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Bacteria-generated PtdIns(3)*P* Recruits VAMP8 to Facilitate Phagocytosis

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Salmonella enterica **serovar Typhimurium invades non-phagocytic cells by inducing macropinocytosis. SopB is involved in modulating actin dynamics to promote *Salmonella*-induced invasion. We report here that SopB-generated PtdIns(3)*P* binds VAMP8/endobrevin to promote efficient bacterial phagocytosis. VAMP8 is recruited to *Salmonella*-induced macropinosomes in a nocodazole-dependent, but Brefeldin A-independent, manner. We found that VAMP8 directly binds to and colocalizes with PtdIns(3)*P*. The inositol phosphatase activity of SopB is required for PtdIns(3)*P* and VAMP8 accumulation, while wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, has no effect. Knockdown of endogenous VAMP8 by small interfering RNA or expression of a truncated VAMP8 (1–79aa) reduces the invasion level of wild-type *Salmonella* to that of the phosphatase-deficient SopB^{C460S} mutant. Our study demonstrates that *Salmonella* exploit host SNARE proteins and vesicle trafficking to promote bacterial entry.**

Key words: invasion, PtdIns(3)*P*, *Salmonella*, SopB, VAMP8

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Bacterial invasion of non-phagocytic cells is essential for *Salmonella* virulence. *Salmonella* encodes a type III secretion system to inject bacterial proteins into host cells to exploit the actin dynamics, leading to macropinosome formation and bacterial entry. *Salmonella*-induced macropinocytosis requires addition of membranes (1,2). The vacuole compartments and the repertoire of SNARE proteins that may be involved in *Salmonella*-induced membrane ruffles remain unknown. VAMP8, a v-SNARE protein that shares 31% amino acid sequence identity with VAMP3, is known to mediate the homotypic fusion of early and late endosomes (3) and is involved in cytokinesis (4) and regulated exocytosis by forming a SNARE complex with syntaxin 4 and SNAP23 (5–8). The syntaxin 4/SNAP23 complex may also be involved in the fusion of lysosomes with the plasma membrane by interacting with VAMP7 during lysosome exocytosis (9). Exocytic vesicles can originate from the *trans* Golgi network (TGN), endosomes (10) or lysosomes (9,11). Ca^{2+}-dependent exocytosis of lysosomes requires synaptotagmin VII by forming a complex with VAMP7, syntaxin 4 and SNAP23 (9). SytVII/VAMP7-mediated lysosome exocytosis plays important roles in membrane repair (11), neurite outgrowth (12) and host defense to bacterial infections (13) and during *Trypanosoma cruzi* invasion into mammalian cells (14).

SopB plays important roles in *Salmonella*-induced actin cytoskeleton rearrangements and bacterial entry (15,16). The PtdIns(4,5)P_2 level is locally increased in *Salmonella*-induced ruffles, accompanied by a SopB-dependent PtdIns(4,5)P_2 decrease in invaginating regions for efficient membrane fission and the formation of *Salmonella*-containing vacuoles (SCVs) (17). SopB can also increase the level of PtdIns(3,4)P_2 and activate Akt/PKB (18). More recent studies have shown the SopB-dependent accumulation of PtdIns(3)*P* in membrane ruffles and nascent SCVs (19). Furthermore, exogenously expressed SopB localizes to endosomes containing PtdIns(3)*P* and inhibits vesicle trafficking from endosomes to lysosomes (20). In this study, we present evidence that supports a role of SopB-generated PtdIns(3)*P* and VAMP8 in *Salmonella* invasion into epithelial cells.

Results and Discussion

VAMP8 is recruited to Salmonella*-induced membrane ruffles*

Salmonella invasion of non-phagocytic cells is marked by pronounced outward extension of membrane ruffles. To examine whether VAMP7 and VAMP8 are involved in *Salmonella* infection, HeLa cells were transfected with plasmid expressing chimeric yellow fluorescent protein (YFP)–VAMP7 and YFP–VAMP8 proteins. Transfected cells were infected with wild-type *Salmonella* to examine the distribution of YFP–VAMP7 and YFP–VAMP8. We found that YFP–VAMP8 is prominently recruited to *Salmonella*-induced membrane ruffles (Figure 1A), while YFP–VAMP7 is not (Figure 1B). In uninfected cells, YFP–VAMP8 is present on vesicles throughout the cytoplasm with a perinuclear concentration. To determine if VAMP8-containing vesicles are actively recruited to the ruffles, we infected

