Supplementary Methods

Immunohistochemistry

HER2 protein expression was evaluated with the DAKO HercepTest kit (product K5207, DAKO, Glostrup) and following the manufacturer's instructions. Scoring values ranged between 0 and 3, as described in (17). A score of 3 was considered as a positive test. The HER2 gene copy number of those samples with a score of 2 was analyzed by fluorescence in situ hybridization (FISH) performed by INFORM (package insert 2001; Ventana Medical Systems, Inc., Tucson, AZ) (≥5 gene copies of HER2/neu considered amplified). HER2-positive breast cancers samples were fixed, paraffin-embedded and immunostained with mouse monoclonal CB11 (BioGenex, San Ramon, CA, USA; 1/500 dilution), an antibody against the cytoplasmic domain of HER2, or with a mouse monoclonal anti-p95HER2/611CTF specific antibody as described in (6) (final dilution 0.2 mg/ml).

Molecular characterization of breast cancers

Expression of 50 selected genes was measured using the Counter platform from 54 formalin-fixed paraffin embedded (FFPE) HER2-positive samples. For each sample, three 2 mm cores enriched with tumor tissue were obtained from the original tumor block, RNA was purified using the High Pure FFPE RNA Micro Kit (Roche Applied Science, Penzburg, Germany) and 100 ng of total RNA was used for the Counter platform to measure gene expression. Counter raw data was log base 2 transformed and normalized using 5 house-keeping genes (ACTB, MRPL19, PSMC4, RPLP0 and SF3A1). All tumors were assigned to an intrinsic molecular subtype of breast cancer

(Luminal A, Luminal B, HER2-enriched and Basal-like) or the Normal-like breast group as described in (18).

Patient tumor samples

Breast tumors used in this study were from 56 surgical resections at Vall d'Hebron University Hospital and were obtained following the institutional guidelines. Written informed consent for the performance of tumor molecular studies was obtained from all patients who provided tissue.

Plasmids

All plasmid constructs of HER2 were derived from a cDNA clone identical to the published sequence gi:183986. HER2 was subcloned in pQCXIH (Clontech Laboratories Inc, Mountain View, CA, USA) and HER2 and p95HER2/611CTF in pLPCX using standard PCR, sequencing and cloning techniques. Primer sequences for HER2 and p95HER2/611CTF are the following: HER2-forward (caccatggagctggcggccttgtgcc), p95HER2/CTF611-forward (caccatgcccatctggaagttccag), HER2- and p95HER2/CTF611-reverse (tcacactggcacgtccagaccc).

Cell culture and infections

BT474, HCC1954, MDA-MD-453 and MCF10A were obtained from ATCC-LGC Standards (Teddington, UK) and 67NR from Barbara Ann Karmanos Cancer Institute (Detroit, MI, USA). All cell lines were routinely maintained as recommended by the providers.

Primary cell lines derived from patient-derived xenografts (PDXs) were obtained from tumors that were excised and cut into small pieces. Then, they were incubated for 30 minutes with collagenase IA (Sigma, St Louis, MO, USA), washed and resuspended in Dulbecco's Modified Eagle's Media (DMEM):F-12, 10% FBS, 4 mmol/L L-glutamine, Penicillin/Streptomycin (all from Gibco, Paisley, UK), 10 mM HEPES (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 1.75 µg/ml Amphotericin B (Fungizone, Gibco, Paisley, UK) for 6 hours. Subsequently, medium was carefully removed and changed to 10% supplemented Mammocult human medium (StemCell Technologies, Vancouver, BC, Canada) with Penicillin/Streptomycin, 10 mM HEPES and 1.75 µg/ml Amphotericin B for one week in order to facilitate the growth of epithelial cells with respect to contaminating mouse fibroblasts.

