

The *Shigella flexneri* effector IpaH1.4 facilitates RNF213 degradation and protects cytosolic bacteria against interferon-induced ubiquitylation

Reviewed Preprint

v2 • October 3, 2025

Revised by authors

Reviewed Preprint

v1 • November 20, 2024

Luz Saavedra-Sanchez, Mary S Dickinson, Shruti Apte, Yifeng Zhang, Maarten de Jong, Samantha Skavicus, Nicholas S Heaton, Neal M Alto, Jörn Coers 

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States •

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, United States • Department

of Integrative Immunobiology, Duke University Medical Center, Durham, United States

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eLife Assessment

In this manuscript, the authors report the **fundamental** finding that a secreted ubiquitin ligase of *Shigella*, called IpaH1.4, mediates the degradation of a host defense factor, RNF213. The data are **convincing** and represent a major contribution to our understanding of cell-autonomous immunity and bacterial pathogenesis as they provide new mechanistic insight into how the cytosolic bacterial pathogen *Shigella flexneri* evades IFN-induced host immunity.

<https://doi.org/10.7554/eLife.102714.2.sa4>

Abstract

A central signal that marshals host defense against many infections is the lymphocyte-derived cytokine interferon-gamma (IFN γ). The IFN γ receptor is expressed on most human cells, and its activation leads to the expression of antimicrobial proteins that execute diverse cell-autonomous immune programs. One such immune program consists of the sequential detection, ubiquitylation, and destruction of intracellular pathogens. Recently, the IFN γ -inducible ubiquitin E3 ligase RNF213 was identified as a pivotal mediator of such a defense axis. RNF213 provides host protection against viral, bacterial, and protozoan pathogens. To establish infections, potentially susceptible intracellular pathogens must have evolved mechanisms that subdue RNF213-controlled cell-autonomous immunity. In support of this hypothesis, we demonstrate here that a causative agent of bacillary dysentery, *Shigella flexneri*, uses the type III secretion system (T3SS) effector IpaH1.4 to induce the degradation of RNF213. *S. flexneri* mutants lacking IpaH1.4 expression are bound and ubiquitylated by RNF213 in the cytosol of IFN γ -primed host cells. Linear (M1-) and lysine-linked ubiquitylation of *S. flexneri* requires RNF213 but is independent of the linear ubiquitin chain assembly complex (LUBAC). We find that ubiquitylation of *S. flexneri* is insufficient to kill intracellular bacteria, suggesting that *S. flexneri* employs additional virulence factors to escape from host

defenses that operate downstream from RNF213-driven ubiquitylation. In brief, this study identified the bacterial IpaH1.4 protein as an inhibitor of mammalian RNF213 and highlights evasion of RNF213-driven immunity as a characteristic of the human-tropic pathogen *Shigella*.

Introduction

The lymphocyte-derived cytokine IFN γ is a strong inducer of cell-autonomous immunity (1). The IFN γ -inducible E3 ubiquitin ligase ring finger protein 213 (RNF213) provides protection against a diverse group of intracellular pathogens that includes viruses, protozoa, and bacteria (2–8). RNF213 binds to bacteria that enter the host cell cytosol. Once bound to Gram-negative bacteria such as *Salmonella*, RNF213 directly ubiquitylates the bacterial surface molecule, lipopolysaccharide (LPS). In addition to RNF213, the linear ubiquitin chain assembly complex (LUBAC) was reported to play a critical role in the ubiquitylation of cytosolic bacteria (9). Whereas LUBAC catalyzes linear ubiquitin chains, the types of ubiquitin linkages deposited on bacterial surfaces by RNF213 are unknown. Following ubiquitylation cytosolic bacteria can be captured by ubiquitin-binding proteins and delivered into autolysosomes for degradation (3).

While the predominantly vacuolar pathogen *Salmonella* is susceptible to RNF213-mediated cytosolic host defense (3), the cytosol-adapted Gram-negative pathogen *Burkholderia thailandensis* is resistant. *B. thailandensis* escapes RNF213-driven host defense in part through the secretion of the virulence factor TssM, a ubiquitin esterase that removes ubiquitin from LPS on the bacterial surface (10). However, whether and how other cytosolic bacterial pathogens escape from RNF213-driven ubiquitylation is unknown.

Shigella spp. are the etiological agent of bacillary dysentery and a leading cause of diarrheal deaths, malnutrition, and growth retardation in children (11–13). *Shigella* spp. invade the intestinal epithelium, enter the host cell cytosol of infected human colonic epithelial cells, and trigger an acute inflammatory response that involves the secretion of IFN γ by lymphocytes (14–17). IFN γ signaling in epithelial cells induces the expression of human guanylate binding protein 1 (GBP1), a defense protein that blocks bacterial intracellular motility and cell-to-cell spread (18–21). To counter this potent cell-autonomous immune program, *Shigella flexneri* employs its type III secretion system (T3SS) to deliver the GBP1 inhibitor IpaH9.8 into the host cell cytosol. Loss of IpaH9.8 results in diminished *S. flexneri* virulence and a potent antibacterial host response (20–23).

IpaH9.8 belongs to a family of T3SS effectors that contain a leucine-rich repeat (LRR) domain involved in substrate recognition and a novel E3 ligase (NEL) domain that transfers K48-linked ubiquitin to its bound substrate (24). K48-linked ubiquitylation tags the substrate for proteasomal degradation. These IpaH proteins belong to a ‘second wave’ of T3SS effectors that are under the control of the transcriptional regulator MxiE. Second wave T3SS effectors have been shown to promote host cell survival, to dampen inflammation, and to counteract cell-autonomous immune programs (25), yet their full repertoire of targets remains to be determined.

In the present work we found that the MxiE-regulated T3SS effector IpaH1.4 facilitates the proteasomal degradation of RNF213. Remarkably, RNF213 functions independent of LUBAC to promote the linear and lysine-linked ubiquitylation of *ipaH1.4*-deficient *S. flexneri* mutants. We observed that ubiquitylated *S. flexneri* continued to replicate inside the host cell cytosol, indicating that *S. flexneri* employs additional unknown virulence mechanisms to interfere with its degradation. Thus, our study demonstrates that the human enteric pathogen *S. flexneri* directly interferes with RNF213 expression and also limits host defense downstream of RNF213-driven ubiquitylation.

Results

Deletion of *mxiE* renders *S. flexneri* susceptible to linear ubiquitylation

Previous studies identified a cell-autonomous host defense program in which mammalian cells attach linear ubiquitin (M1) to the surface of the Gram-negative pathogen *Salmonella enterica* upon bacterial spillage into the host cell cytosol (3, 9). Recently, it was reported that the cytosol-adapted pathogen *B. thailandensis* secretes a ubiquitin esterase that renders the bacteria resistant to this type of cytosolic ubiquitylation (10). We therefore hypothesized that resistance to host-driven linear ubiquitylation is a common feature of bacterial pathogens that are adapted to a cytosolic milieu. To test this concept, we monitored ubiquitylation of the professional cytosolic pathogen *Shigella flexneri* (serotype 2a, strain 257T) by immunofluorescence. We found that wildtype (WT) *S. flexneri* remained largely devoid of M1-linked ubiquitin in both alveolar A549 and colonic HT29 epithelial cell lines under both unprimed and IFN γ -primed conditions (Fig. 1A – C). In contrast to WT *S. flexneri*, a $\Delta mxiE$ mutant strain that has diminished expression of ‘second-wave’ T3SS effector proteins (26, 27), was robustly stained with anti-M1 antibodies in IFN γ -primed host cells (Fig. 1A – C). These observations indicate that *S. flexneri* uses MxiE-dependent effectors to evade an IFN γ -activated ubiquitylation pathway that targets cytosolic bacteria.

Linear and lysine-linked ubiquitin is attached to *S. flexneri* $\Delta mxiE$

The ubiquitin protein contains seven lysine residues. Any of these lysine residues or the N-terminal methionine of the acceptor ubiquitin can form isopeptide bonds with the C-terminal glycine residue of the donor ubiquitin to form chains. To determine which ubiquitin linkage types are present on the surface of *S. flexneri* $\Delta mxiE$ bacteria, we generated A549 cells that overexpressed internally tagged ubiquitin (INT-Ub) variants, which contain only a single lysine residue or lack lysines altogether. As a ubiquitin acceptor, the lysine-less (7KR) variant can only be assembled into ubiquitin chains via its N-terminal methionine. We found that 7KR INT-Ub robustly co-localized with *S. flexneri* $\Delta mxiE$, albeit with diminished efficiency compared to WT INT-Ub (Fig. 2A).

In addition to its incorporation into linear ubiquitin chains, 7KR ubiquitin can also function as donor ubiquitin and be attached as mono-ubiquitin to a substrate or to an existing ubiquitin chain as a chain terminator. To distinguish between 7KR INT-Ub signals originating from linear versus mono-ubiquitylation, we generated an N-terminally HA-tagged 7KR INT-Ub variant. The N-terminal tag prevents linear ubiquitylation but still allows 7KR INT-Ub to be attached as mono-ubiquitin via its C-terminal carboxyl group. We found that the addition of this N-terminal HA-tag significantly reduced but not completely abolished the number of $\Delta mxiE$ bacteria decorated with 7KR INT-Ub (Fig. S1). These data indicate that much of the association of 7KR INT-Ub with cytosolic $\Delta mxiE$ bacteria is due to the formation of M1-linked linear ubiquitin chains.

Collectively, our data confirmed that M1-linked ubiquitin is bound to *S. flexneri* $\Delta mxiE$ but also indicated that other additional ubiquitin linkage types are attached to *mxiE*-deficient bacteria. The functional importance of these lysine-linked ubiquitin chains was demonstrated by improved bacterial targeting of INT-Ub variants that can form additional K27-linkages or both K27- and K63-linkages (Fig. 2A). Linkage-specific antibodies against K27 and K63 confirmed that these two linkage types are present on $\Delta mxiE$ bacteria in IFN γ -primed A549 cells (Fig. 2B–C). Staining with an antibody that detects most ubiquitin linkage types (FK2) showed near complete overlap with anti-M1 staining (Fig. 2D–E). Because the percentage of FK2-positive but M1-negative bacteria

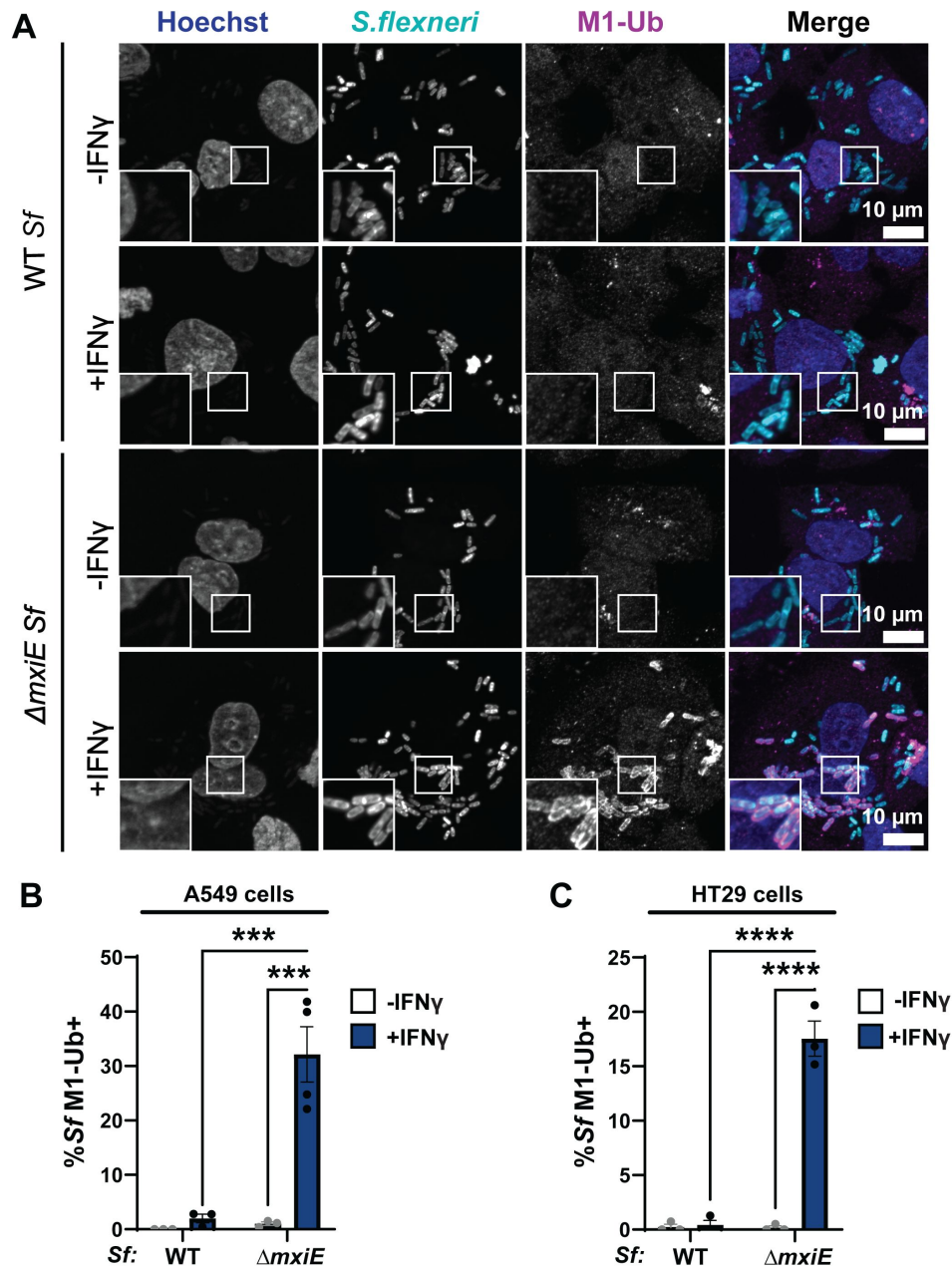


Fig. 1.

Mutation of the bacterial transcription factor MxiE results in ubiquitylation of *S. flexneri* in IFN γ -primed epithelial cells.

