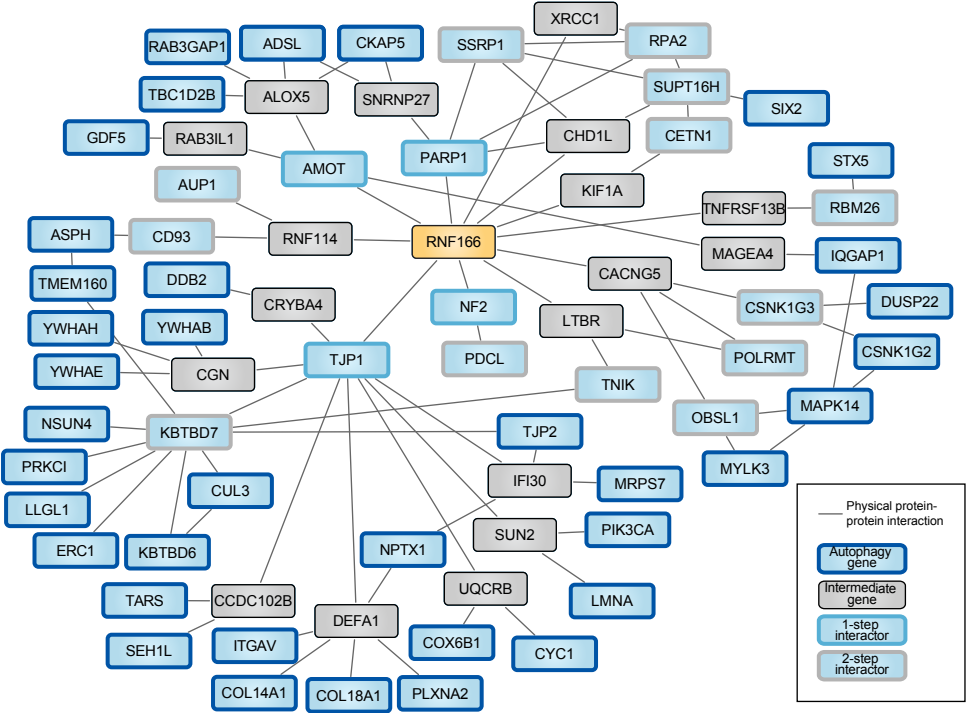# Figure S1

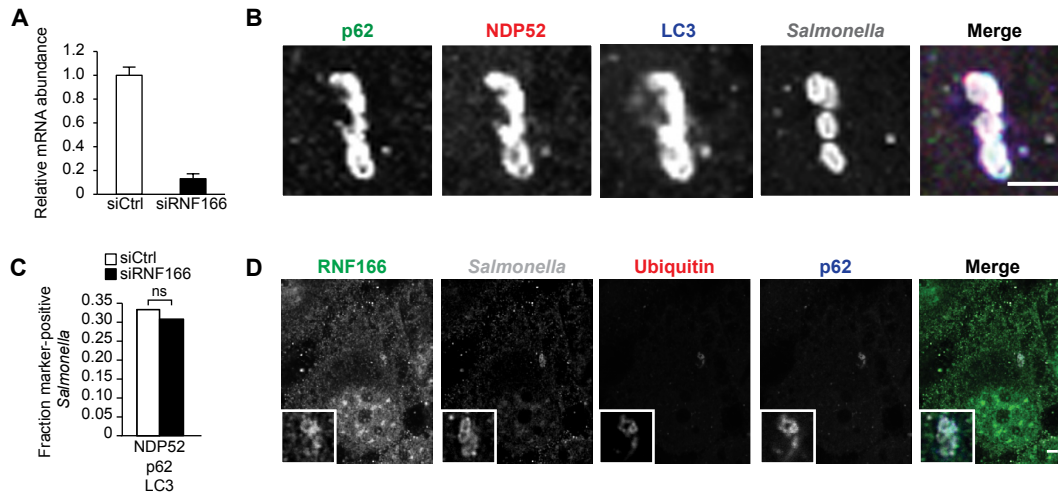**Figure S1. Network of RNF166 and autophagy-related genes, Related to Figure 1.**

Figure S2

Figure S2. Knockdown of *RNF166* alters adaptor recruitment to bacteria; RNF166 colocalizes with bacteria during antibacterial autophagy, Related to Figure 2.
(A) Relative mRNA levels of *RNF166* in HeLa cells transfected with a control siRNA or siRNA against *RNF166*. Data represent means + SD.
(B) Representative confocal images of *Salmonella* colocalizing simultaneously with NDP52, p62, and LC3. Scale bar, 5 μm.
(C) HeLa cells treated with a non-targeting siRNA or siRNA targeting RNF166 for 48 hr were infected with *Salmonella* for 1 hr and co-stained for endogenous LC3, p62, and NDP52. The simultaneous colocalization of each intracellular bacterium with LC3, p62, and NDP52 was scored. N = 50 bacteria pooled from two independent experiments.
(D) Representative confocal images of endogenous RNF166 colocalizing with p62 and ubiquitin around *Salmonella*, Scale bar, 10 μm.

