<u>Virus-like particle capture reveals coordination of actin remodeling during Shigella</u>

<u>flexneri entry by host proteins</u>

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<u>Abstract</u>

Shigella spp. are intracellular bacterial pathogens that enter the host via plasma membrane

insertion of a type 3 secretion system (T3SS) translocon, which triggers signaling cascades that

include modulation of cytoskeletal dynamics, resulting in bacterial uptake. To better understand

translocon insertion-induced host processes, we adapted a method to capture in virus-like

particles (VLP) host proteins that are recruited to the cytosolic face of natively delivered *S.*

flexneri translocons. Proteomic analyses reveal enrichment of 14-3-3ζ, a signaling protein, and

CAP2, a regulator of actin turnover. 14-3-3ζ and CAP2 are necessary for host entry by T3SS

pathogens. 14-3-3ζ dimers function as molecular scaffolds in the formation of bacterial-

associated membrane ruffles. Concurrently, CAP2 localizes to membrane ruffles and

cooperates with 14-3-3ζ to enable the formation of membrane ruffles that function efficiently in

bacterial uptake. The findings define a coordinated role for 14-3-3ζ and CAP2 in cytoskeletal

dynamics during T3SS pathogen infection.

Introduction

S. flexneri is an intracellular bacterial pathogen that gains entry into host cells via the

T3SS[1]. The T3SS, a conserved virulence strategy, encodes a nanomachine that delivers into

the cytosol of host cells effector proteins that facilitate bacterial uptake into host cells and

subsequent infection[2-6]. IpaB and IpaC, the first two proteins secreted through the T3SS upon *S.*

flexneri contact with host cells, are translocases that form a heterooligomeric translocon in the

plasma membrane. The T3SS translocon is highly conserved among T3SS pathogens and

constitutes an early interface between the pathogen and the host cell[4,7,8]. The *S. flexneri*

translocon interacts with host proteins and, in addition to serving as the conduit for effectors,

controls their secretion from the bacterium into the cell[9-11].

Plasma membrane insertion of translocons initiates a cascade of host responses that

culminate in bacterial uptake by plasma membrane ruffling (micropinocytosis)[12]. Upon

membrane insertion, the translocase IpaC interacts with host intermediate filaments, inducing

conformational changes in the translocon that allow docking of the T3SS needle onto the

pore[8,13]. Concurrently, actin polymerization is required to open the pore channel for delivery of

effector proteins into the host cytosol[10]. Several *S. flexneri* T3SS effectors remodel the host

cytoskeleton at the start of infection. IpaA binds to and activates host vinculin, triggering F-actin

polymerization[14,15], IpgB1 stimulates Rac1/Cdc42 to promote actin polymerization at entry sites,

initiating membrane ruffle formation[16], and VirA interacts with calpain to modulate bacterial

entry[17].

To extend our understanding of host processes that are triggered by translocon insertion

and which contribute to bacterial entry, we identified host proteins that localize to sites of *S.*

flexneri engagement with host plasma membranes. To do so, we captured in VLPs host proteins

that accumulated at the cytosolic face of membrane-embedded translocons[18,19]. Proteomic

analysis of these VLPs demonstrated enrichment of 14-3-3ζ, a protein involved in regulating

cytoskeletal dynamics, at the cytosolic face of translocons. We found that upon *S. flexneri*

engagement with host plasma membranes, 14-3-3ζ acts as a molecular scaffold, contributing to

the expansion of the membrane ruffles that enable bacterial entry. Furthermore, we found that

CAP2, an actin capping protein that promotes polymerization of branched actin networks, is

enriched to *S. flexneri* membrane ruffles, dependent on 14-3-3ζ, and like 14-3-3ζ, is required for

efficient *S. flexneri* entry.

Results

Adaptation of virus-like particle generation for capturing membrane-embedded *Shigella*

***flexneri* translocons**

To investigate host protein engagement with membrane-embedded *S. flexneri*

translocons, we modified Virotrap, a method that employs HIV-1 Gag to capture plasma

membrane and associated molecules within virus-like particles (VLPs) (Fig. 1a)[18,19]. Gag is

sufficient to induce the budding of membrane-bound vesicles. HIV-1 Gag-transfected HeLa

monolayers were infected with *S. flexneri* expressing a derivative of the translocase IpaB that is

FLAG-tagged at the C-terminal extracellular domain.

The presence of plasma membrane-localized Gag did not sterically hinder native

delivery, membrane insertion, or function of translocon complexes (Extended Data Fig. 1).

Assembly of translocons and effector delivery into Gag-transfected HeLa cells were comparable

to those of untransfected cells (Extended Data Fig. 1a,b,c,d). VLPs containing FLAG-tagged

translocons were selectively enriched from the culture supernatant of infected cells (Extended

Data Fig. 1e). These data indicate that expression of Gag does not hinder *S. flexneri* translocon

insertion, assembly, or function. Furthermore, this modified Virotrap approach enables targeted

selection of translocon-containing VLPs for downstream analyses.

VLPs generated during *S. flexneri* infection were collected, and the subset that

contained membrane-embedded translocons were enriched from the total VLP population via

FLAG immunoprecipitation. VLP proteomes were analyzed by mass spectrometry.

Cytoskeletal proteins are enriched at the cytosolic face of functionally stalled T3SS

translocons

We collected in parallel VLPs containing functionally wildtype translocons and VLPs

containing functionally stalled translocons. Stalled translocons were achieved by expressing *S.*

flexneri IpaC R362W (Fig. 1b), a mutant IpaC derivative that locks the translocon in a docking

incompetent state[13]. Stalled translocons theoretically promote accumulation of host proteins that

participate in protein interactions at the cytosolic face of translocons[20], as a result of delays in

membrane ruffling and bacterial uptake. Collected VLPs were analyzed by mass spectrometry.

IpaB and IpaC were detected in abundance (32-34 peptides and 10-12 peptides detected,

respectively) in VLPs produced during *S. flexneri* infection compared to VLPs produced by

uninfected HeLa monolayers, indicating the presence of translocons specifically in infection-

associated VLPs. Also identified in infection-associated VLPs, but not in statistically significant

abundances, were host cytoskeletal components known to be involved in *S. flexneri* infection,

including actin (ACTA), vimentin, and spectrin (data available at the Proteomics Identifications

Database, PRIDE), indicating that this approach is an effective method for capturing infection-

associated host proteins.

