**Nε-fatty acylation of multiple membrane-associated proteins by Shigella IcsB effector to modulate host function**

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*Shigella flexneri*, an intracellular Gram-negative bacterium causative for shigellosis, employs a type III secretion system to deliver virulence effectors into host cells. One such effector, IcsB, is critical for *S. flexneri* intracellular survival and pathogenesis, but its mechanism of action is unknown. Here, we discover that IcsB is an 18-carbon fatty acyltransferase catalysing lysine Nε-fatty acylation. IcsB disrupted the actin cytoskeleton in eukaryotes, resulting from Nε-fatty acylation of RhoGTPases on lysine residues in their polybasic region. Chemical proteomic profiling identified about 60 additional targets modified by IcsB during infection, which were validated by biochemical assays. Most IcsB targets are membrane-associated proteins bearing a lysine-rich polybasic region, including members of the Ras, Rho and Rab families of small GTPases. IcsB also modifies SNARE proteins and other non-GTPase substrates, suggesting an extensive interplay between *S. flexneri* and host membrane trafficking. IcsB is localized on the *Shigella*-containing vacuole to fatty-acylate its targets. Knockout of CHMP5—one of the IcsB targets and a component of the ESCRT-III complex—specifically affected *S. flexneri* escape from host autophagy. The unique Nε-fatty acyltransferase activity of IcsB and its altering of the fatty acylation landscape of host membrane proteomes represent an unprecedented mechanism in bacterial pathogenesis.

Bacterial pathogens have sophisticated interplay with their host to promote infection and cause diseases. A critical virulence mechanism commonly used by different pathogens is to secrete effectors or toxins that manipulate various host processes. Many Gram-negative bacteria have evolved a type III secretion system (T3SS) to deliver multiple effectors into host cytosol. These effectors are often endowed with unique and potent biochemical activities to post-translationally modify host proteins1. *Shigella flexneri* is an enteropathogenic bacterium that causes bacillary dysentery by invasion and spread through the colonic epithelium2. *S. flexneri* exploits the type III secretion system (T3SS) to deliver multiple effectors into host cytosol. These effectors are often endowed with unique and potent biochemical activities to post-translationally modify host proteins1. *Shigella flexneri* is an enteropathogenic bacterium that causes bacillary dysentery by invasion and spread through the colonic epithelium2. *S. flexneri* exploits the type III secretion system (T3SS) to deliver multiple effectors into host cytosol. These effectors are often endowed with unique and potent biochemical activities to post-translationally modify host proteins1. *Shigella flexneri* is an enteropathogenic bacterium that causes bacillary dysentery by invasion and spread through the colonic epithelium2. *S. flexneri* employs a type III secretion system to deliver virulence effectors into host cells. One such effector, IcsB, is critical for *S. flexneri* intracellular survival and pathogenicity.

IcsB—one of the first identified *Shigella* T3SS effectors—modulates several host cellular processes, including lysis of the protrusions during *S. flexneri* intercellular spread3,4, evasion of host autophagy5,6,7 and avoiding septin cage entrapment8. The ΔicsB mutant is deficient in provoking keratoconjunctivitis in infected guinea pigs, indicating its critical role in *S. flexneri* pathogenicity. Various models are proposed to account for the functional mechanism of IcsB9,10,11, but the host target(s) that can explain its pleiotropic functions are not identified. While bioinformatic analyses have indicated a possible enzymatic activity in IcsB12, whether and how IcsB catalyses elimination of the phosphothreonine in MAPKs to block the host immunity3,5,8.

**Results**

The putative catalytic motif in IcsB is critical for its functioning. *Saccharomyces cerevisiae* is a useful model for elucidating the molecular function of bacterial effectors. We found that inducible expression of IcsB could severely block *S. cerevisiae* growth (Fig. 1a).
The cytotoxicity was also observed in IcsB-transfected 293T cells (Fig. 1b). IcsB is present in all four pathogenic Shigella species (S. flexneri, S. sonnei, S. dysenteriae and S. boydii), and shares about 24% sequence homology with the Burkholderia pseudomallei T3SS effector BopA as well as the RID (Rho-inactivation domain) domain of Vibrio species multifunctional autoprocessing repeat-toxin (MARTX) toxins (Fig. 1c). The three proteins adopt a similar secondary structure arrangement (Fig. 1c). Previous bioinformatics analysis identifies His-145, Asp-195 and Cys-306 in IcsB that are conserved in BopA and RID (Fig. 1c), which may form a catalytic triad resembling that in the circularly permuted papain-like hydrolytic enzymes (Fig. 1d). Alanine substitution of His-145, Asp-195 or Cys-306 in IcsB diminished its cytotoxicity in yeast, while mutation of two other residues (K183A and Y297A) had no effect (Fig. 1a). Similar findings were obtained with Rac1 and Cdc42 (Fig. 2b).

IcsB disrupts the actin cytoskeleton by inactivating RhoGTPases. IcsB expression could disrupt the actin cytoskeleton in HeLa cells, as is evident from the loss of filamentous actin fibres and the development of cell-rounding responses (Fig. 1d). Such activity was also blocked by mutation of any residue in the His–Asp–Cys motif. The cytoskeleton phenotype caused by IcsB resembles that of the Yersinia T3SS effector YopT (Fig. 1f). YopT is a papain-like cysteine protease that cleaves the carboxy (C)-terminal prenylated cysteine in Rho family small GTPases (RhoA, Rac1 and Cdc42). Notably, the RID of MARTX toxin can also disrupt the actin cytoskeleton by inactivating RhoGTPases, which requires the His–Asp–Cys motif (Fig. 1f). Similar findings were obtained in IcsB-transfected 293T cells (Fig. 1b). IcsB is critical for S. flexneri intercellular spread, particularly in the absence of virA (Fig. 2a). We confirmed this virulence defect caused by ΔicsB in S. flexneri 2457T (Supplementary Fig. 1a), as well as the requirement of the His–Asp–Cys triad of IcsB (Supplementary Fig. 1b,c). Thus, IcsB and its homologous effectors may function as a papain-like hydrolytic enzyme in modulating host function.

IcsB disrupts RhoGTPase membrane cycling by modifying its C-terminal tail. The fact that IcsB could block the Rho–GTP interaction with RhoGDI indicates that IcsB, like YopT, may modulate RhoGTPase cycling onto and off the membrane. Consistent with this idea, IcsB expression abolished the precipitation of Rac1/Cdc42 Q61L (equivalent to RhoA Q63L) by RhoGDI while it had no effect on their binding to the effector domain PBD (The Rac/Cdc42 (p21)-binding domain of RhoGDI), dependent on the Cys-306 (Supplementary Fig. 2a). IcsB also disrupted the interaction between RhoA and Rho GDP-dissociation inhibitor (RhoGDI), which was abolished by mutations of the His–Asp–Cys motif but not Tyr-297 (Supplementary Fig. 2b). Thus, IcsB uses its papain-like activity to inactive RhoA in mammalian cells.

