Guanylate-binding proteins (GBPs) coat *Shigella flexneri* in a GBP1-dependent manner and inhibit actin-dependent cell-to-cell spread of bacteria unless degraded through the bacterial ubiquitin ligase IpaH9.8.
GBPs Inhibit Motility of *Shigella flexneri* but Are Targeted for Degradation by the Bacterial Ubiquitin Ligase IpaH9.8

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https://doi.org/10.1016/j.chom.2017.09.007

SUMMARY

Interferon exposure boosts cell-autonomous immunity for more efficient pathogen control. But how interferon-enhanced immunity protects the cytosol against bacteria and how professionally cytosol-dwelling bacteria avoid clearance are insufficiently understood. Here we demonstrate that the interferon-induced GTPase family of guanylate-binding proteins (GBPs) coats *Shigella flexneri* in a hierarchical manner reliant on GBP1. GBPs inhibit actin-dependent motility and cell-to-cell spread of bacteria but are antagonized by IpaH9.8, a bacterial ubiquitin ligase secreted into the host cytosol. IpaH9.8 ubiquitylates GBP1, GBP2, and GBP4 to cause the proteasome-dependent destruction of existing GBP coats. This ubiquitin coating of *Shigella* favors the pathogen as it liberates bacteria from GBP encapsulation to resume actin-mediated motility and cell-to-cell spread. We conclude that an important function of GBP recruitment to *S. flexneri* is to prevent the spread of infection to neighboring cells while IpaH9.8 helps bacterial propagation by counteracting GBP-dependent cell-autonomous immunity.

INTRODUCTION

Pathogens inhabit specific niches in their host organism to which they are exquisitely adapted. The host cytosol appears a particularly hostile environment, considering the small number of bacteria able to replicate in this compartment despite its high nutrient content. In resting cells, anti-bacterial autophagy, inflammation, and the induction of cell death are all potent effector mechanisms against cytosol-invading bacteria (Randow et al., 2013). Additional resistance to infection is caused by exposure to interferons, which induce a large number of interferon-stimulated genes (ISGs) to enhance cytosolic immunity (MacMicking, 2012). ISGs antagonize both viruses and bacteria, although their contribution to the interferon-induced “anti-viral” state is much better understood than their anti-bacterial action (Boxx and Cheng, 2016).

Among the interferon-induced effector proteins with anti-bacterial function are guanylate-binding proteins (GBPs), which belong to a large IFN-induced GTPase family (Kim et al., 2012; Man et al., 2017). GBPs have been suggested to compromise the structural integrity of bacteria, to release ligands that stimulate inflammasomes, and to activate anti-bacterial effector mechanisms such as xenophagy and the oxidative burst (Kim et al., 2011; Man et al., 2016; Meunier et al., 2015). The target of GBP action remains hotly disputed since evidence has emerged for GBPs attacking host membranes as well as bacterial surfaces (Man et al., 2016; Meunier et al., 2014). It also remains unknown whether individual GBPs perform specific functions or whether their action is largely redundant as suggested by mouse knockout experiments, in which phenotypes emerged only upon large chromosomal deletions encompassing several GBPs (Yamamoto et al., 2012).

A fundamental question in innate immunity is how professionally cytosol-dwelling bacteria avoid clearance by cell-autonomous immunity (Deretic et al., 2013; Huang and Brumell, 2014). *Shigella flexneri* appears to have evolved sophisticated countermeasures against cytosolic effector mechanisms, such as the IpaH family of E3 ubiquitin ligases, characterized by an N-terminal leucine-rich repeat domain and a uniquely folded C-terminal catalytic domain distinct from cellular E3 ligases (Huibregtse and Rohde, 2014; Rohde et al., 2007; Singer et al., 2008; Zhu et al., 2008). Secreted via the type 3 secretion system (T3SS), IpaH proteins target host processes critical for anti-bacterial defense. IpaH1.4, for example, degrades the LUBAC subunit HOIP to prevent the activation of NF-κB and the deposition of M1-linked ubiquitin chains on the bacterial surface, even on co-infecting *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) (de Jong et al., 2016; Noad et al., 2017), while IpaH9.8 degrades the IKK subunit NEMO and the splicing factor U2AF35, resulting in reduced inflammation and higher bacterial counts (Ashida et al., 2010; Okuda et al., 2005). On the other hand, cytosol-dwelling bacteria take advantage of the unique opportunities their cytosolic lifestyle offers and infect neighboring cells via actin-dependent motility, thus avoiding exposure...
Figure 1. The Effect of IFNγ on Ubiquitin Coating of S. flexneri and Actin Tail Formation

(A and M) Percentage of S. flexneri positive for total ubiquitin in HeLa cells treated with the indicated cytokines. Mean ± SEM of triplicate coverslips from three independent repeats, n > 100 (for 1 hr p.i.), n > 200 (for 2 hr p.i.), n > 300 (for 3 hr p.i.) bacteria per coverslip. ns, non-significant; **p < 0.01, one-way ANOVA with Tukey’s multiple comparisons test.

(B and J) Confocal micrographs of HeLa cells treated or untreated with IFNγ and infected with (B) WT or (J) Ruby-expressing S. flexneri taken at 2 hr p.i. and stained for (B) total ubiquitin or (J) actin. Scale bar, 10 μm (B) or 25 μm (J).

(C) Percentage of S. Typhimurium positive for total ubiquitin in HeLa cells. Mean ± SEM of triplicate coverslips from three independent repeats, n > 100 bacteria per coverslip.

(D and E) Percentage of S. flexneri positive for the indicated ubiquitin chain types in HeLa cells treated or untreated with IFNγ at 2 hr p.i. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. **p < 0.01, Student’s t test.

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to the hostile extracellular space, while host cells carefully monitor their cytosolic homeostasis and deploy septins to antagonize such actin-driven bacterial motility (Mostowy et al., 2010). As demonstrated in primate models of infection, cell-to-cell spread is indeed an essential virulence determinant for S. flexneri and inactivating icsA, the gene that mediates actin-based motility, may contribute toward development of a live-attenuated vaccine strain (Bernardini et al., 1989; Sansonetti and Arondel, 1989).

