PNAS
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Supplementary Information for
Macrophage LC3-associated phagocytosis is an immune defense against *Streptococcus pneumoniae* that diminishes with host aging

Megumi Inomata[1,2], Shuying Xu[1,3], Pallavi Chandra[4], Simin N. Meydani[5], Genzou Takemura[6], Jennifer A. Philips[4], John M. Leong[1]

[1] Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA;
[2] Department of Oral Microbiology, Asahi University School of Dentistry, Mizuho, Gifu, Japan;
[3] Graduate Program in Immunology, Tufts Graduate School of Biomedical Sciences, Boston, United States.
[4] Division of Infectious Diseases, Department of Medicine, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA;
[5] Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts, USA;
[6] Department of Internal Medicine, Asahi University School of Dentistry, Mizuho, Gifu, Japan.

Email: john.leong@tufts.edu

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SI Materials and Methods

Bacterial strains and mutants.

These *S. pneumoniae* strains were grown at 37 °C in 5% CO2 in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract (THY) plus Oxyrase® (Oxyrase Inc.) to mid-log phase (optical density [OD] 0.8). Bacteria were then were frozen at -80 °C in the growth media with 25% (v/v) glycerol. Prior to use, bacterial aliquots were thawed, washed once, and diluted in PBS to the appropriate concentration. Bacterial titers were confirmed by plating on Tryptic Soy Agar plates supplemented with 5% sheep blood agar (Northeast Laboratory Services). Encapsulated *S. pneumoniae* was incubated with 10% mouse serum at 37 °C for 15 min on a rotating rack and used for in vitro assay as described previously (1).

Transfection

BMDMs (2×10^6 cells) were transfected with pGFP-LC3 (5 μg) or siRNA (1 μM), using the Nucleofector™ 2b electroporator (Lonza) and the accessory program Y-001. Cells were then were maintained in basal medium for 24 h. Transfection of pGFP-LC3 into mouse RAW264.7 cells was performed by Lipofectamine 2000 reagent (Thermo Fisher Scientific). Mouse RAW264.7 cells stably transfected with pGFP-LC3 were maintained in basal medium with 1 mg/ml G418 (Roche Applied Science, Indianapolis, IN, USA). The Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) and Opti-MEM I medium (Thermo Fisher Scientific) were used to transfect 100 nM of siRNA for mouse RAW264.7 cells, per the manufacturer's protocol. After 12 h of incubation, culture media were changed to DMEM supplemented with 10% of FBS, and incubation continued for 12 h.

Bone-marrow derived macrophages (BMDMs) and mouse RAW264.7 cells.

Bone marrow cells were seeded in 100-mm diameter culture dishes (1×10^6 cells) for incubation for 2 days in 10 ml of the differentiation medium. On day 3, 5 ml of fresh differentiation medium was added, and cultivation continued for 2 days. Nonadherent cells were aspirated, and adherent cells were gently detached, using cell scrapers in the presence of 5 ml of the differentiation medium. The collected cells were plated to the

appropriate density in tissue-culture dishes in antibiotic-free basal medium and incubated overnight prior to infection. RAW264.7 cells (American Type Culture Collection) were cultured in basal medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Western blotting.

BMDMs seeded on 6-well plates (1×10^6 cells/well) were infected with *S. pneumoniae* at a multiplicity of infection (MOI) of 50, washed with PBS, and lysed with 300 μl of the lysis buffer at 4 °C for 15 min. The collected lysates were centrifuged, and the supernatants were boiled with sodium dodecyl sulfate (SDS) sample buffer for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (10–20% gradient gel) under reducing conditions. Separated proteins in gels were transferred to Trans-Blot Turbo Mini PVDF Transfer Pack (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a Trans-Blot Turbo transfer device (Bio-Rad Laboratories; constant 25 V for 10 min). The membranes were blocked in 5% skim milk for 1 hr. Immunoreactive bands were detected using the following primary antibodies with or without a horseradish peroxidase (HRP)-conjugated secondary antibody: anti-LC3 mouse monoclonal Ab (mAb)-HRP-Direct T (M186-7) and anti-tubulin rabbit polyclonal Ab (pAb)-HRP-Direct T (PM054) were obtained from MBL; anti-phospho-p70S6K rabbit pAb (p-p70S6K; 9205), anti-Atg5 rabbit pAb (8540), anti-Atg7 rabbit pAb (2631), autophagy induction (ULK1 Complex) antibody sampler kit (46486), and autophagy vesicle nucleation antibody sampler kit (70751) were obtained from Cell Signaling Technology. The SuperSignal West Pico Substrate (Thermo Fisher Scientific) was used to visualize the blots with a G-Box camera system (SynGene). Densitometric quantification of the immunoblot bands was performed in ImageJ (National Institutes of Health). For LC3 detection, 100 nM Bafilomycin A1 (BafA1) was added to the medium.

