Supporting Information

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SI Text

Chemicals, Antibodies, Plasmids, and Recombinant Proteins. Chemicals were of research grade and purchased from Sigma Aldrich or Carl Roth, unless stated otherwise. Hexokinase, ATP, creatine phosphate, creatine kinase, and pefabloc were from Roche. Wortmannin (WM) was from EMD Millipore. Pik-93 and mouse anti-PI(4)P IgM (No. Z-P004) were from Echelon Bioscience. Mouse anti-TfR was from Zymed (No. 13–6890). Rabbit anti-Rab5b (No. sc-598), goat anti-cathepsin D (No sc-6486), and rabbit anti-GST (No. sc-459) were from Santa Cruz Biotechnology. Rabbit anti-PI4KIIβ was from Pierce (No. PA5-15277). Affinity-purified inhibitory rabbit anti-Vps34 (1) and purified inhibitory mouse anti-type II PI4K (clone 4C5G) (2) were as previously published. Fab fragments of 4C5G were prepared using the Mouse IgG1 Fab and F(ab′)2 preparation kit (Pierce). The rat anti-murine lysosome-associated membrane protein 1 (LAMP1; clone 1D4B) elevated by J. T. August was obtained from the Developmental Studies Hybridoma Bank under the auspices of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biology, Iowa City, Iowa. The following antibodies were kind gifts: rabbit anti-Rab7 (T. Watts, University of Toronto, Toronto, ON, Canada) (3) and rabbit anti-PI4KIIα (P. de Camilli, Yale University School of Medicine, New Haven, CT) (4).

Plasmids encoding 2xFYVE-GST [two copies of FYVE (Fab1p, YOTB, Vac1p, EEA1) domain from Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) in tandem) (5)] or hexahistidine- and GST-tagged MTM1 (myotubularin 1) (6) were from W. Wickner (Geisel School of Medicine, Dartmouth College, Hanover, NH). Plasmid DNA encoding GST-fusions of PH domains of Centaurin β2, TAPP1 (tandem PH domain-containing protein), FAPP1, or PLCδ1 (phospholipase Cδ1) or full-length mouse GRP1 (general receptor for phosphoinositides 1) (7) were from D. Alessi (University of Dundee, Dundee, United Kingdom). Plasmids encoding a GST-fusion of Rab7-interacting lysosomal protein-C33 (RILP-C33) (8) or hexahistidine-fusions of RabGDI (9) or Sac1p (10) were kindly provided by C. Bucci (University of Salento, Lecce, Italy), O. Ullrich (Hamburg University of Applied Sciences, Hamburg, Germany), and C. Barlowe (Geisel School of Medicine, Dartmouth College, Hanover, NH), respectively. Purified SidC P4C [PI(4)P-binding fragment from *Legionella pneumophila* SidC] was as previously published (11).

Cultivation of Mammalian Cells. High mannose receptor cell line J774E from P. D. Stahl (Washington University in St. Louis, St. Louis, MO) and RAW 264.7 mouse macrophages from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) were grown in DMEM/FCS at 37 °C in a humid atmosphere of 7% CO_2.

Coating of Latex Beads with NeutrAvidin, Fluorescent Labeling of BSA, and Preparation of Ferrofluid. BSA-rho was prepared as in ref. 12. For preparation of BSA-rho-bio, 5 mg/mL BSA in 0.1 M $NaHCO_3$ were mixed with 1.2 mg/mL carboxytetramethylrhodamine-NHS-ester and 1.56 mg/mL of biotin-LC-NHS ester (Thermo Scientific), incubated for 2 h at ambient temperature, and dialyzed against PBS for 2 h at ambient temperature. BSA-bio was prepared by mixing 5 mg/mL BSA in 0.1 M $NaHCO_3$ with 1.56 mg/mL of biotin-LC-NHS ester for 2 h at ambient temperature and dialysis against HB.