Generation of MCF10A cells stably expressing vector, HER2/611CTF, HER2 or the combination was performed by retroviral infection. Briefly, viruses were produced by calcium-phosphate transfection of Phoenix cells and target cells were subsequently infected and selected using hygromycin (100 ug/ml; Gibco, Paisley, UK) or/and puromycin (1 ug/ml; Sigma, St Louis, MO, USA).
All experiments were performed using low-passage cell lines (passage 10 to 25).

Proliferation assay

1.10^3 cells per well were seeded in ninety six-well plates and treated as indicated in the corresponding figure. After 7 days, medium was removed and adherent cells were fixed in 10% glutaraldehyde for 10 min, then washed thrice in distilled water and stained with

0.1% crystal violet for 20 min. After washing, the stain was dissolved with 10% acetic acid and subsequently quantified at 570 nm.

Flow cytometry

The percentage of cells in the sub-G1 phase of the cell cycle (i.e., apoptotic cells) was determined based on relative DNA content. Briefly, 1.10^5 cells were treated with different concentrations of doxorubicin for 24 hours. Then cells were detached with trypsin-EDTA (Gibco, Paisley, UK), washed with phosphate-buffered saline (PBS), fixed for 30 min on ice in 70% ethanol, treated for 10 min at 37ºC with a DNA extraction solution (0,19 M Na_2PO_4, 4 mM Citric acid pH 7.8), and stained with a propidium iodide (40 µg/ml)/ RNase (10µg/ml) solution for 30 min at 37ºC (all from Sigma, St Louis, MO, USA). Stained cells were filtered and analyzed on a FACSCalibur cytometer and analyzed with CellQuest software (BD Biosciences, San Jose, CA, USA).

To analyze the expression level of HER2 in the cell surface, cells were harvested with Accutase (Gibco, Paisley, UK), blocked with 5% horse serum and 1% BSA in PBS and incubated with 2.5 µg/ml trastuzumab or human IgG. Next, an Alexa 488-conjugated goat anti-human IgG (#A-11013; 1/500 dilution) (Invitrogen Ltd, Paisley, UK) detection antibody was added. To discard non-viable cells in the analysis, 2 ug/ml of propidium iodide was used. Cells were analyzed on a Navios cytometer (Beckman Coulter, Brea, CA, USA). Expression levels are indicated as fold over non-stimulated cells, after subtracting the fluorescence intensity detected with the isotype-matched control antibody to the specific antibody signal.

Protein extraction and immunoblotting

Cells were washed twice with ice-cold 1X PBS and proteins were extracted with 20 mM Tris-HCl pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40 supplemented with 50 mg/ml leupeptin, 50 mg/ml aprotinin, 0.5 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride (Sigma, St Louis, MO, USA). Breast tumor samples were homogenized in the same lysis buffer with a Polytron homogeneizer. Protein extracts were quantified using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA), resolved by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Proteins were detected with Immobilon western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA). Signals in Western blots were quantified with the software ImageJ 1.38 (NIH, Bethesda, MD, USA). Primary antibodies included mouse monoclonal anti-HER2 (CB11; #MU-134-UC; 1/1000) from BioGenex (San Ramon, CA, USA) and rabbit polyclonal anti-p-HER2 (Y1221/Y1222; #2249; 1/1000), rabbit monoclonal anti-p-ERK1/2 (T202/Y204; #4370; 1/1000), rabbit polyclonal anti-Erk1/2 (#9102; 1/1000), rabbit polyclonal anti-p-Akt (S473; #9271; 1/1000) rabbit polyclonal anti-Akt (#9272; 1/1000) from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-β Tubulin (#sc-9104, Santa Cruz Biotech, Santa Cruz, CA, USA; 1/2000) was used as loading control (LC) where indicated. Anti-p95HER2/611CTF antibody was developed in-house and described previously (6). Secondary antibodies included horseradish peroxidase-linked donkey anti-rabbit IgG and sheep anti-mouse IgG (Amersham GE Healthcare, Piscataway, NJ, USA; 1/5000 both).

