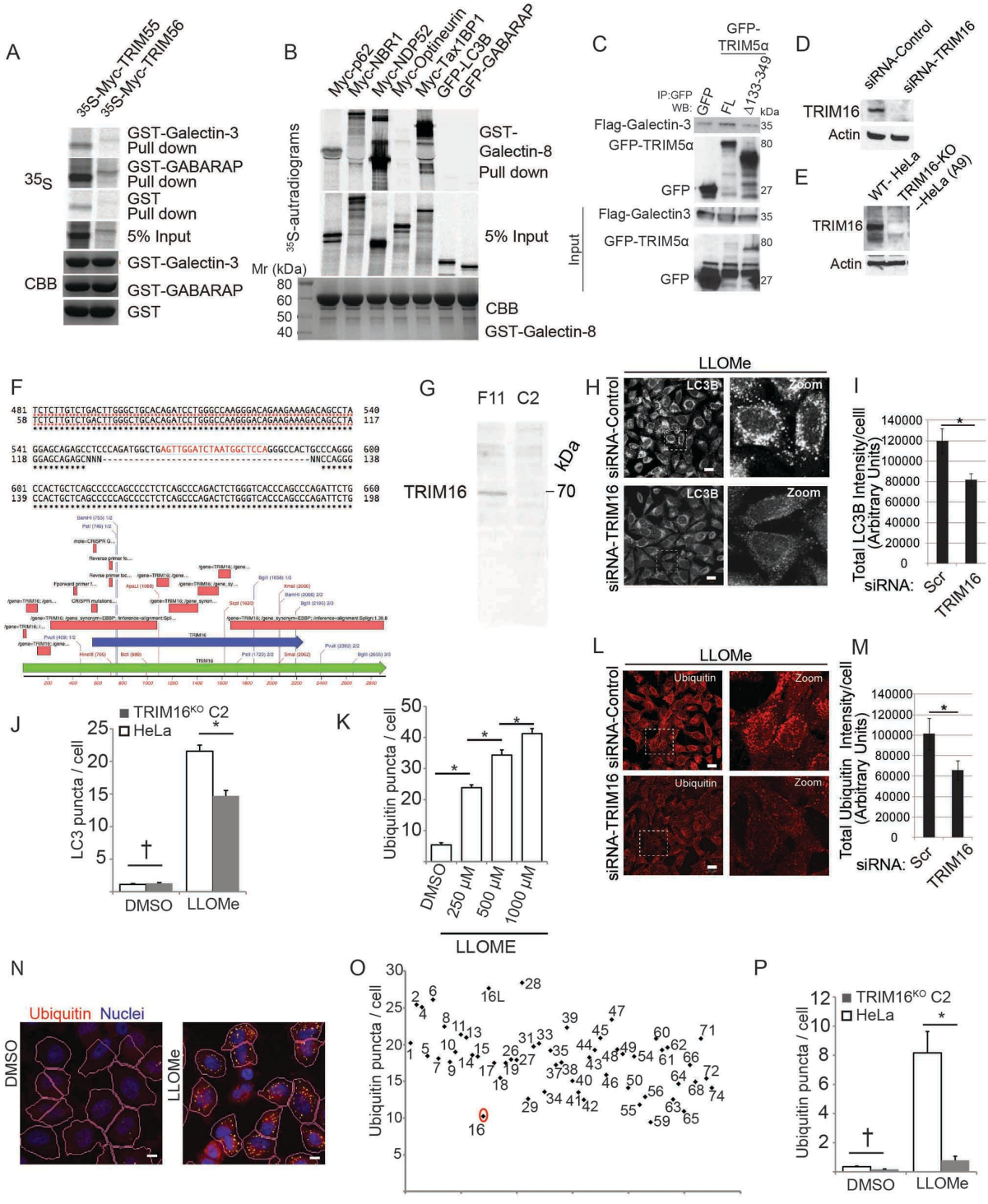


Figure S1 related to Figure 1. Analysis of interactions with Galectins, knockdown and knockout of TRIM16, and screen of TRIM family for effects on ubiquitination response to lysosomal damage. (**A)** GST-galectin-3 pulldowns of TRIM55 and TRIM56. **(B)** GST-galectin-8 pulldowns with Sequestosome 1-like receptors. **(C)** Co-IP analysis of GFP-TRIM5α (full-length vs CCD deletion Δ133-349) and Flag-Galectin-3

(D) Immunoblot analysis of siRNA knock down efficiency of TRIM16 in HeLa cells. (**E**) Immunoblot analysis of CRISPR-Cas9-mediated knock out of TRIM16 in HeLa cells, clone A9. (**F**) The clone A9 was subjected to next generation sequencing to characterize the mutation. Sequence alignment (top human TRIM16 genomic NCBI NM_006470 sequence; bottom, 62,593 bp next generation reads of a 259 bp contig, with 99.81% representation; a minor species with a 0.18% representation had additional changes in the region); The gap represents deletion introduced by Cas9, and it encompasses start codon at position 558 (NM_006470); the target sequence (red font) starts at position 551 (NM_006470). Bottom, a schematic showing TRIM16 exons and location of the Cas9-intorodcuced deletion (orange); blue, TRIM16 coding sequence. (**G**) Independently obtained CRISPR TRIM16 knockout clone C2 in HeLa cells. C2, knockout clone, F11, clone that is not a knockout. **(H,I)** Confocal images in H of HeLa cells transfected with control or TRIM16 siRNA treated with LLOMe, and processed for immunofluorescence with LC3B antibody. Graph in I represents average corrected total fluorescence intensity of cells ± SD. >50 cells from 6 fields from three different experiments were measured using Image J. Student's unpaired t test was used to test for statistical significance: *, $p < 0.05$. (**J**) Autophagic response (HC, LC3 puncta) to LLOMe (0.5 mM, 2 h) in HeLa cells and their CRISPR TRIM16^{KO} mutant derivative C2. **(K)** Dose-dependent increase in the abundance of ubiquitin puncta in HeLa cells upon LLOMe treatment as determined by high content microscopy. **(L,M)** Confocal imaging analysis of HeLa cells transfected with control or TRIM16 siRNA treated with LLOMe and immunofluorescence performed with ubiquitin antibody (M). Graph represents average corrected total cell fluorescence of cells ± standard deviation (SD) (N). >50 cells from 6 fields from three different experiments were measured using Image J. Student's unpaired t test was used to test for statistical significance: *, $p < 0.05$. **(N,O)** Screen for the effects of TRIM family knockdowns (specific TRIM identified indicated by numbers) on ubiquitin response to lysosomal damage by LLOMe. Images of HeLa cells treated or not with 0.5 mM LLOMe, stained with anti-ubiquitin (red), and subjected to high content microscopic imaging and analysis (N). White masks, cell boundaries. Yellow masks, ubiquitin puncta. Expression of a subset of TRIMs was knocked down by siRNAs in HeLa cells (O). Following LLOMe treatment, the number of ubiquitin puncta per cell was determined by high content microscopy. (**P**) Ubiqutination response, revealed with FK2 mouse monoclonal antibody in HeLa vs. TRIM16^{KO} HeLa mutant C2. HC data: means (n>3); t-test (I,O) or ANOVA (J) *, $p < 0.05$.

