FIGURE S1. MS-based phosphopeptide identification and quantitation strategy involving *in vitro* phosphorylation of the FAT domain by Src at pH 5.5, 6.0, 6.5, 7.0, and 7.5, in-solution trypsinization, spike-in of heavy-labeled phosphopeptide standards, and LC-MS/MS-identification/absolute quantitation. SDS-PAGE verification was performed after trypsin digestion to ensure consistent proteolysis of the FAT domain under the different pH conditions.

FIGURE S2. Product ion spectra resulting from CID-MS/MS of FAT phosphopeptides and corresponding spiked-in heavy-labeled synthetic phosphopeptide internal standards. A. pY926 light phosphopeptide. B. pY926 heavy phosphopeptide. Fragment ions corresponding to the unambiguous identification of the phosphorylation site are shown for each peptide.

FIGURE S3. Product ion spectra resulting from CID-MS/MS of FAT phosphopeptides and corresponding spiked-in heavy-labeled synthetic phosphopeptide internal standards. A. pY1008 light phosphopeptide. B. pY1008 heavy phosphopeptide. Fragment ions corresponding to the unambiguous identification of the phosphorylation site are shown for each peptide.

FIGURE S4. Titration of the histidine residues. Titration profiles of A. H981, B. H1026, and C. H1053 used to calculate pK_a values of that residue. D. Residues that titrate with H981 (blue) and H1026 (red) mapped onto the structure of the FAT domain. No residues titrate with H1053.

FIGURE S5. NH residual dipolar couplings as a function of pH. Residual dipolar coupling data were collected on the FAT domain at pH 6.0 and pH 7.5. A. Comparison of two independent datasets collected at pH 6.0. B. Comparison of two independent datasets collected at pH 7.5. C. Comparison of RDCs collected at pH 6.0 and pH 7.5. D. Comparison of second datasets collected at pH 6.0 and pH 7.5.

FIGURE S6. ^{1}H-^{1}H NOEs of the side chain of one of the sites of phosphorylation, Y926, from a simultaneous 3D $^{13}C/^{15}N$-NOESY with a 150 ms mixing time collected on a Varian INOVA 800 MHz spectrometer at 37°C. A. Hδ of Y926 at pH 6.0, B. Hδ of Y926 at pH 7.5, C. Hε of Y926 at pH 6.0, and D. Hε of Y926 at pH 7.5.

FIGURE S7. ^{1}H-^{1}H NOEs of the side chain of one of the sites of phosphorylation, Y1008, from a simultaneous 3D $^{13}C/^{15}N$-NOESY with a 150 ms mixing time collected on a Varian INOVA 800 MHz spectrometer at 37°C. A. Hδ of Y1008 at pH 6.0, B. Hδ of Y1008 at pH 7.5, C. Hε of Y1008 at pH 6.0, and D. Hε of Y1008 at pH 7.5.

