Kinase-independent synthesis of 3-phosphorylated phosphoinositides by a phosphotransferase

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Despite their low abundance, phosphoinositides play a central role in membrane traffic and signalling. PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are uniquely important, as they promote cell growth, survival and migration. Pathogenic organisms have developed means to subvert phosphoinositide metabolism to promote successful infection and their survival in host organisms. We demonstrate that PtdIns(3,4)P2, a major product generated in host cells by the effectors of the enteropathogenic bacteria Salmonella and Shigella, is generated by a phosphotransferase/phosphosomerase mechanism. Recombinant SopB is capable of generating PtdIns(3,4)P2 from PtdIns(4,5)P2 in a cell-free system. Through a remarkable instance of convergent evolution, bacterial effectors acquired the ability to synthesize 3-phosphorylated phosphoinositides by an ATP- and kinase-independent mechanism, thereby subverting host signalling to gain entry and even provoke oncogenic transformation.

By directing vesicular traffic, gating ion channels and orchestrating numerous signal transduction pathways, polyphosphoinositides (PPIns) shape organellar identity and function. The seven PPIns species formed by combinatorial phosphorylation of the inositol ring are in constant flux. Kinases and phosphatases catalyse the addition and removal, respectively, of phosphates at the 3-, 4- or 5-position of the inositol ring, whereas phospholipase C (PLC) detaches the phosphoinositol ring from its glycerol backbone. That numerous human pathologies are the result of mutations in these enzymes is a testament to the central role of PPIns in cellular homeostasis.

PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are among the least-abundant species in quiescent cells, constituting <0.1% of the total PPIns. However, in response to hormones, growth factors and chemokines, phosphoinositide 3-kinases (PI3Ks) can increase their abundance by as much as 100-fold. The PI3K family comprises 15 proteins; a subset—class I and class II PI3Ks—are the sole pathway to generate PtdIns(3,4,5)P3 and PtdIns(3,4)P2. When activated, class I PI3Ks phosphorylate PtdIns(4,5)P2 to yield PtdIns(3,4,5)P3. Class II isoforms phosphorylate the 3-position of PtdIns(4)P to yield PtdIns(3,4)P2 in the plasma membrane (PM) but can also modify the 3-position of PtdIns to yield PtdIns(3)P in endosomes. Stereospecific interactions of 3-phosphorylated inositides with cognate protein domains coordinate cell migration, mitogenesis, growth, metabolism and autophagy via downstream effectors including Rac, AKT and mTOR. However, the inappropriate accumulation of these lipids is a powerful anabolic signal that can drive oncogenesis.

Pathogenic organisms have evolved means to enter eukaryotic cells by receptor-independent pathways. The delivery of effector proteins into host cells can target the actin cytoskeleton to reshape the plasmalemma, driving bacterial internalization and the formation of vacuoles that serve as an intracellular niche for pathogen survival. Remarkably, signalling associated with 3-phosphorylated PPIns has long been noted during entry of several enteropathogenic bacteria but the mechanism(s) underlying their formation remains elusive. It has been assumed that this is a direct consequence of altered PI3K activity but the putative kinase has not been identified. The precise nature of the PPIns generated has also remained uncertain due to the use of dual-specificity biosensors such as the pleckstrin-homology (PH) domain of AKT. New genetically encoded biosensors with high specificity for PtdIns(3,4)P2 or PtdIns(3,4,5)P3 have recently been devised. We took advantage of such improved probes, in combination with live-cell imaging, optogenetics and in vitro lipid analyses to revisit the mechanism underlying 3-PPIns generation by enteropathogens.
Our studies identified an unprecedented mechanism of 3-PPIns generation, independent of PI3Ks.

**Results**

**Formation of 3-phosphorylated PPIns during pathogen entry.** To investigate the possible formation and distribution of PtdIns(3,4)P₂ during bacterial invasion, we employed a genetically encoded biosensor based on the carboxyl-terminal PH domain of TAPP1 (refs. 19,20), which was recently modified to improve its sensitivity17 (Extended Data Fig. 1a). As a model of enteropathogenic invasion, we exposed epithelial cells to *Salmonella enterica* (serovar Typhimurium, hereafter *Salmonella*)—a prevalent world-wide threat to humans21,22.

In the absence of serum, the levels of PtdIns(3,4)P₂ in epithelial cells are low, resulting in a cytosolic distribution of the biosensor NES-EGFP–cPHx3 (hereafter cPHx3; EGFP, enhanced green fluorescent protein; Fig. 1a)17. In contrast, the addition of *Salmonella* was associated with a remarkable enrichment of cPHx3 at sites of contact between bacteria and the host PM; this was followed by the generation of extensive cPHx3-labelled membrane ruffles and the internalization of bacteria into plasmalemmal-derived vacuoles (Fig. 1a, Extended Data Fig. 1a–c and Supplementary Videos 1,2).

Quantification of cPHx3 in the PM revealed enrichment of the biosensor that persisted long after pathogen entry (Fig. 1b and Extended Data Fig. 1e). Although *Salmonella* entered cells within 10 min of addition, elevated levels of plasmalemmal cPHx3 were detected for >1 h post infection, despite the removal of extracellular bacteria. The phosphorylation of the sentinel kinase AKT, which senses 3-PPIns, tracked temporally the increase in membrane cPHx3 (Extended Data Fig. 1e,f).

To validate whether cPHx3 faithfully maps PtdIns(3,4)P₂, we analysed the infection of cells overexpressing PM-targeted monoisotopic polyphosphate 4-phosphatase type II (INPP4B-CAAX)24,25, which specifically hydrolyzes PtdIns(3,4)P₂ (Fig. 1c). The enrichment of cPHx3 within the *Salmonella*-induced ruffles was largely eliminated by coexpressed active INPP4B-CAAX but not by a mutant (C482A) lacking phosphatase activity (Fig. 1d,e). Further analyses of cPHx3 revealed that during pathogen entry, PtdIns(3,4)P₂ became highly enriched on the forming *Salmonella*-containing vacuole (Fig. 1a). Loss of vacuolar cPHx3 correlated tightly with its disengagement from the plasmalemma (Fig. 1g and Extended Data Fig. 1c). Labelling of bystander macropinocytic compartments by aPHx2 (ref. 17), evinced modest enrichment in the invasion ruffles (Fig. 1a, Extended Data Fig. 1a–c and Supplementary Videos 1,2).

**SopB promotes the acute synthesis of PtdIns(3,4)P₂.** *Salmonella* expresses a complement of virulence factors, pre-synthesized and primed for delivery into the host cytosol for invasion. The type III secretion system, a ‘molecular needle’ expressed on the bacterial surface, penetrates the host membrane to translocate the effector secretion system, a ‘molecular needle’ expressed on the bacterial surface, penetrates the host membrane to translocate the effector.

**PI3Ks are not required for SopB-driven PtdIns(3,4)P₂.** Hydrolysis of the predominant cellular phosphoinositides PtdIns(4)P and PtdIns(4,5)P₂ by a phosphatase cannot directly account for the observed PtdIns(3,4)P₂ synthesis during the acute and robust PtdIns(3,4)P₂ response in human cells.
**Fig. 1 | Rapid and sustained PtdIns(3,4)P2 synthesis during *Salmonella* entry and maturation.**

**a**, Confocal imaging of cPHx3 during invasion by red fluorescent protein (RFP)-expressing *Salmonella*. Maximum intensity projections (top) and enlargements of the region in the white box (bottom) are presented, where 0 min indicates the time of bacterial contact with the membrane. **b**, Cells that had been serum-starved for 3 h were infected with *Salmonella*. The extracellular bacteria were removed by washing and the cells were returned to serum-free medium containing gentamycin. The cPHx3 PM intensities (int_{PM}) were quantified in 59 (control), 60 (30 min), 58 (60 min), 75 (120 min) and 66 (240 min) cells from n = 3 independent experiments. ****P < 0.0001 and **P = 0.0027.

**c**, Model of PtdIns(3,4)P2 depletion by INPP4B-CAAX. **d**, INPP4B impairs recruitment of the biosensor cPHx3 to *Salmonella*-induced ruffles. The membrane cPHx3 intensity in invasion ruffles was analysed 10 min post infection of cells co-transfected with TagBFP2-CAAX (Control-CAAX, 106 ruffles), TagBFP2-INPP4B^{WT}-CAAX (112 ruffles) or the catalytically inactive TagBFP2-INPP4B^{C842A}-CAAX (113 ruffles); n = 3 independent experiments. **d**, Maximum intensity projections (top) and the corresponding confocal sections of the invasion ruffle (bottom) are presented. CellMask identifies the PM. TagBFP2 fluorescence is not presented. **e**, Quantification of PM cPHx3 intensities in the ruffles (int_{Ruffle}) from **d**. ***P = 0.0006. **b**, **e**, Data are the trial mean ± s.e.m. (blue, foreground) overlaid on individual cell measurements (grey, background). Dotted horizontal grey lines indicate the level where the cytosolic and PM intensities are identical. NS, not significant. **f**, Airyscan microscopy of cPHx3 within the invasion ruffle induced by *Salmonella* (10 min post infection). Note the continuous cPHx3 (inverted grey) labelling that coincides with a CellMask (right)-stained membrane compartment. **g**, Confocal time-lapse images of cPHx3 during bacterial entry and constriction of the invaginating membrane. Images are maximum intensity projections of a 2-μm optical slice. Bacterial fluorescence is not presented. **h**, PtdIns(3,4,5)P3 during invasion. HeLa cells expressing aPHx2 were imaged live during invasion by *Salmonella*. Confocal sections are presented where 0 min marks the first indication of membrane ruffling induced by the bacteria. Source numerical data are available.
Fig. 2 | SopB is necessary and sufficient for PtdIns(3,4)P₂ biosynthesis in mammalian cells. a, Cells expressing cPHx3 were infected with wild-type or ΔsopB Salmonella expressing RFP for 10 min, followed by PM staining with CellMask. Maximum intensity projections (top) and the corresponding confocal sections of the invasion ruffle (bottom) are presented. b, Intensity of membrane cPHx3 in invasion ruffles from n = 3 independent experiments (wild-type, 113 ruffles; ΔsopB, 87 ruffles). Data are the trial mean ± s.e.m. (blue, foreground) overlaid on cell measurements (grey, background). The dotted horizontal grey line indicates the level where the ruffle and cytosolic intensities are identical. ****P < 0.0001. c, Heterologous expression of SopB generates PtdIns(3,4)P₂. Representative xy and z confocal sections of HeLa cells expressing mCherry-tagged cPHx3 and co-transfected as indicated before live imaging; n = 122 (control, EGFP-transfected), 210 (SopBWT–EGFP) and 107 (SopBC460S–EGFP) cells imaged from five, five and three independent experiments, respectively. d, Model of HCK incorporation at SopB residue 464 and photolysis. Cells are transfected with SopB464TAG and plasmids that encode an Amber stop codon (UAG)-recognizing transfer RNA and a tRNA synthase that incorporates HCK during translation. Illumination by 405-nm light photolyzes the HCK to yield wild-type (active) SopB. e, Photoactivation of SopB induces acute PtdIns(3,4)P₂ formation. Representative confocal time-lapse images of cells expressing cPHx3 before (t − 30 s) and after (t + 90 s, t + 210 s) photoactivation of SopBWT-464TAG and SopBC460S-464TAG. Insets: inverted grayscale images of cPHx3 from the region marked by the box in the left-most frame. f, g, Changes in the cPHx3 (left ordinate axis) and aPHx2 (right ordinate axis) intensities of the PM following photoactivation of SopBWT-464TAG and SopBC460S-464TAG. Data are the baseline-corrected mean ± s.e.m. of n = 29 cells from three independent experiments. f, g, The samples were exposed to 405-nm illumination at the time points indicated by the blue-shaded background. Dotted horizontal grey lines indicate the initial baseline-corrected intensity. WT, wild type; inF₉₉₉, PM intensity. Source numerical data are available.
like Ras, PtdIns(4,5)P₂ is phosphorylated to PtdIns(3,4,5)P₃ by class I PI3Ks. PtdIns(3,4,5)P₃ can be rapidly converted to PtdIns(3,4)P₂ by subsequent dephosphorylation by 5-phosphatases[4] (Fig. 3a). Consistently, the constitutive recruitment of a class I PI3K to the PM (p110α-CAAX) triggered a parallel increase in PtdIns(3,4)P₂ levels (Fig. 3b).

