Loss of the Ceramide Transfer Protein Augments EGF Receptor Signaling in Breast Cancer

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Abstract

Triple-negative breast cancers (TNBC) are especially refractory to treatment due to their negative hormone receptor and ErbB2/HER2 status. Therefore, the identification of cancer-associated deregulated signaling pathways is necessary to develop improved targeted therapies. Here, we show that expression of the ceramide transfer protein CERT is reduced in TNBCs. CERT transfers ceramide from the endoplasmic reticulum to the Golgi complex for conversion into sphingomyelin (SM). We provide evidence that by regulating cellular SM levels, CERT determines the signaling output of the EGF receptor (EGFR/ErbB1), which is upregulated in approximately 70% of TNBCs. CERT downregulation in breast cancer cells enhanced ErbB1 lateral mobility, ligand-induced autophosphorylation, internalization, and chemotaxis. Together, our findings provide a link between lipid metabolism at the Golgi with signaling at the plasma membrane, thereby implicating CERT loss in the progression of TNBCs. Cancer Res; 72(11); 2855–66. ©2012 AACR.

Introduction

Breast cancer is the most common malignancy among women. Based on global gene expression signatures, breast cancers are classified into 3 subgroups: luminal types that express the estrogen receptor (ER) and progesterone receptor (PR), those that overexpress the receptor tyrosine kinase (RTK) ErbB2/HER2, and basal-like cancers that express cytokeratins 5/6, 14, and 17 (1, 2). The latter subgroup comprising approximately 20% of all cases typically lacks ER/PR expression and is referred to as triple negative. These cancers are difficult to treat as they do not respond to hormone and ErbB2/HER2 blocking therapies and are especially aggressive due to their frequent recurrence and high metastatic potential (2). Therefore, the identification of common molecular alterations in triple-negative breast cancers (TNBC) will be necessary to enable the design of effective targeted therapies.

A total of 50% to 70% of TNBCs overexpress the EGF receptor (EGFR)/ErbB1/HER1, which together with ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 constitute the ErbB family of RTKs. Ligands such as EGF, amphiregulin, and TGFα induce the formation of ErbB1 homodimers and heterodimers mainly with ErbB2/HER2, leading to activation of the intrinsic kinase domain and phosphorylation of tyrosine residues within the receptor’s cytoplasmic tail. These phosphorylated residues serve as docking sites for downstream signaling proteins, the recruitment of which leads to activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, which stimulate cellular responses such as survival, proliferation, and migration (3).

At the plasma membrane, sphingolipids together with cholesterol cluster in highly ordered regions termed lipid rafts. These membrane microdomains are thought to sequester certain membrane-associated signaling proteins while excluding others, thereby adding a spatial layer of control to signaling events (4, 5). For example, in T lymphocytes lacking sphingomyelin (SM) synthase 1 (SMS1), Fas/CD95, and T-cell receptor clustering and subsequent activation of downstream signaling were shown to be compromised, suggesting a requirement for lipid rafts in the assembly of signaling complexes downstream of these receptors (6, 7). Both ErbB1 and ErbB2/HER2 were found to be associated with lipid rafts in biochemical studies (8). More recent reports using imaging techniques have shown that ErbB receptors are nonrandomly distributed within the plasma membrane and exist in preformed nanoclusters, the formation of which is sensitive to the lipid environment (9–11).
The steriodogenic acute regulatory related lipid transfer (START) domain protein CERT/StarD11 has emerged as a crucial protein in sphingolipid homeostasis. CERT is responsible for the nonvesicular transport of ceramide from the endoplasmic reticulum to Golgi membranes where ceramide is converted to SM by SM synthases (12). Cells expressing a mutant CERT protein showed impaired ceramide trafficking to the Golgi and contained lower SM levels when grown in delipidated medium (12, 13). Reduced SM and ceramide phosphoethanolamine levels were also observed in CERT-deficient mice and flies, respectively (14, 15). CERT-mediated lipid transfer requires its START domain, which forms a hydrophobic pocket that accommodates a single ceramide molecule. CERT further contains a pleckstrin homology domain specific for phosphatidylinositol 4-phosphate that contributes to Golgi localization and an FFAT motif for endoplasmic reticulum targeting via interaction with VAP-A and VAP-B (16, 17). These targeting motifs localize the protein to closely apposed endoplasmic reticulum and Golgi membranes, so-called membrane contact sites, supporting nonvesicular ceramide transfer between these organelles.

