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Supplementary Materials for

Salmonella Inhibits Retrograde Trafficking of Mannose-6-Phosphate Receptors and Lysosome Function

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Published 16 November 2012, *Science* **338**, 963 (2012)
DOI: 10.1126/science.1227037

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available at www.sciencemag.org/cgi/content/full/338/6109/963/DC1

Supplemental Materials and Methods

Bacterial strains and plasmids. The *S.* Typhimurium strains used in this study were wild-type 12023s and its mutant derivatives: *ssaV::aphT* (*9*), *aroC,purD* (*15*), *sifA,sseJ* (*15*) and *sseJ* (*15*). All *S.* Typhimurium strains harboured the GFP-expressing pFPV25.1 plasmid carrying *gfpmut3A* under the control of the *rpsM* constitutive promoter (*25*) with the exception of those used during experiments to quantify levels of CD-MPR at the TGN, where detection of pSifA-2HA, pSifA$_{L130D}$-2HA or pSseJ-2HA was required. The plasmids pSifA-2HA and pSifA$_{L130D}$-2HA (*18*) and transfection vectors encoding EGFP-SifA and EGFP-SifA$_{L130D}$ were gifts from Dr. Stéphane Méresse (Centre d'Immunologie de Marseille-Luminy; Marseille, France). A gene encoding EGFP-SifB was also ligated in place of EGFP-SifA. Plasmid pSseJ-2HA, which is a derivative of pWSK29 carrying *sseJ-2HA* under the control of its own promoter (*26*), was introduced into the *aroC,purD* mutant to detect effector translocation from this strain.

Antibodies and siRNA oligos. The following antibodies were used for immunofluorescence and immunoblot analysis: sheep polyclonal anti-TGN46 (ABD Serotec, U.K.); rabbit polyclonal anti-Rab6 (Santa Cruz, U.S.A.); mouse monoclonal anti-Syntaxin 6 (BD Biosciences, U.K.); rabbit polyclonal anti-EpsinR (gift from Professor Margaret Robinson, Cambridge Institute for Medical Research, U.K.); mouse monoclonal anti-CD-MPR (22d4, The Developmental Studies Hybridoma Bank, University of Iowa, U.S.A.); mouse monoclonal anti-CI-MPR (2G11, Abcam, U.K.); rat monoclonal anti-HA (3F10, Roche, U.K.); mouse monoclonal anti-Flag (M2, Sigma-aldrich, U.K.); rabbit polyclonal anti-GFP (Life Technologies, U.K.); goat polyclonal anti-cathepsin D (C20, Santa Cruz, U.S.A.); rabbit polyclonal anti-actin (Sigma-aldrich, U.K.); rabbit monoclonal anti-Rab9 (D52G9 XP, Cell Signaling, U.K.); rabbit monoclonal anti-EEA1 (C45B10, Cell Signaling, U.K.); rabbit polyclonal anti-VPS26 (gift from Dr. Matthew Seaman Cambridge Institute for Medical

Research, U.K.) and rabbit polyclonal anti-syntaxin 10 (gift from Dr. Andrew Peden, Cambridge Institute for Medical Research, U.K.) ; rabbit polyclonal anti-SKIP (gift from Dr. Stéphane Méresse, Centre d'Immunologie de Marseille-Luminy; Marseille, France); Alexa Fluor 405-, 488-, 555- and 633- conjugated donkey anti-sheep, anti-mouse, and anti-rabbit antibodies were from Life technologies, U.K.

siRNA oligo duplexes targeted against non-overlapping regions of Syn10 or Syn6 mRNA, were either used singly or in pools of 4 (SMARTpool, Dharmacon Inc., U.K.). The siRNA oligo duplex targeted against SKIP was custom synthesized using a previously described targeting sequence (*16*) (Dharmacon Inc., U.K.). The scramble pool of 4 control oligos (Dharmacon Inc., U.K.) were designed not to target any human mRNA transcript.

Cell growth and infection. Bacteria were grown in Luria Bertani (LB) medium at 37 °C with shaking and supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (25 μg/ml) or chloramphenicol (34 μg/ml) as appropriate. HeLa (human epithelial cell line 93021013) cells or mouse embryonic fibroblast (MEF) cells (Eurocom Consortium) used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life technologies, U.K.) supplemented with 10% foetal calf serum (FCS) (PAA Laboratories, U.K.) at 37 °C in 5% CO_2. For infection, HeLa cells were grown overnight and then infected with *S.* Typhimurium strains as described previously (*10*). RAW264.7 macrophages were obtained from the European Collection of Cell Cultures (Sigma-aldrich, U.K.). Cells were grown in media supplemented with 10% FCS at 37 °C in 5% CO_2. For infection, RAW264.7 macrophages were grown overnight then infected with *S.* Typhimurium strains as described previously (*10*).

Primary bone-marrow macrophages (BMM) were obtained from C57BL/6 WT (Charles River, U.S.A.) mice. Cells were grown in RPMI (Life technologies, U.K.)

supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 50 μM β-mercaptoethanol, 100 U/mL penicillin/streptomycin (Sigma-aldrich, U.K.), and L929 cell–conditioned medium 20% (vol/vol; National Institute for Medical Research, U.K.). After 3 days of culture, further fresh complete medium containing L929 cell–conditioned medium was added to the growing macrophages. On day 7, cells were washed and seeded in complete medium without antibiotic and incubated for 24 h before bacterial challenge.

siRNA transfection. HeLa cells were seeded in 12 well plates and grown until they were 70% confluent. siRNA transfection using RNAiMAX (Life technologies, U.K.) was carried out according to the manufacturer's protocol with a final concentration of pooled or single siRNA oligos of 10 nM. A scrambled pool of 4 oligo sequences was included in all experiments as a negative control. Cells were collected 24 h after transfection and re-seeded in 12 well plates at a concentration of 1 x 10^5 cells/well. Cells were then transfected a second time as above and 24 h after that were seeded again at appropriate concentrations for analysis.

DNA transfection. HeLa or MEF cells were seeded in 24 well, 12 well or 35 mm glass bottomed dishes (Matek, U.S.A.) and grown until they were 70% confluent. DNA transfection procedures were carried out according to the manufacturer's protocol (Lipofectamine™ 2000, Life technologies, U.K.) with 400 ng/1 x 10^5 cells of DNA. Samples were prepared for analysis 24 h after transfection. Only cells with similar levels of expression, as determined from their GFP fluorescence using FACS analysis or fluorescence microscopy, were included for analysis.

Fixation, fluorescence labeling and microscopy. Cell monolayers were fixed, permeabilized and labeled as described previously (*23*). Fluorescently labeled samples were

analysed using either an epi-fluorescence microscope (BX50; Olympus, Melville, NY) or a confocal laser-scanning microscope (LSM510 or LSM710; Zeiss GmBH).

