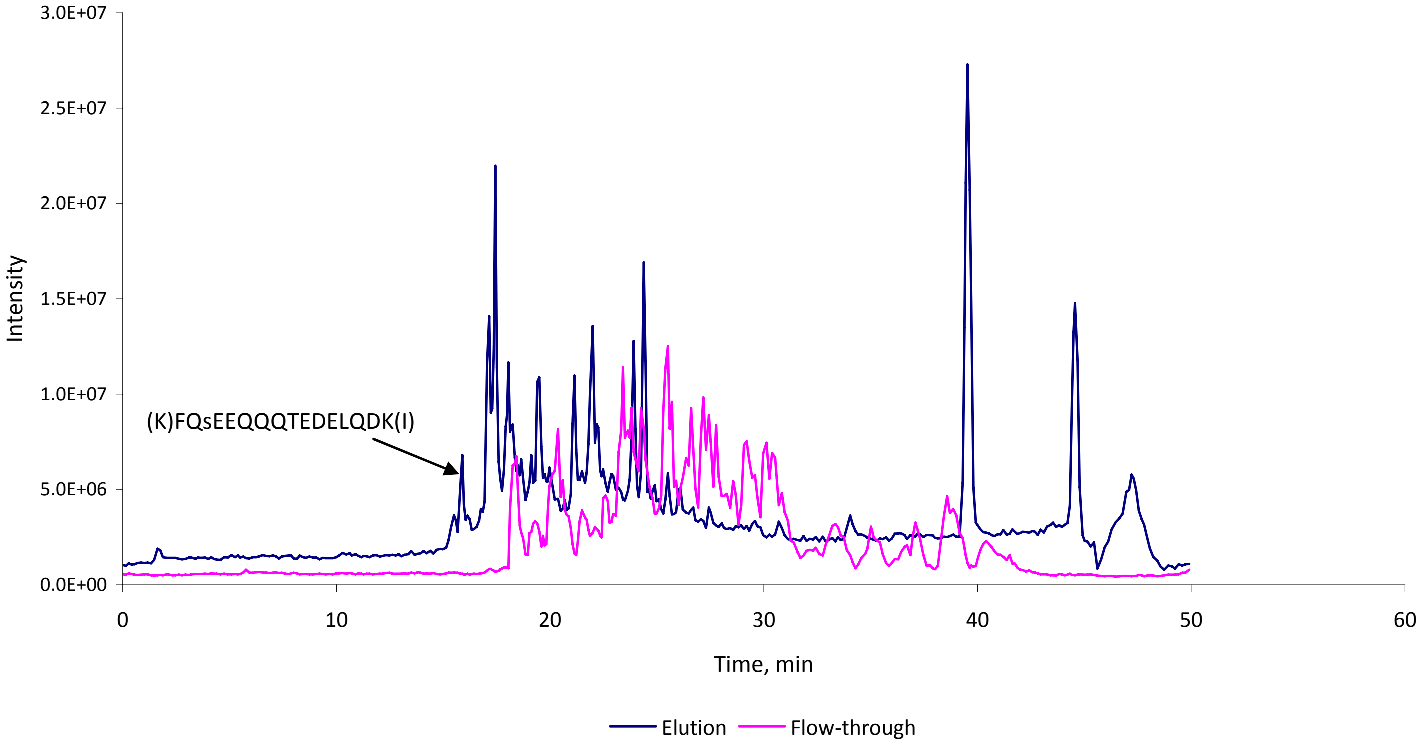
Electronic Supporting Material



Supplement 1. Figure 1. Base peak chromatograms of flow-through and elution operated at 100 nM β-casein and BSA protein mixture obtained by phosphochip® coupled to NanoLC-Chip/ITMS system. As shown in Figure 1 the peak at 16 min which belongs to *β*-casein does not appear at the flow-through chromatogram but on the other hand by injecting elution buffer *β*-casein eluted from TiO_2 column and the *β*-casein peak is observed at the elution chromatogram.

