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METHODS

Strains and plasmids. *Shigella flexneri* strain YSH6000 was used as the wild type, and S325 (*mxiA*::Tn*5*) was used as a type III secretion system-deficient negative control. Construction of the non-polar mutant of *ipaH9.8* of *Shigella flexneri* YSH6000 was carried out as described previously[33]. The pTB-101 vector (under the control of isopropyl-β-D-thiogalactopyranoside, IPTG, inducible ptac promoter) was used to construct the IpaH9.8- or IpaH9.8CA (the 337th cystein residue was replaced by alanine)-overexpressing strains. The *ipaH9.8–Flag* or *ipaH9.8CA–Flag* were cloned into pTB-101, to yield pTB-*ipaH9.8–Flag* and pTB-*ipaH9.8CA*. The resultant plasmids were introduced into *Shigella* wild-type or Δ*ipaH9.8* strain. The pCACTUS-Tp suicide vector was used to construct the *ipaH9.8* complement strains. The *ipaH9.8* or *ipaH9.8CA* were cloned into pCACTUS, to yield pCACTUS-*ipaH9.8* and pCACTUS-*ipaH9.8CA*. pCACTUS-*ipaH9.8* and pCACTUS-*ipaH9.8CA* were integrated into the *ipaH9.8* gene on the *Shigella* 220-kb plasmid creating Δ*ipaH9.8/ipaH9.8* and Δ*ipaH9.8/ipaH9.8CA* strains. The *ipaH9.8* or *ipaH9.8CA* coding sequence was amplified by PCR and cloned into pEGFP, pTB-101–Flag, pGEX-4T-1, pcDNA–Flag, pcDNA–Myc$_6$ (6 × Myc), pcDL-SRα–Myc, pME–Flag or pME–HA vector. Human *NEMO* cDNA was amplified by RT-PCR from HeLa total mRNA and cloned into pEGFP, pGEX-4T-1, pGEX-6P–Flag, pME–GST, pcDNA–Myc$_6$, or pcDNA–Flag vector. Human *ABIN-1* cDNA was amplified by RT-PCR from HeLa total mRNA and cloned into pGEX-4T-1, pcDL-SRα–Myc or pME–HA vector. The E2s cDNA were subcloned into pGEX6P-1, pcDNA–HA or pcDNA–Myc vector. Site directed mutagenesis of *ipaH9.8*, *NEMO* or *ABIN-1* was performed using QuickChange site directed mutagenesis kit (Stratagene).

Materials. Anti-M2 Flag (Sigma), anti-GFP (MBL), GFP-conjugated agarose beads (MBL), anti-Myc 9B11 (Cell signaling), anti-HA (Santa Cruz Biotechnology, Cell signaling), anti-NEMO (Santa Cruz Biotechnology, Cell signaling), anti-actin (Millipore), anti-ubiquitin P4D1, anti-GST, anti-p65 antibody, goat anti-MPO antibody (Santa Cruz Biotechnology), anti-IκBα (BD transduction), anti-phospho-Erk, anti-Erk, anti-phospho-JNK, anti-JNK (Cell Signaling) and anti-ABIN-1 (Zymed) antibodies were obtained commercially. iE-DAP (Invivogen), TNF-α (Peprotech), MG132 (Peptide Inst.), LPS, E64D and pepstatin A (Sigma) were obtained commercially.

Cell culture. HeLa and 293T cells were cultured in Eagle's minimal essential medium (Sigma) and Dulbecco's modified Eagle medium (Sigma), respectively, containing 10% fetal calf serum. Stable HeLa cells expressing IpaH9.8 or control vector were selected with 1 mg ml^{-1} G418 (Roche) after transfection with pcDNA-Flag–IpaH9.8 or pcDNA vector. Wild-type and *Nemo*-deficient MEF cells were described previously[28]. To construct *Nemo*$^{-/-}$ cells stably expressing *NEMO*-wild-type and *NEMO*$^{K309R/K321R}$, cDNAs encoding these genes were subcloned into pMX-puro retroviral expression vectors. Retroviral supernatants were produced in Plat-E cells. Target cells were transduced with supernatants in the presence of DO-TAP (Roche), and then cloned under puromycin selection.

Bacterial infection. HeLa cells were infected with the different strains of *Shigella*, at a multiplicity of infection (MOI) of 100. In the case of the *Shigella* strains expressing afimbrial adhesin (Afa)-expressing strains, cells were infected at MOI = 10. Infection was initiated by centrifuging the plate at 700*g* for 10 min. After incubation for 15 min at 37°C, the plates were washed three times with PBS, transferred into fresh medium containing 100 μg ml^{-1} gentamicin and 60 μg ml^{-1} kanamycin to kill extracellular bacteria. In the case of IpaH9.8 (or IpaH9.8CA)-overexpressing experiments, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After incubation for the indicated times, the cells were washed with PBS and collected using 2 × Laemmli sample buffer. Samples were subjected to western blotting. The density of each band was quantified by measuring the mean intensity with NIH image software version 1.63. The expression levels were normalized to the levels of β-actin.

