Alpha-kinase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose

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Immune recognition of pathogen-associated molecular patterns (PAPMs) by pattern recognition receptors often activates proinflammatory NF-κB signalling. Recent studies indicate that the bacterial metabolite d-glycero-β-d-manno-heptose 1,7-bisphosphate (HBP) can activate NF-κB signalling in host cytosol1-4, but it is unclear whether HBP is a genuine PAMP and the cognate pattern recognition receptor has not been identified. Here we combined a transposon screen in Yersinia pseudotuberculosis with biochemical analyses and identified ADP-β-d-manno-heptose (ADP-Hep), which mediates type III secretion system-dependent NF-κB activation and cytokine expression. ADP-Hep, but not other heptose metabolites, could enter host cytosol to activate NF-κB. A CRISPR–Cas9 screen showed that activation of NF-κB by ADP-Hep involves an ALPK1 (alpha-kinase 1)-TIFA (TRAF-interacting protein with forkhead-associated domain) axis. ADP-Hep directly binds the N-terminal domain of ALPK1, stimulating its kinase domain to phosphorylate and activate TIFA. The crystal structure of the N-terminal domain of ALPK1 and ADP-Hep in complex revealed the atomic mechanism of this ligand–receptor recognition process. HBP was transformed by host adenylyltransferases into ADP-heptose 7-P, which could activate ALPK1 to a lesser extent than ADP-Hep. ADP-Hep (but not HBP) alone or during bacterial infection induced ALPK1-dependent inflammation in mice. Our findings identify ALPK1 and ADP-Hep as a pattern recognition receptor and an effective immunomodulator, respectively.

Gram-negative bacteria such as Yersinia5, Salmonella6, Burkholderia7 and enteropathogenic Escherichia coli8 induce NF-κB-mediated cytokine expression in a type III secretion system (T3SS)-dependent manner. Consistently, infection of 293T cells with Y. pseudotuberculosis Δ6 (lacking the six T3SS effectors; Δ6 is omitted hereafter) robustly activated NF-κB-driven luciferase and eGFP reporters9 (Fig. 1a, b). From 21,000 Y. pseudotuberculosis transposon mutants, we identified 37 defective in activating both reporters. Most mutations were in T3SS-encoding genes. One mutant that was more impaired than the T3SS-deficient ΔyopB strain had a functional T3SS with its transposon inserted in hldE (Fig. 1a–c and Extended Data Fig. 1a). Expression of HldE in the transposon or the ΔhldE mutant restored infection-induced NF-κB activation. HldE, together with Gmha and GmhB, synthesizes ADP-d-glycero-β-d-manno-heptose (ADP-DD-Hep; β is omitted hereafter) from d-sedoheptulose 7-phosphate (S7P) through d-glycero-β-d-manno-heptose 7-phosphate (H7P), HBP and d-glycero-β-d-manno-heptose 1-phosphate (H1P) (Extended Data Fig. 1b). ADP-DD-Hep and ADP-LD-Hep (ADP-1-glycero-β-d-manno-heptose) undergo interconversion, catalysed by HldD. Deletion of gmhB or hldD did not affect Y. pseudotuberculosis-induced activation of NF-κB, whereas deletion of ghmba phenocopied the ΔhldE strain (Fig. 1b). This seems to suggest that HBP, but not H1P or ADP-Hep, determines Y. pseudotuberculosis-dependent activation of NF-κB, echoing the analyses in Neisseria meningitidis.3 However, Y. pseudotuberculosis ΔgmhB, unlike the ΔhldE and Δghmba strains, still supported ADP-Hep-dependent autotransporter heptosylation10 (Extended Data Fig. 1c), because of an unknown redundancy to gmbh in ADP-Hep biosynthesis11. When electroporated into 293T cells, synthetic HBP and ADP-DD-Hep or ADP-LD-Hep—but not S7P—stimulated NF-κB activation, with ADP-Hep being the most potent (Fig. 1d). H1P was even less active than HBP(Extended Data Fig. 1d). When added directly to 293T cells, only ADP-DD-Hep and ADP-LD-Hep induced activation of NF-κB and production of interleukin (IL)-8 (Fig. 1d, e). This explains the use of transfection in recent reports on HBP12,13. Thus, ADP-Hep is a potent and versatile PAMP.

Activation of NF-κB–eGFP reporter by extracellular ADP-Hep enabled us to carry out a fluorescence-activated cell sorting (FACS)-based genome-wide CRISPR–Cas9 screen (Extended Data Fig. 2a). Following a counterscreen against TNF stimulation, we identified ALPK1, TIFA and TRAF6 (each hit by more than one guide RNA (gRNA)) that were required for ADP-Hep-induced NF-κB–eGFP expression (Fig. 2a and Supplementary Table 1). Upon phosphorylation at T9, TIFA forms foci to activate TRAF6-dependent NF-κB signalling12,14. During the preparation of this manuscript, ALPK1 was identified as contributing to activation of NF-κB in Shigella flexneri and Helicobacter pylori15. Deletion of ALPK1 or TIFA (Supplementary Table 2) abolished ADP-LD-Hep–induced activation of NF-κB and expression of IL-8 (Fig. 2b, c and Extended Data Fig. 2b, c). Defective NF-κB activation in ALPK1−/− cells was restored by wild-type ALPK1 but not by its kinase-inactive K1067M mutant (Fig. 2b); TIFA−/− was rescued by wild-type TIFA but not by a T9A mutant (Extended Data Fig. 2c). ADP-LD-Hep–induced phosphorylation of TIFA at T9 and formation of eGFP–TIFA foci dependent upon ALPK1 kinase activity (Fig. 2d and Extended Data Fig. 2d). ADP-LD-Hep did not affect the cytoplasmic localization of ALPK1 and induced no myosin phosphorylation16 (Extended Data Fig. 2e, f). ALPK1 and TIFA were also required for the activation of NF-κB by electroperoration of ADP-Hep (Extended Data Fig. 2g).

ADP-LD-Hep triggered co-immunoprecipitation of TIFA with ALPK1 and TRAF6 (Extended Data Fig. 2h). Deletion of ALPK1 did not affect activation of NF-κB by TNF, NOD1 or NOD2 (mediates Salmonella-induced NF-κB activation16,17) or MYD88 overexpression (Extended Data Fig. 1e). Cells lacking NOD1 and NOD2 showed intact NF-κB responses and TIFA foci following treatment with ADP-LD-Hep (Extended Data Fig. 1f, g). Thus, ADP-Hep activates NF-κB specifically through the ALPK1–TIFA–TRAF6 axis.

Induction of IL-8 expression by Y. pseudotuberculosis, which required hldE and yopB, was blocked by deletion of ALPK1 (Fig. 2e). Infection-induced activation of NF-κB and formation of eGFP–TIFA foci required ALPK1-dependent phosphorylation of TIFA at T9 (Extended Data Fig. 3a–e). Other bacteria, such as diffuse-adhering E. coli (DAEC), enterotoxicigenic E. coli (ETEC) and Burkholderia
**Fig. 2** | CRISPR–Cas9 screens identify an ALPK1–TIFA axis that mediates activation of NF-κB induced by ADP-Hep or Yersinia. 

(a) Top eight gRNA hits from CRISPR–Cas9 screen of ADP-LD-Hep-induced NF-κB activation. 

(b–d, h) Wild-type or ALPK1−/− 293T cells expressing the indicated ALPK1 mutants were treated with ADP-LD-Hep (b–d, h) or HBP (c), or infected with Y. pseudotuberculosis (e, h). 