We employed several potent pan-PI3K inhibitors to probe the role of this pathway: wortmannin, which irreversibly modifies the active site of multiple PI3Ks[5]; PI-103, a multi-target PI3K and mTOR inhibitor[6]; and Pertilisib (GDC-0941), which exhibits selectivity towards class I PI3Ks[7]. Addition of nanomolar concentrations of these compounds to cells expressing p110α-CAAX led to the complete and rapid (<3 min) release of cPHX3 from the PM (Fig. 3b and Extended Data Fig. 4d) and suppressed phosphorylation of AKT (Fig. 3c and Extended Data Fig. 4e), validating their potency towards PI3Ks. Remarkably, despite their obvious efficacy towards PI3Ks, pre-incubation of any of these inhibitors failed to reduce PtdIns(3,4)P₂ biogenesis at the sites of Salmonella invasion (Fig. 3d,e) or block the synthesis of PM PtdIns(3,4)P₂ following photoactivation of SopB (Fig. 3f,g).

As an alternative to SopB stimulation a class I PI3K, we tested the role of class II PI3Ks, which can directly phosphorylate PtdIns(4)P to generate PtdIns(3,4)P₂ (Fig. 3h). It is noteworthy that of the class II isoforms, only PI3K-C2α is (somewhat) refractory to classical PI3K inhibitors[8,9], and may have resisted inhibition in our pharmacological screen. To test the role of PI3K-C2α, we independently targeting short interfering RNA (siRNA) sequences against PIK3C2α (encoding PI3K-C2α) were introduced into the cells in conjunction with the biosensor cPHX3. Despite 85–95% depletion of the PI3K-C2α, as determined by immunoblotting (Fig. 3i,j), the robust recruitment of cPHX3 to Salmonella-induced ruffles persisted (Fig. 3k,l). Moreover, endogenous PI3K-C2α showed no enrichment at the invasion ruffles (Extended Data Fig. 4g). Together, these observations raised the possibility that conventional biosynthetic pathways are not responsible for the PtdIns(3,4)P₂ synthesis induced by SopB.

IpgD generates PtdIns(3,4)P₂ independently of PI3Ks. Based on sequence homology (Extended Data Fig. 6a), we hypothesized that SopB and IpgD (a Shigella flexneri effector) share analogous enzymatic activities. We monitored the localization of cPHX3 when coexpressed with IpgD. Expression of wild-type IpgD—but not IpgD with Cys 439 mutated to Ser—indeed led to a robust re-localization of cPHX3 to the PM (Extended Data Fig. 6d). As in the case of SopB, IpgD-mediated generation of PtdIns(3,4)P₂ was unaffected by PI3K inhibition by either PI-103 or wortmannin (Extended Data Fig. 6d,e). Thus, S. flexneri IpgD and Salmonella SopB are likely to share their mode of action.

SopB and IpgD activity in S. cerevisiae devoid of PI3Ks. The inhibitor studies and silencing of PI3K-C2α suggested that neither class I nor class II PI3Ks are required for SopB- or IpgD-mediated PtdIns(3,4)P₂ generation. To assess this conclusion more definitively, we examined the effect of these effectors in Saccharomyces cerevisiae, an organism lacking class I and class II PI3Ks[10,11]. In otherwise untreated cells, the cPHX3 probe was entirely cytosolic (Fig. 4a, left), consistent with the inability of yeast to intrinsically synthesize PtdIns(3,4)P₂. SopB was expressed acutely under the control of a galactose-inducible promoter because its prolonged expression is deleterious to yeast[12]. Remarkably, induction of SopB in the yeast yielded a robust translocation of cPHX3 to the PM (Fig. 4a). Importantly, SopB Cys 460 was strictly required for this translocation (Fig. 4aa, b). Like SopB, IpgD caused robust translocation in S. cerevisiae, an effect that required Cyst 439 of the S. flexneri effector (Extended Data Fig. 6f).

Despite lacking class I and II PI3Ks, it was conceivable that the sole PI3K expressed in S. cerevisiae—class III Vps34—might be involved in the SopB- or IpgD-induced generation of PtdIns(3,4)P₂. Deletion of VPS34 (encoding Vps34p) and the resultant loss of PtdIns(3)P severely compromise endo-vacuolar protein sorting and growth of the yeast[13,14]. We therefore turned to a temperature-sensitive mutant (vps34Δ) that exhibits acute loss of PtdIns(3)P only at non-permissive temperatures, which we confirmed using a tandem FYVE domain probe[15] recognizing PtdIns(3)P (Fig. 4c). SopB expression was induced 90 min after transferring the vps34Δ strain to a non-permissive temperature before monitoring cPHX3 localization. Despite the loss of Vps34p activity and cellular PtdIns(3)P expression of wild-type SopB (but not the C460S mutant) induced translocation of cPHX3 to the PM (Fig. 4d,e).

SopB requires PtdIns(4,5)P₂ to generate PtdIns(3,4)P₂. Jointly, the preceding results seem to rule out the involvement of known host PI3Ks in the generation of PtdIns(3,4)P₂ induced by SopB and

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**Fig. 3 | Class I PI3Ks and class II PI3K-C2α are not required for PtdIns(3,4,5)P₃ synthesis during Salmonella entry.** a. Class I PI3K-mediated indirect PtdIns(3,4,5)P₃ synthesis. Cells were co-transfected with cPHX3 and p110α-CAAX (main images) or vector control (inset). Representative micrographs of cells from n=3 independent experiments before (left) and after (right) treatment with 500nM PI-103. B. Control and p110α-CAAX-expressing cells were treated with dimethylsulfoxide (DMSO; vehicle), wortmannin (100 nM), PI-103 (500 nM) or GDC-0941 (500 nM) for 20 min, followed by immunoblotsing for pan AKT and AKT phosphorylated at Ser 473 (pAKT). The pAKT/AKT densitometry is presented normalized to p110α. C. cPHX3 was pre-treated as in b before Salmonella exposure (10 min). Representative maximum intensity projections (top) with confocal sections of the invasion ruffle (bottom) are presented for cells treated with DMSO or wortmannin. D. Normalized cPHX3 intensity levels in the invasion ruffles from d following pre-treatment with PI3K inhibitors (20 min) analyzing 81 (DMSO), 80 (wortmannin), 76 (PI-103), 85 (GDC-0941) and 58 (DMSO (ΔsopB)) ruffles. E. Photoactivation of SopBTAG-466TAG in untreated and GDC-0941 (250 nM, 30 min)-treated cells. Data are filtered baseline-corrected mean ± s.e.m. of the cPHX3 intensity from n=69 (control; ten experiments, 40 cells pooled from Fig. 2f) and 39 (GDC-0941; six experiments) cells. The samples were exposed to 405-nm illumination at the time points indicated by the blue-shaded background. F. Area under the curve (AUC) values of the cPHX3 intensities from d. G. Data are the median (horizontal line), 25th-75th percentiles (boxes) and 10th-90th percentiles (whiskers) of n=69 control (ten experiments, 40 cells pooled from Fig. 2f) and 39 GDC-0941 (six experiments) cells. The dotted horizontal grey line indicates the initial baseline-corrected intensity. H. P=0.00075. I. PI3K-C2α-mediated PtdIns(3,4)P₂ synthesis and half maximal inhibitory concentration (IC₅₀) values. J. Cells were treated with the indicated siRNAs and their lysates were immunoblotted against PI3K-C2α and VPS34. K. Vps34p serves as a loading control. L. Densitometric estimation of the level of PI3K-C2α (normalized to vinculin) remaining after the RNA interference treatment. M. Data are the mean ± s.e.m. of n=3 independent experiments. N. Representative maximum intensity projections (top) and invasion ruffle sections (bottom) of siRNA-treated cells transfected with cPHX3 and infected with Salmonella for 10 min. Quantification of the cPHX3 intensity in the invasion ruffles from k. P=0.6826 (control vs PIK3CA 1) and P>0.9999 (control versus PIK3CA 2) analyzing 117 (control), 105 (PIK3CA 1) and 107 (PIK3CA 2) ruffles. E. Data are the mean ± s.e.m. (blue, foreground) overlaid on cell measurements (grey, background) from n=3 independent experiments. Dotted horizontal grey lines indicate the level where the ruffle and cytosolic intensities are identical. **P<0.001; ****P<0.0001; NS, not significant. WT, wild type; infmax, PM intensity; infruffle, ruffle intensity; a.u., arbitrary units. Source numerical data and unprocessed blots are available.
IpgD; a distinct biosynthetic pathway must therefore be invoked. In vitro, SopB can function as a rather promiscuous PPIns and inositol polyphosphate phosphatase, and causes the disappearance of PtdIns(4,5)P₂ from the base of invasion ruffles in vivo. These effects depend on Cys 460. It is noteworthy that the same residue is also essential for the formation of PtdIns(3,4)P₂ by SopB (Figs. 2 and 4). On this basis we hypothesized that rather than activating host kinases, SopB directly causes phosphorylation of the 3-position of the inositol ring through rearrangement of the phosphate groups of pre-existing cellular lipids.
To test this hypothesis, we analysed the fate of PPIns regio-isomers during SopB-induced generation of PtdIns(3,4)P₂. To monitor PtdIns(4)P, we transfected human cells with a biosensor based on tandem P4M domains from the *Legionella* effector SidM₅₆. PtdIns(4)P was abundant in the resting PM as well as *Salmonella*-induced ruffles and could hence serve as a substrate for the formation of PtdIns(3,4)P₂ (Fig. 5a). To test the possible involvement of PtdIns(4)P in the formation of PtdIns(3,4)P₂, we simultaneously monitored the distribution of the 2xP4M and cPHₓ₃ sensors during optogenetic activation of SopB. Plasmalemmal PtdIns(4)P was largely unperturbed under these conditions, despite robust PtdIns(3,4)P₂ generation (Fig. 5b,c). The minute decrease in PtdIns(4)P observed following SopB photo-uncaging was also observed when using C₄₆₀S SopB, implying that it was unrelated to the generation of PtdIns(3,4)P₂ (Fig. 5d,e). Thus, we found no evidence that PtdIns(4)P was directly involved in the process.

We turned our attention to the other major PPIns species of the PM, namely PtdIns(3,4,5)P₃, using the PH domain of human PLCδ₁ (refs. 57,58). Photoactivation of SopB caused a sharp decrease in plasmalemmal PtdIns(3,4,5)P₃ that coincided temporally with the appearance of PtdIns(3,4)P₂ (Fig. 5f). Like the accompanying synthesis of PtdIns(3,4)P₂, the decline in PtdIns(3,4,5)P₃ was dependent on the integrity of the catalytic site of SopB (Fig. 5g,h). Similar results were obtained with IpgD (Extended Data Fig. 6b,c). These observations suggest that PtdIns(3,4,5)P₃ plays a crucial role in the generation of PtdIns(3,4)P₂ during SopB-mediated signaling.

