Regulation of phosphoribosyl ubiquitination by a calmodulin–dependent glutamylase

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The bacterial pathogen Legionella pneumophila creates an intracellular niche permissive for its replication by extensively modulating host-cell functions using hundreds of effector proteins delivered by its Dot/Icm secretion system1. Among these, members of the SidE family (SidEs) regulate several cellular processes through a unique phosphoribosyl ubiquitination mechanism that bypasses the canonical ubiquitination machinery2–4. The activity of SidEs is a unique phosphoribosyl ubiquitination mechanism that bypasses the canonical ubiquitination machinery2–4. The activity of SidEs is not completely understood6,7. Here we demonstrate that SidJ inhibits the activity of SidEs by inducing the covalent attachment of glutamate moieties to SdeA—a member of the SidE family—at E860, one of the catalytic residues that is required for the mono-ADP-ribosyltransferase activity involved in ubiquitin activation2. This inhibition by SidJ is spatially restricted in host cells because its activity requires the eukaryote-specific protein calmodulin (CaM). We solved a structure of SidJ–CaM in complex with AMP and found that the ATP used in this reaction is cleaved at the α-phosphate position by SidJ, which—in the absence of glutamate or modifiable SdeA—undergoes self-AMPylation. Our results reveal a mechanism of regulation in bacterial pathogenicity in which a glutamylation reaction that inhibits the activity of virulence factors is activated by host-factor-dependent acyl-adenylation.

Ubiquitination regulates many aspects of immunity, and pathogens have evolved various strategies through which to co-opt the ubiquitin network to promote their virulence5,6. One such example is the SidE

Fig. 1 | SidJ antagonizes the effects of SdeA in eukaryotic cells. a, SidJ suppresses the yeast toxicity of SdeA (H277A). Top, diluted cells from yeast strains inductively expressing SdeA or SdeA(H277A) that contain the vector (V) or a SidJ construct were spotted onto the indicated media and grown for 2 days. Bottom, the expression of relevant proteins was probed by immunoblotting. The 3-phosphoglyceric phosphokinasae-1 (PGK1) was detected as a loading control. b, SidJ abrogates SdeA-mediated ubiquitination in mammalian cells. Lysates of HEK293T cells expressing the indicated proteins were detected by immunoblotting with a haemagglutinin (HA)-specific antibody to detect 3×HA–Ub–AA and proteins modified by 3×HA–Ub–AA. The expression of Flag–SdeA and Flag–SidJ was also investigated. c, SidJ rescues the degradation of hypoxia-inducible factor 1-α (HIF–1α) that is blocked by SdeA. Lysates of HEK293T cells expressing the indicated proteins were resolved by SDS–PAGE and analysed with antibodies specific for the epitope tags or the relevant proteins. d, SidJ from E. coli or HEK293T cells cannot deubiquitinate proteins modified by SdeA. Proteins modified by 3×HA–Ub–AA obtained by immunoprecipitation were treated with GST–SidJ from E. coli, Flag–SidJ from HEK293T or SdeA(1–193), a truncated form of SdeA containing residues 1–193. Note that none of these proteins caused a reduction in the ubiquitination signals. e, GST–SidJ does not inhibit SdeA-induced ubiquitination in vitro. SidJ was co-incubated with SdeA for 2 h at 37 °C and SdeA activity was assayed. A Flag–specific antibody was used to detect modified and unmodified 4×Flag–Rab33B, judging by a shift in its molecular mass. SdeA and SidJ were analysed with specific antibodies. Experiments in each panel were performed independently at least 3 times with similar results.

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family effectors from *L. pneumophila*, which ubiquitinate structurally diverse proteins that are associated with the endoplasmic reticulum\(^2\). Ubiquitination by SidEs is initiated by means of ADP-ribosylation at R42 of ubiquitin, which is catalysed by mono-ADP-ribosyltransferase (mART)\(^3\). The activated ADP-ribosylated ubiquitin (ADPR-Ub) is then used by a phosphodiesterase-like domain that is also present in SidEs; this domain ligates phosphoribosylated ubiquitin (PR-Ub) to serine residues of substrate proteins\(^2,3\). Because both ADPR-Ub and PR-Ub impair the function of eukaryotic cells by inhibiting canonical ubiquitination\(^1\), which is pivotal for bacterial virulence\(^10\), it is likely that there exist factors of either bacterial or host origin that function to prevent potential cellular damage caused by these molecules.

The activity of members of the SidE family—such as SdeA—is regulated by Sidf\(^5\), which is able to suppress the yeast toxicity of SdeA\(^6\). Sidf purified from *L. pneumophila* also seems to remove ubiquitin from modified substrates\(^7\). Despite these observations, questions about the mechanism of action of Sidf remain. For example, an SdeA mutant with a histidine-to-alanine mutation at residue 277 (SdeA(H277A))—which is defective in phosphodiesterase activity—is still toxic to yeast even though it cannot ubiquitinate substrates\(^8\). However, whether Sidf can suppress its toxicity is unknown. Furthermore, it is not clear why the deubiquitinating activity is observed only in Sidf purified from *L. pneumophila*\(^7\).

We set out to address these questions by constructing a yeast strain that inducibly expressed SdeA(H277A), and found that Sidf effectively suppressed its toxicity (Fig. 1a). Sidf may therefore neutralize the toxicity of ADPR-Ub or target the ADP-ribosylation activity of SdeA. In addition, Sidf substantially reduced protein modification induced by SdeA and effectively relieved SdeA-induced inhibition of the degradation of hypoxia-inducible factor 1 (Fig. 1b, c). However, Sidf that was purified from *Escherichia coli* or from mammalian cells failed to remove ubiquitin from modified proteins, nor did it detectably affect the SdeA-induced ubiquitination of Rab33b (Fig. 1d, e). Together, these results suggest that Sidf affects the function of SdeA, but its activity in cells cannot be recapitulated by biochemical reactions.

Flag-tagged SdeA (Flag–SdeA) that was coexpressed with GFP or with the Sidf(D542A/D545A) mutant (carrying aspartic-acid-to-alanine mutations at residues 542 and 545), which is defective in suppressing SdeA yeast toxicity\(^8\), was found to robustly modify Rab33b. However, Flag–SdeA obtained from cells coexpressing GFP–Sidf (Flag–SdeA\(^*)\) failed to ubiquitinate Rab33b (Fig. 2a). We next examined whether Sidf affects the mART activity by carrying out reactions that measure the ability of Flag–SdeA\(^*)\) to use ubiquitin or ADPR-Ub for ubiquitination. Flag–SdeA\(^*)\) lost the ability to catalyse ubiquitination from ubiquitin, but retained the ability to use ADPR-Ub for ubiquitination (Fig. 2b). Consistently, Flag–mART (SdeA residues 563–910) failed to ubiquitinate Rab33b with ADPR-Ub. ADPR-Ub or ubiquitin was used by a phosphodiesterase-like domain that is also present in SidEs; this domain ligates phosphoribosylated ubiquitin (PR-Ub) to serine residues of substrate proteins\(^2,3\). Because both ADPR-Ub and PR-Ub impair the function of eukaryotic cells by inhibiting canonical ubiquitination\(^1\), which is pivotal for bacterial virulence\(^10\), it is likely that there exist factors of either bacterial or host origin that function to prevent potential cellular damage caused by these molecules.

