



## Supplementary Materials for

### **The *Legionella* Effector RavZ Inhibits Host Autophagy Through Irreversible Atg8 Deconjugation**

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## **Materials and Methods:**

### **Bacterial strains and plasmids**

*Legionella* strains were grown on charcoal yeast extract plates as described previously (16). *L. pneumophila thyA* (Lp02) (17), a thymidine auxotroph derived from the *L. pneumophila* serogroup 1 strain Lp01, was used, along with isogenic strains Lp03 (*dotA*) (17),  $\Delta$ *flaA* (18) and pentuple and single chromosomal deletion strains (12). Bacteria were cultured for 48 h on charcoal-yeast extract agar plates supplemented with thymidine (100  $\mu$ g/ml). The plasmid pJB1806 (19) was used to produce RavZ (AAU27763.1), Ceg23 (AAU27701.1), Lem12, (AAU27705.1), Lpg1639 (AAU27719.1), SidB (YP\_095669.1), Lpg1654 (AAU27734.1), LegL3 (YP\_095687.1), Lpg1663 (AAU27743.1), Ceg24 (AAU27746.1), Lpg1684 (AAU27764.1) and BlaM-RaIF (AAL23711.1) in *Legionella*. These genes were ligated into the vector using BamH1 and Sal1 sites added during the PCR amplification. The plasmid pDEST-17 (Invitrogen) was used for all His-tagged constructs and the plasmid pCDNA-DEST-53 (Invitrogen) was used for all GFP fusion constructs. The plasmid pSR47S (20) was used to generate the RavZ deletion mutant in Lp02.

### **Mice and bone marrow-derived macrophages**

C57BL/6 mice were purchased from Jackson Laboratories. GFP-LC3 transgenic mice were used with the permission of Dr. Mizushima (21), and provided by the laboratory of Akiko Iwasaki (Yale University). Bone marrow was collected from the femurs and tibiae of mice and cultured as described previously (22). Following differentiation,

macrophages were replated in RPMI 1640 from GIBCO (Carlsbad) containing 10% fetal bovine serum (FBS) and 5% macrophage colony-stimulating factor (M-CSF).

### **Cell culture and transfection**

HEK293 and HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) from Gibco (Carlsbad) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco). Cell lines were cultured at 37 °C in 5% CO<sub>2</sub>. For transfection, cells were added to 12-mm coverslips in 24-well plates at a density of  $2.5 \times 10^4$  cells per well. Cells were transfected with 0.8 µg of each plasmid using Lipofectamine 2000 (Invitrogen) and fixed 18 h after transfection in 4% PFA, permeabilized with methanol and processed for immunofluorescence microscopy as described previously (16).

### **Infection of HEK293 cells**

HEK293 FcγRII (23) cells grown to near confluency in 24-well dishes were infected with opsonized *Legionella pneumophila* strain Lp02 (wild type) or the isogenic mutants at an estimated multiplicity of infection (MOI) of 10 bacteria to 1 host cell. For experiments requiring LC3 immunoblots, infections were done in the presence of Bafilomycin A1 [160nM] (Sigma) as indicated.

### **Isolation of injected and uninjected macrophages**

The *Legionella* Lp02-derived  $\Delta flaA$  strain was used for infection of bone marrow-derived macrophages to avoid flagellin-induced pyroptosis. Macrophages were plated on 10 cm dishes and infected with *Legionella* expressing BlaM-RaIF at an MOI of 50 in the

presence of Bafilomycin [160nM] for 2 hours. The media was then removed and cells were loaded with the fluorescent substrate CCF4/AM, using the LiveBLAzer-FRET B/G Loading Kit (Invitrogen) with 15 mM probenecid, in the dark for 2 hours at room temperature. Cells were then washed and sorted based on the ratio of the signal of 460 nm vs. 535 nm when excited at 415 nm using an iCyt Reflection cell sorter. Immunoblot analysis to determine LC3-II levels was conducted on the sorted cells and compared to uninfected cells.

### **Generation of Atg5-deficient macrophages**

To generate myeloid lineage cell-specific Atg5-deficient mice using the Cre/loxP recombination system, *Atg5<sup>fllox/fllox</sup>* mice (24), which contained two loxP sites flanking exon 3 of the *Atg5* gene, were crossed with B6.129P2-*Ly2z<sup>tm1(cre)lfo</sup>*/J mice (The Jackson Laboratory) expressing Cre recombinase under the control of a *Lyzs* promoter. Bone marrow was harvested from these mice and differentiated into macrophages. Non-expression of Atg5 in these macrophages was confirmed by immunoblot analysis using an Atg5-specific antibody (Sigma). All procedures relating to the care and treatment of the animals were performed in accordance with the NIH guidelines.

### **Macrophage growth curves**

Following differentiation, macrophages were replated in RPMI 1640 from GIBCO (Carlsbad) containing 10% fetal bovine serum (FBS) and 5% macrophage colony-stimulating factor (M-CSF) at a density of 200,000 cells/well in a 24-well dish. Macrophages were infected with *Legionella* at an MOI of 1 for 1 hour, washed 3 times with PBS from GIBCO (Carlsbad) and incubated in fresh media. For collection of *Legionella* from the wells, macrophages were lysed with sterile H<sub>2</sub>O at the indicated time

point. Serial dilutions of the lysates were plated to determine colony-forming units (CFUs).

### **Immunoblotting**

Total cell lysates were prepared by adding cells to Laemmli buffer (100mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 6%  $\beta$ -mercaptoethanol, 0.0025% Bromophenol Blue). Equal amount of proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibody for 1 hour. After 3 washes, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 45 minutes. Signals were visualized with ECL on GE's ImageQuant LAS 4000. LC3 antibody (Novus) was used at 1:3000, Actin antibody (Sigma) was used at 1:200, goat anti-mouse HRP antibody (Invitrogen) was used at 1:3000, and goat anti-rabbit HRP (Invitrogen) antibody was used at 1:3000.

### **LC3 puncta assay, immunofluorescence and fluorescence microscopy**

Cells infected or transfected as described above were incubated in DMEM (HEK293 and HeLa) or RPMI (macrophages) with 10% FBS and 160nM Bafilomycin A1 for 2 hours. Cells were then fixed in 4% PFA, permeabilized with methanol, blocked with goat serum, incubated with primary antibody for 1 hour, incubated with secondary antibody for 45 minutes, and mounted using ProLong Gold (Invitrogen). LC3 antibody (Nanotools) was used at 1:200, GFP antibody (Roche) was used at 1:1000. Rhodamine Red goat anti-rabbit (Invitrogen) and Alexa Fluor 488 goat anti-mouse (Invitrogen) were used at 1:1000. Cells were scored as positive for LC3 puncta if they contained more than 5 puncta. For localization of GFP-LC3 and ubiquitin to the *Legionella* vacuole, GFP antibody (Invitrogen) was used at 1:200 and ubiquitin antibody (Enzo) was used at 1:1000. For GFP-RavZ localization studies HeLa cells were stained using antibodies for

LC3 (Nanotools) and Atg16 (CosmoBio) in conjunction with DyLight-conjugated secondary antibodies (Rockland). Digital images were acquired with a Nikon TE300 microscope using a  $\times 100$  1.4 N.A objective lens and a Hamamatsu ORCA-ER camera controlled by IP Lab software.

