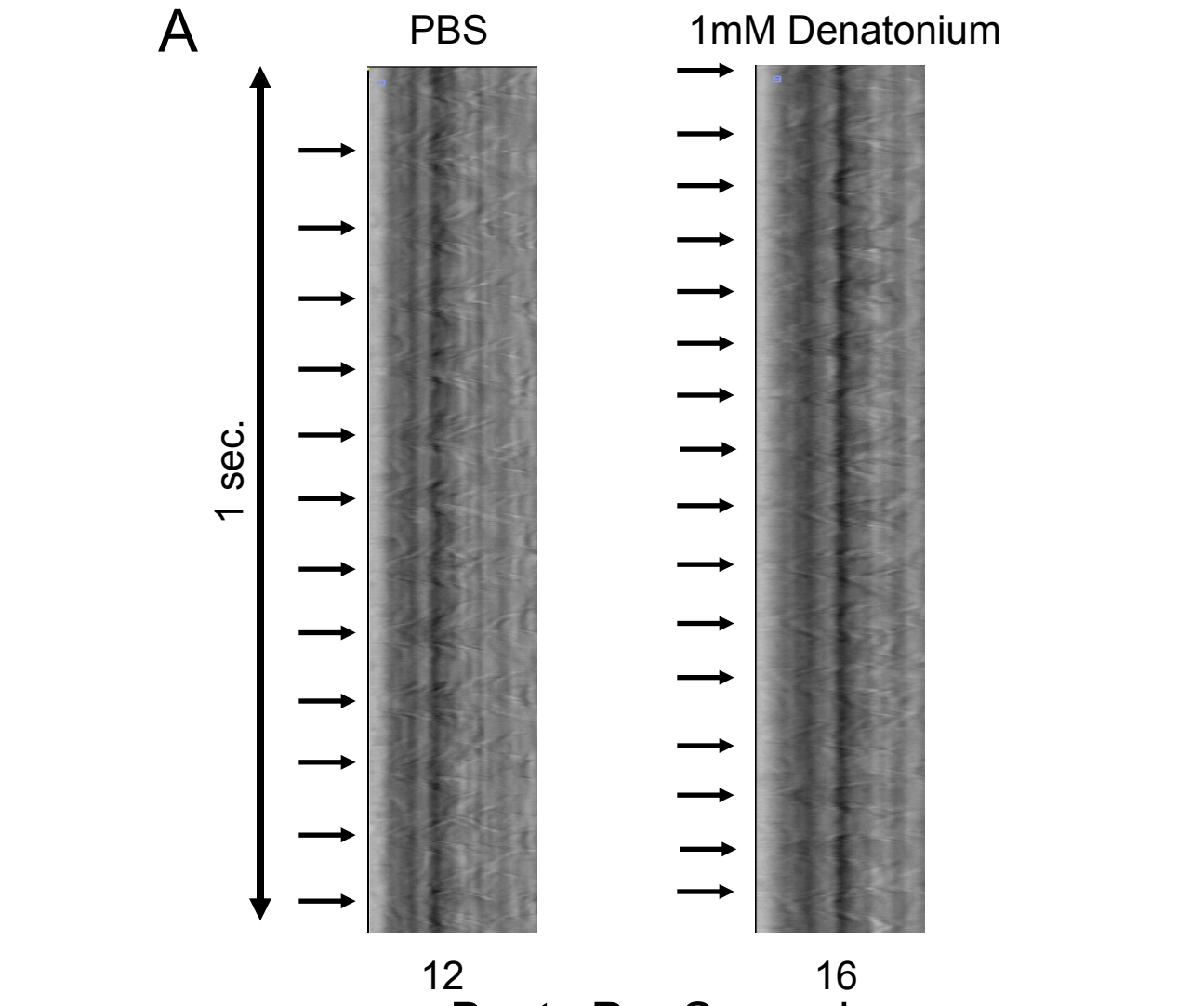

B
20
15
10
5
0
Beats Per Second
*
Basal
Denatonium

Fig. S8



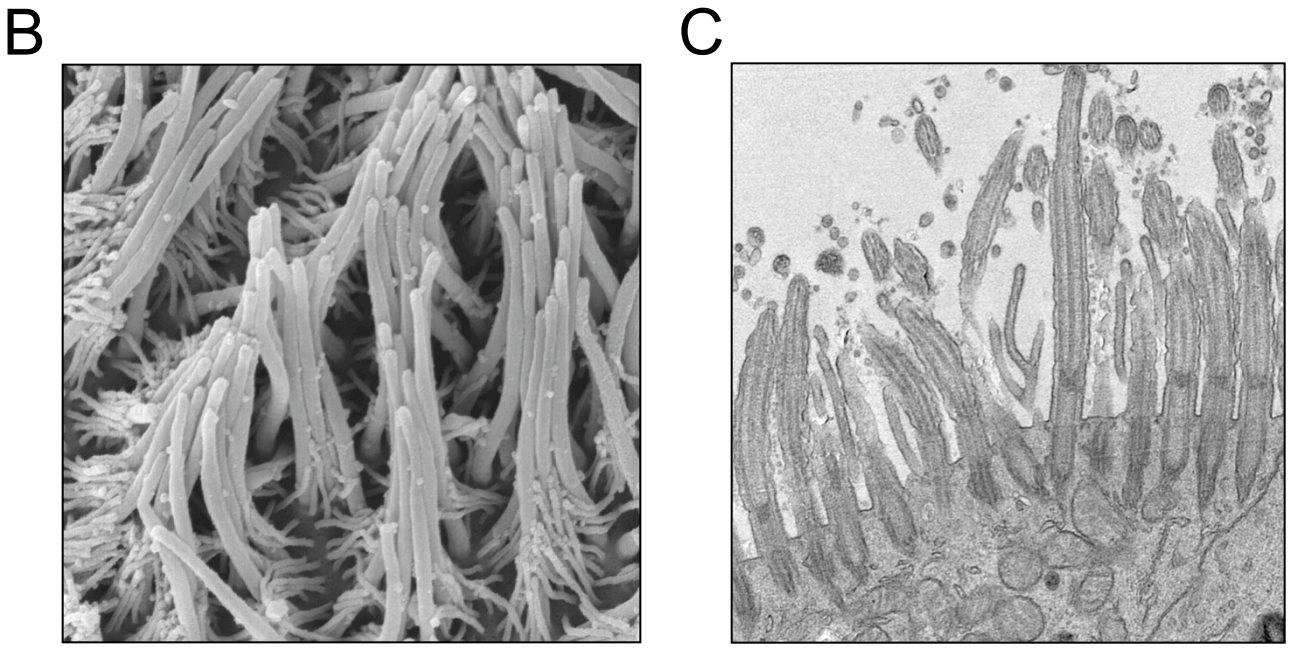




Fig. S9