Reanalyses of gene expression data sets from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) revealed reduced CERT transcript levels in basal-like breast cancers. By immunohistologic analyses of primary breast cancer specimens, we could confirm reduced CERT expression in TNBCs. We therefore sought to investigate the cellular consequences of CERT downregulation and its potential contribution to aberrant signaling and cellular transformation. We provide evidence that reduced SM production due to CERT downregulation is associated with alterations in plasma membrane organization and phospholipase D2 (PLD2) activation, which facilitate ligand-induced ErbB1 signaling and cell motility. Our data support the conclusion that altered sphingolipid homeostasis at the Golgi impacts RTK signaling, thus contributing to the transformed phenotype of TNBC cells.

Materials and Methods

Cell lines

MCF7, T47D, and MDA-MB 157 cells were obtained from Cornelius Knabbe (Institute of Clinical Pharmacology); BT474 and SKBR3 cells from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland); MDA-MB 453 cells from Jane Visvader (The Walter and Eliza Hall Institute, Melbourne, Australia); HS578T, ZR751, and MDA-MB 468 cells from Bernhard Lüscher (RWTH Aachen University) in 2005. Cell lines were authenticated by morphology and growth characteristics, tested for mycoplasma and frozen. For experiments, cells were kept in culture for less than 4 months. MDA-MB 231 cells were obtained from CLS in 2010 and not reauthenticated. Cells were cultured in RPMI (Invitrogen) supplemented with 10% fetal calf serum (FCS; PAA) in a humidified atmosphere of 5% CO₂ at 37°C.

Immunofluorescence, filipin, and cholera toxin B stainings

Cells were grown on collagen-coated glass coverslips (25 μg/mL; SERVA), fixed with 4% paraformaldehyde (PFA), permeabilized with PBS containing 0.1% Triton X-100 and blocked with 5% goat serum in PBS containing 0.1% Tween 20. Cells were incubated with primary and secondary antibodies in blocking buffer and mounted in Fluoromount G (Southern Biotechnology). For cholesterol distribution, fixed cells were incubated with 10 μg/mL filipin in PBS. For GM1 staining, cells were incubated with 1 μg/mL Alexa Fluor 488-labeled cholera toxin subunit B, followed by incubation with anti-CT-B rabbit serum. See Supplementary Information for details on confocal microscopy.

Lysenin sensitivity assays

Cells were incubated with 400 ng/mL lysenin for 2 hours, washed with PBS, and stained with trypan blue. Cells were photographed with a DM IRB microscope (10-fold magnification).

Internalization experiments

Cells were incubated with 200 ng/mL Alexa Fluor 555-labeled EGF or transferrin and fixed with PFA. Confocal images were obtained, and internal fluorescence intensities of cells were determined by ImageJ.

Fluorescence recovery after photobleaching

Photobleaching of cells expressing CFP-ErbB1 or incubated with 2.5 μmol/L C6-NBD-SM for 20 minutes on ice was carried out at room temperature with 405 and 488 nm laser lines, respectively, at full power in a circular region of interest (ROI). Pre- and postbleach scans were monitored at low laser intensity. Fluorescence recoveries were measured for 30 seconds (NBD-SM) and 60 seconds (CFP-ErbB1) with maximal speed and fitted to a nonlinear curve with ZEN 2009 software. The diffusion coefficient (D) was determined from the equation: 

\[ D = \frac{(r^2 - k_1)}{[4 \ln(2)]} \]

with \( k_1 = \frac{1}{T_{1/2}} \) where \( r \) is the radius of the ROI and \( T_{1/2} \) the diffusion half time.

Spreading, migration, and invasion assays

For spreading assays, cells were plated on collagen-coated dishes (25 μg/mL) before PFA fixation and staining with 0.1% crystal violet. Cells were photographed with a DM IRB microscope (10-fold magnification). For migration assays, \( 1 \times 10^5 \) cells in medium with 0.5% FCS were seeded into the top chambers of Transwells (8.0-μm membrane pores; Costar) coated with 2.5 μg/mL collagen on the underside. For invasion assays, \( 5 \times 10^5 \) cells in medium with 0.5% FCS were seeded into BioCoat Growth Factor Reduced MATRIGEL Invasion Chambers (BD Biosciences) and left to invade overnight. Bottom chambers contained medium with 0.5% FCS and 50 ng/mL EGF. Cells on the top surface of the membranes were removed and cells on the underside were fixed in PFA and stained with 0.1% crystal violet.

