PKC\(\alpha\) Attenuates Jagged-1–Mediated Notch Signaling in ErbB-2–Positive Breast Cancer to Reverse Trastuzumab Resistance

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Abstract

**Purpose:** Breast cancer is the second leading cause of cancer mortality among women worldwide. The major problem with current treatments is tumor recurrence, progression, and disease progression. ErbB-2–positive breast tumors are aggressive and often become resistant to trastuzumab or lapatinib. We showed previously that Notch-1 is required for trastuzumab resistance in ErbB-2–positive breast cancer.

**Experimental Design:** Here, we sought to elucidate mechanisms by which ErbB-2 attenuates Notch signaling and how this is reversed by trastuzumab or lapatinib.

**Results:** The current study elucidates a novel Notch inhibitory mechanism by which PKC\(\alpha\) downstream of ErbB-2 (i) restricts the availability of Jagged-1 at the cell surface to transactivate Notch, (ii) restricts the critical interaction between Jagged-1 and Mindbomb-1, an E3 ligase that is required for Jagged-1 ubiquitylation and subsequent Notch activation, (iii) reverses trastuzumab resistance in vivo, and (iv) predicts better outcome in women with ErbB-2–positive breast cancer.

**Conclusions:** The clinical impact of these studies is PKC\(\alpha\) is a potentially good prognostic marker for low Notch activity and increased trastuzumab sensitivity in ErbB-2–positive breast cancer. Moreover, women with ErbB-2–positive breast tumors expressing high Notch activation and low PKC\(\alpha\) expression could be the best candidates for anti-Notch therapy.

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Introduction

Breast cancer is the second leading cause of cancer-related deaths in women. Main hurdles for women are drug resistance, recurrence, and disease progression (1, 2). Breast cancer is a heterogeneous disease comprising at least four major subtypes, that is, luminal A, luminal B, HER2, and basal/triple-negative (3). The majority of breast cancers express estrogen, progesterone receptors (ER, PR), and/or the human epidermal growth factor receptor-2 (HER2 or ErbB-2). The ErbB-2 proto-oncogene is amplified in 15% to 25% of breast cancers (4, 5). ER and/or ErbB-2 are targets of therapies that include selective estrogen receptor modifiers (SERM) such as tamoxifen (6), aromatase inhibitors (7), or selective estrogen receptor disruptor (SERD) fulvestrant (8) for ER/PR-positive breast cancer or trastuzumab (9) for ErbB-2–positive breast cancer.

Despite the efficacy and dramatic effects of trastuzumab on survival, 20% to 50% of women with ErbB-2–positive metastatic breast cancer exhibit intrinsic resistance (10). Furthermore, 10% to 15% of the women treated with trastuzumab plus chemotherapy developed acquired resistance within the first year (11). Thus, understanding resistant mechanisms is critical for identifying novel targets to prevent and/or reverse resistance. Numerous mechanisms are implicated in resistance, including loss of PTEN, overexpression of IGF-1R, truncation of ErbB-2, hyperactivation of PI3K and mTOR signaling, among others (12).

PKC\(\alpha\) is another mediator of the ErbB-2 pathway. ErbB-2 activates phospholipase C \(\gamma\) (PLC\(\gamma\)), which cleaves phospha
diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), triggering an elevation of intracellular Ca\(^{2+}\) and activation of PKC\(\alpha\) (13). PKC\(\alpha\) overexpression promotes tamoxifen resistance (14) and invasion (15).
Translational Relevance

Breast cancer is the second leading cause of cancer mortality among women worldwide. The major problem with current treatments is tumor resistance, recurrence, and disease progression. The current study elucidates a novel mechanism by which ErbB-2 via PKCα attenuates Jagged-1–mediated Notch signaling in ErbB-2–positive breast cancer. The clinical impact of these studies is that PKCα is a potential alternative target for low Notch activity and increased trastuzumab sensitivity in ErbB-2–positive breast cancer. Moreover, women with ErbB-2–positive breast tumors expressing high Notch activity and low PKCα expression could be the best candidates for anti-Notch therapy. These studies provide a preclinical proof of concept for future clinical trials using combinations of trastuzumab/lapatinib plus an anti-Jagged-1–targeted therapy for trastuzumab-resistant, ErbB-2–positive breast cancer expressing low levels of PKCα.

Recently, PKCα was shown to mediate breast cancer stem cell survival (16) and is a therapeutic target in triple-negative breast cancer (17). However, the role of PKCα in the ErbB-2–positive breast cancer and its significance for anti-ErbB-2–targeted therapy remain unclear.