Carboxylated 1-μm latex beads (Polysciences) were coated with NeutrAvidin (Pierce) as in ref. 13, with modifications. In brief, 4.6×10^{10} particles/mL were washed twice in Mes (4-morpholineethanesulfonic acid) buffer (50 mM Mes/NaOH, pH 6.8), resuspended in Mes buffer containing 0.3 mg/mL NeutrAvidin, and incubated for 15 min on a rocker at ambient temperature. *N*-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) was added (final concentration: 0.2 mg/mL). After 60 min at ambient temperature, another 0.2 mg/mL of EDAC were added and particles were incubated for additional 60 min at ambient temperature. Tris buffer (1.5 M Tris/HCl, pH 8.8) was added (final concentration: 10 mM Tris). Particles were washed three times in PBS (16,100 × *g*, 4 °C, 5 min), resuspended in storage buffer (PBS, 10 mg/mL BSA, 1× protease inhibitor mixture 0.01% NaN_3), and stored at 4 °C.

Fluorescent and Paramagnetic Labeling of Endocytic Compartments. Labeling of early endosomes was achieved by adding DMEM/FCS containing 3.5 mg/mL calcein and 6 μL/mL ferrofluid (EMG 508, Ferrotec Europe) for 10 min at 37 °C.

Labeling of late endosomes was achieved by adding DMEM/FCS containing 200 μg/mL BSA-rho-bio and 6 μL/mL ferrofluid (pulse) for 10 min at 37 °C, washing cells twice in PBS, and adding DMEM/FCS containing 200 μg/mL BSA-rho-bio. Cells were incubated for an additional 20 min at 37 °C (chase).

Lysosomes were fluid-phase labeled using BSA-rho-bio or calcein. For labeling with BSA-rho-bio, J774E macrophages were incubated for 16 h at 37 °C in DMEM/FCS containing 100 μg/mL BSA-rho-bio. Fluor-containing DMEM/FCS was removed and 6 μL/mL ferrofluid in DMEM/FCS was added for 5 min at 37 °C (pulse). Cells were rinsed twice in PBS, fresh DMEM/FCS was added, and cells were incubated for 120 min at 37 °C (chase). Labeling with calcein was for 10 min (37 °C) in DMEM/FCS containing 3.5 mg/mL calcein and 6 μL/mL ferrofluid (pulse). Cells were washed twice in PBS and incubated at 37 °C for 120 min in fresh DMEM/FCS (chase).

Biochemical Analysis of Phagosome and Endosome Maturation. For immunoblot analysis, different-aged LBPs were harvested from density gradients, adjusted to identical OD_{600} and to 4.3% (wt/vol) sucrose, and pelleted (100,000 × g, 60 min, 4 °C). LBPs were resuspended in SDS-sample buffer [40 mM Tris/HCl (pH 6.8), 4% (vol/vol) glycerol, 0.64% SDS, 0.01% bromophenol blue] and incubated for 5 min at 95 °C before separation and blotting.

Protein contents of purified endocytic compartments were determined using Bio-Rad protein assay. Purified endosomes were adjusted to identical protein concentration, SDS-sample buffer was added, and samples were incubated for 5 min at 95 °C.

To prepare total cell lysates (TCL), cells were washed twice in PBS, resuspended in RIPA [25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS], and incubated for 30 min at 4 °C. Samples were centrifuged (16,100 × *g*, 4 °C, 30 min). Supernatants (TCL) were adjusted to identical protein content and analyzed by immunoblotting.

Separated phagosome, endosome, or TCL proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell Bioscience) and proteins of interest were detected after incubation in blocking buffer [PBS, 5% (wt/vol) nonfat milk, 0.05% Tween] for 30 min at ambient temperature. Primary antibodies used in this study were diluted in blocking buffer as follows: Mouse anti-TfR (ZYMED, No. 13–6890) 1:200, rabbit anti-Rab5b (Santa Cruz Biotechnolgy, No. sc-598) 1:250, goat anticathepsin D (Santa Cruz Biotechnolgy, No. sc-6486) 1:250, rabbit anti-GST (Santa Cruz Biotechnolgy, No. sc-459) 1:500, rabbit anti-Rab7

(T. Watts, University of Toronto, Toronto, ON, Canada) 1:500, rabbit anti-PI4KIIα (P. de Camilli, Yale University School of Medicine, New Haven, CT) 1:250, and rabbit anti-PI4KIIβ (Pierce), 1:250. Primary antibodies were detected by enhanced chemiluminescence (Pierce) using suitable HRP-coupled secondary antibodies (Dianova) diluted in PBST (PBS, 0.05% Tween, 1:5,000).

