Supplementary Information

Identification of prolidase as a high affinity ligand of the ErbB2 receptor and its regulation of ErbB2 signaling and cell growth

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Supplementary Materials and Methods

Reagents

Recombinant human EGF (236-EG-200) and human NRG-1 (5218) were purchased from R&D Systems and Cell Signaling, respectively. Trastuzumab (Genentech) was obtained from Roswell Park Cancer Institute Pharmacy. APMA, crystal violet, methylthiazolyldiphenyl-tetrazolium bromide (MTT) and vanadate were from Sigma-Aldrich. The following antibodies were used: anti-PEPD (Abcam, ab86507), anti-PTEN (Santa Cruz, sc-7974), anti-p-PTEN (Santa Cruz, sc-101789), anti-p-Tyr (PY99) (Santa Cruz, sc-7020), anti-CK2α (Santa Cruz, sc-12738), anti-ECD of ErbB2 (Santa Cruz, sc-134481), anti-ErbB3 (Santa Cruz, sc-285), anti-TGFα (Santa Cruz, sc-9043), anti-ErbB2 (Cell Signaling, 2165), anti-p-ErbB2 (Y1196) (Cell Signaling, 6942), anti-p-ErbB2 (Y1221/1222) (Cell Signaling, 2243), anti-ErbB1 (Cell Signaling, 2232), anti-ErbB4 (Cell Signaling, 4795), anti-PI3K p85 (Cell Signaling, 4257), anti-p-Src (Cell Signaling, 6943), anti-Src (Cell Signaling, 2123), anti-AKT (Cell Signaling, 4691), anti-p-AKT (Cell Signaling, 4060), anti-ERK (Cell Signaling, 9102), anti-p-ERK (Cell Signaling, 9101), anti-ubiquitin (Santa Cruz, sc-8017), anti-GAPDH (Millipore, MAB374), anti-human IgG_1 for detection of Fc (Santa Cruz, sc-2453), FITC-conjugated anti-His-tag (Abcam, ab1206), biotin-conjugated anti-His-tag (Bethyl, A190-113B), and TRITC-conjugated goat-anti-rabbit (Jackson, 111-025-003). HRP-conjugated streptavidin (N100) was from Thermo Scientific.

Plasmid construction

Construction of plasmids for expressing the wild-type PEPD (pBAD/TOPO-PEPD-His) and its G278D mutant (pBAD/TOPO-PEPD/G278D-His) and expression of the proteins in bacteria, expressed without the N-terminal Thio, were described in our recent publication (ref. 10). All other PEPD mutants were constructed using the pBAD/TOPO-PEPD-His as the template and the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), which include the N-terminal 184 amino acids fragment (pBAD/TOPO-PEPD/R184X-His), the N-terminal 265 amino acids fragment (pBAD/TOPO-PEPD/R265X-His), and the C-terminal 265 amino acids fragment (pBAD/TOPO-PEPD/X265R-His). All primers were designed with the QuikChange primer design software and are listed in Supplementary Table S1. All constructs were sequenced to ensure the correct deletion.

To construct pCMV6-XL5-ERBB2 which expresses human ErbB2, the full length human ERBB2 coding sequence was amplified by PCR from the LNCaP cDNA using NotI-forward primer (5'- ATAAGAAT**GCGGCCGC**AGCTGAGATTCCCCTCCATT-3') and NotI-reverse primer

(5'- ATAGTTTA**GCGGCCGC**CTTGATGCCAGCAGAAGTCA-3'). Amplified PCR products were digested by NotI (New England BioLabs), followed by ligation into pCMV6-XL5 (Origene) which was pre-digested with the same restriction enzyme. The orientation of the insert was determined by colony PCR using forward primer 5'- CAAATGGGCGGTAGGCGTGTA-3' (localized to the plasmid) and reverse primer 5'- ATTGGTGGGCAGGTAGGTGAGTTC-3' (annealed to the beginning of the insert). The construct was sequenced to confirm the integrity of the entire coding sequence. All site-directed mutations and deletions in the ERBB2 gene were generated by the QuikChange Lightning Site-Directed Mutagenesis Kit. These constructs include ErbB2 that carries K753M mutation (pCMV6-XL5-ERBB2/K753M), deletion of ErbB2 ECD subdomain 1 (pCMV6-XL5-ERBB2/delD1, deletion of amino acids 1-195), deletion of ErbB2 ECD subdomain 2 (pCMV6-XL5-ERBB2/delD2, deletion of amino acids 196-320), deletion of ErbB2 ECD subdomain 3 (pCMV6-XL5-ERBB2/delD3, deletion of amino acids 321-488), and deletion of ErbB2 ECD subdomain 4 (pCMV6-XL5-ERBB2/delD4, deletion of amino acids 489-560). All primers were designed with the QuikChange primer design software and are listed in Supplementary Table S1. All constructs were sequenced to ensure the correct mutation/deletion.