RhoGTPases are frequently targeted by bacterial pathogens, which is often mediated by bacterial toxins or effector-catalysed covalent modifications. In vitro pulldown of recombinant MBP (maltose-binding protein)-IcsB C306A from bovine brain extracts identified a specific binding partner of 25 kDa (Fig. 2c), and this band mainly contained Rho, Rac1 and Cdc42, according to mass spectrometry identification. Confirming this observation, RhoA was readily co-immunoprecipitated by IcsB from transfected 293T cells (Fig. 2d). Furthermore, endogenous RhoA in 293T cells often showed a slow migration on a high-concentration sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel upon expression of IcsB, but not IcsB C306A (Fig. 2e). Thus, IcsB probably directly modifies RhoGTPases, rendering them more hydrophobic.

The C-terminal tail of RhoA, bearing the prenylated cysteine, is sufficient for recognition and cleavage by YopT (Fig. 2f). Expression of IcsB, but not IcsB C306A, rendered the enhanced green fluorescent protein (eGFP)-tagged RhoA C-terminal tail resistant to YopT cleavage (Fig. 2f). The tail, when co-expressed with IcsB in 293T cells, exhibited a more evident mobility shift (Fig. 2g). PDE6G, a non-selective prenyl-binding protein, could efficiently pull down RhoA from cell lysates, but this was not altered by IcsB expression (Fig. 2h). Taken together, these data suggest that IcsB probably modifies the C-terminal tail of RhoA without affecting its prenylation.

IcsB is an 18-carbon N-fatty acyltransferase that modifies lysine residues in the C-terminal PBR in RhoGTPases. To reveal the modification on RhoA, we affinity-purified Flag-RhoA Q63L from control or IcsB-expressing cells and performed mass spectrometry analyses. Determination of the total molecular mass revealed that RhoA was modified with 1, 2 or 3 18-carbon fatty acyl groups (stearoyl or oleoyl) (Fig. 3a). Tandem mass spectrometry (MS/MS) analysis showed that the acyl groups were conjugated to Lys-185, Lys-186 and Lys-187 in the PBR (Fig. 3b–d). The PBR, preceding the prenylated cysteine, has electrostatic interactions with phospholipid head groups in the membrane. Thus, fatty acylation of the PBR increased the hydrophobicity and membrane association of RhoA (Fig. 3a). The modification also explains the resistance of IcsB-modified RhoA to YopT cleavage, as an intact PBR is required for recognition by YopT (Fig. 3e). According to the crystal structure of RhoGDI complexed with prenylated Cdc42 (ref. 17), fatty acylation of the PBR should disrupt the binding of RhoGDI to RhoGTPases, also explaining our experimental observations (Supplementary Fig. 2b,d).

We attempted to reconstitute IcsB fatty acylation of RhoA in vitro. Purified farnesylated RhoA (the L193S mutant was used to switch the native geranyleranylation to farnesylation), obtained from co-expression with the farnesyltransferase (FTase) in Escherichia coli, was incubated with recombinant MBP-IcsB in the presence of H3-stearoyl-CoA and inositol hexaphosphate (IP6). Autoradiography showed that the FTase was readily modified by the stearyl group, which did not occur with IcsB C306A (Fig. 3e). We also performed the same reaction using cold stearoyl-CoA. Mass spectrometry then revealed that the molecular mass of RhoA was increased by 535 and 801 Da (Supplementary Fig. 3a), corresponding to the addition of 2 and 3 acyl groups, respectively. MS/MS analyses confirmed the modification on Lys-185, Lys-186 and Lys-187 (Supplementary Fig. 3b). The Yersinia T3SS effector YopT, which also bears a papain-like hydrolytic triad, acetylates serine and threonine residues in MAPK kinases15. Different from YopL, IcsB is an 18-carbon fatty acyltransferase that modifies lysine residues. IcsB-catalysed modification also differs from the known long-chain fatty acylation that generally occurs on a cysteine or the amino (N) terminus of a glycin residue.

We found that the C-terminal tail of RhoA expressed in the absence of the FTase resisted modification by IcsB and was therefore
Fig. 1 | Ectopic expression of IcsB is toxic to yeast and disrupts the actin cytoskeleton in mammalian cells, which requires the putative catalytic motif. 

a, Effects of IcsB expression on yeast growth. *S. cerevisiae* strains harbouring an empty vector or an indicated galactose-inducible IcsB expression plasmid were spotted onto the selective media containing glucose or galactose. Cells cultured in the liquid media were subjected to western blot analysis (bottom).

b, Effects of IcsB expression on mammalian cell viability. 293T cells were transfected with an empty vector or an indicated IcsB expression plasmid. Cell viability was determined by measuring cytosolic ATP levels normalized to the vector control (mean ± s.d. from three replicates).

Bottom: immunoblots of IcsB expression.

c, Sequence alignment of the IcsB family. IcsB_Sf and IcsB_Ss are IcsB of *S. flexneri* and *S. sonnei*, respectively. BopA_Bp and BopA_Bo are BopA of *B. pseudomallei* and *Burkholderia oklahomensis*, respectively. RTX_Vc and RTX_Pm are RIDs of MARTX toxins from *Vibrio cholerae* and *Proteus mirabilis*, respectively. In total, 77 amino acids of RTX_Vc and RTX_Pm that are not conserved were omitted from the alignment. The putative catalytic residues and other conserved residues are coloured in red and grey, respectively. The yellow-coloured residues are not required for cytotoxicity of IcsB in yeast and 293T cells. Secondary structures predicted from IcsB sequence are shown above the sequence. Blue ovals represent α helices and green arrows represent β strands.

d, Effects of IcsB expression on filamentous actin cytoskeleton structure. HeLa cells were co-transfected with eGFP and an indicated Flag-IcsB expression plasmid. F-actin was stained by rhodamine phalloidin. Scale bars, 20 µm. Total cell lysates were subjected to anti-Flag immunoprecipitation followed by anti-Flag immunoblotting (bottom). The data in a, b and d are representative of three independent experiments.
only partitioned into the aqueous phase (Fig. 3f). Consistently, IcsB did not affect the activation state of non-prenylated RhoA C190A (Supplementary Fig. 2c). Thus, prenylation of RhoA is required for its recognition by IcsB. Moreover, IcsB-catalysed H₃-stearoyl labeling of RhoA was barely detectable in the absence of IP₃ (Fig. 3e). This property echoes several other bacterial toxins, including the autoprocessing cysteine protease domain of the large clostridial cytotoxins TcdA and TcdB and the Vibrio MARTX toxin, and a cysteine protease domain-like T3SS effector from V. parahaemolyticus, as well as the YopJ family of acetyltransferase effectors. A previous study reported a cholesterol-binding region in IcsB, but cholesterol was not required for IcsB stearoylation of RhoA in vitro.

