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Protein Kinase C δ is required for ErbB2-driven mammary gland tumorigenesis and negatively correlates with prognosis in human breast cancer

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Abstract

Protein Kinase C delta (PKC δ) regulates apoptosis in the mammary gland, however the functional contribution of PKC δ to the development or progression of breast cancer has yet to be determined. Meta-analysis of ErbB2-positive breast cancers shows increased PKC δ expression, and a negative correlation between PKC δ expression and prognosis. Here we present *in vivo* evidence that PKC δ is essential for the development of mammary gland tumors in a ErbB2-overexpression transgenic mouse model, and *in vitro* evidence that PKC δ is required for proliferative signaling downstream of the ErbB2 receptor. MMTV-ErbB2 mice lacking PKC δ (δ KO) have increased tumor latency compared to MMTV-ErbB2 wild type (δ WT) mice, and tumors show a dramatic decrease in Ki-67 staining. To explore the relationship between PKC δ and ErbB2-driven proliferation more directly, we used MCF-10A cells engineered to express a synthetic ligand-inducible form of the ErbB2 receptor. Depletion of PKC δ with shRNA inhibited ligand-induced growth in both 2D (plastic) and 3D (Matrigel) culture, and correlated with decreased phosphorylation of the ErbB2 receptor, reduced activation of Src, and reduced activation of the MAPK/ERK pathway. Similarly, in human breast cancer cell lines in which ErbB2 is overexpressed, depletion of PKC δ suppresses proliferation, Src, and ERK activation. PKC δ appears to drive proliferation through formation of an active ErbB2/PKC δ /Src signaling complex, as depletion of PKC δ disrupts association of Src with the ErbB2 receptor. Taken together, our studies present the first evidence that PKC δ is a critical regulator of ErbB2-mediated tumorigenesis, and suggest further investigation of PKC δ as a target in ErbB2-positive breast cancer.

Keywords

PKC δ ; breast cancer; ErbB2; Src

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Introduction

The ErbB2 oncogene is amplified or overexpressed in 20–30% of all breast cancers and is associated with decreased survival, faster relapse, and increased lymph node metastasis (1, 2). Overexpression of ErbB2 leads to receptor dimerization and activation of pathways involved in tumor cell proliferation and survival, such as Mitogen Activated Protein Kinase (MAPK), Src, and Phosphatidylinositol 3-kinase (PI3K). Additionally, ErbB2 is the preferred binding partner of other epidermal growth factor receptor (EGFR) family members, thus overexpression of this protein increases receptor interactions, providing an efficient link to mitogenic pathways (3, 4).

The PKC family of serine/threonine kinases consists of 10 isoforms that are structurally related and that regulate many biological functions (5–7). PKC δ is an important regulator of epithelial cell apoptosis, which has led to the speculation that it may play a role in tumorigenesis (8–13). Increased expression of PKC δ has been shown in colon, pancreatic, and high-grade breast tumors (14–16). Conversely, PKC δ expression is decreased in some cancers including bladder and endometrial carcinoma (17, 18). We have recently shown that PKC δ functions as a tumor promoter in Ras-mediated lung tumors *in vivo* and in K-ras addicted human Non-Small Cell Lung Cancer (NSCLC) cells *in vitro* through regulation of the Ras/MAPK pathway (19). Likewise, studies from Keshamouni *et. al.* have implicated PKC δ in regulating Ras signaling downstream of ErbB2 in breast cancer cells *in vitro* (20). PKC δ has also been shown to positively regulate cell migration in several cell types, including EGFR overexpressing breast cancer cells (21–24).

Src is a major mediator of ErbB2 signaling, and a potential mechanism through which cancer cells can become resistant to ErbB2 therapies (25). PKC δ expression is increased in breast cancer cells resistant to tamoxifen and lapatinib, suggesting that both PKC δ and Src may be necessary for ErbB2 mediated signal transduction (26, 27). Our current studies identify PKC δ as a critical regulator of ErbB2-mediated proliferation, and as a tumor promoter in a MMTV-ErbB2 transgenic mouse model of mammary gland cancer. Meta-analysis of ErbB2-positive human breast cancers reveals a negative correlation between PKC δ expression and prognosis, supporting further investigation of PKC δ as a potential therapeutic target.

Results

Increased expression of PKC δ negatively correlates with prognosis in ErbB2 positive human breast cancer

To explore the contribution of PKC δ to human breast cancer, we used the Oncomine database (28), to interrogate 21 ErbB2 positive human breast cancer data sets (n=> 2,000 patients) for PKC δ mRNA expression. Our analysis shows that PKC δ is significantly overexpressed in ErbB2 positive human breast cancers (Figure 1A, red; $P=0.01$). Since ErbB2 is associated with a poor prognosis (1, 2), we determined whether increased PKC δ expression effects clinical outcome specifically in patients with ErbB2 positive human breast tumors. Using the GOBO tool (29), we performed a meta-analysis of relapse-free survival in four breast cancer data sets, where 122 patients were identified as ErbB2 positive

using the PAM50 array (30). Strikingly, high expression of PKC δ (red line) significantly correlated with decreased relapse-free survival compared to low PKC δ expression (gray line) ($P=0.017$) (Figure 1B). Similar results were seen when we examined the correlation between PKC δ mRNA expression and relapse-free survival over all molecular subtypes ($P=0.0002$) (Figure 1C). In contrast, there was no correlation between PKC δ mRNA expression and relapse-free survival in patients with the triple-negative tumor subtype (negative for ER, PR, and ErbB2 ($P=0.488$) (Figure 1D)), moreover, PKC δ mRNA expression was significantly decreased in 13 triple-negative tumor data sets (Oncomine, data not shown). These results suggest that PKC δ may be a significant factor in the progression of ErbB2 driven breast tumors.

Loss of PKC δ increases tumor latency in MMTV-ErbB2 transgenic mice

To directly address the functional role of PKC δ in ErbB2-driven tumors we utilized a transgenic mouse model in which mammary gland tumorigenesis is driven by overexpression of the wild type rat *ERBB2* gene under control of the Mouse Mammary Tumor Virus (MMTV) promoter (31, 32). MMTV-ErbB2 mice were crossed with δ KO mice to generate MMTV-ErbB2; δ WT and MMTV-ErbB2; δ KO mice. Both MMTV-ErbB2; δ WT and MMTV-ErbB2; δ KO mice develop focal mammary tumors consistent with the MMTV-ErbB2 phenotype (31, 32); however, MMTV-ErbB2; δ KO mice had a significant delay in tumor onset, with a mean latency of 293 days compared to 243 days in MMTV-ErbB2; δ WT mice ($P=0.03$) (Figure 2A). Mammary gland tumors were never detected in aged δ KO mice (>1 year of age; data not shown). Histological analysis showed that both MMTV-ErbB2; δ WT and MMTV-ErbB2; δ KO tumors were highly vascularized and consisted of uniform solid sheets of cells (Figure 2B, panels a, b).