Detection of Lipid Species on Endosomes by Lipid Probes. Purified endosomes (0.4 mg protein/mL) were incubated under fusion assay conditions in the presence of 2 μM GST-tagged lipid probe or a PI(4)P-binding antibody (1:200) for 60 min. Reactions were adjusted to 1 mL HB/1 mM DTT, set on a magnet, and compartments were washed twice in 1 mL HB/1 mM DTT at 4 °C. For immunoblot analysis, endosomes were harvested, adjusted to equal protein content, and assayed for bound lipid-binding probes by immunoblotting using a rabbit anti-GST antibody (Santa Cruz Biotechnology, No. sc-459, 1:500 in blocking buffer) and a HRP-coupled secondary antibody (goat anti-rabbit, 1:5,000 in PBST). For fluorescence microscopy analysis, lysosomes were harvested, spun onto glass coverslips (1,800 × *g*, 4 °C, 15 min), and fixed in HB containing 4% (wt/vol) FA for 60 min. Visualization of lysosome-associated lipid-binding probes was performed as described for LBPs (*Materials and Methods*).

Analysis of Phagosome and Lysosome Lipids by RP-HPLC-MS. Purified lysosomes (230 μg protein) were prepared as described above and incubated under fusion assay conditions in a reaction of 10 times the standard volume for 60 min at 37 °C. One milliliter of HB was added and lysosomes were pelleted (100,000 × *g*, 60 min, 4 °C). LBPs (18 OD_{600}) were prepared from J774E cells and incubated in a fusion reaction of 200 times the standard volume for 60 min at 37 °C. Reactions were adjusted to 4.3% (wt/vol) sucrose and LBPs were collected by centrifugation (100,000 × *g*, 60 min, 4 °C). LBP and lysosome lipids were extracted as in ref. 14. In brief, LBP or lysosome suspensions were mixed with 500 μL of chloroform/methanol (1:2) and incubated on ice for 10 min. Next, 300 μL of 20% (vol/vol) HCl were added and the phases were separated by centrifugation. The lower organic phase was collected and solvent was removed in vacuum. To deacylate lipids, methylamine in water/methanol/1-butanol (43:46:11) was added and samples were incubated for 50 min at 53 °C (15). Samples were set on ice, 25 μL cold n-propanol were added, and samples were dried in vaccum. Before RP-HPLC-MS analysis, dried lipids were dissolved in 50 μL of solvent A [water containing 5 mM N,*N*-Dimethylhexylamine (DMHA) and 4 mM glacial acetic acid].

RP-HPLC-MS analysis of monophosphorylated PIPs was performed essentially as in ref. 16. An Agilent 1100 HPLC system (Agilent) was used for the separation of PIPs prepared from purified lysosomes on a 250-mm Synergi 4u Hydro-RP 80A column with a particle size of 3 μm (Phenomenex) and 0.5-mm inner diameter using 10-μL injection volumes. Lipid extracts from purified LBPs were separated using two 150 mm Biobasic C18 columns in line (Thermo Fisher). Both column setups were suitable to separate PI(3)P and PI(4)P. Solvents, gradient profile, and flow rates were similar for both column set-ups and are summarized in Table S1. Full-scan MS data were acquired during the first 80 min of the HPLC separation in the negative ion mode on a Bruker Apex Qe FT-ICR mass spectrometer (Bruker) equipped with a 7 Tesla actively shielded magnet and an Apollo Dual ESI/MALDI ion source. The instrumental parameters were as follows: source temperature, 200 °C; datapoints sampling rate, 512k; accumulation time for each scan, 0.3 s. Data acquisition and analysis was performed using the HyStar 3.2 software (Bruker Daltonics). RP-HPLC-MS data were processed using Bruker's Data Analysis 3.4 software. EIC were obtained for the deacylated PIP species at 413.02555 *m/z*.

