Modulation of membrane phosphoinositide dynamics by the phosphatidylinositol 4-kinase activity of the *Legionella* LepB effector

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*Bacterial pathogens interact intimately with host membrane trafficking to promote infection and pathogenesis. Extracellular bacteria may target the autophagosome membrane transport to inhibit proinflammatory cytokine secretion. Intracellular free-living bacteria escape rapidly from the endosome and avoid being targeted by the autophagy-mediated host defence. For bacteria adapted to live in a vacuole compartment, they often intercept the endosome/phagosome-to-lysosome flux and remodel the endosome/phagosome-derived vacuole into a replication-permissive niche. In these processes, secreted virulence factors or effectors are the major bacterial weapons. These have been well documented in pathogens such as *Salmonella enterica* serovar Typhimurium, *Shigella flexneri* and *Legionella pneumophila*. These effectors, often endowed with unique biochemical activities, commonly target central players in the host membrane trafficking system, particularly small GTPases such as Rab or their effectors and regulators.

Gram-negative *L. pneumophila* has been a prototype for studying bacterial interaction with host membrane trafficking1–5. *L. pneumophila* infects amoebae and macrophages and requires a Dot/Icm type IV secretion system to establish a *Legionella*-containing vacuole (LCV). The LCV membrane mainly contains PtdIns4P, which is important for anchoring many secreted *Legionella* effectors onto the LCV. Here, we identify a cryptic functional domain (LepB_NTD) preceding the well-characterized RabGAP domain in the *Legionella* Dot/Icm type IV secretion system effector LepB. LepB_NTD alone is toxic to yeast and can disrupt the Golgi in mammalian cells. The crystal structure reveals an unexpected kinase fold and catalytic motif important for LepB_NTD function in eukaryotes. Cell biology-guided biochemical analyses uncovered a lipid kinase activity in LepB_NTD that specifically converts PtdIns3P into PtdIns(3,4)P$_2$. PtdIns(3,4)P$_2$ is efficiently hydrolysed into PtdIns4P by another Dot/Icm effector SidF that is known to possess phosphoinositide phosphatase activity. Consistently, SidF is capable of counteracting the cellular functions of LepB_NTD. Genetic analyses show a requirement for LepB kinase activity as well as lipid phosphatase activity of SidF for PtdIns4P biosynthesis on the LCV membrane. Our study identifies an unprecedented phosphatidylinositol 4-kinase activity from bacteria and highlights a sophisticated manipulation of host phosphoinositide metabolism by a bacterial pathogen.

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LepB RabGAP domain functionally resembles the type III secretion RabGAP effectors VirA from *S. flexneri* and EspG from enteropathogenic *E. coli* (EPEC) \(^{2,13,23}\). Ectopic expression of VirA in *Saccharomyces cerevisiae* can block cell growth in a RabGAP activity-dependent manner. Similarly, expression of full-length LepB (ref. 24) or its RabGAP domain also results in lethality in yeast (Fig. 1a). Mutation of the RabGAP catalytic arginine (Arg444) abolished yeast growth inhibition caused by the LepB RabGAP domain, but, surprisingly, the same mutation on full-length LepB showed no such effect (Fig. 1a). This suggested that another cryptic domain in LepB may harbour an independent activity capable of inhibiting yeast growth. Consistent with this prediction, progressive truncation analyses identified residues 1–311 in LepB (designated as LepB_NTD hereafter) that, on their own, could severely inhibit the growth of *S. cerevisiae* (Fig. 1a). Thus, these analyses unexpectedly unveil a cryptic functional domain LepB_NTD in the LepB effector.

LepB_NTD can disrupt the Golgi structure and function in mammalian cells. Many Dot/Icm effectors can alter intracellular vesicle trafficking when expressed in mammalian cells, and this property has been helpful in defining the functional mechanism of the effectors. Prompted by this notion, we examined several intracellular organelles in cells expressing LepB_NTD. Overexpression of LepB_NTD in HeLa cells did not affect the morphology of the ER and the lysosome (Supplementary Fig. 1a), but caused a complete disruption of the cis- and trans-Golgi structures (Fig. 1b). When full-length LepB_NTD (LepB_NTD-TM) was expressed in mammalian cells, it was able to disrupt the Golgi structure and function. LepB_NTD (LepB_NTD-TM) caused the same disruption of medial- and trans-Golgi structures stained by the anti-ManII and anti-TGN46 antibodies, respectively (Fig. 1c). The cis-Golgi was less affected and only a weak and partial dispersion of anti-GM130-stained cis-Golgi structure could be observed. LepB contains a predicted transmembrane (TM) motif (residues 1234–1256) at its C terminus, and fusion of the TM motif to the C terminus of LepB_NTD (LepB_NTD-TM) caused the same disruption of medial- and trans-Golgi structures (Fig. 1c). When full-length

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**Figure 1 | Identification of LepB_NTD, which can inhibit yeast growth and disrupt the Golgi structure in mammalian cells.** a. Yeast toxicity assay, showing a cryptic functional domain (NTD, residues 1–311) in LepB. *S. cerevisiae* W303a is transformed with a plasmid expressing LepB or indicated LepB mutants under a galactose-inducible promoter. FL, full length. Residues 313–618 encode the RabGAP domain in LepB, and R444K is the RabGAP activity-deficient mutant. b. LepB_NTD exhibits prominent activity in disrupting medial- and trans-Golgi structures. RFP-tagged LepB_NTD alone (b) or fused to the C-terminal transmembrane domain (TM) (c) or LepB R444K mutant (b,c) was transfected into HeLa cells. Cells were subjected to immunofluorescence staining using anti-GM130, ManII and TGN46 antibodies (the markers for cis-, medial- and trans-Golgi structures, respectively). Statistics of cells showing Golgi disruption (mean ± s.d.) are listed in the corresponding fluorescence images (at least 100 cells were counted for each experiment). d. LepB_NTD blocks VSV-G trafficking from the Golgi to the plasma membrane. VSV-G-GFP-expressing HeLa cells transfected with a plasmid expressing RFP or RFP-LepB_NTD were incubated at 42 °C for 16 h and then shifted to 32 °C for 30 min or 4 h. Shown are confocal fluorescence images of VSV-G-GFP localization. Scale bars in b–d, 20 μm. Data shown in a–d are representative of three independent experiments.
LepB R444K mutant devoid of RabGAP activity was expressed in HELa cells, fragmentation of medial and trans-Golgi also occurred (Fig. 1c). The latter two constructs showed a slightly more prominent effect than LepB_NTD alone. The Golgi disruption activity of LepB_NTD was further confirmed by using other trans-Golgi markers including GalT-GFP, GalNAc-T2-GFP (N-acetylgalactosaminyltransferase-2) and p230/golgin-245 (Supplementary Fig. 1b).