Stearoylome profiling reveals that IcsB modifies multiple host proteins. The fact that IcsB can target the prenylated C-terminal tail of RhoA without full-length RhoA indicates that IcsB may modify other host proteins. We therefore employed bioorthogonal chemical proteomics to profile IcsB-fatty-acylated proteins in living cells. Specifically, HeLa cells were labelled with alk-16—an alkylene analogue of stearic acid that can be metabolized and covalently installed onto cellular proteins installed onto cellular proteins. Alkyne-labelled proteins in cell lysates were then subjected to the click-chemistry reaction with azide-rhodamine for in-gel fluorescence, or azide-biotin for streptavidin enrichment and mass spectrometry. In the pilot in-gel fluorescence assay, several abundant stearoylated proteins were detected.

Fig. 2 | IcsB disrupts RhoGTPase membrane cycling by modifying its C-terminal tail. a, Effects of IcsB on the hydrophobicity of RhoA. Cytosolic extracts of RhoA-transfected 293T cells were incubated with purified IcsB and ATP followed by Triton X-114 partitioning. b, Effects of IcsB on RhoGTPase cleavage by YopT. 293T cells were co-transfected with an indicated Rho expression plasmid and IcsB (WT or C306A). Cell lysates were treated with or without YopT, then subjected to Triton X-114 partitioning. c, Identification of RhoGTPases as IcsB-binding proteins. Purified MBP-IcsB C306A was incubated with or without bovine brain extracts and then subjected to amylose bead pulldown. Bound proteins were separated on the SDS-PAGE gel and Coomassie blue staining of the gel is shown. The specific band bound by IcsB C306A contains Rho, Rac and Cdc42, revealed by mass spectrometry. d, Interaction between transfected IcsB and RhoA in 293T cells. Shown are immunoblots of anti-Flag immunoprecipitates and total cell lysates (input). e, SDS-PAGE mobility shift of the endogenous RhoA or RhoA C-terminal tail induced by IcsB. 293T cells were transfected with an indicated IcsB expression plasmid alone (e) or co-transfected with a Flag-SUMO-tagged RhoA C-terminal ten-residue tail (C10 aa) (f). Cell lysates were subjected to 15% SDS-PAGE, followed by immunoblotting analyses. The asterisk in (e) indicates the mobility shift of the endogenous RhoA. g, Effects of IcsB on the cleavage of RhoA-transfected 293T cells were incubated with purified IcsB and ATP followed by Triton X-114 partitioning. h, Effects of IcsB on RhoGTPase cleavage by YopT. 293T cells were co-transfected with Flag-RhoA Q63L and an indicated IcsB expression plasmid. Cell lysates were subjected to GST-PDE pulldown or YopT digestion followed by Triton X-114 partitioning. Proteins in the aqueous phase (AP) and detergent phase (DP) were subjected to immunoblotting analyses (a, b, g and h). All data (a–h) are representative of three independent experiments.
Fig. 3 | IcsB is an 18-carbon N-fatty acyltransferase that modifies lysine residues in the C-terminal PBR of RhoGTPases. **a**, Mass spectrometry identification RhoA modification by IcsB in transfected 293T cells. Flag-RhoA Q63L was purified using the anti-Flag affinity resin. The total molecular mass was determined by electrospray ionization mass spectrometry. The asterisk denotes RhoA Q63L without the prenylation modification. **b**, Extracted ion chromatograms of Flag-RhoA Q63L purified from transfected 293T cells. Shown are graphs of the stearoylated peptide RGKKK (m/z = 441.8). **c**, MS/MS mass spectrum of the stearoylated peptide RGKKK in IcsB-modified Flag-RhoA Q63L purified from 293T cells. b and y fragments in (c) are obtained from the MS/MS spectra of the fatty acylated peptide (RGK(ste)KK). **d**, Alignment of the RhoA, Cdc42 and Rac1 C-terminal 13-residue sequences. The prenylated cysteine residues are in red, and the PBR lysines are in blue. **e**, In vitro reconstitution of RhoA stearoylation by IcsB. Purified farnesylated RhoA was incubated with MBP-IcsB, 3H-stearoyl-CoA and the indicated concentration of IP6. The reactions were stopped by adding the SDS sample buffer, and subjected to SDS-PAGE followed by Coomassie blue staining and 3H autoradiography. **f**, Requirement of RhoA prenylation for fatty acylation by IcsB. The farnesylated or unmodified Flag-SUMO-RhoA C-terminal 10-residue (C10 aa) tail was incubated with SUMO-IcsB, ATP and cell extracts. The reactions were stopped by adding the SDS sample buffer, then subjected to 15% SDS-PAGE followed by anti-Flag immunoblotting (left). Cell lysates were also subjected to Triton X-114 partitioning (AP, aqueous phase; DP, detergent phase; right). L/S is an L193S mutation that switches the native geranylgeranylation to farnesylation. Data in **a–c, e** and **f** are representative of three independent experiments.
**Fig. 4** | Chemical proteomics reveals that IcsB targets multiple host membrane proteins for lysine $N^\varepsilon$-fatty acylation. 

**a**, In-gel fluorescence visualization of the stearoylome profile of IcsB (WT or C306A)-transfected (left) and S. flexneri (WT, ΔicsB, ΔvirA or ΔvirAΔicsB)-infected (right) HeLa cells. Coomassie blue staining confirms equal protein loading. 

**b**, Scatter plots of SILAC proteomic data. Hits with an abundance increase of more than threefold ($\log_2[H/L > 1.58]$ in the forward SILAC and $\log_2[H/L < -1.58]$ in the reverse SILAC) in the IcsB group relative to the control group are shown in red. H/L represents the ratio between heavy and light label partners in the indicated (forward or reverse) experiment. 

**c**, Validation of IcsB modification of the proteomic hits. A total of 11 selected candidate substrates were individually transfected into 293T cells. The cells were infected with S. flexneri WT or ΔicsB in the presence of Alk-16, and subjected to in-gel fluorescence assay. Anti-Flag immunoblotting confirms equal loading of the indicated samples. 

