Experimental Procedure

Abbreviations

ACN, acetonitrile; AmBic, ammonium bicarbonate; FA, formic acid; IP, immuno-precipitation; PSM, peptide to spectrum match; RT, room temperature; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; TMT, Tandem Mass Tags.

Materials

SILAC K8/R10 HeLa cell pellet was from Dundee Cell Products (Dundee, UK). TMT10plex isobaric label reagents were from Thermo Fisher Scientific (Rockford, IL, USA). Universal Proteomics Standard Set (UPS1) and all other chemicals were from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated.

Protein digestion and controlled mixture preparation

Heavy SILAC HeLa cell pellet was homogenized in lysis buffer (1% SDS, 8 M urea, 50 mM TEAB pH 8.5, protease inhibitors (cOmplete, Roche) at RT at a concentration of 2.5E4 cells/µl using a tip sonifier (QSonica, Newtown, CT), applying 3 × 10 s bursts (30% amplitude) and cooling the samples between bursts on ice for 1 min. Protein concentration was determined using the bicinchoninic assay (BCA, Pierce). Proteins were precipitated using methanol/chloroform. Briefly, four, one, and three volumes of methanol, chloroform, and water, respectively, were added to the lysate followed by vortexing after each solvent addition, and final centrifugation at 15'000 × *g* at RT for 10 min. After removal of the supernatant, protein pellet was washed twice with cold methanol and air dried. Heavy SILAC HeLa protein pellet and UPS1 proteins were separately re-dissolved in digestion buffer (8 M urea, 50 mM TEAB pH 8.5) at a concentration of 5 and 0.2 µg/µl, respectively, reduced with 10 mM (end concentration) DTT at 56°C for 30 min and alkylated with 20 mM (end concentration) iodoacetamide at RT for 30 min (dark). SILAC HeLa and UPS1 proteins were pre-digested with 1:100 and 1:20 w/w lysyl endopeptidase (Lys-C, Wako Pure Chemical Industries Ltd., Japan), respectively, at 37 °C for 4 h. Samples' urea concentration was diluted to 2 M with 50 mM TEAB pH 8.5 solution, and digestion was continued by the addition of 1:100 and 1:20 w/w trypsin (Promega, Madison, WI), respectively, and incubation at 37 °C overnight. Samples were acidified with FA to a final 5% v/v content, vortexed, and centrifuged at 7'000 × *g* at 4 °C for 10 min to remove insoluble material. Heavy SILAC HeLa and UPS1 peptides were desalted using solid-phase extraction (SPE) (50 mg C18 Sep-Pak, Waters, Milford, MA) and homemade StAGE tips (prepared with C18 material from 3M Empore) (1), respectively, and eluates used to prepare controlled mixtures. 500, 333, 250, and 62.5 fmol UPS1 peptides were combined with 50 µg SILAC HeLa peptides in duplicate in order to generate a dilution series corresponding to 2/3, 1/2, and 1/8 of the highest UPS1 peptide amount (500 fmol). In addition, a reference sample was generated by pooling all four diluted UPS1 peptide samples (286.5 fmol) and combined with 50 µg of SILAC HeLa in duplicate. This was repeated to generate a total of five controlled mixtures replicates and all samples were dried down using a speedvac. Finally, to assess technical variability, three technical replcates were carried out for each mixture.

Peptide Tandem Mass Tag labelling

TMT labeling was performed according to Paulo et al. (2) with some minor modifications. Dried peptides were reconstituted in 100 mM HEPES pH 8.5 buffer (ca. 0.7 µg/µl final concentration) and individually labeled with 150 µg TMT10plex reagents (dissolved in anhydrous ACN, 30% v/v end concentration) at RT for 85 min according to Table 1.

Table 1. TMT10plex labelling scheme of the 5 replicate controlled mixtures. Numbers in the table refer to UPS1 peptides dilution compared to highest amount (1 = 500 fmol)

TMT10plex reagent		126	127N	127C	128N	128C	129N	129C	130N	130C	131
Controlled mixture	1 & 5	Ref	2/3	1/8	1/2	1	1/8	1/2	1	2/3	Ref
	2	Ref	1/2	1	2/3	1/8	1	2/3	1/8	1/2	Ref
	3	Ref	1/8	2/3	1	1/2	1/2	1/8	2/3	1	Ref
	4	Ref	1	1/2	1/8	2/3	2/3	1	1/2	1/8	Ref

The reaction mixtures were quenched with hydroxylamine (0.3% v/v end concentration) at RT for 20 min followed by acidification with FA (5% v/v end concentration). Samples were combined within their respective controlled mixtures 1-5, evaporated to dryness (speedvac), desalted by SPE (50 mg C18 Sep-Pak), dried down and stored at -20 °C until analysis.

