A Rab32-Dependent Pathway Contributes to *Salmonella* Typhi Host Restriction

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Unlike other Salmonellae, the intracellular bacterial human pathogen *Salmonella* Typhi exhibits strict host specificity. The molecular bases for this restriction are unknown. Here we found that the expression of a single type III secretion system effector protein from broad-host *Salmonella* Typhimurium allowed *Salmonella* Typhi to survive and replicate within macrophages and tissues from mice, a nonpermissive host. This effector proteolytically targeted Rab32, which controls traffic to lysosome-related organelles in conjunction with components of the biogenesis of lysosome-related organelle complexes (BLOCs). RNA interference–mediated depletion of Rab32 or of an essential component of a BLOC complex was sufficient to allow *S.* Typhi to survive within mouse macrophages. Furthermore, *S.* Typhi was able to survive in macrophages from mice defective in BLOC components.

The bacterial pathogen *Salmonella enterica* comprises thousands of serovars (i.e., variants that can be distinguished by their surface antigen composition) that as a whole can infect a large number of vertebrate species (*1*, *2*). Some serovars can infect a broad range of hosts, whereas others are extremely host specific. For example, *Salmonella enterica* serovar Typhi (*S.* Typhi) can only infect humans, although experimentally it can also infect higher primates (*3*). *S.* Typhi causes typhoid fever, a life-threatening systemic disease that every year kills 200,000 people worldwide (*4*–*6*). Although the genome sequences of several host-specific and broad-host Salmonellae are available, the molecular bases for this host adaptation are unknown (*7*, *8*). It is believed that genome reduction most likely played a central role in the narrowing of *S.* Typhi's host range (*9*). This host restriction is also manifested at the cellular level because, unlike human macrophages, *S.* Typhi cannot survive within macrophages of mice, a nonpermissive species (*10*, *11*). The interaction of *S. enterica* with host cells is largely determined by the activities of two type III secretion systems (T3SS), which deliver bacterial effector proteins into host cells to modulate a variety of cellular processes (*12*–*15*). Differences in the assortment of the T3SS effector proteins encoded by *S.* Typhi and *S.* Typhimurium result in differences in the composition of the membrane compartment that harbors these bacteria (*16*). Whereas vacuoles containing *S.* Typhi recruit Rab29, those containing the broad-host serovar *S.* Typhimurium do not, because Rab29 is degraded by the T3SS effector GtgE, absent from *S.* Typhi (*16*).

We hypothesized that the presence or absence of a Rab guanosine triphosphatase (GTPase) in the vacuoles harboring *S.* Typhi or *S.* Typhimurium must translate into fundamental differences in the intracellular biology of these pathogens, potentially influencing host-cell restriction. We expressed the *S.* Typhimurium T3SS effector GtgE in *S.* Typhi and examined its ability to survive within primary bone-marrow–derived macrophages (BMDMs) obtained from mice, a nonpermissive species (*17*). Surprisingly, expression of *gtgE* significantly increased the ability of *S.* Typhi to survive within mouse BMDMs (Fig. 1A and fig. S1). Whereas very few bacteria were recovered from mouse macrophages 48 hours after infection with wild-type *S.* Typhi, large numbers of bacteria were recovered from macrophages infected with *S.* Typhi expressing *gtgE*. Indeed, the number of colony-forming units (CFU) recovered from macrophages infected with *S.* Typhi expressing *gtgE* were equivalent to those recovered from macrophages infected with the broad-host serovar *S.* Typhimurium (Fig. 1A and fig. S1). Thus, expression of a single effector protein from broad-host Salmonellae allows *S.* Typhi to overcome host-cell restriction and survive in a nonpermissive host cell.

To investigate the consequences of overcoming host-cell restriction for the ability of *S.* Typhi to replicate within a nonpermissive host, we infected mice with the *S.* Typhi strain expressing *gtgE*. We found a significantly higher number of bacteria in the tissues of mice infected with *S.* Typhi expressing *gtgE* compared to those infected with wild type (Fig. 1, B and C, and fig. S2). Thus, expression of *gtgE* allows *S.* Typhi to overcome some of the host restriction barrier.

