Supplementary materials and methods

Cell surface biotinylation. The methods were performed as described previously (S1) with modifications as shown in Fig. S1A. A549 and CL1-0 cells were grown at 37°C in DMEM medium with 10% FBS until approaching confluency (90%). Four dishes (10 cm) of cells were washed with iced PBS three times, and then added freshly prepared 10 ml PBS containing 2.5 mg EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA). After the cells were incubated at 4°C for 1 h with gentle agitation, the biotinylation reaction was terminated by addition of Tris-HCl (pH 7.5) to a final concentration of 50 mM. The cells were scraped and washed three times with Tris buffered saline (25 mM Tris, 150 mM NaCl, pH 7.2). Cells were lyzed with 0.5 ml of lysis buffer (50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100) with protease inhibitor and incubated at 4°C for 30 min before disruption with 5 strokes using a probe sonicator (Sartorius, Labsonic M, Germany). Unbroken cells and nuclei were pelleted from the cell homogenate by centrifugation at 10000 x g for 2 min at 4°C.

Preparation of purified plasma membrane proteins using affinity chromatography. The clarified supernatant was incubated with 500 µl of NeutrAvidin (Pierce) for 1 h at room temperature. The beads were washed three times

with cell lysis buffer and the biotinylated proteins were incubated with the beads 1 h at room temperature. The biotinylated proteins were eluted with 500 µl SDS sample buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 50 mM DTT) after three washes with cell lysis buffer. Eluates and non-biotinylated cell lysates were analyzed by immunoblotting.

Two-dimensional electrophoresis. Two-dimensional electrophoresis (2DE) was performed using Ettan IPGphor III (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) as previously described (15). Total proteins (400 µg) were mixed with rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTE, 1% IPG Buffer and 0.002% bromophenol blue in a total volume of 315 µl. The mixtures were loaded onto an 18 cm IPG ReadyStrip pH 4-7 (Bio-Rad, Laboratories Inc., Hercules, California, USA). Isoelectric focusing (IEF) parameters were set at 50 µA/strip at 20°C with a rehydration step for 12 h using 50 V. IEF was carried out using the following conditions: (1) 100 V for 2 h; (2) 250 V for 1 h; (3) 500 V for 1 h; (4) 1,000 V for 1 h; (5) 4,000 V for 1 h; (6) and 8,000 V for 65,000 Vh. After reduction with 65 mM DTE and alkylation with 55 mM iodoacetamide, the second-dimension separation was performed on a linear gradient 10–18% polyacrylamide gel. The protein gel was fixed in 10% methanol with 7% acetic acid

and stained using the SYPRO® Ruby method (Invitrogen Corporation, Carlsbad, CA, USA). Gels were then scanned using a Typhoon 9400TM Fluorescence Imager (GE Healthcare Bio-Sciences Corp.) and analyzed using the ImageMasterTM 2D Elite software package (GE Healthcare Bio-Sciences Corp.) in high-quality TIF format.

In-gel digestion and protein identification. The 2DE image of the DMSO-treated cells was set as the reference gel image. After comparing the drug-treated gels to the reference image, the differentially expressed protein spots were excised. These gel pieces were washed with 1:1 (v/v) solution containing 50 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, Missouri, USA) and acetonitrile (ACN, Sigma-Aldrich) and dehydrated with ACN twice. After air drying of the gel pieces, proteins were digested for 16 h at 37°C with sequence-grade trypsin (Promega Corporation, Wisconsin, USA). The digested peptides were extracted from the gel with 2% trifluoroacetic acid (TFA, Sigma-Aldrich) in 100% ACN. The extracts were evaporated to dryness, and the protein fragments were dissolved in 0.1% TFA, mixed with 10 μg/mlα-ciano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich) (1:1, V/V), and directly spotted onto the sample plate of a MALDI-TOF mass spectrometer.

MALDI-TOF mass spectrometry was performed on a dedicated TOF-TOF Ultima MALDI instrument (Micromass, Manchester, UK). All individual MS data were

generated and output as a single MASCOT-searchable peak list file. Subsequently, proteins were identified by searching in the SWISS-PROT version 51.7 database with the MASCOT (http://www.matrixscience.com) search engine, as previously described. The search parameters were as follows: peptide mass tolerance ± 100 ppm, fragment mass tolerance ± 0.25 Da, only tryptic peptides with up to one missed cleavage site, and variable modifications were carbamidomethylation (C) and oxidation of methionine. For positive identification, the score of the result of [-10 Log(P)] had to be significant ($P < 0.05$).

Reference:

S1. DeBlaquiere J and Burgess AW. Affinity purification of the plasma membranes. J Biomol Tech. 1999; 10: 64–71.