The *Salmonella* effectors SseF and SseG inhibit Rab1A-mediated autophagy to facilitate intracellular bacterial survival and replication

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ABSTRACT:

In mammalian cells, autophagy plays crucial roles in restricting further spread of invading bacterial pathogens. Previous studies have established that the *Salmonella* virulence factors SseF and SseG are required for intracellular bacterial survival and replication. However, the underlying mechanism by which these two effectors facilitate bacterial infection remains elusive. Here, we report that SseF and SseG secreted by *Salmonella Typhimurium* (S. Typhimurium) inhibit autophagy in host cells and thereby establish a replicative niche for the bacteria in the cytosol. Mechanistically, SseF and SseG impaired autophagy initiation by directly interacting with the small GTPase Rab1A in the host cell. This interaction abolished Rab1A activation by disrupting the interaction with its guanine nucleotide exchange factor (GEF), the TRAPPIII (abbreviation for the transport protein particle III) complex. This disruption of Rab1A signaling blocked the recruitment and activation of Unc-51–like autophagy-activating kinase 1 (ULK1) and decreased phosphatidylinositol 3-phosphate biogenesis, which ultimately impeded autophagosome formation. Furthermore, SseF- or SseG-deficient bacterial strains exhibited reduced survival and growth in both mammalian cell lines and mouse infection models, and Rab1A depletion could rescue these defects. These results reveal that virulence factor-dependent inactivation of the small GTPase Rab1A represents a previously unrecognized strategy of S. Typhimurium to evade autophagy and the host defense system.

*Salmonella enterica* causes a variety of diseases, including gastroenteritis and typhoid fever, the latter is a life-threatening systemic disease causing more than 200,000 human deaths every year(1). As a facultative intracellular pathogen, *Salmonella enterica* survives and replicates in a variety of hosts. The virulence traits of *Salmonella* include the *Salmonella* pathogenicity-island (SPI)
The Salmonella effectors SseF and SseG inhibit Rab1A-encoded type III secretion systems (T3SSs), which are important for invasion of host cells and intracellular replication(2). After entering the host cells, Salmonella enterica requires the formation of a unique organelle termed Salmonella-containing vacuole (SCV) in order to advance their infection. The T3SS encoded by Salmonella Pathogenicity Island 2 (SPI2–T3SS) is essential for the formation and maintenance of the SCV. The SPI2–T3SS translocates a variety of effector proteins that interfere with host cell functions such as organelle homeostasis and autophagy pathways (2-4).

The molecular targets for most of the SPI2-T3SS effector proteins remain largely unexplored, and mutational analyses indicated that the intracellular replication of Salmonella requires a subset of these proteins, including SifA, SseF, SseG, PipB2 and SopD2(5,6). These effectors share a common subcellular localization after SPI2-T3SS-dependent translocation, and can be found in close association with the membrane of SCV (7). Both SseF and SseG are characterized by large hydrophobic domains that may be responsible for the association of these effectors with membrane structures after translocation(6,8). Defects in either SseF or SseG result in a reduction of systemic pathogenesis and attenuation of intracellular proliferation(5,6,9-11). The recent study showed that SseG and SseF anchor SCV at the Golgi network through interaction with mammalian protein ACBD3(12). However, how SseF and SseG facilitate pathogen replication inside the host cell remains largely unknown.

Autophagy is an evolutionarily conserved process, which plays a key role in a variety of human disorders including infectious diseases(13-17). During autophagy, cellular contents or invading pathogens are engulfed in double-membraned autophagosomes and delivered to lysosomes, leading to the degradation of the internal components. During pathogen infection, a specific role for autophagy has been demonstrated in the encapsulation and degradation of intracellular bacteria and viruses, known as “xenophagy”(13,18-23). Therefore, dysfunction of autophagy may lead to persistent infection. In recent years, evidence of the specific roles of autophagy in selective targeting of bacteria through autophagy machinery has been studied(22). Many bacterial pathogens have evolved mechanisms to interfere with autophagic initiation or flux(14-17,24). Autophagy limits the replication of S. Typhimurium in different cell culture and mouse models(25). Conversely, S. Typhimurium has evolved strategies to subvert the autophagy-mediated host defense system. For example, it has been reported that S. Typhimurium could interfere with host cell signaling cascades and vesicle trafficking, which eventually results in bacteria escaping from degradation through autophagy(26-28). However, the molecular mechanisms remain largely unexplored.

In the present study, we report that the Salmonella SPI2-T3SS effectors, SseF and SseG facilitate intracellular pathogen replication by attenuating autophagy, and this function is mediated by the inactivation of Rab1A, a small GTPase which plays essential roles in Golgi homeostasis and autophagy initiation. Our findings provide mechanistic insights into the schemes adopted by Salmonella to manipulate host functions and to ultimately enable bacterial infection.