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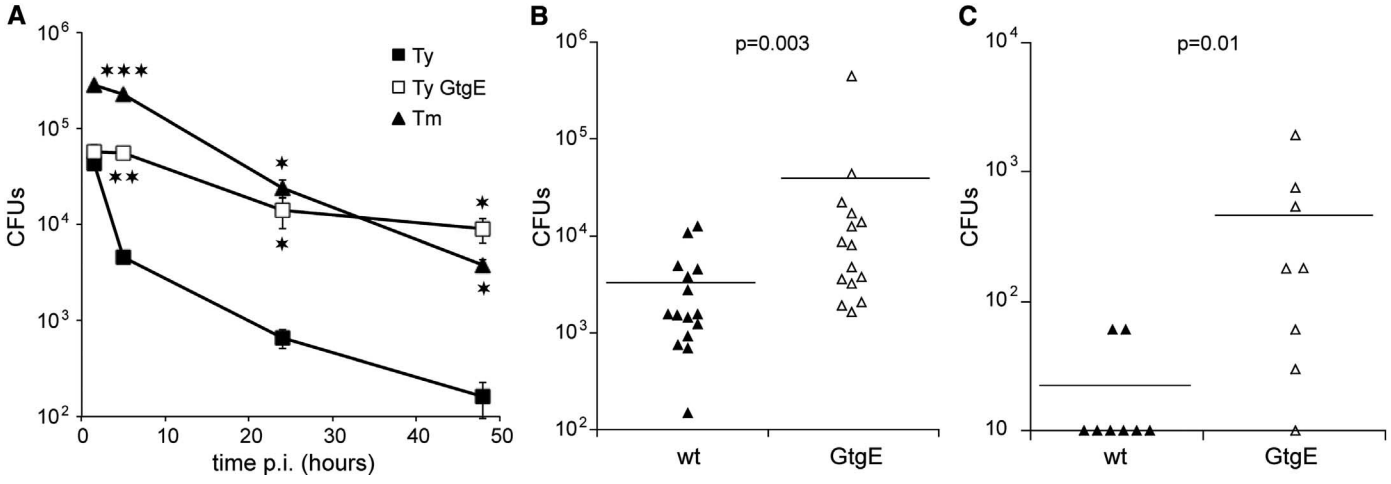

Fig. 1. GtgE expression limits host-cell restriction. (**A**) Survival of *S.* Typhi expressing GtgE in bone-marrow–derived mouse macrophages (BMDMs). Macrophages were infected with *S.* Typhi (Ty), *S.* Typhi expressing GtgE (Ty GtgE), or *S.* Typhimurium (Tm). Cells were lysed at the indicated time points and CFU enumerated. Values are means ± SEM of three independent measurements. *P* values (for the difference relative to values obtained from cells infected with *S.* Typhi) were determined by the Student's *t* test: **P* < 0.05; ** *P* < 0.01; *** *P* < 0.001. (**B** and **C**) CFU recovered from the spleens of C57BL/6 Nramp$^{+/+}$ mice infected with *S.* Typhi (wt, wild type) or *S.* Typhi expressing GtgE (GtgE) 4 days after intraperitoneal (B) or oral (C) inoculation. Horizontal bars indicate the means. The *P* value was determined by the Wilcoxon rank sum test.

Furthermore, the inability of *S.* Typhi to replicate within nonhuman hosts appears at least in part due to its inability to survive within macrophages of the nonpermissive species. However, additional factors must contribute to host restriction, because the virulence of *S.* Typhi expressing GtgE did not match that of *S.* Typhimurium (*18*), which is highly virulent for mice.

To gain insight into the mechanisms by which GtgE allows *S.* Typhi to overcome the host-cell restriction barrier, we examined the effect of depleting Rab29, a target of its protease activity (*16*), on the ability of *S.* Typhi to survive within primary mouse BMDMs. Surprisingly, small interfering RNA (siRNA)–mediated depletion of Rab29 had no effect on the ability of *S.* Typhi to survive in nonpermissive macrophages (fig. S3), which indicated that GtgE might have an additional cellular target(s) for its protease activity. We therefore examined the ability of GtgE to target other Rab GTPases. Despite the broad conservation of residues surrounding the GtgE cleavage site in several GTPases (fig. S4), GtgE could only target Rab32 and Rab38 (Fig. 2, A to C, and fig. S5), which are the GTPases most highly related to Rab29 (Fig. 2D and fig. S6). However, GtgE did not cleave Rab23, the next most highly related GTPase, nor did it cleave other GTPases phylogenetically close to Rab29, Rab32, or Rab38 (Fig. 2, A, C, and D, and figs. S4 to S6). Rab32 and Rab38 (but not Rab23) were cleaved in cells infected with *S.* Typhimurium, which expresses GtgE, but not in cells infected with *S.* Typhi, which does not (Fig. 2C). Thus, GtgE is a specific protease that targets a very restricted subgroup of highly related Rab GTPases.

