Shigella flexneri suppresses NF-kB activation by inhibiting linear ubiquitin chain ligation

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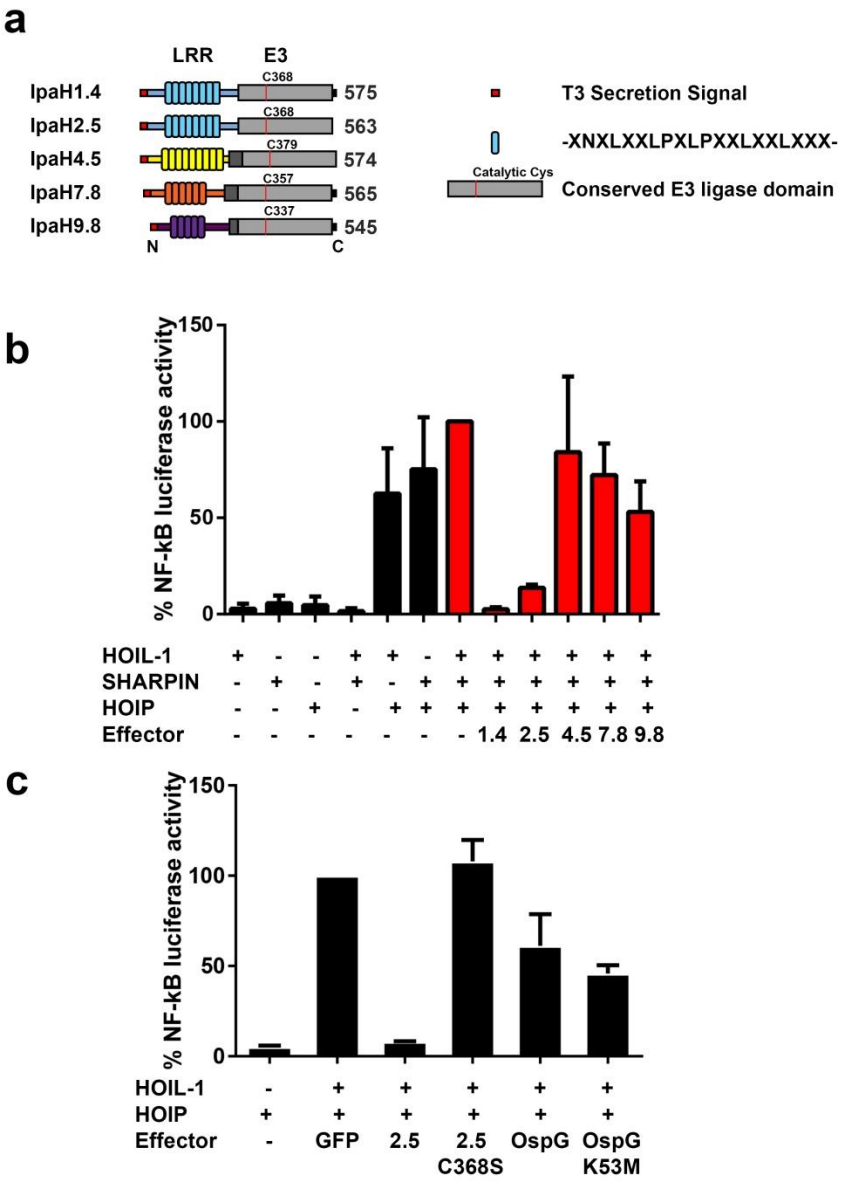
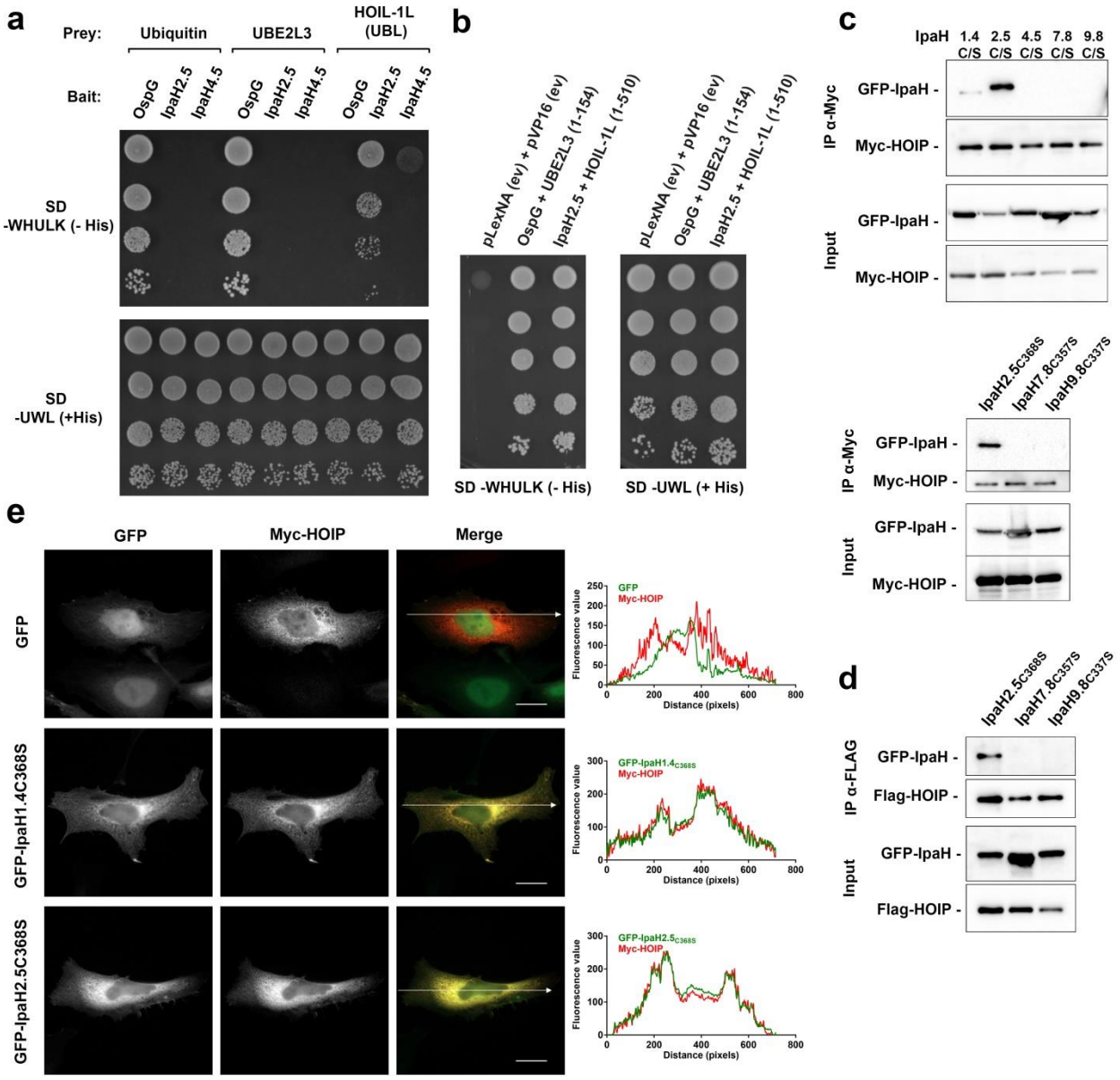
Supplementary Figures and Figure Legends