Figure S1

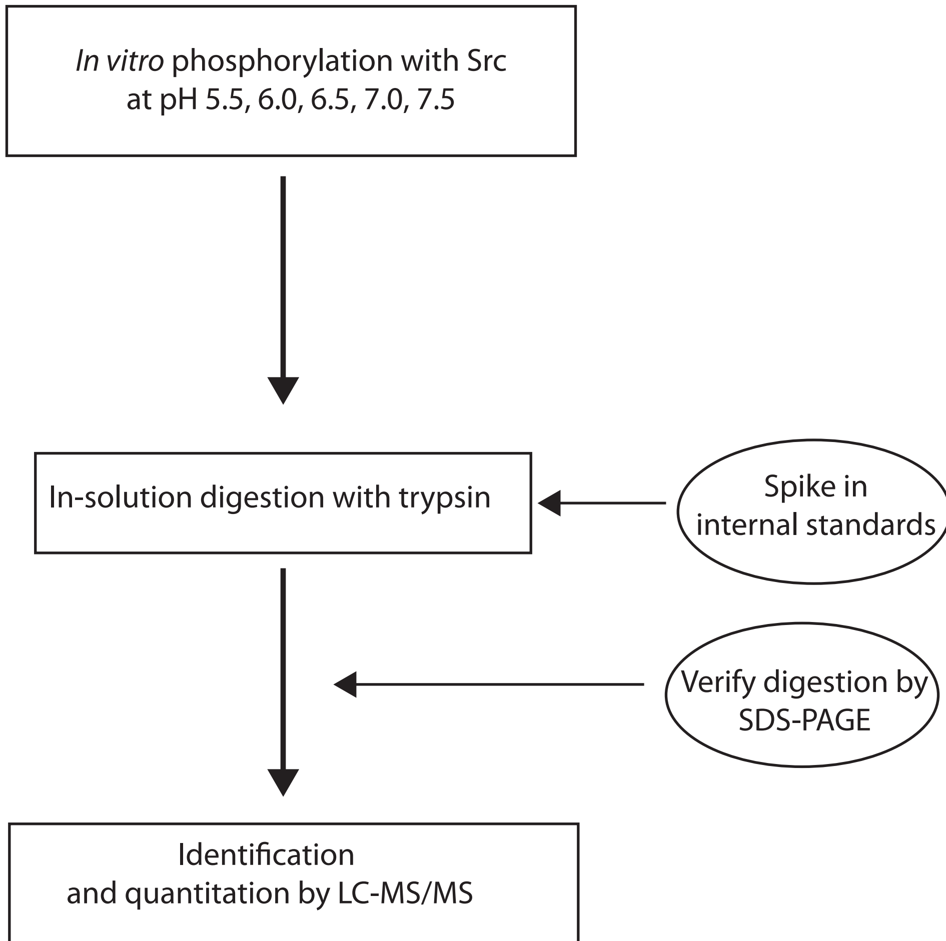


Figure S2

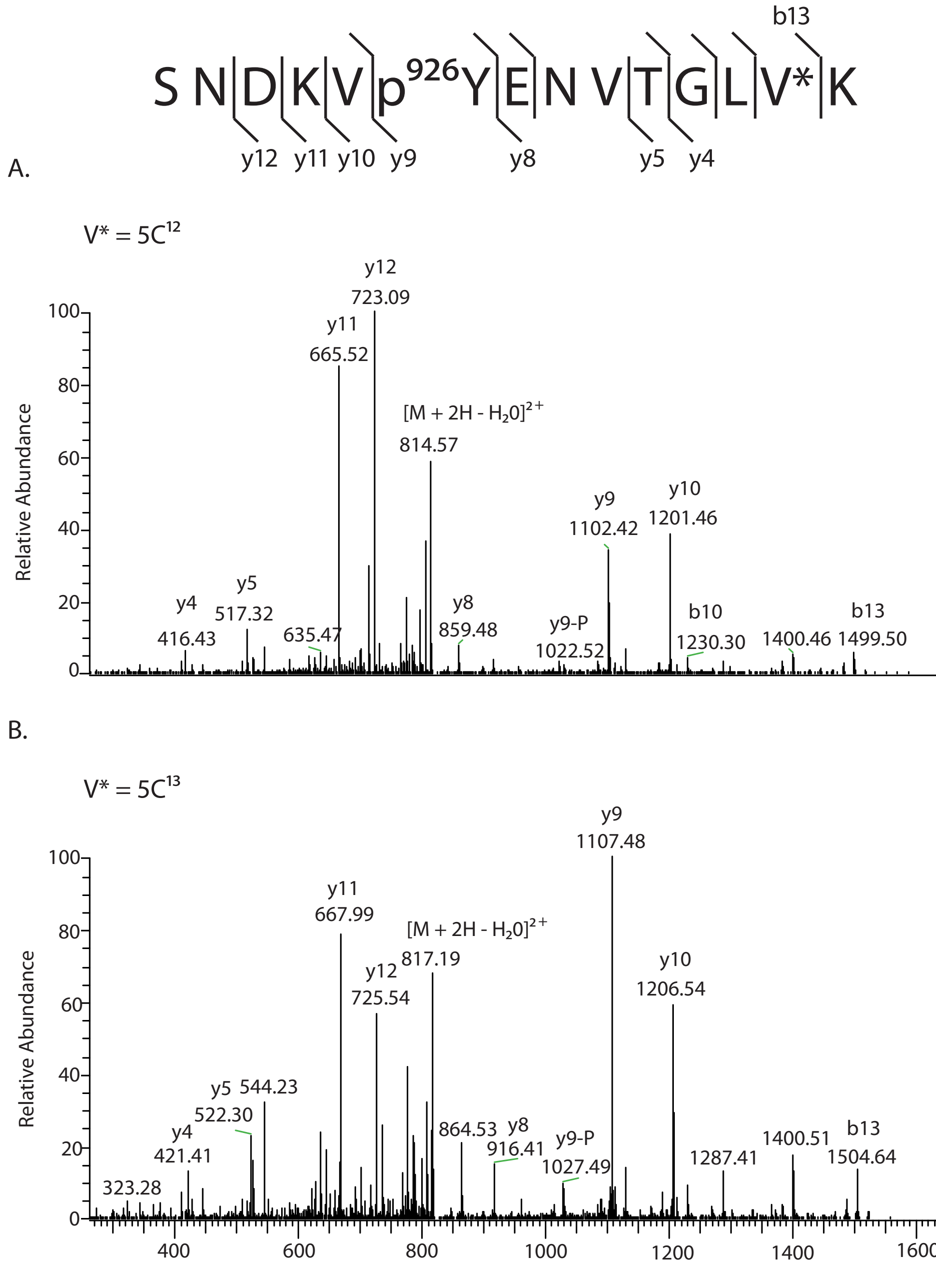
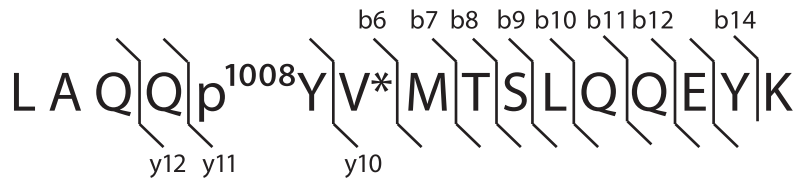
