Supporting Information

Peptide labeling with isobaric tags yields higher identification rates using iTRAQ 4-plex compared to TMT 6-plex and iTRAQ 8-plex on LTQ Orbitrap

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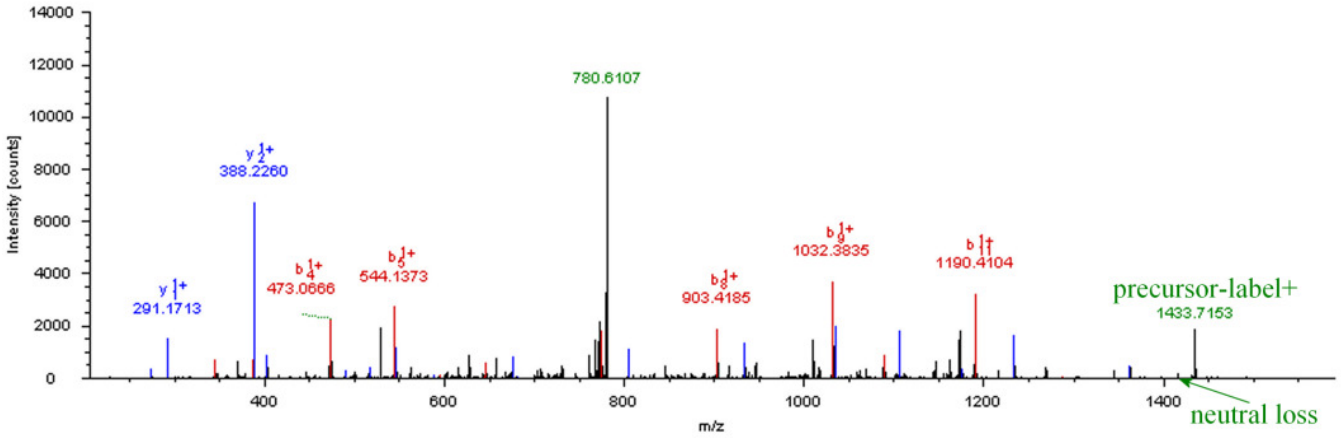
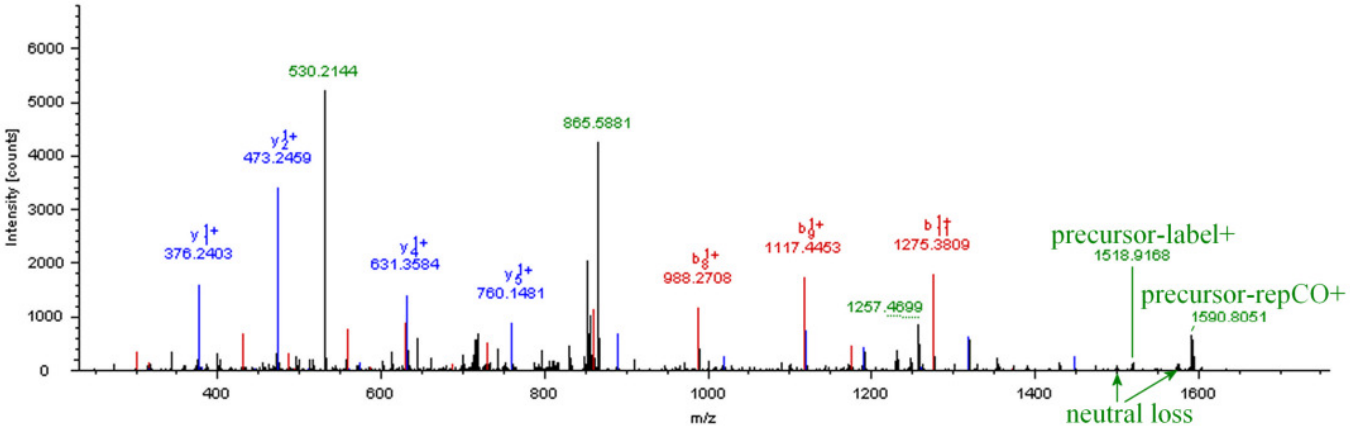
A aEGAATEEEGTPk (BASP-1), Modification iTRAQ 4-plex (N-term & K13)

Precursor *m/z* 789.4016, charge 2+, Mascot Score improved from 48 to 51



B aEGAATEEEGTPk (BASP-1), Modification TMT 6-plex (N-term & K13)

Precursor *m/z* 874.4609, charge 2+, Mascot Score improved from 41 to 48



C aEGAATEEEGTPk (BASP-1), Modification iTRAQ 8-plex (N-term & K13)