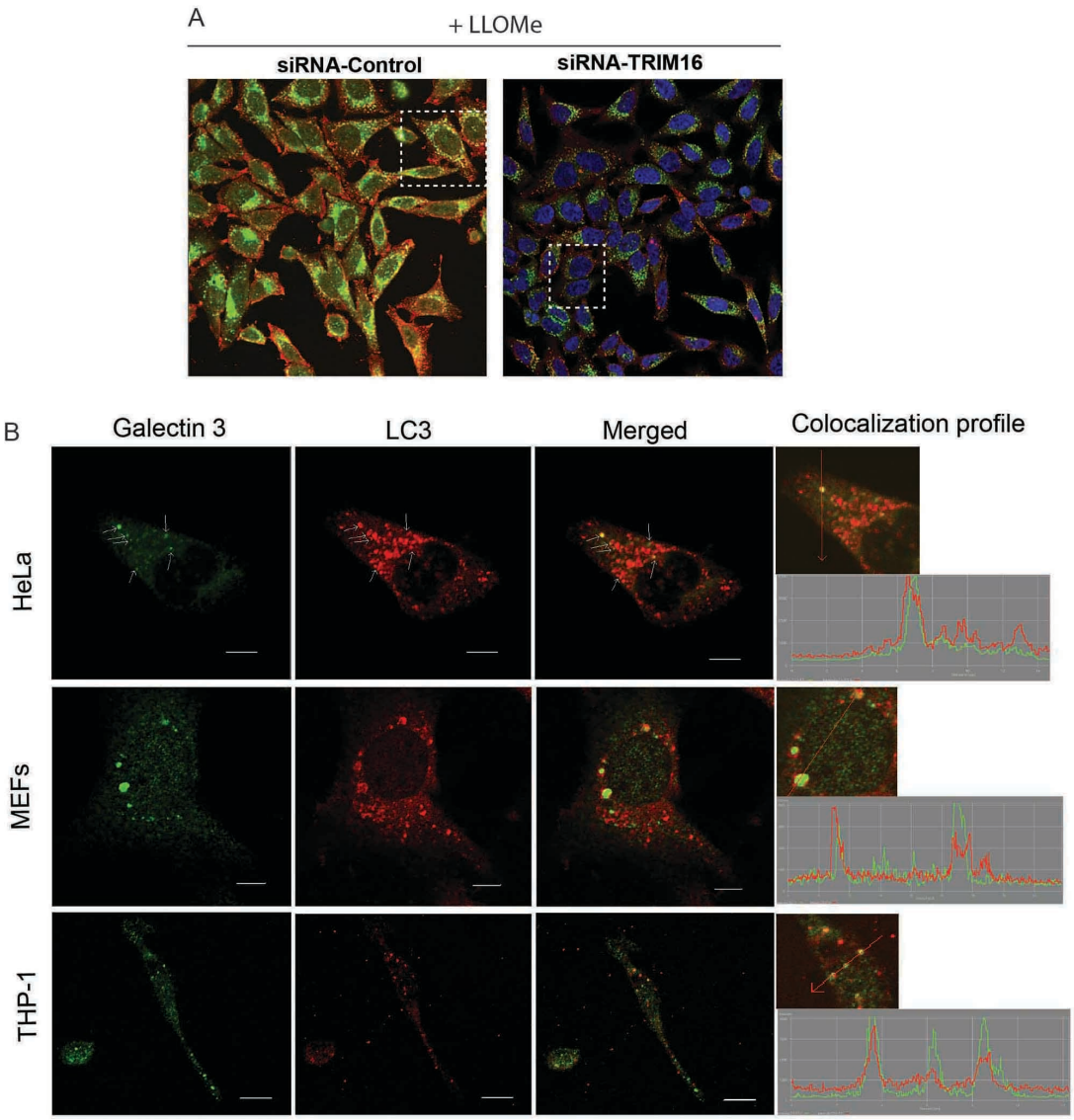
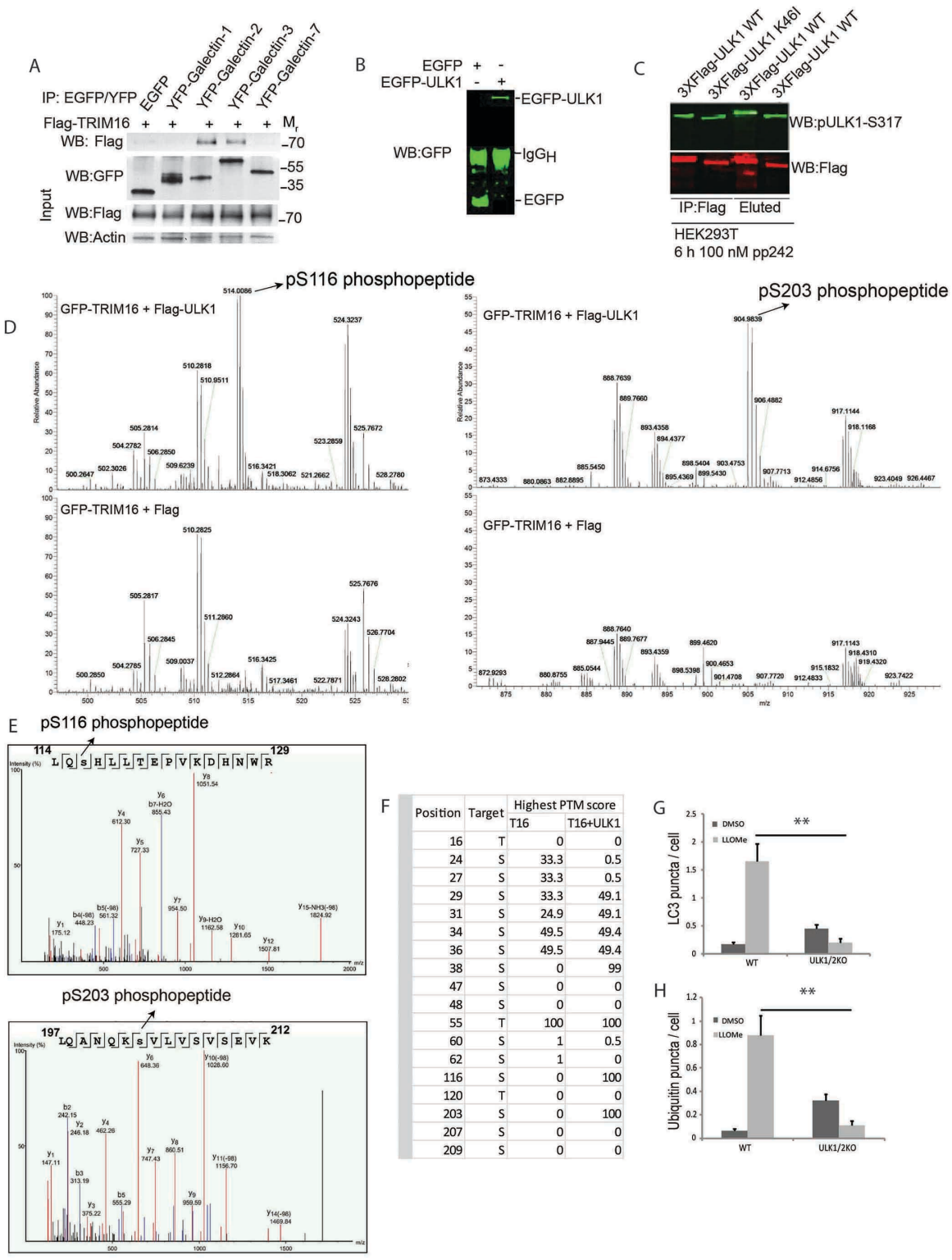