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563 to 910, hereafter denoted SdeA(563–910))12 that was purified from HEK293T cells expressing GFP–SidJ (Flag–mART+) also failed to ubiquitinate Rab33b with the PDE-competent SdeA(E860A/E862A) mutant12 (Fig. 2c). We therefore conclude that SidJ targets the mART activity of SdeA.

Analysis by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) identified a mass shift of 129.04 Da (m/z = 129.04, z = 1) on the peptide –H855GEGTESEFSVYLPEDVALVPVK877 in Flag–mART+ (Fig. 2d, e). The modification—probably the addition of a glutamate—was mapped to E860, one of the catalytic residues of the mART2 (Fig. 2e). Approximately 93.7% of E860 was modified in samples coexpressed with GFP–SidJ, and a modification of 258.09 Da (m/z = 258.09, z = 1)—presumably diglutamate—was also detected on a small portion of the same peptide (Extended Data Fig. 1). Thus, SidJ may be a glutamylase that ligates one or more glutamate moieties to E860 of SdeA.

We did not detect SidJ activity in reactions containing AT—energy transfer for known glutamylases12—and l-glutamate, or its structural isomers N-acetylserine and N-methylaspartate (Extended Data Fig. 2a). Because the inhibitory effects of SidJ are evident only when it is expressed in mammalian cells, we tested the hypothesis that its activity requires one or more factors of eukaryotic origin by including lysates of E. coli or HEK293T cells in the reactions. Lysates of HEK293T cells (native or boiled) caused a decrease in Rab33b modification (Extended Data Fig. 2b), which indicates that one or more heat-stable factors specific to eukaryotic cells are required for the activity of SidJ.

Analysis of the Pfam database13 of protein families revealed an IQ-like motif, which is involved in CaM binding, located near the carboxyl end of SidJ (Fig. 3a). The yeast toxicity of SidJ was suppressed by mutations in I841 and Q842 (Fig. 3b) in CaM-dependent inhibition by SidJ was added to a subset of a series of reactions containing SdeA, GST–SidJ and l-glutamate, N-acetylserine or N-methylaspartate. The activity of SdeA was measured by ubiquitination. SidJ is a CaM-dependent glutamylase that modifies SdeA at E860. A series of reactions containing the indicated proteins, [14C]-glutamate and ATP were allowed to proceed for 2 h at 37°C. The incorporation of [14C]-glutamate was detected by autoradiography. Data shown in b–f are one representative of at least three experiments with similar results.

Fig. 3 | Calmodulin is the host cofactor required for the glutamylase activity of SidJ. a, SidJ contains an IQ motif. The list shows the alignment of the IQ domain of SidJ with that of several CaM-binding proteins. Conserved residues are highlighted in red. The accession numbers for each of the proteins (from NCBI databases) are included. b, The cmd1 gene suppresses the yeast toxicity of SidJ. The top two panels show images of serially diluted yeast cells incubated with lysates of macrophages infected with the indicated strains and allowed to grow for 2 days. The lower two panels show the suppression of SidJ toxicity by cmd1. The expression of SidJ in each strain was examined and PKG1 was detected as a loading control (right). c, The interactions between SidJ and CaM. Beads coated with CaM were incubated with lysates of macrophages infected with the indicated bacterial strains to analyse its binding to SidJ (c, top). SidJ in bacteria (c, middle) or translocated into the host cytosol (c, bottom) was also examined. The bacterial isocitrate dehydrogenase (ICDH) and tubulin were analysed as loading controls, respectively. Lysates of HEK293T cells transfected to express GFP–SidJ or GFP–SidJ(I841D/Q842A) were incubated with CaM-coated beads (d). SidJ or SidJ(I841D/Q842A) bound to CaM was analysed by immunoblotting (bottom). TCL, total cell lysates. e, Inhibition of SdeA activity by SidJ requires glutamate and CaM. CaM was added to a subset of a series of reactions containing SdeA, GST–SidJ and l-glutamate, N-acetylserine or N-methylaspartate. The activity of SdeA was measured by Rab33b ubiquitination. SidJ is a CaM-dependent glutamylase that modifies SdeA at E860. A series of reactions containing the indicated proteins, [14C]-glutamate and ATP were allowed to proceed for 2 h at 37°C. The incorporation of [14C]-glutamate was detected by autoradiography. Data shown in b–f are one representative of at least three experiments with similar results.

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glutamylase that catalyses the ligation of glutamate moieties to E860 of SdeA.

We further investigated the mechanism of the CaM-dependent glutamylation activity of SidJ by structural analysis. A truncated SidJ that lacks the first 99 residues (SidJ(ΔN99)) showed activity that was indistinguishable from that of the full-length protein. Biological analysis indicated that it formed a stable heterodimer with CaM at a ratio of 1:1 (Extended Data Fig. 4). We solved a structure at 2.71 Å resolution of the SidJ(ΔN99)–CaM complex, using a 2.95 Å-resolution structure of the SidJ(Se-Met)–CaM derivative as the search model (Extended Data Table 1). In our structure, two SidJ–CaM heterodimeric complexes are found in one asymmetric unit (Extended Data Fig. 5a). Analysis of the intersubunit contacts in the asymmetric unit suggests that the interface between the two SidJ molecules in the structure results from crystal packing. In the complex, SidJ(ΔN99) folds into three distinct domains that we designated as the N-terminal domain, the central domain and the C-terminal domain (Fig. 4a). CaM docks onto the carboxyl end that contains the IQ motif (Fig. 4b). The interface area between SidJ and CaM is about 1,574 Å², which accounts for 17.6% of the surface of CaM.

SidJ(ΔN99) interacts extensively with CaM through hydrogen bonds and salt bridges. Specifically, Q830 and Q842 of SidJ engage in hydrogen-bonding interactions with E85 and S102 of the CaM C-lobe, respectively. Other hydrogen bonds include S808(SidJ) and R812(SidJ):R38(CaM), R804(SidJ):S39(CaM), R660(SidJ) and R796(SidJ):E15(CaM) (Fig. 4b). Mutations in these residues reduced the binding affinity of SidJ for CaM (Fig. 4c).

