**Supplemental Materials and Methods**

**Cell Culture and Reagents**

BT474 Sensitive and Resistant cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fischer Scientific, Waltham, MA, USA). MDA-MB-453 and HCC1954 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). Mouse fibroblast: LTK-parental and LTK-Jagged-1 cells (a kind gift from Dr. Geraldine Weinmaster) were maintained in Dulbecco's Modified Eagle Medium (DMEM). All cells were tested for mycoplasma contamination within the last three months. All cells were maintained in medium supplemented with 100μM non-essential amino acid (Invitrogen, Carlsbad, CA, USA), 1% L-glutamine (2 mM, Thermo Fischer Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA, USA) and incubated at 37oC with 95% O2 and 5% CO2.

**Confocal Immunofluorescence**

Notch-1 staining was performed using 1μg/ml of the rabbit anti-human Notch-1 C-20 antibody (Cat. SC-6014, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and 5μg/ml of AlexaFluor 555 labelled anti-rabbit IgG (Cat. A31572, Life Technologies, Grand Island, NY, USA). Staining for Jagged-1 was performed using 4μg/ml of goat anti-human Jagged 1 C-20 antibody (Cat. SC-6011, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), 5μg/ml of AlexaFluor 488 labelled anti-goat IgG (Cat. A11055, Life Technologies, Grand Island, NY, USA). Staining for Early Endosomal Antigen-1 (EEA1) was performed using 1μg/ml of EEA1 (Cat. 07292MI, Upstate Biotechnology, Charlottesville, VA, USA) and 20μg/ml of AlexaFluor 555 labelled anti-rabbit IgG (Cat. A31572, Life Technologies, Grand Island, NY, USA). Staining for PKCα was performed using 1μg/ml of PKCα (Cat. SC-208 Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and 5μg/ml of AlexaFluor 555 labelled anti-rabbit IgG (Cat. A31572, Life Technologies, Grand Island, NY, USA). VectaShield mounting media containing DAPI (Cat. H1200, Vector Laboratories, Burlingame, CA, USA) was used to mount the slides after staining for specific protein was completed. Jagged-1 (green), EEA1 (red), Notch-1 (red), PKCα (red), nucleus (blue) were detected with a Zeiss LSM-510 confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

**Chemicals**

N-Ethylmaleimide (NEM) (Sigma Aldrich, St. Louis, MO, USA), Hepes (Fisher Scientific, Waltham, MA, USA), Sodium Chloride (NaCl) (Fisher Scientific, Waltham, MA, USA), Disodium dihydrogen Ethylenediaminetetraacetate (EDTA) (Fisher Scientific, Waltham, MA, USA), Phenylmethylsulfonyl Fluoride (PMSF) (Fisher Scientific, Waltham, MA, USA), Complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Basel, Switzerland), Triton-X 100 (Sigma Aldrich, St. Louis, MO), Sodium Fluoride (NAF) (Fisher Scientific, Waltham, MA, USA), Sodium Vanadate (NaVO4) (Fisher Scientific, Waltham, MA, USA), 2X Laemmli Sample Buffer (Bio-Rad, Hercules, CA), 4X Laemmli Sample Buffer (Bio-Rad, Hercules, CA), 2-Mercaptoethanol (Fisher Scientific, Waltham, MA, USA), Saponin (Sigma Aldrich, St. Louis, MO, USA), Bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA), Paraformaldahyde (Sigma Aldrich St. Louis, MO, USA), Dimethylsulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA).