Shortly after infection, Rab29 is recruited to the *S.* Typhi–containing vacuoles and remains associated with these vacuoles for several hours after infection (*16*). Like Rab29, Rab32 and Rab38 were recruited to the *S.* Typhi–containing vacuole with similar kinetics (Fig. 3A). Similar observations were made in cells infected with the related human-restricted serovar *Salmonella* Paratyphi, which, like *S.* Typhi, does not encode *gtgE* (Fig. 3B). In contrast, Rab32 and Rab38 were not recruited to the *S.* Typhimurium vacuole (Fig. 3B), consistent with the GtgE-mediated degradation of these GTPases in infected cells (Fig. 2C). Similar results were observed in mouse primary BMDMs (Fig. 3C). Thus, there are marked differences between the composition of the intracellular compartments that harbor the human-restricted *S.* Typhi and the broad-host Salmonellae such as *S.* Typhimurium, which may contribute to their differences in host specificity.

To ascertain which of the GtgE targets restricts the survival of *S.* Typhi within nonpermissive macrophages, we investigated the effect of siRNA-mediated depletion of Rab32 or Rab38 on the ability of *S.* Typhi to survive within primary mouse BMDMs. Depletion of Rab38 had no effect on the ability of *S.* Typhi to survive in nonpermissive macrophages (Fig. 4A and fig. S7). However, depletion of Rab32 significantly increased the ability of *S.* Typhi to survive within mouse macrophages, essentially phenocopying *S.* Typhi expressing *gtgE* (Fig. 4A and fig. S7). Thus, GtgE confers on *S.* Typhi the capacity to survive within mouse macrophages by degrading Rab32.

Rab32 and Rab38 have been implicated in the biogenesis of specialized compartments distinct from lysosomes that are collectively known as lysosome-related organelles (LROs) such as melanosomes or specialized granules in platelet and T cells (*19*–*24*). Rab32 and Rab38, in conjunction with BLOC-1, -2, and -3, coordinate the delivery of specific cargo to LROs including enzymes required for melanine synthesis or a variety of antimicrobial proteins (*20*, *23*, *25*, *26*). Intriguingly, the *Salmonella*-containing vacuole (SCV) exhibits features of LROs such as the presence of the lysosomal glycoprotein 1 (Lamp-1), the absence of lysosomal degradative enzymes (*27*, *28*) and, as shown here, the presence of Rab32 and Rab38 (fig. S8). Rab32 may therefore restrict *S.* Typhi growth in mouse macrophages by delivering an antimicrobial activity to the SCV. If this hypothesis is correct, interfering with components