Quantification of proteins localized to the TGN. Confocal three-dimensional Z-stacks were acquired for each sample using a slice increment of 0.4 μm and 63 x optical magnification. Individual TGNs from cells were selected automatically using a protocol established in Volocity image analysis software (Perkin Elmer, U.K.). TGNs were identified as objects within a volume range of 50 to 500 μm^3 according to an arbitrary threshold of fluorescence intensity associated with anti-TGN46 or anti-Rab6 antibody labeling. Once individual objects were identified, the mean fluorescent signal from pixel-to-pixel co-labeled protein was quantified using Volocity software. These values were further partitioned, where appropriate, into those arising from infected and non-infected or transfected and non-transfected cells, determined by GFP or HA labeling. At least 50 TGNs from each condition were quantified for each sample.

CD-MPR co-localization. To determine the co-localization of CD-MPR within compartments containing EEA1, VPS26 or Rab9, confocal three-dimensional Z-stacks were acquired for each sample using a 63x objective with a slice increment of 0.5 μm. At least 50 infected cells were imaged for each condition. Rendered three-dimensional stacks were analysed with Volocity image analysis software (Perkin Elmer, U.K.). The frequency of CD-MPR co-localizing with other markers was determined using an automated identification protocol implemented using Volocity software as follows: compartments were identified as objects within a volume range of 0.01 to 50 μm^3 according to an arbitrary threshold of fluorescence intensity associated with anti-CD-MPR immunolabeling. CD-MPR-positive compartments co-localizing with anti-EEA1, anti-Vps26 or anti-Rab9 antibody labeling were expressed as proportional frequency of total CD-MPR-positive compartments. The Pearson's

correlation coefficient of whole cell co-localization of CD-MPR and Rab9 was determined using Volocity software. Non-infected cells or si Scramble treated cells were used to normalize between different experimental conditions and samples. Results from infected cells or si SKIP-treated cells are represented as a function of their respective normalization controls.

Plasma membrane-TGN transport assays. CI-MPR transport or CTxB transport assays were undertaken as described previously (*11*, *27*) with the following modifications. HeLa cells were transfected with Syn6 or SKIP siRNA as described above or were infected with wild-type or *ssaV* mutant *S.* Typhimurium for 13 h. Then, cells were incubated with culture medium containing either anti-CI-MPR antibody (2G11) or Alexa Fluor 555-CTxB (Life technologies, U.K.) for 45 min followed by a chase of 30 min with medium only. Quantification of the amount of CI-MPR or CTxB localized at the TGN after SKIP siRNA treatment was conducted using quantitative confocal microscopy as described above. Quantification of the number of cells showing CI-MPR or CTxB localization at the TGN after infection or Syn6 siRNA treatment was done by epi-fluorescence microscopy. Quantification was conducted in the fluorescent channels associated with CI-MPR or CTxB and TGN46 or Rab6 labeling. The cells were assessed as to whether there was TGN-associated labeling of CI-MPR or CTxB or not. Next, where appropriate, the fluorescent channel was switched to reveal *Salmonella* and the same cells were assessed according to whether they were infected or not. At least 50 cells were analysed for each condition.

Lysosomal enzyme secretion assays. The activities of secreted β-hexosaminadase and cathepsin D were analysed as described (*28*, *29*) with the following modifications: HeLa cells were grown in 6 well plates, seeded at a concentration of 2 x 10^5 cells/well, for 24 h. Cells were infected with wild-type or *ssaV* mutant *S.* Typhimurium. At 1 h after inoculation and for

every subsequent media exchange DMSO or amiodarone (Sigma-aldrich, U.K.) were added to negative and positive control cells, respectively. Amiodarone was used at a concentration of 8 μM. At 8 h after inoculation cells were washed 4 times with pre-warmed collection media (Optimem, Life technologies, U.K.) supplemented with 10 mM mannose-6-phosphate (Sigma-aldrich, U.K.) and 20 μg/ml gentamicin. Collection medium (1.5 ml) was then added to each well and the cells were incubated once more at 37 °C.

At 14 h after inoculation extracellular β-hexosaminidase activity was assayed in 480 μl of the collection medium by addition of 120 μl of β-hexosaminidase substrate buffer (0.5 M NaOAc pH 4.4, 0.5% Triton X-100, 5 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-d-glucopyranoside (Sigma-aldrich, U.K.)). After 1 h 30 min at 37 °C, reactions were stopped by addition of 600 μl of 0.5 M glycine, 0.5 M Na_2CO_3, pH 10.0. Release of fluorescent substrate was measured by a Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 360 nm and emission wavelength of 447 nm. Intracellular β-hexosaminidase activity was determined by scraping the cells of one well in 500 μl of cell collection buffer (10 mM phosphate buffer, pH 6.0, 0.15 M NaCl, 0.5% Triton X-100) followed by centrifugation at 60,000 *g* for 30 min. The supernatant was transferred into a fresh tube and 120 μl of sample and 120 μl of hexosaminidase substrate buffer were diluted with 360 μl of Optimem. After 1 h 30 min at 37 °C, reactions were stopped by addition of 600 μl of 0.5 M glycine, 0.5 M Na_2CO_3, pH 10.0 and samples were assayed as described for extracellular β-hexosaminidase activity.

At 14 h after inoculation, extracellular cathepsin D activity was assayed in 80 μl of the collection medium by addition of 20 μl of cathepsin D substrate buffer (0.25 M NaOAc, pH 4.2, 0.25% Triton X-100, 100 μM MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH_2 (Peptide Institute, Japan)). After 1 h at 37 °C, reactions were stopped

by addition of 600 µl of 5% TCA. Release of fluorescent substrate was measured by a Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 328 nm and emission wavelength of 398 nm. Intracellular cathepsin D activity was determined by scraping the cells from one well in 500 µl of cell collection buffer (10 mM phosphate buffer, pH 6.0, 0.15 M NaCl, 0.5% Triton X-100) followed by centrifugation at 60,000 *g* for 30 min. The supernatant was transferred into a fresh tube and 20 µl of sample and 20 µl of cathepsin D substrate buffer were diluted with 60 µl of Optimem. After 1 h at 37 °C, reactions were stopped by addition of 600 µl of 5% TCA and samples were assayed as described for extracellular cathepsin D activity. β-hexosaminidase and cathepsin D activity from intracellular and extracellular samples were expressed as a % ratio of total enzyme activity, which equals the sum of intracellular and extracellular activities.