RESULTS:
SseF and SseG inhibits autophagy initiation.

To understand the mechanism of how SseF and SseG subvert host cell function, we asked
The Salmonella effectors SseF and SseG inhibit Rab1A whether they modulate cellular autophagy pathway which play crucial roles in eliminating invading bacterial pathogens(14-17). To test this idea, we ectopically express HA-tagged SseF or SseG in HeLa cells (Fig.1A), and we analyzed the cellular autophagy activity by measuring three parameters: the conversion of LC3-I to LC3-II, the level of the autophagy substrate, p62, and GFP-mCherry-LC3 fluorescence microscopy.

We observed that ectopic expression of either SseF or SseG significantly decreased LC3-II levels, and caused p62 accumulation in both Hela and RAW264.7 cells, indicating that cellular autophagy activity is repressed in the presence of SseF and SseF (Fig. 1A, B, C). To further solidify this conclusion, we also performed confocal microscopy analysis. As shown in Fig. 1D, the number of LC3 puncta, the indicator of autophagic membrane structures, was significantly reduced in both unstressed and starved conditions, which was further confirmed by transmission electron microscopy (TEM) analysis (Fig. 1E). Consistently, SseF/G hampered autophagic flux measured by GFP-mCherry-LC3 fluorescence microscopy. The decreased LC3 lipidation suggested that these two virulence factors may attenuate the early step of autophagy. Indeed, the formation of Atg16 punctum, an indicator of early autophagic structures was significantly impaired (Fig. 1F). Taken together, these results demonstrate that SseF and SseG interrupt the initiation of autophagy.

SseF and SseG interact and colocalize with Rab1A in host cells.

To understand the molecular mechanism of how SseF and SseG impair autophagy, we searched for their interacting proteins by immunoprecipitation assay. Mass spectrometric (MS) analysis of the proteins that co-immunoprecipitated (co-IP) with HA-SseF or HA-SseG identified Rab1A as a potential binding partner (Fig. 2A). Additional co-IP experiment confirmed that endogenous Rab1A interacted with ectopically expressed SseF or SseG (data not shown). To test whether SseF and SseG interact with Rab1A under physiological conditions, infection experiments were performed using Salmonella strains expressing HA-SseF or HA-SseG, which were generated using a similar strategy as described before(6). Indeed, anti-HA co-IP efficiently pulled down endogenous Rab1A (Fig.2B). Furthermore, we found that SseF and SseG interact with different forms of Rab1A with a preference to GTP-bound form (Fig. 2C, D). We then asked whether their interaction is direct by performing in vitro GST pulldown assays. The recombinant proteins utilized in this experiment was purified towards homogeneity from E. Coli that harbors the recombinant expression plasmid (Fig. 2E, F).

We showed that GST-tagged Rab1A but not GST alone efficiently pulled down HA-tagged SseF and SseG in vitro, indicating that Rab1A directly binds to SseF and SseG (Fig. 2G). Furthermore, the presence of SseF or SseG appeared to enhance the association between Rab1A and GDI1 (Fig. 2H). Confocal microscopy analysis showed that SseF and SseG colocalized with Rab1A (Fig. 2J). Together, these results demonstrated that SseF and SseG interact and colocalize with Rab1A under physiological conditions.

SseF and SseG prevent TRAPP III-mediated Rab1A activation.

Previous studies have established that Rab1A is activated by its GEF, the TRAPP III complex, which is conserved from yeast to human(29). Given the fact that SseF and SseG bind to all of three forms of Rab1A, we hypothesized that these virulence factors may
impede Rab1A activation by interrupting the interaction between Rab1A and mammalian TRAPPIII complex (mTRAPPIII). To test these ideas, we first investigated the interaction between Rab1A and mTRAPPIII in the cells ectopically expressing SseF or SseG. We found that the presence of SseF or SseG abolished the association between mTRAPPIII and Rab1A (Fig. 3A). We then investigated whether Rab1A activation would be affected by performing the GTP loading assay using purified recombinant Rab1A. mTRAPPIII complex was immunoprecipitated from HeLa cells using antibody against mTrs85. In our studies, mTrs85-containing beads were washed extensively and then assayed at 25°C for their guanine nucleotide exchange activity on Rab1A. We found that mTRAPPIII stimulated the guanine nucleotide exchange activity of Rab1A in a time-dependent manner. While no stimulation of GTPγS uptake was observed when the control beads were incubated (Fig. 3B). However, the co-presence of recombinant SseG and SseF significantly decreased the nucleotide exchange rate of Rab1A stimulated by mTRAPPIII in a dose dependent manner (Fig. 3B, C). Therefore, we conclude that SseF and SseG active Rab1A by blocking the guanine nucleotide exchange activity of mTRAPPIII for Rab1A.

