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p62 and NDP52 Proteins Target Intracytosolic *Shigella* and *Listeria* to Different Autophagy Pathways^[5]

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Serge Mostowy^{‡§¶1}, Vanessa Sancho-Shimizu^{||**}, Mélanie Anne Hamon^{‡§¶1}, Roxane Simeone^{‡‡}, Roland Brosch^{‡‡}, Terje Johansen^{§§}, and Pascale Cossart^{‡§¶12}

From the [‡]Institut Pasteur, Unité des Interactions Bactéries-Cellules, Paris F-75015, France, [§]INSERM U604, Paris F-75015, France, [¶]Institut National de la Recherche Agronomique (INRA), USC2020, Paris F-75015, France, the ^{||}Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM U980, Necker Medical School, Paris 75015 France, the ^{**}University Paris Descartes, Paris 75015, France, the ^{‡‡}Institut Pasteur, Unité Postulante Pathogénomique Mycobactérienne Intégrée, Paris F-75015, France, and the ^{§§}Molecular Cancer Research Group, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway

Autophagy is an important mechanism of innate immune defense. We have recently shown that autophagy components are recruited with septins, a new and increasingly characterized cytoskeleton component, to intracytosolic *Shigella* that have started to polymerize actin. On the other hand, intracytosolic *Listeria* avoids autophagy recognition by expressing ActA, a bacterial effector required for actin polymerization. Here, we exploit *Shigella* and *Listeria* as intracytosolic tools to characterize different pathways of selective autophagy. We show that the ubiquitin-binding adaptor proteins p62 and NDP52 target *Shigella* to an autophagy pathway dependent upon septin and actin. In contrast, p62 or NDP52 targets the *Listeria* ActA mutant to an autophagy pathway independent of septin or actin. TNF- α , a host cytokine produced upon bacterial infection, stimulates p62-mediated autophagic activity and restricts the survival of *Shigella* and the *Listeria* ActA mutant. These data provide a new molecular framework to understand the emerging complexity of autophagy and its ability to achieve specific clearance of intracytosolic bacteria.

Autophagy is an evolutionarily conserved catabolic pathway that allows eukaryotes to degrade and recycle intracellular components by sequestering proteins and organelles in specialized double-membrane vesicles named autophagosomes (1–3). Although autophagosomes can sequester cytosolic material nonspecifically, e.g. as a response to starvation, there is increasing evidence for selective autophagic degradation of various cellular structures, including protein aggregates, mitochondria, and microbes (4, 5). The mechanism of selective autophagy is not well understood, yet the role of ubiquitin in this process is critical (5, 6). By simultaneous binding to both ubiquitin and the autophagosome-associated ubiquitin-like proteins (i.e. LC3/GABARAP proteins) autophagy receptors can mediate docking of ubiquitinated cargo to the autophagosome, thereby ensuring their selective degradation (5, 6). Of the ubiquitin-

binding proteins in selective autophagy, p62 (sequestosome 1; SQSTM1) has emerged as the prototype autophagy receptor (7). p62 is an LC3 interaction partner *in vivo* and is constantly degraded by autophagy, establishing it as a useful marker for autophagic vesicle turnover (8). NDP52³ has more recently emerged as another autophagy receptor and shares with p62 the ability to bind LC3 and ubiquitinated cargo simultaneously (9). The respective roles of p62 and NDP52 are not understood. Whether these individual autophagy receptors recognize different ubiquitinated proteins and/or perform independent functions in cells may be critical for the complete understanding of autophagy and its therapeutic potential.

Recent evidence has implicated the cytoskeleton as a critical mediator of selective autophagy. We have shown that septins, a novel component of the cytoskeleton (10), are recruited with autophagy proteins to “cage” *Shigella flexneri* in the cytosol of infected cells and restrict bacterial dissemination (11). These results suggest an interdependence between the two evolutionarily conserved processes of septin assembly and autophagy: when septins are absent, autophagy markers accumulate less, and vice versa. However, in the case of *Listeria monocytogenes*, a bacterial pathogen that also escapes to the cytosol, no efficient septin caging has been observed (11). *Listeria* has been reported to avoid autophagic recognition by expressing ActA, a bacterial effector required for actin tail motility (12, 13). Therefore *Listeria* evades both septin caging and autophagy via its surface expression of ActA (11). Given these fundamental differences between *Shigella* and *Listeria*, it is clear that in-depth investigation of these two bacteria will help to describe precisely the coordination among actin, septin, and selective autophagy.

The role of p62 and/or NDP52 in selective autophagy of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) has recently been characterized (9, 14, 15). It has been proposed that p62 and NDP52 act independently to drive efficient bacterial autophagy of *S. typhimurium* within *Salmonella*-containing vacuoles (15). To assess the role of p62 and NDP52 in the autophagy of bacterial pathogens that escape to the cytosol and to determine the role of the actin/septin cytoskeleton in this process, we examined these components in the case of *Shigella*

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S4.

¹ To whom correspondence may be addressed. Tel.: 33-1-40-61-32-69; Fax: 33-1-45-68-87-06; E-mail: smostowy@pasteur.fr.

² International research scholar from the Howard Hughes Medical Institute and recipient of European Research Council Advanced Grant Award 233348. To whom correspondence may be addressed. Tel.: 33-1-40-61-30-32; Fax: 33-1-45-68-87-06; E-mail: pcossart@pasteur.fr.

³ The abbreviations used are: NDP52, 52-kDa nuclear dot protein; CFP, cyan fluorescent protein; IF, immunofluorescence; LC3, light chain 3; SEPT, Septin.

Autophagy of *Shigella* versus *Listeria*

and *Listeria* and reveal that these different intracytosolic bacteria are targeted to different pathways of selective autophagy.

EXPERIMENTAL PROCEDURES

Bacteria and Cell Culture—*L. monocytogenes* EGD (BUG 600) and EGD Δ actA (BUG 2140) were grown overnight at 37 °C in brain heart infusion medium (Difco Laboratories), diluted 15 \times in fresh brain heart infusion medium, and cultured until $A_{600\text{ nm}} = 0.8$. *S. flexneri* M90T (BUG 2505), M90T Δ icsA (BUG 1791), and M90T Δ icsB were cultured overnight in trypticase soy, diluted 80 \times in fresh trypticase soy, and cultured until $A_{600\text{ nm}} = 0.6$. *Mycobacterium marinum* M-DsRed were cultured at 30 °C in Middlebrook 7H9 (BD Biosciences) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADC Enrichment (Fisher Scientific), diluted 48 h prior to infection in fresh medium, and cultured until $A_{600\text{ nm}} = 0.6$.