(d, h) Anti-Flag and anti-pT9-TIFA immunoblots of 293T cells. 

(e) CRISPR–Cas9 screen identified an ALPK1–TIFA axis. 

(f) TIFA in Y. pseudotuberculosis Δ36 was rescued by HBP, ADP-LD-Hep or ADP-LD-Hep + Flag–APLPK1 + HBP treatment. 

(g) ALPK1–/– THP-1 cells were transfected with Flag–APLPK1 or mock and infected with Y. pseudotuberculosis Δ36. Co-immunoprecipitation of ALPK1 N- and C-terminal regions with Flag–TIFA and anti-Flag was determined. 

Notably, ALPK1-(N+K) from E. coli ΔhldE did not phosphorylate TIFA (Fig. 3a). Small-molecule extracts from wild-type but not ΔhldE mutant E. coli-derived ALPK1−/−, when added to 293T cells, potentely stimulated ALPK1-dependent phosphorylation of TIFA and activation of NF-κB, the latter of which correlated with the amount of ALPK1−/− (Fig. 3b, c).

High-performance liquid chromatography (HPLC) of the small-molecule extracts identified one active fraction (no. 6, the only one with high ultraviolet absorption) (Extended Data Fig. 5b–d). Mass spectrometry of fraction 6 uncovered three dominant ions with mass-to-charge ratios (m/z) of 619.8, 347.9 and 427.8, matching those of ADP-Hep, AMP and ADP, respectively (Extended Data Fig. 5e). The presumed ADP-Hep ion showed a similar retention time and fragmentation pattern to synthetic ADP-Hep (Extended Data Fig. 5f, g). The mass of native ALPK1−/− exceeded that of denatured ALPK1−/− by 619.32 Da (one ADP-Hep) (Fig. 3d). Direct binding of E. coli ΔhldE-derived apo-APLPK1-(N+K) to ADP-LD-Hep (but not S7P) was readily detected (Extended Data Fig. 5h).

We determined the 2.59 Å crystal structure of ALPK1−/− purified from wild-type E. coli (Extended Data Table 1a). Each asymmetric

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**Fig. 1** | Transposon screen of *Yersinia* T3SS-dependent NF-κB activation identifies ADP-Hep as a PAMP. 

(a, b) 293T cells were infected with indicated *Yersinia* strains. 

(c) Transposon insertion in hldE. 

(d) 293T (d) or PMA-differentiated THP-1 cells (e) were electroporated (d) or extracellularly treated (d, e) with indicated sugars. 

(e) IL8 mRNA (also known as CXCL8, shown relative to 18S rRNA) and IL-8 secretion were measured by quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. 

(f) NF-κB activation was measured by luciferase activity (a, d) or eGFP reporter expression (b) (scale bar, 50 μm). 

(g) Data shown as mean ± s.d. from three technical replicates (a, d, e), and representative of three (a, b, d) and two (e) independent experiments. 

(h) Two-tailed unpaired Student’s t-test (***P < 0.01, ****P < 0.001).

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*E. coli* (N+K) and *Yersinia* strains. pHldE is a plasmid expressing HldE. Y. pseudotuberculosis T3SS-dependent NF-κB activation identifies ADP-Hep as a PAMP.
unit contains nine molecules (A–I). Molecule A has the highest-quality density map and was used to build the final model, which lacks only the first methionine. The structure bears 18 helices (α1 to α18), forming seven antiparallel pairs (α1–α4, α5–α6, α7–α8, α9–α10, α11–α13, α14–α15, and α16–α17) (Fig. 3e). The α1–α4 pair features an insertion containing α2, α3 and loop L1, α18, which precedes the L2 tail, flanks the outer surface of the α16–α17 pair. The one-by-one-packed seven helix pairs form a right-hand solenoid (Fig. 3e), resembling a...
Alpk1 ADP-Hep, resisted activation by ADP-Hep (Fig. 4b). Similar results with the R116A, R150A, R153A or K233A mutant forms of ALPK1, B activation (Fig. 1d). Full-length ALPK1 purified from κ of binding to ALPK1-NTD (Extended Data Fig. 5h) and failure to or H1P (Figs. 3a, 4a). The inactivity of S7P is consistent with its lack of other vertebrates (Extended Data Fig. 4f).

The concave side of the ALPK1-NTD solenoid has a narrow pocket anchored by Q67 and D231, C2 and C6 are contacted by K233, and R116 (or R153) and β for ADP-LD-Hep (although it is spacious enough for ADP-DD-Hep; Fig. 3e). The pocket has high-quality electron densities specifically to ALPK1-NTD (Extended Data Table 1b).

The two ADP phosphates are clamped by R116, R150, R153 and K233 through a hydrogen-bond network: two bonds between R116 (or R153) and β-phosphate, two between R150 and both phosphates, and one between K233 and α-phosphate (Fig. 3g). The ADP adenosine has a stacking interaction with F295 and two hydrogen bonds with S236 and T237. The histidine C3 and C4 hydroxyls are anchored by Q67 and D231, C2 and C6 are contacted by K233, and C7 is fixed by R153. The heptose backbone has a stacking interaction with F295 and two hydrogen bonds, and one between K233 and R116 (or R153).

The R116A, R150A, R153A or K233A mutant forms of ALPK1, which are expected to have impaired binding to the phosphates of ADP-Hep, resisted activation by ADP-Hep (Fig. 4b). Similar results were obtained with Q67A/D231A, T237E/F295K and F61D mutations, which affect heptose or adenosine binding. These ALPK1 mutants could not mediate activation of NF-κB by ADP-LD-Hep (Fig. 4c). The requirement of AMP-contacting residues for activation of ALPK1 explains why HBP is inactive in vitro. Thus, ALPK1 is a functional receptor specifically for ADP-Hep.

HBP could bind apo-ALPK1-(N+K) but with a lower affinity than ADP-Hep (Extended Data Fig. 6a); a 100-fold excess of HBP was needed for it to compete with ADP-LD-Hep to activate ALPK1 (Fig. 4d and Extended Data Fig. 6b). ALPK1-(N+K) incubated with ADP-LD-Hep could bind and phosphorylate a GST-fused N-terminal 15-residue peptide of TIFA and catalyse ATP hydrolysis (Extended Data Fig. 6c–e). By contrast, ALPK1-(N+K) incubated with HBP showed neither substrate recognition nor catalysis of ATP hydrolysis. Chemical cross-linking coupled with mass spectrometry identified ten peptide pairs between the NTD and KD molecules of apo-ALPK1-(N+K) (Extended Data Fig. 6f and Supplementary Table 3). ALPK1-(N+K) incubated with HBP showed a similar pattern, but ALPK1-(N+K) incubated with ADP-LD-Hep had only six crosslinks. The four connections to K1149 of ALPK1-KD and one to the nearby K1140, which were partially lost in HBP-incubated ALPK1-(N+K), all disappeared in ADP-LD-Hep-incubated ALPK1-(N+K). The two lysine residues predicted to be near the kinase catalytic cleft 18 (Extended Data Fig. 6g), which participates in substrate binding. Thus, binding of ADP-LD-Hep to ALPK1-NTD renders the catalytic cleft more...
exposed, probably by inducing a larger conformational change than the nonproductive HBp binding.