Figure S2 related to Figure 2. TRIM16 is important for endomembrane damaged induced autophagy response. (A) Whole microscopic field of the images analyzed in Figure 2A. **(B)** Galectin-3 puncta formation and colocalization analysis with the autophagosomal marker LC3 in THP-1, Mouse embryonic fibroblast, and HeLa cells**.** HeLa and MEFs were treated with 1mM LLOMe for 2h, while MEFs were treated with 0.5 mM of LLOMe for 45 min.



Position	Target	Highest PTM score	
		T16	T16+ULK1
16	T	0	0
24	S	33.3	0.5
27	S	33.3	0.5
29	S	33.3	49.1
31	S	24.9	49.1
34	S	49.5	49.4
36	S	49.5	49.4
38	S	0	99
47	S	0	0
48	S	0	0
55	T	100	100
60	S	1	0.5
62	S	1	0
116	S	0	100
120	T	0	0
203	S	0	100
207	S	0	0
209	S	0	0

Figure S3 related to Figure 3. Co-immunoprecipitation analyses between TRIM16 and different Galectins and ULK1 preparations and identification of ULK1-dependent phospho-Ser residues on TRIM16. (**A**) Co-immunoprecipitation (Co-IP) analysis of interactions between TRIM16 and Galectins in HEK293T cell lysates

expressing GFP or GFP-Galectin proteins (galectin-1, 2, 3 and 7) andfFlag-TRIM16. (**B**) EGFP-ULK1 preparation. (**C**) 3xflag-ULK1 preparation (wild type and enzymatically dead mutant K46I. (**D**) Mass spectra showing new peaks (514.009 and 904.982) appearing with GFP-TRIM16 in the presence of Flag-ULK1 fusion compared to Flag alone. **(E)** Annotated tandem mass spectra for fragmented precursor ions (514.009 and 904.982) from the scans shown in (D), demonstrating that the peptides are from phosphorylated TRIM16 (S116 and S203). **(F)** Table showing percentage probabilities of specific sites in TRIM16 being phosphorylated in an ULK1-dependent or ULK1-independent manner. (**G**,**H**) Ulk1/2 double knockout MEFs were treated with LLOMe and number of LC3 (G) or ubiquitin (**H**) puncta per cell determined by HC. HC data: means (n>3); t-test **, $p < 0.01$.