**SseF and SseG attenuates Rab1A-mediated ULK1 recruitment and activation**

It has been reported that Rab1A, after mTRAPPIII-mediated activation, recruits Atg1/ULK1 complex to pre-autophagosomal structures to initiate autophagy (30). Therefore, we postulated that SseF and SseG may interfere with this process. First, we immunoprecipitated endogenous Rab1A and then detected for ULK1, and we found that the association of Rab1A and ULK1 complex was largely abolished in the presence of ectopically expressed SseF or SseG. In contrast, we did not detect interaction between Rab1A and Atg2 (Fig. 4A). It appeared that SseF or SseG may also disrupt the interaction of Rab1A and C9orf72 to facilitate ULK1 recruitment and activation(31) (Fig. 4B). In addition, the punctum formation of the components of ULK1 complex, ULK1 and Atg13, was largely repressed under both normal and starved conditions when SseF or SseG is present (Fig. 4 C, D, E, F). These results indicate that SseF and SseG may affect the function of ULK1 complex in autophagy initiation. After accumulating in the pre-autophagosomal membrane structures, ULK1 complex phosphorylates several substrates to propel the progression of autophagy. We further investigated ULK1 activity by measuring the phosphorylation levels of its substrate. We observed that starvation-triggered phosphorylation of Beclin1 (Ser15) was significantly repressed by SseF or SseG (Fig. 4G). Collectively, SseF and SseG impair Rab1A-mediated ULK1 recruitment and activation.

**SseG and SseF repress Atg14L-PI3KC3-mediated PI3P biogenesis and the recruitment of Atg2-WIPI2 complexes.**

The biogenesis of phosphatidylinositol 3-phosphate (PI3P) on the isolation membrane, the early autophagosomal membrane structure by Vps34 is a crucial early event in autophagosome formation (32). The phosphorylation Beclin1 and Atg14L by ULK1 is required for activation of ATG14L-Beclin1-Vps34 subcomplex in response to autophagic stimuli (33,34). Because SseF and SseF suppress ULK1-mediated phosphorylation of Beclin1 and Atg14L, we investigated whether the catalytic activity of Vps34 is altered. We transfected GFP-FYVE2 (FYVE, a domain
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found in Fab 1, YOTB, Vac 1 and EEA1), a PI3P specific probe possessing two tandem PI3P binding domain, to monitor the cellular PI3P levels. Indeed, the presence of SseF or SseG decreased the levels of PI3P by 51% and 63%, respectively (Fig. 5A, B). To provide more quantitative measurement, we assessed the cellular PI3P levels by a well-established ELISA assay. Consistently, ectopic expression of SseF or SseG led to remarkable reduction of PI3P levels (Fig. 5C). The direct effect of these two virulence factors on Vps34 catalytic activity was excluded, since neither SseF nor SseG did not co-immunoprecipitate with Vps34 complex, the direct action of SseF/G on Vps34 is not operative (data not shown).

During autophagy, PI3P generated by Class III PI3K is required for the nucleation by recruiting of Atg2-WIPI2 complex, which possesses direct PI3P binding activity. Therefore, we also measured the acquisition of Atg2 by measuring its punctum formation. We found that the number of Atg2-positive vacuole was significantly reduced in the presence of SseF or SseG under both unstressed and starved cells. In sum, SseF and SseF decrease PI3P biogenesis to inhibit autophagy signaling.

**Intracellular replication defect caused by SseF or SseG-deficiency attributes to Rab1A-mediated autophagy.**

Autophagy plays important roles in restricting the growth of intracellular Salmonella (25,28,35). Since our results indicated inhibition of autophagy by SseF and SseG, we reasoned that SseF and SseG may inhibit cellular autophagy pathway to facilitate bacterial growth inside host cells.

Salmonella Typhimurium is an intracellular bacterial pathogen that infects both epithelial cells and macrophages. To determine if SseF and SseG play a role in cell culture infection model, we generated bacterial mutants for in vivo studies. Deletions of SseF or SseG were generated using the λ-red recombination system(36) Consistent with previous studies, SseF- or SseG-deficient Salmonella mutant strains exhibited intracellular replication defects in RAW 264.7 cells, and the effects of the defects were largely rescued by silencing Rab1A expression (Fig 6A, B). In contrast, the depletion of another GTPase, Rab6A, is ineffective in reversing the replication defects of the mutant strains, as Rab6A is not the interactor of SseF or SseG. (Fig 6C, D).

Conversely, the overexpression of Rab1A, but not Rab6A or Rab33B attenuated the replication of wild type of Salmonella in both cell lines (Fig 6E, F). These data suggested that the blockade of Rab1A-mediated autophagy pathway by SseF or SseG facilitates bacterial intracellular replication.

