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Supplemental Information

PIKfyve Regulates Vacuole Maturation and Nutrient Recovery following Engulfment

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Supplemental Information

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SUPPLEMENTAL FIGURES AND LEGENDS

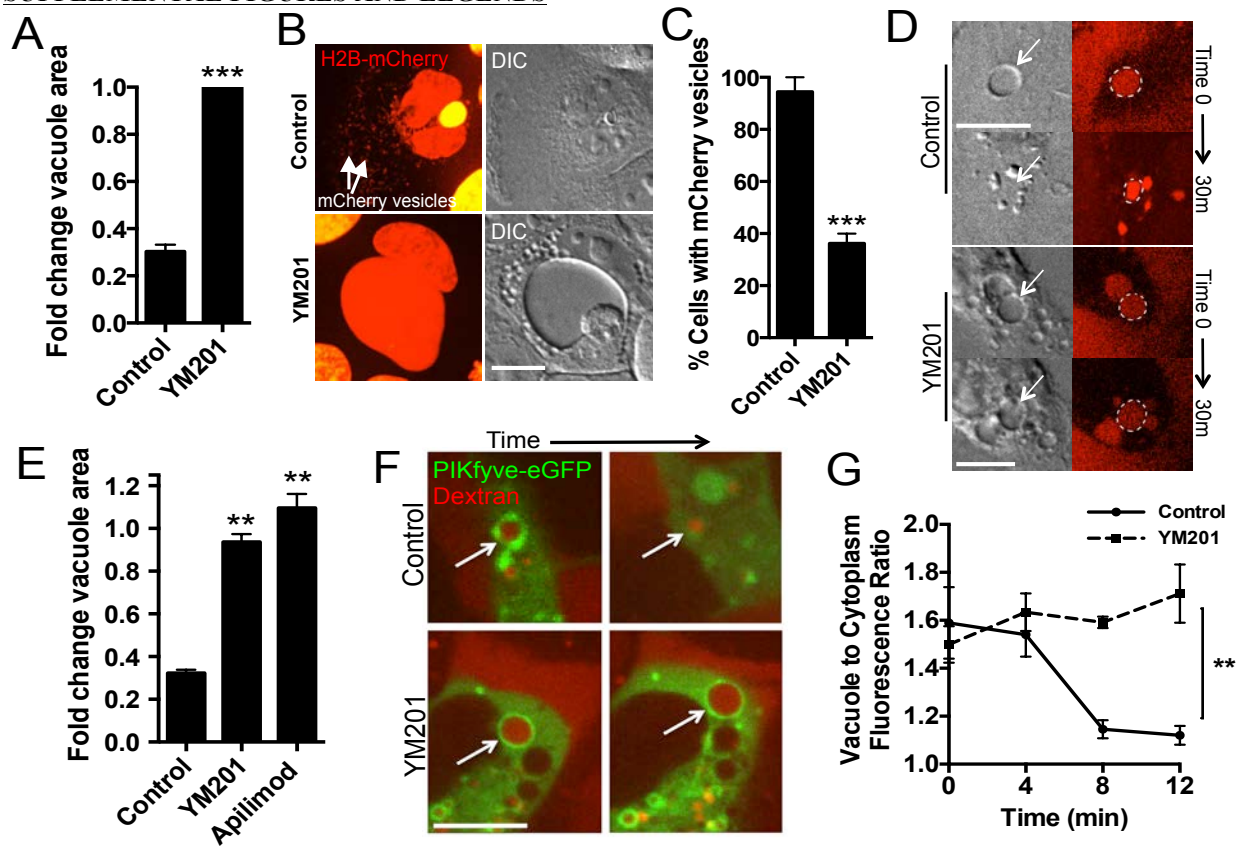

Figure S1. (Related to Figure 1). PIKfyve regulates vacuole shrinkage during entosis and macropinocytosis.

