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ADDENDUM

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The type three secreted effector SipC regulates the trafficking of PERP during *Salmonella* infection

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ABSTRACT

Salmonella enterica Typhimurium employs type III secreted effectors to induce cellular invasion and pathogenesis. We previously reported the secreted effector SipA is in part responsible for inducing the apical accumulation of the host membrane protein PERP, a host factor we have shown is key to the inflammatory response induced by *Salmonella*. We now report that the *S. Typhimurium* type III secreted effector SipC significantly contributes to PERP redistribution to the apical membrane surface. To our knowledge, this is the first report demonstrating a role for SipC in directing the trafficking of a host membrane protein to the cell surface. In sum, facilitation of PERP trafficking appears to be a result of type III secreted effector-mediated recruitment of vesicles to the apical surface. Our study therefore reveals a new role for SipC, and builds upon previous reports suggesting recruitment of vesicles to the cell surface is important for *Salmonella* invasion.

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Introduction

Salmonella enterica subtype Typhimurium (*S. Typhimurium*) is one of several *Salmonella enterica* strains responsible for over a million cases of salmonellosis in the United States each year, and is also a leading cause of diarrheal disease in developing countries. Salmonellosis is typified by gastrointestinal distress, including abdominal cramping, nausea, fever, vomiting, and most prominently, diarrhea that may contain mucus or blood. Intestinal inflammation is further characterized histopathologically by a prevailing influx of polymorphonuclear leukocytes, the non-specific effects of which lead to tissue damage and also contribute to diarrhea.²⁰

Salmonella species, like other Gram-negative pathogens, have evolved a sophisticated virulence mechanism called a type III secretion system (T3SS), which is responsible for the delivery of a series of bacterial effectors into host cells aimed at reprogramming eukaryotic cell functions. While the T3SS apparatus is highly conserved, the translocated effectors are unique proteins with very specialized functions central to virulence. In particular, *Salmonella* effector

proteins impart a variety of effects on the host cell including triggering *Salmonella* invasion by interfering with actin dynamics,²² and inducing inflammatory responses.^{18,28,29}

Our prior studies have revealed that the type III secreted effector SipA is a key regulator of intestinal inflammation during *S. Typhimurium* infection and that it is able to impart this function in the host without being translocated into the host cell via the T3SS.^{13,18,28} That SipA is able to carry out its proinflammatory functions without being translocated via the T3SS indicated to us that SipA might be interacting with a binding partner on the host cell surface. In line with this hypothesis, we recently reported that SipA binds to the mammalian tetraspanning membrane protein PERP, which we show accumulates at the apical surface of colonic epithelial cells during *S. Typhimurium* infection in a SipA-dependent manner.¹³ PERP (p53-effector related to PMP-22) was first identified as a p53 effector,² and has since been shown to have roles in development,¹⁷ caspase activation,⁵ and cancer.²⁴ Functionally, we identified PERP to be involved in proinflammatory pathways required for *S.*

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Typhimurium-induced PMN migration, uncovering a previously unknown role for PERP in *Salmonella* pathogenesis.

While SipA is involved in eliciting PERP accumulation to the apical surface of polarized colonic epithelial cells, we noted that it is not the only *S. Typhimurium* factor to be involved. This is because infection with Δ SipA *S. Typhimurium* shows a significant, but incomplete, reduction in the level of PERP accumulation as compared to wild type infection,¹³ indicating involvement of another virulence factor. Since it has been previously documented that SipA and SipC cooperate to promote *Salmonella* invasion,²² we examined the extent to which SipC influences PERP accumulation at the apical surface of colonic epithelial cells. A prior study indicated that SipC mediates the formation of the exocyst at sites of *Salmonella* invasion via its interaction with multiple components.²³ Given that the exocyst is known to play a critical role in vesicle trafficking, this observation led us to consider that SipC could have a direct role in mediating PERP trafficking to the apical surface of infected cells. Herein, we describe our observations that SipC also contributes to the trafficking of PERP to the cell surface. To date, studies of SipC have largely focused on its role in perturbing actin dynamics and its role in effector translocation during *Salmonella* invasion of host cells. Our study is the first to reveal SipC also plays a role in regulating the trafficking of a host membrane protein to the cell surface during *S. Typhimurium* infection.

