APPENDIX

Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy

Tomonori Kimura*, Jingyue Jia*, Suresh Kumar, Seong Won Choi, Yuexi Gu, Michal Mudd, Nicolas Dupont, Shanya Jiang, Ryan Peters, Farzin Farzam, Ashish Jain, Keith A. Lidke, Christopher M. Adams, Terje Johansen, and Vojo Deretic

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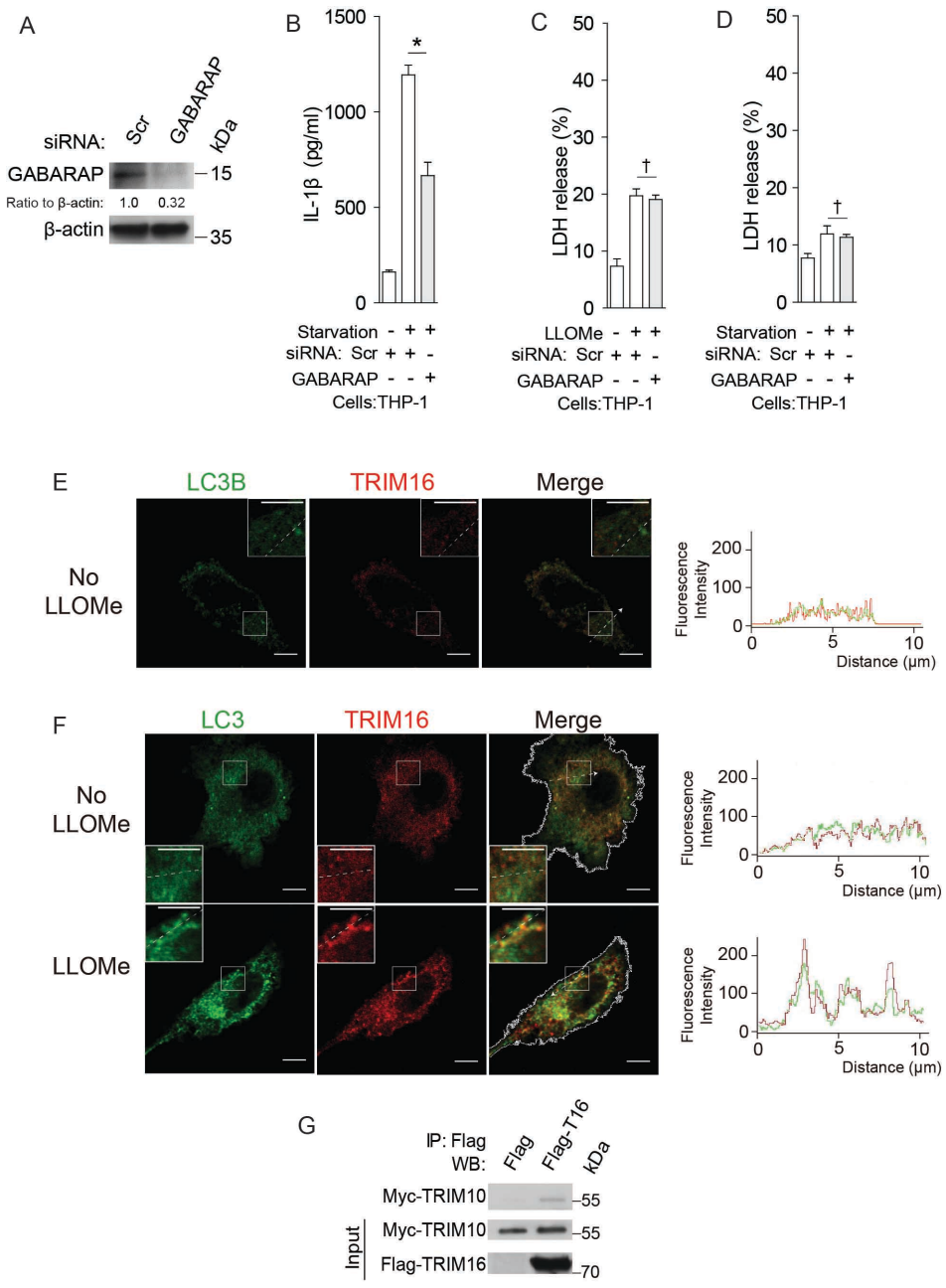
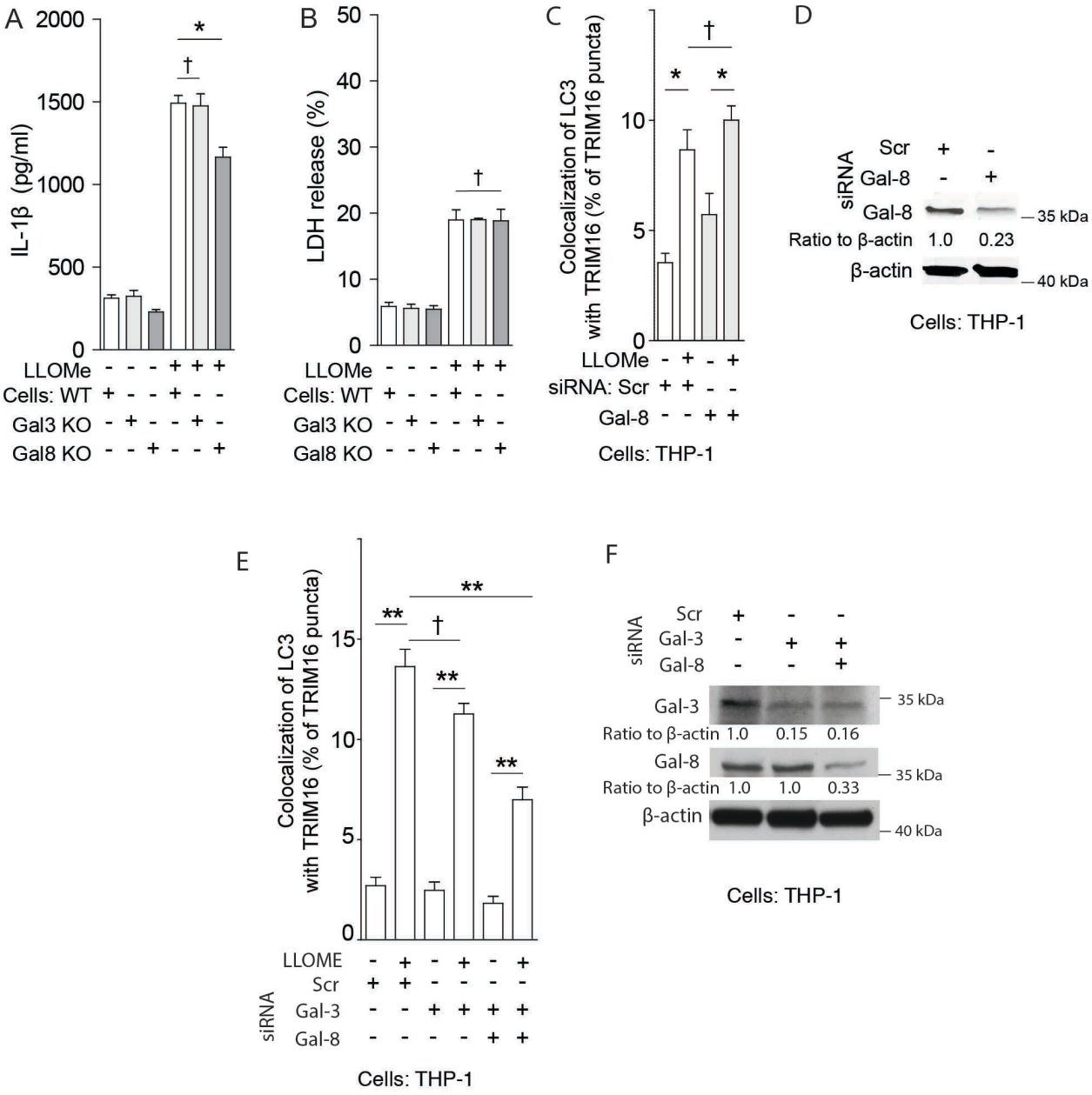
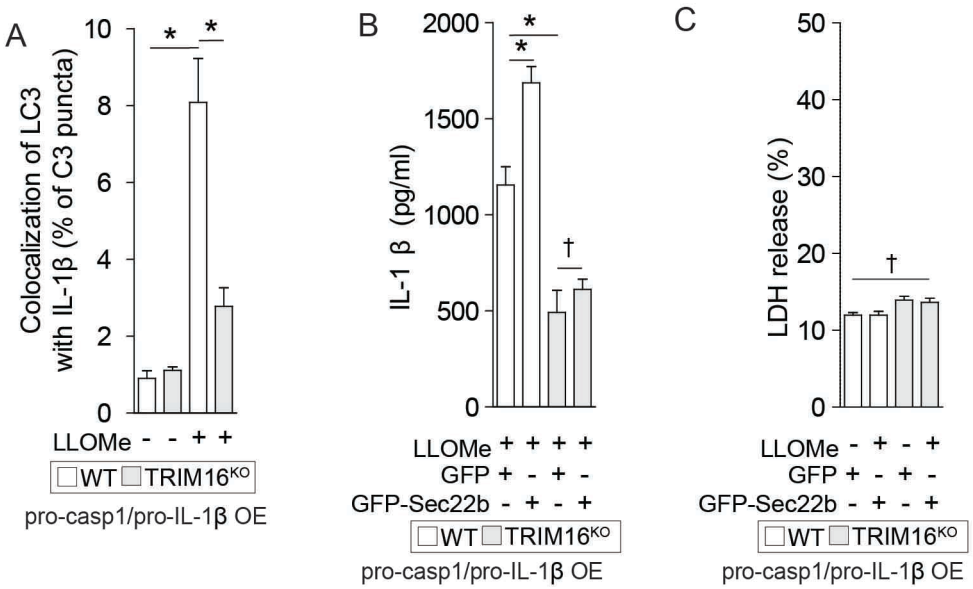