A) PIKfyve inhibition delays entotic vacuole shrinkage in HEK-293 cells. Graph shows fold change in entotic vacuole area after 10 hours for control and PIKfyve-inhibited cells, determined by time-lapse microscopy. Error bars show mean±SEM for n=3 independent experiments. Total cell number analysed for Control n=63, YM201 n=59.
B) mCherry fluorescence from entotic corpses (H2B-mCherry-expressing MCF10A cells) appears in vesicles in the cytoplasm of control cells (H2B-mCherry, left top panel, arrows; DIC right top panel) as a result of fission, which is blocked in PIKfyve-inhibited cells (bottom panel). Scale bars equal 10μm.
C) PIKfyve inhibition reduces the appearance of vacuole-derived mCherry vesicles in the cytoplasm of engulfing cells. Graph shows quantification of percentage of entotic corpses in MCF10A cells containing mCherry vesicles after 10 hours in control and PIKfyve-inhibited conditions. Error bars show mean±SEM for n=3 independent experiments. Total cell number analysed for Control n=18, YM201 n=17.
D) Macropinosome shrinkage is controlled by PIKfyve. Images show TMR-Dextran fluorescence from engulfed macropinosomes in J774.1 macrophages. Note that macropinosomes in control, untreated cells (top panels, left DIC white arrows, right Dextran white circles) undergo rapid shrinkage by 30 minutes, whereas in PIKfyve inhibited cells (YM201-treated, bottom panels, left DIC white arrows, right Dextran white circles) macropinosomes fail to shrink. See Supplemental Movies S2,3. Scale bars equal 10μm.
E) PIKfyve inhibitors YM201 and Apilimod delay macropinosome shrinkage. Graph shows quantification of fold change in macropinosome area after 30 minutes in control, YM201 and Apilimod treated J774.1 macrophages, measured by confocal time-lapse microscopy. Error bars show mean±SEM for n=3 independent experiments, Total cell number analysed for Control n=10, YM201 n=10, Apilimod n=10.
F) PIKfyve localizes to macropinosomes. Images show PIKfyve-eGFP recruitment to dextran-containing macropinosomes in HEK293 cells. Recruitment occurs in a transient manner in control cells (top panel, arrow), and in a prolonged manner in PIKfyve-inhibited cells (bottom panel, arrow). Scale bars equal 10μm.
G) PIKfyve localizes to macropinosomes transiently in control vacuoles and in a prolonged manner in YM201 treated cells. Graph shows ratio of fluorescence intensity of PIKfyve-eGFP on the macropinosome vacuole vs cytoplasm in control and YM201 treated cells. Error bars show mean±SEM, for Control n=3 and YM201 n=3 cells.
For all graphs, **p<0.02, ***p<0.001 (Student's t-test).