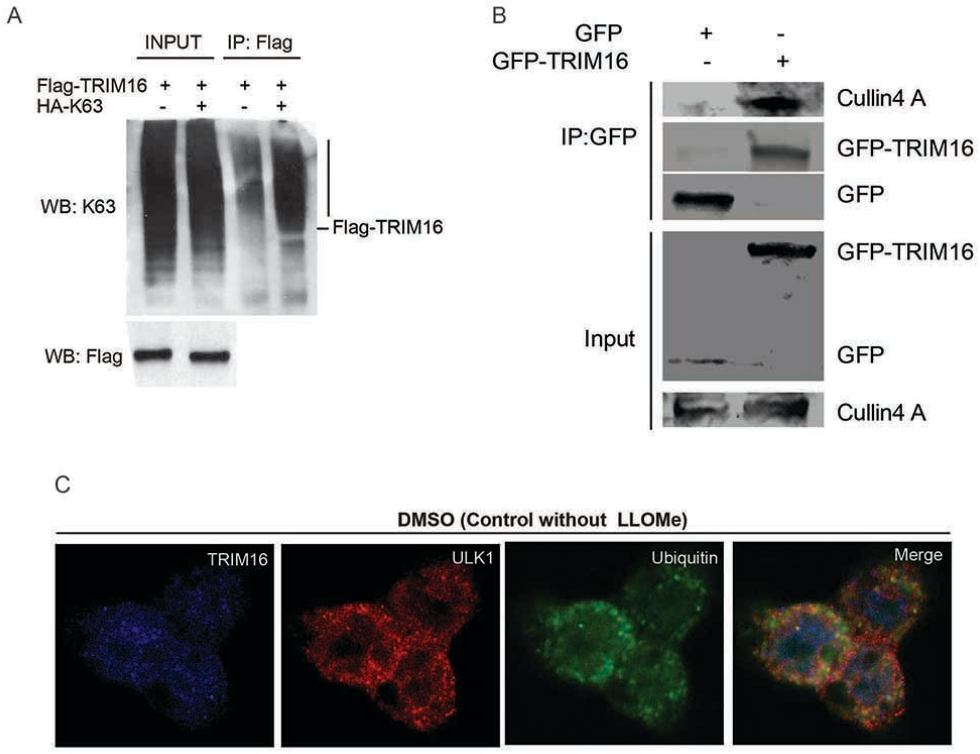


Figure S4 related to Figure 4. Analysis of TRIM16 role in ubiqutination. (A) Analysis of TRIM16 ubiquitination in cells expressing flag-TRIM16 in absence and presence of HA-tagged ubiquitin C mutated for all lysine except Lysine 63 (HA-K63). Immunoprecipitation was performed with flag antibody followed by the Western blotting with indicated antibodies**. (B)** 293T cells transfected with GFP or GFP-TRIM16 were subjected to co-IP analysis as indicated. **(C)** Confocal images of DMSO treated THP-1 cells; immunofluorescence was performed with TRIM16, ULK1 and ubiquitin antibody.

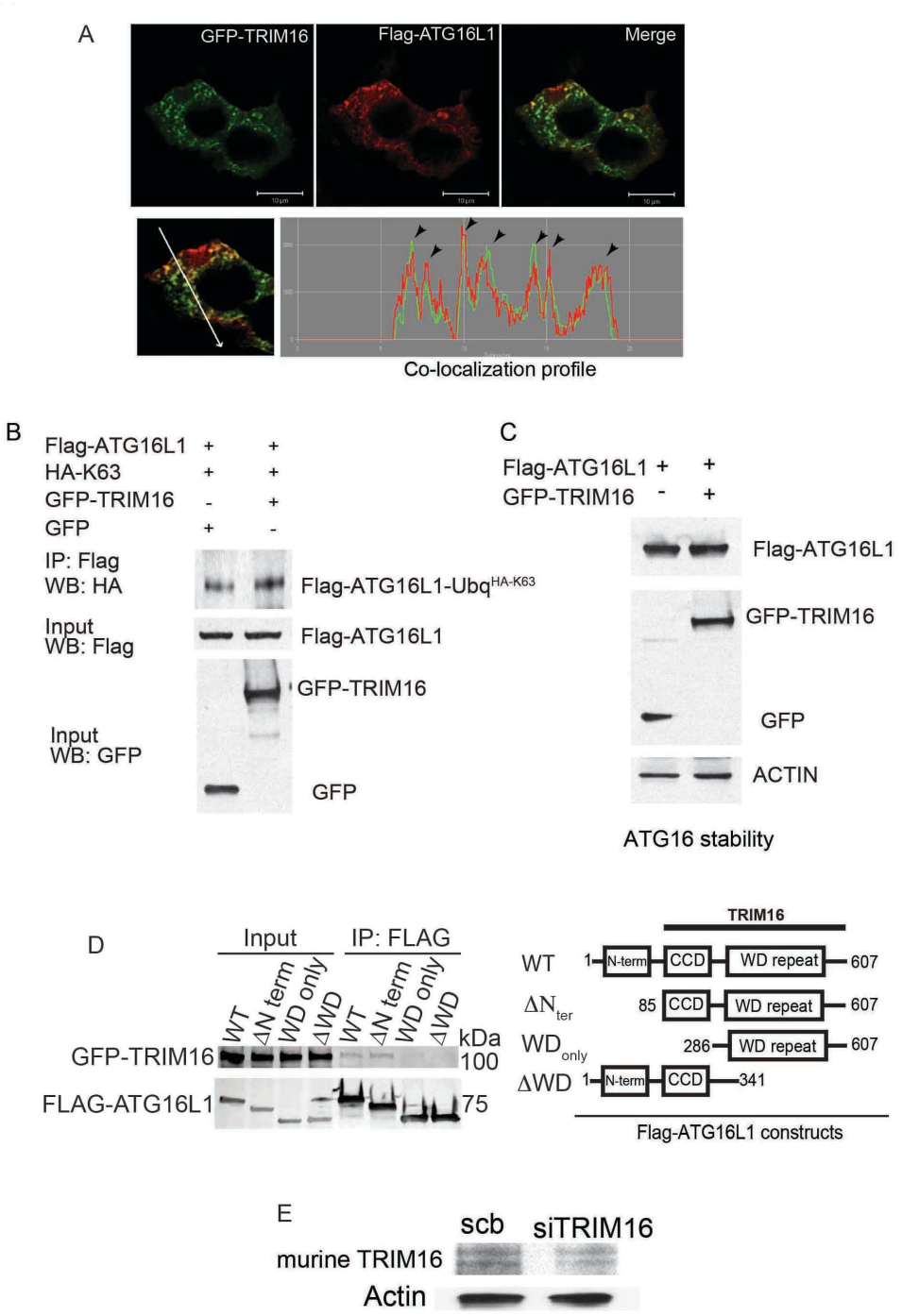


Figure S5 related to Figure 5. TRIM16 does not increase K63-linked ubiquitination and stability of ATG16L1 and domain mapping of TRIM16-ATG16L1 interactions. **(A)** Top confocal microscopy images of HEK293T cells transiently expressing GFP-TRIM16 and flag-ATG16L1. Bottom, co-localization profile tracer along straight line generated using LSM510 software. **(B)** Analysis of ATG16L1 ubiquitination in cells co-expressing flag-ATG16L1, HA-K63 and GFP or GFP-TRIM16. Immunoprecipitation was performed with flag antibody followed by the Western blotting with indicated antibodies.

