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RESEARCH ARTICLE

The GBP1 microcapsule interferes with IcsA-dependent septin cage assembly around *Shigella flexneri*

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One sentence summary: Guanylate-binding protein 1 microcapsules and septin cages are complementary antimotility factors targeting the enteric pathogen *Shigella flexneri*.

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ABSTRACT

Many cytosolic bacterial pathogens hijack the host actin polymerization machinery to form actin tails that promote direct cell-to-cell spread, enabling these pathogens to avoid extracellular immune defenses. However, these pathogens are still susceptible to intracellular cell-autonomous immune responses that restrict bacterial actin-based motility. Two classes of cytosolic antimotility factors, septins and guanylate-binding proteins (GBPs), have recently been established to block actin tail formation by the human-adapted bacterial pathogen *Shigella flexneri*. Both septin cages and GBP1 microcapsules restrict *S. flexneri* cell-to-cell spread by blocking *S. flexneri* actin-based motility. While septins assemble into cage-like structures around immobile *S. flexneri*, GBP1 forms microcapsules around both motile and immobile bacteria. The interplay between these two defense programs remains elusive. Here, we demonstrate that GBP1 microcapsules block septin cage assembly, likely by interfering with the function of *S. flexneri* IcsA, the outer membrane protein that promotes actin-based motility, as this protein is required for septin cage formation. However, *S. flexneri* that escape from GBP1 microcapsules via the activity of IpaH9.8, a type III secreted effector that promotes the degradation of GBPs, are often captured within septin cages. Thus, our studies reveal how septin cages and GBP1 microcapsules represent complementary host cell antimotility strategies.

Keywords: guanylate-binding protein; septin; actin; *Shigella flexneri*; GBP1; interferon

INTRODUCTION

Microbial pathogens escape extracellular immune responses and cell-autonomous immunity by a variety of evasion strategies, which include hijacking the actin cytoskeleton to promote inter- and intracellular motility. To promote actin-based motility, pathogens evolved different strategies to hijack the host actin polymerization machinery (Welch and Way 2013; Lamason and Welch 2017; Koseoglu and Agaisse 2019; Dowd, Mortuza and Ireton 2021). Actin-based motility of the human-adapted

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bacterial pathogen *Shigella flexneri* depends on IcsA, a polar outer membrane protein. IcsA binds to and activates proteins involved in actin polymerization, leading to the formation of comet-like actin structures, referred to as actin tails (Bernardini *et al.* 1989; Goldberg *et al.* 1993; Suzuki *et al.* 1998; Egile *et al.* 1999; Mauricio *et al.* 2017).

The force of polymerizing actin propels *S. flexneri* through the cytosol and enables the formation of bacteria-containing host cell plasma membrane protrusions. These protrusions are engulfed by neighboring cells enabling *S. flexneri* to spread directly from cell to cell, thus evading extracellular immune defenses while disseminating throughout the colonic epithelium (Agaisse 2016). In order to contain *S. flexneri* infections, the host requires effective cell-autonomous immune programs targeting cytosolic bacteria. At least two classes of cytosolic proteins interact with *S. flexneri* inside infected epithelial cells and interfere with actin tail formation: interferon (IFN)-inducible guanylate-binding proteins (GBPs) and ubiquitously expressed septins.

GBPs are dynamin-related large GTPases that show broad antimicrobial functions against viral, protozoan and bacterial pathogens (Daumke and Praefcke 2016; Ngo and Man 2017; Praefcke 2018; Gomes *et al.* 2019; Kutsch and Coers 2020). Gram-negative bacteria are specifically recognized by the founding member of the GBP family, GBP1, which binds directly to bacterial lipopolysaccharide (LPS) (Kutsch *et al.* 2020; Santos *et al.* 2020). LPS consists of a lipid A moiety, inner and outer core sugars, and the O-antigen polysaccharide. LPS molecules are anchored via their lipid A moieties in the bacterial outer membrane, whereas their outward-facing O-antigens form a chemophysical barrier (Simpson and Trent 2019; Giordano, Cian and Dalebroux 2020). Polymerizing GBP1 binds to LPS and forms a uniform protein coat that encapsulates the bacterial cell. This GBP1 microcapsule acts as a surfactant emulsifying the protective LPS barrier and thus interferes with the integrity of the bacterial envelope (Kutsch *et al.* 2020).

