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All chemicals and HPLC-grade solvents were obtained from Sigma-Aldrich (St. Louis, MO). Integra Frits^{TM} 150 µm bore ID and Pico Frits, 50 µm bore ID, 10 µm at the tip, were from New Objective (Cambridge, MA). Microflow tees, PEEK unions and the conductive union were from Upchurch Scientific (Oak Harbor, WA). The stationary phase used for packing the nanoscale LC columns was 3 µm C_{18} from Dr Maisch.

Samples A and B were dissolved at the same concentration, (circa 0.085 mg/mL) in 100 mM NH_4HCO_3, containing 2 mM DTT. The mixture was split in two, and either trypsin or GluC (enzyme/(substrate ratio 1:20) were added to the reaction mixture. After overnight incubation, the peptides were directly injected for nanoscale LC-MS/MS analysis (injected amount: 850 ng).

Chromatography was performed on an Ultimate nano LC system from Dionex (Sunnyvale, CA). The analytical nano LC column used was an in-house packed 50 µm i.d., 10 cm long Pico Frit^{TM}, column. 10 µL of the peptide mixture were loaded onto an in-house packed 150 µm i.d., 1 cm long Integra Frit^{TM} trapping column (packing bed length 1 cm) at 10 µL/min of loading pump solvent, consisting of H_2O/acetonitrile/trifluoroacetic acid (TFA) 97.95:2:0.05 (v/v/v). After 4 minutes washing, the trapping column was switched on-line to the analytical column, and gradient separation started at about 120 nL/min.
A binary gradient was used for peptide elution. Mobile phase A was H_2O/acetonitrile/formic acid/TFA 97.9:2:0.09:0.01 (v/v/v/v); mobile phase B was H_2O/acetonitrile/formic acid/TFA 19.9:80:0.09:0.01 (v/v/v/v). Gradient was from 0 to 45% B in 45 minutes.

MS detection was performed on a QSTAR XL hybrid LC-MS/MS from Applied Biosystems (Foster City, CA) operating in positive ion mode, with nESI potential at 1300 V, curtain gas at 15 units, CAD gas at 3 units. Information-dependent acquisition (IDA) was performed by selecting the two most abundant peaks for MS/MS analysis after a full TOF-MS scan from 400 to 1600 m/z lasting 1 second. Both MS/MS analyses were

performed in enhanced mode (1.5 seconds/scan). Threshold value for peak selection for MS/MS was 20 counts.

Data were searched on the Mascot search engine (www.matrixscience.com) against the IPI database. using the following parameters: MS tolerance 30 ppm; MS/MS tolerance 0.3 Da; variable modifications methionine oxidised; enzyme trypsin (or GluC); max. missed cleavages 1; taxonomy human.

Protein hits based on two successful peptide identifications were considered valid. Error tolerant searches allowed the identification of three phosphorylation sites, together with many putative amino acid substutions/rare modifications, which were not taken into consideration due to their large number and the low experience in the laboratory with error tolerant searches.