Precursor *m/z* 949.5035, charge 2+, Mascot Score improved from 39 to 44

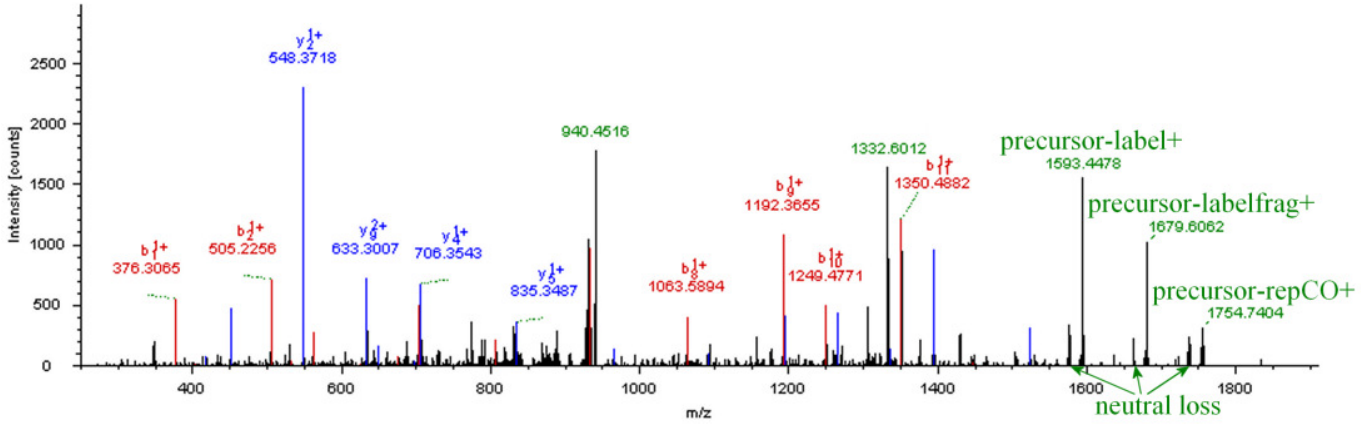
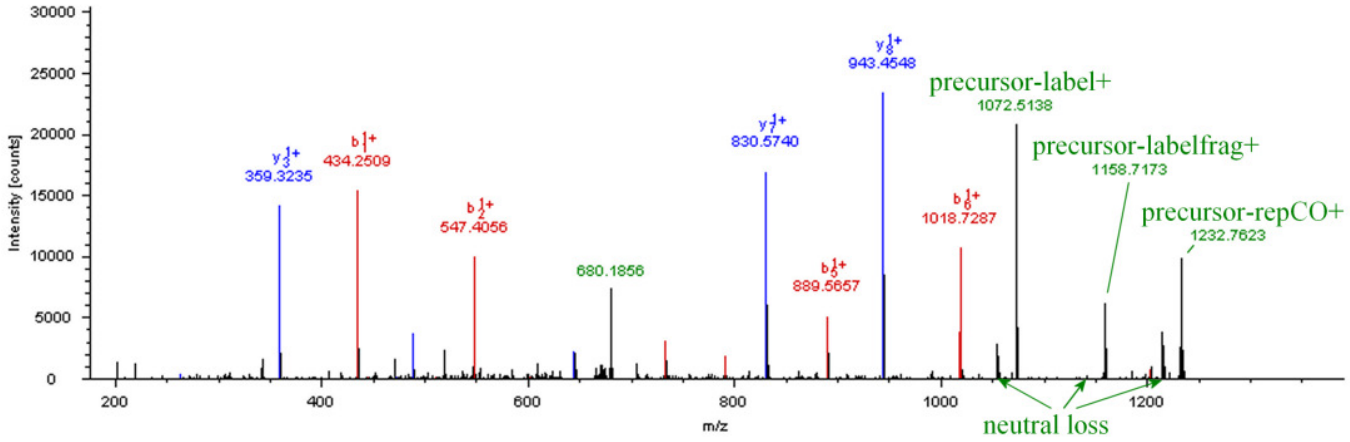


Figure S-1. Ions derived from fragmentation of the label tag.

MS/MS spectra of peptide aEGAATEEEGTPk (HeLa sample) labeled with iTRAQ 4-plex (A), TMT 6-plex (B) and iTRAQ 8-plex (C) respectively. Removal of label-associated fragment ions and cleaning of the b/y-ion free window (between the largest possible b/y-ion and the precursor MH+) improved Mascot ions scores as indicated.

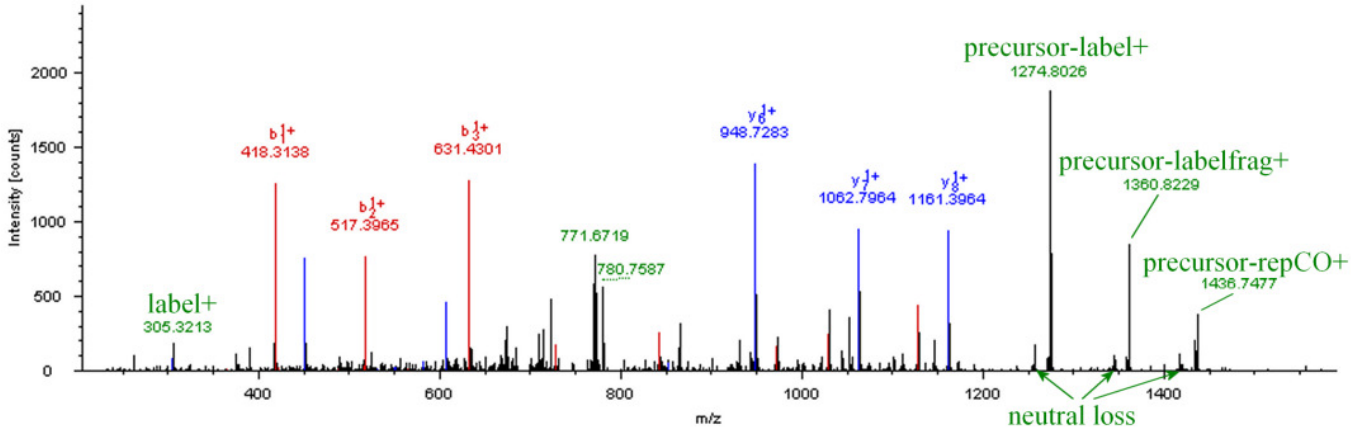
A eIWGVEPSR (Glycogen phosphorylase), Modification iTRAQ 8-plex (N-Term)