By studying the fate of S. flexneri in human cells activated with IFNγ, we discovered that, in a hierarchical manner and dependent on GBP1, bacteria become coated with multiple GBPs. The GBP coat antagonizes actin-dependent bacterial motility and invasion of neighboring cells. However, efficient cell-to-cell spread of S. flexneri is restored by IpaH9.8, which, by ubiquitylating GBPs for proteasome-dependent degradation, removes existing GBP coats and induces a transient ubiquitin coat. The IpaH9.8-derived ubiquitin coat appears morphologically indistinguishable but functionally opposite to the classical host-generated ubiquitin coat on cytosol-invading bacteria in that it promotes the pathogen rather than immunity.

RESULTS

IFNγ Induces Coating of S. flexneri with K48-Linked Ubiquitin Chains

When entering its replicative niche in the host cytosol, S. flexneri avoids becoming coated with polyubiquitin (Figures 1A and 1B), suggesting that lack of ubiquitin coating enables S. flexneri to escape xenophagy and other effector mechanisms of cell-autonomous immunity. By contrast, S. Typhimurium, which is restricted by autophagy (Birmingham et al., 2006), becomes decorated with polyubiquitin (Figure 1C). To investigate whether exposure of cells to interferons or pro-inflammatory cytokines inactivates a pre-existing E3 ubiquitin ligase. GBPs comprise a family of seven GTPases in humans that control cell-autonomous immunity (Man et al., 2017), of which GBP1, GBP2, GBP3, GBP4, and GBP5 were strongly and selectively upregulated by IFNγ (Figure 2B). When investigating the localization of GFP-tagged GBPs in cells stimulated with IFNγ, we observed that GBPs co-localized directly with bacteria and did not stain galectin-8-positive damaged endomembranes in the vicinity of bacteria that had originated from the entry of S. flexneri into the cytosol (Figure S2). Antibodies raised against GBP1 or GBP2, or pan-reactive for GBP1–5 revealed that, in both HeLa and THP1 cells, GBP1 mediates hierarchical GBP recruitment and induces ubiquitin coating of S. flexneri.

GBP1 Induces Coating of S. flexneri with K48-Linked Ubiquitin Chains

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(F) Structured illumination (SI) micrographs of HeLa cells treated with IFNγ and infected with Ruby-expressing S. flexneri at 2 hr p.i. and stained for total (FK2) or K48-linked ubiquitin chains. Scale bar, 1 μm.

(G) Percentage of S. flexneri positive for YFP::galecin-8 in HeLa cells treated or untreated with IFNγ. Mean ± SEM of triplicate coverslips from three independent repeats, n > 100 (for 1 hr p.i.), n > 200 (for 2 hr p.i.), n > 300 (for 3 hr p.i.).

(H) Colony-forming units (CFU) of S. flexneri in HeLa cells treated or untreated with IFNγ. Bacteria were counted based on their ability to grow on agar plates. Mean ± SD of triplicate HeLa cultures and duplicate colony counts of a representative experiment.

(I) Percentage of WT or ΔicsA S. flexneri with actin tails in HeLa cells treated or untreated with IFNγ at 2 hr p.i. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. **p < 0.01, Student’s t test.

(J) Percentage of ΔicsA or ΔicsA complemented with FLAG::IcsA S. flexneri with FLAG-positive poles in HeLa cells treated or untreated with IFNγ at 2 hr p.i. Mean ± SEM of triplicate coverslips from three independent repeats, n > 100 bacteria per coverslip; ns, non-significant; Student’s t test.

(K) Percentage of WT or ΔicsA S. flexneri positive for the indicated GFP-tagged protein in HeLa cells treated or untreated with IFNγ at 2 hr p.i. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip; ns, non-significant; Student’s t test.
Figure 2. GBP1 Is Required for GBP Recruitment and Ubiquitin Coating of S. flexneri

(A and J) Percentage of S. flexneri positive for total ubiquitin at 2 hr p.i. HeLa cells were treated with siRNAs against (A) STAT1 or (J) GBPs as indicated. Cells were treated or untreated with IFNγ as indicated. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. ns, non-significant; **p < 0.01, one-way ANOVA with Tukey’s multiple comparisons test.

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upon stimulation with IFNγ, endogenous GBPs were recruited to *S. flexneri* (Figures 2C and 2D). At 1 hr p.i. between 30% and 60% of *S. flexneri* co-localized with GFP-tagged GBP1, GBP2, and GBP3; 10% with GBP4; and 5% with GBP7, while very few or none were positive for GBP5 and GBP6 (Figure 2E). Over-expressed GBP1 associated with *S. flexneri* even in the absence of IFNγ (Figure 2E). GBP1 recruitment required a catalytically active GTPase domain (Figure 2F), and importantly, GBP1 was essential for the recruitment of GBP2, GBP3, or GBP4 (Figures 2G and S1B). In contrast, depletion of GBP2, GBP3, or GBP4 had no effect on the recruitment of other GBPs. We therefore conclude that in a GTPase-dependent manner, GBP1 performs a catalytically inactive role in initiating the hierarchical recruitment of GBPs to cytolsol-invading bacteria.

We next investigated a potential functional link between GBP recruitment to, and ubiquitin coating of, *S. flexneri*. We found that the majority of GBP1- and GBP3-positive *S. flexneri* were also coated with ubiquitin but that, importantly, only GBP1 occurred on all ubiquitin-coated bacteria (Figures 2H and S3). Structured illumination microscopy revealed an association of both GBP1 and K48-linked ubiquitin chains with the bacterial surface (Figure 2I). Depletion of GBP1 prevented the occurrence of polyubiquitin on *S. flexneri* in cells stimulated with IFNγ (Figure 2J), while overexpression of GBP1 in resting cells was sufficient to induce ubiquitin coating (Figures 2K–2M). Depletion or overexpression of GBP2, GBP3, or GBP4 had no effect. Taken together, we conclude that the ubiquitin coat of *S. flexneri* in cells stimulated with IFNγ is due to the induction of GBP1, which localizes to *S. flexneri*, initiates the hierarchical recruitment of other GBPs, and may recruit an E3 ubiquitin ligase.