HeLa (American Type Culture Collection (ATCC) CCL-2) cells were cultured in minimum essential medium plus GlutaMAX (Invitrogen) supplemented with 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acid solution (Invitrogen), and 10% fetal calf serum (FCS). p62-GFP cells were cultured in DMEM plus GlutaMAX supplemented with 15 μ g/ml hygromycin, 200 μ g/ml blasticidin, and 10% FCS (16). Cells were grown at 37 °C and 10% CO₂. RAW 264.7 cells (ATCC TIB-71) were cultured in DMEM plus GlutaMAX supplemented with 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate, and 10% FCS. Cells were grown at 37 °C and 10% CO₂. Mouse embryonic fibroblasts from C57BL/6 mice were maintained in DMEM containing 10% FCS.

Antibodies—Rabbit polyclonal antibodies used were anti-SEPT2 (R170) and anti-SEPT9 (R69) (11), anti-p62 (Cliniscience, PM045), anti-NDP52 (AbCam, ab68588), and anti-LC3/Atg8 (Novus Biologicals, NB100-2331). The anti-NBR1 (Abnova, H00004077-A01) used was a mouse polyclonal antibody. Mouse monoclonal antibodies used were anti-GAPDH (AbCam, 6C5), FK2 (Enzo Life Sciences, PW8810), FK1 (Enzo Life Sciences, PW8805), and anti-p62 lck ligand (BD Biosciences, 610832). Secondary antibodies used were Cy5- (Jackson ImmunoResearch Laboratories), Alexa Fluor 488-, or Alexa Fluor 546-conjugated goat anti-rabbit or goat anti-mouse (Molecular Probes). F-actin was labeled with Alexa Fluor 488-, 546-, or 647-phalloidin (Molecular Probes).

For immunoblotting, total cellular extracts were blotted with the above-mentioned antibodies followed by peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Biosys Laboratories). GAPDH was used throughout as a loading control. Proteins were run on 8, 10, or 14% acrylamide gels.

Infections and Microscopy— $1\text{--}1.5 \times 10^5$ HeLa cells were plated on glass coverslips in 6-well plates (Techno Plastic Products) and used for experiments 48 h later. Cells on coverslips were fixed for 15 min in 4% paraformaldehyde and then washed with 1 \times PBS and processed for immunofluorescence (IF). After 10 min of incubation in 50 mM ammonium chloride, cells were permeabilized for 4 min with 0.1% Triton X-100 and then incubated in 1 \times PBS. Incubation with primary or secondary antibodies was performed in 1 \times PBS. Vectashield hard set mounting medium with DAPI (Vector Laboratories) or mounting medium for IF (Interchim) was used.

Listeria was added to host cells at a multiplicity of infection of 100. Bacteria and cells were centrifuged at 1000 \times *g* for 1 min at 21 °C and, then incubated at 37 °C and 10% CO₂ for 1 h, washed with minimum essential medium, and incubated with fresh gentamicin-containing complete medium (10 μ g/ml) for an additional 2 h, after which they were washed with 1 \times PBS and fixed and processed for IF. *Shigella* was added to cells at a multiplicity of infection of 100 (for quantification analyses), or 400 μ l of growth ($A_{600\text{ nm}} = 0.6$) was diluted in minimum essential medium and added directly to cells (for imaging analyses). Bacteria and cells were centrifuged at 700 \times *g* for 10 min at 21 °C and then placed at 37 °C and 10% CO₂ for 30 min, washed with minimum essential medium, and incubated with fresh gentamicin-containing complete medium (50 μ g/ml) for 4 h, after which they were washed with 1 \times PBS and fixed and processed for IF. For infection of cells with *M. marinum*, 2×10^5 RAW macrophages or mouse embryonic fibroblasts were plated onto glass coverslips in 6-well plates and used for experiments 48 h later. *M. marinum* was washed twice in 1 \times PBS and passaged through a 26-gauge needle. Bacteria were added to cells in medium without FCS at a multiplicity of infection of 10 (RAW) or 100 (mouse embryonic fibroblasts). Bacteria and cells were centrifuged at 700 \times *g* for 5 min at 21 °C and then placed for 2 h at 32 °C and 5% CO₂. Infected cells were then washed with 1 \times PBS and incubated with 200 μ g/ml amikacin for 2 h at 32 °C and 5% CO₂. After this time, macrophages were washed with 1 \times PBS and incubated in complete medium at 32 °C and 5% CO₂ for 20 or 44 h. After a total of 24 or 48 h, infected cells were washed with 1 \times PBS and fixed and processed for IF.

Images were acquired on a fluorescence inverted microscope Axiovert 200 M (Carl Zeiss MicroImaging, Inc.) equipped with a cooled digital charge-coupled device camera (Cool SNAPHQ, Photometrics) driven by Metamorph Imaging System software (Universal Imaging Corp). For three-dimensional representation, quantitative microscopy (*i.e.* counting autophagy receptor recruitment) was performed using Z-stack image series of infected cells, counting 250–1000 bacteria/experiment. Images were processed using ImageJ. Where mentioned, IF microscopy was performed with SEPT2-YFP (BUG 2444), SEPT6-GFP (BUG 2445), SEPT9-CFP (BUG 2309), SEPT9-tdTomato (BUG 2723), or GFP-LC3 (BUG 3046) (11). Cells were transfected with jetPEI (PolyPlus Transfection).

siRNA, Pharmacological Inhibitors, and Cytokine Treatment— 0.8×10^5 HeLa cells were plated in 6-well plates and transfected the following day using Oligofectamine (Invitrogen). Control siRNA (AM4635) as well as predesigned siRNA for SEPT2 (14709), SEPT9 (18228), NDP52 (s19994), and NBR1 (s8381) were all from Ambion. siRNA sequences for p62 were taken from Refs. 11, 17. Cells were tested 72 h after siRNA transfection.