To analyse protein levels of intracellular and secreted cathepsin D, HeLa cells were grown in 10 cm circular plastic dishes and seeded at a concentration of 2.5 x 10^6 cells/dish. Cells were infected with wild-type or *ssaV* mutant *S.* Typhimurium. At 1 h after inoculation and for every subsequent media exchange DMSO, amiodarone (8 µM) were added as appropriate. At 8 h after inoculation the cells were washed 4 times with pre-warmed collection media (Optimem, Life technologies, U.K.). Collection medium (4 ml) was then added to each dish and the cells were incubated once again at 37 °C. At 14 h after inoculation the collection medium was harvested (extracellular fraction) and concentrated using Amicon Ultra-15 Centrifugal Filter Units according to the manufacturer's protocol (Millipore, U.K.). Cells were collected using a trypsin-EDTA solution (Life technologies, U.K.), then resuspended in equal volume of lysis buffer (20 mM Tris-Cl pH 8.0, 0.5% NP40, 150 mM NaCl) in the presence of protease inhibitors (Complete EDTA-Free, Roche, U.K.). Intracellular samples were normalized by dilution to give the same overall protein

concentration. The same dilution factor was applied to each of the corresponding extracellular fractions, so that samples reflected an equivalent number of cells. Intracellular and extracellular proteins were then separated by SDS-PAGE and analysed by immunoblot using antibodies against cathepsin D and actin (loading control).

Cathepsin B assay using Magic Red-RR. For flow cytometry assays to determine cathepsin B activity at steady state, cells were harvested in Optimem (Life Technologies, U.K.) containing a membrane-permeable substrate, Magic Red cathepsin B peptide (Magic Red™ Cathepsin B, ABD Serotec, U.K.) added to the medium at one-tenth the manufacturer's recommended concentration. Cells were then fixed in 3% PFA prior to data collection. A total of over 10,000 events were analysed on a LSR Fortessa™ flow cytometer (Becton Dickinson, U.K.) for fluorescence intensities using a 530 nm emission filter and a 640-670 nm band pass emission filter. The presence of GFP fluorescence was used to determine whether cells were infected or transfected. Untransfected cells or uninfected cells were used to calibrate the instrument settings. All data were normalized to the fluorescent signal of control samples treated with 50 mM ammonium chloride 10 min prior to the addition of Magic Red cathepsin B peptide. Data were analysed with FlowJo™ 8.6.3 software.

For confocal assays to determine the temporal response of cathepsin B activity following photobleaching, HeLa cells, RAW264.7 macrophages or BMM were grown on 35 mm glass bottomed dishes (Matek, U.S.A.) seeded at a concentration of 1.0-5.0 x 10^5 cells/dish in appropriate media. Cells were then either transfected or infected with *S.* Typhimurium strains. At 1 h 30 min prior to imaging, growth medium was replaced with imaging medium (Optimem) and incubated at 37 °C. Live cell imaging and fluorescence recovery analysis were done using a LSM510 Zeiss confocal microscope. Fluorescence bleaching was done on entire cells using 458, 488, 543, and 633 nm lasers with a 40 x

objective. Approximately 5 to 8 cells per field of view were bleached for a duration of 100 bleaching cycles according to the bleaching module within the LSM510 software (LSM510 version 3.2). Post-bleaching images were acquired every three seconds for 150, 200 or 300 seconds. Quantification of fluorescence was carried out using Volocity software, with each data set having approximately 25 cells in each condition analysed, and each curve representing results from 3 experiments. Fluorescence values were converted to relative values by expressing the first post-bleach value as zero. Inter-sample normalization was done using the fluorescence values of non-infected or non-transfected samples, as appropriate. In all experiments error bars represent +/- SEM of at least 3 independent experiments each with at least triplicate measurements.

Lysosome protease assays using DQ-BSA. HeLa cells were treated with siRNA or were infected with GFP-expressing *S.* Typhimurium strains for 13 h, after which DQ-Red-BSA (0.25 μg/ml) (Life technologies, U.K.) was added for 1 h at 37 °C. Cells were harvested and fixed in 3% PFA for 20 min. A total of over 10,000 events were analysed on a LSR Fortessa^{TM} flow cytometer (Becton Dickinson, U.K.) for fluorescence intensities using a 530 nm emission filter and a 640-670 nm band pass emission filter. All data were normalized to the fluorescent signal of control samples treated with 50 mM ammonium chloride 15 min prior to the addition of DQ-BSA and maintained throughout the assay. Data were analysed with FlowJo^{TM} 8.6.3 software.

Intracellular bacterial replication. HeLa cells were seeded at a concentration of 2 x 10^5 cells/well in 6 well plates and treated with mock, scramble, Syn6 or Syn10 siRNA oligos or exposed to a lysosomal protease inhibitor (Cathepsin Inhibitor III, Merck, U.K.) at a concentration of 0.4 μM, 4 h prior to infection. The inhibitor was maintained through all subsequent media exchanges. Cells were then infected with wild-type *S.* Typhimurium as

described above. Cells were collected at 2 h, 8 h, 10 h and 12 h after inoculation. Following exposure to trypsin-EDTA solution, cells from each well were resuspended in 10 ml of 10% FCS DMEM. Cells were then washed and resuspended in 1 ml of PBS. Cells were fixed in 4% paraformaldehyde-PBS for 20 min, washed twice in PBS and then re-suspended in 1 ml PBS for analysis. A total of over 10,000 events were analysed on a FACS Calibur™ flow cytometer (Becton Dickinson, U.K.) for fluorescence intensities in channel FL-1. Uninfected cells were used to calibrate the instrument settings. Data were analysed with FlowJo™ 8.6.3 software. The median GFP fluorescence per HeLa cell was determined for triplicate samples per condition. Results were expressed as the mean ratio of the median measurements between 2 h and indicated timepoints after inoculation, normalized to mock or DMSO treated cells.

Flow cytometry quantification of cellular protein levels. For flow cytometry assays to determine protein levels by immunolabeling, trypsinized cells were washed once in PBS prior to fixation in 3% PFA for 20 min at room temperature. Cells were then washed twice in PBS and once in permeabilization solution: PBS supplemented with 2% bovine albumin serum (BSA, Sigma-aldrich, U.K.) and 0.1% saponin (Fisher, U.K.). Cells were incubated for 30 min at 4 °C with primary antibodies diluted in permeabilization solution. Cells were washed twice and incubated for 30 min at 4 °C with secondary antibodies. Finally, cells were washed twice in permeabilization solution and resuspended in PBS. IgG isotype controls were used at the same concentration as the primary antibodies. A total of over 10,000 events were analysed on a FACS Calibur™ flow cytometer for fluorescence intensities associated with the labeled protein and where appropriate infected cells. Uninfected cells were used to calibrate the instrument settings. Data were analysed with FlowJo™ 8.6.3 software. To determine the amount of plasma membrane associated CI-MPR in infected cells or si SKIP or scramble-treated HeLa cells the same protocol was applied in the absence of 0.1% saponin.

