Supplemental material and methods

Co-immunoprecipitation and western blotting

Transfected COS-7 cells and LoVo cells were lysed with lysis buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.1% triton X-100) containing a protease-inhibitor cocktail. After 30 min incubation on ice, cell debris was cleared at 20,000g for 30 min at 4°C. The supernatants were then pre-cleared with Protein A/G plus-agarose beads (Santa Cruz Biotechnology) for 30 min at 4°C. Then the lysates were incubated with 1 µg anti-GFP (Sigma), or 1 µg anti-TTYH2 (Prosci) antibodies for 3 h at 4°C with gentle agitation. This was followed by incubation with Protein A/G plus-agarose beads for 1 h. The beads were then washed three times with cell lysis buffer. Protein elutes were separated by 10% SDS–PAGE gels and transferred to PVDF membranes, which were blocked in 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T). The blots were incubated overnight at 4°C with anti-HA (Roche Diagnostics) or anti-β-COP (Santa Cruz Biotechnology) antibodies. Blots were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 35 min at room temperature. This was followed by washing and detection of immunoreactivity with enhanced chemiluminescence (Amersham Biosciences).

Surface expression analysis

For surface expression analysis, expression vectors containing N-terminal hemagglutinin (HA)-tagged TTYH2 and GFP-tagged β-COP or GFP only were co-transfected into COS-7 cells on 35 mm dishes or 12 mm coverslips in 24-well plates. The transfected cells in 35 mm dishes were incubated for 1 h at 4°C with 1 µg/µl rat monoclonal anti-HA antibody (Roche Diagnostics) with 1% BSA in PBS, and then incubated with HRP-conjugated goat anti-rat antibody with 1% BSA in PBS for 1 h at 4°C. The cells were lysed in lysis buffer for 30 min
on ice. Individual cells were placed in 50 µl Supersignal ELISA Femto solution (Pierce Biotechnology). After an equilibration period of 30 sec, chemiluminescence was measured on a luminometer using Plate CHAMELEON™ V (HIDEX).

The transfected cells were incubated on coverslips for 18 h at 4°C with 1 µg/µl rat monoclonal anti-HA antibody (Roche Diagnostics) with 1% BSA in PBS, and then incubated with Alexa Fluor™ 549 conjugated anti-rat IgG antibody with 1% BSA in PBS for 30 min at 4°C. The cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and then rinsed twice with PBS. Then each cell nucleus were stained with DAPI for 5 min. Coverslips were mounted on slides, and after the Z-stack images were compressed to obtain a projection image (Fluoview FV1000; Olympus), fluorescence intensities were measured.

**Bimolecular fluorescence complement (BiFC) assay**

In order to achieve the BiFC, three constructs (TTYH2, TTYH2ΔC and β-COP) were cloned into pBiFC-VN173 and pBiFC-VC155 vectors (Addgene). COS-7 cells were co-transfected with cloned BiFC vectors in all possible pairwise combinations. The cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature, then rinsed twice with PBS. And then each cell nuclei were stained with DAPI for 5 min. Coverslips were mounted on slides, and a confocal microscope (Fluoview FV1000; Olympus) was used to obtain Venus fluorescence signal images.

**Immunocytochemistry**

LoVo cells grown on coverslips were transfected with expression constructs encoding mCherry or mCherry-β-COP. The cells were washed in PBS, and then they were fixed with 4% formaldehyde in PBS for 15 min at room temperature and rinsed twice with PBS. Then, wheat germ agglutinin staining (Alexa Fluor™ 488 Conjugate, Invitrogen) was performed at room
temperature for 5 min to label the membrane. Coverslips were mounted on slides, and cells were imaged with a confocal microscope (Fluoview FV1000; Olympus). Co-localization was analyzed by Image J software.

**RT-PCR**

Total RNA was isolated from LoVo cells using an RNA purification kit (Hybrid-R; GeneAll). cDNA was synthesized from 1 µg total RNA and reverse transcription was performed using a SensiFAST™ cDNA Synthesis Kit (BIOLINE) according to the manufacturer’s instructions. RT-PCR was performed using 2x TOPsimpleTM DyeMIX-Tenuto (Enzynomics). The following primers were used for RT-PCR experiments: β-COP; forward- 5’ AGTCGGAAGAACTGGCTTTC 3’, reverse- 5’ AACATAGCTGTGATGCT 3’, TTYH2; forward- 5’ GTGGACTACATCGCTCCCT 3’, reverse- 5’ TCGTCCAAGGAGTACATCAG 3’, GAPDH; forward- 5’ GTCTTCACCACCAGGAGA 3’, reverse- 5’GCATGGACTGTGGTGCATGAG3’. GAPDH was used as a loading control.

**Electrophysiology**

Whole-cell patch clamp experiments were performed 24 h after transfection in COS-7 cells. The external solution contained (in mM): 70 NaCl, 0.5 MgCl₂, 2 CaCl₂, 10 HEPES and 140 D-mannitol at pH 7.4 (300 mOsm/kg-H₂O). The pipette solution consisted of (in mM): 70 N-methyl-D-glucamine chloride(NMDG-Cl), 1.2 MgCl₂, 10 HEPES, 1 EGTA, 140 D-mannitol and 2 ATP at pH 7.2 (300mOsm/kg-H₂O). A patch clamp amplifier (Axopatch 200B; Axon Instruments) was used to record whole-cell currents at room temperature (22–25°C). The current-voltage relations were measured by applying ramp pulses (-100 to +100 mV over 1 sec)
from a holding potential of -60 mV. Currents were filtered at 5 kHz and digitized at 5 kHz for further analysis. A pClamp 10.2 (Molecular Devices) was used to perform data analysis.

**Statistical analysis**

Numerical data are presented as mean ± SEM. The significance of data for comparison was assessed by Student’s two-tailed unpaired t-test and significance levels were given as: *P < 0.05, **P < 0.01, ***P < 0.001.