Supplementary Information

A Pan-specific Antibody for Direct Detection of Protein Histidine Phosphorylation

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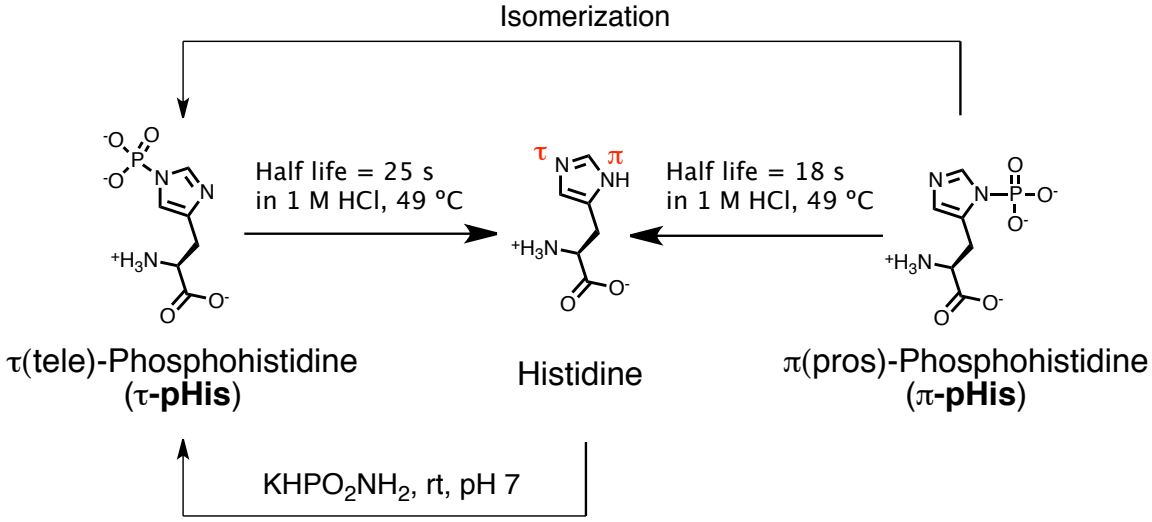
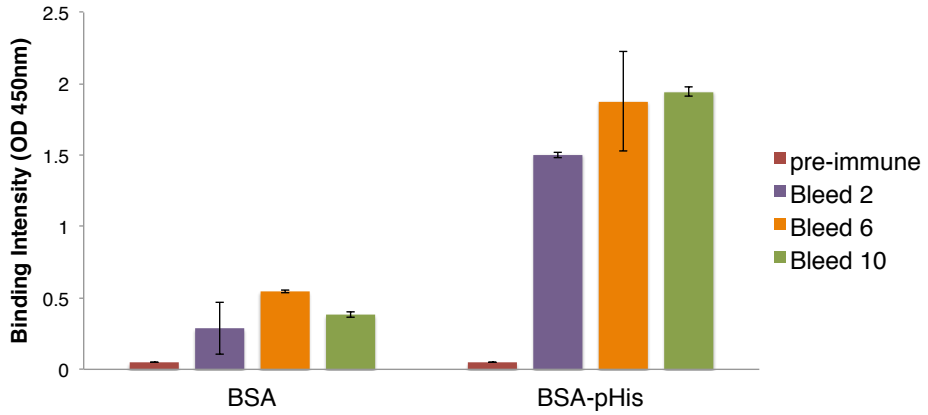
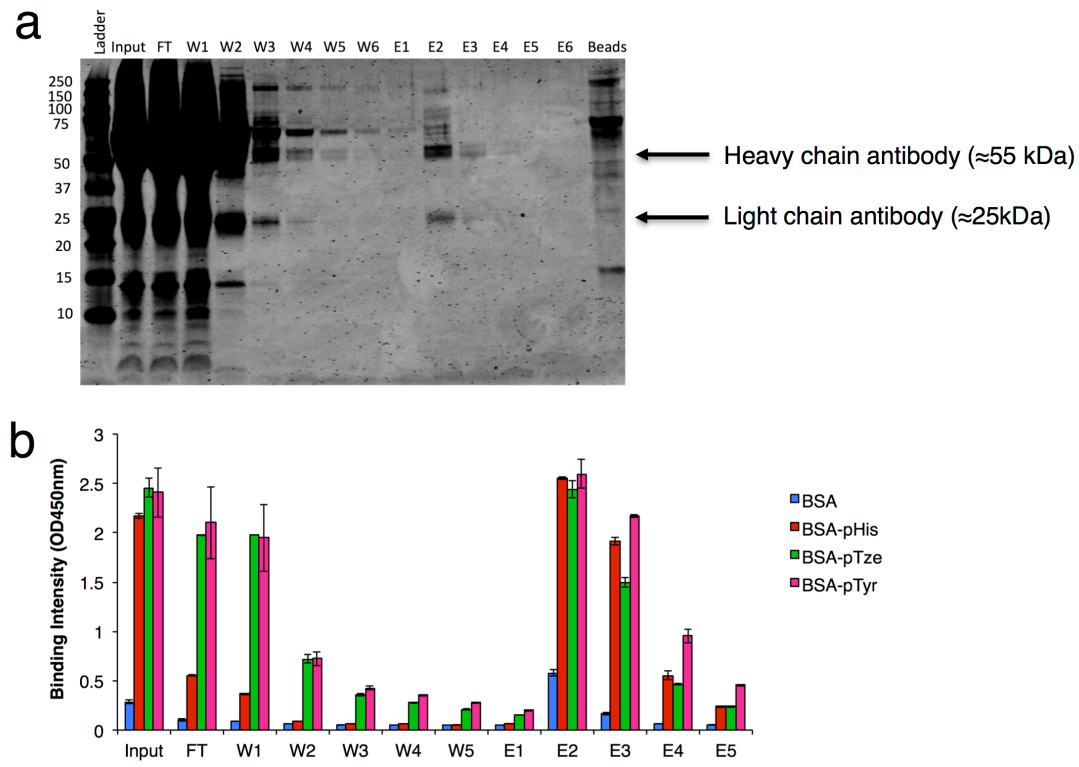


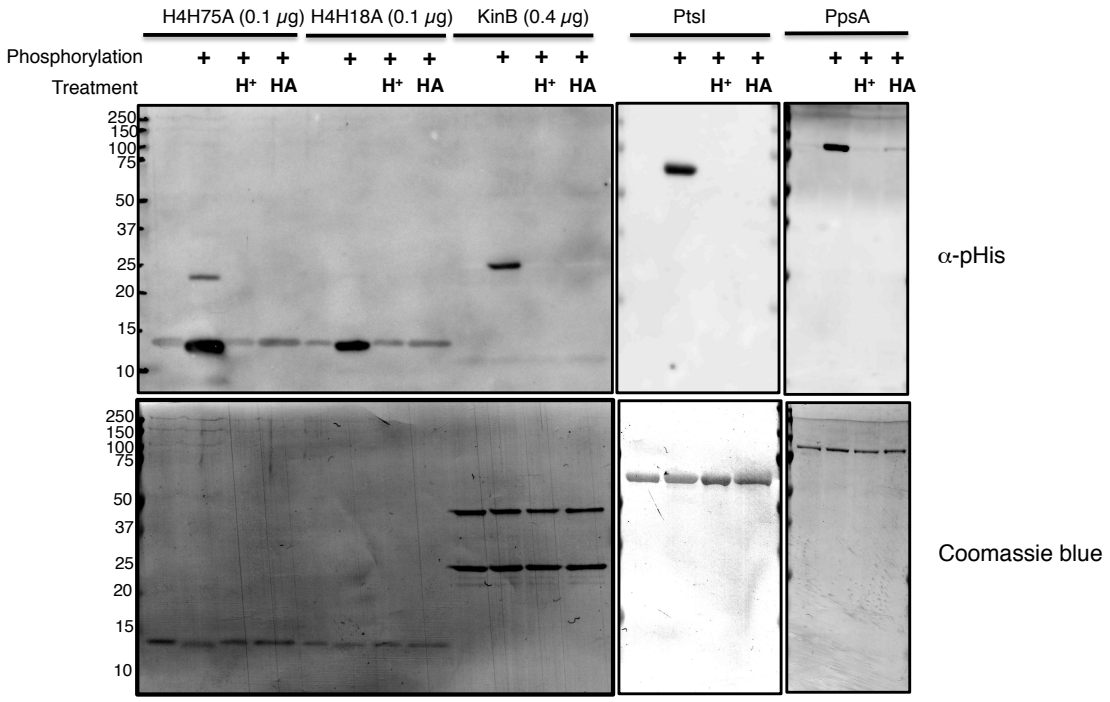
Supplementary Figure 1. Two isomers of phosphohistidine. Both pHis isomers undergo facile dephosphorylation under acidic conditions. The more thermodynamically stable τ-pHis can be selectively formed *via* phosphorylation with potassium phosphoramidate. This chemical phosphorylation specifically phosphorylates histidine residues over other amino acid residues in peptides and proteins.



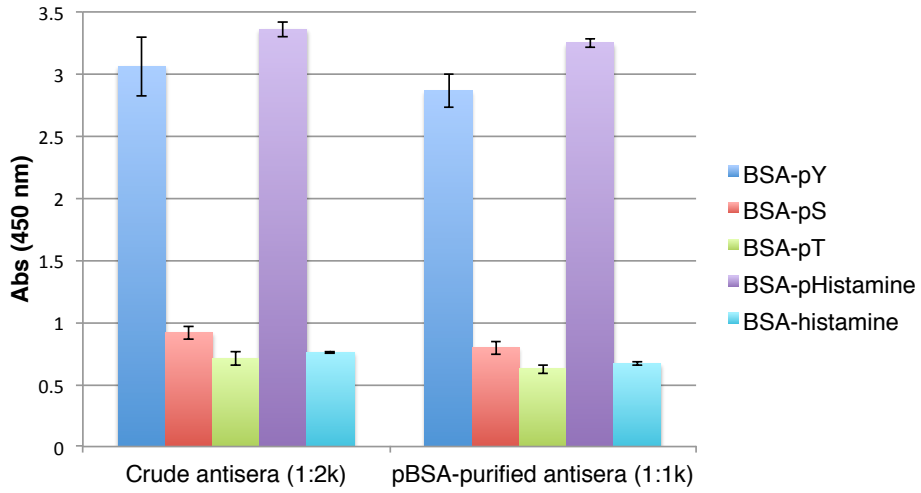
Supplementary Figure 2. Analysis of rabbit anti-serum for pHis binding affinity. Antiserum was obtained from a rabbit just prior to immunization with pTze conjugated to KLH (pre-immune) and then after a series of immunizations (bleed 2, 6, and 10). Anti-serum from bleeds 2, 6 and 10 show increased binding affinity for BSA-pHis over BSA. Pre-immune anti-serum displays no affinity for BSA or BSA-pHis (n = 2, mean ± s.d).



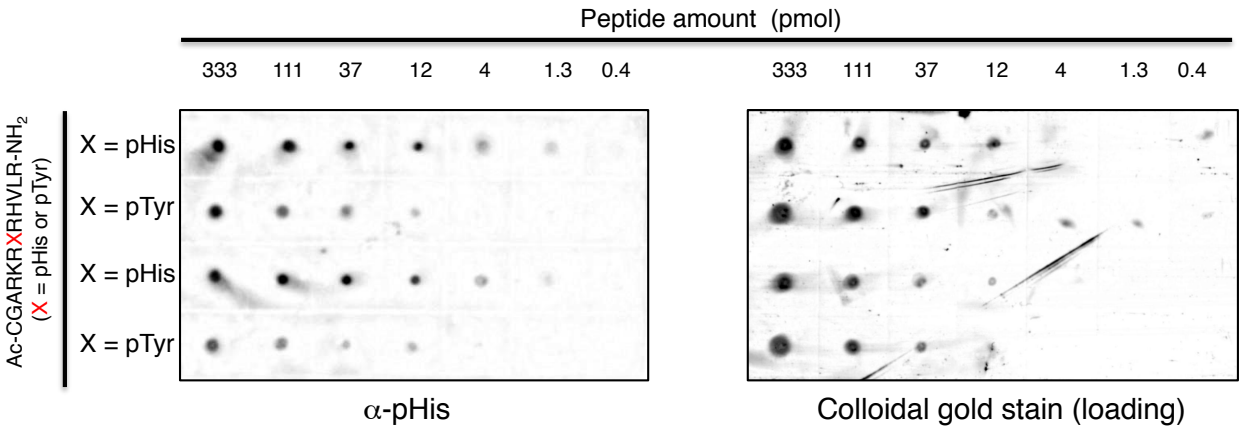
Supplementary Figure 3. SDS-PAGE and ELISA analysis of affinity-purified antibody fractions. Input, flow through (FT), wash fractions (W1, W2, W3, W4, W5, W6), and elution fractions (E1, E2, E3, E4, E5, E6) were analyzed by: a) SDS-PAGE for antibody purity and b) ELISA for pHis binding affinity (n = 2, mean ± s.d). Fractions E2 and E3 are shown by gel electrophoresis to contain antibody. ELISA analysis further shows that fractions E2 and E3 display the highest affinity for phosphohistidine (BSA-pHis). Fractions E2 and E3 also display binding affinity for pTze and pTyr. As noted by the ELISA, a pool of antibody that recognizes pTze and pTyr, but not pHis is removed in the FT and W1 fractions. Based on this analysis, E2 and E3 were pooled together and used as the affinity-purified antibody sample. Note that the BSA-pHis data from panel b are slightly different from Figure 1c because they are from two different sets of experiments.



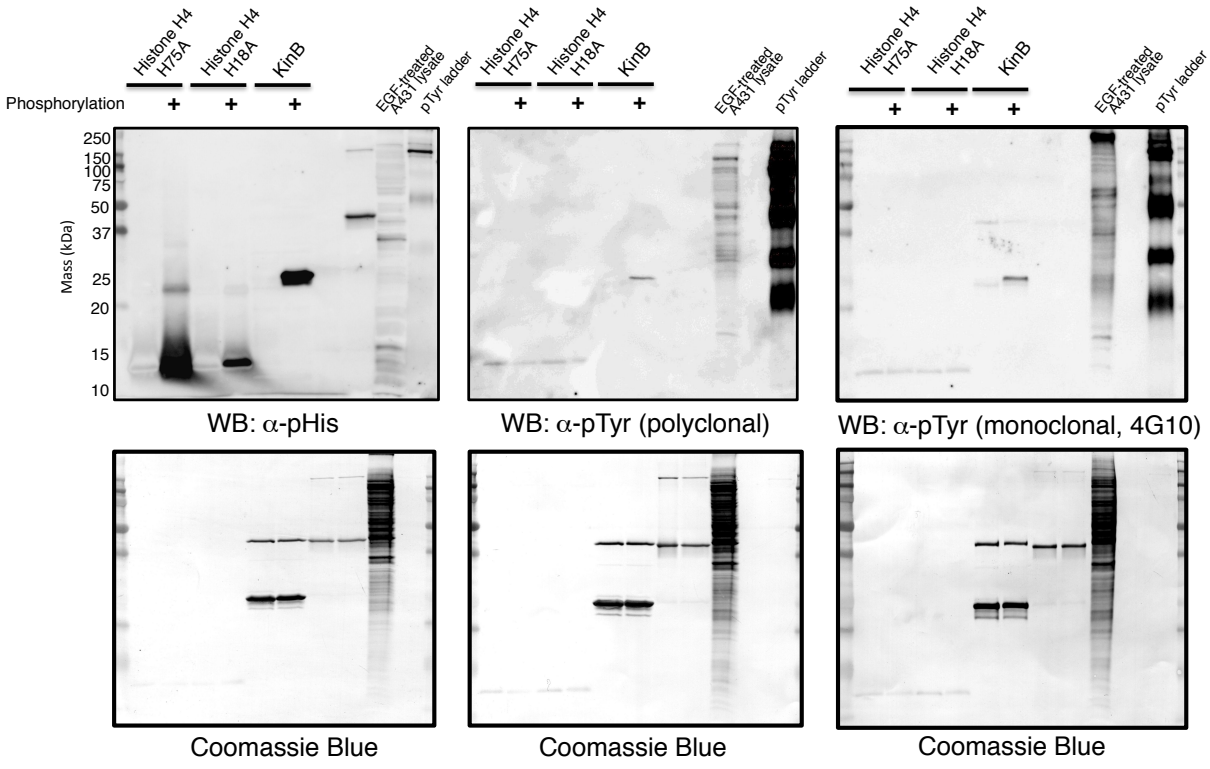
Supplementary Figure 4. Western blot of *in vitro* phosphorylated proteins. Chemically phosphorylated histones, an enzymatically autophosphorylated bacterial histidine kinase (KinB) and enzymatically autophosphorylated bacterial metabolic proteins (PtsI and PpsA) were all recognized by the anti-pHis antibody. Treatment of the phosphoproteins with acid or hydroxylamine abolishes the signal, consistent with the labile nature of pHis. Coomassie blue stain of the same membrane confirms the equal loading of samples.



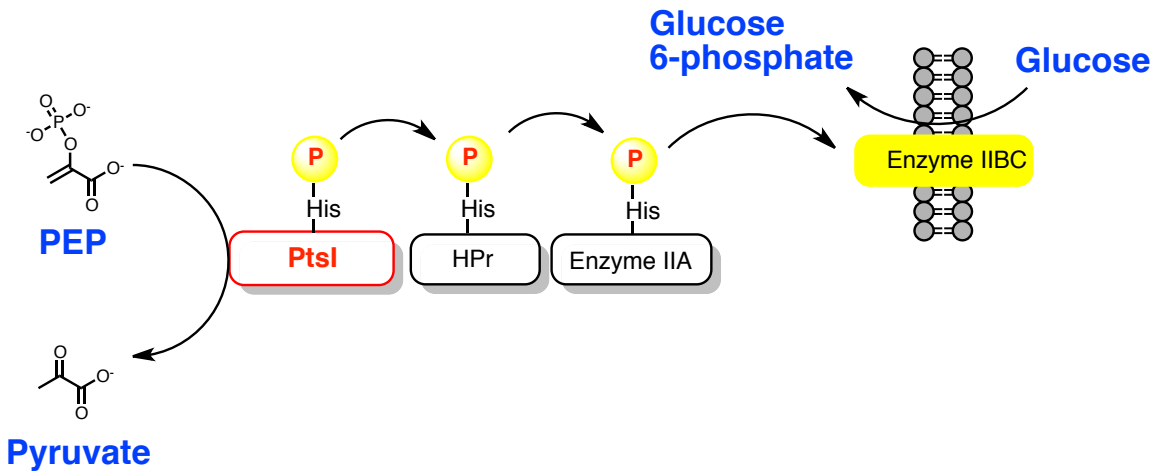
Supplementary Figure 5. Selectivity of α-pHis antibody against other phosphoamino acids. ELISA was carried out using BSA-phosphoamino acid conjugates prepared by conjugating the phosphoamino acid to BSA using a glutaraldehyde crosslinker. The pHis antibody displays higher binding affinity for BSA conjugated to phosphorylated histamine and pTyr over BSA conjugated to pSer and pThr (n = 2, mean ± s.d.).



Supplementary Figure 6. Selectivity of α-pHis antibody for pHis over pTyr. Representative dot blots of peptides derived from the histone H4 sequence. The peptide loading was monitored by staining the nitrocellulose membrane with colloidal gold stain.

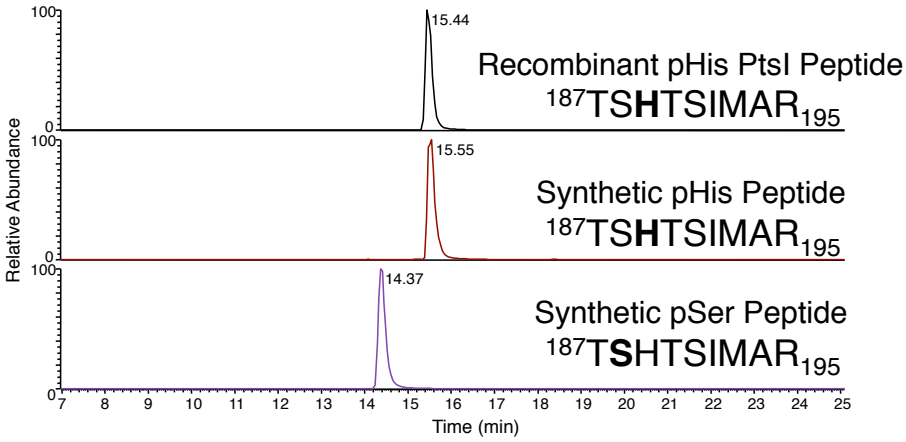


Supplementary Figure 7. Western blots using α-pHis and α-pTyr antibodies. Western blots of *in vitro*-phosphorylated histone H4 and KinB. Coomassie blue stain of each membrane is shown in the bottom row. EGF-stimulated A431 lysates and a pTyr protein ladder containing proteins modified with pTyr was also probed. Histidine-phosphorylated proteins are readily detected with α–pHis. In contrast, proteins probed with α-pTyr antibodies (both monoclonal and polyclonal) show minimal cross-reactivity to phosphorylated KinB. Histone H4 proteins show weak binding to the pTyr antibodies that is independent of phosphorylation. Conversely, the pTyr ladder shows strong signals when probed with α-pTyr antibodies, but not with the α-pHis antibody. In addition, the pattern of recognized bands in the EGF-stimulated lysates are different between α-pHis and α-pTyr antibodies.