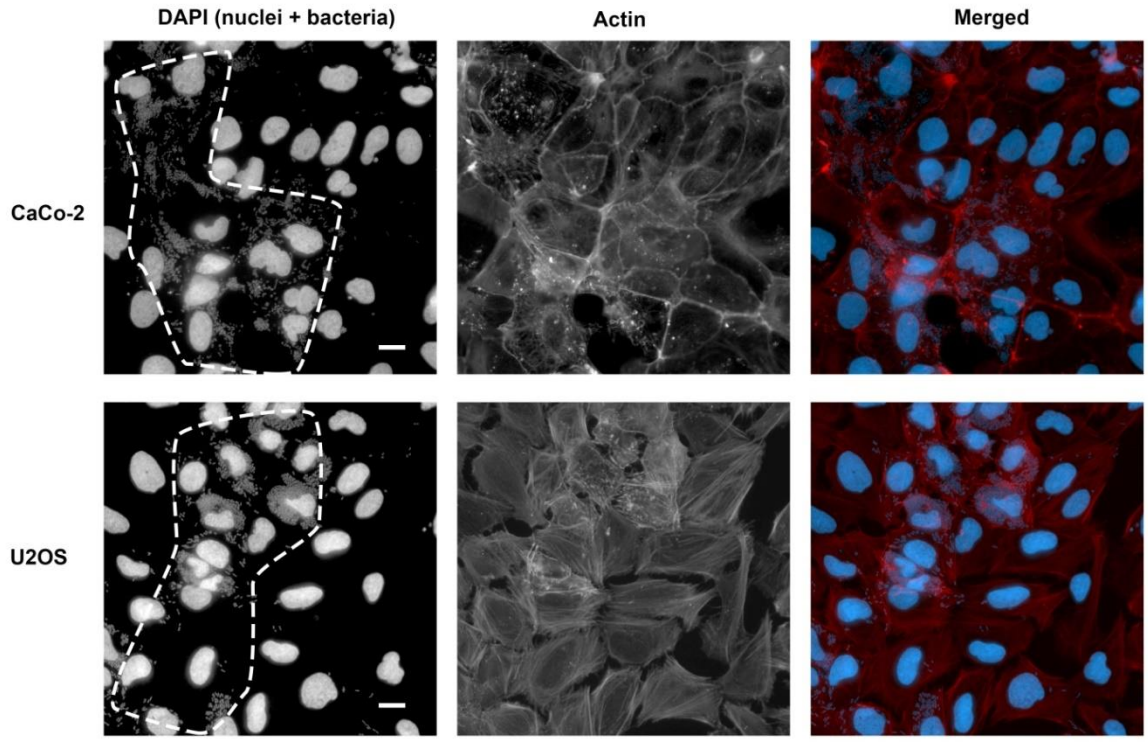


Supplementary fig. 1. **LUBAC induced NF-κB activation is reduced by IpaH1.4/2.5 and OspG**. **a**, Schematic of IpaH proteins of *Shigella flexneri* M90T encoded on its virulence plasmid. Shown are the N-terminal Type III secretion (T3SS) signal sequence followed by the variable leucine rich repeat (LRR) and the conserved novel E3 ligase domain (NEL) containing the catalytic Cys. **b**, Luciferase assay showing activation of NF-κB by overexpression of HOIP + HOIL-1L (LUBAC I), HOIP + SHARPIN (LUBAC-II) or HOIP + HOIL-1L + SHARPIN (LUBAC-III). LUBAC mediated NF-κB activation is inhibited by coexpression with IpaH1.4/2.5 but not with other IpaH proteins. **c**, Inhibition of LUBAC induced NF-κB luciferase reporter by IpaH1.4/2.5 is dependent on the E3 ligase catalytic residue Cys368, but inhibition by OspG is not dependent on kinase catalytic residue Lys53. Data shown in **b** and **c** are presented as the mean +/- sd of three independent experiments. LRR: Leucine rich repeat.



Supplementary fig. 2. IpaH1.4/2.5 interacts with HOIL-1L and HOIP. a, Ubiquitin and UBE2L3 bind to OspG and HOIL-1L residues 1-134 bind to IpaH2.5 in yeast two-hybrid assay. The pLexNA plasmid (containing bait) and pVP16 plasmid (containing prey) provide *Saccharomyces cerevisiae* yeast the ability to grow on Trp^- and Leu^- minimal medium (SD –UWL). An interaction between bait and prey allows for growth on Trp^-, Leu^-, His^- (SD –WHULK) medium. The y-axis represents a 10 fold dilution scheme. SD –WHULK plates contained 3 mM 3-Amino-1,2,4-triazole. **b,** Full length HOIL-1L (residues 1-510) binds to IpaH2.5 in yeast two-hybrid assay. As negative control no growth on SD –WHULK plates was observed for L40 yeast containing empty vector (ev) pLexNA and empty prey vector pVP16. As positive control full length UBE2L3 (residues 1-154) interacted with OspG. The x-axis represents a 10 fold dilution

scheme. In this experiment SD –WHULK plates contained no 3-Amino-1,2,4-triazole. **c,** Catalytically dead GFP-IpaH1.4$_{C368S}$ and IpaH2.5$_{C368S}$, but not *Shigella* IpaH4.5$_{C379S}$, IpaH7.8$_{C357S}$ or IpaH9.8$_{C337S}$ interact with Myc- tagged HOIP in immunoprecipitation assays from HEK293T cell lysates using anti-Myc antibodies. **d,** GFP-IpaH2.5$_{C368S}$ specifically interacts with Flag-HOIP in immunoprecipitation (IP) assays using anti-Flag antibodies. **e**, IpaH1.4 and IpaH2.5 co-localize with HOIP in HeLa cells. GFP-IpaH1.4$_{C368S}$ or GFP-IpaH2.5$_{C368S}$ were expressed in HeLa cells together with Myc-HOIP cells for 24 hours and cells were fixed and stained with anti-Myc antibodies. A Zeiss Observer Z1 fluorescent microscope and Zeiss software Zen were used to obtain and process images. Scale bars, 20µm. Results shown are representative from three independent experiments. IP: Immunoprecipitation.



Supplementary fig. 3. Infection of CaCo-2 and U2OS cells with *S. flexneri* M90T (WT) at MOI 150 shows that not all cells in the monolayer become infected. DAPI was used to stain bacteria and nuclei of cells and phalloidin to stain the actin cytoskeleton. Foci of infection in the monolayer are encircled. Representative results are shown from three independent experiments. Scale bars, 20 μm.