Electron microscopy.

BMDMs cultured on 6-well plates or 2-well glass chamber slide (Eppendorf) at a density of 1×10^6 cells/well were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate

buffer (pH 7.2) or 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), respectively. After this primary fixation, the cells were rinsed three times in fresh fixation buffer for 10 min each time and were secondarily fixed with 1.0% osmium tetroxide in distilled deionized water (ddH_2O) for 1 hr at room temperature. The cell cultures were then washed again three times in ddH_2O; the cells cultured on 6-well plates were then gently scraped from the culture plates and transferred to microfuge tubes. Cells were then dehydrated through a graded series of ethanol (10% to 100%; three changes), and transferred to 100% ethanol with SPI-Pon 812/Araldite 502 resin (50/50 v/v) for 12 h at room temperature. Following infiltration, the cells were transferred three times in pure SPI-Pon 812/Araldite 502 epoxy resin for 1 hr each. Cells were then placed in the embedding resin mixture, pelleted, and polymerized for two days at 68 °C in microfuge tubes. Ultrathin sections (approximately 70 nm thick) were collected onto 200 mesh copper support grids and contrasted with uranyl acetate and lead citrate and then imaged using an HT7700 (Hitachi), under 80 Kv or 100 Kv accelerating voltage. Images were recorded with a Gatan Erlangshen CCD Digital camera. The UMass Medical School Electron Microscopy Core Facility were utilized to generate preliminary images.

Immunofluorescence.

Fixed cells were blocked overnight with 3% BSA/PBS in PBS for 0.5 hr at room temperature. Cells were then stained with pAbs to *S. pneumoniae* and an Alexa 350-conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific) to label only extracellular bacteria. Cells were permeabilized with 50 µg/ml digitonin (sigma)/PBS for 5 min and washed the cells three times with PBS. The cells were blocked with 3% BSA/PBS for 0.5 hr at room temperature, and then stained again with pAbs to *S. pneumoniae* and an Alexa 568-conjugated anti-rabbit IgG antibody to label total bacteria. In this way, intracellular *S. pneumoniae* were only visible in the red channel, while extracellular bacteria were visible in both the red and blue channels. Stained cells were embedded in the presence of the ProLong Diamond Antifade reagent (Thermo Fisher Scientific). Images were captured using a Zeiss Axio Observer.Z1 (Zeiss, Oberkochen, Germany) fluorescent microscope with Colibri.2 LED light source, an Apotome.2 (Zeiss) for optical sectioning, and an ORCA-R^2 digital CCD camera (Hamamatsu). We used the following antibodies:

anti-LC3 mouse mAb (M152-3) from MBL, anti-*S. pneumoniae* rabbit pAb (NB100-64502) from NOVUS biologicals, anti-PLY antibody mouse mAb (sc-80500) and anti-p40 ^{phox} rabbit pAb (sc-1825-R) from Santa Cruz, anti-LAMP-2 rat pAb (ABL-93) from the Developmental Studies Hybridoma Bank, and anti-NOX2/gp91^{phox} rabbit pAb (ab80897) from Abcam. Inhibitors were added to the media 0.5 hr before infection and used during infection: 2.5 mM 3-MA and 10 μg/ml DPI. At least 100 bacteria were counted for each condition in each experiment. BMDMs were challenged with Zymosan A Bioparticle Alexa Fluor 594 conjugate (Z23374; Thermo Fisher Scientific) at 2 μg per well.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