Results

Type three secreted effectors drive PERP accumulation

It did not escape our attention that in addition to the type III secreted effector SipA¹³ other *S. Typhimurium* virulence determinants might be required to induce the full level of PERP accumulation at the apical surface of colonic epithelial cells observed in response to wild type infection. We first considered the T3SS located on *Salmonella* pathogenicity island (SPI)-1, given that the SPI-1 T3SS (T3SS1) is largely associated with early invasion and pathogenicity events.¹⁰ The key regulator of this system is the transcription factor HilA, without which the T3SS1 needle apparatus is not formed and the transcription of several T3SS1 effectors is blocked, thus rendering

Salmonella invasion-deficient.⁶ To examine the importance of the T3SS1 in PERP accumulation at the apical surface, we employed an apical surface biotinylation assay,^{13,27} which allows us to selectively label the apical surface of polarized cells and examine changes in protein expression at this cellular location in response to infection. As shown in Fig. 1A, infection of HCT8 colonic epithelial cells with our HilA-deficient strain nearly abolishes apical PERP accumulation in response to infection. Complementation of the HilA mutant (Fig. 1B) also rescued the ability of *S. Typhimurium* to promote the accumulation of PERP. This result shows that the key factors governing PERP accumulation at the apical surface of colonic epithelial cells infected with *S. Typhimurium* are associated with the T3SS1.

SipC promotes PERP accumulation at the apical surface of colonic epithelial cells

Since there is precedence for the T3SS1 effectors SipA and SipC cooperating to promote *Salmonella* invasion due to interference with actin dynamics,²² we next examined the extent to which SipC functions in PERP trafficking during infection. To observe changes in PERP protein levels at the apical surface in response to infection, we compared wild type *S. Typhimurium* versus an isogenic *Salmonella* Δ SipC strain.²⁸ As shown in Figure 1C, the absence of SipC causes less PERP to accumulate at the apical surface compared to levels observed in response to wild type infection; complementation with a plasmid expressing SipC rescued the effect, confirming the result is SipC-specific (Fig. 1D). To our knowledge, this represents the first time SipC has been shown to have a direct role in regulating the trafficking of a host membrane protein to the surface of polarized colonic epithelial cells during *Salmonella* infection.

We next sought to determine whether SipC and SipA act via the same or independent pathways to induce PERP accumulation in response to *S. Typhimurium* infection. To accomplish this, we generated a Δ SipA Δ SipC isogenic double mutant strain via replacement of SipC with a chloramphenicol resistance cassette⁴ in our SipA mutant strain, and then examined PERP accumulation employing the biotinylation assay. We reasoned that if SipA and SipC act via independent pathways to induce PERP accumulation, one would expect to see an additive

