

## Supporting Information

for

### **A Phosphohistidine Proteomics Strategy Based on Elucidation of a Unique Gas-phase Phosphopeptide Fragmentation Mechanism**

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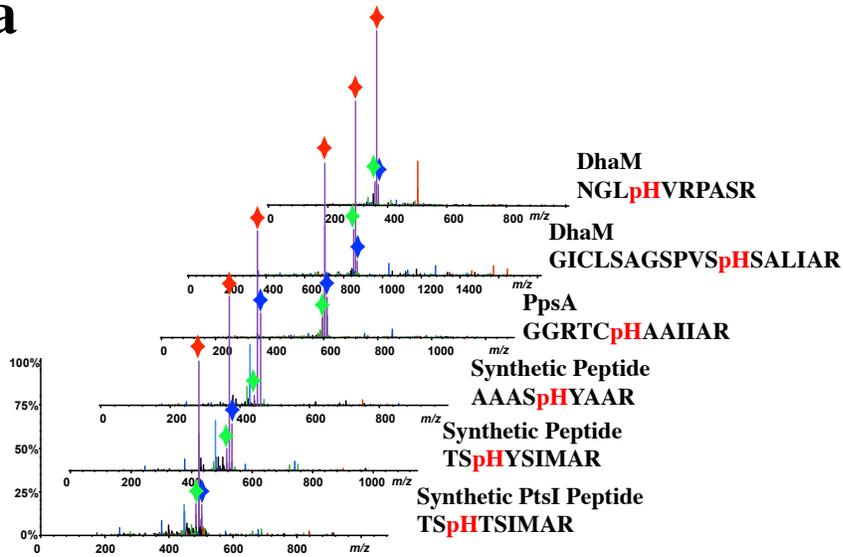
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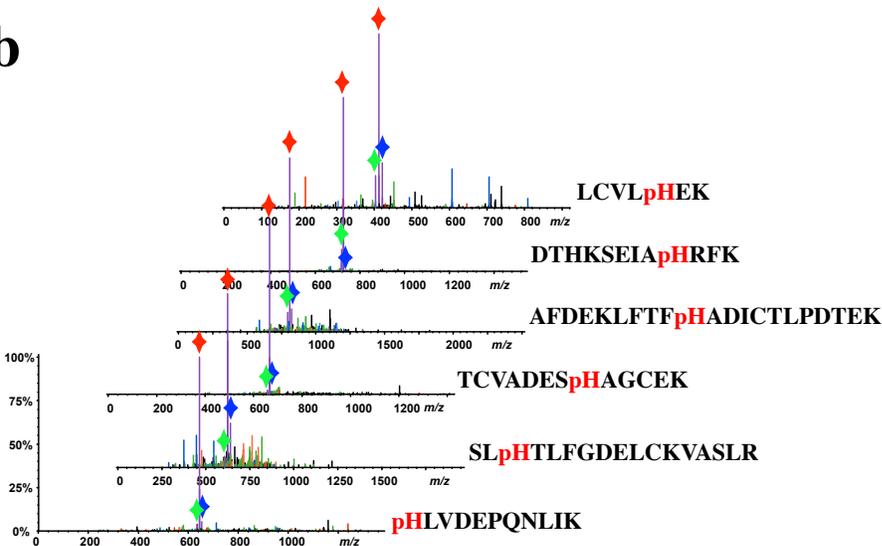
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## Supplemental Figures

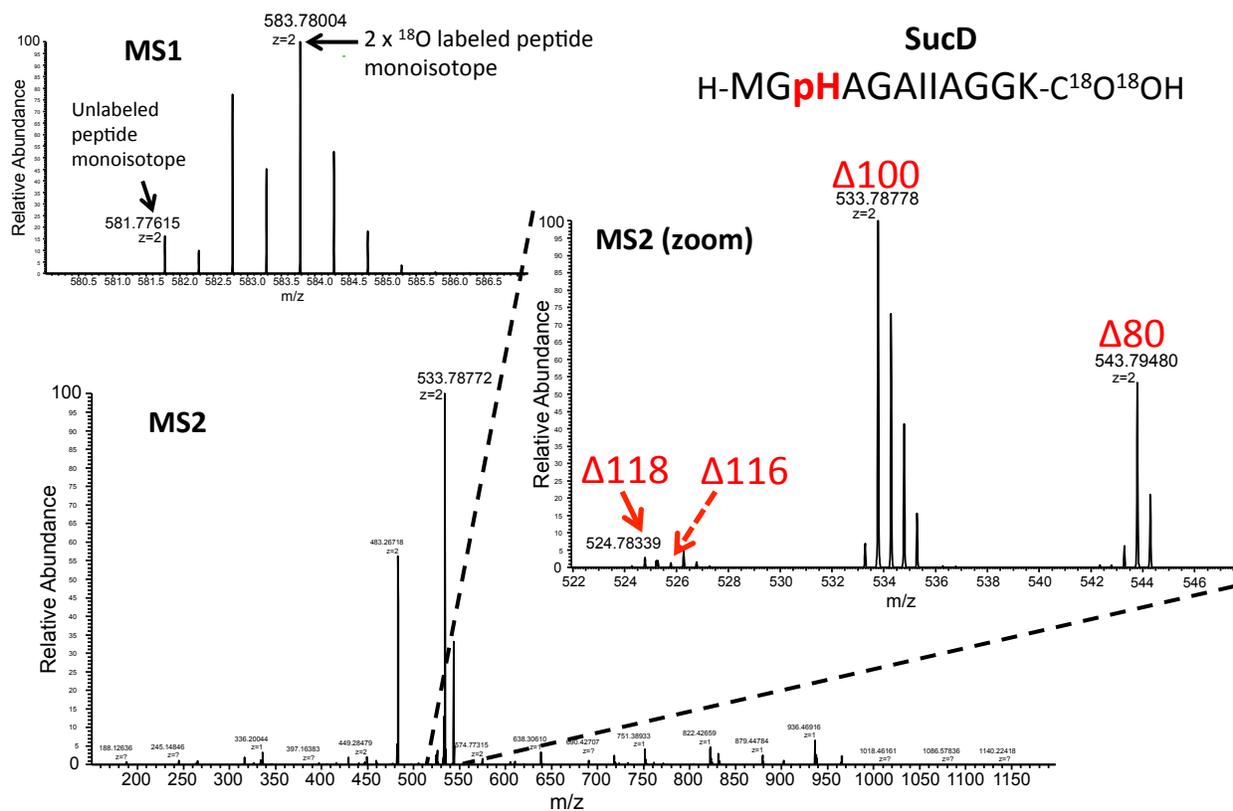
**a**



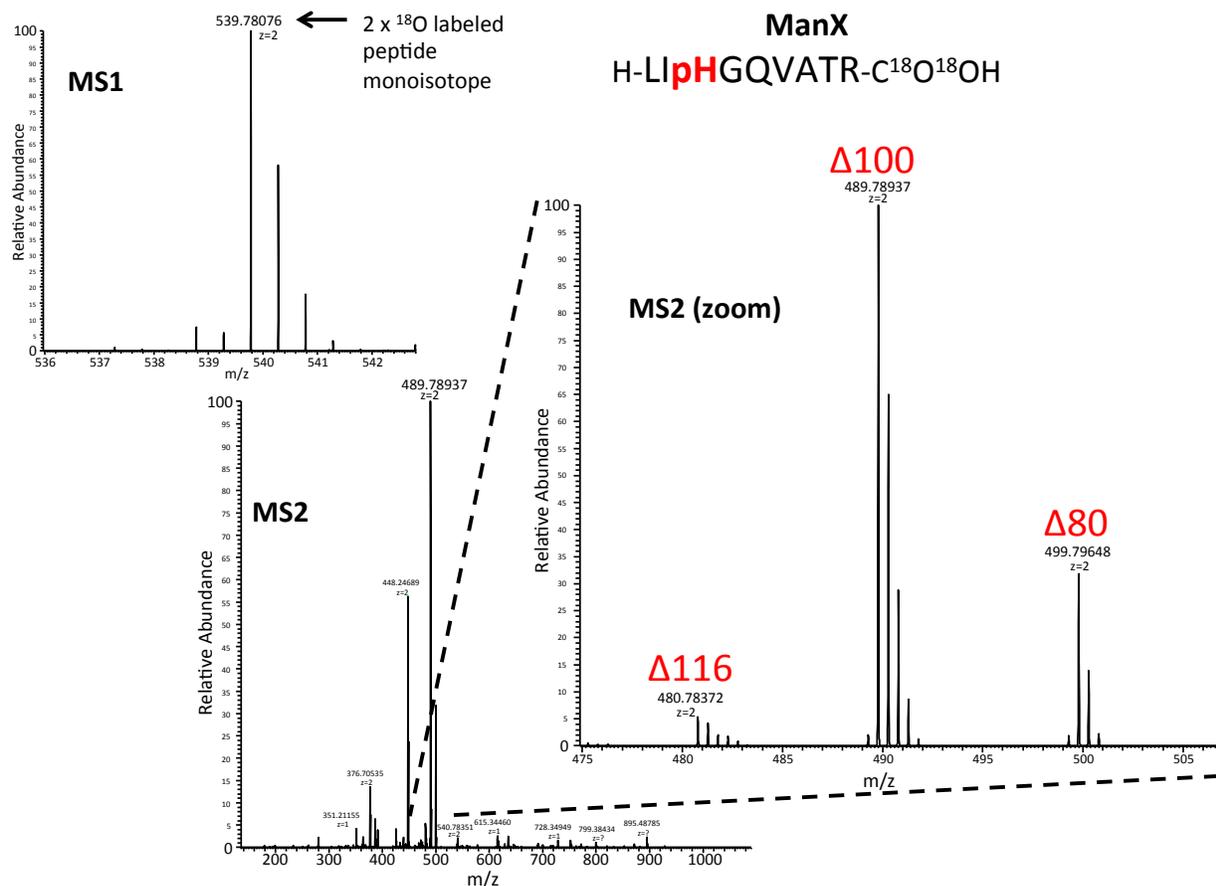
**b**



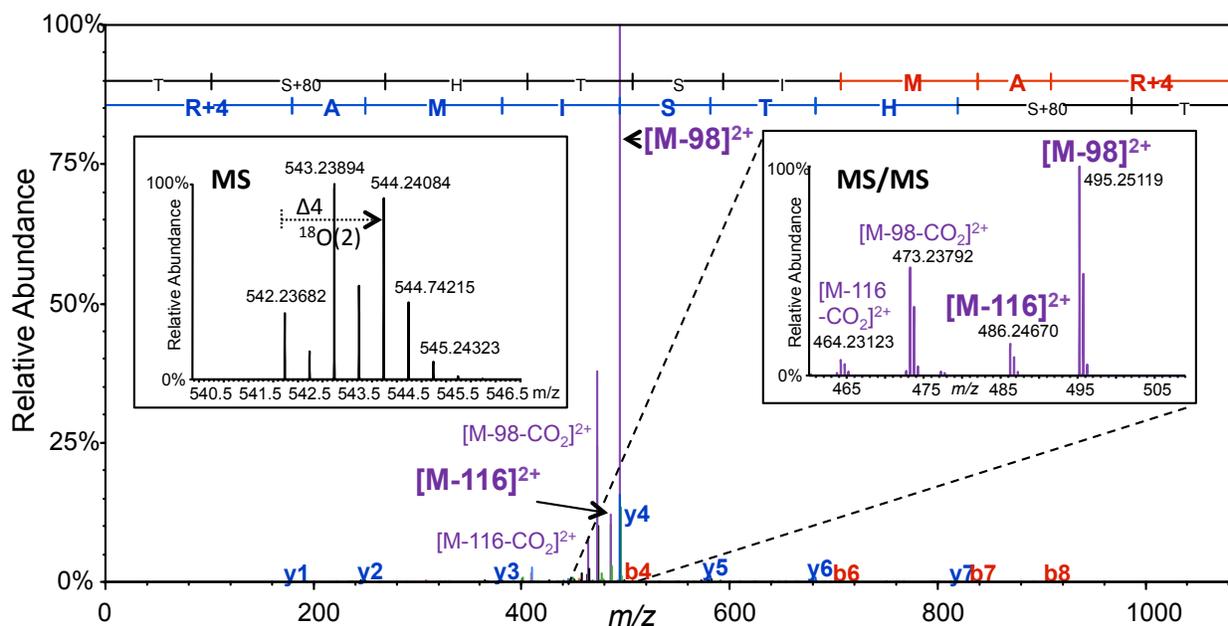
**Figure S1.** MS/MS spectra from Figure 2 with full scale m/z. Prominent neutral loss triplet of 116, 98, and 80 Da was observed for a) tryptic peptides from known pHis proteins (DhaM, PpsA) or chemically phosphorylated synthetic peptides, respectively, and b) pHis peptides derived from chemically phosphorylated BSA. The peaks from 116 Da (green triangles), 98 Da (red triangles), and 80 Da (blue triangles) neutral losses, as well as the peptide sequence and pHis site are indicated for each spectrum.



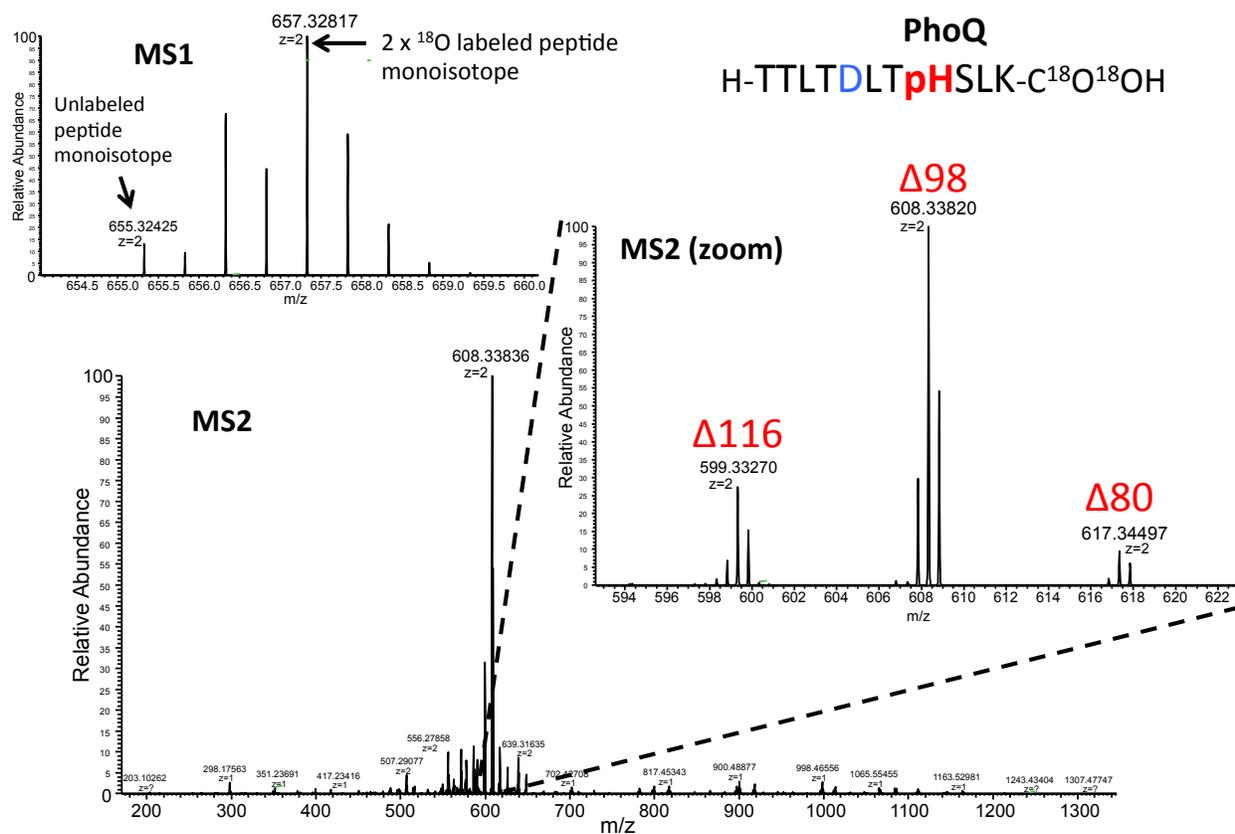
**Figure S2. CID Fragmentation of the peptide, H-MGpHAGAIAGGK-C<sup>18</sup>O<sup>18</sup>OH, results in prominent neutral loss of 100 Da.** CID MS/MS of the doubly <sup>18</sup>O-labeled [M+2H]<sup>2+</sup> pHis peptide from SucD (*m/z* 583.77981). Inset into the spectrum is a high resolution MS spectrum of the precursor species at *m/z* 583.78004 (top left) and a zoomed-in, high resolution MS/MS spectrum showing species corresponding to the primary loss of 100 Da and less prominent losses of 80, 118, and 116 Da (right panel).



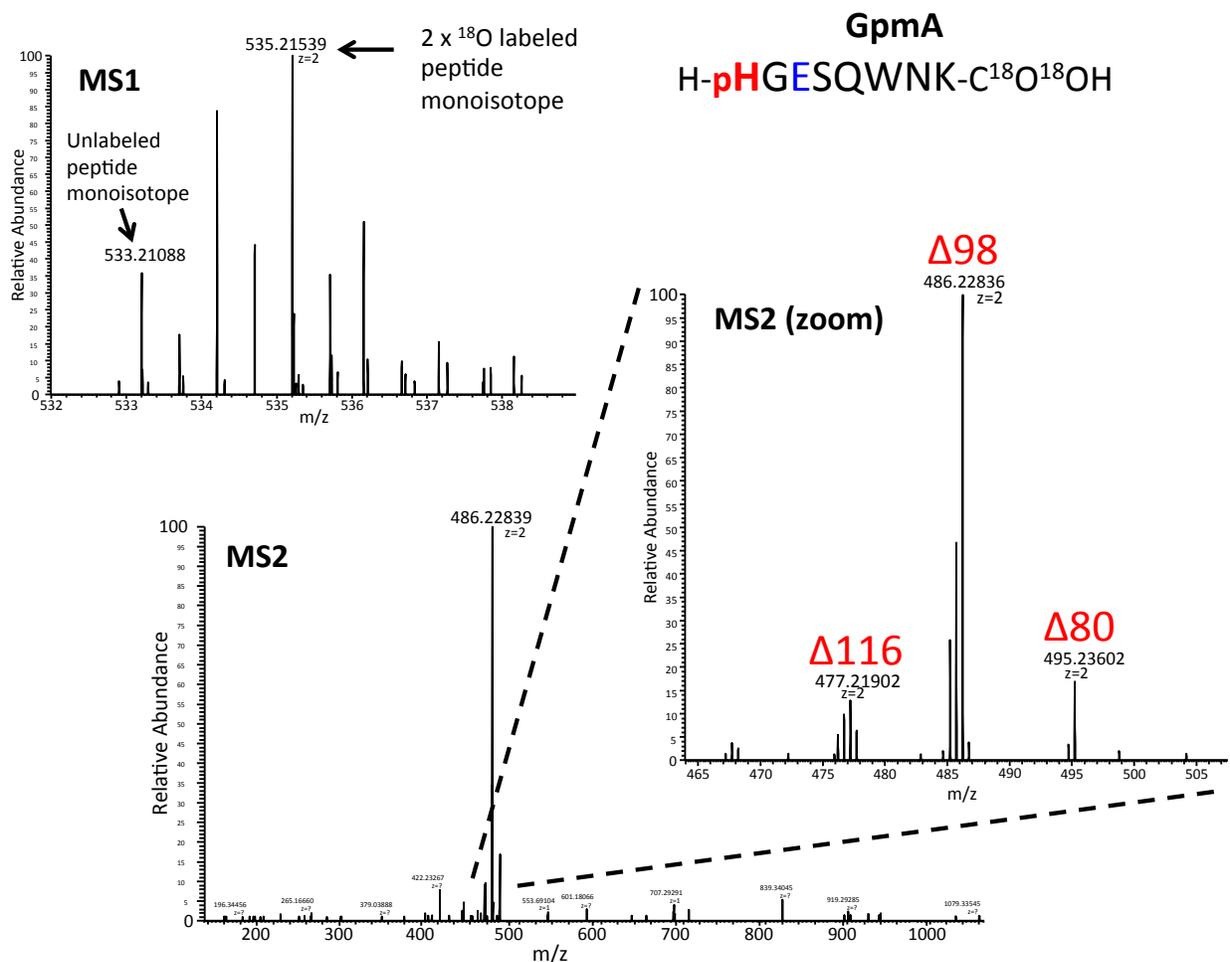
**Figure S3.** CID Fragmentation of the peptide, H-LIpHGQVATR-C $^{18}\text{O}^{18}\text{OH}$ , results in prominent neutral loss of 100 Da. CID MS/MS of the doubly  $^{18}\text{O}$ -labeled  $[\text{M}+2\text{H}]^{2+}$  pHis peptide from ManX ( $m/z$  539.78071). Inset into the spectrum is a high resolution MS spectrum of the precursor species at  $m/z$  539.78076 (top left) and a zoomed-in, high resolution MS/MS spectrum showing species corresponding to the primary loss of 100 Da and less prominent losses of 80, and 116 Da (right panel).