Cell lines and cell culture

Human breast cancer MCF-7 cells and human bladder cancer RT-4 cells, from ATCC, were also used in the study. MCF-7 cells and RT-4 cells were cultured in high-glucose DMEM plus 10% FBS and McCoy'5A medium plus 10% FBS, respectively, in humidified incubators at 37^{o}C with 5% CO_2.

Western blot analysis

Non-reducing gel electrophoresis was used to determine, besides the wild type human PEPD, whether any of its mutants, including G278D-PEPD, R184X-PEPD, R265X-PEPD, and X265R-PEPD, existed as a homodimer. Briefly, each protein sample was mixed with non-reducing sample buffer (sample loading buffer without β-mercaptoethanol) and then resolved by 10% SDS-PAGE, before silver staining using a kit (LC 6070) from Invitrogen.

Immunoprecipitation

For detection of direct and specific binding of PEPD or NRG-1 to ErbB2, ErbB3 or ErbB4, recombinant human PEPD or NRG-1 was incubated with recombinant human ErbB2/ECD-Fc, recombinant human ErbB3/ECD-Fc, recombinant human ErbB4/ECD-Fc or recombinant human

Fc in 0.4 ml binding buffer. All solutions were incubated for 2 h at 37^{o}C, followed by incubation with protein G-sepharose beads for 1 h at room temperature. The beads were washed with IP washing buffer and then subjected to western blot analysis. In experiments using cell lysates, cells were lysed in M-PER Buffer (Thermo Scientific) supplemented with a proteinase inhibitor mix (Roche Applied Science). Cell lysates (0.5 mg of total protein in 0.5 ml binding buffer) were incubated with an antibody overnight at 4^{o}C. The immunocomplexes were pulled down by protein G-agarose (1 h incubation at room temperature). The beads were washed with IP washing buffer and then subjected to western blot analysis. In experiments involving the use of pervanadate, it was prepared fresh by incubating 10 mM vanadate with 10 mM hydrogen peroxide for 15 min at room temperature, followed by addition of catalase (Sigma) at final concentration of 0.2 mg/ml to remove residual hydrogen peroxide, as previously described (ref. 23).

ELISA-based ErbB2-PEPD binding analysis

To measure PEPD binding to human ErbB2 or its deletion mutants, ELISA plates were coated overnight at 4^{o}C with 100 μl/well of an ErbB2 antibody (binding to the cytoplasmic tail of ErbB2) at 10 μg/ml. After washing the wells three times with PBST, residual protein binding sites in the wells were blocked by incubation for 2 h at room temperature with 300 μl/well of 1% BSA in PBS. Following addition of 60 μl of serially diluted recombinant human PEPD into each well, 60 μl of cell lysates (prepared from CHO-K1 cells transfected with the empty vector for 24 h and CHO-K1 cells transfected with wild-type ErbB2 for 24 h), containing 25 μg of total protein per sample (note: a preliminary experiment using up to 250 μg of total protein per sample showed similar outcome of PEPD binding to ErbB2), were added to each well and incubated at 37^{o}C for 2 h. After three washes with PBST, 100 μl of a biotin-conjugated anti-His antibody (1:10,000 dilution; note that PEPD is His-tagged) was added to each well and incubated for 2 h at room temperature. After another round of washing with PBST, 100 μl of streptavidin-conjugated HRP (1:10,000 dilution) was added to each well and incubated for 45 min at room temperature. After another round of washing with PBST, 100 μl/well of 1x substrate solution (3,3'5,5'-tetramethylbenzedine) was added, and after adequate color development, 100 μl/well of stop solution (1 N H_2SO_4) was added and absorbance reading at 450 nm was recorded. In experiments comparing PEPD binding to wild-type ErbB2 and its deletion mutants (deletion of subdomains 1, 2, 3 or 4 in the ErbB2 ECD), an equal amounts of wild-type ErbB2 protein and its mutants were used. The lysates of cells transfected with the plasmid expressing each protein (for 24 h) were first subjected to western blot analysis, followed by densitometry measurement

of the specific protein bands normalized to a loading control, to calculate the amount of lysates that deliver the same amount of each protein (25 μg of total protein/sample were used for lysates carrying the wild-type ErbB2).

RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and after treatment with TURBO DNase to remove potential genomic DNA contamination, 500 ng RNA per sample was reverse transcribed into cDNA in 25 μl reaction using the TaqMan Reverse Transcription Reagents (Invitrogen). The RT reaction was performed at 25^{o}C for 10 min, followed by heating at 48^{o}C for 30 min, and then 95^{o}C for 5 min. Each PCR amplification was carried out in 20 μl volume, containing 10 μl GoTaq Master Mix (2x) (Promega), 0.5-1 μl of the reverse-transcribed mixture (cDNA), 0.25 μM each of specific forward and reverse primers. The primers are as follows: human ERBB2, forward, 5'- CTGTTTGCCGTGCCACCCTGAGT-3', reverse, 5'-CTTCTGCTGCCGTCGCTTGATGAG-3'; human GAPDH, forward, 5'-CCAGGGCTGCTTTTAACTC-3', reverse, 5'- GCTCCCCCCTGCAAATGA-3'; Chinese hamster GAPDH, forward, 5'-TGGAATCTACTGGCGTCTTC-3', reverse, 5'-CACCACCTTCTTGATGTCCT-3'. The PCR conditions used for all reactions are as follows: 94^{o}C for 3 min, 28 cycles (human ERBB2)/25 cycles (GAPDH) at 94^{o}C (denaturation) for 30 sec, 63^{o}C (human ERBB2)/60^{o}C (human GAPDH)/56^{o}C (Chinese hamster GAPDH) for 30 sec (annealing), and 72^{o}C for 30 sec (extension); the final extension was performed at 72^{o}C for 5 min. The PCR products were analyzed by electrophoresis with 1% agarose gel, stained by ethidium bromide, and visualized under UV light.

BrdU assay

BrdU incorporation into DNA was measured using the FITC BrdU Flow Kit (BD Pharmingen). Briefly, cells were grown in 6-well plates (0.15x10^{6} cells/well for CHO-K1 cells and CHO-K1/ErbB2 cells, 0.4x10^{6} cells/well for BT-474 cells; 2 ml medium/well) overnight, treated with vehicle or PEPD for 48 h, and then incubated with BrdU at 10 μM in culture medium for 30 min (CHO-K1 cells and CHO-K1/ErbB2 cells) or 18 h (BT-474 cells). The cells were then harvested, fixed and permeabilized in fixation/permeabilization buffer, treated with NDase for 1 h at 37^{o}C, and incubated with a BrdU antibody for 20 min at room temperature, followed by DNA staining with 7-amino-actinomycin D. The stained cells were resuspended in 0.5-1 ml of staining buffer per sample and analyzed by a flow cytometer (BD FACS Calibur, BD Biosciences), counting 10,000 cells per sample. BrdU incorporation was modeled using the WinMDI 2.8 software.

Soft agar colony formation assay

After 1 ml of 0.8% ultrapure Noble Agar (USB, cat#10907) in culture medium was solidified in each well of 6-well plates, 1 ml of cells ($2x10^4$ BT474 cells, $1x10^3$ CHO-K1 cells or $1x10^3$ CHO-K1/ErbB2 cells) suspended in 0.4% agar in culture medium at 37°C were added to each well, which also solidified afterwards. PEPD or vehicle was then added in 2 ml of medium to each well, which was changed every 3-4 days for a total of 21 days. At the end of this treatment, cell colonies of ≥100 μm in diameter were counted under a dissection microscope (Axiovert 40 CFL, Carl Zeiss) in 10 different fields (10x magnification) per well, aided by ImageJ.

Cell invasion and migration assay

Cell invasion and migration were measured using the BD BioCoat Matrigel Invasion Chambers (BD Biosciences). Briefly, the lower chamber was filled with 0.75 ml medium with 10% FBS, and the upper chamber was placed with $4x10^4$ cells suspended in 0.5 ml of serum-free medium containing vehicle or PEPD. The chambers were placed in a cell culture incubator at 37^{o}C for 48 h. At the end of the incubation, the cells that invaded through a Matrigel matrix layer coated on the filter insert which was placed at the bottom of the invasion chamber were fixed with 100% methanol, stained with 0.5% crystal violet, and counted under a microscope (Eclipse 50i) in 10 different fields (20x magnification) per filter, aided by ImageJ.