The LPS barrier not only protects Gram-negative bacteria from antimicrobials but also reduces outer membrane fluidity, mainly through lateral interactions of O-antigen side chains (Herrmann *et al.* 2015; Rojas *et al.* 2018). The resulting decreased diffusion of outer membrane proteins helps to position IcsA at one bacterial pole, an important prerequisite for efficient actin tail formation and cell-to-cell spread by *S. flexneri* (Robbins *et al.* 2001). Furthermore, direct interaction of IcsA with O-antigen has been proposed to favor an IcsA conformation required for interactions with the host actin polymerization machinery (Van den Bosch and Morona 2003). GBP1 blocks *S. flexneri* actin tail formation and tethers bacteria to immobilized clusters, ultimately blocking bacterial dissemination (Piro *et al.* 2017; Wandel *et al.* 2017). Mechanistically, GBP1 as an LPS-binding surfactant interferes with the polar localization and function of IcsA (Kutsch *et al.* 2020). *Shigella flexneri* can evade GBP1-mediated immunity via the action of IpaH9.8, an E3 ubiquitin ligase, an effector that is directly secreted via its type III secretion system into the cytosol of infected cells. IpaH9.8 promotes the modification of GBPs, including GBP1, with K48-linked polyubiquitin chains that target them for proteasomal degradation (Li *et al.* 2017; Piro *et al.* 2017; Wandel *et al.* 2017).

Septins are GTP-binding proteins that assemble into non-polar filaments as well as higher order structures on the inner surface of eukaryotic plasma membranes. Septins associate with the actin and microtubule cytoskeleton and play a central role in cell division, membrane remodeling and maintenance of cell polarity (Valadares *et al.* 2017; Addi, Bai and Echard 2018; Woods and Gladfelter 2020). More recently, septins have been characterized as important executioners of cell-autonomous immunity (Van Ngo and Mostowy 2019; Robertin and Mostowy 2020). Septins, including the ubiquitously expressed Septin 7 (Sept7), form cage-like structures around bacterial and viral pathogens to facilitate antimicrobial autophagy and to block microbial actin-based dissemination (Mostowy *et al.* 2010; Sirianni *et al.* 2016; Pfanzelter, Mostowy and Way 2018). Septins bind to anionic lipids present in the outer membrane of immobilized *S. flexneri*, which leads to the assembly of septin cages in an actin polymerization-dependent manner (Mostowy *et al.* 2010; Krokowski *et al.* 2018). Septins also form cage-like structures around bacterial actin tails, yet fail to trap and immobilize already motile bacteria (Mostowy *et al.* 2010). In contrast to GBPs, septins are not known to be targeted by bacterial E3 ubiquitin ligases. However, *S. flexneri* is able to break out of its surrounding septin cage, to regain motility, and to escape septin-mediated host defenses by a poorly defined mechanism (Mostowy *et al.* 2010).

Both GBP1 and septins efficiently block *S. flexneri* actin-based motility and restrict cell-to-cell spread. Functional interactions between these antimotility factors have not been reported previously. Here, we demonstrate that the assembly of GBP1 microcapsules and septin cages around cytosolic *S. flexneri* occurs independently. However, we find that GBP1 microcapsules block IcsA-dependent assembly of septin cages. Accordingly, the frequency of septin cage formation is significantly reduced for bacteria that lack bacterially secreted GBP1 inhibitor IpaH9.8 and are therefore efficiently encapsulated by GBP1. Together, our study reveals that GBP1 microcapsules and septin cages function as complementary defense programs to restrict *S. flexneri* actin tail formation.

