Supporting Information

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SI Text

Materials. Angiotensin I was purchased from Bachem, c-Myc peptide from AnaSpec, and β-catenin peptide (116–124) was synthesized by the Proteomics Resource Center of Rockefeller University. Bovine insulin, bovine α-casein, and bovine RNase A were obtained from Sigma and used without further purification. PITC, TFA, biotin-NHS, TCEP, pyridine, triethylamine, methanol, benzene, ether, acetonitrile, benzamidine, α-cyano-4-hydroxycinnamic acid, urea, and cisplatin were also from Sigma. EZ-Link Sulfo-NHS-SS-biotin and neutravidin agarose beads were from Pierce. Sequencing grade-modified trypsin was from Promega. Protease inhibitor mixture tablets were from Roche. z-VAD-fmk was from R&D Systems.

The Jurkat T cell line was obtained from the American Type Culture Collection. Mouse monoclonal vimentin antibody (AMF-17b) developed by Alice B. Fulton (1) and myosin 10 antibody (CMII 23) developed by Gary W. Conrad and Abigail H. Conrad (2) were obtained from Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development, and maintained by the University of Iowa, Department of Biological Sciences. Rabbit polyclonal NAP1L1 antibody was from Abgent, β-actin polyclonal antibody from Santa Cruz Biotechnology, and biotin antibody from Sigma.

Cell Culture and Induction of Apoptosis. Jurkat T cells were cultured in RPMI medium 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 1,500-cm^2 cell culture bottles. When the cell density reached 1×10^6/mL, the cells were either treated with 200 μM cisplatin or DMSO for 8 h. A total of 1×10^8 cells were collected for each experiment and lysed in 1 mL lysis buffer [150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 2 mM ethylenediamine tetraacetic acid, and 1% triton X-100, pH 7.4] by gentle sonication in the presence of protease inhibitor mixture. The lysate was centrifuged at 100,000 × *g* for 30 min in an ultracentrifuge (Beckman, 70.1 Ti rotor) with thick-wall polycarbonate centrifuge tubes, and the supernatant was collected for N-CLAP experiments. For the Western blot analysis, cells were treated with either DMSO, 200 μM cisplatin, or 200 μM cisplatin after 2 h pretreatment of 20 μM z-VAD-fmk.

Chemical Modification of Amines. Peptides and individual proteins (50 μg, 5 μL, sample A) were dissolved in a 100-μL mixed solvent consisting of methanol, pyridine, triethylamine, and distilled water in a ratio of 7:1:1:1. PITC (20 μL) was added into sample A, which was flushed with nitrogen gas, and the reaction was carried out at 55 °C for 1 h with constant shaking. An additional 20 μL PITC was added to the sample, and the reaction was continued for 1 h. Organic solvents were extracted by adding 8 volumes of benzene and centrifuging for 5 min at 3,000 × *g*. This extraction was repeated 3 times. The peptide/protein phase was dried by SpeedVac. Neat TFA (50 μL) was added, flushed with nitrogen gas, and vortexed for 5 min at room temperature to completely dissolve the pellet. The "deblocking" of the PITC-modified N-terminal amino acid was carried out at 45 °C for 15 min with constant shaking. It should be noted that prolonged TFA treatment can cleave N-terminal sides of serine and threonine residues as well as the C-terminal side of aspartic acid residue (3). Therefore, in our experiment the TFA treatment is limited to 15 min, and the small number of these peptides were removed from our analysis, although it is possible that a small amount of bona fide N-terminal peptides were removed as well. The reaction conditions can be fine-tuned by lowering the temperature, reducing the time of TFA treatment, or replacing TFA with other acids to minimize such cleavages. The volume of TFA was reduced by flushing air at room temperature, and the sample was precipitated and washed with anhydrous ether 3 times and dissolved in 0.2 M Hepes buffer (pH 8.5) in the presence of 2% SDS and 150 mM NaCl. EZ-Link Sulfo-NHS-SS-biotin (dissolved in DMSO) was added to the sample to a final concentration of 5 mM, and sample was incubated at 37 °C for 4 h or overnight. At the end of the reaction, the sample was treated with 100 mM hydroxylamine (pH ≈10, adjusted with sodium hydroxide) at room temperature for 30 min. When the above chemical reactions were carried out for Jurkat T cell lysate, the initial sample volume was 1 mL, and the protein concentration was approximately 2 mg/mL, determined by Bradford protein assay. The volume of other organic solvent and reagent was increased proportionally.

Evaluation of PITC Modification and TFA Cleavage. The amine modification by PITC and the TFA cleavage of N-terminal PITC-modified amino acid were evaluated by an assay for amines. Samples were precipitated with ether and dissolved in 0.2 M Hepes (pH 8.5) in the presence of 2% SDS and 150 mM NaCl. Free amines in the samples were reacted with biotin-NHS (5 mM) for 30 min at 37 °C, thus labeling any unmodified amines. The presence of amines was indicated by a signal in Western blotting using streptavidin-HRP and ECL Western blotting reagents.