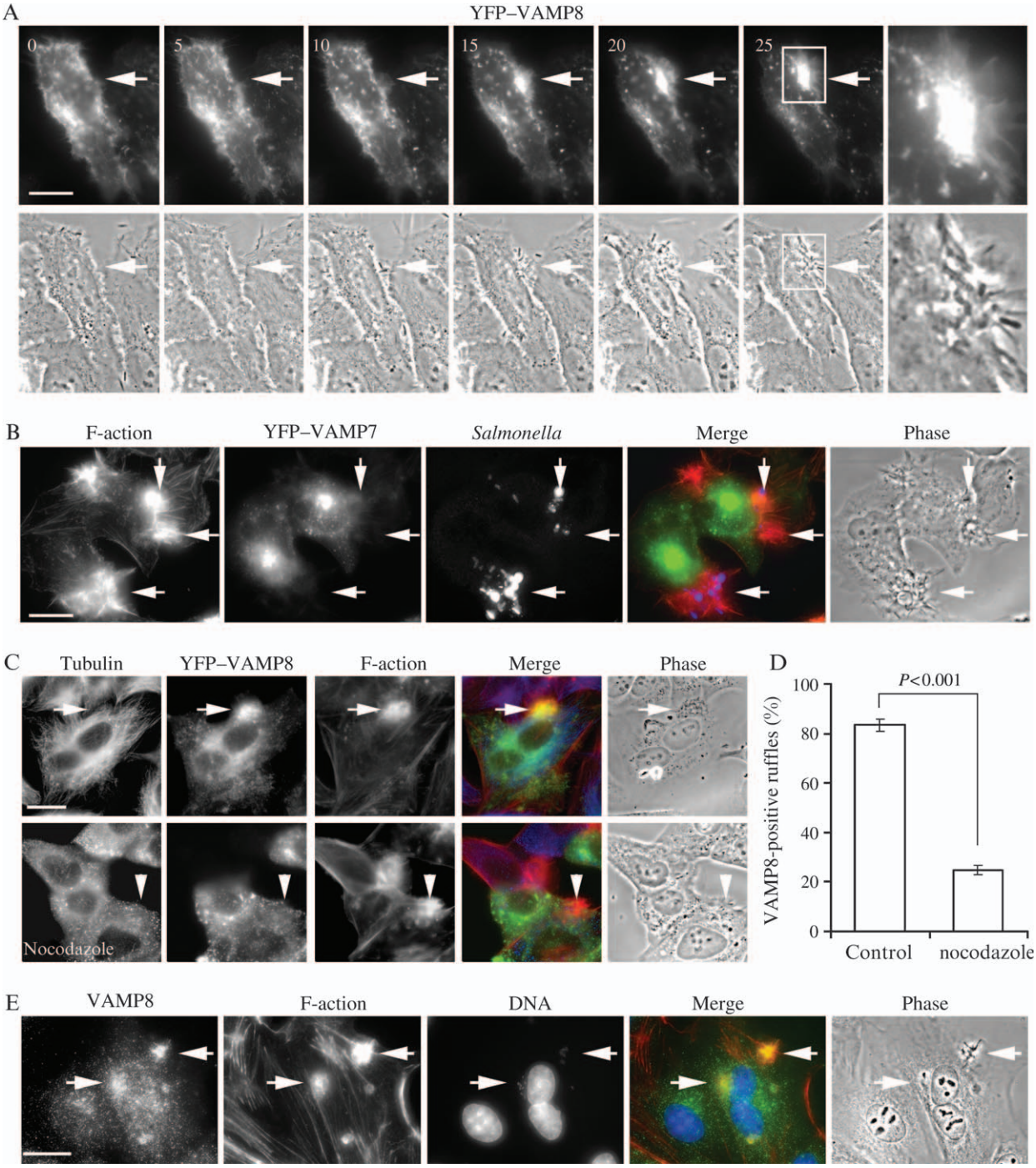

Figure 1: VAMP8-containing vesicles are recruited to *Salmonella*-induced ruffles. A) HeLa cells were transfected with plasmids expressing YFP–VAMP8. Transfected cells were infected with wild-type *Salmonella* at an MOI of 10, and images were taken at 1-min intervals. Selected images of every 5 minutes are shown (top left corner, time in minutes). B) VAMP7 is not recruited to the ruffles. HeLa cells were transfected with plasmids expressing YFP-VAMP7. Transfected cells were infected with wild-type *Salmonella* at an MOI of 10 for 15 min. VAMP7 was monitored by YFP (green), *Salmonella* by anti-LPS antibody (blue) and F-actin by Texas Red-phalloidin (red). C) Nocodazole inhibits VAMP8 recruitment. HeLa cells were transfected with plasmids expressing YFP–VAMP8. Transfected cells were infected with wild-type *Salmonella* at an MOI of 10 for 15 min in the presence of dimethyl sulphoxide (upper panels) or 25 μM nocodazole (lower panels). Tubulin was identified by anti-tubulin antibody (blue), VAMP8 by YFP (green) and F-actin by Texas Red-phalloidin (red). D) Quantification of the effect of nocodazole on VAMP8 recruitment. Only the cells transfected with YFP–VAMP8 (green) were counted. Data from three independent experiments were presented with standard deviation shown. The p value was calculated using the Student's *t*-test. E) MDCK cells were infected with wild-type *Salmonella* at an MOI of 10 and VAMP8 was stained with rabbit anti-VAMP8 antibody (green) and F-actin by Texas Red-phalloidin (red). Arrows and arrowheads point to sites of ruffle formation. Bars represent 20 μm.

YFP–VAMP8-expressing cells in the presence of nocodazole, a microtubule-depolymerizing drug, to disrupt microtubules and to interrupt vesicle trafficking along microtubules. We found that wild-type *Salmonella* failed to recruit YFP–VAMP8 to ruffles in the presence of nocodazole (Figure 1C,D). Furthermore, VAMP8 was not recruited to the site of bacterial attachment in the presence of cytochalasin D (data not shown). To further examine whether endogenous VAMP8 is concentrated in *Salmonella*-induced ruffles, Madin-Darby canine kidney (MDCK) cells were infected with wild-type *Salmonella* and the distribution of VAMP8 was visualized with anti-VAMP8 antibody. We found that endogenous VAMP8 is also recruited to *Salmonella*-induced membrane ruffles in MDCK cells (Figure 1E). To examine whether VAMP8 accumulation at the plasma membrane decreases when ruffles disappear following invasion, we did a short pulse (10 min) of invasion followed by incubation (50 min) in the absence of *Salmonella*. We found that VAMP8 did not colocalize with filamentous actin (F-actin) (Figure S1). Interestingly, it does appear that VAMP8 remains associated with SCVs.

Inositol phosphatase activity of SopB is required for VAMP8 recruitment

Salmonella type III effector, SopB, is an inositol phosphatase involved in host phospholipid metabolism and *Salmonella* invasion (16,17). To examine the role of SopB in the recruitment of VAMP8, YFP–VAMP8-transfected HeLa cells were infected with the wild-type *Salmonella* or inositol phosphatase-deficient SopB^{C460S} mutant strain (16) for 15 min. We found that cells infected with SopB^{C460S} mutant strain are severely defective in VAMP8 recruitment (Figure 2A) for up to an hour and that this is not because of the delayed invasion of the SopB^{C460S} mutant strain (Figure 2B). These data also demonstrate that the recruitment of VAMP8 by wild-type *Salmonella* is not because of membrane accumulation in the ruffles as the SopB^{C460S} mutant strain remains capable of inducing delayed membrane ruffles, yet it is defective in VAMP8 recruitment (Figure 2B). Recruitment of endogenous VAMP8 was also largely defective in MDCK cells when infected with the SopB^{C460S} mutant strain (Figure S2). Previous study has shown that heterologous expression of SopB leads to the accumulation of PtdIns(3)*P*-containing endosomes to