(A – C) Cells were primed with 100 U/ml IFN γ or left unprimed and then infected with WT or Δ *mxiE* *S. flexneri* at an MOI of 50 – 100. Cells were fixed 3 – 4 h post infection (hpi) and stained for linear ubiquitin (M1-Ub) with anti-M1 antibody. Percentage of M1-Ub-positive bacteria was quantified in infected A549 epithelial cells at 4 hpi (**B**) and HT29 epithelial cells at 3 hpi (**C**). Graphs show the average of three independent experiments and depict means $\pm$ SEM. Two-way ANOVA with Tukey's multiple comparison tests were performed; all statistically significant comparisons are shown. *** $p < 0.001$; **** $p < 0.0001$; ns, not significant

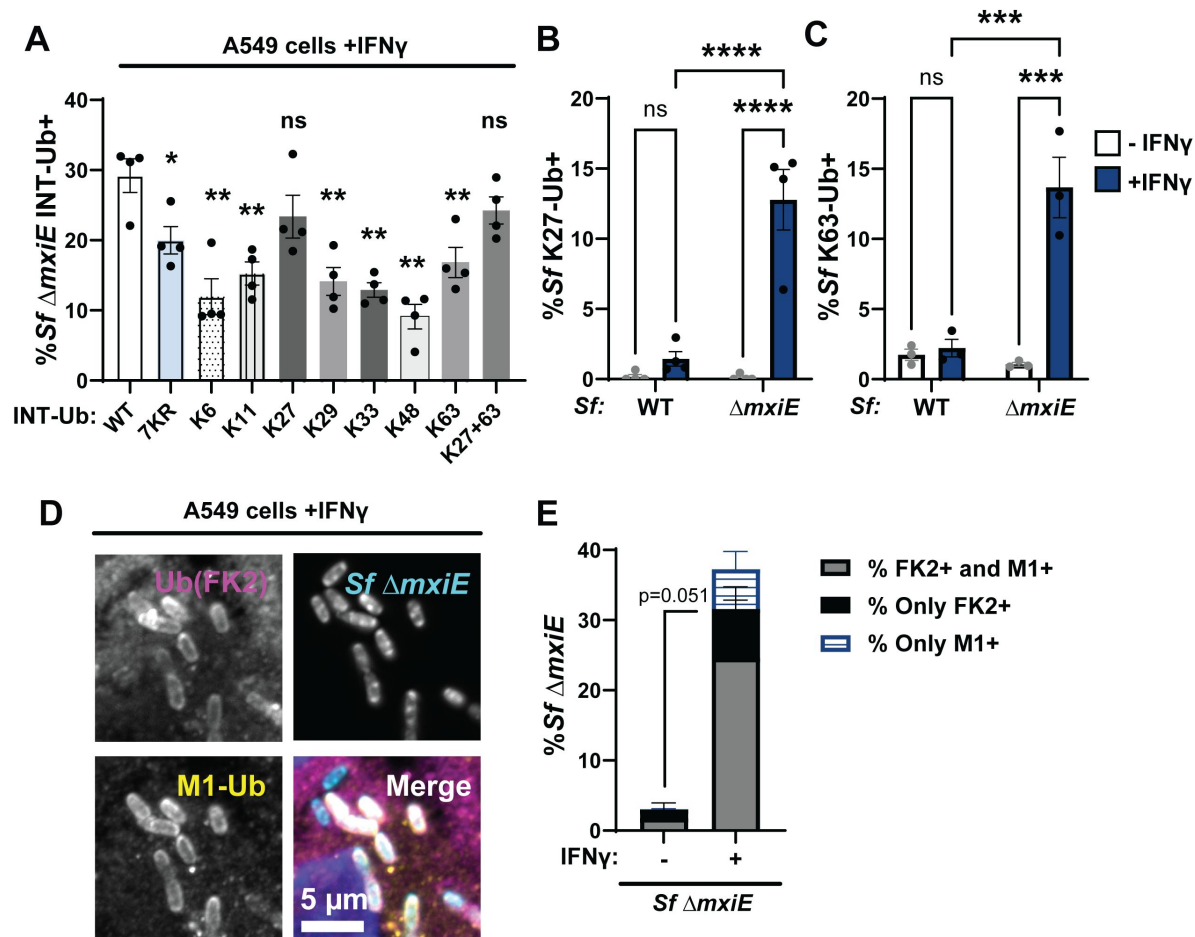


Fig. 2.

***S. flexneri* $\Delta mxiE$ mutants become decorated with linear and lysine-linked ubiquitin in IFN γ -primed A549 cells.**

(A) WT A549 cells expressing the indicated internally Strep-tagged Ubiquitin (INT-Ub) variants were primed with 100 U/ml IFN γ overnight and co-localization of INT-Ub with cytosolic *S. flexneri* (Sf) $\Delta mxiE$ was assessed at 4 hpi. Ubiquitin linkage-specific antibodies were used to determine the percentage of *S. flexneri* $\Delta mxiE$ staining positive for K27-linked ubiquitin (K27-Ub) (B) or K63-linked ubiquitin (C) in IFN γ -primed untransduced WT A549 cells. Co-staining of anti-M1 and anti-ubiquitin (FK2) on the surface of *S. flexneri* $\Delta mxiE$ in IFN γ -primed (100U/ml) A549 cells is shown in (D). Quantification of anti-M1-FK2 co-staining in untreated and IFN γ -primed A549 cells is depicted in (E). All infections were done at an MOI of 50 – 100. Data were generated from at least three independent experiments and shows mean $\pm$ SEM. One-way ANOVA followed by Dunnet's multiple comparisons were performed of all groups against WT-ubiquitin group (A). Two-way ANOVA with Tukey's multiple comparison tests were performed for (B) and (C). An unpaired t-test was performed between "both FK2+M1" groups (gray bars) (E). *p<0.05; **p<0.01; ***p<0.001; **** p<0.0001

(Fig. 2E). was much smaller than the percentage of bacteria that were K27- or K63-positive (Fig. 2B-C), these data suggest that most *S. flexneri* $\Delta mxiE$ bacteria are decorated with both linear and lysine-linked ubiquitin.

The ubiquitylation of *S. flexneri* $\Delta mxiE$ is dependent on RNF213 but not LUBAC

Previous work showed that the multimeric E3 ligase LUBAC can deposit M1-linked ubiquitin on the surface of cytosolic *S. enterica* Typhimurium (9). Moreover, *S. flexneri* secretes the MxiE-regulated effector IpaH1.4 which specifically recognizes and marks the LUBAC components HOIP and HOIL-1 for proteasomal degradation (28–30). Consequently, we hypothesized that LUBAC was essential for the ubiquitylation of $\Delta mxiE$. To test this hypothesis, we infected HOIP- and HOIL-1-deficient A549 cells (HOIP^{KO}, HOIL-1^{KO}, Fig. 3A) with the $\Delta mxiE$ mutant under IFN γ -primed conditions.

Unexpectedly, deletion of either HOIP or HOIL-1, each an essential component of the LUBAC complex, did not diminish linear ubiquitylation of $\Delta mxiE$ when compared to WT cells (Fig. 3B). Similarly, the internally tagged 7KR-Ub variant decorated *S. flexneri* $\Delta mxiE$ independent of the LUBAC component HOIP (Fig. 3C). Collectively, these data demonstrate that LUBAC is dispensable for linear ubiquitylation of $\Delta mxiE$ bacteria in the host cell cytosol of IFN γ -primed A549 cells.

The ubiquitin E3 ligase RNF213 was recently shown to ubiquitylate pathogen-containing vacuoles (2, 4) as well as Gram-positive and Gram-negative bacteria inside the host cell cytosol (3, 6, 10). Like HOIL-1, RNF213 is an IFN γ -inducible protein (Fig. 3A and (2, 4)). Using immunofluorescence microscopy, we detected RNF213 on the surface of $\Delta mxiE$ but not WT bacteria in IFN γ -primed A549 as well as colonic HT29 cells (Fig. 3D-G). The vast majority of ubiquitylated *S. flexneri* $\Delta mxiE$ mutants (~93%) co-stained with anti-RNF213 (Fig. 3G), indicative of a functional role for RNF213 in the ubiquitylation of $\Delta mxiE$ bacteria. To directly probe for RNF213 function, we infected a pool of CRISPR-generated A549 RNF213^{KO} cells (Fig. 3A) with *S. flexneri* $\Delta mxiE$ and stained these IFN γ -primed cells for M1-, K27-, and K63-linked ubiquitin. We found that in the absence of human RNF213, antibodies specific for M1-, K27-, and K63-linked ubiquitin no longer stained the surface of *S. flexneri* $\Delta mxiE$ (Fig. 3B and 3H-I). In agreement with these immunofluorescence data, both WT and lysine-less ubiquitin (7KR-Ub) localized to $\Delta mxiE$ bacteria in WT but not in RNF213^{KO} cells (Fig. 3C). Together, these data demonstrate that human RNF213 is essential for the linear and lysine-linked ubiquitylation of MxiE-deficient *S. flexneri* mutants.

Ectopic expression of *S. flexneri* virulence factors IpaH1.4 or IpaH2.5 induces RNF213 degradation

The transcription factor MxiE induces the expression of several secreted effector proteins of the IpaH family that act as bacterial ubiquitin E3 ligases and tag host proteins for proteasomal degradation (21, 23, 28, 29, 31–35). Since our data revealed that the *S. flexneri* virulence factor MxiE blocks bacterial ubiquitylation by RNF213, we hypothesized that *S. flexneri* infections induce the proteasomal degradation of RNF213 in a MxiE-dependent manner. To test this hypothesis, we measured RNF213 protein levels by immunoblotting in cells that had been infected with WT or $\Delta mxiE$ *S. flexneri*. Our data revealed that RNF213 protein levels are drastically reduced upon infection with WT *S. flexneri* and that the deletion of *mxiE* partially restored RNF213 expression in infected cells (Fig. 4A). Similarly, and as reported previously (9, 28, 29), HOIP protein expression is diminished in cells infected with WT *S. flexneri* but much less so in the case of infections with $\Delta mxiE$ *S. flexneri* (Fig. 4A).

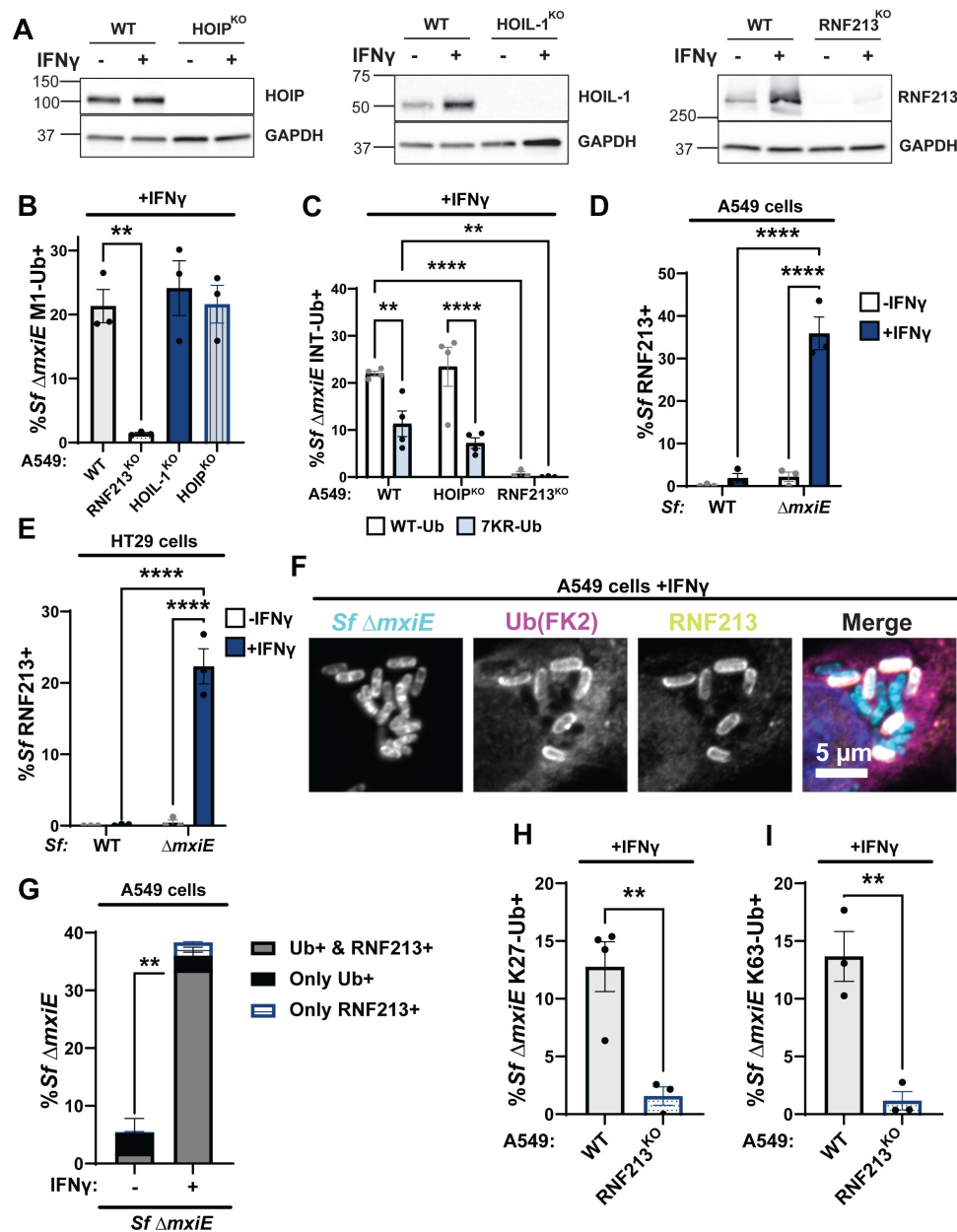


Fig. 3

Ubiquitylation of *S. flexneri* $\Delta mxiE$ is dependent on RNF213 but not LUBAC

