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PKCλ/ι signaling promotes triple-negative breast cancer growth and metastasis

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Triple-negative breast cancer (TNBC) is a distinct breast cancer subtype defined by the absence of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2/*neu*), and the patients with TNBC are often diagnosed with higher rates of recurrence and metastasis. Because of the absence of ER, PR and HER2/*neu* expressions, TNBC patients are insensitive to HER2-directed and endocrine therapies available for breast cancer treatment. Here, we report that expression of atypical protein kinase C isoform, PKCλ/ι, significantly increased and activated in all invasive breast cancer (invasive ductal carcinoma or IDC) subtypes including the TNBC subtype. Because of the lack of targeted therapies for TNBC, we choose to study PKCλ/ι signaling as a potential therapeutic target for TNBC. Our observations indicated that PKCλ/ι signaling is highly active during breast cancer invasive progression, and metastatic breast cancers, the advanced stages of breast cancer disease that developed more frequently in TNBC patients, are also characterized with high levels of PKCλ/ι expression and activation. Functional analysis in experimental mouse models revealed that depletion of PKCλ/ι significantly reduces TNBC growth as well as lung metastatic colonization. Furthermore, we have identified a PKCλ/ι-regulated gene signature consisting of 110 genes, which are significantly associated with indolent to invasive progression of human breast cancer and poor prognosis. Mechanistically, cytokines such as TGFβ and IL1β could activate PKCλ/ι signaling in TNBC cells and depletion of PKCλ/ι impairs NF-κB p65 (RelA) nuclear localization. We observed that cytokine-PKCλ/ι-RelA signaling axis, at least in part, involved in modulating gene expression to regulate invasion of TNBC cells. Overall, our results indicate that induction and activation of PKCλ/ι promote TNBC growth, invasion and metastasis. Thus, targeting PKCλ/ι signaling could be a therapeutic option for breast cancer, including the TNBC subtype.
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Breast cancer is a clinically heterogeneous disease and both intra and inter-tumor heterogeneities provide great challenges for developing successful therapies. Expressions (or absence thereof) of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2)/*neu* are widely used to clinically classify breast tumors into multiple therapeutic groups.[1] The ER/PR-positive and the HER2-positive breast cancer patients could be benefited from endocrine and HER2-targeted therapies.[1] However, triple-negative breast cancers (TNBCs), which represent ~12–17% of all breast cancer,[2] lack ER, PR and HER2/*neu* expressions[2] and are not responsive to therapies targeting these receptors. Therefore, the only systemic therapy available for TNBC is chemotherapy.[3] Furthermore, TNBC is associated with aggressive pathologic features like higher histology grade and mitotic index[4] and often found to be associated with higher rate of metastasis and recurrence leading to limited clinical outcome.[5–8] Recurrence of TNBC tends to recur within a few years after successful initial treatment[6,9] and often develops metastasis to the bone, brain and lungs with poor prognosis.[2,6] Thus, identification of signaling pathways that regulate malignant progression of breast cancer subtypes, especially TNBCs, would be therapeutically important.

In recent years, PKC signaling has been implicated in modulating invasion and metastasis of multiple tumors.[10,11] The PKC family consists of multiple serine/threonine kinases and the relative contribution of individual PKC isoforms during cancer progression varies due to pleiotropism.[12] PKC isoforms regulate diverse cellular functions such as cell-cycle

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Abbreviations: AJC, apical-junctional complex; ADH, atypical ductal hyperplasia; BLI, bioluminescence imaging; BRCA1, breast cancer 1, early onset; DAB2, Dab, mitogen-responsive phosphoprotein, homolog 2; DCIS, ductal carcinoma *in situ*; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, epidermal growth factor receptor 2; HIF1α, hypoxia-inducible factor 1alpha; ICAM1, intercellular adhesion molecule 1; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; IL1β, interleukin-1 beta; MRPL19, mitochondrial ribosomal protein L19; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAR3, partitioning defective 3 homolog; PAR6, partitioning defective 6 Homolog; PKCλ/ι, atypical protein kinase C lamda/iota; PKCζ, atypical protein kinase C zeta; PKCλ/ιIS110, PKCλ/ι invasive signature 110; PLAU, plasminogen activator, urokinase; PR, progesterone receptor; PSMC4, proteasome (prosome, macropain) 26 S subunit, ATPase, 4; PUM1, pumilio RNA-binding family member 1; RNA, ribonucleic acid; RNAi, RNA interference; SMAD3, SMAD family member 3; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor beta; TNBC, triple-negative breast cancer; TP53, tumor Protein p53; VIM, vimentin

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regulation, cellular survival, cell–cell communications and apoptosis.[13] In particular, atypical PKC isoforms, PKCζ and atypical protein kinase C lamda/iota (PKCλ/ι), are known to be important for chemotaxis, cell polarity, migration and wound healing processes.[14,15] Aberrations in all these processes are manifested in tumor progression and metastasis.[14] Consistent with these notions, recent studies indicated that atypical PKCs are associated with various human cancers.[10,11] Importantly, the *PKC*λ/ι gene is located at the 3q26.2 genomic region, which is most frequently amplified in human cancer[16,17], and overexpression of PKCλ/ι has been implicated in cancer development in multiple tissues including the lung,[18,19] pancreas,[20] stomach,[21] colon,[22] esophagus,[23] liver,[24] bile duct,[25] ovary,[17] prostate[26] and brain.[27] Recently, few studies have been reported higher expression of PKCλ/ι in ER/PR- and HER-positive breast cancer and also in lymph node metastases.[28,29] Kojima *et. al.*[28] showed that PKCλ/ι expression is highly induced in the ER/PR- and HER2-positive IDCs compared with ductal carcinoma *in situ* (DCIS) and normal breast. PKCλ/ι forms apical-junctional complexes (AJCs) with other polarity proteins such as partitioning defective 3 homolog (PAR3) and partitioning defective 6 homolog (PAR6),[30–33] and invasiveness of breast tumor cells was shown to be associated with loss of PKCλ/ι localization from their apical domains.[28] In addition, predominant nuclear localization of PKCλ/ι in both normal and atypical ductal hyperplasia (ADH) lesions prompted the concept that PKCλ/ι might be in an inactive state in these lesions.[28] However, expression and activation of PKCλ/ι in TNBCs and the functional importance of PKCλ/ι signaling in relation to invasive breast cancer progression and metastasis are very poorly understood.[10,11]

Here, we studied PKCλ/ι signaling during invasive progression of TNBC. We utilized expression evaluations in triple-negative IDCs as well as metastatic breast cancers of human patients, *in vitro* and *in vivo* functional assays, and global gene expression analysis of human patient samples. We concluded that PKCλ/ι signaling is an important regulator for invasion and metastatic progression of human breast cancers including triple-negative subtypes.

Results

PKCλ/ι is highly active in human TNBCs and metastatic breast cancers. To investigate PKCλ/ι signaling in breast cancer disease progression, we tested expression of PKCλ/ι in human breast sample cohort, consisting of normal breasts, DCIS and IDC samples of ER-positive, HER2-positive and TNBC subtypes (Figures 1a–d, Supplementary Figure S1a and S1b). Immunohistochemical analysis of PKCλ/ι expression indicated significant increase in ER- and HER2-positive IDC samples compared with normal breast and DCIS (Figure 1a, Supplementary Figures S1c and d) and supported previous reports.[28,29] Importantly, we also observed significant higher expression of PKCλ/ι in TNBC subtypes compared with normal breast and DCIS (Figures 1a–c, Supplementary Figures S1c and d). In all IDC samples, the induced PKCλ/ι localization was detected in both cytoplasm and nuclei, (Figure 1d and Supplementary Figure S1b) with a few cases showing focal (5–25%) nuclear staining.

To test the distribution of activated form of PKCλ/ι, we used a previously validated antibody[34–36] that selectively recognizes PKCλ/ι molecules, phosphorylated at threonine 555 (T555) and threonine 563 (T563) residues in the catalytic domain[15,37] (phospho-PKCλ/ι). We observed that phospho-PKCλ/ι levels were also significantly increased from normal breast to DCIS to IDC samples of all clinical subtypes (Figures 1b–d, Supplementary Figures S1e and f). Interestingly, in contrast to total PKCλ/ι, we observed predominant nuclear localization of phospho-PKCλ/ι in all IDC samples (Figure 1d and Supplementary Figure S1b). As phosphorylation event (specifically at T555) is believed to prime activation of PKCλ/ι,[38] our observations indicate predominant localization of active PKCλ/ι in the nuclei of breast tumor cells and oppose the idea that inactive PKCλ/ι localizes within nuclei, as suggested earlier.[28]

Next, we collected multiple human metastatic breast cancer samples ($n = 10$) from distal organs such as the bone, brain, chest wall, colon, gallbladder, liver, lung and ovary to test PKCλ/ι expression and phosphorylation (Figure 1e). We found that similar to IDC and lymph node metastasis,[29] PKCλ/ι is highly expressed and phosphorylated in the metastatic breast cancers with predominant nuclear localization of phospho-PKCλ/ι (Figure 1e). Collectively, these results and previous reports[28,29] strongly correlate higher expression and activation of PKCλ/ι with invasive progression of breast cancer including TNBC subtypes to develop local and distal metastasis.