Supplement 2.

The results for β-casein obtained from both search engines gave the same singly phosphorylated peptide on serine (K)FQsEEQQQTEDELQDK(I) and the phosphorylation was on site 50S of the protein. Also as expected β-casein could not be identified with both search engines using the data obtained from flow-through chromatogram. Spectrum Mill results for BSA using flow-through chromatogram resulted in 9 matching peptides; however Mascot gave 12 matched peptides for the same data. When elution chromatogram was searched with both search engines, Spectrum Mill and Mascot gave 3 and 4 matched peptides, respectively for BSA (Table 1). These results showed that some of the non-phosphorylated peptides could be retained on the TiO_2 column and eluted with elution buffer. Similar results were obtained by different groups and they indicated that these non-phosphorylated peptides in the elution chromatogram are highly acidic in nature and contamination with acidic peptides represents a common issue in phosphorylated peptide enrichment, based on metal co-ordination[21, 30, 31]. Nevertheless it is still advantageous to perform TiO_2 enrichment to improve detection of phosphorylated peptides due to low abundance of many phosphorylated peptides.

References to this supplement 2. (numbers are from the manuscript reference list)

21. S. B. Ficarro, M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt and F. M. White, "Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae," Nature biotechnology **20**, 301-305 (2002).

30. S. Mohammed, K. Kraiczek, M. W. H. Pinkse, S. Lemeer, J. J. Benschop and A. J. R. Heck, "Chip-based enrichment and NanoLC-MS/MS analysis of phosphopeptides from whole lysates," Journal of Proteome Research **7**, 1565-1571 (2008).

31. R. Raijmakers, K. Kraiczek, A. P. De Jong, S. Mohammed and A. J. R. Heck, "Exploring the human leukocyte phosphoproteome using a microfluidic reversed-phase-TiO2reversed-phase high-performance liquid chromatography phosphochip coupled to a quadrupole time-of-flight mass spectrometer," Analytical Chemistry **82**, 824-832 (2010).

Table 1. Comparison of flow-through and elution chromatogram results using Spectrum Mill and Mascot database search engines

Spectrum Mill results							
Flow-through Chromatogram				Elution Chromatogram			
Total Score	# matched peptides	Peptide sequence	Protein name	Score	# matched peptides	Peptide sequence	Protein name
-	-	-	-	20	1	(K)FQsEEQQQTEDELQDK(I)	β-casein
92	9	(K)CCAADDKEACFAVEGPK(L) (K)DAFLGSFLYEYSR(R) (K)DAIPENLPPLTADFAEDK(D) (R)ETYGDMADCCEK(Q) (K)GLVLIAFSQYLQQCPFDEHVK(L) (K)LFTFHADICTLPDTEEK(Q) (R)MPCTEDYLSLILNR(L) (R)RHPYFYAPELLYYANK(Y) (K)SHCIAEVEKDAIPENLPPLTADFAEDK(D)	Serum albumin	38	3	(K)LVNELTEFAK(T) (K)DLGEEHFK(G) (K)AEFVEVTK(L)	Serum albumin
Mascot results							
Flow-through Chromatogram				Elution Chromatogram			
Total Score	# matched peptides	Peptide sequence	Protein name	Score	# matched peptides	Peptide sequence	Protein name
-	-	-	-	74	1	(K)FQsEEQQQTEDELQDK(I)	β-casein
247	12	(K)HLVDEPQNLIK(Q) (K)SLHTLFGDELCK(V) (K)DAFLGSFLYEYSR(R) (R)ETYGDMADCCEK(Q) (R)MPCTEDYLSLILNR(L) (R)HPYFYAPELLYYANK(Y) (R)NECFLSHKDDSPDLPK(L) (K)LFTFHADICTLPDTEK(Q) (K)CCAADDKEACFAVEGPK(L) (K)DAIPENLPPLTADFAEDK(D) (R)RHPYFYAPELLYYANK(Y) (K)GLVLIAFSQYLQQCPFDEHVK(L)	Serum albumin	93	4	(K)LVNELTEFAK(T) (K)DLGEEHFK(G) (K)AEFVEVTK(L) (K)DDPHACYSTVFDK(L)	Serum albumin