Cell lysis, immunoprecipitation, and Western blotting

Cells were lysed in TEB buffer. For immunoprecipitations, cells were lysed in RIPA buffer. Equal amounts of protein were incubated with specific antibodies on ice and immune complexes were collected with protein G-Sepharose (KPL). Proteins were separated by SDS-PAGE and transferred to
polyvinylidene difluoride membrane (Roth). Membranes were blocked with 0.5% blocking reagent (Roche) in PBS containing 0.1% Tween 20 and incubated with primary antibodies, followed by horseradish peroxidase–conjugated secondary antibodies in blocking buffer. Visualization was with the ECL detection system (Pierce).

**PLD activity measurements**

Cells were lysed in PLD buffer and equal amounts of protein were analyzed with the Amplex Red Phospholipase D Assay Kit (Invitrogen).

**Thin layer chromatography and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry**

Lipids extracted by the method of Bligh and Dyer were spotted onto high performance thin layer chromatography (TLC) silica gel 60 plates and separated by chloroform/ethanol/water/triethylamine (30:35:7:35, v/v). Plates were sprayed with PRIMULINE. SM was analyzed from the TLC plate with 0.5 m 2,5-dihydroxybenzoic acid solution in methanol containing 0.1% trifluoroacetic acid as a matrix (18).

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections were cut, mounted, and deparaffinized. For pErbB1 detection, slides were pretreated with phosphatase inhibitor before antigen retrieval. Slides were stained with CERT– and pErbB1-specific antibodies, followed by a biotinylated detection kit (DABMap Kit; Ventana). Slides were counterstained with haematoxylin and bluing reagent, washed and dehydrated with an ascending alcohol series, and then covered with Cytoseal. See Supplementary Information for details on clinical material and immunohistochemistry evaluation.

**Microarray data analysis**

Normalized gene expression microarray data were retrieved from NCBI GEO. Gene expression values for COL4A3BP (CERT) were extracted, and box plots were generated in concordance with published subgroup classifications of each data set.

**Statistical analysis**

\( P \) values were determined by Student \( t \) test: \( * * * , P \leq 0.001; \) \( * * , P \leq 0.01; \) \( * , P \leq 0.05, \) nonsignificant (n.s.) \( P \geq 0.05. \)

See Supplementary Information for antibodies, reagents and buffers, plasmids, and siRNAs.

**Results**

**CERT expression is reduced in TNBCs**

To elucidate CERT expression in breast tumors, we analyzed 2 different publicly available microarray gene expression data sets (19, 20). CERT mRNA levels were significantly lower in the basal breast cancer subtype (\( P < 0.013 \)) compared with the other molecular subtypes (Fig. 1A). In addition, CERT mRNA levels were slightly reduced in basal breast cancer compared with normal breast tissue (Fig. 1A, right). To confirm these observations, immunohistochemical analysis was conducted with 32 invasive ductal breast carcinomas (Table 1). Compared with moderate CERT expression in normal breast tissue (Fig. 1B, a), expression was reduced (absent or weak) in 12 tumors and moderate to strong expression was seen in 20 tumors. The "reduced CERT phenotype" was more frequent in larger tumors with more than 2 cm diameter (T2 and higher: 53%; T1: 15%) and there was a strong and significant correlation with poor differentiation (GIII 57% vs. GI and II 0%), negative hormone receptor and negative ErbB2/HER2 status and consequently triple negativity of the tumors (80%). Restriction of the analysis to tumors with a confirmed basal-like phenotype (CK5/6 or EGFR/ErbB1 positive) also revealed a significant association with the CERT-low phenotype (82%; Fig. 1B, d–f). Of note, none of the luminal A or B tumors showed reduced CERT expression (Fig. 1B, b and c). Furthermore, the levels of phosphorylated ErbB1 were increased in 9 of 11 cases with reduced CERT expression, compared with only 2 of 6 CERT-positive cases (Fig. 1B, g and h). Thus, low CERT expression appears to be associated with the basal-like breast cancer subtype. In addition, CERT transcript levels were also found to be reduced in metastatic prostate cancers (Supplementary Fig. S1).