Binding of CaM, ATP, and CaM to SidJ for 2 h at 37 °C were monitored by autoradiography. In the presence of ATP, the formation of a product of ATP breakdown, induced by SidJ—is coordinated by pyrophosphate and Mg²⁺ ions. The AMP moiety—which is probably a product of ATP breakdown, induced by SidJ—is coordinated by R352(SidJ):E15(CaM), R804(SidJ):S39(CaM), R660(SidJ) and R796(SidJ):E15(CaM) (Fig. 4d). Substitution of R352(SidJ), R804(SidJ), R660(SidJ) or R796(SidJ) by alanine abolished the activity of SidJ, whereas a mutation in the distal Y443 had no effect (Fig. 4e). The binding of AMP does not cause obvious conformational changes in the SidJ(ΔN99)–CaM complex (Extended Data Fig. 5b). In our structures, we observed CaM in a relatively closed conformation with one Ca²⁺ ion coordinated to the EF1 site of CaM (Extended Data Fig. 5a). However, the B-factor of Ca²⁺ was relatively high, indicating partial occupancy of the ion; this is consistent with the partial disorder found in the CaM polypeptide in the crystals. CaM remained active even after dialysis against buffer containing 32P-ATP-P–IB: SdeA, ATP, and CaM for 2 h at 37 °C and measuring the ubiquitin ligase activity of SdeA. ATP analogues. The indicated compounds were incubated with SdeA, GST–SidJ, ATP, CaM and GTP at 37 °C and monitoring the loss of free CaM by the NanoTemper Analysis 2.2.4 software. Data shown are one representative from at least three independent experiments with similar results.
against EGTA or upon inclusion of this chelator in the reactions (Extended Data Fig. 6b, c).

The presence of AMP in the structure suggests that ATP was cleaved at the α site during the reaction. Indeed, the ATP analogues adenyl-
imidodiphosphate and ATP-γ-S—which cannot be effectively hydrolysed at the γ site—stalled activation of SidJ (Fig. 4f). ADP—but not AMP or adenosine—potently induced SidJ activity; in addition, ATP-α-S, which can be slowly hydrolysed at the α site, partially supported SidJ activity. By contrast, ApCp—which cannot be cleaved at the α site—failed to detectably activate SidJ (Fig. 4g). We therefore conclude that the SidJ-catalysed reaction involves the cleavage of ATP between the α and β phosphates.

Because SidJ-induced cleavage of ATP is analogous to the reaction involved in AMPylation\textsuperscript{15,16}, we thus examined whether SidJ catalyses AMPylation using \textsuperscript{32}P-α-ATP. Robust self-AMPylation of SidJ was detected in reactions containing CaM; such modification also occurred in glutamylation reactions that lacked glutamate or modifiable SdeA (Extended Data Fig. 7a, b). Furthermore, residues that are important for binding AMP are required for self-modification activity (Fig. 4h). We detected AMP in reactions containing SidJ, CaM and ATP, and the release of AMP was accelerated by SdeA but not by SdeA(E860A) (Extended Data Fig. 7c). We propose a model in which SidJ activates E860 of SdeA by acyl-adenylation, which is followed by nucleophilic attack of the amino group of free glutamate on the activated carbonyl of the unstable E860–AMP intermediate, leading to glutamylation of E860 and the release of AMP (Extended Data Fig. 7d).

Overexpression of SdeA in the ΔsidJ mutant severely affected intracellular bacterial replication\textsuperscript{8,9}, as did expression of SdeA(M408A/L411A), which is defective in substrate recognition\textsuperscript{11}. Such defects were rescued by simultaneous expression of SidJ (Extended Data Fig. 8). We attempted to separate the ubiquitin ligase activity from being the substrate for SidJ by constructing the mutant protein SdeA(E860D). SidJ can neither modify this mutant nor suppress its yeast toxicity. Similarly, its ubiquitin ligase activity is insensitive to SidJ. Of most relevance, the inhibition of intracellular growth of the ΔsidJ strain by SdeA(E860D) cannot be rescued by coexpressing SidJ (Extended Data Fig. 9).

The AMP-binding site in our structure is essential for the activation step, but it remains unclear how free glutamate is recognized. The E860–AMP intermediate produced at this site may transit to a second nucleotide-binding site in the same domain for glutamylation\textsuperscript{16}. It is also not clear how SidJ selectively targets E860 of SdeA, but not nearby E857 and E862, or whether it modifies other proteins as well as SdeEs by glutamylation or AMPylation. The glutamylation of SdeEs by SidJ expands the strategies used by \textit{L. pneumophila} to ensure balanced modulation of host function\textsuperscript{5}. SidJ is a unique glutamylase that bears no similarity to mammalian glutamylases\textsuperscript{12–15}. The requirement of CaM for its activity ensures that SidEs will not be inactivated prior to modifying host targets\textsuperscript{7}. CaM also activates the oedaema factor of \textit{Bacillus anthracis} and CysA of \textit{Bordetella pertussis}\textsuperscript{21,23}, both catalysing the synthesis of the important signalling molecule cyclic AMP\textsuperscript{24}. Further study of the mechanism of CaM-induced activation of SidJ and the relationship between the AMPylation and glutamylation reactions is likely to reveal insights into the regulation and function of glutamylases.

### Online content

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For protein purification from mammalian cells, HEK293T cells were transfected with corresponding plasmids to express Flag-tagged proteins. Cells were lysed with RIPA buffer, and subject to immunoprecipitation with beads coated in Flag-specific antibody. Proteins were then eluted from the beads by using 3 × Flag peptides following the manufacturer’s protocol (Sigma-Aldrich, F47999).

**Crystallization.** The purity of SidJ(AN99)–CaM was around 95% as assessed by SDS–PAGE, and initial crystallization screens of native SidJ–CaM were conducted by sitting-drop vapour diffusion using commercial crystallization screens. The protein concentration used for crystallization was 5–7 mg ml⁻¹. Hampton Research kits were used in the sitting-drop vapour diffusion method to obtain preliminary crystallization conditions at 16 °C. Crystallization drops contained 0.5 μl of the protein solution mixed with 0.5 μl of reservoir solution. Diffraction-quality crystals of SidJ(AN99)–CaM and its complex with ATP (SidJ(AN99)–CaM–ATP) were grown in the presence of 0.1 M HEPES (pH 6.5–7.3), 20% (v/v) PEG 4000, and 0.2 M NaCl. To solve the phase problem, Se-Met was incorporated into SidJ and the SidJ(Se-Met) was purified similarly to native SidJ except with the addition of 5 mM DTG to the buffer during the purification process. The concentration of SidJ(Se-Met)–CaM used for crystallization was also around 7 mg ml⁻¹. Diffraction quality crystals of SidJ(Se-Met)–CaM were grown and optimized under the same conditions. All crystals were flash-frozen in liquid nitrogen, with the addition of 20–25% (v/v) glycerol as a cryoprotectant.