When compared with VLPs produced from uninfected HeLa monolayers, VLPs

containing functionally wildtype translocons displayed significantly increased abundance of

cytoskeletal components (adenylyl cyclase-associated protein [CAP]1, CAP2), whereas VLPs

containing functionally stalled translocons displayed significantly increased abundance of some

of the same cytoskeletal components (CAP2), additional cytoskeletal components (ACTN1), and

intracellular signaling proteins (ANXA3, S100A14, YWHAB [14-3-3β/ζ]) (Fig. 1c).

14-3-3ζ is required for efficient *S. flexneri* entry into host cells

14-3-3 proteins are present in all eukaryotic cell types and function as molecular

scaffolds, facilitating numerous cellular processes and signaling pathways[21,22]. Their scaffolding

functions depend on dimerization and binding to phosphoserine and phosphothreonine. In

bacterial pathogenesis, 14-3-3 proteins are necessary for activation of *Pseudomonas*

aeruginosa ExoS activity[23].

Seven 14-3-3 isoforms exist, of which 14-3-3β and 14-3-3ζ share high sequence

similarity[24,25]. In the proteome of VLPs from stalled translocons, the m/z ratios and

corresponding sequences identified by mass spectrometry mapped to peptides present in both

14-3-3β and 14-3-3ζ, indicating that either or both isoforms might be associated with *S. flexneri*

translocons. To discriminate which isoform was enriched by mass spectrometry, we generated

HeLa cells lacking 14-3-3ζ. Loss of 14-3-3ζ significantly hindered bacterial adherence and entry.

Compared to wildtype (WT) HeLa monolayers, *S. flexneri* entry into HeLa 14-3-3ζ$^{-/-}$ monolayers

was reduced by 50% ± 12 and 57% ± 5, as determined by gentamicin protection and plaque

assays, respectively (Fig. 2a, Extended Data Fig. 3a,b), whereas bacterial cell-to-cell spread

was not defective (Extended Data Fig. 3c). These data indicate that 14-3-3ζ is specifically

required during *S. flexneri* entry and that 14-3-3β is non-redundant for this function. They also

suggest that 14-3-3ζ is the isoform enriched in the VLPs from stalled translocons.

Complementation of 14-3-3ζ rescued the bacterial entry and adherence phenotypes (Fig. 2a,

Extended Data Fig. 3a). These results indicate that 14-3-3ζ is required for *S. flexneri* entry but

not spread.

14-3-3ζ functions as a molecular scaffold to support formation of bacterial-induced

membrane ruffles

S. flexneri entry occurs within seconds to minutes of cell contact[13]. Membrane-inserted

T3SS translocases interact with host intermediate filaments[13], enabling delivery of effectors into

the cytoplasm that induce membrane ruffle formation and bacterial uptake (Fig. 1b). To identify

the step at which 14-3-3ζ contributes to entry, we tested the impact of the absence of 14-3-3ζ

on specific steps in bacterial entry. In the absence of 14-3-3ζ, *S. flexneri* efficiently delivered and

assembled functional, membrane-embedded translocons, indicating that the observed 14-3-3ζ-

dependent defects in entry were not due to translocase instability or loss of function of

membrane-embedded translocons (Extended Data Fig. 3d,e). However, the formation of

membrane ruffles was defective; whereas membrane ruffles were present at infection foci in

HeLa 14-3-3ζ[-/-] monolayers (Fig. 2b), those ruffles that formed were significantly smaller than

the ruffles that formed at infection foci in WT HeLa monolayers (Fig. 2c,d).

To test whether the scaffolding function of 14-3-3ζ contributes to *S. flexneri* membrane

ruffle formation, we generated 14-3-3ζ mutants defective in dimerization[26], vimentin binding[27],

and binding to phosphorylated proteins[28] (Table 1). Complementation of HeLa 14-3-3ζ[-/-] cells

with HA-14-3-3ζ (WT) rescued the size of membrane ruffles to that observed in WT HeLa cells,

whereas complementation with none of the 14-3-3ζ mutants rescued the defect (Fig. 3,

Extended Data Fig. 2c,d). These data indicate that efficient membrane ruffle formation during *S.*

flexneri entry requires dimerization and protein interaction capabilities of 14-3-3ζ, consistent

with 14-3-3ζ functioning as a molecular scaffold for *S. flexneri* entry.

Host proteins that depend on 14-3-3ζ for recruitment to translocons

To identify proteins that potentially function with 14-3-3ζ in *S. flexneri* entry, we

compared VLP proteomes from WT HeLa monolayers to those of HeLa 14-3-3ζ[-/-] monolayers,

each infected with *S. flexneri* expressing functionally WT translocons, *S. flexneri* expressing

functionally stalled translocons, or *S. flexneri* Δ*ipaC* (which do not form translocons due to lack

of the translocase IpaC) (Fig. 4a, Supplementary Table 1). During infection with *S. flexneri*

expressing WT and stalled translocons, CAP2 abundance was significantly enriched in VLPs

derived from WT HeLa monolayers compared with those derived from HeLa 14-3-3ζ[-/-]

monolayers, indicating that CAP2 accumulation at the cytosolic face of translocons depends at

least in part on 14-3-3ζ. This enrichment was not observed in VLPs from uninfected or *S.*

flexneri ∆ipaC-infected monolayers (Fig. 4b), indicating that CAP2 abundance in the cell cortex

is dependent on the presence of assembled translocons.

CAP2 localizes to membrane-engaged *S. flexneri* in a membrane ruffle-dependent

manner

CAP2 is one of several proteins that control the cellular cytoskeletal actin network[29].

During *S. flexneri* infection, CAP2 colocalized with actin foci in bacteria-associated membrane

ruffles in a 14-3-3ζ-independent manner (Pearson's R = 0.79 ± 0.21 and 0.66 ± 0.19 in WT and

14-3-3ζ$^{-/-}$ cells, respectively) (Fig. 5a,b). However, VLP proteomics (Fig. 4a), which may be more

sensitive than immunofluorescence, showed that CAP2 accumulation is dependent in part upon

the presence of 14-3-3ζ. In uninfected cells, CAP2 was distributed throughout the cytosol, only

weakly colocalized with cortical actin, indicating that CAP2 enrichment in the actin cortex

depends on factors involved in bacterial entry. These results indicate that bacterial engagement

with the plasma membrane triggers CAP2 localization to bacterial-induced membrane ruffles in

a manner that is likely enhanced by 14-3-3ζ.

