Figure S1

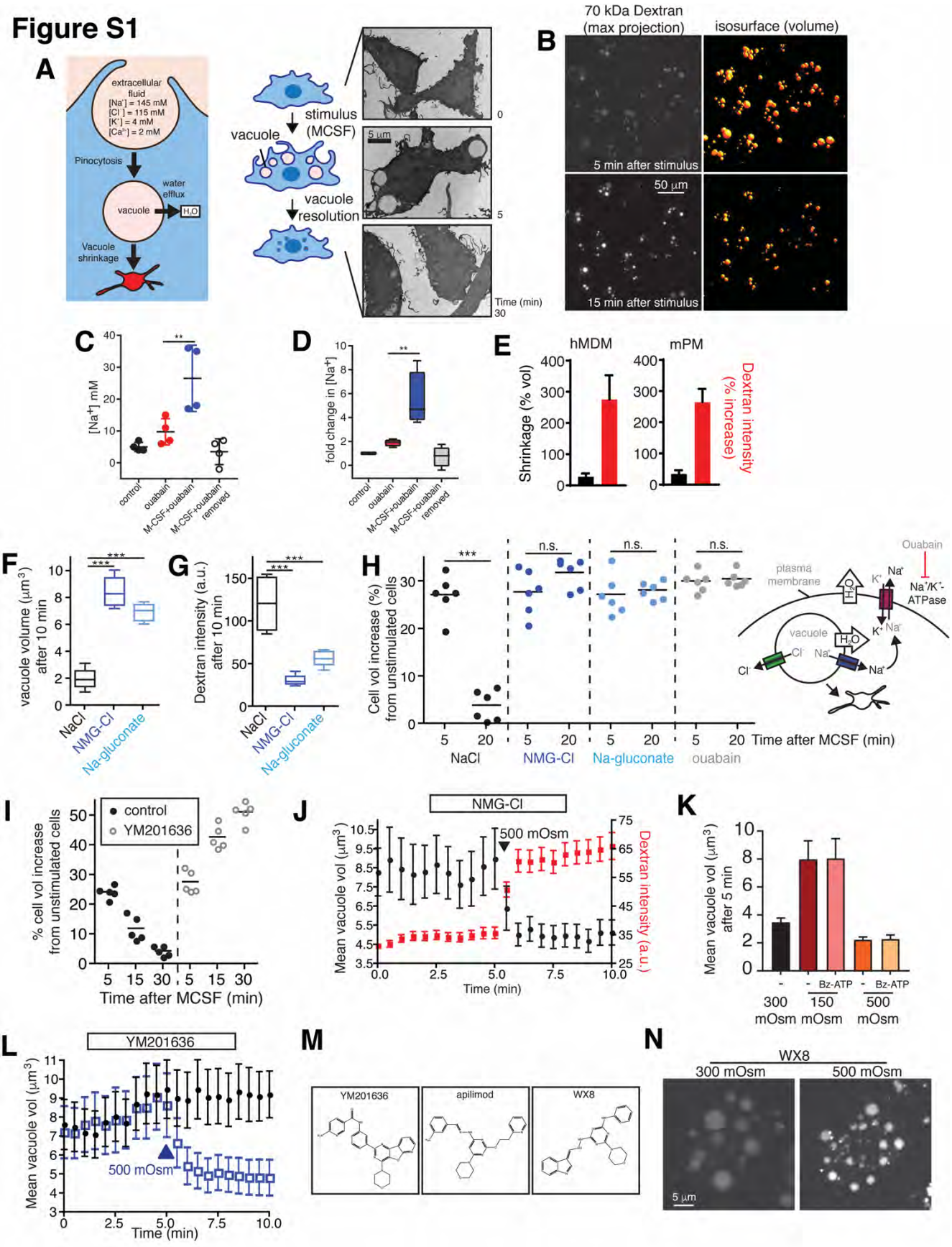
A
extracellular fluid
[Na+] = 145 mM
[Cl-] = 115 mM
[K+] = 4 mM
[Ca2+] = 2 mM
Pinocytosis
vacuole
water efflux
H2O
Vacuole shrinkage
stimulus (MCSF)
vacuole
vacuole resolution
5 µm
0
5
Time (min)
30
B
70 kDa Dextran (max projection)
isosurface (volume)
5 min after stimulus
15 min after stimulus
50 µm
C
[Na+] mM
control
ouabain
M-CSF+ouabain
M-CSF+ouabain removed
D
fold change in [Na+]
E
hMDM
mPM
Shrinkage (% vol)
Dextran intensity (% increase)
F
vacuole volume (µm3) after 10 min
NaCl
NMG-Cl
Na-gluconate
G
Dextran intensity (a.u.) after 10 min
H
Cell vol increase (%) from unstimulated cells
n.s.
NaCl
NMG-Cl
Na-gluconate
ouabain
Time after MCSF (min)
plasma membrane
Ouabain
Na+/K+-ATPase
vacuole
I
control
YM201636
% cell vol increase from unstimulated cells
Time after MCSF (min)
J
NMG-Cl
500 mOsm
Mean vacuole vol (µm3)
Dextran intensity (a.u.)
Time (min)
K
Mean vacuole vol (µm3) after 5 min
Bz-ATP
300 mOsm
150 mOsm
500 mOsm
L
YM201636
Mean vacuole vol (µm3)
500 mOsm
Time (min)
M
YM201636
apilimod
WX8
N
WX8
300 mOsm
500 mOsm
5 µm

