Electronic Supplementary Information

Insights into Chemoselectivity Principles in Metal Oxide Affinity Chromatography Using Tailored Nanocast Metal Oxide Microspheres and Mass Spectrometry-Based Phosphoproteomics†

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ESI Experimental details

Preparation of metal oxide microspheres

The starting silica microspheres used as templates in the nanocasting procedure were purchased from Daiso Co. Ltd., Osaka, Japan. Two types of silica samples with the same average particle size (10 µm), but with different pore diameters were used, SP-120-10P (12 nm/120 Å) and SP-300-10P (30 nm/300 Å). In the preparation of the TiO_2 and ZrO_2 microspheres, the SP-300-10P template was used, while SP-120-10P was used for all the other metal oxides.

For each metal oxide, the pore diameter of the silica starting template was chosen in such a way to produce metal oxide replicas with comparable BET (Brunauer-Emmett-Teller) surface areas. By using the SP-120-10P silica material, BET surface areas in the range of 45–60 m^2/g could be attained for most of the oxides. However, as the interaction of TiO_2 and ZrO_2 with the silica surface is exceptionally strong (producing large surface areas in the etched samples), the larger pore-sized silica template (SP-300-10P, 30 nm pore diameter) was used in this case to produce comparably small surface areas (72 m^2/g and 66 m^2/g for the TiO_2 and ZrO_2 samples, respectively).

In the synthesis of the metal oxide spheres, titanium isopropoxide (97%, Sigma-Aldrich), tin chloride ($SnCl_2$, 98%, Riedel de Haën), zirconyl chloride octahydrate ($ZrOCl_2 \cdot 8\ H_2O$, 99%, Fluka), iron nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9\ H_2O$, 98%, Aldrich), nickel nitrate hexahydrate ($Ni(NO_3)_2 \cdot 6\ H_2O$, ≥ 98.5%, Fluka), cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6\ H_2O$, ≥ 99%, Sigma-Aldrich), and indium nitrate hydrate ($In(NO_3)_3 \cdot x\ H_2O$, ≥ 99%, Aldrich) were used as precursors for the corresponding metal oxides with either de-ionized water (Milli-Q, Millipore), n-hexane (anhydrous, 95%, Sigma-Aldrich) or absolute ethanol (Altia Oyj) as solvents.

The method of nanocasting has been used to synthesise all of the metal oxides used in this work according to four main protocols:

(1) Synthesis of TiO_2 microspheres: The TiO_2 microspheres were made according to a previously published sol-gel process.[30-31] 1.5 mL titanium isopropoxide was dissolved in 1.5 mL ethanol, after which the solution was added to 0.25 g of silica (SP-300-10P). The suspension was stirred at 500 rpm for about 4 h and then centrifuged at 3000 rpm for 2 min to remove the supernatant, and then dried at 95 °C overnight. The impregnated silica particles were then mixed with 1.5 mL ethanol and 1.5 mL water and stirred for 4 h at 500 rpm. The suspension was centrifuged as done previously and then dried at 150 °C overnight. The impregnation/heating cycle described above was repeated once more and then the dried particles were calcined using the following heat ramp: 150 °C for 3 h, 250 °C for 3 h, 550 °C for 5 h with a heating rate of 1 °C/min. In the final etching step, 2 M NaOH was added to the calcined particles and they were heated at 80 °C overnight. The etching process was repeated once more and then the particles were centrifuged and washed, first with water and then with ethanol before drying at 70 °C. After etching the TiO_2 microspheres were further calcined at 650 °C for 2 h at the same heating rate of 1 °C/min in order to partially sinter the material.

(2) Synthesis of ZrO_2 microspheres:[32] For the preparation of the ZrO_2 material, 0.5 g $ZrOCl \cdot 8\ H_2O$ was dissolved in 0.5 g H_2O. Then 0.25 g silica (SP-300-10P) was added to the solution and stirred for 1 h at 500 rpm. The mixture was centrifuged at 3000 rpm and the supernatant was removed. The impregnated spheres were then heated at 70 °C overnight before they were calcined at 150 °C for 3 h, then at 250 °C for 3 h, and finally at 550 °C for 5 h using heating ramps of 1 °C/min. This process was repeated two

additional times before etching twice in 2 M NaOH solution at 80 °C overnight, followed by washing with water and ethanol, and then drying at 70 °C.

