Supplementary Figures

Supplementary Figure 1. Characterization of *Salmonella*-containing phagosomes.

Salmonella-containing phagosomes (SCP) were isolated as described in Experimental Procedures. Previously, we have demonstrated that SCP prepared using this procedure are devoid of any contamination with lysosomes, Golgi and other cellular components (1,2). In Fig. S1, we have compared the presence of indicated markers on early (SCP5) and late *Salmonella*-containing phagosomes (SCP90) to determine the purity of phagosomes. The results presented in the Fig. S1 show that early phagosomes are significantly pure and free from lysosomes, Golgi and cytosolic contamination. The presence of Rab5 and LAMP1 on SCP90 is the property of late phagosomes rather than endosomal or lysosomal contamination.

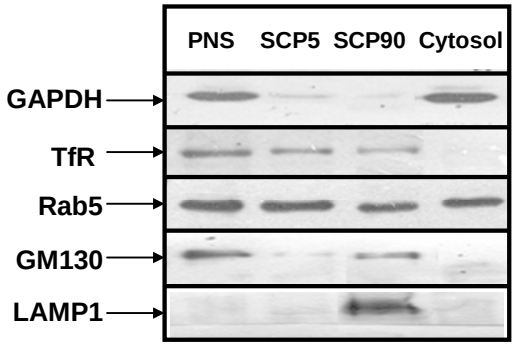


Fig. S1. Characterization of *Salmonella*-containing phagosomes. Western blot analysis was carried using 40 μg of phagosomal protein from each fraction using indicated antibodies.

Supplementary Figure 2. Identification of Syntaxin6 binding protein as SipC by mass spectrometry

To identify the Syntaxin6 binding protein from *Salmonella*, pull out experiment was carried out using immobilized GST-Syntaxin6 and non-biotinylated secretory proteins of *Salmonella* as described in Experimental Procedures. The bound proteins were separated on a 12% SDS-PAGE. Subsequently, the Syntaxin6 binding protein was subjected to tryptic digestion and peptides obtained were purified and sequenced by mass spectrometry in a commercial facility (The Center for Genomic Application, New Delhi). The obtained peptides were mapped into SipC (Fig. S2).

MLISNVGINPAAYLNNHSVENSSQTASQSVSA**KDILNSIGISSSKV**SDLGLSPTLSAPAPGVLTQTPGTI
TSFLKASIQNTDMNQDLNALANNVTT**KANEVVQTQLRE**QQAEVGKFFDISGMSSSAVALLAAANTLMLTL
NQADSKLSG**KLSLVSFDAAKT**TASSMMREGMNALSGSISQSALQLGITGVGAKLEYKGLQNERGALKHNA
AKIDKLTTESHSIKNVLNGQNSV**KLGAEGVDSLKS**LNMKKTGTDATKNLNDATL**KSNAGTSATESLGIK**D

SNKQISPEHQAILS**KRLESVESDIRL**EQNTMDMTRIDAR**KMQMTGDLIMKN**SVTVGGIAGASGQYAATQE
RSEQQISQVNNRVASTASDEARESSR**KSTSLIQEMLKT**MESINQS**KASALAAIAGNIRA**

Fig. S2. Sequences of peptides obtained by mass spectrometry (red) and mapped into SipC sequence.

Supplementary Figure 3. Binding of different concentrations of SipC with immobilized Syntaxin6.

GST-Syntaxin6 (2.5 μg) or equimolar concentration of GST was immobilized on beads and incubated with indicated concentrations of His_6-SipC and the binding of SipC was detected by Western blot analysis using anti-SipC antibody (Fig. S3A) as described in Experimental Procedures. Optimal binding of Syntaxin6 with SipC was observed at 1 μg of SipC and reached steady state plateau at higher concentration of SipC. The results presented in the Fig. S3B show the coomassie blue stain of SDS-PAGE of different concentrations of SipC used in the experiments.