**IpaH9.8 Decorates *S. flexneri* with Polyubiquitin**

We considered the possibility that an E3 ubiquitin ligase encoded by *S. flexneri* rather than a cellular enzyme may generate the bacterial ubiquitin coat. To test our hypothesis, we used *S. flexneri* ΔmxiE, a strain deficient in the upregulation of many effector genes upon contact with host cells, including up to twelve bacterially encoded E3 ubiquitin ligases. *S. flexneri* ΔmxiE did not become coated with K48-linked ubiquitin chains (Figures 3A and 3B). We therefore investigated whether members of the *Shigella*-encoded and MxiE-controlled IpaH family of E3 ubiquitin ligases are recruited to and mediate ubiquitin coating of *S. flexneri* in IFNγ-stimulated cells. Among a panel of catalytically inactive IpaHs, only IpaH9.8 was recruited to *S. flexneri* (Figure 3C). Recruitment of exogenously expressed, catalytically inactive IpaH9.8 occurred specifically to bacteria that were also coated with K48-linked ubiquitin chains (Figures 3D and 3E) and only in cells stimulated with IFNγ (Figure 3F). To test whether IpaH9.8 is required for ubiquitin coating of *S. flexneri* in IFNγ-stimulated cells, we deployed strains lacking specific *ipaH* genes. *S. flexneri* ΔipaH9.8 did not become coated with K48-linked ubiquitin chains, in contrast to *S. flexneri* ΔipaH1.4 or ΔipaH7.8 (Figures 3G and 3H). An antibody against K27-linked ubiquitin also lost reactivity against *S. flexneri* ΔipaH9.8 (Figures 3I and 3J). Complementation with *ipaH9.8*, but not with catalytically inactive *ipaH9.8*<sub>C337A</sub>, restored ubiquitin deposition on *S. flexneri* ΔipaH9.8 (Figure 3K). We therefore conclude that the GBP1-dependent ubiquitin coat of *S. flexneri* in IFNγ-stimulated cells is synthesized by IpaH9.8. The bacterial origin of the ubiquitin coat on *S. flexneri* in cells stimulated with IFNγ suggests that this particular ubiquitin coat may not have antibacterial functions but rather that IpaH9.8 counteracts a GBP-dependent cellular defense pathway, potentially through directly antagonizing GBP recruitment.

**IpaH9.8 Ubiquitylates GBPs for Proteasomal Degradation**

We therefore investigated whether IpaH9.8 negatively controls the accumulation of GBPs on *S. flexneri*. Bacteria lacking IpaH9.8 were much more frequently associated with GBP1, GBP2, GBP3, and GBP4; complementation with IpaH9.8 reversed the effect (Figure 4A). To test whether IpaH9.8 controls GBP accumulation on bacteria by degrading GBPs, we analyzed GBP levels by flow cytometry in cells infected with *S. flexneri*. At 10 min p.i., GBP levels were indistinguishable between infected and uninfected cells, while at 180 min p.i., levels of GBP1, GBP2, and, to a lesser extent, GBP4 were reduced specifically in cells carrying a high burden of *S. flexneri*, i.e., in cells with proliferating bacteria (Figures 4B and S4). GBP3 levels were unaffected. Infection with *S. flexneri* ΔipaH9.8 did not reduce GBP levels, revealing that IpaH9.8 is essential for reducing GBP levels in infected cells. Endogenous GBP1 in cells stimulated with IFNγ was similarly reduced upon overexpression of IpaH9.8, but not other IpaH family members (Figure 4C), thus revealing exquisite
Figure 3. IpaH9.8 Causes Ubiquitin Coats on S. flexneri

(A, E, H, and J) Confocal micrographs of HeLa cells treated with IFN-γ and infected with either WT or the indicated strains of S. flexneri expressing Ruby at 2 hr p.i. Cells were stained for (A and H) K48-linked ubiquitin, (J) K27-linked ubiquitin, or (E) K48-linked ubiquitin and expressed GFP-tagged, catalytically inactive IpaH9.8. Scale bar, 25 μm.

(B and G) Percentage of the indicated S. flexneri strains positive for K48-linked ubiquitin at 2 hr p.i. HeLa cells were treated with IFN-γ as indicated. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. ns, not significant; **p < 0.01, (B) Student’s t test or (G) one-way ANOVA with Dunnett’s multiple comparisons test.

(C, D, and F) HeLa cells expressing inactive alleles of the indicated ipaH genes and (C and D) treated with IFN-γ or (F) treated with IFN-γ as indicated were infected with S. flexneri. (C and F) Bacteria positive for the indicated GFP-tagged IpaHs or (D) percentage of bacteria positive for GFP-tagged IpaHs among those positive for K48-linked ubiquitin at 2 hr p.i. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip.

(I and K) Percentage of the indicated S. flexneri strains positive for (I) K27-linked ubiquitin or (K) K48-linked ubiquitin in HeLa cells treated with IFN-γ at 2 hr p.i. (K) ΔipaH9.8 complemented with empty vector, WT, or catalytically inactive alleles of IpaH9.8. Mean ± SEM of triplicate coverslips from three independent experiments, n > 200 bacteria per coverslip. ns, not significant; *p < 0.05, **p < 0.01, one-way ANOVA with Dunnett’s multiple comparisons test.
specificity of IpaH proteins in the absence of other bacterial proteins. To further investigate the specificity of IpaH9.8 and to reveal how GBPs are degraded, we co-expressed IpaH9.8 and GBPs in 293ET cells. Similar to infection with *S. flexneri*, expression of WT, but not catalytically inactive IpaH9.8, drastically reduced levels of GBP1, GBP2, and GBP4, but not GBP3 (Figure 4D). Such specificity of GBP degradation correlates with and is likely explained by the ability of IpaH9.8 to bind GBP1, GBP2, and GBP4, but not GBP3 (Figures 4E and S5). To investigate whether GBPs are direct substrates for IpaH9.8, we performed *in vitro* ubiquitylation assays using purified proteins. IpaH9.8 catalyzed almost quantitative ubiquitylation of GBP1, resulting in a high molecular weight ubiquitin smear, but was inactive toward GBP3 (Figure 5A), consistent with the binding specificity of IpaH9.8 for GBPs (Figure 4E) and the autoinhibited state of IpaH E3 ligases in the absence of bound substrate (Chou et al., 2012). Co-expression of GBP1 and IpaH9.8 resulted in ubiquitylation of GBP1 and its degradation (Figure 5B).