BMDMs cultured in a 24-well culture plate (1×10^5 cells/well) were infected with *S. pneumoniae* at an MOI of 50. Total RNA was prepared from cells using a NucleoSpin RNA (TaKaRa). One microgram of total RNA was reverse-transcribed using ReverTraAce reverse transcriptase (TOYOBO) with oligo21dT and random hexamer primers. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) on a CFX Connect Real-Time PCR detection system (Bio-Rad Laboratories). Primer sets were obtained from QIAGEN. We confirmed that there was no critical difference between the values normalized to the levels of three house-keeping genes, *Ppia*, *Actb*, and *Gapdh*. The results shown were normalized to the level of *Ppia*.

Lactate dehydrogenase release assay.

Cytotoxicity of *S. pneumoniae* towards BMDMs was assessed by measuring lactate dehydrogenase (LDH) released into culture supernatants. Prior to infection, the culture medium was changed to DMEM without phenol red. Released LDH was colorimetrically measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). Cytotoxicity (%) was calculated as 100 × [(experimental LDH release) - (control LDH release) / (maximum LDH release) - (control LDH release)], where values for control and maximum LDH release were obtained from non-stimulated cells and completely lysed cells, respectively, using 0.9% Triton X-100.

SI Reference

1. Gordon SB, Irving GR, Lawson RA, Lee ME, & Read RC (2000) Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. *Infect Immun* 68(4):2286-2293.