Confocal microscopy

Cells were seeded on glass coverslips, washed with 1X PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 10 min. PBS with 1% bovine serum albumin, 0.1% saponin and 0.02% NaN_3 was used for blocking (1 h), primary antibody binding (2 h) and secondary fluorochrome-conjugated antibody binding (40 min in dark). Preparations were mounted using Vectashield with 40,60-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). All procedures were performed at room temperature. Images were captured using an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

Patient-derived xenografts

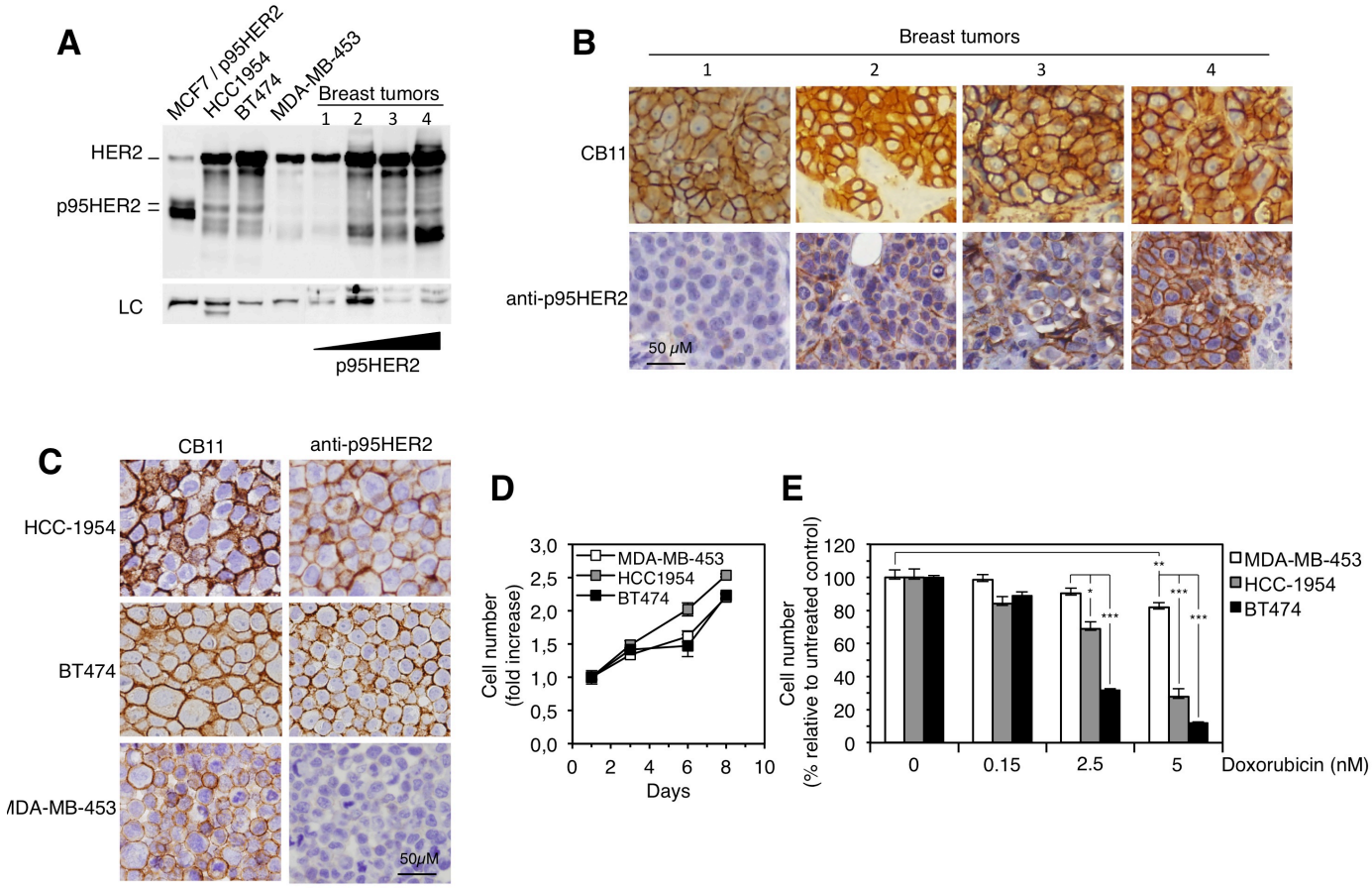
Six to eight-week old NOD.CB17PrkdcSCID/J (NOD/SCID) were purchased from Charles River Laboratories (Paris, France). Fragments of patient samples were implanted orthotopically without matrigel into the number four fat pad of NOD/SCID mice and 17 b-estradiol (1 uM) (Sigma, St Louis, MO, USA) was added to drinking water. When tumor volume reached approximately 250 mm^3, the mice were randomized (7 per group) and treated with trastuzumab (10mg/kg in sterile PBS, twice weekly intraperitoneally), doxorubicin (0.83 mg/kg in sterile PBS1X, thrice weekly i.p every 3 weeks) or the combination. Tumor xenografts were measured with callipers every 3 days, and tumor volume was determined using the formula: (length x $width^2$) x (pi/6). At the end of the experiment, the animals were anesthetized with a 1.5 % isofluorane-air mixture and were killed by cervical dislocation. Mice were maintained and treated in