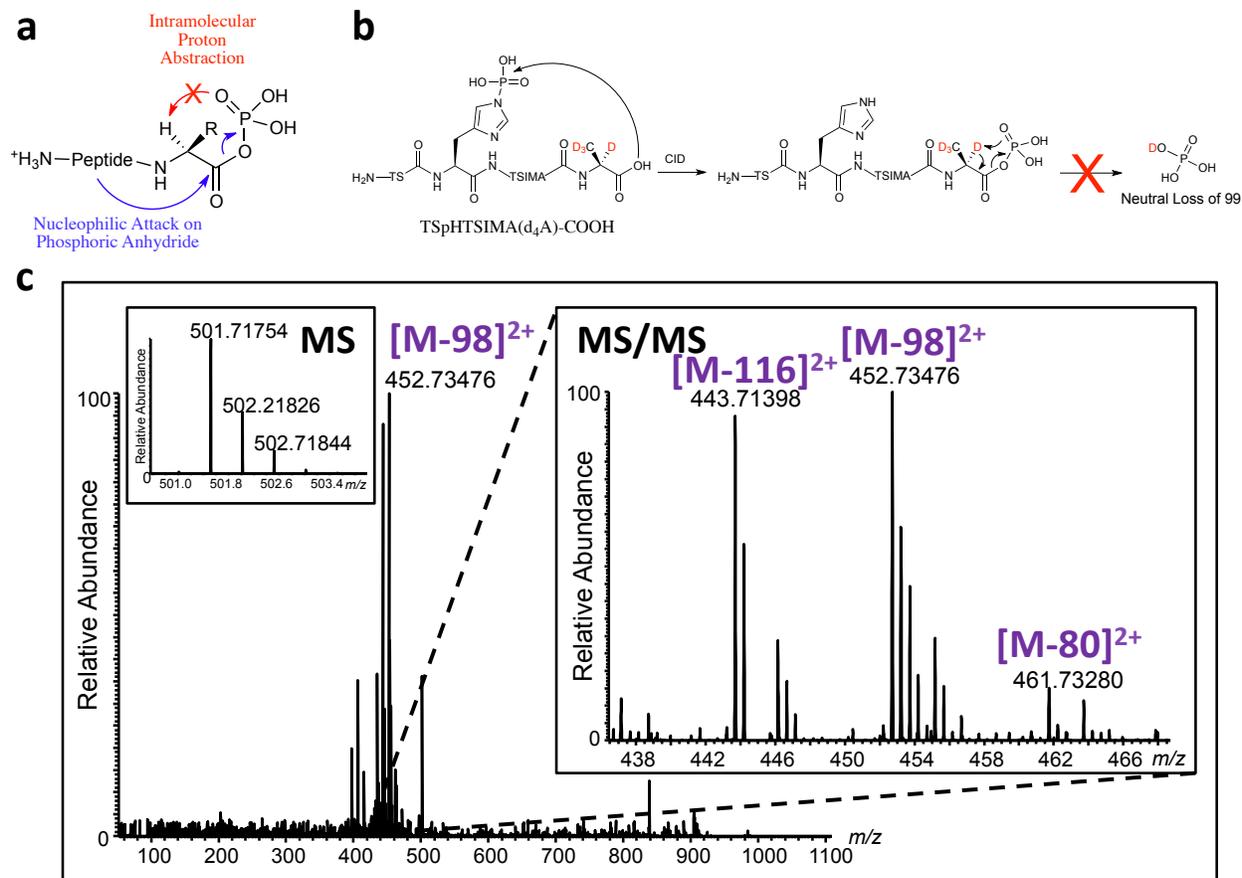
**Figure S4. Neutral loss of 98 Da from the peptide, TpSHTSIMAR, occurs by a different mechanism from pHis peptides.** CID MS/MS of a pSer peptide doubly <sup>18</sup>O-labelled at the C-terminus, TpSHTSIMAR-C<sup>18</sup>O<sup>18</sup>OH, observed as a [M+2H]<sup>2+</sup> ion at m/z 544.24084, shown in the high resolution MS spectrum of the precursor species (left inset). Here, the prominent neutral loss during CID is 98 Da (not 100 Da as in Figure 3b), as displayed in the high resolution MS/MS spectrum (right inset). Secondary losses shown ( $\Delta 16$ , etc.) also do not appear to involve participation of the C-terminus and associated loss of the <sup>18</sup>O label.



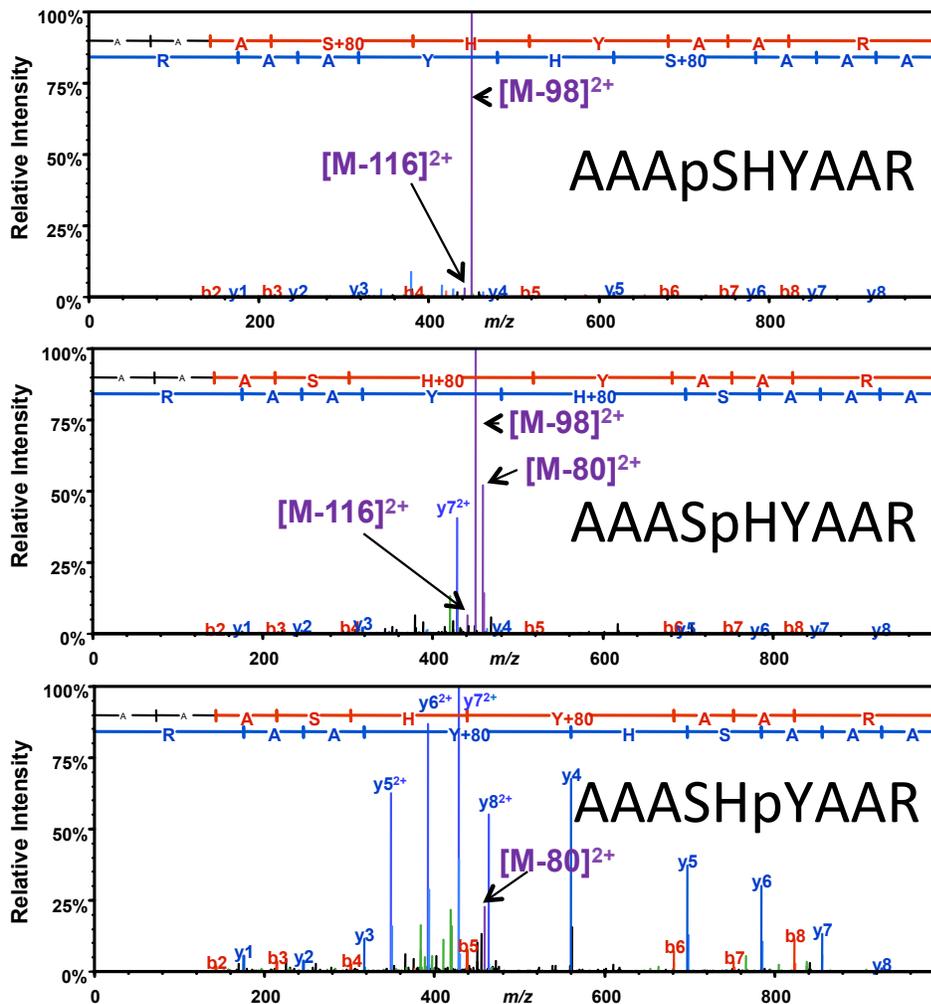
**Figure S5. CID Fragmentation of the peptide, H-TTLTDLT<sup>p</sup>HSLK-C<sup>18</sup>O<sup>18</sup>OH, results in prominent neutral loss of 98 Da.** CID MS/MS of the doubly <sup>18</sup>O-labeled [M+2H]<sup>2+</sup> pHis peptide from ManX (*m/z* 657.32785). Inset into the spectrum is a high resolution MS spectrum of the precursor species at *m/z* 657.32817 (top left) and a zoomed-in, high resolution MS/MS spectrum showing species corresponding to the primary loss of 98 Da and less prominent losses of 80 and 116 Da (right panel). The acidic residue is highlighted in blue to highlight its role in contributing to the 98 Da neutral loss instead of the 100 Da neutral loss as observed for peptides without Asp/Glu residues.



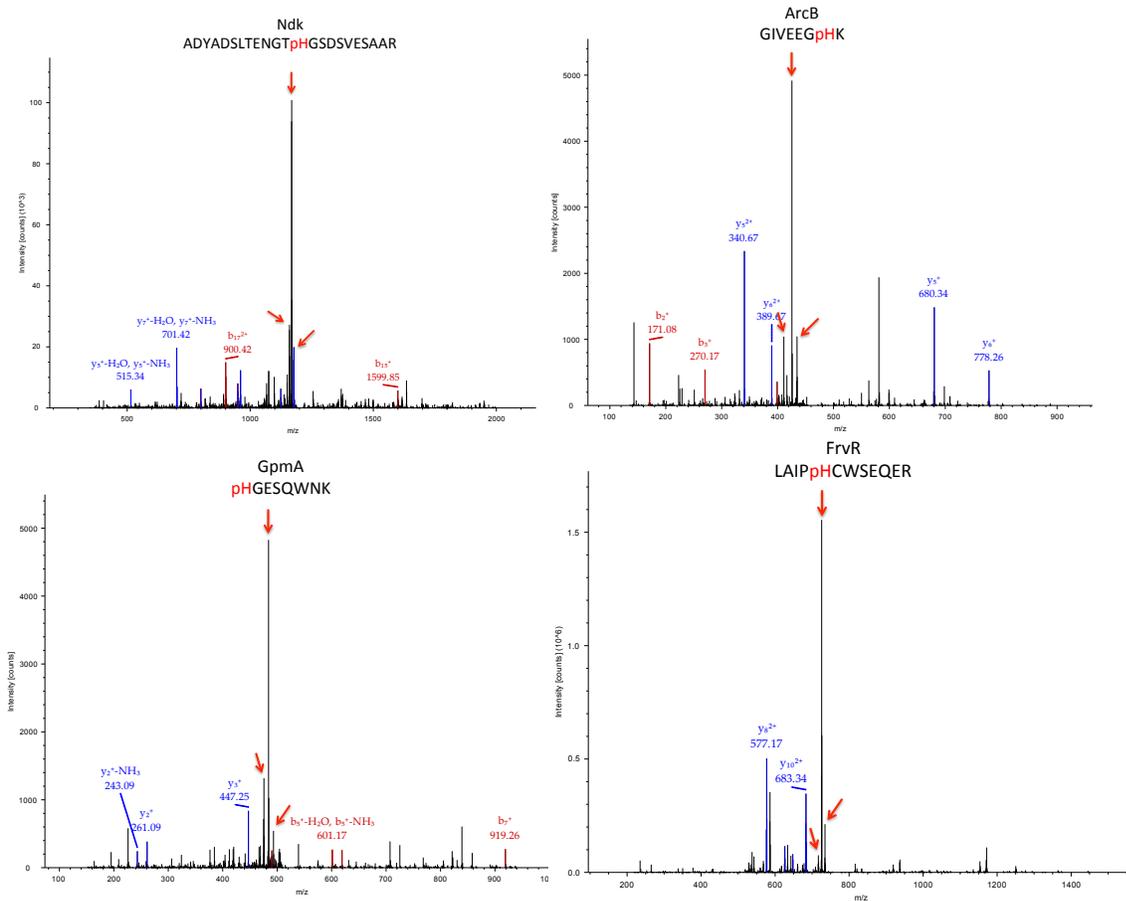
**Figure S6. CID Fragmentation of the peptide, H-TTLTDLT<sub>p</sub>HSLK-C<sup>18</sup>O<sup>18</sup>OH, results in prominent neutral loss of 98 Da.** CID MS/MS of the doubly <sup>18</sup>O-labeled [M+2H]<sup>2+</sup> pHis peptide from ManX (*m/z* 535.21535). Inset into the spectrum is a high resolution MS spectrum of the precursor species at *m/z* 535.21539 (top left) and a zoomed-in, high resolution MS/MS spectrum showing species corresponding to the primary loss of 98 Da and less prominent losses of 80 and 116 Da (right panel). The acidic residue is highlighted in blue to highlight its role in contributing to the 98 Da neutral loss instead of the 100 Da neutral loss as observed for peptides without Asp/Glu residues.



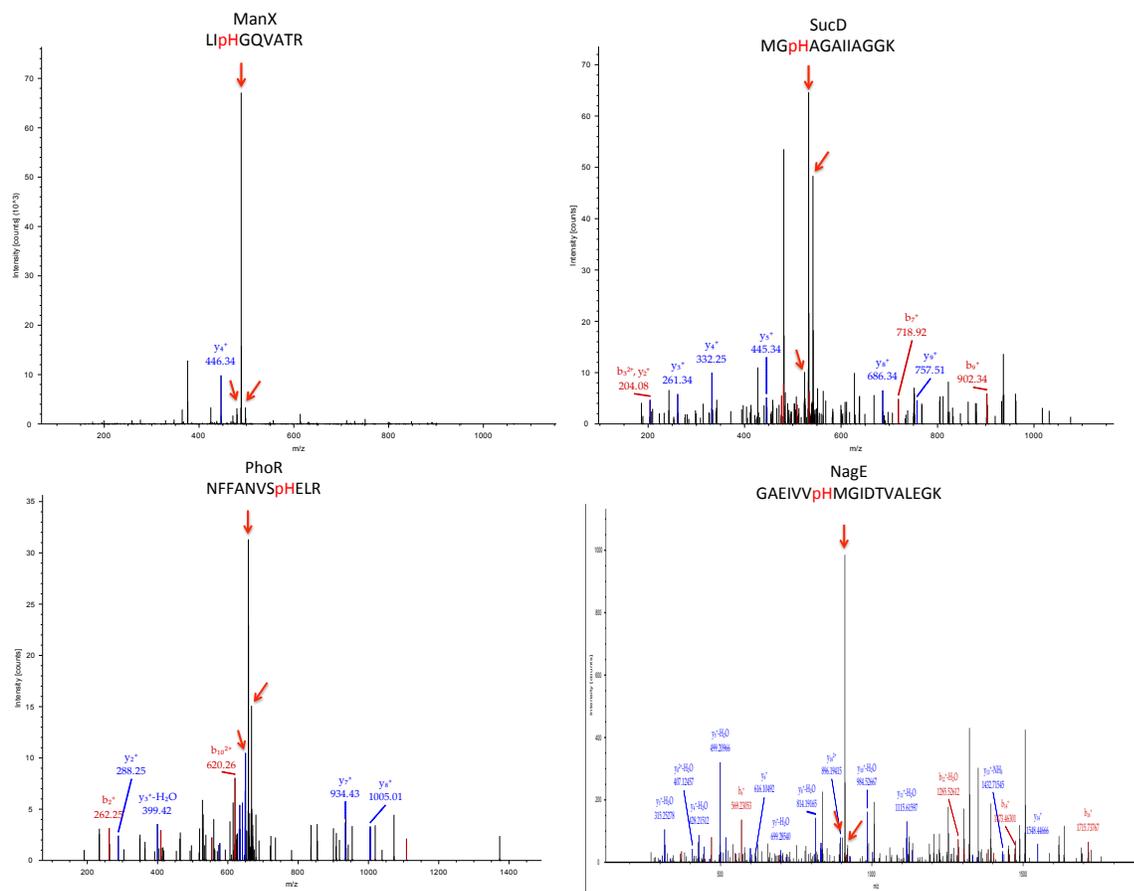
**Figure S7. Investigation of CID-induced neutral loss of 98 Da.** a) Formation of the phosphoric acid neutral loss species likely occurs through nucleophilic attack of the acyl phosphate rather than through intramolecular proton abstraction. b) A PtsI peptide analog with C-terminal deuterated alanine was constructed to investigate whether the neutral loss occurs through intramolecular proton abstraction at the C-terminus. c) MS/MS analysis of the peptide does not result in neutral loss of 99 Da, but rather, 98 Da.



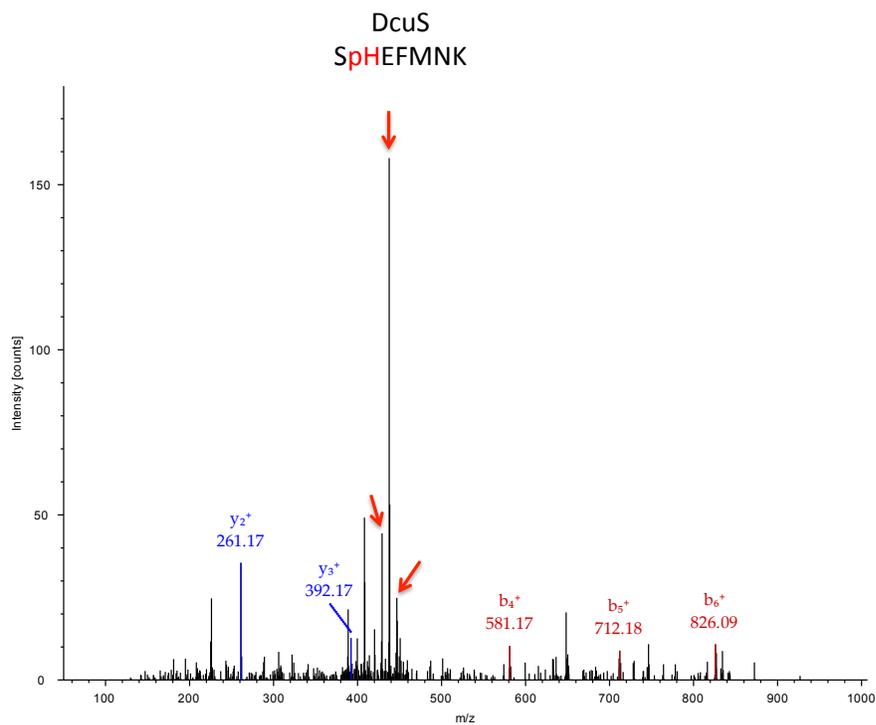
**Figure S8. CID-Induced neutral losses of 98, 80, and 116 Da are characteristic of pHis peptides.** The pHis peptide AAASpHYAAR (middle) undergoes CID-induced neutral losses of 116, 98, and 80 Da that are not observed for the isobaric pSer (AAASpHYAAR, top) and pTyr (AAASHpYAAR, bottom) peptides.



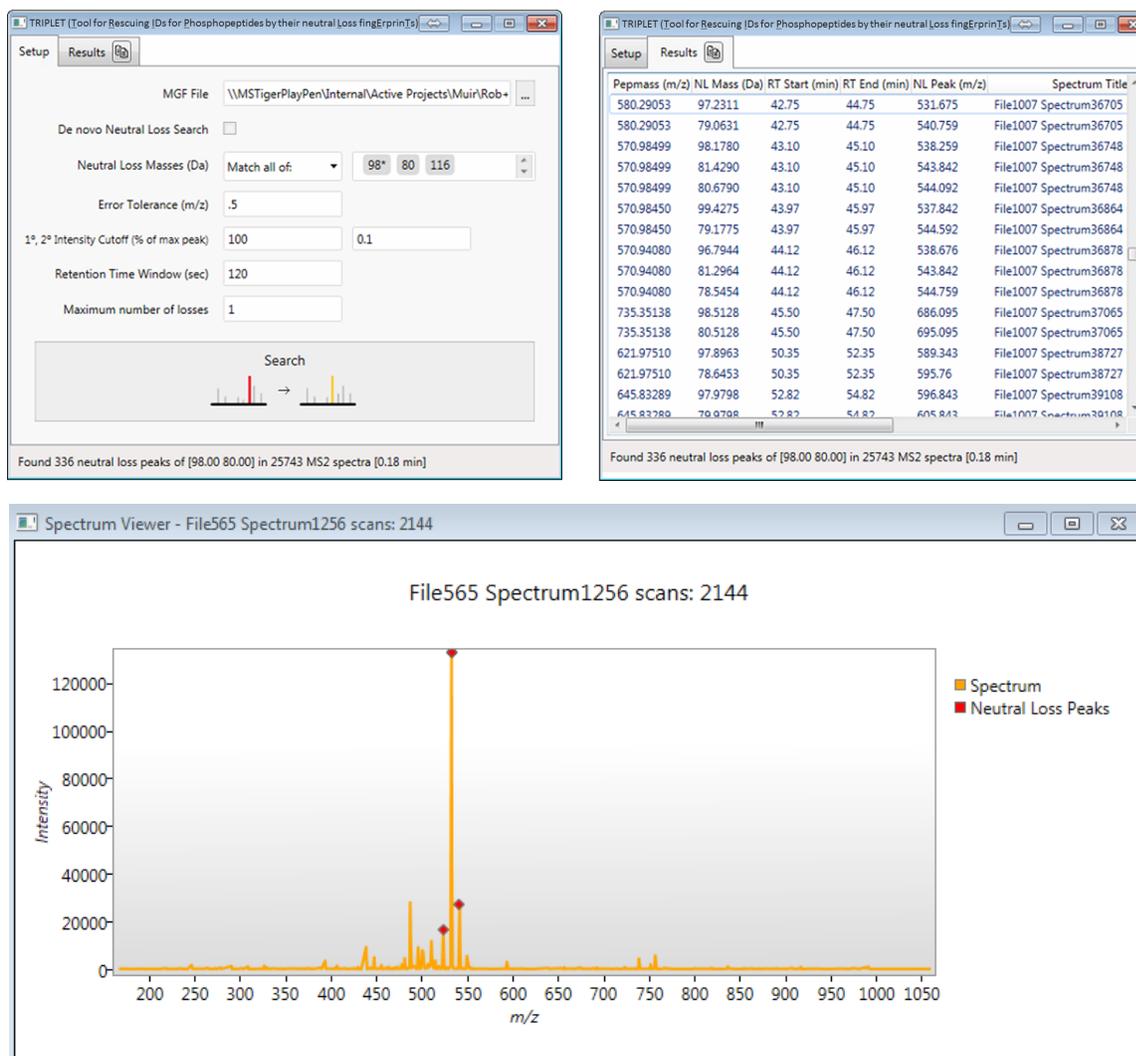
**Figure S9. Triplet neutral loss pattern ( $\Delta 98$ ,  $\Delta 80$ , and  $\Delta 116$  Da) observed in pHis peptides upon CID fragmentation from phosphopeptide library in Figure 5a. Ndk pHis peptide (top left), ArcB pHis peptide (top right), GpmA pHis peptide (bottom left), and FrvR pHis peptide (bottom right). The peaks corresponding to the triplet neutral loss patterns are indicated with red arrows.**



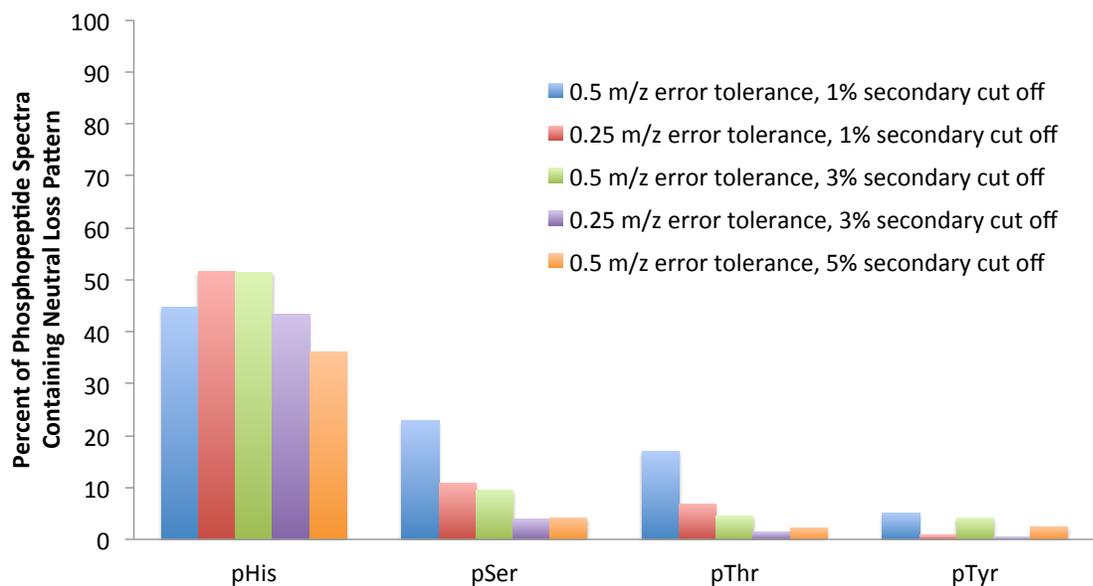
**Figure S10.** Triplet neutral loss pattern ( $\Delta 98$ ,  $\Delta 80$ , and  $\Delta 116$  Da) observed in pHis peptides upon CID fragmentation from phosphopeptide library in Figure 5a. ManX pHis peptide (top left), SucD pHis peptide (top right), PhoR pHis peptide (bottom left), and NagE pHis peptide (bottom right). The peaks corresponding to the triplet neutral loss patterns are indicated with red arrows.