Supplement 3. Table 2. Phosphorylated protein results obtained by Spectrum Mill and Mascot (100µM SeMet + 30µM iAs inoculated cell lysate)

	Spectrum Mill Score	Mascot Score	Access #*	Peptide Sequence	Variable Sites found in the study**	UniprotKB Reported Variable Sites ***	Protein Name	**Function** (this column represents abbreviated and paraphrased information from the UniprotKB database for each protein report and is obtained through the access # link in Row D of this table).
1	21.19	57	P46060	(R)YQDEVFGGFVtEPQEEsEEEVEEPEER(Q) (K)sssPAPADIAQTVQEDLR(T)	S149s T143t S230s, S231s, S232s	**S :** 149, 230, 231,371 **T :** 143, 266	Ras GTPase-activating protein-binding protein 1	GTPase activator for the nuclear Ras-related regulatory protein Ran. Phosphorylated occurs before nuclear envelope breakdown and continues throughout mitosis.
2	13.95	61	P54105	(K)FEEESKEPVADEEEEDsDDDVEPITEFR(F)	S102s	**S :** 102, 193 **T :** 223	Methylosome subunit pICln	Inhibits the binding of survival motor neuron protein (SMN) to Sm proteins. May participate in cellular volume control. Phosphorylated upon DNA damage, probably by ATM or ATR
3	13.61	54	P05388	(K)EEsEEsDEDMGFGLFD(-)	S304s S307s	**S :** 304, 307 **T :** 24	60S acidic ribosomal protein P0 (L10E)	Equivalent function to E.coli protein L10
4	13.37	48	P13861	(K)GDsEsEEDEDLEVPVPSR(F)	S78s, S80s	**S :** 58, 78, 80, 99 **T :** 54	cAMP-dependent protein kinase type II	Type II regulatory chains mediate membrane association by binding to anchoring proteins, including the MAP2 kinase. Phosphorylated by the activated catalytic chain.
5	12.4	42	Q9UQ35	(K)AQtPPGPSLsGSKSPCPQEK (S)	S1010s T1003t	**S** (partial report) **:** 250, 358, 1010,	Serine/arginine repetitive matrix protein 2	Part of pre- and post-splicing multiprotein mRNP complexes. May be involved in pre-mRNA

	11.08			(R)sGSsQELDVKPSASPQER(S)	S1539s S1542s	2740; not included 1539, 1542 **T** (partial report) **:** 1003, 2738 **Y :** 145, 996, 2693		processing events. Binds to RNA. Phosphorylated upon DNA damage, probably by ATM or ATR.
6	11.33	-	O60609	(R)SLGNyELDVSPYEDTVTSKPWKMNLSK(L)	Y129y	Not Reported (NR)	GDNF family receptor alpha-3	Receptor for the glial cell line-derived neurotrophic factor, ARTN (artemin). Mediates the artemin-induced autophosphorylation and activation of the RET receptor tyrosine kinase.
7	11.27	38	O15085	(R)LSsLSSLGDSAPERKSPSHHR (Q)	S14s	**S :** 245, 251, 255, 663, 1155, 1295, 1300, 1457, 1458 , 1480, 1489, 1491 **T :** 254, 668, 672, 1462, 1475	Rho guanine nucleotide exchange factor 11	Acts as guanine nucleotide exchange factor (GEF) for RhoA GTPase and may act as GTPase-activating protein (GAP) for GNA12 and GNA13.
8	11.26	44	Q15185	(K)DWEDDsDEDMSNFDR(F)	S113s	**S :** 113, 118, 148, 151	Prostaglandin E synthase 3	Molecular chaperone that localizes to genomic response elements in a hormone-dependent manner and disrupts receptor-mediated transcriptional activation.
9	11.22	-	P08910	(R)CLNLKsPTAPPDLYFQDSGLsRFLLK(S)	S37s S52s	NR	Abhydrolase domain-containing protein 2	May play a role in smooth muscle cells migration
10	11.12	47	Q14247	(K)TQtPPVsPAPQPTEER(L)	S405s T401t	**S :** 113, 117, 215, 334, 405, 417, 418 **T :** 399, 401, 411 **Y :** 141, 154, 421, 446, 453	Src substrate cortactin	May contribute to the organization of cell structure. Tyrosine phosphorylation in transformed cells may contribute to cellular growth regulation and transformation.