**Drugs and Antibodies**

Trastuzumab was obtained from the pharmacy (Cat. 787237, Genentech, San Francisco, CA, USA). Trastuzumab was resuspended in sterile PBS to obtain a stock concentration of 22 mg/mL. For xenograft studies, 10 mg/Kg trastuzumab in a total volume of 100 L sterile PBS was intraperitoneally injected (i.p) into the mice once a week. For cell culture treatments, the working concentration of 10 or 20µg/mL was used. Lapatinib was purchased from Selleck Chemicals (Cat. S1028, Houston, TX, USA). For cell culture treatments, Lapatinib was dissolved in the solvent dimethylsulfoxide (DMSO) to obtain a stock concentration of 2 mM and was stored at -20oC. The working concentration used for the cell treatment was 2 µM. LY294002 (Cat. L-7962, LC laboratories, Woburn, MA, USA) was dissolved in DMSO to obtain a stock concentration of 20 mM and was stored at -20oC. The working concentration used for the cell treatment was 20 µM. U0126 (Cat. 9903S, Cell Signaling, Danvers, MA, USA) was dissolved in DMSO to obtain a stock concentration of 10 mM and was stored at -20oC. The working concentration used for the cell treatment was 10 µM. 17β- Εstradiol (Cat. E88775 Sigma Aldrich, St. Louis, MO, USA) capsules were prepared as previously described (29) and implanted in mice for xenograft studies. The CTX-033 Probody therapeutic is a prodrug form of an antibody that is engineered to remain inert in healthy tissues but activated by proteases specifically up-regulated in the tumor microenvironment (30-32). CTX-033 Probody is a recombinant, fully human IgG1 antibody with sub-nanomolar affinities for human and mouse Jagged-1/-2. It was engineered to include two additional components; 1) a masking peptide specific to the antigen-binding region, and 2) a substrate linker that is cleavable by several tumor-specific proteases such as UPA or MMP7/9. CTX-033 was kindly provided by Jason Sagert (CytoMx Therapeutics, Inc., San Francisco, CA). It was provided as 10 mg/ml stock in PBS. For xenograft studies, 5 mg/Kg CTX-033 in a total volume of 100 L sterile PBS was injected i.p into mice once a week.

**Expression Vectors**

Plasmid pcMV10 and pcMV10-Flag-Ub expression vectors were kindly provided by Dr. Adriano Marchese (Department of Pharmacology, Loyola University Chicago, Maywood, IL, USA). Wild type ErbB-2 and ErbB-2 kinase dead (K753M) was kindly provided by Dr. Dihua Yu (M.D. Anderson Cancer Center, Houston, TX) (33). Retroviral expression vectors, LRZS, LRZS-PKCα, and LRZS-PKCαΔ22-28 were kindly provided by Dr. Mitchell Denning (Department of Pathology, Loyola University Chicago, Maywood, IL, USA). Cells were transfected with expression constructs were using Lipofectamine 2000 according to the manufactures guidelines. Briefly, 6 g of DNA were transfected with 24 l of Lipofectamine 2000 in a 10 cm cell culture plates, or scaled up or down to adjust for smaller or larger surface area culture plates.

**RNA Interference and Transfection Reagents**

For siRNA transfection, cells were plated at 5x105 cells/ml in regular growth media and transfected with specific siRNA complexes using RNAiMAX (cat. 13778-150, Life Technologies, Grand Island, NY, USA) or Lipofectamine 2000 (cat. 11668-019, Life Technologies, Grand Island, NY, USA). The transfections were performed according to the manufactures guidelines. The scrambled control siRNA was purchased from Qiagen (Cat# 1027281). Jagged-1 (JAG1i-New) siRNA was purchased from Dharmacon (cat# M-0111060-02-0005). The second Jagged-1 siRNA (JAG1i-A) was purchased from Invitrogen (Cat# HSS176254). The Mib-1 siRNA was purchased from Dharmacon (Cat# L-014033-00-0005). The two ErbB-2 siRNAs were purchased from Origene (cat# SR301443-A and SR301443-B). The PKCα siRNA was purchased from Dharmacon (cat# L-003523-00-0005).

**Retroviral Transduction**

Cells were plated at a density whereby they were 70% confluent when transduction was performed. The LRZS-linker and LRZS-PKCα viral supernatant (provided by Dr. Mitchell Denning) was placed into the tissue culture vessel and incubated at 37oC for 2 hours. Viral supernatant was removed and fresh media was placed onto cells for 5 hours. Another aliquot of virus was placed onto cells for 2 additional hours. Viral supernatant was replaced with fresh media. The cells were allowed to proliferate for 2-3 days after transduction. At this time, PKCα protein expression was confirmed in cells by western blotting or immunofluorescence.