PKCλ/ι regulates epithelial morphology, invasion and migration of triple-negative breast cancer cells. On the basis of our observation in human patient samples, we hypothesized that PKCλ/ι signaling could promote invasion and metastasis of TNBC. Therefore, we specifically depleted PKCλ/ι in MDA-MB-231 cells, a highly invasive and metastatic TNBC cell line, via RNA interference (RNAi) (Figure 2a and Supplementary Figure S2). We found that specific depletion of PKCλ/ι induced mesenchymal-to-epithelial transition (MET)-like response in MDA-MB-231 cells (Figures 2b and c). Notably, these morphological changes of MDA-MB-231 also associated with significant inhibition in invasion through Matrigel-coated transwells and also migration during wound closure assays (Figures 2d–g). To test whether PKCλ/ι signaling is a common positive regulator of invasion and migration, we depleted PKCλ/ι in multiple TNBC cell lines (Supplementary Figure S3a). We found that depletion of PKCλ/ι significantly inhibited invasion and migration of multiple triple-negative breast cancer cells including MDA-MB-468 (EGFR-expressing and *TP53* mutant),[39] BT-20 (constitutively active ER carrying deletion of exon 5, EGFR-expressing and *TP53* mutant)[40] and HCC-1937 (EGFR-expressing, null for p53 expression and homozygous breast cancer 1, early onset (BRCA1) mutation carrier)[41] (Supplementary Figures S3b and c).

We checked survival and proliferation of all the PKCλ/ι-depleted TNBC cells and found no significant difference compared with the controls (Supplementary Figure S3d). These results indicate that specific depletion of PKCλ/ι can inhibit the invasive potential of TNBC cells regardless of their *TP53* status, EGFR expression and *BRCA1* mutation without affecting their survival *in vitro*.

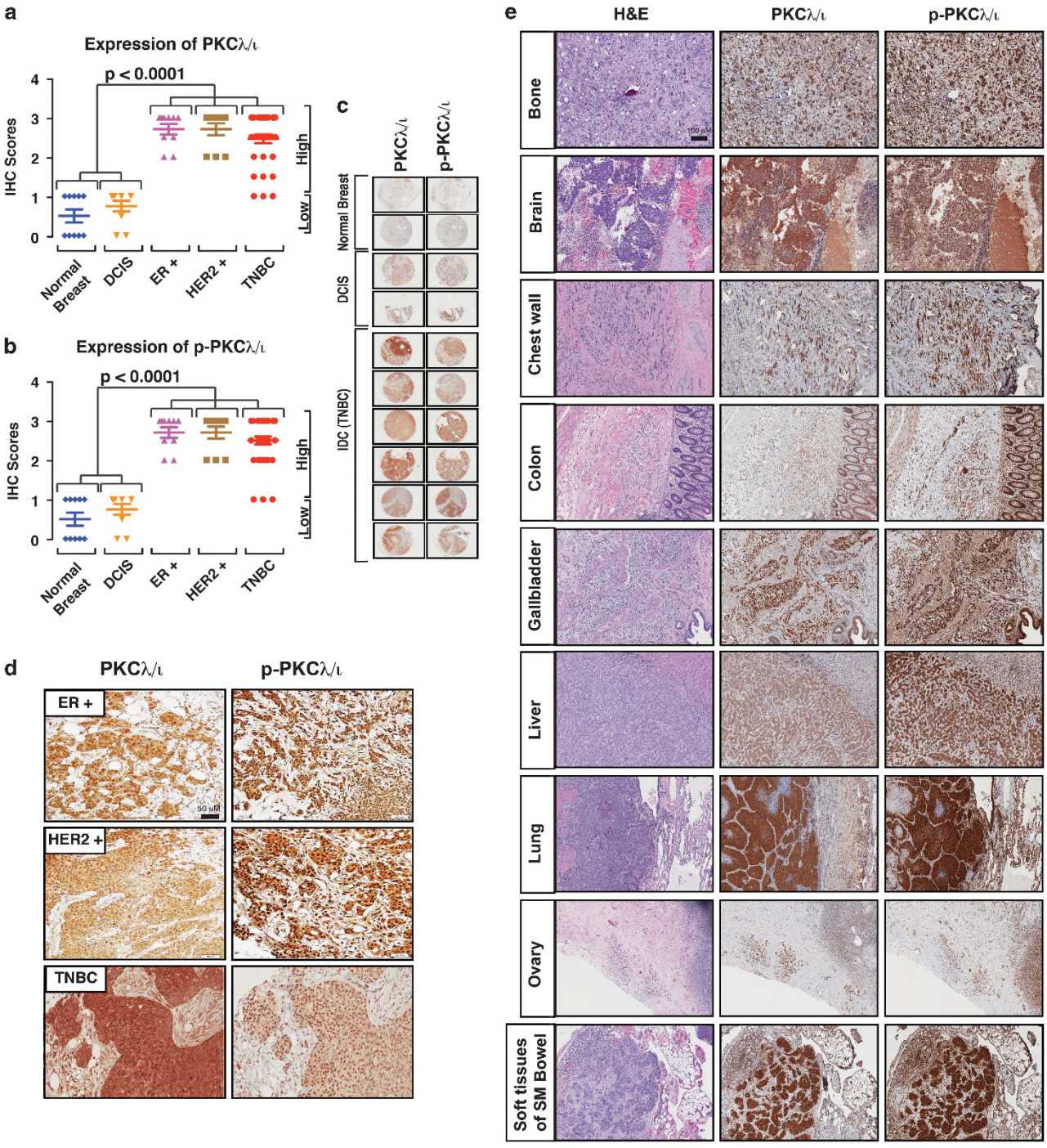


Figure 1 Human breast cancers are associated with higher expression and phosphorylation of PKCλ/ι. Expression of PKCλ/ι (**a**) and phospho- PKCλ/ι (**b**) determined by Immunohistochemistry (IHC) in normal breast ($n = 10$), DCIS ($n = 10$) and IDC samples of ER + subtype ($n = 10$), HER2 + subtype ($n = 10$) and triple-negative subtype ($n = 35$). Results were expressed as IHC scores of individual samples (by two independent pathologists) using a scale 0–3. IHC scores in between 0 to 1 considered as low expression, whereas IHC scores > 1 considered as high expression. Expression of PKCλ/ι and phospho-PKCλ/ι in all IDC subtypes were significantly higher compared with DCIS and normal breast. *P*-values were calculated by two-way ANOVA with Bonferroni post-test (see also Supplementary Figure S1c–f for detailed statistical analysis). (**c**) Representative images of PKCλ/ι and phospho-PKCλ/ι expression in human normal breast, DCIS and triple-negative IDC samples. Expression of PKCλ/ι and phospho-PKCλ/ι gradually increased from normal breast to DCIS, and further increased in triple-negative IDCs. (**d**) Representative images showing localization of PKCλ/ι and phospho- PKCλ/ι in ER +, HER2 + and triple-negative IDCs. (**e**) Expression of PKCλ/ι and phospho-PKCλ/ι in human metastatic breast cancers ($n = 10$)

PKCλ/ι regulates TNBC growth, invasion and metastasis *in vivo*. Our *in vitro* analyses indicated that PKCλ/ι depletion in TNBC cells strongly inhibits their invasive potential (Figure 2 and Supplementary Figure S3). Furthermore, analysis in human patient samples showed that PKCλ/ι expression and phosphorylation are induced in all IDC