Notch ligands (Delta-like 1, 3, and 4 and Jagged-1 and -2) and receptors (Notch-1, -2, -3, and -4) are implicated in breast cancer development and drug resistance (18). They require cell–cell contact for engagement and subsequent cleavage of membrane-bound receptors to intracellular transcriptional activators. The ligand-induced Notch activation is regulated by E3 ubiquitin ligases, Mindbomb-1 (18), andNeutralized (19). Co-overexpression of Notch-1 and Jagged-1 predicts for the poorest overall survival (20). Notch-1, -3, and -4 are breast oncogenes and potent regulators of cell differentiation, proliferation, and apoptosis (21). High Notch-1 and -4 expression correlate to poor prognosis (22) and Notch-3 promotes ErbB-2–negative breast cancer cell proliferation (23). Notch receptor–ligand interactions are important for mammary stem cell differentiation (24) and tumorigenesis (25).

We showed that trastuzumab or a dual EGFR/ErbB-2 inhibitor increased Notch-1 activity, and trastuzumab resistance was reversed by Notch-1 knockdown or a γ-secretase inhibitor (GSI; ref. 26). Subsequently, we demonstrated that dual targeting of ErbB-2 and Notch prevented recurrence and partially reversed resistance to trastuzumab (27). Here, we describe a novel mechanism of action: PKCα attenuates Mib-1–mediated Jagged-1–Notch activation to predict sensitivity to trastuzumab. Furthermore, breast cancers expressing low PKCα might be more sensitive to anti-ErbB-2 agents. Importantly, anti-Jagged-1 therapy could reverse resistance to trastuzumab by attenuating Jagged-1–mediated Notch activity.

Materials and Methods

Cell culture and reagents

MDA-MB-453, BT474, and HCC1954 breast cancer cells were purchased from ATCC within the last 6 years. BT474 trastuzumab-resistant (BT474 Resistant) cells were generated by treating parental BT474 cells with increasing concentrations of trastuzumab for 6 months (26). All cell lines were authenticated using short tandem repeat (STR) allelic profiling (DCC Medical).

Biotinylation assay

MDA-MB-453 cells were seeded in 10-cm plates at a density of 40% to 50% confluency. After 48 hours, cells were treated with either mouse IgG (20 μg/mL in PBS) or trastuzumab (20 μg/mL in PBS) for 48 hours. The cells were washed 3 times in PBS, and a cell-impermeable biotinylation reagent EZ-Link Sulfo-NHS-Biotin (Pierce Chemicals) as previously described (28) was added to cells to label cell surface proteins at 4°C under constant shaking. Cells were scraped, centrifuged at 1,000 rpm for 1 minute, washed twice with PBS, and lysed in RIPA lysis buffer. The biotinylated cell surface proteins were precipitated from the total protein lysate using 30 μL of Immobilized Neutravidin Protein (Cat. 29200, Pierce Chemicals). The beads were washed 4 times with PBS and the protein was eluted with 4 × SDS Laemmli buffer and then heated at 95°C for denaturation. Western blotting was performed to detect Jagged-1 and ErbB-2 as described in the Supplementary Section.

Confocal immunofluorescence microscopy

MDA-MB-453, BT474HS, or BT474HR cells were either transfected with control siRNA, PKCα siRNA, or ErbB-2 siRNA or transfected with LRZS-linker alone or LRZS-PKCα and subsequently plated into chamber slides or untransfected cells were treated with PBS or 20 μg/mL trastuzumab directly in the well. Cells were then fixed in the chamber with 3.7% paraformaldehyde, permeabilized with 0.1% saponin, and nonspecific interactions were blocked with 1% BSA. The staining protocol for specific proteins of interest is provided in the Supplementary Section.

Co-immunoprecipitation

Jagged-1 (Jagged-1 2H28 rabbit monoclonal antibody, Cat. 2620, Cell Signaling) was immunoprecipitated under treatment conditions as described in the figure legends. Lysates at a concentration of 3 to 5 mg/mL were incubated with 6 μg of antibody for the specific protein of interest or rabbit isotype control IgG (Cat. SC-2027, Santa Cruz) overnight with gentle rocking at 4°C. Thirty microliters of protein A-plus beads (sc-2002, Santa Cruz) were added to the immune complexes for 2 hours, the beads were washed with lysis buffer, and the proteins attached to the pellet were eluted with 20 μL of 2× Laemmli sample buffer plus β-mercaptoethanol and heated for 10 minutes at 95°C while vigorously shaking. Immunoblots are described in Supplementary Section.

Coculture assay

MDA-MB-453 cells were plated at a density of 3 × 10^6 cells in a 10-cm plate. Mouse fibroblast (LTK) cells expressing no ligand or overexpressing Jagged-1 were added in equal parts (1:1) to previously plated MDA-MB-453 cells. The cells were then treated with PBS or 20 μg/mL trastuzumab for 18 hours. Cells were then stained with 10 μL of phycoerythrin (PE)-conjugated human ErbB-2 antibody (Cat. 340552, Becton Dickinson). Stained cells were then sorted for ErbB-2 expression at the cell surface by FACS. Total RNA was extracted from sorted cells, reverse transcribed to total cDNA, and real-time PCR was performed as previously described. The PCR primers that were used for detection of specific
transcripts were Hes-1 and Deltex-1 with RPL13a as a loading control.