Activation of NF-κB induced by HBp transfection required phosphorylation of TIFA by ALPK1 (Extended Data Fig. 7a, 3,4) (Extended Data Fig. 7a, b). We considered how HBp activates ALPK1 in cells. After noticing a side reaction of HldE adenylyltransferase with HBP, we found that host-derived adenylyltransferases—such as nicotinamide nucleotide adenylyltransferases—could convert HBp into ADP-heptose 7-P, which was competent (albeit less than ADP-Hep) to activate ALPK1 and the downstream NF-κB response (Fig. 4e and Extended Data Fig. 7c–k; see Supplementary Text). We also found that ALPK1(Q67A/Y68A), ALPK1(Y68A) or the double mutant lost the response to HBp exportation but remained competent to mediate ADP-LDH-hep-induced activation of NF-κB (Extended Data Fig. 8a, b). Cells expressing these mutants also showed defective responses to ADP-heptose 7-P (Extended Data Fig. 8c). Purified ALPK1(Q67A/Y68A) was activated by ADP-LDH-hep but not ADP-heptose 7-P or HBp (Extended Data Fig. 8d). Small-molecule extracts from cells electroporated with HBp could activate wild-type ALPK1 but not ALPK1(Q67A/Y68A) (Extended Data Fig. 8e, f). Thus, cytosolic HBp might be metabolized into ADP-heptose 7-P to activate ALPK1. Moreover, 293T cells expressing ALPK1(Q67A/Y68A) showed a normal NF-κB response to Y. pseudotuberculosis (Extended Data Fig. 8g), confirming the detection of ADP-Hep by the host during infection.

Injection of ADP-LDH-hep, but not of HBp, into the dorsal air pouches of mice induced massive neutrophil recruitment (Fig. 5a). Several NF-κB-controlled cytokines and chemokines, including IL-6, TNF, IP-10, MCP-1, MCP-3, IFNγ, GM-CSF, MIP-1α, MIP-1β and RANTES, were highly elevated in the air pouches (Fig. 5b and Extended Data Fig. 9a). ADP-LDH-hep also increased the serum levels of GRO-α, IP-10 and MCP-1 (Extended Data Fig. 9b, c). HBP injection affected neither local nor systemic productions of these inflammatory mediators (Fig. 5b and Extended Data Fig. 9a–c). Alpk1−/− mice injected with ADP-LDH-hep showed no increase in cytokine production (Fig. 5c and Extended Data Fig. 9d). Mice were also infected with B. cenocepacia, which is known to trigger lung inflammation. Consistent with the cell culture data (Extended Data Fig. 3f, g), infection with B. cenocepacia increased the expression of MCP-3, GM-CSF, MIP-1α and 3, and RANTES in the lungs of wild-type mice, and these responses were compromised in Alpk1−/− mice (Fig. 5d). Alpk1−/− mice showed a higher bacterial load in the lungs than wild-type mice (Fig. 5e). These data emphasize the functional relevance of recognition of ADP-Hep by ALPK1.

We have shown that ADP-Hep is permeable to mammalian cells and can be exploited as an immunomodulator or a vaccine adjuvant. The pattern recognition receptor function of ALPK1 highlights the versatile mechanisms underlying cytosolic detection of bacteria 19 and will modulate responses to other alpha kinases 20. ADP-Hep is present in all Gram-negative and some Gram-positive bacteria, 21 ALPK1 is also widely expressed. Recognition of ADP-Hep by ALPK1, as with recognition of LPS by TLR4 and caspase-11, represents a generic form of cytosolic detection of bacteria 19 and will provide means for the design of new drugs. The ADP-heptosyltransferase activity of ALPK1 may be exploited as an immunomodulator or a vaccine adjuvant. The cytosolic form of ALPK1 may be useful as a standard for the detection of bacterial infections, as the ADP-heptosyltransferase activity is a common feature of all Gram-negative and some Gram-positive bacteria.

## Online content

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0433-3

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METHODS

Plasmids, antibodies and reagents. DNA for hIdE was amplified from Y. pseudotuberculosis IP2666 and inserted into the pBAD24 vector for rescue expression in E. coli. DNAs for gfnA, hIdE and ghmb were amplified from E. coli DH5α and cloned into pET28a-6×His-SUMO or pQE-80L vectors for recombinant expression in E. coli. CDNA for human ALPK1 was amplified from 293T cDNA reverse transcripts. CDNAs for TIFA and MDRN8 were amplified from a HeLa cDNA library. CDNA for ACTG3 (kindly provided by J. Koch, Cancer Research, University of Washington and S. Lory, Harvard Medical School) was used to generate the mutant library of Y. pseudotuberculosis. 293T and THP-1 cells were obtained from the ATCC. 293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. THP-1 cells were grown in RPMI 1640 medium containing 10% FBS and 2 mM L-glutamine. Knockout 293T cell lines were generated by using the CRISPR-Cas9 method as recently described and sequences for gRNAs targeting ALPK1 and TIFA were listed in Supplementary Table 2. All cells were tested for mycoplasma using the standard PCR method. Cell identity was checked frequently by their morphological features but was not authenticated by short tandem repeat (STR) profiling. For luciferase assay, the plasmids were transfected into 293T cells with the VivaTrans (Polyplus). Luciferase activity was determined using the dual luciferase assay kit (Promega) according to the manufacturer’s instructions. Fluorescence microscopy imaging, 293T cells expressing the pNF-B-eGFP reporter were seeded onto glass coverslips in 24-well plates and cultured for 16 h before transfection or infection (MOI 50). The cells were fixed and stained with Hoechst 33342. Fluorescence images were acquired on the Nikon A1-R or Zeiss Meta confocal microscope.

Immunoprecipitation and qRT-PCR. For immunoprecipitation, 293T cells at a confluency of 70–80% in 6-well plates were transfected with a total of 2.5 μg of indicated plasmids. Twenty-four hours after transfection, cells were treated with or without ADP-LD-He (100 μM) for 4 h. The cells were washed once in PBS and lysed in buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 1% Triton X-100, supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals). Pre-cleared cell lysates were then subjected to anti-Flag M2 immunoprecipitation following the manufacturer’s instructions. The beads were washed four times with the lysis buffer and the immunoprecipitates were subjected to standard immunoblots analysis. qRT–PCR was performed as previously described. mRNA levels of the target gene were normalized to that of 18S rRNA. The primers used for human 18S rRNA were 5′-GACGTTCTTGGCCCGTTGTT-3′ (forward) and 5′-CAAGAGTCACACTCGAGCTT-3′ (reverse), and those for human IP2666 were 5′-GACTCTTGGGCGATGTTGTTGTT-3′ (forward) and 5′-CCAGATCAGCTAATGAGGAGT-3′ (reverse), both of which are the same as previously described.

