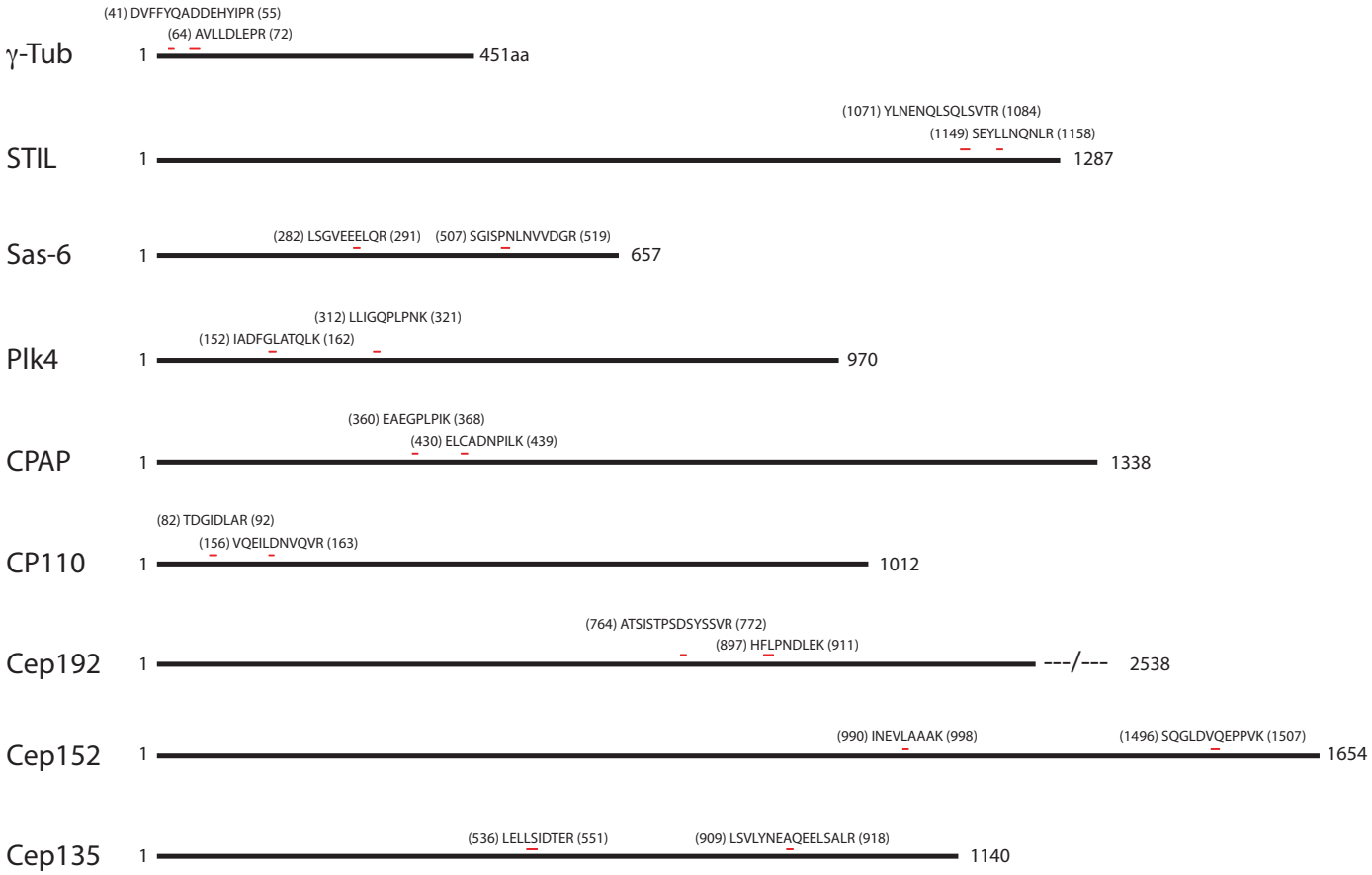
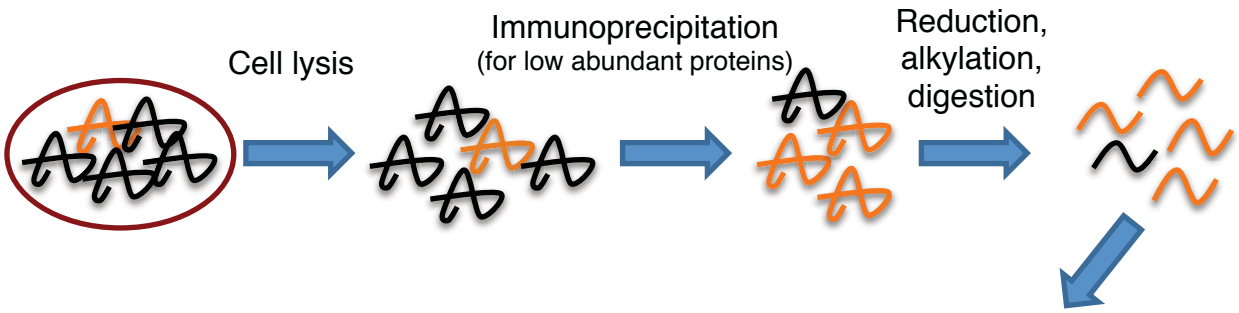
Appendix