**Data collection and structure determination.** X-ray diffraction for SidJ(AN99/Se-Met)–CaM, native SidJ(AN99)–CaM and SidJ(AN99)–CaM–ATP were collected at beamline BL-17U1 of the Shanghai Synchrotron Radiation Facility. All data were indexed and scaled using HKL2000 software. The initial phase of SidJ(AN99)–CaM was determined by potential, molecular replacement by the universal myoglobin detection kit from ATCC (30–1012K).

For infection experiments, *L. pneumophila* strains were used to transfect HEK293T cells to grow about 70% confluence. Different plasmids were transfected into HEK293T cells. Transfected cells were collected and lysed with the radioimmunoprecipitation assay buffer (RIPA buffer, Thermo Fisher Scientific) 16–18 h after transfection. Cells infected with indicated bacterial strains were similarly processed for immunoprecipitation. When needed, immunoprecipitation was performed with lyses of transfected cells using agarose beads coated with HA-specific antibody (Sigma-Aldrich, A2095), Flag-specific antibody (Sigma-Aldrich, F1804), or CaM (Sigma-Aldrich, A6112) at 4 °C for 4 h. Beads were washed with pre-cold RIPA buffer or respective reaction buffers three times. Samples were resolved by SDS–PAGE and followed by immunoblotting analysis with the specific antibodies, or silver staining following the manufacturer’s protocols (Sigma-Aldrich, PROTISIL).

For infection, *L. pneumophila* strains were grown to the post-exponential phase (optical density at 600 nm, OD₅₀₀, of 3.3–3.8) in AYE broth. When needed, 3 ml of reservoir solution. Diffraction-quality crystals of SidJ(Se-Met)–CaM used for crystallization was also around 7 mg ml⁻¹. Diffraction quality crystals of SidJ(Se-Met)–CaM were grown and optimized under the same conditions. All crystals were flash-frozen in liquid nitrogen, with the addition of 20–25% (v/v) glycerol as a cryoprotectant.

**Analytic ultracentrifugation.** Sedimentation velocity experiments were used to assess the molecular size of the SidJ(AN99)–CaM complex at 20°C on a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and an An60 Ti rotor (Beckman Coulter). Samples were diluted to an optical density at 280 nm (OD₂₈₀) of 1 in a 1.2-cm path length. The rotor speed was set to 72,500g for all samples. The sedimentation coefficient was obtained using the (s) method with the Sedfit software.

**In vitro ubiquitination assays.** For the SdeA-mediated ubiquitination reaction, 0.1 μg His₅-SdeA and 1 μg GST–SidJ were preincubated in a 25-μl reaction system containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT and 1 mM β-NAD⁺ for 2 h at 37°C. When needed, 5 mM MgCl₂, 1 mM t-glutamate, 1 mM ATP and 1 μM CaM (Sigma-Aldrich, C4874) were supplemented. After a 2-h preincubation, a cocktail containing 1 mM β-NAD⁺, 0.25 μg 4 × Flag–Rab33B and 5 μg ubiquitin was supplemented into the reactions and the reaction was allowed to proceed for another 2 h at 37°C.

**In vivo glutamylase assays.** His₅-SdeA (0.1 μg) and GST–SidJ (1 μg) were incubated in a 25-μl reaction system containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM t-glutamate, 1 mM ATP and 1 μM CaM for 2 h at 37°C. To measure the glutamylase activity of SidJ using 14C-glutamate, 2 μg His₅-SdeA and 0.5 μg GST–SidJ were incubated in a 25-μl reaction system containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM t-glutamate, 1 mM ATP and 1 μM CaM (Perkin Elmer NEC290E050U), 1 mM ATP and 1 μM CaM for 2 h at 37°C. Products were resolved by SDS–PAGE and stained with Coomassie Brilliant Blue. Gels were then dried and signals were detected with X-ray films with a BioMax TranScreen LE (Kodak) for 3 days at −80°C.

The Sedfit software. Proteins were then eluted from the beads by using 3 × Flag peptides following the manufacturer’s protocol (Sigma-Aldrich, F47999).

**Crystallization.** The purity of SidJ(AN99)–CaM and its complex with ATP (SidJ(AN99)–CaM–ATP) were grown in the presence of 0.1 M HEPES (pH 6.5–7.3), 20% (v/v) PEG 4000, and 0.2 M NaCl. To solve the phase problem, Se-Met was incorporated into SidJ and the SidJ(Se-Met) was purified similarly to native SidJ except with the addition of 5 mM DTG to the buffer during the purification process. The concentration of SidJ(Se-Met)–CaM used for crystallization was also around 7 mg ml⁻¹. Diffraction quality crystals of SidJ(Se-Met)–CaM were grown and optimized under the same conditions. All crystals were flash-frozen in liquid nitrogen, with the addition of 20–25% (v/v) glycerol as a cryoprotectant.

**Data collection and structure determination.** X-ray diffraction for SidJ(AN99/Se-Met)–CaM, native SidJ(AN99)–CaM and SidJ(AN99)–CaM–ATP were collected at beamline BL-17U1 of the Shanghai Synchrotron Radiation Facility. All data were indexed and scaled using HKL2000 software. The initial phase of SidJ(AN99)–CaM was determined by potential, molecular replacement by the universal myoglobin detection kit from ATCC (30–1012K).

For infection, *L. pneumophila* strains were grown to the post-exponential phase (optical density at 600 nm, OD₅₀₀, of 3.3–3.8) in AYE broth. When needed, 3 ml of reservoir solution. Diffraction-quality crystals of SidJ(Se-Met)–CaM used for crystallization was also around 7 mg ml⁻¹. Diffraction quality crystals of SidJ(Se-Met)–CaM were grown and optimized under the same conditions. All crystals were flash-frozen in liquid nitrogen, with the addition of 20–25% (v/v) glycerol as a cryoprotectant.

**Analytic ultracentrifugation.** Sedimentation velocity experiments were used to assess the molecular size of the SidJ(AN99)–CaM complex at 20°C on a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and an An60 Ti rotor (Beckman Coulter). Samples were diluted to an optical density at 280 nm (OD₂₈₀) of 1 in a 1.2-cm path length. The rotor speed was set to 72,500g for all samples. The sedimentation coefficient was obtained using the (s) method with the Sedfit software.

**In vitro ubiquitination assays.** For the SdeA-mediated ubiquitination reaction, 0.1 μg His₅-SdeA and 1 μg GST–SidJ were preincubated in a 25-μl reaction system containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT and 1 mM β-NAD⁺ for 2 h at 37°C. When needed, 5 mM MgCl₂, 1 mM t-glutamate, 1 mM ATP and 1 μM CaM (Sigma-Aldrich, C4874) were supplemented. After a 2-h preincubation, a cocktail containing 1 mM β-NAD⁺, 0.25 μg 4 × Flag–Rab33B and 5 μg ubiquitin was supplemented into the reactions and the reaction was allowed to proceed for another 2 h at 37°C.