**Trastuzumab-resistant xenografts**

Five million BT474 trastuzumab-resistant cells were injected bilaterally into mammary fat pads of ovariectomized FoxN1 nu/nu athymic nude mice (Harlan Sprague-Dawley) followed by implantation of a 17β-estradiol–containing silastic capsule of 0.3 cm in length with a constant release providing 83 to 100 pg/mL as described previously (29). The identity of each mouse and tumor was tracked by an ear tag. Once tumors grew to a mean cross sectional area (CRA) of 0.30 to 0.50 cm², mice were randomized to four treatment groups: (i) vehicle control (PBS), (ii) trastuzumab (10 mg/kg in a total volume of 100 µl sterile PBS, intraperitoneally once weekly), (iii) CTX-033 (5 mg/kg, intraperitoneally once weekly), or (iv) trastuzumab plus CTX-033. In case of BT474HR cells retrovirally transduced with the LRZS-linker or LRZS-PKCo, these tumors were randomized to two treatment groups each: (i) PBS or (ii) trastuzumab. Tumor area (l x w) was measured weekly using Vernier calipers and cross sectional area [(l x w)/2] was calculated and graphed. All animal study protocols were approved by Loyola University's Institutional Animal Care and Use Committee.

**Flow cytometry**

MDA-MB-453 cells were treated with indicated dose of lapa
tinib/trastuzumab for 48 hours. DMSO and IgG were used as negative controls. Cells were harvested using Cellstripper (Cat. 25–056-CI Corning Cellgro) and were stained with fluorescein isothiocyanate (FITC)–conjugated Jagged-1 antibody as per the manufacturer's protocol. The cells were then resuspended in Flow Cytometry Staining Buffer (Cat. FC001 R&D Systems) and analyzed with BD FACS Canto II. Data acquisition was done using BD FACSDiva software and data analysis was performed using FlowJo software.

**Proliferation assay**

MDA-MB-453 cells were seeded into 10-cm dishes at 5 x 10⁶ cells. HCC-1954 cells were seeded into 10-cm dishes at 3.5 x 10⁶ cells. The cells were transfected as described in the RNA interference of Methods. After completion of the siRNA transfection, the cells were seeded in triplicate into 6-well plates at a density of 50,000 for MDA-MB-453 cells or HCC-1954 cells. BT474HS sensitive cells were seeded into 6-well plates at a density of 60,000 cells. BT474HR resistant cells were seeded into 6-well plates at a density of 60,000 cells. After plating into the 6-well plates, cells were treated with PBS or 20µg/mL trastuzumab on a daily basis. Cells were counted at days 2, 4, 6, and 8 posttreatment and were trypsinized into single-cell suspensions and counted using the Countess Automated Cell Counter (Cat. C10310, Life Technologies). The live cell number was then used to calculate the total number of live cells over the number of live cells plated.

**Immunohistochemical staining of human ErbB-2–positive breast tumors**

Tissue microarray (TMA) sections were placed in a 58°C to 60°C oven overnight for tissue to adhere. The sections were deparaffinized in xylene, rehydrated through graded ethanol, and washed with PBS before being treated with 1× Reveal in a Decloaking Chamber (Biocare Medical) for antigen retrieval following the manufacturer's protocol. After rinsed in PBS for 15 minutes, the sections were soaked in 3% H₂O₂ in PBS for 20 minutes to quench endogenous peroxidase activity. Sections were incubated for 60 minutes in 3% normal rabbit serum (Vector Laboratories) in PBS at room temperature to block nonspecific binding sites and then probed with primary antibodies (PKCo antibody was Santa Cruz C20 at 1 µg/mL), with nonimmune IgG used as controls. The details of the staining protocol are in the Supplementary Section.

**Statistical analysis**

Most experiments were performed at least three times. Means ± SDs were calculated on at least n = 3 experiments. A two-tailed Student t test was performed on results with two comparisons. ANOVA was performed on results with multiple comparisons. Linear regression analysis was performed on tumor growth studies, as each mouse was tagged with a number and each tumor was measured independently followed by ANOVA for multiple comparisons. A Kaplan–Meier curve for recurrence-free survival or overall survival of human patients was generated by the Kaplan–Meier Plotter software (30) or generated by GraphPad Prism software and analyzed with a log-rank (Mantel—Cox) test.

**Supplementary Materials and Methods**

More detailed methods are provided in the Supplementary Section.