Analysis of Colocalization of LBPs with Lysosome Contents in Intact Cells. J774E macrophages were incubated with 50 μg/mL BSA-rho in DMEM/FCS for 16 h. Cells were washed twice in PBS and fresh DMEM/FCS was added for additional 120 min at 37 °C to deliver BSA-rho to lysosomes. Cells were washed once in PBS, incubated for 10 min in a suspension of 1 μm NeutrAvidin-coated latex beads in DMEM (6×10^8 particles/mL), washed three times in PBS, and fresh DMEM/FCS was added for 20 min at 37 °C. Cells were washed once in cold PBS, DMEM containing 100 nM WM (or DMSO) was added, and cells were incubated for 30 min at 15 °C. Then, cells were either set on ice or incubated at 37 °C for additional 60 min in fresh DMEM containing either 100 nM WM or DMSO. Eventually, cells were harvested and postnuclear supernatants (PNSs) were prepared. PNSs were adjusted to identical protein concentration and 2xFYVE-GST (final concentration: 4 μM) was added. After 15 min at 4 °C, PNSs were adjusted to 35% (wt/vol) sucrose by addition of HB/62%, overlaid with 1 mL of HB/25% and 200 μL of HB. The resulting density gradients were centrifuged (1,600 × *g*, 4 °C, 30 min) and LBPs were harvested from the HB/25%/HB interface, adjusted to 500 μL HB and 2 mg/mL BSA, and spun and fixed onto glass coverslips using HB/4% FA for 16 h at 4 °C. Samples were quenched by addition of HB/50 mM NH_4Cl and immunostained for phagosome-associated 2xFYVE-GST as described above. The proportions of LBPs colocalizing with the lysosomal tracer or 2xFYVE-GST were determined using Zeiss Axio Observer.Z1 epifluorescence microscope and image analysis by ImageJ.

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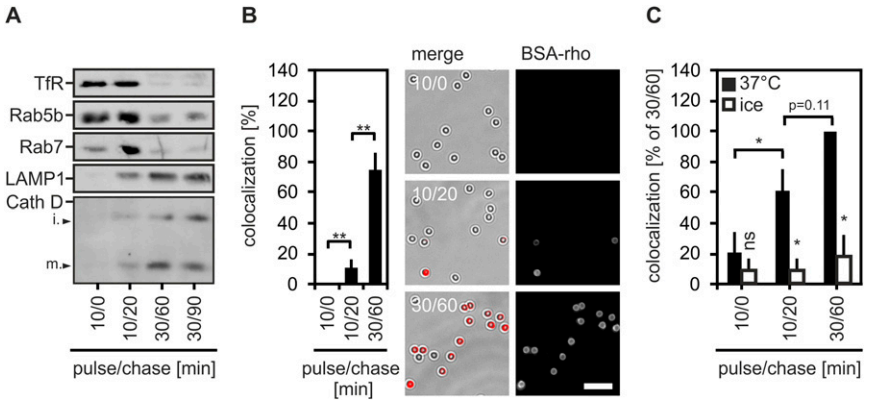

Fig. S1. Biochemical analysis of LBP preparations. (*A*) LBPs were purified from J774E macrophages after pulse-chase periods indicated. Identical numbers (identical OD_{600}) of LBPs were analyzed by immunoblotting. TfR (transferrin receptor); LAMP1 (lysosome-associated membrane protein 1); Cath D (cathepsin D). Positions of Cath D intermediate (i.) and mature (m.) forms are indicated. (*B*) After pulse-chase periods specified, LBPs were purified from J774E macrophages containing BSA-rho-loaded lysosomes, fixed onto coverslips, and analyzed by fluorescence microscopy: per sample, at least 100 LBPs were assayed for co-localization with the lysosomal fluor. (Scale bar, 5 μm.) (*C*) LBPs of different maturation stages were tested for fusion with purified lysosomes in a cell-free assay under conditions specified. Colocalization frequency in samples containing 30 min/60 min-LBPs was set as 100%. Data are means ± SEM from three independent experiments. $*P < 0.05$, $**P < 0.01$.