Severe perturbation of the Golgi structure by LepB_NTD is anticipated to inhibit the exocytic pathway. This idea was confirmed by the observation that LepB_NTD expression in HELa cells inhibited the secretion of human growth hormone (hGH) (Supplementary Fig. 1c). The effect of LepB_NTD on the anterograde transport was also investigated by assaying the trafficking of the temperature-sensitive mutant (ts045) of vesicular stomatitis virus glycoprotein (VSV-G) in 293T cells. At 32 °C, which restricted the correct folding of VSV-G, VSV-G-GFP showed expected accumulation in the ER. When cells were shifted to the permissive temperature of 37 °C for 30 min, most VSV-G-GFP proteins were transported to the Golgi and post-Golgi compartments (Supplementary Fig. 1d). In contrast, the sensitivity of VSV-G in LepB_NTD-expressing cells remained constant even after 2 h release into the permissive temperature (Supplementary Fig. 1d), which agrees with its retention in the ER and cis-Golgi (Fig. 1d). The defective intra- and post-Golgi trafficking of VSV-G caused by LepB_NTD supports the disruption of medial- and trans-Golgi structures observed already.

Crystal structure reveals a kinase fold of LepB_NTD. To understand the mechanism of LepB_NTD function, we determined a 2.75 Å crystal structure of LepB (residues 1–618) containing both the NTD and RabGAP domain (Fig. 2a and Supplementary Table 1). There are two LepB molecules in an asymmetric unit, and the structures of the two chains are nearly identical (Chain A was chosen for further analyses). The NTD and RabGAP domain are connected by a long loop (residues 307–326), among which the electron densities for residues 311–318 are missing due to the flexible conformation of the loop. The relative orientation of the two domains is fixed to a certain degree by two crystal-packing contacts (Supplementary Fig. 2a). One is the back-to-back contact on the RabGAP domains of the two LepB molecules in the same asymmetric unit, as a result of which the concavities formed by the NTD and the RabGAP domain in each LepB face opposite directions. The orientation between the two domains is also stabilized by a head-to-tail contact between two neighbouring LepB molecules related by the crystallographic two-fold axis (Supplementary Fig. 2a).

The structure of the apo-RabGAP domain in LepB (1–618) is identical to that in the Rab1 complex11–13. The NTD adopts an independent fold completely separate from the RabGAP domain. Three flexible loop regions (Lys15–Asp23, Ser106–Asn133 and Glu164–Glu167) are missing due to the lack of clear electron density. At the primary amino acid level, LepB_NTD shares no conspicuous sequence homology to known proteins in the database. Interestingly, when the structure of the NTD was subjected to DALI searches, hits returned from the DALI server with a Z-score >5 are all kinases. The top two unique ones (Z-score of ~10) are CTKA, a Ser/Thr kinase from Helicobacter pylori22 and the actin-fragmin kinase from a plasmid of Phasus marinus28 (Supplementary Fig. 2b). CTKA and the actin-fragmin kinase bear little sequence homology to protein kinases in eukaryotes and are considered to be atypical kinases. Similar to CTKA and the actin-fragmin kinase, LepB_NTD adopts a kinase-like fold consisting of two lobes with a possible ATP-binding groove in between (Fig. 2b). The N-terminal lobe (Ile9–Pro92) is mainly formed by four antiparallel β-strands (β1–β4) and a large (a1) and small helix (α2). The a1 helix (also referred to as αC) is highly conserved among most known kinases. The C-terminal lobe (Phe96–Met310) contains a three-stranded antiparallel β-sheet (β5–β7) flanked by α3 and the α4–α11 helical bundle. The main structural units in LepB_NTD, along with their topological arrangements, resemble of CTKA and the prototypical kinase PAK (Fig. 2b and Supplementary Fig. 2c), although the C-lobe helical bundle in CTKA and PAK is packed more tightly with the N-lobe helices than that in LepB_NTD.

The kinase catalytic centre and its importance for LepB_NTD function in eukaryotic cells. We also compared the catalytic motif structure of LepB_NTD with that of CTKA (Fig. 2c). Lys37 in CTKA, similar to that in most kinases, has strong ionic interactions with Glu57 and Asp179 (in the conserved DFG motif) and also forms direct contact with the α-phosphate moiety of ATP27. These interactions are critical for stabilizing the active-site structure. Equivalent residues in the structure of LepB_NTD, including Lys39, Glu49 and Asp176 (also in a DFG motif), adopt similar structural arrangements and side-chain interactions (Fig. 2c). Asp179 and Asn160 in CTKA coordinate the Mg2+ ion in the nucleotide ligand, and two structurally equivalent residues (Asp176 and Asn157) are also present in LepB_NTD. Interestingly, as in other kinases, Asp155 in CTKA serves as the general base to activate the to-be-phosphorylated substrate, but the corresponding residue in the primary sequence of LepB_NTD is a serine (Ser152). At the three-dimensional level, a histidine residue (His154) in LepB_NTD occupies the position corresponding to Asp155 in CTKA (Fig. 2c).