Supplementary Figure 8. Schematic diagram of phosphoenolpyruvate:sugar transferase system (PTS). Phosphoenolpyruvate (PEP) is utilized as the phosphoryl group donor to phosphorylate enzyme I (PtsI) on a histidine residue. The phosphoryl group gets transferred, *via* a phosphorelay between downstream proteins, to incoming glucose to form glucose-6-phosphate.

a



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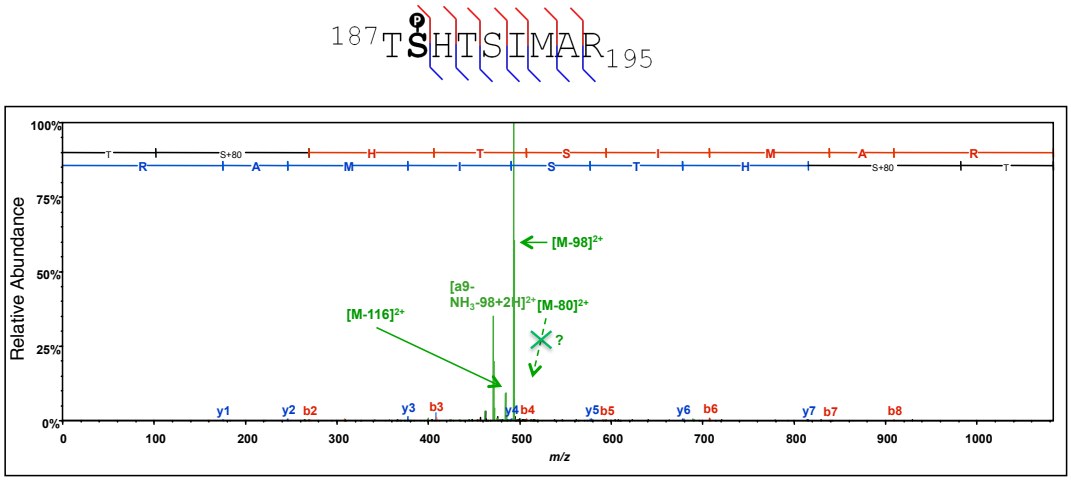
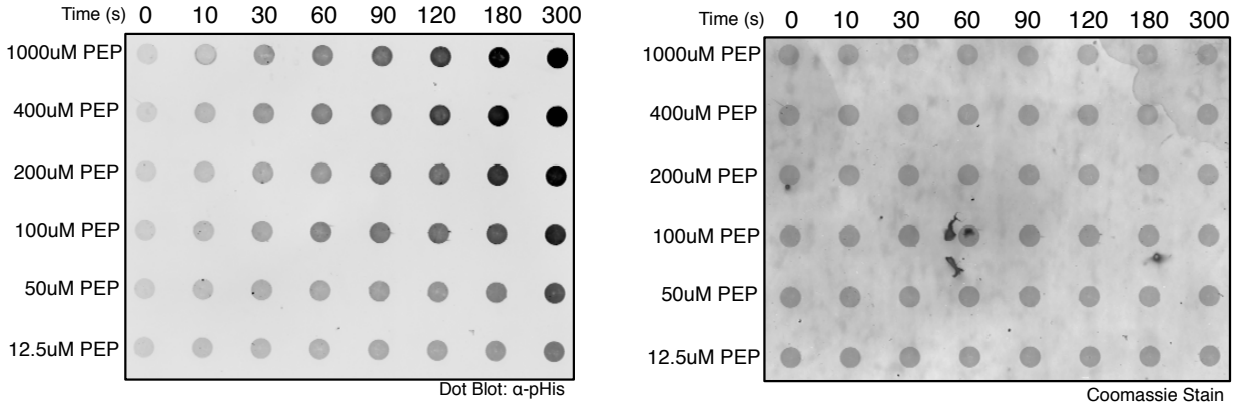
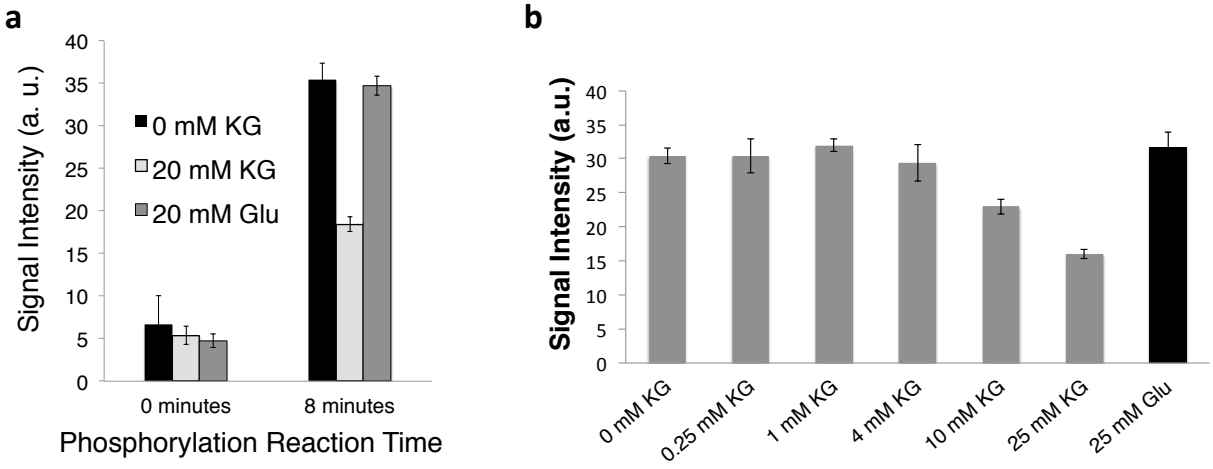


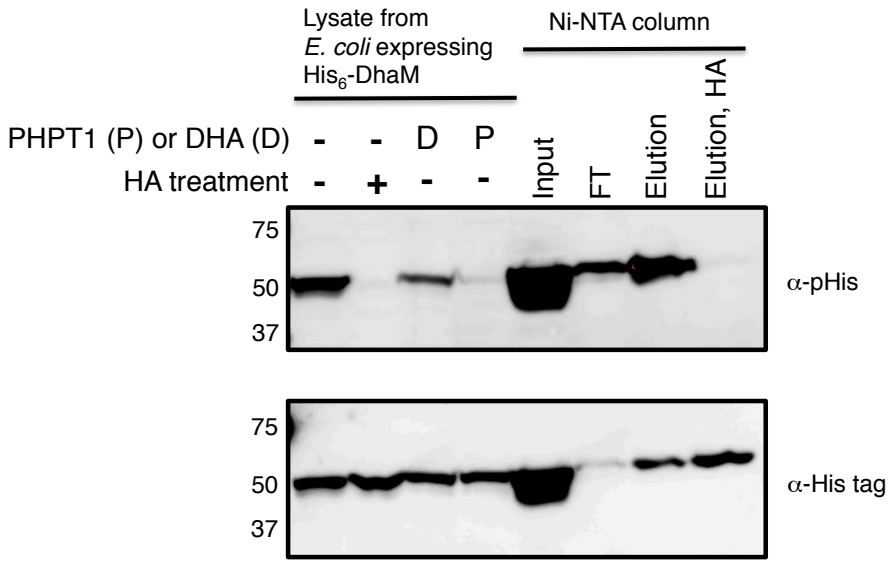
Supplementary Figure 9. Evidence for phosphorylation on histidine residue. Histidine phosphorylation of the PtsI phosphopeptide in Figure 4b was supported by an Ascore-derived localization probability of 84%. To further demonstrate the properties of this pHis peptide, its LC chromatographic profile was compared to that of the synthetic pHis peptide and a synthetic pSer version. Extracted-ion chromatograms (panel a) for the recombinant PtsI tryptic peptide (top), synthetic pHis PtsI peptide (middle), and synthetic pSer PtsI peptide (bottom) show that the recombinant PtsI peptide and pHis PtsI peptide have essentially identical retention times, but the pSer PtsI peptide elutes markedly earlier. (Note: phosphorylated residues are in bold). b) MS/MS spectrum of the pSer PtsI peptide. Note that the neutral loss of 80 amu is not observed.



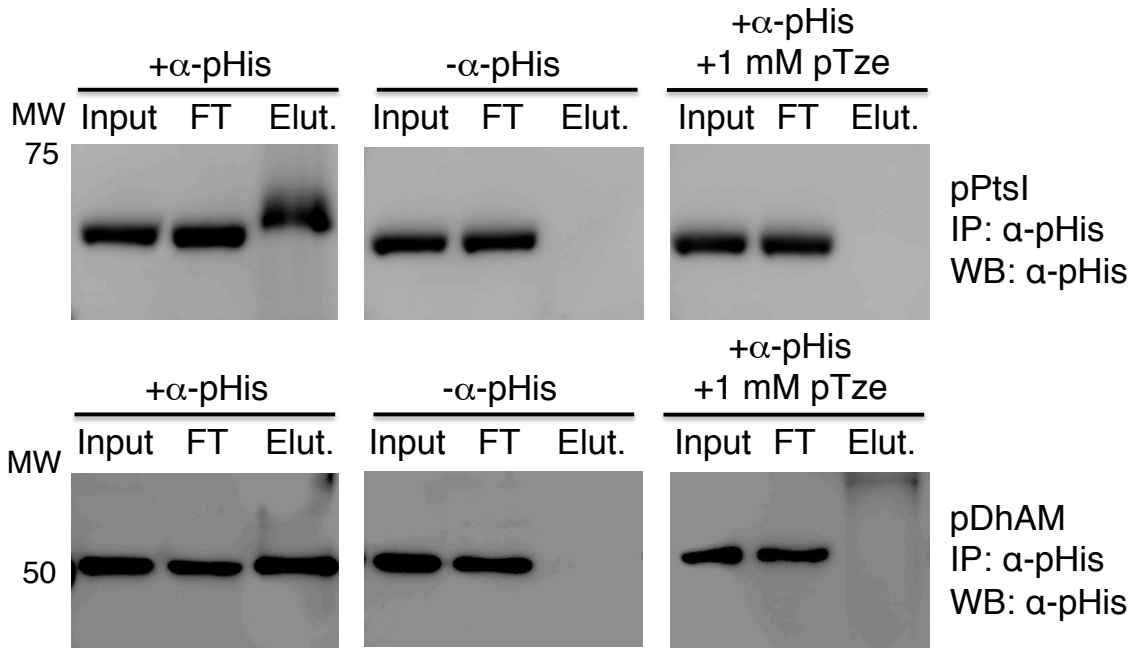
Supplementary Figure 10. Dot blot assay of PtsI autophosphorylation using PEP. Representative dot blot showing that the intensity of each time point increases as a function of time and PEP concentration indicating substrate dependent autophosphorylation (left panel). The Coomassie stained membrane is shown as a loading control (right panel).



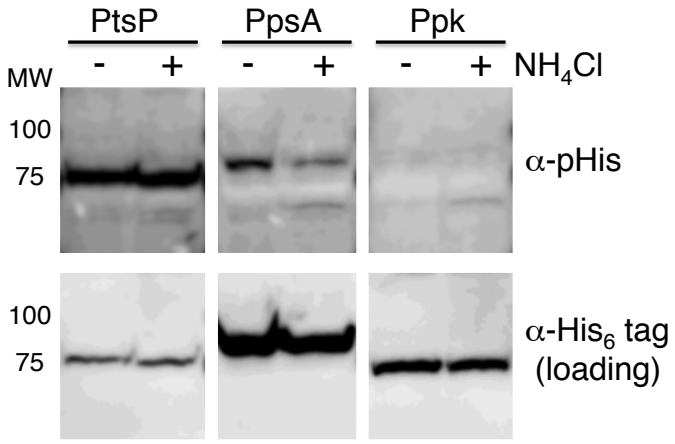
Supplementary Figure 11. α-KG inhibits PtsI autophosphorylation by PEP. a) Recombinant PtsI was treated with PEP in the presence or absence of α-KG (Sigma cat. no. 75890) and monitored by dot-blot. α-KG inhibits autophosphorylation of PtsI but glutamate (Glu) does not (n = 4, mean ± s.d.). b) PEP-dependent autophosphorylation of PtsI shows dose dependence on α-KG concentration (gray bars) and is not inhibited by Glu (black bar) (n = 3, mean ± s.d).



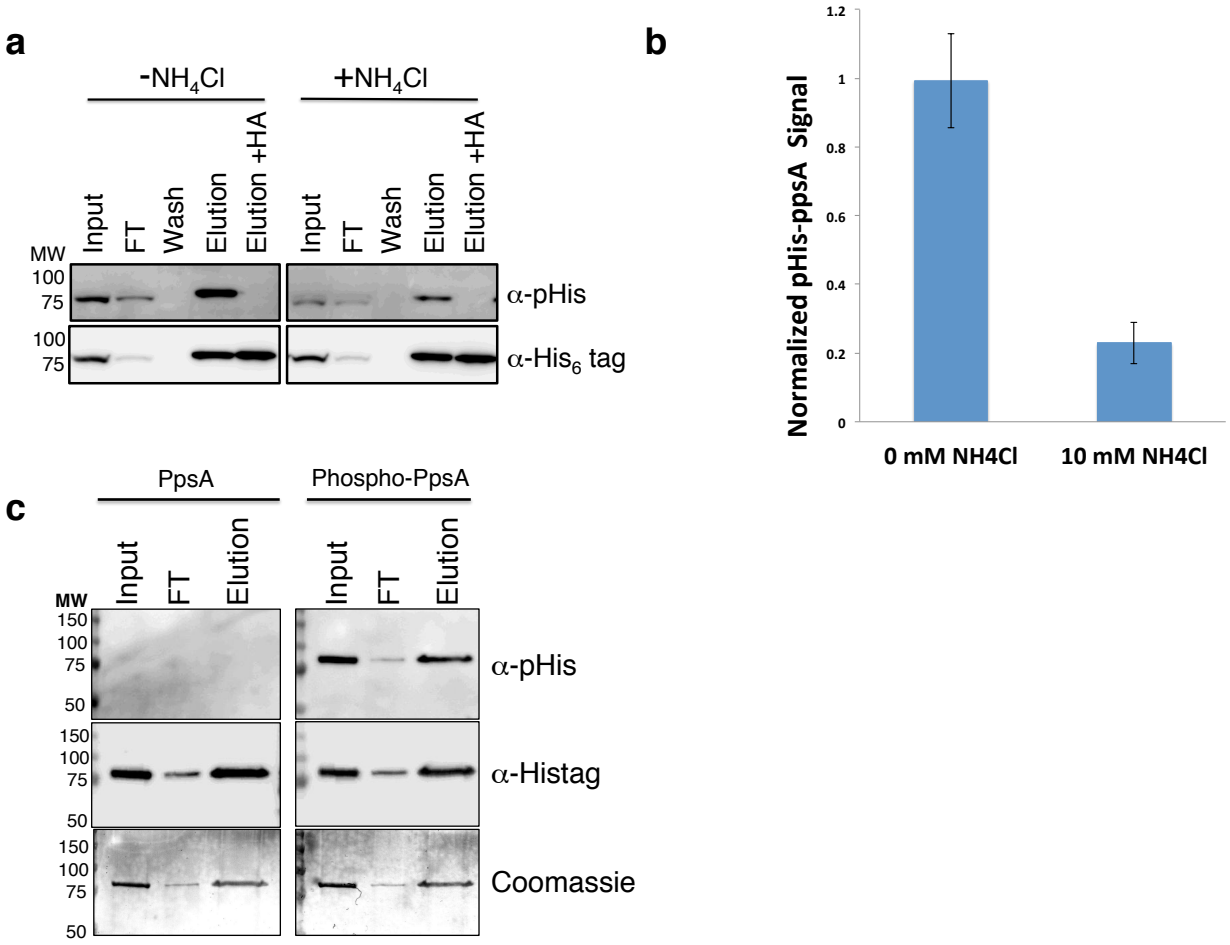
Supplementary Figure 12. Western blot of the *E. coli* lysate expressing His-tagged DhaM. The lysate was treated with hydroxylamine (HA), dihydroxyacetone (DHA), and phosphohistidine phosphatase (PHPT1). In all cases, the signal from pHis decreased. The tagged DhaM can be purified using Ni-NTA affinity chromatography preserving the pHis signal. Bottom: Re-blot with anti-His tag antibody shows the total amount of tagged DhaM.



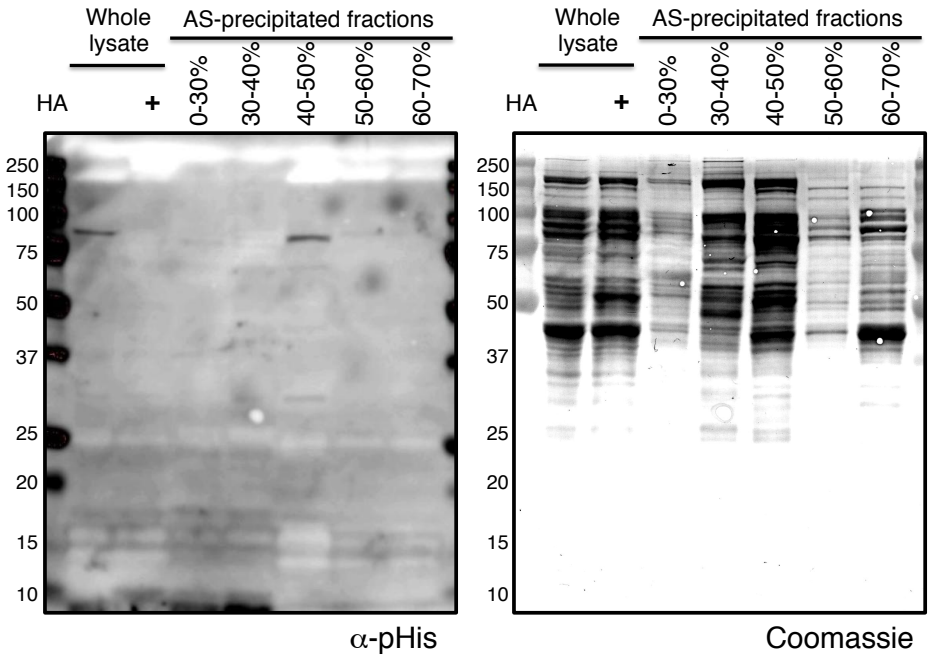
Supplementary Figure 13. Immunoprecipitation of pHis Proteins. E. coli lysate overexpressing phosphorylated PtsI (pPtsI) (top) or phosphorylated DhaM (pDhaM) (bottom) was immunoprecipitated with the α-pHis antibody. Protein bands corresponding to PtsI and DhaM were observed in the elution fractions from lysate treated with antibody (+α-pHis). PtsI and DhaM and were not observed in the elution fractions when the immunoprecipitations were performed in the absence of antibody (-α-pHis) or the presence of antibody and pTze (+α-pHis, 1 mM pTze).



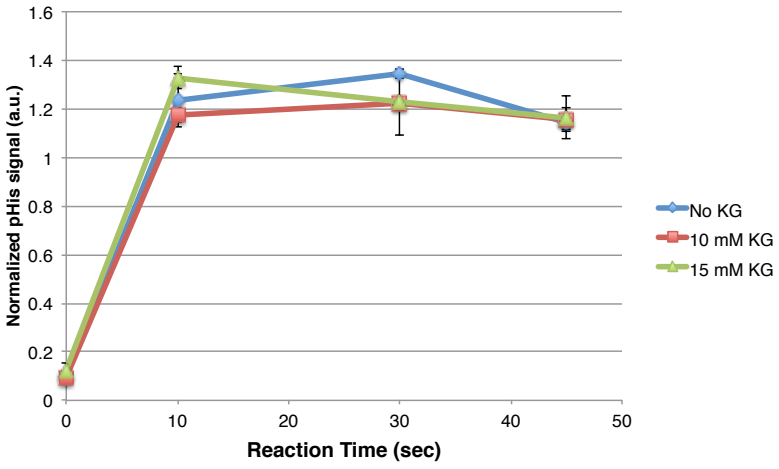
Supplementary Figure 14. PpsA pHis levels are sensitive to nitrogen upshift. NCM 3722 cells overexpressing PtsP, PpsA, or Ppk were treated with or without ammonia chloride and probed for histidine phosphorylation using the pHis antibody. Only PpsA pHis levels showed sensitivity to ammonia chloride. Interestingly, we did not detect histidine phosphorylation on endogenous PpsA in cells overexpressing Ppk.



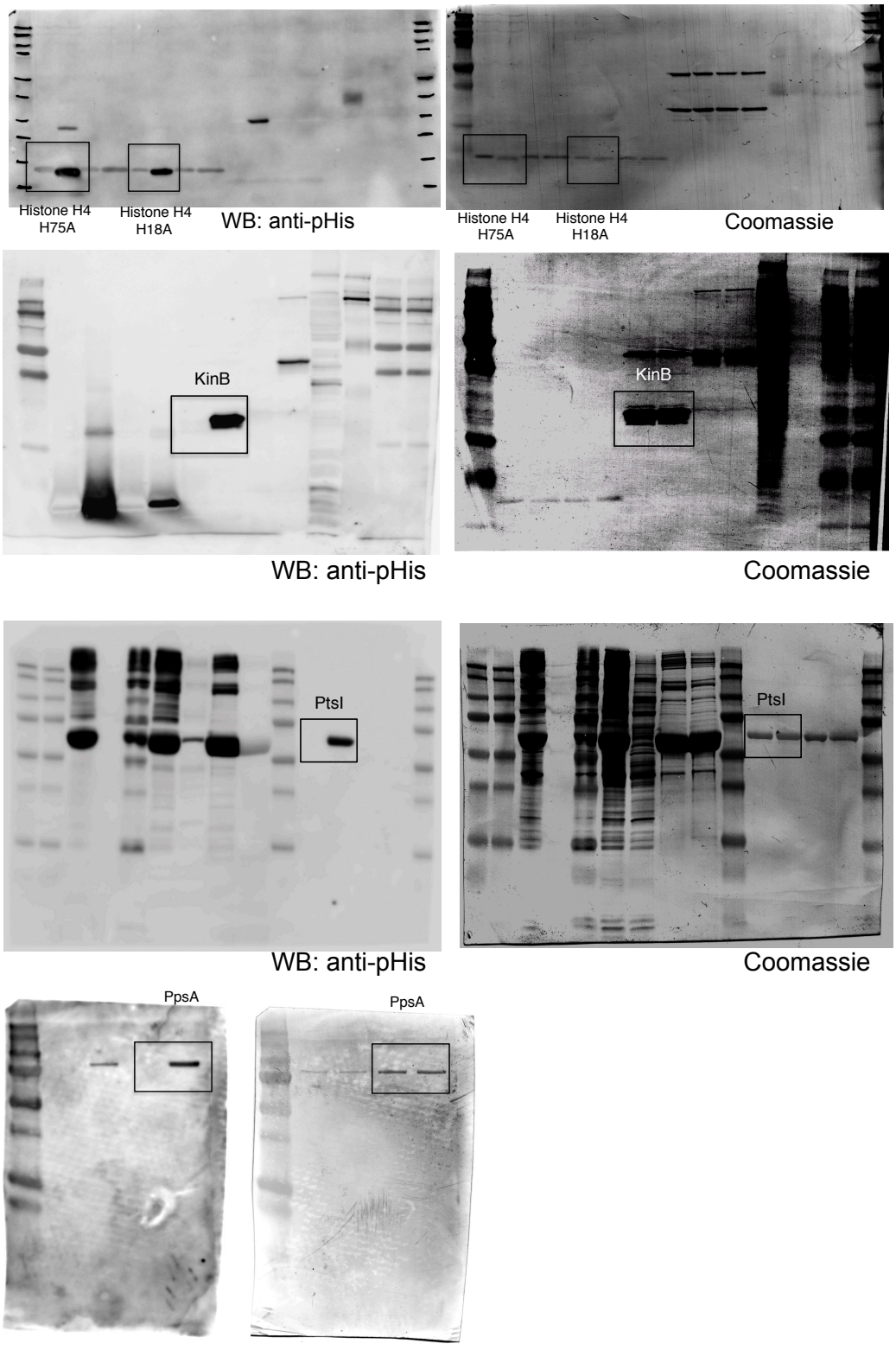
Supplementary Figure 15. pHis levels from His_6-tag purified PpsA show sensitivity to nitrogen upshift. This is a follow up experiment to Supplementary Figure 14 where overexpressed PpsA was purified from the lysate and then analyzed for pHis levels (in Supplementary Figure S14, overexpressed PpsA pHis levels were analyzed directly from the lysate without additional purification). FT refers to the supernatant after nickel pull down and Elution +HA refers to the elution sample treated with hydroxylamine (HA) to dephosphorylate the sample indicating presence of pHis. a) His_6-PpsA was overexpressed in NCM 3722 cells and then purified from the lysate using Ni-NTA resin. Cells grown on minimal media with arginine as the nitrogen source were treated with IPTG to induce protein overexpression followed by addition of ammonia. Treatment with ammonia caused a substantial loss of pHis signal in purified His_6-PpsA. b) pHis signal intensity from the purified PpsA protein (elution fraction) treated with or without ammonia was measured by densitometry and plotted as a histogram with the pHis signal normalized to the His tag signal (n = 3, mean ± s.d.). The histogram shows that the pHis levels in PpsA are strongly dependent on the presence or absence of ammonia. c) As a control, the effect of PpsA histidine phosphorylation on the binding to Ni-NTA resin was tested using purified His_6-PpsA. Phosphorylated and non-phosphorylated PpsA exhibit identical binding to Ni-NTA resin. FT refers to the supernatant after nickel pull down.