Figure S1. *Monovalent ion efflux from macropinosomes maintains cell volume*. **A**, Model (left) and bone marrow-derived macrophages (BMDM) imaged by TEM at the indicated times after addition of M-CSF. **B**, BMDM were imaged for 5-10 min after stimulation with M-CSF in the presence of 70 kDa rhodamine-labeled dextran. The mean macropinosomal volumes and dextran intensities were determined from features (macropinosomes) identified by software that applied suitable thresholds (see Figures 1 and 2). **C-D**, BMDM, treated with vehicle (control) or ouabain (5 mM) for 10 min, ouabain and M-CSF for 10 min, or treated with ouabain and M-CSF followed by a 10 min chase. $[Na^+]$ was determined by atomic absorption spectrometry, considering the volume determined with a Coulter counter. Both the concentration (C) and the fold-change normalized to control (D) are shown. n=4. **E**, M-CSF-induced vacuole volume and dextran fluorescence intensity of human monocyte-derived macrophages (hMDM) and murine peritoneal macrophages (mPM) measured 10 min after stimulation. Data are presented as % of the value recorded immediately after stimulation; >1000 vacuoles, n=3. **F-G**, BMDM were stimulated for 5 min with M-CSF in the presence of 70 kDa rhodamine-dextran in the indicated media, such that sealed macropinosomes contained 150 mM sodium chloride (NaCl), N-methylglucamine chloride (NMG-Cl), or sodium gluconate along with the dextran. The macropinosomal volume and dextran intensity from >15 fields containing 5-10 BMDM each, from 3 independent experiments (totaling >1000 macropinosomes per condition) is graphed. Boxes represent the upper and lower quartiles and horizontal lines within the box represent the median. **H**, BMDM were stimulated with M-CSF in the indicated media for 5 min. Cell volume was measured at the indicated times after stimulation. Each dot represents the median cell volume as calculated by electronic (Coulter counter) determinations of >10,000 cells. Measurements were made in 3-5 independent experiments. Experimental model, right. **I,** BMDM in vehicle or YM201636 were stimulated with M-CSF for 5 min. M-CSF was removed and cells were maintained in vehicle or YM201636. Cell volume was measured electronically at the indicated times after stimulation. Each dot represents the median cell volume of >10,000 cells. Measurements were made in 3-5 independent experiments. **J**, Macropinosomes from 3 fields of BMDM loaded with NMG-Cl and 70 kDa rhodamine-dextran were recorded for 10 min after M-CSF stimulation. At 5 min, hypertonic solution was added to increase the osmolarity of the medium to 500 mOsm (arrowhead). Means ± SEM are shown. **K**, BMDM in Ca^{2+}/Mg^{2+} free solutions were stimulated with M-CSF for 5 min and subsequently given 10 μM Bz-ATP. At 5 min, hyper- or hypotonic solution was added to increase or decrease the osmolarity of the medium to 500 mOsm or 150 mOsm. 3-5 fields were imaged. Means ± SEM are shown. n=3. **L**, BMDM were treated with vehicle control or with 500 nM YM201636 (PIKfyve inhibitor) at the time of M-CSF addition. At 5 min, either a hypertonic (blue) or equi-osmotic (black) solution was added to increase the osmolarity of the medium to 500 mOsm (arrow) or maintain 300 mOsm respectively. Means ± SEM are shown. **M**, chemical structures of PIKfyve inhibitors used in Fig 2G. **N**, representative macrophages treated with PIKfyve inhibitor WX8 (relates to Fig 2G). Macropinosomes loaded with 70 kDa dextran. Hypertonic solution added to the cell for 30 s (right).