11	11.06	59	P46821	(K)VLsPLRsPPLIGsEsAYESFLSADDK(A) (K)LGDVsPtQIDVSQFGSFK(E) (R)EsSPLYsPTFSDSTSAVK(E)	S1396s S1400s S1406s S1408s S1501s T1503t S1792s S1797s	**S** (partial) **:** 336, 1396, 1400, 1408, 1501, 1792, 1797, 2289 **T** (partial) **:** 527, 1282, 2334 **Y** (partial) **:** 1062, 1762, 2025	Microtubule-associated protein 1B (MAP 1B)	The function is essentially unknown. Phosphorylated MAP1B may involve in the cytoskeletal changes that accompany neurite extension. Phosphorylated upon DNA damage, probably by ATM or ATR.
12	10.85	38	Q96JZ2	(R)GYtDPCVATSLKsPSQPQAPKDR(K)	S276s T266t	NR	Hematopoietic SH2 domain-containing protein	May modulate any apoptotic response because it can affect mitochondrial stability. It is an adapter protein associated with tyrosine kinase signaling. May be phosphorylated by FES and ACK1.
13	10.83	40	Q8NEA6	(R)LGLISPPAsQVSTACNQIsPSLQR(A)	S13s S23s	NR	Zinc finger protein GLIS3	Acts as both a repressor and activator of transcription.
14	10.72	38	Q9NT68	(K)VPWSLKNSSIDsGEAEVGR(R)	S448s	**S :** 523 **Y :** 1150	Teneurin-2	May function as a cellular signal transducer.
15	10.55	-	Q9Y2Q0	(R)AEGyEKTDDVSEKTSLADQEEVR(T)	Y18y	**S :** 9, 25, 29 **T :** 28 **Y:** 269	Probable phospholipid-transporting ATPase IA	May play a role in the transport of aminophospholipids from the outer to the inner leaflet of various membranes and the maintenance of asymmetric distribution of phospholipids.
16	10.48	-	Q09019	(R)YHSLGNIsRGGSGGSGSGGEKPSGPVPR(S)	S535s	**S :** 545	Dystrophia myotonica WD repeat-containing protein	May have a regulatory function in meiosis.