accordance with institutional guidelines of Vall d'Hebron University Hospital Care and Use Committee.

Antibody-dependent cell cytotoxicity (ADCC) assay

ADCC was analysed by measuring the lactate dehydrogenase (LDH) released from the cancer cells as a result of ADCC activity of peripheral blood mononuclear cells (PBMC). PBMCs from healthy donors obtained by Ficoll–Paque separation (GE HealthCare, Uppsala, Sweden) were used as effector cells, while cancer cells (target) were previously seeded and stimulated with doxorubicin for 7 days. Cancer cells (5,000 to 10,000 per well) and PMBCs were co-incubated at 1:10 target:effector ratio in 100 µL DMEM containing 5% heat-inactivated FBS in a 96-well U-bottomed plate in quadruplicate for six hours at 37°C with or without 20 µg/ml trastuzumab. ADCC was measured in a LDH release assay following manufacturer's instructions (CytoTox Non-Radioactive Cytotoxicity Assay, Promega Corporation, Madison, WI, USA). The negative control sample (target spontaneous) was prepared identically, contained trastuzumab, but did not contain PBMCs; effector spontaneous sample contained no target cells. Tumor cells killed by freezing at -80°C for one hour and then warmed up to 37°C served as positive control (target maximum).

Supplementary Figures



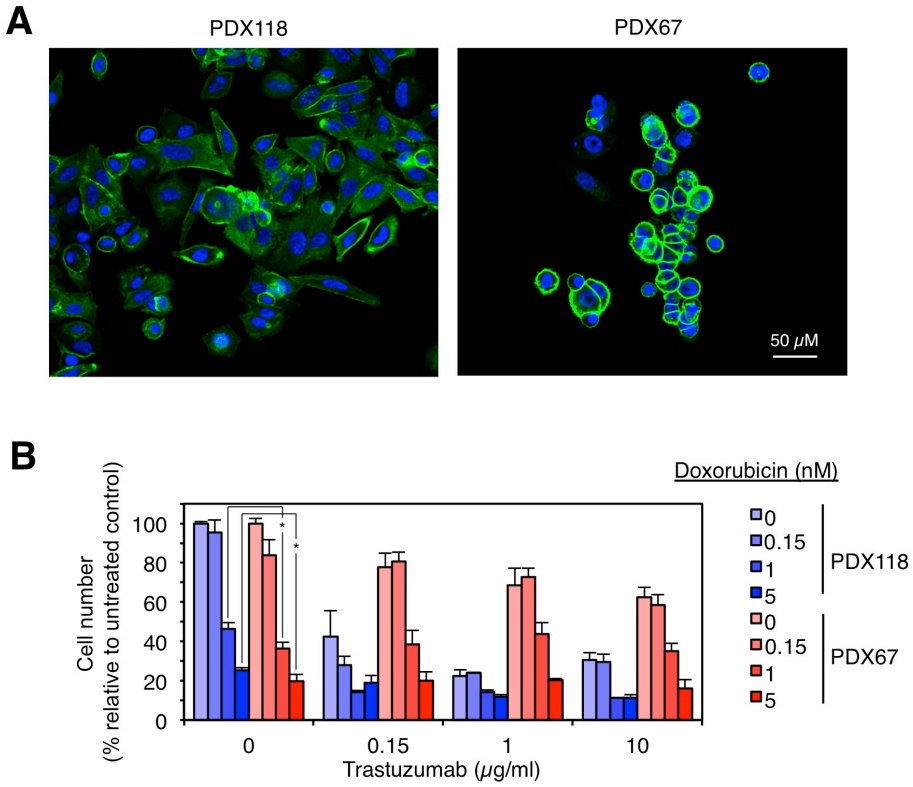
Supplementary Figure 1. Effect of doxorubicin in breast cancer cell lines expressing different levels of p95HER2/611CTF