(C) Western blot analysis of total amount of ATG16L1 in presence and absence of TRIM16 in HEK293T cells transiently expressing Flag-ATG16L1 and GFP or GFP-TRIM16. (**D)** Left panel, lysates of HEK293T cells co-expressing GFP-TRIM16 and the indicated Flag-ATG16L1 variants (see right panel) were subjected to immunoprecipitation with anti-GFP and blots were probed as indicated. Right panel, schematic of ATG16L1 domain structure along with deletion constructs used in Co-IP analysis. **(E)** Western blot analysis of siRNA knock down efficiency of TRIM16 in RAW264.7 cells.

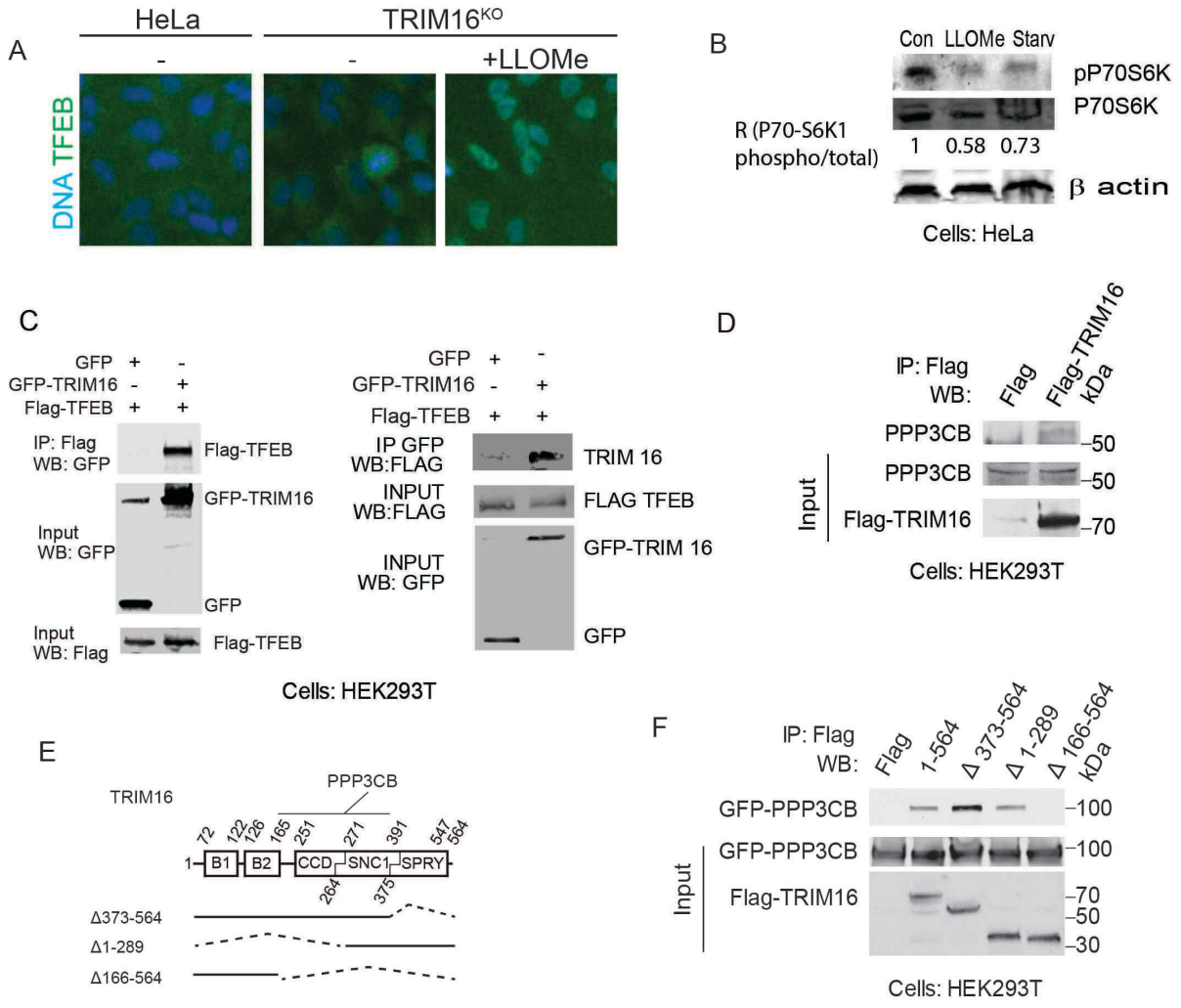


Figure S6 related to Figure 6. Comparison of nuclear TFEB partition in CRISPR TRIM16^{KO} vs HeLa cells, detection and mapping of TRIM16 interactions with TFEB and calcineurin, and analysis of the role of Galecin-3, TRIM16 and ATG16L1 in acquisition of LAMP1 by *M. tuberculosis* phagosomes. **(A)** HC images with masks removed, corresponding to Figure 6D. **(B)** Analysis of S6K1 phosphorylation (mTOR target) in HeLa cells treated with LLOMe. Con, solvent DMSO control; LLOMe, 1 mM LLOMe, 2h incubation; Starv, starvation in EBSS for 2h. (**C**) Co-IP and reverse Co-IP analysis of interactions between GFP-TRIM16 and Flag-TFEB in HEK293T cells. (**D**) Co-IP analysis of interactions between Flag-TRIM16 and endogenous calcineurin catalytic subunit PPP3CB in HEK293T cells. (**E,F**) Mapping of the TRIM16 region required for it being in complexes with PPP3CB.