Sample Preparation for Mass Spectrometric Analysis. Samples were resolved on a 4–12% polyacrylamide NuPage gel, and the gel was divided into 5 strips. Each strip was cut into ≈1 mm × 1 mm small pieces, and in-gel digestion was carried out in 37 °C for 24 h with sequencing grade-modified trypsin without reduction and alkylation. Peptides were extracted with 5% formic acid/50% acetonitrile in aqueous solution 3 times using a 30-min incubation and 30-min sonication in a water bath sonicator, followed by extraction with 100% acetonitrile. The sample was combined and dried by SpeedVac. The peptides were dissolved in 800 μL resuspension buffer (100 mM Hepes, 1 M NaCl, pH 7.4) in the presence of 250 mM benzamidine, incubated for 30 min at room temperature, and centrifuged to remove any undissolved material. Neutravidin agarose beads (30 μL of 50% slurry) was added to each fraction and incubated at 4 °C for 2 h and washed sequentially with 1 mL of each of the following buffers for 10 min each with constant shaking: 5 M NaCl in phosphate buffer (pH 7.4); 3 M NaCl in PBS; 1 M NaCl in PBS; 2 M urea in PBS; PBS; 20% methanol; followed by 2 washes with water. The beads were incubated with 100 μL 10 mM TCEP (pH was adjusted to 7 by ammonium hydroxide) for 2 h at room temperature with constant shaking. The beads were spun down, washed once with Milli-Q H_2O, and the supernatant was combined and filtered through a 0.2-μm spin column (Pall). The sample was dried down and resuspended in 20 μL 5% acetonitrile/0.1% TFA aqueous solvent before mass spectrometry analysis. Each sample was analyzed twice.

MALDI-TOF-MS and LC-MS/MS Analysis. For MALDI-TOF-MS of peptides and RNase A, samples were desalted by Millipore C18 ZipTip according to the manufacturer's protocol. The masses of

the samples were analyzed in the reflector mode by MALDI-TOF-MS (Applied Biosystems).

Samples from Jurkat T cell lysate after avidin purification were analyzed by nano LC Q-TOF MS/MS to obtain peptide identification. For each sample, 8 μL was injected onto an LC/MS system consisting of an 1100 Series HPLC, HPLC-Chip Cube MS interface, and 6520 Series Q-TOF mass spectrometer (Agilent Technologies). The HPLC-Chip contains a 40-nL enrichment column and a 43 mm $\times$ 75 μm analytical column packed with Zorbax 300SB-C18 (5-μm particles). Peptides were loaded onto the enrichment column with 97% solvent A and 3% solvent B with a flow rate of 4 μL/min. Solvent A consists of 0.1% formic acid and solvent B of 90% acetonitrile and 0.1% formic acid. Peptides were eluted with a gradient from 3% to 45% solvent B in 45 min, followed by a steep gradient to 90% solvent B for 5 min at a flow rate of 0.3 μL/min. Mass spectra were acquired in positive-ion mode with automated data-dependent MS/MS on the 5 most intense ions from precursor MS scans, and every selected precursor peak was analyzed twice within 2 min.

Spectrum Mill Database Search. PKL files were created with the Spectrum Mill (Rev A.03.03.080) Data Extractor for MS/MS spectra that could be assigned to at least 4 *y*- or *b*-series ions. Scans with the same precursor $\pm$0.4 *m*/*z* were merged within a time frame of $\pm$15 s, charges up to a maximum of 5 were assigned to the precursor ion, and the ^{12}C peak was determined by the Data Extractor. Swiss-Prot database (v55.6, July 1, 2008) with concatenated reverse database with same entries and same protein lengths was searched for N-terminal nonspecific tryptic peptides with a mass tolerance of $\pm$20 ppm for the precursor ions and a mass tolerance of $\pm$40 ppm for the fragment ions with two fixed modifications: PITC-modified lysine and N-terminal modification of a remaining group (-$COCH_2CH_2SH$) from EZ-Link Sulfo-NHS-SS-biotin after reduction of disulfide bond during the elution step. Four missed cleavages were allowed during the search. The threshold used for peptide identification was a score of $\geq$10, an SPI (the percentage of assigned spectrum intensity of total spectrum intensity) of $\geq$50%, and a value of forward score minus reverse score larger than 2. These stringent criteria resulted in only 2 possible false-positive hits during the database search, and the search result gives a $<$1% false positive rate. In the peptide list, only the highest-scoring member of each peptide group was shown, and only peptides with a charge state of 2, 3, and 4 were reported. Finally, all MS/MS spectra were manually validated and the spectra with low-quality fragmentations were discarded.

1. Isaacs WB, Cook RK, Van Atta JC, Redmond CM, Fulton AB (1989) Assembly of vimentin in cultured cells varies with cell type. *J Biol Chem* 264:17953–17960.
2. Conrad AH, Clark WA, Conrad GW (1991) Subcellular compartmentalization of myosin isoforms in embryonic chick heart ventricle myocytes during cytokinesis. *Cell Motil Cytoskeleton* 19:189–206.
3. Hulmes JD, Pan YC (1991) Selective cleavage of polypeptides with trifluoroacetic acid: Applications for microsequencing. *Anal Biochem* 197:368–376.

Other Supporting Information Files

Table S1
Table S2
Table S3
Table S4
SI Appendix