PI3 kinase assay

A PI3-Kinase Activity ELISA Kit (Echelon, K-1000s) was used. Briefly, PI3K was pulled down from whole cell lysates (prepared from approximately $1x10^6$ cells per sample) using an PI3K antibody (anti-PI3K p85), and the immunoprecipitates were mixed with 30 μl of KBZ reaction buffer, which was then mixed with 30 μl of 10 μM PI(4,5)P_2 substrate, followed by incubation for 2 h at 37^{o}C. The kinase reaction was terminated by adding 90 μl of kinase stop solution to each reaction solution, and 60 μl of each stopped kinase reaction solution was transferred together with 60 μl of PIP_3 detector to each well in the incubation plate. After incubation at room temperature for 60 min, 100 μl per sample from the incubation plate was transferred to the corresponding wells of the detection plate and incubated for 60 min at room temperature. The plates were washed with TBST and then incubated with the HRP-conjugated secondary detector for 30 min, followed by washing with TBST, and the immobilized HRP was measured by a standard colorimetric assay, using 3,3',5,5'-tetramethylbenzedine as a substrate.

Src kinase assay

Src activity in cell lysates was measured using the Universal Tyrosine Kinase Assay Kit (TaKaRa, #MK410). Briefly, lysates (prepared from approximately $1x10^6$ cells per sample) were pre-cleared with protein A-agarose beads prior to IP with a Src antibody. The immunoprecipitates were washed and incubated with 10 mM β-mercaptoethanol in 150 µl of kinase reacting solution. Each sample (40 µl) was mixed with 10 µl of 40 mM ATP-2Na solution, which was transferred to microtiter plate wells coated with a PTK substrate, followed by incubation at 37°C for 30 min. After wash with TBST, an HRP-conjugated anti-phosphotyrosine (PY20) solution was added to each well and incubated for 30 min at 37°C. After another round of wash with TBST, the immobilized HRP was measured by a standard colorimetric assay, using 3,3',5,5'-tetramethylbenzedine as a substrate.

MTT cell proliferation assay

Cells were grown in 96-well plates (500 CHO-K1 cells or CHO-K1/ErbB2 cells per well, 2,000 BT-474 cells per well; 150 µl medium per well) overnight and then treated with vehicle, PEPD or trastuzumab in 200 µl medium per well for 72 h, followed by incubation with MTT (9.2 mM in medium) at 37°C for 3 h. The cells were then washed with PBS and mixed with dimethyl sulfoxide (150 µl per well), and the cell density was determined by measuring the reduction of MTT to formazan spectroscopically at 570 nm.

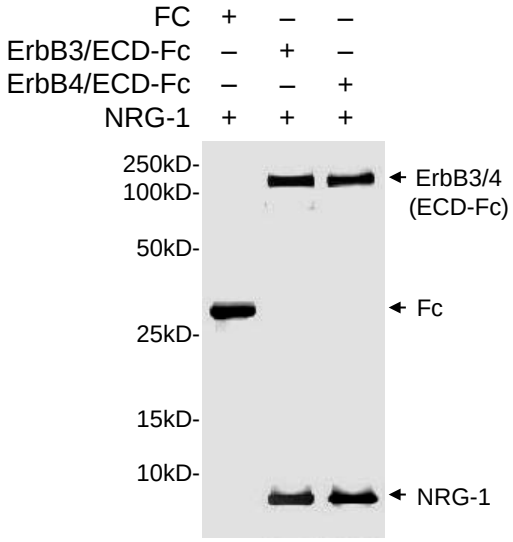

Figure S1. Binding of NRG-1 to the ECDs of ErbB3 and ErbB4. NRG-1 at 0.2 μM was incubated with Fc (0.04 μM), ErbB3/ECD-Fc (0.04 μM) or ErbB4/ECD-Fc (0.04 μM), pulled down with protein G-sepharose, separated by SDS-PAGE (15%), and stained with silver.