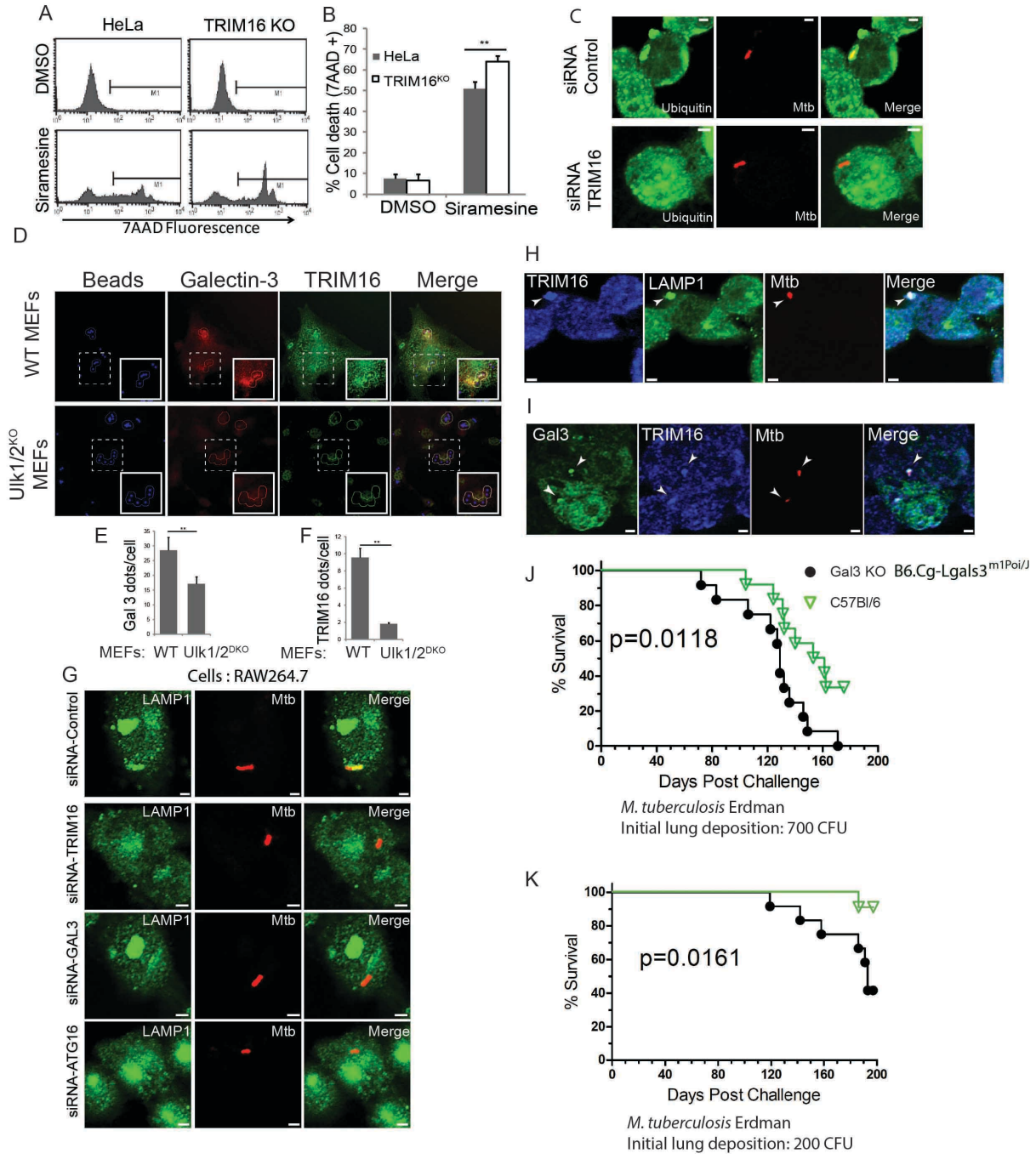


Figure S7 related to Figure 7. TRIM16, Galectin-3 and ATG16L1 colocalization on *M. tuberculosis* autophagolysosomes, and reduced survival of galectin-3 knockout mice infected with *M. tuberculosis* Erdman. (A, B) HeLa or CRISPR TRIM16^{KO} HeLa cells A9 were incubated with siramesine and cell death was measured using 7AAD nuclear staining. Data, means; n>3; **, p < 0.01 (t-test). **(C)** RAW264.7 macrophages were transfected with siRNAs against TRIM16 or control siRNAs for 48 h. Cells were then infected with Alexa-568-labeled wild-type Mtb Erdman for 4 h and processed for confocal microscopy analysis for the colocalization of Mtb with ubiquitin.

Images correspond to the graph in Figure 7I. **(D)** Effectene-coated beads (fluorescence detected at 485 nm; rendered blue) were phagocytosed by wt or Ulk1/Ulk2 double KO MEFs in 96 well plates, incubated for 24 h, stained with antibodies (Galectin 3, fluorescence detected at 568 nm, rendered green; and TRIM16, far red fluorescence detected at 647 nm, rendered red), and imaging carried out and data processed by automated HC microscopy and analysis (graphs with data are shown in Figure 7K. Arrows, Galectin-3 profiles colocalizing with TRIM16 on Effectene-beads. **(E,F)** Wild type and ULK1/2 KO MEFs were transfected with effectene coated beads for 24 h and incubated with Galectin 3 (E) or TRIM 16 (F) and imaging and data analysis carried out by automated HC microscopy and data analysis**. (G) (**Representative images for the data in Figure 7L. Confocal images of RAW264.7 cells infected with Alexa-568-labeled wild-type Erdman and immunostained for LAMP1. Bar, 2 μm. (**H,I**) **C**onfocal images of RAW264.7 cells infected with Alexa 568-labeled wild-type Erdman and immunostained for TRIM16, LAMP1, and Galectin-3, as indicated. Bar, 2 μm. **(J,K)** Survival curves of C57BL mice and their galectin-3 knockout derivative in a chronic model of respiratory infection with *M. tuberculosis*; medium dose, 600 CFU (J) and low dose, 200 CFU (K) of initial bacterial *M. tuberculosis* Erdman lung deposition following exposure to aerosols.

Supplementary experimental procedures

GST pull downs and ULK1 phosphorylation assay

GST-fusion proteins were expressed in *Escherichia coli* SoluBL21 (Amsbio). GST fusion proteins were purified and immobilized on glutathione-coupled sepharose beads (Amersham Bioscience, Glutathione-sepharose 4 Fast Flow) and pull-down assays with in vitro translated [^{35}S]-labeled proteins were done as described previously (Pankiv et al., 2007). The [^{35}S] labeled proteins were produced using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in the presence of [^{35}S] L-methionine. The proteins were eluted from washed beads by boiling in SDS-PAGE gel loading buffer, separated by SDS-PAGE, and radiolabeled proteins were detected in a Fujifilm bioimaging analyzer BAS-5000 (Fuji).