To investigate whether LepB_NTD functions as a kinase in eukaryotic cells, mutagenesis of Lys39, His154, Asn157 and Asp176 in LepB_NTD was performed. Mutation of any of the four residues abolished the activity of LepB_NTD in blocking yeast cell growth (Fig. 2d). We also noted that Arg174, near Asp176 within the catalytic site, could be substituted by a lysine but not an alanine. Mutation of any of the above residues resulted in functional loss of LepB_NTD in disrupting the medial- and trans-Golgi structure as well as blocking anterograde transport of VSV-G (Fig. 2e,f and Supplementary Fig. 2d,e). The strict requirement of His154 for the cellular function of LepB_NTD supports the idea that the histidine may serve as the general base to activate the substrate either directly or indirectly through a water molecule. A similar situation has been noted in the structure of mammalian PI3Kγ, in which His948 rather than Asp946 is proposed to act as the general base29. These results indicate that LepB_NTD functions as a kinase in disrupting the Golgi and modulating membrane trafficking in eukaryotic cells.

The kinase activity of LepB_NTD modulates cellular phosphoinositides distribution. When the structure of LepB (1–618) was superimposed onto that of the Rab1-bound RabGAP domain, it was evident that the space between the kinase domain and the RabGAP domain, to a maximal extent, could only accommodate a Rab1-size substrate (Supplementary Fig. 3a). An intuitive idea is that LepB_NTD may phosphorylate Rab1 or another Rab protein. To this end, we employed the yeast lethality assay and examined whether overexpression of a Rab protein could reverse the toxicity of LepB_NTD. The results showed that Rab1, as well as its S25N and Q70L mutants locked in the GTP- and GDP-bound states,
Figure 2 | Crystal structure reveals that LepB_NTD is an active atypical kinase. 

a, Overall structure of LepB (1–618) determined by X-ray crystallography. The structure shows two well-separated NTD and RabGAP domains. 
b, Structural comparison of LepB_NTD with those of CTKA (cell-translocating kinase A) and PKA (protein kinase A). The secondary structures of LepB_NTD are labelled as shown. 
c, Comparison of the putative catalytic centre in LepB_NTD with those of CTKA and PKA. Top: catalytic loop sequence comparison of LepB, CTKA and PKA. Bottom: structures of residues involved in catalysis and binding to the nucleotide (CTKA structure, Protein Data Bank accession code 3AKL). The structures are shown as sticks, and Mg\(^{2+}\) as green spheres. Hydrogen/salt bonds are indicated by dashed lines. The structure of AMP-PNP (adenylyl-imidodiphosphate, a nonhydrolyzable ATP analogue) is from the CTKA structure. 
d, Role of putative catalytic residues in LepB_NTD in inhibiting yeast growth. S. cerevisiae W303a cells were transformed with a plasmid expressing LepB_NTD or an indicated point mutant of LepB_NTD under a galactose-inducible promoter. 
e, Effects of His154 mutation on LepB_NTD disruption of the Golgi structure. HeLa cells were transfected with a plasmid expressing RFP alone or RFP-LepB_NTD (wild type (WT) or the H154A mutant). The medial- and trans-Golgi were stained by the anti-ManII and TGN46 antibody, respectively; confocal fluorescence images of the cells are shown. 
f, Effects of His154 mutation on LepB_NTD blocking of VSV-G trafficking. Scale bars in e, f, 20 µm. Experiments were performed in a manner similar to that in Fig. 1d except that the H154A mutant of LepB_NTD was included in the assay. Data shown in d–f are representative of at least three independent experiments.
respectively, could not counteract the growth inhibition caused by LepB_NTD (Supplementary Fig. 3b). In contrast, overexpression of wild-type Rab1 could effectively block the yeast toxicity of the LepB RabGAP domain. We also profiled all the yeast Rab1s and found that none of them showed a rescue activity (Supplementary Fig. 3b). These data argue against the possibility that LepB_NTD directly phosphorylates a Rab protein for its functioning.

During the course of analysing the cell biological function of LepB_NTD, we noticed that selective disruption of mediat- and trans-Golgi structures by LepB_NTD resembles that caused by overexpression of the PtdIns4P-binding pleckstrin-homology (PH) domain from the oxysterol binding protein (PHOSBP)30. We confirmed that overexpression of GFP-PH_{PHOSBP} or a similar PtdIns4P-binding PH domain from FAPP1 (GFP-PH_{FAPP1}) in HeLa cells resulted in disruption of the TGN46-stained trans-Golgi but not GM130-stained cis-Golgi structure (Supplementary Fig. 4a). Phosphatidylinositol phosphates or phosphoinositides play pivotal roles in defining the organelle identity and controlling membrane trafficking by recruiting specific effectors to the membrane. We therefore checked the effects of LepB_NTD on the distribution of various cellular phosphoinositides imaged by fluorescent probes31. Three probes were found to exhibit a perturbed staining pattern upon RFP-LepB expression in HeLa cells. PHAKT-GFP, which binds to both PtdIns(3,4)P_{2} and PtdIns(3,4,5)P_{3}, and GFP-PH_{TAPP1}, which is highly specific for PtdIns(3,4)P_{2}, showed a prominent change from a diffused localization to bright punctate dots (Fig. 3a). Staining of the PtdIns3P-specific probe GFP-2xFYVE_His was nearly abolished by LepB expression (Fig. 3a). The effects on the distribution of these phosphoinositides were similarly observed with LepB_NTD alone (Supplementary Fig. 4b). LepB_NTD expression also altered the distribution of the PtdIns(3,4,5)P_{3}-specific probe GFP-PH_{TAPP1} (Supplementary Fig. 4b). Importantly, LepB_NTD did not affect the distribution of GFP-PH_{FAPP1}, GFP-PH_{PHOSBP}, and GFP-PH_{cisGolgi}, and GFP-PH_{transGolgi}, which stain PtdIns4P, PtdIns(4,5)P_{2}, PtdIns3P and phosphatidylinerine (PS), respectively (Supplementary Fig. 4c). Alteration of PtdIns3P, PtdIns(3,4)P_{2} and PtdIns(3,4,5)P_{3} distribution by LepB_NTD was more prominent than that by full-length LepB (Fig. 3a and Supplementary Fig. 4b), and this phenomenon is consistent with the higher expression level of LepB_NTD (Supplementary Fig. 4d). Notably, the kinaseinactive H154A mutants of LepB and LepB_NTD were inactive in disrupting the distribution of PtdIns3P, PtdIns(3,4)P_{2} and PtdIns(3,4,5)P_{3} (Fig. 3a and Supplementary Fig. 4b). Thus, LepB relies on its putative kinase activity to modulate the distribution of cellular phosphoinositides.