Semi-quantitative RT-PCR analysis. Total RNA was prepared with ISOGEN (Nippon gene), and cDNA was generated with Superscript II reverse transcriptase (Invitrogen) and amplified by PCR. Primer pairs were as follows; IL-8, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ and 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′; and GAPDH, 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. The density of each band was quantified by measuring mean intensity with NIH image software version 1.63. The expression levels were normalized to the levels of GAPDH.

Luciferase reporter assays. 293T cells were seeded in 24-well plates. After 24 h, cells were transfected with reporter plasmid (pNF-κB–Luc, pElK-1-luc, or pAP-1–Luc), *Renilla* luciferase constructs (phRL-TK; promega), and various combinations of expression plasmids using FuGENE6 transfection reagent (Roche). Total amounts of plasmid DNA were equalized using empty vector. After 24 h, cells were infected with *Shigella* wild-type (MOI = 30) or treated with TNF-α (10 ng ml^{-1}) or LPS (100 ng ml^{-1}) for 3 h. For investigating the MyD88 signalling pathway, TLR4 and MD2 expressing vectors were additionally transfected. Cell extracts were prepared and reporter activity was determined using the luciferase assay system (Promega). Results are presented as fold relative to the activity of uninfected or unstimulated cells. Data are representative of three independent experiments.

NF-κB nuclear translocation. HeLa cells were transiently transfected with GFP-, GFP–IpaH9.8- or GFP–IpaH9.8CA-expressing plasmids. After 24 h, cells were infected with *Shigella* harbouring an Afa-expressing plasmid to induce NF-κB activation signalling. After infection for 1 h, cells were fixed and subjected to immunohistochemical staining using an anti-p65 antibody and TO-PRO3. To quantify the percentage of nuclear translocated p65, p65 co-localized with nuclear was determined by counting at least 300 GFP-, GFP–IpaH9.8- or GFP–IpaH9.8CA-expressing cells using fluorescence microscopy. Cells infected with *Shigella* was also confirmed by observing the bacteria which invaded the cells with fluorescence microscopy.

Yeast two-hybrid screening. The yeast two-hybrid screen was performed with Matchmaker GAL4 Two-Hybrid System 3 (Clontech) or Pro-Quest (Invitrogen). The cDNA encoding full-length *ipaH9.8* was cloned in-frame into the GAL-4 DNA binding domain vector pGBKT7 (Clontech) or pDB-Leu (Invitrogen). The resulting plasmid, pGBKT7-IpaH9.8 or pDB-Leu-IpaH9.8, was used as bait in yeast two-hybrid screening. Screening with a HeLa cDNA library (Clontech) or human brain cDNA library (Invitrogen) was performed according to the manufacturer's protocol.

Expression and purification of recombinant proteins. For GST fusion proteins, *E. coli* BL21 (DE3) strain harbouring pGEX4T-1 or pGEX-6P-1 derivatives were cultivated in L-broth supplemented with ampicillin (50 μg ml^{-1}) 3 h at 30 °C. Expression was induced by the addition of 1 mM IPTG and incubation for 3 h at 30 °C. Bacteria were disrupted by sonication and lysozyme treatment. Purification of the GST fusion proteins with glutathione-Sepharose 4B (GE Healthcare) was performed according to the manufacturer's protocol. For E2s, ubiquitin, NEMO or Flag–IpaH9.8 protein purification, GST was removed from GST–E2s, GST–ubiquitin, GST–NEMO, or GST-Flag–IpaH9.8 with PreScission protease (GE Healthcare) treatment[34].

Pulldown assay. GST–NEMO or GST–ABIN-1 bound to glutathione Sepharose 4B beads was mixed with recombinant IpaH9.8–Flag for 2 h at 4 °C. After centrifugation, the beads were washed five times with 1% Triton X-100-PBS and subjected to western blotting.

Immunoprecipitation. 293T cells were transiently transfected using the calcium phosphate precipitation method. Cells were washed with PBS and lysed for 30 min at 4 °C in a lysis buffer containing 150 mM NaCl, 50 mM HEPES at pH7.5, 1 mM EDTA, 0.5% NP-40 and Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and proteins were immunoprecipitated for 2 h with anti-GFP conjugated beads or anti-M2 Flag and Protein G beads (Sigma) at 4 °C. Immunoprecipitates were washed five times with lysis buffer and subjected to immunoblotting.

***In vitro* ubiquitylation assays.** Autoubiquitylation assays were performed in 40 μl reaction mixture containing reaction buffer (25 mM Tris-HCl at pH 7.5, 50 mM NaCl, 5 mM ATP, 10 mM $MgCl_2$ and 0.1 mM DTT), 0.5 μg E1, 2 μg UbcH5b purified from *E. coli* as E2, and 2 μg ubiquitin purified from *E. coli* in the presence or absence of GST–IpaH9.8 or GST–IpaH9.8C337A. For *in vitro* NEMO ubiquitylation by IpaH9.8, reactions were performed as for autoubiquitylation assays except 1 μg recombinant NEMO was included. Reactions were incubated at 37 °C for 1 h and stopped by the addition of 5 × Laemmli sample buffer.