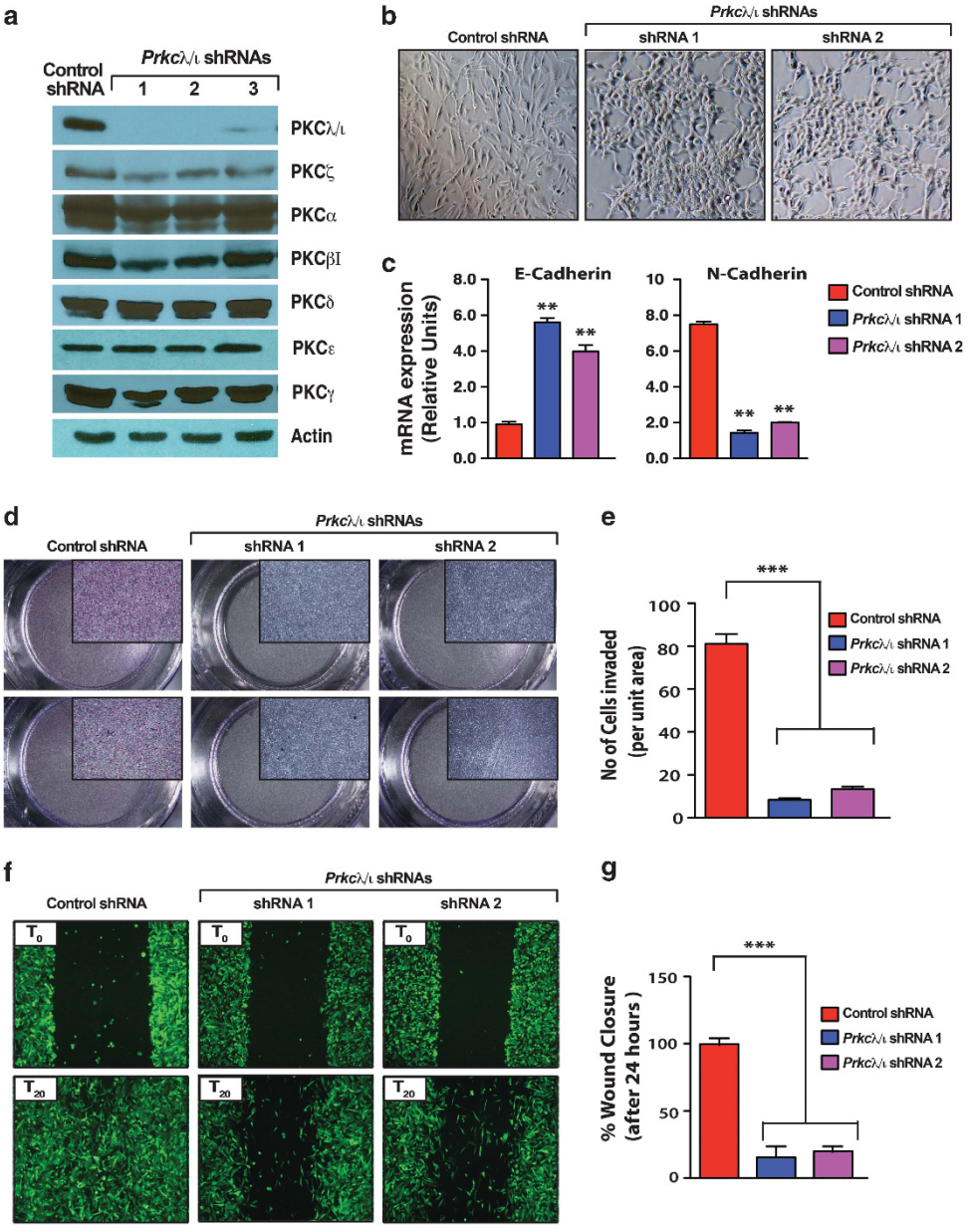


Figure 2 Depletion of PKCλ/ι signaling promotes MET and inhibits invasion and migration of breast cancer cells. (**a**) Specific knockdown of PKCλ/ι in MDA-MB-231 cells. (**b**) Morphologies of PKCλ/ι-depleted MDA-MB-231 cells. (**c**) Expression of epithelial marker E-Cadherin and basal marker N-Cadherin in PKCλ/ι-depleted MDA-MB-231 cells ($n=3$). (**d**) Transwell invasion of PKCλ/ι-depleted MDA-MB-231 cells. Inset represents higher magnification image of the corresponding transwell filter. (**e**) Quantification of invasion assays (each field was divided into nine unit areas and three fields per condition). (**f**) Wound closure assays of PKCλ/ι-depleted MDA-MB-231 cells. (**g**) Quantification of wound closure assays ($n=3$). For all quantifications, results represent means ± S.E.M. *P*-values were calculated by two-tailed unpaired Student's *t*-test. $^{**}P\leq 0.01$, $^{***}P\leq 0.001$

subtypes including TNBC and metastatic breast cancers (Figure 1). Thus, we tested whether PKCλ/ι signaling could promote TNBC growth and invasion *in vivo*. We orthotopically transplanted luciferase reporter expressing MDA-MB-231 (MDA-MB-231-luc) cells with or without PKCλ/ι depletion, into the mammary glands of immunodeficient mice ($n=5$) and monitor tumor growth via bioluminescence imaging (BLI) (Figure 3a). We found that loss of PKCλ/ι resulted in significant inhibition in primary tumor growth within 5 weeks (Figure 3b–f and Supplementary Figure S4). Importantly, similar to our observation in human IDC samples and metastatic breast cancers (Figures 1d and e), we observed that PKCλ/ι predominantly localized in the cytoplasm and phospho-PKCλ/ι predominantly localized in the nuclei of the xenografted MDA-MB-231 cells (Figure 3g).

Although PKCλ/ι depletion does not induce overt changes of TNBC cell proliferation during *in vitro* culture conditions (Supplementary Figure S3d), our *in vivo* results suggest that the growth of primary tumors from orthotopically transplanted TNBC cells was dependent upon a cell-autonomous PKCλ/ι signaling. We reasoned that, during *in vitro* studies, a plethora of signaling molecules was present in high concentrations

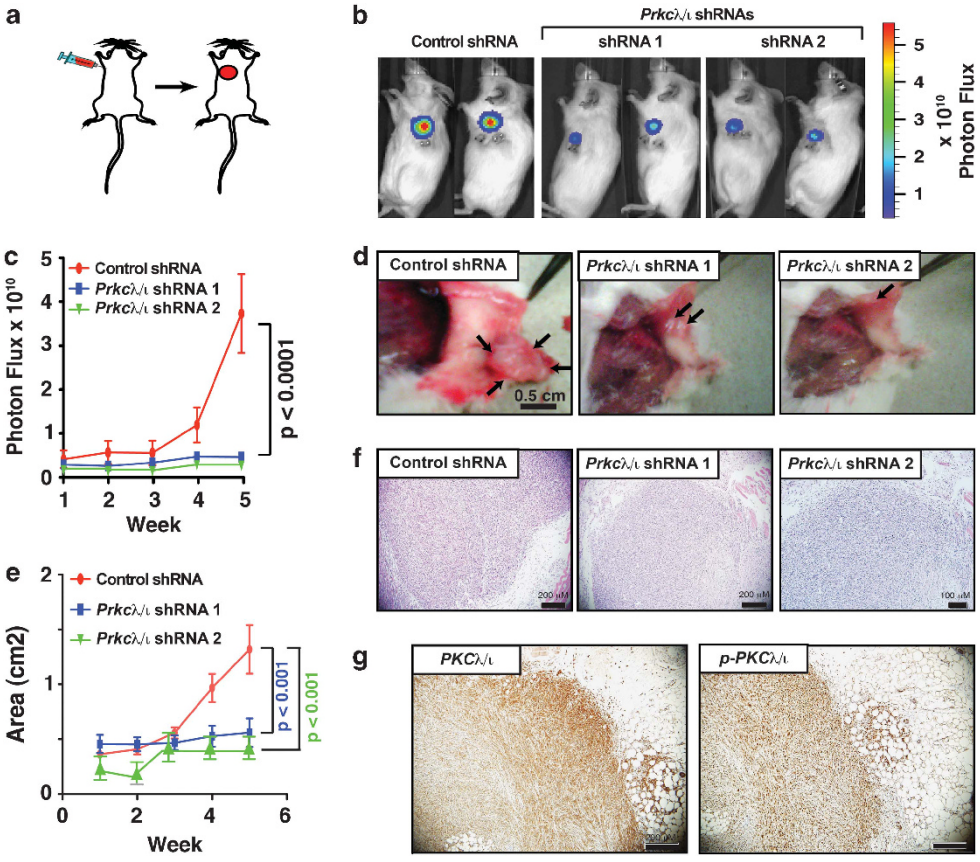


Figure 3 Depletion of PKCλ/ι inhibits orthotopic breast tumor growth. (**a**) Schematic of orthotopic breast tumor formation assay with PKCλ/ι-depleted MDA-MB-231-luc cells. (**b**) Representative whole-animal images at 5 weeks post-transplantation. (**c**) Quantification of tumor growth via luminescence measurements ($n = 5$). (**d**) Representative tumors at 5 weeks post-transplantation. Arrow heads indicating tumor regions on second mammary glands. (**e**) Quantification of tumor area ($n = 5$). (**f**) H&E staining of breast tissues at 5 weeks post-transplantation. (**g**) Immunohistochemical analysis of PKCλ/ι and phospho-PKCλ/ι expression and localization in control MDA-MB-231 xenograft breast tumor. For all quantifications, results represent means ± S.E.M. *P*-values were calculated two-way ANOVA with Bonferroni post-test (see also Supplementary Figure S4 for detailed statistical analysis)

in serum-supplemented growth media and these signaling molecules might simultaneously activate multiple signaling cascades, thereby compensating the loss of PKCλ/ι signaling pathway. However, that compensation was absent *in vivo* as transplanted cells only had limited access to tissue specific growth factors or signaling molecules, which are highly regulated in a spatio-temporal manner within a complex environment and are present at their physiological concentrations.

