SUPPLEMENTARY MATERIALS

Material and Methods

Cell Lines and Cell Culture Conditions

The MCF10A human mammary epithelial cell line (obtained from the Michigan Cancer Foundation, Ann Arbor, MI) was maintained in DMEM/F12 medium (Gibco® Cell Culture Systems, Invitrogen Corporation, Carlsbad, CA) containing 5% horse serum (Invitrogen Corporation), 0.5 µg/ml hydrocortisone (Sigma Chemicals, St. Louis, MO), 100 ng/ml cholera toxin (Sigma Chemicals), 10 µg/ml insulin (Sigma Chemicals), 2.5 mM L-Glutamine (Biofluids, Biosource International, Camarillo, CA), and 20 ng/ml epidermal growth factor (EGF; Sigma Chemicals). MCF-7 and SK-Br3 human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were routinely grown in Improved MEM (IMEM; Biosource International) supplemented with 5% fetal bovine serum and 2 mM L-Glutamine. MCF-7/*neo* and MCF-7/HER2-18 transfectants (gift from Prof. Mien-Chie Hung, The University of Texas M.D. Anderson Cancer Center, Houston, TX) were grown as indicated for MCF-7 cells, except that 450 µg/ml G418 was added to the culture medium. Cells were maintained at 37 ^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

Generation of HER2 ECD Deletion Mutants

Deletion mutations within subdomain III of the HER2 ECD were generated by a two-step PCR method using a 3,938-bp wt HER2 cDNA as the starting template. This 3,938-bp insert corresponds to nucleotides 34–3,972 of the human HER2 cDNA deposited with GenBank (accession #M11730). For Δ447-466, the upstream portion was generated using the following primers: 5'-TCCTCCCTGCAGGATTAATCCA-3' (HER2 5' flanking) and 5'-TGTTCTGGTGGTGATGCCCAG-3' (HER2 upstream deletion). The downstream portion was generated using the following primers: 5'-CTGGGCATCCACCATAACA-3' (HER2 downstream deletion) and 5'-TACTTCCGGATCTTCTGCTGC-3' (HER2 3' flanking). Wild-type HER2 cDNA and the mutants were cloned into the *Xho*I site of the pLXSN retroviral vector (Clontech, Palo Alto, CA) for expression in mammalian cells.

Deletions were confirmed by forward and reverse sequencing (Gene Gateway, Hayward, CA) using primers bracketing sub-domain III of the HER2 ECD.

Generation of MCF10A Cells Expressing HER2 ECD Deletions

Retroviral stocks were generated by co-transfection of the vector plasmid DNA with a packaging plasmid into a high-efficiency transient amphotropic packaging system (TSA54 cell line) using the FuGENE 6 transfection reagent (Roche, Indianapolis, IN) as per manufacturer's instructions. Medium containing infectious retroviruses was collected from transfected cells after 48 h, filtered, and stored at -80 ^{o}C until utilization. MCF10A cells were infected with replication-incompetent amphotropic retroviruses containing either full-length HER2 or HER2 ECD deletion mutant cDNAs cloned into the pLXSN (LTR gene X–simian virus 40–*neo*) retroviral vector for 24 h in the presence of polybrene (4 µg/ml; Sigma Chemicals). Infected MCF10A cells were grown for an additional 24 h in standard medium, and stable cell lines were selected in G418 (500 µg/ml). Expression levels of the wild-type form of HER2 and the HER2 ECD deletion mutants were assessed by RT-PCR using the Gene Amp Kit (Promega Corp., Madison, WI), as per manufacturer´s instructions.

Immunoblotting Analyses

For assaying levels of protein expression and the phosphorylation status of HER1, HER2, ERK1/2, and AKT, cells were cultured in 24-well dishes. Cells were then washed with cold PBS, placed on ice, and lysed in non-denaturing 1X lysis buffer (Cell Signaling, Beverly, MA) containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO4, 1 µg/ml leupeptin, and 1 mM PMSF. Cells were scraped, added to Eppendorf tubes, and incubated on ice for 20 min before debris was removed by a 15-min spin at 14,000 rpm at 4 ^{o}C. A BCA protein reagent kit (Pierce, Rockford, IL) was used to determine total protein levels.