Western blotting analysis of CERT expression in a panel of breast cancer cell lines revealed lower protein amounts in the basal-like breast cancer cell lines, whereas expression levels were higher in most of the luminal breast cancer cell lines (Fig. 1C), correlating with the results obtained in primary breast cancer specimens.

**Depletion of the lipid transfer protein CERT affects plasma membrane properties**

To analyze the cellular consequences of CERT loss at the level of the plasma membrane, we depleted CERT in MCF7 cells with specific siRNAs. To verify reduction of plasma membrane SM levels, we used the pore-forming toxin lysenin that specifically binds SM (21). Cells were typically analyzed 48 to 72 hours posttransfection after serum starvation overnight. Indeed, cells in which CERT was depleted were resistant to lysenin whereas control cells transfected with siRNA against β-galactosidase (siLaCZ) stained positively with trypan blue upon lysenin incubation (Fig. 2A, top). Reduction of cellular SM by 44% resulting from CERT knockdown was further confirmed by TLC of total lipid extracts, and the identity of SM was verified by direct matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) analyses from the developed TLC plate (Fig. 2B). It should be noted that phosphatidylethanolamine (PC) and phosphatidylethanolamine profiles as determined by mass spectrometry were comparable in CERT knockdown and control cells (Supplementary Fig. S2), ruling out dramatic effects on overall phospholipid metabolism by SM depletion. To further characterize the impact of CERT depletion on the plasma membrane lipid composition, we stained cells with filipin, which binds cholesterol, and choleratoxin B, which binds the ganglioside GM1 that is known to accumulate in cholesterol-rich membrane microdomains. In cells lacking CERT, plasma membrane staining with filipin was diminished in comparison with control cells, indicating reduced cholesterol levels at the cell surface (Fig. 2A, middle). Instead
cholesterol appeared to accumulate internally leading to a net increase in cholesterol levels, which was also observed in the positive ion MALDI-TOF mass spectra of total cellular lipids (Supplementary Fig. S2). Similarly, plasma membrane staining intensities of cholera toxin B were weaker and appeared less patchy in the absence of CERT (Fig. 2A, bottom).

Cholesterol in particular determines the rigidity and viscosity of membranes. To investigate how CERT depletion impacts on plasma membrane viscosity, we carried out fluorescence recovery after photobleaching (FRAP) experiments with a fluorescently labeled SM analog, C6-NBD-SM, that spontaneously inserts into membranes (Fig. 2C). These experiments revealed an increase in the lateral mobility of NBD-labeled SM in cells depleted of CERT compared with control cells, indicating greater fluidity of the plasma membrane. Similar observations were made with fluorescently labeled PC (data not shown). Together these data provide evidence that CERT-mediated ceramide transfer to the Golgi impacts the precise lipid composition and physical properties of the plasma membrane.

CERT expression modulates EGF-induced Akt activation
To obtain insight how SM loss from the plasma membrane influences growth factor–induced signaling, we analyzed activation of the serine/threonine kinase Akt in response to EGF stimulation. Phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] produced by PI3K recruits Akt to the plasma membrane, in which the kinase becomes activated by phosphorylation (22). Upon CERT knockdown, breast cancer cell lines stimulated with EGF showed increased Akt activation as judged by immunoblotting of cell lysates with an antibody that recognizes phosphorylated Akt (Fig. 3A). Enhanced Akt activation was also seen with a second CERT-specific siRNA, ruling out off-target effects (Supplementary Fig. S3A). Of note, EGF mainly activated Akt1 in both control and CERT-depleted MCF7 cells (data not shown). We also observed a moderate increase in EGF-induced phosphorylation of Erk1/2 as a read-out for MAPK activation, indicating that augmented ErbB1 signaling was not limited to the PI3K-Akt pathway, however, the effect on Erk1/2 was less pronounced (data not shown). To ascertain that SM depletion was responsible for the observed differences, we downregulated the 2 SM synthase isoforms,
SMS1 and SMS2 (23, 24), using specific siRNAs (Supplementary Fig. S4A). Compared with control cells, EGF-induced Akt activation was also enhanced in MCF7 cells lacking SMS1/2 (Supplementary Fig. S4B), recapitulating the effects observed in the absence of CERT. Finally, pretreatment of MCF7 cells with the ErbB inhibitor PD168393 blocked EGF-induced Akt activation, showing that Akt hyperphosphorylation in CERT-depleted cells depends on ErbB receptor activation (Fig. 3B).