Immunoprecipitation HeLa cells stably expressing GFP-Rab9 and Flag-SKIP were grown on 150 mm dishes (Corning, U.K.) seeded at a concentration of 5 x 10^7 cells/dish in DMEM supplemented with 10% FCS. Cells were then infected with *S.* Typhimurium strains expressing either SifA-2HA or $SifA_{L130D}$-2HA for 14 h or left uninfected. Cells were lysed in GTPase lysis buffer (50 mM Tris.Cl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100) supplemented with complete protease inhibitor cocktail (Roche, U.K.) on ice for 20 min. Lysates were then centrifuged at 16100 *g* and the supernatants were harvested and incubated with 30 µl of anti-GFP beads (Chromotek, GmBH), or protein G control beads where appropriate, for a total of 30 min at 4 °C. Samples were then centrifuged at 1500 *g* and the supernatant was removed. Pellets were washed 3 times with lysis buffer and 3 times with wash buffer (50 mM Tris.Cl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100). Sample proteins were then separated by SDS-PAGE and analysed by immunoblot using anti-Flag, anti-GFP and anti-HA antibodies.

Virus production and transduction of HeLa cells and MEF cells. Viral supernatants were produced by transfection of 293ET cells with a proviral plasmid based on the M5P plasmid (*30*) together with helper plasmids encoding the vesicular stomatitis virus glycoproteins (pMD-VSVG) and the gag/pol proteins (pMD-OGP). Two days after transfection supernatants were collected. HeLa or MEF cells were spin-transduced (650 *g* for 2 hours, at room temperature) with a volume of virus that gave rise to a 30% transduction efficiency containing 8 µg/ml polybrene (Sigma-aldrich, U.K.). To select for transduced cells, cells were selected in 1.1 µg/ml puromycin (Sigma-aldrich, U.K.) two days post-transduction.

Generation of stable *Skip*$^{-/-}$ MEFs. MEF cells from *Skip*$^{-/-}$ mice (Eurocom Consortium) were immortalized with SV40 large T antigen. Following immortalization, cells were sub-cloned to obtain cells with a normal distribution of Magic Red-RR peptide fluorescence.

Three clones were used for further experiments.

Quantitative reverse-transcriptase PCR 1 x 10^6 HeLa cells were harvested following a 14 h infection with wild-type *Salmonella* for RNA extraction (Qiagen RNAeasy mini kit, U.K.). 500 ng of RNA was used to synthesize complementary DNA (cDNA) (QuantiTect RT kit, Qiagen, U.K.). 0.5 µl of cDNA was used in qPCRs (SensiMix dT kit, Quantace, U.K.) containing 0.2 µM gene-specific primers. The cycle threshold (Ct) value was determined as the number of cycles necessary to reach a significant increase in the emission intensity of the SybR Green reporter dye. Data are represented as relative amounts of mRNA normalized to an actin control.

Mouse Infections. 8 to 12-week-old BALB/c mice (Jackson Laboratory, U.S.A.) were left uninfected or inoculated by intraperitoneal injection with GFP-expressing bacteria comprizing 5x10^5 c.f.u. (wt) or 5x10^6 c.f.u. (*ssaV*) mutant or 1x10^6 c.f.u. (*sifAsseJ*) double mutant in a volume of 200 µL. Mice were sacrificed 48 h later. Spleens were harvested, homogenized and splenic CD11b(+) cells enriched using magnetic beads following manufacturer instructions (Miltenyi Biotec, U.K.). Purified cells were then incubated with Optimem containing Magic Red cathepsin B peptide as described above. Data were collected using LSR Fortessa flow cytometer (Becton Dickinson, U.K.). A total of over 10,000 events were analysed for each sample. Cathepsin B activity in uninfected CD11b(+) cells from infected mice was also measured and compared to activity in CD11b(+) cells from uninfected animals. Non-infected cells from within each sample were used to calibrate the instrument settings. All data were normalized to the fluorescent signal of control samples treated with 50 mM ammonium chloride 5 min prior to the addition of Magic Red cathepsin B peptide. Data were analysed with FlowJo™ 8.6.3 software.

Statistical analysis. Statistical differences were determined using a two-tailed student *t*-Test

on the means of at least three independent experiments with the exception of data utilized in figure S10, which was analyzed by two-way ANOVA. Probability values of less than 0.05, 0.01 and 0.005 were used to show statistically significant differences and are represented with *, ** or *** respectively.

Supplemental Figure Legends

Figure S1. (A and B) *S.* Typhimurium does not redistribute Rab6, TGN46, EpsinR or Syn6. (A) HeLa cells were infected for 14 h with a GFP-expressing wild-type (wt) *Salmonella* (blue), and were immunolabeled for TGN46 (green) and either Rab6, EpsinR or Syn6 (red). Scale bars, 10 μm. (B) Quantitative 3-D confocal microscopy of mean TGN-associated fluorescent signals of TGN46, Rab6, EpsinR and Syn6 in HeLa cells infected for 14 h. Data were normalized to uninfected cells within the same sample. (C) HeLa cells were infected for 14 h with an *aroC,purD* mutant strain expressing SseJ-HA and were immunolabeled, without permeabilizing bacteria, for HA (green), TGN46 (blue) and CD-MPR (red). Scale bar, 10 μm. (D) Redistribution of CD-MPR from TGN begins approximately 6 h after infection with GFP-expressing wild-type *Salmonella*. Infected HeLa cells were fixed at the indicated time-points following invasion and immunolabeled for CD-MPR and Rab6, then analysed by confocal microscopy. Data were normalized to uninfected cells from the same sample.

Figure S2. (A and B) Levels of plasma membrane-associated CI-MPR. HeLa cells were (A) non-infected (ni), infected for 14 h with GFP-expressing wild-type (wt) or *ssaV* mutant *Salmonella* or (B) depleted of SKIP by siRNA, then immunolabeled for plasma membrane (PM) levels of CI-MPR without permeabilizing the cell membrane and analysed by flow cytometry. Data were normalized to non-infected cells or si Scramble treated cells. n.s., not significantly different. The results fail to provide evidence for a change in the rate of endocytosis during infection or after depletion of SKIP.