**Fig. 4 | PtdIns(3,4)P₂ generation in *S. cerevisiae* that are devoid of PI3K activity.** a, PtdIns(3,4)P₂ synthesis in the PM of *S. cerevisiae*. GAL1 promoter-driven (schematic, top) empty vector (control; left), SopBWT (middle), and SopBC₄₆₀S (right) were induced by growth in galactose for 2 h in yeast expressing cPHₓ₃. Concanavalin A (ConA) and trypsin blue staining demarcate both the cell wall and non-viable yeast. b, Yeast from a were scored for plasmalemmal cPHₓ₃ localization analyzing 929 (control and SopBWT) and 1,116 (SopBC₄₆₀S) yeast cells. c, PtdIns(3)P loss in *vps34* mutant *S. cerevisiae*. Cells expressing EGFP–2xFYVE were maintained at 28–30 °C (permissive) during growth or transferred, where indicated (schematic; bottom), to 38 °C (non-permissive) for 90 min before switching the carbon source and continued growth at 38 °C. d, PtdIns(3,4)P₂ synthesis by SopB persisted despite a loss of Vps34p activity. Yeast expressing galactose-inducible plasmids were moved to a non-permissive temperature as in c before imaging. Non-viable cells were excluded using trypan blue (not shown). e, Scoring of cPHₓ₃ translocation to the PM from the experiments in d. Control, 55 (wild-type) and 510 (*vps34*”) cells; SopBWT, 597 (WT) and 475 (*vps34*”) cells; and SopBC₄₆₀S, 518 (WT) and 331 (*vps34*”) cells analyzed. b, e, Data are the mean ± s.e.m. from n = 3 independent experiments. Dotted horizontal grey lines indicate y = 0 (%). ****P < 0.0001. WT, wild-type. Source numerical data are available.
**Fig. 5 | PtdIns(4,5)P2 levels correlate inversely with SopB-mediated PtdIns(3,4)P2 formation.**

a. PtdIns(4)P during bacterial invasion. Representative time-lapse images of a HeLa cell expressing 2xP4M during wild-type *Salmonella* invasion (top; 0 min indicates the time of bacterial contact with the membrane). Magnified views of the region denoted by the white box are shown (bottom). Inset: linear RGB intensity scale from 0 (black) to 255 (white). PtdIns(4)P remains abundant within ruffles but is rapidly cleared from the forming vacuole. b. PtdIns(4)P in the PM is stable during optogenetic activation of SopB. Representative micrographs of cPHx3 and 2xP4M before and after photoactivation of SopBWT-464TAG. Left bars, corresponding linear RGB intensity scales from 0 (black) to 255 (white). Illumination of the sample with 405-nm light began at \( t = 30 \) s. Insets: magnified views of the region denoted by the white box in frame 1 (bottom). c. Changes in the 2xP4M (left ordinate axis) and cPHx3 (right ordinate axis) intensities of the PM following photoactivation of SopBWT-464TAG; \( n = 21 \) cells from three independent experiments. d. Comparison of the 2xP4M responses (change in intensity) to the photoactivation of SopBWT-464TAG and SopBC460S-464TAG; \( n = 21 \) (SopBWT-464TAG) and 27 (SopBC460S-464TAG) cells from three independent experiments. e. Calculated AUC of the 2xP4M intensities from d. \( P = 0.064; n = 21 \) (SopBWT-464TAG) and 27 (SopBC460S-464TAG) cells from three independent experiments. f. PtdIns(4,5)P2 depletion during PtdIns(3,4)P2 synthesis. Changes in the PH-PLCδ1 (left ordinate axis) and cPHx3 (right ordinate axis) intensities of the PM before and after photoactivation of SopBWT-464TAG. g. Data from f comparing the response of PH-PLCδ1 (change in intensity) to photoactivation of SopBWT-464TAG and SopBC460S-464TAG. h. Data from g. Data are the median (horizontal line), 25th–75th percentiles (boxes) and 10th–90th percentiles (whiskers). Dotted horizontal grey lines indicate \( y = 0 \). i. Calculated AUC of the PH-PLCδ1 intensities from g. \( P = 0.0297; n = 40 \) (SopBWT-464TAG) and 10 (SopBC460S-464TAG) cells from seven independent trials (unfiltered cPHx3 data presented in Fig. 2f). Source numerical data are available.
tions suggested that the decline in PtdIns(4,5)P₂, previously noted to occur at the base of invasion ruffles⁵⁵, might be required for the generation of PtdIns(3,4)P₂. To probe its requirement as a precursor for SopB-mediated PtdIns(3,4)P₂ biosynthesis, we depleted PtdIns(4,5)P₂ by recruiting PLC₃ to the PM using a heterodimerization system⁵⁹,⁶⁰. In this context PLCs are particularly useful due to their low activity towards PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂⁶¹, while extensively depleting cellular PtdIns(4,5)P₂ when recruited to the PM by a Lyn₁₁⁻tagged FRB domain (Fig. 6a,b). Critically, the pre-recruitment of FKBP-tagged PLC₃ to the PM led to a virtually complete inhibition of PtdIns(3,4)P₂ formation in Salmonella-induced ruffles, as monitored by cPHx₃ (Fig. 6c,d). Similarly, rapamycin-induced recruitment of the 5-phosphatase INPP5E—but not its catalytically inactive mutant—led to a marked reduction in PtdIns(3,4)P₂ generation at the invasion ruffles (Extended Data Fig. 7a,b). That INPP5E

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**Fig. 6 | SopB requires a PLC-sensitive inositide to generate PtdIns(3,4)P₂.**

**a**, Chemically induced recruitment of PLC₃ to deplete PtdIns(4,5)P₂ from the PM. HeLa cells expressing mRFP–FKBP (control) or mRFP–FKBP-PLC₃ together with PM-targeted Lyn₁₁⁻FRB and the biosensor PH-PLCδ₁ (inverted grey scale) were treated with 1μM rapamycin before live imaging. Insets: re-localization of FKBP conjugates to the PM induced by rapamycin. **b**, Plasmalemmal PH-PLCδ₁ intensity from the experiments in a. ***P=0.0005; 55 (control) and 56 (PLC₃) cells analyzed.**

**c**, Inhibition of PtdIns(3,4)P₂ generated during invasion by pre-recruitment of PLC₃. Cells transfected as in a but expressing cPHx₃ (main images; maximum intensity projections) were incubated with 1μM rapamycin for 2 min before the addition of wild-type blue fluorescent protein (BFP)-expressing Salmonella for an additional 10 min. The PM was stained with CellMask (inset; confocal sections of invasion ruffle) before fixation and imaging. **d**, Resulting PM cPHx₃ intensities in the invasion ruffles from c. ****P<0.0001; 115 (control) and 76 (PLC₃) cells analyzed.**

**b, d**, Data are the mean±s.e.m. (red or blue, foreground) of n=3 independent experiments overlaid on individual cell measurements (grey, background). Dotted horizontal grey lines indicate the level where cytosolic and PM intensities are identical. **e, f**, Activation of endogenous PLC₃ precludes SopB-mediated PtdIns(3,4)P₂ synthesis. HeLa cells overexpressing SopBWT-464TAG and muscarinic M3 receptor were subjected to treatment with medium control (e) or 50μM carbachol (f) at 30 s to activate endogenous PLC₃, followed by 405-nm light illumination at 270 s (blue-shaded region) to optogenetically activate SopB. The decrease in PtdIns(4,5)P₂ and response in PtdIns(3,4)P₂ were monitored with PH-PLCδ₁ and cPHx₃, respectively. Data are the mean±s.e.m. of the baseline-corrected measurements of individual cells; n=33 (medium control) and 41 (carbachol) cells from three independent experiments. Dotted horizontal grey lines indicate the initial baseline-corrected intensity. Note that a slight decrease in PM cPHx₃ occurs following carbachol treatment, probably due to the inhibition of class I PI3K signalling constitutively stimulated by the presence of serum. Rapa, rapamycin. Source numerical data are available.
Fig. 7 | In vitro reconstitution reveals SopB phosphotransferase activity. a. Inorganic phosphate release following treatment (30 min) of liposomes of the indicated compositions with recombinant SopB<sup>WT</sup>. Data are the mean ± s.e.m. of duplicate wells from a representative experiment. b. Confocal micrographs of GUVs treated with SopB<sup>WT</sup> or an equal volume of dialysis buffer (control) for 30 min. The liposome composition was PtdCho:PtdSer:PtdIns(4,5)P<sub>2</sub>:PtdEth-Rhodamine B:DSPE-PEG-biotin (76.8:20:3:0.1:0.1 molar percentage (mol%)). Recombinant EGFP–PH-PLCδ<sub>1</sub> (1 µM) was added before microscopy. c. Normalized EGFP–PH-PLCδ<sub>1</sub> liposome intensity from b. *P = 0.0269; n = 142 (control) and 155 (SopB<sup>WT</sup>) GUVs from four independent experiments. d. Normalized EGFP–PH-PLCδ<sub>1</sub> liposome intensity. ***P = 0.0006; 86 (0% PtdIns), 84 (3% PtdIns(4,5)P<sub>2</sub>) and 83 (3% PtdIns(3,4)P<sub>2</sub>) GUVs analyzed from n = 3 independent experiments. e. Representative micrographs of recombinant EGFP–cPHx1 (0.5 µM) incubated with increasing mol% PtdIns(3,4)P<sub>2</sub> (top). The background composition of liposomes was PtdCho:PtdIns(4,5)P<sub>2</sub>:PtdIns(4)P:PtdIns(3,4)P<sub>2</sub>:PE-Rhodamine B:DSPE-PEG-biotin (77-X:1.5:1.5:X:0.1:0.1). The corresponding normalized intensity profiles of EGFP and Rhodamine B channels are plotted (bottom). f. Normalized EGFP–cPHx1 liposome intensity from e. Inset: liposome measurements (background points) and paired trial averages (foreground points) from 0% and 0.1% PtdIns(3,4)P<sub>2</sub>-containing GUVs (red box); 53 (0%), 64 (0.1%), 80 (0.5%), 68 (1.0%) and 78 (3.0%) GUVs analyzed from n = 3 independent experiments. g. Confocal analysis of GUVs treated with dialysis buffer (control), SopB<sup>WT</sup> or SopB<sup>WTTM</sup> (enzyme pre-treated with a molar excess of N-ethylmaleimide (NEM)) for 30 min (top). Representative EGFP–cPHx1 localization with normalized intensity profiles (bottom). The GUV compositions (mol%) were PtdCho:PtdIns(4,5)P<sub>2</sub> or PtdIns(4)P:PtdEth-Rhodamine B:DSPE-PEG-biotin (76.8:20:3:0.1:0.1). h. Normalized EGFP–cPHx1 liposome intensity from g. Substrate: PtdIns(4,5)P<sub>2</sub>: PtdIns(4)P. The numbers on the images are the liposome/medium EGFP intensity for the representative liposome. b, e, g, h, The numbers on the images are the liposome/medium EGFP intensity for the representative liposome. b. Normalized EGFP–cPHx1 liposome intensity from g. PtdIns(4,5)P<sub>2</sub> substrate: 130 GUVs from n = 4 independent experiments. c.d.f.h. Data in the box-and-whisker plots are the median (horizontal line), 25th–75th percentiles (boxes) and 10th–90th percentiles (whiskers) of individual liposome measurements. Dotted horizontal grey lines indicate the level where the liposome and medium intensities are identical. ****P < 0.0001. Source numerical data are available.
preserves PM PtdIns(4)P (Extended Data Fig. 7c–f) and does not generate additional second messengers (that is, InsP₃) provides an argument for a direct role of PtdIns(4,5)P₂ in the generation of PtdIns(3,4)P₂ by SopB.

We also took advantage of endogenous PLCβ enzymes that were activated by the addition of carbachol to cells overexpressing the M3 muscarinic receptor. Thus, carbachol-mediated depletion of cellular PtdIns(4,5)P₂ could be timed with the optogenetic activation of SopB (Fig. 6f). Treatment of carbachol strongly depleted PtdIns(4,5)P₂, as indicated by the detachment of plasma-membrane PH-PLCδ₁ (Fig. 7j), and blocked the subsequent generation of PtdIns(3,4)P₂ in response to phototoactivated SopB. PtdIns(3,4)P₂ was formed normally in these cells when carbachol pre-treatment was omitted (Fig. 7e). These results suggest that SopB consumes PtdIns(4,5)P₂ as it generates PtdIns(3,4)P₂. However, they do not rule out a requirement for additional lipids or host factors.