CERT downregulation augments ligand-induced ErbB1 activation and mobility

We next analyzed how altered plasma membrane lipid composition impacts ligand-induced ErbB receptor activation. Ligand binding induces ErbB receptor homodimerization and heterodimerization. Although ErbB2 has no ligand, it is the preferred heterodimerization partner of the other ErbB receptors and is activated upon EGF binding (25, 26). We therefore immunoprecipitated ErbB1 and ErbB2 from lysates of MCF7 cells stimulated with EGF and detected the receptors with a phosphotyrosine-specific antibody. Figure 4A reveals that in cells lacking CERT, basal receptor phosphorylation was reduced, but EGF-induced receptor phosphorylation was increased, providing an explanation for enhanced Akt activation upon ligand stimulation. Similar results were obtained when cells were treated with heregulin, which triggers activation of the ErbB2/ErbB3 heterodimer, and this was also associated with elevated Akt activation (Supplementary Fig. S5A and S5B). It has been reported that changes in lipid composition (e.g., cholesterol depletion with methylcyclodextrin) affect ErbB1 availability on the cell surface (27, 28), and work from our own laboratory has revealed a function of CERT in Golgi secretory activity (29), raising the possibility that CERT knockdown may result in altered plasma membrane receptor levels. However, by fluorescence-activated cell sorting analysis, we did not observe any significant differences in surface ErbB receptor levels (Supplementary Fig. S5C shown for ErbB2 and ErbB3).

To investigate the lateral mobility of ErbB1, siLacZ control and CERT-depleted cells were transiently transfected with CFP-tagged ErbB1 and FRAP experiments were carried out. The mean diffusion coefficient of CFP-ErbB1 increased from 0.130 μm²/s in control cells to 0.156 μm²/s in CERT-depleted cells, indicating elevated mobility of the receptor in plasma membranes with a reduced SM content (Fig. 4B). Upon ligand binding, ErbB1 is rapidly internalized, ubiquitylated, and lysosomally degraded. Although this serves to shut off signaling, it is well appreciated that the internalized receptor continues to transduce signals (30). To study how alterations of plasma membrane lipid composition impact on ligand-induced receptor internalization, we incubated cells with fluorescently labeled EGF for different periods of time. EGF was found to rapidly accumulate intracellularly in CERT-depleted cells. Quantification of internal fluorescence intensities revealed a 2.3-fold increase after 15 minutes of EGF administration, suggesting that the receptor is internalized at a higher rate (Fig. 4C, left). Because internal fluorescence intensities remained high at 60 minutes in CERT-depleted cells, receptor degradation may also be affected. Of note, constitutive

Table 1. Analysis of immunohistochemical CERT expression in primary breast cancers with relation to standard clinical parameters and cancer subtype

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<th>N-total</th>
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<th>CERT, normal or high score 2-3</th>
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<td>11 (100%)</td>
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<td>12 (63%)</td>
<td>7 (37%)</td>
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<td>0 (0%)</td>
<td>13 (100%)</td>
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<td></td>
<td>11</td>
<td>0 (0%)</td>
<td>11 (100%)</td>
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<td>9 (82%)</td>
<td>2 (18%)</td>
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</table>

SM1 and SM2 (23, 24), using specific siRNAs (Supplementary Fig. S4A). Compared with control cells, EGF-induced Akt activation was also enhanced in MCF7 cells lacking SMS1/2 (Supplementary Fig. S4B), recapitulating the effects observed in the absence of CERT. Finally, pretreatment of MCF7 cells with the ErbB inhibitor PD168393 blocked EGF-induced Akt activation, showing that Akt hyperphosphorylation in CERT-depleted cells depends on ErbB receptor activation (Fig. 3B).
Internalization of transferrin was not elevated in cells lacking CERT (Fig. 4C, right), indicating a specific effect on ligand-induced ErbB1 trafficking.