In vitro phosphorylation assays were performed by incubating the recombinant proteins with FLAG-ULK1 kinase immunoprecipitated from the transfected HEK293 cells in a standard kinase buffer containing 50 µM of cold ATP and 2.5 µCi [γ-^{32}P]-ATP per reaction at 30°C for 30 min. The reaction was stopped by adding SDS sample buffer and boiling, and then subjected to SDS–PAGE gel and autoradiography. For pull-downs assays without ^{32}P radiolabeling (i.e. cold-phosphorylation), cold ATP was used in a mixture with TRIM16 and then the standard in-vitro pull-downs were performed.

In vivo phosphorylation and Liquid Chromatography – Mass Spectrometry

For in vivo phosphorylation analyses, subconfluent HEK293 cells in 10 cm dishes were transfected with total 5 µg plasmids (i.e. pDest-eGFP-TRIM16, plus pDest-3xFLAG or pDest-3xFLAG-ULK1) using Metafectene Pro (Biontex) following the supplier's instructions. Twenty-four hours after transfection cells were rinsed with ice-cold PBS prior to lysis in RIPA buffer (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 1 mm EDTA, 1% Nonidet P-40 (v/v), 0.25% Triton X-100) supplemented with Complete Mini EDTA-free protease inhibitor mixture tablets (1 tablet per 10 ml) (Roche Applied Science) and phosphatase inhibitor mixture set II (Calbiochem). Lysates were cleared by centrifugation and the cleared lysates were then incubated with the Anti-GFP MicroBeads (Miltenyi Biotec Norden AB) for 30 min at 4°C. The GFP-precipitated immunocomplexes were washed five times with RIPA lysis buffer and eluted following the supplier's instructions. The eluted samples were subjected to SDS-PAGE and gel bands containing TRIM16 were excised and subjected to in-gel reduction, alkylation, and tryptic digestion with 6 ng/µl trypsin (Promega). OMIX C18 tips (Varian, Inc.) was used for sample cleanup and concentration. Peptide mixtures containing 0.1% formic acid were loaded onto a Thermo Fisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2µm, 100 Å, 50µm, 50 cm). Peptides were fractionated using a 2-100% acetonitrile gradient in 0.1 % formic acid over 50 min at a flow rate of 200 nl/min. The separated peptides were analyzed using a Thermo Scientific Q-Exactive mass spectrometer. Data was collected in data dependent mode using a Top10 method. The raw data were processed using the Proteome Discoverer 1.4 software (Thermo Scientific) and the PEAKS Studio 7 software (v. 7.0, Bioinformatics Solutions). The

fragmentation spectra from Proteome Discoverer was searched against the Swissprot database using an in-house Mascot server (Matrix Sciences). The phosphoRS 3.0 tool in the Proteome Discoverer software was used to validate potential phosphosites in the samples. A human Swissprot database was used for the de novo peptide sequencing assisted search engine database searching by the PEAKS software. Peptide mass tolerances used in the searches were 10 ppm, and fragment mass tolerance was 0.02 Da. Both software's identified the S203 and S116 phosphosites in TRIM16 in the samples with ULK1, but not in the FLAG control. The presence of these phosphorylations in the ULK1 samples but not in the FLAG control was manually verified by inspecting the LC-MS spectra in the Excalibur 2.2 software (Thermo Scientific).

Antibodies

The following antibodies and dilutions were used: TRIM16 (goat polyclonal antibody, Santa Cruz; sc-79770; 1:50-1:100 for immunofluorescence (IF) and 1:200-1:1,000 for Western blots (WB)); Galectin-3 (rabbit polyclonal Abcam; cat# ab53082; 1:100, IF; or mouse monoclonal Santa Cruz sc-32790 1:100 (IF) and 1:250 (WB)); ubiquitin (mouse monoclonal FK2, MBL; cat# D058-3; 1:100-1:500, IF); LAMP1 (mouse monoclonal Abcam; cat# ab25630; 1:100; IF); LAMP2 (mouse monoclonal Hybridoma Bank, University of Iowa; 1:500 (IF)); ULK1 (rabbit polyclonal Santa Cruz, sc33182; 1:100 (IF); 1:500 (WB)); ATG16L1 (rabbit polyclonal MBL (PM040); 1:2,000 (WB)) K63 ubiquitin (rabbit monoclonal Millipore 05-1308; 1:100 (IF); 1:500 (WB); TFEB (rabbit polyclonal anti-human; Cell Signaling CST 4240, 1:200 (IF)); phospho p70s6K (rabbit polyclonal Cell Signaling CST 9205; 1:750 (WB)) p70s6k (rabbit polyclonal Cell Signaling CST 9202; 1:1,000 (WB)); RagB (rabbit monoclonal Cell Signaling CST 8150; 1:500); Rag D (rabbit polyclonal Cell Signaling CST 4470; 1:500); DEPTOR (Rabbit monoclonal Cell Signaling CST 11816; 1:1000); Cullin4A (rabbit monoclonal antibody Abcam #ab92554 1:500 (WB)); Cullin5 (rabbit polyclonal antibody #ab34840; 1:500). GFP (rabbit polyclonal Abcam; cat# ab290; 0.5 µg/ml IP and 1:5,000 (WB)); PPP3CB (rabbit polyclonal antibody Abcam, ab96573 1:500 (WB)) Flag (mouse monoclonal Sigma; cat# F1804, used at 1:1,000); Myc (mouse monoclonal Santa Cruz, sc-40; 1:200 (IF); 1:500 (WB)); HA (mouse monoclonal Millipore 05-904; 1:1,000 (WB)); and actin (mouse monoclonal Abcam; cat# ab8226, used at 1:4,000).

Immunoblots and co-immunoprecipitation assays

Immunoblots and co-IPs assays for endogenous or exogenously expressed proteins were carried out as described previously (Chauhan et al., 2015). For immunoprecipitation experiments with exogenously expressed proteins, 293T cells were transfected with 5 µg of expression constructs by calcium phosphate for 24 h and lysed on ice using NP-40 buffer (Invitrogen) containing protease inhibitor cocktail (Roche, cat# 11697498001) and PMS (Sigma, cat# 93482). Lysates were mixed with antibody (2-3 µg) incubated at 4°C for 2 h followed by incubation with protein G Dynabeads (Life technologies) for 2 h at 4°C. Beads were washed four times with PBS and then boiled with SDS-PAGE buffer for analysis of interacting protein by Immunoblotting. Input lanes contained 10% of material unless otherwise indicated.