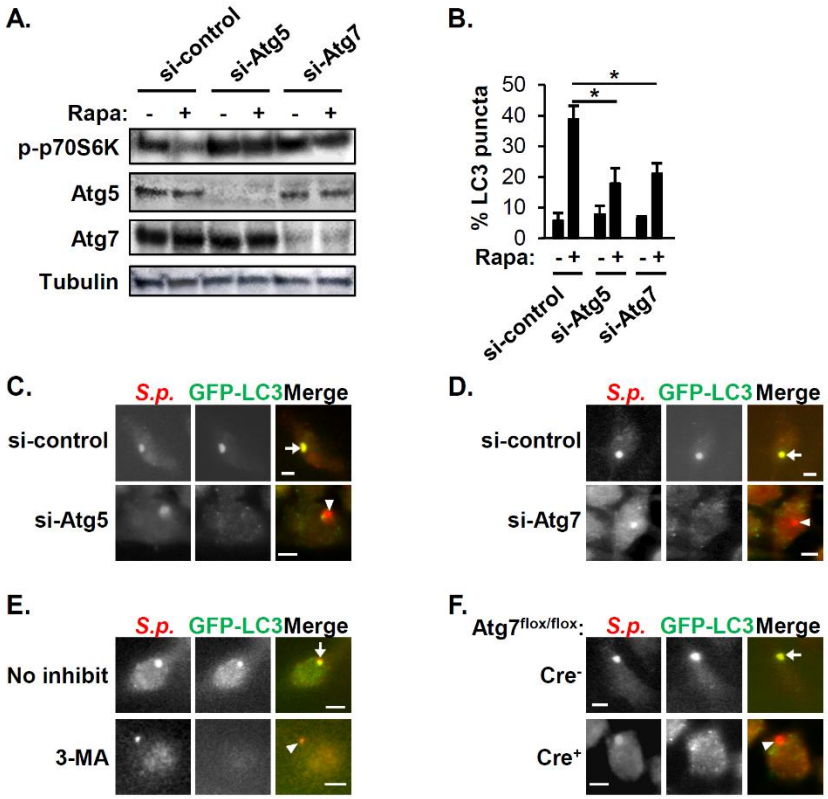

Fig. S1. Gene silencing of Atg5 or Atg7 in RAW264.7 macrophages eliminates rapamycin-induced autophagic responses.
A and B. RAW264.7 macrophages stably expressing GFP-LC3 were transfected with indicated siRNAs for 48 h. These cells were treated with 200 nM Rapa for 6 hr. Cell lysates were immunoblotted to detect p-p70S6K, Atg5, Atg7, or tubulin **(A).** The percentage of LC3 puncta was quantified **(B)**. * $p < 0.05$, one-way ANOVA with Dunnett's test.
C and D. IF images of LC3 and *S.p*. at 1 hr post-infection in RAW264.7 macrophages stably expressing GFP-LC3 transfected with siRNA for control, *Atg5* **(C)**, or *Atg7* **(D)** for 48 h. Shown are representative of three experiments. Arrows: intracellular *S.p.* colocalized with LC3; arrowheads: intracellular *S.p.* not colocalized with LC3. Scale bar = 5 µm.
E. IF images of LC3 and *S.p*. at 1 hr post-infection in RAW264.7 macrophages stably expressing GFP-LC3 untreated or pretreated with 3-MA for 0.5 hr. Shown are representative of three experiments. Arrows: intracellular *S.p.* colocalized with LC3; arrowheads: intracellular *S.p.* not colocalized with LC3. Scale bar = 5 µm.
F. IF images of LC3 and *S.p*. at 1 hr post-infection in BMDMs isolated from *Atg7*-KO (*Atg7*$^{flox/flox}$-LysM-Cre^{+}) or control (*Atg7*$^{flox/flox}$-LysM-Cre^{-}) mice transfected with GFP-LC3. Shown are representative of three experiments. Arrows: intracellular *S.p.* colocalized with LC3; arrowheads: intracellular *S.p.* not colocalized with LC3. Scale bar = 5 µm.

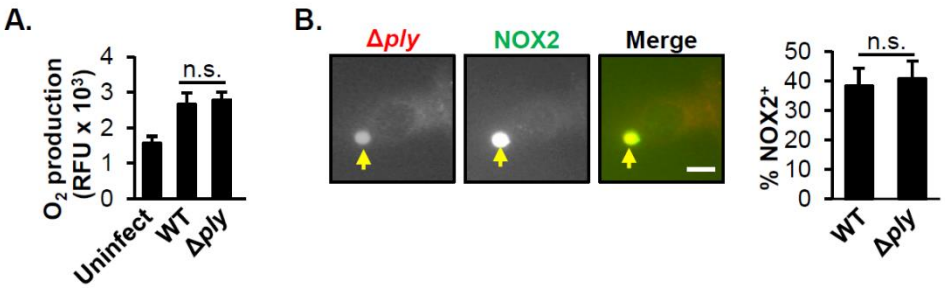

Fig. S2. Δ*ply* produces ROS equivalent to WT.
A. BMDMs were treated with WT or Δ*ply* for 1.5 hr. The cells were stained with CellROX reagent. Fluorescense was read on a microplate reader. Shown are the mean ± SEM from one of three independent experiments. "n.s.", not significant by Student's *t* test.
B. BMDMs were incubated with WT or Δ*ply* for 1 hr and subjected to immunofluorescence microscopy. Arrows indicate *S.p.* colocalized with NOX2 ($gp91^{phox}$). Scale bar = 5 μm. The percentage of *S.p.* that colocalized with NOX2 ($gp91^{phox}$) was quantified. Shown are the mean ± SEM from one representative experiment from at least two independent experiments. "n.s.", not significant by Student's *t* test.