(3) Synthesis of SnO_2 microspheres:[32] For the preparation of SnO_2 microspheres, 1.35 g of $SnCl_2$ was dissolved in 0.75 mL H_2O, to which 0.25 g silica (SP-120-10P) was added and stirred for 1 h at 500 rpm. The mixture was centrifuged at 3000 rpm and the supernatant was subsequently removed. The impregnated spheres were heated directly at 150 °C for 3 h, then at 250 °C for 3 h, and finally at 550 °C for 5 h with a heating ramp of 1 °C/min. The impregnation/heating cycle described above was repeated one more time. The silica template was finally etched using 2 M NaOH solution at 80 °C two times overnight, after which the particles were washed with water and ethanol and dried at 70 °C.

(4) Synthesis of Fe_2O_3, Co_3O_4, NiO, and In_2O_3 microspheres: Fe_2O_3, Co_3O_4, NiO, and In_2O_3 microspheres were all prepared from hydrated nitrate salts using the double solvent method wherein a predetermined amount (calculated from the total pore volume of silica) of the nitrate salt solution is added to the silica particles earlier dispersed in n-hexane.[33] For example, in the synthesis of the Fe_2O_3 material, 0.25 g of silica (SP-120-10P) was dispersed in 2 mL of n-hexane and stirred at 500 rpm. 300 µL of a 2 M solution of iron nitrate nonahydrate was added to the suspension with continuous stirring for 1 h after which the n-hexane was removed after centrifugation. The resulting wet particles were allowed to dry at room temperature for about 4 h, after which they were heated as follows: 140 °C for 1 h, and then 300 °C for 2 h, with heating ramps of 1 °C/min. This impregnation step was repeated 10 or more times (in order to obtain well-defined and smooth particles), after which the particles were calcined at 550 °C for 5 h with a heating rate of 1 °C/min. Thereafter, the particles were etched twice in 2 M NaOH solution at 80 °C overnight. For Co_3O_4, NiO and In_2O_3, a similar procedure was followed but 3 M solutions of cobalt nitrate hexahydrate, nickel nitrate hexahydrate, and indium nitrate hydrate were used for same amount of silica particles.

Characterization of metal oxide microspheres

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS): SEM images of the starting silica and the nanocast metal oxide microspheres were taken using a Jeol JSM-6335F instrument equipped with a Link Inca 300 (Oxford Instruments) EDS unit. The samples were sputtered with platinum prior to analysis.

Nitrogen physisorption: Nitrogen physisorption measurements were carried out at 77 K using an ASAP 2010 sorptometer (Micromeritics Co). The metal oxide materials were degassed at 423 K for more than 8 h. The BET surface area was determined from the pressure range 0.05-0.20 P/P_0, the pore volume was determined from the adsorption branch at $P/P_0 = 0.98$, and the pore diameter was determined from the desorption branch using the BJH (Barrett-Joyner-Halenda) model.

Zeta potential (ZP) measurements: Isoelectric points for the metal oxide materials were determined by zeta potential measurements using a Malvern Zetasizer NanoZS instrument. The samples were suspended in deionized water (1 mg/mL) and dispersed by sonication before each point was measured at a designated pH after it had been adjusted with either 0.5 M HCl or 0.5 M KOH at 298 K.

X-ray diffraction (XRD): XRD measurements were carried out on a Bruker AXS D8 Discover instrument with a HI-Star 2-D detector using a Cu K_α X-ray source (for the Fe_2O_3 and Co_3O_4 samples a dynamic scintillation detector was used).