FACS-based genome-wide CRISPR-Cas9 screen. Human CRISPR knockout pooled gRNA plasmid library (GeCKO v2) encompassing 123,411 different gRNAs targeting 19,050 human genes was generated by the Zhang laboratory and obtained from Addgene. Amplification of the library and preparation of the lentivirus were performed as recently described. To perform the screen, 293T cells stably expressing Cas9 and pNF-B-eGFP reporter were seeded in the 15-cm dish (7.5 × 106 cells per dish) and a total of 1.5 × 107 cells were infected with the gRNA lentivirus library at an MOI of 0.3. Forty-eight hours after infection, the cell media were collected and mixed with 108 puromycin-resistant cells were left untreated to obtain the control sample. About 2.5 × 106 puromycin-resistant cells were treated overnight with 10 μM ADP-LD-He and sorted for eGFP-negative cells (~1%) on a BD Biosciences FACSAria II Flow Cytometer. After culturing for about one week, the sorted cells were re-treated with ADP-LD-He until the percentage of eGFP-negative cells reached more than 95%. The resulting ADP-LD-He unresponsive cells were cultured and treated overnight with 20 mg ml−1 TNF followed by FACs sorting of the eGFP-positive cells. After expansion, the TNF-responsive cells, together with the control cells, were subjected to DNA extraction. Two parallel screens were performed. Amplification of the gRNA sequences was performed using a two-step PCR method similar to that described recently. The first step, twenty-four 50-μl PCR reactions (each containing 10 μg of genomics DNA were performed with the forward primer 50bp-F (5′-CTCCTTCTCTACACCAGCGCTCTCGG-3′) and reverse primer 50bp-R (5′-GTGACTGAGGTCAGGTGTCGTCGAGCTC-3′); the PCR product was used 95°C for 3 min, 18 cycles of 95°C for 30 s, 56°C for 20 s and 68°C for 20 s, and a final 3-min extension at 68°C.
Products of the first-step PCR were pooled together and used as the template for the second-step PCR. Four μl PCR reactions (each containing 2.5 μl of the first-step PCR product) were performed with the forward primer Index-F (5'-AATGATACGGCGGTCTTCTCTCTCTCTCTTTCTACTACGCT-3') and one of the reverse primers: Index-R1 (5’-CAAGCAGAAGACGCCGATACGAGCTCGTGAGTGTACGGAGCTGTTCT-3') for the control sample, Index-R4 (5’-CAAGCAGAAGACGGCTACGAGTGGTCGATCGACTGAGGTCT-3') and Index-R8 (5’-CAAGCAGAAGACGGGCTACGAGTGGTCGATCGACTGAGGTCT-3') for the screen sample. The PCR program used was 95°C for 3 min, 21 cycles of 95°C for 30 s, 56°C for 20 s and 68°C for 20 s, and a final 3-min extension at 68°C. The products of the second-step PCR reactions were subjected to electrophoresis on 2% agarose gel; the 274-bp DNA bands were extracted and sequenced at the HiSeq2500 platform (illumina). The first 19 nucleotides from each sequencing read are the gRNA sequence recovered. The frequency of each gRNA was obtained by dividing the gRNA read number by the total sample read number; the fold change was calculated by comparing the frequency of each gRNA in the screen sample with that in the control sample. The fold change ranking was obtained based on the smaller fold change value of each individual gRNA in the two parallel screens.

Recombinant protein purification. Protein expression was induced in *E. coli* BL21 (DE3) strain (wild-type or Δ*hldE)*. Enzymatic synthesis of H1P and ADP-heptose 7-P. For enzymatic synthesis of H1P, 1 mM HBP and 10 μM GmbH protein purified from *E. coli* BL21 (DE3) Δ*hldE* were buffer-exchanged into 100 mM ammonium acetate (pH 7.5) using a centrifugal buffer exchange column (Micro Bio-Spin, 6-Bio-Rad), and one aliquot was denatured by adding 0.1% formic acid (final concentration). Both the native and denatured protein samples were analysed by direct infusion. Specifically, 3 μl of each protein sample was loaded into a nano-flow capillary (borosilicate emitter, Thermo Scientific) and sprayed into a high-resolution mass spectrometer Q-Exactive HF through the nanospray ionization probe. Samples were separated using a synergi Hydro-RP column (2.0 × 100 mm, 2.5 μm, Phenomenex). A binary solvent system (mobile phase A, 10 mM tributylamine adjusted with 15 mM acetic acid in water; mobile phase B, methanol) was used. A 15-min gradient with a flow rate of 250 μl min⁻¹ was applied as follows: 1–7 min at 15% B, 7–12 min, 15–98% B; 12–12.1 min, 98–15% B; 12.1–12.2 min, 15% B; 12.2–15 min, 15% B. The column was set to 35°C and the flow rate was 0.4 ml min⁻¹. The following gradient was applied: 0–4 min 0% B, 4–6.5 min from 0% to 50% B, 6.5–6.6 min from 50% to 100% B, 6.6–8 min 100% B, 8–8.2 min from 100% to 0% B, and 8.2–10 min re-equilibration at 0% B. Six microlitres of each sample was injected into the instrument and the mass spectrometry data were collected in positive and negative ionization modes for detecting ADP-Hep and ADP-heptose 7-P, respectively. A collision energy of 20 V was applied for the MS/MS acquisition of ADP-Hep.

Native mass spectrometry analysis. His₄–ALPK1-NTD protein purified from *E. coli* BL21 (DE3) was used for native-ESI analysis. Purified recombinant proteins (10 μM) were buffer-exchanged into 100 mM ammonium acetate (pH 7.5) using a centrifugal buffer exchange column (Micro Bio-Spin, 6-Bio-Rad), and one aliquot was denatured by adding 0.1% formic acid (final concentration). Both the native and denatured protein samples were analysed by direct infusion. Specifically, 3 μl of each protein sample was loaded into a nano-flow capillary (borosilicate emitter, Thermo Scientific) and sprayed into a high-resolution mass spectrometer Q-Exactive HF through the nanospray ionization probe. Samples were separated using a synergi Hydro-RP column (2.0 × 100 mm, 2.5 μm, Phenomenex). A binary solvent system (mobile phase A, 10 mM tributylamine adjusted with 15 mM acetic acid in water; mobile phase B, methanol) was used. A 15-min gradient with a flow rate of 250 μl min⁻¹ was applied as follows: 1–7 min at 15% B, 7–12 min, 15–98% B; 12–12.1 min, 98–15% B; 12.1–12.2 min, 15% B; 12.2–15 min, 15% B. The column was set to 35°C and the flow rate was 0.4 ml min⁻¹. The following gradient was applied: 0–4 min 0% B, 4–6.5 min from 0% to 50% B, 6.5–6.6 min from 50% to 100% B, 6.6–8 min 100% B, 8–8.2 min from 100% to 0% B, and 8.2–10 min re-equilibration at 0% B. Six microlitres of each sample was injected into the instrument and the mass spectrometry data were collected in positive and negative ionization modes for detecting ADP-heptose 7-P, respectively. A collision energy of 20 V was applied for the MS/MS acquisition of ADP-Hep.

Ultra-performance liquid chromatography with tandem mass spectrometry analysis of ADP-heptose 7-P generated from HBP by NMNAT1. The Dionex Ultimate 3000 UPLC system was coupled to a TSQ Quantiva Ultra triple-quadrupole mass spectrometer (Thermo Fisher), equipped with a heated electrospray ionization probe. Samples were separated using a synergi Hydro-RP column (2.0 × 100 mm, 2.5 μm, Phenomenex). A binary solvent system (mobile phase A, 10 mM tributylamine adjusted with 15 mM acetic acid in water; mobile phase B, methanol) was used. A 15-min gradient with a flow rate of 250 μl min⁻¹ was applied as follows: 1–7 min at 15% B, 7–12 min, 15–98% B; 12–12.1 min, 98–15% B; 12.1–12.2 min, 15% B; 12.2–15 min, 15% B. The column was set to 35°C and the flow rate was 0.4 ml min⁻¹. The following gradient was applied: 0–4 min 0% B, 4–6.5 min from 0% to 50% B, 6.5–6.6 min from 50% to 100% B, 6.6–8 min 100% B, 8–8.2 min from 100% to 0% B, and 8.2–10 min re-equilibration at 0% B. Six microlitres of each sample was injected into the instrument and the mass spectrometry data were collected in positive and negative ionization modes for detecting ADP-heptose 7-P, respectively. A collision energy of 20 V was applied for the MS/MS acquisition of ADP-Hep.