Equal amounts of protein were heated in SDS sample buffer (Laemmli) for 10 min at 70 ^{o}C, subjected to electrophoresis on either 3–8% Tris-Acetate NuPAGE [HER2, phospho-HER2 (pHER2), HER1, phospho-HER1 (pHER1), and HER3] or 10% SDS-PAGE [ERK1/2, phospho-ERK1/2 (pERK1/2), AKT, and phospho-

AKT (pAKT)] gels, and transferred to nitrocellulose membranes. For immunoblot analyses of HER2, pHER2, HER1, pHER1, and HER3, nonspecific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at room temperature (RT) with TBS-T [25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween 20] containing 5% (*w/v*) nonfat dry milk. The treated filters were washed in TBS-T and then incubated with primary antibodies for 2 h at RT in TBS-T containing 1% (*w/v*) nonfat dry milk. The membranes were washed in TBS-T, and then horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) in TBS-T were added for 1 h. Immunoreactive bands were visualized with ECL detection reagent (Pierce, Rockford, IL). For immunoblot analyses of ERK1/2, pERK1/2, AKT, and pAKT, membranes were blocked as described above and incubated overnight at 4 ^{o}C with primary antibody in TBS-T containing 5% bovine serum albumin. The membranes were washed in TBS-T, and then horseradish peroxidase-conjugated secondary antibodies in TBS-T containing 5% (*w/v*) nonfat dry milk were added for 1 h. Primary antibody binding was detected with ECL detection reagent (Pierce, Rockford, IL). Blots were re-probed with an antibody for β-actin to control for protein loading and transfer. Densitometric values of protein bands were quantified by Scion imaging software (Scion Corp., Frederick, MD).

The following primary antibodies were used at the concentrations indicated: HER2 (Ab-3; mouse monoclonal, Oncogene Research Products, Cambridge, MA; 2.5 μg/ml), HER2 (rabbit polyclonal, Cell Signaling, 1:1000); pHER2 (Ab-18; mouse monoclonal, Lab Vision Corp., Fremont, CA; 2.5 μg/ml), pHER2 (rabbit polyclonal, Cell Signaling, 1:1000), HER1 (rabbit polyclonal, Upstate USA, Chicago, IL; 1 μg/ml), EGFR (rabbit polyclonal, Cell Signaling, 1:1000), phospho-HER1 (rabbit polyclonal, Upstate; 1 μg/ml) and pEGFR (Y1045, rabbit polyclonal, Cell Signaling), AKT (rabbit polyclonal, Cell Signaling; 1:500), pAKT (rabbit polyclonal, Cell Signaling; 1:500), p44/p42 (rabbit polyclonal, Cell Signaling; 1:500), phospho-p44/p42 (rabbit polyclonal, Cell Signaling; 1:500), HER3 and pHER3 (both rabbit polyclonal, Cell Signaling, 1:1000)). To verify equivalent protein loading, β-actin (mouse monoclonal, Sigma Chemicals) was included at a concentration of 0.2 μg/ml. HRP-conjugated anti-rabbit or anti-mouse secondary antibodies were used at a concentration of 1:5,000 (Cell Signaling).

Metabolic Status Assessment Cell viability was determined using a modified MTT reduction assay (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega Corp.). Briefly, cells in exponential growth were harvested by trypsinization and seeded at a concentration of 3 x 10^3 cells/200 µl/well into 96-well plates and allowed an overnight period for attachment. Then the cells were washed twice with pre-warmed PBS and cultured in serum-free medium overnight. Then, the medium was removed, and fresh medium in the absence or presence of EGF or graded concentrations of paclitaxel (Sigma Chemicals) was added to the cultures as specified. Following a 4-5 day treatment, 96-well plates were centrifuged at 200 x *g* for 10 min, and MTS/PMS solution was added to each well at 1/5 volume. After incubation for 3 h at 37 °C in the dark, absorbance was measured at 490 nm using a multiwell plate reader. Dose-response curves were plotted as percentages of the control cell absorbance, which was obtained from control cells treated with appropriate concentrations of vehicles (*v/v*) and processed simultaneously. For each treatment, cell viability was measured as a percentage using the following equation: (A_{490} of treated sample/A_{490} of untreated sample) *x* 100. Each experiment was performed in triplicate.