### **Long-lived protein degradation assay**

HeLa cells were pulse-labeled with 1 mCi/mL of  $^{14}\text{C}$ -Valine (Moravek Biochemicals Inc.) for 4 hours in RPMI medium supplemented with 10% FCS. Cells were then incubated in chase media (DMEM + 10% FCS + 10 mM L-Valine) for 15 hours before being washed three times in HBSS and incubated either in full media (FM, same as chase media) or in starvation media (Starv.: HBSS + 50 mM Hepes pH 7.4 + 10 mM L-Valine) for four hours. Media and cells scraped in PBS + 1% Triton X-100 were then respectively precipitated in 10% Trichloroacetic acid (TCA) by incubation on ice for 10 minutes. TCA-precipitates were spun 10 minutes at 20 000g at 4°C. TCA-soluble and TCA-insoluble fractions (resuspended in 0.2N NaOH) were separately submitted to scintillation counting. Percent of long-lived protein degradation was obtained by dividing the counts for the TCA-soluble fractions by the combined TCA-soluble and TCA-insoluble fractions counts.

### **Protein expression and purification for in vitro biochemistry**

To reconstitute the ubiquitin-like lipidation reaction of Atg8 homologues in vitro, the mammalian homologues of Atg7, Atg3, and Atg8 proteins were expressed and purified essentially as previously described for the yeast forms of these proteins (13). In brief, a baculoviral expression plasmid carrying mouse Atg7 with an amino-terminal 6 histidine tag was a kind gift from Xuejun Xiang (25). SF9 cells were infected with virus at  $1 \times 10^6$  to  $1 \times 10^7$  pfu/ml and collected after 72-96 hours. Cells were resuspended in Lysis Buffer

(20mM Tris, pH 7.6, 0.5M NaCl, 10% glycerol, 20 mM Imidazole, 1mM  $\beta$ -mercaptoethanol ( $\beta$ -Me) (Sigma)) containing complete EDTA-free Protease Inhibitor Cocktail (Roche) and lysed by sonication. The lysate was cleared by centrifugation and incubated with Ni-NTA Agarose (Qiagen) beads for 2 hours at 4°C. Beads were washed three times against a 10 bead-volume of Wash Buffer (20 mM Tris, pH 7.6, 10 mM NaCl, 20 mM Imidazole, 1 mM  $\beta$ -Me), and protein was eluted with Atg7 Elution Buffer (20mM Tris, pH 7.6, 10mM NaCl, 500mM Imidazole, 1mM  $\beta$ -Me). Purified protein was centrifuged at 900 rpm and 4°C for 5 minutes and the soluble material was stored at -80°C in 20% glycerol.

Mouse Atg3, human Atg4B and each of the mammalian homologues of Atg8 tested were cloned into pGEX-6p. The Atg8 homologues (human GABARAP, human GABARAP L1, human GABARAP L2, human LC3A and rat LC3B) are ordinarily expressed in a pro-form that is processed by Atg4 to cleave off the COOH-terminus and reveal a glycine that is used in the lipidation reaction. To facilitate lipidation in vitro, each protein was expressed as a COOH-terminal deletion such that the reactive glycine is the last residue. Atg3, Atg4B, and each Atg8 homologue were expressed in BI21-DE3 bacteria. Cells were lysed in thrombin cleavage buffer (20 mM Tris, pH 7.6, 1 mM DTT, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>) in the presence of protease inhibitor cocktails and the protein was collected on glutathione beads. The beads were washed with 20 column volumes of thrombin-cleavage buffer. Thrombin was added at 10 units/ml and cleavage was performed for 4 hours at 4°C. Thrombin cleavage removes the protein from the GST tag and results in a protein with wild type sequence (i.e. no additional amino acids). Purified proteins were stored in 20% glycerol at -80°C.

For proteolysis experiments involving soluble protein, three additional proteins were generated. Atg4-mediated processing of full-length GABARAP L1 to expose the COOH-terminal glycine removes only a single COOH-terminal lysine. In order to detect this processing event *in vitro*, we have generated a full-length GABARAP L1 fused to a COOH terminal YFP (GR-YFP). Cloning to generate this construct included the introduction of a *Sall* restriction site such that the amino acid sequence spanning the fusion of the two proteins includes an insertion of the sequence “VD” between the GABARAP L1 and YFP sequences to yield “...DESVYGK**V**DMVSKGEEL...”. We also tested whether rat LC3B proteins carrying short COOH-extensions were substrates for proteolysis. In this case, the plasmid coding for Rat MAP1LC3B<sup>G120</sup> was mutated to introduce a tail beyond the terminal glycine of sufficient length such that its cleavage would be visible on a 12% Bis-Tris SDS-Page gel. Two tail sequences were introduced. The tail on LC3-E1 is unrelated to the natural tail sequence in MAP1LC3B, with the sequence EFIVTD being added after the glycine. The tail on LC3-E2 has the sequence TALGFSDDLPRAFR, which includes the first three natural amino acids after the glycine found in unprocessed MAP1LC3B. Each protein was expressed and purified as described above.

### **Liposomes**

Dioleoyl-phosphatidylethanolamine (DOPE), palmitoyl-oleyl-phosphatidylcholine (POPC), and dioleoyl-phosphatidylserine (DOPS) were mixed in organic solvent at a 55:35:10 ratio and dried to a thin film. The lipids were resuspended in liposome buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 5 mM MgCl<sub>2</sub>), exposed to 7 rounds of snap freezing in liquid nitrogen and thawing in a 37°C water bath and finally were sonicated to produce small unilamellar liposomes. Liposomes were stored on ice for up to 7 days, and freshly sonicated before each use.



### **Lipidation reactions**

Coupling of GR or other homologues was performed essentially as described (13). In brief, GR (1-20  $\mu\text{M}$ ), Atg3 (1-3  $\mu\text{M}$ ), Atg7 (1-3  $\mu\text{M}$ ), and liposomes (1-3 mM lipid) were mixed in microcentrifuge tubes in reaction buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT). Lipidation was initiated by the addition of 1 mM ATP and the samples were moved to 37°C for the indicated time. In some experiments, additional components were added during the reaction (including RavZ and Atg4). Completed reactions were either stored on ice (before being applied to nycodenz gradients) or were immediately mixed with SDS-loading buffer and boiled to stop further reaction.

### **Nycodenz flotation gradients**

To isolate lipidated GR from unlipidated starting protein and other reaction components, completed lipidation reactions were loaded onto a nycodenz gradient. In brief, reactions were mixed 1:1 with 80% nycodenz. The resulting 40% nycodenz mixture (350  $\mu\text{l}$ ) formed the basis of a step gradient that also included a 30% nycodenz (250  $\mu\text{l}$ ) and 0% nycodenz step (50  $\mu\text{l}$ ; each nycodenz solution made up in reaction buffer). Gradients were run for 4 hours at 48000 rpm in an SW-55 Beckman rotor. Proteo-liposomes were recovered as a tight band at the 0%/30% interface.