Appendix Figure S1. GABARAP participates in IL-1β secretion and TRIM16 colocalizes with LC3 and interacts with TRIM10. (A) Knockdown of GABARAP in THP1 cells corresponding to studies in Fig 1B, and panels B-D. (B) Effects of GABARAP knockdown on IL-1β secretion elicited by starvation in EBSS. (C) LDH control corresponding to data in Fig 1B. (D) LDH control corresponding to data in panel A. (E) Confocal microscopy of THP-1 cells in response to LLOMe. No LLOMe treated control for Fig 1C. (F) Confocal images from another set of experiments related to Fig. 1C. Line tracings correspond to arrows. Scale bars, 5 μm. (G) Co-imunoprecipitation analysis of tagged TRIM16 and TRIM10 expressed in 293T cells. Data, means ± SEM; n≥5. *P < 0.05 (t test).



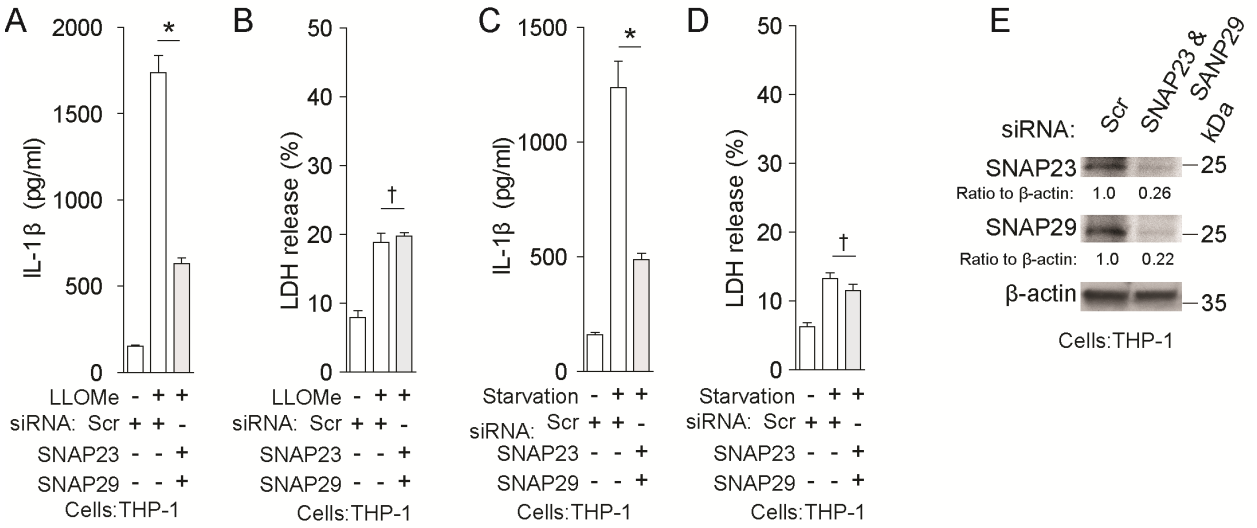
Appendix Figure S2. Galectin-8 participates in IL-1β secretion.