T3SS-induced membrane ruffle formation is coordinately regulated by 14-3-3ζ and CAP2

Using HeLa CAP2$^{-/-}$ and 14-3-3ζ$^{-/-}$ CAP2$^{-/-}$ cells, we tested the effect of CAP2 on bacterial

entry. Bacterial entry into CAP2$^{-/-}$ and 14-3-3ζ$^{-/-}$ CAP2$^{-/-}$ monolayers was reduced by 39% ± 9

and 48% ± 11, respectively, compared with HeLa WT monolayers, similar to the entry defect

observed for 14-3-3ζ$^{-/-}$ monolayers (41% ± 5) (Fig. 5c). This result indicates that both 14-3-3ζ

and CAP2 are required for efficient *S. flexneri* entry. The finding that the entry defect observed

during infection of 14-3-3ζ$^{-/-}$ CAP2$^{-/-}$ cells was not additive of the defects that occurred during

infection of 14-3-3$\zeta^{-/-}$ and CAP2$^{-/-}$ cells suggests that CAP2 and 14-3-3ζ act in the same

signaling pathway during T3SS pathogen entry.

Membrane ruffles formed during *S. flexneri* entry of HeLa 14-3-3$\zeta^{-/-}$ monolayers were

significantly smaller than those formed in WT HeLa monolayers (Fig. 2d). While absence of

CAP2 alone did not lead to significant changes in membrane ruffle size, bacterial-associated

membrane ruffles in HeLa 14-3-3$\zeta^{-/-}$ CAP2$^{-/-}$ monolayers were significantly larger than HeLa WT,

14-3-3$\zeta^{-/-}$, and CAP2$^{-/-}$ monolayers (Fig. 5d). Altogether these results suggest that both 14-3-3ζ

and CAP2 are involved in regulating membrane ruffle formation during bacterial uptake and

support a model where CAP2 activity in membrane ruffle formation is independent of 14-3-3ζ

but that CAP2 accumulation in membrane ruffles is enhanced by the scaffolding activity of 14-3-

3ζ dimers.

To determine whether our observations are relevant to other T3SS pathogens, we tested

the requirement for 14-3-3ζ and CAP2 during entry of *Salmonella enterica* Typhimurium SL1344

(*S.* Typhimurium). As with entry of *S. flexneri*, the absence of 14-3-3ζ and CAP2, both

individually and together, reduced *S.* Typhimurium entry by 49% ± 6, 39% ± 9, and 47% ± 9,

respectively, compared with WT HeLa monolayers (Fig. 5e). These data indicate that 14-3-3ζ

and CAP2 are required for efficient bacterial entry not only for *S. flexneri* but also for another

T3SS pathogen. Of note, where *S. flexneri*-induced membrane ruffles were significantly larger in

HeLa 14-3-3$\zeta^{-/-}$ CAP2$^{-/-}$ monolayers than in WT HeLa monolayers, *S.* Typhimurium-induced

membrane ruffles were significantly smaller in 14-3-3$\zeta^{-/-}$, CAP2$^{-/-}$, and 14-3-3$\zeta^{-/-}$ CAP2$^{-/-}$

monolayers than in WT HeLa monolayers (Fig. 5f).

Discussion

Among the challenges in identifying novel intracellular protein-protein interactions

relevant to bacterial infection of eukaryotic cells are the capture of transient or dynamic

interactions and discrimination of target complexes from the sheer volume of the cellular

proteome[30,31]. A tool that combats these challenges is Virotrap, a method that fuses the protein

target of interest to HIV Gag, which coopts the HIV budding mechanism, encapsulating both

stable and transient target protein complexes in extracellular VLPs that can be

immunoprecipitated for proteomic analyses[18,19]. Use of this approach to identify host factors

required for bacterial pathogenesis has been limited[32]. Whereas these previously published

Virotrap methods utilized FLAG-VSVG for VLP immunoprecipitation, here, to identify host

proteins specifically recruited to T3SS translocons during bacterial entry, we adapted Virotrap to

capture *S. flexneri* IpaB-FLAG-containing VLPs following native delivery of bacterial T3SS

translocons to HeLa cells that express Gag. Gag-expressing HeLa cells support translocon

insertion and production of translocon-containing VLPs, and the resulting VLPs yielded new

insights into host proteins that function in translocon-induced bacterial uptake, establishing that

this adapted Virotrap method is feasible and productive in the study of interactions between

bacterial transmembrane proteins and host proteins.

The plasma membrane-embedded T3SS translocon is a dynamic structure that

undergoes conformational changes upon interaction with the host cytoskeleton, leading to

membrane ruffling and bacterial internalization by micropinocytosis[10,13]. Several host factors that

contribute to these interactions have been described[10,13,14,16,17,33], yet the process remains

incompletely understood. By capturing in VLPs host proteins present at the cytosolic face of *S.*

flexneri translocons, we identified additional host factors required for *S. flexneri* manipulation of

host membranes and the cortical cytoskeleton (Fig. 1a)[4,34,35]. To maximize proteomic recovery of

biologically relevant host proteins recruited to the translocon, we leveraged our prior

identification of a translocon point mutant that forms functionally stalled translocons, thereby

locking the translocon in a docking incompetent state (Fig. 1b)[13].

Among the proteins we found enriched in the proteome of VLPs containing functionally

stalled translocons, and which was required for efficient bacterial entry and membrane ruffle

formation, was the 14-3-3 protein 14-3-3ζ. 14-3-3 proteins are small, soluble, intracellular

proteins that participate in signaling pathways via binding to phosphoserine and/or

phosphothreonine when in monomer or dimer form[21,22,36,37]. 14-3-3ζ is required for activation of

P. aeruginosa ExoS during infection[23], by mechanisms unrelated to its role in *S. flexneri* and *S.*

Typhimurium entry. Whereas 14-3-3 isoforms have been thought to be functionally redundant,

individual 14-3-3 isoforms display differential tissue specificity and subcellular localization[38]. All

isoforms are present in HeLa cells (Extended Data Fig. 2b), and we found a specific

requirement for 14-3-3ζ in *S. flexneri* and *S.* Typhimurium entry. Thus, with this study, we

attribute a novel function to 14-3-3ζ that is non-redundant with other isoforms.