**SopB functions as a phosphotransferase in vitro.** We next considered the possibility that SopB could generate PtdIns(3,4)P₂ directly through remodelling of pre-existing PPIPs, probably PtdIns(4,5)P₂. To this end, we purified a recombinant construct consisting of residues 33–554 of SopB that retains the putative catalytic site and has catalytic activity in vivo (Extended Data Fig. 3d). The phosphatase activity of this recombinant protein was initially validated by measuring the release of inorganic phosphate from large unilamellar vesicles (LUVs) containing PtdIns(4,5)P₂ and PtdSer; no phosphate was released from vesicles containing PtdSer only (Fig. 7a), confirming the specificity of the recombinant enzyme towards PPIPs.

The activity of the recombinant SopB was also validated using giant unilamellar vesicles (GUVs), which are amenable to analysis by confocal microscopy using fluorescent biosensors. The GUVs were immobilized onto streptavidin-coated coverslips by incorporating biotinylated PtdEth (DSPE-PEG-biotin) and detected by immobilized recombinant EGFP–PH-PLCδ₁. This effect was observed when using SopBWT (Fig. 7g,h) but not with SopBC460S (Fig. 7h). An example HPLC–MS trace is presented for each derived from the (SopB)₅₀ reaction. –

**Time-resolved HPLC–MS analysis of SopB phosphotransferase-phosphatase activities.** To assess the role of SopB in the generation of PtdIns(3,4)P₂, we purified recombinant EGFP–cPHx₁ to probe the formation of PtdIns(3,4)P₂. EGFP–cPHx₁ bound to GUVs in a manner proportional to the concentration of PtdIns(3,4)P₂; no binding was detected in the absence of PtdIns(3,4)P₂, even when other PPIPs were present (Fig. 7c,f). The lower limit of detection of PtdIns(3,4)P₂ with this recombinant sensor was 0.1 mol% (Fig. 7f, inset). Using this system, we tested whether SopB was capable of generating PtdIns(3,4)P₂ in a recombinant system of defined composition. Liposomes containing PtdCho:PtdSer:Rhodamine:DSPE-PEG-biotin (76.8:20:0.1:0.1) plus 3% of the PPIPs of interest were incubated with SopB. EGFP–cPHx₁ was added immediately before imaging to probe for the formation of PtdIns(3,4)P₂. As shown in Fig. 7g,h, the addition of recombinant SopB to PtdIns(4,5)P₂-containing liposomes caused translocation of EGFP–cPHx₁ to the liposome surface. This effect was dependent on SopB activity, as it was abolished by pre-treating SopB with N-ethylmaleimide to modify Cys residues, including Cys₄₆⁰. The ability of SopB to produce PtdIns(3,4)P₂ in this assay required PtdIns(4,5)P₂; pre-incubation of SopB with liposomes containing an equimolar amount of PtdIns(4)P did not result in the recruitment of EGFP–cPHx₁ (Fig. 7g,h).

To gain quantitative insight into the flux of PPIPs species under the influence of SopB, we analysed enzyme-treated LUVs using high-performance liquid chromatography with mass spectrometry (HPLC–MS)⁶₄,⁶₅. Liposomes containing PtdCho:PtdSer:PtdIns(4,5)P₂ (75:20:5) were incubated with wild-type or mutant enzyme. Treatment of liposomes with SopB⁶₄,⁶₅ (grey, Fig. 8), which caused no detectable phosphate release (Extended Data Fig. 8a), served as negative control in these assays, as did LUVs incubated without enzyme (dotted lines). As expected, incubation of LUVs with SopB⁶₄,⁶₅ decreased PtdIns(4,5)P₂ in a time-dependent manner (Fig. 8a).

That SopB functions as a phosphatase capable of dephosphorylating the inositol ring at both the 4- and 5-positions was confirmed by the progressive appearance of PtdIns(4)P/PtdIns(5)P (not differentiated by HPLC–MS; Fig. 8d), whereas the remainder (Fig. 8e), followed by PtdIns (Fig. 8e). Importantly, the HPLC–MS analyses also revealed that SopB⁶₄,⁶₅ acutely generates 3-phosphorylated PPIPs including PtdIns(3,4,5)P₃ (Fig. 8b), PtdIns(3,4)P₂ (Fig. 8b,h), PtdIns(3,4,5)P₃ (Fig. 8g and Extended Data Fig. 8b) and PtdIns(3,4,5)P₃ (Fig. 8c). Gradual accumulation of PtdIns(3,4,5)P₃ was observed when using a low (14 nM) concentration of SopB (Fig. 8b), whereas only a progressive decrease after rapid generation was seen with higher concentrations of SopB⁶₄,⁶₅ (72 nM; Fig. 8g). The peak corresponding to PtdIns(3,4,5)P₃ was detected when using SopB⁶₄,⁶₅ (Fig. 8g and Extended Data Fig. 8b) but not with SopB⁴⁶⁰S (Fig. 8h). This peak could not be accurately quantified due to its closeness to two other larger peaks.

It is noteworthy that the cell-free systems we used were devoid of high-energy phosphates (for example, ATP) as well as the divalent cations.
cations generally required by kinases. Thus, we concluded that, in addition to functioning as a polyphosphoinositide phosphatase in host cells, SopB possesses phosphotransferase activity, relocating the phosphate groups of PtdIns(4,5)P$_2$ to yield 3-phosphorylated PPIns such as PtdIns(3,4)P$_2$ (Fig. 8i). Whether this enzyme possesses an intramolecular transferase (that is, a phosphoisomerase) activity in addition to its intermolecular transferase function (Fig. 8i) remains unclear.
Discussion
Several Enterobacteriaceae species diverged from harmless symbionts to parasites. A key evolutionary driver of this feat was the acquisition of virulence factors that facilitate cellular adhesion, invasion and manipulation of host signalling \cite{22,26}. The activation of the host pro-survival kinase AKT by Salmonella and Shigella effectors was described more than 20 years ago \cite{13,14,15,16}. By regulating the survival and proliferation of the infected host cells, AKT counters the pro-apoptotic nature of co-secreted effectors, thereby supporting intracellular bacterial growth \cite{4,46,47,49,50}. In this context, AKT also modulates the inflammatory response \cite{50,51,52}, drives the expansion of M cells \cite{53} and clinically promotes infection-associated carcinoma \cite{54}. Although AKT was known to be activated by PtdIns(3,4,5)P$_3$ and PtdIns(3,4,5)P$_4$, how accumulation of the responsible lipid is induced by the enteropathogens had not been delineated. A previous unbiased screen identified 52 kinases that, when silenced, partially reduced AKT phosphorylation during Salmonella invasion \cite{55}. How these kinases impinge on AKT regulation is unclear and may reflect indirect pleiotropic effects.

Our data indicate that SopB and IpgD are sufficient to acutely stimulate the synthesis of 3-phosphorylated PPIPs in host cells, particularly PtdIns(3,4)P$_2$. They accomplish this without the need to harness any of the previously recognized 3-PPIPs biosynthetic pathways, namely class I, class II or class III PI3Ks. Rather, the lipid arises by an effector-catalysed phosphotransferase reaction.

Several lines of evidence indicate that PtdIns(4,5)P$_2$ is the sole substrate required for the generation of PtdIns(3,4)P$_2$. First, PtdIns(3,4)P$_2$ production during infection is restricted to the PM (Fig. 1), the main PtdIns(4,5)P$_2$ reservoir in host cells. Second, the invariable Cys residue (Cys460 in SopB and Cys439 in IpgD) that is essential for the effector-induced disappearance of PtdIns(4,5)P$_2$ is also essential for PtdIns(3,4)P$_2$ production (Figs. 2, 5 and Extended Data Fig. 6). Third, depletion of PtdIns(4,5)P$_2$ by PLCβ or INPP5E obliterated the generation of PtdIns(3,4)P$_2$ by SopB (Fig. 7 and Extended Data Fig. 7). Fourth, and most important, the presence of PtdIns(4,5)P$_2$—but not of other phospholipids, including other PPIPs—was absolutely required for SopB to generate PtdIns(3,4)P$_2$ in a cell-free system (Figs. 7 and 8).

SopB had been reported earlier to function as a PPIPs phosphatase \cite{44,47}, an observation we confirmed (Fig. 7a and Extended Data Fig. 8a). This activity involves Cys460, which resides in a Cys-X$_5$-Arg motif—similar motifs are prevalent throughout the protein tyrosine phosphatase superfamily \cite{56}. It is noteworthy that Cys460 is also essential for the generation of PtdIns(3,4)P$_2$ from PtdIns(4,5)P$_2$; we therefore propose that a common intermediate step underlies the phosphatase and phosphotransferase activities of SopB (and presumably IpgD). In the case of simple phosphatases, Cys-mediated nucleophilic attack on a scissile phosphate generates a cysteinyl-phosphate intermediate that is normally hydrolyzed by water to complete the phosphatase cycle \cite{57}. We envisage three possible catalytic mechanisms to account for the observed phosphotransferase reaction (Fig. 8i): Cys460 could act as a nucleophile, attacking one of the phosphates (for example D-5) on PtdIns(4,5)P$_2$, breaking the bond between the phosphate and inositol ring and forming a high-energy phospho-Cys intermediate. Rather than abstracting a proton from water, as would occur during an ordinary phosphatase reaction, proton abstraction would occur from the 3-position of the inositol ring. The neighbouring Asp465 of SopB could conceivably donate a proton to the leaving group (D-5) of the inositol ring. Thus, the D-3 hydroxyl group nucleophile would be prompted to attack the phospho-Cys intermediate, generating PtdIns(3,4)P$_2$ and regenerating the free Cys. By catalysing such an intramolecular rearrangement, SopB would operate as a phosphoisomerase (Fig. 8i, reaction (iii)).

Transfer of a phosphate from one inositol to another (that is, intermolecular) by an analogous mechanism is also plausible (Fig. 8i, reactions (i) and (iii)) and could operate in combination with the phosphatase activity of SopB. By this putative mechanism, the phospho-Cys intermediate would attach a second phosphate onto position D-3 of PtdIns(4)P$_2$ generated previously by dephosphorylation of PtdIns(4,5)P$_2$. This would require sequential phosphatase and phosphotransferase reactions. Last, the reverse sequence can also be contemplated (Fig. 8i, reaction (ii)): the phospho-Cys intermediate could generate PtdIns(3,4,5)P$_3$ by inserting an additional phosphate into PtdIns(4,5)P$_2$. That PtdIns(3,4,5)P$_3$ was readily detected by our HPLC–MS analyses in vitro (Fig. 8b,f) and previously observed in HPLC analyses of infected cells \cite{58} argues in favour of intersubstrate phosphate transfer. This mechanism would also be consistent with the generation of PtdIns(3,5)P$_2$ previously detected in host cells \cite{59}. However, we hypothesize that PtdIns(3,4,5)P$_3$ is a fleeting intermediate in the SopB-induced biogenesis of PtdIns(3,4)P$_2$ during infection. The multiplicity of active host 5-phosphatases makes it probable that PtdIns(3,4,5)P$_3$, generated by SopB would be rapidly converted to PtdIns(3,4)P$_2$ (Extended Data Fig. 8e). This may account for the minute amount of PtdIns(3,4,5)P$_3$ detected (Figs. 1, 2 and Extended Data Fig. 1,2,8c,d) and would also explain why biochemical measurements of infected cells revealed PtdIns(3,4)P$_2$ to be much more abundant (approximately sevenfold) than PtdIns(3,4,5)P$_3$ \cite{59,60}.