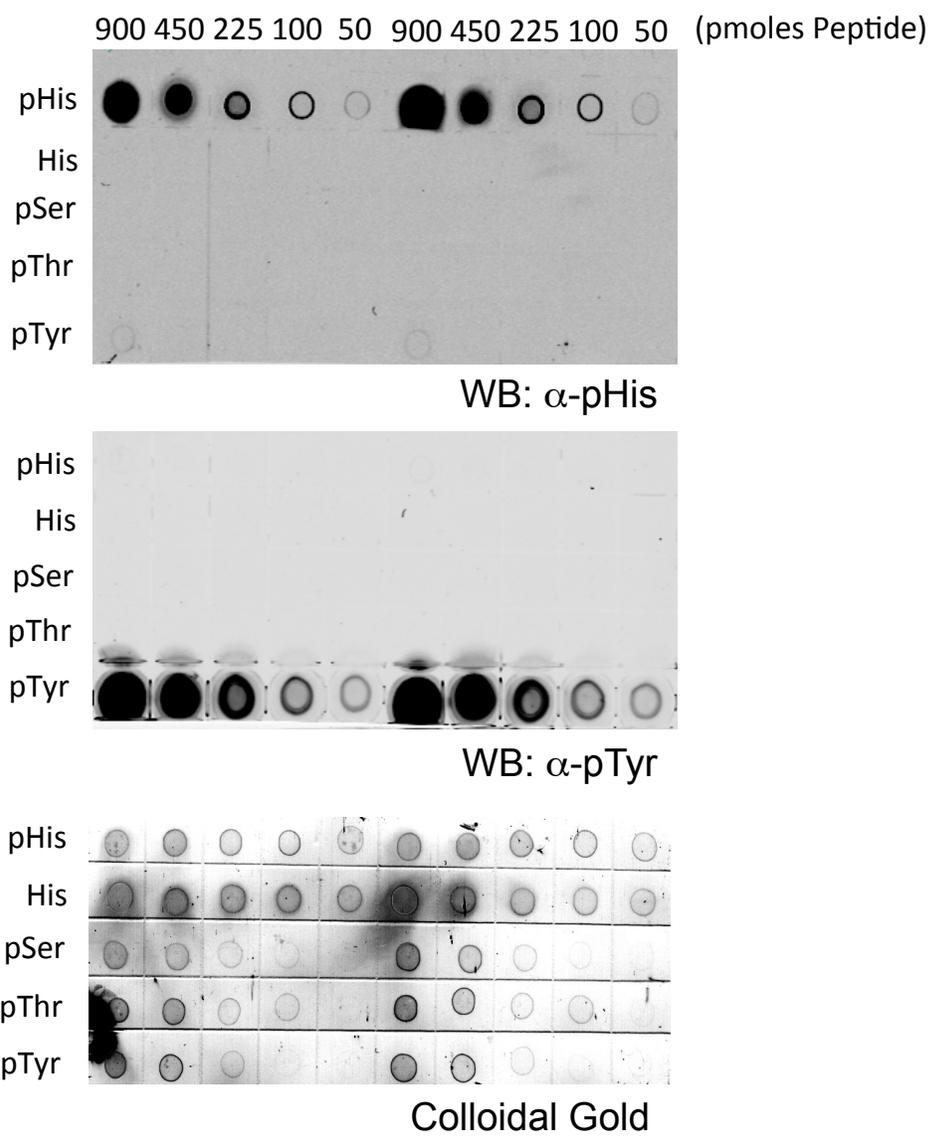
**Figure S11.** Triplet neutral loss pattern ( $\Delta 98$ ,  $\Delta 80$ , and  $\Delta 116$  Da) observed in the DcuS pHis peptide upon CID from phosphopeptide library in Figure 5a. The peaks corresponding to the triplet neutral loss patterns are indicated with red arrows.



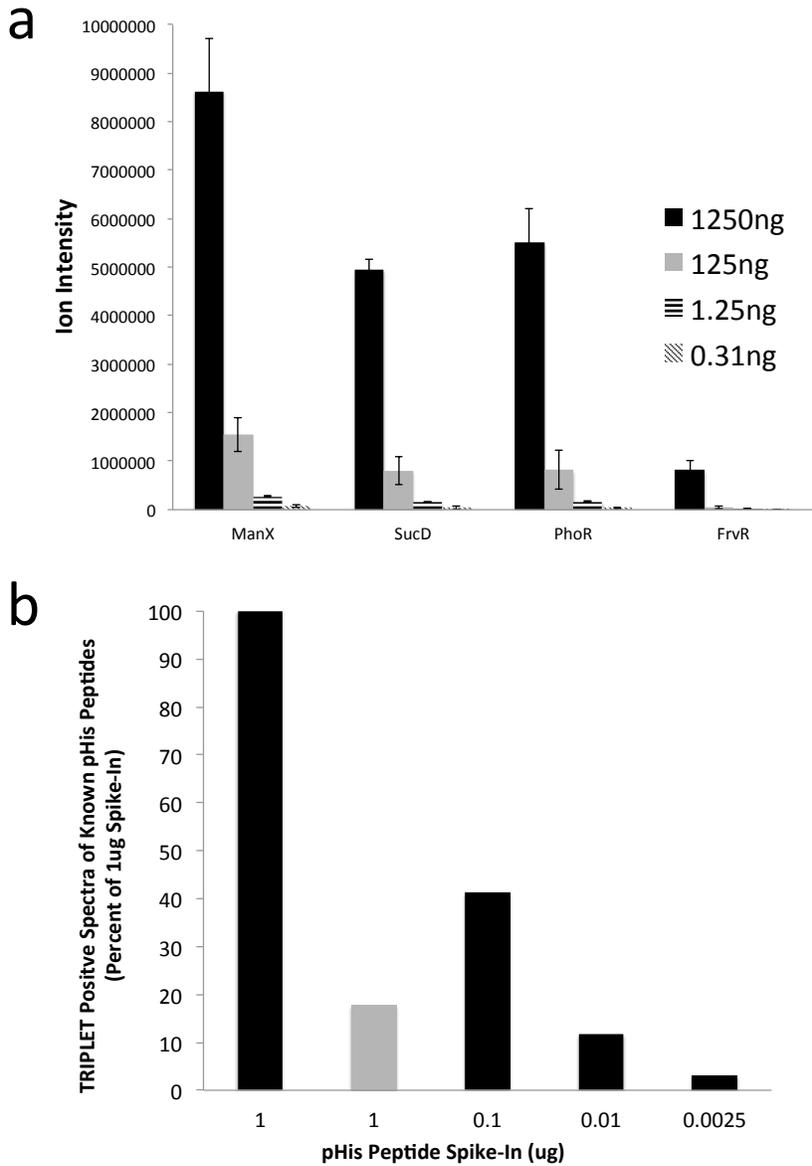
**Figure S12. TRIPLET software functionality and user interface.** Top left panel: the MS/MS peaklist file is specified and the neutral loss fingerprint parameters are entered and searched. Top right panel: after searching the peaklist file, the program displays a precursor ion list of the MS/MS spectra containing the neutral loss triplet. Bottom panel: double-clicking on any of the precursor ion entries listed (in the top right panel) will launch a new window containing the MS/MS spectrum in which the neutral loss fingerprint ions (indicated with red diamonds) are found. This allows for visual inspection of spectral quality, signal to noise, etc.



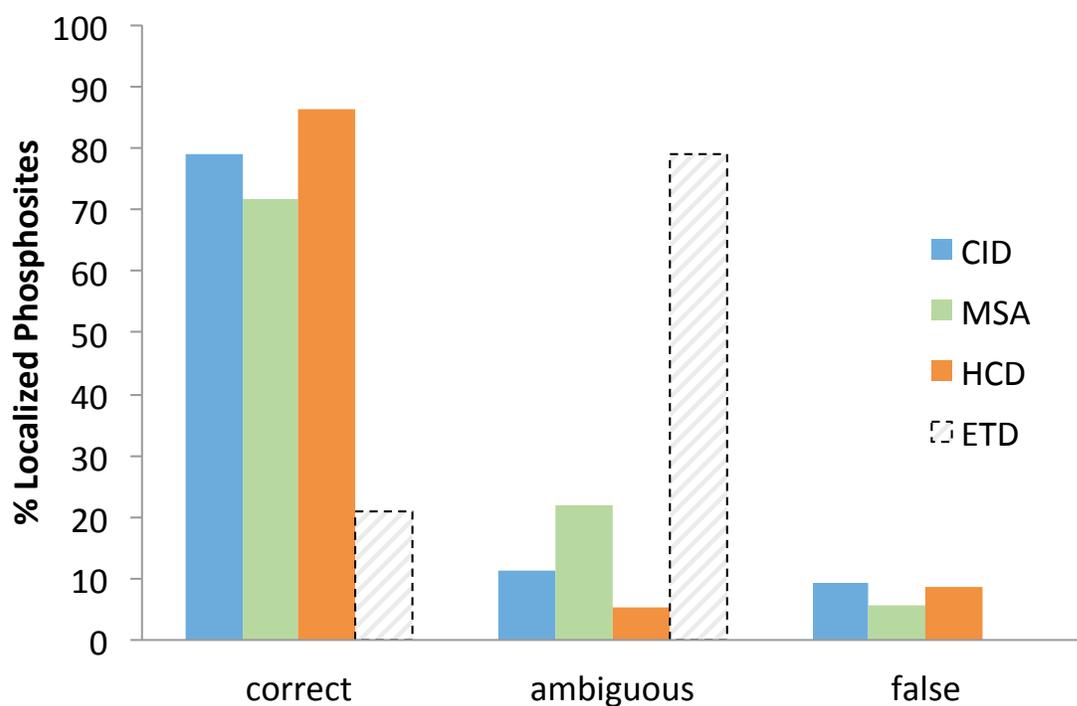
**Figure S13. TRIPLET software analysis of phosphopeptides.** Peptides in the phosphopeptide library were analyzed by CID MS/MS and the percentage of each MS/MS spectra displaying the  $\Delta 98$ ,  $\Delta 80$  and  $\Delta 116$  Da neutral loss pattern was calculated for each phosphotype. The m/z error tolerance and secondary peak intensity cutoff parameters were adjusted in the TRIPLET software prior to filtering the MS/MS spectra. pHis peptide spectra can be selectively filtered by increasing the secondary peak intensity cutoff and/or the m/z error tolerance.



**Figure S14. Dot blot from Figure 5c with loading control.** Dot blot membrane was stained with colloidal gold.

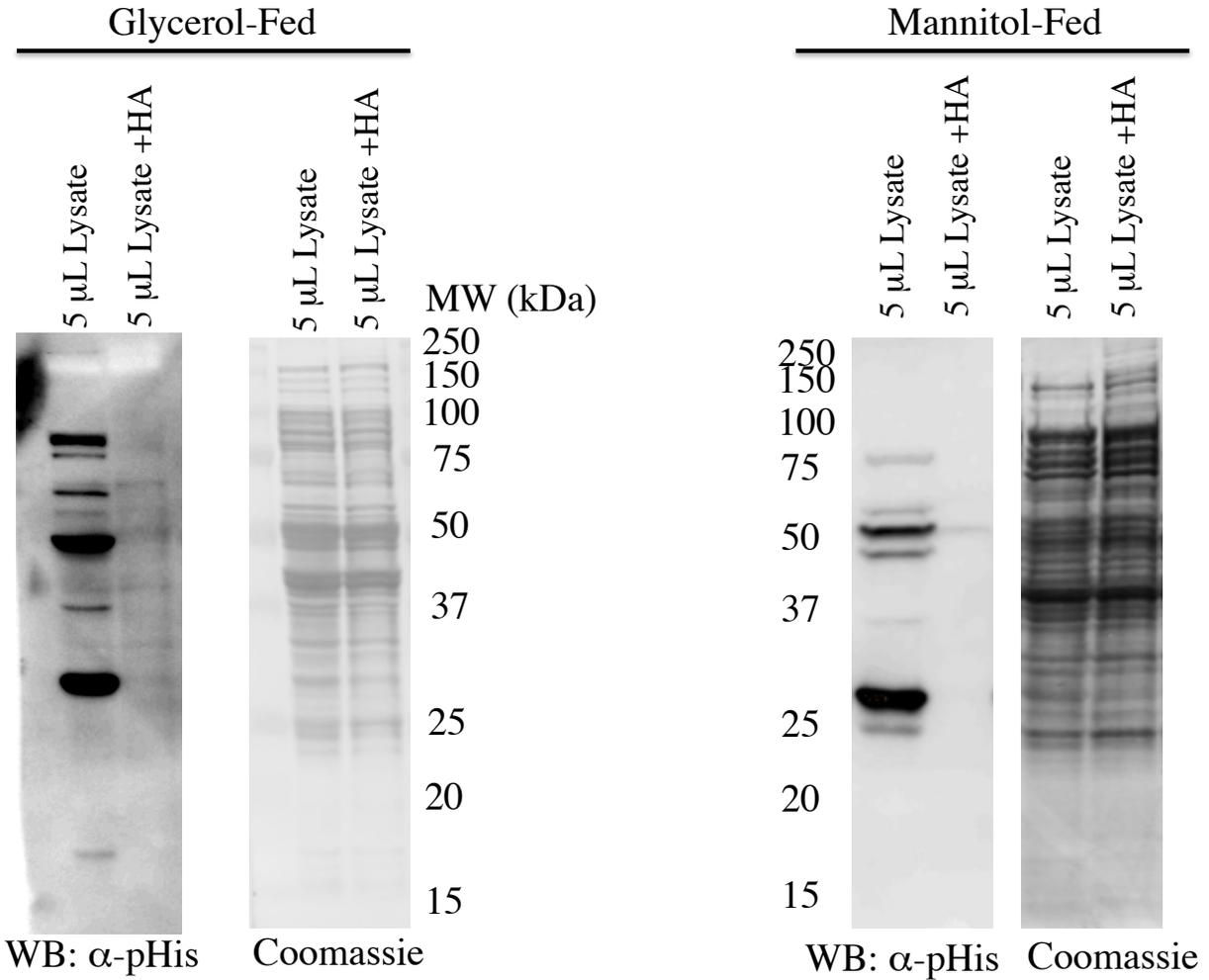


**Figure S15. Immunoprecipitation of pHis peptides spiked into *E. coli* lysate.** a) pHis peptides from the phosphopeptide library were spiked into trypsin-digested *E. coli* lysate (treated with acid overnight to remove endogenous pHis peptides) and then subsequently immunoprecipitated with the  $\alpha$ -pHis antibody. The immunoprecipitated peptides were analyzed by CID MS/MS and the ion intensity for each detected pHis peptide was obtained from the extracted ion chromatogram and graphed for each of the spike-in amounts ( $n = 2$ ; mean  $\pm$  s.d.). b) MS/MS spectra from immunoprecipitated samples were analyzed by the TRIPLET software. Triplet neutral loss positive spectra corresponding to spiked-in pHis peptides were obtained and graphed as a percent of the triplet positive spectra detected in the 1  $\mu$ g spike in sample (black bars). The gray bar indicates the percentage of triplet neutral loss positive spectra detected in a mock immunoprecipitation (-antibody) control experiment.

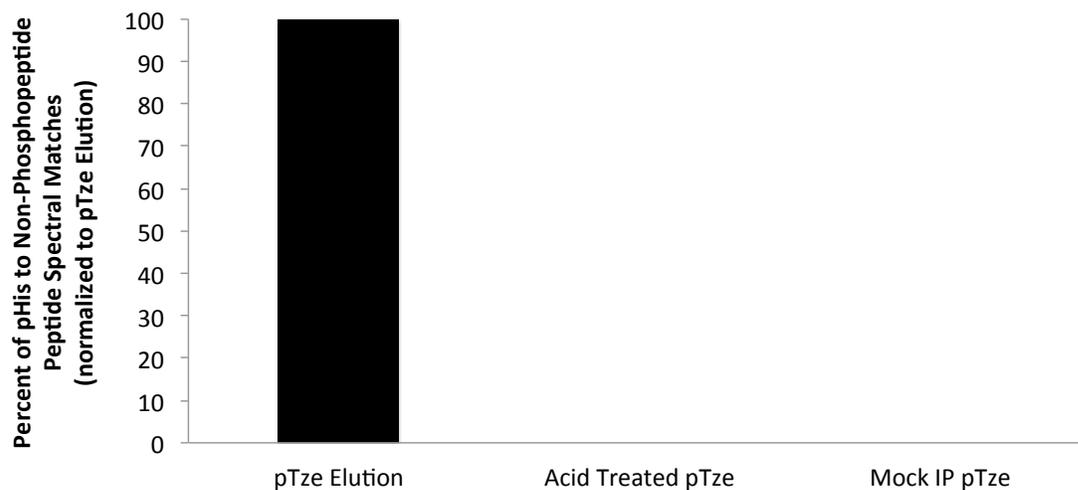


**Figure S16. Use of alternative fragmentation methods to facilitate phosphosite localization.**

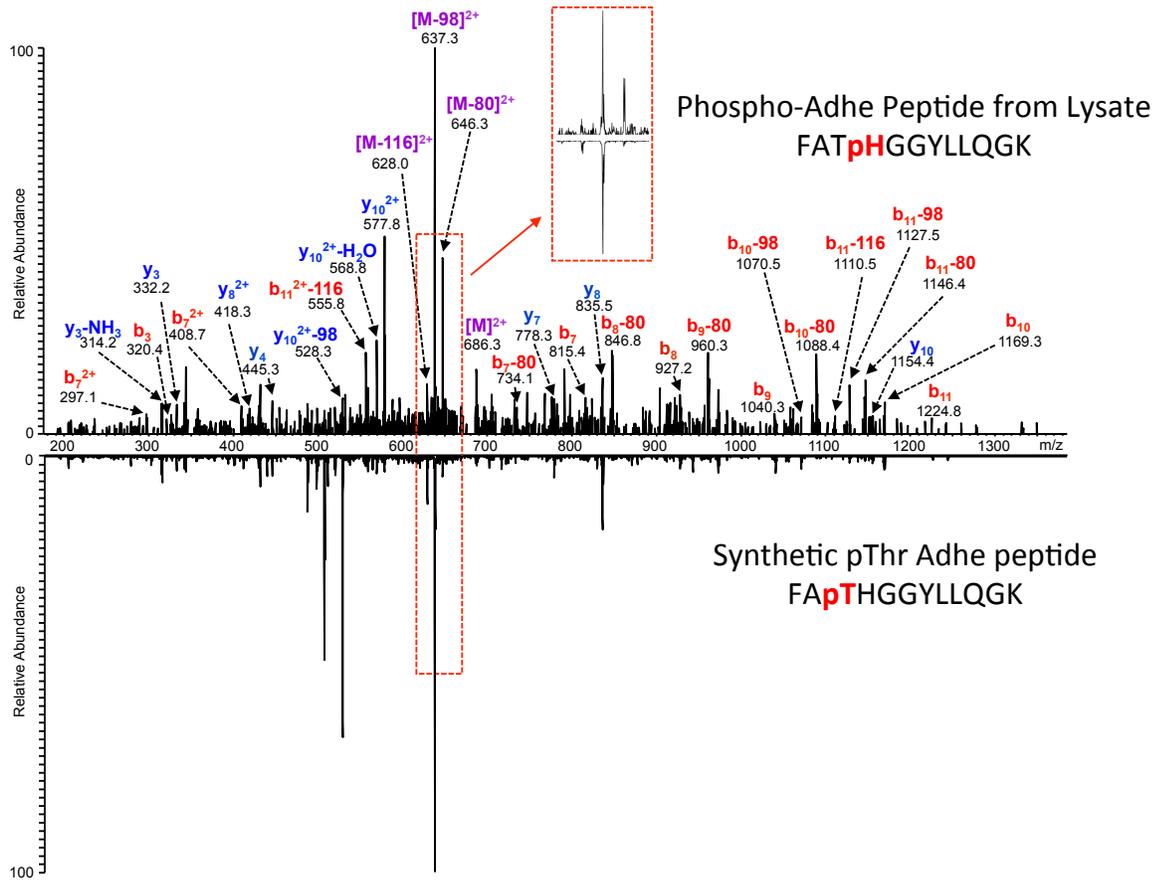
pHis peptides from the phosphopeptide library were analyzed by CID, MSA, HCD, or ETD MS/MS and the PhosphoRS algorithm<sup>1</sup> was used to determine the site of phosphorylation. The percent of correct (100-75% probability), ambiguous (74-40% probability), or false (< 40% probability) phosphosite localizations were calculated for each pHis peptide (correct pHis peptide assignments were known *a priori* from the precursor ion m/z value). The percentages were calculated by dividing the number of correct, ambiguous, or false phosphosite assignments by the total number of phosphosite predictions. Note that in this analysis we only used pHis peptides from the library containing additional sites of phosphorylation (Ser, Thr, or Tyr). The poor ETD results are likely due to our focus on 2+ and 3+ charge state peptides.