Peptide synthesis and preparation of cell lysates

Synthetic phosphopeptides peptides were synthesized using conventional Fmoc solid phase synthesis and were available from earlier studies.[28-29] The following peptides were used (with phosphorylated residues in bold): N**S**VEQGRRL, LIEDNE**Y**TAR, ENIMR**S**ENSESQLTSK, APPDNLPSPGG**S**R, SVENLPEAGI**T**HEQR, WWGSGPSGSGG**S**GGGK, WWGSGPSG**S**GG**S**GGGK, TASDTDSS**Y**AIPTAGMSPSR. Human embryonic kidney (HEK)293 cells were cultured on Dulbecco's Modified Eagle medium containing 10% fetal calf serum to 80-90% confluence. Pelleted cells were lysed in 8.9 M urea/100 mM ammonium bicarbonate by vortexing and sonication. Aggregated DNA was removed with a pipette tip and the resulting sample was centrifuged for 10 min at 16 900 × g. Protein concentration in the resulting supernatant was determined using a bicinchoninic acid (BCA) assay (Thermo Scientific).

Protein digestion

HEK lysate (prepared as above) or a mixture of the model proteins (bovine serum albumin, human transferrin, bovine lactoferrin, all obtained from Sigma-Aldrich) were digested using a two-step procedure with endoproteinase Lys-C (Wako) and trypsin (Promega). All steps except the alkylation step were carried out with mild shaking (750 rpm) on a Thermomixer (Eppendorf). First, protein mixtures were adjusted to a concentration of 1 mg/mL in 8 M urea/150 mM NH_4HCO_3. Disulfide bonds were reduced by addition of tris(2-carboxyethyl) phosphine (final concentration 2.5 mM) and incubation at 37 °C for 30 minutes. Free cysteine thiol groups were subsequently alkylated by iodoacetamide (final concentration 5 mM) and incubation at room temperature in the dark for 30 minutes. The samples were diluted to 5.5 M urea with 150 mM NH_4HCO_3 and Lys-C was added at an enzyme-to-substrate ratio of 1:100. After 3 h incubation at 37 °C, samples were further diluted to 1 M urea and trypsin was added at an

enzyme-to-substrate ratio of 1:50. Samples were incubated at 37 °C overnight. Digestion was stopped by adding pure formic acid to a final concentration of 2% (v/v), and acidified samples were purified by solid-phase extraction using SepPak tC18 cartridges (Waters). The SPE eluate was evaporated to dryness in a vacuum centrifuge.

Generic MOAC enrichment protocol

The batch enrichment protocol followed published procedures.[30,34] For material screening experiments, we used 5 mg of metal oxides and 1 µg per synthetic phosphopeptide dissolved in 100 µL of the respective binding/washing solution (see below). For experiments with spiked-in unphosphorylated background we used 5 mg of metal oxides, 100 ng of each synthetic phosphopeptide and 100 µg of digested model protein mixture. For the enrichment of phosphopeptides from digested HEK293 lysate, we used 5 mg metal oxides and 200 µg peptides (based on protein amounts from the BCA assay and assuming no further losses during sample preparation).

Phosphopeptides were enriched either in the presence (+LA) or absence (-LA) of lactic acid as a competitor to reduce nonspecific binding. For the +LA protocol, peptides were bound to the metal oxides using 200 µL of binding/washing solution A (50% acetonitrile, 0.1% trifluoroacetic acid, 300 mg/mL lactic acid in water), the particles were washed once with 200 µL of solution A and once with 200 µL of solution B (50% acetonitrile, 0.1% trifluoroacetic acid in water). Elution was performed with 2 × 100 µL 50 mM $(NH_4)_2HPO_4$ in water adjusted to pH 10.5 with ammonium hydroxide. For the -LA protocol, binding and washing steps were carried out with solution B only, the elution procedure was the same as above.

The elution solution was immediately acidified using concentrated trifluoroacetic acid to a pH of 2-3 (determined by pH indicator paper) and purified by solid-phase extraction using SepPak tC18 cartridges. The SPE eluate was evaporated to dryness in a vacuum centrifuge.

Enrichment experiments were performed in technical triplicates and each sample was analysed by LC-MS/MS twice, with the exception of the preliminary experiments carried out with synthetic phosphopeptides only, for which single injections were made.