**Rab1A-dependent autophagy pathway restricts the replication of Salmonella in Mouse Tissues**

To investigate whether Rab1A is responsible for the regulation of bacterial infection in vivo, we prepared the Rab1A-knockout mouse line by deleting the first exon. The mouse embryonic fibroblast cell line of Rab1A-/ was generated, and both the lipidation of LC3 and the degradation of the autophagy substrate p62 were inhibited in Rab1A-/ cells, indicating the depletion of Rab1A caused autophagic defects (Fig 7A). On the other hand, SseF-deletion stain appeared to stimulate higher level of host autophagy (Fig 7B). Next, we examined the contribution of these factors to S. Typhimurium virulence in mouse infection model. Mice infected with S. Typhimurium deficient for either SseF or SseG had lower bacterial loads in systemic tissues than those infected with wild-type (Fig 7C). Furthermore, the markedly reduced virulence exhibited by the S. Typhimurium mutant strains was partially restored in Rab1A-deficient mice (Fig 7C). Taken together, these results demonstrate that S. Typhimurium targets the
The Salmonella effectors SseF and SseG inhibit Rab1A-dependent pathogen-restriction pathway with two T3SS effector proteins, SseF and SseG, and that the Rab1A-mediated autophagy pathway plays a crucial role in host defense against *S. Typhimurium*.

**DISCUSSION**

The co-evolution of hosts and pathogens led to both the development of immune defense systems for higher organisms to eliminate invading microbes, and the evolution of mechanisms for pathogens to infect eukaryotic cells and counteract these host defenses(37). Autophagy has emerged as a critical host defense function against intracellular bacterial pathogens. Recent progress has made towards the elucidation of the fact that bacterial virulence factors target specific host autophagy regulators for intracellular bacterial survival(38,39).

In eukaryotic cells, autophagy is regulated by a variety of protein machineries including the small GTPases. The Rab protein constitute a superfamily of small GTPases that define membrane identity and play central roles in regulating various aspects of membrane traffic (40). GTPases are molecular switches that functionally oscillate between an active (GTP-bound) and an inactive (GDP-bound) state, a process that is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Activated Rab proteins recruit specific effector proteins, forming protein complexes on specified membranes to provide directionality for membrane transport processes. Recent studies have established that different steps of autophagy process require different RAB proteins such as Rab1A, Rab5, Rab6, Rab7, Rab9 and Rab33 (38,39,41).

GTPases are the major targets for bacterial virulence factor for a variety of bacterial pathogens, including but not limited to *Legionella pneumophila, Listeria monocytogenes, Chlamydia spp.*, *S. Typhimurium* and *Brucella spp.* et al(37,42). Of all of the GTPases, Rab1A is the most frequent target for many different bacterial pathogens. For example, *Legionella pneumophila* effector AnkX Catalyzes posttranslational addition of a PC moiety to Rab1A to prevent binding of host effectors and deactivation by GAPs(43). While Lem3 reverses the activity of AnkX by removal of phos-phocholine from the switch II loop of Rab1A, which renders the protein susceptible to deactivation by GAPs(44). LepB acts as Rab1A GAP that catalyzes inactivation through GTP hydrolysis and promotes its subsequent removal from the Legionella-containing vacuole (LCV) (45).

*Chlamydia pneumoniae* effector Cpn0585 Interacts with Rab1A, Rab1A0, and Rab1A1 and is required for modulating Rab dynamics during infection(46). However, it is not known whether Rab1A is also targeted by the virulence factors from *S. Typhimurium*. Rab1A was shown to be crucial for autophagy-mediated restriction of intracellular *S. Typhimurium*(47), however, the molecular mechanism is not known.

In the present study, we have described a previously uncharacterized mechanism of autophagy blockade by the intracellular bacterial pathogen *S. Typhimurium*. This mechanism involves the delivery of two T3SS effector proteins, SseF and SseG, which are able to specifically neutralize Rab1A-dependent autophagy pathway. Furthermore, *S. Typhimurium* strains lacking either of these two effectors exhibited a drastic virulence-attenuation phenotype demonstrating the loss of the ability to neutralize the Rab1A-dependent pathogen-restriction mechanism is most likely crucial to the bacterial evolutionary process. Consistently, the virulence phenotype was
partially reversed in both Rab1A-deficient cell lines and mice indicating the specific functional and physical interplay between Rab1A of host cells and SseF or SseG of S. Typhimurium. It remains unknown why S. Typhimurium evolves two different proteins performing the same autophagy blockade.

