Table S1: Selected crystallographic data

Crystal	pHipA ($P3_121$)	pHipA ($P2_1$)	HipA(S150A)	pHipA-ADP	HipA(D309Q)-ADP
Space group	$P3_121$	$P2_1$	$P2_1$	$P6_5$	$P2_1$
Cell dimensions	a = b = 68.6 Å,	a = 48.5 Å	a = 48.4 Å	a = b = 133.7 Å	a = 68.6 Å
		b = 84.1 Å	b = 84.5 Å		b = 84.4 Å
	c = 170.5 Å	c = 50.1 Å	c = 50.0 Å	c = 48.5 Å	c = 69.6 Å
		β = 90.1°	β = 90.0°		β = 91.7°
Resolution (Å)	85.1-1.90	50.1-1.50	84.5-1.88	67.2-2.30	89.7-2.30
R_{sym}(%)[a]	5.0 (21.0)[b]	2.5 (29.0)	3.9 (20.4)	5.8 (34.8)	7.3 (42.4)
I/σ(I)	17.0 (3.4)	16.5 (2.4)	18.0 (3.7)	13.9 (3.4)	7.0 (1.9)
Total Reflections (#)	122359	100452	97397	81388	98866
Unique Reflections (#)	36801	52283	29361	14821	11060
Multiplicity	3.3	1.9	3.3	3.5	2.7
R_{work}/R_{free}(%)[c]	18.4/24.3	21.2/24.8	19.1/24.2	23.8/28.0	22.1/26.3
rmsd					
Bond angles (°)	1.55	1.55	1.53	1.10	1.23
Bond lengths (Å)	0.014	0.013	0.009	0.015	0.007
Ramachandran analysis					
Most favored (%/#)	91.0/335	94.2/324	93.6/324	86.6/316	90.1/657
Additionally allowed (%/#)	8.4/31	5.5/19	6.1/21	12.6/46	9.5/69
Gen allowed (%/#)	0.5/2	0.3/1	0.3/1	0.5/2	0.1/1
Disallowed (%/#)	0/0	0/0	0/0	0.3/1	0.3/2

[a] $R_{sym} = \sum\sum|I_{hkl(j)} - I_{hkl)}|/ \sum\sum I_{hkl}$, where $I_{hkl(j)}$ is the observed intensity and I_{hkl} is the final average intensity value.
[b] values in parentheses are for the highest resolution shell.
[c] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$ and $R_{free} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$; where all reflections belong to a test set of 5% randomly selected data.

Extended Experimental Procedures

Crystallization of pHipA, HipA(S150A), pHipA-ADP and HipA(D309Q)-ADP. The coding regions of wild type HipA and HipA(S150A) were inserted into the pET28a vector and induced and overexpressed in BL21(DE3) cells at 37 °C following standard protocols. The proteins were purified using Ni^{2+}-NTA affinity chromatography and concentrated to 50 mg/mL. Wild type phosphorylated HipA (pHipA) was crystallized at room temperature from solutions containing 25% PEG 6000, 20 mM MES pH 5.6. The crystals take the trigonal space group $P3_121$. A second, monoclinic crystal form of pHipA, space group $P2_1$, was grown from solutions containing 16% PEG 8000, 15% glycerol and 0.04 M potassium phosphate pH 7.5. Crystals of HipA(S150A) were grown with 14% PEG 8000, 20% glycerol and 0.04 M potassium phosphate pH 7.5. The pHipA-ADP crystals were grown by addition of 3 mM ADP (final concentration) and using 0.17 M ammonium sulphate, 25.5% PEG 5000 MME, 1.0 M MES pH 6.5 and 15% glycerol. These crystals are hexagonal, space group $P6_5$. HipA(D309Q)-ADP crystals were grown using 2 mM ADP and 40% PEG 600, 0.1 M CHES pH 9.5 as the crystallization reagent. Cryo-protection of the trigonal pHipA crystals was carried out using the crystallization reagent supplemented with 45% glycerol. Monoclinic pHipA, HipA(S150A), pHipA-ADP and HipA(D309Q)-ADP were cryo-cooled straight from the drops in a nitrogen gas stream. Intensity data were processed with MOSFLM.

Structure determinations and refinement. All structures were solved by molecular replacement using MolRep and the apo HipA(D309Q) structure as the search model (Schumacher et al., 2009). Model building was carried out using O and the structures

were refined using CNS (Jones et al., 1991; Brünger et al., 1998). The final trigonal pHipA structure, which contains one molecule per asymmetric unit (ASU), includes residues 2-105, 114-134, 142-437 of the 440 residue protein and 370 water molecules. The monoclinic pHipA structure contains one molecule per ASU and includes residues 2-108, 114-32, 154-437 and 410 water molecules. The HipA(S150A) structure contains one molecule of HipA per ASU and includes residues 2-132, 155-437, 2 phosphates and 244 water molecules. The pHipA-ADP structure contains one subunit in the ASU and includes residues 2-105, 113-133, 139-437, 1 sulphate ion, 156 solvent molecules and the adenine moiety of the ADP. There was no electron density for the ribose and phosphate groups. The HipA(D309Q)-ADP structure contains two HipA molecules per ASU and includes residues 2-105, 114-437 of one subunit and residues 3-435 of the second subunit, 1 ADP molecule per protein, 218 water molecules, 3 magnesium ions and 2 sulphate ions (Table 1). Figures 1A-C, Figure 2A-D and Figure 4A and 4C were made with PyMOL (Delano, 2002).