Precursor *m/z* 688.8771, charge 2+, Mascot Score improved from 21 to 46



B iVNPIMGVk (Argininosuccinate synthase), Modifications iTRAQ 8-plex (N-Term & K9)

Precursor *m/z* 789.9984, charge 2+, Mascot Score improved from 17 to 23



C kDSGFQMNQLR (Serotransferrin), Modification iTRAQ 8-plex (N-Term & K1)

Precursor *m/z* 644.6891, charge 3+, Mascot Score improved from 23 to 35

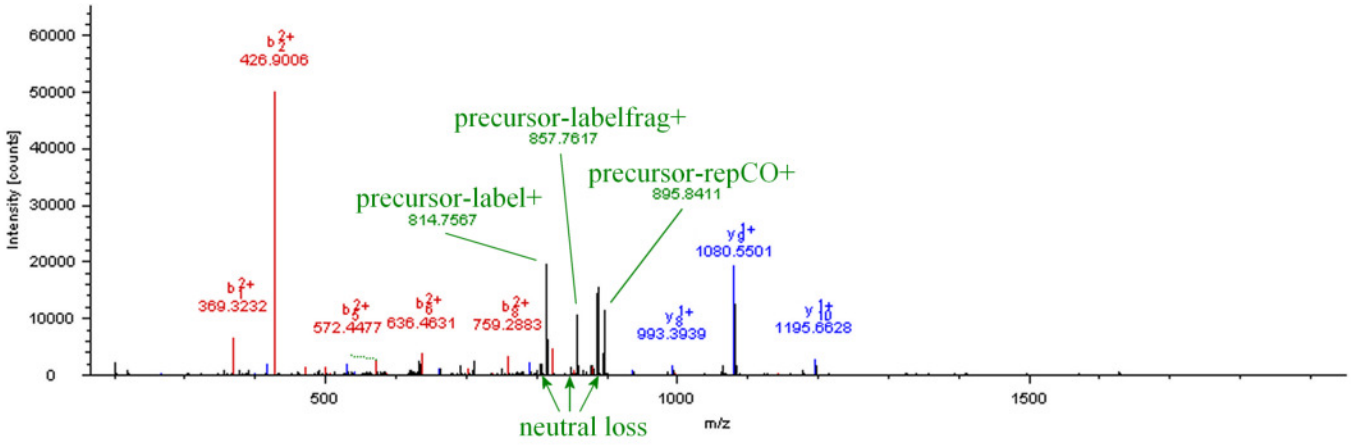


Figure S-2. Score improvement in initially unidentified iTRAQ 8-plex spectra upon filtering of label-associated fragment ions.

All of these spectra initially failed to be identified in the iTRAQ 8plex-labeled protein mix when the stringent filter criteria were applied, but were identified using a combination of relaxed filter criteria, manual inspection of spectra and information on predicted retention time. Removal of label-associated ions and cleaning of the b/y-ion free window led to an improvement in Mascot ions scores as indicated.

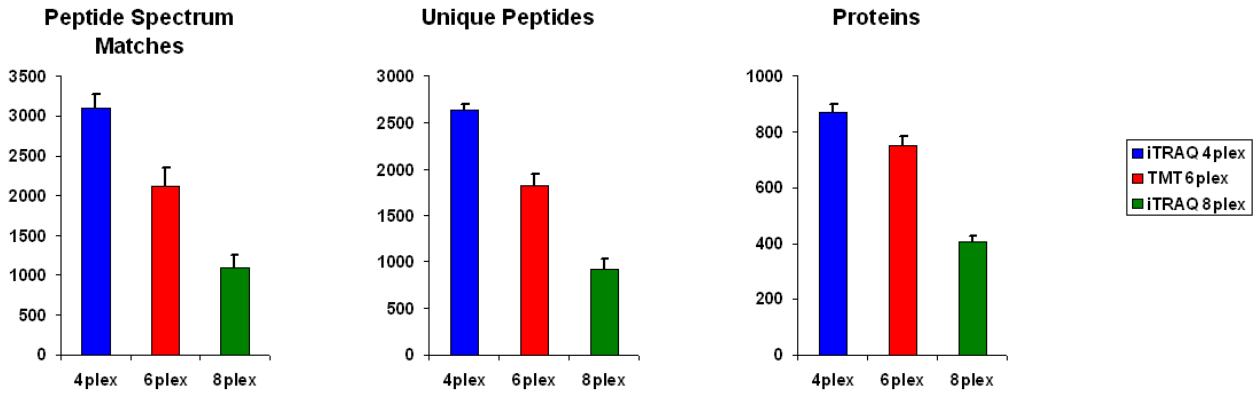


Figure S-3. Peptide identification rates in labeled HeLa samples searched with Sequest.

CID spectra from the HeLa samples labeled with three types of isobaric tags were re-searched with Sequest. Similar to the Mascot results, the highest numbers of peptide-spectrum matches, unique peptides and protein groups were identified when iTRAQ 4-plex was used for labeling, followed by TMT 6-plex and then iTRAQ 8-plex. Column heights reflect the arithmetic means of two replicates with one-sided error bars indicating half the range.