**d**, Effects of lysine mutation in the PBR of selected substrates on their modification by IcsB. 293T cells were co-transfected with IcsB and Flag-tagged RhoA, VAMP8 or Rab13, or the indicated lysine mutant. The cells were metabolized with Alk-16 and subjected to in-gel fluorescence assay. Data are representative of two (a and b) or three (c and d) independent experiments.
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RNAi, RNA interference.
specifically in the lysates of cells expressing wild-type (WT) IcsB (Fig. 4a). To quantify the differences in stearoylated proteins between WT IcsB and IcsB C306A-expressing cells, double SILAC (stable isotope labelling with amino acids in cell culture) proteomic analyses were also performed (Supplementary Fig. 4a). The quantification identified about 60 proteins that were stearoylated in an IcsB-dependent manner (Fig. 4b, Table 1 and Supplementary Table 1), most of which belong to the Ras superfamily of small GTPases, including the Ras family (R-Ras, K-Ras, H-Ras, Rap1B, RaLα, RaB and RhoB), Rho family (Rac1, Rac2, RhoA, RhoC and RhoG) and Rab family (Rab1A, Rab5B, Rab8A, Rab10, Rab11A, Rab11B, Rab13, Rab22A, Rab23, Rab34 and Rab35). The list also contains non-small GTPase proteins, including SNARE proteins (YKT6, VAMP3, VAMP8, SEC22B and BNI1/SEC20), N-myristoylated proteins (MARCKS, MARCKSL1 and BASP1), septins (SEPT7 and SEPT11) and some other proteins. Gene Ontology-based clustering analysis suggested that these proteins regulate the actin cytoskeleton, membrane trafficking, cell–cell adhesions junctions, and endocytosis and endosomal recycling (Supplementary Fig. 4b).

We also performed the proteomic profiling under S. flexneri infection conditions. For this, HeLa cells were infected with the icsB-sufficient or -deficient S. flexneri strains. An evident increase of total stearoylated proteins was observed with icsB-positive infections (Fig. 4a). SILAC quantitative proteomics identified more than 40 proteins that were stearoylated in an icsB-dependent manner (Fig. 4b). The presence or absence of VirA, which affects autophagy in intracellular S. flexneri32, had little influence on the stearoylated proteins identified (Fig. 4b). The list of stearoylated proteins during infection largely overlapped with that of the transfection experiment (Table 1 and Supplementary Table 2). In-gel fluorescence assay of a dozen selected candidates, including small GTPases (Rho, Ras, Rap and Rab) and the non-small GTPase protein VAMP8 showed that they could all be labelled with azide-rhodamine upon icsB-positive infections (Fig. 4c), validating the proteomic data. Thus, IcsB alters the fatty acylation landscape of the host cell proteome to modulate multiple host cellular processes.

Using the alk-16 labelling assay, we found that only mutations of all three lysines (Lys-185, Lys-186 and Lys-187) in the PBR of RhoA could completely block its stearoylation by IcsB in 293T cells (Fig. 4d). Results of a similar nature were obtained with VAMP8 and Rab13—two other IcsB substrates that also have a PBR preceding their C-terminal membrane insertion or association sequences (Fig. 4d).

IcsB is localized on Shigella-containing vacuoles and modifies its substrates there. The substrates of IcsB are all known to be membrane associated or within a membrane complex (Table 1 and Fig. 5a). Consistently, ectopically expressed IcsB in 293T cells was in the membrane fraction (Fig. 5b). To investigate the localization of IcsB during S. flexneri infection, we used the recently developed SunTag system, which is extremely sensitive in imaging a cellular protein tagged with a GCN4 protein-derived peptide (SunTag)33,34. Specifically, eGFP-fused single-chain anti-SunTag antibody was stably expressed in HeLa cells, and the cells were then infected with S. flexneri expressing IcsB harbouring 24 copies of the SunTag. The 24x SunTag did not affect T3SS-dependent secretion of IcsB (Supplementary Fig. 5a). The data revealed that T3SS-delivered IcsB, before bacterial escape from the vacuole into the cytosol, was localized on S. flexneri-containing vacuole membranes (Fig. 5c,d).

Many of the IcsB substrates, including the Ras superfamily of small GTPases, rely on their C-terminal cysteine prenylation to be membrane associated. Serine substitution of the cysteine in representative members of the Rho, Ras, Rab and Rap families abolished their stearoylation by transfected IcsB in 293T cells (Fig. 5e). Similar results were obtained with a prenylated non-GTPase substrate CNP, a microtubule-associated 2′,3′-cyclic nucleotide-3′-phosphodiesterase (Fig. 5e). Deletion of the C-terminal transmembrane domain in VAMP8 also diminished its stearoylation by IcsB (Fig. 5e). The membrane insertion motif in these IcsB substrates is often preceded by a PBR (Fig. 5f). We generated PBR-mutated RabH, Rab13 and VAMP8 containing only one lysine, with all other lysines mutated into arginine (with PBR) or with all other lysines or arginines mutated into alanine (without PBR). Mutations to alanine abolish or reduce the membrane association. When subjected to IcsB-catalysed stearoylation in cells, the one-lysine-with-PBR mutants were still modified by IcsB, in contrast with their cognate without-PBR mutants (Fig. 5f). Thus, membrane localization of IcsB targets is required for their encounter with and subsequent fatty acylation by IcsB from S. flexneri.

Fatty acylation of CHMP5 by IcsB is important for Shigella evasion of autophagy. Loss of icsB in S. flexneri results in trapping of the bacterium into the autophagosome31,32,33. We confirmed that few WT S. flexneri were positive for the autophagosome marker eGFP-LC3 when nearly 80% of ΔicsB-infected cells contained eGFP-LC3-decorated bacteria (Supplementary Fig. 5b,c). The increased autophagy of S. flexneri ΔicsB was suppressed by expression of WT IcsB, but not its acyltransferase-inactive H145A, D195A and C306A mutants in the bacteria (Supplementary Fig. 5b,c). As expected, eGFP-LC3 decoration of S. flexneri ΔicsB was diminished in ATG16LΔ or ATG5Δ HeLa cells (Supplementary Fig. 5d,e). It is necessary to note that the impact of IcsB deficiency on anti-Shigella autophagy was not that robust, particularly when the percentage of intracellular bacteria decorated with eGFP-LC3 was counted. Thus, the overall relevance of IcsB inhibition of autophagy to Shigella pathogenesis requires further investigation, which is consistent with our finding of IcsB targeting of multiple different host proteins.