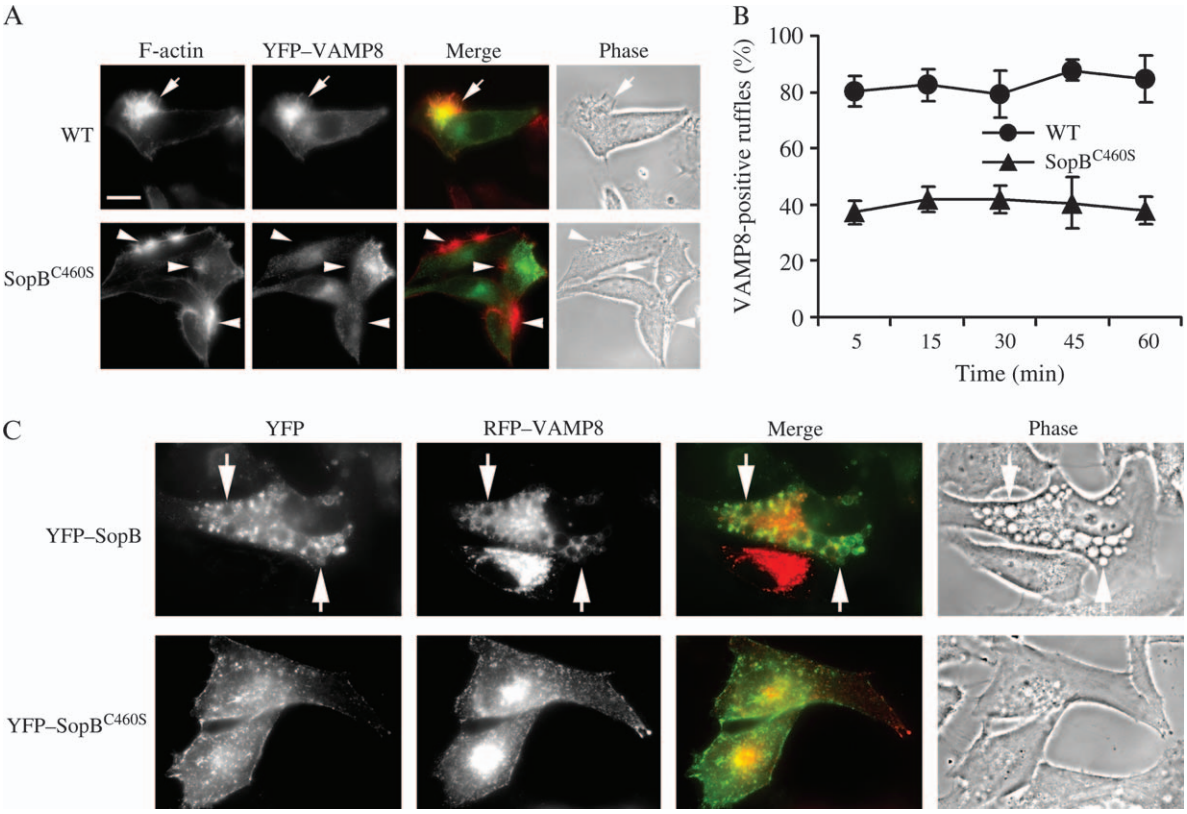

Figure 2: The inositol phosphatase activity of SopB is required for VAMP8 recruitment. A) The SopB^{C460S} mutant strain is deficient in VAMP8 recruitment. HeLa cells were transfected with plasmids expressing YFP–VAMP8. Transfected cells were infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain at an MOI of 10 for 15 min. Recruitment of VAMP8-containing vesicles was monitored by YFP and F-actin by Texas Red-conjugated phalloidin. B) The SopB^{C460S} mutant strain is deficient in VAMP8 recruitment over a period of 60 min. Data from three independent experiments were presented with standard deviation shown. C) SopB and VAMP8 colocalize near SopB-induced vacuoles. HeLa cells were cotransfected with plasmid expressing RFP–VAMP8 together with plasmids expressing either YFP-SopB or YFP-SopB^{C460S}. SopB was monitored by YFP and VAMP8 by RFP. Bars represent 20 μm. WT, wild-type.

which SopB localizes (19,20). To further investigate the role of SopB in VAMP8 recruitment, we cotransfected HeLa cells with plasmids expressing YFP–SopB and red fluorescent protein (RFP)–VAMP8. We found that YFP–SopB and RFP–VAMP8 colocalize well near the SopB-induced vacuoles (Figure 2C), suggesting that SopB, but not the phosphatase-deficient SopB^{C460S}, is capable of inducing VAMP8 accumulation.

VAMP8 is required for efficient Salmonella *invasion*

We next assessed the role of VAMP8 in *Salmonella* invasion using small interfering RNA (siRNA) knockdown. We constructed a bicistronic expression plasmid, which simultaneously expresses siRNA and enhanced green fluorescent protein (EGFP) to identify transfected cells. The knockdown effect of VAMP8 was examined by immunofluorescence microscopy and Western blot in HeLa cells (Figure 3A,B). Transfected cells were then infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain for 15 min. Expression of VAMP8 siRNA significantly decreased the invasion of wild-type *Salmonella* compared with the control siRNA (Figure 3C, $p = 0.0045$, two-tailed *t*-test). In contrast, VAMP8 siRNA had no obvious effect on the invasion by the SopB^{C460S} mutant strain (Figure 3C). Two-tailed *t*-tests indicated that the invasion of control cells by the wild-type *Salmonella* is significantly different from three other sample groups (Figure 3C). These results were further supported by expression of a cytosolic VAMP8$^{1–79}$ fragment, which had a dominant-negative effect on VAMP8 function (4). Expression of VAMP8$^{1–79}$ results in a significant reduction of *Salmonella* invasion (Figure 3D). Furthermore, expression of the wild-type VAMP8 significantly increased wild-type *Salmonella* invasion but not the SopB^{C460S} mutant strain. These results are consistent with the notion that VAMP8-containing vesicles are recruited to the *Salmonella*-induced membrane ruffles in a SopB-dependent manner to facilitate bacterial invasion.