For experiments involving pharmacological inhibitors, HeLa cells were infected and treated for 30 min prior to fixation with dimethyl sulfoxide or cytochalasin D (5 μ M). Cytochalasin D was suspended in dimethyl sulfoxide and handled as suggested by the manufacturer (Sigma). To monitor autophagic flux, cells were treated with bafilomycin A1 (Sigma, B1793) for 12 h (160 nM). For experiments involving TNF- α (R&D Systems, 210-

TA), cells were treated with 20 ng/ml TNF- α for 12, 20, or 24 h. Treatment was continued throughout infection. For experiments involving IL-1 β (R&D Systems, 201-LB) or IFN- γ (Imukin, Roche Applied Biosciences), cells were treated with 20 ng/ml or 5000 units/ml, respectively, for 12 or 24 h.

Gentamicin Survival Assays—Gentamicin survival assays were adapted from Refs. 18, 19. Cells treated with or without TNF- α were incubated with *Shigella* or *Listeria* as detailed above. Cells were washed and then lysed with distilled H₂O. The number of viable bacteria released from the cells was assessed by plating on Luria Bertani (LB) or brain heart infusion agar plates.

Flow Cytometry—Gene expression for p62-GFP cells was induced (i.e. “On”) by adding 1 μ g/ml tetracycline (Sigma, T7660) to the culture medium for 24 h (16). After these 24 h, gene expression was turned off (i.e. “Off”) by washing cells twice with DMEM and incubating with fresh tetracycline-free complete medium for 16 h (16). After these times, treated p62-GFP cells were washed in 1 \times PBS, detached with 0.05% EDTA-trypsin, and permeabilized/fixed using BD Cytotfix/Cytoperm Fixation/Permeabilization kit (BD Biosciences, 554714). Samples were analyzed using a FACSCalibur instrument (BD Biosciences). Dead cells were excluded on the basis of forward and side scatter, and a minimum of 10,000 events were acquired per sample. Results were analyzed using FlowJo software.

Immunoprecipitation Studies—HeLa cells (uninfected) treated or not with bafilomycin, or HeLa cells infected with *Shigella* or the *Listeria* ActA mutant in the absence and presence of TNF- α , were lysed in Igepal buffer (20 mM Tris, pH 8.0, 1% (v/v) Igepal CA-630 (Sigma), 150 mM NaCl, 10% (v/v) glycerol, and protease inhibitors mixture). p62, NDP52, SEPT2, or SEPT9 was immunoprecipitated from 800 μ g of the total protein extracts by the addition of 1 μ g of anti-p62, anti-NDP52, anti-SEPT2, or anti-SEPT9 antibody and 40 μ l of a 50% slurry suspension of protein A-Sepharose beads (Amersham Biosciences). Samples were analyzed by SDS-PAGE and immunoblotted with anti-p62, anti-NDP52, anti-SEPT2, or anti-SEPT9 antibody. HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies were subsequently used. Protein input was evaluated by probing blots of cell lysates prior to the immunoprecipitation step using antibodies specific to p62, NDP52, SEPT2, or SEPT9.

Gene Expression Studies—Cells were treated with TNF- α , IL-1 β , or IFN- γ for 0, 12, or 24 h. After this time RNA was extracted using an RNeasy mini-kit (Qiagen) and converted to cDNA using the High Capacity RNA-to-cDNA mastermix (Applied Biosystems 4375575). Gene expression probes (Applied Biosystems) included GUS (4326320E), p62 (Hs00177654_m1), and NDP52 (Hs00414663_m1). Gene expression was evaluated using the ddCt method, and levels of p62 and NDP52 were normalized to GUS.

RESULTS

The Recruitment of p62 and NDP52 to *Shigella* Is Interdependent—We have reported previously that p62 is recruited to intracytosolic *Shigella* entrapped by septin cages, and the recruitment of p62 is dependent upon SEPT2 and SEPT9 (11). NDP52 shares with p62 the ability to bind ubiquitin-coated *S. typhimurium* (9, 14, 15). We thus addressed the recruitment of NDP52 to *Shigella*. We first observed the

co-localization of NDP52 with ubiquitinated proteins around *Shigella* (Fig. 1A) and showed that $84 \pm 3\%$ of *Shigella*-septin cages recruited NDP52 (means \pm S.E. from $n = 3$ experiments) (Fig. 1B). To determine whether NDP52 recruitment is dependent upon septins, we used siRNA to deplete cells of SEPT2 or SEPT9 and evaluated NDP52 recruitment to *Shigella* in these siRNA-treated cells. In both cases, NDP52 recruitment was significantly reduced (3.8 ± 0.4 -fold or 3.0 ± 0.6 -fold, respectively) (Fig. 1C), concordant with the view that septins play a role in the recruitment of autophagic markers (11).

We then asked whether p62 and NDP52 were recruited to the same *Shigella*-septin cage. Indeed, $83 \pm 2\%$ of *Shigella*-septin cages recruited both p62 and NDP52 (mean \pm S.E. from $n = 3$ experiments) (Fig. 1D). To determine whether p62 and NDP52 have independent roles at the *Shigella*-septin cage, we used siRNA to deplete cells of p62 or NDP52 and evaluated adaptor recruitment to *Shigella* in these siRNA-treated cells. Strikingly, p62 or NDP52 recruitment was significantly reduced in either case (31.1 ± 5.1 -fold or 2.4 ± 0.3 -fold in p62-depleted cells, respectively, or 6.6 ± 2.3 -fold or 32.6 ± 14.6 -fold in NDP52-depleted cells, respectively) (Fig. 1E). These results strongly suggest an interdependent relationship between p62 and NDP52 to target *Shigella* toward autophagy.

NDP52 Regulates p62-mediated Autophagic Activity and Vice Versa—p62 is constantly degraded by autophagy and has thus been established as a marker for autophagic activity, i.e. autophagic flux (8). To address the role of NDP52 in autophagic activity, we examined the steady-state levels of p62 in the absence and presence of bafilomycin (an inhibitor of autophagosome-lysosome fusion) in control cells or in cells depleted of NDP52. Levels of p62 were significantly reduced in cells depleted for NDP52 in the absence (2.1 ± 0.4 -fold) and presence (1.5 ± 0.1 -fold) of bafilomycin (Fig. 2), suggesting that p62 and NDP52 are interdependent to promote autophagy. We next examined steady-state levels of NDP52. Levels of NDP52 accumulated in control cells treated with bafilomycin (3.9 ± 0.5 -fold), indicating that NDP52, like p62, can be used as a marker of autophagic vesicle turnover (Fig. 2). In cells depleted for p62, levels of NDP52 were significantly reduced in the absence (1.5 ± 0.1 -fold) and presence (1.2 ± 0.1 -fold) of bafilomycin (Fig. 2). Thus, p62 can significantly regulate NDP52 activity and vice versa.

p62 and NDP52 Are Recruited Independently to *Listeria*—Different pathogens have evolved different ways to escape autophagy (20). In the case of *L. monocytogenes*, these bacteria avoid ubiquitination, p62 recognition, and septin caging by expressing the surface protein ActA, a bacterial effector required to polymerize actin (11, 12). We addressed the role of NDP52 and showed that *L. monocytogenes* EGD Δ actA, but not the wild-type strain EGD, is recognized by ubiquitin, p62, and NDP52 (Fig. 3A). $95 \pm 2\%$ of EGD Δ actA recruiting p62 also recruited NDP52 (mean \pm S.E. from $n = 3$ experiments), showing that p62 and NDP52 were recruited to the same bacteria (Fig. 3B).