CERT expression modulates cell motility and focal adhesion clustering

To explore how the reduction of CERT expression affects biological responses downstream of ErbB1, we carried out migration experiments with EGF as a chemotactic stimulus. Compared with siLacZ control cells, CERT knockdown in MCF7 cells led to a 4-fold increase in the number of migrated cells (Fig. 5A). This result was confirmed with a second CERT-specific siRNA and by downregulating SMS1 and SMS2 (Supplementary Fig. S3B and S4C). A significant increase in migration was also observed in MDA-MB 231 cells despite their higher basal migratory potential (Fig. 5A). Vice versa, ectopic expression of CERT in MDA-MB 231 and MDA-MB 468 basal-like breast cancer cells inhibited both migration and invasion through Matrigel-coated filters (Fig. 5A).

For cells to migrate, they first require attachment to the substratum, followed by cell spreading and polarization, processes that go along with profound cytoskeletal rearrangements. We therefore analyzed how reduced CERT expression affects cell adhesion and spreading by freshly plating cells onto collagen-coated dishes. MCF7 cells with downregulated CERT were found to adhere and spread more rapidly in comparison with control cells (Fig. 5B). Cell attachment is mediated by integrin receptors that recruit intracellular adaptor proteins to form so-called focal adhesions, which establish connections with the actin cytoskeleton. To visualize focal adhesions, we stained cells for the adaptor proteins talin and paxillin, which displayed increased clustering in CERT-depleted cells in

Figure 2. CERT depletion alters plasma membrane composition and fluidity. MCF7 cells were transiently transfected with CERT-specific siRNA or siLacZ. A, cells were treated with lysenin and stained with trypane blue (top; scale bar, 200 μm), or stained with filipin or Alexa Fluor 488-labeled cholera toxin B (middle and bottom; scale bar, 20 μm). B, lipids were separated by TLC and SM (arrow) was analyzed by matrix-assisted laser desorption/ionization-time-of-flight. The TLC plate was quantified by densitometry. Spot volumes of the different lipid species were normalized to that of PC, values for the siLacZ control were set as 1. LPC, lysophosphatidylcholine. C, cells were incubated with C6-NBD-SM and FRAP studies of 15 cells per sample were carried out. Mean diffusion coefficients were 0.292 ± 0.009 μm²/s (siLacZ) and 0.339 ± 0.012 μm²/s (siCERT); P = 0.0054. Error bars, SEM.
comparison with siLacZ control cells (Fig. 5C). This was associated with elevated signal intensities of phosphotyrosine at sites reminiscent of focal adhesions and phosphorylated, active focal adhesion kinase (Fig. 5C). Together, these data support the conclusion that CERT downregulation induces cytoskeletal changes that facilitate cellular chemotactic responses.

Increased EGF signaling upon CERT downregulation is mediated by PLD2

PLD, which is activated in various cell types in response to EGF, hydrolyzes PC to produce the lipid second messenger phosphatidic acid (PA; refs. 31, 32). It was recently reported that PLD2 activation controls the ligand-induced signaling output of ErbB1 (11). We therefore investigated the contribution of PLD2 in our cellular system. Interestingly, silencing of PLD2 abrogated increased ErbB1 phosphorylation in CERT-depleted cells (Fig. 6A, top). This was also observed with a second PLD2-specific siRNA (Supplementary Fig. S6). Due to the lack of commercially available antibodies that detect the endogenous protein, the efficiency of PLD2 silencing was confirmed by cotransfecting a GFP-tagged PLD2 expression construct in HEK293T cells, followed by immunoblotting of cell lysates with a GFP-specific antibody (Fig. 6A, bottom). PLD2 downregulation also blocked increased Akt activation in CERT-depleted cells (Fig. 6B and Supplementary Fig. S6). Indeed, measurement of basal PLD activity revealed a 30% increase in cells lacking CERT compared with control cells (Fig. 6C). Because endogenous PLD2 could not be detected, possible changes in protein expression could not be addressed. PLD has further been reported to contribute to ErbB1 internalization (33). We therefore investigated whether PLD2 downregulation also prevented the increased uptake of fluorescently labeled EGF in cells with diminished SM levels. Indeed, PLD2 knockdown significantly reduced EGF accumulation in CERT-depleted cells almost to the levels seen in siLacZ control cells (Fig. 6B, bottom).
Finally, PLD2 knockdown also reverted both increased spreading and migration of cells lacking CERT (Fig. 6E and F). Taken together, our data reveal that the loss of CERT triggers aberrant ligand-induced ErbB1 signaling and chemotaxis through PLD2 activation.