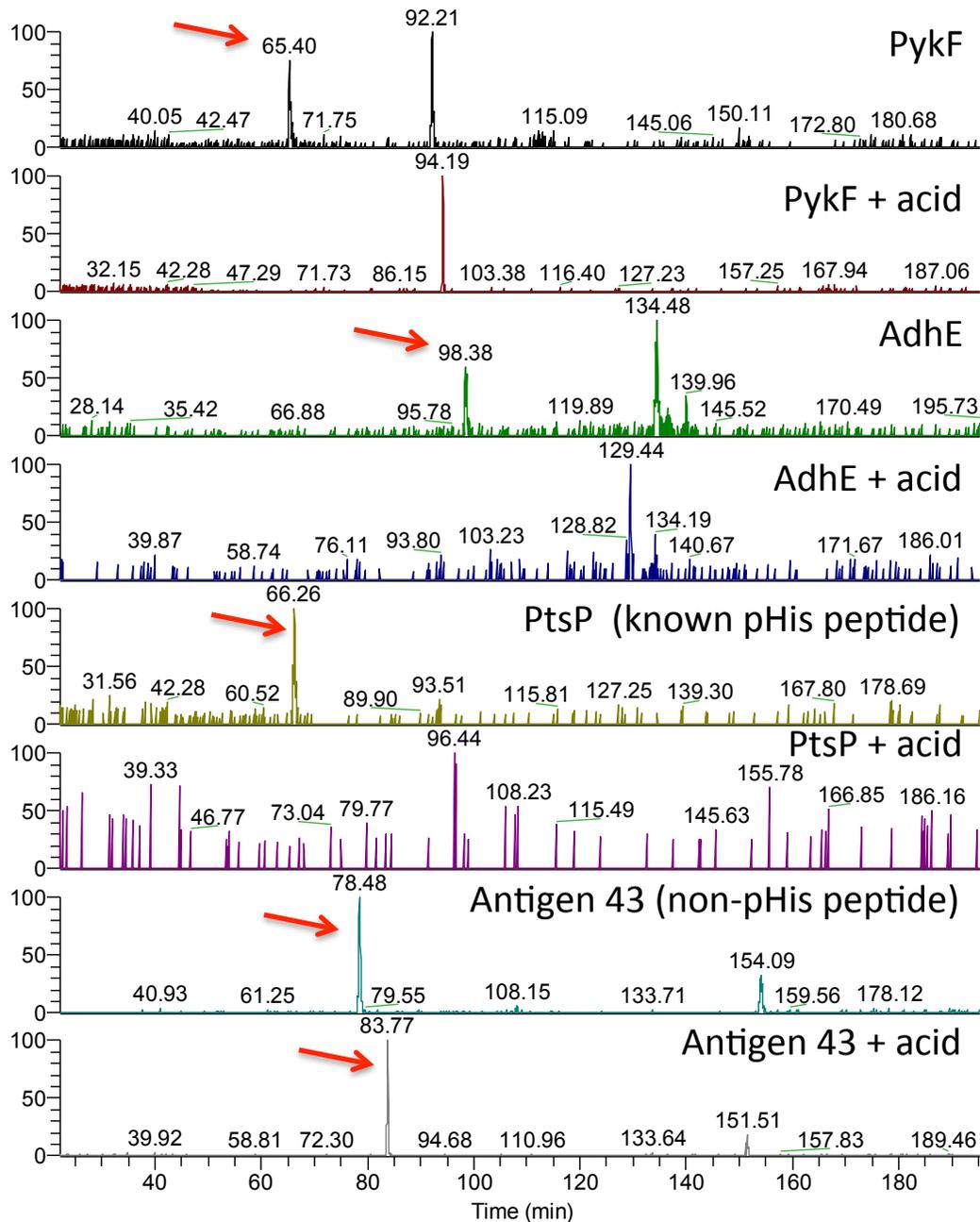
**Figure S17. Western blot of nitrogen limited, glycerol-fed and mannitol-fed *E. coli* lysate.** pHis containing proteins are detected in the *E. coli* lysate but not in *E. coli* lysate treated with 500 mM hydroxylamine (HA). HA treatment results in loss of the pHis mark.<sup>2</sup> The Coomassie stain for each of the blotted membranes shows equal loading.



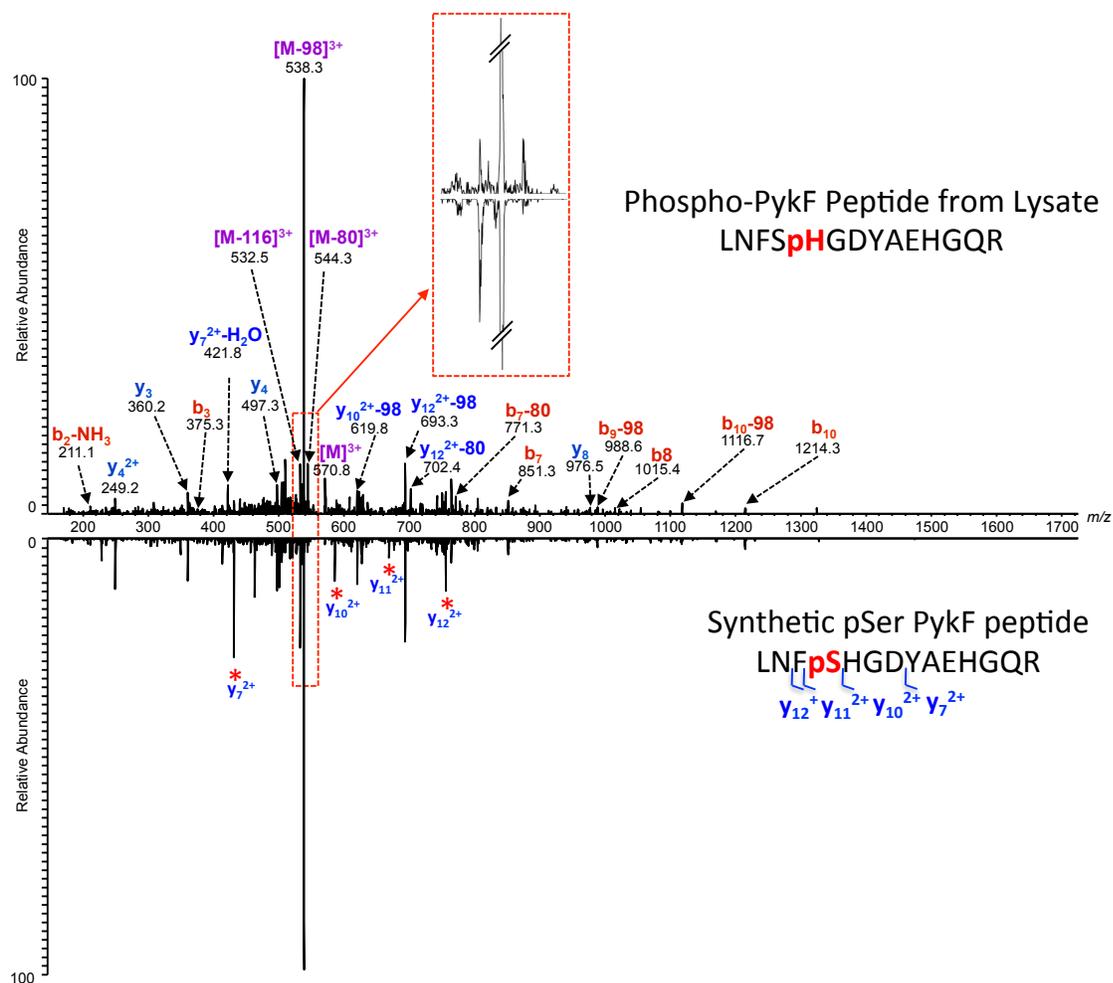
**Figure S18. Immunoprecipitation of nitrogen limited, glycerol-fed *E. coli* peptides with mock and acid treatment controls.** pHis peptides were immunoprecipitated with a pan  $\alpha$ -pHis antibody or no antibody (mock) and then eluted using pTze. The elution samples: pTze elution, pTze elution + overnight acid-treatment in 1% TFA (Acid Treated pTze), and a mock immunoprecipitation control (Mock IP pTze) were analyzed by MS/MS. Peptide spectral matches to known pHis peptides were obtained for each sample and plotted as a percent of the pTze elution sample. No pHis peptides are detected in the acid treated sample indicating that acid treatment removed the pHis mark. Also, no pHis peptides were detected in the mock IP sample indicating that the pan  $\alpha$ -pHis antibody is required for successful enrichment of pHis peptides.



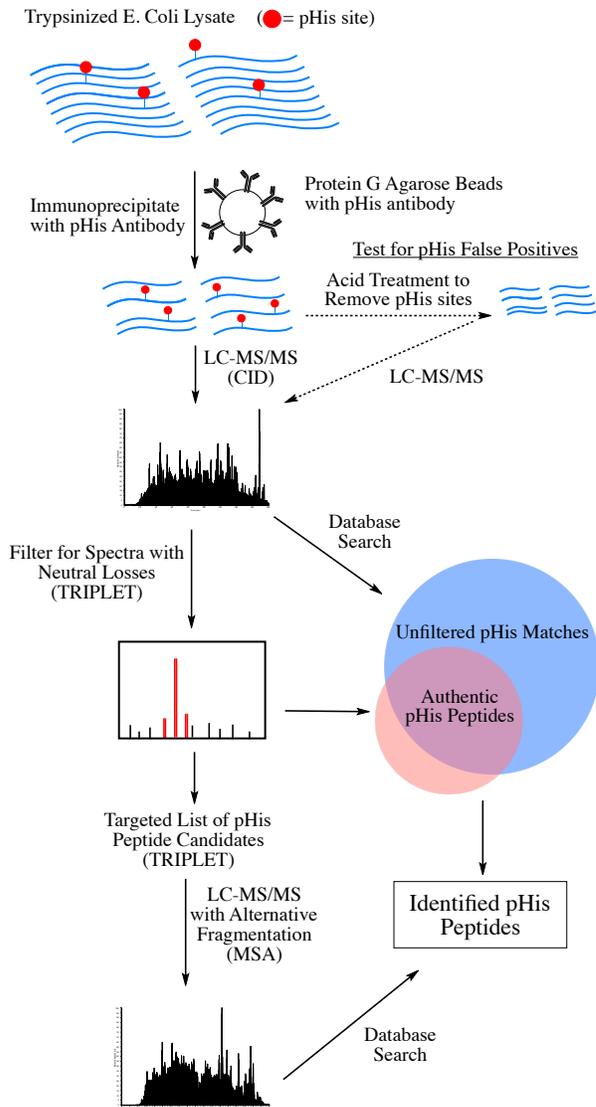
**Figure S19. MS/MS spectra comparison of a pHis AdhE peptide from lysate and a synthetic pThr AdhE peptide.** The synthetic pThr AdhE peptide is reflected as a mirror image to the pHis AdhE peptide identified from the lysate. Note that the majority of the fragments do not match. Inset into the figure is a zoom-in of the triplet neutral loss region showing a difference in the neutral loss species.



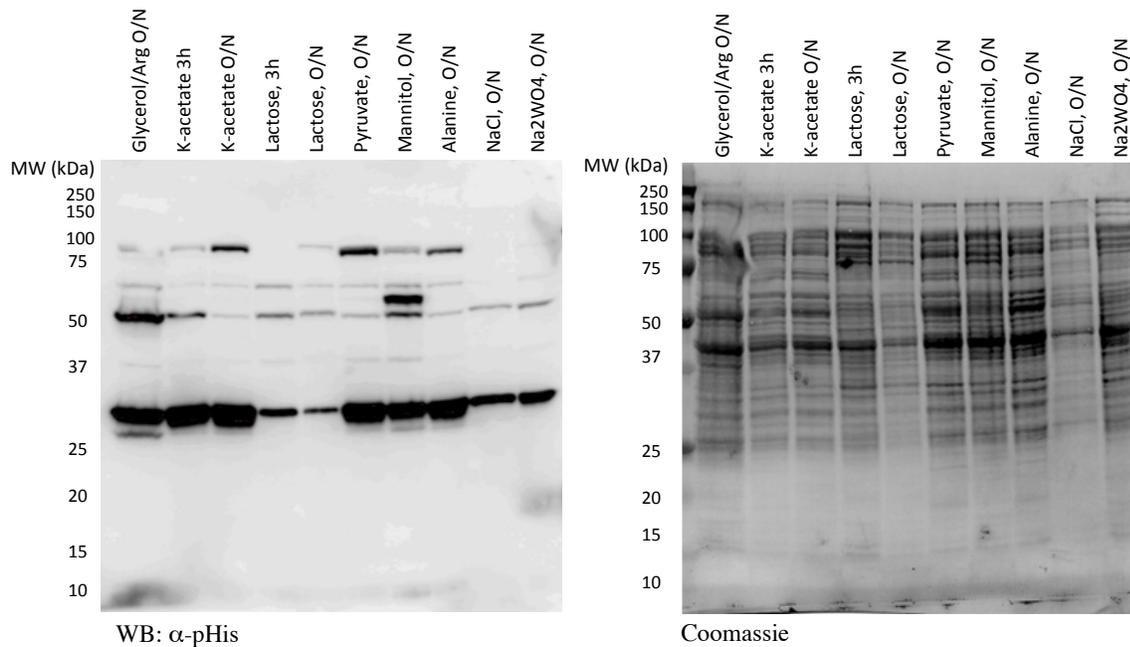
**Figure S20. Extracted ion chromatograms of peptides immunoprecipitated from mannitol-fed *E. coli* lysate.** pHis peptide ions from PykF, AdhE, and PtsP (known pHis peptide) were extracted from the immunoprecipitated mannitol lysate LC-MS/MS chromatogram (peptide peaks are indicated with a red arrow). Overnight acid treatment in 1% TFA followed-by LC-MS/MS re-analysis resulted in complete detection loss of all three peptides. In contrast, a non-pHis peptide identified in the sample from Antigen 43 was still detected post acid treatment.



**Figure S21. MS/MS spectra comparison of a pHis PykF peptide from lysate and a synthetic pSer PykF peptide.** The synthetic pSer PykF peptide is reflected as a mirror image to the pHis PykF peptide identified from the lysate with key differences highlighted. Notable y- ion fragment differences indicative of a pSer peptide are highlighted with a red asterisk in the synthetic peptide spectrum. In the synthetic pSer peptide,  $y_7$ ,  $y_{10}$ ,  $y_{11}$ , and  $y_{12}$  ion fragments are detected without the loss of water, 80 or 98 Da, whereas in the pHis peptide from the lysate, those ions are detected as missing 80 Da ( $y_{12}$ ), 98 Da ( $y_{10}$  and  $y_{11}$ ) or water ( $y_7$ ). Inset into the figure is a zoom-in of the triplet neutral loss region showing a difference in the neutral loss species.



**Figure S22.** Workflow for global phosphohistidine proteomics using pan  $\alpha$ -pHis peptide IP, LC-MS/MS by CID, followed by TRIPILET neutral loss screening and TRIPILET-directed MSA.



**Figure S23. Growth of *E. coli* under different carbon sources causes differences in protein pHis levels.** *E. coli* were grown for 3 hours (3 h) or overnight (O/N) in different carbon sources. The cells were then lysed and analyzed by Western blot for pHis levels (left panel). The coomassie stained membrane is shown as a loading control (right panel).

**Table S1.** Full list of *E. coli* pHis proteins annotated in the SwissProt database (as of November 2013). Those pHis proteins of which pHis sites had been experimentally verified are shown in italics. Proteins highlighted in yellow indicate those observed in our experiment (see Table 2).

	Uniprot Accession #	Protein Name	
PTS system proteins	P32670	Fructose-like phosphotransferase enzyme IIA component (PtsA)	
	P77439	Fructose-like phosphotransferase enzyme IIA component 2 (FryA)	
	<b>P23538</b>	<b><i>Phosphoenolpyruvate synthase (PpsA)</i></b>	
	<b>P37177</b>	<b><i>PTS system enzyme I-Ntr (PtsP)</i></b>	
	P54745	Putative PTS system EIIBC component (HrsA)	
	<b>P09323</b>	<b><i>PTS system N-acetylglucosamine-specific EIICBA component (NagE)</i></b>	
	<b>P00550</b>	<b><i>PTS system mannitol-specific EIICBA component (MtlA)</i></b>	
	P08722	PTS system beta-glucoside-specific EIIBC component (BglF)	
	P32152	Putative PTS system EIIA component (FrvR)	
	<b>P08839</b>	<b><i>Phosphotransferase system, enzyme I (PtsI)</i></b>	
	<b>P37349</b>	<b><i>PTS-dependent dihydroxyacetone kinase, phosphotransferase subunit (DhaM)</i></b>	
	<b>P69811</b>	<b><i>Fructose-specific phosphotransferase enzyme IIA component (FruB)</i></b>	
	<b>P69797</b>	<b><i>PTS system mannose-specific EIIB component (ManX)</i></b>	
	<b>P69783</b>	<b><i>Glucose-specific phosphotransferase enzyme IIA component (Crr)</i></b>	
	<b>P69829</b>	<b><i>Nitrogen regulatory protein Enzyme IIA-NTR (PtsN)</i></b>	
	P42909	N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1 (AgaB)	
	P42904	N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 2 (AgaV)	
	P69820	Ascorbate-specific phosphotransferase enzyme IIA component (UlaC)	
	P69828	Galactitol-specific phosphotransferase enzyme IIA component (GatA)	
	P32155	Fructose-like phosphotransferase enzyme IIA component (FrvA)	
	P69824	Mannitol-specific cryptic phosphotransferase enzyme IIA component (CmtB)	
	P36881	Putative phosphotransferase enzyme IIA component (YadI)	
	P39363	Putative phosphotransferase IIA component (SgcA)	
	P05706	Glucitol/sorbitol-specific phosphotransferase enzyme IIA component (SrlB)	
	P69791	N,N'-diacetylchitobiose-specific phosphotransferase enzyme IIA component (ChbA)	
	P0A9N0	Phosphocarrier protein NPr (PtsO)	
	<i>P0AA04</i>	<i>Phosphocarrier protein HPr (PtsH)</i>	
	Histidine kinases	P30855	Sensor protein (EvgS)
		P14376	Capsular synthesis regulator component C (RcsC)
		P0AEC5	Signal transduction histidine-protein kinase (BarA)
		P39453	Sensor protein (TorS)
		P21865	Sensor protein (KdpD)
<i>P39838</i>		<i>Sensor-like histidine kinase (RcsD)</i>	
<i>P0AEC3</i>		<i>Aerobic respiration control sensor protein (ArcB)</i>	
<i>P07363</i>		<i>Chemotaxis protein (CheA)</i>	
Q06067		Signal transduction histidine-protein kinase (AtoS)	
P0AFA2		Nitrate/nitrite sensor protein (NarX)	
P27896		Nitrate/nitrite sensor protein (NarQ)	
P0AA93		Sensor histidine kinase (YpdA)	
P77510		Sensor histidine kinase (DpiB or CitA)	
<i>P0AEC8</i>		<i>Fumarate sensor histidine kinase (DcuS)</i>	
P09835		Sensor protein (UhpB)	
<i>P23837</i>		<i>Sensor protein (PhoQ)</i>	
P77485		Sensor kinase (CusS)	
P52101		Sensor histidine kinase (GlrK)	
P08401		Sensor protein (CreC)	
P30847		Signal transduction histidine-protein kinase (BaeS)	
P14377		Sensor protein (ZraS)	
P0AE82		Sensor protein (CpxA)	
P76339		Probable sensor-like histidine kinase (YedV)	
<i>P0AEJ4</i>		<i>Osmolarity sensor protein (EnvZ)</i>	
P40719		Sensor protein (QseC)	
P18392		Sensor protein (RstB)	
P08400		Phosphate regulon sensor protein (PhoR)	
P30844		Sensor protein (BasS)	
<i>P0AFB5</i>		<i>Nitrogen regulation protein NR(II) (GlnL)</i>	
Other metabolic proteins		P00936	Adenylate cyclase (CyaA)
		<i>P0A7B1</i>	<i>Polyphosphate kinase (Ppk)</i>
		<b>P0AGE9</b>	<b><i>Succinyl-CoA synthetase subunit alpha (SucD)</i></b>
	<b>P62707</b>	<b><i>2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (GpmA)</i></b>	
	P0A7A2	Probable phosphoglycerate mutase (GpmB)	
	P52086	Alpha-ribazole phosphatase (CobC)	
<i>P0A763</i>	<i>Nucleoside diphosphate kinase (Ndk)</i>		

## General Materials

All buffering salts were purchased from Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA) was purchased from Halocarbon (North Augusta, SC). Dimethyl formamide (DMF), dichloromethane (DCM), piperidine,  $\beta$ -mercaptoethanol (BME), Coomassie brilliant blue, *N,N*-diisopropylethylamine (DIPEA), bovine serum albumin (BSA), hydroxylamine (HA), dithiothreitol (DTT), iodoacetamide, arginine, ammonium chloride, and Fmoc-Ala-OH-2,3,3,3- $d_4$  (product number: 616044) were purchased from Sigma-Aldrich (St. Louis, MO). HATU and HBTU were purchased from GenScript (Piscataway, NJ). HOBt and *N*-methyl pyrrolidone (NMP) were purchased from AGTC Bioproducts (Wilmington, MA). Wang resin, Fmoc-Arg(Pbf) Wang resin, Fmoc-Lys(Boc) Wang resin, and all amino acids used in peptide synthesis (unless otherwise noted) were purchased from Novabiochem (Hohenbrunn, Germany). Fmoc-Ala-OH-2,3,3,3- $d_4$  was purchased Bachem (Bubendorf, Switzerland). SulfoLink resin, ECL substrate, nitrocellulose membrane, and Protein G agarose resin were purchased from Thermo Scientific (Rockford, IL). Criterion XT 12% Tris TGX gels, goat anti rabbit HRP secondary antibody, Colloidal Gold Total Protein Stain, and 0.2  $\mu$ m PVDF membrane were purchased from BioRad (Hercules, CA). Goat anti-rabbit 800CW and goat anti-mouse 680RD secondary antibodies were from Li-Cor. Spin columns were from Sartorius Stedim Biotech.  $H_2^{18}O$  was purchased from Cambridge isotope laboratories (Tewksbury, MA). 4G10 monoclonal pTyr antibody was from Millipore (Billerica, MA). Complete protease inhibitor tablets were purchased from Roche Diagnostics (Mannheim, Germany). Trypsin (Sequencing grade modified trypsin) was from Promega. NCM 3722 *E. coli* strain<sup>3</sup> was a kind gift from the Rabinowitz lab (Lewis-Sigler Institute for Integrative Genomics, Princeton University, New Jersey, USA).

## General Methods

Analytical RP-HPLC was performed on Agilent 1100 and 1200 series instruments equipped with a C18 Vydac column (5  $\mu$ m, 4.6 x 150 mm) at a flow rate of 1 mL/min. All analytical RP-HPLC 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 analytical RP-HPLC 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). We performed preparative RP-HPLC on a Waters prep LC system that consisted of a Waters 2545 Binary Gradient Module and a Waters 2489 UV detector. A C18 Vydac 218TP1022 column (10  $\mu$ m; 22 x 250 mm) was used for sample purifications at a flow rate of 18 mL/min. All preparative RP-HPLC 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 preparative RP-HPLC runs a five-minute isocratic period in initial conditions was followed by a 60-minute linear gradient with increasing solvent B concentration. Electrospray ionization mass spectrometric analysis (ESI-MS) was performed on synthesized peptides by direct infusion on a Bruker Daltonics MicrOTOF-Q II mass spectrometer. Western blot membranes were imaged using a GE ImageQuant LAS 4010 Imager or a Li-COR Odyssey Infrared Imager. Coomassie stained membranes were imaged on an Epson Scanner. Cells were lysed using a Branson Sonifier. Samples were lyophilized on a Millrock technology MD85 lyophilizer.