### **Proteolysis of soluble Atg8 proteins**

To test whether RavZ or Atg4B can process GR-YFP, 5 $\mu\text{M}$  of GR-YFP was incubated with 100nM Atg4B or 700nM RavZ at 37°C in SN buffer (20mM Tris pH 7.6, 100mM NaCl, 5mM  $\text{MgCl}_2$ ). Samples of each reaction were collected at 1, 2, 4, and 16 minutes for Atg4 treated samples; and 10, 30, 60, and 120 minutes for RavZ treated samples. These samples were immediately quenched by mixing with SDS-PAGE buffer and

boiling. To test whether RavZ or Atg4B can process rat LC3B proteins with COOH-terminal extensions, 25  $\mu$ M of each LC3 protein was incubated with either 1.9  $\mu$ M Atg4B or 875 nM RavZ in SN Buffer at 37°C for 30 minutes. The products were run out on SDS-PAGE and imaged by Coomassie staining.

### **Protein labeling**

GR was mutated to introduce an amino-terminal cysteine (forming cys-GR) to allow site-specific labeling with a maleimide-associated fluorophore. Cys-GR was diluted into conjugation buffer (20mM Tris pH 7.10, 100mM NaCl, 5mM MgCl<sub>2</sub>) to a final concentration of 0.5 mg/ml. A hundred-fold excess of TCEP was added to this mixture and the reaction vial incubated at room temperature for one hour. 5 $\mu$ L of Texas Red dye (Invitrogen) dissolved in DMF to a concentration of 8mM was added per 500  $\mu$ L of the conjugation reaction. The reaction was covered with aluminum foil and incubated with shaking at room temperature for one hour, and then moved to 4°C overnight. Following incubation, the reaction was loaded onto an illustra NAP size exclusion column. Labeled GABARAP-L1 protein (TxRed-GR) was separated from unconjugated dye by elution with the conjugation buffer. The protein was passed twice through the column and purity of the dye-labeled GABARAP-L1 was confirmed by SDS-PAGE and fluorescent gel imaging.

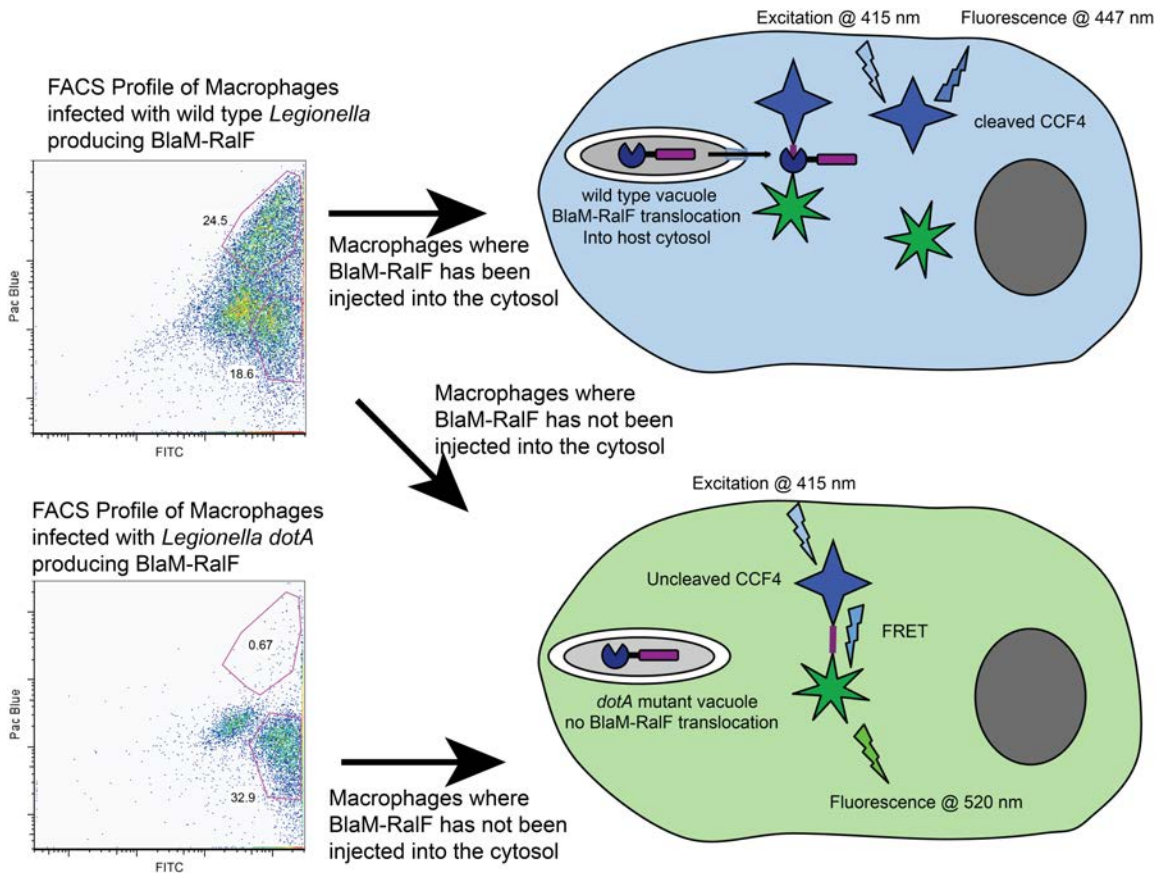
### **Fluorescence anisotropy**

TxRed-GR was coupled to liposomes in vitro by incubation of the reaction components (10 $\mu$ M TxRed-GR, 2.5 $\mu$ M human Atg7, 2.5 $\mu$ M mouse Atg3, 1mM DTT, 1mM ATP, 3mM 55% DOPE sonicated liposomes) at 37°C for 90 minutes. Proteo-liposomes were floated up and collected in SN buffer. For anisotropy measurements, proteoliposomes equivalent to 67.5nM TxRed-GR were added to a quartz cuvette and constant traces of

anisotropy and polarization were recorded on a PC1 benchtop spectrofluorimeter (ISS; Champaign, IL) at 575 nm excitation with a 625 nm longpass emission filter. Once a baseline anisotropy was obtained, RavZ or Atg4 was added at different concentrations and the delipidation reaction was followed in real-time for 10 minutes.