Notably, deletion of residues 520–554 of SopB completely abrogated its phosphotransferase activity (Extended Data Fig. 3d). Multiple cationic residues encoded within this region could conceivably form an electrostatic counterpart to stabilize the negatively charged inositol head group during phosphate transfer. Mutation of two of these sites, Lys525 and Lys528, decreased AKT activation in response to SopB \cite{61} and suppressed the phosphotransferase activity of SopB (Extended Data Fig. 5a–d). Consistent with an accessory role, these residues are structurally predicted to neighbour the Cys-X$_5$-Arg motif of SopB (Extended Data Fig. 5e,f) \cite{62}.

In summary, this work identifies an unprecedented PtdIns(4,5)P$_2$-to-PtdIns(3,4)P$_2$ conversion mechanism mediated by a phosphotransferase. Through a remarkable instance of convergent evolution, pathogenic organisms have acquired the ability to manipulate host signalling pathways that are central to endocytosis and survival signalling, and can even provoke cellular transformation, as illustrated by the development of gallbladder carcinomas during Salmonella infection \cite{63}.

Online content
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Methods

Plasmids and siRNA. The plasmids utilized in this study are summarized in Supplementary Table 1 and were verified by Sanger sequencing. The plasmids were constructed using In-Fusion HD EcoDry cloning kits (Takara, 121416), NEBuilder (New England Biosci, E3520A) or other traditional restriction enzyme methods. Site-directed mutagenesis was performed using targeted pairs of oligonucleotides that centrally housed the mutation. The codon corresponding to Lys 464 of wild-type and C460S SopB was substituted with the infrequently used Amber STOP codon (TAG) for phototransactivation studies. The plasmids HAX3-AC8R-M3 was provided by J. Wes, SopB(+)-EGFP by D. Zhou and complementary DNA to generate pUG3-GFP-2xFYYE' by S. B. Emr.13 The plasmids Myc-p110e-CAAX', Myc-Pi3K-C2A-CAAX', mRFP-FKBP-PLCβ3 (ref. 14), Lynm, FRR-HA and p8pCas9 (BB)-2A-Puro' were previously generated. Custom basic RNA oligos (Thermo Fisher, 10620310) targeting PKA2A were used to transfect HeLa, U2-1AUAAUUU-3' (sequence 1) and 5'-GGGAAACCCAGGCUAUUU-3' (sequence 2). Phosphorylated oligonucleotides were suspended in water to 20μM. An equimolar amount of ON-TARGETplus non-targeting control (Dharmacon, D-00180-10-20) was used as the control siRNA.

Reagents, lipids and antibodies. Commercially available inhibitors, stains, lipids and other reagents used in this study are summarized in Supplementary Table 2. Commercially available primary and secondary antibodies and their dilutions are summarized in Supplementary Table 3. Unless otherwise stated, antibodies were incubated during immunoblotting in 2.5% (wt/vol) milk powder in Tris-buffered saline (TBS, pH 7.4) or 1% (vol/vol) Tween 20. Commercially purchased diC6 PIPNs were solubilized in 1 ml of 2:1:0.01 CHCl3: methanol:1 M HCl, vortexed and dried under a N2 stream before three resuspension-and-drying cycles in CHCl3. Finally, diC6 PIPNs were resuspended in CHCl3 at a final concentration of 0.5 mg·ml−1. C18-20:4 PtdIns(4,5)P2 (synthesized by the Biological Chemistry Department at Barraham Institute) was dissolved in 20:9:1 CHCl3:methanol:1 M HCl, vortexed and dried under a N2 stream before harvesting by centrifugation (5,000 g for 5 min) for downstream purification.

Cell culture and transfection. HeLa cells were cultured in low-glucose DMEM (Life Technologies, 10567022) containing 10% fetal bovine serum (Gibco, 12483-020). The cells were incubated at 37 °C in a humidified atmosphere 95% air/5% CO2. For RNA interference, cells were seeded in 12-well plates at 1.1 × 105 cells per well 2 d before bacterial invasion on 18-mm Number 1.5 glass coverslips (Fisher Scientific, 12-1551-05). Where indicated, concanavalin A (5 μg ml−1) was added 90 min before inducing SopB with galactose for an additional 60–90 min. The cells were then fixed and stained for further analysis.

CRISPR–Cas9-mediated genome editing. Cdc42-specific single-guide RNAs were designed using the Graphical User Interface for DNA Editing Screens available at http://guides.sanjanalab.org/ and are summarized in Supplementary Table 4. Annealed oligonucleotides were ligated into pSpCas9 (BB)-2A-Puro’. A CACC 5′ flanking sequence was added to each sense oligonucleotide and an AAAC 5′ flanking sequence to each antisense oligonucleotide for ligation into the BbsI site. At 48 h post transfection, a mixture of the ligated vectors was transfected and puromycin (2 μg ml−1) was added to the culture medium for 48 h. Single cell dilutions were transferred into 96-well plates and allowed to recover to near confluency, at which time the knock out efficiency was determined by immunoblotting.

Salmonella strains, culture and invasion. The Salmonella strains used are derivatives of wild-type S. enterica subspecies I serovar Typhimurium strain SL1344 (ref. 85). Isogenic ΔsopB SL1344 (ref. 85), ΔsopE/Fop2 SL1344 (ref. 85) and ΔsopB SL1344 transformed with pACEDE-SopB-myc were generated previously19. BFP- and mRFP-expression strains were generated by electroporating low-copy plasmids that encoded each fluorophore (pFPV25.1 where EGFP was replaced by BFP and pR8322 encoding mRFP1) under the control of the rpsM promoter47.

Epithelial cells were exposed to late-log-phase Salmonella cultures to induce optimum for bacterial invasion37. Several colonies were inoculated into 2 ml LB medium with antibiotic selection and cultured overnight at 37 °C with shaking. Stationary-phase cultures were diluted 1:33 into fresh LB without antibiotics for 3.5 h at 37 °C and inocula were prepared by centrifugation at 10,000g for 2 min before resuspending in an equivalent volume of D-PBS. Each inoculum was diluted 1:50 (BFP and mRFP-expressing strains) to 1:100 in D-PBS and 1 ml was added per well for 10 min at 37 °C. During the time-course studies, extracellular bacteria were removed by washing extensively and 100 μg ml−1 gentamycin was added to the cell culture medium 30 min post infection. The concentration of gentamycin was reduced to 10 μg ml−1 2 h post infection for the remainder of the experiment. Stationary-phase cultures during invasion were routinely monitored using inverted microscopes, and infected coverslips were submerged for 5 min in pre-chilled D-PBS containing CellMask (1 μg·ml−1), followed by two gentle washes and paraformaldehyde (2% wt/vol) fixation. The fixed cells were imaged immediately following PBS washes. To label the PM with a fluorescently labelled lectin, infected cells were incubated for 10 min in pre-chilled Tyrode’s buffer containing 10 μg ml−1 WGA-Texas Red before washing and fixing.

Yeasts and strains culture. SEY6210 wild-type S. cerevisiae (MATα leu2-3,112 ura3-5 his3-A200 rpl9-A902 pyr2-801 suc2-AY G4)10 and SEY6210 vps34Δ1-TRP1 (PHY102)11 transformed with a low-copy plasmid (CEN URA3) encoding the vps34Δ mutant allele were generated previously19. Plasmids were transformed by the lithium-acetate method and strains were maintained on appropriate minimal medium containing 1.7 g·l−1 yeast nitrogen base without amino acids, 5 g·l−1 ammonium sulphate, amino acids, 2% (wt/vol) d-glucose or 2% (wt/vol) d-galactose and 2% (wt/vol) bacto-agar. Yeast strains cultured overnight in the appropriate medium at 28–30 °C were subcultured into fresh glucose-containing medium at an optical density of 0.25 at 600 nm and allowed to grow for 3 h. Yeast cultures were allowed growing on plates in YEPD for 2 days before being diluted into galactose-containing medium for 2 h to induce SopB or PldG before imaging. Vps34p was inactivated in the vps34Δ strain by moving the cultures to 38 °C (non-permissive temperature) for 90 min before inducing SopB with galactose for an additional 60–90 min. The cells were collected by centrifugation and resuspended in medium containing 0.1% triton X-100, 0.1% BSA followed by 1.8-cm Number 1.5 glass coverslips. Where indicated, concanavalin A (5 μg ml−1) was added for 10 min before centrifugation to demarcate the cell wall.

Immunostaining. Paraffin-dehydrate (2% wt/vol)-fixed cells were permeabilized in 0.1% (vol/vol) Triton X-100 in PBS for 5 min, blocked in 2% (wt/vol) BSA in PBS for 20 min and overlaid consecutively for 1 h with primary and secondary antibodies in 1% BSA, separated by PBS and BSA washes. To stain epitope-tagged SopB, the cells were permeabilized for 30 min with 0.2% saponin in PBS supplemented with 10% goat serum (vol/vol). Primary and secondary antibodies were overlaid in permeabilizing solution for 60 min, separated by PBS washes. Immunofluorescence was assessed by differential inside–outside staining: coverslips were sequentially blocked and stained in PBS containing 10% goat serum before proceeding with saponin permeabilization and staining internalized bacteria.

Recombinant protein production. Recombinant proteins were purified from BL21 (DE3) E. coli strain (NEB, C5257) using isopropyl β-D-1-thiogalactopyranoside-inducible expression systems and amino-terminal hexa-histidine tags. Colonies were inoculated into Terrific Broth containing 4 ml l−1 glycerol (Multicell, cat no. 800-067-LG; total volume, 5 ml), 50 μg ml−1 kanamycin and 0.8% (wt/vol) d-glucose. Starter cultures were incubated overnight at 37 °C with shaking before 1:50 dilution into 300 ml pre-warmed Terrific Broth with antibiotics selection. When the culture reached an optical density of 0.6–0.8 at 600 nm, they were equivalibrated (23 °C) and induced with 0.05–0.1 mM isopropyl β-D-1-thiogalactopyranoside for 20 h at 23 °C (210 r.p.m.) before harvesting by centrifugation (5,000g for 15 min at 4°C) and freezing for downstream purification.
Pellets containing His<sub>E</sub>, EGF-PH-PI(3)- and SMT3-SopB<sub>33–554</sub> were resuspended in 25 µl of 300 mM NaCl, 40 mM Tris, pH 7.4 (10 °C), 1 µl EDTA-free protease inhibitors (Thermo Fisher, A32955) and DNase I (Sigma-Aldrich, DN25). Pellets containing His<sub>E</sub>, EGF-PH-PI(4,5)-P<sub>2</sub> and SMT3-SopB<sub>33–554</sub> were resuspended in 20 µl of 200 mM NaCl, 40 mM Tris, 15 mM imidazole, 5% (vol/vol) glycerol, pH 7.4 (10 °C), 5 mM β-mercaptoethanol, protease inhibitors and DNase. Bacteria were disrupted by French press (SLM Aminco Spectronic Electronic Instruments) before centrifugation (15,000 g for 20 min at 4 °C). Clarified supernatants were incubated with TALON metal affinity resin (Takara, 635602) and rotated at 4 °C for 30 min (PhaseLock<sup>®</sup> Gel gravity flow [PH-PLCS1, SUMO-SopB]). The resin was washed twice with 10–15 mM imidazole in lysis buffer before loading into a TALON 2 ml gravity column (Takara, 635606). The columns were washed once before elution in a single step (150 mM imidazole, 2 mM βME) or a stepwise gradient (15, 30, 40, 60, 180, 180 and 240 mM imidazole; PH-PLCS1, SUMO-SopB). Peak fractions analyzed by SDS–PAGE were pooled.