MATERIALS AND METHODS

Cell lines

GBP1-deficient HeLa cells ($HeLa^{GBP1\text{-}KO}$) were previously described (Piro *et al.* 2017). $HeLa^{GBP1\text{-}KO}$ cells were stably transduced with an anhydrotetracycline (aTc)-inducible gene expression system to drive the expression of mCherry-GBP1. $HeLa^{GBP1\text{-}KO}$ cell lines and parental wild-type HeLa cells (Piro *et al.* 2017) were cultivated in Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Corning, Corning, NY, USA), 1% non-essential amino acids (Gibco, Thermo Fisher Scientific) and 55 μM β-mercaptoethanol (Gibco, Thermo Fisher Scientific) at 37°C and 5% CO_2.

Bacterial strains and culture conditions

Shigella flexneri 2547T strains were grown on tryptic soy broth (TSB) agar supplemented with Congo Red and antibiotics, as needed (50 μg/ml carbenicillin, 30 μg/ml kanamycin), for 12–16 h at 37°C. For infection experiments, overnight cultures of *S. flexneri* were diluted 1:30 in fresh TSB and grown for an additional 1–1.5 h to an OD_{600} of 0.3–0.7. Bacteria were then harvested at 2400 × *g* for 3 min, washed once with 1× phosphate buffered saline (PBS) (Gibco, Thermo Fisher Scientific) and treated with 100 ng/ml poly-D-lysin hydrobromide (Millipore, Sigma-Aldrich, Burlington , MA, USA) in 1× PBS for 15 min to enhance infectivity.