Liquid chromatography-tandem mass spectrometry (material screening stage)

LC-MS/MS analysis was performed using an Easy-nLC HPLC system connected to a LTQ Orbitrap XL mass spectrometer (both Thermo Scientific). Samples were reconstituted in 5% acetonitrile, 0.1% formic acid in water and injected onto an 11 cm × 75 µm column packed in-house with 3 µm Magic C18 beads (Michrom). Peptides were separated using a linear gradient from 5% A, 95% B to 30% A, 70% B over 40 min (synthetic peptides only) or 60 min (synthetic peptides in background), where A = 5% acetonitrile, 0.1% formic acid in water and B = 5% water, 0.1% formic acid in acetonitrile. The flow rate was set to 300 nL/min. For enrichment of synthetic phosphopeptides only, the sample was reconstituted in 500 µL and 1 µL of sample was injected, for the spike-in experiments, 1 µL of 50 µL was used.

The mass spectrometer was operated in data-dependent mode, with one cycle consisting of an Orbitrap full scan at 60 000 resolution (mass range m/z 350-1600) followed by MS/MS scans of the five most abundant precursors in the linear ion trap at normal scan rate. Only multiply charged precursors were selected for fragmentation using CID at 32% normalized collision energy. Dynamic exclusion was enabled with a window of 30 s after one sequencing event.

Data analysis (material screening stage)

Orbitrap XL data were first converted into mzXML format using ProteoWizard (version 3.0.3316) and then into mgf (Mascot generic format) using MzXML2Search (part of the Trans-Proteomic pipeline). Mgf files were searched against a custom database of the synthetic phosphopeptides and the three background proteins using Mascot (version 2.4.01). The following search parameters were used: Enzyme = trypsin, allow up to 2 missed cleavages, fixed modifications = carbamidomethylation on Cys, variable modifications = oxidation on Met and phosphorylation on Ser/Thr/Tyr, peptide tolerance ± 15 ppm, MS/MS tolerance ± 0.6 Da, instrument = ESI-TRAP and decoy search = on. Results were filtered to a false discovery rate of > 1% and only peptides with an ion score of ≥ 20 were used for quantification. Label-free quantification was performed with Skyline (version 2.5.0.6157)[35] using the Mascot search results in dat format as input.

Liquid chromatography-tandem mass spectrometry (cell lysate)

LC-MS/MS analysis was carried out on an Easy-nLC 1000 HPLC system connected to an Orbitrap Elite mass spectrometer (both Thermo Scientific). Samples were reconstituted in 20 µL of 5% acetonitrile, 0.1% formic acid in water and injected onto a 15 cm × 75 µm PepMap RSLC column (2 µm particle size, Thermo Scientific). Peptides were separated using a linear gradient from 5% A, 95% B to 25% A, 75% B over 180 min, where A = 5% acetonitrile, 0.15% formic acid in water and B = 5% water, 0.15% formic acid in acetonitrile. The flow rate was set to 300 nL/min. The injection volume was set to 2 µL with the exception of SnO_2 +LA, NiO +LA and Co_3O_4 +LA samples, for which the injection volume was 4 µL to compensate for the expected reduced recoveries.

The mass spectrometer was operated in data-dependent mode, with one cycle consisting of an Orbitrap full scan at 120 000 resolution (mass range m/z 350-1600) followed by MS/MS scans of the 15 most abundant precursors in the linear ion trap at normal scan rate. Only multiply charged precursors were selected for fragmentation using CID at 35% normalized collision energy. Dynamic exclusion was enabled with a window of 30 s after one sequencing event.

Data analysis (cell lysate)

Orbitrap Elite data in raw format were searched using MaxQuant[36] (version 1.5.2.8) against human entries of the UniProt/SwissProt database (version 2014_11) and the contaminant protein database provided with MaxQuant. Six replicate measurements of individual materials/conditions were searched together. Search settings included: Digestion mode = trypsin/P, fixed modification = carbamidomethylation on Cys, variable modifications = oxidation on Met and phosphorylation on Ser/Thr/Tyr, first search (MS1) tolerance = 20 ppm, ITMS MS/MS match tolerance = 0.6 Da, decoy mode = reversed, match between runs = on, minimum peptide length = 6, PSM FDR = 0.01. Label-free quantification was also performed with MaxQuant with the following parameters: minimum peptide count = 1, use unmodified and modified peptides for quantification.