(A) Immunoblotting for HOIP, HOIL-1, and RNF213 protein expression in untreated and IFN γ -primed WT and the corresponding gene deletion (KO) A549 cells. (B) Percentage $\Delta mxiE$ *S. flexneri* bacteria stained with anti-M1-linked ubiquitin in IFN γ -primed WT, HOIP^{KO}, HOIL-1^{KO} and RNF213^{KO} A549 cells. (C) Percentage of WT and 7KR INT-Ub-positive $\Delta mxiE$ *S. flexneri* in IFN γ -primed WT, HOIP^{KO}, and RNF213^{KO} A549 cells. (D – G) Untreated and IFN γ -primed A549 and HT29 cells were infected with the indicated *S. flexneri* strains and immuno-stained for RNF213 and ubiquitin (FK2). Representative immunofluorescence microscopy images are shown for A549 infections in (F). RNF213-*S. flexneri* colocalization percentages were quantified in A549 (D) and HT29 (E) cells. (G) Quantification of ubiquitin and RNF213 colocalization with $\Delta mxiE$ *S. flexneri* in A549 cells. Percentages of $\Delta mxiE$ *S. flexneri* staining positive for antibodies specific for K27-linked (H) and K63-linked ubiquitin (I) are also provided. All data are represented by the mean $\pm$ SEM from at least three independent experiments. One-way ANOVA followed by Dunnett's multiple comparisons were performed of all groups against WT A549 group in (A). Two-way ANOVA with Tukey's multiple comparison tests were performed in (C, D and F). an unpaired t-test was performed between the "Ub+ & RNF213+" groups (gray bars) in (G). For (H and I), an unpaired-t test was performed. Comparisons not shown are non-significant. **p<0.01; ****p<0.0001

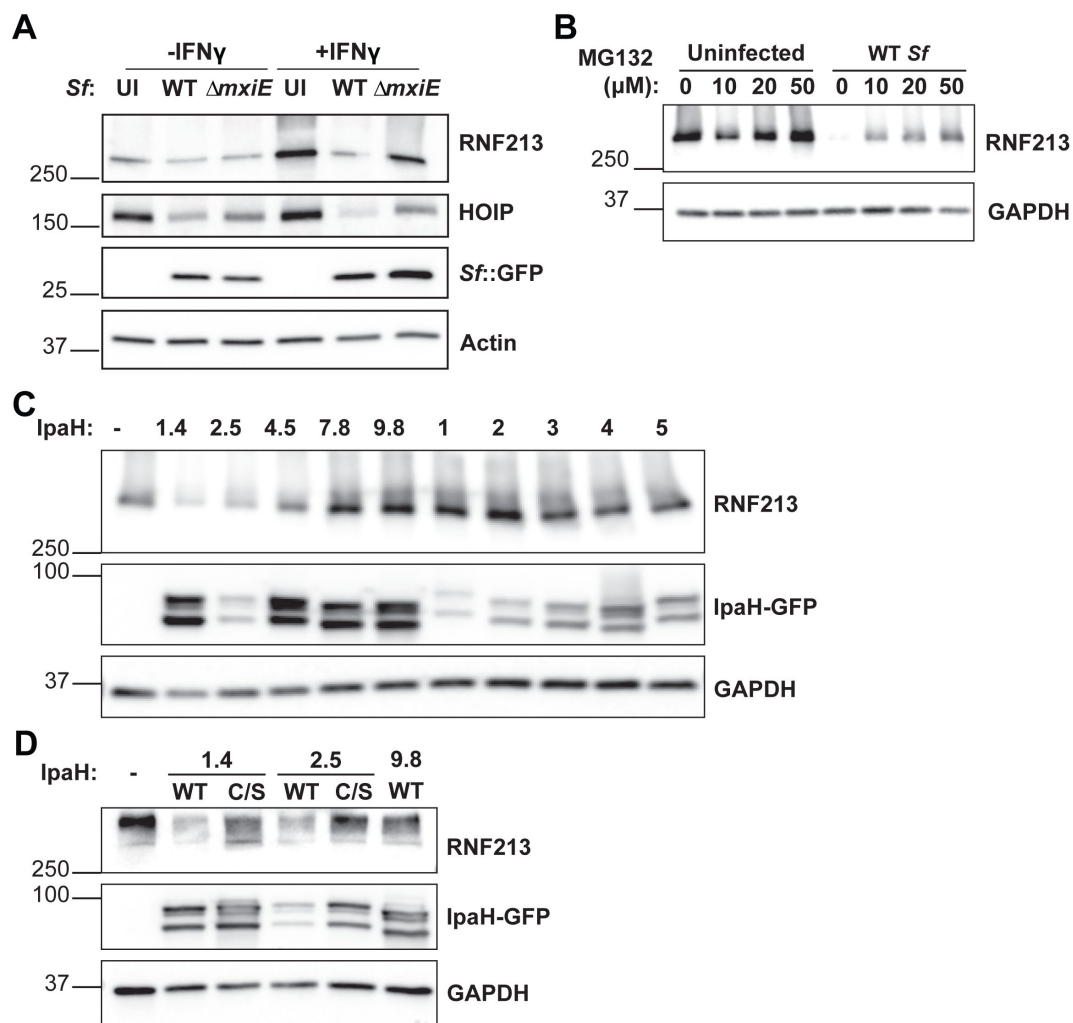


Fig. 4.

***S. flexneri* virulence factors IpaH1.4 and IpaH2.5 induce proteasomal degradation of RNF213**

(A-B) All *S. flexneri* strains express PilT to enhance adhesion and infection rates and infections were carried out at an MOI of 5 – 25 for 3 hours **(A)** Untreated and IFN γ -primed (100 U/ml) A549 cells were infected with indicated *S. flexneri* strains expressing GFP. Protein lysates were probed for expression of RNF213, HOIP, and GFP. GFP expression serves as a bacterial protein loading control. **(B)** Infected and uninfected A549 cells were cultured in the presence of different concentrations of the proteasomal inhibitor MG132 and RNF213 expression was monitored by immunoblotting. **(C)** HEK 293T cells stably expressing mCherry-RNF213 were transiently transfected with individual GFP-tagged IpaH effectors for 24 hours and RNF213 expression was assessed. **(D)** WT and catalytically inactive C368S mutants of IpaH1.4 and IpaH2.5 were transiently transfected into HEK 293T cells expressing mCherry-RNF213 and cell lysates were subjected to immunoblotting. **(C-D)** Denaturation of cell lysates at lower temperature (56°C) was required for RNF213 detection but also resulted in the formation of double bands for all IpaH-GFP constructs. Images are representative of three independent experiments UI: Uninfected.

To directly test whether *S. flexneri* promote proteasomal degradation of RNF213, we conducted infections in the presence of the proteasomal inhibitor MG132. As expected, MG132 largely restored RNF213 total protein levels in *S. flexneri*-infected cells (Fig. 4B). We next tested whether a specific IpaH effector facilitates the degradation of RNF213. To this end, we transiently expressed GFP-fusion proteins of individual IpaH family members in HEK 293T cells that ectopically expressed RNF213 and measured their effects on RNF213 protein levels by immunoblotting. Our mini-screen revealed that the ectopic expression of the two closely related paralogs IpaH1.4 and IpaH2.5 were sufficient to promote RNF213 degradation in HEK 293T cells (Fig. 4C). Of note, the substrate-binding LRR domains of IpaH2.5 and IpaH1.4 are 98.9% identical in DNA sequence and were previously shown to have comparable substrate specificity and functional redundancy (28). Both IpaH1.4 and 2.5 share a highly conserved NEL E3 ubiquitin ligase domain that contains an essential catalytic cysteine residue at position 368 (29). Catalytically inactive forms of IpaH1.4 or IpaH2.5, in which Cys368 is mutated to a serine (C/S), failed to degrade RNF213 (Fig. 4D). As an additional control we included overexpression of IpaH9.8, an inhibitor of GBPs, which also did not impact RNF213 expression (Fig. 4D), as anticipated.

To investigate potential physical interactions between IpaH1.4 and IpaH2.5, we reanalyzed a previously generated dataset that employed a method known as ubiquitin-activated interaction traps (UBAITS) (32). As depicted in Fig. S2A, the human ubiquitin gene was fused to the 3' end of IpaH2.5, producing a C-terminal IpaH2.5-ubiquitin fusion protein. When incubated with ATP, a ubiquitin-activating enzyme E1, and a ubiquitin-conjugating enzyme E2, the IpaH2.5-ubiquitin “bait” protein is capable of binding to and ubiquitylating target substrates. This ubiquitylation creates an iso-peptide bond between the IpaH2.5 bait and its substrate, thereby enabling purification via a Strep affinity tag incorporated into the fusion construct (32). IpaH2.5-ubiquitin bait and IpaH3-ubiquitin control proteins were incubated with lysates from murine intestinal tissue. To detect interaction partners in a physiologically relevant setting, we used intestinal lysates derived from mice infected with *Salmonella*, which in contrast to *Shigella* causes pronounced inflammation in WT mice and therefore better simulates human *Shigellosis* in an animal model. Using UBAIT we identified HOIP (Rnf31) as a likely IpaH2.5 binding partner (Fig. S2B), thus confirming previous observations (28) and validating the effectiveness of our approach. Strikingly, we identified mouse Rnf213 as the most abundant interaction partner of the IpaH2.5-ubiquitin bait protein (Fig. S2B). Collectively, our data and concurrent reports showing direct interactions between IpaH1.4 and human RNF213 (36, 37) indicate that the virulence factors IpaH1.4 and IpaH2.5 directly bind and degrade mouse as well as human RNF213.

Loss of IpaH1.4 is sufficient to render *S. flexneri* susceptible to RNF213-driven ubiquitylation in human and murine host cells

Given that ectopic expression of either IpaH1.4 or IpaH2.5 was sufficient to degrade RNF213, we expected these two endogenous effectors to be functionally redundant. However, we found that genetic deletion of *ipaH1.4* in either *S. flexneri* serotype 2a strain 257T (Fig. 5A-B) or in serotype 5a strain M90T (Fig. S3A) was sufficient to restore RNF213 protein expression and RNF213 recruitment to cytosolic bacteria. A deletion mutant of *ipaH2.5* was as resistant to RNF213 targeting as co-isogenic WT bacteria and a $\Delta ipaH1.4$ -*ipaH2.5* double mutant was indistinguishable from the $\Delta ipaH1.4$ single deletion mutant (Fig. S3A). These data suggested that endogenous IpaH2.5 is non-functional, which agrees with a previous characterization of *ipaH2.5* as a minimally expressed pseudogene (38).

Previous work showed that IpaH1.4 binds to and degrades the LUBAC subunit HOIP (9, 28, 29). To determine whether the degradation of RNF213 could be indirect and HOIP-dependent, or vice versa, we monitored RNF213 expression in HOIP^{KO} cells and HOIP expression in RNF213^{KO} cells. We found that expression of RNF213 remained at WT levels in the absence of HOIP and that the degree of IpaH1.4-dependent RNF213 degradation was comparable in WT and HOIP^{KO} cells

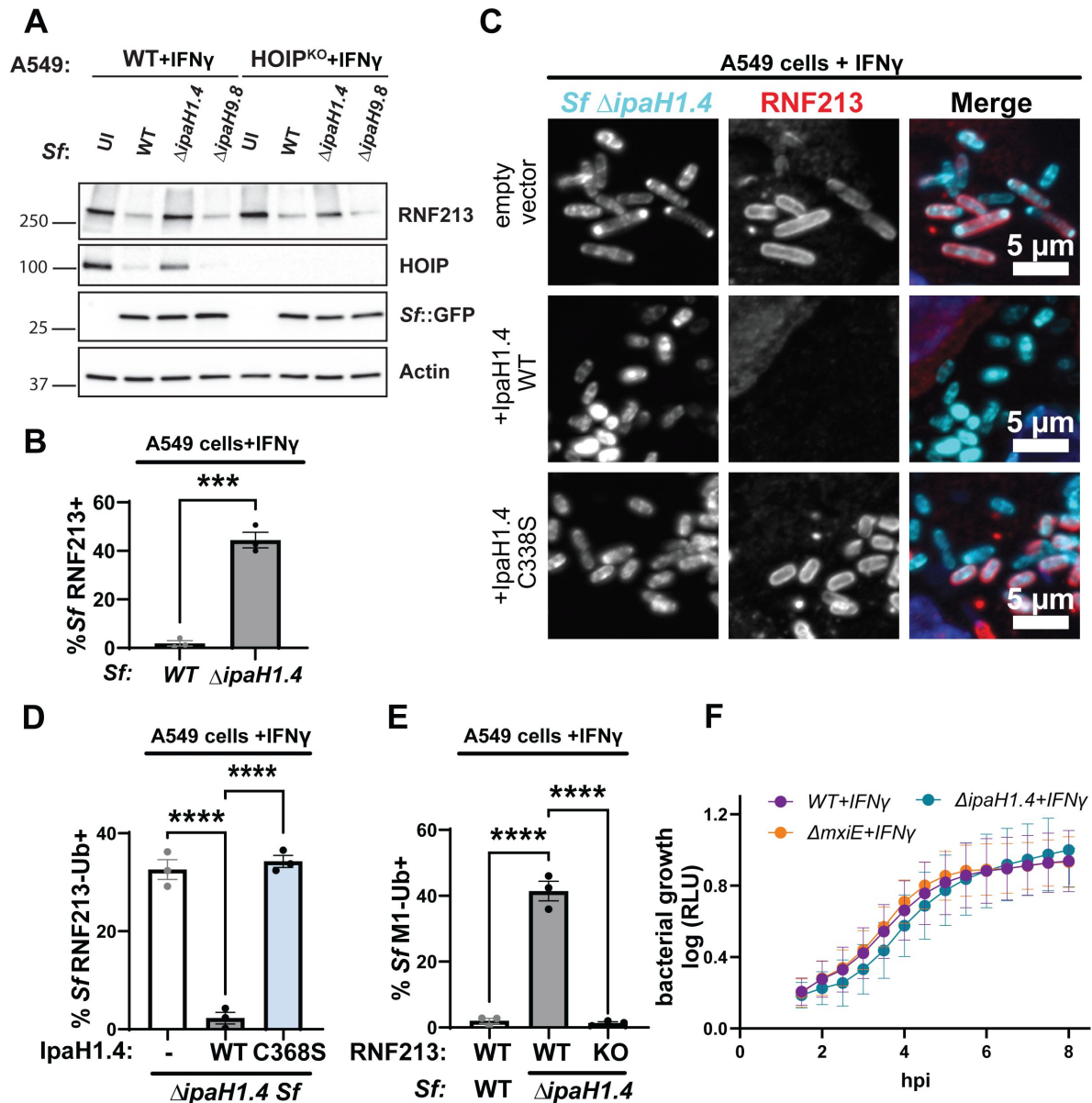


Fig. 5.