Figure S3

A

B RNF166 | DNA | *Shigella* | Merge

C RNF166 | DNA | Merge

D WT *RNF166* null — RNF166, Tubulin

E RNF166-V5 | RNF166 C33A,C36A-V5 — V5, Actin

F WT | *RNF166* null — Torin / E64D/PepA — LC3-I, LC3-II, p62, Actin

G V5 | Ubiquitin | DNA/*Shigella* | Merge
RNF166-*V5*
RNF166-C33A, C36A-V5

H RNF166-V5 (1 hr) — Fraction marker-positive *Shigella ΔicsB*
RNF166 WT | RNF166 C33A, C36A

I NDP52 (1 hr) — Fraction marker-positive *Shigella ΔicsB*
RNF166 null | RNF166 WT | RNF166 C33A, C36A

J NDP52 (4 hr) — Fraction marker-positive *Shigella ΔicsB*
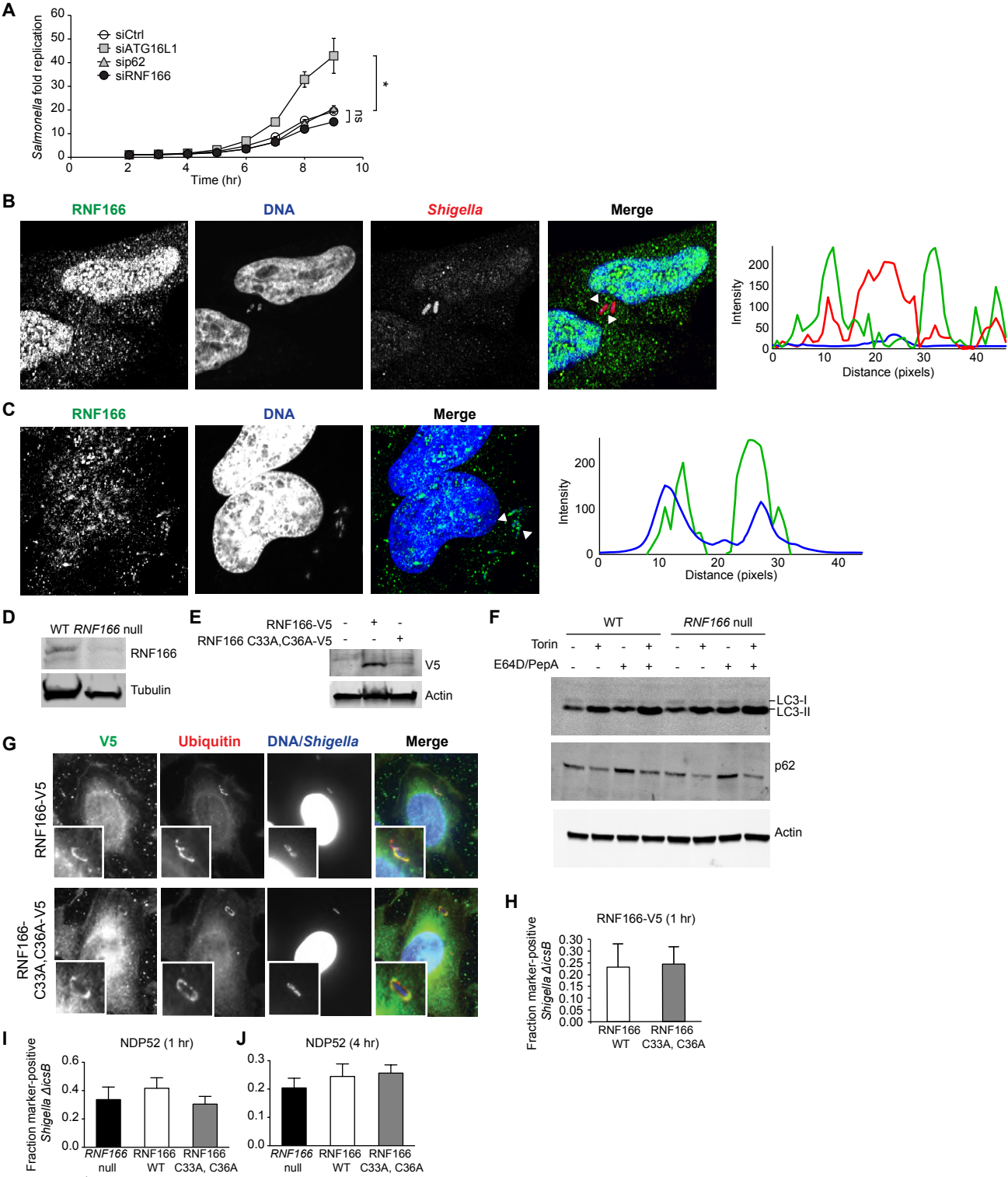*RNF166* null | RNF166 WT | RNF166 C33A, C36A