We used siRNA to deplete cells of p62, NDP52, SEPT2, or SEPT9 and evaluated adaptor recruitment to EGD Δ actA in these siRNA-treated cells. Unlike what was observed for *Shi-*

Autophagy of *Shigella* versus *Listeria*

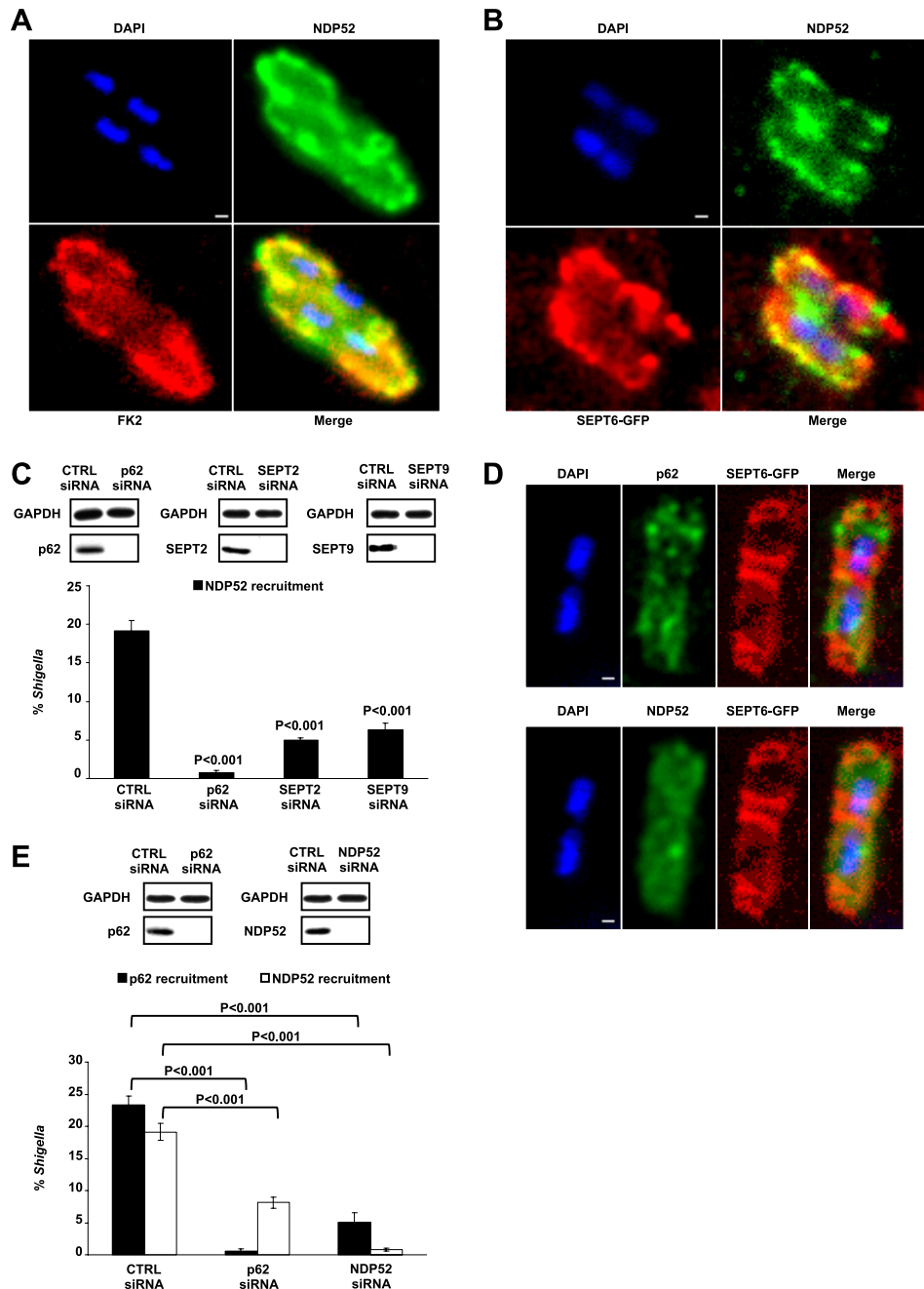


FIGURE 1. NDP52 recruitment to *Shigella*. *A*, HeLa cells were infected with *S. flexneri* for 4 h 40 min, fixed for fluorescent light microscopy, and stained with antibodies to NDP52 and ubiquitin (*FK2*). *Scale bar*, 1 μ m. Similar images were obtained labeling for FK1. *B*, HeLa cells were transfected with SEPT6-GFP, infected with *S. flexneri* for 4 h 40 min, fixed for fluorescent light microscopy, and stained with antibodies to NDP52. *Scale bar*, 1 μ m. Similar images were obtained for cells transfected with SEPT2 or SEPT9 fluorescent constructs. *C*, HeLa cells were treated with control (*CTRL*), p62, SEPT2, or SEPT9 siRNA. Whole cell lysates of siRNA-treated cells were immunoblotted for GAPDH, p62, SEPT2, or SEPT9 to show the efficiency of p62, SEPT2, or SEPT9 depletion (*top*). After 4 h 40 min of infection with *S. flexneri*, cells were fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. (*error bars*) of *Shigella* with NDP52 recruitment from three independent experiments per treatment. *p* values, Student's *t* test. *D*, HeLa cells were transfected with SEPT6-GFP, infected with *S. flexneri* for 4 h 40 min, fixed for fluorescent light microscopy, and stained with antibodies to p62 and NDP52. *Scale bar*, 1 μ m. Similar images were obtained for cells transfected with SEPT2 or SEPT9 fluorescent constructs. *E*, HeLa cells were treated with control (*CTRL*), p62, or NDP52 siRNA. Whole cell lysates of siRNA-treated cells were immunoblotted for GAPDH, p62, or NDP52 to show the efficiency of p62 or NDP52 depletion (*top*). After 4 h 40 min of infection with *S. flexneri*, cells were fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. of *Shigella* with p62 or NDP52 recruitment from three independent experiments per treatment. *p* values, Student's *t* test.