**Results**

**Jagged-1 is required for trastuzumab-induced Notch activation**

We demonstrated that inhibiting ErbB-2 increased NICD1 expression, CBF-1–driven reporter activity, Deltex-1 and Hey-1 transcripts, and thus most likely canonical Notch activation (26). Jagged-1 predicts poor outcome in women with breast cancer (31). Here, we sought to understand whether ErbB-2 attenuates Notch signaling by regulating Jagged-1 expression and/or cellular localization in breast cancer cells. Figure 1A confirms that ErbB-2 inhibits Notch transcriptional activity. Conversely, ErbB-2 inhibition using a kinase-dead mutant, ErbB-2 siRNA, or lapatinib increased RNA transcripts of Notch gene targets, Hes-1, Deltex-1, and/or Hey-1 (Fig. 1A). Figure 1B shows that trastuzumab or lapatinib increased Jagged-1 protein expression on the cell surface by flow cytometry. Furthermore, this increase in Jagged-1 protein expression by trastuzumab treatment is restricted to the cell surface as measured by a cell surface biochemical assays (Fig. 1C). These results indicate that ErbB-2 inhibition promoted the accumulation of Jagged-1 at the cell surface.

To confirm the supporting cell surface biochemical assays from Fig. 1B and C that ErbB-2 overexpression may restrict cell surface expression of Jagged-1, we performed confocal fluorescence microscopy to visualize Jagged-1 cellular localization. The IgG control cells showed that a portion of Jagged-1 colocalized with early endosomal antigen-1 (EEA-1; Fig. 1D). In contrast, trastuzumab-treated cells showed that Jagged-1 was no longer retained in EEA-1–positive endosomes and primarily accumulated at the cell surface (Fig. 1D). Furthermore, Notch-1 colocalized with Jagged-1 in control cells (Fig. 1D). However, trastuzumab treatment induced accumulation of Notch-1 throughout the cell and Jagged-1 at sites of cell–cell contacts (Fig. 1D). These results indicate that ErbB-2 overexpression possibly trapps both Jagged-1 and Notch-1 in subplasma membrane compartments, including...
early endosomes. However, anti-ErbB-2 treatment with trastuzu-
mab disrupts the Jagged-1–Notch-1 colocalization. If Jagged-1–mediated transactivation of Notch is attenuated by ErbB-2, we predicted that supplying the Notch-expressing breast cancer cells with stromal cells expressing abundant surface Jagged-1 would restore Notch activation. We cocultured ErbB-2-expressing MDA-MB-453 breast cancer cells with mouse fibroblasts expressing no ligand (LTK-Parental) or overexpressing Jagged-1 (LTK-JAG1) and then treated with vehicle or trastuzumab. Trastuzumab increased both Hes-1 (4-fold; Fig. 1E, right) and Deltex-1 mRNAs (7-fold; Fig. 1E, left) in the presence or absence of trastuzumab. Interestingly, trastuzumab treatment in the presence of LTK-JAG1 did not increase Deltex-1 levels compared with LTK-P cells. It is possible that there are cis-inhibitory mechanisms within the breast cancer cells that limited the activation of the Notch gene target. These results indicate that ErbB-2 most likely restricts Jagged-1 cell surface localization and limits transacti-

**Figure 1.** ErbB2 inhibits Notch signaling and Jagged-1 cell surface expression. A, ErbB2 expression and activity inhibits Notch activation. Left, MDA-MB-453 cells were stably transfected with vector alone or kinase dead ErbB2 (ErbB2KD) in an expression plasmid. Middle, MDA-MB-453 cells were transfected with a scrambled control siRNA (SCBi) or an ErbB2 siRNA. Right, MDA-MB-453 cells were treated with DMSO or lapatinib (LAP) for 6 days. Total protein from cell lysates were subjected to 5% PAGE followed by Western blotting to detect tyrosine phosphorylated ErbB2 (PY-ErbB2), total ErbB2, and actin proteins. Bottom, similar studies were performed as described in A, and real-time PCR was performed to detect RNA transcript levels for Hes-1, Hey-1, and/or Deltex-1. Bar graphs, means ± SDs of three independent experiments. *, statistical significance $P < 0.05$ using a nonpaired Student t test. B, trastuzumab (Trast) or lapatinib increases cell surface expression of Jagged-1 protein. The results are representative of at least three independent studies. C, inhibition of ErbB-2 increases cell surface Jagged-1 expression. MDA-MB-453 cells were treated with IgG or trastuzumab for 48 hours. Cell surface proteins were biotinylated as described in experimental procedures followed by streptavidin precipitation and Western blotting to detect the following proteins: Jagged-1 (JAG1) and total ErbB-2 in both precipitates and total lysates. D, trastuzumab promotes Jagged-1 surface localization and Notch-1 activation as shown by confocal immunofluorescence. Top, scale bars = 20 μm; bottom, scale bars = 10 μm. E, Jagged-1 mediated transactivation of Notch. MDA-MB-453 cells were cocultured with mouse fibroblasts expressing no ligand (LTK-Parental) or overexpressing Jagged-1 (LTK-JAG1) in a 1:1 ratio and then treated with PBS or trastuzumab for 18 hours. Cells were sorted by flow cytometry for ErbB-2–positive cells. RNA was extracted and real-time PCR was performed to detect relative transcript levels: Hes-1 (right) or Deltex-1 (left). Western blotting was performed on LTK cells to detect levels of Jagged-1 protein (middle). **, $P < 0.05$ between PBS and trastuzumab; ***, statistical significance between LTK-P and LTK-JAG1; and ****, statistical significance between LTK-P and LTK-JAG1 in response to trastuzumab using an ANOVA.
To determine whether Jagged-1 is necessary for trastuzumab-induced Notch activation, we used a genetic approach to downregulate Jagged-1 in two ErbB-2–positive breast cancer cell lines. Different Jagged-1 siRNAs (JAG1iA or JAG1iNew) decreased Jagged-1 protein expression (Figs. 2A, 2B; Supplementary Fig. S1A) and significantly inhibited the trastuzumab-induced increase of Notch gene targets, Hes-1, Deltex-1, Hey-1, and/or Notch-4 transcripts (Figs. 2A and 2B; Supplementary Fig. S1A). These results show that Notch activation mediated by ErbB-2 inhibition is most likely Jagged-1–dependent.