Immunoprecipitation *(In vivo* ubiquitylation assay). 293T cells were transfected as described above with the indicated constructs. Twelve hours before cell

collection, cells were treated with MG132 (10 μM). Cells were collected, lysed in 1% SDS, followed by tenfold dilution with RIPA buffer (150 mM NaCl, 50 mM HEPES at pH 7.5, 1mM EDTA, 1% Triton X-100 and 0.1% SDS) and lysates were cleared by centrifugation as described above. Flag–NEMO was immunoprecipitated with anti-M2 Flag and Protein G beads. Samples were washed and prepared for western blot analysis.

Cycloheximide chase assay. 293T cells were treated with cycloheximide (25 μg ml^{-1}; Wako) at 24 h after transfection, then were collected at the indicated time. Nod1-dependent NF-κB activation was stimulated by transfection with pcDNA-Nod1 and iE-DAP (10 ng ml^{-1}; (Invivogen). Samples were subjected to western blotting. The density of each band was quantified by measuring mean intensity with NIH image software version 1.63. The expression levels were normalized to the levels of β-actin.

RNAi. The human *ABIN-1*-specific siRNA sequence was as follows: 5′-CAGGAGAGCGUUACCAUGUGG-3′ and 5′-ACAUGGUAACGCUCUCC-UGAG-3′ (Sigma). Cells were transfected using RNAiMax (Invitrogen). After 72 h, siRNA-treated cells were used for further analysis.

Ubiquitin binding assay. 293T cells were transiently transfected with pME-GST–NEMO, pcDNA-Nod1 and pcDNA-HA-Ub (or empty vector) by a calcium phosphate precipitation method. After 36 h, cells were treated with iE-DAP (10 ng ml^{-1}) to promote the Nod1-mediated polyubiquitylation of NEMO, and collected. The cell lysates were incubated with glutathione Sepharose 4B beads at 4 °C for 2 h. The beads were then washed five times with RIPA buffer. To examine the association between polyubiquitylated NENO and IpaH9.8 in the presence or absence of ABIN-1, GST-$(Ub)_n$-NEMO Sepharose beads were incubated with Myc–IpaH9.8CA or HA–ABIN-1 (or HA–ABIN-1ER/AA) isolated from 293T cells for *in vitro* GST pulldown assay at 4 °C for 2 h. The beads were washed as described above, then subjected to western blotting.

Murine pneumonia model. Pulmonary infection was modelled in the mice as described previously[11]. Five-week-old female C57BL/6 mice (CREA Japan) were housed for a week in the animal facility of the Institute of Medical Science, University of Tokyo, in accordance with guidelines drafted by the university. *S. flexneri* were suspended in sterile saline, and 20 μl of the bacterial suspension was administered intranasally at 5×10^6 cfu. For histological analysis, mice were killed at the indicated time points after infection, and their lungs were removed and fixed in 4% paraformaldehyde in PBS. The tissue embedded was frozen in liquid nitrogen and sectioned with a Leica cryostat (model CM1900). The sections were stained with hematoxylin and eosin and examined under a microscope. Viable bacteria in the lung tissue were counted by culturing homogenized tissue for 18 h on LB agar plates. Each data point is the mean of the values for four infected mice in each group. MPO in the lung tissue was measured as described previously[12]. Relative MPO values are shown as the mean of pixels with saline infected mice deviation. Cytokine levels in lung homogenates were measured with an ELISA kit (R & D Systems).

Statistical analysis. Values are reported as the mean ± s.d. or mean ± s.e.m. of data obtained in independent experiments. *P* values were calculated using a one-way ANOVA test.

33. Toyotome, T. *et al*. *Shigella* protein IpaH(9.8) is secreted from bacteria within mammalian cells and transported to the nucleus. *J. Biol. Chem.* **276,** 32071–32079 (2001).
34. Kim, M., Tezuka, T., Tanaka, K. & Yamamoto, T. Cbl-c suppresses v-Src-induced transformation through ubiquitin-dependent protein degradation. *Oncogene*. **23,** 1645–1655 (2004).