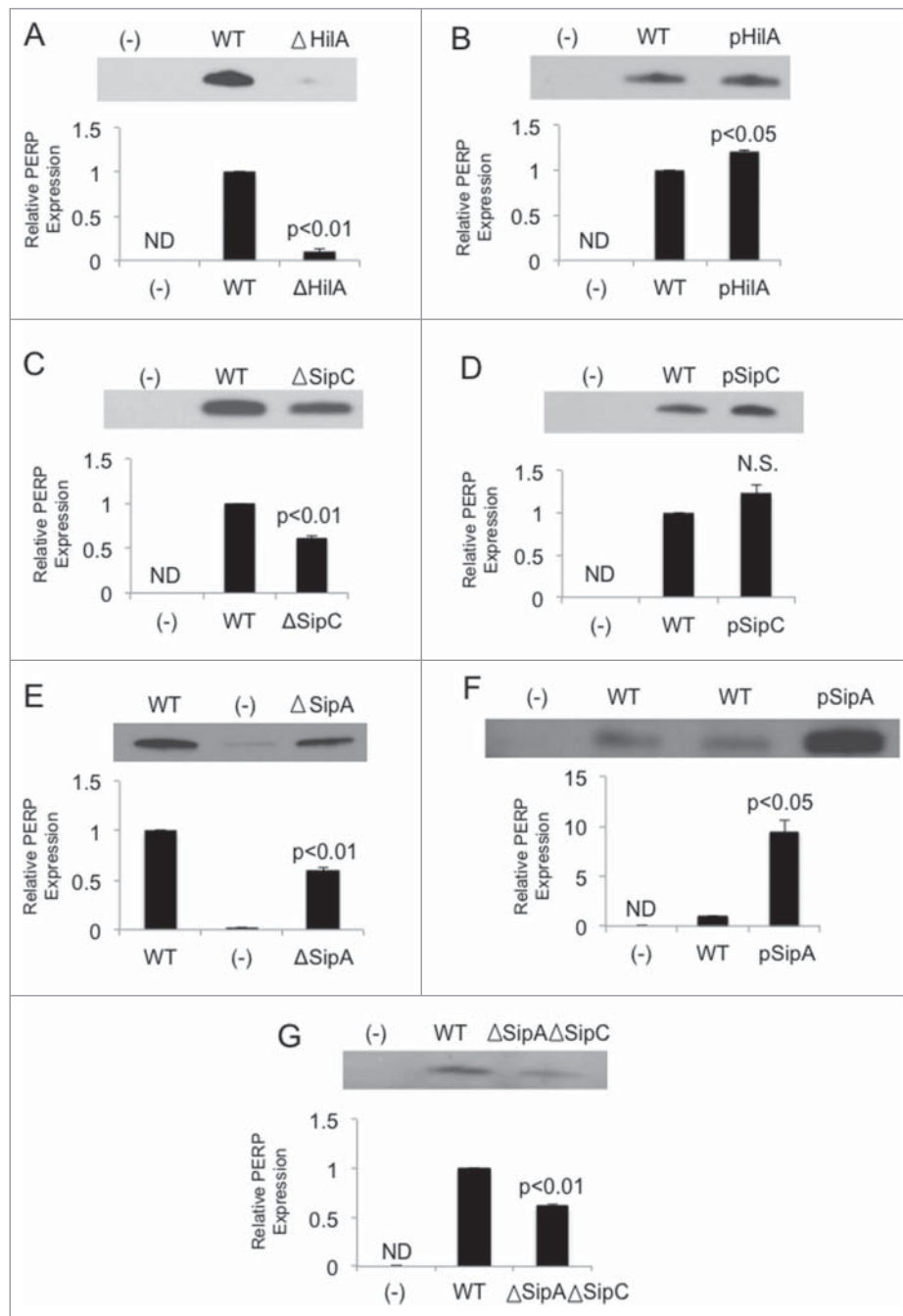


Figure 1. PERP Accumulation at the Apical Membrane Surface is Mediated by the Type Three Secretion System (A-G) Polarized HCT8 monolayers were infected with wild-type (WT), HilA-deficient (Δ HilA), HilA complemented (pHilA), SipC-deficient (Δ SipC), SipC complemented (pSipC), SipA-deficient (Δ SipA), SipA complemented (pSipA), or a double SipA/SipC-deficient (Δ SipA Δ SipC) strains of *Salmonella*, or left uninfected (–) in HBSS+ for 1 hour. The apical cell surfaces were biotinylated, and whole cell lysates were normalized via a Bradford assay then pulled down with streptavidin beads, and Western blotted for PERP. The level of PERP intensity in cells following infection with mutant strains or complemented strains was compared to the level of PERP intensity following WT infection to obtain a ratio of PERP expression. WT PERP expression level was set to 1. Densitometric measurements are from 3 replicates showing reproducible results. Error bars show \pm standard error. P-values less than 0.05 were considered statistically significant. “ND” indicates no detection of protein. Although we have previously reported on SipA’s role in the apical accumulation of PERP,¹³ the data reported for this observation here are independent of those reported in our previous publication.

effect from the double mutant compared to our observations with either individual mutant. However, if SipA and SipC were to act via the same

pathway, we would envision the Δ SipA Δ SipC double mutant not to cause any additional loss of PERP accumulation as compared to either

individual mutant. As shown in Fig. 1G, we observed a comparable reduction in PERP accumulation from the double Δ SipA Δ SipC mutant as compared to either single mutant (Fig. 1C-F; ¹³). This result suggests that SipA and SipC may use the same pathway to induce PERP accumulation to the apical surface of colonic epithelial cells during *S. Typhimurium* infection, although further experimentation is required to fully validate this proposal.