Dual blockade of ErbB-2 and Jagged-1 inhibits proliferation in vitro and reverses resistance in vivo

We tested whether decreasing Jagged-1 by siRNA would enhance the effectiveness of trastuzumab in a panel of ErbB-2–positive breast cancer cell lines. Different Jagged-1 siRNAs (JAG1iA or JAG1iNew) decreased Jagged-1 protein expression (Figs. 2A, 2B; Supplementary Fig. S1A) and significantly inhibited the trastuzumab-induced increase of Notch gene targets, Hes-1, Deltex-1, Hey-1, and/or Notch-4 transcripts (Figs. 2A and 2B; Supplementary Fig. S1A). These results show that Notch activation mediated by ErbB-2 inhibition is most likely Jagged-1–dependent.

Because ubiquitinylation of Jagged-1 by Mib-1 is required to activate Notch (35, 36), we hypothesized that ErbB-2 attenuates expression of Mib-1 and/or its association with Jagged-1 to inhibit Notch activity. To test this hypothesis, Jagged-1 was immunoprecipitated from MDA-MB-453 cells treated with trastuzumab or lapatinib, and Western blotting was performed to detect Jagged-1 and Mib-1 proteins. Co-immunoprecipitation (co-IP) demonstrated that trastuzumab or lapatinib increased the amount of

Erbb-2 limits Mib-1–mediated ubiquitinilation of Jagged-1 and subsequent transactivation of Notch

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Mib-1 in a complex with Jagged-1 (Fig. 4A). From these results, we conclude that ErbB-2 possibly limits the interaction between Jagged-1 and Mib-1.

To investigate the role of Mib-1 on Notch-1 activity, the effect of a Mib-1 siRNA on the expression of Hes-1 protein levels was measured. As measured by Western blot analysis, Mib-1 protein levels were decreased upon Mib-1 knock down (Supplementary Fig. S2). Either trastuzumab or lapatinib increased Hes-1 protein (Supplementary Fig. S2). Importantly, Mib-1 siRNA decreased the induction of Hes-1 (Supplementary Fig. S2), indicating that Mib-1 is necessary for Notch activation by ErbB-2 inhibitors.

To determine whether ErbB-2 or downstream mediators such as PI3K or MEK attenuate Jagged-1 ubiquitinylation, we performed an IP of Jagged-1 similar to Fig. 4A except we used the PI3K inhibitor, LY294002, or the Mek1/2 inhibitor, U0126, in addition to lapatinib. Lapatinib increased Jagged-1 ubiquitin bylation at 150 kDa and enhanced the recruitment of Mib-1 to a Jagged-1 complex (Fig. 4B). Neither LY294002 nor U0126 induced recruitment of Mib-1 to Jagged-1 or Jagged-1 ubiquitin bylation compared with lapatinib (Fig. 4B). Western blot analyses confirmed that the kinase inhibitors inhibited their targets (Supplementary Fig. S3A). These results suggest that ErbB-2 restricts the interaction of Mib-1 to Jagged-1 and its ubiquitin bylation possibly through a different kinase downstream of ErbB-2.

ErbB-2 promotes an association between Jagged-1 and PKCα

To determine the mechanism by which ErbB-2 attenuates the association between Jagged-1 and Mib-1, the protein sequences of Jagged-1 and Mib-1 were scanned using Prosite Expasy and several high scoring putative PKC phosphorylation sites were identified. ErbB-2 activates PKCα in breast cancer cells via PLCγ (37) or by upregulating c-Src (37). Co-IP demonstrated that Jagged-1 was associated with PKCα when ErbB-2 was active but failed to bind PKCα when cells were treated with lapatinib (Fig. 4C). Down-regulation of PKCα using an siRNA facilitated the association of Mib-1 with Jagged-1 and subsequent ubiquitinylation of Jagged-1.
similarly to lapatinib treatment (Fig. 4D). Control Western blot analyses were performed to detect protein levels of active ErbB-2 (PY), total ErbB-2, and PKCα to confirm the efficiency of the PKCα siRNA and that it had little effect on the expression and activity of ErbB-2 (Fig. 4D).