To explore the mechanism underlying increased tumor latency in MMTV-ErbB2; δ KO mice, we assayed apoptosis and proliferation by immunohistochemistry for cleaved caspase-3 and Ki-67, respectively. Very few apoptotic cells were observed in tumors from either genotype (Figure 2C). In contrast, Ki-67 positive cells were reduced by >50% in MMTV-ErbB2; δ KO tumors compared to MMTV-ErbB2; δ WT tumors (Figure 2D), suggesting that increased tumor latency is due at least in part to reduced ErbB2-driven proliferation. Consistent with this finding, tumor lysates from MMTV-ErbB2; δ KO mice show decreased levels of ErbB2 receptor phosphorylation compared to WT, suggesting that PKC δ regulates ErbB2 signal transduction (Figure 2E).

PKC δ is required for ErbB2-driven proliferation

To explore more directly the contribution of PKC δ to ErbB2-driven proliferation and tumorigenesis, we utilized a derivative of the non-transformed human breast epithelial line, MCF-10A, engineered to express a synthetic ligand-inducible form of the ErbB2 receptor (10A.ErbB2) (33, 34). In this model, the addition of an ErbB2 dimerization ligand induces ErbB2 signaling, resulting in abnormal acinar growth that resembles human ductal carcinoma *in situ* (35). To ask if PKC δ contributes to this ErbB2-induced morphogenesis, 10A.ErbB2 cells were depleted of PKC δ using lentiviral delivered shRNA targeted to PKC δ (sh δ 193 and sh δ 203), or a scrambled control (shSCR), and grown on Matrigel for 6 days (Figure 3A, panels a, b, c). In the absence of the ligand, all cells formed small, round,

organized acini typical of normal MCF-10A growth (Figure 3A, panels a, b, c) (36). Acini were then treated with ligand for 3–8 days. Dimerization of ErbB2 resulted in misshapen acini in shSCR, sh δ 193, and sh δ 203 cells (Figure 3A, panels g, h, I, m, n, o, insets), however no consistent changes were seen in acini derived from sh δ 193 and sh δ 203 cells compared to shSCR cells. In contrast, acinar size appeared to be reduced in sh δ 193 and sh δ 203 cells treated with ligand compared to shSCR cells (Figure 3A, panels g, h, i, m, n, o, insets). Indeed, quantification of structure area showed a significant decrease in acinar size in cells depleted of PKC δ as early as 3 days, which persisted through at least 8 days of growth (Figure 3A, panels g, h, i, m, n, o and 2B). In the absence of ligand, there were no significant differences in acinar size between sh δ 193, sh δ 203 and shSCR cells, suggesting that PKC δ is required specifically for ErbB2 driven proliferation (Figure 3B).

To ask if the decrease in acinar area correlated with reduced proliferation, control 10A.ErbB2 cells (shSCR), or 10A.ErbB2 cells depleted of PKC δ (sh δ 193 or sh δ 203) were plated on Matrigel (3D culture), or as monolayers on plastic (2D culture) and cell number was quantified. The addition of ligand significantly increased the total cell number of shSCR cells grown as 3D acini (Figure 3C) and as monolayers (Figure 3D) compared to untreated cells. For shSCR cells, the ligand-induced increase in cell number (Figure 3C and D) was considerably more robust than the increase in acinar area (Figure 3B), suggesting that additional mechanisms may contribute to acinar size. In contrast, in ligand-treated cells depleted of PKC δ (sh δ 193 and sh δ 203), no increase in cell number was detected under either growth condition (Figure 3C and D), suggesting an absolute requirement for PKC δ in ErbB2-induced proliferation.

Loss of PKC δ attenuates ErbB2 signal transduction

Our studies suggest that PKC δ is necessary to activate ErbB2 proliferative pathways; to determine how PKC δ regulates ErbB2 signaling, we treated 10A.ErbB2 cells in 3D culture (Figure 4A) and 2D culture (Figure 4B) with ligand and analyzed the activation of key signaling molecules. Addition of ligand to 10A.ErbB2 shSCR cells in 3D or 2D culture resulted in robust phosphorylation at two major ErbB2 autophosphorylation sites, Y1221/1222 and Y1248, which couple ErbB2 to the Ras/MAPK pathway through the binding of Shc and companion adaptor proteins, and at Y877, which has been shown to regulate the intrinsic kinase activity of ErbB2 (37–40). In cells depleted of PKC δ (sh δ 193 and sh δ 203), ligand-induced autophosphorylation of ErbB2 Y1221/1222 and Y1248 was slightly reduced, while phosphorylation of ErbB2 at Y877 was more dramatically reduced (Figure 4A and 4B, graphs) (40). Of note, total ErbB2 receptor protein was decreased in the absence of ErbB2 dimerization in shSCR cells, consistent with the findings that ErbB2 activation can decrease receptor turnover and stability (41–43).

Since depletion of PKC δ reduced phosphorylation of ErbB2 Y1221/1222, Y1248, and Y877, phosphorylation of downstream proteins was analyzed to determine signaling pathways regulated by PKC δ . Phosphorylation of ErbB2 has been shown to recruit the adaptor protein Shc, which can also signal through the Ras/MAPK pathway (39, 44, 45). However, only a small decrease in the level of pShc Y239/40 was observed in sh δ 193 and sh δ 203 cells compared to shSCR cells in 3D culture (Figure 4A), while no consistent decrease was

observed under 2D culture conditions (Figure 4B). Further studies have not detected a difference in the phosphorylation of Shc Y317 or GAB2, both of which have been implicated in downstream signaling through ErbB2 (data not shown). These results suggest that decreased signaling through pShc is unlikely to contribute to the decrease in pERK and proliferation seen with the depletion of PKC δ .