A) The indicated cell lines and selected breast cancer samples expressing different levels of p95HER2/611CTF were lysed and the cell lysates analyzed by Western blot.

B), C) The same cell lines and tumor samples as in A were analyzed by immunohistochemistry with the indicated antibodies.

D) The indicated cell lines were cultured and counted at different time points. Averages of three independent experiments are shown, each containing triplicates.

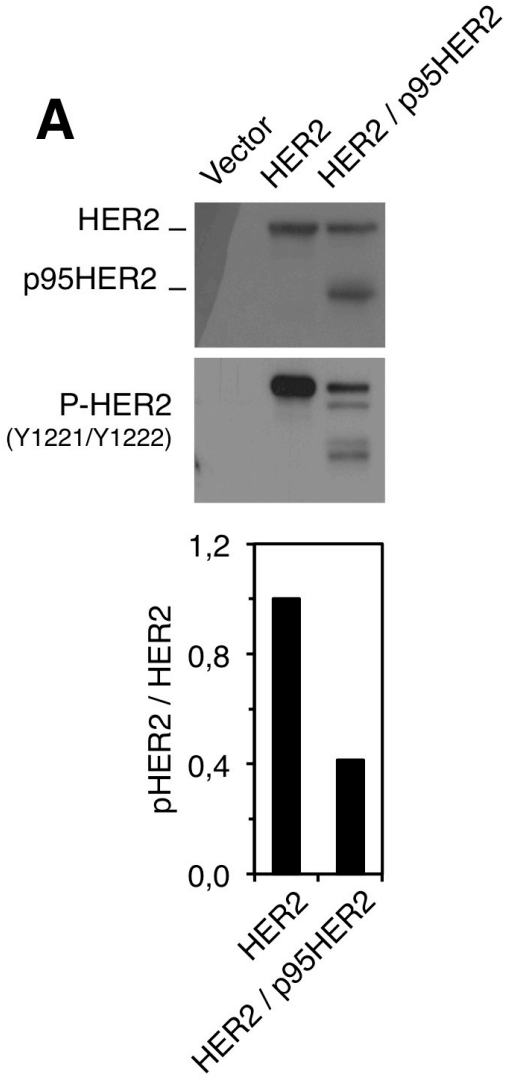
E) The same cell lines were treated with different concentrations of doxorubicin. Cell number was estimated with the crystal violet assay. Averages and 95% confidence intervals of three independent experiments are shown. **P* < .05, ***P* < .01 and ****P* < .001 using the two-sided Student *t* test.



Supplementary Figure 2. Characterization of cell cultures established from PDXs and sensitivity to different combinations of trastuzumab and doxorubicin.