14-3-3 proteins are maintained at steady levels in the cytoskeletal network via binding to

phosphorylated vimentin[27], an intermediate filament. *S. flexneri* T3SS translocons also bind

intermediate filaments[13], consistent with engagement of the same cytoskeletal compartment.

Our finding that 14-3-3ζ was significantly enriched in VLPs and was required for efficient

membrane ruffle formation during *S. flexneri* entry (Fig. 2) indicates that 14-3-3ζ contributes to

bacterial entry through modulation of the membrane ruffle cytoskeletal architecture.

Among their roles in signaling pathways, 14-3-3 proteins act as molecular scaffolds that

drive protein-protein interactions and signaling mediated by these interactions[21]. Several

mutations in 14-3-3, including defects in dimerization and vimentin binding, and abrogation of

the phospho-binding pocket, are known to cause specific functional defects (Table 1)[28].

Complementation of HeLa 14-3-3ζ[-/-] with each mutant construct did not rescue *S. flexneri-*

induced membrane ruffle morphology (Fig. 3, Extended Data Fig. 2c,d), indicating that 14-3-3ζ

functions as a molecular scaffold in membrane ruffles during infection and that 14-3-3ζ

interactions with other cytoskeletal components are critical for membrane ruffle formation.

To identify proteins that interact with 14-3-3ζ during bacterial entry, we compared the

proteomes of translocon-containing VLPs from HeLa WT cells against VLPs from 14-3-3ζ$^{-/-}$ cells

(Fig. 4, Supplementary Table 2). Among the proteins we found to be enriched and required for

efficient bacterial entry is CAP2, a capping protein that accelerates actin turnover by promoting

release of G-actin from the ends of actin filaments[39,40] and nucleotide exchange on G-actin[41,42],

which prepares the monomers for rapid incorporation into growing filaments. Cells lacking CAP2

exhibit increased membrane protrusions and focal adhesions and show defects in cell

migration[29]. CAP2 is best understood in the context of cardiac disease, as CAP2 mutations

and/or deletions are associated with dilated cardiomyopathy and dysfunction of the

sarcomere[43,44]. A role for CAP2 in bacterial infection has not been previously reported.

Based on our findings that absence of 14-3-3ζ or CAP2 alone lead to bacterial entry

defects, but that the dual absence of 14-3-3ζ and CAP2 did not result in an additive entry defect

(Fig. 5c), we propose that 14-3-3ζ and CAP2 act in the same pathway during bacterial entry.

Although we found by immunofluorescence microscopy that CAP2 localized to membrane

ruffles even in the absence of 14-3-3ζ (Fig. 5a), the marked decrease in enrichment of CAP2 in

VLPs from 14-3-3ζ$^{-/-}$ cells (Fig. 4a) indicates that CAP2 recruitment to sites of *S. flexneri* entry is

at least in part dependent on 14-3-3ζ, suggesting that 14-3-3ζ may act upstream of CAP2

during membrane ruffle formation. It is possible that in the absence of 14-3-3ζ, CAP2 is

recruited into the active cortical actin network, and that 14-3-3ζ brings CAP2 closer to the

plasma membrane, leading to improved incorporation into VLPs. Altogether these data are

consistent with a model in which upon translocon insertion, 14-3-3ζ and CAP2 coordinately

contribute to the formation of membrane ruffles capable of bacterial uptake.

We tested whether the roles of 14-3-3ζ and CAP2 in bacterial entry are conserved

among T3SS translocon homologs using *S*. Typhimurium. As for *S. flexneri*, both 14-3-3ζ and

CAP2 were necessary for efficient *S*. Typhimurium entry (Fig. 5c,e). In contrast, whereas only

14-3-3ζ modulated *S. flexneri* membrane ruffle size, *S*. Typhimurium infection of CAP2$^{-/-}$

monolayers resulted in smaller membrane ruffles comparable to those produced in WT

monolayers (Fig. 5d,f). Membrane ruffle expansion during *S*. Typhimurium infection requires

vesicular delivery of plasma membrane reservoirs via Rab10 and LRRK2, without which the

ruffles remain stunted and unable to support bacterial internalization[45,46]. Although it is not

known whether Rab10 and/or LRRK2 are required during *S. flexneri* infection, these findings

raise the possibility of multiple 14-3-3 functions during *S*. Typhimurium infection giving rise to

infection phenotypes distinct from those observed during *S. flexneri* infection. 14-3-3 dimers

negatively regulate LRRK2 activity[47], yet whether this specific activity of 14-3-3 is altered during

pathogen infection is unknown.

14-3-3ζ, either directly or indirectly, promotes recruitment of CAP2 to bacterial entry

sites. 14-3-3ζ is known to promote lamellipodia and membrane ruffle formation through the

cytoskeletal binding partner ARHGEF7 (Rho guanine nucleotide exchange factor 7, βPIX)[48].

Rac1, the small GTPase that activates lamellipodia formation, is targeted by ARHGEF7 and is

activated by *Shigella* and *Salmonella* effectors in a manner that enhances entry[16,49]. In the

presence of a dominant-negative derivative of 14-3-3ζ, instead of promoting lamellipodia,

ARHGEF7 promotes filopodia[48], suggesting that in the absence of 14-3-3ζ, activation of Rac1

by the pathogen may lead to misdirection of actin polymerization into filopodia instead of

lamellipodia, which would be expected to hamper bacterial entry.