gella (Fig. 1*E*), NDP52 was recruited to bacteria in p62-depleted cells and vice versa (Fig. 3*C*). Septin depletion did not affect autophagy receptor recruitment to EGD Δ *actA* (Fig. 3*C*), in agreement with our previous work showing no septin caging of these bacteria (11). These data reveal that p62 and NDP52 can

be recruited independently of each other, and independently of septins, during autophagy of *Listeria*.

Autophagy Receptor Recruitment Is Dependent on Shigella Effectors IcsA and IcsB—As an interdependence between p62 and NDP52 was clearly identified in cells infected with *Shigella*

(Fig. 1E), we analyzed more precisely the signals that recruit p62 and NDP52. *Shigella* avoids autophagy via the bacterial effector protein IcsB which prevents the binding of Atg5, a protein critical for autophagosome maturation (21), to IcsA (22). We thus addressed the role of IcsA and IcsB in the recruitment of p62 and NDP52.

S. flexneri M90T Δ icsA, i.e. an isogenic mutant strain unable to polymerize actin or recruit septin cages in the cytosol of cells (11), failed to recruit ubiquitin, p62, and NDP52 (data not shown). In agreement with this result, the inhibition of actin polymerization by cytochalasin D significantly reduced the recruitment of ubiquitin, p62, and NDP52 to wild-type bacteria (3.1 \pm 0.5-fold, 2.3 \pm 0.6-fold, and 2.2 \pm 0.5-fold, respectively) (Fig. 4A). Moreover, SEPT2 or SEPT9 depletion similarly reduced the recruitment of ubiquitin (data not shown), p62

(11), and NDP52 (Fig. 1C) to wild-type bacteria. Therefore, accumulation of ubiquitinated proteins and the targeting of *Shigella* to autophagy by p62 and NDP52 are dependent on actin and septin.

It has been shown previously that the IcsB mutant is more efficiently targeted to autophagy (22, 23) and is more efficiently entrapped in septin cages (11). Interestingly, *S. flexneri* M90T Δ icsB recruited significantly more ubiquitin, p62, and NDP52 than did wild-type bacteria (1.8 \pm 0.1-fold, 1.9 \pm 0.2-fold, and 1.9 \pm 0.2-fold, respectively) (Fig. 4, B and C). Taken together, these data highlight the role of IcsB in preventing ubiquitin protein recruitment/formation and the recruitment of p62 and NDP52 and confirm the critical role of actin and septin in this process.

p62-mediated Autophagic Activity Is Stimulated by TNF- α —We next considered host factors that control the recruitment of autophagy receptors during *Shigella* infection. TNF- α is a pleiotropic cytokine that orchestrates a wide range of biological functions, including host defense against pathogens (24), and is prominently induced upon *Shigella* infection (25). TNF- α is also known to stimulate septin caging (11). We therefore tested p62 and NDP52 recruitment to *Shigella* in TNF- α -treated cells. In agreement with the increase observed for septin caging, p62 and NDP52 recruitment to *Shigella* significantly increased (1.9 \pm 0.2-fold and 1.9 \pm 0.2-fold, respectively) upon treatment with TNF- α (Fig. 5A). In the case of EGD Δ actA, TNF- α did not affect the recruitment of p62 and NDP52 (data not shown), in agreement with our previous work showing no septin caging of these bacteria (11).

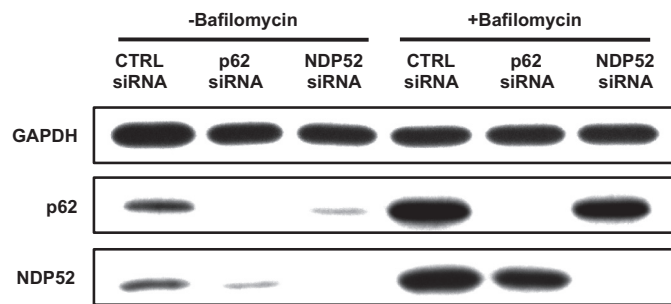


FIGURE 2. **NDP52 is required for p62-mediated autophagic activity and vice versa.** HeLa cells were treated with control (CTRL), p62, or NDP52 siRNA, treated or not with bafilomycin, and immunoblotted for GAPDH, p62, or NDP52. Representative blots from three independent experiments are shown.

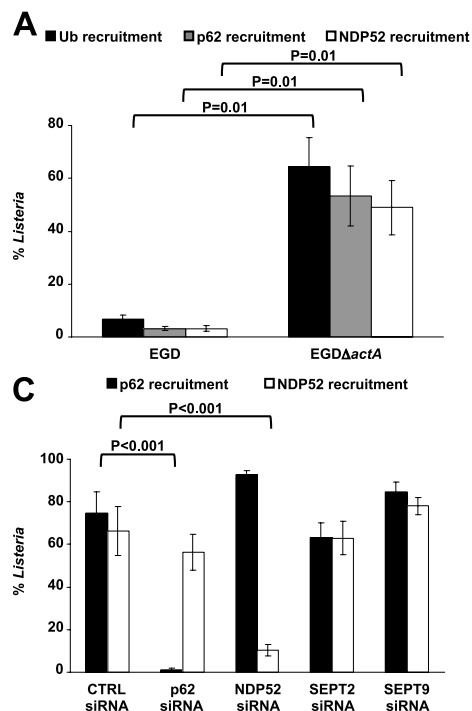
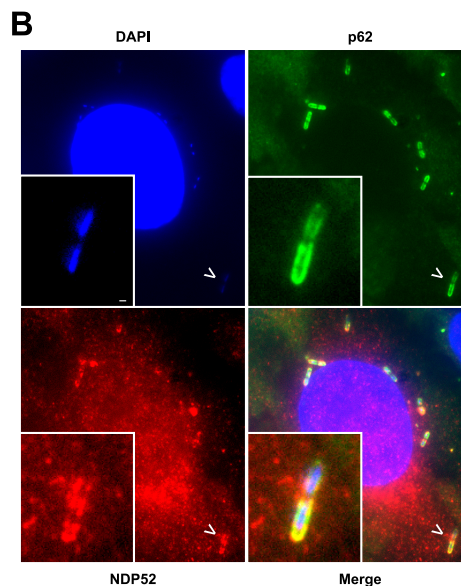