Discussion

TNBCs compose a heterogeneous group of cancers for which very few common molecular markers have been identified. Here, we report downregulation of the Golgi-localized lipid transfer protein CERT in TNBCs. Due to its critical role in SM production CERT determines the lipid composition and organization of the plasma membrane, thereby impacting the behavior of transmembrane proteins and membrane proximal signaling events including those connecting to the actin cytoskeleton.

Sphingolipids are being recognized to play important roles in various diseases (34). Alterations in sphingolipid metabolism have also been shown to contribute to chemoresistance and tumor survival. Interestingly, CERT expression was reported to be induced in ovarian tumors treated with paclitaxel, thereby protecting cells from ceramide-induced apoptosis (35). This indicates that CERT is dynamically regulated and, depending on the situation, both its up- and downregulation can be beneficial for a tumor cell. Molecular regulatory mechanisms for CERT have been uncovered, for example, involving posttranslational modification by phosphorylation (29, 36) and complex formation with the oxysterol binding protein, whereby SM synthesis is linked to cholesterol homeostasis (37). The exact nature of membrane microdomains is a matter of ongoing debate, but it is possible that the altered ErbB1 signaling characteristics observed are a consequence not only of SM reduction but the disruption of SM- and cholesterol-enriched microdomains. Because CERT depletion reduced surface cholesterol as judged by filipin staining, our data are in agreement with the hypothesis that SM synthesis is linked to cholesterol transport. Although generally perceived as platforms that amplify signaling, our results rather point toward restriction of ErbB1 activation by sphingolipid-enriched membrane microdomains.

We provide evidence that CERT downregulation enhances PLD2 activity, which is responsible for the potentiation of ligand-induced ErbB1 activation. PLD activity is implicated in various biological processes, including membrane trafficking, actin cytoskeletal reorganization, and cell migration (32). PLD activity is increased in different cancer types, including those of the breast (38, 39), and PLD was shown to transform fibroblasts overexpressing c-Src or ErbB1 (40, 41). Intriguingly, MDA-MB 231 cells contain high PLD activity, whereas MCF7 cells have low levels (42), inversely correlating with their CERT expression. The precise molecular mechanism by which CERT controls PLD2 activity will require further investigation but could perhaps involve...
enhanced membrane recruitment. PA formed by PLD activates phosphatidylinositol 4-phosphate 5-kinases, whereby phosphatidylinositol 4,5-bisphosphate [PI(4, 5)P₂] is generated as a substrate for PI3K, ultimately leading to PI(3, 4, 5)P₃ production and Akt activation. This also implies a positive feedback as PI(4, 5)P₂ recruits and activates PLD (32). Dominant-negative PLD2 was shown to inhibit ligand-induced ErbB1 nanoclustering and Erk activation (11). PLD2 expression modulates breast cancer cell motility and spreading and focal adhesion clustering. A, top, cells were transiently transfected with siCERT and siLacZ and subjected to migration assays (18 hours for MCF7 cells, 3 hours for MDA-MB 231 cells). Middle and bottom, MDA-MB 231 and MDA-MB 468 cells were nucleofected with a CERT expression plasmid and subjected to migration and invasion assays. Cells were counted in 5 independent fields at a 20-fold magnification. The mean of duplicate filters is plotted. Error bars, SEM. CERT knockdown in MDA-MB 231 cells and overexpression in MDA-MB 231 and MDA-MB 468 cells was verified by immunoblotting of cell lysates. B and C, MCF7 cells were transiently transfected with siCERT and siLacZ. B, cells were replated onto collagen-coated dishes for the indicated times, fixed, and stained with crystal violet. C, cells were immunostained with antibodies directed against the indicated proteins. Scale bars, 20 μm.