Total Ion Chromatograms

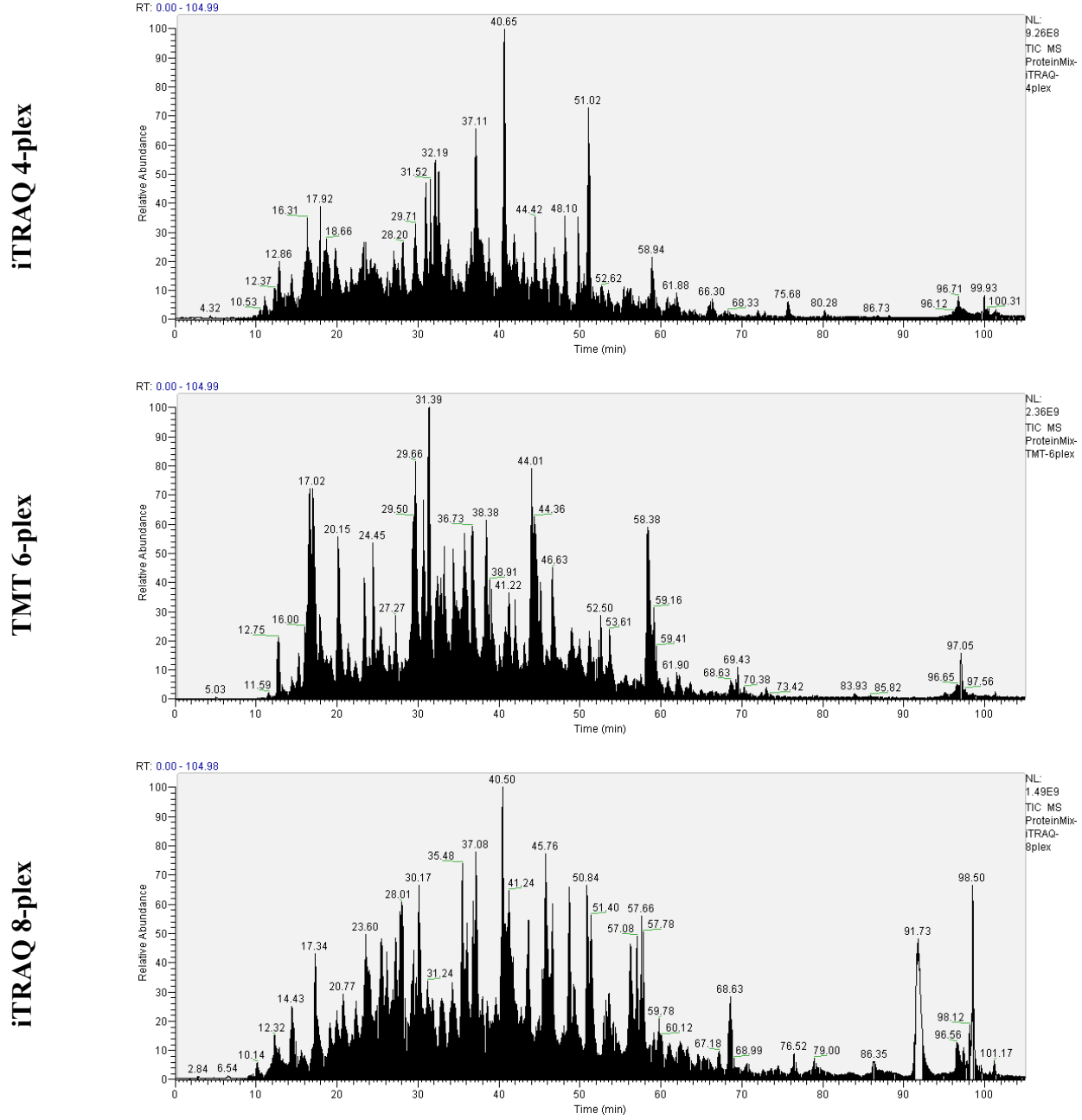


Figure S-4. Total Ion Chromatograms of protein mix labeled with iTRAQ 4-plex, TMT 6-plex and iTRAQ 8-plex.

TIC (total ion current chromatogram) for a set of LC-runs of the protein mix labeled with iTRAQ 4-plex, TMT 6-plex and iTRAQ 8-plex respectively.