An Amicon Ultra-15 centrifugal filter unit (Millipore, UFC030008) was used to dialyse pH<sub>3</sub>-fractions several hundred-fold into imidazole-free buffer (150 mM NaCl and 20 mM Tris, pH 7.4) during concentration. PH-PLCS<sub>1</sub> fractions were dialysed (Thermo Fisher, 66388; Slide-A-Lyzer Dialysis Cassettes) into imidazole-free buffer (150 mM NaCl, 20 mM Tris, 5% (vol/vol) glycerol and 0.5 mM β-mercaptoethanol, pH 7.4). SMT3-SopB<sub>33–554</sub>-fractions were diluted with additional buffer before dialysis. The products were aliquoted and snap-frozen in liquid N<sub>2</sub> for storage.

Unilamellar vesicle assays. The GUVs were generated on a film of agarose<sup>1</sup>. Glass slides overlaid with 1% (wt/vol) ultra-low gelling temperature agarose were gently heated until translucent. Liposome compositions were prepared by adding chloroform-suspended lipids to glass vials, drying under N<sub>2</sub> gas and resuspending in a small volume of chloroform (generally ~1.33 mol total lipid in 35 µl chloroform). Lipids were spread over the agarose, chloroform was thoroughly evaporated under N<sub>2</sub> gas and the slides were submerged in assay buffer (2000 CHCl<sub>3</sub>:1 M HCl (484:242:23.55) and 70 μl: d6-18:0-20:4 PtdIns(3,4,5)P<sub>3</sub>). GUVs harvested 3–20 h later were used directly or centrifuged (4000g, 5 min) to concentrate. The preparations were stored at 4 °C and used within 7 d.

To stabilize GUVs for fluorescence microscopy, acetic-acid washed coverglasses were passivated with a 3:1 mixture of PLL(20)-g[3.5]-PEG(5) to PLL(20)-g[3.5]-PEG(2)/PLL(20)-g[3.5]-PEG(2)/PLL(20)-g[3.5]-PEG(2) (150 mM NaCl and 20 mM Tris, pH 7.4) during concentration. PH-PLCS<sub>1</sub>-GUVs were dialysed (Thermo Fisher, 66388; Slide-A-Lyzer Dialysis Cassettes) into chloroform-free buffer (150 mM NaCl, 20 mM Tris, 5% (vol/vol) glycerol and 0.5 mM β-mercaptoethanol). GUVs were snap-frozen in liquid N<sub>2</sub> at the indicated time points and stored at −80 °C for analysis.

PM internal standard was used to correct both 18:0-20:4 PtdIns(4)P and 18:0-20:4 PtdIns(3)P. The 1:1 mixture of PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> was used, allowing assessment of regio-isomer separation and correction for endogenous (substrate) 18:0-20:4 PtdIns(4,5)P<sub>2</sub>.

Image acquisition and photoinactivation. Spinning disk confocal microscopy was performed on a Quorum WaveFX spinning disk system (Quorum Technologies Inc.) consisting of an Axiosview 200M microscope (Carl Zeiss) equipped with a 63×/1.3 NA objective and x2/0.8 NA immersion objective multiplied by a x1.53 magnifying lens. Scanning was performed by the CSU10 confocal multi-beam scanner (Yokogawa). Fluorophores were excited consecutively by a four-line laser module (405, 491, 561 and 640 nm; Spectral Applied Research) and filtered by a corresponding emission filter wheel (447/40, 515/40, 515 LP, 594/40, 624/40, 670/40). Signal was detected by a back-thinned EM–CCD camera (Hamamatsu ImageEM C9100-13). This system is driven by a motorized xy stage (Applied Scientific Instrumentation) and Piezo Focus Drive (Ludl Instruments). Acquisition settings and capture were controlled by Volocity v6.2.1 (PerkinElmer).

For live-cell imaging, coverslips were mounted within a Chamlide magnetic chamber (Live Cell Instrument Inc.) overlaid with pre-warmed phenol red-free Tyrode's buffer (pH 7.4). Using an environmental chamber (Live Cell Instruments Inc.) up to ten fields were acquired consecutively with 0.3 µm Z-steps to capture entire bacterial invasion ruffles.

Airyscan microscopy was performed on the LSM880 Airyscan system (Carl Zeiss), which is equipped with a x63/1.4 NA oil objective and three detectors: two x63/1.4 NA two-channel tubes and an x63/1.4 NA widefield tube. Laser lines were 488 nm (Argon), 552 nm and 642 nm under Airyscan mode. Acquisitions are driven by a motorized XY stage and Z-Piezo Drive (Carl Zeiss), and settings and capture controlled by Zen Black software v2.3 (Carl Zeiss).

Optogenetic experiments were imaged on a Nikon TiE inverted stand confocal microscope with an A1R resonant scan head and fibre-coupled four-line excitation (Ex) / LU-UV laser configuration (405, 488, 561 and 642 nm). Each 16-frame averaging was used to improve the signal-to-noise ratio. A x10 1.4 NA plan-achromat oil immersion objective was used. To minimize crosstalk, blue (425–475 nm) and yellow/orange (emission, 570–620 nm) fluorescence were acquired on a separate excitation scan to the green (emission, 500–550 nm) and far-red (663–737 nm) channels. Optogenetic activation of SopB was performed after acquiring approximately 30 s of data with 405 nm illumination set to zero power, at which time the transmission was increased to 20% of the maximum available power from the LU-UV unit. The acquisition settings and capture were controlled by Nikon Elements AR-5.21.02. Cells were imaged in FluoroBrite DME medium (Life Technologies, A196702) supplemented with 25 mM HEPES (pH 7.4), chemically defined lipid supplement (1:1,000, Life Technologies, 11905031) and 10% fetal bovine serum.

Western blots were imaged digitally on an Odyssey FC System (LI-COR) equipped with 685 and 785 nm lasers and acquisition–controlled by the Image Studio v4.0 software (LI-COR).

Image analysis. Nikon (n2d) and Volocity (m2vd) files were exported and analyzed in Fiji v1.53d1. To quantify the biosensor intensity in the bulk PM or invasion ruffles, an index was produced by normalizing the fluorescence intensity in the membrane to the cytosol after background correction. Data analysis was presented as the fluorescence in the PM compartment (PM<sub>flu</sub>) to the fluorescence in the cytosol (Cytosol<sub>flu</sub>) before photoactivation from the ratio along the time-lapse. The PM was defined by the signal of CellMask staining (see below) or by manually selecting a region of interest (ROI) based on lipid biosensors enriched at the PM before photoinactivation. Except for the direct comparison of CH3<sub>3</sub> in cells expressing SopB<sup>d6-18:0-20:4</sup>-EGFP and SopB<sup>d6-18:0-20:4</sup>-CH<sub>3</sub>-EGFP (Fig. 2f), optogenetic activation data were filtered for optimal SopB uncaging by monitoring cells with baseline (time, t = 0) PCh3<sub>flu</sub> and/or PCh3<sub>flu</sub> at t = 300 s.

To estimate the fluorescence intensity in the bulk PM, a binary mask of the compartment was generated from a tousled wave decomposition<sup>44</sup> using CellMask fluorescence as a PM marker. The resulting wavelet product was thresholded to include pixel intensities several-fold larger than the s.d. of the wavelet product (empirically determined per dataset and ranging from 0.8x to 1.5x the s.d.). After manually producing ROIs that encompassed single transfected cells and restricting measurement to within that region, the resulting binary mask served to quantify the fluorescence intensity of a given biosensor in the PM compartment.

To capture biosensor recruitment to bacterial invasion ruffles, individual circular ROIs (pixel<sub>radius</sub> = 100 or ±13.2 µm) encompassing single invasion sites were manually annotated. As described earlier, the fluorescence intensity of a binary mask was quantified by the signal of CellMask after conversion of CellMask. Binary masks of ruffles were generated by first smoothing CellMask images with a 3×3 neighbour pixel averaging. After converting to an 8-bit grey scale, a local threshold was applied to images using Bernsen's method for computing a sliding circular window with pixel<sub>radius</sub> = 3. Biosensor intensity was then analysed within the circular binary mask of the invasion membrane.
To enumerate the number of SopB–EGFP puncta per cell, the wavelet product of maximum intensity projections was first calculated by a trous wavelet decomposition. This product was thresholded using Bernsen’s method (pixel≥3) and parametric fitting of the following equation were annotated: size: 0–200 pixel²; circularity, 0.5–1.0 and mean particle intensity of 2×fold the total cellular median intensity. Transfected cells reaching a mean EGFP intensity of ≥1.25-fold that of the background intensity were included in the analysis and identified puncta were reported per transfected cell.

To quantify biosensor recruitment to liposomes, an annulus of the liposome surface was generated and compared with an annulus of the surrounding medium. Otto’s method was applied to the PE–Rhodamine channel to threshold the pixel intensities of individual liposomes, and the binary mask was filled and iteratively eroded. The resulting binary shape conforming to the shape of the liposome was outlined and dilated to create a 4 pixel-wide annulus of the liposome surface. A corresponding ‘medium’ measurement was generated by dilating this annulus ten pixels beyond the liposome surface. Data are presented as the resulting liposome/medium intensity ratio per liposome.

Immunoblot densitometric calculations were performed in Image Studio Lite v5.2 (LI-COR).

Statistics and reproducibility
Data were imported into GraphPad Prism 9 for statistical analysis and presentation. Superplots95 were generated to communicate statistical comparisons. Data were assessed using an unpaired one-tailed Mann–Whitney test of trial means in Fig. 7c. A non-parametric one-tailed t-test was applied on separate passages of cells. Except for the photoactivation assays, trial averages or independent experimental values, non-parametric data were subjected to statistical analyses. Shapiro–Wilk normality tests (n≥3) were consistent with a normal distribution, so parametric tests were applied. Phosphate release and HPLC–MS data are plotted directly and fit to a curve by the smoothing spline (four knots) feature. To compare photoactivation datasets, baseline-corrected data were sorted into individual groups of curves, each comprising measurements of a normalized ROI across an entire time-lapse. The AUC value was calculated for each time-lapse and the resulting net AUCs were sorted by condition to compare groups statistically. Shapiro–Wilk normality tests were applied to each group, and parametric or non-parametric t-tests were applied accordingly, as described above.

Significance was assessed using an ordinary one-way analysis of variance with Bonferroni’s multiple comparisons test in Figs. 1b, c, e, f, j, l, 4b, 4d, and Extended Data Figs. 1d, 4c, 5c, 6e, 7c.e. Significance was assessed using an ordinary two-way analysis of variance with Bonferroni’s multiple comparisons test in Fig. 4e. Significance was assessed using an unpaired two-tailed t-test in Figs. 2b, 6b, and Extended Data Figs. 2c, 3g, 5d, 6c, 7b. Significance was assessed using an unpaired one-tailed t-test with Welch correction in Fig. 5e and a two-tailed ratio paired t-test of trial means in Fig. 7c. A non-parametric one-tailed Mann–Whitney test of ranks was utilized to assess significance in Figs. 3g and 5h. Exact P values are provided in the figure legends. P>0.05 was considered not significant.

Representative images were chosen based on being phenotypical, possessing good signal-to-noise ratio and being quantitatively representative of the dataset. Merging and cropping fluorescent channels was performed in Fiji. To aid visualization, linear adjustments were made to the brightness and contrast across the entire image or alternative look-up tables were applied. Exported RGB format tiff images were sized once in Adobe Photoshop v21.1.1 to the final publication format before assembly in Adobe Illustrator CS6 v16.0.0.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data supporting the findings of this study are available from the corresponding author on reasonable request. Source data are provided with this paper.

Code availability
Custom macros used in this study have been deposited on GitHub and are available for download at the manuscript repository (https://github.com/walpoleg/Walpole-et-al-Nature-Cell-Biology-2022).