Src is recruited to ErbB2 through sequences surrounding the ErbB2 Y877 residue and is thought to be responsible for phosphorylation at this site (40, 46). (47, 48). Concomitant with reduced phosphorylation of ErbB2 at Y877, activation of Src, as indicated by phosphorylation at Y416, was dramatically suppressed in ligand treated sh δ 193 and sh δ 203 cells compared to shSCR cells (Figure 4A and 4B). Quantitation by densitometry showed that in 3D and 2D culture, phosphorylation of Src Y416 was reduced 50–80% in sh δ 193 and sh δ 203 cells compared to shSCR cells, which correlated with a 30–60% decrease in ErbB2 phosphorylation at Y877 (Figure 4A and 4B, graphs). Activation of Src regulates proliferative signaling through the Ras/MAPK pathway (49–51). As seen in Figure 4A and B, phosphorylation of ERK is dramatically reduced in cells depleted of PKC δ , suggesting a mechanism by which PKC δ suppresses ErbB2 driven proliferation.

Decreased activation of Src, combined with the reduced phosphorylation of ErbB2 Y877, suggests that PKC δ may be required for interaction of Src with the ErbB2 receptor. To address this possibility, 10A.ErbB2 cells depleted of PKC δ were treated with ligand, immunoprecipitated with antibodies to the HA-tagged ErbB2 receptor, and probed for HA, PKC δ , and Src. In the presence of ligand, Src was found to associate with the ErbB2 receptor in shSCR cells, supporting previous findings that activation of ErbB2 requires Src (Figure 4C). PKC δ was also found to immunoprecipitate with the ErbB2 receptor, and the binding of Src to the ErbB2 receptor was disrupted in cells depleted of PKC δ , demonstrating that PKC δ is required for formation of an active ErbB2 signaling complex.

PKC δ regulates proliferation in ErbB2 overexpressing human breast cancer cells

We next examined the contribution of PKC δ to ErbB2 regulated proliferation in human breast cancer cells that overexpress ErbB2. Depletion of PKC δ with sh δ 193 or sh δ 203 in MDA-MB-361, BT-474, and ZR-75-30 cells significantly reduced proliferation compared to shSCR control cells (Figure 5A). The largest decrease in proliferation was observed in BT-474 cells, where proliferation was reduced 59% and 38% in sh δ 193 or sh δ 203 cells, respectively. These results correspond to the relative depletion of PKC δ (Figure 5A, PKC δ insets). Analysis of ErbB2 mediated signaling showed that both Src and ERK phosphorylation were decreased in sh δ 193 and sh δ 203 cells compared to shSCR in all three cell lines, similar to that seen in 10A.ErbB2 cells (Figure 5B). Interestingly, in all cell lines the total level of Src appeared to be slightly reduced, a result that was not seen in 10A.ErbB2 cells, suggesting that PKC δ may regulate Src stability or turnover in breast cancer cells.

To determine if PKC δ regulates ErbB2 driven proliferation through the MAPK pathway, MDA-MB-361 and BT-474 cells with PKC δ knockdown were transduced with an adenovirus that expresses constitutively activated MEK (CA-MEK). Expression of CA-MEK activated ERK, and significantly rescued the reduction in proliferation seen with the

loss of PKC δ in sh δ 193 and sh δ 800 cells (Figure 5C, panel a). Similarly, when PKC δ was re-expressed in MDA-MB-361 cells depleted of PKC δ (sh δ 800), proliferation was rescued (Figure 5C, panel b), and this rescue was significantly reduced by pre-treatment with the ERK inhibitor, PD98059. Together these results indicate that PKC δ specifically regulates ErbB2 signaling through activation of the MAPK pathway.

Discussion

Our studies reveal PKC δ as a major regulator of ErbB2 mediated proliferation in breast cancer. Analysis of human ErbB2 positive breast cancer tumor samples shows a negative correlation between PKC δ expression and relapse free survival. Using a mouse model of ErbB2-dependent mammary gland tumorigenesis, we show that loss of PKC δ significantly increases tumor latency and suppresses tumor cell proliferation. Further, studies in human breast cancer cell lines support these findings and suggest that PKC δ is essential for activation of Src and ERK downstream of the ErbB2 receptor. Together these findings implicate PKC δ as a tumor promoter and a potential therapeutic target in ErbB2 overexpressing breast cancer.

We show that tumors from MMTV-ErbB2; δ KO mice have reduced proliferation and altered ErbB2 activation compared to MMTV-ErbB2; δ WT controls (Figure 2), and that PKC δ is necessary for induction of ErbB2 driven proliferation in 2D and 3D culture (Figure 3). An interesting observation is that depletion of PKC δ has a much more dramatic effect on 3D growth of MCF10A.ErbB2 cells, compared to 2D growth. Presumably, 3D culture provides a more complex signaling system by allowing not only cell-cell interactions, but also cell-matrix interactions, that are lacking in cells grown on plastic. We observed a similar phenomenon in lung cancer, where PKC δ was required for lung tumorigenesis *in vivo*, and for growth of NSCLC lines in soft agar, but not for growth on plastic (19).

Characterization of the proteins involved in ErbB2 signaling in the context of PKC δ depletion revealed a decrease in phosphorylation of the ErbB2 receptor at Y1221/1222, Y1248, and Y877 (Figure 4). We were unable to detect a change in activation of the adaptor protein, Shc; however, loss of PKC δ significantly suppressed Src activation, which likely contributes to the suppression of ERK and downstream proliferation. Phosphorylation at ErbB2 Y877 has been linked to the regulation of ErbB2's intrinsic kinase activity and loss of this phosphorylation results in a corresponding decrease in ErbB2 Y1221/1222 and Y1248 phosphorylation (40, 46, 52). These results suggests that the ErbB2 receptor may have decreased activity in cells lacking PKC δ , and that decreased auto-phosphorylation of ErbB2 at Y1221/1222 and Y1248 may be secondary to the decrease at ErbB2 Y877. Additionally, total ErbB2 levels decreased in response to EGF starvation (Figure 4A, -Ligand), and addition of the ErbB2 dimerization ligand restored ErbB2 expression. Cells with PKC δ knockdown have decreased expression of the ErbB2 receptor in response to dimerization compared to scrambled controls, indicating that either PKC δ regulates the stability of the receptor or that PKC δ effects ErbB2 translation. Further studies will need to be performed to directly explore this regulation.