Table of contents

Appendix Figure S1



Appendix Figure S2

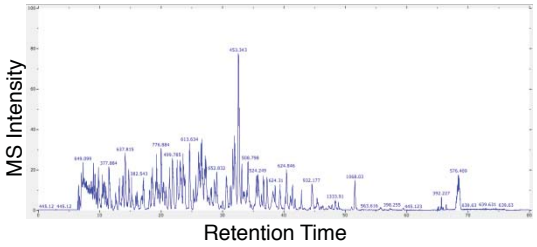


Filtered and ranked peptide list

Peptide	Precursor Ion Intensity
EAEGPLPIK	3.32E+07
VILFPNGTR	6311090
LIEFNNGQR	3240160
ELCADNPILK	2677990
EITFPDQTVK	2219460

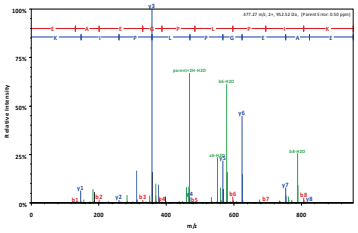
Database searching

Shotgun LC-MS/MS Analysis

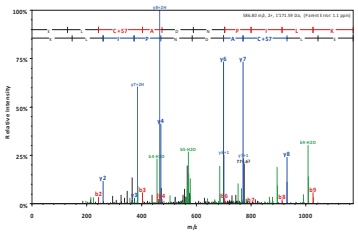


Spectral library generation and SRM assay generation

EAEGPLPIK



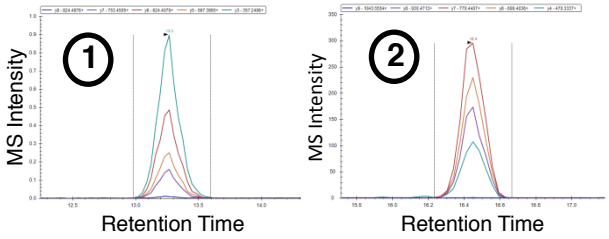
ELCADNPILK

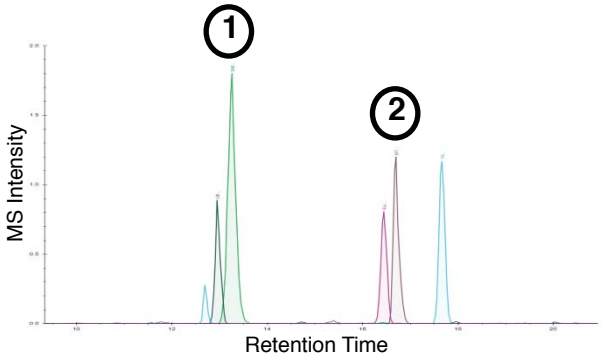


SRM analysis in complex sample

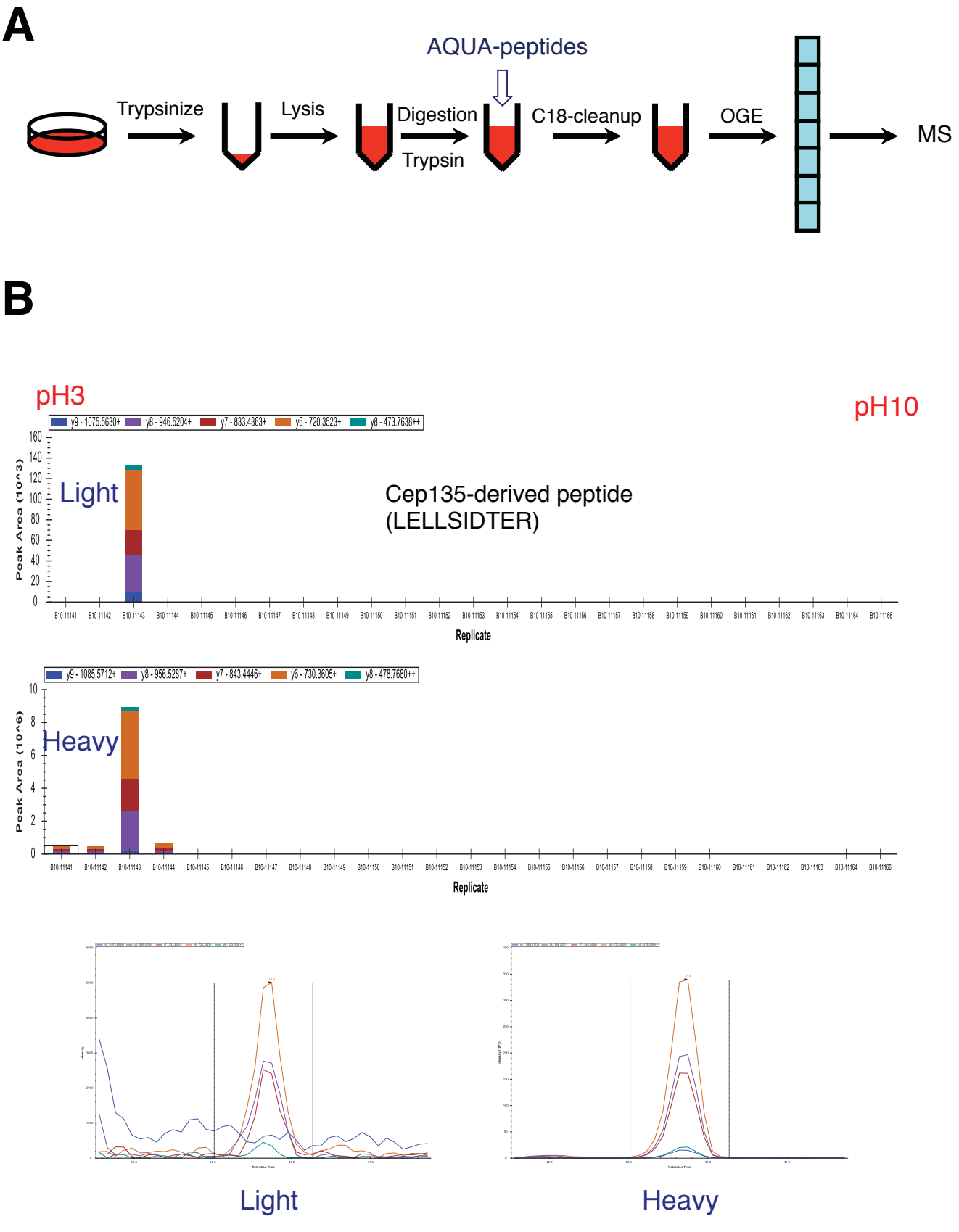
Selection of the most suited SRM assays

EAEGPLPIK ***ELCADNPILK***





Appendix Figure S3



Appendix Figure S1: Proteotypic peptides.

Schematic illustrates positioning of proteotypic peptides (and corresponding AQUA peptides) used for SRM with stable isotope dilution. Indicated are sequences and amino acid positions relative to the corresponding proteins (major isoforms). Optimal proteotypic peptides were selected in extensive pilot experiments (summarized in detail in Appendix Figure S2). In brief, each protein of interest was purified by immunoprecipitation and analyzed by shotgun LC-MS. Identified peptides were first filtered (full tryptic enzymatic cleavage, unique sequence in the human protein database, etc.) and then ranked according to their fragment ion intensities, with the most intense being considered as most suited. To make fragment ion intensities comparable across all identified peptides of a protein, adjustments had to be made for their point of acquisition during chromatographic peptide elution. To this end, we determined precursor ion intensities at the apex and at the point of triggering the tandem mass spectrum, calculated ratios and then applied these to the corresponding fragment ion intensities. The two peptide sequences with the highest fragment ion intensities (transitions) were selected for the final SRM assays. Furthermore, the five highest transitions for each peptide could also be selected from this dataset; these were optimized and used for quantification (see also (Bauer et al, 2014)).