Soft Agar Colony Formation Assays

A bottom layer of 1 ml 2X complete DMEM/F12 medium containing 0.7% agar and 10% horse serum was prepared in 35-mm multi-well cluster dishes. After the bottom layer solidified, 10,000 cells/dish were added in a 1-ml top layer of 2X complete DMEM/F12 medium containing 0.35% agar and 10% horse serum. All samples were prepared in triplicate. Dishes were incubated in a humidified 5% CO_2 incubator at 37 ^oC, and after ~14 d, colonies measuring ≥50 μm were counted using a cell colony counter (Ommias 3600, Imaging Products Inter. Inc., Charley, VA) after staining with nitroblue tetrazolium (Sigma Chemicals).

Cell Cycle Analysis

Adherent and detached cells were collected after trypsinization, washed in cold PBS, and centrifugated at 1,500 rpm. Cells were resuspended at 2 x10^6 cells/ml in PBS and fixed in ice-cold 80% ethanol for at least 24 h. Fixed cells were centrifugated at 300 x *g*, and each sample was resuspended in propidium iodide staining buffer (0.1% Triton X-100, 200 µg DNase-free RNase A, 20 µg propidium iodide) in PBS for 30 min. Samples were then analyzed using a FACScalibur (Becton Dickinson, San Diego, CA, USA) and ModFiT LT (Verity Software House).

Phosphoproteome Profiling

Cells were rinsed with cold PBS and immediately solubilized in NP-40 lysis buffer [1% NP-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin] by rocking the lysates gently at 4 ^{o}C for 30 min. Following microcentrifugation at 14,000 *x* g for 5 min, supernatants were transferred to a clean test tube, and sample protein concentrations were determined by the Pierce Protein Assay Kit (Rockford, IL). 500 µg of lysates were diluted and incubated with the Human Phospho-RTK Array Kit (Proteome Profiler™; R&D Systems, Minneapolis, MN) as per manufacturer's instructions. Phospho-RTK Array data were developed on x-ray films following exposure to chemiluminescent reagents.

Surface biotinylation

Surface biotinylation was performed as described previously (1). Briefly, cells were incubated with 0.5 mg/ml biotin for 30 min at 4°C, lysed in RIPA buffer and subjected to an o/n precipitation with Streptavidin (SA)-beads followed by Western blot analysis of HER2 and actin as loading control.