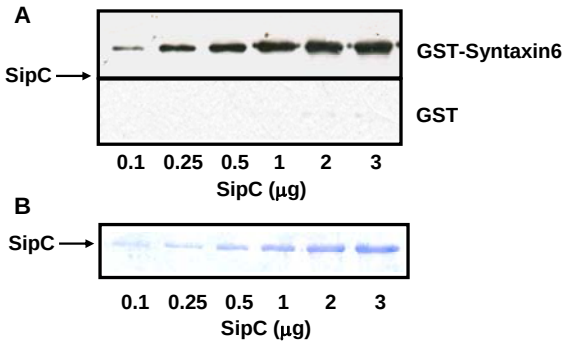


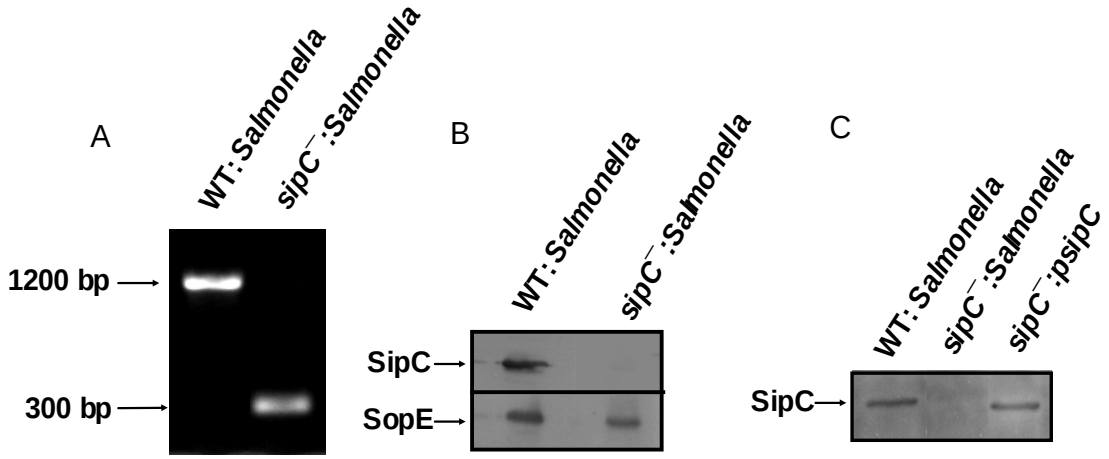
Fig. S3. A. Binding of different concentrations of SipC with immobilized GST-Syntaxin6 and GST. B. Coomassie blue stain of SDS-PAGE of indicated concentrations of SipC used in pull out experiment.

Supplementary Figure 4. Generation and characterization of *sipC*⁻ *Salmonella*.

To understand the role of SipC in the trafficking of *Salmonella* in macrophages, *sipC* knockout bacteria (*sipC*⁻*:Salmonella*) was generated by a suicide vector based allelic exchange method of homologous recombination (3) using 1 kb upstream and downstream regions of *sipC*. In addition, *sipC* was complemented into *sipC*⁻*:Salmonella* using an arabinose inducible *Salmonella* expression vector, pBAD24 (*sipC*⁻*:*p*sipC Salmonella*). PCR analysis carried out using gene specific primers which amplified a 300 bp fragment from *sipC*⁻*:Salmonella* genomic DNA in comparison to 1200 bp fragment from WT:*Salmonella* (Fig. S4A). Similarly, no SipC was

detected from secretory protein preparation of s*ipC*¯*:Salmonella* by Western blot analysis using a specific antibody. Presence of SopE in *sipC*¯*:Salmonella* secreted proteins indicated that deletion of SipC does not interfere with the secretion of other effectors (Fig. S4B). These results demonstrated the successful deletion of *sipC* from *Salmonella*. Moreover, similar levels of SipC were obtained in comparison to WT:*Salmonella* when s*ipC*¯:p*sipC Salmonella* was induced with 0.0005% of L-arabinose for 2 hrs at 37°C (Fig. S4C) indicating the successful complementation of *sipC* into s*ipC*¯:p*sipC Salmonella*.

To compare the intracellular trafficking of WT and *sipC*¯*:Salmonella* in macrophages, J774E cells were infected with respective GFP-*Salmonella* for 5 min at 37°C, fixed and incubated with Alexa-546 phalloidin (3.3 nM) for 45 min to stain F-actin. Cells were analyzed by confocal microscopy and results are representative of three independent experiments. Our results showed that WT:bacteria induce actin rearrangements and membrane ruffling in close vicinity of SCP. In contrast, actin localization was distinctly more diffuse and distant from s*ipC*¯:SCP (Fig. S4D) as shown previously in HeLa cells (4).