Figure 4. IpaH9.8 Causes GBP Degradation
(A) Percentage of the indicated *S. flexneri* strains positive for GFP-tagged GBP1–4 in IFNγ-stimulated HeLa cells at 4 hr p.i. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. **p < 0.01, one-way ANOVA with Tukey’s multiple comparisons test (WT against ∆9.8 for each sample).
(B) HeLa cells expressing GFP-tagged GBP alleles were analyzed by flow cytometry and gated for intracellular *S. flexneri* expressing Ruby. Infected cells with reduced GBP levels were quantified. Mean ± SD of three independent repeats, n > 10,000 cells per sample, ns, non-significant; **p < 0.01, one-way ANOVA with Tukey's multiple comparisons test. Dot plots and gating strategy of a representative repeat are shown in Figure S4.
(C) Lysates from HeLa cells mock transduced or transduced with the indicated Flag-tagged *ipaH* constructs were treated with IFNγ and probed with antibodies against GBP1, Flag, and PCNA.
(D) 293ET cells were co-transfected with GFP-tagged GBP alleles and Flag-tagged *ipaH* constructs as indicated. Lysates were probed for GFP, Flag, and β-actin.
(E) LUMIER binding assay. Luciferase-tagged GBP1–4 transfected in 293ET cells were pulled down using recombinant GST or GST::IpaH9.8 coupled to beads. Proteins were eluted from beads with glutathione. Left panel: protein binding was determined as luciferase activity over GST control. Mean ± SD of two independent repeats. Right panel: coupling to beads was assessed by SDS-PAGE and Coomassie staining. See also Figure S4.
Treatment with the proteasome inhibitor Carfilzomib antagonized the effects of IpaH9.8 on GBPs — Carfilzomib caused accumulation of polyubiquitin in immunoprecipitated GBP1 samples (Figure 5B), it rescued the degradation of endogenous GBP1 in cells infected with \textit{S. flexneri} (Figure 5C), and it restored GBP1 coats on \textit{S. flexneri} (Figure 5D). Taken together, we conclude that \textit{S. flexneri} secretes IpaH9.8 to ubiquitylate specific GBPs and cause their proteasome-mediated degradation, resulting in reduced GBP levels in infected cells and escape of bacteria from labeling by GBPs.

\textbf{IpaH9.8 Degrades GBPs to Promote Bacterial Spreading}

To investigate whether the inhibition of actin tail formation on \textit{S. flexneri} observed upon IFN\textsubscript{γ} treatment is mediated by GBPs and whether GBP degradation by IpaH9.8 precedes actin-driven motility of \textit{S. flexneri}, we performed live microscopy in cells expressing GFP::GBP1 and labeled with an F-actin binding peptide known as Lifeact (Movies S1 and S2; Figure 6A) (Riedl et al., 2008). Upon infection, both WT bacteria and \textit{S. flexneri ΔipaH9.8} became coated with GBP1, a status that, once established, was sustained throughout division by bacteria of either strain. However, while \textit{S. flexneri ΔipaH9.8}, due to lack of actin-driven motility, formed tightly clustered micro-colonies and failed to...
Figure 6. IpaH9.8 Antagonizes GBP Effects on Actin-Dependent Motility and Bacterial Spread

(A) Representative frames from Movies S1 and S2 of HeLa cells co-expressing GFP::GBP1 and Lifeact::CFP infected with WT or ΔipaH9.8 Ruby-expressing S. flexneri. Time p.i. as indicated; scale bar, 10 μm.

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invade neighboring cells. Quantification confirmed the absence of GBP1-positive bacteria displaying actin tails in WT and ΔipaH9.8 strains, as well as the lower and higher percentage of actin tails and GBP1 coats, respectively, in S. flexneri ΔipaH9.8 compared to WT bacteria (Figure 6B). Complementation with ipaH9.8 reversed the actin phenotype (Figure 6C) and treatment with the proteasome inhibitor Carfilzomib antagonized the IpaH9.8 effect on actin tail formation (Figure S5). To test whether GBP1 mediates the IFNγ-induced suppression of actin tail formation, we depleted cells of GBPs using small interfering RNAs (siRNAs) (Figure 6D) and discovered that GBP1 was essential for IFNγ to antagonize actin tail formation on S. flexneri. Finally, to investigate the functional consequences of IpaH9.8-mediated degradation of IFNγ-induced GBPs and its effect on actin tail formation, we measured proliferation of S. flexneri and bacterial load in host cells. Bacterial proliferation in either resting or IFNγ-treated cells was unaffected by deletion of IpaH9.8 (Figure 6E). However, bacteria lacking IpaH9.8 did not spread efficiently between cells, resulting in a much smaller number of cells becoming infected (Figures 6F, 6G, and S7) and those cells carrying a much higher bacterial load (Figures 6F, 6H, and S7). Taken together, we conclude that GBP1 restricts actin-driven motility of S. flexneri, thus impairing the spread of bacteria into neighboring, infected cells and containing the bacterial burden in a smaller number of infected cells. IpaH9.8 antagonizes GBP-mediated cell-autonomous immunity by targeting GBPs for proteasomal degradation, resulting in unrestricted spread of bacteria.

**DISCUSSION**

GBPs provide important but poorly understood immunity against invasive bacteria in interferon-stimulated cells. Here we provide evidence for the GBP1-dependent hierarchical recruitment of multiple GBPs to cytosol-exposed S. flexneri, where they form a dense GBP coat surrounding the bacterium that inhibits actin-dependent bacterial motility and, consequently, cell-to-cell spread. S. flexneri antagonizes GBP-mediated cellular defenses by secreting the E3 ubiquitin ligase IpaH9.8, which ubiquitylates and degrades GBPs in a proteasome-dependent manner, causing existing GBP coats to dissolve and actin-dependent motility to become re-established. The existence of an E3 ubiquitin ligase in the genome of S. flexneri that efficiently degrades multiple GBP1-dependent GBPs provides compelling evolutionary evidence for the importance of GBPs in antibacterial defense.

GBPs clearly play an important role in defending the cell interior against parasites and bacteria, although the target of GBP attack and their mode of action remain poorly characterized (Kim et al., 2012; Man et al., 2017). While both host membranes and bacterial surfaces have been found to be targeted by GBPs, our SIM super-resolution data provide unequivocal evidence for the accumulation of GBPs on the bacterial surface with no observable enrichment on galectin-8-positive remnants of Shigella-containing vacuoles. Importantly, the accumulation of GBPs on S. flexneri required catalytically active GBP1, suggesting a clear hierarchy in GBP recruitment and the possibility that GBP1 either serves as a receptor for S. flexneri and possibly other pathogens or that other cellular proteins are required to load GBPs in a hierarchical manner onto the bacterial surface. Similar to the ubiquitin coat formed by cellular E3 ubiquitin ligases when they encounter bacteria in the host cytosol (Franco et al., 2017; Huett et al., 2012; Manzanillo et al., 2013; Noad et al., 2017; Perrin et al., 2004), the GBP coat also represents a highly polyvalent display of host proteins on the bacterial surface. Therefore, just as the ubiquitin coat, the GBP coat may also transform the bacterial surface into a signaling platform (Noad et al., 2017; van Wijk et al., 2017). Considering the time required to upregulate IFNγ-dependent GBP expression and the model character of the cells used in this study, follow-up investigations will be required to test the importance of the GBP coat for the defense against S. flexneri in vivo.