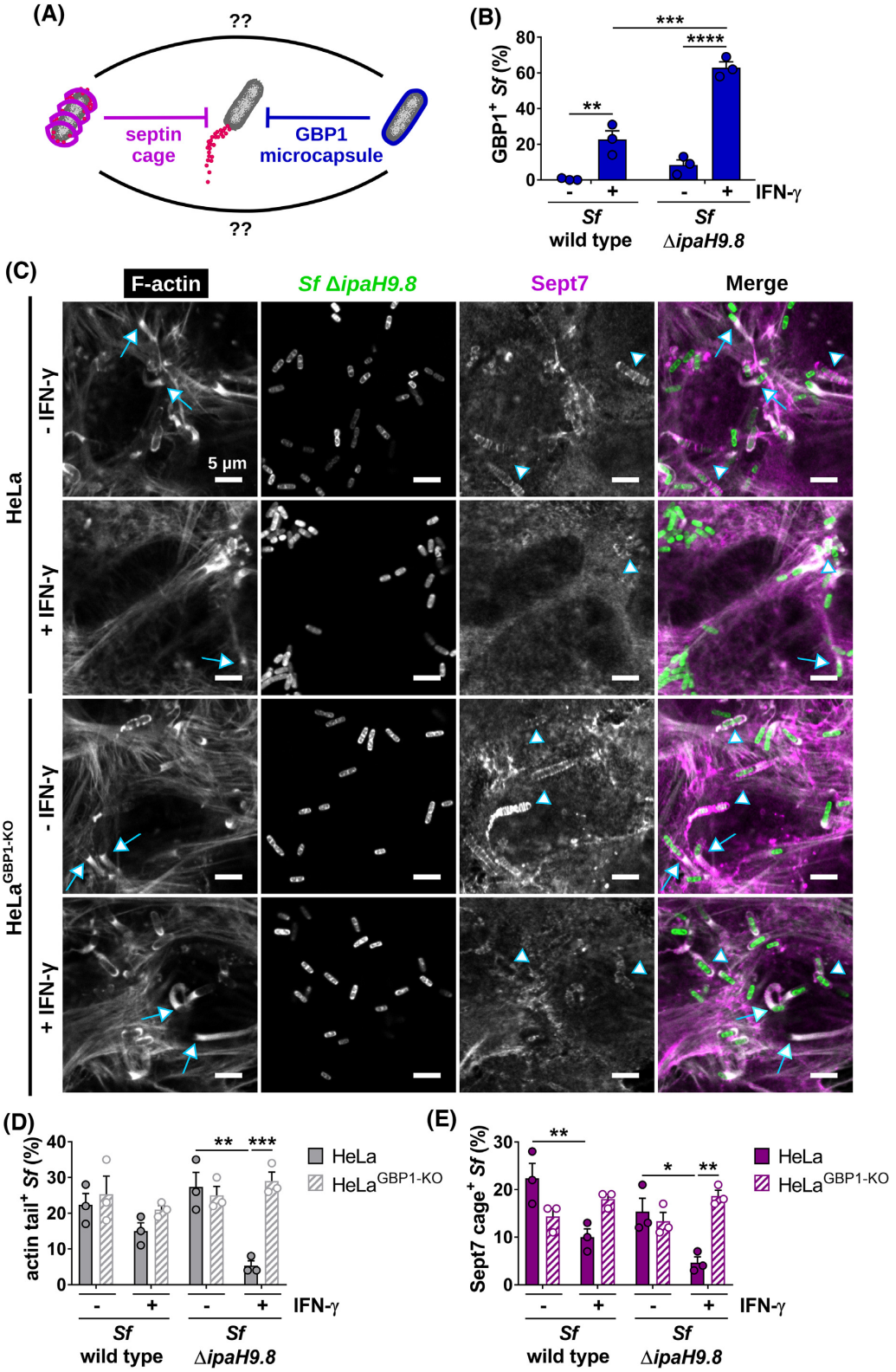

Figure 1. IFNγ priming reduces septin caging in a GBP1-dependent manner. **(A)** Septins and GBP1 block *S. flexneri* actin tail formation by entrapping the bacteria in septin cages or GBP1 microcapsules. **(B)** HeLa cells either left untreated or treated with IFNγ to stimulate GBP1 expression were infected with either *S. flexneri* wild type or coisogenic *S. flexneri* Δ*ipaH9.8* for 2.5 h, and immunostained for endogenous GBP1. Bacteria targeted by GBP1 were quantified. **(C–E)** HeLa wild-type and GBP1-deficient (HeLa$^{GBP1\text{-}KO}$) cells were either left untreated or treated with IFNγ and infected for 2.5 h with *S. flexneri* wild type or Δ*ipaH9.8* (only representative images of Δ*ipaH9.8* infections are shown in panel C), and immunostained for filamentous actin and endogenous Sept7. Bacteria with actin tails **(D)** and Sept7 cages **(E)** were quantified. **(B, D, E)** Mean frequencies ± SEM (standard error of the mean) of combined data from three independent experiments are shown. Significance was determined by two-way ANOVA with Tukey's multiple comparison test. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. Actin tails were classified as tails when ≥2.5 µm. Arrows point at actin tails; arrow heads point at septin cages. All scale bars equal 5 µm.

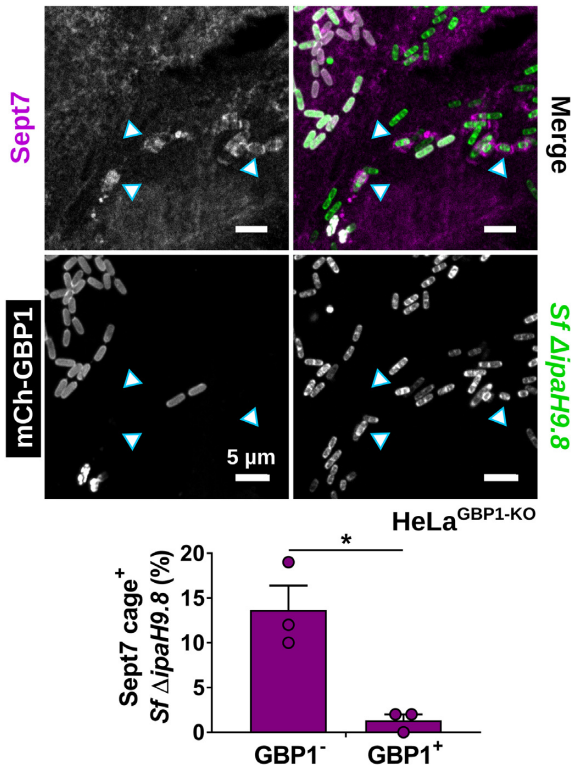

Figure 2. GBP1 microcapsules around *S. flexneri* interfere with septin caging. HeLa$^{GBP1\text{-}KO}$ cells stably expressing mCherry-GBP1 were infected with *S. flexneri* Δ*ipaH9.8*, fixed after 2.5 hpi, and immunostained for endogenous Sept7. Co-localization of GBP1 and Sept7 on bacteria was quantified. Mean frequencies ± SEM of combined data from three independent experiments are shown. Significance was determined by unpaired two-tailed *t*-test. $^{*}P \leq 0.05$. Arrow heads point at septin cages. All scale bars equal 5 μm.