Figure S2

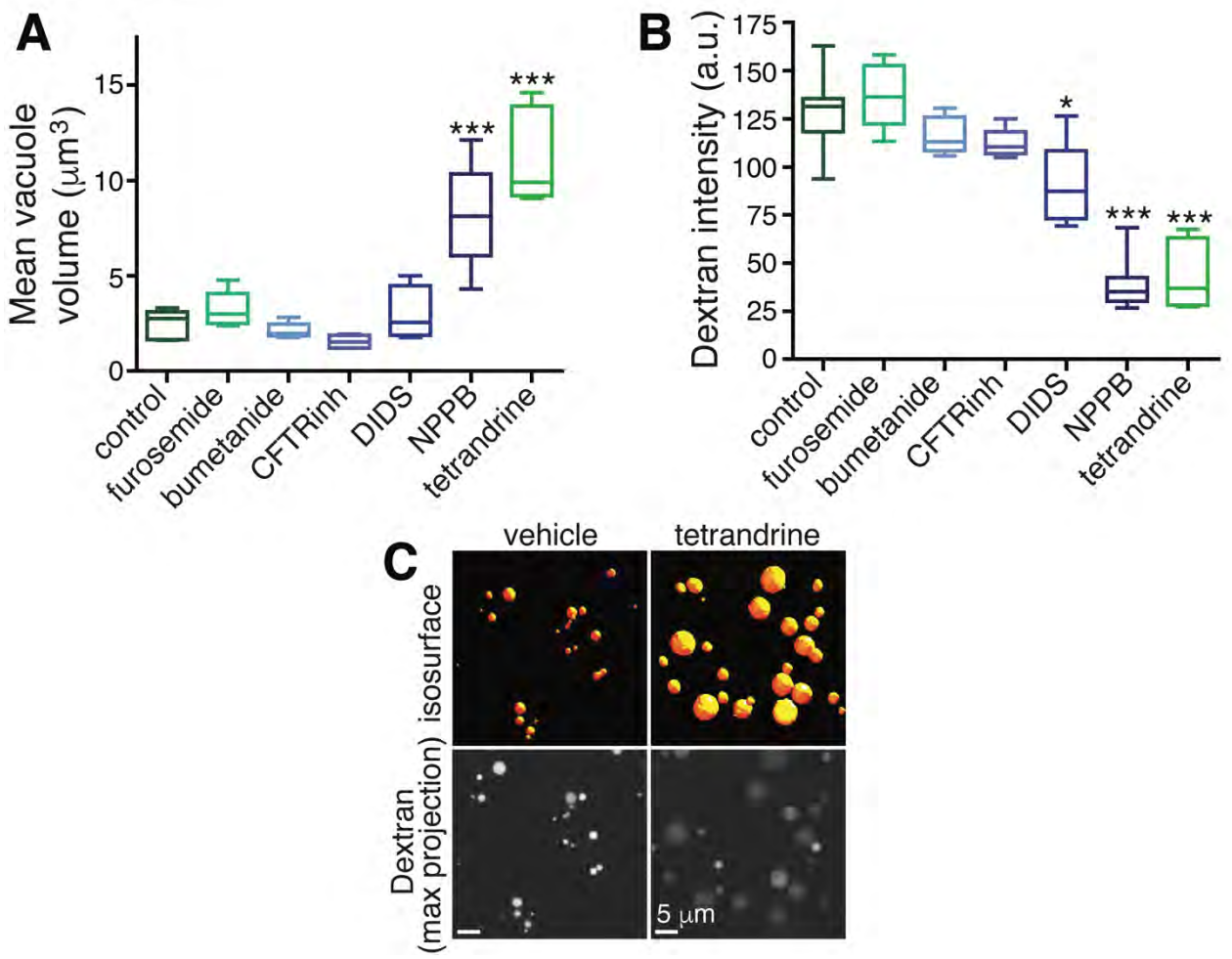

Figure S2. *Inhibitors tested for macropinosome resolution*. **A-B,** volume (A) and dextran fluorescence intensity (B) of >1500 macropinosomes after 10 min of resolution in the presence of the indicated compounds; n=3. See *Methods* for concentrations. **C** Macropinosomes of M-CSF-stimulated BMDM in media containing vehicle control or 5 μM tetrandrine and 70 kDa dextran. Representative images acquired at 5 min. See also **Video S4**.

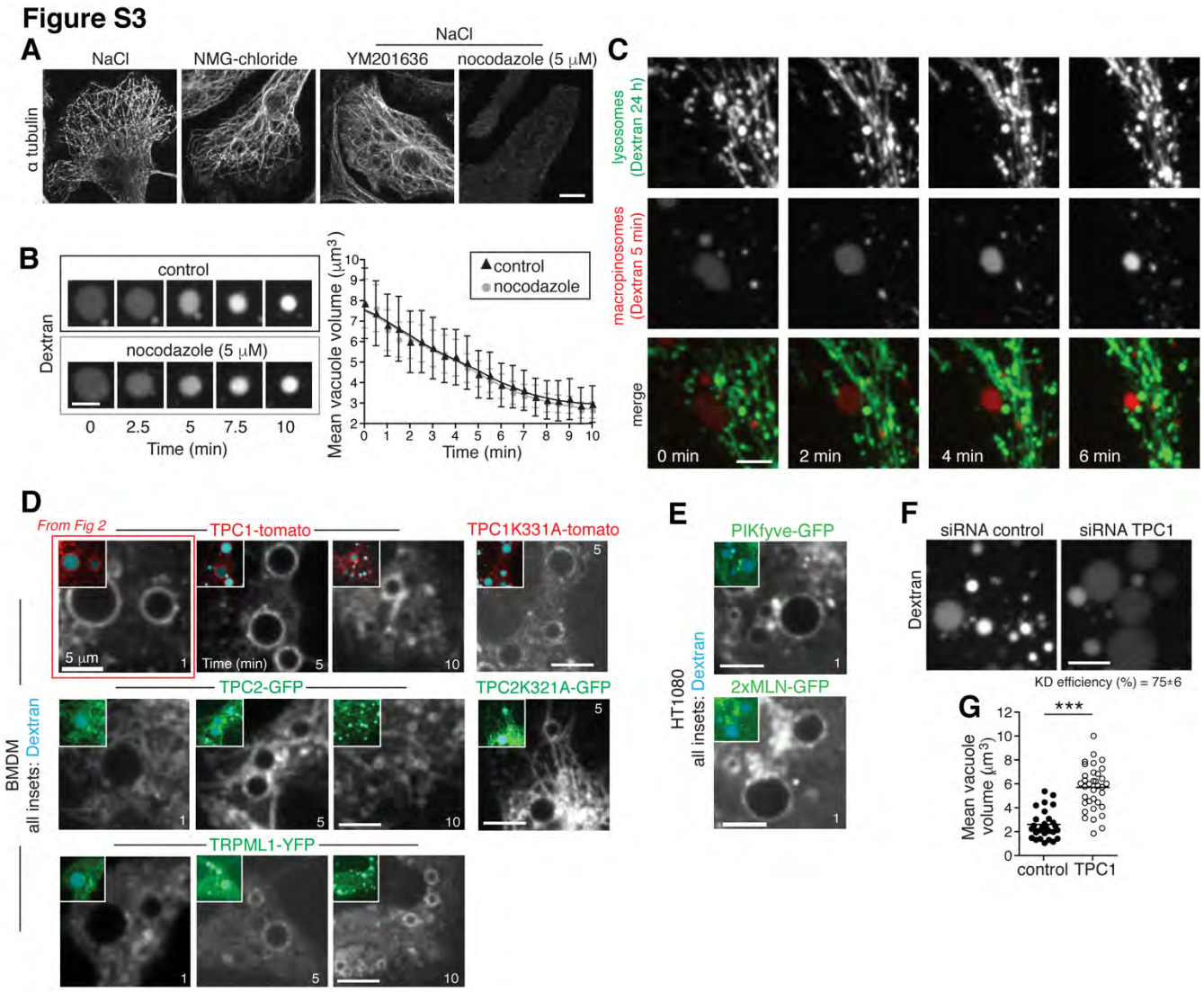

Figure S3. *Macropinosome shrinkage is microtubule-independent and starts before fusion with lysosomes and the incorporation of TPC2*. **A**, immunostaining of microtubules in BMDM fixed after 5 min of M-CSF stimulation in the presence or absence of the PIKfyve inhibitor YM201636 or of nocodazole, in media containing either 150 mM NaCl or 150 mM N-methylglucamine chloride. **B**, representative macropinosomes loaded with 70 kDa rhodamine-dextran were monitored over the indicated times (left) by video recording. Means ± SEM from >15 cells from 3 independent experiments are shown to the right. **C**, BMDM were pulsed with 10 kDa dextran-647 (pseudo-coloured green) for 2 h, then medium was removed overnight at which time the cells were stimulated with M-CSF in the presence of 70 kDa dextran-rhodamine (red) for 5 min. Cells were then washed and subsequently imaged every 30 s for >5 min. Representative frames are shown. **D**, BMDM expressing wildtype and mutant forms of TPC or wildtype TRPML1 were stimulated with M-CSF and imaged at indicated times after macropinosome sealing. **E**, Distribution of 2xMLN-GFP or PIKfyve-GFP expressed in HT1080 cells loaded with 70 kDa dextran for 10 min. **F-G**, macropinosomes in control and TPC1 siRNA-treated cells imaged 10 min after resolution started. Knockdown efficiency, indicated below the image, was determined by qRT-PCR; n=3. In **G**, macropinosomal volume assessed 10 min after resolution started; dots represent means per field of 5-15 cells. All scale bars, 5 μm.