Figure S3. Cathepsin B activity in cells depleted of Syn10 or Syn6 by siRNA. (A) Total HeLa cell levels of Syn10 and Syn6 were analysed by immunoblotting in mock-treated cells,

or cells treated with scrambled siRNA oligos, or pools of 4 oligos for Syn10 or Syn6. Intracellular levels of actin were used as a loading control. (B) HeLa cells depleted of Syn10 or Syn6 by indicated siRNA or exposed to scrambled siRNA were analysed for recovery of Magic Red-RR CatB activity after photobleaching. (C) HeLa cells treated with the indicated siRNA oligos were immunolabeled and analysed by quantitative 3-D confocal microscopy for mean TGN-associated fluorescence signals of Rab6, TGN46 or CD-MPR. Statistically significant relationships are denoted (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.005$)

Figure S4. CatB activity in infected macrophages. (A) RAW264.7 cells were infected for 14 h with GFP-expressing wild-type (wt) or *ssaV* mutant *Salmonella* and were analysed for Magic Red-RR CatB activity following photobleaching. (B and C) C57BL/6 mouse bone marrow-derived macrophages (BMM) were infected for 48 h (B) or 72 h (C) with wt or *ssaV* mutant *Salmonella* and were analysed by Magic Red-RR CatB activity following photobleaching. Data were normalized to the mean fluorescence of uninfected cells. Statistically significant relationships are denoted (* = $P<0.05$)

Figure S5. CD-MPR localization and CatB activity in SifA-expressing HeLa cells. HeLa cells expressing similar levels of SifA or SifA-$_{L130D}$ following transfection, were analysed for TGN-associated CD-MPR (A) or Magic Red-RR CatB activity following photobleaching (B). Data were normalized to non-transfected cells within the same sample. Statistically significant relationships are denoted (* = $P<0.05$; ** = $P<0.01$)

Figure S6. Analysis of SKIP depleted cells**.** (A) Total levels of SKIP were analysed by SDS-PAGE and immunoblotting in scramble or SKIP-specific siRNA-treated HeLa cells using an anti-SKIP antibody. Intracellular levels of actin were used as a loading control. (B) The

absence of SKIP was verified in *Skip*$^{-/-}$ MEFs using an anti-SKIP antibody. n.s. denotes a non-specific band used as a loading control. (C) Immunoblot analysis of *Skip*$^{-/-}$ MEFs transfected with vectors encoding full length Flag-SKIP or a SKIP variant lacking its PH domain (Flag-SKIP(N550)). (D) Flow cytometry analysis of *Skip*$^{-/-}$ MEFs to quantify levels of Flag-SKIP or Flag-SKIP(N550) by antibody labeling.

Figure S7. *Salmonella* depletes and redistributes CD-MPR. (A) HeLa cells were infected for 14 h with GFP-expressing wild-type *Salmonella* and immunolabeled for intracellular levels of CD-MPR and Rab6 prior to analysis by flow cytometry. Data were normalized to non-infected cells within the sample. (B) HeLa cells were infected with GFP-expressing wild-type *Salmonella* and immunolabeled for CD-MPR and either EEA1 or VPS26. The relative frequency of CD-MPR co-localizing with either EEA1 or VPS26 is shown with values normalized to non-infected cells within the same sample. There was no difference in the relative frequency of CD-MPR co-localizing with EEA1 when infected cells were compared to non-infected cells. (C) HeLa cells were infected for 14 h with GFP-expressing wild-type (wt) *Salmonella* or *ssaV* mutant strain and analysed by quantitative RT-PCR for relative levels of CD-MPR mRNA. Data were normalized to non-infected cells. Statistically significant relationships are denoted (* = $P<0.05$; ** = $P<0.01$)

Figure S8. *S.* Typhimurium does not redistribute Syn10. (A) HeLa cells were infected for 14 h with GFP-expressing wild-type (wt) *Salmonella* (blue), and were immunolabeled for TGN46 (green) and Syn10 (red). Scale bar, 10 μm. (B) Quantitative 3-D confocal microscopy of mean TGN-associated fluorescence signals of TGN46 and Syn10 in HeLa cells infected for 14 h. Data were normalized to uninfected cells from the same sample.

Figure S9. SifA, SKIP and Rab9 form a complex in infected cells. HeLa cells stably expressing Flag-SKIP and GFP-Rab9 were infected for 14 h with *sifA,sseJ* double mutant *Salmonella* expressing $SifA_{wt}$-HA or not infected. Cells were lysed and proteins immunoprecipitated with anti-GFP conjugated beads or anti-protein G (prG) conjugated beads. Proteins in samples before (Input) and after immunoprecipitation (IP) were separated by SDS-PAGE and immunoblotting.

Figure S10. Overexpression of $Rab9_{CA}$ inhibits the ability of *Salmonella* to reduce lysosome function. HeLa cells stably expressing GFP-$Rab9_{Q66L}$ ($Rab9_{CA}$) or GFP-Galectin3 (Gal3) were infected for 14 h with wild-type *Salmonella* and exposed to Magic Red-RR and the percentage of fluorescent product intensity of infected cells compared to uninfected cells from the same sample was measured at steady state by flow cytometry. Statistically significant relationships are denoted (** = $P<0.01$).

Figure S11. HeLa cells depleted of Syn6 or Syn10 or treated with scrambled control siRNA were infected with GFP-expressing wild-type *Salmonella*. Bacterial load, expressed as GFP fluorescence per cell, was measured by flow cytometry (n>10,000) for each sample at each timepoint. Results are expressed as the mean ratio of the median measurements between 2 h and indicated timepoints after inoculation, normalized to si Scramble-treated cells. Statistically significant relationships with respect to controls by two-way ANOVA are denoted (*= $P<0.05$)

Movies S1 and S2. HeLa cells were infected for 14 h with GFP-expressing wild-type (S1, wt) or *ssaV* mutant (S2, *ssaV*) *Salmonella* and exposed to CatB substrate (Magic Red-RR). Entire cells were selected for analysis using differential interference contrast (DIC)

microscopy. Infected and uninfected cells in the same field of view were analysed before and after bleaching and were imaged every 3 sec over a 150 sec period of analysis.