LepB_NTD is a phosphatidylinositol 4-kinase specifically acting on PtdIns3P. The above observation prompted us to hypothesize that LepB_NTD is a phosphoinositide kinase. This idea was supported by the facts that LepB_NTD is an atypical kinase and also that the eukaryotic phosphatidylinositol 3-kinase catalytic subunit was ranked the third top hit among structural homologues of LepB_NTD retrieved from the DALI server (Supplementary Fig. 2b). To test this hypothesis, a panel of phosphoinositides were subjected to in vitro kinase assays32 using bacterially purified LepB_NTD or its catalytic-site mutant (H154A). Strikingly, LepB_NTD could readily and robustly phosphorylate PtdIns3P. The specific activity of LepB_NTD was comparable to that of human PI4KIIIβ, which, as expected, only phosphorylated phosphatidylinositol (PtdIns) (but not PtdIns3P) on the D4 hydroxyl of the inositol ring (Fig. 3b). The H154A mutant showed no PtdIns3P kinase activity. LepB_NTD exhibited no kinase activity towards PtdIns, PtdIns4P, PtdIns5P, PtdIns(3,4)P_{2}, PtdIns(3,5)P_{2} or PtdIns(3,4,5)P_{3} (Fig. 3b,c). An extremely weak but detectable activity was observed with PtdIns(4,5)P_{2} (Fig. 3c). These data highlight a substrate selectivity of LepB_NTD kinase for PtdIns3P. To further investigate the enzymatic nature of LepB_NTD phosphoinositide kinase activity, a fluorescent phosphoinositide-based thin-layer chromatography (TLC) method was used to determine the product phosphoinositide generated by LepB_NTD. Incubation of PtdIns3P with LepB_NTD, but not its H154A mutant, generated a phosphoinositide species exhibiting slower migration on the TLC plate (Fig. 3d). The mobility of this phosphoinositide product was reversed back to the position of a phosphatidylinositol monophosphate upon further reaction with Sdf, a L. pneumophila Dot/Icm effector known to have lipid phosphorylase activity specifically hydrolysing the D3 phosphate of PtdIns(3,4,5)P_{3} and PtdIns(3,4)P_{2} (ref. 33). The product of Sdf could be further hydrolysed by a yeast lipid phosphatase Sac1p that dephosphorylates both PtdIns3P and PtdIns4P into PtdIns (Fig. 3d). Meanwhile, the phosphoinositide product generated by LepB_NTD resisted dephosphorylation by another yeast lipid phosphatase Fsp4p that selectively targets the D5 phosphate of PtdIns(3,5)P_{3} (ref. 34; Fig. 3d). These analyses firmly establish that LepB_NTD is a phosphatidylinositol 4-kinase (PI4K) that specifically converts PtdIns3P into PtdIns(3,4)P_{2}. Such enzymatic activity agrees well with the cell biology observation that the kinase activity of LepB_NTD could disrupt PtdIns3P localization and alter the distribution of PtdIns(3,4)P_{2} in HeLa cells (Fig. 3a).

Mammalian PI4Ks are classified into two major classes—type-II PI4K (PI4KII) and type-III PI4K (PI4KIII)34—both of which adopt the two-lobe canonical kinase fold35–38 and use PtdIns as the substrate. Despite the different phosphoinositide substrates used, structure-based alignment shows a sequence homology within the catalytic domains of mammalian PI4Ks and LepB_NTD, including most of the catalytic residues examined above (Supplementary Fig. 5). Kinetic analyses further revealed that the K_{m} (the Michaelis constant) values of LepB_NTD for PtdIns3P and ATP were ~26 μM and 72 μM, respectively (Fig. 3e,f), which are comparable to those of human PI4KII (ref. 35).

LepB_NTD works together with the PtdIns(3,4)P_{2} 3-phosphatase effector Sdf in PtdIns4P synthesis on the LCV. It is well established that kinases and phosphatases work together to generate a particular phosphoinositide and precisely regulate phosphoinositide metabolism. Bioinformatics analyses suggest that L. pneumophila encodes more than 20 putative phosphatase effectors, and some of them might function in establishing the LCV (refs 33,39). We then experimentally profiled the activity of these effectors using a panel of the seven different phosphoinositide substrates, but could only detect the phosphoinositide phosphatase activity in Sdf (Lp2584) and SdfP (Lp0130) (Supplementary Fig. 6). Consistent with previous reports33,39, both Sdf and SdfP showed phosphatidylinositol 3-phosphatase activity; SdfP targeted PtdIns3P and PtdIns(3,5)P_{2}, while Sdf preferred PtdIns(3,4)P_{2} and PtdIns(3,4,5)P_{3} as substrates. Consistent with these observations, in vitro studies showed that Sdf could act on the product of LepB_NTD kinase activity and convert LepB_NTD-synthesized PtdIns(3,4)P_{2} into PtdIns4P (Fig. 3d), a Golgi-enriched phosphoinositide. At the cellular level, only Sdf, but not SdfP or any of the other putative Legionella phosphatase effectors, was capable of suppressing the toxicity of LepB_NTD in yeast cells (Fig. 4a). Accordingly, expression of Sdf but not SdfP in HeLa cells could rescue LepB_NTD-induced abnormal distribution of PHAKT-GFP, GFP-PH_{TAPP1} and GFP-PH_{FAPP1}-probed phosphoinositides (Fig. 4b). Thus, Sdf could function downstream of LepB_NTD and hydrolyse LepB_NTD-synthesized PtdIns(3,4)P_{2} into PtdIns4P.