Loss of IpaH1.4 is sufficient to render *S. flexneri* susceptible to RNF213-driven ubiquitylation

(A) IFN γ -primed WT and HOIP^{KO} A549 cells were infected with the indicated *PilT*⁺, GFP⁺ *S. flexneri* strains for 3 hours at an MOI of 25 and RNF213 protein levels were assessed by Western blotting. Bacterial GFP and host actin are used as host protein loading controls. (B) Percentages of RNF213⁺ WT and Δ ipaH1.4 *S. flexneri* in IFN γ -primed A549 cells are shown. (C) Representative microscopy images depicting RNF213 recruitment to cytosolic Δ ipaH1.4 bacteria complemented with empty vector, WT IpaH1.4, or catalytically inactive IpaH1.4 (C368S) in IFN γ -primed WT A549 cells. (D) Percentages of RNF213⁺ indicated *S. flexneri* strains in IFN γ -primed WT A549 cells are shown. (E) Percentages WT and Δ ipaH1.4 *S. flexneri* stained positive for anti-M1-linked antibody in IFN γ -primed WT and RNF213^{KO} A549 cells are depicted. (F) The *ilx* operon was introduced into *S. flexneri* strains to use bioluminescence relative light units (RLU) as a proxy for bacterial growth. RLU was measured in IFN γ -primed WT A549 cells infected with the indicated *S. flexneri* strains at an MOI of 5. (B, D, E) Data represent the mean $\pm$ SEM from at least three independent experiments. An unpaired t-test was conducted for (B). One-way ANOVA followed by Dunnett's multiple comparisons were performed between all groups against the group Δ ipaH1.4 expressing WT IpaH1.4 (D) and WT A549 cells (E). *p<0.05; ***p<0.001; **** p<0.0001

(Fig. 5A). Similarly, infections with WT but not *ΔipaH1.4* mutant *S. flexneri* resulted in a substantial reduction of HOIP protein expression in RNF213^{KO} cells (Fig. S3B). These observations show that IpaH1.4 targets RNF213 and HOIP for degradation independent from each other.

The sustained RNF213 expression in cells infected with *S. flexneri* *ΔipaH1.4* (Fig. 5A) correlated with staining of cytosolic *ΔipaH1.4* bacteria with RNF213 (Fig. 5B–C). As expected, the complementation of the *ΔipaH1.4* mutant with WT IpaH1.4 efficiently prevented RNF213 recruitment to cytosolic bacteria, whereas complementation with catalytically inactive IpaH1.4 did not (Fig. 5C–D). Through the use of linkage-specific antibodies, we also observed M1-, K27-, and K63-linked ubiquitin on cytosolic *ΔipaH1.4* bacteria in IFN γ -primed WT A549 cells (Figs. 5E, S3C–E). In contrast to IFN γ -primed WT A549 cells, IFN γ -primed RNF213^{KO} A549 cells lacked linear ubiquitylation of *ΔipaH1.4* (Fig. 5E). This ubiquitylation of *S. flexneri* *ΔipaH1.4* was independent of LUBAC, as it occurred in HOIL-1^{KO}, HOIP^{KO}, and WT A549 cells at comparable frequencies (Fig. S3F).

Although *S. flexneri* is a human-adapted pathogen, some IFN γ -inducible host defense programs targeting *S. flexneri* and the corresponding bacterial evasion mechanisms transcend host barriers. For example, the *Shigella* virulence factor IpaH9.8 inhibits both human and murine guanylate binding proteins (GBPs) (23, 39). We therefore asked whether RNF213 ubiquitylates *S. flexneri* *ΔipaH1.4* in at least one other mammalian species, the mouse. To do so, we infected unprimed and IFN γ -primed WT and Rnf213^{KO} mouse embryonic fibroblasts (MEFs, Fig. S4A) with *S. flexneri* WT and *ΔipaH1.4* mutant. We observed that *ΔipaH1.4* bacteria became decorated with mouse Rnf213 (Fig. S4B) and linear ubiquitin chains (Fig. S4C). The IFN γ -dependent increase in the percentage of M1-ubiquitin decorated *ΔipaH1.4* *S. flexneri* bacteria was reversed in Rnf213^{KO} MEFs (Fig. S3C). Collectively, these data depict a conserved role for the mammalian ubiquitin E3 ligase RNF213 in cytosolic immune surveillance and demonstrate that IpaH1.4 can evade both mouse and human RNF213-driven linear ubiquitylation.

Ubiquitylated microbial cargo can be delivered into microbicidal lysosomes in an autophagy-like process commonly referred to as xenophagy (40). Previous work showed that linear ubiquitylation of *S. enterica* leads to bacterial clearance by xenophagy (3). In contrast to the xenophagic killing of ubiquitylated *S. enterica*, we found that ubiquitylated *S. flexneri* *ΔipaH1.4* and *ΔmxlE* mutant bacteria continued to replicate inside IFN γ -primed A549 cells (Fig. 5H), reminiscent of the continued growth of ubiquitylated *B. thailandensis* *tssM* mutants inside the host cell cytosol of human IFN γ -primed cells (10). Thus, our data indicate that *S. flexneri*, like *B. thailandensis*, can block ubiquitin-dependent xenophagy both at the stage of ubiquitylation and at another so far undetermined downstream step of this cell-autonomous host defense pathway.

Discussion

Xenophagy is a cell-autonomous defense program in which intracellular pathogens are captured and delivered into degradative lysosomes. Ubiquitylation of intracellular pathogens is the most common initiating step for xenophagy (40). Like other steps of the xenophagy cascade, pathogen ubiquitylation is augmented in cells primed with the lymphocyte-derived cytokine IFN γ (2, 4, 7, 41, 42). An antimicrobial E3 ubiquitin ligase, RNF213, is itself an IFN γ -inducible protein (2–7). Here, we report that the enteric human pathogen *S. flexneri* employs the T3SS effector IpaH1.4 to induce the proteasomal degradation of RNF213. Accordingly, loss of IpaH1.4 results in RNF213-dependent ubiquitylation of *S. flexneri*. However, IpaH1.4-deficient *S. flexneri* mutants, despite their ubiquitylation, continue to replicate inside the cytosol of IFN γ -primed human cells. These observations suggest that *S. flexneri* not only evades RNF213-driven ubiquitylation but also the downstream degradation of ubiquitylated bacteria.

Accompanying previous reports on *Chlamydia* and *Burkholderia* (4 [↗](#), 10 [↗](#)), our study on *Shigella* provides evidence that host-adapted intracellular pathogens escape from RNF213-driven host defense. Each of these pathogens evolved a mechanistically distinct strategy to escape ubiquitylation by RNF213. The soil-dwelling accidental pathogen *B. thailandensis* is recognized and ubiquitylated by RNF213 but instantly removes ubiquitin from its surface through the activity of its secreted ubiquitin esterase TssM (10 [↗](#)). The human pathogen *Chlamydia trachomatis* decorates its surrounding vacuole with the secreted T3SS effector Gard, which renders the *Chlamydia*-containing vacuole invisible to RNF213 (4 [↗](#)). In contrast to TssM and Gard, the *Shigella* effector IpaH1.4 is a direct inhibitor of RNF213, which is further supported by recently published biochemical studies demonstrating direct interactions between the LRR domain of IpaH1.4 and the RING domain of RNF213 (36 [↗](#), 37 [↗](#)).

IpaH1.4 and IpaH2.5 have nearly 99% sequence identity in their substrate-binding LRR domains and overexpression of either paralog is sufficient to trigger RNF213 degradation. However, only IpaH1.4 appears to be essential for *S. flexneri*-triggered RNF213 degradation – as shown in the present work – or for HOIP degradation (28 [↗](#)). While these observations agree with a previous report demonstrating low expression of IpaH2.5 under standard culture conditions (38 [↗](#)), it remains to be seen whether IpaH2.5 is expressed and possibly redundant with IpaH1.4 for virulence during *in vivo* infections.

The mechanism by which IpaH1.4 and its paralog IpaH2.5 promote RNF213 degradation can be gleaned from previous studies characterizing IpaH1.4 and IpaH2.5 as ubiquitin E3 ligases that directly conjugate K48-linked ubiquitin to the LUBAC subunit HOIP leading to HOIP degradation by the proteasome (28 [↗](#)). Indeed, studies published during the revisions of our manuscript show that IpaH1.4 and IpaH2.5 similarly bind to and directly ubiquitylate RNF213 to facilitate RNF213's proteasomal degradation (36 [↗](#), 37 [↗](#)). Such direct interactions are additionally supported by our data showing that transient IpaH2.5-RNF213 interactions can be trapped via formation of covalent bonds between these proteins in a UBAIT assay.

The deletion of *ipaH1.4* leads to *S. flexneri* ubiquitylation in IFN γ -primed host cells. Different ubiquitin linkage types and their incorporation into heterotypic conjugates create a rich diversity of ubiquitin modifications with unique functional outputs, which is commonly referred to as the 'ubiquitin code.' (43 [↗](#), 44 [↗](#)). We found that at least three linkage types are attached to *S. flexneri* Δ *ipaH1.4* in an RNF213-dependent manner: K27, K63, and linear (M1). Linear ubiquitylation is a head-to-tail inter-ubiquitin linkage through the amino group of the N-terminal methionine (M1) and the C-terminal carboxyl group (45 [↗](#)). The LUBAC complex is the only recognized ubiquitin E3 ligase able to conjugate M1-linked ubiquitin chains (46 [↗](#), 47 [↗](#)). However, we observed that LUBAC is dispensable for M1-linked ubiquitylation of cytosolic *S. flexneri* Δ *ipaH1.4*. We found that lysine-less internally tagged ubiquitin or an M1-specific antibody bound to *S. flexneri* Δ *ipaH1.4* in cells lacking LUBAC (HOIL-1^{KO} or HOIP^{KO}) but failed to bind bacteria in RNF213-deficient cells. These data corroborate our previous observations, which suggested that IFN γ -induced linear ubiquitylation of *Chlamydia*- and *Toxoplasma*-containing vacuoles occur independent of LUBAC in human cells (2 [↗](#), 4 [↗](#)). While RNF213 itself may catalyze such linear ubiquitin conjugation, it is equally possible that this reaction is carried out by additional ubiquitin E3 ligases that are being recruited to the bacterial surface alongside RNF213.

It was previously reported that RNF213 ubiquitylates LPS on the surface of *S. enterica* prompting the recruitment of LUBAC, which catalyzes the conjugation of linear ubiquitin to the surface of *S. enterica* (3 [↗](#)). Whereas LUBAC was found to be essential for M1-linked ubiquitylation of cytosolic *S. enterica* (9 [↗](#)), we find that LUBAC is dispensable for M1-linked ubiquitylation of *S. flexneri* Δ *ipaH1.4* bacteria. While differences between *S. enterica* and *S. flexneri*, for example in the composition of their bacterial cell walls, could possibly contribute to these discrepant results, it is also important to note that the previous *S. enterica* infection studies were conducted in naïve cells (3 [↗](#), 9 [↗](#)), whereas our studies were performed under IFN γ priming conditions. We therefore

propose that a boost in the expression of RNF213 and other IFN-stimulated genes (ISGs) renders LUBAC obsolete for M1-linked ubiquitylation of Gram-negative bacteria. Future studies are needed to determine how RNF213 co-operates with LUBAC and other host factors to ubiquitin-tag intracellular pathogens.

Shigella is a human adapted pathogen that secretes T3SS effectors to directly interfere with type I and III IFN signaling in epithelial cells (48 [↗](#)). Additionally, *Shigella* employs virulence factors that inhibits ISGs which act downstream from IFN signaling. For example, the T3SS effector IpaH9.8 blocks the activity of IFN γ -inducible antimicrobial ISGs such as GBP1 (20 [↗](#)–23 [↗](#)). Here we report that IpaH1.4 inactivates another ISG, *i.e.* RNF213. Because ubiquitylated *S. flexneri* Δ *IpaH1.4* and Δ *mxiE* mutants continue to replicate inside the cytosol of IFN γ -primed host cells, we propose that *S. flexneri* also uses a *mxiE*-independent T3SS effector to interfere with host defenses occurring downstream from RNF213. Collectively, these data indicate that *Shigella* is equipped with redundant counter-immune mechanisms. This type of bacterial effector redundancy is not unique to *Shigella*. It was recently shown that the rodent pathogen *Citrobacter rodentium* can tolerate various combinations of individual T3SS effector deletions *in vivo*, an observation that led to the ‘T3SS effector networks’ hypothesis (49 [↗](#), 50 [↗](#)). Further characterization of the *Shigella* T3SS effector network that renders bacteria resistant to IFN γ -inducible cell-autonomous immune program will provide a deeper understanding of the pathogenesis of this important human enteric pathogen. Given the rise of extensively drug-resistant *Shigella* strains (51 [↗](#)–54 [↗](#)), such an understanding may prove to be essential for the development of critically needed novel therapeutic approaches to treat Shigellosis.

Materials and methods

Cell culture

A549 (ATCC #CCL-185), HT-29 (ATCC #HTB-38), and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 1% MEM non-essential aminoacids (Gibco), 55 μ M 2-Mercaptoethanol (Gibco) and 10% heat inactivated fetal bovine-serum. MEFs were generated from E12-E14 embryos as previously described (55 [↗](#)). Cells were grown at 37°C in 5% CO $_2$. Cell lines were authenticated using GenePrint10 (Promega) by the Duke University DNA Analysis Facility. All cell lines were routinely tested for mycoplasma.