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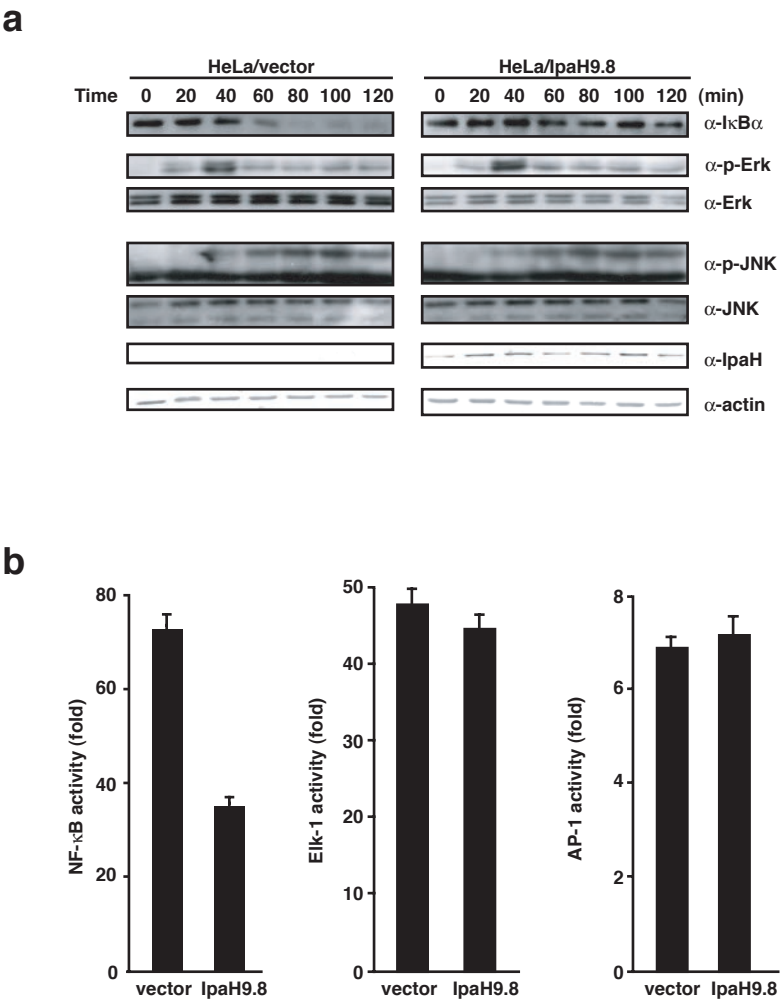


Figure S1 IpaH9.8 specifically inhibits NF-κB pathway. (**a**) IpaH9.8 specifically targets NF-κB pathway. HeLa cells stably expressing IpaH9.8 or control vector were infected with *Shigella* harboring Afa expression plasmid (moi=10). After *Shigella* invasion, cell lysates were prepared at the indicated time points and subjected to immunoblotting analysis. (**b**) IpaH9.8 specifically inhibits NF-κB activity. Luciferase assays were performed after *Shigella* infection for 3 h of 293T cells transiently transfected with an NF-κB, Elk-1, or AP-1 reporter plasmid and empty vector or IpaH9.8 expressing plasmid. Results are presented as 'fold increase' relative to the activity of non-infected cells. Data are mean ± s.e.m. ($n = 3$).

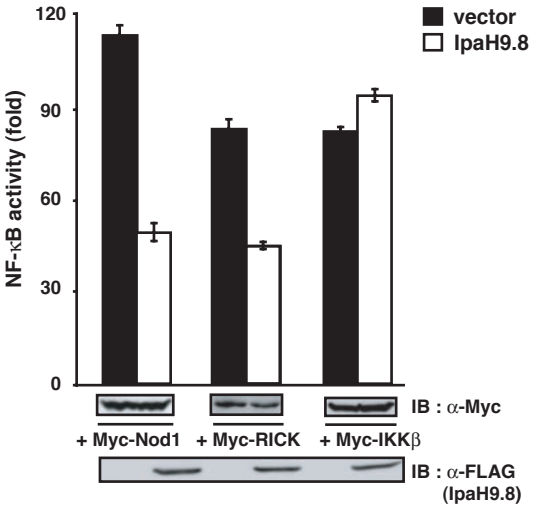


Figure S2 IpaH9.8 acts at a level between RICK and IKKβ in the Nod1-RICK-NF-κB pathway. Luciferase assays of 293T cells transiently transfected with an NF- κB reporter plasmid and empty vector or FLAG-IpaH9.8 expressing plasmid, plus stimulation plasmids expressing Myc-Nod1, Myc-RICK, and Myc-IKKβ. Results are presented as 'fold increase' relative to the activity of non-stimulated cells. Data are mean ± s.e.m. ($n = 4$).