It is known that mature LCV contains mainly PtdIns4P. Many of the Dot/Icm effectors such as SidC, SdcA and SidM/DrrA are correctly localized onto the LCV surface through binding to PtdIns4P (refs 12,20,40). The PtdIns4P-mediated targeting is essential for appropriate functioning of these effectors during L. pneumophila infection. The functional relationship between the PtdIns3P 4-kinase activity of LepB_NTD and the PtdIns(3,4)P_{2} 3-phosphatase activity of Sdf suggests a
plausible route for synthesizing the PtdIns4P of the LCV from PtdIns3P. To test this, we made use of the SidC reporter system and examined its LCV localization in L. pneumophila-infected bone marrow macrophage cells. Similar to reports from a previous study, the recruitment of SidC onto the LCV surface was markedly impaired by genetic deletion of sidF from L. pneumophila (Fig. 4c,d). Deletion of lepB caused a similar reduction in SidC localization on the LCV, which could be fully restored by expression of wild-type

Figure 3 | LepB_NTD functions as a phosphatidylinositide 4-kinase to convert PtdIns3P into PtdIns(3,4)P2. a. Disruption of cellular phosphoinositides distribution by the kinase activity of LepB_NTD. RFP or RFP-tagged LepB or the LepB H154A mutant was co-transfected into HeLa cells with a fluorescence phosphoinositide probe (PHACT for PtdIns(3,4,5)P3/PtdIns(3,4)P2, PHAPP1 for PtdIns(3,4)P2, and 2xFYVEHrs for PtdIns3P). Shown are confocal fluorescence images of transfected cells. Scale bar, 20 μm. b–d. In vitro phosphoinositide kinase assay of LepB_NTD. Purified LepB_NTD (WT or H154A) or human PI4KIIIβ (residues 81–801) was reacted with the indicated phosphoinositide substrates. The ADP-Glo assay was used to measure the transfer of the phosphate. PI, phosphatidylinositol or PtdIns; RLU, relative luminescence units. d. TLC assay of the reaction product of LepB_NTD phosphoinositide kinase activity; di-C8-Bodipy-FL-PI(3)P was reacted with LepB_NTD. SidF that can dephosphorylate PI(3,4)P2 or Fig4P, which acts on PI(3,5)P2, was then added into the reaction. The SidF-reacted samples were further treated with Sac1p, which dephosphorylates both PtdIns3P and PtdIns4P into PtdIns. A schematic diagram illustrating the enzymatic reactions of different enzymes is shown under the fluorescence image. PI, phosphatidylinositol or PtdIns; PIP, phosphatidylinositol monophosphate; PIP2, phosphatidylinositol bisphosphate. e,f. Kinetic parameters of LepB phosphatidylinositide 4-kinase activity. Reactions were performed with purified LepB_NTD and varying concentrations of the PtdIns3P (e) or ATP (f) substrate. Data are plotted as means from six replicate assays and the Km values were determined. V, velocity. Data shown in a–d are representative of at least three independent experiments.
LepB but not the kinase-inactive H154A mutant (Fig. 4c,d). This indicates a critical role of the PtdIns3P 4-kinase activity of LepB in establishing PtdIns4P on the LCV. Moreover, double deletion of sidF and lepB caused no additive effect on blocking SidC recruitment onto the LCV (Fig. 4c,d), supporting that the two effectors function sequentially in the same pathway. Moreover, LepB and SidF contributed little to disruption of the Golgi structure in L. pneumophila-infected macrophages (Supplementary Fig. 1e). Thus, the phosphoinositide kinase activity of LepB appeared to only affect local phosphoinositide metabolism on the LCV.

Discussion

Taken together, our studies illustrate a possible model for PtdIns4P biosynthesis on the LCV alongside its maturation (Supplementary Fig. 7). On entry into host macrophages, vacuole-residing L. pneumophila deploys the Dot/Icm effector LepB to phosphorylate PtdIns3P at the D4 hydroxyl position to generate PtdIns(3,4)P2. The phosphoinositide phosphatase effector SidF then converts PtdIns(3,4)P2 into PtdIns4P. It is necessary to point out that this model is incomplete regarding the source of PtdIns3P on the LCV. The LCV is derived from the plasma membrane and resists...
fusion with PtdIns3P-enriched early endosomes. Inactivation of Rab5 is indeed positive for L. pneumophila infection, and the bacterium has even evolved a phospholipase effector VipD to consume PtdIns5P-enriched early endosomes. The LCV has extensive interactions with ER-derived vesicles, and it has been shown that L. pneumophila subverts the function of host proteins, including lipid kinase and phosphatase, that regulate ER–plasma membrane contact sites. Therefore, host lipid kinase or other Dot/Icm effector kinases may contribute to generate PtdIns3P at the ER–plasma membrane contact sites, and the dynamic of PtdIns4P generation on the LCV must be complex (Supplementary Fig. 7). Moreover, homologues of LepB and SidF are present in most L. pneumophila clinical isolates, but do not always coexist in other Legionella species such as L. longbeachae. This further highlights the complexity of how Legionella modulate host phosphoinositide metabolism.

Phosphoinositides have recently emerged as key regulators of pathogen infection. Many bacterial effectors rely on binding to a specific phosphoinositide for subcellular targeting and appropriate functioning. L. pneumophila have evolved both phosphoinositide phosphatase and kinase activities. LepB is the first bacterial virulence factor shown to have phosphoinositide kinase activity (despite the fact that L. longbeachae also encodes a protein predicted to have PtdIns kinase activity). In contrast, phosphoinositide phosphatase activity has been found in several bacterial pathogens including Mycobacterium tuberculosis, S. enteric serovar Typhimurium, S. flexneri and Vibrio paraenemolyticus. Thus, it is likely that there are other yet to be identified bacterial phosphoinositide kinase effectors playing important functions in host cells. LepB_NTD is a unique phosphoinositide kinase in that it selectively modifies PtdIns3P and adds a phosphate on the D4 position phosho of the inositol ring. LepB_NTD could serve as a useful biochemical and cell biological tool for studying phosphoinositide dynamics and membrane transport. Given the scarce knowledge about the lipid substrate recognition of known phosphoinositide kinases and that LepB_NTD is an atypical kinase, how LepB_NTD achieves specific recognition of the PtdIns3P substrate remains an interesting question for future studies. The biochemical property of LepB_NTD is most similar to that of PIP4Ks, but the latter are homo- or heterodimers with the most divergent structure from that of canonical protein kinase. LepB_NTD has higher sequence similarity to PI4KIII than to P4KII (the former is more related to PI3K and protein kinases in sequence and structure than PI4KII). The putative catalytic histidine in LepB_NTD (His154) is conserved in PI4KIIIs and PI3K but not in PI4KII (ref. 37), and a similar catalytic histidine has been proposed for PIP4K (ref. 29). LepB_NTD also exhibited a weak PI3K activity on PtdIns(4,5)P2 (Fig. 3c). These findings indicate a possible evolutionary connection between LepB_NTD and eukaryotic PI4K family.