PERP accumulation is due to an exocytic trafficking event

We next investigated whether PERP accumulation is dependent on actin-mediated mechanisms associated with *Salmonella* invasion by treating cells with the actin-disrupting drug cytochalasin D. Treatment of host cells with cytochalasin D is well documented to reduce *Salmonella* invasion by blocking the ability of actin to polymerize and form membrane ruffles, an event that is mediated during *Salmonella* invasion via the activities of SipA and SipC among other effectors.⁹ We confirmed this observation in the HCT8 cell line (Fig. 2A). Further, we inferred that if the actin-modulating activities of SipA and SipC were to be critical for PERP accumulation, then interfering with actin dynamics via cytochalasin D treatment should cause a reduction in PERP accumulation. Rather, we found that treatment with cytochalasin D caused an additional increase in PERP accumulation at the apical surface in response to *S. Typhimurium* infection (Fig. 2B). Although this result was not statistically significant, the observed trend was reproducible. Since cytochalasin D blocks *Salmonella* invasion by interfering with actin-mediated endocytosis, this observation suggests that invasion of *S. Typhimurium* into host cells is not required for PERP accumulation. Further, since SipA and SipC have been shown to manipulate actin dynamics to promote endocytic uptake of *Salmonella*,²² our cytochalasin D results also imply that the actin-modulating domains of these effectors may also be dispensable for PERP accumulation. Although further investigation is required to achieve full understanding of the precise roles each effector plays in inducing PERP accumulation, our studies start to shed light on the molecular mechanisms by which PERP accumulates at the apical surface.

We previously reported that PERP accumulation at the apical surface of colonic epithelial cells infected

with *S. Typhimurium* was likely due to a trafficking event involving existing cellular PERP stores, as inhibiting new protein synthesis did not block PERP accumulation.¹³ Building upon this observation, we next sought to determine whether *S. Typhimurium* was preventing endocytosis, consequently causing PERP to accumulate at the apical surface, or inducing exocytosis, and thus targeting PERP-containing vesicles to the apical surface.

To distinguish between these 2 pathways, we treated HCT8 cells with 80 μ M of the dynamin-blocking drug dynasore. Dynamin is an integral part of clathrin-mediated endocytosis, and potentially caveolae-mediated endocytosis, as it functions to essentially “pinch off” newly forming vesicles.⁷ Dynasore is routinely used to block endocytosis at the cell surface.¹⁹ If *Salmonella* prevents endocytosis, we would expect that treatment with dynasore would have no additional effect on the level of PERP accumulation. As shown in Fig. 2C, blocking endocytosis alone by treating cells with dynasore was insufficient to induce PERP accumulation at the apical surface. However, we observed an additional increase in PERP accumulation in infected cells treated with dynasore compared to those not treated with dynasore (Fig. 2C), suggesting that *S. Typhimurium* induces PERP accumulation via an endocytosis-independent pathway. Indeed, this is in line with the cytochalasin D observations, as this drug has also been shown to block clathrin-mediated endocytosis at the apical surface of polarized epithelial cells,¹² and we also see an additive effect on PERP expression levels in cells infected and treated with cytochalasin D. Given our previous observations that PERP co-localizes with apical recycling endosomes,¹³ which transport new intracellular material to the surface of polarized cells¹¹ we propose that *S. Typhimurium* causes PERP to accumulate at the apical surface by triggering PERP-containing endosomes to traffic to the apical surface.

Discussion

Previous studies of SipA and SipC have mainly focused on their roles in regulating *Salmonella* invasion and inflammatory responses to infection and, in the case of SipC, effector translocation. Such studies have shown that both effectors are capable of perturbing actin dynamics in ways that promote growth of the host cell plasma membrane outward and around