To investigate whether any IcsB substrate is involved in S. flexneri autophagy, knockout or knockdown analyses in HeLa cells were performed with nearly all the substrates identified from the proteomic screen. These extensive analyses (Table 1) only identified one gene, CHMP5 (charged MVB protein 5), whose deficiency could inhibit eGFP-LC3 decoration of S. flexneri ΔicsB (Fig. 6a,b and Supplementary Fig. 6a,b). The defective autophagy of S. flexneri ΔicsB was restored by re-expression of CHMP5 in the knockout cells. Consistent with the functional data, CHMP5 was found to be stearoylated by IcsB in S. flexneri-infected 293T cells (Fig. 6c). The N-terminal region in CHMP5 contains three lysines (Lys-7, 9 and 11) and one arginine (Arg-3), resembling the PBR in other IcsB substrates. Mutation of Lys-7 or the three lysines together severely inhibited or completely blocked CHMP5 stearoylation by IcsB, respectively (Fig. 6d), whereas mutation of other lysine residues in CHMP5 showed no effects (Supplementary Fig. 7a). Thus, IcsB mainly targets Lys-7 in CHMP5 for stearoylation, resulting in suppression of antibacterial autophagy in host cells. CHMP5 is a component of the ESCRT-III complex35,36, CHMP5ΔΔ did not affect S. flexneri invasion into HeLa cells (Supplementary Fig. 6c,d). Thus, CHMP5 is probably involved in regulating the trafficking and property of S. flexneri-containing vacuoles, which indirectly affects development of the autophagic response to S. flexneri. Supporting this notion, CHMP5ΔΔ in HeLa cells disrupted autophagosome formation in response to both ΔicsB and WT S. flexneri infection (Fig. 6a,b). Endogenous CHMP5 could be identified on Shigella-containing vacuoles (Fig. 6e).

We also infected CHMP5ΔΔ HeLa cells with other bacterial pathogens, including Salmonella typhimurium, Yersinia pseudotuberculosis (the effector-less ΔHEMOJ(T) IP2666 strain) or Listeria monocytogenes, which are known to be targeted by host autophagy. Interestingly, eGFP-LC3 decoration of these bacteria was not affected by the loss of CHMP5 (Supplementary Fig. 7b,c). This agrees with the hypothesis that CHMP5 and the ESCRT-III complex may have a differential role in endocytosis-mediated entry for
**Fig. 5 | IcsB is localized on Shigella-containing vacuoles and modifies its substrates on the membrane location.**

**a.** Classification of IcsB substrates by their membrane-targeting mechanisms.  
Sub-cellular fractionation of IcsB. 293T cells transfected with Flag-eGFP-IcsB were lysed in hypotonic buffer, and the cell lysates (S10) were ultracentrifuged (100,000 g) to obtain the plasma membrane (PM) and the S100 cytoplasmic fraction. Shown are the anti-Flag, anti-cadherin and anti-Erk2 immunoblots.

**b.** Sub-cellular fractionation of IcsB. 293T cells transfected with Flag-eGFP-IcsB were lysed in hypotonic buffer, and the cell lysates (S10) were ultracentrifuged (100,000 g) to obtain the plasma membrane (PM) and the S100 cytoplasmic fraction. Shown are the anti-Flag, anti-cadherin and anti-Erk2 immunoblots.

**c.** Localization of IcsB during *S. flexneri* infection. HeLa cells stably expressing scFV-SunTag-eGFP were infected with *S. flexneri ΔicsB* complemented with 24× SunTag-fused IcsB (WT or C306A). IPTG was used to induce IcsB expression. Fluorescence images taken at 4 h post-infection are shown in c (scale bars, 3 μm), and percentages of intracellular bacteria containing SunTag-positive signals are shown in d. At least 200 infected cells were examined for each experiment, and data are means ± s.d. from three replicates. A two-tailed unpaired Student’s t-test was performed (*P < 0.05; **P < 0.01).

**d.** Membrane localization of the substrates is required for their modification by IcsB. 293T cells were co-transfected with IcsB and an indicated Flag-tagged substrate. The mutants assayed in e are cysteine mutants devoid of lipid modification or deletion of the transmembrane helix for VAMP8 (D76–96). RhoA, Rab13 and VAMP8 mutants with or without the PBR, both of which bear a single modifiable lysine, were assayed. Cells were metabolized with Alk-16 and harvested for in-gel fluorescence assay. Data (b–f) are representative of three independent experiments.
Fig. 6 | Fatty acylation of CHMP5 by IcsB is important for Shigella escape from host autophagy. **a, b.** Effects of CHMP5 deficiency on bacterial autophagosome formation in response to _S. flexneri_ infection. Indicated HeLa cells expressing eGFP-LC3 were infected with _S. flexneri_ WT or ΔicsB. **a.** Percentages of infected cells containing LC3-positive _S. flexneri_ at indicated time points after infection. **b.** Representative fluorescence images taken at 2 h post-infection (scale bars, 3 μm). At least 200 infected cells were examined for each experiment and the data are means ± s.d. from three replicates. A two-tailed unpaired Student’s t-test was performed (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, 0.0734). **c.** Stearoylation of CHMP5 by IcsB during _S. flexneri_ infection. 293T cells stably expressing Flag-CHMP5 were infected with _S. flexneri_ WT, ΔicsB, ΔvirA or ΔvirAΔicsB in the presence of Alk-16. Cells were harvested 2 h after infection for in-gel fluorescence assay. **d.** Effects of lysine mutation on CHMP5 stearoylation by IcsB. 293T cells were transfected with IcsB and Flag-CHMP5 (WT or an indicated K to R mutant). Cells were metabolized with Alk-16 for 24 h and subjected to in-gel fluorescence assay. **e.** Localization of endogenous CHMP5 by immunofluorescence staining in _S. flexneri_-infected HeLa cells (scale bars, 1 μm). Data shown in **a–e** are representative of three independent experiments.
different bacteria. Thus, CHMP5 functions specifically in anti-
Shigella autophagy, and its fatty acylation by IcsB contributes to Shigella escape from host autophagy.

Discussion
In summary, we show that the T3SS effector IcsB from S. flexneri targets a large set of host proteins for fatty acylation during infection. This property differs from other bacterial effectors that often act on one or a few host proteins. Most IcsB targets, such as the small GTPases, bear the CAAX-mediated prenylation. IcsB modifies RhGTPases and disrupts their membrane cycling, which causes cell rounding in transfected cells, but not in S. flexneri-infected cells. This is different from the RID of MARTX toxin whose inactiva-
tion of Rh causes cell rounding during infection. It is possible that IcsB modification of RhGTPases is not extensive enough to generate a gross cytoskeleton phenotype. Thus, the functional con-
sequence of IcsB modification of its other targets during infection is a subject of future studies. Despite this, IcsB appears to be a master effector that alters the fatty acylation landscape of host membrane proteomes during S. flexneri infection.

Autophagy is important for host defence against bacterial infec-
tions. Different bacterial pathogens enter the host cell through different routes with distinct trafficking routes. Successful pathogens have evolved effectors to hijack different steps in bacterial autoph-
yagy. The Legionella pneumophila RavZ effector cleaves and decon-
jugates LC3-PE (LC3-phosphatidylethanolamine conjugate) 32. S. flexneri VirA inactivates Rab1 at the endoplasmic reticulum–Golgi exit sites to block autophagosome membrane formation. 37 We show that IcsB modifies the endosomal ESCRT-III complex protein CHMP5, and CHMP5 deficiency suppresses anti-Shigella autophagy. These results suggest that IcsB functions during the traf-
ficking of endosome-derived Shigella-containing vacuoles, and its suppression of autophagy is an indirect result of CHMP5 inactiva-
tion. CHMP5 deficiency does not affect autophagy of other intracellular bacteria, consistent with the distinct endosomal machineries exploited by different bacteria.