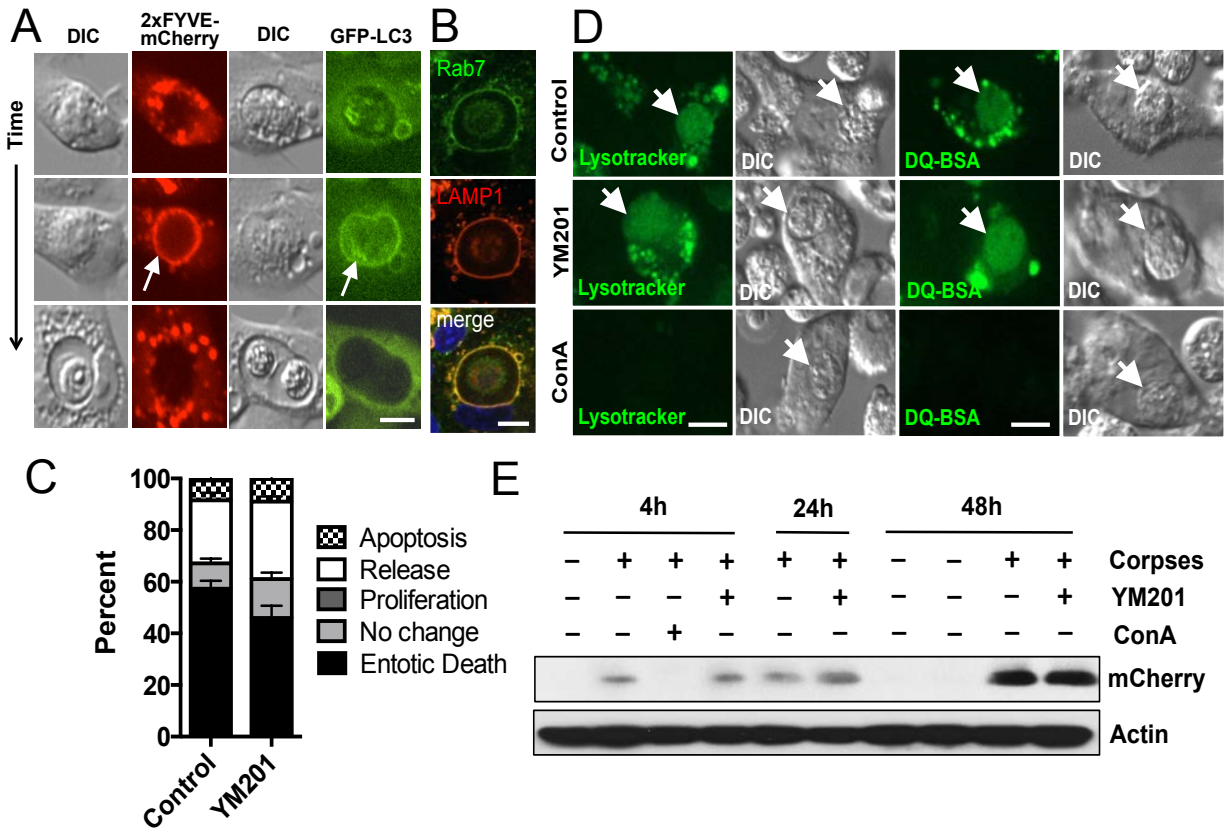

Figure S2. (Related to Figure 1). PIKfyve regulates vacuole shrinkage independent of upstream maturation events and corpse degradation.

A) PIKfyve inhibition has no effect on upstream PI3P formation and LC3 recruitment to the entotic vacuoles. PI3P reporter 2xFYVE-mCherry (DIC first panel, 2xFYVE-mCherry in red second panel, arrow) and GFP-LC3 (DIC third panel, GFP-LC3 in green fourth panel, arrow) localize to entotic vacuoles in PIKfyve-inhibited MCF10A cells. Scale bars equal 10μm.
B) PIKfyve inhibition has no effect on recruitment of Rab7 (green, top panel) and Lamp1 (red, middle panel) in PIKfyve-inhibited MCF10A cells. Scale bars equal 10μm.
C) PIKfyve inhibition has no effect on internalized cell fate during entosis. Graph shows quantification of cell fate as entotic death, no change, proliferation, release and apoptosis in control and PIKfyve-inhibited MCF10A cells. Error bars show mean±SEM for n=6 independent experiments. Total cell number analysed for Control n=387, YM201 n=426. No significant differences in cell fate between control and PIKfyve-inhibited cultures were observed.
D) PIKfyve inhibition has no effect on lysotracker positivity (Left panel, Green, DIC, arrow) and DQ-BSA fluorescence (Right panel, Green, DIC, arrow) in J774.1 macrophage phagosomes. Note that lysotracker and DQ-BSA fluorescence are blocked by treatment with Concanamycin A (ConA) (bottom panel). Scale bars equal 10μm.
E) PIKfyve inhibition has no effect on apoptotic corpse degradation in J774.1 macrophages. Western blot shows kinetics of mCherry derived from digestion of H2B-mCherry of apoptotic corpses at 4 hours, 24 hours and 48 hours, in the presence or absence of YM201 and ConA.