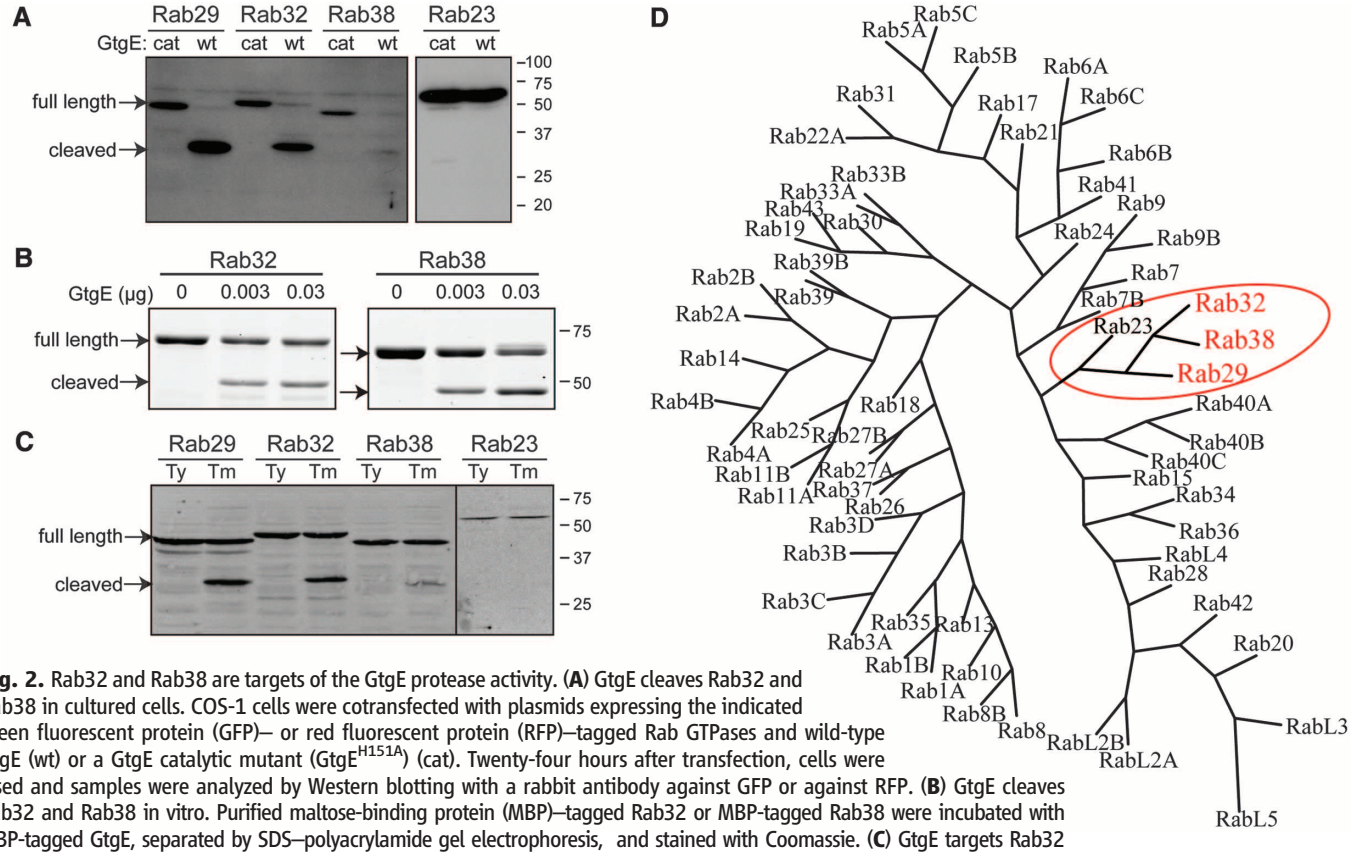

Fig. 2. Rab32 and Rab38 are targets of the GtgE protease activity. (**A**) GtgE cleaves Rab32 and Rab38 in cultured cells. COS-1 cells were cotransfected with plasmids expressing the indicated green fluorescent protein (GFP)– or red fluorescent protein (RFP)–tagged Rab GTPases and wild-type GtgE (wt) or a GtgE catalytic mutant (GtgE^{H151A}) (cat). Twenty-four hours after transfection, cells were lysed and samples were analyzed by Western blotting with a rabbit antibody against GFP or against RFP. (**B**) GtgE cleaves Rab32 and Rab38 in vitro. Purified maltose-binding protein (MBP)–tagged Rab32 or MBP-tagged Rab38 were incubated with MBP-tagged GtgE, separated by SDS–polyacrylamide gel electrophoresis, and stained with Coomassie. (**C**) GtgE targets Rab32 and Rab38 during *Salmonella* infection. COS-1 cells expressing GFP-Rab29, yellow fluorescent protein (YFP)–Rab32, YFP-Rab38, or RFP-Rab23 were infected with *S.* Typhi (Ty) or *S.* Typhimurium (Tm). Two and a half hours after infection, cells were lysed and samples were analyzed by Western blotting with a rabbit antibody against GFP or against RFP. (**D**) Phylogenetic tree of the human Rab and Rab-like GTPases. The locations of Rab29, Rab32, and Rab38 within the tree are indicated in red.