17	10.43	49	Q9NYF8	(K)SSATSGDIWPGLSAYDNsPR (S)	S222s	**S** (partial) **:** 102, 222, 760 **T** (partial) **:** 341,526, 840 **Y :** 284, 408, 511	Bcl-2-associated transcription factor 1	Death-promoting transcriptional repressor.
18	10.43	41	P25391	(K)SPGTANVLDVNNstLMFVGGLGGQIKKSPAVK(V)	S2245s T2246t	NR	Laminin subunit alpha-1	A glycoprotein that most likely affects the organization of cells into tissues during embryonic development by interacting with other extracellular matrix components.
19	10.42	-	Q6P9A3	(R)IHTGERPYVCMECGKSFIHsyDR(I)	S373s Y374y	NR	Zinc finger protein 549	May be involved in transcriptional regulation.
20	10.4	50	Q15365	(K)QICLVMLETLSQsPQGR(V)	S173s	**S :** 173, 189, 190, 246, 262, 263, 264	Poly(rC)-binding protein 1 (Alpha-CP1)	Single-stranded nucleic acid binding protein that binds preferentially to oligo dC and lowers poly(rC)-binding activity.
21	10.36	-	Q7Z5N4	(R)VIQTLQAPPDVAPtSVTVRTASETSLRLR(W)	T1178t	NR	Protein sidekick-1	Cell adhesion protein that guides axonal terminals to specific synapses in developing neurons.
22	10.25	44	P52292	(R)NVSSFPDDATsPLQENR(N)	S62s	**S :** 24, 62, 490 **T :** 3, 9, 61	Importin alpha-2 subunit	An adapter protein to nuclear receptor KPNB1 for nuclear protein import.
23	10.25	40	Q9HCP6	(-) MGIKtALPAAELGLYSLVLSGALAyAGR(G)	T5t Y25y	NR	Protein-cysteine N-palmitoyltransferase HHAT-like protein	Negatively regulates N-terminal palmitoylation of SHH by HHAT/SKN .
24	10.12	61	Q99102	(K)sLEPFTLEILARsAKIGLASALQPR(T)	S1763s S1775s	NR	Mucin-4	Assoctitated with a possible role in tumor progression. This may be because apoptosis is repressed rather than cell proliferation
25	10.09	35	Q9HBF4	(R)RHPVTVyNVsNLQESLEAEEMDEETK(R)	S124s Y121y	NR	Zinc finger FYVE domain-containing protein 1	Binds to phosphatidylinositol-3-phosphate (PtdIns3P) through its FYVE-type zinc finger.

26	10.02	48	Q86YT6	(R)VQQIYSDSDLKVEVCGTSWTyNPAAVsK(V)	S398s Y392y	NR	E3 ubiquitin-protein ligase MIB1	Positively regulates the Delta-mediated Notch signaling by ubiquitinating the intracellular domain of Delta, leading to endocytosis of Delta receptors.
27	9.82	34	Q9UDY8	(K)RALTDPIQGTEySAESLVRNLQWAK(A)	Y569y	**S :** 135	Mucosa-associated lymphoid tissue lymphoma translocation protein 1	Has ubiquitin ligase activity. Enhances BCL10-induced activation of NF-kappa-B. Involved in nuclear export of BCL10.
28	9.75	46	Q96MP8	(R)CYEDTMLAAMFSGRHyIPTDsEGR(Y)	S91s Y86y	**S :** 162, 163	BTB/POZ domain-containing protein KCTD7	Cause progressive myoclonic epilepsy type 3 (EPM3) if defects in KCTD.
29	9.73	43	Q8IWB6	(R)sQFPVLHMEVIVHLLLQISDALR(Y)	S343s	NR	Testis-expressed protein 14	May be required for normal structure of the intercellular bridge that connects spermatocytes and spermatogonia. Has no protein kinase activity (in vitro)
30	9.66	42	Q86U17	(R)HQLsEPAPAYHRItPTITNFALRLYK(E)	S41s T51t	NR	Serpin A11	Glycoprotein
31	9.63	47	Q9ULC6	(K)tWRWGPEGyGAILLVNCDR (D)	T138t Y146y	NR	Protein-arginine deiminase type-1	Catalyzes the deimination of arginine residues of proteins
32	9.61	36	Q58A45	(K)SSNFGYITsCYKAVNsKDDLPYCLR(R)	S359s S366s	NR	PAB-dependent poly(A)-specific ribonuclease subunit 3	Functions in cytoplasmic mRNA decay. As part of the Pan nuclease complex, recruits polyadenylate-binding protein which in turn stimulates PAN2 nuclease activity.
33	9.46	-	Q8TAK6	(K)LSKIATLLLARNyILLLGsSLQELR(R)	S166s Y160y	NR	Oligodendrocyte transcription factor 1 (Oligo1)	Promotes formation and maturation of oligodendrocytes. Cooperates with OLIG2 to establish the pMN domain of the embryonic neural tube