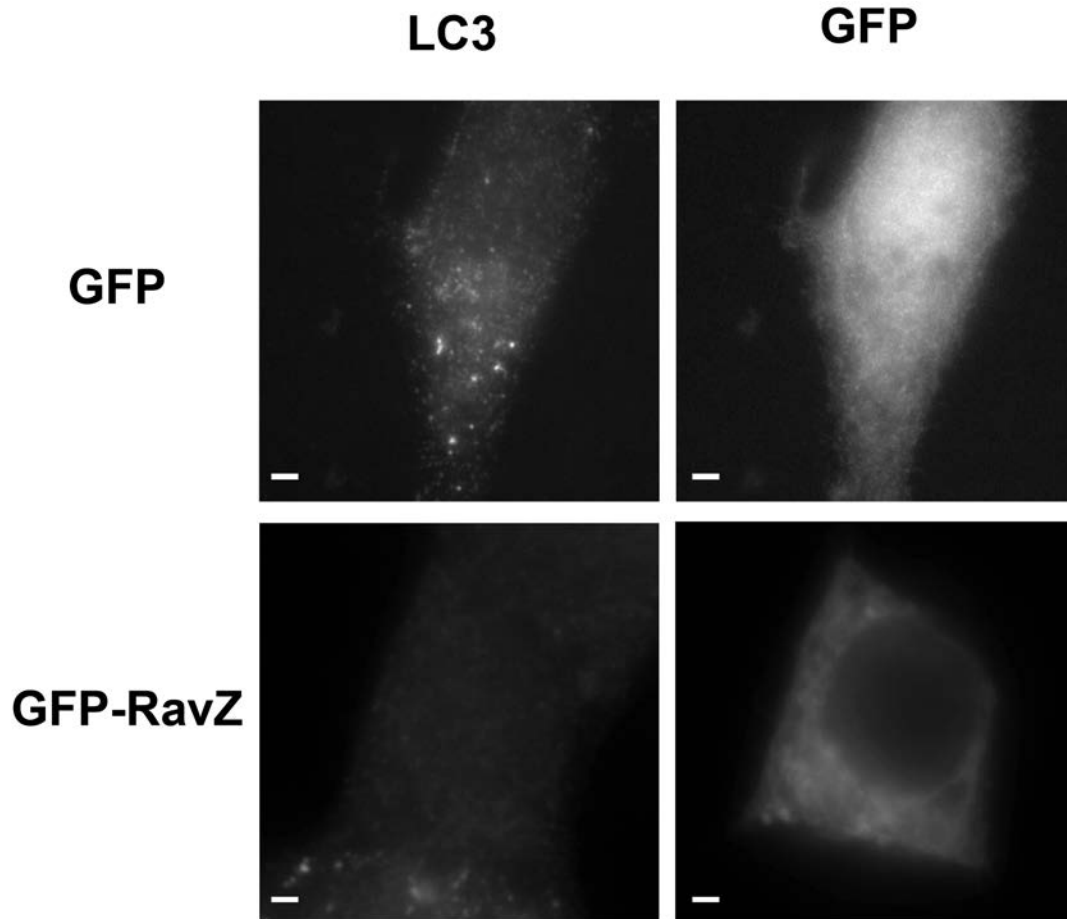
### **Mass spectrometry of in vitro decoupling reactions**

The sequence exposed at the COOH terminus of GR following RavZ mediated deconjugation was determined by mass spectrometry (LC-MS/MS). RavZ (350 nM) was mixed with free GR (GR with a COOH-terminal glycine; 13  $\mu$ M) or with GR-decorated liposomes isolated from a nycodenz gradient (approximately 13  $\mu$ M total GR protein). Digestion continued for 60 minutes at 37°C. Samples were then mixed with SDS-PAGE sample buffer, boiled, and run on a 12% Bis-Tris gel. Controls lacking RavZ were also included. Each lane contained approximately 7  $\mu$ g of GR. GR bands were cut out of the gel with a methanol-rinsed, previously unused razor blade and stored in methanol-rinsed microcentrifuge tubes at -20°C until processing. LC-MS/MS and subsequent analysis of each sample was carried out at the Yale W.M. Keck Foundation Proteomics center.



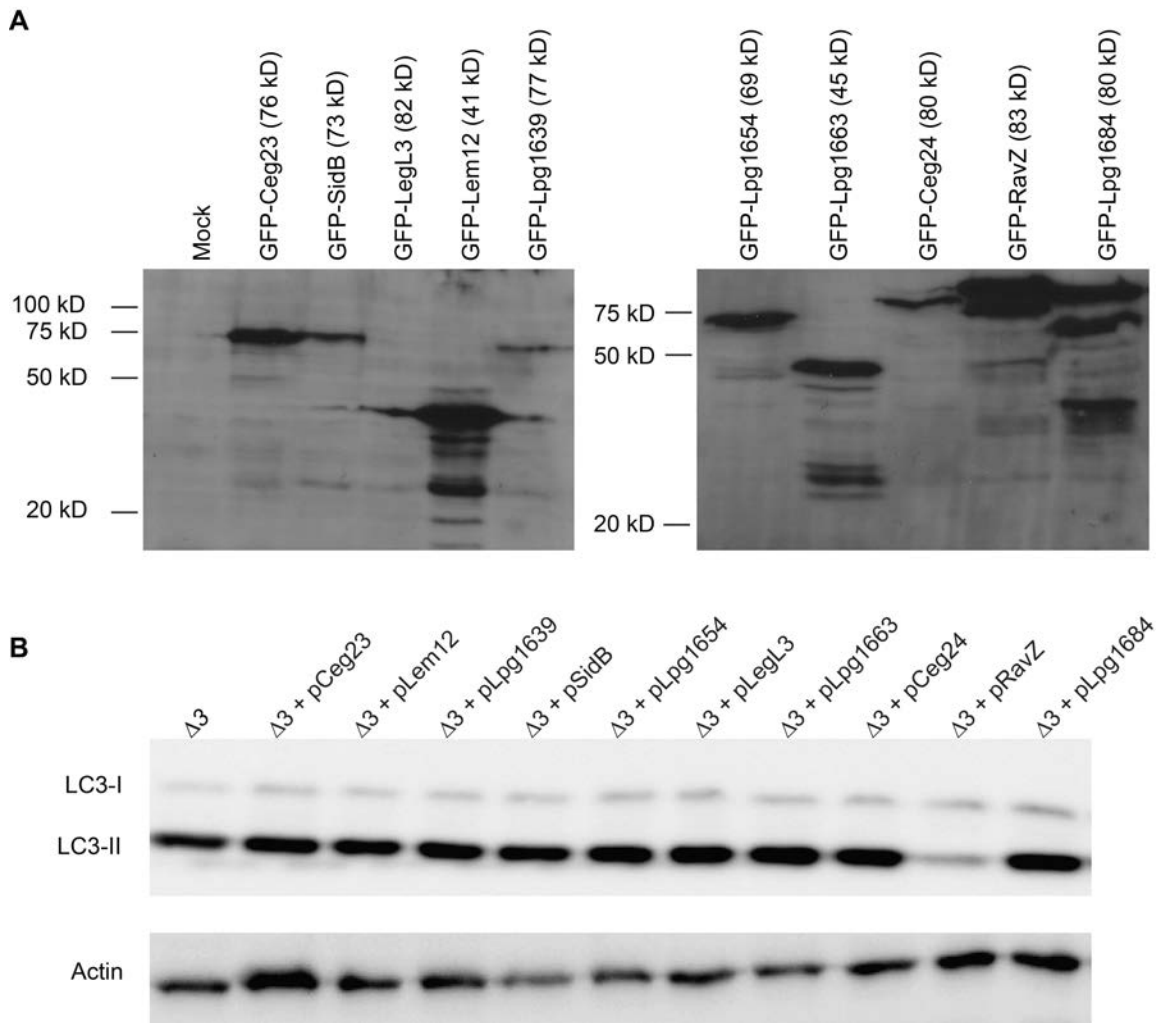
**Fig. S1**

Isolation of macrophages that have received effector proteins translocated into the cytosol by the Dot/Icm system. Illustration represents *Legionella* that are producing a hybrid protein having  $\beta$ -lactamase fused to the effector protein RaIF (BlaM-RaIF). When this protein is produced in a strain having a functional Dot/Icm system, the BlaM-RaIF protein is delivered into the host cytosol during infection. Detection of BlaM-RaIF in the cytosol of mammalian cells can be detected using CCF4, which has two fluorescent tags and a linker that can be cleaved by  $\beta$ -lactamase. When excited at 415 nm, the uncleaved CCF4 exhibits strong FRET-based fluorescence at 520 nm. When this probe is cleaved by  $\beta$ -lactamase, the FRET signal is no longer detectable and fluorescence shifts to 447 nm. The fluorescence ratio can be used to differentiate cells where BlaM-RaIF has been delivered into the host cytosol during infection from cells that are uninfected or that have not received injected proteins. The FACS plots on the left show how a cell sorter was used to distinguish between the injected and uninjected macrophage populations. This approach was used to isolate the macrophages in Figure 1D. After infection with *Legionella* producing BlaM-RaIF, two populations of cells were identified. The outlined population marked 24.5 (percent) had a high Blue:Green ratio and the isolated cells were labeled as “injected”. The population marked 18.6 (percent) had a low Blue:Green ratio and the isolated cells were labeled as “uninjected”. The lower FACS plot shows a control experiment done in parallel using a *dotA* mutant producing BlaM-RaIF. As shown in the FACS plot, the population of cells showing a high Blue:Green ratio was absent because the *dotA* mutant is unable to deliver BlaM-RaIF into the cytosol of macrophages. Thus, this control validates that the cells with a high Blue:Green ratio are injected cells.