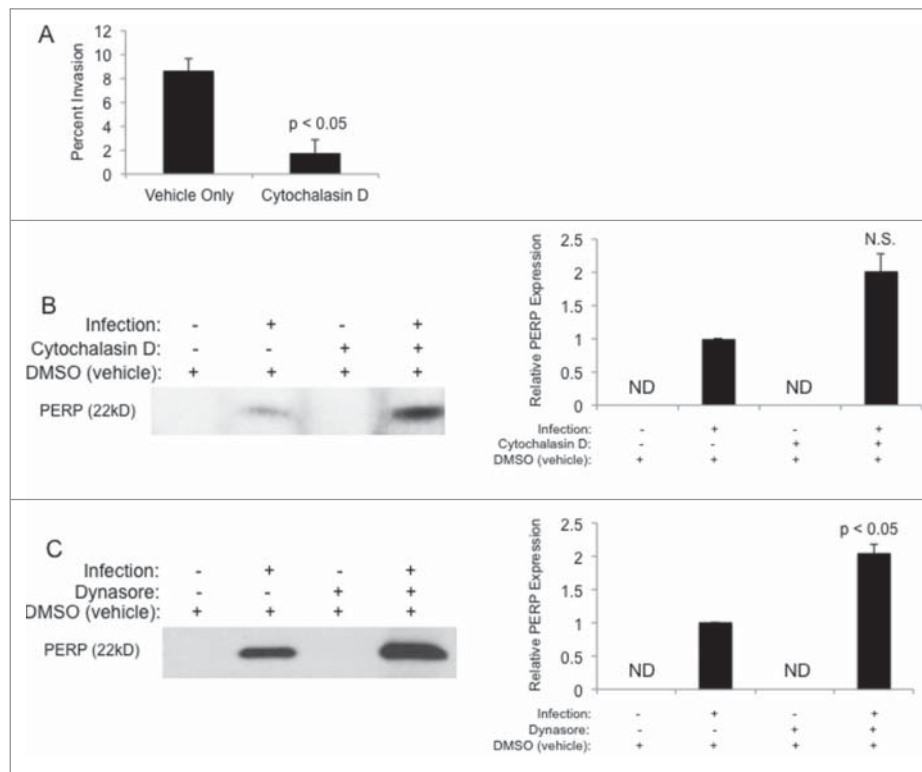


Figure 2. PERP Accumulation Occurs Independently of *Salmonella* Invasion, and Dynamin-mediated Endocytosis. **A.** Polarized HCT8 monolayers were treated with 1 μ g/mL of cytochalasin D or DMSO only (vehicle only) prior to infection and during infection. The amount of invading bacteria in each condition was quantified via a gentamicin protection assay (see Methods). **B-C.** Polarized HCT8 monolayers were treated with cytochalasin D (**B**) or dynasore (**C**) or with DMSO only (vehicle) prior to and during a 1 hour *Salmonella* infection. The apical cell surfaces were then biotinylated, and whole cell lysates were normalized via a Bradford assay then pulled down with streptavidin beads, and Western blotted for PERP. The level of PERP intensity in infected cells following treatment with either drug was compared to the level of the PERP intensity in infected cells treated with the vehicle to obtain a ratio of PERP expression. PERP expression level from infected, vehicle-treated cells was set to 1. Densitometric measurements are from 3 replicates showing reproducible results. Error bars show \pm standard error. P-values less than 0.05 were considered statistically significant. "ND" indicates no detection of protein.

Salmonella, thus permitting the bacteria to invade the cell.²² Furthermore, SipA, and to some extent SipC, has been shown to be a key regulator of the inflammatory response to *Salmonella* infection.^{18,28} However, our work investigating the role of SipA¹³ and now SipC in PERP trafficking is the first to show these effectors induce the apical accumulation of a host transmembrane protein.

Perhaps not surprising was our observation that PERP trafficking to the apical surface is almost entirely dependent on the type III secretion system. The type III secreted effectors promote disease by facilitating cell attachment and entry, suppressing the host immune/defense response, and modulating other aspects of host cell biology. Consequently, type III secreted effectors play a prominent role in bacterial pathogenesis and host-association. In the absence of HilA, and thus in the absence of a key regulator of the

T3SS1 and its effectors, *Salmonella* invasion is significantly reduced.⁶ It is notable that treatment with cytochalasin D does not inhibit PERP accumulation at the cell surface, but rather fosters more accumulation, as cytochalasin D is known to block invasion (⁹; this report). Multiple conclusions may be drawn from this observation. First, although cytochalasin D treatment inhibits invasion, most likely by interfering with the actin polymerization required for *Salmonella* engulfment,⁸ it does not prevent either the secretion or translocation of type 3 secreted effectors.¹ This observation suggests that invasion is dispensable for PERP trafficking as long as the effectors are still able to access the cell, and further supports the notion that type III secreted effectors, including SipA and SipC, play an active role in PERP accumulation.