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                 **   *** **   ** *** ****                        ***  **  *
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                 *   *  **  **   **** *    **  *** *      *** *  ** **    *
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ipaH2.5         ----TGA
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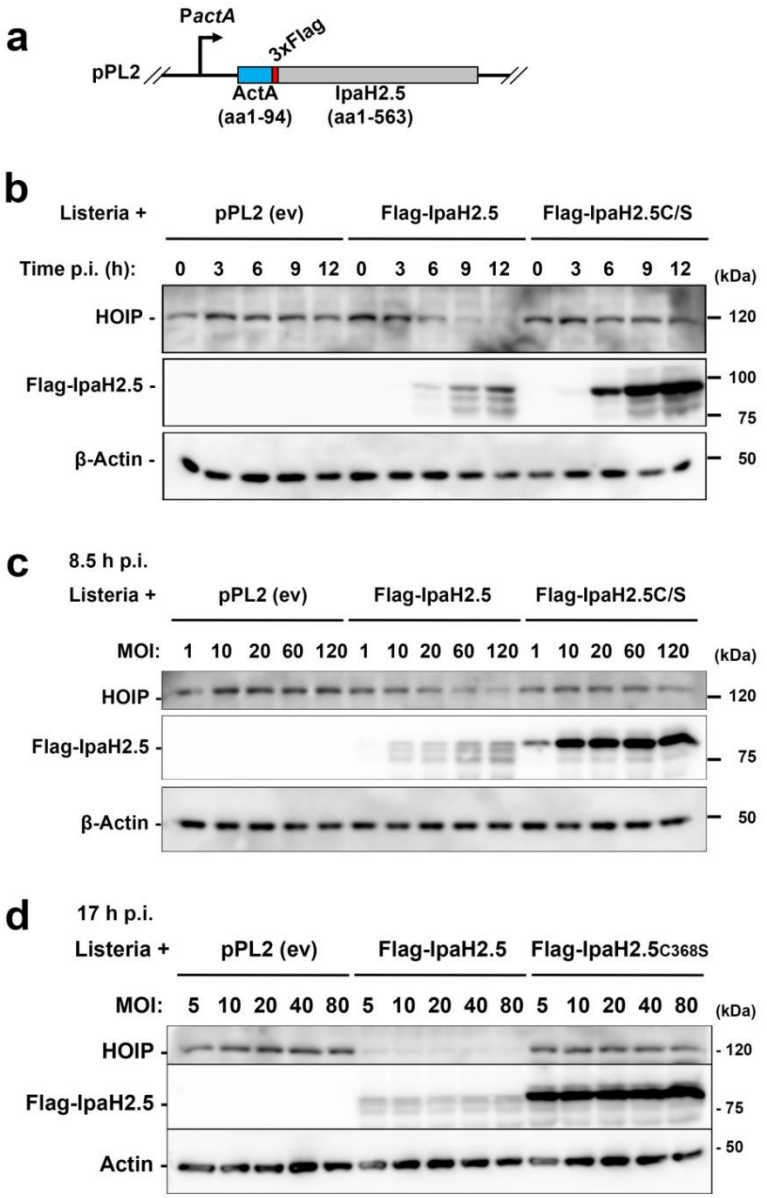
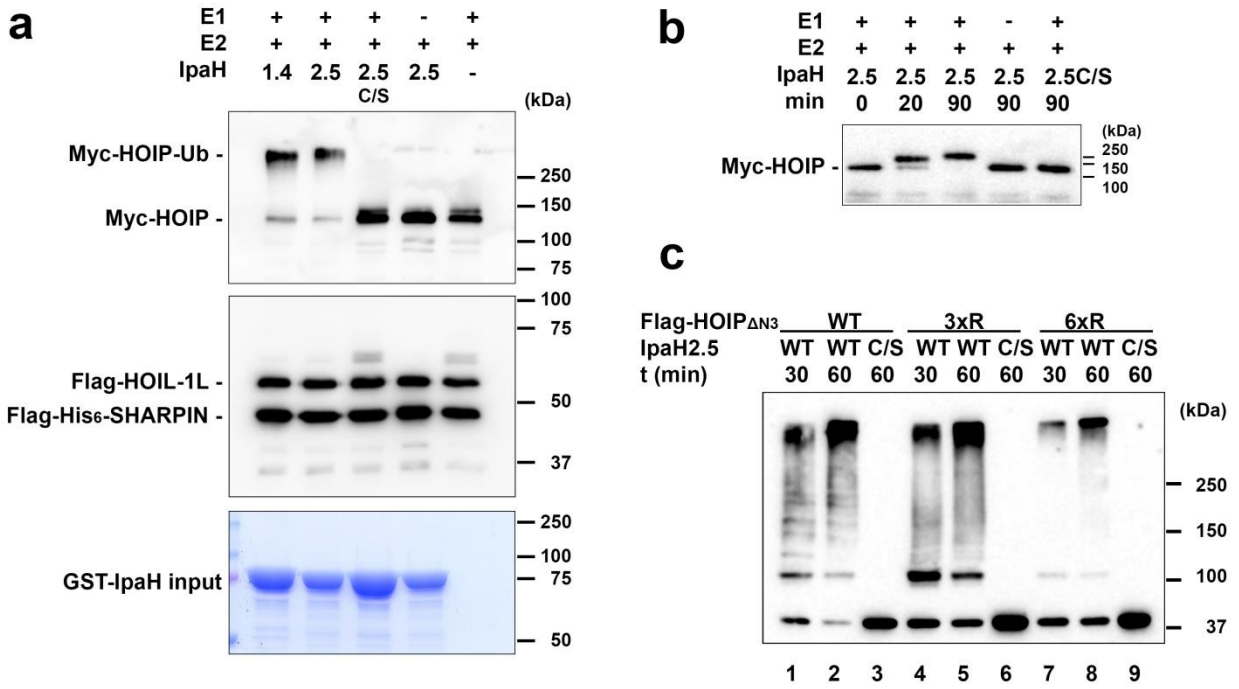
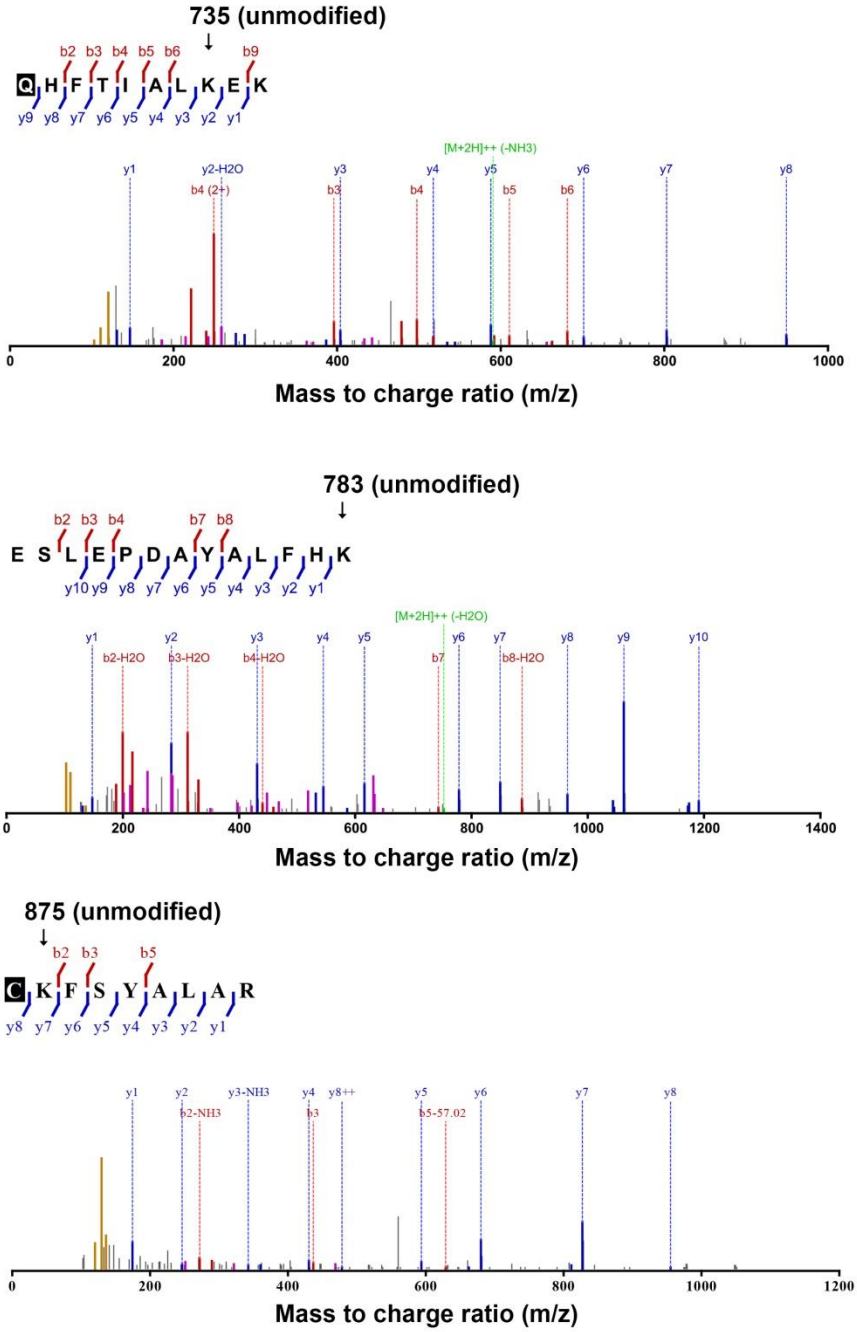
Supplementary fig. 4. Alignment of nucleotide sequences of *ipaH1.4* and *ipaH2.5*. *S. flexneri ipaH1.4* and *ipaH2.5* open reading frames, including 500 bp upstream of predicted start codon, were aligned using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Start and stop codons are highlighted yellow.