Along with growth of primary tumors, we also assessed invasive progression of MDA-MB-231 cells *in vivo* upon PKCλ/ι depletion. We tested spontaneous metastasis of orthotopically transplanted MDA-MB-231 cells with and without PKCλ/ι depletion ($n = 5$). We transplanted cells into the fourth mammary gland of immunodeficient mice, removed primary tumor at 5 weeks and observed for spontaneous metastasis to the lung (Figure 4a). Again, we observed inhibition in orthotopic tumor growth upon PKCλ/ι depletion within 5 weeks (Figures 4b and 4c) and also observed lung metastasis of control MDA-MB-231 cells 10 weeks after orthotopic transplantation (Figures 4d and e). However, such an event was never observed after transplantation of PKCλ/ι-depleted MDA-MB-231 cells (Figures 4d and e). As, the primary tumor growth after orthotopic transplantation was itself inhibited upon PKCλ/ι depletion, the lack of metastatic lung colonization of PKCλ/ι-depleted MDA-MB-231 cells could be a secondary effect due to lack of primary tumor growth. Therefore, to further confirm the functional importance of PKCλ/ι during breast cancer metastasis, we transplanted both control and PKCλ/ι-depleted MDA-MB-231-luc cells intravenously via tail vein ($n = 5$) and monitored for metastatic lung colonization via BLI (Figure 4f). We observed significant inhibition in lung colonization with PKCλ/ι-depleted cells within 3 weeks (Figures 4g–i and Supplementary Figure S5). Collectively, our functional analyses in animal models strongly indicate that PKCλ/ι signaling is important to promote TNBC growth, invasion and metastasis *in vivo*.

PKCλ/ι-regulated signature genes are associated with breast cancer invasive progression and poor clinical outcome. Although PKCλ/ι is highly expressed in IDC and metastatic breast cancers (Figure 1), we were unable to significantly correlate the PKCλ/ι mRNA expression data with clinicopathological parameters (Supplementary Figure S1a). These results suggest that active, phosphorylated-PKCλ/ι forms (significantly high in IDC and metastatic breast cancers

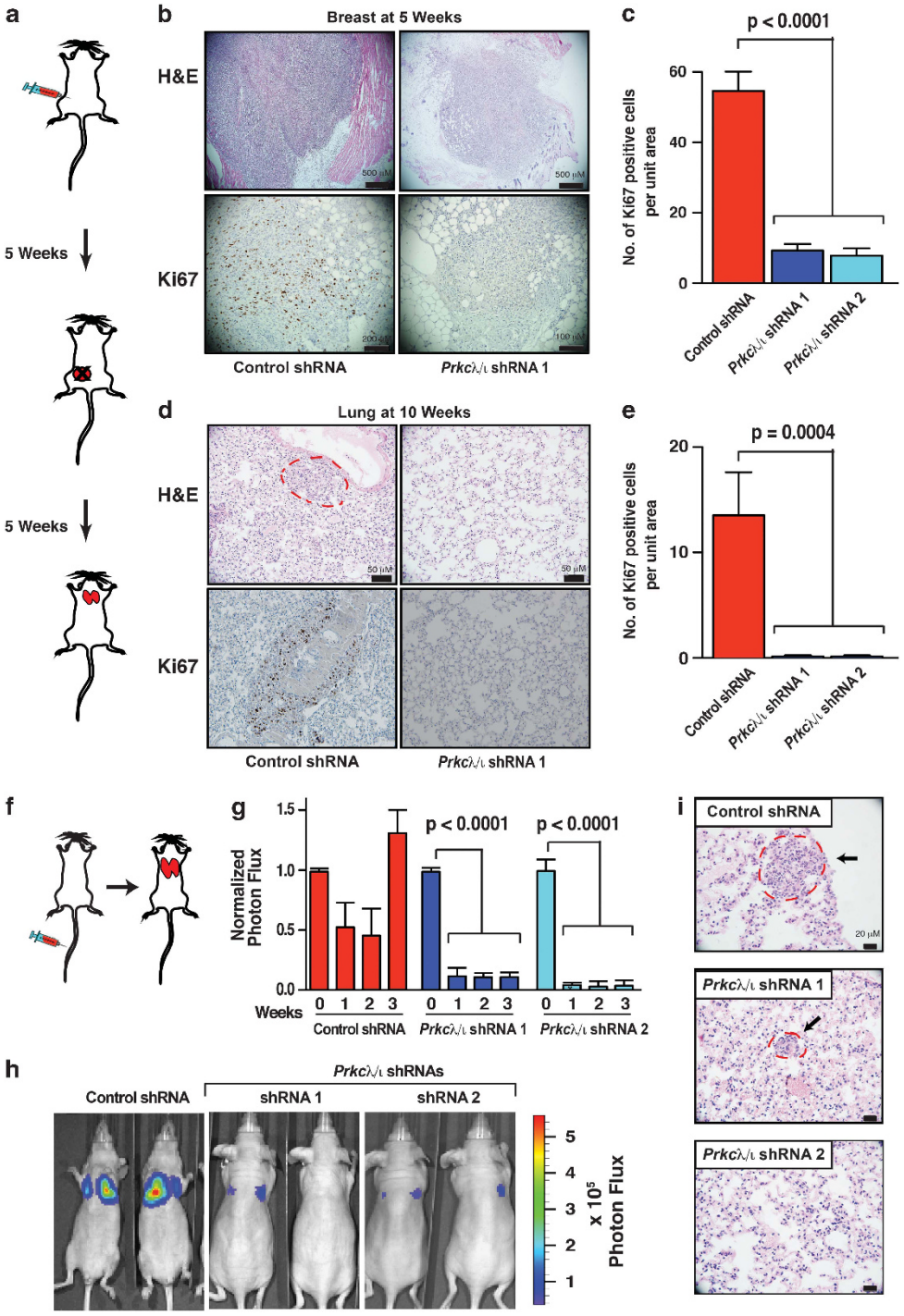


Figure 4 Depletion of PKCλ/ι inhibits breast cancer metastasis. (**a**) Schematic of spontaneous metastasis assay using PKCλ/ι-depleted MDA-MB-231 cells ($n = 5$). H&E and Ki67 staining (**b**) and quantification of Ki67 staining ($n = 18$) (**c**) of breast tissues transplanted with MDA-MB-231 cells with or without PKCλ/ι depletion at 5 weeks after orthotopic transplantation. Results represent means ± S.E.M. *P*-values were calculated using one-way ANOVA with Bonferroni post-test. H&E and Ki67 staining (**d**) and quantification of Ki67 staining ($n = 18$) (**e**) of lung tissues at 10 weeks after orthotopic transplantation of MDA-MB-231 cells with or without PKCλ/ι depletion. Indicated region by perforated red lines showed metastatic growth at lung. Results represent means ± S.E.M. *P*-values were calculated one-way ANOVA with Bonferroni post-test. (**f**) Schematic of metastatic lung colonization assay after intravenous transplantation of MDA-MB-231-luc cells with or without PKCλ/ι depletion. (**g**) Quantification of metastatic lung colonization via luminescence measurements ($n = 5$). Results represent means ± S.E.M. *P*-values were calculated using one-way ANOVA with Bonferroni post-test (see also Supplementary Figure S5 for detailed statistical analysis) (**h**) Representative whole-animal images at 3 weeks post-intravenous transplantation. (**i**) H&E staining of lung tissues 3 weeks after intravenous transplantation. Indicated regions by perforated red lines and arrows showed lung colonization