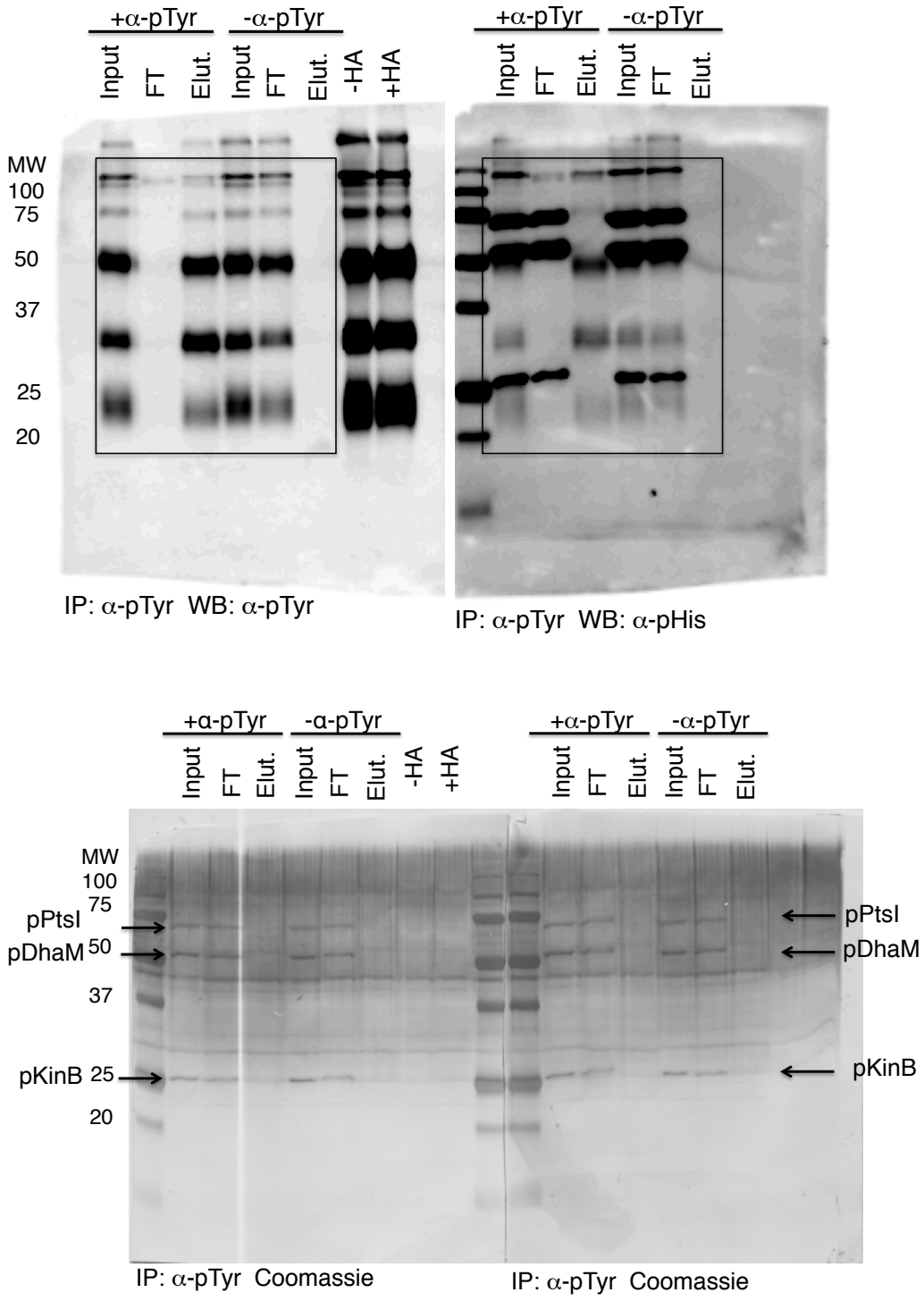
Supplementary Figure 16. **Fractionation of *E. coli* lysate by ammonium sulfate (AS) precipitation**. Lysates from NCM 3722 cells grown on minimal media containing glucose as the carbon source and arginine as the nitrogen source were fractionated by ammonium sulfate precipitation. The gel region corresponding to a 87 kDa band identified by Western blot (left panel, 40-50% fraction) was excised and analyzed by mass spectrometry. A pHis-containing peptide fragment from PpsA was identified (Figure 5b).



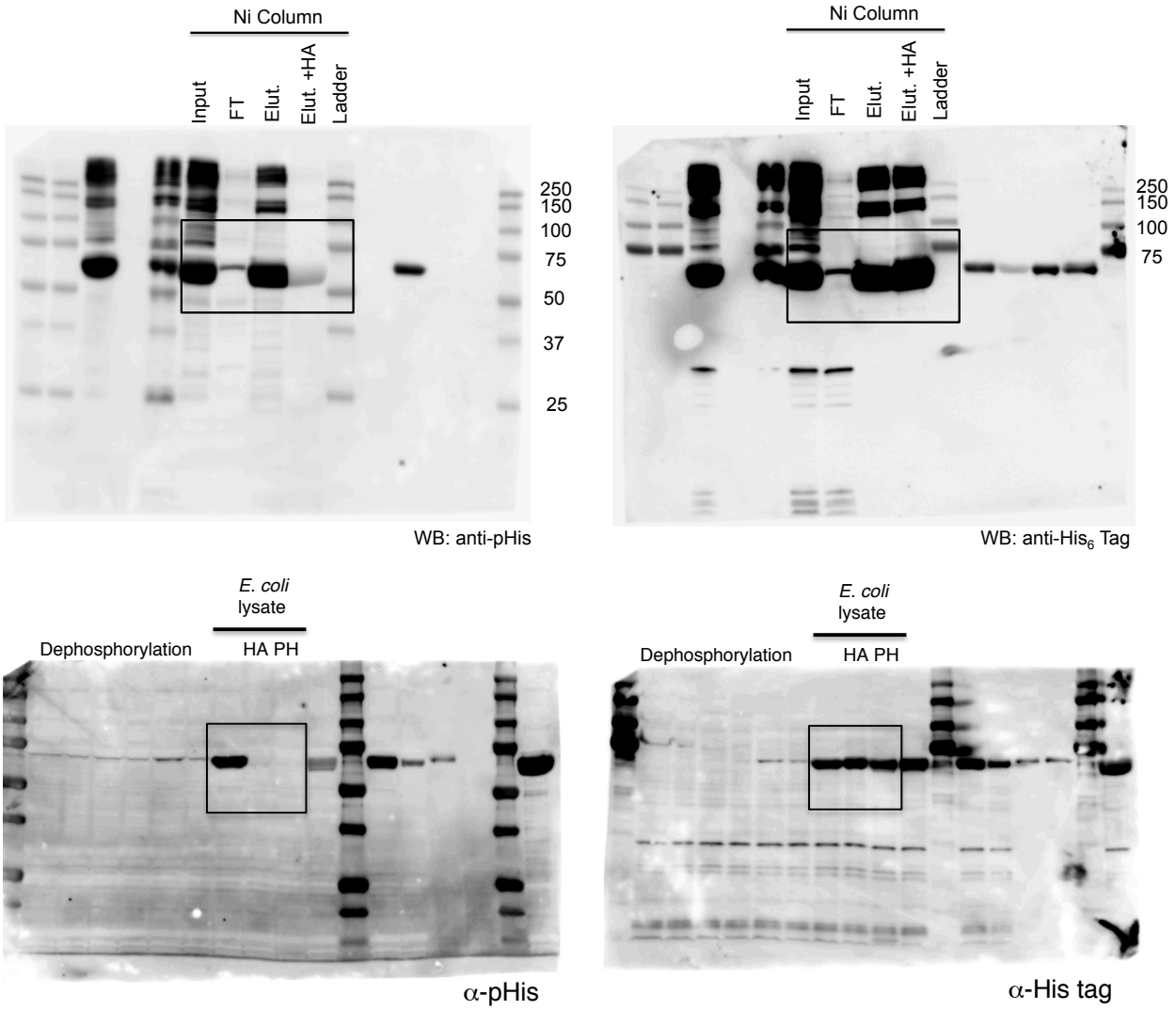
Supplementary Figure 17. Phosphorylation of PpsA. Autophosphorylation of PpsA by ATP in the absence of pyruvate substrate was monitored by dot blot assay. Millimolar concentration of α-KG had no effect on the phosphorylation (n = 2, mean ± s.d).



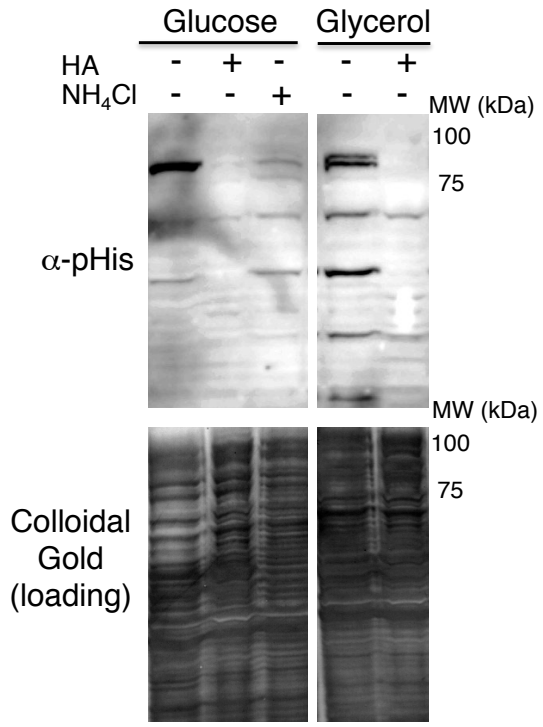
Supplementary Figure 18. Full Western blots of Figure 2b. The boxed image depicts what is shown in figure 2b.



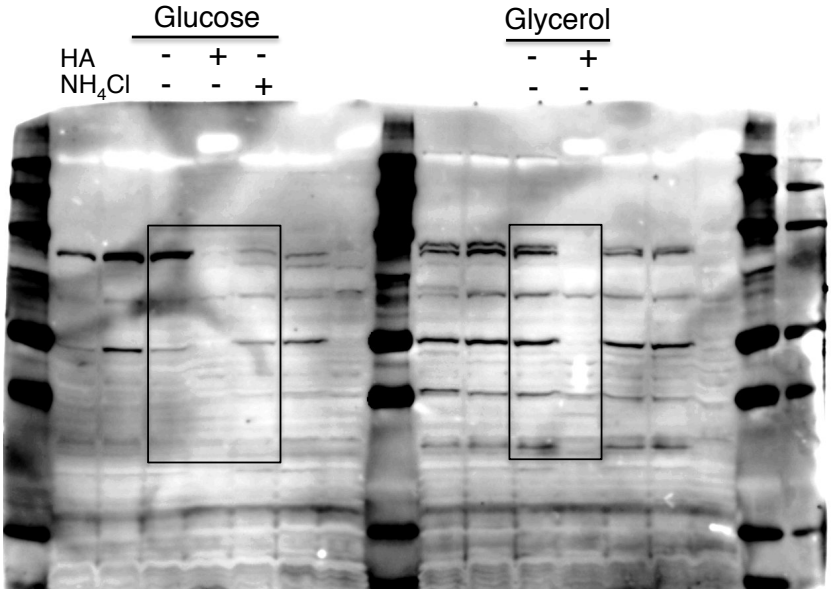
Supplementary Figure 19. Full Western blots and gels of Figure 3b. In the top panel the boxed image depicts the portion of the Western blot that is actually shown in figure 3b. Note that treatment of pTyr protein sample with hydroxylamine (HA) (left panel) does not cause loss of pTyr signal. The bottom panel is the coomassie stain of the membranes. Note that the phosphorylated proteins (pPtsI, pDhaM, and pKinB) are visible by coomassie stain as indicated by the arrows. The Western blot and coomassie stained membrane both show that these three proteins are not immunoprecipitated by the α-pTyr antibody.



Supplementary Figure 20. Full Western blots of Figure 4a. The boxed image depicts what is actually shown in figure 4a.



Supplementary Figure 21. Western blot and colloidal gold stain from Figure 5a. The colloidal gold stain of the membrane serves as the loading control.



Supplementary Figure 22. Full Western blot of Figure 5a. The boxed image depicts what is actually shown in figure 5a.

Accession	Description	Score	# of Unique Peptides	Sequence Coverage (%)	MW [kDa]
P0AAI3	ATP-dependent zinc metalloprotease (FtsH)	453.13	9	19.88	70.7
Q59385	Copper-exporting P-type ATPase A (CopA)	351.95	8	13.31	87.8
P0ABJ0	Ubiquinol oxidase subunit 1 (CyoB)	297.29	3	6.79	74.3
P23538	***Phosphoenolpyruvate synthase (PpsA)***	***283.44***	***5***	***7.70***	***87.4***
P37177	***Phosphoenolpyruvate-protein phosphotransferase (PtsP)***	***237.69***	***5***	***7.89***	***83.7***
P00582	DNA polymerase I (PolA)	228.94	6	8.84	103.1
P06612	DNA topoisomerase 1 (TopA)	206.71	7	8.55	97.3
P0ABB9	Magnesium-transporting ATPase, P-type 1 (MgtA)	204.49	9	16.15	99.4
P15877	Quinoprotein glucose dehydrogenase (Gcd)	167.81	5	7.29	86.7
P0A7B2	***Polyphosphate kinase (Ppk)***	***151.41***	***4***	***7.12***	***80.4***
B1XAJ3	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (MetE)	105.95	4	9.56	84.6
P06971	Ferrichrome-iron receptor (FhuA)	94.04	4	7.76	82.1
P62518	Glucans biosynthesis glucosyltransferase H (MdoH)	92.56	3	2.95	96.9
P0AG22	GTP pyrophosphokinase (RelA)	84.64	1	2.15	83.8
P05825	Ferrienterobactin receptor (FepA)	84.02	2	4.16	82.1
A1AIC1	Catalase-peroxidase (KatG)	79.53	1	1.93	80.0
P00579	RNA polymerase sigma factor (RpoD)	47.28	1	1.79	70.2
A1A7M1	Outer membrane protein assembly factor (YaeT)	38.92	2	3.46	90.6
P45800	Putative membrane protein igaA homolog(YrfF)	37.53	2	3.52	79.4
P0AEC3	Aerobic respiration control sensor protein (ArcB)	34.60	1	1.29	87.9
A7ZHI9	Protein translocase subunit (SecA)	31.79	2	4.99	102.0

Supplementary Table 1. List of proteins immunoprecipitated with anti-pHis antibody from *E. coli* lysate grown under nitrogen deficiency. The immunoprecipitated proteins were separated by SDS-PAGE, and the gel region around 85 kDa was excised and analyzed by mass spectrometry. Proteins of molecular weight between 70 and 100 kDa are chosen from the initial hits, and proteins obtained by mock-immunoprecipitation without the antibody (total of 9 proteins) are discarded from the list. Proteins with known pHis sites are shown in red.

Supplementary Note 1

General Materials

All buffering salts and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Fischer Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA) was purchased from Halocarbon (North Augusta, SC). Kanamycin sulfate (Kan), β-mercaptoethanol (BME), Coomassie brilliant blue, *N,N*-diisopropylethylamine (DIPEA), bovine serum albumin (BSA), adenosine triphosphate (ATP), phosphotyrosine, phosphoserine, phosphothreonine, histidine, glutaraldhyde, phosphatase inhibitor cocktails 2 and 3, phosphoenolpyruvic acid cyclohexylammonium salt (PEP), hydroxylamine (HA), sodium 2-sulfanylethanesulfonate (MESNA), dithiothreitol (DTT), arginine, ammonium chloride, glutamic acid, and α-ketoglutaric acid (cat. no. 75890) were purchased from Sigma-Aldrich (St. Louis, MO). SulfoLink Resin, Ultra TMB ELISA substrate, ECL substrate, Restore Western Blot Striping Buffer, Protein G Agarose Resin, Nunc-Immuno Maxisorp 96-well plates, Nitrocellulose membrane, and Phusion High–Fidelity PCR kit were purchased from Thermo Scientific (Rockford, IL). Criterion XT 12% Bis-Tris Polyacrylamide Gels, Criterion XT 15% Tris Polyacrylamide Gels, Criterion XT 10.5-14% Tris Polyacrylamide Gels, 12% TGX Criterion Gels, 12% Mini-Protean TGX gels, goat anti rabbit HRP secondary antibody, goat anti mouse HRP secondary antibody, 0.2 μm PVDF membrane, cation exchange chromatography AG 50W-X8 resin (pre-packaged in column cartridges containing 2 mL of resin), and colloidal gold total protein stain were purchased from BioRad (Hercules, CA). EGF-stimulated A431 whole cell lysate was from Rockland (Gilbertsville, PA). Phosphotyrosine molecular weight marker and rabbit anti-phosphotyrosine polyclonal antibody was from Calbiochem (San Diego, CA). Mouse anti-His tag antibody, Protein A-HRP and 4G10 anti-phosphotyrosine monoclonal antibody were purchased from Millipore (Billerica, MA). Complete protease inhibitor tablets were purchased from Roche Diagnostics (Mannheim, Germany). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Subcloning efficiency DH5α competent cells and One Shot BL21(DE3) chemically competent *E. coli* were purchased from Invitrogen (Carlsbad, CA) and used to generate "in-house" high competency cell lines. NCM 3722 *E. coli* strain[1] was a kind gift from the Rabinowitz lab (Lewis-Sigler Institute for Integrative Genomics, Princeton University, New Jersey, USA).

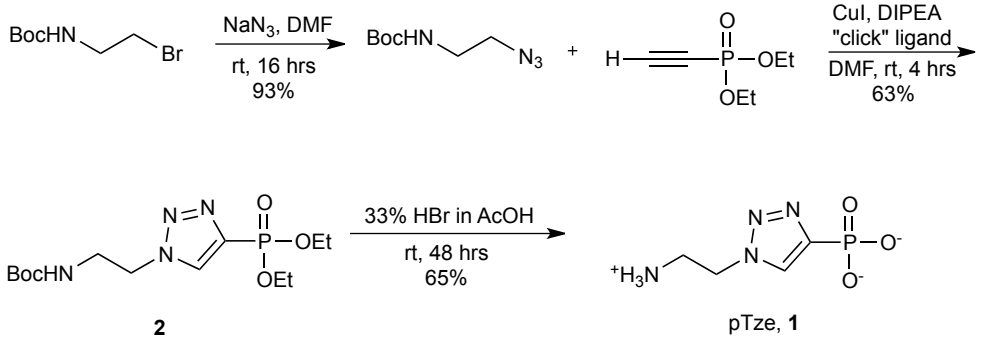
General Equipment

Size-exclusion chromatography was carried out on an ÄKTA FPLC system from GE Healthcare on a Superdex 75 10/300 column. Analytical RP-HPLC was performed on Hewlett-Packard 1100 and 1200 series instruments equipped with a C18 Vydac column (5 μm, 4.6 x 150 mm) at a flow rate of 1 mL/min. All runs were carried out employing gradients of solvent A (0.1% TFA in water) and solvent B (90% acetonitrile in water with 0.1% TFA). For all runs a two-minute isocratic period in initial conditions was followed by a 30-minute linear gradient with increasing solvent B concentration. The solvent gradients are specified in each of HPLC experiments (see below). Electrospray ionization mass spectrometric analysis (ESI-MS) was performed on pTze, pTze precursors, Histone H4 Proteins, and PHPT1 by direct infusion on a Bruker Daltonics MicrOTOF-Q II mass spectrometer. Coomassie stained gels were imaged on a LI-COR Odyssey Infrared Imager. ELISA absorbance measurements at 450 nm were recorded on a Molecular Devices Spectramax M3 micro plate reader. Western blot membranes were imaged using the GE ImageQuant LAS 4010 Imager. Dot blots were imaged using the GE ImageQuant LAS 4010 Imager or on a LI-COR Odyssey Infrared Imager. PCR was performed on a MJ Mini™ thermal cycler (Bio-Rad). All plasmids used in this study were sequenced by GENEWIZ (South Plainfield, NJ) to verify the correct DNA sequence. Cells were lysed using a Branson Sonifier. Samples were lyophilized on a Millrock technology MD85 lyophilizer.

Supplementary Note 2

Synthesis of pTze

Supplementary Figure 23. Synthetic scheme for pTze.



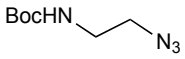
General Synthetic Materials and Methods

All chemicals and solvents for the synthesis of pTze were obtained from Sigma-Aldrich unless otherwise noted and used without further purification unless otherwise noted. Silica gel (pore size 60 Å, mesh particle size 200-400) was purchased from Sigma-Aldrich (St. Louis, MO). Silica gel 60 aluminum-backed thin-layer chromatography (TLC) plates were purchased from EMD Chemicals (Billerica, MA). $CDCl_3$, C_6D_6, and deuterium oxide NMR solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Anhydrous solvents were obtained by passage through an activated alumina column. Unless otherwise noted, all reactions were carried out in an oven-dried (>100 °C) round-bottom flask equipped with a Teflon™ coated magnetic stir bar and a rubber septum under a positive pressure of argon. Rotary evaporation was performed using a Büchi rotary evaporator equipped with a vacuum pump. Flash Chromatography over silica gel was performed according to the method described by Still.[2] Compounds were visualized on TLC plates by irradiation with UV light, or by treatment with a solution of ninhydrin in ethanol followed by heating. Product yields refer to pure compounds, unless otherwise indicated.