VAMP8 colocalizes with SopB-generated PtdIns(3)P during Salmonella *invasion*

SopB-dependent accumulation of PtdIns(3)*P* in membrane ruffles suggests that PtdIns(3)*P* may play a role in

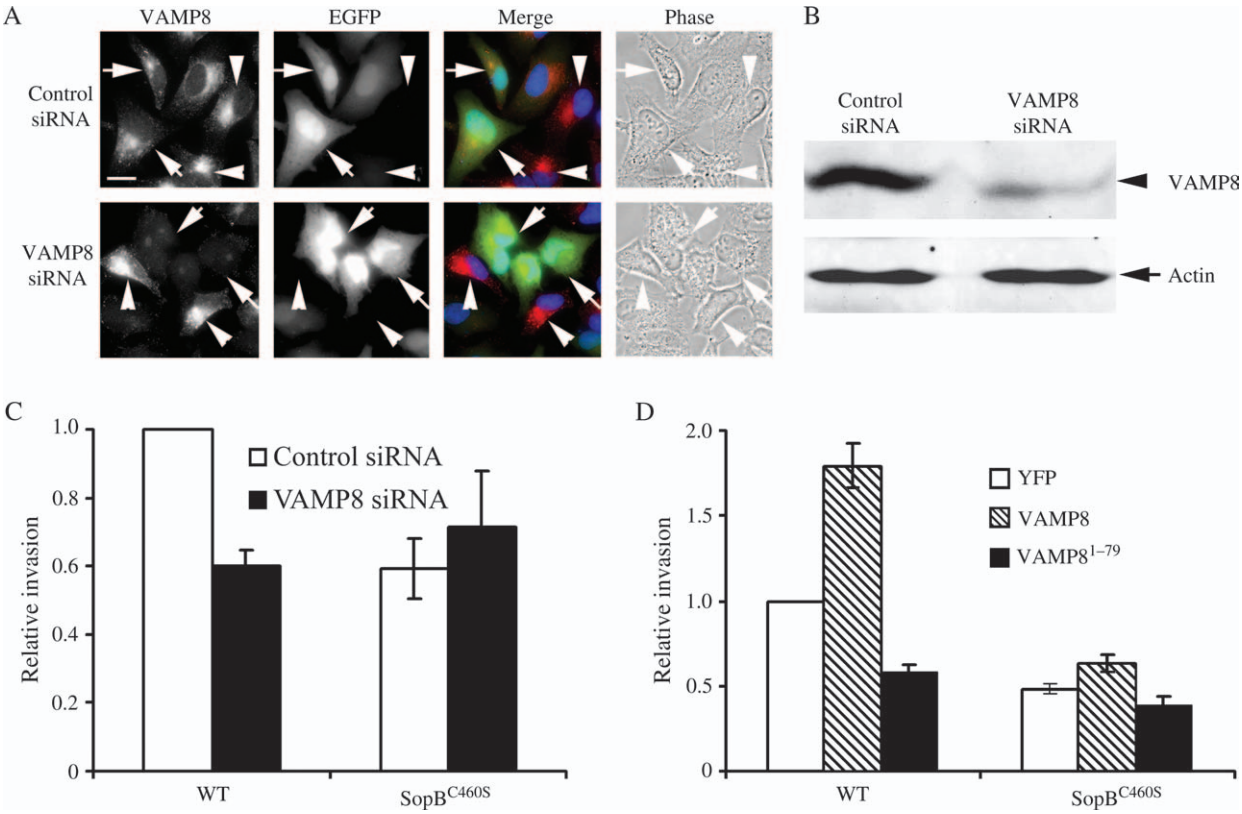

Figure 3: VAMP8 is required for efficient *Salmonella* invasion. A) HeLa cells were transfected with plasmid expressing VAMP8 or control siRNA. VAMP8 was examined by immunofluorescent microscopy (A) or Western blot (B) using anti-VAMP8 and anti-actin antibodies. Arrows and arrowheads indicate transfected cells and non-transfected cells, respectively. Bars represent 20 μm. C) VAMP8 siRNA-expressing cells were infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain at an MOI of 1 for 15 min. D) HeLa cells were transfected with plasmids expressing YFP, YFP–VAMP8 or YFP–VAMP8$^{1–79}$ and were infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain at an MOI of 1 for 15 min. The invasion rates were enumerated by inside/outside differential staining. The invasion of control cells by wild-type *Salmonella* was normalized as one for each independent experiment. Data from three independent experiments were presented with standard deviation shown. WT, wild-type.

VAMP8 recruitment. To examine VAMP8 and PtdIns(3)*P* distribution during *Salmonella* invasion, HeLa cells were transfected with plasmids expressing hemagglutinin (HA)-VAMP8 and p40-phox homology (PX)-green fluorescent protein (GFP), which specifically binds to PtdIns(3)*P* (21). Transfected cells were infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain. We found that both HA-VAMP8 and p40-PX-GFP were recruited to membrane ruffles and to vacuoles around the invading bacteria in more than 90% of the cells (among 500 cells) infected with wild-type *Salmonella* (Figure 4A). In contrast, the SopB^{C460S} mutant strain failed to recruit HA-VAMP8 and p40-PX-GFP (Figure 4A). HA-VAMP8 also colocalized with p40-PX-GFP, suggesting that SopB-generated PtdIns(3)*P* might be responsible for the recruitment of VAMP8-containing vesicles.