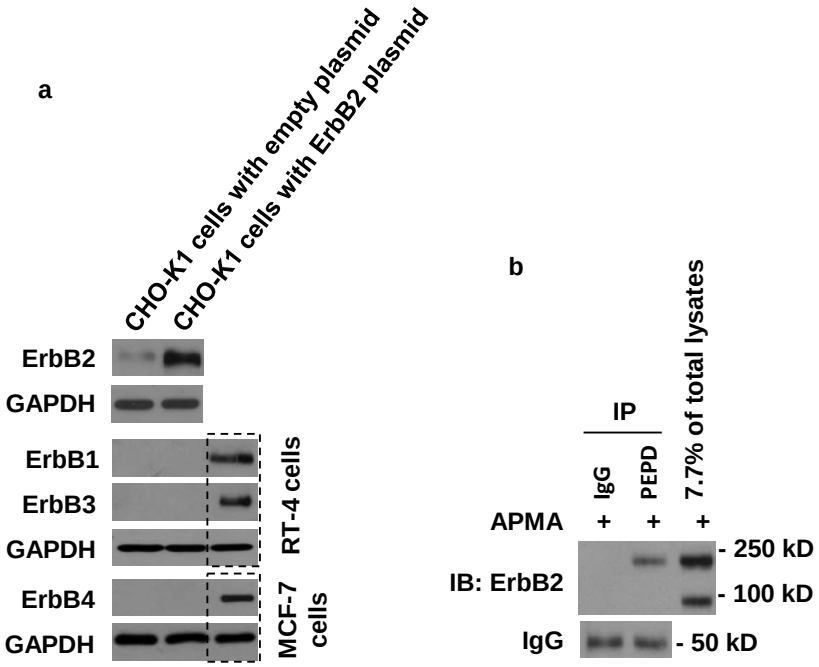

Figure S2. Validation of the main cell line used in the present study, and lack of binding of PEPD to the transmembrane and intracellular regions of human ErbB2. (**a**) CHO-K1 cells were transiently transfected with an ErbB2-expressing plasmid (pCMV6-XL5-ERBB2) or the empty plasmid for 24 h. Human bladder cancer RT-4 cells and human breast cancer MCF-7 cells were untreated and were used as positive controls for ErbB1, ErbB3 and ErbB4. Cell lysates were analyzed by western blotting. GAPDH is a loading control. (**b**) CHO-K1 cells stably expressing ErbB2 (CHO-K1/ErbB2 cells) were treated with 1 mM APMA for 0.25 h. Cell lysates were then prepared and analyzed by western blotting (the first lane on the right). The same cell lysates were mixed with 1 μM PEPD, which were then incubated with a PEPD antibody or an isotype-matched IgG, pulled down with protein G-agarose, and analyzed by western blotting. The result shows that APMA causes ErbB2 ECD cleavage, as expected, generating the p95 fragment (minus ECD), but PEPD only co-precipitates with the intact ErbB2, not the p95 fragment, indicating that PEPD does not bind to the trans-membrane or intracellular regions of ErbB2.