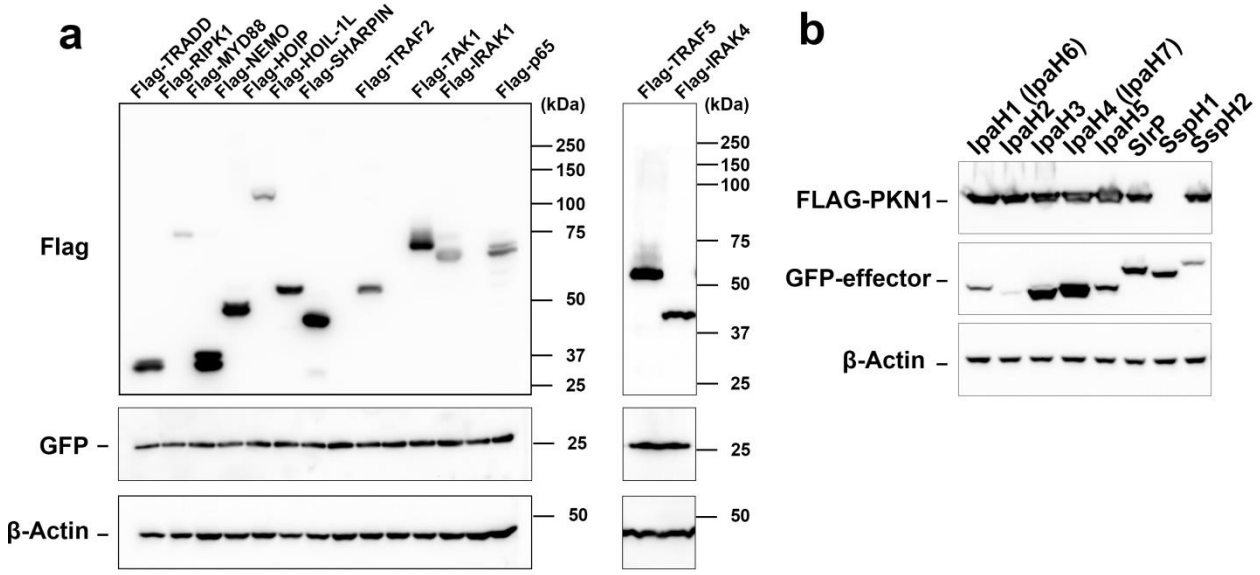
Supplementary fig. 5. Construction of a *Listeria monocytogenes* (Lm) strain expressing and secreting IpaH2.5 or IpaH2.5C368S into host cells. a Illustration of pPL2 plasmid containing P*actA* promoter driving expression of $ActA_{1-94}$-3xFlag-*ipaH2.5*$_{1-563}$, integrated into the *L. monocytogenes* chromosome. **b-d** Heterologous expression and secretion of IpaH2.5, but not of $IpaH2.5_{C368S}$, by *L. monocytogenes* results in time and multiplicity of infection (MOI) dependent degradation of endogenous HOIP in U2OS cells. h p.i.: hours post infection, ev: empty vector. Representative results are shown from three independent experiments. h p.i.: hours post infection, ev: empty vector.



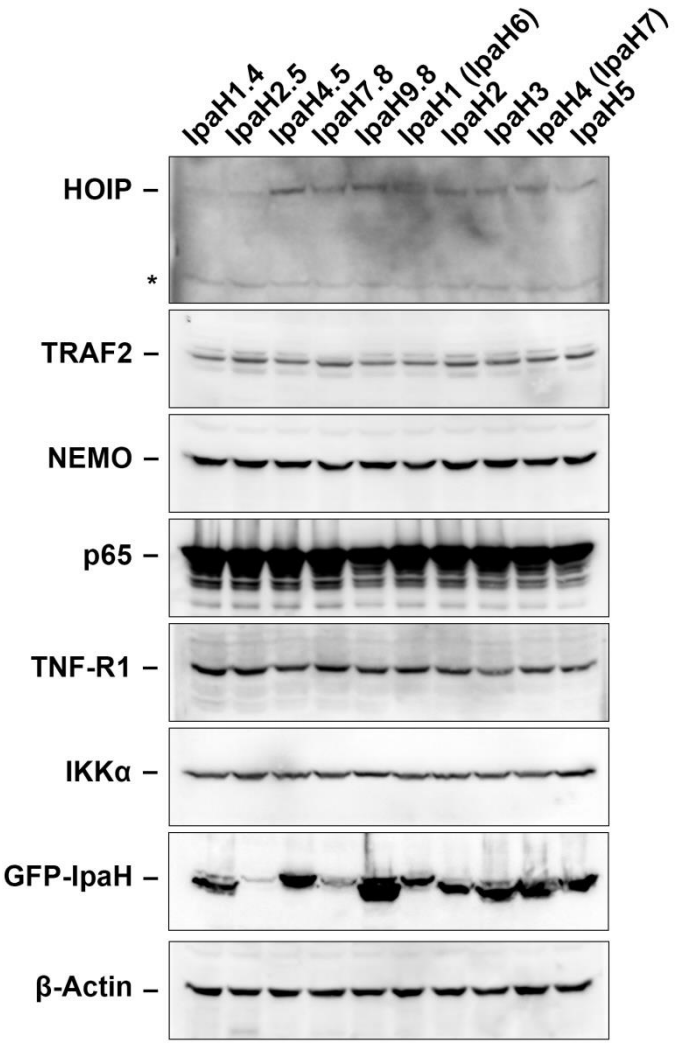
Supplementary fig. 6. IpaH1.4/2.5 catalyzes the ligation of ubiquitin chains on lysines in HOIP. **a**, *In vitro* ubiquitination reaction using purified LUBAC (Flag-His_6-SHARPIN, Myc-HOIP and Flag-HOIL-1L), UbE1, UbcH5b, ATP and HA-Ub shows that HOIP, but not HOIL-1L or SHARPIN, is specifically ubiquitinated by addition of IpaH1.4/2.5. Top panel is a Western blot probed with anti-Myc antibodies. Second panel shows a Western blot probed with anti-Flag antibodies and the third panel shows GST-IpaH input samples in Coomassie stained SDS-PAGE gel. **b**, Time course *in vitro* ubiquitination reaction shows the progress of ubiquitin chains ligation on full length Myc-HOIP by GST-IpaH2.5. Both catalytic Cys 368 in IpaH2.5 and the presence of UbE1 (E1 enzyme) in the reaction are required for HOIP ubiquitination. **c**, Ubiquitination assay using Ni-NTA purified Flag-His_6-HOIP fragments (ΔN3, aa 687-1072) shows reduced IpaH2.5 ubiquitination of fragments containing 3xR and 6xR mutations compared to wild type (WT) fragments. Representative results are shown from three independent experiments.