Figure 5. CERT expression modulates breast cancer cell motility and spreading and focal adhesion clustering. A, top, cells were transiently transfected with siCERT and siLacZ and subjected to migration assays (18 hours for MCF7 cells, 3 hours for MDA-MB 231 cells). Middle and bottom, MDA-MB 231 and MDA-MB 468 cells were nucleofected with a CERT expression plasmid and subjected to migration and invasion assays. Cells were counted in 5 independent fields at a 20-fold magnification. The mean of duplicate filters is plotted. Error bars, SEM. CERT knockdown in MDA-MB 231 cells and overexpression in MDA-MB 231 and MDA-MB 468 cells was verified by immunoblotting of cell lysates. B and C, MCF7 cells were transiently transfected with siCERT and siLacZ. B, cells were replated onto collagen-coated dishes for the indicated times, fixed, and stained with crystal violet. C, cells were immunostained with antibodies directed against the indicated proteins. Scale bars, 20 μm.

example, PLD2 can contribute to ErbB1 endocytosis by different mechanisms: (i) PA promotes negative membrane curvature, thereby facilitating vesicle budding, (ii) PtdIns(4, 5)P₂ generated by phosphatidylinositol 4-phosphate 5-kinases recruits endocytic proteins and activates the GTPase dynamin, and (iii) PLD possesses GAP activity shown to directly activate dynamin (43). PLD activation is required for integrin-mediated cell spreading and migration of leukocytes (44). In the absence of CERT, MCF7 cells showed increased motility and proliferative activity, accompanied by prominent focal adhesion clustering. This may be due to direct effects on membrane-spanning integrins and/or by inside-out signaling triggered by PLD2. Because CERT knockdown did not favor proliferation of cells, downregulation of the protein may
occur at later stages during carcinogenesis when growth promoting signals are already activated. In this respect, advances in the development of specific PLD inhibitors, shown to inhibit invasion of metastatic breast cancer cells, hold special promise (44).

Recently, the tyrosine phosphatase PTPN12 was identified as a tumor suppressor in TNBC (45). Loss of PTPN12 prevented dephosphorylation and thus inactivation of multiple kinases, including ErbB1 and ErbB2/HER2. Similarly, reduced CERT expression will affect RTKs and receptor classes other than ErbB1. Indeed, heregulin-stimulated ErbB2 and ErbB3 activation were also augmented in CERT-depleted MCF7 cells. Furthermore, our findings may not be restricted to TNBC because metastatic prostate cancers also displayed reduced CERT transcript levels.

Due to their aberrant activation in various cancer types, ErbB receptors are clinically targeted with small-molecule inhibitors and blocking antibodies. ErbB1 and ErbB2/HER2 inhibitors have failed to show clinical efficacy for TNBC, even though many of these breast cancers display high ErbB1 levels (1, 46). c-Kit and c-Src activation are also implicated in this breast cancer subtype (2), therefore, inhibition of multiple tyrosine kinases may be required or common aberrantly activated downstream pathways need to be identified. The PI3K/Akt pathway is particularly interesting because increasing evidence points to its importance in determining sensitivity and resistance of tumor cells to targeted therapies (46). Thus, alterations in lipid metabolism facilitating PLD2 and Akt activation may make important contributions to cellular transformation and may

Figure 6. PLD2 mediates increased ligand-induced ErbB1 signaling in CERT-depleted cells. MCF7 cells were transiently transfected with CERT and PLD2 siRNAs. A, top, cells were stimulated with 10 ng/mL EGF. Lysates were analyzed by immunoblotting with pErbB1(Y1068)-, CERT-, and tubulin-specific antibodies. Bottom, HEK293T cells were transfected with a GFP-PLD2 vector along with the indicated siRNAs. Lysates were immunoblotted with GFP- and tubulin-specific antibodies. B, serum-starved cells were stimulated with 10 ng/mL EGF for the indicated times. Lysates were immunoblotted with the indicated antibodies. The panels are from the same gel. C, cells were lysed and PLD activity was measured. The activity of siLacZ control cells was set as 1. D, cells were incubated with 200 ng/mL Alexa Fluor 555-labeled EGF for 15 minutes. The mean internal fluorescence of at least 25 cells per sample was quantified. Error bars, SEM. E, migration assay as described in Fig. 5. F, spreading assay as described in Fig. 5.
be relevant to the design of therapeutic interventions targeting TNBCs.

Disclosure of Potential Conflicts of Interest

T.N. Fehm is a consultant and is an advisory board member of Roche. No potential conflicts of interest were disclosed by the other authors.

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Loss of the Ceramide Transfer Protein Augments EGF Receptor Signaling in Breast Cancer

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