While several effector molecules of the ubiquitin coat have been identified, including pro-inflammatory signaling molecules such as the Nemo-recruited IKK complex (Noad et al., 2017) and autophagy cargo receptors such as NDP52, Optineurin, and p62 (Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009), GBP effectors remain to be identified, although IRGB10, recruited to Francisella novicida via GBPs and contributing to the liberation of bacterial ligands for the inflammasome, may represent an important effector molecule of the GBP coat (Man et al., 2016).

Since the bacterial ubiquitin coat serves anti-bacterial purposes, S. flexneri, as a professional cytosol-dwelling pathogen, has evolved sophisticated countermeasures against ubiquitin coating, such as, for example, degrading anti-bacterial E3 ligases (de Jong et al., 2016; Noad et al., 2017). However, in cells stimulated with IFNγ, S. flexneri does become ubiquitin coated.

(B) S. flexneri positive for actin tails and/or GBP1 at 2 hr p.i. in HeLa cells expressing GFP::GBP1. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. ns, not significant; **p < 0.01 one-way ANOVA with Tukey’s multiple comparisons test.

(C and D): S. flexneri positive for actin tails at 2 hr p.i. in HeLa cells treated with IFNγ as indicated (C) and treated with indicated siRNAs against human GBPs. Mean ± SEM of triplicate coverslips from three independent repeats, n > 200 bacteria per coverslip. ns, not significant; **p < 0.01, Student’s t test (control) or one-way ANOVA with Tukey’s multiple comparisons test (control versus GBP siRNAs).

(E–H) Replication of S. flexneri in HeLa cells treated with or without IFNγ and infected with WT or ΔipaH9.8 S. flexneri for the indicated times.

(F) Replicate replication of S. flexneri. Bacteria were counted based on their ability to grow on agar plates. Mean ± SD of triplicate HeLa cultures and duplicate colony counts.

(G) Spread of infection by Ruby-expressing S. flexneri through HeLa culture scored by flow cytometry as illustrated by red gate in (F).

(H) Percentage of infected HeLa cells with high levels of Ruby as illustrated by black gate in (F).

(G and H) Mean ± SEM of triplicate HeLa cultures of at least three independent repeats. *p < 0.05, **p < 0.01, Student’s t test; ns, not significant.

See also Figures S5 and S6 and Movies S1 and S2.
although not due to the action of cellular E3 ubiquitin ligases, but as a result of IpaH9.8 ubiquitylating the GBP coat. Therefore, rather than promoting anti-bacterial defense mechanisms by providing “eat-me” signals for cargo receptors of xenophagy (Boyle and Randow, 2013), the coating of S. flexneri with polyubiquitin by IpaH9.8 results from the bacterial attempt to counteract a cellular defense pathway. Care must therefore be taken when attempting to assign anti-bacterial function to the ubiquitin coat on cytosolic bacteria. Our results on the ubiquitin coating of S. flexneri by a bacterially encoded E3 ubiquitin ligase are congruent with a growing body of literature suggesting that S. flexneri possesses multiple mechanisms to evade anti-bacterial autophagy. Previous studies have shown that Toca1 recruitment to intracellular S. flexneri antagonizes xenophagy and that IcsB and VirA are bacterially encoded factors that help in evading anti-bacterial autophagy (Baxt and Goldberg, 2014; Campbell-Valois et al., 2015; Ogawa et al., 2005).

IpaH9.8 is an important pathogenicity factor for S. flexneri since, in a murine pneumonia model, S. flexneri ΔipaH9.8 proliferates less and induces higher levels of pro-inflammatory cytokines (Okuda et al., 2005). Degradation of both U2AF35, a splicing factor, and Nemo, an essential subunit of the NF-κB-inducing IKK complex, has been suggested to cause the observed phenotype (Ashida et al., 2010; Okuda et al., 2005). Our work revealed that IpaH9.8, among a panel of twelve IpaH proteins, specifically targets GBP1, GBP2, and GBP4, but not GBP3. As demonstrated for GBP1 and GBP3, binding of GBPs to IpaH9.8 parallels the ability of IpaH9.8 to ubiquitylate GBPs in vitro, consistent with the autoinhibitory state of IpaH E3 ligases in the absence of bound substrate (Chou et al., 2012), and the ability of IpaH9.8 to induce proteasome-dependent degradation in cells. GBP degradation does not require GBP oligomerization on the bacterial surface, since expression of IpaH9.8 in uninfected cells was sufficient to deplete GBP from cells, suggesting sufficiently high affinity of IpaH9.8 for soluble GBPs consistent with binding of IpaH9.8 to GBP1, GBP2, and GBP4 in LUMIER interaction assays. The ability of IpaH9.8 and potentially other IpaH proteins to selectively degrade key host defense molecules suggests that IpaH proteins hold significant potential for the experimental manipulation of host-pathogen interactions, although it will be important to first gain a better understanding of the structural basis of IpaH specificity.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, two tables, and two movies and can be found with this article online at https://doi.org/10.1016/j.chom.2017.09.007.

**AUTHOR CONTRIBUTIONS**

M.P.W., C.P., E.I.W., C.J.E., K.B.B., and A.v.d.M. performed experiments and analyzed data. J.R. provided knockout strains and reviewed the manuscript. M.P.W., C.P., and F.R. wrote the manuscript.

**ACKNOWLEDGMENTS**

We thank Janet Deane for providing a FLAG-tagged icsA cDNA and Paul Elliott for providing E1, UBE2D1, and ubiquitin. This work was supported by the MRC (U105170648), the Wellcome Trust (WT104752MA), and a Boehringer Ingelheim Fonds PhD fellowship (to C.P.).