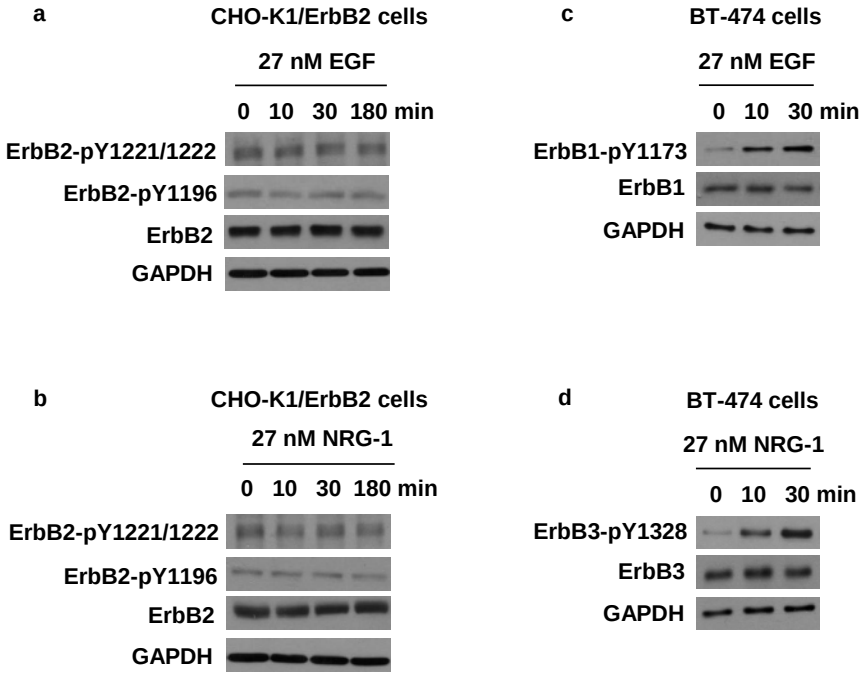

Figure S3. No effects of EGF and NRG-1 on ErbB2. (**a, b**) CHO-K1 cells stably overexpressing human ErbB2 (CHO-K1/ErbB2 cells) were treated with recombinant EGF or recombinant NRG-1. Cells lysates were then prepared and analyzed by western blotting. (**c, d**) In order to ensure that both EGF (ligand of ErbB1) and NRG-1 (ligand of ErbB3 and ErbB4) that were used in our experiments were bioactive, they were evaluated in BT-474 cells, which expressed relatively low levels of ErbB1 and ErbB3. As expected, EGF significantly stimulated ErbB1 phosphorylation, and NRG-1 significantly stimulated ErbB3 phosphorylation.

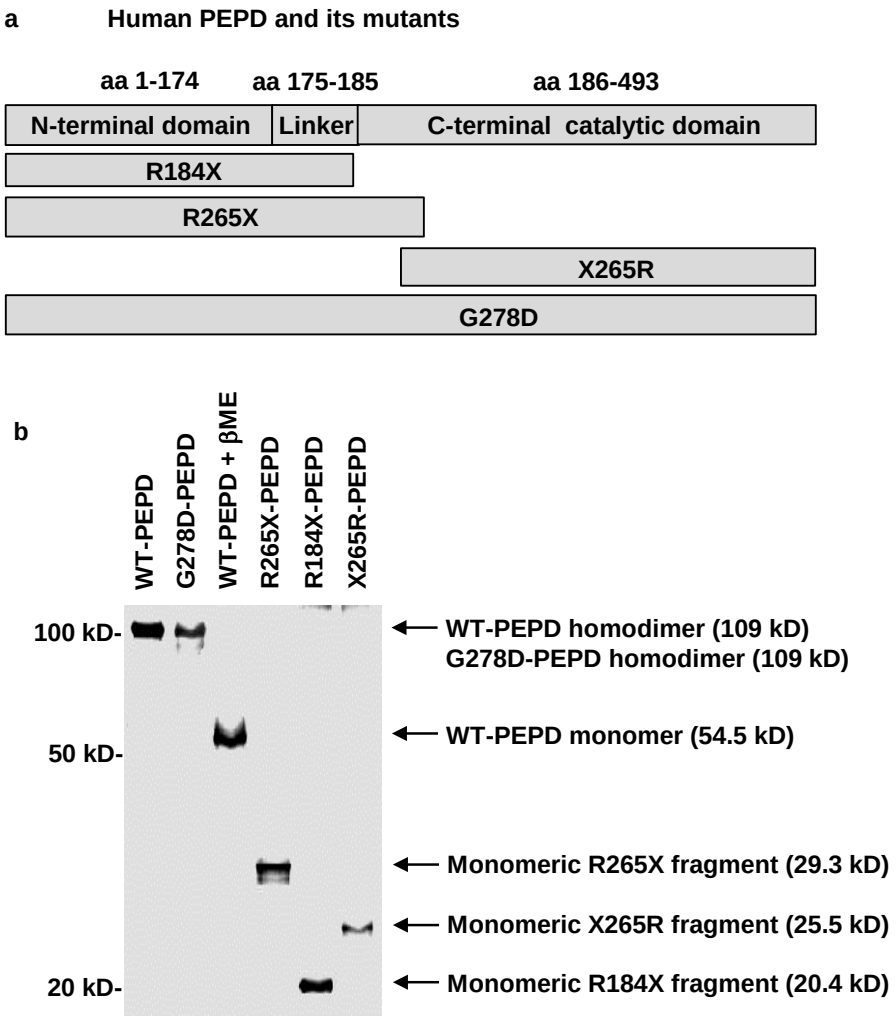

Figure S4. Human PEPD and its mutants. (**a**) Wild-type human PEPD and amino acid (aa) changes in its mutants. (**b**) Recombinant wild-type human PEPD and its mutants were generated in bacteria, purified using nickel affinity chromatography and analyzed by non-reducing SDS-PAGE and silver staining. Note: Protein loading varied across the lanes. In the lane indicated by "WT-PEPD + βME", the wild-type PEPD was incubated with 10% β-mercaptoethanol in PBS before non-reducing gel electrophoresis.