Several reports have intimately linked Src with the phosphorylation of ErbB2 Y877 (40, 46). Marcotte et. al. demonstrated that Src associates with the ErbB2 receptor through sequences surrounding ErbB2 Y877 and is required for ErbB2 Y877 phosphorylation (46). Correspondingly, in our studies depletion of PKC δ results in a loss of Src activation (phosphorylation at Y416) and binding to the ErbB2 receptor, suggesting that PKC δ is required for formation of an active ErbB2 signaling complex with Src (Figure 4C). Reduced signaling through ErbB2/Src is likely the mechanism by which depletion of PKC δ suppresses ERK activation and proliferation. It is not clear from our current studies whether ErbB2 or Src are PKC δ kinase substrates; alternatively PKC δ may function as a scaffold in this complex. In NSCLC cell lines ERK activation can be inhibited by expression of a kinase-inactive form of PKC δ , suggesting that the kinase activity of PKC δ is important for activation of this proliferative pathway (19).

Limited studies in human breast cancer tissue, and in human breast cancer cell lines, have suggested that elevated PKC δ may correlate with more aggressive breast cancer (14, 22). Our analysis shows that PKC δ expression is significantly increased in ErbB2 positive versus negative human breast tumors. High expression of PKC δ also significantly correlates with decreased relapse-free survival in ErbB2 positive human breast cancer patients (Figure 1), supporting a role for PKC δ in the regulation of ErbB2 driven tumor formation. Further, a broad analysis of relapse-free survival over all molecular subtypes indicates that high PKC δ expression persistently correlates with a significant negative clinical outcome. Notably, this correlation does not appear to hold true across all subtypes, as our meta-analysis indicates that PKC δ expression is decreased in triple-negative breast cancers with no significant change in relapse-free survival (Figure 1). Interestingly, it has been proposed that ErbB2 overexpressing breast cancer cells can confer therapeutic resistance through an upregulation of Src activation (25). Moreover, Rexer et. al. show that lapatinib resistant cells contain increased levels of PKC δ phosphorylation at Y313, a site known to be phosphorylated by Src, compared to sensitive cells (26). Similarly, our current data indicate that PKC δ increases the occurrence of tumor relapse in ErbB2 positive tumors.

In contrast to numerous studies from our lab and others that demonstrate a pro-apoptotic function for PKC δ (12, 53–55), our current studies indicate a pro-proliferative role for PKC δ in breast cancer. Likewise PKC δ has also been shown to regulate proliferation in murine mammary cells, in mouse models of pancreatic and lung cancer, and in human NSCLC cells (19, 56, 57). Our studies specifically implicate PKC δ in regulating the MAPK pathway downstream of ErbB2 (Figure 5C). We propose that in some oncogenic contexts, the function of PKC δ may switch from pro-apoptotic to pro-survival. In this regard, NSCLC cells addicted to oncogenic K-ras appear to utilize PKC δ as a proliferative signal, while non-K-ras addicted NSCLC cell lines may preferentially utilize PKC δ for apoptosis ((19) and unpublished data).

Taken together, our studies suggest a unique regulatory mechanism by which PKC δ regulates ErbB2 signal transduction (Figure 5D). We show that in cells in which ErbB2 is activated, Src and ErbB2 are found in a complex. This association correlates with increased phosphorylation of ErbB2 at Y877, Y1221/1222, and Y1248 and increased proliferation. Loss of PKC δ disrupts this complex, resulting in decreased association of Src with the

ErbB2 receptor, as well as a decrease in ErbB2 and Src activation. Since Src is responsible for the phosphorylation of ErbB2 Y877, a site that has been associated with maintaining ErbB2 activity, loss of Src binding to the ErbB2 receptor ultimately leads to an attenuation of ErbB2 driven proliferation. Together these studies support a critical role for PKC δ in the regulation of ErbB2 signaling and proliferation both *in vivo* and *in vitro* and support future investigation of PKC δ as a therapeutic target in human breast cancer.

Materials and Methods

Animal Model

FVB PKC δ KO mice (δ KO) have been previously described (19). MMTV-ErbB2 (line 202) transgenic mice (32) were obtained from the Jackson Laboratory, Bar Harbor, ME, USA and crossed to FVB δ KO mice. Genotypes were determined by PCR using primers to the ErbB2 transgene (forward primer (Neu-3) 5'-CGGAACCCACATCAGGCC-3'; reverse primer (Neu-4) 5'-TTTCCTGCAGCAGCCTACGC-3'). Animals were maintained at the University of Colorado Denver Anschutz Medical Campus in accordance with Laboratory Animal Care guidelines and protocols and approved by the Institutional Animal Use and Care Committee. Virgin MMTV-ErbB2; δ WT and MMTV-ErbB2; δ KO mice were palpated weekly for mammary tumors, starting at 120 days of age. MMTV-ErbB2; δ WT littermates were used for all studies. Tumors were harvested at 1 cm in diameter, fixed in 10% neutral buffered formalin, processed and paraffin embedded for sectioning.

Cell lines and reagents

MCF10A.ErbB2 (10A.ErbB2) cells were a generous gift from Senthil Muthuswamy and previously described (33, 34). 10A.ErbB2 cells were kept in growth media (Dulbecco's modified Eagle medium/F12 (DMEM/F12) (HyClone, Thermo Fisher Scientific, Logan, UT, USA) supplemented with 5% donor horse serum (Gibco, Grand Island, NY, USA), 20 ng/mL epidermal growth factor (EGF) (BD Transduction Laboratories, Bedford, MA, USA), 10 mg/mL insulin (Gibco), 1 ng/mL cholera toxin (Thermo Fisher Scientific), 100 mg/mL hydrocortisone, and 200 mg/mL active-G418 (Gibco)). The ErbB2 dimerizers (ligand), AP1510 (Ariad, Cambridge, MA, USA) and B/B homodimer (Clontech, Mountain View, CA, USA) were used at 250–500 nM for 2D cell culture and 1 μ M for 3D cell culture. Growth factor reduced Matrigel was purchased from BD Transduction Laboratories. Human breast cancer lines were obtained from the University of Colorado Denver Cancer Center Tissue Culture Core.