Shigella flexneri strain construction

Shigella flexneri Δ*ipaH9.8* was previously reported (Piro *et al.* 2017). To generate *S. flexneri* Δ*ipaH9.8*Δ*icsA*, the KAN^R cassette from Δ*ipaH9.8*::*FRT-KAN^R-FRT* (Piro *et al.* 2017) was removed by expressing Flp recombinase via cell transduction with plasmid pCP20 (Cherepanov and Wackernagel 1995). Next, the FRT::KAN^R::FRT from Δ*icsA*::*FRT-KAN^R-FRT* was PCR amplified and introduced into Δ*ipaH9.8* using the lambda red recombination system (Datsenko and Wanner 2000). To transform *S. flexneri* Δ*ipaH9.8*Δ*icsA* with a fluorescent plasmid, bacteria were prepared for electroporation and transformed with pEGFPmut2 (Cormack, Valdivia and Falkow 1996), as described previously (Warren 2011).

Bacterial cell culture infections

Cell lines were cultivated on glass coverslips in 24-well plates and were either treated with 200 U/ml IFNγ (Millipore, Sigma-Aldrich) or 1–2 μg/ml aTc (Takara), or left untreated for 20–22 h. Cells were then infected at a multiplicity of infection (MOI) of 6. Poly-D-lysine was used to coat bacteria and thereby enhance bacterial attachment to host cells, as previously described (Enninga *et al.* 2005). Poly-D-lysine-treated bacteria were resuspended in prewarmed cell culture media and spun on cells for 10 min at 700 × *g*. Infected cells were incubated for 45 min at 37°C and 5% CO_2. Following two washes with 1× Hanks' Balanced Salt Solution (Gibco, Thermo Fisher Scientific), cells were incubated with cell culture media containing 25 μg/ml gentamycin for 2.5 h at 37°C and 5% CO_2. At this point, cells were fixed with 4% formaldehyde at room temperature (RT) and washed three times with 1× PBS for immunofluorescence staining.

Immunofluorescence microscopy

Endogenous GBP1 and Sept7 were stained with rabbit monoclonal anti-GBP1 immunoglobulin G (IgG) (Abcam; ab131255, diluted 1:150) or rabbit anti-human Septin 7 (C) IgG (IBL America; catalog no. 18 991, 5 μg/ml final concentration). Cells were permeabilized with 0.25% Triton X-100 in 1× PBS for 10 min at RT and washed three times with 1× PBS. Permeabilized cells were blocked with blocking buffer (1× PBS, 50 mg/ml BSA, 300 mM glycine) for at least 45 min. Cells were treated with primary antibodies diluted in blocking buffer at 4°C overnight. Following incubation with primary antibody, cells were washed with 0.05% Triton X-100 in 1× PBS three times for 5 min and then treated with 1:1000 dilution of anti-rabbit IgG conjugated with Alexa Fluor 568 or Alexa Fluor 660 in blocking buffer for 1 h at RT. F-actin was stained with Alexa Fluor 660 phalloidin (Thermo Fisher Scientific) diluted 1:40 in blocking buffer for 1 h at RT. Cells were washed three times with 0.05% Triton X-100 for 5 min and then mounted on microscopy slides with mounting media (100 mM Tris–HCl, pH 8.5, 25% glycerol, 125 μg/ml Mowiol) diluted 9:1 with 0.1 mg/ml *para*-phenylenediamine antifading agent. Processed slides were imaged with either a Leica STED and Confocal on Leica DMi8 motorized inverted microscope using a Zeiss Plan-Apochromat 40×/1.3 oil objective or a Zeiss 880 AiryScan Fast Inverted Confocal on AxioObserver Z1 microscope using a Zeiss Plan-Apochromat 63×/1.4 oil objective. Z-stacks were acquired with an interval of 0.5 μm. Images were processed with Fiji.