Chemical cross-linking coupled with mass spectrometry. To obtain well-behaved apo–ALPK1-(N-K) complexes for chemical cross-linking coupled with mass spectrometry analysis, GST–ALPK1-NTD protein was co-expressed with SUMO–ALPK1-KD in *E. coli* BL21 (DE3) Δ*hldE*. The GST–ALPK1-(N-K) complexes were immobilized on a glutathione sepharose column and washed with 20 bed volumes of 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl. GST was removed by overnight digestion with a homogenous HRV 3C protease at 4°C. The supernatant containing the released apo-ALPK1-(N⁰-K) was passed through fresh glutathione sepharose beads, further purified by gel filtration chromatography, and concentrated to 1 mg ml⁻¹. The precursor and fragment ions were collected at the resolution of 0.7 FWHM. The source parameters are as follows: spray voltage, 3,000 V; ion transfer tube temperature, 350°C; vaporizer temperature, 300°C; sheath gas flow rate, 35 ArB; auxiliary gas flow rate, 12 ArB; CID gas, 1.5 mTorr. Data analysis and quantification were performed using the software Xcalibur 3.0.63 (Thermo Fisher).

HPLC–MS analysis, fractionation and LC–MS/MS. Enzymatic reactions and extracts of *E. coli*-purified His₄–ALPK1-NTD were subjected to HPLC-MS analysis or fractionation performed on Waters HPLC (Column: Atlantis T3, eluents: 0.1% NH₄HCO₃/H₂O and CH₃CN with 2998PD A and 3100 MS detectors (ESI ionization). Liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis was performed on an Agilent 1290 Infinity HPLC coupled with an Agilent 6540 quadrupole time of flight mass spectrometer. A Phenomenex Kinetex F5 column (2.1 × 100 mm, 2.6 μm) was used for separation. Mobile phases A and B were 0.1% formic acid-containing water and acetonitrile, respectively. Column temperature was set to 35°C and the flow rate was 0.4 ml min⁻¹. The following gradient was applied: 0–4 min 0% B, 4–6.5 min from 0% to 50% B, 6.5–6.6 min from 50% to 100% B, 6.6–8 min 100% B, 8–8.2 min from 100% to 0% B, and 8.2–10 min re-equilibration at 0% B. Six microlitres of each sample was injected into the instrument and the mass spectrometry data were collected in positive and negative ionization modes for detecting ADP-Hep and ADP-heptose 7-P, respectively. A collision energy of 20 V was applied for the MS/MS acquisition of ADP-Hep.
were isolated for HCD MS2 (resolution 15,000; normalized collision energy 27°) with a dynamic exclusion time of 45 s. Precursors with 1+, 2+, 2+ or above, or unassigned charge states were excluded. Each sample was analysed twice and the two technical repeats were combined for data analysis. pLlink software was used to identify cross-linked peptides with precursor and fragment ion mass accuracy at 20 p.p.m., and the results were filtered by applying a 5% FDR cutoff at the spectral level and then an F-value cutoff at 0.001. The inter-domain crosslinks were further filtered by requiring >3 spectra and the best F-value < 1.0 × 10⁻² in at least one of the samples to eliminate false identifications.

In vitro ALPK1 kinase assay. Two hundred nanograms of ALPK1-(N+K) complex purified from E. coli BL21 (DE3) ΔhldE or 3×-Flag-ALPK1 (full-length) immunopurified from E. coli BL21 (DE3) 293T cells were mixed with 2 μg TIF–His protein (also purified from the ΔhldE strain) in a 50-μl reaction containing 45 mM HEPES (pH 7.4) and 4 mM MgCl₂. ALPK1 K/M and TIF9A mutant proteins were used as negative controls. ADP-LH-Hep or HBP (20 μmol) was added to test their ability to activate ALPK1. Kinase reactions were initiated by adding 100 μM ATP and allowed to proceed for 30 min or 1 h at 30°C. To assay HBP and its activation by NMNAT-mediated modification, 5 μg of NMNAT1 or NMNAT3 protein purified from E. coli BL21 (DE3) ΔhldE were mixed with 20 μM HBP also in a 50-μl reaction in the presence of 100 μM ATP; the reaction was incubated at 30°C for 1 h. Following addition of 200 ng of ALPK1-(N+K) and 2 μg of TIF–His, the reaction was allowed to proceed for another 1 h. The reactions were stopped by adding 4× SDS loading buffer and subjected to SDS–PAGE analyses. Phosphorylated TIF–His protein was detected by immunoblotting using the anti-pT9-TIFA antibody as described above.

Protein structural determination. Purified proteins were concentrated to 10 mg ml⁻¹ for crystallization screens at 20°C using the sitting-drop vapour diffusion method. The drop, containing 1 μl of protein solution and 1 μl of reservoir solution, was equilibrated over 100 μl reservoir solution. Initial crystallization hits of SeMet-labelled ALPK1 (1–451) and native ALPK1 (1–446) appeared from the PEG-ion Kit and the Crystal Screen Kit (Hampton Research), respectively. Qualified crystals of SeMet-labelled ALPK1 (1–451) were obtained in the reservoir solution containing 6% Tacsimate (pH 5.6) and 6.8% PEG 3550 within 1 week, and the best-diffracted crystals of native ALPK1 (1–446) were grown from the reservoir solution containing 0.1 M CH₃COONa (pH 4.0) and 1.4 M HCOONa. For data collection, the crystals were soaked in cryo-protectant solution containing the reservoir buffer supplemented with 30% ethylene glycol for SeMet-labelled ALPK1 (1–451) or 15% glycerol for native ALPK1 (1–446) followed by flash-freezing with liquid nitrogen. Diffraction data were collected at the Shanghai Synchrotron Radiation Facility (Shanghai) beamline BL18U1 for SeMet-labelled ALPK1 (1–451) and BL19U1 for native ALPK1 (1–446) under the wavelengths of 0.97776 Å and 0.97853 Å, respectively. Data were processed in X-ray Detector Software. The phase was determined by the single wavelength anomalous dispersion method and the MAD data collection was performed in PHENIX. The rest of the model was manually built with Coot. The structure of ALPK1-NTD (residues 1–466) was refined in PHENIX, and manual modelling was performed using Extended Data Table 1a. Ramachandran statistics indicated that all the residues are in the allowed region, and 98.2% fall into the favoured region.

The quality of the final model was validated by MolProbity.

Mouse experiments and measurements of cytokines. Wild-type C57BL/6 mice were purchased from Vital River Laboratory Animal Technology (Beijing). To generate Alpk1−/− mice, a gRNA (GGCCCTCTGCGCGGCTAAAGG) targeting exon 3 of Alpk1 was designed with an online gRNA designing tool (http://crispr.mit.edu/). In vitro transcribed guide RNA and Cas9 mRNA were co-microinjected into C57BL/6 mice-derived zygotes. The tail-end genomic DNA of each offspring was amplified with the forward primer 5′-CCGTAGGGCAGGCTAGGCGG-3′ and the reverse primer 5′-TCCAAGGAGCAGGTACGTTG-3′. Sanger sequencing was performed to analyse the PCR products and identify the founders with out-of-frame indels. Founders with the same out-of-frame indels were intercrossed to obtain homozygous Alpk1−/− mice (Supplementary Table 2). All mice were maintained in the specific pathogen-free facility at National Institute of Biological Sciences, Beijing. All mouse experiments were carried out in accordance with the national guidelines for housing and care of laboratory animals (Ministry of Health, China) and the protocol is in accordance with institutional regulations after review and approval by the Institutional Animal Care and Use Committee at National Institute of Biological Sciences, Beijing.