Knock out cell lines and mammalian expression systems

The production and characterization of A549 knockout cell lines (pooled RNF213KO, HOIP^{KO} clone and HOIL-1^{KO} clone) was previously reported (2 [↗](#), 4 [↗](#)). Various GFP-tagged IpaH expression constructs including the IpaH1.4 and IpaH2.5 C/S mutants were previously described (28 [↗](#)). Internally Strep-tagged ubiquitin (INT-Ub) and Internally Strep-tagged & N-Terminally HA-tagged ubiquitin (NTerm-HA-Ub_ INT-Strep) open reading frames were cloned via Gateway cloning into the lentiviral expression plasmid pLEX307, a gift from Dr. David Root (Addgene plasmid # 41392; <http://n2t.net/addgene:41392> [↗](#); RRID: Addgene_41392). DNA of full length human RNF213 with N-terminal mCherry was a gift from Daisuke Morito (56 [↗](#)). RNF213 was amplified in segments using PCR and cloned using Golden Gate assembly with the enzyme kit BsmBI-v2 (New England Biolabs). Endogenous BsmBI sites were removed from RNF213 by introducing silent mutations in primers used to add enzyme overhangs for Golden Gate assembly. RNF213 was first cloned into a modified Gateway donor vector pDONR221 (Thermo Fisher Scientific) containing a golden gate cloning site in between ATTP sites to make pENTR-mCherry-RNF213. mCherry-RNF213 was transferred into piggybac vector PB-TA-ERN using LR clonase enzyme (Thermo Fisher Scientific) for dox-inducible expression of mCherry-RNF213. PB-TA-ERN (57 [↗](#)) was a gift from Knut Woltjen (Addgene plasmid #80474; <http://n2t.net/addgene:80474> [↗](#); RRID: Addgene_80474). Whole plasmid sequencing of PB-TA-ERN-mCherry-RNF213 and the INT-Ub vectors was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation. The INT-Ub protein sequences are

provided in [table 1](#) in supplemental material. For mCherry-RNF213 expression in human cells, 293T cells (ATCC CRL-3216) were transfected with PB-TA-ERN and piggybac transposase (pRP[Exp]-EGFP-EF1A>hyPBase, VectorBuilder) using lipofectamine 2000 transfection reagent (Thermo Fisher Scientific), according to manufacturer's instructions. Cells were grown for approximately 8 days to allow for loss of the transiently transfected vectors, while mCherry-RNF213 that had integrated into the 293T genome was being maintained.

Production of Rnf213 knockout mouse and derived cells

RNF213 knockout mice were generated using a modified iGONAD methodology ([58](#)–[60](#)). Briefly, 1-day time mated, vaginal-plug-positive 8 – 24-week-old C57BL/6J female mice were put under isoflurane anesthesia. A dorsal surgical incision was made, and the ovary/oviduct was exposed. The oviduct was injected with a 0.5 μ L Cas9/CRISPR mix which included 6.1 μ M Cas9 protein (IDT, Cat. #1081058), 30 μ M sgRNAs (IDT, Alt-R CRISPR-Cas9 sgRNA), and 0.02% Fast Green FCF (VWR, Cat. #AAA16520-14) in Opti-MEM (Thermo Fisher Scientific, Cat. #11058021). The 30 μ M sgRNA concentration included the following two sgRNAs targeting exon 28 of Rnf213: 5'-TTAAATACTGGTAAGGTCGT(TGG)-3' and 5'-AGTCGGAGTAGCAAAATCCC(TGG)-3'. After the Cas9/sgRNA mix injection, the oviducts were covered with tissue and then electroporated using a CUY21EDIT II (BEX Co., Ltd.) electroporator with the settings: Square mA mode, Pd V: 60 V, Pd A: 100 mA, Pd on: 5 ms, Pd off: 50 ms, Pd N: 3, and Decay: 10%. This procedure was done on both set of oviducts in the mouse. After surgery, female mice were housed together and at 19-21 days post-surgery monitored for pup birth. Pups were genotyped and three founder mice with out-of-frame 101 bp, 284 bp, or 407 bp deletions within exon 28, respectively, were selected and bred to homozygosity. Loss of Rnf213 protein expression in cells derived from these knockout mice was validated by Western blotting.

Lentivirus production

Plasmids were transfected into HEK 293T cells using the TransIT 293 transfection reagent (Mirus). Wells were transfected with 1 μ g plasmid, 750 ng pGAGpol, 250 ng VSVG. 6 hours post transfection, media was aspirated and replaced with fresh DMEM. 24 hours post transfection, media was aspirated and replaced with pseudoparticle DMEM (3% fbs, 1% MEM non-essential aminoacids, 20mM HEPES). Supernatant was collected 48 and 72 hours after transfection. Supernatants containing lentivirus were filtered using 0.45 μ m filters (corning) and later mixed with 10 μ g/ml polybrene (Millipore Sigma). Virus was frozen at -80° C until use. For cell transduction, A549 cells were diluted to a concentration of 3.33×10^4 cells/ml and mixed with 10 μ g/ml polybrene. 500 μ L of lentivirus was added to each well of a 6 well plate, and 1.5 ml of diluted cells were added on top. Cells were selected in media with 1 μ g/ml of puromycin and expanded.

IpaH mini-screen

HEK 293T that express PiggyBac RNF213 were seeded at 10^5 cells/well. Sixteen hours before transfection, aTc was added to cells to induce expression of RNF213. The next morning, 2.5 μ g of IpaH effector-encoding plasmids were used to transfect HEK 293T using the TransIT 293 transfection reagent (Mirus). 24 hours after transfection, cells were lysed in RIPA buffer and prepared for immunoblotting, as described ([20](#)).

Bacterial strains

All *S. flexneri* strains were grown on Tryptic Soy Broth (TSB, Millipore Sigma) agar plates in presence of 0.01% of Congo Red (Millipore Sigma) and red colonies were cultured in TSB broth culture. *S. flexneri* 2457T wildtype and mutant strains (Δ *mxiE* and Δ *ipaH1.4*) were previously described ([20](#)). *S. flexneri* M90T strains (wildtype, Δ *ipaH2.5*, Δ *ipaH1.4* and Δ *ipaH1.4-ipaH2.5*) were also previously reported ([28](#)). For immunofluorescence studies, we used fluorescent *S. flexneri* strains carrying the pGFPmut2 ([61](#)) or the pJUMP45-2A (sfGFP) ([62](#)) plasmids. Bacteria

INT-Ub Construct	Amino acid sequence (ORF)
WT	MQIFV ~~K~~ TLTG ~~K~~ TITLEVEPSDTIENV ~~K~~ AKIQD ~~K~~ EGIPPDQQLIFAG ~~K~~ QLEDGS AWSHPPQFEKSATLSDYNIQ ~~K~~ ESTLHLVLRRLGG
7KR	MQIFVRTLTLTGRTITLEVEPSDTIENVRAIRIQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG
6KR+K6	MQIFV ~~K~~ TLTGRTITLEVEPSDTIENVRAIRIQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG*
6KR+K11	MQIFVRTLTLTG ~~K~~ TITLEVEPSDTIENVRAIRIQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG*
6KR+K27	MQIFVRTLTLTGRTITLEVEPSDTIENV ~~K~~ ARIQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG*
6KR+K29	MQIFVRTLTLTGRTITLEVEPSDTIENVRA ~~K~~ IQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG*
6KR+K33	MQIFVRTLTLTGRTITLEVEPSDTIENVRAIRIQD ~~K~~ EGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG*
6KR+K48	MQIFVRTLTLTGRTITLEVEPSDTIENVRAIRIQDREGIPPDQQLIFAG ~~K~~ QLEDGS AWSHPPQFEKSATLSDYNIQRESTLHLVLRRLGG*
6KR+K63	MQIFVRTLTLTGRTITLEVEPSDTIENVRAIRIQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQ ~~K~~ ESTLHLVLRRLGG*
5KR+K27-63	MQIFVRTLTLTGRTITLEVEPSDTIENV ~~K~~ ARIQDREGIPPDQQLIFAGRQLEDGS AWSHPPQFEKSATLSDYNIQ ~~K~~ ESTLHLVLRRLGG*

Table 1

Internally STREP-tagged Ubiquitin (INT-Ub) constructs.

Ubiquitin lysines are shown in red. Strep tag sequence in highlighted in green

were transformed with the ilux pGEXO plasmid (Addgene plasmid #107879) (63 [DOI](#)) to generate bioluminescent bacteria, as previously reported (64 [DOI](#)). Where specified, *S. flexneri* strains were used that harbor the pILT plasmid which encodes an adhesin (65 [DOI](#)) and enhances bacterial adhesion to host cells. To complement the $\Delta ipaH1.4$ strain, full length IpaH1.4 and its catalytically inactive variant C368S were cloned into the bacterial expression vector pDSW206 using Takara In-Fusion Snap Assembly Master Mix (Cat# 638948) following the manufacturer's instructions. Bacterial transformation was performed as previously described (20 [DOI](#)). Antibiotics were used as needed at the following concentration: carbenicillin (50 ug/ml), kanamycin (50 ug/ml), spectinomycin (50ug/ml), and chloramphenicol (100 ug/ml).

Infection procedures

For immunofluorescence staining A549, HT29, and MEF cells were seeded at 1×10^5 cells per well on cover slips in 24-well plates. HT29 cells were plated at 2×10^5 cells per well. For Western blotting cells were plated at 2×10^5 cells per well in 24-well plates. Cells were either left untreated or treated overnight with human or mouse cytokine IFN γ at a final concentration of 100 U/ml, as indicated. The day before infection, *S. flexneri* was inoculated into TSB with antibiotics as needed and grown overnight at 37°C with aeration. The next day bacteria were diluted (1:20) in TSB with antibiotics and grown for 1h 30 minutes. To induce the expression of WT and C368S mutant IpaH1.4 in complemented $\Delta ipaH1.4$ bacteria, 1mM IPTG (Invitrogen 15529-019) was added 1 hour after subculturing. Subsequently, the optical density of the broth culture was measured and pelleted bacteria were washed in sterile PBS and resuspended in DMEM. Infections were carried out at an approximate multiplicity of infection (MOI) of 50 to 100, unless otherwise specified. Cells were centrifuged at 1000 g for 10 minutes at the time of infection and infected cells were incubated at 37°C with 5% CO $_2$. Cells were washed with warm HBSS two times after 90 minutes (A549 and HT29 cells) or 60 minutes (MEFs) post infection. Following these washes gentamicin at a final concentration of 50 ug/ml (for A549 and MEFs) or 100 ug/ml (for HT29) was added to the media. For fluorescence microscopy infected cells were fixed with 4%PFA in phosphate-buffered saline (PBS) at 4 hpi (A549 cells), 3 hpi (HT29), or 2 hpi (MEFs). All *S. flexneri* strains carried the plasmid pGFPmut2 to visualize bacteria by fluorescence microscopy. For Western blotting, bioluminescence experiments, and MEF infections, *S. flexneri* strains expressed pILT for improved adhesion and infection rates.

Immunofluorescence microscopy

Cells were fixed in 4% PFA in PBS and then permeabilized either with 0.1%triton in PBS for 15 minutes or with ice-cold methanol for 1 minute followed by 0.05% saponin in blocking buffer. Blocking buffer was composed of 5% BSA, 2.2% glycine +/-0.05% saponin or 10% goat serum and 0.05 % saponin in PBS. Following permeabilization, coverslips were blocked for 30 minutes at room temperature (RT). Next, coverslips were incubated with the respective primary antibody diluted in blocking buffer overnight at 4°C. The following primary antibodies were used: rabbit monoclonal anti-RNF213 (Sigma HPA026790; 1:1000), rabbit monoclonal anti-linear (M1) ubiquitin; clone 1E3 (Sigma ZRB2114; 1:250), mouse polyclonal anti-ubiquitin FK2 (Cayman Chemical 14220; 1:100), rabbit monoclonal anti-K63 ubiquitin, clone Apu3 (Sigma 05-1308. 1:100), rabbit monoclonal anti K27 (Abcam ab181537, 1:100), mouse monoclonal anti-Strep (Genscript A01732, 1:200), custom rabbit anti-mRnf213 antibody from an animal immunized with peptides RKSNEGNTQPEDQRKPGEGR (aa296-316) and KDTVEYEFIYEQAQKKGE (aa429-446) (Life Technologies, 1:100). Coverslips were then washed three times with 0.05% Triton in PBS or 0.05% saponin and incubated with an Alexa Fluor-conjugated secondary antibody (1:1000, Invitrogen) and nuclear dye Hoechst 33258 (2ug/ml; Invitrogen) for 1 hour at RT. Next, coverslips were washed three times with 0.05% Triton or 0.05% saponin in PBS and mounted on glass slides using a mix of with mowiol 4-88 combined with para-phenylenediamine antifading agent (9:1). Coverslips were imaged at a Zeiss Axio Imager microscope with Apotome 3 using a 63 $\times$ /1.4 NA Oil immersion lens. Two to eleven fields of view were captured per coverslip to account for at least 100 bacteria, and z

slices with an interval of 0.5 μm were taken per field of view. Images were processed on the software program Fiji. Bacteria that had protein signal surrounding at least 50% of its visible surface were considered as targeted.

Bacterial luminescence assay

Infections were conducted with bacterial strains carrying the *pilT* plasmid to promote cell adhesion and the *ilux* plasmid as a bioluminescence reporter. Host cells were seeded in 96-well plates at a density of 2.5×10^4 cells per well. An approximate MOI of 5 in an infection volume of 100 μl per well was used. Twenty-five minutes post infection, wells were washed once with 200 μl of warm HBSS and then fresh DMEM containing 50 $\mu\text{g}/\text{ml}$ gentamicin was added to each well. Bacterial bioluminescence was measured on a multimode microplate reader (Biotek Synergy H1, Agilent) every 30 minutes, starting at 1 hpi and finishing at 8 hpi.

Western blotting

After infection, cells were washed two times with cold HBSS and lysed in RIPA buffer (Sigma) in the presence of 4U/ml DNase I (NEB) and protease inhibitor cocktail (Sigma). Lysates were incubated at 4°C for 30 min and centrifuged at 20,000g for 12 minutes at 4°C. Protein concentrations were measured using the BCA kit (ThermoFisher) and normalized. Normalized samples were mixed with Laemmli buffer that contained 5% β -mercaptoethanol and heated at 95°C for 10 minutes. Samples were heated at 56°C for 10 minutes when samples were prepared for RNF213 immunoblotting. Ten to thirty μg of protein sample were resolved on a 4-20% Mini-Protean TGX stain-free gels (Biorad) and then blotted onto a PVDF membrane using a tank transfer system overnight at 4°C. After the transfer, PVDF membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20 (Sigma-Aldrich) (TBS-T) for 1 hour at RT. Subsequently, membranes were incubated with primary antibody diluted in blocking solution overnight at 4°C. After primary antibody incubation, blots were washed three times with TBST and incubated with the respective secondary antibody in blocking solution for 1 hour at RT. Afterwards, membranes were washed with TBS-T and developed with Clarity ECL (Cytiva). Blots were imaged on an Azure 500 visualization system. Primary antibodies used in this study include rabbit monoclonal anti-RNF213 (Sigma HPA026790 1:2000), rabbit polyclonal anti-GAPDH (Abcam ab9845; 1:10000); mouse monoclonal anti-HOIL-1, clone E3E (Sigma MABC576; 1:500); custom rabbit anti-mRnf213 (Life Technologies, 1:500); rabbit polyclonal anti-HOIP (Abcam ab46322; 1:500); monoclonal Anti- β -Actin (Sigma A2228, 1:6000). Images were processed in Fiji.