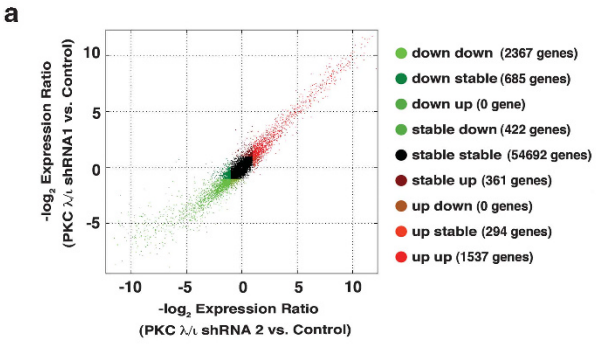
as shown in Figure 1) might be functionally more significant during breast cancer progression. Hence, we hypothesized that alteration of gene expression owing to PKCλ/ι signaling could be an indicative parameter to assess clinical outcome in breast cancer patients. To test this hypothesis, we investigated global gene expression changes in PKCλ/ι – depleted MDA-MB-231 cells via RNA sequencing (Figure 5a and Supplementary Table S1). We found that 2367 genes were downregulated and 1537 genes were upregulated upon PKCλ/ι depletion. Out of these PKCλ/ι-regulated genes, we selected 2234 genes, whose expressions were significantly altered in MDA-MB-231 cells after PKCλ/ι depletion and to be regulated by known transcription factors (via IPA resources www.ingenuity.com; Supplementary Table S2). We evaluated expressions of those genes against publicly available breast cancer patient microarray data sets. We refined these genes to a set of 110 genes by a factor analysis-based filtering procedure (see details in Methods section) performed on a human breast cancer patient cohort of 31 DCIS, 36 IDC and 6 normal breast samples (Gene Expression Omnibus under accession GSE 26304).[42] The set of 110 signature genes (Supplementary Table S3) was subsequently termed the PKCλ/ι invasive signature 110 (PKCλ/ιIS110), and their expression patterns stratified patients into three distinct clusters; normal breast (group 1), DCIS (group2) and IDC (group3) with cluster independence *P*-value of 0.0108 and empirical *P*-value of 0.0026 based on Rand Index (Figures 5b and c). Notably, a few basal-like (triple negative) DCIS cases clustered very closely with IDC groups indicating the similarity in molecular signatures between basal-like DCIS and IDC as reported earlier.[42] We observed that, among PKCλ/ιIS110, 45 genes are favorably overexpressed in the normal breast group (NB specific), 3 genes are specifically overexpressed in the DCIS group (DCIS-specific) and 62 genes are overexpressed in the IDC group (IDC specific) (Figures 5b and c).

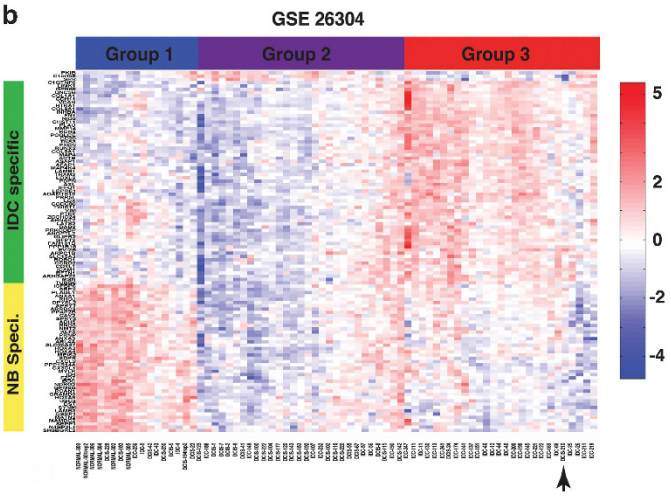
Next, we performed validation analysis with another independent human breast cancer patient cohort of seven DCIS and seven matched IDC samples (GSE3893-GPL570).[43] Although these matched samples have inherent tendency to cluster together, we observed that PKCλ/ιIS110 stratified almost all IDC patients (six out of seven) in one group and all DCIS patients into another distinct group (cluster independence *P*-value 0.0031 and empirical *P*-value based on Rand Index is 0.0031) (Figures 5d and e). These data strongly indicate that altered expressions of PKCλ/ιIS110 genes could contribute to the invasive progression of breast cancer and PKCλ/ιIS110 gene set might serve as potential biomarkers to predict the DCIS to invasive breast cancer transition.

Consequently, we investigated whether downregulation of normal breast-specific genes or upregulation of IDC-specific genes of PKCλ/ιIS110 gene set could predict clinical outcome such as relapse or death in human breast cancer. We analyzed clinical relevance of the PKCλ/ιIS110 genes in a cohort of 159 human primary tumor samples representing breast cancer heterogeneity (25 basal-like or TNBC, 15 HER2-positive, 39 luminal-A, 23 luminal-B, 37 normal breast-like and 20 unclassified).[44] The PKCλ/ιIS110 genes showed clinically relevant clustering of these patients into three subgroups independent of the breast cancer molecular subtypes (Figure 5f). Importantly, patients with loss of NB-specific genes and higher expression of IDC-specific genes predominantly clustered into the groups with poor clinical outcome (Group 2 and 3) (Figure 5g–i). Similar observations were also observed in another independent data set of 286 lymph-node-negative patients with known ER status (GSE 2034).[45] The PKCλ/ιIS110 genes clustered patients into subgroups with significantly different clinical outcomes independent of ER status (Supplementary Figure S6), and, again, loss of normal breast-specific genes or higher expression of IDC-specific genes indicated poor outcome (Supplementary Figures S6b and c). Thus, our RNA-seq and multi-step bioinformatic analyses with gene expression data sets of human breast cancer patients have identified a PKCλ/ι-regulated gene signature predictive of invasive progression of human breast cancer and poor clinical outcome.

Cytokine-PKCλ/ι-RelA regulatory axis positively modulates progression of breast cancer. As our analysis in microarray data sets of human breast cancer patients indicated clinical significance of differential expressions of PKCλ/ιIS110 genes, we performed bio-function pathway analysis via ingenuity resources (IPA). We found that the expression pattern of PKCλ/ιIS110 genes in MDA-MB-231 cells indicated significant inhibition of various cellular pathways involved in cancer progression such as cell movement, migration, invasion and metastasis (Supplementary Figure S7a). Consequently, we wanted to understand potential signaling cascades regulating PKCλ/ιIS110 genes via activation of PKCλ/ι. Our analysis for upstream regulators potentially associated with the regulation of PKCλ/ιIS110 genes (via IPA) indicated that a majority of the PKCλ/ιIS110 genes could be regulated through various cytokines including TGFβ, IL1β and TNFα (Supplementary Table S4 and Supplementary Figure S7b). All these cytokines have been implicated in breast cancer progression previously.[46–48] To test the effect of these cytokines on PKCλ/ι activation, we treated MDA-MB-231 cells with different cytokines and tested phosphorylation of PKCλ/ι. Intriguingly, we found that both TGFβ1 and IL1β, individually and in combination, induced PKCλ/ι phosphorylation in MDA-MB-231 cells (Figure 6a and Supplementary Figure S8a). However, we did not observe induction of PKCλ/ι phosphorylation upon TNFα treatment (Supplementary Figure S8a). Interestingly, we also observed that cytokine-induced phospho-PKCλ/ι molecules are predominantly localized within the nuclei of MDA-MB-231 cells (Figure 6b). Taken together, these data strongly indicate that cytokines, like TGFβ and IL1β, might induce PKCλ/ι activation in TNBC cells and facilitate nuclear translocation of phospho-PKCλ/ι as observed in our xenogtraft models as well as in IDC and metastatic breast cancers (Figure 1 and Figure 3g).

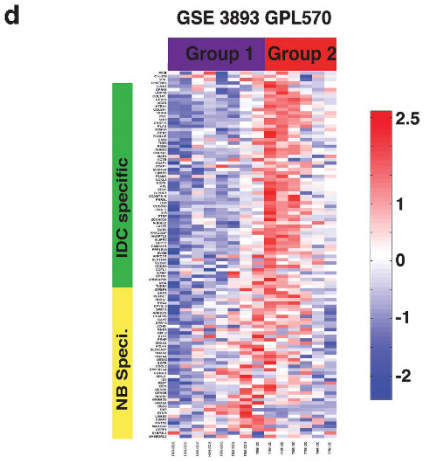
To link different cytokine signaling nodes and PKCλ/ι, we looked for a common downstream regulator. Interestingly, earlier reports implicated atypical PKC signaling in regulating NF-κB p65 (RelA) function in multiple cell types,[49,50] and RelA has a known association with various cytokine networks[51,52] (Supplementary Figure S7b). As RelA is one of the major transcription factors that inhibited upon PKCλ/ι depletion in MDA-MB-231 cells (as predicted by IPA in Supplementary

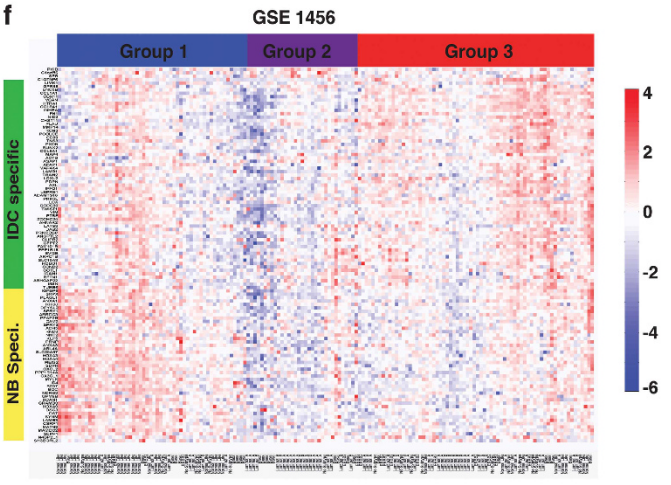




c

	Mean Expression			Expression polarization p-value		
	Group1	Group 2	Group 3	Gr.1 vs Gr.2	Gr.1 vs Gr.3	Gr.2 vs Gr.3
Normal Breast specific 45 genes	0.9683	- 0.3628	- 0.22	0.0062	0.0206	0.6827
DCIS specific 3 genes	- 0.3870	0.5319	- 0.3277	0.0616	0.8921	0.0332
IDC specific 62 genes	- 0.3928	- 0.4150	0.6930	0.9597	0.0290	0.0107