Appendix Figure S2: SRM assay development

Overview of SRM assay development: to exemplify validation of the workflow, data for one low abundant target protein (CPAP; product of *CENPJ* gene) are summarized in a schematic. In a first step, proteins were prepared by cell lysis, and a comprehensive list of MS-suitable peptides was collected for each target protein by shotgun LC-MS/MS (see Materials and Methods). For relatively abundant proteins, we could observe several suitable peptides in whole cell extracts, but to achieve reasonable sequence coverage for less abundant proteins (specifically CPAP, Sas-6, STIL and Plk4), protein concentration had to be enhanced by immunoprecipitation (see below). Next, we discarded peptides unsuitable for absolute quantification (Picotti & Aebersold, 2012), including those containing missed or non-tryptic cleavages, glutamate at the N-termini, non-unique amino acid sequences and, if possible, methionine. Then, we ranked all remaining peptides according to their precursor ion intensity (MS1 level) and selected the 5 most intense peptides per protein for SRM assay development. To this end, we generated spectral libraries from the shotgun LC-MS datasets and selected the 5 most intense fragments with a mass higher than the precursor ion as transitions, using Skyline ((MacLean et al, 2010)MacLean B. et al, Bioinformatics, 2010, version 1.3). Subsequently, the fragment ion intensity (MS2 level), peak shape and reproducibility of the single SRM assays were evaluated by analyzing the same samples on a QQQ instrument in replicates. Additionally, we assessed the quality of these assays in the presence of a complex human peptide mixture, by spiking in immuno-purified proteins at low concentrations into whole cell lysates. We only considered assays that showed no transition interferences by background peptides. Based on this, we picked the two most suited peptides per protein and ordered corresponding quantified heavy reference peptides

(Aqua peptides; Thermo Scientific, Waltham, MA, USA). Finally, we optimized collision energies for all assays using the Skyline software and the heavy reference peptides. Further details of assay development, including transition lists with optimized collision energies for all peptides, as well as a thorough evaluation of quantification limits and quantitative linearity for the generated assays can be found in a recent report (Bauer et al, 2014).

For immunoprecipitation, cells were washed once with ice-cold PBS, and resuspended in ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.5 % IGEPAL CA- 630, 1 mM DTT, 30 µg/ml RNAse, 30 µg/ml DNAse, protease inhibitor cocktail (Roche, 1 EDTA- free tablet for 10 ml lysis buffer) and phosphatase inhibitors cocktail (cocktails 2 and 3; Sigma- Aldrich) and incubated for 30 min on ice. After cell lysis, suspensions were cleared by centrifugation at 14'000 rpm for 15 min. Immuno-purifications of endogenous proteins were performed using 50 µl of solid Affi-Prep protein G matrix beads (Bio-Rad Laboratories) chemically cross-linked to 1 µg/µl of antibody against 1-2 mg of clarified cell lysate for 2 h at 4°C. Afterwards the resin was washed with lysis buffer followed by washing with HNN buffer (50 mM Hepes pH7.5, 150 mM NaCl, 5 mM EDTA, 50 mM NaF). Proteins were eluted with 100 mM glycine pH 2.8, neutralized by the addition of Tris buffer (pH 8,0). Protein samples were diluted with lysis buffer to a final concentration of 2.5 µg/µl.

Appendix Figure S3:

A) Schematic illustration of whole cell lysate sample preparation for SRM/mass spectrometry. In a typical experiment, $5x10^6$ cells were lysed and trypsinized (resulting in ca. 1 mg of peptides). To reduce complexity of peptide samples, tryptic digests were then subjected to off gel

electrophoresis (OGE). This resulted in 24 fractions to be analyzed by mass spectrometry (MS). Note that heavy-isotope labeled (tryptic) AQUA peptides (250 fmol) were added to peptide mixtures prior to C18 cleanup and OGE. B) Fractionation of a Cep135-derived peptide illustrating the partitioning of this peptide during OGE. Also shown are the transitions observed for the light and heavy versions of this peptide.

References

Bauer M, Ahrne E, Baron AP, Glatter T, Fava LL, Santamaria A, Nigg EA, Schmidt A (2014) Evaluation of data-dependent and -independent mass spectrometric workflows for sensitive quantification of proteins and phosphorylation sites. *Journal of proteome research* **13:** 5973-5988

MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **26:** 966-968

Picotti P, Aebersold R (2012) Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nature Methods* **9:** 555-566