Microarray data analysis

OncomineTM (Compendia Bioscience, Ann Arbor, MI) was used for analysis of mRNA expression data from 21 human breast cancer studies (28). Methods of normalization and statistical calculations are provided on the Oncomine website (www.oncomine.org). Gene expression was compared within ErbB2 positive versus negative tumors. Differential expression of the individual genes analyzed was then ranked by p-value across each data set, with a rank of one being the most significantly changed gene in ErbB2 positive tumors. The median rank of PKC δ was calculated across all 21 data sets and analyzed for significance of overexpression or underexpression. Similar analysis was used to determine PKC δ expression

in triple-negative tumors versus all other molecular subtypes. GEO accession numbers or paper references for the 21 datasets are as follows: 1. [GSE3143](#) 2. [GSE2109](#) 3. [GSE5847](#) 4. [GSE6861](#) 5. [GSE22358](#) 6. [GSE25066](#) 7. [GSE8465](#) 8. [GSE20685](#) 9. [GSE18728](#) 10. [GSE5460](#) 11. Ma Breast (58) 12. [GSE1379](#) 13. [GSE14548](#) 14. [GSE2603](#) 15. Perou Breast (59) 16. [GSE3744](#) 17. [GSE5325](#) 18. [GSE3893](#) 19. The Cancer Genome Atlas Project (TCGA) Breast <http://tcga-data.nci.nih.gov/tcga/> 20. [GSE21921](#) 21. [GSE3971](#).

Meta-analysis of PKC δ gene expression and correlation with clinical relapse-free survival was analyzed over four data sets (GSE7390, GSE3494, GSE1456, and GSE6432) encompassing 914 individual tumors using the GOBO tool (<http://co.bmc.lu.se/gobo>) as previously described (29). ErbB2 enriched tumors were identified from the four data sets (listed above) through the PAM50 gene set signature and represent 122 individual tumors, while basal tumors represent 143 tumors (30).

PKC δ depletion and reconstitution

Stable depletion of PKC δ was achieved using lentiviral constructs containing short hairpin RNA (shRNA) to human PKC δ (pLKO-TRC00010193 (sh δ 193), pLKO-TRC00010203 (sh δ 203), or pLKO-TRCN0000284800 (sh δ 800)) or a shRNA control (pLKO-scrambled (shSCR)) (Open Biosystems, Huntsville, AL, USA or Sigma-Aldrich, St. Louis, MO) as previously described (19). Adenovirus expression of LacZ, PKC δ or CA-MEK was done as previously described (60). Adenovirus transduction efficiency was >95% as verified by visualization of GFP-tagged protein by fluorescent microscopy.

Immunohistochemistry

For immunohistochemistry, sections were exposed to citrate buffer and heat antigen retrieval and then blocked with avidin and biotin. Endogenous peroxidase was quenched with 3% H₂O₂. Sections were incubated with primary antibody for 1 hour at room temperature (Anti-cleaved caspase-3 and Ki-67). Primary antibodies include anti-cleaved caspase-3 (1:100; Cell Signaling Technology, Beverly, MA, USA) and anti-Ki-67 (1:50; DakoCytomation, Carpinteria, CA, USA). Sections were incubated in appropriate secondary for 30 min at room temperature. Sections were incubated with Vectastain ABC Reagent (Vector Laboratories) for an additional 30 minutes at room temperature. The signal was detected with diaminobenzadine substrate (Sigma, St. Louis, MO, USA) and tissues were counterstained with Gill's hematoxylin. For quantification of Ki-67 and cleaved caspase-3 staining, tumor sections were scanned using Aperio hardware and software (Vista, CA, USA). Six-eight fields were outlined (per tumor, per genotype), which encompassed the majority of non-border and non-necrotic regions of each tumor section. Staining was quantified and expressed as the percent of positive cells utilizing the Aperio color deconvolution algorithm. n=5–8 MMTV-ErbB2; δ WT, 4–7 MMTV-ErbB2; δ KO.

3D Morphogenesis Assay

Growth factor reduced Matrigel (40 μ l) was plated on eight well chamber slides (#354230, #354118; BD Transduction Laboratories) and allowed to solidify for >15 minutes at 37°C. 10A.ErbB2 cells (5,000 cells/well) were trypsinized and resuspended in plating media (DMEM/F12 supplemented with 2% donor horse serum, 10 mg/ml insulin, 1 ng/ml cholera

toxin, 100 mg/ml hydrocortisone, 5 ng/ml EGF, 200 mg/mL active-G418) with 2% Matrigel. On day 5 cells were switched to assay media without EGF and 24 hours later cells were left untreated or treated with ligand (1 μ M). Ligand was replenished every two days. Cells were imaged live on day 6, 9, and 14 using the Zeiss Observer.Z1 microscope (Thornwood, NY, USA) and captured using Volocity 6.0 software (Perkin Elmer, Waltham, MA, USA). 5X binary pictures of each condition were then used to quantify structure area using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA); $n \geq 200$ structures, representative of 3 experiments shown.

2D and 3D growth curves

For 2D culture growth curves, BT-474, MDA-MB-361, ZR-75-30 and 10A.ErbB2 cells expressing either shRNA to PKC δ (sh δ 193 and sh δ 203) or a scrambled control (shSCR) and were plated at equal densities. 10A.ErbB2 cells were plated in growth media. Twenty-four hours after plating, media was changed to assay media and 48 hours after plating cells were treated with ligand in assay media. For 3D growth curves, 10A.ErbB2 cells were grown as described above (3D morphogenesis assay). Three days after the addition of ligand, acini were treated with 0.25% trypsin for 1 hour at 37°C and cells were counted by hemocytometer.

Immunoblot analysis

For 3D cultures, acini were washed in ice cold PBS then treated with lysis buffer for 1 hour at 4°C with continuous shaking. Lysates were spun at 12,500 rpm for 20 minutes at 4°C, and supernatants from 2D and 3D culture was then separated by SDS/PAGE and immunoblotting as previously described (61). Anti-PKC δ (C-20) was purchased from Santa Cruz Biotechnology (sc-937 Santa Cruz, CA, USA). The following antibodies were purchased from Cell Signaling Technologies: Phospho-ErbB2 (Tyr877) (#2241; pErbB2 Y877); phospho-ErbB2 (Tyr1221/1222) (#2249; pErbB2 Y1221/1222); ErbB2 (#2165); phospho-Src Family (Tyr416) (#2101; pSrc); Src (#2108); phospho-Shc (Tyr239/240) (#2434; pShc); Shc (#2432); phospho-ERK1/2 (#9101; pERK1/2); and ERK1/2 (#4695). Phospho-ErbB2 (Tyr1248) (ab47755) and actin-HRP (ab49900) were purchased from Abcam (Cambridge, MA, USA). Densitometry was performed using UVP VisionWorks LS software.