Accession	Protein Group Name	iTRAQ 4-plex L/N ratio	iTRAQ 4-plex #PSMs	TMT 6-plex L/N ratio	TMT 6-plex #PSMs	iTRAQ 8-plex L/N ratio	iTRAQ 8-plex #PSMs	**geometric mean L/N**	iTRAQ4 -fold reg	TMT6 -fold reg	iTRAQ8 -fold reg	**geo mean -fold reg**	iTRAQ4 rel error	TMT6 rel error	iTRAQ8 rel error
B3KWB2	Protein NDRG1	0.35	6	0.24	4	0.26	3	**0.28**	2.90	4.10	3.80	**3.56**	0.81	1.15	1.07
P00558	Phosphoglycerate kinase 1	0.36	22	0.37	17	0.53	13	**0.41**	2.81	2.69	1.90	**2.43**	1.16	1.11	0.78
Q969I0	KRT8 protein	0.46	9	0.39	5	0.43	3	**0.43**	2.16	2.55	2.35	**2.35**	0.92	1.09	1.00
Q8WM99	MHC class I	0.46	1	0.36	1	0.49	2	**0.43**	2.19	2.79	2.03	**2.32**	0.95	1.20	0.88
Q0QEN7	ATP synthase subunit beta	0.61	10	0.47	4	0.36	3	**0.47**	1.64	2.14	2.80	**2.14**	0.77	1.00	1.31
B4DEK5	Proactivator polypeptide	0.53	3	0.73	1	0.29	1	**0.48**	1.90	1.37	3.45	**2.08**	0.92	0.66	1.66
Q96CF4	Histone 1, H2bj, isoform CRA_b	0.54	5	0.55	6	0.47	5	**0.51**	1.87	1.83	2.15	**1.94**	0.96	0.94	1.10
P05783	Keratin, type I cytoskeletal 18	0.55	5	0.69	1	0.41	1	**0.54**	1.83	1.46	2.44	**1.87**	0.98	0.78	1.31
B4DUM6	Beta-enolase	0.54	2	0.54	2	0.54	2	**0.54**	1.84	1.86	1.85	**1.85**	0.99	1.01	1.00
B4E3K9	Superoxide dismutase (Mn)	0.49	5	0.86	1	0.40	2	**0.55**	2.06	1.16	2.52	**1.82**	1.13	0.64	1.38
Q8IXH2	MSTP057	0.59	2	0.47	2	0.71	1	**0.58**	1.69	2.12	1.41	**1.72**	0.99	1.24	0.82
A8K092	ATP synthase subunit alpha, mitochondrial	0.54	5	0.60	5	0.63	2	**0.58**	1.87	1.68	1.60	**1.71**	1.09	0.98	0.93
B3KTS5	Voltage-dep. anion-selective channel protein 1	0.64	3	0.54	1	0.61	1	**0.59**	1.56	1.87	1.65	**1.69**	0.92	1.11	0.98
B4E2X0	CD44 antigen	0.62	2	0.81	2	0.47	1	**0.62**	1.61	1.23	2.15	**1.62**	0.99	0.76	1.33
Q15084	Protein disulfide-isomerase A6	0.67	4	0.77	5	0.50	3	**0.63**	1.50	1.30	2.02	**1.58**	0.95	0.82	1.28
P30041	Peroxiredoxin-6	0.60	5	0.66	4	0.70	3	**0.65**	1.67	1.51	1.44	**1.54**	1.09	0.98	0.93
P06733	Alpha-enolase	0.67	31	0.70	25	0.59	9	**0.65**	1.49	1.42	1.69	**1.53**	0.97	0.93	1.10
B4DNK4	Pyruvate kinase isozymes M1/M2	0.73	19	0.67	13	0.57	12	**0.65**	1.38	1.49	1.74	**1.53**	0.90	0.97	1.14
P60174	Triosephosphate isomerase	0.66	12	0.65	7	0.66	7	**0.66**	1.52	1.53	1.53	**1.52**	1.00	1.00	1.00
B4DZY9	T-complex protein 1 subunit epsilon	1.53	4	1.47	4	1.55	1	**1.51**	1.53	1.47	1.55	**1.51**	1.01	0.97	1.02
P07437	Tubulin beta chain	1.46	6	1.43	3	1.69	2	**1.52**	1.46	1.43	1.69	**1.52**	0.96	0.94	1.11
P78371	T-complex protein 1 subunit beta	1.39	19	1.53	9	1.66	5	**1.52**	1.39	1.53	1.66	**1.52**	0.91	1.01	1.09
Q5TCU3	Tropomyosin 2 (Beta)	1.38	3	1.54	1	1.74	1	**1.54**	1.38	1.54	1.74	**1.54**	0.89	1.00	1.12
P11142	Heat shock cognate 71 kDa protein	1.56	18	1.60	14	1.48	13	**1.55**	1.56	1.60	1.48	**1.55**	1.01	1.04	0.96
B4DMA2	Heat shock protein HSP 90-beta	1.53	8	1.53	7	1.72	4	**1.59**	1.53	1.53	1.72	**1.59**	0.96	0.96	1.08
P07900	Heat shock protein HSP 90-alpha	1.48	19	1.65	13	1.72	6	**1.62**	1.48	1.65	1.72	**1.62**	0.92	1.02	1.07
P10809	60 kDa heat shock protein, mitochondrial	1.66	41	1.70	32	2.04	13	**1.79**	1.66	1.70	2.04	**1.79**	0.93	0.95	1.14
B3KNT8	Nucleosome Assembly Protein 1-like 1	1.99	2	1.80	1	1.79	3	**1.86**	1.99	1.80	1.79	**1.86**	1.07	0.97	0.96
Q9UNM1	Chaperonin 10-related protein	2.58	5	1.67	3	1.84	1	**2.00**	2.58	1.67	1.84	**2.00**	1.29	0.84	0.92
P12277	Creatine kinase B-type	1.79	7	2.16	3	2.68	4	**2.18**	1.79	2.16	2.68	**2.18**	0.82	0.99	1.23
Q6NVI1	MARCKS protein	1.75	1	2.83	2	2.40	1	**2.28**	1.75	2.83	2.40	**2.28**	0.77	1.24	1.05
A8MX94	Protein GSTP1	2.15	5	2.34	3	2.79	3	**2.41**	2.15	2.34	2.79	**2.41**	0.89	0.97	1.16
			9.0 iTRAQ4		**6.3** TMT6		**4.1** iTRAQ8					average relative error (protein level)	**0.97**	**0.98**	**1.09**
												standard deviation	0.11	0.14	0.18
			average number of peptide spectrum matches used for protein ratio calculation										iTRAQ4	TMT6	iTRAQ8

Table S-1. Proteins regulated more than 1.5-fold.