Received: June 12, 2017

Revised: August 11, 2017

Accepted: September 18, 2017

Published: October 11, 2017

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## Key Resources Table

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| GFP (JL8) mouse monoclonal | Clontech | Cat# 632381; RRID: AB_2313808 |
| Ubiquitin (FK2) mouse monoclonal | Enzo Life Science | Cat# BML-PW8810; RRID: AB_10541840 |
| Ubiquitin M1 (1E3) rabbit monoclonal | Merck Millipore | Cat# MABS199; RRID: AB_2576212 |
| Ubiquitin K11 (2A3/2E6) rabbit monoclonal | Merck Millipore | Cat# MABS107-I; RRID: AB_2713901 |
| Ubiquitin K48 (Apu2) rabbit monoclonal | Merck Millipore | Cat# 05-1307; RRID: AB_1587578 |
| PCNA (PC10) mouse monoclonal | Santa Cruz Biotechnology | Cat# sc-56; RRID: AB_628110 |
| Galectin-8 goat polyclonal | R&D Systems | Cat# AF1305; RRID: AB_2137229 |
| GBP1 rabbit polyclonal | Proteintech | Cat# 15303-1-AP; RRID: AB_2247448 |
| GBP1 (1B1) rat monoclonal | Santa Cruz Biotechnology | Cat# sc-53857; RRID: AB_2109333 |
| GBP1-5 (G-12) mouse monoclonal | Santa Cruz Biotechnology | Cat# sc-166960; RRID: AB_10611378 |
| GBP2 (G-9) mouse monoclonal | Santa Cruz Biotechnology | Cat# sc-271568; RRID: AB_10655677 |
| Ubiquitin K27 rabbit monoclonal | Abcam | Cat# ab181537; RRID: AB_2713902 |
| Flag-tag (M2) mouse monoclonal | Sigma | Cat# F1804; RRID: AB_262044 |
| β-actin rabbit polyclonal | Abcam | Cat# ab8227; RRID: AB_2305186 |
| Alexa-conjugated anti-mouse | Thermo Fisher Scientific | N/A |
| Alexa-conjugated anti-rabbit | Thermo Fisher Scientific | N/A |
| HRP-conjugated reagents | Dabco | N/A |
| **Bacterial and Virus Strains** | | |
| *Shigella flexneri* strain M90T | Gift from Chris Tang | N/A |
| *Shigella flexneri* strain M90T ΔicsA | Gift from Chris Tang | N/A |
| *Shigella flexneri* strain M90T ΔmxiE | Sidik et al., 2014 | N/A |
| *Shigella flexneri* strain M90T ΔipaH1.4 | Sidik et al., 2014 | N/A |
| *Shigella flexneri* strain M90T ΔipaH7.8 | Sidik et al., 2014 | N/A |
| *Shigella flexneri* strain M90T ΔipaH9.8 | Sidik et al., 2014 | N/A |
| *Salmonella Typhimurium* strain 12023 | Gift from David Holden | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Phalloidin Alexa488 | Thermo Fisher Scientific | Cat# A12379 |
| Phalloidin Alexa568 | Thermo Fisher Scientific | Cat# A12380 |
| Human IFNγ | R&D Systems | Cat# 285-IF |
| Human IFNβ | PBL | Cat# 11415-1 |
| Human TNFα | R&D Systems | Cat# 210-TA |
| Human IL1-β | R&D Systems | Cat# 201-LB |
| Human IL-22 | R&D Systems | Cat# 782-IL |
| Lipofectamine RNAiMAX | Thermo Fisher Scientific | Cat# 13778150 |
| VECTASHIELD HardSet Antifade Mounting Medium with DAPI | Vector Laboratories | Cat# H-1500 |
| ProLong Gold Antifade Mountant | Thermo Fisher Scientific | Cat# P36930 |
| Carfilzomib (PR-171) | Selleck Chemicals | Cat# S2853 |
| Polyethyleneimine (PEI) | Polysciences | Cat# 23966-2 |
| Saponine | Thermo Fisher Scientific | Cat# AC419231000 |
| E1 enzyme (UB reagent) | Gift from Paul Elliott, MRC LMB Cambridge | N/A |
| UBE2D1 (UB reagent) | Gift from Paul Elliott, MRC LMB Cambridge | N/A |
| Ubiquitin (UB reagent) | Gift from Paul Elliott, MRC LMB Cambridge | N/A |
| cOmplete Protease Inhibitor Cocktail | Roche | Cat#000000011697498001 |

(Continued on next page)
## Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Renilla Luciferase Assay System | Promega | Cat# E2820 |
| Fix & Perm | Thermo Fisher Scientific | Cat# GAS004 |
| RNeasy Plus Mini Kit | QIAGEN | Cat# 74134 |
| Amersham ECL | GE Healthcare Life Sciences | Cat# RPN2106 |

## Deposited Data

| EXPERIMENTAL MODEL | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| DNA microarray | This study | GEO: GSE103363 |

## Experimental Models: Cell Lines

| EXPERIMENTAL MODEL | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| HeLa | Lab strain | N/A |
| 293ET | Lab strain | N/A |
| THP1 | Lab strain | N/A |

## Oligonucleotides

| EXPERIMENTAL MODEL | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| See Table S2 for siRNAs | This study | N/A |

## Recombinant DNA

| EXPERIMENTAL MODEL | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| pFPV25.1.ipaH9.8 | This study | CP75 |
| pFPV25.1.ipaH9.8.C337A | This study | CP76 |
| M6P.GFP::ipaH1.4.C368A | This study | CP37 |
| M6P.GFP::ipaH2.5.C368A | This study | CP38 |
| M6P.GFP::ipaH4.5.C379A | This study | CP39 |
| M6P.GFP::ipaH7.8.C357A | This study | CP40 |
| M6P.GFP::ipaH9.8.C337A | This study | CP41 |
| M6P.GFP::ipaH1.C379A | This study | CP48 |
| M6P.GFP::ipaH2.C400A | This study | CP42 |
| M6P.GFP::ipaH3.C340A | This study | CP43 |
| M6P.GFP::ipaH4.C375A | This study | CP44 |
| M6P.GFP::ipaH5.C339A | This study | CP45 |
| M6P.GFP::ipaH6.C339A | This study | CP46 |
| M6P.GFP::ipaH7.C400A | This study | CP47 |
| M6P.Flag::ipaH1.4 | This study | CP02 |
| M6P.Flag::ipaH2.5 | This study | CP03 |
| M6P.Flag::ipaH4.5 | This study | CP04 |
| M6P.Flag::ipaH7.8 | This study | CP05 |
| M6P.Flag::ipaH9.8 | This study | CP06 |
| M6P.Flag::ipaH2 | This study | CP07 |
| M6P.Flag::ipaH3 | This study | CP08 |
| M6P.Flag::ipaH4 | This study | CP09 |
| M6P.Flag::ipaH5 | This study | CP10 |
| M6P.Flag::ipaH6 | This study | CP11 |
| M6P.Flag::ipaH7 | This study | CP12 |
| M6P.Flag::ipaH1 | This study | CP13 |
| M6P.Flag::ipaH9.8.C337A | This study | CP67 |
| pETM30.HIS::GST::ipaH9.8 | This study | CP77 |
| M6P.GFP::GBP1 | This study | MW478 |
| M6P.GFP::GBP2 | This study | MW479 |
| M6P.GFP::GBP3 | This study | MW480 |
| M6P.GFP::GBP4 | This study | MW482 |
| M6P.GFP::GBP5 | This study | MW483 |
| M6P.GFP::GBP6 | This study | MW484 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Felix Randow (randow@mrc-lmb.cam.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture
HeLa, 293ET and THP1 cells, as well as all stable cell lines, were grown in IMDM supplemented with 10% FCS at 37°C in 5% CO2. HeLa and 293ET are of female, THP1 of male origin. Cell lines have not been authenticated. All cell lines were tested to be Mycoplasma free.