(A) Analysis of IL-1β secretion from primary macrophages derived from bone marrows of C57BL and their derivative galectin-3 (B6.Cg-Lgals3m1Poi/J) and galectin-8 (B6;129S5-*Lgals8*$^{Gt(neo)406Lex}$/Mmucd) knockout mice. (B) LDH measurements from experiments in A. (C-F) High content microscopy analysis of effects of galectin-8 (C,D), galectin-3 or combined galectin-3 and galectin-8 (E,F) knockdown on colocalization between TRIM16 profiles and LC3 puncta (% of total TRIM16 puncta) in THP cells. Data, means ± SEM; n≥5. *$P < 0.05$, ** $P < 0.01$, † $P \geq 0.05$ (ANOVA).



Appendix Figure S3. TRIM16 is required for IL-1β delivery to IL-1β -sequestration membranes.

(A) High content imaging and quantitative colocalization analysis of IL-1β and endogenous LC3B in HeLa cells (TRIM16 wild type; WT) and their TRIM16^{KO} derivative A9 reconstituted with pro-caspase 1 and pro-IL-1β by transfection. (B) IL-1β secretion form HeLa TRIM16^{wt} or TRIM16^{KO} cells reconstituted with pro-caspase 1 and pro-IL-1β and overexpressing GFP-Sec22b or GFP. (C) LDH release from cells in B. Data, means ± SEM; n≥5. *P < 0.05 (ANOVA).



Appendix Figure S4. Q_{bc}-SNAREs participate in IL-1β secretion.
(A-D) (A and C) IL-1β levels and (B and D) LDH release controls in supernatants of THP-1 cells subjected to knockdowns, treated with LPS, and then with LLOMe (A and B) or starved in EBSS (C and D). (E) Double SNAP23/SNAP29 knockdown corresponding to Appendix Figure S4A-D. Data, means ± SEM; n≥5. * $P < 0.05$, † $P \geq 0.05$ (t test).

Supplementary Materials and Methods

Cells, plasmids, siRNA, and transfection

THP-1, HeLa and HEK293T cells were from ATCC. Human peripheral blood monocytes were from StemCell Technologies or from healthy individual donors and cultured as previously described (Kimura, Jain et al., 2015), as approved by the Human Research Review Committee at the University of New Mexico. Informed consent was obtained from all subjects and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and Department of Health and Human Services Belmont Report. Atg9 KO MEFs and their matching wild type MEFs were from Sharon Tooze and Shizuo Akira. HeLa cells stably expressing mRFP-GFP-LC3B (tandem HeLa) were from D. Rubinsztein, Cambridge University. Murine bone-marrow macrophage cells were prepared from femurs of $Atg5^{fl/fl}$ LysM-Cre mice and their Cre-negative littermates (Zhao, Fux et al., 2008), and from C57BL mice and their galectin-3 (B6.Cg-Lgals3m1Poi/J. Jackson Laboratory) or galectin-8 knockout derivatives (B6;129S5-*Lgals8*$^{Gt(neo)406Lex}$/Mmucd*;* Mutant Mouse Resource & Research Centers), as previously described (Ponpuak, Davis et al., 2010). The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of New Mexico and it followed the NIH Policy on Humane Care and Use of Laboratory Animals. THP-1 cells were differentiated with 50 nM PMA for overnight before use. TRIM16, pro-IL-1β, and galectin-8 were cloned by PCR and Gateway BP reaction into pDONR221 or pENTR vectors (Invitrogen). Expression vectors were made by the LR reaction (Gateway; Invitrogen). The TRIM16 and galectin-8 mutants were generated by site-directed mutagenesis and confirmed by sequencing. Sec22b was from DNASU. pCI-Caspase 1 was from K. Fitzgerald (University of Massachusetts Medical School). siRNAs were from GE Healthcare/Dharmacon, and were delivered to cells by either RNAiMax (Life Technologies) or nucleoporation (Amaxa). Plasmid transfections were performed by either calcium phosphate or nucleoporation (Amaxa).