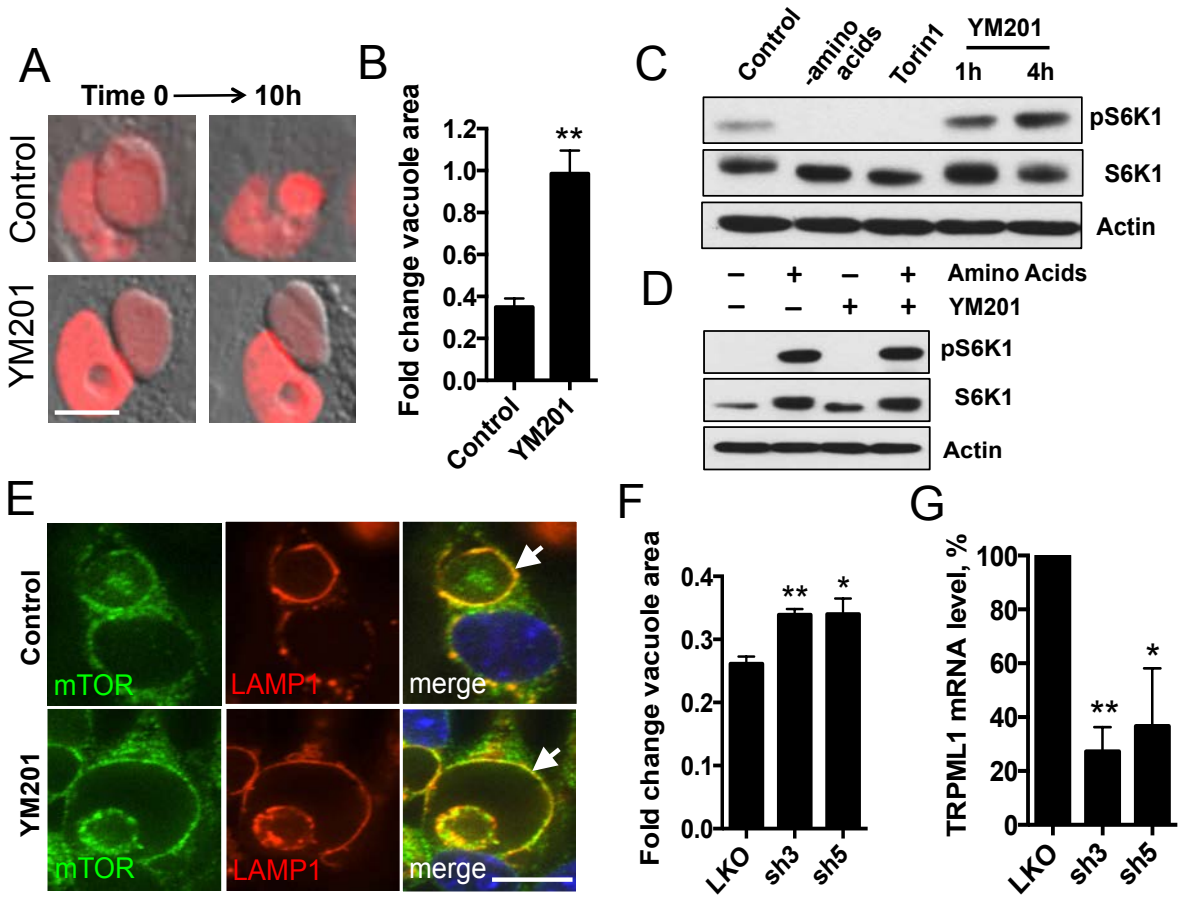

Figure S3. (Related to Figure 2 and 3). PIKfyve regulates vacuole shrinkage in an mTORC1 independent manner through TRPML1.

A) Entotic vacuoles in H2B-mcherry expressing MCF10A cells with digested corpses generated by 16h pre-treatment with Torin1 undergo rapid shrinkage in control cells (upper panel), which is blocked in PIKfyve-inhibited cells (bottom panel). Scale bars equal 10μm.

B) PIKfyve inhibition blocks the shrinkage of vacuoles resulting from prior mTOR inhibition. Graph shows fold change in area of vacuoles pre-treated with Torin1, and then subjected to Torin1 washout and treatment with vehicle control or YM201 for 10 hours. Error bars show mean±SEM for n=3 independent experiments. Total cell number analysed for Control n=74, YM201 n=80.