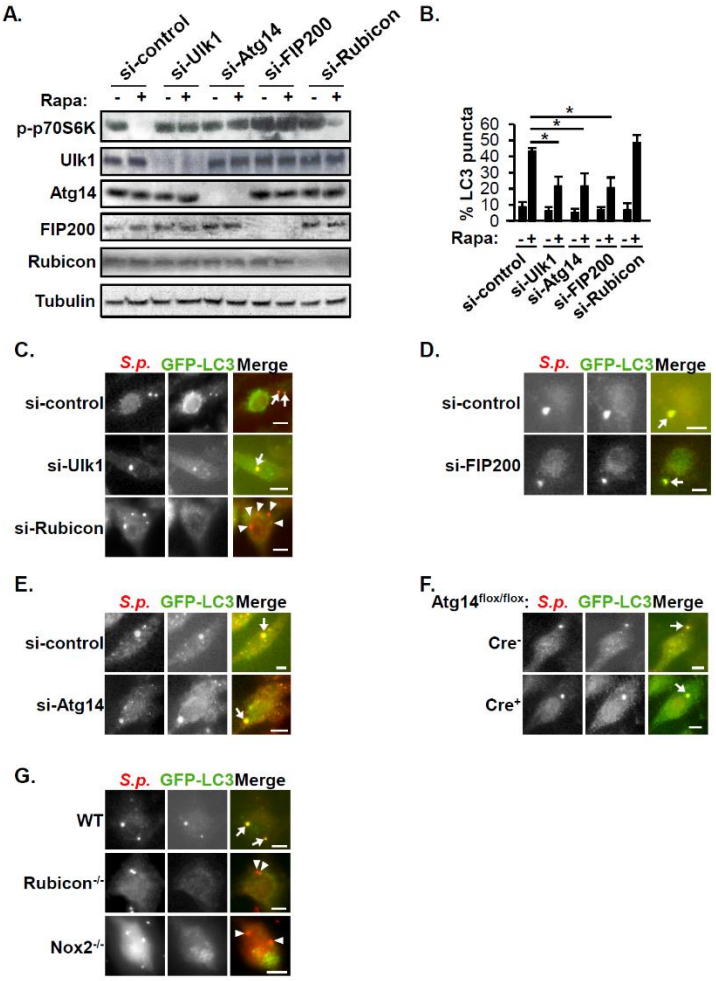

Fig. S3. Gene silencing of Ulk1, Atg14, or FIP200 in RAW264.7 macrophages eliminates rapamycin-induced autophagic responses.
A and B. RAW264.7 macrophages stably expressing GFP-LC3 were transfected with indicated siRNAs for 48 h. These cells were treated with 200 nM Rapa for 6 hr. Cell lysates were immunoblotted to detect p-p70S6K, or tubulin (**A**). The percentage of LC3 puncta was quantified (**B**). * $p < 0.05$, one-way ANOVA with Dunnett's test.
C-E. IF images of LC3 and *S.p*. at 1 hr post-infection in RAW264.7 macrophages stably expressing GFP-LC3 transfected with control siRNA, *Ulk1* siRNA or *Rubicon* siRNA (**C**); control siRNA or *FIP200* siRNA (**D**); control siRNA or *Atg14* siRNA (**E**) for 48 h. Shown are representative of three experiments. Arrows: intracellular *S.p.* colocalized with LC3; arrowheads: intracellular *S.p.* not colocalized with LC3. Scale bar = 5 μm.
F and G. IF images of LC3 and *S.p*. at 1 hr post-infection in BMDMs isolated from *Atg14*-KO (*Atg14*$^{flox/flox}$-LysM-Cre^{+}) or control (*Atg14*$^{flox/flox}$-LysM-Cre^{-}) mice (**F**); WT, *Rubicon*$^{-/-}$, and *Nox2*$^{-/-}$ (**G**) transfected with GFP-LC3. Shown are representative of three experiments. Arrows: intracellular *S.p.* colocalized with LC3; arrowheads: intracellular *S.p.* not colocalized with LC3. Scale bar = 5 μm.