Figure S4

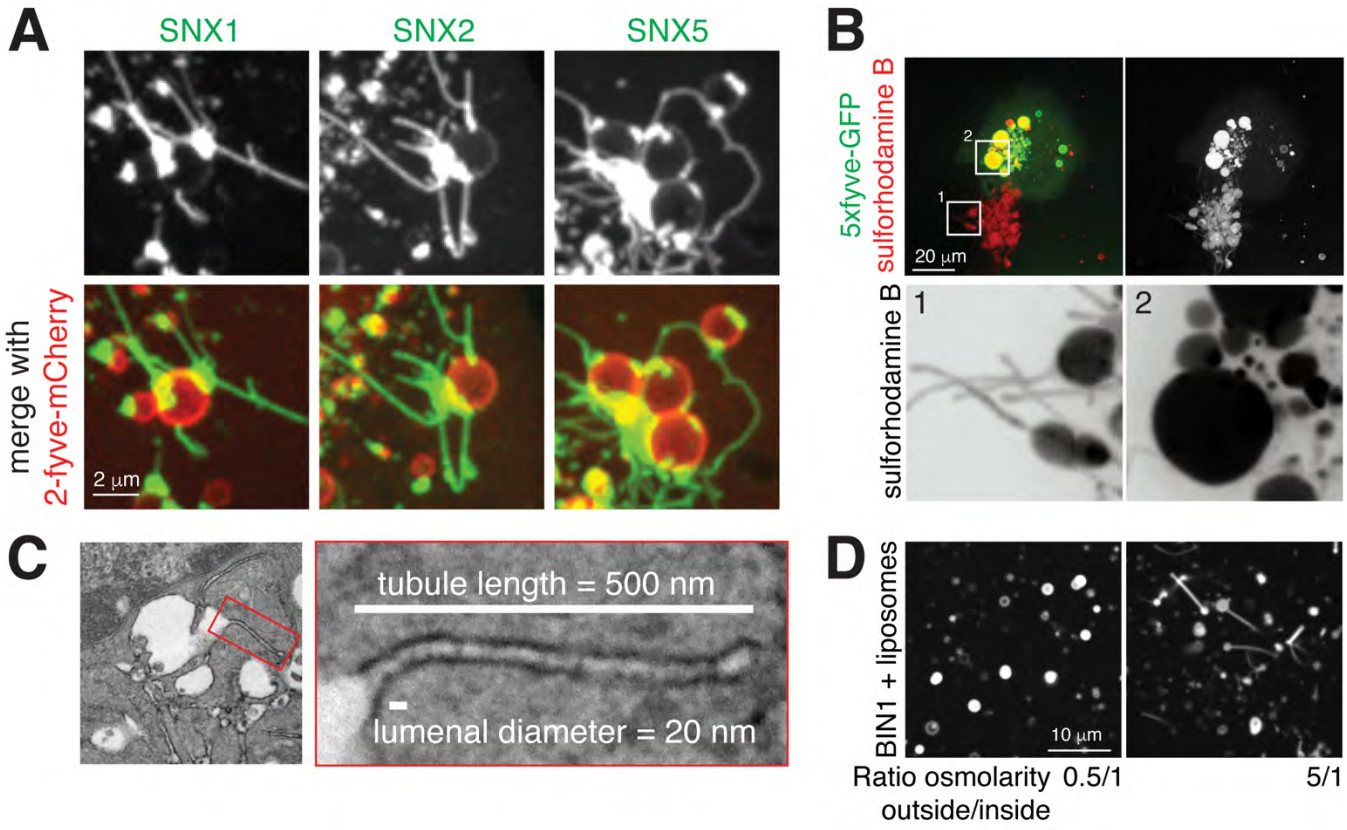

Figure S4. *The tubulation of liposomes by BAR domain-containing proteins is dependent on hydrostatic tension.* **A**, SNX-GFP chimeras were expressed in HT1080 cells together with 2xfyve-mCherry to indicate macropinosomes. **B**, HT1080 cells expressing 5xfyve-GFP were stimulated with phorbol 12-myristate 13-actetate in medium containing sulforhodamine B for 10 min then imaged. Two representative images are shown, n=3. **C**, transmission electron micrograph of representative macropinosome 5 min after M-CSF stimulation. Multiple similar tubules were quantified in Fig. 3F. **D**, liposomes, made with whole brain lipid and PtdIns(4,5)P_2 and visualized by rhodamine-PE, were formed in 20 mOsm solution along with recombinant BIN1, a BAR-domain protein. Liposomes were subsequently imaged before and then after the tonicity of the medium was changed as indicated. The same conditions were used for Fig. 3G.