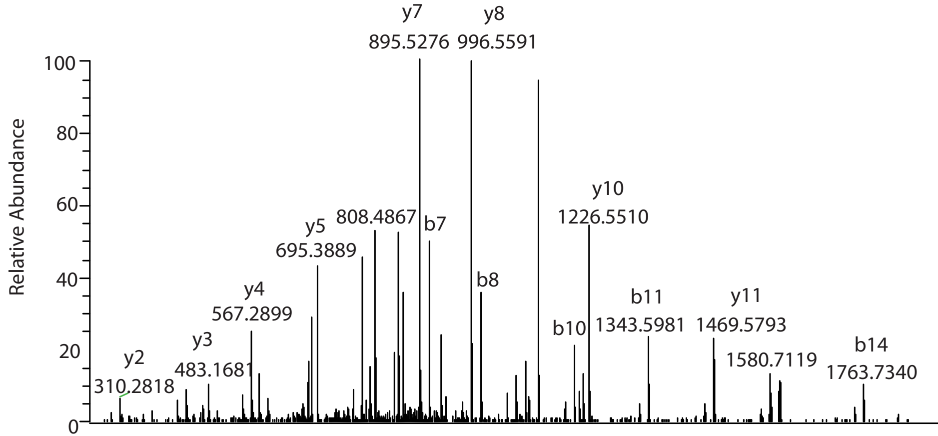


Figure S3



A.



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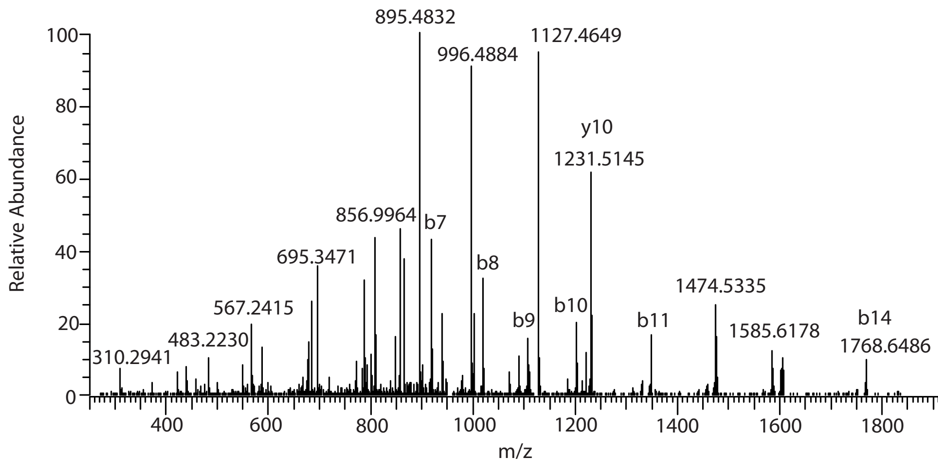