Jagged-1 and PKCα colocalize in ErbB-2–positive breast cancer cells and dissociate upon trastuzumab treatment

We performed confocal fluorescence microscopy to explore whether Jagged-1 and PKCα colocalize in ErbB-2–overexpressing breast cancer cells. Jagged-1 colocalizes with PKCα (Fig. 4E, left) in PBS-treated cells. In contrast, Jagged-1 was no longer colocalized with PKCα (Fig. 4E, left) in response to trastuzumab. Similar, results were observed in trastuzumab-sensitive BT474 cells (Fig. 4E, right). Interestingly, trastuzumab-resistant BT474 cells express less PKCα and Jagged-1 is primarily near the cell surface (Fig. 4E, right, top). PKCα knockdown in BT474 sensitive cells disrupts the Jagged-1–PKCα colocalization similar to what we observed in resistant cells (Fig. 4E, right, bottom).

PKCα inhibits Jagged-1–mediated Notch activation and restores trastuzumab sensitivity in vivo

To determine whether PKCα is sufficient to restrict Notch activation, we tested whether a constitutive active PKCα...
(Δ22–28) would prevent the lapatinib-induced Mib-1 and Jagged-1 interaction, Jagged-1 ubiquitylation, and increase in Notch gene targets. Figure 5A showed that lapatinib increased recruitment of Mib-1 to a Jagged-1 complex and increased Jagged-1 ubiquitylation in cells transduced with control LRZS-linker. In contrast, lapatinib treatment of cells transduced with LRZS-PKCαΔ22–28, which increased PKC activation as shown in Fig. 5A, right, failed to increase Mib-1 recruitment to Jagged-1 and ubiquitylation (Fig. 5A). Furthermore, Fig. 5C showed that lapatinib significantly increased 3 of 5 Notch gene targets (Deltex-1, Hey-1, and Notch-4) in cells transduced with LRZS-linker but not in cells expressing PKCαΔ22–28. In contrast, knockdown of PKCα increased expression of Hes-1 and Hey-1 proteins (Fig. 5B, left) and Deltex-1 transcripts (Fig. 5B, right), respectively. Taken together, these results indicate that ErbB-2 facilitates a PKCα–Jagged-1 association to limit Mib-1–mediated ubiquitylation of Jagged-1 and activation of Notch.

To determine whether PKCα expression limits Notch-1 nuclear localization, we performed confocal immunofluorescence. The results showed that cellular localization of Notch-1 and Jagged-1 was relatively unchanged in cells expressing LRZS-linker alone or LRZS-PKCα upon vehicle treatment (Fig. 5D, top). In contrast, trastuzumab treatment of cells transduced with the LRZS-linker induced nuclear localization of a proportion of the Notch-1 protein, whereas Jagged-1 accumulated in the cytoplasm or near the cell surface (Fig. 5D, bottom left). PKCα overexpression
PKCα Regulates Notch to Prevent Trastuzumab Resistance

PKCα overexpression reverses trastuzumab resistance and predicts better survival in women with ErbB-2–positive breast cancer. A, overexpression of PKCα inhibits growth of trastuzumab-resistant BT474 tumor xenografts. Five million trastuzumab-resistant BT474 cells retrovirally transduced with LRZS-linker or LRZS-PKCα were injected into mammary fat pads of 20 female nude athymic mice, respectively. Tumors were allowed to grow to a mean cross-sectional area of approximately 10 to 20 mm² and then mice were randomized to PBS or trastuzumab, injected intraperitoneally once weekly for up to 10 weeks. Statistical significance was calculated by a two-way ANOVA. B, low PKCα protein predicts poorer overall survival in women with ErbB-2–positive breast cancer. The Nottingham cohort of 100 ErbB-2–positive breast tumor tissues was stained for PKCα protein and scored 0.00 for negative staining or 1.00 for positive staining. Overall survival follow-up results were collected prospectively up to 400 months. Kaplan–Meier analysis for overall survival was performed on negative and positive expression of PKCα. Statistical significance was calculated using log-rank (Mantel–Cox) test. Immunohistochemistry was performed to detect PKCα protein in ErbB-2–positive breast cancer tissue. The three panels are representative of negative expression (top), high expression (middle), and moderate expression (lower). C, model summarizes conclusion of current study. ErbB-2 through PKCα–mediated Notch regulation is responsible for ErbB-2–positive breast cancer resistance.