Figure S5

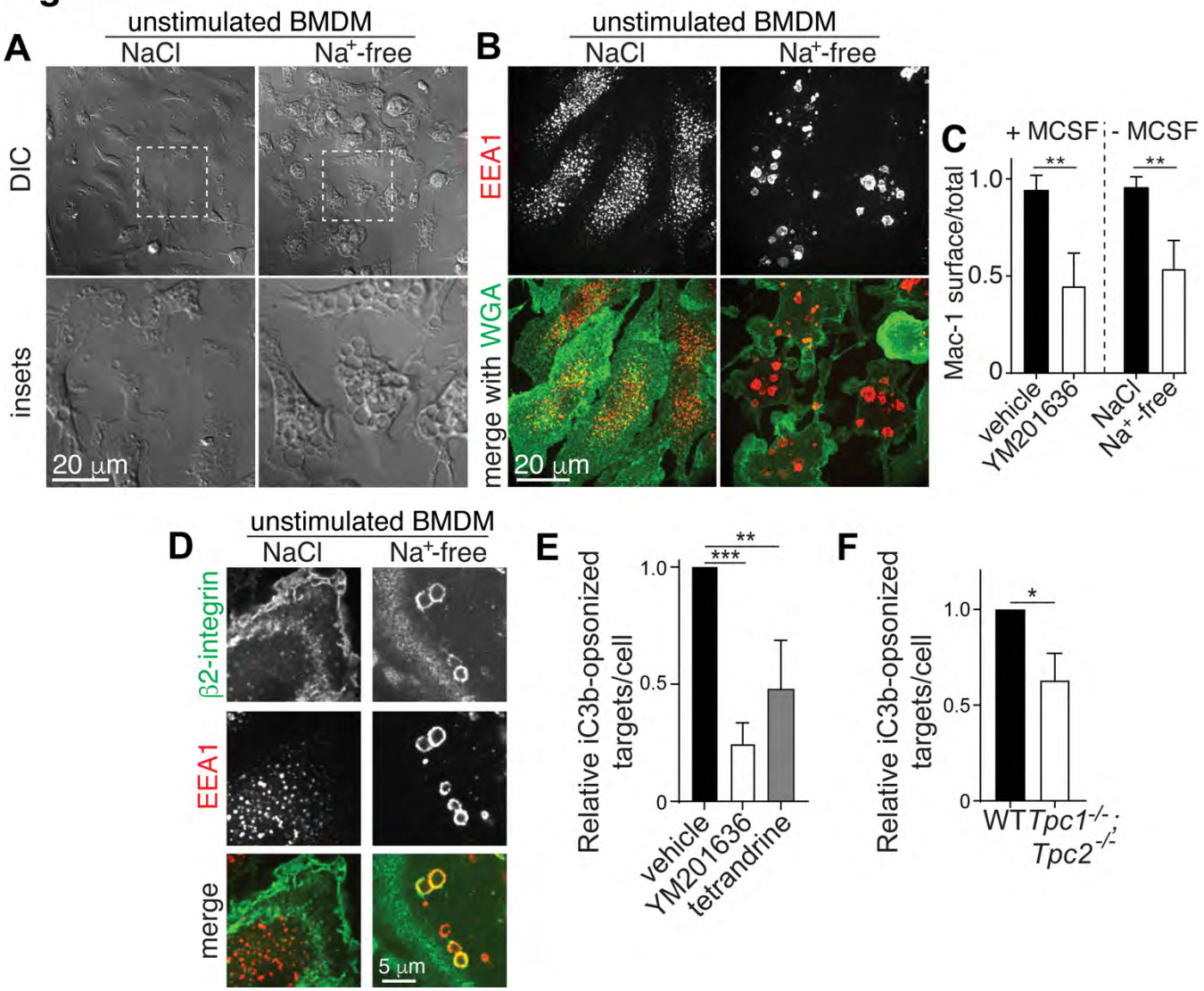

Figure S5. *A gradient of Na⁺ between the endocytic pathway lumen and the cytosol is required for vacuole resolution, receptor recycling, and phagocytosis*. **A-B**, BMDM incubated in NaCl or Na⁺-free (NaCl substituted for KCl) solution for 1 h. In **B**, cells were fixed, permeabilized and stained with the indicated antibodies or lectins. Wheat germ agglutinin (WGA) was used as a surface glycoprotein marker. Early endosome antigen 1 (EEA1) was used as an early endosome marker. **C**, BMDM were stimulated for 30 min with M-CSF, with or without YM201636, or maintained in Na⁺-containing or Na⁺-free solution for 1 hour, fixed and stained for Mac-1. Quantitation of surface over total Mac-1, from >30 cells, n=3. **D**, BMDM as in **B**, stained also for Mac-1/β2-integrin. **E-F**, BMDM challenged with iC3b-opsonized sheep red blood cells for 20 min. Phagocytic efficiency was quantified under the indicated conditions in 3-5 fields of >10 cells each; n=3. All *p* values determined by unpaired, two-sided t-tests.