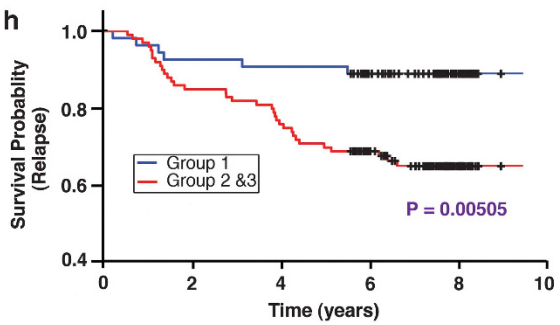


e

	Mean Expression		Expression polarization p-value
	Group1	Group 2	Gr.1 vs Gr.2
Normal Breast specific 45 genes	-.0491	0.0655	0.8787
DCIS specific 3 genes	0.0219	- 0.0292	0.9455
IDC specific 62 genes	- 0.4676	0.6234	0.05

g

	Mean Expression			Expression polarization p-value		
	Group1	Group 2	Group 3	Gr.1 vs Gr.2	Gr.1 vs Gr.3	Gr.2 vs Gr.3
Normal Breast specific 45 genes	0.4386	- 0.5215	- 0.1050	0.0319	0.0754	0.1497
DCIS specific 3 genes	- 0.1220	0.3309	- 0.0584	0.2429	0.8187	0.1727
IDC specific 62 genes	- 0.1098	- 0.4676	0.3083	0.3531	0.1586	0.0290



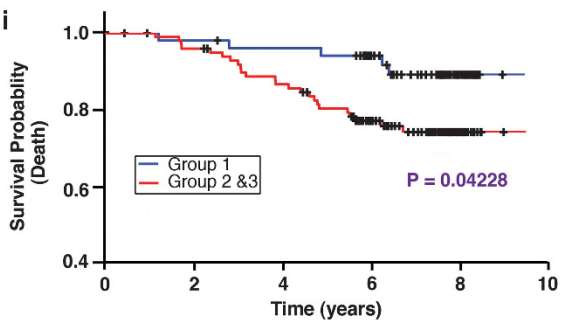


Table S3), we tested RelA expression in multiple TNBC cells along with HER2-positive BT474 and ER + MCF-7 cells. We found that RelA was highly abundant in all these cell types (Supplementary Figure S8b). Thus, we hypothesized that a PKCλ/ι-RelA signaling axis might regulate the expression of at least some of the PKCλ/ιIS110 genes during breast cancer disease progression. For confirmation, we tested RelA nuclear localization and activity in MDA-MB-231 cells after PKCλ/ι depletion. We observed significant reduction of RelA nuclear localization in MDA-MB-231 cells after PKCλ/ι depletion (Figures 6c, 6d and Supplementary Figure S8c). We confirmed no significant changes in the total RelA expression level in MDA-MB-231 cells after PKCλ/ι depletion (Figure 6d). To validate compromised RelA function, we performed NF-κB reporter gene analysis and observed significant repression of NF-κB transcription activity in the PKCλ/ι-depleted MDA-MB-231 cells (Figure 6e). For further confirmation, we measured mRNA expression levels of known RelA target genes from PKCλ/ιIS110 signature panel such as *ICAM1* (intercellular adhesion molecule 1)[53] and *PLAU* (plasminogen activator, Urokinase)[54] and also the genes that contain consensus RelA-binding motifs at the promoter regions such as *DAB2* (Dab, mitogen-responsive phosphoprotein, homolog 2 (Drosophila)) and *VIM* (Vimentin) (Supplementary Figure S8d). We observed significant downregulation of these genes in PKCλ/ι-depleted cells compared with control (Figure 6f). Furthermore, RelA chromatin occupancy at the consensus RelA-binding motifs present in the promoter regions of these genes was also impaired (Figure 6g).

To confirm further that RelA transcriptional activity downstream to PKCλ/ι signaling might contribute to gene regulation and invasiveness of TNBC cells, we ectopically expressed a constitutively active RelA mutant (RelA-S536E)[55] in PKCλ/ι-depleted MDA-MB-231 cells (Supplementary Figure S9). We observed that ectopic expression of RelA-S536E partially rescues expression of RelA target genes in PKCλ/ι-depleted MDA-MB-231 cells and promotes their invasiveness when tested via *in vitro* invasion assay (Supplementary Figure S9). These results strongly indicate that a cytokine-PKCλ/ι-RelA signaling axis might be involved in gene regulation to positively modulate invasive and metastatic progression of breast cancer cells (Figure 6h).

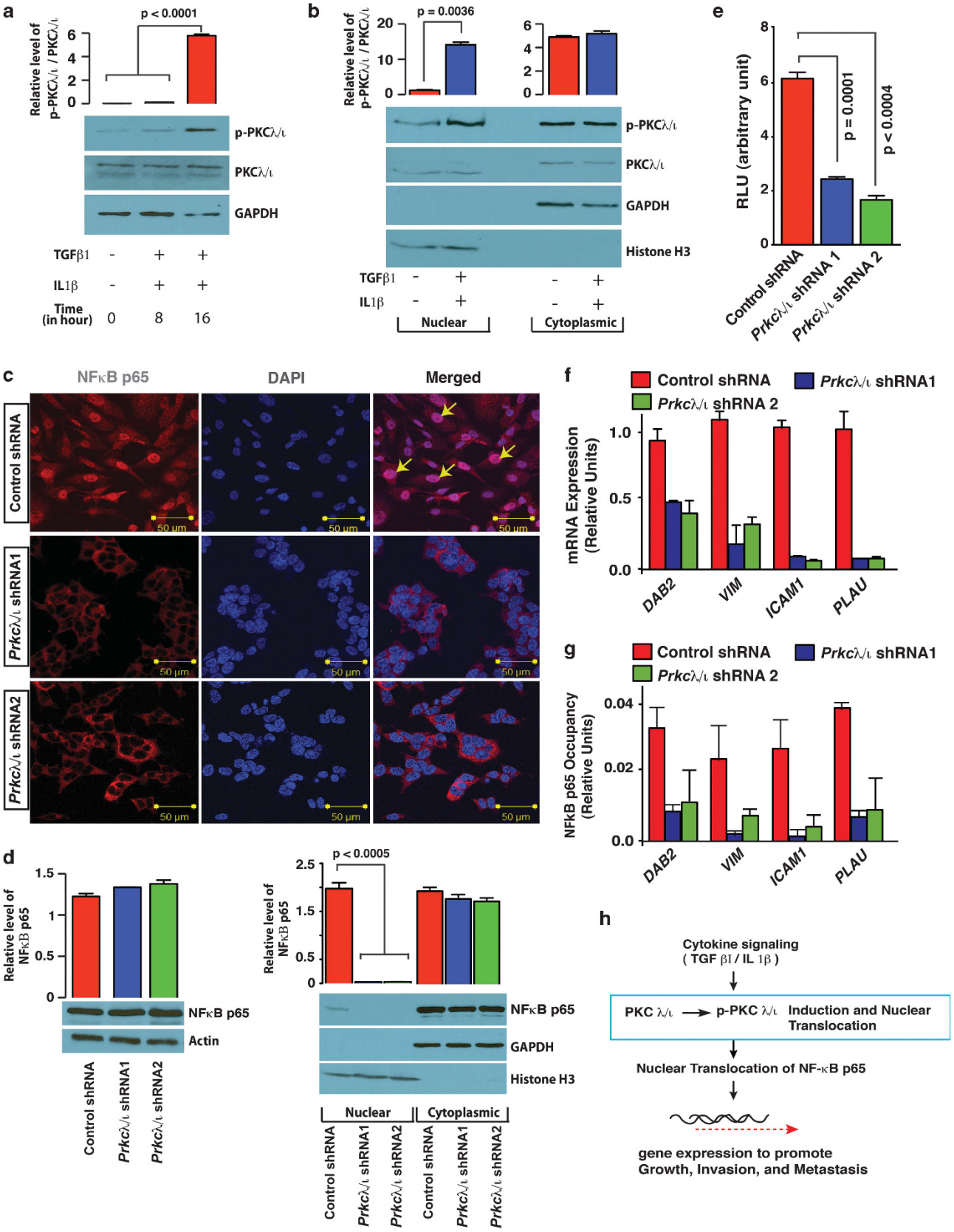
Discussion

In this study, we provided evidence that PKCλ/ι signaling positively regulates TNBC invasion and metastasis. Specific depletion of PKCλ/ι inhibited invasiveness of multiple TNBC breast cancer cell types with mutant *TP53*, EGFR overexpression and *BRCA1* mutation. All these genetic abnormalities are significantly associated with poor prognosis.[8,56] Importantly, depletion of PKCλ/ι significantly inhibited TNBC growth and metastatic lung colonization in experimental animal models. These observations indicate that targeting PKCλ/ι signaling might have therapeutic potential for TNBC relapse and metastasis.