Fatty acylation modification is present in all kingdoms of life, and long-chain fatty acylation is commonly manifested as N-myristoylation and S-palmitoylation of the N-terminal glycine and cysteine residues, respectively. 32–41 Lysine N-fatty acylation has only been found on aquaporin-0 (ref. 35) and some secreted cytokines, 19,10,41 but their functional significance is unclear. IcsB catalyses N-fatty acylation to modulate multiple host cellular processes. IcsB adopts a papain-like fold that is common in the protein structure space. It is not unexpected that N-fatty acylation might be widely used in regulating eukaryotic biology. A recent study shows that SRT6 can defatty-acylate PBR lysines in R-Ras. 41 While our manuscript was under revision, a follow-up study reported that the RID of MARTX toxin also functions as a long-chain N-fatty acyltransferase to mod-
ify PBR lysine residues of RhGTPase, which explains the known functions of RID, such as inducing host cell rounding and inhibiting phagocytosis. 19,47

Methods
Plasmids, antibodies and reagents. DNA for the ibcB gene was amplified from the genomic DNA of the S. flexneri 2a strain 2457T. For recombinant expression in E. coli, ibcB DNA was constructed into the pSU30 vector with an N-terminal SUMO (small ubiquitin-like modifier) tag and the pMAL vector with an N-terminal MBP tag. For expression in mammalian cells, ibcB DNA was cloned into the pCS2 vector with an N-terminal 3xFlag tag or a modified pEGFP-C1 or pLKO1 vector containing a Flag tag preceding eGFP. IgA DNA was cloned into the pCS2 vector with an N-terminal RFP (red fluorescent protein) or GFP (blue fluorescent protein) tag. For complementary expression in S. flexneri, ibcB was cloned into the pME9032 vector under the control of a tac promoter. For expression in yeast, ibcB and igpA were cloned into the p414 and p413-Gal vector, respectively, and protein expression was induced by galactose in the media. EGF-tagged 13 Cterminal residues of RhoA were generated using a standard PCR cloning strategy. RhoA was cloned into pET21a with an N-terminal Flag-6xHis tandem tag for recombinant bacterial expression. Complementary DNAs for the α and β subunits of human FTase were amplified from the pRSF-FTase α/β plasmid and 24x SunTag and single α/β variable fragment (scFV)-SunTag eGFp sequences were synthesized at our gene synthesis facility and cloned into the pGEM6032 and FUIPWM vectors, respectively. Point mutations were generated by QuickChange Site-Directed Mutagenesis. All plasmids were verified by DNA sequencing.

Antibodies for CHMP5 (F-17; sc-743238), RhoA (sc-418), eGFp (sc-8334), HA (Y11; sc-805) and Erk2 (C14; sc-154) were purchased from Santa Cruz Biotechnology. Antibodies for Myc (9E10) were from Covance. Anti-Flag M2 mouse monoclonal antibody (F4049) and anti-Flag rabbit polyclonal antibody (F7425) were from Sigma–Aldrich. Anti-paro caderin antibody (ab5298) was from Abcam. Streptavidin-biotinylated horseradish peroxidase complex was from GE Healthcare. All the yeast cell lines and transformation reagents were from BD Biosciences. Cell culture reagents were Invitrogen products. All other chemicals and reagents used were Sigma–Aldrich products unless otherwise noted.

Yeast, cell culture, bacterial strains and infection. Culture and transformation of the S. cerevisiae W303a strain and assays of IcsB toxicity in yeast cells were performed as described recently. 15 Expression of IcsB and IgpA proteins was induced using 2% galactose as the carbon source in the medium. 293T and HeLa cells were obtained from the American Type Culture Collection. All cell lines were tested to be mycoplasma-free by PCR analyses. The identity of the cells was routinely checked by assaying their morphological features, but has not been authenticated by the short tandem repeat profiling. 293T and HeLa cells were maintained in DMEM supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C in a humidified 5% CO2 incubator. Transient transfection was performed using the VigoFect (Vigor) or jetPRIME (Polyplus) reagents following the manufacturers’ instructions.

S. flexneri 2a strain 2457T was transformed into S. flexneri to facilitate the infection. ΔicsB, ΔvirA and ΔvirAΔicsB mutant strains were constructed using the suicide vector pCVD442. For bacterial infection, S. flexneri 2457T, S. typhimurium SL1344 and Y. pseudotuberculosis IP2666 (∆HemO(T)) were cultured overnight at 37 °C in 2xYT broth with shaking. Before infection, bacteria cultured with BFP were induced by 1:100 in fresh 2xYT broth, and incubated in a shaker until the absorbance of a wavelength of 600 nm reached 1.5. L. monocytogenes EGD strain was cultured overnight at 30 °C in brain heart infusion broth with shaking until the absorbance of a wavelength of 600 nm reached 1.5. For fluorescence microscopy, HeLa cells were seeded on glass coverslips in 24-well plates and cultured for 16 h before infections with the indicated multiplicity of infection (MOI) values. The infection was facilitated by centrifugation at 800 g for 5 min at room temperature followed by another hour of incubation at 37 °C in a 5% CO2 incubator. Cells were subsequently washed three times with phosphate-buffered saline (PBS) and fresh DMEM containing 100 μg ml−1 gentamycin (to kill extracellular bacteria). After another 2 h of incubation, infected cells were washed three times with PBS, then incubated to immunofluorescence staining. Cells cultured on the coverslips were fixed with 4% parafomaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100, blocked with bovine serum albumin or FBS, then incubated with the primary antibody for one hour and the secondary antibody for another hour. Rhodamine-labelled phalloidin was used to stain the F-actin structures.

Plaque formation assay. HeLa cells were seeded onto 6-well plates and cultured for 24 h before the infection (MOI = 0.05). Bacteria were cultured at 37 °C in 2x YT broth containing 100 μg ml−1 streptomycin with shaking at 250 r.p.m. until the absorbance at a wavelength of 600 nm reached 2.0. The infection was facilitated by centrifugation at 800 g for 5 min at room temperature, and proceeded for 1.5 h at 37 °C in a 5% CO2 incubator. Cells were subsequently washed three times with PBS and incubated in 2 ml fresh DMEM containing 10% FBS, 2 mM L-glutamine, 100 μg ml−1 gentamycin and 0.5% (v/v) agarose. For S. flexneri ΔvirAΔicsB harbouring pME9032-icsB-Flag or other catalytically inactive mutants, bacteria were cultured at 37 °C in 2xYT broth containing 100 μg ml−1 streptomycin, 25 μg ml−1 tetracycline and 200 μM isopropyl [β-D-1-thiogalactoside (IPTG). The infected cells were incubated in fresh DMEM containing 10% FBS, 2 mM L-glutamine, 100 μg ml−1 gentamycin, 100 μM IPTG and 0.5% (v/v) agarose. Afimbrial adhesin transformed into S. flexneri was not recommended for this assay. The plaque area (A) was defined as A = πd²/4, where d was obtained by measuring the diameter (d) of a plaque. The average plaque area was calculated by analysing at least 15 plaques per group.