We propose a model in which CAP2 generates pools of G-actin for *Shigella*-directed (or

Salmonella-directed) actin polymerization for membrane ruffle formation at entry sites. In the

absence of CAP2, the pool of G-actin is reduced, leading to less effective bacterial direction of

membrane ruffle generation. We found that *S. flexneri* formed significantly larger membrane

ruffles during infection of HeLa monolayers lacking both 14-3-3ζ$^{-/-}$ and CAP2$^{-/-}$ (Fig. 5c). The

absence of both might be expected to be associated with both a reduction in the CAP2-

generated G-actin pool and hampered 14-3-3ζ-directed lamellipodia formation, which together

with actin polymerization-promoting bacterial translocase IpaC[50] and effectors IpaA[51] and

IpgB1[52], would likely promote misdirected filopodia formation; our findings in the dual absence

of 14-3-3ζ and CAP2 are consistent with this. Although larger, the ruffles present in the HeLa

14-3-3ζ$^{-/-}$ CAP2$^{-/-}$ monolayers did not support recovery of bacterial entry compared with

monolayers lacking each protein individually (Fig. 5c), consistent with these large ruffles lacking

the structural capacity for efficient *S. flexneri* internalization and highlighting that the precise

coordination of bacterially-induced membrane ruffles is required for pathogenesis.

Altogether our data show that 14-3-3ζ and CAP2 work together to regulate membrane

ruffle dynamics during *S. flexneri* infection. These data are consistent with a model in which

CAP2 is recruited to sites of bacterial entry (in part by 14-3-3ζ), promoting bacterial effector-

induced lamellipodia and associated membrane ruffles, which support bacterial entry. Our data

indicate that T3SS pathogens co-opt 14-3-3ζ and CAP2-associated signaling pathways during

host entry. In the context of infection, it is likely that these two events happen rapidly upon T3SS

translocon insertion into host plasma membranes and the resulting cytoskeletal remodeling

triggers 14-3-3ζ dimer scaffolding and CAP2 recruitment and activation to manipulate the

cytoskeletal network within membrane ruffles towards bacterial uptake. These mechanisms are

likely to be broadly relevant to T3SS pathogens and non T3SS pathogens that manipulate host

cytoskeletal networks during host cell entry.

Online Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Supplementary Table

3. Wildtype *S. flexneri* serotype 2a strain 2457T has been described[53]. All mutants are isogenic

derivatives of 2457T. The Δ*ipaB* Δ*ipaC* mutant was generated using the Wanner method[54]. IpaB

and IpaC derivatives were encoded by genes inserted into the plasmid pDSW206[55], for which

protein expression is driven by the ColE1 promoter and induced with 1mM isopropyl thio-β-

galactoside (IPTG); these plasmids were introduced into *S. flexneri* Δ*ipaB* Δ*ipaC* and its

derivatives, as appropriate. Unless otherwise noted, to enhance translocon delivery into cell

membranes, all *S. flexneri* strains expressed the *E. coli* adhesion Afa-1[56]. The sequences of

primers used in PCR and sequencing are available from the authors upon request.

Cell Culture

HeLa (CCL2) cells were obtained from ATCC, cultured in Dulbecco's Modified Eagles

media (DMEM) supplemented with 0.45% glucose and 10% heat-inactivated fetal bovine serum

(FBS), and grown at 37°C with 5% CO_2 and humidity.

Quantification of T3SS translocases in plasma membranes

Membrane isolation and detection of translocon proteins was performed as described

previously[8,57]. Briefly, 3×10^5 HeLa cells were seeded into each well of a 6-well plate. Two wells

were used for each strain tested. *S. flexneri* cultures were diluted to an MOI of 100 in PBS with

1mM IPTG. Bacteria were centrifuged onto HeLa monolayers at 25°C for 10 minutes at 2,000 x

g, and infected cells were incubated at 37°C in 5% CO2 with humidified air for 20 minutes.

Infected cells were subsequently harvested, and plasma membrane-enriched fractions, which

contains translocons, were isolated via sequential membrane fractionation with 0.2% saponin

then 0.5% TritonX-100, as previously described[9,58]. Western blots were performed using rabbit

anti-caveolin-1 (Sigma, C4490), rabbit anti-GroEL (Sigma, G6532), rabbit anti-IpaC (gift of R.

Kaminski), and mouse anti-IpaB (gift of R. Kaminski).

Bacterial effector translocation assay

Effector translocation was performed as previously described[10]. Briefly, 3×10^5 HeLa

cells were seeded into each well of a 6-well plate. Two wells were used for each strain tested. *S.*

flexneri cultures expressing OspB-FLAG were diluted to an MOI of 100 in HBSS with 4% FBS

and 1mM IPTG. Infection was carried out as described in Methods (Quantification of T3SS

translocases in plasma membranes), and infected cells were incubated 37°C in 5% CO2 for 60

minutes before harvesting and lysing with RIPA buffer (50 mM Tris, pH 8 containing 150 mM

NaCl, 1% Nonidet-P40, 0.1% SDS, 10 mM NaF, and EDTA-free protease inhibitor cocktail,

Roche). Cellular debris and bacteria were separated by centrifugation and collected as the

bacterial fraction, and the soluble fraction was collected as the cell cytosol. Abundance of OspB-

FLAG delivered to the cytosol was determined by western blot using mouse anti-FLAG (Sigma,

F1804), mouse anti-HIV1 p24 (Abcam, ab9071), mouse anti-β-actin-peroxidase (Sigma, A3854).

Pore formation

Pore formation in nucleated cells was performed as described previously[59]. HeLa cells

seeded in 96-well flat-bottom plates at 1.5×10^4 per well, were preloaded with BCECF, AM [2'-7'-

Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (Invitrogen, B1170)]

and Hoechst (Invitrogen) in HBSS at 37°C with 5% CO_2. The cells were infected with

exponential phase bacteria at an MOI of 100 in RPMI. Entry was synchronized by centrifugation

at 800x*g* for 10 minutes at 25°C, and the infection was continued at 37°C with 5% CO_2 for 50

minutes. Fluorescent microscopy was used to image the total number of cells (blue filter) and

BCECF-positive cells (green filter).