Immunofluorescence and image acquisition

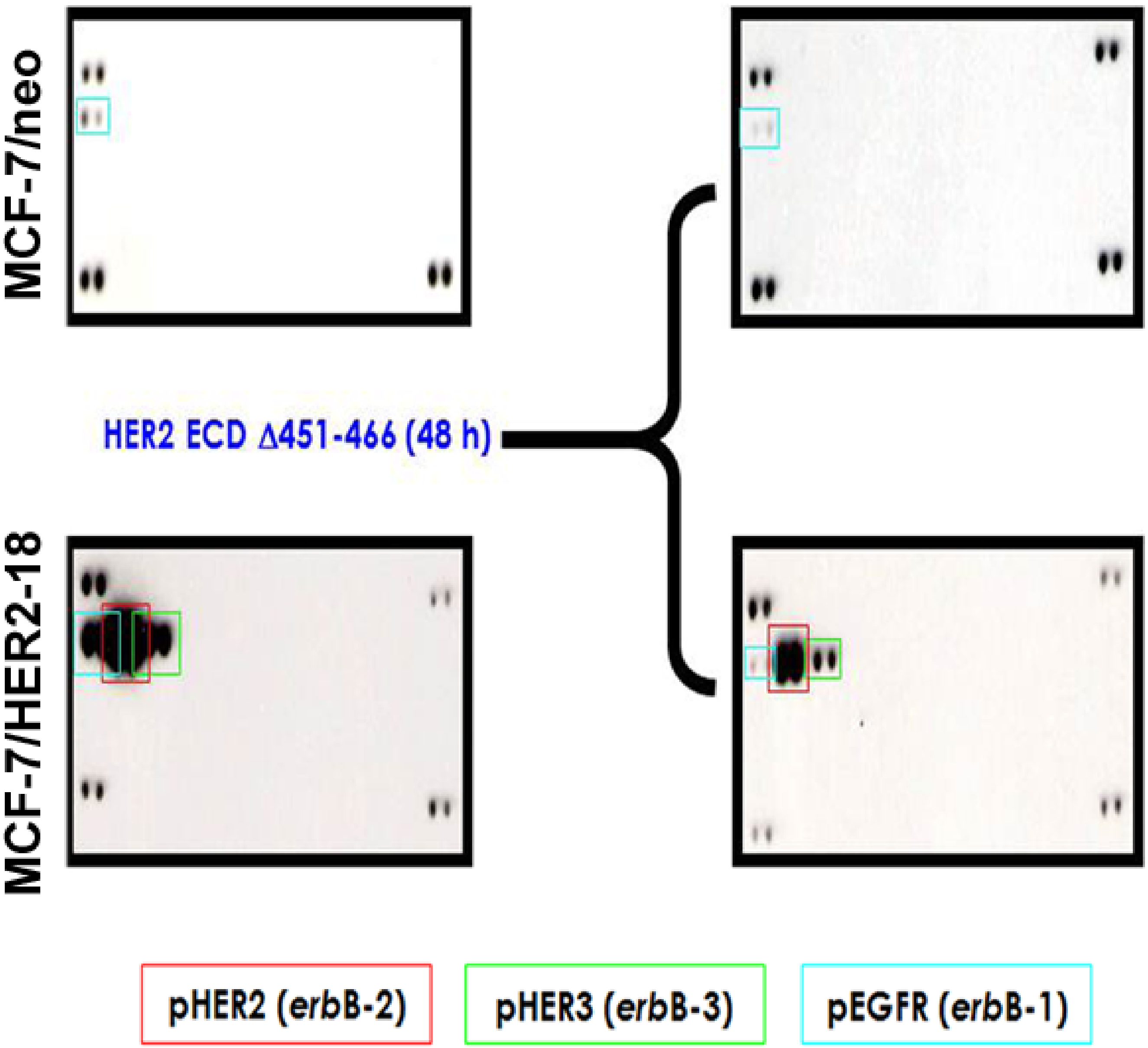
Indirect immunofluorescence, image acquisition and manipulations were performed as described previously (2). All images were taken at the same exposure and process in parallel using *Adobe Photoshop* software. For HER2 surface staining, a HER2-ECD specific mouse monoclonal antibody from proteintech^{TM} was used in a 1:300 dilution without cell permeabilization.

References

1. Schroeder B, Srivatsan S, Shaw A, et al. CIN85 phosphorylation is essential for EGFR ubiquitination and sorting into multivesicular bodies. Mol Biol Cell 2012;23(18):3602-11.
2. Schroeder B, Weller SG, Chen J, et al. A Dyn2-CIN85 complex mediates degradative traffic of the EGFR by regulation of late endosomal budding. EMBO J 2010;29(18):3039-53.

Supplementary Figure 1

MCF-7/Her2-18 transfectants and MCF-7/*neo* control cells were transiently transduced with retroviruses to express HER2-ECD-Δ451-466. 500 µg total cell lysates were incubated with membranes of the Human p-RTK Array Kit as per manufacturer's instructions. Shown is a p-proteome analysis representative of three independent experiments.



MCF-7/neo
HER2 ECD Δ451-466 (48 h)
MCF-7/HER2-18
pHER2 (erbB-2)
pHER3 (erbB-3)
pEGFR (erbB-1)