Proteins that were detected in each of the three HeLa experiments using the labeling reagents iTRAQ 4-plex, TMT 6-plex and iTRAQ 8-plex respectively and for which the geometric mean of the three protein ratios (log-phase:nocodazole = L/N) - as the best estimate of true regulation - indicated regulation more than 1.5-fold. Relative errors were calculated by dividing the –fold regulation values obtained with a certain labeling reagent by the geometric mean of all three values.

Additional Experimental Section

Chemicals and Reagents

All chemicals were purchased at the highest purity available. Acetonitrile (ACN), isopropanol, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 2,2,2-trifluoroethanol (TFE), beta-glycerophosphate, Na-pyrophosphate, NaF, Na_3VO_4, phenylmethanesulfonyl fluoride (PMSF), protease inhibitor mix (leupeptin, pepstatin and chymostatin) and nocodazole were purchased from Sigma-Aldrich (Steinheim, Germany); triethlyammonium bicarbonate (TEAB), S-methyl-thiomethanesulfonate (MMTS), KCl and Triton X-100 from Fluka (Buchs, Switzerland); ethanol from Merck (Darmstadt, Germany); ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and glycerol from AppliChem (Darmstadt, Germany); formic acid (FA) from SAFC Biosciences (Andower, UK); dithiothreitol (DTT) from Roche (Mannheim, Germany); okadaic acid from Alexis (Lausen, Switzerland); phosphate buffered saline (PBS) and Dulbecco's modified Eagle's Medium (DMEM) were made in-house; iTRAQ 4-plex and iTRAQ 8-plex kits were purchased from Applied Biosystems (Foster City, CA); TMT 6-plex kits were from PierceNet/Thermo Fisher Scientific (Bremen, Germany); and mass spectrometry-grade Trypsin was from Promega (Madison, USA). Ultrapure 18-MΩ water was obtained from an in-house Millipore MilliQ-system (Bedford, USA).

Sample preparation

The standard protein mix sample was prepared as follows: All proteins were purchased from Sigma and were dissolved in 0.5 M TEAB at a concentration of 5 mg/ml. Seven proteins were mixed (Carbonic anhydrase, Myoglobin, Alcohol Dehydrogenase, beta-Lactoglobulin A, beta-Galactosidase, Lysozyme C, Ovalbumin) and this stock solution was split into samples A and B in a 1:1 ratio so that A and B contained 30 µg of each protein. Three proteins (Apo-Transferrin, Aldolase, Conalbumin) were mixed likewise and were added to samples A and B in a 5:1 ratio so that A contained 30 µg and B 6 µg of each of these proteins. Three further proteins (Phosphorylase b, Catalase, alpha Casein) were mixed and were added to samples A and B in a 1:40 ratio so that A contained 0.75 µg and B contained 30 µg of each protein. After tryptic digest, 6 pmol of a mixture of synthetic peptides was spiked into A and 30 pmol into B for a 1:5 ratio. The equimolar peptide mixture consisted of the following peptides:

HLVDEPQNLIK, IGSEISSLTLEEAR, VGPPPAPSGGLPGTDNSDQAR, FGpSpSNTDSAGALGpTLR, WWGpSGPpSGpSGGpSGGGK, TASDTDSSpYAIPTAGMSPSR, SVENLPEAGIpTHEQR, APPDNLPSPGGSR, LIEDNEpYTAR, APPDNLPSPGGpSR, RpSDGGHTVLHR, ENIMRpSENSESQLTSK, QLGEPEKpSQDSSPVLpSELK, QLGEPEKpSQDpSSPVLpSELK, KFLpSLASNPELLNLPSpSVIK, SVpSDYEGK, THILLFLPKpSVSDYEGK, EGVNDNEEGFFSAR, NpSVEQGRRL, WWGSGPSGSGGSGGGK. Samples A and B were each split into six equal parts for duplicate labeling with the three types of isobaric labeling reagents respectively. Two aliquots of sample A were labeled with iTRAQ 4-plex reagents 114 and 115, and two aliquots of sample B were labeled with reagents 116 and 117. The four channels were mixed, and 1/500 of the mixture was injected per LC-run. iTRAQ 8-plex labeled samples were prepared in an analogous manner. For TMT 6-plex labeled samples, two aliquots of sample A were labeled with reagents 126 and 127, and two aliquots of sample B were labeled with reagents 128 and 129 respectively. Reduction of disulfide bonds (TCEP), alkylation of cysteines (MMTS) and overnight tryptic digest at 37 °C were performed according to the iTRAQ protocol for all samples. iTRAQ 4-plex and iTRAQ 8-plex labeling was performed according to the manufacturer's protocol. TMT 6-plex samples were diluted to the volume recommended in the TMT protocol and labeling was performed as described in the TMT protocol. All labeling reactions were performed according to manufacturer's recommendations, but for twice the labeling time. Labeling efficiency was evaluated by a Mascot search where the respective isobaric modifications were set as variable modifications on N-term and lysine.