## Synthesis of Potassium Phosphoramidate

Synthesis of potassium phosphoramidate was prepared as described previously.<sup>2</sup>

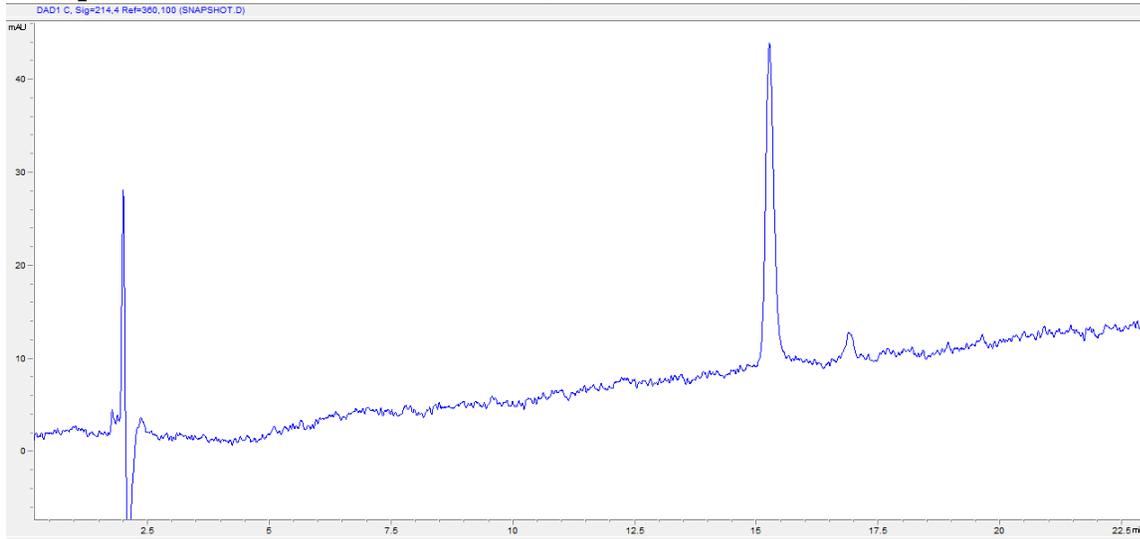
## Preparation of pHis peptides for Figure 2a and 2b

The peptides corresponding to the individual MS/MS spectra in Figures 2a and 2b were obtained as described below or in Kee et al.<sup>2</sup> MS/MS analysis of each peptide was performed as described below.

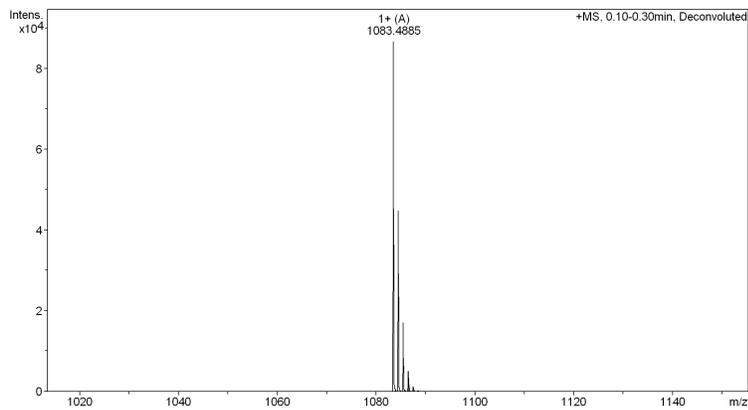
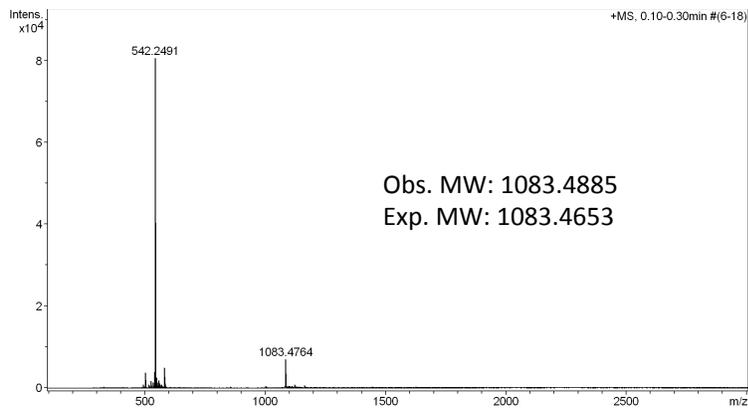
## Synthesis of H-TSpHTSIMAR-OH

The peptide was synthesized on an Fmoc-Arg (Pbf) pre-loaded Wang resin (Novabiochem) on a 0.1 mmol scale using an automated peptide synthesizer (CEM Liberty) via Fmoc chemistry. Fmoc deprotections were carried out using a 20% piperidine in DMF solution (5 mL) at 75 °C under 30 W microwave power for 30 s, followed by an additional 3 min at 30 W. Amino acids were coupled using HBTU/HOBt/DIPEA as the activating agents. This was done by dissolving the desired amino acid (0.5 mmol) in DMF (2.5 mL) and then adding it to the reaction vessel followed by subsequent addition of HBTU (0.49 mmol) and HOBt (0.49mmol) in DMF (1 mL), and DIPEA (1 mmol) in NMP (0.5 mL). The reaction mixture was then heated to 75°C with 20W power for 5 min. For coupling of the histidine residue, the reaction was carried out at 75°C with 30W power for 3 min. The peptide was cleaved from the resin by treating with a mixture of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5) for 1 h at r.t. The cleaved peptide was precipitated in ice-cold ethyl ether. The peptide precipitate was then dissolved in 100% solvent A and separated from the resin. The crude peptide was then purified by preparative RP-HPLC. Phosphorylation on histidine was carried out using potassium phosphoramidate which is known to selectively label histidine residues.<sup>4,5,6</sup> This was done by dissolving the peptide in 100 mM Tris pH 8.0 to a final concentration of 2.3 mM and treating with 90 mM potassium phosphoramidate for 36 hours at r.t. The phosphopeptide was purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. The purified peptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

### H-TSpHTSIMAR-OH RP-HPLC Trace (5-25%B over 30 min, 1 mL/min, 214 nm detection)



### H-TSpHTSIMAR-OH ESI-MS (Calculated MW: 1083.4653, Observed MW: 1083.4885)



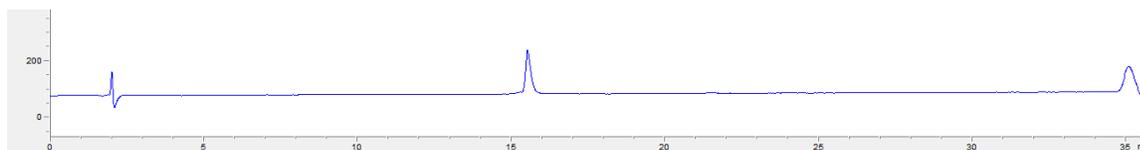
### **<sup>18</sup>O-Labeling of H-TSpHTSIMAR-OH**

10 ng of **H-TSpHTSIMAR-OH** was added to 10  $\mu$ L of 10 mM Tris pH 8 and lyophilized to dryness. The sample was resuspended in 10  $\mu$ L of H<sub>2</sub><sup>18</sup>O followed by addition of 10 ng of trypsin. The sample was then incubated overnight at r.t and analyzed by LC-MS/MS as described below.

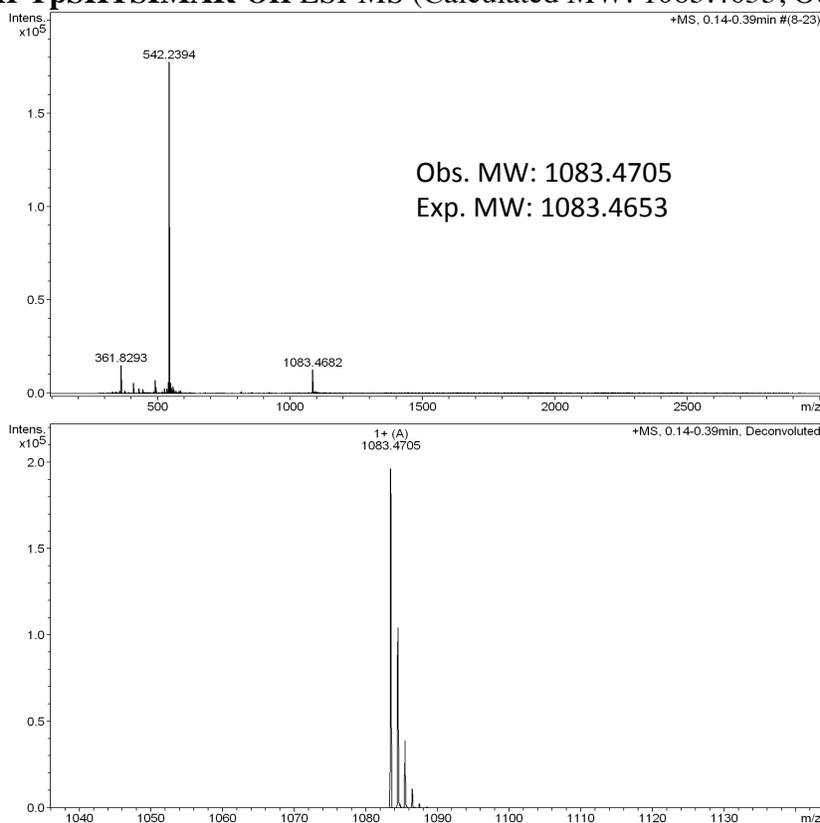
### **Synthesis of H-TpSHTSIMAR-OH**

The peptide sequence HTSIMAR was synthesized on an Fmoc-Arg (Pbf) pre-loaded Wang resin on a 0.1 mmol scale using an automated peptide synthesizer (CEM Liberty) via Fmoc chemistry as described above for **H-TSpHTSIMAR-OH**. The remaining amino acids were added manually using Fmoc chemistry. For manual Fmoc chemistry, deprotections were carried out using 20% piperidine in DMF (5 mL) at r.t. with N<sub>2</sub> bubbling for 20 min. Amino acids were coupled using HBTU/HOBt/DIPEA as the activating agents. This was done by dissolving the desired amino acid (0.16 mmol) in DMF (1.0 mL) followed by addition of HBTU (0.15 mmol) and HOBt (0.15mmol). DIPEA (0.33 mmol) was then added and the reaction mixture was then added to the reaction vessel and incubated for 30 min at r.t. with N<sub>2</sub> bubbling. For phosphoserine, the monobenzyl-protected phosphoamino acid was used and HATU was used in place of HBTU/HOBt. Also, following the coupling of phosphoserine, subsequent deprotection reactions were performed for 10 min and the resin was washed with 3 x 5 mL 5% DIPEA in DMF prior to the next coupling reaction to promote piperidine counter ion exchange. The peptide was cleaved from the resin by treating with a mixture of trifluoroacetic acid, triisopropylsilane, water, ethanedithiol (94:2.5:2.5:1) for 1 h at r.t. The cleaved peptide was precipitated in ice-cold ethyl ether, dissolved in 100% solvent A and separated from the resin. The crude peptide was then purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified peptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

### **H-TpSHTSIMAR-OH RP-HPLC Trace (5-25%B over 30 min, 1 mL/min, 214 nm detection)**



## H-TpSHTSIMAR-OH ESI-MS (Calculated MW: 1083.4653, Observed MW: 1083.4705)



### <sup>18</sup>O-Labeling of H-TpSHTSIMAR-OH

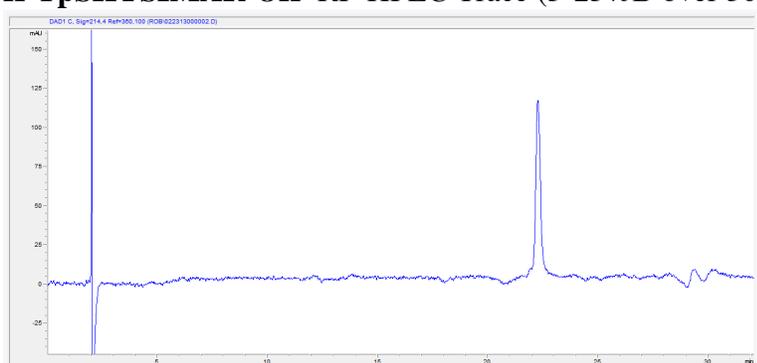
30 ng of H-TpSHTSIMAR-OH was added to 10  $\mu$ L of 10 mM Tris pH 8 and lyophilized to dryness. The sample was resuspended in 10  $\mu$ L of H<sub>2</sub><sup>18</sup>O followed by addition of 50 ng of trypsin. The sample was incubated overnight at r.t. and then analyzed by LC-MS/MS as described below.

### Synthesis of H-TpSHYSIMAR-OH, H-TSHpYSIMAR-OH, H-TSpHYSIMAR-OH

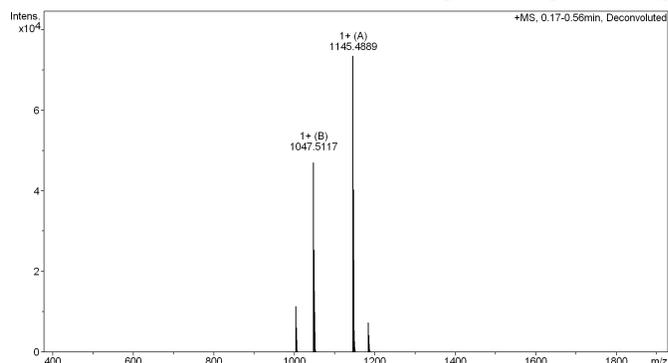
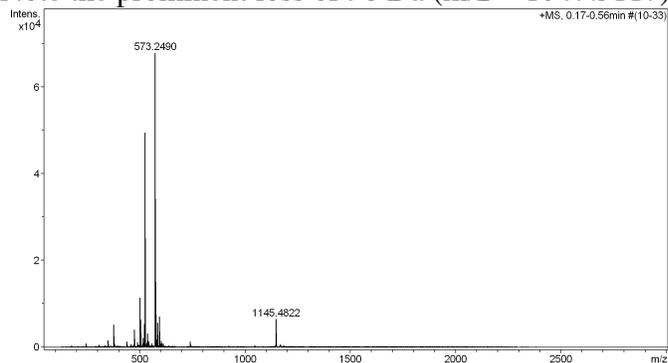
The peptide sequence SIMAR was synthesized on an Fmoc-Arg (Pbf) pre-loaded Wang resin on a 0.1 mmol scale using an automated peptide synthesizer (CEM Liberty) via Fmoc chemistry as described above for H-TSpHTSIMAR-OH. The resin was then split equally into three separate reaction vessels and the remaining sequences were added manually using Fmoc chemistry. For manual Fmoc chemistry, deprotections were carried out using a 20% piperidine in DMF solution (5 mL) at r.t. with N<sub>2</sub> bubbling for 20 min. Amino acids were coupled using HBTU/HOBt/DIPEA as the activating agents. This was done by dissolving the desired amino acid (0.16 mmol) in DMF (1.0 mL) followed by addition of HBTU (0.15 mmol) and HOBt (0.15 mmol). DIPEA (0.33 mmol) was then added and the reaction mixture was then added to the reaction vessel and incubated for 30 min at r.t. with N<sub>2</sub> bubbling. For phosphoserine and

phosphotyrosine residues, the monobenzyl-protected phosphoamino acids were used and HATU was used in place of HBTU/HOBT. Also, following the coupling of phosphoamino acids, subsequent deprotection reactions were performed for 10 min and the resin was washed with 3 x 5 mL 5% DIPEA in DMF prior to the next coupling reaction to promote piperidine counter ion exchange. The peptide was cleaved from the resin by treating with a mixture of trifluoroacetic acid, triisopropylsilane, water, ethanedithiol (94:2.5:2.5:1) for 1 h at r.t. The cleaved peptide was precipitated in ice-cold ethyl ether, dissolved in 100% solvent A and separated from the resin. For **H-TpSHYSIMAR-OH** and **H-TSHpYSIMAR-OH**, the crude peptides were then purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified peptides were analyzed by analytical RP-HPLC and ESI-MS to confirm their identity.

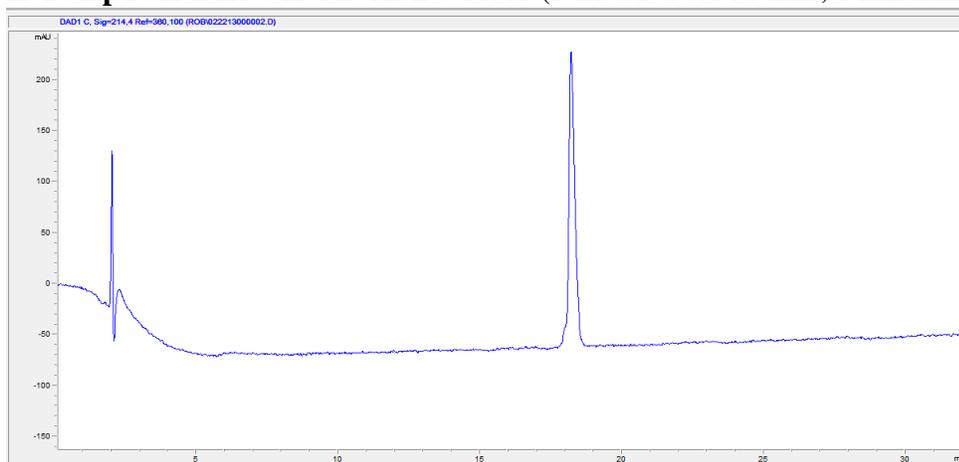
**H-TpSHYSIMAR-OH** RP-HPLC Trace (5-25%B over 30 min, 1 mL/min, 214 nm detection)



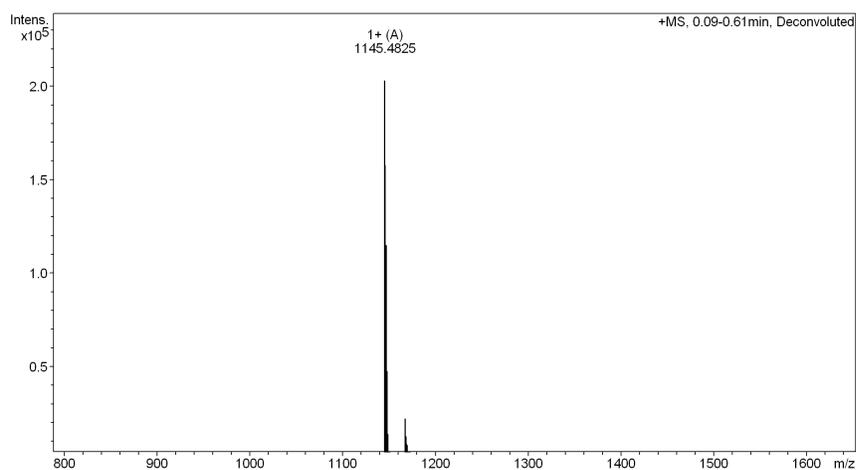
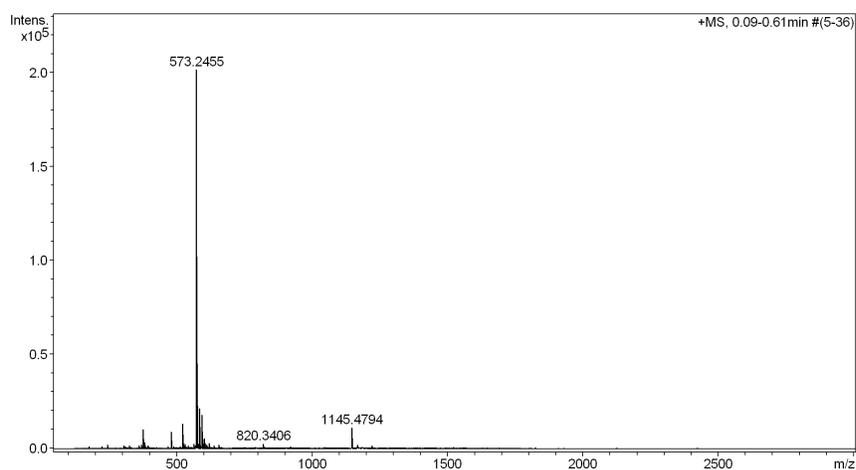
**H-TpSHYSIMAR-OH** ESI-MS (Calculated MW: 1145.4809, Observed MW: 1145.4889)  
 Note the prominent loss of 98 Da ( $m/z = 1047.5117$ ).