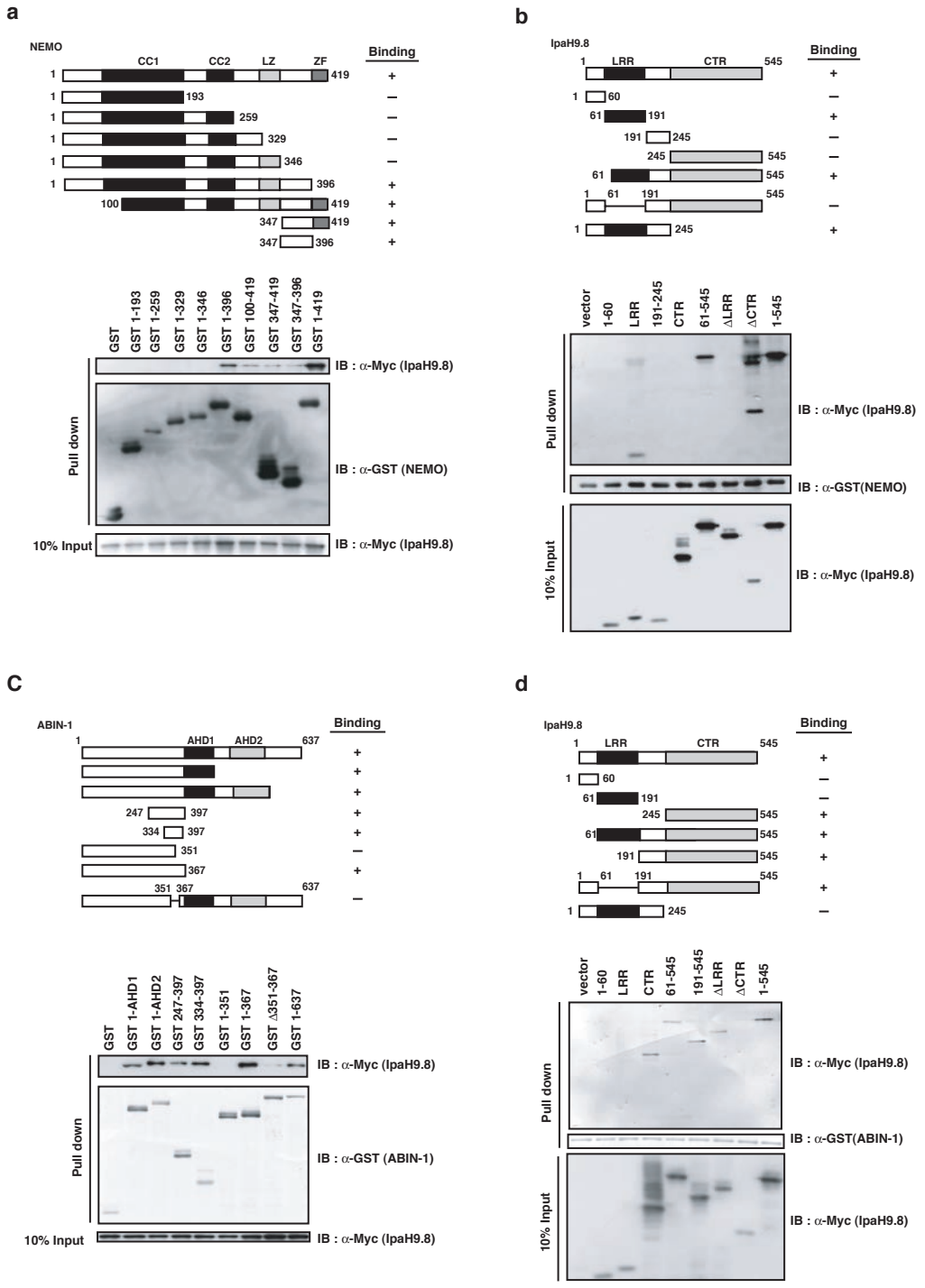
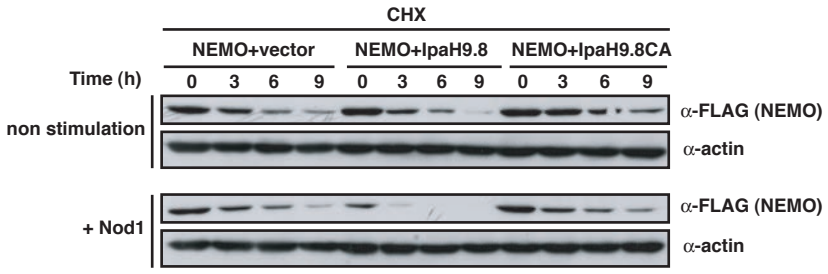


Figure S3 Binding domain of IpaH9.8 and NEMO or ABIN-1. (**a**) Deletion mutants of NEMO used in domain-mapping experiments. Numbers in parentheses indicate amino acids included in constructs (upper panel). GST-NEMO deletion mutants or GST were mixed with cell lysates expressing Myc_6-IpaH9.8CA and the bound proteins were immunoblotted with the antibodies indicated (lower panel). (**b**) Deletion mutants of IpaH9.8 used in domain-mapping experiments (upper panel). GST-NEMO beads were mixed with cell lysates expressing several deletion mutants of Myc_6-IpaH9.8CA and the bound proteins were immunoblotted with the antibodies indicated (lower panel). (**c**) Deletion mutants of ABIN-1 used in domain-mapping experiments (upper panel). GST-ABIN-1 deletion mutants or GST were mixed with cell lysates expressing Myc_6-IpaH9.8CA and the bound proteins were immunoblotted with the antibodies indicated (lower panel). (**d**) Deletion mutants of IpaH9.8 used in domain-mapping experiments (upper panel). GST-ABIN-1 beads were mixed with cell lysates expressing several deletion mutants of Myc_6-IpaH9.8CA and the bound proteins were immunoblotted with the antibodies indicated (lower panel). Interaction (+), absence of interaction (-).