Immunoprecipitation

10A.ErbB2 cells were placed in media without EGF for 24 hours, and then treated with ErbB2 ligand (B/B homodimer, 250 nM) for 2 hours. immunoprecipitation was performed as previously described (61). Five-hundred μ g of protein was incubated with primary antibody (2 μ g; anti-HA; #MMS-101, Covance) or control (2 μ g; anti mouse IgG; #2025; Santa Cruz Biotechnology) for three hours at 4°C. Immunoblots were probed with the following antibodies: anti-HA (#MMS-101, Covance), Src (#8056, Santa Cruz) and PKC δ (#sc-937 Santa Cruz Biotechnology).

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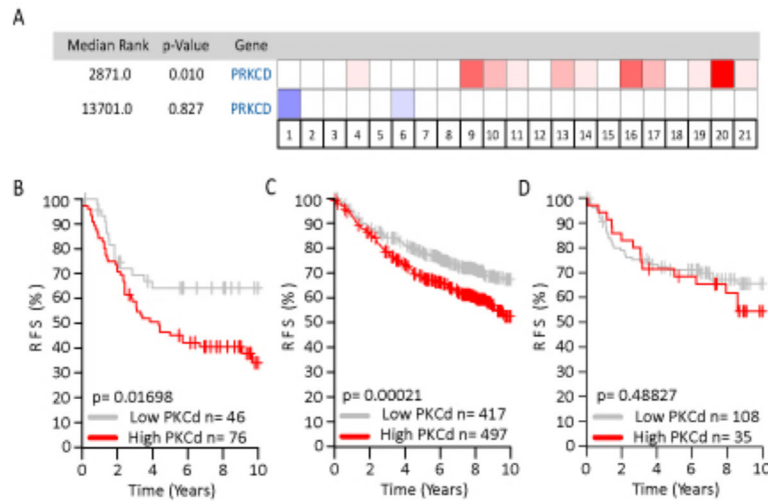


Figure 1. Increased expression of PKC δ negatively correlates with prognosis in ErbB2 positive human breast cancer

A. Overexpression (red) and underexpression (blue) of PKC δ in 21 human breast cancer data sets. Intensity of the cell color corresponds to the top percentage of ranked genes, with 50% (white) representing no change between tumor sets. The median rank for PKC δ overexpression in ErbB2 positive tumors across each data set is 2,871 ($P=0.01$), while the underexpression median rank is 13,701 ($P=0.827$). **B, C, and D.** Kaplan-Meier analysis, using relapse-free survival as endpoint, for **(B)** ErbB2 positive tumors (four data sets, $n=122$, $P=0.017$), **(C)** All molecular subtypes (four data sets, $n=914$, $P=0.0002$), and **(D)** Triple-negative (ER-/PR-/ErbB2-)(four data sets, $n=143$, $P=0.488$), stratified into two quantiles base on high (red line) or low (gray line) PKC δ expression.

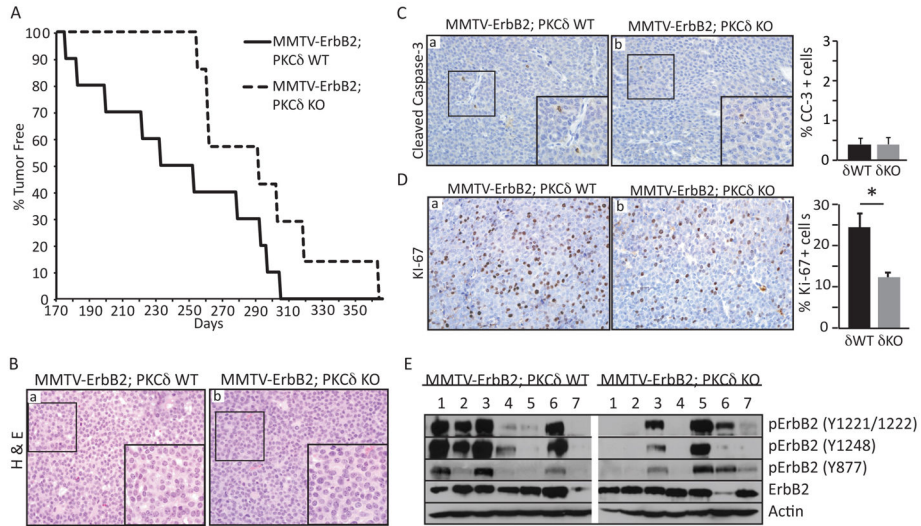


Figure 2. Loss of PKC δ increases tumor latency in MMTV-ErbB2 transgenic mice
A. MMTV-ErbB2; δ WT (n=10) and δ KO (n=7) mice were palpated weekly to detect mammary tumor growth. Graph shows the number of days to tumor detection versus the percentage of tumor-free mice for each genotype; $P=0.03$. **B.** MMTV-ErbB2; δ WT and MMTV-ErbB2; δ KO tumor sections were stained with hematoxylin and eosin (a, b). Histology- 20X, insets depict 60X enlargement of boxed region. **C and D.** Cleaved caspase-3 (**C**) and Ki-67 (**D**) immunohistochemistry on MMTV-ErbB2; δ WT and MMTV-ErbB2; δ KO tumor sections. Histology- 20X, insets depict 60X enlargement of boxed region. Graphs depict quantification of the percent of cleaved caspase-3 or Ki-67 positive cells over 6–8 fields per tumor, per genotype; n=5–8 MMTV-ErbB2; δ WT, 4–7 MMTV-ErbB2; δ KO; $*P=0.01$. **E.** Tumor lysates from MMTV-ErbB2; δ WT (7) and δ KO (7) mice were immunoblotted with the following antibodies to determine the levels of ErbB2 receptor phosphorylation: pErbB2 (Y877, Y1221/1221, Y1248); ErbB2; and actin.