It is interesting to note that human PI4KIIIβ binds to the active form of a Rab protein (Rab11) using a helical domain adjacent to the kinase domain, which is important for the recruitment of Rab11 effectors to the Golgi and membrane transport from the Golgi to the plasma membrane. Architecturally, the large size of LepB and the transmembrane motif at its extreme C terminus should allow its N-terminal half region lying on the LCV membrane with the NTD to be accessible to membrane lipid substrates.

### Methods

**Plasmids, antibodies and reagents.** DNA for LepB, SidF and SidP was PCR-amplified from the genomic DNA of L. pneumophila Philadelphia-1 strain and inserted into the pCMV-ADP vector. DNA fragments for ADP-Glo assay (Promega) were purchased from the ATCC. Antibodies and reagents were from BioRad, SeraLab and Echelon Biosciences. The ADP-Glo kinase assay kit (V9102) was purchased from Promega.

**Cell culture, transfection and immunofluorescence assay.** HeLa and 293T cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% FBS and 2 mM l-glutamine at 37 °C in a 5% CO2 incubator. Transfection was carried out using Lipofectamine 2000 reagent. Primary bone marrow macrophages were prepared from 6-week to 3-month-old male A/J mice and differentiated in bone marrow macrophage media (RPMI, 1 mM glutamine, 20% FBS and 5892 cell-conditioned medium) for 7–8 days. All cell lines were tested for mycoplasma using the commonly used PCR method. The cells were frequently checked for their mycoplasma status, but not authenticated by short tandem repeat (STR) profiling. All animal experiments were conducted following the Ministry of Health national guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Use Committee at the National Institute of Biological Sciences.

For fluorescence staining, HeLa cells cultured on the coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), washed with PBS and then permeabilized for 10 min in PBS containing 0.5% Triton X-100. Cells were blocked with PBS for 30 min, followed by blocking with the primary and secondary antibodies for at least 1 h each. After removing the extracellular PtdIns(4,5)P2 in the extracellular fluid, bone marrow macrophages in culture were fixed, permeabilized for 10 min in PBS containing 0.5% Triton X-100, blocked with FBS for 30 min and stained with the anti-Legionella antibody and the Alexa Fluor-conjugated secondary antibody (Life Technology). To stain the total bacterial cells were further stained with 10 min 0.5% DAPI. All animal experiments were conducted following the Ministry of Health national guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Use Committee at the National Institute of Biological Sciences.

**Expression and purification of recombinant proteins.** E. coli BL21 (DE3) cells (Novagen) were used for the host for all recombinant expression. Protein expression was induced for 18 h at 16 °C with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after achieving a optical density at 600 nm (OD600) of 0.6–1.0. For purification of GST-tagged proteins, bacteria were centrifuged and resuspended in a phosphate buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by sonication and the cell lysates were centrifuged at 45,000g for 20 min at 4 °C. Supernatants were incubated with glutathione Sepharose 4B beads (GE Healthcare) for 1 h at 4 °C. The beads were washed three times and bound proteins were eluted with 20 mM reduced glutathione. For purification of His-tagged proteins, bacteria were collected and lysed in a buffer containing 10 mM Tris–HCl (pH 7.5), 300 mM NaCl and 2 mM imidazole supplemented with protease inhibitors. Centrifuged bacterial lysates were incubated with the Ni-NTA agarose (Qiagen) followed by washing with 25 mM imidazole. Proteins were eluted by 250 mM imidazole and then further purified by size-exclusion chromatography (GE Healthcare). Protein concentrations were determined spectrophotometrically using the theoretical molar extinction coefficient at 280 nm, and protein purity was examined by Coomassie blue staining of SDS–PAGE gels.

**In vitro ADP-Glo kinase and phosphatase assays.** White 96-well polystyrene plates were used for the ADP-Glo assay (Promega). Phosphoinositides were dissolved in reconstituted expression in E. coli and into the pI25-GPD vector for rescue expression in yeast. Expression in L. pneumophila LepB and SidP were inserted into the pBl908 vector. CDNA for D128 for OSBP (87–185 aa) and FAPPI (1–100 aa) were amplified from human liver cDNA and cloned into the pEGFP-C1 vector (Clontech Laboratories). Expression plasmid for VSV-G GFP was purchased from Addgene. Truncation and deletion mutants were constructed by the standard PCR cloning strategy. Point mutants were generated by the overlapping PCR cloning strategy. All plasmids were confirmed by DNA sequencing. Plasmids were prepared by GoldHi Endofree Plasmid Maxi Kit (Beijing CoWin Bioscience, product code CW2104) for transfection.

**Antibodies for GFP (sc-8334) and Legionella (sc-5025) were purchased from Santa Cruz Biotechnology, anti-Flag (M2) and TGN46 (T7576) antibodies from Sigma Aldrich, anti-GM130 (6108822) and p230 (611280) antibodies from BD Transduction Laboratories, anti-ManII antibody (ab12277) from Abcam, and anti-calcinein antibody (10427-2-AP) from Proteintech. The anti-SidA antibody was generated in house by the Antibody Core Facility at the National Institute of Biological Sciences, Beijing. All diC8 phosphoinositide, Bodipy FL C5, Alexa Fluor-conjugated secondary antibody (Life Technology) and the monoclonal mouse anti-HA (K-1500) were purchased from Echelon Biosciences.**