During breast cancer progression, transitions from normal breast to DCIS and then to IDC are associated with important developmental changes. However, to date, the molecular basis of these developmental changes is poorly understood. Intriguingly, we observed a gradual increase in PKCλ/ι signaling from normal breast to DCIS to IDC samples of multiple clinical subtypes, indicating that gradual increase in PKCλ/ι activity might be important for altered gene expression circuitry associated with breast cancer progression. Consistently, our global gene expression analysis in breast cancer cells and human breast cancer patient data sets identified a PKCλ/ι dependent gene signature (PKCλ/ιIS110) differentially expressed between normal breast, DCIS and IDC samples. We also observed that differential expression of IDC-specific genes of PKCλ/ιIS110 is significantly associated with poor clinical outcome such as relapse and death of breast cancer patients. Thus, PKCλ/ιIS110 could be of tremendous clinical significance and might serve as a platform to identify specific biomarkers, which could predict invasive progression of breast cancer. Altogether, these observations and previous reports[28,29] indicate significance of PKCλ/ι signaling during the breast cancer disease progression including growth, invasion, relapse/metastasis and survival.

Mechanistically, we have identified cytokine-PKCλ/ι-NF-κB signaling axis, which could regulate key genes associated with breast cancer progression and metastasis. IPA analysis identified that depletion of PKCλ/ι resulted in inhibition of multiple cytokine signaling nodes such as TGFβ1 and IL-1β; both cytokines are known to control expressions of various migration- and metastasis-related genes.[57,58] Supporting IPA

Figure 5 PKCλ/ι-regulated genes are differentially expressed in the normal breast, DCIS and IDC patients and associated with poor clinical outcome. (**a**) Schematic representation of global transcriptome changes in MDA-MB-231 cells with and without PKCλ/ι depletion as determined by RNA sequencing. (**b**) Heatmap representing the unsupervised *k*-means clustering of human patient data set GSE 26304 ($n = 73$) using PKCλ/ιIS110 feature vector. Patient samples are distinctly grouped according to their disease type. The color gradient of the heatmap represents normalized expression values from high (red) to low (blue). A set of 62 genes specifically overexpressed in group 3 or IDC cluster (indicated as IDC specific, green bar along left side), 45 genes overexpressed in group 1 or normal breast cluster (indicated as NB Speci, yellow bar along left side), and three genes overexpressed in group 2 or DCIS cluster. One basal-like (triple negative) DCIS patient clustered in Group 3 indicated by black arrow. (**c**) Mean expression and expression polarization *P*-values of PKCλ/ιIS110 genes between patient groups of data set GSE 26304. (**d**) Heatmap representing the unsupervised *k*-means clustering of an independent human patient data set GSE3893-GPL570 containing matched DCIS and IDCs ($n = 14$) using PKCλ/ιIS110 feature vector. While these samples show a natural tendency to cluster by patient, *k*-means clustering with PKCλ/ιIS110 feature vector distinctly groups them according to their disease type. The color gradient of the heatmap represents normalized expression values from high (red) to low (blue). All DCIS patients clustered in Group 1 (purple bar along the top), and six out of seven IDC patients clustered in Group 2 (red bar) indicating the validity of PKCλ/ιIS110 signature gene set to predict invasive phenotype of breast cancer. (**e**) Mean expression and expression polarization *P*-values of PKCλ/ιIS110 genes between patient groups of data set GSE3893-GPL570. (**f**) Heatmap representing unsupervised *k*-means ($k = 3$) clustering in human patient data set GSE1456 ($n = 159$) using PKCλ/ιIS110 feature vector. The color gradient of the heatmap represents normalized expression values from high (red) to low (blue). Group 1 (blue bar along the top), Group 2 (purple bar) and Group 3 (red bar). (**g**) Mean expression and expression polarization *P*-values of PKCλ/ιIS110 genes between patient groups of data set GSE1456. (**h**) Kaplan–Meier estimates of the time-to-relapse of patient samples (from GSE1456 cohort) of groups 2 (purple) and 3 (red) were compared with group 1 (blue). The *P*-values were calculated using the log-rank test. Patient group 1 (blue) showed better prognosis compared with groups 2 and 3. (**i**) Kaplan–Meier estimates of time-to-death due to breast cancer of patient samples (from GSE1456 cohort) of groups 2 (purple) and 3 (red) were compared with group 1(blue). The *P*-values were calculated using the log-rank test. Patient group1 (blue) showed better prognosis compared with groups 2 and 3


a
p < 0.0001
Relative level of p-PKCλ/ι / PKCλ/ι
p-PKCλ/ι
PKCλ/ι
GAPDH
TGFβ1 - + +
IL1β - + +
Time (in hour) 0 8 16
b
p = 0.0036
Relative level of p-PKCλ/ι / PKCλ/ι
p-PKCλ/ι
PKCλ/ι
GAPDH
Histone H3
TGFβ1 - + - +
IL1β - + - +
Nuclear
Cytoplasmic
e
RLU (arbitrary unit)
p = 0.0001
p < 0.0004
Control shRNA
Prkcλ/ι shRNA 1
Prkcλ/ι shRNA 2
c
NFκB p65
DAPI
Merged
Control shRNA
Prkcλ/ι shRNA1
Prkcλ/ι shRNA2
50 μm
f
Control shRNA
Prkcλ/ι shRNA1
Prkcλ/ι shRNA 2
mRNA Expression (Relative Units)
DAB2
VIM
ICAM1
PLAU
g
Control shRNA
Prkcλ/ι shRNA1
Prkcλ/ι shRNA 2
NFκB p65 Occupancy (Relative Units)
DAB2
VIM
ICAM1
PLAU
d
Relative level of NFκB p65
NFκB p65
Actin
Control shRNA
Prkcλ/ι shRNA1
Prkcλ/ι shRNA2
p < 0.0005
Relative level of NFκB p65
NFκB p65
GAPDH
Histone H3
Nuclear
Cytoplasmic
Control shRNA
Prkcλ/ι shRNA1
Prkcλ/ι shRNA2
Control shRNA
Prkcλ/ι shRNA1
Prkcλ/ι shRNA2
h
Cytokine signaling
(TGF βI / IL 1β)
PKC λ/ι → p-PKC λ/ι Induction and Nuclear Translocation
Nuclear Translocation of NF-κB p65
gene expression to promote
Growth, Invasion, and Metastasis

analysis, we found that TGFβ1 and IL-1β could induce phosphorylation as well as nuclear translocation of PKCλ/ι in TNBC cells. Previously, various lipids and growth factors, such as ceramide, phosphoinositide 3-kinase (PI3K), 3 phosphoinositide-dependent protein kinase-1 (PDK-1), EGF, NGF, PDGF and HER2, have been implicated as the upstream regulators to activate PKCλ/ι.[15,31] Thus, it is possible that TGFβ1 and IL-1β could modulate these known activators of PKCλ/ι to induce phosphorylation at Thr555/Thr563. However, further research is required to investigate specific molecular mechanism.

We also observed that NF-κB p65 or RelA transcriptional activity in TNBC cells could be regulated by PKCλ/ι signaling. Furthermore, rescue of invasive phenotype of PKCλ/ι-depleted MDA-MB-231 cells upon ectopic expression of constitutively active RelA-S536E molecules strongly indicates that a PKCλ/ι-RelA signaling axis, at least in part, might contribute to the invasive progression of TNBC cells. However, our global gene expression analysis also indicated that depletion of PKCλ/ι signaling could repress additional transcriptional networks, such as hypoxia-inducible factor 1alpha (HIF1α), signal transducer and activator of transcriptions (STATs) and SMAD3, known to function downstream of various cytokine and growth factor signaling networks to promote breast cancer invasion and metastasis.[14] These observations imply the existence of overlapping regulatory mechanisms involving PKCλ/ι and other signaling cascades to promote invasive progression of breast cancer, and future studies in this direction could provide important information.