### H-TSHpYSIMAR-OH RP-HPLC Trace (5-25%B over 30 min, 1 mL/min, 214 nm detection)

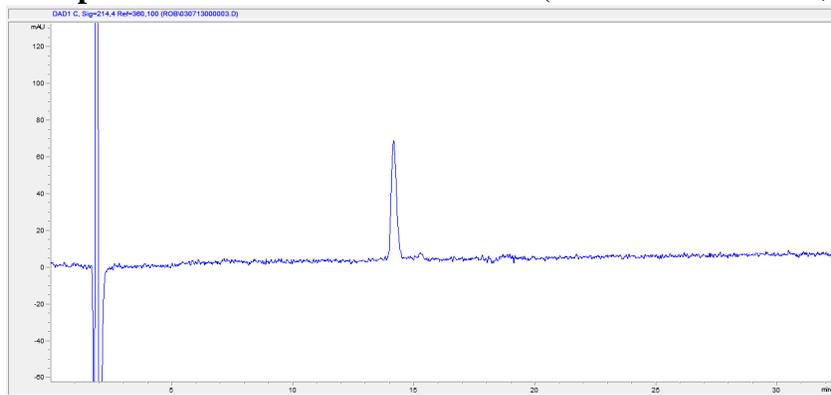


### H-TSHpYSIMAR-OH ESI-MS (Calculated MW: 1145.4809, Observed MW: 1145.4825)

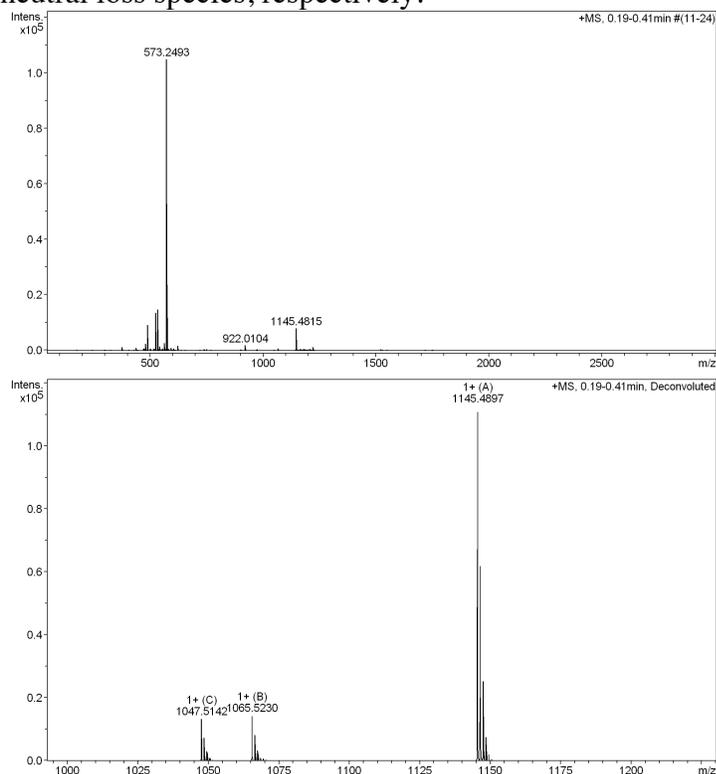


For **H-TSpHYSIMAR-OH**, the crude peptide was purified by preparative RP-HPLC. Phosphorylation on histidine was carried out using potassium phosphoramidate which is known to selectively label histidine residues.<sup>4,5,6</sup> For phosphorylation on the histidine residue, the peptide was dissolved in 100mM Tris pH 8.0 to a final concentration of 560  $\mu$ M and treated with 35 mM potassium phosphoramidate for 96 h at r.t. The phosphopeptide was purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified phosphopeptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

### **H-TSpHYSIMAR-OH** RP-HPLC Trace (5-25%B over 30 min, 1ml/min, 214nm detection)



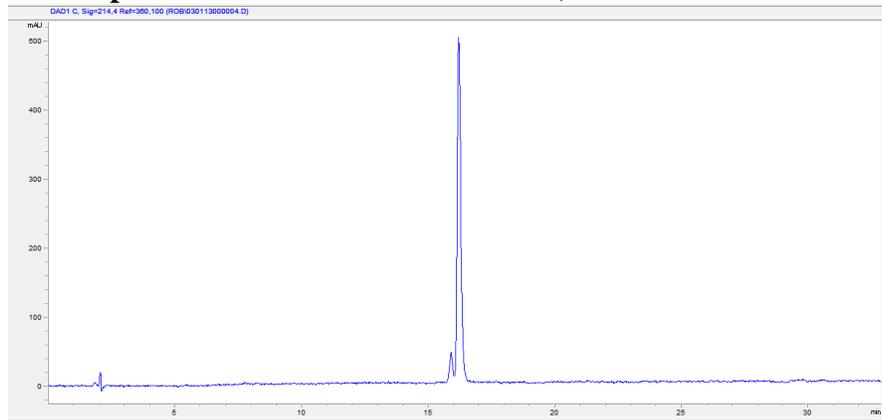
**H-TSpHYSIMAR-OH** ESI-MS (Calculated MW: 1145.4809, Observed MW: 1145.4897). Note that 1047.5142 and 1065.5230 in the deconvoluted MS spectra are from the 98 and 80 Da neutral loss species, respectively.



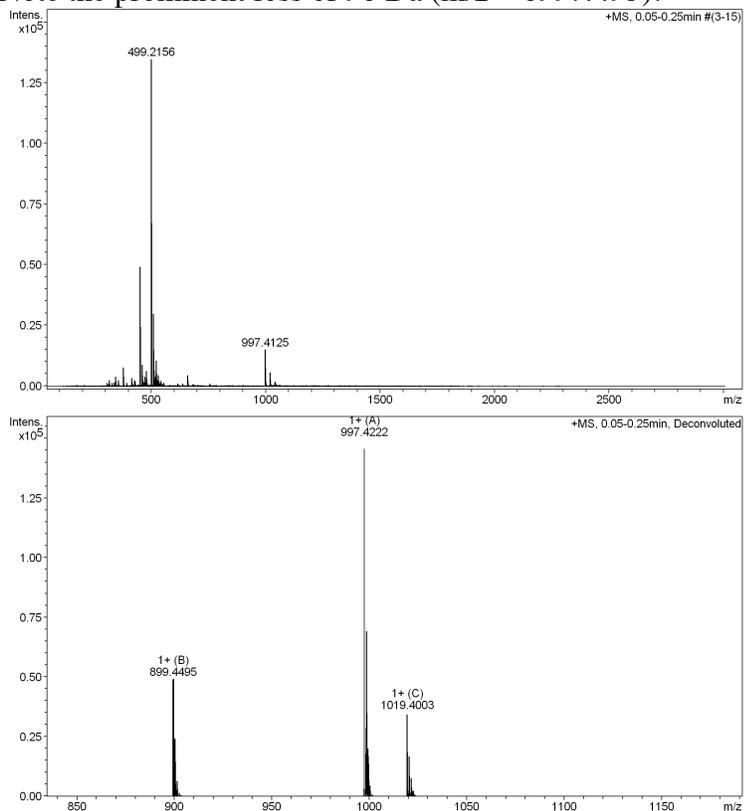
## Synthesis of H-AAApSHYAAR-OH, H-AAASHpYAAR-OH, H-AAASpHYAAR-OH

The synthesis of these peptides was carried out using a similar Fmoc chemistry method as described above for H-TpSHYSIMAR-OH, H-TSHpYSIMAR-OH, and H-TSpPHYSIMAR-OH. For H-AAApSHYAAR-OH and H-AAASHpYAAR-OH, the crude peptides were then purified by analytical RP-HPLC and used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified peptides were analyzed by analytical RP-HPLC and ESI-MS to confirm their identity.

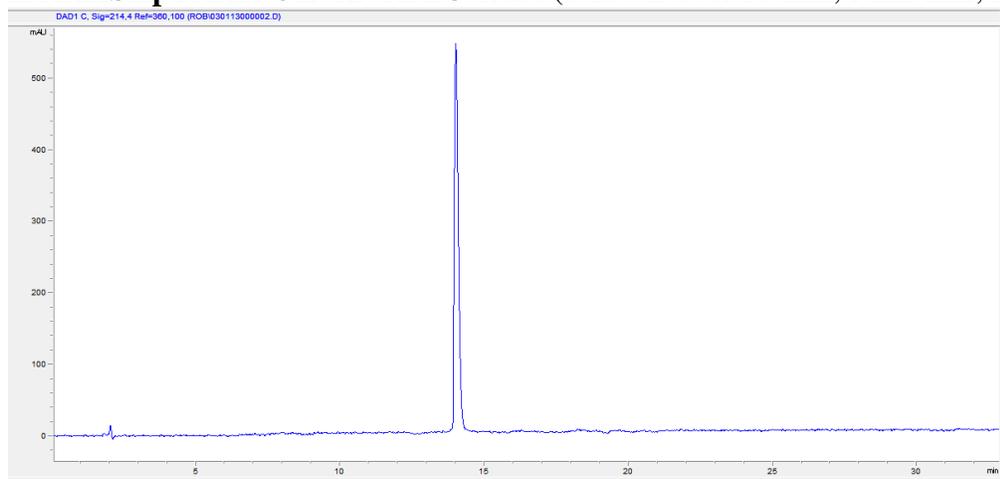
### H-AAApSHYAAR-OH RP-HPLC Trace (5-25%B over 30 min, 1mL/min, 214nm detection)



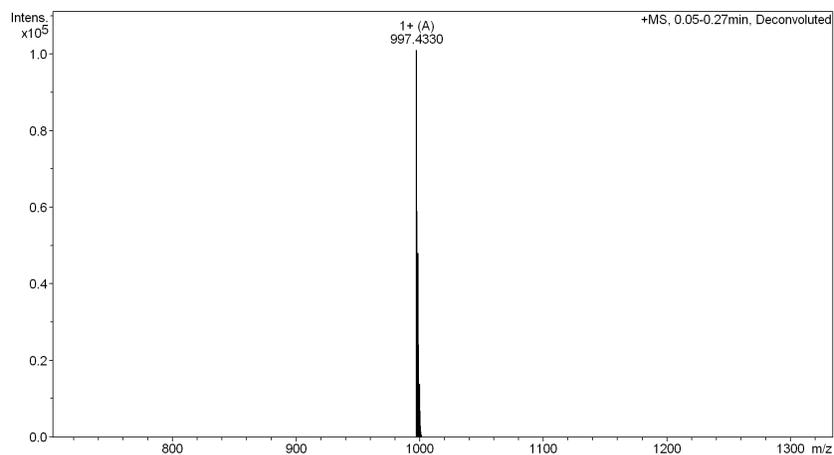
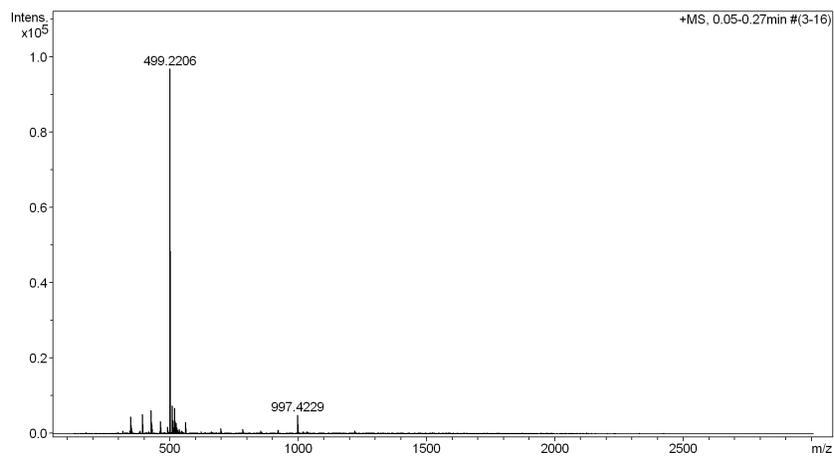
### H-AAApSHYAAR-OH ESI-MS (Calculated MW: 997.4251, Observed MW: 997.4222) Note the prominent loss of 98 Da ( $m/z = 899.4495$ ).



### H-AAASHpYAAR-OH RP-HPLC Trace (0-25%B over 30 min, 1 mL/min, 214 nm detection)

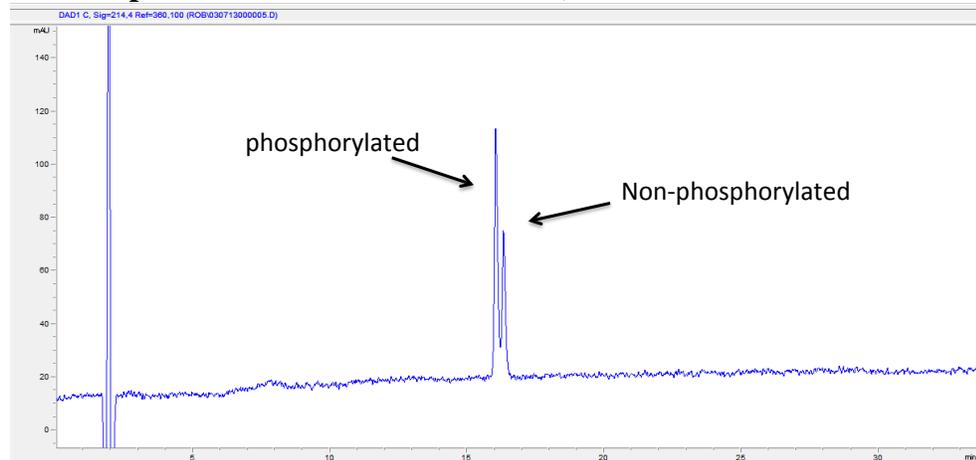


### H-AAASHpYAAR-OH ESI-MS (Calculated MW: 997.4251, Observed MW: 997.4330)

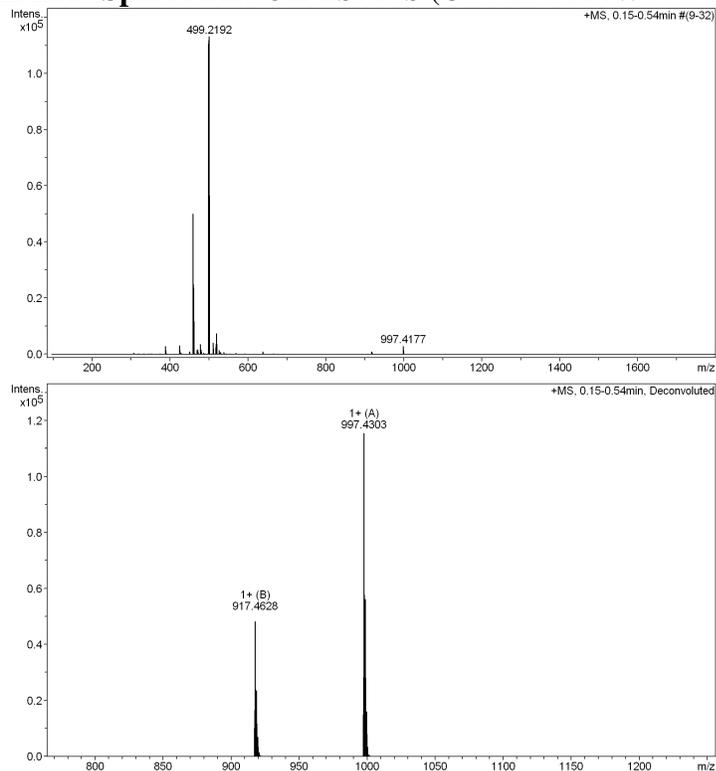


For **H-AAASpHYAAR-OH**, the crude peptide was purified by preparative RP-HPLC prior to histidine phosphorylation. Phosphorylation on histidine was carried out using potassium phosphoramidate which is known to selectively label histidine residues.<sup>4,5,6</sup> The peptide was dissolved in 100 mM Tris pH 8.0 to a final concentration of 1.1 mM and treated with 64 mM potassium phosphoramidate for 96 h at r.t. The phosphopeptide was purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified phosphopeptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

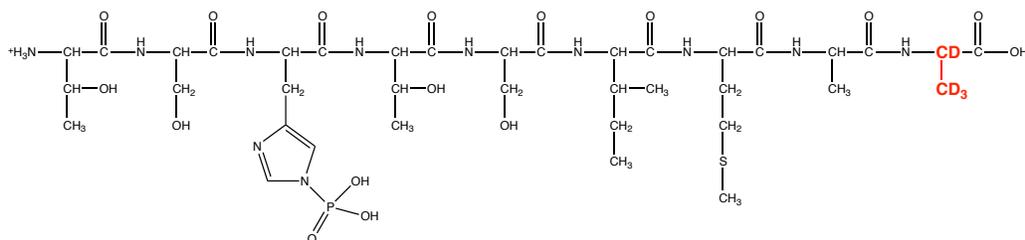
#### **H-AAASpHYAAR-OH** RP-HPLC Trace (0-25%B over 30 min, 1 mL/min, 214 nm detection)



#### **H-AAASpHYAAR-OH** ESI-MS (Calculated MW: 997.4251, Observed MW: 997.4303)

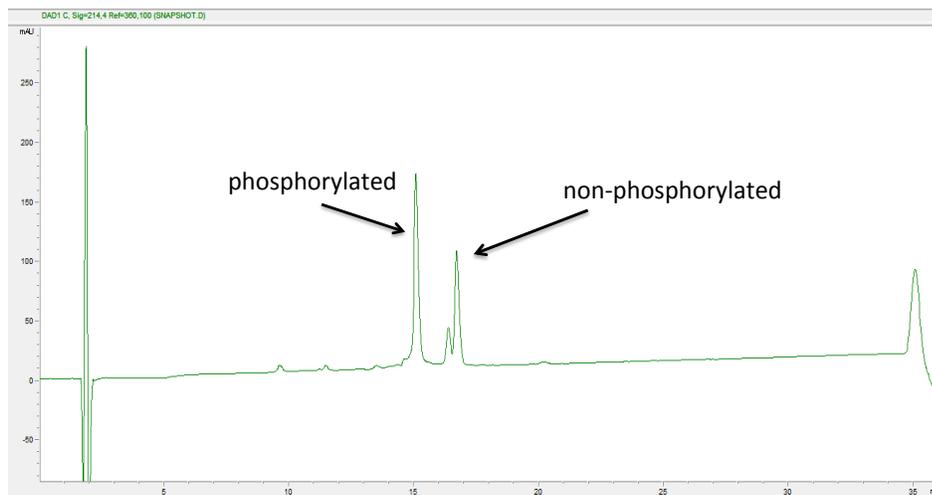


## Synthesis of C-terminal deuterated PtsI analog H-TSpHYSIMA(d<sub>4</sub>-A)-OH

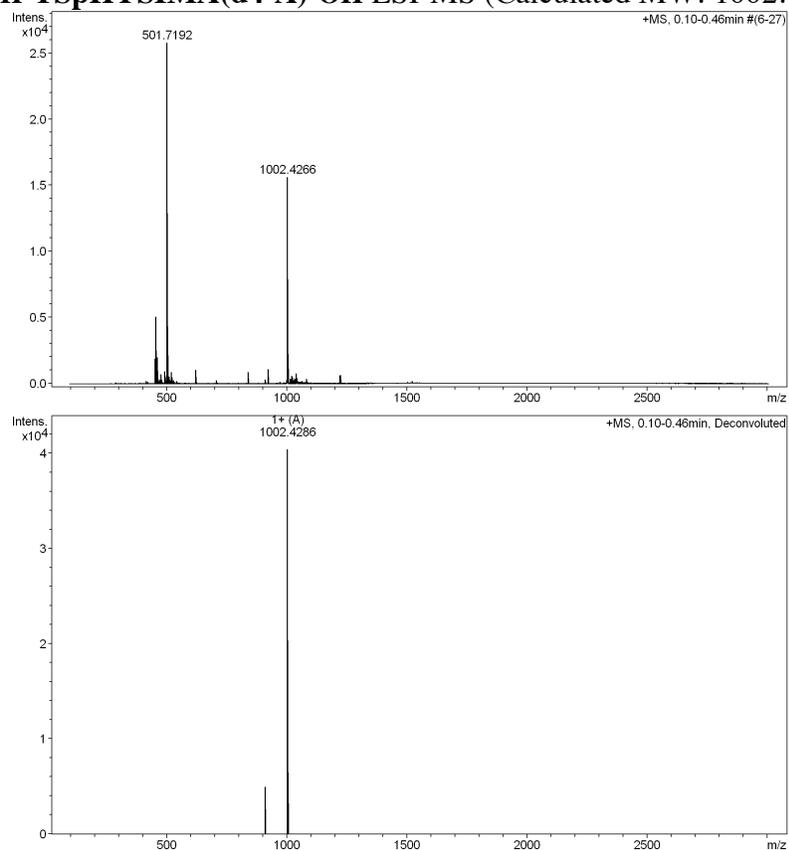


Deuterated alanine was coupled onto Wang resin using MSNT/MeIm. Briefly, 0.08 g Wang resin (0.1 mmol) was washed and swelled in DCM. The resin was then covered with DCM and flushed with nitrogen gas. 5 equivalents (0.5 mmol, 157 mg) of Fmoc-protected deuterated alanine was transferred to a glass vial and dissolved in 1.5 mL DCM with a few drops of THF. MeIm (15 eq., 1.5 mmol, 120  $\mu$ L) was added to the DCM solution followed by 5 eq. of MSNT (0.5 mmol, 150 mg). The sample was then stirred at r.t. until all of the components were fully dissolved. The reaction mixture was then added to the swelled resin and the sample was then incubated at r.t. for 1 h. After stirring for 1 h, another 3 equivalents of deuterated alanine, 3 equivalents of MSNT, and 10 eq. of MeIm in 1 ml DCM were added to the reaction vessel. The reaction was then incubated for 1.5 h at r.t. with N<sub>2</sub> bubbling. After 2.5 h total reaction time, the resin was washed with DMF and deprotected using 20% piperidine in DMF. The remaining amino acids were coupled to the resin using Fmoc chemistry. For manual Fmoc chemistry, deprotections were carried out using a 20% piperidine in DMF solution (5mL) at r.t. with N<sub>2</sub> bubbling for 20 min. Amino acids were coupled using HATU/DIPEA as the activating agents. This was done by dissolving the desired amino acid (0.12 mmol) in DMF (1.0 mL) followed by addition of HATU (0.1 mmol) and DIPEA (1 mmol). The reaction mixture was then added to the reaction vessel and incubated for 30 min at r.t. with N<sub>2</sub> bubbling. The peptide was cleaved from the resin by treating with a mixture of trifluoroacetic acid, triisopropylsilane, water, ethanedithiol (94:2.5:2.5:1) for 1 h at r.t. The cleaved peptide was precipitated in ice-cold ethyl ether. The peptide precipitate was then dissolved in 100% solvent A and separated from the resin. The crude peptide was then purified by preparative RP-HPLC prior to histidine phosphorylation. Phosphorylation on histidine was carried out using potassium phosphoramidate.<sup>4,5,6</sup> The peptide was dissolved in 100 mM Tris pH 8.0 to a final concentration of 1.1 mM and treated with 110 mM potassium phosphoramidate for 48 h at r.t. The phosphorylated peptide was then analyzed by analytical RP-HPLC and ESI-MS to confirm identity.