Rab1A is not only importantly for autophagy initiation, but also crucial for the homeostasis of Golgi apparatus by engaging a variety of different factor, including p115, TRAPP II, Golgin45 and GM130(29,48-51). Previous studies have also shown that Golgi may be tightly connected to Salmonella infection(5,9,10,12,52). Therefore, it is also possible that the functional and physiological association of Rab1A, SseF and SseG may affect the membrane dynamics of Golgi apparatus, which requires future investigation. In addition, SseF and SseG bind to different forms of Rab1A although exhibiting higher affinity to the GTP-bound form. Whether SseF and SseG affect Rab1A recycle by targeting its GAPs or GEFs is unknown. Furthermore, it would be interesting to study how the host cells antagonize the inhibitory effects of SseF and SseG on Rab1A.

In conclusion, the identification of Rab1A as the host target molecule of these T3SS-III effectors SseF and SseG will further deepen our understanding of the mechanisms that govern the localization of Salmonella in the Golgi network and the contribution of each effector to the intracellualar replication of this important pathogen. Our results demonstrate the importance of a cell-autonomous, Rab1A-dependent defense mechanism against the pathogen. These findings constitute a novel mechanism of autophagy-mediated host defense against bacterial pathogen and may provide the insights for the development of therapeutic strategies to combat infectious diseases by vacuolar pathogens.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies-Anti-ULK1 (ab206612, ab200980) anti-actin (ab8226), anti-LC3 (ab48394), anti-Flag (M2), anti-Atg16L1 (D6D5), anti-p62(ab56416), anti-Rab1A, anti-Rab6A(ab97956), Anti-TRAPPC8 (ab122692) were purchased from Abcam (Cambridge, MA). Anti-HA (HA.11) antibody were purchased from Covance (Emeryville, CA). HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa-flour conjugated Phospho-Atg14 (Ser29) Antibody (13155), anti-phospho-Beclin-1 (Ser15) (13825), anti-Beclin-1 (D40C5) were purchased from Cell Signaling Technology (Beverly, MA). PIP3 Mass ELISA(K-3300) were purchased from Echelon Biosciences (Salt Lake City, UT).

Cell Culture and Growth Conditions—The human epithelial cell lines HEK293T (ATCC CRL-11268) and HeLa (ATCC CCL-2) were grown in DMEM medium (Gibco). The human macrophage-like cell line THP-1 (ATCC TIB-202) and the mouse macrophage cell line RAW264.7(ATCC TIB-71) were cultivated in RPMI-1640 medium (Gibco). Cell culture media were supplemented with 10% fetal calf serum (Gibco), and cells were grown under standard tissue culture conditions of 37 °C and 5% CO2. All experiments in the various cell lines were performed within six passages after seeding of the original frozen stocks.

Bacterial Stains and Plasmids—The wild-type Salmonella Typhimurium (S. Typhimurium) strain SL1344 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). All of the genetically modified strains of S. Typhimurium were constructed using λ-Red recombination and allelic exchange procedures as previously.
The Salmonella effectors SseF and SseG inhibit Rab1A described(53). Bacteria were grown in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/ml), or chloramphenicol (30 μg/ml) as appropriate. The recombinant plasmids were constructed using standard molecular cloning procedures.

**Immunoprecipitation**- Cell pellets were resuspended in 5 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl, 0.2% NP-40, protease inhibitor cocktails). The mixture was centrifuged at 16,000 rpm for 20 min. The resulting supernatant was incubated for 4-8 hours at 4°C (with rotation) with purified antibodies as appropriate or nonspecific IgG, and protein G-Agarose beads. The beads were washed three times with lysis buffer. The beads were analyzed by Western blot analysis or assayed for the ability to stimulate Rab1A GEF activity.

**Immunofluorescence and Transmission Electron Microscopy**- For immunofluorescence analyses, the cells were grown in 6-well tissue culture plates on glass coverslips. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then washed three times with PBS. The antibodies were diluted in a blocking solution consisting of 5% goat normal serum in PBS. The coverslips were incubated with various antibodies as detailed, and were washed three times with PBS after each incubation step. The coverslips were mounted and sealed with coverslips. Samples were analyzed using a confocal laser scanning microscope (Zeiss, LSM 800). For transmission electron microscopy analysis, cells were fixed in 3% PFA glutaraldehyde, 1% sucrose, and 0.028% CaCl2 in 0.1 N sodium cacodylate, pH 7.4, overnight at 4°C. Samples were then incubated in 0.5% osmium tetroxide for 1 h and in half-saturated aqueous uranyl acetate for 30 min at room temperature, dehydrated in a graded series of ethanol, and embedded in Durcupan (Fluka) according to the manufacturer’s recommendations. 70-nm sections were stained in Reynolds lead citrate and viewed on a JEM-1011 transmission electron microscope (Jeol).