LC-MS/MS

LC-MS/MS was performed using an EASY-nLC 1200 ultrahigh pressure liquid chromatography (UHPLC) connected to an Orbitrap Fusion Lumos Tribrid and equipped with an EASY-spray source (Thermo Fisher Scientific, San Jose, CA). Samples were re-suspended in 5% FA/2% ACN, and concentrated on an Acclaim PepMap C18 trapping column (75 µm × 20 mm, 5 µm particle size) at a controlled maximum backpressure of 500 bar. Approximately 2 µg sample (SILAC HeLa peptides) containing 2, 1.333, 1, 0.25, and 1.146 fmol UPS1 peptides corresponding to the 1, 2/3, 1/2, 1/8, and reference samples dilutions (see Table 1) was loaded and run in triplicate for each controlled mixture. Peptides were separated on an Acclaim PepMap C18 EASY-spray column (75 µm × 750 mm, 2 µm particle size) heated at 45 °C and using the following gradient at 300 nl/min: 5% B for 5 min, 5-20% B in 120 min, 20-45% B in 120 min, 45-100% B in 5 min, 100% B for 20 min, corresponding to a total acquisition time of 270 min (buffer A: 0.1% FA; buffer B: 0.1% FA/80% ACN). The spray voltage used was 1.9-2.2 kV.

The data were acquired using an MS2/MS3 (also called "multinotch MS3" or Synchronous Precursor Selection, SPS) (3) or MS2-only strategies. For the MS2/MS3, the instrument was operated in the data-dependent mode, collecting Orbitrap full MS1 scans over a mass range from *m/z* 300 to 1400 using quadrupole isolation, a resolution of 120k (at *m/z* 200), an automatic gain control (AGC) target value of 2E5, and a maximum injection time (IT) of 50 ms. Data were on-the-fly recalibrated using ambient air hexacyclodimethylsiloxane at *m/z* 445.12002. During a cycle time of 3 sec (top speed), the most intense precursor ions, with charge states between 2 and 6, a minimum intensity of 5E3, were mono-isotopically selected for collision induced dissociation (CID), using a quadrupole isolation of *m/z* 0.7, AGC target of 1E4, maximum IT of 50 ms, collision energy of 35%, and ion trap readout with turbo scan rate. Only a single charge state per precursor was selected for MS2. Interrogated precursor ions were dynamically excluded for 75 s using a ±10 ppm mass tolerance. TMT reporter ions were generated using SPS, a quadrupole isolation of *m/z* 2, high-energy collision dissociation (HCD) at a normalized collision energy of 65%, and readout in the Orbitrap with a resolution of 60k, scan range of *m/z* 100 to 500, an AGC target of 5E4, and a maximum IT of 105 ms. The mass range for selecting the SPS (MS3) precursors was from *m/z* 400 to 2000, excluding the MS2 precursor with a tolerance of *m/z* 40 (low) and 5 (high), and any TMT neutral loss from it. The number of SPS precursors was set to 10. Alternatively, the instrument was operated in MS2-only mode using HCD at

a normalized collision energy of 40%, AGC target of 5E4, and a maximum IT of 115 ms. All the other relevant parameters were essentially the same as above.