The primary output from MaxQuant was retrieved from the evidence.txt files and further processed as follows: Hits to the contaminant database, hits with scores <50 or a posterior error probability >0.05 were removed.

To determine the specificity of the enrichment procedure, the following convention was used: Both phosphorylated and unphosphorylated peptides were counted as multiple identifications if they were observed in both unmodified and (Met-)oxidized forms. For phosphopeptides, every phosphorylation “state” (1 phosphate, 2 phosphates, etc.) was counted only once to avoid ambiguities introduced by site localization scores. This conservative approach likely underestimates the total number of phosphopeptides, but avoids a bias against highly phosphorylated states: MS/MS spectra of many triply and quadruply phosphorylated peptides do not contain sufficient evidence for confidently localizing all modification sites although sequence identity can be assigned with high certainty.

To derive final lists of non-redundant phosphopeptides in a data set, we also considered the site localization confidence as calculated by MaxQuant. We removed all hits for which the n-th localization score was <0.8 (80% confidence), where n is the number of phosphate groups. For example, for a triply phosphorylated peptide, the localization confidence of all three sites must be 0.8 or higher.

Additional data analysis was performed using Excel 2013 (Microsoft) and R 3.1.3 (The R Foundation for Statistical computing). Isoelectric points of phosphorylated and unphosphorylated peptides were calculated using the tool pI calculator.[37]

Data deposition

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier <PXD002646>.

ESI Figures

Fig. S1 XRD patterns of the different nanocast metal oxide materials. The inverted triangle (❑) indicates trace amounts of monoclinic ZrO_2, while asterisks (*) indicate reflections from the sample holder. The ZrO_2 sample consists mainly of the tetragonal phase (JCPDS card number: 01-072-7115), but traces of monoclinic ZrO_2 can also be detected (JCPDS card number: 00-037-1484). The diffraction pattern of the TiO_2 replica displays the typical anatase crystal structure (JCPDS card number: 03-065-5714), while the SnO_2 sample fits well to the cassiterite structure (JCPDS card number: 01-070-6995). The In_2O_3, Fe_2O_3, NiO and Co_3O_4 samples can be indexed to the JCPDS card numbers: 00-006-0416, 00-033-0664, 00-047-1049, and 01-073-1701, respectively.