**Nucleotide Exchange Assays**- For the purification of Rab1A recombinant proteins, *Escherichia coli* strain BL21(DE3), transformed with a plasmid (pGEX4T-1) that contained the Rab1A WT or mutants, was grown at 37°C to an OD600 of 0.6. Protein expression was induced during an overnight incubation at 16°C in the presence of 0.2 mM isopropyl β-D-thiogalactoside. The next day, cells were harvested and resuspended in lysis buffer (PBS, 1 mM β-mercaptoethanol, 5 mM MgCl2, 10 mM CaCl2, and 2 mM phenylmethylsulfonyl fluoride). The cells were lysed by sonication and centrifuged at 10,000 × g for 15 min. The cleared lysate was then loaded onto a glutathione-Sepharose column, washed extensively with lysis buffer, and eluted with lysis buffer containing 10 mM glutathione. For GDP preloading, the Rabs were incubated at 30°C for 20 min in preloading buffer (20 mM Tris pH 8.0, 100 mM KCl, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT and 0.2 mg/ml BSA), then fivefold excess guanosine diphosphate (GDP) was added to the mixture. Nucleotide binding was stabilized by the addition of MgCl2 (10 mM, final concentration) and then incubated for an additional 60 min. Free GDP was removed by a desalting column. For the guanosine triphosphate uptake assay, 20 pmol of [35S] guanosine 5′-O-(3-thio) triphosphate (GTPγS) was added to 50 pmol of Rab-GDP in assay buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, and 0.1 mM EDTA). Nucleotide exchange was initiated by transferring 20 pmol of the Rab proteins to a tube containing the mTRAPP complex immobilized on beads. The reaction was terminated by the addition
stop buffer (50 mM Tris, pH 8.0, and 50 mM MgCl2) at the indicated time points. The reaction mixture was filtered through a nitrocellulose membrane (Millipore, Billerica, MA) and extensively washed with stop buffer. Protein-bound--radiolabeled nucleotide was retained on the membrane and measured by scintillation counting.

**Cell Culture Infection Experiments**- Overnight cultures of the different *Salmonella* strains were diluted 1/20 in LB broth containing 0.3 M NaCl and grown until they reached an OD600 of 0.9. Cultured cells were infected with the different strains of *S. Typhimurium* Hank’s balanced salt solution (HBSS). One hour after infection, cells were washed three times with HBSS and incubated in normal culture medium supplemented with 100 mg/ml gentamicin for 30 min to eliminate extracellular bacteria. Cells were then washed, and fresh medium containing 10 mg/ml gentamicin was added to avoid reinfection.

**Animal Infection Experiments**- All animal experiments were conducted according to protocols approved by Jiangsu Normal University’s Institutional Animal Care and Use Committee. Rab1A-knockout mice were generated by CRISPR/Cas9 technology. pX330-sgRNA targeting Rab1A was introduced into ES cells. Rab1A-mutated ES cell clones were microinjected into C57BL/6 blastocysts to generate chimeric mice, which were further bred following the standard procedure (Model Animal Research Center of Nanjing University, Nanjing, China). Eight- to twelve-week-old C57BL/6- or Rab1A-deficient mice were infected intraperitoneally with different *S. Typhimurium* strains. Mice were sacrificed 4–5 days after infection, organs were homogenized in PBS, and bacteria were recovered and counted after plating a dilution series onto LB agar and LB agar with the antibiotics.

**Conflict of interest**- The authors declare that they have no conflicts of interest with the contents of this article.

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**Author contributions**- X.P., Z.F. and A.J. designed the experiments. Z.F., A.J., A.M., Y.F., W.W., J.L., and X.Z. and K.X. performed the experiments. X.P., Z.F. and A.J. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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FIGURE LEGEND:
FIGURE 1. SseF and SseG attenuate cellular autophagy initiation. A, Western-blot analysis of LC3-II and p62 levels in the HeLa cell lines ectopically expressing either HA-SseF or HA-SseG, cell line harboring empty vector as the negative control. Data are shown as mean ± SD (**p < 0.01, *p < 0.05). B, Western-blot analysis autophagy activities of RAW264.7 cell lines which ectopically express HA-GST (Glutathione S transferase), HA-SseF or SseG. Data are shown as mean ± SD (**p < 0.05) C, Western-blot analysis of autophagy activities in Hela cells expressing...
HA-SseF or empty vector. Data are shown as mean ± SD (*p < 0.05). D, Confocal microscopy analysis of LC3-positive autophagic membrane structures. Scale bars, 10 μm. Data are shown as mean ± SD (**p < 0.01). D, Quantification was made according to the relative average number of LC3 punctum. E, Autophagy activity was further measured by transmission electron microscopy. Black arrow heads indicate autophagic vacuoles; “N” represents Nucleus. Quantification was made according to the average number of autophagic vacuoles per cell. Scale bars, 1 μm. Data are shown as mean ± SD (**p < 0.01). F, Confocal microscopy analysis of Atg16L-positive autophagic membranes structures. Quantification was made according to the relative average number of Atg16L punctum. Scale bars, 10 μm. Data are shown as mean ± SD (**p < 0.01).