Immunostaining and fluorescence confocal microscopy

Immunostaining and fluorescence confocal microscopy was carried out as described previously (Kimura et al., 2015). Images were acquired using a microscope (META; Carl Zeiss) equipped with a 63×/1.4 NA oil objective, camera (LSM META; Carl Zeiss) and AIM software (Carl Zeiss). Fluorochromes associated with secondary antibodies were Alexa Fluor 488, 568, or 647. Perason correlation coefficient was calculated using ImageJ from > 5 fields per experiment.

High content analysis of puncta in subpopulations of transfected cells

HeLa cells were transfected with plasmids and cultured in media. Cells were fixed and stained to detect LC3 (Alexa Fluor 568 as fluorochromes), GFP, and nuclei (Hoechst 33342). High content imaging and automated analysis were performed using a Cellomics V^{TI} HCS scanner and iDEV software (ThermoScientific); > 500 primary

objects (transfected cell subpopulations) were analyzed. Overlapping area corresponding to colocalization between GFP-Sec22 and LC3, and between GFP and mRFP, was determined by iDEV software program.

Generation of knockouts using CRISPR/Cas9

To generate TRIM16 CRISPR knockout cell lines, HeLa cells were transfected with a PX458 (Ran, Hsu et al., 2013) encoding the U6 promoter, human TRIM16 target sequence located within the first exon (AGTTGGATCTAATGGCTCCA, with the 5' nucleotide A changed into a G; this sequence is followed on the chromosome by a protospacer adjacent motif GGG and was selected using crispr.mit.edu/guides site) fused to a chimeric guide RNA, S. *pyrogenes* Cas9, and GFP. One day after transfection, GFP-positive cells were sorted by flow cytometry and plated in 96-well plates. Single colonies were expanded into 24-well plates before screening for depletion of the targeted gene product by immunoblotting.

Immunoblotting, co-immunoprecipitation and GST pull-downs

Immunoblotting and Co-immunoprecipitations were performed as previously described (Kimura et al., 2015). In brief, cells were lysed with NP-40 buffer (Life Technologies) containing 1mM PMSF and protease inhibitor cocktail (Roche) for 45 min followed by centrifugation. Supernatants were incubated for 2 h with antibodies at 4ºC. The immune complexes were captured with Dynabeads (Life Technologies). Immunoprecipitates were washed three times with PBS, eluted with Laemmli SDS-PAGE sample buffer, and subjected to immunoblots analysis. 10% of extract used for immunoprecipitations were loaded as inputs. GST and GST-tagged proteins were expressed in Escherichia coli BL21 (DE3) or SoluBL21 (Amsbio). GST and GST-fusion proteins were purified and immobilized on glutathione-coupled sepharose beads (Glutathione-sepharose 4 Fast Flow: GE Healthcare) and pull-down assays with in vitro translated [^{35}S]-labeled proteins were done as described previously (Pankiv, Clausen 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 for 5 min in SDS-PAGE gel loading buffer, separated by SDS-PAGE, and radiolabeled proteins detected in a Fujifilm bioimaging analyzer (BAS-5000; Fuji film).

Super-resolution fluorescence microscopy analysis details

After fixation as previously described (Valley, Liu et al., 2015), cells were labeled with anti-flag-AF647 at 5 µg/ml in PBS + 2% BSA + 0.05% Triton X-100 (PBS/BSA/TX) overnight and washed in PBS/BSA/TX. Then, cells were mounted in 1% agarose gel for sample stabilization. 1% gel was made from 4% gel heated to 80°C and mixed with heated 1X-PBS at 80°C then brought down to 40°C. 75 µL of 1% gel was added on coverslip and the resulting sample placed in a refrigerator at 4°C for 5 minutes to solidify. The coverslip was mounted on an Attofluor cell chamber (A-7816, life technologies) with 1.1 ml of the imaging buffer. Imaging buffer consisted of an enzymatic oxygen scavenging system and primary thiol: 50 mM Tris, 10 mM NaCl, 10% w/v glucose, 168.8 U/ml glucose oxidase (Sigma #G2133),1404 U/ml catalase (Sigma #C9332), and 20 mM 2-aminoethanethiol (MEA), pH 8. The chamber was sealed by

placing an additional coverslip over the chamber and the sample the oxygen scavenging reaction was allowed to proceed for 20 min at room temperature before the imaging started.