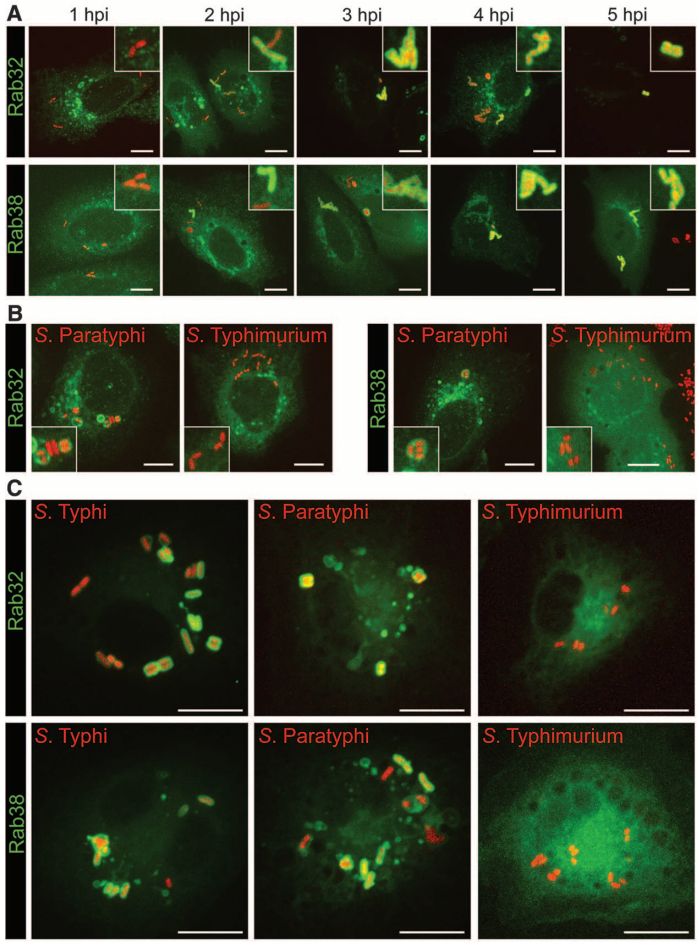

Fig. 3. Rab32 and Rab38 are recruited to the *Salmonella* Typhi–containing vacuole. Henle-407 cells (**A** and **B**) or mouse primary BMDMs (**C**) expressing YFP- or CFP-tagged Rab32 or Rab38 (green) were infected with *S.* Typhi (A and C), *S.* Paratyphi (B and C), or *S.* Typhimurium (B and C) expressing mCherry (red) and imaged at the indicated times (A) or 2 hours (B and C) after infection. The images shown represent maximum intensity projections of Z-stacks. Bars, 10 μm.

of the BLOC complexes should allow *S.* Typhi to survive in mouse macrophages. Depletion of HPS-7 or HPS-6, essential components of BLOC-1 and BLOC-2, respectively, did not affect the ability of *S.* Typhi to survive within mouse macrophages (Fig. 4B and fig. S9). In contrast, depletion of HPS-4, an essential component of BLOC-3, allowed *S.* Typhi to survive within mouse macrophages (Fig. 4B and fig. S9). Furthermore, *S.* Typhi survived in BMDMs obtained from a mouse (*pa/co/le*) simultaneously defective in BLOC-1, -2, and -3 (*29–31*) (Fig. 4C). Thus, *S.* Typhi restriction in cells from a nonpermissive host is due to an antimicrobial activity delivered to its intracellular vacuole by a machinery akin to that used in the genesis of specialized compartments such as melanosomes and T cell granules. The rapid loss of *S.* Typhi CFU after infection of nonpermissive cells (Fig. 1) suggests that this activity must kill *S.* Typhi rather than restrict its growth. Perhaps differences in the antimicrobial activity of human macrophages may have relieved *S.* Typhi from the need to acquire (or retain) *gtgE*, thus contributing to its host specialization.