FIGURE 2. SseF and SseG are associated with Rab1A. A, Identify Rab1A as an interactor of SseF. Potential cellular binding partners of SseF were communoprecipitated (co-IP) by anti-HA beads, the protein complexes were eluted and analyzed by SDS-PAGE and mass spectrometry. B, Rab1A interacts with SseF or SseG under physiological conditions. Salmonella stains were engineered to replace the gene locus of SseF or SseG by HA-tagged SseF and SseG. The resultant stains were applied to infect the macrophage cell line RAW264.7. Cellular protein complexes associated with HA-tagged SseF or SseG were co-IP-ed and analyzed by Western-blot. C, Test the interaction between SseF and different forms of Rab1A. Flag-tagged Rab1A-WT, Rab1A-Q70L (GTP-bound mimetics) or Rab1A-S25N (GDP-bound mimetics) were transfected into cells stably expressing HA-SseF or HA-SseG. Co-IP and Western-blot analysis were performed. Data are shown as mean ± SD (*p < 0.05). D, Test the interaction between SseG and different forms of Rab1A using a protocol described in C. Data shown as mean ± SD (*p < 0.05). E, Purification of recombinant proteins for GST-tagged Rab1A-WT, Rab1A-Q70L or Rab1A-S25N. F, Purification of recombinant proteins for HA-tagged SseF and SseG from Hela cells. G, In vitro GST-pulldown assay. Data are shown as mean ± SD (*p < 0.05). H, Purified recombinant GST-GDI1 pulldown Rab1A from the cell lysates as indicated. Data are shown as mean ± SD (*p < 0.05). I, Subcellular localization of SseF and SseG. Confocal microscopy analysis was performed to visualize the localization of SseF and SseG. Quantification of the overlap percentage.

FIGURE 3. SseF and SseG inhibit mTRAPPIII-mediated Rab1A activation. A, The interaction of mTRAPPIII and Rab1A in the presence of SseF or SseG. The association of mTRAPPIII and Rab1A in the cells expressing either HA-SseF or HA-SseG was measured by co-IP assay using the anti-mTrs85 antibody, the IP-ed protein complexes were analyzed by Western-blot. Data are shown as mean ± SD (**p < 0.01). B, Recombinant Rab1A was incubated with mTRAPPIII complex purified from HeLa cells, which was followed by the addition of GTPγS to the reaction. For testing the inhibitory effect of SseF or SseG, recombinant Rab1A was firstly incubated with mTRAPPIII complex, then purified recombinant SseF and SseG were added into the reaction, which was followed by the addition of GTPγS. The guanine nucleotide exchange activity of Rab1A was measured by determination of GTPγS at different time point. C, For testing the inhibitory effect of SseF or SseG in a dose-dependent manner, recombinant Rab1A was firstly incubated with mTRAPPIII complex, then purified recombinant SseF and SseG were added into the reaction at different ratio versus Rab1A, which was followed by the addition of GTPγS. The guanine nucleotide exchange activity of Rab1A was measured by determination of GTPγS at different time point, and the relative GTPγS loading was calculated.
FIGURE 4. SseF and SseG impair Rab1A-mediated ULK1 recruitment and activation. A, The interaction of ULK1 and Rab1A in the presence of SseF or SseG. The association of ULK1 and Rab1A in the cells expressing either HA-SseF or HA-SseG was analyzed by co-IP and Western-blot using antibodies as indicated. Data are shown as mean ± SD (*p < 0.05). B, The interaction of C9orf72 and Rab1A under bacterial infection. The association of C9orf72 and Rab1A in the bacteria-infected cells was measured by co-IP and Western-blot using antibodies as indicated. Data are shown as mean ± SD (*p < 0.05). C, Confocal microscopy analysis of ULK1-positive autophagic membranes structures. D, Quantification was made according to the average number of ULK1 punctum shown in C. Scale bars, 10 μm. Data are shown as mean ± SD (**p < 0.01). E, Confocal microscopy analysis of Atg13-positive autophagic membranes structures. Scale bars, 10 μm. F, Quantification was made according to the average number of Atg13 punctum shown in E. Data are shown as mean ± SD (**p < 0.01). G, Western-blot analysis of Phospho-Beclin1 (Ser15). Data are shown as mean ± SD (*p < 0.05, **p < 0.01).