Eight-week-old female wild-type or Alpk1−/− C57BL/6 mice were used for air pouch model construction, followed by HBP or ADP-LH-Hep administration. Five mice were assayed for each group. The air pouch was constructed as previously described, with slight modifications. In brief, 3 ml of sterile air was injected into the subcutaneous tissue of the back of the mice. Three days later, another 2.5 ml sterile air was injected into the same pouch to maintain patency. Six days later, synthetic HBP or ADP-LH-Hep (2 mg kg⁻¹ body weight) dissolved in 300 μl of saline was injected into the well-developed pouch (saline alone was injected into the control mice). Another 3 h later, blood samples from mouse tail end were collected for serum isolation, the mice were then killed, and the pouch was carefully excised from a small hole. Immediately afterwards, 600 μl of PBS was injected into the pouch, and the fluids were gently sucked into and out of the bulb to mix the contents. All fluids within the pouch were collected and centrifuged; the supernatants and the cell pellets were used for ELISA analysis and counting the total leukocyte number, respectively. Cytokine levels in the sera and air pouch fluid supernatants were determined using ELISA kits (IL-6, IP-10, GRO-α and MCP1). ProcartaPlex multiplex immunoassay (ebioscience) was also performed to measure up to 36 cytokines in both the air pouch washes and the sera.