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Author contributions
G.F.W., S.G., G.R.V.H. and G.D.C. conceived experiments and developed methods. G.F.W. conducted experiments and analysed the resulting data. J.P. conducted photoactivation assays and analysed the resulting data with G.R.V.H. K.E.A. and J.C. performed HPLC–MS measurements and analysed the resulting data with L.R.S. and P.T.H. D.B. performed inversion efficiency assays, N.C., Y.M.A., M. – R., Z.L., H.Z., J.H.B. and A.D. made and/or provided critical reagents. G.F.W. wrote the original draft of the manuscript. All authors reviewed and edited the manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | See next page for caption.
**Extended Data Fig. 1** Rapid and sustained PtdIns(3,4)P₂ synthesis during Salmonella entry and maturation. (a) Model of PtdIns(3,4)P₂ biosensors based on single, double-, or triple-tandem carboxy-terminal PH domains from TAPP1 (PLEKHA1). NES, nuclear export signal. Right, gel electrophoresis of PCR amplicons generated by primers that house the open reading frames. (b) Cells expressing cPHx1, cPHx2, or cPHx3 were infected for 10 min with wild-type Salmonella prior to staining the PM with CellMask. Representative maximum intensity projections (main) and a corresponding confocal section of the invasion ruffle (bottom panels) are presented. (c) Confocal imaging of cells (three examples in I, II, and III) expressing the PtdIns(3,4,5)P₃ sensor aPHx2 during invasion by wild-type Salmonella. Bottom vertical panels are expanded from the white box region and correspond to the minute-by-minute time series. cPHx3 is also presented in a grey inverted lookup table (RGB intensity 0=white, 255=black). (d) As in (c), three examples (I, II, and III) of cells expressing the PtdIns(3,4,5)P₃ sensor aPHx2 during invasion by wild-type Salmonella. (e) Cells serum-starved for 3 h were infected by Salmonella. Extracellular bacteria were removed, and cells returned to serum-free medium containing gentamycin. PM cPHx3 intensities were quantified in the following number of cells: 59 (control), 60 (WT, 30 min), 42 (ΔsopB, 30 min), 58 (WT, 60 min), 53 (ΔsopB, 60 min), 75 (WT, 120 min), 48 (WT, 240 min), and 36 (ΔsopB, 240 min) across n=3 independent experiments. Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (background). Data from Fig. 1c are presented with ΔsopB infections. ****P < 0.0001; **P = 0.0054 (UI-vs-WT60), **P = 0.0044 (WT120-vs-ΔsopB120); ns, not significant. (f) Cells serum-starved for 3 hours, were exposed to Salmonella (wild-type or ΔsopB) for 10 min. Extracellular bacteria were removed, and cells were returned to serum-free medium with gentamycin. Lysates were collected at the indicated time points and analysed on parallel membranes for pAKT (S473) or pAKT (T308) prior to stripping and re-probing for total AKT or GAPDH (loading control). Representative immunoblots are presented from n=2 independent experiments. UI, uninfected. Source numerical data and unprocessed blots are available in source data.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | PtdIns(3,4,5)P3 analysis during Salmonella invasion or during optogenetic activation of SopB. (a) Model of the PtdIns(3,4,5)P3 biosensor NES-EGFP-aPHx2 derived from tandem ARNO PH domains (2G splice variant, I303E mutation). NES, nuclear export signal. (b) Cells were exposed to invasive RFP-expressing wild-type or isogenic ΔsopB Salmonella and the PM was stained with CellMask prior to imaging. Maximum intensity projections (main) and single confocal sections of the invasion ruffle are presented at right for each. (c) Quantification from (b). Normalized intensity of aPHx2 in the PM of invasion sites from n=3 independent experiments analysing 71 (WT) and 63 (ΔsopB) invasion sites. *P = 0.0294. (d) Normalized intensity of aPHx2 in the PM during optogenetic activation of SopBWT-464TAG or SopBC460S-464TAG. Filtered baseline-corrected time-lapse data are mean ± s.e.m. of individual cell measurements from 3 independent experiments quantifying n=29 cells (WT) and n=13 cells (C460S). (e) AUC calculations of aPHx2 intensities from (d). Data are median, box (25th-75th) and whisker (10th-90th) percentiles of n=29 cells (WT) and n=13 cells (C460S) from 3 independent experiments. P = 0.4415, ns. (f,g) SopB-mediated synthesis of PtdIns(3,4)P2 is not accompanied by a robust PtdIns(3,4,5)P3 response. Representative confocal sections of aPHx2 and cPHx3 localization during optogenetic activation of WT or C460S SopB. Left, corresponding linear RGB intensity scales are presented. The indicated times are prior to (t - 30 s) or after illumination with 405 nm light to photolyze hydroxycoumarin lysine in SopB. See corresponding quantification of (f) presented in Fig. 2g. Source numerical data are available in source data.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Sequence determinants of SopB-mediated PtdIns(3,4)P$_2$ generation. (a) Bacterially injected SopB localizes to the vacuolar membrane and PM invaginations. HeLa cells were exposed to $\Delta$sopB (control) or $\Delta$sopB + SopB-c-myc Salmonella for 10 min prior to labelling the PM (WGA) and immunostaining against c-myc and Salmonella. Inset panels are expanded from the hashed box region. Representative maximum intensity projections are from n=3 independent experiments. (b) Anti-myc fluorescence is not contaminated by non-secreted SopB or anti-Salmonella immunostaining. $\Delta$sopB + SopB-c-myc Salmonella were centrifuged onto poly-L-lysine coated coverslips. Bacteria were processed as in (a) and imaged with equivalent acquisition settings. The representative maximum intensity projection is from n=3 independent experiments. (c) Heterologous SopB coalesces within puncta that abut cortical membranes. Full-length SopB constructs were co-transfected with cPHx3 and imaged live. Amino-terminally tagged SopB was enriched along cytosolic reticular structures; amino- and carboxy-terminal EGFP fusions also enriched on the basal footprint of cells. Representative confocal sections are presented for each construct, from more than three similar experiments. CellMask served as a PM marker. (d) SopB requires amino acids 68-172 and 520-554 for PtdIns(3,4)P$_2$ generation. The indicated SopB plasmids (top panels) were co-transfected with cPHx3 (bottom panels) and imaged live in HeLa cells. Representative confocal sections are presented. Note that membrane-targeting of SopB is disrupted following the deletion of amino acids 68-172 while PM-targeting is preserved following the deletion of amino acids 520-554. (e) CRISPR-Cas9-mediated deletion of Cdc42. Total lysates from control sgRNA- or Cdc42-specific sgRNA-treated Henle 407 cells were immunoblotted against Cdc42. Alpha tubulin served as a loading control. (f) Cdc42 regulates SopB targeting but is not strictly required for PtdIns(3,4)P$_2$ generation. Representative maximum intensity projections are presented of SopB (1-561)-EGFP localization in parental wild-type Henle (top) or Cdc42 KO Henle cells (bottom). Inset images depict localization of cPHx3 (confocal section). cPHx3 was markedly enriched in the PM with a concomitant decrease in cytosolic fluorescence in (mean ± s.e.m.) 94.1 ± 2.92% of parental WT and 88.5 ± 7.28% of Cdc42 KO cells across n=3 independent experiments. (g) Quantification of SopB-EGFP puncta per cell from n=3 independent experiments analysing the following number of Henle 407 cells: 91 (WT) and 59 (Cdc42 KO). Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). **P = 0.0014. Source numerical data and unprocessed blots are available in source data.
Extended Data Fig. 4 | 3-phosphorylated phosphoinositides promote bacterial invasion of host cells. (a) Cells were exposed to wild-type or ΔsopB Salmonella for 10 min and extracellular bacteria were removed by extensive washing before returning to growth medium for an additional 20 min. Extracellular and intracellular bacteria were differentially stained. Data are mean ± s.e.m. from n=3 independent experiments quantifying ≥300 infected cells per strain. **P = 0.0022, ***P = 0.0007. (b,c) PtdIns(3,4)P2 and/or PtdIns(3,4,5)P2 promote bacterial invasion. (b) Model of PTEN-catalysed reactions: membrane-associated PTEN dephosphorylates both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 at the 3-position of the inositol ring. (c) Cells transfected with EGFP (control) or the A4 mutant of PTEN were left untreated or pre-treated with LY294002 (10 μM, 30 min) prior to invasion. Twofold excess of ΔsopE/sopE2 bacteria was used to obtain sufficient infected cells. Data are mean ± s.e.m. (normalized to respective control) from n=3 independent experiments quantifying ≥300 infected cells per strain. WT: **P = 0.0022, ***P = 0.0007; ΔsopE/E2: *P = 0.0125, **P = 0.0039. (d) Cells were co-transfected with cPHx3 and PM-targeted catalytic subunit of class IA PI3K (p110α-CAAX). Images were acquired immediately before (left) or 3 min after addition of DMSO (vehicle, right). (e) HeLa cells expressing vector control or p110α-CAAX were treated for 20 min with DMSO, wortmannin (100 nM), PI-103 (500 nM), or GDC-0941 (500 nM) prior to collection of cell lysates and immunoblotting using phospho-AKT (S473) and pan AKT antibodies. The immunoblot presented is representative of n=3 independent experiments. Corresponding quantification, Fig. 3c. (f) Confirmation of anti-PI3K-C2α polyclonal antibody labelling of heterologously-expressed PI3K-C2α. HeLa cells transfected with PM-targeted myc-PI3K-C2α were co-stained for the myc epitope tag and PI3K-C2α. Panels right depict enlargement of hashed box regions (I, II), where the non-transfected cells (I) were overexposed to visualize endogenous staining. (g) Class II PI3K-C2α is not enriched at the site of Salmonella invasion. Confocal sections of uninfected and wild-type Salmonella infected HeLa cells stained for endogenous PI3K-C2α. Source numerical data and unprocessed blots are available in source data.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Lysine residues within the carboxyl-terminus of SopB support optimal phosphotransferase activity. (a) Alignment of SopB amino acids 504-554 with other PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 5-phosphatases. Basic residues in SopB are highlighted red along with conserved basic residues in the other phosphatases; additional conserved residues are highlighted purple, and regions of highest similarity are boxed (grey). Notably, residues that share sequence similarity with Mus musculus Synaptojanin-1 (SYNJ1) 534-584 fall outside of the SYNJ1 5-phosphatase domain and have not been implicated in its catalytic activity. Local homology between SopB and INPP5B or OCRL failed to be identified by NCBI BLAST. (b) Mutation of lysines 525 and 528 blunt PtdIns(3,4)P_2 generation by SopB. Wild-type, K525A, K528A, and C460S SopB amino-terminally tagged with EGFP (bottom panels) were transiently expressed with cPHx3 (inverted grey, main panels). The PM was stained with CellMask before imaging live. Representative confocal sections are presented for each. Note that expression of the K528A mutant led to notable perturbation of cellular morphology including rounding, cortical membrane ‘crumpling’, and blebbing. (c) Normalized intensity of PM cPHx3 from (b) was quantified across n=3 independent experiments (WT, 78 cells; K525A, 81 cells; K528A, 86 cells; C460S, 57 cells). Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). ****P < 0.0001. (d) cPHx3 intensities from K528A- and C460S SopB-transfected cells (red box, panel c) plotted on an expanded axis. Subtle PM-enrichment of cPHx3 was evident in K528A-transfected cells relative to the C460S-transfected mutant. Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). **P = 0.0017. (e,f) Lysine 525 and 528 of SopB are structurally predicted to neighbour the C(X)R motif. The primary sequence of SopB (residues 1-561) was analysed by RoseTTAFold and the resulting highest ranked model is presented in the surface filling view (grey). The location of lysine 525 and 528 (green) and the C(X)R motif of SopB (blue) are annotated. Source numerical data are available in source data.
Extended Data Fig. 6 | Generation of PtdIns(3,4)P_2 by the S. flexneri effector IpgD does not require class I or class II PI3Ks. (a) Sequence alignment of the phosphate-binding (P-loop) sequence from SopB (Salmonella enterica) and IpgD (Shigella flexneri). Residue 439 of IpgD encodes the cysteine of the C(X)_5R motif. (b) IpgD reduces PM PtdIns(4,5)P_2 levels in a C(X)_5R-dependent manner. Heterologous expression of EGFP-IpgD (WT or C439S) and PH-PLCδ_1 (inverted grey, main panels). The PM was stained with CellMask prior to imaging live. (c) Quantification of PH-PLCδ_1 intensity in the PM from (b) across n=3 independent experiments quantifying 82 cells (IpgDWT) and 74 cells (IpgDC439S). Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). ****P < 0.0001. (d) Live cell imaging of heterologously-expressed EGFP-IpgD (WT or C439S) and NES-mCherry-cPHx3 following PM staining with CellMask. Cells were treated with the indicated inhibitors (PI-103, 500 nM; wortmannin, 100 nM) for 20 min prior to and throughout imaging. (e) PM cPHx3 intensity was quantified from (d) across 3 independent trials (IpgDWT, 70 cells; IpgDC439S, 62 cells; IpgDWT(PI-103), 68 cells; IpgDWT(Wortmannin), 76 cells). Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). ****P < 0.0001. (f) PM PtdIns(3,4)P_2 synthesis in S. cerevisiae. Galactose-inducible empty vector (control), IpgDWT, or IpgDC439S were induced for 2 hours in yeast that expressed cPHx3. Trypan Blue staining demarcates the cell wall and non-viable yeast. (g) Yeast from (f) were scored for plasmalemmal cPHx3 localization across n=4 independent experiments analysing the following number of yeast: control, 881 cells; IpgDWT, 1165 cells; IpgDC439S, 938 cells. Data are mean ± s.e.m. ****P < 0.0001. Source numerical data are available in source data.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | SopB requires an INPP5E-sensitive plasmalemmal inositide for the generation of PtdIns(3,4)P₂. (a) Inhibition of PtdIns(3,4)P₂ generated during invasion by pre-recruitment of INPP5E. HeLa cells expressing mRFP-FKBP-INPP5E (WT or D556A) together with PM-targeted LynF11-FRB and the biosensor cPHx1 (grey) were treated with 1 μM rapamycin for 2 min before addition of wild-type Salmonella (BFP, red) for an additional 10 min. The PM was stained (CellMask) and cells fixed before imaging. Maximum intensity projections (main panels) and confocal sections of the boxed region (bottom panels) are presented. Re-localization of mRFP-FKBP-conjugates to the PM is depicted in the bottom panels. (b) Normalized PM intensity of cPHx1 was quantified from part (a) across n=2 independent trials (INPP5EWT, 90 cells; INPP5ED556A, 85 cells). Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). **P = 0.0019. (c) Comparison of PtdIns(4,5)P₂ depletion by chemically induced recruitment of PLCβ3 and INPP5E. Normalized PM intensity of PH-PLCδ1 was quantified from n=3 independent experiments (control, 55 cells; PLCβ3, 56 cells; INPP5E, 74 cells). Data from Fig. 6a,b are re-plotted with INPP5E (acquired in parallel) and support that INPP5E results in a modest PtdIns(4,5)P₂-depletion relative to PLCβ3. Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). ***P = 0.0002, *P = 0.0477. (d) PtdIns(4,5)P₂-depletion by INPP5E recruitment depends on its catalytic activity. Comparison of HeLa cells co-transfected as in (a) but expressing the biosensor PH-PLCδ1-EGFP. Representative confocal sections are presented following 5-min rapamycin treatment (1 μM) and PM staining with CellMask. (e,f) Chemically induced INPP5E recruitment modestly increases PM PtdIns(4)P. HeLa cells expressing mRFP-FKBP-(control), -PLCβ3, or -INPP5E (wild-type) together with PM-targeted LynF11-FRB and EGFP-2xP4M were imaged live 5 min after treatment with 1 μM rapamycin and staining the PM (CellMask). (e) The normalized PM intensity of 2xP4M was quantified from n=3 independent experiments (control, 55 cells; PLCβ3, 41 cells; INPP5E, 53 cells). Data are trial means ± s.e.m. (foreground) overlaid on cell measurements (grey, background). **P = 0.0020. (f) Representative confocal sections of each channel are presented. Source numerical data are available in source data.
Extended Data Fig. 8 | Time-resolved HPLC–MS analysis of SopB phosphotransferase-phosphatase activities. (a) Inorganic phosphate release assessed following treatment of liposomes with 2.5 µg recombinant SopBWT or SopBC460S. Liposome composition was (mol%) POPS:PtdIns(4,5)P2 (90:10) and 2.5 nmol PtdIns(4,5)P2 was provided per reaction. At the indicated time points, reactions were terminated by addition of 50 mM NEM. Data are mean ± s.e.m. of duplicate wells. Time 0 min corresponds to a no enzyme control. (b) Separation of PtdIns(3,4)P2 and PtdIns(4,5)P2 regio-isomers in a sample treated with SopBWT. An example HPLC–MS trace derived from LUVs treated with 0.5 µg (71.5 nM) SopBWT for 5 min. Note the appearance of PtdIns(3,4)P2 at ≈20.6 min. (c) Model of the PtdIns(3,4,5)P3 biosensor designed with tandem Bruton’s Tyrosine Kinase (BTK) PH domains (bPHx2). Each component was separated by flexible, serine- and glycine-rich linker sequences. NES, nuclear export signal. (d) Heterologous expression of wild-type SopB induces PM-translocation of bPHx2. Representative confocal micrographs of mCherry-tagged bPHx2 (inverted grey) co-transfected with EGFP-SopBC460S or EGFP-SopBWT. (e) Hypothesized PPIns conversions catalysed by SopB and potential modulation by host phosphatases. In the presence of PtdIns(4,5)P2 in vitro, SopB generates the species PtdIns(3,4,5)P3, PtdIns(3,4)P2, and PtdIns(3)P. The latter species are hypothesized to arise, at least in part, by sequential dephosphorylation of PtdIns(3,4,5)P3. INPP5B/E, SYNJ1/2, and SHIP1/2. In vivo, Salmonella infection favours the accumulation of PtdIns(3,4,5)P3 likely due to the high basal activity of host 5-phosphatases (INPP5B, SYNJ1/2, SHIP1/2, and others) that rapidly convert PtdIns(3,4,5)P3 to PtdIns(3,4)P2. It remains unclear if the rapid clearance of PtdIns(3,4)P2 following fission of the Salmonella-containing vacuole from the PM is due to the intrinsic activity of SopB or to the activity of host 4-phosphatases (INPP4A/B). Nonetheless, SopB is sufficient to give rise to PtdIns(3)P in vitro from PtdIns(4,5)P2 by SopB, or indirectly by dephosphorylation of products of the phosphotransferase reaction. Source numerical data are available in source data.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
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- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Acquisition settings and capture were controlled by Volocity software v6.2.1 (PerkinElmer), by Zen Black software v2.3 (Carl Zeiss), and by NIS-Elements AR-5.21.02 (Nikon Instruments Inc.). Li-COR Image Studio v4.0 software