PKCα overexpression significantly decreased BT474 breast tumor growth compared with LRZS-linker. This result is not surprising, as we have shown previously that Notch-1 knockdown alone in trastuzumab-resistant BT474 cells was necessary to inhibit cell proliferation in vitro in the absence or presence of trastuzumab (26). These current results would suggest that PKCα overexpression could also be necessary to inhibit ErbB-2–positive breast tumor growth after acquired resistance to trastuzumab possibly by suppressing Notch signaling. PKCα overexpression partially restored trastuzumab sensitivity, as the tumors almost completely regressed upon trastuzumab treatment (Fig. 6A). These results demonstrate that PKCα could limit Notch-1 activation and thus possibly trastuzumab resistance in ErbB-2–positive breast cancer cells.

PKCα and Hey-1 inversely predict ErbB-2–positive breast cancer recurrence and survival

These results suggest that ErbB-2–positive breast cancer patients with high PKCα expression have better outcomes, as PKCα can attenuate Notch signaling to promote sensitivity to anti-ErbB-2 therapy. To address this, we used survival analysis via the Kaplan–Meier Plotter software, version 2014 (30) and searched for expression of PRKCA and Hey-1 transcripts in a
cohort of 208 patients with ErbB-2–positive breast cancer to predict recurrence-free survival (RFS) outcome. Results showed that high PRKCA transcript expression based on an average of four probes predicted better RFS outcome compared with low with an HR of 0.64 (log-rank $P = 0.037$). In contrast, high expression of a Notch gene target, Hey-1, predicted poor RFS outcome (HR, 1.72; log-rank $P = 0.011$) using an average of two Hey-1 probes (Supplementary Fig. S3). Interestingly, when we used the multigene analyzer and asked whether the same breast cancer samples that express high Hey-1 and low PRKCA predict worse RFS outcomes compared with the individual genes, the results showed a slightly worse outcome with HR = 1.77 and log-rank $P = 0.0074$ (Supplementary Fig. S3).

We examined another cohort, the Nottingham primary breast cancer series (the Tenovus cohort) prepared as TMA. 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 breast cancer-related death (38, 39). One hundred nine ErbB-2–positive breast cancer tissues were stained for PKCα protein by immunohistochemistry, blindly scored for staining intensity (0–3). Samples were classified into two groups: Group 1.0 with high PKCα (staining intensity, 2–3) and group 0.0 with low or negative PKCα (staining intensity, 0–1) and labeled as “0.0.” The results demonstrated that high PKCα protein expression predicted better BCSS compared with low expressing patients (log-rank $P = 0.019$; Fig. 6B). Figure 6B showed a sample of the immunohistochemistry for PKCα protein stained as negative (top), positive sample #1 (middle), and positive sample #2 (bottom). Therefore, on the basis of the results of our current study, we propose a model where PKCα attenuates the association between Jagged-1 and Mib-1 to restrict Jagged-1 ubiquitylation and subsequent transactivation of Notch-1 to prevent Notch-1–driven resistance to ErbB-2–targeted therapies (summarized in Fig. 6C).

**Discussion**

ErbB-2–positive breast cancer is currently treated with trastuzumab, lapatinib, pertuzumab, or trastuzumab emtansine. Many patients will not initially respond to these drugs and, among responders, more than 25% develop resistance within the first year of therapy (40). Thus, resistance remains a clinical problem that requires identification of new targets for future therapeutic strategies and novel prognostic biomarkers. Our results reveal that PKCα downstream of ErbB-2 limits an association between Mib-1 and Jagged-1 to attenuate Notch-1 activation. The PKCα–mediated attenuation of Notch-1 signaling is necessary and sufficient to enhance sensitivity to trastuzumab and possibly prevent resistance to potentially improve survival for women with ErbB-2–positive breast cancer.

Notch signaling promotes breast cancer progression (41, 42) and is critical for survival and self-renewal of breast cancer stem cells (24, 25). These cells are hypothesized to be the dormant or slowly self-replicating cell population resistant to standard therapies. We showed that Notch-1 is required for trastuzumab resistance (26) and ErbB-2 breast tumor recurrence (27). Others have shown that Notch cooperates with ErbB-2 to promote a more invasive phenotype in ductal carcinoma in situ (DCIS; ref. 43). Furthermore, Notch expression and activity have been implicated in ErbB-2–driven cancer stem cells (44). Recently, Abravanel and colleagues showed that Notch promotes recurrence of breast tumor cells after anti-ErbB-2 therapy (45). Thus, Notch could promote breast tumor development during early stages of breast tumorigenesis, and at later stage, its activity and function is attenuated as other pathways become dominant. From our studies, we conclude that ErbB-2 attenuates Notch signaling via PKCα under conditions whereby ErbB-2 is the primary growth driver. However, when ErbB-2 is inhibited by trastuzumab or lapatinib, PKCα is inhibited and Jagged-1 is made competent to transactivate Notch to promote a compensatory survival signal.