Virotrap

10^7 HeLa cells were transiently transfected with pMET7-GAG-GW and pcDNA3-FLAG-

VSVG or pcDNA3-VSVG (this study) at a 9:1 ratio using FuGENE HD Transfection Reagent

(Promega). pMET7-GAG-GW was a gift from Sven Eyckerman (Addgene plasmid # 194634;

http://n2t.net/addgene:194634; RRID: Addgene_194634) and pcDNA3-FLAG-VSVG was a gift

from Sven Eyckerman (Addgene plasmid # 80606; http://n2t.net/addgene:80606; RRID:

Addgene_80606). DNA mixed with FuGENE HD at a 1:3.5 ratio in Opti-MEM (Gibco) was

incubated at room temperature for 5 minutes, then added dropwise onto HeLa cells. pcDNA3-

FLAG-VSVG was used in the DNA mix to generate VLPs for uninfected controls. pcDNA3-

VSVG was used in the DNA mix to generate VLPs for infected samples. After 48 hours,

transfected monolayers were washed with PBS and infected with *S. flexneri* expressing IpaB-

FLAG at MOI 100. After 30 minutes of infection, gentamicin, which kills extracellular but not

intracellular bacteria, was added to final concentration of 25µg/ml, and infected cells were

incubated for an additional 2 hours. Cell culture supernatants, which contain VLPs, were

collected and processed as previously described[19]. Briefly, cell debris was removed from

supernatants by centrifugation and passage through 0.45µm filters. Supernatants were then

filter concentrated using Amicon ultra centrifugal filters, 100kDa MWCO (Millipore), loaded onto

anti-FLAG M2 magnetic beads (Millipore), and incubated for 2 hours at 4°C with end-over-end

rotation. VLPs captured with anti-FLAG beads were washed three times, resuspended in SDS

sample buffer and boiled for 10 min. at 95°C. Samples were then loaded in 12.5% SDS PAGE

gels, and gel bands were excised and submitted to the Taplin Mass Spectrometry Facility

proteomics core for analyses.

Mass spectrometry/Proteomics

At the Taplin Mass Spectrometry Facility, excised gel bands were cut into approximately

1 mm^3 pieces. Gel pieces were then subjected to a modified in-gel trypsin digestion procedure[60].

Gel pieces were washed and dehydrated with acetonitrile for 10 min. followed by removal of

acetonitrile. Pieces were then completely dried in a speed-vac. Rehydration of the gel pieces

was with 50 mM ammonium bicarbonate solution containing 12.5 ng/µl modified sequencing-

grade trypsin (Promega, Madison, WI) at 4ºC. After 45 min., the excess trypsin solution was

removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces.

Samples were then placed in a 37ºC room overnight. Peptides were later extracted by removing

the ammonium bicarbonate solution, followed by one wash with a solution containing 50%

acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac (~1 hr). The

samples were then stored at 4ºC until analysis.

On the day of analysis, the samples were reconstituted in 5 - 10 µl of HPLC solvent A

(2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was

created by packing 2.6 µm C18 spherical silica beads into a fused silica capillary (100 µm inner

diameter x ~30 cm length) with a flame-drawn tip[61]. After equilibrating the column each sample

was loaded via a Thermo EASY-LC (Thermo Fisher Scientific, Waltham, MA). A gradient was

formed, and peptides were eluted with increasing concentrations of solvent B (90% acetonitrile,

0.1% formic acid).

As peptides eluted, they were subjected to electrospray ionization and then entered into

a Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides

were detected, isolated, and fragmented to produce a tandem mass spectrum of specific

fragment ions for each peptide. Peptide sequences (and hence protein identity) were

determined by matching protein databases with the acquired fragmentation pattern by the

software program, Sequest (Thermo Fisher Scientific, Waltham, MA)[62]. All databases include a

reversed version of all the sequences and the data was filtered to between a one and two

percent peptide false discovery rate.

Generation of CRISPR knockouts

To generate the HeLa 14-3-3$\zeta^{-/-}$ cell line, guide RNA sequence ("CD.Cas9 HRNB5585

AA" AGGAGATTACTACCGTTACT/GGGATTGTCGATCAGTCACA) was subcloned into a

lentiviral construct, and HeLa cells were transduced with lentivirus packaged with CRISPRMAX.

To generate the HeLa CAP2$^{-/-}$ cell line, RNP complexes were assembled with pooled

guide RNA sequences ("guide 1" UGACUGCUUACAGAAUGACG, "guide 2"

CUUUCAGAGAGAGAAACCGG, "guide 3" ACAGCUAUCCAUCCAAGGGC) and Cas9 at a ratio

of 3:1 in Resuspension buffer R. 2×10^5 HeLa cells were pelleted, resuspended in

Resuspension Buffer R, added to the assembled RNP complex or scrambled control. HeLa WT

and HeLa 14-3-3$\zeta^{-/-}$ were electroporated using the Neon Transfection System

(Invitrogen) according to the manufacturer's instructions. Using a 10 µL Neon tip, cells were

pulsed once at 1005 V and 35 ms pulse width. Electroporated cells were immediately

resuspended in 2.5 mL of DMEM with 10% FBS and placed into wells of a 6-well plate. Cells

were incubated for 72 hours at 37°C before analysis with the Inference for CRISPR edits tool

(Synthego) and limiting dilution for selection of a clonal homozygous CAP2 deletion lineage.

Levels of 14-3-3ζ transcripts were determined by quantitative reverse transcriptase

polymerase chain reaction (qRT-PCR). Transcripts were prepared using RNeasy Micro kit

(Qiagen), cDNA was generated using the SuperScript IV VILO kit (Thermo Fisher Scientific),

and PCR was performed with SsoFast EvaGreen Supermix kit (Bio-Rad) for 40 cycles. Primers

used in qRT-PCR were YWHAZ F: TTCTTGATCCCCAATGCTTC and YWHAZ R:

AGTTAAGGGCCAGACCCAGT. The housekeeping genes used for reference were MAP3K14

(F: ACAACGAGGGTGTCCTGCTC, and R: TCAGCTCCTCTGCCCGAAA) and HPRT (F:

CCTGGCGTCGTGATTAGTGAT, and R: AGACGTTCAGTCCTGTCCATAA).

14-3-3ζ and CAP2 proteins were detected with rabbit anti-14-3-3ζ (LSBio #LS-C353025)

and rabbit anti-CAP2 antibodies (Proteintech #15865-1-AP), with β-actin as a loading control.