C) mTORC1 activity in full media is not affected by PIKfyve inhibition in macrophages. Western blot from J774.1 cell lysates shows phosphorylation of mTORC1 target S6-kinase threonine 389 (pS6K) in full media, amino acid-starved, mTOR-inhibited (Torin1), and PIKfyve-inhibited (YM201) conditions, as indicated. Note that PIKfyve inhibition for 1 hour or 4 hours does not reduce pS6K levels.

D) mTORC1 reactivation by amino acids is not affected by PIKfyve inhibition. Western blot of J774.1 lysates shows restimulation of pS6K in amino acid-starved cells, by refeeding with full media for 4 hours, in the presence or absence of YM201. Note that PIKfyve inhibition does not block pS6K restimulation.

E) mTORC1 localization on apoptotic phagosomes is not affected by PIKfyve inhibition. Top panels, mTOR (green) is recruited to apoptotic phagosome where it colocalizes with Lamp1 (red) in J774.1 macrophages. Bottom panels, PIKfyve inhibition for 4 hours has no effect on mTOR localization to Lamp1-positive phagosome (arrow). Panels show confocal microscopic imaging of immunofluorescence staining. Scale bars equal 10μm.

F) shRNA-mediated TRPML1 knockdowns delay entotic vacuole shrinkage in MCF10A cells. Graph shows fold change in entotic vacuole area after 10 hours in control and knockdown cells. Error bars show mean±SEM for n=3 independent experiments. Total cell number analysed for LKO n=60, sh3 n=85, sh5=74.

G) Fold knockdowns of TRPML1 expression by the shRNAs from S3F, determined by quantitative RT-PCR. Error bars show mean±SEM for n=3 independent experiments. For all graphs, *p<0.05, **p<0.02 (Student's t-test).