Figure S6

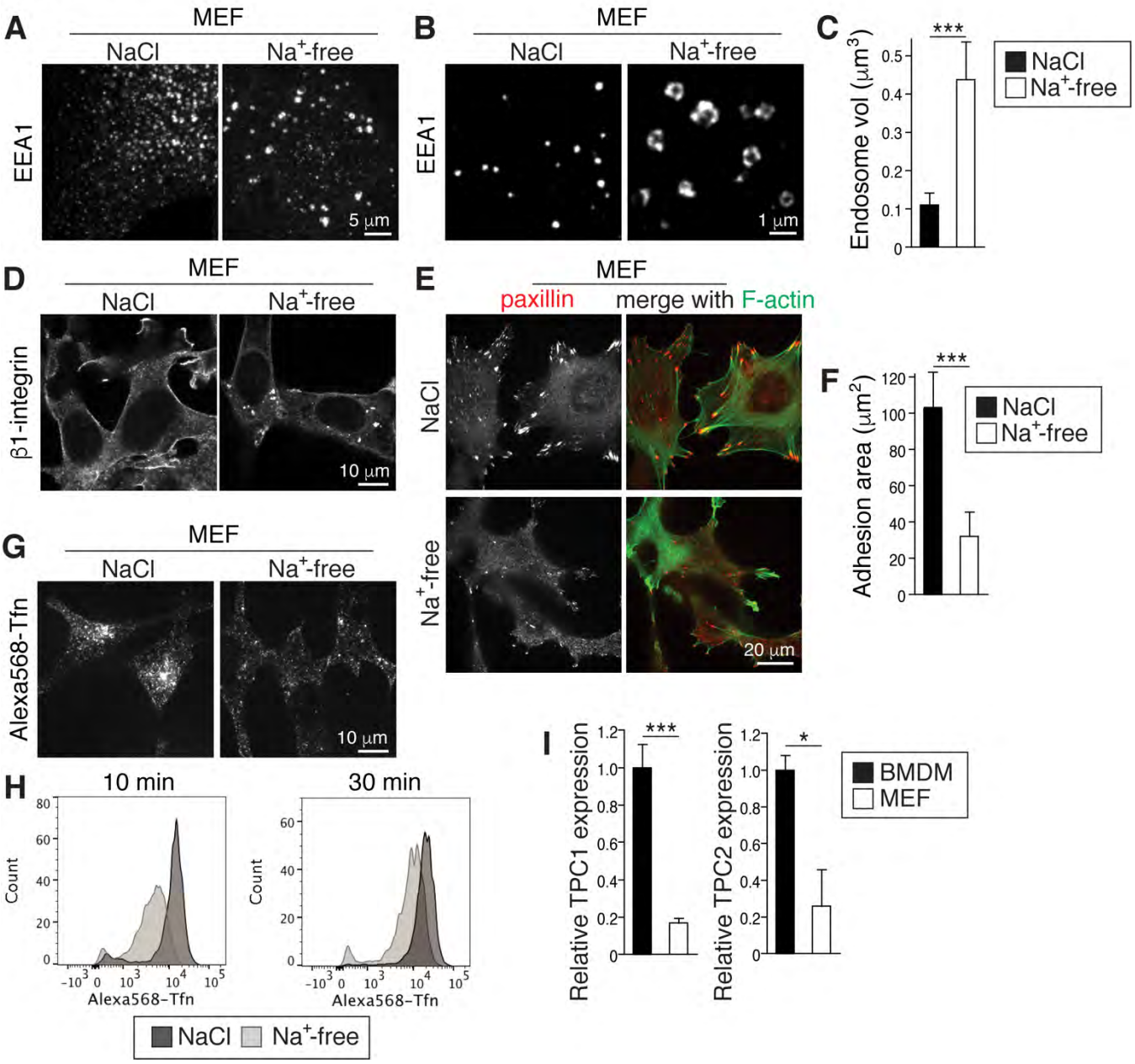

Figure S6. *Na⁺-efflux along the endocytic pathway orchestrates canonical receptor recycling pathways*. **A-F**, mouse embryonic fibroblasts (MEFs) were incubated in NaCl or Na⁺-free (NaCl substituted for KCl) solution for 2 h. Cells were fixed and immunostained with the indicated antibodies. In **B**, cells were imaged by STED microscopy. STED images were quantified for endosome volume in **C** for 3-5 cells; n=2. In **F**, adhesion area was quantified as the paxillin signal above a fixed threshold for >25 cells; n=2. **G-H**, MEFs were cultured in NaCl or Na⁺-free solution for 1.5 h then incubated with Alex568-transferrin (Tfn) for the indicated times. Cell-associated Tfn was analyzed by flow cytometry in **H**. **I**, qRT-PCR of TPC1 and TPC2 in BMDM and MEF; normalized mean ± SE of n=3. All *p* values determined by unpaired, two-sided t-tests.

Figure S7

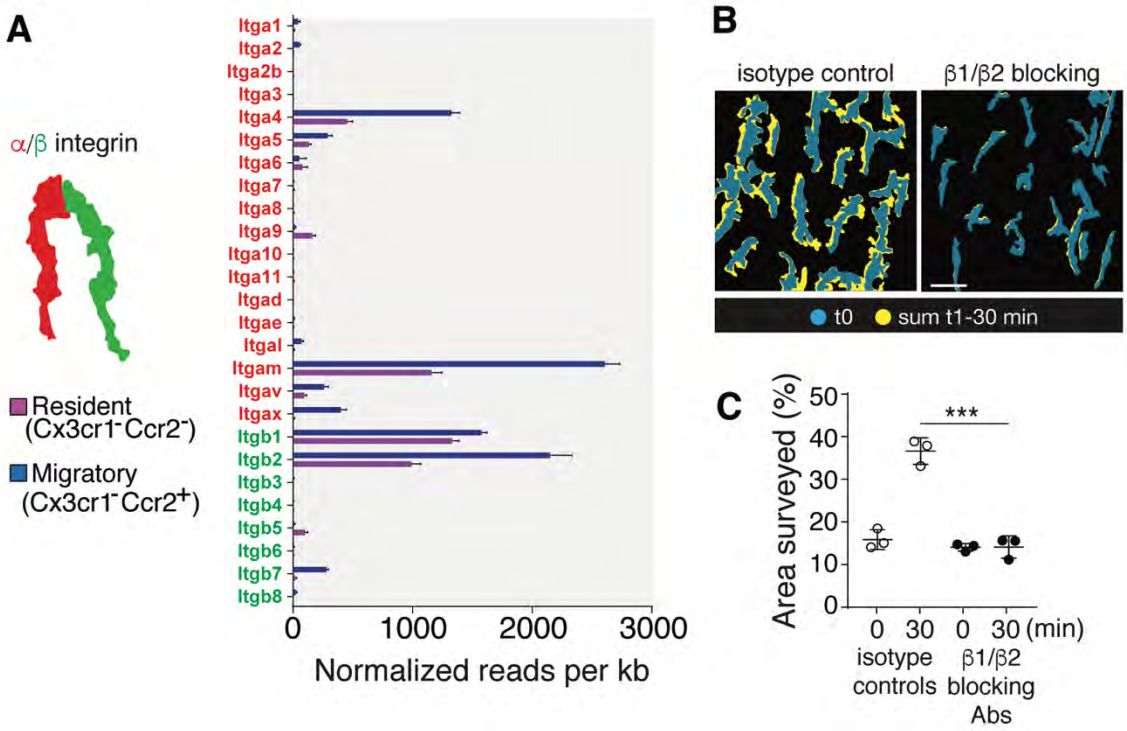

Figure S7. *Resident macrophages express β1 and β2 integrins to survey local tissue*. **A**, Left: diagrammatic representation of integrin structure. Right: RNA-seq of integrin message of Cxcr1^{-}/Ccr2^{-} (resident) or Cxcr1^{-}/Ccr2^{+} (migratory) myeloid cells isolated from the peritoneal serosa of mice. **B-C**, resident tissue macrophages stained with β1 (αCD29, clone HMβ1-1) and β2 (αCD11b, clone M1/70) blocking antibodies or isotype controls were imaged for 30 min. Surveillance area over time is shown in **B** and graphed in **C**. A Mann-Whitney test was used to determine significance.