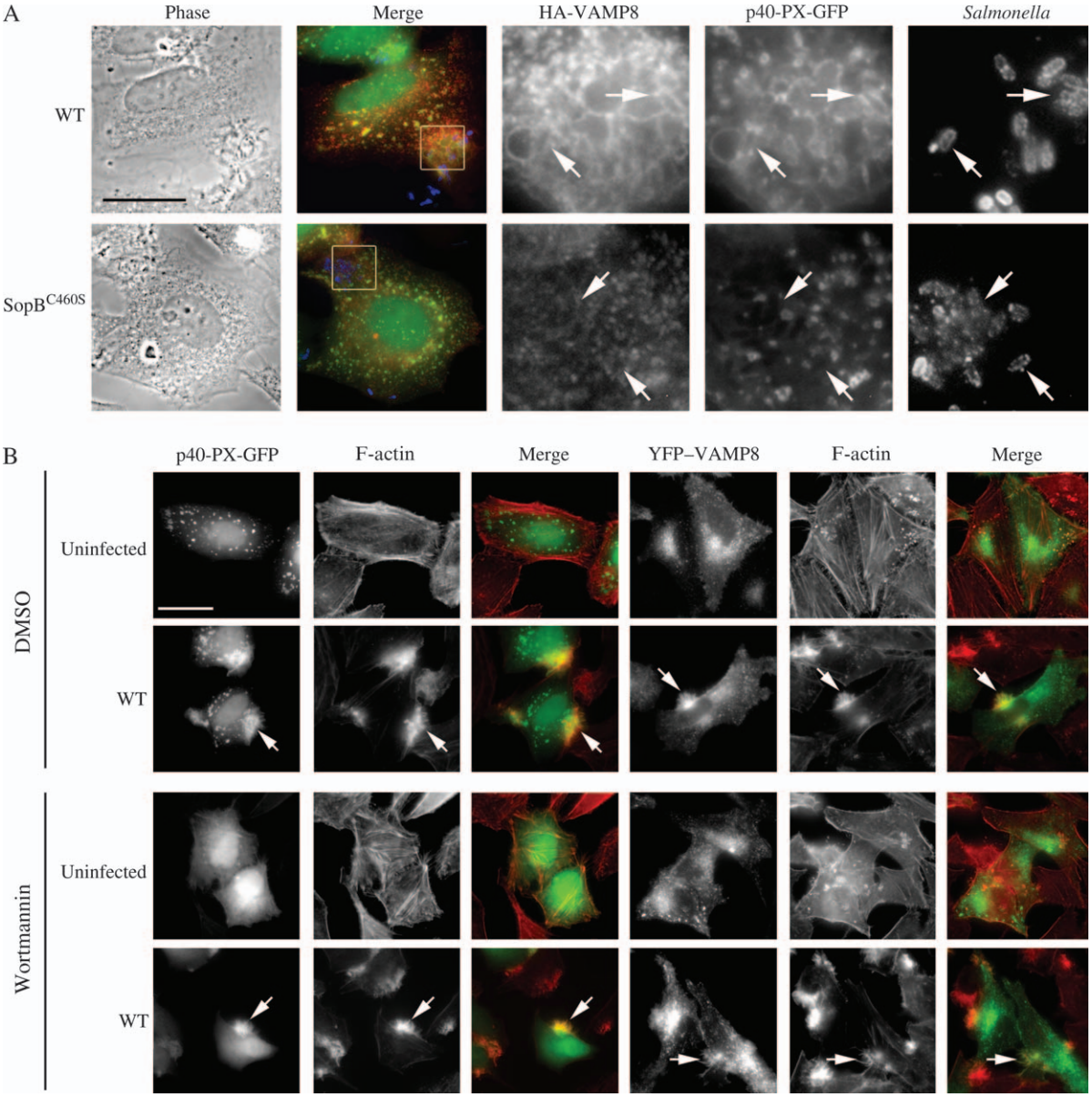

Figure 4: VAMP8 colocalizes with SopB-generated PtdIns(3)*P* during *Salmonella* invasion. A) Colocalization of PtdIns(3)*P* and VAMP8 requires the phosphatase activity of SopB. HeLa cells were cotransfected with plasmids expressing HA-VAMP8 and p40-PX-GFP. Transfected cells were infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain at an MOI of 10 for 15 min. HA-VAMP8 was identified with monoclonal anti-HA antibody and bacteria with anti-LPS antibody. Bars represent 10 μm. B) Wortmannin does not affect SopB-generated PtdIns(3)*P* during *Salmonella* invasion. HeLa cells were transfected with p40-PX-GFP or YFP–VAMP8 and then infected with wild-type *Salmonella* at an MOI of 10 for 15 min in the presence of dimethyl sulphoxide or wortmannin (100 nM). Distribution of p40-PX-GFP or YFP–VAMP8 was monitored by GFP or YFP (green), F-actin by Texas Red-phalloidin (red) and *Salmonella* by anti-LPS antibody (blue). Arrows point to ruffles. Bars represent 20 μm. WT, wild-type.

To investigate whether host phosphatidylinositol (PI) 3-kinase contributes to the PtdIns(3)*P* accumulation, we studied PtdIns(3)*P* distribution in the presence of wortmannin, a specific PI3 kinase inhibitor. HeLa cells expressing YFP–VAMP8 or p40-PX-GFP were infected with wild-type *Salmonella* or the SopB^{C460S} mutant strain in the presence or absence of 100 nM wortmannin. As expected, wortmannin treatment resulted in the disappearance of the punctate localization of p40-PX-EGFP (Figure 4B) (22). However, we found that wortmannin had no effect on the accumulation of PtdIns(3)*P* and YFP–VAMP8 upon *Salmonella* invasion (Figure 4B). Wortmannin also did not affect the invasion rate of either the wild-type *Salmonella* or the SopB^{C460S} mutant strain (data not shown), consistent with a previous report that PI3 kinase does not affect *Salmonella* invasion (23). To further exclude the possibility that PtdIns(3)*P* is actively recruited to ruffles, we infected p40-PX-GFP-expressing cells in the presence of nocodazole. We found that nocodazole treatment had no obvious effect on the accumulation of PtdIns(3)*P* in ruffles (data not shown). These data indicate that the phosphatase activity of SopB is responsible for generating PtdIns(3)*P* in *Salmonella*-induced ruffles.

VAMP8 binds PtdIns(3)P

PtdIns(3)*P* functions in signal transduction and membrane trafficking by interacting with PtdIns(3)*P*-binding protein modules. The PX domain is a 130 amino acid module that is present in the p40phox and the p47phox subunit of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (24,25). The classic FYVE zinc-finger domains also recognize PtdIns(3)*P* and share the basic (R/K)(R/K)HHCR amino acid consensus sequence (26). Etf1, a type II transmembrane protein important in autophagy without any PX or FYVE domain, is known to bind PtdIns(3)*P* through its basic cytosolic region (27). It is possible that SopB-dependent PtdIns(3)*P* binds VAMP8 to recruit VAMP8 to the ruffles. To test whether PtdIns(3)*P* binds VAMP8, we performed a lipid overlay assay (22) with lysates from HeLa cells expressing YFP–VAMP8, YFP–VAMP8$^{1–79}$ or lysates from *Escherichia coli* expressing glutathione S-transferase (GST)–VAMP8$^{1–79}$. We found that YFP–VAMP8, YFP–VAMP8$^{1–79}$ and GST–VAMP8$^{1–79}$ interacted strongly with PtdIns(3)*P* (Figure 5A). The binding of VAMP8 to PtdIns(3)*P*, but not to PtdIns, is comparable to that of the p40-PX domain as shown by YFP–VAMP8 and His-YFP–VAMP8 (Figure 5B). We further tested the direct interaction between VAMP8 and PtdIns(3)*P* with a liposome-binding assay. We found that VAMP8, but not VAMP7, interacted directly with liposomes containing PtdIns(3)*P* but not with control liposomes in the presence or absence of PtdIns (Figure 5C).