UBAIT Substrate Capture Assays

UBAIT assays were performed essentially as described before (32). Briefly, unknown substrates were captured from colon and cecum lysates from 6-8 weeks old wild type C57BL/6 (Charles River) mice. All mouse infection experiments conducted were in accordance with the policies of the Institutional Animal Care and Use Committee at UT Southwestern. Mice were orally infected by gavage with 1×10^9 *Salmonella enterica* Typhimurium S1344 WT and after 4 days mice were euthanized and colon and cecum were removed, washed and homogenized in Lysis Buffer (1x Ubiquitination buffer (Boston Biochem), 1x protease inhibitor (Sigma), 1mM DTT). Homogenates were then centrifuged at 13,000g at 4°C for 10 minutes and supernatants were added to UBAIT reactions. The supernatant were incubated with 25 μg of purified Strep-IpaH2.5-3xFLAG-Ub or Strep-IpaH3-3xFLAG-Ub and rotated at 4°C. After 1 hour, His-UbE1 (100nM), His-UbcH5b (2000nM), energy regeneration buffer (Boston Biochem), MgCl (1mM) and 1 x ubiquitination buffer were added to a final volume of 500 μl . The reaction was run at 30°C for 10 minutes and then 300 μl TBS buffer (25 mM Tris-HCL pH7.4, 150 mM NaCl, 1mM DTT) and 30 μl of washed Strep-Tactin Superflow beads (Iba LifeSciences) were added and rotated for 2 hours at 4°C. The beads were washed 4 times in TBS-T (TBS+ 0.5% Triton-X100) and 2 times in TBS. Strep-IpaH2.5-3xFLAG-Ub was eluted from the beads in 150 μl Strep-tag elution buffer (Iba LifeSciences). Eluted sample and beads were centrifuged, and supernatant moved to a new tube. SDS (0.25% final concentration)

and DTT (5mM final concentration) were added, and samples were heated at 95°C for 5 minutes. Next, 1.2 ml TBS was added, followed by 20 µl of M2-FLAG beads (Sigma). The mixture was rotated for 2 hours at 4°C. M2-FLAG beads were washed 4 times in TBS-T followed by 2 times in TBS. Finally, 35 µl of hot (95°C) SDS-PAGE Loading Buffer was added and the beads were heated for an additional 5 minutes at 95°C. Samples were electrophoresed on a gradient precast SDS-PAGE gel (Biorad) and Coomassie stained. A lane of gel above the band with unmodified Strep-IpaH2.5-3xFLAG-Ub or Strep-IpaH3-3xFLAG-Ub was excised, and proteins were digested overnight with trypsin (Pierce) following reduction and alkylation with DTT and iodoacetamide (Sigma–Aldrich). Following solid-phase extraction cleanup with an Oasis HLB µelution plate (Waters), the resulting peptides were reconstituted in 10 µL of 2% (v/v) acetonitrile (ACN) and 0.1% trifluoroacetic acid in water. Two µL of each sample were injected onto an Orbitrap Fusion Lumos (Thermo) mass spectrometer, coupled to an Ultimate 3000 RSLC-Nano liquid chromatography systems (Thermo) at the Mass Spectrometry Core at University of Texas Southwestern. Samples were injected onto a 75 µm i.d., 75-cm long, (Lumos) EasySpray column (Thermo), and eluted with a gradient from 0-28% buffer B over 90 minutes. Buffer A contained 2% (v/v) ACN and 0.1% formic acid in water, and buffer B contained 80% (v/v) ACN, 10% (v/v) trifluoroethanol, and 0.1% formic acid in water. The Orbitrap Fusion Lumos mass spectrometer operated in positive ion mode with a source voltage of 2.0-2.4 kV and an ion transfer tube temperature of 275 °C. MS scans were acquired at 120,000 resolution in the Orbitrap and up to 10 MS/MS spectra were obtained in the Orbitrap for each full spectrum acquired using higher-energy collisional dissociation (HCD) for ions with charges 2-7. Dynamic exclusion was set for 25 seconds after an ion was selected for fragmentation. Raw MS data files were analyzed using Proteome Discoverer v.2.4 (Thermo), with peptide identification performed using Sequest HT searching against the human reviewed protein database from UniProt. Fragment and precursor tolerances of 10 ppm and 0.6 Da (Lumos) was specified, and three missed cleavages were allowed. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was set as a variable modification. The false-discovery rate (FDR) cutoff was 1% for all peptides. The abundance of identified peptides found for Strep-IpaH2.5 UBAIT were compared to those found for control IpaH3 UBAIT that was included in the same experiment. Hits for IpaH2.5 were included when abundance levels were at least 2-fold higher than UBAIT controls.

Statistical analysis

Graphpad Prism 10.2.2 was used to generate graphs and to perform statistical analyzes. Experiments were conducted in three to five biological replicates. Data in graphs are displayed as mean ± standard error of the mean (SEM). Statistical analyses included student t-test one-way and two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Significance was considered when p values were <0.05

Data storage

A large UBAIT mass spectrometry dataset broadly probing for IpaH protein family interaction partners was originally generated and published in (28 [DOI](#)). The numerical values of all other quantified data depicted in data panels in this manuscript are openly available in the Digital Repositories at Duke under the digital object identifier doi.org/10.7924/r4vq36v59.

Supplementary figure legends

Fig. S1

Addition of an N-terminal HA-tag significantly reduces targeting of 7KR ubiquitin to *S. flexneri* Δ *mxjE*.

N-terminally untagged and N-terminally HA-tagged versions of the internally Strep-tagged Ubiquitin (INT-Ub) 7KR mutant variant were expressed in A549 cells. Cells were primed with 100 U/ml IFN γ overnight and co-localization of INT-Ub with cytosolic *S. flexneri* (Sf) Δ *mxjE* was assessed at 4 hpi. An unpaired t-test was conducted. **p<0.01

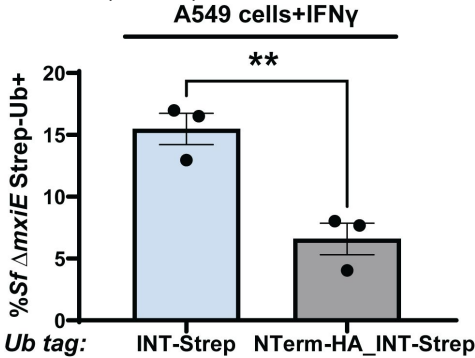
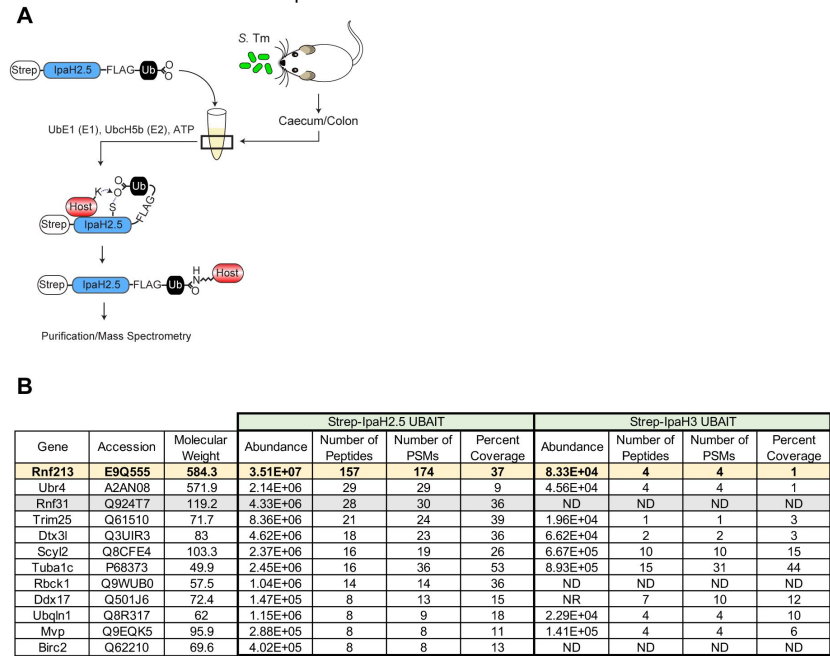


Fig. S2

Ubiquitin-Activated Interaction Trap (UBAIT) identifies mouse Rnf213 as likely interaction partner of IpaH2.5.

(A) Diagram showing the experimental procedure for UBAIT detection of IpaH2.5 and IpaH3 (control) substrates from the colon and caecum of mice infected with *Salmonella enterica* Typhimurium (*S. Tm*). (B) Mass spectrometry of hits identified in the Strep-IpaH2.5 UBAIT (left) as compared to Strep-IpaH3 UBAIT (right). The top 12 proteins identified in the Strep-IpaH2.5 UBAIT samples as determined by peptide abundance are shown. The corresponding peptide abundances from Strep-IpaH3 UBAIT are shown. RNF213 and the previously identified IpaH2.5 substrate RNF31 (HOIP) are highlighted. PSM = peptide spectrum match. ND = Not Detected. NR = Not Reported.



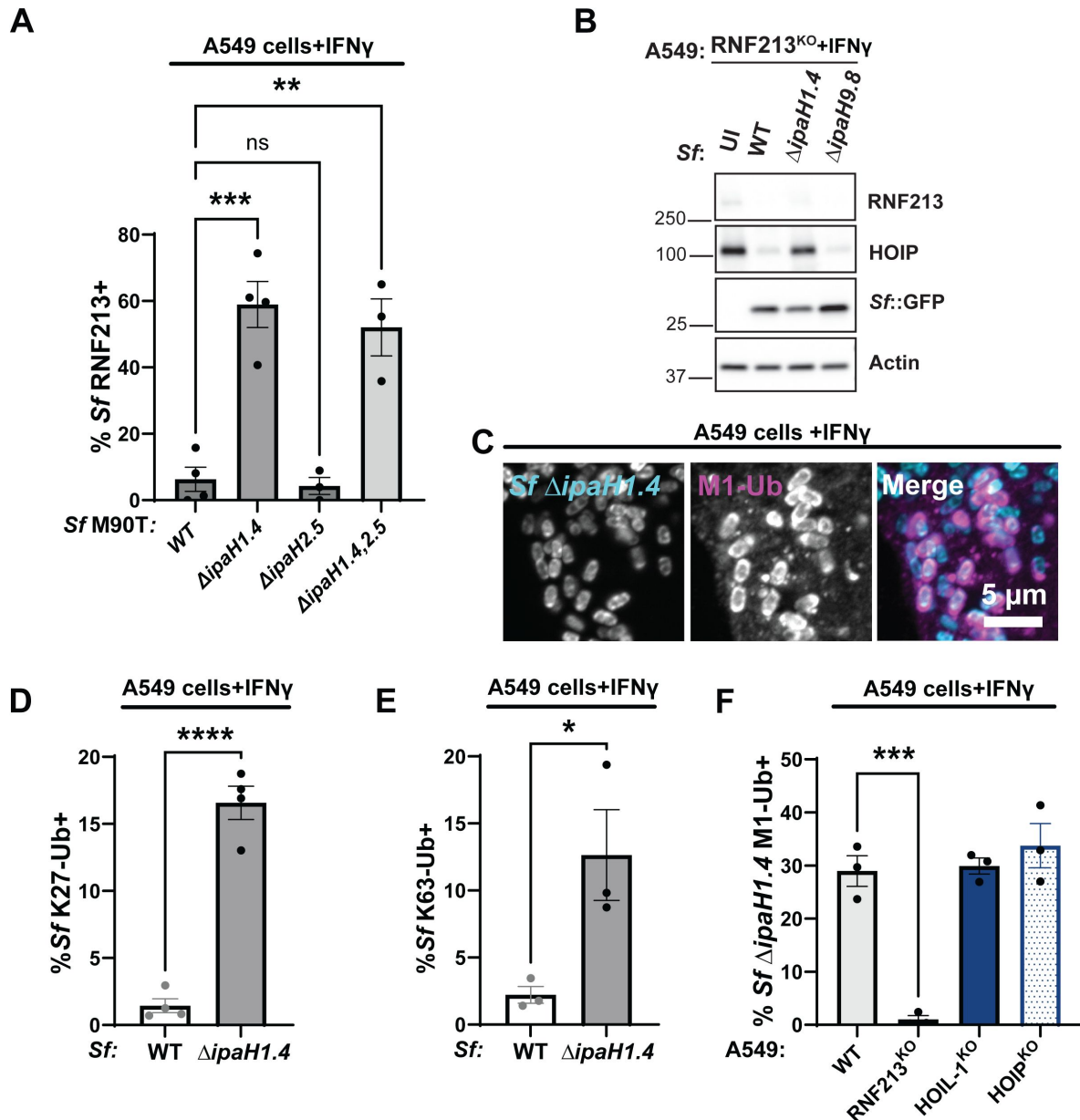


Fig. S3

Deletion of *ipaH1.4* but not *ipaH2.5* results in RNF213 recruitment to cytosolic *S. flexneri*.