Bacteria
S. flexneri (strain M90T) was grown in tryptic soy broth (TSB) or on tryptic soy agar containing 0.003% Congo red.
S. Typhimurium (strain 12023) was grown in Luria broth (LB) or on LB agar plates.
E. coli strains MC1061, BL21 and Rosetta2 were grown on tryptic yeast extract (TYE) agar plates or in LB.

METHOD DETAILS

Cytokine Treatment
Cytokines were added for 10-20 hours before experiments at the following concentrations: IFNγ 1 ng/ml, IFNβ 100 U/ml, TNFα 10 ng/ml, IL-1β 10 ng/ml and IL-22 10 ng/ml.

Plasmid Generation
M5P or closely related plasmids were used to produce recombinant MLV for the expression of proteins in mammalian cells (Randow and Sale, 2006). Open reading frames encoding IpaH proteins, N-WASP, WIP, ARP2, ARP3 and GBP1-7 were amplified by PCR.
Mutations were generated by PCR and verified by sequencing. For complementation IpaH9.8 and FLAG::IcsA (Mauricio et al., 2017) were amplified by PCR and cloned into pFPV25.1.

**Bacterial Infections and Enumeration of Intracellular Bacteria**

*S. flexneri* was grown overnight in tryptic soy broth (TSB) and sub-cultured (1:100) in fresh TSB for 2.5 h before infection. Such cultures were consecutively washed in PBS and re-suspended in antibiotic-free IMDM plus 10% FCS immediately before 100 μL was used to infect HeLa cells in 24-well plates. Samples were centrifuged for 10 min at 670 g followed by incubation at 37°C for 20 or 30 min. Following two washes with warm PBS, cells were cultured in 100 μg/ml gentamycin for 2 h and 20 μg/ml gentamycin thereafter. For FACS-based infection assays, bacteria were diluted 1:3 in warm antibiotic-free IMDM before infection and cells were washed 10 min after infection.

S. Typhimurium was grown overnight in Luria broth (LB) and sub-cultured (1:33) in fresh LB for 3.5 h before infection. Such cultures were further diluted (1:5) in antibiotic-free IMDM plus 10% FCS immediately before 20 μL was used to infect HeLa cells in 24-well plates for 15 min at 37°C. Following two washes with warm PBS, cells were cultured in 100 μg/ml gentamycin.

To enumerate intracellular bacteria, cells from triplicate wells were lysed in 1 ml cold PBS containing 0.1% Triton X-100. Serial dilutions were plated in duplicate on TYE agar.

**Microarray**

Total RNA from HeLa cells was extracted using the RNeasy Plus Mini Kit. RNA samples were prepared using the Ambion WT Expression Kit and Affymetrix GeneChip WT Terminal Labeling and Hybridization Kit. The generated cocktails were hybridized to Human Affymetrix Gene ST 1.0 cartridge arrays. The arrays were then scanned on the Affymetrix GCS3000 by the Addenbrooke’s Hospital Genomics Core Laboratory.

**RNA Interference**

5 × 10⁵ cells per well were seeded in 24-well plates. The following day, cells were transfected with 40 pmol of siRNA using Lipofectamine RNAiMAX. Experiments were performed after 3 days.

The non-targeting negative control was used as control.

**Microscopy**

HeLa cells were grown on glass coverslips before infection. After infection, cells were washed twice with warm PBS and fixed in 4% paraformaldehyde for 20 min. Cells were washed twice in PBS and then simultaneously permeabilized and blocked in PBSB (PBS, 0.01% saponin, 2% BSA). Coverslips were incubated with primary followed by secondary antibodies for 1 h in PBSB. Samples were mounted either in mounting medium with DAPI or Prolong Antifade mounting medium for confocal imaging and super resolution microscopy, respectively. Marker positive bacteria were scored by eye among at least 200 bacteria per coverslip. Confocal images were taken with a ×63, 1.4 numerical aperture objective on either a Zeiss 710 or a Zeiss 780 microscope. Live imaging was performed on a Nikon Eclipse Ti equipped with an Andor Revolution XD system and a Yokogawa CSU-X1 spinning disk unit. Super resolution images were acquired using an Elyra S1 structured illumination microscope (Carl Zeiss Microscopy Ltd, Cambridge, UK). The system has four laser excitation sources (405nm, 488nm, 561nm and 640nm) with fluorescence emission filter sets matched to these wavelengths. SIM Images were obtained using a 63X 1.4 NA oil immersion lens with grating projections at 3 rotations and 5 phases in accordance with the manufacturers instructions. The number of Z planes varied with sample thickness. Super resolution images were calculated from the raw data using Zeiss ZEN software.

**Immunoprecipitation and Western Blot**

293ET cells were grown on 6-well plates and transfected with 2 μg plasmid DNA (1.5 μg GFP:GBP1 and 0.5 μg IpaH9.8) using PEI. 24h after transfection the proteasome was inhibited for 18h using 100 nM Carfilzomib.