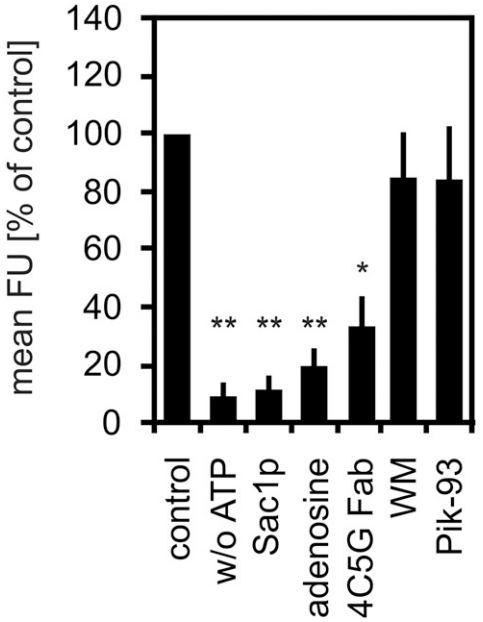

Fig. S2. Impact of PI4K inhibitors on phagosome $PI(4,5)P_2$ levels. Purified LBPs were incubated under fusion-promoting conditions in absence of ATP or in the presence ATP and 32 μM Sac1p, 8 mM adenosine, 12.5 μg/mL 4C5G Fab, 1 μM WM, or 250 nM Pik-93 and analyzed for $PI(4,5)P_2$ content using PLCδ1 PH domain as in Fig. 2*D*. Data are means ± SEM from at least three independent experiments. $*P < 0.05$, $**P < 0.01$ compared with control.

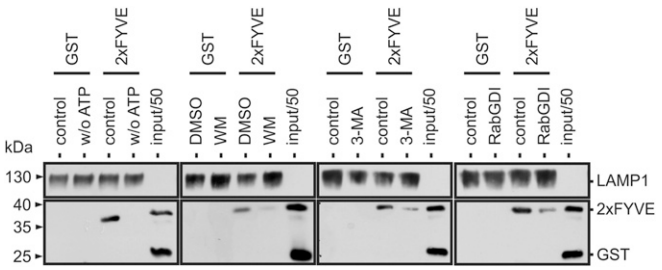

Fig. S3. Analysis of PI3K activity associated with lysosomes. Purified lysosomes were incubated under fusion assay conditions in the presence of 2 μM 2xFYVE or 2 μM GST. Either ATP was omitted (w/o ATP) or WM, 3-MA, or RabGDI were added at the same concentrations as in Fig. 4. Lysosomes were washed and assayed for bound 2xFYVE domain by immunoblotting. LAMP1 staining was used as loading control. Input samples contained both, 2xFYVE and GST.