FIGURE 3. **NDP52 and p62 are recruited independently to *Listeria*.** A, HeLa cells were infected with *L. monocytogenes* for 3 h and fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. (error bars) of *Listeria* EGD or EGD Δ actA having recruited ubiquitin (Ub), p62, or NDP52 from three independent experiments per strain. *p* values, Student's *t* test. B, HeLa cells were infected with *L. monocytogenes* EGD Δ actA for 3 h, fixed for fluorescent light microscopy, and stained with antibodies to p62 and NDP52. White arrowheads point to representative bacteria shown in inset. Scale bar, 1 μ m. C, HeLa cells were treated with control (CTRL), p62, NDP52, SEPT2, or SEPT9 siRNA, infected with *L. monocytogenes* EGD Δ actA for 3 h and fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. of *Listeria* with p62 or NDP52 recruitment from three independent experiments per treatment. *p* values, Student's *t* test.



Autophagy of *Shigella* versus *Listeria*

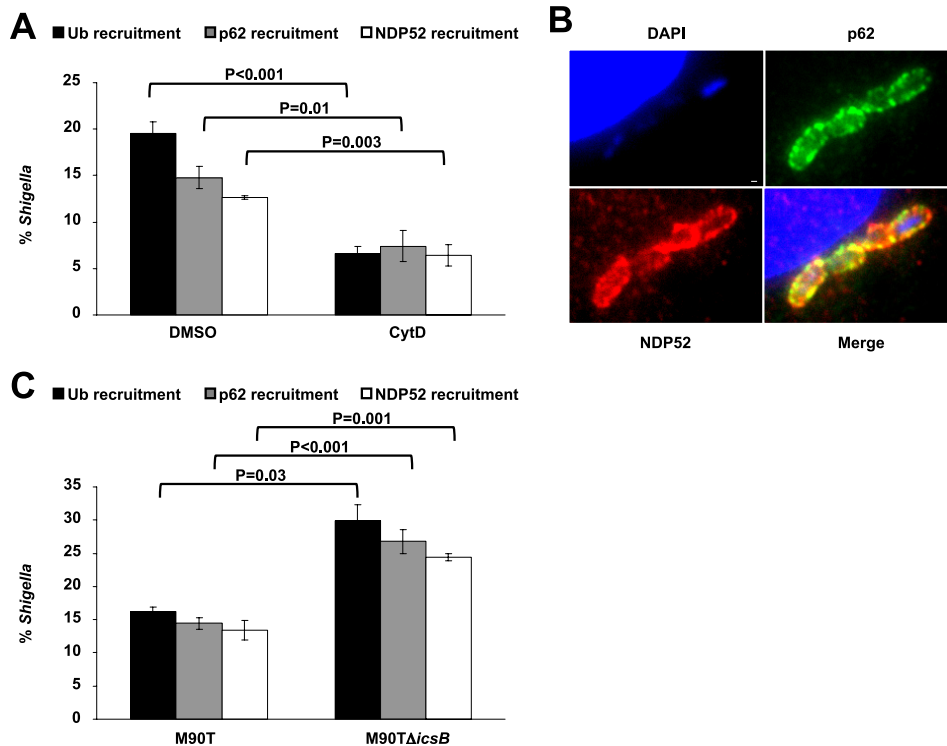


FIGURE 4. NDP52 and p62 recruitment to *Shigella* depends on actin and *Shigella* IcsB. *A*, HeLa cells were infected with *S. flexneri*, treated with dimethyl sulfoxide (DMSO) or cytochalasin D (CytD), and after 4 h 40 min were fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. (error bars) of *Shigella* having recruited ubiquitin (Ub), p62, or NDP52 from three independent experiments per treatment. *p* values, Student's *t* test. *B*, HeLa cells were infected with *S. flexneri* M90TΔicsB for 4 h 40 min, fixed for fluorescent light microscopy, and stained with antibodies to p62 and NDP52. Scale bar, 1 μ m. *C*, HeLa cells were infected with *S. flexneri* for 4 h 40 min and fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. of *Shigella* M90T or M90TΔicsB having recruited ubiquitin, p62, or NDP52 from three independent experiments per strain. *p* values, Student's *t* test.

To determine the impact of increased autophagy receptor recruitment on the fate of *Shigella*, we performed bacterial survival assays in cells treated or not with TNF- α . TNF- α treatment resulted in $28.0 \pm 4.1\%$ reduced bacterial counts (Fig. 5B). Interestingly, similar results were obtained using EGDΔactA, *i.e.* TNF- α treatment resulted in $36.3 \pm 2.2\%$ reduced bacterial counts (Fig. 5C), raising the possibility that TNF- α treatment is a general stimulant of autophagic activity, not excluding, however, that other events could lead to *Listeria* reduced counts.

To test whether the impact of TNF- α on autophagic activity could be extended to uninfected cells, we used the autophagic flux assay (8) on cells treated or not with TNF- α . Strikingly, levels of p62 and LC3-II were significantly increased in cells treated with TNF- α in the absence (p62 by 2.3 ± 0.3 -fold and LC3-II by 2.4 ± 0.5 -fold) and presence (p62 by 1.4 ± 0.2 -fold and LC3-II by 2.0 ± 0.4 -fold) of bafilomycin (Fig. 5D and supplemental Fig. S1A). To confirm this, we applied a novel reporter cell system based on induced expression of p62-GFP, where the degradation of GFP can be followed after promoter shutoff by flow cytometry (16). In accordance with the autophagic flux assay (Fig. 5D), TNF- α induced significantly more GFP before (1.5 ± 0.0 -fold) and after (1.5 ± 0.1 -fold) promoter shutoff than did untreated cells (Fig. 5E and supplemental Fig. S1B).