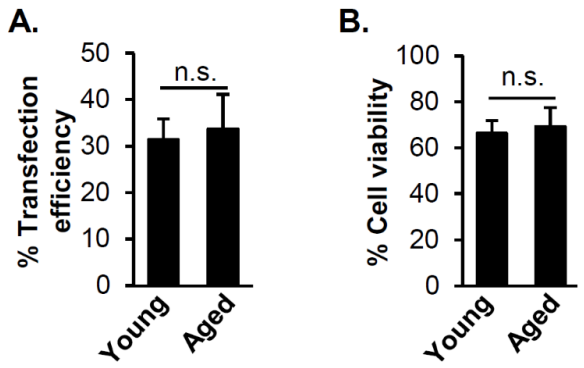

Fig. S4. Young and aged BMDMs display no differences in transfection efficiency or post-transfection cell viability.
A. BMDMs (1× 10^6 cells) were transfected with GFP-LC3 (2 µg) using the Nucleofector™ 2b electroporator (Lonza) and the accessory program Y-001. Cells were then were maintained for 24 h. Total and GFP-expressing cells were counted, and then the transfection efficiency was expressed as percentage of cells expressing GFP. Data show the mean ± SEM of one representative experiment from at least three independent experiments. “n.s.”, not significant, Mann–Whitney U test.
B. Cell viability was assessed by measuring lactate dehydrogenase, and expressed as percentage of live BMDMs. Data show the mean ± SEM of one representative experiment from three independent experiments. “n.s.”, not significant, Mann–Whitney U test.

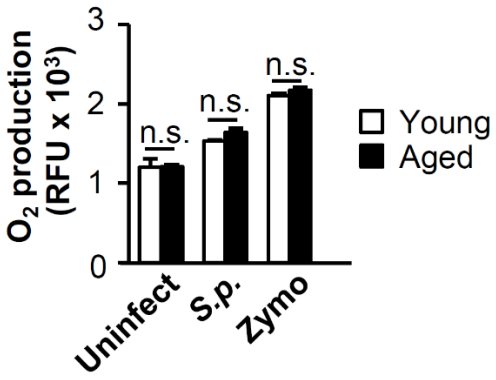

Fig. S5. Young and aged BMDMs produce equivalent ROS upon infection by *S. pneumoniae*. Young and aged BMDMs were treated with *S.p.* or Zymo for 1.5 hr. The cells were stained with CellROX reagent and fluorescense was read on a microplate reader. “n.s.”, not significant, Mann–Whitney U test.

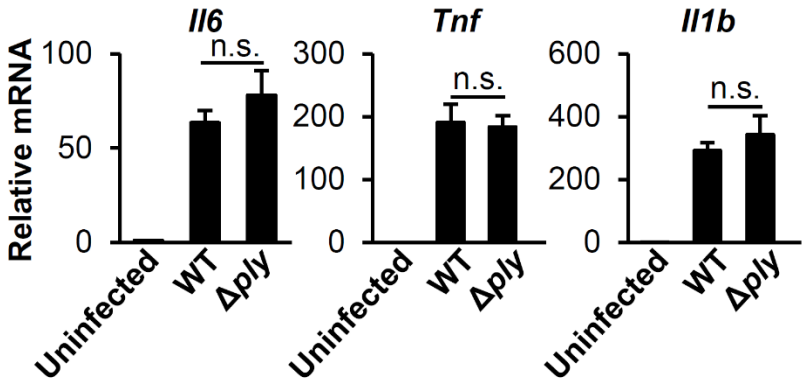

Fig. S6. *S. pneumoniae* TIGR4 Δ*ply* infection of BMDMs induces wild-type levels of cytokines. BMDMs were infected with WT or Δ*ply* for 1.5 hr. RNA was extracted and the levels of cytokines relative to uninfected BMDM were determined by qRT-PCR. Shown are the mean ± SEM of one of three independent experiments. "n.s.", not significant by Student's *t* test.

Table S1. *S. pneumoniae* are found in single membrane vacuoles in BMDMs.

Induction method	Number of cells examined	Total vacuoles	Single membrane (% of total)	Double membrane (% of total)
Zymosan	6	19	19 (100%)	0 (0%)
Rapamycin	6	8	0 (0%)	8 (100%)
S.p.	6	11	11(100%)	0 (0%)

Six cells were randomly selected from each group and all vacuoles contained in those cells were scored for single- or double-membranes. The scoring could not be performed blindly due to our inability to blind the scorer to identity the induction method, which was apparent visually in each image. Nevertheless, *p* values, calculated using the Chi-square test, would be < 0.05 for comparison of *S.p.*-induced with Rapa-induced vacuoles.