Figure S3. Characterization of RNF166 ligase dead mutant, Related to Figure 4.
(A) HeLa cells treated with a non-targeting siRNA or siRNA targeting RNF166, p62, or
ATG16L1 for 48 hr were infected with *Salmonella* expressing luciferase. Cells were treated with gentamicin to remove extracellular bacteria and relative light units were monitored over the indicated time course. Fold replication represents light units over time relative to light units at 2 hr post-infection. Data represent means ± SEM, n = 8. *p < 0.05; ns, not significant; one way ANOVA.
(B, C) Representative confocal images and histograms of pixel intensity of endogenous RNF166 colocalizing with *Shigella ΔicsB* (B) and *Listeria ΔactA* (C) at 1 hr postinfection. Histogram profiles were generated along a line connecting the triangles in merged images. Scale bar, 10 μm.
(D) Western blot analysis of RNF166 levels in WT HeLa cells or a *RNF166*-null HeLa cell line generated using CRISPR.
(E) Immunoblot of *RNF166*-null HeLa cells stably expressing the indicated RNF166-V5 tagged construct.
(F) Western blot analysis of LC3 lipidation in response to mTOR inhibition in WT HeLa cells or *RNF166*-null HeLa cells. Cells were treated for 3 hr with complete media or media containing Torin, Torin and the lysosomal protease inhibitors E64d and pepstatin A, or E64d and pepstatin A alone.
(G) Representative images of WT or ligase-dead RNF166 colocalization with *Shigella ΔicsB* at 1 hr postinfection.
(H) Quantification of WT or ligase-dead RNF166 colocalization with *Shigella ΔicsB* at 1 hr postinfection. n = 150 infected cells. Data represent means + SEM, n = 3 independent experiments.
(I, J) Quantification of NDP52 colocalization with *Shigella ΔicsB* at 1 hr (I) or 4 hr (J) postinfection. n = 180 infected cells. Data represent means + SEM, n = 3 independent experiments.
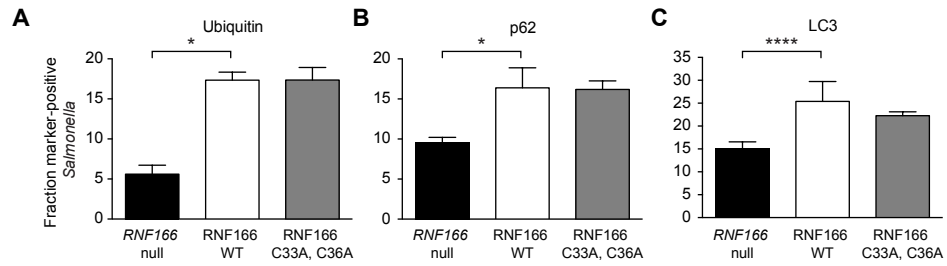
Figure S4

Figure S4. RNF166 ligase activity if not required for *Salmonella*-adapter colocalization, Related to Figure 4.
(A-C) Quantification of LC3 (A), p62 (B), and ubiquitin (C) colocalization with *Salmonella* at 1 hr postinfection. n = 240 infected cells. Data represent means + SEM, n = 3 independent experiments.

Table S1 (XLS). Normalized Z-scores for siRNAs against each gene compared to control siRNA for bacteria-marker colocalization at the indicated time point, Related to Figure 1.

Table S2 (XLS). Autophagy interaction network compiled from published datasets, Related to Figure 1. Identified gene and corresponding PMID from the publication in which they were identified are listed.