Imaging was performed using a custom built microscope controlled by custom-written software in MATLAB (MathWorks Inc.). A sCMOS camera (C11440-22CU-Hamamatsu) was used to collect super-resolution data and a second camera (DMK 31AU03-imagingsource) for active stabilization (McGorty, Kamiyama et al., 2013). A 642 nm Laser (80 mW-HL6366DG-Thorlabs) is used as the excitation laser and a 405 nm Laser (40 mW-DL5146-101S-Thorlabs) was used to accelerate the dark to fluorescent state transition. The objective was NA=1.49 (APON 60XOTIRF-Olympus) and the filters consisted of a 835/70 nm (FF01-835/70-25-Semrock) for IR stabilization emission path, 708/75 nm (FF01-708/75-25-Semrock) for SR image emission path, and 640/8 nm (LD01-640/8-12.5-Semrock) laser diode clean up filter.

When imaging the first label (TRIM16), a brightfield reference image was taken and saved. During the data acquisition, the 642 nm laser was used at ~ 1.5 kW/cm^2 to take 20 sets of 2,000 frames at 20 Hz. After imaging all cells on the coverslip, the dye was removed using bleaching and $NaBH_4$ as described (Valley et al., 2015). GFP-Sec22 was labeled by first blocking the cell with Signal Enhancer (136933-Molecular Probes, Life Technologies) for 30 minutes followed by relabeling with GFP binding protein (gt-250-Chromotech) conjugated with AF647 at 1 μg/ml for 2 hours. For the second round of imaging, each cell was re-aligned using the saved brightfield reference image as described (Valley et al., 2015).

Data were analyzed via a 2D localization algorithm based on maximum likelihood estimation (MLE) (Smith, Joseph et al., 2010). The localized emitters were filtered through thresholds of a maximum background photon counts at 200, a minimum photon counts per frame per emitter at 250, and a minimum *P* value at 0.01. The accepted emitters were used to reconstruct the SR image. Each emitter was represented by a 2D-Gaussian with σ_x and σ_y equal to the localization precisions, which were calculated from the Cramér-Rao Lower Bound (CRLB). For cluster analysis, the data was first processed by H-SET (Hierarchical Single Emitter hypothesis Test), a top-down hierarchical clustering algorithm designed to collapse clusters of observations of blinking fluorophores into single estimates of the true locations of the fluorophores using a log-likelihood hypothesis test (Lin, Wester et al., 2016). Next, the pairwise cross-correlation function was computed from this data via a reconstructed histogram image using a pixel size of 10.4 nm. The two-dimensional pairwise cross-correlation function was averaged over eight 3000 nm x 3000 nm regions of interest chosen to emphasize the co-clustering of the two proteins. The results were then angularly averaged to produce the figure displaying g(r) versus r. The computation of the cross-correlation function was based on MATLAB code originally developed (Veatch, Machta et al., 2012).

_N_-ethylmaleimide (NEM) treatment

NEM treatment was performed as previously described (Galli, Zahraoui et al., 1998). Cells were washed twice in PBS supplemented with 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ (PBS^+) and were treated in PBS^+ on ice either with 1 mM NEM for 15 min followed by quenching with 2 mM DTT for 15 min or with 1mM NEM plus 2 mM DTT for 30min. Cells were washed in PBS^+ and further incubated in culture medium for 30 min at 37 °C.

Methanol precipitation

For chloroform methanol precipitation, cells were stimulated in OptiMEM. Culture media were cleared by centrifugation at 2,000 rpm. Proteins were precipitated with equal volume of methanol and 1/3 volume of chloroform, and then harvested by centrifugation. Pellets were resuspended in Laemmli buffer.

Bacterial strains and microbiological procedures

M. tuberculosis wild-type Erdman and ESX-1 mutant were cultured in Middlebrook 7H9 broth supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% oleic acid, albumin, dextrose, and catalase (OADC; BD Biosciences) at 37 °C and homogenized to generate single-cell suspension for macrophage infection studies. THP-1 cells were infected with mycobacteria as previously described (Ponpuak, Delgado et al., 2009). THP-1 cells were plated onto each well were infected with single cell suspension of mycobacteria in complete media at MOI of 10 for 1 h. Cells were then washed with PBS to remove un-internalized mycobacteria. After 17 h infection, supernatants of cells were harvested using 96-well filter plates (Agilent Technologies) and the levels of indicated proteins were measured.

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