Dephosphorylation of pHipA by λ phosphatase. Purified wild type HipA is typically 65-90% phosphorylated on residue Ser150 (Correia et al., 2006 and our results). pHipA was dephosphorylated by overnight treatment of 1 mg of protein with 8000 units of λ phosphatase in 600 μL of a buffer of 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35 with $MnCl_2$. The dephosphorylated protein was repurified using Ni^{2+}-NTA affinity chromatography.

Interaction studies of HipA proteins with ATP, ADP or the ATP analogue AMPPNP. UV thermal denaturation experiments were conducted with a Beckman DU

800 or a PerkinElmer Lambda 25 UV/Vis spectophotometer. HipA proteins (0.5 to 2 μM) were incubated for 30 minutes at 22 °C with various concentrations of nucleotide in 25 mM Tris pH 7.5, 150 mM NaCl and 5 mM $MgCl_2$ followed by heating at a rate of 1 °C/min. The change in absorbance was monitored at 310 nm. Data points were taken at 30 sec intervals. The data were normalized using the absorbance values at 22 °C and 70 °C as 0% and 100% change. There was no confounding absorbance from ATP or ADP at 310 nm.

HipA kinase autophosphorylation and peptide phosphorylation assays. The kinase activity of λ phosphatase-treated pHipA was evaluated by its ability to autophosphorylate. Briefly, 1 μL of dephosphorylated HipA (~1 mg/mL) was added to 19 μL of a reaction solution, which contained 20 mM Hepes pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1.0 mM DTT, 50 μM ATP, [γ-^{32}P]ATP (10 μCi) and phosphatase inhibitors, β-glycerophosphate (20 mM), orthovanadate (0.1 mM), and okadaic acid (1 mM). Solutions were incubated at 30 °C over a 24 hr period. Autophosphorylated HipA was visualized by autoradiography after SDS-PAGE. This same kinase assay using non-radiolabeled ATP was carried out using dephosphorylated HipA in the presence of the HipA peptide, EENDFRISVAGAQEK (see below). As a control the same components were mixed without HipA and analyzed.

Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (LC/ESI-MS) of HipA peptide samples. Phosphorylation of the HipA 15mer peptide by dephosphorylated HipA or pHipA (control) was analyzed by reverse phase liquid chromatography (RP-LC) coupled with electrospray ionization mass spectrometry,

operated in the positive ion mode. The LC/MS instruments used were a Shimadzu Scientific Instruments (Columbia, MD) LC system (comprising a solvent degasser, two LC-10A pumps, and a SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Foster City, CA) equipped with an electrospray source. The LC was operated at a flow rate of 200 μL/min with a linear gradient as follows: 100% A was run isocratically for 2 min and then linearly increased to 60% B over 18 min and then increased to 100% B over 5 min. Mobile phase A consisted of water:acetonitrile (98:2 v/v) with 0.1% acetic acid. Mobile phase B consisted of acetonitrile:water (90:10 v/v) with 0.1% acetic acid. For analysis of the peptides, A Zorbax C8 column (SB-C8, 2.1mm ID × 50 mm (5μm), Agilent Technology) was used. The acquired spectra were then reprocessed using Analyst QS software (with the BioAnalyst extension). The doubly charged (protonated) species is observed at m/z 848.912, corresponding to the measured molecular weight (mono-isotopic) of 1691.81 Da. The calculated value (mono-isotopic) for peptide (EENDFRISVAGAQEK) is: 1691.81 Da. With the addition of HipA, a new peak at m/z 886.894 appeared, corresponding to the measured molecular weight of 1771.77 Da. The calculated value (mono-isotopic) for the phosphorylated peptide (EENDFRIpSVAGAQEK) is 1771.77 Da. The deconvoluted molecular weight (average) of the intact HipA protein from the multiply charged ESI ions is 50021.0 Da.

in vivo **phosphorylation of HipA(D309Q) by coexpressed wild type HipA.** A codon-optimized HipA-HipA(D309Q) co-expression system was purchased from Genscript Corporation, Piscatway, NJ, USA. DNA encoding HipA(D309Q) with an extra N-

terminal tail, MGSSHHHHHHSQHHHHHHH, was cloned into the BamHI/HindIII restriction sites of the pETDuet-1 plasmid. The N-terminal tail was added to allow the facile purification of HipA(D309Q), the kinase dead protein, from wild type HipA protein, which was not tagged. The DNA encoding the wild type HipA protein was cloned into the same vector using NdeI/KpnI sites. The construct was transformed into BL21(DE3) cells. Overnight cultures were inoculated into 18 litres of LB media with the addition of 100 mg/L of ampicillin and grown to an OD_{600} of 0.6. Protein expression was induced by the addition of 1 mM IPTG and the cells grown an additional 3.5 hrs. The cells were harvested and the HipA(D309Q) protein was purified from wtHipA using Ni^{2+}-NTA affinity chromatography. Two methods were used to ascertain if the HipA(D309Q) was phosphorylated. First purified HipA(D309Q) was stained with Pro-Q Diamond Phosphoprotein Gel Stain, which will stain phosphoserine, phosphothreonine and phosphotyrosine containing proteins, as described (Molecular Probes). Second, the samples were analyzed by mass spectrometry. In this procedure, the samples were digested with trypsin, dried and subjected to automated phosphopeptide enrichment using TiO_2. The enriched samples were then acidified with neat formic acid, dried and subjected to LC-MS analysis. Phosphorylated peptides were identified based on retention time and mass.

Additional References:

Delano, W.L. The PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, California, 2002).