NMR Spectroscopy

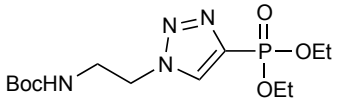
^{1}H NMR spectra were recorded on a Bruker 500 Avance-III equipped with a Cryo-QNP probe (Bruker BioSpin, Billerica, MA). ^{1}H NMR chemical shifts are reported in parts per million (ppm) and are referenced relative to the residual solvent proton signal for $CDCl_3$ at 7.26 ppm, C_6D_6 at 7.16 ppm, and D_2O at 4.79 ppm. ^{1}H NMR data are tabulated in the following format: chemical shift, multiplicity [singlet (s), broad singlet (brs), doublet (d), triplet (t), multiplet (m)], coupling constant [Hz], number of protons, and structural assignments. ^{13}C NMR spectra were recorded on a Bruker 500 Avance-III equipped with a Cryo-QNP probe or a Cryo-DCH probe (Bruker BioSpin, Billerica, MA). ^{13}C NMR chemical shifts are reported in ppm relative to carbon signals for $CDCl_3$ at 77.16 ppm, C_6D_6 at 128.06 ppm or, in D_2O, they are reported relative to the solvent deuterium signal.[3] ^{13}C NMR data are tabulated in the following format: chemical shift, multiplicity [doublet (d)] (if applicable), coupling constant [Hz], and structural assignments. ^{31}P NMR spectra were recorded on a Bruker 500 Avance-III equipped with a Cryo-QNP probe (Bruker BioSpin, Billerica, MA). ^{31}P NMR chemical shifts are reported relative to the solvent deuterium signal.[3] A 2D NOESY spectrum for *tert*-butyl (2-(4-(diethoxyphosphoryl)-1*H*-1,2,3-triazol-1-yl)ethyl)carbamate was recorded on a Bruker 500 Avance-III equipped with a Cryo-TCI probe (Bruker-Bio-spin, Billerica, MA) with a 700 ms mixing time. The NOESY spectrum is reported as 1D slices taken at 3.86 ppm and 7.81 ppm (see Supplementary Figure 24).

Synthesis of 2-(Boc-amino)ethyl azide



In a round bottom flask equipped with a magnetic stir bar, 2-(Boc-amino)ethyl bromide (250 mg, 1.1 mmol) was dissolved in 4 mL DMF followed by addition of NaN_3 (215 mg, 3.3 mmol). The reaction mixture was then stirred for 15 hours at room temperature under argon. The mixture was then poured into 100 mL EtOAc and washed with 4 x 50 mL H_2O. The wash layers were combined and extracted with 1x 50 mL EtOAc. The EtOAc layers were then combined, dried over $NaSO_4$, filtered and the solvent was removed by rotary evaporation to give 2-(Boc-amino)ethyl azide as a clear oil (192 mg, 93% yield). This product was used in the next reaction without further purification. Analytical data for this compound are consistent with previous reports.[4]

Synthesis of *tert*-butyl (2-(4-(diethoxyphosphoryl)-1*H*-1,2,3-triazol-1-yl)ethyl) carbamate (**2**)



2

In a round bottom flask equipped with a magnetic stir bar, 2-(Boc-amino)ethyl azide (190 mg, 1.0 mmol) and diethyl ethynylphosphonate[5] (165 mg, 1.00 mmol) were dissolved in 4 mL DMF and stirred at room temperature under Argon. Ascorbic acid (80 mg, 0.45 mmol), *tris*-(benzyltriazolylmethyl)amine (TBTA)[6] (40 mg, 0.075 mmol) and diisopropylethylamine (540 μL, 3.1 mmol) were then added to the reaction mixture followed by addition of CuI (40 mg, 0.2 mmol). The reaction mixture was then stirred for 4 hours at room temperature until TLC indicated the consumption of 2-(Boc-amino)ethyl azide (R_f = 0.9 eluted with 100% EtOAc). The reaction mixture was then diluted into 100 mL EtOAc and washed with 3 x 50 mL H_2O with the pH pre-adjusted to 3 with 1M HCl. The organic layer was then washed once with 50 mL brine. The aqueous layers were combined and extracted with 2 x 50 mL EtOAc. All of the EtOAc layers were combined, dried over $MgSO_4$, filtered, and the solvent was then removed by rotary evaporation to give a clear oil. The clear oil was purified by flash chromatography over silica gel (100% EtOAc) to give the product as a white solid (220 mg, 63% yield).

R_f = 0.1 (eluted with 100% EtOAc, a dark brown spot visualized with ninhydrin stain).

^{1}H NMR (500MHz, C_6D_6): 7.85 (s, 1H, triazole), 4.84 (brs, 1H, NH), 4.20-4.26 (m, 4H, -OCH_2CH_3), 3.88 (t, J = 5 Hz, 2H, triazoleCH_2CH_2), 3.14 (m, 2H, triazoleCH_2CH_2), 1.47 (s, 9H, t-butyl), 1.21 (t, J = 5 Hz, 6H, -OCH_2CH_3) ppm.

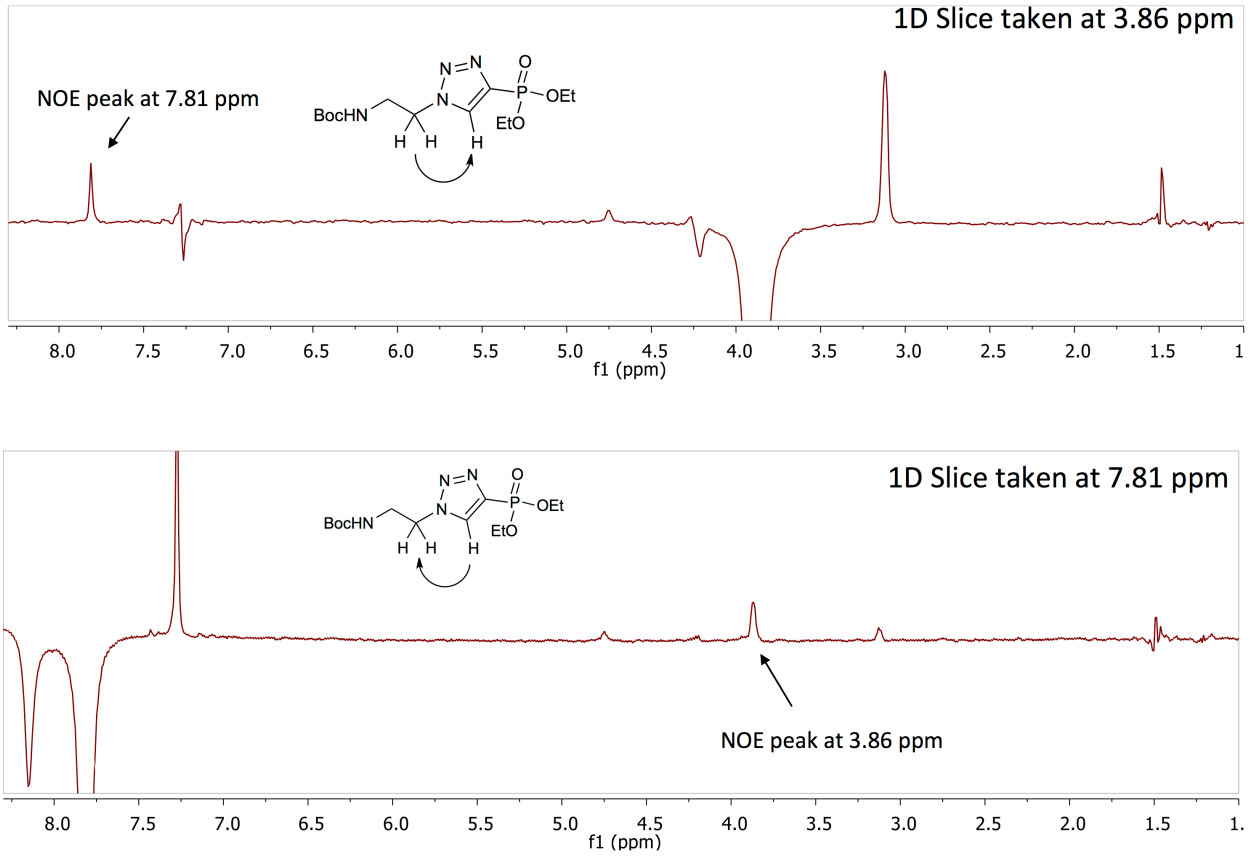
^{13}C NMR (C_6D_6): 155.84 (-CONH), 138.09 (d, J_{C-P} = 235 Hz, -C=C-P), 131.66 (d, J_{C-P} = 32 Hz, -C=C-P), 79.14 ($(CH_3)_3C$-), 62.86 (-OCH_2CH_3), 49.55 (-$NHCH_2CH_2$-triazole), 40.53 (-$NHCH_2CH_2$-triazole), 28.36 ($(CH_3)_3C$-), 16.35 (-OCH_2CH_3) ppm

^{31}P NMR (C_6D_6): 20.41 ppm.

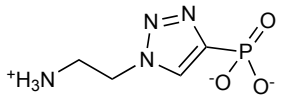
ESI-MS: 371.1460 (calculated for $[MW+Na]^+$), 371.1529 (observed).

HPLC retention time = 17.7 minutes (C18 analytical column, 0-73% solvent B over 30 mins, 1 mL/min).

Supplementary Figure 24. NOE measurements for determination of triazole regiochemistry



Synthesis of (1-(2-aminoethyl)-1*H*-1,2,3-triazol-4-yl)phosphonic acid (pTze, 1)



1

In a round bottom flask equipped with a magnetic stir bar, *tert*-butyl (2-(4-(diethoxyphosphoryl)-1*H*-1,2,3-triazol-1-yl)ethyl)carbamate (155 mg, 0.4 mmol) was dissolved in 2.3 mL 33% HBr in acetic acid. The reaction mixture was then capped with a glass stopper and stirred at room temperature for 48 hours until ESI-MS indicated complete loss of Boc- and ethyl protecting groups. The reaction mixture was then concentrated to a solid by rotary evaporation. The crude solid was then dissolved in 2 mL of 0.1% AcOH and purified by cation exchange chromatography. This was done by loading the crude product onto 2 mL of resin pre-equilibrated with 0.1% AcOH. The sample was then washed with 6 mL 0.1% AcOH. The product was then eluted off of the resin with 500 mM ammonium acetate and collected in 3 mL fractions. Fractions containing product were identified by ESI-MS and then flash frozen in liquid nitrogen and lyophilized to dryness to give pTze as a white powder (54 mg, 65% yield). ^{1}H NMR analysis indicated that ≤ 5% AcOH is present in the final product.

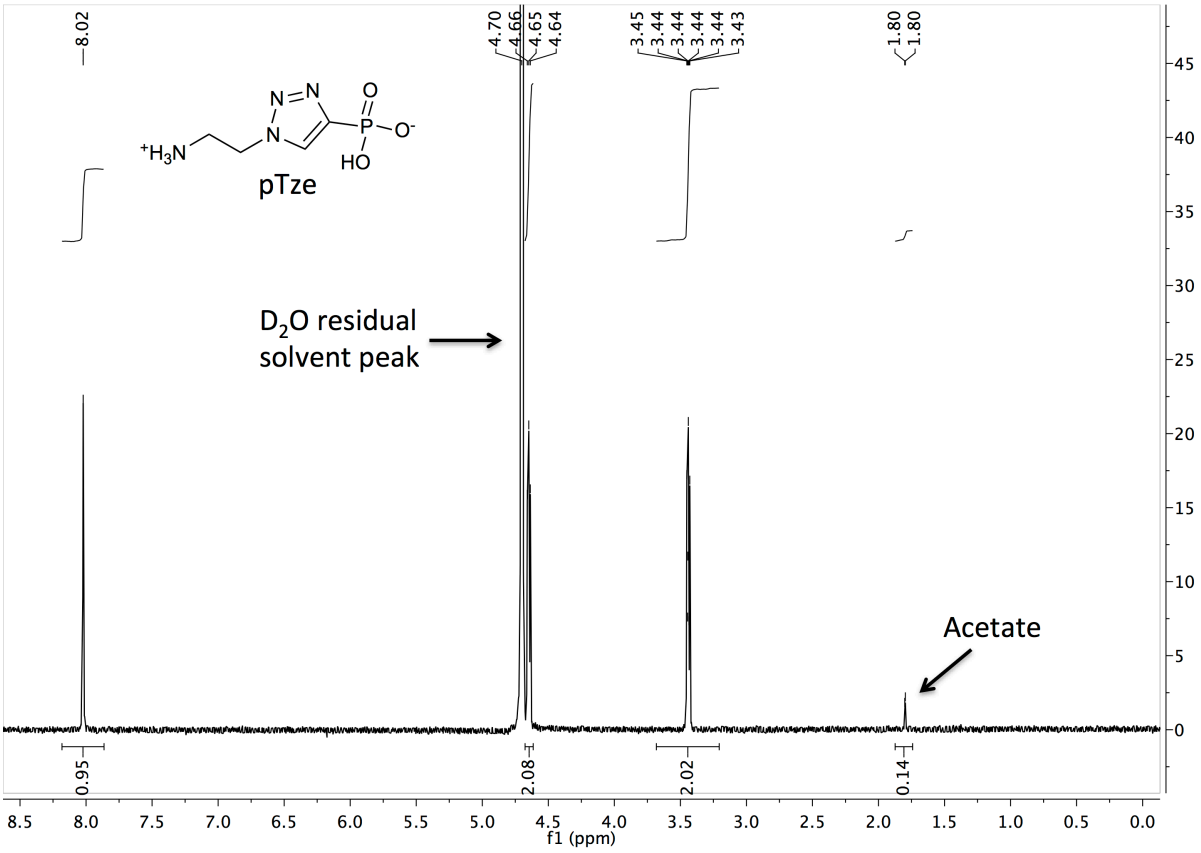
^{1}H NMR (500MHz, D_2O): 8.02 (s, 1H, triazole), 4.65 (t, J = 5 Hz, 2H, triazoleCH_2CH_2), 3.44 (t, J = 5 Hz, 2H, triazoleCH_2CH_2) ppm.

^{13}C NMR (D_2O): 143.68 (d, J_{C-P} = 235 Hz, -C=C-P), 129.25 (d, J_{C-P} = 36.25 Hz, -C=C-P), 47.06 (-$NHCH_2CH_2$-triazole), 38.84 (-$NHCH_2CH_2$-triazole) ppm.

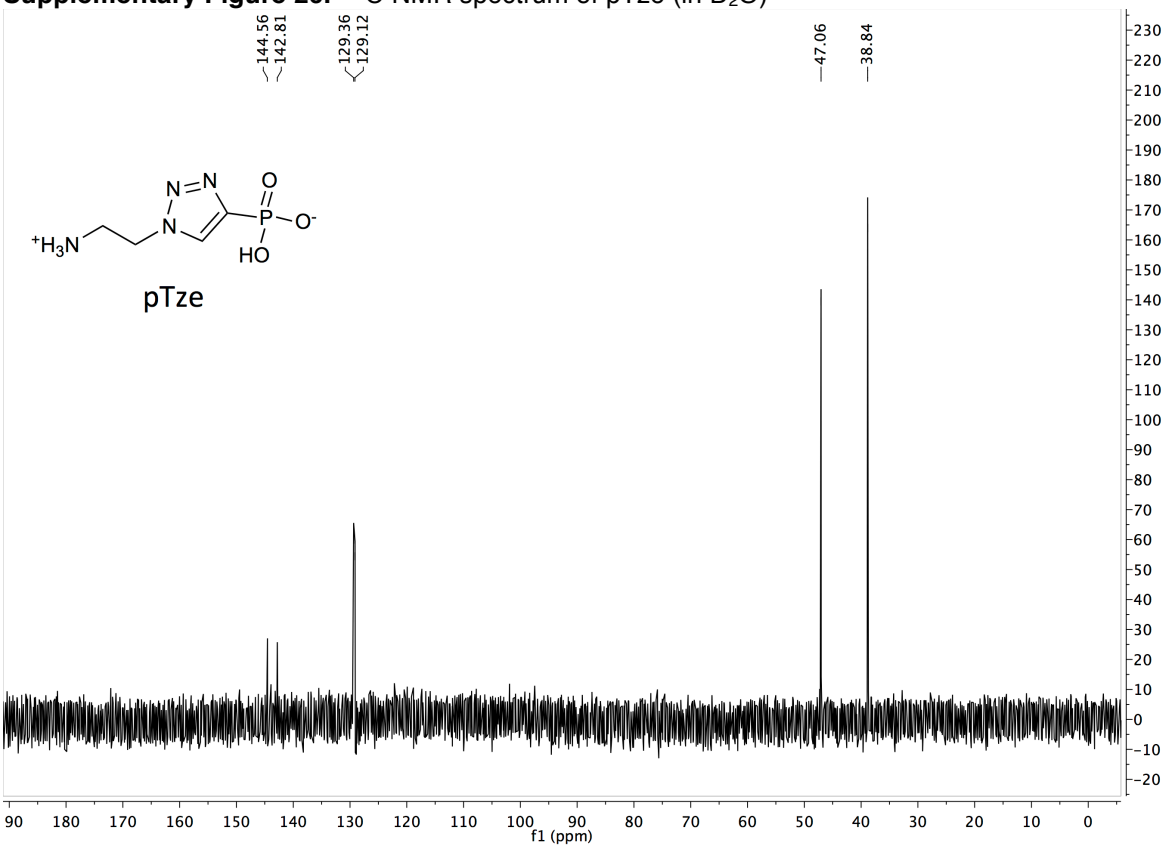
^{31}P NMR (D_2O): 0.72 ppm.

ESI-MS: 193.0485 (calculated for $[MW+H^+]^+$), 193.0516 (observed).

Supplementary Figure 25. ^{1}H NMR spectrum of pTze (in D_2O)



Supplementary Figure 26. ^{13}C NMR spectrum of pTze (in D_2O)



Supplementary Note 3

Synthesis of potassium phosphoramidate

Potassium phosphoramidate was prepared according to the procedure by Wei and Matthews.[7] In a fume hood, 9.15 mL (100 mmol) of $POCl_3$ was added dropwise to a stirred solution of ice-cold 10% ammonia (150 mL, made from 50 mL of 30% aq. ammonia) via a syringe over 2 min. Some fuming and heating was observed. This was stirred for 15 min on ice until a clear solution was obtained. To this, 500 mL of acetone was added, upon which some precipitate formation was observed in the bottom (aqueous) layer. The bottom phase (including the precipitates) was collected and acidified to pH 6 using acetic acid. The suspension was kept at -20 ˚C for 1 h to induce crystallization of the solid (ammonium hydrogen phosphoramidate). The precipitate was filtered on a pre-weighed filter paper. The solid was washed with 100% EtOH (15 mL) and ether (15 mL), and dried under vacuum overnight to afford a white solid (6.62 g). This solid was added in small portions to a stirred solution of 50% KOH (11.6 mL) in a fume hood. (*CAUTION*: Vigorous bubbling and exotherm were observed. Make sure to add in small portions to avoid bumping.) The opaque suspension was heated in the fume hood to 70 ˚C (in a heated water bath) for 20 min to expel ammonia gas. The solution was cooled to RT and neutralized to pH 6 with acetic acid. Precipitation was observed on neutralization. This suspension was poured onto 500 mL of 100% EtOH and it was kept at -20 ˚C overnight to induce further precipitation. The suspension was filtered using a pre-weighed filter paper to collect the precipitates. The solids were washed with 100% EtOH (15 mL) and ether (15 mL) and dried under vacuum to afford a white solid (6.0 g, 44% yield). ^{31}P NMR shows about 4% contamination with phosphate.

^{31}P NMR (D_2O): -3.49 ppm

Preparation of τ-phosphohistidine

τ-phosphohistidine (τ-pHis) was prepared according to the method of Besant *et al.*[8] with slight modifications. L-histidine hydrochloride (84 mg, 0.54 mmol) was dissolved in 1.5 mL of water and the pH was adjusted to 7. Potassium phosphoramidate (200 mg) was added to this solution and the mixture was incubated at RT for 4 h. A silica gel column was packed with an EtOH/H_2O mixture (80:20) and the reaction mix was loaded onto the column. The column was first eluted with an EtOH/H_2O/NH_4OH mixture (80:18:2) to separate unreacted histidine and then with an EtOH/H_2O/NH_4OH mixture (65:22:8) to collect pHis. Histidine and phosphohistidine were separated out. The collected fractions (~100 mL) were concentrated down to ~10 mL using a rotary evaporator at RT. During the evaporation, the pH dropped from 10 to 8. The pH was re-adjusted to 10 with 2 N NaOH and the mixture was lyophilized. The solid residue (~120 mg) was dissolved in 1 mL of water, and an aliquot was diluted in D_2O for quantification by ^{1}H NMR using 1,4-dioxane as the internal standard. The concentration of 1 mL stock was 322 mM (64% yield) with ~5 % contamination with histidine. The analytical data are consistent with previous reports.[8] The stock solution was aliquoted and stored frozen at -20 ˚C until further use. Prior to each use, the purity of the sample was assessed using TLC to check its decomposition to histidine.

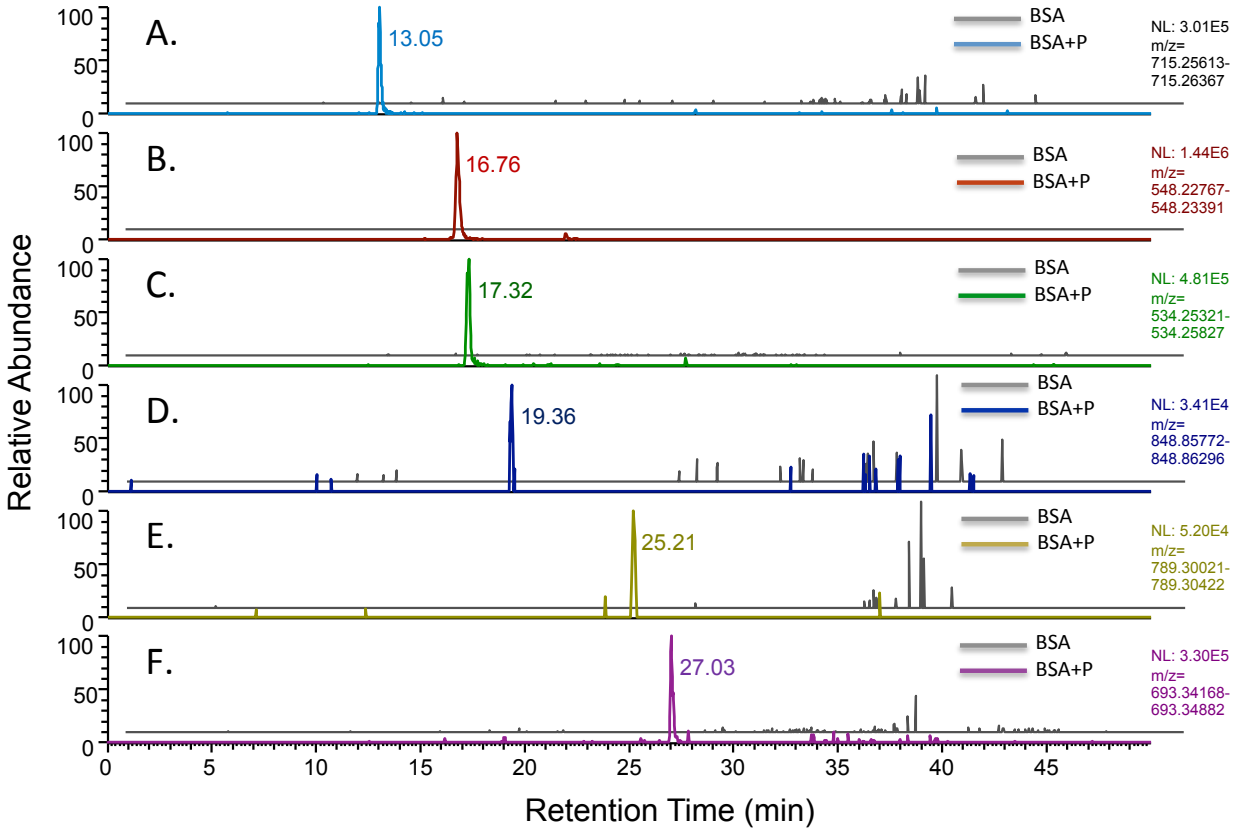
R_f = 0.3 (eluted with EtOH:H_2O:NH_4OH = 65:22:8, A streaky purple spot when stained with ninhydrin). R_f of histidine is 0.7 under the same condition.