Interestingly, TNF- α treatment did not increase levels of NDP52 in the absence and presence of bafilomycin (Fig. 5D), suggesting that TNF- α may particularly stimulate the autophagic activity of p62. To test this, we measured levels of

p62 and NDP52 gene expression in response to TNF- α over time. p62 gene expression progressively increased in the presence of TNF- α (1.8 ± 0.9 -fold at 12 h and 4.0 ± 1.9 -fold at 24 h), unlike NDP52 gene expression (1.2 ± 0.4 -fold at 12 h and 1.6 ± 0.3 -fold at 24 h) (Fig. 5F). These data, together with results obtained from the autophagic flux assay (Fig. 5D) and the induced expression of p62-GFP (Fig. 5E), highlight TNF- α as a potent stimulant of p62-mediated autophagic activity.

DISCUSSION

Autophagy has recently emerged as an important mechanism for controlling intracellular pathogens (26, 27). A variety of different bacteria, including mycobacteria, *Salmonella*, *Shigella*, and *Listeria*, are recognized by autophagy (9, 11, 12, 14, 22, 28), yet the specific signals that mediate recognition of intracellular pathogens by the autophagy machinery have not been defined thus far. A role for ubiquitin in this process has become increasingly recognized (5, 6), and it is currently thought that ubiquitin associated with intracellular pathogens promotes targeted autophagosome formation and pathogen destruction. Here, we use *Shigella* and *Listeria* to characterize different pathways of selective autophagy. We show that the ubiquitin-binding adaptor proteins p62 and NDP52 targeted *Shigella* to an autophagy pathway dependent upon septin and actin. In contrast, p62 or NDP52 targeted the *Listeria* ActA mutant to an autophagy pathway independent of septin or actin. Taken together, these results reveal that p62 and NDP52 mediate different pathways of selective autophagy for *Shigella* and *Listeria*

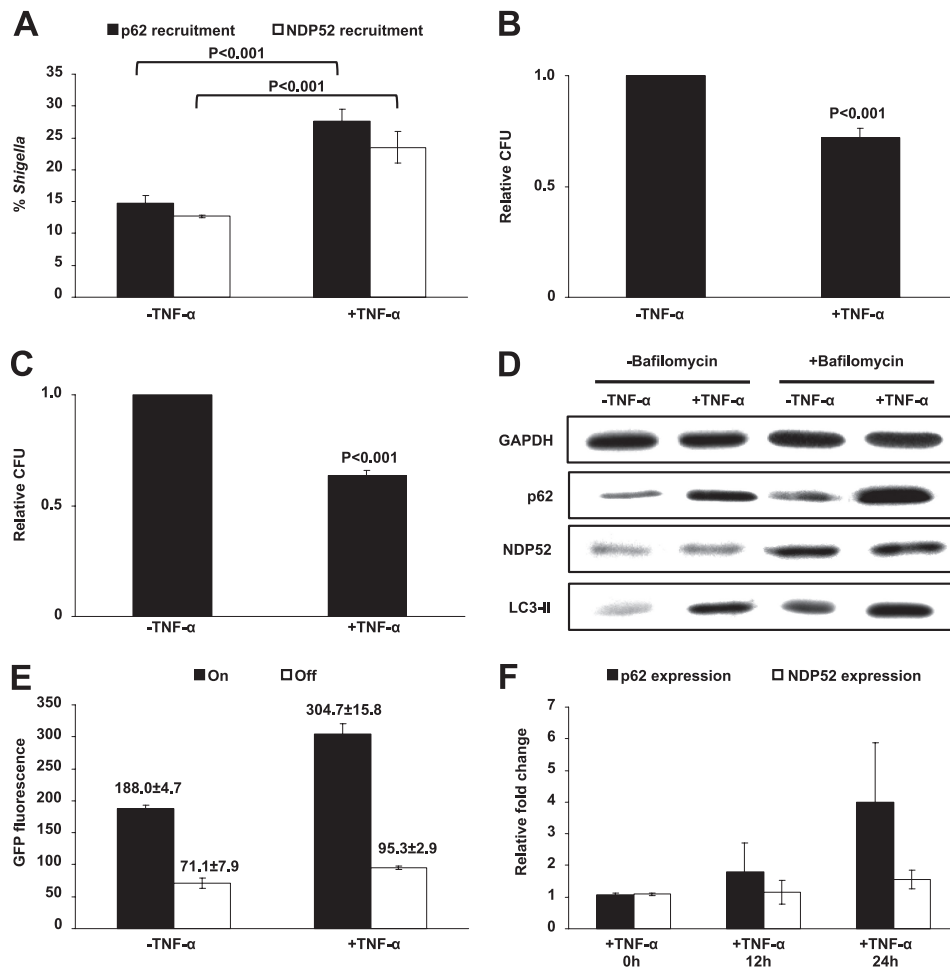


FIGURE 5. Autophagic activity is stimulated by TNF- α . *A*, untreated or TNF- α -treated HeLa cells were infected with *S. flexneri* for 4 h 40 min and fixed and labeled for quantitative microscopy. Graphs represent the mean percent \pm S.E. (error bars) of *Shigella* having recruited p62 or NDP52 from three independent experiments per treatment. *p* values, Student's *t* test. *B*, gentamicin survival assays for *S. flexneri* were performed in HeLa cells treated or not TNF- α . Graphs represent the relative number (i.e. normalized to -TNF- α cells) of colony-forming units (CFU) counted from cells after 4 h 40 min. On the graph -TNF- α is figuratively presented as 1, and data represent the mean \pm S.E. from $n \geq 3$ experiments. *p* values, Student's *t* test. *C*, gentamicin survival assays for *L. monocytogenes* EGD Δ actA were performed in HeLa cells treated or not with TNF- α . Graphs represent the relative number (i.e. normalized to -TNF- α cells) of colony-forming units counted from cells after 4 h 40 min. On the graph -TNF- α is figuratively presented as 1, and data represent the mean \pm S.E. from $n \geq 3$ experiments. *p* values, Student's *t* test. *D*, untreated or TNF- α -treated HeLa cells were treated or not with bafilomycin and immunoblotted for GAPDH, p62, NDP52, or LC3-II. Representative blots from three independent experiments are shown. A representative blot showing the ratio of LC3-II:III for TNF- α -treated cells in the presence and absence of bafilomycin is provided as supplemental Fig. S1A. *E*, p62-GFP reporter cells were treated or not with TNF- α . Cells were treated for 24 h with tetracycline (i.e. On), and after 16 h in the absence of tetracycline (i.e. Off), cells were fixed and labeled for flow cytometry to measure levels of p62-GFP. Values provided are the mean \pm S.E. from three triplicate experiments. One representative analysis comparing untreated and TNF- α -treated cells of three is shown, and the others are shown as supplemental Fig. S1B. *F*, p62 and NDP52 mRNA expression following TNF- α stimulation are shown. p62 and NDP52 cDNA expression was determined by real-time PCR in HeLa cells following 0, 12, or 24 h of stimulation with TNF- α . Data are represented as relative-fold change (ddCt units), and the housekeeping gene used for normalization was *GUS*. Values provided are mean \pm S.E. from $n \geq 3$ independent experiments per treatment.

and provide novel insight into the mechanisms by which adaptor proteins target bacteria to autophagy.