Figure S4

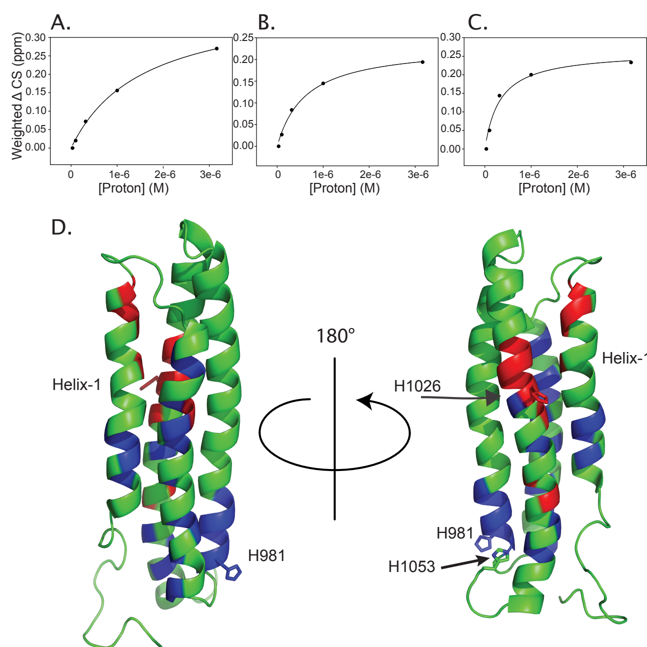

Figure S5

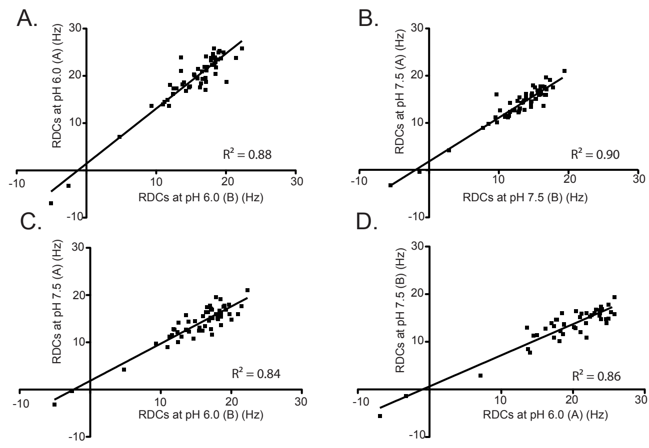

Figure S6

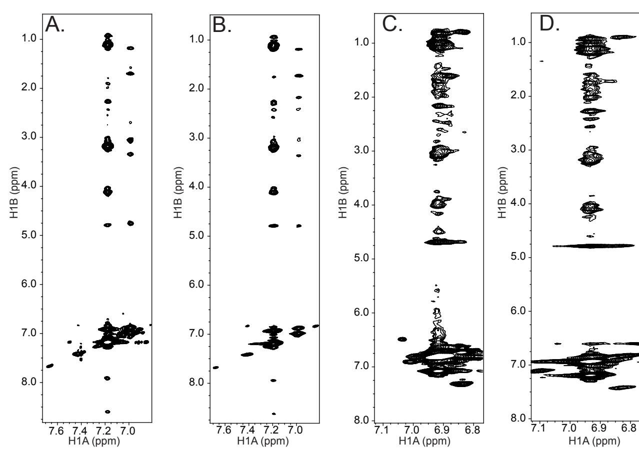

Figure S7