Supplemental Experimental Procedures

Cell Culture and siRNA Knockdown
HeLa and HEK293T cells were obtained from ATCC and grown in Iscove's modified Dulbecco's Medium (IMDM) supplemented with GlutaMAX (Life Technologies), 10% fetal calf serum, and 20 μg/ml gentamicin. HeLa cells stably expressing LC3-GFP have been described previously (Rioux et al., 2007). Routine testing for mycoplasma infection occurred at 6-month intervals. HeLa cells were plated in 12-well plates at a density of 6 x 10^4 cells per well in IMDM (Gibco) the day before transfection. For siRNA knockdown, 20 pmol modified RNA (Stealth RNAi, Life Technologies) was added using RNAiMax Lipofectamine (Life Technologies) according to the manufacturer's guidance. Autophagy assays and knockdown validation were performed 48 hr post transfection. For LC3-GFP screening purposes, 6 x 10^3 HeLa cells stably expressing LC3-GFP were reverse-transfected using Lipofectamine RNAiMax (Life Technologies) in a 96-well glass-bottomed plate (Corning) with pools of 3 targeting sequences per gene at a concentration of 2.5pmol/siRNA (7.5 pmol total siRNA/well) (Ambion), in accordance with the manufacturer's guidelines. 48 hr post transfection, plates were infected with *S. enterica* serovar Typhimurium expressing dsRed at an MOI of 100 (Huett et al., 2012), fixed with 4% paraformaldehyde, and stained for 30 min with Hoechst 33342 (Life Technologies). For secondary screening localizing endogenous NDP52, p62, or ubiquitin to *Salmonella*, cells were transfected as above. Postinfection, cells were fixed with -20°C methanol for 3 min and rehydrated with phosphate buffered saline (PBS). Cells were then stained with primary antibodies against a CSA-1 (KPL) in conjunction with anti-LC3B (Sigma-Aldrich), anti-p62 (American Research Products), anti-NDP52 (Abcam), or FK2 (Enzo Lifesciences).

To validate knockdown, RNA was harvested using RNeasy (Qiagen) and cDNA generated using iScript Synthesis kit (Bio-Rad). Quantitative PCR was performed using SYBR Green Supermix (Bio-Rad) with 40 ng cDNA and sequence-specific primers with expression levels normalized to 18s rRNA levels.

Screen Analysis and Hit Prioritization
Effect size colocalization data obtained from three different secondary screens (NDP52, P62, and ubiquitin co-localization rate with *Salmonella*) at two distinct time points (1 hour and 4 hours post-infection) were normalized to their respective negative control (scrambled siRNA). Z-score normalization was performed with respect to the mean and standard deviation of the negative controls. A negative Z-score implied decrease in colocalization and presumably increased bacterial replication. Data from duplicate runs was normalized independently and showed reasonable correlation. Average Z-scores of each gene (averaged over duplicate runs) from each of the six secondary screens were pooled and ordered based on number of times the average Z-score was negative across six secondary screens and the magnitude of Z-score. Twelve genes that consistently scored below zero in all six assays were prioritized.

Next we performed a network analysis to evaluate which of the 12 candidate genes regulated known autophagy genes. Using the Bioplex protein interactome database (Huttlin et al., 2015) to identify first, second, and third degree interactors of each candidate gene. We enumerated the number of autophagy-associated genes (curated from previous studies) that could be linked via protein-protein interactions with each of our twelve genes of interest in a 1-, 2- and 3-step fashion. We found four genes that directly interacted with one or more autophagy-associated genes while three genes that could not be linked to any of the autophagy genes even with third degree interactions. The Complete RNF166 network was derived from Bioplex.

Plasmids
RNF166 was cloned into pCMV with an N-terminal 3x FLAG tag or a C-terminal V5 tag. HA-p62 has been described previously (Huett et al., 2012). p62 and RNF166 mutations were generated using a Phusion site-directed mutagenesis kit (NEB). All constructs were sequence verified prior to use. Mutagenesis primers are shown below.

p62 K13R
P62_A38GF, gccgcgtcctccctgcccagaaggtag
P62_A38GR, ctaccttctgggcagggaggacgcggc

p62 K91R
P62_A272GF, ggaagatgtcatccctcacgtaggacatggccat
P62_A272GR, atggccatgtcctacgtgagggatgacatcttcc

p62 K141R
P62_A422GF, gcagacgctgcacctgtagcgggttcc

P62_A422GR, ggaacccgctacaggtgcagcgtctgc

p62 K157R
P62_A470GF, gtgcaagccccttccctcgcagacgc
P62_A470GR, gcgtctgcgagggaaggggcttgcac

p62 K165R
P62_A494GF, ggaatgcgagcctggtgtgccccccg
P62_A494GR, cgggggcacaccaggctcgcattcc