Rab GTPases are highly conserved across vertebrate species (*32*). However, Rab32 exhibits relatively more amino acid sequence variation across mammalian species than most other members of this family (Fig. 4, D and E). This variation may have been driven by the action of virulence factors that, like GtgE, may target this host-defense pathway. Mutations in the components of the machinery involved in melanosome

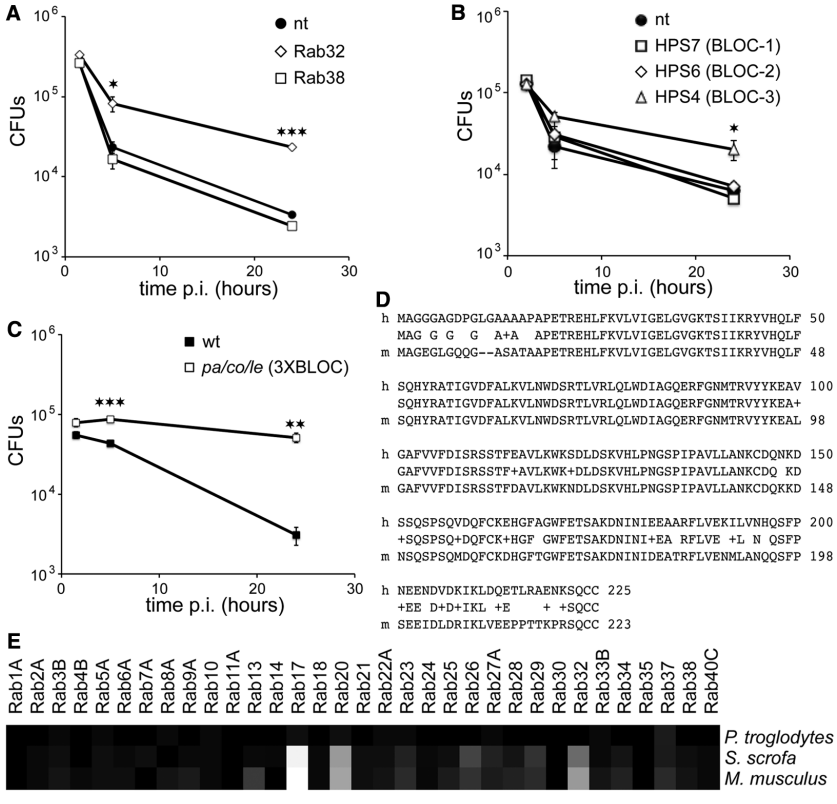

Fig. 4. Rab32 and BLOC-3 are required for *S.* Typhi host-cell restriction. (**A**) Intracellular survival of *S.* Typhi in BMDMs depleted of Rab32 and Rab38. BMDMs from mice were transfected with a nontargeting siRNA smart pool (nt) or siRNA smart pools targeting Rab32 or Rab38. Three days after transfection, cells were infected with *S.* Typhi and then lysed at the indicated time points after infection, and CFU were enumerated. Values are means ± SEM of three independent measurements. (**B**) Intracellular survival of *S.* Typhi in BMDMs depleted of BLOCs. BMDMs from mice were nucleoporated with a nontargeting siRNA smart pool (nt) or specific siRNA smart pools targeting HPS7 (subunit of BLOC-1), HPS6 (subunit of BLOC-2), or HPS4 (subunit of BLOC-3). Three days after transfection, cells were infected with *S.* Typhi and then lysed at the indicated time points, and CFU were enumerated. Values are means ± SEM of three independent experiments. (**C**) Intracellular survival of *S.* Typhi in BMDMs defective for BLOC-1, -2, and -3. BMDM cells from mice simultaneously defective in BLOC-1, 2, and -3 (*pa/co/le*) were infected with *S.* Typhi and then lysed at the indicated time points after infection, and CFU were enumerated. Values are means ± SEM of three independent measurements. (**D**) Alignment of human (h) and mouse (m) Rab32 shows sequence variation in the N-terminal and C-terminal regions. (**E**) Heat-map of the degrees of identity of the human Rab GTPases with the orthologs in three mammalian species: *Pan troglodytes*, *Sus scrofa*, *Mus musculus*. Black represents 100% identity, and white 75% identity. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t* test).