**In vivo glutamylase assays.** His₅-SdeA (0.1 μg) and GST–SidJ (1 μg) were incubated in a 25-μl reaction system containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM t-glutamate, 1 mM ATP and 1 μM CaM (Perkin Elmer NEC290E050U), 1 mM ATP and 1 μM CaM for 2 h at 37°C. Products were resolved by SDS–PAGE and stained with Coomassie Brilliant Blue. Gels were then dried and signals were detected with X-ray films with a BioMax TranScreen LE (Kodak) for 3 days at −80°C.
resolved by SDS–PAGE and stained with Coomassie Brilliant Blue. Gels were then dried and signals were detected with X-ray films.

**HPLC analysis of glutamylation reactions.** SidJ(AN99) (40 μg) was incubated with 1 mM ATP in a 100-μl reaction system containing 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl for 4 h at 37 °C. When needed, 1 mM CaM, 2 mM l-glutamate and 80 μg SdeA or SdeA(E860A) were supplemented. Samples were injected into a Waters Acquity UPLC system equipped with a C18 reversed-phase column and a UV detector. Components were eluted isocratically with 100% H2O for 2 min followed by a 10-min gradient to 95% H2O and 5% acetonitrile. ATP and (1 mM) were run as standards.

**Antibodies and immunoblotting.** Purified His6-GFP was used to raise rabbit-specific antibodies using a standard protocol (Porcono Rabbit Farm and Laboratory). The antibodies were affinity-purified as previously described. Antibodies specific for SidJ and SdeA have been previously described; commercial antibodies used are as follows: anti-Flag (Sigma-Aldrich, F1804; 1:2,000); anti-HA (Roche, 11867423001; 1:5,000); anti-ICDH (1:10,000); anti-tubulin (DSHB, E7; 1:10,000); anti-HIF-1α (R&D Systems, MAB1536; 1:1,000); anti-PGK1 (Abcam, ab13687; 1:2,500); anti-CaM (Millipore, 05-173; 1:2,000). Membranes were then incubated with an appropriate IRDye infrared secondary antibody and scanned using an Odyssey infrared imaging system (Li-Cor's Biosciences).

**Constitution of the SidJ–CaM complex and size–exclusion chromatography.** Proteins purified as described above were further purified using a size-exclusion chromatography column (Superdex 200 increase 10/300; GE Healthcare) equilibrated with a washing buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl) on an AKTA pure system (GE Healthcare). To constitute the protein complex, purified SidJ and CaM were mixed at a 1:1.2 molar ratio at 4 °C for 1 h on a rotary shaker, and the complex was purified by size-exclusion chromatography using the above column. In each case, the proteins were eluted with the washing buffer. Fractions containing the protein of interest were pooled and used for further analysis.

**Liquid chromatography–tandem mass spectrometry analysis.** The Flag–mART domain was purified from HEK293T cells coexpressing GFP–SidJ or GFP. After separation by SDS–PAGE, gel slices containing the protein detected by silver staining were digested as described previously. The digested peptides were analysed on a C18 reversed-phase column connected to a UPLC (Acquity, Waters) coupled to an Orbitrap mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific), using the same conditions as described previously. Tandem mass spectra were converted to peak lists using DeconMSn and submitted for blind posttranslational modification search using MODa against the Swiss-Prot database. Tandem mass spectra were then incubated with an appropriate IRDye infrared secondary antibody and scanned using an Odyssey infrared imaging system (Li-Cor's Biosciences).

**Microscale thermophoresis.** The interaction between SidJ and CaM and the ATP-binding activity of SidJ were measured by microscale thermophoresis using the NanoTemper Monolight NT.115 instrument set at 20% LED and 20–40% IR-laser power. Laser on and off times were set at 30 s and 5 s, respectively. Each measurement consists of 16 reaction mixtures in which the concentration of fluorescent-labelled SidJ was set to be constant at 150 nM and the concentration of two-fold-diluted CaM ranged from 20 μM to 0.61 nM. For ATP binding, the concentrations of ATP used were from 100 μM to 3.05 μM. The NanoTemper Analysis 2.2.4 software was used to fit the data and to determine the Kd.

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**Author contributions.** N.G. and Z.-Q.L. conceived the ideas for this work. Unless specified, N.G. and Yao Liu performed the experiments. Yao Liu, Yancheng Liu, N.G. and J.Q. performed the yeast experiments; G.M.F. and E.S.N. performed mass spectrometric analyses. X.Z., X.X., C.H., B.Z., L.Z. and S.O. determined the structures and analysed protein properties using biophysical tools. K.P. and C.D. performed HPLC analysis of nucleotide products. N.G., Yao Liu, E.S.N., S.O. and Z.-Q.L. interpreted the results. N.G., Yao Liu, S.O. and Z.-Q.L. wrote the manuscript and all authors provided editorial input.