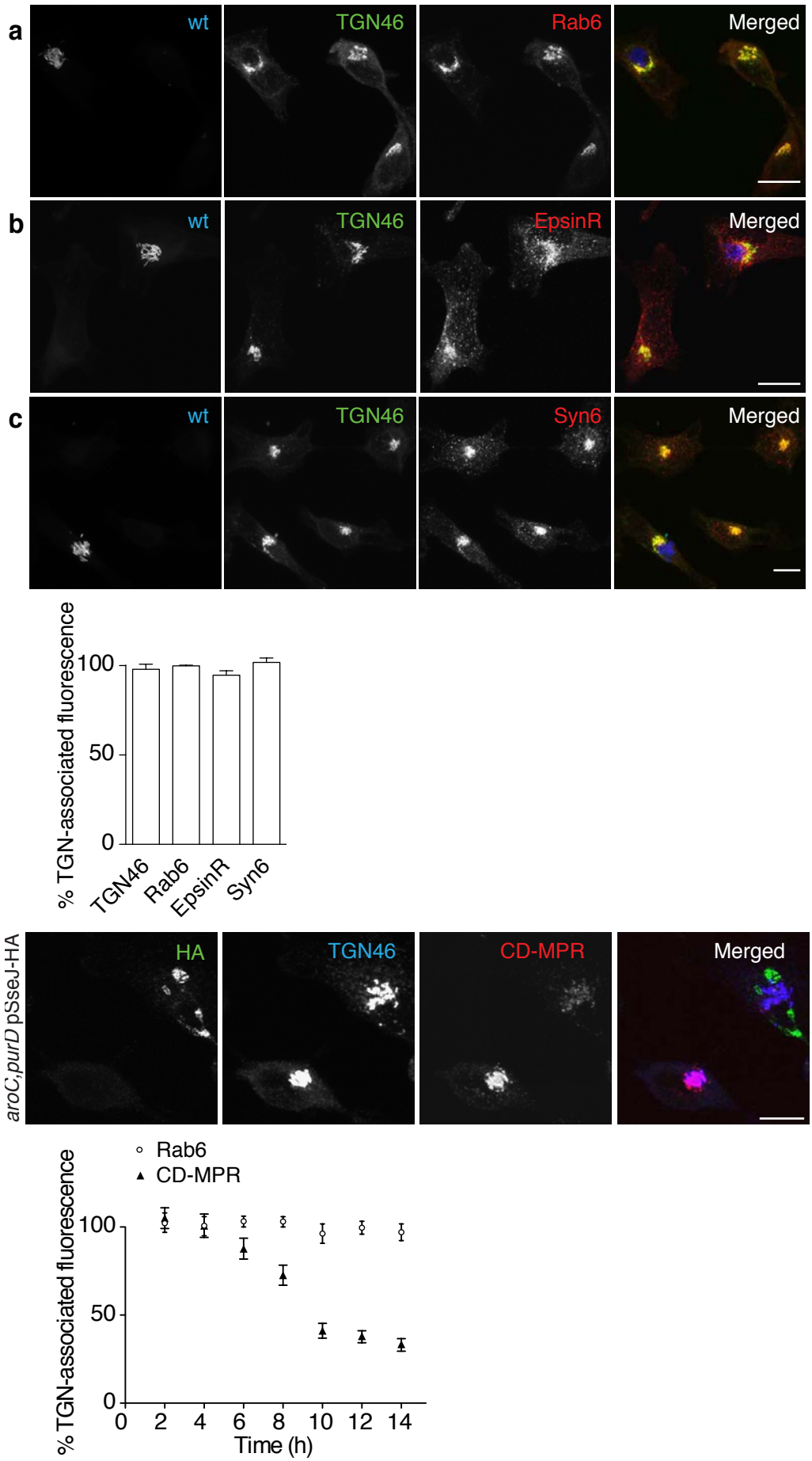

a
wt
TGN46
Rab6
Merged
b
wt
TGN46
EpsinR
Merged
c
wt
TGN46
Syn6
Merged
% TGN-associated fluorescence
100
50
0
TGN46
Rab6
EpsinR
Syn6
aroC,purD pSseJ-HA
HA
TGN46
CD-MPR
Merged
Rab6
CD-MPR
% TGN-associated fluorescence
100
50
0
0
2
4
6
8
10
12
14
Time (h)

fig. S2

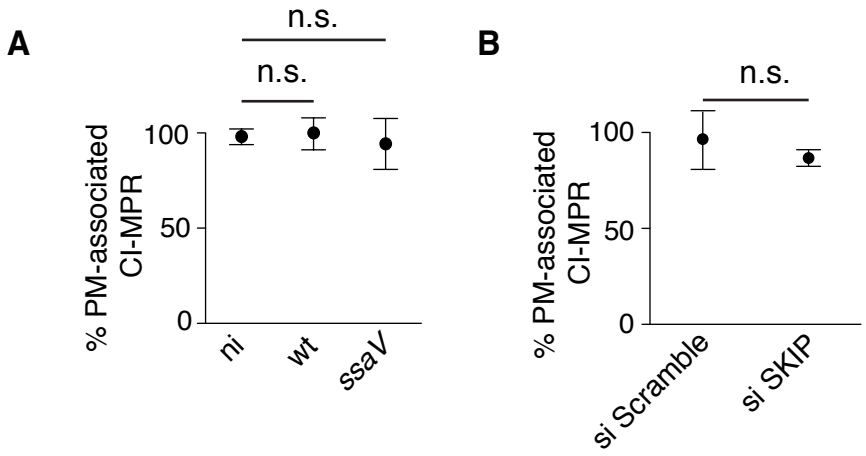
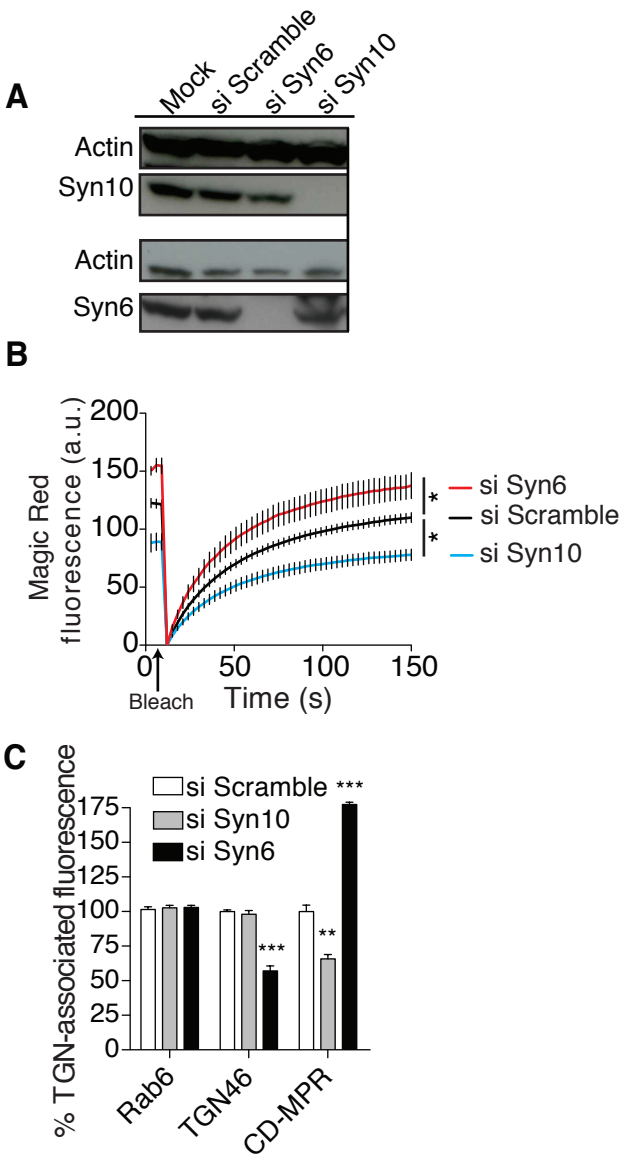

A
n.s.
n.s.
% PM-associated CI-MPR
100
50
0
ni
wt
ssaV
B
n.s.
% PM-associated CI-MPR
100
50
0
si Scramble
si SKIP