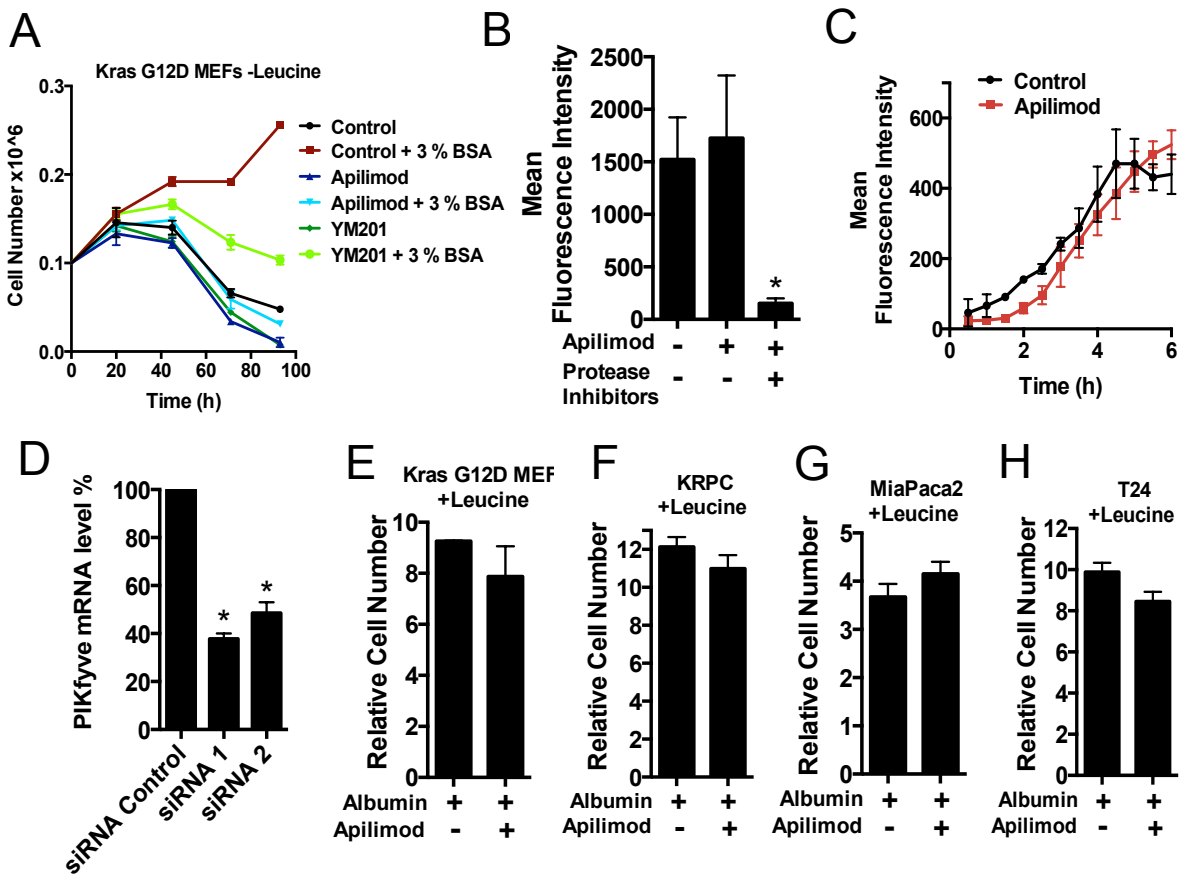

Figure S4. (Related to Figure 5): PIKfyve regulates albumin-dependent growth of mutant Ras cells during starvation while lysosomal degradation and cell growth in full media are unaffected.

A) Rescue of leucine-starved Kras G12D MEF cell growth by engulfment of albumin requires PIKfyve activity. Graph shows kinetics of change in absolute cell number in leucine-free conditions in the presence or absence of 3% albumin supplementation, and treatment with Apilimod (0.1μM) or YM201 (4μM).
B) PIKfyve inhibition does not affect lysotracker staining or BSA degradation in Ras-transformed. Quantification of mean fluorescence intensity of DQ-BSA for Kras G12D MEFs treated as in Figure 5B.
C) Quantification of the kinetics of mean fluorescence intensity of DQ-BSA over 6 hours between control and Apilimod-treated Kras G12D MEFs.
D) Fold knockdowns of PIKfyve expression by the shRNAs from Figure 5C, determined by quantitative RT-PCR.
E) PIKfyve inhibition does not significantly affect the growth of mutant Ras cells in full media conditions. Graphs show relative change in cell number of Kras G12D MEFs, **F)** KRPCs **G)** MiaPaca2 and **H)** T24 in leucine-replete media from day 0 to day 2 with albumin supplementation, in the presence or absence of the PIKfyve inhibitor Apilimod (0.1μM for Kras G12D MEFs and at 0.6μM for KRPC, MiaPaca2 and T24 cells).
All graphs show representatives of three independent experiments, error bars show mean±SD. *$p<0.05$, (Student's t-test).