Protein expression and purification. Recombinant expression in E. coli BL21 cells was induced by 0.4 mM IPTG overnight at 25 °C unless otherwise noted. Expression and purification of GST-RBD and GST (glutathione S-transferase)-peGFP were performed. 13 C-terminal residues of the GST-tagged fusion proteins were purified by Ni-NTA resin (Qiagen). MBP-tagged proteins were purified by the amylase resin (NEB). The proteins were further purified by ion exchange chromatography followed by gel filtration chromatography. All the proteins were
concentrated and stored at −20 °C with 20% glycerol in the final volume. The protein concentration was determined by Coomassie blue staining of SDS-PAGE gels using bovine serum albumin standards.

Immunoprecipitation, GST pulldown and western blot analysis. For immunoprecipitation, approximately 1 × 10^6 cells were collected 24 h after transfection, and lysed in 1 ml of cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Roche) for 15 min on ice. The cell lysates were centrifuged at 13,000 rpm for 15 min to collect the supernatants. Then, 20 μl of Anti-Flag M2 agarose affinity gel (F2426; Sigma) was added to the supernatants. After 2 h rotation at 4 °C, the beads were washed three times with the cell lysis buffer. For GST pulldown assay, cell lysates were incubated with about 30 μg of GST-RBD/PBD/GDI proteins pre-bound on the glutathione beads. After 1 h rotation at 4 °C, the GST beads were collected and washed three times with the cell lysis buffer. Protein bound on the beads was eluted by boiling in 1× sodium dodecyl sulfate (SDS) sample buffer or by adding 300 ng/ml Flag peptides to the lysis buffer before immunoblotting analysis, as indicated.

YopT cleavage and Triton X-114 partition assay. Indicated cells were lysed in an ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol (DTT) and 1% Triton X-114 supplemented with the protease inhibitor cocktail. Then, 20 μl of clear lysates were incubated with 2 μg of recombinant GST-YopT protein at 30 °C for 1 h. The total lysates were then centrifuged at 13,000 rpm for 10 min and partitioned into the aqueous and detergent phase followed by immunoblotting analysis as indicated. For the reconstitution assay, the reactions were supplemented with 1% Triton X-114 (final concentration) and treated with GST-YopT for 1 h before performing the phase partition assay.

In vitro acyltransferase assay. To prepare the prenylated substrates, equal molar amounts of human F13ase complex and Flag-6xHis-RhoA or SUMO-RhoA C-terminal 13-residue tail proteins (1:935 version) were mixed in a reaction buffer containing 25 mM HEPES (pH 7.4), 200 mM farnesyl pyrophosphate, 5 mM MgCl₂, 5 μM ZnCl₂, and 2 mM DTT. The reactions were carried out at 30 °C for 3 h, then loaded onto a HiTrap Q column (GE Healthcare) to collect farnesylated substrate or F13ase complex. The farnesylation modification was confirmed by matrix-assisted laser desorption/ionization mass spectrometry analysis. To assay the fatty acyltransferase activity of IcsB, 494 μg of farnesylated substrates was incubated with 2 μg of purified IcsB protein in the presence of 0.5 μCi of [3H]-stearyl coenzyme A (Moravek Biochemicals) at 30 °C for 1 h in the reaction buffer containing 40 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM ZnCl₂, and 10 μM FPP. The reactions were stopped by adding 5× SDS sample buffer. Protein samples were separated on 15% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane followed by Coomassie blue staining. Incorporation of the [3H]-stearyl group was visualized by X-ray autoradiography.

Mass spectrometry analyses of RhoA modification by IcsB. To prepare RhoA samples for total molecular weight measurement by mass spectrometry, 293T cells cultured in 15 cm dishes were transfected with 3×Flag-RhoA Q63L alone or co-transfected with IcsB. Transfected cells were harvested and resuspended in ice-cold buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 1% octyl β-D-glucopyranoside and the protease inhibitor cocktail. The collected cells were lysed by sonication, and the cleared lysates were loaded onto 40 μl of 60 μM Anti-Flag beads for 4 h. The beads were washed extensively with the buffer containing 25 mM HEPES (pH 7.4), 500 mM NaCl and 1% octyl β-D-glucopyranoside. The bound proteins were eluted by 300 ng/ml Flag peptide in the elution buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl and 0.1% octyl β-D-glucopyranoside. For mass spectrometry, the eluted RhoA protein was loaded onto a homemade capillary column (75 μm inner diameter; 6 cm long) packed with POROS R1 medium (Applied Biosystems). Subsequently, the proteins were eluted by an Agilent 1100 binary pump system with the following gradient: 0–80% B in 30 min (A = 0.1 M acetic acid in water; B = 0.1 M acetic acid in 80% acetonitrile/water), then sprayed into a QSTAR XL mass spectrometer (AB Sciex) equipped with a Nano Electrospray ion source. The instrument was operated in a positive mode under 2,100 volts of spray voltage. The protein charge envelope was averaged across the mass spectrometry. The LC-MS/MS (LC-MS/MS) analysis.

Chemical proteome profiling of IcsB-modified proteins. HeLa cells were grown in DMEM supplemented with 10% FBS (HyClone; Thermo Fisher Scientific). For the SILAC experiments, cells were cultured in arginine- and lysine-deficient DMEM (Thermo Fisher Scientific) supplemented with 10% dialysed FBS. For the culture of ‘light’-labelled cells, SILAC medium containing 0.1%[13C6]-L-lysine-2HCl (Lys0; 80.0 μM; Sigma) and 0.1%[13C6]-L-arginine-HCl (Arg0; 40.0 μM; Sigma) was used. For the culture of ‘heavy’-labelled cells, SILAC medium containing 0.1%[13C6]-L-lysine-2HCl (Lys0; 80.0 μM; Cambridges) and 0.1%[13C6]-L-arginine-HCl (Arg0; 40.0 μM; Cambridge Isotope) was used. After seven cell doublings, the incorporation of the heavy isotope was estimated to be>98% as determined by liquid chromatography MS/MS (LC-MS/MS) analysis.