The role of PKCα in breast cancer remains controversial, as it is not clear whether PKCα is a tumor promoter or a suppressor. PKCα is implicated in human breast cancer progression (46). However, there are conflicting data, as some have shown PKCα is decreased in human breast tissue (47). Ectopic expression of PKCα in cell lines promotes a more aggressive phenotype. For example, PKCα overexpression induces tamoxifen resistance in ER-positive cell lines (14). Furthermore, PKCα is required for invasion of breast cancer cells (48). In addition, PKCα activates Notch-4 expression in ER-positive breast cancer cells to promote tamoxifen resistance (49). PKCα is also a mediator of breast cancer stem cell expansion (16), regulates recycling of ErbB2 in breast cancer cells (50), and a therapeutic target in triple-negative breast cancer (17). One possible explanation for the conflicting results could be that the activation status of PKCα, its subcellular localization, and its immediate substrates in breast cancer remain unclear. As breast cancer is a heterogeneous disease, it would not be surprising that PKCα could have pleiotropic functions in different subtypes of breast cancer. For example, patients with ErbB-2–positive tumors expressing low PKCα have poorer RFS than high expressors. These data included both ER-positive and -negative tumors. Ideally, if we could stratify and separate these data based on ER expression, results could determine whether ER status changes the prediction. However, because of the low number of ErbB-2+ patients from the Kaplan–Meier Plotter dataset and the Nottingham cohort, this could not be done. Increasing the number of retrospective samples and/or a prospective clinical trial will help increase power and answer some of these critical questions. Results from clinical trials using PKC inhibitors have been disappointing. The ISIS 3521 (aprinocarsen or LY900003) trial failed to show a response and this could be due to multifactorial roles of PKCs. More than 20 trials from phase I to III have been conducted using various PKC inhibitors in many solid tumors (51). However, results of trials have been disappointing in women with metastatic breast cancer (52), suggesting that targeting PKCα might depend on the cell context.

The mechanism by which PKCα attenuates Jagged-1 vesicular trafficking and recruitment of Mib-1 to Jagged-1 is under investigation. Both Jagged-1 and Mib-1 contain putative PKC phosphorylation sites. Therefore, Jagged-1 and/or Mib-1 could be direct substrates of PKCα. Phosphorylation events regulate recruitment of E3 ligases and thus PKCα could directly phosphorylate Jagged-1 and/or Mib-1 to prevent their interaction. Alternatively, PKCα could regulate vesicular trafficking of Jagged-1 possibly sequestering Jagged-1 in endosomes targeted for recycling. Classical PKCs such as α and βI regulate proteins that are normally recycled by sequestering them in the pericentriol (53). Our results are consistent with this as we showed that Jagged-1 is localized to endosomes when ErbB-2 was hyperactive.
but accumulated at the cell surface upon ErbB-2 blockade. Thus, PKCα might limit the cell surface availability of Jagged-1 by facilitating sequestration of Jagged-1 in recycling endosomes.

We describe a critical function for PKCα downstream of ErbB-2, which is to attenuate the competency of Jagged-1 to activate Notch-1. Notch ligands are made competent by Mib-1 and subsequent ubiquitylation that drives endocytosis. We show that Jagged-1 is required for trastuzumab resistance in breast cancer (Fig. 3) providing the first preclinical proof of concept for the use of an anti-Jagged-1, such as the Probody CTX-033, targeted therapy for prevention or reversal of resistance possibility in breast cancers expressing low PKCα levels (Fig. 6) and/or high Hey-1 levels (Supplementary Fig. S3). An anti-Jagged Probody therapeutic may be well positioned for such a strategy given that it demonstrates less systemic toxicity compared with other Notch targeting approaches because it is only activated within the tumor microenvironment (34). The human data from two cohorts of ErbB-2–positive breast tumors predict that women with breast tumors expressing low PKCα mRNA (Supplementary Fig. S4) or protein (Fig. 6B) have poorer RFS or BCSS, respectively. Conversely, high Hey-1 expression predicts poor RFS outcome in women with ErbB-2–positive breast cancer (Supplementary Fig. S4). To determine whether the same tumors that express low PKCα also express high Hey-1 would be better targets for anti-Notch therapy, a prospective clinical trial should be conducted using the Jagged-1 Probody or a Notch-specific inhibitor in combination with trastuzumab. These data are consistent with the overall model where the ErbB-2–PKCα axis attenuates Notch activation and this subset of tumors could predict better sensitivity to anti-ErbB-2–targeted agents. Conversely, low PKCα tumors could require concomitant Notch inhibition to prevent or reverse trastuzumab resistance. These results could have clinical impact for the treatment of women with ErbB-2–positive breast cancer. Future clinical trials need to be designed to test whether PKCα protein levels will predict for trastuzumab sensitivity.

Disclosure of Potential Conflicts of Interest
J. Sager has ownership interest in CytoMx. L. Miele is a consultant/advisory board member for CytoMx. No potential conflicts of interest were disclosed by the other authors.

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