The molecular events underlying the autophagy of *Shigella* have not been completely elucidated, although a number of contributing factors are herein identified. Given the interdependence between p62 and NDP52 observed for *Shigella* (Fig. 1E), there could be a single mechanism for autophagy activation in this pathway, and the concentration of autophagic components to the substrates by septin cages enable autophagy to achieve specific and efficient clearance of bacteria. The mechanisms by which septins modulate properties of the autophagy receptors are not yet clear. Immunoprecipitation experiments on cells using antibodies for SEPT2/SEPT9 or p62/NDP52 did not reveal a direct interaction between p62/NDP52 and SEPT2/

SEPT9.⁴ However, very few direct interaction partners have been identified for septins (19, 29), possibly because septin-binding partners interact with specific conformations of higher order septin assemblies and not with individual septins or septin complexes (30).

Remarkably, autophagy activation for *Listeria* is actin- and septin-independent. Together with the finding that p62 and NDP52 are not interdependent for autophagy of *Listeria* (Fig. 3C), these results demonstrate that *Listeria* induces a different autophagy process than *Shigella* and reinforces the view that different bacterial pathogens invoke different pathways of

⁴ S. Mostowy and P. Cossart, unpublished observations.

Autophagy of *Shigella* versus *Listeria*

selective autophagy. In particular, by expressing ActA to polymerize actin, *Listeria* prevents ubiquitin protein recruitment/formation, autophagy, and septin caging (11, 12, 31). On the other hand, by expressing IcsA to polymerize actin, *Shigella* allows ubiquitin protein recruitment/formation, autophagy, and septin caging (11, 22, 31). We suggest that autophagosomes acting on *Shigella* and *Listeria* are intrinsically distinct, and the differential requirement of the actin cytoskeleton could reflect the diverse nature of the two autophagic pathways. In the future, a detailed comparative survey of the composition of *Shigella*- versus *Listeria*-induced autophagosomes would be crucial to elucidate the molecular and biochemical basis that establishes features unique to these autophagic pathways. Investigation of infection by other intracytosolic bacteria will also help to describe precisely the different autophagy mechanisms. In this respect, preliminary data show that *M. marinum*, which is similar to *Shigella* in that it also recruits WASP family proteins for actin tail polymerization (32), promotes septin cage formation (11) and is recognized by autophagy markers (supplemental Fig. S2).

To examine whether physiological stimuli induce selective autophagy, we treated cells with TNF- α , a host cytokine associated with protective immunity against bacterial pathogens (24). TNF- α has been proposed to induce autophagic destruction of the obligate intracellular pathogen *Toxoplasma gondii* (33, 34), and our study reveals a general role for TNF- α to stimulate p62-mediated autophagic activity. This novel feature of TNF- α will likely be of central importance for infections caused by a variety of intracellular pathogens including *Shigella* and pathogenic mycobacteria for which TNF- α signaling represents a key host defense mechanism (11, 25, 35). The precise role of autophagy and other TNF- α -mediated mechanisms of host defense to contain bacterial dissemination *in vivo* is currently under investigation. The role of other cytokines and their ability to induce autophagy receptor activity will also be critical. In this respect, preliminary data have shown that p62 and NDP52 gene expression can be increased in response to IL-1 β (3.4 ± 2.2 -fold and 2.0 ± 1.2 -fold at 12 h, respectively; 5.5 ± 3.7 -fold and 2.2 ± 1.1 at 24 h, respectively) and IFN- γ treatment (3.7 ± 2.5 -fold and 3.2 ± 1.5 -fold at 12 h, respectively; 5.1 ± 3.2 -fold and 8.3 ± 5.2 at 24 h, respectively) (supplemental Fig. S3).

How autophagy is activated and how targeted cargo is specifically recognized by the autophagy machinery are poorly understood. Identification of the E3 ubiquitin ligase(s) responsible for the accumulation of ubiquitinated proteins at the bacteria will be critical. Our study highlights the role of p62 and NDP52 in the autophagic recognition of different intracytosolic bacteria. p62 has been reported to act in conjunction with other adaptors, including NBR1 (neighbor of *BRCA1* gene 1) (36). NBR1 and p62 interact directly via their PB1 domains (37). NBR1 is recruited to intracytosolic *Shigella* (supplemental Fig. S4A), and its depletion significantly reduced the recruitment of p62 and NDP52 (5.0 ± 2.5 -fold and 3.5 ± 0.3 -fold, respectively) (supplemental Fig. S4B). The underlying reasons for the interdependence of these three adaptors remain elusive. It may be that each adaptor brings unique components necessary for the ubiquitin-dependent autophagy of bacteria. Different adaptors

may detect ubiquitin chains of different linkage types, recruit distinct signaling molecules, and/or determine the specific autophagy pathway (5, 6, 9). Alternatively, through their binding to LC3 or different LC3 proteins (38), different adaptors may help recruit membrane, potentially from different sources (39, 40), to generate autophagosomes around bacteria. Although the exact relationship between actin polymerization and septin assembly in regulating autophagy is not yet clear, we hypothesize that septins are required to scaffold ubiquitinated proteins and autophagy receptors around actin-polymerizing substrates for clearance by autophagy. It is thus of great interest to study the respective role of different autophagy receptors, actin polymerization, and septin assembly in the different pathways of selective autophagy.

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p62 and NDP52 Proteins Target Intracytosolic *Shigella* and *Listeria* to Different Autophagy Pathways

Serge Mostowy, Vanessa Sancho-Shimizu, Mélanie Anne Hamon, Roxane Simeone, Roland Brosch, Terje Johansen and Pascale Cossart

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