Supporting Information

EXPERIMENTAL PROCEDURES

The Online 2D LC System

The comprehensive 2D SCX-RP (reverse phase) LC system (UltiMate 3000, Thermo Fisher Scientific, Waltham, MA) was equipped with one gradient pump for strong cation exchange (SCX), one additional gradient pump for RP, one isocratic pump for online dilution, one 10-port valve with two RP-trapping columns for alternating trapping, one six-port valve for controlling the trapping column in either washing or eluting mode, and a manual injector for sample loading. Such a combination allowed us to introduce an organic solvent (acetonitrile) in the first-dimensional SCX separation without affecting the second-dimensional RP separations, by using an online dilution design. Briefly, a tryptic digest dissolved in 50% acetonitrile containing 0.1% formic acid was loaded onto the SCX column (0.5×150 mm, packed with Luna-SCX particles from Phenomenex, Torrance, CA, USA) using a manual injector. The first-dimensional separation was operated at a flow rate of 1.5–0.5 μL/min, and peptides were eluted using a continuous ammonium chloride gradient in the presence of 0.1% formic acid and 30% acetonitrile. The salt gradient was segmented in 44 steps, 65 min for each, and matched with the second-dimensional reverse-phase separations. The flow rate and slope of the gradient of each segment were tuned to prevent the overloading of the

RP-trapping column. A flow of solvent A (50 μL/min, 0.1% formic acid in water) delivered by an isocratic loading pump was used to dilute the effluent of the SCX column through a T union and a mixing tube before it reached the trapping column (0.5×5 mm, packed with Source 15RPC particles). Concomitantly, the other RP-trapping column, which was installed on the same 10-port valve, was connected with the RP-separating column, and its contents were analyzed using a mass spectrometer. Five minutes before each salt gradient step was completed, the binary pump used for SCX separation was stopped, and the six-port valve was switched to allow the loading pump to wash away the residual salt solution in the flow path of the RP-trapping column. After the trapping column was switched to the RP analytical column again, the bound peptides were eluted using a complete acetonitrile gradient (elution, regeneration, and reequilibration) in the presence of 0.05% formic acid over 65 min.

MS

All data were acquired using an LTQ Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific). The instrument was calibrated using standard compounds, and the lock mass function was enabled using a cyclosiloxane signal of *m/z* 445.120025 as an internal standard over the whole data acquisition time. Mass spectrometric data were acquired in a data-dependent mode and

controlled by the Xcalibur software (version 2.0.7 SR 2, Thermo Fisher Scientific). Each full MS spectrum scan (*m*/*z*, 420–2000; resolution, 30,000 at *m*/z 400) was acquired using the Orbitrap system, from which the six most intense ions (with intensities higher than 20,000) were selected for fragmentation using low-energy collision-induced dissociation in the linear ion trap. The preview scan function was disabled. The repeat count was set to 1, and the dynamic exclusion limit was set to 45 s. Fragmentation was performed at a normalized collision energy of 39% with an activation q of 0.25 and an activation time of 30 ms. Other mass spectrometric parameters were set as follows: spray voltage, 1.8 kV; capillary voltage, 49 V; capillary temperature, 200°C; and tube lens voltage, 120 V. The automatic gain control was set to 1×10^6 ions with a maximum fill time of 1000 ms for the Orbitrap full scan, and 4000 ions with a maximum fill time of 150 ms for the LTQ MS/MS scan.

Analysis of Mass Spectrometric Data

The identification and quantification of proteins were carried out using the MaxQuant v1.0.13.13 software package developed by Jürgen Cox and Matthias Mann (Max Planck Institute of Biochemistry, Planegg, Germany). SILAC doublets were extracted, recalibrated, and quantified in the raw data files with Quant.exe using the following parameters: Arg6 and Lys6 were set as labels; maximum of three labeled amino acids per peptide; carbamidomethylcysteine was set as a fixed modification; and methionine

oxidation, protein amino-terminal acetylation, and N-terminal modification of glutamine to pyroglutamic acid were set as variable modifications. Two missed cleavages were allowed, enzyme specificity was trypsin/P, and the MS/MS tolerance for precursor ions and fragment ions was set to 7 ppm and 0.5 Da, respectively. The generated peak lists (msm files) were submitted to a MASCOT search engine (Daemon.exe version 2.2.2, MatrixScience, Boston, MA) and searched against a database which contains 20,294 human protein sequence entries extracted from UniProtKB/Swiss-Prot Database, Release 2010_06, 262 commonly observed contaminants provided by the MaxQuant and the reversed sequences of all proteins. Peptide lists were further used by Identify.exe to identify and to quantify proteins relatively using the following parameters: peptide and protein false-discovery rates were set to 0.01; the maximum peptide posterior error probability (PEP) was set to 1; the minimum peptide length was set to 6; PEP was based on Mascot score; the minimum number of peptides for identification and quantification of proteins was set to 2, of which at least one must be unique; and identified proteins were requantified. Protein groups containing matches to proteins from the reversed database or contaminants were discarded. For correcting the effects of arginine-to-proline conversion on protein quantification, peptide ratios of non- and single-proline-containing peptides were extracted from the Evidence.txt files and the

median ratios of these two populations were used to estimate the overall conversion rate of each heavy isotope labeled sample. The conversion rates of all the experiments were found to fall in the range of 14.7–17.3%. The ratio of each peptide was then recalculated using the following equation: Rcorr = Robs / (1-r)n, where Rcorr is the corrected ratio, Robs is the observed ratio, r is the arginine-to-proline conversion rate, and n is the number of proline residues in the peptide sequence. Finally, the protein ratios were recalculated and normalized using the same methods as in MaxQuant.