Supplementary experimental procedures

Analytical 2-DE (150 µg of extracts) using broad pH gradient IPG strips.

Briefly, samples were diluted 1:1 with rehydration buffer including DTT, and the IPG strips were rehydrated overnight in the same buffer but with 0.5% IPG buffer and 12 µL/mL of DeStreak (GE Healthcare). Samples were loaded using the cup loading method onto universal strip holders, and the strips were subjected to electrophoresis using an Ettan IPGphor IEF system (GE Healthcare). The focusing conditions were: (i) 300 V, 3 h, step; (ii) 1000 V, 3 h, gradient; (iii) 8000 V, 3 h, gradient; and (iv) 8000 V, 3 h, step. After equilibration, the IPG strips were transferred onto 12.5% homogeneous polyacrylamide gels. Electrophoresis was conducted in an Ettan-DALT six electrophoresis system for 30 min at 2 W/gel and at 20 W/gel constant current at 15ºC until electrophoresis was complete. Gels were stained with a PlusOne Silver Staining Kit using the protocol recommended by suppliers for further MS (GE Healthcare) or Sypro Ruby (Bio Rad) following the instructions of the suppliers.

MALDI identification of 2-DE gel protein spots

The spot digestion was performed overnight with sequencing grade trypsin (Roche Molecular Biochemicals) as described in (20). After digestion, the supernatant was collected and 1 mL was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 mL of a 3 mg/mL of CHCA matrix (Sigma) in 50% v/v ACNe was added to the dried peptide digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (PerSeptives Biosystems, Framingham, MA). The instrument was operated in reflector mode, with an accelerating voltage of 20000

V. All mass spectra were calibrated externally using a standard peptide mixture (Sigma). Peptides from the auto digestion of the trypsin were used for the internal calibration. MALDI-TOF MS analysis produces peptide mass fingerprints and the peptides observed can be collected and represented as a list of monoisotopic molecular weights with an S/N greater than 20. Some suitable precursors for MS/MS sequencing analyses were selected and fragmentation was carried out using the CID on (atmospheric gas was used) 1 KV ion reflector mode and precursor mass Windows 6 10 Da. The plate model and default calibration were optimized for the MS/MS spectra processing. Parameters used for peak-picking were the following: minimum S/N filter was 15; peak density filter was 50 peaks per 200 Da; maximum number of peaks searched was 65 and the mass exclusion list corresponding to peptides from trypsin included the following masses 805.41, 906.505, 996.5, 1153.574, 1168.57, 1433.72, 1490.9, 1493.75, 2163.05, 2273.16, 2289.165, 2295.2, 2530.3

Digestion in solution

Proteins were reduced for 1 hour in 10 mM DTT, 25 mM ammonium bicarbonate, pH 8.25, at 37ºC and alkylated in 50 mM iodoacetamide for 30 min at room temperature in the dark. Samples were diluted to reduce urea concentration below 2 M, and digested with trypsin (Promega, Madison, WI, USA) at 37ºC overnight in a 1:50 ratio. The protease reaction was stopped by acidifying the solution with TFA. Then, the sample was cleaned up by passing through a homemade column packed with Poros 50 R2 resin (Applied Biosystems, Foster City, CA, USA). Peptides were eluted with 95% ACN in 0.1% TFA. Finally, the sample was dried in a Savant SpeedVac, and dissolved in 0.1% formic acid just before analysis.