Data processing

Raw data were processed using Proteome Discoverer 2.2.0.388 (Thermo Fisher Scientific) and Mascot Server 2.6.1 (Matrix Science, London). Processing workflows were designed to perform a two-pass search on the MS2 data for the MS2/MS3 and MS2-only files (Supplemental Figure S1). First, the data were searched against the UniProt/SwissProt human protein database (07.2018 release, 20'398 entries) using trypsin/P as an enzyme, a maximum of two missed cleavage sites, and 10 ppm, as the precursor ion mass tolerance. 0.5, And 0.1 Da were specified as the fragment ion mass tolerances for the MS2/MS3, and MS2-only data, repectively. Carbamidomethylated cysteines (+57.02146 Da), TMT10 and SILAC K8 labeled lysines (+237.177131 Da), SILAC R10 arginines (+10.008269 Da), and TMT10 labeled peptide N-termini (+229.162932 Da) were set as static, while oxidized methionines (+15.99492 Da) were set as dynamic modifications. Second, the data were searched against the human protein database for which the endogenous were replaced with UPS sequences (protein entries marked with _ups, 20'395 entries), using carbamidomethylated cysteines, TMT10 labeled lysines and peptide N-termini as static, and oxidized methionines as dynamic modifications. The peptide-to-spectrum matches (PSMs) false discovery rates (FDRs) were controlled using Percolator and setting a max. delta Cn of 0.05 and a q-Value threshold of 0.01. Reporter ion quantitation was performed using the MS2, and MS3 data order for the MS2 and MS3 approaches, respectively, 3 mmu peak integration and most confident centroid tolerances. Reporter ion intensities were adjusted to correct for the isotopic impurities of the different TMT reagents (manufacturer specifications). Reporter ions intensities or signal to noise (S/N) ratio (3) were used to express abundances.

A consensus workflow was defined to group PSMs into peptide and proteins (Supplemental Figure S2). PSMs from all ranks were considered, peptide FDRs were controlled by setting a q-Value threshold of 0.01 and allowing the software to automatically select PSM q-Value or ion score for the grouping (PSM FDR was 0.89%, peptide group FDR was 1.21%). High confidence peptides with a minimal length of 6 residues were further grouped into proteins and protein FDR was set to fulfill a q-Value threshold of 0.01. At this level, protein FDR was 1.09%. For protein grouping, strict parsimony principle was applied. Peptide and protein quantitation was performed by summing intensities or S/N for each channel and normalizing each value with the highest TMT channel total. For protein quantitation, unique peptides only were considered. Finally, individual peptide and protein S/N were scaled to an average of 100.

References

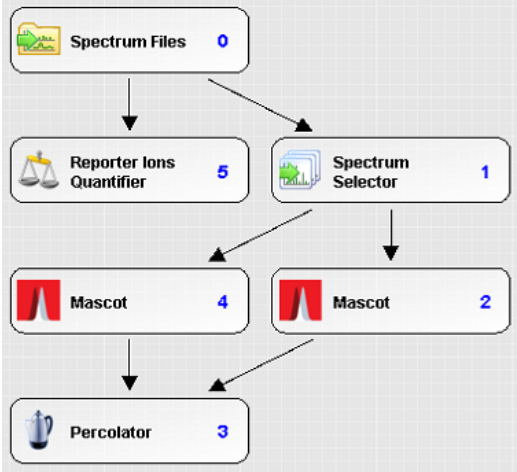
1. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* 2, 1896-1906

2. Paulo, J. A., McAllister, F. E., Everley, R. A., Beausoleil, S. A., Banks, A. S., and Gygi, S. P. (2015) Effects of MEK inhibitors GSK1120212 and PD0325901 in vivo using 10-plex quantitative proteomics and phosphoproteomics. *Proteomics* 15, 462-473

3. McAlister, G. C., Nusinow, D. P., Jedrychowski, M. P., Wuhr, M., Huttlin, E. L., Erickson, B. K., Rad, R., Haas, W., and Gygi, S. P. (2014) MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal. Chem.* 86, 7150-7158

Supplemental Figure S1

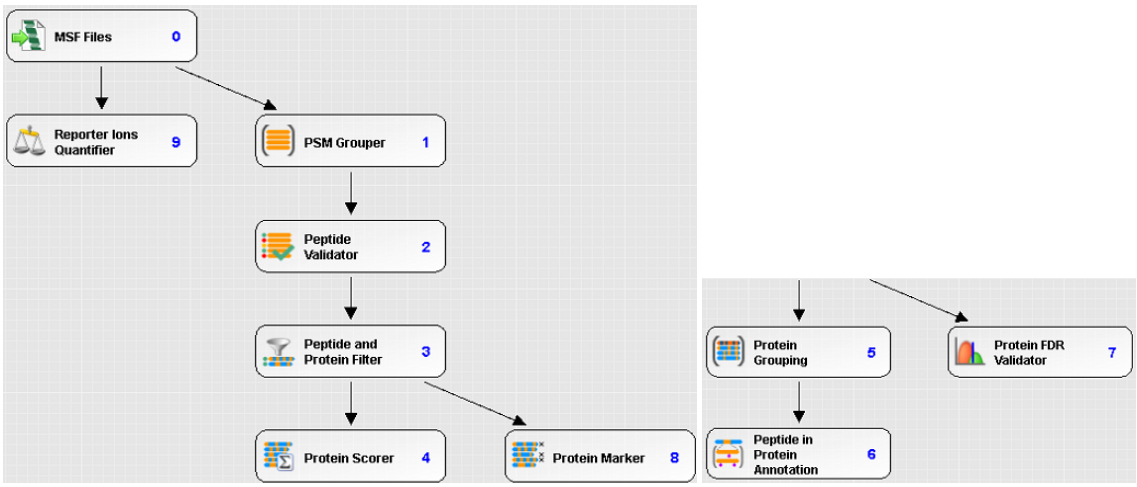
Proteome Discoverer 2.2 Processing Workflow