SopB has limited similarity to inositol polyphosphate 4-phosphatases and mammalian type II inositol 5-phosphatase synaptojanin (28,29). It hydrolyzes a number of inositol phosphates and PI lipids including PtdIns(3,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (29). It is possible that

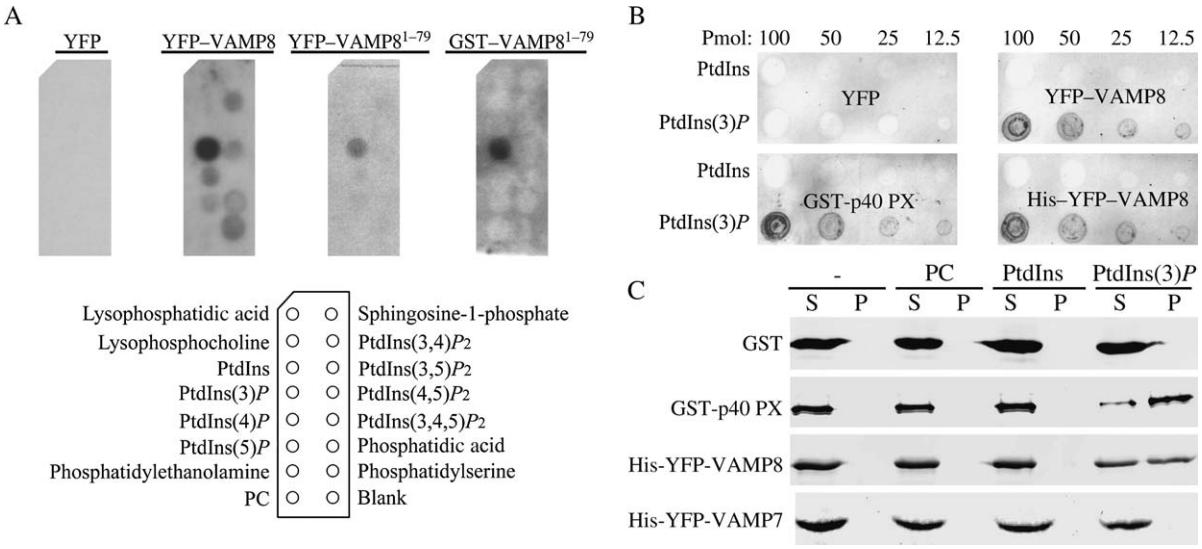

Figure 5: VAMP8 binds PtdIns(3)*P*. A) YFP–VAMP8, YFP–VAMP8$^{1–79}$ and GST–VAMP8$^{1–79}$ interact with PtdIns(3)*P*. Phospholipid microstrips were incubated with HeLa cell lysate expressing YFP, YFP–VAMP8 and YFP–VAMP8$^{1–79}$ or *E. coli* lysate containing GST–VAMP8$^{1–79}$. Proteins bound were detected by Western blot with anti-GST or anti-YFP antibody. B) Decreasing concentrations of PtdIns and PtdIns(3)*P* on Hybond-C membranes were incubated with *E. coli* lysate containing GST-p40-PX or His-YFP–VAMP8, HeLa cell lysate containing YFP or YFP–VAMP8. Proteins bound were detected by Western blot with anti-GST or anti-YFP antibody. C) Purified His-YFP–VAMP8 binds liposome containing PtdIns(3)*P*. Purified proteins were incubated with liposomes that contain 5% PtdIns or PtdIns(3)*P*. Binding to the liposome was analyzed by ultracentrifugation followed by Western blot using anti-YFP and anti-GST antibodies. PC, phosphatidylcholine.

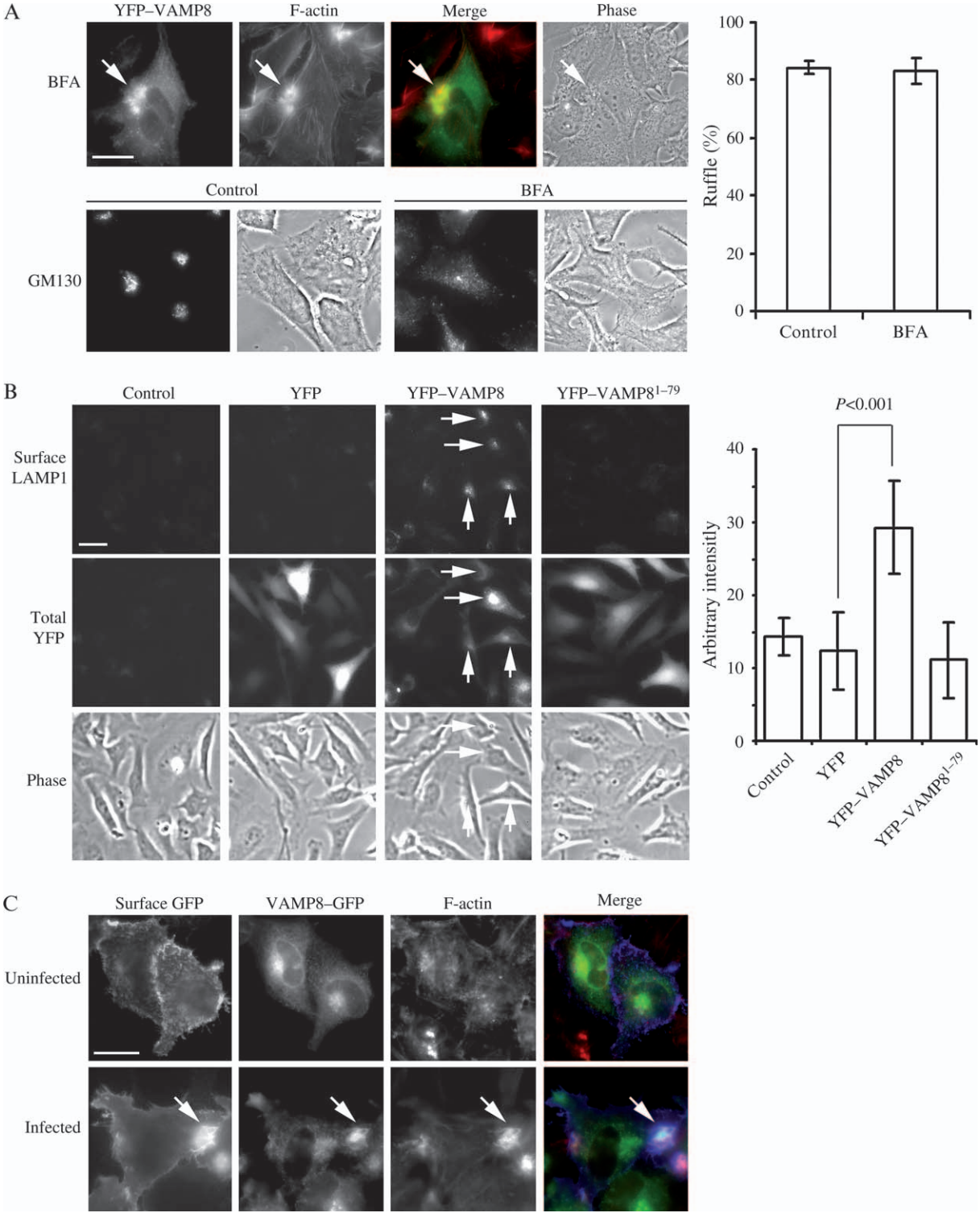

Figure 6: Legend on next page.