formation such as the BLOCs or a geranylgeranyl transferase that modifies Rab32 lead to a variety of pathologies such as Hermansky Pudlak syndrome (*33*, *34*). These deficiencies lead not only to albinism but also to other clinical manifestations, including increased susceptibility to infections (*35*). Deficiencies in macrophage microbial killing functions may contribute to the observed increased susceptibility to bacterial infections. Indeed, a recent genome-wide association study has uncovered a genetic polymorphism in Rab32 that is linked to increased susceptibility to *Mycobacterium leprae* infection (*36*). Furthermore, Rab32 has been reported to be present in the *Mycobacterium tuberculosis*–containing vacuole (*37*). It is thus likely that the mechanism described here may be important in the control of other intracellular pathogens that, like *Salmonella*, reside in this specialized membrane compartment.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S9
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Salmonella Inhibits Retrograde Trafficking of Mannose-6-Phosphate Receptors and Lysosome Function

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Salmonella enterica is an intracellular bacterial pathogen that replicates within membrane-bound vacuoles through the action of effector proteins translocated into host cells. *Salmonella* vacuoles have characteristics of lysosomes but are reduced in hydrolytic enzymes transported by mannose-6-phosphate receptors (MPRs). We found that the effector SifA subverted Rab9-dependent retrograde trafficking of MPRs, thereby attenuating lysosome function. This required binding of SifA to its host cell target SKIP/PLEKHM2. Furthermore, SKIP regulated retrograde trafficking of MPRs in noninfected cells. Translocated SifA formed a stable complex with SKIP and Rab9 in infected cells. Sequestration of Rab9 by SifA-SKIP accounted for the effect of SifA on MPR transport and lysosome function. Growth of *Salmonella* increased in cells with reduced lysosomal activity and decreased in cells with higher lysosomal activity. These results suggest that *Salmonella* vacuoles undergo fusion with lysosomes whose potency has been reduced by SifA.

Lysosomes are membrane-bound acidic organelles that contain over 50 different hydrolytic enzymes. In the trans-Golgi network (TGN), the majority of newly synthesized hydrolytic enzymes bind the cation-dependent (CD-) and cation-independent (CI-) mannose phosphate receptors (MPRs), which then deliver the hydrolases to endosomes. Endosome acidification dissociates the hydrolases from their MPRs, which recycle back to the TGN. As early endosomes mature into late endosomes, the hydrolases are converted into their active forms and transported to lysosomes (*1*). Lysosomes fuse with vacuoles containing microbes and other exogenous material, causing their degradation.

Salmonella enterica proliferates within mammalian cells in membrane-bound compartments: *Salmonella*-containing vacuoles (SCVs) (*2*). SCVs interact extensively with the endocytic pathway, resulting in acidic compartments (*3*) whose membranes are enriched in lysosomal membrane glycoproteins (*4*, *5*) and which remain accessible to late endosomal and lysosomal content (*6*). However, the CD- and CI-MPRs, as well as their associated hydrolases, do not accumulate in SCVs (*4*, *5*, *7*, *8*). To investigate this paradox, we studied the consequences of *Salmonella* infection on the distribution of MPRs. Human epithelial (HeLa) cells were infected with wild-type *S. enterica* serovar Typhimurium (*Salmonella*), and the amounts of TGN-localized CD-MPR and CI-MPR were quantified by three-dimensional confocal microscopy. In uninfected cells, the proteins accumulated as expected at the TGN (Fig. 1A). In infected cells, the CD- and CI-MPRs were distributed diffusely throughout the cell (Fig. 1, A and B), whereas the localization of other TGN-associated proteins was unaffected (fig. S1, A and B).

Salmonella translocates effector proteins across the SCV membrane via the *Salmonella* pathogenicity island 2 (SPI-2)–encoded type III secretion

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A Rab32-Dependent Pathway Contributes to *Salmonella* Typhi Host Restriction
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Editor's Summary

Removing Typhoid Restriction

Some bacterial pathogens exhibit exquisite host adaptation and can only infect a single host. For example, *Salmonella enterica* serovar Typhi (*S.* Typhi), the cause of typhoid fever, can only infect humans. The host restriction is manifested at the cellular level because *S.* Typhi is unable to survive within macrophages of species other than human. **Spanò and Galán** (p. 960) found that expression of a single type-III secretion effector protein from a broad host *Salmonella* in S. Typhi, allowed this human-exclusive pathogen to survive within macrophages from a nonpermissive host. Furthermore, *S.* Typhi expressing this effector was able to replicate within mice, a nonpermissive host.