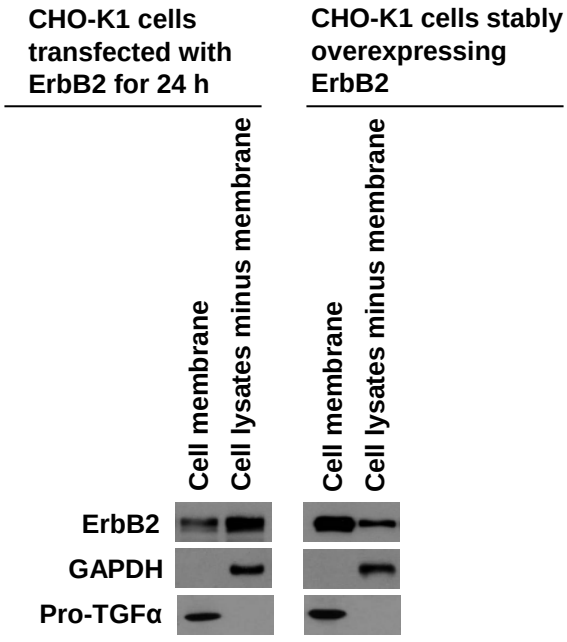

Figure S5. ErbB2 distribution in cells. Membrane fraction and lysates minus membrane were prepared from CHO-K1 cells that either stably overexpressed human ErbB2 (CHO-K1/ErbB2 cells) or were transiently transfected with ErbB2 (pCMV6-XL5-ERBB2) for 24 h, and were analyzed by western blotting. GAPDH and pro-TGF-α were used as loading controls.

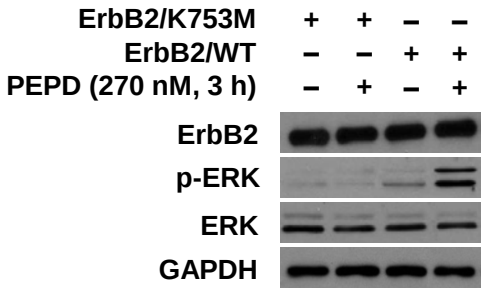

Figure S6. Induction of ERK phosphorylation by PEPD in CHO-K1 cells depends on ErbB2. CHO-K1 cells were transiently transfected with wild-type human ErbB2 or a kinase-dead mutant (ErbB2/K753M) for 24 h and then treated with PEPD or vehicle, followed by western blotting.