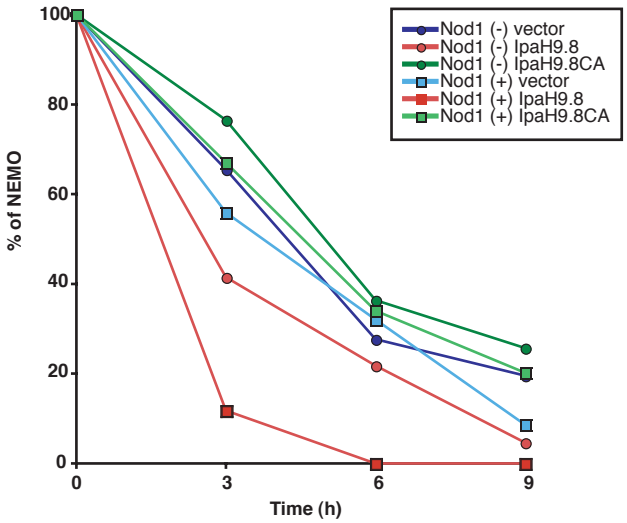


Figure S4 IpaH9.8 promotes NEMO degradation. 293T cells were transfected with FLAG-NEMO with or without FLAG-IpaH9.8 or FLAG-IpaH9.8CA. Twenty-four hours after transfection, cells were treated with CHX (25 μg/ml) and cell extracts were prepared at the indicated time points. Nod1-dependent NF-κB activation was stimulated by the transfection with pcDNA-Nod1 and iE-DAP (10 ng/ml). Samples were subjected to immunoblotting. The remaining NEMO was quantified (lower graph).