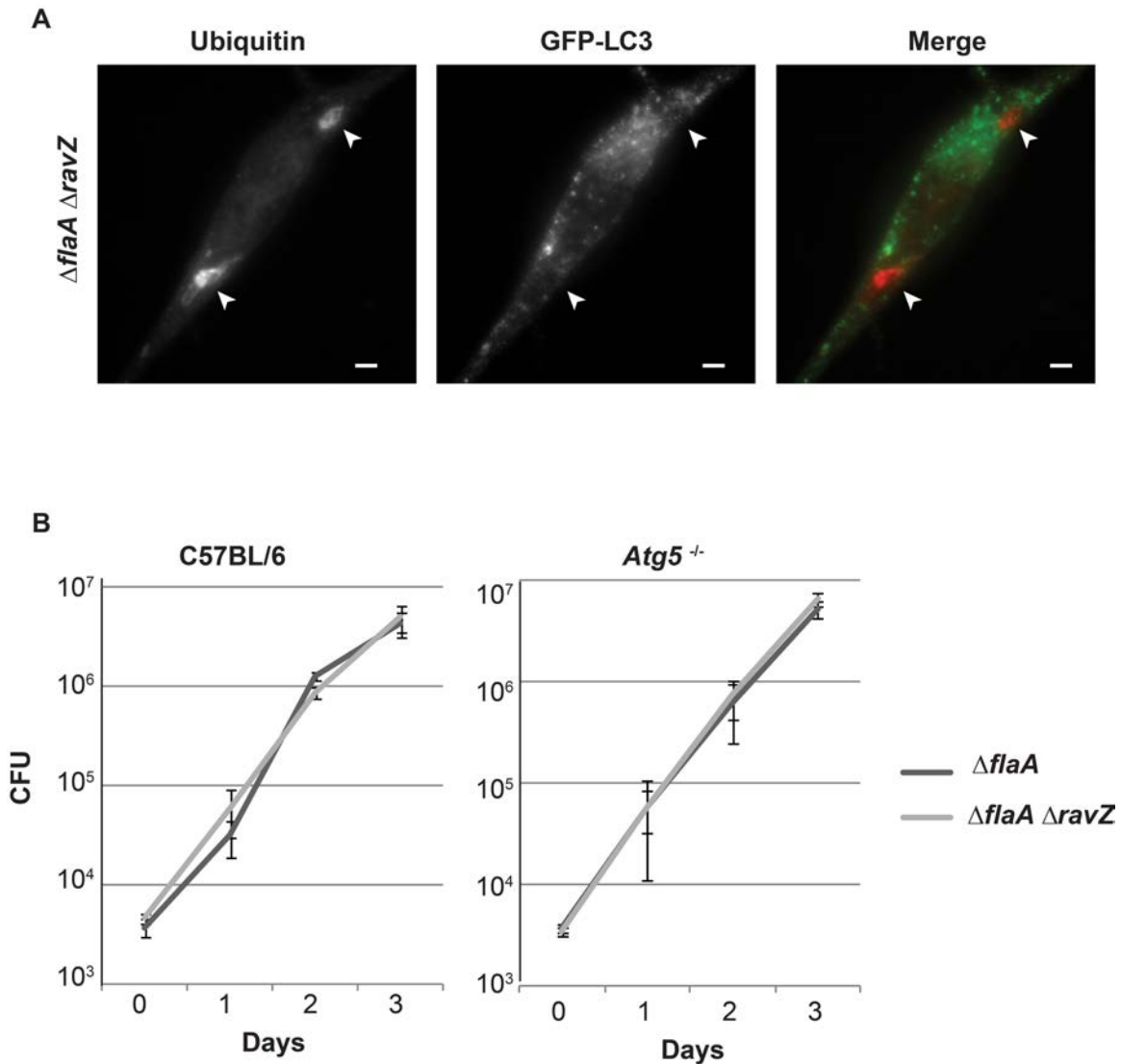
**Fig. S2**

RavZ is sufficient to inhibit autophagy in mammalian cells. Representative images from HEK293 cells transfected with a plasmid producing GFP (top) or GFP-RavZ (bottom). Cells were stained using an antibody specific for LC3. Single channel images show LC3 puncta in the control cells (GFP) but not in the cells transfected with GFP-RavZ. Scale bar, 1  $\mu$ m.



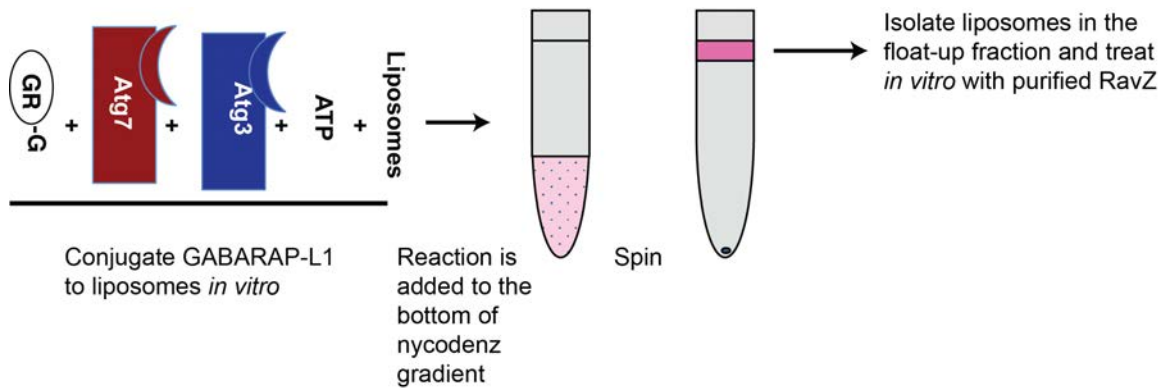
**Fig. S3**

The effector protein RavZ mediates autophagy inhibition. **(A)** Mammalian expression vectors encoding GFP fusions to each of the effector proteins deleted in the  $\Delta 3$  mutant were transfected into HEK293 cells to evaluate LC3-II levels as shown in Figure 2B. Blots shown here evaluate the production of the GFP effector by immunoblot analysis using an anti-GFP antibody. Indicated above each well are the GFP fusion protein encoded on the plasmid transfected into the cells and the predicted molecular weight of the GFP-effector hybrid protein. Mock cells received transfection reagent but no plasmid DNA. The locations of molecular weight markers are shown on the left side of each blot. Plasmid transfection was also confirmed by fluorescence microscopy to detect GFP expression. **(B)** Plasmids encoding each of the effectors deleted in the  $\Delta 3$  mutant were introduced into this strain and autophagy inhibition was evaluated after infection of HEK293 cells for 2-hours. Lysates from HEK293 cells infected with the  $\Delta 3$  mutant containing the plasmid indicated above each lane were assayed for LC3-I and LC3-II levels by immunoblot analysis using an LC3-specific antibody. The actin immunoblot panel shows the level of protein loading in each lane.