RESULTS

Both GBP1 encapsulation and septin caging block *S. flexneri* actin tail formation, but their functional relationship has remained unexplored (Fig. 1A). GBP1-mediated inhibition of actin-based motility is inhibited by *S. flexneri* IpaH9.8. Confirming previous reports (Li *et al.* 2017; Piro *et al.* 2017; Wandel *et al.* 2017), we observed that a *S. flexneri* mutant lacking IpaH9.8, Δ*ipaH9.8* *S. flexneri*, is more frequently enveloped by IFNγ-induced GBP1 protein than wild-type bacteria (Fig. 1B). Affirming the anti-motility effect of the GBP1 microcapsule, we detected substantially reduced actin tail formation by GBP1-susceptible Δ*ipaH9.8* compared with wild-type *S. flexneri* at 2.5 h post-infection (hpi) (Fig. 1C and D; Figs S1 and S2, Supporting Information). Actin tail formation by Δ*ipaH9.8* *S. flexneri* is restored in GBP1-deficient cells (Fig. 1C and D; Fig. S2, Supporting Information), demonstrating that the IFNγ-induced loss of actin-based motility by a Δ*ipaH9.8* *S. flexneri* mutant is due to an effective GBP1-mediated cell-autonomous host defense pathway.

Since the surfactant activity of GBP1 promotes the recruitment of other host factors such as lipid A-binding caspase-4 to the bacterial surface (Fisch *et al.* 2019, 2020; Kutsch *et al.* 2020; Santos *et al.* 2020; Wandel *et al.* 2020), we hypothesized that GBP1 microcapsules could similarly promote the recruitment of septins to *S. flexneri*, possibly through the unmasking of bacterial outer membrane-resident anionic lipids such as cardiolipin that were previously shown to serve as septin-binding substrates (Krokowski *et al.* 2018). To test this hypothesis, we stained cells with antibodies against Sept7, a septin cage subunit required for the incorporation of the additional septins