(A) A549 cells were primed with IFN γ (100 U/ml) and infected with the indicated strains expressing GFP for 4 hours at an MOI of 100. RNF213 recruitment to cytosolic bacteria was quantified. (B) IFN γ -primed A549 RNF213^{KO} cells were infected with the indicated *PilT*⁺ *S. flexneri* strains for 3 hours at an MOI of 25 and HOIP protein levels were assessed by Western blotting. (C) Representative microscopy images showing anti-M1-linked ubiquitin staining of Δ ipaH1.4 *S. flexneri* bacteria in IFN γ -primed WT A549 cells. Quantified data are shown in Fig. 5E. Percentages of WT and Δ ipaH1.4 *S. flexneri* bacteria stained with anti-K27-linked ubiquitin (D) or anti-K63-linked ubiquitin (E) in IFN γ -primed WT A549 cells. (F) Percentages of anti-M1-linked ubiquitin positive Δ ipaH1.4 *S. flexneri* bacteria in IFN γ -primed WT, RNF213^{KO}, HOIL-1^{KO} and HOIP^{KO} A549 cells. Data are represented by the mean $\pm$ SEM from three independent experiments. An unpaired t-test was conducted for (D, E). Data are represented by the mean $\pm$ SEM from three independent experiments. Two-way ANOVA with Tukey's multiple comparison tests were performed for (A, F); all statistically significant comparisons are shown. **** p<0.0001; *** p<0.001; ** p<0.01; ns, not significant

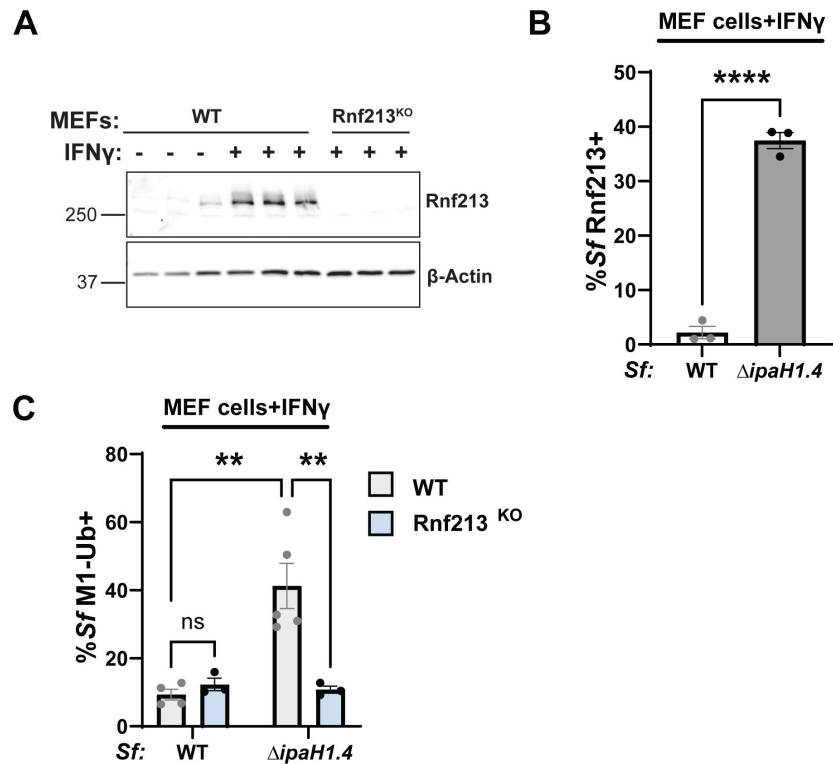


Fig. S4

Mouse Rnf213 binds to Δ ipaH1.4 *S. flexneri* and facilitates linear ubiquitylation of cytosolic bacteria

(A) Immunoblotting for mouse Rnf213 protein expression in untreated and IFN γ -primed WT and IFN γ -primed Rnf213^{KO} MEF cells. Each lane represents MEFs from separate mouse embryos that were used for infections. (B and C) MEF cells were primed with mouse IFN γ (100 U/ml) and infected with the indicated strains expressing GFP and PilT for 2 hours at an MOI of 100. Rnf213-*S. flexneri* colocalization percentages were quantified and are shown in (B). (C) Percentage of M1-linked ubiquitin positive *S. flexneri* in IFN γ -primed WT and Rnf213^{KO} MEF cells. All data are represented by the mean $\pm$ SEM from at least three independent experiments. An unpaired t-test was performed in (B). Two-way ANOVA with Tukey's multiple comparison tests were performed in (C). **p<0.01; ****p<0.0001

Acknowledgements

This work was supported by National Institutes of Health grants AI139425 (to JC), AI083359 (to NMA), AI137031 (to NSH), and AI168107 (to NSH); and the Welch Foundation (grant I-1704 to NMA). NSH holds an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. We would like to thank members of the Coers lab as well as the labs of Drs. Clare Smith, David Tobin, and Edward Miao for providing valuable feedback.

Additional information

Funding

National Institute of Health, U.S. (AI139425)

National Institute of Health, U.S. (AI083359)

National Institute of Health, U.S. (AI137031)

National Institute of Health, U.S. (AI168107)

Welch Foundation (I-1704)

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Author information

Luz Saavedra-Sanchez

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States

Mary S Dickinson

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States

Shruti Apte

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States

Yifeng Zhang

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States

Maarten de Jong

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, United States
ORCID iD: [0000-0003-2278-286X](#)

Samantha Skavicus

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States

Nicholas S Heaton

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, United States

Neal M Alto

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, United States
ORCID iD: [0000-0002-7602-3853](#)

Jörn Coers

Department of Molecular Genetics and Microbiology, Duke University Medical Center,
Durham, United States, Department of Integrative Immunobiology, Duke University Medical
Center, Durham, United States
ORCID iD: [0000-0001-8707-4608](https://orcid.org/0000-0001-8707-4608)

For correspondence: jorn.coers@duke.edu

Editors

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Sophie Helaine

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Reviewer #1 (Public review):

Shigella flexneri is a bacterial pathogen that is an important globally significant cause of diarrhea. *Shigella* pathogenesis remains poorly understood. In their manuscript, Saavedra-Sanchez et al report their discovery that a secreted E3 ligase effector of *Shigella*, called IpaH1.4, mediates the degradation of a host E3 ligase called RNF213. RNF213 was previously described to mediate ubiquitylation of intracellular bacteria, an initial step in their targeting to xenophagosomes. Thus, *Shigella* IpaH1.4 appears to be an important factor to permit evasion of RNF213-mediated host defense. Strengths: The work is focused, convincing, well-performed and important, and the manuscript is well-written. The revised version addressed all the concerns raised during the initial review.

<https://doi.org/10.7554/eLife.102714.2.sa3>

Reviewer #2 (Public review):

Summary:

The authors find that the bacterial pathogen *Shigella flexneri* uses the T3SS effector IpaH1.4 to induce degradation of the IFN γ -induced protein RNF213. They show that in the absence of IpaH1.4, cytosolic *Shigella* is bound by RNF213. Furthermore, RNF213 conjugates linear and lysine-linked ubiquitin to *Shigella* independently of LUBAC. Intriguingly, they find that *Shigella* lacking ipaH1.4 or mxiE, which regulates the expression of some T3SS effectors, are not killed even when ubiquitylated by RNF213 and that these mutants are still able to replicate within the cytosol, suggesting that *Shigella* encodes additional effectors to escape from host defenses mediated by RNF213-driven ubiquitylation.

Strengths:

The authors take a variety of approaches, including host and bacterial genetics, gain-of-function and loss-of-function assays, cell biology, biochemistry, . Overall, the experiments are elegantly designed, rigorous, and convincing.

<https://doi.org/10.7554/eLife.102714.2.sa2>

Reviewer #3 (Public review):

Summary:

In this study the authors set out to investigate whether and how *Shigella* avoids cell autonomous immunity initiated through M1-linked ubiquitin and the immune sensor and E3 ligase RNF213. The key findings are that the *Shigella flexneri* T3SS effector, IpaH1.4 induces degradation of RNF213. Without IpaH1.4, the bacteria are marked with RNF213 and ubiquitin following stimulation with IFN γ . Interestingly, this is not sufficient to initiate the destruction of the bacteria, leading the authors to conclude that *Shigella* deploys additional virulence factors to avoid this host immune response. The second key finding of this study is that M1 chains decorate the mxiE/ipaH *Shigella* mutant independent of LUBAC, which is by and large, considered the only enzyme capable of generating M1-linked ubiquitin chains. These findings are fundamental in nature and of general interest.

Strengths and weaknesses:

The data is well-controlled and clearly presented with appropriate methodology. The authors provide compelling evidence that demonstrates that IpaH1.4 is the effector responsible for the degradation of RNF213 via the proteasome and their conclusions are well supported. They have clearly demonstrated how *Shigella* disarms RNF213-mediated immunity.

This work builds on prior work from the same laboratory that suggests that M1 ubiquitin chains can be formed independently of LUBAC (in the prior publication this related to *Chlamydia* inclusions). Two key pieces of evidence support this statement - fluorescence microscopy-based images and accompanying quantification in Hoip and Hoil knockout cells for association of M1-ub, using an M1 specific antibody, and the use of an internally tagged Ub-K7R mutant. Whilst it remains possible that the M1 antibody is non-specific, as acknowledged by the authors, the data in supplementary figure 1, comparing K7R-ub and the N-terminally tagged K7R ub variant, provides evidence that during *Shigella* infection, LUBAC independent M1-ubiquitin chains are indeed formed. This represents an important new angle in ubiquitin biology.

The importance of IFN γ priming for RNF213 association to the mxiE or ipaH1.4 remains an interesting question that awaits future studies that compare different intracellular bacteria and the role of RNF213.

Overall, the findings are important for the host-pathogen field, cell autonomous/innate immune signaling fields and microbial pathogenesis fields and the work is a very valuable addition to the recent advances in understanding the role of RNF213 in host immune responses to bacteria.

<https://doi.org/10.7554/eLife.102714.2.sa1>

Author response:

The following is the authors' response to the original reviews

Reviewer #1 (Public review):

Shigella flexneri is a bacterial pathogen that is an important globally significant cause of diarrhea. *Shigella* pathogenesis remains poorly understood. In their manuscript, Saavedra-Sanchez et al report their discovery that a secreted E3 ligase effector of *Shigella*, called IpaH1.4, mediates the degradation of a host E3 ligase called RNF213. RNF213 was previously described to mediate ubiquitylation of intracellular bacteria, an

initial step in their targeting of xenophagosomes. Thus, Shigella IpaH1.4 appears to be an important factor in permitting evasion of RNF213-mediated host defense.

Strengths:

The work is focused, convincing, well-performed, and important. The manuscript is well-written.

We would like to thank the reviewer for their time evaluating our manuscript and the positive assessment of the novelty and importance of our study. We provide a comprehensive response to each of the reviewer's specific recommendations below and highlight any changes made to the manuscript in response to those recommendations.

Reviewer #1 (Recommendations for the authors):

(1) In the abstract (and similarly on p.10), the authors claim to have shown "IpaH1.4 protein as a direct inhibitor of mammalian RNF213". However, they do not show the interaction is direct. This, in my opinion, would require demonstrating an interaction between purified recombinant proteins. I presume that the authors are relying on their UBAIT data to support the direct interaction, but this is a fairly artificial scenario that might be prone to indirect substrates. I would therefore prefer that the 'direct' statement be modified (or better supported with additional data). Similarly, on p.7, the section heading states "S. flexneri virulence factors IpaH1.4 and IpaH2.5 are sufficient to induce RNF213 degradation". The corresponding experiment is to show sufficiency in a 293T cell, but this leaves open the participation of additional 293T-expressed factors. So I would remove "are sufficient to", or alternatively add "...in 293T cells".

We agree with the reviewer and made the recommended changes to the text in the abstract, in the results section on page 7, and in the Discussion on page 11. During the revision of our manuscript two additional studies were published that provide convincing biochemical evidence for the direct interaction between IpaH1.4 and RNF213 (PMID: 40205224; PMID: 40164614). These studies address the reviewer's concern extensively and are now briefly discussed and cited in our revised MS.

(2) In the abstract the authors state "Linear (M1-) and lysine-linked ubiquitin is conjugated to bacteria by RNF213 independent of the linear ubiquitin chain assembly complex (LUBAC)." However, it is not shown that RNF213 is able to directly perform M1-ubiquitylation. It is shown that RNF213 is required for M1-linked ubiquitylation in IpaH1.4 or MxiE mutants, this is different than showing conjugation is done by RNF213 itself. This should be reworded.

We agree and edited the text accordingly

(3) Introduction: one of the main points of the paper is that RNF213 conjugates linear ubiquitin to the surface of bacteria in a manner independent of the previously characterized linear ubiquitin conjugation (LUBAC) complex. This is indeed an interesting result, but the introduction does not put this discovery in much context. I would suggest adding some discussion of what was known, if anything, about the type of Ub chain formed by RNF213, and specifically whether linear Ub had previously been observed or not.

We now provide context in the Introduction on page 3 and briefly discuss previous work that had implicated LUBAC in the ubiquitylation of cytosolic bacteria. We emphasize that LUBAC specifically generates linear (M1-linked) ubiquitin chains, while the types of ubiquitin

linkages deposited on bacteria through RNF213-dependent pathways had remained unidentified.

(4) Figure 3C: is the difference in 7KR-Ub between WT and HOIP KO cells significant? If so, the authors may wish to acknowledge the possibility that HOIP partially contributes to M1-Ub of MxiE mutant Shigella

The frequencies at which bacteria are decorated with 7KR-Ub is not statistically different between WT and HOIP KO cells. We have included this information in the panel description of Figure 3.

(5) On page 11, the authors state that "...we observed that LUBAC is dispensable for M1-linked ubiquitylation of cytosolic S. flexneri ΔipaH1.4. We found that lysine-less internally tagged ubiquitin or an M1-specific antibody bound to S. flexneri ΔipaH1.4 in cells lacking LUBAC (HOIL-1KO or HOIPKO) but failed to bind bacteria in RNF213-deficient cells". In fact, what is shown is that M1-ubiquitylation in ΔipaH1.4 infection is RNF213-dependent (5E), but the work with lysine mutants, HOIP or HOIL-1 KOs are all with ΔmxiE, not ΔipaH1.4 (3B) in this version of the manuscript. Ideally, the data with ΔipaH1.4 could be added, but alternatively, the conclusion could be re-worded.

We now include the data demonstrating that staining of ΔipaH1.4 with an M1-specific antibody is unchanged from WT cells in HOIL-1 KO and HOIP KO cells. These data are shown in supplementary data (Fig. S3E) and referred to on page 9 of the revised manuscript.

(6) The UBAIT experiment should be explained in a bit more detail in the text. The approach is not necessarily familiar to all readers, and the rationale for using Salmonella-infected ceca/colons is not well explained (and seems odd). Some appropriate caution about interpreting these data might also be welcome. Did HOIP or HOIL show up in the UBAIT? This perhaps also deserves some discussion.