^{1}H NMR (500 MHz, D_2O): 7.58 (s, 1H), 6.91 (s, 1H), 3.64-3.60 (dd, *J* = 9.6, 4.2 Hz, 2H), 3.03-2.89 (dd, *J* = 15.1, 4.2 Hz, 2H), 2.80-2.61 (dd, *J* = 15.0, 9.6 Hz, 1H).

Chemical phosphorylation of BSA, histone H4 H74A, histone H4 H18A

BSA was chemically phosphorylated using potassium phosphoramidate, which is known to selectively phosphorylate histidine residues.[5,9,10] Briefly, BSA was dissolved in PBS (final concentration 1 mg/mL) and treated with potassium phosphoramidate (final concentration 100 mM). The mixture was incubated at RT overnight and stored frozen at -20 ˚C until further use. Phosphorylated BSA hereafter referred to as BSA-pHis. For LC-MS and MS/MS analyses, solution containing 10 ug protein was subjected to reduction with 20 mM DTT for 30 min at RT, alkylation using 50 mM iodoacetamide for 30 min at RT in the dark, followed by quenching with 100 mM DTT. The sample was then subjected to buffer exchange into 100mM Tris, pH 8.5 and endoprotease digestion with 1 ug Lys-C for 4 h at RT. The sample was then rapidly de-salted using STAGE-tips, and analyzed by nano-UPLC MS on an Orbi Elite platform using the parameters described below.

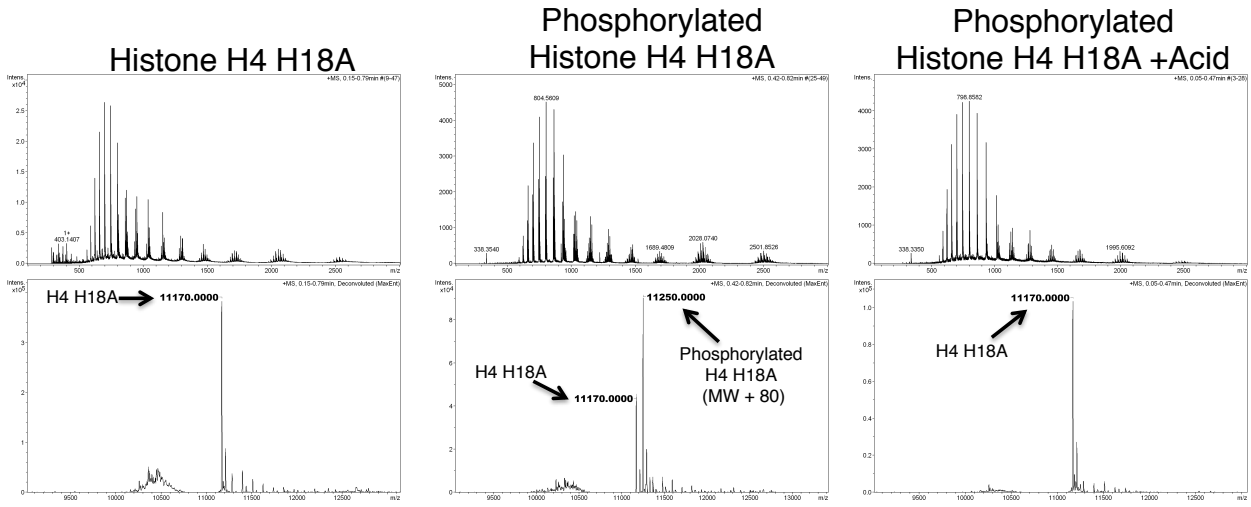
	BSA Sequence	Retention Time	m/z
A	TCVADES**pH**AGCEK	13.05	715.2592
B	S**pH**CIAEVEK	16.76	548.2318
C	SEIA**pH**RFK	17.32	534.2556
D	QEPERNECFLS**pH**K	19.36	848.8604
E	DDP**pH**ACYSTVFDK	25.21	789.3029
F	**pH**LVDEPQNLIK	27.03	693.345



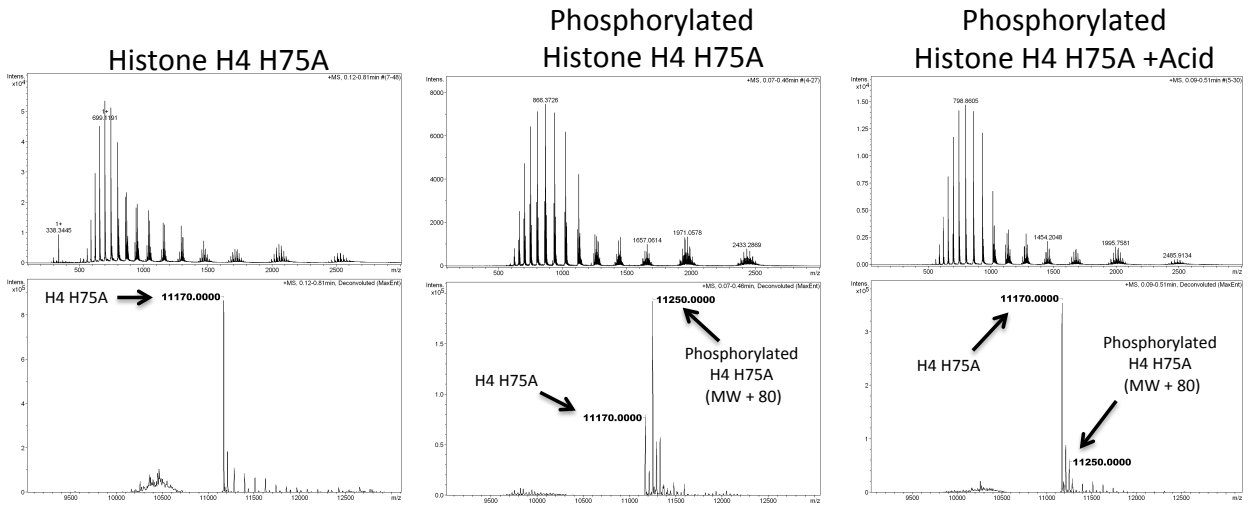
Supplementary Figure 27. Detection of multiple pHis-bearing peptides from BSA treated with phosphoramidate that characterize several distinct sites of histidine phosphorylation. BSA treated with or without phosphoramidate was analyzed by LC-MS and MS/MS. Numerous peptides containing phosphorylation on histidine were detected (six shown, see A-E). Shown in the bottom panel are extracted ion chromatograms for each of the peptides from either BSA treated with phosphoramidate (BSA+P, colored line) or untreated (BSA, gray line). As the chromatograms display, the phosphorylated peptides were only detected in the phosphoramidate-treated BSA sample. This analysis indicates that phosphoramidate treatment of BSA results in phosphorylation at multiple histidine residues.

Human histone H4 and the mutants (H18A and H75A) were recombinantly expressed and chemically phosphorylated using potassium phosphoramidate as described previously.[5] Briefly, the histone was dissolved in PBS (final concentration 1 mg/mL) and treated with potassium phosphoramidate

(final concentration 100 mM). The mixture was incubated at RT overnight and stored frozen at -20 ˚C until further use. Phosphorylated H4 H75A hereafter referred to as H4(H75A)-pHis and phosphorylated H4 H18A hereafter referred to as H4(H18A)-pHis.



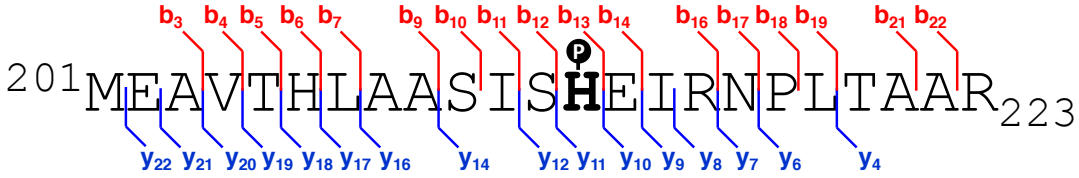
Supplementary Figure 28. Mass spectrometric analysis of H4(H18A)-pHis. Left panel, MS analysis of H4 H18A. Middle panel, MS analysis of H4 H18A treated with phosphoramidate as described in supplementary methods. Right panel, MS analysis of phosphorylated H4 H18A treated with acid (pH = 2) for 24 hours at room temperature. The peak corresponding to the phosphorylated mass is not detected after acid treatment indicating that the phosphorylation is acid-labile.



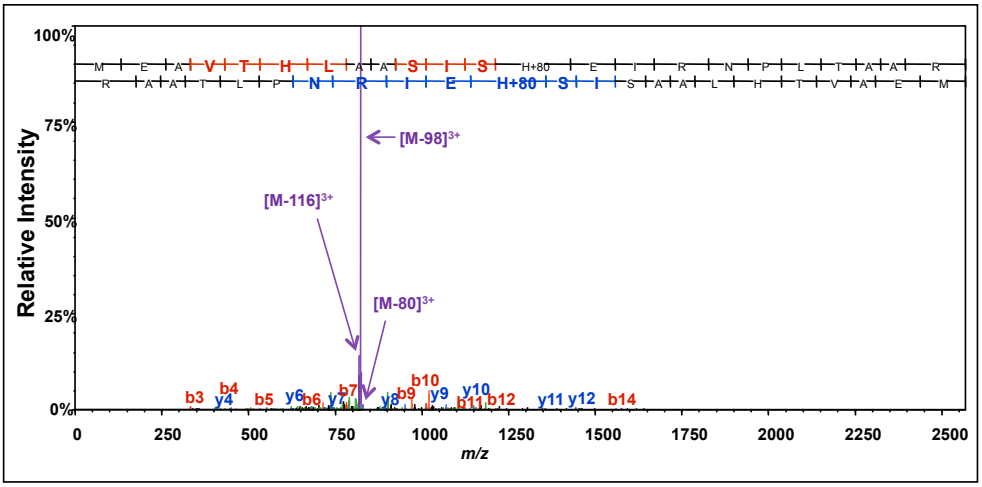
Supplementary Figure 29. Mass spectrometric analysis of H4(H75A)-pHis. Left panel, MS analysis of H4 H75A. Middle panel, MS analysis of H4 H75A treated with phosphoramidate as described in supplementary methods. Right panel, MS analysis of phosphorylated H4 H75A treated with acid (pH = 2) for 24 hours at room temperature. The peak corresponding to the phosphorylated mass is barely detected after acid treatment indicating that the phosphorylation is acid-labile.

In vitro autophosphorylation of KinB

The soluble fraction of KinB was expressed, purified, and *in vitro* phosphorylated as described previously (the plasmid for *B. stearothermophilus* KinB was a gift from Seth Darst, The Rockefeller University).[11] Briefly, 15 μL of the KinB stock (1 mg/mL = 35 μM, in 100 mM NaCl, 20 mM Tris, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 15 % glycerol) was diluted with 75 μL of the reaction buffer (50 mM EPPS (pH 8.5), 50 mM KCl, 20 mM $MgCl_2$, 5% glycerol) and treated with 5 μL of 100 mM ATP. The reaction mixture was incubated at 37 ˚C for 1 h and quenched with 30 μL of 4x gel loading buffer (160 mM Tris, pH 8.5, 40% (v/v) glycerol, 4% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 8% (v/v) BME). The sample was flash frozen and then stored at -80 ˚C until further use. For LC-MS and MS/MS analyses, solution containing 5 ug protein was subjected to reduction with 20 mM DTT for 30 min at RT, alkylation using 50 mM iodoacetamide for 30 min at RT in the dark, followed by quenching with 100 mM DTT. The sample was then subjected to buffer exchange into 100mM Tris, pH 8.5 and endoprotease digestion with 500 ng trypsin for 4 h at RT. The sample was then rapidly de-salted using STAGE-tips, and analyzed by nano-UPLC MS on an Orbi Elite platform using the parameters described below.



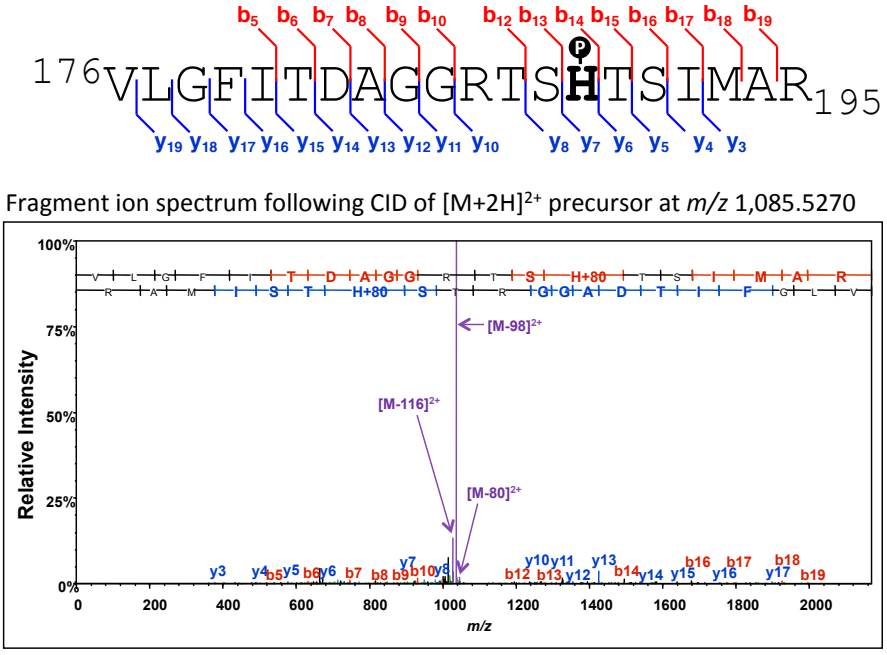
Fragment ion spectrum following CID of $[M+3H]^{3+}$ precursor at *m/z* 856.7650



Supplementary Figure 30. Detection of histidine phosphorylation on KinB by MS. Phosphorylation on the canonical histidine site (His-213) was detected and characterized by LC-MS and MS/MS analyses of enzymatically phosphorylated KinB. This result confirms our detection of phosphohistidine on KinB by Western blot (Figure 2b).

In vitro autophosphorylation of PtsI

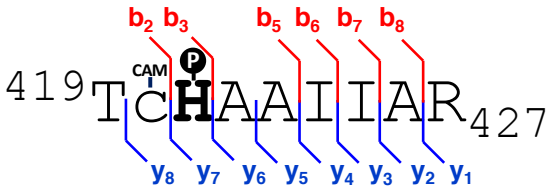
Recombinant PtsI (see expression and purification below) was diluted to a final concentration of 0.3 μM in assay buffer (25 mM sodium phosphate pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT) in a siliconized Eppendorf tube. The reaction was initiated by adding PEP to the PtsI enzyme solutions at a final concentration of 500 μM. The sample was incubated for 30 minutes at 26 ˚C. 10 μL of this sample was then analyzed by Western blot using the general protocol described above. For LC-MS and MS/MS analyses, solution containing 15 ug protein was subjected to reduction with 20 mM DTT for 30 min at RT, alkylation using 50 mM iodoacetamide for 30 min at RT in the dark, followed by quenching with 100 mM DTT. The sample was then subjected to buffer exchange into 100mM Tris, pH 8.5 and endoprotease digestion with 500 ng trypsin for 4 h at RT. The sample was then rapidly de-salted using STAGE-tips, and analyzed by nano-UPLC MS on an Orbi Elite platform using the parameters described below.



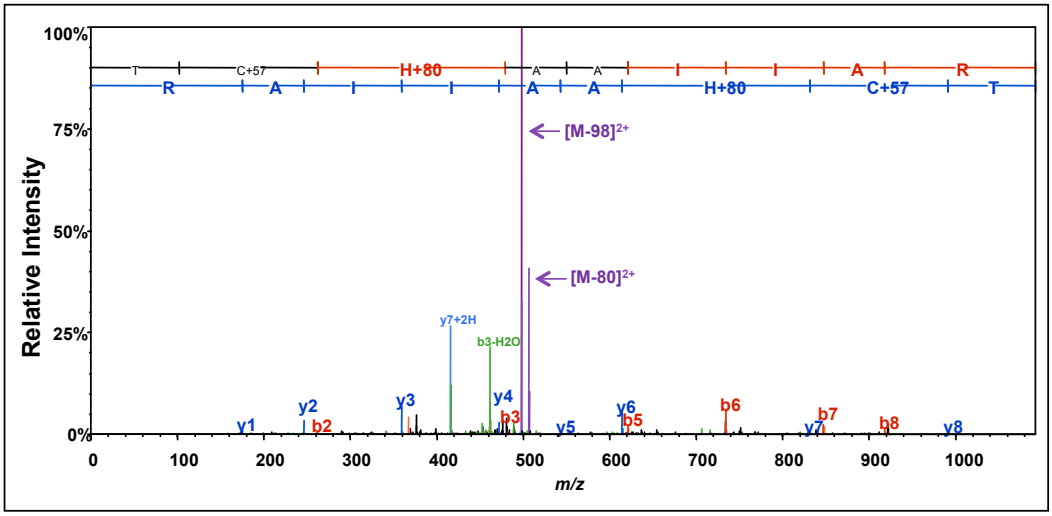
Supplementary Figure 31. Detection of histidine phosphorylation on PtsI by MS. Phosphorylation on the canonical histidine site (His-189) was detected and characterized by LC-MS and MS/MS analyses of enzymatically phosphorylated PtsI. This result confirms our detection of phosphohistidine on PtsI by Western blot (Figure 2b).

In vitro autophosphorylation of PpsA

Recombinant PpsA (see expression and purification below) was diluted to a final concentration of 0.3 μM in assay buffer (100 mM Tris (pH 8.0), 5 mM $MgCl_2$) in a siliconized Eppendorf tube. The reaction was initiated by adding ATP to the PpsA enzyme solutions at a final concentration of 10 μM. The sample was incubated for 10 minutes at 26 ˚C. 10 μL of this sample was then analyzed by Western blot using the general protocol described above. For LC-MS and MS/MS analyses, solution containing 5 ug protein was subjected to reduction with 20 mM DTT for 30 min at RT, alkylation using 50 mM iodoacetamide for 30 min at RT in the dark, followed by quenching with 100 mM DTT. The sample was then subjected to buffer exchange into 20mM Tris, pH 8.5 in 1 M urea and endoprotease digestion with 1 ug trypsin for 4 h at RT. The sample was then rapidly de-salted using STAGE-tips, and analyzed by nano-UPLC MS on an Orbi Elite platform using the parameters described below.



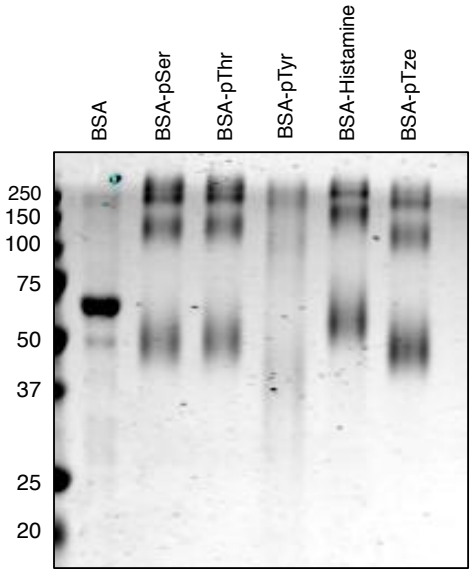
Fragment ion spectrum following CID of $[M+2H]^{2+}$ precursor at *m/z* 546.7557



Supplementary Figure 32. Detection of histidine phosphorylation on PpsA by MS. Phosphorylation on the canonical histidine site (His-421) was detected and characterized by LC-MS and MS/MS analyses of enzymatically phosphorylated PpsA. This result confirms our detection of phosphohistidine on PpsA by Western blot (Figure 2b).

Conjugation of pTze or phosphoamino acids to BSA

Conjugation of BSA to pTze (BSA-pTze), histamine (BSA-Histamine), phosphotyrosine (BSA-pTyr), phosphoserine (BSA-pSer), and phosphothreonine (BSA-pThr) was performed using glutaraldehyde.[12]



Supplementary Figure 33. SDS-PAGE of BSA-conjugates. Conjugates run smeary on a SDS-PAGE gel, indicating it is a complex mixture.

Generation of τ-pHis rabbit polyclonal antibodies

Rabbit polyclonal antiserum recognizing τ-pHis was generated using pTze as the hapten conjugated to KLH using glutaraldehyde as the crosslinker (21st Century Biochemicals, Marlboro, Massachusetts).

Supplementary Note 4

General notes for assays with anti-pHis antibody

Purified pHis antibodies were stored at 4°C for at least three months without any detected loss in binding affinity. Tris buffered saline pH 8.5 was used in all pHis antibody assays. Phosphate-based buffers or lower pH buffers led to weaker antibody binding. Also, we used a basic gel-loading buffer with a pH of 8.5 and we did *NOT* boil the samples after mixing with loading buffer prior to loading them onto a gel. We found that boiling of samples in loading buffer led to significant/complete loss of phosphohistidine. Tris-HCl gels were preferred over bis-tris gels due to the more basic running buffer (pH 8.3 vs. pH 7.3). We also found that Bis-tris gels heat up more than Tris-HCl gels, which may affect pHis levels. 3% BSA was sufficient as a blocking agent in ELISAs and Western blots. For this antibody, non-fat dry milk should not be used since we found that it causes significant loss of signal.