### H-TSpHYSIMA(d<sub>4</sub>A)-OH RP-HPLC Trace (5-25%B over 30 min, 1mL/min, 214nm detection)



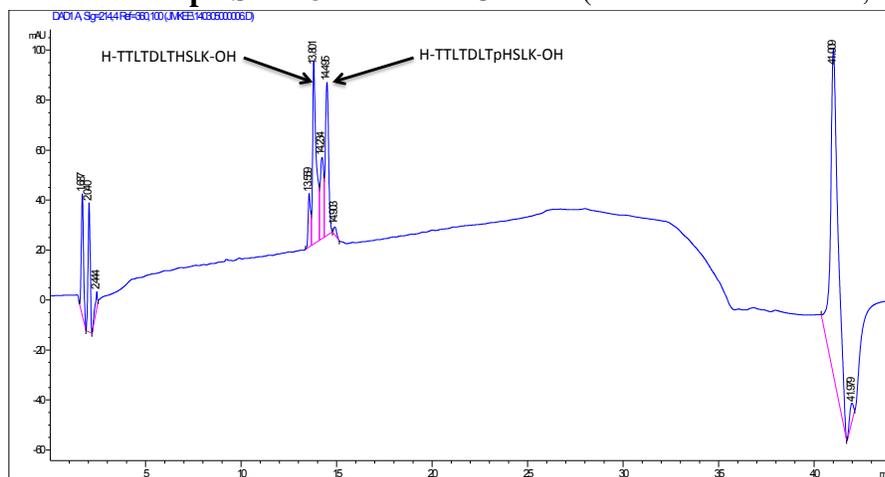
### H-TSpHYSIMA(d<sub>4</sub>A)-OH ESI-MS (Calculated MW: 1002.4264, Observed MW: 1002.4286)



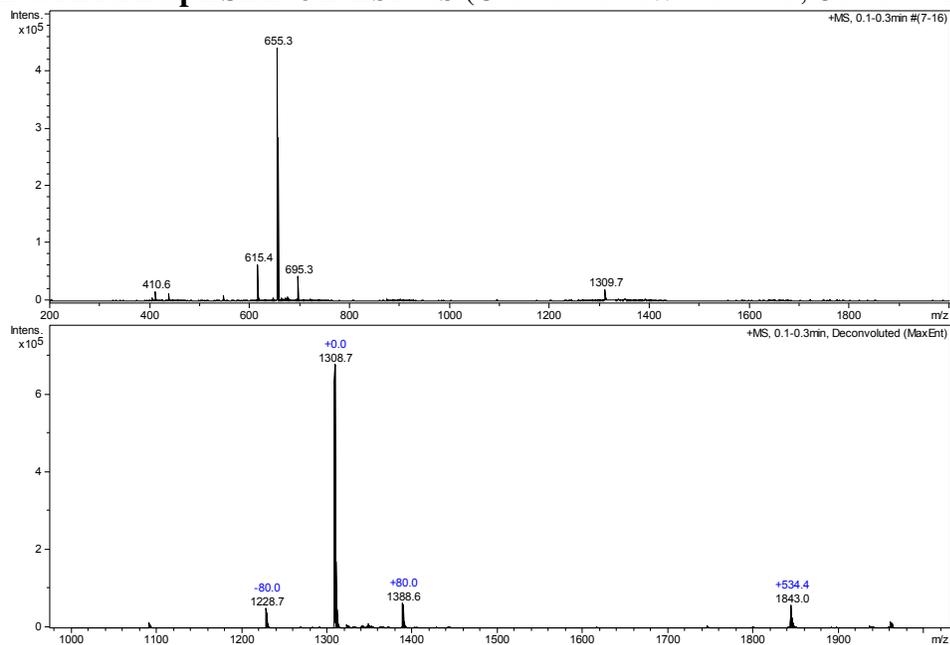
## Synthesis of H-TTLTDLTpHSLK-C<sup>18</sup>O<sup>18</sup>OH

The synthesis of this peptide was carried out using similar Fmoc chemistry as described above for **H-TpSHYSIMAR-OH**, **H-TSHpYSIMAR-OH**, and **H-TSpHYSIMAR-OH** using Fmoc-Lys(Boc) Wang resin. The crude peptide was purified by semi-preparative RP-HPLC and then phosphorylated by dissolving in 100 mM Tris pH 8.0 to a final concentration of 1 mM and treating with 200 mM potassium phosphoramidate for 48 h at r.t. Prior to <sup>18</sup>O-labeling, the purified phosphopeptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

### H-TTLTDLTpHSLK-OH RP-HPLC Trace (0-73%B over 30 min, 1mL/min, 214nm detection)



### H-TTLTDLTpHSLK-OH ESI-MS (Calculated MW: 1308.63, Observed MW: 1308.7)



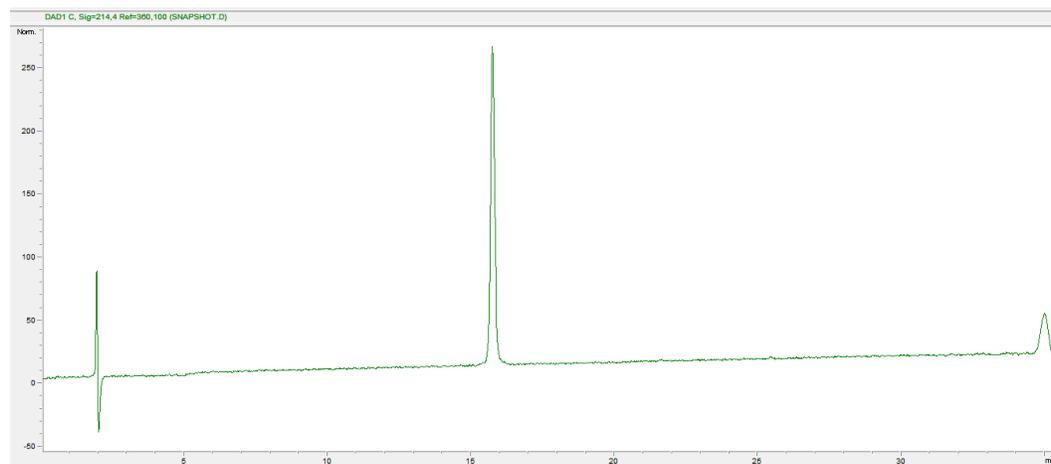
### **<sup>18</sup>O-Labeling of H-TTLTDLTpHSLK-OH**

50 ng of H-TTLTDLTpHSLK-OH was added to 10 mL of 10 mM Tris pH 8 and lyophilized to dryness. The sample was resuspended in 10 mL of H<sub>2</sub><sup>18</sup>O followed by addition of 50 ng of trypsin. The sample was incubated overnight at r.t. and then analyzed by LC-MS/MS as described below.

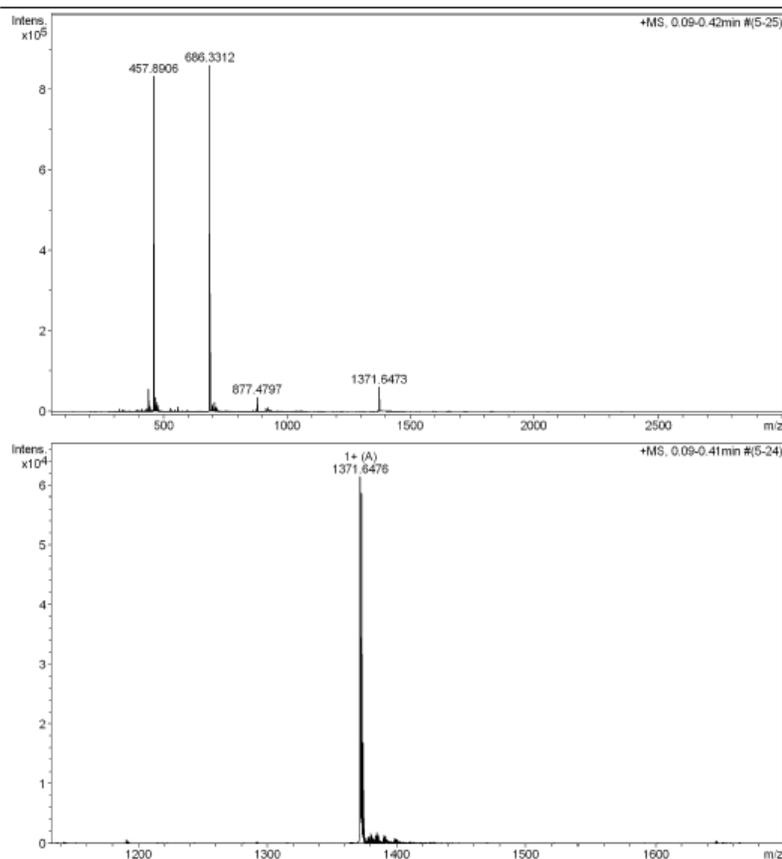
### **Synthesis of H-FApTHGGYLLQGK-OH Peptide Standard**

The synthesis of this peptide was carried out using similar Fmoc chemistry as described above for **H-TpSHYSIMAR-OH**, **H-TSHpYSIMAR-OH**, and **H-TSpHYSIMAR-OH** using Fmoc-Lys(Boc) Wang resin. The crude peptide was purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified phosphopeptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

**H-FApTHGGYLLQGK-OH** RP-HPLC Trace (10-40%B over 30 min, 1mL/min, 214nm detection)



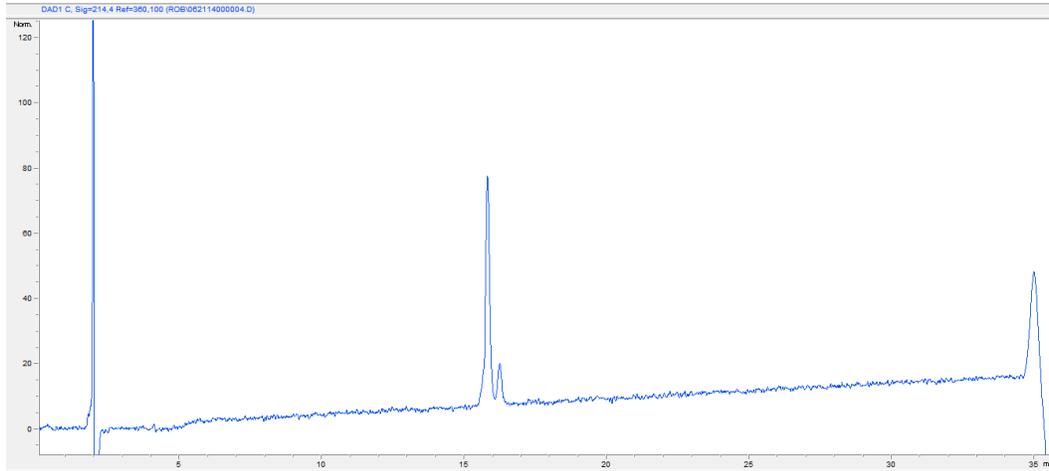
## H-FApTHGGYLLQGK-OH ESI-MS (Calculated MW: 1371.6457, Observed MW: 1371.6476)



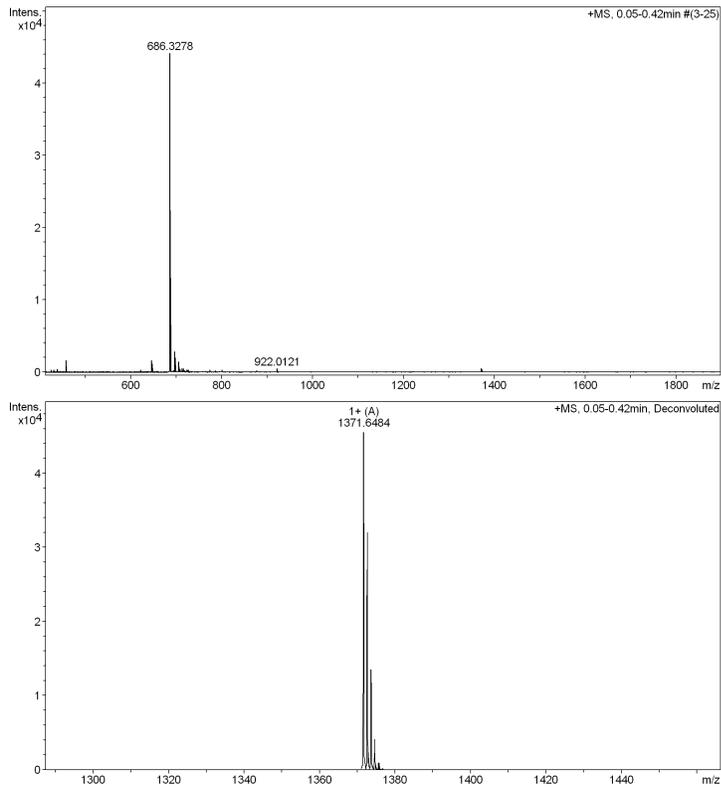
### Synthesis of H-FATpHGGYLLQGK-OH Peptide Standard

The synthesis of this peptide was carried out using similar Fmoc chemistry as described above for **H-TpSHYSIMAR-OH**, **H-TSHpYSIMAR-OH**, and **H-TSpHYSIMAR-OH** using Fmoc-Lys(Boc). The crude peptide was purified by analytical RP-HPLC and then phosphorylated by dissolving in 100 mM Tris pH 8.0 to a final concentration of 100  $\mu$ M and treating with 200 mM potassium phosphoramidate for 24 h at r.t. The phosphopeptide was purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified phosphopeptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

**H-FATpHGGYLLQGK-OH RP-HPLC Trace (10-40%B over 30 min, 1mL/min, 214nm detection)**



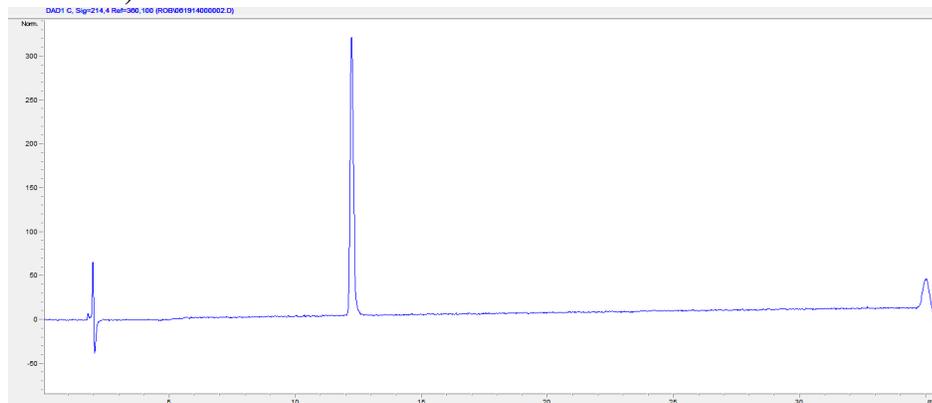
**H-FATpHGGYLLQGK-OH ESI-MS (Calculated MW: 1371.6457, Observed MW: 1371.6484)**



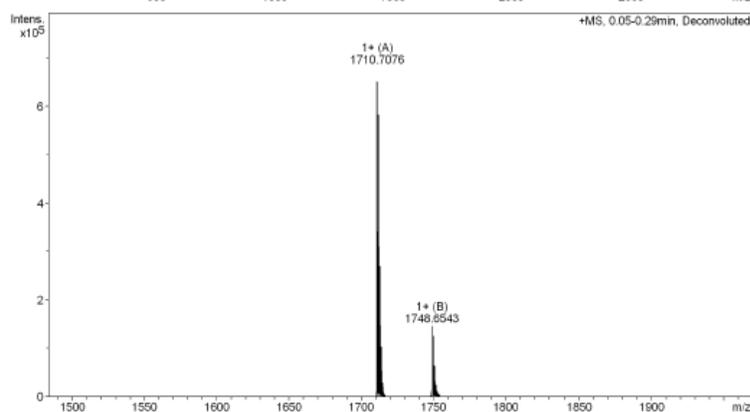
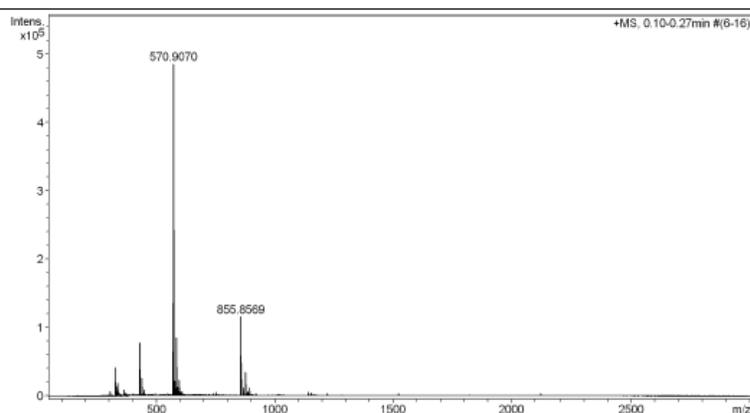
## Synthesis of H-LNFpSHGDYAEHGQR-OH Peptide Standard

The synthesis of this peptide was carried out using similar Fmoc chemistry as described above for **H-TpSHYSIMAR-OH**, **H-TSHpYSIMAR-OH**, and **H-TSpHYSIMAR-OH** using Fmoc-Arg (Pbf) Wang resin. The crude peptide was purified by analytical RP-HPLC and then used for further LC-MS/MS analysis as described below. Prior to LC-MS/MS analysis, the purified phosphopeptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

### H-LNFpSHGDYAEHGQR-OH RP-HPLC Trace (10-40%B over 30 min, 1mL/min, 214nm detection)



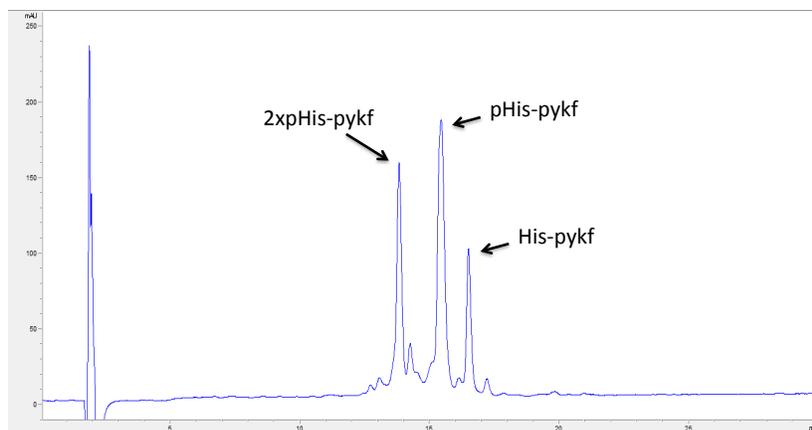
### H-LNFpSHGDYAEHGQR-OH ESI-MS (Calculated MW: 1710.7020, Observed MW: 1710.7076)



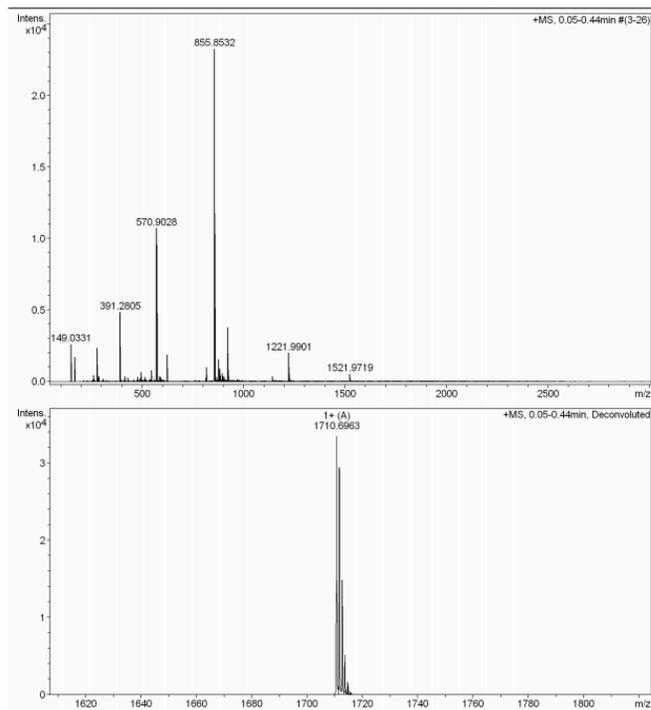
### Synthesis of Histidine phosphorylated H-LNFSHGDYAEHGQR-OH Peptide Standard

The synthesis of this peptide was carried out using similar Fmoc chemistry as described above for **H-TpSHYSIMAR-OH**, **H-TSHpYSIMAR-OH**, and **H-TSpHYSIMAR-OH** using Fmoc-Arg (Pbf) Wang resin. The crude peptide was purified by analytical RP-HPLC and then phosphorylated by dissolving in 100 mM Tris pH 8.0 to a final concentration of 100  $\mu$ M and treating with 100 mM potassium phosphoramidate for 24 h at r.t. The sample was then diluted 10-fold with MQ-H<sub>2</sub>O and then analyzed by LC-MS/MS as described below. Note that, under these phosphorylation conditions, we observed doubly phosphorylated peptide product likely from phosphorylation of both histidine residues. Also, we assume that the mono-phosphorylated product is a mixture of both pHis isomers. We analyzed the mono-phosphorylated pHis peptide by LC-MS/MS as described below and allowed PhosphoRS<sup>1</sup> to determine the phosphorylation assignment. Prior to LC-MS/MS analysis, the purified mono-phosphorylated peptide was analyzed by analytical RP-HPLC and ESI-MS to confirm its identity.

**Histidine Phosphorylated H-LNFSHGDYAEHGQR-OH RP-HPLC Trace (10-30%B over 30 min, 1mL/min, 214nm detection).**



**Hisitidine phosphorylated H-LNFSHGDYAEHGQR-OH** ESI-MS (Calculated MW: 1710.7020, Observed MW: 1710.6963)



### Preparation of Phosphopeptide library

Peptides were synthesized on an Intavis Multiprep peptide synthesizer. Novabiochem NovaSyn TGR amide resin and standard Fmoc protected amino acids were used for synthesis. Fmoc deprotection was carried out using 20% Piperidine and residue coupling carried out using HBTU and 4-methyl morpholine. After each coupling cycle a capping step was incorporated using Acetic Anhydride. After synthesis the peptides were cleaved using 87.5% trifluoroacetic acid, 2.5% water, 2.5% triisopropylsilane, 2.5% 1,2-ethanedithiol, 5% dimethyl sulfide then precipitated and spun down twice with chilled diethyl ether. The peptide pellets were dried then dissolved in water containing 0.1% trifluoroacetic acid, 0.1% acetonitrile and lyophilized. The histidine, pSer, pThr, and pTyr peptides from this library were then individually resuspended in 25mM Tris pH 8.0 to a final concentration of 0.5 mg/ml and treated with 5  $\mu$ g Trypsin for 16 hrs at r.t. to convert peptides to the C-terminal carboxylate. The samples were then passed over a spin column (10,000 MWCO) to remove the trypsin and the flow through was collected. All peptides were analyzed by analytical RP-HPLC to assess purity and determine peptide concentration by  $A_{214}$  absorbance using previously determined extinction coefficients.<sup>7</sup>

### Phosphorylation of Histidine Peptides from Phosphopeptide Library

500  $\mu$ L of each Histidine peptide from the library (corresponding to 300-1000  $\mu$ g of peptide) was treated with 100 mM phosphoramidate for 48 hrs. The samples were then analyzed by analytical RP-HPLC to determine both the % phosphorylation and concentration of each peptide. Under these phosphorylation conditions we obtained 45-85% histidine phosphorylation yields depending on the peptide sequence. Note that we took this incomplete phosphorylation into

account when equimolar amounts of pHis, pSer, pThr, and pTyr peptide mixtures were prepared for LC-MS/MS analysis. To determine whether the pHis peptides from this library displayed the characteristic triplet neutral loss pattern upon CID fragmentation, we combined 20 µg of each pHis peptide and de-salted the sample using a Sep-pak tC18 Vac RC 100 mg cartridge (Waters) using the following protocol. Just prior to de-salting, the column was equilibrated with 0.1% TFA followed by addition of the peptide sample. The collected flow through was added a second time to the column to maximize peptide binding. The cartridge was then washed with 0.01% TFA and the peptides were eluted off with 80% acetonitrile containing 0.01% TFA. The sample was concentrated down to dryness using a speed vacuum and promptly stored at -80 °C until experimentation. For LC-MS/MS analysis as described below, the peptides were resuspended in 10 mM ammonium bicarbonate pH 8.2.