For infection, single clones of B. cenocepacia J2315 strain were cultured at 37°C in LB broth with shaking for 18 h. The bacterial cultures were diluted to 1:50 in fresh LB broth, and grown until OD₆₀₀ reached 0.8. The bacteria were washed with PBS and then diluted in 1% gelatin-containing PBS. Indicated 6-week-old male mice (C57BL/6 background) were anaesthetized by intraperitoneal injection of 0.8% pentobarbital sodium and injected intratracheally with 30 μl of bacteria suspension (5 × 10⁴ c.f.u. for each mouse) or 1% gelatin-containing PBS. The mice were killed 24 h post-infection. The lungs were removed and homogenized to measure bacterial burden or cytokine concentration by ProcartaPlex multiplex immunoassay (ebioscience Inc.).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability. All data supporting the findings of this study are included in this manuscript and its Supplementary Information files. The atomic coordinates and structure factors of the ALPK1-NTD–ADP-heptose complex have been deposited in the Protein Data Bank under the accession code 5ZZC2.
Extended Data Fig. 1 | Analyses of the ADP-Hep biosynthesis pathway and ADP-Hep-induced NF-κB activation. a, Low calcium-induced secretion of the T3SS substrates in the \textit{hldE} transposon mutant. \textit{yscC} is required for T3SS assembly. \textbf{b}, Schematic of the classical ADP-Hep biosynthesis pathway in Gram-negative bacteria. \textbf{c}, ADP-Hep-dependent autotransporter heptosylation in \textit{ΔgmhB} mutant. A fragment of the AIDA-I autotransporter (GST–AIDA-I50–600–Flag) and its heptosyltransferase (AAH) were expressed in \textit{Y. pseudotuberculosis} \textit{Δ6} deleted of a gene in the ADP-Hep biosynthesis pathway. AIDA-I heptosylation was assessed using the ECL glycoprotein detection kit. \textbf{d}, NF-κB activation in 293T cells electroporated with HBP or H1P (obtained by treating HBP with recombinant GmhB for the indicated times). \textbf{e}, Effects of \textit{ALPK1} knockout on other known NF-κB pathways. NOD1, NOD2 and MYD88 were transfected into the cells. Wild-type cells and two \textit{ALPK1} \textit{−/−} 293T clones (KO-1/2) were treated with ADP-LD-Hep (100 μM), TNF (20 ng ml\(^{-1}\)), C12-iE-DAP (10 ng ml\(^{-1}\)) or MDP (10 ng ml\(^{-1}\)). \textbf{f}, Effects of NOD1/2 deficiency on ADP-Hep-induced NF-κB activation and TIFA foci formation. Wild-type HeLa cells and two NOD1/2 double-knockout clones (DKO-1/2) were assayed. eGFP–TIFA was transfected into the cells (g). Scale bar, 20 μm. \textbf{d–f}, NF-κB activation was assessed by luciferase reporter assay (mean ± s.d. from three technical replicates); two-tailed unpaired Student\'s \textit{t}-test was performed (\(*P < 0.05\), \(**P < 0.01\), \(***P < 0.001\), NS, not significant). All data are representative of three independent experiments.
Extended Data Fig. 2 | FACS-based genome-wide CRISPR-Cas9 screen identifies the ALPK1–TIFA–TRAF6 axis that mediates ADP-Hep-induced NF-κB activation. a, Flow chart of the CRISPR-Cas9 screen for genes required for activation of NF-κB induced by extracellular ADP-LD-Hep but not TNF in 293T cells. b, Immunoblots of Flag–ALPK1 (wild type or its K/M mutant) and Flag–TIFA (wild type or its T9A mutant) expressed in knockout 293T cells. c, Requirement of T9 of TIFA for ADP-Hep-induced NF-κB activation. Flag–TIFA (wild type or T9A) was transfected into two TIFA−/− 293T clones (KO-1/2). d, Requirement of ALPK1 kinase activity for ADP-LD-Hep-induced formation of TIFA foci. eGFP–TIFA was stably expressed in wild-type or ALPK1−/− 293T cells expressing Flag–ALPK1 (wild type or the kinase-dead K/M mutant). e, Fluorescence imaging of mCherry–ALPK1 and eGFP–TIFA in 293T cells treated with or without ADP-LD-Hep. Scale bar, 10 μm. f, Phos-tag gel assay of myosin I phosphorylation in ADP-LD-Hep-treated cells. Flag–myosin I was transfected into indicated 293T cells. Immunoblots of total cell lysates separated on phos-tag gel or regular SDS gels are shown. g, Effects of ALPK1 or TIFA knockout on activation of NF-κB induced by ADP-LD or DD-Hep electroporation. ALPK1−/− and TIFA−/− 293T cells were complemented as indicated. h, ADP-LD-Hep-induced co-immunoprecipitation of TIFA with ALPK1 and TRAF6 in transfected 293T cells. c, g, NF-κB activation was assessed by luciferase reporter assay (mean ± s.d. from three technical replicates); two-tailed unpaired Student’s t-test was performed (*P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant). d, e, Confocal images with Hoechst-stained nuclei. All data are representative of three independent experiments.
Extended Data Fig. 3 | See next page for caption
Extended Data Fig. 3 | The ALPK1–TIFA pathway mediates T3SS-dependent and -independent activation of NF-κB by various bacterial pathogens. a, b, Requirement of ALPK1 kinase activity and TIFA for *Y. pseudotuberculosis*-induced activation of NF-κB. Wild-type or ALPK1−/− 293T cells expressing Flag–ALPK1 (wild type or K/M) or TIFA−/− 293T cells expressing Flag–TIFA were infected with indicated *Y. pseudotuberculosis* strains. NF-κB activation was assessed by luciferase reporter assay (a) or eGFP reporter expression (b). Scale bar, 50 μm (b). c–e, Requirement of ALPK1 kinase activity for *Y. pseudotuberculosis* (or *S. flexneri* 2457T)-induced phosphorylation of TIFA (c, d) or formation of eGFP–TIFA foci (e). Flag– or eGFP–TIFA was expressed in wild-type or ALPK1−/− 293T cells expressing Flag–ALPK1 (wild type or K/M). TIFA phosphorylation was assessed by anti-pT9-TIFA immunoblotting (c, d). *Y. pseudotuberculosis* Δ6 was labelled with mCherry (scale bar, 20 μm) (e). f, g, NF-κB luciferase reporter activation and formation of eGFP–TIFA foci induced by T3SS-negative bacteria. Wild-type, ALPK1−/− or Flag–ALPK1-complemented ALPK1−/− 293T cells were infected with DAEC 2787, ETEC H10407, *B. cenocepacia* J2315 or their ΔhldE mutants. Scale bar, 20 μm. a, f, Luciferase data are shown as mean ± s.d. from three technical replicates; two-tailed unpaired Student’s *t*-test was performed (*P < 0.05, **P < 0.01, ***P < 0.001). b, e, g, Confocal images with Hoechst-stained nuclei. All data shown are representative of three independent experiments.
Extended Data Fig. 4 | Co-expression of ALPK1-NTD and ALPK1-KD can support ADP-Hep and Y. pseudotuberculosis-induced activation of NF-κB and conservation of ADP-Hep-binding residues in ALPK1-NTD. a, c, Immunoblots of ALPK1 N- and C-terminal truncation mutants expression in indicated 293T cells. NF-κB luciferase reporter activation induced by ADP-LD-Hep and Y. pseudotuberculosis Δ6 are in Fig. 2h. b, Mapping the minimal N- and C-terminal regions of ALPK1 sufficient for ADP-LD-Hep-induced activation of NF-κB. Fluorescence images of NF-κB–eGFP expression are shown. Scale bar, 50 μm. d, e, Assay of ALPK1-NTD and ALPK1-KD co-expression in mediating ADP-LD-Hep (d) or Y. pseudotuberculosis (e)-induced TIFA phosphorylation. Immunoblots of total cell lysates using indicated antibodies are shown. f, Multiple sequence alignment of the NTDs of ALPK1 from indicated organisms. Red residues are those involved in ADP-Hep binding, revealed by the crystal structure of human ALPK1-NTD–ADP-LD-Hep complex (Fig. 3g). Data shown are representative of three (a–c) or two (d, e) independent experiments.
Extended Data Fig. 5 | See next page for caption
Extended Data Fig. 5 | Direct binding of ADP-Hep to ALPK1-NTD.  
a, E. coli-purified ALPK1-(N+K) complex on a Coomassie blue-stained SDS–PAGE gel.  
b–e, HPLC–MS fractionation, NF-κB-inducing activity and mass spectrometry of small-molecule extracts from His₆–ALPK1-NTD purified from wild-type E. coli BL21 (DE3). The small-molecule extracts, obtained by protein denaturing and precipitation (95 °C for 5 min), were analysed by HPLC–MS with 17 fractions obtained (b). The 17 fractions were used to treat 293T cells (c) or cells expressing Flag–TIFA (d); NF-κB luciferase activity (mean ± s.d. from three technical replicates) and anti-pT9-TIFA immunoblotting are in c and d, respectively.  
e, Mass spectrometry of fraction 6 identified three major ions corresponding to AMP, ADP and ADP-Hep.  
f, LC–MS/MS of ADP-Hep in fraction 6 (b, e) or synthetic ADP-LD-Hep standard.  
g, MS/MS spectra of the [M+H]+ product ions of ADP-Hep in fraction 6 in comparison with those of synthetic ADP-LD-Hep. The heptose of ADP-Hep was not shown owing to neutral loss.  
h, BLI assay of ADP-LD-Hep or S7P binding to ALPK1-(N+K). ALPK1-(N+K) complexes purified from E. coli BL21 (DE3) ΔhldE were biotinylated in vitro. Sensorgrams of the binding to ALPK1-(N+K) by different concentrations of the indicated sugar (colour lines) are shown. Grey lines are from model fits. Data shown are representative of three (a, h) or two (b–g) independent experiments.
Extended Data Fig. 6 | HBP binding is insufficient to render ALPK1-KD competent for substrate recognition and phosphate transfer. a, BLI assay of HBP binding to in vitro-biotinylated apo-ALPK1-(N+K). Sensorgrams of the binding in different concentrations of HBP (colour lines) are shown. Grey lines are from model fits. b, Excess HBP competitively inhibits activation of ALPK1 by ADP-Hep. Flag–ALPK1 purified from 293T cells was incubated with purified TIFA–His6 and ADP-Hep in the presence of a titrating amount of HBP. c, d, Effects of HBP binding on ALPK1 phosphorylation and recognition of a 15-residue peptide substrate derived from TIFA. The N-terminal 15-residue sequences of TIFA were fused to GST (GST–TIFA.N15). c, GST-pulldown assay of ALPK1-(N+K) binding to GST–TIFA.N15. d, Effects of HBP binding on ATP hydrolysis activity of ALPK1. Apo-ALPK1-(N+K) (wild type or K/M) was incubated with HBP or ADP-LD-Hep, and further reacted with ATP. Percentages of ATP consumption at indicated reaction conditions are shown (mean ± s.d. from three technical replicates). Two-tailed unpaired Student’s t-test was performed (*P < 0.05; **P < 0.01; ***P < 0.001). e, Graphical representation of DSS-crosslinked residues between ALPK1-NTD and ALPK1-KD identified by chemical crosslinking coupled with mass spectrometry. Apo-ALPK1-(N+K) was left untreated, or incubated with HBP or ADP-LD-Hep. Crosslinking connections are depicted by straight lines, and the corresponding raw mass spectrometry data are in Supplementary Table 3. g, Crystal structure of TRPM7 kinase domain in complex with AMPPNP (PDB code, 1IA9). TRPM7 is shown in cartoons and AMPPNP is in sticks. The loop containing K1727 and I1736 (shown in sticks, corresponding to K1140 and K1149 in human ALPK1, respectively) is in yellow. Phosphorylation of TIFA and GST–TIFA.N15 was assayed by anti-pT9-TIFA immunoblotting (b, c). Apo-ALPK1-(N+K), TIFA–His6, and GST–TIFA.N15 were purified from E. coli BL21 (DE3) ΔhldE (a–f). Data shown are representative of three (a–e) and two (f) independent experiments.
Extended Data Fig. 7 | See next page for caption
Extended Data Fig. 7 | HBP can be transformed into ALPK1 activation-competent ADP-heptose 7-P by bacterial or host adenylyltransferase. a, b, Requirement of ALPK1 kinase activity for cytosolic HBP-induced phosphorylation of TIFA (a) and activation of NF-κB (b). HBP was electroporated into wild-type or ALPK1−/− 293T cells expressing Flag–ALPK1 (wild-type or K/M). c, d, Enzymatically synthesized HBP could induce ALPK1-mediated NF-κB activation (c) and TIFA phosphorylation in vitro (d). S7P was reacted with recombinant GmhA or HldE or both. Following protein denaturing and precipitation, reaction supernatants were added to wild-type or ALPK1−/− 293T cells containing an empty vector or Flag–ALPK1 (c). Enzymatically synthesized HBP product (HBP_{enzymatic}), synthetic ADP-LD-Hep or HBP was incubated with ALPK1-(N+K) in the presence of TIFA–His6 (d). e, Schematic of HldE adenylyltransferase synthesis of ADP-Hep and ADP-heptose 7-P from H1P and HBP, respectively. f, g, NF-κB activation by (f) and LC–MS of (g) enzymatically synthesized HBP product. Indicated reaction products were added to wild-type or ALPK1−/− 293T cells (f) or analysed by LC–MS (g). h, i, NF-κB (h) and in vitro ALPK1 activation (i) by ADP-heptose 7-P. HPLC-purified ADP-heptose 7-P was added to wild-type or ALPK1−/− 293T cells expressing Flag–ALPK1 (wild-type or K/M). j, Equal amounts of the indicated sugars were incubated with ALPK1-(N+K) in the presence of TIFA–His6. Ultra-performance liquid chromatography with tandem mass spectrometry of the reaction product of HBP and NMNAT1. Purified ADP-heptose 7-P, synthesized by reacting HBP with HldE, was used as the standard. k, Effect of NMNAT1 overexpression on activation of NF-κB by electroporation of HBP into 293T cells. The immunoblots show NMNAT1 expression. Apo-ALPK1-(N+K) was purified from E. coli BL21 (DE3) ΔhldE and phosphorylation of TIFA was assessed by anti-pT9-TIFA immunoblotting (d, i). b, c, f, h, k, NF-κB activation was assessed by luciferase reporter assay (mean ± s.d. from three technical replicates); two-tailed unpaired Student’s t-test was performed (*P < 0.05; **P < 0.01; ***P < 0.001). Data shown are representative of three (a–d, h, i, k) or two (f, g, j) independent experiments.
Extended Data Fig. 8 | ALPK1 Q67A/Y68A mutants that can discriminate cytosolic HBP from ADP-Hep resist activation by ADP-heptose 7-P. a–c, Effects of ALPK1 Q67A, Y68A or Q67A/Y68A double mutations on activation of NF-κB by HBP, ADP-Hep and ADP-heptose 7-P. ALPK1−/− 293T cells expressing the indicated Flag–ALPK1 mutants were electroporated with excess HBP or ADP-LD-Hep (a), or treated with excess ADP-heptose 7-P or ADP-LD-Hep (c). Anti-Flag immunoblot (b) show expression of the ALPK1 mutants. d, In vitro TIFA phosphorylation assay of ALPK1 Q67A/Y68A activation by HBP, ADP-heptose 7-P, or ADP-LD-Hep. Flag–ALPK1 Q67A/Y68A was purified from 293T cells. e, f, In vitro TIFA phosphorylation assay of ALPK1 Q67A/Y68A activation by small-molecule extracts from HBP-electroporated cells (SE HBP). 293T cells electroporated with HBP were used to prepare the small-molecule extracts, and synthetic HBP was included as the control. Apo–ALPK1-(N+K) (e) and Flag–ALPK1 (f) were purified from E. coli BL21 (DE3) ΔhldE and 293T cells, respectively. g, Effects of ALPK1 Q67A/Y68A double mutations on Y. pseudotuberculosis-induced activation of NF-κB. ALPK1−/− 293T cells expressing Flag–ALPK1 (wild-type or the Q67A/Q68A mutant) were left uninfected or infected with indicated Y. pseudotuberculosis strains. a, c, g, NF-κB activation was assessed by luciferase reporter assay (mean ± s.d. from three technical replicates); two-tailed unpaired Student’s t-test was performed (*P < 0.05; **P < 0.01; ***P < 0.001). All data are representative of at least two independent experiments.
Extended Data Fig. 9 | ADP-Hep but not HBP induces Alpk1-dependent cytokine expression in mice. HBP or ADP-LD-Hep (2 mg kg\(^{-1}\)) or the saline control were injected into the dorsal air pouches of wild-type (a–d) or Alpk1\(^{-/-}\) (d) mice (C57BL/6). \(n\) (biologically independent animals) = 5 for each group of treatment. Cytokine profiling was determined by the multiplex immunoassay (a–c) or ELISA (d). a, b, Heat maps of indicated cytokine concentrations in the air pouch (a) and the serum (b) of injected mice. c, d, Cytokine concentrations in the serum shown as mean ± s.e.m. (two-tailed unpaired Student’s \(t\) test, *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), ns, not significant). All data are representative of two independent experiments.