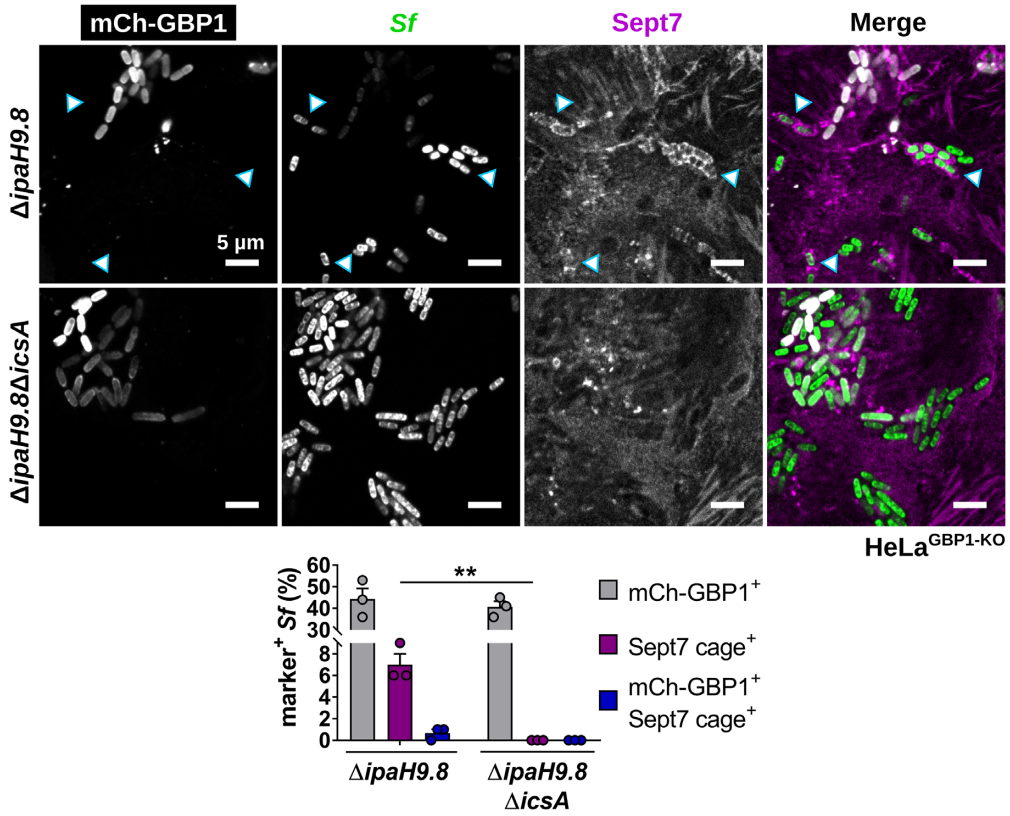

Figure 3. Septin caging but not GBP1 encapsulation of *S. flexneri* depends on IcsA. HeLa$^{GBP1\text{-}KO}$ cells stably expressing mCherry-GBP1 were infected with *S. flexneri* Δ*ipaH9.8* or Δ*ipaH9.8*Δ*icsA*, fixed after 2.5 hpi, and immunostained for endogenous Sept7. Bacteria caged by Sept7 and encapsulated by GBP1 as well as co-localization of Sept7 and GBP1 on bacteria were quantified. Mean frequencies ± SEM of combined data from three independent experiments are shown. Significance was determined by multiple unpaired two-tailed *t*-tests. $^{**}P \leq 0.01$. Arrow heads point at septin cages. All scale bars equal 5 µm.

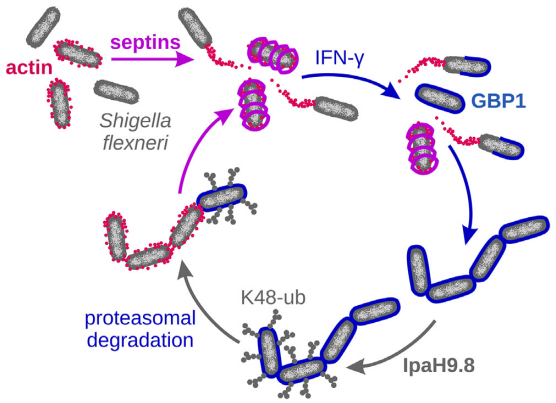

Figure 4. GBP1 microcapsules and septin cages are complementary antimotility factors targeting *S. flexneri*. Actin halos around immobile *S. flexneri* promote the assembly of septin cages. In IFNγ-primed human epithelial cells, GBP1 forms microcapsules around both motile and immobile bacteria. These GBP1 microcapsules block IcsA-mediated actin halo and actin tail formation and supersede IcsA-dependent septin caging. Wild-type *S. flexneri* secretes the type III secreted effector IpaH9.8 that tags GBP1 with K48-linked ubiquitin for proteasomal degradation. The IpaH9.8-mediated evasion of GBP1-driven host defense enables *S. flexneri* to form actin halos and thereby renders the bacteria again susceptible for septin caging.

Sept2 and Sept9 (Sirianni *et al.* 2016). We observed that the frequency of septin cage formation around the Δ*ipaH9.8 S. flexneri* was not increased but rather diminished in IFNγ-primed wild-type cells, thus refuting our initial hypothesis. Furthermore, we found that Sept7 cage formation around the *S. flexneri* Δ*ipaH9.8* mutant was restored in GBP1-deficient cells (Fig. 1C and E). Together, these results strongly indicated that, instead of promoting septin cage assembly, GBP1 microcapsules block septin cage formation around *S. flexneri*. To further test this hypothesis, we ectopically expressed mCherry-GBP1 and monitored co-localization of GBP1 with Sept7 cages. We found that bacteria staining positive for GBP1 mostly lacked Sept7 cages (Fig. 2), lending further support that GBP1 microcapsules surrounding cytosolic *S. flexneri* impede septin cage assembly.