Cytochalasin D treatment is also well known to block endocytosis as discussed above. Therefore,

our observation that PERP accumulation increases in the presence of cytochalasin D is in agreement with our finding that blocking dynamin-mediated endocytosis with dynasore (Fig. 2C) also causes increased PERP accumulation. Since inhibition of endocytosis by treating with either of these 2 drugs causes an additive effect on *S. Typhimurium*-induced PERP accumulation, and given we have previously ruled out synthesis of new protein as a possibility,¹³ we postulate that *S. Typhimurium* does not facilitate PERP accumulation at the apical surface through a mechanism that disrupts its endocytic uptake. Rather, we propose that *S. Typhimurium* prompts PERP redistribution to the apical surface by inducing increased exocytosis. This assertion is consistent with previous reports indicating that blocking endocytosis counters *Salmonella*'s strategies for invasion and pathogenesis.^{25,21} We infer that when this occurs in the presence of dynasore or cytochalasin D, the PERP trafficked to the apical surface in response to infection is unable to be endocytosed, thus causing the additive level of PERP accumulation in infected cells treated with either drug.

In considering how exocytosis is regulated at the cell surface, involvement of the exocyst complex becomes a key event. The exocyst is an octomeric protein complex that regulates tethering of vesicles to the new membrane location, thus promoting their eventual fusion.¹⁵ This process becomes important at the cell surface during *Salmonella* infection as incorporation of new vesicles at the cell surface provides an extra store of membrane at this cellular location. This is thought to help promote the membrane ruffling events that promote *Salmonella* engulfment into the intestinal epithelial cell.²³ Indeed, involvement of the exocyst in *Salmonella* invasion has been demonstrated, and it is known that exocyst components accumulate around areas of *Salmonella* invasion.²³ Furthermore, SipC likely mediates such directed accumulation given that this effector binds to at least 3 exocyst components.²³ These observations favor the hypothesis that SipC provides a "docking site" for the exocyst to form and thus direct the trafficking of vesicles to the cell surface.²³

SipA can be coupled to exocyst trafficking as well. This is supported by reports showing SipA induces the apical accumulation of the GTPase Arf6,³ and also GTP-bound Arf6 directs the exocyst complex to the

cell surface via its interaction with Sec10.²⁶ Moreover, Arf6-mediated recruitment of the exocyst appears to be directed to areas of plasma membrane growth and remodeling.²⁶ These observations are consistent with SipA directing the apical recruitment of Arf6,³ and thereafter Arf6 eliciting exocyst recruitment to sites of apical membrane ruffling during *Salmonella* invasion. Therefore, that both SipA and SipC can be networked to exocyst formation at the cell surface is in keeping with our proposal that these effectors trigger PERP accumulation at the cell surface via the same pathway (Fig. 1).

While we identified SipA¹³ and SipC to play key roles in PERP trafficking to the apical surface, our data do not exclude the possibility that at least one other type III secreted effector is involved with PERP trafficking, especially since SipC is critical to the translocation of effectors into infected cells. In this respect, the effector SopE is an interesting consideration. This effector is important for *Salmonella* invasion by promoting the cytoskeletal rearrangements that lead to membrane ruffling and has been shown to function as a guanine nucleotide exchange factor, or GEF.¹⁴ Furthermore,²³ it was previously shown that SopE is required for the activation of RalA, the GTPase known to regulate exocyst activity. If other secreted effectors are indeed shown to be required for PERP accumulation at the apical surface, it is then worth considering that SipC's role in this process is indirect. In this case rather than mediating the exocyst, SipC may instead be required for the translocation of effectors that mediate PERP accumulation. Nevertheless, additional experimentation is necessary to identify the other T3SS effector(s) responsible for PERP accumulation, as well as their mechanisms of action.