Fig. S3


A
Mock
si Scramble
si Syn6
si Syn10
Actin
Syn10
Actin
Syn6
B
Magic Red fluorescence (a.u.)
200
150
100
50
0
0
50
100
150
Bleach
Time (s)
si Syn6
si Scramble
si Syn10
*
*
C
% TGN-associated fluorescence
si Scramble ***
si Syn10
si Syn6
175
150
125
100
75
50
25
0
***
**
Rab6
TGN46
CD-MPR

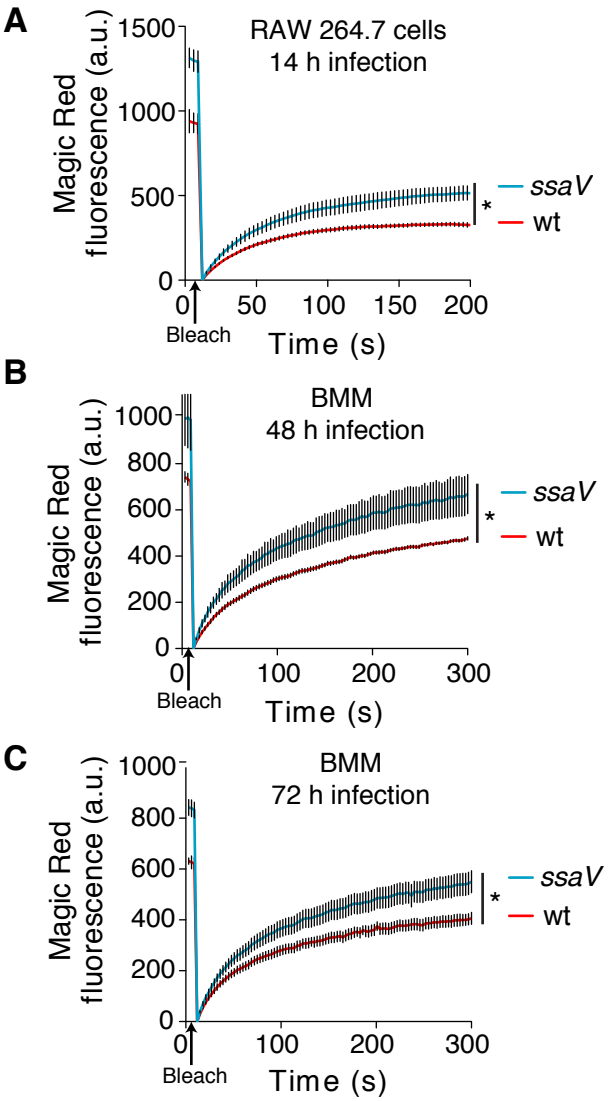
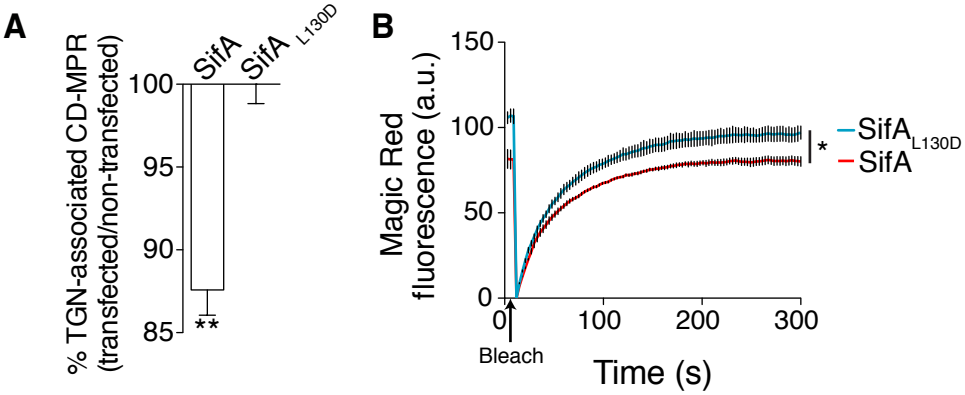

A
RAW 264.7 cells
14 h infection
Magic Red fluorescence (a.u.)
1500
1000
500
0
0
50
100
150
200
Bleach
Time (s)
ssaV
wt
*
B
BMM
48 h infection
Magic Red fluorescence (a.u.)
1000
800
600
400
200
0
0
100
200
300
Bleach
Time (s)
ssaV
wt
*
C
BMM
72 h infection
Magic Red fluorescence (a.u.)
1000
800
600
400
200
0
0
100
200
300
Bleach
Time (s)
ssaV
wt
*

fig. S5


A
% TGN-associated CD-MPR
(transfected/non-transfected)
100
95
90
85
SifA
SifA L130D
**
B
Magic Red
fluorescence (a.u.)
150
100
50
0
0
100
200
300
Bleach
Time (s)
*
SifA L130D
SifA