**Competing interests.** The authors declare no competing interests.

**Additional information.**

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Extended Data Fig. 1 | Determination of the modification rate of E860 of SdeA. a, Peak areas of the extracted-ion chromatograms (XIC) were normalized on the basis of the area of the unmodified peptide –I608IQQILANPDCIHDDHVLINGQK630–. The occupancy rate of glutamylation on the residue was calculated on the basis of the consumption of the unmodified –H855GEGTESEFSVYLPEALVPVK877– in samples from cells cotransfected to express GFP–SidJ compared to those of controls from cells transfected to express GFP. b, SidJ induces a 258.09-Da post-translation modification on E860 within the mART motif of SdeA. 4× Flag–mART purified from HEK293T cells coexpressing SidJ detected by silver staining (Fig. 2d) was analysed by mass spectrometric analysis. The tandem mass spectrum shows the fragmentation profile of the modified peptide –H855GEGTEGluGluSEFSVYLPEALVPVK877–, including ions b5 and b6, which confirms the modification site at the E860 residue. In each case, similar results were obtained in three independent experiments.
Extended Data Fig. 2 | The effects of cell lysates, ATP and heat treatment of CaM on the activity of SidJ and its inhibition of the activity of all members of the SidE family. a, Inhibition of SdeA activity does not occur in in vitro reactions containing l-glutamate or each of its two structural isomers, N-acetylserine or N-methylaspartate was incubated with SdeA, SidJ and ATP for 2 h before assaying for the activity of SdeA. b, One or more factors from mammalian cells are required for SidJ to inhibit SdeA. Lysates from E. coli or HEK293T cells were added to reactions containing SdeA and SidJ for 2 h before measuring the activity of SdeA. c, Heat treatment does not completely abolish CaM activity. CaM or CaM treated by heating at 100 °C for 5 min was included in reactions that allow glutamylation of SdeA for 2 h. A cocktail containing 4× Flag–Rab33b, NAD⁺ and ubiquitin was added to each reaction. d, The activity of SidJ requires ATP. His₅-SdeA was incubated with GST–SidJ, l-glutamate and CaM in reactions with or without 1 mM ATP for 2 h; 4× Flag–Rab33b, NAD⁺ and ubiquitin were added to each reaction. After another 2-h incubation, the activity of SdeA was evaluated by the production of ubiquitinated Rab33b. Protein components in the reactions were detected by immunoblotting with specific antibodies. The binding of ATP by SidJ. Binding of ATP by purified SidJ was evaluated using microscale thermophoresis in which the concentration of SidJ was kept constant. K_D was determined by the NanoTemper Analysis 2.2.4 software. f, SidJ inhibits the activity of members of the SidE family. A recombinant protein of each member of the SidE family was incubated with ATP, l-glutamate and GST–SidJ in the presence or absence of CaM for 2 h, and a cocktail containing 4× Flag–Rab33b, NAD⁺ and ubiquitin was added to the reactions. After an additional 2-h incubation, modification of Rab33b was detected by immunoblotting with a Flag-specific antibody. The formation of Ub–4× Flag–Rab33b is indicated by a shift in molecular mass. In each panel, data shown are one representative from at least three independent experiments with similar results.
Extended Data Fig. 3 | The IQ motif of SidJ is required for its optimal response to CaM. a, b. The IQ motif is required for the optimal activity of SidJ in response to CaM. Serially diluted CaM was preincubated with SidJ (a) or the SidJ(I841D/Q842A) mutant (b) and SdeA in the glutamylation buffer at 37 °C for 2 h. A cocktail containing 4×Flag–Rab33b, NAD⁺ and ubiquitin was added to the reactions. After incubation for another 2 h at 37 °C, proteins separated by SDS–PAGE were assessed using the indicated antibodies. In each panel, data shown are one representative from at least three independent experiments with similar results. c. The SidJ(I841D/Q842A) mutant complements the intracellular growth defect of the ΔsidJ mutant. A. castellanii was infected with the indicated bacterial strains and intracellular bacteria were determined at the indicated time points. Experiments on each strain were performed in triplicate and similar results were obtained in two independent experiments. Results are from one representative experiment performed in triplicate from three independent experiments; error bars represent s.e.m. (n = 3).
Extended Data Fig. 4 | SidJ forms a stable heterodimer with CaM at a molar ratio of 1:1. a, SidJ(ΔN99) maintains the ability to inhibit SdeA activity, to a similar extent to that of full-length SidJ. SdeA was incubated with GST–SidJ or SidJ(ΔN99) at indicated molar ratios in reactions containing ATP, l-glutamate, CaM for 2 h at 37 °C. A cocktail containing 4× Flag–Rab33b, NAD⁺ and ubiquitin was added to each reaction for an additional 2 h at 37 °C, and the proteins resolved by SDS–PAGE were analysed with the indicated antibodies. SdeA activity was measured by the production of ubiquitinated Rab33b as indicated by a shift in molecular mass. b, Size-exclusion chromatography profiles of SidJ–CaM. Left, purified proteins were separated by a Superdex 200 Increase 10/300 column (GE Healthcare) on an AKTA pure system. Right, fractions with strong absorbance at an optical density of 260 nm (OD₂₆₀) were collected and analysed by SDS–PAGE followed by detection with Coomassie brilliant blue staining. c, The heterodimer formed between SidJ(ΔN99) with CaM is a monomer. Analytical ultracentrifugation analysis yielded a sedimentation coefficient of 5.770 S, and a molecular mass of approximately 96.12 kDa, which is indicative of the heterodimer of SidJ(ΔN99) and CaM. In each panel, data shown are one representative from at least three independent experiments with similar results.
Extended Data Fig. 5 | Overall structure of the SidJ–CaM complex in one asymmetric unit and the comparison of complex structures with or without AMP. 

**a**, Two views of the structure of the SidJ–CaM heterodimer in the asymmetric unit displayed as a ribbon diagram (top) and with surface rendering (bottom); one of the SidJ–CaM heterodimers is coloured as shown in Fig. 4 and the other one is coloured in grey. 

**b**, Superimposition of the structures of SidJ–CaM and SidJ–CaM–AMP. The SidJ–CaM–AMP ternary complex is coloured as shown in Fig. 4d and the SidJ–CaM binary complex is coloured in grey.
Extended Data Fig. 6 | Interactions between CaM and Ca\(^{2+}\) from the crystal structures and the role of Ca\(^{2+}\) in the activation of SidJ by CaM.