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Extended Data Table 1 | Data collection and refinement statistics for ALPK1-NTD structure (a) and Dali-search results of the structure (b)

### a

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| Refinement      |                  |                      |
| Resolution (Å)  | 48.89-2.59       |                      |
| No. of reflections | 173,963         |                      |
| Rmerge/Rfree    | 0.242/0.2628     |                      |

| No. of atoms    |                  |                      |
| Protein         | 30,723           |                      |
| Water           | 0                |                      |

| B factors       |                  |                      |
| Protein         | 62.91            |                      |
| Water           | 0                |                      |

| r.m.s deviations | Bond lengths (Å) | 0.005 |
| Bond angles (°)  | 0.599            |       |

### b

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<td>PINS</td>
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<tr>
<td>3</td>
<td>5g05-O</td>
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<td>4.8</td>
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</tr>
<tr>
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<td>5dbk-A</td>
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<td>3.5</td>
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<td>13</td>
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<td>5</td>
<td>3txm-A</td>
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<td>4.2</td>
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<td>26S proteasome regulatory complex subunit P42IB</td>
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<tr>
<td>6</td>
<td>4ila-B</td>
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<td>3.2</td>
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<td>Response regulator aspartate phosphatase I</td>
</tr>
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<td>7</td>
<td>5o01-A</td>
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<td>13</td>
<td>BKLC (Bacterial Kinesin-Light Chain-like)</td>
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<td>4.0</td>
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<td>Anaphase-promoting complex subunit 1</td>
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<tr>
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<td>3.9</td>
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<td>26S proteasome non-ATPase regulatory subunit 4</td>
</tr>
</tbody>
</table>

*Values in parentheses are for the highest-resolution shell.

In a, single SeMet or native crystal was used for data collection and structure determination.
Experimental design

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

2. Data exclusions
   - Describe any data exclusions.
   - There were no data exclusions.

3. Replication
   - Describe the measures taken to verify the reproducibility of the experimental findings.
   - All attempts at replication are successful.

4. Randomization
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - No randomization was used in this study.

5. Blinding
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - No blinding was done in this study.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

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

n/a Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

- A statement indicating how many times each experiment was replicated

- The statistical test(s) used and whether they are one- or two-sided

- Only common tests should be described solely by name; describe more complex techniques in the Methods section.

- A description of any assumptions or corrections, such as an adjustment for multiple comparisons

- Test values indicating whether an effect is present

- Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.

- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

- Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.
Software
Policy information about availability of computer code
7. Software

Describe the software used to analyze the data in this study.

Not relevant.

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

Materials and reagents
Policy information about availability of materials
8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All unique materials used are readily available from the authors.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The anti-AAH antibody was described previously (Reference 20). The rabbit polyclonal antibody for ALPK1 was from GeneTex Inc. (#GTX87015). Antibodies for tubulin (T5168) and Flag (M2) were from Sigma-Aldrich. Anti-Myc monoclonal antibody (9E10) was from Covance Inc. The rabbit polyclonal antibody (anti-pT9-TIFA) against Thr-9-phosphorylated human TIFA was developed by Abcam as a collaborative project using a synthetic phospho-peptide antigen, from which a monoclonal antibody was generated (ab214815).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

293T, HeLa and THP-1 cells were obtained from the American Type Culture Collection (ATCC).

b. Describe the method of cell line authentication used.

Identity of the cell lines were frequently checked by their morphological features but have not been authenticated by the short tandem repeat (STR) profiling.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were tested to be mycoplasma-negative by the standard PCR method.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines are used in this study.

Animals and human research participants
Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines
11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

8 week-old female mice (C57BL/6 background, wild-type or Alpk1-/- as indicated) were used for ADP-Heptose and HBP injection experiments; and 6-week old male mice (C57BL/6 background) were used for B. cenocepacia infection assays.

Policy information about studies involving human research participants
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

There are no human subjects involved in this study.