### **Preparation of Phosphopeptide library mixtures for *in vitro* immunoprecipitation**

10 µg of each His, pHis, pSer, pThr, and pTyr peptide from the library (excluding the NagE peptide set due to insolubility) or 20 µg of each pHis peptide from the library (excluding NagE pHis peptide due to insolubility) were combined into a single Eppendorf tube and then de-salted using a Sep-pak tC18 Vac RC 100 mg cartridge (Waters) using the protocol described in the previous section. The sample was concentrated down to dryness using a speed vacuum and promptly stored at -80 °C until experimentation.

### **<sup>18</sup>O-Labeling of pHis peptides from Phosphopeptide library**

8 µg of the desalted pHis peptide mixture was resuspended in 100 µl of 10 mM Tris pH 8.0. 10 µl of this sample was lyophilized to dryness and resuspended in 10 µl of H<sub>2</sub><sup>18</sup>O followed by addition of 200 ng of trypsin. The sample was incubated overnight at rt. The sample was diluted 10-fold in H<sub>2</sub><sup>18</sup>O and analyzed by LC-MS/MS as described below. MS/MS spectra from H-MGpHAGAIAGGK-C<sup>18</sup>O<sup>18</sup>OH, LIpHGQVATR-C<sup>18</sup>O<sup>18</sup>OH, SpHEFMNK-C<sup>18</sup>O<sup>18</sup>OH, and pHGESQWNK-C<sup>18</sup>O<sup>18</sup>OH library peptides are reported in this study.

### **Immunoprecipitation of phosphohistidine peptides spiked into *E. coli* lysate**

#### **Preparation of Acid-treated *E. Coli* Lysate for pHis peptide Spike-In Experiments**

NCM3722 cells were grown in 100 mL LB media at 37 °C to an OD of 2.3. The cells were pelleted by centrifugation. The supernatant was removed and the pellet was then resuspended in 1.2 mL of 8 M Urea in TBS pH 8.5. The cell suspension was then sonicated (4x5 seconds at 45% power with 30 second rest in between sonication steps on ice). The cells were then centrifuged for 5 minutes at 17000 xg to pellet the cell debris. The supernatant protein concentration was determined by BCA to be 15 mg/ml.

10 mg of the lysate sample was treated with 25 mM DTT for 30 minutes at rt. The sample was then treated with 75 mM IAA for 30 minutes at rt in the dark. After IAA treatment, 50 mM DTT was added to the sample. The sample was then diluted to 1.7 M Urea with 100 mM Tris pH 8.0, 137 mM NaCl, 2mM KCl. The sample was then treated with 400 µg of trypsin (Promega) for 4

hours at rt. The sample was de-salted using an Oasis HLB cartridge following manufacturer's instructions with a slight modification. Prior to desalting, the digested lysate was treated with 0.1% formic acid and immediately loaded onto the column, washed with 0.01% formic acid, and eluted off with 50% acetonitrile in 10 mM ammonium bicarbonate, pH 8.2. After desalting, the sample was concentrated down to dryness using a speed vacuum. The sample was then dissolved in 50% acetonitrile with 2% TFA and incubated overnight. The sample was then aliquoted into 500 µg aliquots and lyophilized to dryness.

### **Immunoprecipitation of pHis peptides spiked in to *E. coli* peptide lysate**

500 µg of the acid-treated peptide lysate was resuspended in 0.2 mL of TBS pH 8.5 followed by addition of 1, 0.1, 0.01, 0.0025 µg of total pHis peptide (the NagE pHis peptide was not included in the pHis peptide library due to poor solubility). 30 µg of affinity purified pHis antibody was then added to the sample and incubated overnight at 4 °C (final volume of sample is 300 µL). 25 µL of protein G beads was then added to the sample and incubated for 1.5 hrs at 4 °C. The samples were then centrifuged on a table top centrifuge to pellet the beads. The supernatant was removed and the beads were washed with 2 x 200 µL cold TBS pH 8.5. pHis peptides were eluted from the beads by incubation with 25 µL 25mM pTze in TBS pH 8.5 for 2 min at rt followed by elution through a fritted micro bio-spin chromatography column (this elution step was repeated a second time and the two elution samples were combined). The sample was desalted using a homemade C18 zip tip. The zip tips were constructed by placing a small piece of C18 solid phase extraction disk (Empore, 3M) into a gel loading tip. The tip was washed with 80% acetonitrile in 10 mM ammonium bicarbonate buffer and then equilibrated in 10 mM ammonium bicarbonate buffer in MQ-H<sub>2</sub>O. The sample was then loaded onto the zip tip and washed with 10mM ammonium bicarbonate followed by elution of the peptides in 80% acetonitrile with 10mM ammonium bicarbonate buffer. The sample was concentrated to near dryness in a speed vac and then resuspended in 10 mM ammonium bicarbonate buffer for LC MS/MS analysis as described below.

### **Immunoprecipitation of phosphopeptide library**

10 µg of the desalted phosphopeptide library consisting of the His, pHis, pSer, pThr, and pTyr peptides (excluding the NagE peptide set due to insolubility) was dissolved in 300 µl TBS pH 8.5 and incubated with or without 25 µg of pHis antibody (final sample volume is 350 µL). The sample was then incubated overnight at 4 °C. 50 µL of the sample was removed for MS/MS analysis as the pre-enrichment sample. 25 µL of protein G beads was then added to the sample and incubated for 1.5 hrs at 4 °C. The samples were then centrifuged on a table top centrifuge to pellet the beads. The supernatant was removed and the beads were washed with 2 x 200 µL cold TBS pH 8.5. pHis peptides were eluted from the beads by incubation with 25 µL 25 mM pTze in TBS pH 8.5 for 2 min at rt followed by elution through a fritted micro bio-spin chromatography column (this elution step was repeated a second time and the two elution samples were combined). The input and elution samples were desalted using the method described above in the previous section.

### **Dot Blot analysis of Phosphopeptide library**

Each peptide from the library (excluding the NagE pHis peptide set due to insolubility) was combined into His, pHis, pSer, pThr, or pTyr peptide samples and serially diluted to 300, 150, 75, 33, and 16.7  $\mu\text{M}$  total peptide in MQ-H<sub>2</sub>O. 3  $\mu\text{L}$  from each dilution was spotted onto a nitrocellulose membrane to give 900, 450, 225, 100, and 50 pmol of total peptide. The membrane was then either analyzed by Western blot or colloidal gold staining.

For Western blotting, the membrane was incubated in TBST (pH 8.5) with 3% BSA for 1 hr. The membrane was then incubated with affinity purified pHis antibody (prepared as described below) or monoclonal pTyr antibody that was diluted 1:250 (pHis) or 1:500 (pTyr) in TBST with 3% BSA for 1 hr. The membrane was then washed three times with TBST for 5 minutes each and then incubated with secondary antibody (Li-Cor, goat anti-rabbit 800CW for pHis, or goat anti mouse 680RD for pTyr) for 1 hr in TBST with 3% BSA. The membrane was then washed 3x with TBST for 5 minutes each and then washed 3x with MQ-H<sub>2</sub>O for 10 seconds each. The membrane was then imaged using a Li-COR Odyssey Infrared Imager. To serve as a loading control, a similarly spotted membrane that was not submitted through the Western blot protocol was stained with Colloidal gold following the manufacturer's instructions.

### **Preparation of pHis peptides from trypsinized phospho-BSA**

1mL BSA (2 mg/mL in PBS) was phosphorylated with 250 mM phosphoramidate for 36 h at r.t. The sample was then treated with 50 mM DTT for 30 min followed by treatment with 75 mM iodoacetamide (IAA) for 30 min in the dark at r.t. The sample was then treated with 50 mM DTT and buffer exchanged into 20 mM Tris pH 8 using a 10,000 MWCO spin column. The sample was then treated with 100  $\mu\text{g}$  of trypsin (1/20 ratio) for 5.5 h at r.t. on a shaker and then collected from the spin column without filtering. The sample volume was adjusted to 1 mL with 20 mM Tris buffer (pH 8). 500  $\mu\text{L}$  of this sample was de-salted on an Oasis HLB cartridge according to manufacturer's instructions. However, the peptides were treated with 0.1% formic acid and immediately loaded onto the column, washed with 0.01% formic acid, and eluted off of the column with 50% acetonitrile in 10 mM ammonium bicarbonate, pH 8.2. The sample was then analyzed by LC-MS/MS as described below.

### **Development of rabbit polyclonal anti-pHis antibody for pHis peptide immunoprecipitation**

The pHis antibody was co-developed with Cell Signaling Technology (CST) using a similar strategy to our previous pan pHis antibody.<sup>2</sup>

### **Affinity purification of pHis antibody**

Rabbit anti-serum raised against the pTze hapten was affinity purified using phosphorylated BSA covalently attached to agarose. Affinity resin consisting of phosphorylated BSA immobilized onto agarose was prepared by covalently attaching BSA to agarose beads using SulfoLink Resin according to the manufacturer's instructions. Chemical phosphorylation of the immobilized BSA-agarose resin was performed by pre-equilibrating 1 mL of resin in TBS (25 mM Tris pH

8.5, 137 mM NaCl, 2.7 mM KCl) 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  $\mu$ L of the crude polyclonal pHis antiserum was diluted 5-fold into TBS and then added to the resin and incubated for 1 h 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 (100 mM Glycine pH 2.5). Elution fractions were immediately neutralized by adding 100  $\mu$ L of 1 M Tris (pH 8.0). Fractions containing pHis antibody (determined by ELISA, as described previously<sup>2</sup>) were pooled together and the concentration of antibody was determined to be 0.06 mg/mL by measuring the  $A_{280}$  ( $A_{280}$  extinction coefficient assumed to be 14 for a 10 mg/mL antibody solution).

### **Western blot of glycerol-fed *E. coli* lysate**

NCM 3722 cells were grown in 5 mL of starter culture (Gutnick minimal salts<sup>8</sup>, 2.5 mM arginine, 2 mM  $\text{NH}_4\text{Cl}$ , 0.4% w/v glycerol) overnight to an OD<sub>600</sub> of 1.25. 1.5 mL from the starter culture was added to 40 mL of nitrogen-limited culture medium (Gutnick minimal salts<sup>8</sup>, 2.5 mM arginine, 0.4% w/v glycerol) containing glycerol as the sole carbon source. The cells were grown overnight at 37 °C to an OD<sub>600</sub> of 1.2. The cells were then pelleted by centrifugation (4500 xg for 5 min at 4 °C). The pellets were then resuspended in 1 mL 8 M urea in TBS (25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl) and sonicated (4 x 5 sec at 45% power) on ice. The sample was then centrifuged (17,000 xg for 5 min at 4 °C) to pellet the cellular debris. Protein concentration was measured by the BCA protein assay (Pierce) to be 5  $\mu$ g/ $\mu$ L. 100  $\mu$ L of the lysate was treated with 4x SDS-PAGE 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). 50  $\mu$ L of this sample was then incubated with 500 mM hydroxylamine for 30 min at 37 °C. 5  $\mu$ L of each sample (-HA and +HA) were loaded onto a 12% TGX gel and resolved by electrophoresis for 15 minutes at 120 V and then 60 min at 175 V. Electroblothing of the proteins to a 0.2  $\mu$ m PVDF membrane, and Western blotting with affinity-purified pHis antibody were both carried out as described previously.<sup>2</sup> Western blot analysis of the lysate is shown in Figure S17.

### **Western blot of mannitol-fed *E. coli* lysate**

NCM 3722 cells were grown in 5 mL of LB starter culture overnight to an OD of 4.0. 250  $\mu$ L of the starter culture was centrifuged (4500 xg for 5 min at 4 °C) to pellet the cells. The cells were resuspended in 1 mL of mannitol minimal media (Gutnick minimal salts<sup>8</sup>, 20 mM mannitol, 10 mM arginine) and transferred to 50 mL of mannitol media. The cells were grown at 37 °C to an OD<sub>600</sub> of 1.3. The cells were then pelleted by centrifugation (4500 xg for 5 min at 4 °C) and then resuspended in 1 mL 8 M urea in TBS (25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl) and sonicated (4 x 5 sec at 45% power) on ice. The sample was then centrifuged (17,000 xg for 5 min at 4 °C) to pellet the cellular debris. Protein concentration was measured by the BCA protein assay (Pierce) to be 5  $\mu$ g/ $\mu$ L. 100  $\mu$ L of the lysate was treated with 4x SDS-PAGE 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) and analyzed by Western blot as described above for the glycerol fed cells. Western blot analysis of the lysate is shown in Figure S17.

### **Western blot of *E. coli* lysate from cultures of different carbon sources**

NCM 3722 cells were grown in 5 mL of starter culture (Gutnick minimal salts, 2.5 mM arginine, 2 mM NH<sub>4</sub>Cl, 0.4% w/v glycerol) overnight to an OD600 of 1.7. The starter culture was centrifuged (4500 xg for 5 min at 4 °C) to pellet the cells, and the cells were resuspended in 5 mL of Gutnick minimal media (10 mM NH<sub>4</sub>Cl) without carbon source. 50 μL of this stock was added to 5 mL of culture media (Gutnick minimal salt, 10 mM NH<sub>4</sub>Cl, 20 mM of each carbon source (potassium acetate, alanine, serine, lactose, sodium pyruvate, and mannitol). The cells were grown at 37 °C for 3 h or overnight and then pelleted by centrifugation (4500 xg for 5 min at 4 °C). The cell pellet from each 5 mL culture was resuspended in 400 μL of 4x SDS-PAGE 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) and sonicated (3 x 5 sec at 40% power). The lysates were analyzed by Western blot as described above for the glycerol fed cells. Western blot analysis of the lysate is shown in Figure S23.

### **Immunoprecipitation of trypsinized glycerol-fed or mannitol-fed *E. coli* lysates**

5 mg of the glycerol-fed or mannitol-fed *E. coli* lysate was treated with 25 mM DTT for 30 min at r.t. The sample was then treated with 75 mM iodoacetamide (IAA) for 30 min at r.t. in the dark. After IAA treatment, 50 mM DTT was added to the sample. The lysate was then diluted five-fold with TBS (25 mM Tris pH 8.5, 137 mM NaCl, 2.7 mM KCl) to a final urea concentration of 1.6 M Urea. The sample was then treated with 250 μg of trypsin (1/20 wt/wt ratio) for 4.5 h at r.t., then desalted with a Sep-pak C18 Vac RC 100 mg cartridge (Waters), and handled using the following minor adjustments to standard protocols. One by one, just prior to de-salting, the columns were equilibrated with 0.1% TFA followed by addition of individual peptide samples in original TBS/Urea buffers. The collected flow-throughs of unbound peptides were added a second time to columns to maximize binding. The cartridges were washed rapidly with 0.01% TFA and peptides were eluted off with 80% acetonitrile containing 0.01% TFA. The samples were rapidly concentrated down to 100 μL using a speed vacuum and promptly frozen at -80 °C. For immunoprecipitation, the samples were thawed and diluted to final volumes of 1 mL using TBS pH 8.5 with protease inhibitor cocktail (Roche). 30 μg of affinity-purified pHis antibody or no antibody (mock antibody control) was added to each sample, and they were then incubated overnight at 4°C with constant mixing. 25 μL protein G agarose beads was then added to each sample and incubated for 1.5 hours at 4°C. IP and mock IP samples were centrifuged and the supernatant removed. Resin was washed twice with 200 μL cold TBS pH 8.5 and then eluted twice with 30 μL of 25 mM pTze in TBS for 2 min at r.t. The eluted peptide samples were desalted using STAGE-Tips<sup>9</sup> under basic pH conditions. Briefly, tips were wetted with 80% acetonitrile in 10 mM ammonium bicarbonate buffer, pH 8.2 ammonium bicarbonate buffer and then equilibrated in 10 mM ammonium bicarbonate buffer. The samples were loaded onto the tips and washed with 10mM ammonium bicarbonate buffer, followed by elution of the peptides in 50% acetonitrile in 10mM ammonium bicarbonate buffer. Samples were concentrated to near

dryness in a speed vac and then resuspended in 1 mM ammonium bicarbonate buffer for LC MS/MS analysis as described below. For acid sensitivity experiments, a portion of the eluates were treated with 1% TFA for overnight prior to LC-MS/MS analysis.

### ***TRIPLET Software Development***

TRIPLET is a Windows Presentation Foundation (WPF) application written in C#.NET 4.0 and was developed in the Microsoft Visual Studio 2010 environment (<http://www.visualstudio.com/>). TRIPLET takes as its input MS/MS spectra peak list files from LC-MS runs. It is MS instrument vendor neutral, however the current version requires that the peaklists be formatted according to the Mascot generic format (.mgf). Users can specify the delta mass value of a single primary neutral loss, and any number of secondary neutral losses, with independent minimal peak intensity thresholds (specified as percent of tallest ion peak) for the primary and secondary losses. An error window is specified for the neutral loss calculation and can be either wide, to accommodate low resolution MS/MS mass measurements made in an ion trap, or very narrow to accommodate high resolution, accurate mass MS/MS mass measurements made in an Orbitrap or TOF. Output of TRIPLET is a list of parent ions whose MS/MS spectra contain the neutral loss species as defined by the user's parameters. This list may be exported into a clipboard for pasting into other applications, such as database search results filtering software, or exported as an inclusion list (parent ion list) to be imported into an MS instrument method file for targeted follow-up analysis of samples (e.g., MSA analysis). Additionally, when the user double-clicks on any single parent ion entry in the output list within TRIPLET, a pop-up window appears containing a plot of the MS/MS spectrum for that parent ion species with the detected neutral loss ions indicated with red diamonds. TRIPLET is available for download as an .exe file to run on Windows platforms via an FTP file-share at <FTP://128.112.112.51/> (Login name: TRIPLET Password: pHis). TRIPLET is also released as open source software and can be found at the following web address: <https://github.com/vnbhatia/triplet>

### **High-resolution nano-UPLC-MS**

LC-MS/MS analyses were performed on a high-resolution, high-mass-accuracy, reversed-phase nano-UPLC-MS platform, consisting of an Easy nLC Ultra 1000 nano-UPLC system coupled to a VelosPro-Orbi Elite hybrid mass spectrometer (ThermoFisher Scientific) equipped with a Flex Ion source (Proxeon Biosystems, Odense, Denmark). LC was conducted using a trapping capillary column (150  $\mu\text{m}$  x ca. 40 mm, packed with 3  $\mu\text{m}$ , 100 Å Magic AQ C18 resin, Michrom, Auburn, CA) at a flow rate of 4  $\mu\text{L}/\text{min}$  for 4 min, followed by an analytical capillary column (75  $\mu\text{m}$  x ca. 45 cm, packed with 3  $\mu\text{m}$ , 100 Å Magic AQ C18 resin, Michrom) under a linear gradient of A and B solutions (solution A: 3% acetonitrile/ 0.1% formic acid; solution B: 97% acetonitrile/ 0.1% formic acid) from 5%-35% B over 70, 180, or 300 min at a flow rate of 300 nL/ min. Nanospray was achieved using commercial sprayer tips (New Objective, Woburn, MA) at a voltage of 2.4 kV, with the Elite heated capillary at 275 °C. Full-scan (typically, m/z 335–1800) positive-ion mass spectra were acquired in the Orbitrap at a resolution setting of 120,000. MS/MS spectra were simultaneously acquired using CID (for initial pHis neutral loss screening experiments and for analyses of synthetic and recombinant-derived peptides) in the

LTQ for the fifteen most abundant multiply charged species in the full-scan spectrum, or using CID-MSA (with MSA settings to target the neutral losses of 98 and 80 Da from 2+ and 3+ peptides only) for the 10 most abundant entries in a parent ion list derived from TRIPLET analysis (see below), having signal intensities of >1000 NL. For synthetic peptide and parent ion list directed LC-MS analyses, dynamic exclusion was typically not enabled. Lockmass was employed, maintaining calibration to 2-3 ppm of accurate mass.

## Mass Spectrometry Data Analysis

Resultant LC-MS/MS data were subjected to preprocessing into mgf peaklist files using ProteomeDiscoverer (v. 1.4, ThermoFisher), which were searched against the SwissProt *E. coli* database, or a BSA subset database using the Mascot search engine (v. 2.4, Matrix Science, London, UK.), allowing for a parent ion mass window of  $\pm 6$  ppm,  $\leq 3$  missed trypsin cleavages, histidine phosphorylation, methionine oxidation and *N*-terminal protein acetylation as variable modifications, and carbamidomethylation of cysteines as a fixed modification. Searches were conducted within the Proteome Discoverer framework using the Mascot and phosphoRS nodes. Mass spectra were visualized using Xcalibur (v. 2.2, ThermoFisher) and Scaffold (v. 4.3.4, Proteome Software, Portland, OR). Raw Mascot search results from initial CID runs were exported from the Mascot as CSV files with an ion score cutoff of zero and were compared to the output list from TRIPLET analysis of the same mgf files used for the Mascot search, in order to populate the intersection of both. Also, output lists from TRIPLET analysis of CID MS/MS data were used as “parent ion lists” for follow-up CID-MSA experiments. TRIPLET parameters settings were: a primary neutral loss of 98 Da as the most abundant peak in the spectrum (100% of base peak), secondary losses of 80 and 116 Da (with the lower threshold of 1% of base peak) and *m/z* error tolerance of 0.5. All phosphohistidine-bearing peptide assignments were further validated by manual inspection.

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