Figure S8

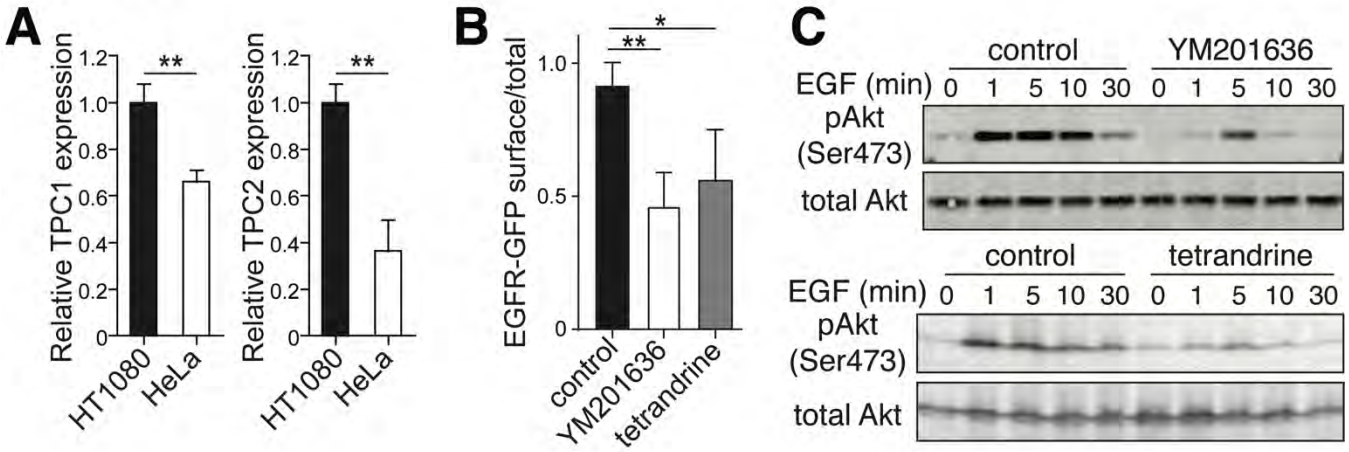

Figure S8. *HT1080 cells express TPC*. **A**, qPCR of TPC1 and 2 comparing HT1080 vs. HeLa cells; normalized mean ± SE of n=3. **B**, quantitation of surface over total EGFR-GFP in HT1080 cells treated as indicated, from >10 cells per experiment, mean ± SE of n=3. All *p* values determined by unpaired, two-sided t-tests. **C** Assessment of phospho-Akt formation in response to EGF in serum starved HT1080 cells.