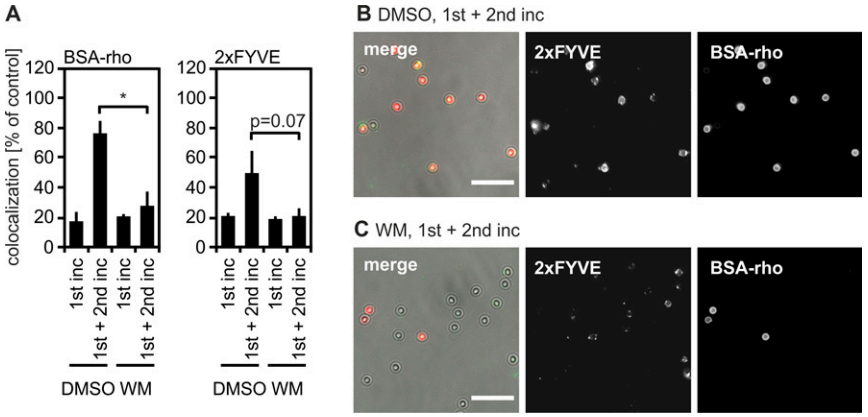

Fig. S4. Late phagosome-to-phagolysosome transition in intact macrophages requires PI3K activity. Lysosomes of J774E cells were pulse-chase labeled with BSA-rho. Cells were incubated with latex beads for 10 min, rinsed three times in PBS, fresh DMEM/FCS was added, and cells were incubated for additional 20 min at 37 °C delivering latex beads into late phagosomes. WM (or DMSO) was added (final concentration: 100 nM WM) and cells were incubated for 30 min at 15 °C. Cells were either set on ice [first incubation (inc)] or incubated for 60 min in DMEM containing 100 nM WM or DMSO (first + second inc). PNSs were prepared, adjusted to identical protein concentration, and 2xFYVE domain was added (4 μM, 15 min, 4 °C). LBPs were purified from the PNSs and assayed for colocalization with the BSA-rho and the lipid-binding probe by fluorescence microscopy. (*A*) Mean colocalization of phagosomes with BSA-rho (phagosome-lysosome fusion) or 2xFYVE-domain (PI(3)P quantification) ± SEM from three independent experiments. (*B* and *C*) Representative micrographs. (Scale bars, 5 μm.) $*P < 0.05$.

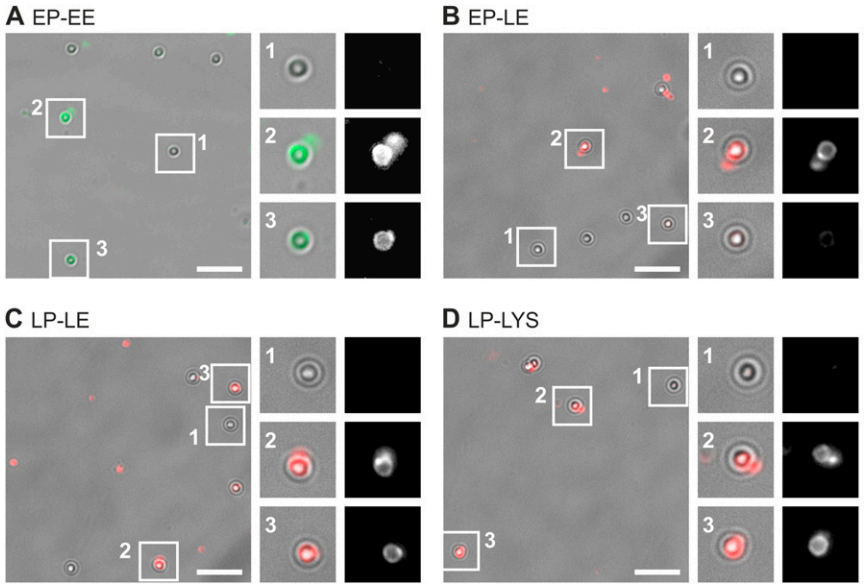

Fig. S5. Micrographs showing LBPs from cell-free fusion reactions between (*A*) early phagosomes and early endosomes, (*B*) early phagosomes and late endosomes, (*C*) late phagosomes and late endosomes, or (*D*) late phagosomes and lysosomes. Fusion of LBPs with endocytic compartments results in colocalization between LBPs and endosomal calcein (*A*) or BSA-rho-bio (*B–D*). Enlargements show LBPs colocalizing (no. 2, 3) or not colocalizing (no. 1) with the endosomal fluor. (Scale bars, 5 μm.) Enlargements (2.2-fold magnification) show PLBs colocalizing (3 and 4) or not colocalizing with the endosomal fluor.

Table S1. Gradient used for the separation of lipids by RP-HPLC-MS

Time (min)	Solvent A (%)	Solvent B (%)
0	85	15
2	85	15
60	65	35
61	0	100
76	0	100
79	85	15
120	85	15

Flow rates: 10 μL/min using (Synergi column), 5 μL/min (Two Biobasic C18 columns in line). Solvents: A, water containing 5 mM N,*N*-Dimethylhexylamine (DMHA) and 4 mM glacial acetic acid; B, methanol containing 5 mM DMHA and 4 mM glacial acetic acid (50:49:1).