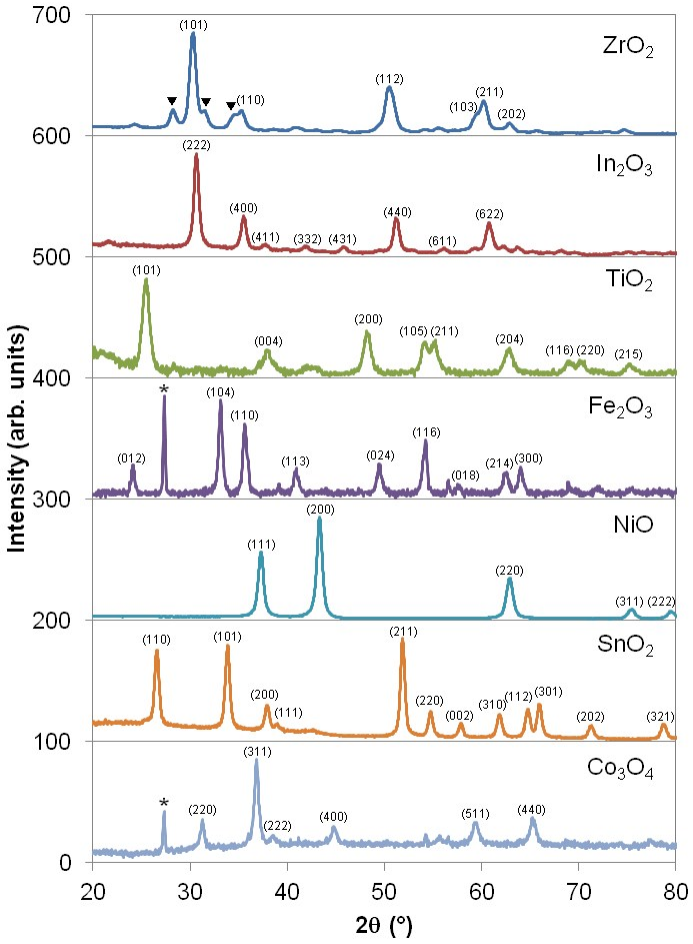
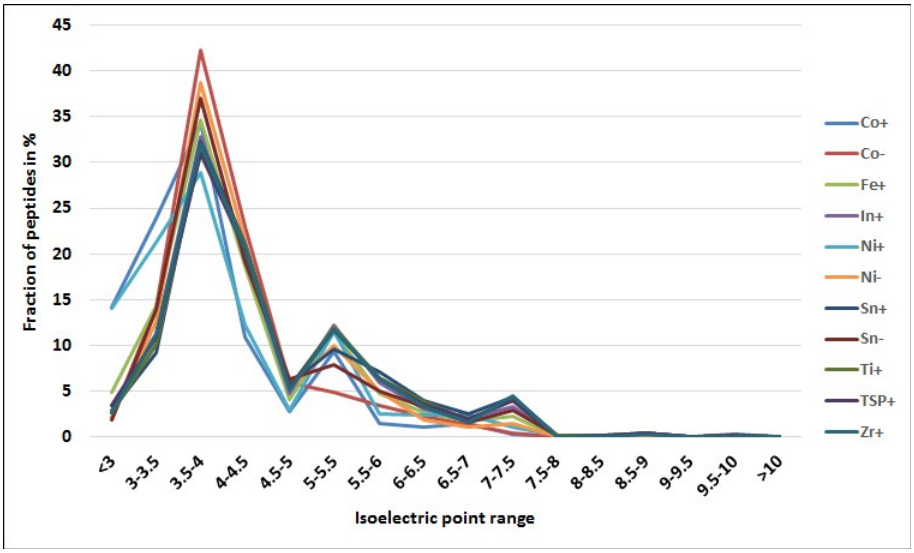
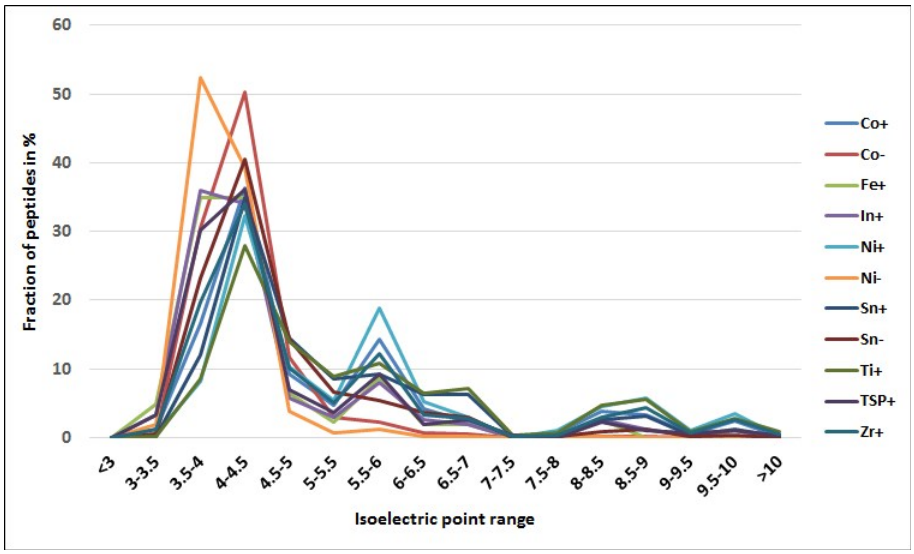


Fig. S2 Isoelectric point distribution of (top) phosphorylated and (bottom) non-phosphorylated peptides enriched from digested HEK lysate with different metal oxide materials. Only peptides with non-oxidized Met residues were included. For phosphorylated peptides every peptide sequence was counted only once, even if multiple phosphopeptides with the same sequence were identified.

PHOSPHOPEPTIDES



NON-PHOSPHORYLATED PEPTIDES



ESI Tables

Table S1 Detailed results of phosphopeptide enrichment experiments from digested HEK lysate. Shown are the numbers of identified peptides for every single injection and specificities based on peptide counts and peak areas. Provided as a separate file in Microsoft Excel format.