Other 14-3-3 isoforms were detected with rabbit anti-YWHAB (Invitrogen, MA5-32419), rabbit

anti-YWHAE (Abcam, ab92311), rabbit anti-YWHAG (Invitrogen, PIMA535769), rabbit anti-

YWHAH (Abcam, ab206292), rabbit anti-SFN (Abcam, ab193667), and rabbit anti-YWHAQ

(Abcam, ab124909). HA-14-3-3ζ was detected with mouse anti-HA (BioLegend, 901501).

Quantification of adherent and intracellular bacteria

HeLa cells were seeded at 10^5 cells per well in 24-well plates the day prior to infection.

S. flexneri not expressing *E. coli* Afa-1 was grown as described above and added to cell

monolayers in DMEM at an MOI of 100. Bacteria were centrifuged onto cells for 10 min. at

2,000 rpm and incubated at 37°C with 5% CO2 for 30 min. Bacteria that did not invade the cells

were removed by washing with phosphate-buffered saline (PBS). To quantify the number of

adherent bacteria, infected monolayers were then treated with 0.25% trypsin and lysed in PBS

containing 0.5% Triton X-100. Lysates containing the bacteria were plated on agar plates to

quantify the number of adherent bacteria. To quantify the number of intracellular bacteria,

infected HeLa cells were cultured for an additional hour at 37°C with fresh DMEM containing 25

μg/ml gentamicin. Infected monolayers were then washed with PBS, treated with trypsin, and

lysed in PBS containing 0.5% Triton X-100 to release the intracellular bacteria. Lysates

containing the bacteria were plated on agar plates to quantify the number of intracellular

bacteria.

Quantification of bacterial spread

The plaque assay was performed by seeding 5.5 × 10☐ cells per well in a 6-well plate,

the day prior to infection, followed by infection with WT *S. flexneri* not expressing *E. coli* Afa-1 at

a multiplicity of infection (MOI) of 0.001. The plates were centrifuged at 2,000 rpm for 10 min. at

room temperature to promote infection and subsequently incubated at 37°C for 1.5 hours.

Following incubation, the media was removed, and an agarose overlay containing DMEM, 10%

fetal bovine serum (FBS), 25 µg/mL gentamicin, and 1% agarose was added to each well. The

overlay was allowed to solidify at room temperature before incubating the plates at 37°C for 72

hours to allow plaque formation. For visualization, a neutral red agarose overlay was applied,

consisting of DMEM, 10% FBS, 25 µg/mL gentamicin, 1.4% agarose, neutral red solution, and

6N HCl. Plates were incubated for an additional 6 hours before plaques were scanned. The

plaques were subsequently analyzed by quantifying the number and area of each plaque to

assess infection efficiency and spread.

Immunofluorescence microscopy of membrane ruffles, CAP2, and *S. flexneri*

HeLa cells were seeded onto glass coverslips at $3x10^5$ cells per well of a 6-well plate.

After 24 hours, HeLa monolayers were infected with *S. flexneri* at an MOI of 2 by centrifugation

at 800x*g* for 10 min. at room temperature, followed by an additional 10 min. at 37°C with 5%

CO2. Infected cells were washed 3x with PBS then fixed with 4% paraformaldehyde for 20 min.

at room temperature. HeLa membranes were permeabilized with 0.5% TritonX-100 for 7 min. at

room temperature. DNA was stained with Hoechst, actin was stained with Alexa Fluor 568

conjugated to phalloidin (Invitrogen, A12380) or Alexa Fluor 647 conjugated to phalloidin

(Invitrogen, A22287), CAP2 was stained with rabbit anti-CAP2 and an Alexa Fluor 488

secondary antibody (Invitrogen, A-11034), and *S. flexneri* was stained with rabbit anti-*Shigella*

FITC (Virostat, 0903) conjugated to FITC. Coverslips were mounted onto glass slides with

ProLong Diamond (Invitrogen) and images were collected by epifluorescence microscopy.

Microscopy and image analysis

Images were collected using a Nikon TE-300 microscope equipped with Q-Imaging Exi

Blue Cameras (Q-imaging), Chroma Filters, and IVision Software (BioVision Technologies).

Unless noted otherwise, images were randomly collected across a coverslip. Single channel

images were pseudo-colored and assembled in ImageJ or Illustrator (Adobe).

Signal from Western blots was captured by film or with an Azure 300 Chemiluminescent

Imager (Azure Biosystems). Film was digitized using an Epson Perfection 4990 photo scanner,

and the density of bands was determined using ImageJ (National Institutes of Health).

Statistical analysis

Except where specifically noted, all data are from three independent experiments and

the means ± standard errors of the means (SEM) are presented. Dots within graphs represent

independent experimental replicates. The means between groups were compared by a one-way

analysis of variance (ANOVA) using GraphPad Prism 8 (GraphPad Software, Inc.). Analysis of

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702 **Tables**

703 **Table 1.** 14-3-3ζ mutants.

Native sequence	Mutation	Mutant designation	Loss of function
$L_{12}AE$	$Q_{12}QR$	Monomer	Dimerization[26]
E_5, $L_{12}AE$, $Y_{82}REKIE$	K_5, $Q_{12}QR$, $Q_{82}RENIQ$	Vimentin	Vimentin binding[27]
K_{49}, R_{56}, R_{127}, Y_{128}	A_{49}, A_{56}, A_{127}, A_{128}	Binding pocket	Protein-protein interactions via phospho-binding pocket[28]

Figures

Fig. 1. Proteomic analysis of VLPs that capture host factors proximal to type 3

translocons during *S. flexneri* invasion. a, Schematic of incorporation of *S. flexneri* type 3

translocons into VLPs. Following insertion of translocons into the plasma membrane, Gag-

mediated membrane budding leads to incorporation of translocons into VLPs. *S. flexneri*

translocase IpaB is tagged with FLAG at its C-terminus, which remains extracellular both

following membrane insertion and on VLPs. The diameter of VLPs is approximately 140 nm,

whereas that of translocons is approximately 13.5 nm. **b,** Schematic of type 3 translocons

during cell invasion. The secretion system needle docks on plasma membrane-embedded

translocons in a process that depends on interaction of IpaC with intermediate filaments[9].