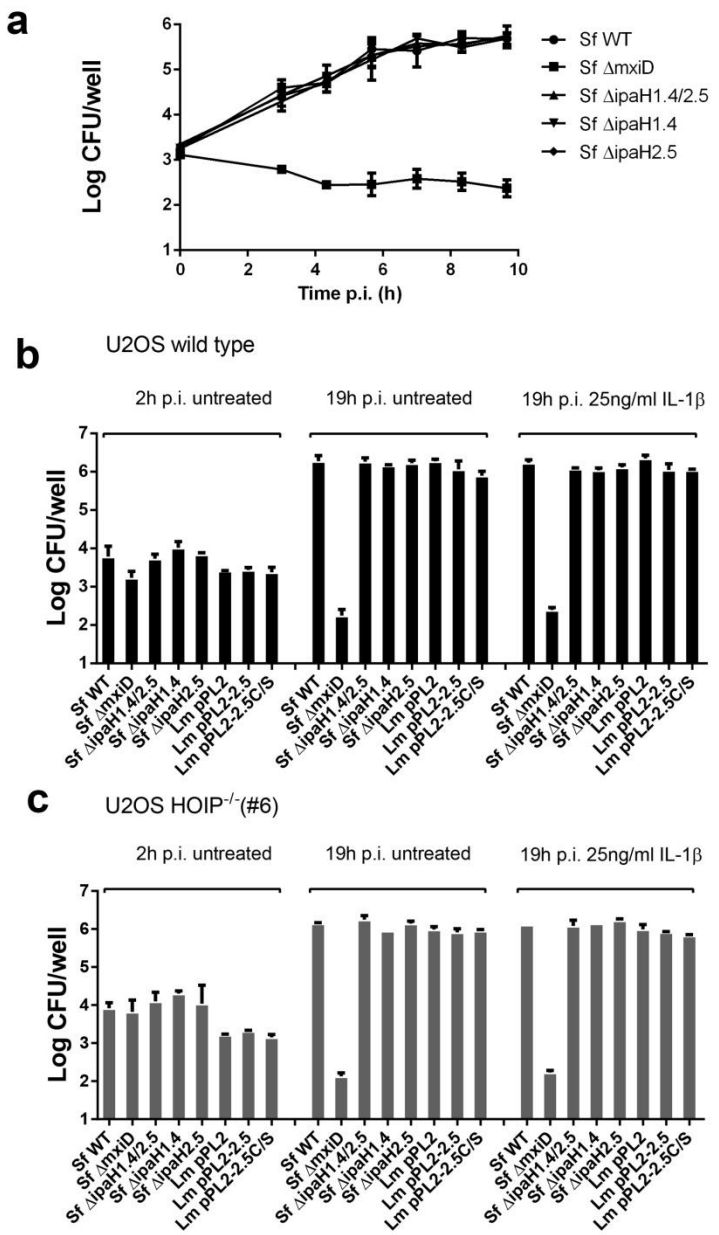
Supplementary fig. 7. Catalytic Cys 368 in IpaH1.4/2.5 is required to post-translationally modify Lys residues 735, 783 and 875 in HOIP with Ubiquitin. Tandem mass (MS/MS) spectra of IpaH2.5$_{C368S}$ treated HOIP sample showing that no GlyGly residues on Lys 735, 783 and 875 were detected.



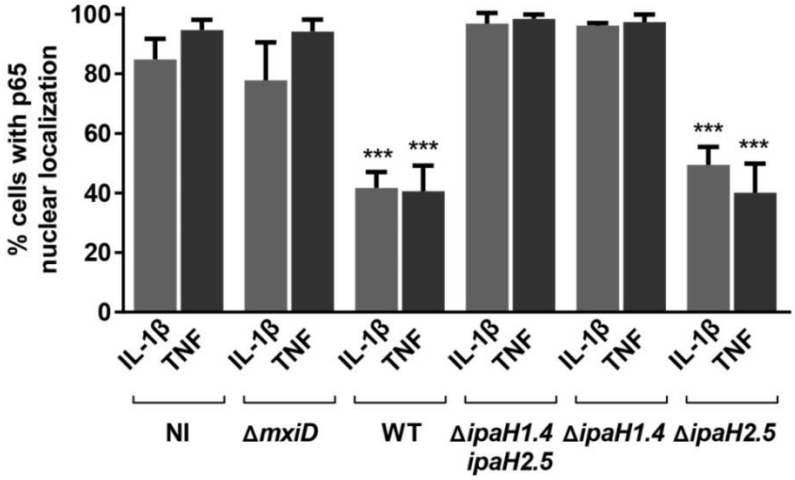
Supplementary fig. 8. a. GFP expressed from empty vector pEGFP-C2 does not result in destabilization of Flag-tagged TNF or IL-1 RSC components. **b**, *Salmonella* SspH1 specifically mediates degradation of PKN1. Results from a cellular degradation assay where HEK293T cells were transfected with GFP-tagged E3 ligases and Flag-tagged PKN1. SspH1 from *Salmonella enterica* serovar Typhimurium leads to absence of PKN1 in cell lysates, while other IpaH effectors had no effect on PKN1 stability. Results shown are representative blots from three independent experiments.