Table S2 List of all identified phosphopeptides enriched from digested HEK lysate with different metal oxide materials. Provided as a separate file in Microsoft Excel format.

Table S3 Amino acid frequency distribution (in %) of phosphorylated and non-phosphorylated peptides enriched from digested HEK lysate with different metal oxide materials. Only peptides with non-oxidized Met residues were included. For phosphorylated peptides every peptide sequence was counted only once, even if multiple phosphopeptides with the same sequence were identified. Reference data from UniProt/SwissProt (SwPr) is from version 2015_05.

Phosphopeptides

	Zr+	TSP	In+	Ti+	Fe+	Ni-	Sn+	Ni+	Sn-	Co+	Co-	SwPr
Ala	7.0	6.5	6.9	6.8	6.5	6.9	6.2	6.3	6.7	5.6	6.9	8.3
Arg	4.8	5.2	5.1	5.0	5.1	4.5	5.0	5.8	4.0	5.3	4.1	5.5
Asn	2.6	2.8	2.6	2.6	2.6	2.5	2.7	2.2	2.4	2.2	2.5	4.1
Asp	7.6	7.9	7.5	7.4	8.6	8.6	8.0	7.9	8.9	9.8	9.2	5.5
Cys	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.4	0.5	0.5	1.4
Glu	11.6	11.6	11.5	11.4	12.6	12.9	13.1	12.2	13.2	15.1	14.2	6.7
Gln	3.9	4.0	3.8	4.0	3.6	3.9	3.8	3.3	3.9	3.1	4.1	3.9
Gly	6.6	6.6	6.7	6.7	6.7	6.7	6.1	6.6	6.6	5.9	6.5	7.1
His	1.5	1.6	1.5	1.6	1.4	1.4	1.7	1.2	1.5	1.0	1.6	2.3
Ile	2.4	2.3	2.2	2.3	2.1	2.3	2.0	2.1	2.4	2.0	2.3	5.9
Leu	5.7	5.3	5.3	5.5	5.1	5.4	4.5	4.4	5.5	3.8	5.1	9.7
Lys	5.3	5.6	5.5	5.3	5.6	5.3	7.0	5.5	5.7	6.7	5.7	5.8
Met	0.1	0.4	0.2	0.5	0.3	0.5	0.1	0.1	0.5	0.1	0.7	2.4
Phe	1.6	1.5	1.4	1.5	1.4	1.6	1.2	1.1	1.7	1.0	1.6	3.9
Pro	12.1	12.1	12.6	12.6	11.5	11.7	11.8	13.6	12.0	11.5	11.5	4.7
Ser	15.0	15.5	15.8	14.9	15.8	14.2	15.8	17.2	13.2	16.9	12.7	6.6
Thr	5.2	4.7	4.9	5.3	4.9	5.0	4.7	4.8	5.0	4.2	4.6	5.3
Trp	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.1	0.3	1.1
Tyr	1.4	1.3	1.3	1.3	1.3	1.3	1.2	1.2	1.4	1.2	1.5	2.9
Val	4.5	4.3	4.4	4.4	4.2	4.5	4.2	3.9	4.5	4.0	4.5	6.9