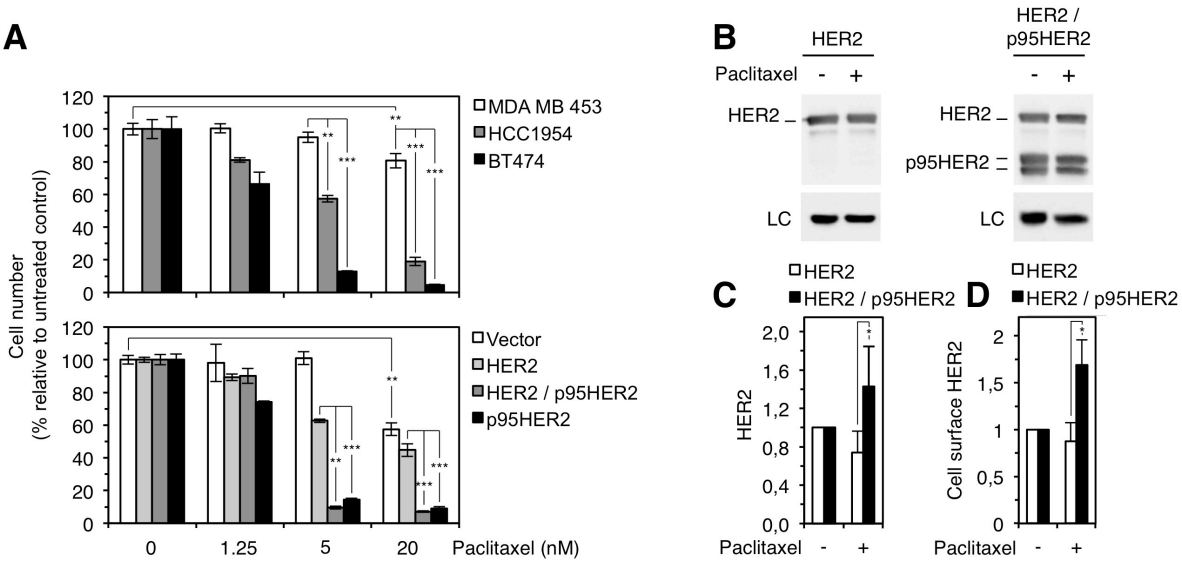
A) Cell cultures from the indicated PDXs were analyzed by indirect immunofluorescence with monoclonal antibodies against the extracellular domain of HER2.

B) The same cell cultures as in A were treated with different concentrations of doxorubicin and trastuzumab. Cell number was estimated with the crystal violet assay. Averages and 95% confidence intervals of three independent experiments are shown, each containing triplicates. $P < .05$ using the two-sided Student *t* test.



Supplementary Figure 3.

A) 67NR cells stably transduced with an empty viral vector or the same vector encoding HER2 or p95HER2/611CTF, individually or together, were lysed and the cell lysates analyzed by Western blot. The bands corresponding to HER2 and p95HER2/611CTF are indicated in the upper panel. The signals corresponding to HER2 or phospho-HER2 were quantified.



Supplementary Figure 4.

Effect of paclitaxel in breast cancer cell lines expressing different levels of p95HER2/611CTF

A) The indicated cell lines were treated with different concentrations of paclitaxel. Cell number was estimated with the crystal violet assay. Averages and 95% confidence intervals of three independent experiments are shown, each containing triplicates. ***P* < .01 and ****P* < .001 (two-sided Student's *t* test).

B) MCF10A cells expressing HER2 or HER2 and p95HER2/611CTF were treated with 2.5 nM paclitaxel for one week, lysed and cell lysates analyzed by Western blot. Results are representative of three independent experiments.

C) The signals of Fig. 4A corresponding to full-length HER2 were quantified. Averages and 95% confidence intervals of three independent experiments are shown. **P* < 0.05 using the two-sided Student *t* test.

D) The same cells as in B were treated were treated with 2.5 nM paclitaxel for one week and analyzed by flow cytometry with antibodies against the extracellular domain of HER2. Averages and 95% confidence intervals of three independent experiments are shown. $*P < 0.05$ using the two-sided Student *t* test.