**Co-Immunoprecipitation (Co-IP)**

Upon completion of treatments, cells were lysed with lysis buffer (1% Triton-X , 50 mM Hepes NEM, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1mM PMSF, 1mM NaVO4, and 1 Complete mini protease inhibitor cocktail tablet per 10ml of lysis solution), scraped, and collected in 1.7 mL Eppendorf tubes. The collected samples were allowed to incubate on ice for 15 minutes. The lysates were sonicated and centrifuged at 14,000 rpm at 4oC for 20 minutes. The supernatant was removed and the concentration of total protein was ascertained using the BCA protein assay (Cat. 23225, Fisher Scientific, Waltham, MA, USA). Proteins that were co-immunoprecipitated as well as the total protein lysate were detected by western blotting as described in the immunoblotting section of these methods. Antibodies used for detection are also described in the immunoblotting section of these methods

**Antibodies and Immunoblotting**

Cells were lysed with lysis buffer and lysates were prepared as described in the immunoprecipitation section of these methods. Protein extracts and/or immunoprecipitated samples were resolved through 7% SDS-PAGE, transferred to PVDF membranes, and probed at 1:1000 (unless otherwise denoted) with antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) against Jagged-1 C-20 (Cat. SC-6011), Hey-1 C-20 (1:500) (Cat. sc-28746), Hes-1 H-140 (Cat. sc-25392), Notch-1 C-20 (Cat. SC-6014), Ub (P4D1) (Cat. SC-8017), AKT1 (C-20) (1:2000) (Cat. SC-1618), ErbB-2 (c-erbB2/HER-2/neu Ab-17 (clone e2-4001+3B5), Cat. MS-730-P, Thermoscientific, Waltham, MA, USA), Anti-phosphotyrosine ErbB-2 (Y1248, Cat. AF1768, R&D Systems, Minneapolis, MN, USA), Pan-phosphotyrosine (Cat. 610000, BD Transduction, Franklin Lakes, NJ, USA), Mib-1 (Cat. ab124929, Abcam, Cambridge, MA, USA), PKCα (Cat. Ab32122, Abcam, Cambridge, MA, USA), Flag M2 (Cat. F3165 Sigma Aldrich, St. Louis, MO, USA), DLL1 (Cat. 2588), phosphoSer-PKC substrate (Cat. 2261), p-AKT Ser473 (Cat. 9271), p-ERK1/2 (Cat. 4370), ERK1/2 (Cat. 4695) (Cell Signaling). β –Actin (Cat. A5441, Sigma Aldrich, St. Louis, MO, USA) was used at 1:5000 as a loading control for all Western blots. Secondary antibodies, donkey anti-mouse IgG-HRP (1:5000, Cat. SC-2314), donkey anti-rabbit IgG-HRP (1:2000, Cat. SC-2313), and donkey anti-goat IgG-HRP (1:2000, Cat. SC-2020) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