Node name (WF node #)	Parameter Cat.	Parameter	Setting or value
Spectrum Selector (1)	3. Scan Event Filters	MS Order	Is MS2
Mascot (2)	1. Input Data	Protein Database	SwissProt
		Enzyme Name	Trypsin/P
		Max. Miss. Cleav. Sites	2
		Instrument	ESI-Trap
		Taxonomy	Homo sapiens
	2. Tolerances	Prec. Mass Tolerance	10 ppm
		Frag. Mass Tolerance	0.5 Da (MS2/MS3); 0.1 Da (MS2 only)
	4. Dynamic Modifications	1. Dynamic Modification	Oxidation (M)
	5. Static Modifications	1. Static Modification	Carbamidomethyl (C)
		2. Static Modification	Label:13C(6)15N(2)+TMT10 (K)
		3. Static Modification	Label:13C(6)15N(4) (R)
		4. Static Modification	TMT10plex (N-term)
Percolator (3)	2. Decoy Database Search	Target FDR (Strict)	0.01
		Validation based on	q-Value
Mascot (4)	1. Input Data	Protein Database	Sigma_UPS_replaced_human
	5. Static Modifications	2. Static Modification	TMT10plex (K)
		3. Static Modification	TMT10plex (N-term)
Reporter Ions Quant. (5)	1. Peak Integration	Integration Tolerance	0.03 mmu
		Integration Method	Most Confident Centroid
	2. Scan Event Filters	Mass Analyzer	FTMS
		MS Order	MS3 (MS2/MS3); MS2 (MS2 only)
		Activation Type	HCD

Supplemental Figure S2

Proteome Discoverer 2.2 Consensus Workflow



Node name (WF node #)	Parameter Cat.	Parameter	Setting or value
MSF Files (**0**)	1. Spectra Storage Set.	Spectra to Store	Identified or Quant.
	4. PSM Filters	Max. Rank	0
PSM Grouper (**1**)	1. Peptide Group Mod.	Site Prob. Threshold	25
Peptide Validator (**2**)	1. General Validation Settings	Validation Mode	Automatic (Control peptide level error rate if possible)
		Target FDR (Strict) for PSMs	0.01
		Target FDR (Strict) for Peptides	0.01
Peptide and Protein Filter (**3**)	1. Peptide Filters	Peptide Conf. At Least	High
		Min. Peptide Length	6
Protein Grouping (**5**)	1. Protein Grouping	Apply strict parsimony principle	True
Peptide in Protein Annotation (**6**)	2. Mod. in Peptide	Protein Mod. Reported	Only for Master Proteins
	3. Mod. in Protein	Mod. Sites Reported	All And Specific
	4. Positions in Protein	Protein Pos. for Peptides	Only for Master Proteins
Protein FDR Validator (**7**)	1. Confidence Thresholds	Target FDR (Strict)	0.01 (protein)
Peptide and Protein Quantifier (**9**)	1. General Quantification Settings	Peptides to Use	Unique
		Consider Protein Groups for Peptide Uniqueness	True
		Replace Missing Values with Minimum Value	False
		Reject Quan Results with Missing Channels	False
	2. Reporter Quantification	Reporter Abundance Based On	Intensity or S/N
		Apply Quan Value Corr.	True
		Co-Isolation Threshold	100

		Average Reporter S/N Threshold	0
	3. Normalization and Scaling	Normalization Mode	Tot. Pep. Amount
		Scaling Mode	On All Average