**Fig. S4**

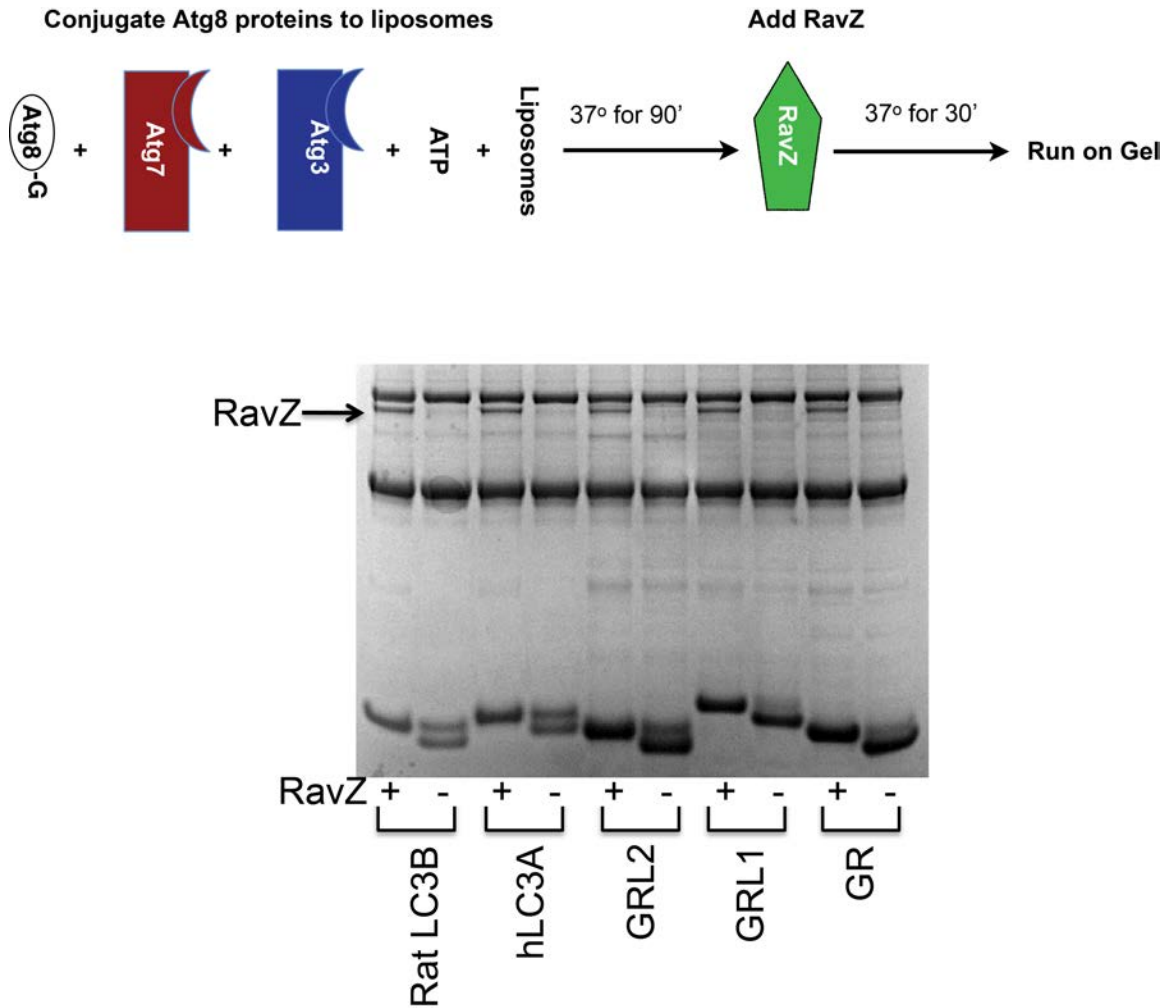
*Legionella* requires *ravZ* to block autophagy but not to prevent recruitment of LC3-positive vesicles to vacuoles supporting bacterial replication. **(A)** Bone marrow-derived macrophages from a transgenic GFP-LC3 mouse were infected with *Legionella*  $\Delta flaA \Delta ravZ$  for 10 hours. Cells were stained with an antibody specific for ubiquitin (red) to identify vacuoles supporting bacterial replication, and with an antibody specific for GFP (green) to identify LC3-positive vesicles. Arrowheads shown in the representative images demonstrate that GFP-LC3 does not colocalize circumferentially around the vacuole membrane that stains positive for ubiquitin. Scale bar, 1  $\mu$ m. **(B)** Bone marrow-derived macrophages from a control C57BL/6 mouse (left graph) and an *Atg5*-deficient mouse (right graph) were infected with RavZ-deficient *Legionella* ( $\Delta flaA \Delta ravZ$ ) or the isogenic control *Legionella* ( $\Delta flaA$ ). Bacterial replication was measured by determining colony-forming units (CFU) over a period of three days. Graphs show no significant difference in the replication of the two strains in these macrophages. Data are the average  $\pm$  s.d. calculated from three different wells for each time point.



**Fig. S5**

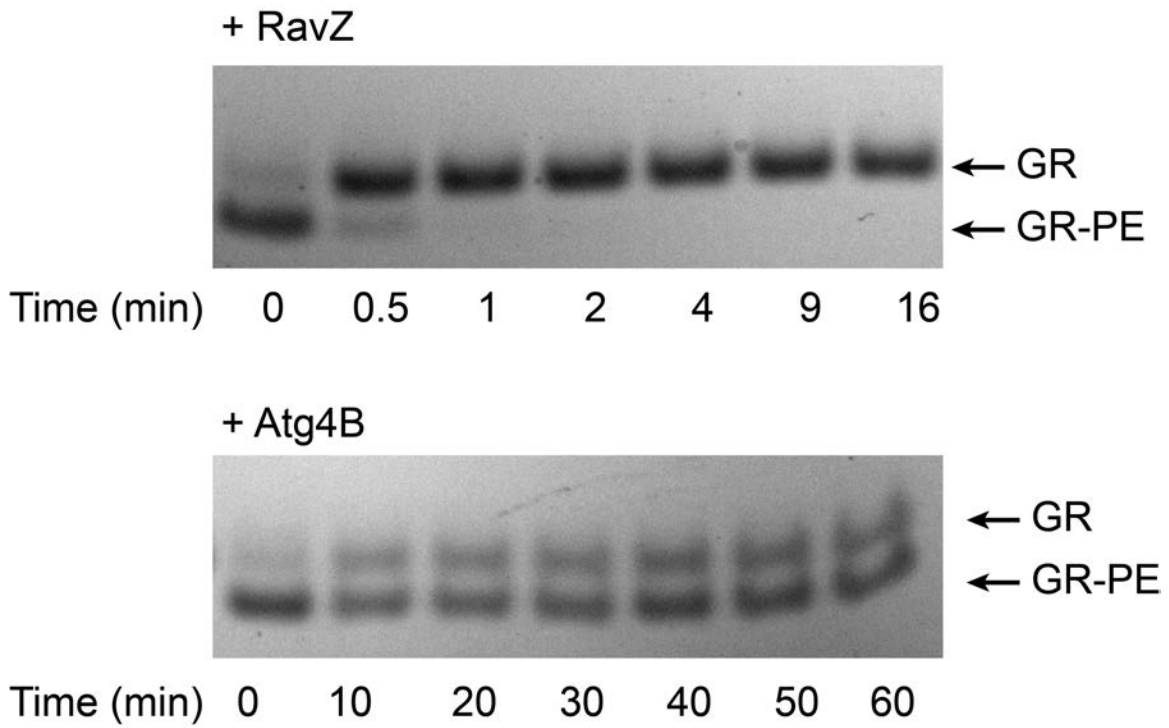
Strategy to purify lipidated GR away from reaction components prior to RavZ treatment. After the *in vitro* lipidation reaction, GR-PE decorated liposomes are floated on a 0/30/40% nycodenz step gradient. Other reaction components, including unreacted GR and free Atg3 and Atg7 collect as a pellet at the bottom of the gradient. The proteo-liposome band is collected and stored on ice until use in RavZ or Atg4 delipidation assays.