SopB generates PtdIns(3)*P* by hydrolyzing PtdIns(3,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 resulting in the accumulation of PtdIns(3)*P* in ruffles (19). Vam7p, the vacuolar SNAP-23 homologue in yeast, has been shown to bind PtdIns(3)*P* and target to membrane through its PX domain (30). Etf1, another type II transmembrane protein without PX or FYVE domains, has been shown to interact with PtdIns(3)*P* through a basic cytosolic region. It was

suggested that the Etf1–PtdIns(3)*P* interaction functions in the localization of Etf1 and regulates autophagosome trafficking (27). We showed here that VAMP8 binds PtdIns(3)*P* through its cytosolic N-termini. Further experiments are needed to examine whether the VAMP8–PtdIns(3)*P* interaction affects VAMP8-mediated SNARE complex formation or membrane fusion process.

VAMP8-containing vesicles are recruited to the Salmonella*-induced ruffles through the endocytic pathway*

VAMP8 is mainly present on early and late endosomes and the trans-Golgi network (TGN) and is responsible for homotypic fusion of early endosomes and late endosomes (3). VAMP8-containing vesicles at the *Salmonella*-induced ruffles might originate from either the endocytic pathway or the secretory pathway. To examine if the recruited VAMP8 originates from the endocytic pathway, we repeated the infection in the presence of Brefeldin A (BFA) to disrupt the Golgi complex and block the secretory pathway. We found that YFP–VAMP8 recruitment was similar to that of untreated cells (Figure 6A). Thus, the recruitment of VAMP8 is likely from the endocytic pathway rather than the secretory pathway during *Salmonella* invasion.

It was reported that *Salmonella* induces cell surface exposure of lysosomal-associated membrane protein 1 (LAMP1) (13), a marker for late endosomes and lysosomes. To investigate the role of VAMP8 in LAMP1 exposure, HeLa cells were infected with adenovirus expressing YFP–VAMP8 or YFP–VAMP8$^{1-79}$. Cells were then stained with anti-LAMP1 antibody without permeabilization. We found that cells expressing YFP–VAMP8 had increased the surface level of LAMP1. In contrast, expression of YFP, YFP–VAMP8$^{1-79}$ had no obvious effect on the surface level of LAMP1 (Figure 6B).

VAMP8 is a type II transmembrane SNARE protein with its N-terminus facing the cytoplasm. If VAMP8-containing vesicles are recruited to membrane ruffles, we anticipate that the C-termini of VAMP8 may be exposed, while the N-termini remain facing the cytosol in *Salmonella*-induced ruffles. We tested this by using a VAMP8–GFP hybrid protein, with GFP fused to the C-termini of VAMP8. HeLa cells expressing VAMP8–GFP were infected with wild-type *Salmonella* and examined for surface GFP using anti-GFP antibody without permeabilization. We detected surface GFP on uninfected cells and prominent surface GFP in *Salmonella*-induced ruffles (Figure 6C). Furthermore, we did not observe any surface YFP when the above experiment was repeated using YFP–VAMP8, which had the YFP fused to the N-terminus of VAMP8. This suggests that the C-termini of VAMP8 may be exposed in *Salmonella*-induced ruffles.

Lysosome exocytosis was observed during bacteria invasion and was believed to serve as a host-killing mechanism to protect eukaryotic cells from pathogens (13). VAMP8 mainly localizes on early and late endosomes and the TGN involved in homotypic fusion and regulated exocytosis (3). Our data are consistent with VAMP8-containing vesicles being recruited to *Salmonella*-induced ruffles by recycling the VAMP8-containing endosomes. Both phagocytosis and pathogen-induced macropinocytosis require membrane addition to facilitate the increase in membrane surface area (10). Our study demonstrates a novel cellular mechanism in which SopB functions to generate PtdIns(3)*P* leading to the VAMP8 recruitment, thereby facilitating *Salmonella* invasion. The membrane addition may also synergize with the actin cytoskeleton rearrangements mediated by SipA, SipC and SopE to promote robust bacterial invasion into the host cells. This represents a major advancement in our current working model of *Salmonella* invasion. These data may have broader implications that vesicle exocytosis and bacterial phagocytosis are closely linked and suggest that phospholipids play an essential role in recruiting endosomes to facilitate the formation of lamellipodia and filopodia.

Materials and Methods

Bacterial strains, mammalian cell lines and bacterial infection

Wild-type *Salmonella typhimurium* SL1344 and a chromosomal point mutant derivative, which expresses the phosphatase-defective SopB^{C460S}, have been described previously (16,28,31). Mammalian cell infection was conducted as described previously (31) at the multiplicity of infection (MOI) of 1 or 10, as indicated. Brefeldin A (5 μg/mL), wortmannin (100 nM) or nocodazole (25 μM) was added 1 h before infection, and infections were carried out in the presence of these drugs, where indicated. Bacterial invasion was quantified using the inside/outside differential staining protocol as described previously (31). HeLa (CCL-2) and human embryonic kidney (HEK)293 (CRL-1573) cells were from the American Type Culture

Figure 6: VAMP8-containing vesicles are recruited to *Salmonella*-induced ruffles through exocytosis. A) Brefeldin A does not inhibit VAMP8 recruitment. The effect of BFA is shown by staining with anti-GM130, indicating the disruption of the Golgi complex. Arrows point to sites of ruffle formation. Data from three independent experiments were presented on the right panel with standard deviation shown. B) Expression of YFP-VAMP8 increases the surface LAMP1. HeLa cells expressing YFP, YFP–VAMP8 or YFP–VAMP8$^{1-79}$ were analyzed for surface LAMP1 with anti-LAMP1 antibody without permeabilization. Images were taken at a fixed exposure time. Data from three independent experiments were presented on the right panel with standard deviation shown. The p value was calculated using Tukey's test. C) The GFP moiety of VAMP8–GFP is surface exposed during *Salmonella* invasion. HeLa cells expressing VAMP8–GFP were infected with wild-type *Salmonella* at an MOI of 10 for 15 min. Surface VAMP8–GFP was detected with anti-GFP antibody without permeabilization (blue), and total VAMP8–GFP was monitored by GFP (green) and F-actin by Texas Red-phalloidin (red). Bars represent 20 μm.