ELISA of crude anti-pHis antiserum

Buffers used in ELISA Protocol
Coating Buffer: 0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6
Wash Buffer: 25 mM Tris, pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% v/v Tween 20

BSA and BSA-pHis in 1 mg/mL stock solutions were diluted 10-fold into coating buffer and 50 μL of each sample was added to a Nunc-Immuno Maxisorp 96-well plate to give 5 μg protein/well. The plate was incubated for 2 hours at room temperature on a nutator. The wells were then washed three times with wash buffer. The wells were then blocked by adding 1% BSA in wash buffer at 50 μL/well and incubating for 45 minutes at room temperature on a nutator. The blocking solution was then removed from each well. Crude antiserum from the pre-immune, bleed 2, 6 and 10 samples were diluted 1000-fold in wash buffer and then added to the wells at 50 μL/well and incubated for 45 minutes at room temperature on a nutator. The wells were then emptied and washed three times with wash buffer. Goat-anti rabbit-HRP secondary antibody was diluted 5000-fold into wash buffer and added to the wells at 50 μL/well and incubated for 45 minutes at room temperature on a nutator. The wells were emptied and washed four times with wash buffer. 50 μL of Ultra TMB ELISA substrate was added to each well and incubated for 5 minutes at room temperature followed by addition of 50 μL of 2 N H_2SO_4 to quench the reaction. The absorbance at 450 nm was measured on a Spectramax M3 plate reader.

Affinity purification of anti-pHis polyclonal antiserum

Buffers used in affinity purification:
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
Elution Buffer: 100 mM Glycine pH 2.5

Affinity resin consisting of phosphorylated BSA immobilized onto agarose was prepared by covalently attaching BSA to agarose beads using SulfoLink Resin according to the manufacturers instructions. Chemical phosphorylation of the immobilized BSA-agarose resin was performed by pre-equilibrating 1 mL of resin in TBS followed by incubation of the resin in 500 mM potassium phosphoramidate in TBS overnight at room temperature with mixing on a nutator. The resin was then washed with 4 column volumes of TBS. 600 μL of crude polyclonal pHis antiserum diluted 5-fold into TBS was then added to the resin and incubated for 1 hour at room temperature on a nutator. The flow through was collected and the resin was washed with 6 column volumes of TBS. Antibodies were eluted from the column in 1 mL fractions with elution buffer. After elution off of the column, the elution fractions were immediately neutralized by adding 100 μL of 1 M Tris (pH 8.0). Fractions containing pHis antibody (determined by SDS-PAGE and ELISA, see below) were pooled together and the concentration of antibody was determined to be 0.03 mg/ml by measuring the A_{280} (A_{280} extinction coefficient assumed to be 14 for a 10 mg/ml antibody solution).

SDS-PAGE of affinity purification fractions

10 μL of the input and flow through fractions, and 25 μL of the wash and elution fractions were mixed 1:1 with 2x loading buffer (80 mM Tris pH 6.8, 20% v/v Glycerol, 2% w/v SDS, 0.04% w/v Bromophenol Blue, 4% v/v 2-mercaptoethanol) and boiled at 100 °C for 10 minutes. The samples were loaded onto a Criterion XT 12% Bis-Tris polyacrylamide gel and the proteins were resolved by electrophoresis by running at 175V for 1 hour. The gel was stained with Coomassie and imaged on a LI-COR Odyssey Infrared Imager.

ELISA of affinity purification fractions

Buffers used in ELISA Protocol
Coating Buffer: 0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6
Wash Buffer: 25mM Tris pH 8.5, 137 mM NaCl, 2.7mM KCl, 0.1% v/v Tween 20

BSA, BSA-pHis, BSA-pTze and BSA-pTyr in 1 mg/mL stock solutions were diluted 10-fold into coating buffer and 50 μL of each sample was added to a Nunc-Immuno Maxisorp 96-well plate to give 5 μg protein/well. The plate was incubated for 2 hours at room temperature on a nutator. The wells were then washed three times with wash buffer. The wells were then blocked by adding 1% BSA in wash buffer at 50 μL/well and incubating for 45 minutes at room temperature on a nutator. The blocking solution was then removed from each well. The input, flow through, wash, and elution fractions were diluted 200-fold into wash buffer and then added to the wells at 50 μL/well and incubated for 45 minutes at room temperature on a nutator. The wells were then processed following the exact protocol described above for ELISA of crude antiserum.

ELISA of pHis antibody against BSA-pHis and H4 H75A-pHis

Buffers used in ELISA Protocol
Coating Buffer: 0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% v/v Tween 20

BSA, BSA-pHis, H4 H75A, and H4(H75A)-pHis in 1 mg/mL stock solutions were diluted 10-fold into coating buffer and 50 μL of each sample was added to a Nunc-Immuno Maxisorp 96-well plate to give 5 μg protein/well. As controls, BSA-pHis and H4(H75A)-pHis were pre-treated with 0.2N HCl or 500 mM hydroxylamine (HA) for 2 hours at 45 °C and then added the plate at 5 μg protein/well. The acid treated samples were neutralized with 2 N NaOH prior to adding to wells. The ELISA was then carried out in an analogous manner to the procedure described above for the affinity purification fractions. Briefly, after adding the proteins in coating buffer, the plate was incubated for 2 hours at room temperature and then washed with wash buffer. The wells were then blocked by adding 1% BSA in wash buffer. The blocking solution was then removed from each well and affinity-purified antibody was diluted 250-fold into wash buffer and then added to the wells and incubated for 45 minutes at room temperature. As controls, 2 mM histidine or phosphohistidine were included with the affinity-purified antibody incubation step. The wells were then washed with wash buffer. Goat-anti rabbit-HRP secondary antibody diluted 5000-fold into wash buffer was then added to the wells and incubated for 45 minutes at room temperature. The wells were washed with wash buffer and then treated with Ultra TMB ELISA substrate. 2 N H_2SO_4 was then added to each well and the absorbance at 450 nm was measured on a Spectramax M3 plate reader.

ELISA of pHis antibody against phosphoamino acids

Buffers used in ELISA Protocol
Coating Buffer: 0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% v/v Tween 20

BSA-pTyr, BSA-pSer, BSA-pThr in 1 mg/mL stock solutions were individually diluted 10-fold into coating buffer and 50 μL of each sample was added to a Nunc-Immuno Maxisorp 96-well plate to give 5 μg protein/well. The ELISA was then carried out in an analogous manner to the procedure described above for the ELISA of crude antiserum.

General procedure for Western blot of pHis-containing proteins

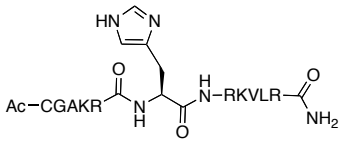
Buffers used in SDS-PAGE and Western blots
Tris-Glycine running buffer: 25 mM Tris base, 192 mM glycine, pH 8.3.
Towbin buffer: 25 mM Tris base, 192 mM glycine, pH 8.3, 10 % (v/v) methanol.
Wash Buffer: 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween-20, pH 8.5

Samples were diluted into 4x basic loading buffer (160 mM Tris, pH 8.5, 40% (v/v) glycerol, 4% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 8% (v/v) BME) and resolved by SDS-PAGE. Samples were loaded onto a Tris-HCl gel (15% or 10.5-14% acrylamide) or 12% Bis-Tris gel and run for 10 min at 120 V and then for 50 min at 180 V.

The resolved proteins were electroblotted onto a PVDF membrane in Towbin buffer at 100 V for 60 minutes. The membrane was blocked with 3% BSA in wash buffer for 1 h at RT. The membrane was then incubated with affinity-purified anti-pHis antibody diluted 1:100 in wash buffer with 3% BSA for 1 h at RT. The membrane was washed with wash buffer (3 x 5 min) and then incubated with goat anti-rabbit IgG-HRP conjugate (diluted 1:5000 in wash buffer with 3% BSA) or Protein A-HRP (diluted 1:8000 in wash buffer with 3% BSA) for 1 h at RT. The membrane was washed with wash buffer (3 x 5 min), drained, and was incubated with ECL chemiluminescence solution for 1 min at RT. The chemiluminescence from the membrane was imaged using ImageQuant LAS 4000. The membrane was washed with water and stained with Coomassie blue or colloidal gold (Bio-Rad) for the visualization and quantification of total protein.

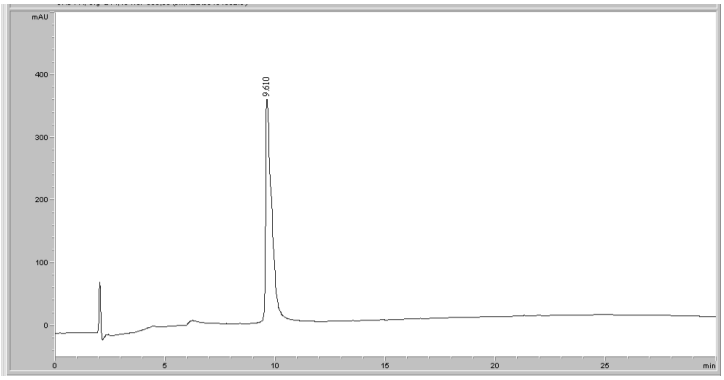
Peptide dot blots comparing pHis and pTyr affinity

Histone H4-pTyr18 peptide: Ac-CGARKRpYRKVLR-NH_2

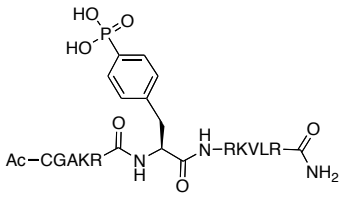


ESI-MS: 1363.80 (calculated); 1364.34 ± 0.15 (observed).

HPLC retention time: 9.61 min (C18 Vydac column, 0-73% solution B over 30 min, 1 mL/min)

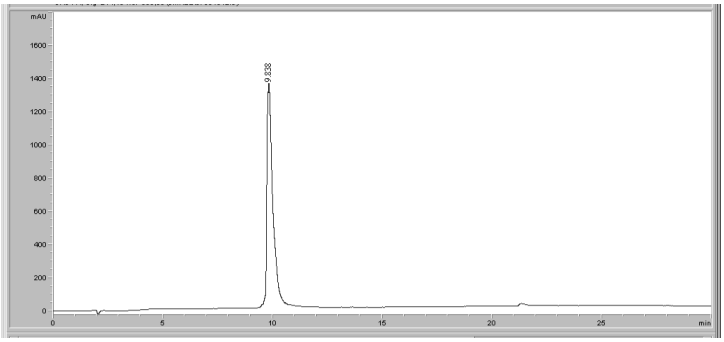


Histone H4-His18 peptide: Ac-CGARKRHRKVLR-NH_2



ESI-MS: 1470.68 (calculated); 1471.02 ± 1.01 (observed).

HPLC retention time: 9.84 min (C18 Vydac column, 0-73% solution B over 30 min, 1 mL/min)



Both peptides were synthesized as previously reported.[5] The H4-His18 peptide (1 mM solution) was chemically phosphorylated at the histidine with potassium phosphoramidate (final 100 mM) overnight at RT. The 1 mM stock of each peptide was serially diluted 3-fold with ddH_2O to give solutions of 333, 111, 37, 12, 4, 1.3, 0.4 μM. Each solution was spotted onto two nitrocellulose membranes, and the membranes were air-dried. One membrane was stained with colloidal gold, dried, and scanned. The other membrane was analyzed by Western blot with affinity-purified pHis antibody following the general Western blot procedure above. The signal intensity from the blot was measured using the ImageJ software.

Western blotting using anti-pTyr antibodies

Phosphotyrosine molecular weight marker (2 μL), EGF-stimulated A431 cell lysate (20 μL), and phosphorylated and non-phosphorylated Histone H4(H75A) and H4(H18A) (0.1 μg), and KinB (1 μg) proteins diluted 4-fold with 4x basic loading buffer (160 mM Tris, pH 8.5, 40% (v/v) glycerol, 4% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 8% (v/v) BME) were loaded onto a 15% Tris-HCl gel and run for 75 min at 150 V. Electroblotting of the proteins to a 0.2 μm PVDF membrane and probing of the membrane with anti-pHis or anti-pTyr antibody were carried out as described in the general Western blot procedure described above except that membranes imaged with anti-pTyr antibodies were done so with the 4G10 anti-pTyr antibody monoclonal antibody (1:500 dilution in wash buffer with 3% BSA) or anti-pTyr antibody polyclonal antibody (1:5000 dilution in wash buffer with 3% BSA) in the primary antibody incubation step.

Supplementary Note 5

Cloning and expression of His_6-tagged DhaM

Sequence of His_6-DhaM

MGSSHHHHHHGENLYFQGMVNLVIVSHSSRLGEGVGELARQMLMSDSCKIAIAAGIDDPQNPIGTDAVKVMEAIESVADADHVLVMM
DMGSALLSAETALELLAPEIAAKVRLCAAPLVEGTLAATVSAASGADIDKVIFDAMHALEAKREQLGLPSSDTEISDTCPAYDEEARSLA
VVIKNRNGLHVRPASRLVYTLSTFNADMLLEKNGKCVTPESINQIALLQVRYNDTLRLIAKGPEAEEALIAFRQLAEDNFGETEEVAPPT
LRPVPPVSGKAFYYQPVLCTVQAKSTLTVEEEQDRLRQAIDFTLLDLMTLTAKAEASGLDDIAAIFSGHHTLLDDPELLAAASELLQHEH
CTAEYAWQQVLKELSQQYQQLDDEYLQARYIDVDDLLHRTLVHLTQTKEELPQFNSPTILLAENIYPSTVLQLDPAVVKGICLSAGSPV
SHSALIARELGIGWICQQGEKLYAIQPEETLTLDVKTQRFNRQG

Primer A:

5'-CATCATCATCATCACGGCGAGAACCTGTATTTTCAGGGCATGGTAAACCTGGTCATAGTTTCACATAGCAGC-3'

Primer B:

5'-CACCAGGCCGCTGTTGAAAGCAGCTTAACCCTGACGGTTGAAACGTTGCG-3'

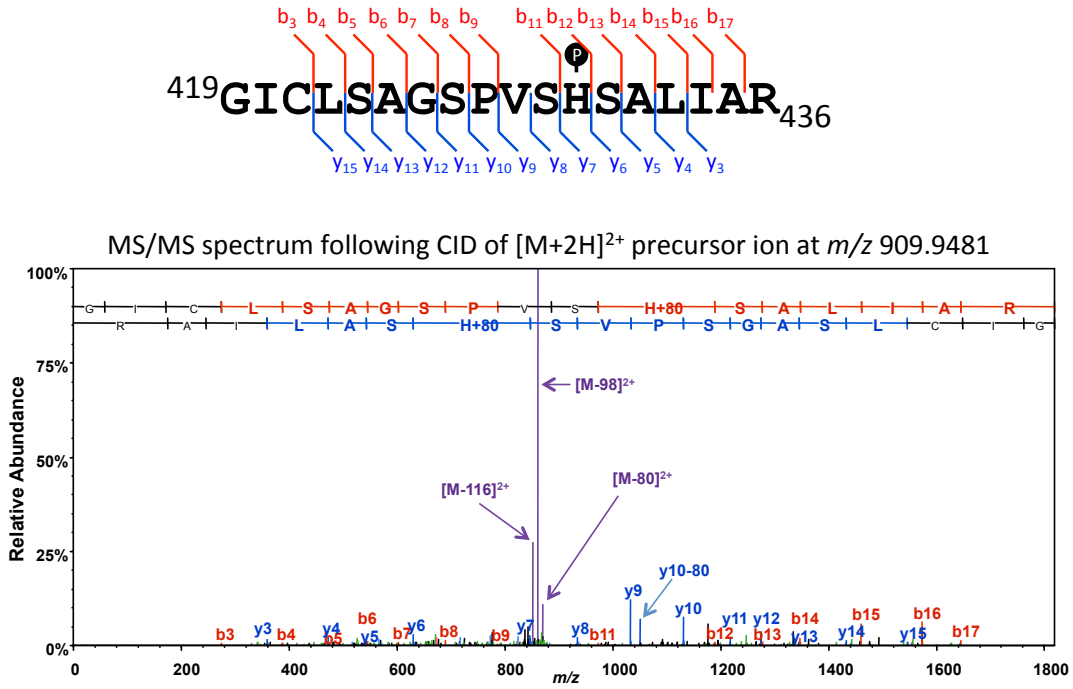
The plasmid pET-dhaM was prepared in two steps by overlap-extension PCR.[13] First, the DhaM gene was amplified from *E. coli* DNA (extracted using Trizol according to manufacturer's instructions) by Phusion polymerase using primers A and B that contain 5' and 3' sequences that overlap with regions of a modified pET-SUMO plasmid.[14] The resulting product DNA was purified by agarose gel electrophoresis followed by gel extraction using QIAquick® gel extraction kit according to the manufacturer's instructions.

In the second step, the DhaM gene product was inserted into a modified pET-SUMO plasmid[14] that contains a TEV-protease cleavage site between the hexa-histidine tag and the protein sequence to be inserted, using Phusion polymerase. Then the template plasmid was globally digested using DpnI at 37 ˚C for 1 h. The resulting product plasmid was used to transform *E. coli* DH5α cells by heat shock (42 ˚C, for 45 seconds). The transformed bacteria were grown in LB media (no antibiotics) for 1 h at 37 ˚C and plated onto a kanamycin-containing LB-agar plate. The plate was incubated at 37 ˚C overnight, and the colonies were individually picked and inoculated into 5 mL LB with kanamycin (50 μg/mL). The bacteria were grown overnight at 37 ˚C in a shaker and the plasmid DNA was extracted using QIAprep spin Miniprep kit according to the manufacturer's instructions. The obtained pET-dhaM plasmids were sequenced and used to transform *E. coli* BL21(DE3) cells.

To express the tagged DhaM, *E. coli* BL21 cells transformed with pET-DhaM were grown in 100 mL of LB medium containing kanamycin (50 μg/mL) at 37 ˚C until OD600 = 0.6. Then the expression was induced by addition of 0.1 mM IPTG for 30 min. After harvesting the cells by centrifugation, the cell pellet was resuspended in 1 mL of cold lysis buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 8.5) supplemented with Complete® protease inhibitor cocktail and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich, each diluted 100-fold from stock solution). The cells were lysed by sonication on ice (45% power, 3 x 5 second pulses) and centrifuged (15000 xg, 10 min) at 4 ˚C to remove the cell debris.

For the Ni-NTA affinity purification, 100 μL of the lysate was loaded onto 50 μL of Ni-NTA resin and incubated at 4 ˚C for 30 min. After discarding the flow-through, the column was washed with 5 column volumes (CV) of lysis buffer and eluted with 2 CV of elution buffer (lysis buffer with 200 mM EDTA).

The lysate and fractions from the Ni-NTA column were treated with 4x gel loading buffer (160mM Tris, pH 8.5, 40% (v/v) glycerol, 4% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 8% (v/v) 2-mercaptomenthanol). After the addition of the gel-loading buffer, an aliquot was taken out, treated with HA (final concentration 500 mM), and incubated at 37 ˚C for 1 h. Then the samples were resolved by SDS-PAGE and analyzed by Western blotting following the general Western blot procedure described above. For LC-MS and MS/MS analyses, solution containing 10 ug protein was subjected to reduction with 20 mM DTT for 30 min at RT, alkylation using 50 mM iodoacetamide for 30 min at RT in the dark, followed by quenching with 100 mM DTT. The sample was then subjected to buffer exchange into 20mM Tris, pH 8.5 in 1 M urea and endoprotease digestion with 1 ug trypsin for 4 h at RT. The sample was then rapidly de-salted using STAGE-tips, and analyzed by nano-UPLC MS on an Orbi Elite platform using the parameters described below.



Supplementary Figure 34. Detection of histidine phosphorylation on DhaM by MS. Phosphorylation on the canonical histidine site (His-430) was detected and characterized by LC-MS and MS/MS analyses of recombinantly expressed and endogenously phosphorylated DhaM. This result confirms our detection of phosphohistidine on DhaM by Western blot (Supplementary Figure 12).

Cloning and expression of His_6-tagged PtsI

Sequence of His_6-PtsI

MGSSHHHHHHGENLYFQGMISGILASPGIAFGKALLLKEDEIVIDRKKISADQVDQEVERFLSGRAKASAQLETIKTKAGETFGEEKEAI
FEGHIMLLEDEELEQEIIALIKDKHMTADAAAHEVIEGQASALEELDDEYLKERAADVRDIGKRLLRNILGLKIIDLSAIQDEVILVAADLTP
SETAQLNLKKVLGFITDAGGRTSHTSIMARSLELPAIVGTGSVTSQVKNDDYLILDAVNNQVYVNPTNEVIDKMRAVQEQVASEKAELA
KLKDLPAITLDGHQVEVCANIGTVRDVEGAERNGAEGVGLYRTEFLFMDRDALPTEEEQFAAYKAVAEACGSQAVIVRTMDIGGDKEL
PYMNFPKEENPFLGWRAIRIAMDRREILRDQLRAILRASAFGKLRIMFPMIISVEEVRALRKEIEIYKQELRDEGKAFDESIEIGVMVETPA
AATIARHLAKEVDFFSIGTNDLTQYTLAVDRGNDMISHLYQPMSPSVLNLIKQVIDASHAEGKWTGMCGELAGDERATLLLLGMGLDEF
SMSAISIPRIKKIIRNTNFEDAKVLAEQALAQPTTDELMTLVNKFIEEKTIC

Primer C:

5'-CATCATCATCATCACGGCGAGAACCTGTATTTTCAGGGCATGATTTCAGGCATTTTAGCATCCCCG -3'

Primer D:

5'-CACCAGGCCGCTGTTGAAAGCAGCTTAGCAGATTGTTTTTTCTTCAATGAACTTGTTAACC-3'

The plasmid pET-ptsI was prepared analogously to pET-dhaM. Briefly, the ptsI gene was amplified using primers C and D, and the resulting product was inserted into a modified pET-SUMO plasmid. To express the tagged PtsI, *E. coli* BL21 cells were transformed with pET-ptsI and grown in 1 L of LB medium containing kanamycin (50 μg/mL) at 37 ˚C until OD600 = 0.6. Then the expression was induced by addition of 0.3 mM IPTG for 1 hour. After harvesting the cells by centrifugation (5000 xg, 20 min, 4 ˚C) the cell pellet was resuspended in 10 mL of cold lysis buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 8.5) supplemented with Complete® protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich, each diluted 100-fold from stock solution). The cells were lysed

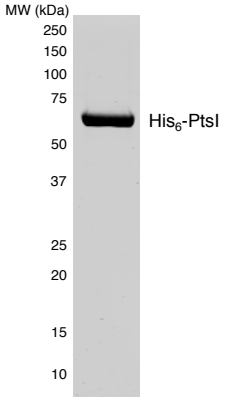
by sonication on ice (50% power, 10 x 10 second pulses) and then centrifuged (15000 xg, 10 min) at 4 ˚C to remove the cell debris.

Purification of PtsI for pHis Detection by Western Blot and MS Analysis:

Expression of PtsI was performed according the method described above for DhaM. Western blot analysis of cell lysate and Ni-NTA purified protein were carried out following the general Western blot procedure described above. The lysate and fractions from the Ni-NTA column were treated with a modified 4x gel-loading buffer (160mM Tris, pH 8.5, 40% (v/v) glycerol, 4% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 8% (v/v) 2-mercaptomenthanol). After the addition of the gel-loading buffer, an aliquot was taken out, treated with hydroxylamine (final concentration 500 mM), and incubated at 37 ˚C for 1 h. Then the samples were resolved by SDS-PAGE and analyzed by Western blot following the general Western blot procedure described above. A fresh aliquot of the elution fraction was also subjected to LC-MS analyses (*vide infra*).