As expected, HOIP (listed under its official gene name Rnf31 in the table of Fig.S2B) was identified as a candidate IpaH1.4 interaction partner as the third most abundant hit from the UBAIT screen. Remarkably, Rnf213 was the hit with the highest abundance in the IpaH1.4 UBAIT screen. To address the reviewer's comments, we now explain the UBAIT approach in more detail and provide the rationale for using intestinal protein lysates from *Salmonella* infected mice. The text on page 8 reads as follows: "To investigate potential physical interactions between IpaH1.4 and IpaH2.5, we reanalyzed a previously generated dataset that employed a method known as ubiquitin-activated interaction traps (UBAITs) (32). As shown in Fig. S2A, the human ubiquitin gene was fused to the 3' end of IpaH2.5, producing a C-terminal IpaH2.5-ubiquitin fusion protein. When incubated with ATP, ubiquitin-activating enzyme E1, and ubiquitin-conjugating enzyme E2, the IpaH2.5-ubiquitin "bait" protein is capable of binding to and ubiquitylating target substrates. This ubiquitylation creates an isopeptide bond between the IpaH2.5 bait and its substrate, thereby enabling purification via a Strep affinity tag incorporated into the fusion construct (32). IpaH2.5-ubiquitin bait and IpaH3-ubiquitin control proteins were incubated with lysates from murine intestinal tissue. To detect interaction partners in a physiologically relevant setting, we used intestinal lysates derived from mice infected with *Salmonella*, which in contrast to *Shigella* causes pronounced inflammation in WT mice and therefore better simulates human *Shigellosis* in an animal model. Using UBAIT we identified HOIP (Rnf31) as a likely IpaH2.5 binding partner (Fig. S2B), thus confirming previous observations (28) and validating the effectiveness of our approach. Strikingly, we identified mouse Rnf213 as the most abundant interaction partner of the IpaH2.5-ubiquitin bait protein (Fig. S2B). Collectively, our data and concurrent reports showing direct interactions between IpaH1.4 and human RNF213 (36, 37) indicate that the

virulence factors IpaH1.4 and IpaH2.5 directly bind and degrade mouse as well as human RNF213.”

(7) It would be helpful if the authors discussed their results in the context of the prior work showing IpaH1.4/2.5 mediate the degradation of HOIP. Do the authors see HOIP degradation? If indeed HOIP and RNF213 are both degraded by IpaH1.4 and IpaH2.5, are there conserved domains between RNF213 and HOIP being targeted? Or is only one the direct target? A HOIP-RNF213 interaction has previously been shown (<https://doi.org/10.1038/s41467-024-47289-2>). Since they interact, is it possible one is degraded indirectly? To help clarify this, a simple experiment would be to test if RNF213 degraded in HOIP KO cells (or vice-versa)?

We appreciate the reviewer’s suggestions. We conducted the proposed experiments and found that WT *S. flexneri* infections result in RNF213 degradation in both WT and HOIP KO cells. Similarly, we found that HOIP degradation was independent of RNF213. We have included these data in Figs. 5A and S3B of our revised submission. A study published during revisions of our paper demonstrates that the LRR of IpaH1.4 binds to the RING domains of both RNF213 and LUBAC (PMID: 40205224). We refer to this work in our revised manuscript.

Reviewer #2 (Public review):

Summary:

The authors find that the bacterial pathogen Shigella flexneri uses the T3SS effector IpaH1.4 to induce degradation of the IFNγ-induced protein RNF213. They show that in the absence of IpaH1.4, cytosolic Shigella is bound by RNF213. Furthermore, RNF213 conjugates linear and lysine-linked ubiquitin to Shigella independently of LUBAC. Intriguingly, they find that Shigella lacking ipaH1.4 or mxiE, which regulates the expression of some T3SS effectors, are not killed even when ubiquitylated by RNF213 and that these mutants are still able to replicate within the cytosol, suggesting that Shigella encodes additional effectors to escape from host defenses mediated by RNF213-driven ubiquitylation.

Strengths:

The authors take a variety of approaches, including host and bacterial genetics, gain-of-function and loss-of-function assays, cell biology, and biochemistry. Overall, the experiments are elegantly designed, rigorous, and convincing.

Weaknesses:

The authors find that ipaH1.4 mutant S. flexneri no longer degrades RNF213 and recruits RNF213 to the bacterial surface. The authors should perform genetic complementation of this mutant with WT ipaH1.4 and the catalytically inactive ipaH1.4 to confirm that ipaH1.4 catalytic activity is indeed responsible for the observed phenotype.

We would like to thank the reviewer for their time evaluating our manuscript and the positive assessment of our work, especially its scientific rigor. We conducted the experiment suggested by the reviewer and included the new data in the revised manuscript. As expected, complementation of the ΔipaH1.4 with WT IpaH1.4 but not with the catalytically dead C338S mutant restored the ability of Shigella to efficiently escape from recognition by RNF213 (Figs. 5C-D).

Reviewer #2 (Recommendations for the authors):

The authors should perform genetic complementation of the ipaH1.4 mutant with WT ipaH1.4 and the catalytically inactive ipaH1.4 to confirm that ipaH1.4 catalytic activity is indeed responsible for the observed phenotype.

We performed the suggested experiment and show in Figs. 5C-D that complementation of the Δ ipaH1.4 mutant with WT IpaH1.4 but not with the catalytically dead C338S mutant restored the ability of *Shigella* to efficiently escape from recognition by RNF213. These data demonstrate that the catalytic activity of IpaH1.4 is required for evasion of RNF213 binding to the bacteria.

Reviewer #3 (Public review):

Summary:

*In this study, the authors set out to investigate whether and how *Shigella* avoids cell-autonomous immunity initiated through M1-linked ubiquitin and the immune sensor and E3 ligase RNF213. The key findings are that the *Shigella flexneri* T3SS effector, IpaH1.4 induces degradation of RNF213. Without IpaH1.4, the bacteria are marked with RNF213 and ubiquitin following stimulation with IFN γ . Interestingly, this is not sufficient to initiate the destruction of the bacteria, leading the authors to conclude that *Shigella* deploys additional virulence factors to avoid this host immune response. The second key finding of this paper is the suggestion that M1 chains decorate the mxiE/ipaH *Shigella* mutant independent of LUBAC, which is, by and large, considered the only enzyme capable of generating M1-linked ubiquitin chains.*

Strengths:

The data is for the most part well controlled and clearly presented with appropriate methodology. The authors convincingly demonstrate that IpaH1.4 is the effector responsible for the degradation of RNF213 via the proteasome, although the site of modification is not identified.

Weaknesses:

- (1) The work builds on prior work from the same laboratory that suggests that M1 ubiquitin chains can be formed independently of LUBAC (in the prior publication this related to *Chlamydia* inclusions). In this study, two pieces of evidence support this statement -fluorescence microscopy-based images and accompanying quantification in Hoip and Hoil knockout cells for association of M1-ub, using an antibody, to *Shigella* mutants and the use of an internally tagged Ub-K7R mutant, which is unable to be incorporated into ubiquitin chains via its lysine residues. Given that clones of the M1-specific antibody are not always specific for M1 chains, and because it remains formally possible that the Int-K7R Ub can be added to the end of the chain as a chain terminator or as mono-ub, the authors should strengthen these findings relating to the claim that another E3 ligase can generate M1 chains de novo.*
- (2) The main weakness relating to the infection work is that no bacterial protein loading control is assayed in the western blots of infected cells, leaving the reader unable to determine if changes in RNF213 protein levels are the result of the absent bacterial protein (e.g. IpaH1.4) or altered infection levels.*
- (3) The importance of IFN γ priming for RNF213 association to the mxiE or ipaH1.4 strain could have been investigated further as it is unclear if RNF213 coating is enhanced due to increased protein expression of RNF213 or another factor. This is of interest as*

IFN γ priming does not seem to be needed for RNF213 to detect and coat cytosolic Salmonella.

Overall, the findings are important for the host-pathogen field, cell-autonomous/innate immune signaling fields, and microbial pathogenesis fields. If further evidence for LUBAC independent M1 ubiquitylation is achieved this would represent a significant finding.

We would like to thank the reviewer for their time evaluating our manuscript and the positive assessment of our work and its significance. We provide a comprehensive response to the main three critiques listed under ‘weaknesses’ and also have responded to each of the reviewer’s specific recommendations below. We highlight any changes made to the manuscript in response to those recommendations.

(1) As the reviewer correctly pointed out, 7KR ubiquitin cannot only be used for linear ubiquitylation but can also function as a donor ubiquitin and can be attached as mono-ubiquitin to a substrate or to an existing ubiquitin chain as a chain terminator. To distinguish between 7KR INT-Ub signals originating from linear versus mono-ubiquitylation, we followed the reviewer’s advice and generated a N-terminally tagged 7KR INT-Ub variant. The N-terminal tag prevents linear ubiquitylation but still allows 7KR INT-Ub to be attached as a mono-ubiquitin. We found that the addition of this N-terminal tag significantly reduced but not completely abolished the number of Δ *mxlE* bacteria decorated with 7KR INT-Ub. These data are shown in a new Fig. S1 and indicate that 7KR lacking the N-terminal tag is attached to bacteria both in the form of linear (M1-linked) ubiquitin and as donor ubiquitin, possibly as a chain terminator. While we cannot rule out that the anti-M1 antibodies used here cross-react with other ubiquitin linkages, we reason that the 7KR data strongly argues that linear ubiquitin is part of the ubiquitin coat encasing IpaH1.4-deficient cytosolic *Shigella*. Collectively, our data show that both linear and lysine-linked (especially K27 and K63) ubiquitin chains are part of the RNF213-dependent ubiquitin coat on the surface of IpaH1.4 mutants. And furthermore, our data strongly indicate that this ubiquitylation of IpaH1.4 mutants is independent of LUBAC.

(2) We used GFP-expressing strains of *S. flexneri* for our infection studies and were therefore able to use GFP expression as a loading control. We have incorporated these data into our revised figures. These new data (Figs. 4A, 5A, and S3B) show that bacterial infection levels were comparable between WT and mutant infections and that therefore the degradation of RNF213 (or HOIP – see new data in Fig. S3B) is not due to differences in infection efficiency.

(3) We agree with the reviewer that the mechanism by which RNF213 binds to bacteria is an important unanswered question. Similarly, whether other ISGs have auxiliary functions in this process or whether binding efficiencies vary between different bacterial species are important questions in the field. However, these questions go far beyond the scope of this study and were therefore not addressed in our revisions.

Reviewer #3 (Recommendations for the authors):

(1) An N-terminally tagged K7R-ub should be used as a control to test whether the signal found around the mutant shigella is being added via the N terminal Met into chains. As it is known that certain batches of the M1-specific antibodies are in fact not specific and able to detect other chain types, the authors should test the specificity of the antibody used in this study (eg against different di-Ub linkage types) and include this data in the manuscript.

We agree with the reviewer in principle. The anti-linear ubiquitin (anti-M1) monoclonal antibody, clone 1E3, prominently used in this study was tested by the manufacturer (Sigma) by Western blotting analysis and according to the manufacturer “this antibody detected ubiquitin in linear Ub, but not Ub K11, Ub K48, Ub K63.” However, this analysis did not

include all possible Ub linkage types and thus the reviewer is correct that the anti-M1 antibody could theoretically also detect some other linkage types. To address this concern, we added new data during revisions demonstrating that 7KR INT-Ub targeting to *S. flexneri* is largely dependent on the N-terminus (M1) of ubiquitin. Our combined observations therefore overwhelmingly support the conclusion that linear (M1-linked) as well as K-linked ubiquitin is being attached to the surface of IpH1.4 *S. flexneri* bacteria in an RNF213-dependent and LUBAC-independent manner.

(2) The M1 signal detected on bacteria with the antibody is still present in either Hoip or Hoil KO's but due to the potential non-specificity of the antibody, the authors should test whether K7R ub is detected on bacteria in the Hoil ko (in addition to Hoip KO). This would strengthen the authors' data on LUBAC-independent M1 and is important because Hoil can catalyze non-canonical ubiquitylation.

The specific linear ubiquitin-ligating activity of LUBAC is enacted by HOIP. We show that linear ubiquitylation of susceptible *S. flexneri* mutants as assessed by anti-M1 ubiquitin staining or 7KR INT-Ub recruitment occurs in HOIPKO cells at WT levels (Figs. 3B, 3C, S3E [new data]). In our view, these data unequivocally show that the observed linear ubiquitylation of cytosolic *S. flexneri* ipaH1.4 and mxiE mutants is independent of LUBAC.

(3) For Figure 4A, do mxiE bacteria show similar invasion - authors should include a bacterial protein control to show levels of bacteria in WT and mxiE infected conditions. A similar control should be included in Figure 5A.

We used GFP-expressing strains of *S. flexneri* for our infection studies and were therefore able to use GFP expression as a loading control. We have incorporated these data into our revised figures. These new data (Figs. 4A, 5A, and S3B) show that bacterial infection levels were comparable between WT and mutant infections and that therefore the degradation of RNF213 (or HOIP – see new data in Fig. S3B) is not due to differences in infection efficiency.

(4) Can the authors speculate why IFNγ priming is needed for the coating of *Shigella* mxiE mutant but not in the case of *Salmonella* or *Burkholderia*? Is this just amounts of RNF213 or something else?

In our studies we did not directly compare ubiquitylation rates of cytosolic *Shigella*, *Burkholderia*, and *Salmonella* bacteria with each other under the same experimental conditions. However, such a direct comparison is needed to determine whether IFNγ priming is required for RNF213-dependent bacterial ubiquitylation of some but not other pathogens. Two papers published during the revisions of our manuscript (PMID: 40164614, PMID: 40205224) reports robust RNF213 targeting to IpaH1.4 *Shigella* mutants in unprimed cells HeLa cells (whereas we used A549 and HT29 cells). Therefore, differences in reagents, cell lines, and/or other experimental conditions may determine whether IFNγ priming is necessary to observe substantial RNF213 translocation to cytosolic bacteria.

(5) Typos - there are several, but this is hard to annotate with line numbers so the authors should proofread again carefully.

We proofread the manuscript and corrected the small number of typos we identified

<https://doi.org/10.7554/eLife.102714.2.sa0>