Collection stock centre. Cells were maintained in DMEM supplemented with 10% FBS. Antilipopolysaccharide (anti-LPS) antibodies were from Difco Laboratories. Anti-human LAMP1 antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Affinity-purified anti-VAMP8 antibody was described previously (32).

Plasmids and recombinant proteins

The full-length *VAMP8* gene was amplified from a human kidney complementary DNA (cDNA) library by polymerase chain reaction (PCR) using primers: 5′-GGAGATCTATGGAGGAAGCCAGTGAAGG-3′ and 5′-GCGGTACCTAAGAGAAGGCACCAGTGGCAAAG-3′. Polymerase chain reaction products were cloned into enhanced yellow fluorescent protein (pEYFP)-C1, pEGFP-N2 (Clontech) and pJ3H to yield plasmids expressing YFP–VAMP8, VAMP8–GFP and HA-VAMP8, respectively. Genes encoding the truncated cytosolic fragment of VAMP8 (1–79aa) was amplified by PCR using primers: 5′-GGAGATCTATGGAGGAAGCCAGTGAAGG-3′ and 5′-GCGGTACCTAGTTCTTCCACCAGAATTTCC-3′. Yellow fluorescent protein–VAMP8 was cloned into pET28 to produce His-YFP–VAMP8 recombinant protein. Plasmids pGEX-p40-PX and pGFP-p40-PX, expressing chimeric proteins of GST or GFP fused with the PX domain of p40phox subunit of NADPH oxidase complex, were described previously (22). To construct the VAMP8 siRNA knockdown plasmid, pSilencer 3.1-H1 neo (Ambion) was used to target VAMP8 (5′-AAGCCACATCTGAGCACTT CA-3′). The bicistronic siRNA and EGFP expression plasmids were constructed by replacing the neomycin gene with the EGFP cassette from pEGFP-N2. Recombinant proteins were expressed and purified from lysates in *E. coli* BL-21 using Ni_2+-nitrilotriacetate (Ni-NTA) agarose (Qiagen) or immobilized glutathione (Pierce).

Adenovirus production

pAdTrack cytomegalovirus vectors containing cDNAs of interest were electroporated into BJ5183 cells containing the Adeasy plasmid. Recombined adenovirus clones were screened, packaged, amplified and titred in HEK293 cells, as described previously (33).

Transfection and immunofluorescence microscopy

Transfection of HeLa cells was carried out using FuGene 6 (Roche). Infection of HeLa cells with adenovirus was performed in DMEM supplemented with 10% FBS and 8 μg/mL polybrene at an MOI of 10. Twenty-four hours after transfection or adenoviral infection, cells were infected with *Salmonella* and processed as described previously (16). Filamentous actin was visualized by staining with Texas Red-conjugated phalloidin (Molecular Probes). Images were obtained on a Zeiss AxioVert 200M deconvolution microscope.

Protein–lipid overlay assay

Phospholipid-strip overlay assay was performed using phospholipid Microstrips (Echelon Biosciences) following manufacturer's protocol. Protein–lipid overlay assay was carried out as previously described (22). Briefly, lipid solution containing 12.5–100 pmol of phospholipids dissolved in chloroform:methanol:water (1:1:0.3 volume ratio) was spotted onto Hybond-C extramembranes (GE Healthcare) and dried at room temperature. The membrane was blocked in 4% fat-free milk in Tris-Buffered Saline Tween-20 (TBST) [50 mM Tris (pH 7.5), 150 mM NaCl and 0.1% Tween-20]. Membranes were incubated with the same solution containing desirable proteins of the same concentration at 4°C overnight, washed five times in TBST and preceded for Western blot. The expression level of GST-p40-PX, GST–$VAMP8^{1–79}$ and His-YFP–VAMP8 were comparable in *E. coli* lysate (Figure S3). The expression level of YFP, YFP–VAMP8 and YFP–$VAMP8^{1–79}$ were also similar in HeLa cell lysates (Figure S3).

Liposome–protein interaction assay

Liposome–protein interaction assay was modified, as described previously (22). Lipids were from Matreya Inc. Mixtures of phosphatidylcholine with and without the indicated phospholipids (5% of total) in methanol:chloroform:H_2O (1:1:0.3 volume ratio) were dried under N_2 and hydrated at room temperature in 20 mM HEPES (pH 7.5) and 100 mM NaCl. Liposomes were formed by multiple passages (>11) through a Mini-Extruder (Avanti) equipped with a polycarbonate membrane (0.1 μm) at 45°C. Purified His-YFP-VAMP8, GST or GST-p40-PX were incubated with liposomes for 1 h at room temperature in a total volume of 100 μL, and liposomes were pelleted by centrifugation at 400,000 x ***g*** for 30 min. Pellets were washed three times in HEPES, and the amount of proteins in the supernatant and pellet was separated by SDS–PAGE, followed by Western blot.

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Supplementary Materials

Figure S1: VAMP8 accumulation decreases when ruffles decrease following invasion. HeLa cells were infected for 10 min, and bacteria were washed away followed by incubating in fresh media for 50 min in the absence of bacteria. Filamentous actin was visualized by Texas Red-phalloidin (red), VAMP8 by YFP (green) and *Salmonella* by anti-LPS antibody (blue). Bars represent 20 μm.

Figure S2: The $SopB^{C460S}$ mutant strain is deficient in VAMP8 recruitment. MDCK cells were infected with the $SopB^{C460S}$ mutant strain at an MOI of 10, VAMP8 was stained with rabbit anti-VAMP8 antibody (green) and F-actin by Texas Red-phalloidin (red). Arrows point to sites of ruffle formation.

Figure S3: Expression levels of recombinant proteins. The expression level of GST-p40-PX, GST–$VAMP8^{1–79}$ and His-YFP–VAMP8 were comparable in *E. coli* lysate (left panel). The expression level of YFP, YFP–VAMP8 and YFP–$VAMP8^{1–79}$ were also similar in HeLa cell lysates (right panel).

Supplemental materials are available as part of the online article at http://www.blackwell-synergy.com

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