34	9.38	46	Q96AY2	(R)RAGPSEDREDWVEEPtVLVLLR(A)	T311t	**S :** 12, 15, 84, 85, 87, 111, 117 **T :** 150	Crossover junction endonuclease EME1	Interacts with MUS81 to form a DNA structure-specific endonuclease. May be required in mitosis for the processing of stalled or collapsed replication forks.
35	9.29	-	O60733	(K)GDGEILVELVQYCHtQMDVTDyK(G)	T177t Y184y	**S :** 587	85 kDa calcium-independent phospholipase A2	Catalyzes the release of fatty acids from phospholipids. May participate in fas mediated apoptosis and in regulating transmembrane ion flux in glucose-stimulated B-cells. Has a role in cardiolipin (CL) deacylation.
36	-	80	O00193	(R)SAsPDDDLGSSNWEAADLGNEER(K)	S17s	**S :** 15, 17, 25, 105, 132, 136, 138, 140, 142, 147 **Y:** 84	Small acidic protein	Phosphoprotein with acetylation
37	-	72	P26232	(R)tPEELEDDsDFEtEDFDVR(S) (R)TLAVERLLEPLVTQVTTLVNTNSKGPsNK(K)	S640s T632t T644t S47s	**S :** 6, 151, 262, 640, 654, 901 **T :** 5, 261, 653, 65	Catenin alpha-2	May function as a linker between cadherin adhesion receptors and the cytoskeleton to regulate cell-cell adhesion and differentiation in the nervous system. Regulates morphological plasticity of synapses and cerebellar and hippocampal lamination during development
38	-	70	Q13765	(K)VQGEAVSNIQENTQtPtVQEEsEEEEVDEtGVEVK(D)	S166s T159t T161t T174t	**S :** 43, 166, 186, 191 **T :** 92, 159, 161	Nascent polypeptide-associated complex subunit alpha	Prevents inappropriate targeting of non-secretory polypeptides to the endoplasmic reticulum (ER). May act as a specific coactivator for JUN.Phosphorylated upon DNA damage, probably by ATM or ATR.

39	-	66	P35221	(R)TPEELDDsDFETEDFDVR(S)	S641s	**S :** 641, 652, 655 **T :** 645, 654, 658 **Y :** 177, 691	Catenin alpha-1	Associates with the cytoplasmic domain of a variety of cadherins. May play a crucial role in cell differentiation.
40		53	P05387	(K)KEEsEEsDDDMGFGLFD(-)	S102 S105s	**S :** 16, 17, 79, 86, 102, 105	60S acidic ribosomal protein P2	Plays an important role in the elongation step of protein synthesis
41	-	49	P29692	(K)KPAtPAEDDEDDDIDLFGsDNEEEDKEAAQLR (E)	S162s T147t	**S :** 60, 119, 133, 162 **T :** 129, 147	Elongation factor 1-delta	stimulate the exchange of GDP bound to EF-1-alpha to GTP. Phosphorylated upon DNA damage, probably by ATM or ATR
42	-	48	Q13895	(R)MPQDGsDDEDEEWPTLEK(A)	S98s	**S :** 98, 167	Bystin	Required for processing of 20S pre-rRNA precursor and biogenesis of 40S ribosomal subunits. May be required for trophinin-dependent regulation of cell adhesion during implantation of human embryos.
43	-	44	Q08945	(K)EGMNPSYDEYADsDEDQHDAYLER(M)	S444s	**S** (partial) **:** 437, 444, 688 **T :** 170 **Y :** 311, 441, 554	FACT complex subunit SSRP1	a general chromatin factor that acts to reorganize nucleosomes

*Access numbers are active links allowing access to the full information provided by UniprotKB.

**Variable site(s) newly found in this study are labled in green, while those found in this study and also reported by UniprotKB, are given in orange.

***The sites underline in column G are to show coincidence with those reported by UniprotKB.