p62 K187R
P62_A560GF, ccgtgtttcaccctccggagccagcgg
P62_A560GR, ccgctggctccggagggtgaaacacgg

p62 K189R
P62_A566GF, gtgtccgtgtctcaccttccggagccagc
P62_A566GR, gctggctccggaaggtgagacacggacac

p62 K264R
P62_A791GF, caggcggcttcttctccctccgtgctc
P62_A791GR, gagcacggagggagaagaagccgcctg

p62 K281R
P62_A842GF, gctgtgagctgctcctctcctctgtgctg
P62_A842GR, cagcacagaggagaggagcagctcacagc

p62 K295R
P62_A884GF, cacccggcctgctggggtcagagc
P62_A884GR, gctctgaccccagcaggccgggtg

p62 K313R
P62_A938GF, gactccaaggcgatcctcctcatctgctccg
P62_A938GR, cggagcagatgaggaggatcgccttggagtc

p62 K420R
P62_A1259GF, ccgatgtcatagttcctggtctgcaggagcc
P62_A1259GR, ggctcctgcagaccaggaactatgacatcgg

p62 K435R
P62_A1304GF, gcgggggatgccttgaatactggatggtgtcc
P62_A1304GR, ggacaccatccagtattcaaggcatcccccgc

RNF166 C33A, C36A
97a_t106a_F, gacctccaggctgatggggctggtgtactgcg
97a_t106a_R, cgcagtacaccagccccatcagcctggaggtc

Gentamycin Protection Assay
Bacterial survival assays were performed as previously described (Lassen et al., 2014). Briefly, 96-well plates of HeLa cells were transfected with siRNA or cDNA as appropriate, and infected with a bioluminescent *S.* Typhimurium, *Listeria ΔactA*, or *Shigella ΔicsB* (Perkin Elmer, Xen26) and incubated for the indicated times postinfection. 30 min postinfection, cells were washed twice in warm PBS and incubated in IMDM containing 40 μg/ml gentamicin for 1.5 hr. Cells were then washed twice in 37°C PBS and IMDM containing 20 μg/ml gentamicin was added. At this point initial luminescent readings were taken. Bioluminescent readings were subsequently taken at the indicated time points. Fold replication was calculated by normalizing individual wells to their respective initial 2-hr time point readings.

Co-Immunoprecipitation

3 x 10^5 HEK293T cells co-expressing FLAG-RNF166 and HA-tagged autophagy proteins were lysed in TNN lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10% glycerol, plus complete mini protease inhibitors [Roche]), for 30 mins at 4°C and the insoluble fraction removed by centrifugation at 15,000 x g for 10 mins at 4°C. Lysates were then incubated with M2 FLAG beads (Sigma-Aldrich) for 2 hr and stringently washed.

For ubiquitination co-immunoprecipitations, cells were lysed in TNN lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10% glycerol, plus complete mini protease inhibitors [Roche], plus 10mM N-ethylmaleimide and 5 μM PR-619) for 30 mins at 4°C. SDS was then added to a final concentration of 1% and lysates heated at 95°C for 10 mins. Denatured lysates were diluted with TNN lysis buffer to a final SDS concentration of ≤ 0.1% and incubated on ice for a further 20 mins and the remaining insoluble fraction removed by centrifugation at 15,000 x g for 10 mins at 4°C. Cleared lysates were incubated with 5 μg anti-HA antibody (Sigma) for 2 hr at 4°C followed by incubation with Protein A magnetic beads (NEB) for a further 2 hr at 4°C and subsequent stringent washing with TNN lysis buffer. Beads were boiled in loading dye and immunoprecipitated proteins were blotted using appropriate antibodies and fluorescent secondary antibody detection (Odyssey, LI-COR Biosciences).

Classical Autophagy Assay
Autophagy was induced in HeLa cells by treatment for 4 hr with 100 nM Torin-1, 10 μg/ml E64d-Pepstatin A (Sigma-Aldrich), or mock treatment with DMSO. Cells were treated with siRNA for 48 hr as described above and autophagy induced with subsequent cell lysis (25 mM Tris [pH 7.5], 0.5% NP-40, 150 mM NaCl, and protease inhibitors [Roche]). Western blotting to demonstrate LC3 lipidation was performed after equalization of protein amounts and SDS-PAGE on AnyKD polyacrylamide gels (Bio-Rad). Following transfer to Immobilon-P membranes (Millipore), detection was performed using rabbit anti-LC3 primary (Sigma-Aldrich), mouse anti-actin (Sigma-Aldrich), and appropriate fluorescent secondary antibodies (LI-COR Biosciences).