Data analysis

Data were imported into the open access image analysis software Fiji (ImageJ 1.53c) for analysis utilizing previously described, open access plugins and features. Data were imported into GraphPad Prism 9 software for statistical analysis and the presentation of data. Images were generated using Adobe Illustrator CSG v16.0.0 and Adobe Photoshop v21.1.1. Li-COR Image Studio Lite v5.2

Data were imported into the open access image analysis software Fiji v1.53f1 for analysis and utilized previously described, open access plugins and features. Custom-written macros have been uploaded to [https://github.com/walpoleg/Walpole-et-al-Nature-Cell-Biology-2022](https://github.com/walpoleg/Walpole-et-al-Nature-Cell-Biology-2022). Resulting data were imported into GraphPad Prism 9 software for statistical analysis and graphical presentation.

For manuscripts utilizing custom algorithms or software that are not central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

As listed in the main manuscript, all data sets generated during and/or analysed during this study are available from the corresponding authors on reasonable request.
The large number of files, folder structure and file type do not make these readily compatible with available repositories.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.
☒ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No statistical method was used to predetermine sample size. Sample sizes were determined based on standard practices in the host-pathogen field (PMID: 31339948, 31851926, 34349110, 30002242, 28426838), quantitative studies of lipid sensors (PMID: 32211894, 30591513, 32713893), and previous HIP-C-MS studies (PMID: 29056375, 21728744). This was further guided by variability of measurements within and across trial repeats. For transparency, both trial averages and individual measurements are presented as ‘SuperPlots’ (PMID: 32346721) whenever possible.

Data exclusions
No data were excluded.

Replication
The main findings of this study were reproduced using pharmacological, RNA-interference, bacterial infection, heterologous expression and optogenetic approaches in mammalian cell culture models. Findings were validated in additional model organisms and cell-free assays. All experiments were repeated in the number of replicates specified in the Methods and individual Figure Legends and replicates were successful based on statistical analysis.

Randomization
Samples were allocated from a common cell population into experimental groups (RNAi, infection, transfection, treatment) or were allocated by genotype.

Blinding
Investigators were not blinded to group allocation during data collection or analysis. Blinding was not utilized due to the large number of experimental modalities (see above) that required knowledge of inherent variables (i.e. fluorophores, strains, temperature, duration) and were demanding on available resources.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|------------------------|
| ☒   | Antibodies             |
| ☒   | Eukaryotic cell lines  |
| ☒   | Palaeontology and archaeology |
| ☐   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data          |
| ☒   | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|------------------------|
| ☒   | ChIP-seq               |
| ☒   | Flow cytometry         |
| ☒   | MRI-based neuroimaging |
Antibodies

The Materials and Methods section contains the commercial source and catalogue numbers of all utilized antibodies. Additional information including dilutions and the antibody registry number is included in supplementary table 3.

Validation

Antibodies used in this study have Research Resource Identifiers (RRIDs) for transparency and are commercially available.

Mouse anti-AKT mAb #2920 (40D4) >1000 citations on manufacturers website.

Phospho-Akt (Ser473) [DE3] XP® Rabbit mAb #4050. As expected the signal is abolished with PI3K inhibitors in both our experiments and on the manufacturers website.

Phospho-Akt (Thr308) Antibody #9275 >1100 citations on manufacturers website.

Mouse anti-GAPDH (EMD Biosciences) has 248 citations on the antibody registry.

Rabbit pAb anti-PI3K-C2α [ProteinTech]. Validated by manufacturer using siRNA.

Anti-c-Myc antibody [Thermo Fisher] validated by manufacturer by heterologous expression in cells that do not express c-myc and blocked by specific peptide.

Anti-vinculin (EMD) manufacturer provides 5 references on website. We previously used in Ostrowski PP, et al. (2019) Dynamic Podosome-Like Structures in Nascent Phagosomes Are Coordinated by Phosphoinositides. Developmental cell, 50(4), 357-410.e3.

Rabbit anti-Salmonella O antigen (Fisher) has not been validated. Although in our experiments it only reacts with bacteria and not host cells as predicted. No information from manufacturer.

Mouse anti-Cdc42 (BD Biosciences) not validated by manufacturer used by one other paper Lin et al., 2019 Immunity

Mouse anti-alpha-tubulin (Sigma) from manufacturer’s website enhanced validation by independent antibodies.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

HeLa CCL-2 cell stocks of low passage number (<5) were obtained from the American Type Culture Collection (ATCC).

Authentication

As cell lines were obtained directly from the American Type Culture Collection, authentication was not performed.

Mycoplasma contamination

"HeLa (CCL-2) and Henle 407 (CCL-6) were tested by ATCC prior to receipt and tested negative upon receipt from the manufacturer. No additional screening was performed before experimentation."

Commonly misidentified lines

No cell lines listed in the ICLAC register (v10) were used in the current study.