Non-phosphorylated peptides

	Zr+	TSP	In+	Ti+	Fe+	Ni-	Sn+	Ni+	Sn-	Co+	Co-	SwPr
Ala	7.5	6.1	6.8	6.7	6.3	6.8	6.8	8.7	6.8	8.2	6.9	8.3
Arg	3.6	3.4	3.1	3.8	3.1	3.2	3.5	4.0	3.3	3.5	3.3	5.5
Asn	3.8	5.7	4.2	4.7	4.2	3.3	4.1	4.5	3.8	4.4	3.8	4.1
Asp	10.4	14.4	12.9	9.3	13.9	14.0	10.2	7.5	10.7	9.7	10.7	5.5
Cys	1.1	1.0	0.9	1.3	1.0	1.1	1.3	1.5	1.3	1.3	1.4	1.4
Glu	13.3	13.7	17.4	11.0	17.1	17.5	14.0	10.1	14.4	12.7	15.2	6.7
Gln	4.3	4.4	4.1	5.4	4.1	4.4	4.9	4.9	4.9	4.6	4.9	3.9
Gly	7.4	7.8	7.9	7.3	8.2	6.7	7.0	7.9	6.3	8.3	6.3	7.1
His	1.6	2.1	1.0	3.6	0.9	1.0	3.1	1.2	2.3	1.1	1.6	2.3
Ile	4.2	3.9	3.9	4.2	4.2	3.4	4.0	4.9	3.9	4.5	3.8	5.9
Leu	6.9	6.7	6.5	7.0	6.2	6.6	6.6	8.2	7.3	7.3	7.7	9.7
Lys	5.6	5.5	5.6	6.9	5.3	4.5	7.7	5.7	5.7	5.8	4.6	5.8
Met	0.1	0.5	0.2	0.6	0.3	1.0	0.1	0.1	1.0	0.0	1.4	2.4
Phe	2.9	2.9	2.2	2.8	2.3	2.4	2.2	2.9	2.7	2.6	2.8	3.9
Pro	5.9	4.0	5.0	5.9	4.1	5.7	4.9	4.6	5.6	4.3	5.6	4.7
Ser	7.6	5.8	5.7	6.6	6.0	6.6	6.9	7.0	7.1	6.7	7.0	6.6
Thr	4.9	4.5	4.8	4.6	5.1	4.4	4.7	5.9	4.8	5.7	4.9	5.3
Trp	0.5	0.8	0.4	0.6	0.4	0.6	0.4	0.4	0.7	0.2	0.7	1.1
Tyr	2.5	2.5	2.4	2.3	2.6	1.9	2.3	2.9	2.2	2.5	2.2	2.9
Val	5.8	4.6	5.1	5.4	4.8	4.9	5.4	7.2	5.3	6.6	5.4	6.9

Table S4. Quantitation of selected phosphopeptides with common sequence, but different numbers of phosphorylation sites. Averaged peak areas as reported by MaxQuant are shown. Only identifications with at least one identification with confident site localization for a given material/condition are listed. Provided as a separate file in Microsoft Excel format.

Table S5. Comparison of confidently localized phosphopeptides in 13 highly phosphorylated proteins from digested HEK lysate. Materials/conditions that identified the highest number of peptides from a particular protein are marked in bold green.

	Material >>>	Zr+	TSP+	In+	Ti+	Fe+	Ni-	Sn+	Ni+	Sn-	Co+	Co-	All
Protein	Protein name												
ACINU	Apoptotic chromatin condensation inducer in the nucleus	**20**	18	19	**20**	**20**	14	13	9	5	8	6	32
BCLF1	Bcl-2-associated transcription factor 1	**34**	**34**	30	30	33	27	20	10	18	9	18	55
CHAP1	Chromosome alignment-maintaining phosphoprotein 1	24	**27**	26	25	19	18	11	6	5	3	5	38
MAP1B	Microtubule-associated protein 1B	**26**	21	18	24	16	13	9	3	1	4	0	40
MINT	Msx2-interacting protein	**27**	25	21	23	20	18	14	8	6	7	5	43
NUCKS	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	**22**	20	19	16	19	18	18	11	11	13	10	24
PININ	Pinin	12	14	13	11	**19**	11	8	6	6	4	7	22
SRRM1	Serine/arginine repetitive matrix protein 1	**64**	59	**64**	48	51	39	41	56	30	42	23	112
SRRM2	Serine/arginine repetitive matrix protein 2	140	140	144	138	**151**	94	63	102	37	71	37	285
TCOF	Treacle protein	25	**26**	25	23	20	16	19	7	9	5	5	33
TR150	Thyroid hormone receptor-associated protein 3	**26**	23	20	23	24	20	17	9	12	11	8	34
TP53B	Tumor suppressor p53-binding protein 1	29	**31**	**31**	27	26	23	11	7	9	4	7	54
ZC3HD	Zinc finger CCCH domain-containing protein 13	25	**29**	26	22	27	13	13	17	4	13	5	44
	All proteins	**474**	467	456	430	445	324	257	251	153	194	136	816