HeLa cells were cultured in DMEM medium. When cells reached 70% confluency, nocodazole was added at a concentration of 330 nM. Sample N was derived from cells exposed to nocodazole for 14 h, and sample L from cells in log–phase. Cells were harvested by scraping, centrifuged for 15 min at 5,000 rpm at 4°C, washed twice in ice-cold PBS and shock-frozen in liquid nitrogen. Lysis buffer (twice the pellet volume: 50 mM HEPES-KOH pH 7.5, 5 mM EDTA pH 8.0, 150 mM KCl, 10% glycerol, 1% Triton X-100, 20 mM beta-glycerophosphate, 10 mM Na-pyrophosphate, 10 mM NaF, 1 mM DTT, 1 mM Na_3VO_4, 0.1 mM PMSF, protease inhibitor mix 20 µg/ml of leupeptin, pepstatin and chymostatin each, 1 µM okadaic acid) was added to frozen pellets. Cell membranes were disrupted by passage through 22 to 27 gauge needles. The suspension was cleared by centrifugation at 4°C for 15 min (20,000 rpm, SS-34 rotor Sorvall RC plus). Soluble protein concentration was measured by Bradford assay before and after protein purification by acetone precipitation. Extracts were redissolved in 0.5 M TEAB and brought to a calculated protein concentration of 2.5 mg/ml.

Reduction of disulfide bonds (TCEP), alkylation of cysteines (MMTS) and overnight tryptic digest at 37 °C were performed according to the iTRAQ protocol for all samples. Samples L and N were each split into 6 identical parts for duplicate labeling with the three types of isobaric labeling reagents. iTRAQ 4-plex and iTRAQ 8-plex labeling was performed according to the manufacturer's protocol. TMT 6-plex samples were diluted to the volume recommended by the TMT protocol and labeling was performed as described in the TMT protocol. Labeling reactions were performed according to the manufacturer's recommendations, but for twice the labeling time. Labeling efficiency was evaluated with a Mascot search where the respective isobaric modifications were set as variable modifications on N-term and lysine. Samples N were labeled with iTRAQ 4-plex 114 and 115, TMT 6-plex 126 and 128, and iTRAQ 8-plex 114 and 115 reagents. Samples L were labeled with iTRAQ 4-plex 116 and 117, TMT 6-plex 127 and 129, and iTRAQ 8-plex 116 and 117 reagents.

nano-HPLC

Peptide mixtures were separated on an UltiMate 3000 nano-HPLC system (Dionex, Amsterdam, The Netherlands) using a C18 column (Acclaim, 15 cm x 75 µm x 3 µm, 100 Å, Dionex). HPLC flow was coupled online to an LTQ-Orbitrap XL ETD mass analyzer via a Proxeon (Odense, Denmark) nano-source. Buffers were A: 5% ACN, 0.1% FA; B: 30% ACN, 0.08% FA; C: 80% ACN, 0.08% FA, 10% TFE. Standard protein mix samples were separated by a 110 min ternary gradient (0 min: 100%A; 86 min: 100%B; 90 min: 85%B, 15%C; 95 min: 10%B, 90%C continued for 10 min and 100%C for 5min, MS on at 5 min, acquisition for 105 min) and HeLa samples by a 3.5 h ternary gradient (0 min: 100%A; 182 min: 100%B; 183 min: 85%B, 15%C; 192 min: 10%B, 90%C continued for 10 min; MS on at 7 min, acquisition for 221 min).

Mass Spectrometry

LTQ Orbitrap XL ETD (Thermo Fisher Scientific, Bremen, Germany) parameters were as follows: Nano-electrospray ion source (Proxeon) with spray voltage 2 kV, capillary temperature 200 °C. Survey MS1 scan *m/z* range 300-2000, resolution 60,000 with AGC target 5E5, maximum inject time 500 ms. Lock mass Siloxane 445.120024 for internal calibration. Preview mode for FTMS master scans: on, injection waveforms: on, monoisotopic precursor selection: on, rejection of charge state: 1. Selection of the four most prominent precursor ions exceeding a signal of 500 for both CID (collision-induced dissociation) and HCD so that one full MS survey scan was followed by a maximum of 4 CID scans

alternated with a maximum of 4 HCD scans. The chromatography feature was set on for peak detection and fragmentation close to the apex of the HPLC elution profile, with settings 9.0 s FWHM and correlation 0.8. Dynamic exclusion of *m/z* values that triggered MS/MS for 180 s with exclusion mass window 5 ppm. CID isolation window 3 Da, AGC target 1E4, maximum inject time 400 ms, activation time 30 ms, activation Q 0.25, normalized collision energy 35%, multistage activation on with neutral loss list 32.6, 49 and 98 *m/z* within top 3 ions. HCD isolation window 3 Da, AGC target 3E5 with maximum inject time 500 ms, activation time 30 ms and HCD collision energy 75%. This rather high collision energy ensures high intensities in the low *m/z* range of the reporter ions, however in such spectra fragment ions in the medium and high m/z range are typically of weak intensity or may be absent so that these HCD spectra are not suitable for identification and were used exclusively for the extraction of reporter ions. HCD spectra were recorded at 7500 resolution. For gas phase fractionation experiments, MS1 spectra were confined to *m/z* 350-552, 548-702, 698-852 and 848-2000 *m/z*. MS1 data were recorded in profile mode whereas MS/MS data were recorded in centroid mode.