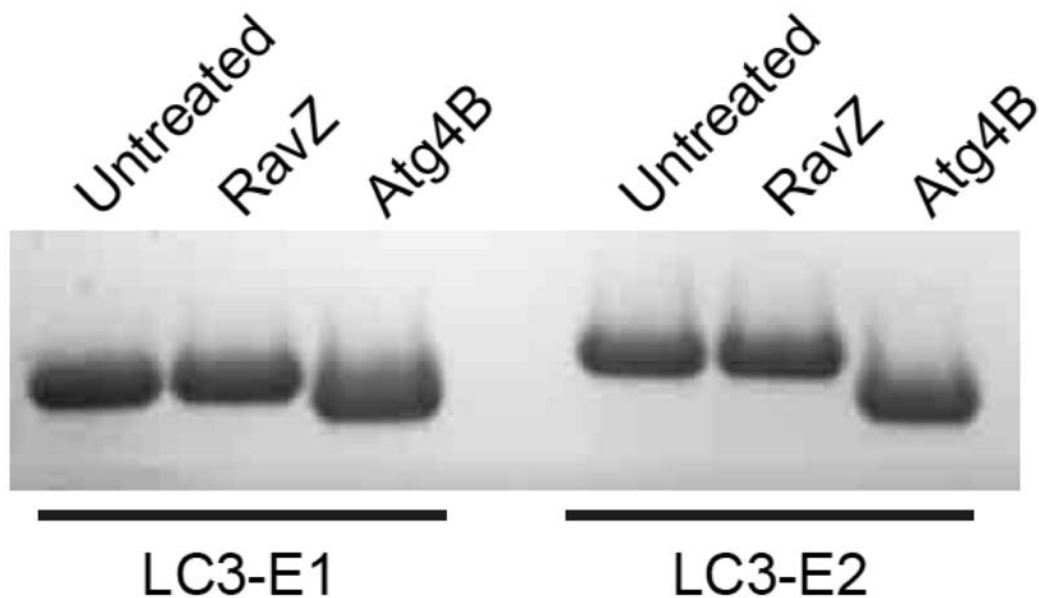
**Fig. S6**

RavZ processes each of the mammalian Atg8 homologues. In addition to human GABARAP L1 (used throughout Fig. 3 and here shown as GRL1), human GABARAP (GR), human GABARAP L2 (GRL2), human LC3A and rat LC3B were lipidated as in Fig. 3. After 90 minutes, aliquots of each reaction were moved to ice (-RavZ lanes). RavZ (252 nM) was added to remaining reactions and the samples were left at 37 degrees for an additional 30 minutes. All samples were mixed with SDS sample buffer and boiled for 5 min and the products were run out on SDS-PAGE and imaged by coomassie staining.



**Fig. S7**

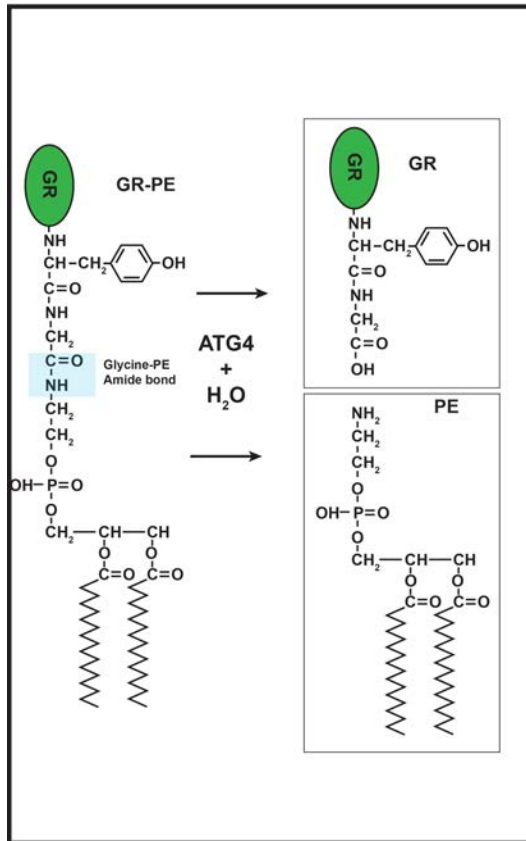
RavZ delipidates GR-PE much more efficiently than Atg4B. Lipidation reactions were run as in figure 3. GR-decorated liposomes were purified away from unlipidated GR and other reaction components by floatation on a nycodenz gradient. Floated liposomes were incubated with either RavZ (322 nM) or Atg4B (255 nM) at 37 degrees. Samples were removed at the indicated times and immediately mixed with SDS sample buffer and boiled. The products were run out on SDS-PAGE and imaged by coomassie staining.



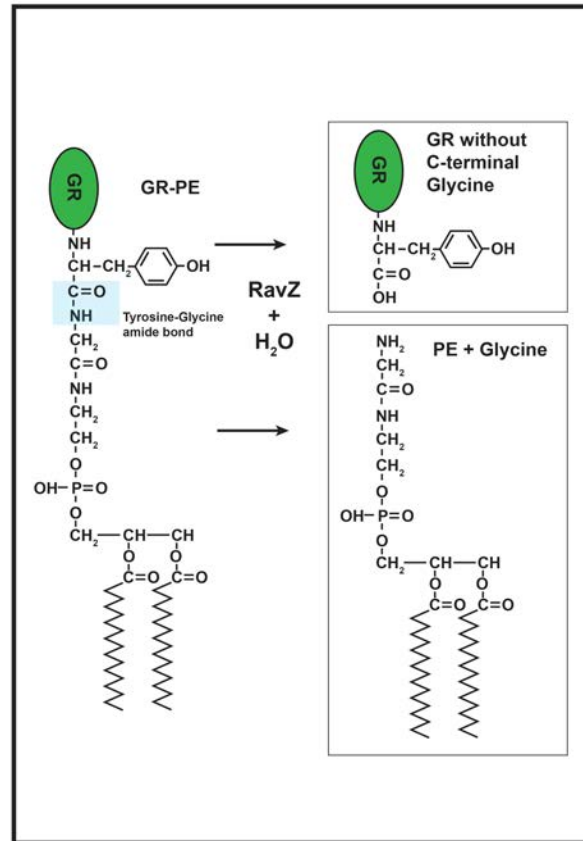
**Fig. S8**

RavZ does not cleave LC3 in solution. The protease activity of RavZ and Atg4B was assayed in solution using full-length LC3 that has the unrelated sequence EFIVT after the reactive C-terminal glycine (LC3-E1), and using an LC3 protein with the extension TALGFSDDLPRAFR after the reactive glycine (LC3-E2), which has similar residues following the glycine as those found in the native LC3 protein (TALAV). Stained SDS PAGE gels show there was no detectable LC3 cleavage in untreated samples and samples incubated with RavZ, whereas, both proteins were efficiently cleaved by Atg4B, resulting in a truncated product.