In light of the above conclusions, Fig. 3 depicts a working model for how we envision *S. Typhimurium* effectors trigger PERP accumulation at the cell surface. First, *S. Typhimurium* invades at the apical surface of colonic epithelial cells where SipA (red) and SipC (blue) act to trigger the formation of the exocyst complex (yellow) (Fig. 3A). The exocyst complex permits the tethering (and thus eventual fusion) of vesicles from apical recycling endosomes containing Rab25 and PERP (purple M's) to the apical surface (Fig. 3B; ¹³). The newly recruited vesicles then fuse to the apical plasma membrane, as indicated by the darker shading at the apical surface in Figure 3C. This fusion leads to the incorporation of PERP at the apical surface. Additionally, with the extra store of membrane provided

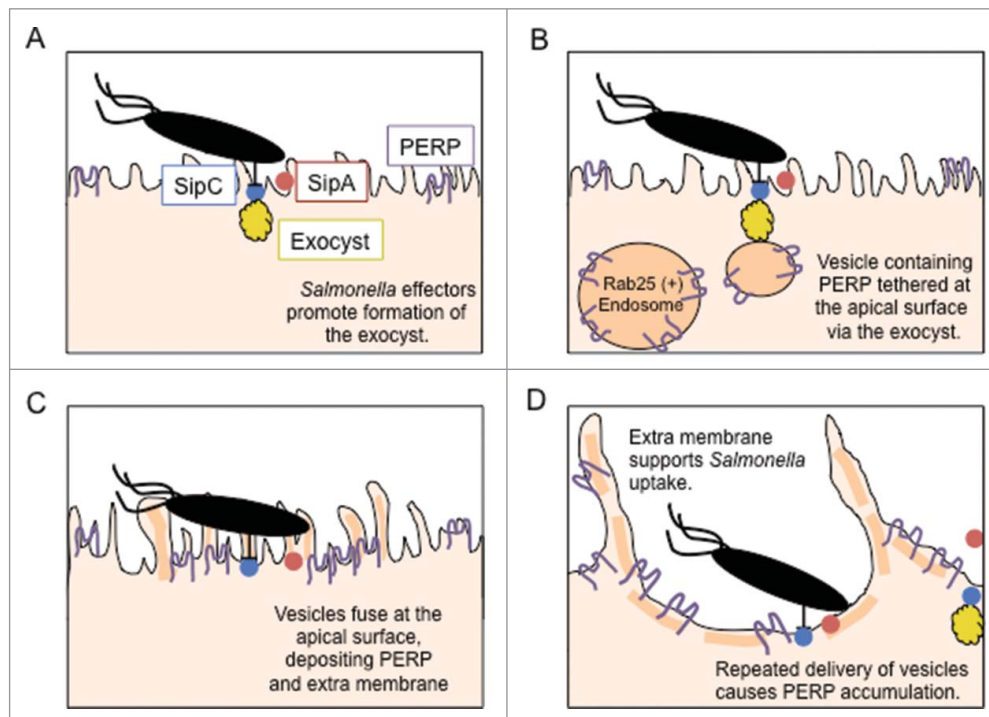


Figure 3. *Salmonella* Induces PERP Accumulation by Triggering Increased Exocytosis. (A) *Salmonella* invades at the apical surface of colonic epithelial cells, where it secretes and translocates type III secreted effectors including SipC (blue dot) and SipA (red dot). These effectors trigger the formation of the exocyst complex (yellow dot) at the apical surface. (B) The fully formed exocyst mediates increased trafficking of vesicles from apical recycling endosomes containing Rab25¹³ and PERP (purple M) to the cell surface, and their eventual fusion to the plasma membrane. (C) The fused vesicles (shaded regions), as well as PERP, become incorporated into the plasma membrane at the apical surface. (D) The extra membrane provided by vesicle fusion permits membrane ruffles to form, thus allowing for *Salmonella* invasion. Repeated delivery of vesicles in response to infection causes increased PERP levels at the apical surface.

by the fusion of vesicles at the cell surface, membrane ruffling occurs thus permitting *Salmonella* to enter the cell (Fig. 3D). As multiple bacteria infect a cell, more vesicles will traffic to the apical surface, thus causing an accumulation of PERP at this cellular location.

Several questions remain to be asked of this model, including the identity of the remaining T3SS effectors responsible for PERP trafficking, as well as the timing of each event with respect to *S. Typhimurium*'s invasion strategy and overall induction of pathogenesis. Nevertheless, the data presented in this report suggest that *S. Typhimurium* exploits type III secreted effectors, namely SipA and now SipC, to induce exocytic trafficking events that lead to the accumulation of PERP at the cell surface.