D.

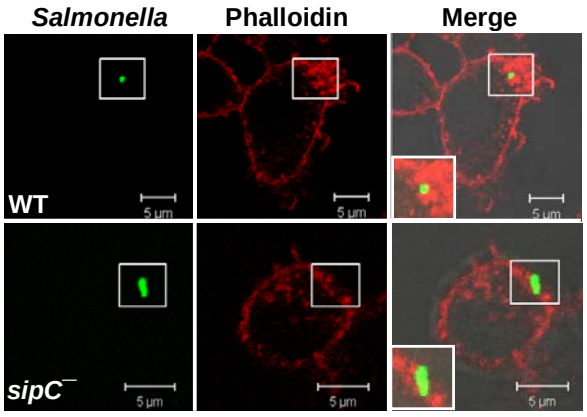


Fig. S4. Generation and characterization of s*ipC*$^{-}$: *Salmonella*. (A) PCR analysis was carried out by *sipC* gene specific primers using genomic DNA prepared from WT:*Salmonella* and s*ipC*$^{-}$*:Salmonella*. (B) WT:*Salmonella* and s*ipC*$^{-}$*:Salmonella* were grown in the presence of high salt to induce the secretion of secretory proteins from the respective *Salmonella*. Subsequently, indicated proteins were detected in the secretory protein preparations by Western blot analysis using specific antibodies. (C) Similar analysis was carried out using secretory proteins prepared from WT:*Salmonella*, s*ipC*$^{-}$ *:Salmonella* and s*ipC*$^{-}$:p*sipC Salmonella*. (D) Confocal micrographs showing the localization of actin in macrophages infected with respective *Salmonella* where green shows the GFP-*Salmonella* and actin appears in red.

Supplementary Figure 5. Comparative analysis of intracellular trafficking of WT: and s*ipC*$^{-}$:SCP in macrophages.

The recruitment of endocytic markers like Rab5, Rab7 and Transferrin receptor (TfR) on respective bacteria containing phagosomes was determined by Western blot analysis during their maturation in macrophages. Our results revealed that both WT:SCP and s*ipC*$^{-}$SCP even after 90 min of internalization in macrophages retain significant amounts of Rab5 and TfR; and show reduced level of Rab7 (Fig. S5). Whereas, latex bead-containing phagosomes after 90 min of internalization showed relatively low amounts of Rab5 and TfR but contained significant amount of Rab7 in comparison to early phagosomes. These results demonstrated that both WT and mutant bacteria enter into Rab5 and TfR positive early endosomal compartment but inhibit their transport towards late endocytic/lysosomal compartment.

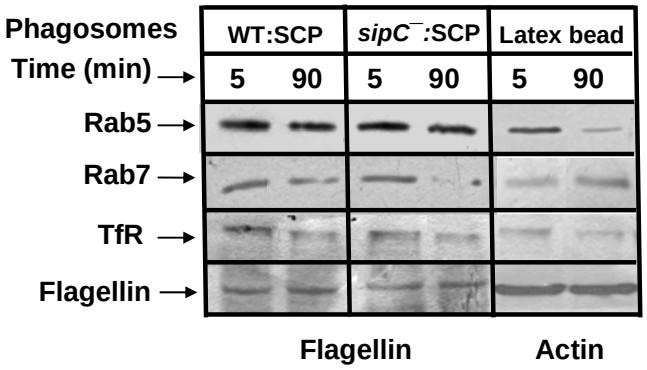


Fig. S5. Comparative analysis of endocytic markers on WT:SCP, s*ipC*$^{-}$:SCP and Latex bead phagosomes in macrophages.

Supplementary Figure 6. Overexpression and synchronization of LAMP1-GFP in Golgi in macrophages.

LAMP1-GFP was overexpressed in RAW 264.7 macrophages and synchronized in GM130 labelled Golgi by appropriate temperature block as described in Experimental Procedures. Our results showed that LAMP1 is predominantly present in the GM130-labeled Golgi along with several exocytic vesicles in the cytoplasm 20 hrs post transfection (Fig. S6, upper panel) which is subsequently synchronized in Golgi (Fig. S6, middle panel). Budding of LAMP1-containing vesicles from Golgi was observed when these cells were further incubated for 10 min at 37°C (Fig. S6, lower panel).