**The ADP-Glo Kinase Assay** (V9102) was purchased from Promega. Cell culture products were from Life Technology and Legionella agar base (#218301) was from BD Biosciences. All other chemicals were Sigma Aldrich products, unless otherwise noted.
vector-based complementation plasmids were introduced into homologous recombination method using the suicide plasmid pSR47. pJB908 medium with a starting OD600 of 0.1. When the OD600 reached above 3.5 after plates 2 days before infection. Bacteria patches were then inoculated into liquid AYE cultured in the absence of thymidine. For macrophage infection, fresh single charcoal yeast extract agar plates supplemented with 0.1 mg ml
DNA encoding LepB (1 galactose to assay the effect of LepB variants on yeast growth. For rescue experiments, the GAL1 promoter. The integration strain was then transformed with the p425 vector expressing an indicated Legionella CX,R-containing effector under the GFP promoter. The transformants were selected for survival on synthetic defined medium lacking base and leucine supplemented with 2% galactose and 1% raffinose as the carbon sources.

hGH trafficking and VSV-G transport assays. hGH trafficking assays were performed as described previously.2 HeLa cells plated in six-well dishes were transfected with 1.6 µg of 4×FKBP-hGH (Ariad Pharmaceutical) and 0.9 µg of pCS2-RFP vector expressing the target protein. At 18 h later, cells were switched into fresh medium containing 2 µM AP21998 compound or ethanol as a control and incubated for 2 h. The supernatant was collected and the released hGH was measured using an hGH ELISA kit (Roche Life Science) according to the manufacturer's instruction.

To monitor VSV-G trafficking from the ER to the plasma membrane, HeLa cells cultured on coverslips in six-well dishes were transfected with a plasmid expressing VSV-G-GFP (iso45) together with a plasmid expressing GFP-LepB, NTD (wild type or indicated mutants) or RFP alone. Transfected cells were incubated at 42 °C for 16 h and then were shifted to 32 °C to initiate VSV-G-GFP release from the ER. At indicated time points after release, the cells were fixed with 3.7% paraformaldehyde and fluorescence images were captured. To examine VSV-G sensitivity to Endo H digestion, the released VSV-G-GFP cells were collected in lysis buffer and the lysates were treated with 20 units of Endo H (New England Biolabs) at 37 °C for 1 h. Reactions were stopped by adding SDS loading buffer. Boiled samples were then loaded onto an 8% SDS–PAGE gel and analysed by anti-GFP immunoblotting.

Bacterial strains and infection. L. pneumophila strains were cultured on buffered charcoal yeast extract agar plates supplemented with 0.1 mg ml

References
9. Schouela, S., Oesterlin, L. K., Blankenhoff, W., Goody, R. S. & Itzen, A. RabGDI displacement by DrA from Rab1 inactivation is a consequence of its guanine nucleotide exchange activity. Mol. Cell 36, 1060–1070 (2009).
ARTICLES


36. Acknowledgements

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Author contributions

F.S. and N.D. conceived the study. N.D. and M.N. designed and performed the functional experiments. L.H. and Q.Y. determined the structure. R.Z. provided technical assistance. F.S. and N.D. analysed the data and wrote the manuscript. All authors discussed the results and should be addressed to N.D. and F.S.

Additional information

Supplementary information is available for this paper.

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Competing interests

The authors declare no competing financial interests.
Modulation of membrane phosphoinositide dynamics by the phosphatidylinositide 4-kinase activity of the *Legionella* LepB effector

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This file includes:

Supplementary Table 1
Supplementary Figures 1-8
# Supplementary Table 1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Crystal</th>
<th>LepB (1-618)</th>
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<td><strong>Data collection</strong></td>
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<td>I/σI</td>
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<tr>
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<tr>
<td>Redundancy</td>
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</tr>
</tbody>
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| **Refinement statistics** |              |
| R\(_{\text{work}}$/R\(_{\text{free}}\) (%)\(^b\) | 0.20/0.24 |
| No. of protein atoms | 8,850 |
| B-factors (Å\(^2\)) | 77.2 |
| RMSD bond lengths (Å) | 0.003 |
| RMSD bond angles (°) | 0.854 |

| **Ramachandran plot statistics** |              |
| Most favored regions (%) | 96.94 |
| Additional allowed regions (%) | 2.69 |
| Outlier regions (%) | 0.37 |

\(^a\) The data for the highest resolution shell are shown in parentheses

\(^b\) $R_{\text{free}}$ is calculated by omitting 5% of the total number of reflections in model refinement
Supplementary Figure 1. LepB_NTD specifically disrupts the Golgi structure. a, b, HeLa cells expressing RFP or RFP-LepB_NTD were stained with anti-calnexin (ER), LAMP2 (lysosome) or p230 (trans-Golgi) antibodies. The trans-Golgi was also imaged by co-transfected GalT-GFP or GalNAc-T2-GFP reporters. Shown are confocal fluorescence images. Scale bar, 20 μm. c, Inhibition of hGH secretion by LepB_NTD. Drug-induced secretion of hGH from cells expressing indicated effectors were measured by ELISA and normalized to that from RFP-expressing control cells (mean±S.D.). d, Inhibition of VSV-G trafficking by LepB_NTD. VSV-G-GFP-expressing HeLa cells transfected with RFP or RFP-LepB_NTD were incubated at 42°C for 16 h and then shifted to 32°C for indicated time periods. Cell lysates was treated with Endo H, and analyzed by anti-GFP immunoblotting. R and S mark the Endo H-resistant and sensitive forms of VSV-G. e, Effects of LepB on Golgi structure during L. pneumophila infection. Bone marrow macrophages (A/J mouse) were infected with indicated L. pneumophila strains (MOI, 10). Anti-GM130 and anti-Legionella fluorescence staining images are shown. Scale bar, 5 μm. Data in (a, b, c, d, e) are representative of at least three independent experiments.
No: Chain  Z rmsd Description
1: 3akk-A  10.0  3.3  CTKA
2: 1cja-B  9.7  3.3  Protein( actin-fragmin kinase)
3: 3ls8-A  9.2  3.8  Phosphatidylinositol 3'-kinase catalytic subunit
4: 3tpd-A  8.6  4.4  Serine/threonine-protein kinase HIPK
5: 4kqa-B  8.3  3.8  Protein h03a11.1
6: 3hhm-A  8.2  3.7  Phosphatidylinositol-4,5-bisphosphate 3'-kinase
7: 4jsp-B  8.0  3.9  Serine/threonine-protein kinase MTOR
8: 2jd5-B  7.1  3.9  Serine/threonine-protein kinase SKY1
9: 1e07-B  7.1  3.8  SR protein kinase
10: 3ttg-A  7.1  3.8  Serine/threonine-protein kinase WR216c
11: 4agu-C  6.9  3.7  Cyclic-dependent kinase-like 1
12: 4gl9-A  6.9  5.3  Tyrosine-protein kinase
13: 4f0f-A  6.9  5.0  Serine/threonine-protein kinase ROC04
14: 4bbe-C  6.7  4.8  Tyrosine-protein kinase JAK2