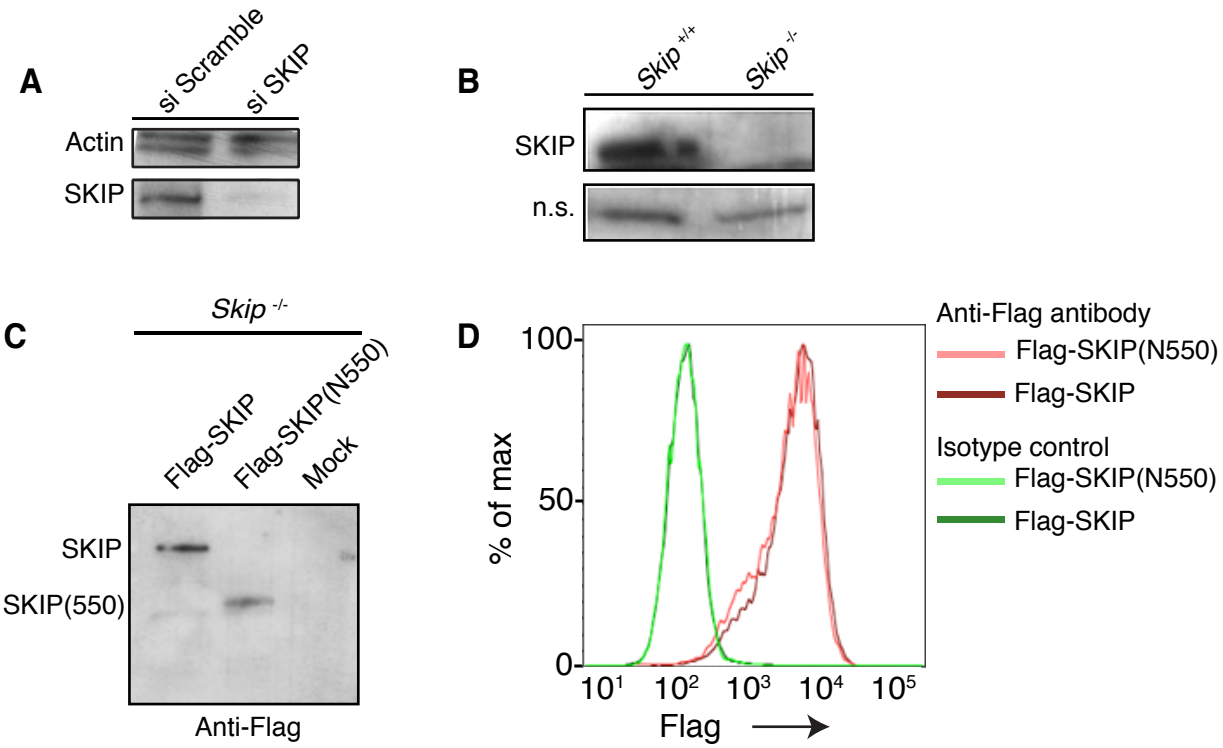

A
si Scramble
si SKIP
Actin
SKIP
B
Skip +/+
Skip -/-
SKIP
n.s.
C
Skip -/-
Flag-SKIP
Flag-SKIP(N550)
Mock
SKIP
SKIP(550)
Anti-Flag
D
100
50
0
% of max
10¹ 10² 10³ 10⁴ 10⁵
Flag
Anti-Flag antibody
Flag-SKIP(N550)
Flag-SKIP
Isotype control
Flag-SKIP(N550)
Flag-SKIP

fig. S7

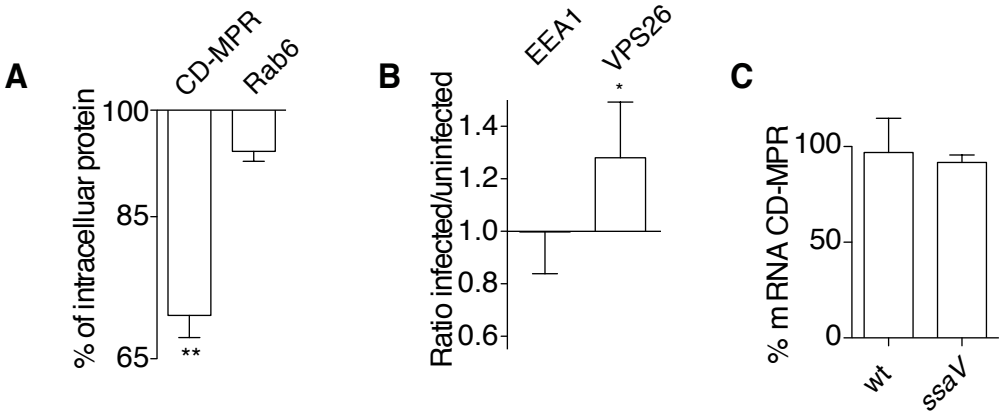
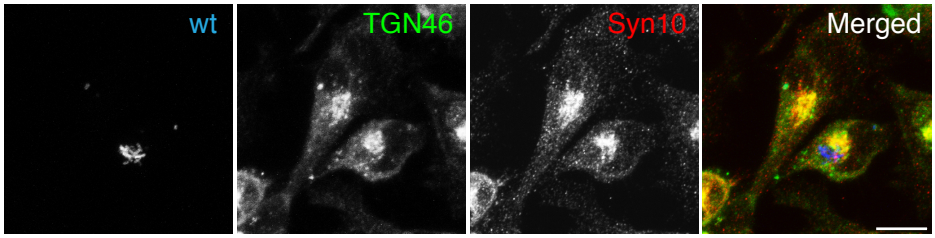

A
CD-MPR
Rab6
% of intracelluar protein
100
85
65
**
B
EEA1
VPS26
Ratio infected/uninfected
1.4
1.2
1.0
0.8
0.6
*
C
% m RNA CD-MPR
100
50
0
wt
ssaV

fig. S8

A


wt
TGN46
Syn10
Merged

B

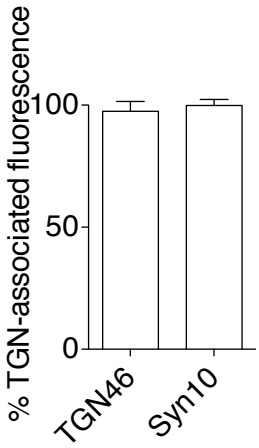

% TGN-associated fluorescence
100
50
0
TGN46
Syn10

fig. S9

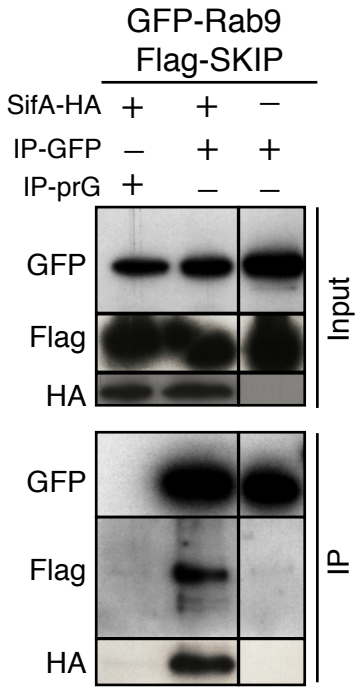

GFP-Rab9
Flag-SKIP
SifA-HA + + −
IP-GFP − + +
IP-prG + − −
GFP
Flag
HA
Input
GFP
Flag
HA
IP

fig. S10

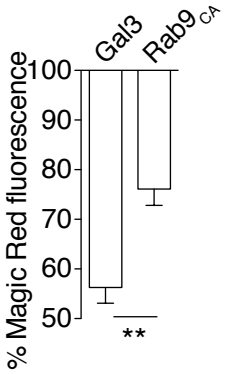
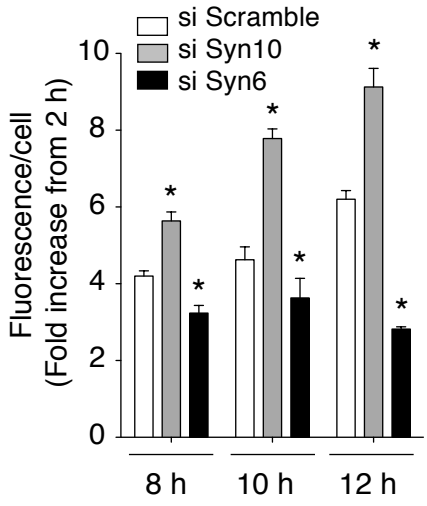


fig. S11


si Scramble
si Syn10
si Syn6
Fluorescence/cell
(Fold increase from 2 h)
0
2
4
6
8
10
*
*
*
*
*
*
8 h
10 h
12 h

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