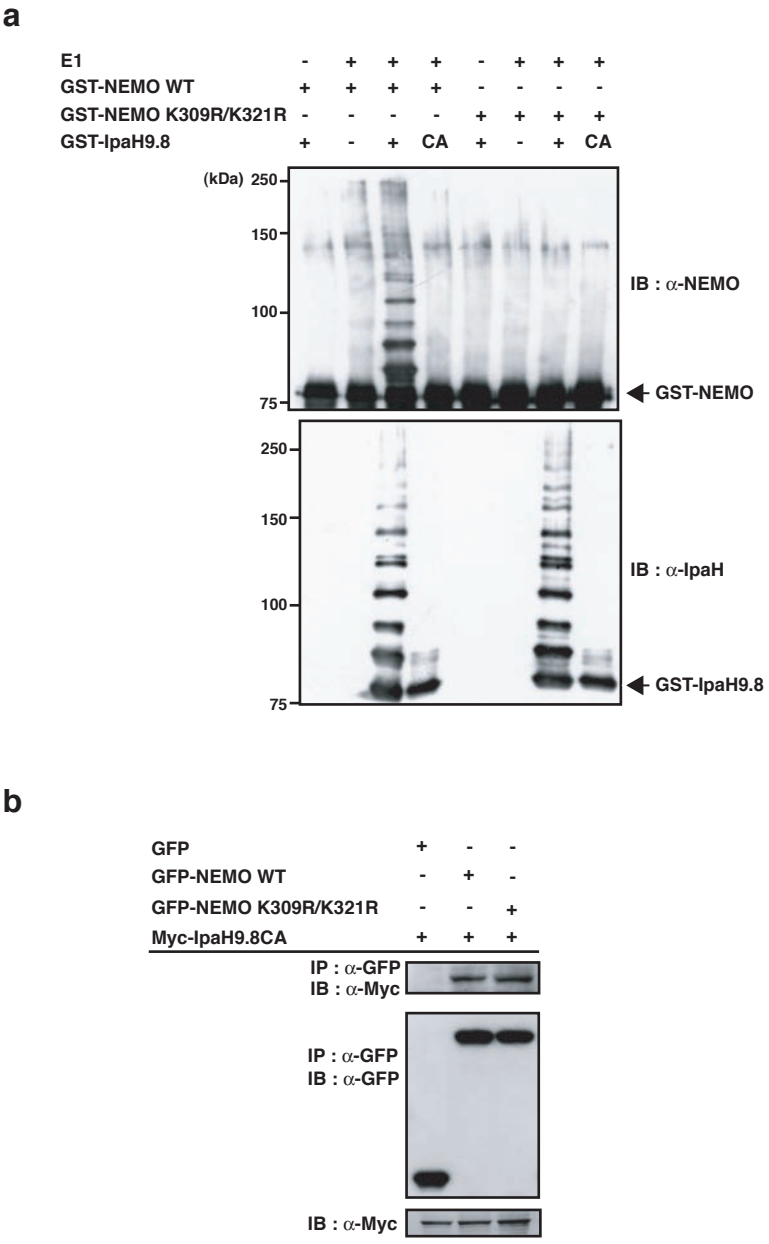


Figure S5 NEMO K309 and K321 residues are ubiquitination sites mediated by IpaH9.8. (**a**) *In vitro* ubiquitination assay with GST-NEMO-WT or GST-NEMO-K309R/K321R and a mixture of E1, UbcH5b, ATP, ubiquitin, in the presence of GST-IpaH9.8 or GST-IpaH9.8CA. Samples were subjected to immunoblotting with anti-NEMO and -IpaH antibody. (**b**) IpaH9.8 equally binds to NEMO-WT and NEMO-K309R/K321R. Immunoprecipitation (IP) analysis of lysates of 293T cells expressing GFP-NEMO-WT or GFP-NEMO-K309R/K321R and Myc_6-IpaH9.8CA. Proteins that were immunoprecipitated were immunoblotted with anti-Myc antibody.