Data analysis and processing

Raw files were analyzed with the Proteome Discoverer 1.1.0.221 software suite (Thermo). Parameters for the Spectrum Selector node were set to generate peak lists of CID spectra with essentially no filtering (activation type: CID, s/n cut-off: 0, total intensity threshold: 0, minimum peak count: 1, precursor mass 300-10,000 Da). Parameters for Proteome Discoverer triggered searches submitted to an in-house Mascot 2.2.04 server were as follows: precursor tolerance 25 ppm; MS/MS tolerance 0.5 Da; Trypsin; 2 missed cleavages; instrument type: matching of b- and y-ions; fixed modification of lysines and peptide N-termini with the respective isobaric label (iTRAQ 4-plex, TMT 6-plex or iTRAQ 8-plex) and of cysteines to methylthio-cysteine; variable modifications oxidation of methionine, deamidation of asparagine and glutamine, and modification of tyrosine with the respective isobaric label. Only peptides with a minimum Mascot ions score of 20 were stored in .msf result files (except for the re-searches of the 8-plex labeled protein mixture where this cut-off was deactivated in order to permit the detection of peptides with lower Mascot scores). For the standard protein mixture, variable phosphorylation of serine, threonine and tyrosine was also included. All searches were performed against a concatenated forward and reversed database to control for global false discovery rate. For the standard protein mixture, sequences of human, mouse, bovine, rabbit, horse, chicken, yeast and E.coli proteins were extracted from the 090420 Swiss-Prot database for the following reasons: First, commercially available proteins often contain isoforms, related proteins or contaminants in

addition to the predominant protein form. Therefore it was necessary to cover not only the 13 standard proteins but also potential co-purified proteins of lower abundance or contaminants. Second, only a database of adequate size provides a large enough search space to ensure that database search results in particular calculated E-values and FDR (false discovery rate) are meaningful. The database was supplemented with sequences from a set of contaminant proteins, and concatenated with its reversed version. The combined database comprised in total 113,784 protein sequences. For HeLa data searches, human sequences extracted from the UniProt 090826 database were fused with sequences from a set of contaminant proteins. The resulting database was likewise concatenated with its reversed version. The combined database comprised in total 171,990 protein sequences. All searches were performed without species restriction. For the Sequest searches of HeLa data that were used in the generation of Figure S-3, the database and search-related settings were analogous to the Mascot searches.

Search results were opened in Proteome Discoverer with the following filter criteria: maximum Mascot peptide rank 1 without including matches with identical score, minimum sequence length 8 amino acids, Mascot significance cut-off of 0.05. After these initial filtering steps during import, the mass tolerance was set to 8 ppm for the standard protein mixture experiments. For the Sequest searches of HeLa raw data (Figure S-3), import filters were as in the Mascot searches with regard to peptide rank and length, with additional filters XCorr/Charge state (thresholds: 2.5 for 2+, 3.0 for 3+ and 3.5 for ≥ 4+) and Probability Score ≥ 20. Decoy hits were recognized as peptide-spectrum matches mapped only to decoy protein sequences. With the above-named filter criteria, the number of decoy hits in the combined forward and reversed database was less than 1% of the forward hits on both the peptide and the protein levels in all experiments. Protein grouping was switched on to reduce redundancy in accordance with the principle of maximum parsimony (Occham's razor). Identified peptide sequences differing in at least one amino acid residue were counted as unique. Covalent modifications including N-terminal or C-terminal prolongation (i.e. missed cleavages) were also counted as additional unique peptides. In contrast, different charge states or different site localization of modifications or multiple fragmentation did not count as additional unique peptides.

The quantification method in Proteome Discoverer defines how reporter ion areas are extracted from .raw files and how calculations of peptide and protein ratios are performed. Quantification method settings were as follows: reporter ion integration window tolerance: 5 mmu, integration method: centroid sum, no minimum threshold, no outlier removal, use only unique peptides for quantification, consider only proteins from different protein groups for peptide uniqueness, replace missing quan

values with minimum intensity: off, apply quan value correction (isotope impurity correction): on, reject all quan values if not all quan channels are present: off, maximum allowed fold change: 100, use extreme ratios for quantitation: off. Experimental bias correction based on protein median was set on. Isotope correction factors for iTRAQ 4-plex and iTRAQ 8-plex were taken from batch certificates supplied with the kits, whereas correction factors for TMT 6-plex were set according to the "updated TMT6 correction factors for batch GA0603/1A obtained on Orbitrap XL", which were transformed so that the sum adds up to 100% (rather than the +0 isotope peak alone). For iTRAQ experiments, ratios 115:114, 116:114, 117:115 and 117:116 were calculated. For TMT experiments, ratios 127:126, 128:126, 129:127 and 129:128 were calculated. Proteome Discoverer 1.1.0.221 calculates the protein ratio as the median (i.e. the least extreme) ratio of all the "unique PSM" ratios of a protein ("unique" reflecting a peptide-spectrum match to a single protein group); in case the number of unique PSM ratios is even, Proteome Discoverer calculates the geometric mean of the two least extreme unique PSM ratios. Protein ratios (Supporting Table S-1) were derived from exported Proteome Discoverer results and calculated as the geometric mean of the two duplicate log-phase:nocodazole (L/N) ratios available for each protein i.e. as the geometric mean of ratios 116:114 & 117:115 for iTRAQ 4-plex and iTRAQ 8-plex and as the geometric mean of ratios 127:126 & 129:128 for TMT 6-plex.