**a.** Key residues of CaM involved in the interaction with Ca\(^{2+}\). Ca\(^{2+}\) is coordinated by D21, D23, D25 and T27 of CaM, which are shown as red sticks. Ca\(^{2+}\) is shown as a pink sphere. Electron density of a simulated annealing \(F_o - F\) omit map for Ca\(^{2+}\) contoured at 3.0\(\sigma\). **b.** Dialysis against 20 mM EGTA does not abolish the activity of SidJ. All proteins used in the reactions were dialysed against a buffer containing 20 mM EGTA for 14 h. SdeA was incubated with SidJ in reactions containing ATP and EGTA-dialysed CaM for 2 h at 37 °C. Reactions without SidJ were established as a control. A cocktail containing 4×Flag–Rab33b, NAD\(^+\) and ubiquitin was added to each reaction. After further incubation for 2 h at 37 °C, proteins resolved by SDS–PAGE were analysed with the indicated antibodies. SdeA activity was measured by the production of ubiquitinated Rab33b as indicated by a shift in molecular mass. **c.** The activity of SidJ is not sensitive to 10 mM EGTA. SdeA was first incubated with SidJ for glutamylation with the indicated amounts of EGTA for 2 h at 37 °C. NAD\(^+\), 4×Flag–Rab33b and ubiquitin were then supplemented to the reactions, which were allowed to proceed for 2 h at 37 °C before resolution by SDS–PAGE. Rab33b modification was detected as described in **b.** Proteins in the reactions were detected by immunoblotting with specific antibodies. In **b, c,** similar results were obtained in at least three independent experiments.
Extended Data Fig. 7 | The mechanism of SidJ-induced CaM-dependent self-AMPylation and SdeA glutamylation. a, SidJ induces self-AMPylation in a CaM-dependent manner. SidJ was incubated with $^{32}$P-$\alpha$-ATP and Mg$^{2+}$, with or without CaM for 2 h at 37 °C. After separation by SDS–PAGE, the incorporation of $^{32}$P-$\alpha$-ATP was detected by autoradiography. b, SdeA glutamylation by SidJ interferes with SidJ self-AMPylation. SidJ was incubated with $^{32}$P-$\alpha$-ATP, Mg$^{2+}$ and CaM for 2 h at 37 °C. l-glutamate, SdeA and SdeA(E860D) were supplemented as stated. After separation by SDS–PAGE, the incorporation of $^{32}$P-$\alpha$-ATP was detected by autoradiography. c, SdeA glutamylation by SidJ accelerates ATP hydrolysis and the release of AMP. SidJ was incubated with the indicated components for 2 h at 37 °C. Samples were analysed by HPLC. AMP and ATP were used as standards. In a–c, data shown are one representative from at least three independent experiments with similar results. d, Schematic model of SidJ-induced glutamylation and AMPylation. SidJ induces glutamylation on SdeA(E860D) when ATP and l-glutamate are supplemented into the reaction. In reactions in which l-glutamate or modifiable SdeA are not present, SidJ undergoes self-AMPylation.
Extended Data Fig. 8 | Intracellular growth phenotypes associated with the ΔsidJ mutant expressing SdeA and its mutants. a, Intracellular defects of the *L. pneumophila* ΔsidJ mutant can be complemented by SidJ expressed from a multicopy plasmid. The indicated strains were used to infect *A. castellanii* at an MOI of 0.05 and the growth of the bacteria was evaluated at 24-h intervals. Fold growth was calculated on the basis of total bacterial counts at the indicated time points and those of the 2-h time point. b, Overexpression of a SdeA mutant defective in substrate recognition inhibits intracellular growth of the ΔsidJ mutant. Intracellular growth of the indicated *L. pneumophila* strains in *A. castellanii* was evaluated as described in a. In each panel, the expression of SidJ, SdeA and its mutants in bacterial cells and their translocation into infected cells was determined by immunoblotting from total bacterial cell lysates and the saponin-soluble fraction of infected cells, with isocitrate dehydrogenase and tubulin as loading controls, respectively (right). In each case, results are from one representative experiment performed in triplicate from three independent experiments; error bars represent s.e.m. (*n* = 3).
Extended Data Fig. 9 |  SidJ functions to regulate the activity of SdeA during L. pneumophila infection. a, SdeA(E860D) is resistant to glutamylation catalysed by SidJ. SdeA, SdeA(E860A) or SdeA(E860D) was added to reactions containing GST–SidJ, 14C-glutamate ATP and CaM and the reactions were allowed to proceed for 2 h at 37 °C. After separation by SDS–PAGE, the incorporation of 14C-glutamate was detected by autoradiography. b, Yeast toxicity induced by SdeA(E860D) cannot be suppressed by SidJ. A plasmid that directs the expression of SidJ was introduced into yeast strains expressing SdeA or SdeA(E860D) from a galactose inducible promoter, serially diluted yeast cells were spotted onto glucose or galactose medium for 2 days and the growth of the cells was evaluated by imaging (top). The expression of SidJ, SdeA and SdeA(E860D) was determined by immunoblotting with specific antibodies. The PGK1 (3-phosphoglyceric phosphokinase-1) was analysed as a loading control (bottom). c, SdeA(E860D) still ubiquitinates Rab33b. Reactions containing the indicated components were allowed to proceed for 2 h at 37 °C, samples were then resolved by SDS–PAGE and ubiquitination of Rab33b was assessed by immunoblotting with a Flag-specific antibody to detect the production of modified Rab33b with a higher molecular mass. d, SdeA(E860D)-mediated protein ubiquitination in mammalian cells is insensitive to SidJ. HEK293T cells were transfected to express the indicated proteins for 16–18 h. Cleared cell lysates were subjected to SDS–PAGE and immunoblotting with an HA-specific antibody to detect proteins ubiquitinated by 3 × HA–Ub–AA. The amounts of SdeA, SdeA(E860D) and SidJ were assessed by antibodies specific for these proteins. Note that coexpression of SidJ reduced the ubiquitination induced by SdeA but not by SdeA(E860D). In a–d, data shown are one representative from at least three independent experiments with similar results. e, The effects of SidJ on intracellular growth defect caused by overexpression of SdeA or SdeA(E860D). The indicated L. pneumophila strains were used to infect A. castellanii at an MOI of 0.05 and the growth of the bacteria was evaluated at 24-h intervals. Fold growth was calculated on the basis of total bacterial counts at the indicated time points. Note the difference between strain ∆sidJ (pSdeA) and ∆sidJ (pSdeA, pSidJ). The growth defect caused by overexpressing the SdeA(E860D) mutant cannot be rescued by SidJ. The amounts of relevant proteins in bacterial cells and in infected cells were analysed by immunoblotting from total bacterial cell lysates and the saponin-soluble fraction of infected cells, with isocitrate dehydrogenase and tubulin as loading controls, respectively (right). Results shown are from one representative experiment performed in triplicate from three independent experiments; error bars represent s.e.m. (n = 3).
### Extended Data Table 1 | Data collection and refinement statistics

|                       | SidJ$_{Se}$-Met-CaM (PDB 6K4L) | SidJ-CaM (PDB 6K4K) | SidJ-CaM-AMP (PDB 6K4R) |
|-----------------------|-------------------------------|---------------------|-------------------------|
| **Data Collection**   |                               |                     |                         |
| Space group           | $P 1 2_1 1$                   | $P 1 2_1 1$         | $P 1 2_1 1$             |
| Cell dimensions       |                               |                     |                         |
| $a, b, c$ (Å)         | 61.06, 159.25, 135.81         | 60.96, 159.53, 135.61 | 60.85, 159.18, 135.03 |
| $\alpha, \beta, \gamma$ (°) | 90.00, 101.68, 90.00          | 90.00, 101.89, 90.00 | 90.00, 101.78, 90.00   |
| Wavelength (Å)        | 0.9792                        | 0.9792              | 0.9792                  |
| Resolution (Å)        | 66.50-2.95 (3.01-2.95) *      | 55.46-2.71 (2.81-2.71) | 66.09-3.11 (3.22-3.11) |
| $R_{merge}$           | 0.158 (0.959)                 | 0.176 (1.401)       | 0.230 (1.131)           |
| $I / \sigma I$        | 12.8 (2.5)                    | 12.1 (2.2)          | 12.5 (2.3)              |
| Completeness (%)      | 99.90 (100.00)                | 96.87 (97.82)       | 92.45 (99.80)           |
| Redundancy            | 6.8 (7.1)                     | 12.9 (12.2)         | 6.5 (5.4)               |
| **Refinement**        |                               |                     |                         |
| Resolution (Å)        | 2.95                          | 2.71                | 3.11                    |
| No. reflections       | 53475                         | 66262               | 41852                   |
| $R_{work} / R_{free}$ | 0.252/0.278                   | 0.205/0.243         | 0.239/0.279             |
| No. atoms             |                               |                     |                         |
| Protein               | 12936                         | 12738               | 12640                   |
| Ligand/ion            | 2                             | 2                   | 100                     |
| Water                 | 16                            | 2                   | 0                       |
| B factors (Å$^2$)     |                               |                     |                         |
| Protein               | 58.30                         | 69.39               | 65.00                   |
| Ligand/ion            | 64.10                         | 128.39              | 75.65                   |
| R.m.s. deviations     |                               |                     |                         |
| Bond lengths (Å)      | 0.005                         | 0.006               | 0.003                   |
| Bond angles (°)       | 0.89                          | 0.93                | 0.63                    |