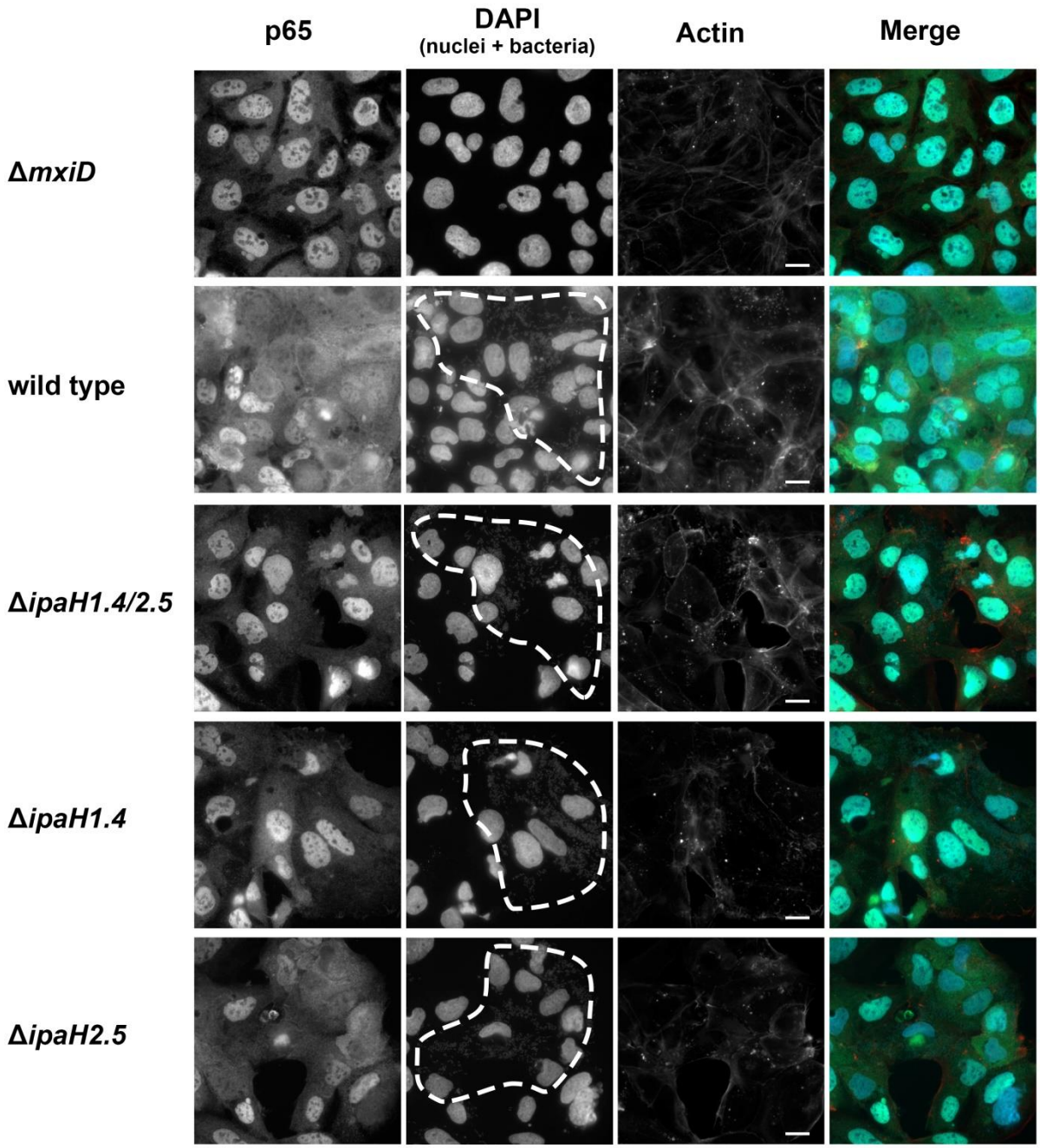
Supplementary fig. 9. Degradation assay in HEK293T cells transfected with GFP-IpaH proteins. As almost all cells in the well expressed the GFP-IpaH proteins as determined with immunofluorescence microscopy (not shown), degradation of endogenous HOIP in IpaH1.4/2.5 expressing cells was visible by Western blotting. No reduction in stability of endogenous TRAF2, NEMO (IKKγ), p65 (RelA), TNF-R1 or IKKα was detected. Band indicated with * on HOIP Western blot is a nonspecific protein detected with the anti-HOIP antibody. Representative results are shown from two independent experiments. The gene encoding IpaH1 is nearly identical to the gene encoding IpaH6 and the gene encoding IpaH4 is nearly identical to the gene encoding IpaH7. IpaH1/IpaH6 = IpaH2610/IpaH0887 (see ref (*1*)) (*Shigella flexneri* M90T ORFs SF5M90T_2545/SF5M90T_744), IpaH2 = IpaH1880 (SF5M90T_1825), IpaH3 = IpaH1383 (SF5M90T_1355), IpaH4/IpaH7 = IpaH2022/IpaH2202 (SF5M90T_1966/SF5M90T_2130) and IpaH5 = IpaH0722 (SF5M90T_2665).



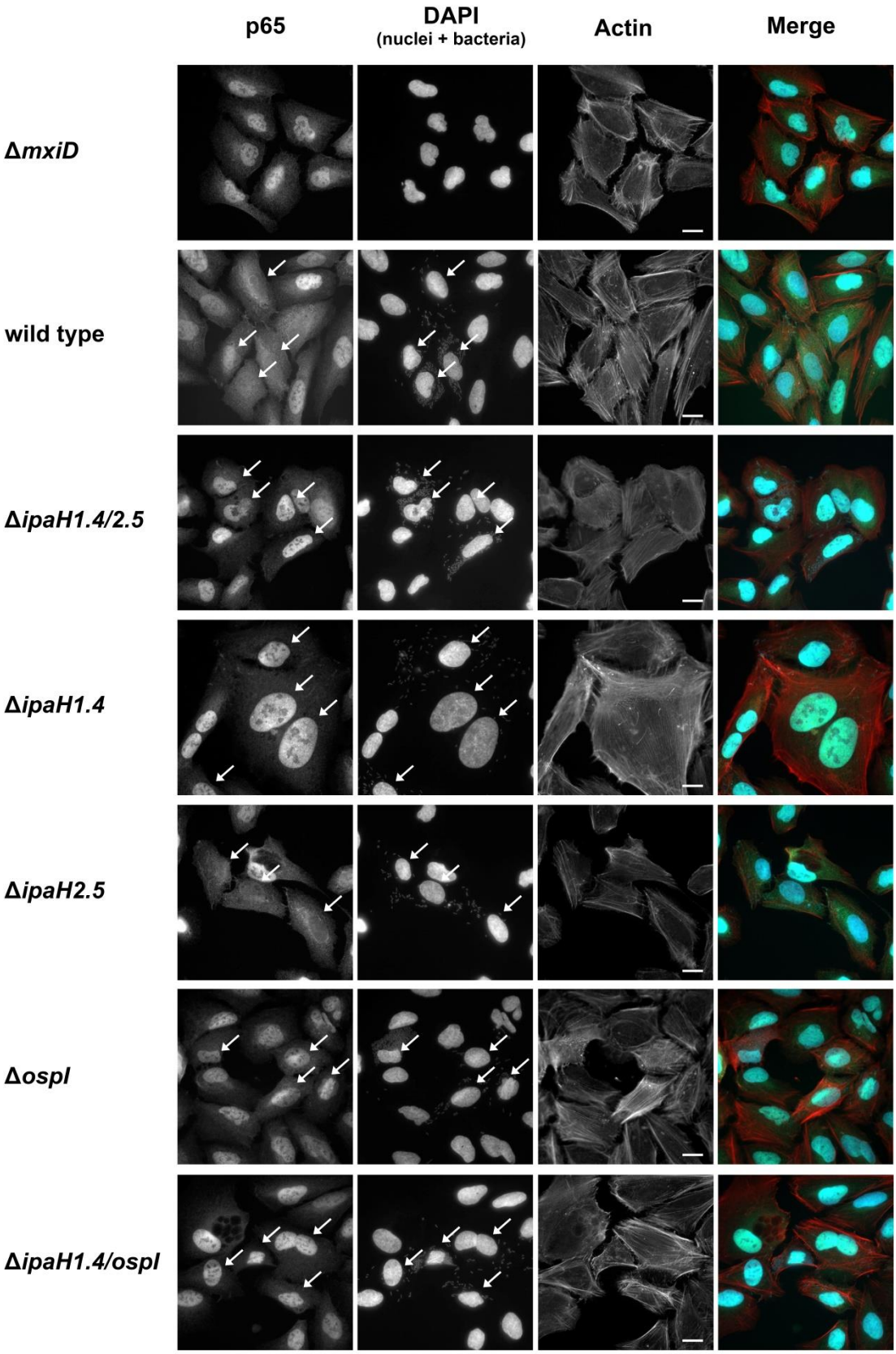
Supplementary fig. 10. *Shigella flexneri* M90T (Sf) strains lacking *ipaH1.4 and ipaH2.5* genes are not attenuated for intracellular growth. **a,** Time course of infection of Sf strains in U2OS cells. After indicated time points cells were lysed in PBS + 1% Triton X-100 and serial dilutions were plated on BHI plates for CFU enumeration. **b-c,** Sf (MOI 0.25) and Listeria (Lm) (MOI 1) strains with or without the *ipaH1.4/2.5* genes all have a similar intracellular growth phenotype in wild type or in HOIP$^{-/-}$ U2OS cells, untreated or treated with 25ng/ml IL-1β. WT: wild type, h p.i.: hours post infection. Data shown in **a**, **b** and **c** are representative of three independent experiments and are presented as the mean +/- sd from three replicates.