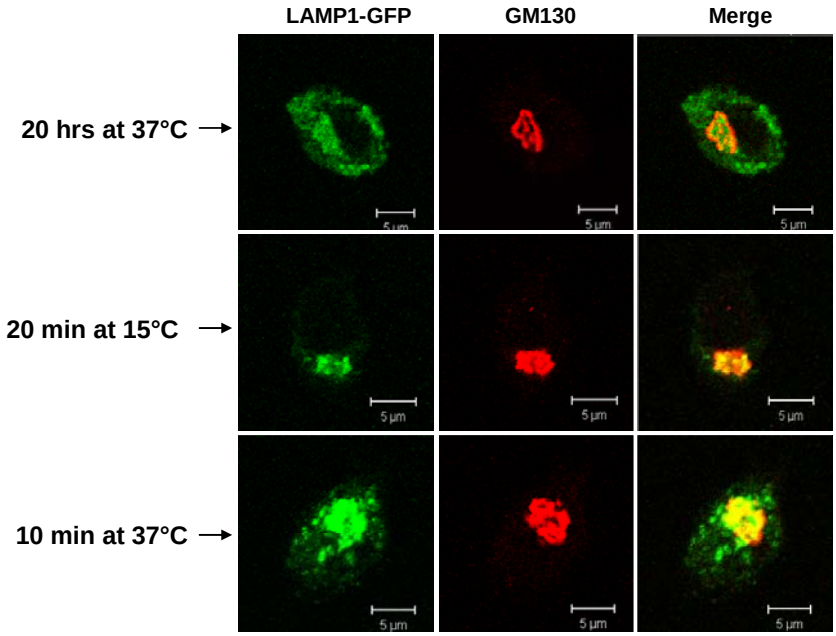


Fig. S6. Synchronization of LAMP1-GFP in Golgi of RAW 264.7 macrophages.

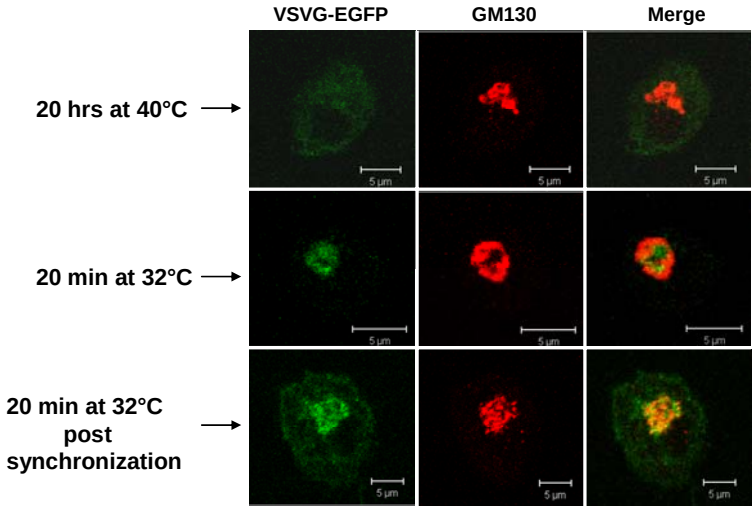
Supplementary Figure 7. SCP recruit VSVG from Golgi-derived vesicles.

To determine the interaction of WT:SCP with the secretory pathway, RAW264.7 macrophages were transfected with temperature sensitive mutant of VSVG-EGFP (kindly provided by Dr. Jennifer L. Schwatrz, NIH, Bethesda) and cells were incubated for 20 hrs at 40°C to overexpress this protein. Subsequently, VSVG-EGFP overexpressing cells were further incubated at 32°C for 20 min to synchronize VSVG in Golgi (Fig. S7A).