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

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

### Software and code

Policy information about availability of computer code

#### Data collection

- Odyssey Loric imaging system, GE Healthcare AKTA, Orbitrap mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific), NanoTemper Monolith NT.115, Beamline BL-17U1 of the Shanghai Synchrotron Radiation Facility (SSRF), Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and an An60 Ti rotor, Waters Acquity UPLC equipped with a C18 reversed-phase column and a UV detector

#### Data analysis

- Microsoft Excel 2016, and Prism Graphpad 7.0, GE Healthcare Unicorn V7.3, HKL2000 software, AutoSol implemented in PHENIX V 1.5, PROCHECK V 3.5.4, PDBePISA v1.52, Coot V 0.8.9, Sedfit Software V16.1c, Odyssey Image Studio V 5.2, DeconMSn V2.2, MODa V1.03, MaxQuant v1.6.6.0, NanoTemper Analysis 2.2.4 software, Pymol V1.8.6.2

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the conclusions of this study are included in this published article along with its Supplementary Information files, and are also available from the corresponding author upon request. The atomic coordinates and structure factors of the SidJS-Met-CaM, Sidi-CaM and Sidi-CaM-AMP have been deposited in the Protein Data Bank (PDB) under the accession codes 6K4L, 6K4K and 6K4R, respectively.
### Life sciences study design

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

| Sample size       | Sample size is 3 |
|-------------------|------------------|
| Data exclusions   | No data were excluded |
| Replication       | For growth curve experiments, infections with each bacterial strain was performed in triplicate and similar results were obtained in two independent experiments. All other experiments were performed for at least 3 times. |
| Randomization     | Randomisation was not required as no human participants or animal models were reported in this manuscript. The experiments were performed on matched cell lines or specific biochemical reactions. |
| Blinding          | Blinding was not used as the present study is not a clinical research trial |

### Reporting for specific materials, systems and methods

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| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              | n/a     |
| Antibodies                       | Involved in the study |
| Eukaryotic cell lines            |         |
| Palaeontology                    |         |
| Animals and other organisms      |         |
| Human research participants      |         |
| Clinical data                    |         |
| ChIP-seq                         |         |
| Flow cytometry                   |         |
| MRI-based neuroimaging           |         |

### Antibodies

**Antibodies used**

Purified His6-GFP was used to raise rabbit specific antibodies using a standard protocol (Pocono Rabbit Farm & Laboratory). The antibodies were affinity purified as describe Antibodies specific for SidJ and SdeA had been described. Commercial antibodies used are listed as below: anti-Flag (Sigma, Cat# F1804), 1: 2000; anti-HA (Roche, cat# 11867423001 1:5,000), anti-ICDH3, 1:10,000, anti-tubulin (DSHB, E7) 1:10,000, anti-HIF-1α (R&D systems, cat#3A815361 1:1,000), anti-PGK1 (Abcam, cat# ab113687 1:2,500), anti-CaM (Millipore, cat#05-173 1:2,000). Membranes were then incubated with an appropriate IRDye infrared secondary antibody ( Invitrogen Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 680 CAT#A21057 1:10,000; Invitrogen Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680, cat#A21109, 1:10,000; LICOR IRDye® 800CW Goat anti-Rabbit IgG (H + L), CAT#926-32211 1:10,000; LICOR IRDye® 800CW Goat anti-Mouse IgG (H + L), CAT#926-32210 1:10,000) and scanned using an Odyssey infrared imaging system (Li-Cor’s Biosciences).

**Validation**

GFP and ICDH antibodies were described in: Xu, L. et al. Inhibition of host vacuolar H+-ATPase activity by a Legionella pneumophila effector. PLoS Pathog. 6, e1000822 (2010).

SdeA antibody was described in: Qiu, J. et al. Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. Nature 533, 120-124, doi:10.1038/nature17657 (2016).

SidJ antibody was described in:  Liu, Y. & Luo, Z. Q. The Legionella pneumophila effector SidJ is required for efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome. Infect Immun 75, 592-603 (2007).

Antibody, catalogue number, manufacturer information for commercial antibodies:

- anti-Flag (Sigma, Cat# F1804): [https://www.sigmaaldrich.com/catalog/product/sigma/f1804?lang=en&region=US](https://www.sigmaaldrich.com/catalog/product/sigma/f1804?lang=en&region=US)
- anti-HA (Roche, cat# 11867423001): [https://www.sigmaaldrich.com/catalog/product/roche/roahaha?lang=en&region=US](https://www.sigmaaldrich.com/catalog/product/roche/roahaha?lang=en&region=US)
- anti-tubulin (DSHB, E7): [http://dshb.biology.uiowa.edu/tubulin-beta_-2](http://dshb.biology.uiowa.edu/tubulin-beta_-2)
anti-HIF-1α (R&D systems, cat#MAB1536): https://www.rndsystems.com/products/human-mouse-rat-hif-1alpha-antibody-241809_mab1536
anti-PGK1 (Abcam, cat#ab113687): https://www.abcam.com/pgk1-antibody-22c5d8-ab113687.html
anti-CaM (Millipore, cat#05-173): http://www.emdmillipore.com/US/en/product/Anti-Calmodulin-Antibody,MM_NF-05-173
Invitrogen Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 680 CAT#A21057: https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21057
Invitrogen Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680, cat#A21109: https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21109
LICOR IRDye® 800CW Goat anti-Rabbit IgG (H + L), CAT#926-32211: https://www.licor.com/bio/reagents/irdye-800cw-goat-anti-rabbit-igg-secondary-antibody
LICOR IRDye® 800CW Goat anti-Mouse IgG (H + L), CAT#926-32210: https://www.licor.com/bio/reagents/irdye-800cw-goat-anti-mouse-igg-secondary-antibody

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) HEK293T cells, Acanthamoeba castellanii cells, Yesat W303 and BY4741 cells were purchased from ATCC
Authentication Authenticated by ATCC. ATCC uses morphology, karyotyping, and PCR based approaches to confirm the identity of human cell lines and to rule out both intra- and interspecies contamination. These include an assay to detect species specific variants of the cytochrome C oxidase I gene (COI analysis) to rule out inter-species contamination and short tandem repeat (STR) profiling to distinguish between individual human cell lines and rule out intra-species contamination.
Mycoplasma contamination Contamination tested by using the universal mycoplasma detection kit from ATCC (cat# 30-1012K). All cell lines tested are confirmed as negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register) No commonly misidentified cell lines were used