ManII Merge

0 min  30 min  2 h

RFP-LepB_NTD

RFP  WT

RFP-LepB_NTD

RFP  WT

RFP-LepB_NTD

D176A
Supplementary Figure 2. Structural analyses of LepB_NTD and functional importance of the putative kinase-catalytic residues. a, Crystal contacts in the structure of LepB (1-618). The black and grey circles mark the back-to-back contact between the RabGAP domains in the same asymmetric unit and the head-to-tail contact between two LepB molecules in the adjacent asymmetric units, respectively. b, DALI search results using LepB_NTD structure as the bait. c, Comparison of structural topology of LepB_NTD, CTKA and PKA. \( \alpha \)-helices and \( \beta \)-strands are shown as cylinders and arrows, respectively. d, Mutational analyses of the putative catalytic residues in LepB_NTD on its disruption of the Golgi structure. HeLa cells were transfected as indicated. The medial-Golgi was stained by the anti-ManII antibody and confocal fluorescence images are shown. e, Mutational analyses of the putative catalytic residues in LepB_NTD on blocking VSV-G trafficking. Experiments were performed similarly as that in Fig. 1d except that indicated LepB_NTD mutants were assayed. Images shown in (d, e) (scale bar, 20 \( \mu \)m) are representative of at least three independent experiments.
Supplementary Figure 3. Exploring the possibility of a Rab protein being the kinase substrate of LepB_NTD. **a**, Superimposition of the structures of LepB (1-618) and LepB RabGAP-Rab1 complex. The LepB RabGAP-Rab1 complex structure is shown in cartoon models. The structure of LepB (1-618) is shown in both cartoon and surface models. **b**, Assaying the ability of various Rab proteins in suppressing the yeast toxicity of LepB_NTD and LepB RabGAP domain. Indicated LepB and Rab proteins were co-expressed in *S. cerevisiae* W303a strain. Expression of LepB variants was under a galactose inducible promoter. Data shown are representative of at least three independent experiments.
Supplementary Figure 4. LepB_NTD expression can alter cellular distribution of certain phosphoinositides. a, Disruption of the trans- but not cis-Golgi structure by overexpression of the PtdIns4P-binding PH domains of OSBP and FAPP1. HeLa cells were transfected with EGFP-PH\textsubscript{OSBP} or EGFP-PH\textsubscript{FAPP1} and stained with the anti-GM130 or TGN46 antibody. Scale bar, 20 μm. b, c, Assays of cellular distribution of various phosphoinositides in LepB_NTD-expressing HeLa cells. RFP or RFP-LepB_NTD (WT or H154A) was co-expressed with an indicated EGFP-tagged phosphoinositide probe (PH\textsubscript{BTK} for PtdIns(3,4,5)\textsubscript{P}3, PH\textsubscript{AKT} for PtdIns(3,4,5)\textsubscript{P}3/PtdIns(3,4)\textsubscript{P}2, PH\textsubscript{FAPP1} for PtdIns(3,4)\textsubscript{P}2, 2xFYVE\textsubscript{Hrs} for PtdIns3\textsubscript{P}, PH\textsubscript{FAPP1} for PtdIns4\textsubscript{P}, PH\textsubscript{PLC\delta1} for PtdIns(4,5)\textsubscript{P}2, PH\textsubscript{ING2} for PtdIns5\textsubscript{P}, and PH\textsubscript{Lact-C2} for phosphatidylserine). Scale bar, 10 μm. d, The expression level of RFP-LepB_NTD and RFP-LepB_FL (full length). HeLa cells were transfected with indicated expression constructs and the lysates were analyzed by anti-GFP immunoblotting. Data shown in (a, b, c, d) are representative of at least three independent experiments.
Supplementary Figure 5. Multiple sequence alignment of LepB_NTD and the catalytic domains of mammalian PI4Ks. The alignment was performed in GENEDOC and ESPript3.0. Identical and conserved residues are highlighted by black background and box, respectively. The red triangles mark the catalytic residues in LepB_NTD assayed in the study. Secondary structures determined from LepB crystal are on top of the sequence.
Supplementary Figure 6. Phosphoinositide phosphatase assays of putative *L. pneumophila* phosphatase effectors. Indicated purified recombinant *L. pneumophila* effector containing a CX_R motif was incubated with each of a panel of seven different phosphoinositides. Shown are Malachite Green assays of the released phosphate in the reaction system. Data shown are representative of at least three independent experiments.
Supplementary Figure 7. A working model for LepB and SidF-mediated synthesis of PtdIns4P from PtdIns3P on the LCV membrane. Following pinocytosis-mediated entry into host macrophages, *L. pneumophila* stay in the *Legionella*-containing vacuole (LCV) that has extensive interactions with ER-derived vesicles. The phosphoinositides on the LCV also undergo dynamic changes during LCV maturation into a replication-permissive niche. LepB and SidF deployed by the *Legionella* Dot/Icm secretion system contribute to LCV phosphoinositide metabolism by catalyzing sequential phosphorylation of PtdIns3P and dephosphorylation of PtdIns(3, 4)P_2, respectively, generating PtdIns4P that is known to be the most enriched phosphoinositide on the LCV membrane. PtdIns4P is important for anchoring many Dot/Icm effectors on the LCV.
Supplementary Figure 8. Uncropped gels and blots.