293T cells were transfected with the indicated eGFP-IcsB plasmid for 6 h and labelled with DMSO or Alk-16 (25 μM) overnight. Alternatively, HeLa cells labelled with Alk-16 (50 μM) for 1 h were infected with S. flexneri WT or ΔicsB for 1.5 h. The media were then replaced with fresh media containing 100 μM genamycin and 50 μM Alk-16. Infected cells were further incubated for 1 h, then washed with PBS twice, incubated with 4% SDS (50 mM triethanolamine with 150 mM NaCl pH 7.4) with vigorous vortexing and sonication. The cell lysates were centrifuged at 16,000 rpm for 20 min at room temperature to remove cellular debris. Protein concentrations were determined using the BCA assay (Pierce). For in-gel fluorescence visualization, whole cell lysates (50 μg) diluted with SDS lysis buffer (1 mg/ml) were reacted with a freshly prepared click-chemistry reaction cocktail containing azido-rhodamine, TCEP, CuSO₄, and 0.1 M acetic acid in 80% acetonitrile/20% Tris-HCl gels (Bio-Rad) for SDS-PAGE separation. Gels were de-stained in 40% methanol and 10% acetic acid for at least 1 h, then scanned on a GE Healthcare Typhoon 9400 variable mode imager with excitation and emission at 532 and 580 nm, respectively. The gels were then also stained with Coomassie Brilliant Blue (Bio-Rad).

For the SILAC proteomics studies, ‘heavy’- and ‘light’-labelled cells were transfected with eGFP-tagged WT or the C306A mutant IcsB in the ‘forward’ experiment and vice versa in the ‘reverse’ experiment. For the infection assay, ‘heavy’- and ‘light’-labelled cells were infected with S. flexneri WT or ΔicsB strain and vice versa in the ‘reverse’ experiment. Infected cells were labelled with Alk-16 and lysed with 4% SDS as described above. After estimating the protein concentration, ‘heavy’-labelled cell lysates (1 mg) were mixed with ‘light’-labelled cell lysates (1 mg) and treated with freshly prepared NH₄OH (0.75 M) at room temperature with rotation for 1 h. Proteins were then precipitated with ice-cold MeOH, resuspended in 4% SDS and clicked with azido-biotin using CuAAC reactions as above described. Methanol-precipitated and washed protein pellets were again resuspended in 4% SDS. Equal amounts of each protein were diluted 1/4 by volume with 50 mM triethanolamine buffer (final 1% SDS). Then, 60 μl of precooled streptavidin agarose beads (Invitrogen) were added to each sample. The protein and bead mixtures were incubated for 1 h at room temperature on a rotating mixer. The beads were then washed once with PBS containing 0.2% (w/v) SDS, three times with PBS and twice with 250 mM ammonium bicarbonate. Beads were resuspended in 500 μl of 8 M urea, reduced with 10 mM DTT for 30 min, alkylated with 50 mM iodoacetamide in the dark for another 30 min. Finally, the beads were washed with 25 mM ammonium bicarbonate and digested with 1 μg of trypsin at 37 °C for 2 h. The supernatant was collected, dried and pre-fractionated with SCX StageTips for LC-MS analysis.

LC-MS/MS analysis for proteomic profiling was carried out at the Proteomics Resource Center at The Rockefeller University, New York, NY, USA. LC-MS analysis was performed with a Dionex 3000 nano-HPLC coupled to an Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Peptide samples were loaded onto a C18 reverse-phase column (75 μm diameter; 15 cm length) at 300 nl/min with a linear gradient increasing from 5% buffer A (water with 0.1% formic acid) and 5% buffer B (acetonitrile with 0.1% formic acid) to 75% buffer B in 13 min was used at 0.2 μl/min to inject the samples into the mass spectrometer. The
Orbitrap XL was operated in top-8-CID-mode with MS spectra measured at a resolution of 60,000 FWHM (full width at half maximum) at m/z = 400. One full MS scan (300–2,000 molecular weight) was followed by three data-dependent scans of the 10 highest m/z ions with dynamic exclusion enabled. Peptides fulfilling a Percolator-calculated 1% false discovery rate threshold were reported. Acquired tandem MS spectra were extracted and quantified using MaxQuant software[1]. The search results from MaxQuant were analysed by Perseus (http://www.perseus-framework.org). Enzyme specificity was set to trypsin, allowing two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and N-terminal acetylation were set as variable modifications. Mass deviation for MS/MS peaks was set at a maximum of 0.5 m/z units, and maximum false discovery rates were set to 0.01, both at the peptide and the protein levels. Peak lists generated by MaxQuant were searched with Andromeda against the UniProt complete human database concatenated with common known contaminants. Only unique and razor peptides were used for quantification with a minimum of two ratio counts. The ‘re-quantify’ feature of MaxQuant was used to correct the quantification of proteins with high ratios. Before analysis, known contaminants and reverse hits were removed.

In this study, no statistical methods were used to predetermine the sample size. The experiments were not randomized, and the investigators were not blinded to allocation during the experiments and outcome assessment.

**Data availability.** The data that support the findings of this study are included in this published article along with its Supplementary Information files, and are also available from the corresponding author upon request.

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**References**


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Author contributions
F.S. conceived the study. Y.Z. performed initial studies on the identification of RhoGTPases as the substrate of IcsB and its fatty acyltransferase activity. W.L. established the SunTag labelling of T3SS effectors, analysed the proteomic hits of IcsB, and performed the localization and autophagy studies. P.Z. and Z.L. provided technical assistance to Y.Z. and W.L. H.Z. and Y.X. performed the plaque assay. T.P. and H.C.H. were responsible for the chemical proteomic analyses. X.D. and S.C. carried out the mass spectrometry experiments. Y.Z., W.L., T.P., S.C., H.C.H. and F.S. analysed the data. W.L., Y.Z. and F.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing interests.

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▶ Experimental design

1. Sample size
   Describe how sample size was determined. No statistical methods were used to predetermine sample size

2. Data exclusions
   Describe any data exclusions. There were no data exclusions in this study.

3. Replication
   Describe whether the experimental findings were reliably reproduced. All attempts at replication were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups. No randomization was used as none of the experiments described in this study involve random allocation of experimental groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis. No blinding was done as none of the experiments described in this study involve group allocation during data collection or analyses.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   - The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

There were no special softwares used to analyze the data in this study.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

All unique materials used are readily available from the corresponding authors.

Antibodies for CHMP5 (F-7, sc-374338), RhoA (sc-418), EGFP (sc-8334), HA (Y-11, sc-805) and Erk2 (C-14, sc-154) were purchased from Santa Cruz Biotechnology Inc. Antibodies for Myc (9E10) was from Covance. Anti-Flag M2 mouse monoclonal antibody (F4049) and anti-Flag rabbit polyclonal antibody (F7425) were from Sigma-Aldrich. Anti-pan Cadherin antibody (ab6529) was from Abcam. Streptavidin-biotinylated Horseradish peroxidase (HRP) complex was from GE Healthcare.

Animals and human research participants

There are no animal experiments in this study.

There are no human subjects involved in this study.