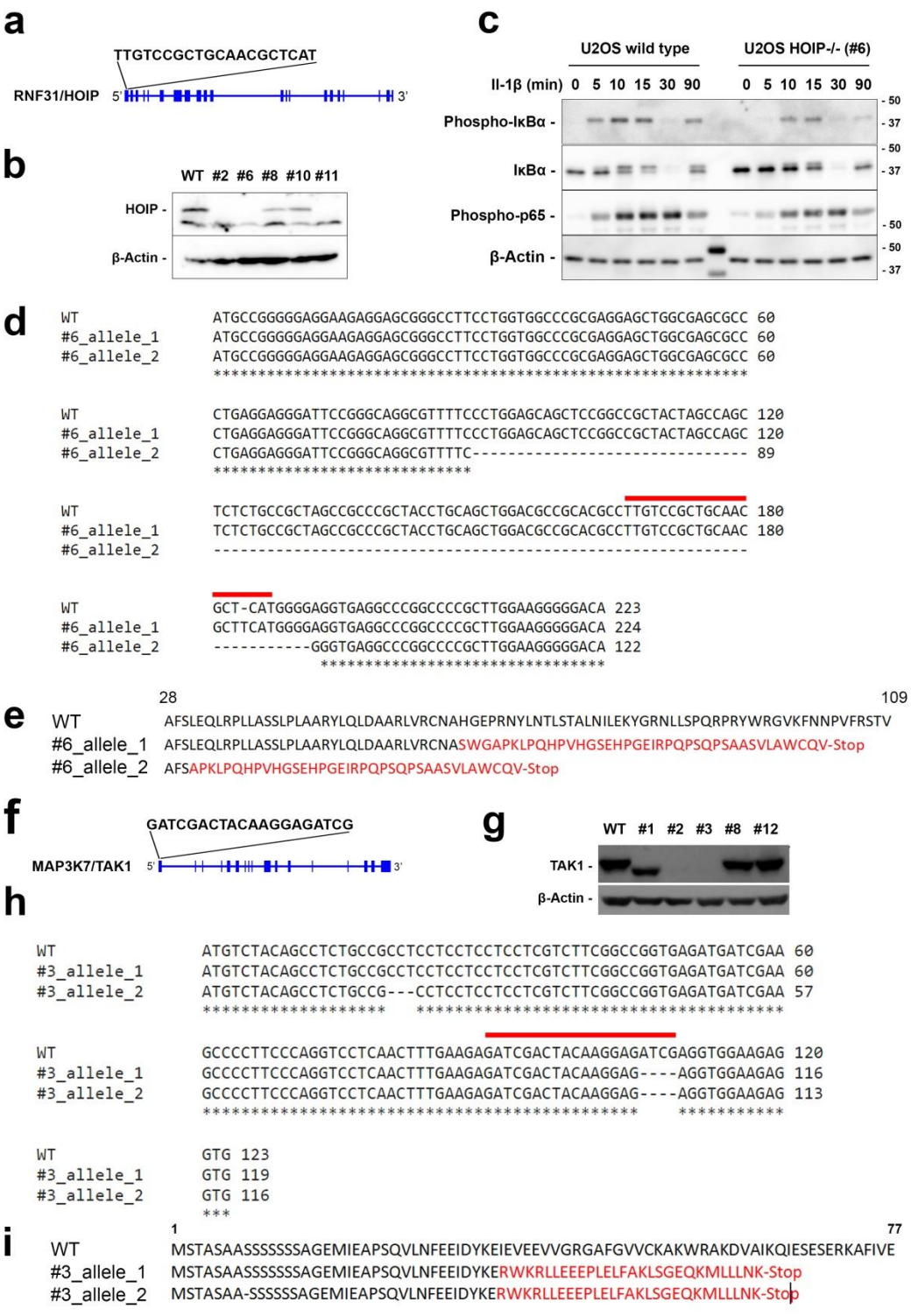
Supplementary fig. 11. Innate immune pathways are disrupted by IpaH1.4 during infection. *S*. *flexneri* M90T prevents nuclear translocation of p65 in infected cells treated with 25 ng/ml IL-1β or TNF for 20 mins, and this depended on the presence of the *ipaH1.4* gene. Bar graphs represent results from three independent experiments presented as the mean +/- s.d. *** P<0,001 (unpaired two-tailed t-test). For *S*. *flexneri* WT (wild type), Δ*ipaH1.4/2.5*, Δ*ipaH1.4* and Δ*ipaH2.5* treated cells, only infected cells were quantified. For not infected (NI) and *mxiD* all cells were quantified.



Supplementary fig. 12. Representative fluorescent microscope images from three independent experiments show p65 nuclear translocation in a monolayer of CaCo-2 cells treated with 25 ng/ml IL-1β. In a majority of *Shigella* wild type and Δ*ipaH2.5* infected wild type CaCo-2 cells p65 translocation to the nucleus is suppressed, while in nearly all *Shigella* Δ*ipaH1.4/2.5* and Δ*ipaH1.4* infected cells p65 translocation to the nucleus is visible. Cells were treated with 25ng/ml IL-1β 20 mins prior to fixing the cells. Cells were stained with anti-p65 antibodies (green), DAPI (blue) and phalloidin (red). Foci of infection in the monolayer are encircled. Scale bars, 20μm.