**Reverse Transcription Real-Time Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted from breast cancer cells using the RiboPure Kit (Cat. AM1924, Ambion, Inc., Grand Island, NY, USA) according to the manufacturer’s instructions. Total RNA was reverse transcribed to total cDNA using the TaqMan reverse transcription Kit (Cat. N8080234, Applied Biosystem., Grand Island, NY, USA) according to the manufacturer’s instructions. Real-time PCR was performed using cDNA as a template to detect relative transcript levels. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) or 60S ribosomal protein L13a (RPL13a) were used as loading controls. Real-time PCR was performed using the StepOnePlus™ System (Applied Biosystems) as previously described ([28](#_ENREF_28)). The sequences of primers are as follows:Jagged-1 forward primer: 5’-GACTCATCAGCCGTGTCTCA-3’, reverse primer: 5’-TGGGGAACACTCACACTCAA-3’; Notch-1: forward: 5’-ATCAACGCCGTAGATGACC-3' and reverse: 5'-TTGTTAGCCCCGTTCTTCAG-3'; Notch-4: forward: 5’-TGCAGGCATATGGGATGTAA-3’and reverse: 5’-CATCCCCACAGTGGAGTTCT-3’; Deltex-1: forward: 5’-CAGTTTCGCCAGGACACAG-3’and reverse 5’-GCAGATGTCCATATCGTAGGC-3’; Hes-1: forward: 5’-CGGACATTCTGGAAATGACA-3’and reverse: 5’-CATTGATCTGGGTCATGCAG-3’;Hey-1: forward: 5’-TCATTTGGAGTGTTGGTGGA-3’and reverse: 5’-CTCGCACACCATGATCACTT-3’; HRPT: 5’-ATGAACCAGGTTATGACCTTGAT-3’ and reverse: 5’-CCTGTTGACTGGTCATTACAATA-3’;RPL13a: forward: 5’-CATAGGAAGCTGGGAGCAAG-3’ and reverse: 5’-ACAAGATAGGGCCCTCCAAT-3’. Transcripts levels were calculated relative to control samples after normalization to a loading control using ΔΔCt.

**Human ErbB2 positive breast cancer Cohort**

Nottingham primary breast cancer series (Tenovus cohort) were prepared as a tissue microarray (TMA). All patients were mean 55 years old, stage (I–III) primary operable BC cases at presentation. Clinical and pathological information were available in this consecutive series regarding patients’ age, menopausal status, tumor characteristics in the form of tumor histological grade, tumor type, tumor size, lymph node stage, lymphovascular invasion and Nottingham Prognostic Index (NPI) ([30](#_ENREF_30), [31](#_ENREF_31)). This series is characterized by its uniform protocol in terms of patients’ management and their prospective assessment. Outcome data were collected on a prospective basis. These include breast cancer-specific survival (BCSS) defined as the time in months from the date of surgery to the BC-related death. Median age was 53; 62.4% of patients had tumors larger than 2 cm; Grade 1 (3.7%), Grade 2 (12.8%) and Grade 3 (82.6%); 97.2 % were ductal carcinomas (including mixed); Stage I (50.5%), Stage II (34.9%), Stage III (13.8%); Vascular invasion definite (33.9%), probable (66.1%); Survival status (alive 43.1%; deceased 48.6%, missing 8.3%).

**Immunohistochemical staining of Human ErbB-2 positive breast tumors**

Pilot experiments determined the best antibody concentration to minimize background. Sections were incubated with diluted primary antibodies and prepared in PBS containing 1.5% normal rabbit serum for one hour in a hydrated chamber at room temperature. TMA sections were incubated with 1μg/ml normal goat IgG (Santa Cruz Biotechnologies, CA), run in parallel with each stain, were used as negative controls. Following extensive washing, antigen-antibody complexes were detected using the Vectastain Elite ABC kit (Vector Laboratories, CA) according to the manufacturer’s protocol. Staining was performed with ImmPactTM DAB peroxidase substrate kit (Vector Laboratories, CA). Sections were then counterstained in Gill’s hematoxylin and dehydrated in ascending grades of ethanol before clearing in xylene and mounting under a coverslip using Cytoseal XYL. The levels of PKCα protein expression in each specimen were scored according to the extent (percent of stained cells) and intensity of staining. The score for the extent of the IHC stained area was scaled as 0 for no IHC signal at all and 1 for 10–80 of tumor cells stained. Stained slides were sent to Nottingham, England where they were scanned. High-resolution images were uploaded to the Nottingham web-accessible scoring site. High resolution images were scored by 2 independent investigators (He Zhu and Lucio Miele). Intensity scores (0-1), percent staining scores and H-Scores were uploaded as Excel spreadsheets and survival analysis was performed using SPSS.