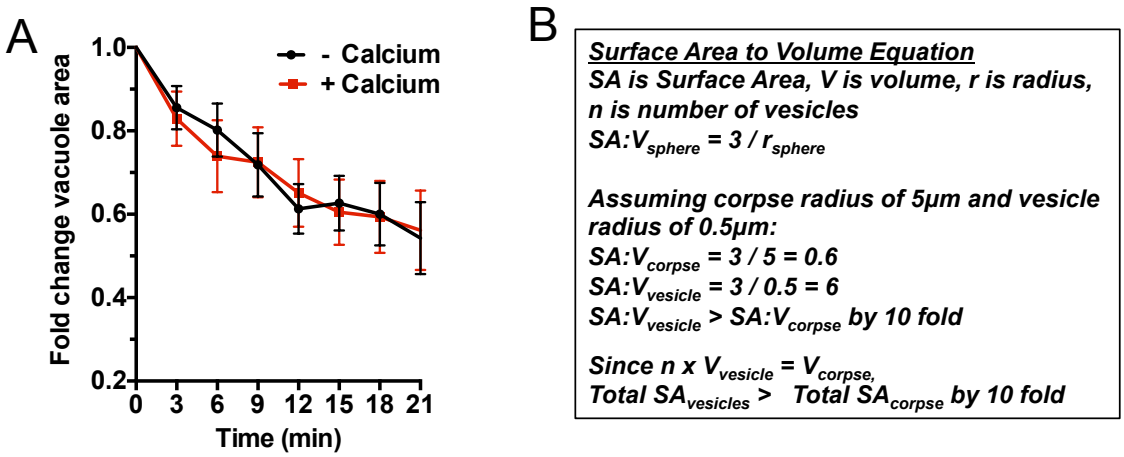

Figure S5. (Related to Figure 3, 4 and Discussion): Role of extracellular calcium during vacuole shrinkage.

A) Calcium free media has no effect on macropinosome shrinkage in J774.1 macrophages. Graph shows fold change in vacuole area over time in calcium free and calcium replete media. Error bars show mean±SD, graph is representative of independent experiments. Total cell number analysed for –calcium n= 5 and +calcium n=5.
B) Surface Area-to-Volume (SA:V) ratio of a vesicle is larger than that of a corpse-containing vacuole, by the fold difference in their radii. Equation shows calculations of SA:V of a sphere with radius of 5µm (approximate size of an entotic vacuole or phagosome) compared to one with radius of 0.5µm (similar to, or larger than, a vesicle budded from a large vacuole), where SA:V ratio is 10-fold larger. Since the total volume of all vesicles is predicted to be similar to the volume of the corpse-containing vacuole, the total surface area of all the vesicles would increase by at least 10-fold. If vesicle size is smaller than 0.5µm radius, then SA:V would increase by more than 10-fold.

SUPPLEMENTAL MOVIE LEGENDS:

Movie S1. (Related to Figure 1). **PIKfyve regulates entotic vacuole shrinkage.** Images show representative time-lapse analyses of entotic vacuoles containing engulfed cell corpses in control and PIKfyve-inhibited cells (YM201 treatment, middle panel; shRNA knockdown, right panel). Note that vacuoles in PIKfyve-inhibited cells fail to shrink over 24 hours despite the degradation of cell corpses. Red fluorescence = H2B-mCherry. Time is shown as hours (hrs).

Movie S2. (Related to Figure 1). **Macropinosome shrinkage in control J774.1 macrophages**. Images show TMR-Dextran fluorescence (DIC left panel, dextran middle panel, white arrow) from engulfed macropinosomes in J774.1 macrophages. Note that macropinosomes undergo rapid shrinkage after acquiring lysotracker positivity (Lysotrocker right panel, white arrow). Images were captured at one minute intervals, total duration of movie is 42 minutes.

Movie S3. (Related to Figure 1). **Macropinosomes fail to shrink in PIKfyve-inhibited J774.1 macrophages.** Images show TMR-Dextran fluorescence (DIC left panel, dextran middle panel, white arrow) from engulfed macropinosomes in J774.1 macrophages treated with YM201. Note that macropinosomes fail to undergo rapid shrinkage after acquiring lysotracker positivity (Lysotrocker right panel, white arrow). Images were captured at one minute intervals, total duration of movie is 44 minutes.

Movie S4. (Related to Figure 2). **TRPML1 overexpression rescues entotic vacuole shrinkage in PIKfyve-inhibited MCF10A cells**. Note that entotic vacuole in TRPML1-eGFP-expressing cell (green, right cell; note arrow from Fig. 2E) shrinks rapidly while control vacuole in adjacent, non-expressing cell (left cell, note arrow from Fig. 2E) fails to shrink as corpse is degraded. Red = H2BmCherry; green = TRPML1-eGFP. Times are shown as hours:minutes:seconds:milliseconds.

Movie S5. (Related to Figure 3). Hypertonic medium rescues vacuole shrinkage and fission in PIKfyve-inhibited MCF10A cells. Images show vacuoles generated by YM201 pretreatment, in YM201 regular media conditions (left) or YM201 in hypertonic medium (right). Note the vacuole in hypertonic medium undergoes rapid shrinkage and membrane fission as seen by appearance of mCherry vesicles in the cytoplasm. Time is shown as hours.