Next, we investigated whether septin cages block GBP1 microcapsule formation. To address this question, we took advantage of previous observations demonstrating that septin cage formation not only requires septins to interact with bacterial lipids such as cardiolipin but is also dependent on bacterially driven actin recruitment and polymerization (Mostowy *et al.* 2010). Specifically, it had previously been reported that septins fail to encase *S. flexneri* in cells treated with actin capping or sequestering factors, and furthermore that septin cages were absent from *S. flexneri* mutants deficient for the actin polymerization factor IcsA (Mostowy *et al.* 2010). We therefore revisited the role of IcsA in septin cage and GBP1 microcapsule assembly by using *S. flexneri* strains deficient for either just the GBP1 inhibitor IpaH9.8 or both IpaH9.8 and IcsA. As expected, based on previous reports (Mostowy *et al.* 2010), we found that septin cages were not detectable when cells were infected with the IcsA-deficient Δ*ipaH9.8*Δ*icsA* mutant (Fig. 3). Because *S. flexneri* Δ*ipaH9.8*Δ*icsA* lacks the GBP1 inhibitor IpaH9.8 and is deficient for septin cage assembly, we were able to investigate whether

the absence of septin cages impacts GBP1 microcapsule assembly. We found that Δ*ipaH9.8* and Δ*ipaH9.8*Δ*icsA S. flexneri* were encapsulated by GBP1 at comparable frequencies, therefore indicating that septin cages do not interfere with GBP1 recruitment. Collectively, these data demonstrate that GBP1 microcapsules form independently of septin cages and that GBP1 microcapsules act as the dominant host defense program directed at inhibiting *S. flexneri* motility in IFNγ-primed human epithelial cells.

DISCUSSION

Septins and GBPs have previously been identified as host defense proteins that bind to cytosolic *S. flexneri* and interfere with the ability of the pathogen to disseminate via actin-based motility. Here, we explored the functional relationship between these two classes of host defense proteins and demonstrate that GBP1 microcapsules around *S. flexneri* form independent of septin cages, but once formed, block the IcsA-dependent assembly of septin cages. We further show that *S. flexneri* mutants deficient for the GBP1 inhibitor IpaH9.8 become more susceptible to septin caging in IFNγ-primed cells, thus revealing how a complementary septin-mediated host defense is directed at *S. flexneri* that escape GBP1-mediated immunity.

Septins assemble into polymeric supramolecular structures, including rings and filaments. Other components of the cytoskeleton such as actin polymers and microtubuli often serve as templates for the formation of long septin filaments (Valadares *et al.* 2017; Addi, Bai and Echard 2018; Woods and Gladfelter 2020). Previous work by Mostowy *et al.* (2010) demonstrated that IcsA-mediated actin assembly around the bacterial cell, often referred to as actin halos or actin clouds, is required for septin caging. We confirm these observations and show that septin cages fail to assemble around IcsA-deficient bacteria simultaneously deficient for the GBP1 inhibitor IpaH9.8. Our present study further shows that GBP1 microcapsules block septin cage formation, most likely through interference with IcsA function and the consequential lack of a bacteria-associated actin template needed for septin cage formation. While GBP1 impedes septin caging, the absence of septin cages from Δ*ipaH9.8*Δ*icsA S. flexneri* mutants does not result in an increase in the number of bacteria trapped inside GBP1 microcapsules, arguing that septins do not interfere with the recruitment of GBP1 to the bacterial surface.

Collectively, our observations reveal that septin- and GBP1-mediated inhibition of actin-based *S. flexneri* motility function as complementary defense programs (Fig. 4). Septins form cage-like structures around invading *S. flexneri*, thereby blocking actin tail formation. Following activation of innate immunity signaling and subsequent upregulation of GBP1 expression, the formation of GBP1 microcapsules supersedes septin-mediated defenses. However, bacteria that escape from GBP1-mediated immunity through the activity of the *S. flexneri* virulence factor IpaH9.8 become targets for the formation of septin cages. These observations underpin the fitness advantages provided by the evolution of functionally overlapping host defense programs.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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AUTHOR CONTRIBUTION

MK and JC conceived the project. MK conducted the experiments. MK and JC wrote the manuscript. CG-P and CFL provided reagents. JC supervised the project. JC and CFL acquired funding related to the project.

Conflict of Interest. None declared.

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