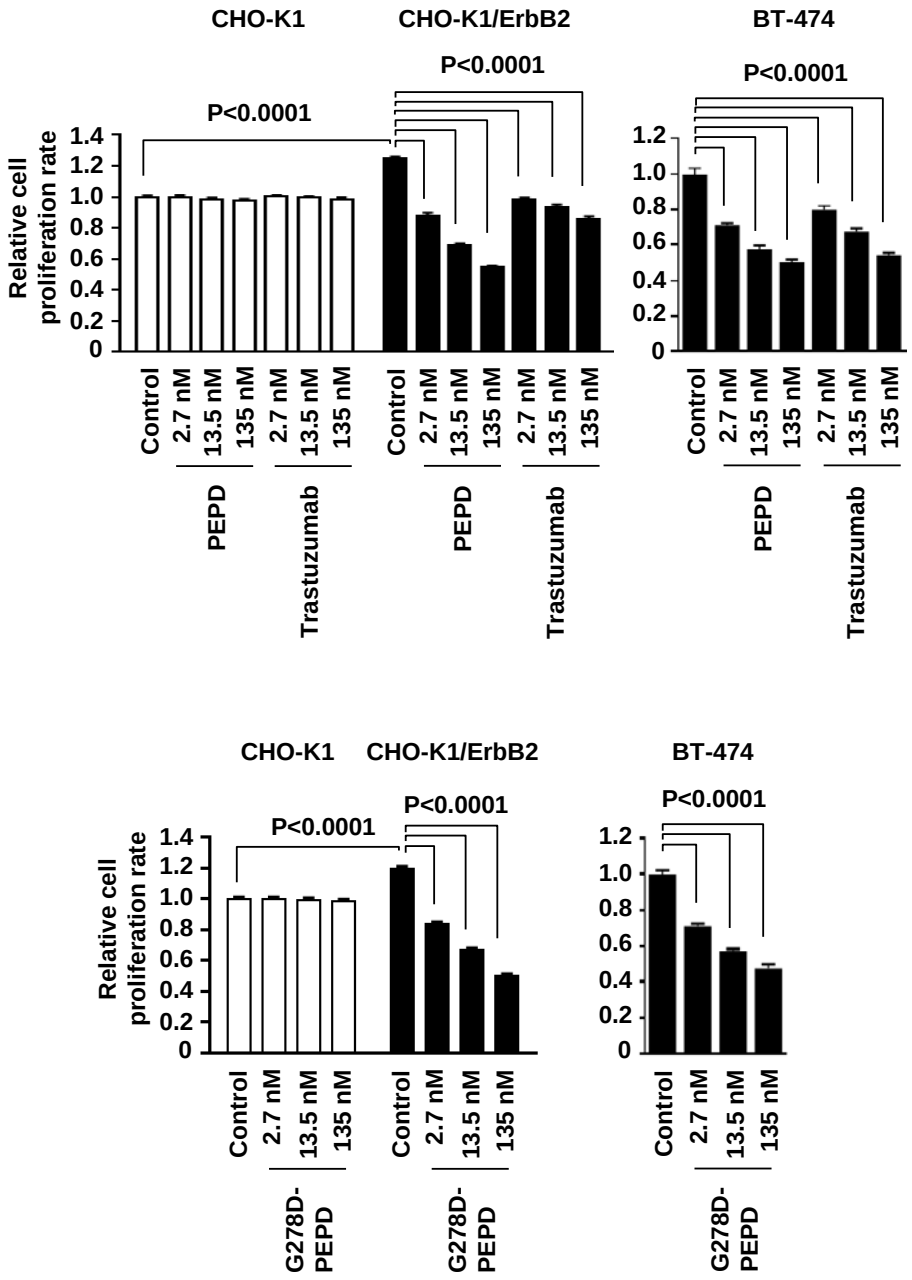

Figure S7. The growth-inhibitory effects of PEPD, G278D-PEPD and trastuzumab on cells with or without ErbB2 overexpression. Cells were grown in 96-well plates (500 CHO-K1 cells or CHO-K1/ErbB2 cells per well or 2,000 BT-474 cells per well; 150 μl medium per well) overnight and then treated with vehicle, PEPD, G278D-PEPD or trastuzumab in 200 μl medium per well for 72 h, followed by incubation with MTT (9.2 mM in medium) at 37°C for 3 h. After removing the medium, the cells were treated with dimethyl sulfoxide (150 μl per well), and the cell density was determined by measuring formazan formed from MTT spectroscopically at 570 nm (n=3). The error bars indicate SD.

Table S1. The sequences of primers used for site-directed mutations and deletions.

Target vector	Primer	Sequence
pBad/Topo-PEPD/R184X-His	For	5’-gatcgttgagtgccgaaagggcgagcttgaag-3’
	Rev	5’-cttcaagctcgccctttcggcactcaacgatc-3’
pBad/Topo-PEPD/R265X-His	For	5’-gctcccaacgaccgaaagggcgagcttgaa-3’
	Rev	5’-ttcaagctcgccctttcggtcgttgggagc-3’
pBad/Topo-PEPD/X265R-His	For	5’-gtgccgggcgaacatgcgaacgatccagaatg-3’
	Rev	5’-cattctggatcgttcgcatgttcgcccggcac-3’
pCMV6-XL5-ERBB2-K753M	For	5’-gaaaattccagtggccatcatggtgttgagggaaaacacatc-3’
	Rev	5’-gatgtgttttccctcaacaccatgatggccactggaattttc-3’
pCMV6-XL5-ERBB2/delD1	For	5’-cgcagtgagcaccatgtctccgatgtgtaagg-3’
	Rev	5’-ccttacacatcggagacatggtgctcactgcg-3’
pCMV6-XL5-ERBB2/delD2	For	5’-tctgctgtcacctcacaggggtggcagg-3’
	Rev	5’-cctgccacccctgtgaggtgacagcaga-3’
pCMV6-XL5-ERBB2/delD3	For	5’-tgccccctgcacaaccaaccgcaccaag-3’
	Rev	5’-cttggtgcggttggttgtgcaggggca-3’
pCMV6-XL5-ERBB2/delD4	For	5’-cagctctttcggaacttgccgtgccaccct-3’
	Rev	5’-agggtggcacggcaagttccgaaagagctg-3’