Large Scale Purification of De-Phosphorylated PtsI for Immunoassays:

5 mL of the lysate was treated with 1 μM PHPT1 (prepared as described below) for 1 hour at 37 ˚C. This step was carried out to dephosphorylate the PtsI sample. The sample was then loaded onto 1 mL of Ni-NTA resin and incubated at room temperature for 10 min. After discarding the flow-through, the column was washed with 5 column volumes (CV) of lysis buffer containing 10 mM imidazole and then eluted with 3 CV of elution buffer consisting of lysis buffer with 25 mM, 50 mM, 100 mM, and 200 mM imidazole. Fractions containing purified protein (as determined by SDS-PAGE analysis) were pooled together and dialyzed into buffer containing: 25 mM phosphate Buffer pH 7.5, 500 mM NaCl, 1 mM BME. Final concentration of the protein was determined to be 24 μM by UV A_{280nm} and by the Bradford assay. Aliquots of the protein were flash frozen and stored at -80˚C until used in assays. See Supplementary Figure 33 for purity analysis of PtsI.



Supplementary Figure 35. SDS-PAGE analysis of purified His_6-PtsI.

Cloning and expression of His_6-PpsA

Sequence of His_6-PpsA

MRGSHHHHHHTDPALRASNNGSSPLVLWYNQLGMNDVDRVGGKNASLGEMITNLSGMGVSVPNGFATTADAFNQFLDQSGVNQRI
YELLDKTDIDDVTQLAKAGAQIRQWIIDTPFQPELENAIREAYAQLSADDENASFAVRSSATAEDMPDASFAGQQETFLNVQGFDAVLV
AVKHVFASLFNDRAISYRVHQGYDHRGVALSAGVQRMVRSDLASSGVMFSIDTESGFDQVVFITSAWGLGEMVVQGAVNPDEFYVH
KPTLAANRPAIVRRTMGSKKIRMVYAPTQEHGKQVKIEDVPQEQRDIFSLTNEEVQELAKQAVQIEKHYGRPMDIEWAKDGHTGKLFI
VQARPETVRSRGQVMERYTLHSQGKIIAEGRAIGHRIGAGPVKVIHDISEMNRIEPGDVLVTDMTDPDWEPIMKKASAIVTNRGGRTCH
AAIIARELGIPAVVGCGDATERMKDGENVTVSCAEGDTGYVYAELLEFSVKSSSVETMPDLPLKVMMNVGNPDRAFDFACLPNEGVG
LARLEFIINRMIGVHPRALLEFDDQEPQLQNEIREMMKGFDSPREFYVGRLTEGIATLGAAFYPKRVIVRLSDFKSNEYANLVGGERYE
PDEENPMLGFRGAGRYVSDSFRDCFALECEAVKRVRNDMGLTNVEIMIPFVRTVDQAKAVVEELARQGLKRGENGLKIIMMCEIPSNA
LLAEQFLEYFDGFSIGSNDMTQLALGLDRDSGVVSELFDERNDAVKALLSMAIRAAKKQGKYVGICGQGPSDHEDFAAWLMEEGIDSL
SLNPDTVVQTWLSLAELKK

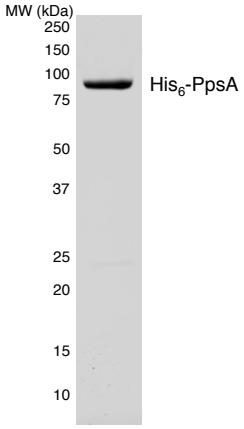
Primer C

5'- CGG ATC CGG CCC TGA GGG CC ATG TCC AAC AAT GGC TCG TCA CCG -3'

Primer D

5'- GA CCC TTA GCG GCC GCA TAG GCC TTA TTT CTT CAG TTC AGC CAG GCT TAA CCA GG -3'

The plasmid pCA24N-PpsA was prepared analogously to pET-DhaM. Briefly, the ppsA gene was amplified from *E. coli* genomic DNA using primers C and D, and the resulting product was inserted into a modified pCA24N plasmid. To express the tagged PpsA, *E. coli* BL21 cells were transformed with pCA24N-ppsA. Expression of PpsA was performed according the method described above for DhaM. Western blot analysis of cell lysate and Ni-NTA purified protein were carried out following general Western blot procedure described above. See Supplementary Figure 34 for purity analysis of PpsA.



Supplementary Figure 36. SDS-PAGE analysis of purified His_6-PpsA.

Preparation of Phosphohistidine Phosphatase 1 (PHPT1)

PHPT1 was cloned and expressed as PHPT1-GyrA-His_6 and then thiolyzed at the PHPT1-GyrA thioester bond to generate PHPT1 with a C-terminal carboxylate.

Sequence of PHPT1-GyrA-His_6:

MAVADLALIPDVDIDSDGVFKYVLIRVHSAPRSGAPAAESKEIVRGYKWAEYHADIYDKVSGDMQKQGCDCECLGGG
RISHQSQDKKIHVYGYSMAYGPAQHAISTEKIKAKYPDYEVTWANDGYCITGDALVALPEGESVRIADIVPGARPNSDN
AIDLKVLDRHGNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLVDVAGVPTLLWKLIDEIKPGDYAVIQRSAFS
VDCAGFARGKPEFAPTTYTVGVPGLVRFLEAHHRDPDAQAIADELTDGRFYYAKVASVTDAGVQPVYSLRVDTADHA
FITNGFVSHAHHHHHH

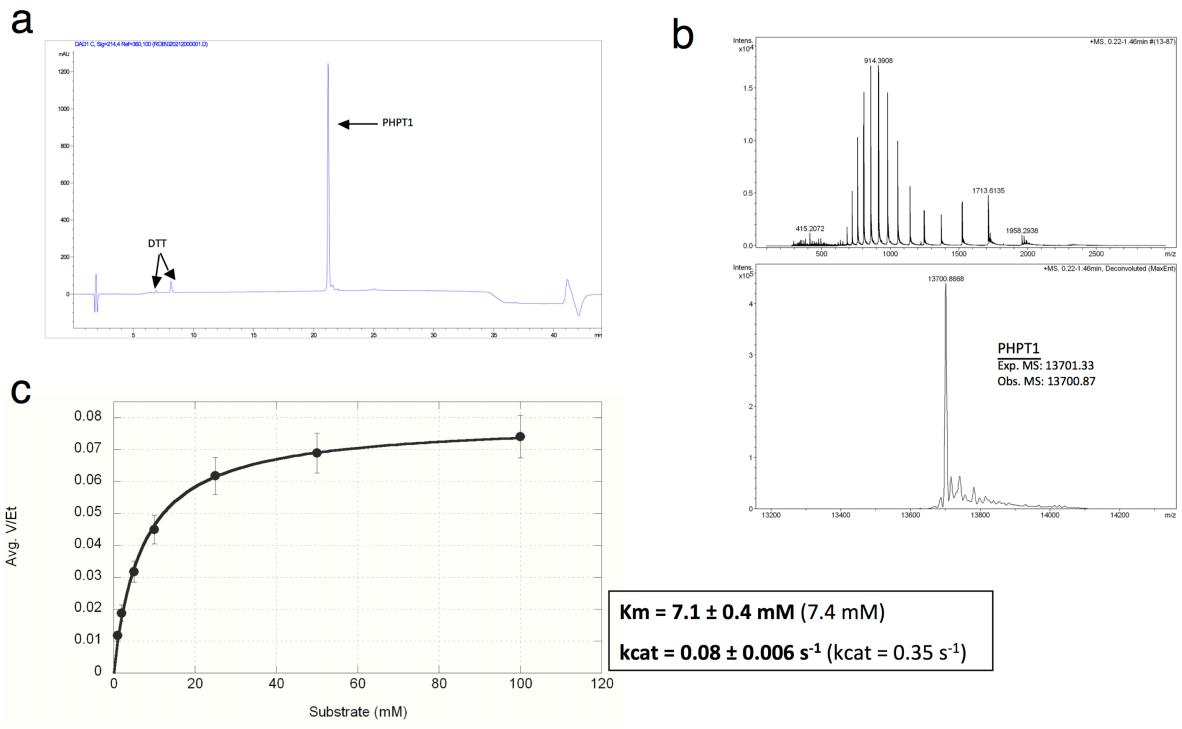
PHPT1-GyrA-His_6 cloning and expression:

The PHPT1 gene was purchased from Genewiz (South Plainfield, NJ) and amplified by PCR using Phusion Polymerase and primers with overhangs that annealed to the desired insertion point into a modified pTXB1 vector (the modified pTXB1 vector contains a His_6 tag and stop codon between the GyrA and the chitin binding domain). The PHPT1 gene was inserted into the modified pTXB1 plasmid using overlap extension PCR.[13]

E. coli BL21(DE3) cells transformed with the pTXB1-PHPT1-GyrA-His_6 plasmid were grown in 1 L of LB containing 100 μg/mL of ampicillin at 37 °C until OD_{600} = 0.6. The cells were induced by addition of

0.5 mM IPTG for 3 hours at 37 °C. After harvesting the cells by centrifugation (10,500 rcf, 30 min), the cell pellets were transferred to 50 mL conical tubes with 5 mL of Lysis Buffer (50 mM phosphate pH 8, 300 mM NaCl, 5 mM imidazole) and stored at -80°C until further use. The cell pellets were resuspended by adding an additional 15 mL of Lysis Buffer supplemented with Complete® protease inhibitor cocktail. Cells were lysed by sonication (35% power, 8 x 15 second pulses). The soluble fraction was recovered by centrifugation (35,000 rcf, 30 min). The soluble fraction was mixed with 2 mL of Ni-NTA resin and incubated at 4 °C for 30 minutes. After incubation, the slurry was loaded onto a fritted column. After discarding the flow-through, the column was washed with 5 column volumes (CV) of Lysis Buffer, 5 CV of Wash Buffer 1 (Lysis Buffer with 20 mM imidazole), and 5 CV of Wash Buffer 2 (Lysis Buffer with 50 mM imidazole). The protein was eluted with Ni Column Elution Buffer (Lysis Buffer with 250 mM imidazole) in four 1.5 CV elution fractions. The wash and elution fractions were analyzed by SDS-PAGE and fractions containing the desired protein were pooled together to give a crude protein yield of 60 mg.

6 mL of 5 mg/mL crude PHPT1-GyrA-His_6 in 50 mM phosphate pH 8, 300 mM NaCl, 250 mM imidazole was treated with 1 mM TCEP and 200 mM MESNA and incubated at room temperature for 7 hours. The sample was then transferred to a 3 mL dialysis cassette (MWCO: 7000) and dialyzed into 1 L 50 mM phosphate pH 8, 300 mM NaCl, 250 mM imidazole overnight at room temperature. The sample was then removed from the dialysis cassette, treated with 10 mM TCEP, and then placed into another dialysis cassette (MWCO: 7000) and dialyzed into 50 mM phosphate pH 8, 300 mM NaCl, 5 mM imidazole, 0.5 mM DTT for 2 hours. Fresh dialysis buffer was replaced after 2 hours and the dialysis was repeated for a second time. After dialysis, the protein sample was mixed with 2 mL of Ni-NTA resin and incubated at 4 °C for 20 minutes. After incubation, the slurry was loaded onto a fritted column and the flow through was collected. The column was washed with 4 x 2 mL wash buffer (50 mM phosphate pH 8, 300 mM NaCl, 5 mM imidazole, 0.5 mM DTT). The flow-through and wash fractions were combined and concentrated to 3 mL total volume using a Centricon® concentrator (MWCO: 3000). The crude PHPT1 was then purified by size-exclusion chromatography (SEC) on an S75 10/300 gel filtration column (3 x 1 mL injections) and eluted over 1.35 column volumes at 0.5 mL/min flow-rate in freshly prepared buffer consisting of 50 mM Tris pH 8, 50 mM NaCl, 1 mM DTT. SEC fractions were analyzed by SDS-PAGE, and the purest fractions were pooled and analyzed by analytical RP-HPLC (0-73% solvent B over 1mL/min, retention time = 21.2 min), and mass spectrometry (Supplementary Figure 35). The final concentration of purified PHPT1 was determined by UV A_{280nm} and by the Bradford assay to be 60 μM. The protein was aliquoted, flash frozen and stored at -80 °C. The activity of PHPT1 was verified using a previously reported colorimetric assay that employs para-nitrophenyl phosphate as substrate.[15] Curve fitting to determine and k_{cat} values was performed using Kaleidagraph (see Supplementary Fig. 35).



Supplementary Figure 37. Analysis of PHPT1 purity and enzyme activity.
(a) RP-HPLC analysis of PHPT1 (0-73% solvent B over 30 min). (b) ESI-MS analysis of the major HPLC chromatogram peak from (a) corresponding to PHPT1. c) Michaelis-Menten plot of PHPT1-catalyzed hydrolysis of para-Nitrophenyl phosphate. K_m and k_{cat} values are reported in bold (n = 3, mean ± s.d.). These values are similar to previously reported K_m and k_{cat} values for this assay shown in parenthesis.[15]

Supplementary Note 6

Immunoprecipitation of DhaM

Buffers used for immunoprecipitation:
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
Wash Buffer: 25mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20
4x Basic Loading Buffer: 160 mM Tris pH 8.5, 40% v/v glycerol, 4% w/v SDS, 0.08% w/v Bromophenol Blue, 8% v/v 2-mercaptoethanol

Immunoprecipitation experiments were carried using anti-pHis bound to protein G agarose beads. The binding of anti-pHis antibody to protein G agarose was achieved by mixing 20 μL of protein G agarose slurry to 500 μL of TBS that contained 5 μg of affinity purified anti-pHis antibody or no antibody as a mock IP control. The samples were then incubated for 1 hour at 4 °C on a nutator. After incubation the sample was centrifuged for 10 seconds at 17,000 xg at room temperature and the supernatant was removed. The resin was then resuspended in 250 μL of wash buffer. 5 μL of DhaM overexpression lysate (obtained as described above) was diluted with 250 μL of wash buffer (25 μL of this sample was removed

as the input sample, treated with 5 μL 4x basic loading buffer and stored at -80 °C until SDS-PAGE analysis) and then added to the 250 μL of wash buffer containing the antibody-bound agarose resin. This sample was then incubated at 45 minutes at 4 °C on a nutator. After incubation, the samples were then centrifuged at 17,000 xg for 10 seconds at room temperature to precipitate the resin. The supernatant was removed and 50 μL of the supernatant was collected, flash frozen in liquid nitrogen, and then lyophilized to reduce the volume to 20 μL so that it could be loaded onto a gel. This sample (flow-through) was then treated with 5 μL of 4x loading buffer and stored on ice till SDS-PAGE. The resin was then washed four times with 200 μL TBS. To elute DhaM off of the resin, the resin was treated with 20 μL of 4x loading buffer for 2 minutes at room temp. The sample was then transferred to a fritted micro bio-spin chromatography column and centrifuged to separate the lysate from the resin. The filtrate was then collected as the elution fraction and stored on ice until SDS-PAGE analysis. Alternatively, DhaM may be eluted off of the resin by treating the resin with 25 μL of 20 mM pTze in wash buffer for 15 minutes at room temperature followed by centrifugation to collect the filtrate.

The input, flow through, and elution samples were loaded onto a 10.5-14% Tris-HCl gel and resolved by electrophoresis for 15 minutes at 120 V and then 60 minutes at minutes at 180 V. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody were both carried out as described in the general Western blot procedure above.

Immunoprecipitation of PtsI

Buffers used for immunoprecipitation:
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20

BL21(DE3) cell lysate overexpressing PtsI (obtained as described above) was immunoprecipitated following the procedure described for immunoprecipitation of DhaM with a few minor changes. Briefly, 5 μL of cell lysate was diluted into 500 μL of wash buffer and then added to Protein G agarose beads pre-treated with affinity-purified anti-pHis antibodies. The sample was incubated for 45 minutes at 4 °C. The sample was centrifuged to pellet the resin and the supernatant was discarded. The resin was washed two times with wash buffer and two times with TBS. The elution was carried out by treating the resin with 25 μL 20 mM pTze for 15 minutes at room temperature. The supernatant was then collected by centrifugation of the sample through a fritted micro bio-spin chromatography column. The input, flow through, and elution samples were analyzed by SDS-PAGE as described for DhaM. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody were both carried out as described in the general Western blot procedure above.

Selective immunoprecipitation of pTyr proteins using α-pTyr mAb

Buffers used for immunoprecipitation:
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
TBST: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20

2.0 μg of phosphorylated KinB (pKinB), DhaM (pDhaM), and PtsI (pPtsI) were combined and diluted into 250 μL in TBST buffer containing 3 μL pTyr protein ladder (Calbiochem) (25 μl was removed from each sample for analysis as the input sample by SDS-PAGE). The lysates were then added to 250 μL TBST containing 20 μL protein G resin that had been pre-bound with 20 μg anti-pTyr mAb antibody (Millipore). Binding of antibody to the protein G agarose resin was performed by incubating the resin with antibody or

TBS (mock IP control) for 1 h at 4 °C on a nutator. After adding protein mixture to the protein G resin, the samples were incubated for 1 hour at 4 °C on a nutator. The samples were centrifuged to pellet the resin and 50 μL of the supernatant was collected for analysis as the flow through sample (the volume of the flow through sample was reduced to about 20 μL by lyophilization so that it could fit into a gel lane for SDS-PAGE). The pelleted resin was then washed with 5 x 200 μL of TBST buffer. The resin was then treated with 30 μL 50 mM phosphotyrosine for 15 minutes at RT. The sample was then transferred to a micro bio-spin chromatography column and centrifuged. The filtrate was collected and split into two parts. Each of the splits was treated with 4x loading buffer and stored on ice till SDS-PAGE. To measure pTyr stability in the presence of HA, 3 μL of pTyr ladder was added to 20 μL of 1x loading buffer and treated with or without 500 mM HA for 1 h at 37 °C .

The input, flow through, and elution samples were loaded onto a 15% Tris-HCl gel and resolved by electrophoresis for 20 minutes at 110V and then 60 minutes at 180 V. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody or pTyr were both carried out as described in the general Western blot procedures above.

Supplementary Note 7

Western blot of NCM 3722 *E. coli* lysate under varying nitrogen sources

Buffers used for procedure:
Growth Medium:

Starter Culture Medium: Gutnick minimal salts[16], 2.5 mM arginine, 2 mM NH_4Cl, 0.4% w/v glucose.

Nitrogen-Limited Culture Medium: Gutnick minimal salts, 2.5 mM arginine, 0.4% w/v glucose.

Loading Buffer: 160 mM Tris pH 8.5, 40% v/v glycerol, 4% w/v SDS, 0.08% w/v Bromophenol Blue, 8% v/v 2-mercaptoethanol.
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20.

A colony of NCM 3722 cells growing on an LB agar plate was picked and transferred to 5 mL of starter culture and grown overnight to an OD600 of 1.0. 1.5 mL of the starter culture was added to 40 mL of Nitrogen-limited culture medium (starting OD600: 0.04). The cells were grown overnight at 37 °C to an OD600 of 0.6. The cells were then split into 2 x 10 mL aliquots in sterile 50 mL conical plastic tubes, and one aliquot was treated with NH_4Cl (final concentration: 10 mM). Both aliquots were then grown at 37 °C for 30 minutes. The cells were then pelleted by centrifugation (4500 xg for 5 minutes at 4 °C). The pellets were then resuspended in 400 μL loading buffer and sonicated (3 x 5 seconds at 45% power) on ice. 50 μL of lysate not treated with NH_4Cl was incubated with 500 mM hydroxylamine for 1 hour at 37 °C.

15 μL of each sample (-NH_4Cl, -NH_4Cl +HA, and +NH_4Cl) were loaded onto a 10.5-14% Tris-HCl gel and resolved by electrophoresis for 15 minutes at 120 V and then 60 minutes at minutes at 180 V. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody were both carried out as described in the general Western blot procedure above.

Western blot of NCM 3722 E. coli lysate grown with glycerol as the carbon source

Buffers used for procedure:
Growth Medium:

Starter Culture Medium: Gutnick minimal salts[16], 2.5 mM arginine, 2 mM NH_4Cl, 0.4% w/v glycerol.

Nitrogen-Limited Culture Medium: Gutnick minimal salts, 2.5 mM arginine, 0.4% w/v glycerol.

Loading Buffer: 160 mM Tris pH 8.5, 40% v/v glycerol, 4% w/v SDS, 0.08% w/v Bromophenol Blue, 8% v/v 2-mercaptoethanol
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20

A colony of NCM 3722 cells growing on an LB agar plate was picked and transferred to 5 mL of starter culture containing glycerol as the carbon source. The culture was grown overnight to an OD600 of 1.25. 1.5 mL from the starter culture was added to 40 mL of Nitrogen-limited culture medium containing glycerol as the carbon source (starting OD600: 0.04). The cells were grown overnight at 37 °C to an OD600 of 0.6. The cells were then pelleted by centrifugation (4500 xg for 5 minutes at 4 °C). The pellets were then resuspended in 400 μL loading buffer and sonicated (3 x 5 seconds at 45% power) on ice. 50 μL of lysate was incubated with 500mM hydroxylamine for 1 hour at 37 °C.

15 μL of each sample (–HA and +HA) were loaded onto a 10.5-14% Tris-HCl gel and resolved by electrophoresis for 15 minutes at 120 V and then 60 minutes at minutes at 180 V. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody were both carried out as described in the general Western blot procedure above.

Immunoprecipitation of NCM 3722 *E. coli* lysate

Buffers used for immunoprecipitation:
Growth Medium:
Starter Culture Medium: Gutnick minimal salts[16], 2.5 mM arginine, 2 mM NH_4Cl, 0.4% w/v glucose.
Nitrogen-Limited Culture Medium: Gutnick minimal salts, 2.5 mM arginine, 0.4% w/v glucose.
Lysis Buffer: Tris Buffered Saline (TBS) supplemented with Complete® protease inhibitor (Roche) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich, diluted 200-fold from stock solution).
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20

NCM 3722 cells were grown in 5 mL of starter culture containing glucose as the carbon source overnight at 37 °C to an OD of 1.25. 1.5 mL from the starter culture was added to 40 mL of Nitrogen-limited culture medium containing glucose as the carbon source (starting OD600: 0.06). The cells were grown overnight at 37 °C to an OD600 of 1.0. The cells were then pelleted by centrifugation (4500 xg for 5 minutes at 4 °C). The pellets were then resuspended in 1.2 mL lysis buffer and sonicated (3 x 5 seconds at 45% power) on ice. The lysate was then centrifuged at 17,000 xg for 10 minutes at 4 °C to remove cell debris.