In summary, we discovered that PKCλ/ι signaling mediates survival adaptability of breast cancer cells in their natural milieu, that is, in the breast, and promotes metastasis in distal tissues such as the lung by regulating NF-κB and other cytokine and growth factor-associated transcription factors. Thus, our study raised a possibility to treat highly heterogeneous breast cancer disease by targeting PKCλ/ι signaling and its upstream and downstream regulatory molecules.

Materials and Methods

Analysis of human primary breast tumor samples. The tissue microarray slides consisting of 55 IDC samples (10 ER positive, 10 HER2 positive, 35 TNBC), 10 DCIS samples and 10 normal breast tissues were prepared by the University of Kansas Medical Center Department of Pathology from archival material following IRB approval. Expressions of both PKCλ/ι and phospho-PKCλ/ι were analyzed by immunohistochemistry (IHC). Digital images of the stained slides were taken using Aperio TMA software, and expressions of PKCλ/ι and phospho-PKCλ/ι were analyzed by two independent pathologists in a double-blind manner. The expression levels (IHC scores) were indicated in a scale of 0 to 3, where 3 indicates highest and 0 indicates lowest expressions. IHC scores 0 to 1 and >1 were considered as low and high expression, respectively. For significance, *P*-values were calculated by two-way ANOVA with Boneferroni post-test (Supplementary Figures S1c–f).

Cell lines. All breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to the recommended protocol. For knockdown of PKCλ/ι, cell lines were infected with lentiviral pGIPZ shRNAmir vector containing short hairpins and GFP reporter (Supplementary Table S5) (Open Biosystems, Pittsburgh, PA, USA).

Animal studies. All animal works were done in accordance with approved protocol by the Institutional Animal Care and Use Committee. Female NU/NU nude and NOD-SCID NSG mice (Charles River) of 4–6 weeks old ($n=5$ for cell line for each *in vivo* experimental set) were used in xenograft studies for lung metastasis and orthotopic mammary fat pad tumor assays. Two different shRNA constructs showed most effective knockdown of PKCλ/ι and subsequently used to knockdown PKCλ/ι in TNBC cells including MDA-MB-231-luc or MDA-MB-231 cells that were used for animal studies.

For orthotopic tumor assays, cells were collected in PBS. Mice were anesthetized, a small incision was made to reveal the mammary gland and 2×10^6 cells were injected directly into the second or fourth mammary glands. The incision was closed with wound clips, and primary tumor outgrowth was monitored weekly. For spontaneous metastasis assays, primary tumors were surgically removed after 5 weeks and kept for another 5 weeks for the development of metastasis in the lungs.

For lung colonization assay, 1×10^6 viable cells were injected into the lateral tail vein in a volume of 0.1 ml. Following isoflurane-induced anesthesia, mice were imaged for luciferase activity immediately after injection to exclude any that were not successfully xenografted. Lung colonization was monitored weekly and continued up to 3 weeks.

Bioluminescent imaging. Mice were anesthetized and injected intraperitoneally (IP) with D-luciferin (15 mg/ml in PBS and 0.01 ml/g bodyweight). Imaging was performed after injection with a Xenogen IVIS system coupled to Living Image acquisition and analysis software version 4.0 (Xenogen, Hopkinton, MA, USA). Region of interest (ROI) boxes were drawn around the entire body (excluding tail) of the animals. Measurements were expressed as flux (i.e., photons/second) and scaled to a comparable background value (from a luciferin-injected mouse with no tumor cells). For normalization, the flux values obtained immediately after xenografting (day 0) were considered as 100 so that all mice had an arbitrary starting BLI signal of 100.

RNA-Seq analysis and Gene set Enrichment Analysis. We used whole-transcriptome shotgun sequencing (RNA-Seq) to obtained the global gene expression profile of MDA-MB-231 cells after PKCλ/ι depletion by RNAi carried out independently using two different shRNA clones (shRNA1 and shRNA2, Supplementary Table S5) and compared it with the expression profile under control conditions (control shRNA, Supplementary Table S5). Detailed methods were described in Supplementary Materials and Methods.

Patient data set analysis. All genes were normalized to the geometric average of the three housekeeping genes *MRPL19*, *PSMC4* and *PUM1*, as these genes showed minimal variation in expression across a variety of experimental conditions.[59] Genes with multiple probesets were represented by the probeset with maximal variance across samples. The significance of the partitions generated by k-means clustering for the 110 gene feature vector (PKCλ/ιIS110) was evaluated as an empirical *P*-value calculated on the χ^2-statistic of cluster independence as follows: $P_{Chi}=(c+1)/(N+1)$, where c is the number of clusterings for randomly selected genes of the same size as the signature gene set that generated a χ^2-statistic equal to or greater than the observed χ^2-statistic and N (10,000) the number of random tests performed. The empirical *P*-value based on the Rand

Figure 6 Involvement of Cytokine-PKCλ/ι-NF-κB regulatory axis in TNBC cells. (**a**) Phosphorylation of PKCλ/ι in MDA-MB-231 cells was significantly induced after TGFβI and IL1β treatment. For quantification, results represent means ± S.E.M. ($n=3$), and *P*-value was calculated using one-way ANOVA with Bonferroni post-test. (**b**) Induction of phospho-PKCλ/ι predominantly noticed in the nucleus. For quantification, results represent means ± S.E.M. ($n=3$) and *P*-value was calculated using two-tailed unpaired Student's *t* test. (**c**) Localization of NF-κBp65 (RelA) in MDA-MB-231 cells with and without PKCλ/ι depletion. RelA expression showed in red and nuclear staining showed by DAPI. Arrows indicated nuclear localization of RelA. (**d**) Western blot analysis indicating no significant changes in RelA expression level after PKCλ/ι depletion, rather, impaired RelA nuclear translocation. For quantification, results represent means ± S.E.M. ($n=3$) and *P*-value was calculated using one-way ANOVA with Bonferroni post-test. (**e**) NF-κB reporter gene assay of MDA-MB-231 cells with and without PKCλ/ι depletion ($n=3$). Results represent means ± S.E.M. *P*-values were calculated using two-tailed unpaired Student's *t*-test. (**f**) Quantitative RT-PCR measurements of RelA target genes *DAB2, VIM, ICAM1* and *PLAU* in MDA-MB-231 cells with and without PKCλ/ι depletion ($n=3$). Results represent means ± S.E.M. (**g**) Quantitative PCR-based measurements of RelA chromatin occupancy at the promoter regions of *DAB2, VIM, ICAM1* and *PLAU* genes in MDA-MB-231 cells with and without PKCλ/ι depletion ($n=3$). Results represent means ± S.E.M. (**h**) Schematic of cytokine signaling-PKCλ/ι-NF-κB p65 regulatory axis for breast cancer growth, invasion and metastasis

index was calculated similarly giving an alternate assessment of the significance of the generated clusters. Here $P_{Rand} = (r+1)/(N+1)$ where r is the number of clusterings for randomly selected genes of the same size as the signature gene set that generated a Rand measure equal to or greater than the observed Rand measure and N (10,000) the number of random tests performed. Population survival or remission curves were compared using the log-rank test. The survival function was estimated using the Kaplan–Meier procedure. Gene expression levels were standardized (mean 0 and S.D. 1) across samples for visualization. Expression polarization was defined as the absolute difference between the mean expression levels over the genes and patients in each group, and *P*-values of the observed expression polarization was calculated as described earlier.[60]

Among the 3904 significantly differentially expressed genes in the RNA-Seq experiments, 2234 genes were identified that were associated with significantly inhibited or activated upstream regulators as predicted by IPA. With these genes as observations and samples as variables, the data set GSE26304 was factor analyzed with three common factors. The factor solution was rotated using the 'varimax' procedure for better interpretation. Genes with an absolute correlation coefficient ≥ 0.6 with the factor loadings of any one of the factors were selected to comprise the signature set of 110 genes (PKCλ/ιIS110). These 110 signature genes formed the feature vector for further k-means clustering on the data sets GSE26304, GSE3893-GPL570, GSE1456 and GSE 2034. Kaplan–Meier estimation was performed using time-to-death due to breast cancer or censoring and time-to-relapse or last follow-up for GSE1456 and GSE 2034 data sets. Genes from the data set GSE26304 were hierarchically clustered using the Euclidian pair-wise distance between rows and the 'ward' method for the linkage function to establish the signature gene order.

Statistics. All statistical analyses were performed using GraphPad Prism5 statistical software (GraphPad Software Inc., San Diego, CA, USA) unless mentioned otherwise. All data are expressed as means ± S.E.M. unless mentioned otherwise. *P*-values were calculated by two-tailed unpaired Student's *t*-test, one-way or two-way ANOVA with Bonferroni post-test unless mentioned otherwise, and $P < 0.05$ was considered as significant.

Conflict of Interest

The authors declare no conflict of interest.

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