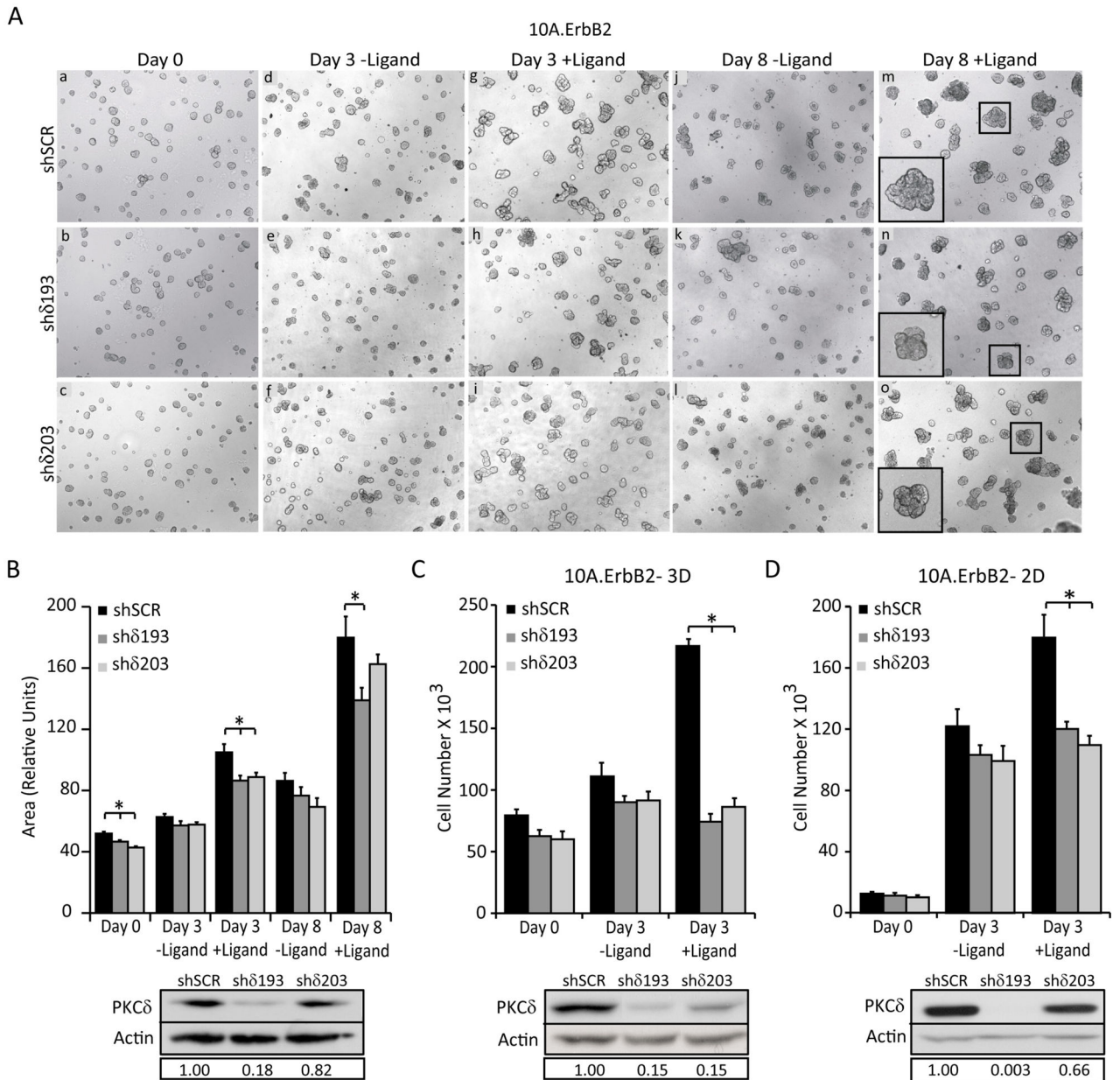


Figure 3. PKC δ is required for ErbB2-driven proliferation

For all panels: PKC δ was depleted using lentiviral shRNA constructs (sh δ 193 and sh δ 203) and compared to control shRNA (shSCR) as described in Materials and Methods. **A.** 10A.ErbB2 cells depleted of PKC δ using shRNA (sh δ 193 and sh δ 203) were grown on Matrigel for 6 days (a, b, c). Cells were then left untreated (d, e, f; j, k, l) or treated with 1 μ M ligand for 3–8 days (g, h, i; m, n, o). Representative images of three separate experiments taken at 5X magnification are shown. Inset shows digital enlargement to show structure morphology. **B.** Quantification of structure area from Figure 2A, using Metamorph software. Representative of three experiments shown; * P < 0.02. **D. and E.** 10A.ErbB2 cells depleted of PKC δ were plated at equal densities in 3D (**D**) or 2D (**E**) growth assays.

For 3D growth, cells were plated on Matrigel and grown for 6 days. Ligand was added for 3 days after which cells were trypsinized and counted; $*P < 0.0001$. For 2D growth, cells were treated with or without ligand for 4 days, trypsinized, and counted; $*P < 0.04$. **B, C, and D.** Insets show depletion of PKC δ by immunoblot with actin as a loading control. Boxed region below blots shows relative PKC δ knockdown compared to shSCR controls. Representative graphs of three separate experiments are shown.