Analysis of Effectene-bead phagosomes

Wild type (WT) and Ulk1/Ulk2 double KO mouse embryonic fibroblasts (McAlpine et al., 2013) in 96-well plates were incubated with Fluoresbrite bead (3 μm; Polysciences Inc. cat#21637-1; fluorescence detected at 485 nm) treated with effectene transfection reagent as described previously (Fujita et al., 2013). The beads were not spun onto the cells but were allowed to spontaneously uptake the beads, which required times > 3h. After up to 24 h of incubation, cells were processed for immunofluorescence microscopy with Galectin-3 and TRIM16 antibodies for 2 h followed by treatment with secondary antibodies (goat anti-mouse Alexa Flour 568 and goat anti-rabbit Alexa Flour 647). High content microscopy was carried out in a Cellomics HCS scanner and images analyzed and objects quantified using iDEV software (Thermo) (Mandell et al., 2014).

For intracellular mycobacterial survival assays see Supplementary experimental procedures. RAW264.7 cells were infected with mycobacteria and quantification of mycobacterial survival carried out as previously described (Ponpuak et al., 2009). In brief, 3×10^5 cells of RAW264.7 macrophages were plated onto each well of 12-well plates 12 h before infections. Cells were then infected with single cell suspension of mycobacteria in complete media at MOI of 10 for 1 h. Cells were then washed three times with PBS to remove un-internalized mycobacteria. Infected cells were then lysed to determine the number of intracellular mycobacteria at t=0 by plating onto Middlebrook 7H11 agar supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% OADC (BD Biosciences) and grown at 37^{o}C or infected cells were continued to grow until harvesting at t=24 for CFU analysis. Percent mycobacteria survival was calculated by dividing the number of intracellular mycobacteria at t=24 over that of t=0 multiply by 100 and relative to control cells set to 100%.

Bacterial phagosomes, intracellular mycobacterial survival, and fluorescence microscopy of infected macrophages

For immunofluorescence microscopy with mycobacteria-infected macrophages, 3×10^5 cells of RAW264.7 macrophages were plated onto coverslips in 12-well plates 12 h before infections. Cells were then infected with 3×10^6 Alexa-568-labeled mycobacteria per well in complete media at 37^{o}C for 15 min, washed three times in PBS, and chased for 1 h in complete media as previously described (Ponpuak et al., 2009). Cells were then washed three times with PBS and incubated in complete media for the indicated times. Cells were then fixed with 4% paraformaldehyde/PBS for 15 min followed by permeabilization with 0.1% Triton X-100/PBS for 5 min. Coverslips were then blocked in PBS containing 3% BSA and then stained with primary antibodies according to manufacturer's recommendation. Cells were washed three times with PBS and then incubated with appropriate secondary antibodies (Invitrogen) for 2 h at room temperature. Coverslips were then mounted using ProLong Gold Antifade Mountant (Invitrogen) and analyzed by confocal microscopy using the Zeiss LSM510 Laser Scanning Microscope. At least 50 phagosomes per experimental condition in three independent experiments were quantified. For quantification, % mycobacteria-marker

colocalization was fraction of total mycobacterial phagosomes examined counted as positive when one or more puncta were observed on or in contact with the mycobacterial phagosomes.

RAW264.7 cells were infected with mycobacteria and quantification of mycobacterial survival carried out as previously described (Ponpuak et al., 2009). In brief, 3 × 10^5 cells of RAW264.7 macrophages were plated onto each well of 12-well plates 12 h before infections. Cells were then infected with single cell suspension of mycobacteria in complete media at MOI of 10 for 1 h. Cells were then washed three times with PBS to remove un-internalized mycobacteria. Infected cells were then lysed to determine the number of intracellular mycobacteria at t=0 by plating onto Middlebrook 7H11 agar supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% OADC (BD Biosciences) and grown at 37^{o}C or infected cells were continued to grow until harvesting at t=24 for CFU analysis. Percent mycobacteria survival was calculated by dividing the number of intracellular mycobacteria at t=24 over that of t=0 multiply by 100 and relative to control cells set to 100%.

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Plasmids, siRNAs, cell transfections

TRIMs, SLRs, Galectins, and GABARAP cDNA were first cloned into pENTR or pDONR221 vectors from Invitrogen, and then (Gateway) cloned into either pDestMyc or pDest53 (GFP) using LR-II enzyme from Invitrogen. (GST-Galectin-3, GST-Galectin-8 and GST-GABARAP are described in Figure-1). pENTR clones of different TRIM5α deletion constructs (ΔRING, ΔB.Box, ΔCCD and ΔSPRY) including the full-length TRIM5α were generated using Phusion DNA Polymerase (from NEB) and T4-DNA ligase (from NEB), and then (Gateway) cloned into pDestMyc using LR-II enzyme from Invitrogen. Constructs containing cDNAs encoding Flag-ATG16L1 and its deletions and Flag-Galectin-3 were cloned by amplifying with primer pairs

5′caccatggcccaactgaggattaag3′ (forward) and 5′tcagcgtctcccaaagatattagtgataga3′ (reverse) and 5′caccatggcagacaatttttcgctccat3′ (forward) and 5′ttatatcatggtatatgaagcact3′ (reverse), respectively, followed by subcloning into pENTRY (Invitrogen) and recombined into pDEST 3X Flag. pTRIM16wt and pTRIM16S116A/S203A were generated as Gateway clones in _pDEST_ vector encoding Flag-TRIM16 fusions.

All siRNAs were from Dharmacon. TRIM screens were carried out as previously described (Mandell et al., 2014). TRIM RAW264.7 cells were transfected with 1.5 µg of siRNAs as previously described (Ponpuak et al., 2009); 10^7 cells were resuspended in 100 µl of Nucleofector solution kit V (Amaxa), siRNAs were then added to the cell suspension and cells were nucleoporated using Amaxa Nucleofector apparatus with program D-032. Cells were re-transfected with a second dose of siRNAs 24 h after the first transfection, and assayed after 48 h.

Supplementary references

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