Figure S9

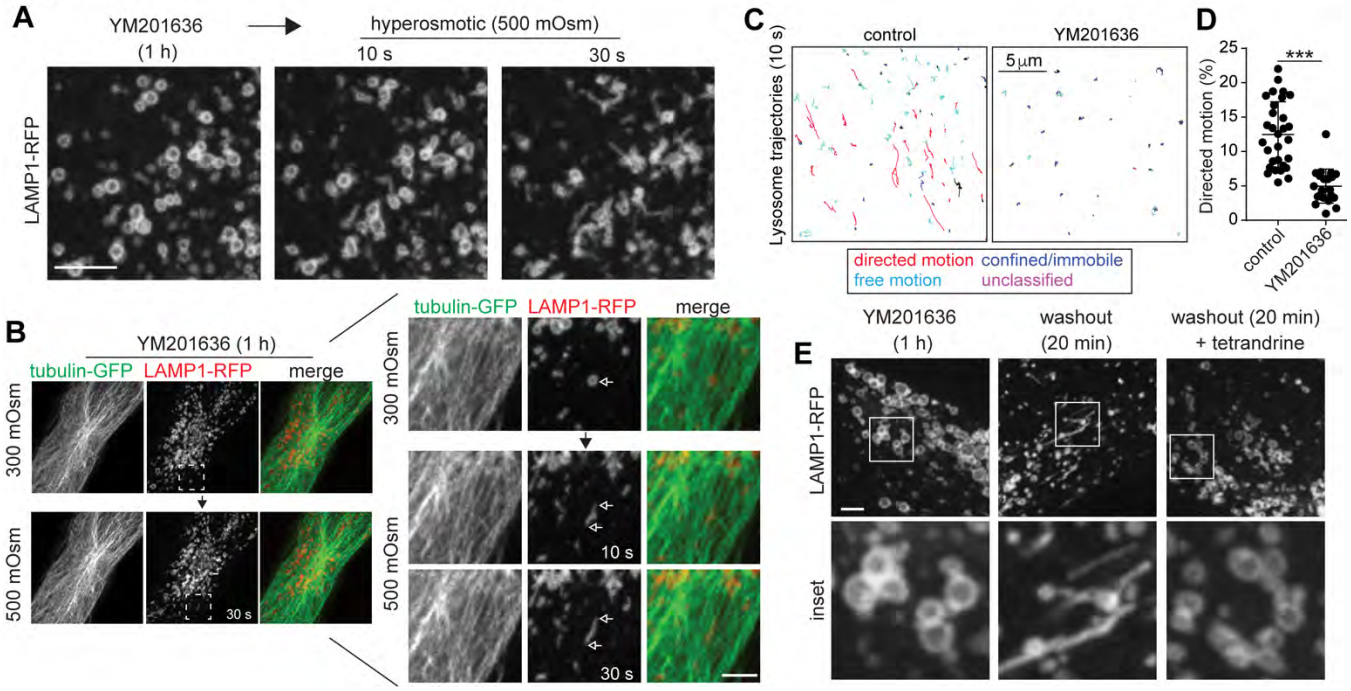

Figure S9. *Lysosome swelling upon inhibition of PIKfyve is an osmotic effect and prevents motor-based movement of the organelles*. **A-B**, HeLa cells expressing LAMP1-RFP with or without tubulin-GFP were treated with the PIKfyve inhibitor YM201636 (500 nM) for 1 h, causing swelling of the LAMP1-RFP-positive vesicles to the extent that their lumen could be resolved by confocal microscopy. The vesicles were monitored before and at the indicated times after addition of sucrose to bring the osmolarity to 500 mOsm. Images in **A** are taken from **Video S4**. Open arrows in **B** (right side) point to tubules emanating from one lysosome. **C**, LAMP1-RFP-expressing HeLa cells given vehicle alone or the PIKfyve inhibitor YM201636 for 1-2 h were recorded at 10 Hz for 10 s. The trajectories of lysosomes were analyzed by moment scaling spectrum (MSS) to determine whether their motion was confined (blue), free (cyan), directed (red), or unclassified (magenta). Representative trajectories are displayed (left). **D**, The percentage of trajectories identified as undergoing directed motion for >25 videos (each video represented by one data point), along with the median and upper and lower quartiles, are shown (right). A Mann-Whitney test was used to determine the *p* value. **E**, LAMP1-RFP-expressing HeLa cells treated with PIKfyve inhibitor for 1 h were washed and complete medium with or without tetrandrine (5 μM) was then added and images acquired at the indicated times. All scale bars, 5 μm.

Figure S10

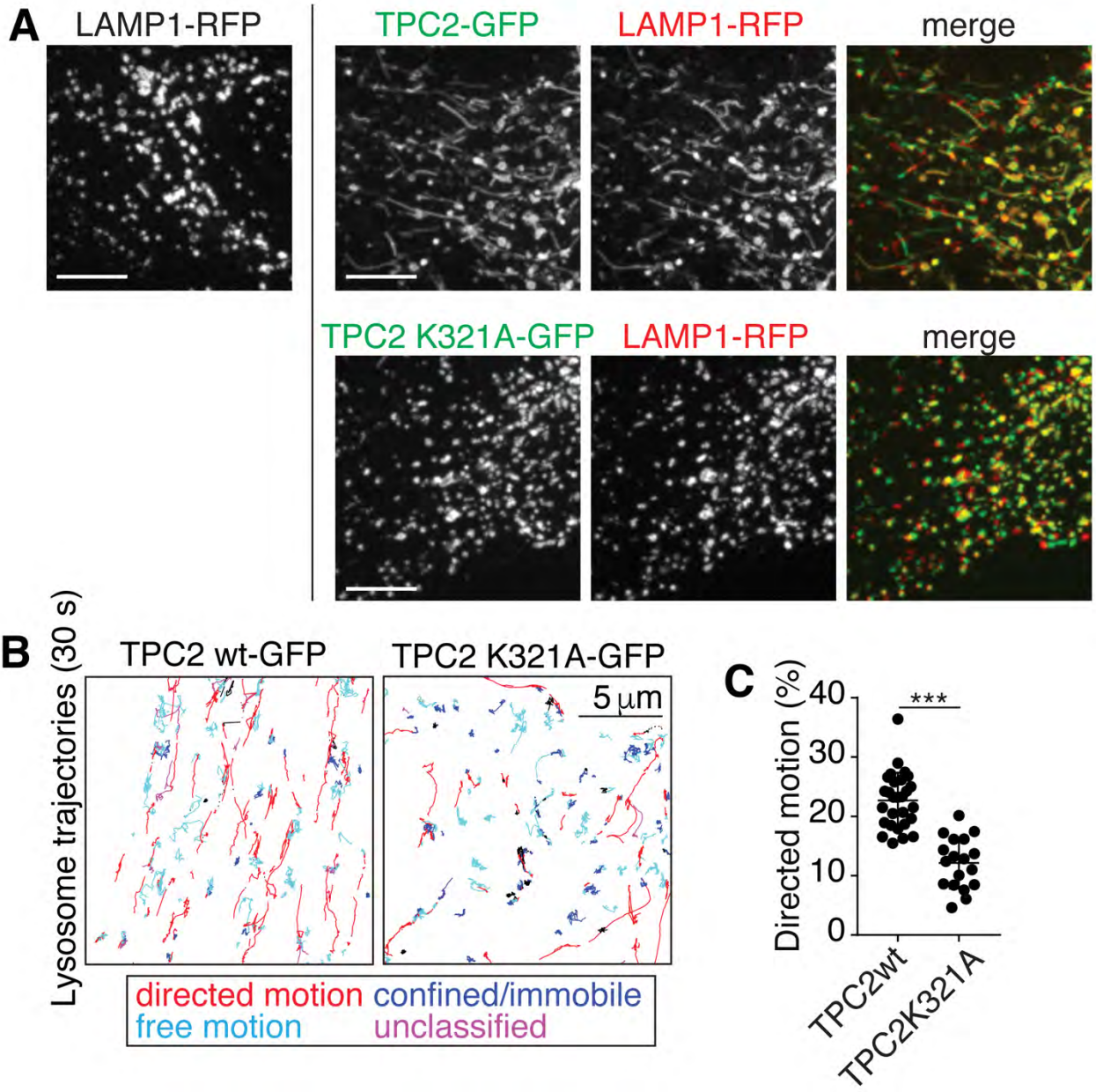

Figure S10. *TPC2-GFP localizes to LAMP1 vesicles and fosters increased motor-based movement*. **A**, HeLa cells expressing LAMP1-RFP alone or along with TPC2wt-GFP or TPC2K321A-GFP. Cells were imaged 8 h after transfection. Scale bar, 5 μm. **B-C**, HeLa cells expressing TPC2wt-GFP to TPC2K321A-GFP were recorded at 10 Hz for 30 s. The trajectories of lysosomes were analyzed by MSS to define their motion as confined (blue), free (cyan), directed (red), or unclassified (magenta). Representative trajectories are displayed in **B**. In **C,** the percentage of trajectories identified as undergoing directed motion in >25 videos of individual cells (each video is represented by one data point), along with the median and upper and lower quartiles are graphed. A Mann-Whitney test was used to determine the *p* value.