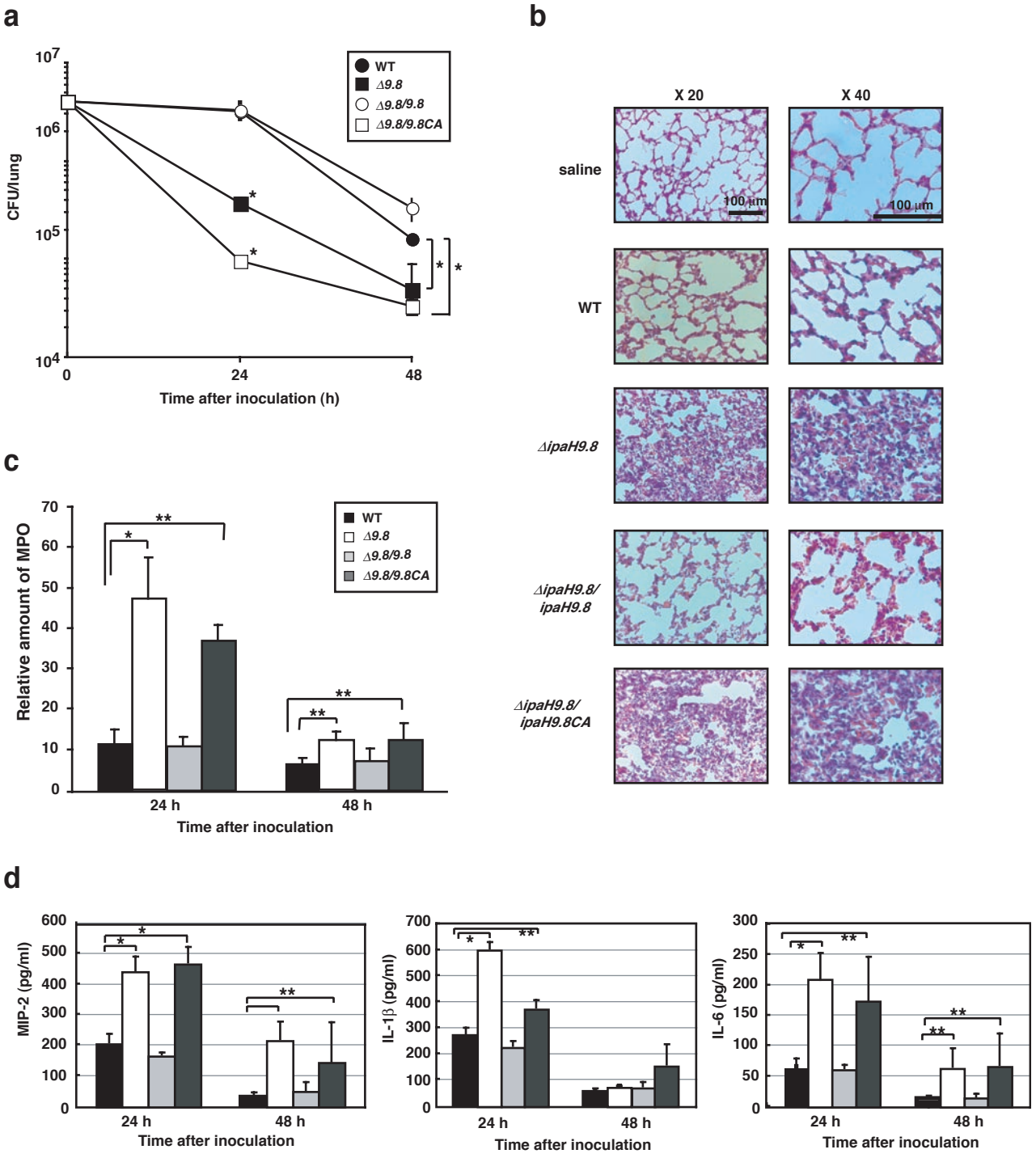


Figure S6 IpaH9.8 modulates the host inflammatory response in a murine pneumonia infection model. (**a**) The number of viable bacteria in the lungs of mice infected with the *S. flexneri* WT strain, Δ*ipaH9.8* mutant, Δ*ipaH9.8/ipaH9.8* complementation strain, or Δ*ipaH9.8/ipaH9.8CA* complementation strain at 24 h and 48 h after inoculation. Data are means of values from 4 mice (± s.d.). **P*<0.001 (**b**) Histological analysis of mouse lungs at 48 h after inoculation. Sections of infected lung were stained with hematoxylin and eosin. (**c**) Quantification of MPO in the lungs of mice 24 and 48 h after inoculation.Data are means of values from 4 mice (± s.d). **P*<0.01, ** *P*<0.05 (**d**) Measurement of cytokines in lung tissue infected with WT (closed bar), Δ*ipaH9.8* (open bar), Δ*ipaH9.8/ipaH9.8* (light gray bar), or Δ*ipaH9.8/ipaH9.8CA* (gray bar). Data are means of values from 4 mice (± s.d). **P*<0.01, ** *P*<0.05.

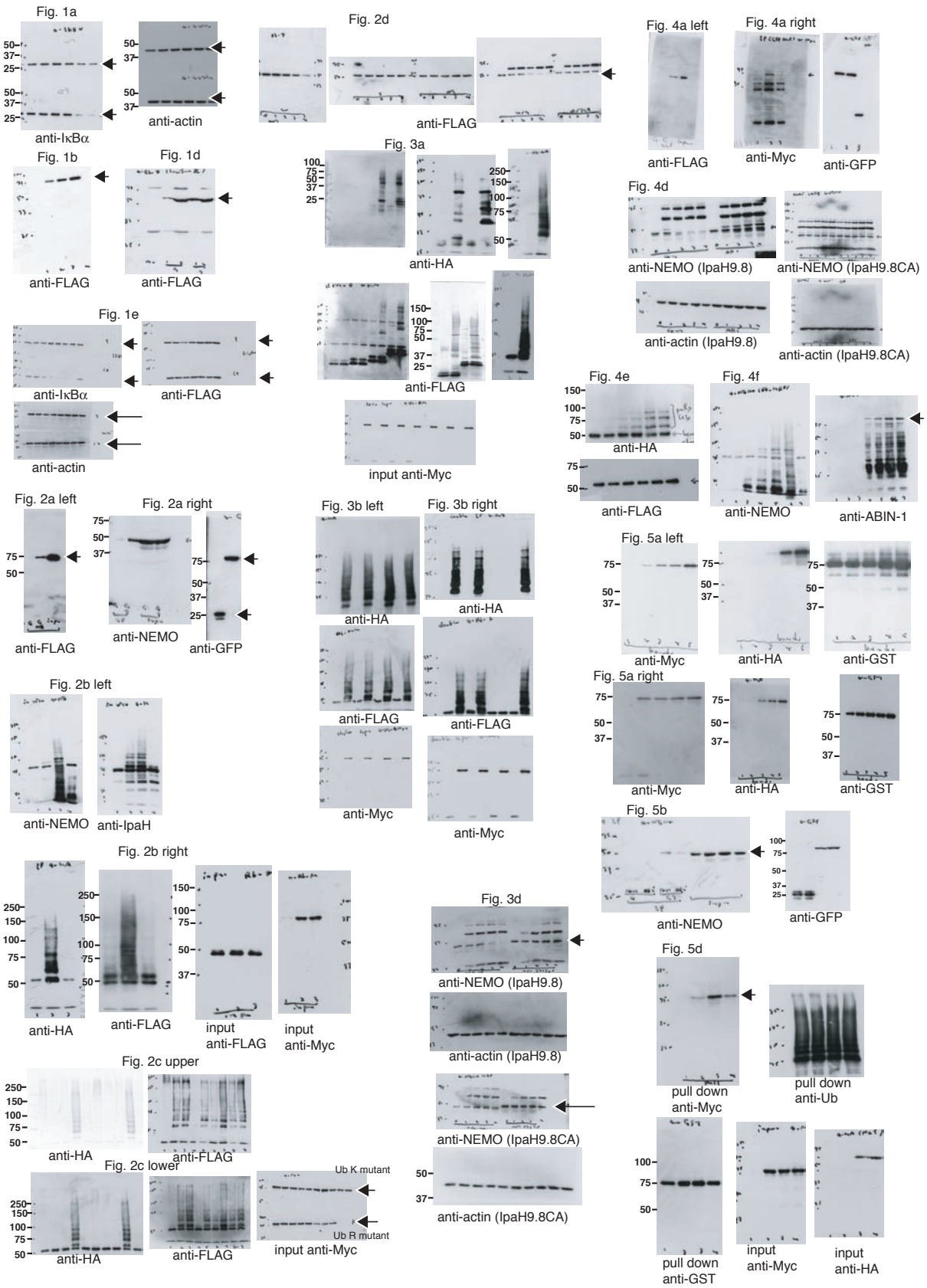


Figure S7 Full scans