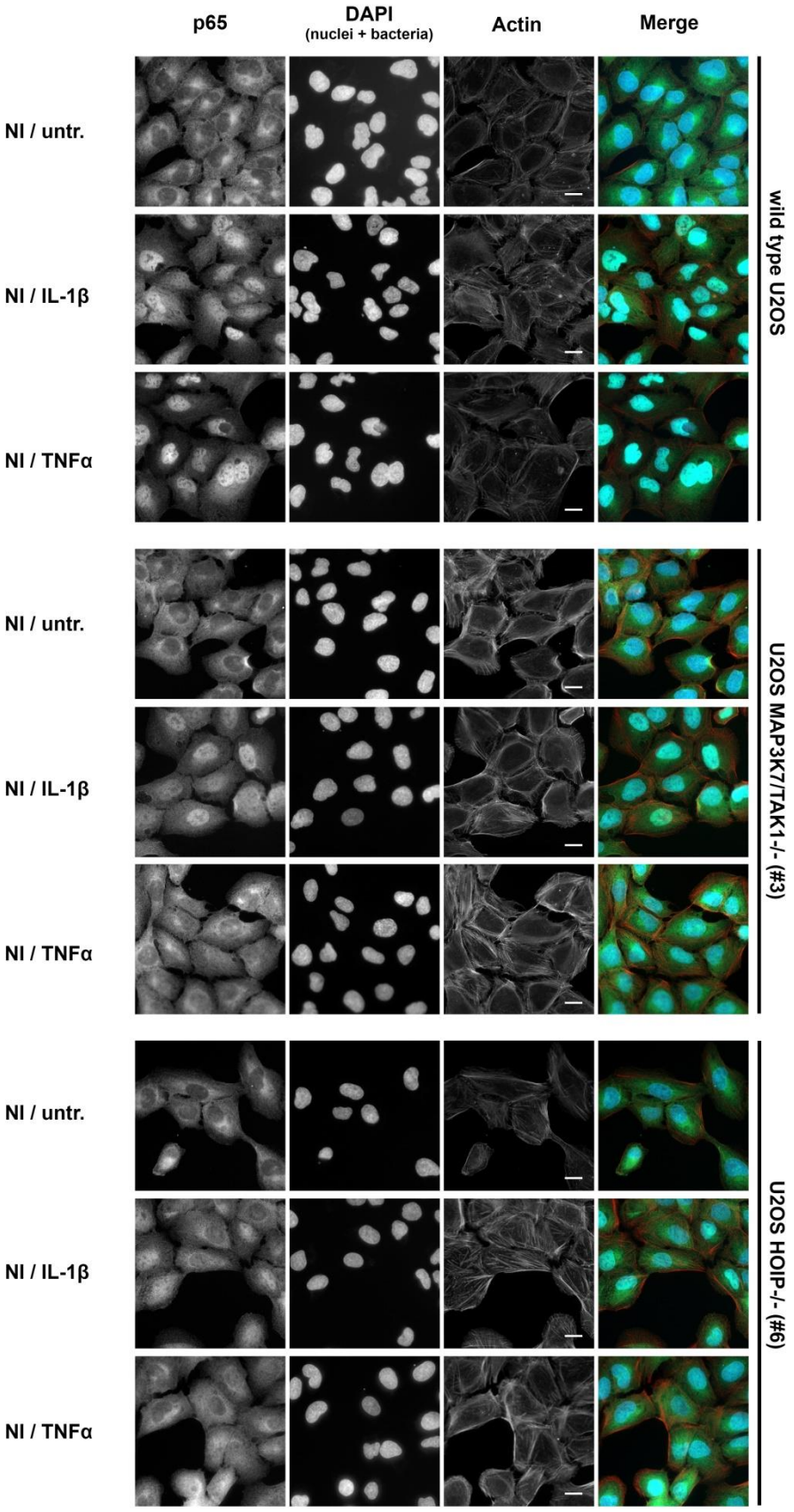

p65
DAPI
(nuclei + bacteria)
Actin
Merge
ΔmxiD
wild type
ΔipaH1.4/2.5
ΔipaH1.4
ΔipaH2.5
ΔospI
ΔipaH1.4/ospI

Supplementary fig. 13. Representative fluorescent microscope images from three independent experiments show p65 nuclear translocation in U2OS cells treated with 25 ng/ml IL-1β. In a majority of *Shigella* wild type and Δ*ipaH2.5* infected wild type U2OS cells p65 translocation to the nucleus is suppressed, while in nearly all *Shigella* Δ*ipaH1.4/2.5*, Δ*ipaH1.4,* Δ*ospI* and Δ*ipaH1.4/*Δ*ospI* infected cells p65 translocation to the nucleus is visible. Cells were treated with 25ng/ml IL-1β 20 mins prior to fixing the cells. Cells were stained with anti-p65 antibodies (green), DAPI (blue) and phalloidin (red). White arrows indicate infected cells. Scale bars, 20μm.



Supplementary fig. 14. CRISPR-Cas9 mediated knockout of RNF31/HOIP and MAP3K7/TAK1 in U2OS cells. a, Schematic representation of RNF31 encoding HOIP showing introns and exons. The sequence of the guide RNA used for targeting Cas9 to HOIP exon 1 is

shown. **b**, Western blot probed with anti-HOIP (top) and anti-Actin antibodies shows absence of HOIP expression is 3 out of 5 cell lines tested. **c**, HOIP$^{-/-}$ (#6) cells stimulated with IL-1β show a delayed and reduced phosphorylation and degradation of IκBα, compared to wild type cells. **d**, PCR of RNF31 exon 1 from genomic DNA extracted from cell line 6, and subsequent sequencing, shows allele 1 containing a 1 nt insertion and allele 2 containing a 101 nt deletion compared to wild type RNF31. The red bar shows the sequence targeted by the guide RNA. **e**, Translation of sequences show that mutations in both alleles lead to frameshift and early stop codons. **f**, CRISPR-Cas9 mediated knockout of MAP3K7/TAK1 in U2OS cells**.** Schematic representation of MAP3K7 encoding TAK1 showing introns and exons. The sequence of the guide RNA used for targeting Cas9 to TAK1 exon 1 is shown. **g**, Western blot probed with anti-TAK1 (top) and anti-Actin antibodies shows absence of TAK1 expression in 2 out of 5 cell lines tested. **h**, PCR of MAP3K7/TAK1 exon 1 on genomic DNA extracted from cell line 3, and subsequent sequencing, shows both alleles contain a 4 nt deletion compared to wild type MAP3K7/TAK1. The red bar sequence targeted by the guide RNA. **i**, Translation of sequences show that these deletions lead to frameshift and early stop codons. In panels **b**, **c** and **g** representative results from two independent experiments are shown.


p65
DAPI
(nuclei + bacteria)
Actin
Merge
NI / untr.
NI / IL-1β
NI / TNFα
wild type U2OS
NI / untr.
NI / IL-1β
NI / TNFα
U2OS MAP3K7/TAK1-/- (#3)
NI / untr.
NI / IL-1β
NI / TNFα
U2OS HOIP-/- (#6)

Supplementary fig. 15. Representative fluorescent microscope images from three independent experiments. Compared to wild type cells (**a**) IL-1β or TNF induced nuclear p65 translocation in uninfected MAP3K1/TAK1$^{-/-}$ (**b**) and HOIP$^{-/-}$ (**c**) cells is reduced. Cells were treated with 25ng/ml IL-1β or TNF for 20 mins prior to fixing the cells. Cells were stained with anti-p65 antibodies (green), DAPI (blue) and phalloidin (red). Scale bars, 20μm. NI: not infected.

Supplementary fig. 16. Uncropped Western blots. Brackets indicate lanes that were used in figures.