Post-nuclear supernatants from 2 × 10⁸ 293ET cells expressing tagged proteins were obtained following lysis (150 mM NaCl, 0.1% Triton X-100, 20 mM Tris-HCl pH 7.4, 5 mM EDTA and proteinase inhibitors). Proteins were precipitated for 2 h with anti-GFP or Flag agarose before washing. Samples were eluted with Flag peptide or Laemmli buffer and separated on 4%–12% denaturing Bis-Tris gels (Thermo Fisher Scientific). Visualization following immunoblotting was performed using ECL detection reagents.

**Protein Expression for LUMIER Assays**

Proteins were expressed from pETM30 in E.coli BL21. Bacteria were grown to an OD600 of 0.7 at 37°C before overnight induction at 16°C in the presence of 100 μM IPTG. Cells were mechanically lysed in lysis buffer (20mM Tris (pH 8.0), 150mM NaCl, 1mM DTT, protease inhibitors) and cleared by centrifugation. Lysates were snap frozen and stored at −80°C.

**LUMIER Assays**

LUMIER binding assays with pairs of putative interactors, one fused to luciferase and the other fused to GST, were performed in LUMIER lysis buffer (150 mM NaCl, 0.1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 5% glycerol, 5 mM EDTA and proteinase inhibitors) as previously described (Ryzhakov and Randow, 2007). GST-fusion proteins were immobilized on beads before incubation with the luciferase tagged binding partner for 2 h. Luciferase-tagged proteins were expressed in 293ET cells. After washing in lysis buffer,
proteins were eluted with glutathione in *Renilla* lysis buffer (Promega). Relative luciferase activity represents the ratio of activity eluted from beads and present in lysates.

**Protein Expression and Purification for In Vitro Ubiquitination Assay**

His<sub>6</sub>-GBP1 and His<sub>6</sub>-GBP3 (both in the pETM-11 vector) and His<sub>6</sub>-GST-IpaH9.8 (in pETM-30 vector) were expressed in Rosetta2 (DE3) cells. Cells were grown at 30°C in 2xTY medium supplemented with 30 μg/ml kanamycin and 34 μg/ml chloramphenicol. Cultures were induced with IPTG (400 μM) at 18°C and harvested after 16 hours. Proteins were purified by immobilized metal-affinity chromatography and the His<sub>6</sub>-GST tag was removed from IpaH9.8 by incubation with TEV protease. His<sub>6</sub>-GBP1, His<sub>6</sub>-GBP3 and untagged IpaH9.8 were further purified by anion exchange chromatography using a Resource Q column (GE Healthcare) and size exclusion chromatography using a Superdex 200 16/60 column (GE Healthcare), the latter in buffer 20 mM Tris pH 8.5, 200 mM NaCl and 4 mM DTT.

**In Vitro Ubiquitination Assay**

Reactions were set up in 40 mM Tris pH 8.5, 10 mM MgCl<sub>2</sub>, 0.6 mM DTT with combinations of the following reagents: E1 (0.5 μM), UBE2D1 (3 μM), IpaH9.8 (5 μM), His<sub>6</sub>-GBP1 (30 μM), His<sub>6</sub>-GBP3 (30 μM), ubiquitin (300 μM) and ATP (10 mM). The reaction was incubated at 37°C for 30 min after which, Ni<sup>2+</sup> resin was added for 15 minutes at r.t.. Following binding, resin was washed three times in 20 mM Tris pH 7.4, 300 mM NaCl, 50 mM Imidazole, 2 mM β-mercaptoethanol and 0.01% tween.

**FACS**

After infection, cells were washed twice with warm PBS, detached with trypsin and fixed in 4% paraformaldehyde for 20 min. Cells were washed twice in PBS and quenched with PBSG (PBS, 1M Glycine). For intracellular staining samples were fixed and stained using Fix & Perm (Life Technologies). All samples were analyzed on a BD LSR ii flow cytometer, using the high throughput system (HTS) to score spread of bacteria. Data were analyzed in FlowJo.

**Transformation of *S. flexneri***

A Tryptic Soy Broth (TSB) overnight culture was diluted 1:100 in 10 mL of TSB and grown at 37°C to an OD<sub>600</sub> of 0.6 to 0.8. Bacteria were cooled on ice for 10 min, centrifuged (4,300 g x 4 min, 4°C) and washed once in 10 mL then twice in 1 mL of ice cold electroporation buffer (1 mM MOPS, 20% glycerol, pH 7.2). Bacteria were pelleted a final time, resuspended in 100 μL of buffer and mixed with 150 ng of plasmid DNA. The mixture was electroporated in a chilled 2mm cuvette (Flowgen Bioscience) using 2,500V, 600 Ω and 10 μF. Electroporated bacteria were regenerated for 1 h at 37°C in 1 mL of Super Optimal Broth (SOB) medium and plated on TSB agar supplemented with Ampicillin (100 μg/mL) and Congo red (0.003%).

## QUANTIFICATION AND STATISTICAL ANALYSIS

All data were tested for statistical significance with Prism software (GraphPad Prism 7). The unpaired Student’s t test was used to test whether two samples originate from the same population. For more than two samples with only one variable an analysis of variance (one-way ANOVA) was performed. Either Dunnett’s multiple comparison test (to compare all samples against a control) or Tukey’s multiple comparison test (to compare all samples against each other) was applied. Performed tests are indicated in Figure Legends. No specific method was used to determine whether the data met assumptions of the statistical approach. Unless otherwise stated, all experiments were performed at least three times and the data were combined for presentation as mean ± SEM. All differences not specifically indicated as significant were not significant (ns, p > 0.05). Significant value are indicated as *, p < 0.05; **, p < 0.01. Statistical details, including sample size (n), are reported in the Figures and Figure Legends.

**Microscopy**

For scoring marker positive bacteria three independent experiments with three replicates each were performed. Bacteria were scored by visual enumeration as n > 100 (for 1h p.i.), n > 200 (for 2h p.i.) or n > 300 (for 3h p.i.) bacteria per replicate. Graphs show mean ± SEM.

**Scoring Intracellular Bacteria**

To score bacterial burdens, cells from triplicate wells were lysed and bacteria were plated in duplicate on TYE agar. Each experiment was performed three times. Bacterial colonies were counted using the aCOLyte3 system (Synbiosis).

Graphs show mean ± SD of a representative experiment or mean ± SEM for combined datasets.

## DATA AND SOFTWARE AVAILABILITY

The accession number for the DNA microarray data reported in this paper is NCBI Gene Expression Omnibus, GEO: GSE103363.