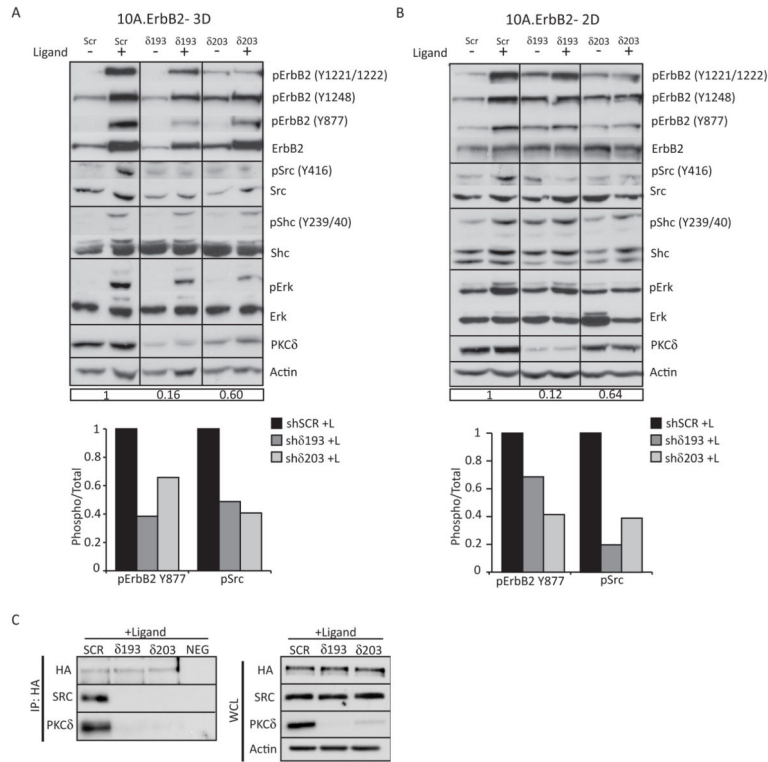


Figure 4. Loss of PKCδ attenuates ErbB2 signal transduction

For all panels: PKCδ was depleted using lentiviral shRNA constructs (shδ193 and shδ203) and compared to control shRNA (shSCR) as described in Materials and Methods. **A. and B.** 10A.ErbB2 cells depleted of PKCδ were plated in 3D culture (**A**) or 2D culture (**B**) and then treated for 3 days or 24 hours with ligand respectively. Protein expression and phosphorylation in whole cell lysates was evaluated by immunoblotting. The following antibodies were used: pErbB2 (Y877, Y1221/1221, Y1248); ErbB2; pSrc (Y416); Src; pShc (Y239/240); Shc, pErk1/2 (T202/Y204); Erk1/2; PKCδ; and actin. Immunoblots probed for phosphorylated proteins were stripped and probed with antibodies to total protein, as indicated. Blots were probed for actin as a loading control. Relative PKCδ knockdown compared to shSCR is shown in boxed region below blots. Immunoblots were quantified by densitometry; graphs depict the levels of phosphorylated pErbB2 Y877 and pSrc Y416 over total protein levels upon ligand treatment. Representative experiments shown; experiments were repeated a minimum of three times. **C.** 10A.ErbB2 cells depleted of PKCδ were treated for 24 hours with ligand. Cells were lysed and ErbB2 was immunoprecipitated with anti-HA primary antibody. Immunoprecipitated protein was resolved on an SDS-page gel and probed for HA, PKCδ and Src. Lysates were incubated with a non-specific mouse IgG as a negative control (NEG). Whole cell lysates (WCL) were probed for HA, PKCδ, and Src. Actin was used as a loading control. Representative experiments shown; experiments were repeated a minimum of three times.

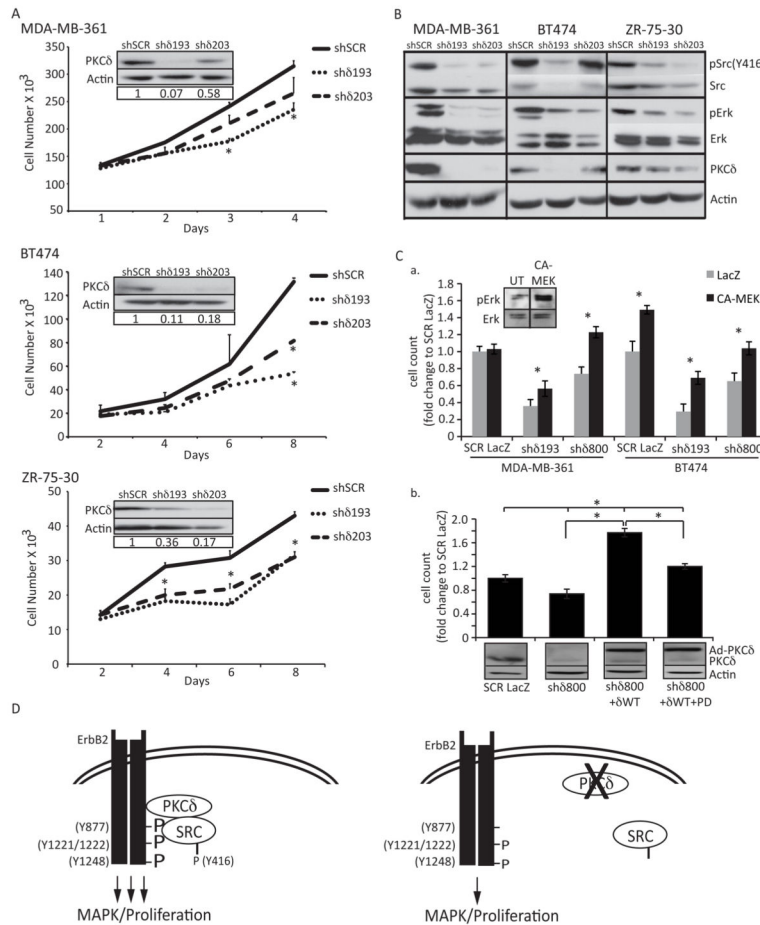


Figure 5. PKC δ regulates proliferation in ErbB2 overexpressing human breast cancer cells
A. MDA-MB-361, BT-474, and ZR-75-30 cells were depleted of PKC δ as described in Materials and Methods. Cells were plated at equal densities and counted at four time points. Representative experiments shown; experiments were repeated a minimum of three times, $p < 0.05$. Insets show depletion of PKC δ by immunoblot with actin as a loading control. **B.** Whole cell lysates from MDA-MB-361, BT-474, and ZR-75-30 cells were resolved on an SDS-page gel and probed with the following antibodies: pSrc (Y416); Src; pERK1/2 (T202/Y204); ERK1/2; PKC δ . Immunoblots probed for phosphorylated proteins were stripped and probed with antibodies to total protein, as indicated. Blots were probed for actin as a loading control. Representative experiments are shown; experiments were repeated a minimum of three times. **C., panel a,** MDA-MB-361 and BT474 cells with PKC δ knockdown (sh δ 193 or sh δ 800) were transduced with an adenovirus encoding either LacZ or a constitutively activated MEK (CA-MEK) MOI=100). Cells were counted on day 5 (BT-474) or day 6 (MDA-MB-361). Inset shows pERK activation upon 48 hours of CA-MEK expression in BT-474 cells. **Panel b,** MDA-MB-361 cells (SCR and sh δ 800) were transduced with adenovirus encoding either LacZ or GFP-PKC δ WT (MOI=100). On the subsequent day media was replaced and 10 μ M PD98059 was added to the indicated wells; cells were counted on day 6. Lysates were probed for PKC δ and actin. **D.** Model: Loss of PKC δ decreases the phosphorylation and binding of Src to the ErbB2 receptor, resulting in

decreased ERK signaling and abrogated ErbB2 driven proliferation. See text for detailed discussion.