Infection with *S. flexneri* expressing the IpaC derivative R362W, which is defective for

interaction with intermediate filaments, leads to reduced bacterial docking but wildtype levels of

translocon insertion[9]. Effector translocation depends on de novo actin polymerization[10]. **c,**

Enrichment of cellular proteins in VLPs from HeLa cells infected with *S. flexneri* IpaC R362W

(top) or WT *S. flexneri* (bottom). Mass spectrometry analysis of VLPs. Shown are significant ($p \leq$

0.05) $\log_2$ fold-changes (Log_2FC) in abundance of human proteins compared with uninfected

cells. Labeled are cytoskeletal proteins, proteins involved in calcium signaling, and proteins

known to be involved in *S. flexneri* infection. Bold, proteins investigated further in this study.

Fig. 2. 14-3-3ζ is required for efficient membrane ruffle formation and *S. flexneri* entry

into cells. a, Reduction of *S. flexneri* entry into HeLa cells lacking 14-3-3ζ, which is rescued by

complementation with 14-3-3ζ. Left graph, entry efficiency defined as the number of plaques

formed in cell monolayers upon low MOI infection (see Methods), normalized to WT *S. flexneri*.

Right graph, rescue of entry in 14-3-3ζ[-/-] HeLa cells with complementation *in trans* by

transfection of HA-tagged 14-3-3ζ. N = 4 independent biological replicates. **b,** 14-3-3ζ[-/-] HeLa

cells support bacterial-induced membrane formation. Number of ruffles per 100 HeLa cells,

normalized to WT cells. N = 3 independent biological replicates. **c,** Reduction in area of plasma

membrane ruffles induced during *S. flexneri* infection of cells lacking 14-3-3. Box plot of median

value and interquartile range (IQR)1-IQR3 (25th-75th percentile range of values). Whiskers

represent 10th-90th percentile of ruffles quantified. Individually plotted data points fall outside of

736   percentile whiskers. Circles, HeLa WT; triangles HeLa 14-3-3$\zeta^{-/-}$. **d,** Visualization of reduction in

737   size of ruffles during *S. flexneri* infection of cells lacking 14-3-3ζ. Fluorescence microscopy of

738   infected 14-3-3$\zeta^{-/-}$ (top) or WT (bottom) HeLa cells, labeled for bacteria (green), polymerized

739   actin (F-actin, phalloidin, red), and DNA (Hoechst, blue). Arrowheads, plasma membrane ruffles

740   associated with bacteria. Mean ± SEM (**a**). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Fig. 3. Derivatives of 14-3-3ζ with known functional defects support defective *S. flexneri*-induced membrane ruffles. a, Membrane ruffles induced during *S. flexneri* infection of HeLa cells expressing 14-3-3ζ derivatives defective in 14-3-3ζ dimerization (Monomer), in 14-3-3ζ

interaction with vimentin (Vimentin), or in 14-3-3ζ binding to phosphorylated ligands (Binding

pocket). Fluorescence microscopy of infection of HA-tagged WT or mutant 14-3-3ζ derivatives

transfected into 14-3-3ζ$^{-/-}$ HeLa cells. Bacteria (green), polymerized actin (phalloidin, red), and

DNA (Hoechst, blue). **b,** Functionally defective 14-3-3ζ derivatives are associated with

reductions in area of plasma membrane ruffles induced by *S. flexneri* infection of cells. WT

HeLa cells, filled circles. HeLa 14-3-3ζ$^{-/-}$ cells complemented with each of the following HA-

tagged 14-3-3ζ derivatives: empty vector, filled triangles; WT 14-3-3ζ, filled diamonds;

dimerization-defective 14-3-3ζ derivative, open circles; 14-3-3ζ derivative defective in vimentin

binding, open triangles; 14-3-3ζ derivative defective in phospho-binding pocket, open hexagons.

Whiskers represent 10th-90th percentile of ruffles quantified. Individually plotted data points fall

outside of percentile whiskers.

Fig. 4. Proteomic comparison of VLPs from 14-3-3ζ$^{-/-}$ cells to WT cells during *S. flexneri*

entry. a, Enrichment of cellular proteins in VLPs from WT HeLa cells relative to HeLa 14-3-3ζ$^{-/-}$

cells, infected with *S. flexneri* producing functional or stalled type 3 translocons or not producing

intact translocons due to lack of the translocase IpaC. Mass spectrometry analysis of VLPs.

Shown are significant (p ≤ 0.05) Log$_2$FC in protein abundance. Labeled are cytoskeletal proteins

and proteins known to participate in *Shigella* pathogenesis. Bold, CAP2. **b,** Increased

abundance of CAP2 in VLPs from WT cells relative to VLPs from 14-3-3ζ$^{-/-}$ cells during infection

764   with WT or docking incompetent *S. flexneri*, each of which inserts translocons efficiently into the

765   plasma membrane. Shown is Log$_2$FC in CAP2 abundance.

Fig. 5. CAP2 and 14-3-3ζ act in the same pathway to promote membrane ruffle formation

during T3SS pathogen infection. a, Fluorescence microscopy localization of CAP2 and

polymerized actin with and without *S. flexneri* infection. CAP2 (green), bacteria (red),

polymerized actin (far red). b, Peason's correlation coefficient of CAP2 colocalization with

polymerized actin in the indicated conditions. c, Absence of 14-3-3ζ and/or CAP2 lead to

defects in *S. flexneri* entry. Entry is defined as the number of bacteria recovered from the

monolayer at 90 mins of infection in the presence of gentamicin (see Methods). Normalized to

WT HeLa. N = 5-6 independent biological replicates. **d,** Area of membrane ruffles induced

during *S. flexneri* infection. Box plot representing median values, IQR1, and IQR3. Whiskers

represent 10th-90th percentile of ruffles quantified. Individually plotted data points fall outside of

percentile whiskers. **e,** Absence of 14-3-3ζ and/or CAP2 leads to defects in *S.* Typhimurium

entry. N = 3-6 independent biological replicates. **f,** Area of membrane ruffles induced during *S.*

Typhimurium infection. Defined as in panel **d**. HeLa cells (**c-f**): WT, filled circles; 14-3-3ζ$^{-/-}$, filled

triangles; CAP2$^{-/-}$, filled diamonds; 14-3-3ζ$^{-/-}$ CAP2$^{-/-}$, open circles.