50 μL of the lysate was diluted into 500 μL wash buffer. This protein mixture was then added to 30 μL of protein G agarose resin pre-bound with 30 μg anti-pHis antibody (IP sample) or protein G agarose with no antibody (mock IP sample). The antibody had been bound to the resin by incubating in 1 mL of TBS with 30 μL of protein G agarose resin for 2 hours at 4 °C. After adding the lysate to the resin, it was incubated for 45 minutes at 4 °C on a nutator and then centrifuged (10,000 xg at room temperature for 10 seconds) to pellet the resin. The supernatant was removed and the resin was then washed with 4 x 200 μL wash buffer. Bound proteins were dissociated from the resin by adding 25 μL of 20 mM pTze in wash buffer and incubating for 15 minutes at room temperature. The sample was then transferred to a micro bio-spin chromatography column, centrifuged, and the filtrate was collected as the elution fraction. The elution fraction was then treated with 5 μL 4x loading buffer and then placed on ice till SDS-PAGE analysis.

20 μL of the IP and mock IP samples were boiled at 100 ˚C and loaded onto a 12% Bis-Tris Gel and resolved by electrophoresis for 60 minutes at 165 V. The gel region between 70 kDa to 100 kDa was excised from the gel and submitted for proteomic analysis by the Proteomics Resource Center at the Rockefeller University. (Note: Boiling of these samples precluded the detection and analysis of pHis-containing peptides by mass spectrometry. Thus, this analysis primarily focused on the identification of immunoprecipitated proteins, not necessarily on the detection of actual pHis sites on these proteins.)

Fractionation of NCM 3722 *E. coli* lysate

Lysis Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, supplemented with phosphatase inhibitor cocktail 2 and 3 (1:100 dilution, Sigma) and Complete® protease inhibitor cocktail (1 tablet/50 mL, Roche).
Growth Medium:

Starter Culture Medium: Gutnick minimal salts[16], 2.5 mM arginine, 2 mM NH_4Cl, 0.4% w/v glucose.

Nitrogen-Limited Culture Medium: Gutnick minimal salts, 2.5 mM arginine, 0.4% w/v glucose.

A culture of NCM 3722 was grown overnight to an OD600 of ~1.2. 1.2 mL from the starter culture was added to 60 mL of Nitrogen-limited culture medium containing glucose or glycerol as the carbon source (starting OD600: 0.06). The cells were grown overnight at 37 °C to an OD600 of 0.4. The cells were then pelleted by centrifugation (4500 xg for 10 minutes at 4 °C). The pellets were then resuspended in 1000 μL lysis buffer and sonicated (3 x 5 seconds at 45% power) on ice. The lysates were spun down (10000 xg, 10 min) to remove the cell debris and DNA. The supernatant was collected. 60 μL of the lysate was taken out and treated with 20 μL of 4x SDS loading buffer. 36 μL of the SDS-treated lysate was treated with 4 μL of 5 M hydroxylamine at 37 ˚C for 1 h.

The rest (1 mL) of lysate (pH 7.5) was pH adjusted to 8.5 and ammonium sulfate 176 mg was added to the final concentration of 30%. The mixture was centrifuged (15700 xg, 5 min) and the pellet was dissolved in 250 μL of TBS (This is designated as the 0-30% fraction). The supernatant was transferred to a new Eppendorf tube and treated with 62 mg of ammonium sulfate to the final concentration of 40%. The mixture was centrifuged (15700 xg, 5 min) and the pellet was redissolved in 250 μL of TBS (This is designated as the 30-40% fraction). The supernatant was transferred to a new Eppendorf tube and the precipitation process was repeated to obtain the 40-50% fraction, 50-60% fraction, and 60-70% fraction. The re-dissolved fractions were stored at -80 ˚C and the final supernatant was discarded.

15 μL of each fraction was treated with 5 μL of 4x SDS loading buffer, resolved by SDS-PAGE on a 12% TGX gel and analyzed by Western blot using the anti-pHis antibody as described in the general Western blot procedure above. The fractions that were found to contain pHis proteins were again analyzed by SDS-PAGE in a 12% TGX gel. The molecular weight regions that correspond to the pHis bands in the Western blot were excised out from the gel and subjected to mass spectrometric analyses (see below).

Transformation and overexpression of His_6-tagged Ppk and PtsP in WT *E. coli*

ASKA strains harboring pCA24N plasmids[17] for N-terminally His_6-tagged Ppk and PtsP were grown in LB overnight at 37 ˚C. The cells were individually harvested by centrifugation and the plasmid DNAs were extracted using QIAprep spin Miniprep kit according to the manufacturer's instructions. The identity of each plasmid was confirmed by DNA sequencing.

Chemically competent NCM3722 (wildtype) *E. coli* strain was prepared. Briefly, NCM3722 strain *E. coli* was grown overnight in LB and was harvested by centrifugation (3000 g, 10 min, 4 ˚C). The supernatant was removed and the cell pellet was suspended in ice-cold $CaCl_2$ (0.1 M) and incubated on ice for 30 min. The cells were centrifuged (3000 xg, 10 min, 4 ˚C) and resuspended in 0.1 M $CaCl_2$, 10% glycerol. The cell suspension was aliquoted into Eppendorf tubes (140 μL/tube), flash-frozen with liquid nitrogen, and stored at -80 ˚C until further use.

The competent NCM3722 cells were individually transformed with the pCA24N plasmids of His_6-Ppk, His_6-PtsP and His_6-PpsA (see section below for preparation of this plasmid). Briefly, the frozen competent cells were thawed on ice, and ~300 ng of each plasmid was added to the cell suspension. The

cells were incubated on ice for 15 min and heat-shocked at 42 ˚C for 45 seconds. The cells were immediately cooled on ice for 2 min, and 1 mL of fresh sterile LB was added to each tube. The tubes were incubated at 37 ˚C in a shaker for 1 h, and 200 μL of each cell suspension was plated on LB-chloramphenicol agar plate. The plates were incubated at 37 ˚C overnight and colonies were picked for further experiments.

Western blot of lysate from His_6-tagged PpsA, PtsP, and Ppk NCM 3722 *E. coli* overexpression cells grown under varying nitrogen sources

Buffers used for procedure:
Growth Medium:

Starter Culture Medium: Gutnick minimal salts[16], 2.5 mM arginine, 2 mM NH_4Cl, 0.4% w/v glucose.

Nitrogen-Limited Culture Medium: Gutnick minimal salts, 2.5 mM arginine, 0.4% w/v glucose.

Loading Buffer: 160 mM Tris pH 8.5, 40% v/v glycerol, 4% w/v SDS, 0.08% w/v Bromophenol Blue, 8% v/v 2-mercaptoethanol
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20

Colonies of NCM 3722 cells overexpressing PpsA, PtsP, or Ppk growing on an LB Agar Plate were individually picked and transferred to 5 mL of starter culture and then grown overnight at 37 °C to an OD600 of about 1.3. 500 μL from each starter culture was added to 17 mL of Nitrogen-limited culture medium (starting OD600: 0.07). The cells were grown overnight at 37 °C to an OD600 of about 1.0. IPTG (0.02 mM final concentration) was added to each culture and the cells were then incubated for 12 minutes at 37 °C. The cells were then split into 2 x 7.5 mL aliquots in sterile 50 mL conical plastic tubes. One aliquot from each of the cell samples was treated with NH_4Cl (10 mM final concentration). All of the samples were then grown at 37 °C for 10 minutes. The cells were then pelleted by centrifugation (4500 xg for 5 minutes at 4 °C). The pellets were then resuspended in 500 μL of 2x loading buffer and sonicated (3 x 5 seconds at 45% power) on ice.

10 μL of each sample was loaded onto a 12% TGX gel and resolved by electrophoresis for 55 minutes at 180V. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody were both carried out as described in the general Western blot procedure above.

Western blot of overexpressed PpsA purified from NCM 3722 cells grown under varying nitrogen sources

Buffers used for procedure:
Growth Medium:

Starter Culture Medium: Gutnick minimal salts[16], 2.5 mM arginine, 2 mM NH_4Cl, 0.4% w/v glucose.

Nitrogen-Limited Culture Medium: Gutnick minimal salts, 2.5 mM arginine, 0.4% w/v glucose or 0.4% w/v glycerol.

Loading Buffer: 160 mM Tris pH 8.5, 40% v/v glycerol, 4% w/v SDS, 0.08% w/v Bromophenol Blue, 8% v/v 2-mercaptoethanol
Wash Buffer: 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20

A colony of NCM 3722 cells overexpressing PpsA growing on an LB agar plate was picked and transferred to 5 mL of starter culture. The culture was grown overnight at 37 °C to an OD600 of about 1.3. 1 mL from the starter culture was added to 40 mL of nitrogen-limited culture medium (starting OD600: 0.06). The cells were grown overnight at 37 °C to an OD600 of about 0.8. IPTG (0.02 mM final

concentration) was added to each culture and the cells were then incubated for 12 minutes at 37 °C. The cells were then split into 2 x 15 mL aliquots in sterile 50 mL conical plastic tubes, and one of the aliquots was treated NH_4Cl (10 mM final concentration). Both cell samples were then grown at 37 °C for 10 minutes. The cells were then pelleted by centrifugation (4500 xg for 5 minutes at 4 °C). The pellets were then resuspended in 1 mL TBS buffer and sonicated (3 x 5 seconds at 45% power) on ice. The cell lysate was then centrifuged (17,000 xg for 10 min at 4 °C) to pellet the cell debris.

950 μL of each sample lysate was loaded onto a fritted micro bio-spin column containing 100 μL of Ni-NTA agarose resin pre-equilibrated with TBS. The lysate was incubated with the resin for 5 minutes at room temperature on a nutator. The flow through was collected, and the resin was then washed with 5 column volumes of TBS. Elution of bound proteins was performed by adding 250 μL of TBS containing 250 mM EDTA. 50 μL of the elution fraction was incubated with 500 mM HA for 1 hour at 37 °C.

15 μL of each sample (lysate, flow through, wash, elution, and elution + HA) was mixed with 5 μL of 4x loading buffer, loaded onto a 12% TGX Criterion gel and then resolved by electrophoresis for 55 minutes at 180 V. Electroblotting of the proteins to a 0.2 μm PVDF membrane, and Western blot of membrane with affinity-purified pHis antibody were both carried out as described in the general Western blot procedure above.

Supplementary Note 8

Dot blot assays for the activity measurement of PtsI

Buffers used for PtsI assay:
Assay buffer: 25 mM sodium phosphate pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT
Quenching buffer: 6 M guanidine hydrochloride in TBS.
Phosphoenolpyruvate (PEP) stock: 200 mM in in dd H_2O. Neutralized with NaOH.
Alpha-ketoglutarate (α-KG) stock: 500 mM in dd H_2O. Neutralized with NaOH.
L-Glutamate stock: 500 mM in dd H_2O. Neutralized with NaOH.
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
TBS-tween: TBS with 0.1% Tween-20 (v/v)

Recombinant PtsI (see expression and purification above) was diluted to a final concentration of 0.3 μM in assay buffer in a siliconized Eppendorf tube. The reaction was initiated by adding PEP to the PtsI enzyme solutions at final concentrations of 1000 μM, 400 μM, 200 μM, 50 μM, 20 μM, or 5 μM at 26 ˚C. At time points (t=0, 10, 30, 60, 90, 120, 180, and 300 s), 25 μL of the reaction mixture was taken out and added into 200 μL of Quenching Buffer pre-loaded into a well of a Hybri-dot® dot blot apparatus (Bethesda Research Laboratories, Maryland) assembled with a nitrocellulose membrane (Thermo scientific). The reaction mixtures were blotted onto the membrane using vacuum and the wells were washed with 200 μL of TBS. The apparatus was disassembled and the membrane was blocked with 3% BSA in TBS-tween for 1 h at RT. The membrane was then incubated with anti-pHis antibody (1:100 in TBS-tween containing 3% BSA) for 1 h at RT, washed with TBS-tween (3 x 5 min), and incubated with anti-Rabbit fluorescent antibody (Li-Cor, 800 nm, 1:15000 in TBS-tween with 3% BSA) for 1 h at RT. The membrane was washed (3 x 5 min) with TBS-tween, rinsed with ddH_2O, and imaged on Li-Cor Odyssey Infrared Imager at 800 nm. The signal intensity of each dot was quantified using the Odyssey software. Curve fitting to determine the K_m was performed using Graphpad Prism Software.

Inhibition of PtsI phosphorylation by α-KG was performed by adding α-KG (25, 10, 4, 1, 0.25 mM final concentrations) or glutamate (final concentration 25 mM) to 200 μL of assay buffer containing 0.3 μM PtsI. PEP (150 μM final concentration) was added to the reaction mixture and it was incubated for 8 minutes at 26 ˚C. At 0 and 8 minutes, 25 μL of reaction mixture was added into 200 μL of Quenching

Buffer pre-loaded into a well of a Hybri-dot dot blot apparatus (Bethesda Research Laboratories, Maryland) assembled with a nitrocellulose membrane (Thermo scientific). The membrane was washed and imaged exactly as described for the PtsI phosphorylation assay above.

Dot blot assays for the activity measurement of PpsA

Buffers used for the PpsA assay
Reaction Buffer: 100 mM Tris (pH 8.0), 5 mM $MgCl_2$
Quenching buffer: 6 M guanidine hydrochloride in 100 mM Tris buffer (pH 8)
Alpha-ketoglutarate (α-KG) stock: 200 mM in dd H_2O. Neutralized with NaOH.
Tris Buffered Saline (TBS): 25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl
TBS-tween: TBS with 0.1% Tween-20 (v/v)

Phosphorylation Assay of PpsA

PpsA (~25 μM) was diluted in assay buffer (100 mM Tris (pH 8.0), 5 mM $MgCl_2$) to a final concentration of 500 nM. 170 μL of the PpsA sample was then placed into a siliconized Eppendorf tube followed by addition of 10 μL α-KG (final concentration of 10 or 15 mM) or 10 μL of assay buffer. The mixture was cooled to 15 $^{\circ}$C and the reaction was initiated by adding 20 μL of 100 μM ATP (final 10 μM). At time points (t=0, 10, 30, and 45 s), 20 μL of the reaction mix was taken out and added into 100 μL of quenching buffer (6 M guanidine hydrochloride in 100 mM Tris buffer (pH 8)). The quenched reaction mixtures were loaded into the wells of a dot-blot apparatus (Bethesda Research Laboratories, Maryland) assembled with a nitrocellulose membrane (Thermo scientific). The reaction mixtures were blotted onto the membrane using vacuum and the wells were washed with 200 μL of TBS (25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl). The apparatus was disassembled and the membrane was blocked with 3% BSA in TBST (25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) for 1 hour at room temperature. The membrane was then incubated with anti-pHis antibody (1:100 dilution in TBST with 3% BSA) for 1 h at RT, washed with TBST (3 x 5 min), and incubated with anti-Rabbit fluorescent antibody (Li-Cor, 800 nm, 1:15000 dilution in TBST with 3% BSA) for 1 hour at room temperature. The membrane was washed (3 x 5 min) with TBST, rinsed with ddH_2O, and imaged on Li-Cor Odyssey Infrared Imager at 800 nm. The signal intensity of each dot was quantified using the Odyssey software.

Dephosphorylation Assay of PpsA

50 μL of PpsA stock (~25 μM) was diluted with 50 μL of Reaction Buffer (final ~12 μM) followed by addition of 3 μL of 1 mM ATP (final 33 μM). The reaction mixture was incubated for 10 min at room temperature and the reaction mixture was diluted with 500 μL of Reaction buffer. The mixture was then concentrated back to 100 μL using a Vivaspin® 500 microcolumn (MWCO 30000). The protein concentration was determined by measuring the absorbance at 280 nm. The phosphorylated PpsA sample was then diluted with Reaction Buffer to a final concentration 500 nM. 170 μL of this phosphorylated PpsA stock solution was transferred to a siliconized Eppendorf tube followed by addition of 10 μL of 200 mM α-KG stock (final 10 mM), 10 μL of 200 mM glutamate stock (final 10 mM) or 10 μL of Reaction Buffer. The sample was then incubated at RT for 10 min. The reaction was initiated by adding 20 μL of 100 μM sodium pyruvate (final 10 μM). At time points (t=0, 10, 30, and 45 s), 20 μL of the reaction mix was taken out and added into 100 μL of Quenching Buffer. The quenched reaction mixtures were loaded into the wells of a dot-blot apparatus (Bethesda Research Laboratories, Maryland) assembled with a nitrocellulose membrane (Thermo scientific). The reaction mixtures were blotted onto the membrane using vacuum and the wells were washed with 200 μL of TBS. The apparatus was disassembled and the membrane was blocked with 3% BSA in TBS-tween for 1 h at RT. The membrane was then incubated with anti-pHis antibody (1:100 in 3% BSA/TBS-tween) for 1 h at RT, washed with TBS-tween (3 x 5 min), and incubated with anti-Rabbit fluorescent antibody (Li-Cor, 800 nm, 1:15000 in 3% BSA/TBS-tween) for 1 h at RT. The membrane was washed (3 x 5 min) with TBS-tween, rinsed with

ddH2O, and imaged on Li-Cor Odyssey Infrared Imager at 800 nm. The signal intensity of each dot was quantified using the Odyssey software.

Supplementary Note 9

LC-MS analysis of pHis proteins

Preparation of synthetic PtsI peptides:

Synthetic peptides containing the PtsI tryptic peptide sequence, TSpHTSIMAR or TpSHTSIMAR, were prepared for use as standards for LC-MS analysis. The peptides were prepared using synthetic methods similar to those previously reported[5]. Histidine phosphorylation of the TSHTSIMAR peptide was carried out by mixing 2.3 mM peptide in 200 mM Tris pH 8.0 with 90 mM potassium phosphoramidate and incubating at room temperature for 48 hours. Prior to LC-MS analysis, both phosphorylated peptides were purified by HPLC on a C18 column (5-25% solvent B over 30 minutes with a flow rate of 1 mL/min). The fraction containing the desired phosphorylated peptide was collected and immediately neutralized by adding 1 M basic Tris to a final concentration of 50 mM. The samples were then stored at -80 ˚C until LC-MS preparation.

Preparation of pHis bearing peptides from recombinant and endogenous E. coli proteins:

Purified His-tagged proteins, produced as described above, were for prepared for peptide MS analyses by dilution of the protein elution fraction directly into 50 mM Tris, pH 8.5 digestion buffer containing 1 μg trypsin, and conducting digestion at room temperature for 4 hours. For consistency, the synthetic PtsI pHis and pSer peptides were subjected to equivalent trypsin digestion treatment. Samples were diluted into distilled water prior to loading into autosampler vials for LC-MS analysis. Note that acidic conditions were avoided until the sample was subjected to exposure to the LC mobile phase on-column to reduce loss of the pHis signal.

SDS-PAGE gel bands from ammonium sulfate precipitated fractions were excised and subjected to in-gel reduction and alkylation and trypsin digestion in a manner adapted from the procedure of Shevchenko, et al.[18] with the following alterations. All steps were conducted as rapidly as possible at room temperature, reduction consisted of a 30 min treatment with DTT (10 mM dissolved in 20 mM Tris, pH 8.5) and alkylation consisted of a 30 min treatment with iodoacetamide (20 mM in 20 mM Tris, pH8.5). Trypsin digestion was accomplished over 4 hours, using 2 ug enzyme in 20 mM Tris, pH 8.5. Extraction of peptides from the gel was achieved using acetonitrile and 20 mM Tris pH 8.5, samples were concentrates and resuspended in water prior to loading in autosampler vials. Desalting was not performed prior to LC-MS analysis.

High-resolution nano-UPLC-MS:

LC-MS and MS/MS analyses were performed on high-resolution, high-mass-accuracy, reversed-phase nano-UPLC-MS platforms, consisting of either a nano-flow capillary ultra high performance LC system (Nano Ultra 2D Plus, Eksigent, Dublin, CA) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) outfitted with a Triversa NanoMate ion source robot (Advion, Ithaca, NY), or an Easy nLC Ultra 1000 nano-UPLC system (ThermoFisher Scientific) coupled to a Velos Pro-Orbi Elite hybrid mass spectrometer (ThermoFisher Scientific) equipped with a Flex Ion source (Proxeon Biosystems, Odense, Denmark). Sample concentration and washing was accomplished online using a trapping capillary column (150 μm x ca. 40 mm, packed with 3 μm, 100 Å Magic AQ C18 resin, Michrom, Auburn, CA) at a flow rate of 4 μL/min for 4 min, while separation was achieved using an analytical capillary column (75 μm x ca. 15 cm, packed with 1.7 μm 100 Å BEH C18 resin, Waters, Milford, MA), under a linear gradient of A and B solutions (solution A: 3% acetonitrile/ 0.1%

formic acid/ 0.1% acetic acid; solution B: 97% acetonitrile/ 0.1% formic acid/ 0.1% acetic acid) over 90 min at a flow rate of 250-300 nL/ min. On the Orbi XL platform, nanospray ionization was carried out using the NanoMate ion source at 1.74 kV, with the LTQ heated capillary set to 200 °C, while on the Orbi Elite platform, nanospray was achieved using sprayer tips made from PicoFRIT capillaries (New Objective, Woburn, MA) using a voltage of 2.1 kV, with the Velos heated capillary at 200 °C. Full-scan mass spectra were acquired in the Orbitrap in positive-ion mode over the m/z range of 335–1800 at a resolution of 100,000 (Orbi XL) or 120,000 (Orbi Elite). MS/MS spectra were simultaneously acquired using CID in the LTQ for the seven (Orbi XL) or fifteen (Orbi Elite) most abundant multiply charged species in the full-scan spectrum having signal intensities of >1000 NL. For protein identification experiments, dynamic exclusion was set such that MS/MS was acquired only once for each species over a period of 120 s. All spectra were acquired in profile mode.

Mass Spectrometry Data Analysis:

Resultant LC-MS and MS/MS data were subjected to preprocessing into mgf peaklist files using ProteomeDiscoverer (v. 1.4, ThermoFisher), which were searched against the UniProt *E. coli* databases, using the Mascot search engine (v. 2.2.7, Matrix Science, London, UK.), allowing for a precursor mass window of ±6 ppm, ≤ 3 missed trypsin cleavages, histidine phosphorylation, methionine oxidation and N-terminal protein acetylation as variable modifications, and carbamidomethylation of cysteines a fixed modification. Aggregate search results for each sample were consolidated according to the PeptideProphet and ProteinProphet parsimony algorithms (ISB, Seattle WA) using the Scaffold software (Proteome Software, Portland, OR), and filtered to contain 95% protein/ 90% peptide confidence, requiring 2 peptides per protein assignment, which corresponded to an estimated protein false discovery rate of ≤ 0.1%. Primary MS and MS/MS spectra were visualized using Xcalibur (v. 2.1, ThermoFisher) along with Scaffold, Ascore-based probability localization scoring was accomplished through the use of ScaffoldPTM software (Proteome Software), and all phosphohistidine-bearing peptide assignments were further validated by manual inspection.

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