To monitor the acquisition of VSVG by WT or s*ipC*$^{-}$: SCP from Golgi derived vesicles, RAW264.7 macrophages were infected with RFP:WT or RFP:s*ipC*$^{-}$: *Salmonella* for 5 min at

40°C after 18 hrs transfection with VSVG-EGFP. After infection, uninternalized bacteria were removed by extensive washes with RPMI medium and the infected cells were incubated at 40°C for 120 min to allow bacterial transport towards the appropriate late compartments inside the cells. Subsequently, cells were incubated at 32°C for 20 min to synchronize the transport of VSVG at Golgi (5). Finally, cells were incubated for various periods of time at 32°C to allow the budding of VSVG-containing vesicles from Golgi and the presence of VSVG-EGFP on respective SCP was determined at indicated time points. Our results showed (Fig. S7B) that WT:SCP fuse with VSVG-containing Golgi-derived vesicles whereas s*ipC*$^{-}$:SCP failed to recruit VSVG. These results further confirmed that SipC is necessary for the interaction of *Salmonella*-containing phagosomes with Golgi-derived vesicles.

A.



B.

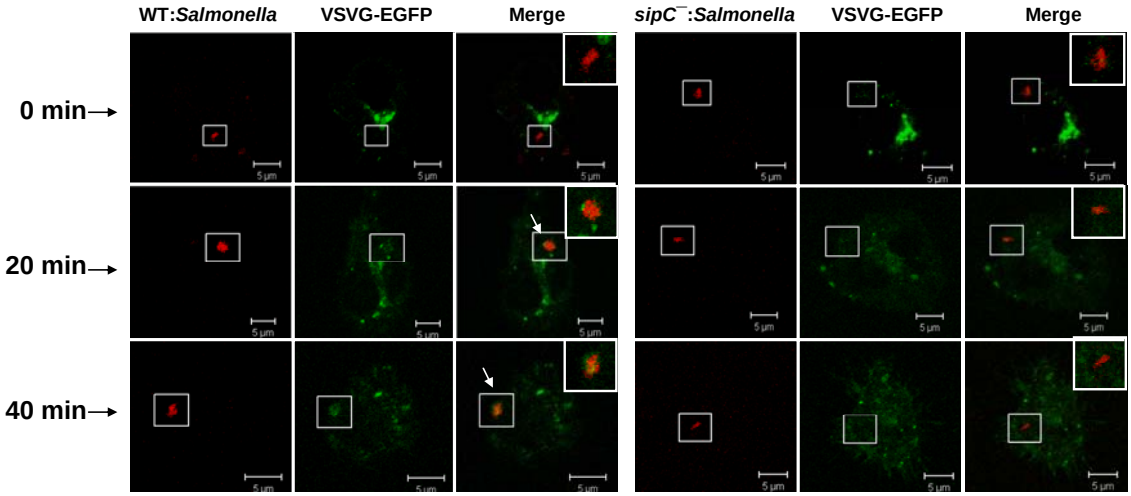


Fig. S7. *Salmonella*-containing phagosomes recruit VSVG from Golgi-derived vesicles. (A) Overexpression and synchronization of VSVG in Golgi. (B) Recruitment of VSVG by respective *Salmonella*-containing phagosomes.

Supplementary Figure 8. Recruitment of various markers by *sipC*⁻:p*sipC* SCP at different stages of maturation in macrophages.

To confirm that the recruitment of Syntaxin6, VAMP2, Rab6 by WT:SCP is a SipC driven process, we determined the levels of these proteins on s*ipC*⁻:p*sipC* SCP at different stages of maturation in macrophages. Our results showed that both WT:SCP and s*ipC*⁻:p*sipC*:SCP recruit comparable amounts of Syntaxin6, VAMP2 and Rab6.

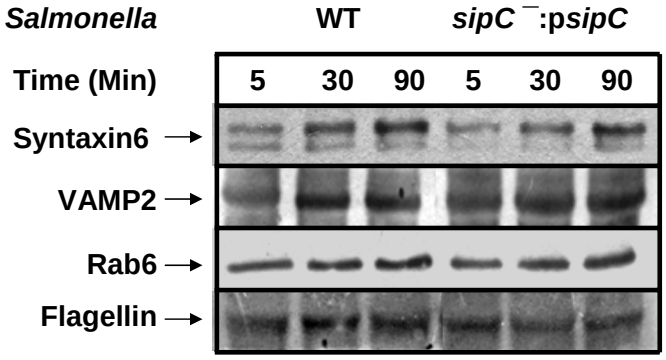


Fig. S8. Recruitment of Syntaxin6, VAMP and Rab6 by s*ipC*⁻:p*sipC* SCP at different stages of maturation in macrophages.

References

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