FIGURE 5. SseF and SseG affect ULK1 downstream signaling in autophagy pathway. A, The biogenesis of PI3P in the presence of SseF or SseG. The biogenesis of PI3P in the cells expressing either HA-SseF or HA-SseG was measured by transfecting the cells with the PI3P probe GFP-FYVE2 at low levels. Confocal microscopy analysis was performed to quantify the punctum positive for GFP-FYVE2, which was shown in B. Scale bars, 10 μm. Data are shown as mean ± SD (*p < 0.05). C, Measure the cellular PI3P levels by ELISA assay. The ELISA assay was performed as described by the manufacture. Data are shown as mean ± SD (**p < 0.01, *p < 0.05). D, Confocal microscopy analysis of Atg2-positive membranes structures. HeLa cells were grown on coverslip, after fixation and permeabilization, the cells were sequentially stained with anti-Atg2 antibody as the primary antibody, and Alexa Fluor 488-conjugated IgG as the secondary antibody. The average number of Atg2 punctum was quantified and shown in E. Scale bars, 10 μm. Data are shown as mean ± SD (**p < 0.01).

FIGURE 6. SseF and SseG antagonize Rab1A-mediated autophagy for intracellular bacterial replication. A, Knockdown of Rab1A in RAW264.7 cell line. B, Role of SseF and SseG for intracellular proliferation of S. Typhimurium. RAW 264.7 macrophages were infected with S. Typhimurium wild type (WT) and various mutant strains at a multiplicity of infection of about 5. Extracellular bacteria were washed away followed by addition of gentamicin to kill remaining bacteria. At 2 h and 12 h after infection, cells were lysed and the number of intracellular CFU (colony formation unit) was determined. Intracellular replication was expressed as the increase in intracellular CFU from 2 h to 12 h after infection. Quantification was first made by the fold increase in intracellular CFU at 2h versus that at 12 h after infection, was then further normalized by that of WT RAW264.7 cells infected by the WT Salmonella strain. Data are shown as mean ± SD (**p < 0.01), ns means no significance. C, Knockdown of Rab6 in RAW264.7 cell line. D, Infection assay was performed as described in B. Data are shown as mean ± SD (**p < 0.01). E, Establishing RAW 264.7 cell lines overexpressing Rab1A, Rab6A or Rab33B. Infection assay was performed as described in B. Data are shown as mean ± SD (**p < 0.01).

FIGURE 7. Rab1A-mediated autophagy is required for the restriction of systemic infection of Salmonella in the mouse model. A, Mouse embryonic fibroblast cell lines for Rab1A+/+ and Rab1A−/− were established, the cellular levels of Rab1A, LC3-I, LC3-II, and p62 were analyzed by Western-blot using anti-Rab1A, LC3, p62 or Actin as the primary antibodies, and HRP-conjugated IgG as the secondary antibody. B,
WT or ΔSseF Salmonella strain was applied to infect mice, small intestines were isolated for Western-blot analysis. Data are shown as mean ± SD (**p < 0.05). C57BL/6- or Rab1A-deficient mice were intraperitoneally infected with 10⁵ CFU of different strains of S. Typhimurium as indicated above, and 4 days after infection, the levels of the different strains in the spleen of infected mice were enumerated. Each diamond represents the bacterial load for an individual animal, and horizontal bars indicate medians of the CFU. The significant p values of the differences in bacterial loads between the indicated conditions determined by the Wilcoxon-Mann-Whitney test are shown.
The Salmonella effectors SseF and SseG inhibit Rab1A

**Fig 1**

(A) Western blot analysis of Rab1A in cells expressing SseF, SseG, or a control vector. (B) Quantification of Rab1A expression levels. (C) Western blot analysis of Rab1A in cells expressing SseG with or without treatment with specific inhibitors. (D) Immunofluorescence staining for LC3 in cells treated with DMSO or Rapamycin, showing the effect on autophagy. (E) Electron microscopy images demonstrating the autophagic vacuoles in control and SseF/SseG expressing cells. (F) Fluorescence microscopy showing the relative anti-AAP1 signals in cells expressing different constructs.
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**Fig 7**

A. Western blot analysis of Rab1A, LC3-I, LC3-II, p62, and actin levels in WT and ΔSseF strains with Rab1A+/+ and Rab1A−/− background.

B. Graph showing relative p62 levels in WT and ΔSseF strains. * indicates statistical significance.

C. Scatter plot showing bacterial load in spleen (CFU) for WT, ΔSseF, and ΔSseG strains with Rab1A+/+ and Rab1A−/− background. ** and * indicate statistical significance.
The *Salmonella* effectors SseF and SseG inhibit Rab1A-mediated autophagy to facilitate intracellular bacterial survival and replication

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