### Atg4-mediated deconjugation of GR-PE

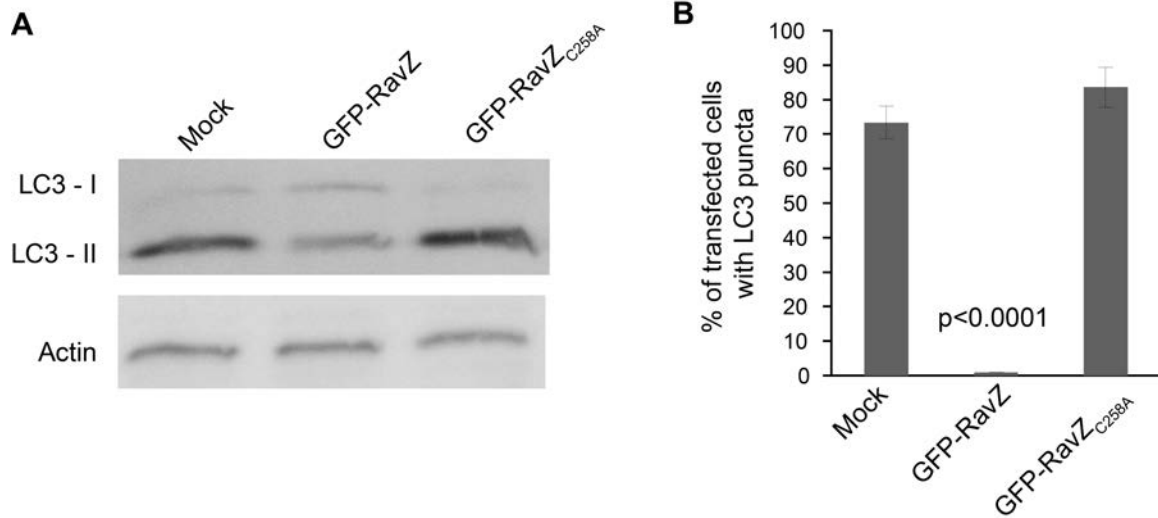


### RavZ-mediated deconjugation of GR-PE



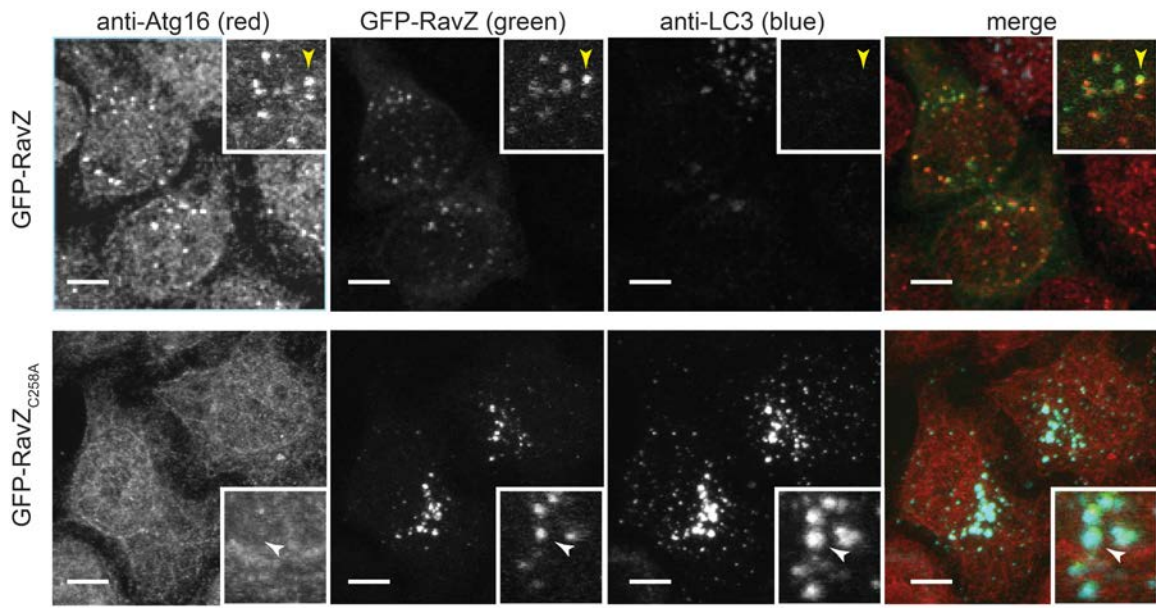
**Fig. S9**

RavZ and Atg4 deconjugate lipidated Atg8 proteins by hydrolyzing different amide bonds. Schematic shows the chemical structure surrounding the bonds that attach Atg8 proteins to PE. The left panel shows the reaction products generated after Atg4-mediated deconjugation of the Atg8 protein GR from PE. This reaction reverses the forward reaction that links Atg8 proteins to PE, and generates a delipidated Atg8 protein that retains the C-terminal glycine required for re-conjugation. The right panel shows the reaction products generated after RavZ-mediated deconjugation of the Atg8 protein GR from PE. This reaction differs from the Atg4 deconjugation reaction in that the amide bond linking the tyrosine and glycine residues in GR is hydrolyzed. The resulting products include an Atg8 protein that has the C-terminal glycine removed, preventing re-conjugation, and a novel PE-derived lipid with a glycine attached.



**Fig. S10**

The RavZ C258 residue is essential for autophagy inhibition. **(A)** Lysates from HEK293 cells were assayed for LC3-I and LC3-II levels by immunoblot analysis using an LC3-specific antibody. Lysates from mock transfected (mock) and cells transfected with plasmids encoding either GFP-RavZ or GFP-RavZ<sub>C258A</sub> are shown. The actin immunoblot panel shows the level of protein loading in each lane. **(B)** LC3 puncta were measured in the HEK293 cells transfected with plasmids encoding GFP-RavZ or GFP-RavZ<sub>C258A</sub> as indicated. The percent of cells containing LC3-puncta was calculated from three independent assays where a total of 100 cells were scored in each assay. Data represent the average  $\pm$  s.d.,  $p < 0.0001$  compared to the mock control.



**Fig. S11**

RavZ and RavZ<sub>C258A</sub> show differential localization to early and late AP structures. HeLa cells were transfected with either GFP-RavZ (top panel) or GFP-RavZ<sub>C258A</sub> (bottom panel) 24-hours before the cells were stained and fixed to localize endogenous LC3 and Atg16. Images show representative fluorescence micrographs. Yellow arrowheads point to an example of a structure where GFP-RavZ and Atg16 colocalize. White arrowheads point to an example of a structure where GFP-RavZ<sub>C258A</sub> and LC3 colocalize. Scale bar, 10  $\mu$ m.