Materials and methods

Tissue culture

HCT8 polarized monolayers were seeded on transwells in 100 mm tissue culture dishes (Costar 3419) and used 6–8 d later.

Use of bacterial strains

Wild type *S. Typhimurium* (SL1344) was grown as previously described,¹⁸ as were the Δ SipA and pSipA strains.¹³ HilA-deficient (Δ HilA), SipC-deficient (Δ SipC), and the double SipA-SipC-deficient (Δ SipA Δ SipC) strains are isogenic to SL1344, and were grown in the same manner. The pSipC and pHilA isogenic complementing strains were grown in the same manner in the presence of 50 μ g/mL ampicillin. Cells were infected for 1 hour at an MOI of 100:1.

Construction of mutants and complemented strains

The SipC mutant has been previously described.²⁸ The pSipC complemented strain was constructed by placing the whole SipC gene into the pBH expression vector (described in¹⁸) via the EcoRI and HindIII restriction digest sites. The gene with the appropriate digest sites was constructed via IDT. The SipA/SipC double mutant was constructed by replacing SipC with a chloramphenicol resistance cassette in the SipA-mutant strain as described in.⁴ Briefly, primers

were designed to amplify the chloramphenicol cassette from pKD3 with end regions of homology to the N and C termini of SipC. This method leads to replacement of SipC with the chloramphenicol cassette following recombination. The resulting PCR product was gel-purified using the QiaQuick Gel Extraction Kit (Qiagen, 28704). The SipA deletion strain was then transformed with the pKD46 plasmid, which expresses the lambda Red system for recombination in the presence of arabinose, and then transformed with the purified PCR product via electroporation. Transformants were selected on chloramphenicol-containing media, and sequenced for verification of replacement of SipC with the chloramphenicol cassette. The HilA-deficient strain has previously been described (vv341; ¹⁶). The pHilA complemented strain was constructed as described above for the pSipC complement, with the exception that the EcoRI and KpnI sites were used.

Biotinylation

Apical cell surface biotinylation was performed using the protocol described by.¹³ Following infection, the apical surface of HCT8 monolayers was labeled with biotin (Thermo Scientific, 21335) at 4C. Labeling of the basolateral surface was blocked with acetate (Thermo Scientific, 26777). The cells were then lysed, normalized for protein concentration via a Bradford assay, and incubated with streptavidin beads (Thermo Scientific, 20347) overnight at 4C in order to pull down apically-labeled proteins. The apically-enriched lysates were then immunoblotted for PERP (Santa Cruz, SC-67184). For drug treatments, cells were exposed to 1 ug/mL of cytochalasin D (Sigma Aldrich, C8273-1MG) ⁹ OR 80 uM dynasore (Tocris, 2897) ^{21,25} in HBSS+, or DMSO in HBSS+ (vehicle control) for 1 hour prior to infection and during infection.

Invasion assay

To confirm the invasion-blocking effects of cytochalasin D in the HCT8 cell line, we performed a gentamicin protection assay generally following the guidelines in.⁹ Cells were pretreated with 1 ug/mL of cytochalasin D or the vehicle (DMSO) for 1 hour prior to infection and maintained during the 1 hour infection with SL1344. After washing away adherent bacteria, the cells were incubated with 0.1 mg/mL of gentamicin for 90 minutes, then

washed and lysed with 1% Triton X in PBS. 10-fold serial dilutions were then plated in triplicate. Serial dilutions of the bacterial suspension used to infect the cells were also plated to determine percent invasion as a result of each condition.

Data presentation

Densitometric analyses were performed via the FIJI software (NIH), and are presented as the results from 3 replicates showing reproducible results. For all biotinylations, the level of PERP expression from cells infected with wild-type *Salmonella* was normalized to 1 (to allow for comparisons across experiments) and the ratio of PERP expression from the non-infected, or mutant or complemented-strain infected cells was obtained. Error bars show +/- standard error, and p-values were obtained using a One Sample T-test between the normalized wild-type infection value (set to 1) and the PERP expression ratios described above. Blots are one representative of the replicate experiments. The p-value for the invasion assay was obtained via Student's T-test. For all experiments, a p-value of less than 0.05 was considered statistically significant.

Abbreviations

T3SS Type III secretion system
PERP p53 effector related to PMP-22

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest.

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